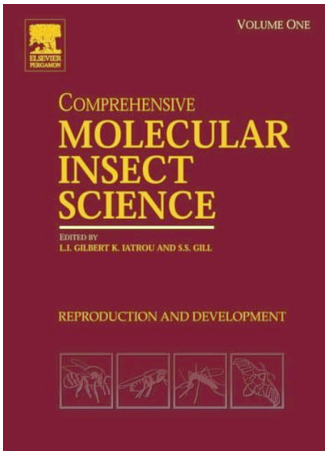
ELSEVIER
PERGAMON

COMPREHENSIVE MOLECULAR INSECT SCIENCE

EDITED BY
L.I. GILBERT, K. IATROU AND S.S. GILL





Comprehensive Molecular Insect Science

Seven-Volume Set, Volume 1-7

by Kostas Iatrou, Sarjeet S. Gill

Product Details

- **Hardcover:** 3300 pages
- **Publisher:** Pergamon (December 20, 2004)
- **Language:** English
- **ISBN:** 044451516X
- **Product Dimensions:** 12.0 x 9.5 x 9.9 inches
- **Shipping Weight:** 29.3 pounds.

List Price: $2,650.00

Book Description

The important developments and achievements in modern insect science since the first edition have led to this new revised, expanded and retitled reference work. New chapters in this edition include the ecdysone receptor, lipocalins, bacterial toxins, etc. Each article may also be read independently, as a review of that particular subject.

Comprehensive Molecular Insect Science also covers new technologies that have been developed since 1985. The Index Volume will help researchers to track specific subjects and particular species of insects cited in several volumes.

Available Online in 2005 via ScienceDirect!
' Extensive browse and search across subject, thematic, alphabetical, author and cited author indexes - as applicable to the wor k
' Basic and advanced search functionality within volumes, parts of volumes, or across the whole work
' Build, save and re-run searches as well as combine saved searches
' Internal cross-referencing between articles in the work, plus dynamic linking to journal articles and abstract databases, maki ng navigation flexible and easy
' All articles are available as full text HTML files, and as PDF files that can be viewed, downloaded or printed out in their or iginal print format

The online edition of this title is kept under regular editorial review, and enhanced as warranted to reflect the state of current knowledge. Enhancements may take place as frequently as annually and may consist of new versions of existing articles, new articles, or additional material.

Key Features:

' Revised edition of critically acclaimed **Comprehensive Insect Physiology, Biochemistry and Pharmacology** (1985, Pergamon Press, 13 Volume Set)
' Written by a distinguished international group of contributors
' Well-organized format provides for concise, readable entries, easy searches, and thorough cross-references
' Complete up-to-date coverage of many important topics - essential information for scientists, students, and professionals alik e

About the Author

Kostas Iatrou is the Director of the "Insect Molecular Genetics and Biotechnology" Research Group at the Institute of Biology, NCSR "Demokritos" and an adjunct Professor of the University of Calgary. He is the former Professor of Biochemistry and Molecular Biology at the Faculty of Medicine, University of Calgary, Canada and Director of the Institute of Biology, National Institute of Scientific Research "Demokritos", Athens Greece. His major research interests are in the areas of insect cell differentiation, development and reproduction. His parallel research activities include the development of insect, insect cell and insect virus-based functional genomics tools and technologies that can be used for a wide variety of biotechnological applications impacting on the fields of insect pest control human disease treatment.

Sarjeet S. Gill is Professor of Cell Biology in the Department of Cell Biology and Neuroscience at the University of California, Riverside, and Entomologist in the Agricultural Experimental Station on the same campus. His research interests are in biochemistry, molecular biology and insect toxicology, with a focus on mechanisms of bacterial and viral pathogenesis, and the role of the midgut and Malpighian tubules in insect homeostasis. Dr. Gill's research also employs the use of whole genome tools to analyze gene function. His research has been continually funded through the NIH and USDA, he continues to serve on national and international committees related to his research interests, and is an elected fellow of the American Association for the Advancement of Science.

Comprehensive Molecular Insect Science

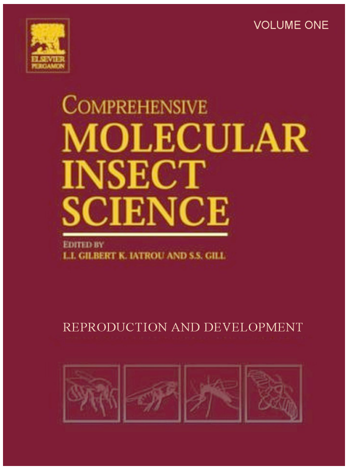
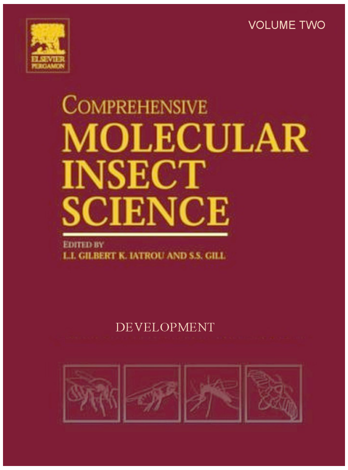


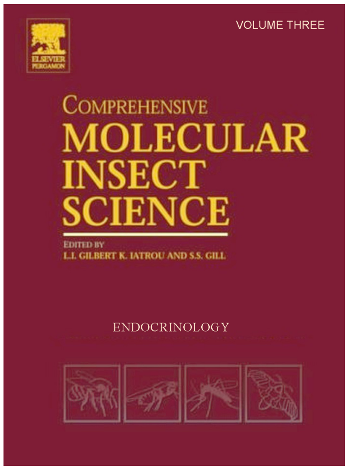
Table of Contents

Volume 1: Reproduction and Development

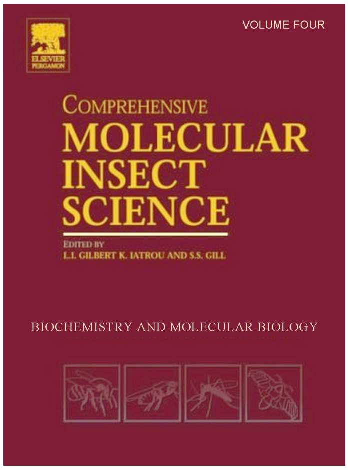
Volume 2: Development



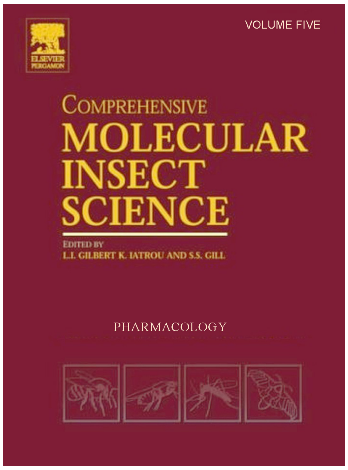
Volume 3: Endocrinology



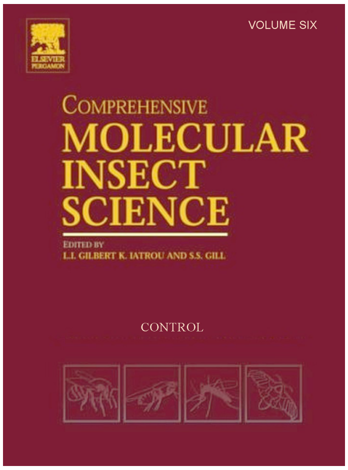
Volume 4: Biochemistry and Molecular Biology



Volume 5: Pharmacology



Volume 6: Control



Volume 7: Indexes

EDITOR-IN-CHIEF

LAWRENCE I. GILBERT
University of North Carolina
Chapel Hill, NC
USA

Editors

KOSTAS IATROU
National Center for Scientific Research "Demokritos"
Athens, Greece

SARJEEET S. GILL
University of California
Riverside, CA
USA

INTRODUCTION

The first edition of this treatise, entitled *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* and edited by Gerald A. Kerkut (see p. ix for the Dedication to Prof. Kerkut) and Lawrence I. Gilbert, was published in 1985. The preliminary meetings regarding that 13-volume series began in 1980 and it was agreed that there would be 12 volumes "that would provide an up-to-date summary and orientation on the physiology, biochemistry, pharmacology, behavior, and control of insects that would be of value to research workers, teachers, and students." It was felt that the volumes would provide a classical background to the literature, include all the critical basic material, and then emphasize the literature from 1950 to the present day (1984). By mid-1981 most of the chapters were assigned to authors and the project was underway. It was agreed upon that there would be a final Volume 13, an Index volume, that combined the subject, species, and author indexes for all 12 volumes so that all material in those volumes could be located easily. This was a monumental undertaking and the 13 volumes finally appeared in 1985. It received plaudits from reviewers and from researchers around the world. One of us (LG.) was approached by Carrol Williams, an internationally renowned insect physiologist, at an International Congress of Entomology following the publication of the first edition, and Professor Williams noted that he had a choice between purchasing the 13-volume set for his laboratory and buying a new centrifuge. He then stated that he chose the 13-volume series and had never regretted it for a moment. There were more than 50 000 references to the literature in the first edition and more than 10 000 species of insects were referred to, and all of this information was readily available through the thirteenth volume (index). It is of interest that volumes of that original series are still being purchased some 19 years after this series was published.

Over the past 5–6 years, one of us (LG.) was approached many times by colleagues around the world asking when a second edition of this series would be available. There was an obvious need because of the dramatic explosion of knowledge in insect science due to the utilization of molecular biological paradigms and techniques culminating in the elucidation of the *Drosophila* genome (fly database), the *Anopholes* genome, and of course this explosion continues. The use of this technology was really only beginning when the first series was developed and in 2000, 15 years after the publication of the first edition, we and Elsevier agreed that a new edition was necessary. This would be a series in which each chapter would begin by devoting a few pages summarizing the work in a particular research area up to the publication of the first series (1984–1985), and the remainder of the chapter would be a review of the literature from 1985 to 2003. It was agreed upon that each chapter would make full use of data arising from the utilization of molecular technologies if such data were applicable to that particular chapter subject. We chose to fulfill that premise in six volumes plus an index volume and that both a printed version and an electronic version would be available. As is evident, the title of the series has been changed, both to reflect the emphasis on molecular approaches and to alleviate the use of the obviously cumbersome title *Comprehensive Insect Physiology, Biochemistry, Pharmacology, and Molecular Biology,* which would have to be typed every time a researcher refers to a chapter in the series. We do not pretend, however, that this is a truly comprehensive treatise, since critical areas of research, e.g., ecology, evolutionary biology, and behavior, have not been included, but we have chosen to retain the use of the word "Comprehensive" for continuity with the first series.

The reader will note that the number of chapters in the six volumes is certainly well below the number of chapters in the original 12 volumes. The editors made very subjective decisions based on their own experience and knowledge regarding those areas where the most progress had been made over the past 18 years and omitted areas that have not grown significantly. Surely, there are areas of omission that many readers will feel were due to arbitrary decisions or lack of knowledge of the editors. That is bound to be the case although we spent a great deal of time deciding which chapters should be included, but there is little doubt that personal research bias played a role in those decisions. We are certainly confident that these volumes will be of great value to the research community, including postdoctoral investigators and graduate students. The new publishing technology will allow upgrades by the publisher every several years for those areas of research in which significant breakthroughs occur. This, of course, will only occur in the electronic format of the series and it is hoped that at least for the first few upgrades the original authors will take part.

Lawrence I. Gilbert
Kostas Iatrou
Sarjeet Gill

DEDICATION

Professor Gerald Allan Kerkut: 19 August 1927–6 March 2004

Gerald Kerkut began his scientific career in Cambridge, graduating with a first class degree in Natural Sciences (1945–1948). He then elected to remain at Cambridge for his Ph.D. (1948–1951), studying locomotion in starfish under the supervision of Professor Eric Smith in the Department of Zoology. Gerald continued at Cambridge for a further three years (1951–1954) as a junior fellow of his college, Pembroke. During this period, he studied the electrical activity of the gastropod central nervous system, initially selecting slugs but, due to problems of identification, changed to the garden snail, *Helix aspersa*. A species he was to use for nearly 30 years.

In 1954 Gerald moved to the University of Southampton to take up a lectureship in Animal Physiology within the Department of Zoology. Together with Professor Kenneth A. Munday, Gerald established the Department of Physiology and Biochemistry in 1959 and in 1966 was appointed to the second chair of Physiology and Biochemistry. He remained at Southampton throughout his academic life, retiring in 1992, when he was appointed Emeritus Professor. Gerald continued an active association with the University up until his death. He was invited by a number of universities to become chairman of their zoology or physiology departments but he always declined, preferring to remain at Southampton.

Gerald Kerkut had a first class analytical mind, a prodigious capacity for hard work and an extensive knowledge of the scientific literature. He was always ready to challenge accepted dogma and this was very well illustrated in his inaugural lecture entitled "The Missing Pieces," delivered in December 1968 (Kerkut, 1969). In this he reviewed his research on a number of topics, including the variable ionic composition of neurons, the role of the sodium–potassium pump in the maintenance of the resting potential, fast orthodromic and slow antidromic axon transport and amino acids as transmitters. Although much of his earlier research used the snail, Gerald also worked on insects where he was interested in the effect of sudden temperature changes on the nervous system. Using the cockroach leg preparation he observed that a sudden fall in temperature resulted in a transient increase in activity. Intracellular recordings showed that a decrease in temperature resulted in a depolarization of the membrane potential, which was responsible for the transient increase in activity. *Q*-10 values for changes in membrane potential of insect muscle were greater than those predicted from the Nernst equation. This led to his work on electrogenic metabolic pumps, summarized in his book with Barbara York, "*The Electrogenic Sodium Pump*" (Kerkut and York, 1971). Gerald also employed insect preparations for his research on glutamic acid as a transmitter and that certain insect neuron cell bodies possessed overshooting action potentials, for example, the octopamine-containing dorsal unpaired median (DUM) cells.

Having been a great advocate of the use of isolated invertebrate central nervous system preparations, Gerald turned his attention to isolated mammalian preparations in the late 1970s. In particular, he developed the use of the isolated spinal cord in conjunction with Jeff Bagust. Gerald was particularly interested in sensory integration in the dorsal horn of the spinal cord. Although a great protagonist of

isolated preparations, Gerald stressed that observations using isolated preparations must always be related to the living animal.

Gerald Kerkut was also very involved in the publication of scientific data both in terms of books and journals. In the late 1950s he met Robert Maxwell, the founder of Pergamon Press. They immediately formed an excellent rapport, and Maxwell encouraged Gerald to explore his idea of starting a journal in comparative physiology and biochemistry. To cut a long story short, this resulted in the publication of *Comparative Biochemistry and Physiology* with its first number in 1960 and Gerald as co-editor with Bradley T. Scheer. The journal proved a great success and eventually expanded into three sections, namely, Physiology, Biochemistry, and Pharmacology. Gerald continued to edit the journal until 1994. Another of his editing successes was *Progress in Neurobiology*, which he co-edited with John Phillis. Gerald also edited a very successful series of monographs in Zoology, which were published by Pergamon Press. Gerald's interest in publishing work on insects is illustrated by his co-editing with Lawrence Gilbert in 1985 of the first edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology.* He was particularly proud of the high standard of scholarship in these volumes.

Gerald was always interested in evolution and with Maxwell's support published "*The Implications of Evolution*" in 1960 (Kerkut, 1960). In this he examined the problem of the evolution and interrelationships of the animal phyla. This was a topic of lasting interest to him and one to which he returned in a mini-review entitled "*Possible Evolutionary Futures for Mankind*" (Kerkut, 1988). In 1988 a Festschrift was organized in Gerald's honor and the proceedings published as a special edition of *Comp. Biochem. Physiol.* Gerald provided the opening chapter, in which he reviewed his research career up to that time (Kerkut, 1989).

Gerald Kerkut enjoyed music, art, and travel. He was an accomplished pianist and had an extensive collection of art books. Up until 6–7 years ago, Gerald traveled widely in the Americas and the Far East. Gerald enjoyed teaching and interacting with undergraduates and postgraduates. Many will retain enduring memories of his humor and concerned interest in their welfare. During his active research period, Gerald trained over 80 postgraduates and their success will provide a lasting legacy to his memory.

References

Kerkut, G.A., **1960**. The Implications of Evolution. Oxford, Pergamon, p. 174.

Kerkut, G.A., **1969**. The Missing Pieces. University of Southampton, Southampton, UK, p. 15.

Kerkut, G.A., **1988**. Possible evolutionary futures for mankind. *Comp. Biochem. Physiol. 90A*, 5–10.

Kerkut, G.A., **1989**. Studying the isolated central nervous system; a report on 35 years: more inquisitive than aquisitive. *Comp. Biochem. Physiol. 93A*, 9–24.

Kerkut, G.A., York, B., **1971**. The Electrogenic Sodium Pump. Scientechnica, Bristol, UK, p. 182.

Robert Walker
Head of Honours School
School of Biological Sciences
University of Southampton

PERMISSION ACKNOWLEDGMENTS

The following material is reproduced with kind permission of Nature Publishing Group

Figure 11 of Chapter 1.8 Insect Homeotic Complex Genes and Development, Lessons from *Drosophila* and Beyond

Figures 4 and 11 of Chapter 1.12 The Development of the Olfactory System

Figures 3, 6, 7, 8 and 9 of Chapter 3.5 The Ecdysteroid Receptor

Figure 5 of Chapter 3.11 Circadian Organization of the Endocrine System

Figure 9 of Chapter 5.4 GABA Receptors of Insects

http://www.nature.com/nature

The following material is reproduced with kind permission of American Association for the Advancement

Figure 8 of Chapter 5.4 GABA Receptors of Insects

http://www.sciencemag.org

Notes on the Subject Index

To save space in the index the following abbreviations have been used:

ETH – ecdysis triggering hormone
GPCRs – G protein-coupled receptors
PBAN – pheromone biosynthesis activating neuropeptide
PDV – polydnaviruses
PTTH – prothoracicotropic hormone
QSAR – qualitative structure-activity relation
RDL – resistance to dieldrin

1.1 Sex Determination and the Development of the Genital Disc

L Sánchez, Centro de Investigaciones Biológicas, Madrid, Spain
N Gorfinkiel and I Guerrero, Universidad Autonoma de Madrid, Spain

1.1.1. Sex Determination in *Drosophila*

1.1.1.1. Introduction and Historical Overview

Perpetuation by sexual reproduction is the rule within the animal kingdom. Males and females are different at the morphological, physiological, and behavioral levels. This sexual dimorphism results from the integration of the sex determination developmental program, which commits the embryo to either the male or the female pathway, and the developmental program which determines the type of cellular structure that will be differentiated in specific regions in the animal.

This review deals with the genetic and molecular bases controlling sex determination and the sexual dimorphic development of the genital disc that gives rise to the terminalia, the most sexual dimorphic structure of the animal. It is focused on *Drosophila* since it is in this organism where a coherent picture about these developmental processes is now emerging. This has become possible thanks to the combination of sophisticated genetic and molecular biology techniques available in this model insect. However, sex determination and development of the genital disc in other insects will be also reviewed here.

In *Drosophila melanogaster*, 2X;2A individuals (X, X chromosome; A, autosomal set) are females and XY;2A individuals (Y, Y chromosome) are males. Since females and males differ in the number of X chromosomes, a process has evolved to eliminate the difference in the doses of the X-linked genes in the two sexes. This process is called dosage compensation (see **Chapter 1.7**). In *D. melanogaster*, the two X chromosomes in females are active and dosage compensation is achieved in males by hypertranscription of the single X chromosome.

A series of results led to the discovery that in *D. melanogaster* sex is determined by the ratio of the X chromosomes to the sets of autosomes. First, XXY and X0 flies are female and male, respectively. This indicates that the Y chromosome plays no role in sex determination. Second, gynandromorphs are sexually mosaic individuals with some portions of the body typically male and others typically female. Such individuals arise from the loss of one X chromosome during the early development of XX flies. The sharp borderline between female and male tissues indicates that each individual cell chooses its sex autonomously, i.e., according to its chromosome constitution. Third, 2X;3A flies are mosaics with male and female structures. Collectively, these results indicate that gender in *D. melanogaster* is not determined by the absolute number of X chromosomes but by the ratio of X chromosomes to the sets of autosomes (X/A ratio signal). Supporting this contention, clones with one X chromosome and one set of autosomes (1X;1A clones) develop into female structures (Santamaría and Gans, 1980; Santamaría, 1983). In 2X;3A mosaic flies, the X/A ratio is at a threshold level between a normal female

and a normal male signal. Some cells interpret this ambiguous signal as female while others interpret it as male.

Based on genotypes with variable X/A ratios, Bridges (1921, 1925) proposed his "balance concept" of sex determination. This hypothesis considers gender as a quantitative character, with continuous variation, under the control of two opposing polygenic signals, whose component factors have a small effect. The female-determining factors are located on the X chromosome and the male-determining factors are located on the autosomes. The opposing action of the two sets of signals would determine the gender of the fly, according to the stoichiometric assumption that two doses of the female-determining factors (two X chromosomes) outweigh the effect of two doses of male-determining factors (two sets of autosomes), leading to female development. However, two doses of male-determining factors (two sets of autosomes) outweigh the effect of one dose of female-determining factors (one X chromosome), leading to male development. In the case of 2X;3A flies, the stoichiometry of the interaction between female- and male-determining factors is such that, within the same fly, in some cells the male development is imposed and in others the female factors prevail leading to female development.

A breakthrough in the understanding of the genetic basis underlying sex determination in *Drosophila* was the work of Cline (1978), who found that *Sex-lethal* (*Sxl*) – a gene whose function depends on the X/A signal – is the key gene controlling both sex determination and dosage compensation processes, in such way that *Sxl* is activated in females (2X;2A) but not in males (X;2A). The function of *Sxl* would determine female sexual development and autosome-like basal transcription of each of the two X chromosomes in 2X;2A flies, whereas the lack of *Sxl* function would cause male sexual development and hypertranscription of the single male X chromosome (dosage compensation).

Gadagkar *et al*. (1982) and Chandra (1985) proposed a model based on noncoding DNA to explain how cells would assess the X/A ratio signal. They proposed the existence of noncoding DNA sequences in the X chromosome (locus π) that would bind a repressor Ω encoded by an autosomal gene ω. Moreover, they incorporated in their model Cline's proposition about *Sxl* as the key gene controlling sex determination (Cline, 1978). In addition, they also incorporate Cline's finding that the maternal product of gene *daughterless* (*da*) is also required for *Sxl* activation (Cline, 1978).

The "noncoding DNA" model consists of the following components. First, the maternal Da product that is stored in the egg; second, the Ω repressor that is encoded by the autosomal gene ω and is activated in the zygote by the maternal Da product; and third, the gene *Sxl* and the locus π, which have affinity for the Ω repressor and RNA polymerase. A central assumption in this model is that the Ω repressor and RNA polymerase compete with each other for binding sites on both the *Sxl* and π loci in such a way that RNA polymerase binds to both loci with a lower affinity than that of the Ω repressor. It was further assumed that *Sxl* has a lower affinity than π for the repressor as well as the polymerase. According to this model, the amount of repressor in male and female embryos would be the same, since both have two copies of the gene ω and both inherit the same amount of maternal Da product. The male embryo has only one X chromosome and therefore only one dose of the low affinity *Sxl* gene and one dose of the high-affinity locus π. The Ω repressor would bind significantly to both sites thus preventing the binding of RNA polymerase to *Sxl*. Consequently, little or no Sxl protein product would be produced. In the female embryo, there are two doses of both *Sxl* and π loci. Most of the Ω repressor will be sequestered by the π sites, so that the *Sxl* loci will be free to bind RNA polymerase. Synthesis of the Sxl product will then take place.

The role of the X/A signal as the primary input for sex determination and dosage compensation could be visualized in two different ways. One possibility is that the X/A signal may be continuously needed during development for the cells to stay in the chosen sexual pathway and maintain the dosage compensation process properly adjusted. Under this hypothesis, X0 clones induced at any time during the development of XX flies would survive and differentiate male structures. Alternatively, the cells could use the X/A signal at a certain time in their development to set up their sex and dosage compensation processes. Under this second hypothesis, X0 clones induced before that time would survive and differentiate male structures, whereas X0 clones induced later would die because their dosage compensation process would be upset.

To answer this question, a clonal analysis strategy was used. Genotypes were constructed that allowed the removal, by mitotic recombination induced by X-irradiation, of one of the two X chromosomes from an XX cell at different times in development. The results demonstrated that the X0 clones induced at around the blastoderm stage survive and differentiate male structures, while clones induced later in development die. However, the X0 clones survive and differentiate male structures if they carry a loss-of-function *Sxl* mutant allele, independent of

the time in development that the clones are induced. These results indicate that the X/A signal irreversibly sets, in a cell-autonomous manner, the state of activity of *Sxl* sometime around the blastoderm stage. Once this is achieved, the X/A signal is no longer needed and the activity of *Sxl* remains fixed (Sánchez and Nöthiger, 1983; Bachiller and Sánchez, 1991).

The capacity of the *Sxl* gene to function as a stable switch is due to a positive autoregulatory function of *Sxl* (Cline, 1984; Bell *et al*., 1991). Genetic evidence for this autoregulatory function came from the discovery of a new class of *Sxl* mutations that affected the sex determination process but still retained enough *Sxl* function to properly control dosage compensation. These *Sxl* mutant alleles showed the property that they do not need the maternal Da product for its expression in the zygote. In addition, they had the capacity of acting in *trans* causing the activation of a wild-type Sxl^+ allele, which otherwise would not be activated because of the absence of maternal Da product (Cline, 1984). The posterior cloning and molecular characterization of *Sxl* demonstrated that this positive autoregulation is due to the requirement of the Sxl protein in the female-specific splicing of its own primary transcript (Bell *et al*., 1991).

The gene *Sxl* controls the expression of two independent sets of regulatory genes (Lucchesi and Skripsky, 1981). The sex determination genes form one set, and mutations in these genes affect sex determination while having no effect on dosage compensation. The other set of genes is formed by the generically called *male-specific lethal* (*msl*) genes; mutations in these genes affect dosage compensation whilst having no effect on sex determination.

This chapter is focused on sex determination in the somatic component of the animal (reviews: Cline and Meyer, 1996; Schütt and Nöthiger, 2000). The germline exhibits sexual dimorphism, as does the somatic tissue. Cells with the 2X;2A chromosomal constitution will follow the oogenic pathway, while XY;2A cells will develop into sperm. Sex determination in the germline will not be reviewed here, and the interested reader is referred to the reviews of Cline and Meyer (1996) and Oliver (2002). Finally, Lucchesi reviews the dosage compensation process elsewhere in this Encyclopedia (see **Chapter 1.7**).

1.1.1.2. The X/A Signal and the Activation of Gene *Sex-lethal*

1.1.1.2.1. Genetic basis of the X/A signal The identification of a set of genes involved in the initial step of *Sxl* activation indicates that a conventional genetic system is at the basis of the X/A signal. The isolation of X-linked genes involved in the formation of the X/A signal was approached by selection of sex-specific lethal mutations. This selection was based on the prediction that any relevant X-linked genes should function as numerator elements of the X/A signal. A numerator element should display several properties:

1. Reduction of its zygotic doses should specifically be lethal to 2X;2A flies (females) as a consequence of a failure to activate *Sxl*, which causes a hypertranscription of the two X chromosomes. This female-specific lethality should be suppressed by Sxl^M mutations. These are constitutive mutations that express female *Sxl* function independently of the X/A signal (Cline, 1978).
2. Increase of its zygotic doses should specifically be lethal to X;2A flies (males), because *Sxl* is inappropriately activated, leading to an alteration of dosage compensation. This male-specific lethality should be suppressed by loss-of-function *Sxl* mutations.
3. The activation of *Sxl* requires the maternal Da product (Cline, 1978). Therefore, mutations and any X-linked numerator of the X/A signal and a lower amount of maternal Da product are expected to display female-specific lethal synergistic interaction. Such interaction is also expected between mutations in different X-linked numerator genes of the X/A signal. In both cases, female lethality should be suppressed by Sxl^M mutations.
4. A variation of the zygotic doses of X-linked numerator genes should alter the sexual phenotype of triploid intersex (2X;3A) flies. An increase should feminize, whereas a reduction should masculinize these individuals.

The expected properties for an autosomal denominator gene of the X/A signal are the opposite of those expected for an X-linked numerator gene.

Genetic analyses have identified a set of zygotic and maternal genes that form the X/A signal. They fall into three classes. First, the X-linked numerator genes *scute* (*sc*, also called *sisterless-b, sis-b*) (Torres and Sánchez, 1989; Parkhurst *et al*., 1990; Erickson and Cline, 1991; Torres and Sánchez, 1991), *sisterless-a* (*sis-a*) (Cline, 1986), *unpaired* (*upd*, also called *sisterless-c, sis-c*) (Jinks *et al*., 2000; Sefton *et al*., 2000), and *runt* (*run*) (Duffy and Gergen, 1991; Torres and Sánchez, 1992). Second, the gene *deadpan* (*dpn*), the only autosomal denominator gene of the X/A signal that has been found so far (Younger-Shepherd *et al*., 1992). Finally, the maternal effect genes are *daughterless* (*da*) (Cline, 1978), *hermaphrodite* (*her*) (Pultz and

Baker, 1995), *extramacrochaetae (emc)* (Younger-Shepherd *et al*., 1992) and a gene(s) located in chromosomal region 7D10;7F12 of the chromosome (Sánchez *et al*., 1994).

Not all of the genes involved in early *Sxl* activation play the same role. Among the X-linked numerator genes required for *Sxl* activation, only the *sc* and *sis-a* genes fulfil all criteria for numerator elements of the X/A signal. The autosomal gene *dpn* is the single known denominator element. The X-linked genes *run* and *upd* also participate in the X/A signal, yet they do not play the predominant role played by *sc* and *sis-a*. Thus:

1. Simultaneous duplications of *run* and *sc* show very low *Sxl*-dependent male-specific lethality (Torres and Sánchez, 1992) compared with the lethal effect produced by *sis-a* and *sc* duplications (Cline, 1988; Torres and Sánchez, 1989).
2. The gene *run* is only expressed in the trunk region of the embryo, whereas *Sxl* is activated in every cell of the embryo (Duffy and Gergen, 1991).
3. The majority of triploid intersexes heterozygous for *run* show normal male terminalia though some of them show a mixture of male and female structures (Duffy and Gergen, 1991; Torres and Sánchez, 1992). In contrast, all triploid intersexes heterozygous for *sc*, for example, show completely normal male terminalia (Torres and Sánchez, 1992). In the case of triploid intersexes heterozygous for *sis-a*, the degree of masculinization is almost complete (Cline, 1986). The terminalia are produced by the genital disc, and this is the most sexual dimorphic region of the fly (see below).
4. While mutations in *sis-a* and *sc* strongly down-regulate Sxl_{Pe}*::lacZ* expression, removal of *upd* activity or its downstream components has a significantly weaker effect, and residual Sxl_{Pe}*::lacZ* expression is still seen in most mutant female embryos (Jinks *et al*., 2000; Sefton *et al*., 2000).
5. The effect of upd^+ transgenes is comparable to that of a chromosomal duplication of upd^+, but is smaller than that seen for extra doses of $sis\text{-}a^+$ or sc^+. Eliminating *upd* activity reduces expression of Sxl_{Pe} but to a lesser extent than eliminating *sis-a* or *sc* (Sefton *et al*., 2000).
6. The female-specific lethal synergistic effect between *run* and a deficiency of *upd* is weaker than the one between *run* and either *sc* or *sis-a*, or that between *upd* and either *sc* or *sis-a* (Sánchez *et al*., 1994, 1998).
7. Males doubly duplicated for a chromosomal region carrying *upd* and either *sc* or *sis-a* are as viable as their brothers carrying only one of the duplications. This means that there is no male-specific lethal synergism between duplications at *upd* and either *sc* or *sis-a* (Sánchez *et al*., 1994).

1.1.1.2.2. Molecular nature of the X/A signal The X/A signal acts on the early *Sxl* promoter and controls *Sxl* expression at the transcription level (Torres and Sánchez, 1991; Keyes *et al*., 1992). By definition, the X/A signal is strictly zygotic and the numerator X-linked products play the key role in forming this signal since the autosomal and maternal products are equally present in the male and female zygotes. Concerning the molecular nature of the X/A signal, only those products are considered for which a clear role has been found.

The Sc (Villares and Cabrera, 1987; Murre *et al*., 1989), Da (Cronmiller *et al*., 1988; Murre *et al*., 1989), and Dpn (Bier *et al*., 1992) proteins contain a basic helix–loop–helix domain (bHLH). The HLH domain allows these proteins to form homodimers or heterodimers, while the basic domain is required for their binding to specific DNA sequences (Murre *et al*., 1989). Emc also belongs to the HLH family but is devoid of a basic domain (Ellis *et al*., 1990; Garrell and Modolell, 1990). SisA is a basic leucine-zipper (bZIP) protein (Erickson and Cline, 1993). Members of the bHLH and bZIP protein families are usually involved in transcriptional regulation (review: Massari and Murre, 2000).

In vitro methods and yeast two-hybrid assays have allowed the analyses of protein–protein interactions. Experimental evidence supports the formation of the following homo- and heterodimers: Sc–Da (Cabrera and Alonso, 1991; Van Doren *et al*., 1991; Deshpande *et al*., 1995; Liu and Belote, 1995); SisA–Da (Liu and Belote, 1995); SisA–Dpn (Liu and Belote, 1995); Dpn–Dpn (Winston *et al*., 1999); Emc–Sc and Emc–Da (Van Doren *et al*., 1991). No interaction has been observed between Sc and Dpn (Deshpande *et al*., 1995; Liu and Belote, 1995) or between Sc and SisA (Liu and Belote, 1995). Nothing is known about the formation of higher-order multimers.

1.1.1.2.3. Role of the products that form the X/A signal The molecular characterization of the X/A signal components has clarified how they act on the early *Sxl* promoter (see **Figure 1**). The Sc and maternal Da products form complexes that induce early *Sxl* transcription by binding to a set of regulatory sites called E-boxes (Massari and Murre, 2000) within the early *Sxl* promoter (Estes *et al*., 1995; Hoshijima *et al*., 1995; Yang *et al*., 2001). The Emc protein forms heterodimer complexes

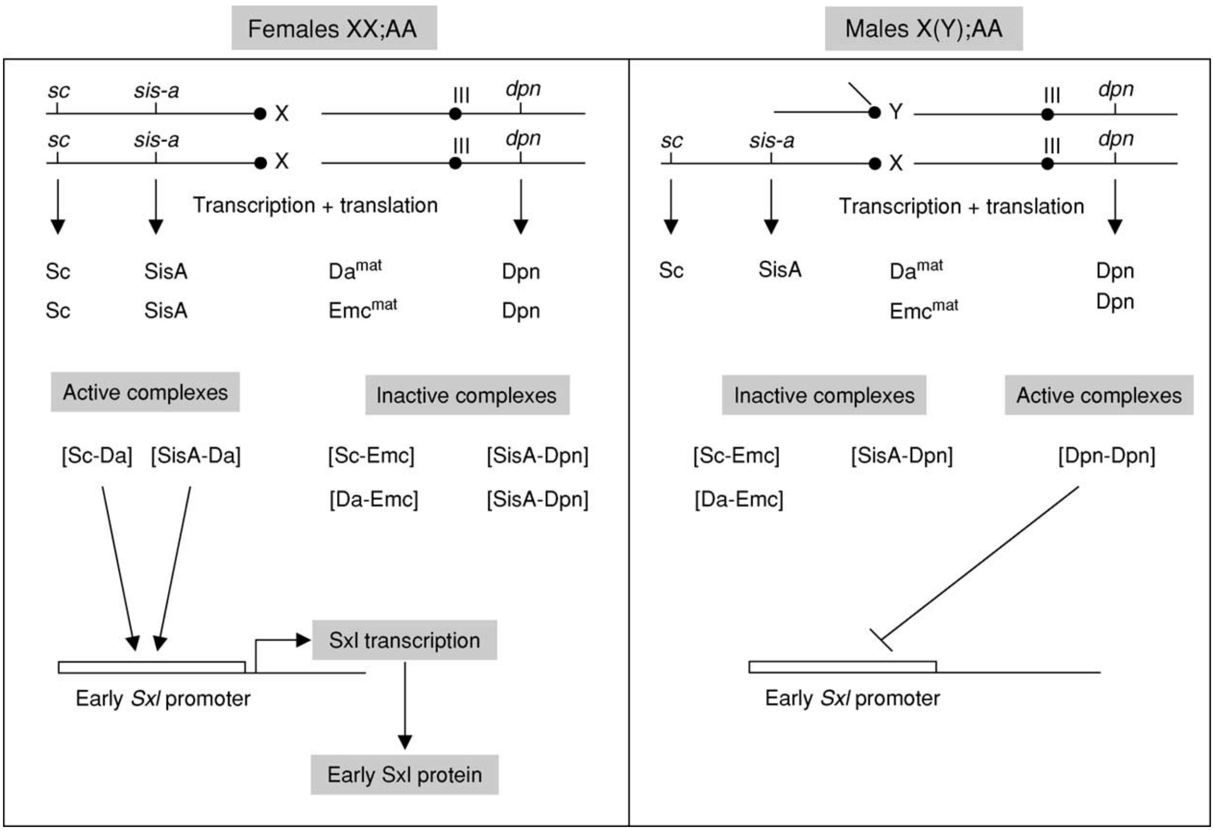


Figure 1 Molecular basis of the X/A signal. The signal represents the ratio between activator and repressor complexes of the early *Sxl* promoter. For simplicity, it is assumed that in females (XX;AA), activators but not repressors are produced, whereas in males (X;AA), repressors but nor activators are formed, so that *Sxl* is only activated in females. Also, for the sake of clarity, representatives of each of the three classes of genes are chosen – X-linked zygotic, autosomal zygotic, and maternal genes – involved in early *Sxl* activation. X, X chromosome; III, third autosome; Y, Y chromosome. See text for explanation.

with either Sc or Da that are unable to bind to DNA (Davis *et al.*, 1990; Voronova and Baltimore, 1990). Therefore, Emc prevents the formation of active Sc–Da complexes.

The SisA protein is required for efficient induction of early *Sxl* transcription (Erickson and Cline, 1991; Estes *et al.*, 1995) although specific binding sites for this protein on the early *Sxl* promoter have yet to be determined. Since the formation of SisA–Da (Liu and Belote, 1995) and SisA–Dpn (Liu and Belote, 1995) complexes has been reported, it is possible that SisA exerts a dual function: on one hand, it forms SisA–Da complexes that activate *Sxl*, and on the other, it sequesters Dpn through the formation of inactive SisA–Dpn complexes. Consequently, it lowers the amount of Dpn that remains free and capable of forming Dpn–Dpn dimers, which repress *Sxl* transcription by binding to a pair of adjacent regulatory sites in the early *Sxl* promoter (Estes *et al.*, 1995; Hoshijima *et al.*, 1995).

1.1.1.2.4. The organization of the early *Sxl* promoter The molecular organization of the early *Sxl* promoter has been determined (Estes *et al.*, 1995; Hoshijima *et al.*, 1995; Yang *et al.*, 2001). It has been shown that a fragment of 1400 bp upstream of the early *Sxl* transcription initiation site contains all the *cis*-acting sequences required for the control of early *Sxl* expression by the X/A signal. Deletion experiments suggest that the upstream distal region (−1.4 to −0.8 kbp) is not essential for sex specificity. In contrast, the proximal region (−390 bp to the transcription initiation site) is necessary and sufficient for transcriptional induction in presence of sufficient amounts of activators. The ostensible function of the distal region is to enhance transcription once it has been initiated by the proximal region; control of early *Sxl* transcription is mainly exerted through the proximal region (Yang *et al.*, 2001).

Binding sites controlled by the repressor have been termed D-boxes. Two D-boxes that bind Dpn dimers lie upstream and close to the transcription initiation site (Hoshijima *et al.*, 1995; Yang *et al.*, 2001). Due to their location close to the transcription start site, binding of the D-boxes is very

likely to inhibit the formation of the transcription machinery.

1.1.1.2.5. The action of the X/A signal on the early *Sxl* promoter The X/A ratio is primarily transduced by the relative amount of positive and negative regulators of *Sxl* (Parkhurst *et al*., 1990; Parkhurst and Ish-Horowicz, 1992). Although early Sxl protein has not been detected in males, suggesting that *Sxl* remains silent in males, it is still possible that *Sxl* transcription occurs in both sexes, but much more efficiently in females (activators outweigh repressors) than in males (repressors outweigh activators). As a result, early Sxl protein is abundantly produced in females whereas it remains undetectable in males.

To explain in molecular terms how the X/A signal could exert its function, for simplicity it is considered that the X/A signal is such that, in females, activators but not repressors are formed whereas in males (X;2A), repressors but not activators are produced, so that early *Sxl* transcription occurs in females, whereas in males this gene remains silent (**Figure 1**). Since the interactions between some of the positive and negative regulators of *Sxl* have been shown, their relative abundance is regulated by a titration mechanism, where the existence of sequestering complexes like Sc–Emc and Da–Emc lowers the availability of free Sc and Da proteins for assembly into active complexes. Alternatively, the formation of SisA–Dpn would lower the amount of free Dpn and the formation of Dpn–Dpn repressors. Because the autosomal zygotic Dpn and the maternal Da and Emc are present in equal amounts in the two sexes, they have no discriminative power in sex determination. The sole difference between males (X;2A) and females (XX;2A) is the number of X chromosomes and, thus, the dosage of the X-linked gene products Sc and SisA. In females, the two doses of *sc* and *sis-a* produce sufficient Sc and SisA products to form Sc–Da and SisA–Da activator complexes, whereas negative Dpn–Dpn complexes are not formed due to the sequestration of Dpn by SisA into inactive SisA–Dpn complexes. Consequently *Sxl* is activated (**Figure 1**). In males, the amount of Sc and SisA products is half of that found in females. Emc sequesters Sc and Da, thus preventing formation of activator complexes. In addition, no SisA is available to sequester Dpn, so that the negative Dpn–Dpn complex is formed that prevents activation of *Sxl*.

As mentioned above, other genes are also known to be required for early activation of *Sxl*:

1. Experimental evidence supports the idea that Run activates *Sxl* transcription by direct interaction with the early *Sxl* promoter. First, Run-binding sites have been identified in the early *Sxl* promoter. Second, a mutant Run protein that cannot bind DNA fails to activate the early *Sxl* promoter. Finally, a modified Run protein containing a heterologous transcription activation domain activates rather than represses the early *Sxl* promoter, indicating that Run does not activate *Sxl* indirectly by repressing a *Sxl*-repressor. Nevertheless, it is not possible to exclude the possibility that the binding of Run to the early *Sxl* promoter interferes with the binding of a *Sxl* repressor (Kramer *et al*., 1999).
2. Both Upd and downstream components – the JAK kinase and the signal transducer and activator of transcription (STAT) – are needed for proper early activation of *Sxl* (Jinks *et al*., 2000; Sefton *et al*., 2000). Interestingly, the X/A signal acts cell-autonomously: the sexual pathway chosen by each cell depends on the cell's chromosome constitution (X/A ratio). This is clearly observed in gynandromorphs (mosaic individuals with portions of the body typically male and others typically female) and in clones where the X/A ratio of the cells has been changed (see above). The question arises as to the function of the JAK/STAT signaling pathway (a noncell-autonomous component) in a cell-autonomous decision process. It has been hypothesized that JAK/STAT signaling between adjacent cells may constitute a mechanism for stochastic compensation of differences in the activation of the early *Sxl* promoter by the X/A signal (Jinks *et al*., 2000). Indeed, gene transcription is a process of an intrinsically probabilistic nature (Zlokarnik *et al*., 1998; Fiering *et al*., 2000; Hume, 2000).
3. The maternal Her product is also necessary for early *Sxl* activation (Pultz and Baker, 1995), although it is not known whether specific binding sites for this protein exist in the early *Sxl* promoter or whether the protein forms complexes with any of the other positive regulators of *Sxl*.

1.1.1.2.6. Developmental meaning of the X/A signal The gene *Sxl* produces two temporally-distinct sets of transcripts corresponding to the function of its two promoters (**Figure 2**). The early set is produced as a response to the X/A signal, which controls *Sxl* expression at the level of transcription. The early promoter functions only around the blastoderm stage, when the X/A signal is formed. Later on, the late promoter starts functioning at the same level in both sexes and remains active throughout development and during adult life in both males and

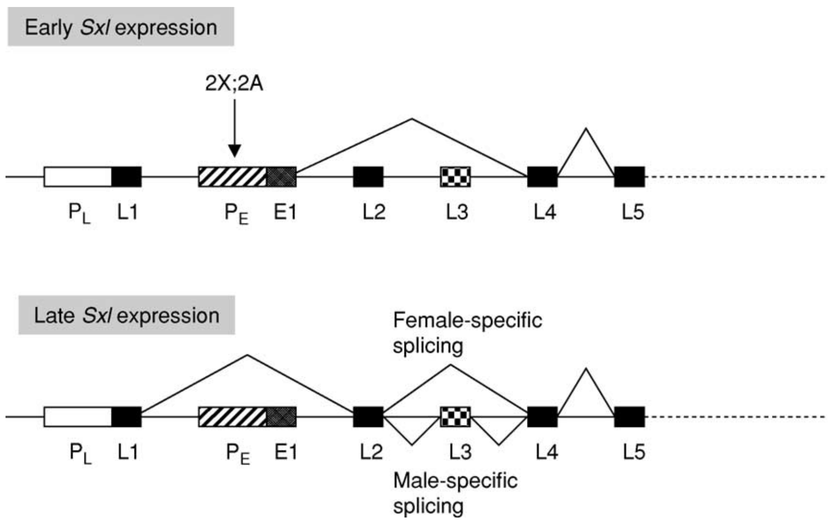


Figure 2 Scheme showing the two expression phases of *Sxl* related to its two promoters. P_L and P_E indicate the late and early promoters. E1 and L1–L5 indicate exons. The boxes and the thin lines represent exons (E1 and L1–L5) and introns, respectively. There are more exons (dotted line) that are included in the early and late *Sxl* mRNAs but these are not involved in the sex-specific and temporally specific splicing regulation of *Sxl* primary transcripts. Note that the early *Sxl* pre-mRNA follows a unique splicing pathway, whereas the late *Sxl* pre-mRNA follows two alternative splicing pathways.

females. In this case, the control of *Sxl* expression occurs by sex-specific splicing of its primary transcript. Male and female processed transcripts differ by the inclusion or exclusion of a male-specific exon (exon L3), which introduces a stop codon in the open reading frame, giving rise to a truncated, nonfunctional Sxl protein. In females, this exon is spliced out and normal, functional Sxl protein is produced (Bell *et al*., 1988; Bopp *et al*., 1991). Female-specific splicing of late *Sxl* pre-mRNA requires the presence of the functional Sxl product, and this constitutes the *Sxl* autoregulatory function (Bell *et al*., 1991).

The early and late *Sxl* transcripts are distinct not only because of the different promoters used for their generation but, also, because of the different splicing pathways they follow (**Figure 2**). The early *Sxl* primary transcripts follow a fixed splicing pattern, where exon L2 and the male-specific exon L3 are excluded, and with the early specific exon E1 being directly spliced to exon L4. Exon L4 and the exons downstream from it are present in both early and late *Sxl* mRNAs. Splicing of the early *Sxl* primary transcript does not require the presence of the Sxl protein (Horabin and Schedl, 1996).

The developmental meaning of the X/A signal is related to the existence of the two *Sxl* gene promoters. In females, early Sxl protein is abundantly produced, because the X/A signal (activators prevail over repressors) efficiently activates the early *Sxl* promoter (**Figure 3**). After the blastoderm stage, when the late *Sxl* promoter begins to function, the abundant early Sxl protein directs the first *Sxl* pre-mRNA to the female-specific splicing pathway that gives rise to late functional Sxl protein, which subsequently ensures that the female-specific splicing state of *Sxl* is set up. In males, early Sxl protein is scarcely produced because the X/A signal (repressors prevail over activators) does not activate efficiently the early *Sxl* promoter. Consequently, when the late *Sxl* promoter starts functioning in males, the first *Sxl* pre-mRNA follows the male-specific splicing pathway that produces a truncated, nonfunctional Sxl protein. The result is the establishment of the male-splicing state of *Sxl*.

Thus, the developmental meaning of the X/A signal is to provide the cells, at the beginning of development, with a sufficient quantity of early Sxl protein, which ascertains the female-splicing control of late *Sxl* pre-mRNA producing functional Sxl protein.

1.1.1.3. The Sex Determination Genetic Cascade

The epistatic relationships between the sex determination genes revealed that a hierarchical interaction exists among these genes (Baker and Ridge, 1980). The characterization of these genes showed that their expression during development is controlled by sex-specific splicing of their primary transcripts: the product of a gene controls the sex-specific splicing of the pre-mRNA of the downstream gene in the genetic cascade.

Sxl is at the top of this cascade: its product controls the splicing of its own pre-mRNA and the splicing of the pre-mRNA from the downstream gene *transformer* (*tra*). The Tra product and the

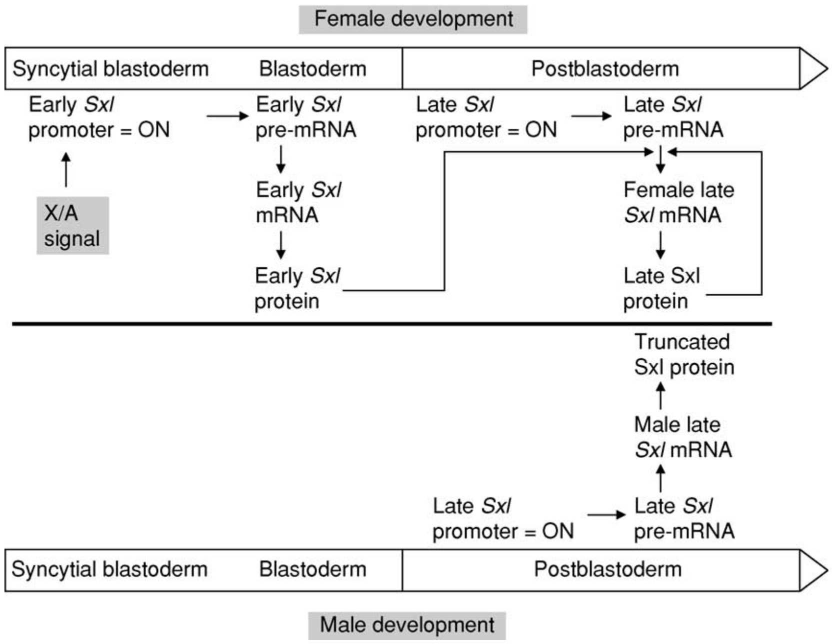


Figure 3 Scheme showing the developmental meaning of the X/A signal. For simplicity, it is assumed that the X/A signal is only produced in females. See text for details.

product of the constitutive gene *transformer-2* (*tra-2*) control the sex-specific splicing of pre-mRNA from gene *doublesex* (*dsx*), which is transcribed in both sexes but gives rise to two different proteins, Dsx^F and Dsx^M, in females and males, respectively. The genes *intersex* (*ix*) and *her* are expressed in both sexes and in females, their products assist Dsx^F to activate female terminal differentiation and to repress male terminal differentiation, whereas Her helps Dsx^M to activate male terminal differentiation and to repress female terminal differentiation (**Figure 4**).

The gene *dsx* is the last gene in the genetic cascade that controls the development of sexual somatic characters, yet there are exceptions. These concern the sex-specific characteristics of the central nervous system and the neuronal induction of a male-specific abdominal muscle, which depend on the function of *tra* and not *dsx* through the genes *fruitless* and *dissatisfaction* (**Figure 4**). The gene *takeout*, which is expressed in the fat body tissue, is closely associated with the adult brain function and is involved in male courtship behavior (Dauwalder *et al.*, 2002). The determination of sexual behavior will not be reviewed here. The interested reader is referred to other reviews on the subject (Yamamoto *et al.*, 1998; O'Kane and Asztalos, 1999; Billeter *et al.*, 2002).

1.1.1.3.1. Control of *Sxl* expression by sex-specific splicing of its primary transcript The mechanism by which Sxl precisely controls the skipping of its male-specific exon L3 is not totally understood. In contrast to most examples of exon-skipping events described in the literature, Sxl promotes a 100% switch between the two alternatively spliced forms of its own pre-mRNA, suggesting the existence of a complex mechanism of regulation. The role of the Sxl protein in the splicing of its own pre-mRNA is highlighted in brief. More extensive details have been reviewed in Penalva and Sánchez (2003).

The *Sxl* gene encodes one of the best-characterized members of the family of RNA-binding proteins. It has two segments, located in tandem in the central portion, with similarity to the RNA-binding domain sequences (RBD) or RNA recognition motifs (RRM). The amino terminus of Sxl is very rich in glycine. It has been shown that this domain is implicated in protein–protein interactions (Sxl multimerization) and absolutely required for proper control of *Sxl* RNA alternative splicing (Wang and Manley, 1997; Waterbury *et al.*, 2000; Lallena *et al.*, 2002). Deletions of the N- and C-termini does not interfere with the ability of the Sxl RBDs to properly bind *in vitro* to their target sequences, while both RNA-binding domains are required for site-specific RNA binding (Wang and Bell, 1994; Kanaar *et al.*, 1995; Sakashita and Sakamoto, 1996; Samuels *et al.*, 1998).

The Sxl-binding sequence consists of long stretches of poly(U) interrupted by two to four guanosines (Sosnowski *et al.*, 1989; Inoue *et al.*, 1990;

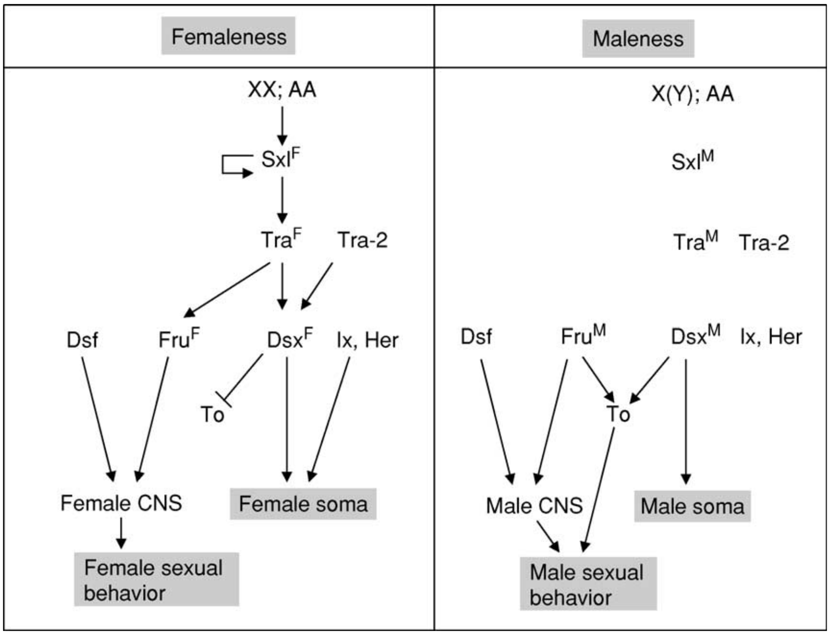


Figure 4 Sex determination gene network. Sxl^F and Sxl^M stand for functional female and nonfunctional truncated male Sxl protein, respectively. Tra^F and Tra^M stand for functional female and nonfunctional truncated male Tra protein, respectively. Dsx^F and Dsx^M stand for functional Dsx protein in females and males, respectively. Fru^F and Fru^M stand for functional Fru protein in females and males, respectively. Dsf is *dissatisfaction*. To is *takeout*. In the absence of the X/A signal in males, the default state corresponds to the production of Sxl^M, Tra^M, and Dsx^M proteins. Ix and Her proteins are produced in both sexes. CNS, central nervous system. For description of the genes and their regulation see text.

Sakamoto *et al.*, 1992; Horabin and Schedl, 1993a, 1993b; Sakashita and Sakamoto, 1994; Samuels *et al.*, 1994; Singh *et al.*, 1995; Wang and Manley, 1997). It has been observed that Sxl binds to RNAs containing two or more poly(U) sequences in a cooperative manner (Wang and Bell, 1994; Kanaar *et al.*, 1995). Different models have been proposed for the molecular mechanism underlying the role of Sxl protein in controlling the female-specific splicing of its own pre-mRNA (Sakamoto *et al.*, 1992; Horabin and Schedl, 1993a, 1993b; Wang and Bell, 1994; Wang and Manley, 1997; Waterbury *et al.*, 2000; Penalva *et al.*, 2001; Lallena *et al.*, 2002; Nagengast *et al.*, 2003). Nevertheless, there is a common aspect to all of them, namely, the fact that the Sxl protein participates in the skipping of the male-specific exon L3 (female-specific splicing of *Sxl* pre-mRNA) through binding to multiple U-rich sequences placed in introns 2 and 3, surrounding the male-specific exon L3 (**Figure 5**). Multiple poly(U) sequences are also present in the adjacent introns of the male-specific exon L3 of the *Sxl* genes of *D. virilis* (Bopp *et al.*, 1996) and *D. subobscura* (Penalva *et al.*, 1996).

The female-specific splicing of late *Sxl* pre-mRNA requires, in addition to the Sxl protein, the function of other genes, such as *sans fille* (*snf*) (Albrecht and Salz, 1993; Flickinger and Salz, 1994; Salz and Flickinger, 1996; Samuels *et al.*, 1998), *female-lethal-2-d* (*fl(2)d*) (Granadino *et al.*, 1990, 1992; Penalva *et al.*, 2000; Ortega *et al.*, 2003), and *virilizer* (*vir*) (Hilfiker and Nöthiger, 1991; Hilfiker *et al.*, 1995; Niessen *et al.*, 2001). These genes, however, do not play any role in the splicing pattern of early *Sxl* transcripts (Horabin and Schedl, 1996).

1.1.1.3.2. *Sxl* protein as its own translation repressor *Sxl* gives rise to a variety of transcripts, some of them stage- and tissue-specific (Salz *et al.*, 1989; Samuels *et al.*, 1991; Keyes *et al.*, 1992; Penalva *et al.*, 1996). The *Sxl* mRNAs also contain several Sxl-binding sites at the 3′ unstranslated region (UTR). It has been shown that the association of Sxl protein with the 3′ UTR of *Sxl* mRNA significantly decreases Sxl protein expression (Yanowitz *et al.*, 1999). Transcripts having a short 3′ UTR are more abundant in the germline and during the early stages of embryogenesis (5–8 h postfertilization) (Salz *et al.*, 1989). In later stages of development and during adult life, the most abundant transcripts have a long 3′ UTR (up to 3600 nt) containing several Sxl binding sites (13 in the largest transcript) (Samuels *et al.*, 1991).

It was speculated that the negative autoregulatory loop of *Sxl* might play an important homeostatic role and that the presence of short transcripts at

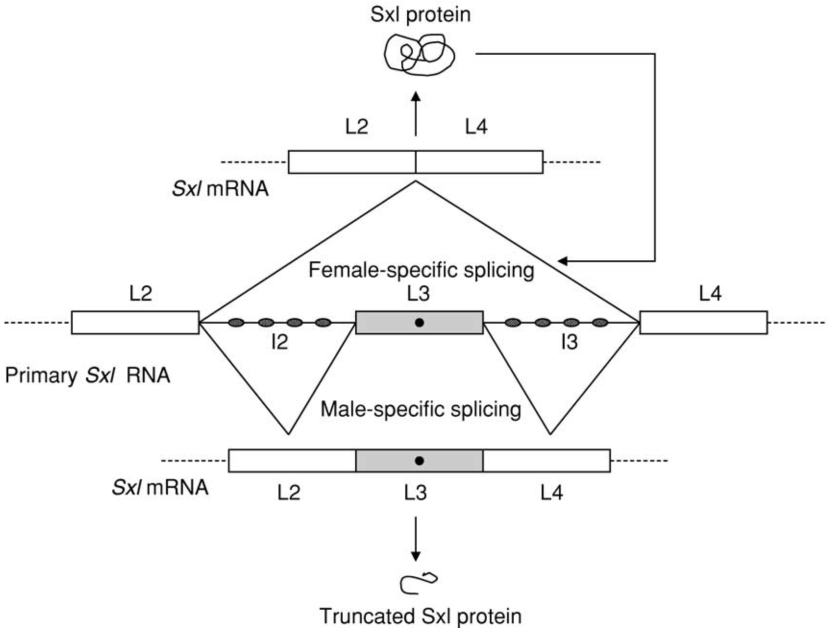


Figure 5 Sex-specific splicing of late *Sxl* pre-mRNA (autoregulatory *Sxl* function throughout development and adult life). Only the key exons (L2–L4) and introns (I2–I3) of the pre-mRNA are shown. The dot inside exon L3 represents translation stop codons. The hatched ellipsoids in introns I2 and I3 represent the sequences bound by the Sxl protein. Exons and introns are not drawn to scale and the numbers and distribution of Sxl-binding sequences are also not drawn to scale.

early stages of development allows a substantial amount of Sxl protein to be produced and a positive autoregulatory loop to be established via splicing. Once accumulation of the Sxl protein is no longer required, short mRNAs are substituted for larger ones whose expression can be repressed by Sxl. The balance between the negative and the positive posttranscriptional controls keeps the concentration of Sxl at levels that do not interfere with other cellular functions but are sufficient for regulation of *Sxl* splicing and splicing of its target genes (Yanowitz *et al*., 1999). Indeed, it has been shown that high levels of Sxl protein have toxic consequences for the cells (Meise *et al*., 1998; Saccone *et al*., 1998).

1.1.1.3.3. The role of transformer in sex determination The *tra* gene is transcribed in both sexes, but its RNA follows alternative splicing pathways (**Figure 6**). Intron 1 of *tra* has two alternative 3′ splice sites. A non-sex-specific transcript is generated when the proximal 3′ splice site is used. Use of this splice site introduces a stop codon in the open reading frame, leading to the production of a truncated, nonfunctional peptide. In females, approximately half of the *tra* pre-mRNA is spliced differently due to the intervention of the Sxl protein. In this case, the distal 3′ splice site is used. As a result, the stretch containing the termination codon is not included in the mature transcript and synthesis of full length Tra protein occurs (Boggs *et al*., 1987; Belote *et al*., 1989) (**Figure 6**).

It has been determined that Sxl regulates female-specific *tra* pre-mRNA splicing by a blockage mechanism rather than by enhancing the use of the female specific 3′ splice site (distal 3′ss). There is a Sxl binding site at the polypyrimidine tract of the non-sex-specific splice site (proximal 3′ss) (Sosnowski *et al*., 1989; Inoue *et al*., 1990). This region is also the binding site for the U2 auxiliary factor (U2AF), an essential splicing factor important for the recognition of the 3′ splice site. U2AF but not Sxl binds also to the polypyrimidine tract associated with the female-specific 3′ splice site, but with 100 times less affinity (Valcárcel *et al*., 1993). Chimeric proteins containing the effector domain of U2AF fused to the complete RNA binding domain of Sxl inhibit rather than promote splicing to the non-sex-specific 3′ splice site. This suggests that Sxl and U2AF compete for binding to the polypyrimidine tract associated with the non-sex-specific 3′ splice site. Binding of Sxl to this sequence displaces U2AF, diverting it to the low affinity distal polypyrimidine tract and promoting the usage of the female-specific 3′ splice site (Valcárcel *et al*., 1993; Granadino *et al*., 1997) (**Figure 6**).

The chimeric U2AF–Sxl protein, however, does not disrupt *Sxl* pre-mRNA splicing regulation, in

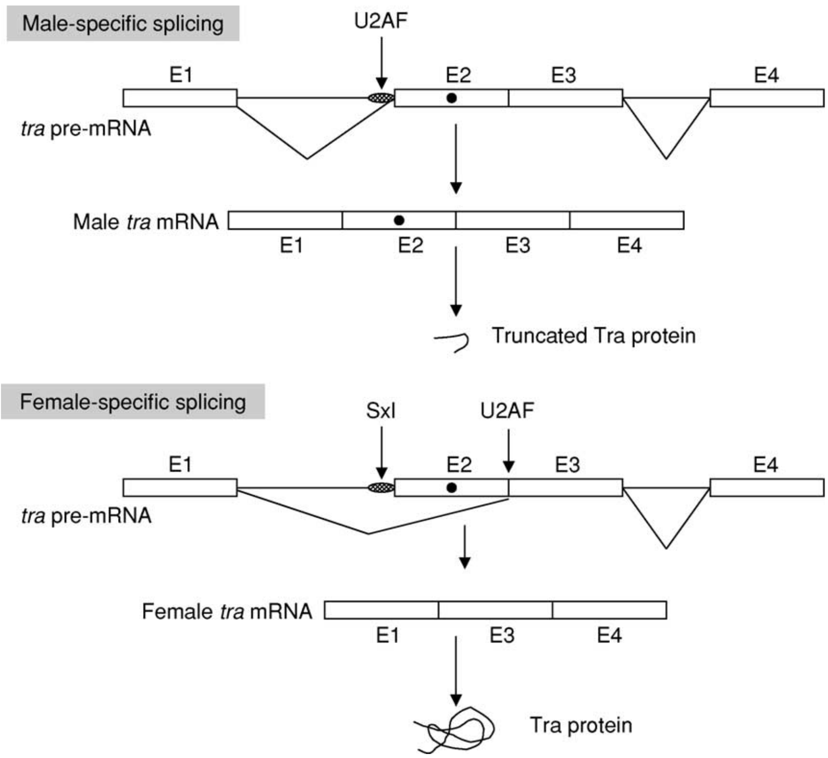


Figure 6 Sex-specific splicing of *tra* pre-mRNA. Boxes and thin lines represent exons (E1–E4) and introns, respectively. The dot inside exon E2 represents translation stop codons. The hatched ellipsoid in front of exon E2 represents the sequence to which both Sxl and U2AF proteins bind.

contrast to what occurs with *tra* splicing (Granadino *et al.*, 1997). These data suggest that Sxl controls *tra* and *Sxl* pre-mRNA alternative splicing by different mechanisms. Notwithstanding, there is controversy concerning the function of the N-terminal region in *tra* RNA alternative splicing regulation. While it has been proposed that this region is not necessary for *tra* regulation (Granadino *et al.*, 1997), others have proposed the opposite (Yanowitz *et al.*, 1999). Differences in how the different groups have performed the experiments, which include different constructs (40aa deletion of the N-terminal region in one case and 94aa in the other), use of different promoters, and different real-time polymerase chain reaction (RT-PCR) methods to detect the splice forms, might explain the discrepancy.

The genes *female-lethal-2-d* (*fl(2)d*) (Granadino *et al.*, 1996) and *virilizer* (*vir*) (Hilfiker *et al.*, 1995) are also required for female-specific splicing of the *tra* pre-mRNA. Genetic analyses have ruled out a direct role of *sans-fille* (*snf*) in *tra* pre-mRNA splicing (Cline *et al.*, 1999).

1.1.1.3.4. The role of *doublesex, hermaphrodite,* and *intersex* in sex determination At the bottom of the sex determination gene cascade are the genes *dsx*, *her*, and *ix*, which are expressed in both sexes.

The *dsx* pre-mRNA contains six exons: three common exons (E1–E3), a female-specific exon (E4), and two male-specific exons (E5–E6) (**Figure 7**). In females, the Tra^F protein, together with the constitutive Tra-2 protein (Goralski *et al.*, 1989; Amrein *et al.*, 1990; Mattox *et al.*, 1990), directs the splicing of the *dsx* pre-mRNA to the female mode. The mRNA produced contains exons E1, E2, E3, and E4 and gives rise to the female Dsx^F protein, which promotes female sexual development. In males, where no functional Tra protein is available, the *dsx* pre-mRNA follows the male mode of splicing that gives rise to mRNA containing exons E1, E2, E3, E5, and E6, which produces male Dsx^M protein, which promotes male sexual development (Burtis and Baker, 1989; Hoshijima *et al.*, 1991) (**Figure 7**). A further component that is crucial in the sex-specific splicing of *dsx* pre-mRNA, is the splicing enhancer *dsx*RE (*doublesex* repeat element) placed in the female-specific exon E4, 300 nt downstream of the 3′ splice site (review: Maniatis, 1991). Six copies of a 13 nt repeat together with a purine-rich element (PRE) located between repeats 5 and 6, make up the *dsx*RE enhancer (Burtis and Baker, 1989; Lynch and Maniatis, 1996).

The sequent of the female-specific 3′ splice site in intron 3 departs significantly from the consensus 3′

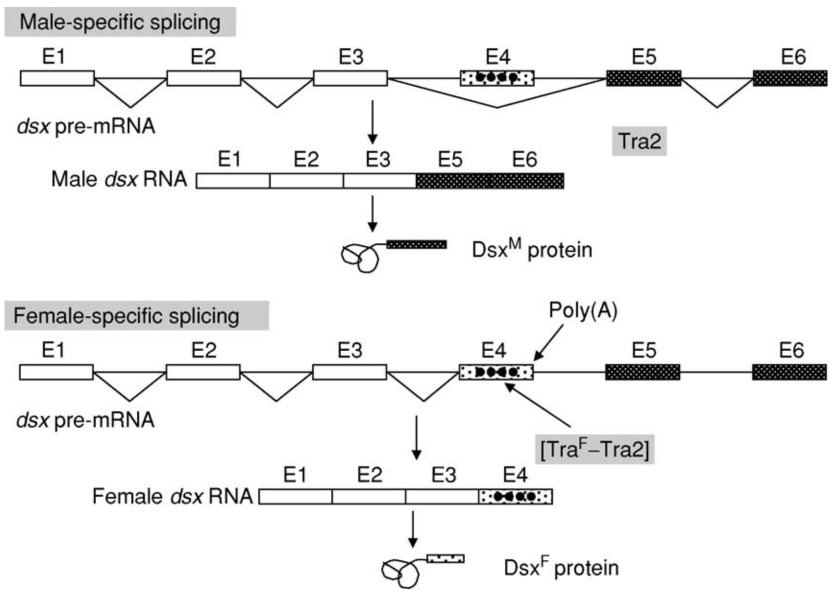


Figure 7 Sex-specific splicing of *dsx* pre-mRNA. Boxes and thin lines represent exons (E1–E6) and introns, respectively. The dots inside exon E4 represent the *dsx*RE enhancer to which the Tra^F–Tra2 complex binds. Poly(A) indicates the polyadenylation site of the mRNA arising from female-specific splicing.

splice site and is weaker than the 3′ splice site of the downstream intron 4. In males, the splicing machinery recognizes the 3′ splice site of intron 4 instead of the 3′ splice site of intron 3. Consequently, the male splicing follows and Dsx^M protein is produced. In females, however, because of the presence of Tra^F protein, Tra^F and Tra2, as well as the SR-protein RBP1, form a complex that binds to the *dsx*RE enhancer. This complex recruits U2AF and possibly other components of the general splicing machinery to the *dsx* pre-mRNA causing the splicing machinery to recognize the 3′ splice site of intron 3 instead of the 3′ splice site of intron 4. Consequently, female splicing follows and Dsx^F protein is produced (Hedley and Maniatis, 1991; Inoue *et al*., 1992; Tian and Maniatis, 1993, 1994; Zuo and Maniatis, 1996; Du *et al*., 1998; Nikolakaki *et al*., 2002). The activity and localization of the Tra^F, Tra-2, and RBD1 proteins depend on the activity of the LAMMER kinase encoded by the gene *Darkener of apricot* (*Doa*) (Du *et al*., 1998; Yun *et al*., 2000; Nikolakaki *et al*., 2002).

The two Dsx proteins, Dsx^F and Dsx^M, are transcription factors controlling the activity of the final target genes involved in sexual differentiation. The two proteins share the N-terminal domain, which contains a DNA-binding domain (DM domain), whereas they differ at their C-terminal domains, which endow these proteins with their specific function (Burtis and Baker, 1989; Hoshijima *et al*., 1991).

The *her* gene encodes a zinc finger protein, which endows to this protein the capacity to bind DNA, so that Her can function as a transcription factor (Li and Baker, 1998a). This agrees with the dual function shown by the *her* gene. Its maternal expression is necessary for early *Sxl* activation (see Section 1.1.1.2.5), while its zygotic expression is necessary for female terminal differentiation and some aspects of male terminal differentiation (Pultz and Baker, 1995; Li and Baker, 1998b).

The Dsx and Her products act either independently or interdependently to control different sexual differentiation processes. Thus, for example, the *yolk protein* (*yp*) genes *yp1* and *yp2* are the best-characterized female terminal differentiation genes. The expression of the *yp1* and *yp2* genes is under the control of gene *dsx* in the fat body (review: Bownes, 1994). Dsx^F and Dsx^M directly activate and inhibit the *yp* genes, respectively. They exert their function through the fat body specific enhancer (FBE). It has been found that in females, Dsx^F and Her independently activate the *yp* genes and that Her acts through sequences different than those of the FBE enhancer (Li and Baker, 1998b). In males, Dsx^M prevents the activation of *yp* genes by Her (Li and Baker, 1998b). However, the genes *dsx* and *her* also function interdependently to control the female differentiation of the sexual dimorphic foreleg bristles and the pigmentation of the 5th and 6th tergites (Li and Baker, 1998b).

The *ix* gene is transcribed in both sexes and its pre-mRNA does not follow a sex-specific splicing, indicating that the Ix protein is present in both sexes. Ix shows similarity to proteins thought to

act as transcriptional activators, although a DNA-binding domain has yet to be identified (Garrett-Engele *et al*., 2002). By means of the yeast-2 hybrid and coimmunoprecipitation analyses, it has been possible to demonstrate that Ix interacts with Dsx^F but not Dsx^M. In addition, by gel shift analysis it has been shown that Ix and Dsx^F form a DNA-binding complex (Garrett-Engele *et al*., 2002).

Ix also participates in the control of *yp* gene expression through the FBE enhancer (Garrett-Engele *et al*., 2002). Ectopic expression of Dsx^F in *ix* mutant males is not sufficient to induce expression of the *yp* genes, indicating that Dsx^F requires *ix* function to activate the *yp* genes (Waterbury *et al*., 1999). Collectively, these results suggest that Ix and Dsx^F form a complex to control female terminal differentiation.

1.1.1.4. Sex Determination Genes in Other Dipteran and Nondipteran Species

The search for genes homologous to sex determination genes of *D. melanogaster* has been undertaken. Among the genes that form the X/A signal, gene *sc* of *D. subobscura* (Botella *et al*., 1996) and gene *sis-a* of *D. pseudoobscura* and *D. virilis* (Erickson and Cline, 1998) have been characterized. These genes display a significant conservation in their structure and function.

The *Sxl* gene has also been characterized in different *Drosophila* species. The structure and sequence organization of *Sxl* of *D. virilis* (Bopp *et al*., 1996) and *D. subobscura* (Penalva *et al*., 1996) have been determined. Like in *D. melanogaster*, *Sxl* regulation occurs by sex-specific alternative splicing: the *Sxl* transcripts in males have an additional exon containing stop translation codons. The *Sxl* of *D. virilis*, however, is unusual due to the presence in males of an open reading frame, downstream of the last stop codon in the male-specific exon, which encodes an Sxl protein. This is identical to the female Sxl protein except for the first 25 amino acids of the amino terminal region, which are encoded by differentially spliced exons. The male Sxl protein is predominantly accumulated in the embryonic ectoderm, suggestive of a putative role in the development of the central nervous system (Bopp *et al*., 1996). Sxl protein was also detected in males of other species (*D. americana, D. flavomontana*, and *D. borealis*) of the *virilis* radiation (Bopp *et al*., 1996).

Outside the genus *Drosophila*, *Sxl* has been characterized in *Chrysomya rufifacies* (Müller-Holtkamp, 1995), *Megaselia scalaris* (Sievert *et al*., 1997, 2000), *Musca domestica* (Meise *et al*., 1998) and *Ceratitis capitata* (Saccone *et al*., 1998), which belong to the Brachycera Suborder, and in *Sciara ocellaris* (Ruiz *et al*., 2003) which belongs to the Nematocera Suborder. Particularly interesting is the case of *S. ocellaris*, where as in *D. melanogaster*, gender depends on chromosome constitution: females are XX and males are X0 (Gerbi, 1986). Further, dosage compensation in *Sciara* appears to be achieved by hypertranscription of the single male X chromosome (da Cunha *et al*., 1994). The *Sxl* gene of all these species shows two main properties. First, *Sxl* is not regulated in a sex-specific fashion. Second, the Sxl proteins of the nondropsophilid and drosophilid species show a great degree of conservation in the two RBD domains and the few amino acids that separate them (the linker region). However, a high degree of conservation is not found outside these two domains. Collectively, these results suggest that the *Sxl* gene may not play the master regulatory role in sex determination in the non-drosophilids, as is the case with the *Drosophila* genus. This further suggests that *Sxl* was coopted to become the master regulatory gene in sex determination and dosage compensation during the evolution of the *Drosophila* lineage.

The *tra* genes in the species that belong to the *melanogaster* group, *D. simulans, D. mauritiana*, *D. sechellia*, and *D. erecta* (O'Neil and Belote, 1992; Kulathinal *et al*., 2003), and *D. hydei* and *D. virilis* (O'Neil and Belote, 1992) have also been characterized. Its comparison with *tra* of *D. melanogaster* revealed an unusually high degree of divergence, yet the heterologous genes can rescue *tra* mutations in *D. melanogaster*. The gene *tra-2* of *D. virilis* has been characterized as well (Chandler *et al*., 1997). It encodes a set of protein isoforms analogous to those of *D. melanogaster*, and it can rescue the *tra-2* mutations in this species.

Outside the genus *Drosophila*, *tra* has been characterized in *C. capitata* (Pane *et al*., 2002). Like in *D. melanogaster*, alternative splicing regulates the *Ceratitis tra* gene, so that only females contain a full-length protein. In contrast to *Drosophila* where *Sxl* regulates *tra*, however, the *tra* gene of *Ceratitis* shows an autoregulatory function that produces functional protein specifically in females. Therefore, *tra* in *Ceratitis* constitutes a cellular memory devise that maintains the female developmental pathway.

The *dsx* genes of *Megaselia scalaris* (Sievert *et al*., 1997), *Bactrocera tryoni* (Shearman and Frommer, 1998), *Bombyx mori* (Ohbayashi *et al*., 2001; Suzuki *et al*., 2001), and *Musca domestica* (Hediger and Bopp, cited in Schütt and Nöthiger, 2000) and *C. capitata* (Saccone *et al*., cited in Pane *et al*., 2002) have been characterized as well. In these species, *dsx* encodes male- and female-specific RNAs, which encode putative male- and female-specific Dsx

proteins sharing the N-terminal region and differing at their C-terminal regions, like in *Drosophila*. Unlike the *Drosophila* case, however, the female-specific intron in *Bombyx dsx* does not show a weak 3′ splice site, and the Tra–Tra2 binding sequences (corresponding to the *dsx*RE enhancer in *Drosophila*; see Section 1.1.1.3.4) were not found. This suggests that the female-specific splicing of *dsx* pre-mRNA is the default splicing mode in *Bombyx*, in contrast to *Drosophila*, where the default splicing is male-specific.

Collectively, these results agree with the model of Wilkins (1995), who proposed that during evolution, the sex-determining cascades were built from bottom to top, with the genes at the bottom being more conserved than the more upstream genes in the cascade.

1.1.2. The Development of the Genital Disc of *Drosophila*

1.1.2.1. Historical Overview

The imaginal discs of *D. melanogaster* are good experimental models for the study of developmental processes (Nöthiger, 1972). Imaginal discs are formed by invagination from the embryonic ectoderm as pouches of cells and remain connected to the larval tissue until metamorphosis. They grow by cell division during the larval stages, whereby they acquire their patterning and typical shape. At the end of the larval period, each mature imaginal disc appears as an assembly of sets of spatially arranged cells with distinct determination states (patterning). These differentiate during metamorphosis as a response to hormonal stimulus (ecdysone). The result is the formation of the adult epidermal structures, such as the eye, antenna, leg, wing, and terminalia. Although these structures differ in appearance, the mechanisms controlling their growth and patterning, and even the genes involved, are very similar.

The genital disc forms the terminalia, which comprise the entire set of internal and external genitalia and analia, the most sexually dimorphic structures in the adult (**Figure 8**). This disc shows certain characteristics that distinguish it from other imaginal discs. There are two imaginal discs, one for the right and one for the left, for each of the adult appendage except for the terminalia, which are derived from a single genital disc. The cells that form the genital disc are recruited from different segments in the embryo (see Section 1.1.2.3.1), whereas the other discs (with the exception of the eye–antenna disc) are formed by cells derived from single segments. Because of these characteristics, the genital disc is well suited for studying developmental processes, such as the recruitment of cells to form complex structures and the control of patterning. Most importantly, however, the genital disc shows a unique feature. All imaginal discs except the genital one produce the same specific single adult appendage in both males and females. Thus, the eye, antenna, leg, wing, and haltere imaginal discs produce the corresponding adult structures in both sexes. In contrast, the genital disc has the potential to develop into either male or female terminalia, which are composed of different structures (**Figure 8**). Thus, the genital disc is a very good experimental model for the study of how sex determination genes, together with the homeotic ones – which determine cell identity – form an integrated genetic input that directs the development of a population of cells into two distinct, male or female, adult structures (reviews: Sánchez and Guerrero, 2001; Christiansen *et al*., 2002).

The complex organization of the genital disc was revealed through the analysis of gynandromorphs, flies composed of male (X0) and female (XX) cells. These animals allow the construction of fate maps concerning the relative position of the blastoderm founder cells for the different structures that compose the adult cuticle. The use of gynandromorphs to construct fate maps is based on an idea developed by Sturtevant (1929), who performed an analysis of gynandromorphs and calculated the frequencies with which two parts in the adult fly had different sexual phenotypes; i.e., whether they were formed by X0 or XX cells. The main conclusion was that the frequency with which the X0/XX border run between two parts was directly related to the distance of these parts in the adult and to the proximity of their founder blastoderm cells in the embryo (Janning, 1978).

Dobzhansky (1931) and Kröger (1959) used gynandromorphs for the first time to determine the origin of the sexually dimorphic structures derived from the genital disc (**Figure 8**). These authors assumed that the male and female terminalia were homologous; i.e., the cells that composed the genital disc primordium have the capacity of developing along two alternative, male or female, pathways. Thus, cells with a X0 genetic constitution formed male terminalia, whereas cells having an XX constitution produced female terminalia. Under this assumption, it was expected to find a set of gynandromorphs that would cover a whole spectrum of terminal structures, ranging from pure male to pure female terminalia, through cases displaying different mixtures of pure male and female structures. The prerequiste to be fulfilled was that in the

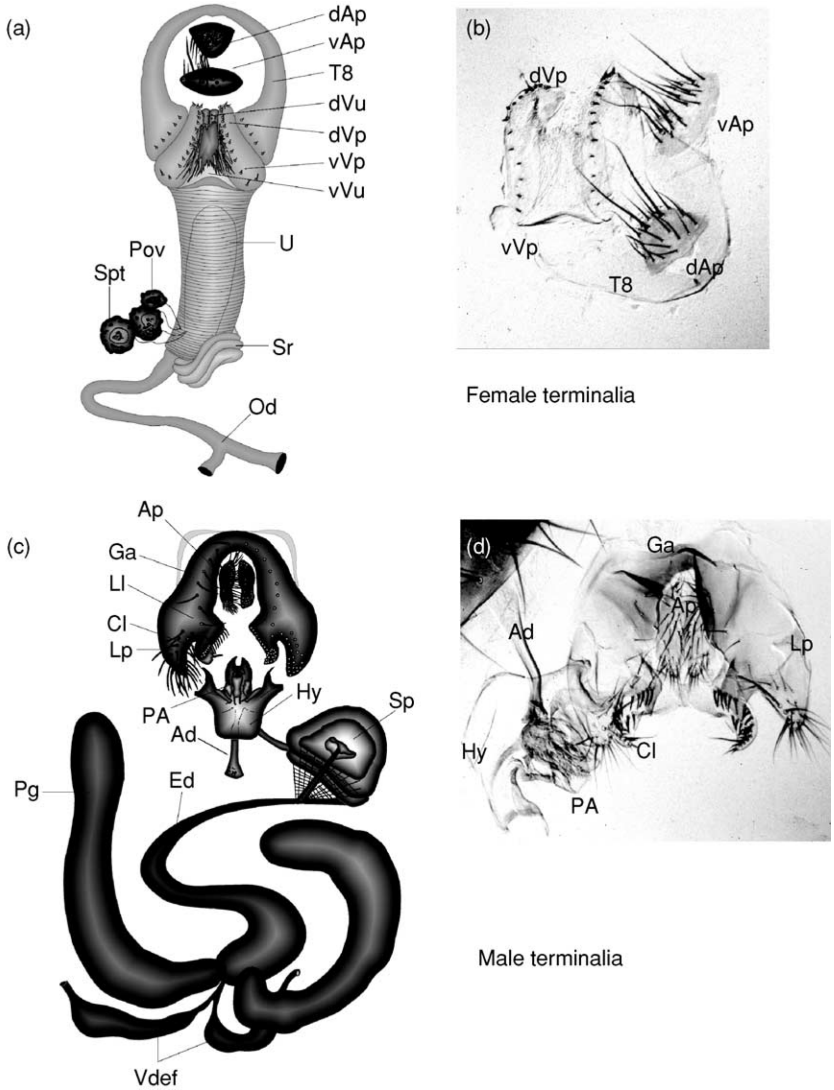


Figure 8 Scheme showing all the adult derivatives of the genital disc in both sexes ((a) female; (c) male) and photographs of external adult structures ((b) female; (d) male). External female terminalia: dAp, dorsal anal plate; vAp, ventral anal plate; T8, tergite eight; dVu, dorsal vulva; vVu, ventral vulva; dVp, dorsal vaginal plate; vVp, ventral vaginal plate. Internal female terminalia: U, uterus; Sr, seminal receptacle; Spt, spermatheca; Pov, parovaria; Od, oviduct (it is connected to the ovaries). Male external terminalia: Ap, anal plate; Ga, genital arch; Ll, lateral lobe; Lp, lateral plate; Cl, clasper; PA, penis apparatus; Ad, apodeme; Hy, hypandrium. Internal male terminalia: Ed, ejaculatory duct; Sp, sperm pump; Pg, paragonia (male accessory gland); Vdef, *vas deferens* (it is connected to the testes).

gynandromorphs the male and female structures should add up to a complete terminalia. However, Dobzhansly and Kröger noticed that this was not the case.

Nöthiger *et al.* (1977) addressed this question again more recently by analyzing the derivatives of mosaic genital discs of gynandromorphs. Their results constituted a benchmark in the comprehension of the organization and development of the genital disc. They found that, in the majority of the gynandromorphs, the male and female analia behave as expected if both were homologous and derived from a common population of cells: the male and female anal structures added up to a complete analia. In contrast, the male and female genitalia yielded different results, as any female external genital structure could be combined with any male external structure. Furthermore, gynandromorphs were found that either lacked completely an external genital structure or had two external genitalia – a complete set of male and female structures. These observations applied not only to the external terminalia but also to the internal genital structures. The data were explained by the proposal

that the genital disc is composed of three primordia: two separate primordia for the male and female genitalia, respectively, and a third one for the analia. The development of each genital primordium is either allowed or blocked depending on the chromosome constitution of their component cells. In X0 individuals, the male primordium develops to form the male genitalia and the development of the female primordium is blocked. In XX individuals, the female primordium develops to form the female genitalia, while the development of the male genital primordium is inhibited. However, the analia will always develop, giving rise to either male or female anal structures depending on the chromosome constitution of their cells, X0 or XX, respectively (but see below).

According to this hypothesis the relative position of the three primordia that form the genital disc would be such that the female genital primordia occupies the most anterior position, the anal primordium occupies the most posterior position, and the male genital primordium is placed in the middle. This organization was suggested by the appearance of gynandromorphs lacking genitalia and having a female analia, and gynandromorphs that had complete female and male genitalia as well as male analia. In both classes of gynandromorphs, the X0/XX border runs between the female and the male genital primordia (**Figure 9**). In the first class, X0 cells populate the female genital primordium, whereas XX cells populate the male genital and anal primordia. Consequently, the female and male genital primordia do not develop, whereas the anal primordium develops and produces a female analia. In the second class of gynandromorphs, the XX cells populate the female genital primordium whereas X0 cells populate the male genital and anal primordia. Consequently, the female genital primordium develops to produce complete female genitalia, whereas the male genital primordium develops and produces complete male genitalia, while the anal primordium develops and produces male analia.

The hypothesized organization of the three primordia in the genital disc was confirmed when the disc's embryonic origin was analyzed (Schüpbach *et al*., 1978). It was found that the genital disc arises from a group of cells in the ventral side of the embryo. Within this group, three regions can be distinguished that are present in both sexes: an anterior region that gives rise to the female genitalia, a medial region that gives rise to the male genitalia, and a posterior one that produces the analia. Collectively, these data support the model that the genital disc is composed of three primordia: the female genital primordium (FGP) located anteriorly, the anal primordium (AP) located posteriorly, and the male genital primordium (MGP) placed in between (**Figure 9**).

In each sex, only one of the two genital primordia develops depending on its sexual genotype, whereas the other remains in a so-called "repressed state," and

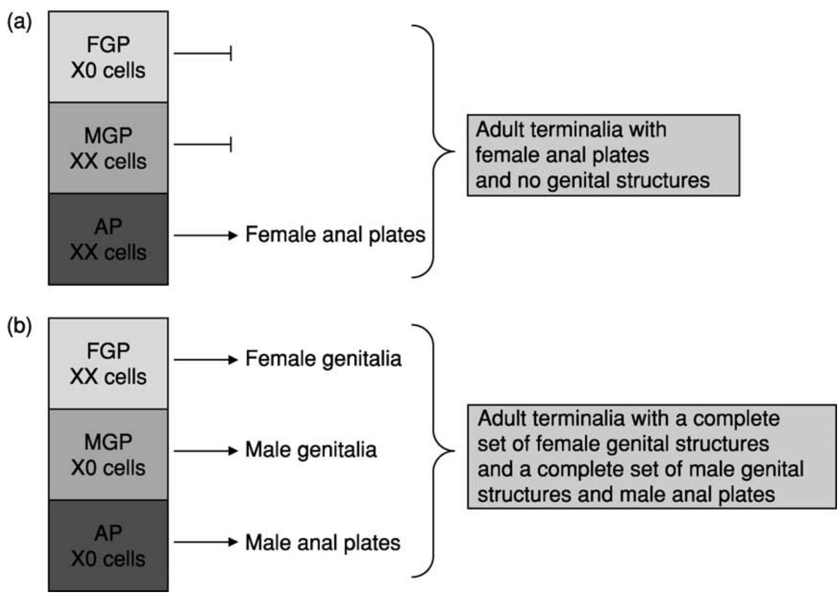


Figure 9 Gynandromorphs lacking genitalia and having female analia (a), and gynandromorphs that have complete female and male genitalia plus male analia (b). In both classes of gynandromorphs, the X0/XX border runs between the female and the male genital primordia. FGP, female genital primordium; MGP, male genital primordium; AP, anal primordium. See text for explanation.

does not form a recognizable adult structure (but see below). The "repressed" FGP in a male genital disc is termed RFP, and the "repressed" MGP in a female genital disc is termed RMP. In females, Dsx^F promotes the development of both the female genital and anal primordia, giving rise to the female genitalia and analia respectively; whereas the development of the male genital primordium is brought into the "repressed state" (RMP) (**Figure 10**). In normal males, Dsx^M promotes development of both the male genital and anal primordia, giving rise to the male genitalia and analia respectively, whereas the development of the female genital primordium is brought into the "repressed state" (RFP) (**Figure 10**). Mutations of the *dsx* gene, that do not produce Dsx^F and Dsx^M products, result in the generation of genital discs that show the same type of intersexual development in both 2X;2A and XY;2A flies. The latter is characterized by the development of both male and female genital primordia (though neither produce a complete set of genital structures) and by the anal primordium forming intersexual analia (Ehrensperger, 1983). The same intersexual development is observed when both Dsx^F and Dsx^M are simultaneously present within a cell (Epper, 1981) (**Figure 10**). This situation is encountered in 2X;2A flies of genotype dsx^+/dsx^D, where the dsx^+ allele produces the Dsx^F protein and the dsx^D allele produces the Dsx^M protein (Nagoshi and Baker, 1990).

The demonstration that the RFP corresponds to the FGP (i.e., the localization of the RFP in the male genital disc) and that the RMP corresponds to the MGP (i.e., the localization of the RMP in the female genital disc) was performed by the analysis of intersexual genital discs and the clonal analysis of *tra* mutations. Thus:

1. The XX; $dsx^D/+$ (Epper, 1981) and XX; *dsx/dsx* or XY; *dsx/dsx* or XX; *ix/ix* (Ehrensperger, 1983) intersexual genital discs contain three major regions (ventral, anterior dorsal, and posterior dorsal), which were isolated by fragmentation and transplanted into mature larvae ready to pupate, such that they underwent metamorphosis. The ventral fragment produced female genital structures, the dorsal anterior fragment produced male genital structures, and the dorsal posterior fragment produced anal structures. The ventral fragment of the intersexual genital disc corresponds to the region of the normal male genital disc that does not develop; i.e., to the RFP. The dorsal anterior fragment of the intersexual genital disc corresponds to the region of the normal female genital disc that does not develop; i.e., to the RMP.
2. The *tra* mutation transforms chromosomal (XX) females into males (see above). Clones homozygous for *tra* were induced by X-irradiation of

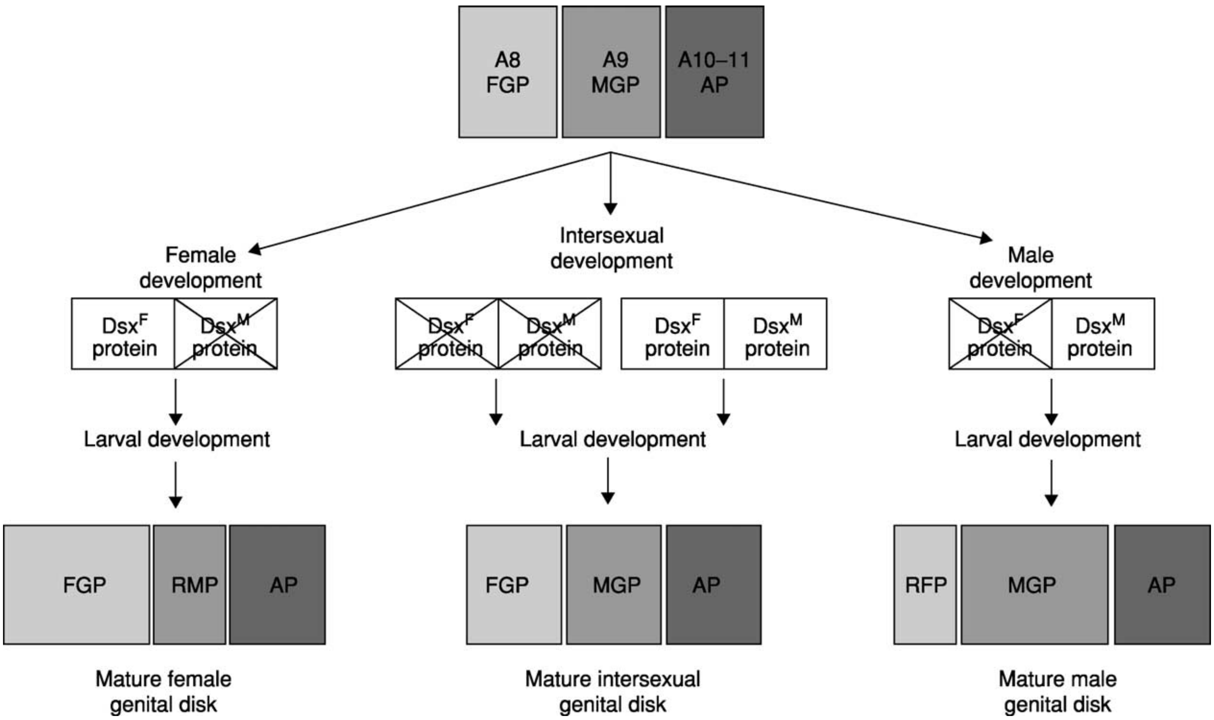


Figure 10 Sexually dimorphic development of the genital disc. A8, A9, and A10–11 indicate the corresponding abdominal segments. For further explanations on the role of Dsx, see text.

XX; *tra*/+ larvae at late third larval instar. The genital discs were dissected and cultured *in vivo* in the abdomen of fertilized females to allow them to grow further before metamorphosis. Thereafter, the implants were dissected and, in a significant number of cases, the genital discs showed local overgrowths, at the site of the RMP in the female genital disc. These overgrowths were cut out of the discs and transplanted into larvae that underwent metamorphosis. All of them produced male genital structures. These results suggest that the RMP corresponds to the male genital primordium of the female genital disc that does not develop and that the *tra* function is needed to prevent development of the RMP (Epper and Nöthiger, 1982).

The cell lineage relationships between the primordia that compose the genital disc were studied by clonal analysis in the male and female genital discs. It was shown that clones overlapped in the right and left sides in both genital discs, but no clones were found that comprised genital and anal structures. This indicates that there is a cell lineage restriction between the analia and the genitalia in both sexes (Schüpbach *et al*., 1978; Dübendorfer and Nöthiger, 1982; Janning *et al*., 1983). No cell lineage restriction between the two genital primordia could be determined, since in each sex only one of the primordia develops (but see Section 1.1.1.2.2).

1.1.2.2. Embryonic Organization of the Genital Disc

As mentioned above, the embryonic region of the genital disc contains three different regions (**Figure 11**). The anterior region that contains precursors for the female genital primordium (FGP) corresponds to abdominal segment A8. The central region that contains precursors for the male genital primordium (MGP) corresponds to segment A9. And the most posterior region that gives rise to the analia of both males and females corresponds to segments A10 and A11 (Schüpbach *et al*., 1978) (**Figure 11**).

The embryonic genital disc can be distinguished at about 12 h of development by the expression of *headcase* (*hdc*) and *escargot* (*esg*), which mark all imaginal cells (Whiteley *et al*., 1992; Weaver and White, 1995; Casares *et al*., 1997). According to genetic mosaic analysis conclusions, the embryonic genital disc contains 12–15 cells (Hartenstein and Jan, 1992) that are organized as three clusters: two of them located to the left and right of the midline, and the other located posteriorly. These clusters do not correspond to the three primordia of the genital

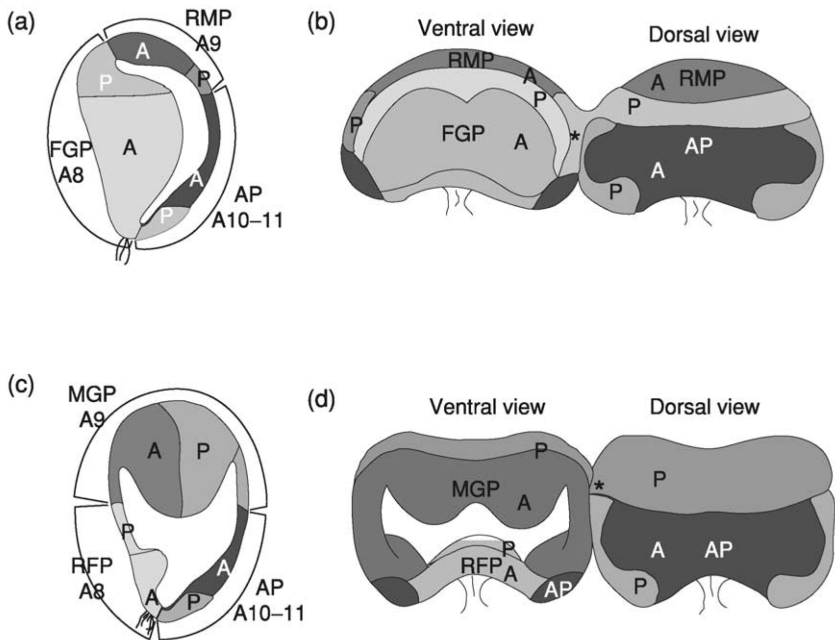


Figure 11 Schematic representation of the compartmental organization of the female and male genital disc. Transverse sections (a, c) and dorsal and ventral view of (b, d) of the female (a, b) and male (c, d) genital disc respectively. Developing female genital primordium (FGP) or repressed female genital primordium (RFP) is in light gray; developing male genital primordium (MGP) or repressed male genital primordium (RMP) is in gray, and anal primordium (AP) is in dark gray. In each case, the anterior compartment (A) is lighter than the posterior compartment (P). The asterisk indicates that the P compartment cells of the three genital primordia are contiguous. For the rest of the symbols see legend to **Figures 8** and **9**.

disc; in fact, the tripartite organization of the genital disc is not obvious in the embryo (Casares *et al.*, 1997). The genital disc grows during larval stages to give the sexually dimorphic disc characteristic of the third instar larvae.

1.1.2.3. Organization and Patterning of the Genital Disc during Larval Development

1.1.2.3.1. Segmental and compartmental organization of the genital disc As was previously described, early studies have demonstrated the existence of a cell lineage restriction between the analia and male or female genitalia (Schüpbach *et al.*, 1978; Dübendorfer and Nöthiger, 1982; Wieschaus and Nöthiger, 1982). Initially, the question of a clonal restriction between male and female genitalia could not be addressed, because only one of the two genital primordia gives rise to adult structures in any one sex. Although it is now known that the RMP in females gives rise to the parovaria and that the RFP in males gives rise to a miniature 8th tergite (Keisman and Baker, 2001; see also below), these structures cannot be marked in the adult and are not suitable for analysis of clonal relationships. A cell lineage restriction analysis using dsx^1 genital discs, where both primordia develop, has shown that clones could not cross the border between female and male genitalia (Gorfinkiel *et al.*, 2003). Therefore, it was concluded that a cell lineage restriction exists between female and male genitalia, and between genitalia and analia. The evidence suggests that the three primordia of the genital disc represent three originally separate abdominal segments.

Each primordium of the genital disc has an anterior and a posterior compartment, which can be marked by the reciprocal expression of the *cubitus interruptus* (*ci*) and *engrailed* (*en*) genes (Freeland and Kuhn, 1996; Casares *et al.*, 1997; Chen and Baker, 1997) (**Figure 12**). The *ci* and *en* mRNAs label the anterior and posterior compartments, respectively, and mark correspondingly the cells in all imaginal discs (Kornberg *et al.*, 1985; Eaton and Kornberg, 1990). This agrees with *en* mutations affecting both male and female genitalia plus analia (Lawrence and Struhl, 1982; Epper and Sánchez, 1983). The analysis of *en* loss-of-function clones has shown that the majority of external genital structures are of anterior origin (Casares *et al.*, 1997; Emerald and Roy, 1998). Clones induced at the second larval stage do not cross between *en* mRNA- and *ci* mRNA-expressing cells (Chen and Baker, 1997) suggesting that at least by the beginning of the second instar, the genital disc has already subdivided into anterior and posterior compartments.

A striking recent finding has challenged the idea that imaginal genital discs have, like all other imaginal discs, only anterior and posterior compartments. Ahmad and Baker (2002) have found a group of cells in the male genital primordium that express the *breathless* (*btl*) gene, which encodes the fibroblast growth factor receptor. These cells define a novel (third) compartment, because they do not express either *ci* or *en* and clones do not cross between *btl*-expressing cells and the rest of the disc. Surprisingly, these *btl*-expressing cells are not originally part of the male genital disc but are recruited into it during

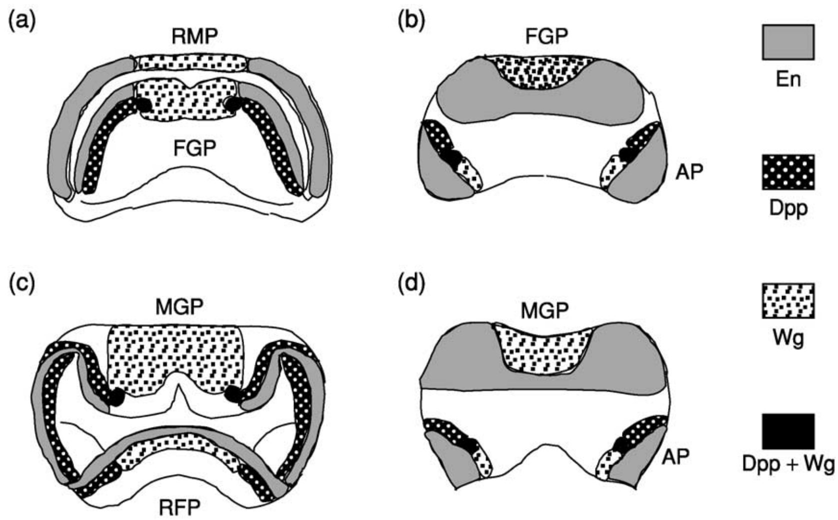


Figure 12 Expression patterns of En, Dpp, and Wg in the female (a, b) and male (c, d) genital discs. The cells coexpressing Wg and Dpp are shown in black. (a) and (c) are ventral views, (b) and (d) are dorsal views of the corresponding genital discs. For the symbols see legend to **Figures 9** and **11**.

the larval stages. Cells in the male genital primordium expressing the gene *branchless* (*bnl*), which encodes the fibroblast growth factor, induce the *btl*-expressing mesodermal cells to migrate into the male disc. In this process, they lose the mesodermal marker *twist* (*twi*), acquire the ectodermal marker *coracle* (*cora*) and, eventually, form the paragonia. Therefore, three compartments compose the male genital primordium.

Are the segmental borders different than the compartment borders? The genital disc is a good model to address this question, since the three primordia are contiguous to each other, like in the abdomen. It has been shown that in the wing disc, the Hedgehog (Hh) signaling pathway is responsible for the maintenance of the compartment border (see **Chapter 1.9**). *smoothened* (*smo*) anterior clones, which are unable to transduce the Hh signal, mix freely with posterior cells (Blair and Ralston, 1997; Rodriguez and Basler, 1997). In the genital disc, *smo* anterior clones can also mix with posterior cells but only from the same segment; *smo* anterior cells from one segment cannot mix with posterior cells from another segment (although they are contiguous). This lineage restriction does not depend on homeotic function either, although it cannot be ruled out that homeotic genes fix the restriction border much earlier during embryonic development (Gorfinkiel *et al.*, 2003). Altogether, it can be concluded that segmental borders are different from the compartment borders.

1.1.2.3.2. Inductive signals in the development of the genital disc In the past decade, a paradigm has arisen about the genetic organization of imaginal discs. The selector gene *en* is expressed in posterior cells and activates the signal molecule Hh, which diffuses into the anterior compartment and elicits the activation of target genes. In the wing disc, Hh activates the expression of *decapentaplegic* (*dpp*), while in the leg and antennal disc, it activates de expression of *dpp* in dorsal anterior cells and *wingless* (*wg*) in ventral anterior cells. Both *wg* and *dpp* are signaling molecules that specify different identities in a concentration-dependent manner (reviews: Campbell and Tomlinson, 1995; Lawrence and Struhl, 1996) (see also **Chapter 1.9**).

The same interactions take place in the genital disc, where each primordium is organized in a manner similar to that of the leg and antennal discs (Freeland and Kuhn, 1996; Casares *et al.*, 1997; Chen and Baker, 1997; Sánchez *et al.*, 1997; Gorfinkiel *et al.*, 1999). In the genital and anal primordia, *en* in the posterior compartment activates the expression of *hh*, which, in turn, activates the expression of *wg* and *dpp* in mutually exclusive and complementary domains, except in the A9 of female discs where *dpp* is not expressed (Freeland and Kuhn, 1996; Casares *et al.*, 1997; Sánchez *et al.*, 1997) (**Figure 12**). As it has been demonstrated in the leg disc (see **Chapter 1.9**), *wg* and *dpp* inhibit each other's expression (Sánchez *et al.*, 1997).

The analysis of the expression domains of *wg* and *dpp* in the adult and the phenotypes obtained from lack of function and/or ectopic function of these signaling molecules have allowed the determination of the genital structures specified by these genes. Thus, in males, *wg* is expressed in the penis apparatus, the claspers, and the genital arch (Sánchez *et al.*, 1997). A requirement for *wg* in the development of these structures has been confirmed by the phenotype of *wg* hypomorphic alleles (Chen and Baker, 1997; Gorfinkiel *et al.*, 2003). In females, the dorsal vaginal plates express high levels of *wg* and can be duplicated by ectopic expression of *wg*, suggesting that Wg specifies these structures (Chen and Baker, 1997; Sánchez *et al.*, 1997). The vulva has been found to require *wg* function also (Gorfinkiel *et al.*, 2003). In addition, *wg* is required for the development of the internal genitalia in both sexes (Chen and Baker, 1997). The *dpp* gene is expressed also in the vaginal plates in females and in a subset of structures of the male genitalia like the genital arch, the hypandrium, and also the claspers (Sánchez *et al.*, 1997).

The analysis of hypomorphic combinations of *dpp* has shown that *dpp* is required for all the genital structures except for the penis apparatus (Gorfinkiel *et al.*, 2003). Furthermore, it has been shown that *optomotor-blind* (*omb*), a downstream gene in the *dpp* pathway (Grimm and Pflugfelder, 1996), is required for the formation of the dorsal bristles of the claspers and the hypandrium bristles (Gorfinkiel *et al.*, 1999). It is also known that *dachshund* (*dac*), which is a downstream gene of *wg* in females but a downstream gene of *dpp* in males (Keisman and Baker, 2001; Sánchez *et al.*, 2001) (see Section 1.1.2.4.1), is required for proper formation of the internal ducts that connect the spermathecae to the uterus in females, and for correct formation of the claspers in males (Gorfinkiel *et al.*, 1999; Keisman and Baker, 2001).

The assignment of a particular signaling pathway to a specific adult structure is difficult because the Wg and Dpp pathways also act coordinately to activate downstream genes that, in turn, specify different structures. For example, the expression of *Distal-less* (*Dll*) is activated by the coordinated action of *wg* and *dpp* (Gorfinkiel *et al.*, 1999), as happens in the leg disc (Díaz-Benjumea *et al.*, 1994)

(see **Chapter 1.9**). *Dll* has been identified as a gene required for leg development and, in the terminalia, it is required for the development of the anal plates in both males and females (Sunkel and Whittle, 1987; Cohen and Jürgens, 1989; Gorfinkiel *et al*., 1997, 1999).

1.1.2.3.3. Homeotic identity of the genital disc primordia The homeotic (Hox) genes specify structures along the anterior–posterior axis in most multicellular organisms (see **Chapter 1.8**). In *Drosophila*, the homeotic genes *abdominal-A* (*abd-A*), *Abdominal-B* (*Abd-B*), and *caudal* (*cad*) specify the identity of the three posterior primordia (**Figure 13**). The *abd-A* gene is expressed in part of the A8 primordium; in females this region correspond to the internal genitalia (Freeland and Kuhn, 1996). Based on its expression domain, it appears that *abd-A* is not required for the formation of the external genitalia (Sánchez-Herrero *et al*., 1985; Tiong *et al*., 1985). The *Abd-B* gene has two transcripts: *Abd-Bm* and *Abd-Br*, which encode two proteins differing from each other in their N-terminal region. The m transcript is expressed in the A8 primordium, while the r transcript is expressed in the A9 primordium (Casares *et al*., 1997). The complete lack of *Abd-B* expression transforms the genitalia into leg or distal antenna, and this transformation is accompanied by ectopic expression of *Dll* and *dachshund* (*dac*), two genes required for leg development (Estrada and Sánchez-Herrero, 2001) (see **Chapters 1.8** and **1.9**). Conversely, ectopic *Abd-B* expression transforms parts of the head into genitalia (Postlethwait *et al*., 1972; Kuhn *et al*., 1995). It has been suggested that the role of *Abd-B* is to specify genitalia by repressing *Dll* activity (Estrada and Sánchez-Herrero, 2001). As *Dll* is activated by the combined action of *wg* and *dpp* in the genital disc, the role of *Abd-B* would be to counteract the activating function of these two signaling molecules. Because high levels of ectopic Dll are able to induce ventral appendages, legs, or antennae

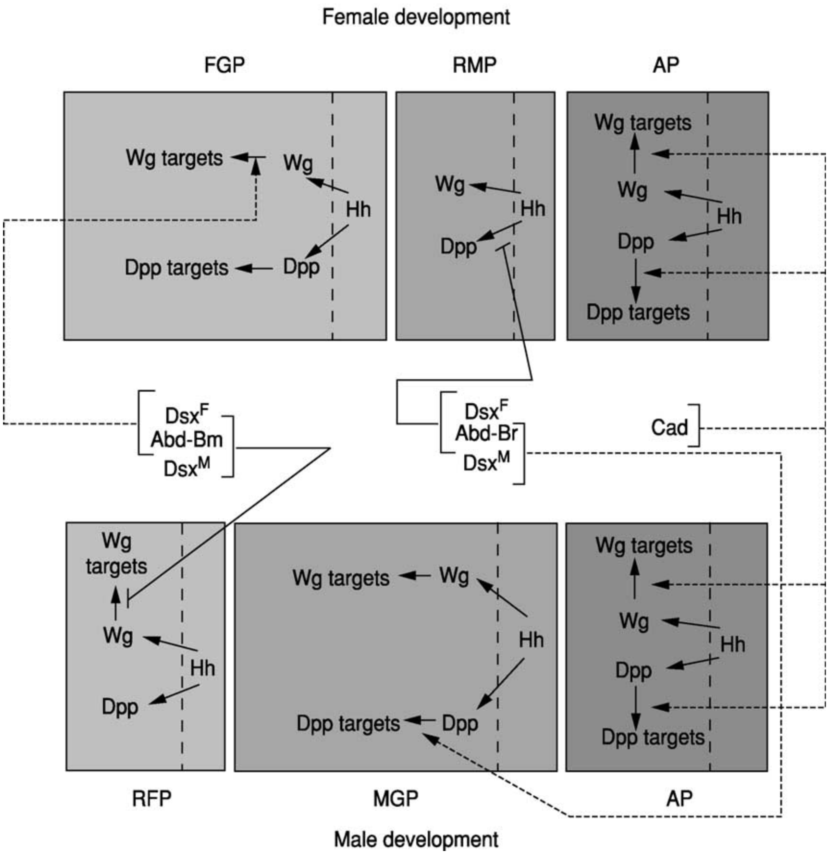


Figure 13 Schematic representation of the control of dimorphic sexual development of male and female genital primordia by the *dsx*, *Abd-B*, and *cad* genes. A8, A9, and A10–11 indicate the 8th and 9th abdominal segments, and the fusion of 10th and 11th abdominal segments, respectively. The repression of Dpp expression in the RMP and the repression of the Wg pathway in the RFP are indicated by a continuous line. The activation of Wg and Dpp pathways are indicated by stippled lines in the FGP and MGP, respectively. See text for explanation. The broken line represents the anteroposterior compartment border in each primordia.

(Gorfinkiel *et al*., 1997), the counteracting effect of Abd-B would be required to avoid leg development in the genitalia, and the *Dll* regulatory region would integrate the activating and repressing signals to control *Dll* transcription (Estrada and Sánchez-Herrero, 2001).

The *cad* gene (**Figure 13**) is expressed in the anal primordium and gives rise to two different structures: the hindgut and the anal plates. This gene is required for the development of anal structures and, when ectopically expressed, can induce ectopic analia (Moreno and Morata, 1999). Because of this and despite the fact that *cad* is not physically linked to the Hox cluster, it is the fly's most posterior Hox gene (Moreno and Morata, 1999). Consistently, *cad* clones show ectopic expression of *Abd-B* and concomitant transformation to genital structures in males (Moreno and Morata, 1999). Cad also activates *Dll* in the anal primordium; *brachyenteron* (*byn*), which is required for the development of the larval hindgut (Wu and Lengyel, 1998); and *even-skipped* (*eve*), another homeobox gene that is expressed in the presumptive region of the hindgut but is not required for hindgut development. *Dll* is also activated in the anal primordium by the combined action of Wg and Dpp, and is required for the formation of the anal plates (Gorfinkiel *et al*., 1999). Curiously, a mutually repressive interaction exists between *Dll* and *eve* in the anal primordium (Gorfinkiel *et al*., 1999).

Thus, in the anal primordium, Cad acts in combination with Wg and Dpp to activate *Dll* (Moreno and Morata, 1999), while in the genital primordia, the Hox gene *Abd-B* counteracts the Wg and Dpp signals in order to downregulate *Dll* (Estrada and Sánchez-Herrero, 2001) (see Section 1.1.2.3.4) (**Figure 13**).

1.1.2.3.4. Do the analia show a complex organization? Two chitinous plates, one dorsal and the other ventral to the anal opening, form the female analia. Two chitionous plates also form the male analia, one to the right and the other to the left of the anal opening (**Figure 8**). As mentioned above, the male and female analia behave as homologous structures derived from a common population of cells. Some experimental results, however, support the notion that the analia of both sexes may be derived from two distinct primordia, one giving rise to the dorsal analia in females or the complete analia in males (homologous primordium), while the other produces the ventral analia in females and no structure in males (nonhomologous primordium) (Andersen, 1979). A similar relationship has been described for the male and female analia in *D. virilis* (Newby, 1942).

First, XX;*dsx*^{D}/+, XX;*dsx*/*dsx*, or XX;*ix*/*ix* intersexes show one large horseshoe-shaped anal plate or separated left and right drop-shaped lobes, and a rudimentary plate with only two long bristles located ventral to the large plate (Andersen, 1979). Second, diplo-X homozygous *tra-2*^{ts} animals reared at 21 °C (intermediate temperature between female- and male-determining temperature) develop a dorsal anal plate with the appearance of dorsally fused right and left male anal plates, while the ventral anal plate is greatly reduced or absent (Belote and Baker, 1982).

Third, various degrees of sexual transformation in the analia were exhibited by diplo-X homozygous *tra-2*^{ts} animals reared at temperatures intermediate between 16 and 29 °C, or in animals that were shifted from 29 to 16 °C at progressively later stages of larval development. With increasing degrees of masculinization (progressively later shifts), the ventral female anal plate becomes reduced, while the dorsal plate changes into the horseshoe-shaped form that was described above for intersexual flies, becoming split into a left and right plate in the most extreme transformation (Epper and Bryant, 1983). Fourth, the shift of XX; *tra-2*^{ts}/*tra-2*^{ts} animals from 16 to 29 °C resulted in the quick formation of pure male anal plates. In the opposite shift, however, the formation of pure female anal plates occurred gradually. Moreover, the time course, required for the dorsal and ventral anal plates to show normal female phenotype, was different: when the dorsal anal plates were completely normal, it was still possible to find incomplete ventral anal plates (Sánchez and Granadino, 1992). Finally, in flies mutant for *Dll* alleles with different degrees of functionality, the dorsal female anal plate and the two male anal plates are reduced, whereas the ventral female anal plate is normal. Furthermore, *Dll*$^{-}$ clones do not develop anal plates in males or dorsal anal plates in females, but the development of the ventral female anal plates is not affected (Gorfinkiel *et al*., 1999). Thus, the dorsal female and male anal plates show the same genetic requirement for their development, supporting the idea that both are derived from a common primordium. It remains unknown, however, whether the segmental origin of this primordium is A10, and whether that of the ventral female analia forming primordium is A11.

1.1.2.3.5. Interaction between genital disc primordia The working model that emerges for the development of the genital disc is that each primordium develops as an independent unit in response to the general genetic cues that are also valid for other types of discs. However, there are also some results

suggesting that the development of one primordium affects the development of the others.

First, the regeneration of the genital disc, when different fragments of it are mixed (Littlefield and Bryant, 1979), suggests that some information is exchanged between primordia. Second, when cells of the anal or the genital primordia are killed by ectopic expression of the toxic *ricin A* gene, the development of the whole genital disc is impaired (Gorfinkiel *et al*., 2003). Similarly, mutations in *Abd-Br*, which is expressed only in A9 (see Section 1.1.2.4.4), produce a lack of female genitalia and analia (Casanova *et al*., 1986), and *Abd-B* mutations that transform the A8 and A9 segments into abdomen produce an absence of analia (Estrada and Sánchez-Herrero, 2001). Third, because the genital disc results from the fusion of three adjacent segments, the posterior compartment of one segment is contiguous with the anterior compartment of the corresponding segment and the anterior compartment of the following one. Thus, it has been found that Hh in the posterior compartment diffuses in both directions and induces different target genes for and aft. While *wg* and/or *dpp* are induced towards the anterior, *en* expression is induced towards the following segment. These results suggest that signals can diffuse across the different primordia of the genital disc (Gorfinkiel *et al*., 2003).

Accordingly, the ectopic expression of *hh*, *wg*, or *dpp* in the anal primordium induces duplications of genital structures. Also, *dpp* and *wg* mutant phenotypes that affect mainly the genitalia, can be rescued by expressing these genes in the analia. While the nonautonomous effect of Dpp is mediated by Dpp itself, the effect of Wg may be mediated by an as yet unidentified signal (Gorfinkiel *et al*., 2003).

In summary, it can be concluded that the development of the different primordia of the genital disc is interdependent and that specific signals may diffuse across the segmental boundaries.

1.1.2.4. Genetic Control of the Sexual Dimorphic Development of the Genital Disc

1.1.2.4.1. Interaction of sex determination genes with signaling pathways Sexual differentiation deeply affects patterning and growth of the genital disc, suggesting that sex influences not only the terminal differentiation genes but also the early events in the morphogenesis of the genital structures. Accordingly, the development of the male and female genital disc requires the integration of the information supplied by the sex determination gene *dsx*, the morphogenetic signaling pathways, and the Hox genes. How this integration of patterning genes and Dsx takes place is starting to be elucidated.

The RMP of the Dsx^F-containing female genital disc does not express *dpp* (Freeland and Kuhn, 1996; Casares *et al*., 1997) (**Figure 13**). The lack of *dpp* expression in the RMP cannot be attributed to the failure of cells to receive the Hh signal, because Hh induces expression of *wg* in these cells. Rather, the *dsx* gene renders the cells of the male genital primordium capable or incapable of activating *dpp*. The cells of the male genital primordium are able to activate *dpp* in response to the Hh signal in male (absence of Dsx^F) but not female (presence of Dsx^F) genital discs (**Figure 13**). Thus, clones mutant for *tra-2* (which is required for Dsx^F protein production) induced in the RMP of female genital discs, are always associated with *dpp* activation and show nonautonomous overgrowth (Sánchez *et al*., 2001).

Furthermore, it was also shown that the sex of the cells in the anterior compartment close to the anteroposterior compartment border (Hh responding cells), and not the sex of the whole genital disc, was the major factor controlling the growth of the genital primordia. When these cells are feminized in a male disc or masculinized in a female disc, both genital primordia respond by switching to growth patterns that reflect the sex in the induced anteroposterior compartment cells. Furthermore, these sexual changes in the Hh responding cells can change the expression of *dpp* in these cells (Keisman and Baker, 2001).

Collectively, these results indicate that the developing or repressed status of the male genital primordium may be controlled by Dsx at the level of *dpp* expression, thereby dictating the sex specific patterns of growth within the genital disc (Keisman and Baker, 2001; Sánchez *et al*., 2001). Moreover, it has been shown that Dsx^F is continuously required during female larval development to prevent activation of *dpp* in the RMP (Sánchez *et al*., 2001) (see Section 1.1.2.4.2).

The low proliferation rate of RFP in male genital discs cannot be attributed to the lack of Dpp or Wg, since both genes are expressed. Neither can it be due to a failure to respond to the Dpp signal, because the RFP expresses the Dpp-target gene *omb* (Gorfinkiel *et al*., 1999). Moreover, *Dll*, a Wg and Dpp target gene in the genital disc (Gorfinkiel *et al*., 1999), is not expressed in the RFP, where there is Dsx^M product, but is induced in the growing female genital primordium of genital discs that lack Dsx^M (Sánchez *et al*., 2001). Thus, the repressed status of the female genital primordium seems to be controlled by blockage of the Wg signaling pathway by Dsx^M. Moreover, it has been also observed that Dsx^M is not continuously required during larval

development for blocking the Wg-signaling pathway in the FGP (Sánchez *et al.*, 2001) (see Section 1.1.2.4.2).

The sex determination genes also control the fibroblast growth factor (FGF) signaling cascade. The *branchless* (*bnl*) and the *breathless* (*btl*) genes, which encode FGF and its receptor, respectively, in *Drosophila*, are each expressed in two bilaterally symmetrical and adjacent groups of cells in the mature male but not female genital disc (Ahmad and Baker, 2002). The *btl*-expressing cells give rise to paragonia and *vas deferens*, which are major parts of the internal male genitalia (**Figure 8**). The induction of ectopic Tra^{+} clones in the MGP of the male genital disc represses *bnl* expression. Conversely, in *dsx* mutant discs, where neither Dsx^{F} nor Dsx^{M} proteins are present, Bnl is expressed in the A8 and A9 segments. This indicates that Dsx^{F} represses the FGF pathway in the female genital disc and maybe Dsx^{M} activates this pathway in males (Ahmad and Baker, 2002).

1.1.2.4.2. The Dsx^{M} and Dsx^{F} products play positive and negative roles in the development of both the male and female genital primordia The established model for the development of the genital disc states that the male and female genital primordia, which are derived from two different cell populations, develop normally unless Dsx^{F} and Dsx^{M}, respectively, repress them (review: Steinmann-Zwicky *et al.*, 1990). Accordingly, these two proteins play a negative, rather than positive, role in the developmental control of the genital disc. This model is primarily based on the observation that intersexual *dsx* mutant flies – simultaneously lacking Dsx^{M} and Dsx^{F} proteins – develop both male and female adult genital structures. These male and female genital structures are derived from the male and female genital primordia, respectively (recall that in these intersexual flies both the male and female genital primordia develop) (see Section 1.1.2.4.1). However, the inventory of these structures is not complete. This cannot be attributed to the mutual interference of male and female genital primordia, which develop simultaneously, because gynandromorphs (flies in which X0 male and XX female cells are observed within the same individual) with complete male and female adult genitalia have been found (Nöthiger *et al.*, 1977). Rather, it seems that in the *dsx* intersexual condition, both the male and female genital primordia fail to develop completely. Indeed, the number of cells in the mature *dsx* intersexual genital disc does not add up to the complete male plus female genital disc complement (Ehrensperger, 1983). This suggests that in the absence of Dsx^{M} and Dsx^{F} products, both male and female genital primordia proliferate, albeit incompletely, and points to a positive role for Dsx^{M} and Dsx^{F} in the development of male and female genital primordia, respectively.

Recent results concerning the expression of the gene *dachshund* (*dac*) in the genital disc (Sánchez *et al.*, 2001) support the standard model but also raise some uncertainties including one related to the positive role of the Dsx products in the development of the genital disc. In normal female genital discs, which have the Dsx^{F} product (**Figure 13**), *dac* is expressed in the Wg but not in the Dpp domain. In normal male genital discs, which have the Dsx^{M} product (**Figure 13**), *dac* is expressed in the Dpp but not in the Wg domain (Keisman and Baker, 2001; Sánchez *et al.*, 2001). In genital discs simultaneously lacking or having both Dsx^{M} and Dsx^{F} products, *dac* shows low levels of expression in both the Wg and Dpp domains. This agrees with the idea that *dac* is regulated by Wg in the female genital disc and by Dpp in the male genital disc, and that this differential regulation requires the function of *dsx*. This is also compatible with the idea that Dsx^{M} and Dsx^{F} have dual roles with respect to the expression of *dac* in the genital disc. In male genital discs, Dsx^{M} positively and negatively regulates *dac* expression in the Dpp and Wg domains, respectively. In female genital discs, Dsx^{F} positively and negatively regulates *dac* expression in the Wg and Dpp domains, respectively.

At another level, Dsx has been also found to have an instructive and restrictive function. It was recently found that both the male and female primordia give rise to part of the adult structures in both sexes (Keisman and Baker, 2001). Using the expression patterns of genes such as *en* or *wg* as markers to track different genital primordia during metamorphosis, it was shown that each primordium gives rise to specific structures in each sex. In females, the male primordium forms a pair of female-specific accessory glands, whereas in males it produces a miniature eighth tergite. These findings indicate that, in one case, Dsx directs the female genital primordium to form either female genitalia or eighth tergite, and the male genital primordium to produce either male genitalia or the parovaria.

Such a dual role of the Dsx products is not unique to the genital disc. A similar role has been observed in the regulation of the female-specific *yolk protein* (*yp*) genes: Dsx^{M} represses the *yp* genes in males, whereas Dsx^{F} activates the *yp* genes in females (see **Chapters 1.2** and **1.3**). In flies that lack both Dsx products, the *yp* genes are expressed at a basal level (Bownes and Nöthiger, 1981; Coschigano and

Wensink 1993; Waterbury *et al*., 1999). A positive role for Dsx^M has been also observed in the development of the sex comb in males (Jurnish and Burtis, 1993).

1.1.2.4.3. Different time requirements for the Dsx^M and Dsx^F products The following set of results suggests that the development of male and female genital primordia has different time requirements for Dsx^M and Dsx^F products:

1. Analysis of the growth of genital primordia and their capacity to differentiate adult structures in homozygous *tra-2^ts^* genital discs with two X chromosomes was performed using pulses of the male- and the female-determining temperatures in both directions during development. Regardless of the stage in development at which the female-determining temperature pulse was given (transient presence of functional $Tra\text{-}2^{ts}$ product, i.e., transient presence of Dsx^F product and absence of Dsx^M product), the male genital disc developed normal male adult genital structures and not female ones. This occurred even when the pulse was applied during pupation. Pulses at the male-determining temperature (temporal absence of functional $Tra\text{-}2^{tts}$ product, i.e., transient absence of Dsx^F product and presence of Dsx^M product), before the end of first larval stage produced female and not male genital structures. However, later pulses always gave rise to male genital structures, except when the pulses were given close to pupation. Further, the capacity of the female genital disc to differentiate adult genital structures was also reduced when the temperature pulse was applied during metamorphosis (Sánchez *et al*., 2001).
2. When the effect of the male-determining temperature pulses was analyzed in the genital disc, it was found that overgrowth of the RMP was always associated with the activation of *dpp* in this primordium. However, this activation and the associated overgrowth only occurred when the temperature pulse was given after the end of first larval instar. This suggests that there is a time requirement for induction of *dpp*. The activation of this gene in the RMP and the cell proliferation that resumed in this primordium, as well as its capacity to differentiate into adult structures, was irreversible, since these were maintained upon return of the larvae to the female-determining temperature, when functional $Tra\text{-}2^{ts}$ product (i.e., presence of Dsx^F product and absence of Dsx^M product) was again available (Sánchez *et al*., 2001).
3. The conclusion for the time requirement for induction of *dpp* was also supported by the fact that dsx^{11} clones (which lack Dsx^M) induced in the developing male genital primordium of XY; dsx^{11}/+ male genital discs (which express only Dsx^M) after 24 h of development, differentiated normal male adult genital structures. However, when the dsx^{11} clones were induced in the time period between 0 and 24 h of development, they did not differentiate normally and gave rise to incomplete adult male genital structures.

   The different developmental capacity shown by the dsx^{11} clones depending on their induction time was explained as follows. When the clones were induced after 24 h of development, *dpp* was already activated. Indeed, these clones showed no change in the expression pattern of *dpp* or its targets (Sánchez *et al*., 2001). Accordingly, these clones displayed normal proliferation and capacity to differentiate male adult genital structures. On the contrary, when the clones were induced early in development, *dpp* was not yet activated, as this gene is not expressed in the male genital primordium of male genital discs early in development (Casares *et al*., 1997). Therefore, when the male genital disc reaches the state in development when *dpp* is induced, the cells that form the clones activate this gene as in *dsx* mutant intersexual flies, because the clones have neither Dsx^M nor Dsx^F products. Consequently, these clones do not achieve a normal proliferation rate and do not produce normal adult male genital structures (Sánchez *et al*., 2001).
4. The expression of the *dac* gene was analyzed in genital discs of diplo-X homozygous *tra-2^ts^* flies using pulses of the male- and the female-determining temperatures in both directions (recall that in male genital discs, Dsx^M positively and negatively regulates *dac* expression in the Dpp and Wg domains, respectively; and in female genital discs, Dsx^F positively and negatively regulates *dac* expression in the Wg and Dpp domains, respectively). It was found that the *dac* expression pattern switched from a “female type” to a “male type,” when male-determining temperature pulses were applied to *tra-2^ts^* larvae after the first larval instar. However, when the pulse was given during the first larval instar, *dac* was not activated in the Dpp domain of RMP, in spite of the fact that there was also a transient presence of Dsx^M instead of Dsx^F (Sánchez *et al*., 2001). This was explained by the lack of competence of cells to express Dpp, which is acquired after the first larval instar (Casares *et al*., 1997).

When the *tra-2*^{ts} larvae reach such a developmental stage, these cells produce Dsx^F because they are returned to the female-determining temperature. Dsx^F prevents activation of *dpp* in the RMP and, consequently, no induction of *dac* expression occurs. In the female genital primordium, *dac* expression is strongly reduced in the Wg domain and is absent from the Dpp domain.

Taken together, these results suggest that the development of male and female genital primordia have different time requirements for the Dsx^F and Dsx^M products. In females, Dsx^F is continuously required during female larval development for preventing activation of *dpp* in the RMP and, during pupation, for female cytodifferentiation. In males, Dsx^M seems not to be continuously required during larval development for blocking the Wg signaling pathway in the female genital primordium nor during pupation for male cytodifferentiation.

1.1.2.4.4. Integration of signaling pathways, sex determination, and homeotic information Homeotic and sex determination genes have to interact, along with the Hh, Dpp, and Wg signals, for normal development of the genitalia to occur (Keisman and Baker, 2001; Sánchez *et al*., 2001). The *dsx* gene controls which of the two genital primordia will develop and which one will be repressed. Nevertheless, since Dsx^M and Dsx^F are equally active in each cell of the A8 and A9 segments of the male and female, respectively, another gene(s) is required to distinguish between female and male genitalia. The female genitalia develop from the A8 segment and the male from A9 (Freeland and Kuhn, 1996; Casares *et al*., 1997). Since the *Abdominal-B* (*Abd-B*) gene is responsible for the specification of these posterior segments (review: Duncan, 1996) (see **Chapter 1.8**), it has been proposed that Abd-Br and Abd-Bm participate, together with *dsx*, in the sexually dimorphic development of the genital disc (Sánchez *et al*., 1997, 2001).

The genetic model that has been proposed for the control of the sexually dimorphic development of the genital disc (**Figure 13**), suggests that Dsx^M and Dsx^F combine with Abd-Bm and Abd-Br to make up the determining signals (Sánchez *et al*., 2001). First, it has been reported that Abd-B reduces the response to Wg and Dpp signals in the genital disc (Estrada and Sánchez-Herrero, 2001). Abd-B seems to repress *dac* and *Dll* expression (perhaps by antagonizing the Dpp and Wg signaling) in some cells of the male and female primordia. It is still unknown if this multiple input acts directly on the *dac* and *Dll* regulatory regions. Second, in the absence of both Dsx^M and Dsx^F products, there is basal expression of *dpp* and a basal functional level of the Wg signaling pathway in both the male and female genital primordia. This leads to both genital primordia developing, albeit incompletely. In normal females, the concerted input of Dsx^F and Abd-Br causes repression of the development of the male genital primordium by preventing the expression of *dpp* and resulting in the RMP of female genital discs. In normal males, the concerted input of Dsx^M and Abd-Bm represses the female genital primordium by blocking the Wg signaling pathway, which gives rise to the RFP of the male genital discs. It is further proposed that Dsx^M plus Abd-Br increase *dpp* expression and the response to the Dpp signaling pathway in the MGP of male genital discs, and that Dsx^F plus Abd-Bm enhance the Wg signaling pathway function in the FGP of female genital discs. Therefore, Dsx^M plays a positive and a negative role in male and female genital primordia, respectively, while Dsx^F plays a positive and a negative role in female and male genital primordia, respectively.

Such integration of the *dsx* and *Abd-B* functions seems not to be exclusive to the genital disc. The *Drosophila* 5th and 6th abdominal tergites are formed in both sexes, but in females they show dark pigmentation restricted to the posterior region (non-sex-specific pigmentation), whereas in males the pigmentation covers the whole tergite. The gene *bric-a-brac* (*bab*) is involved in the sexually dimorphic pigmentation of these tergites by integrating regulatory inputs from *dsx* and *Abd-B* (Kopp *et al*., 2000). Another integrated genetic input made up by *dsx* and the homeotic gene *Sex combs reduced* (*Scr*) has been invoked for sex-specific differentiation of the basitarsus of the prothoracic leg (see **Chapter 1.9**). This contains the sex comb, another male-specific structure (Jurnish and Burtis, 1993). However, it is not known at which level this interaction takes place.

1.1.2.5. Gradual Acquisition of the Developmental Capacity to Differentiate Adult Structures

At the end of the larval period, each mature imaginal disc represents a set of cells with different determination states that will be realized (differentiation) during metamorphosis as a response to the hormonal stimulus (ecdysone), resulting in the adult pattern that characterizes each imaginal disc (Nöthiger, 1972). It has been reported, however, that imaginal discs acquire their capacity to differentiate into adult structures gradually throughout their larval growth; i.e., there is a specific temporal sequence in which the structures from imaginal discs

of increasing age differentiate, this sequence being unique for each disc. These temporal sequences have been reported for the imaginal discs of the leg (Schubiger, 1974), the eye–antenna (Mindek and Nöthiger, 1973; Gateff and Schneiderman, 1975), and the wing (Bownes and Roberts, 1979) (see **Chapter 1.9**). This was determined through transplantation of imaginal discs, from larvae at different stages of development, into mature larvae ready to pupate, and analyses of the inventories of adult structures derived from the implanted discs upon eclosion.

The genital disc also shows a gradual acquisition of the developmental capacity to differentiate adult structures. The strategy used for the demonstration of the latter (Sánchez and Granadino, 1992) involved a temperature-sensitive allele *tra-2^{ts1}* that was characterized because XX flies homozygous for this mutation develop as females at the permissive temperature (16 °C), whereas they develop as males at the restrictive temperature (29 °C) (Belote and Baker, 1982) (see Section 1.1.2.4.3 for the function of gene *tra-2*). The rationale of the strategy was as follows. When *tra-2^{ts1}* homozygous diplo-X larvae are raised at 16 °C, when the Tra-2 product is active, the FGP develops while the development of the MGP is blocked. If, during larval development, these larvae are shifted to 29 °C, when no functional Tra-2 product will be available, the cells of the MGP will enter into a normal proliferation rate (Belote and Baker, 1982; Epper and Bryant, 1983). The degree of growth that the MGP reaches will depend on the time during development when the temperature shift is performed. Therefore, it is possible to establish a correlation between the development attained by the MGP and its capacity to differentiate adult structures. At the same time that the shift from 16 to 29 °C prompts the MGP to develop, the cells of the FGP, which are developing at a normal rate, are brought into the slow proliferation rate that characterizes the RFP of the male genital disc, as a consequence of the inactivation of the Tra-2 product at the high temperature. Hence, it is possible to asses the developmental capacity attained by the FGP for differentiation of adult structures, when its normal growth is blocked at certain times before metamorphosis. An analogous situation occurs when the temperature shift goes from the 29 to 16 °C but, in this case, the behavior of the female and male genital primordia is reversed.

The results showed that the male genital primordium (male genital disc) follows a temporal sequence in its capacity to differentiate male external adult structures: the genital arch and lateral plates were the first structures that the male genital primordium differentiated, followed by the clasper, hypandrium, and penis apparatus (see **Figure 8** for the structures). Also, the female genital primordium (female genital disc) exhibits a sequence of capacity to differentiate external adult structures (8th tergite before vaginal plate), although in this case it is difficult to define a clear-cut sequence due to the reduced morphological elements that form the external adult female genital pattern. With respect to the analia, where cells proliferate independently of the state of *tra-2* activity (Belote and Baker, 1982; Epper and Bryant, 1983), the temperature shift between 16 and 29 °C, in both directions, indicated that the anal cells show a plasticity to change their sexual developmental program. Nevertheless, the change from female to male development occurred quickly, whereas that from male to female occurred gradually (Sánchez and Granadino, 1992).

There is a correlation between the sequence of structures differentiated by the genital disc and their topography in the fate map of the mature genital disc (Littlefield and Bryant, 1979; Ehrensperger, 1983; Epper and Bryant, 1983), as well as their cell lineage relationship (Dübendorfer and Nöthiger, 1982). It was suggested that this sequence is related to the dynamics of growth of the genital disc. It was further suggested that, at the end of the larval period, the mature genital disc is a mosaic of cells with different states of determination which, through metamorphosis, will differentiate into the distinct adult structures that form the terminalia. This mosaic pattern of the mature genital disc is not attained as an all-or-nothing event at the end of the larval period. Rather, during larval development, the proliferating genital disc is a mixture of cells already determined and cells that are still on their way to acquire a state of determination. The temporal sequence in the capacity to differentiate adult structures would reflect the temporal sequence of determination events in the genital primordia.

In the temperature shift experiments of XX; *tra-2^{ts}/tra-2^{ts}* animals, duplicated male genital structures corresponding to the penis apparatus were found. A similar duplication is observed in *dsx* or *dsx^{D}* intersexual flies (Epper, 1981). What is the origin of these duplicated structures? The remaining vaginal plates always enclose the secondary set of male genitalia, whenever these female structures are formed. This location suggests that the duplicated male structures derive from the female genital primordium. Several results support this interpretation. Fate mapping of the *dsx^{D}* intersexual genital discs showed that the small penis duplication maps on the female genital primordium (Epper, 1981). Moreover, Epper and Bryant (1983), in their experiments

of culturing *in vivo*, at the male-determining temperature, fragments of XX; *tra-2^ts^/tra-2^ts^* genital discs that had been developing at the female-determining temperature, observed that the female genital fragment differentiated small penis structures.

It is possible to argue that cutting errors during disc sectioning are the cause for the presence of the duplicated male structure. However, an independent observation supporting the notion that the penis duplication originates from the female genital primordium, stems from the analysis of the effect of *en* alleles in the genital disc (Epper and Sánchez, 1983). The en^1/en^2 combination affects the female genital primordium, more severely, while the male genital primordium is most affected by the en^2/en^3 combination. In intersexual en^2/en^3; $dsx^D/+$ flies, despite the lack of the entire penis apparatus in the male genital set, the small duplicated penis is always present. This rudimentary duplication, however, is absent from intersexual en^1/en^2; *dsx/dsx* flies, while the penis of the male genital set is always present. Thus, the secondary penis seems to be recognized by the *en* combinations as an element of the female genital primordium. Finally, in the above mentioned cases, the duplicated male structure is always accompanied by a reduction in the size of the ventral region of the vaginal plates. Collectively, these results suggest that a group of cells from the female genital primordium have the potential of following two alternative sexual pathways. In wild-type females, this group of cells gives rise to the ventral region of the vaginal plate, while in wild-type males it forms part of the "repressed" RMP primordium. In intersexual flies, when both female and male genital primordia develop, this group of cells produces the secondary penis.

1.1.2.6. Evolutionary Considerations

Evolutionary changes in morphology do not necessarily involve the generation of novel cell types but rather changes in morphogenesis, i.e., in the processes that are responsible for the generation of three-dimensional arrangements of various cell types (Raff and Kauffman, 1983). What is the genetic basis for morphological evolution? The genital disc, in addition to being a good experimental model in which to examine the control of pattern formation, is also well suited for the study of morphological evolution. In this respect, two aspects of the genital disc can be highlighted. First, the female external terminalia are very similar in the *Drosophila* species. The external male terminalia, however, display great differences; indeed, these are some of the main features used in taxonomy (Tsacas and Bocquet, 1976). Therefore, the terminalia are particularly well suited for studying the great morphological variety observed in the male genitalia. The second aspect is related to the origin of the genital disc.

1.1.2.6.1. The genital disc in interspecific hybrids

The genetic basis for the morphological evolution of the genital disc has been addressed through the analysis of interspecific hybrids of *Drosophila*. This analysis has focused on the effect of quantitative trait loci (QTL) on the shape of a particular structure of the male terminalia, the lateral lobe, in the sibling species *D. melanogaster, D. simulans*, *D. mauritiana*, *D. sechellia*, and their hybrids (Coyne, 1983; Coyne *et al*., 1991; Liu *et al*., 1996). The morphology of the lateral lobe differs markedly in these species. The genetic basis underlying the formation of this structure was studied through the production of first generation-interspecific hybrids. In the cases where the female hybrids were fertile, F_2 backcrosses to males of either generation could be performed and it was possible to analyze hybrids that contained different combinations of genes of the two parental species. Evidence was presented supporting a polygenic basis for the shape of this male structure.

Analysis of the morphological variation in other interspecific hybrids cannot be carried out because, in the majority of the cases, the species never interbreed, due to ethological isolation or other causes. A way of circumventing this problem is to cross-transplant germ cells. If the germ cells from the donor can develop in the soma of a host, it may be possible to obtain interspecific hybrids when the chimeric adult hosts are mated with individuals of their own species (Santamaria, 1977; Sánchez and Schmid, 1984; Schmid *et al*., 1984; Lawrence *et al*., 1993). Following this strategy, germ cells from *D. yakuba* or *D. teissieri* – both belonging to the *melanogaster* subgroup (Lachaise *et al*., 1988) and not breeding with *D. melanogaster* – were transplanted into *D. melanogaster* host embryos, and the chimeric hosts were mated to *D. melanogaster* males. The germ cells of both donor species were capable of producing functional *yakuba* or *teissieri* oocytes, respectively, which, when fertilized by the *melanogaster* sperm, gave rise to *yakuba–melanogaster* and *teissieri–melanogaster* adult hybrids, whose external terminalia were characterized (Sánchez and Santamaria, 1997).

The female external terminalia are very similar in the three species, *D. melanogaster*, *D. yakuba*, and *D. teissieri*. The external male terminalia, however, display significant differences (Tsacas and Bocquet, 1976). The morphological differences in

the external terminalia of *yakuba–melanogaster* and *teissieri–melanogaster* male hybrids, and those of their parental species, can be either qualitative or quantitative and could be ascribed to four main categories:

1. There were structures in the three species that showed a different shape in their hybrids; for example, the edeagus, the hypandrium, and the lateral lobe.
2. There were structures that in the hybrids showed the morphology of one of the parental species; for example, the dorsal parameters in *teissieri–melanogaster* resembled those of *D. teissieri*.
3. There were structures present in the hybrids and in only one of the parental species; e.g., the penis mantle is absent in *D. teissieri* and present in *D. melanogaster* and in *teissieri–melanogaster* hybrids.
4. There were structures absent in the hybrids but present in the two parental species; e.g., the ventral parameres are present in *D. melanogaster* and *D. teissieri* but absent in their hybrids.

It was speculated that these morphological differences correspond to changes in two basic genetic mechanisms that control the generation of morphological patterns. Qualitative differences, which refer to the presence of absence of a given structure of the pattern, probably reflect a simple genetic basis, in which a single gene is at work (or a combination of a few genes, each contributing a qualitative effect). Quantitative differences, however, are also characterized, because the structures display more or less continuous variation or intermediate phenotypes, suggesting that a polygenic system is at work (each gene in the system has a minor effect and shows additivity) (Sánchez and Santamaria, 1997).

The *dsx* gene is the last gene in the sex determination cascade (**Figure 4**). The dsx^D mutation transforms females into intersexes while having no effect in males (Gowen, 1942; Denell and Jackson, 1972). The XX; dsx^D/+ intersexual flies contain both the Dsx^M (from the dsx^D allele) and Dsx^F (from the dsx^+ allele) products (Nagoshi and Baker, 1990). The dsx^D allele was introduced into the *teissieri–melanogaster* hybrids via *D. melanogaster* males crossed to chimeric *D. melanogaster* females carrying exclusively *D. teissieri* germ cells (Sánchez and Santamaria, 1997). The most conspicuous feature of the intersexual terminalia of XX; dsx^D/+ *D. melanogaster* flies is the presence of both male and female genital structures. These are reduced and arranged in two separate genitalia. Some intersexes show a reduction of genital structures to such an extent that only traces of male and/or female genitalia are present. The anal plates are always present and their shape resembles neither the wild-type male nor the normal female. Rather they display an intermediate sexual phenotype (Gowen, 1942; Denell and Jackson, 1972; Epper, 1981). The most salient characteristic of the intersexual terminalia of XX; dsx^D/+ *teissieri–melanogaster* hybrids was the great difference in the inventories of male versus female genital structures: in general terms, the external genitalia of these hybrids could be defined as male-like more than intersexual. There was an almost completely normal set of male genital structures. The analia, however, were intersexual (Sánchez and Santamaria, 1997).

As mentioned earlier, the *dsx* gene must act in concert with another regulatory gene(s) to determine the genital primordia that will develop in each sex. It has been proposed that this additional gene is *Abdominal-B* (*Abd-B*) (Sánchez *et al*., 1997, 2001). To explain the male-like phentoype of the genitalia of XX; dsx^D/+ *teissieri–melanogaster* hybrids, it was speculated that during the evolution of the *D. melanogaster* and *D. teissieri* species, genetic changes have occurred in the regulatory genes, such as *dsx* and/or *Abd-B*, and/or in the genes controlled by these regulators, which are responsible for the development of the terminalia (Sánchez and Santamaria, 1997). These species-specific variations could be responsible for the morphological changes observed in the terminalia of these species. When the genotypes of the two species are put together within a single hybrid cell, divergent coadapted gene complexes are confronted. This might result in the formation of hybrid patterns different from those of their parental species; i.e., in the production of morphological diversity.

1.1.2.6.2. The genital disc in other dipteran species The expression of pattern-forming genes in the genital disc indicates that this disc is organized like the ventral, leg, and antenna discs (see **Chapter 1.9**). Evolutionary studies have suggested that the terminalia arose as a modification of a primitive appendage of a common ancestor to all arthropods (Matsuda, 1976). It has also been suggested that *Dll* expression along the proximal–distal axis arose once in an appendage-bearing ancestor, and has been utilized repeatedly in body wall outgrowths ever since (Panganiban *et al*., 1997). It can be hypothesized that the *Abd-B* function has modified this "ground state" in order to allow genitalia differentiation. This modification appears to be concomitant with the tendency towards a reduction of the structures (through the fusion of corresponding segments)

posterior to the 5th abdominal segment. Such reductions have occurred to different extents in males and females, leading to an increase in sexual dimorphism (Crampton, 1942; Matsuda, 1976). Two major features characterize this evolution: a decrease in the number and size of posterior-most tergites and sternites, and the fusion of posterior segments into a single genital disc (Crampton, 1942; Matsuda, 1976).

Insects such as *Musca* (Dübendorfer, 1971) and *Calliphora* (Emmert 1972a, 1972b), which are more primitive than *Drosophila*, have two lateral and one median genital discs. The primordium of the lateral discs corresponds to A8, and that of the single median disc to the fusion of A9 to A11 (**Figure 14**). In females, the lateral discs form the female genitalia, except for the parovaria. The median disc forms the parovaria (A9) and the female analia (A10–A11). In males, the lateral discs produce a reduced eighth tergite. The median disc develops the male genitalia (A9) and the male analia (segments A10–A11) (**Figure 14**). A further level of fusion has occurred in the *Drosophila* lineage, where segments A8 to A11 form a single genital disc (**Figure 14**).

1.1.3. Concluding Remarks and Perspectives

Perpetuation by sexual reproduction is the rule within the animal kingdom. The sex determination mechanisms have long been of major interest from a developmental and evolutionary point of view. There is a plethora of mechanisms whereby formation of males and females occurs through a species' ontogeny. This is more evident in insects, where all types of sex determination mechanisms are represented.

The characterization of the genes that control sexual development in *Drosophila* contributed significantly to the understanding of the molecular strategies behind sex determination, sex behavior, and the development of the germline (see **Chapters 1.2** and **1.4**). This knowledge paved the way for the study of the sex-determination mechanisms in other insects, so that the understanding of evolution of the sex-determining mechanisms is now possible. This will undoubtedly also contribute to an increased understanding of the evolution of other genetic pathways. In addition, results from studies on the mechanisms that control sexual development, are likely to contribute to the development of novel

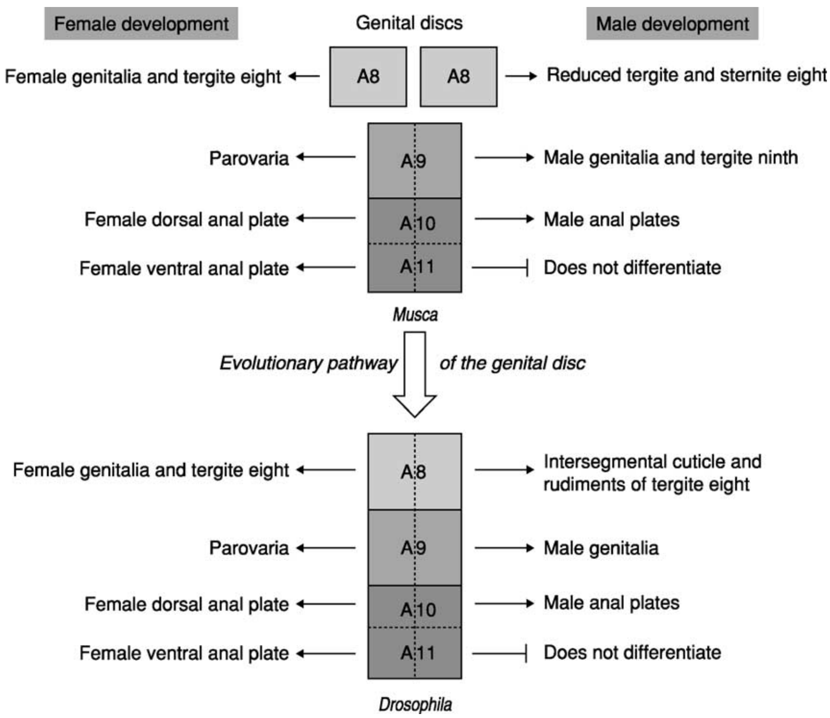


Figure 14 Hypothetical evolutionary pathway of the genital disc. Comparison of the genital discs of *Musca* (upper) and *Drosophila* (lower). A8–11 stands for abdominal segments 8–11. Strong experimental evidence supports the idea that the anal primordium of the *Drosophila* genital disc shows a complex organization (see text for explanation). Therefore, it is illustrated that two cell populations, assumed to be derived from segments A10 and A11, respectively, form the anal primordium (the horizontal broken line indicates the different segmental origin). It is further assumed that this organization is already present in *Musca*. The dotted line running from top to bottom indicates the bilateral symmetry organization of the genital disc.

strategies for the control or eradication of harmful insects that fhave a serious detrimental economic impact in agriculture and affect human health.

Traditionally, the control of pest insects has been performed by the use of insecticides. Although this strategy is quite effective, it has important disadvantages including the pollution of the environment, a significant risk for human health, and the appearance of insecticide resistance in the pest population. This is the reason why biological control of pest insects is receiving increasing attention. The biological control of insect pests has been carried out by the release of sterilized adults into the pest population, as is the case with the fruit flies. Although this method can be quite effective, there are certain aspects that are undesirable. Only the sterile males contribute positively to pest control, yet both males and females are mass-reared and released into the environment. Although females are sterile, they are undesirable because they can still cause crop damage through oviposition. Consequently, the selective elimination of females would be extremely important for biological control of insect pests. Two potential strategies can accomplish this goal. One of them is based on the selective expression, in females, of a harmful gene, which would be under the control of female-specific regulatory sequences. The second strategy is based on the transformation of females into sterile males or intersexes lacking the ovipositor structure, due to the expression of a gene that causes male instead of female development. Both strategies require the isolation and further characterization of the genes controlling sex determination and/or sex-specific regulatory sequences, as well as the development of vectors (transposable elements) that would allow the formation of transgenic fruit flies (see also **Chapters 4.12**, **4.13**, and **5.12**).

Drosophila has a single genital disc that produces the terminalia, the most sexually dimorphic structure in the adult. The founder cells are recruited from different segments to form the two genital primordia, female and male, plus the anal primordium that constitute the genital disc. In addition to being a good experimental model for the study of the control of pattern formation, the genital disc is also well suited for the study of morphological evolution. In this respect, two aspects of the genital disc can be highlighted. First, the fact that the female external terminalia are very similar in the *Drosophila* species. The external male terminalia, however, display great differences. Therefore, the terminalia are a good experimental model for understanding the mechanisms controlling morphogenesis. In addition, they are particularly well suited for studying the causes determining the relative morphological simplicity observed in the female genitalia, and the great morphological variety observed in the male genitalia. The second aspect is related to the origin of the genital disc. The expression of pattern-forming genes in the genital disc supports the idea that the terminalia arose as the result of the modification of a primitive appendage of a common ancestor to all arthropods. This modification appears to be concomitant with the tendency towards the reduction of structures (through the fusion of corresponding segments) posterior to segment A5. Such reductions have occurred to different extents in males and females, leading to an increase in sexual dimorphism. The knowledge acquired from the study of the *Drosophila* genital disc allows us to address questions about the organization and development of the genital disc in other insects. This will aid us to understand the evolution of the genital disc and, ultimately, morphological evolution.

Acknowledgments

This work was financed by grant BMC2002-02858 awarded to L.S. and grant BMC2002-03839 awarded to I.G. by the Comisión Interministerial de Ciencia y Tecnología (CICYT), and an institutional grant from the Fundación Areces. N.G. was supported by a research contract I3P from Consejo Supeior de Investigaciones Científicas (CSIC).

References

Ahmad, S.M., Baker, B.S., **2002**. Sex-specific deployment of FGF signaling in *Drosophila* recruits mesodermal cells into the male genital imaginal disc. *Cell 109*, 651–661.

Albrecht, E.B., Salz, H.K., **1993**. The *Drosophila* sex determination gene *snf* is utilized for the establishment of the female-specific splicing pattern of *Sex-lethal*. *Genetics 134*, 801–807.

Amrein, H., Maniatis, T., Nöthiger, R., **1990**. Alternatively spliced transcripts of the sex determining gene *tra-2* of *Drosophila* encode functional proteins of different size. *EMBO J. 9*, 3619–3629.

Andersen, N., **1979**. Der Einfluss des Alleles intersex auf die Geschlechtsdifferenzierung von *Drosophila melanogaster.* Diploma thesis, Zoological Institute, University of Zurich, Switzerland.

Bachiller, D., Sánchez, L., **1991**. Production of XO clones in XX females of *Drosophila*. *Genet. Res. 57*, 23–28.

Baker, B., Ridge, K., **1980**. Sex and the single cell. I. On the action of the major loci affecting sex determination in *Drosophila melanogaster. Genetics 94*, 383–423.

Bell, L.R., Horabin, J.I., Schedl, P., Cline, T.W., **1991**. Positive autoregulation of *Sex-lethal* by alternative splicing maintains the female determined state in *Drosophila*. *Cell 65*, 229–239.

Bell, L.R., Maine, E.M., Schedl, P., Cline, T.W., **1988**. *Sex-lethal*, a *Drosophila* sex determination switch gene, exhibits sex-specific RNA splicing and sequence similar to RNA binding proteins. *Cell 55*, 1037–1046.

Belote, J.M., Baker, B.S., **1982**. Sex determination in *Drosophila melanogaster*: analysis of *transformer-2*, a sex-transfroming locus. *Proc. Natl Acad. Sci. USA 79*, 1568–1572.

Belote, J.M., McKeown, M., Boggs, R.T., Ohkawa, R., Sosnowski, B.A., **1989**. The molecular genetics of *transformer*, a genetic switch-controlling sexual differentiation in *Drosophila*. *Devel. Genet. 10*, 143–154.

Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L., Jan, Y., **1992**. *Deadpan*, an essential pan-neural gene in *Drosophila*, encodes a helix–loop–helix protein similar to the hairy gene product. *Genes Devel. 6*, 2137–2151.

Billeter, J., Goodwin, S., O'Dell, K.M.C., **2002**. Genes mediating sex-specific behaviors in *Drosophila*. *Adv. Genet. 47*, 87–150.

Blair, S.S., Ralston, A., **1997**. Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior–posterior lineage restriction in the developing wing of *Drosophila*. *Development 124*, 4053–4063.

Boggs, R.T., Gregor, P., Idriss, S., Belote, J.M., McKeown, M., **1987**. Regulation of sexual differentiation in *Drosophila melanogaster* via alternative splicing of RNA from the *transformer* gene. *Cell 50*, 739–747.

Bopp, D., Bell, L.R., Cline, T.W., Schedl, P., **1991**. Developmental distribution of female-specific Sex-lethal proteins in *Drosophila melanogaster. Genes Devel. 5*, 403–415.

Bopp, D., Calhoun, G., Horabin, J.I., Samuels, M., Schedl, P., **1996**. Sex-specific control of *Sex-lethal* is a conserved mechanism for sex determination in the genus *Drosophila*. *Development 122*, 971–982.

Botella, L.M., Doñoro, C., Sánchez, L., Segarra, C., Granadino, B., **1996**. Cloning and characterization of the *scute (sc)* gene of *Drosophila subobscura*. *Genetics 144*, 1043–1051.

Bownes, M., **1994**. The regulation of the yolk protein genes, a family of sex differentiation genes in *Drosophila melanogaster. BioEssays 16*, 745–752.

Bownes, M., Nöthiger, R., **1981**. Sex determining genes and vitellogenin synthesis in *Drosophila melanogaster. Mol. Gen. Genet. 182*, 222–228.

Bownes, M., Roberts, S., **1979**. Acquisition of differentiative capacity in imaginal discs of *Drosophila melanogaster. J. Embryol. Exp. Morphol. 49*, 103–113.

Bridges, C.B., **1921**. Triploid intersexes in *Drosophila melanogaster. Science 54*, 252–254.

Bridges, C.B., **1925**. Sex in relation to chromosomes and genes. *Am. Nat. 59*, 127–137.

Burtis, K.C., Baker, B.S., **1989**. *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell 56*, 997–1010.

Cabrera, C., Alonso, M., **1991**. Activation by heterodimers of the achaete-scute and daughterless gene products of *Drosophila*. *EMBO J. 10*, 2965–2973.

Campbell, G., Tomlinson, A., **1995**. Initiation of proximal–distal axis in insect legs. *Development 121*, 619–628.

Casanova, J., Sánchez-Herrero, E., Morata, G., **1986**. Identification and characterization of a parasegment specific regulatory element of the *Abdominal-B* gene of *Drosophila*. *Cell 47*, 627–636.

Casares, F., Sánchez, L., Guerrero, I., Sánchez-Herrero, E., **1997**. The genital disc of *Drosophila melanogaster.* I. Segmental and compartmental organization. *Devel. Genes Evol. 207*, 216–228.

Chandler, D., McGuffin, M., Piskur, J., Yao, J., Baker, B., *et al.*, **1997**. Evolutionary conservation of regulatory strategies for the sex determination factor transformer-2. *Mol. Cell Biol. 17*, 2908–2919.

Chandra, H.S., **1985**. Sex determination: a hypothesis based on noncoding DNA. *Proc. Natl Acad. Sci. USA 82*, 1165–1169.

Chen, E.H., Baker, B.S., **1997**. Compartmental organization of the *Drosophila* genital imaginal discs. *Development 124*, 205–218.

Christiansen, A.E., Keisman, E.L., Ahmad, S.M., Baker, B.S., **2002**. Sex comes in from the cold: the integration of sex and pattern. *Trends Genet. 18*, 510–516.

Cline, T.W., **1978**. Two closely-linked mutations in *Drosophila melanogaster* that are lethal to opposite sexes and interact with *daughterless*. *Genetics 90*, 683–698.

Cline, T.W., **1984**. Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state. *Genetics 107*, 231–277.

Cline, T.W., **1986**. A female specific lethal lesion in an X-linked positive regulator of the *Drosophila* sex determination gene *Sex-lethal*. *Genetics 113*, 641–663.

Cline, T.W., **1988**. Evidence that "sisterless-a" and "sisterless-b" are two of several discrete "numerator elements" of the X:A sex determination signal in *Drosophila* that switch *Sex-lethal* between two alternative stable expression states. *Genetics 119*, 829–862.

Cline, T.W., Meyer, B.J., **1996**. Vive la différence: males vs. females in flies vs. worms. *Annu. Rev. Genet. 30*, 637–702.

Cline, T.W., Rudner, D.Z., Barbash, D.A., Bell, M., Vutien, R., **1999**. Functioning of the *Drosophila* integral U1/U2 protein Snf independent of U1 and U2 small nuclear ribonucleoprotein particles is revealed by snf^{+} gene dose effects. *Proc. Natl Acad. Sci. USA 96*, 14451–14458.

Cohen, S.M., Jürgens, G., **1989**. Proximal–distal pattern formation in *Drosophila*: cell autonomous requirements for *Distal-less* gene activity in limb development. *EMBO J. 8*, 2045–2055.

Coschigano, K.T., Wensink, P.C., **1993**. Sex-specific transcriptional regulation by the male and female doublesex proteins of *Drosophila*. *Genes Devel. 7*, 42–54.

Coyne, J.A., **1983**. Genetic basis of differences in genital morphology among three sibling species of *Drosophila*. *Evolution 37*, 1101–1118.

Coyne, J.A., Rux, J., David, J.R., **1991**. Genetics of morphological differences and hybrid sterility between *Drosophila sechellia* and its relatives. *Genet. Res. 57*, 113–122.

Crampton, G.C., **1942**. The external morphology of the Diptera. *Conneticut Geol. Nat. Hist. Survey Bull. 64*, 10–165.

Cronmiller, C., Schedl, P., Cline, T.W., **1988**. Molecular characterization of daughterless, a *Drosophila* sex determination gene with multiple roles in development. *Genes Devel. 2*, 1666–1676.

da Cunha, P.R., Granadino, B., Perondini, A.L.P., Sánchez, L., **1994**. Dosage compensation in sciarids is achieved by hypertranscription of the single X chromosome. *Genetics 138*, 787–790.

Dauwalder, B., Tsujimoto, S., Moss, J., Mattox, W., **2002**. The *Drosophila takeout* gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. *Gene Devel. 16*, 2879–2892.

Davis, R.L., Cheng, P., Lassar, A., Weintraub, H., **1990**. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell 60*, 733–746.

Denell, R.E., Jackson, J., **1972**. A genetic analysis of *transformer-Dominant*. *Drosophila Inform. Serv. 48*, 45.

Deshpande, G., Stukey, J., Schedl, P., **1995**. *Scute* (*sis*-b) function in *Drosophila* sex determination. *Mol. Cell Biol. 15*, 4430–4440.

Díaz-Benjumea, F.J., Cohen, B., Cohen, S.M., **1994**. Cell interaction between compartments establishes the proximal–distal axis of *Drosophila* legs. *Nature 372*, 175–179.

Dobzhansky, T., **1931**. Interaction between female and male parts in gynandromorphs of *Drosophila simulans*. *Roux's Arch. Entwickl. Mech. Org. 123*, 719–746.

Du, C., McGuffin, M.E., Dauwalder, B., Rabinow, L., Mattox, W., **1998**. Protein phosphorylation plays an essential role in the regulation of alternative splicing and sex determination in *Drosophila*. *Mol. Cell 2*, 741–750.

Dübendorfer, A., **1971**. Untersuchungen zum Anlageplan und determinationszustand der weiblichen Genital- und Analprimordien von *Musca domestica* L. *Roux's Arch. Devel. Biol. 168*, 142–168.

Dübendorfer, K., Nöthiger, R., **1982**. A clonal analysis of cell lineage and growth in the male and female genital disc of *Drosophila melanogaster*. *Roux's Arch. Devel. Biol. 191*, 42–55.

Duffy, J.B., Gergen, J.P., **1991**. The *Drosophila* segmentation gene *runt* acts as a position-specific numerator element necessary for the uniform expression of the sex-determinating gene *Sex-lethal*. *Genes Devel. 5*, 2176–2187.

Duncan, I., **1996**. How do single homeotic genes control multiple segment identities? *BioEssays 18*, 91–94.

Eaton, S., Kornberg, T., **1990**. Repression of *ci-D* in posterior compartments of *Drosophila* by *engrailed*. *Genes Devel. 4*, 1068–1077.

Ehrensperger, P.C., **1983**. Die Entwicklung der bisexuallen Anlage der Genitalien und Analien, untersucht an verschiedenen Geschlechtsmutanten der taufliege *Drosophila melanogaster*. Ph.D. thesis, University of Zurich, Switzerland.

Ellis, H.M., Apann, D.R., Posakony, J.W., **1990**. *extramacrochaete*, a negative regulator of sensory organs development in *Drosophila*, defines a new class of helix–loop–helix protein. *Cell 61*, 27–38.

Emerald, B.S., Roy, J.K., **1998**. Organising activities of *engrailed, hedgehog, wingless* and *decapentaplegic* in the genital discs of *Drosophila melanogaster*. *Devel. Genes Evol. 208*, 504–516.

Emmert, W., **1972a**. Entwicklungsleistungen abdominaler Imaginalscheiben von *Calliphora erytrocephala*: Experimentelle Untersuchungen zur Morphologie des Abdomens. *Roux's Arch. Devel. Biol. 169*, 87–133.

Emmert, W., **1972b**. Experimente zur Bestimmung des Anlageplans der männlichen und der weiblichen Genital-imaginalscheibe von *Calliphora*. *Roux's Arch. Devel. Biol. 171*, 109–120.

Epper, F., **1981**. Morphological analysis and fate map of the intersexual genital disc of the mutant *doublesex-dominant* in *Drosophila melanogaster*. *Devel. Biol. 88*, 104–114.

Epper, F., Bryant, P.J., **1983**. Sex-specific control of growth and differentiation in the *Drosophila* genital disc, studied using a temperature-sensitive transformer-2 mutation. *Devel. Biol. 100*, 294–307.

Epper, F., Nöthiger, R., **1982**. Genetic and developmental evidence for a repressed genital primordium in *Drosophila melanogaster*. *Devel. Biol. 94*, 163–175.

Epper, F., Sánchez, L., **1983**. Effect of *engrailed* in the genital disc of *Drosophila melanogaster*. *Devel. Biol. 100*, 387–398.

Erickson, J.W., Cline, T.W., **1991**. Molecular nature of the *Drosophila* sex determination signal and its link to neurogenesis. *Science 251*, 1071–1074.

Erickson, J., Cline, T.W., **1993**. A bZIP protein, sisterless-a, collaborates with bHLH transcription factors early in *Drosophila* development to determine sex. *Genes Devel. 7*, 1688–1702.

Erickson, J.W., Cline, T.W., **1998**. Key aspects of the primary sex determination mechanism are conserved across the genus *Drosophila*. *Development 125*, 3259–3268.

Estes, P., Keyes, L.N., Schedl, P., **1995**. Multiple response elements in the *Sex-lethal* promoter ensure its female-specific expression pattern. *Mol. Cell Biol. 15*, 904–917.

Estrada, B., Sánchez-Herrero, E., **2001**. The Hox gene *Abdominal-B* antagonizes appendage development in the genital disc of *Drosophila*. *Development 128*, 331–338.

Fiering, S., Whitelaw, E., Martin, D.I.K., **2000**. To be or not to be active: the stochastic nature of enhancer action. *BioEssays 22*, 381–387.

Flickinger, T.W., Salz, H.K., **1994**. The *Drosophila* sex determination gene *snf* encodes a nuclear protein with

sequences and functional similarity to the mammalian U1A snRNP protein. *Genes Devel. 8*, 914–925.

Freeland, D.E., Kuhn, D.T., **1996**. Expression patterns of developmental genes reveal segment and parasegment organization of *Drosophila melanogaster* genital discs. *Mech. Devel. 56*, 61–72.

Gadagkar, R., Nanjundiah, V., Joshi, N., Chandar, H., **1982**. Dosage compensation and sex determination in *Drosophila*: mechanisms of measurement of the X/A ratio. *J. Biosci. 4*, 377–390.

Garrell, J., Modolell, J., **1990**. The *Drosophila extramacrochaetae* locus, an antagonist of proneural genes that, like these genes, encodes a helix–loop–helix protein. *Cell 61*, 39–48.

Garrett-Engele, C.M., Siegal, M.L., Manoli, D.S., Williams, B.C., Li, H., *et al.*, **2002**. *intersex*, a gene required for female sexual development in *Drosophila*, is expressed in both sexes and functions together with doublesex to regulate terminal differentiation. *Development 129*, 4661–4675.

Gateff, E., Schneiderman, H., **1975**. Developmental capacities of immature eye–antennal imaginal disc of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 176*, 171–189.

Gerbi, S.A., **1986**. Unusual chromosome movements in sciarid flies. In: Hennig, W. (Ed.), Germ Line – Soma Differentiation. Springer-Verlag, Berlin, pp. 71–104.

Goralski, T.J., Edström, J.E., Baker, B.S., **1989**. The sex determination locus *transformer-2* of *Drosophila* encodes a polypeptide with similarity to RNA binding proteins. *Cell 56*, 1011–1018.

Gorfinkiel, N., Morata, G., Guerrero, I., **1997**. The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. *Gene Devel. 11*, 2259–2271.

Gorfinkiel, N., Sánchez, L., Guerrero, I., **1999**. *Drosophila* terminalia as an appendage-like structure. *Mech. Devel. 86*, 113–123.

Gorfinkiel, N., Sánchez, L., Guerrero, I., **2003**. Development of the *Drosophila* genital disc requires interactions between its segmental primordia. *Development 130*, 295–305.

Gowen, J.W., **1942**. Hermaphrodites in *Drosophila melanogaster. Drosophila Inform. Serv. 16*, 63.

Granadino, B., Campuzano, S., Sánchez, L., **1990**. The *Drosophila melanogaster fl(2)d* gene is needed for the female-specific splicing of *Sex-lethal* RNA. *EMBO J. 9*, 2597–2602.

Granadino, B., Penalva, L.O.F., Green, M.R., Valcárcel, J., Sánchez, L., **1997**. Distinct mechanisms of splicing regulations *in vivo* by the *Drosophila* protein Sex-lethal. *Proc. Natl Acad. Sci. USA 94*, 7343–7348.

Granadino, B., Penalva, L.O.F., Sánchez, L., **1996**. The gene *fl(2)d* is needed for the sex-specific splicing of *transformer* pre-mRNA but not for *double-sex* pre-mRNA in *Drosophila melanogaster. Mol. Gen. Genet. 253*, 26–31.

Granadino, B., San Juán, A.B., Santamaría, P., Sánchez, L., **1992**. Evidence of a dual function in *fl(2)d* a gene needed for *Sex-lethal* expression in *Drosophila melanogaster. Genetics 130*, 597–612.

Grimm, S.Y., Pflugfelder, G.O., **1996**. Control of the gene *optomotor-blind* in *Drosophila* wing development by *decapentaplegic* and *wingless*. *Science 271*, 1601–1604.

Hartenstein, V., Jan, Y., **1992**. Studying *Drosophila* embryogenesis with P-*lacZ* enhancer trap lines. *Roux's Arch. Devel. Biol. 183*, 85–100.

Hedley, M., Maniatis, T., **1991**. Sex-specific splicing and polyadenylation of *dsx* pre-mRNA requires a sequence that binds specifically to tra-2 protein *in vitro*. *Cell 65*, 579–586.

Hilfiker, A., Amrein, H., Dübendorfer, A., Nöthiger, R., **1995**. The gene *virilizer* is required for female-specific splicing controlled by *Sxl*, the master gene for sexual development in *Drosophila*. *Development 121*, 4017–4026.

Hilfiker, A., Nöthiger, R., **1991**. The temperature-sensitive mutation vir^{ts} (*virilizer*) identifies a new gene involved in sex determination of *Drosophila*. *Roux's Arch. Devel. Biol. 200*, 240–248.

Horabin, J.I., Schedl, P., **1993a**. Regulated splicing of the *Drosophila Sex-lethal* male exon involves a blockage mechanism. *Mol. Cell Biol. 13*, 1408–1414.

Horabin, J.I., Schedl, P., **1993b**. *Sex-lethal* autoregulation requires multiple *cis*-acting elements upstream and downstream of the male exon and appears to depend largely on controlling the use of the male exon 5′ splice site. *Mol. Cell Biol. 13*, 7734–7746.

Horabin, J.I., Schedl, P., **1996**. Splicing of the *Drosophila Sex-lethal* early transcripts involves exon skipping that is independent of Sex-lethal protein. *RNA 2*, 1–10.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H., Shimura, Y., **1991**. Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in *Drosophila*. *Science 252*, 833–836.

Hoshijima, K., Kohyama, A., Watakabe, I., Inoue, K., Sakamoto, H., *et al.*, **1995**. Transcriptional regulation of the *Sex-lethal* gene by helix–loop–helix proteins. *Nucl. Acids Res. 23*, 3441–3448.

Hume, D., **2000**. Probability in transcriptional regulation and its implications for leukocyte differentiation and inducible gene expression. *Blood 96*, 2323–2328.

Inoue, K., Hoshijima, K., Higuchi, I., Sakamoto, H., Shimura, Y., **1992**. Binding of the *Drosophila* Transformer and Transformer-2 proteins to the regulatory elements of *doublesex* primary transcripts for sex-specific RNA processing. *Proc. Natl Acad. Sci. USA 89*, 8092–8096.

Inoue, K., Hoshijima, K., Sakamoto, H., Shimura, Y., **1990**. Binding of the *Drosophila Sex-lethal* gene product to the alternative splice site of *transformer* primary transcript. *Nature 344*, 461–463.

Janning, W., **1978**. Gynandromorphs fate maps in *Drosophila*. In: Gehring, W.J. (Ed.), Genetic Mosaics and Cell Differentiation. Springer-Verlag, Berlin, pp. 1–28.

Janning, W., Labahrt, C., Nöthiger, R., **1983**. Cell lineage restrictions in the genital disc of *Drosophila* revealed by Minute gynandromorphs. *Roux's Arch. Devel. Biol. 192*, 337–346.

Jinks, T.M., Polydorides, A.D., Calhoun, G., Schedl, P., **2000**. The JAK/STAT signaling pathway is required for the initial choice of sexual identity in *Drosophila melanogaster*. *Mol. Cell 5*, 581–587.

Jurnish, V.A., Burtis, K.C., **1993**. A positive role in differentiation for the male doublesex protein of *Drosophila*. *Devel. Biol. 155*, 235–249.

Kanaar, R., Lee, A.L., Rudner, D.Z., Wemmer, D.E., Rio, D.C., **1995**. Interaction of the *Sex-lethal* RNA binding domains with RNA. *EMBO J. 14*, 4530–4539.

Keisman, E.L., Baker, B.S., **2001**. The *Drosophila* sex determination hierarchy modulates *wingless* and *decapentaplegic* signaling to deploy dachshund sex-specifically in the genital imaginal disc. *Development 128*, 1643–1656.

Keyes, L.N., Cline, T.W., Schedl, P., **1992**. The primary sex determination signal of *Drosophila* acts at the level of transcription. *Cell 68*, 933–943.

Kopp, A., Duncan, I., Carroll, S.B., **2000**. Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature 408*, 553–559.

Kornberg, T., Siden, I., O'Farrel, P., Simon, M., **1985**. The *engrailed* locus of *Drosophila*: *in situ* localization of transcripts reveals compartment specific expression. *Cell 40*, 45–53.

Kramer, S.G., Jinks, T.M., Schedl, P., Gergen, J.P., **1999**. Direct activation of *Sex-lethal* transcription by the *Drosophila* Runt protein. *Development 126*, 191–200.

Kröger, H., **1959**. The genetic control of genital morphology in *Drosophila*. A study of the external genitalia of sex mosaics. *Wilhelm Roux's Arch Entwickl Mech. Org. 151*, 301–322.

Kuhn, D.T., Turenchalk, G., Mack, J.A., Packert, G., Kornberg, T.B., **1995**. Analysis of the genes involved in organizing the tail segments of the *Drosophila melanogaster* embryo. *Mech. Devel. 53*, 3–13.

Kulathinal, R.J., Skwarek, L., Morton, R.A., Singh, R.S., **2003**. Rapid evolution of the sex-determining gene, *transformer*: structural diversity and rate heterogeneity among sibling species of *Drosophila*. *Mol. Biol. Evol. 20*, 441–452.

Lachaise, D., Cariou, M.L., David, J.R., Lemeunier, F., Tsacas, L., *et al.*, **1988**. Historical biogeography of the *Drosophila melanogaster* species subgroup. *Evol. Biol. 22*, 159–225.

Lallena, M.J., Chalmers, K., Llamazares, S., Lamond, A., Valcárcel, J., **2002**. Splicing regulation at the second catalytic step by *Sex-lethal* involves 3′ splice site recognition by SPF45. *Cell 109*, 85–296.

Lawrence, P.A., Ashburner, M., Johnston, P., **1993**. An attempt to hybridize *Drosophila* species using pole cell transplantation. *Genetics 134*, 1145–1148.

Lawrence, P.A., Struhl, G., **1982**. Further studies of the *engrailed* phenotype in *Drosophila*. *EMBO J. 1*, 827–833.

Lawrence, P.A., Struhl, G., **1996**. Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell 85*, 951–961.

Li, H., Baker, B.S., **1998a**. *her*, a gene required for sexual differentiation in *Drosophila*, encodes a zinc finger protein with characteristics of ZFY-like proteins and is expressed independently of the sex determination hierarchy. *Development 125*, 225–235.

Li, H., Baker, B.S., **1998b**. *hermaphrodite* and *doublesex* function both dependently and independently to control various aspects of sexual differentiation in *Drosophila*. *Development 125*, 2641–2651.

Littlefield, C.L., Bryant, P.J., **1979**. Sexual homologies and intercalation between parts of the male and female genital discs of *Drosophila melanogaster*. *Devel. Biol. 70*, 180–194.

Liu, J., Mercer, J.M., Stam, L.F., Gibson, G.C., Zeng, Z.B., *et al.*, **1996**. Genetic analysis of a morphological shape difference in the male genitalia of *Drosophila simulans* and *Drosophila mauritiana*. *Genetics 142*, 1129–1145.

Liu, Y., Belote, J., **1995**. Protein–protein interactions among components of the *Drosophila* primary sex determination signal. *Mol. Gen. Genet. 248*, 182–189.

Lucchesi, J.C., Skripsky, T., **1981**. The link between dosage compensation and sex differentiation in *Drosophila melanogaster*. *Chromosoma 82*, 217–227.

Lynch, K.W., Maniatis, T., **1996**. Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila doublesex* splicing enhancer. *Genes Devel. 10*, 2089–2101.

Maniatis, M., **1991**. Mechanisms of alternative pre-mRNA splicing. *Science 251*, 33–34.

Massari, M., Murre, C., **2000**. Helix–loop–helix proteins: regulators of transcription in eucaryotic organisms. *Mol. Cell Biol. 20*, 429–440.

Matsuda, R., **1976**. Morphology and Evolution of the Insect Abdomen. Pergamon, Oxford.

Mattox, W., Palmer, M., Baker, B., **1990**. Alternative splicing of the sex determination gene *transformer-2* is sex-specific in the germ line but not in the soma. *Genes Devel. 4*, 789–805.

Meise, M., Hilfiker-Kleiner, D., Dübendorfer, A., Brunner, C., Nöthiger, R., *et al.*, **1998**. *Sex-lethal*, the master sex-determining gene in *Drosophila*, is not sex-specifically regulated in *Musca domestica*. *Development 125*, 1487–1494.

Mindek, G., Nöthiger, R., **1973**. Parameters influencing the acquisition of competence for metamorphosis in imaginal discs of *Drosophila*. *J. Insect Physiol. 19*, 1711–1720.

Moreno, E., Morata, G., **1999**. *Caudal* is the Hox gene that specifies the most posterior *Drosophila* segment. *Nature 400*, 873–877.

Müller-Holtkamp, F., **1995**. The *Sex-lethal* gene in *Chrysomya rufifacies* is highly conserved in sequence and exon–intron organization. *J. Mol. Evol. 41*, 467–477.

Murre, C., McCaw, P., Vaessin, H., Caudy, M., Jan, L., *et al.*, **1989**. Interactions between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell 58*, 537–544.

Nagengast, A.A., Stitzinger, S.M., Tseng, C.H., Mount, S.M., Salz, H.K., **2003**. *Sex-lethal* splicing autoregulation in vivo: interactions between SEX-LETHAL, the U1 snRNP and U2AF underlie male exon skipping. *Development 130*, 463–471.

Nagoshi, R.N., Baker, B.S., **1990**. Regulation of sex-specific RNA splicing at the *Drosophila doublesex* gene: *cis*-acting mutations in exon sequences alter sex-specific RNA splicing pattrens. *Genes Devel. 4*, 89–97.

Newby, W.W., **1942**. A study of intersexes produced by a dominant mutation in *Drosophila virilis*. *Univ. Texas Publ. 4228*, 113–145.

Niessen, M., Schneiter, R., Nöthiger, R., **2001**. Molecular identification of *virilizer*, a gene required for the expression of the sex-determining gene *Sex-lethal* in *Drosophila melanogaster. Genetics 157*, 679–688.

Nikolakaki, E., Du, C., Lai, J., Giannakouros, T., Cantley, L., *et al.*, **2002**. Phosphorylation by LAMMER protein kinases: determination of a consensus site, identification of *in vitro* substrates, and implications for substrate preferences. *Biochemistry 41*, 2055–2066.

Nöthiger, R., **1972**. The larval development of imaginal discs. In: Ursprung, H., Nöthiger, R. (Eds.), The Biology of Imaginal Discs. Springer, Berlin, pp. 1–34.

Nöthiger, R., Dübendorfer, A., Epper, F., **1977**. Gynandromorphs reveal two separate primordia for male and female genitalia. *Roux's Arch. Devel. Biol. 181*, 367–373.

Ohbayashi, F., Suzuki, M., Mita, K., Okano, K., Shimada, T., **2001**. A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol. 128*, 145–158.

O'Kane, C., Asztalos, Z., **1999**. Sexual behaviour: courting dissatisfaction. *Current Biol. 9*, R289–R292.

Oliver, B., **2002**. Genetic control of germline sexual dimorphism in *Drosophila*. *Int. Rev. Cytol. 219*, 1–60.

O'Neil, M.T., Belote, J.M., **1992**. Interspecific comparison of *transformer* gene of *Drosophila* reveals an unusually high degree of evolutionary divergence. *Genetics 131*, 113–128.

Ortega, A., Niksic, M., Bachi, A., Wilm, M., Sánchez, L., *et al.*, **2003**. Biochemical function of female-lethal (2)D/Wilms' tumor suppressor-1-associated proteins in alternative pre-mRNA splicing. *J. Biol. Chem. 278*, 3040–3047.

Pane, A., Salvemini, M., Bovi, P.D., Polito, C., Saccone, G., **2002**. The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development 129*, 3715–3725.

Panganiban, G., Irvine, S.M., Lowe, C., Roehl, H., Corley, L.S., *et al.*, **1997**. The origin and evolution of animal appendages. *Proc. Natl Acad. Sci. USA 94*, 5162–5166.

Parkhurst, S., Bopp, D., Ish-Horowicz, D., **1990**. X:A ratio, the primary sex determination signal in *Drosophila*, is transduced by helix–loop–helix proteins. *Cell 63*, 1179–1191.

Parkhurst, S.M., Ish-Horowicz, D., **1992**. Common denominators for sex. *Current Biol. 2*, 629–631.

Penalva, L.O.F., Lallena, M.J., Valcárcel, J., **2001**. Switch in 3′ splice site recognition between exon definition and splicing catalysis is important for *Sex-lethal* autoregulation. *Mol. Cell Biol. 21*, 1986–1996.

Penalva, L.O.F., Ruiz, M.F., Ortega, A., Granadino, B., Vicente, L., *et al.*, **2000**. The *Drosophila fl(2)d* gene, required for female-specific splicing of *Sxl* and *tra* pre-mRNAs, encodes a novel nuclear protein with a HQ-rich domain. *Genetics 155*, 129–139.

Penalva, L.O.F., Sakamoto, H., Navarro-Sabate, A., Sakashita, E., Granadino, B., *et al.*, **1996**. Regulation of the gene *Sex-lethal*: a comparative analysis of *Drosophila melanogaster* and *Drosophila subobscura*. *Genetics 144*, 1653–1664.

Penalva, L.O.F., Sánchez, L., **2003**. The RNA binding protein Sex-lethal (Sxl) and the control of *Drosophila* sex determination and dosage compensation. *Microbiol. Mol. Biol. Rev. 67*, 343–359.

Postlethwait, J.H., Bryant, P.J., Schubiger, G., **1972**. The homeotic effect of "*tumorous-head*" in *Drosophila melanogaster. Devel. Biol. 29*, 337–342.

Pultz, M., Baker, B., **1995**. The dual role of *hermaphrodite* in the *Drosophila* sex determination regulatory hierarchy. *Development 121*, 99–111.

Raff, R.A., Kauffman, T.C., **1983**. Embryos, Genes and Evolution: The Developmental Genetic Basis of Evolutionary Change. Macmillan Publishers, London.

Rodriguez, I., Basler, K., **1997**. Control of compartmental affinity boundaries by *hedgehog*. *Nature 389*, 614–618.

Ruiz, M.F., Goday, C., González, P., Sánchez, L., **2003**. Molecular analysis and developmental expression of the *Sex-lethal* gene of *Sciara ocellaris* (Diptera Order, Nematocera Suborder). *Gene Exp. Patt. (Mech. Devel.) 3*, 341–346.

Saccone, G., Peluso, I., Artiaco, D., Giordano, E., Bopp, D., *et al.*, **1998**. The *Ceratitis capitata* homologue of the *Drosophila* sex-determining gene *Sex-lethal* is structurally conserved, but not sex-specifically regulated. *Development 125*, 1495–1500.

Sakamoto, H., Inoue, K., Higuchi, I., Ono, Y., Shimura, Y., **1992**. Control of *Drosophila Sex-lethal* pre-mRNA splicing by its own female-specific product. *Nucl. Acids Res. 20*, 5533–5540.

Sakashita, E., Sakamoto, H., **1994**. Characterization of RNA binding specificity of the *Drosophila* Sex-lethal protein by *in vitro* ligand selection. *Nucl. Acids Res. 22*, 4082–4086.

Sakashita, E., Sakamoto, H., **1996**. Protein–RNA and protein–protein interactions of the *Drosophila Sex-lethal* mediated by its RNA-binding domains. *J. Biochem. 120*, 1028–1033.

Salz, H.K., Flickinger, T.W., **1996**. Both loss-of-function and gain-of-function mutations in *snf* define a role for snRNP proteins in regulating *Sex-lethal* pre-mRNA splicing in *Drosophila* development. *Genetics 144*, 95–108.

Salz, H.K., Maine, E.M., Keyes, L.N., Samuels, M.E., Cline, T.W., *et al.*, **1989**. The *Drosophila* female-specific sex-determination gene, *Sex-lethal*, has stage-, tissue-, and sex-specific RNAs suggesting multiple modes of regulation. *Genes Devel. 3*, 708–719.

Samuels, M.E., Bopp, D., Colvin, R.A., Roscigno, R.F., García-Blanco, M., *et al.*, **1994**. RNA binding by Sxl proteins *in vitro* and *in vivo*. *Mol. Cell Biol. 14*, 4975–4990.

Samuels, M.E., Deshpande, G., Schedl, P., **1998**. Activities of the Sex-lethal protein in RNA binding and protein:-protein interactions. *Nucl. Acids Res. 26*, 2625–2637.

Samuels, M.E., Schedl, P., Cline, T.W., **1991**. The complex set of late transcripts from the *Drosophila* sex determination gene *Sex-lethal* encodes multiple related polypeptides. *Mol. Cell Biol. 11*, 3584–3602.

Sánchez, L., Casares, F., Gorfinkiel, N., Guerrero, I., **1997**. The genital disc of *Drosophila melanogaster.* II. Role of the genes *hedgehog, decapentaplegic* and *wingless*. *Devel. Genes Evol. 207*, 229–241.

Sánchez, L., Gorfinkiel, N., Guerrero, I., **2001**. Sex determination genes control the development of the *Drosophila* genital disc modulating the response to Hedgehog, Wingless, and Decapentaplegic signals. *Development 128*, 1033–1043.

Sánchez, L., Granadino, B., **1992**. Gradual acquisition of the developmental capacity to differentiate adult structures by the genital disc of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 201*, 105–112.

Sánchez, L., Granadino, B., Torres, M., **1994**. Sex determination in *Drosophila melanogaster*: X-linked genes involved in the initial step of *Sex-lethal* activation. *Devel. Genet. 15*, 251–264.

Sánchez, L., Guerrero, I., **2001**. The development of the *Drosophila* genital disc. *BioEssays 23*, 698–707.

Sánchez, L., López, P., Granadino, B., **1998**. Early events associated with sex determination in *Drosophila melanogaster.* In: Chatterjee, R., Sánchez, L. (Eds.), Genome Analysis in Eukaryotes: Developmental and Evolutionary Aspects. Narosa Publishing House (Springer-Verlag), India, pp. 98–119.

Sánchez, L., Nöthiger, R., **1983**. Sex determination and dosage compensation in *Drosophila melanogaster*: production of male clones in XX females. *EMBO J. 2*, 485–491.

Sánchez, L., Santamaria, P., **1997**. Reproductive isolation and morphogenetic evolution in *Drosophila* analyzed by breakage of ethological barriers. *Genetics 147*, 231–242.

Sánchez, L., Schmid, H., **1984**. Analysis of the sterility of male hybrids between *Drosophila simulans* and *Drosophila mauritiana*. *Genetics 107* (Suppl.), s93–s94.

Sánchez-Herrero, E., Vernós, I., Marco, R., Morata, G., **1985**. Genetic organization of *Drosophila* bithorax complex. *Nature 313*, 108–113.

Santamaría, P., **1977**. On the causes of sterility in some interspecific hybrids from the *melanogaster* subgroup of *Drosophila*. *Roux's Arch. Devel. Biol. 182*, 305–310.

Santamaría, P., **1983**. Analysis of haploid mosaics in *Drosophila*. *Devel. Biol. 96*, 285–295.

Santamaría, P., Gans, M., **1980**. Chimeras of *Drosophila melanogaster* obtained by injection of haploid nuclei. *Nature 287*, 143–144.

Schmid, H., Sánchez, L., Nöthiger, R., **1984**. Heterospecific combinations of germ cells and gonadal soma between *Drosophila melanogaster*, *D. mauritiana* and *D. ananassae*. *Roux's Arch. Devel. Biol. 194*, 99–106.

Schubiger, G., **1974**. Acquisition of differentiative competence in the imaginal leg disc of *Drosophila*. *Roux's Arch. Devel. Biol. 174*, 303–311.

Schüpbach, T., Wieschaus, E., Nöthiger, R., **1978**. The embryonic organization of the genital disc studied in genetic mosaics of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 185*, 249–270.

Schütt, C., Nöthiger, R., **2000**. Structure, function and evolution of sex-determing system in Dipteran insects. *Development 127*, 667–677.

Sefton, L., Timmer, J.R., Zhang, Y., Beranger, F., Cline, T.W., **2000**. An extracellular activator of the *Drosophila* JAK/STAT pathway is a sex-determination signal element. *Nature 405*, 970–973.

Shearman, D., Frommer, M., **1998**. The *Bactrocera tryoni* homologue of the *Drosophila melanogaster* sex-determination gene *doublesex*. *Insect Mol. Biol. 7*, 355–366.

Sievert, V., Kuhn, S., Paululat, A., Traut, W., **2000**. Sequence conservation and expression of the *Sex-lethal* homologue in the fly *Megaselia scalaris*. *Genome 43*, 382–390.

Sievert, V., Kuhn, S., Traut, W., **1997**. Expression of the sex determination cascade genes *Sex-lethal* and *doublesex* in the phorid fly *Megaselia scalaris*. *Genome 40*, 211–214.

Singh, R., Valcárcel, J., Green, M.R., **1995**. Distinct binding and functions of higher eucaryotic polypirimidine tract-binding proteins. *Science 268*, 1173–1176.

Sosnowski, B.A., Belote, J.M., McKeown, M., **1989**. Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence dependent splice site blockage. *Cell 58*, 449–459.

Steinmann-Zwicky, M., Amrein, H., Nöthiger, R., **1990**. Genetic control of sex determination in *Drosophila*. In: Wright, T.R.F. (Ed.), Advances in Genetics. Academic Press, San Diego, CA, pp. 189–337.

Sturtevant, A., **1929**. The *claret* mutant type of *Drosophila simulans*: a study of chromosome elimination and cell-lineage. *Z. Wiss. Zool. 135*, 323–356.

Sunkel, C.E., Whittle, J.R.S., **1987**. *Brista*: a gene involved in the specification and differentiation of distal cephalic and thoracic structures in *Drosophila melanogaster. Roux's Arch. Devel. Biol. 196*, 124–132.

Suzuki, M.G., Ohbayashi, F., Mita, K., Shimada, T., **2001**. The mechanism of sex-specific splicing at the *doublesex* gene is different between *Drosophila melanogaster* and *Bombyx mori*. *Insect Biochem. Mol. Biol. 31*, 1201–1211.

Tian, M., Maniatis, T., **1993**. A splicing enhancer complex controls alternative splicing of *doublesex* pre-mRNA. *Cell 74*, 105–114.

Tian, M., Maniatis, T., **1994**. A splicing enhancer exhibits both constitutive and regulated activities. *Genes Devel. 8*, 1703–1712.

Tiong, S., Bone, L.M., Whittle, J.R., **1985**. Recessive lethal mutations within the bithorax complex in *Drosophila*. *Mol. Gen. Genet. 200*, 335–342.

Torres, M., Sánchez, L., **1989**. The *scute (T4)* gene acts as a numerator element of the X:A signal that determines the state of activity of *Sex-lethal* in *Drosophila melanogaster. EMBO J. 10*, 3079–3086.

Torres, M., Sánchez, L., **1991**. The *sisterless-b* function of the *Drosophila* gene *scute* is restricted to the state when the X:A ratio signal determines the activity of *Sex-lethal*. *Development 113*, 715–722.

Torres, M., Sánchez, L., **1992**. The segmentation gene *runt* is needed to activate *Sex-lethal*, a gene that controls sex determination and dosage compensation in *Drosophila*. *Genet. Res. 59*, 189–198.

Tsacas, L., Bocquet, C., **1976**. L'espèce chez les *Drosophilidae*. *Mém. Soc. Zool. Fr. 39*, 203–247.

Valcárcel, J., Singh, R., Zamore, P.D., Green, M.R., **1993**. The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. *Nature 362*, 171–175.

Van Doren, M., Ellis, H., Posakony, J., **1991**. The *Drosophila* extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes. *Development 113*, 245–255.

Villares, R., Cabrera, C., **1987**. The *achaete–scute* gene complex of *D. melanogaster*: conserved domains in a subset of genes required for neurogenesis and their homology to *myc*. *Cell 50*, 415–424.

Voronova, A., Baltimore, D., **1990**. Mutations that disrupt DNA binding and dimer formation in the E47 helix–loop–helix protein map to distinct domains. *Proc. Natl Acad. Sci. USA 87*, 4722–4726.

Wang, J., Bell, L.R., **1994**. The Sex-lethal amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. *Genes Devel. 8*, 2072–2085.

Wang, J., Manley, J.L., **1997**. Regulation of pre-mRNA splicing in metazoa. *Curr. Opin. Genet. Devel. 7*, 205–211.

Waterbury, J.A., Horabin, J.I., Bopp, D., Schedl, P., **2000**. Sex determination in the *Drosophila* germline is dictated by the sexual identity of the surrounding soma. *Genetics 155*, 1741–1756.

Waterbury, J.A., Jackson, L.L., Schedl, P., **1999**. Analysis of the doublesex female protein in *Drosophila melanogaster*: role in sexual differentiation and behavior and dependence on intersex. *Genetics 152*, 1653–1667.

Weaver, T.A., White, R.A.H., **1995**. *headcase*, an imaginal specific gene required for adult morphogenesis in *Drosophila melanogaster. Development 121*, 4149–4160.

Whiteley, M., Noguchi, P., Sensabaugh, S., Odenwald, W., Kassis, J., **1992**. The *Drosophila* gene *escargot* encodes a zinc finger motif found in snail-related genes. *Mech. Devel. 36*, 117–127.

Wieschaus, E., Nöthiger, R., **1982**. The role of the *transformer* genes in the development of the genitalia and analia of *Drosophila melanogaster. Devel. Biol. 90*, 320–334.

Wilkins, A., **1995**. Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. *BioEssays 17*, 71–77.

Winston, R.L., Millar, D., Gottesfeld, J., Kent, S., **1999**. Characterization of the DNA binding properties of the bHLH domain of Deadpan to single and tandem sites. *Biochemistry 38*, 5138–5146.

Wu, L., Lengyel, J., **1998**. Role of *caudal* in hindgut specification and gastrulation suggests homology between *Drosophila* amnioproctodeal invagination and vertebrate blastopore. *Development 125*, 2433–2442.

Yamamoto, D., Fujitani, K., Usui, K., Ito, H., Nakano, Y., **1998**. From behavior to development: genes for sexual behavior define the neuronal sexual switch in *Drosophila*. *Mech. Dev. 73*, 135–146.

Yang, D., Lu, H., Hong, Y., Jinks, T., Estes, P., *et al.*, **2001**. Interpretation of X chromosome dose at *Sex-lethal* requires non-E-box sites for the basic helix–loop–helix proteins SisB and Daughterless. *Mol. Cell. Biol. 21*, 1581–1592.

Yanowitz, J.L., Deshpande, G., Calhoun, G., Schedl, P., **1999**. An N-terminal truncation uncouples the sex-transforming and dosage compensation functions of *Sex-lethal*. *Mol. Cell Biol. 19*, 3018–3028.

Younger-Shepherd, S., Vaessin, H., Bier, E., Jan, L.Y., Jan, Y.N., **1992**. *deadpan*, an essential pan-neural gene encoding an HLH protein, acts as a denominator in *Drosophila* sex determination. *Cell 70*, 911–922.

no>Yun 2000Yun, B., Lee, K., Farkas, R., Hitte, C., Rabinow, L., **2000**. The LAMMER protein kinase encoded by the *Doa* locus of *Drosophila* is required in both somatic and germline cells and is expressed as both nuclear and cytoplasmic isoforms throughout development. *Genetics 156*, 749–761.

Zlokarnik, G., Negulescu, P., Knapp, T., Mere, L., Burres, N., *et al.*, **1998**. Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. *Science 279*, 84–88.

Zuo, P., Maniatis, T., **1996**. The splicing factor U2AF^{35} mediates critical protein–protein interactions in constitutive and enhancer-dependent splicing. *Genes Devel. 10*, 1356–1368.

1.2 Oogenesis

D A Dansereau and P Lasko,
McGill University, Montreal, QC, Canada
D McKearin, University of Texas,
Dallas, TX, USA

1.2.1. Introduction

Drosophila have polytrophic ovarioles: each oocyte develops in an egg chamber (follicle), containing a cluster of interconnected germline cells (cyst) surrounded by a layer of mesodermal follicle cells. In principle, whatever applies to *Drosophila* also broadly applies to many other insects with polytrophic ovarioles (e.g., *Anopheles* and *Bombyx*; see also **Chapter 1.3**).

The *Drosophila* oocyte develops within a cyst of 16 interconnected germline cells, all derived from one cystoblast through mitotic divisions. Egg chambers are assembled in the germarium, a mitotically active zone at the anterior tip of each ovariole. Here, mitotic division of a germline stem cell (GSC) renews the GSC and produces a cystoblast, which undergoes four synchronous mitotic divisions to generate a 16-cell cyst (Figure 1). Cystocyte cell cycles are rapid and uncoupled from cell growth, leading to a progressive reduction in cell volume. Older cysts migrate posteriorly through the germarium, and are individually surrounded by mesodermal follicle cells, thus forming egg chambers. Follicle cell migration is associated with the flattening and lengthening of the cyst perpendicular to the anterior–posterior (A–P) axis so that it spans the width of the germarium.

Each germarium, which can be further subdivided into four consecutive and morphologically and functionally distinct regions (1, 2A, 2B, and 3; Figure 2), typically contains 7–12 developing 16-cell cysts. There are 6–7 egg chambers per ovariole connected to each other by stalks of follicle cells (Figure 2). As

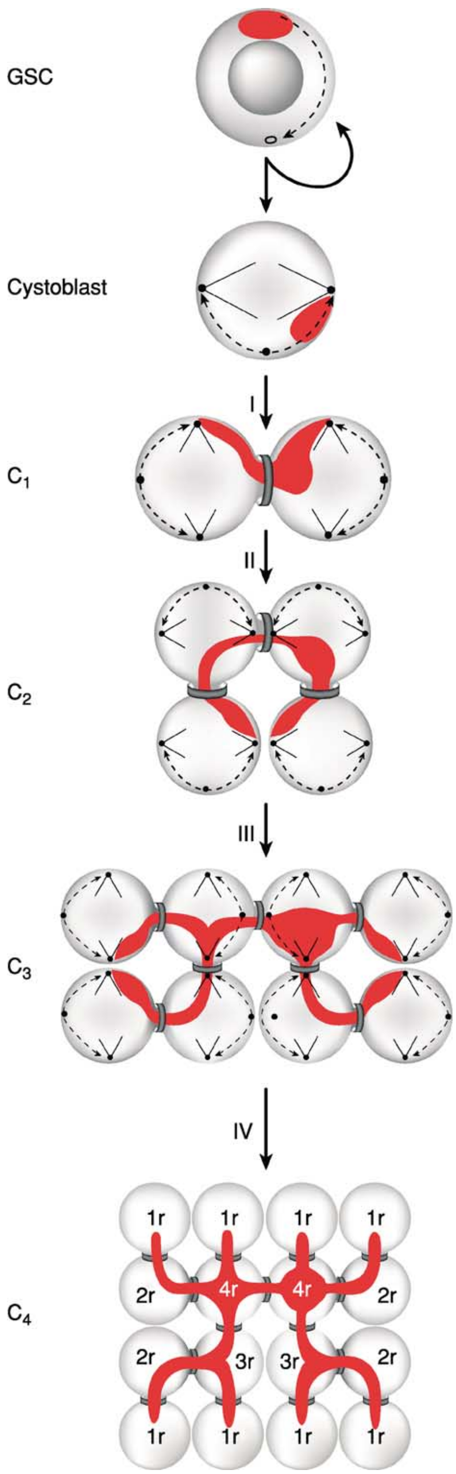


Figure 1 Cyst divisions in region 1 of the germarium. Oogenesis begins with the asymmetric division of a germline stem cell (GSC) at the anterior tip of germarial region 1. One daughter remains in the stem cell niche and is maintained as a GSC, while the second leaves the stem cell niche and differentiates as a cystoblast (discussed in Section 1.2.2). Each cystoblast divides four times (I–IV) with incomplete cytokinesis to give rise to a cyst of 16 cells connected to each other by cytoplasmic bridges called ring canals (discussed in Section 1.2.3). This leads to the production of a cyst containing only two cells with four ring canals (marked 4r), one of which becomes the oocyte and undergoes meiosis (discussed in Section 1.2.4.1). Broken arrows denote centriole migration and open arrowheads represent mitotic spindles. During these divisions, a membranous cytoplasmic organelle called the fusome, shown in red, anchors one pole of each mitotic spindle, orienting and synchronizing each cyst division (discussed in Sections 1.2.3.2 and 1.2.3.3). These organelles are spherical in GSCs, where they are called spectrosomes, but grow and fuse to become elongated, branched structures that extend through each ring canal during cyst divisions. The cystocyte that is chosen to become the oocyte might be determined as early as the first cystoblast division, when one daughter inherits more fusome than the other. This initial asymmetry is propagated through subsequent divisions and likely plays a central role in specifying oocyte fate (discussed in Section 1.2.3.2).

development proceeds, a rhythmically contracting muscular epithelial sheath pushes egg chambers progressively toward the posterior. Thus, each ovariole can be considered as a linear assembly line for the formation of eggs, with progressively older egg chambers toward the posterior. Seventeen to 20 discrete tubular ovarioles are packaged together in parallel, in each of the two ovaries. Each ovary is surrounded by a network of muscle fibers called the peritoneal sheath and both are connected to a common oviduct with associated accessory sex glands (see **Chapter 1.5**). As mature eggs pass from the common oviduct into the uterus, they are fertilized by sperm stored in the ventral receptacle and spermathecae. The germline-derived cell types are the GSCs and the cystocytes of individual egg chambers, which develop into nurse cells and the oocyte. Somatic tissue surrounding the egg chambers is derived from the gonadal mesoderm. All other structures of the reproductive system differentiate from the genital imaginal disc (see **Chapter 1.1**).

Oogenesis can been divided into 14 stages based on morphological criteria (King, 1970). Stage 1 refers to a newly formed egg chamber and stage 14 is a mature egg that is ready to be fertilized and oviposited. The mature egg is visibly asymmetric along its A–P and dorsal–ventral (D–V) axes (**Figure 2**). It has an anterior micropyle and anterior dorsal appendages, a posterior aeropyle, and is more curved on the ventral surface relative to the dorsal one. This polarity is defined and maintained during oogenesis through communication between the follicle cells and the developing nurse cell-oocyte complex.

1.2.2. Stem Cells

1.2.2.1. Germline Stem Cells and the Stem Cell Niche

Stem cells are defined by their ability to self-renew and produce daughters that differentiate. There are

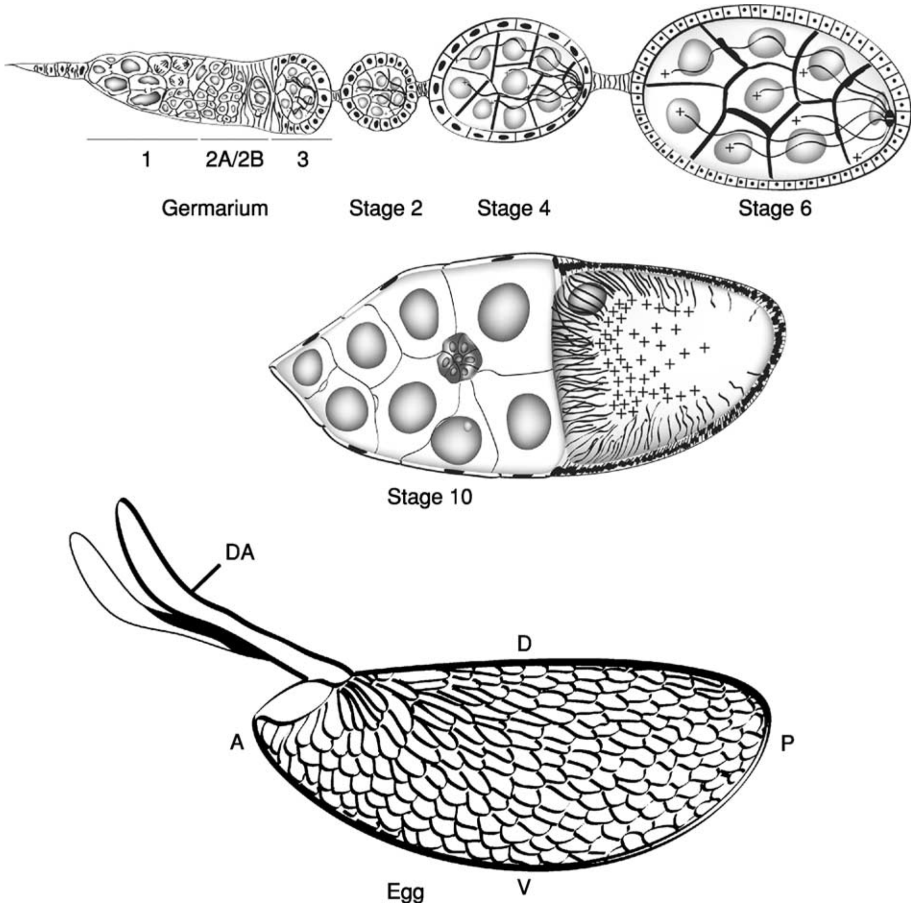


Figure 2 *Drosophila* ovarioles and egg chambers. *Drosophila* ovaries are organized into approximately 17 tubular structures called ovarioles that contain progressively maturing egg chambers. Different developmental stages from a single ovariole are pictured here. Egg chamber development initiates in the germarium at the anterior tip of each ovariole, which can be divided into three sections based on different stages of cyst development. Region 1 is filled with mitotically active cells: germline stem cells (GSCs), somatic stem cells (SSCs), and dividing cystocytes (Sections 1.2.2 and 1.2.3). In region 2, 16-cell cysts change shape to take up the width of the germarium, and the oocyte is determined (discussed in Section 1.2.2.4.1). Cysts are encapsulated by somatic follicle cells in region 2, and a subpopulation of follicle cells between adjacent cysts intercalates in region 3 to form a stalk, causing the newly formed stage 1 egg chamber to bud from the germarium (discussed in Section 1.2.5.1). Oogenesis can be further subdivided into 14 stages based on morphological criteria, where stage 1 refers to a newly formed egg chamber and stage 14 is a mature egg that is ready to be fertilized and oviposited. During stages 1–6, a stable microtubule organizing center (MTOC, marked with a black oval and minus sign in the stage 6 egg chamber pictured here) is present at the posterior of the oocyte (discussed in Section 1.2.4.1.1). It nucleates a single microtubule network (depicted as black lines) emanating through the ring canals into each nurse cell, with most MT minus ends focused in the oocyte. Until the cytoskeleton is reorganized during stages 6–7, mRNAs and proteins are steadily transported toward the MT minus ends, so that they accumulate in the oocyte. Major cytoskeletal reorganization occurs during stages 7–8, after which the posterior MTOC is no longer detected. A reorganized cytoskeleton emerges with the bulk of the MT network at the anterior, creating a higher density of MT in the anterior of the oocyte than the posterior. MT minus ends appear to be nucleated along the anterior and lateral cortices, with MT plus ends pointing into the center of the oocyte (discussed in Section 1.2.6.1 and portrayed in the stage 10 egg chamber drawn here). This cytoskeletal organization is required to direct the posterior localization of *osk* mRNA, the anterior localization of *bcd* mRNA, and the dorsal-anterior relocalization of the oocyte nucleus and *grk* mRNA. The mature egg (bottom drawing) is visibly asymmetric along its anterior–posterior (A–P) and dorsal–ventral (D–V) axes. It has an anterior micropyle and anterior dorsal appendages, a posterior aeropyle, and the egg is more curved on the ventral than on the dorsal surface.

two types of stem cells in the ovary: GSCs and somatic follicle stem cells (SSCs, **Figure 3**; Margolis and Spradling, 1995; Spradling *et al*., 1997, 2001). The anterior of each developing germarium hosts on average 2 or 3 GSCs, each of which is located adjacent to two or three somatic cap cells (Forbes *et al*., 1996a; Spradling *et al*., 1997; Xie and Spradling, 2000). Anterior to the cap cells in each ovariole are about six terminal filament (TF) cells which are arranged in a single-file array resembling a stack of pancakes. To the posterior of the cap cells and surrounding the GSCs and newly formed cystocytes are

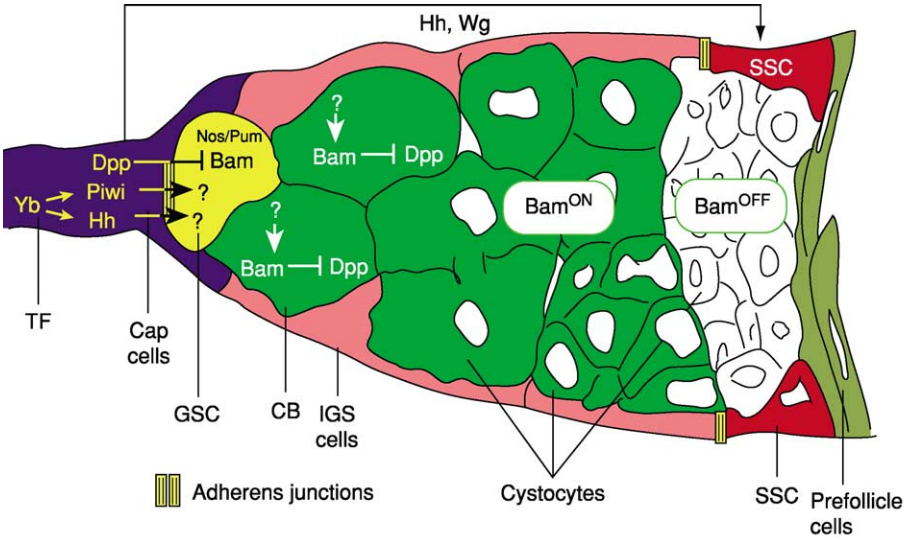


Figure 3 The ovarian germline and somatic stem cell niche. The germline stem cells (GSCs, yellow) and somatic stem cells (SSCs, red) are maintained in a somatic niche in germarial region 1, illustrated here. The stem cell niche is composed of terminal filament cells (TFs, blue), cap cells (blue), and inner germarial sheath cells (IGS, pink). TF and cap cells express Yb, Piwi, and the signaling molecules Dpp, Wg, and Hh, which act together to coordinate the two stem cell populations. GSCs maintain close contact with the cap cells through *adherens* junctions. Dpp signaling activity is restricted to GSCs where it maintains GSC fate by repressing Bam transcription, thereby preventing differentiation. When a GSC divides, one daughter maintains contact with the cap cells and remains a GSC, while the other daughter (the cystoblast – CB) escapes the niche and activates Bam expression (Bam^{ON}, shown in green). Bam is required to execute the cystoblast division program of four incomplete divisions, forming 16-cell cysts that no longer express Bam (Bam^{OFF}). In addition to intercellular signaling pathways, cell-autonomous factors like the translational repressors Nanos and Pumilio (Nos/Pum) are required in the germline for the maintenance of GSCs. SSCs are located at the boundary between germarial regions 1 and 2, and serve as the source of prefollicle cells (brown) that will encapsulate individual cysts. *Adherens* junctions between the SSCs and IGS cells retain SSCs in their niche. Hh and Wg are the only known signals for maintaining SSCs. Yb is expressed in the TF and cap cells where it activates the Piwi pathway for GSC maintenance, and the Hh pathway for both GSC and SSC maintenance. Molecular targets of Piwi, Hh, and Wg in stem cell maintenance have not been identified.

the inner germarial sheath (IGS) cells (**Figure 3**; Godt and Laski, 1995; Chen *et al*., 2001).

The TF cells, cap cells, and IGS cells constitute the stem cell niche – the cellular microenvironment that regulates, induces and supports the development of stem cells (**Figure 3**; review: Spradling *et al*., 2001). The TF and somatic cap cells are the likely sources of several important signaling molecules, and the survival of GSCs is dependent on their close association with these cells. Niche function also depends on the continued presence of stem cells, since empty niches eventually degenerate (Xie and Spradling, 2000). Thus, the GSCs and the stem cell niche function together to maintain the size of the stem cell pool, and allow the regular production of new egg chambers (Spradling *et al*., 2001; Lin, 2002; Gonzalez-Reyes, 2003; Kai and Spradling, 2003).

Cap cells and TF cells can be visualized with a *hedgehog-lacZ* enhancer trap and the IGS cells can be identified by *patched-lacZ* expression (Forbes *et al*., 1996a). The GSCs can be recognized because of their large size, location, and the presence of a cytoplasmic organelle called a spectrosome that is invariably anchored to the cap cell-GSC contact site (**Figure 1**; Lin *et al*., 1994; de Cuevas and Spradling, 1998).

1.2.2.2. Somatic Stem Cells

Continued generation of follicle cells is ensured by the maintenance of two or three somatic stem cells that are found at the surface of the germarium at the 2A/2B boundary (**Figures 2 and 3**). The immediate progeny of the SSCs, the follicle cell progenitors, divide to generate different classes of follicle cells that encapsulate each germline cyst. No positive marker for the SSCs has been described so far and they cannot be recognized by morphology or position, thus complicating their study. The SSCs have been identified as the only mitotically active somatic cells that remain in the germarium and continue to divide after being genetically marked (Margolis and Spradling, 1995). Zhang and Kalderon (2001) noted that the anterior-most mitotically active somatic cells fail to stain with Fasciclin III (FasIII) antibodies, which mark differentiating follicle cells, and have suggested that these are the SSCs.

1.2.2.3. Stem Cell Niche Cell Adhesion

During late larval and early pupal development, the somatic mesoderm starts to differentiate, dividing the ovary into individual ovarioles. The TFs form during the second half of the third larval instar. *Adherens* junctions form between the cap cells and the newly established GSCs during early pupation (**Figure 3**). After the formation of the TFs and the cap cells, the anterior primordial germ cells (PGCs) are selected to become GSCs while the rest of the PGCs are thought to enter directly the cyst differentiation pathway (Bhat and Schedl, 1997; Song *et al*., 2002; Zhu and Xie, 2003).

Cell adhesion mediated by DE-Cadherin plays an important role during late larval development in the recruitment of PGCs to the stem cell niche where they become GSCs (Song and Xie, 2002). Without DE-Cadherin, germline cells are not efficiently incorporated into the niche as stem cells. Contact between germline cells and newly differentiated cap cells seems to initiate the establishment of stable *adherens* junctions between them. The *adherens* junction components, DE-Cadherin and Armadillo (Arm; β-Catenin), are concentrated at the interfaces between the cap cells and GSCs. These *adherens* junctions are essential to hold the GSC in the niche and, therefore, maintain its stem cell identity (**Figure 3**; Lin and Spradling, 1993; Xie and Spradling, 1998). Removal of DE-Cadherin or Arm from the GSCs allows them to leave the stem cell niche and differentiate (Song and Xie, 2002).

The GSCs divide to produce cystoblasts that differentiate into germline cysts (**Figure 1**). GSCs divide along the A–P axis of the germarium so that the anterior daughter remains anchored to the cap cells and retains stem cell identity. The other daughter, now one cell diameter from the cap cells, differentiates as a cystoblast and begins oogenesis (Lin and Spradling, 1997). Stem cells and cystoblasts are connected by a transient ring canal (Carpenter, 1981; de Cuevas and Spradling, 1998). Before either cell divides again, the ring canal shrinks and the connection between the two cells is severed. The GSCs are randomly lost to differentiation, with a half-life of about 4–5 weeks (Margolis and Spradling, 1995; Xie and Spradling, 1998). When a GSC is lost, the remaining stem cells can divide perpendicular to the A–P axis so that both daughters remain attached to the cap cells (Xie and Spradling, 2000; Zhu and Xie, 2003). This division pattern suggests that symmetrical GSC division replenishes any vacant niche spaces.

As with GSCs, Cadherin-mediated cell adhesion is probably required for anchoring SSCs to their stem cell niche (Song and Xie, 2002, 2003). The SSCs interact directly with the IGS cells, which may function as the SSC niche. DE-Cadherin and Arm accumulate at the border between SSCs and IGS cells, and removal of DE-Cadherin or Arm from the SSCs allows them to move away from their niche and differentiate. While the asymmetry of GSCs divisions is clearly marked by asymmetric cytoplasmic inheritance of the spectrosome (Section 1.2.3.3), the division of a SSC is not necessarily asymmetric: the different behavior of the SSC daughters can be explained if the stem cell niche has a limited capacity and one daughter cell must leave the niche after division.

1.2.2.4. Stem Cell Niche Communication

Signals emanating from the GSC niche are required for the maintenance of both the GSCs and the SSCs. TF and cap cells express the genes *Yb*, *piwi*, *decapentaplegic* (*dpp*), *wingless* (*wg*), and *hedgehog* (*hh*), which are part of an intercellular signaling network that coordinates the two stem cell populations (**Figure 3**). Intrinsic factors, including the Decapentaplegic (Dpp) receptor, Pumilio (Pum) and Nanos (Nos), are required in the GSCs for maintenance.[1]

1.2.2.4.1. Dpp Dpp, the *Drosphila* homolog of TGF-β/BMP, is secreted by the somatic cells in the stem cell niche and received by the GSCs, where the Dpp signaling pathway is activated (Xie and Spradling, 1998, 2000; Song *et al*., 2004). The *dpp* mRNA is expressed in the cap cells, and the Dpp receptor, and downstream proteins in the signaling pathway, are required in the GSCs. Overexpressing *dpp* in somatic cells is sufficient to induce more GSCs, and misexpression of *dpp* or ectopic activation of the Dpp pathway in GSCs results in germ cell overproliferation, indicating that Dpp signaling stimulates GSC division and represses differentiation of the daughter cells. GSCs lacking Dpp receptors are replaced by wild-type germline cells, which come to lie next to the cap cells and behave as GSCs, indicating that the Dpp signal maintains GSCs in a niche that is able to program newly introduced cells to become stem cells. In fact, Dpp appears to be sufficient to cause four- and eight-cell germline cystocystes to de-differentiate and repopulate the GSC niche (Kai and Spradling, 2004).

The activity of *dpp* maintains GSCs in their niche because it represses the expression of *bag of marbles* (*bam*) (Chen and McKearin, 2003b; Song *et al*., 2004), a factor required to resume a program of

[1]An appendix at the end of this chapter includes a list of gene names, their abbreviations, and a short description of their functions.

cyst cell division and differentiation (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995). The *bam* gene is expressed in the cystoblasts but not in the GSCs (**Figure 3**; McKearin and Ohlstein, 1995; Chen and McKearin, 2003b). Overexpression of *bam* in GSCs effectively eliminates the GSC fate, similar to the loss of *dpp* signal transduction in the GSCs (Ohlstein and McKearin, 1997). Significantly, loss of *dpp* expression in the stem cell niche quickly allows the expression of *bam* in the GSCs (Song *et al*., 2004). Conversely, broad expression of *dpp*, directly prevents *bam* transcription and blocks the differentiation of GSC daughter cells (Chen and McKearin, 2003a). *bam* appears to be the principal Dpp target because ectopic Bam expression is sufficient to restore differentiation to Dpp-overexpressing cells, and because the *bam* promoter contains a transcriptional silencer element that binds the Mothers against Dpp (Mad) and Medea proteins (Chen and McKearin, 2003a, 2003b; Song *et al*., 2004). The simplest explanation for these results is that Dpp functions as a short-range signal that promotes GSC survival by repressing *bam* expression specifically in the GSCs and preventing their differentiation as cystoblasts.

1.2.2.4.2. Nanos and Pumilio The RNA binding proteins Nanos (Nos) and Pumilio (Pum) are required in the germline for the maintenance of GSCs (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Bhat, 1999; Parisi and Lin, 1999). Ovaries mutant for *nos* or *pum* have very few germ cells, and the number of germ cells decreases with age, suggesting that these two genes are required for GSC maintenance (Forbes and Lehmann, 1998; Bhat, 1999; Wang and Lin, 2004). *nos* is expressed at a high level in all PGCs in the third instar larval ovary. If *nos* function is removed during this period, a large proportion of PGCs, including those in contact with the stem cell niche, prematurely differentiate into cysts (Wang and Lin, 2004). These results indicate that *nos* activity maintains GSC self-renewal by preventing differentiation. The ovarian phenotype of a *nos pum* double mutant is similar to either *nos* or *pum* alone (Wang and Lin, 2004), suggesting they act as partners in GSC maintenance. As a Nos/Pum complex acts as a translational repressor in later stages of oogenesis (review: Parisi and Lin, 2000), these molecules are presumed to maintain GSC fate by repressing translation of one or more mRNAs required for differentiation.

1.2.2.4.3. Hedgehog and Wingless Hedgehog (Hh) and Wingless (Wg), expressed by the TF and cap cells, are the only known signals required for the maintenance of somatic stem cells (SSCs) (Forbes *et al*., 1996a, 1996b; Zhang and Kalderon, 2000, 2001; King *et al*., 2001; Song and Xie, 2003). Loss of Wg signal transduction or constitutive activation of the Wg pathway in SSCs results in their rapid loss, suggesting that correct intermediate levels of Wg signaling are important for proper regulation of SSCs. In addition, constitutive Wg signaling causes overproliferation and increased differentiation of polar cells and stalk cells (Section 1.2.5.3), causing defects in egg chamber budding and abnormal egg chamber morphology. Hh also regulates directly SSC maintenance and proliferation. Unlike *wg*, however, overexpression of *hh* induces SSC proliferation; like *wg*, a reduction in Hh signaling causes rapid SSC loss and arrests egg chamber budding.

Hh may also have a partially redundant function in GSC maintenance, since loss of *hh* causes loss of about 20% of the GSCs, while its overexpression stimulates a slight increase in the number of GSCs. The hypothesis that Hh positively regulates GSC survival is further supported by the observation that *hh* overexpression restores GSC maintenance to both *piwi* and *Yb* mutants (see below).

1.2.2.4.4. Piwi and Yb Two other genes known to be involved in maintaining stem cell survival are *piwi* (Cox *et al*., 1998, 2000), and *Yb* (King and Lin, 1999; King *et al*., 2001). Overexpression of either gene allows the specification of extra GSCs, while loss of either leads to the differentiation of GSCs into a few dividing cystoblasts, thus depleting the germline. Both are expressed and autonomously required in the TF cells and cap cells. *piwi* is also expressed in the germline, but is required specifically in the somatic cells for GSC maintenance. In *Yb* mutant germaria, *piwi* expression is specifically eliminated in the TF and cap cells, suggesting that *Yb* acts through *piwi* to maintain GSCs.

Piwi is likely to be of fundamental importance as it is the founding member of a conserved class of proteins (the Piwi, Argonaute, or PPD family) that function in stem cell maintenance in both the animal and plant kingdoms (reviews: Benfey, 1999; Cerutti *et al*., 2000). The step of gene expression on which Piwi may exert regulation is unclear, as Piwi-related proteins have been implicated in several DNA- and RNA-related processes including RNA interference (Smulders-Srinivasan and Lin, 2003).

Yb is a positive regulator of both *hh* and *piwi* expression. Since *hh* is required primarily for SSC maintenance, and *piwi* is required primarily for GSC maintenance, Yb appears to coordinately regulate SSCs, through *hh*, and GSCs, through *piwi*, *hh*, and an uncharacterized signal (King *et al*., 2001). Yb

regulates SSC proliferation by autonomously activating *hh* expression in the stem cell niche. Ectopic *hh* expression can induce SSC proliferation and partially restore GSC maintenance to *Yb* mutants, suggesting that *Yb* acts upstream of *hh* to regulate both stem cell populations.

While *piwi* and *hh* are both involved in maintaining GSCs, it is believed that they function in separate parallel pathways, with *piwi* having the more important role in normal development. However, *hh* overexpression increases the number of GSCs even in *piwi* mutants, therefore ectopic *hh* is able to rescue the GSC loss phenotype of *piwi*. It is unlikely that this effect comes about through an Hh-dependent increase in the capacity of the niche, as overexpression of *dpp* does not rescue the effects of *piwi* on GSC number (King *et al.*, 2001).

1.2.3. Germline Cyst Formation

The mitotically active region at the anterior of each ovariole is termed the germarium. It can be divided into four regions organized along the A–P axis (1, 2A, 2B, and 3) that correspond to maturing cyst stages (**Figures 2** and **3**). Region 1 contains 2–3 GSCs, cystoblasts and interconnected cysts of 2, 4, or 8 cells. Two or three GSCs occupy the apical position in each ovariole and are in close contact with several anterior somatic cells (**Figure 3**). GSC division produces a new GSC that remains in the position of the mother cell, and a second posterior daughter that develops as a cystoblast (King, 1970). The cystoblast divides four times with incomplete cytokinesis at each division, producing a syncytial cluster of 16 interconnected cystocytes (the cyst; **Figure 1**). Comparative analyses of germ cell development suggest that similar cell lineages arise during gametogenesis in diverse organisms (de Cuevas and Spradling, 1998; Pepling *et al.*, 1999). Cytokinesis is incomplete, since cleavage furrows are arrested and become stabilized as ring canals (see below). Similar ring canals form in most germ cells, including those from vertebrate models, and may serve to synchronize germ cell maturation (Pepling *et al.*, 1999).

Prefollicle cells, produced by 2–3 SSCs that reside on the side of the germarium at the regions 2A/2B (**Figure 3**), surround the germline cysts to form an epithelium and separate cysts from one another (**Figures 2** and **3**). The newly formed follicles or egg chambers change from spherical to elliptical so that they span the entire width of the germarium. The oocyte is centrally located in the cyst at this time. In regions 2B and 3, the newly specified oocyte takes up a posterior position as the cyst again changes shape to become spherical as it buds from the germarium as a stage 1 egg chamber (**Figure 2**). Under controlled conditions, it takes 3 days for a cystoblast to develop into a 16-cell cyst (King, 1970), and 7 days to reach stage 1 egg chamber (Wieschaus and Szabad, 1979). Then development of an egg progresses rapidly and is complete within 3 additional days (Lin and Spradling, 1993).

1.2.3.1. Ring Canals

During oogenesis of many insects, including *Drosophila*, cytoplasmic bridges called ring canals allow the flow of materials among the nurse cells and the oocyte. Ring canals can be visualized by staining with fluorescently labeled phalloidin, which binds to filamentous Actin, or with antibodies directed against ring canal components. The division pattern of the germline cyst leads to only two cells with four ring canals (**Figure 1**). The ring canal connecting the two daughter cells of the first division is the largest in diameter, and those arising at the second, third, and fourth divisions are each smaller than the previous ones. Ring canals have an electron-opaque outer ring associated with membranes and an electron-dense inner rim of Actin, the Hu-Li Tai Shao ring canal (Hts-RC) protein, and Kelch. Their assembly involves the sequential addition of proteins that transform the arrested cleavage furrow into a stable intercellular bridge (de Cuevas and Spradling, 1998). Although the inner rim is established coordinately with the degeneration of the fusome (see below) and the addition of Actin to the ring canal, the fusome is not required for arrest of the contractile ring or to initiate ring canal formation (Section 1.2.3.2; Lin *et al.*, 1994).

An outer-rim component that is recognized by anti-phosphotyrosine antibodies begins to mark ring canals in the early germarium, as the cleavage furrows arrest in the final round of cyst mitosis (Robinson *et al.*, 1994). Next, the electron-dense inner ring is formed as Cheerio (Filamin), then Actin and Hts-RC, are added starting in region 2A (Yue and Spradling, 1992; Theurkauf *et al.*, 1993; Robinson *et al.*, 1994). Female sterile *cheerio* mutants fail to recruit Hts-RC and Actin to the ring canals (Yue and Spradling, 1992; Robinson *et al.*, 1997). Kelch is the last known component to be added to the ring canals and it is added only after the Actin filament structure of the ring canals is complete (Tilney *et al.*, 1996). After Kelch is added, it is required through oogenesis for the continued expansion of the ring canals. *kelch* mutants initially form normal ring canals, which then become disorganized, resulting in female sterility due to transport blockage from the nurse cells into

the oocyte (Section 1.2.6.4; Xue and Cooley, 1993; Robinson and Cooley, 1997).

1.2.3.2. The Fusome

Reconstruction of the GSC, cystoblast, and cystocyte divisions by electron microscopy (EM) and confocal microscopy demonstrated that mitotic spindles are coordinately oriented during these divisions (King, 1970; Lin and Spradling, 1995). This is accomplished by anchoring one spindle pole of each dividing cell to a complex organelle termed the fusome (**Figure 1**; McGrail and Hays, 1997). Fusomes can be found in many insect germ cells (Büning, 1994), and similar organelles may exist in mammalian lymphocytes (Gregorio *et al*., 1992; McKearin, 1997), and germ cells (Pepling and Spradling, 1998). The organelles are spherical in GSCs and cystoblasts but grow to become elongated, branched structures that extend through each ring canal as cysts form (**Figure 1**).

Mutations in genes encoding fusome proteins, such as *hts* or *α-spectrin*, block fusome formation and cause premature termination of cyst divisions (Lin *et al*., 1994; de Cuevas *et al*., 1996). Thus, fusome integrity is essential for proper execution of cyst divisions. In the absence of fusomes, cystocyte spindles are disoriented, mitoses are not synchronized, and cystocytes usually do not complete four full divisions, emerging with unusual number of cystocytes such as 3, 5, or 6. Cysts are approximately of normal size because each cystocyte assumes a volume greater than the average wild-type cystocyte volume. Although cysts containing fewer than 15 nurse cells can complete oogenesis (Mata *et al*., 2000; D. McKearin, unpublished data), females carrying fusome-inactivating mutations are almost always sterile because cysts fail to form an oocyte. This observation reveals a second role for fusomes in cyst formation – oocyte determination depends on the fusome, probably because it establishes polarity within the cyst (Section 1.2.4.1).

Genetic and immunolocalization studies in *Drosophila* have identified a large group of proteins found associated with fusomes (**Table 1**). Our current view of the organelle is that membrane skeleton proteins form a meshwork within which membranous cisternae are embedded. Ultrastructural features suggest that fusome cisternae represent a germ cell-specific modification of the endoplasmic reticulum (ER) (Büning, 1994). The finding that an ER-associated protein, Ter94, is a fusome component was consistent with this hypothesis. Furthermore, green fluorescence protein (GFP) that carries ER-targeting signals localizes to the fusome (N. Liu and D. McKearin, unpublished), adding additional support to the idea that the fusome is the site of ER assembly in germ cells.

1.2.3.3. The Fusome Cycle

As indicated above, an asymmetric GSC division produces another GSC and a cystoblast, while asymmetric cystocyte divisions produce 15 nurse cells and a single oocyte. The intrinsic factors that underlie the asymmetric fates of GSC and cystocyte divisions are largely unknown but some features of the asymmetry are clear. Unequal fusome distribution is one prominent manifestation of intrinsic asymmetry, since the future cystoblast inherits a smaller fraction of fusome material than the GSC (**Figure 1**) (Deng and Lin, 1997; de Cuevas and Spradling, 1998). During cyst divisions, the oldest cystocyte always retains the largest fraction of

Table 1 Fusome proteins

Protein	*Function*	*Mutant phenotype*	*Reference*
α-Spectrin	Membrane skeleton	No fusome	de Cuevas *et al.* (1997)
β-Spectrin	Membrane skeleton	No fusome	Lin *et al.* (1994)
Ankyrin	Scaffold or linker protein	n.d.	de Cuevas *et al.* (1997)
Actin	Cytoskeleton structure, diverse	n.d.	Hime *et al.* (1996), Kai and Spradling (2003)
Bag of marbles	Novel	Reticulum disrupted	McKearin and Ohlstein (1995)
Cyclin A	CDK component	Cell lethal	Eberhart *et al.* (1996)
Dynein	Motor	Disoriented mitoses	McGrail and Hays (1997)
Encore	Cullin-binding protein	>16 cystocytes	Ohlmeyer and Schüpbach (2003)
Hu-Li Tai Shao	Adducin related, cytoskeleton	No fusome	Lin *et al.* (1994)
KLP61	Kinesin-like motor	Disoriented mitoses	Wilson (1999)
Lis-1	Lissencephaly ortholog	<16 cystocytes	Liu *et al.* (1999)
Orbit	Microtubule binding protein, CLASP ortholog	Poor cyst formation, GSC loss	Mathe *et al.* (2003)
Par-1	Microtubule binding protein	No oocyte	Cox *et al.* (2001a), Huynh *et al.* (2001b)
Spectraplakin	Cytoskeletal linker protein	No oocyte	Roper and Brown (2004)
Ter94	ER assembly, vesicle fusion	Cell lethal	Leon and McKearin (1999)

fusome material, perhaps anticipating which cell will become the oocyte (de Cuevas and Spradling, 1998; see below). The recognition of Bag of marbles (Bam), as both a fusome-associated protein (McKearin and Ohlstein, 1995) and a regulator of cystoblast differentiation, underscores a role for the fusome in germline asymmetric divisions (McKearin, 1997).

de Cuevas and Spradling described the fusome cycle in considerable detail and proposed that the remarkable dynamics of fusome morphogenesis could account for oocyte specification (de Cuevas and Spradling, 1998). Starting with the cystoblast as an example, the centriole at one spindle pole is anchored to the fusome, and the spindle body extends away from this point. Cytokinesis arrests as the contractile ring approaches the spindle midbody, and fusome proteins appear to aggregate in the gap separating the two daughters. This ring canal-associated piece of fusome is connected to the parent fusome by microtubules, and gradually the distance between the fusome pieces closes until they fuse. This process is almost certainly mediated by microtubules (MTs) and MT-associated motor proteins, since mutations affecting several MT-binding proteins disrupt fusome assembly and leave fusomes fragmented (e.g., *dynein* or *orbit* mutations). The cycle of gathering ring canal-associated fusome aggregates into a single coherent organelle is repeated at each cystocyte division until the fusome is large and branched.

The GSC fusome is unique in several ways. In electron micrographs, it contains only a sparse collection of small, spherical vesicles compared to cyst fusomes. The GSC fusome also occupies a stereotypical position in stem cells – at the anterior-most pole where the GSC is anchored to the cap cells via *adherens* junctions (Xie and Spradling, 2000). The unique features of the GSC fusome are recognized by giving it a special name, the spectrosome. Viewed with Hts antibodies, the spectrosome is a large dot during interphase but elongates to stretch across the entire A–P axis of the GSC near the end of mitosis. The GSC and cystoblast remain connected for some time through an intercellular bridge that is unstable and eventually closes to separate the cells. One end of the stretched spectrosome remains anchored at the anterior end of the GSC while the other end traverses the intercellular bridge and enters the presumptive cystoblast's cytoplasm. When the transient-bridge closes, it splits the resulting single fusome into unequal portions with the stem cell retaining a greater portion (Deng and Lin, 1997). The spectrosome in the GSC returns to the anterior of the cell and regains its spherical shape.

It is always one of the two cystocytes arising from the first cystoblast division that is determined to become the oocyte, while the remaining cells become nurse cells. Throughout cyst divisions, one of the two cells from the first division retains more fusomal material than any other cell (Lin and Spradling, 1995; de Cuevas and Spradling, 1998; Deng and Lin, 2001). Persistent asymmetry in the fusome indicates the presence of early cues capable of distinguishing the two pro-oocytes by cytoplasmic inheritance (de Cuevas and Spradling, 1998). In *Drosophila*, oocyte markers accumulate after the fusome starts to break down, so a direct lineage cannot be established between the cell retaining the most fusomal material and the oocyte. In *Dytiscus*, however, a visible ring of highly amplified ribosomal DNA is inherited by the future oocyte at the first cyst division, and this cell also inherits the fusome (de Cuevas and Spradling, 1998, and references therein). These results support the model that the initial asymmetry of the first cyst division is maintained through subsequent divisions and plays an central role in specifying oocyte fate (Section 1.2.4.1; Lin *et al*., 1994; Theurkauf, 1994a).

1.2.3.4. Counting Cystocyte Cell Divisions

The strict reproducibility of the number of cystocyte divisions is a remarkable feature of germline cysts. In *Drosophila melanogaster*, the cystoblast divides precisely four times with incomplete cytokinesis to produce a syncytium of 16 cells. Wild-type cysts rarely vary from this pattern of divisions. Despite the regularity of the number of cystocyte divisions, the counting mechanism can be manipulated or overridden by misexpression or inactivation of several cell cycle regulators. Lilly *et al*. (2000) showed that the induction of high levels of a degradation-resistant form of Cyclin A (CycA) causes a significant fraction of cysts to form with 32 cells. The same is true for misexpression of stabilized Cyclin B (CycB), albeit at a lower frequency. While misexpression of the G1 Cyclin E (CycE) does not produce 32-cell cysts, females heterozygous for an inactivating *cycE* allele produce cysts with only eight cells. Perhaps the simplest explanation for these observations is that the cystocyte counting mechanism is sensitive to the absolute length of each cell cycle and that the mitotic rate is controlled, in part, by the absolute concentration of the G1 and G2 cyclins. This suggests that cystocytes must complete their divisions within a specific time or region of the germarium as they move posteriorly.

While the biochemistry of counting the cystocyte divisions is unknown, the process is clearly controlled

genetically, since several mutations that disrupt the counting mechanism have been identified. Several of these genes have been implicated in the control of cyclin stability and it is likely that they exert their effects by premature destabilization or inappropriate stabilization of one or more cyclins. For example, inactivating alleles of the *UbcD1* E2 ubiquitin-conjugating enzyme cause extra cystocyte divisions and thus phenocopy the effect of CycA overexpression (Lilly *et al*., 2000). The extra rounds of cystocyte mitoses can be suppressed by removing one copy of the *cycA* gene, suggesting that the *UbcD1* effect is mediated by inappropriate perdurance of mitotic cyclins.

Using a screening strategy that permits gene misexpression, Mata *et al*. (2000) determined that cystocyte counting mechanisms can be altered by overexpressing the *Drosophila* Cyclin-dependent kinase Cdc25 homologs, *string* (*stg*) and *twine* (*twe*). The activity of Cdc25-like proteins is a rate-limiting step in *Drosophila* cell cycles as the *string* and *twine* phosphatases derepress the Cyclin-dependent kinase Cdk1 to initiate the G2/M transition. Surprisingly, overexpression of *string* causes cysts to form with only eight cystocytes, in contrast to the extra cystocyte divisions caused by CycA overexpression. The fact that ectopic Cdk1 activity causes fewer cystocyte divisions, while stable expression of one of its obligate partners, CycA, causes supernumerary divisions, indicates that critical features of the mitotic counting mechanism remain mysterious.

The misexpression screen for mitotic dysregulation also revealed two genes that caused extra cystocyte divisions, *tribbles* (*trbl*) and *grapes* (*grp*) (Mata *et al*., 2000). Grapes (Grp) was identified previously as an inhibitory kinase of Cdc25-like proteins (Ohi and Gould, 1999), and, therefore, its overexpression phenotype in cysts can be interpreted as a super-repression of String activity. The effects of Tribbles misexpression are particularly striking as ~90% of the cysts contained 32 cells. Elegant biochemical and genetic experiments showed that Tribbles inhibits String and Twine in embryonic and cystocyte cell cycles by causing protein degradation (Mata *et al*., 2000). Thus, mitotic entry is delayed in cystocytes expressing too much Tribbles.

Inactivating mutations in the *encore* (*enc*) gene also produce 32-cell cysts with high frequency (Hawkins *et al*., 1996). While Encore (Enc) is novel, a recent study clarified the *encore* loss-of-function effect by showing that the protein is necessary for proper assembly of the SCF-complex (Skp/Cullin/Rbx1/F-box) on the fusome (Ohlmeyer and Schüpbach, 2003). In germ cells lacking *encore*, failure of the fusome-associated SCF-complex to degrade CycE in a timely fashion leads to CycA overactivity, which can cause extra cystocyte divisions (see above). Regulation of the counting mechanism by *encore* demonstrates that the fusome plays a critical role in cystocyte divisions. Mutations in core fusome components, such as *hts* or *α-spectrin*, cause cysts with fewer than 16 cystocytes to form (Yue and Spradling, 1992; de Cuevas *et al*., 1996). The effect of mutations affecting fusome structure is somewhat different from those in *cycE* and *tribbles* since cystocyte divisions are largely asynchronous in *hts* and *α-spectrin* mutants. Mitotic spindle misorientation probably accounts for the more profound effect of mutations that block fusome formation (Lin *et al*., 1994; de Cuevas *et al*., 1996).

It is not surprising that dysregulation of cell cycle regulators would affect cystocyte counting mechanisms, but the expression patterns of these proteins do not correlate with the timing of the four cystocyte divisions. On the other hand, Bam protein begins to accumulate in the cystoblast, its abundance increases throughout the first three cystocyte cycles, and then it disappears at or shortly after the fourth division (**Figure 3**). Thus, Bam has exactly the profile expected of a protein that acts as a meter of the four-division cycle. The female germline *Bam* mutant phenotype did not reveal a role as a cystocyte division factor since the gene is necessary for cystoblast differentiation. Mutations in *Bam*, however, are able to suppress the supernumerary divisions observed in ovaries lacking *encore* (Hawkins *et al*., 1996) or overexpressing stable CycA (Lilly *et al*., 2000). Finally, discontinuous *Bam* expression from heat-inducible transgenes can rescue cystoblast and cyst formation but often leads to cysts with fewer than 16 cells, suggesting that *Bam* is required throughout the cyst division cycles to complete four mitoses (Ohlstein and McKearin, 1997; D. McKearin, unpublished data).

As Bam is a novel and rare protein, the biochemical mechanism by which it regulates cyst formation remains unclear. Bam is cytoplasmic (McKearin and Ohlstein, 1995) and works together with Benign gonial cell neoplasm (Bgcn), an unusual RNA helicase-like protein, suggesting that the Bam : Bgcn complex may act by regulating translation of cystoblast promoting mRNAs (Ohlstein *et al*., 2000). Genetic and immunolocalization studies suggest that the mitotic cyclins and their regulators drive cystocyte divisions only as long as Bam is present. Perhaps Bam is degraded in newly formed 16-cell cysts in response to a signal received from somatic cells at the posterior of region 1. Once Bam is degraded, cystocyte divisions cease, and

cystocytes destined to be nurse cells switch to endoreduplicative cycles while the oocyte enters meiosis 1.

1.2.4. Early Oocyte Differentiation and Polarity

Once the oocyte fate has been specified, the oocyte condenses its DNA into a compact karyosome, arrested in prophase I until late in oogenesis. At the same time, the nurse cells endoreplicate their DNA, eventually reaching 2048C in those cells closest to the oocyte. While transcription from the oocyte nucleus remains relatively quiescent, the nurse cells transcribe and translate very actively, and steadily transfer mRNAs and proteins into the oocyte on a polarized microtubule (MT) cytoskeleton. The determination and maintenance of the oocyte fate depends on: (1) the launching of meiosis and its restriction to the oocyte; (2) the acquisition and maintenance of a single microtubule organizing center (MTOC) in the oocyte, for the collection of the mRNAs and proteins that are necessary to continue oogenesis; and (3) karyosome formation.

1.2.4.1. Establishing the Oocyte and Early Polarity

The oocyte-localized proteins Oo18 RNA binding protein (Orb), Bicaudal-D (Bic-D), Egalitarian (Egl), and Par-1 are required for oocyte differentiation; recessive mutations at these loci cause female sterility because egg chambers form 16 nurse cells and no oocyte (Schüpbach and Wieschaus, 1991; Vaccari and Ephrussi, 2002). All four proteins become highly enriched in several cells as early as the middle of germarial region 2A, as they move along the MT network and localize to the oocyte by region 2B (Lantz *et al.*, 1992; Lantz and Schedl, 1994; Mach and Lehmann, 1997; Pare and Suter, 2000). By this time, the nurse cell centrosomes have also migrated on the fusome to a single focus in the oocyte (a detailed description of centrosome migration can be found in Grieder *et al.*, 2000 and Section 1.2.4.1.1). Between region 2B and 3 of the germarium, the centrosome cluster, together with Orb, Egl, Bic-D, and Par-1, migrates from an anterior position to one between the oocyte nucleus and the posterior terminal follicle cells. Until the cytoskeleton is reorganized during stage 7, a single MT cytoskeleton emanates from this posterior MTOC through the ring canals into each nurse cell (**Figure 2**; Theurkauf *et al.*, 1993). mRNAs and proteins are steadily transported toward the MT minus-ends, so that they accumulate in the oocyte. This is a critical polarization event, and is the first indication of polarity within the oocyte itself. Without this posterior relocalization, the oocyte exits meiotic arrest and reverts to a nurse cell fate.

1.2.4.1.1. Centrosome migration A large MT network extends through all the cells of the early cyst before the centrosomes and MTOC are restricted to the oocyte (Grieder *et al.*, 2000). The fusome is embedded in this MT network throughout region 2A and 2B of the germarium. The MT minus ends, marked by a No distributive disjunctions (Nod) : β-Gal fusion protein, are enriched along the fusome, and by region 2B become concentrated in the central cells, then in the oocyte (**Figure 2**). In the absence of a fusome, in *hts* mutant cysts, the clustering of MTs to the fusome and oocyte was not observed at any time, so the fusome is necessary to organize MTs and focus them into the oocyte (Grieder *et al.*, 2000).

Centrosome migration into the oocyte does not depend on Bic-D, Orb, Egl, or Par-1 (Huynh and St. Johnston, 2000; Bolivar *et al.*, 2001; Vaccari and Ephrussi, 2002). It is Dynein-dependent, suggesting directed transport on a MT network, but it is also resistant to MT depolymerizing drugs such as colcemid, suggesting that centrosome migration is either MT-independent, or that it depends on a pool of stable MTs that are resistant to these drugs. The discovery of a new fusome component encoded by *short stop* (*shot*) supports the latter alternative (Roper and Brown, 2004). *shot* encodes a large cytoskeletal linker protein called spectraplakin that binds MTs, perhaps protecting them from depolymerizing drugs. Shot localizes to the fusome and is essential for organizing the cyst MT cytoskeleton, but is not required for fusome structure or stability, nor for the control of the number of cyst divisions. Loss of *shot* results in a 16-nurse cell phenotype in which Bic-D, Orb, and Dynein do not accumulate in a single cell. Therefore, *shot* acts downstream of the structural components of the fusome to specify oocyte fate. Interestingly, the cystocyte centrosomes do not migrate into the oocyte of *shot* mutants, but instead remain spread throughout the cyst. Acetylated α-tubulin, a marker for stable microtubules, clearly localizes to the fusome and colocalizes with Shot (Roper and Brown, 2004). In *shot* mutants, the level of acetylated MTs is reduced, and the association of these MTs with the fusome is lost. These results indicate that Shot mediates the association of stable MTs with the fusome. Without Shot, MTs fail to assemble on the fusome, the centrosomes do not localize to one cell, and the oocyte fails to become specified. The disruption of centrosome migration in shot mutants

can be explained by the migration of centrosomes on a MT network that is stabilized by Shot on the fusome (Grieder *et al*., 2000; Roper and Brown, 2004).

1.2.4.1.2. Restricting meiosis to the oocyte As the oocyte is the only cell that contributes genetic information to the egg, meiosis must be established within it, and repressed in the other germline cells that will become nurse cells. An initial stage of meiosis is synaptonemal complex (SC) formation. Meiosis, the mechanisms of SC formation, and meiotic recombination in *Drosophila* have been recently reviewed in detail (McKim *et al*., 2002). The behavior of SCs in the cyst suggests that oocyte fate is restricted gradually to one cell. The SC appears in two cells in the early region 2A, then spreads to the two cells with three ring canals, before it is restricted to two cells, and then finally to the oocyte in region 2B (Huynh and St. Johnston, 2000). The region 3 oocyte remains arrested at diplotene of meiotic prophase I (Carpenter, 1994), while the other 15 cells lose SCs and differentiate into nurse cells – via a series of endoreplication cycles. In later development, the oocyte completes prophase I, but undergoes a second meiotic arrest at metaphase I, which is released upon egg activation. In metazoans, meiosis is arrested at metaphase II, largely through the activity of the Mos kinase, and this arrest is released upon fertilization. Perhaps surprisingly, a recent analysis of a *mos* mutant in *Drosophila* does not support an essential role for this kinase in maintaining metaphase arrest (Ivanovska *et al*., 2004).

The first sign of oocyte identity, even before the SC has been restricted to the oocyte, is the specific accumulation of Bic-D, Egl, and Orb to one cell (Suter *et al*., 1989; Huynh and St. Johnston, 2000; Oh and Steward, 2001). Bic-D, Egl, and Orb seem to be involved in MT-dependent, Dynein-mediated transport of mRNA and protein from the nurse cells into the oocyte (Suter *et al*., 1989; Wharton and Struhl, 1989; Ran *et al*., 1994; Mach and Lehmann, 1997; Bullock and Ish-Horowicz, 2001; Chang *et al*., 2001). Orb is the *Drosophila* ortholog of cytoplasmic polyadenylation element binding (CPEB) protein, and is believed to stimulate translation of specific mRNAs that contain a cytoplasmic polyadenylation element (CPE) sequence by mediating an extension of their poly-A tails (Castagnetti and Ephrussi, 2003). Bic-D and Egl appear to function as core components of a Dynein motor complex that mediates minus-end directed transport (Mach and Lehmann, 1997; Karki and Holzbaur, 1999; Bullock and Ish-Horowicz, 2001; Matanis *et al*., 2002). In cultured human cells, the Bic-D ortholog also binds Dynein, and is capable of inducing rapid minus-end directed transport of experimentally tethered organelles such as mitochondria (Hoogenraad *et al*., 2003).

The *Bic-D*, *egl*, and *orb* mutant phenotypes indicate that these three proteins play an important role in restricting and maintaining SC formation to one cell. Loss of any one of these genes results in the formation of cysts with 16 nurse cells and no oocyte. In *egl* mutants, all 16 cells enter meiosis, reach full pachytene, and then revert to the nurse cell fate by region 2 of the germarium (Carpenter, 1994; Huynh and St. Johnston, 2000). Strong *orb* alleles also cause all cyst cells to form a complete SC, and most mutant cysts to degenerate in region 2. A hypomorphic *Bic-D* mutation shows a similar but weaker phenotype, where too many cells build synaptonemal complexes that disappear in region 3. In the complete absence of *Bic-D*, almost no SC formation can be detected using an SC antibody, though weak and diffuse staining is observed in all cells of some cysts (Huynh and St. Johnston, 2000). These results are most readily explained if Bic-D, Egl, and Orb are directly involved in restricting oocyte fate and SC formation to a single cell.

Individual mutations in *Bic-D*, *egl*, or *orb* also prevent the localization of the other two proteins to the oocyte (Suter and Steward, 1991; Lantz and Schedl, 1994; Machand and Lehmann, 1997). In *shot* mutants, they all fail to localize, the SC fails to refine to one cell, and is always lost by region 2B of the germarium (Roper and Brown, 2004). These data suggest that the effects of Bic-D, Egl, and Orb are mediated by their own localization to the oocyte. However, in the presence of drugs that depolymerize dynamic MTs, the SC becomes restricted to one cell of a wild-type cyst, even in the absence of localized Bic-D, Egl, or Orb accumulation (Huynh and St. Johnston, 2000), indicating that the function of these proteins in SC restriction does not depend on their own localization to the oocyte. Interestingly, SC restriction to the oocyte is not maintained in the presence of colcemid, therefore maintaining oocyte fate does depend on the MT-dependent localization of these determinants into the oocyte (see below).

Several other genes can mutate to give a 16-nurse cell phenotype, but their precise functions and relationships to *Bic-D*, *egl*, and *orb* are unclear. Hypomorphic alleles of *stonewall* (*stwl*) cause a 16-nurse cell phenotype, while in null alleles, cystoblasts and cysts are formed, oocyte differentiation fails, and all germ cells eventually undergo apoptosis. *stwl* encodes a DNA-binding protein with partial similarities to the mammalian Myb and Adf1 transcription

factors (Clark and McKearin, 1996), and domains that suggest its involvement in the regulation of chromatin remodelling. Targets for Stwl-mediated transcriptional regulation remain unidentified. The *shutdown* (*shu*) gene appears to function to maintain oocyte fate. In *shu* mutant ovaries, cysts usually have 16 cells, and one Bic-D positive cell begins to differentiate as an oocyte (Munn and Steward, 2000). However, these cells stop expressing oocyte markers during stages 1–3, and the cysts degenerate shortly thereafter. Mutations in a newly-identified gene, *soft boiled*, also result in a 16-nurse cell phenotype, but this gene has not yet been molecularly characterized (Morris *et al.*, 2003).

In addition to Bic-D, Egl, and Orb, many other RNAs and proteins accumulate in the early oocyte. Most of these have no known role in oocyte specification, but some are involved in later steps of axis patterning (Section 1.2.6). It appears, however, that mechanisms for localizing RNAs to the early oocyte are not especially selective, as one study indicates that almost 10% of randomly selected ovarian mRNAs become enriched in the early oocyte (Dubowy and Macdonald, 1998).

1.2.4.1.3. Maintaining oocyte fate The establishment of stable polarity within the oocyte, marked by the anterior-to-posterior relocalization of the oocyte centrosomes, together with Bic-D, Egl, Orb, and Par-1, is required to maintain MT-dependent transport of mRNAs and proteins into the oocyte and, therefore, maintain oocyte fate. Polarization of the oocyte requires the previous localization of at least Bic-D, Egl, Orb, and Par-1 (Bolivar *et al.*, 2001; Cox *et al.*, 2001a, 2001b; Huynh *et al.*, 2001a, 2001b). Par-1 posterior relocalization requires *Bic-D* and *orb* (and by association *egl*). In a *par-1* mutant, Bic-D, Orb, Egl, and the centrosomes still localize to one cell, and transiently localize together at the anterior of the oocyte. However, they do not relocalize to the posterior cortex, but instead remain associated with the remnants of the fusome, completely disrupting formation of a stable polarized MT network. Without posterior relocalization, Bic-D, Egl, and Orb localization in the cyst becomes diffuse, and the oocyte loses its fate, creating a cyst of 16 nurse cells. Therefore, establishing a stable posterior MTOC maintains meiotic arrest in the oocyte.

Essentially identical phenotypes have been observed for *par-1*, *bazooka* (*baz; par-3*), *par-5* (*14-3-3*), *par-6*, and *aPKC* (Cox *et al.*, 2001a, 2001b; Huynh *et al.*, 2001a, 2001b; Benton *et al.*, 2002; Vaccari and Ephrussi, 2002; Benton and Johnston, 2003). This family of genes, originally identified in *Caenorhabditis elegans* as cell polarity mutants, has an essential conserved role in establishing cell polarity (Ahringer, 2003; Etienne-Manneville and Hall, 2003). As in the *C. elegans* embryo, Par-1 and Baz are localized to complementary domains in the early *Drosophila* oocyte, and the anterior localization of Baz depends on posterior Par-1 localization (Vaccari and Ephrussi, 2002). Par-1 binds to the 14-3-3 proteins 14-3-3ε and 14-3-3ζ, and since the *14-3-3ε*; 14-3-3ζ double mutant phenotype is identical to the *par-1* phenotype, 14-3-3 proteins are thought to mediate Par-1 function (Benton *et al.*, 2002). Par-1 is a serine-threonine kinase and one of its targets is Baz. Par-1 can phosphorylate Baz, thus creating 14-3-3 binding sites. The PDZ domain proteins Baz and Par-6 normally form a tripartite complex with Atypical protein kinase C (aPKC) at the anterior of the oocyte (Benton *et al.*, 2002). Since 14-3-3 binding can inhibit interactions between aPKC and Baz, Par-1 can inhibit the assembly of Baz/Par-6/aPKC complexes (Benton and Johnston, 2003).

Taken together, this information suggests that Par-1 establishes cortical domains in the *Drosophila* oocyte by locally inhibiting Baz/Par-6/aPKC complex assembly, thus excluding Baz from the posterior of the oocyte. However, the mechanism by which these proteins affect MT polarity or dynamics is currently unknown. Furthermore, this model does not provide an explanation for how the oocyte is initially polarized. In *C. elegans*, Par-1 is recruited to the posterior cortex after the sperm centrosome defines the posterior pole. As such a cue is missing in *Drosophila*, it would be interesting to explore a possible relationship between establishing a posterior cortical Par-1 domain in the oocyte, and the cell adhesion surface established between the oocyte and the posterior follicle cells (Section 1.2.5.2).

1.2.4.2. The Spindle Genes, Meiotic Checkpoint, and Patterning

Mutations have been identified in several genes (*okra*, *spindle-A*, *-B*, *-C*, and *-D*, for example) that are required for repair of double-strand DNA breaks (DSBs) during meiosis (Ghabrial and Schüpbach, 1999; Staeva-Vieira *et al.*, 2003). In these mutants, the oocyte chromatin appears fragmented and thread-like, suggesting the presence of unrepaired DSBs. Thus, as in many other systems, recognition of DSBs activates a pathway that stops the progression of the cell cycle until the damaged DNA is repaired. This pathway is dependent on the *mei-41* function in *Drosophila*. Surprisingly, mutations in these genes also result in defects in axial patterning and in accumulation of the TGF-α

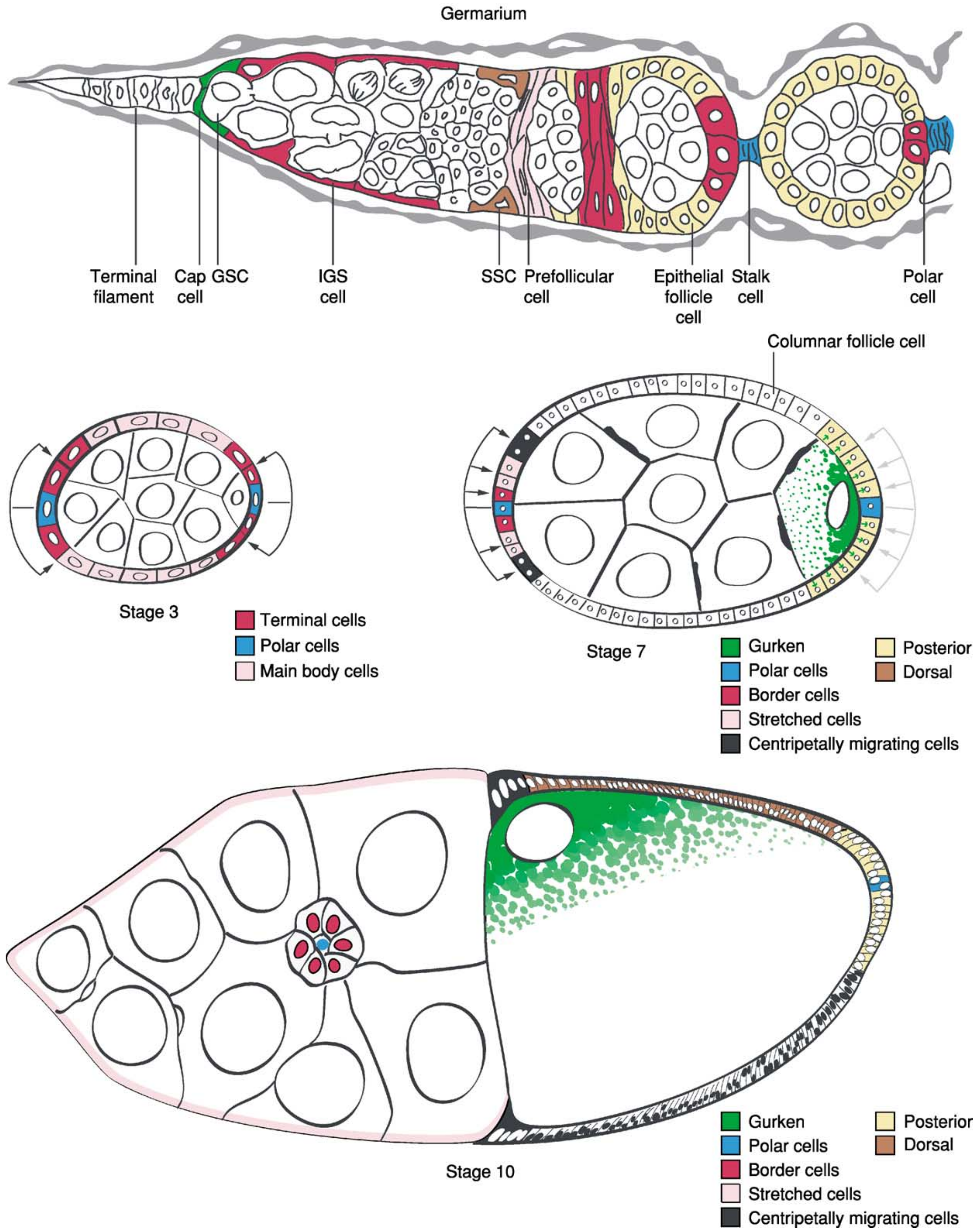


Figure 4 Cell types in the developing ovary. Top panel: There are two types of stem cells in the ovary: germline stem cells (GSCs) and somatic follicle stem cells (SSCs). The terminal filament cells, cap cells and inner germarial sheath (IGS) cells comprise the stem cell niche – the cellular microenvironment that regulates and supports the development of stem cells (discussed in Section 1.2.2). In germarial region 2B, the prefollicular cells invaginate from the germarium wall and encapsulate 16-cell cysts in order to complete the construction of individual egg chambers. These intercyst cells, the precursors of the polar cells and the intercyst stalk cells separate from the general follicle cell lineage and stop dividing by germarial region 3. Intercyst cells interdigitate to form a stalk during cyst separation from the germarium, and persist, anchored to the polar cells, so that adjacent egg chambers remain connected but not fused. Prefollicular cells divide and differentiate into a cuboidal monolayer of about 80 epithelial follicle cells that

ortholog Gurken (Grk) in the oocyte (Ghabrial and Schüpbach, 1999). This appears to occur because the *mei-41* dependent pathway downregulates Vasa (Vas) by posttranslationally modifying it. Vas, a probable translational activator (Section 1.2.7.1), is required for normal Grk accumulation (Styhler *et al.*, 1998), and presumably regulates the translation of one or more other mRNAs involved in DSB repair. *vas*-null mutant oocytes have fragmented chromosomes, similar to those in *okra* and *spindle* mutants that are not suppressible by *mei-41* (Ghabrial and Schüpbach, 1999). Phenotypes similar to those of the spindle mutations, including decreased Grk accumulation and aberrant posttranslational modification of Vas, have also been observed in a mutant Cop9 signalosome subunit 5 (*Csn5*) that affects a component of the Cop9 signalosome (Doronkin *et al.*, 2002). These results suggest that Csn5 might regulate the stability of one or more components of the DSB repair pathway.

There is controversy over whether Chk2, a kinase implicated in the response to DNA damage during mitotic replication (Takada *et al.*, 2003) and in the regulation of irradiation-induced p53-mediated apoptosis (Brodsky *et al.*, 2004), also has a role in meiotic DSB repair. Abdu *et al.* (2002) reported that a *chk2* mutation suppresses the effects of spindle-class mutations on Grk accumulation, and that Chk2 is phosphorylated and activated by the *mei-41* dependent pathway. They further report that Chk2 modifies two downstream proteins, Vas and Wee1, thus mediating the effects of DSBs on cell cycle progression, Grk accumulation, and oocyte nuclear morphology. In contrast, Masrouha *et al.* (2003) reported that *chk2* is not essential for normal meiosis and recombination, based on an analysis of X-linked nondisjunction, in which no *chk2*-dependent effects were observed. These workers did observe, however, accumulation of *chk2* RNA to the oocyte in early germarial stages, an expression pattern consistent with a meiotic function. Further work will be required to resolve this apparent paradox.

1.2.5. Follicle Cells

In germarial region 2B, the precursors of the follicle cells invaginate from the germarium wall and encapsulate 16-cell cysts in order to complete the construction of individual egg chambers (**Figure 4**; Margolis and Spradling, 1995). Groups of cells defining the anterior and posterior pole of each egg chamber separate from the general follicle cell lineage and stop dividing by germarial region 3 (Tworoger *et al.*, 1999). These intercyst cells are the precursors of the polar cells and the intercyst stalk cells (**Figure 4**). Intercyst cells interdigitate to form a stalk during cyst separation from the germarium, and persist, anchored to the polar cells, so that adjacent egg chambers remain connected, but not fused. The common precursors to stalk and polar cells are distinct from the precursors of the main body follicle cells and can be identified in the germarium using antibodies to the Big brain (Bib) protein (Larkin *et al.*, 1996). Bib is a positive regulator of Notch (N) signal reception and is expressed in the follicle cells that will intercalate to form the stalk, as well as fully developed stalk cells. The enhancer trap line P{lacW}B1-93F acts as a differentiated stalk cell marker (Ruohola *et al.*, 1991).

Prefollicular cells (**Figure 4**) divide and differentiate into a cuboidal monolayer of about 80 polarized follicle cells that surround the germline cyst to create the stage 1 egg chamber. As the nurse cells and oocyte increase in volume, the follicle cells divide to reach about 1000 cells per egg chamber by stage 6. Then, during stages 7–9, the follicle cells

surround the germline cyst to create the stage 1 egg chamber. Middle left panel: By stage 3 three distinct populations of follicle cells have become specified. Polar cells are adjacent to the anterior and posterior ends of the germline cyst, and the posterior polar cell is adjacent to the posterior pole of the oocyte. Terminal cells, specified by the Upd signal from the polar cells (arrows) flank the polar cells. Main body cells occupy lateral regions of the egg chamber. Middle right panel and bottom panel: During stages 7 and 8, the anterior follicle cells begin to migrate posteriorly toward the oocyte. The nature of their migration and their final destination has already been determined at this time, by a gradient of Jak activity. Upd, the Jak ligand, is secreted by the polar cells and establishes this gradient (denoted by arrows; discussed in Section 1.2.5.1). The most anterior cells are the anterior polar cells and the border cells. These squeeze between the anterior nurse cells and begin migrating posteriorly (illustrated here are the central polar cells (blue) surrounded by border cells (red) among the nurse cells). Border cell migration finishes at the beginning of stage 10 when they reach the anterior of the oocyte. Later, they create the micropylar pore through which sperm passes at fertilization. The next-most anterior cells form a thin layer of squamous cells (the stretched cells) that remain associated with the nurse cells until late oogenesis. The follicle cells immediately posterior to these (the centripetally migrating cells) will migrate posteriorly to the nurse cell/oocyte interface, then will migrate inward until they cover the anterior of the oocyte, where they will later produce the anterior vitelline membrane and chorion. More posterior follicle cells become increasingly columnar, and migrate posteriorly to cover only the oocyte by stage 10. During this same period of development, the oocyte nucleus migrates to the dorsal-anterior corner of the oocyte. Gurken (Grk) is secreted from that location, activates Egfr in adjacent follicle cells, and specifies them as dorsal (discussed in Section 1.2.6.5.1).

cease mitosis and become polyploid by endocycling, before chorion gene amplification takes place during stages 10–13. The transition from mitosis to endocycle is controlled by the Notch-pathway signaling from the germline. Just before follicle transition from mitosis to endocycle, germline Delta (Dl) expression increases to activate Notch in all follicle cells. Notch activation represses the expression of the CDC25 phosphatase encoded by *string*, allowing the follicle cells to exit mitosis and differentiate (Deng *et al.*, 2001; Lopez-Schier and St. Johnston, 2001).

Until stage 6, the follicle cells surrounding the cyst are relatively uniform in size and shape. During stages 7 and 8, the anterior follicle cells begin to migrate posteriorly around the enlarging oocyte (**Figure 4**). The posterior follicle cells become increasingly columnar, while those covering the nurse cells at the anterior begin to flatten and form a thin layer of squamous cells (the stretched cells). By the beginning of stage 10, most follicle cells cover the oocyte, which takes up the posterior half of the egg chamber. The oocyte nucleus has migrated to the dorsal-anterior corner of the oocyte and the dorsal follicle cells (those closest to the oocyte nucleus) become taller than those on the ventral side. Next, the anterior-most columnar follicle cells (the centripetally migrating cells) start to migrate between the nurse cells and oocyte until they cover the anterior of the oocyte, where they will produce the anterior vitelline membrane and chorion.

During stage 9, 6–8 follicle cells and the adjacent anterior polar cell (the border cells) squeeze between the anterior nurse cells and begin migrating posteriorly. Border cell migration finishes at the beginning of stage 10 when they reach the anterior of the oocyte. Here, they create the micropylar pore through which sperm passes at fertilization. The different populations of migrating follicle cells can be visualized with enhancer-trap reporter transgenes: centripetally migrating cells (P{LacW}BB127; Roth *et al.*, 1995), border cells (P{LacZ}5A7; Roth *et al.*, 1995), and stretched cells (P{LacZ}MA33; Gonzalez-Reyes and St. Johnston, 1998). The genes identified by these P-element insertions have not been characterized.

1.2.5.1. The Organizing Activity of Polar Cells

The polar cells are a signaling source that specifies different follicle cell fates through the Janus kinase (Jak) pathway. Its substrate, Unpaired (Upd), is specifically expressed in the polar cells at both poles (Xi *et al.*, 2003). Upd interacts with the cell-surface receptor Domeless (Dome) in nearby follicle cells to activate Jak (Hopscotch, Hop) and Stat (Stat92E). Without polar cells, no stalk cells are specified and the three distinct anterior follicle cell types are missing (Grammont and Irvine, 2002).

Polar cells refine the polar cell domain and induce stalk cell fate through the Jak pathway (Zhang and Kalderon, 2000; Lopez-Schier and St. Johnston, 2001; Baksa *et al.*, 2002; McGregor *et al.*, 2002). Loss of *Stat92E* or *hop* in the follicle cells blocks stalk cell specification and leads to an increase in the number of polar cells, while overexpression of *upd* causes all of the polar and stalk cell precursors to differentiate as stalk cells. Upd from the polar cells also induces a wider field of follicle cells at each pole to adopt a terminal fate (**Figure 4**; Xi *et al.*, 2003), thus separating the epithelial layer into a main body region and two terminal regions. *mirror* (*mirr*) expression can be used as a marker for main body fate since it is expressed in the main body cells, with a graded reduction toward the egg chamber poles. Ectopic *mirr* expression is observed in *hop* mutant cells regardless of position, and ectopic expression of *hop* or *upd* activates the Jak pathway and is sufficient to repress *mirr* expression. Ectopic *upd* represses *mirr* expression nonautonomously and in a graded fashion, while cell-autonomous activation of the Jak pathway, achieved by overexpressing *hop*, autonomously represses *mirr* expression. These results indicate a direct role for Jak in establishing terminal domains by repressing main body fate.

Within the anterior terminal region, graded Jak activity plays an important role in specifying the border, stretched, and centripetal follicle cells (**Figure 4**; Silver and Montell, 2001; Beccari *et al.*, 2002; Xi *et al.*, 2003). Jak activity can be measured by assessing nuclear accumulation of Stat, the Jak pathway transcriptional activator, and increasing levels are observed near the polar cells. Also, the graded expression of Jak-responsive reporters in the polar domains can be ectopically recapitulated in response to ectopic *upd* expression. At the highest levels of Jak activation, adjacent to the polar cells, Jak activity is necessary and sufficient to specify border cell fate. In addition, reducing Jak signaling reduces the adoption of border, centripetal, and stretch cell fate, while ectopic expression of *upd* or *hop* can induce ectopic adoption of these fates (Xi *et al.*, 2003). These results suggest that Upd acts as a morphogen to induce different cell fates at a distance and in a concentration-dependent manner. However, specification of stretched and centripetal cells appears to involve additional mechanisms. For instance, Grammont and Irvine (2002) have demonstrated that stretched and centripetal cells can form normally in the absence of Upd signaling. This could

be explained if other Upd-like ligands are involved (three such genes are present and functional; Hombria and Brown, 2002). Also, there are contradictory reports about the effects of *hop* and *stat92E* mutants, and therefore Jak signaling, on the specification of stretched and centripetal cells (Beccari *et al*., 2002; Xi *et al*., 2003). Denef and Schüpbach (2003) have raised the interesting suggestion that the gradient of Jak activity may be overlaid on cells with different responsiveness at different developmental stages, thus explaining these apparent contradictions, and perhaps providing a mechanism to achieve greater precision in patterning.

Loss of polar cells or Jak activity at the posterior end of the egg chamber disrupts the oocyte A–P polarity (Deng and Ruohola-Baker, 2000; Grammont and Irvine, 2002; Xi *et al*., 2003). Initially, the terminal follicle cell populations are equivalent; anterior and posterior cells that are at a similar distance from the polar cells have similar identities. In mosaics, small groups of *epidermal growth factor receptor* (*egfr*) mutant posterior cells interpret their distance from the posterior pole and autonomously adopt the appropriate anterior fate, indicating a mirror-image pre-pattern along the A–P axis (Gonzalez-Reyes and St. Johnston, 1998). This pattern is disrupted during stage 6, when Grk, secreted from the posteriorly localized oocyte, activates Egfr and specifies posterior fate in adjacent follicle cells (**Figure 4**; reviews: Nilson and Schüpbach, 1999; Lopez-Schier, 2003). Using *punt* (*put*) expression as a marker for posterior follicle cell fate, Xi *et al.* (2003) have shown that the coordinated activity of the Egfr and Jak pathways are necessary and sufficient to induce posterior follicle cell identity. Clones of cells that express activated Egfr can induce posterior fate in anterior follicle cells, but not in the main body cells. However, the coexpression of activated Egfr and Jak is sufficient to induce posterior fate in all follicle cells. Therefore, Jak activation induces competence to respond to Egfr activation in both polar domains, and localization of the oocyte nucleus determines which polar domain is specified as posterior.

1.2.5.2. Polar Cells Transmit Polarity to Adjacent Cysts

When a cyst leaves the germarium, the oocyte is always found at the most posterior position of the egg chamber. By germarial region 2B, the oocyte is specified and expresses higher levels of the adhesion molecules DE-Cadherin, α- and β-Catenin relative to the nurse cells. Prior to differentiating into the polar and stalk cells, the intercyst somatic cells face the flattened germline cyst and also express high levels of these adhesion molecules. When egg chambers or follicle cells do not express DE-Cadherin or β-Catenin, the oocyte is normally specified but is often mislocalized. Therefore, an adhesion-mediated sorting process establishes oocyte positioning (Godt and Tepass, 1998; Gonzalez-Reyes and St. Johnston, 1998; review: van Eeden and St. Johnston, 1999).

Polar cells have been implicated in oocyte localization because their loss leads to A–P polarity defects in the egg chamber, and the oocyte is often found apposing the polar cells even when the latter are not at the posterior end (Grammont and Irvine, 2002). In addition to disrupting polar cell specification (see below), *Notch* mutants cause egg chamber A–P polarity defects. In mosaics, when *Notch* is missing from the follicle cells, or *Dl* is missing from the germline, polar cells are not specified. However, stalk cells always differentiate between a mutant egg chamber and the next older wild-type egg chamber, while the stalk between the mutant follicle and the next younger follicle to the anterior is not specified, indicating that only anterior polar cells induce stalk cells (Torres *et al*., 2003). In addition, the anterior egg chamber frequently mislocalizes its oocyte, independent of genotype (Torres *et al*., 2003). These results indicate the transmission of polarity between egg chambers from posterior to anterior in the germarium. In this model, Dl is required in the germline to activate Notch and specify polar cell fate, while the anterior polar cells specify the anterior stalk through Jak signaling. The polar or stalk cells would then induce DE-Cadherin expression in the posterior follicle cells of the next younger egg chamber through an unknown mechanism, thereby orienting the oocyte at the posterior of the egg chamber.

1.2.5.3. Determining the Polar Cells

Clearly, polar cells are important organizing centers that pattern the follicular epithelium. They are specified through a complex multi-step process beginning with the subdivision of the polar-stalk lineage from the main body follicle cells. Then, localized Notch activity in this lineage represses the expression of the main body determinant *eyes absent* (*eya*), therefore allowing polar cell specification. Localized Notch activity is ensured through the localized expression of *mirror* (*mirr*) at the anterior of the germarium, but the mechanisms that localize *mirr* expression are not well understood. Mirror (Mirr) represses expression of the Notch signaling regulator Fringe (Fng), and this is thought to localize Notch activity to the polar-stalk cell lineage.

1.2.5.3.1. Epidermal growth factor receptor The mechanism that subdivides the polar-stalk cell precursors from the main body cells has not been characterized, though it might depend on an inductive signal from mature 16-cell cysts (Spradling *et al.*, 1997). Cyst encapsulation requires the function of the genes that encode the Epidermal growth factor receptor (Egfr), its ligand Gurken (Grk), and at least two additional genes, *brainiac* (*brn*), and *egghead* (*egh*) (Goode *et al.*, 1992, 1996a, 1996b; Rubsam *et al.*, 1998). Loss of *grk*, *brn*, or *egh* from the germline or *egfr* from the follicle cells leads to the production of fused follicles that are made up of multiple sets of nurse cells and oocytes surrounded by a single layer of follicle cells. These fused egg chambers are formed in the germarium because follicle cells often fail to migrate between cysts.

brn is first expressed in the germline, when the follicle cells begin to surround the cyst. The *brn* phenotype can be enhanced by reducing the dose of *egfr* or *grk*, suggesting that *brn* is required for *egfr* activation in the follicle cells, and that *egfr* activation is required for follicle cell migration during encapsulation. *brn* encodes a glycolipid-specific β1,3*N*-acetylglucosaminyltransferase (Muller *et al.*, 2002), and *egh* encodes a Golgi-located β1,4-mannosyltransferase predicted to form the precursor glycolipid for Brn (Wandall *et al.*, 2003). These enzymes function in the biosynthesis of glycolipids that might mediate extracellular signal-receptor function through direct interactions between glycolipids and receptors, including, but not limited to, Egfr.

1.2.5.3.2. Notch, Delta, and Fringe Polar cell specification is progressive, since early stage egg chambers contain at each pole an excess of cells that express polar cell markers, while by stage 4, they invariably contain only two pairs of such cells (Ruohola *et al.*, 1991). For example, the cell adhesion molecule Fasciclin III (FasIII) is first detected in all follicle cells in region 2 of the germarium (except the SSCs, Section 1.2.2). Its expression begins to restrict to the polar cell precursors by region 3 of the germarium, and is fully restricted to two cells only after the egg chamber has completely separated from the germarium.

Loss-of-function alleles of *Notch* (*N*) or *Delta* (*Dl*) cause a cyst encapsulation defect allowing polycystic fusions to accumulate in the germarium (Ruohola *et al.*, 1991). In weak *Notch*^{ts} mutants, the polar expression domain of FasIII is expanded (Goode *et al.*, 1996a), and in mosaics of strong *Notch* alleles, mutant cells, but not their neighbors, maintain FasIII expression regardless of their location (Grammont and Irvine, 2001; Lopez-Schier and St. Johnston, 2001). These results have been interpreted to suggest that Notch suppresses polar cell specification, and extra polar cells are produced in its absence. However, Notch target genes, including those of the *Enhancer of split Complex* (*E(spl)-C*), are highly expressed specifically in polar cells, confirming that Notch is active in these cells. Polar cells can also be identified by the specific expression of *neuralized* (*neur; P{lacZ}A101*) (Ruohola *et al.*, 1991), and *Notch* mutant follicle cells never express *neur* (Lopez-Schier and St. Johnston, 2001). These results indicate that *Notch* mutant cells do not differentiate as polar cells, but instead arrest as undifferentiated precursors.

Loss of *fng* also causes fusion of adjacent egg chambers. *fng* encodes a glycosyltransferase that cell-autonomously modifies the Notch receptor to potentiate Dl–Notch signaling. *Dl* is required in the germline, while *fng* and *Notch* are required in the follicle cells, for the specification of polar cell fate (Grammont and Irvine, 2001; Lopez-Schier and St. Johnston, 2001). In contrast to *Dl* and *Notch*, which are generally expressed, *fng* expression is restricted to the intercyst cells of the stage 2B germarium by *mirr* (see below), then to the polar cells and stalk cells, as they differentiate. From stages 2–6, *fng* is specifically expressed in the polar cells. Mosaic analysis indicates that *fng* is exclusively required in the polar cell-stalk cell lineage, for establishing polar cell fate, but not stalk cell fate. In fact, only two *fng*$^{+}$ cells are required to begin or end a compound egg chamber.

1.2.5.3.3. Mirror *mirror* mutant germaria are phenotypically similar to *fng*, and *mirr* expression is complementary to *fng* expression: in the germarium, *mirr* is expressed in the IGS cells and the anterior-most follicle cells, whereas *fng* is expressed in the more posterior follicle cells (Jordan *et al.*, 2000). In a young egg chamber, *mirr* is expressed in the main body follicle cells and *fng* in the polar cells and stalk cells. These complementary expression patterns are controlled by the ability of the Mirr transcription factor to repress *fng* expression. Thus, *mirr* expression must be excluded from the polar cell lineage for their specification, and *mirr* mutant ovaries likely have compound follicles because derepressed *fng* expression prevents Notch activation and polar cell specification.

The mechanisms that control *mirr* expression in the germarium are less well understood. Activation of the Jak pathway represses polar *mirr* expression in young egg chambers (Xi *et al.*, 2003), and the Jak ligand *unpaired* (*upd*) is expressed in the polar/stalk cell precursors in region 3 of the germarium

(McGregor *et al*., 2002). This suggests that Jak signaling is already active before polar cell pairs are specified, and that Jak might repress *mirr* expression in the stalk-polar cell lineage.

Since *mirr* expression in dorsal follicle cells of late-stage egg chambers depends on *grk* (Jordan *et al*., 2000), it is tempting to speculate that *grk* also regulates *mirr* expression in the germarium. However, in a *grk* hypomorphic background, early *mirr* expression seems unaffected (Jordan *et al*., 2000), suggesting that another pathway induces *mirr* expression in the IGS cells and anterior-most follicle cells. The proteins encoded by *wg* and *hh* are proposed to cooperate to activate *mirr* expression in the eye-antennal disc (Lee and Treisman, 2001), and these signals are both active within the *mirr* expression domain in the germarium (Section 1.2.2.4.3), suggesting they may have a similar function in the germarium. However, the relationships among the stem cell niche signals, *Egfr*, and the signaling mechanisms operating in the polar-stalk cell lineage have not yet been wholly explored.

1.2.5.3.4. Eyes absent Expression of *eyes absent* (*eya*) might determine main body follicle cell fate. *eya* encodes a transcription cofactor that is expressed in prefollicular cells (**Figure 4**), then is specifically turned off in the intercyst cells as these intercalate in region 2B/3 of the germarium (Bai and Montell, 2002; Torres *et al*., 2003). *eya* is not expressed in the differentiated polar cells or stalk cells. *eya* functions cell-autonomously, and its absence allows main body follicle cells to assume the polar cell fate. Ectopic expression of *eya* is sufficient to suppress normal polar cell specification, thus, *eya* expression must be repressed in the polar cells to allow their specification.

Cells not expressing *eya* are not found in *Notch* or *fng* mutant follicles. Overexpression of *fng* or a constitutively active Notch receptor is sufficient to cell-autonomously repress *eya* expression, but only within the polar-stalk lineage, and this effect is limited to the generation of one or two extra polar cells (Grammont and Irvine, 2001; Bai and Montell, 2002). Therefore, Notch signaling is required exclusively in the polar cells for repressing *eya* expression and allowing specification of polar cells, but not for the specification of the intercyst precursors of polar and stalk cells.

In addition to its role in regulating SSC proliferation and maintenance (Section 1.2.2.4.3), Hh regulates *eya* expression. Overexpression of *hh*, or loss of *hh* pathway inhibitors, is sufficient to repress *eya* expression and generate ectopic polar cells outside of the intercyst lineage (Bai and Montell, 2002). However, *hh* expression is normally limited to the terminal filament and cap cells at the anterior end of the germarium. Although the Hh signal is received at a distance by the SSCs and the prefollicle cells, there is evidence that polar cell fate can be specified, with an abnormal delay, in cells that cannot directly transduce the Hh signal (Zhang and Kalderon, 2000; Bai and Montell, 2002). This indicates the presence of other signals, acting downstream or independently of Hh, that repress *eya* expression and allow polar and stalk cell fates to be specified. Wingless is a good candidate for such a signal since it is expressed in the same cells as Hh and also affects SSC maintenance, while for reasons already discussed, the Jak–Stat pathway does not appear to be involved in polar cell specification.

1.2.6. Establishing Embryonic Polarity

Localization of maternal *gurken* (*grk*), *bicoid* (*bcd*), *oskar* (*osk*), and *nanos* (*nos*) mRNAs to the egg cortex during oogenesis is critical to embryonic axes establishment. These mRNAs are synthesized in the nurse cells, transported into the oocyte, and localized within the oocyte to their cortical destinations. Many of the genes involved in these processes were identified through mutations that disrupt egg polarity in the now famous maternal-effect lethal and female sterile screens (Nusslein-Volhard *et al*., 1987; Schüpbach and Wieschaus, 1989; Schüpbach and Wieschaus, 1991). These screens could only recover mutations that were not lethal, so although they were very successful in identifying genes required for axis determination, other genes involved in this process that are also essential for viability must have been missed. Genetic screens using germline clones promise to identify these genes, and will be important to a deeper understanding of the cell biology of axis determination (Martin *et al*., 2003).

The A–P polarity of the cyst and the oocyte is defined in the germarium (Sections 1.2.4.1 and 1.2.5.3). The oocyte localizes to the posterior of the cyst because oocyte and the posterior-most follicle cells express highest levels of DE-Cadherin. A posterior cortical domain is defined through *par* gene activity, and a posterior MTOC that nucleates MT minus ends is established between the oocyte nucleus and the posterior cortex (**Figure 2**). Posterior oocyte localization in the egg chamber is maintained through the early stages of oogenesis, and during stages 6–7, posterior follicle cell fate is induced in the follicle cells that overlay the oocyte (**Figure 4**, reviews: Nilson and Schüpbach, 1999; Riechmann and Ephrussi, 2001). This induction is

mediated by Egfr in the follicle cells, and the germline TGF-α like signal encoded by *grk*. *grk* mRNA and protein are already localized in the oocyte, when egg chambers bud from the germarium. It is controversial whether *grk* is transcribed in nurse cells and transported toward the posterior MTOC, like many other proteins and RNAs that localize in the early oocyte, or if *grk* is primarily transcribed from the oocyte nucleus (Saunders and Cohen, 1999; Thio *et al.*, 2000). Grk preferentially accumulates between the oocyte nucleus and the follicle cells adjacent to the posterior of the oocyte, from where it activates Egfr signaling (**Figure 4**), which specifies those follicle cells as posterior. The posterior follicle cells then signal back to the oocyte, to induce the cytoskeletal reorganization that is required to direct the posterior localization of *osk* and *nos* mRNAs, the anterior localization of *bcd* mRNA, and the dorsal-anterior relocalization of the oocyte nucleus and *grk* mRNA.

1.2.6.1. Oocyte Cytoskeletal Polarity and *oskar* Localization

Major cytoskeletal reorganization occurs during mid-oogenesis (stages 7–8), after which the posterior MTOC is no longer detected (review: Riechmann and Ephrussi, 2001). In later stages, cytoskeletal polarity has been inferred from the localization in oocytes of reporter proteins whose properties are known in other polarized cells, such as epithelial cells or neuronal axons. Specifically, Nod : β-Gal is a microtubule minus-end reporter that is localized anteriorly, while Kinesin : β-Gal is a microtubule plus-end reporter that localizes posteriorly in stage 8–9 egg chambers (review: Riechmann and Ephrussi, 2001). Posterior localization of *osk* mRNA, which also occurs during stage 8–9, requires the *kinesin heavy chain* (*khc*) gene, which encodes a component of the conventional Kinesin motor complex. In addition, posterior *osk* localization is disrupted by MT depolymerization or in mutants that disturb oocyte polarity (e.g., *grk* mutants). The simplest model to account for these results is that *osk* mRNA is localized by Kinesin on a highly polarized MT cytoskeleton oriented with its minus ends at the anterior and its plus ends at the posterior. In further support of this model, cytoplasmic Dynein, the oocyte's minus-end directed motor, is required for anterior localization of *bcd* mRNA (Duncan and Warrior, 2002; Januschke *et al.*, 2002).

Dynein function is mediated by the multi-protein Dynactin complex, made up of p150^{Glued}, p50 Dynamitin (Dmn), and Lissencephaly-1 (Lis-1). Overexpressing Dmn causes Dynein to dissociate from Dynactin, and disrupts anterior *bcd* localization (Brendza *et al.*, 2002; Duncan and Warrior, 2002; Januschke *et al.*, 2002).

However, several experimental observations are not readily accommodated by this model. One is the ability of MT minus-ends and *osk* mRNA to associate with most of the oocyte cortex (Shulman *et al.*, 2000; Cha *et al.*, 2002; Vaccari and Ephrussi, 2002). After cytoskeletal reorganization, the bulk of the MT network accumulates at the anterior cortex of the oocyte, creating a higher density of MTs in the anterior of the oocyte than the posterior (**Figure 2**; Theurkauf *et al.*, 1992; Clark *et al.*, 1994). Microtubule nucleation is mediated by the γ-Tubulin Ring Complex (γTuRC), which associates with centrosomes and the minus-ends of MTs (Moritz *et al.*, 2000). In stage 10 oocytes, components of γTuRC are uniformly distributed along the entire oocyte cortex, suggesting that the cortex is uniform in its capacity to nucleate MT minus-ends (Cha *et al.*, 2002). Consistent with this observation, loss of *khc* disrupts posterior *osk* mRNA localization, but instead of localizing diffusely or at the center of the oocyte, *osk* mRNA associates with the anterior and lateral cortices (Cha *et al.*, 2002). These results indicate that most of the oocyte cortex can support *osk* localization, and that plus-end directed transport normally carries *osk* away from the cortex except at the MT-poor posterior pole.

Par-1 localization to the posterior cortex might account for the low MT density observed at the posterior pole (Shulman *et al.*, 2000; Vaccari and Ephrussi, 2002). Loss of *par-1* leads to the nucleation of MT minus-ends along the entire cortex of mid-stage oocytes, resulting in abnormal localization of *osk* mRNA to the center of the oocyte together with the MT plus ends (Riechmann *et al.*, 2002; Shulman *et al.*, 2000; Tomancak *et al.*, 2000). Par-1 acts in a conserved pathway that regulates cell polarity and MT dynamics, and may function similarly to its mammalian orthologs, the microtubule-affinity regulating kinases (MARKs). MARKs phosphorylate microtubule-associated proteins (MAPs) and disrupt their binding to MTs. MAPs normally bind along the length of the MTs to stabilize them, so MARK activity destabilizes MTs (Drewes *et al.*, 1997; Ebneth *et al.*, 1999). A similar function could destabilize posterior MTs in the *Drosophila* oocyte and allow posterior localization of *osk* mRNA.

1.2.6.2. Dynein and Kinesin: Dueling Motors

Although Dynein is the major minus-end directed MT motor, both Dynein heavy chain : β-Gal (Dhc : β-Gal), p150^{Glued}, and Dmn are enriched at the posterior pole (McGrail and Hays, 1997; Wilkie

and Davis, 2001; Duncan and Warrior, 2002). Additionally, although Kinesins are generally plus-end directed motors and concentrate at the posterior pole, in germline clones mutant for *khc*, anterior localization of *bcd* and *grk* mRNA and posterior *osk* localization are all disrupted (Brendza *et al.*, 2000; Duncan and Warrior, 2002; Januschke *et al.*, 2002). Thus, the distribution of both motor complexes is similar, and *khc* is required for transport to many different locations in the oocyte. If MT plus-ends are pointed posteriorly, the requirement for Kinesin in anterior transport, and the posterior localization of Dhc : β-Gal must be indirect. Importantly, in *khc* mutant egg chambers, Dhc : β-Gal moves to the anterior cortex, indicating that Kinesin normally relocates Dynein from the anterior cortex to the posterior (Brendza *et al.*, 2000, 2002; Duncan and Warrior, 2002; Januschke *et al.*, 2002). These results suggest that Kinesin and Dynein are both components of the same complex, and that they might cooperate to recycle one another for renewed rounds of cargo loading and transport.

Motor recycling could play an essential role in the efficient transport of a population of small cargoes. In this model, cargoes associate with a complex that includes two opposing motors and can move efficiently in one direction depending on the regulatory state of the opposing motors (Duncan and Warrior, 2002; Gross *et al.*, 2002; Januschke *et al.*, 2002). The dual motor model implies that the cargo to be localized contains proteins capable of directing intracellular transport, so understanding how mRNAs are packaged into transport-competent complexes is important.

1.2.6.3. RNA Recognition Complexes

1.2.6.3.1. Posterior *oskar* localization *Cis*-acting elements in the *osk* mRNA 3′ untranslated region (3′UTR) are essential for *osk* mRNA transport (Ephrussi and Lehmann, 1992; Kim-Ha *et al.*, 1993). *Oskar* mRNA travels from the nurse cells to the posterior of the oocyte as part of a ribonucleoprotein (RNP) complex, that includes at least two RNA binding proteins, Staufen (Stau) and Tsunagi (Tsu), that control the localization and translation of *osk*. Until its posterior localization, *osk* translation is repressed by several trans-acting factors that bind to its 3′UTR (Kim-Ha *et al.*, 1995; Gunkel *et al.*, 1998; Lie and Macdonald, 1999). Repressing *osk* translation during transport from the nurse cells to the oocyte requires Me31B, a DEAD-box RNA helicase (Nakamura *et al.*, 2001). The RNA-binding protein Bruno (Bru) is also required for repression of unlocalized *osk* in the oocyte, although complete repression also requires Apontic (Apt) and p50 (review: Johnstone and Lasko, 2001). Mutations in *cup* block posterior *osk* mRNA localization, and also allow translation of unlocalized *osk*, indicating that Cup is an important link between *osk* localization and translational control (Wilhelm *et al.*, 2003; Nakamura *et al.*, 2004). The mechanisms that relieve *osk* translational repression upon localization remain elusive, but they involve a de-repressor element in the *osk* 5′UTR (Gunkel *et al.*, 1998), Stau (Micklem *et al.*, 2000), the eIF2C-related protein Aubergine (Aub; Wilson *et al.*, 1996), and Orb. In particular, Orb maintains the length of the *osk* poly-A tail to enhance *osk* translation (Lantz *et al.*, 1992; Christerson and McKearin, 1994; Castagnetti *et al.*, 2000).

While in normal development *osk* translation requires posterior localization of its mRNA, these two processes can be uncoupled experimentally. For example, treatment of ovaries with MT depolymerizing drugs results in the ectopic association of *osk* mRNA to the oocyte cortex, and a broad cortical accumulation of Osk and Vas, indicating that most of the oocyte cortex can accommodate the first steps of pole plasm assembly and support *osk* translation (Cha *et al.*, 2002). Similarly, centrally mislocalized *osk* mRNA is translated in *encore* or *par-1* mutant egg chambers (Tomancak *et al.*, 2000; Van Buskirk *et al.*, 2000), or when *laminin A* (Deng and Ruohola-Baker, 2000) mutant posterior follicle cell clones are produced.

Several lines of evidence suggest that the nuclear history of *osk* mRNA can determine its fate in the cytoplasm. The nuclear shuttling proteins Mago Nashi (Mago), Barentsz (Btz), and Tsunagi (Tsu) are required for posterior *osk* localization. In *btz* mutants or in hypomorphic alleles of *mago* or *tsu*, *osk* mRNA fails to accumulate at the posterior, and instead localizes to the anterior margin (Newmark and Boswell, 1994; Micklem *et al.*, 1997; Hachet and Ephrussi, 2001; Mohr *et al.*, 2001; van Eeden *et al.*, 2001). Mago, Btz, and Tsu are components of the exon-junction complex (EJC), and remain with the mRNA after its export from the nucleus, marking the site of intron removal. Although Mago and Tsu are predominately nuclear, while Btz associates with the nuclear envelope, all three proteins shuttle and localize with *osk* mRNA and Stau at the posterior of stage 9 oocytes. These results are consistent with the function of Mago, Btz, and Tsu as components of the *osk* mRNA transport complex. The binding of EJC proteins to *osk* mRNA in the nucleus is thought to imprint the mRNA, and may be a critical step in its posterior targeting (Hachet and Ephrussi, 2001).

Observations of injected *osk* mRNA suggest an additional MT-independent step in *osk* localization, suggesting that cytoplasmic flows or vesicle trafficking are an important final stage in carrying *osk* to the posterior (Glotzer *et al.*, 1997; Dollar *et al.*, 2002). Once *osk* mRNA reaches the posterior of the oocyte, it is stably anchored and translated to allow the assembly of polar granules (Section 1.2.7.1). Actin-dependent anchoring of *osk* mRNA at the posterior has been inferred from the loss of *osk* localization in *tropomyosin II* and *moesin* mutants, and the partial release of *osk* from the posterior upon Actin depolymerization (Erdelyi *et al.*, 1995; Cha *et al.*, 2002; Jankovics *et al.*, 2002; Polesello *et al.*, 2002). Osk protein is also required to anchor its own mRNA at the posterior, since *osk* mRNA is not maintained at the posterior in protein-null *osk* mutants (Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991).

1.2.6.3.2. Anterior *bicoid* localization The *bcd* transcripts are also modified prior to their transport into the oocyte from adjacent nurse cells such that they specifically associate with the anterior cortex (Cha *et al.*, 2001). When injected directly into the oocyte, *bcd* mRNA forms particles that localize to the nearest anterior or lateral cortical surface. Only after *bcd* mRNA has been injected into the nurse cell cytoplasm, withdrawn, and then injected into the oocyte, does it specifically associate with the anterior cortex. Endogenous *bcd* mRNA is found by stage 9 in the nurse cells in large perinuclear clusters of mRNA and proteins (mRNPs). Exuperantia (Exu) protein is also a component of these clusters, which are transported into the oocyte. *bcd* mRNA then localizes to the anterior cortex between stages 10 and 12. In *exu* mutants, the perinuclear clusters fail to form and *bcd* mRNA moves into the oocyte but does not localize to the anterior cortex. Injection of labelled *bcd* mRNA into the nurse cells allows the formation of large (0.5–1 μm) Exu-containing mRNPs which are capable of localizing specifically to the anterior (Cha *et al.*, 2001).

Exhaustive deletion analyses of the *bcd* 3′UTR has identified a minimal localization element that is sufficient for nurse cell to oocyte transport and anterior cortical localization (Macdonald and Kerr, 1997, and references therein). Using a gel-mobility shift assay, Arn *et al.* (2003) have identified a multiprotein complex that binds to the minimal *bcd* mRNA localization signal. It includes a member of the Kinesin family of motor proteins, Nod, and at least four proteins with RNA binding motifs, Modulo (Mod), Swallow (Swa), Smooth (Sm), and Poly-A binding protein (Pabp). Of these, only Swa had been previously implicated in *bcd* localization. In *mod* mutant egg chambers, *bcd* mRNA is often abnormally associated with the lateral cortex of the oocyte. Furthermore, *bcd* mRNA injected into *mod* mutant nurse cells rarely forms RNP particles, and those that do form usually fail to localize to the anterior cortex.

Swa is required for the anterior localization and anchoring of *bcd* mRNA, but is not required for the localization of *bcd* from the nurse cells into the oocyte (Schnorrer *et al.*, 2000). Swa is localized to the anterior, and this depends on MTs but not Exu or *bcd* (Stephenson and Mahowald, 1987; Stephenson *et al.*, 1988). Swa includes an RNA binding motif, and a coiled-coil domain that interacts with Dynein light chain (Dlc). Since disrupting Dynein function causes frequent defects in *bcd* mRNA localization (Duncan and Warrior, 2002; Januschke *et al.*, 2002), and mutations that block the association of Swa with Dlc also block anterior *bcd* localization, Swa appears to act as an adaptor between the Dynein motor complex and *bcd* mRNA (Berleth *et al.*, 1988; St. Johnston *et al.*, 1989; Schnorrer *et al.*, 2000). Anterior *bcd* localization is also diffuse in *khc* mutants, suggesting that repeated cycles of posterior to anterior transport are required to maintain *bcd* localization (Januschke *et al.*, 2002; see dual motor model, Section 1.2.6.2).

A final redistribution of *bcd* mRNA is presumed to occur at the end of oogenesis since its distribution in stage 12 oocytes differs slightly from that in freshly laid eggs (St. Johnston *et al.*, 1989). In late-stage oocytes, *bcd* is cortically localized to an anterior cap, while in very early embryos, it does not appear specifically bound to the cortex and is often located slightly dorsally. Stau binds to the *bcd* 3′UTR and, in the absence of *stau* function, *bcd* is localized normally during oogenesis, but becomes partially delocalized in the embryo, suggesting that Stau mediates this late *bcd* mRNA localization step (St. Johnston *et al.*, 1989; Ferrandon *et al.*, 1994).

1.2.6.4. Nurse Cell-Oocyte Cytoplasmic Transfer and Late-Localizing mRNAs

The later stages of oogenesis (stages 8–14) are marked by a 100-fold increase in the protein content of the oocyte. This is accomplished by the synthesis and uptake of yolk proteins, and an Actin-dependent contraction of the nurse cells that results in the bulk transfer of nurse cell cytoplasm into the oocyte (see also **Chapter 1.3**; Gutzeit, 1986). This process of cytoplasmic transfer is often referred to as "dumping" for simplicity. Most of the nurse cell cytoplasm, which is rich in RNA and protein, ribosomes, lipid droplets, and mitochondria, is transferred through the ring canals into the oocyte

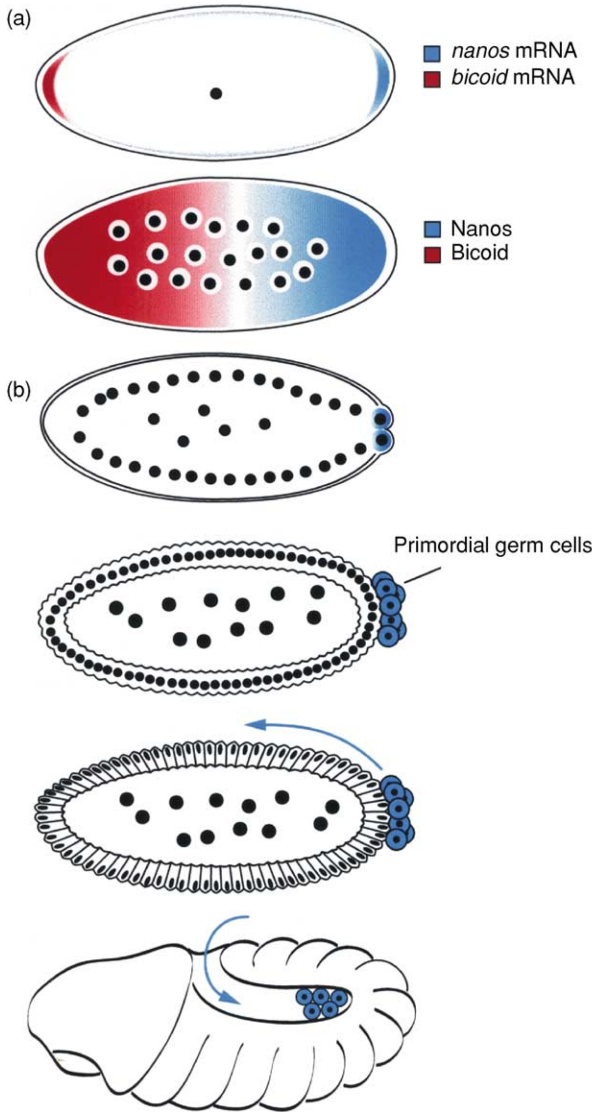


Figure 5 Embryonic Anterior–posterior polarity and primordial germ cell (PGC) specification. (a) Maternal gradients of Bicoid (Bcd) and Nanos (Nos) are established at opposite poles of the postfertilization embryo (discussed in Section 1.2.6.5) These proteins establish an anterior-to-posterior gradient of the Hunchback (Hb) transcription factor, a morphogen whose local concentration defines the position of blastoderm nuclei along the anterior–posterior (A–P) axis. Zygotic *hb* transcription is activated in a concentration-dependent manner by an anterior-to-posterior gradient of Bcd, and *hb* translation is repressed by a posterior-to-anterior gradient of Nos, producing a single anterior-to-posterior Hb gradient. Bcd also establishes a posterior-to-anterior gradient of the transcription factor Caudal (Cad), by repressing translation of *cad* mRNA in anterior regions. (b) The germline is the first distinct cell lineage established in the *Drosophila* embryo. During early syncytial divisions, nuclei form an ellipsoid array that expands from the interior of the embryo to its periphery, and during nuclear cycle 9, a few nuclei at the posterior pole contact the posterior pole, protrude, and form pole buds (top panel). Pole buds divide twice, cellularize, then the PGCs divide asynchronously to form 30–35 germ cells at the embryonic posterior (second panel from top). The PGCs then cease dividing. As the germ band extends dorsally and anteriorly during gastrulation, the posterior midgut (PMG) invaginates and the PGCs are carried into the PMG lumen (third and fourth panels).

during stages 10 and 11. Several well-characterized genes including *chickadee* (*chic*) (Cooley *et al.*, 1992), *quail* (Mahajan-Miklos and Cooley, 1994), and *singed* (*sn*) (Paterson and O'Hare, 1991), and structural components of ring canals (Section 1.2.3.1), affect the transfer of nurse cell contents into the oocyte. The oocytes in these mutants are only about one-half of wild-type size because nurse cells fail to dump their contents into the oocyte. In *chic* mutants, the nurse cell Actin filaments fail to polymerize during late stage 10. *chic* encodes Profilin, a protein that binds Actin and phospholipids, suggesting a role in anchoring microfilaments to membranes. The *singed* gene encodes a Fascin-related protein, and *quail* encodes a Villin-related protein; both of these proteins are thought to cross-link Actin filaments to form bundles.

In the oocyte, parallel arrays of microtubules assemble next to the oocyte cortex just prior to nurse cell dumping (Theurkauf *et al.*, 1992). These subcortical microtubules are required for the rapid circular flow of the oocyte cytoplasm called ooplasmic streaming, which begins during mid-oogenesis and continues until stage 13 (Gutzeit, 1986). Ooplasmic streaming is thought to complete the recruitment of macromolecules to the cortex (Theurkauf, 1994b). Cappuccino (Capu), Spire (Spir), and Profilin are required to repress ooplasmic streaming, and in their absence, premature streaming disrupts *osk*, *nos*, and *grk* mRNA localization (reviews: Manseau *et al.*, 1996; Mahowald, 2001). Both Spir, a putative G-Actin binding protein, and Capu, a Formin homology protein, have been implicated in Actin cytoskeletal dynamics (review: Hudson and Cooley, 2002). Their phenotypes, and the induction of MT-based ooplasmic streaming after Actin depolymerization, strongly suggest that the Actin and MT-based cytoskeletons are functionally interconnected.

1.2.6.4.1. Posterior *nanos* localization Localization of *nos* mRNA to the posterior of the oocyte ensures sufficient localized Nos translation to direct abdominal development and ensures the incorporation of *nos* RNA into the pole cells (**Figure 5**; Wang and Lehmann, 1991; Gavis *et al.*, 1996; Wilhelm *et al.*, 2000). *Nanos* is one of a group of late-localizing RNAs, including *germ cell-less* (*gcl*), and *polar granule component* (*pgc*), that repress transcription in the embryonic germ cells (Section 1.2.7.3; Gavis and Lehmann, 1992; Jongens *et al.*, 1992; Gavis

et al., 1996; Nakamura *et al.*, 1996). Posterior localization of these mRNAs depends on the prior establishment of polar granules at the posterior pole of the oocyte (Section 1.2.7.1). In the absence of polar granule assembly (e.g., in *osk* and *vas* mutants), *nos* mRNA remains unlocalized and its translation is repressed, so the resulting embryos fail to develop abdominal segments (Gavis and Lehmann, 1994; review: Mahowald, 2001).

Starting at stage 11, the oocyte becomes impermeable to whole-mount immunolabelling, so studies of molecular localization in these final stages of oogenesis have been hindered by the need to section or manually remove the chorion and vitelline membrane. To overcome this limitation, Forrest and Gavis (2003) have fluorescently tagged *nos* mRNA *in vivo* in order to visualize its localization in real time. They found that nurse cell dumping is required to drive *nos* mRNA into the oocyte (Forrest and Gavis, 2003). Once in the oocyte, ooplasmic streaming increases the efficiency of *nos* transfer to the posterior, but this is not absolutely necessary. Actin filaments maintain *nos* mRNA, associated with the polar granules at the posterior cortex, which detach from the cortex and are swept away as an aggregate when the Actin filaments are disrupted (Lantz *et al.*, 1999; Forrest and Gavis, 2003).

Posterior *nos* localization is inefficient, as only 4% of the total *nos* mRNA pool is found at the posterior of the embryo (Bergsten and Gavis, 1999). Translational repression of unlocalized *nos* mRNA is mediated by a 90-nucleotide translational control element (TCE) in the *nos* 3′UTR, which is bound by a multi-protein complex that includes the RNA-binding protein Smaug (Smg) (Smibert *et al.*, 1996, 1999; Dahanukar *et al.*, 1999; Crucs *et al.*, 2000). Smg function requires an interaction with the eIF4E-binding protein Cup, which mediates an indirect interaction between Smg and eIF4E (Nelson *et al.*, 2004). Cup blocks the binding of the translation initiation factor eIF4G to eIF4E, so Smg represses *nos* translation by blocking eIF4G recruitment (Nelson *et al.*, 2004). Since Smg and Osk can interact directly, posteriorly localized Osk may capture posterior *nos* mRNA and release Smg-mediated translational repression (Dahanukar *et al.*, 1999). Posterior localization of *nos* mRNA requires recognition of multiple sequence elements within a 540 nucleotide localization signal in the *nos* 5′UTR that is distinct from, yet overlaps, the TCE (Gavis *et al.*, 1996). Based on their ability to genetically separate the translational repression and localization functions of these overlapping elements, Crucs *et al.* (2000) suggest that translational activation of *nos* results when Smg binding is prevented by the direct interaction of localization factors with *nos* 5′UTR sequences.

1.2.6.5. Embryonic Anterior/Posterior Polarity

In the postfertilization embryo, positional information along the A–P axis is defined by the local concentration of the Hunchback (Hb) protein, which is highest at the anterior pole and absent from the posterior pole (reviews: Nusslein-Volhard, 1991; Riechmann and Ephrussi, 2001). Hb fits the classic definition of a morphogen (see **Chapters 1.8** and **1.9**), as it directly defines different cell fates at a distance and in a concentration-dependent manner. The Hb gradient is generated through the antagonistic activities of the maternal-effect genes *bcd* and *nos* (**Figure 5**). *bicoid* mRNA, tightly localized to the anterior pole of the embryo, encodes a transcription factor that patterns the head and thorax. Bcd acts as a transcriptional activator of *hb* (reviews: St. Johnston and Nusslein-Volhard, 1992; Rivera-Pomar *et al.*, 1995), and represses the translation of *caudal* (*cad*) mRNA in the anterior of the embryo (Dubnau and Struhl, 1996; Rivera-Pomar *et al.*, 1996; Niessing *et al.*, 2002). Like Hb, Cad is a transcription factor that activates expression of zygotic segmentation genes, such as *knirps* (*kni*) and *giant* (*gt*), which are required for posterior development (Schulz and Tautz, 1995) in a concentration-dependent manner. *nos* mRNA, localized to the posterior pole of the oocyte, encodes an RNA-binding protein that determines the primordial germ cells (Section 1.2.7) and the abdomen. Zygotic *hb* expression is activated in a concentration-dependent manner by an anterior-to-posterior gradient of Bcd, and *hb* translation is repressed by a posterior-to-anterior gradient of Nos, producing a single anterior-to-posterior Hb protein gradient.

1.2.6.6. Dorsal–Ventral Polarity

1.2.6.6.1. Dynamic *gurken* localization In *grk* or *egfr* mutants, both the oocyte and egg become ventralized, because Egfr activity specifies a dorsal fate (review: Nilson and Schüpbach, 1999). Egfr is uniformly expressed in the somatic follicle cells and its activity is restricted by the strict subcellular localization of Grk, and by the feedback mechanisms outlined below. Localization of *grk* RNA and protein changes dramatically when the oocyte cytoskeleton is reorganized. Grk is closely associated with the oocyte nucleus, and D–V polarity is first apparent at about stage 8, when the oocyte nucleus and Grk localize to the future dorsal-anterior corner of the oocyte (**Figures 4** and **6a**). During cytoskeletal reorganization, *grk* mRNA first localizes around the oocyte's anterior cortex, then concentrates dorsally

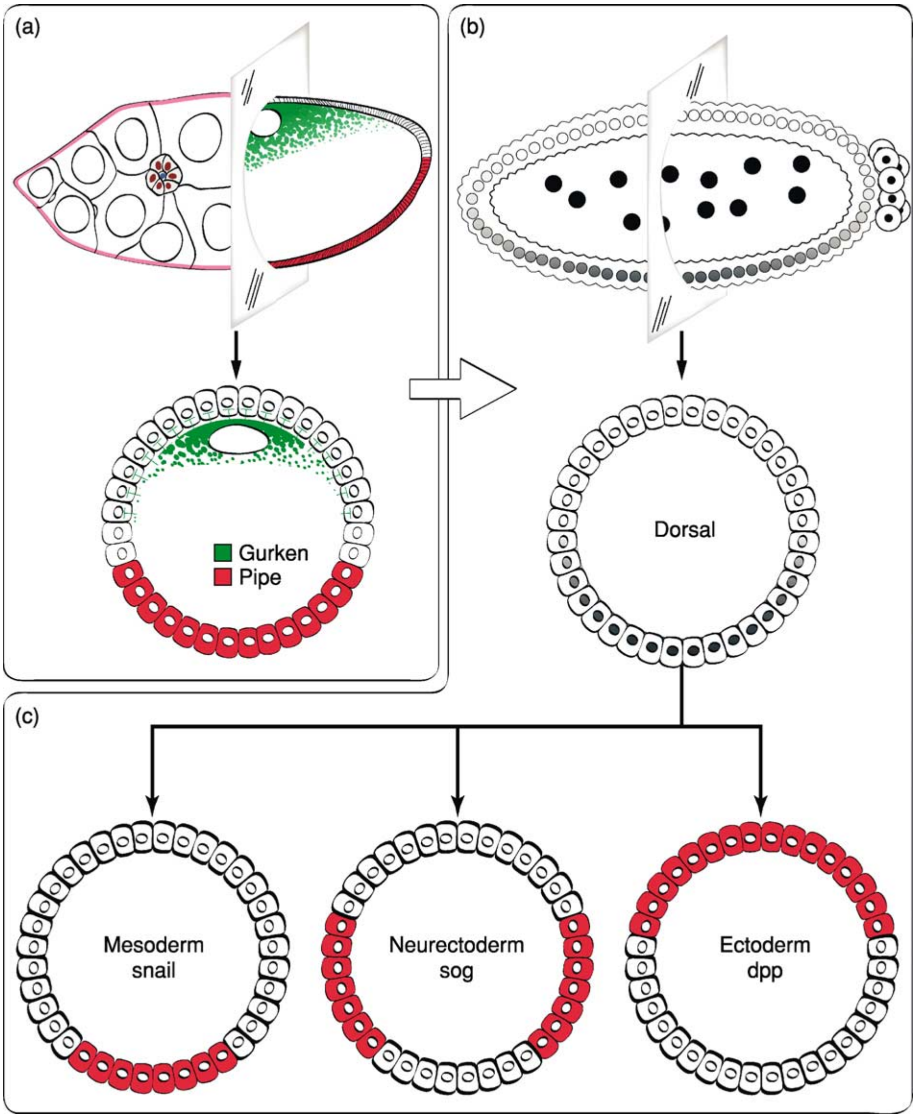


Figure 6 Localized Pipe expression in the ovary establishes dorsal–ventral polarity in the embryo. (a) In the stage-10 egg chamber, Gurken (Grk), an Egfr ligand, is restricted to the anterodorsal corner of the oocyte, and Pipe is expressed only in the ventral follicle cells (discussed in Section 1.2.6.6.2). (b) Pipe-modified ECM proteins are thought to locally initiate a proteolytic cascade that activates the Toll (Tl) receptor on the ventral surface of the now-fertilized egg (discussed in Section 1.2.6.6.4). Nuclei that migrate into ventral regions, where Tl is active, accumulate Dorsal, an NF-κB type transcription factor, while in dorsal regions, Dorsal is sequestered in the cytoplasm and is thus inactive. Nuclear import of the Dorsal protein occurs in a ventral to dorsal gradient (depicted as degrees of shading of the round nuclei) and is restricted to cells in the ventral 40% of the embryo. (c) Different concentration thresholds of nuclear Dorsal initiate the differentiation of the mesoderm, neurectoderm, and mesectoderm by activating expression of *snail* (*sna*), *short gastrulation* (*sog*), *decapentaplegic* (*dpp*) and other genes (discussed in Section 1.2.6.6.4).

between the oocyte nucleus and the overlying follicle cells. The position of the oocyte nucleus defines the new site of *grk* mRNA and protein accumulation, so the critical first step in generating D–V polarity is nuclear migration.

The nature of the asymmetry that targets oocyte nuclear migration is not fully understood, but it relies on the first Grk signal to the posterior follicle cells. Unexpectedly, the oocyte nucleus and *grk* mRNA localize normally in *par-1* hypomorphs, that allow uniform cortical MT nucleation, indicating that an anterior-to-posterior concentration gradient of MTs is not required for oocyte migration (Shulman *et al*., 2000; Vaccari and Ephrussi, 2002). However, other indicators suggest that MT polarity is important. Stable nuclear positioning and *grk* localization requires microtubules, and both Dynein and Kinesin I (Koch and Spitzer, 1983; Roth *et al*., 1999; Saunders and Cohen, 1999; Guichet *et al*., 2001). In addition, mutations affecting the

Dynein-interacting proteins Bic-D and Lis-1 are associated with mispositioning of the oocyte nucleus (Liu *et al.*, 1999; Swan *et al.*, 1999). Disrupting Dynein function by overexpressing Dmn allows the nucleus to leave the cortex during, or after localization, indicating an important function for the Dynein motor complex in cortical anchoring of the oocyte nucleus (Duncan and Warrior, 2002; Januschke *et al.*, 2002).

Dynein–dynactin complexes accumulate around the nucleus in a MT-dependent manner (Januschke *et al.*, 2002), suggesting that the nucleus may have its own perinuclear cytoskeleton that directs anterior-dorsal transport. Supporting this are observations indicating that a scaffold of MTs, or a MT cage, surrounds the oocyte nucleus (Theurkauf 1992), and that treatments which disrupt the cage also disrupt *grk* localization (Duncan and Warrior, 2002; Januschke *et al.*, 2002). For example, Dmn overexpression causes the cage to disappear, and this is associated with diffusion of *grk* mRNA and protein from the D-A corner, even when the nucleus is normally positioned. Grk protein becomes anterior or dispersed, but is not able to specify dorsal fate, leading to the production of ventralized eggs. This suggests that Dynein is additionally required for Grk protein transport to the oocyte cell membrane (Januschke *et al.*, 2002). Recent analysis of *grk* mRNA localization also suggests the presence of a special MTOC at the nucleus as a requirement for dorsal-anterior transport (MacDougall *et al.*, 2003). When labelled *grk* mRNA is injected into the oocyte, it forms particles that generally move toward the oocyte anterior, then appear to change direction and move dorsally. MacDougall *et al.* (2003) suggest that *grk* mRNA might be transported toward the minus ends of a MT skeleton that emanates from the oocyte nuclear cage. However, this model implies an as yet unclear mechanism to distinguish subpopulations of MTs.

Deletion analysis of *grk* indicates the presence of multiple *cis*-acting sequences required for *grk* mRNA transport into the oocyte (Thio *et al.*, 2000), but no *trans*-acting factors required for its early transport have been identified. The 3′UTR of *grk* mRNA, and two *trans*-acting factors, K10 and Squid (Sqd), are required for the movement of *grk* mRNA from the anterior cortex to the D-A of the oocyte (Kelley, 1993; Neuman-Silberberg and Schüpbach, 1993; Serano and Cohen, 1995; Thio *et al.*, 2000). K10 can directly interact with Sqd, which encodes a heterogenous nuclear RNA-binding protein (hnRNPA1 or Hrp40), that binds to the *grk* 3′UTR and regulates both *grk* localization and translation (Norvell *et al.*, 1999). In *K10* or *sqd* mutants, *grk* mRNA is consistently mislocalized and translated along the entire anterior cortex of the oocyte, leading to the production of severely dorsalized eggs. *sqd* produces three alternatively spliced transcripts that produce three protein isoforms with nonidentical functions. Of these, SqdS is localized within both the nurse cell and oocyte nuclei, and is the only Sqd isoform that is sufficient to both localize *grk* mRNA and rescue the dorsalized phenotype of *sqd* mutants (Norvell *et al.*, 1999). Since *sqd* encodes a nuclear shuttling protein, it may connect the nuclear export and MT-dependent localization of *grk* mRNA, analogous to the role of the exon junction complex in *osk* mRNA localization (Section 1.2.6.3.1). In support of this model, although naked (protein-free) pair rule transcripts fail to localize following injection into a blastoderm-stage embryo, pre-incubating these transcripts with any of the Sqd isoforms is sufficient to promote their MT-dependent apical localization (Lall *et al.*, 1999).

In addition to the *spindle* (*spn*) class mutations (Section 1.2.4.1.3), several other genes have been implicated in regulating *grk* translation, but their mechanisms of action remain uncharacterized. *aubergine* (*aub*) was originally identified by mutations that cause ventralized eggs, and loss of *aub* reduces *grk* translation (Schüpbach and Wieschaus, 1991; Wilson *et al.*, 1996). The weakest *orb* allele, orb^{mel}, is associated with reduced *grk* translation, suggesting that Orb may regulate *grk* translation by maintaining its poly-A tail length (Chang *et al.*, 2001). In addition to regulating *osk* translation, Bru may also suppress *grk* translation (Filardo and Ephrussi, 2003). While the *grk* mRNA expression pattern appears normal in *arrest* (*aret*) mutants (confusingly, *arrest* encodes Bru), there is some reduction in Grk protein. Although *arrest* mutants have only a mild effect on dorsal–ventral patterning, they can be strongly enhanced by a reduction in *grk* dose. Using the *grk* 3′UTR as a probe, UV-cross-linked complexes that are immunoprecipitated with anti-Bru include *grk* mRNA, thus suggesting that Bru may regulate *grk* translation directly, similar to its regulation of *osk*. This conclusion, however, does not explain the observation that phenotypes caused by ectopic Bru can be suppressed completely by overexpressing the *osk* 3′UTR. Finally, the Grk protein levels are reduced in *enc* mutants, suggesting that Enc may also influence *grk* translation or protein stability (Hawkins *et al.*, 1996, 1997).

1.2.6.6.2. Egfr directly represses *pipe* expression Localized Egfr activity in ventral follicle cells (**Figure 6**) has two distinct consequences: (1) the restriction of *pipe* expression to ventral follicle cells

to establish embryonic polarity, and (2) the specification of dorsal follicle cell fates that pattern the eggshell. Loss-of-function and ectopic expression data indicate that *pipe* is both necessary and sufficient to induce embryonic ventral fates (Nilson and Schüpbach, 1998; Sen *et al*., 1998). *pipe* is the only gene in the *dorsal* pathway with a ventrally restricted expression pattern, supporting a role in transferring D–V information from the ovary to the embryo. Follicle cells without activated Egfr take up the default ventral follicle cell fate and express *pipe* (Nilson and Schüpbach, 1998; Sen *et al*., 1998). In follicle cell mosaics, *ras* or *Raf* mutant cells that cannot transmit the Egfr signal express *pipe*, but their wild-type neighbors do not (James *et al*., 2002; Peri *et al*., 2002). Therefore, Egfr directly restricts *pipe* expression to the ventral follicle cells.

How Egfr is activated in ventral follicle cells is unclear, as Grk is restricted to an anterior-dorsal domain. Either Grk is secreted and acts as a long-range signal, or a different Egfr ligand is involved. Spitz (Spi), another Egfr ligand, is an obvious candidate as ectopic Spi activity is sufficient to repress *pipe* expression in ventral follicle cells (Peri *et al*., 2002). Spi activity is regulated by the Rhomboid (Rho) protease, which cleaves Spi in the Golgi, allowing its secretion (Urban *et al*., 2001; Wasserman and Freeman, 1998). Grk and Dpp cooperate to activate *rho* expression in the dorsal-anterior follicle cells, and Rho activates Spi secretion from these cells (Twombly *et al*., 1996; Peri and Roth, 2000). These results are consistent with a role for Spi, i.e., by acting as a secondary signal downstream of Grk, in expanding the domain of Egfr activity to a broad dorsal-anterior zone (Wasserman and Freeman, 1998). However, large dorsal clones that are mutant for *rho*, and therefore do not secrete Spi, also do not misexpress *pipe*, as would be expected if Spi repressed *pipe* in dorsal cells that are too far away to directly receive the Grk signal. So, although ectopic Spi activity is sufficient to repress *pipe* expression ventrally, it is not required to repress *pipe* dorsally. These results support a direct role for Grk in specifying dorsal fate at a distance, without requiring secondary signaling events.

The homeodomain transcription factor Mirr is expressed in response to Grk in dorsal anterior cells (Jordan *et al*., 2000; Zhao *et al*., 2000). Loss of *mirr* in mosaics leads to variable ventralization defects in the eggshell, suggesting a role in defining dorsal fate. Ectopic *mirr* expression is sufficient to repress ventral *fng* and *pipe* expression, suggesting that *mirr* might act through *fng*, and by implication the Notch signaling pathway, to repress *pipe* expression in dorsal cells (Jordan *et al*., 2000; Zhao *et al*., 2000). However, loss of *mirr* or *fng* from dorsal or ventral cells has no effect on *pipe* expression (Tran and Berg, 2003). While the role of *fng* in this process remains to be clarified, the effects of ectopic dorsal *mirr* on *pipe* expression are likely mediated through Spi and Egfr, although independently of Grk. Since ectopic *mirr* is sufficient to induce *rho* expression in ventral cells, *mirr* may normally act downstream of Egfr to activate dorsal anterior expression of *rho*, thereby affecting eggshell patterning but not embryonic patterning.

1.2.6.6.3. Refining Egfr activity A predicted consequence of the Spi positive feedback loop is that without refinement, the ensuing signal amplification would spread ventrally. Ventral broadening of Egfr activity appears to be limited by several independent means, including general repression of Egfr activity by Cbl, and Egfr-dependent expression of the *egfr* repressors Argos (Aos), Kekkon (Kek), and Sprouty (Sty), which act in negative feedback loops to refine Egfr activity (review: Leevers, 1999).

The highest level of Egfr activity occurs along the dorsal midline and depends on the Spi positive feedback loop (Wasserman and Freeman, 1998). *argos* expression is induced in these cells, and Aos is secreted, diffuses locally, and directly inhibits Egfr activity along the dorsal midline, thereby refining Egfr activity to two lateral peaks (Wasserman and Freeman, 1998). Cells within these two peaks of Egfr activity later differentiate a pair of dorsal appendages near the anterior of the egg (**Figure 2**), which are thought to mediate gas exchange for the enclosed embryo. Since *rho* expression requires the synergistic interaction of Egfr activity and a Dpp signal from the anterior follicle cells, the position of the dorsal appendages is thought to be regulated in part by limiting *rho* expression to an anterior-dorsal domain (Peri and Roth, 2000).

The function of *kek* is distinct from that of *argos*. Compared to *argos*, *kek* is induced in a broader domain and in a graded fashion, and *kek* is not required to define inter-dorsal appendage fate (Ghiglione *et al*., 1999). *kek* encodes a single-pass transmembrane protein that can directly interact with Egfr, so Kek may directly prevent ligand–receptor interactions (Ghiglione *et al*., 2003). Loss of *kek* is associated with a modest increase in Egfr activity, while ectopic *kek* expression suppresses Egfr activity, consistent with its role as an Egfr repressor. A much more pronounced dorsalized phenotype is caused by loss of *sprouty* (*sty*) (Reich *et al*., 1999). Like *kek*, *sty* expression is more broadly activated by Egfr and its overexpression can suppress Egfr

activity (Casci *et al.*, 1999; Reich *et al.*, 1999). Genetic evidence indicates that Sty acts cell-autonomously to impede Egfr signal transduction in the cytoplasm, although its mechanism of action is not well understood (reviews: in Leevers, 1999; Li *et al.*, 2003b).

A general inhibitor of Egfr activity in the follicle cells is the RING-finger E3 ubiquitin ligase Cbl. In mosaics, loss of *cbl* allows cell-autonomous ectopic Egfr activity, even in ventral follicle cells where *pipe* is repressed (Pai *et al.*, 2000). These effects depend on the presence of Grk, indicating that Cbl acts downstream of Egfr activation. Interesting connections exist between the human orthologs of Cbl, Sty, and Egfr: Cbl poly-ubiquitinates Egfr and hSpry2, and targets them to the proteasome for degradation (Wong *et al.*, 2002; Fong *et al.*, 2003; Hall *et al.*, 2003; Rubin *et al.*, 2003, and references therein). The Cbl-Egfr and Cbl-hSpry2 interactions require the phosphorylation of Egfr and hSpry2 to create Cbl-binding sites. Both phosphorylation events depend on Egfr activation, which induces its own degradation and that of Sty, while at the same time replenishing both proteins by increasing their transcription (Clark *et al.*, 1985; Reich *et al.*, 1999). In the presence of high levels of hSpry2, Egfr is not ubiquitinated or degraded, but instead remains on the cell surface in an activated state. Taken together, this information supports a model in which hSpry2 acts as an Egfr activator by sequestering Cbl, allowing sustained Egfr signaling activity.

However, this model cannot be easily assimilated with data in *Drosophila* and other organisms that implicate Sty in Egfr repression. A highly conserved carboxy-terminal cysteine-rich domain in hSpry2 mediates Egfr inhibition, while a highly conserved amino-terminal phosphotyrosine site bound by Cbl potentiates Egfr signaling (review: Li *et al.*, 2003b). Therefore, Sprouty proteins such as Sty and hSpry2 appear to have dual opposing functions in regulating Egfr activity, depending on their interaction with Cbl, which in turn is regulated by Egfr activity.

Different levels of Egfr activity delimit the domains of four types of follicle cells along the D–V axis of the egg: *argos*-expressing dorsal midline cells with low Egfr activity; adjacent domains with peaks of Egfr activity with *rho*, *kek*, and *sty* expression; lateral domains of decreasing Egfr activity which do not express *pipe* or Egfr-responsive genes; and ventral cells with little or no Egfr activity that express *pipe*. Different levels of Egfr activity appear to be induced by restricting Grk to the dorsal side of the oocyte, and fine-tuning and stabilizing Egfr activity through a complex set of regulatory feedback mechanisms that are only partly understood.

1.2.6.6.4. Embryonic dorsal/ventral polarity The expression of *pipe* is limited to the ventral follicle cells by dorsal Egfr activity in the ovary, and its localized activity establishes dorsal–ventral polarity in the embryo (**Figure 6**; reviews: Roth, 2003; Stathopoulos and Levine, 2002). *pipe* encodes a putative heparin sulfate 2-*O*-sulfotransferase that modifies extracellular matrix (ECM) proteins that localize to the perivitelline space of the egg (Sen *et al.*, 1998, 2000). Pipe-modified ECM proteins are thought to locally initiate a proteolytic cascade, involving Nudel (Ndl), Gastrulation defective (Gd), Snake (Snk), Easter (Ea), and the Serpin Spn27A, that locally activates Spätzle (Spz) in the ventral perivitelline space (review: LeMosy *et al.*, 1999). Spz activates the Toll (Tl) receptor, and the Tl signal is transduced by the Pelle (Pll) kinase, which leads to the phosphorylation and degradation of Cactus (Cact). Cactus normally binds to Dorsal and retains it in the cytoplasm, so Tl activity releases Dorsal and allows its nuclear import. Nuclear import of the Dorsal protein occurs in a ventral to dorsal gradient and is restricted to cells in the ventral 40% of the embryo. Dorsal is a member of the NF-κB family of transcription factors, and different thresholds in its nuclear concentration initiate the differentiation of the mesoderm, mesectoderm, and neurectoderm (review: Stathopoulos and Levine, 2002). At low concentrations, nuclear Dorsal restricts dorsal fate by repressing the expression of dorsal-specific genes such as *dpp*, while it activates the expression of *short gastrulation* (*sog*) and other genes necessary for neurectoderm fate (**Figure 6**). High concentrations of nuclear Dorsal specify mesoderm fate in ventral cells by activating expression of at least two target genes, *twist* (*twi*) and *snail* (*sna*), which then repress the expression of *sog* to restrict neurectoderm fate to two lateral domains.

1.2.7. Primordial Germ Cells

1.2.7.1. PGC Fate Specification

The germline is the first distinct cell lineage established in the *Drosophila* embryo when the pole cells, or PGCs, form at the posterior of the embryo during nuclear cycle 8 (**Figure 5**). PGCs are specified by a unique mechanism that involves cytoplasmic inheritance of RNAs and proteins that are localized to the posterior germ plasm during oogenesis.

The existence of morphologically distinct types of cytoplasm in the posterior region of the egg has been recognized for well over 100 years (review: Mahowald, 1992, 2001). The importance of germ plasm in PGC specification was first described in the beetles *Leptinotarsa* and *Calligrapha*, where experimental removal of the posterior oocyte cytoplasm resulted in larvae lacking germ cells (Hegner, 1908, 1911). In *Drosophila*, cytoplasmic transplantation of posterior pole plasm to the anterior of a recipient egg is sufficient to induce ectopic pole cells that are capable of forming germ cells when implanted to a host posterior (Illmensee and Mahowald, 1974). These important experiments firmly established the role of the germ plasm as the morphogenetic determinant of PGC fate.

Germ plasm contains specialized organelles called polar granules in *Drosophila* – electron-dense fibrous bodies of RNA and protein found only in the posterior oocyte (or early embryonic) cytoplasm or in germ cells (review: Mahowald, 2001). Polar granules are thought to store and regulate the translation of maternal mRNA required for embryonic germ cell determination (see below). Some of the maternal-effect mutations that block PGC formation, namely *capu*, *spir*, *osk*, *stau*, *valois* (*vls*), *tudor* (*tud*), and *vasa* (*vas*), result in the disappearance of polar granules, supporting the direct involvement of the respective gene products in PGC specification. Although in most cases the molecular mechanisms by which polar granule components regulate PGC fate remain obscure, some polar granule components appear to have direct roles in defining the PGC lineage.

Polar granule construction begins with the posterior localization of the first components of polar granules, *osk* mRNA and Stau, and is completed late in oogenesis with the addition of late-localizing mRNAs like *nos* (review: Mahowald, 2001). The critical role for *osk* in forming PGCs was demonstrated by experiments in which a chimeric mRNA containing the *osk* coding region fused to the *bcd* 3′UTR was ectopically localized to the oocyte anterior. A high level of localized *osk* activity was thus produced at the anterior pole, sufficient to direct the assembly of ectopic polar granules, and resulting in the formation of functional PGCs at the anterior (Ephrussi and Lehmann, 1992), reminiscent of those produced by transfer of posterior cytoplasm (Illmensee and Mahowald, 1974). Formation of *osk*-induced ectopic PGCs depends on the function of *vas* and *tud*, but not *capu*, *spir*, or *stau*. This indicates that *osk*, *vas*, and *tud* are centrally important for PGC formation, and that the other three genes function indirectly, perhaps through involvement in localizing Osk. Later work confirmed that Stau functions directly in the transport and posterior anchoring of *osk* mRNA. As discussed in Section 1.2.6.4, *capu* and *spir* encode Actin-binding proteins, and mutations in these genes affect several localization processes. Once produced, Osk is phosphorylated by the Par-1 kinase to stabilize it at the posterior (Bullock and Ish-Horowicz, 2002; Riechmann *et al*., 2002).

Vas is also a key component of polar granules. *vas* mRNA is abundant and uniformly distributed in the cytoplasm of the nurse cells and the oocyte, but Vas protein accumulates at the posterior pole of the oocyte beginning at early stage 10 (Hay *et al*., 1990; Lasko and Ashburner, 1990). Osk is required for posterior Vas accumulation, and Osk and Vas can directly interact, suggesting a direct function for Osk in anchoring Vas to the posterior (Breitwieser *et al*., 1996). Vas arrives a the posterior pole by a mechanism distinct from that of Osk, which is dependent on a second Vas-interacting protein, Gustavus (Gus) (Styhler *et al*., 2002). Vas is stabilized in the pole plasm through an association with the deubiquitination enzyme Fat facets (Faf), which also localizes to the posterior pole dependent on Osk (Fischer-Vize *et al*., 1992; Liu *et al*., 2003).

Vas is a DEAD-box RNA helicase related to the translation factor eIF4A. In *vas*-null mutants, Grk protein levels are reduced (Styhler *et al*., 1998; Tomancak *et al*., 1998). Because Vas can also interact with the general translation factor, dIF2/eIF5B (Carrera *et al*., 2000), it is a candidate translational activator of *grk* and, by extension, at least one of the polar granule mRNAs (review: Johnstone and Lasko, 2001). However, a direct role for Vas in regulating translation has not been absolutely established for any mRNA, nor have any specific Vas-binding RNA sequences or structures yet been identified. Prior to its localization at the posterior, Vas accumulates in punctate perinuclear RNP structures, referred to as the nuage (the French word for "cloud"), near the nurse cell nuclear pores. Other polar granule components, such as Tud and Aub, colocalize with Vas in the nuage (Harris and Macdonald, 2001). Prior to its own accumulation into polar granules, Aub is involved in RNA interference-mediated translational repression of unlocalized *osk* mRNA, potentially implicating the nuage in this process (Kennerdell *et al*., 2002; Findley *et al*., 2003). Thus, the nuage may well be the site where mRNAs are assembled with the proteins that control their localization and translation, a critical

step in the assembly of the transport and translational control machinery (Section 1.2.6.3).

Less is known about the third central gene implicated in germ cell specification, *tud*. PGCs do not form in *tud* mutants, even though some small polar granules have been observed in possibly hypomorphic alleles (Amikura *et al*., 2001). Tud contains 11 repeated copies of a domain of unknown function, termed the Tudor domain. Two related proteins in mouse, Mouse Tudor Repeat-1 (MTR-1), which is the probable Tudor ortholog, and the Survival of Motor Neuron (SMN) protein, have been implicated in direct protein–protein interactions with Sm proteins, the components of the small nuclear ribonucleoproteins (snRNPs) that are involved in RNA splicing (Selenko *et al*., 2001; Chuma *et al*., 2003, and references therein). The snRNPs are assembled in the cytoplasm, but are usually transported to and confined within the nucleus (Will and Luhrmann, 2001). In germline cells of other organisms, Sm proteins colocalize with *vasa* ortholog. This occurs in the perinuclear nuage of *Panorpa* nurse cells (Batalova and Parfenov, 2003), in *C. elegans* P-granules (Barbee *et al*., 2002), and with mouse *Mtr-1* in chromatoid bodies, large RNA and protein complexes that probably represent the mouse nuage (Chuma *et al*., 2003). The distribution of Sm proteins in *Drosophila* nurse cells and oocytes has not been reported. Accumulation of Sm proteins in the cytoplasm is unusual, and may reflect an association with translationally silent mRNAs during oogenesis.

In many species including *Drosophila*, the germ plasm is distinguished not just by the presence of polar granules, but also by a high concentration of mitochondria. Individual mitochondria are often closely juxtaposed with polar granules (Mahowald, 1992, 2001). Surprisingly, two mitochondrial ribosomal RNAs, *mtlrRNA* and *mtsrRNA*, are transported from the mitochondria and localized to the surface of polar granules (Kobayashi *et al*., 1993; Kashikawa *et al*., 1999). While these RNAs are not readily amenable to genetic studies, several lines of evidence support a function for them in germ cell specification. For example, irradiation of pole plasm with UV-light inactivates it (Geigy, 1931), and this inactivation is relieved by injection of *mtlrRNA* (Kobayashi and Okada, 1989). Furthermore, injection into the pole plasm of a ribozyme that specifically cleaves *mtlrRNA* significantly reduces subsequent PGC formation (Kobayashi *et al*., 1993; Iida and Kobayashi, 1998). Accumulation of *mtlrRNA* on the polar granules is greatly reduced in *tud* mutants, leading to the suggestion that Tud might mediate the transport of mitochondrial ribosomal RNAs to the polar granules (Amikura *et al*., 2001).

1.2.7.2. Cell Cycle Control and PGC Migration

The first 13 nuclear divisions in *Drosophila* embryos are synchronized and occur in a syncytium under maternal control. During syncytial nuclear divisions, nuclei form an ellipsoid array that expands from the interior of the embryo to its periphery (**Figure 5**), and during nuclear cycle 9, a few nuclei at the posterior pole contact the germ plasm, protrude and form pole buds. Pole buds divide twice during somatic cycles 9 and 10, and form complete cells at the end of cycle 10, well before somatic cellularization (see below). Polar granules are closely associated with dividing pole bud nuclei, and are specifically inherited by the PGCs upon cellularization. Some polar granule components, most notably Osk, disappear at this stage and are not inherited by the PGCs. Once formed, PGCs divide asynchronously, zero to two times, to form 30–35 large cells at the embryonic posterior. During the same interval, the somatic nuclei divide synchronously three more times (cycles 11–13). After the 13th division, extensions of the common cell membrane pull down between each of the somatic nuclei, and fuse basally to produce individual blastoderm cells (Wieschaus and Sweeton, 1988; Schüpbach and Wieschaus, 1989). At this time, the PGCs cease dividing, and remain quiescent throughout their migration and incorporation into the gonads. Germ cells outside the gonads or that migrate inappropriately are eliminated by apoptosis (review: Coffman, 2003).

As the germ band extends dorsally and anteriorly during gastrulation, the posterior midgut (PMG) invaginates and the PGCs are carried into the PMG lumen (**Figure 5**). They become amoeboid and migrate through the epithelium of the midgut, crawl dorsally over the basal surface of the midgut, then divide into two groups and move laterally to contact the somatic portion of the gonad in parasegments 10–12 (reviews: Starz-Gaiano and Lehmann, 2001; Muller, 2002). The somatic and germline cells coalesce to form the larval gonad. Each female embryonic gonad houses on average 12 PGCs that proliferate without differentiating during larval stages to produce a population of over 100 undifferentiated PGCs. They stop dividing, and some PGCs become germline stem cells at the larval-pupal transition after the development of the somatic GSC niche, while the remainder may directly differentiate as cystoblasts (Section 1.2.2).

In a large-scale screen of the third chromosome, Lehmann and colleagues have identified 25 zygotically required genes that affect germ cell

migration (Moore *et al.*, 1998), including *slow as molasses* (*slam*), *hedgehog* (*hh*), and *HMG-CoA reductase* (*hmgcr*). The novel protein Slam is proposed to guide PGCs off the midgut into the mesoderm (Stein *et al.*, 2002). *hh* is expressed in the somatic gonadal precursor cells, and is sufficient to attract PGCs to the gonad or to sites of ectopic *hh* expression (Deshpande *et al.*, 2001). One or more products of a lipid metabolism pathway involving Hmgcr, Farnesyl-diphosphate synthase, and Geranyl-geranyl-diphosphate synthase provide an attractive cue to PGCs, guiding them towards the gonadal mesoderm (Santos and Lehmann, 2004). Like *hh*, ectopic expression of *hmgcr* can alter the migration path of PGCs. These proteins are proposed to produce extracellular PGC guidance cues by protein geranylation, but the attractant itself has yet to be identified. The attractive signal operates antagonistically with the products of two other genes, *wunen* and *wunen2*. These phospholipid phosphatases repel PGCs from the ventral PMG to ensure dorsal migration (Zhang *et al.*, 1997; Van Doren *et al.*, 1998; Starz-Gaiano and Lehmann, 2001). These studies indicate that PGC migration is a multi-step process, controlled at individual stages by genetically separable mechanisms.

The receptor tyrosine kinase (RTK) encoded by *torso* (*tor*) is somatically expressed and is required for the activation of the Ras/MAP kinase signaling cascade and Stat in anterior and posterior embryonic cells (review: Duffy and Perrimon, 1994). Surprisingly, Ras and Stat are also activated in the PGCs, where they are required for the initial PGC mitotic divisions, and their migration (Li *et al.*, 2003a). Loss of maternal Stat or Ras causes a reduction in PGC numbers, while extra Stat activity dramatically increases their numbers, yet alterations in Stat or Ras activity does not affect the number of pole buds, strongly suggesting that they stimulate PGC divisions. Quiescent PGCs can be induced to divide by overexpression of String, an activator of Cdk1, or an activated form of Cdk1 that cannot be inhibited by phosphorylation, suggesting that inhibitory phosphorylation of Cdk1 induces cell cycle arrest in PGCs (Su *et al.*, 1998). How Stat and Ras cooperate to regulate the PGC cell cycle is not known. The Jak/Stat pathway has also been implicated in PGC migration (Li *et al.*, 2003a). It is interesting that Stat activation is also required for the migration of border follicle cells from the anterior of the egg chamber to the anterior of the oocyte (Beccari *et al.*, 2002; Ghiglione *et al.*, 2002), and like in PGCs, migration of border cells also depends on activated RTK signaling (Duchek *et al.*, 2001). There appears to be highly conserved associations between Stat activity and both increased cell proliferation and cell motility, but the molecular mechanisms are not clear (review: Hou *et al.*, 2002).

1.2.7.3. Transcriptional Repression in PGCs

During the later stages of oogenesis, polar granules mature with the addition of *nos*, *gcl*, and *pgc* RNAs. While *nos* is essential for posterior patterning, *gcl* and *pgc* have no known function besides germline development. In embryos produced by females mutant for any one of these genes, transcription in the PGCs, which is normally postponed until the PGCs approach the gonad, begins prematurely. Loss of transcriptional repression disrupts normal cell cycle control and PGC migration into the gonad.

Even prior to cellularization, the pole buds have greatly reduced levels of active RNA polymerase II that is phosphorylated on its C-terminal domain (CTD) (Leatherman *et al.*, 2002). However, the level of Pol II CTD phosphorylation is increased dramatically in most pole buds of *gcl* mutants (Leatherman *et al.*, 2002). Gcl is detectable at the posterior of early embryos, and it specifically associates with the nuclei that enter the germ plasm (Jongens *et al.*, 1992). Gcl is present at the nucleoplasmic face of the PGC nuclear envelopes, where it is detected until the PGCs migrate through the posterior midgut primordium. Its subcellular localization and phenotype suggest that Gcl may direct chromatin compaction (Jongens *et al.*, 1994), but its mechanism of action remains to be determined. While most *gcl* mutant embryos do not form PGCs, they do form pole buds, and some embryos form a few PGCs and develop into weakly fertile adults. The majority of *gcl* mutant pole bud nuclei misexpress somatic genes, such as *sisterless-a* and *scute*, and ectopic Gcl expression at the anterior of the embryo is sufficient to downregulate *sisterless-a* and *tailless* expression in anterior somatic cells (Leatherman *et al.*, 2002). The *gcl* phenotype suggests that transcriptional quiescence might be a prerequisite to PGC cellularization, while the few fully functional PGCs that form without *gcl* strongly suggest redundancy in the mechanisms that silence transcription in the germline. Transcriptional repression in PGCs is very robust since even the strong Gal4 : VP16 transcriptional activator is not sufficient to overcome it (Van Doren *et al.*, 1998).

Embryos produced from *nos* mutant females fail to establish or maintain transcriptional quiescence in their PGCs and fail to develop functional germ cells. Because *nos* is also required maternally for posterior patterning and thus embryonic viability,

its role in PGC development only became apparent through experiments whereby PGCs lacking *nos* were transplanted to a wild-type host (Kobayashi *et al*., 1996). PGCs mutant for *nos* abnormally transcribe a subset of somatic genes such as *Sex-lethal* (*Sxl*), and the segmentation genes *fushi-tarazu* and *even-skipped* (Kobayashi *et al*., 1996; Asaoka *et al*., 1998; Deshpande *et al*., 1999).

PGCs mutant for *nos* also fail to establish mitotic arrest and thus undergo extra divisions. Both the cell cycle and migration defects of *nos* mutant PGCs can be alleviated by the removal of the *Sxl* gene, indicating that *Sxl* must be silenced by Nos in order to properly specify PGCs. In the soma, Nos directly represses translation of maternal *hb* mRNA (review: Parisi and Lin, 2000), so its effect on transcription in PGCs is thought to be mediated indirectly through translational repression. Zygotic *hb* expression can be induced in ectopic PGCs by misexpressing *bcd* at the anterior, even in a wild-type *nos* background (Deshpande *et al*., 2004). Ectopic *bcd* expression can cause PGC migration and division defects, stimulate Pol II CTD phosphorylation, and induce *Sxl* expression, indicating that Nos-dependent translational repression is very important to transcriptional silencing in PGCs. Translational repression by Nos appears to be a highly conserved mechanism required for PGC development from worms to vertebrates (Subramaniam and Seydoux, 1999; Koprunner *et al*., 2001; Jaruzelska *et al*., 2003; Tsuda *et al*., 2003).

Polar granule component (*pgc*), which is believed to be a noncoding RNA, is also required to build functional PGCs through transcriptional silencing (Nakamura *et al*., 1996; Deshpande *et al*., 2004; Martinho *et al*., 2004). As for *gcl*, germ cells in embryos from females mutant for *pgc* have elevated levels of Polymerase II CTD phosphorylation, and express somatic genes such as *zerknüllt*, *tailless*, and *slam*. Loss of *pgc* also leads to increased levels of methylated Histone H3, which is associated with open chromatin state and suggests transcriptional activity. Again, these data suggest that transcriptional repression in PGCs might be established through regulating RNA Pol II function, but a direct role for *pgc* has not been defined. In contrast to *gcl*, *pgc* mutations do not affect the formation of germ cells, and unlike *nos*, *pgc* appears to have no role in somatic patterning (Martinho *et al*., 2004).

Not all somatic genes tested are misexpressed in *nos*, *gcl*, or *pgc* mutants, and a gene that is misexpressed in one mutant is not necessarily misexpressed in the others. Therefore, transcriptional repression may be accomplished in PGCs through partially redundant mechanisms. Although current evidence indicates the importance of transcriptional and translational repression in the PGC development, it is important to note that a single germ cell determinant has not yet been identified. Since the PGCs are undifferentiated until late in larval development, the germline may be specified by protecting PGCs from somatic patterning signals.

Acknowledgments

We would like to thank Jean-Paul Acco from the Department of Neurophotography at the Montreal Neurological Institute for his expert assistance in creating figures, and Lucia Caceres for critical comments on the manuscript.

Appendix: Gene Products and Functions in Oogenesis

Atypical protein kinase C (aPKC) Serine/threonine kinase. Asymmetric aPKC protein localization is involved in cell fate commitment and maintenance of epithelial apical–basal polarity and oocyte anterior–posterior polarity.

Apontic (Apt) RNA binding protein that, together with Bruno, is involved in negative regulation of *oskar* mRNA translation.

Argos (Aos) Secreted protein that directly inhibits Egfr activity in follicle cells along the dorsal midline, required to define interdorsal appendage fate.

Armadillo (Arm; β-Catenin) Adherens junction component required to maintain the germline and somatic stem cells in the stem cell niche.

***arrest* (*aret*)** See Bruno.

Aubergine (Aub) An eIF2C-related protein, originally identified by mutations that cause ventralized eggs, found in the perinuclear nuage and polar granules, involved in RNA interference-mediated translational repression.

Bag of marbles (Bam) Novel cytoplasmic protein that works together with Bgcn, mitotic cyclins, and their regulators drive cystocyte divisions only as long as Bam is present.

Barentsz (Btz) Nuclear shuttling protein, component of the exon-junction complex (EJC), required for posterior *osk* localization.

Bazooka (Baz) PDZ domain protein, *Drosophila* Par-3 ortholog, phosphorylated and negatively regulated by Par-1, binds aPKC.

Benign gonial cell neoplasm (Bgcn) An unusual RNA helicase-like protein, works together with Bam to promote cystocyte divisions.

Bicaudal-D (Bic-D) Required for oocyte differentiation, mutant egg chambers have 16 nurse cells

and no oocyte, involved in MT-dependent, Dynein-mediated transport of mRNA and protein from the nurse cells into the oocyte.

Bicoid (Bcd) Homeodomain-containing transcription factor defines cell fates along the anterior–posterior axis by activating *huchback* expression and repressing *caudal* translation in the embryo.

Big brain (Bib) Cell membrane protein, positive regulator of Notch signal reception, expressed in stalk cells.

Brainiac (Brn) Glycolipid-specific β1,3N-acetylglucosaminyltransferase, functions in the biosynthesis of glycolipids that might regulate extracellular signal-receptor interactions.

Bruno (Bru) RRM-containing RNA-binding protein encoded by *arrest (aret)*, binds to the Bruno response elements (BREs) of *osk* 3′UTR to repress translation of unlocalized *osk* in the ovary.

Cactus (Cact) Component of the Toll signaling cascade required for dorsal–ventral axis determination. Cact, an I-κB ortholog, inhibits nuclear translocation of Dorsal protein in ventral cells of the embryo by binding to and retaining Dorsal in the cytoplasm.

Cappuccino (Capu) Formin homology protein implicated in Actin cytoskeletal dynamics, required to repress ooplasmic streaming, maternal-effect mutations block PGC formation.

α-Catenin (α-Cat) Component of adherens junctions, has filamentous-Actin and Cadherin binding activity.

β-Catenin (β-Cat) See Armadillo (Arm).

Caudal (Cad) Homeodomain-containing transcription factor required for posterior patterning.

Cbl RING-finger E3 ubiquitin ligase, general inhibitor of Egfr activity in the follicle cells, polyubiquitinates Egfr and hSpry2 and targets them to the proteasome for degradation.

Cdc2/Cdk1 Cyclin-dependent kinase, regulates G2/M transition.

Cheerio Filamin, recruits Hts-RC and Actin to the ring canals.

Chickadee (Chic) *Drosophila* profilin, Actin-binding protein required to polymerize Actin filaments during nurse cell dumping.

Chk2 Serine/threonine protein kinase, transducer of the meiotic checkpoint that is activated by unrepaired double-strand DNA breaks.

Cop9 complex homolog subunit 5 (Csn5) Regulator of specific protein degradation, required in the oocyte for axis determination.

Cup Represses translation of unlocalized *osk* and *nos,* and is required for posterior localization of *osk* mRNA.

Cyclin A (CycA) Regulates G2/M transition, gain-of-function causes a significant fraction of cysts to form with 32 cells.

Cyclin B (CycB) Regulates G2/M transition, gain-of-function causes some cysts to form with 32 cells.

Cyclin E (CycE) G1 Cyclin , loss-of-function leads to cells with only eight cells.

DE-Cadherin Adherens junction component required to maintain the germline and somatic stem cells in the stem cell niche.

Decapentaplegic (Dpp) (TGF-β/BMP) extracellular ligand secreted by the somatic cells of the stem cell niche, represses the expression of *bag of marbles* and maintain GSC fate.

Delta (Dl) Extracellular membrane-bound cell surface ligand for the Notch receptor, required in the germline to activate Notch and specify polar cell fate.

Domeless (Dome) Cell-surface receptor in the Jak pathway.

Dorsal Member of the NF-κB family of transcription factors, regulated nuclear import and activity occurs in a ventral to dorsal gradient in ventral cells, and different thresholds in its nuclear concentration initiate the differentiation of the embryonic mesoderm, mesectoderm, and neurectoderm.

Dynactin Multiprotein complex including Dynamitin, Glued, and Lissencephaly-1, activates Dynein microtubule motor activity.

Dynamitin (Dnm) Component of the Dynein–Dynactin complex that activates Dynein microtubule motor activity.

Dynein Minus-end directed microtubule-dependent motor, its function is mediated by the multiprotein dynactin complex, made up of $p150^{Glued}$, p50 Dynamitin, and Lissencephaly-1.

Dynein heavy chain (Dhc) Protein component of the plus-end directed microtubule motor Dynein.

Easter (Ea) Serine endopeptidase, a component of the protease cascade that produces the Toll ligand during embryonic dorsal–ventral patterning.

Egalitarian (Egl) Required for oocyte differentiation, mutant egg chambers have 16 nurse cells and no oocyte, involved in MT-dependent, Dynein-mediated transport of mRNA and protein from the nurse cells into the oocyte.

Egghead (Egh) A Golgi-located β1,4-mannosyltransferase predicted to form the precursor glycolipid for Brn, functions in the biosynthesis of glycolipids that might regulate extracellular signal–receptor interactions.

Enhancer of split (E(spl)) Canonical Notch pathway target gene, encodes a helix–loop–helix-type transcription factor.

Encore (Enc) Novel protein, mutants produce 32-cell cysts with high frequency necessary for proper assembly of the SCF-complex (Skp/Cullin/Rbx1/F-box) on the fusome, which degrades CycE, negative regulator of Bam.

Epidermal growth factor receptor (Egfr) Important membrane-tethered extracellular receptor that is activated by TGF-α family ligands (e.g., Grk, Spi).

Eyes absent (Eya) A transcription cofactor whose expression must be repressed in the polar cells to allow their specification.

Even-skipped (Eve) Transcription factor, from the pair-rule class of segmentation genes.

Exuperantia (Exu) Protein is also a component of the *bcd* mRNP complex that is required for the anterior cortical localization of *bcd* Mrna.

Fasciclin III (FasIII) Cell adhesion molecule, expression marks undifferentiated follicle cells, and differentiated polar cells.

Farnesyl-diphosphate synthetase Enzyme proposed to modify the extracellular matrix to guide migrating germ cells during embryogenesis.

Fat facets (Faf) Deubiquitination enzyme that localizes to the posterior pole dependent on Osk, deubiquitinates and stabilizes Vas in the pole plasm.

Fringe (Fng) A glycosyltransferase that cell-autonomously modifies the Notch receptor to potentiate Dl to Notch signaling.

Fushi-tarazu (Ftz) Transcription factor, from the pair-rule class of segmentation genes.

Gastrulation defective (Gd) Serine endopeptidase, a component of the protease cascade that produces the Toll ligand during embryonic dorsal–ventral patterning.

Geranyl–geranyl diphosphate synthetase Enzyme proposed to modify the extracellular matrix to guide migrating germ cells during embryogenesis.

Germcell-less (Gcl) Late-localizing RNA, repress transcription in the embryonic germ cells, localizes to the nucleoplasmic face of PGC nuclear envelopes, might direct chromatin compaction.

Giant (Gt) Transcription factor, from the gap class of segmentation genes.

Glued (Gl) A component of the Dynein–Dynactin complex that activates Dynein microtubule motor activity.

Grapes (Grp) Inhibitory kinase of Cdc25-like proteins.

Gurken (Grk) Germline TGF-α-like secreted extracellular Egfr ligand.

Gustavus (Gus) VAS-interacting protein required for posterior localization of VAS.

Hedgehog (Hh) Extracellular ligand, expressed by the TF and cap cells, maintains somatic stem cell fate.

HMG-CoA reductase (Hmgcr) Component of a lipid metabolism pathway involving Farnesyl–diphosphate synthase, and Geranyl–geranyl–diphosphate synthase that provides an attractive cue to PGCs, guiding them toward the gonadal mesoderm.

hnRNPA1/Hrp40 See Squid.

Hopscotch (Hop) The *Drosophila* Janus kinase (Jak).

Hu-Li Tai Shao (Hts) Ring canal protein (Hts-RC) and fusome component.

Hunchback (Hb) Transcription factor, activates gap gene expression along the A/P axis of the embryo in a concentration-dependent manner.

K10 Can directly interact with Sqd, required for the movement of *grk* mRNA from the anterior cortex to the dorsal–anterior of the oocyte.

Kekkon (Kek) Single-pass transmembrane protein that repressed Egfr, can directly interact with Egfr, expected to prevent ligand–receptor interactions.

Kelch The final ring canal component to be added, required through oogenesis for the continued expansion of the ring canals.

Kinesin heavy chain (Khc) Component of conventional Kinesin, plus-end directed microtubule mediated motor complex.

Knirps (Kni) Transcription factor from the gap class of segmentation genes.

Laminin A (LanA) Structural component of the basal lamina extracellular matrix, required for substrate cell adhesion, cell migration, and survival.

Leonardo (14-3-3ζ) Protein kinase C inhibitor required for oocyte fate maintenance, and to establish oocyte cytoskeletal polarity.

Lissencephaly-1 (Lis-1) WD-repeat protein, interacts with the molecular motor cytoplasmic Dynein and its cofactor Dynactin, regulates Dynein function.

Mago Nashi (Mago) Nuclear shuttling protein, component of the exon-junction complex (EJC), required for posterior *osk* localization.

Mirror (Mirr) Homeodomain transcription factor, represses expression of the Notch signaling regulator *fringe*, and this is expected to localize Notch activity to the polar-stalk cell lineage.

Me31B DEAD-box RNA helicase that represses *osk* translation during transport from the nurse cells to the oocyte.

Medea (Med) Transcription factor required for the intracellular transduction of the Dpp (TGF-β) signal.

Meiotic-41 (Mei-41) Serine–threonine protein kinase, delays entry into mitosis in response to DNA damage.

Microtubule-associated protein/microtubule affinity-regulating kinase (MARK) Human Par-1 kinase ortholog.

Modulo (Mod) RRM-domain containing RNA-binding protein, required for *bcd* mRNA localization.

Moesin (Moe) Actin-binding protein, anchors microfilaments to the cell cortex, required to maintain epithelial cell polarity.

Mothers against Dpp (Mad) Component of the Dpp (TGF-β) intracellular signaling cascade, nuclear translocation is essential for Medea function.

mtlrRNA and mtsrRNA Two mitochondrial ribosomal RNAs that localize to polar granules and function in germ cell specification.

Nanos (Nos) Interacts with Pumilio to repress translation of *hb* and presumably other RNAs required to repress transcription in PGCs.

Neuralized (Neur) RING-finger DNA-binding protein and ubiquitin ligase that promotes degradation of Delta. Notch target gene, specifically expressed in polar cells.

No distributive disjunction (Nod) Kinesin-family motor, required for meiotic chromosome segregation.

Notch (N) Important membrane-bound extracellular receptor protein.

Nudel (Ndl) Extracellular serine protease in the Toll dorsal–ventral signaling pathway, locally activates the serine protease cascade that produces the Toll ligand.

Oo18 RNA-binding protein (Orb) *Drosophila* ortholog of Cytoplasmic polyadenylation element-binding protein (CPEB), believed to stimulate translation of specific mRNAs that contain a CPE sequence by mediating an extension of their poly(A) tails.

Okra DEAH-type DNA helicase with an SNF2 domain, ortholog of RAD52 in other organisms.

Orbit/Mast Microtubule-binding protein, required for spindle assembly during mitosis, stem cell and cystocyte divisions and oocyte differentiation.

Oskar (Osk) Protein sufficient to direct the assembly of ectopic polar granules, polar granule component, maternal-effect mutations block PGC formation.

Par-1 Serine–threonine kinase, phosphorylates Osk and anchors it at the posterior. Acts in a conserved pathway that regulates cell polarity and MT dynamics, may function similarly to its mammalian orthologs, the microtubule-affinity regulating kinases (MARKs), which phosphorylate microtubule-associated proteins (MAPs) to destabilize MTs.

Par-3 See Bazooka.

Par-5 (14-3-3ϵ) Protein Kinase C inhibitor, required for oocyte fate maintenance, and to establish oocyte cytoskeletal polarity.

Par-6 Required to establish epithelial and oocyte polarity, Baz and Par-6 proteins are interdependent for their asymmetric localization at the apical cell cortex of epithelial cells and neuroblasts in the embryo.

Patched (Ptc) Transmembrane receptor for Hedgehog.

PDZ domain Approximately 90 residue protein-recognition domain.

Pelle (Pll) Serine/threonine protein kinase required for transduction of the Toll signal in dorsoventral embryonic patterning.

Pipe Putative heparin sulfate 2-O-sulfotransferase that modifies extracellular matrix (ECM) proteins modified ECM proteins are expected to locally initiate a proteolytic cascade is both necessary and sufficient to induce embryonic ventral fates.

Piwi Autonomously required in the TF cells and cap cells for stem cell survival, founding member of a conserved class of proteins (the Piwi, Argonaute, or PPD family) that function in stem cell maintenance. Involved in RNA interference pathway.

***polar granule component* (*pgc*)** Late-localizing nontranslated RNA that is required for transcriptional repression in the PGCs.

Poly-A binding protein (Pabp) RRM-domain RNA-binding protein, binds to the 3′poly-A tail of mRNA, positive regulator of mRNA stability and translation.

Profilin See Chickadee.

Pumilio (Pum) RNA-binding protein, complexes with Nanos, required in the germline for the maintenance of GSCs, mutants fail to establish or maintain transcriptional quiescence in their PGCs.

Punt (Put) Type II transmembrane Dpp (TGF-β) receptor.

Quail Villin-related protein thought to cross-link Actin filaments to form bundles.

Ras GtPase, key intermediate in G-protein coupled receptor signaling pathways.

Raf Serine/threonine protein kinase, key intermediate in receptor tyrosine kinase-mediated intercellular signaling pathways.

Rhomboid (Rho) Protease that cleaves Spitz in the Golgi, allowing its secretion.

SCF-complex Multiprotein complex of SKP1, Cul1, and an F-box protein that catalyzes the polyubiquitination of proteins, a modification required for their degradation by the 26S proteasome.

Scute (Sc) Transcription factor, first expressed in early embryogenesis in cells with neurogenic potential.
Sex-lethal (Sxl) RNA-binding protein, expressed in females but not males, controls sex-specific alternative splicing and translation required for sex determination.
Short gastrulation (Sog) Secreted BMP-binding protein that narrows the domain of dorsal Dpp (TGF-β/BMP) activity during dorsal–ventral axis patterning in the embryo.
Short stop (Shot) Large cytoskeletal linker protein called spectraplakin, fusome component that binds and stabilizes microtubules.
Shutdown (Shu) Similar to the heat-shock protein-binding immunophilins, required for the production of 16-cell cysts during oogenesis.
Serpin47A Serine protease inhibitor, mutants show uniform high levels of Toll signaling.
Singed (Sn) Fascin-related protein expected to cross-link Actin filaments to form bundles.
Sisterless-a (SisA) Transcription factor required for endoderm migration, midgut formation, and sex determination.
Slow as molasses (Slam) Novel protein required for cellularization and proposed to guide PGCs off the midgut into the mesoderm.
Smaug (Smg) RNA-binding protein, binds to the 90-nucleotide translational control element (TCE) in the *nos* 3′UTR and represses *nos* translation by blocking eIF4G recruitment.
Smooth (Sm) RNA-binding protein, component of *bicoid* mRNA–protein complex.
Snail (Sna) Transcription factor, required for ventral furrow formation at gastrulation.
Snake (Snk) Serine endopeptidase, a component of the protease cascade that produces the Toll ligand during embryonic dorsal–ventral patterning.
Soft-boiled female sterile mutation causing the production of cysts with 16 nurse cells and no oocyte.
Spätzle (Spz) extracellular ligand for the Toll receptor, Easter cleaves and activates SPZ on the ventral side of the embryo.
α-Spectrin (α-Spec) Membrane skeletal protein, structural component of the fusome, which is required for ovarian cyst formation.
Spindle (Spn) A group of proteins involved in DSB repair, mutations cause decreased Grk accumulation and fragmented chromosomes in the oocyte nucleus.
Spire (Spir) Putative G-Actin-binding protein implicated in Actin cytoskeletal dynamics, required to repress ooplasmic streaming, maternal-effect mutations block PGC formation.
Spitz (Spi) A secreted Egfr ligand.
Sprouty (Sty) Negative regulator of EGF signaling
Squid (Sqd) RRM-domain containing heterogenous nuclear RNA-binding protein (hnRNPA1 or Hrp40) that binds *grk* RNA.
Stat (*Stat92E)* Intracellular mediator of Jak signaling, transcription regulator.
Staufen (Stau) Double-stranded RNA-binding protein, required for posterior *osk* mRNA localization and a late *bcd* mRNA localizaton step, polar granule component, maternal-effect mutations block PGC formation.
Stonewall (Stwl) Transcription factor required for oocyte fate determination.
String (Stg) Drosophila Cdc25 kinase homolog, derepress the Cyclin-dependent kinase Cdk1 to initiate the G2/M transition, overexpression causes cysts to form with only eight cells.
Swallow (Swa) RRM-domain and coiled-coil domain containing protein that interacts with Dynein light chain (Dlc), possible adaptor between the Dynein motor complex and *bcd* mRNA, required for the anterior localization and anchoring of *bcd*.
Tailless (Tll) transcription factor required to determine embryonic posterior and anterior terminal regions.
Ter94 an AAA-ATPase, a fusome component also associated with the endoplasmic reticulum.
Toll (Tl) transmembrane receptor required to define positional information along the dorsal–ventral embryonic axis.
Torso (Tor) somatically expressed receptor tyrosine kinase (RTK) that activates the Ras/MAP kinase signaling cascade and Stat in anterior and posterior embryonic cells and PGCs, required for the initial PGC mitotic divisions, and PGC migration.
Tribbles (Trbl) cell cycle regulator, inhibits STG in embryonic and cystocyte cell cycles by inducing its degradation, misexpression causes the formation of 32 cell cysts.
Tropomyosin II (TmII) Actin-binding protein required to anchor *oskar* mRNA to the posterior cortex of the oocyte.
Tsunagi (Tsu) nuclear shuttling hnRNP RNA binding protein, ortholog of vertebrate Y14, component of the exon junction complex (EJC), required for posterior *osk* mRNA localization.
γ-Tubulin Ring Complex (γTuRC) Associates with centrosomes and the egg cortex, mediates nucleation of microtubule minus ends.
Tudor (Tud) Centrally important for PGC formation, Tud contains 11 repeated copies of the Tudor domain, thought to mediate protein–protein interactions, maternal-effect mutations block PGC formation.

Twine (Twe) CDC25 protein tyrosine phosphatase, essential for meiotic G2/MI transition during oogenesis and has a role in regulating mitosis in the syncytial embryo.
Twist (Twi) Transcription factor required to specify mesodermal muscle fate, target of the Toll signaling pathway.
UbcD1 E2 ubiquitin-conjugating enzyme.
Unpaired (Upd) Extracellular Jak ligand, expressed in the polar cells.
Valois (Vls) Maternal-effect mutations affect posterior patterning and block PGC formation.
Vasa (Vas) DEAD-box RNA helicase related to the translation factor eIF4A, polar granule component, centrally important for PGC formation, probable translational activator of *grk* in early oocytes.
Wingless (Wg) Extracellular WNT-family ligand, expressed by the TF and cap cells, maintains somatic stem cell fate.
Wunen and Wunen2 Phospholipid phosphatases required to repel PGCs from the ventral PMG to ensure dorsal migration.
Yb P-loop containing nucleoside triphosphate hydrolase, autonomously required in the TF cells and cap cells for stem cell survival, positive regulator of both *hh* and *piwi* expression.

References

Abdu, U., Brodsky, M., Schüpbach, T., **2002**. Activation of a meiotic checkpoint during *Drosophila* oogenesis regulates the translation of Gurken through Chk2/Mnk. *Curr. Biol. 12*, 1645–1651.

Ahringer, J., **2003**. Control of cell polarity and mitotic spindle positioning in animal cells. *Curr. Opin. Cell Biol. 15*, 73–81.

Amikura, R., Hanyu, K., Kashikawa, M., Kobayashi, S., **2001**. Tudor protein is essential for the localization of mitochondrial RNAs in polar granules of *Drosophila* embryos. *Mech. Devel. 107*, 97–104.

Arn, E.A., Cha, B.J., Theurkauf, W.E., Macdonald, P.M., **2003**. Recognition of a bicoid mRNA localization signal by a protein complex containing Swallow, Nod, and RNA binding proteins. *Devel. Cell. 4*, 41–51.

Asaoka, M., Sano, H., Obara, Y., Kobayashi, S., **1998**. Maternal Nanos regulates zygotic gene expression in germline progenitors of *Drosophila melanogaster. Mech. Devel. 78*, 153–158.

Bai, J., Montell, D., **2002**. Eyes absent, a key repressor of polar cell fate during *Drosophila* oogenesis. *Development 129*, 5377–5388.

Baksa, K., Parke, T., Dobens, L.L., Dearolf, C.R., **2002**. The *Drosophila* Stat protein, stat92E, regulates follicle cell differentiation during oogenesis. *Devel. Biol. 243*, 166–175.

Barbee, S.A., Lublin, A.L., Evans, T.C., **2002**. A novel function for the Sm proteins in germ granule localization during *C. elegans* embryogenesis. *Curr. Biol. 12*, 1502–1506.

Batalova, F., Parfenov, V., **2003**. Immunomorphological localization of Vasa protein and pre-mRNA splicing factors in *Panorpa communis* trophocytes and oocytes. *Cell Biol. Int. 27*, 795–807.

Beccari, S., Teixeira, L., Rorth, P., **2002**. The Jak/Stat pathway is required for border cell migration during *Drosophila* oogenesis. *Mech. Devel. 111*, 115–123.

Benfey, P.N., **1999**. Stem cells: A tale of two kingdoms. *Curr. Biol. 9*, R171–R172.

Benton, R., Johnston, D.S., **2003**. *Drosophila* Par-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell 115*, 691–704.

Benton, R., Palacios, I.M., St. Johnston, D., **2002**. *Drosophila* 14-3-3/PAR-5 is an essential mediator of Par-1 function in axis formation. *Devel. Cell. 3*, 659–671.

Bergsten, S.E., Gavis, E.R., **1999**. Role for mRNA localization in translational activation but not spatial restriction of nanos RNA. *Development 126*, 659–669.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., *et al.*, **1988**. The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J. 7*, 1749–1756.

Bhat, K.M., **1999**. The posterior determinant gene nanos is required for the maintenance of the adult germline stem cells during *Drosophila* oogenesis. *Genetics 151*, 1479–1492.

Bhat, K.M., Schedl, P., **1997**. Establishment of stem cell identity in the *Drosophila* germline. *Devel. Dyn. 210*, 371–382.

Bolivar, J., Huynh, J.R., Lopez-Schier, H., Gonzalez, C., St. Johnston, D., *et al.*, **2001**. Centrosome migration into the *Drosophila* oocyte is independent of BicD and egl, and of the organisation of the microtubule cytoskeleton. *Development 128*, 1889–1897.

Breitwieser, W., Markussen, F.H., Horstmann, H., Ephrussi, A., **1996**. Oskar protein interaction with Vasa represents an essential step in polar granule assembly. *Genes Devel. 10*, 2179–2188.

Brendza, R.P., Serbus, L.R., Duffy, J.B., Saxton, W.M., **2000**. A function for kinesin I in the posterior transport of oskar mRNA and Staufen protein. *Science 289*, 2120–2122.

Brendza, R.P., Serbus, L.R., Saxton, W.M., Duffy, J.B., **2002**. Posterior localization of dynein and dorsal-ventral axis formation depend on kinesin in *Drosophila* oocytes. *Curr. Biol. 12*, 1541–1545.

Brodsky, M.H., Weinert, B.T., Tsang, G., Rong, Y.S., McGinnis, N.M., *et al.*, **2004**. *Drosophila melanogaster* MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. *Mol. Cell. Biol. 24*, 1219–1231.

Bullock, S.L., Ish-Horowicz, D., **2001**. Conserved signals and machinery for RNA transport in *Drosophila* oogenesis and embryogenesis. *Nature 414*, 611–616.

Bullock, S.L., Ish-Horowicz, D., **2002**. Cell polarity: Oskar seeks PARtner for a stable relationship. *Nat. Cell Biol. 4*, E117–E118.

Büning, J., **1994**. The Insect Ovary: Ultrastructure, Previtellogenic Growth, and Evolution. Chapman and Hall, New York.

Carpenter, A.T., **1981**. EM autoradiographic evidence that DNA synthesis occurs at recombination nodules during meiosis in *Drosophila melanogaster* females. *Chromosoma 83*, 59–80.

Carpenter, A.T., **1994**. Egalitarian and the choice of cell fates in *Drosophila melanogaster* oogenesis. *Ciba Found. Symp. 182*, 223–254.

Carrera, P., Johnstone, O., Nakamura, A., Casanova, J., Jackle, H., *et al.*, **2000**. VASA mediates translation through interaction with a *Drosophila* yIF2 homolog. *Mol. Cell. 5*, 181–187.

Casci, T., Vinos, J., Freeman, M., **1999**. Sprouty, an intracellular inhibitor of Ras signaling. *Cell 96*, 655–665.

Castagnetti, S., Ephrussi, A., **2003**. Orb and a long poly(A) tail are required for efficient oskar translation at the posterior pole of the *Drosophila* oocyte. *Development 130*, 835–843.

Castagnetti, S., Hentze, M.W., Ephrussi, A., Gebauer, F., **2000**. Control of oskar mRNA translation by Bruno in a novel cell-free system from *Drosophila* ovaries. *Development 127*, 1063–1068.

Cerutti, L., Mian, N., Bateman, A., **2000**. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem. Sci. 25*, 481–482.

Cha, B.J., Koppetsch, B.S., Theurkauf, W.E., **2001**. *In vivo* analysis of *Drosophila* bicoid mRNA localization reveals a novel microtubule-dependent axis specification pathway. *Cell 106*, 35–46.

Cha, B.J., Serbus, L.R., Koppetsch, B.S., Theurkauf, W.E., **2002**. Kinesin I-dependent cortical exclusion restricts pole plasm to the oocyte posterior. *Nat. Cell Biol. 4*, 592–598.

Chang, J.S., Tan, L., Wolf, M.R., Schedl, P., **2001**. Functioning of the *Drosophila* orb gene in gurken mRNA localization and translation. *Development 128*, 3169–3177.

Chen, D., McKearin, D., **2003a**. Dpp signaling silences Bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol. 13*, 1786–1791.

Chen, D., McKearin, D.M., **2003b**. A discrete transcriptional silencer in the Bam gene determines asymmetric division of the *Drosophila* germline stem cell. *Development 130*, 1159–1170.

Chen, J., Godt, D., Gunsalus, K., Kiss, I., Goldberg, M., *et al.*, **2001**. Cofilin/ADF is required for cell motility during *Drosophila* ovary development and oogenesis. *Nat. Cell Biol. 3*, 204–209.

Christerson, L.B., McKearin, D.M., **1994**. orb is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis. *Genes Devel. 8*, 614–628.

Chuma, S., Hiyoshi, M., Yamamoto, A., Hosokawa, M., Takamune, K., *et al.*, **2003**. Mouse Tudor Repeat-1 (MTR-1) is a novel component of chromatoid bodies/nuages in male germ cells and forms a complex with snRNPs. *Mech. Devel. 120*, 979–990.

Clark, A.J., Ishii, S., Richert, N., Merlino, G.T., Pastan, I., **1985**. Epidermal growth factor regulates the expression of its own receptor. *Proc. Natl Acad. Sci. USA 82*, 8374–8378.

Clark, I., Giniger, E., Ruohola-Baker, H., Jan, L.Y., Jan, Y.N., **1994**. Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr. Biol. 4*, 289–300.

Clark, K.A., McKearin, D.M., **1996**. The *Drosophila* stonewall gene encodes a putative transcription factor essential for germ cell development. *Development 122*, 937–950.

Coffman, C.R., **2003**. Cell migration and programmed cell death of *Drosophila* germ cells. *Ann. NY Acad. Sci. 995*, 117–126.

Cooley, L., Verheyen, E., Ayers, K., **1992**. chickadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell 69*, 173–184.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., *et al.*, **1998**. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Devel. 12*, 3715–3727.

Cox, D.N., Chao, A., Lin, H., **2000**. piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development 127*, 503–514.

Cox, D.N., Lu, B., Sun, T.Q., Williams, L.T., Jan, Y.N., **2001a**. *Drosophila* par-1 is required for oocyte differentiation and microtubule organization. *Curr. Biol. 11*, 75–87.

Cox, D.N., Seyfried, S.A., Jan, L.Y., Jan, Y.N., **2001b**. Bazooka and atypical protein kinase C are required to regulate oocyte differentiation in the *Drosophila* ovary. *Proc. Natl Acad. Sci. USA 98*, 14475–14480.

Crucs, S., Chatterjee, S., Gavis, E.R., **2000**. Overlapping but distinct RNA elements control repression and activation of nanos translation. *Mol. Cell. 5*, 457–467.

Dahanukar, A., Walker, J.A., Wharton, R.P., **1999**. Smaug, a novel RNA-binding protein that operates a translational switch in *Drosophila*. *Mol. Cell. 4*, 209–218.

de Cuevas, M., Lee, J.K., Spradling, A.C., **1996**. alpha-spectrin is required for germline cell division and differentiation in the *Drosophila* ovary. *Development 122*, 3959–3968.

de Cuevas, M., Lilly, M.A., Spradling, A.C., **1997**. Germline cyst formation in *Drosophila*. *Annu. Rev. Genet. 31*, 405–428.

de Cuevas, M., Spradling, A.C., **1998**. Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development 125*, 2781–2789.

Denef, N., Schüpbach, T., **2003**. Patterning: Jak–Stat signalling in the *Drosophila* follicular epithelium. *Curr. Biol. 13*, R388–R390.

Deng, W., Lin, H., **1997**. Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized

microtubule array for oocyte specification in *Drosophila*. *Devel. Biol. 189*, 79–94.

Deng, W., Lin, H., **2001**. Asymmetric germ cell division and oocyte determination during *Drosophila* oogenesis. *Int. Rev. Cytol. 203*, 93–138.

Deng, W.M., Althauser, C., Ruohola-Baker, H., **2001**. Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in *Drosophila* follicle cells. *Development 128*, 4737–4746.

Deng, W.M., Ruohola-Baker, H., **2000**. Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior–posterior axis in *Drosophila*. *Curr. Biol. 10*, 683–686.

Deshpande, G., Calhoun, G., Schedl, P., **2004**. Overlapping mechanisms function to establish transcriptional quiescence in the embryonic *Drosophila* germline. *Development 131*, 1247–1257.

Deshpande, G., Calhoun, G., Yanowitz, J.L., Schedl, P.D., **1999**. Novel functions of nanos in downregulating mitosis and transcription during the development of the *Drosophila* germline. *Cell 99*, 271–281.

Deshpande, G., Swanhart, L., Chiang, P., Schedl, P., **2001**. Hedgehog signaling in germ cell migration. *Cell 106*, 759–769.

Dollar, G., Struckhoff, E., Michaud, J., Cohen, R.S., **2002**. Rab11 polarization of the *Drosophila* oocyte: a novel link between membrane trafficking, microtubule organization, and oskar mRNA localization and translation. *Development 129*, 517–526.

Doronkin, S., Djagaeva, I., Beckendorf, S.K., **2002**. Csn5/Jab1 mutations affect axis formation in the *Drosophila* oocyte by activating a meiotic checkpoint. *Development 129*, 5053–5064.

Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E.M., Mandelkow, E., **1997**. MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell 89*, 297–308.

Dubnau, J., Struhl, G., **1996**. RNA recognition and translational regulation by a homeodomain protein. *Nature 379*, 694–699.

Dubowy, J., Macdonald, P.M., **1998**. Localization of mRNAs to the oocyte is common in *Drosophila* ovaries. *Mech. Devel. 70*, 193–195.

Duchek, P., Somogyi, K., Jekely, G., Beccari, S., Rorth, P., **2001**. Guidance of cell migration by the *Drosophila* PDGF/VEGF receptor. *Cell 107*, 17–26.

Duffy, J.B., Perrimon, N., **1994**. The torso pathway in *Drosophila*: lessons on receptor tyrosine kinase signaling and pattern formation. *Devel. Biol. 166*, 380–395.

Duncan, J.E., Warrior, R., **2002**. The cytoplasmic dynein and kinesin motors have interdependent roles in patterning the *Drosophila* oocyte. *Curr. Biol. 12*, 1982–1991.

Eberhart, C.G., Maines, J.Z., Wasserman, S.A., **1996**. Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature 381*, 783–785.

Ebneth, A., Drewes, G., Mandelkow, E.M., Mandelkow, E., **1999**. Phosphorylation of MAP2c and MAP4 by MARK kinases leads to the destabilization of microtubules in cells. *Cell. Motil. Cytoskeleton 44*, 209–224.

Ephrussi, A., Dickinson, L.K., Lehmann, R., **1991**. Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. *Cell 66*, 37–50.

Ephrussi, A., Lehmann, R., **1992**. Induction of germ cell formation by oskar. *Nature 358*, 387–392.

Erdelyi, M., Michon, A.M., Guichet, A., Glotzer, J.B., Ephrussi, A., **1995**. Requirement for *Drosophila* cytoplasmic tropomyosin in oskar mRNA localization. *Nature 377*, 524–527.

Etienne-Manneville, S., Hall, A., **2003**. Cell polarity: Par6, aPKC and cytoskeletal crosstalk. *Curr. Opin. Cell Biol. 15*, 67–72.

Ferrandon, D., Elphick, L., Nusslein-Volhard, C., St. Johnston, D., **1994**. Staufen protein associates with the 3′UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. *Cell 79*, 1221–1232.

Filardo, P., Ephrussi, A., **2003**. Bruno regulates gurken during *Drosophila* oogenesis. *Mech. Devel. 120*, 289–297.

Findley, S.D., Tamanaha, M., Clegg, N.J., Ruohola-Baker, H., **2003**. Maelstrom, a *Drosophila* spindle-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage. *Development 130*, 859–871.

Fischer-Vize, J.A., Rubin, G.M., Lehmann, R., **1992**. The fat facets gene is required for *Drosophila* eye and embryo development. *Development 116*, 985–1000.

Fong, C.W., Leong, H.F., Wong, E.S., Lim, J., Yusoff, P., *et al.*, **2003**. Tyrosine phosphorylation of Sprouty2 enhances its interaction with c-Cbl and is crucial for its function. *J. Biol. Chem. 278*, 33456–33464.

Forbes, A., Lehmann, R., **1998**. Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development 125*, 679–690.

Forbes, A.J., Lin, H., Ingham, P.W., Spradling, A.C., **1996a**. Hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. *Development 122*, 1125–1135.

Forbes, A.J., Spradling, A.C., Ingham, P.W., Lin, H., **1996b**. The role of segment polarity genes during early oogenesis in *Drosophila*. *Development 122*, 3283–3294.

Forrest, K.M., Gavis, E.R., **2003**. Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr. Biol. 13*, 1159–1168.

Gavis, E.R., Curtis, D., Lehmann, R., **1996**. Identification of *cis*-acting sequences that control nanos RNA localization. *Devel. Biol. 176*, 36–50.

Gavis, E.R., Lehmann, R., **1992**. Localization of nanos RNA controls embryonic polarity. *Cell 71*, 301–313.

Gavis, E.R., Lehmann, R., **1994**. Translational regulation of nanos by RNA localization. *Nature 369*, 315–318.

Geigy, R., **1931**. Action de l'ultraviolet sur le pole germinal dans l'oeuf de *Drosophila* melanogaster. *Rev. Suisse Zool. 38*, 187–288.

Ghabrial, A., Schüpbach, T., **1999**. Activation of a meiotic checkpoint regulates translation of Gurken during *Drosophila* oogenesis. *Nat. Cell Biol. 1*, 354–357.

Ghiglione, C., Amundadottir, L., Andresdottir, M., Bilder, D., Diamonti, J.A., *et al.*, **2003**. Mechanism of inhibition of the *Drosophila* and mammalian EGF receptors by the transmembrane protein Kekkon 1. *Development 130*, 4483–4493.

Ghiglione, C., Carraway, K.L., 3rd, Amundadottir, L.T., Boswell, R.E., Perrimon, N., *et al.*, **1999**. The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the *Drosophila* EGF receptor during oogenesis. *Cell 96*, 847–856.

Ghiglione, C., Devergne, O., Georgenthum, E., Carballes, F., Medioni, C., *et al.*, **2002**. The *Drosophila* cytokine receptor Domeless controls border cell migration and epithelial polarization during oogenesis. *Development 129*, 5437–5447.

Glotzer, J. B., Saffrich, R., Glotzer, M., Ephrussi, A., **1997**. Cytoplasmic flows localize injected oskar RNA in *Drosophila* oocytes. *Curr. Biol. 7*, 326–337.

Godt, D., Laski, F.A., **1995**. Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of bric a brac. *Development 121*, 173–187.

Godt, D., Tepass, U., **1998**. *Drosophila* oocyte localization is mediated by differential cadherin–based adhesion. *Nature 395*, 387–391.

Gonzalez-Reyes, A., **2003**. Stem cells, niches and cadherins: a view from *Drosophila. J. Cell. Sci. 116*, 949–954.

Gonzalez-Reyes, A., St. Johnston, D., **1998**. Patterning of the follicle cell epithelium along the anterior–posterior axis during *Drosophila* oogenesis. *Development 125*, 2837–2846.

Goode, S., Melnick, M., Chou, T.B., Perrimon, N., **1996a**. The neurogenic genes egghead and brainiac define a novel signaling pathway essential for epithelial morphogenesis during *Drosophila* oogenesis. *Development 122*, 3863–3879.

Goode, S., Morgan, M., Liang, Y.P., Mahowald, A.P., **1996b**. Brainiac encodes a novel, putative secreted protein that cooperates with Grk TGF alpha in the genesis of the follicular epithelium. *Devel. Biol. 178*, 35–50.

Goode, S., Wright, D., Mahowald, A.P., **1992**. The neurogenic locus brainiac cooperates with the *Drosophila* EGF receptor to establish the ovarian follicle and to determine its dorsal–ventral polarity. *Development 116*, 177–192.

Grammont, M., Irvine, K.D., **2001**. fringe and Notch specify polar cell fate during *Drosophila* oogenesis. *Development 128*, 2243–2253.

Grammont, M., Irvine, K.D., **2002**. Organizer activity of the polar cells during *Drosophila* oogenesis. *Development 129*, 5131–5140.

Gregorio, C.C., Kubo, R.T., Bankert, R.B., Repasky, E.A., **1992**. Translocation of spectrin and protein kinase C to a cytoplasmic aggregate upon lymphocyte activation. *Proc. Natl Acad. Sci. USA 89*, 4947–4951.

Grieder, N.C., de Cuevas, M., Spradling, A.C., **2000**. The fusome organizes the microtubule network during oocyte differentiation in *Drosophila*. *Development 127*, 4253–4264.

Gross, S.P., Welte, M.A., Block, S.M., Wieschaus, E.F., **2002**. Coordination of opposite-polarity microtubule motors. *J. Cell. Biol. 156*, 715–724.

Guichet, A., Peri, F., Roth, S., **2001**. Stable anterior anchoring of the oocyte nucleus is required to establish dorsoventral polarity of the *Drosophila* egg. *Devel. Biol. 237*, 93–106.

Gunkel, N., Yano, T., Markussen, F.H., Olsen, L.C., Ephrussi, A., **1998**. Localization-dependent translation requires a functional interaction between the 5′ and 3′ ends of oskar mRNA. *Genes Devel. 12*, 1652–1664.

Gutzeit, H.O., **1986**. The role of microfilaments in cytoplasmic streaming in *Drosophila* follicles. *J. Cell. Sci. 80*, 159–69.

Hachet, O., Ephrussi, A., **2001**. *Drosophila* Y14 shuttles to the posterior of the oocyte and is required for oskar mRNA transport. *Curr. Biol. 11*, 1666–1674.

Hall, A.B., Jura, N., DaSilva, J., Jang, Y.J., Gong, D., *et al.*, **2003**. hSpry2 is targeted to the ubiquitin-dependent proteasome pathway by c-Cbl. *Curr. Biol. 13*, 308–314.

Harris, A.N., Macdonald, P.M., **2001**. Aubergine encodes a *Drosophila* polar granule component required for pole cell formation and related to eIF2C. *Development 128*, 2823–2832.

Hawkins, N.C., Thorpe, J., Schüpbach, T., **1996**. Encore, a gene required for the regulation of germ line mitosis and oocyte differentiation during *Drosophila* oogenesis. *Development 122*, 281–290.

Hawkins, N.C., Van Buskirk, C., Grossniklaus, U., Schüpbach, T., **1997**. Post-transcriptional regulation of gurken by encore is required for axis determination in *Drosophila*. *Development 124*, 4801–4810.

Hay, B., Jan, L.Y., Jan, Y.N., **1990**. Localization of vasa, a component of *Drosophila* polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development 109*, 425–433.

Hegner, R.W., **1908**. The effects of removing the germ cell determinants from the eggs of some chrysomelid beetles. *Biol. Bull. 16*, 19–26.

Hegner, R.W., **1911**. Experiments with chrysomelid beetles. iii. The effects of killing parts of the eggs of *Leptinotarsa decemlineata*. *Biol. Bull. 20*, 237–251.

Hime, G.R., Brill, J.A., Fuller, M.T., **1996**. Assembly of ring canals in the male germ line from structural components of the contractile ring. *J. Cell. Sci. 109*, 2779–2788.

Hombria, J.C., Brown, S., **2002**. The fertile field of *Drosophila* Jak/Stat signalling. *Curr. Biol. 12*, R569–575.

Hoogenraad, C.C., Wulf, P., Schiefermeier, N., Stepanova, T., Galjart, N., *et al.*, **2003**. Bicaudal D induces selective

dynein-mediated microtubule minus end-directed transport. *EMBO J. 22*, 6004–6015.

Hou, S.X., Zheng, Z., Chen, X., Perrimon, N., **2002**. The Jak/Stat pathway in model organisms: emerging roles in cell movement. *Devel. Cell. 3*, 765–778.

Hudson, A.M., Cooley, L., **2002**. Understanding the function of Actin-binding proteins through genetic analysis of *Drosophila* oogenesis. *Annu. Rev. Genet. 36*, 455–488.

Huynh, J.R., Petronczki, M., Knoblich, J.A., St. Johnston, D., **2001a**. Bazooka and PAR-6 are required with Par-1 for the maintenance of oocyte fate in *Drosophila*. *Curr. Biol. 11*, 901–906.

Huynh, J.R., Shulman, J.M., Benton, R., St. Johnston, D., **2001b**. Par-1 is required for the maintenance of oocyte fate in *Drosophila*. *Development 128*, 1201–1209.

Huynh, J.R., St. Johnston, D., **2000**. The role of BicD, Egl, Orb and the microtubules in the restriction of meiosis to the *Drosophila* oocyte. *Development 127*, 2785–2794.

Iida, T., Kobayashi, S., **1998**. Essential role of mitochondrially encoded large rRNA for germ-line formation in *Drosophila* embryos. *Proc. Natl Acad. Sci. USA 95*, 11274–11278.

Illmensee, K., Mahowald, A.P., **1974**. Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. *Proc. Natl Acad. Sci. USA 71*, 1016–1020.

Ivanovska, I., Lee, E., Kwan, K.M., Fenger, D.D., Orr-Weaver, T.L., **2004**. The *Drosophila* Mos ortholog is not essential for meiosis. *Curr. Biol. 14*, 75–80.

James, K.E., Dorman, J.B., Berg, C.A., **2002**. Mosaic analyses reveal the function of *Drosophila* Ras in embryonic dorsoventral patterning and dorsal follicle cell morphogenesis. *Development 129*, 2209–2222.

Jankovics, F., Sinka, R., Lukacsovich, T., Erdelyi, M., **2002**. MOESIN crosslinks Actin and cell membrane in *Drosophila* oocytes and is required for OSKAR anchoring. *Curr. Biol. 12*, 2060–2065.

Januschke, J., Gervais, L., Dass, S., Kaltschmidt, J.A., Lopez-Schier, H., *et al.*, **2002**. Polar transport in the *Drosophila* oocyte requires Dynein and Kinesin I cooperation. *Curr. Biol. 12*, 1971–1981.

Jaruzelska, J., Kotecki, M., Kusz, K., Spik, A., Firpo, M., *et al.*, **2003**. Conservation of a Pumilio-Nanos complex from *Drosophila* germ plasm to human germ cells. *Devel. Genes Evol. 213*, 120–126.

Johnstone, O., Lasko, P., **2001**. Translational regulation and RNA localization in *Drosophila* oocytes and embryos. *Annu. Rev. Genet. 35*, 365–406.

Jongens, T.A., Ackerman, L.D., Swedlow, J.R., Jan, L.Y., Jan, Y.N., **1994**. Germ cell-less encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Devel. 8*, 2123–2136.

Jongens, T.A., Hay, B., Jan, L.Y., Jan, Y.N., **1992**. The germ cell-less gene product: a posteriorly localized component necessary for germ cell development in *Drosophila*. *Cell 70*, 569–584.

Jordan, K.C., Clegg, N.J., Blasi, J.A., Morimoto, A.M., Sen, J., *et al.*, **2000**. The homeobox gene mirror links EGF signalling to embryonic dorso-ventral axis formation through notch activation. *Nat. Genet. 24*, 429–433.

Kai, T., Spradling, A., **2003**. An empty *Drosophila* stem cell niche reactivates the proliferation of ectopic cells. *Proc. Natl Acad. Sci. USA 100*, 4633–4638.

Kai, T., Spradling, A., **2004**. Differentiating germ cells can revert into functional stem cells in *Drosophila melanogaster* ovaries. *Nature 428*, 564–569.

Karki, S., Holzbaur, E.L., **1999**. Cytoplasmic dynein and dynactin in cell division and intracellular transport. *Curr. Opin. Cell Biol. 11*, 45–53.

Kashikawa, M., Amikura, R., Nakamura, A., Kobayashi, S., **1999**. Mitochondrial small ribosomal RNA is present on polar granules in early cleavage embryos of *Drosophila* melanogaster. *Devel. Growth Differ. 41*, 495–502.

Kelley, R.L., **1993**. Initial organization of the *Drosophila* dorsoventral axis depends on an RNA-binding protein encoded by the squid gene. *Genes Devel. 7*, 948–960.

Kennerdell, J.R., Yamaguchi, S., Carthew, R.W., **2002**. RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on aubergine and spindle-E. *Genes Devel. 16*, 1884–1889.

Kim-Ha, J., Kerr, K., Macdonald, P.M., **1995**. Translational regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. *Cell 81*, 403–412.

Kim-Ha, J., Smith, J.L., Macdonald, P.M., **1991**. oskar mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell 66*, 23–35.

Kim-Ha, J., Webster, P.J., Smith, J.L., Macdonald, P.M., **1993**. Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. *Development 119*, 169–178.

King, F.J., Lin, H., **1999**. Somatic signaling mediated by fs(1)Yb is essential for germline stem cell maintenance during *Drosophila* oogenesis. *Development 126*, 1833–1844.

King, F.J., Szakmary, A., Cox, D.N., Lin, H., **2001**. Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the *Drosophila* ovary. *Mol. Cell. 7*, 497–508.

King, R.C., **1970**. Ovarian Development in *Drosophila melanogaster*. Academic Press, New York.

Kobayashi, S., Amikura, R., Okada, M., **1993**. Presence of mitochondrial large ribosomal RNA outside mitochondria in germ plasm of *Drosophila melanogaster*. *Science 260*, 1521–1524.

Kobayashi, S., Okada, M., **1989**. Restoration of pole-cell-forming ability to u.v.-irradiated *Drosophila* embryos by injection of mitochondrial lrRNA. *Development 107*, 733–742.

Kobayashi, S., Yamada, M., Asaoka, M., Kitamura, T., **1996**. Essential role of the posterior morphogen nanos for germline development in *Drosophila*. *Nature 380*, 708–711.

Koch, E.A., Spitzer, R.H., **1983**. Multiple effects of colchicine on oogenesis in *Drosophila*: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. *Cell Tissue Res. 228*, 21–32.

Koprunner, M., Thisse, C., Thisse, B., Raz, E., **2001**. A zebrafish nanos-related gene is essential for the development of primordial germ cells. *Genes Devel. 15*, 2877–2885.

Lall, S., Francis-Lang, H., Flament, A., Norvell, A., Schüpbach, T., *et al.*, **1999**. Squid hnRNP protein promotes apical cytoplasmic transport and localization of *Drosophila* pair-rule transcripts. *Cell 98*, 171–180.

Lantz, V., Ambrosio, L., Schedl, P., **1992**. The *Drosophila* orb gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Development 115*, 75–88.

Lantz, V., Schedl, P., **1994**. Multiple *cis*-acting targeting sequences are required for orb mRNA localization during *Drosophila* oogenesis. *Mol. Cell. Biol. 14*, 2235–2242.

Lantz, V.A., Clemens, S.E., Miller, K.G., **1999**. The Actin cytoskeleton is required for maintenance of posterior pole plasm components in the *Drosophila* embryo. *Mech. Devel. 85*, 111–122.

Larkin, M.K., Holder, K., Yost, C., Giniger, E., Ruohola-Baker, H., **1996**. Expression of constitutively active Notch arrests follicle cells at a precursor stage during *Drosophila* oogenesis and disrupts the anterior-posterior axis of the oocyte. *Development 122*, 3639–3650.

Lasko, P.F., Ashburner, M., **1990**. Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. *Genes Devel. 4*, 905–921.

Leatherman, J.L., Levin, L., Boero, J., Jongens, T.A., **2002**. Germ cell-less acts to repress transcription during the establishment of the *Drosophila* germ cell lineage. *Curr. Biol. 12*, 1681–1685.

Lee, J.D., Treisman, J.E., **2001**. The role of Wingless signaling in establishing the anteroposterior and dorsoventral axes of the eye disc. *Development 128*, 1519–1529.

Leevers, S.J., **1999**. What goes up must come down. *Nat. Cell Biol. 1*, E10–E11.

LeMosy, E.K., Hong, C.C., Hashimoto, C., **1999**. Signal transduction by a protease cascade. *Trends Cell Biol. 9*, 102–107.

Leon, A., McKearin, D., **1999**. Identification of Ter94, an AAA ATPase protein, as a Bam-dependent component of the *Drosophila* fusome. *Mol. Biol. Cell 10*, 3825–3834.

Li, J., Xia, F., Li, W.X., **2003a**. Coactivation of Stat and Ras is required for germ cell proliferation and invasive migration in *Drosophila*. *Devel. Cell 5*, 787–798.

Li, X., Wheldon, L., Heath, J.K., **2003b**. Sprouty: a controversial role in receptor tyrosine kinase signalling pathways. *Biochem. Soc. Trans. 31*, 1445–1446.

Lie, Y.S., Macdonald, P.M., **1999**. Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in *Drosophila* ovarian extracts. *Development 126*, 4989–4996.

Lilly, M.A., de Cuevas, M., Spradling, A.C., **2000**. Cyclin A associates with the fusome during germline cyst formation in the *Drosophila* ovary. *Devel. Biol. 218*, 53–63.

Lin, H., **2002**. The stem-cell niche theory: lessons from flies. *Nat. Rev. Genet. 3*, 931–940.

Lin, H., Spradling, A.C., **1993**. Germline stem cell division and egg chamber development in transplanted *Drosophila* germaria. *Devel. Biol. 159*, 140–152.

Lin, H., Spradling, A.C., **1995**. Fusome asymmetry and oocyte determination in *Drosophila*. *Devel. Genet. 16*, 6–12.

Lin, H., Spradling, A.C., **1997**. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development 124*, 2463–2476.

Lin, H., Yue, L., Spradling, A.C., **1994**. The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development 120*, 947–956.

Liu, N., Dansereau, D.A., Lasko, P., **2003**. Fat facets interacts with vasa in the *Drosophila* pole plasm and protects it from degradation. *Curr. Biol. 13*, 1905–1909.

Liu, Z., Xie, T., Steward, R., **1999**. Lis1, the *Drosophila* homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation. *Development 126*, 4477–4488.

Lopez-Schier, H., **2003**. The polarisation of the antero-posterior axis in *Drosophila*. *Bioessays 25*, 781–791.

Lopez-Schier, H., St Johnston, D., **2001**. Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during *Drosophila* oogenesis. *Genes Devel. 15*, 1393–1405.

Macdonald, P.M., Kerr, K., **1997**. Redundant RNA recognition events in bicoid mRNA localization. *RNA 3*, 1413–1420.

MacDougall, N., Clark, A., MacDougall, E., Davis, I., **2003**. *Drosophila* gurken (TGFalpha) mRNA localizes as particles that move within the oocyte in two dynein-dependent steps. *Devel. Cell 4*, 307–319.

Mach, J.M., Lehmann, R., **1997**. An Egalitarian-BicaudalD complex is essential for oocyte specification and axis determination in *Drosophila*. *Genes Devel. 11*, 423–435.

Mahajan-Miklos, S., Cooley, L., **1994**. The villin-like protein encoded by the *Drosophila* quail gene is required for Actin bundle assembly during oogenesis. *Cell 78*, 291–301.

Mahowald, A.P., **1992**. Germ plasm revisited and illuminated. *Science 255*, 1216–1217.

Mahowald, A.P., **2001**. Assembly of the *Drosophila* germ plasm. *Int. Rev. Cytol. 203*, 187–213.

Manseau, L., Calley, J., Phan, H., **1996**. Profilin is required for posterior patterning of the *Drosophila* oocyte. *Development 122*, 2109–2116.

Margolis, J., Spradling, A., **1995**. Identification and behavior of epithelial stem cells in the *Drosophila* ovary. *Development 121*, 3797–3807.

Martin, S.G., Leclerc, V., Smith-Litiere, K., St. Johnston, D., **2003**. The identification of novel genes required for *Drosophila* anteroposterior axis formation in a germline clone screen using GFP-Staufen. *Development 130*, 4201–4215.

Martinho, R.G., Kunwar, P.S., Casanova, J., Lehmann, R., **2004**. A noncoding RNA is required for the repression of RNApolII-dependent transcription in primordial germ cells. *Curr. Biol. 14*, 159–165.

Masrouha, N., Yang, L., Hijal, S., Larochelle, S., Suter, B., **2003**. The *Drosophila* chk2 gene loki is essential for embryonic DNA double-strand-break checkpoints induced in S phase or G2. *Genetics 163*, 973–982.

Mata, J., Curado, S., Ephrussi, A., Rorth, P., **2000**. Tribbles coordinates mitosis and morphogenesis in *Drosophila* by regulating string/CDC25 proteolysis. *Cell 101*, 511–522.

Matanis, T., Akhmanova, A., Wulf, P., Del Nery, E., Weide, T., *et al.*, **2002**. Bicaudal-D regulates COPI-independent Golgi-ER transport by recruiting the dynein–dynactin motor complex. *Nat. Cell. Biol. 4*, 986–992.

Mathe, E., Inoue, Y.H., Palframan, W., Brown, G., Glover, D.M., **2003**. Orbit/Mast, the CLASP orthologue of *Drosophila*, is required for asymmetric stem cell and cystocyte divisions and development of the polarised microtubule network that interconnects oocyte and nurse cells during oogenesis. *Development 130*, 901–915.

McGrail, M., Hays, T.S., **1997**. The microtubule motor cytoplasmic dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in *Drosophila*. *Development 124*, 2409–2419.

McGregor, J.R., Xi, R., Harrison, D.A., **2002**. Jak signaling is somatically required for follicle cell differentiation in *Drosophila*. *Development 129*, 705–717.

McKearin, D., **1997**. The *Drosophila* fusome, organelle biogenesis and germ cell differentiation: if you build it. *Bioessays 19*, 147–152.

McKearin, D., Ohlstein, B., **1995**. A role for the *Drosophila* bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development 121*, 2937–2947.

McKearin, D.M., Spradling, A.C., **1990**. bag-of-marbles: a *Drosophila* gene required to initiate both male and female gametogenesis. *Genes Devel. 4*, 2242–2251.

McKim, K.S., Jang, J.K., Manheim, E.A., **2002**. Meiotic recombination and chromosome segregation in *Drosophila* females. *Annu. Rev. Genet. 36*, 205–232.

Micklem, D.R., Adams, J., Grunert, S., St. Johnston, D., **2000**. Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. *EMBO J. 19*, 1366–1377.

Micklem, D.R., Dasgupta, R., Elliott, H., Gergely, F., Davidson, C., *et al.*, **1997**. The mago nashi gene is required for the polarisation of the oocyte and the formation of perpendicular axes in *Drosophila*. *Curr. Biol. 7*, 468–478.

Mohr, S.E., Dillon, S.T., Boswell, R.E., **2001**. The RNA-binding protein Tsunagi interacts with Mago Nashi to establish polarity and localize oskar mRNA during *Drosophila* oogenesis. *Genes Devel. 15*, 2886–2899.

Moore, L.A., Broihier, H.T., Van Doren, M., Lunsford, L.B., Lehmann, R., **1998**. Identification of genes controlling germ cell migration and embryonic gonad formation in *Drosophila*. *Development 125*, 667–678.

Morris, J.Z., Navarro, C., Lehmann, R., **2003**. Identification and analysis of mutations in bob, Doa and eight new genes required for oocyte specification and development in *Drosophila* melanogaster. *Genetics 164*, 1435–1446.

Muller, H.A., **2002**. Germ cell migration: as slow as molasses. *Curr. Biol. 12*, R612–614.

Muller, R., Altmann, F., Zhou, D., Hennet, T., **2002**. The *Drosophila* melanogaster brainiac protein is a glycolipid-specific beta 1,3*N*-acetylglucosaminyltransferase. *J. Biol. Chem. 277*, 32417–32420.

Nakamura, A., Amikura, R., Hanyu, K., Kobayashi, S., **2001**. Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during *Drosophila* oogenesis. *Development 128*, 3233–3242.

Nakamura, A., Amikura, R., Mukai, M., Kobayashi, S., Lasko, P.F., **1996**. Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. *Science 274*, 2075–2079.

Nakamura, A., Sato, K., Hanyu-Nakamura, K., **2004**. *Drosophila* Cup is an eIF4E binding protein that associates with Bruno and Regulates oskar mRNA translation in oogenesis. *Devel. Cell. 6*, 69–78.

Nelson, M.R., Leidal, A.M., Smibert, C.A., **2004**. *Drosophila* Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression. *EMBO J. 23*, 150–159.

Neuman-Silberberg, F.S., Schüpbach, T., **1993**. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell 75*, 165–174.

Newmark, P.A., Boswell, R.E., **1994**. The mago nashi locus encodes an essential product required for germ plasm assembly in *Drosophila*. *Development 120*, 1303–1313.

Niessing, D., Blanke, S., Jackle, H., **2002**. Bicoid associates with the 5′-cap-bound complex of caudal mRNA and represses translation. *Genes Devel. 16*, 2576–2582.

Nilson, L.A., Schüpbach, T., **1998**. Localized requirements for windbeutel and pipe reveal a dorsoventral prepattern within the follicular epithelium of the *Drosophila* ovary. *Cell 93*, 253–262.

Nilson, L.A., Schüpbach, T., **1999**. EGF receptor signaling in *Drosophila* oogenesis. *Curr. Top. Devel. Biol. 44*, 203–243.

Norvell, A., Kelley, R.L., Wehr, K., Schüpbach, T., **1999**. Specific isoforms of squid, a *Drosophila* hnRNP, perform distinct roles in Gurken localization during oogenesis. *Genes Devel. 13*, 864–876.

Nusslein-Volhard, C., **1991**. Determination of the embryonic axes of *Drosophila*. *Development* (Suppl. 1), 1–10.

Nusslein-Volhard, C., Frohnhofer, H.G., Lehmann, R., **1987**. Determination of anteroposterior polarity in *Drosophila*. *Science 238*, 1675–1681.

Oh, J., Steward, R., **2001**. Bicaudal-D is essential for egg chamber formation and cytoskeletal organization in *Drosophila* oogenesis. *Devel. Biol. 232*, 91–104.

Ohi, R., Gould, K.L., **1999**. Regulating the onset of mitosis. *Curr. Opin. Cell. Biol. 11*, 267–273.

Ohlmeyer, J.T., Schüpbach, T., **2003**. Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during *Drosophila* oogenesis. *Development 130*, 6339–6349.

Ohlstein, B., Lavoie, C.A., Vef, O., Gateff, E., McKearin, D.M., **2000**. The *Drosophila* cystoblast differentiation factor, benign gonial cell neoplasm, is related to DExH-box proteins and interacts genetically with bag-of-marbles. *Genetics 155*, 1809–1819.

Ohlstein, B., McKearin, D., **1997**. Ectopic expression of the *Drosophila* Bam protein eliminates oogenic germline stem cells. *Development 124*, 3651–3662.

Pai, L.M., Barcelo, G., Schüpbach, T., **2000**. D-cbl, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in *Drosophila* oogenesis. *Cell 103*, 51–61.

Pare, C., Suter, B., **2000**. Subcellular localization of Bic-D :: GFP is linked to an asymmetric oocyte nucleus. *J. Cell. Sci. 113*, 2119–2127.

Parisi, M., Lin, H., **1999**. The *Drosophila* pumilio gene encodes two functional protein isoforms that play multiple roles in germline development, gonadogenesis, oogenesis and embryogenesis. *Genetics 153*, 235–250.

Parisi, M., Lin, H., **2000**. Translational repression: a duet of Nanos and Pumilio. *Curr. Biol. 10*, R81–R83.

Paterson, J., O'Hare, K., **1991**. Structure and transcription of the singed locus of *Drosophila melanogaster. Genetics 129*, 1073–1084.

Pepling, M.E., de Cuevas, M., Spradling, A.C., **1999**. Germline cysts: a conserved phase of germ cell development? *Trends Cell. Biol. 9*, 257–262.

Pepling, M.E., Spradling, A.C., **1998**. Female mouse germ cells form synchronously dividing cysts. *Development 125*, 3323–3328.

Peri, F., Roth, S., **2000**. Combined activities of Gurken and decapentaplegic specify dorsal chorion structures of the *Drosophila* egg. *Development 127*, 841–850.

Peri, F., Technau, M., Roth, S., **2002**. Mechanisms of Gurken-dependent pipe regulation and the robustness of dorsoventral patterning in *Drosophila*. *Development 129*, 2965–2975.

Polesello, C., Delon, I., Valenti, P., Ferrer, P., Payre, F., **2002**. Dmoesin controls Actin-based cell shape and polarity during *Drosophila melanogaster* oogenesis. *Nat. Cell. Biol. 4*, 782–789.

Ran, B., Bopp, R., Suter, B., **1994**. Null alleles reveal novel requirements for Bic-D during *Drosophila* oogenesis and zygotic development. *Development 120*, 1233–1242.

Reich, A., Sapir, A., Shilo, B., **1999**. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development 126*, 4139–4147.

Riechmann, V., Ephrussi, A., **2001**. Axis formation during *Drosophila* oogenesis. *Curr. Opin. Genet. Devel. 11*, 374–383.

Riechmann, V., Gutierrez, G.J., Filardo, P., Nebreda, A.R., Ephrussi, A., **2002**. Par-1 regulates stability of the posterior determinant Oskar by phosphorylation. *Nat. Cell. Biol. 4*, 337–342.

Rivera-Pomar, R., Lu, X., Perrimon, N., Taubert, H., Jackle, H., **1995**. Activation of posterior gap gene expression in the *Drosophila* blastoderm. *Nature 376*, 253–256.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W.J., Jackle, H., **1996**. RNA binding and translational suppression by bicoid. *Nature 379*, 746–749.

Robinson, D.N., Cant, K., Cooley, L., **1994**. Morphogenesis of *Drosophila* ovarian ring canals. *Development 120*, 2015–2025.

Robinson, D.N., Cooley, L., **1997**. *Drosophila* kelch is an oligomeric ring canal Actin organizer. *J. Cell. Biol. 138*, 799–810.

Robinson, D.N., Smith-Leiker, T.A., Sokol, N.S., Hudson, A.M., Cooley, L., **1997**. Formation of the *Drosophila* ovarian ring canal inner rim depends on cheerio. *Genetics 145*, 1063–1072.

Roper, K., Brown, N.H., **2004**. A spectraplakin is enriched on the fusome and organizes microtubules during oocyte specification in *Drosophila*. *Curr. Biol. 14*, 99–110.

Roth, S., **2003**. The origin of dorsoventral polarity in *Drosophila*. *Phil. Trans. R. Soc. Lond. B Biol. Sci. 358*, 1317–1329.

Roth, S., Jordan, P., Karess, R., **1999**. Binuclear *Drosophila* oocytes: consequences and implications for dorsal-ventral patterning in oogenesis and embryogenesis. *Development 126*, 927–934.

Roth, S., Neuman-Silberberg, F.S., Barcelo, G., Schüpbach, T., **1995**. cornichon and the EGF receptor signaling process are necessary for both anterior–posterior and dorsal–ventral pattern formation in *Drosophila*. *Cell 81*, 967–978.

Rubin, C., Litvak, V., Medvedovsky, H., Zwang, Y., Lev, S., *et al*., **2003**. Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops. *Curr. Biol. 13*, 297–307.

Rubsam, R., Hollmann, M., Simmerl, E., Lammermann, U., Schafer, M.A., *et al*., **1998**. The egghead gene product influences oocyte differentiation by follicle cell-germ cell interactions in *Drosophila melanogaster. Mech. Devel. 72*, 131–140.

Ruohola, H., Bremer, K.A., Baker, D., Swedlow, J.R., Jan, L.Y., *et al*., **1991**. Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in *Drosophila*. *Cell 66*, 433–449.

Santos, A.C., Lehmann, R., **2004**. Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Devel. Cell 6*, 283–293.

Saunders, C., Cohen, R.S., **1999**. The role of oocyte transcription, the 5′UTR, and translation repression and derepression in *Drosophila* gurken mRNA and protein localization. *Mol. Cell. 3*, 43–54.
Schnorrer, F., Bohmann, K., Nusslein-Volhard, C., **2000**. The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of *Drosophila* oocytes. *Nat. Cell. Biol. 2*, 185–190.
Schulz, C., Tautz, D., **1995**. Zygotic caudal regulation by hunchback and its role in abdominal segment formation of the *Drosophila* embryo. *Development 121*, 1023–1028.
Schüpbach, T., Wieschaus, E., **1989**. Female sterile mutations on the second chromosome of *Drosophila melanogaster.* I. Maternal effect mutations. *Genetics 121*, 101–117.
Schüpbach, T., Wieschaus, E., **1991**. Female sterile mutations on the second chromosome of *Drosophila melanogaster.* II. Mutations blocking oogenesis or altering egg morphology. *Genetics 129*, 1119–1136.
Selenko, P., Sprangers, R., Stier, G., Buhler, D., Fischer, U., *et al.*, **2001**. SMN tudor domain structure and its interaction with the Sm proteins. *Nat. Struct. Biol. 8*, 27–31.
Sen, J., Goltz, J.S., Konsolaki, M., Schüpbach, T., Stein, D., **2000**. Windbeutel is required for function and correct subcellular localization of the *Drosophila* patterning protein Pipe. *Development 127*, 5541–5550.
Sen, J., Goltz, J.S., Stevens, L., Stein, D., **1998**. Spatially restricted expression of pipe in the *Drosophila* egg chamber defines embryonic dorsal–ventral polarity. *Cell 95*, 471–481.
Serano, T.L., Cohen, R.S., **1995**. A small predicted stem-loop structure mediates oocyte localization of *Drosophila* K10 mRNA. *Development 121*, 3809–3818.
Shulman, J.M., Benton, R., St. Johnston, D., **2000**. The *Drosophila* homolog of *C. elegans* Par-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. *Cell 101*, 377–388.
Silver, D.L., Montell, D.J., **2001**. Paracrine signaling through the Jak/Stat pathway activates invasive behavior of ovarian epithelial cells in *Drosophila*. *Cell 107*, 831–841.
Smibert, C.A., Lie, Y.S., Shillinglaw, W., Henzel, W.J., Macdonald, P.M., **1999**. Smaug, a novel and conserved protein, contributes to repression of nanos mRNA translation *in vitro*. *RNA 5*, 1535–1547.
Smibert, C.A., Wilson, J.E., Kerr, K., Macdonald, P.M., **1996**. smaug protein represses translation of unlocalized nanos mRNA in the *Drosophila* embryo. *Genes Devel. 10*, 2600–2609.
Smulders-Srinivasan, T.K., Lin, H., **2003**. Screens for piwi suppressors in *Drosophila* identify dosage-dependent regulators of germline stem cell division. *Genetics 165*, 1971–1991.
Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., *et al.*, **2004**. Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the *Drosophila* ovary. *Development 131*, 1353–1364.
Song, X., Xie, T., **2002**. DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the *Drosophila* ovary. *Proc. Natl Acad. Sci. USA 99*, 14813–14818.
Song, X., Xie, T., **2003**. Wingless signaling regulates the maintenance of ovarian somatic stem cells in *Drosophila*. *Development 130*, 3259–3268.
Song, X., Zhu, C.H., Doan, C., Xie, T., **2002**. Germline stem cells anchored by adherens junctions in the *Drosophila* ovary niches. *Science 296*, 1855–1857.
Spradling, A., Drummond-Barbosa, D., Kai, T., **2001**. Stem cells find their niche. *Nature 414*, 98–104.
Spradling, A.C., de Cuevas, M., Drummond-Barbosa, D., Keyes, L., Lilly, M., *et al.*, **1997**. The *Drosophila* germarium: stem cells, germ line cysts, and oocytes. *Cold Spring Harbor Symp. Quant. Biol. 62*, 25–34.
St. Johnston, D., Driever, W., Berleth, T., Richstein, S., Nusslein-Volhard, C., **1989**. Multiple steps in the localization of bicoid RNA to the anterior pole of the *Drosophila* oocyte. *Development 107*(Suppl.), 13–19.
St. Johnston, D., Nusslein-Volhard, C., **1992**. The origin of pattern and polarity in the *Drosophila* embryo. *Cell 68*, 201–219.
Staeva-Vieira, E., Yoo, S., Lehmann, R., **2003**. An essential role of DmRad51/SpnA in DNA repair and meiotic checkpoint control. *Embo J. 22*, 5863–5874.
Starz-Gaiano, M., Lehmann, R., **2001**. Moving towards the next generation. *Mech. Devel. 105*, 5–18.
Stathopoulos, A., Levine, M., **2002**. Dorsal gradient networks in the *Drosophila* embryo. *Devel. Biol. 246*, 57–67.
Stein, J.A., Broihier, H.T., Moore, L.A., Lehmann, R., **2002**. Slow as molasses is required for polarized membrane growth and germ cell migration in *Drosophila*. *Development 129*, 3925–3934.
Stephenson, E.C., Chao, Y.C., Fackenthal, J.D., **1988**. Molecular analysis of the swallow gene of *Drosophila melanogaster. Genes Devel. 2*, 1655–1665.
Stephenson, E.C., Mahowald, A.P., **1987**. Isolation of *Drosophila* clones encoding maternally restricted RNAs. *Devel. Biol. 124*, 1–8.
Styhler, S., Nakamura, A., Swan, A., Suter, B., Lasko, P., **1998**. vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. *Development 125*, 1569–1578.
Su, T.T., Campbell, S.D., O'Farrell, P.H., **1998**. The cell cycle program in germ cells of the *Drosophila* embryo. *Devel. Biol. 196*, 160–170.
Subramaniam, K., Seydoux, G., **1999**. nos-1 and nos-2, two genes related to *Drosophila* nanos, regulate primordial germ cell development and survival in *Caenorhabditis elegans*. *Development 126*, 4861–4871.
Suter, B., Romberg, L.M., Steward, R., **1989**. Bicaudal-D, a *Drosophila* gene involved in developmental asymmetry: localized transcript accumulation in ovaries and

sequence similarity to myosin heavy chain tail domains. *Genes Devel. 3*, 1957–1968.

Suter, B., Steward, R., **1991**. Requirement for phosphorylation and localization of the Bicaudal-D protein in *Drosophila* oocyte differentiation. *Cell 67*, 917–926.

Swan, A., Nguyen, T., Suter, B., **1999**. *Drosophila* Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. *Nat. Cell. Biol. 1*, 444–449.

Takada, S., Kelkar, A., Theurkauf, W.E., **2003**. *Drosophila* checkpoint kinase 2 couples centrosome function and spindle assembly to genomic integrity. *Cell 113*, 87–99.

Theurkauf, W.E., **1994a**. Microtubules and cytoplasm organization during *Drosophila* oogenesis. *Devel. Biol. 165*, 352–360.

Theurkauf, W.E., **1994b**. Premature microtubule-dependent cytoplasmic streaming in cappuccino and spire mutant oocytes. *Science 265*, 2093–2096.

Theurkauf, W.E., Alberts, B.M., Jan, Y.N., Jongens, T.A., **1993**. A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development 118*, 1169–1180.

Theurkauf, W.E., Smiley, S., Wong, M.L., Alberts, B.M., **1992**. Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. *Development 115*, 923–936.

Thio, G.L., Ray, R.P., Barcelo, G., Schüpbach, T., **2000**. Localization of gurken RNA in *Drosophila* oogenesis requires elements in the 5′ and 3′ regions of the transcript. *Devel. Biol. 221*, 435–446.

Tilney, L.G., Tilney, M.S., Guild, G.M., **1996**. Formation of Actin filament bundles in the ring canals of developing *Drosophila* follicles. *J. Cell. Biol. 133*, 61–74.

Tomancak, P., Guichet, A., Zavorszky, P., Ephrussi, A., **1998**. Oocyte polarity depends on regulation of gurken by Vasa. *Development 125*, 1723–1732.

Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K.C., Kemphues, K.J., *et al.*, **2000**. A *Drosophila melanogaster* homologue of *Caenorhabditis elegans* par-1 acts at an early step in embryonic-axis formation. *Nat. Cell. Biol. 2*, 458–460.

Torres, I.L., Lopez-Schier, H., St. Johnston, D., **2003**. A Notch/Delta-dependent relay mechanism establishes anterior–posterior polarity in *Drosophila*. *Devel. Cell 5*, 547–558.

Tran, D.H., Berg, C.A., **2003**. bullwinkle and shark regulate dorsal-appendage morphogenesis in *Drosophila* oogenesis. *Development 130*, 6273–6282.

Tsuda, M., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., *et al.*, **2003**. Conserved role of nanos proteins in germ cell development. *Science 301*, 1239–1241.

Twombly, V., Blackman, R.K., Jin, H., Graff, J.M., Padgett, R.W., *et al.*, **1996**. The TGF-beta signaling pathway is essential for *Drosophila* oogenesis. *Development 122*, 1555–1565.

Tworoger, M., Larkin, M.K., Bryant, Z., Ruohola-Baker, H., **1999**. Mosaic analysis in the *Drosophila* ovary reveals a common hedgehog-inducible precursor stage for stalk and polar cells. *Genetics 151*, 739–748.

Urban, S., Lee, J.R., Freeman, M., **2001**. *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell 107*, 173–182.

Vaccari, T., Ephrussi, A., **2002**. The fusome and microtubules enrich Par-1 in the oocyte, where it effects polarization in conjunction with Par-3, BicD, Egl, and dynein. *Curr. Biol. 12*, 1524–1528.

Van Buskirk, C., Hawkins, N.C., Schüpbach, T., **2000**. Encore is a member of a novel family of proteins and affects multiple processes in *Drosophila* oogenesis. *Development 127*, 4753–4762.

Van Doren, M., Williamson, A.L., Lehmann, R., **1998**. Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr. Biol. 8*, 243–246.

van Eeden, F., St. Johnston, D., **1999**. The polarisation of the anterior–posterior and dorsal–ventral axes during *Drosophila* oogenesis. *Curr. Opin. Genet. Devel. 9*, 396–404.

van Eeden, F.J., Palacios, I.M., Petronczki, M., Weston, M.J., St. Johnston, D., **2001**. Barentsz is essential for the posterior localization of oskar mRNA and colocalizes with it to the posterior pole. *J. Cell. Biol. 154*, 511–523.

Wandall, H.H., Pedersen, J.W., Park, C., Levery, S.B., Pizette, S., *et al.*, **2003**. *Drosophila* egghead encodes a beta 1,4-mannosyltransferase predicted to form the immediate precursor glycosphingolipid substrate for brainiac. *J. Biol. Chem. 278*, 1411–1414.

Wang, C., Lehmann, R., **1991**. Nanos is the localized posterior determinant in *Drosophila*. *Cell 66*, 637–647.

Wang, Z., Lin, H., **2004**. Nanos maintains germline stem cell self-renewal by preventing differentiation. *Science 303*, 2016–2019.

Wasserman, J.D., Freeman, M., **1998**. An autoregulatory cascade of EGF receptor signaling patterns the *Drosophila* egg. *Cell 95*, 355–364.

Wharton, R.P., Struhl, G., **1989**. Structure of the *Drosophila* BicaudalD protein and its role in localizing the posterior determinant nanos. *Cell 59*, 881–892.

Wieschaus, E., Sweeton, D., **1988**. Requirements for X-linked zygotic gene activity during cellularization of early *Drosophila* embryos. *Development 104*, 483–493.

Wieschaus, E., Szabad, J., **1979**. The development and function of the female germ line in *Drosophila melanogaster*: a cell lineage study. *Devel. Biol. 68*, 29–46.

Wilhelm, J.E., Hilton, M., Amos, Q., Henzel, W.J., **2003**. Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. *J. Cell Biol. 163*, 1197–1204.

Wilhelm, J.E., Mansfield, J., Hom-Booher, N., Wang, S., Turck, C.W., *et al.*, **2000**. Isolation of a ribonucleoprotein complex involved in mRNA localization in *Drosophila* oocytes. *J. Cell Biol. 148*, 427–440.

Wilkie, G.S., Davis, I., **2001**. *Drosophila* wingless and pair-rule transcripts localize apically by dynein-mediated transport of RNA particles. *Cell 105*, 209–219.

Will, C.L., Luhrmann, R., **2001**. Spliceosomal UsnRNP biogenesis, structure and function. *Curr. Opin. Cell. Biol. 13*, 290–301.

Wilson, J.E., Connell, J.E., Macdonald, P.M., **1996**. aubergine enhances oskar translation in the *Drosophila* ovary. *Development 122*, 1631–1639.

Wilson, P.G., **1999**. BimC motor protein KLP61F cycles between mitotic spindles and fusomes in *Drosophila* germ cells. *Curr. Biol. 9*, 923–926.

Wong, E.S., Fong, C.W., Lim, J., Yusoff, P., Low, B.C., *et al.*, **2002**. Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. *EMBO J. 21*, 4796–4808.

Xi, R., McGregor, J.R., Harrison, D.A., **2003**. A gradient of Jak pathway activity patterns the anterior-posterior axis of the follicular epithelium. *Devel. Cell. 4*, 167–177.

Xie, T., Spradling, A.C., **1998**. decapentaplegic is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell 94*, 251–260.

Xie, T., Spradling, A.C., **2000**. A niche maintaining germ line stem cells in the *Drosophila* ovary. *Science 290*, 328–330.

Xue, F., Cooley, L., **1993**. kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell 72*, 681–693.

Yue, L., Spradling, A.C., **1992**. hu-li tai shao, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes Devel. 6*, 2443–2454.

Zhang, N., Zhang, J., Purcell, K.J., Cheng, Y., Howard, K., **1997**. The *Drosophila* protein Wunen repels migrating germ cells. *Nature 385*, 64–67.

Zhang, Y., Kalderon, D., **2000**. Regulation of cell proliferation and patterning in *Drosophila* oogenesis by Hedgehog signaling. *Development 127*, 2165–2176.

Zhang, Y., Kalderon, D., **2001**. Hedgehog acts as a somatic stem cell factor in the *Drosophila* ovary. *Nature 410*, 599–604.

Zhao, D., Woolner, S., Bownes, M., **2000**. The Mirror transcription factor links signalling pathways in *Drosophila* oogenesis. *Devel. Genes Evol. 210*, 449–457.

Zhu, C.H., Xie, T., **2003**. Clonal expansion of ovarian germline stem cells during niche formation in *Drosophila*. *Development 130*, 2579–2588.

1.3 Vitellogenesis and Post-Vitellogenic Maturation of the Insect Ovarian Follicle

L Swevers, National Centre for Scientific Research ''Demokritos,'' Athens, Greece
A S Raikhel, University of California, Riverside, CA, USA
T W Sappington, Iowa State University, Ames, IA, USA
P Shirk, USDA ARS CMAVE, Gainesville, FL, USA
K Iatrou, National Centre for Scientific Research ''Demokritos,'' Athens, Greece

1.3.1. Introduction

Female insects typically produce prodigious numbers of eggs to assure the propagation of their genes, and invest considerable resources towards this end. Ultimately, the egg of an insect must contain a haploid set of chromosomes, sufficient nutrients to supply the growing embryo with resources to last until the larva or nymph ecloses and begins feeding, and a set of determinants to direct the organization and progression of embryogenesis, including the differentiation of a new cluster of germ cells. As with all organs, the morphology of the ovary reflects the physical and genetic requirements of its physiological role, which in this case is the functional assembly of the various components of the oocyte.

Visual inspection shows that the polytrophic ovary of holometabolus insects, which represent a major focus of this chapter, is comprised of a series of ovarioles that contain linear arrays of progressively developing follicles starting with dividing germ stem cells at one end and ending with mature oocytes ready for fertilization at the other (Figure 1). Essentially, the ovariole can be considered an assembly line leading to the production of the egg. How this assembly line operates within different species to produce similar end products, i.e., the mature oocytes, depends on the insect, its life style, and its evolutionary history.

1.3.2. Previtellogenesis: The Development of the Ovarian Structure

In this section, the development of the ovary and oocyte in the fruit fly, *Drosophila melanogaster*, is examined briefly and then extend the discussion to other insects, mainly Lepidoptera and non-*Drosophila* Diptera, where most of the additional relevant information is available.

1.3.2.1. *Drosophila melanogaster*

The organogenesis of the ovary and the production of eggs in *Drosophila* is one of the most completely examined developmental systems. From the formation of germ cells in the embryo to the localization

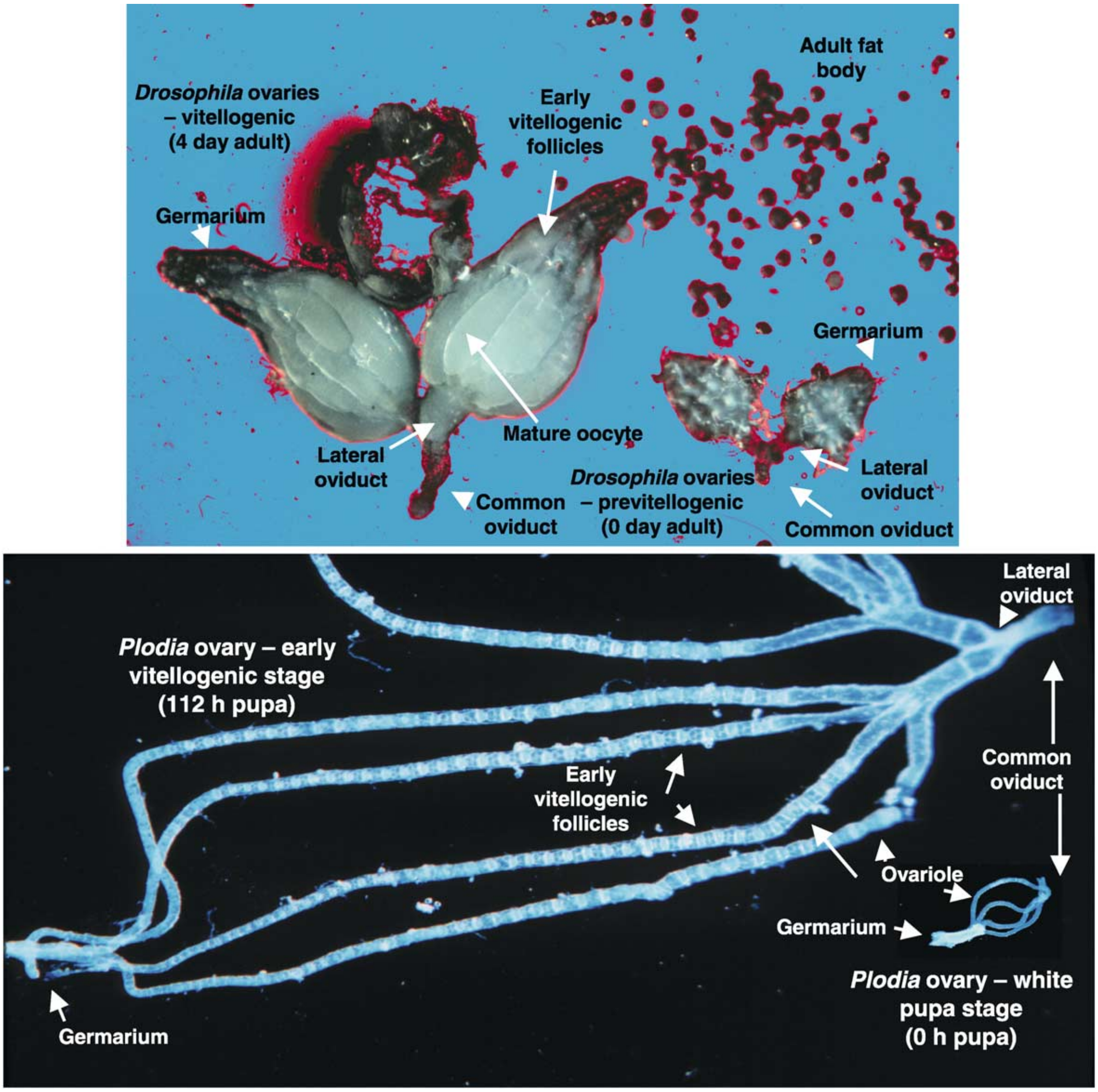


Figure 1 Ovaries of holometaboulous insects. The upper panel shows ovaries dissected from previtellogenic (newly eclosed) and vitellogenic (four day old) *Drosophila melanogaster* adult females. The increase in size of the ovaries is due to the uptake of yolk proteins and other nutrients by the maturing follicles. The lower panel shows ovaries dissected from early vitellogenic (112 h) and prefollicular (white or 0 h) *Plodia interpunctella* female pupae. The increase in size is due to the growth of the germ and somatic tissues of the ovary. The linear array of developing follicles can be seen within each ovariole extending from the germarium to the lateral oviduct. The size of the ovary will increase again as the follicles complete vitellogenesis.

of germ cell determinants in the oocytes within the ovary of the adult female, the growth and activities of the ovary and follicles have been examined extensively to determine the fundamental genetic and developmental processes that are involved in the production of an egg. As a result, the structural organization of the ovary and the developmental processes controlling its formation have been studied in-depth for over three-quarters of a century (Dobzhansky, 1930; Kerkis, 1930; Demerec, 1950; King *et al.*, 1968; King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993; Lasko, 1994; Mahajan-Niklos and Cooley, 1994; Dobens and Raftery, 2000; Houston and King, 2000) (see **Chapter 1.2**).

In *Drosophila*, the female molts into the adult stage with an immature ovary but, under the influence of juvenile hormone (JH), it initiates yolk protein production (vitellogenesis) and follicle maturation within the first day of adulthood (reviews: Postlethwait and Shirk, 1981; Bownes, 1986; Lasko, 1994). The female continues producing eggs throughout its life, provided a sufficient supply of nutrients is available (Bownes, 1986). Because the process of egg production is continuous, plentiful experimental material is always at hand, providing

a rich resource for the manipulation and analysis of oocyte production and maturation.

Under the influence of determinants localized within the posterior pole plasm of the embryo, the germ cells are the first to cellularize in the syncytial embryos (reviews: Spradling, 1993; Houston and King, 2000) (see **Chapter 1.2**). The formation of the pole plasm within the oocyte is dependent upon the maternal contribution and posterior localization of transcripts for *oskar* (*osk*; Lehman and Nüsslein-Volhard, 1986), *nanos* (*nos*; Nüsslein-Volhard *et al*., 1987), and *germ cell-less* (*gcl*; Jongens *et al*., 1992, 1994), as well as the proteins Staufen (Stau; Schüpbach and Wieschaus, 1986), Valois (Vls; Schüpbach and Wieschaus, 1986), Vasa (Vas; Schüpbach and Wieschaus, 1986), Tudor (Tud; Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986), and Mago-nashi (Mago; Boswell *et al*., 1991). In addition to these determinants, normal germ cell development and dorsoventral patterning are also dependent on the posterior localization of Cappuccino (Capu) and Spire (Spir) (Manseau and Schüpbach, 1989) through the organization of microtubules and regulation of cytoplasmic streaming. Furthermore, germ cell development and posterior somatic patterning require posterior localization of the *nos* and *pumilio* (*pum*) transcripts (Lehman and Nüsslein-Volhard, 1987). These transcripts and proteins are produced in the nurse cells and sequentially localized in the posterior of the embryo. The transcripts include a 630-nucleotide sequence within the 3′ untranslated region, which specifies the localization signal that interacts with microtubules to facilitate translocation (Macdonald and Struhl, 1988; Pokrywka and Stephenson, 1991, 1995; review: **Chapter 1.2**). Once associated with the microtubules, the translocation of these determinants from the nurse cells to the oocyte and their stable localization within the embryo is dependent on activities of the microtubules, which function both via minus- and plus-end-directed motors driven by dynein and kinesin, respectively (reviews: Pokrywka, 1995; Stebbings *et al*., 1995) (see **Chapter 1.2**).

The number of germ cells formed in the embryo is influenced by the levels of Osk (Smith *et al*., 1992). A threshold level of Osk is required for normal germ cell formation and establishment of normal body patterning, while its over-expression leads to ectopic formation of pole cells as well as formation of additional posterior pole cells (for additional details, see **Chapter 1.2**). After cellularization of the germ cells, their migration and eventual localization within the gonads can be visualized using antibody staining for the Vas protein (Lasko and Ashburner, 1990), an RNA helicase that is constitutively expressed in germ cells (Hay *et al*., 1988; Lasko and Ashburner, 1988). Nearly half of the originally formed pole cells are lost during embryogenesis, as they migrate from the posterior of the germ band to their position within the embryonic gonads, and only 10–15 germ cells are present within the late embryonic gonad (Lasko and Ashburner, 1990; Mueller, 2002) (see **Chapter 1.2**).

Throughout most of the larval stages, the germ cells divide mitotically to produce an unorganized mass of primordial germ cells mixed with somatic cells (Kerkis, 1930). Midway through the third instar, however, the metamorphosis of the ovary is initiated, leading to the organization of the somatic and germ cells into the adult organ (King *et al*., 1968) (see **Chapter 1.2**). The morphogenetic changes in the undifferentiated ovary begin approximately 12 h after the molt to the third (last) instar, when the anterior somatic cells of the ovary express Ultraspiracle (USP; Oro *et al*., 1990) and the A isoform of the ecdysteroid (or ecdysone) receptor (EcR-A; Hodin and Riddiford, 1998). In the presence of low levels of ecdysteroids, these two proteins interact to activate downstream gene expression including that of the *bric à brac* (*bab*) gene. Under the influence of the BAB protein, approximately 20 terminal filament stacks begin to differentiate from the somatic cells (Godt and Laski, 1995; Sahut-Barnola *et al*., 1995).

After initiating differentiation, the terminal filament cells maintain high levels of USP but no detectable EcR (Hodin and Riddiford, 1998). The first two terminal filament stacks form on the medial side of the ovary, with new stacks forming more laterally, as progressively more are added until 32 h after the molt (Godt and Laski, 1995). By the time of pupariation, the 13–20 terminal filaments, which consist of stacks of 8–9 disk-shaped cells, have been formed in the ovary. These terminal filament stacks establish the positions for the formation of each of the ovarioles within the ovary.

Within 48 h from pupariation, the morphogenesis of the ovary is completed. A small cluster of cells forms at the base of the terminal filaments, in direct contact with the 2–3 germ cells localized within each ovariole (Lin, 2002) (see **Chapter 1.2**). The somatic cap cells and inner sheath cells surround the germ cells and form a niche that provides the signaling that controls germ cell divisions during the adult reproductive period (see **Chapter 1.2**). The primary germ cells function as stem cells and divide asymmetrically to produce one daughter cell that becomes a cystoblast, which enters follicle differentiation, and another daughter cell that retains a stem cell function (reviews: Spradling *et al*., 2001;

Lin, 2002) (see **Chapter 1.2**). The cap cells, alternatively, express several genes, e.g., *hedgehog* (*hh*), *piwi*, *fs(1)Yb*, and *decapentapalegic* (*dpp*), whose function is required for the maintenance of stem cell function (Cox *et al*., 1998; Xie and Spradling, 1998; King and Lin, 1999; Cox *et al*., 2000; Xie and Spradling, 2000; King *et al*., 2001) (see **Chapter 1.2**). Of these genes, *dpp* is the major regulator for clonal expansion of germ cells during the formation of the niche at the early pupal stage (Zhu and Xie, 2003).

The establishment of the stem cells within the niche requires fewer germ cells than are present at pupation, and stem cells that are not included in the niche differentiate directly into cystoblasts (Bhat and Schedl, 1997). Establishment of the germ stem cells is also dependent on the formation of *adherens* junctions between the cap cells and germ stem cells (Song *et al*., 2002). In addition to establishing their identity, they also provide a further physical constraint leading to asymmetric division of the germ stem cells, which is important for the renewal of the stem cells (Zhu and Xie, 2003).

Each ovariole in a mature ovary is divided into three regions, the terminal filament, the germarium, and the vitellarium (**Figures 1** and **2**). Within the germarium, four functional regions, 1, 2A, 2B, and 3, have been described (Koch and King, 1966; Mahowald and Strassheim, 1970; Lasko, 1994) (see **Chapter 1.2**), where the previtellogenic follicle forms (**Figure 2**). A cluster of 2–3 germ stem cells is found within a niche in the anterior-most section of the germarium (region 1), immediately adjacent to the terminal filaments (Wieschaus and Szabad, 1979). During egg production by the adult female, germ stem cells divide asymmetrically to produce cystoblasts and new stem cells (review: Lin, 2002; **Chapter 1.2**). The asymmetry of the cell division is necessary for maintenance of the stem cells, so that eggs can be produced throughout the adult stage.

In addition to anchoring the germ stem cells to the cap cells, polarized division of the daughter cells is maintained by the asymmetric segregation of the fusome (or spectrosome) in the germ stem cell. The fusome is a ring-shaped organelle replete with membrane skeletal proteins including actin (Warn *et al*., 1985; Theurkauf *et al*., 1992), α- and β-spectrins (de Cuevas *et al*., 1996), filamin and ankyrin (Li *et al*., 1999), motor molecules such as cytoplasmic dynein (McGrail and Hays, 1997), and the unique fusome proteins Hu-Li Tai Shao (HTS, an adducin-like protein; Yue and Spradling, 1992; Ding *et al*., 1993), Bag-of-marbles (BAM; McKearin and Ohlstein, 1995), TER94 (León and McKearin, 1999), and Kelch (Xue and Cooley, 1993). The fusome acts in

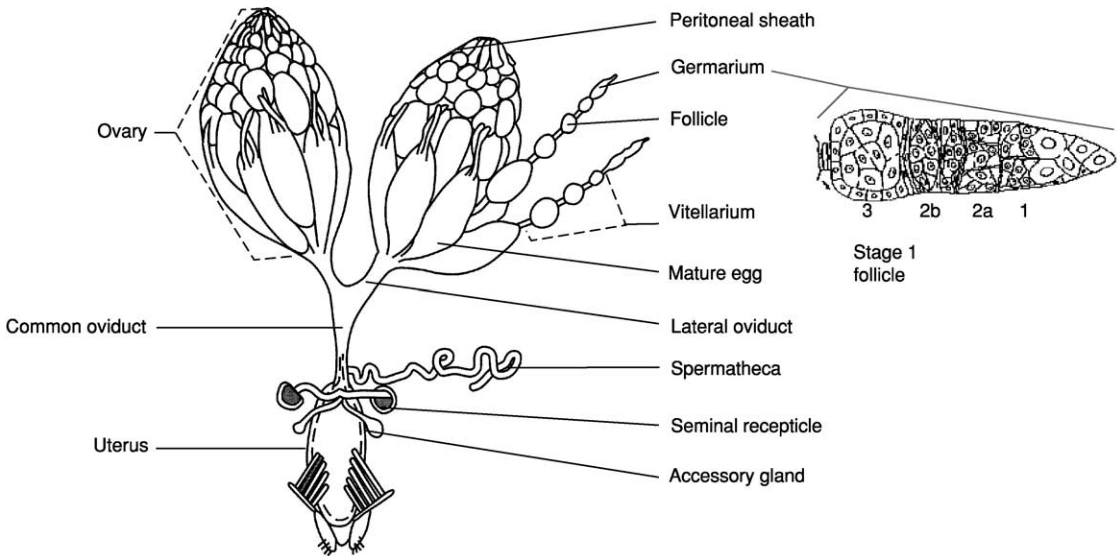


Figure 2 The reproductive system of *Drosophila melanogaster* contains two ovariole clusters that connect to a common oviduct. Eggs pass through the oviduct and are momentarily stored in the uterus until egg deposition. The broken line indicates the position of the mature egg in the uterus (the posterior end faces down). (Redrawn from Miller, A., **1950**. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: M. Demerec, M. (Ed.), Biology of *Drosophila*. Wiley, New York, pp. 420–534). The germarium contains germline stem cells (region 1) and mitotically active 2-, 4-, and 8-cell clusters. Clusters of 16 germline cells are present in 2a, where follicle cells begin to migrate around the cluster. Young egg chambers completely surrounded by follicle cells flatten into a lens shape in region 2b. The egg chamber reaches region 3 of the germarium (stage 1), where it becomes spherical and prepares for departure from the germarium as a stage 2 egg chamber. (Redrawn in part from Robinson, D.N., Cant, K., Cooley, L., **1994**. Morphogenesis of *Drosophila* ovarian ring canals. *Devel. 120*, 2015–2025).

determining the asymmetrical division of the dividing germ stem cells by associating with the cap cell-anchored germ stem cell pole of the mitotic spindle in subsequent mitoses of the cystocytes (Storto and King, 1989; Lin and Spradling, 1995; McGrail and Hays, 1997; Máthé *et al.*, 2003) (see **Chapter 1.2**).

The polarization of fusome localization is accomplished under the influence of *pum* whose product is involved in the organization of the microtubule structure (Lin and Spradling, 1997), and the Orbit/Mast and CLIP-190 proteins, which mediate interactions between microtubules and cytoskeletal organelles (Máthé *et al.*, 2003). The Orbit/Mast protein is found initially at the poles of the mitotic spindle but subsequently moves, coincident with the arrested cleavage furrow. This guides the migration of the fusome plugs toward the site of the pre-existing fusome (Storto and King, 1989), and assures the polarized positioning of the fusome and the asymmetry of germ stem cell division.

Following its separation from the germ stem cell, a cystoblast divides four times, with incomplete cytokinesis, within region 2A. This produces the cystocyte complex. The complex is composed of the oocyte that is connected by cytoplasmic bridges to the 15 nurse cells. The asymmetric division of the cystocytes leads to the assignment of one cell as the oocyte, with the remaining daughters of the cystocyte progeny becoming nurse cells (Storto and King, 1989; Lin and Spradling, 1995; McGrail and Hays, 1997; Máthé *et al.*, 2003; review: **Chapter 1.2**). After the formation of the oocyte–nurse cell complex, the dividing mesodermal cells, associated with the *tunica propria* in region 2B, separate from this membrane and attach to the oocyte–nurse cell complex as follicle cells (also known as follicular epithelium cells; this term is used predominantly in this chapter). These eventually intercalate between the germ cells (Margolis and Spradling, 1995). The formation of the differentiated previtellogenic follicle (oocyte–nurse cell complex surrounded by the follicular epithelium cell monolayer) is completed within region 3, and the assembled follicle is expelled into the vitellarium. There it undergoes the transition from previtellogenesis to vitellogenesis and, subsequently, the transition to choriogenesis and ovulation.

During vitellogenesis, the oocyte receives material from the nurse and follicle cells and from the hemolymph. Some of these components represent differentially distributed determinants that establish pattern formation during early embryonic development, while others are nutrients necessary for the completion of embryogenesis. Upon completion of provisioning the oocyte and before the follicle is expelled into the oviduct, the follicle undergoes the transition into the choriogenic period. During this period, the oocyte is first sealed within a vitelline membrane before being covered by the chorion (see Section 1.3.3.8 below).

1.3.2.2. Lepidoptera

Many lepidopteran species have a relatively short, nonfeeding adult stage, which requires the adult female to emerge with most of her eggs ready to be fertilized and oviposited within hours. This life style constrains these insects to a program of ovarian organogenesis and follicle development that must occur at stages earlier than in *Drosophila*. Although the information base for ovarian and follicular cell development in the Lepidoptera is limited, this section will summarize the information that is available, and compare it with that obtained from *Drosophila*.

In the embryos of the domesticated silkworm, *Bombyx mori*, the primordial germ cells form a single cluster in the lateral and ventral region, which is associated with the localization of the *vasa* transcript (Nakao, 1999) rather than posteriorly, as in *Drosophila* (Hay *et al.*, 1990; Lasko and Ashburner, 1990). The lateral location of the primordial germ cell cluster was suggested previously from electron microscopy observations (Miya, 1958, 1959) but, due to the absence of specific molecular markers, its position could not been confirmed. Nevertheless, the existing information suggests that the determinants required for germ cell formation are similar in moths and *Drosophila*, but there are spatial differences in their localization within the presumptive germ band.

The germ cells of *Manduca sexta* are first detected after germ band formation with a monoclonal antibody, 3B11, that specifically immunostains the large primordial germ cells (Nardi, 1993). These are observed as a loose aggregate in the midline of the embryo near the posterior pole, as is the case with *B. mori*, rather than directly posterior as in *Drosophila*. After segmentation of the embryo, the germ cells are distributed between three abdominal segments, and segregate laterally into two groups. Subsequently, the primordial germ cells form two tight clusters of 18–30 cells each that are localized within the fifth abdominal segment.

Ovary development in the Indian meal moth, *Plodia interpunctella*, was examined during the larval, pupal, and adult stages using an immunostain for the α-crystallin 25 kDa protein (αcp25), which is found primarily in the germ cells (Zimowska *et al.*, 1991; Shirk and Zimowska, 1997; Shirk *et al.*, 1998). In the newly hatched first instar *Plodia* larva, each gonad contains a single cluster of 28 germ cells (Beckemeyer and Shirk, in press). The

gonads retain this morphology until the end of the first instar, when the germ cells of each ovary are segregated into four clusters of seven germ cells each (Figure 3, upper left; Beckemeyer and Shirk, in press). This segregation of the germ cells is completed in the early second instar, when the morphology of the four ovarioles within each gonad can be discerned (Figure 3, upper right; Beckemeyer and Shirk, in press). The organization of the ovarioles within the *Plodia* ovaries occurs much earlier than in *Drosophila*, where it begins at the end of the last larval instar. Whether the organization of the terminal filaments in *Plodia* is dictated by the localized expression of a *bab*-like gene within the undifferentiated somatic cells, as is the case with *Drosophila* (Godt and Laski, 1995), has yet to be determined.

No divisions of the primordial germ cells are observed until after the formation of the ovarioles is complete in the late second instar. Soon after the molt to the third instar, however, germ cell divisions begin, but there appears to be a separation between the seven founding primordial germ cells, which are maintained in the apical region of the ovarioles (Figure 3, lower left). The segregation of these seven cells may represent physically the niche of the germ stem cells in this moth. By the end of the penultimate instar, each ovariole contains about 56 germ cells. The cluster of seven germ cells is maintained at the

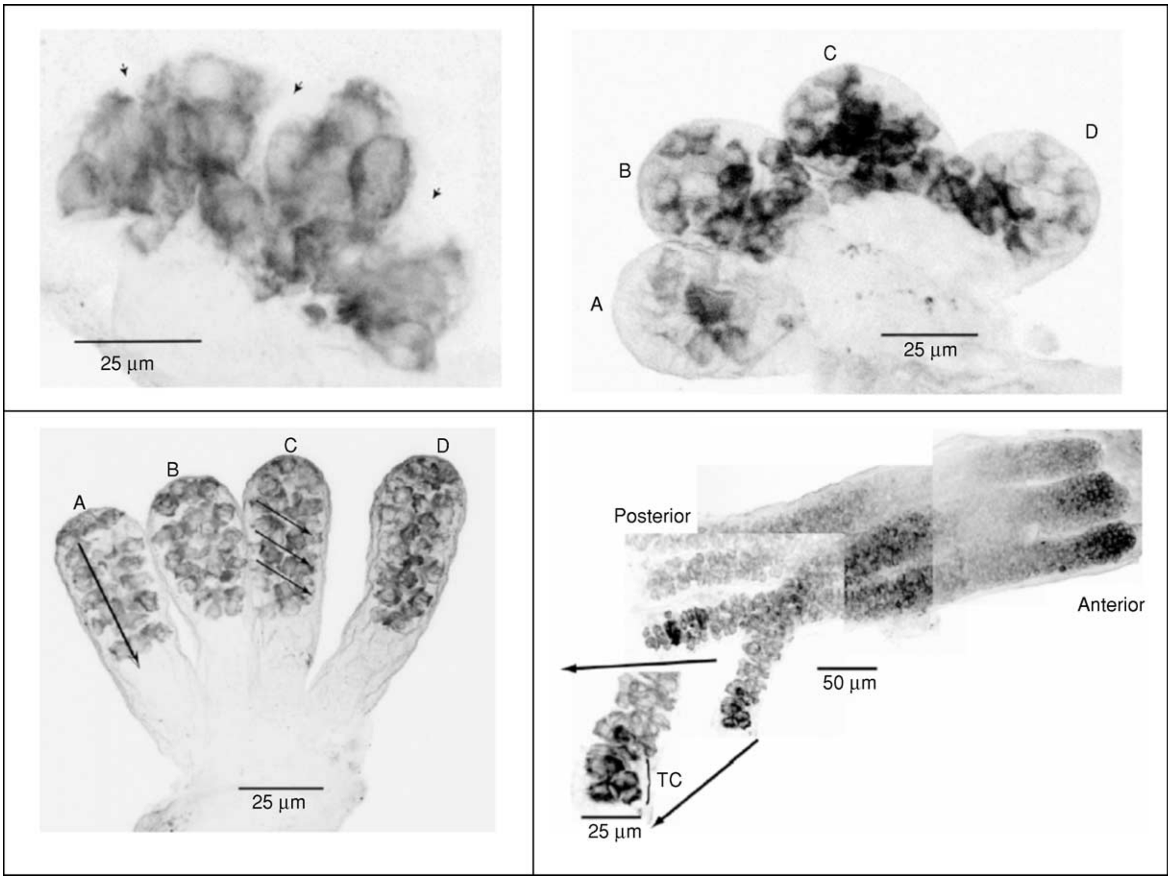


Figure 3 Upper left: a gonad from a late first instar *Plodia* larva immuno-fluorescently stained with α-crystallin (αcp25) antibodies. Somatic tissue segregation of the founding germ cells into separate ovarioles is apparent (marked by arrow heads). The confocal image is a compilation of optical sections through the specimen. Upper right: a gonad (ovary) from an early second instar *Plodia* larva stained with the same antibody. Segregation of the gonad into four ovarioles (A, B, C, and D) is complete and the number of cystoblasts has increased from the earlier stages. The confocal image is a compilation of optical sections through the specimen. Lower left: an ovary from a late third instar *Plodia* larva. The number of cystoblasts has increased and the cells show three levels of organization within the ovarioles. The confocal image is a compilation of half the optical sections through the specimen. The separation between the distal-most cluster of germ cells and the other germ cells within each ovariole is maintained. In ovariole A, a central space can be observed that does not contain immuno-fluorescently stained germ cells (paralleled by the half-open headed arrow). In ovariole C, a diagonal alignment of the germ cells can be seen (paralleled by the three arrows). Lower right: An ovary from a newly pupated *Plodia*. The image is a reassembly of three confocal scanning regions that cover the entirety of the ovary. Each region is the compilation of the optical sections through the region. The arrows show a projection of the most posterior or terminal clusters of cystocytes (TC).

apical tip of each ovariole, while the majority of the germ cells are arranged in a spiral around the perimeter of the ovariole. No cystocyte clusters are observed at this stage, however, suggesting that in moths the divisions to form all of the primary cystoblasts occur before the onset of the cystocyte divisions. This is supported by the clear presence of eight-cell cystocyte clusters discernable in the posterior termini of the ovarioles at the end of the last instar (Zimowska *et al*., 1991; Beckemeyer and Shirk, in press).

The division of the cystoblasts and cystocytes in *Plodia* takes a path considerably different than that taken by *Drosophila*, where the primordial germ cells within the germarium give rise to the cystoblasts, which divide to form the 16-cell cystocyte cluster independent of the formation of other cystoblasts and throughout the adult stage (King *et al*., 1968). At the end of the third larval instar in *Plodia*, the divisions necessary to form all of the cystoblasts have taken place. This appears to happen before the three divisions of the cystoblasts to cystocytes begin. Thus, at the end of the third larval instar, the ovary contains all the cystoblasts necessary for the formation of the full complement of eggs that the female will lay during her adult life. Using a comparison to industry, *Plodia* mass-produces each stage of its germ cell/follicle production line before advancing to the next assembly stage, while *Drosophila* takes the on-time supply approach, by producing and assembling all components of a single follicle, given an adequate food supply and before initiating the production of a subsequent follicle.

The presence of the cluster of the seven primordial germ cells at the apical tip of each ovariole, even at the white pupa stage of *Plodia* (**Figure 3**, lower right) (Zimowska *et al*., 1991; Beckemeyer and Shirk, in press), suggests that there is a potential for producing more cystoblasts, even though all cystocyte clusters necessary for adult egg laying are already present. At this stage, however, the follicles are not complete, because no follicular epithelium cells are observed in association with the cystocyte clusters (Zimowska *et al*., 1991). Follicle cells begin to surround each oocyte–nurse cell complex during the ecdysteroid peak that occurs 28–36 h after pupation (Shaaya *et al*., 1993). By comparison, the organization of the ovarioles and germ cells in the early fifth (last) instar larva of *B. mori* (Miya *et al*., 1970) is similar to that observed in the white pupa of *P. interpunctella*, suggesting an even earlier completion of primordial germ cell development in *Bombyx*.

Following the peak of ecdysteroids that occurs during the early pupal stage (Shaaya *et al*., 1993), pharate adult development is initiated, and the terminal follicles become fully differentiated within the ovarioles of *Plodia* (Zimowska *et al*., 1991). The progress of the female toward the initiation of vitellogenesis is regulated by the declining levels of the ecdysteroids (Shirk *et al*., 1990). After final assembly by 36 h post-pupation, the follicles remain in a pre-vitellogenic state until 92 h after pupation. After this time, the physiology of the female shifts to vitellogenesis, and each ovariole contains a linear array of sequentially developing follicles (**Figure 1**).

1.3.3. Vitellogenesis

During vitellogenesis, the growth of the oocyte in the ovarian follicle accelerates relative to that of the nurse cell complex (Büning, 1994). Growth of the oocyte is enhanced via endocytosis of yolk proteins, which may be derived from two sources, the fat body (vitellogenins) and the follicular epithelial cells that surround the oocyte–nurse cell complex (Raikhel and Dhadialla, 1992; Izumi *et al*., 1994; Melo *et al*., 2000). The oocyte surface is not directly exposed to the hemolymph but is separated from it by the follicular epithelium. Wide channels form between the epithelial cells, a condition termed patency (Telfer *et al*., 1982; Raikhel and Lea, 1991; Davey, 1993; Zimowska *et al*., 1994), allowing passage of hemolymph-derived proteins through the follicular epithelium toward the oocyte surface and into the oocyte.

1.3.3.1. The Transition from Previtellogenesis to Vitellogenesis

In this section, the hormonal control of the transition from previtellogenesis to vitellogenesis in the insects, mostly Diptera and Lepidoptera is discussed, that have been most intensely investigated, including the fruit fly *D. melanogaster*, the yellow fever mosquito, *Aedes aegypti*, and the silkmoth, *B. mori* is discussed. In these three insects, most of the data addressing the mechanism of action of hormones (predominantly ecdysone) in the ovarian tissue, has accumulated. The regulation of the transition in other insects will be only briefly discussed, using the models above as a basis for comparisons and, wherever possible, emphasis will be given to the molecular mechanisms controlling ovarian follicle development.

1.3.3.1.1. Dipteran insects

1.3.3.1.1.1. **Drosophila melanogaster** In *Drosophila*, previtellogenic growth of follicles, corresponding to stages 1–7 of egg chamber (follicle) development, is initiated at the end of adult development, while vitellogenesis (corresponding to stages 8–10) starts after eclosion. The initiation of

vitellogenesis constitutes a control point of oogenesis that integrates internal (hormonal) and external (environmental) signals, to allow efficient progression of follicle development (Spradling, 1993).

1.3.3.1.1.1.1. JH and ecdysone physiological data Hormonal control of vitellogenesis in *Drosophila* is complex and requires both JH (see **Chapter 3.7**) and ecdysteroids (Riddiford, 1993). JH stimulates yolk protein (YP) synthesis in the ovary, and is necessary for the continuation of development of stages 8 and 9 vitellogenic follicles (Jowett and Postlethwait, 1980; Wilson, 1982; Bownes *et al.*, 1988a). In addition, JH stimulates the production of ovarian ecdysteroids (Schwartz *et al.*, 1989).

Ecdysteroids play (see **Chapters 3.3**, **3.4**, and **3.7**) both positive and negative roles in the control of vitellogenesis. In diapausing female adults, oogenesis is arrested shortly before vitellogenesis but can be relieved by the application of 20-hydroxyecdysone (20E) (Richard *et al.*, 1998). Based on this and other observations, it was proposed that in the early stages following adult eclosion, ecdysteroids derived from the ovary or from other sources (Bownes, 1989) stimulate YP synthesis in the fat body as well as YP uptake by the oocyte (Richard *et al.*, 1998).

During the later stages of oogenesis, alternatively, 20E has a negative effect on follicle development. In virgin females, 20E induces apoptosis, causing the disappearance of nearly all vitellogenic follicles, and this effect can be counteracted by administration of JH or sex peptide (SP; Soller *et al.*, 1999). The SP is a 36 amino acid long peptide synthesized by the accessory glands of *Drosophila* males, and transferred to the female fly during mating (Chen *et al.*, 1988; Kubli, 1996) (see also **Chapters 1.5** and **3.9**). It acts on the corpora allata (CA) to stimulate the production of JH (Moshitzky *et al.*, 1996), which, in turn, prevents the 20E-dependent induction of apoptosis in vitellogenic follicles. Thus, a correct balance between JH and ecdysteroids seems to be essential for the progression of vitellogenic follicle development in the *Drosophila* ovary (Soller *et al.*, 1999; Gruntenko *et al.*, 2003). The induction/prevention of apoptosis by 20E/JH shows strict developmental regulation and is restricted to follicles of stage 9, while follicles of earlier or later stages (stages 8 and 10, respectively) are not affected.

1.3.3.1.1.1.2. Genetic analysis role for ovarian ecdysone? Genes implicated in the ecdysone regulatory hierarchy show stage-specific expression patterns during *Drosophila* oogenesis. The levels of the mRNAs for the nuclear receptor E75 and the E twenty-six (ETS) domain-containing transcription factor E74 become upregulated at stage 8 (beginning of vitellogenesis) and peak in abundance at stage 10B (end of vitellogenesis), in both the nurse and the follicular epithelium cells (Buszczak *et al.*, 1999). The E74 and E75 transcription factors function as primary response genes in the ecdysone regulatory cascade that is triggered at the beginning of metamorphosis (Thummel, 1996). Another primary ecdysone-responsive gene, which encodes the various Broad-Complex (BR-C) zinc finger protein-containing transcription factor isoforms, is also induced in the cells of the follicular epithelium during stages 5 and 6, and its expression continues throughout vitellogenesis (Deng and Bownes, 1997). Furthermore, both components of the ecdysone receptor heterodimer, EcR and USP, are expressed throughout oogenesis in both the germline (nurse cells and oocyte) and the soma (follicular epithelium cells) (Khoury-Christianson *et al.*, 1992; Buszczak *et al.*, 1999; Carney and Bender, 2000). Finally, the expression of *E75* and *BR-C* in the ovarian follicles seems to be sensitive to the levels of 20E: higher expression is observed when 20E is added to ovarian cultures, while lower expression occurs at the restrictive temperature in *ecdysoneless* (*ecd*) mutant flies (Buszczak *et al.*, 1999).

Follicles that are germline clones of *E75* and *EcR* show developmental arrest and degeneration at stages 8–9 (Buszczak *et al.*, 1999). A similar arrest in oogenesis was also observed for germline clones of the *dare* gene, which encodes the *Drosophila* homolog of adrenodoxin reductase, an enzyme implicated in steroid hormone biosynthesis (Buszczak *et al.*, 1999; Freeman *et al.*, 1999). Characterization of temperature-sensitive *ecdysoneless*^{1} (*ecd*^{1}) mutants, which have low ecdysteroid levels at the restrictive temperature, also indicate that 20E may be required for the progression of oogenesis beyond stage 8 (Audit-Lamour and Busson, 1981; Walker *et al.*, 1987). Because *dare* is expressed in the nurse cells, it was proposed that *dare* is involved in ecdysone biosynthesis carried out by the nurse cell complex.

Based on the analysis of the available mutants and the expression pattern of genes implicated in ecdysone biosynthesis and the ecdysone regulatory hierarchy during oogenesis, a model was proposed in which ecdysteroids, produced by the germline oocyte–nurse cell complex at the beginning of vitellogenesis, act in an autocrine manner (i.e., within the developing follicle) to activate the ecdysone regulatory cascade. The activation of the ecdysone regulatory cascade is considered necessary for the subsequent progression of oogenesis (vitellogenesis)

beyond stages 8 and 9 (Buszczak *et al.*, 1999; Freeman *et al.*, 1999).

1.3.3.1.1.1.3. Integration of physiological and genetic data From the discussion above, it is clear that much work still needs to be done, in order to elucidate the precise role of ecdysone in the regulation of vitellogenesis in *Drosophila*. In particular, conflicting data exist between the results obtained from the physiological and genetic experiments that requires resolution. Indeed, the physiological experiments suggest that 20E stimulates previtellogenic growth, while it induces apoptosis of vitellogenic follicles (Richard *et al.*, 1998; Soller *et al.*, 1999; Gruntenko *et al.*, 2003), and that a correct balance between ecdysteroids and JH is required for the progression through vitellogenesis (Soller *et al.*, 1999). The genetic studies, alternatively, indicate a requirement for genes such as *EcR*, *E74*, *E75*, and *BR-C* in the transduction of the 20E response during metamorphosis (Buszczak *et al.*, 1999; Carney and Bender, 2000). However, the fact that the *EcR*, *E74*, *E75*, and *BR-C* genes are involved in the control of oogenesis (vitellogenesis), does not necessitate that the regulatory pathway initiated by the hormone 20E is actually involved in this process. In fact, the data that support the model of autocrine control of follicle development progression, by the induction of the required regulatory cascade by 20E, may not be entirely conclusive.

Thus, the function of the *dare* gene may not be entirely limited to ecdysone biosynthesis, but could instead be involved in the catalysis of other enzymatic reactions. Of relevance to this is previous research in other insects, which has shown that the cells of the follicular epithelium, rather than the nurse cell complex, are involved in ecdysone biosynthesis (Lagueux *et al.*, 1977). This has been confirmed through the expression pattern of the *disembodied* (*dis*) gene, which encodes a specific hydroxylase in the ecdysone biosynthetic pathway (Warren *et al.*, 2002) and is expressed in the follicular epithelium (Chávez *et al.*, 2000). The differential expression pattern suggests that the DARE and DIS enzymes are involved in distinct biosynthetic pathways. Therefore, it would be interesting to examine whether mutant clones for the *dis* gene (in the follicular epithelium) would affect the progression of vitellogenesis. Additonally, the fact that mutations of the *E75*, *E74*, *EcR*, and *BR-C* genes affect the progression of vitellogenesis, does not necessarily imply that the ecdysone regulatory pathway is involved. The expression of these genes could be regulated by other pathways. For example, during oogenesis, the expression of the *E75* and *BR-C* genes is controlled by the epidermal growth factor (EGF) pathway during oogenesis (Deng and Bownes, 1997; Buszczak *et al.*, 1999). Furthermore, the requirements for *EcR* may reflect the function of this receptor as a repressor rather than as a ligand(20E)-dependent activator.

In view of these reflections, the view presented by the physiological experiments is favored, i.e., that 20E may be involved in the stimulation of previtellogenic growth but causes apoptosis in vitellogenic follicles. In addition, although genes implicated in the ecdysone regulatory pathway (*E75*, *E74*, *EcR*, and *BR-C*) are required for sustainance of vitellogenic growth, these genes may very well be induced and act in an ecdysone-independent manner.

1.3.3.1.1.1.4. Nutritional control through the insulin signaling pathway The quality of nutrition (absence/presence of protein diet) has profound effects on the rate of follicle development during oogenesis, which can vary by as much as 60-fold (Drummond-Barbosa and Spradling, 2001). Variations in the rates of follicle development are due to differences in rates of cell proliferation in both the germline and the follicle cell lineage, as well as to the different frequencies of cell death at two developmental stages, first, during egg chamber or follicle assembly, when the region of the germarium containing the germline cysts are engulfed by the precursors of the follicle cells, and, second, at the time of initiation of vitellogenesis (stage 8 of follicle maturation; Drummond-Barbosa and Spradling, 2001) (see also **Chapter 1.2**).

The insulin signaling pathway is conserved between *Drosophila* and vertebrates (Edgar, 1999), and *Drosophila* mutants for components of this pathway show a growth-deficiency phenotype (Garofalo, 2002). Mutants for the *Drosophila* insulin receptor, the insulin receptor substrate (IRS)-like gene *chico*, and the ribosomal protein S6 kinase homolog also show female sterility due to poor ovarian growth (Chen *et al.*, 1996; Böhni *et al.*, 1999; Montagne *et al.*, 1999). These results suggest that the insulin signaling pathway is functional in the ovaries. Furthermore, the effects of nutrition on oogenesis are mediated through the insulin pathway. Thus, even in the presence of abundant nutrients, mutations in *chico* partially impair the increase in the proliferation rate of the follicular cells and almost completely prevent the progression of the follicles into vitellogenic stages (Drummond-Barbosa and Spradling, 2001).

Seven insulin-like peptide sequences have been identified in the *Drosophila* genome (DILPs), which show specific and unique patterns of expression in a variety of tissues, including the cells of the ovarian follicular epithelium (Brogiolo *et al.*, 2001; Ikeya

et al., 2002). The expression levels of DILPs in the brain respond to variations in nutrition, and overexpression of DILP2 results in an increase in body weight and organismal size (Brogiolo *et al.*, 2001). The picture that emerges is that signals generated by high nutrition levels result in the release of DILPs from neurosecretory cells at the corpora cardiaca (CC) of the ring gland (Leevers, 2001); DILPs secreted in the hemolymph subsequently stimulate cell proliferation and growth. At the level of the ovary, DILPs are proposed to increase the proliferation rate of the follicular cells and promote the transition from previtellogenesis to vitellogenesis. Furthermore, the expression of DILP5 in follicular cells of vitellogenic follicles (Ikeya *et al.*, 2002) also suggests the existence of a parallel autocrine/paracrine pathway that regulates follicle growth and maturation.

With respect to the possible conservation of the signaling systems that regulate reproduction in insects and vertebrates, it is also worth mentioning that two orphan G protein-coupled receptors (GPCRs) have been identified in the *Drosophila* genome, which are homologs of the mammalian follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH) receptors (Hauser *et al.*, 1997; Eriksen *et al.*, 2000). However, no data exists for addressing the possible involvement of these receptors in the regulation of insect reproduction. In fact, flies mutant for one of these receptors die at the time of hatching, thus implicating this receptor in the control of embryonic development (Eriksen *et al.*, 2000). Moreover, no FSH, LH, or TSH-like hormones could be identified in the *Drosophila* genome and, consequently, the two orphan *Drosophila* GPCRs may have other types of peptides as ligands (De Loof *et al.*, 2001).

1.3.3.1.1.2. **Aedes aegypti** In contrast to the Lepidoptera (discussed in Section 1.3.3.1.2 below) and *Drosophila*, vitellogenesis and follicle maturation in female mosquitoes occur synchronously following a blood meal (Raikhel and Lea, 1990). At the time of adult eclosion, each ovary consists of the germarium and one follicle per ovariole. JH is required for the follicle to mature to a resting stage, at which time it becomes competent to respond to a blood meal and initiate vitellogenesis (Bownes, 1986). Vitellogenesis consists of two phases, first, of an initiation phase (immediately after the blood meal), during which the cells of the follicular epithelium resume mitosis and separate from the oocyte, while low levels of vitellogenins are produced by the ovary and, second, of a trophic phase (starting 5–6 h after the blood meal), during which the fat body produces large amounts of vitellogenins, and rapid growth of the follicle occurs in the ovary (Riehle and Brown, 2002).

During the initiation phase, neurosecretory hormones, termed egg development neurosecretory hormones (EDNHs) or ovary ecdysteroidogenic hormones (OEHs), are released from the brain and stimulate ecdysteroid production by the ovary (Brown *et al.*, 1998; see also **Chapters 1.5** and **3.9**). The ecdysteroids released from the ovary subsequently initiate the trophic phase by acting on the fat body and stimulating the first vitellogenic cycle (Deitsch *et al.*, 1995; Zhu *et al.*, 2000). Ecdysteroids have also been proposed to be involved in a feedback mechanism that causes the pinching off of the second follicle from the germarium and the shutdown of the high synthetic activity in the fat body at the end of the cycle (Bownes, 1986).

As is the case with *Drosophila*, the insulin signaling pathway is involved in the regulation of ovarian growth (vitellogenesis). Mosquito ovaries contain high levels of the mosquito insulin receptor homolog (MIR; Graf *et al.*, 1997). MIR is expressed in both the nurse and the follicular epithelium cells of the maturing follicles, and its expression levels in the cells of the follicular epithelium increase during the first phase of vitellogenesis (Helbling and Graf, 1998; Riehle and Brown, 2002).

Interestingly, the ovarian MIR can be stimulated by micromolar quantities of bovine insulin, a finding that allowed the elucidation of the signaling pathway in some detail. Bovine insulin was shown to be capable of stimulating tyrosine phosphorylation in the ovary (Riehle and Brown, 2002), and the downstream signaling was shown to involve the phosphatidyl-inositol 3-kinase (PI3K) and protein kinase B (PKB) pathways, but not that of the mitogen-activated protein kinase (MAPK) (Riehle and Brown, 1999, 2003). Furthermore, the action of bovine insulin results in the biosynthesis and release of ecdysteroids by the ovary (Riehle and Brown, 1999). Thus, insulin-like peptides have the same effect on ovarian tissue as the OEH hormones. To what extent the transduction pathways of these two types of hormones overlap or are integrated is unknown at present.

Although the ovary itself is not considered to be a direct target for 20E (Bownes, 1986), data exist with respect to the expression of ecdysone-responsive genes in the mosquito ovary. Of particular interest is the differential accumulation of various *usp* mRNA isoforms during previtellogenesis and vitellogenesis (Wang *et al.*, 2000). During previtellogenesis, the mRNA for the A-isoform predominates, but this isoform becomes rapidly downregulated within 1 h after a blood meal, and the decline persists throughout

vitellogenesis. The mRNA for the USP-B isoform, alternatively, shows an opposite pattern of expression, becoming induced in the ovary after the blood meal. In addition, the expression of *EcR* mRNA increases at the initiation of vitellogenesis (Cho *et al.*, 1995; Wang *et al.*, 2000). Relevant to the differential regulation of target genes in follicular cells may be the observation that EcR/USP-B complexes bind more strongly than EcR/USP-A ones to target sites in the DNA, and are also stronger transcriptional activators in heterologous systems (Wang *et al.*, 2000).

The expression patterns of mRNAs for early and early–late ecdysone response genes (*E75A*, *E75B*, *E75C*, *HR3*, and *E74B*) are nearly identical to that of the vitellogenin receptor (*VgR*) gene, suggesting a function for these genes in the regulation of vitellogenesis in the ovary (Pierceall *et al.*, 1999; Kapitskaya *et al.*, 2000; Sun *et al.*, 2002). Although of unknown functional relevance, the B isoform of the mosquito HNF-4 nuclear receptor (see also discussion on lepidopteran HNF-4 expression below) is also present in ovarian tissue, and its expression is induced following a blood meal (Kapitskaya *et al.*, 1998).

Taken together, these results suggest that genes historically implicated in the ecdysone response have an expression pattern consistent with their having roles as regulators of vitellogenesis in the ovary. Whether the expression of these genes in the ovary is regulated by ecdysteroids, however, remains to be investigated. As was discussed for the case of *Drosophila* (Section 1.3.3.1.1.1.3), the expression of the "ecdysone response" genes may be under the control of other signal transduction pathways.

While uncertainty exists in the case of ovarian tissue, the induction of early and early–late response genes by 20E in the fat body has been demonstrated beyond doubt, and additional data suggest that the regulatory cascade induced by 20E in the fat body of the adult female shows great resemblance to the cascade induced by 20E in other tissues during metamorphosis (Raikhel *et al.*, 1999; Li *et al.*, 2000; Zhu *et al.*, 2000).

1.3.3.1.2. Lepidoptera Lepidopterans can be grouped in different categories based on their dependence for ovarian follicle maturation on metamorphic events (Ramaswamy *et al.*, 1997). The silkmoth *B. mori* exemplifies the first category, in which follicle development is dependent on 20E, occurs during pupal and adult development, and is completed before the emergence of the adult. The second category, is typified by the Indian meal moth, *P. interpunctella*, in which vitellogenesis is triggered by the declining titers of ecdysteroids during adult development (Shirk *et al.*, 1992). Follicle development in this category is also completed before adult eclosion. In a third category, exemplified by the tobacco hornworm, *M. sexta*, initiation of vitellogenesis during metamorphosis is independent of ecdysteroids (Satyanaryana *et al.*, 1994), while choriogenesis is controlled by JH and completed in the adult moth (Nijhout and Riddiford, 1974).

In other lepidopterans such as *Heliothis virescens* (fourth category), the initiation and completion of vitellogenesis and choriogenesis are independent of metamorphic events, with follicle development being initiated in adults and completely dependent on JH (Zeng *et al.*, 1997). Finally, an additional version of hormonal control has been described recently in the fall armyworm, *Spodoptera frugiperda* (Sorge *et al.*, 2000). In the adults of this species, JH is crucial for the regulation of the incorporation of yolk proteins in vitellogenic follicles, and for the induction of ecdysteroid production by the ovary, in a manner reminiscent of the one occurring in *Drosophila*. Ecdysteroids produced by the ovary in turn regulate the production of vitellogenins by the fat body (Sorge *et al.*, 2000).

***1.3.3.1.2.1.* Bombyx mori** In *Bombyx*, oogenesis occurs almost exclusively during pupal and pharate adult development and is completed before adult eclosion (Tsuchida *et al.*, 1987). The moulting hormone 20E was shown to be essential and sufficient for initiating previtellogenic and vitellogenic development in ovarian follicles. Thus, when late spinning larvae are ligated between the thorax and the abdomen, 20E that is produced by and secreted from the prothoracic glands is prevented from entering the abdominal cavity, and developmental arrest ensues. Adult development, including ovarian development, can be induced in the arrested abdomens by injections of microgram quantities of 20E (**Figure 4**) (Tsuchida *et al.*, 1987; Swevers and Iatrou, 1999).

The other major hormone that regulates development in insects, JH, has not been found to be involved in the stimulation of ovarian development in *Bombyx*. Thus, allatectomy of the 4th instar larvae does not prevent ovarian development and egg maturation (vitellogenesis and choriogenesis) in the resulting precocious pupae, suggesting that JH does not play a role during oogenesis (Izumi *et al.*, 1984).

The rise in ecdysteroid titer, during pupal and pharate adult development, initially results in important morphological changes in the ovarian structure. The ovarian capsule, in which the ovarioles are assembled and enclosed during larval and pupal development, ruptures and the developing ovarioles emerge gradually in the abdominal cavity (see **Figure 12** below) (Yamauchi and Yoshitake,

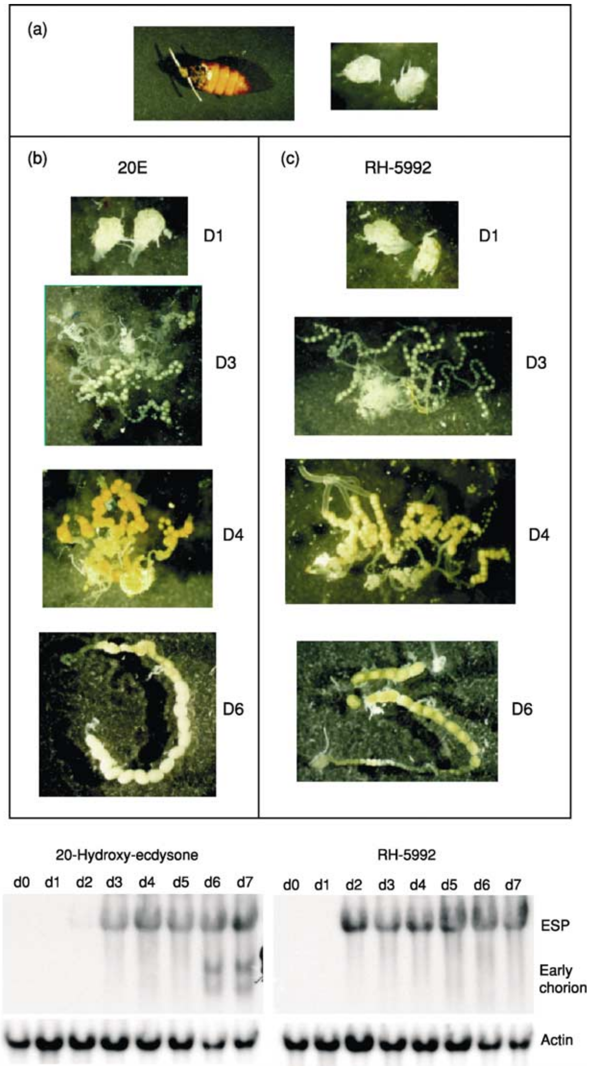


Figure 4 The nonsteroidal ecdysone agonist RH-5992 (tebufenozide) induces ovarian development (previtellogenic growth and vitellogenesis) in the silkmoth, *Bombyx mori*, that is followed by developmental arrest prior to choriogenesis. Upper panel: photographs of a developmentally arrested abdomen (panel (a), left) and of ovaries and ovarioles from abdomens that were either uninjected (panel (a), right), injected with 20E (panel (b)), or injected with RH-5992 (panel (c)). Ovaries and ovarioles are either in dissection medium (panel (a), right, and series D1, D3, and D4 from panels (b) and (c) or mounted in glycerol (D6 in panels (b) and (c). Indicated are the time intervals (in days, D) between injection and dissection. Note that for ovarioles mounted in glycerol, oocytes in choriogenic follicles are prevented from collapsing by the protective eggshell. Color also distinguishes vitellogenic (yellow) from choriogenic (white) follicles. Choriogenic follicles are only apparent in D6 ovarioles from 20E-injected abdomens. Lower panel: expression of egg-specific protein (ESP) and early chorion mRNAs in ovaries at different time intervals (in days, d) following injection of 20E or RH-5992 (tebufenozide) in pupal developmentally-arrested abdomens of *B. mori*. Hybridizing mRNA species in Northern blot analysis are 2 kb (ESP), 0.8 and 0.6 kb (early chorion), and 1.3 kb (actin). Note that ESP mRNA is induced in both 20E- and tebufenozide-injected abdomens, while early chorion mRNA only appears in the former. (Reprinted with permission from Swevers, L., Iatrou, K., **1999**. The ecdysone agonist tebufenozide (RH-5992) blocks progression of the ecdysteroid-regulated gene expression cascade during silkmoth oogenesis and causes developmental arrest at mid-vitellogenesis. *Insect Biochem. Mol. Biol.* 29, 955–963; © Elsevier.)

1984). The removal of the ovarian capsule allows the follicles in the ovarioles to take up yolk proteins from the hemolymph and facilitates their direct exposure to growth factors that are found in it.

At the molecular level, the silkmoth ovary responds to the circulating 20E through the induction of a gene expression cascade that is practically identical to the one observed in other insect tissues (the ecdysone regulatory cascade; Henrich and Brown, 1995; Thummel, 1996). The ovarian tissue expresses the nuclear receptors that constitute the functional ecdysone receptor, which is composed of the heterodimer complex between EcR and USP (Tzertzinis *et al*., 1994; Swevers *et al*., 1995), suggesting that the ovary is a direct target of 20E. Similar to other insect tissues, 20E induces, within a few hours, the

expression of the primary response genes, which encode the nuclear receptors BmHR3 (B and C isoforms) and BmE75 (A and C isoforms) (Eystathioy *et al.*, 2001; Swevers *et al.*, 2002a). During the induction of expression of the mRNAs for these receptors, the mRNA for another nuclear receptor, BmFTZ-F1 (Sun *et al.*, 1994), becomes down-regulated (Swevers and Iatrou, 2003).

The expression of a sixth nuclear receptor, BmHNF-4, is also induced in ovarian tissue (follicular epithelium cells) upon exposure to 20E (Swevers and Iatrou, 1998). In contrast to the BmE75 and BmHR3 receptors, however, the accumulation of BmHNF-4 occurs more slowly and appears to represent a delayed response to the hormone. To date, BmHNF-4 has not been associated with the induction of the ecdysone response in other tissues (Thummel, 1995).

The expression of the yolk protein, egg-specific protein (ESP; Sato and Yamashita, 1991), which is produced by the cells of the follicular epithelium, is initiated approximately two days after larval–pupal ecdysis, when ecdysteroid titers reach peak levels of accumulation in the hemolymph (Tsuchida *et al.*, 1987), and the time of first expression of ESP can be viewed as a timing marker for the initiation of vitellogenesis (Sato and Yamashita, 1991). Moreover, when 20E is injected in developmentally arrested abdomens, ESP expression occurs with similar kinetics as during normal development (2–3 days after injection; **Figure 4**; Swevers and Iatrou, 1999), indicating that ESP expression represents a late response in the ecdysone regulatory cascade. The expression of the *esp* gene does not seem to be directly regulated by 20E, however, because it can occur both during the period of ecdysteroid accumulation to high titers (normal development) and in the *quasi* absence of 20E (developmentally arrested abdomens injected with 20E, in which the hormone is neutralized within 24 h from the injection; Ohnishi and Chatani, 1977).

The expression of two types of transcription factors, BmHR3 and BmGATAβ, shows remarkable changes in abundance in ovarian follicles at the transition from previtellogenesis to vitellogenesis. The A isoform of the nuclear receptor BmHR3 becomes expressed in the cells of the follicular epithelium of vitellogenic follicles (Eystathioy *et al.*, 2001), while there is also a remarkable increase in the accumulation of the mRNAs for the other two isoforms of BmHR3, B and C. The expression of BmHR3 and ESP during vitellogenesis coincides remarkably, and retinoic acid receptor-related orphan receptor response elements (ROREs) capable of binding BmHR3A have been identified in the *esp* gene promoter (Eystathioy *et al.*, 2001), implicating BmHR3A as a potential regulator of *esp* expression (Swevers and Iatrou, 2003). In addition, the expression of the zinc finger-containing transcription factor BmGATAβ (Drevet *et al.*, 1994) is down-regulated during the transition from previtellogenesis to vitellogenesis. In this case, however, immunocytochemistry experiments have shown that expression of BmGATAβ is restricted to the cells of the ovariole sheath rather than the follicular epithelium (Lunke, 2000). Thus, while the expression of BmGATAβ has been correlated with the transcriptional competence of chorion genes (see Sections 1.3.3.3.2 and 1.3.4.1.3), it does not seem to play a role in the differentiation of the follicular epithelium during the transition from previtellogenesis to vitellogenesis. Instead, it may have a specialized (but as yet undefined) role in the ovariole sheath cells that overlay the previtellogenic follicles (Swevers and Iatrou, 2003).

1.3.3.1.2.2. **Plodia interpunctella** Vitellogenesis in *Plodia interpunctella* begins during the pupal stage, which lasts 136 h, and eggs are completely chorionated and ready for fertilization by 6 h after adult eclosion (Zimowska *et al.*, 1991). As is typical of most Lepidoptera, each of the four ovarioles within the ovary contains a linear array of progressively developing follicles, with the most posterior or terminal follicle being the most advanced in development.

During vitellogenesis, oocytes accumulate two major yolk proteins: vitellin (Vn), which consists of two yolk protein (YP) subunits, YP1 ($M_r = 153\,000$) and YP3 ($M_r = 43\,000$), produced and secreted as vitellogenin (Vg) by the fat body cells (see also Section 1.3.3.2 below); and follicular epithelium yolk protein (FEYP), which consists of two subunits, YP2 ($M_r = 69\,000$) and YP4 ($M_r = 33\,000$), produced and secreted by the follicular epithelium cells (Shirk *et al.*, 1984; Bean *et al.*, 1988). The presence of vitellogenin in the hemolymph can be detected by immuno-staining of Western blots with antisera for YP1 or YP3 at 80–83 h after pupation (Shirk *et al.*, 1992). However, Vn cannot be detected by immuno-gold staining with anti-YP1 or anti-YP3 antisera in the terminal follicles until after 112 h after pupation (Zimowska *et al.*, 1994, 1995). Repeated treatments of pharate adult female pupae with 20E leads to the suppression of the oocyte growth and yolk protein accumulation (Shirk *et al.*, 1990), suggesting that the developmental progress of female *P. interpunctella* toward vitellogenesis is regulated by the declining titers of ecdysteroids.

Between the previtellogenic and vitellogenic stages, the terminal follicles enter the provitellogenic stage, during which the follicles become competent to produce FEYP within the follicular epithelium cells, initiate the formation of yolk spheres, and develop patency, the opening of spaces between the follicular epithelium cells that allows penetration of the hemolymph proteins into the oocyte (Zimowska *et al*., 1994). Using immuno-gold staining with anti-YP2 serum, it was established that the follicular epithelium cells begin production of YP2 at 92 h after pupation and, at the same time, the oocytes begin formation of YP2-containing yolk spheres (Zimowska *et al*., 1994). However, none of the other yolk protein subunits is detectable at this time. By culturing early vitellogenic ovaries in varying concentrations of 20E, it was shown that the continuation of YP2 synthesis requires low concentrations of 20E but is inhibited by high levels of the hormone (Shirk *et al*., 1990). These findings suggest that the action of the ecdysteroids is to regulate the progression of vitellogenesis by acting directly on the synthetic activity of the ovarian tissue.

The formation and accumulation of early yolk spheres containing only YP2 and the increased thickness of the brush borders of the follicular cells are both indicators of the progression towards vitellogenesis (Zimowska *et al*., 1994). By 105 h after pupation, the follicular epithelium of the terminal follicles begins to show signs of patency. By 120 h after pupation, full patency is achieved, and both yolk proteins, Vn and FEYP, are accumulated in the yolk spheres (Zimowska *et al*., 1994, 1995; see also Section 1.3.3.2).

1.3.3.1.2.3. Other Lepidoptera While progress has been made regarding the effects of 20E on the silkmoth ovary at the molecular level, no such data exist regarding the hormonal control of vitellogenesis by JH or 20E in the other categories of lepidopterans mentioned earlier. It can be anticipated, however, that regulatory cascades other than the ecdysone cascade are involved. For instance, in the case in which vitellogenesis is triggered by a decline in ecdysteroid titer (*P. interpunctella*; Shirk *et al*., 1992), the involvement of the nuclear receptor FTZ-F1 is expected, since this receptor functions as a competence factor during developmental periods characterized by low ecdysteroid titers (Broadus *et al*., 1999). However, regarding the response to JH, little is known, and a “JH-induced regulatory cascade” has yet to be characterized (Gilbert *et al*., 2000). It remains to be seen to what extent the regulation of vitellogenesis by 20E or JH during follicle development, in lepidopterans belonging to the different categories mentioned above, is carried out by common regulatory factors. It is obvious, however, that no unifying model for the control of the transition from previtellogenesis to vitellogenesis can be proposed at the moment.

1.3.3.1.3. Other insects In other insects where oogenesis has been studied extensively, such as the African migratory locust, *Locusta migratoria*, the cockroaches *Blattella germanica* and *Leucophaea maderae*, and the hemipteran, *Rhodnius prolixus*, JH has been implicated as the major hormone for the control of vitellogenesis (Bownes, 1986; Wyatt, 1991; Davey, 1993; Cruz *et al*., 2003). However, very little information exists with respect to the action of JH in developing follicles at the molecular level. In addition to the action of JH, a few peptide hormones have been also isolated that stimulate or inhibit vitellogenesis (De Loof *et al*., 2001).

The action of JH on ovarian follicle cells has been elucidated to the greatest extent in *R. prolixus* (Davey, 2000). In *Rhodnius*, as in many other insects, JH is essential for vitellogenin uptake by the oocyte. The target cells in the ovary are the cells of the follicular epithelium: the action of JH causes a great reduction in their cellular volumes, and this results in the creation of intercellular channels between the follicular cells (patency), which allows passage of vitellogenin from the hemolymph toward the oocyte surface (Telfer and Woodruff, 2002). At the molecular level, JH acts by binding to an as yet undefined membrane receptor, the subsequent activation of calcium-dependent protein kinase C, and phosphorylation of a polypeptide resembling in size the 100 kDa subunit of Na^+/K^+-ATPase (Sevala and Davey, 1993; Sevala *et al*., 1995). Presumably this leads to a change in the osmotic pressure of the follicular epithelium cells, concomitant shrinkage of their volume, and establishment of patency.

In addition to its action at the membrane level, JH has effects also at the transcription level in the cells of the follicular epithelium. In *B. germanica*, JH stimulates the accumulation of transcripts for the Ca^{2+}-dependent regulatory protein Calmodulin (CAM) in the cells of the follicular epithelium (Iyengar and Kunkel, 1995). The mechanism of action of JH at the level of regulation of gene transcription in the nucleus is unknown at present (Gilbert *et al*., 2000).

In *L. migratoria*, a peptide gonadotropic hormone, called ovary maturating parsin (OMP), has been identified, which is active during the same period of vitellogenesis as JH (Girardie *et al*., 1991; see also **Chapters 1.5** and **3.9**). OMP, however, acts independently of JH, since it is capable of inducing

vitellogenesis in allatectomized animals (Girardie *et al.*, 1996). OMP stimulates vitellogenesis via two distinct hydrophilic domains (Girardie *et al.*, 1998a). On the one hand the C-terminal part presumably acts on ovarian tissue to stimulate ovarian ecdysteroidogenesis, with the produced ecdysteroids subsequently stimulating vitellogenin production by the fat body and concomitant ovarian growth (Girardie and Girardie, 1996; Girardie *et al.*, 1998a). The N-terminal part of OMP, on the other hand, acts on the fat body and stimulates vitellogenin production by increasing its mRNA stability and translatability (Girardie *et al.*, 1998a). The mode of action of either domain of OMP at the molecular level is unknown.

Other peptides derived from neural tissue that may play a role in the regulation of ovarian growth include neuropeptide F-like peptides in locusts and a beetle (Cerstiaens *et al.*, 1999), neuroparsins in locusts (Girardie *et al.*, 1998b; Janssen *et al.*, 2001), and a cAMP-generating peptide in fleshflies (Schoofs *et al.*, 1994). Neuropeptide F-like peptides stimulate ovarian growth and act through GPCRs (Mertens *et al.*, 2002). Neuroparsins, however, have anti-JH effects and are inhibitory to ovarian growth (Girardie *et al.*, 1998b). For both the neuropeptide F-like peptides and the neuroparsins, it is not known whether the ovary is the primary target organ. The cAMP-generating peptide alternatively, has been isolated by its capacity to enhance cAMP production in the ovaries of the flesh fly, *Neobellieria bullata* (Schoofs *et al.*, 1994), but no data exists regarding a correlation between cAMP levels and ovarian differentiation in this species.

Recently, a second type of neuroparsin has been identified in locusts, whose expression in the brain is upregulated during the gonadotropic cycle (Janssen *et al.*, 2001). Because of its expression pattern, this second type of neuroparsin may act as a stimulator, rather than an inhibitor, of ovarian development. It should also be noted that locust neuroparsins show homology to the ovary ecdysteroidogenic hormone (OEH) of the mosquito, which has gonadotropic and ecdysteroidogenic activity on ovarian tissue (Brown *et al.*, 1998; Janssen *et al.*, 2001). As can be hypothesized for mosquito OEH, neuroparsins may function as modulators (negative or positive, dependent on type) of the putative insulin response in locusts.

From the discussion above, it is evident that oogenesis in insects is not regulated by a single, general hormonal mechanism. For all insects, an involvement of at least one of the two major insect morphogenetic hormones, 20E and JH, is apparent. However, the relative contribution of these hormones to the control of oogenesis differs from species to species. Furthermore, the action of 20E or JH in different insect species is exerted on different control points of oogenesis.

More recently, the involvement of neuropeptides in the control of oogenesis could be clearly demonstrated, but again no common "master neuroregulatory circuit" could be identified that is conserved in all insects. This contrasts the situation in vertebrates, where the control of ovarian development is carried out by a conserved set of hormones such as gonadotropins and sex steroids.

1.3.3.2. Ovarian Yolk Proteins

The fat body is the principal site of production of the major yolk protein precursors (YPPs), which are vitellogenin (Vg) in most insects, and yolk peptides (YPs) in cyclorrhaphan (higher) Diptera. It is also the production site of additional YPPs, which are synthesized in a unique fashion in different insect species depending on their life histories and developmental requirements. Remarkably, considering the extreme specialization of this tissue, the fat body of several insects also produces pro-proteases, which are deposited in the developing oocytes. These proteolytic enzymes are involved in the degradation of yolk proteins during embryogenesis.

In most insects, the major protein component of the yolk is vitellin (Vn), the crystallized storage form of Vg. Insect Vgs are large, conjugated proteins. They are translated as primary products with masses of ~200 kDa, which are usually cleaved into subunits (apoproteins) ranging in size from 50 to 180 kDa, although cleavage is not universal (Sappington and Raikhel, 1998b; Maruta *et al.*, 2002; Sappington *et al.*, 2002). Following extensive co- and posttranslational modification, the Vg subunits form high molecular weight oligomeric phospholipoglycoproteins (400–700 kDa), which are secreted into the hemolymph of vitellogenic females (Sappington and Raikhel, 1998b; Tufail *et al.*, 2004). For example, in *P. interpunctella*, the major yolk protein component, Vn, is comprised of two subunits, YP1 ($M_r = 153$ kDa) and YP3 ($M_r = 43$ kDa). It is produced and secreted as Vg by the fat body cells and accumulates in the oocyte during vitellogenesis (Shirk *et al.*, 1984; Bean *et al.*, 1988).

In cyclorrhaphan Diptera such as *Drosophila*, the major YPPs belong to a class of proteins evolutionarily distinct from Vg (Sappington and Raikhel, 1998b; Sappington, 2002), which is not produced at all. Designated "yolk polypeptides" or YPs, they are relatively small proteins (~45 kDa) that are glycosylated, phosphorylated, and sulfated (Bownes and Pathirana, 2002). Interestingly, the YPs of

cyclorrhaphan Diptera are evolutionarily related to *B. mori* ESP and the YP2 subunit of the follicular epithelium yolk protein (FEYP) of pyralid moths, both YPPs that are produced as supplements to Vg (Inagaki and Yamashita 1989; Shirk and Perera 1998; Sappington 2002). They are also homologous to the mammalian triacylglycerol lipase, but lack the functional enzymatic center (Baker and Wolfner, 1988; Bownes *et al.*, 1988b; Terpstra and Ab, 1988; Bownes, 1992).

A number of supplemental proteins have been described that accumulate selectively in developing oocytes. Some of them originate from the fat body and some from the cells of the follicular epithelium. These proteins include both female-specific and bisexually distributed members and, normally, they represent minor components of the yolk. For example, eggs of the stick insect, *Carausius morosus*, contain a minor YP that is secreted by the fat body and sulfated by the follicle cells (Giorgi *et al.*, 1995). However, in a few cases they are almost as abundant as vitellogenin. Thus, in *P. interpunctella*, FEYP, which is comprised of two subunits, YP2 ($M_r = 69$ kDa) and YP4 ($M_r = 33$ kDa), is produced and secreted by the follicular epithelium cells and taken up by the oocyte (Shirk *et al.*, 1984; Bean *et al.*, 1988; see also Section 1.3.3.1.1.2 above). Though not as abundant as Vg, it comprises 25–40% of the yolk protein (Shirk and Perera, 1998). A 30 kDa YPP, called microvitellogenin, is produced by the fat body in *Hyalophora cecropia* and *M. sexta* (Kawooya *et al.*, 1986; Telfer and Pan, 1988; Pan *et al.*, 1994). The fat body of the domesticated silkworm *B. mori* also produces multiple 30 kDa proteins homologous to microvitellogenin (Wang *et al.*, 1989). These proteins are principal components of the hemolymph of both males and females during the late larval and pupal stages of this insect (Izumi *et al.*, 1981). The 30 kDa proteins of *Bombyx* constitute 35% of the egg's total soluble protein, while Vn comprises 40% (Zhu *et al.*, 1986). In *L. migratoria* females, a 21 kDa and a 25 kDa protein are produced by the fat body in synchrony with the synthesis and secretion of Vg (Zhang *et al.*, 1993; Zhang and Wyatt, 1996). These additional YPPs are significant constituents of the yolk in *Locusta* eggs, with the molar concentration of the 21 kDa protein in eggs being close to that of Vn.

Lipids are a critical source of energy during insect embryogenesis (Beenakkers *et al.*, 1985; Van Antwerpen *et al.*, 2004). They represent 30–40% of the insect egg's dry weight (Troy *et al.*, 1975; Kawooya and Law, 1988; Briegel, 1990). Although Vg has a lipid content of 8–15%, its contribution to the total lipid content of the egg apparently is minor (Kawooya *et al.*, 1988; Maruta *et al.*, 2002; Van Antwerpen *et al.*, 2004). The role of lipophorin (Lp), the insect lipid transport protein, in lipid accumulation in insect oocytes is much greater, and was recently reviewed by Van Antwerpen *et al.* (2004). In addition to its role as a lipid carrier, Lp plays a role as a YPP. In saturniid and sphingid moths, Lp is the second most abundant YP behind Vg (Telfer and Pan, 1988). Lipophorin is also present in the yolk of the fall webworm, *Hyphantria cunea* (Yun *et al.*, 1994), and the mosquito *A. aegypti* (Sun *et al.*, 2000).

Biochemical and molecular characterization of two female-specific, fat body-secreted proteins that are deposited in the yolk of *A. aegypti* mosquitoes has revealed that they are proenzymes. The first is a glycosylated 53 kDa protein secreted by the female fat body and accumulated in the developing oocyte in synchrony with Vg (Hays and Raikhel, 1990; Snigirevskaya *et al.*, 1997a). Analysis of the deduced amino acid sequence has shown that it is the proenzyme for a serine carboxypeptidase. This stored proenzyme, vitellogenic carboxypeptidase (VCP), is activated during embryogenesis by cleavage to a 48 kDa polypeptide (Cho *et al.*, 1991). The second protein, a 44 kDa fat body product with sequence similarities to vertebrate cathepsin B, is also incorporated into the yolk of *A. aegypti* (Cho *et al.*, 1999). It is converted to a 42 kDa protein after internalization in the developing oocytes, and further reduced to 33 kDa in developing embryos, coincident with the onset of Vn degradation. The 33 kDa peptide exhibits enzymatic properties of a thiol cathepsin B-like protease, and has been shown to degrade purified mosquito Vn; hence it was named vitellogenic cathepsin B (VCB) (Cho *et al.*, 1999).

An acid cysteine proteinase purified from *B. mori* eggs was subsequently named *Bombyx* cysteine proteinase (BCP). It has a broad substrate specificity and hydrolyzes yolk proteins. The proform of BCP accumulates in the hemolymph, suggesting a fat body origin. Indeed, Northern blot analysis clearly showed that *bcp* mRNA is expressed in the fat body (Kageyama and Takahashi, 1990; Yamahama *et al.*, 2004).

In several studied insects, many other proteins are incorporated into the yolk (Telfer, 2002; Masuda *et al.*, 2004). For example, the iron transport protein transferrin (~65 kDa) is selectively deposited in the yolk of the flesh fly, *Sarcophaga peregrina* (Kurama *et al.*, 1995), and a similar protein is deposited in the bean bug *Riptortus clavatus* (Hirai *et al.*, 2000). The hemolymph and oocytes of *R. prolixus* also contain a 15 kDa heme-binding protein (Oliveira *et al.*, 1995). In Lepidoptera, a blue biliprotein

called insecticyanin, a component of larval hemolymph, is deposited in developing oocytes and is present in mature eggs (Chino *et al.*, 1969; Kang *et al.*, 1995). Biliverdin is a chromophore covalently bound to Vg in *Spodoptera litura*, coloring the eggs green or blue, presumably providing camouflage against green vegetation (Maruta *et al.*, 2002). Finally, an arylphorin-like cyanoprotein, which is a hemolymph storage hexamer, also is deposited in eggs of the bean bug, *R. clavatus*, as a YPP (Chinzei *et al.*, 1990; Miura *et al.*, 1994). For more detailed information on insect yolk proteins and their precursors, see Bownes and Pathirana (2002), Sappington *et al.* (2002), Telfer (2002), Masuda *et al.* (2004), Tufail *et al.* (2004), and Yamahama *et al.* (2004).

1.3.3.2.1. Yolk protein gene transcription factors In *Drosophila*, the gene structures of the three yolk protein genes, *yp1–3*, have been elucidated. *Cis*-regulatory elements were determined in the promoter regions, which are necessary for expression in ovarian follicular cells and in the fat body (Logan and Wensink, 1990; An and Wensink, 1995). Almost all studies have focused on the *yp1/yp2* gene pair whose members are divergently transcribed from a common promoter region. Only very recently has the analysis been extended to the unlinked *yp3* gene (Hutson and Bownes, 2003). To date, most studies have focused on the regulation of these genes in the fat body, with only a limited number of studies addressing their regulation in the ovary.

With regard to expression in the ovarian follicular epithelium cells, genetic analysis in transgenic flies identified two enhancer regions, ovarian enhancer 1 (OE1) and OE2, that are located in the intergenic region and the first exon of *yp2*, respectively (Logan *et al.*, 1989; Logan and Wensink, 1990). The OE regions are distinct from the fat body enhancer (FBE) region, which mediates expression of the yolk protein genes in this tissue (An and Wensink, 1995). The *trans*-acting factors that regulate transcription through OE1 or OE2 have yet to be identified.

Biochemical methods, such as gel retardation assays and *in vitro* transcription assays, have revealed two *trans*-acting factors implicated in the regulation of yolk protein expression in follicular cells. The first factor, yolk protein factor 1 (YPF1), is a homolog of the Ku autoantigen that corresponds to the DNA-binding subunit of DNA-dependent protein kinase (Mitsis and Wensink, 1989; Jacoby and Wensink, 1994). YPF1 binds to a 31 bp sequence located immediately downstream of the *yp1* transcription start site; deletion of this site results in a marked decrease in expression levels of *yp1* mRNA, suggesting that YPF1 is a regulator of *yp1* gene expression (Jacoby and Wensink, 1994). The expression of *ypf1* mRNA is upregulated in egg chambers during vitellogenesis and choriogenesis (Jacoby and Wensink, 1994).

The second factor, dGATAb, belongs to the GATA family of transcriptional activators. An ovary-specific isoform of dGATAb is specifically expressed in the follicular epithelium cells and functions as stimulator of yolk protein gene expression through binding to a palindromic GATA element in the intergenic region of *yp1* and *yp2* (Lossky and Wensink, 1995). It has been proposed that dGATAb is not a stimulatory transcription factor by itself, but rather acts through the formation of partnerships with other proteins, presumably the ones that bind the OE1 and OE2 enhancers (Lossky and Wensink, 1995).

In contrast to the fat body, expression of yolk protein genes in the follicular epithelium cells is not dependent on the sex determination hierarchy (Wolfner, 1988). Experiments using a temperature-sensitive mutation of *transformer-2* (*tra-2*), a component in the sex determination hierarchy, have shown that this hierarchy (see **Chapter 1.1**) functions only during the formation of the ovary. Once the ovarian follicle cells have formed, however, expression of female-specific genes (such as the *yp* genes) in the ovary is regulated by tissue (ovary)-specific factors and is independent of the sex determination hierarchy (Bownes *et al.*, 1990).

In *B. mori*, the orphan nuclear receptor BmHR3A is expressed in the cells of the follicular epithelium of vitellogenic follicles concomitant with the expression of the follicle cell-specific yolk protein ESP (see Section 1.3.3.1.2.1), an analog of *Drosophila* YPs (Sappington, 2002). BmHR3A functions as a transcriptional activator in tissue culture cells and a HR3-binding element has been identified within the *esp* promoter (Eystathioy *et al.*, 2001). It should also be noted that the mosquito AaHR3 is expressed in the ovaries during vitellogenesis together with other transcriptional regulators implicated in the ecdysone regulatory hierarchy. Thus, although functional studies are lacking, the data suggest that HR3 may be a regulator of follicle cell yolk protein gene expression in insects.

1.3.3.2.2. Receptor-mediated endocytosis of yolk proteins in insect oocytes

1.3.3.2.2.1. Early studies of yolk protein uptake in insect oocytes Insect oocytes accumulate enormous amounts of yolk protein precursors that are mainly produced by the fat body. In turn, oocytes are extraordinarily specialized for the specific uptake of these proteins. The first observations related

to the specific accumulation of YPPs were made by Telfer (1960) in the developing oocytes of the silkmoth, *Hyalophora cecropia*, while the first interpretation of endocytosis came from electron microscopic studies of the developing oocytes of the mosquito *A. aegypti* (Roth and Porter, 1964). This latter study revealed the presence of a large number of membrane pits and vesicles in the cortical layer of the oocyte cytoplasm of female mosquitoes after a blood meal. Roth and Porter (1964) described a "bristle layer" on the cytoplasmic side of these vesicles, referred to these vesicles as "coated," and attributed their presence to YPP uptake. They also suggested that coated vesicles carrying YPPs lose their bristle coat, form larger vacuoles, and then grow into "protein droplets," the yolk bodies (**Figure 5**).

Numerous electron microscopic studies of developing oocytes in various insect orders have subsequently shown the presence of these vesicular compartments – coated pits, coated vesicles, and uncoated vesicles – in the oocyte cortex, and studies utilizing electron-dense dyes have confirmed Roth and Porter's hypothesis about the role of coated vesicles in protein uptake (review: Raikhel and Dhadialla, 1992).

Various binding and uptake studies have indicated that Vg is internalized via receptor-mediated endocytosis (RME), and have suggested the presence of specific Vg receptors in the oocytes of *A. aegypti* (Raikhel and Dhadialla, 1992; Sappington *et al.*, 1995), *L. migratoria* (Röhrkasten and Ferenz, 1985; Röhrkasten *et al.*, 1989), *Nauphoeta cinerea* (König and Lanzrein, 1985), *Blattella germanica* (König *et al.*, 1988), *R. prolixus* (Oliveira *et al.*, 1986), *M. sexta* (Osir and Law, 1986), and *H. cecropia* (Kulakosky and Telfer, 1987). However, despite these pioneering studies, the mechanisms of endocytosis have been largely elucidated in other systems, by a combination of animal biochemistry and cell biology, and yeast genetics.

1.3.3.2.2.2. General characteristics of receptor-mediated endocytosis Endocytosis is the cellular process of membrane vesicular transport between the plasma membrane (PM) and cytoplasmic membrane compartments, as well as within the intracellular membrane system. Receptor-mediated endocytosis controls many essential cellular activities, including internalization of essential macromolecules, transmission of neuronal, metabolic and proliferative signals, and defense against invading microorganisms (Goldstein *et al.*, 1985; Bu and Schwartz, 1994).

In RME, an extracellular macromolecule (ligand) is recognized by a specific cell surface receptor. Specific recognition results in high-affinity binding between the ligand and the receptor, which, in turn, triggers endocytosis. The endocytic pathway comprises functionally and physically distinct compartments involved in the internalization of the

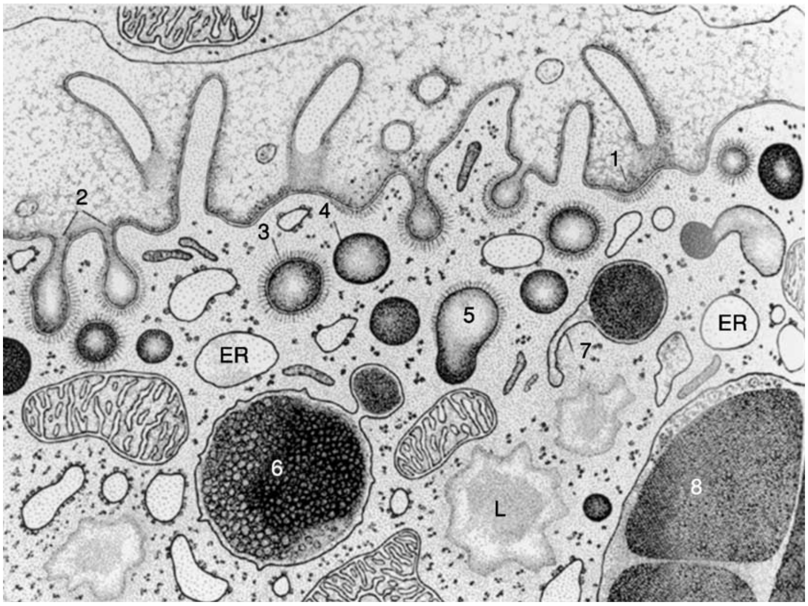


Figure 5 A schematic drawing of protein uptake in the mosquito oocyte: (1) the coated invagination in the plasma membrane of the oocyte; (2) the fully developed coated pit; (3) the coated vesicle; (4) the vesicles after losing their bristles to form dense spheres of similar size; (5) the vesicles fused with other dense spheres; (6) larger droplets coalesced with one another; (7) flattened empty tubular compartment attached to the droplet; (8) larger droplets coalesced to form the large crystalline protein yolk bodies of the oocyte. ER, endoplasmic reticulum; L, lipid droplet. (Reproduced from Roth, T.F., Porter, K.R., **1964**. Yolk protein uptake in the oocytes of the mosquito Aedes aegypti. *J. Cell Biol. 20*, 313–332 by copyright permission of the Rockfeller University Press.)

ligand–receptor complex, the accumulation of ligands, and the recycling of receptors, plasma membrane proteins, and lipids. There are two types of RME, clathrin-dependent and clathrin-independent, defined by the presence or absence of a protein coat on the cytoplasmic side of the vesicular compartments. The main molecular complex of this coat is clathrin (Pearse and Robinson, 1990).

Clathrin-dependent RME, which was first observed in mosquito oocytes, is the major pathway for selective internalization of various macromolecules in insect oocytes through cognate plasma membrane receptors. Goldstein and Brown (1974) were the first to describe RME in mammalian cells and to postulate its general features. In a series of remarkable studies, they characterized the first receptor for RME, the low density lipoprotein (LDL) receptor (LDLR), and identified the pathway for LDL internalization (Goldstein *et al*., 1985). Amongst the other common macromolecular ligands that animal cells internalize by RME, are transferrin (an iron-binding protein), insulin, and most other peptide hormones (Goldstein *et al*., 1979, 1985; Mellman, 1996; Simonsen *et al*., 2001). In insect oocytes, clathrin-dependent RME is responsible for the massive accumulation of Vg and other YPPs (Raikhel and Dhadialla, 1992; Snigirevskaya and Raikhel, 2004). Below, the term RME is used for describing clathrin-dependent RME.

1.3.3.2.2.3. Formation of ligand–receptor complexes, internalization and intracellular fate Receptor–ligand complexes accumulate in coated invaginations of the PM, called clathrin-coated pits (CCPs) (**Figure 6**, A). These invaginations serve to concentrate cargo proteins into the forming vesicles, and provide a mechanical means to deform a membrane into a vesicular bud. During the progressive assembly of clathrin-coated buds and their maturation, the membrane acquires increasing curvature, until a deeply – invaginated coated pit forms (Takei and Haucke, 2001). When this bud matures, it pinches off giving rise to a clathrin-coated vesicle (CCV).

The clathrin coat has been the subject of extensive biochemical characterization (Pearse and Robinson, 1990; Marsh and McMahon, 1999; Brodsky *et al*., 2001). Its basic constituent, the clathrin functional unit, is the triskelion consisting of three 180 kDa clathrin heavy chains (CHCs), each complexed with a 30–35 kDa light chain (CLC) (**Figure 6**, B). When triskelions polymerize, they form a curved structure. It has been calculated that each vesicle consists of

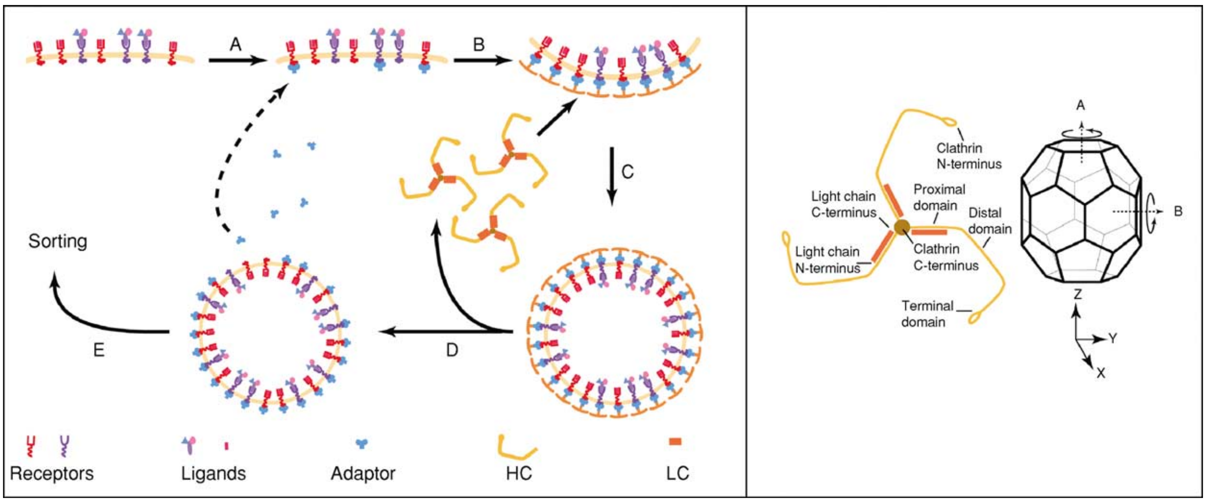


Figure 6 Left panel: early sequential steps of receptor-mediated endocytosis. (A) Receptors are present in the plasma membrane (PM); binding of ligands to their cognate receptors activates recruitment of adaptors that interact with the cytoplasmic domains of receptors. (B) Clathrin triskelions are recruited from the cytoplasmic pool, and assembly of the clathrin lattice on the PM cytoplasmic side is triggered. (C) The clathrin coated vesicle pinches off from the PM and migrates to the cytoplasm. (D) The first step of vesicle uncoating involves detachment of clathrin, which is reutilized for formation of other coated vesicles. In the second step, adaptors are removed and recycled to the PM. (E) The uncoated vesicle fuses with an acceptor compartment, where uncoupling of ligand–receptor complexes and ligand sorting occur. HC, heavy chain of clathrin; LC, light chain of clathrin. (Based on Brodsky, F.M., Chen, C.-Y., Knuehl, C., Towler, M.C., Wakeham, D.E., **2001**. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Devel. Biol. 17*, 517–586.) Right panel: Schematic diagrams of the clathrin triskelion (left) and the clathrin cage (right). Left: Three clathrin heavy chains (CHC) come together to form a triskelion. The clathrin light chains (CLC) are located at the CHC proximal portions that are being connected at their C-termini. CLCs facilitate triskelion formation. Right: Location of the symmetry axes and the asymmetry unit of the hexagonal clathrin cage. The dashed arrows (A and B) mark the sixfold symmetry axes. The *X*, *Y*, and *Z* axes are defined so that they intersect at the center of the barrel (Smith *et al*., 1998).

60 triskelions assembled into 20 hexagons and 12 pentagons to achieve the curvature of a CCP (**Figure 6**).

An insect CHC has been characterized in the mosquito *A. aegypti* (Kokoza and Raikhel, 1997; Kokoza *et al*., 1997). The authors showed that the *chc* gene spans approximately 45 kb, with its coding region divided into seven exons, five of which encode the protein (**Figure 7**). Three distinct mature *chc* transcripts were identified in various mosquito tissues. Two of them are specifically expressed in female germ-line cells encoding isoforms of the CHC polypeptide (M_r of ~191 500) that differ from each other in their NH_2-terminal sequences. The third transcript has a 3′-untranslated region about 1 kb longer than the other variants, and is found only in somatic cells. Tissue-specific 5′-exon splicing and alternative polyadenylation of the primary transcript combine to give rise to these mRNA variants.

The mosquito CHC isoforms are highly conserved, with full-sequence identities of 88% with *D. melanogaster*, 81% with mammal (rat, cow, and human), 71% with *Caenorhabditis elegans*, 58% with *Dictyostelium discoideum*, and 49% with yeast CHC polypeptides. The highest degree of conservation is in the middle portion of the CHC molecule, which includes the linker region and the extended triskelion arm, with decreasing conservation through the N-terminal domain, trimerization domain, and the relatively diverged C-terminal region.

Polyclonal antibodies raised against a bacterially-expressed AaCHC fusion protein recognized one major band of about 180 kDa in whole vitellogenic ovary lysates, while immuno-gold labeling of the AaCHC polypeptide localized it to the coat of CCPs and CCVs of oocytes of vitellogenic follicles (see below). Finally, Northern blot and *in situ* hybridization analyses have suggested that the regulation of *chc* gene expression in the mosquito ovary is complex, and likely involves both developmental and hormonal signals (Kokoza *et al*., 1997).

After internalization, CCVs rapidly lose their clathrin coats and deliver their contents to early endosomes (EEs), where molecular sorting occurs (**Figure 6**). The assembly and uncoating of CCVs are both energy-dependent processes (Takei and Haucke, 2001). Early endosomes represent a dynamic network of tubules and vesicles dispersed throughout the cytoplasm (Geuze *et al*., 1983a, 1983b; Tooze and Hollinshead, 1991; Wilson *et al*., 1991; Gruenberg and Maxfield, 1995; Mukherjee *et al*., 1997). Their slightly acidic pH (6.0–6.8), which is maintained by an ATP-driven proton pump (Al-Awqati, 1986; Mellman, 1996; Sheff *et al*., 2002), facilitates the dissociation of many of the ligands contained in them from their receptors.

Free receptors accumulate in the EE's tubular extensions (see below) and are recycled back to the PM (Gruenberg and Maxfield, 1995; Mellman, 1996). The vesicular portions of EEs containing the dissociated ligands traverse to the perinuclear

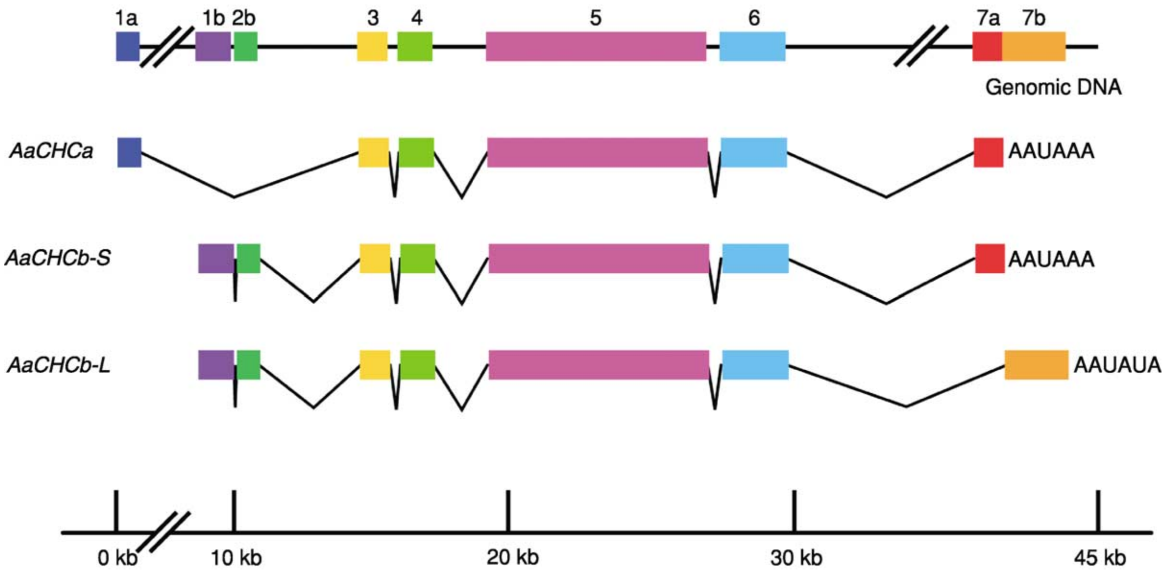


Figure 7 Schematic deduced structure of the *Aedes aegypti chc* gene and its relation to alternatively spliced mRNAs. The *A. aegypti chc* gene is depicted as *boxes* and *lines* representing exons and introns, respectively. Exons are numbered. Three putative transcripts generated from the *A. aegypti chc* gene are shown. Two alternative polyadenylation signals, (AAUAAA) and (AAUAUA), are indicated. (Modified from Kokoza, V.A., Raikhel, A.S., **1997**. Ovarian- and somatic-specific transcripts of the mosquito clathrin heavy chain gene generated by alternative 5′-exon splicing and polyadenylation. *J. Biol. Chem. 272*, 1164–1170, with permission.)

cytoplasm and sort toward late endosomes (LEs) and lysosomes. The LEs accumulate and concentrate internalized contents and partially degraded ligands. From the endosomes, proteins may move to the lysosomes, where they can be degraded by an even lower pH (5.0) and a high concentration of lysosomal enzymes. Some proteins may recycle back to the *trans*-Golgi network or the cell surface for further rounds of transport (Mellman, 1996).

*1.3.3.2.2.4. **Receptor-mediated endocytotic pathways in insect oocytes*** In recent years, many details have been elucidated concerning YPP internalization pathways and specific endocytic compartments in insect oocytes (Raikhel and Dhadialla, 1992; Sappington *et al*., 1995; Sappington and Raikhel, 1995; Snigirevskaya *et al*., 1997a, 1997b; Raikhel and Snigirevskaya, 1998; Snigirevskaya and Raikhel, 2004). These studies have revealed that, in general, the sequential steps of RME in oocytes are the same in different insect species. Furthermore, the cellular organelles involved in oocyte RME are similar to those of somatic cells. However, unique features of RME in developing oocytes include an extraordinarily high level of endocytotic organelles in the oocyte cortex, as well as the final routing of internalized YPPs to specialized accumulation organelles, the mature yolk bodies (MYBs), in which YPPs are only minimally processed and are stored until the onset of embryonic development (Telfer *et al*., 1982; Raikhel, 1984; Raikhel and Dhadialla, 1992; Opresko and Wiley, 1987; Giorgi *et al*., 1993a; Raikhel and Snigirevskaya, 1998; Warrier and Subramoniam, 2002; Snigirevskaya and Raikhel, 2004).

1.3.3.2.2.4.1. Receptor-mediated endocytotic pathway of vitellogenin Most studies have been devoted to the internalization of the major YPP, Vg. Studies, at the electron microscopy (EM) level, particularly those that utilized electron dense tracers, have provided the foundation for our understanding of the ultrastructural morphology underlying the RME of YPPs in insect oocytes (see Telfer *et al*., 1982; Raikhel and Dhadialla, 1992).

The purification of Vgs from different insects facilitated the introduction of immunocytochemical methods at the ultrastructural level. A detailed immunocytochemical study was first conducted using developing oocytes of the mosquito *A. aegypti* (Raikhel, 1984). It was shown that Vg binds only along the lower third of oocyte microvilli and between them, suggesting the presence of Vg-specific receptors (VgRs) in these regions of the ooplasm. In adddition, Vg was localized in the organelles of the endocytotic pathway, the CCPs, CCVs, and uncoated vesicular compartments (Raikhel, 1984).

Vitellogenin receptors were purified from the oocytes of *L. migratoria* (Ferenz, 1993) and *A. aegypti* (Sappington *et al*., 1995), and specific anti-VgR antibodies were raised against them. Vitellogenin receptors have been localized subsequently in the oocyte CCPs and CCVs of these insects (Ferenz, 1993; Snigirevskaya *et al*., 1997a). A similar localization of VgR has been described in other oviparous animals, including the chicken *Gallus gallus* and the crab *Scylla serrata* (Schneider, 1992; Warrier and Subramoniam, 2002).

A detailed EM immunocytochemical study of mosquito oocytes has demonstrated the co-localization of Vg and VgR in compartments of the endocytotic pathway (Snigirevskaya *et al*., 1997a). Following initiation of vitellogenesis, the intracellular spaces in the follicular epithelium dilate, facilitating access of macromolecules to the surface of the oocyte. Vitellogenin and its receptor co-localized to specific microdomains at the base of the oocyte microvilli (Snigirevskaya *et al*., 1997a). They were also present in the same compartments of the endocytotic pathway, the CCPs, CCVs, uncoated small vesicular compartments, and EEs (**Figure 8**). Both Vg and VgR were found close to the membrane of these organelles, suggesting that Vg was still bound to its receptors at these stages of internalization (**Figure 8a**).

Uncoated EEs have been observed fusing with one another as well as with larger vesicular compartments, called transitional yolk bodies (TYBs). However, in the TYBs, Vg is localized predominantly in the lumen. Remarkably, numerous tubular compartments are connected to the TYBs, which also are labeled positively with anti-VgR antibodies but not with anti-Vg ones (Snigirevskaya *et al*., 1997a). The differential localization of the ligand and its receptor clearly indicates that the TYB is the organelle for ligand–receptor dissociation, accumulation of Vg, and receptor recycling via tubular compartments (**Figure 8b**). Similar tubular compartments were observed previously in developing vitellogenic insect oocytes (Telfer *et al*., 1982; Raikhel, 1984 Van Antwerpen *et al*., 1993), but the role of these compartments remained unclear, until the direct EM immunolabeling studies with anti-VgR antibodies were conducted with the *A. aegypti* oocytes (Snigirevskaya *et al*., 1997a).

The TYBs are simultaneously in contact with both Vg-containing EEs and VgR-containing tubular compartments. Thus, these data clearly demonstrate that the dissociation of VgR and Vg occurs in the TYBs during coalescence with the EEs, which serve as sorting compartments in somatic cells.

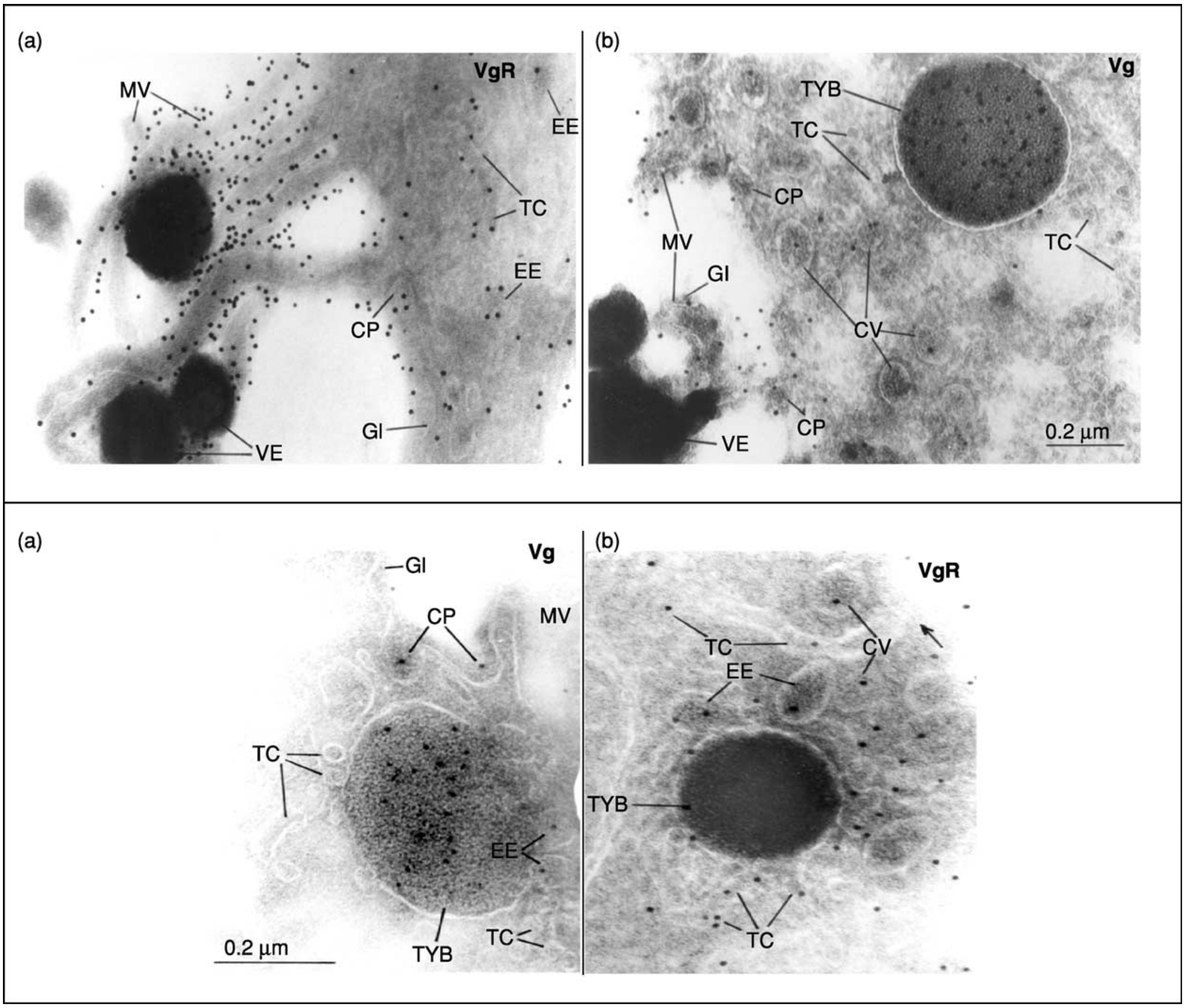


Figure 8 Internalization of vitellogenin (Vg) in the mosquito oocyte visualized through labeling of Vg and vitellogenin receptor (VgR) in ultra-thin frozen sections with 10 nm colloidal gold. Upper panel: The early stages of internalization. (a) Anti-Vg antibodies and protein-gold immunocytochemistry reveals the presence of Vg on the ooplasm membrane at the base of the microvilli (MV), in coated pits (CP), coated vesicles (CV), and the transitional yolk body (TYB), but not in the tubular compartments (TC). (b) Localization of VgR by anti-VgR antibodies and protein-gold immunocytochemistry shows its abundant presence in the oocyte microvillus membrane, CPs, early endosomes (EE), and TCs. Lower panel: The late stages of receptor-mediated endocytosis of vitellogenin in the mosquito oocyte. (a) Anti-Vg antibodies and protein-gold immunocytochemistry reveals the presence of Vg in the lumen of the transitional yolk body (TYB), but not in the tubular compartments (TC). (b) Localization of VgR by anti-VgR antibodies and protein-gold immunocytochemistry shows its abundant presence in TCs. However, in the TYB, VgR label can be found only in TYB membrane. CP, coated pit; EE, early endosome; GL, glycocalyx (surface extracellular coat); MV, microvillus; VE, plaques of the forming vitelline envelope. Scale bar = 0.2 µm. (From Snigirevskaya, E.S., Sappington, T.W., Raikhel, A.S., **1997b**. Internalization and recycling of vitellogenin receptor in the mosquito oocyte. *Cell Tissue Res. 290*, 175–183, with permission.)

Eventually, the TYBs are transformed into mature yolk bodies (MYBs), where vitellin, the crystallized storage form of Vg, is stored (Snigirevskaya *et al*., 1997a, 1997b).

*1.3.3.2.2.5. **Accumulation of nonvitellogenin yolk proteins*** Several yolk protein precursors (YPPs) are deposited in developing insect oocytes. In addition, some other female-specific proteins are important for the development of insect oocytes. However, detailed analysis of their internalization is limited.

1.3.3.2.2.5.1. RME of YPs in Drosophila As mentioned earlier, the YPs of cyclorrhaphan Diptera are small (~45 kDa) glycosylated, phosphorylated, and sulfated (Bownes and Pathirana, 2002). Using the *Drosophila* mutant *yolkless* (*yl*), the gene encoding the YP receptor (Yl) has been cloned and characterized (Schonbaum *et al*., 1995; Bownes and Pathirana, 2002). In wild-type vitellogenic oocytes, the receptor is located at the cortex of the egg, and YPs accumulate through the CCPs (Schonbaum *et al*., 2000).

1.3.3.2.2.5.2. Uptake of lipophorin Lipophorin (Lp) serves as a lipid shuttle between the fat body and the developing oocyte, and in some insects, such as lepidopterans and mosquitoes, is sequestered in the yolk (Kawooya and Law 1988; Sun *et al*., 2000; Telfer, 2002; Van Antwerpen *et al*., 2004). Competition experiments with cultured *M. sexta* ovaries indicate that Vg and Lp are internalized by two different receptors as well (Osir and Law, 1986; Kawooya *et al*., 1988). In *H. cecropia*, however, evidence suggests that only a single receptor is involved in endocytosis of these two ligands (Kulakosky and Telfer, 1990). The Lp receptor (LpR) has been cloned and sequenced from both the locust, *L. migratoria* (Dantuma *et al*., 1999) and the mosquito, *A. aegypti* (Cheon *et al*., 2001; Seo *et al*., 2003).

Transient expression studies using vertebrate cell lines demonstrated that the *Locusta* receptor is capable of mediating the endocytic uptake of the *Locusta* high density lipopoprotein (HDLp), lipophorin (Lp). Sun *et al*. (2000) and Cheon *et al*. (2001) have demonstrated direct binding of mosquito Lp to the LpR. It is uncertain, however, how much of the lipid content of *L. migratoria* and *A. aegypti* eggs is derived from receptor-mediated uptake of Lp by the oocyte. A preliminary estimate of the amount of Lp in *Aedes* oocytes indicates that the contribution of internalized Lp to the lipid reserves of the oocyte is limited (Sun *et al*., 2000), suggesting that an additional mechanism exists for lipid delivery to the oocyte. Van Antwerpen *et al*. (1993) conducted a detailed analysis of developing *M. sexta* oocytes with respect to the endocytic pathway for protein incorporation. Three major (lipo)protein components of the mature eggs of this insect, Lp, Vg, and microvitellogenin, were localized along this pathway by immuno-fluorescence and immuno-gold labeling techniques. Labeling of the antigens was initially observed in the extracellular space of the follicle, and the three YPPs have been detected in CCPs and CCVs near the plasma membrane of the oocyte. All three YPPs were observed subsequently in endosomes in the cortex of the oocyte.

The morphology and labeling patterns of the endosomes indicate that this organelle represents the compartment where uncoupling of the receptor from the ligand takes place. In contrast, tubular elements at the surface of the endosome, interpreted to be involved in the recycling of receptors and membranes to the oocyte surface, were not labeled. Strong labeling of Lp, Vg, and microvitellogenin was observed in the developing yolk bodies, the main protein storage compartment of the oocyte. A similar study conducted on *Aedes* oocytes indicates that Vg and Lp are internalized and trafficked together through the same RME pathway (E.S. Snigirevskaya and A.S. Raikhel, unpublished data). Interestingly, both lipoproteins co-localize in the crystalline portion of MYBs.

1.3.3.2.2.5.3. RME of proenzymes serving as YPPs Localization studies of the female-specific YPPs, vitellogenic carboxypeptidase (VCP) and vitellogenic cathepsin B (VCB), in developing mosquito oocytes, have shown that the internalization of these proteins is performed through the same endocytic pathway as Vg. All three YPPs are found on the oocyte microvillus membrane, in CCPs, CCVs, and EEs, and are also co-localized in TYBs (Snigirevskaya *et al*., 1997b). The study, which was based on immuno-gold EM of the developing oocytes of *A. aegypti*, revealed the co-localization of VCP and VCB in most compartments of the RME pathway, from CCPs to TYBs (Snigirevskaya *et al*., 1997a, 1997b). However, VCP and VCB co-localize in the noncrystalline matrix surrounding the compartment where Vn and Lp accumulate (**Figure 9**).

The VCP and VCB proteins are precursors of enzymes participating in yolk degradation during embryogenesis (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1995). Mature yolk granules can be viewed as a delayed lysosomal compartment particular to oocytes (Wall and Meleka, 1985; Fagotto, 1995). However, in contrast to somatic cells, oocytes do not contain typical lysosomes. In somatic cells, lysosomal enzymes (hydrolases) are delivered from the Golgi complex and initiate the degradation of most ligands in LEs (Gruenberg and Maxfield, 1995; Mellman, 1996; Mukherjee *et al*., 1997). However, in the oocytes of some animals, the presence of enzymes in yolk granules has been described. The enzymes include β-N-acetylglucosaminidase in *Xenopus* (Wall and Meleka, 1985), and serine proteases and cathepsin B and L in *Drosophila* and *Bombyx* (Indrasith *et al*., 1988; Medina *et al*., 1988; Medina and Vallejo, 1989). Giorgi *et al*. (1993b) have suggested that Golgi-derived vesicles deliver hydrolytic enzymes from the Golgi complex to the yolk bodies.

Thus far, the screening of mosquito ovary cDNA libraries has revealed only two RME receptors, VgR and LpR. Ligand-overlay experiments have suggested that VgR is highly specific for Vg, while LpR binds Lp, VCP, and VCB. While binding of Lp occurs by itself, binding of VCP and VCB requires the presence of Lp. Moreover, in co-immunoprecipitation experiments, both VCP and VCB bind Lp (X. Wu, T. Hiraoka, and A.S. Raikhel, unpublished data). This suggests that Lp serves as a carrier for VCP

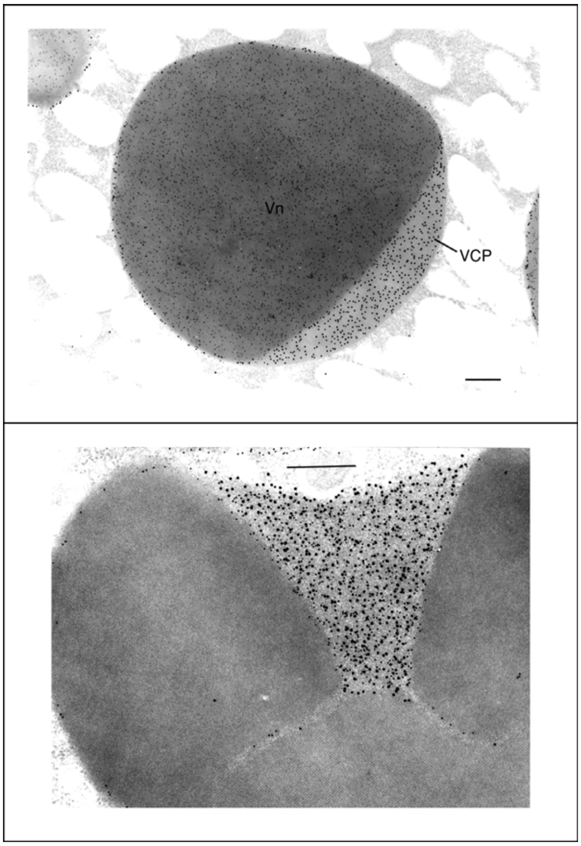


Figure 9 Upper panel: Localization of yolk proteins (vitellin and two processing proteases) in oocyte mature yolk bodies from mosquito females. Double immunolabeling of vitellin (Vn; 10 nm gold particle) and vitellogenic carboxypeptidase (VCP; 15 nm particle) in the mature yolk body of an oocyte from a female mosquito at 24 h post-blood meal. Note the differential localization of these two yolk proteins. Lower panel: Double immunolabeling of vitellogenic carboxypeptidase (VCP; 10 nm gold) and vitellogenic cathepsin B (VCB; 15 nm gold) in the mature yolk body of an oocyte from a female mosquito at 24 h post-blood meal. Note that both these yolk proteins are co-localized in the noncrystalline matrix of the mature yolk body. Bar: 0.5 μm. (Reproduced from Snigirevskaya, E.S., Hays, A.R., Raikhel, A.S., **1997a**. Secretory and internalization pathways of mosquito yolk protein precursors. *Cell Tissue Res. 290*, 129–142, with permission.)

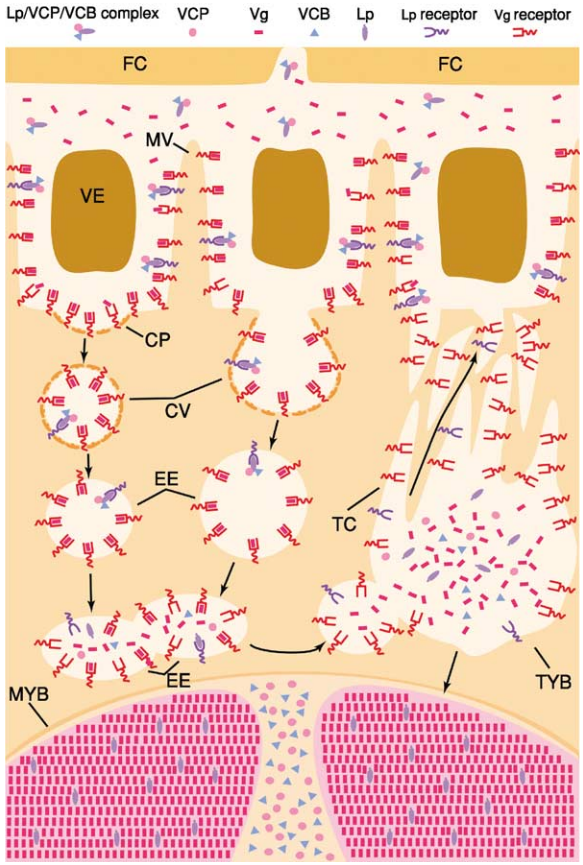


Figure 10 Schematic interpretation of the endocytic pathways and subsequent routing of Vg and VgR, Lp and LpR, VCP and VCB during the internalization of yolk proteins by mosquito oocytes. Ligand–receptor complexes cluster in clathrin-coated pits (CPs) of the vitelline envelope (VE), which invaginate into the cytoplasm and pinch off to form coated vesicles (CVs). VgR binds Vg exclusively, while LpR binds Lp, which presumably transports VCP and VCB via a piggyback mechanism. These complexes are internalized via the same CVs. Upon losing their clathrin coat, the CVs are transformed into early endosomes (EEs), which fuse with one another to form the late endosomes or transitional yolk bodies (TYBs). Early endosomes contain Vg/VgR and Lp/LpR, whereas segregation of the Vg and VgR occurs in the TYBs. Likewise, similar segregation of Lp and LpR is observed. The lumen of the TYB contains four yolk proteins: Vg, Lp, VCP, and VCB. Presumably, Lp, VCP, and VCB are dissociated at this point. VgR and LpR are present only in numerous tubular compartments, which are connected to the TYB and also observed in contact with the ooplasm. The mature yolk bodies (MYBs) contain two parts: the crystalline yolk, composed of modified Vg (vitellin-Vn), and Lp and the non-crystalline matrix, which surrounds Vn/Lp crystals and contains VCP and VCB. (Based on Snigirevskaya *et al*., 1997a, 1997b; E.S. Snigirevskaya and A.S. Raikhel, unpublished data.)

and VCB, and that internalization of these YPPs most likely occurs via a "piggyback" mechanism. The overall picture that has emerged from studies of RME trafficking of YPPs in the mosquito oocytes is presented in Figure 10.

1.3.3.2.3. Oocyte receptors for yolk protein endocytosis

1.3.3.2.3.1. Insect yolk protein receptors Several biochemical studies preceded the cloning and molecular characterization of vitellogenin receptors (VgRs) (reviews: Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998b). The *L. migratoria* VgR has not been cloned, but its size of 180 kDa was determined on SDS gels (Hafer and Ferenz, 1991). By way of comparison, the mosquito VgR was shown to have a mass of 205 kDa using the ligand-overlay technique (Dhadialla *et al*., 1992; Sappington and Raikhel, 1995).

As of the writing of this chapter, there are only three insects from which cloned VgR sequences have been reported, the yellow fever mosquito, *A. aegypti* (Sappington *et al.*, 1996), the fire ant, *Solenopsis invicta* (Chen *et al.*, 2004), and the cockroach, *P. americana* (Acc. no. BAC02725). A database search of the recently published genome sequence of the malaria mosquito, *Anopheles gambiae* (Holt *et al.*, 2002), yielded a predicted amino acid sequence (Acc. no. EAA06264) with 54% identity to the *A. aegypti* VgR sequence. This will be referred to here as the *A. gambiae* VgR (Sappington and Raikhel, 2004).

There is no evidence for Vg production or Vg genes in cyclorrhaphan flies like *Drosophila*, which instead utilize smaller unrelated YPs as their major yolk proteins (Bownes and Pathirana, 2002). The YP receptor in *D. melanogaster* was identified and cloned by taking advantage of the *yolkless* (*yl*) mutation (Schonbaum *et al.*, 1995), and is referred to as the yolkless protein, Yl. Despite their unrelated ligands, the Yl shares high sequence identity with insect VgRs (Sappington *et al.*, 1996; Sappington and Raikhel, 2004), and polyclonal antibodies raised to *A. aegypti* VgR recognize the *D. melanogaster* Yl (Richard *et al.*, 2001) indicating similar tertiary structures.

Dantuma *et al.* (1999) cloned the *L. migratoria* Lp receptor (LpR) and determined that it is a member of the LDL receptor (LDLR) family. The locust LpR is expressed primarily in the fat body, but it is also present in the ovary. The cDNA for a similar lipoprotein receptor (LpR), which is likely responsible for the uptake of Lp by *A. aegypti* oocytes (Sun *et al.*, 2000), has been cloned by Cheon *et al.* (2001). Two splice variants of *LpR* mRNA have been identified that give rise to AaLpRfb and AaLpRov, which are specific for the fat body and the ovary, respectively (Seo *et al.*, 2003). In addition to recognizing Yl, anti-*Aedes* VgR polyclonal antibodies recognize an abundant 80 kDa protein in *D. melanogaster* ovaries, which possibly represents an ovarian LpR, in addition to Yl (Richard *et al.*, 2001). Genes encoding putative LpRs also have been identified in the *Drosophila* genome (Culi and Mann, 2003). Thus, two distinct receptors appear to exist for YPs and Lp in this species.

1.3.3.2.3.2. The superfamily of low-density lipoprotein receptors The insect VgRs, Yl, and LpRs are all members of the low-density lipoprotein receptor (LDLR) superfamily, named after the LDLR first described in humans (Yamamoto *et al.*, 1984). LDLR-superfamily members are characterized by various arrangements of five structural domains (**Figure 11**): (1) *class A complement-type imperfect repeats*, often referred to as ligand-binding repeats (LRs), each containing six cysteine residues that form an invariant pattern of disulfide bonds (Bieri *et al.*, 1995a, 1955b); (2) *class B EGF-type imperfect repeats*, also containing six cysteine residues, which, however, form a pattern of disulfide bonding different than that of the cysteines in the class A repeats (Campbell and Bork, 1993); (3) *YWXD imperfect repeats*, invariably occurring in groups of six, which fold to form a compact 6-bladed β-propeller structure (Springer, 1998; Jeon *et al.*, 2001) that is involved in acid dissociation of the receptor and its ligand in the endosome (Innerarity, 2002) – this domain is often considered part of a larger EGF precursor homology domain, which includes the class B repeats (Nimpf and Schneider, 1998; Hussain *et al.*, 1999); (4) a *single-pass transmembrane domain* near the C-terminus; and (5) a *cytoplasmic tail* that includes one or more tyrosine or dileucine-based internalization signals. In addition, there is often an extracellular *O*-linked sugar region proximal to the membrane, and in some cases, the presence or absence of this domain is determined by differential splicing (e.g., Bujo *et al.*, 1995). Besides the presence of these domains, Hussain *et al.* (1999) list other characteristics of LDLR-superfamily members, including cell-surface expression, a requirement of Ca^{2+} for ligand binding, and internalization of ligands by receptor-mediated endocytosis.

One of the most striking structural characteristics noticed when comparing LDLR-superfamily members, is the clustering of class A modules into 1–4 distinct ligand-binding domains, with a characteristic number of modules per domain (**Figure 11**). Complete VgR sequences are known from five species of vertebrates (Bujo *et al.*, 1994; Okabayashi *et al.*, 1996; Davail *et al.*, 1998; Li *et al.*, 2003; Hiramatsu *et al.*, 2004), and are characterized by a single ligand-binding domain containing eight class A modules. Similarly, the mosquito ovarian LpR (AaLRov) has eight class A modules in a single cluster (Cheon *et al.*, 2001). Alternatively, the fat body-specific LpR isoform (AaLpRfb) consists of seven complement-type, cysteine-rich repeats in its putative ligand-binding domain, which are identical to the second through eighth repeats of AaLpRov. The *AaLpRov* transcripts are present exclusively in ovarian germ-line cells, nurse cells, and oocytes, while the *AaLpRfb* transcripts are fat body-specific (Seo *et al.*, 2003). The differences between these two isoforms suggests that they internalize Lps with different apo-Lp compositions.

Finally, insect VgRs/Yl have two clusters of class A modules (**Figure 11**). The first cluster contains five modules in all except for the *S. invicta* VgR, which only has four. The second cluster contains

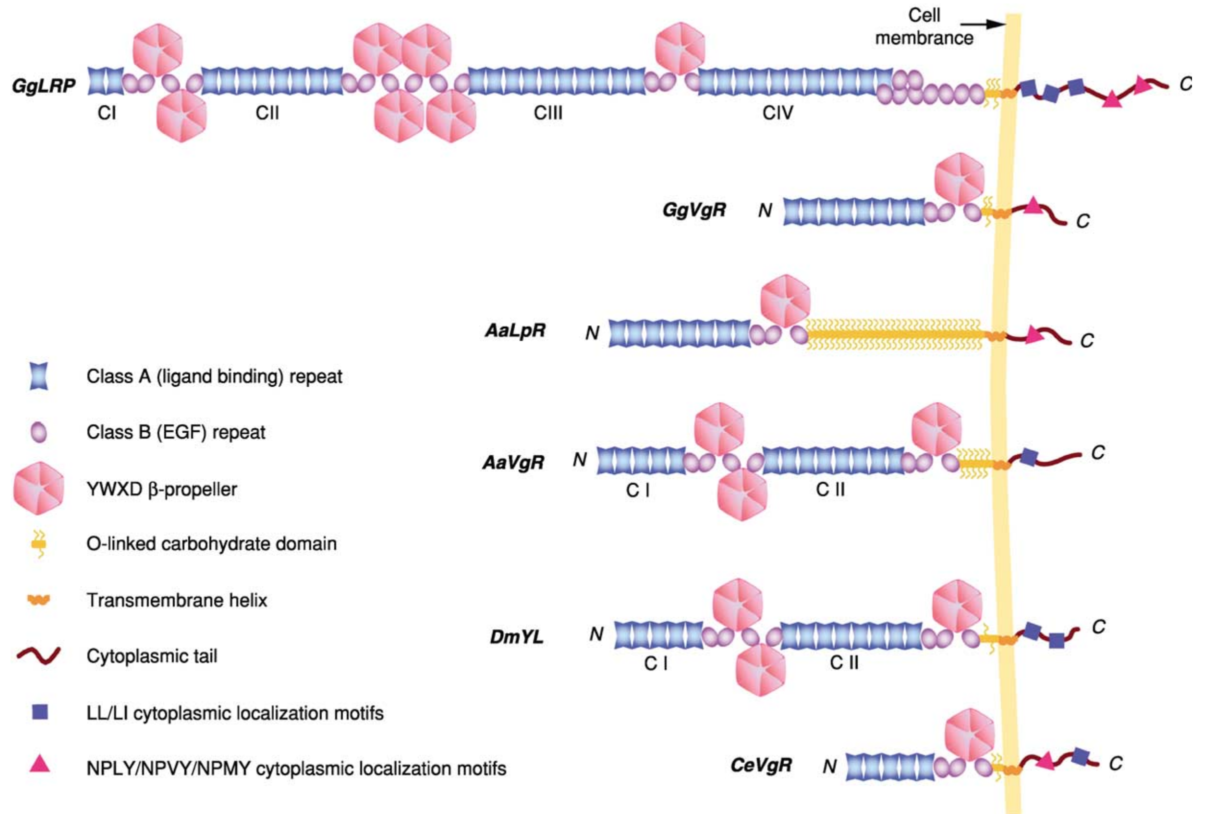


Figure 11 Schematic illustration of receptors of the LDLR superfamily, which are known to internalize yolk protein precursors into oocytes, emphasizing the arrangement of various modular domains. The C-terminus of each receptor is located on the cytoplasmic side of the cell membrane, while the N-terminus is extracellular. CI, first cluster of class A (ligand-binding) repeats in multi-cluster receptors; CII, second cluster, etc. The relative locations of tyrosine-based (NPXY) or dileucine-based (LL or LI) internalization signals in the cytoplasmic tail are also indicated. *GgLRP*, chicken (*Gallus gallus*) LDLR-related protein; *GgVgR*, chicken (*G. gallus*) vitellogenin receptor; *AaLPR*, mosquito (*Aedes aegypti*) lipophorin receptor; *AaVgR*, mosquito (*A. aegypti*) vitellogenin receptor; *DmYl*, fruit fly (*Drosophila melanogaster*) yolkless protein (or YP receptor); *CeVgR*, nematode (*Caenorhabditis elegans*) vitellogenin receptor. (Modified from Sappington, T.W., Raikhel, A.S., **2004**. Insect vitellogenin/yolk protein receptors. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 229–264.)

either eight (*A. aegypti* VgR and *D. melanogaster* Yl) or seven repeats (*P. americana* and *A. gambiae* VgRs). A search of the *D. melanogaster* genome (Adams *et al*., 2000) revealed the presence of seven LDLR superfamily members (Culi and Mann, 2003), including homologs of the very large LDLR-Related Protein-2 receptors of vertebrates (Herz and Bock, 2002). The role of these large receptors in insects is unknown. For a more detailed analysis of insect RME receptors, the characteristics of their domains and their functional relevance, the reader is referred to a recent review by Sappington and Raikhel (2004).

1.3.3.2.4. Concluding remarks In addition to the physiological and functional importance of yolk accumulation in the oocyte to any reproducing insect, the processes and proteins involved are fundamental to many aspects of cell biology in animals in general. For example, RME of macromolecules is a ubiquitous process of eukaryotic cells (Goldstein *et al*., 1979; Schwartz, 1995). Insect Vgs are related to the Vgs of vertebrates and nematodes (Chen *et al*., 1997), and belong to a yet larger superfamily of proteins called large lipid transfer proteins (LLTP) (Babin *et al*., 1999). The apolipoprotein component of vertebrate LDL, a molecule involved in cholesterol homeostasis (Brown and Goldstein, 1986), is an LLTP (Byrne *et al*., 1989; Chen *et al*., 1997). Insect VgRs, Yl, and LpRs are related to vertebrate and nematode VgRs, and all belong to the larger LDLR superfamily, which bind a variety of ligands including LLTPs (Sappington and Raikhel, 1998a, 1998b, 2004; Babin *et al*., 1999). The YPs of *Drosophila* are related to lipoprotein lipases (Bownes *et al*., 1988b), a ligand of certain vertebrate LDLR superfamily receptors (Beisiegel, 1996). Thus, the evolutionary connections among these proteins in widely divergent animal taxa are pervasive. The importance of this cannot be overemphasized, because the commonalities in these systems

make studies on the mechanisms of insect yolk accumulation relevant to mechanisms of cholesterol homeostasis and LDLR-based genetic diseases in humans (Gliemann, 1998; Herz and Farese, 1999; Hussain *et al.*, 1999; Willnow, 1999; Nykjaer and Willnow, 2002; Strickland *et al.*, 2002), and *vice versa*. Thus, those working in these normally disjunct fields of research have much to offer and learn from one another (Sappington and Raikhel 1998b, 2004; Sappington *et al.*, 2002).

1.3.3.3. The Transition from Vitellogenesis to Choriogenesis

At the end of vitellogenesis, the cells of the follicular epithelium lose their patency, and this results in a cessation of yolk protein transport to the oocyte surface (Wang and Telfer, 1996). Concurrently, gene expression in the cells of the follicular epithelium switches from yolk protein gene transcription to the activation of eggshell or chorion protein-encoding genes (Kafatos *et al.*, 1977). Prior to choriogenesis, the nurse cells also empty their cytoplasmic contents into the oocyte and undergo apoptosis (Mahajan-Niklos and Cooley, 1994).

During the vitellogenic stages that precede the onset of choriogenesis, other important events occur in follicles, including the synthesis of vitelline membrane proteins and ovarian (maternal) ecdysteroids by the cells of the follicular epithelium (Kadono-Okuda *et al.*, 1994; Kendirgi *et al.*, 2002), the reinitiation of meiosis in the oocytes (Lanot *et al.*, 1990), and morphogenetic movements of the follicular epithelium prior to their commitment to chorion protein gene expression (Spradling, 1993; Montell, 2001).

1.3.3.3.1. Diptera As is the case for the transition from previtellogenesis to vitellogenesis, most of the information regarding the transition from vitellogenesis to choriogenesis comes from the two major insect models, the fruit fly *D. melanogaster*, and the mosquito *A. aegypti*.

***1.3.3.3.1.1.* Drosophila melanogaster** Particular elements of the process of transition from vitellogenesis to choriogenesis, such as the patterning and movements of the follicular epithelium and the nurse cell dumping process, have been analyzed in detail by genetic methods in *Drosophila*. These processes will be discussed briefly in Sections 1.3.3.6 and 1.3.3.7, respectively, and are also reviewed in more detail in **Chapter 1.2**.

No detailed *in vitro* culture experiments of *Drosophila* ovarian follicles have been carried out to determine whether the transition from vitellogenesis to choriogenesis occurs as a follicle-autonomous process or whether it requires the presence of extra-ovarian factors (see also discussion in Section 1.3.3.3.2.1). Detailed measurements of ecdysteroid levels with respect to follicle development have also not been carried out in order to deduce whether the transition to choriogenesis is accompanied by a decline of ecdysteroid production in the cells of the follicular epithelium (see also discussion for *A. aegypti* and *B. mori* in Sections 1.3.3.3.1.2 and 1.3.3.3.2.1, respectively). Furthermore, genetic analysis has yet to reveal the components of a "master" regulatory pathway that governs the transition from vitellogenesis to choriogenesis in *Drosophila*. Therefore, the mechanism by which the cells of the follicular epithelium differentiate from a patent state that is involved in the synthesis of yolk proteins and allows free passage of hemolymph yolk proteins to the oocyte surface, to one that becomes specialized for chorion protein production, remains unknown in *Drosophila*.

1.3.3.3.1.2.* Aedes aegypti *and anautogenous flies In *A. aegypti*, the transition from vitellogenesis to choriogenesis is accompanied by a decline in ecdysteroid levels (Hagedorn, 1985). A decline in the abundance of the mRNAs of nuclear receptors involved in the ecdysone regulatory pathway, i.e., *AaEcR*, *AaUSP*, *AaE74B*, *AaE75A/B/C*, *AHR3*, also occurs in the ovary at the time of termination of vitellogenesis (Cho *et al.*, 1995; Pierceall *et al.*, 1999; Kapitskaya *et al.*, 2000; Wang *et al.*, 2000; Sun *et al.*, 2002). Also, the levels of the nuclear receptor AaHNF4b are down-regulated at this transition (Kapitskaya *et al.*, 2000). By contrast, the levels of *AaE74A* mRNA increase at the end of vitellogenesis and during choriogenesis (Sun *et al.*, 2002). The changes in the expression levels of the above factors suggest their involvement in the regulation of the transition from vitellogenesis to choriogenesis. However, as has been discussed in Section 1.3.3.1.1.2, it is not clear whether the expression levels of the above nuclear receptors in ovarian tissue are under control of the ecdysone regulatory pathway.

In the mosquito, a peptide hormone involved in the termination of vitellogenesis, trypsin-modulating oostatic factor (TMOF), is produced during vitellogenesis by the cells of the follicular epithelium (Borovsky *et al.*, 1990, 1994b). The target of TMOF is the gut, where it inhibits biosynthesis of trypsin and stops the breakdown of blood proteins that are essential for vitellogenesis (Borovsky *et al.*, 1990, 1994a, 1994b) (see also **Chapters 1.5** and **3.9**). Thus, the action of TMOF blocks the availability of nutrients necessary for

continuation of vitellogenesis. Because TMOF is produced by the cells of the follicular epithelium, a model emerges, in which ovarian follicles produce TMOF upon reaching a certain size, in order to prevent further growth and initiate post-vitellogenic events (De Loof *et al.*, 1995).

A functional analog of TMOF (Neb-TMOF) has also been isolated from *Neobellieria* (*Sarcophaga*) *bullata*, a fly that requires a protein meal to initiate vitellogenesis (anautogenous flies; Huybrechts and De Loof, 1977). Neb-TMOF is produced by vitellogenic follicles but, in this case, the oocyte rather than the cells of the follicular epithelium is the major source of the peptide (Bylemans *et al.*, 1996). The effects of Neb-TMOF include inhibition of trypsin biosynthesis in the gut and concomitant lowering of yolk protein concentration in the hemolymph (Bylemans *et al.*, 1994). A second oostatic factor or folliculostatin of *Neobellieria*, Neb-colloostatin, however, does not inhibit the synthesis of trypsin-like molecules in the gut and does not terminate viellogenesis in vitellogenic follicles. Instead, the peptide prevents previtellogenic follicles from entering vitellogenesis, and may be involved in the feedback mechanism that blocks vitellogenic growth of the penultimate oocyte before completion of vitellogenesis in the terminal follicle (Bylemans *et al.*, 1995).

In the female blowfly, *Phormia regina*, ovarian ecdysteroidogenesis is regulated by antagonistic activities of cAMP (positive regulation) and cGMP (negative regulation) second messengers (Manière *et al.*, 2000, 2003). Calcium signaling is also involved, since calcium ions inhibit steroidogenesis, presumably by activating a calmodulin-sensitive (type I) phosphodiesterase (Manière *et al.*, 2002). Cyclic GMP levels in the blowfly ovary reach their maximal levels at the end of vitellogenesis, in close association with a decrease in ecdysteroidogenesis. Thus, cGMP may signal the end of vitellogenesis through inhibition of ovarian ecdysone production and concomitant inhibition of ecdysone-regulated yolk protein production. The regulation of cGMP levels does not seem to be dependent on extra-follicular factors but may involve paracrine or autocrine factors, possibly nitric oxide (Manière *et al.*, 2003).

1.3.3.3.2. Lepidoptera Information regarding the regulation of the transition from vitellogenesis to choriogenesis in lepidopteran insects has been derived almost exclusively from one species, the domesticated silkmoth, *B. mori*.

***1.3.3.3.2.1.* Bombyx mori** The developing ovariole of the silkmoth, *B. mori*, represents an excellent system for the study of changes in gene expression during follicle development (Swevers and Iatrou, 2003). Because of the asynchronous nature of oogenesis (**Figure 12**), follicles at all different stages of development are simultaneously present and arranged in a linear array in each of the animal's 8 ovarioles (Bock *et al.*, 1986; Swevers and Iatrou, 1992), with each follicle differing from its immediate neighbor by 2–2.5 h of developmental distance. This feature allows the isolation of all stages of oogenesis by dissection of a single female pharate adult.

Because the transition between vitellogenic and choriogenic follicles is easily visible by eye, a

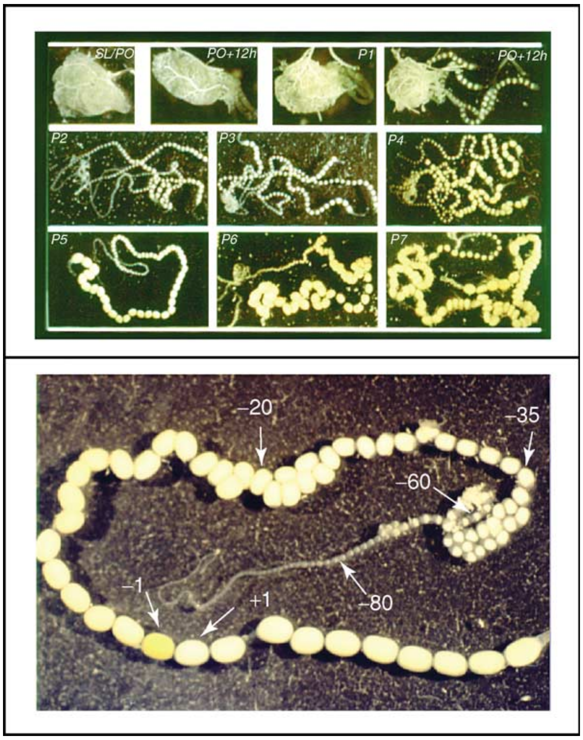


Figure 12 Structure of the developing ovary of the silkmoth, *Bombyx mori* at different stages of development. Upper panel: Development of the ovary of the silkmoth, *B. mori*, in spinning larvae, pupae and pharate adults. In spinning larvae (SL) and pupae until 12 h after larval–pupal ecdysis (P0 and P0 + 12 h), the ovarioles are still enclosed within a protective capsule. When ecdysteroid titers rise in the hemolymph, the capsule ruptures and the ovarioles emerge in the abdominal cavity (P1, P1 + 12 h). Two to three days after ecdysis, vitellogenesis is initiated (P2 and P3) while choriogenesis starts 5 days after ecdysis (P5). During the second half of pharate adult development, choriogenic follicles accumulate in the ovarioles and ovulation ensues (P7). SL, spinning larvae; Pn (+12 h), pupae or pharate adults, n days (+12 h) after larval pupal ecdysis. Lower panel: Structure of a silkmoth ovariole, 6–7 days after larval–pupal ecdysis. Indicated are the various stages of vitellogenesis (−1 to −nn) and choriogenesis (+1 to +nn). In this numbering system, vitellogenesis is initiated at −60, while choriogenesis starts at +1. (Lower panel: Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development is lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol.* 33, 1285–1297; © Elsevier.)

numbering system has been devised to stage the development of follicles (Kafatos *et al.*, 1977). In this numbering system, the first choriogenic follicle acquires the number +1 and successive later stages of choriogenic development (differing by 2.5 h of developmental distance) are given correspondingly higher positive numbers; vitellogenic follicles, alternatively, are numbered with negative numbers, with follicle −1 representing the last stage of vitellogenesis (**Figure 12**).

The developing ovariole is especially suitable for the study of program of follicular cell differentiation from middle vitellogenesis to choriogenesis (stages −30 to −35 and later), because at these stages individual follicles have reached a size sufficiently large to allow easy manipulation for physiological, biochemical, and gene expression studies. Thus, Northern blot analysis has been used to elucidate the changes in the expression patterns of transcription factors during the transition from vitellogenesis to choriogenesis, and correlate such expression patterns with those of structural genes, e.g., genes encoding yolk, vitelline membrane, and chorion proteins (Swevers and Iatrou, 1999, 2003). In many cases, the expression analysis of the mRNA has been coupled with the detection of the corresponding proteins in the follicles by Western blot analysis and immunocytochemistry.

1.3.3.3.2.1.1. In vitro *culture experiments* Mid to late vitellogenic follicles (stages from approximately −35 onwards) are capable of developing in organ culture in a chemically defined medium, completing vitellogenesis and, also, entering and completing the choriogenic program autonomously (Swevers and Iatrou, 1992). Previously, it was also shown that the program of chorion gene expression in the American silkmoth, *Antheraea polyphemus* is also follicle-autonomous (Paul and Kafatos, 1975)

However, it was also observed in *Bombyx* that only a limited number of middle vitellogenic follicles (stages −30/−35 and later) have the capacity to develop autonomously *in vitro* (Swevers and Iatrou, 1992). This finding suggested that follicles of earlier vitellogenic stages require the presence of an extraovarian (hemolymph) factor(s), which triggers the activation of a regulatory cascade allowing the follicles to complete vitellogenesis, and enter and complete choriogenesis autonomously. According to the proposed model, follicles at stages −35/−30 and beyond (at the time of dissection), have acquired the competence to respond to a circulating signal (e.g., a hormone present in the hemolymph) that allows them to develop further autonomously. The term competence implied the stage-specific appearance in the follicles (probably the cells of the follicular epithelium) of a receptor that allows the binding of the circulating signal and the triggering of a relevant regulatory cascade, which establishes the autonomy of the differentiation program. Thus, follicles lacking this capacity (at stages earlier than −35) are unable to respond to the putative signal and cannot proceed autonomously through the execution of the autonomous part of the differentiation program (Swevers and Iatrou, 1992).

To elucidate the nature of the postulated extraovarian factor(s), two approaches have been considered useful: (1) to culture ovarioles in the presence of hemolymph (and, eventually, purified hemolymph factors) or known hormones, and compare the developmental potential of follicles in supplemented versus nonsupplemented culture media; and (2) to search for genes encoding putative receptors and expressed in follicular cells at or around stages −30/−35 but not at earlier stages, because genes implicated in the transduction of the “autonomy signal” would be expected to be differentially expressed during these stages.

Following upon these two approaches, it has been demonstrated that the hormone 20E does not play a role in the induction of the choriogenic program in the follicular cells at mid vitellogenesis. Thus, addition of 20E to ovariole cultures did not change the capacity of vitellogenic follicles to develop autonomously toward subsequent stages (Swevers and Iatrou, 1992). Furthermore, it was determined that the components of the ecdysone receptor heterodimer, BmEcR and BmUSP, as well as the known downstream signaling components in the ecdysone regulatory pathway, are not expressed differentially in the cells of the follicular epithelium with respect to the stages of establishment of follicle autonomy (**Figure 13**) (Swevers *et al.*, 1995; Swevers and Iatrou, 2003).

Subsequent studies to address the issue of the establishment of developmental autonomy in the ovarian follicles have focused on bombyxins, a class of insulin-like peptides that have been isolated from *B. mori* brain extracts based on their prothoracicotropic activity (capacity to activate the prothoracic glands) in the moth *Samia cynthia ricini* (Nagasawa *et al.*, 1984; Jhoti *et al.*, 1987). The functions of bombyxins in *Bombyx* relate to the regulation of glucose metabolism and growth control (Satake *et al.*, 1997; Masumura *et al.*, 2000). High titers of bombyxins are also found in the hemolymph of female pharate adults suggesting an (as yet undefined) role in the regulation of female reproduction, possibly ovarian development (Saegusa *et al.*, 1992). Bombyxin-binding activity is detected in ovarian follicle membrane extracts,

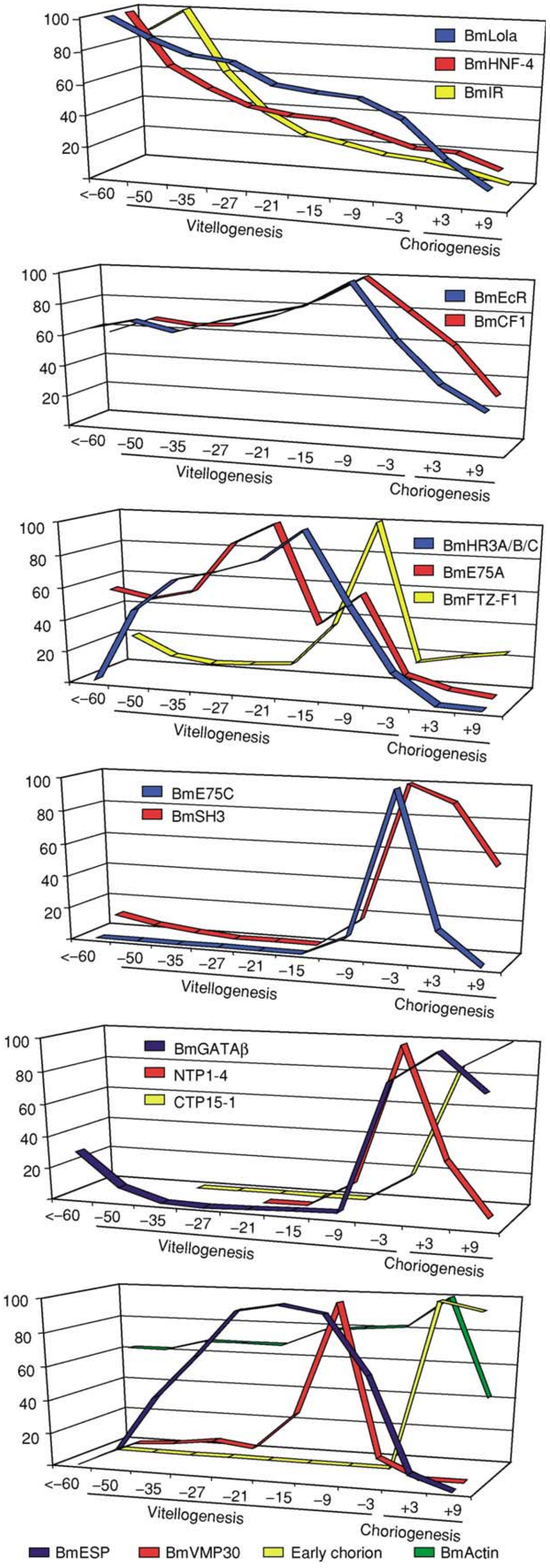


Figure 13 Schematic overview of the expression of the mRNAs of regulatory factors during follicular development in *Bombyx mori* as detected by Northern blot and quantified by Phosphorimager analysis. Expression levels for each gene are plotted relative to the highest signal (100%). Genes were grouped to show similarity in expression levels (first, second, fourth, and fifth panels), to illustrate cross-regulation (third panel) or to indicate broad developmental periods (structural genes in sixth panel). First panel: Genes whose expression levels decline gradually as oogenesis progresses: BmHNF-4, BmIRL, and BmLola. BmLola is the homolog of the *Drosophila* transcription factor Lola that is characterized by an N-terminal BTB-domain and C-terminal C_2H_2 zinc fingers (Giniger *et al.*, 1994). Second panel: Expression of the mRNAs of the components of the ecdysone receptor heterodimer, BmEcR and BmUSP. Both mRNAs are present at similar levels throughout most of vitellogenesis, peak at the end of vitellogenesis, and decline during choriogenesis. Third panel: Expression of the nuclear receptors BmHR3A, BmE75A, and BmFTZ-F1. Note that BmFTZ-F1 becomes induced at maximal values of BmHR3A, concomitantly with a drop in expression levels of BmE75A. Fourth panel: Induction of the mRNAs of the nuclear receptor BmE75C and the adaptor protein BmSH3 at the end of vitellogenesis. The two factors interact in yeast two-hybrid assays and may form a functional complex in the follicular cells. Fifth panel: Induction of the mRNAs of BmGATAβ and two putative interacting factors (NTP 1–4 and CTP 15–1; Glushek, 2001) in the follicular epithelium at the end of vitellogenesis. Sixth panel: Expression of structural genes and developmental markers. ESP is the yolk protein produced by the follicular cells and is a marker of vitellogenesis, BmVMP30 is a vitelline membrane protein expressed during mid-late vitellogenesis, and early chorion genes are induced at the beginning of choriogenesis. BmActin corresponds to the cytoplasmic actin gene; note that its expression shows a peak at the beginning of choriogenesis followed by a sharp decline. Numbers at the bottom of each graph indicate the stages of oogenesis with respect to the start of choriogenesis (+1). The expression levels that are indicated are obtained from pools of 6 follicles (+9, +3, −3, −9, −15, −21, −27), 10 follicles (−35), 20 follicles (−50), or represent the remainder of follicles earlier than −60 (<−60). The numbers indicate the follicle that is the midpoint of each series of 6 follicles (for instance, +9 = +7/+12), 10 follicles (−35 = −31/−40), or 20 follicles (−50 = −41/−60). ''<−60'' represents a large number (> 50) of early follicles. The broad developmental periods of vitellogenesis and choriogenesis are also indicated. (Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297; © Elsevier.)

indicating that ovarian tissue is a direct target tissue of bombyxin peptides (Fullbright *et al*., 1997). Furthermore, the expression of bombyxin mRNAs in ovarian tissue (Iwami *et al*., 1996) also indicates a possible autocrine or paracrine role for bombyxins during follicle development. A gene encoding a putative receptor for bombyxins, the *Bombyx* insulin receptor-like (BmIRL) protein, was cloned (**Figure 14**; I. Lindström-Dinnètz and K.I., unpublished data), and the expression pattern of its mRNA during follicle development determined (Swevers

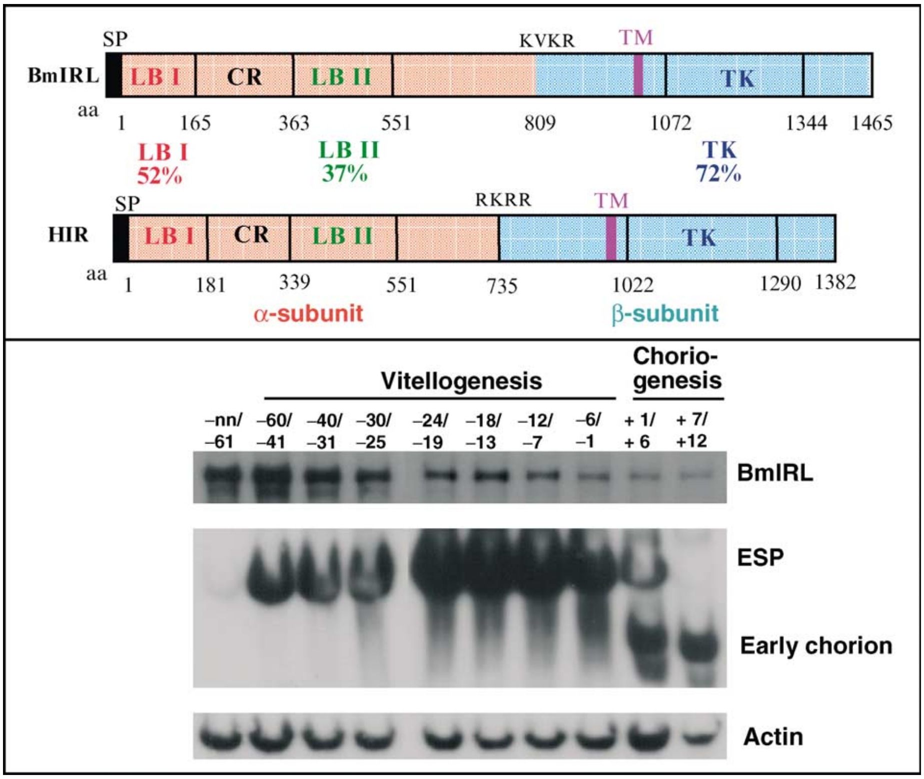


Figure 14 Structure and expression pattern of the *Bombyx* insulin-like receptor (BmIRL; GenBank accession number AF025542). Upper panel: Schematic drawing of the BmIRL protein and comparison with the human insulin receptor (HIR). Indicated are the signal peptide (SP), the ligand-binding domains I and II (LB I and II), the cysteine-rich region (CR) of the α-subunit, the documented cleavage site (RKRR) for processing of HIR into α- and β-subunits, the transmembrane region (TM), and the tyrosine kinase (TK) domain of the β-subunit. Note that the proteolytic processing at the putative cleavage site (KVKR) of BmIRL has yet to be demonstrated. Percentages indicate % identity of selected domains with the corresponding domains of the human insulin receptor. Lower panel: Expression of *BmIRL* mRNA during follicle development. Expression of *Esp* and early chorion mRNA indicates the developmental period of vitellogenesis and early choriogenesis, respectively, and actin hybridizations are shown as control for the integrity of the RNA samples. Molecular weight sizes are 12 kb (BmIRL), 2 kb (ESP), 0.6 and 0.4 kb (early chorion), and 1.3 kb (actin). (Lower panel: Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297; © Elsevier.)

and Iatrou, 2003). The mRNA expression data, however, did not support the prediction for a role for BmIRL in the establishment of the autonomous portion of the program of differentiation at mid-vitellogenesis, as no differential expression was observed with respect to the stages, at which the autonomous capacity is induced in the follicular epithelium (Swevers and Iatrou, 2003). Because the *BmIRL* gene is mainly expressed in previtellogenic and early vitellogenic follicles (**Figures 13 and 14**), signaling through BmIRL is predicted to contribute to the early growth of ovarian follicles. The caveat for this conclusion, however, is that work for the detection of the receptor protein itself through Western analysis or immunolocalization, has not been carried out. Subsequent binding studies, to demonstrate that BmIRL is indeed the receptor for bombyxins, have also been inconclusive (E. Bullesbach, L.S., and K.I., unpublished data). Thus, the question of whether bombyxins are the sought-after inducing signal that establishes autonomy in follicular cells, remains unanswered.

1.3.3.3.2.1.2. Requirement for a decline in 20E signaling In *B. mori*, the transition from vitellogenesis to choriogenesis occurs during those stages of pharate adult development, when the hemolymph titers of ecdysteroids have already declined to low, nearly undetectable levels (Tsuchida *et al*., 1987). Thus, it can be anticipated that the transition from vitellogenesis to choriogenesis is triggered by a decline in the primary 20E signaling in the developing vitellogenic follicles.

Experiments using the nonsteroidal ecdysone agonist tebufenozide have indeed shown that continuous signaling of the 20E pathway blocks ovarian development at mid to late vitellogenesis (Swevers and Iatrou, 1999). Because tebufenozide activates

the 20E signaling pathway at lower concentrations than 20E and persists in tissues much longer than the naturally occurring hormone, it causes prolonged 20E signaling that results in developmental arrest (Retnakaran *et al*., 1995).

When tebufenozide was injected in *Bombyx* developmentally arrested abdomens, ovarian development was induced and the follicles entered the developmental program of vitellogenesis with similar kinetics as in 20E-injected abdomens (Swevers and Iatrou, 1999). However, the transition from vitellogenesis to choriogenesis, monitored through the activation of chorion protein-encoding genes, an event associated with a decline in ecdysteroid titer, did not occur in tebufenozide-injected abdomens (see **Figure 4**). Thus, the experiments with tebufenozide have demonstrated unequivocally that vitellogenic follicles require a decline in 20E-signaling in order to progress towards choriogenesis.

1.3.3.3.2.1.3. Expression patterns of regulatory factors Comparison of the patterns of expression of regulatory factors between normally developing follicles obtained from intact animals and those of arrested follicles obtained from tebufenozide-injected abdomens has led to the identification of a regulatory cascade that is induced in follicular epithelium cells by the declining 20E titers and controls the transition from vitellogenesis to choriogenesis (Swevers and Iatrou, 1999).

At the top of the regulatory cascade stands the orphan nuclear receptor BmFTZ-F1 (Sun *et al*., 1994), which becomes expressed in the follicles at vitellogenic stages −18 to −7 (**Figure 13**). In *Drosophila*, βFTZ-F1 was shown to act as a competence factor during periods of low ecdysteroid signaling; between the periods of high ecdysteroid titers, tissues require low ecdysteroid titers and/or expression of FTZ-F1 in order to regain their capacity to respond properly to a consecutive pulse of 20E (Woodard *et al*., 1994; Broadus *et al*., 1999). Because BmFTZ-F1 seems to be the first factor induced by the declining ecdysone titers in *Bombyx* follicles, it may also function as a competence factor to control the transition from vitellogenesis to choriogenesis.

Interestingly, the induction of BmFTZ-F1 during vitellogenesis coincides with the highest expression levels of BmHR3A as well as with a drop in the expression levels of BmE75A (**Figure 13**; Swevers and Iatrou, 1999; Eystathioy *et al*., 2001; Swevers *et al*., 2002a). Moreover, in transient expression experiments employing *Bombyx*-derived tissue culture cells, BmHR3A and BmE75A function as activators and repressors, respectively, of reporter genes that harbor retinoic acid receptor-related orphan receptor elements (ROREs; binding elements for the HR3 and E75 orphan nuclear receptors) in their upstream regions (**Figure 15**; Swevers *et al*., 2002b). Because of the antagonistic activities displayed by BmHR3A and BmE75A, it was proposed that activation of RORE-dependent target genes depends on the relative expression levels of BmHR3A and BmE75A. It could indeed be demonstrated that during *Drosophila* metamorphosis, prolonged expression of E75 can prevent the activation of the *βFTZ-F1* gene by HR3 (White *et al*., 1997; Kageyama *et al*., 1997; Lam *et al*., 1997). The action of *Drosophila* HR3 and E75 is mediated through their interaction with RORE sites in the *βFTZ-F1* promoter. Whether similar sites also exist in the *Bombyx BmFTZ-F1* promoter is unknown at present, but it is tempting to speculate that the relative levels of BmHR3A and BmE75A determine the induction of BmFTZ-F1 during middle vitellogenesis in *Bombyx* follicles, according to a similar mechanism as that observed during metamorphosis in *Drosophila*.

The induction of BmFTZ-F1 is followed by changes in the expression of several other regulatory factors (**Figure 13**). Thus, at stages −12/−7, induction of the genes encoding the orphan nuclear receptor BmE75C and the adaptor protein BmSH3 occurs, while a concomitant strong decline is observed in the expression of the BmHR3 receptors (all isoforms). Furthermore, a strong decline in the levels of BmFTZ-F1 mRNA is observed at stages −6/−1, while the gene encoding the transcriptional regulator BmGATAβ becomes strongly upregulated at the same time (Drevet *et al*., 1995; Swevers and Iatrou, 1999; Eystathioy *et al*., 2001; Swevers *et al*., 2002a).

For most of the factors described above, however, their exact function in the program of follicular cell differentiation is unknown at present. *BmE75C* is initially induced in ovaries as an early response gene at the beginning of pupation but its expression is not sustained, and the transcription of this mRNA is rapidly downregulated (Swevers *et al*., 2002a). The subsequent (ecdysteroid-independent) increase in the expression of *BmE75C* mRNA (and protein) at stages −12 to +12 is consistent with a function as a regulator of the transition from vitellogenesis to choriogenesis. Because BmE75C acts as a transcriptional repressor in tissue culture cells (**Figure 15**; Swevers *et al*., 2002b), its function in follicular cells may be related to the shut-down of the vitellogenic program rather than the onset of choriogenesis.

BmSH3, a member of a family of conserved adaptor proteins characterized by three SH3 domains at the C-terminus and a lipid raft-targeting “SoHo” domain located at the N-terminus, was isolated

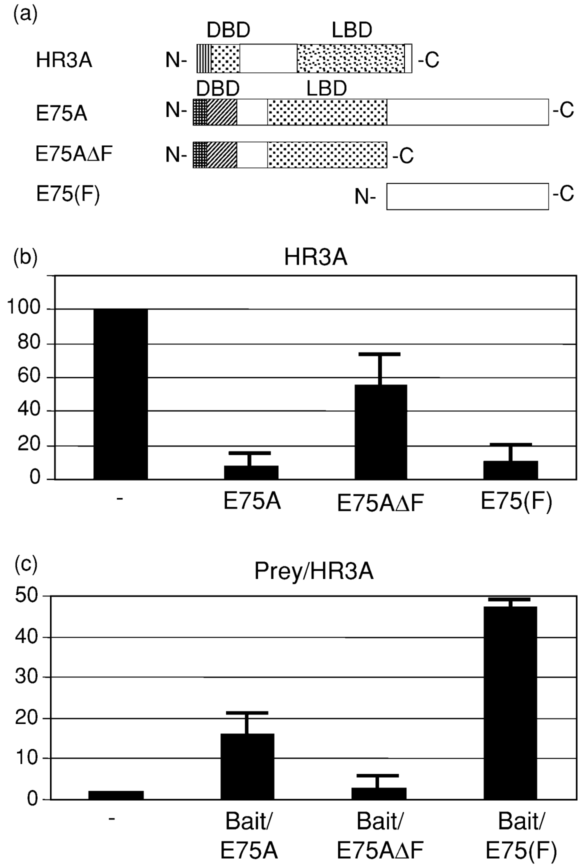


Figure 15 Functional interaction between the nuclear receptors BmHR3A and BmE75A. Panel (a): Schematic drawing of the nuclear receptors BmHR3A, BmE75A, and deletion mutants of BmE75A. In BmE75AΔF the C-terminal F-domain is deleted from the full-length receptor while BmE75(F) only consists of the F-domain. The two conserved domains of nuclear receptors, the DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated. Panel (b): BmE75A represses RORE-dependent reporter gene activation by BmHR3A in a silkmoth-derived cell line. Cells were transfected with RORE-dependent reporter plasmid and expression plasmids of *BmHR3A, BmE75A* and deletion mutants of *BmE75A*. ''–'': Transactivation by BmHR3A only; ''BmE75A'': Transactivation in the presence of BmHR3A and BmE75A; ''BmE75AΔF'': Transactivation in the presence of BmHR3A and BmE75AΔF; ''BmE75(F)'': Transactivation in the presence of BmHR3A and BmE75(F). Values of reporter gene activity are expressed as % of the activity obtained in the presence of BmHR3A only (''–'' column). Note that the F-domain of BmE75 is capable to repress transactivation by BmHR3A, due to interactions with the C-terminal activation function of BmHR3A (Swevers *et al.*, 2002b). Panel (c): Interactions between BmE75A and BmHR3A in yeast two-hybrid assays. Fusions between *BmE75A* or its deletion mutants and the DNA-binding domain of *Gal4* (''Bait'' proteins) were tested for interaction with a fusion between *BmHR3A* and the activation domain of *Gal4* (''Prey'' protein). In ''–,'' the empty ''bait'' vector was tested for interaction with Prey/HR3A. Relative reporter gene activities are expressed as percentages of the activity observed for the control interaction between p53 and SV40 large T-antigen. Note that the interaction between BmE75A and BmHR3A occurs via the F-domain of BmE75A; other experiments have shown that the F-domain forms a complex with the C-terminal activation domain of BmHR3A (data not shown; Swevers *et al.*, 2002b).

in a yeast two-hybrid screen using as bait the proline-rich N-terminus of BmE75C (K. Ito, L. Swevers, and K. Iatrou, unpublished data). Mammalian homologs of BmSH3 are involved in the transduction of tyrosine kinase signaling and the regulation of actin cytoskeleton organization (Baumann *et al.*, 2000; Kioka *et al.*, 2002). Thus, based on its structural features, BmSH3 may be involved in the regulation of the changes in the actin cytoskeleton in the cells of the follicular epithelium at the transition from vitellogenesis to choriogenesis (e.g., loss of patency, assembly of the chorion secretory machinery).

Finally, the BmGATAβ transcription factor has been hypothesized to be a major regulator of choriogenesis (Skeiky and Iatrou, 1991; Skeiky *et al.*, 1994; Drevet *et al.*, 1994, 1995) (see Section 1.3.4). Regarding a possible mechanism for the regulation of *BmGATAβ* gene expression, it should be noted that RORE binding sites exist in its promoter, which are bound by the BmHR3A orphan nuclear receptor in gel retardation assays. Because BmGATAβ and BmHR3A show reciprocal expression patterns during follicle development, it has been suggested that BmHR3A could act as a repressor of BmGATAβ expression (Eystathioy *et al.*, 2001). Although BmHR3A is known to act as a constitutive activator, as was mentioned earlier, its activation function can be repressed by the co-expression of BmE75A (Swevers *et al.*, 2002b). Thus, a possible mechanism for the silencing of the *BmGATAβ* gene during vitellogenesis may involve the recruitment of BmHR3A/BmE75A complexes to the RORE sites in the *BmGATAβ* promoter.

1.3.3.3.3. Other insects In insects, in which vitellogenin uptake by ovarian follicles is regulated by JH, termination of vitellogenesis can, in principle, be accomplished by two mechanisms: first, by a decrease in JH production by the CA or, second, by an increase in JH degradation in the hemolymph.

In the cockroach, *Diploptera punctata*, termination of vitellogenesis can be associated with a decline in JH titer (Sutherland *et al.*, 1998). Ovarian follicles that are in different stages of oogenesis exert different effects on the activity of the CA. Thus, follicles in active vitellogenesis stimulate JH production, while those near maturity inhibit it (Sutherland *et al.*, 2000). During the progression of vitellogenesis, the target for the ovarian inhibitory hormonal factor in the cells of the CA is proposed to be the CYP4C7 cytochrome P450 enzyme, which metabolizes JH and JH precursors by β-hydroxylation (Sutherland *et al.*, 1998, 2000). Thus, the decline in JH titer that is necessary to initiate post-vitellogenic events in

Diploptera, is mediated through a feedback signal from the ovary to the CA, which represses JH synthesis. By contrast, mechanisms that involve JH degradation by esterases in the hemolymph are thought to play a more minor role in the termination of vitellogenesis in this species.

In other cockroaches such as *L. maderae*, control of vitellogenesis is thought to be dependent on the fluctuation of the JH titer through the interplay among JH-binding proteins and JH degrading enzymes (for a relevant discussion, see Engelmann and Mala, 2000).

1.3.3.4. Ecdysteroidogenesis and Resumption of Meiosis

During the progression of vitellogenesis, ecdysteroid synthesis occurs in the cells of the follicular epithelium (Lagueux *et al*., 1977). However, in lepidopteran and orthopteran insects, most of the ecdysteroids produced by the follicular epithelium are inactive conjugates of ecdysone precursors and metabolites, and only very small amounts of active 20E are detected (Mizuno *et al*., 1981; Tawfik *et al*., 1999). In these insects, the ecdysteroids are not secreted to the hemolymph but are transported to the developing oocyte, where they are presumably stored as "maternal ecdysteroids" for use during embryonic development (Lagueux *et al*., 1981; Mizuno *et al*., 1981).

In the orthopteran *L. migratoria*, *in vitro* experiments have also shown that ecdysone promotes the reinitiation of meiosis in the oocyte at the end of vitellogenesis (Lanot *et al*., 1988, 1990). During oocyte growth, the oocyte nucleus (germinal vesicle) is arrested at the prophase of the first meiotic division. At middle to late vitellogenesis, however, germinal vesicle breakdown (GVBD) is initiated by the action of ecdysone produced by the follicular cells. Ecdysone is proposed to act at the level of the oocyte membrane, where it stimulates the production of cAMP (Lanot *et al*., 1988, 1990). After GVBD, the mature egg arrests again and remains arrested, until fertilization or some other signal triggers meiotic completion and further development.

In *B. mori*, ovarian ecdysteroid synthesis is initiated during day 4 of adult development (Ohnishi and Chatani, 1977), corresponding to the mid to late vitellogenic stages −20 to −25. Experiments using the ecdysteroid synthesis inhibitor KK-42 have shown that decreased ovarian ecdysteroid production does not affect ovarian growth, while it has profound effects on fertilization, embryogenesis, and larval hatching (Kadono-Okuda *et al*., 1994). These results suggest that, as is the case with *Locusta*, ovarian ecdysteroids are involved in the promotion of meiotic maturation in *Bombyx*. By contrast, since the experiments using the ecdysone agonist tebufenozide have shown that termination of vitellogenesis and initiation of choriogenesis requires a decline in 20E signaling (Swevers and Iatrou, 2003; Section 1.3.3.3.2.1.2), it is clear that the ecdysteroids produced by the follicular epithelium are not involved in the (autocrine or paracrine) regulation of follicle growth.

Interestingly, ecdysteroid synthesis in silkmoth follicles coincides with the expression of the nuclear receptor BmFTZ-F1, which also becomes expressed in follicular cells around stage −20 (**Figure 13**) (Swevers and Iatrou, 2003). BmFTZ-F1 is the insect homolog of the vertebrate nuclear receptor steroidogenic factor 1 (SF-1), which has essential roles in the differentiation of steroidogenic organs (gonads and adrenals) in vertebrates (Val *et al*., 2003). The SF-1 protein was originally isolated by its capacity to interact with and activate the promoters of steroidogenic enzymes (Rice *et al*., 1991; Morohashi *et al*., 1992). To deduce whether BmFTZ-F1 has a similar role in the regulation of ecdysteroidogenesis in ovarian tissue of *Bombyx*, the isolation of the genes encoding ecdysteroidogenic enzymes and the characterization of their promoter elements will be required.

In contrast to orthopteran and lepidopteran insects, the vitellogenic ovaries of dipteran insects (*Drosophila* and *Aedes*) synthesize significant amounts of 20E that are secreted into the circulation to stimulate vitellogenin synthesis in the fat body (Bownes, 1986; Raikhel *et al*., 1999; see also **Chapters 1.2**, **1.5**, and **3.9**). In *Drosophila*, the site of synthesis is the cells of the follicular epithelium, as demonstrated by the detection of the mRNA of the ecdysteroidogenic enzyme encoded by the *disembodied* (*dis*) gene through *in situ* hybridization (Warren *et al*., 2002) (see **Chapter 1.2**). In *Aedes* and *Phormia*, the synthesis of 20E is stimulated by neurohormones (see Sections 1.3.3.1.1.2 and 1.3.3.3.1.2), presumably through a cAMP-dependent mechanism (Brown *et al*., 1998; Manière *et al*., 2002). It has also been proposed that 20E stimulates ovarian growth by an autocrine/paracrine mechanism, although the evidence for this is not entirely convincing (Buszczak *et al*., 1999; see Section 1.3.3.1.1.1.3). However, whether ecdysteroids produced by the ovary of dipteran insects are also involved in the regulation of reinitiation of meiosis is not known. In *Drosophila*, as in *Locusta*, ovarian oocytes are arrested in prophase I during vitellogenic growth and undergo GVBD upon maturation (Page and Orr-Weaver, 1997; Chen *et al*., 2000) (see **Chapter 1.2**). The mature oocytes become arrested in metaphase I, but are released from the arrest when the eggs pass through the oviduct into the uterus (Heifetz *et al*., 2001) (see also Section

1.3.5). Thus, it can be hypothesized that the ecdysteroids produced by the follicular epithelium in *Drosophila* are also involved in the stimulation of the transition from prophase I to metaphase I during oocyte maturation.

1.3.3.5. Osmotic Swelling and Loss of Patency

The mechanism that triggers the loss of patency at the end of vitellogenesis has been studied mostly and is best understood in the lepidopteran *H. cecropia* (Telfer and Woodruff, 2002). In this species, loss of patency occurs through osmotic swelling (50% increase in cellular volume) of the cells of the follicular epithelium and concomitant closure of the intercellular spaces among the epithelial cells. This process is triggered by a rise in the cAMP content of the follicular cells (Wang and Telfer, 1996), which results in the activation of protein kinase A, and changes in the electrophysiological properties of the cells (increase of chloride ion conductance and potassium ion uptake, acidification of the cytoplasm, hyperpolarization) that result in fluid uptake (Wang and Telfer, 1998a). Interestingly, the rise in cAMP levels and loss of patency can also be effected by pertussis toxin, an inhibitor of the α subunit of inhibitory G proteins (Giα) (Wang and Telfer, 1998b). Thus, based on the assumption that Giα is a component of a conventional heterotrimeric G protein, it has been suggested that during vitellogenesis, cAMP levels in the epithelium remain low through signaling by an unknown GPCR that couples with Giα-containing G protein complexes. At the end of vitellogenesis, however, a decrease in signaling (loss of ligand or decrease in ligand sensitivity) through the hypothetical GPCR is postulated to lead to increased levels of cAMP, which result in osmotic swelling and loss of patency in the follicular epithelium (Wang and Telfer, 1998b). The transformation of the follicular epithelium, however, is thought not to be triggered by extrafollicular factors circulating in the hemolymph but by developmental changes occurring spontaneously in each follicle as it reaches a length of 2 mm (Wang and Telfer, 1996, 1998b).

The activation of protein kinase A in homogenates of follicles also results in the phosphorylation of a 32 kDa protein that has been speculated to represent ribosomal protein S6 (Wang and Telfer, 1996, 2000). However, cell-permeable cAMP analogs that inhibit phosphorylation of the 32 kDa protein, were found to be capable of triggering termination of vitellogenesis in intact follicles, suggesting that the phosphorylation of this protein is not involved in the pathway that terminates vitellogenesis.

Because the cells of the follicular epithelium and the oocyte communicate through gap junctions that allow exchange of ions and small solutes (Telfer and Woodruff, 2002), osmotic swelling occurs both in the cells of the follicular epithelium and the oocyte. However, the process seems to be triggered in the follicular cells because of their high levels of protein kinase A. Osmotic swelling occurs immediately (within 30 min after the rise in cAMP) in the follicular cells, while a significant delay exists for the oocyte. The delay in the swelling of the oocyte probably contributes to the requirement for a mechanical loosening of the vitelline envelope (see Section 1.3.3.8 below), which would otherwise prevent the immediate expansion of the oocyte volume (Wang and Telfer, 1998a).

1.3.3.6. Patterning and Movements of the Follicular Epithelium

The patterning and morphogenetic movements of the follicular epithelium at the transition from vitellogenesis to choriogenesis in *D. melanogaster* have been subject to several excellent recent reviews (Ray and Schüpbach, 1996; Van Eeden and St. Johnston, 1999; Dobens and Raftery, 2000; Riechmann and Ephrussi, 2001; Rørth, 2002) (see **Chapter 1.2**). Only a brief outline will be presented here. However, very little to no information on these important processes exists for other insects.

At the beginning of *Drosophila* vitellogenesis (stage 9), the follicular epithelium is organized in three domains: (1) a small cluster of border cells that has delaminated from the anterior pole of the follicle and migrates between the cells of the nurse cell complex towards the oocyte surface, (2) a squamous epithelium that covers the nurse cell cluster, and (3) a columnar epithelium that covers the oocyte. At the end of vitellogenesis (stage 10B), the nurse cells start dumping their contents into the oocyte (see below), while the most anterior cells of the columnar epithelium move centripetally between the nurse cell cluster and the oocyte to seal the anterior surface of the oocyte. The centripetally migrating cells stop their migration when they meet the border cells. The leading cells of the centripetally moving population of follicular epithelium cells, together with the border cells, form the micropyle of the eggshell, while the remainder of the centripetally moving cells build the operculum. During stage 11, the main body follicle cells also stretch along the anterior–posterior axis to accommodate the increase in oocyte volume during nurse cell dumping (Spradling, 1993; Dobens and Raftery, 2000) (see **Chapter 1.2**).

The patterning and movements of the follicular epithelium are controlled by several autocrine and paracrine signaling pathways. Thus, the "Janus Kinase/Signal Transducer and Activator of Transcription" (JAK/STAT) pathway is responsible for border cell specification and migration between the cells of the nurse cell complex (Castelli-Gair Hombría and Brown, 2002). Furthermore, migration toward the oocyte is guided by spatial cues that are detected by the *Drosophila* homologs of the mammalian platelet-derived growth factor receptor/vascular endothelial growth factor receptor (PDGFR/VEGFR) and the epidermal growth factor receptor (EGFR) pathways (Rørth, 2002). The best known transcription factor necessary for border cell migration is Slow border cells (SLBO), a basic region/leucine zipper transcriptional activator related to the mammalian CCAAT/enhancer-binding protein (C/EBP) transcription factors (Montell, 2001). Interestingly, temporal cues for border cell migration seem to be initiated by components of the ecdysone receptor signaling, such as the USP nuclear receptor and the p160 class of the nuclear receptor coactivator Taiman (Tai; Montell, 2001). Nevertheless, the findings that *tai* or *usp* mutants show deficiencies in border cell migration do not necessarily prove an involvement of ecdysone, since both USP and Tai could act as components of other signaling pathways (see discussion in Section 1.3.3.1.1.1.3; also Swevers and Iatrou, 2003). From a comparative viewpoint, it should be noted that the specification of a group of apical follicular cells that migrate through the cells of the nurse cell complex to reach the oocyte surface (border cells), is specific to the order of the Diptera and does not occur in species of other insect orders (Büning, 1994).

The formation of anterior structures in the eggshell, mediated by the centripetally migrating and border cells, is controlled by the transforming growth factor β/Decapentaplegic (TGFβ/Dpp) and the epidermal growth factor (EGF) pathways (Twombly *et al*., 1996) (see **Chapter 1.2**). The cells at the leading edge of the centripetally migrating population produce Dpp during migration and micropyle formation (Twombly *et al*., 1996; Dobens *et al*., 2000). EGF signals, alternatively, emanate initially from the oocyte nucleus at the dorsal-anterior part of the oocyte, and are subsequently modulated by autocrine signaling in the dorsal-anterior follicular epithelium (Stevens, 1998; Van Buskirk and Schüpbach, 1999). Overexpression experiments have shown that correct patterning of the anterior part of the follicular epithelium/eggshell is dependent on the integration of both Dpp and EGF signaling (Dobens and Raftery, 2000).

1.3.3.7. Nurse Cell Dumping and Apoptosis

The process of rapid transport of the contents of the nurse cells to the oocyte at the end of vitellogenesis that is accompanied by programmed cell death in the nurse cells, has been described in detail and subjected to genetic analysis in *D. melanogaster* (review: **Chapter 1.2**). In other insect species, it is not known whether these processes are regulated by mechanisms analogous to those occurring in *Drosophila* or by different pathways.

In *Drosophila*, the nurse cell cytoplasm is rapidly transported to the oocyte during stages 10B and 11 of oogenesis. This results in the doubling of the oocyte volume in about 30 min. Concomitantly, circular streaming is initiated in the cytoplasm of the oocyte that effects the rapid mixing of the nurse cell contents with the cytoplasm of the oocyte (Mahajan-Niklos and Cooley, 1994).

The transport of the contents of the nurse cell cytoplasm is regulated by the actin cytoskeleton, which performs two functions. First, the cortical actin cytoskeleton together with a myosin motor generates the driving force required to expel the nurse cell cytoplasm (Wheatley *et al*., 1995; Edwards and Kiehart, 1996). Second, actin bundles anchor the nurse cell nuclei to the membrane, thus preventing them from blocking the path of cytoplasmic flow through the ring canals (Cooley *et al*., 1992; Cant *et al*., 1994; Matova *et al*., 1999).

The process of dumping of the cytoplasmic contents of the nurse cells to the oocyte is closely associated with the initiation of programmed cell death (apoptosis) in the nurse cell complex. Loss of function of the *dcp-1* gene, which encodes an effector caspase involved in apoptosis, results in defects in nurse cell dumping, suggesting that apoptosis is a necessary event for the final transport of the nurse cell contents into the oocyte (McCall and Steller, 1998). After the majority of the cytoplasm is lost from the nurse cells, DNA breakdown occurs in the nurse cell nuclei during stage 13, and the nurse cell remnants are removed via phagocytosis by the follicular cells at stage 14 (Foley and Cooley, 1998; Cavaliere *et al*., 1998; Nezis *et al*., 2000). The activities of the regulators of the death process (interplay between caspases and caspase inhibitors) are proposed to be temporally and spatially controlled, since the oocyte must be protected from the death process that occurs in the connected nurse cells (Peterson *et al*., 2003).

Nurse cell dumping and apoptosis must also be tightly coordinated with the morphogenetic movements in the follicular epithelium, to allow the appropriate formation of a continuous epithelium that

will seal the oocyte completely for deposition of the chorion or eggshell. The signaling pathways, between the germline and the cells of the follicular epithelium that coordinate these processes, include the c-Jun-NH_2-terminal kinase (JNK) pathway (Dequier *et al.*, 2001; Dobens *et al.*, 2001) and others that may involve GPCRs and cAMP signaling (Schneider and Spradling, 1997; Lannutti and Schneider, 2001; Pathirana *et al.*, 2001).

1.3.3.8. Vitelline Membrane Synthesis

1.3.3.8.1. Dipteran insects

1.3.3.8.1.1. ***Drosophila melanogaster*** To date, four *Drosophila* vitelline membrane protein (VMP) genes have been cloned, *VM26A.1, VM26A.2, VM34C*, and *VM32E* (Higgins *et al.*, 1984; Mindrinos *et al.*, 1985; Burke *et al.*, 1987; Popodi *et al.*, 1988; Gigliotti *et al.*, 1989). While *VM26A.1, VM34C*, and *VM26A.2* are expressed continuously from stages 8 to 10, *VM32E* is considered to be a “late” gene and is expressed only at stage 10. Furthermore, *VM34C* and *VM32E* are not expressed in the polar follicular cells, in contrast to *VM26A.1* and *VM26A.2*. However, upon synthesis and secretion, both proteins have been postulated to diffuse towards the poles of the follicles (Andrenacci *et al.*, 2001).

For the *VM26A.1* and *VM32E* genes, analysis of their promoter regions has been carried out in transgenic flies, and *cis*-regulatory elements for temporal and spatial regulation as well as expression levels have been delineated (Gargiulo *et al.*, 1991; Jin and Petri, 1993; Cavaliere *et al.*, 1997; Andrenacci *et al.*, 2000). However, no *trans*-acting factors that regulate the expression of the *VMP* genes have been identified thus far.

Immuno-electron microscopy and confocal immunofluorescence studies have shown that yolk proteins (Yps) and VMPs are co-secreted by the cells of the follicular epithelium (stages 9 and 10 of oogenesis). While the VMPs are incorporated in the incomplete vitelline membrane (VM) matrix, the YPs diffuse through gaps in the vitelline layer to reach the oocyte surface. During stage 10B, the incomplete parts of the VM fuse to form a continuous layer that covers completely the oocyte surface (Margaritis, 1985; Trougakos *et al.*, 2001).

All VMPs become localized in the VM during choriogenesis. However, during the later stages, VM32E (but not the other VMPs) is capable of moving from the VM layer to the endochorion (which is secreted during stages 11–14), indicating its participation in the formation of endochorion structures (Andrenacci *et al.*, 2001). The VMPs also undergo proteolytic processing during choriogenesis (Pascucci *et al.*, 1996), but the functional relevance of this finding remains obscure.

The VMPs are characterized by a conserved hydrophobic domain of 38 aa, the VM domain (Scherer *et al.*, 1988) which is necessary for their assembly in the VM. Alternatively, the domain responsible for the localization of the VM32E protein to the endochorion during choriogenesis resides at the C-terminus of the protein (Andrenacci *et al.*, 2001).

In the female sterile mutation *fs(2)QJ42*, the follicles fail to accumulate VM26A.2, and this results in the formation of an altered VM, onto which the endochorion layer collapses during stage 14 (Savant and Waring, 1989). Thus, the preformation of a properly assembled VM is a prerequisite to the formation of a stable chorion structure (Pascucci *et al.*, 1996).

Another genetic locus implicated in vitelline membrane and chorion formation in *Drosophila* is the *defective chorion-1* (*dec-1*) locus (Waring *et al.*, 1990). The *dec-1* gene encodes multiple protein products that are generated by alternative splicing and protein processing. Three proproteins, fc177, fc125, and fc106, are produced that share a common N-terminus but are distinguished by differential C-termini. Following their secretion, the proproteins undergo distinct extracellular maturation pathways: fc106 is processed during stage 10B into s80, which undergoes an additional cleavage that generates s60 during stage 14; fc125 is initially cleaved to a 125 kDa protein at stage 10A and subsequently to 110 and 95 kDa proteins during stage 10B; and fc177 undergoes a cleavage to a 125 kDa and a second one that yields an 85 kDa protein during stages 12 and 13, respectively (Noguerón and Waring, 1995; Noguerón *et al.*, 2000). The DEC-1 proteins are synthesized concomitantly with the VMPs and accumulate extracellularly between the follicular epithelium and the oocyte. The VM functions both as the site where the proteolytic cleavage products are generated and as a reservoir for the release of the different cleavage products to the oocyte and distinct regions of the chorion, where they presumably exert unique functions (Noguerón *et al.*, 2000). Mutants of *dec-1* fail to organize the endochorion, which, as is the case of the *fs(2)QJ42* mutation, collapses into the VM during late choriogenesis (Waring *et al.*, 1990).

Recently, the distinct functions of the three DEC-1 proproteins were also dissected genetically. Using specific *dec-1* mutations, in conjunction with introduced *dec-1* transgenes, it was shown that gross morphological abnormalities occur only in the absence of fc177, although all three proproteins are essential for female fertility. It was proposed that fc177 acts as a scaffolding protein necessary for the

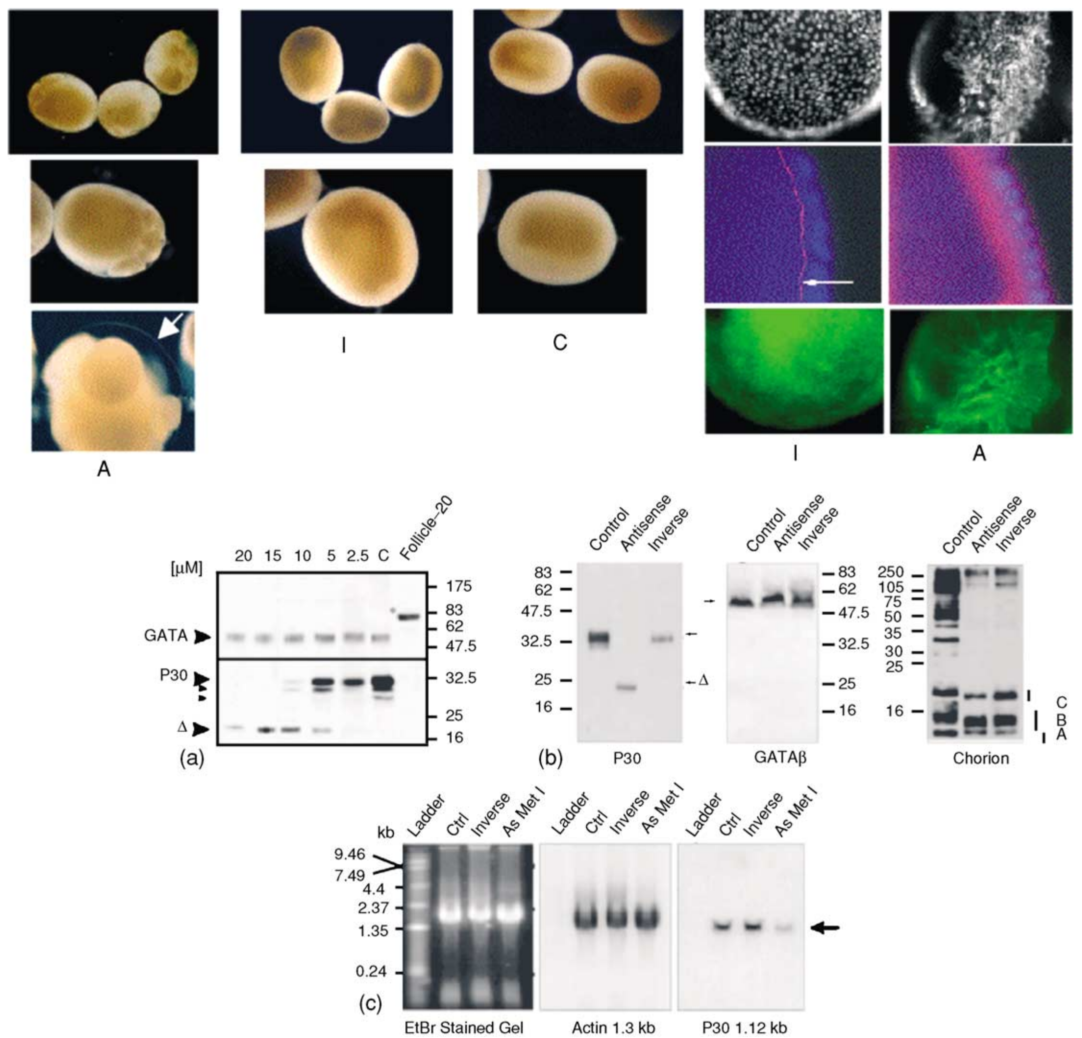


Figure 16 Functional analysis of the *BmVMP30* gene of *Bombyx mori* that encodes a putative member of the insect vitelline membrane protein family. Follicles were treated with antisense or control (inverse) oligonucleotides and the effects of the treatments on the morphology of the follicles and the expression of *BmVMP30* and other genes were assessed. Upper left panel: Light microscopy observations of the integrity of the follicular epithelium and the oocyte of developing follicles following treatment with 15 μM of antisense oligonucleotide (panel A) or control (inverse) oligonucleotide (panel I) or without treatment (panel C) for 48 h *in vitro*. Note the abnormal distribution of the yolk within the antisense oligonucleotide-treated follicles (white arrow in bottom panel of panel A). Upper right panel: Fluorescence microscopy observations of the integrity of the follicular epithelium and the oocyte of developing follicles following treatment with 15 μM of antisense oligonucleotide (panel A) or inverse oligonucleotide (panel I) for 48 h *in vitro*. Follicles were stained with DAPI (top) or stained with fluorescent anti-BmVMP30 (middle) or anti-tubulin (bottom) antibody complexes. While follicles treated with inverse oligonucleotide retain their wild-type appearance, disruptions of the follicular epithelium occur after treatment with antisense oligonucleotide. In panel A, note the disruption of the follicular epithelium characterized by gaps in the nuclear staining (top right), diffuse localization of BmVMP30 epitopes within the follicular cells (middle right), and a nonhomogeneous tubulin staining (bottom right). The normal location of BmVMP30 and the vitelline membrane (wild-type appearance) is marked by a white arrow in the left middle panel (bar = 50 μm). Bottom panel: Western and Northern blot analysis of the expression of *BmVMP30* and other genes after treatment of follicles with BmVMP30 antisense and control oligonucleotides. (a) Western blot demonstrating the effects of different concentrations (2.5 to 20 μM) of BmVMP30 antisense oligonucleotide on BmVMP30 (P30) protein expression after *in vitro* culture of middle/late (stage ''−20'') vitellogenic follicles for 48 h. No BmVMP30 was initially detected in mid-vitellogenic follicle ''−20'' prior to culture (right-most lane). Arrows point to truncated BmVMP30 proteins presumed to originate from the use of alternative translation start sites; ''Δ'' indicates the presence of the putative truncated ΔBmVMP30 protein. While the antisense oligonucleotides had profound effects on BmVMP30 protein expression (lower), no changes were observed in the expression of the 55 kDa BmGATAβ protein (upper). Note that the BmGATAβ antiserum recognizes an additional unknown ∼70 kDa protein in ''−20'' follicles (star). C, control ''−20'' follicles cultured *in vitro* for 48 h in the absence of antisense oligonucleotide. Molecular weight markers are indicated at right. (b) Western blot demonstrating the effects of 15 μM of antisense or inverse oligonucleotide on BmVMP30 (P30) (left), BmGATAβ (middle), or chorion protein (right) expression after *in vitro* culture of middle/late vitellogenic follicles (stage ''−20'') for 48 h. The presence of BmVMP30, ΔBmVMP30 (Δ), and BmGATAβ proteins are indicated by arrowheads while the different chorion protein families (A, B, C) are indicated by bars. Numbers refer to molecular weights (kDa) of protein markers. (c) Northern blot analysis demonstrating the effects of 15 μM of

erection of the chorion structure (Mauzy-Melitz and Waring, 2003).

The VM represents also a storing site for secreted follicular epithelium cell products involved in embryonic pattern formation (see **Chapter 1.2**). Extracellular proteins that signal the patterning of the embryo are anchored in the VM and participate actively in the VM assembly. The Nudel protease, for example, a large modular protein with a trypsin-like serine protease domain, functions both in the generation of the extracellular signal that determines the dorsoventral axis of the embryo, and the cross-linking of the vitelline membrane (LeMosy *et al*., 1999; LeMosy and Hashimoto, 2000). Similarly, the *fs(1)Nasrat* and *fs(1)polehole* genes are not only required for the generation of the ligand for the local activation of the Torso (TOR) receptor at the termini of embryos, but also have structural roles in the biogenesis of the VM (Cernilogar *et al*., 2001).

1.3.3.8.1.2. **Aedes aegypti** Three genes encoding VMPs have been cloned in *A. aegypti*, *15a-1*, *15a-2*, and *15a-3* (Lin *et al*., 1993; Edwards *et al*., 1998). All are characterized by a short 46 aa sequence that bears similarities with the VM domain of the *Drosophila* VMPs. The expression patterns of the vitelline membrane proteins during oogenesis overlap those of the YPs. *In situ* hybridization has also shown that the different *VMP* genes are expressed in spatially distinct domains of the follicular epithelium: *15a-1* and *15a-3* are expressed in the middle and the posterior regions of the follicle, while *15a-2* is expressed over the entire surface (Edwards *et al*., 1998). Finally, a moderate stimulation of expression levels of the VMPs could be achieved by addition of high concentrations 20E (Lin *et al*., 1993; Edwards *et al*., 1998).

1.3.3.8.2. Lepidopteran insects In general, and in contrast to chorion formation (Section 1.3.4), very little is known about vitelline membrane formation in lepidopteran insects at the molecular level. In the best studied lepidopteran model, the domesticated silkmoth *B. mori*, for example, only one vitelline membrane protein gene has been isolated and characterized in detail (see below).

1.3.3.8.2.1. **Bombyx mori** A cDNA clone, *BmVMP30*, encoding a putative VMP has been isolated from a screen for genes that are differentially expressed with respect to stages −35/−1 (Kendirgi *et al*., 2002). BmVMP30 is a 30 kDa protein that is synthesized and secreted by the cells of the follicular epithelium during late vitellogenesis (stages −18 to −1). The protein accumulates between the oocyte surface and the follicular epithelium, where the VM is located (**Figure 16**), and remains associated with the egg envelope during choriogenesis and after egg-laying. However, after fertilization no BmVMP30 protein could be detected by Western blot analysis in the eggshell fraction of the developing embryos. Thus, the temporal and spatial expression pattern of BmVMP30 during and after oogenesis is consistent with the behavior expected for a VMP (Kendirgi *et al*., 2002). Although BmVMP30 does not have significant sequence identity with any *Drosophila* or mosquito VMPs, including the VM domain, it does share with them a distinct overall proline distribution, a similar general hydropathy profile and the lack of introns in the gene structure (Kendirgi *et al*., 2002).

Strong knockdowns of *BmVMP30* gene expression, achieved by antisense oligonucleotide technology, resulted in the appearance of a severe phenotype suggesting disruption of the integrity of the follicular epithelium, while no interference was observed with the initial steps of the program that controls the activation of chorion protein genes (**Figure 16**; Kendirgi *et al*., 2002). Because the knockdown experiments were carried out in *in vitro* culture, the long-term effects on follicle development and eggs (e.g., completion of choriogenesis) could not be assessed.

The expression of *BmVMP30* requires downregulation of the 20E signaling, as its mRNA is not observed in ovaries that are treated with the 20E agonist tebufenozide (Swevers and Iatrou, 1999; see also Section 1.3.4.1.1.2). Interestingly, *BmVMP30* gene expression becomes upregulated during follicular development concomitantly with that of the orphan nuclear receptor BmFTZ-F1, suggesting a possible regulation of the expression of *BmVMP30* by BmFTZ-F1 (Swevers and Iatrou, 1999, 2003).

1.3.4. Choriogenesis

The deposition of the eggshell or chorion occurs when the cells of the follicular epithelium undergo their terminal differentiation and become devoted to the

antisense (AsMet1) or inverse oligonucleotide on BmVMP30 (P30) and actin mRNA expression after *in vitro* culture of middle/late vitellogenic follicles (stage "−20") for 48 h. The specific reduction of BmVMP30 mRNA by antisense oligonucleotide treatment is indicated by an arrow. RNA molecular weight markers are indicated on the left. EtBr, ethidium bromide; Ctrl, control. (Reprinted by permission of Federation of the European Biochemical Societies from An ovarian follicular epithelium protein of the silkworm (*Bombyx mori*) that associates with the vitellise membrane and contributes to the structural integrity of the follicle, by Kendirgi, F., Swevers, L., Iatrou, K., *FEBS Letters* 524, 59–68, © 2002.)

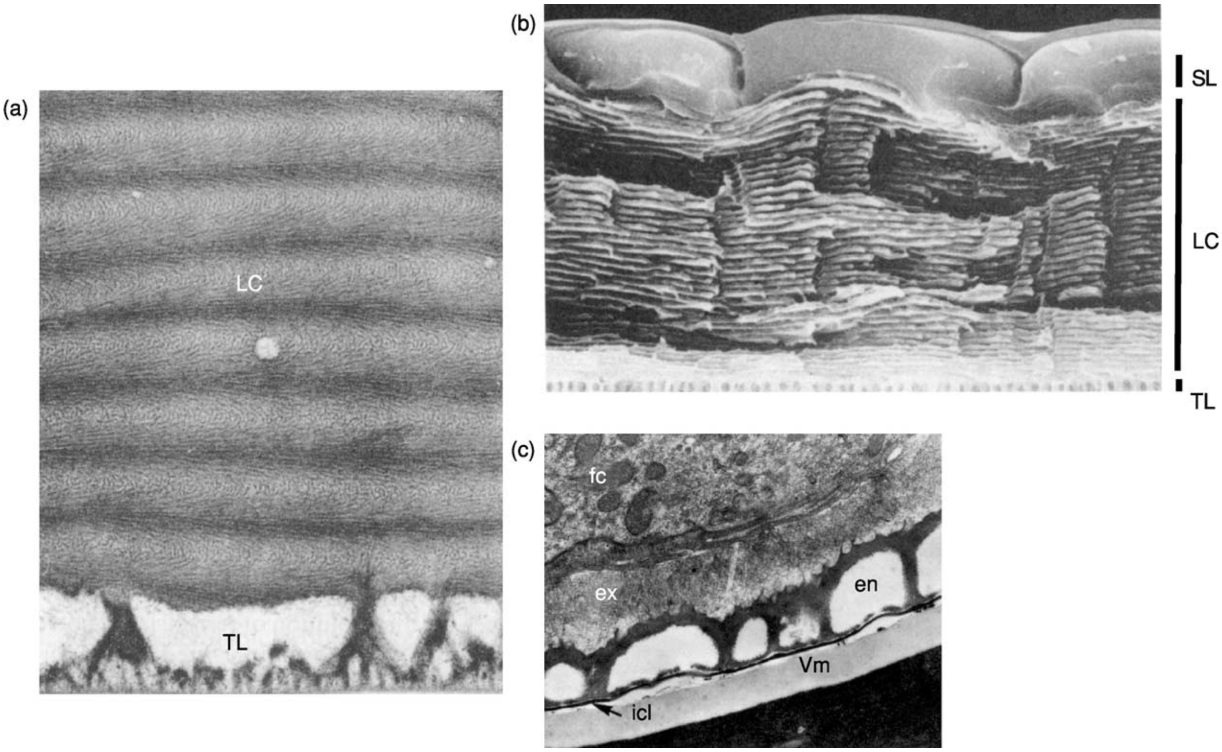


Figure 17 Chorion structure of eggs from *Hyalophora cecropia* (a), *Bombyx mori* (b), and *Drosophila melanogaster* (c). (a) Transmission electron micrograph of an immature chorion from the silkmoth *H. cecropia*. The trabecular layer (TL) is at the bottom with a portion (approximately 6% of total) of the helicoidal lamellae (lamellar chorion, LC) above. Magnification = 30 000×. (Reprinted from Regier, J.C., Friedlander, T., Leclerc, R.F., Mitter, C., Wiegmann, B.M., **1995**. Lepidopteran phylogeny and applications to comparative studies of development. In: Goldsmith, M.R., Wilkins, A.S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 107–137, with permission from Cambridge University Press.) (b) Scanning electron micrograph of a transverse rip through the mature eggshell of *B. mori*. Indicated are the different chorion layers: the small trabecular layer (TL) is located at the side of the vitellin membrane and the oocyte, while the bulk of the chorion consists of multiple horizontal lamellae (LC). At the top (external surface), a few specialized surface lamellae (SL) are deposited. Magnification = 2000×. (Reprinted from Kafatos *et al*., 1985, with permission from Cold Spring Harbor Laboratory Press.) (c) Transmission electron micrograph of a section of a stage 14 follicle of *D. melanogaster* showing the structure of the eggshell. The oocyte (o), vitellin membrane (Vm), inner chorionic layer (icl), endochorion (en), exochorion (ex), and overlying follicle cell (fc) are indicated. Magnification = 150 000×. (Reprinted from Spradling, A.C., **1993**. Developmental genetics of oogenesis. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster* vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–70, with permission from Cold Spring Harbor Laboratory Press. Photo courtesy of Drs. G. Waring and A. Mahowald.)

synthesis and secretion of the chorion proteins, which subsequently become assembled extracellularly into a large macroscopic structure (Orr-Weaver, 1991; Kafatos *et al*., 1995). The insect eggshell is a complex structure that protects the developing embryo in a hostile environment (**Figure 17**). Although water loss should be minimized to prevent desiccation, the eggshell should provide sufficient gas exchanges to allow embryo respiration. Eggshells therefore contain specialized regions that facilitate gas exchange between the embryo and the environment. Examples of such specialized structures for air transport include the dorsal appendages at the dorsal-anterior part of the egg surface, and the aeropyles at the posterior pole of the *Drosophila* egg. The eggshell also contains other specialized structures: at the anterior end are located both the micropyle, that forms a channel through the eggshell that allows the sperm to enter the oocyte surface for fertilization, and the operculum, which functions as the door through which the larva will exit the eggshell (Spradling, 1993).

Choriogenesis has been studied mostly in dipteran and lepidopteran insects, most notably in *D. melanogaster* and *B. mori* (Kafatos *et al*., 1995). The process of choriogenesis differs substantially between flies and moths: the structure of the *Drosophila* chorion is relatively simple and is composed of six major and 14 minor proteins (Kafatos *et al*., 1985, 1995); the silkmoth chorion, alternatively, is a more complex structure, consisting of more than 100 different polypeptides and becoming assembled in a stepwise fashion over a period of approximately 2 days (Kafatos *et al*., 1977, 1985, 1995). Similar complex chorion structures are

observed in other lepidopteran insects of the Bombycoidea superfamily (Regier *et al.*, 1995). Recently, the structure of the chorion of a coleopteran insect, the Colorado potato beetle *Leptinotarsa decemlineata*, has been also presented (Papassideri *et al.*, 2003).

The evolution of the structure of the chorion in lepidopteran insects has also been reviewed (Regier *et al.*, 1995), and the principles that govern the molecular architecture of helicoidal proteinaceous eggshells of the Lepidoptera have described in detail by Hamodrakas (1992). Interestingly, Hamodrakas and his collaborators have provided evidence that the silkmoth eggshell may be a natural protective amyloid (Iconomidou *et al.*, 2000), and described how these amyloids are formed *in vitro* via a liquid crystalline mesophase (Hamodrakas *et al.*, 2004; see also **Chapter 4.2**).

After secretion of the chorion proteins and assembly of the eggshell, the latter becomes stabilized through an extensive crosslinking process that involves peroxidase activity. Peroxidase catalyzes the formation of di- and tri-tyrosyl crosslinks among chorion proteins, a process that strengthens the eggshell. The peroxidase activity is initiated through the production of hydrogen peroxide by the cells of the follicular epithelium (Margaritis, 1985; Han *et al.*, 2000). In mosquitos, the process of chorion tanning (melanin formation) requires tyrosine as well as the action of the enzymes phenol oxidase and dopa decarboxylase (Li, 1994; Ferdig *et al.*, 1996).

1.3.4.1. Chorion Genes and Regulation of Chorion Gene Expression

The early studies on the topic have been reviewed extensively in the past (Regier *et al.*, 1995; Eickbush and Izzo, 1995; Goldsmith and Kafatos, 1984; Kafatos *et al.*, 1987, 1995). In this section, most recent information related to the structure of chorion genes and the regulation of their expression is reviewed, which has been obtained through the study of the two major insect models, the fruit fly *D. melanogaster*, and the silkmoth, *B. mori*.

1.3.4.1.1. *Drosophila melanogaster* Starting from the VM and moving in an outward fashion, the *Drosophila* chorion consists of four layers, the wax layer, the innermost chorionic layer (ICL), the endochorion, and the exochorion (**Figure 17**; Spradling, 1993; Trougakos and Margaritis, 1998). The chorion consists mainly of six major and 14 minor proteins. The genes that encode two major and four minor proteins (s36, s37, s38, A, B, C) are clustered on the X chromosome (region 7F1; Parks *et al.*, 1986; Orr-Weaver, 1991), while those for the four other major proteins (s15, s16, s18, s19) are encoded in a cluster on chromosome 3 (region 66D11-15; Kafatos *et al.*, 1985, 1995). The general features of the chorion genes are their organization in tandem arrangement in both clusters (**Figure 18**), the presence of a small intron near the 5′-end of each gene, and the presence of repeated sequences in the protein coding regions.

The genes on the X cluster are considered "early" genes, since they are expressed during stages 11–13 of oogenesis, when the ICL and endochorion components are produced. The genes on the third chromosome cluster, however, are expressed during stages 13 and 14, when the exochorion is deposited and, thus, represent "late" genes (Mariani *et al.*, 1988; Tolias and Kafatos, 1990). While the chorion proteins play a structural role in chorion assembly, it has also been proposed that s38 corresponds to the eggshell peroxidase enzyme (Keramiris *et al.*, 1991). The organization of the chorion gene clusters is conserved in the Drosophilidae family and in the more distantly related medfly *Ceratitis capitata* (Vlachou and Komitopoulou, 2001).

Recently, a novel chorion protein of *Drosophila*, Femcoat, was isolated. The *femcoat* gene is expressed in the follicular cells during stages 12B to 14A of oogenesis. Double-stranded RNA-mediated RNA interference has shown that Femcoat is essential for the formation of the endochorion (Kim *et al.*, 2002).

1.3.4.1.1.1. Chorion gene amplification To accommodate the high levels of gene expression that are required for the production of the chorion during a period of only 1–2 h (stages 11–14 of oogenesis), gene amplification occurs in both chorion gene clusters of *Drosophila* (Kafatos *et al.*, 1985; Kalfayan *et al.*, 1985). The X-chromosome and third chromosome clusters are amplified 15- to 20-fold and 60- to 100-fold, respectively, above the remainder of the genome of the already polyploid (see below) cells of the follicular epithelium (Calvi and Spradling, 1999).

The selective increase in gene copy number occurs through repeated initiation of replication forks at selected sites in these loci (**Figure 18**). Because replication forks continue to move outwards as choriogenesis progresses, a gradient of amplified DNA extending 40–50 kb to either side of the chorion gene cluster is generated that can be observed as "onion-like" structures in the electron microscope (**Figure 18**) (Orr-Weaver, 1991; Royzman and Orr-Weaver, 1998).

Genetic analysis in transgenic flies identified the *cis*-regulatory elements that are required for chorion gene amplification, termed amplification controlling

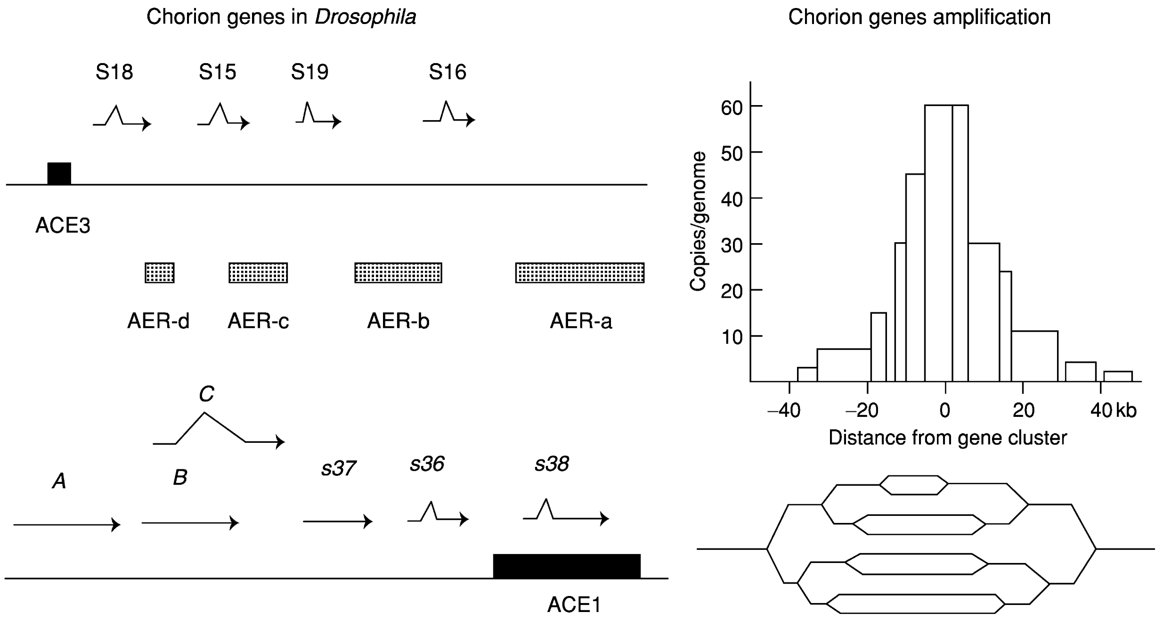


Figure 18 Chorion gene organization and mechanism of chorion gene amplification in *Drosophila*. Left panel: Structure of the chorion gene clusters. The third chromosome cluster above contains the genes for four major chorion proteins, s18, s15, s19, and s16. The X chromosome cluster below contains two major chorion genes, *s36* and *s38*, as well as several genes that encode minor chorion proteins (*A*, *B*, *C*, and *s37*). The positions of the transcripts are shown by the lines with arrows. Introns are indicated except for *A*, *B*, and *s37*, for which introns have not been mapped. The position of the amplification regulatory regions, AERs a–d (amplification enhancing regions a–d), and ACE1 (amplification control element 1), are also indicated. Right panel: Chorion gene amplification results from multiple rounds of reinitiation of DNA replication within the chorion gene cluster and progressive movement of replication forks to either side. Above is shown the level of amplification of DNA fragments at various distances from the origin of replication. Below is illustrated the ''onion-like'' structure of the nested bi-directional replication forks that are observed during chorion gene amplification. (Reprinted from Orr-Weaver, T.L., **1991**. *Drosophila* chorion genes: cracking the eggshell's secrets. *BioEssays 13*, 97–105.)

elements and amplification-enhancing regions (ACEs and AERs, respectively; **Figure 18**) (Kalfayan *et al*., 1985; Spradling *et al*., 1987; Delidakis and Kafatos, 1987; Swimmer *et al*., 1989; Lu *et al*., 2001). The origin of replication (Ori-β) within the third chromosomal cluster was also identified by two-dimensional gel electrophoresis (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Analysis of thin chorion mutants has revealed the involvement of components of the DNA replication complex, such as members of the origin recognition complex (ORC), Cdc7-Dbf4 kinase, double-parked protein (Dup)/Cdt1, and the minichromosome maintenance (MCM) protein complex (Landis *et al*., 1997; Landis and Tower, 1999; Claycomb *et al*., 2002; Schwed *et al*., 2002).

The initiation of chorion gene amplification is under cell cycle control. During their developmental career, two major switches in cell cycle control occur in the cells of the follicular epithelium. The follicular cells undergo mitotis until stage 6 of oogenesis, after which they undergo three endocycles (cycles consisting only of an S phase and a gap phase) and become polyploid (16N; Calvi *et al*., 1998; see **Chapter 1.2**). At the beginning of choriogenesis, the endocycles cease and DNA amplification is initiated at specific sites in the genome where the chorion gene clusters are located. The cyclin E cell cycle regulator plays a major role in orchestrating this transition: at the transition to choriogenesis, cyclin E switches from a cycling pattern of expression to continuous high expression in all follicle cell nuclei. It has been proposed that continuous high levels of cyclin E prevent general DNA replication in the follicular cell genome, while specific factors recruited at the chorion gene origins allow the latter to escape this negative replication control (Calvi *et al*., 1998). Factors that are involved in restricting or recruiting the replication machinery, specifically to the chorion origins of replication, are proposed to include the S-phase regulator E2F/DP/Rbf (Royzman *et al*., 1999; Cayirlioglu *et al*., 2001; Bosco *et al*., 2001) and a Myb (Myeloblastosis)-containing protein complex (Beall *et al*., 2002).

1.3.4.1.1.2. cis-Regulatory elements and factors that bind chorion gene promoter elements Promoter analysis in transgenic flies has been carried

out mainly for the *s15* and *s36* chorion genes, and *cis*-regulatory elements for temporal, spatial, and quantitative control have been delineated (Spradling, 1993; Kafatos *et al*., 1995). Short (less than 100–400 bp) segments of 5′-flanking DNA are sufficient to confer correct tissue-specific expression and precise temporal regulation of the transgenes (Mariani *et al*., 1988; Romano *et al*., 1988). *Cis*-acting regulatory elements, identified by mutational analysis, include an essential positive element, characterized by the sequence TCACGT that is conserved in all the chorion gene promoters, negative and positive elements that confer temporal (early or late) specificity, and elements defining spatially restricted expression (review: Orr-Weaver, 1991; Tolias *et al*., 1993; Mariani *et al*., 1996). In the latter case, the elements delineate expression in specific subregions of the epithelial surface, notably at the anterior and posterior poles and at the dorsal-anterior surface, where the dorsal appendages are formed (Parks and Spradling, 1987; Tolias and Kafatos, 1990).

Gel mobility shift assays were used to isolate putative *trans*-acting factors that bind and activate chorion gene regulatory regions (Kafatos *et al*., 1995). After a binding site was defined using nuclear extracts of choriogenic follicles, oligonucleotides encompassing the binding site were subsequently used to screen expression libraries and isolate binding proteins. This strategy resulted in the isolation of seven cDNAs that encode putative chorion gene transcription factors (chorion factors (CF) 1–7; Kafatos *et al*., 1995). Of these factors, CF1 and CF2 have been analyzed in more detail (Shea *et al*., 1990).

CF1 binds to the essential hexamer-element TCACGT. This factor was shown to correspond to the nuclear receptor USP (Henrich *et al*., 1990; Oro *et al*., 1990; Khoury-Christianson *et al*., 1992), the homolog of the vertebrate RXR receptor that functions as the heterodimer partner of the ecdysone receptor, EcR (Thomas *et al*., 1993; Yao *et al*., 1993). However, genetic analysis has shown that the involvement of CF1/USP in chorion gene expression may be more complicated than initially suggested by the DNA binding studies. Eggs laid by mutant flies that were usp^- in the germline cells (nurse cell oocyte complex) but wild-type (usp^+) in the somatic tissues (including follicular cells), showed a chorion defect, indicating that CF1/USP is required in the germline (but not in the follicular epithelium), in order to trigger the release of a signal that acts on the follicular cells and promotes chorion gene expression. *In situ* hybridization also showed that CF1/USP mRNA is abundant in the oocyte/nurse cell complexes and only barely detectable in follicular cells (Oro *et al*., 1992). The other possible roles of the EcR/USP complex during *Drosophila* oogenesis have already been discussed (Section 1.3.3.1.1.1.3).

CF2 was isolated by its property to bind the positive regulatory region that confers late temporal specificity to the *s15* promoter. It is a C_2H_2 zinc finger protein belonging to the class defined by the *Drosophila* regulatory gene *Krüppel* (*Kr*) and the mouse genes that encode the transcription factors MKR1 and MKR2 (Hsu *et al*., 1992). Genetic analysis, however, has shown that CF2 is involved in the dorsoventral patterning of the follicular epithelium (Hsu *et al*., 1996, Mantrova and Hsu, 1998). Upon activation of the epidermal growth factor receptor (EGFR) by the oocyte-derived Gurken (GRK) signal at the dorsal-anterior region of the follicular epithelium, CF2 is down-regulated by a posttranslational mechanism that involves phosphorylation by mitogen-activated protein kinase (MAPK) and subsequent cytoplasmic retention and degradation (Hsu *et al*., 1996, 2001; Mantrova and Hsu, 1998). Thus, CF2 **protein expression becomes restricted to the ventral** region of the follicular epithelium. One target gene of CF2 is *rhomboid* (*rho*), which encodes a transmembrane protein acting as a positive cofactor in EGFR signaling (Mantrova and Hsu, 1998). Because CF2 acts as a transcriptional repressor, degradation of CF2 in the dorsal cells of the follicular epithelium results in enhanced expression of RHO, which, in turn, results in the amplification of EGFR signaling levels in the dorsal-anterior follicular cells (Hsu *et al*., 1996; Mantrova and Hsu, 1998). The high levels of EGFR signaling are necessary for the formation of the dorsal appendages, the specialized chorion structures that mediate gas exchange between the environment and the embryo. In conclusion, however, as was the case with CF1, genetic analysis did not confirm a role for CF2 as a direct transcriptional regulator of the chorion genes.

Other chorion gene promoter binding factors that have been isolated and characterized include CF3, a zinc finger protein that interacts with the negative regulatory region in the *s15* promoter that is required for repression of its expression during early choriogenesis; three more factors (CF5–7) that bind the late activating region of the *s15* promoter; and CF4, which binds the early temporal element in the *s36* promoter (Orr-Weaver, 1991; Kafatos *et al*., 1995). CF5 has been shown to represent the *Drosophila* homolog of the human structure-specific recognition protein (SSRP), which belongs to the class of high-mobility-group (HMG) proteins (Hsu *et al*., 1993). HMG box-containing proteins are thought to function in processes such as DNA replication, DNA repair, and chromatin modeling, which may be involved in the regulation of transcription. The

localization of CF5 in the nucleolus and the observation that it binds single-stranded nucleic acid suggests that it may be involved in rRNA transcription through an RNA or single-stranded DNA binding mechanism (Hsu *et al.*, 1993). Therefore, the role of factor CF5 in the regulation of chorion genes, as well as that of the other factors, CF3–4 and CF6–7, is not clear at present (Kafatos *et al.*, 1995).

Recently, genetic analysis has indicated a role for the transcriptional regulator Tramtrack 69 (TTK69) in the regulation of chorion gene expression (French *et al.*, 2003). Carriers of the female sterile allele of *tramtrack* (*ttk*) called *twin peaks* (ttk^{twk}), which affects the TTK69 transcription factor, are characterized by thin eggshells, presumably due to low levels of accumulation of mRNAs of the major chorion genes. However, the defects due to loss of TTK69 are considered to be indirect, because TTK proteins are known to function as transcriptional repressors, and no binding sites could be identified within 1 kb of any of the major chorion genes (French *et al.*, 2003). It is, therefore, likely that TTK69 regulates the expression of factors upstream of the chorion genes rather than the chorion genes themselves. Besides influencing general chorion gene expression, TTK69 also has an independent genetic function in the formation of the dorsal appendages of the eggshell (French *et al.*, 2003; see also below).

1.3.4.1.1.3. Signaling pathways Formation of the anterior and dorsal structures in the eggshell, such as the operculum, the micropyle, and the dorsal appendages, are subject to multiple signaling pathways (see Section 1.3.3.6). What will be discussed briefly here are transcription factors involved in the execution of the program of the formation of the dorsal appendages, two structures at the dorsal-anterior end of the eggshell that are responsible for gas exchange.

Signals that stimulate the EGFR in the follicular cells originally emanate from the Gurken (GRK, a TGFβ homolog) signal produced by the oocyte nucleus that is located at the dorsal-anterior end of the oocyte. Positive and negative feedback loops of signaling at the dorsal-anterior surface ultimately result in two patches of follicular cells, where high levels of EGFR signaling (Ras and MAPK activity) occur. These two patches of follicular cells are destined to form the dorsal appendages of the eggshell (Wasserman and Freeman, 1998; reviews: Stevens, 1998; Van Buskirk and Schüpbach, 1999).

Dorsal appendage formation can be divided in two stages; first, an initiation phase, during which the two groups of dorsal-anterior follicular cells move outward from the rest of the follicular epithelium and form a short tube overlying the junction between oocyte and nurse cells (stages 10B to 12 of oogenesis); and second, an elongation phase, during which the two tubes extend to their full lengths (French *et al.*, 2003). The transcription factors Broad-Complex (BR-C), Pointed (PNT), and CF2 seem to have a role during the initiation phase of dorsal appendage formation.

Broad-Complex is a transcription factor characterized by C-terminally located C_2H_2 zinc fingers and an N-terminal Broad-Complex/Tramtrack/Bric-à-Brac (BTB) or poxvirus and zinc finger (POZ) domain, which appears to be involved in protein–protein interactions (DiBello *et al.*, 1991; Collins *et al.*, 2001; see also **Chapter 1.8**). The Z1 isoform of BR-C becomes expressed in the patches of follicular cells destined to form the dorsal appendages, and may be an upstream regulator of this process (Huang and Orr, 1992; Deng and Bownes, 1997).

Pointed is an ETS domain-containing transcription factor that is induced at the stage, when the highest levels of EGFR signaling occur. The expression of PNT results in a downregulation of the EGFR pathway, possibly through the induction of the expression of Argos (AOS), a putative extracellular inhibitor of EGFR (Schweitzer *et al.*, 1995; Morimoto *et al.*, 1996). The result of the action of PNT is the splitting of the original EGFR signaling area into two symmetrical patches across the dorsal midline of the egg, which will generate the two dorsal appendages.

As already mentioned earlier, CF2 is a transcriptional repressor whose expression is downregulated by EGFR signaling. Downregulation of CF2 results in the induction of RHO expression, which is involved in the amplification of the EGFR signal (Hsu *et al.*, 1996; Mantrova and Hsu, 1998).

Finally, the Jun N-terminal kinase (JNK) cascade is involved in the correct execution of the extension phase of dorsal appendage formation (Dequier *et al.*, 2001; Suzanne *et al.*, 2001; French *et al.*, 2003). Members of the JNK cascade include the *Drosophila* Jun N-terminal kinase kinase (DJNKK) or Hemipterous (HEP), DJNK or Basket (BSK), the transcription factors Djun and Dfos, and the JNK phosphatase or Puckered (PUC). In addition, the TTK69 transcriptional repressor (see above) also acts at the extension phase of dorsal appendage formation. Finally, the JNK signaling cascade is also required in the process of micropyle formation (Suzanne *et al.*, 2001).

1.3.4.1.2. Lepidopteran insects The eggshell of lepidopteran insects constitutes a tripartite structure, consisting of the inner vitelline membrane, the chorion, and a very thin outer sieve layer (**Figure 17**;

Kafatos *et al*., 1977). The thickness of the vitelline membrane usually is <2 μm, while the chorion can vary, depending on the species, from <1 μm to >50 μm (Regier and Vlachos, 1988; Leclerc and Regier, 1993). Two regions of the chorion are easily discernible, an inner trabecular layer and a much thicker overlaying lamellar chorion (**Figure 17**). Because the lamellar zone consists of helicoidally arranged planes of fibers, it is referred to as a "helicoidal lamellar chorion" (Mazur *et al*., 1982; Regier *et al*., 1995).

The helicoidal lamellar chorion structure is restricted to ditrysian lepidopteran insects. It is absent from the other insect orders, including the sister order of the Lepidoptera, the Trichoptera (Regier *et al*., 1995). Helicoidal lamellar chorion structures and protein compositions have been studied in detail in the superfamilies of Bombycoidea (mainly *A. polyphemus* and *B. mori*), Sphingoidea (*M. sexta*), and Noctuidea (*Lymantria dispar*) (Regier and Vlachos, 1988; Leclerc and Regier, 1993; Regier *et al*., 1995).

In the superfamily of Bombycoidea, the lamellae in the helicoidal lamellar chorion can number more than 100, and they are assembled by chorion proteins that can be grouped into two branches, α and β, and that belong to six families (ErA and ErB, A and B, HcA and HcB) (Regier *et al*., 1980, 1982; reviews: Kafatos *et al*., 1995; Eickbush and Izzo, 1995; see also Section 1.3.4.1.3 below). Other substructures of the chorion, the trabecular layer at the inner surface and the specialized structures at the external surface, generally contribute to no more than 5% of the mass of the chorion and contain unique subsets of proteins.

The formation of the helicoidal lamellar chorion is proposed to occur in four temporally discrete steps, framework formation (small number of lamellae are deposited), expansion (thickening of existing lamellae), densification (thickening of fibers within lamellae), and surface sculpturing (deposition of additional lamellae that can be sculpted into surface structures, e.g., the aeropyle crowns of *A. polyphemus* eggs; Regier *et al*., 1982; Mazur *et al*., 1989; Leclerc and Regier, 1993).

The different steps can be correlated with the expression of different types of chorion proteins that are synthesized during different periods in choriogenesis. Thus, in *B. mori*, ErA and ErB proteins are produced during framework formation, A and B proteins are secreted during the expansion and densification processes, and the formation of specialized outer lamellae is dependent on HcA and B proteins (see Section 1.3.4.1.3 below). The evolution of the chorion structure within ditrysian lepidopteran insects is proposed to have occurred by shifts in the timing and the intensity of protein synthesis in the four steps of assembly of the helicoidal lamellar chorion (Regier *et al*., 1995).

1.3.4.1.3. *Bombyx mori* The chorion or eggshell of *B. mori* (**Figure 17**) is an elaborate structure consisting of more than 100 polypeptides that are produced and secreted by the follicular cells during their terminal differentiation (Nadel *et al*., 1980; Iatrou *et al*., 1982). The chorion polypeptides are of low molecular weight (10–25 kDa) and have a tripartite structure consisting of a highly conserved central domain that is class-specific (A or B, see below), flanked by more variable left and right arms (Tsitilou *et al*., 1983). Sequence analysis reveals that chorion genes belong to a single gene superfamily that can be divided into two branches, α and β (A-type and B-type members). Each branch can be subdivided into three families or ErA/ErB, A/B, and HcA/HcB (Lecanidou *et al*., 1986).

Choriogenesis in *Bombyx* occurs in four broad developmental periods. Thus, early choriogenesis is characterized by the production of the ErA and ErB proteins (stages +1 to +8 in an ovariole with a choriogenic segment of 40 follicles). Most A and B proteins are synthesized during the early-middle and late-middle periods of choriogenesis (stages +6 to +24 and +10 to +28, respectively). Finally, late choriogenesis (stages +26 to +35) involves the production of high-cysteine (Hc) proteins HcA and HcB (Bock *et al*., 1986; Spoerel *et al*., 1986).

The chorion proteins that are produced during different developmental periods have specialized functions. Early proteins form an initial scaffold, into which middle proteins intercalate to add mass and density, while late (high-cysteine) proteins percolate throughout the previously deposited lamellae to crosslink the whole chorion and, in addition, form the outer lamellate crust (Mazur *et al*., 1982; Regier *et al*., 1982, 1995).

Classical genetic analysis, using protein electrophoretic variants as genetic markers, revealed that chorion genes are organized in two clusters on chromosome 2, Ch1–2, and Ch3 (**Figure 19**), that are separated from each other by 3–4 map units (Goldsmith, 1989). The organization of the chorion genes in two clusters was confirmed after isolation of overlapping chromosomal DNA clones, corresponding to the chorion locus, from genomic libraries of *Bombyx* (**Figure 19**). From cluster Ch1–2, two contiguous chromosomal DNA segments with sizes of 270 kb and 50 kb were isolated. The 270 kb fragment has a threefold organization, in which a central 140 kb region that contains the late Hc chorion genes, is flanked by segments that contain chorion genes of early-middle and late-middle specificity

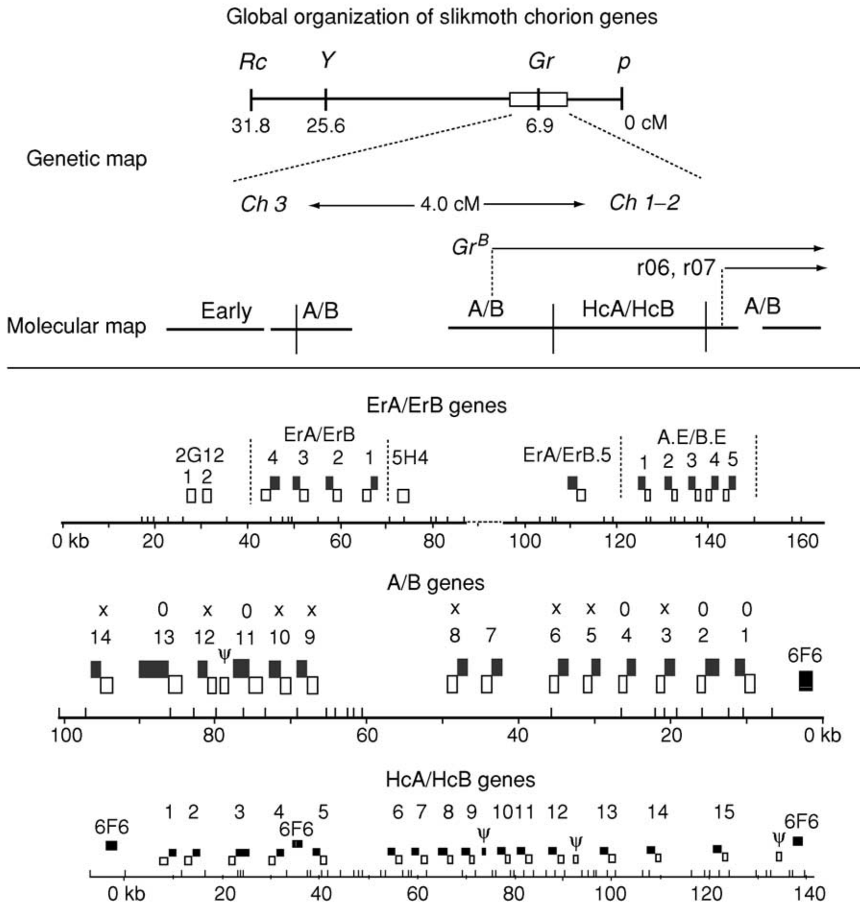


Figure 19 Chorion gene organization in *Bombyx*. Top panel: A portion of the genetic map of chromosome 2 is shown, with the genetic distances in centiMorgans (cM), for the following markers: *p*, plain larval markings; *Gr*, gray eggs; *Y*, yellow hemolymph and cocoons; and *Rc*, rusty cocoon color. The structural genes of the chorion proteins have been mapped to the gene complexes *Ch1–2* and *Ch3* (Goldsmith, 1989). Below the genetic map an overview of the molecular map is shown: the thick horizontal lines correspond to chromosomal segments from *Ch1–2* and *Ch3* that have been recovered as series of overlapping recombinant clones. The major families of chorion genes (early, A, B, HcA, and HcB) present on each cloned segment are indicated. Vertical lines on these chromosomal segments correspond to the boundaries between these gene families. An additional 80 kb of chromosomal DNA (not shown in the figure) has been recovered as a series of unlinked genomic clones containing A and B genes. The orientation and distance of the cloned segments from *Ch3* relative to that of the segments from *Ch1–2* are unknown. The segments of the *Ch1–2* complex deleted by the mutation, Gr^B, or translocated in mutants r06 and r07, are shown by the arrows above the cloned segments. Bottom panel: Organization of the early chorion gene complex (upper), the A/B gene cluster immediately to the left of the HcA/B gene cluster (middle), and the HcA/B gene cluster (lower). Filled boxes are α genes (ErA, A.E, A, and HcA) while open boxes represent β genes (ErB, B.E, B, HcB, 5H4, and 2G12). Almost all α and β genes are paired, with the exception of the 5H4 and two 2G12 genes. Pseudogenes are indicated by Ψ and the unpaired 6F6 (β branch type) genes are indicated by stippled boxes. Numbers refer to distances in kb. (Compiled from Eickbush, T.H., Izzo, J.A., **1995**. Chorion genes: molecular models of evolution. In: Goldsmith, M.R., Wilkins, S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 217–247.)

(A and B genes). The 50 kb fragment also contains genes of middle developmental specificity (Eickbush and Kafatos, 1982; Spoerel *et al*., 1989). From cluster Ch3, two segments of 77 kb and 25 kb were characterized, in which early and early-middle chorion genes are present (Hibner *et al*., 1991).

The clustered organization of chorion genes on the chromosome according to their developmental specificity is intriguing, since it suggests that the different clusters may represent gene assemblies subject to common regulatory control, e.g., chromatin domains that become accessible to transcription factors at specific developmental stages (Eickbush and Kafatos, 1982; Goldsmith, 1989). Alternatively, the clustering of the chorion genes may be coincidental; it has been amply documented that diversification in the chorion locus has occurred by multiple duplication, deletion, inversion, and gene conversion events that involved relatively small patches of chromosomal DNA (Iatrou *et al*., 1984; Spoerel *et al*., 1986; Xiong *et al*., 1988;

Hibner *et al.*, 1991; review: Eickbush and Izzo, 1995). In addition, it can also be noted that three chorion genes with early developmental specificity (e.g., the 6F6 genes) are found interspersed in the Ch1–2 locus (Kravariti *et al.*, 1995), suggesting that opening of the Ch1–2 locus should occur concomitantly with the Ch3 locus and, therefore, arguing against the regulation of early versus middle and late chorion gene expression at the level of the gene cluster.

As a rule, chorion protein-encoding genes are organized in the *Bombyx* genome as gene pairs (**Figure 19**). Each gene pair consists of an A-type and B-type genes (ErA/ErB, A/B, and HcA/HcB), that undergo transcription in opposite and divergent direction from a common short (300 ± 30 bp) promoter region (Iatrou *et al.*, 1984; Spoerel *et al.*, 1986; Hibner *et al.*, 1988). Paired genes are coordinately expressed during choriogenesis (Spoerel *et al.*, 1986). Furthermore, through the use of heterologous functional assays, it has been demonstrated that the short spacer region between the two transcripts is necessary and sufficient for correct sex-, tissue-, and stage-specific expression (Mitsialis and Kafatos, 1985). A limited number of cases deviating from this rule of gene organization in pairs were also found. These involved some genes encoding early chorion genes, found in both the Ch1–2 and Ch3 clusters, which exist as single transcription units (Hibner *et al.*, 1988; Lecanidou and Rodakis, 1992; Kravariti *et al.*, 1995).

High-cysteine chorion proteins, that are synthesized during late choriogenesis, appear to be unique to *B. mori* and are not found in other silkmoths (Nadel and Kafatos, 1980). The production of high-cysteine proteins is a specialized (recently acquired during evolution) function of the *Bombyx* follicular epithelium: these proteins probably provide the eggshells with greater hardness and reduced permeability, both necessary for viability over the long periods of winter diapause (Regier *et al.*, 1995).

1.3.4.1.3.1. cis-Regulatory elements and factors that regulate chorion gene promoter elements Analysis of chorion gene expression in moths has been carried out using two different strategies. First, lepidopteran chorion promoters were cloned upstream of reporter cassettes and the activities of the chorion promoter–reporter cassettes and their mutant derivatives were assayed in transgenic *Drosophila* flies. Second, oligonucleotides encompassing chorion promoter segments were used in gel retardation assays to identify binding elements; the identification of binding sites subsequently allowed isolation of corresponding transcription factors or DNA binding proteins by homology cloning or screening of expression libraries (review: Kafatos *et al.*, 1995).

When a genomic fragment containing the early-middle gene pair *A/B.L12* was introduced into the genome of *D. melanogaster* by P-element transformation, the silkmoth genes were expressed with correct sex, tissue, and cell specificity (Mitsialis and Kafatos, 1985). Thus, *A* and *B* genes were expressed coordinately in *Drosophila*, as in *Bombyx*, and their expression patterns resembled that of the endogenous *Drosophila* late gene *s15*. Comparable results were obtained with constructs, in which the common promoter of the gene pair (300 ± 30 bp) was placed upstream of reporter genes, encoding chloramphenicol acetyl transferase (CAT) or β-galactosidase, in either orientation (Mitsialis *et al.*, 1987). It should be noted, however, that the temporal specificity was not strictly conserved between *Bombyx* and *Drosophila*, because the *A/B.L12* promoter, which is an early-middle promoter in *Bombyx*, behaved as a late promoter in *Drosophila* (Mitsialis *et al.*, 1989). Opposite shifts in temporal expression (from late to early), after introduction into the *Drosophila* genome, were also observed for chorion promoters from the moths *A. polyphemus* and *A. pernyi* (Mitsialis *et al.*, 1989).

The *cis*-regulatory elements of the bi-directional promoter of the *A/B.L12* chorion genes were defined after deletion and linker scanning mutagenesis in combination with P element-mediated genomic transformation of *Drosophila* (Mitsialis *et al.*, 1987, 1989; Spoerel *et al.*, 1993). Three important regulatory regions were detected, when mutant promoters were used in the heterologous functional assays. The regions were located asymmetrically in the promoters, and found to function in an orientation-independent manner and to have similar effects on the expression of both the A and the B genes in the pair. Thus, a TCACGT hexamer-element, located 60 bp upstream of the transcription start site of the *B* gene and found in the promoters of all silkmoth and *Drosophila* chorion genes, except the *ErA/B* genes of *Bombyx* (Hibner *et al.*, 1991; Kafatos *et al.*, 1995), was found to be absolutely necessary for expression; disruption of this element abolished expression of both the *A* and the *B* genes. Second, mutations in region A1, a 35 bp sequence located 185–220 bp upstream of the transcription start site of the *B* gene, resulted in a decrease of expression levels and alterations in temporal specificity. The complex behavior of this region could best be explained in terms of it having three distinct functional properties, acting as a general activator, an early repressor, and a late activator. Lastly, region A2, a 35 bp sequence located 140–175 bp upstream

of the transcription start site of the *B* gene, was shown to have a general activator function; in contrast to region A1, mutations in this region did not result in altered temporal expression.

To isolate *trans*-acting factors involved in the regulation of chorion gene expression, gel retardation assays, using regions of the chorion promoters as probes, were first carried out to define DNA elements that interact with proteins from follicular cell nuclear extracts. In a few cases, the sequence features of the element allowed an accurate prediction of the type of transcription factor capable of binding the element (Skeiky and Iatrou, 1991; Sourmeli *et al.*, 2003). In other cases, oligonucleotides encompassing the elements were used to screen expression libaries from choriogenic follicles to isolate interacting factors (Kafatos *et al.*, 1995). This strategy was used in order to identify binding factors in early, early-middle, and late chorion gene promoters (Skeiky and Iatrou, 1991; Kafatos *et al.*, 1995; Sourmeli *et al.*, 2003).

1.3.4.1.3.2. Early chorion gene regulators Bandshift assays, in combination with recognition by specific antibodies, have revealed that a CAAT/enhancer binding protein (C/EBP)-like transcription factor may be an important regulator of early chorion gene expression (Sourmeli *et al.*, 2003). However, although C/EBP binding sites are more prevalent in early chorion gene promoters, such sites also occur in the chorion gene promoters of all developmental specificities. The C/EBP-like binding activity is also pronounced in nuclear extracts from early to middle choriogenic follicles, while its expression is reduced in late follicles. Thus, while the data indicate a predominant (positive) role for the C/EBP-like factor during the early stages of choriogenesis, other roles during the later stages of choriogenesis are also likely for this factor. It has been hypothesized that the C/EBP-like factor forms stage-specific complexes with other factors (e.g., the BmGATAβ factor, see below) during choriogenesis to direct stage-specific patterns of chorion gene expression (Sourmeli *et al.*, 2003).

1.3.4.1.3.3. Early-middle chorion gene regulators The strategy of screening a choriogenic follicular library with probes corresponding to binding elements in the early-middle chorion gene promoter has resulted in the isolation of five classes of cDNAs, BmMCBP1–5, which encode putative regulatory factors (Kafatos *et al.*, 1995). Of these factors, BmMCBP1–4 were found to bind the temporal regulatory region A1 (see previous section), while BmMCBP5 bound immediately upstream of the general activator region A2.

BmMCBP2, BmMCBP3, and BmMCBP5 each possess HMG-box (nucleic acid binding) regions; in addition, BmMCBP2 is characterized by a cysteine-rich region that resembles the zinc binding domain of the Trithorax (TRX) factor of *Drosophila*. BmMCBP4 has a central region that is homologous to the yeast SNF2 transcriptional activator. Finally, BmMCBP1 does not show significant homology to any transcription factors or other DNA binding proteins (Kafatos *et al.*, 1995).

In *Drosophila*, the CF1 factor was isolated by its capacity to bind the essential TCACGT element in chorion gene promoters (Shea *et al.*, 1990). Since the TCACGT element is also a prominent feature of all but the early *Bombyx* chorion gene promoters, it may be bound by the *Bombyx* homolog of CF1, BmCF1. BmCF1, which corresponds to the *Bombyx* homolog of USP, has been cloned (Tzertzinis *et al.*, 1994) and the expression pattern of its mRNA was studied during follicle development (Swevers *et al.*, 1995). The mRNA for BmCF1/USP is abundant in vitellogenic follicles, but its expression is down-regulated at the beginning of choriogenesis. Because data regarding the accumulation of the BmCF1/USP protein during oogenesis have not been reported, the role of BmCF1/USP in the control of chorion gene expression remains undetermined.

1.3.4.1.3.4. Late chorion gene regulators Gel retardation analysis using nuclear extracts of follicular cells resulted in the definition of two important binding sites in the common promoter region of one of the late gene pairs, HcA/B.12 (Skeiky and Iatrou, 1991). The DNA-binding proteins that bind these sites were designated *Bombyx* chorion factor I (BCFI; not to be confused with BmCF1/USP), and BCFII. BCFI has been considered to be the major determinant of late chorion gene expression, since binding of this factor to the late promoter region is prerequisite to the binding of BCFII and the formation of higher order complexes on the late promoters and, presumably, transcriptional activation (Skeiky and Iatrou, 1991). Both BCFI and BCFII factors have been cloned: BCFI corresponds to the BmGATAβ protein, a member of the GATA family of zinc finger motif-containing transcription factors (Drevet *et al.*, 1994), while BCFII has been shown recently to correspond to a C/EBP-like transcription factor (Sourmeli, 2004).

BmGATAβ transcription is initiated during late vitellogenesis (stage "−6"), consistent with the postulated role of the encoded protein as a major regulator of choriogenesis. Regulation of *BmGATAβ* gene expression is complex and occurs both post-transcriptionally and posttranslationally (Skeiky *et al.*, 1994; Drevet *et al.*, 1995) (**Figure 20**). Three

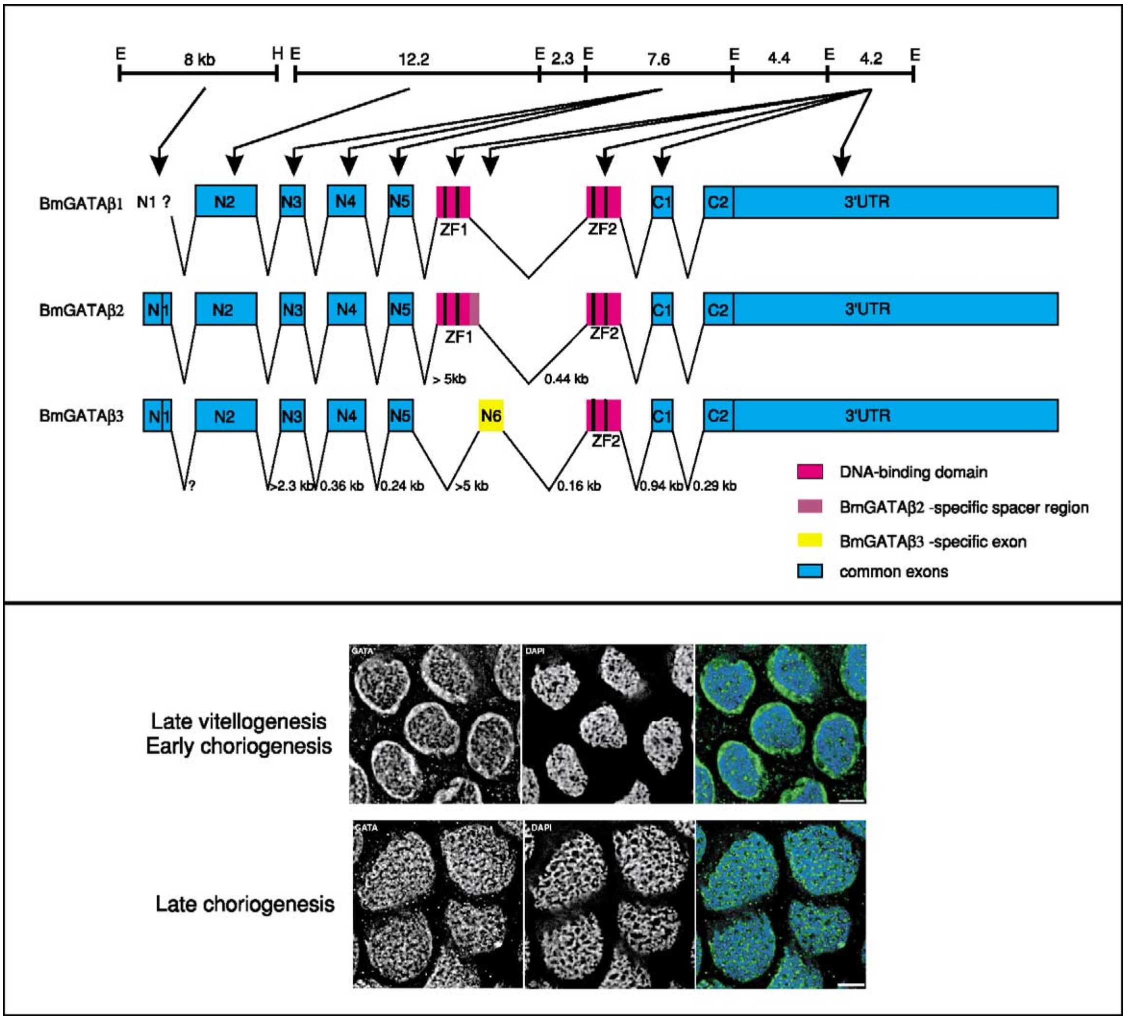


Figure 20 BmGATAβ transcription factors in *Bombyx mori*: structural features and subcellular localization in the cells of the follicular epithelium. Upper panel: Structure of the BmGATAβ gene and of the three major mRNA isoforms (β1, β2, and β3). The BmGATAβ gene consists of 10 exons, of which 9 exons could be located in a single contiguous DNA fragment that was assembled from overlapping genomic clones. The 10th exon, containing the N-terminal part of the β2 and β3 isoforms, belongs to a genomic clone that is not contiguous with the other genomic clones. E and H correspond to *Eco*RI and *Hin*dIII restriction sites, respectively, and the sizes of restriction fragments are indicated in kb. The positions of the exons of the BmGATAβ isoforms in the genomic DNA fragments are also indicated. The two exons that correspond to the N-terminal and C-terminal zinc fingers of the DNA-binding domain are in pink while the exons encoding protein regions N-terminal or C-terminal to the DNA-binding domain (N1-5 and C1-2, respectively) are in blue. In the BmGATAβ3 isoform, the exon of the first Zn finger is replaced by a unique sequence that is encoded by exon N6 (in yellow). Note that BmGATAβ1 and β2 isoforms differ by the spacing between the two zinc fingers (purple) and that the N-terminus of BmGATAβ1 is unknown. If known, the sizes of the introns that separate the different exons are indicated. Lower panel: Immunocytochemical localization of BmGATAβ protein in the follicular epithelial cells of ovarian follicles (Lunke, 2000). Shown is staining of follicular epithelia from late vitellogenic/early choriogenic follicles (top) and of follicular epithelia from late choriogenic follicles (bottom). Epithelia were treated with BmGATAβ antibody to localize BmGATAβ protein (left) or DAPI to stain nuclei (middle). At the right is shown an overlay of the left and middle images. Note that in late vitellogenic and early choriogenic follicles, BmGATAβ protein has both nuclear and perinuclear localization. In late follicles, however, BmGATAβ staining is exclusively nuclear. (Lower panel: Reprinted with permission from Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297; © Elsevier.)

major BmGATAβ mRNA isoforms are generated that differ in the organization of their DNA binding domains: BmGATAβ1 and BmGATAβ2 differ by the spacing between their zinc fingers while BmGATAβ3 has the N-terminal zinc finger replaced by a new domain (Drevet *et al.*, 1995). Because the zinc fingers of GATA transcription factors can have distinct functions in DNA binding and protein interaction (Omichinsky *et al.*, 1993; Whyatt *et al.*, 1993; Fox *et al.*, 1999), the different isoforms are probably involved in the regulation of different sets of target genes. BmGATAβ2 seems to be the most

likely candidate to represent BCFI and regulate late chorion gene expression, because its expression is gonad-specific and its transcripts accumulate in ovarian follicular cells preferentially during late choriogenesis. By contrast, BmGATAβ1 and β3 have more ubiquitous expression patterns (Drevet *et al.*, 1995), and may thus regulate gene expression in many different cell types.

The function of BCFI was also found to be regulated by posttranslational modification. Bandshift assays, using cytoplasmic and nuclear extracts of follicular cells, showed that BCFI remains predominantly in the cytoplasm of the follicular cells during early and middle choriogenesis, while during late choriogenesis, the GATA element (the late chorion promoter binding sequence for BCFI) binding activity is more concentrated in nuclear extracts (Skeiky *et al.*, 1994). Furthermore, the distribution between cytoplasm and nucleus and the DNA binding specificity of BCFI were shown to be dependent of the phosphorylation status of this factor. However, immunocytochemistry experiments using an antibody that recognizes all BmGATAβ isoforms revealed that the BmGATAβ protein is present in the nuclei of follicular cells at all stages of choriogenesis, although cytoplasmic staining could also be observed in the cytoplasm of follicular cells of early and middle choriogenic follicles (**Figure 20**; Lunke, 2000; Swevers and Iatrou, 2003). A possible explanation may be that, of the existing multiple BmGATAβ isoforms, only one corresponds to BCFI and is involved in late chorion gene expression. While BCFI undergoes stage-specific nucleocytoplasmic shuttling, other isoforms may have a constitutive nuclear localization (Lunke, 2000) and be involved in the regulation of the expression of other target genes.

Because BmGATAβ binding sites (GATA elements) and C/EBP binding elements are present in all chorion gene promoters, and variable forms of the BmGATAβ and C/EBP-like factors accumulate in the nuclei of the cells of the follicular epithelium with different temporal specificities, it has been also proposed that the regulation of chorion gene batteries of different temporal specificities is achieved by the accumulation of different types of heteromeric complexes that contain stage-specific combinations of the C/EBP-like and BmGATAβ factors (Sourmeli *et al.*, 2003). A complete understanding of the precise relationships among the various BmGATAβ isoforms, C/EBP proteins, and BCFI and BCFII-type factors, will require the availability and use of factor (and isoform)-specific antibodies in immunocytochemistry and gel retardation assays.

1.3.5. Ovulation and Egg Activation

Completion of choriogenesis is followed by ovulation. During ovulation, the layer of follicle cells is removed from the mature egg. The mature egg is subsequently squeezed out of the ovary into the lateral oviduct (Bloch Qazi *et al.*, 2003).

In *Drosophila* and other insects, ovulation is stimulated by mating and, specifically by sperm and some male accessory gland proteins (Heifetz *et al.*, 2000, 2001). Through a positive feedback mechanism, increased ovulation results in the stimulation of oogenic progression of follicles. The coupling of both processes ensures that high numbers of mature oocytes are produced and high numbers of fertilized eggs are deposited (Spradling, 1993).

After ovulation, egg activation is initiated, when the egg undergoes hydration in the oviduct (Heifetz *et al.*, 2001). Features of egg activation include reinitiation of meiotic divisions (relief of arrest at metaphase I), impermeabilization of the egg coverings to small solutes (through crosslinking of the vitelline membrane and chorion) and initiation of translation (Page and Orr-Weaver, 1997; Chen *et al.*, 2000; Bloch Qazi *et al.*, 2003). All these changes will prepare the egg for subsequent embryogenesis, should fertilization occur in the uterus (Bloch Qazi *et al.*, 2003). In *Drosophila* and many other insects, therefore, egg activation occurs independently of fertilization.

While the mature egg is activated in the oviduct, little is known about the fate of the follicular epithelium that was removed from the mature egg at ovulation. Most likely, these cells undergo necrosis or apoptosis and their remnants are removed by phagocytosis.

1.3.6. Conclusions and Perspectives

From the small number of insects in which oogenesis has been studied in sufficient detail, it is clear that considerable divergence exists in the regulation of oogenesis between different insects, e.g., the variety in the hormonal mechanisms that are involved in the control of vitellogenesis (Section 1.3.3.1). In some aspects, however, remarkable conservation can occur, as illustrated by the fact that lepidopteran chorion genes can become expressed with correct temporal and spatial specificity in transgenic *Drosophila* (Section 1.3.4.1.1.2). Presumably, the evidence related to the variety of control mechanisms that operate during insect oogenesis will increase even more, should oogenesis be studied in detail in more insect species. To have a more thorough understanding of the process of oogenesis in insects, many more models are therefore needed.

In this regard, it should be also noted that the insects that have been studied in detail thus far belong mainly to two orders, the Lepidoptera and the Diptera. Moreover, from these model insects, by far the greatest wealth of information is derived from the fruit fly, *D. melanogaster*. This is due to the fact that the methods of genetic analysis are very advanced in the fly and that *Drosophila* has functioned as a model not only for insects but, also, for many other multicellular eukaryotes, including humans.

Because methods of genetic analysis have not been used in other insects until very recently, data regarding the *in vivo* function of oogenesis-related factors in other insect species are lacking. Thus, for *B. mori*, only one report exists in the literature regarding the temporal specificity of chorion gene promoters after introduction of artificial promoter-reporter constructs into the cells of the follicular epithelia by the biolistic method (Kravariti *et al.*, 2001). In this study, a small 5′-flanking region of the 6F6 early gene and the small intergenic region of the late HcA/B.12 gene pair were shown to be able to direct expression of the reporter gene in the follicular epithelia with correct temporal specificity.

Recently, however, genetic transformation of non-drosophilid insects by transposable elements has become possible in a number of other dipteran insects, including *A. aegypti*, and the lepidopteran *B. mori* (Kidwell and Wattam, 1998; Tamura *et al.*, 2000; Horn and Wimmer, 2000; Handler, 2001; Atkinson, 2002). In addition, a technique for gene targeting in the silkmoth has been developed through the use of baculovirus vectors (Yamao *et al.*, 1999). Pantropic retroviral vectors and sindbis viruses are also regarded as promising tools for foreign gene delivery in somatic insect tissues (Jordan *et al.*, 1998; Foy *et al.*, 2004). Therefore, the availability of genetic transformation techniques for additional model insects will allow the *in vivo* functional testing of the involvement of particular factors that were identified by *in vitro* methods, in oogenesis. As long as these types of experiments have not been carried out, the function of the identified factors (e.g., in *Aedes* or in *Bombyx*) remains speculative.

Another recent development is the application of the technique of RNA interference (RNAi) to knock down the function of genes in cells and tissues (Hannon, 2002). RNAi is based on the introduction into the cells of double-stranded RNA, which becomes processed by the endogenous cytoplasmic enzyme Dicer into small (20–22 nt) double-stranded RNA species known as "small interfering RNAs" (siRNAs). siRNAs subsequently become integrated into a multiprotein complex known as the RNA-induced silencing complex (RISC), where they function as guide RNAs to target homologous mRNAs for cleavage and degradation (Dykxhoorn *et al.*, 2003). The advantage of the technique of RNAi is that it can be easily applied to knock down gene expression and that no extensive knowledge of the genetic make-up of a particular organism is required. The technique of RNAi has already been applied successfully in several insect species (Marie *et al.*, 1999; Lam and Thummel, 2000; Quan *et al.*, 2002; Bettencourt *et al.*, 2002; Blandin *et al.*, 2002; Rajagopal *et al.*, 2002; Valdes *et al.*, 2003; Uhlirova *et al.*, 2003), therefore it holds great promise for the genetic analysis of gene function in insects, in general, including oogenesis.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster*. *Science 287*, 2185–2195.

Al-Awqati, Q., **1986**. Proton-translocating ATPases. *Annu. Rev. Cell Biol. 2*, 179–199.

An, W., Wensink, P.J., **1995**. Three protein binding sites form an enhancer that regulates sex- and fat body-specific transcription of *Drosophila yolk protein* genes. *EMBO J. 14*, 1221–1230.

Andrenacci, D., Cavaliere, V., Cernilogar, F.M., Gargiulo, G., **2000**. Spatial activation and repression of the *Drosophila* vitelline membrane gene *VM32E* are switched by a complex *cis*-regulatory system. *Devel. Dyn. 218*, 499–506.

Andrenacci, D., Cernilogar, F.M., Taddei, C., Rotoli, D., Cavaliere, V., *et al.*, **2001**. Specific domains drive VM32E protein distribution and integration in *Drosophila* eggshell layers. *J. Cell Sci. 114*, 2819–2829.

Audit-Lamour, C., Busson, D., **1981**. Oogenesis defects in the *ecd-1* mutant of *Drosophila melanogaster*, deficient in ecdysteroid at high temperature. *J. Insect Physiol. 27*, 829–837.

Atkinson, P.W., **2002**. Genetic engineering in insects of agricultural importance. *Insect Biochem. Mol. Biol. 32*, 1237–1242.

Babin, P.J., Bogerd, J., Kooiman, F.P., Van Marrewijk, W.J.A., *et al.*, **1999**. Apolipophorin II/I, apolipoprotein B, vitellogenin and microsomal triglyceride transfer protein genes are derived from a common ancestor. *J. Mol. Evol. 49*, 150–160.

Baker, B.S., Wolfner, M.F., **1988**. A molecular analysis of doublesex, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster*. *Genes Devel. 2*, 477–489.

Baumann, C.A., Ribon, V., Kanzaki, M., Thurmond, D.C., Mora, S., *et al.*, **2000**. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature 407*, 202–207.

Beall, E.L., Manak, J.R., Zhou, S., Bell, M., Lipsick, J.S., *et al.*, **2002**. Role for a *Drosophila* Myb-containing protein complex in site-specific DNA replication. *Nature 420*, 833–837.

Bean, D.W., Shirk, P.D., Brookes, V.J., **1988**. Characterization of yolk proteins from the eggs of the Indianmeal moth, *Plodia interpunctella*. *Insect Biochem. 18*, 199–210.

Beckemeyer, E.F., Shirk, P.D., Development of the larval ovary in the moth, *Plodia interpunctella*. *J. Insect Physiol.* (in press).

Beenakkers, A.M., Van der Horst, D.J., Van Marrewijk, W.J., **1985**. Insect lipids and lipoproteins, and their role in physiological processes. *Prog. Lipid Res. 24*, 19–67.

Beisiegel, U., **1996**. New aspects on the role of plasma lipases in lipoprotein catabolism and atherosclerosis. *Atherosclerosis 124*, 1–8.

Bettencourt, R., Terenius, O., Faye, I., **2002**. *Hemolin* gene silencing by ds-RNA injected into Cecropia pupae is lethal to next generation embryos. *Insect Mol. Biol. 11*, 267–271.

Bhat, K.M., Schedl, P., **1997**. Establishment of stem cell identity in the *Drosophila* germline. *Devel. Dyn. 210*, 371–382.

Bieri, S., Djordjevic, J.T., Daly, N.L., Smith, R., Kroon, P.A., **1995a**. Disulfide bridges of a cysteine-rich repeat of the LDL receptor ligand-binding domain. *Biochemistry 34*, 13059–13065.

Bieri, S., Djordjevic, J.T., Jamshidi, N., Smith, R., Kroon, P.A., **1995b**. Expression and disulfide-bond connectivity of the second ligand-binding repeat of the human LDL receptor. *FEBS Lett. 371*, 341–344.

Blandin, S., Moita, L.F., Kocher, T., Wilm, M., Kafatos, F.C., *et al.*, **2002**. Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene. *EMBO Rep. 3*, 852–856.

Bloch Qazi, M.C., Heifetz, Y., Wolfner, M.F., **2003**. The developments between gametogenesis and female sperm storage in *Drosophila melanogaster*. *Devel. Biol. 256*, 195–211.

Bock, S.C., Campo, K., Goldsmith, M.R., **1986**. Specific protein synthesis in cellular differentiation. VI. Temporal expression of chorion gene families in *Bombyx* strain C108. *Devel. Biol. 117*, 215–225.

Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., *et al.*, **1999**. Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. *Cell 97*, 865–875.

Borovsky, D., Carlson, D.A., Griffin, P.R., Shabanowitz, J., Hunt, D.F., **1990**. Mosquito oostatic factor: a novel decapeptide modulating trypsin-like biosynthesis in the midgut. *FASEB J. 4*, 3015–3020.

Borovsky, D., Powel, P.A., Nayar, J.K., Blalock, J.E., Hayes, T.K., **1994a**. Characterization and localization of mosquito-gut receptors for trypsin modulating oostatic factor using a complementary peptide and immunocytochemistry. *FASEB J. 8*, 350–355.

Borovsky, D., Song, Q., Ma, M., Carlson, D.A., **1994b**. Biosynthesis, secretion and immunocytochemistry of trypsin modulating oostatic factor of *Aedes aegypti*. *Arch. Insect Biochem. Physiol. 27*, 27–38.

Bosco, G., Du, W., Orr-Weaver, T.L., **2001**. DNA replication control through interaction of E2F-RB and the origin recognition complex. *Nat. Cell Biol. 3*, 285–295.

Boswell, R.E., Mahowald, A.P., **1985**. *tudor*, a gene required for assembly of the germ plasm in *Drosophila*. *Cell 43*, 97–104.

Boswell, R.E., Prout, M.E., Steichen, J.C., **1991**. Mutations in a newly-identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation and result in the formation of mirror-image symmetrical double abdomen embryos. *Development 113*, 373–384.

Bownes, M., **1986**. Expression of the genes coding for vitellogenin (yolk protein). *Annu. Rev. Entomol. 31*, 507–531.

Bownes, M., **1989**. The roles of juvenile hormone, ecdysone and the ovary in the control of *Drosophila* vitellogenesis. *J. Insect Physiol. 35*, 409–413.

Bownes, M., **1992**. Why is there sequence similarity between insect yolk proteins and vertebrate lipases? *J. Lipid Res. 33*, 777–790.

Bownes, M., Pathirana, S., **2002**. The yolk proteins of higher Diptera. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Volume XII – Recent Progress in Vitellogenesis, Part A. Science Publishers, Inc., Enfield, NH, pp. 103–130.

Bownes, M., Scott, A., Shirras, A., **1988a**. Dietary components modulate yolk protein gene transcription in *Drosophila melanogaster*. *Development 103*, 119–128.

Bownes, M., Shirras, A., Blair, M., Collins, J., Coulson, A., **1988b**. Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *Proc. Natl Acad. Sci. USA 85*, 1554–1557.

Bownes, M., Steinmann-Zwicky, M., Nöthiger, R., **1990**. Differential control of yolk protein gene expression in fat bodies and gonads by the sex-determining gene *tra-2* of *Drosophila*. *EMBO J. 9*, 3975–3980.

Briegel, H., **1990**. Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. *J. Insect Physiol. 36*, 165–172.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., Woodard, C.T., **1999**. The *Drosophila* βFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell 3*, 143–149.

Brodsky, F.M., Chen, C.-Y., Knuehl, C., Towler, M.C., Wakeham, D.E., **2001**. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Devel. Biol. 17*, 517–586.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., *et al.*, **2001**. An evolutionary conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr. Biol. 11*, 213–221.

Brown, M.R., Graf, R., Swiderek, K.M., Fendley, D., Stracker, T.H., *et al.*, **1998**. Identification of a steroidogenic hormone in female mosquitoes. *J. Biol. Chem. 273*, 3967–3971.

Brown, M.S., Goldstein, J.L., **1986**. A receptor-mediated pathway for cholesterol homeostasis. *Science 232*, 34–47.
Bu, G., Schwartz, A.L., **1994**. Receptor-mediated endocytosis. In: Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Schachter, D.A., Shafritz, D. (Eds.), The Liver: Biology and Pathobiology. Raven Press Ltd., NY, pp. 259–274.
Bujo, H., Hermann, M., Kaderli, M.O., Jacobsen, L., Sugawara, S., *et al.*, **1994**. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. *EMBO J. 13*, 5165–5175.
Bujo, H., Lindstedt, K.A., Hermann, M., Dalmau, L.M., Nimpf, J., *et al.*, **1995**. Chicken oocytes and somatic cells express different splice variants of a multifunctional receptor. *J. Biol. Chem. 270*, 23546–23551.
Büning, J., **1994**. The Insect Ovary. Ultrastructure, Previtellogenic Growth and Evolution. Chapman and Hall, London.
Burke, T., Waring, G.L., Popodi, E., Minoo, P., **1987**. Characterization and sequence of follicle cell genes selectively expressed during vitelline membrane formation in *Drosophila melanogaster. Devel. Biol. 124*, 441–450.
Buszczak, M., Freeman, M.R., Carlson, J.R., Bender, M., Cooley, L., *et al.*, **1999**. Ecdysone response genes govern egg chamber development during mid-oogenesis in *Drosophila*. *Development 126*, 4581–4589.
Bylemans, D., Borovsky, D., Hunt, D.F., Shabanowitz, J., Grauwels, L., *et al.*, **1994**. Sequencing and characterization of trypsin modulating oostatic factor (TMOF) from the ovaries of the grey fleshfly, *Neobellieria bullata. Regul. Pept. 50*, 61–72.
Bylemans, D., Proost, P., Samyn, L., Grauwels, J., Van Damme, B., *et al.*, **1995**. Neb-colloostatin, the second folliculostatin of the grey fleshfly, *Neobellieria bullata*. *Eur. J. Biochem. 228*, 45–49.
Bylemans, D., Verhaert, P., Janssen, I., Vanden Broeck, J., Borovsky, D., *et al.*, **1996**. Immunolocalization of the oostatic and prothoracicostatic peptide, Neb-TMOF, in adults of the fleshfly, *Neobellieria bullata*. *Gen. Comp. Endocrinol. 103*, 273–280.
Byrne, B.M., Gruber, M., Ab, G., **1989**. The evolution of egg yolk proteins. *Prog. Biophys. Mol. Biol. 53*, 33–69.
Calvi, B.R., Lilly, M.A., Spradling, A.C., **1998**. Cell cycle control of chorion gene amplification. *Genes Devel. 12*, 734–744.
Calvi, B.R., Spradling, A.C., **1999**. Chorion gene amplification in *Drosophila*: a model for metazoan origins of DNA replication and S-phase control. *Methods 18*, 407–417.
Campbell, I.D., Bork, P., **1993**. Epidermal growth factor-like modules. *Curr. Opin. Struct. Biol. 3*, 385–392.
Cant, K., Knowles, B.A., Mooseker, M.S., Cooley, L., **1994**. *Drosophila* singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. *J. Cell Biol. 125*, 369–380.
Carney, G.E., Bender, M., **2000**. The *Drosophila* ecdysone receptor (EcR) gene is required maternally for normal oogenesis. *Genetics 154*, 1203–1211.
Castelli-Gair Hombría, J., Brown, S., **2002**. The fertile field of *Drosophila* JAK/STAT signalling. *Curr. Biol. 12*, R569–R575.
Cavaliere, V., Spanò, S., Andrenacci, D., Cortesi, L., Gargiulo, G., **1997**. Regulatory elements in the promoter of the vitelline membrane gene *VM32E* of *Drosophila melanogaster* direct gene expression in distinct domains of the follicular epithelium. *Mol. Gen. Genet. 254*, 231–237.
Cavaliere, V., Taddei, C., Gargiulo, G., **1998**. Apoptosis of nurse cells at the late stages of oogenesis of *Drosophila melanogaster. Devel. Genes Evol. 208*, 106–112.
Cayirlioglu, P., Bonnette, P.C., Dickson, M.R., Duronio, R.J., **2001**. *Drosophila E2f2* promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells. *Development 128*, 5085–5098.
Cernilogar, F.M., Fabbri, F., Andrenacci, D., Taddei, C., Gargiulo, G., **2001**. *Drosophila* vitelline membrane cross-linking requires the *fs(1)Nasrat*, *fs(1)polehole* and chorion genes activities. *Devel. Genes Evol. 211*, 573–580.
Cerstiaens, A., Benfekih, L., Zouiten, H., Verhaert, P., De Loof, A., *et al.*, **1999**. Led-NPF-1 stimulates ovarian development in locusts. *Peptides 20*, 39–44.
Chávez, V.M., Marqués, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., *et al.*, **2000**. The *Drosophila* disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126.
Chen, B., Harms, E., Chu, T., Henrion, G., Strickland, S., **2000**. Completion of meiosis in *Drosophila* oocytes requires transcriptional control by Grauzone, a new zinc finger protein. *Development 127*, 1243–1251.
Chen, C., Jack, J., Garofalo, R.S., **1996**. The *Drosophila* insulin receptor is required for normal growth. *Endocrinology 137*, 846–856.
Chen, J.-S., Sappington, T.W., Raikhel, A.S., **1997**. Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *J. Mol. Evol. 44*, 440–451.
Chen, M.-E., Lewis, C.K., Keeley, L.L., Pietrantonio, P.V., **2004**. cDNA cloning and transcriptional regulation of the vitellogenin receptor from the imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Insect Mol. Biol. 13*, 195–204.
Chen, P.S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M., *et al.*, **1988**. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster. Cell 54*, 291–298.
Cheon, H., Seo, S., Sun, J., Sappington, T.W., Raikhel, A.S., **2001**. Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 31*, 753–760.
Chino, H., Murakami, S., Harashima, K., **1969**. Diglyceride-carrying lipoproteins in insect hemolymph.

Isolation, purification and properties. *Biochim. Biophys. Acta 176*, 1–26.

Chinzei, Y., Haruna, T., Miura, K., Numata, H., Nakayama, S., **1990**. Purification and characterization of biliverdin-associated cyanoprotein from eggs and hemolymph of the bean bug, *Riptortus clavatus* (Heteroptera: Alydidae). *Insect Biochem. 20*, 545–555.

Cho, W.L., Deitsch, K.W., Raikhel, A.S., **1991**. An extraovarian protein accumulated in mosquito oocytes is a carboxypeptidase activated in embryos, *Proc. Natl Acad. Sci. USA 88*, 10821–10824.

Cho, W.L., Kapitskaya, M.Z., Raikhel, A.S., **1995**. Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. *Insect Biochem. Mol. Biol. 25*, 19–27.

Cho, W.L., Tsao, S.M., Hays, A.R., Walter, R., Chen, J.S., *et al.*, **1999**. Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *J. Biol. Chem. 274*, 13311–13321.

Claycomb, J.M., MacAlpine, D.M., Evans, J.G., Bell, S.P., Orr-Weaver, T.L., **2002**. Visualization of replication initiation and elongation in *Drosophila*. *J. Cell Biol. 159*, 225–236.

Collins, T., Stone, J.R., Williams, A.J., **2001**. All in the family: the BTB/POZ, KRAB and SCAN domains. *Mol. Cell. Biol. 21*, 3609–3615.

Cooley, L., Verheyen, E., Ayers, K., **1992**. *chickadee* encodes a profiling required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell 69*, 173–184.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., *et al.*, **1998**. A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. *Genes Devel. 12*, 3715–3727.

Cox, D.N., Chao, A., Lin, H., **2000**. *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development 127*, 503–514.

Cruz, J., Martín, D., Pascual, N., Maestro, J.L., Piulachs, M.D., *et al.*, **2003**. Quantity does matter. Juvenile hormone and the onset of vitellogenesis in the German cockroach. *Insect Biochem. Mol. Biol. 33*, 1219–1225.

Culi, J., Mann, R.S., **2003**. Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in *Drosophila*. *Cell 112*, 343–354.

Dantuma, N.P., Potters, M., De Winther, M.P.J., Tensen, C.P., Kooiman, F.P., *et al.*, **1999**. An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins. *J. Lipid Res. 40*, 973–978.

Davail, B., Pakdel, F., Bujo, H., Perazzolo, L.M., Waclawek, M., *et al.*, **1998**. Evolution of oogenesis: the receptor for vitellogenin from the rainbow trout. *J. Lipid Res. 39*, 1929–1937.

Davey, K.G., **1993**. Hormonal integration of egg production in *Rhodnius prolixus*. *Am. Zool. 33*, 397–402.

Davey, K.G., **2000**. The modes of action of juvenile hormones: some questions we ought to ask. *Insect Biochem. Mol. Biol. 30*, 663–669.

De Cuevas, M., Lee, J.K., Spradling, A.C., **1996**. α-Spectrin is required for germline cell division and differentiation in the *Drosophila* ovary. *Development 122*, 2781–2789.

Deitsch, K.W., Chen, J.-S., Raikhel, A.S., **1995**. Indirect control of yolk protein genes by 20-hydroxyecdysone in the fat body of the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 25*, 449–454.

Delidakis, C., Kafatos, F.C., **1987**. Amplification of a chorion gene cluster in *Drosophila* is subject to multiple *cis*-regulatory elements and to long-range position effects. *J. Mol. Biol. 197*, 11–26.

Delidakis, C., Kafatos, F.C., **1989**. Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila*. *EMBO J. 8*, 891–901.

De Loof, A., Baggerman, G., Breuer, M., Clays, I., Cerstiaens, A., *et al.*, **2001**. Gonadotrophins in insect: an overview. *Arch. Insect Biochem. Physiol. 47*, 129–138.

De Loof, A., Bylemans, D., Schoofs, L., Janssen, I., Spittaels, K., *et al.*, **1995**. Folliculostatins, gonadotropins and a model for control of growth in the grey fleshfly, *Neobellieria* (*Sarcophaga bullata*). *Insect Biochem. Mol. Biol. 25*, 661–667.

Demerec, M., **1950**. Biology of *Drosophila*. John Wiley & Sons Inc., New York.

Deng, W., Bownes, M., **1997**. Two signaling pathways specify localized expression of the Broad-Complex in *Drosophila* eggshell patterning and morphogenesis. *Development 124*, 4639–4647.

Dequier, E., Souid, S., Pál, M., Maróy, P., Lepesant, J.-A., *et al.*, **2001**. Top-DER- and Dpp-dependent requirements for the *Drosophila fos/kayak* gene in follicular epithelium morphogenesis. *Mech. Devel. 106*, 47–60.

Dhadialla, T.S., Hays, A.R., Raikhel, A.S., **1992**. Characterization of the solubilized mosquito vitellogenin receptor. *Insect Biochem. Mol. Biol. 22*, 803–816.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., Guild, G.M., **1991**. The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics 129*, 385–397.

Ding, D., Parkhurst, S.M., Lipshitz, H.D., **1993**. Different genetic requirements for anterior RNA localization revealed by the distribution of adducin-like transcripts during *Drosophila* oogenesis. *Proc. Natl Acad. Sci. USA 90*, 2512–2516.

Dobens, L.L., Martín-Blanco, E., Martínez-Arias, A., Kafatos, F.C., Raftery, L.A., **2001**. *Drosophila puckered* regulates Fos/Jun levels during follicle cell morphogenesis. *Development 128*, 1845–1856.

Dobens, L.L., Peterson, J.S., Treisman, J., Raftery, L.A., **2000**. *Drosophila* bunched integrates opposing DPP and EGF signals to set the operculum boundary. *Development 127*, 745–754.

Dobens, L.L., Raftery, L.A., **2000**. Integration of epithelial patterning and morphogenesis in *Drosophila* ovarian follicle cells. *Devel. Dyn. 218*, 80–93.

Dobzhansky, T., **1930**. Studies on the intersexes and supersexes in *Drosophila melanogaster. Bull. Bur. Genet. Leningrad 8*, 91–158.

Drevet, J.R., Skeiky, Y.A.W., Iatrou, K., **1994**. GATA-type zinc finger motif-containing sequences and chorion gene transcription factors of the silkworm *Bombyx mori*. *J. Biol. Chem. 269*, 10660–10667.

Drevet, J.R., Swevers, L., Iatrou, K., **1995**. Developmental regulation of a silkworm gene encoding multiple GATA-type transcription factors by alternative splicing. *J. Mol. Biol. 246*, 43–53.

Drummond-Barbosa, D., Spradling, A.C., **2001**. Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. *Devel. Biol. 231*, 265–278.

Dykxhoorn, D.M., Novina, C.D., Sharp, P.A., **2003**. Killing the messenger: short RNAs that silence gene expression. *Nature Rev. Mol. Cell. Biol. 4*, 457–467.

Edgar, B.A., **1999**. From small flies come big discoveries about size control. *Nat. Cell Biol. 1*, E191–E193.

Edwards, K.A., Kiehart, D.P., **1996**. *Drosophila* non-muscle myosin II has multiple essential roles in imaginal disc and egg chamber morphogenesis. *Development 122*, 1499–1511.

Edwards, M.J., Severson, D.W., Hagedorn, H.H., **1998**. Vitelline envelope genes of the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 28*, 915–925.

Eickbush, T.H., Izzo, J.A., **1995**. Chorion genes: molecular models of evolution. In: Goldsmith, M.R., Wilkins, S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 217–247.

Eickbush, T.H., Kafatos, F.C., **1982**. A walk in the chorion locus of *Bombyx mori*. *Cell 29*, 633–643.

Engelmann, F., Mala, J., **2000**. The interactions between juvenile hormone (JH), lipophorin, vitellogenin, and JH esterases in two cockroach species. *Insect Biochem. Mol. Biol. 30*, 793–803.

Eriksen, K., Hauser, F., Schiott, M., Pedersen, K.-M., Søndergaard, L., *et al.*, **2000**. Molecular cloning, genomic organization, developmental regulation, and a knock-out mutant of a novel Leu-rich repeats-containing G protein-coupled receptor (DLGR-2) from *Drosophila melanogaster. Genome Res. 10*, 924–938.

Eystathioy, T., Swevers, L., Iatrou, K., **2001**. The orphan nuclear receptor BmHR3A of *Bombyx mori*: hormonal control, ovarian expression and functional properties. *Mech. Devel. 103*, 107–115.

Fagotto, F., **1995**. Regulation of yolk degradation, or how to make sleepy lysosomes. *J. Cell Sci. 108*, 3645–3647.

Ferdig, M.T., Li, J., Severson, D.W., Christensen, B.M., **1996**. Mosquito dopa decarboxylase cDNA characterization and blood meal-induced ovarian expression. *Insect Mol. Biol. 5*, 119–126.

Ferenz, H.-J., **1993**. Yolk protein accumulation in *Locusta migratoria* (R&F) (Orthoptera: Acrididae) oocytes. *Int. J. Insect Morphol. Embryol. 22*, 295–314.

Foley, K., Cooley, L., **1998**. Apoptosis in late stage *Drosophila* nurse cells does not require genes within the *H99* deficiency. *Development 125*, 1075–1082.

Fox, A.H., Liew, C., Holmes, M., Kowalski, K., Mackay, J., *et al.*, **1999**. Transcriptional cofactors of the FOG family interact with GATA proteins by means of multiple zinc fingers. *EMBO J. 18*, 2812–2822.

Foy, B.D., Myles, K.M., Pierro, D.J., Sanchez-Vargas, I., Uhlirova, M., *et al.*, **2004**. Development of a new Sindbis virus transducing system and its characterization in three Culicine mosquitoes and two Lepidopteran species. *Insect Mol. Biol. 13*, 89–100.

Freeman, M.R., Dobritsa, A., Gaines, P., Segraves, W.A., Carlson, J.R., **1999**. The *dare* gene: steroid hormone production, olfactory behavior, and neural degeneration in *Drosophila*. *Development 126*, 4591–4602.

French, R.L., Cosand, K.A., Berg, C., **2003**. The *Drosophila* female sterile mutation *twin peaks* is a novel allele of *tramtrack* and reveals a requirement for Ttk69 in epithelial morphogenesis. *Devel. Biol. 253*, 18–35.

Fullbright, G., Lacy, E.R., Bullesbach, E.E., **1997**. The prothoracicotropic hormone bombyxin has specific receptors on insect ovarian cells. *Eur. J. Biochem. 245*, 774–780.

Gargiulo, G., Gigliotti, S., Malva, C., Graziani, F., **1991**. Cellular specificity of expression and regulation of *Drosophila vitelline membrane protein 32E* gene in the follicular epithelium: identification of *cis*-acting elements. *Mech. Devel. 35*, 193–203.

Garofalo, R.S., **2002**. Genetic analysis of insulin signaling in *Drosophila*. *Trends Endocrinol. Metabol. 13*, 156–162.

Geuze, H.J., Slot, J.W., Strous, G.J., Lodish, H.F., Schwartz, A.L., **1983a**. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell 32*, 277–287.

Geuze, H.J., Slot, J.M., Strous, G.J., Schwartz, A.L., **1983b**. The pathway of the asialoglycoprotein ligand during receptor-mediated endocytosis: a morphological study with colloidal gold/ligand in the human hepatoma cell line, Hep G2. *Eur. J. Cell Biol. 32*, 38–44.

Gigliotti, S., Graziani, F., De Ponti, L., Rafti, F., Manzi, A., *et al.*, **1989**. Sex-, tissue-, and stage-specific expression of a vitelline membrane protein from region 32 of the second chromosome of *Drosophila melanogaster. Devel. Genet. 10*, 33–41.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Giniger, E., Tietje, K., Jan, L.Y., Jan, Y.N., **1994**. *lola* encodes a putative transcription factor required for axon growth and guidance in *Drosophila*. *Development 120*, 1385–1398.

Giorgi, F., Ceccchettini, A., Locci, M., Masetti, M., Bradley, J., **1995**. A fat body-derived protein is selectively sulfated during transit to ovarian follicles in the stick insect *Carausius morosus*. *Devel. Biol. 167*, 379–387.

Giorgi, F., Luchesi, P., Morelli, A., Bownes, M.A., **1993a**. Ultrastructural analysis of *Drosophila* ovarian follicles differing on yolk polypeptide (yps) composition. *Development 117*, 319–328.

Giorgi, F., Maqsetti, M., Ignacchiti, M., Cecchettini, A., Bradley, J., **1993b**. Post-endocytotic processing in ovarian follicles of the stick insect *Carausius morosus*. *Arch. Insect Biochem. Physiol. 24*, 93–111.

Girardie, J., Geoffre, S., Delbecque, J.-P., Girardie, A., **1998a**. Arguments for two distinct gonadotropic activities triggered by different domains of the ovary maturating parsin of *Locusta migratoria*. *J. Insect Physiol. 44*, 1063–1071.

Girardie, J., Girardie, A., **1996**. Lom OMP, a putative ecdysiotropic factor for the ovary in *Locusta migratoria*. *J. Insect Physiol. 42*, 215–221.

Girardie, J., Huet, J.-C., Atay-Kadiri, Z., Ettaouil, S., Delbecque, J.-P., *et al.*, **1998b**. Isolation, sequence determination, physical and physiological characterization of the neuroparsins and ovary maturating parsins of *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 28*, 641–650.

Girardie, J., Richard, O., Girardie, A., **1996**. Detection of vitellogenin in the haemolymph of larval female locusts (*Locusta migratoria*) treated with the neurohormone, Lom OMP. *J. Insect Physiol. 42*, 107–113.

Girardie, J., Richard, O., Huet, J.C., Nespoulous, C., Van Dorsselaer, A., *et al.*, **1991**. Physical characterization and sequence identification of the ovary maturating parsin. A new neurohormone purified from the nervous corpora cardiaca of the African locust (*Locusta migratoria migratorioides*). *Eur. J. Biochem. 202*, 1121–1126.

Gliemann, J., **1998**. Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol. Chem. 379*, 951–964.

Glushek, M., **2001**. The transcriptional regulator BmGATAβ of the silkmoth *Bombyx mori*: isolation of putative interacting factors from ovarian follicular cells. M.Sc. thesis, University of Calgary, Canada.

Godt, D., Laski, F.A., **1995**. Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of bric à brac. *Development 121*, 173–187.

Goldsmith, M.R., **1989**. Organization and developmental timing of the *Bombyx mori* chorion gene clusters in strain C108. *Devel. Genet. 10*, 16–23.

Goldsmith, M.R., Kafatos, F.C., **1984**. Developmentally regulated genes in silkmoths. *Annu. Rev. Genet. 18*, 443–487.

Goldstein, J.L., Brown, M.S., **1974**. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem. 249*, 5153–5162.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W., Schneider, W.J., **1985**. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol. 1*, 1–39.

Goldstein, J.S., Anderson, R.G.W., Brown, M.S., **1979**. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.) 279*, 679–685.

Graf, R., Neuenschwander, S., Brown, M.R., Ackerman, U., **1997**. Insulin-mediated secretion of ecdysteroids from mosquito ovaries and molecular cloning of the insulin receptor homologue from ovaries of bloodfed *Aedes aegypti*. *Insect Mol. Biol. 6*, 151–163.

Gruenberg, J., Maxfield, F.R., **1995**. Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol. 7*, 552–563.

Gruntenko, N.E., Bownes, M., Terashima, J., Sukhanova, M.Zh., Raushenbach, I.Y., **2003**. Heat stress affects oogenesis differently in wild-type *Drosophila virilis* and a mutant with altered juvenile hormone and 20-hydroxyecdysone levels. *Insect Mol. Biol. 12*, 393–404.

Hafer, J., Ferenz, H.-J., **1991**. Locust vitellogenin receptor: an acidic glycoprotein with N and O-linked oligosaccharides. *Comp. Biochem. Physiol. 100B*, 579–586.

Hagedorn, H.H., **1985**. The role of ecdysteroids in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, NewYork, pp. 205–261.

Hamodrakas, S.J., **1992**. Molecular architecture of helicoidal proteinaceous eggshells. In: Case, S.T. (Ed.), Results and Problems in Cell Differentiation, vol. 19. Springer-Verlag, Berlin, pp. 116–186.

Hamodrakas, S.J., Hoenger, A., Iconomidou, V.A., **2004**. Amyloid fibrillogenesis of silkmoth chorion protein peptide-analogues via a liquid crystalline intermediate phase. *J. Struct. Biol. 145*, 226–235.

Han, Q., Li, G., Li, J., **2000**. Chorion peroxidase-mediated $NADH/O_2$ oxidoreduction cooperated by chorion malate dehydrogenase-catalyzed NADH production: a feasible pathway leading to H_2 O_2 formation during chorion hardening in *Aedes aegypti* mosquitos. *Biochim. Biophys. Acta 1523*, 246–253.

Handler, A.M., **2001**. A current perspective on insect gene transformation. *Insect Biochem. Mol. Biol. 31*, 111–128.

Hannon, G.J., **2002**. RNA interference. *Nature 418*, 244–251.

Hauser, F., Nothacker, H.-P., Grimmelikhuijzen, C.J.P., **1997**. Molecular cloning, genomic organization, and developmental regulation of a novel receptor from *Drosophila melanogaster* structurally related to members of the thyroid-stimulating hormone, follicle stimulating hormone, luteinizing hormone/choriogonadotropin receptor family from mammals. *J. Biol. Chem. 272*, 1002–1010.

Hay, B., Jan, L.Y., Jan, Y.N., **1988**. A protein component of *Drosophila* polar granules is encoded by *vasa* and has extensive sequence similarity to ATP-dependent helicases. *Cell 55*, 577–587.

Hay, B., Jan, L.Y., Jan, Y.N., **1990**. Localization of *vasa*, a component of *Drosophila* polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development 109*, 425–433.

Hays, A.R., Raikhel, A.S., **1990**. A novel protein produced by the vitellogenic fat body and accumulated in mosquito oocytes, *Wilhelm. Roux's Arch. Devel. Biol. 199*, 114–121.

Heck, M.M.S., Spradling, A.C., **1990**. Multiple replication origins are used during *Drosophila* chorion gene amplification. *J. Cell Biol. 110*, 903–914.

Heifetz, Y., Lung, O., Frongillo, E.A., Wolfner, M.F., **2000**. The *Drosophila* seminal fluid protein Acp26a stimulates release of oocytes by the ovary. *Curr. Biol. 10*, 99–102.

Heifetz, Y., Yu, J., Wolfner, M.F., **2001**. Ovulation triggers activation of *Drosophila* oocytes. *Devel. Biol. 234*, 416–424.

Helbling, P., Graf, R., **1998**. Localization of the mosquito insulin receptor homolog (MIR) in reproducing yellow fever mosquitoes (*Aedes aegypti*). *J. Insect Physiol. 44*, 1127–1135.

Henrich, V., Sliter, T.J., Lubahn, D.B., McIntyre, A., Gilbert, L.I., **1990**. A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. *Nucleic Acids Res. 18*, 4143–4148.

Henrich, V.C., Brown, N.E., **1995**. Insect nuclear receptors: a developmental and comparative perspective. *Insect Biochem. Mol. Biol. 25*, 881–897.

Herz, J., Bock, H.H., **2002**. Lipoprotein receptors in the nervous system. *Annu. Rev. Biochem. 71*, 405–434.

Herz, J., Farese, R.V., Jr., **1999**. The LDL receptor gene family, apolipoprotein B and cholesterol in embryonic development. *J. Nutr. 129* (2S Suppl.), 473S–475S.

Hibner, B.L., Burke, W.D., Eickbush, T.H., **1991**. Sequence identity in a early chorion multigene family is the result of localized gene conversion. *Genetics 128*, 595–606.

Hibner, B.L., Burke, W.D., Lecanidou, R., Rodakis, G.C., Eickbush, T.H., **1988**. Organization and expression of three genes from the silkmoth early chorion locus. *Devel. Biol. 125*, 423–431.

Higgins, M.J., Walker, V.K., Holden, J.A., White, B.N., **1984**. Isolation of two *Drosophila melanogaster* genes abundantly expressed in the ovary during vitelline membrane synthesis. *Devel. Biol. 105*, 155–165.

Hirai, M., Watanabe, D., Chinzei, Y., **2000**. A juvenile hormone-repressible transferrin-like protein from the bean bug *Riptortus clavatus*: cDNA sequence analysis and protein identification during diapause and vitellogenesis. *Arch. Insect Biochem. Physiol. 44*, 17–26.

Hiramatsu, N., Chapman, R.W., Lindzey, J.K., Haynes, M.R., Sullivan, C.V., **2004**. Molecular characterization and expression of vitellogenin receptor from white perch (*Morone Americana*). *Biol. Reprod. 70*, 1720–1730.

Hodin, J., Riddiford, L.M., **1998**. The ecdysone receptor and ultraspiracle regulate the timing and progression of ovarian morphogenesis during *Drosophila* metamorphosis. *Devel. Genes Evol. 208*, 304–317.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Horn, C., Wimmer, E.A., **2000**. A versatile vector set for animal transgenesis. *Devel. Genes Evol. 210*, 630–637.

Houston, D.W., King, M.L., **2000**. Germ plasm and molecular determinants of germ cell fate. *Curr. Top. Devel. Biol. 50*, 155–181.

Hsu, T., Bagni, C., Sutherland, J.D., Kafatos, F.C., **1996**. The transcription factor CF2 is a mediator of EGF-R-activated dorsoventral patterning in *Drosophila* oogenesis. *Genes Devel. 10*, 1411–1421.

Hsu, T., Gogos, J.A., Kirsh, S.A., Kafatos, F.C., **1992**. Multiple zinc finger forms resulting from developmentally regulated alternative splicing of a transcription factor gene. *Science 257*, 1946–1950.

Hsu, T., King, D.L., LaBonne, C., Kafatos, F.C., **1993**. A *Drosophila* single-strand DNA/RNA-binding factor contains a high-mobility-group box and is enriched in the nucleolus. *Proc. Natl Acad. Sci. USA 90*, 6488–6492.

Hsu, T., McRackan, D., Vincent, T.S., Gert de Couet, H., **2001**. *Drosophila* Pin1 prolyl isomerase Dodo is a MAP kinase signal responder during oogenesis. *Nat. Cell Biol. 3*, 538–543.

Huang, R.-Y., Orr, W.C., **1992**. Broad-Complex function during oogenesis in *Drosophila melanogaster. Devel. Genet. 13*, 277–288.

Hussain, M.M., Strickland, D.K., Bakillah, A., **1999**. The mammalian low-density lipoprotein receptor family. *Annu. Rev. Nutr. 19*, 141–172.

Hutson, S.F., Bownes, M., **2003**. The regulation of *yp3* expression in the *Drosophila melanogaster* fat body. *Devel. Genes Evol. 213*, 1–8.

Huybrechts, R., De Loof, A., **1977**. Induction of vitellogenin synthesis in male *Sarcophaga bullata* by ecdysterone. *J. Insect Physiol. 23*, 1359–1362.

Iatrou, K., Tsitilou, S.G., Kafatos, F.C., **1982**. Developmental classes and homologous families of chorion genes in *Bombyx mori*. *J. Mol. Biol. 157*, 417–434.

Iatrou, K., Tsitilou, S.G., Kafatos, F.C., **1984**. DNA sequence transfer between two high-cysteine chorion gene families in the silkmoth *Bombyx mori*. *Proc. Natl Acad. Sci. USA 81*, 4452–4454.

Iconomidou, V.A., Vriend, G.A., Hamodrakas, S.J., **2000**. Amyloids protect the silkmoth oocyte and embryo. *FEBS Lett. 479*, 141–145.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E., **2002**. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr. Biol. 12*, 1293–1300.

Inagaki, S., Yamashita, O., **1989**. Complete amino acid sequence of *Bombyx* egg-specific protein deduced from

cDNA clone. *Arch. Insect Biochem. Physiol. 10*, 131–139.

Indrasith, L., Sasaki, T., Yaginuma, T., Yamashita, O., **1988**. The occurrence of a premature form of egg-specific protein in vitellogenic follicles of *Bombyx mori*. *J. Comp. Physiol. B 158*, 1–7.

Indrasith, L.S., Sasaki, T., Yamashita, O., **1988**. A unique protease responsible for selective degradation of a yolk protein in *Bombyx mori*. *J. Biol. Chem. 263*, 1045–1051.

Innerarity, T.L., **2002**. LDL receptor's β-propeller displaces LDL. *Science 298*, 2337–2339.

Iwami, M., Tanaka, A., Hano, N., Sakurai, S., **1996**. Bombyxin gene expression in tissues other than brain detected by reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization. *Experientia 52*, 882–887.

Iyengar, A.R., Kunkel, J.G., **1995**. Follicle cell calmodulin in *Blattella germanica*: transcript accumulation during vitellogenesis is regulated by juvenile hormone. *Devel. Biol. 170*, 314–320.

Izumi, S., Fikie, J., Yamada, S., Tomino, S., **1981**. Molecular properties and biosynthesis of major plasma proteins in *Bombyx mori*. *Biochim. Biophys. Acta 670*, 222–229.

Izumi, S., Kiguchi, K., Tomino, S., **1984**. Hormonal regulation of biosynthesis of major plasma proteins in *Bombyx mori*. *Zool. Sci. 1*, 223–228.

Izumi, S., Yano, K., Yamamoto, Y., Takahashi, S.Y., **1994**. Yolk proteins from insect eggs: structure, biosynthesis and programmed degradation during embryogenesis. *J. Insect Physiol. 40*, 735–746.

Jacoby, D.B., Wensink, P.C., **1994**. Yolk protein factor 1 is a *Drosophila* homolog of Ku, the DNA-binding subunit of a DNA-dependent protein kinase from humans. *J. Biol. Chem. 269*, 11484–11491.

Janssen, T., Claeys, I., Simonet, G., De Loof, A., Girardie, J., *et al.*, **2001**. cDNA cloning and transcript distribution of two different neuroparsin precursors in the desert locust, *Schistocerca gregaria*. *Insect Mol. Biol. 10*, 183–189.

Jeon, H., Meng, W., Takagi, J., Eck, M.J., Springer, T.A., *et al.*, **2001**. Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nat. Struct. Biol. 8*, 499–504.

Jhoti, H., McLeod, A.N., Blundell, T.L., Ishizaki, H., Nagasawa, H., *et al.*, **1987**. Prothoracicotropic hormone has an insulin-like tertiary structure. *FEBS Lett. 219*, 419–425.

Jin, J., Petri, W.H., **1993**. Developmental control elements in the promoter of a *Drosophila* vitelline membrane gene. *Devel. Biol. 156*, 557–565.

Jongens, T.A., Ackerman, L.D., Swedlow, J.R., Jan, L.Y., Jan, Y.N., **1994**. *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Devel. 8*, 2123–2136.

Jongens, T.A., Hay, B., Jan, L.Y., Jan, Y.N., **1992**. The *germ cell-less* gene product: a posteriorly localized component necessary for germ cell development in *Drosophila*. *Cell 70*, 569–584.

Jordan, T.V., Shike, H., Boulo, V., Cedeno, V., Fang, Q., *et al.*, **1998**. Pantropic retroviral vectors mediate somatic cell transformation and expression of foreign genes in dipteran insects. *Insect Mol. Biol. 7*, 215–222.

Jowett, T., Postlethwait, J.H., **1980**. The regulation of yolk polypeptide synthesis in *Drosophila* ovaries and fat body by 20-hydroxyecdysone and a juvenile hormone analog. *Devel. Biol. 80*, 225–234.

Kadono-Okuda, K., Amornsak, W., Yamashita, O., **1994**. Controlled ecdysteroid accumulation in eggs of the silkworm, *Bombyx mori*, by an imidazole compound (KK-42), and embryogenesis in these eggs. *Arch. Insect Biochem. Biophys. 25*, 121–135.

Kafatos, F.C., Mitsialis, S.A., Spoerel, N., Mariani, B., Lingappa, J.R., *et al.*, **1985**. Studies on the developmentally regulated expression and amplification of insect chorion genes. In: Molecular Biology of Development, Symposia on Quantitative Biology, vol. L. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 537–547.

Kafatos, F.C., Regier, J.C., Mazur, G.D., Nadel, M.R., Blau, H.M., *et al.*, **1977**. The eggshell of insects: differentiation-specific proteins and the control of their synthesis and accumulation during development. In: Beerman, W. (Ed.), Results and Problems in Cell Differentiation, vol. 8. Springer-Verlag, Berlin, pp. 45–145.

Kafatos, F.C., Spoerel, N., Mitsialis, S.A., Nguyen, H.T., Romano, C., *et al.*, **1987**. Developmental control and evolution in the chorion gene families of insects. *Adv. Genet. 24*, 223–242.

Kafatos, F.C., Tzertzinis, G., Spoerel, N.A., Nguyen, H.T., **1995**. Chorion genes: an overview of their structure, function and transcriptional regulation. In: Goldsmith, M.R., Wilkins, A.S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 181–215.

Kageyama, T., Takahashi, S.Y., **1990**. Purification and characterization of a cysteine protease from silkworm eggs. *Eur. J. Biochem. 193*, 203–210.

Kageyama, Y., Masuda, S., Hirose, S., Ueda, H., **1997**. Temporal regulation of the mid-prepupal gene FTZ-F1: DHR3 early late gene product is one of plural positive regulators. *Genes Cells 2*, 559–569.

Kalfayan, L., Levine, J., Orr-Weaver, T., Parks, S., Wakimoto, B., *et al.*, **1985**. Localization of sequences regulating *Drosophila* chorion gene amplification and expression. In: Molecular Biology of Development, Symposia on Quantitative Biology, vol. L. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 527–536.

Kang, Y., Kulakosky, P.C., Van Antwerpen, R., Law, J.H., **1995**. Sequestration of insecticyanin, a blue hemolymph protein, into the egg of the hawkmoth, *Manduca sexta*. Evidence for receptor-mediated endocytosis. *Insect Biochem. Mol. Biol. 25*, 503–510.

Kapitskaya, M.Z., Dittmer, N.T., Deitsch, K.W., Cho, W.-L., Taylor, D.G., *et al.*, **1998**. Three isoforms of a hepatocyte nuclear factor-4 transcription factor with tissue- and stage-specific expression in the adult mosquito. *J. Biol. Chem. 273*, 29801–29810.

Kapitskaya, M.Z., Li, C., Miura, K., Segraves, W., Raikhel, A.S., **2000**. Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol. Cell. Endocrinol. 160*, 25–37.

Kawooya, J., Osir, E., Law, J., **1986**. Physical and chemical properties of microvitellogenin, a protein from the egg of the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem. 261*, 10844–10849.

Kawooya, J.K., Law, J.H., **1988**. Role of lipophorin in lipid transport to the insect egg. *J. Biol. Chem. 263*, 8748–8753.

Kawooya, J.K., Osir, E.O., Law, J.H., **1988**. Uptake of the major hemolymph lipoprotein and its transformation in the insect egg. *J. Biol. Chem. 263*, 8740–8747.

Kendirgi, F., Swevers, L., Iatrou, K., **2002**. An ovarian follicular epithelium protein of the silkworm (*Bombyx mori*) that associates with the vitelline membrane and contributes to the structural integrity of the follicle. *FEBS Lett. 524*, 59–68.

Keramiris, K.E., Stravopodis, D., Margaritis, L.H., **1991**. A structural protein that plays an enzymatic role in the eggshell of *Drosophila melanogaster. Cell Biol. Int. Rep. 15*, 151–159.

Kerkis, J., **1930**. Development of the gonads in *Drosophila melanogaster. Genetics 16*, 212–224.

Khoury-Christianson, A.M., King, D.L., Hatzivassiliou, E., Casas, J.E., Hallenbeck, P.L., *et al.*, **1992**. DNA binding and heterodimerization of the *Drosophila* transcription factor chorion factor 1/ultraspiracle. *Proc. Natl Acad. Sci. USA 89*, 11503–11507.

Kidwell, M.G., Wattam, A.R., **1998**. An important step forward in the genetic manipulation of mosquito vectors of disease. *Proc. Natl Acad. Sci. USA 95*, 3349–3350.

Kim, C., Han, K., Kim, J., Yi, J.S., Kim, C., *et al.*, **2002**. Femcoat, a novel eggshell protein in *Drosophila*: functional analysis by double stranded RNA interference. *Mech. Devel. 110*, 61–70.

King, F.J., Lin, H., **1999**. Somatic signaling mediated by *fs(1Yb)* is essential for germline stem cell maintenance during *Drosophila* oogenesis. *Development 126*, 1833–1844.

King, F.J., Szakmary, A., Cox, D.N., Lin, H., **2001**. *Yb* modulates the divisions of both germline and somatic stem cell through piwi- and *hh*-mediated mechanisms in the *Drosophila* ovary. *Mol. Cell 7*, 497–508.

King, R.C., **1970**. Ovarian Development in *Drosophila melanogaster.* Academic Press, New York.

King, R.C., Aggarwal, S.K., Aggarwal, U., **1968**. The development of the female *Drosophila* reproductive system. *J. Morphol. 124*, 143–166.

Kioka, N., Ueda, K., Amachi, T., **2002**. Vinexin, CAP/ponsin, ArgBP2: a novel adaptor protein family regulating cytoskeletal organization and signal transduction. *Cell Struct. Funct. 27*, 1–7.

Koch, E.A., King, R.C., **1966**. The origin and early differentiation of the egg chamber of *Drosophila melanogaster. J. Morphol. 119*, 283–304.

Kokoza, V.A., Raikhel, A.S., **1997**. Ovarian- and somatic-specific transcripts of the mosquito clathrin heavy chain gene generated by alternative 5′-exon splicing and polyadenylation. *J. Biol. Chem. 272*, 1164–1170.

Kokoza, V.A., Snigirevskaya, E.S., Raikhel, A.S., **1997**. Mosquito clathrin heavy chain: analysis of protein structure and developmental expression in the ovary during vitellogenesis. *Insect Mol. Biol. 6*, 357–368.

König, R., Lanzrein, B., **1985**. Binding of vitellogenin to specific receptors in oocyte membrane preparations of the ovoviviparous cockroach *Nauphoeta cinera*. *Insect Biochem. 15*, 735–747.

König, R., Nordin, J.H., Gochoco, C., Kunkel, H., **1988**. Studies on ligand recognition by vitellogenin receptors in follicle membrane preparations of the German cockroach, *Blatella germanica*. *Insect Biochem. 18*, 395–404.

Kravariti, L., Lecanidou, R., Rodakis, G.C., **1995**. Sequence analysis of a small early chorion gene subfamily interspersed within the late gene locus in *Bombyx mori*. *J. Mol. Evol. 41*, 24–33.

Kravariti, L., Thomas, J.-L., Sourmeli, S., Rodakis, G.C., Mauchamp, B., *et al.*, **2001**. The biolistic method as a tool for testing the differential activity of putative silkmoth chorion gene promoters. *Insect Biochem. Mol. Biol. 31*, 473–479.

Kubli, E., **1996**. The *Drosophila* sex-peptide: a peptide pheromone involved in reproduction. In: Wassarman, P. (Ed.), Advances in Developmental Biochemistry, vol. 4. JAI Press, NewYork, pp. 99–108.

Kulakosky, P.C., Telfer, W.H., **1987**. Selective endocytosis, *in vitro*, by ovarian follicles from *Hyalophora cecropia*. *Insect Biochem. 17*, 845–858.

Kulakosky, P.C., Telfer, W.H., **1990**. Lipophorin as a yolk precursor in *Hyalophora cecropia*: uptake kinetics and competition with vitellogenin. *Arch. Insect Biochem. Physiol. 14*, 269–285.

Kurama, T., Kurata, S., Natori, S., **1995**. Molecular characterization of an insect transferrin and its selective incorporation into eggs during oogenesis. *Eur. J. Biochem. 228*, 229–235.

Lagueux, M., Harry, P., Hoffmann, J.A., **1981**. Ecdysteroids are bound to vitellin in newly laid eggs of *Locusta*. *Mol. Cell. Endocrinol. 24*, 325–338.

Lagueux, M., Hirn, M., Hoffmann, J.A., **1977**. Ecdysone during ovarian development in *Locusta migratoria*. *J. Insect Physiol. 23*, 109–119.

Lam, G., Thummel, C.S., **2000**. Inducible expression of double-stranded RNA directs specific genetic interference in *Drosophila*. *Curr. Biol. 10*, 957–963.

Lam, G.T., Jiang, C., Thummel, C.S., **1997**. Coordination of larval and prepupal gene expression by the DHR3

orphan receptor during *Drosophila* metamorphosis. *Development 124*, 1757–1769.

Landis, G., Kelley, R., Spradling, A.C., Tower, J., **1997**. The *k43* gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the *Drosophila* homolog of yeast origin recognition complex subunit 2. *Proc. Natl Acad. Sci. USA 94*, 3888–3892.

Landis, G., Tower, J., **1999**. The *Drosophila chiffon* gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. *Development 126*, 4281–4293.

Lannutti, B.J., Schneider, L.E., **2001**. *Gprk2* controls camp levels in *Drosophila* development. *Devel. Biol. 233*, 174–185.

Lanot, R., Sellam, M.O., Bucher, B., Thiebold, J.J., **1990**. Ecdysone-controlled meiotic reinitiation in oocytes of *Locusta migratoria* involves a decrease in cAMP levels. *Insect Biochem. 20*, 639–644.

Lanot, R., Thiebold, J., Costet-Corio, M.F., Benveniste, P., Hoffmann, J.A., **1988**. Further experimental evidence for the involvement of ecdysone in the control of meiotic reinitiation in oocytes of *Locusta migratoria* (Insecta, Orthoptera). *Devel. Biol. 126*, 212–214.

Lasko, P.F., **1994**. Molecular Genetics of *Drosophila* Oogenesis. R.G. Landes Company, Austin, TX.

Lasko, P.F., Ashburner, M., **1988**. The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. *Nature 335*, 611–617.

Lasko, P.F., Ashburner, M., **1990**. Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. *Genes Devel. 4*, 905–921.

Lecanidou, R., Rodakis, G.C., **1992**. Three copies of the early gene 6F6 are interspersed in and around the late chorion gene cluster of *Bombyx mori. J. Mol. Evol. 34*, 304–314.

Lecanidou, R., Rodakis, G.C., Eickbush, T.H., Kafatos, F.C., **1986**. Evolution of the silk moth chorion gene superfamily: Gene families CA and CB. *Proc. Natl Acad. Sci. USA 83*, 6514–6518.

Leclerc, R.F., Regier, J.C., **1993**. Choriogenesis in the Lepidoptera: morphogenesis, protein synthesis, specific mRNA accumulation, and primary structure of a chorion cDNA from the gypsy moth. *Devel. Biol. 160*, 28–38.

Leevers, S.J., **2001**. Growth control: invertebrate insulin surprises. *Curr. Biol. 11*, R209–R212.

Lehman, R., Nüsslein-Volhard, C., **1986**. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in *Drosophila*. *Cell 47*, 141–152.

Lehman, R., Nüsslein-Volhard, C., **1987**. Involvement of the *pumilio* gene in the transport of an abdominal signal in the *Drosophila* embryo. *Nature 329*, 167–170.

LeMosy, E.K., Hashimoto, C., **2000**. The Nudel protease of *Drosophila* is required for eggshell biogenesis in addition to embryonic patterning. *Devel. Biol. 217*, 352–361.

LeMosy, E.K., Hong, C.C., Hashimoto, C., **1999**. Signal transduction by a protease cascade. *Trends Cell Biol. 9*, 102–107.

León, A., McKearin, D., **1999**. Identification of TER94, an AAA ATPase protein as a bam-dependent component of the *Drosophila* fusome. *Mol. Biol. Cell 11*, 3825–3834.

Li, A., Sadasivam, M., Ding, J.L., **2003**. Receptor–ligand interaction between vitellogenin receptor (VtgR) and vitellogenin (Vtg), implications on low density lipoprotein receptor and apolipoprotein B/E. The first three ligand-binding repeats of VtgR interact with the amino-terminal region of Vtg. *J. Biol. Chem. 278*, 2799–2806.

Li, C., Kapitskaya, M.Z., Zhu, J., Miura, K., Segraves, W., *et al.*, **2000**. Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. *Devel. Biol. 224*, 96–110.

Li, J., **1994**. Egg chorion tanning in *Aedes aegypti* mosquito. *Comp. Biochem. Physiol. A (Physiol.) 109*, 835–843.

Li, M.G., Serr, M., Edwards, K., Ludmann, S., Yamamoto, D., *et al.*, **1999**. Filamin is required for ring canal assembly and actin organization during *Drosophila* oogenesis. *J. Cell Biol. 146*, 1061–1073.

Lin, H., **2002**. The stem-cell niche theory: lessons from flies. *Nature Rev. Genet. 3*, 931–940.

Lin, H., Spradling, A.C., **1995**. Fusome asymmetry and oocyte determination in *Drosophila*. *Devel. Genet. 16*, 6–12.

Lin, H., Spradling, A.C., **1997**. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development 124*, 2463–2476.

Lin, Y., Hamblin, M.T., Edwards, M.J., Barillas-Mury, C., Kanost, M.R., *et al.*, **1993**. Structure, expression, and hormonal control of genes from the mosquito, *Aedes aegypti*, which encode proteins similar to the vitelline membrane proteins of *Drosophila melanogaster. Devel. Biol. 155*, 558–568.

Logan, S.K., Garabedian, M.J., Wensink, P.C., **1989**. DNA regions that regulate the ovarian transcriptional specificity of *Drosophila* yolk protein genes. *Genes Devel. 3*, 1453–1461.

Logan, S.K., Wensink, P.C., **1990**. Ovarian follicle cell enhancers from the *Drosophila yolk protein* genes: different segments of one enhancer have different cell-type specificities that interact to give normal expression. *Genes Devel. 4*, 613–623.

Lossky, M., Wensink, P.C., **1995**. Regulation of *Drosophila yolk protein* genes by an ovary-specific GATA factor. *Mol. Cell. Biol. 15*, 6943–6952.

Lu, L., Zhang, H., Tower, J., **2001**. Functionally distinct, sequence-specific replicator and origin elements are required for *Drosophila* chorion gene amplification. *Genes Devel. 15*, 134–146.

Lunke, M., **2000**. Molecular characterization of BmGATAβ, a gene involved in choriogenesis in the

silkworm *Bombyx mori*. Ph.D. thesis, University of Calgary, Canada.
Macdonald, P.M., Struhl, G., **1988**. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature 324*, 537–545.
Mahajan-Niklos, S., Cooley, L., **1994**. Intercellular cytoplasm transport during *Drosophila* oogenesis. *Devel. Biol. 165*, 336–351.
Mahowald, A.P., Kambyselis, M.P., **1980**. Oogenesis. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*. Academic Press, New York, pp. 141–224.
Mahowald, A.P., Strassheim, J.M., **1970**. Intercellular migration of centrioles in the germarium of *Drosophila melanogaster.* An electron microscopic study. *J. Cell Biol. 45*, 306–320.
Manière, G., Vanhems, E., Delbecque, J.-P., **2000**. Cyclic AMP-dependent and independent stimulations of ovarian ecdysteroidogenesis by brain factors in the blowfly, *Phormia regina*. *Mol. Cell. Endocrinol. 168*, 31–40.
Manière, G., Vanhems, E., Gautron, F., Delbecque, J.-P., **2002**. Calcium inhibits ovarian ecdysteroidogenesis in the blowfly *Phormia regina*. *J. Endocrinol. 173*, 533–544.
Manière, G., Vanhems, E., Gautron, F., Delbecque, J.-P., **2003**. Inhibition of ovarian steroidogenesis in a fly. *J. Endocrinol. 177*, 35–44.
Manseau, L.J., Schüpbach, T., **1989**. *cappuchino* and *spire*. Two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. *Genes Devel. 3*, 1437–1452.
Mantrova, E.Y., Hsu, T., **1998**. Down-regulation of transcription factor CF2 by *Drosophila* Ras/MAP kinase signaling in oogenesis: cytoplasmic retention and degradation. *Genes Devel. 12*, 1166–1175.
Margaritis, L.H., **1985**. Structure and physiology of the eggshell. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 1. Pergamon Press, NewYork, pp. 153–173.
Margolis, J., Spradling, A.C., **1995**. Identification and behavior of epithelial stem cells in the *Drosophila* embryo. *Development 121*, 3797–3807.
Mariani, B.D., Lingappa, J.R., Kafatos, F.C., **1988**. Temporal regulation in development: negative and positive *cis* regulators dictate the precise timing of expression of a *Drosophila* chorion gene. *Proc. Natl Acad. Sci. USA 85*, 3029–3033.
Mariani, B.D., Shea, M.J., Conboy, M.J., Conboy, I., King, D.L., *et al.*, **1996**. Analysis of regulatory elements of the developmentally controlled chorion *s15* promoter in transgenic *Drosophila*. *Devel. Biol. 174*, 115–124.
Marie, B., Bacon, J.P., Blagburn, J.M., **1999**. Double-stranded RNA interference shows that Engrailed controls the synaptic identity of identified sensory neurons. *Curr. Biol. 10*, 289–292.
Marsh, M., McMahon, H.T., **1999**. The structural era of endocytosis. *Science 285*, 215–220.
Maruta, K., Yoshiga, T., Katagiri, C., Ochiai, M., Tojo, S., **2002**. Purification and characterization of biliverdin-binding vitellogenin from the hemolymph of the common cutworm, *Spodoptera litura*. *Arch. Insect Biochem. Physiol. 50*, 97–106.
Masuda, H., Braz, G.R.C., Paiva-Silva, G.O., Silva-Neto, M.A.C., Oliveira, P.L., **2004**. Non-vitellin yolk proteins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 289–314.
Masumura, M., Satake, S.I., Saegusa, H., Mizoguchi, A., **2000**. Glucose stimulates the release of bombyxin, an insulin-related peptide of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrin. 118*, 393–399.
Máthé, E., Inoue, Y.H., Palframan, W., Brown, G., Glover, D.M., **2003**. Orbit/Mast, the CLASP orthologue of *Drosophila*, is required for asymmetric stem cell and cystocyte divisions and development of the polarized microtubule network that interconnects oocyte and nurse cells during oogenesis. *Development 130*, 901–915.
Matova, N., Mahajan-Miklos, S., Mooseker, M.S., Cooley, L., **1999**. *Drosophila* Quail, a villin-related protein, bundles actin filaments in apoptotic nurse cells. *Development 126*, 5645–5657.
Mauzy-Melitz, D., Waring, G.L., **2003**. fc177, a minor dec-1 proprotein, is necessary to prevent ectopic aggregation of the endochorion during eggshell assembly in *Drosophila*. *Devel. Biol. 255*, 193–205.
Mazur, G.D., Regier, J.C., Kafatos, F.C., **1982**. Order and defects in the silkmoth chorion, a biological analogue of a cholesteric liquid crystal. In: Akai, H., King, R.C. (Eds.), Insect Ultrastructure, vol. 1. Plenum Publishing Corp., New York, pp. 150–183.
Mazur, G.D., Regier, J.C., Kafatos, F.C., **1989**. Morphogenesis of silkmoth chorion: sequential modification of an early helicoidal framework through expansion and densification. *Tissue Cell 21*, 227–242.
McCall, K., Steller, H., **1998**. Requirement for DCP-1 caspase during *Drosophila* oogenesis. *Science 279*, 230–234.
McGrail, M., Hays, T.S., **1997**. The microtubule motor cytoplasmic Dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in *Drosophila*. *Development 124*, 2409–2419.
McKearin, D., Ohlstein, B., **1995**. A role for the *Drosophila* bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development 121*, 2937–2947.
Medina, M., Leon, P., Vallejo, C.G., **1988**. *Drosophila* cathepsin B-like protinase: a suggested role in yolk degradation. *Arch. Biochem. Biophys. 263*, 355–363.
Medina, M., Vallejo, C.G., **1989**. A serine proteinase in *Drosophila* embryos: yolk localization and developmental activation. *Insect Biochem. 19*, 687–691.
Mellman, I., **1996**. Endocytosis and molecular sorting. *Annu. Rev. Cell Devel. Biol. 12*, 575–626.

Melo, A.C.A., Valle, D., Machado, E.A., Salerno, A.P., Paiva-Silva, G.O., *et al.*, **2000**. Synthesis of vitellogenin by the follicle cells of *Rhodnius prolixus*. *Insect Biochem. Mol. Biol. 30*, 549–557.

Mertens, I., Meeusen, T., Huybrechts, R., De Loof, A., Schoofs, L., **2002**. Characterization of the short neuropeptide F receptor from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 297*, 1140–1148.

Miller, A., **1950**. The internal anatomy and histology of the imago of *Drosophila melanogaster.* In: Demerec, M. (Ed.), Biology of *Drosophila*. Wiley, New York, pp. 420–534.

Mindrinos, M.N., Scherer, L.J., Garcini, F.J., Kwan, H., Jacobs, K.A., *et al.*, **1985**. Isolation and chromosomal localization of putative vitelline membrane genes in *Drosophila melanogaster. EMBO J. 4*, 147–153.

Mitsialis, S.A., Kafatos, F.C., **1985**. Regulatory elements controlling chorion gene expression are conserved between flies and moths. *Nature 317*, 453–456.

Mitsialis, S.A., Spoerel, N., Leviten, M., Kafatos, F.C., **1987**. A short 5′-flanking DNA region is sufficient for developmentally correct expression of moth chorion genes in *Drosophila*. *Proc. Natl Acad. Sci. USA 84*, 7987–7991.

Mitsialis, S.A., Veletza, S., Kafatos, F.C., **1989**. Transgenic regulation of moth chorion gene promoters in *Drosophila*: tissue, temporal, and quantitative control of four bi-directional promoters. *J. Mol. Evol. 29*, 486–495.

Mitsis, P.G., Wensink, P.C., **1989**. Identification of yolk protein factor 1, a sequence-specific DNA-binding protein from *Drosophila melanogaster. J. Biol. Chem. 264*, 5188–5194.

Miura, K., Nakagawa, M., Chinzei, Y., Shinoda, T., Nagao, E., *et al.*, **1994**. Structural studies of biliverdin-associated cyanoprotein from the bean bug, *Riptortus clavatus. Zool. Sci. 11*, 537–545.

Miya, K., **1958**. Studies on the embryonic development of the gonads in the silkworm, *Bombyx mori*. L. I. Differentiation of germ cells. *J. Fac. Agric. Iwate Univ. 3*, 436–467.

Miya, K., **1959**. Studies on the embryonic development of the gonads in the silkworm, *Bombyx mori*. L. Part II. Formation of the gonad. *J. Fac. Agric. Iwate Univ. 4*, 126–151.

Miya, K., Kurihara, M., Tanimura, I., **1970**. Electron microscope studies on the oogenesis of the silkworm, *Bombyx mori*. III. Fine structure of the ovary in the early stage of fifth instar larvae. *J. Fac. Agric. Iwate Univ. 10*, 59–83.

Mizuno, T., Watanabe, K., Ohnishi, E., **1981**. Developmental changes of ecdysteroids in the eggs of the silkworm, *Bombyx mori*. *Devel. Growth Diff. 23*, 543–552.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., *et al.*, **1999**. *Drosophila* S6 kinase: a regulator of cell size. *Science 285*, 2126–2129.

Montell, D.J., **2001**. Command and control: regulatory pathways controlling invasive behavior of the border cells. *Mech. Devel. 105*, 19–25.

Morimoto, A.M., Jordan, K.C., Tietze, K., Britton, J.S., O'Neill, E.M., *et al.*, **1996**. Pointed, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in *Drosophila* oogenesis. *Development 122*, 3745–3754.

Morohashi, K., Honda, S., Inomata, Y., Handa, H., Omura, T., **1992**. A common *trans*-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *J. Biol. Chem. 267*, 17913–17919.

Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klauser, S., *et al.*, **1996**. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol. 32*, 363–374.

Mueller, H.A., **2002**. Germ cell migration: as slow as molasses. *Curr. Biol. 12*, R612–R614.

Mukherjee, S., Ghosh, R.N., Maxfield, F.R., **1997**. Endocytosis. *Physiol. Rev. 77*, 759–803.

Nadel, M.R., Goldsmith, M.R., Goplerud, J., Kafatos, F.C., **1980**. Specific protein synthesis in cellular differentiation. V. A secretory defect of chorion formation in the Gr^{col} mutant of *Bombyx mori*. *Devel. Biol. 75*, 41–58.

Nadel, M.R., Kafatos, F.C., **1980**. Specific protein synthesis in cellular differentiation. IV. The chorion proteins of *Bombyx mori* and their program of synthesis. *Devel. Biol. 75*, 26–40.

Nakao, H., **1999**. Isolation and characterization of a *Bombyx* vasa-like gene. *Devel. Genes Evol. 209*, 312–316.

Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., *et al.*, **1984**. Amino-terminal amino acid sequence of the silkworm prothoracicotropic hormone: homology with insulin. *Science 226*, 1344–1345.

Nardi, J.B., **1993**. Modulated expression of a surface epitope on migrating germ cells of *Manduca sexta* embryos. *Development 118*, 967–975.

Nezis, I.P., Stravopodis, D.J., Papassideri, I., Robert-Nicoud, M., Margaritis, L.H., **2000**. Stage-specific apoptotic patterns during *Drosophila* oogenesis. *Eur. J. Biol. 79*, 610–620.

Nijhout, M.M., Riddiford, L.M., **1974**. The control of egg maturation by juvenile hormone in the tobacco hornworm moth, *Manduca sexta*. *Biol. Bull. 146*, 377–392.

Nimpf, J., Schneider, W.J., **1998**. The VLDL receptor: an LDL receptor relative with eight ligand binding repeats, LR8. *Atherosclerosis 141*, 191–202.

Noguerón, M.I., Mauzy-Melitz, D., Waring, G.L., **2000**. *Drosophila dec-1* eggshell proteins are differentially distributed via a multistep extracellular processing and localization pathway. *Devel. Biol. 225*, 459–470.

Noguerón, M.I., Waring, G., **1995**. Regulated processing of *dec-1* eggshell proteins in *Drosophila*. *Devel. Biol. 172*, 272–279.

Nüsslein-Volhard, C., Fronhöfer, H.G., Lehmann, R., **1987**. Determination of anteroposterior polarity in the *Drosophila* embryo. *Science 238*, 1675–1681.

Nykjaer, A., Willnow, T.E., **2002**. The low-density lipoprotein receptor gene family: a cellular Swiss army knife? *Trends Cell Biol. 12*, 273–280.

Ohnishi, E., Chatani, F., **1977**. Biosynthesis of ecdysone in the isolated abdomen of the silkworm, *Bombyx mori*. *Devel. Growth Diff. 19*, 67–70.

Okabayashi, K., Shoji, H., Nakamura, T., Hashimoto, O., Asashima, M., *et al.*, **1996**. cDNA cloning and expression of the *Xenopus laevis* vitellogenin receptor. *Biochem. Biophys, Res. Commun. 224*, 406–413.

Oliveira, P.L., Gondim, K.C., Guedes, D.M., Masuda, H., **1986**. Uptake of yolk proteins in *Rhodnius prolixus*. *J. Insect Physiol. 32*, 859–866.

Oliveira, P.L., Kawooya, J.K., Ribeiro, J.M., Meyer, T., Poorman, R., *et al.*, **1995**. A heme-binding protein from hemolymph and oocytes of the blood-sucking insect, *Rhodnius prolixus*. Isolation and characterization. *J. Biol. Chem. 270*, 10897–10901.

Omichinski, J.G., Clore, G.M., Schaad, O., Felsenfeld, G., Trainor, C., *et al.*, **1993**. NMR structure of a specific DNA complex of Zn-containing DNA binding domain of GATA-1. *Science 261*, 438–446.

Opresko, L., Wiley, H.S., **1987**. Receptor-mediated endocytosis in Xenopus oocytes. I. Characterization of the vitellogenin receptor system. *J. Biol. Chem. 262*, 4109–4115.

Oro, A.E., McKeown, M., Evans, R.M., **1990**. Relationship between the product of the *Drosophila ultraspiracle* locus and vertebrate retinoid X receptor. *Nature 347*, 298–301.

Oro, A.E., McKeown, M., Evans, R.M., **1992**. The *Drosophila* retinoid X receptor homolog *ultraspiracle* functions in both female reproduction and eye morphogenesis. *Development 115*, 449–462.

Orr-Weaver, T.L., **1991**. *Drosophila* chorion genes: cracking the eggshell's secrets. *BioEssays 13*, 97–105.

Osir, E.O., Law, J.H., **1986**. Studies on binding and uptake of vitellogenin by follicles of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 3*, 513–528.

Page, A.W., Orr-Weaver, T.L., **1997**. Activation of the meiotic divisions in *Drosophila* oocytes. *Devel. Biol. 183*, 195–207.

Pan, M., Wiemerslage, L., Telfer, W., **1994**. Male-grown eggs in Hyalophora: deficient follicle cell secretion as well as protein and lipid yolk deposition. *J. Insect Physiol. 40*, 765–773.

Papassideri, I.S., Trougakos, I.P., Leonard, K.R., Margaritis, L.H., **2003**. Structural and biochemical analysis of the *Leptinotarsa decemlineata* (Coleoptera); Chrysomeloidea crystalline chorionic layer. *J. Insect Physiol. 49*, 377–384.

Parks, S., Spradling, A.C., **1987**. Spatially regulated expression of chorion genes during *Drosophila* oogenesis. *Genes Devel. 1*, 497–509.

Parks, S., Wakimoto, B., Spradling, A.C., **1986**. Replication and expression of an X-linked cluster of *Drosophila* chorion genes. *Devel. Biol. 117*, 294–305.

Pascucci, T., Perrino, J., Mahowald, A.P., Waring, G.L., **1996**. Eggshell assembly in *Drosophila*: processing and localization of vitelline membrane and chorion proteins. *Devel. Biol. 177*, 590–598.

Pathirana, S., Zhao, D., Bownes, M., **2001**. The *Drosophila* RGS protein Loco is required for dorsal/ventral axis formation of the egg and embryo, and nurse cell dumping. *Mech. Devel. 109*, 137–150.

Paul, M., Kafatos, F.C., **1975**. Specific protein synthesis in cellular differentiation. II. The program of protein synthetic changes during chorion formation by silkmoth follicles, and its implementation in organ culture. *Devel. Biol. 42*, 141–159.

Pearse, B.M.F., Robinson, M.S., **1990**. Clathrin, adaptors and sorting. *Annu. Rev. Cell. Biol. 6*, 151–171.

Peterson, J.S., Barkett, M., McCall, K., **2003**. Stage-specific regulation of caspase activity in *Drosophila* oogenesis. *Devel. Biol. 260*, 113–123.

Pierceall, W.E., Li, C., Biran, A., Miura, K., Raikhel, A.S., *et al.*, **1999**. E75 expression in mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol. Cell. Endocrinol. 150*, 73–89.

Pokrywka, N.J., **1995**. RNA localization and the cytoskeleton in *Drosophila* oocytes. *Curr. Top. Devel. Biol. 31*, 139–166.

Pokrywka, N.J., Stephenson, E.C., **1991**. Microtubules mediate the localization of *bicoid* RNA during oogenesis. *Development 113*, 55–66.

Pokrywka, N.J., Stephenson, E.C., **1995**. Microtubules are a general component of mRNA localization systems in *Drosophila* oocytes. *Devel. Biol. 167*, 363–370.

Popodi, E., Minoo, P., Burke, T., Waring, G.L., **1988**. Organization and expression of a second chromosome follicle cell gene cluster in *Drosophila*. *Devel. Biol. 127*, 248–256.

Postlethwait, J.H., Shirk, P.D., **1981**. Genetic and endocrine regulation of vitellogenesis in *Drosophila*. *Am. Zool. 21*, 687–700.

Quan, G.X., Kanda, T., Tamura, T., **2002**. Induction of the *white egg 3* mutant phenotype by injection of the double-stranded RNA of the silkworm *white* gene. *Insect Mol. Biol. 11*, 217–222.

Raikhel, A.S., **1984**. The accumulative pathway of vitellogenin in the mosquito oocyte; a high resolution immuno- and cytochemical study. *J. Ultrastruct. Res. 87*, 285–302.

Raikhel, A.S., Dhadialla, T.S., **1992**. Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol. 37*, 217–251.

Raikhel, A.S., Lea, A.O., **1990**. Juvenile hormone controls previtellogenic proliferation of ribosomal RNA in the mosquito fat body. *Gen. Comp. Endocrinol. 77*, 423–434.

Raikhel, A.S., Lea, A.O., **1991**. Control of follicular epithelium development and vitellin envelope formation in the mosquito: role of juvenile hormone and 20-hydroxyecdysone. *Tissue Cell 23*, 577–591.

Raikhel, A.S., Miura, K., Segraves, W.A., **1999**. Nuclear receptors in mosquito vitellogenesis. *Am. Zool. 39*, 722–735.

Raikhel, A.S., Snigirevskaya, E.S., **1998**. Vitellogenesis. In: Harrison, F.W., Locke, M. (Eds.), Microscopic

Anatomy of Invertebrates, Vol. 11C: Insecta. Wiley-Liss, New York, pp. 933–955.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P., Bhatnagar, R.K., **2002**. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *J. Biol. Chem. 277*, 46849–46851.

Ramaswamy, S.B., Shengqiang, S., Park, Y.I., Zeng, F., **1997**. Dynamics of juvenile hormone-mediated gonadotropism in the Lepidoptera. *Arch. Insect Biochem. Physiol. 35*, 539–558.

Ray, R.P., Schüpbach, T., **1996**. Intercellular signalling and the polarization of body axes during *Drosophila* oogenesis. *Genes Devel. 10*, 1711–1722.

Regier, J.C., Friedlander, T., Leclerc, R.F., Mitter, C., Wiegmann, B.M., **1995**. Lepidopteran phylogeny and applications to comparative studies of development. In: Goldsmith, M.R., Wilkins, A.S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, Cambridge, pp. 107–137.

Regier, J.C., Mazur, G.D., Kafatos, F.C., **1980**. The silkmoth chorion: morphological and biochemical characterization of four surface regions. *Devel. Biol. 76*, 296–304.

Regier, J.C., Mazur, G.D., Kafatos, F.C., Paul, M., **1982**. Morphogenesis of silkmoth chorion: initial framework formation and its relation to synthesis of specific proteins. *Devel. Biol. 92*, 159–174.

Regier, J.C., Vlachos, N.S., **1988**. Heterochrony and the introduction of novel modes of morphogenesis during the evolution of moth choriogenesis. *J. Mol. Evol. 28*, 19–31.

Retnakaran, A., Hiruma, K., Palli, S.R., Riddiford, L.M., **1995**. Molecular analysis of the mode of action of RH-5992, a lepidopteran-specific, non-steroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol. 25*, 109–117.

Rice, D.A., Mouw, A.R., Bogerd, A.M., Parker, K.I., **1991**. A shared promoter element regulates the expression of three steroidogenic enzymes. *Mol. Endocrinol. 5*, 1552–1561.

Richard, D.S., Gilbert, M., Crum, B., Hollinshead, D.M., Schelble, S., *et al*., **2001**. Yolk protein endocytosis by oocytes in *Drosophila melanogaster*: immunofluorescent localization of clathrin, adaptin and the yolk protein receptor. *J. Insect Physiol. 47*, 715–723.

Richard, D.S., Watkins, N.L., Serafin, R.B., Gilbert, L.I., **1998**. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol. 44*, 637–644.

Riddiford, L.M., **1993**. Hormones and *Drosophila* development. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 899–939.

Riechmann, V., Ephrussi, A., **2001**. Axis formation during *Drosophila* oogenesis. *Curr. Opin. Genet. Devel. 11*, 374–383.

Riehle, M.A., Brown, M.R., **1999**. Insulin stimulates ecdysteroid production through a conserved signalling cascade in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 29*, 855–860.

Riehle, M.A., Brown, M.R., **2002**. Insulin receptor expression during development and a reproductive cycle in the ovary of the mosquito *Aedes aegypti*. *Cell Tissue Res. 308*, 409–420.

Riehle, M.A., Brown, M.R., **2003**. Molecular analysis of the serine/threonine kinase Akt and its expression in the mosquito *Aedes aegypti*. *Insect Mol. Biol. 12*, 225–232.

Robinson, D.N., Cant, K., Cooley, L., **1994**. Morphogenesis of *Drosophila* ovarian ring canals. *Development 120*, 2015–2025.

Röhrkasten, A., Ferenz, H.-J., **1985**. *In vitro*-study of the selective endocytosis of vitellogenin by locust oocytes. *Wilhelm Roux's Arch. Devel. Biol. 195*, 411–416.

Röhrkasten, A., Ferenz, H.-J., Buschmann-Gebhardt, B., Hafer, J., **1989**. Isolation of the vitellogenin-binding protein from locust ovaries. *Arch. Insect Biochem. Physiol. 10*, 141–149.

Romano, C.P., Bienz-Tadmor, B., Mariani, B.D., Kafatos, F.C., **1988**. Both early and late *Drosophila* chorion gene promoters confer correct temporal, tissue and sex specificity on a reporter Adh gene. *EMBO J. 7*, 783–790.

Rørth, P., **2002**. Initiating and guiding migration: lessons from border cells. *Trends Cell Biol. 12*, 325–331.

Roth, T.F., Porter, K.R., **1964**. Yolk protein uptake in the oocytes of the mosquito *Aedes aegypti*. *J. Cell Biol. 20*, 313–332.

Royzman, I., Austin, R.J., Bosco, G., Bell, S.P., Orr-Weaver, T.L., **1999**. ORC localization in *Drosophila* follicle cells and the effects of mutations in *dE2F* and *dDP*. *Genes Devel. 13*, 827–840.

Royzman, I., Orr-Weaver, T.L., **1998**. S phase and differential DNA replication during *Drosophila* oogenesis. *Genes Cells 3*, 767–776.

Saegusa, H., Mizoguchi, A., Kitahora, H., Nagasawa, H., Suzuki, A., *et al*., **1992**. Changes in the titer of bombyxin-immunoreactive material in hemolymph during the postembryonic development of the silkmoth *Bombyx mori*. *Devel. Growth Diff. 34*, 595–605.

Sahut-Barnola, I., Godt, D., Laski, F.A., Couderc, J.L., **1995**. *Drosophila* ovary morphogenesis: analysis of terminal filament formation and identification of a gene required for this process. *Devel. Biol. 170*, 127–135.

Sappington, T.W., **2002**. The major yolk proteins of higher Diptera are homologs of a class of minor yolk proteins in Lepidoptera. *J. Mol. Evol. 55*, 470–475.

Sappington, T.W., Hays, A.R., Raikhel, A.S., **1995**. Mosquito vitellogenin receptor: purification, developmental and biochemical characterization. *Insect Biochem. Mol. Biol. 25*, 807–817.

Sappington, T.W., Kokoza, V.A., Cho, W.-L., Raikhel, A.S., **1996**. Molecular characterization of the mosquito vitellogenin receptor reveals unexpected high homology to the *Drosophila* yolk protein receptor. *Proc. Natl Acad. Sci. USA 93*, 8934–8939.

Sappington, T.W., Raikhel, A.S., **1995**. Receptor-mediated endocytosis of yolk proteins by insect oocytes. In: Ohnishi,

E., Sonobe, H., Takahashi, S.Y. (Eds.), Recent Advances in Insect Biochemistry and Molecular Biology. University of Nagoya Press, Nagoya, Japan, pp. 235–257.

Sappington, T.W., Raikhel, A.S., **1998a**. Ligand-binding domains in vitellogenin receptors and other LDL-receptor family members share a common ancestral ordering of cysteine-rich repeats. *J. Mol. Evol. 46*, 476–487.

Sappington, T.W., Raikhel, A.S., **1998b**. Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem. Mol. Biol. 28*, 277–300.

Sappington, T.W., Raikhel, A.S., **2004**. Insect vitellogenin/yolk protein receptors. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 229–264.

Sappington, T.W., Oishi, K., Raikhel, A.S., **2002**. Structural characteristics of insect vitellogenin. In: Raikhel, A.S., Sappington, T.W. (Eds.), Recent Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII. John Wiley, Chichester, UK, pp. 69–101.

Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., *et al.*, **1997**. Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comp. Biochem. Physiol. 118B*, 349–357.

Sato, Y., Yamashita, O., **1991**. Structure and expression of a gene coding for egg-specific protein in the silkworm, *Bombyx mori*. *Insect Biochem. 21*, 495–505.

Satyanarayana, K., Bradfield, J.Y., Bhaskaran, G., Dahm, K.H., **1994**. Stimulation of vitellogenin production by methoprene in prepupae and pupae of *Manduca sexta*. *Arch. Insect Biochem. Physiol. 25*, 21–37.

Savant, S.S., Waring, G.L., **1989**. Molecular analysis and rescue of a vitelline membrane mutant in *Drosophila*. *Devel. Biol. 135*, 43–52.

Scherer, L.J., Harris, D.H., Petri, W.H., **1988**. *Drosophila* vitelline membrane genes contain a 114 base pair region of highly conserved coding sequence. *Devel. Biol. 130*, 786–788.

Schneider, L.E., Spradling, A.C., **1997**. The *Drosophila* G-protein-coupled receptor kinase homologue *Gprk2* is required for egg morphogenesis. *Development 124*, 2591–2602.

Schneider, W.J., **1992**. Lipoprotein receptors in oocyte growth. *Clin. Invest. 70*, 385–390.

Schonbaum, C.P., Lee, S., Mahowald, A.P., **1995**. The *Drosophila* yolkless gene encodes a vitellogenin receptor belonging to the low density lipoprotein receptor superfamily. *Proc. Natl Acad. Sci. USA 92*, 1485–1489.

Schonbaum, C.P., Perrino, J.J., Mahowald, A.P., **2000**. Regulation of the vitellogenin receptor during *Drosophila melanogaster* oogenesis. *Mol. Biol. Cell 11*, 511–521.

Schoofs, L., Spittaels, K., Janssen, I., Devreese, B., Van Beeumen, J., *et al.*, **1994**. Isolation of a peptide that stimulates camp production in the ovaries of *Neobellieria bullata*. In: Konopinska, D., Goldsworthy, G., Nachman, R.J., Nawrot, I., Orchard, I., Rosinski, G., Sobota, W. (Eds.), Insects: Chemical, Physiological, and Environmental Aspects. Wydawnictwa Uniwerssytetu, Wroclawskiego, pp. 155–160.

Schüpbach, T., Weischaus, E., **1986**. Maternal-effect mutations altering the antero-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Devel. Biol. 195*, 302–317.

Schwartz, A.L., **1995**. Receptor cell biology: receptor-mediated endocytosis. *Ped. Res. 38*, 835–843.

Schwartz, M.B., Kelly, T.J., Woods, C.W., Imberski, R.B., **1989**. Ecdysteroid fluctuations in adult *Drosophila melanogaster* caused by elimination of pupal reserves and synthesis by early vitellogenic ovarian follicles. *Insect Biochem. 19*, 243–249.

Schwed, G., May, N., Pechersky, Y., Calvi, B.R., **2002**. *Drosophila* minichromosome maintenance 6 is required for chorion gene amplification and genomic replication. *Mol. Biol. Cell 13*, 607–620.

Schweitzer, R., Howes, R., Smith, R., Shilo, B.-Z., Freeman, M., **1995**. Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature 376*, 699–702.

Seo, S.J., Cheon, H.M., Sun, J., Sappington, T., Raikhel, A.S., **2003**. Tissue- and stage-specific expression of two lipophorin receptor variants with 7 and 8 ligand binding repeats in the adult female mosquito, *Aedes aegypti*. *J. Biol. Chem. 278*, 41954–41962.

Sevala, V.L., Davey, K.G., **1993**. Juvenile hormone dependent phosphorylation of a 100 kDa polypeptide is mediated by protein kinase C in the follicle cells of *Rhodnius prolixus*. *Inverteb. Reprod. Devel. 23*, 1898–1903.

Sevala, V.L., Davey, K.G., Prestwich, G.D., **1995**. Photoaffinity labelling and characterization of a juvenile hormone binding protein in the membranes of follicle cells of *Locusta migratoria*. *Insect Biochem. Mol. Biol. 25*, 267–273.

Shaaya, E., Shirk, P.D., Zimowska, G., Plotkin, S., Young, N.J., *et al.*, **1993**. Declining ecdysteroid levels are temporally correlated with the initiation of vitellogenesis during pharate adult development in the Indianmeal moth, *Plodia interpunctella*. *Insect Biochem. Mol. Biol. 23*, 153–158.

Shea, M.J., King, D.L., Conboy, M.J., Mariani, B.D., Kafatos, F.C., **1990**. Proteins that bind to *Drosophila* chorion *cis*-regulatory elements: a new C2H2 zinc finger protein and a C2C2 steroid receptor-like component. *Genes Devel. 4*, 1128–1140.

Sheff, D., Pelletier, L., O'Connell, B., Warren, G., Mellman, I., **2002**. Transferrin receptor recycling in the absence of perinuclear recycling endosomes. *J. Cell Biol. 156*, 797–804.

Shirk, P.D., Bean, D., Millemann, A.M., Brookes, V.J., **1984**. Identification, synthesis, and characterization of the yolk polypeptides of *Plodia interpunctella*. *J. Exp. Zool. 232*, 87–98.

Shirk, P.D., Bean, D.W., Brookes, V.J., **1990**. Ecdysteroids control vitellogenesis and egg maturation in pharate adult females of the Indianmeal moth, *Plodia interpunctella*. *Arch. Insect Biochem. Physiol. 15*, 183–199.

Shirk, P.D., Broza, R., Hemphill, M., Perera, O.P., **1998**. α-Crystallin protein cognates in eggs of the moth, *Plodia interpunctella*: possible chaperones for follicular epithelium yolk protein. *Insect Biochem. Mol. Biol. 28*, 151–161.

Shirk, P.D., Zimowska, G., **1997**. An α-crystallin protein cognate in germ cells of the moth, *Plodia interpunctella*. *Insect Biochem. Mol. Biol. 27*, 149–157.

Shirk, P.D., Zimowska, G., Silhacek, D.L., Shaaya, E., **1992**. Initiation of vitellogenesis in pharate adult females of the Indianmeal moth, *Plodia interpunctella*. *Arch. Insect Biochem. Physiol. 21*, 53–63.

Shirk, P.D., Perera, O.P., **1998**. 5′ Coding region of the follicular epithelium yolk polypeptide 2 cDNA in the moth, *Plodia interpunctella*, contains an extended coding region. *Arch. Insect Biochem. Physiol. 39*, 98–108.

Simonsen, A., Wurmser, A.E., Emr, S.D., Stenmark, H., **2001**. The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol. 13*, 485–492.

Skeiky, Y.A.W., Drevet, J.R., Swevers, L., Iatrou, K., **1994**. Protein phosphorylation and control of chorion gene activation through temporal mobilization of a promoter DNA binding factor from the cytoplasm into the nucleus. *J. Biol. Chem. 269*, 12196–12203.

Skeiky, Y.A.W., Iatrou, K., **1991**. Synergistic interactions of silkmoth chorion promoter-binding factors. *Mol. Cell. Biol. 11*, 1954–1964.

Smith, C.J., Grigorieff, N., Pearse, B., **1998**. Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors. *EMBO J. 17*, 4943–4953.

Smith, J.L., Wilson, J.E., Macdonald, P.M., **1992**. Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in *Drosophila* embryos. *Cell 70*, 849–859.

Snigirevskaya, E.S., Hays, A.R., Raikhel, A.S., **1997a**. Secretory and internalization pathways of mosquito yolk protein precursors. *Cell Tissue Res. 290*, 129–142.

Snigirevskaya, E.S., Raikhel, A.S., **2004**. Receptor-mediated endocytosis in insect oocytes. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 199–228.

Snigirevskaya, E.S., Sappington, T.W., Raikhel, A.S., **1997b**. Internalization and recycling of vitellogenin receptor in the mosquito oocyte. *Cell Tissue Res. 290*, 175–183.

Soller, M., Bownes, M., Kubli, E., **1999**. Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol. 208*, 337–351.

Song, X., Zhu, C.H., Doan, C., Xie, T., **2002**. Germline stem cells anchored by adherens junctions in the *Drosophila* ovary niches. *Science 296*, 1855–1887.

Sorge, D., Nauen, R., Range, S., Hoffmann, K.H., **2000**. Regulation of vitellogenesis in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *J. Insect Physiol. 46*, 969–976.

Sourmeli, S., **2004**. Molecular analysis of differential gene expression in lepidopteras insects. Ph.D. thesis, Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, Greece.

Sourmeli, S., Kravariti, L., Lecanidou, R., **2003**. In vitro analysis of *Bombyx mori* early chorion gene regulation: stage specific expression involves interactions with C/EBP-like and GATA factors. *Insect Biochem. Mol. Biol. 33*, 525–540.

Spoerel, N.A., Nguyen, H.T., Eickbush, T.H., Kafatos, F.C., **1989**. Gene evolution and regulation in the chorion complex of *Bombyx mori*. Hybridization and sequence analysis of multiple developmentally middle A/B chorion gene pairs. *J. Mol. Biol. 209*, 1–19.

Spoerel, N.A., Nguyen, H.T., Kafatos, F.C., **1986**. Gene evolution and regulation in the chorion complex of *Bombyx mori*. Structural and developmental characterization of four eggshell genes and their flanking DNA regions. *J. Mol. Biol. 190*, 23–35.

Spoerel, N.A., Nguyen, H.T., Towne, S., Kafatos, F.C., **1993**. Negative and positive regulators modulate the activity of a silkmoth chorion gene during choriogenesis. *J. Mol. Biol. 230*, 151–160.

Spradling, A.C., **1993**. Developmental genetics of oogenesis. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–70.

Spradling, A.C., de Cicco, D.V., Wakimoto, B.T., Levine, J.F., Kalfayan, L.J., Cooley, L., **1987**. Amplification of the X-linked *Drosophila* chorion gene cluster requires a region upstream from the *s38* chorion gene. *EMBO J. 6*, 1045–1053.

Spradling, A.C., Drummond-Barbosa, D., Kai, T., **2001**. Stem cells find their niche. *Nature 414*, 98–104.

Springer, T.A., **1998**. An extracellular (β-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. *J. Mol. Biol. 283*, 837–862.

Stebbings, H., Lane, J.D., Talbot, N.J., **1995**. mRNA translocation and microtubules: insect ovary models. *Trans. Cell Biol. 5*, 361–365.

Stevens, L., **1998**. Twin peaks: Spitz and Argos Star in the patterning of the *Drosophila* egg. *Cell 95*, 291–294.

Storto, P.D., King, R.C., **1989**. The role of polyfusomes in generating branched chains of cystocytes during *Drosophila* oogenesis. *Devel. Genet. 10*, 70–86.

Strickland, D.K., Gonias, S.L., Argraves, W.S., **2002**. Diverse roles for the LDL receptor family. *Trends Endocrin. Metab. 13*, 66–74.

Sun, G., Zhu, J., Li, C., Tu, Z., Raikhel, A.S., **2002**. Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, *Aedes aegypti*. *Mol. Cell. Endocrinol. 190*, 147–157.

Sun, G.-C., Hirose, S., Ueda, H., **1994**. Intermittent expression of BmFTZ-F1, a member of the nuclear hormone receptor superfamily during development

of the silkworm *Bombyx mori*. *Devel. Biol. 162*, 426–437.

Sun, J., Hiraoka, T., Dittmer, N.T., Cho, K.-H., Raikhel, A.S., **2000**. Lipophorin as a yolk protein in the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 30*, 1161–1171.

Sutherland, T.D., Unnithan, G.C., Andersen, J.F., Evans, P.H., Murataliev, M.B., *et al.*, **1998**. A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA 95*, 12884–12889.

Sutherland, T.D., Unnithan, G.C., Feyereisen, R., **2000**. Terpenoid ω-hydroxylase (CYP4C7) messenger RNA levels in the corpora allata: a marker for ovarian control of juvenile hormone synthesis in *Diploptera punctata*. *J. Insect Physiol. 46*, 1219–1227.

Suzanne, M., Perrimon, N., Noselli, S., **2001**. The *Drosophila* JNK pathway controls the morphogenesis of the egg dorsal appendages and the micropyle. *Devel. Biol. 237*, 282–294.

Swevers, L., Drevet, J.R., Lunke, M.D., Iatrou, K., **1995**. The silkmoth homolog of the *Drosophila* ecdysone receptor (B1) isoform: cloning and analysis of expression during follicular cell differentiation. *Insect Biochem. Mol. Biol. 25*, 857–866.

Swevers, L., Eystathioy, T., Iatrou, K., **2002a**. The orphan nuclear receptors BmE75A and BmE75C of the silkmoth *Bombyx mori*: hormonal control and ovarian expression. *Insect Biochem. Mol. Biol. 32*, 1643–1652.

Swevers, L., Iatrou, K., **1992**. Early establishment and autonomous implementation of a developmental program controlling silkmoth chorion gene expression. *Devel. Biol. 150*, 12–22.

Swevers, L., Iatrou, K., **1998**. The orphan receptor BmHNF-4 of the silkmoth *Bombyx mori*: ovarian and zygotic expression of two mRNA isoforms encoding polypeptides with different activating domains. *Mech. Devel. 72*, 3–13.

Swevers, L., Iatrou, K., **1999**. The ecdysone agonist tebufenozide (RH-5992) blocks progression of the ecdysteroid-regulated gene expression cascade during silkmoth oogenesis and causes developmental arrest at mid-vitellogenesis. *Insect Biochem. Mol. Biol. 29*, 955–963.

Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297.

Swevers, L., Ito, K., Iatrou, K., **2002b**. The BmE75 nuclear receptors function as dominant repressors of the nuclear receptor BmHR3A. *J. Biol. Chem. 277*, 41637–41644.

Swimmer, C., Delidakis, C., Kafatos, F.C., **1989**. Amplification control element ACE3 is important but not essential for autosomal chorion gene amplification. *Proc. Natl Acad. Sci. USA 86*, 8823–8827.

Takei, K., Haucke, V., **2001**. Clathrin-mediated endocytosis: Membrane factors pull the trigger. *Trends Cell Biol. 11*, 385–391.

Tamura, T., Thibert, C., Royer, C., Toshio, K., Eappen, A., *et al.*, **2000**. Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnol. 18*, 81–84.

Tawfik, A.I., Vedrova, A., Sehnal, F., **1999**. Ecdysteroids during ovarian development and embryogenesis in solitary and gregarious *Schistocerca gregaria*. *Arch. Insect Biochem. Physiol. 41*, 134–143.

Telfer, W.H., **1960**. The selective accumulation of blood proteins by the growing oocytes of saturniid moths. *Biol. Bull. 118*, 338–351.

Telfer, W.H., **2002**. Insect yolk proteins: a progress report. In: Raikhel, A.S., Sappington, T.W. (Eds.), Recent Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII. Science Publishers, Inc., Enfield, NH, pp. 29–67.

Telfer, W.H., Huebner, E., Smith, D.S., **1982**. The cell biology of vitellogenic follicles in *Hyalophora* and *Rhodnius*. In: King, R.C., Akai, H. (Eds.), Insect Ultrastructure, vol. 1. Plenum, New York, pp. 118–149.

Telfer, W.H., Pan, M., **1988**. Adsorptive endocytosis of vitellogenin, lipophorin, and microvitellogenin during yolk formation in Hyalophora. *Arch. Insect Biochem. Physiol. 9*, 339–355.

Telfer, W.H., Woodruff, R.I., **2002**. Ion physiology of vitellogenic follicles. *J. Insect Physiol. 48*, 915–923.

Terpstra, P., Ab, G., **1988**. Homology of *Drosophila* yolk proteins and the triacylglycerol lipase family. *J. Mol. Biol. 202*, 663–665.

Theurkauf, W.E., Smiley, S., Wong, M.L., Alberts, B.M., **1992**. Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. *Devel. 115*, 923–936.

Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., **1993**. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. *Nature 362*, 471–475.

Thummel, C.S., **1995**. From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell 83*, 871–877.

Thummel, C.S., **1996**. Flies on steroids – *Drosophila* metamorphosis and the mechanism of steroid hormone action. *Trends Genet. 12*, 306–310.

Tolias, P.P., Kafatos, F.C., **1990**. Functional dissection of an early *Drosophila* chorion gene promoter: expression throughout the follicular epithelium is under spatially composite regulation. *EMBO J. 9*, 1457–1464.

Tolias, P.P., Konsolaki, M., Halfon, M.S., Stroumbakis, N.D., Kafatos, F.C., **1993**. Elements controlling follicular expression of the *s36* chorion gene during *Drosophila* oogenesis. *Mol. Cell. Biol. 13*, 5898–5906.

Tooze, J., Hollinshead, M., **1991**. Tubular early endosomal networks in AtT20 and other cells. *J. Cell Biol. 115*, 635–654.

Trougakos, I.P., Margaritis, L.H., **1998**. Immunolocalization of the temporally "early" secreted major structural chorion proteins, Dvs38 and Dvs36, in the eggshell layers and regions of *Drosophila virilis*. *J. Struct. Biol. 123*, 111–123.

Trougakos, I.P., Papassideri, I.S., Waring, G.L., Margaritis, L.H., **2001**. Differential sorting of constitutively co-secreted proteins in the ovarian follicle cells of *Drosophila*. *Eur. J. Cell Biol. 80*, 271–284.

Troy, S., Anderson, W.A., Spielman, A., **1975**. Lipid content of maturing ovaries of *Aedes aegypti* mosquitoes. *Comp. Biochem. Physiol. B. 50*, 457–461.

Tsitilou, S.G., Rodakis, G.C., Alexopoulou, M., Kafatos, F.C., Ito, K., *et al.*, **1983**. Structural features of B family chorion sequences in the silkmoth *Bombyx mori*, and their evolutionary implications. *EMBO J. 2*, 1845–1852.

Tsuchida, K., Nagata, M., Suzuki, A., **1987**. Hormonal control of ovarian development in the silkworm, *Bombyx mori*. *Arch. Insect Biochem. Biophys. 5*, 167–177.

Tufail, M., Raikhel, A.S., Takeda, M., **2004**. Biosynthesis and processing of insect vitellogenins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 1–32.

Twombly, V., Blackman, R., Jin, H., Graff, J., Padgett, R., Gelbart, W., **1996**. The TGF-beta signalling pathway is essential for *Drosophila* oogenesis. *Development 122*, 1555–1565.

Tzertzinis, G., Malecki, A., Kafatos, F.C., **1994**. BmCF1, a *Bombyx mori* RXR-type receptor related to the *Drosophila ultraspiracle*. *J. Mol. Biol. 238*, 479–486.

Uhlirova, M., Foy, B.D., Beaty, B.J., Olson, K.E., Riddiford, L.M., *et al.*, **2003**. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proc. Natl Acad. Sci. USA 100*, 15607–15612.

Val, P., Lefrançois-Martinez, A.-M., Veyssière, G., Martinez, A., **2003**. SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl. Recept. 1*, 8–30.

Valdes, V.J., Sampieri, A., Sepulveda, J., Vaca, L., **2003**. Using double-stranded RNA to prevent *in vitro* and *in vivo* viral infections by recombinant baculovirus. *J. Biol. Chem. 278*, 19317–19324.

Van Antwerpen, R., Conway, R., Law, J.H., **1993**. Protein and lipoprotein uptake by developing oocytes of the hawkmoth, *Manduca sexta*: an ultrastructural and immunocytochemical study. *Tissue Cell 25*, 205–218.

Van Antwerpen, R., Pham, D.Q.-D., Ziegler, R., **2004**. Accumulation of lipids in insect oocytes. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 265–288.

Van Buskirk, C., Schüpbach, T., **1999**. Versatility in signaling: multiple responses to EGF receptor activation during *Drosophila* oogenesis. *Trends Cell Biol. 9*, 1–4.

Van Eeden, F., St. Johnston, D., **1999**. The polarization of the anterior–posterior and dorsal–ventral axes during *Drosophila* oogenesis. *Curr. Opin. Genet. Devel. 9*, 396–404.

Vlachou, D., Komitopoulou, K., **2001**. The chorion genes of the medfly. II. DNA sequence evolution of the autosomal chorion genes *s18*, *s15*, *s19* and *s16* in Diptera. *Gene 270*, 41–52.

Walker, L.L., Watson, K.L., Holden, J.J.A., Steel, C.G.H., **1987**. Vitellogenesis and fertility in *Drosophila* females with low ecdysteroid titers; the *L(33^{DTS})*. *J. Insect Physiol. 33*, 137–143.

Wall, D.A., Meleka, I., **1985**. An unusual lysosome compartment involved in vitellogenin endocytosis by Xenopus oocytes. *J. Cell Biol. 101*, 1651–1664.

Wang, S.-F., Li, C., Zhu, J., Miura, K., Miksicek, R.J., Raikhel, A.S., **2000**. Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. *Devel. Biol. 218*, 99–113.

Wang, W.-Y., Cole, K., Law, J., **1989**. The nucleotide sequence of microvitellogenin encoding gene from the tobacco hornworm, *Manduca sexta*. *Gene 80*, 259–268.

Wang, Y., Telfer, W.H., **1996**. Cyclic nucleotide-induced termination of vitellogenin uptake by *Hyalophora cecropia* follicles. *Insect Biochem. Mol. Biol. 26*, 85–94.

Wang, Y., Telfer, W.H., **1998a**. Cyclic-AMP-induced water uptake in a moth ovary: inhibition by bafilomycin and anthracene-9-carboxylic acid. *J. Exp. Biol. 201*, 1627–1635.

Wang, Y., Telfer, W.H., **1998b**. Pertussis toxin-sensitive G protein that supports vitellogenin uptake by promoting patency. *Arch. Insect Biochem. Physiol. 39*, 36–45.

Wang, Y., Telfer, W.H., **2000**. Cyclic nucleotide-dependent protein phosphorylation in vitellogenic follicles of *Hyalophora cecropia*. *Insect Biochem. Mol. Biol. 30*, 29–34.

Waring, G.L., Hawley, R.J., Schoenfeld, T., **1990**. Multiple proteins are produced from the dec-1 eggshell gene in *Drosophila* by alternative RNA splicing and proteolytic cleavage events. *Devel. Biol. 142*, 1–12.

Warn, R., Gutzeit, H., Smith, L., Warn, A., **1985**. F-actin rings are associated with the ring canals of the *Drosophila* egg chamber. *Exp. Cell Res. 157*, 355–363.

Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048.

Warrier, S., Subramoniam, T., **2002**. Receptor mediated yolk protein uptake in the crab *Scylla serrata*: crustacean vitellogenin receptor recognizes related mammalian serum lipoproteins. *Mol. Reprod. Devel. 61*, 536–548.

Wasserman, J.D., Freeman, M., **1998**. An autoregulatory cascade of EGF receptor signalling patterns the *Drosophila* egg. *Cell 95*, 355–364.

Wheatley, S., Kulkarni, S., Karess, R., **1995**. *Drosophila* nonmuscle myosin II is required for rapid cytoplasmic transport during oogenesis and for axial nuclear migration in embryos. *Development 121*, 1937–1946.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., **1997**. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science 276*, 114–117.

Whyatt, D.J., de Boer, E., Grosveld, F., **1993**. The two zinc finger-like domains of GATA-1 have different DNA binding specificities. *EMBO J. 12*, 4993–5005.

Wieschaus, E., Szabad, J., **1979**. The development and function of the female germ line in *Drosophila melanogaster. Z. Zellforsch. 102*, 129–152.

Willnow, T.E., **1999**. The low-density lipoprotein receptor gene family: multiple roles in lipid metabolism. *J. Mol. Med. 77*, 306–315.

Wilson, C., Wardell, M.R., Weisgraber, K.H., Mahley, R.W., Agard, D.A., **1991**. Three-dimensional structure of the LDL receptor-binding domain of human *apolipoprotein* E. *Science 252*, 1817–1822.

Wilson, T.G., **1982**. A correlation between juvenile hormone deficiency and vitellogenic oocyte degeneration in *Drosophila melanogaster. Wilhelm Roux's Arch. Devel. Biol. 191*, 257–263.

Wolfner, M.F., **1988**. Sex-specific gene expression in somatic tissues of *Drosophila melanogaster. Trends Genet. 4*, 333–337.

Woodard, C.T., Baehrecke, E.H., Thummel, C.S., **1994**. A molecular mechanism for the stage-specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell 79*, 607–615.

Wyatt, G.R., **1991**. Gene regulation in insect reproduction. *Invert. Reprod. Devel. 20*, 1–35.

Xie, T., Spradling, A.C., **1998**. *decapentaplegic* is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell 94*, 251–260.

Xie, T., Spradling, A.C., **2000**. A niche maintaining germline stem cells in the *Drosophila* ovary. *Science 290*, 328–330.

Xiong, Y., Sakaguchi, B., Eickbush, T.H., **1988**. Gene conversion can generate sequence variants in the late chorion gene multigene families of *Bombyx mori. Genetics 120*, 221–231.

Xue, F., Cooley, L., **1993**. *kelch* encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell 72*, 681–693.

Yamahama, Y., Yamamoto, Y., Watabe, S., Takahashi, S.Y., **2004**. Yolk protein degradation during insect embryogenesis. In: Raikhel, A.S., Sappington, T.W. (Eds.), Progress in Vitellogenesis. Reproductive Biology of Invertebrates, vol. XII, Part B. Science Publishers, Inc., Enfield, NH, pp. 315–354.

Yamamoto, T., Davis, C.G., Brown, M.S., Schneider, W.J., Casey, M.L., *et al.*, **1984**. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell 39*, 27–38.

Yamao, M., Katayama, N., Nakazawa, H., Yamakawa, M., Hayashi, Y., *et al.*, **1999**. Gene targeting in the silkworm by use of a baculovirus. *Genes Devel. 13*, 511–516.

Yamauchi, H., Yoshitake, N., **1984**. Developmental stages of ovarian follicles of the silkworm, *Bombyx mori* L. *J. Morphol. 179*, 21–31.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., *et al.*, **1993**. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature 366*, 476–479.

Yue, L., Spradling, A., **1992**. *hu-li tai shao*, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes Devel. 6*, 2443–2454.

Yun, H., Kim, W., Kim, H., **1994**. Immunological analysis of lipophorin in the haemolymph, ovaries and testes of the fall webworm, *Hyphantria cunea* (Drury). *Arch. Insect Biochem. Physiol. 27*, 153–167.

Zeng, F., Shu, S., Park, Y.I., Ramaswamy, S.B., **1997**. Vitellogenin and egg production in the moth, *Heliothis virescens. Arch. Insect Biochem. Physiol. 34*, 287–300.

Zhang, J., McCracken, A., Wyatt, G.R., **1993**. Properties and sequence of a female-specific, juvenile hormone-induced protein from locust hemolymph. *J. Biol. Chem. 268*, 3282–3288.

Zhang, J., Wyatt, G.R., **1996**. Cloning and upstream sequence of a juvenile hormone-regulated gene from the migratory locust. *Gene 175*, 193–197.

Zhu, C.H., Xie, T., **2003**. Clonal expansion of ovarian germline stem cells during niche formation in *Drosophila*. *Development 130*, 2579–2588.

Zhu, J., Indrasith, L., Yamashita, O., **1986**. Characterization of vitellin, egg-specific protein and 30-kDa protein from Bombyx eggs, and their fates during oogenesis and embryogenesis. *Biochim. Biophys. Acta 882*, 427–436.

Zhu, J., Miura, K., Chen, L., Raikhel, A.S., **2000**. AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti. EMBO J. 19*, 253–262.

Zimowska, G., Shirk, P.D., Silhacek, D.L., Shaaya, E., **1994**. Yolk sphere formation is initiated in oocytes before development of patency in follicles of the moth *Plodia interpunctella. Wilhelm Roux's Arch. Devel. Biol. 203*, 215–226.

Zimowska, G., Shirk, P.D., Silhacek, D.L., Shaaya, E., **1995**. Vitellin and formation of yolk spheres in vitellogenic follicles of the moth, *Plodia interpunctella. Arch. Insect Biochem. Physiol. 29*, 71–85.

Zimowska, G., Silhacek, D.L., Shaaya, E., Shirk, P.D., **1991**. Immuno-fluorescent analysis of follicular growth and development in whole ovaries of the Indianmeal moth. *J. Morphol. 209*, 215–228.

1.4 Spermatogenesis

R Renkawitz-Pohl and L Hempel, Philipps-University, Marburg, Germany
M Hollmann and M A Schäfer, Universität Kassel, Kassel, Germany

1.4.1. Introduction

Spermatogenesis is a highly specialized process of cellular differentiation resulting in the formation of functional spermatozoa for successful reproduction. In principle, the process of spermatogenesis is well conserved in all sexually proliferating organisms, although the size and shape of the mature sperm vary considerably among different species. Many details are comparable between mammals and *Drosophila* making the fly a very good model system to study fertility defects. *Drosophila* germ cells, like those of mammals, are set aside early in embryonic development and migrate through the primordium of the hindgut into the interior of the embryo where they join the somatic parts of the embryonic gonads (review: Zhao and Garbers, 2002). At the end of the third larval instar and the onset of pupariation, the first germ cells enter meiosis (**Figure 1**).

Spermatogenesis is a continuous process during adult life and, thus, the adult testes contain all stages from stem cells to mature sperm (**Figure 1**). As in mammals, the germ cells develop in close contact with somatic cells, in this case the cyst cells, which are of mesodermal origin. At the very tip of the testis tube, the so-called hub is formed by somatic support cells (asterisk in **Figure 1**) to which the germline stem cells (GSCs) and the cyst cell progenitors (somatic stem cells, SSCs) are physically connected. In close contact to the hub, both stem cell types divide asymmetrically depending on the JAK-STAT signaling pathway (see Section 1.4.3.1 for details). One daughter cell remains connected to the hub and maintains stem cell characteristics. The other daughter cell becomes disconnected from the hub and enters the differentiation process. This germ cell, now called spermatogonium, is surrounded by two cyst cells, thus forming a cyst in which the germ cell undergoes four mitotic divisions, meiosis, and sperm morphogenesis until individualization (see **Figure 3** for an overview).

The ultrastructure and cytology of *Drosophila melanogaster* spermatogenesis has been extensively reviewed by Fuller (1993). This complex differentiation process from round cells to the highly specialized structure of spermatozoa is controlled by a large number of genes (up to 1500) affecting spermatogenesis, which is reflected in the large number of male sterile mutants (reviews: Lindsley and Tokuyasu, 1980; Hackstein *et al.*, 2000). The focus here is on the recent advances in understanding the molecular basis for the dramatic changes in

cell morphology during germ cell development starting with the asymmetric division of stem cells into a stem cell and a spermatogonium as the entry point to germ cell differentiation. Control of mitotic proliferation and entry into meiosis are discussed and various aspects of sperm morphogenesis highlighted, such as the formation of the axoneme and the mitochondrial derivative, the Nebenkern. Then the importance of cell interactions during the process is discussed (**Figure 4**), and finally transcriptional and translational control mechanisms during meiotic prophase, and their relevance for sperm morphogenesis (**Figure 3**).

1.4.2. Morphology and Development

At the cellular blastoderm stage of embryogenesis, the somatic cells and the primordial germ cells (PGCs), visible as pole cells, are separated. During gastrulation, pole cells migrate into a pocket of the posterior endoderm primordium through which they migrate into the interior of the embryo. This invasion and the guided migration together with mesodermal cells is followed by aggregation of both cell types to form the gonad. It has been reently shown that the receptor tyrosine kinase Torso (Tor) activates both signal transducer and activator of transcription (STAT) and Ras, a monomeric GTPase, during the early phase of PGC development, and that coactivation of STAT and Ras is required for invasive migration (Li *et al.*, 2003). Then germ cells comigrate with cells of mesodermal origin to the abdominal segment A5, which surround the germ cells and give rise to the gonad sheath. The *fear of intimacy* (*foi*) gene is expressed in the gonadal mesoderm and encodes a transmembrane protein. Foi and E-cadherin encoded by the *shotgun* (*shg*) locus are similarly required for gonad coalescence, the final step in gonad formation (Jenkins *et al.*, 2003; Van Doren *et al.*, 2003). In *foi* mutants, E-cadherin levels are dramatically decreased indicating that Foi is a positive modulator of E-cadherin expression or function. Thus, Foi is needed to maintain high levels of E-cadherin and thereby proper cell adhesion for gonad formation.

Already during gonad formation, sex-specific effects can be observed in the somatic part of the gonad and the germline. During gonad coalescence a group of cells, termed the male-specific somatic gonadal precursors (msSGPs), is specified. These cells express Sox100B, a *Drosophila* homolog of Sox9, which is an essential transcription factor for testis development in mammals. The msSGPs are incorporated into the male gonad only; in the female sex, they are eliminated by sex-specific apoptosis leading to a difference in gonad morphology between the sexes (DeFalco *et al.*, 2003). Furthermore, even though both sexes start out with the same number of pole cells formed, the gonads incorporate a sex-specific number of germ cells. Male gonads contain 14,2 germ cells, on average, compared to 12,2 in female gonads (Poirié *et al.*, 1995).

In abdominal segment A8, the genital disc is established. This differentiates during metamorphosis into the genital ducts and their accessory structures such as seminal vesicles and accessory glands, which are also called paragonia (see Hartenstein, 1993 and **Figure 1** for an overview of the male reproductive tract). Sex determination in *Drosophila* has been well studied and shown to be a cell autonomous process in somatic cells (review: Lalli *et al.*, 2003; see also **Chapters 1.1** and **1.7**). The germ cells are determined in the gonads to enter spermatogenesis or oogenesis. XX germ cells are in contact with XX somatic cells of the gonad which leads to repression of male-specific gene transcription by a short-range signal (Heller and Steinmann-Zwicky, 1998). In XY germ cells, cell autonomous and somatic signals lead to male-specific gene expression and spermatogenesis (Janzer and Steinmann-Zwicky, 2001).

1.4.2.1. Spermatogonia

Spermatogonia go through a mitotic amplification phase with incomplete cytokinesis such that the germ cells remain interconnected and, thus, establish cytoplasmic bridges with ring canals and fusomes. Cessation of mitoses and transition into meiotic prophase is dependent upon the genes *bag of marbles* (*bam*) and *benign gonial cell neoplasm* (*bgcn*) in the germ cells and transforming the growth factor-β (TGF-β) signaling from the cyst cells (Gönczy *et al.*, 1997; review: Fuller, 1998). The molecular analysis revealed that Bgcn is related to DexH-box proteins and may be involved in translational control (Ohlstein *et al.*, 2000). Bam is localized in the cytoplasm, but no further clues to its function exist (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995). The genes *bam* and *bgcn* also play a role in oogenesis. There they are essential for the distinction between stem cell and oogonium during asymmetric stem cell division, as discussed in Section 1.4.3.2. (review: Deng and Lin, 2001; see **Chapter 1.2**).

1.4.2.2. Spermatocytes

After four mitotic divisions, the cells within the cyst enter meiosis as primary spermatocytes (**Figure 1a**) and remain in the G2 phase of the cell cycle for ~3.5

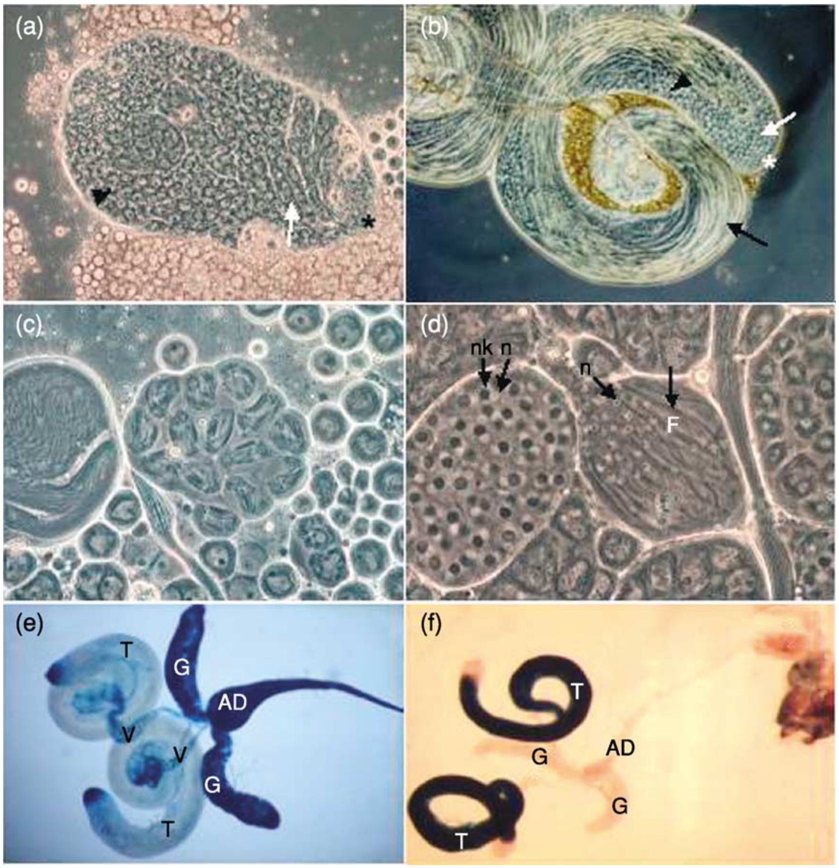


Figure 1 Stages of spermatogenesis in testes of late third instar larvae and adult males. (a) Testis anlage of a larva showing the hub and stem cell region at the apex (asterisk), a cyst with spermatogonia (white arrow), and a cyst with primary spermatocytes (black arrowhead). (b) Testis of an adult male showing the apical tip with hub and stem cells (asterisk), spermatogonia (white arrow), spermatocytes (arrowhead), and elongated spermatids (black arrow). (c) The cyst shows synchronous meiotic divisions. (d) The left cyst shows a Nebenkern stage shortly after the second meiotic division. In the phase contrast optics the nucleus (n) appears light, the Nebenkern (nk) dark. The right cyst contains young spermatids with a round nucleus (n) and an elongating flagellum (F). (e) β1-LacZ reporter gene expression in the male reproductive tract (Wβ1K-carrying transgenic line; Buttgereit and Renkawitz-Pohl, 1993). Within the testes (T), stem cells and spermatogonia express β-galactosidase. In addition, β-galactosidase expression is also observed in the *vas deferens* (V), in accessory glands (G), and in the ejaculatory duct (AD). (f) β2-LacZ reporter gene expression in the male reproductive tract (Michiels *et al.*, 1989).

days (Lindsley and Tokuyasu, 1980). This stage is characterized by extensive transcription, leading to a 25-fold size increase of the nuclei, and by the formation of lampbrush loops from the Y chromosome, which are essential for male fertility (review: Hackstein and Hochstenbach, 1995). At least some of these Y chromosome fertility genes encode dynein subunits (see Section 1.4.2.4.1). Meiotic arrest mutants show developmental arrest at the transition from G2 to meiosis I as they control the accumulation of Twine (Twe), a Cdc25 homolog (White-Cooper *et al.*, 1998). The meiotic arrest mutants – *always early* (*aly*), *cannonball* (*can*), *meiotic arrest* (*mia*), *cookie monster* (*comr*), and *spermatocyte arrest* (*sa*) – fall into two classes (Fuller, 1998; White-Cooper *et al.*, 1998; Jiang and White-Cooper, 2003). Aly and Comr are proposed to belong to chromatin remodeling complexes, while Can, Mia, and Sa form the second class. At least for Can, it has been shown that it interacts directly with the transcription initation complex of sperm differentiation genes, which will be discussed in detail below (for a model, see Jiang and White-Cooper, 2003).

1.4.2.3. Meiosis

Clearly, many features are common between mitosis and male as well as female meiosis (see **Chapter 1.2**). A peculiar feature of *Drosophila* male meiosis, however, is the lack of synaptonemal complex formation with the consequence that meiotic recombination does not occur in male germ cells. After the extended meiotic prophase I, all further stages of the meiotic divisions proceed rapidly, following induction by specific pathways and appropriate actions of cell cycle regulators such as Twe, a Cdc25-type phosphatase, and Boule (Bol), an RNA binding protein.

Both are essential for G2/M transition and Boule controls Twine translation (Maines and Wasserman, 1999). The meiotic spindle has to be assembled and, later, the contractile ring must assemble in order for cytokinesis to take place (review: Maines and Wasserman, 1998). The first 16 spermatocytes within one cyst enter the meiotic divisions at the white prepupal stage. During the first meiotic division the two nuclear membranes remain until telophase, then they disintegrate and stepwise reform. For the rapidly ensuing second division the situation is less well analyzed but seems to be similar (Tates, 1971; Church and Lin, 1982). In both divisions, mitochondria line up and are distributed equally among the daughter cells (Fuller, 1993). Furthermore, as in every meiosis, chromosome segregation has to be tightly controlled. Recently, the *Drosophila* mutants *fusolo* and *solofuso* have been analyzed in male meiosis. Homozygous mutants are severely defective in chromosome segregation and produce secondary spermatocytes that are devoid of chromosomes (Bucciarelli *et al*., 2003). The occurrence of these secondary spermatocytes without chromosomes shows that spindle formation and cytokinesis are independent of the presence of chromosomes (Bucciarelli *et al*., 2003). From the study of mutants in specific genes there is clear evidence that formation of the contractile ring constriction and its disassembly require different genes (review: Giansanti *et al*., 2001).

Several genes have been identified that interfere with one or several aspects of meiosis, but a general network of control has not emerged yet. Mutations in the genes – *sa*, *can*, *aly*, and *mia* – lead to meiotic arrest in cell cycle progression and spermiogenesis. Lin *et al*. (1996) therefore suggested the existence of a control point that may serve to coordinate the male meiotic cell cycle with the spermatid differentiation program. Time-lapse microscopy revealed the existence of a spindle checkpoint in male meiosis, where a large number of misaligned chromosomes or severe disruption of the spindle leads to delays in the entry into anaphase (Rebollo and González, 2000). As demonstrated by the phenotype of mutants in *pelota (pelo),* this gene is involved in spindle formation and nuclear envelope modification but not in chromatin condensation, which proves that various aspects of meiosis are controlled by different genes (Eberhart and Wasserman, 1995). During germ cell divisions, cytokinesis is incomplete, resulting in clusters of cells with stable intercellular bridges which interconnect mitotically or meiotically related germ cells. These stable intercellular bridges are called ring canals and the total set of ring canals between the germ cells in one cyst is termed the fusome (see **Chapter 1.2**). In males, the cohort of cells remains interconnected until sperm individualization. Actin organization and the formation of intercellular bridges are different in the male and female germline (Hime *et al*., 1996). Mature ring canal walls in males lack actin, but contain three septins (Pnut, Sep1, and Sep2) and anillin. Anillin and septins are missing in ovarian ring canals, in which filamentous actin is a major component. A mutation in *four wheel drive (fwd)*, a PI4-kinase, affects the formation of these intercellular bridges during meiosis. This PI4-kinase is required during cytokinesis for tyrosine phosphorylation in the cleavage furrow and organization of actin filaments in the constricting contractile ring. Loss of PI4-kinase activity leads to unstable cleavage furrows and to fusion of daughter cells resulting in multinucleate spermatids (Brill *et al*., 2000); consequently, ring canals are not established.

Genes of general importance may also have a specific function in spermatogenesis. The gene *fumble (fbl)* encodes a pantothenate kinase homolog that is required for mitosis and meiosis during spermatogenesis and affects spindle organization, chromosome segregation, and contractile ring formation. Since it localizes to areas of membrane deposition, its function could be related to synthesis and redistribution of membranous material (Afshar *et al*., 2001). Centrosomes contain the protein Centrosomin (Cnn) and organize the spindle assembly. After male meiosis the centriole is transformed into the sperm basal body. A novel isoform of Cnn that is expressed during spermatogenesis is essential for spindle organization and for the organization of the sperm axoneme (Li *et al*., 1998). During mitotic spindle assembly, the kinase aurora-A interacts with the C-terminal sequence of Cnn and thereby regulates centrosome assembly. The N-terminal half of Cnn then interacts with γ-tubulin. Thus, this kinase controls the ability of Cnn to target and/or anchor γ-tubulin to the centrosome (Terada *et al*., 2003).

During spermatogenesis several special formations of the endoplasmic reticulum and the Golgi can be observed. When spermatocytes enter meiosis they develop parafusorial membranes as well as astral membranes that surround the nucleus on the outside of its double membrane. During spermiogenesis the Golgi forms the acrosome which is necessary for sperm–egg recognition and interaction on the sperm head, and an acrosomal sheath generated from the endoplasmic reticulum (ER) extends along the elongating sperm tail (Lindsley and Tokuyasu, 1980; Fuller, 1993). Thus, it is not surprising that mutations in many genes, with roles in ER formation or processes related to it, would lead to a male

sterile phenotype during spermatogenesis. Syntaxin 5 (Syx5) is a Golgi-localized soluble NSF attachment receptor (SNARE) protein shown to be required for ER–Golgi vesicle traffic. Homozygous flies for a hypomorphic allele are male sterile. This results both from the failure of germ cells to complete cytokinesis and from defects in spermatid elongation and maturation. Thus, during spermatogenesis, Syx5 is required for cytokinesis and for spermatid differentiation, which demonstrates the importance of proper establishment of the Golgi apparatus and its special derivatives during spermatogenesis (Xu *et al.*, 2002). Similarly, mutations in *four way stops* (*fws*) cause dramatic alterations in cell morphology related to Golgi architecture, including disruption of the formation and/or stability of the Golgi-based spermatid acroblast. The *fws* gene encodes the *Drosophila* Cog5 homolog, a subunit of the multisubunit complex COG (conserved oligomeric Golgi). This allows the hypothesis that vesicle traffic is essential during sperm morphogenesis (Farkas *et al.*, 2003). A testis-specific vesicle-associated membrane protein-associated protein (VAP)-like protein, Farinelli (Fan), has been identified, which also associates with derivatives of the ER during various stages of spermatogenesis. Mutations in *fan* result in individualization defects, which could be related to defective transport processes (U. Renner, M. Hollmann, and M.A. Schäfer, in preparation).

1.4.2.4. Sperm Morphogenesis

1.4.2.4.1. Axoneme: β2-tubulin and dynein Motility of the sperm is dependent on the axoneme, which is composed of nine outer microtubule doublets and two inner microtubuli as well as a number of other proteins. The axoneme structure is highly conserved in cilia and flagella from protists to mammals, suggesting a common assembly mechanism. The cylinder of nine doublet microtubules is initiated from the triple microtubules of the basal body, while the inner two microtubules are formed separately. The microtubules are characterized by the presence of the testis-specific isotype β2-tubulin (for a review on the genetics of tubulin genes, see Fuller, 1993).

The β2-tubulin isotype shares a highly conserved C-terminal sequence motif with β-tubulin isotypes from the axoneme of other species. This region is called the axoneme-specific sequence motif (Nielsen *et al.*, 2001). Testing of this motif in the context of the β1-tubulin isotype in transgenic flies showed, in particular, that this β2-tubulin isotype is essential for the specification of the central pair of microtubules in the axoneme (Nielsen *et al.*, 2001). It has been suggested that cooperativity in the function of internal β2-tubulin sequences is needed for the assembly of the exceptionally long *Drosophila* axoneme (Nielsen and Raff, 2002).

Dyneins are the major motor proteins responsible for sperm motility. It has first been shown by Goldstein *et al.* (1982) that the loss of the Y chromosome fertility factors Kl-3 and Kl-5 causes the loss of the outer arms on the microtubule doublets in the axoneme and the lack of three large proteins, presumably dynein heavy chains. Sequence analysis of one of the Y chromosome fertility factor genes, *kl-5* in *D. melanogaster* (Gepner and Hays, 1993) and *threads* in *D. hydei* (Kurek *et al.*, 1998), indeed showed that a dynein heavy chain β-polypeptide is encoded by a giant transcription unit of a Y chromosome fertility factor in both species. Furthermore, it was recently shown that the fertility factor genes *kl-2* and *kl-3* of the *D. melanogaster* Y chromosome encode a β- and a γ-dynein heavy chain, respectively (Carvalho *et al.*, 2000).

In addition to motility, the sperm tail needs flexible stability to allow movement but prevent breakage of the tail. In mammalian systems, this seems to be achieved by nine large structures around the axoneme, the outer dense fibers (ODFs) (Baccetti *et al.*, 1973; Baltz *et al.*, 1990; Haidl *et al.*, 1991). They contain specific ODF proteins (Burfeind and Hoyer-Fender, 1991; Morales *et al.*, 1994), which were identified using the *Drosophila Mst87F* gene as probe, a Male-specific transcript (Mst) at polytene band 87F. Thus, it is proposed that *Mst87F* and related proteins (see below) are the major components of the satellites on the outside of the outer microtubule doublets in *Drosophila* (Kuhn *et al.*, 1988; Schäfer *et al.*, 1995), which represent the ODF homologous structures in the fly flagellum (Baccetti *et al.*, 1973).

1.4.2.4.2. Mitochondrial derivatives Sections through the sperm tail reveal the axoneme and, along the axoneme, the two mitochondrial derivatives, the major and minor mitochondrial derivative, which are needed to supply the axoneme with ATP for motility. These mitochondrial derivatives originate from the fused mitochondria. Immediately after meiosis, they form an electron dense structure next to the nucleus (see **Figure 1d**). Because of its nucleus-like shape the structure at this stage was named Nebenkern (**Figure 1d**). The two mitochondrial derivatives are wrapped into each other during the onion stage of the Nebenkern (for morphological details see review by Lindsley and Tokuyasu, 1980). During spermatid development this sphere, with a diameter of 7 μm, changes to a cylinder, 1.8 mm in length. First, the mitochondrial derivatives

unwrap into one major and one minor derivative, and then these huge mitochondria elongate along the axoneme. In elongated spermatids, a basic protein termed Don Juan (DJ) is found in the mitochondrial derivatives. This protein may be involved in the final steps of mitochondrial differentiation (Santel *et al.*, 1998), in addition to its proposed function in the nucleus (see Section 1.4.2.4.3). As there are no mutants available so far, the function of DJ in the mitochondrial derivatives remains to be clarified.

At a molecular level, the function of the gene *fuzzy onion* (*fzo*) gave a first insight into the mechanism of Nebenkern development. This gene encodes a GTPase that is expressed in a stage-specific fashion and is essential for fusion of the mitochondria (Hales and Fuller, 1997). This GTPase belongs to the well-conserved mitofusin GTPases, of which *Drosophila* contains two isoforms: the specific form for Nebenkern morphogenesis and a ubiquitously expressed isoform named *Drosophila* Mitofusion (Mfn) (Santel and Fuller, 2001; Hwa *et al.*, 2002 and literature cited therein). Its homolog in *Sacharomyces cerevisiae*, yFzo1, has been studied at the functional level and shown to be required for mitochondrial fusion after mating and also for the continuously ongoing mitochondrial fusion events that maintain the dynamic network of mitochondrial filaments in vegetatively growing yeast. Recently, the human homologs Mfn1 and Mfn2 have also been characterized (Santel *et al.*, 2003 and literature cited therein).

1.4.2.4.3. Chromatin condensation and nuclear shaping In mammals, it is well known that the somatic set of histone proteins is modified during meiotic prophase (reviews: Hecht, 1998; Braun, 2001). After meiosis, histones are replaced first by the transition proteins (TP1 and TP2), which are largely redundant in function. TP1 is a small basic protein of 54 amino acids, while TP2 has the size of a core histone (for a review on chromosomal proteins in spermatogenesis in different animal species, see Wouters-Tyrou *et al.*, 1998). Transition proteins are then replaced by the highly basic protamines to ensure the remodeling of chromatin to the typical highly condensed and transcriptionally silent state of the mature sperm (review: Sassone-Corsi, 2002). At the level of the light microscope, the *Drosophila* spermatid nucleus is round after meiosis and then becomes a needle-like structure with highly condensed chromatin by an as yet unknown mechanism (see **Figure 2** for a detailed description of postmeiotic nuclear stages in several *Drosophila* species).

Unfortunately, little is known about the molecular processes leading to chromatin reorganization and condensation in *Drosophila*. An autosomal gene, *Mst77F*, has been identified by its male-specific transcription (Russell and Kaiser, 1993), which encodes a presumptive histone 5 belonging to the histone H1 group (Thomas, 1984). This histone H5 is involved in global transcriptional inactivation in the nucleated erythrocytes of chickens. It has been proposed to be involved in sperm chromatin condensation, but no further investigations were carried out (Russell and Kaiser, 1993). A histone-3 variant (H3.3) has been shown to be highly expressed during meiotic prophase in the male germline, where it is associated with some of the lampbrush loops. After meiosis, H3.3 associates in a nonrandom fashion with the chromatin of early spermatid nuclei but mainly colocalizes with the chromatin close to the nuclear membrane. The somatic H3 isoform, however, remains associated with the majority of the chromatin. Although this distribution is stable during the elongation phase of the nuclei, histones are undetectable by immunohistology in the nuclei of mature sperm (Akhmanova *et al.*, 1997).

In spite of histochemical evidence (Das *et al.*, 1964), it still remains an open question as to whether histones are replaced by protamine-like basic proteins. In the fly genome, six testis-specific proteasome subunits have been described. Two of them have been localized to the sperm nuclei from the elongated spermatid stage onward. It has been proposed that these nuclear proteasomes degrade histones (Ma *et al.*, 2002). Recently, a 160 kDa basic protein, RADHA, encoded by the *radha* (*rha*) gene, with a C-terminal leucine zipper has been described as a testis-specific nuclear protein. RADHA has been localized to the nuclei of primary spermatocytes, round spermatids, and elongating sperm heads (Harhangi *et al.*, 1999). Unfortunately, mutants that could help to elucidate the function of RADHA do not exist. A role as a histone replacement protein seems unlikely, however, due to its large size of 160 kDa compared to mammalian protamines of 10 kDa or less.

Another candidate for a histone replacement protein is the lysine-rich protein DJ with a molecular mass of 29 kDa and a predicted isoelectric point of 9.84 (Santel *et al.*, 1997). Its size and structural homology to histone H1 suggest that DJ may be a chromatin organizing protein. DJ is exclusively expressed in the male germline and its mRNA is under translational repression until the elongated spermatid stage (see Section 1.4.4.3). It is first detectable in spermatid nuclei at the end of nuclear transformation and, shortly after, in the mitochondrial

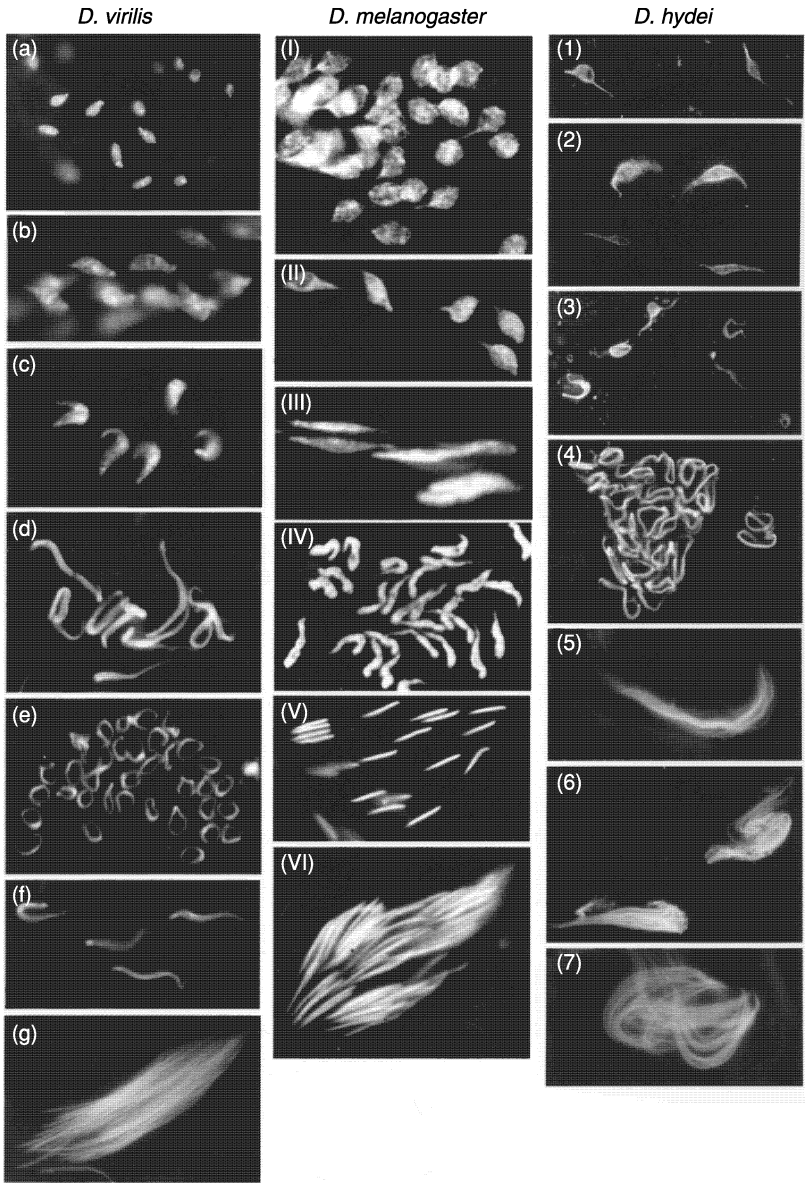


Figure 2 Chromatin condensation during spermiogenesis of *D. melanogaster, D. hydei, and D. virilis*. The panels show nuclear morphogenesis followed by Hoechst 33258 staining of DNA. (a, I, 1) The nucleus is round and the chromatin is distributed throughout the nucleus. (b, II, 2) The chromatin starts to change its distribution as the nucleus starts to elongate. (c, III, 3) The nucleus is ellipsoid and the chromatin concentrates on one side. (d, e, IV, 4, 5) The nucleus further elongates and the chromatin concentrates further. (f, V, 6) The chromatin starts to compact accompanied by further elongation of the nucleus and reduction of nuclear volume. Nuclei are already slim and the chromatin forms a needle-like structure. (g, VI, 7) The nuclei of one cyst are visible as thin and needle shaped structures shortly before individualization.

derivatives, immediately at the onset of individualization (Santel *et al*., 1998). Using immunohistological techniques, DJ is no longer detectable within the nucleus at the onset of individualization, but it cannot be excluded that the protein is not accessible to the antibodies in the highly condensed chromatin at this stage. It is very tempting to postulate that DJ transiently replaces histones, as it exhibits a nuclear localization at a stage when histones are no longer detectable. DJ may act together with DJ-like, a closely related basic protein, which is expressed at the elongated spermatid stage (L. Hempel, C. Rathke and R. Renkawitz-Pohi, in preparation). The establishment of a clear functional role for DJ and DJ-like with respect to chromatin remodeling during spermiogenesis requires the analysis of relevant mutants.

Recent studies give evidence for two true protamines in *Drosophila*. The analysis of the *Drosophila* genome sequence (Adams *et al*., 2000; Celniker *et al*., 2002) led to the identification of two genes with homology to protamines, for which testis-specific transcripts have been described (Ashburner *et al*., 1999; Andrews *et al*., 2000). When these protamines are fused to Green fluorescent protein (GFP) and analyzed in transgenic flies, they represent the major chromosomal proteins in *Drosophila* sperm (**Figure 3**; S. Jayaramaiah Raja and R. Renkawitz-Pohi, submitted). Thus, *Drosophila* sperm contain two protamines as do mammalian sperm. After fertilization and before zygote formation, the protamines have to be removed from the paternal pronucleus, and a nucleosomal configuration of the chromatin has to be established again (see **Chapter 1.6**). In the maternal effect mutant *sésame* (*ssm*), histones have no access to the paternal pronucleus suggesting that sésame is an essential component of the chromatin reorganization machinery in the male pronucleus (Loppin *et al*., 2001).

1.4.2.4.4. Individualization After the axoneme is assembled, the minor and major mitochondrial derivatives are appropriately positioned, the chromatin is tightly packed, the acrosome is established, and all other processes of sperm morphogenesis are completed, but the spermatids are still present in one bundle. As a final step of spermiogenesis, the surplus of cytoplasm needs to be removed and the 64 spermatids have to be separated, so that individual membranes encase each of the 64 mature spermatozoa. This is achieved by the so-called individualization complex (IC), which is assembled at the spermatid heads. Its assembly begins with the formation of actin cones around the nuclei, to which additional proteins are recruited (see below). The IC migrates as a cystic bulge along the sperm tail towards the tip of the tail. It removes excess cytoplasmic material by an apoptosis-based process (see below), and remodels the membranes as it moves. The complexity of the process is reflected by the large number of mutants with defects in individualization, including mutations in a Clathrin heavy

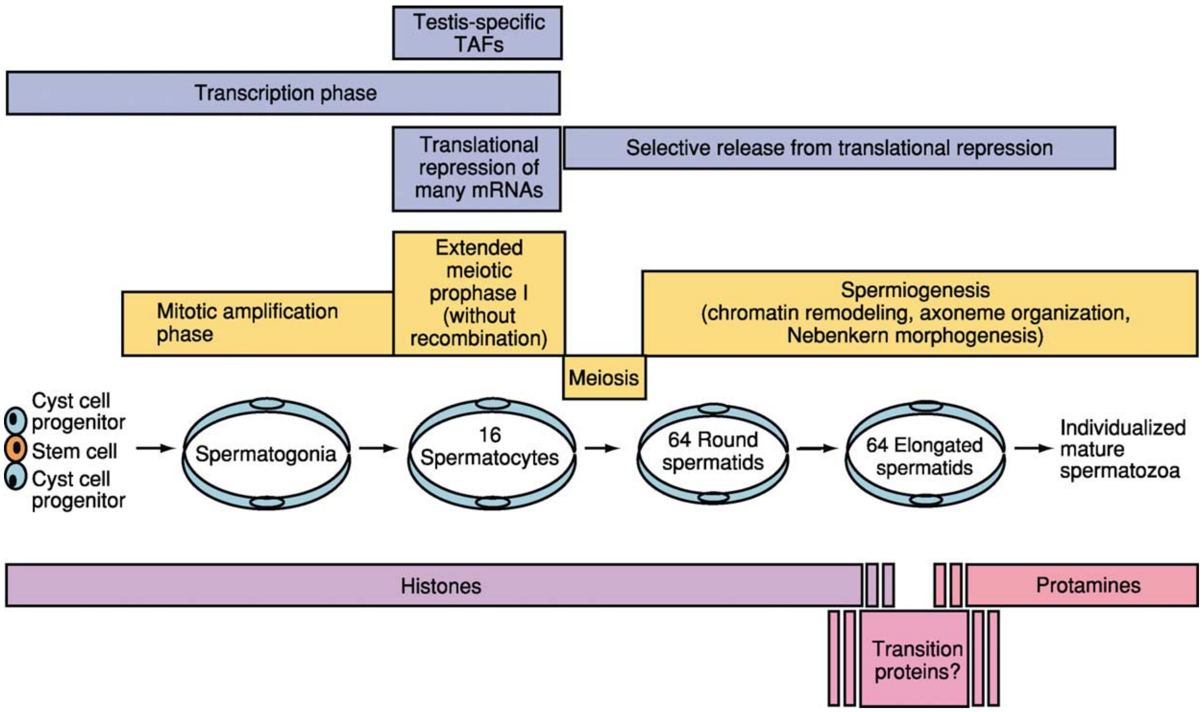


Figure 3 Major developmental and regulatory features during *D. melanogaster* spermatogenesis. The highly regulated developmental program leads from the asymmetric division of a stem cell to the spermatogonium, which is committed to sperm differentiation. Individual spermatogonia amplify enclosed by two cyst cells and enter the meiotic prophase synchronously. Primary spermatocytes are transcriptionally active with the help of stage and tissue-specific transcription initiation complexes. From this stage onward, many transcripts become translationally repressed and remain repressed until the appropriate time during sperm morphogenesis occurs. Shortly after meiosis, spermatids are characterized by a light round nucleus and an adjacent dark Nebenkern (see **Figure 1d**). The 64 spermatids mature in close association with the head and the tail cyst cell until individualization. Before meiosis the chromatin is in a nucleosomal conformation, but after meiosis histones disappear and the chromatin becomes tightly compacted, presumably through the action of basic proteins such as transition proteins and protamines, as in mammals.

chain (Chc) and a calmodulin binding protein encoded by *purity of essence* (*poe*) (Fabrizio *et al*., 1998). These have been grouped into four classes according to their phenotypic characteristics.

The moving IC structure is actin/myosin VI based. In this complex structure, myosin VI colocalizes with the actin polymerization regulatory protein cortactin and the actin-related protein 2/3 (Arp2/3) complexes as well as the actin capping protein at the front (Hicks *et al*., 1999; Rogat and Miller, 2002). The movement of the IC can be followed in *in vitro* cultures (Nogushi and Miller, 2003). Recently, it was shown that an apoptosis-like process is involved in individualization (Arama *et al*., 2003). Caspase activity is required for the translocation of individualization complexes and cytoplasm removal from the elongated spermatids. This activity is dependent on cytochrome-c-d activity, encoded by one of the two *cytochrome c* genes of *Drosophila*. Apoptotic processes must be restricted to the cytoplasmic compartment, however. This might be effected by the local action of dBruce, a giant E2 ubiquitin-conjugating enzyme, which could bind directly to caspases and degrade them (Arama *et al*., 2003).

As has been found for meiosis, control points seem to exist during sperm morphogenesis leading to similar phenotypes for a number of different mutations, which resulted in the classification of the mutants (see above and Fabrizio *et al*., 1998). For example, mutations in *ance* (angiotensin converting enzyme gene homolog) cause misalignment of nuclei, an abnormal structure of the mitochondrial derivatives, and a drastic reduction of individualization complexes. Similar phenotypes are produced by other individualization mutants (Hurst *et al*., 2003), which have not been analyzed at the molecular level as yet.

1.4.3. Cell–Cell Interactions during Spermatogenesis

The first analysis of possible interactions between the germline and somatic cells was performed by Gönczy and DiNardo (1996), who studied the behavior of the somatic cells, in agametic testes and testes containing reduced number of germ cells, using specific cell markers. Their experiments showed that the cells of the hub differentiate independently of the germ cells, whereas the cyst cells react to the absence of germ cells. Thus, although the number of cyst progenitor cells and daughter cells is often reduced in agametic testes, these cells can differentiate in the absence of germ cells. It was further demonstrated that germ cells restrict the proliferation of differentiated cyst cells, as in the absence of germline cells the number of cyst cells in old testes is significantly higher than in younger ones (Gönczy and DiNardo, 1996). The cyst cells of agametic testes incorporate BrdU, indicative of their mitotic activity, and some of them adopt a hub cell fate later on. In mutants for the actin binding protein coding genes *chickadee* (*chic*) and *diaphanous* (*dia*), the number of germ cells severely decreases during adult life. Also in these mutants, the cyst cells undergo similar alterations in the testes of adult flies after all germ cells have been eliminated. This does not happen in the larval testis anlagen, where the germ cells are still present. This indicates that persistent signaling as opposed to a signaling pulse from the germ cells to the cyst cells is necessary during the early steps of spermatogenesis.

1.4.3.1. Stem Cells and the JAK-STAT Pathway

At the tip of each testis tube, a group of nondividing somatic cells, the hub, is surrounded by the germline stem cells (GSCs, **Figure 1**). These GSCs are accompanied by somatic stem cells (SSCs). Both stem cell types are defined by their undifferentiated state and their unlimited proliferation potential. The hub cells form the so-called stem cell niche and, like the two stem cell types, they also express *piwi*, a gene that encodes a nucleoplasmic protein that is essential for the determination of the number and division rate of the germline stem cells (Lin and Spradling, 1997; Cox *et al*., 2000). The *piwi* gene is conserved even between the plant and animal kingdom (Cox *et al*., 1998), but the pathway through which it acts has not been determined as yet.

Oriented divisions of the stem cell lines generate daughter cells that remain as stem cells and daughter cells that differentiate into gonialblasts (germ cells that are committed to undergo differentiation) or cyst cells. Spindle positioning during germline stem cell division has been analyzed in detail (Yamashita *et al*., 2003). Its orientation is always perpendicular to the hub–GSC interface, which ensures that one daughter cell remains in contact with the hub, whereas the other daughter cell is displaced from the hub and, therefore, can receive signals that promote differentiation. Spindle positioning is directed by cell adhesion via *Drosophila* epithelial cadherin (E-cadherin) that is restricted to the interface between hub cells and GSCs. E-cadherin then provides a platform of binding sites for the β-catenin Armadillo (Arm), and one of the two *Drosophila* homologs of the mammalian adenomatous polyposis coli (APC2). APC2 can, in turn, orient the spindle through the microtubules and an intrinsic protein complex containing APC1 and the centrosome component Cnn. Thus, spindle orientation

plays a critical role in the mechanism that maintains the correct balance between stem cell self-renewal and initiation of differentiation throughout adult life (Yamashita *et al.*, 2003). The gonialblast that dislocates from the hub after the stem cell division is enclosed by two cyst cells that are derived from the SSCs (**Figure 3**). These cyst cells are essential for the differentiation of the blast cell and its progeny throughout spermatogenesis (see Section 1.4.3.2).

The stem cell lineage requires the function of the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway for its maintenance (Kiger *et al.*, 2001; Tulina and Matunis, 2001), a pathway that is highly conserved and involved in a variety of biological processes (review: Hou *et al.*, 2002). The JAK ligand Unpaired (Upd) is expressed in the somatic hub cells and activates the JAK-STAT pathway in the adjacent stem cell daughter by binding to the Upd receptor Domeless (Dome), which in turn activates the Janus kinase Hopscotch (Hop) and the transcription factor Stat92E (**Figure 4**). This activation causes the cell to retain its stem cell identity. Mutations in the *stat92E* gene lead to loss of stem cells after 5 days, whereas overexpression of *upd* results in more stem cells and prevention of differentiation. Interestingly, activation of *upd* also results in the generation of excess somatic stem cells, indicating that the maintenance of both stem cell populations is dependent on hub signaling (reviews: Castelli-Gair Hombria and Brown, 2002; Lin, 2002). Additional gene activities affecting stem cells are being identified but are yet to be recognized as part of a signaling pathway. *Nucleolar protein at 60B* (*Nop60B*), for example, encodes a pseudouridine synthase and is required intrinsically for germline stem cell survival. Specific alleles of the gene cause severe loss of germ cells, including stem cells and testicular atrophy (Kauffman *et al.*, 2003).

1.4.3.2. The Interaction between Germ Cells and Cyst Cells

After the stem cell divisions, the two cyst cells surround the presumptive gonialblast, thus forming a cyst (**Figure 3**). This process requires a complex interaction by different signaling cascades. The first step is the encapsulation of the gonialblast by the somatic cyst cells, which is dependent on epidermal growth factor receptor (EGFR) signaling (**Figure 4**). The EGFR pathway components Stem cell tumor (Stet) and Star (S), required for ligand activation in the cyst cells, are expressed in the gonialblast, indicating that the germ cell is the source of the signal (Schulz *et al.*, 2002). The EGF receptor and the downstream acting proteins monomeric GTPase Ras, the serine threonine kinase Raf, and the mitogen activated protein kinase Mek are expressed in the cyst cells, where they help to specify the cyst cell fate. Later on, EGFR activity in the cyst cells is necessary to promote the differentiation of the germline cell(s) and to control the number of mitotic divisions (**Figure 4**). Without the activity of the EGFR pathway, the stem cells divide symmetrically and both daughters retain stem cell identity (Kiger *et al.*, 2000; Tran *et al.*, 2000).

In a screen for mutants, a second signaling pathway was identified in the cyst cells, which prevents germ cells from overproliferation. The *punt* (*put*) and *schnurri* (*shn*) genes were shown to delimit this transient amplification of the germ cells (Matunis *et al.*, 1997). Both genes act within the somatic cyst cells, regulating a signal from the cyst cells to the germ cells (**Figure 4**). They are known components of the Decapentaplegic (Dpp)/transforming growth factor-β (TGF-β)-signaling pathway, where punt is one possible receptor for the ligand Dpp, and schnurri is the transcription factor acting downstream of the pathway. In testes, Dpp is apparently not involved, but there are two other TGF- β homologs, screw (scw) and 60A, one of which might be the ligand for punt in this system (Matunis *et al.*, 1997). This, however, needs to be tested experimentally.

The genes *bam* and the helicase *bgcn* code for germ cell intrinsic components of this pathway, and it is suggested that Bam and Bgcn are the targets of the signal from the cyst cells. Mutations in *bam* and *bgcn* lead to an accumulation of undifferentiated germ cells in both sexes, male and female (Gateff, 1982; McKearin and Spradling, 1990). Many details are known about their activity in the female system during oogenesis (see **Chapter 1.2**). However, in spermatogenesis, Bam and Bgcn seem to have different functions: in mutant females, the undifferentiated cells behave like stem cells, whereas in mutant males they exhibit characteristics of spermatogonial cells, too. In addition, overexpression of *bam* in females causes loss of stem cells (review: Deng and Lin, 2001; see **Chapter 1.2**), whereas in males there is no effect on the stem cells. Here, Bam and Bgcn participate in the assignment of spermatogonial cell fate, but the main signals are extrinsic (see above).

Recently, it was found that an additional signal between cyst cells and germ cells is transmitted via the TGF-β signaling pathway. The bone morphogenic protein (BMP5/8) ortholog glass bottom boat (Gbb), which represents a ligand for the TGF-β signaling pathway is expressed in the cyst cells. It provides a short-range signal to the GSC or the spermatogonia that are enclosed by these cyst cells, which suppresses Bam activity and, thus, maintains the spermatogonia

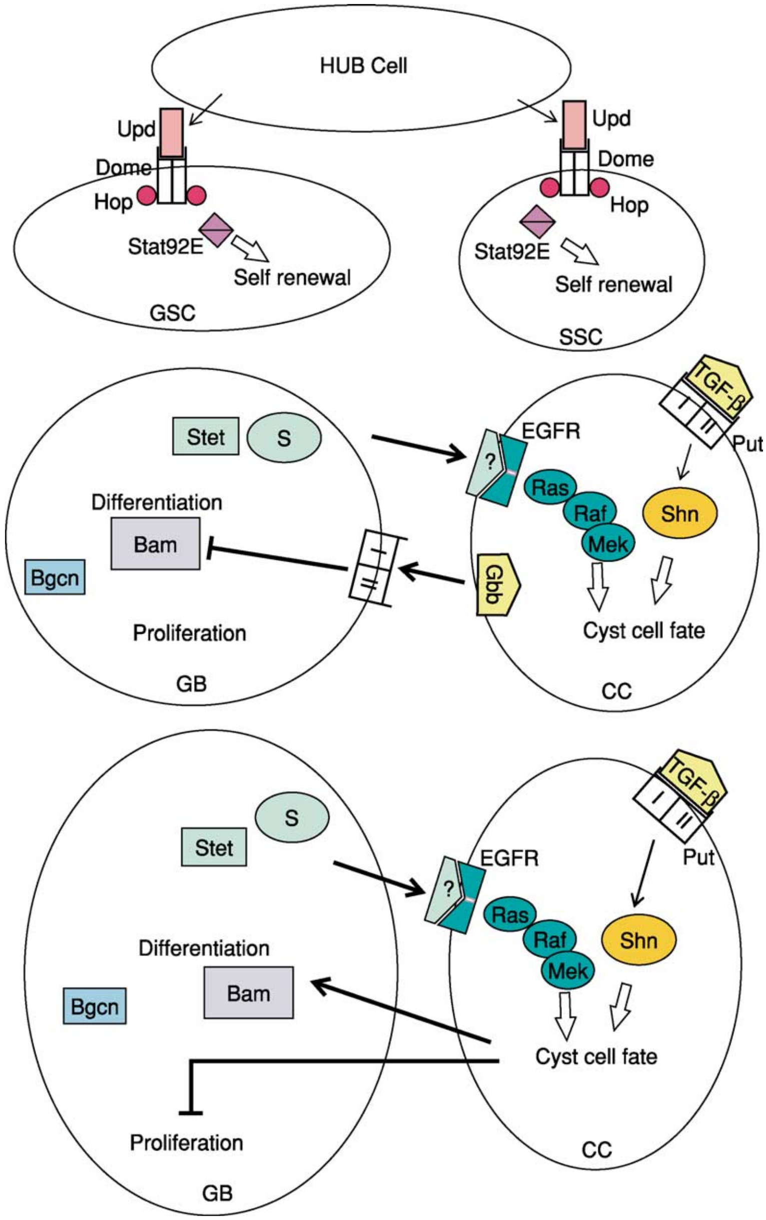


Figure 4 Signaling pathways and interactions between somatic and germ cells in the stem cell niche. The model summarizes the known interactions between somatic and germ cells in the stem cell niche. Only proteins that are genetically or experimentally shown to be expressed or involved in the testis are shown (see text for details). Bam, bag of marbles; Bgcn, benign gonial cell neoplasm; CC, cyst cell; Dome, Domeless; EGFR, epidermal growth factor receptor; GB, gonial blast; Gbb, Glass bottom boat; GSC, germline stem cell; Hop, Hopscotch; Stet, stem cell tumor; Mek, Mitogen activated protein kinase; Shn, Schnurri; SSC, somatic stem cell; Stat, signal transducer and activator of transcription protein; TGF-β, transforming growth factor β; Upd, Unpaired; I and II, type I and type II receptor of TGF-β.

in a proliferative state. With ongoing divisions, TGF-β signaling is proposed to decrease, resulting in an increased Bam activity, which leads to the block of proliferation and induction of differentiation of the enclosed germ cells (Shivdasani and Ingham, 2003).

In addition to signaling pathways, gap junctions may provide another important means of communication between germ and cyst cells. The germline specific innexin 4 zero population growth (Zpg) has been described, which is necessary for the survival of differentiating early germ cells in both sexes (Tazuke *et al*., 2002) and is found on the surface of spermatogonia primarily contacting the surrounding cyst cells.

In summary, at least two different signaling pathways are necessary to specify the somatic cyst

cell and to ensure the so far unknown signal for germ cell differentiation and proliferation control. It should be mentioned that after meiosis the cyst cells adopt two distinct functional fates and become oriented in a specific manner. The head cyst cell surrounds the region of the sperm heads and later becomes engulfed in the terminal epithelium, which seems to initiate the coiling and individualization process. The tail cyst cell encloses the entire tail region of the sperm bundle (Tokuyasu *et al*., 1972). A future research goal will be the identification of the signal cascade(s) needed to assign these different cell fates to the two cyst cells as well as the identification of additional germ cell intrinsic factors.

1.4.4. Regulation of Gene Activity

The enormous reorganization of cell shape during sperm morphogenesis requires a strictly regulated supply of proteins both with respect to time and space (see **Figure 3** for a summary of the regulatory processes). As the vast majority of transcription ceases after meiosis (Oliveri and Oliveri, 1965), transcripts required for spermiogenesis have to be synthesized during the extended primary spermatocyte phase. This requires stability of the mRNAs over several days. Furthermore, many mRNAs are translationally repressed until the encoded proteins are required for a distinct step of sperm morphogenesis (see below for molecular details). At the transcriptional level, mRNAs are differentially regulated by distinct transcription initiation complexes depending on their time of translation (see below).

1.4.4.1. Very Short Regulatory Regions for Transcriptional Control

Transcriptional regulation can be studied *in vivo* through the employment of reporter gene assays (**Figure 1e** and **f**) and the P-element transformation system. This approach has revealed that, in general, the control regions of genes which are expressed specifically in the testis, are extremely short. Genes expressed in the soma and the germline often contain a short testis-specific promoter, as exemplified by the male-specific promoter of the *dihydroorotate dehydrogenase* (*dhod*) gene (Yang *et al*., 1995). Genes, that are expressed in the male and female germline often contain sex-specific promoters, as exemplified by *gonadal* (*gdl*) (Schulz *et al*., 1990). For simplicity, we first discuss here the transcription properties of the *β tubulin* genes, *βTub56D* (*β1 tubulin* gene) and *βTub85D* (*β2 tubulin* gene). These are expressed in germ cells, while the somatic cyst cells express β1 tubulin and β3 tubulin (Kaltschmidt *et al*., 1991). The β1-tubulin isotype is also expressed in stem cells and spermatogonia, while the male germline-specific β2-tubulin isotype is the only β2-tubulin isotype expressed from the meiotic prophase onwards (Hoyle and Raff, 1990; Kaltschmidt *et al*., 1991).

In the early, mitotically active stem cell and spermatogonial stages, *β1 tubulin* gene transcription is dependent on cap site upstream gene sequences located between −45 and −191, while expression in the somatic cyst cells is guided by elements in the large intron of the gene (Buttgereit and Renkawitz-Pohl, 1993). In spermatocytes, the *β1 tubulin* gene is silenced and the *β2 tubulin* gene (*βTub85D*) is activated instead (see **Figure 1e** and **f** for reporter gene assay). Loss-of-function mutants of the *β2 tubulin* gene show that this β2-tubulin isotype is essential for the formation of the meiotic spindle and the axoneme (Kemphues *et al*., 1982). The β2-tubulin mRNA is translated immediately after transcription and is very stable.

Michiels *et al*. (1989) showed that very short upstream regions (53 bp) and the 5′untranslated region of the β2-tubulin gene are sufficient to drive tissue specific and temporally regulated expression, a feature characteristic of genes that are transcribed during the spermatocyte phase. A 14 bp regulatory element β2UE1 (region −51 to −38), which is conserved in function between the distantly related species *D. melanogaster* and *D. hydei*, is indispensable for tissue specific and temporal expression, while a second element, β2UE2 (region −32 to −26), close to the transcription initiation site is responsible for high level of expression. Alternatively, however, the β2UE1-element was found to be unable to drive spermatocyte-specific expression of a reporter gene efficiently upon linkage to the hsp70 promoter or the β3-tubulin promoter, suggesting that different transcription initiation mechanisms are used in spermatocytes (Michiels *et al*., 1991).

Another 18 bp element in the 5′ UTR of the β2-tubulin mRNA, β2DE1 (+48 to +65), forms a predicted stem and loop structure and is required for mRNA stability (Michiels *et al*., 1993). It is very likely that this β2DE1 element has dual function as it regulates promoter strength in addition to mRNA stability in combination with an initiator (Inr) element. The Inr, a 6–8 bp sequence encompassing the transcription initiation site, is thought to function either as a TATA box analog in TATA-less core promoters or as an enhancer of TATA box function in TATA box containing promoters (Santel *et al*., 2000 and literature cited therein).

The *Drosophila Mst(3)CGP* gene family members encode the putative satellite protein components

mentioned above (Section 1.4.2.4.1). These proteins contain large clusters of cysteine–glycine–proline repeats (Kuhn *et al*., 1991). Their genes are also characterized by short upstream transcriptional control regions (Kuhn *et al*., 1988; Gigliotti *et al*., 1997). In this case, the 5′ UTR contains a 12-nucleotide long translational control element (TCE) with regulatory potential both at the RNA and the DNA level (Kempe *et al*., 1993). It mediates negative translational control (see Section 1.4.4.3) and is also capable of mediating correct transcriptional activation *in vivo* of a silent β2-tubulin gene fragment, which lacks the β2UE1 promoter element. Protein binding occurs at the TCE both at the RNA and the DNA level (Kempe *et al*., 1993). Finally, as is the case with the *Mst(3)CGP* gene family, expression of *don juan* is controlled by short upstream regions (23 bp together with the 5′ UTR are sufficient) in combination with downstream sequences, located between +54 and +115 (Blümer *et al*., 2002). This part of the UTR also harbors the translational repression element (TRE) of the *don juan* mRNA (see Section 1.4.4.3).

In summary, all testis-specific promoters achieve high tissue-specific expression by a combination of short upstream sequences mediating tissue specificity and downstream elements necessary for achieving high transcript levels and mRNA stability or translational repression. Messenger RNAs that are translated before and after meiosis are differentially regulated by distinct transcriptional initiation complexes.

1.4.4.2. Distinction between mRNAs that will be Translated Directly and those that will be Translationally Repressed

White-Cooper *et al*. (1998) analyzed the presence of mRNAs that are subject to premeiotic translational repression in a collection of meiotic arrest mutants (block of the transition from G2 to meiosis I) of the genes *aly*, *can*, *mia*, and *sa*. In contrast to the wild-type situation, the translationally controlled mRNAs of the *don juan*, *Mst87F*, *Mst84Da-d*, *Mst98Ca/b*, and *janus B* genes are not transcribed in these mutants. This suggests that genes encoding translationally repressed mRNAs are under differential transcriptional control that is dependent on Aly, Can, Mia, and Sa (review: Fuller, 1998). This hypothesis is strongly supported by the fact that *can* encodes a spermatocyte-specific $TAF_{II}80$ (Hiller *et al*., 2001). TAFs are components of the general transcription factor complex TFIID, which interacts with RNA polymerase II and additional transcription factors. This interaction is essential to initiate transcription. Thus, in *can* mutants, the target genes mentioned above cannot be transcribed. This regulatory mechanism allows the spermatocyte to distinguish at the transcriptional level, through the use of distinct transcription initiation complexes, between the mRNAs that will be immediately transferred to the ribosomes for translation and those that will be translationally repressed for several days until the terminal differentiation program of spermiogenesis is initiated.

Besides the regulation exerted by Can at the individual gene level for transcription initiation, it has been suggested by White-Cooper *et al*. (2000) that modulation of chromatin conformation may also play a major role in selection during spermiogenesis. They proposed that *aly* may play a role at the chromatin level by repressing certain genes and allowing the activation of others, which are essential for meiosis and spermiogenesis. This notion is supported by the chromatin localization of Aly during the spermatocyte stage. As there is no evidence for direct binding of Aly to DNA, it is likely that this protein plays a role in chromatin structure modulation, perhaps in response to intrinsic or extrinsic signals, as suggested by its translocation from the cytoplasm to the nucleus during the early spermatocyte stage. Thus, it is envisaged that Aly reorganizes the chromatin so that the genes for the meiotic cell cycle and the sperm differentiation program become accessible to specific transcription complexes including male germline-specific TAFs like cannonball.

Recently, *comr* mutants were characterized, and were found to cause defects indistinguishable from those of the *aly* mutants. As is the case with the latter, a large number of genes like *Mst87F* (mRNAs under translational repression) are not transcribed in the *comr* mutants. The Comr protein is also nuclear, and it has been proposed that it regulates transcription at the chromatin level in concert with Aly. Both proteins are degraded when germ cells enter the meiotic divisions concomitant with the general turn-off of transcription (Jiang and White-Cooper, 2003).

In addition, a homeodomain containing transcription factor TGIF (TG-interacting factor), encoded by the nearly identical and tandemly arranged genes *achintya* (*achi*) and *vismay* (*vis*), is essential for male fertility and for the activation of the developmentally regulated program of transcription in spermatogenesis (Ayyar *et al*., 2003; Wang and Mann, 2003). Again, spermiogenesis-relevant genes like *don juan*, *fuzzy onion*, *gonadal*, *janus B*, and *Mst87F* are not transcribed in mutants lacking TGIF. Null mutants exhibit the same meiotic arrest phenotype as the *aly* mutation (Ayyar *et al*., 2003), and the protein coimmunoprecipitates with Aly and Comr

suggesting that complex formation is required for proper regulation (Wang and Mann, 2003).

1.4.4.3. Translational Repression of mRNAs and Differential Release from Repression

Since transcriptional activity ceases after the long meiotic prophase, the meiotic divisions and the entire process of sperm morphogenesis rely on presynthesized mRNAs. In particular, the complex morphological alterations necessary for the formation of fully differentiated sperm require a large number of proteins to be synthesized in a correct temporal and spatial manner in order for proper development of sperm to occur. Therefore, a large number of mRNAs must be stored and translationally silenced after synthesis during the spermatocyte stage, and selectively translationally activated at specific time points during spermiogenesis. This implies that translational repression should be a very general phenomenon during spermatogenesis, and allows the assumption that common control elements (*cis* and *trans* acting) may be used for many mRNAs.

The first case studied was that of the *Mst(3)CGP* gene family, which encompasses seven members encoding structural proteins of the sperm tail (see Sections 1.4.2.4.1 and 1.4.4.1). They are all transcribed during the spermatocyte stage but translational activity is only observed during late spermiogenesis, when spermatids have elongated fully (Kuhn *et al*., 1988; Schäfer *et al*., 1993; Gigliotti *et al*., 1997). Concomitant with translational activation, secondary polyadenylation of the mRNAs in the cytoplasm has been observed leading to a longer and heterogeneous transcript size (Schäfer *et al*., 1990, 1993; Kuhn *et al*., 1991). This phenomenon of secondary (or cytoplasmic) polyadenylation has been observed in a number of cases with translational control (Richter, 1999). Surprisingly, however, in mammalian spermatogenesis, where translational control phenomena have also been described, translationally controlled transcripts diminish in size due to shortening of the poly(A) tail when they are translationally activated (Braun, 1990).

Sequence comparisons revealed a conserved 12-nucleotide long element termed TCE in the 5′ UTR of all these mRNAs, which is invariably located at nucleotides +28 to +39 relative to the transcription cap site. Sequence modifications as well as mislocalization of this TCE lead to loss of translational repression. Thus, premature translation is observed in the spermatocyte stage, and cytoplasmic polyadenylation does not occur (Schäfer *et al*., 1990; Kempe *et al*., 1993). However, when the TCE is correctly placed in the *β2-tubulin* gene, it does not confer translational control to the mRNA encoded by this gene (Kempe *et al*., 1993). Instead, it is transcriptionally activated but translated concomittant with transcription. This supports the statement made earlier that translational control can only occur if the RNA has been transcriptionally activated from an appropriate promoter (see Section 1.4.4.2).

Even though all seven members of the *Mst(3)CGP* gene family contain the same TCE, negative translational control apparently is achieved in different ways. In a study that compared the expression of the divergently transcribed genes of the *Mst84D* cluster, *Mst84Da* and *Mst84Db*, it was found that *Mst84Da* is translationally regulated by a short segment containing the TCE as is the case with *Mst87F*. In the case of *Mst84Db*, however, a short segment within the open reading frame, which is totally unrelated to the TCE, mediates translational repression. Furthermore, translational activation is only achieved when the 3′ UTR sequences are present as well. This gene region confers mRNA stability, which is a prerequisite for translational activation late in spermiogenesis (Gigliotti *et al*., 1997). Thus, while the function of the TCE in this case remains to be elucidated, this gene offers the chance to uncouple the phenomenon of translational repression from that of translational activation. Alternatively, translational activation in this case could be a default mechanism after the RNA has persisted until this late developmental stage.

TCE-like sequences could be detected within the 5′ UTR of several other genes. The *janusB* gene (Yanicostas and Lepesant, 1990) contains a TCE close to the translation initiation codon that is necessary for translational control of the mRNA. Similarly, the gene *Mst59D* contains a bipartite TCE sequence, which also proved necessary for translational repression (Huang *et al*., in preparation).

Since it has been hypothesized that many, if not all, genes that are subject to translational control in spermatogenesis may contain a TCE (Schäfer *et al*., 1995), we searched for this element in the *don juan* gene mRNA, which has also been shown to be translationally controlled (see Section 1.4.2.4.3, Santel *et al*., 1997). Even though the 5′ UTR contains such a TCE sequence, at a different position compared to the *Mst(3)CGP* gene family, the relevant sequence does not mediate translational control in this case. Instead, a neighboring region that is capable of forming a secondary structure confers translational repression to the *don juan* mRNA.

Because it is functionally distinct from the TCE sequence, it has been termed TRE (translational repression element; Blümer *et al.*, 2002).

Individual translational activation of the different mRNAs is an essential prerequisite for correct sperm development. Analyses in various mutant strains indeed have shown that the time point of translational activation for the Mst98C mRNAs is preceded by that of the *don juan* mRNA (Blümer *et al.*, 2002). So far, nothing is known about this aspect of translational regulation in *Drosophila* spermatogenesis.

Because translational control is mandatory for proper sperm development, RNA binding proteins have been implicated in male fertility (Venables and Eperon, 1999). Several genes encoding relevant to RNA binding proteins have been described, some operating in both sexes (Lantz *et al.*, 1992; Webster *et al.*, 1997) and others being specific for the male germline (Karsch-Mizrachi and Haynes, 1993; Heatwole and Haynes, 1996; Haynes *et al.*, 1997) but, with the exception of *boule*, a homolog of the mammalian *Deleted in Azoospermia* (*DAZ*) gene (Eberhart *et al.*, 1996), their targets have yet to be determined.

The *boule* gene has been found to encode an RNA binding protein (Eberhart *et al.*, 1996). Mutations in this gene cause a meiotic entry defect. It was shown that this is due to lack of Twe synthesis, a meiotic Cdc25 protein, since expression of Twine from a chimeric *β2 tubulin* gene construct, in which the Twine coding sequence substituted for that of *β tubulin*, could rescue this mutation. Therefore, *boule* must encode a translational activator necessary for Twine translation (Maines and Wasserman, 1999).

In accordance with such a function, Boule has been shown to localize to a specific region of the nucleus and to translocate to the cytoplasm at the onset of meiosis (Cheng *et al.*, 1998). The authors speculate that Boule may have additional RNA targets that encode proteins for sperm morphogenesis, and that Boule may, therefore, coordinate the process of meiosis and spermatid differentiation (Maines and Wasserman, 1999). This raises the possibility that Boule may also be involved in the derepression phenomena discussed above. This can be further analyzed once the RNA sequences to which Boule binds are identified. The central importance of translational control during spermatogenesis is underlined by the fact that *boule* has been found to be highly conserved not only in sequence but also in function. Indeed, the newly identified human *boule* gene, an additional member of the *DAZ* gene family, can rescue a *boule* mutant fly to the same extent as a fly *boule* transgene (Xu *et al.*, 2003).

1.4.5. Insights from Other Insect Species

1.4.5.1. Control of Spermatogenesis

Besides *D. melanogaster*, spermatogenesis in insects has been best analyzed in *D. hydei*, particularly the Y chromosome fertility factors (review: Hackstein and Hochstenbach, 1995). Instead of the four mitotic divisions that occur in *D. melanogaster*, the spermatogonia in *D. hydei* go through only three mitotic divisions before entering meiotic prophase I, a typical example of the species-specific number of mitotic amplification steps. The sperm are unusually long, i.e., 10 mm in length (**Figure 2**). The axoneme as well as the meiotic spindle contain the testis specific β2-tubulin isotype and even the tissue-specific regulation of this gene is conserved between the two species (Michiels *et al.*, 1989).

The number of genes needed for correct sperm development is still unclear. In microarray analyses, 14% of the genome (2063 genes) were found to be testis-biased in their expression compared to only 8.5% (1226 genes) that have ovary-biased expression, and another 3% of the genes that are expressed in both germlines (M. Parisi and B. Oliver, submitted). In contrast to genes that have female-biased expression, genes that exhibit selective or preferential expression in the male germline are underrepresented on the X chromosome of *Drosophila*. Using comparative genomics, X chromosome linked genes were shown to be exceptionally poorly conserved in the mosquito *Anopheles gambiae*, suggesting that the X chromosome, in general, is not a favored location for genes expressed selectively in males (Parisi *et al.*, 2003). The reason could be either that in males, which only have one X chromosome, a mutation would immediately become effective, or that the X chromosome is inactivated early during spermatogenesis preventing effective transcription.

1.4.5.2. Sperm Maturation and Function

In several species of the *D. obscura* group, sperm of different lengths have been observed. In *D. subobscura*, for example, motile sperm show a clear length dimorphism but have similar morphology (Pasini *et al.*, 1996). It was shown that females preferentially store long sperm in their spermathecae and seminal receptacle (Bressac and Hauschteck-Jungen, 1996; see **Chapter 3.9**). Furthermore, only long sperm were present within *Drosophila* eggs upon fertilization of six different species, indicating that only the long class of sperm is fertilization-competent (Snook and Karr, 1998; see **Chapter 1.6**). In addition, it has been found that sperm size can vary significantly among closely related species of insects,

suggesting a rapid evolution of this sexual trait (Swallow and Wilkinson 2002; Hosken, 2003). It has been suggested that this occurs as a sperm-female coevolution, in which sperm size is a correlated response to a changing female reproductive tract size (Miller and Pitnick, 2002).

In the Lepidoptera, dimorphic sperm have also been analyzed (Friedländer, 1997). Only eusperm are capable of fertilization, while parasperm are not, as their DNA is fragmented and degraded. It has been proposed that parasperm have auxilliary functions for the eusperm, such as a nutritive role, facilitation of maturation, and help with the transport from the testes to the female reproductive tract and sperm storage organs. Nonfertile parasperm, which in the case of the tobacco hornworm, *Manduca sexta*, can account for as much as 96% of the ejaculate, may also act as a factor that delays the remating of females, thus reducing the potential for sperm competition (Cook and Wedell, 1999; see also Hodgson, 1998).

Sperm maturation has been found to be under circadian clock control, which is established several days after pupation (Giebultowicz and Joy, 1992; see **Chapters 3.12** and **4.11**). In moths, sperm bundles are released in a two-step circadian rhythm. Release of sperm to the *vas deferens* occurs during a few hours in the evening, and transfer to the seminal vesicles occurs for a few hours in the morning. The *period* (*per*) gene expression has been demonstrated to occur in the region of the terminal epithelium of the moth *vas deferens*, a region that is important for sperm maturation and release (Giebultowicz *et al*., 1997). In *Drosophila*, *per* expression was also detected in the terminal epithelium suggesting the existence of a circadian rhythm for sperm release in flies also (review: Giebultowicz, 2000).

1.4.6. Conclusions and Perspectives

Most of the progress in understanding insect spermatogenesis in recent years has come from the identification of regulatory networks within the germ cells, on the one hand, and the analysis of cell–cell interactions, on the other. For both of these aspects, however, many questions remain unanswered.

With respect to cell interactions, the role of the hub cells and additional signals required for the maintenance of the stem cell fate remain to be elucidated. In particular, the role played by other cell contact regions like gap junctions has yet to be studied in detail. In addition, as mentioned earlier, the very distinct interaction between the germ cells and the two different types of cyst cells, the head and tail cyst cells, has not been studied at all thus far. Among others, it remains to be shown how this specific interaction is regulated and what is the significance of the alignment. After meiosis, the mechanisms underlying the degradation of histones and the switch to a protamine based chromatin structure remain to be elucidated.

From more recent studies, the conclusion is emerging that control points exist at various stages of spermatogenesis. Like in many other developmental processes, the entry into meiosis and initiation of the differentiation program are coordinated and controlled by a number of genes. It is evident that all the players have not been revealed yet. The identification of some of the control genes, however, will undoubtedly facilitate the identification of others and should permit the unravelling of the entire network. The immense difference in detailed knowledge of the molecular processes that are involved in the control of oogenesis and spermatogenesis has diminished in recent years. As it turns out that more genes are preferentially or specifically expressed in the male gonad than in the female one indicating that the regulatory network and the detailed cell–cell communications during the development of the male germ cell line process may exceed the complexity found in the female one.

Recently, the scientific interest in understanding the regulation of insect spermatogenesis has increased greatly for two very different reasons. First, *Drosophila* turns out to be a suitable model for studying human infertility problems. Second, manipulation of male fertility is a potential way of preventing the growth of insect pest populations, which represent a threat for mankind either by spreading diseases or by causing immense destruction of agricultural crops. The large amount of molecular information gathered in recent years should speed up progress in both these areas.

Acknowledgments

We thank Dr Ulrich Schäfer for critical reading of the manuscript, and Heike Sauer and Anke Eberhardt for excellent secretarial assistance. We also thank Richard Bekemeier for providing Figure 2. This work was supported by the Deutsche Forschungsgemeinschaft (RE 628/9-6 and GRK 767 to R.R-P. and SFB 271 to M.A.S.) and the Zentrale Forschungsförderung Universität Kassel to M.A.S. and M.H.

References

Adams, M.D., Celniker, S.E., Holt, R.A., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Afshar, K., Gönczy, P., DiNardo, S., Wasserman, S.A., **2001**. *fumble* encodes a pantothenate kinase homolog required for proper mitosis and meiosis in *Drosophila melanogaster. Genetics 157*, 1267–1276.

Akhmanova, A., Miedema, K., Wang, Y., van Bruggen, M., Berden, J.H.M., *et al.*, **1997**. The localization of histone H3.3 in germ line chromatin of *Drosophila* males as established with a histone H3.3-specific antiserum. *Chromosoma 106*, 335–347.

Andrews, J., Bouffard, G.G., Cheadle, C., Lü, J., Becker, K.G., *et al.*, **2000**. Gene discovery using computational and microarray analysis of transcription in the *Drosophila melanogaster* testis. *Genome Res. 10*, 2030–2043.

Arama, E., Agapite, J., Steller, H., **2003**. Caspase activity and a specific cytochrome c are required for sperm differentiation in *Drosophila*. *Devel. Cell 4*, 687–697.

Ashburner, M., Misra, S., Roote, J., *et al.*, **1999**. An exploration of the sequence of a 2.9-Mb region of the genome of *Drosophila melanogaster*: the *Adh* region. *Genetics 153*, 179–219.

Ayyar, S., Jiang, J., Collu, A., White-Cooper, H., White, R.A.H., **2003**. *Drosophila* TGIF is essential for developmentally regulated transcription in spermatogenesis. *Development 130*, 2841–2852.

Baccetti, B., Pallini, V., Burrini, A.G., **1973**. Accessory fibers of the sperm tail I. Structure and chemical composition of the bull "coarse fibers". *J. Submicr. Cytol. 5*, 237–256.

Baltz, J.M., Williams, P.O., Cone, R.A., **1990**. Dense fibers protect mammalian sperm against damage. *Biol. Reprod. 43*, 485–491.

Blümer, N., Schreiter, K., Hempel, L., Santel, A., Hollmann, M., *et al.*, **2002**. A new translational repression element and unusual transcriptional control regulate expression of *don juan* during *Drosophila* spermatogenesis. *Mech. Devel. 110*, 97–112.

Braun, R.E., **1990**. Temporal translational regulation of the protamine 1 gene during mouse spermatogenesis. *Enzyme 44*, 120–128.

Braun, R.E., **2001**. Packaging paternal chromosomes with protamine. *Nat. Genet. 28*, 10–12.

Bressac, C., Hauschteck-Jungen, E., **1996**. *Drosophila subobscura* females preferentially select long sperm for storage and usage. *J. Insect Physiol. 42*, 323–328.

Brill, J.A., Hime, G.R., Scharer-Schuksz, M., Fuller, M.T., **2000**. A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis. *Development 127*, 3855–3864.

Bucciarelli, E., Giansanti, M.G., Bonaccorsi, S., Gatti, M., **2003**. Spindle assembly and cytokinesis in the absence of chromosomes during *Drosophila* male meiosis. *J. Cell Biol. 160*, 993–999.

Burfeind, P., Hoyer-Fender, S., **1991**. Sequence and developmental expression of a mRNA encoding a putative protein of rat sperm outer dense fibers. *Devel. Biol. 148*, 195–204.

Buttgereit, D., Renkawitz-Pohl, R., **1993**. Expression of β1 tubulin (βTub56D) in *Drosophila* testis stem cells is regulated by a short upstream sequence while intron elements guide expression in somatic cells. *Mol. Gen. Genet. 241*, 263–270.

Castelli-Gair Hombria, J., Brown, S., **2002**. The fertile field of *Drosophila* JAK/STAT signalling. *Curr. Biol. 12*, R569–R575.

Carvalho, A.B., Lazzaro, B.P., Clark, A.G., **2000**. Y chromosomal fertility factors *kl-2* and *kl-3* of *Drosophila melanogaster* encode dynein heavy chain polypeptides. *Proc. Natl Acad. Sci. USA 97*, 13239–13244.

Celniker, S.E., Wheeler, D.A., Kronmiller, B., *et al.*, **2002**. Finishing a whole-genome shotgun: release 3 of the *Drosophila melanogaster* euchromatic genome sequence. *Genome Biol. 3*, RESEARCH 0079.1–0079.14.

Cheng, M.H., Maines, J.Z., Wasserman, S.A., **1998**. Biphasic subcellular localization of the DAZL-related protein boule in *Drosophila* spermatogenesis. *Devel. Biol. 204*, 567–576.

Church, K., Lin, H.P., **1982**. Meiosis in *Drosophila melanogaster*. II. The prometaphase-I kinetochore microtubule bundle and kinetochore orientation in males. *J. Cell Biol. 93*, 365–373.

Cook, P.A., Wedell, N., **1999**. Non-fertile sperm delay female remating. *Nature 397*, 486.

Cox, D.N., Chao, A., Baker, J., Chang, L., Quiao, D., *et al.*, **1998**. A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. *Genes Devel. 12*, 3715–3727.

Cox, D.N., Chao, A., Lin, H., **2000**. *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development 127*, 503–514.

Das, C.C., Kaufmann, B.P., Gay, H., **1964**. Histone-protein transition in *Drosophila melanogaster.* I. Changes during spermatogenesis. *Exp. Cell Res. 35*, 507–514.

DeFalco, T.J., Verney, G., Jenkins, A.B., McCaffery, J.M., Russel, S., *et al.*, **2003**. Sex-specific apoptosis regulates sexual dimorphism in the *Drosophila* embryonic gonad. *Devel. Cell 5*, 205–216.

Deng, W., Lin, H., **2001**. Asymmetric germ cell division and oocyte determination during *Drosophila* oogenesis. *Int. Rev. Cytol. 203*, 93–138.

Eberhart, C.G., Maines, J.Z., Wasserman, S.A., **1996**. Meiotic cell cycle requirement for a fly homologue of human *Deleted in Azoospermia*. *Nature 381*, 783–785.

Eberhart, C.G., Wasserman, S.A., **1995**. The *pelota* locus encodes a protein required for meiotic cell division: an analysis of G_2/M arrest in *Drosophila* spermatogenesis. *Development 121*, 3477–3486.

Fabrizio, J.J., Hime, G., Lemmon, S.K., Bazinet, C., **1998**. Genetic dissection of sperm individualization in *Drosophila melanogaster. Development 125*, 1833–1843.

Farkas, R.M., Giansanti, M.G., Gatti, M., Fuller, M.T., **2003**. The *Drosophila Cog5* homologue is required for

cytokinesis, cell elongation, and assembly of specialized Golgi architecture during spermatogenesis. *Mol. Biol. Cell 14*, 190–200.

Friedländer, M., **1997**. Control of the eupyrene-apyren sperm dimorphism in Lepidoptera. *J. Insect Physiol. 43*, 1085–1092.

Fuller, M.T., **1993**. Spermatogenesis. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. I. Cold Spring Harbor Laboratory Press, New York, pp. 71–147.

Fuller, M.T., **1998**. Genetic control of cell proliferation and differentiation in *Drosophila* spermatogenesis. *Semin. Cell Devel. Biol. 9*, 433–444.

Gateff, E., **1982**. Gonial cell neoplasm of genetic origin affecting both sexes of *Drosophila melanogaster*. In: Weber, R., Burger, M.M. (Eds.), Embryonic Development, Part B: Cellular Aspects. Alan Liss, New York, pp. 621–632.

Gepner, J., Hays, T.S., **1993**. A fertility region on the Y chromosome of *Drosophila melanogaster* encodes a dynein microtubule motor. *Proc. Natl Acad. Sci. USA 90*, 11132–11136.

Giansanti, M.G., Bonaccorsi, S., Bucciarelli, E., Gatti, M., **2001**. *Drosophila* male meiosis as a model system for the study of cytokinesis in animal cells. *Cell Structure and Function 26*, 609–617.

Giebultowicz, J.M., **2000**. Molecular mechanism and cellular distribution of insect circadian clocks. *Annu. Rev. Entomol. 45*, 769–793.

Giebultowicz, J.M., Joy, J.E., **1992**. Ontogeny of the circadian system controlling release of sperm from the insect testis. *J. Biol. Rhythms 7*, 203–212.

Giebultowicz, J.M., Weyda, F., Erbe, E.F., Wergin, W.P., **1997**. Circadian rhythm of sperm release in the gypsy moth, *Lymantria dispar*: ultrastructural study of transepithelial penetration of sperm bundles. *J. Insect Physiol. 43*, 1133–1147.

Gigliotti, S., Balz, V., Malva, C., Schäfer, M.A., **1997**. Organisation of regulatory elements in two closely spaced *Drosophila* genes with common expression characteristics. *Mech. Devel. 68*, 101–113.

Gönczy, P., DiNardo, S., **1996**. The germ line regulates somatic cyst cell proliferation and fate during *Drosophila* spermatogenesis. *Development 122*, 2437–2447.

Gönczy, P., Matunis, E., DiNardo, S., **1997**. *bag-of-marbles* and *benign gonial cell neoplasm* act in the germ line to restrict proliferation during *Drosophila* spermiogenesis. *Development 124*, 4361–4371.

Goldstein, L.S., Hardy, R.W., Lindsley, D.L., **1982**. Structural genes on the Y chromosome of *Drosophila* melanogaster. *Proc. Natl Acad. Sci. USA 79*, 7405–7409.

Hackstein, J.H.P., Hochstenbach, R., **1995**. The elusive fertility genes of *Drosophila*: the ultimate haven for selfish genetic elements. *Trends Genet. 11*, 195–200.

Hackstein, J.H.P., Hochstenbach, R., Pearson, P.L., **2000**. Towards an understanding of the genetics of human male infertility: lessons from flies. *Trends Genet. 16*, *565–572*.

Haidl, G., Becker, A., Henkel, R., **1991**. Poor development of outer dense fibers as a major cause of tail abnormalities in the spermatozoa of asthenoteratozoospermic men. *Human Reprod. 6*, 1431–1438.

Hales, K.G., Fuller, M.T., **1997**. Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell 90*, 121–129.

Harhangi, H.R., Sun, X., Wang, Y.X., Akhmanova, A., Miedema, K., *et al.*, **1999**. RADHA – a new male germ line-specific chromosomal protein of *Drosophila*. *Chromosoma 108*, 235–242.

Hartenstein, V., **1993**. Atlas of *Drosophila* Development. Cold Spring Harbor Laboratory Press, New York.

Haynes, S.R., Cooper, M.T., Pype, S., Stolow, D.T., **1997**. Involvement of a tissue-specific RNA recognition motif protein in *Drosophila* spermatogenesis. *Mol. Cell. Biol. 17*, 2708–2715.

Heatwole, V.M., Haynes, S.R., **1996**. Association of RB97D, an RRM protein required for male fertility, with a Y chromosome lampbrush loop in *Drosophila* spermatocytes. *Chromosoma 105*, 285–292.

Hecht, N.B., **1998**. Molecular mechanisms of male germ cell differentiation. *BioEssays 20*, *559–561*.

Heller, A., Steinmann-Zwicky, M., **1998**. In *Drosophila*, female gonadal cells repress male-specific gene expression in XX germ cells. *Mech. Devel. 73*, 203–209.

Hicks, J.L., Deng, W.M., Rogat, A.D., Miller, K.G., Bownes, M., **1999**. Class VI unconventional myosin is required for spermatogenesis in *Drosophila*. *Mol. Biol. Cell 10*, 4341–4353.

Hiller, M.A., Lin, T.-Y., Wood, C., Fuller, M.T., **2001**. Developmental regulation of transcription by a tissue-specific TAF homolog. *Genes Devel. 15*, 1021–1030.

Hime, G.R., Brill, J.A., Fuller, M.T., **1996**. Assembly of ring canals in the male germ line from structural components of the contractile ring. *J. Cell Sci. 109*, 2779–2788.

Hodgson, A.N., **1998**. Paraspermatozoa. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction, vol. 3. Academic Press, London, pp. 656–668.

Hosken, D.J., **2003**. Sperm biology: size indeed matters. *Curr. Biol. 13*, R355–R356.

Hou, S.X., Zheng, Z., Chen, X., Perrimon, N., **2002**. The JAK/STAT pathway in model organisms: emerging roles in cell movement. *Devel. Cell 3*, 765–778.

Hoyle, H.D., Raff, E.C., **1990**. Two *Drosophila* beta tubulin isoforms are not functionally equivalent. *J. Cell Biol. 111*, 1009–1026.

Hurst, D., Rylett, C.M., Isaac, R.E., Shirras, A.D., **2003**. The *Drosophila* angiotensin-converting enzyme homologue Ance is required for spermiogenesis. *Devel. Biol. 254*, 238–247.

Hwa, J.J., Hiller, M.A., Fuller, M.T., Santel, A., **2002**. Differential expression of the *Drosophila* mitofusin genes *fuzzy onions* (*fzo*) and *dmfn*. *Mech. Devel. 116*, 213–216.

Janzer, B., Steinmann-Zwicky, M., **2001**. Cell-autonomous and somatic signals control sex-specific gene

expression in XY germ cells of *Drosophila*. *Mech. Devel. 100*, 3–13.

Jenkins, A.B., McCaffery, J.M., Van Doren, M., **2003**. *Drosophila* E-cadherin is essential for proper germ cell-soma interaction during gonad morphogenesis. *Development 130*, 4417–4426.

Jiang, J., White-Cooper, H., **2003**. Transcriptional activation in *Drosophila* spermatogenesis involves the mutually dependent function of *aly* and a novel meiotic arrest gene *cookie monster*. *Development 130*, 563–573.

Kaltschmidt, B., Glätzer, K.H., Michiels, F., Leiss, D., Renkawitz-Pohl, R., **1991**. During *Drosophila* spermatogenesis β1, β2 and β3 tubulin isotypes are cell-type specifically expressed but have the potential to coassemble into the axoneme of transgenic flies. *Eur. J. Cell Biol. 54*, 110–120.

Karsch-Mizrachi, I., Haynes, S.R., **1993**. The Rb97D gene encodes a potential RNA-binding protein required for spermatogenesis in *Drosophila*. *Nucl. Acids Res. 21*, 2229–2235.

Kauffman, T., Tran, J., DiNardo, S., **2003**. Mutations in Nop60B, the *Drosophila* homolog of human Dyskeratosis congenita 1, affect the maintenance of the germ-line stem cell lineage during spermatogenesis. *Devel. Biol. 253*, 189–199.

Kempe, E., Muhs, B., Schäfer, M., **1993**. Gene regulation in *Drosophila* spermatogenesis: analysis of protein binding at the translational control element TCE. *Devel. Genet. 14*, 449–459.

Kemphues, K.J., Kaufman, T.C., Raff, R.A., Raff, E.C., **1982**. The testis-specific beta-tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis. *Cell 31*, 655–670.

Kiger, A.A., Jones, D.L., Schulz, C., Rogers, M.B., Fuller, M.T., **2001**. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science 294*, 2542–2545.

Kiger, A.A., White-Cooper, H., Fuller, M.T., **2000**. Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature 407*, 750–754.

Kuhn, R., Kuhn, C., Börsch, D., Glätzer, K.H., Schäfer, U., *et al.*, **1991**. A cluster of four genes selectively expressed in the male germ line of *Drosophila melanogaster*. *Mech. Devel. 35*, 143–151.

Kuhn, R., Schäfer, U., Schäfer, M., **1988**. Cis-acting regions sufficient for spermatocyte-specific transcriptional and spermatid-specific translational control of the *Drosophila* melanogaster gene *mst(3)gl-9*. *EMBO J. 7*, 447–454.

Kurek, R., Reugels, A.M., Glätzer, K.H., Bünemann, H., **1998**. The Y chromosomal fertility factor Threads in *Drosophila hydei* harbors a functional gene encoding an axonemal dynein β heavy chain protein. *Genetics 149*, 1363–1376.

Lalli, E., Ohe, K., Latorre, E., Bianchi, M.E., Sassone-Corsi, P., **2003**. Sexy splicing: regulatory interplays governing sex determination from *Drosophila* to mammals. *J. Cell Sci. 116*, 441–445.

Lantz, V., Ambrosio, L., Schedl, P., **1992**. The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Development 115*, 75–78.

Li, J., Xia, F., Li, W.X., **2003**. Coactivation of STAT and Ras is required for germ cell proliferation and invasive migration in *Drosophila*. *Devel. Cell 5*, 787–798.

Li, K., Xu, E.Y., Cecil, J.K., Turner, F.R., Megraw, T.L., *et al.*, **1998**. *Drosophila* centrosomin protein is required for male meiosis and assembly of the flagellar axoneme. *J. Cell Biol. 141*, 455–467.

Lin, H., **2002**. The stem-cell niche theory: lessons from flies. *Nature Rev. Genet. 3*, 931–940.

Lin, H., Spradling, A., **1997**. A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development 124*, 2463–2476.

Lin, T.Y., Viswanathan, S., Wood, C., Wilson, P.G., Wolf, N., *et al.*, **1996**. Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in *Drosophila* males. *Development 122*, 1331–1341.

Lindsley, D., Tokuyasu, K.T., **1980**. Spermatogenesis. In: Ashburner, M., Wright, T.R.F. (Eds.), Genetics and Biology of Drosophila, vol. 2d. Academic Press, New York, pp. 225–294.

Loppin, B., Berger, F., Couble, P., **2001**. The *Drosophila* maternal gene sésame is required for sperm chromatin remodeling at fertilization. *Chromosoma 110*, 430–440.

Ma, J., Katz, E., Belote, J.M., **2002**. Expression of proteasome subunit isoforms during spermatogenesis in *Drosophila melanogaster*. *Insect Mol. Biol. 11*, 627–639.

Maines, J., Wasserman, S., **1998**. Regulation and execution of meiosis in *Drosophila* males. *Curr. Top. Devel. Biol. 37*, 301–332.

Maines, J.Z., Wasserman, S.A., **1999**. Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule. *Nature Cell Biol. 1*, 171–174.

Matunis, E., Tran, J., Gönczy, P., Caldwell, K., DiNardo, S., **1997**. punt and schnurri regulate a somatically derived signal that restricts proliferation of committed progenitors in the germline. *Development 124*, 4383–4391.

McKearin, D.M., Ohlstein, B., **1995**. A role for the *Drosophila bag-of-marbles* protein in the differentiation of cystoblasts from germline stem cells. *Development 121*, 2937–2947.

McKearin, D.M., Spradling, A., **1990**. *bag-of-marbles*: a *Drosophila* gene required to initiate both male and female gametogenesis. *Genes Devel. 4*, 2242–2251.

Michiels, F., Buttgereit, D., Renkawitz-Pohl, R., **1993**. An 18 bp element in the 5′untranslated region regulates the level of the *Drosophila β2 tubulin* mRNA during post-meiotic stages of spermatogenesis. *Eur. J. Cell Biol. 62*, 66–74.

Michiels, F., Gasch, A., Kaltschmidt, B., Renkawitz-Pohl, R., **1989**. A 14 bp promoter element directs the testis-specificity of the *β2 tubulin* gene. *EMBO J. 8*, 1559–1565.

Michiels, F., Wolk, A., Renkawitz-Pohl, R., **1991**. Further sequence requirements for male germ cell-specific expression under the control of the 14 bp promoter element (β 2UE1) of the *Drosophila β2 tubulin* gene. *Nucl. Acids Res. 19*, 4515–4521.

Miller, G.T., Pitnick, S., **2002**. Sperm-female coevolution in *Drosophila*. *Science 298*, 1230–1233.

Morales, C.R., Oko, R., Clermont, Y., **1994**. Molecular cloning and developmental expression of an mRNA encoding the 27 kDa outer dense fiber protein of rat spermatozoa. *Mol. Reprod. Devel. 37*, 229–240.

Nielsen, M.G., Raff, E.C., **2002**. The best of all worlds or the best possible world? Developmental constraint in the evolution of β tubulin and the sperm tail axoneme. *Evol. Devel. 4*, 303–315.

Nielsen, M.G., Turner, F.R., Hutchens, J.A., Raff, E.C., **2001**. Axoneme-specific β-tubulin specialization: a conserved C-terminal motif specifies the central pair. *Curr. Biol. 11*, 529–533.

Nogushi, T., Miller, K.G., **2003**. A role for actin dynamics in individualization during spermatogenesis in *Drosophila melanogaster. Development 130*, 1805–1816.

Ohlstein, B., Lavoie, C.A., Vef, O., Gateff, E., McKearin, D.M., **2000**. The *Drosophila* cystoblast differentiation factor, *benign gonial cell neoplasm*, is related to DExH-box proteins and interacts genetically with *bag-of-marbles*. *Genetics 155*, 1809–1819.

Oliveri, G., Oliveri, A., **1965**. Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster. Mutat. Res. 2*, 366–380.

Parisi, M., Nuttall, R., Naiman, D., Bouffard, G., Malley, J., *et al.*, **2003**. Paucity of genes on the *Drosophila* X chromosome showing male-biased expression. *Science 299*, 697–700.

Pasini, M.E., Redi, C.A., Caviglia, O., Perotti, M.E., **1996**. Ultrastructural and cytochemical analysis of sperm dimorphism in *Drosophila subobscura*. *Tissue Cell. 28*, 165–175.

Poirié, M., Niederer, E., Steinmann-Zwicky, M., **1995**. A sex-specific number of germ cells in embryonic gonads of *Drosophila*. *Development 121*, 1867–1873.

Rebollo, E., González, C., **2000**. Visualizing the spindle checkpoint in *Drosophila* spermatocytes. *EMBO Rep. 1*, 65–70.

Richter, J.D., **1999**. Cytoplasmic polyadenylation in development and beyond. *Microbiol. Mol. Biol. Rev. 63*, 446–456.

Rogat, A.D., Miller, K.G., **2002**. A role for myosin VI in actin dynamics at sites of membrane remodeling during *Drosophila* spermatogenesis. *J. Cell Sci. 115*, 4855–4865.

Russell, S.R.H., Kaiser, K., **1993**. *Drosophila melanogaster* male germ line-specific transcripts with autosomal and Y-linked genes. *Genetics 134*, 293–308.

Santel, A., Blümer, N., Kämpfer, M., Renkawitz-Pohl, R., **1998**. Flagellar mitochondrial association of the male-specific Don Juan protein in *Drosophila* spermatozoa. *J. Cell Sci. 111*, 3299–3309.

Santel, A., Frank, S., Gaume, B., Herrler, M., Youle, R.J., *et al.*, **2003**. Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. *J. Cell Sci. 116*, 2763–2774.

Santel, A., Fuller, M.T., **2001**. Control of mitochondrial morphology by a human mitofusin. *J. Cell Sci. 114*, 867–874.

Santel, A., Kaufmann, J., Hyland, R., Renkawitz-Pohl, R., **2000**. The initiator element of the *Drosophila* β2 tubulin gene core promoter contributes to gene expression *in vivo* but is not required for male germ line specific expression. *Nucl. Acids Res. 28*, 1439–1446.

Santel, A., Winhauer, T., Blümer, N., Renkawitz-Pohl, R., **1997**. The *Drosophila don juan* (*dj*) gene encodes a novel sperm specific protein component characterized by an unusual domain of a repetitive amino acid motif. *Mech. Devel. 64*, 19–30.

Sassone-Corsi, P., **2002**. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science 296*, 2176–2178.

Schäfer, M., Börsch, D., Hülster, A., Schäfer, U., **1993**. Expression of a gene duplication encoding conserved sperm tail proteins is translationally regulated in *Drosophila melanogaster. Mol. Cell. Biol. 13*, 1708–1718.

Schäfer, M., Kuhn, R., Bosse, F, Schäfer, U., **1990**. A conserved element in the leader mediates post-meiotic translation as well as cytoplasmic polyadenylation of a *Drosophila* spermatocyte mRNA. *EMBO J. 9*, 4519–4525.

Schäfer, M., Nayernia, K., Engel, W., Schäfer, U., **1995**. Translational control in spermatogenesis. *Devel. Biol. 172*, 344–352.

Schulz, C., Wood, C.G., Jones, D.L., Tazuke, S.I., Fuller, M.T., **2002**. Signaling from germ cells mediated by the *rhomboid* homolog *stet* organizes encapsulation by somatic support cells. *Development 129*, 4523–4534.

Schulz, R.A., Xie, X., Miksch, J.L., **1990**. *cis*-acting sequences required for the germ line expression of the *Drosophila gonadal* gene. *Devel. Biol. 140*, 455–458.

Shivdasani, A.A., Ingham, P.W., **2003**. Regulation of stem cell maintenance and transit amplifying cell proliferation by TGF-*β* signaling in *Drosophila* spermatogenesis. *Curr. Biol. 13*, 2065–2072.

Snook, R.R., Karr, T.L., **1998**. Only long sperm are fertilization-competent in six sperm-heteromorphic *Drosophila* species. *Curr. Biol. 8*, 291–294.

Swallow, J.G., Wilkinson, G.S., **2002**. The long and short of sperm polymorphism in insects. *Biol. Rev. Camb. Philos. Soc. 77*, 153–182.

Tates, A.D., **1971**. Cytodifferentiation during Spermatogenesis in *Drosophila melanogaster*: an Electron Microscope Study. Ph.D. thesis, Rijksuniversiteit Leiden.

Tazuke, S.I., Schulz, C., Gilboa, L., Fogarty, M., Mahowald, A.P., *et al.*, **2002**. A germline-specific gap junction protein required for survival of differentiating early germ cells. *Development 129*, 2529–2539.

Terada, Y., Uetake, Y., Kuriyama, R., **2003**. Interaction of Aurora-A and centrosomin at the microtubule-

nucleating site in *Drosophila* and mammalian cells. *J. Cell Biol. 162*, 757–763.

Thomas, J.O., **1984**. The higher order structure of chromatin and histone H1. *J. Cell Sci. Suppl. 1*, 1–20.

Tokuyasu, K.T., Peacock, W.J., Hardy, R.W., **1972**. Dynamics of spermiogenesis in *Drosophila melanogaster.* II. Coiling process. *Z. Zellforsch. Mikrosk. Anat. 127*, 492–525.

Tran, J., Brenner, T.J., DiNardo, S., **2000**. Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. *Nature 407*, 754–757.

Tulina, N., Matunis, E., **2001**. Control of stem cell self-renewal in *Drosophila* spermatogenesis by JAK-STAT signaling. *Science 294*, 2546–2549.

Van Doren, M., Mathews, W.R., Samuels, M., Moore, L.A., Broihier, H.T., *et al.*, **2003**. *fear of intimacy* encodes a novel transmembrane protein required for gonad morphogenesis in *Drosophila*. *Development 130*, 2355–2364.

Venables, J.P., Eperon, I.C., **1999**. The roles of RNA-binding proteins in spermatogenesis and male infertility. *Curr. Opin. Genet. Devel. 9*, 346–354.

Wang, Z., Mann, R.S., **2003**. Requirement for two nearly identical TGIF-related homeobox genes in *Drosophila* spermatogenesis. *Development 130*, 2853–2865.

Webster, P.J., Liang, L., Berg, C.A., Lasko, P., Macdonald, P.M., **1997**. Translational repressor bruno plays multiple roles in development and is widely conserved. *Genes Devel. 11*, 2510–2521.

White-Cooper, H., Leroy, D., MacQueen, A., Fuller, M.T., **2000**. Transcription of meiotic cell cycle and terminal differentiation genes depends on a conserved chromatin associated protein, whose nuclear localisation is regulated. *Development 127*, 5463–5473.

White-Cooper, H., Schäfer, M.A., Alphey, L.S., Fuller, M.T., **1998**. Transcriptional and post-transcriptional control mechanisms co-ordinate the onset of spermatid differentiation with meiosis I in *Drosophila*. *Development 125*, 125–134.

Wouters-Tyrou, D., Martinage, A., Chevaillier, P., Sautiere, P., **1998**. Nuclear basic proteins in spermiogenesis. *Biochimie 80*, 117–128.

Xu, E.Y., Lee, D.F., Klebes, A., Turek, P.J., Kornberg, T.B., *et al.*, **2003**. Human boule gene rescues meiotic defects in infertile flies. *Human Mol. Genet. 12*, 169–175.

Xu, H., Brill, J.A., Hsien, J., McBride, R., Boulianne, G.L., *et al.*, **2002**. Syntaxin 5 is required for cytokinesis and spermatid differentiation in *Drosophila*. *Devel. Biol. 251*, 294–306.

Yamashita, Y.M., Jones, D.L., Fuller, M.T., **2003**. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science 301*, 1547–1550.

Yang, J., Porter, L., Rawls, J., **1995**. Expression of the *dihydroorotate dehydrogenase* gene, dhod, during spermatogenesis in *Drosophila melanogaster. Mol. Gen. Genet. 246*, 334–341.

Yanicostas, C., Lepesant, J.A., **1990**. Transcriptional and translational cis-regulatory sequences of the spermatocyte-specific *Drosophila janusB* gene are located in the 3′ exonic region of the overlapping *janusA* gene. *Mol. Gen. Genet. 224*, 450–458.

Zhao, G.Q., Garbers, D.L., **2002**. Male germ cell specification and differentiation. *Devel. Cell 2*, 537–547.

1.5 Gonadal Glands and Their Gene Products

M F Wolfner, Cornell University, Ithaca, NY, USA
Y Heifetz and S W Applebaum, The Hebrew University of Jerusalem Rehovot, Israel

1.5.1. Introduction

The reproductive tracts of male and female animals contain a variety of glands and tissues whose products aid in reproduction. These gonadal glands and their products are crucial in various aspects of insect reproduction. Male reproductive glands provide proteins, peptides, and other molecules that are transferred to females during mating and contribute directly to female reproductive events. Female reproductive glands produce substances that are thought to support the gametes in their storage and meeting, to protect eggs, and, possibly, to interact with male-derived products.

Here, studies of discrete glands or glandular tissues associated with the reproductive system of insects are reviewed. Gonadal glands vary from primitive secretory epithelial cells along the wall of the reproductive tract to complicated glandular structures (reviews: Adiyodi and Adiyodi, 1975; Gillott, 1988). In this chapter, the term "male gonadal glands" is used to refer to male accessory glands (sometimes called paragonia) and also the secretory epithelia of the ejaculatory duct, ejaculatory bulb, and testis sheath (Figure 1). For females, the term "gonadal glands" includes the female accessory glands (sometimes called parovaria) and glandular structures or tissue associated with the spermatheca (otherwise called spermathecal glands) (Figure 1). Various modifications of glands, glandular tissues, or glandular functions occur in different insect orders, and species-specific functions have evolved. The original names of the organs/tissues and/or functions have been used, when expedient.

Early studies established the composition and role of gonadal gland gene products and their putative functions. Others provided ecological considerations of presumptive roles of gonadal gland products,

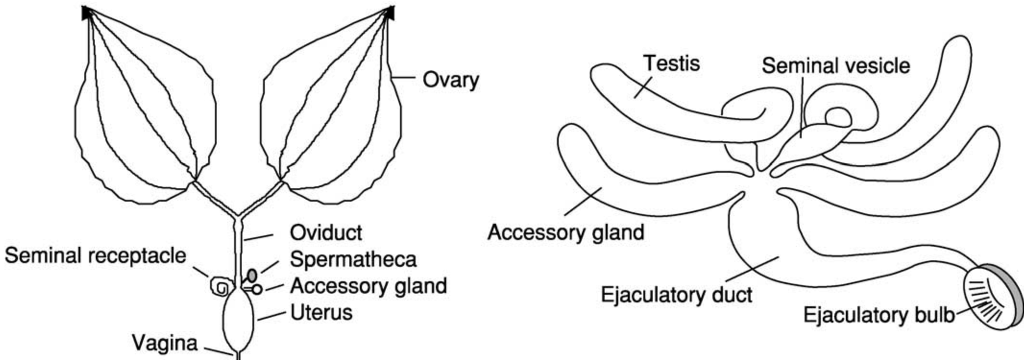


Figure 1 Reproductive tract of *Drosophila melanogaster*, showing the locations of gonadal glands including in females (left panel) the female accessory glands and spermathecae and in males (right panel) the male accessory glands, ejaculatory duct and ejaculatory bulb. (Reprinted with permission from Wolfner, M.F., **1997**. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem. Mol. Biol. 27*, 179–192; © Elsevier.)

either produced in such tissues or sequestered therein. More recently, studies have focused on identifying the factors made in the gonadal glands, and their actions in reproduction. In this chapter, the gonadal glands are discussed, and then, selected examples of male-derived products that are transferred to the female during mating and mediate male–female interactions in reproduction are presented, as well as examples of female-derived products known or likely to be involved in subsequent reproductive events. This material will also assist in viewing current studies on the molecular biology and genetics of gonadal gland products in a broader perspective.

1.5.1.1. Historical Perspectives

1.5.1.1.1. The discovery of important roles of gonadal gland secretions Insect females exhibit a variety of changes after mating. Depending on the species, they either initiate or increase their production of eggs after mating (for examples of reviews and references for *Drosophila melanogaster*, see Boulétreau-Merle, 1974; Wolfner, 1997; Chapman, 2001; Wolfner, 2002; Kubli, 2003; also **Chapters 1.3** and **3.9** and below). Mated insect females also store sperm, as do all animals that carry out internal fertilization (e.g., reviews: Neubaum and Wolfner, 1999b; Bloch Qazi *et al.*, 2003). Female insects often undergo changes in post-mating behavior, such that they no longer attract, or actively reject, subsequent courting males (for examples of reviews and references for *D. melanogaster*, see above and Manning, 1962, 1967; Swanson, 2003; and below). Factors transferred from males' gonadal glands during copulation often induce these changes. Such substances have been reported in Diptera, Orthoptera, Lepidoptera, and Coleoptera (reviews: Gillott, 1988, 2003). For example, in reproductively-mature female moths, biosynthesis and release of sex-pheromones indicate a female's receptivity to mating, and attracts conspecific males (see **Chapter 3.14**). Mated females arrest sex-pheromone production and cease attracting males. This has been demonstrated in many moth species, e.g., *Bombyx mori* (Ando *et al.*, 1996); *Choristoneura fumiferana* and *C. rosaceana* (Delisle *et al.*, 2000); *Epiphyas postvittana* (Foster, 1993); *Lymantria dispar* (Giebultowicz *et al.*, 1991); *Argyrotaenia velutinana* (Jurenka *et al.*, 1993; Kingan *et al.*, 1993); *Plodia interpunctella* (Rafaeli and Gileadi, 2000); *Helicoverpa zea* (Raina, 1989); and *Heliothis virescens* (Ramaswamy *et al.*, 1996). Cessation is permanent or transient, correlated with whether a species exhibits single or multiple mating. In addition to terminating sex pheromone production, mated female moths cease "calling" behavior – specific postures and wing fanning – and start ovipositing. Changes in post-mating willingness to re-mate are also seen in Diptera, including *D. melanogaster* and *Aedes aegypti*, and here too have been shown to be triggered by substances transferred from male gonadal glands (Fuchs and Hiss, 1970; Chen *et al.*, 1988), as will be discussed later.

Tissue transplantation, and the injection of extracts from gonadal glands, first provided an indication that the substances that trigger, and in some cases further regulate, these changes derive from glands of the male reproductive tract, and are donated to the female during mating. The male origin of these regulators was shown by the fact that virgin females did not mate after male reproductive tissues were transplanted into their body cavity. Such females showed physiological and behavioral

characteristics normally associated with mated females. For example, extracts of male *Helicoverpa armigera* reproductive accessory glands inhibited pheromone biosynthesis when injected into unmated females (Fan *et al*., 1999b), and transplantation of male accessory glands into unmated *D. melanogaster* caused the females to increase egg production/laying and to reject the advances of courting males (Garcia-Bellido, 1964; Merle, 1968). These results suggested strongly that molecules from the male must act through the female's hemolymph to affect her behavior and physiology. In another example, the deposition of fully developed and mature oocytes of many locusts and grasshoppers is stimulated by mating and can also be induced by implantation of male accessory glands, or injection of their extracts, into females (*Melanoplus sanguinipes* (Pickford *et al*., 1969); *Schistocerca gregaria* (Leahy, 1973)).

Further evidence that the products of male gonadal glands were important in females' post-mating responses came from studies in which the male tissues were depleted of, or made deficient in, those secretions. Most of these studies were carried out in *D. melanogaster*, in order to take advantage of its genetic techniques. *Drosophila* males that have mated multiple times still transfer sperm, but they have deflated accessory glands, suggesting that the tissue has been depleted of contents (Hihara, 1981). Mates of these males did not show the normal elevation of egg deposition or depression of receptivity to remating. This suggested that some component of the male reproductive tract other than sperm, possibly from the male's accessory glands, was responsible for the changes in mated females. Transgenic males deficient in accessory gland function were created by expression of a transgene encoding an intracellular toxin (Kalb *et al*., 1993), or by expression of particular forms of the *paired* (*prd*) gene, that allowed viability but prevented accessory gland development (Xue and Noll, 2000). Mates of these males did not show the behavioral or physiological changes associated with mating, and had deficient sperm storage. They also utilized the sperm they had stored inefficiently, such that they produced a few progeny unless accessory gland products were supplied by a subsequent mating. Experiments with males singly mutant in some accessory gland or ejaculatory duct products (see below) confirmed the roles of products of these tissues in post-mating responses (Gilbert *et al*., 1981; Cavener and MacIntyre, 1983; Whalen and Wilson, 1986; Ludwig *et al*., 1991; Herndon and Wolfner, 1995; Neubaum and Wolfner, 1999a; Heifetz *et al*., 2000; Saudan *et al*., 2002; Liu and Kubli, 2003).

The presumptive function of male accessory gland secretions was also evaluated by surgical manipulation of female responsive tissues. Protracted post-mating nonreceptivity is imposed in female grasshoppers (*Gomphocerus rufus*) by a component of the "white secretory tubule 1" of the male's accessory gland. This male-derived component is assumed to stimulate chemoreceptors located at the entry of the spermathecal duct into the endbulb (Hartmann and Loher, 1999). Ablation of the presumptive chemoreceptors, or surgical intervention in the neural pathway to the central nervous system (CNS), prevented the induction of nonreceptivity. Although this suggests the involvement of a direct neural pathway, caution must be exercised to rule out the possibility that behavioral responses after such surgical procedures were not due to stimulation of mechanoreceptors.

Although their study has been less extensive than that of male gonadal substances, female gonadal gland substances have also been shown to be important in reproductive physiology. For example, removal of *Musca domestica* female accessory glands (colleterial glands) reduced fertility by over 99% (Leopold and Degrugil, 1973). In another example, a volatile compound (pentanoic acid) produced by the female accessory glands (Comstock-Kellog glands) of the desert locust *S. gregaria* (Slifer, 1940, cited in Uvarov, 1966) is active in the stimulation of premating behavior of conspecific males (Njagi and Torto, 2002).

1.5.1.1.2. Identification of gonadal gland substances Two general methods have been used to identify gonadal gland products.

In several cases, extracts of gonadal glands have been fractionated chemically, and functions have been assigned on the basis of bioassays. Perhaps the best known molecule found in this manner is the "sex peptide" (also called SP; Acp70A) of *D. melanogaster*, which was identified by assaying fractions of male accessory gland methanolic extracts for their ability to stimulate egg deposition or decrease receptivity when injected into the hemolymph of unmated females (Chen *et al*., 1988). Fractionated extracts from mosquito accessory glands have also yielded peptides that affect behaviors (Lee and Klowden, 1999). In some cases, gel electrophoresis or biochemical assays of gonadal gland products provided functional information. For example, electrophoretic analysis of proteins from gonadal glands and from spermatophores of the beetle *Tenebrio molitor* indicated that spermatophore proteins were made in male accessory glands (Happ *et al*., 1977; Black *et al*., 1982; Grimnes *et al*., 1986). Similar analyses showed that a major component of the mating plug in

D. melanogaster derives from the ejaculatory bulb (Ludwig *et al*., 1991), and that a lysine-rich 12 kDa protein transferred with the spermatophore to female *Locusta migratoria* came from the opalescent gland of the male accessory gland (Lange and Loughton, 1985). Biochemical analysis of gonadal gland extracts led to the identification of protease inhibitors in the male accessory gland of *D. funebris* (Schmidt *et al*., 1989) and *S. gregaria* (Hamdaoui *et al*., 1997, 1998) (review: Simonet *et al*., 2002), lipases in male gonadal glands of sand flies *Phlebotomus papatasi* (Rosetto *et al*., 2003a) and *D. melanogaster* (Smith *et al*., 1994) and esterases and dehydrogenases in the *D. melanogaster* male's ejaculatory duct and some parts of the female reproductive tract (Gilbert *et al*., 1981; Cavener and MacIntyre, 1983; Feng *et al*., 1991). Finally, gel assays have identifed antimicrobial peptides in extracts of male or female gonadal glands of *D. melanogaster* and the medfly *Ceratitis capitata*, respectively (Marchini *et al*., 1991; Samakovlis *et al*., 1991; Lung *et al*., 2001a).

With the advent of gene technologies, hybridization or expressed sequence tag (EST) methods have been used to identify products of gonadal glands. In *D. melanogaster*, screens for differential gene expression have identified male-specific RNAs expressed in male accessory glands (Schäfer, 1986; DiBenedetto *et al*., 1987; Wolfner, 1997), eventually leading to a comprehensive EST screen for these "*Acp*" genes in *Drosophila* (Swanson *et al*., 2001). Genes identified by these screens can then be tested for function, either by *in vitro* or injection methods analogous to the ones described above or by expression or RNA interference (RNAi) (e.g., Chen *et al*., 1988; Aigaki *et al*., 1991; Chapman *et al*., 2003b; Brown *et al*., 2003) or, in *D. melanogaster*, by genetic techniques (e.g., Herndon and Wolfner, 1995; Neubaum and Wolfner, 1999a; Heifetz *et al*., 2000; Liu and Kubli, 2003). Finally, gonadal products of known function in one insect have been tested for functions in others, to determine whether sequence- and functionally-related molecules might exist in the other insect (Fan *et al*., 1999b, 2000; see Section 1.5.1.2).

1.5.1.2. Nomenclature

Structure-, function-, or family-based rules and conventions for nomenclature of lipids and enzymes, and genome-based conventions for naming genes are firmly established and usually unambiguous. However, the situation is more complex for bioactive peptides and proteins. For these, names are often assigned based on the first functions detected by *in vivo* assays. For example, among *D. melanogaster* paragonadal products, the peptide mentioned earlier, that is sex (male)-specific and affects egg production and mating-receptivity in mated females was named sex peptide (SP) (Chen *et al*., 1988), and a polypeptide that regulates ovulation, was called ovulin to reflect its function (Heifetz *et al*., 2000). As evocative and therefore useful as these common names are, their use can do a disservice, when additional important functions for the same molecule are discovered by subsequent assays. As a case in point, adipokinetic hormone (not a paragonadal product; simply used here to illustrate nomenclature issues) was named for its ability to mobilize lipids from the fat body (see **Chapter 4.6**), but this name masks its additional function in inhibiting protein synthesis (Carlisle and Loughton, 1979). Names are sometimes assigned based on tissue of origin, e.g., pEB-me: protein from the Ejaculatory Bulb of *D. melanogaster* (Ludwig *et al*., 1991; Lung *et al*., 2001b), or tissue of origin plus chromosomal position, e.g., Acp62F for *D. melanogaster* Accessory gland protein from chromosome position 62F (Wolfner *et al*., 1997), or Dup99B for *Ductus ejaculatorius* protein from chromosomal position 99B (Saudan *et al*., 2002).

Confusion can result when the same molecule has a double designation. For example, *D. melanogaster* SP (Chen *et al*., 1988) is also designated Acp70A (e.g., Wolfner, 2002) and *D. melanogaster* ovulin is also called Acp26Aa (Monsma and Wolfner, 1988; Monsma *et al*., 1990; Heifetz *et al*., 2000). Further confusion results when molecules identified in one species are assayed in another species and their cross-reactivity suggests that the recipient species may have a molecule structurally and/or functionally similar. For example, SP elicits post-mating reactions of the adult female African bollworm, *H. armigera*, analogous to those elicited in *D. melanogaster* (Fan *et al*., 1999b, 2000), suggesting that *H. armigera* makes a similar molecule that elicits these effects (see Section 1.5.1.2).

To unify the nomenclature, the authors propose that each gene be referred to with a single name. When all experiments are conducted on the species in which the gene was identified, species designation need not be indicated (apart from an initial statement of the species used in the paper) but when cross-genera or cross-species studies are carried out, an italicized three lettered species designation should precede the name of the gene or peptide. For example, in the study noted above, using the designation *Drm*-SP (= *Drm*-Acp70A) to identify the origin of the peptide that elicits post-mating responses in the moth *H. armigera* is suggested. Cross-reactivity of *Drm*-SP suggests that an ortholog

Hea-SP will eventually be found in the moth (Fan *et al*., 1999b). Designations based on specific function (e.g., SP), where known, are less cumbersome than ones based only on isolation method or genetic map position in a given organism (e.g., Acp70A), but may be confusing if functions differ in different organisms. Therefore it is proposed that functional names be used with caution and always with co-designation of their systematic or generic name, the first time they are mentioned in the text of a report. Since the functions of many gonadal gland products remain to be determined, their systematic names should be used exclusively until after functions are determined.

1.5.1.3. What are Gonadal Glands?

The gonadal glands of males (**Figure 1**) are the male accessory glands, the seminal vesicle (where sperm are stored prior to transfer to females), the ejaculatory duct (through which ejaculate passes on its way from the male to the female), and the ejaculatory bulb (a pump that also produces some components of the ejaculate). In females, the gonadal glands include the female accessory glands and the spermathecal glands. Often, these glands are simple single layers of secretory cells surrounding a lumen in which their secretions accumulate. There can be differentiation among the secretory cells. For example, the *D. melanogaster* male accessory gland includes two visibly distinct secretory cell types – main cells (96% of the cells) and larger, spherical secondary cells, interspersed among the main cells near the distal end of the gland. The two cell types make different, but overlapping sets of products (DiBenedetto *et al*., 1990; Monsma *et al*., 1990; Bertram *et al*., 1992; Wolfner, 1997). Similarly, in *T. molitor*, the male's accessory glands have several lobes, each of which makes a protein of a particular layer of the spermatophore (Grimnes *et al*., 1986). In many cases the secretory cells are themselves surrounded by muscle sheaths whose contraction can cause expulsion of the gonadal gland contents from the gland.

Gonadal glands have evolved in structure and function and these tissues may differ substantially in appearance in different insect and other arthropod groups, varying from secretory epithelia along the wall of the reproductive tract to complicated glandular structures (reviews: Adiyodi and Adiyodi, 1975; Gillott, 1988). Since the origin, structure, biochemistry, and functions of specific glands vary, their classification can be problematic: some glands with different names in different insects probably secrete similar types of substances, and conversely, some glands whose homologies are not certain have been grouped under the same name. Even within one genus, *Drosophila*, different morphologies are seen in some tissues, such as the ejaculatory duct, whose production of glucose dehydrogenase correlates with different morphological duct types seen in the different species (Cavener, 1985, 1987). Interestingly, in addition to modifications of gonadal glands that still preserve a reproductive function, in some cases, derivatives of these glands have been co-opted for other functions. Examples include defensive, or trail-marking functions in ants (review: Blum and Hermann, 1978) or sting apparati in bees and wasps, which are derived from a nonfunctional ovipositor and fed from venom glands that are homologous to certain female gonadal glands (D'Rozario, 1942, cited in Packer, 2003). These modifications have no bearing on reproductive functions and will not be elaborated on here.

1.5.1.4. Development of Gonadal Glands

Insect gonadal glands develop from cells in genital imaginal discs, usually corresponding to abdominal segments A8, A9, and A10 (Sendi *et al*., 1993; Keisman and Baker, 2001; Keisman *et al*., 2001; Christiansen *et al*., 2002). The signals for this development are best understood in *D. melanogaster*. Here, under the influence of sex determination genes (see **Chapter 1.1**) that mediate responses to *decapentaplegic* (*dpp*) and *wingless* (*wg*) signaling pathways, cells in the genital imaginal disc develop into different genital and gonadal gland structures in males and in females. In females, A8 cells normally comprise a large part of the genital primordium and A9 forms only a small part. A8 cells develop into female genital tissues – except for the female accessory glands which develop from A9 cells. In males, the bulk of the genital primordium derives from A9 cells, which ultimately form the internal and external male genital structures, including the male gonadal glands and the ejaculatory duct and ejaculatory bulb. The A8 cells in the male's genital disc form a small tergite, but do not contribute to the gonadal glands in males (see **Chapter 1.1**).

Insect gonadal glands attain their final form in late pharate adults (Chapman and Wolfner, 1988; Sendi *et al*., 1993) and become functionally mature in the adult stage. Hormonal triggers are important in these events. For example, development of the male accessory glands in the migratory locust, *L. migratoria*, is dependent in the presence of juvenile hormones (JHs; see **Chapter 3.7**). Elimination of allatal JH production by ethoxyprecocene, and its recovery after application of a JH analog, supports this model, as does the presence of JH-binding proteins in male accessory glands (Braun

and Wyatt, 1995) and the existence of hemolymph JH-esterases (Paterson and Weaver, 1997) that can modulate JH hydrolysis.

Insects that begin reproducing immediately after the imaginal molt eclose with gonadal glands that appear to be fully differentiated. In contrast, insects with a preoviposition period between ecdysis and reproduction, or those exhibiting adult diapause (see **Chapter 3.12**), exhibit a lag, after which gonadal gland development proceeds from a functionally immature state to complete differentiation (Sturm, 2002). Even if gonadal glands are fully formed at the time of eclosion, a maturation period is required for them to accumulate their full amount of secretion. For example, although *D. melanogaster* males become fertile during their first posteclosion day, it takes 3 days for their accessory gland protein levels to be maximal, and from that point their levels remain stable unless the animal mates (Chapman and Wolfner, 1988; DiBenedetto *et al*., 1990; Monsma *et al*., 1990; Bertram *et al*., 1992). Mating stimulates male accessory gland protein biosynthesis, in a process that also requires JH (Chen, 1984, 1991; Fernandez and Klowden, 1995; Herndon *et al*., 1997; Wilson *et al*., 2003).

Mating can affect the expression of genes in the gonadal glands. For example, during mating of the German cockroach, *Blatella germanica*, formation of the spermatophore and its transfer to the female correlates with the depletion of most of the proteins from the male's accessory glands. The activity of the male's corpora allata (CA) also decreases, indicating that JH production is inhibited during this process (see **Chapter 3.7**). This has been hypothesized to be necessary to allow for a subsequent round of production of secretions by the accessory gland (Vilaplana *et al*., 1996). Consistent with this, following mating and consequent depletion of accessory gland proteins (Acps) in *D. melanogaster*, Acp RNA and protein synthesis are stimulated in the main cells of the male's accessory gland in a process that requires JH (Herndon *et al*., 1997).

1.5.2. Products of Male Gonadal Glands

As shown by the studies noted above, male gonadal glands produce molecules that, upon entry into females, cause post-mating changes in females. In some insects, male gonadal glands also can sequester molecules made in other places in the animal, or can be taken up from the environment (Dussourd *et al*., 1991; Smedley and Eisner, 1995, 1996). These sequestered molecules can be transferred to females during mating along with molecules made in the gonadal glands themselves. In this section, the identities and functions of products made in, or sequestered by, gonadal glands of males are reviewed. First, examples of presumptive nutritive products of molecules transferred from male gonadal glands are presented. The benefit to the donor male in this scenario might be to attract the female, supplement her nutrition, and/or distract her from feeding on him. Second, male-derived products that occlude the female gonopore, thereby contributing to the reproductive success of the male's sperm are described. Third, the presence and contributions of antimicrobial peptides or immune modifiers are discussed. Finally, molecules that influence the behavior or physiology of mated females are described. Although we will discuss many molecules, we are not presenting a comprehensive list. Rather, we have selected examples to illustrate each important aspect of the functions of male gonadal gland products.

An important consideration in the role of male gonadal secretions is their site of action in female insects. With the exception of nuptial gifts, which are consumed orally by the female, male gonadal secretions enter the female through her reproductive tract. This allows them to act from within the reproductive tract, e.g., mating plug proteins (Ludwig *et al*., 1991; Lung *et al*., 2001b), sperm-storage proteins (Bertram *et al*., 1996; Neubaum and Wolfner, 1999a). Experiments, in which effects on females were seen upon transplantation of male gonadal tissues (Garcia-Bellido, 1964; Merle, 1968) and effects of gonadal secretions on levels of JH in mated females (Moshitzky *et al*., 1996; Fan *et al*., 1999a, 1999b, 2000; Eliyahu *et al*., 2003), indicate, that at least some of these proteins must be able to access the hemolymph and affect the neuroendocrine system of the female. Indeed, experiments with *D. melanogaster* have demonstrated that male gonadal secretions enter the circulation of the female shortly after mating (Meikle *et al*., 1990; Monsma *et al*., 1990; Lung and Wolfner, 1999; Lung *et al*., 2002). Seminal proteins enter the circulation through a transiently-permeable section of the vaginal wall (Lung and Wolfner, 1999). Hemolymph entry begins during mating, but rapidly (within minutes) ceases. Inspection of sequences of male proteins that enter the female's circulation in this way has failed to identify a specific primary sequence responsible for targeting. Tertiary structure, or differences in accessibility of male proteins (for example, whether or not they are bound to sperm) may be responsible for their ability to cross this part of the female anatomy to enter the female's circulation.

1.5.2.1. Female Dietary Supplement

Males from many insect species present nuptial gifts to females before or during mating encounters (Vahed, 1998). These gifts are frequently comprised of gonadal secretions associated with transferred spermatophores, and they often include substances of presumptive nutritional value. In many crickets, where such nuptial gifts are common, the male spermatophore is accompanied by a gelatinous material (spermatophylax) upon which the female feeds after mating. Other nuptial gifts are molecules sequestered by, rather than made in, insect gonadal glands. For example, males of some butterfly and moth species exhibit "puddling" behavior, in which they cluster around puddles or damp areas and drink large amounts of liquid. They sequester sodium from this liquid, which they presumably transfer to females during mating (e.g., Arms *et al.*, 1974; Smedley and Eisner, 1995).

Several hypotheses have been proposed for the role of nuptial gifts. They may enhance the number and/or quality of eggs, as described for sodium above. Several elements in addition to sodium have been reported to be transferred to female insects during mating. Phosphorus, transferred to female *D. melanogaster* and *D. nigrospiracula* upon mating, is incorporated into their eggs (Markow *et al.*, 2001), although whether it also provides a nutritional supplement to the female herself is not yet known. Calcium phosphate is transferred to the female in the spermatophore of the butterfly *Calpodes ethlius* (Lai-Fook, 1991), and zinc to the female moth *H. virescens* (Engebretson and Mason, 1980). Nuptial gifts may also provide a dietary supplement that might contribute to female fecundity (this also benefits the male, by enhancing his fitness as a consequence), or may distract the female's attention for a time sufficient for the sperm to be transferred from the spermatophore, or for the male to escape. Of the male gonadal gland proteins transferred to females, many act in the female to modulate behavior, physiology, and sperm storage (see below), but in principle it is also possible that their amino acids provide nutrition to the female as well. There is a trade-off between using nutrients for somatic maintenance and for eggs (e.g., see Salmon *et al.*, 2001); a variety of strategies are used by female insects to allocate nutrients received from their mates to these functions (e.g., see Rooney and Lewis, 1999).

In some cases the main function of nuptial gifts may not be nutritive. While they are consuming nuptial gifts, female insects are distracted from the mating male long enough for him to ensure optimal sperm (or seminal protein) transfer e.g., the spermatophylax of the cricket *Gryllodes sigillatus*, on which the female feeds after mating, lacks nutritive value (Will and Sakaluk, 1994).

Thus, gonadal gland-derived nuptial gifts, and the nutrients they contain, may serve diverse and multiple purposes in insects. There has also been speculation about the role of nuptial gifts in the evolution of female polyandry, by direct nutritional benefit or otherwise (e.g., Kondoh, 2001; Arnqvist and Nillson, 2001). However, detailed consideration of such ecological and evolutionary questions are beyond the scope of this subsection.

1.5.2.2. Components of a Gonopore Plug

During mating, males of many insect species transfer gonopore plugs, or material derived from products of their gonadal glands, which subsequently congeal, and may harden into "plugs" or "masses" within the female reproductive tract. Such structures are assumed to increase the likelihood of paternity in different, but not necessarily exclusive ways (e.g., Patterson and Stone, 1952; Bairati and Perotti, 1970; Duvoisin *et al.*, 1999; Sauter *et al.*, 2001; Brown *et al.*, 2002). Among many possible functions, they may physically obstruct the reproductive tract and thereby interfere with subsequent copulation, indicate that the female has already mated, trap the sperm within the genital tract and thereby prevent sperm backflow, and/or provide a scaffold for sperm movement.

In an example of the first function, male bumblebees (*Bombus terrestris*) transfer a mating plug after they transfer sperm (Duvoisin *et al.*, 1999; Sauter *et al.*, 2001). This sticky plug is digested within the female after several days. Composed of linoleic, oleic, palmitic, and stearic acids (in decreasing order of amount) and of a cyclic dipeptide (cycloprolylproline), it does not contribute components of exceptional nutritional significance to the female (Baer *et al.*, 2000). It is thought that the mating plug may assist in limiting the number of matings by a female. (This is important to increase the average relatedness of workers in a colony – which can positively affect their level of cooperation.) Consistent with this proposed role for the mating plug, the plugs are more rapidly digested in a related bumblebee species (*B. hypnorum*), in which multiple re-matings (as many as six) can occur (Brown *et al.*, 2002). The limited persistence of the mating plug in *B. hypnorum* may permit its re-mating frequency. A similar hypothesis has been proposed in mosquitoes. In *Culex pipiens*, prevention of multiple-mating has been attributed to the formation of a

mucoid plug in the mated female (Bullini *et al.*, 1976).

Experiments in *Drosophila* suggest a similar role for mating plugs and also additional roles in providing a scaffold for sperm movement and a barrier to backflow of sperm (Bairati and Perotti, 1970; Polak *et al.*, 1998). The *D. melanogaster* mating plug is composed of secretions from the ejaculatory bulb that coagulate at the posterior of the uterus, remaining there until being expelled by the first egg deposited after mating. Gels of mating plug proteins show a major band or series of bands, corresponding to electrophoretic variants (Ludwig *et al.*, 1991). This 37 kDa band is an ultraviolet-fluorescent protein called *Drm*-pEB-me; it is encoded by a single gene on chromosome 2 (Ludwig *et al.*, 1991; Lung *et al.*, 2001b). The sequence of pEB-me contains amino acid motifs resembling those in spider silk and mollusc byssal threads (Lung *et al.*, 2001b). The presence of these motifs suggests that self-association of PEB-me might contribute to solidification of the mating plug in the uterus.

In matings in some *Drosophila* species, a more elaborate "insemination mass" is seen to engorge the female's vaginal tissues after mating. This mass is thought to be derived from secretions of female tissues and from sperm and male-derived seminal proteins (Patterson and Stone, 1952). In some cases this mass appears analogous to the mating plug discussed above: it remains soft and is expelled within a few hours of mating. But in other cases, particularly in certain inter-species matings, the mass hardens and remains for days after copulation, e.g., in crosses of *D. buzzati* females with *D. arizonensis* males masses have been seen 192 h post-mating (Patterson and Stone, 1952). The presence of this hardened mass within the female's uterus and oviducts is thought to interfere with sperm storage and/or use, and even with movement of eggs through the oviducts. That interaction between male and female secretions in interspecific matings can result in a hardened, impenetrable, and persistent mass has been suggested to be part of species-isolation mechanisms.

1.5.2.3. Defensive Molecules

Reproductive tracts are (topologically) open to the outside and, thus, potentially subject to microbial invasion. Moreover, the fertilized eggs deposited after a mating are potentially vulnerable to attack by microbes or by animals. Perhaps because of these considerations, gonadal glands make molecules that can potentially defend these reproductive tissues, and gametes, from attack. Male gonadal glands produce several antimicrobial peptides. In *Drosophila* at least four peptides have been detected. Andropin is a 57-amino acid peptide (including its signal peptide) that is related to cecropins. It is made in the male's ejaculatory duct (Samakovlis *et al.*, 1991) and transferred to females during mating (Lung *et al.*, 2001a). Three additional antimicrobial activities were detected as products of male accessory glands (Lung *et al.*, 2001a), but their molecular identity is not known. Whether any of these antimicrobial molecules enter the circulation of the mated female by crossing the wall of the reproductive tract has yet to be determined. Promoter analysis suggests that the antimicrobial peptides drosomycin, attacin, drosocin, and cecropin are expressed constitutively in selected gonadal tissues of *Drosophila* males (as well as females) (Charlet *et al.*, 1996; Ferrandon *et al.*, 1998; Tzou *et al.*, 2002).

Molecules provided from the male can also protect the eggs after deposition. One example occurs in the beetle *Neopyrochroa flabellata*. Male *N. flabellata* ingest cantharidin, and sequester it in their accessory glands. This irritant molecule is then transferred to the female in the spermatophore, later accumulating in the eggs, where its presence protects the progeny from predation (Eisner *et al.*, 1996). Another example involves alkaloids taken up by caterpillars of the moth *Utetheisa ornatrix* and sequestered in the male's reproductive tissues (Dussourd *et al.*, 1988, 1991; Eisner *et al.*, 2000; Rossini *et al.*, 2001). Male moths display modified forms of the alkaloids (hydroxydanaidal) on "brushes" extended from their reproductive tracts. Female moths can thereby assess the relative alkaloid content of their potential mates; males with high alkaloid content are preferred. The alkaloids are transferred to the female during mating and are thought to protect her eggs from subsequent predation. Similarly, in many arctiid moths, pyrrolizidine alkaloids (PA) are among bioactive compounds sequestered from host plants (review: Weller *et al.*, 1999). PA can be transferred from males to females during mating and/or from females to developing oocytes (Hare and Eisner, 1993; Eisner *et al.*, 2000; Rossini *et al.*, 2001), again imparting protection from predation. As with the alkaloids noted above, PA plays a secondary role in mating strategy, since female prefer males with higher levels of PA.

1.5.2.4. Behavioral Modifiers

Molecules made in, or sequestered by, male gonadal glands and transferred to females during mating can affect the females' subsequent behavior. Female attractiveness to males and her reproductive performance (via pheromones and/or behaviors) can be modulated (Blomquist *et al.*, 1995; Jin and Gong,

2001; Jang, 2002), as can her willingness to mate with subsequent courting males (active rejection behavior) (Bastock and Manning, 1955; Garcia-Bellido, 1964; Manning, 1967; Merle, 1968, Chen *et al*., 1988; Hall, 1994; Kubli, 2003).

1.5.2.4.1. Modification of female attractiveness Female moths exhibit a "calling" behavior in which they emit pheromones to attract males, sometimes from a considerable distance. After mating, calling ceases – sometimes permanently – as a result of transfer of gonadal gland products from males. For example, a pheromonostatic peptide (PSP) (*Hez*-PSP-1), produced in the male accessory glands of the moth *H. zea* and transferred to the female during mating, has been isolated and sequenced (Kingan *et al*., 1995). Pheromonostatic peptide prevents activation of the female moth sex-pheromone gland by PBAN (pheromone biosynthesis activating neuropeptide), a cerebral neurohormone whose secretion is environmentally regulated (review: Rafaeli, 2002). Although bio-activity of synthetic *Hez*-PSP-1 was not assayed in the moth *H. zea*, a partial synthetic C-terminal sequence (*Hez*-PSP-135-57) was shown to be pheromonostatic when injected into gravid females of the closely related *H. armigera* (Eliyahu *et al*., 2003).

cis-Vaccenyl acetate (cVA), a lipid that is transferred from male *D. melanogaster* to females during mating, has also been suggested to play a role in modulating female attractiveness in this species. While its gonadal origin (ejaculatory bulb; Butterworth, 1969) is unequivocal, the exact function of cVA is still unclear. It has been suggested that cVA is hydrolyzed in the female to an alcohol by male-derived esterase-6, concurrently transferred during mating (Meikle *et al*., 1990), and that the alcohol serves as a repellent to subsequent mating (Mane *et al*., 1983). This presumptive function may be circumstantial: cVA can be hydrolyzed by this esterase, and topically applied *cis*-vaccenyl alcohol does deter male *D. melanogaster*, but it is not certain that this is one of its functions in the normal mating situation. cVA does function as an aggregation pheromone. Recently, it has been reported that mated females emit cVA on the host fruit, attracting other males and females and inducing aggregated oviposition (Bartelt *et al*., 1985; Wertheim *et al*., 2002).

Another modulator of female attractiveness is methyl salicylate. Topical application of this compound to virgin female moths deters males (Andersson *et al*., 2000). Methyl salicylate can be made from phenylalanine by male moths (*Pieris napi* and *P. rapae*), or taken up from plant hosts and sequestered, and then transferred to females during mating (in *P. napi* and *P. rapae*, in the spermatophore). Marking of mated females by this compound (or related molecules) curtails their mating with subsequent conspecific males. In *P. brassicae*, phenylananine is used by the male to synthesize benzyl cyanide, also called phenylacetonitrile (PAN), which serves a similar anti-aphrodisiac function to methyl salicylate in the other *Pieris* species (Andersson *et al*., 2003). Interestingly, both methyl salicylate and PAN serve other roles in insect chemical communication (Seidelmann *et al*., 2000; Seidelmann and Ferenz, 2002; James, 2003; Ferenz and Seidelmann, 2003; Ninkovic *et al*., 2003); it is intriguing to think that they may have been co-opted for a mating-related function (or vice versa).

1.5.2.4.2. Inducers of rejection behaviors Male gonadal gland products cause females of some insects to actively reject subsequent courting males. For example, *D. melanogaster* females that have mated reject the advances of subsequent males by kicking, running away, and extruding their ovipositor, which physically prevents intromission (reviews: Wolfner, 1997, 2002; Chapman, 2001; Kubli, 2003; Swanson, 2003; and below). Tissue transplantation experiments showed that molecules made by males' accessory glands caused this change in behavior (Garcia-Bellido, 1964; Manning, 1967; Merle, 1968). A 36-amino-acid peptide made by male accessory glands was shown to induce rejection behavior (Chen *et al*., 1988). This molecule, called "sex peptide" (SP, also called *Drm*-SP and Acp70A), was identified by injecting fractions of male accessory gland extracts into unmated females. SP can induce rejection behavior (and increases in egg production) when introduced into the circulation of female flies (reviews: Kubli, 2003; Swanson, 2003; see also Chen *et al*., 1988; Aigaki *et al*., 1991; Soller *et al*., 1999). Recent knockout (Liu and Kubli, 2003) or RNAi knockdown (Chapman *et al*., 2003b) analyses confirmed these functions – but also indicated that SP is not the sole mediator of these phenomena. Indeed, the *D. melanogaster* ejaculatory duct produces a 31-amino acid peptide related to SP, Dup99B (Saudan *et al*., 2002) that can also induce rejection behavior when injected into the circulation of females. Dup99B is transferred to females during mating. However, when males lack Dup99B, their mates still show full changes in receptivity, suggesting that SP plays a larger role in modulating receptivity than does Dup99B. A third regulator of female receptivity, a 27-amino acid peptide (called PS-1) is made by male accessory glands in *D. funebris* (Baumann, 1974a, 1974b; Baumann *et al*., 1975).

In another dipteran, the mosquito *A. aegypti*, "matrone," a complex of two proteins produced by the male's accessory gland, has been reported to modulate post-mating behaviors (Hiss and Fuchs, 1972; Williams *et al.*, 1978).

Synthetic peptides corresponding to parts of SP have been used to show that its rejection-mediating activity derives from the SP's conserved C-terminal 12 amino acids, including a disulfide-bonded region. Although the target site for the SP's action in inducing the receptivity changes is as yet unknown, it binds to many tissues throughout the fly, including the brain (primarily peripheral nerves) and parts of the subesophageal and thoracic ganglia (Ottiger *et al.*, 2000). These binding sites are found in males as well as females, although the peptide can only reach them in mated females. Tethered versions of SP are active when expressed locally in the head, suggesting that access to neural targets may be important for SP function (Nakayama *et al.*, 1997). Blot overlay assays identified different-sized SP-binding complexes in heads and reproductive tracts of flies, suggesting that the former is a receptivity-related receptor while the latter is related to egg-production (Ding *et al.*, 2003). SP binds to sperm in the mated female (E. Kubli, personal communication; Liu and Kubli, 2003). Kubli and colleagues have suggested that such binding could contribute to the long-term effect (Manning, 1967; Kalb *et al.*, 1993) of sperm on receptivity and egg production (Kubli, 1992), if the sperm provided a source for continued release of SP in the mated female. Recent experiments support this hypothesis (Chapman *et al.*, 2003b; Liu and Kubli, 2003).

Interestingly, the same C-terminal region of SP that induces rejection behavior in *D. melanogaster* females causes behavioral changes in female *H. armigera* moths: when synthetic *Drm*-SP (or *Drm*-SP partial peptides) are injected into unmated female moths, PBAN-stimulated female sex-pheromone production is inhibited (Fan *et al.*, 1999b, 2000). This functional cross-reactivity is intriguing: the peptide essentially serves the same purpose (mating inhibition), but in different manners in the two organisms. The effect of *Drm*-SP on *H. armigera*, and that *H. armigera* females normally show inhibition of pheromone production after mating, suggests that *H. armigera* males may produce a functional homolog to *Drm*-SP.

1.5.2.5. Modifiers of the Mated Female's Reproductive Physiology

The physiology of the female is also modified by molecules made in, or sequestered by, her mate's gonadal glands and transferred to her during mating. Levels of egg production by females and the female's hormonal milieu are manipulated by products she receives from her mate's gonadal glands.

1.5.2.5.1. Regulators of the egg-laying process Molecules made by, or sequestered in, the male's gonadal glands and transferred to the female along with sperm have been shown to increase egg production and deposition by mated females. Coupling mating to the initiation of, or increase in, egg production seems advantageous to both sexes. By having males provide the proximate stimulators of egg production, an unmated female need not expend extensive resources on making eggs that will not be fertilized. Having molecules and/or sperm from the male trigger or stimulate egg production means that large numbers of eggs are produced when there are sperm to fertilize them, thus guaranteeing a good supply of fresh eggs for this joint endeavor.

Deposited eggs are the end result of a stepwise "egg-laying process," defined herein based on studies of *D. melanogaster* (Heifetz *et al.*, 2000). The existence of these steps allows separate controls at each level of the process, potentially allowing highest efficiency or guarding against complete disruption of egg production in the event that a single key regulator is absent or disabled. The first of several interdependent steps is the initiation (or completion) of oogenesis. The second step is ovulation, in which oocytes are released from the ovary. Third, the eggs must move down the oviducts. Fourth, they come to rest in the uterus/bursa, where they can be fertilized by sperm released from storage. Finally, the eggs are deposited on the substratum. Although all steps likely occur in all insect reproductive systems, in most cases studies have focused on oviposition (the final step) as a general response. Results from such examples will be discussed below under "oviposition" though the actual locus of the response may be in a prior step or steps.

1.5.2.5.1.1. Oogenesis Oogenesis, the process of egg production by the ovary (see **Chapters 1.2** and **1.3**), involves a series of sequential stages in which the egg initiates and continues meiosis (which then completes or arrests), fills with RNAs and proteins that will support subsequent embryo development, and acquires a vitelline envelope and chorion (eggshell). In unmated females of many insect species, oogenesis is arrested completely, or is delayed at particular stages. In many cases, this arrest is overcome by substances transferred during mating, resulting in an increase in egg deposition.

Control of oogenesis by factors from the male gonadal glands has been well-documented in *Drosophila* (e.g., see Wolfner, 1997, 2002; Hall, 1994; Chapman, 2001; Kubli, 2003 for review). Each *D. melanogaster* ovary (**Figure 1**) contains 16 ovarioles, each of which contains a series of egg chambers at specific, sequential, stages of development. In unmated *Drosophila*, oogenesis ordinarily arrests with a maximum of one mature oocyte per ovariole. There appears to be a "checkpoint" in virgin *Drosophila* females at approximately stage 9 of oogenesis. Mating overcomes this block, allowing oogenesis to proceed. SP injection also overcomes this block (Soller *et al.*, 1997, 1999), indicating that SP is a modulator of egg production (Kubli, 2003).

SP appears to affect egg production in at least two ways. First, *in vitro* studies suggest that SP acts by regulating the levels of JH in *D. melanogaster*: incubation of corpora allata *in vitro* with SP causes increased levels of JHB3 (Moshitzky *et al.*, 1996; see **Chapters 1.3**, **3.6**, and **3.9**). JH is an important regulator of oogenesis in *Drosophila*. Specifically, it regulates the production and uptake of yolk proteins (vitellogenins; made in the fat body and ovarian follicle cells) into oocytes beginning at oogenic stage 7 (e.g., see Riddiford, 1993; Spradling, 1993; **Chapters 1.3** and **3.9** for reviews; also Schmidt *et al.*, 1993; Soller *et al.*, 1997, 1999). Kubli, Applebaum and colleagues have proposed that mating introduces SP into females, which increases JH levels in those females, leading to increased yolk protein levels, and to overcoming the block to oogenesis (Moshitzky *et al.*, 1996; Soller *et al.*, 1997, 1999; Kubli, 2003). The region of SP required for its effects on JH is its N-terminal part (Fan *et al.*, 2000); this differs from the region active in its second effect on egg production and on receptivity (Section 1.5.2.4.2).

The C-terminal region of SP also affects egg production, possibly by acting at another step – expulsion of uterine eggs (Aigaki *et al.*, 1991). This same region of SP induces changes in receptivity behavior, and the same effective concentration of SP is needed for both responses. However, SP binds to at least two different receptor complexes, one in heads and another in female reproductive tracts. This second complex is in the right place to represent a receptor through which SP modulates effects on egg production (Ding *et al.*, 2003). Effects of sperm in keeping oogenesis levels high (e.g., Kalb *et al.*, 1993; Xue and Noll, 2000; Heifetz *et al.*, 2001a) is suggested to result at least in part from SP's binding to, and slow release from, sperm, as discussed above under "receptivity" (Kubli, 1992, 2003; Chapman *et al.*, 2003b; Liu and Kubli, 2003).

As with receptivity, a multitude of male gonadal factors can affect egg production in *Drosophila*. In addition to SP, several other male-derived peptides regulate egg production in various *Drosophila* species, although it is not yet known whether they specifically stimulate oogenesis or the next step, ovulation. Two are relatives of SP. One is *Drm*-Dup99B, the ejaculatory-duct relative of SP noted above to cause receptivity changes upon injection into unmated females (Saudan *et al.*, 2002). Like SP, injection of Dup99B causes increased egg production in *D. melanogaster*, but similar to the situation noted above for receptivity, it appears to play a minor role *in vivo*. *Drosophila biarmipes* males' ejaculatory ducts produce ED-OSS, a 32-amino acid relative of *Drm*-SP (Imamura *et al.*, 1998). Injection of ED-OSS into unmated females stimulates egg production. Two other male – accessory gland-derived molecules that stimulate egg production in *Drosophila* species have been found by injection-fractionation assays, but little is known of their detailed structure, or the steps at which they act. PS-2 is a glycine carbohydrate derivative from *D. funebris* with egg-production stimulatory activity (Baumann, 1974b, 1974a, Baumann *et al.*, 1975), and OSS from *D. suzukii* also has this activity (Ohashi *et al.*, 1991).

A similar molecular basis may modulate oogenesis after mating in heliothine species of noctuid moths. In these insects, mating induces oogenesis from a completely arrested state. As in *Drosophila*, oogenesis, including choriogenesis, in heliothine moths is regulated by JH (Ramaswamy *et al.*, 1990; Satyanarayana *et al.*, 1992; reviewed in **Chapter 1.3**). JH titers in the hemolymph of mated *H. virescens* females are significantly higher compared to titers in virgin females (Zeng *et al.*, 1997). Elevation of JH production by male-derived accessory gland factors in heliothine noctuid moths would explain the high egg production in mated females. Interestingly, injection of *Drm*-SP into *H. armigera* causes an increase in egg production and JH levels consistent with the presence and action of a related molecule in this moth (Zeng *et al.*, 1997); as for *D. melanogaster*, the N-terminal region of *Drm*-SP induces JH production when injected into unmated *H. armigera* females (Fan *et al.*, 2000). These findings are consistent with the postulate in Section 1.5.2.4.2, above, that *H. armigera* males may produce and transfer to their mates a functional homolog of *Drm*-SP. In another parallel between *D. melanogaster* and *H. armigera* females' responses to male gonadal gland products, it appears that there may be multiple male-derived molecules in *H. armigera*, each of which can increase egg

production. In addition to the likelihood that *H. armigera* possesses a functional homolog of *Drm*-SP, male accessory glands of this moth also produce proteins of larger molecular masses (55–66 kDa) that affect reproductive maturation (oogenesis and egg deposition) in female *H. armigera* (Jin and Gong, 2001).

1.5.2.5.1.2. Ovulation Ovulation is the process by which an egg is released from the ovary. As such, its rate is limited by the rate of oogenesis, though it can in theory feed back to regulate oogenic rate by controlling the rate at which mature oocytes leave the ovary and hence the feedback controls. In *Drosophila* (Heifetz *et al.*, 2001b), and probably Hymenoptera (Went and Krause, 1974), ovulation also causes oocytes to activate, meaning that they resume meiosis, initiate translation, alter the cross-linking state of their vitelline envelope, and essentially transition into a state that is competent to initiate embryonic development.

In mated *D. melanogaster* females, ovulation is stimulated by male gonadal gland proteins received during mating. In particular, a 264-amino acid prohormone called Acp26Aa (or ovulin, *Drm*-Acp26Aa), from the male's accessory glands, stimulates ovulation. Ovulation is not fully stimulated in females that had mated to mutant males that do not produce Acp26Aa (Herndon and Wolfner, 1995; Heifetz *et al.*, 2000); that ovulation, specifically, is dependent on Acp26Aa was determined by assessing the efficiency of each stage in the egg production process in these females (Heifetz *et al.*, 2000). Unmated females engineered to express Acp26Aa on their own ovulate at elevated levels (Y. Heifetz, L.N. Vandenberg, H.I. Cohn, and M.F. Wolfner, in preparation), confirming the role of Acp26Aa in this process. Its normal action has been suggested to be to "clear" old eggs from ovaries, which also ultimately allows egg production to be coordinated with release of sperm from storage (Chapman *et al.*, 2001). Although Acp26Aa's sequence is variable across and within species (see Section 1.5.4 for discussion), a short sequence within one of its active regions is similar to that of egg-laying stimulatory hormones in the mollusc *Aplysia californica* that stimulate muscular contractions and behavioral changes, thus causing egg release (Scheller *et al.*, 1982; Kaldany *et al.*, 1985; Rothman *et al.*, 1986; Monsma and Wolfner, 1988; Heifetz *et al.*, 2000). The mollusc egg-laying hormones (califins, ELH), are cleaved from a prohormonal precursor, and Acp26Aa undergoes comparable proteolytic processing upon entry into female *D. melanogaster* (Monsma and Wolfner, 1988; Monsma *et al.*, 1990; Park and Wolfner, 1995).

Like most male accessory gland proteins in *D. melanogaster*, about 1/3 of a male's Acp26Aa is transferred to his mate (Monsma *et al.*, 1990). Transfer begins early (~3 min into a 20 min mating, before sperm start being transferred) and is largely complete by mid-mating. Once in the female, Acp26Aa targets to the base of the ovary (Heifetz *et al.*, 2000), and also enters the female's circulation (Monsma *et al.*, 1990). The region to which it targets at the ovary base is one at which changes occur in vesicle release and neuromodulator levels after mating (Heifetz and Wolfner, 2004; Y. Heifetz and M.F. Wolfner, unpublished data), suggesting the triggering, by mating, of muscle contractions that move the egg from the ovary into the oviducts. The targeting of Acp26Aa into the circulation gives it access to the neuroendocrine system, from which it could also modulate ovulation.

1.5.2.5.1.3. Egg deposition As noted earlier, in many insects oviposition has been used as a measure for the entire egg laying process. Several regulators of egg deposition that are products of male gonadal glands are discussed below. It is not yet known whether they directly modulate the efficiency of deposition of eggs onto the substratum, or regulate a step leading up to it.

Among Orthoptera, male gonadal glands have been shown to provide some stimulators of egg deposition. For example, the accessory glands of male migratory locusts (*L. migratoria*) produce a myotropic peptide that exhibits potent activity on the oviduct of conspecific females and cross-reactivity on the oviduct of the cockroach, *Leucophaea maderae* (Paemen *et al.*, 1991). It is proposed to stimulate oviposition, though the details of its action are as yet unknown. In migratory grasshoppers, *M. sanguinipes*, an oviposition-stimulating protein (OSP) is produced in the long hyaline tubules of the male's accessory glands. OSP is composed of two subunits with combined molecular mass is about 60 kDa (Yi and Gillott, 1999). When applied to the oviduct, it stimulates an increased frequency of oviduct contraction (Yi and Gillott, 2000).

1.5.2.5.2. Modifiers of hormonal levels in females An important way by which male gonadal gland secretions modulate the responses of mated females is by modulating the female's hormonal levels. Given the important roles of JH in female reproductive physiology (see above; **Chapters 1.3** and **3.9**), an effective way for the male to modulate his

mate's hormonal levels is to transfer to her JH or modulators of JH levels. This is accomplished in several ways in the insect world.

For example, the corpora allata (CA) of adult male saturniid moths, *Hyalophora cecropia*, synthesize JH acids which are then taken up by their accessory glands and methylated therein to JH-I and JH-II. Male accessory gland-derived JH-I and JH-II are subsequently transferred to the female reproductive tract during mating (Shirk *et al.*, 1980). The possibility exists of a similar strategy in the grasshopper *M. sanguinipes*. Extracts of male accessory glands, when injected into reproductively mature virgin females, induce oviposition a day later, similar to the effect of mating (Friedel and Gillot, 1976). A high-affinity JH-binding protein (see **Chapter 3.7**) with molecular mass of 40 kDa is present in the long hyaline tubules of accessory glands of male *M. sanguinipes* (Ismail and Gillott, 1994). Since JH-binding proteins facilitate the transport of JH, it is tempting to speculate that these proteins cause the sequestration of humoral JH into the accessory glands, allowing its transfer to females during mating and thus causing a stimulation of oogenesis.

In another strategy, several homologs of JH are synthesized *de novo* in the male accessory glands of the mosquito *A. aegypti* (Borovsky *et al.*, 1994). JH is necessary for oocytes to advance beyond an early (teneral) stage in development in nutritionally-deprived *A. aegypti* females. Topical application of JH to such females can bypass this arrest (Klowden and Chambers, 1991), as can implantation of a male accessory gland or injection of male accessory gland homogenates – presumably because they provide JH. Upon mating, then, a female can receive JH to mature her ovarian follicles to a stage competent to deposit yolk (yolk deposition requires that the female have taken a blood meal). A maturational change analogous to that occasioned by exogenous provision of JH (e.g., from the male) occurs following a sugar meal (Feinsod and Spielman, 1980); this meal apparently provides a way for the female to elevate her own production of JH, providing an alternative strategy to achieve a hormonal milieu supportive of further ovarian development.

As noted earlier (Section 1.5.2.5.1.1), *Drm*-SP can elevate JH levels in *D. melanogaster* CA incubated *in vitro*, leading to the suggestion that SP carries out a similar function *in vivo*, thereby stimulating oogenesis (Moshitzky *et al.*, 1996; Soller *et al.*, 1997, 1999). Studies of the effects of *Drm*-SP on *H. armigera* have suggested that male *H. armigera* might transfer a molecule related to *Drm*-SP that could increase JH levels in their mates (Fan *et al.*, 1999a, 1999b, 2000). Specifically, the CA of newly emerged *H. armigera* adults are functionally immature and require a day after eclosion to fully acquire competence to produce JH; this maturation can be hastened by addition of *Drm*-SP to the incubation media. At least the first five N-terminal amino acid residues of *Drm*-SP are essential for CA stimulation in both *Drosophila* and *Heliothis* (Fan *et al.*, 2000), whereas *Drm*-Dup99B, which differs from *Drm*-SP in its N-terminal region, does not cause allatal stimulation.

1.5.2.6. Mediators of Sperm Transfer, Storage, and/or Sperm Competition

Female insects store sperm after mating. For example, *D. melanogaster* females receive ~4000–6000 sperm in a mating and store ~1000 of these. *Drosophila melanogaster* females use up to ~50% of the sperm they have stored to fertilize eggs (see Gilbert, 1981; Neubaum and Wolfner, 1999b; Bloch Qazi *et al.*, 2003 for review). Insect sperm are stored in specialized organs (seminal receptacles and/or spermathecae; **Figure 1**) that branch out from the oviducts and bursa/uterus. The walls of these organs themselves (especially spermathecae) and/or glands in the female associated with these organs (e.g., female accessory glands, spermathecal glands) are thought to produce molecules that assist the sperm in staying viable in storage (see Section 1.5.3.2, below).

Storage of sperm by mated females is important for several reasons (see Neubaum and Wolfner, 1999b; Bloch Qazi *et al.*, 2003 for review). First, it gives a mated female an abundant supply of sperm with which to fertilize her eggs, even when males are not plentiful. Second, it allows the male's (as well as female's) progeny to be produced over a long period, which can provide some protection if conditions immediately following mating are not conducive to progeny or egg production. Third, the persistence of several post-mating behaviors is dependent upon the presence of sperm. As noted earlier, one mechanism for this has been suggested to be the binding to, and gradual release from, the sperm of male-derived gonadal gland products (Kubli, 1992; Liu and Kubli, 2003; Swanson, 2003). The sperm's role in prolonging behavioral effects after mating seems most obviously advantageous to the male in preventing his mate from additional mating. However, it also can be advantageous to the female, by allowing her to avoid costs of mating (see Section 1.5.4.2.1) including increased exposure to pathogens or toxic proteins transferred during the mating, risk of predation, and loss of foraging time. Finally, the storage of sperm by female insects, and the fact that many insects mate more than once despite the decreased propensity to mate after a first mating, can be an important

selective process. Specifically, there can be competition between the sperm of different males (Parker, 1970) or, alternatively, the female can exert choice (preference) to select for use the sperm of a more advantageous male (Eberhard, 1996).

In some insects, secretions from male gonadal glands are required (or presumably required) for transfer of sperm to the female. For example, in *T. molitor*, Happ and colleagues have shown that different portions of the male beetle's accessory gland synthesize proteins of different layers of the spermatophore, the structure in which sperm are transferred to females during mating (Grimnes *et al*., 1986). In another example, proteins made by conglobate glands (also called phallic glands; these glands are associated with the reproductive tracts of male *B. germanica*) have been suggested to contribute to the formation of the spermatophore by these male cockroaches. This suggestion is based on the temporal pattern of accumulation of these proteins, as well as the effect of JH on their accumulation and on reproductive maturation (Vilaplana *et al*., 1996). A role for male gonadal gland secretions in sperm transfer may not be universal, however. In *Drosophila* (a nonspermatophore-using insect), secretions of the male's accessory gland are not required for sperm transfer (Tram and Wolfner, 1999; Xue and Noll, 2000), although actions of products of other gonadal glands in this process have not been tested.

Even after sperm have been transferred to females, their dynamics can be affected by male gonadal gland secretions that were transferred with them. For example, experiments in *Drosophila* showed that the products of male reproductive tissues are important for sperm storage, utilization, or competition. The role of gonadal gland products in sperm storage is demonstrated by the severely impaired levels of storage of sperm transferred to *D. melanogaster* females in the near absence of male accessory gland proteins (Kalb *et al*., 1993; Tram and Wolfner, 1999). Two experiments demonstrated the role of male gonadal gland products in the utilization of stored sperm. First, female *D. pulchrella* mated to males of a different but related species of *Drosophila* had few or no progeny, but progeny-production could be rescued to some extent by injecting the females with extracts of reproductive organs from their conspecific males (Fuyama, 1983). Second, *D. melanogaster* females mated to males without accessory glands did not produce progeny until they were post-mated with (spermless) males that could provide accessory gland products (Xue and Noll, 2000). The role of gonadal gland proteins in sperm competition is shown by differences in sperm competition levels in matings with or without accessory gland function (Harshman and Prout, 1994) and by the association of alleles of some accessory gland protein genes with levels of sperm competitive ability (Clark *et al*., 1995).

Several *Drosophila* male gonadal gland products have been suggested to play a role in sperm storage. *Drm*-Acp36DE, a 912 amino acid novel glycoprotein produced in *D. melanogaster* male accessory glands, is essential for sperm storage by mated females (Wolfner *et al*., 1997; Neubaum and Wolfner, 1999a). Acp36DE enters the female's sperm-storage organs and binds to sperm; it does not enter the female's circulation (Bertram *et al*., 1996; Neubaum and Wolfner, 1999a; Bloch Qazi and Wolfner, 2003). Within the female's reproductive tract it also localizes to the anterior end of the mating plug, near the openings of the storage organs, and along the wall of the oviduct just above the openings of the sperm storage organs. Without Acp36DE, sperm are stored poorly (only ~10–15% as many as normal are stored; Neubaum and Wolfner, 1999a); similar to the number that are stored in the absence of all Acps (Tram and Wolfner, 1999), and post-mating effects that depend on stored sperm do not persist (Neubaum and Wolfner, 1999a). Acp36DE is needed to get sperm into storage, but not to maintain them there or release them from storage (Neubaum and Wolfner, 1999a; Bloch Qazi and Wolfner, 2003). The presence of Acp36DE from a prior mating appears to help subsequent sperm resist competition (Chapman *et al*., 2000).

These findings have led to several models for action of Acp36DE, involving corraling of sperm into a storage site, forming a trellis along which sperm can move, triggering changes in the storage organs that pull, or attract, sperm into them, or assisting sperm in bundling or in moving along a "trail" into storage (Bertram *et al*., 1996; Neubaum and Wolfner, 1999a; Bloch Qazi and Wolfner, 2003). Variation in sperm competition levels in wild caught *D. melanogaster* strains correlates with allelic variation at Acp36DE and four other genes encoding male accessory gland proteins (Acp26Aa/Ab, Acp29B, Acp53E, and Acp76A) (Clark *et al*., 1995). This suggests that the male proteins produced from all these loci may play important roles in the dynamics of sperm after transfer to the female. One additional male accessory gland protein, the protease inhibitor Acp62F, localizes to the sperm storage organs of a mated female. It has thus been suggested to contribute to the regulation of the proteolytic environment surrounding the sperm, and thereby to contribute to the activation of sperm or, alternatively, to protecting their surface

proteins from proteolysis (Lung *et al*., 2002). Finally, two products of the ejaculatory duct, esterase 6 and glucose dehydrogenase – also produced in the ejaculatory bulb (Cox-Foster *et al*., 1990) – have been suggested to play roles in sperm dynamics in mated females (Gromko *et al*., 1984; Iida and Cavener, 2004).

1.5.2.7. Modification Enzymes

Seminal fluid in insects, as in mammals, is rich in proteases (Swanson *et al*., 2001; Mueller *et al*., 2004) and protease inhibitors (Schmidt *et al*., 1989; Lung *et al*., 2002; Swanson *et al*., 2001; Mueller *et al*., 2004). Trypsin inhibitors have been identified among accessory gland proteins in *D. funebris* (Schmidt *et al*., 1989) and *D. melanogaster* (Lung *et al*., 2002), and six others are predicted by bioinformatic criteria (Swanson *et al*., 2001; Mueller *et al*., 2004); at least two (*Drm*-Acp62F and the predicted serpin *Drm*-Acp76A) are known to be transferred to the female during mating (Coleman *et al*., 1995; Wolfner *et al*., 1997; Lung *et al*., 2002). Six proteases also are predicted to be produced in, and secreted by, the male's accessory gland, based on EST screening and bioinformatic analysis (Swanson *et al*., 2001; Mueller *et al*., 2004). The roles of these enzymes have not been established, but several models have been proposed for their function, based in part on studies in mammals (He *et al*., 1999; Murer *et al*., 2001). These include action in, or regulation of, proteolytic cascades that activate or protect sperm, or prohormones, and protection of the female reproductive tract. Interestingly, at least one protease inhibitor (the *D. melanogaster* male-derived *Drm*-Acp62F) also enters the circulation of the female (Lung and Wolfner, 1999). Its presence there may be, counterintuitively, harmful: ectopic expression of this protein such that it is present in the circulation causes toxicity to *D. melanogaster* (see Section 1.5.4.2.1). Other modification enzymes found or predicted among the secretion of male gonadal glands include acid lipase activity found in the *D. melanogaster* male accessory gland (Smith *et al*., 1994), and at least six lipases predicted bioinformatically to be expressed in male accessory glands (Swanson *et al*., 2001; Mueller *et al*., 2004). Functions of these molecules are unknown, but they may provide an energy source for sperm.

1.5.3. Products of Female Gonadal Glands

Products derived from female gonadal glands are known or believed to play a variety of roles in fertility. These include facilitating the passage of eggs through the reproductive tracts, modulating the storage or maintenance of sperm, contributing to reproductive structures such as hardened plugs or hardened and tanned egg cases that place and protect eggs subsequent to deposition, and defending the reproductive tract or gametes from attack by microbes or other organisms. Products of female gonadal glands can also elicit long-term behavioral effects on filial generations (e.g., McCaffery and Simpson, 1998). Finally, female gonadal glands of certain hymenopterous parasitoids appear to produce substances that compromise their host's development or immune systems. These roles are discussed below.

Less is known at the molecular level about female gonadal gland secretions, as compared to male gonadal gland secretions. However, the positions of female gonadal glands can sometimes provide an indication of the likely functions of their products in reproduction. Gonadal glands located near the ovary likely produce secretions that facilitate the passage of eggs through the reproductive tract, and possibly the activation of those eggs. Gonadal glands located near the exterior opening of the reproductive tract are likely to be the source of molecules that coat the egg surface, or construct pods in which eggs are deposited. Products of gonadal glands located between these two regions likely facilitate the passage of sperm or eggs through the oviducts, and may also function in aiding sperm storage, and/or facilitate sperm-egg interaction.

1.5.3.1. Lubrication of Female Reproductive Tract

Three reproductive processes are likely to require lubrication of the insect female's reproductive tract. In each case a role for secretions of female gonadal glands has been postulated or demonstrated, although the biochemical nature of the lubricants has not yet been determined. First, lubrication may be needed to support the act of copulation. Such lubrication is one of the functions proposed for the secretion of female accessory glands of the yellow dung fly *Scathophaga stercoraria* (Hosken and Ward, 1999). Second, a supportive medium must be available to assist sperm in transiting through the uterus and oviducts into storage, and from storage into sites for fertilization. In addition, the composition and the viscosity of the medium could facilitate or inhibit sperm movement (Yanagimachi and Bhattacharyya, 1988; Moore *et al*., 2002). Given the location of the openings of the ducts of the female accessory glands (alternatively termed colleterial glands or parovaria) – close to

the sperm storage organs – it seems likely that these glands supply secretions that assist sperm movement within the oviduct and into storage. Studies in Lepidoptera, such as noctuid moths, support the idea that female accessory gland secretions provide lubrication that assists sperm movement through the reproductive tract into the spermathecal duct (Callahan and Cascio, 1963). Third, the smooth passage of an egg from ovary to deposition on the substratum is likely to be assisted by lubrication from secretions from female gonadal glands that facilitate movement of the large egg through the narrow-diameter oviducts and ovipositor. For example, in the cricket *T. commodus*, proteinaceous secretions from the female accessory glands accumulate after adult ecdysis, with kinetics that correlate with increased egg-deposition activity. These secretions have thus been suggested to function as a lubricant facilitating passage of eggs through the narrow ovipositor (Sturm, 2002). Similarly, proteinaceous secretions of female accessory glands (cement glands) have been suggested to play a lubricating role in the female *Rodnius prolixus* (Lococo and Huebner, 1980).

1.5.3.2. Maintenance of Sperm Viability and "Quality"

When they enter the female, sperm are in a male-provided supportive environment. However, once in the female, their continued survival likely depends on female-supplied molecules. After transfer to the female reproductive tract, sperm are retained in most insects in the sperm storage organs (spermathecae, seminal receptacles). Some of these organs appear to have secretory capacity (e.g., often, spermathecae); others are associated with secretory glands (e.g., spermathecal glands), producing lipoproteins, phospholipids, carbohydrates, and proteins (Davey and Webster, 1967; Bhatnagar and Musgrave, 1971; Filosi and Perotti, 1975; see Gillott, 1988). The secretions of these tissues may support the viability of stored sperm (Anderson, 1945) and help to maintain the sperm in an optimal state that maximizes their fertilization potential.

Several aspects of the storage situation could impact the viability or "quality" (fertilizing potential) of the sperm. First, as suggested from studies in non-insect systems (Visconti *et al*., 2002; Hirohashi and Vacquier, 2003), the ionic environment may be important. Female gonadal tissues have been suggested to assist in providing a supportive ionic environment in the honeybee *A. mellifera*. Spermathecal fluid of *A. mellifera* includes ions such as K^+, Na^+, Ca^{2+}, H^+, Cl^-, PO_4^{3-} (Gessner and Gessner, 1976). The ultrastructure of honeybee spermathecal glands suggest that they play a role in ion transport to maintain a physicochemical environment conducive to sperm viability (Kressin *et al*., 1996). In addition to providing a supportive ionic environment, other components of the gonadal glands might help to provide an optimal environment for sperm survival or maintenance. This role has been suggested for glucose dehydrogenase (Gld), given its expression in the female's sperm-storage organs (seminal receptacle and spermathecae) as well as the female accessory glands (Cox-Foster *et al*., 1990; Keplinger *et al*., 1996, 2001; Iida and Cavener, 2004).

Second, secretions of female gonadal tissues might provide energy sources for sperm motility and metabolism. Energy sources for sperm motility are likely initially provided by the male secretions that accompany the sperm into the female. Subsequently, the female must provide such molecules. For example, honeybee spermatozoa rapidly oxidize free sugars of the seminal plasma (Blum *et al*., 1967) and thereafter require a replacement energy source that may be provided by epithelial cells of the female spermathecal ducts. Dense aggregates of glycogen are found at the basal side of these cells in non-ovipositing queen bumblebees. Once egg deposition starts, the glycogen stores gradually move towards the apical side of the epithelium cells. Moreover, bumblebee spermatozoa metabolize glucose as an energy source shortly before fertilization (Seth *et al*., 2002), and sugars are present in the spermathecal duct of the bumblebee queen. In addition to energy-rich substrates, other contributors to sperm metabolism are provided by female gonadal glands. For example, in the tomato moth *Lacanobia oleracea*, angiotensin I-converting enzyme (ACE) activity is found in the male accessory glands, and in the spermatheca/bursa copulatrix of the female (Ekbote *et al*., 2003); some of the activity in the female may have been transferred from the male, but some is endogenous. It appears that the spermatophore/bursa copulatrix of mated females also produces an aminopeptidase, a carboxypeptidase, and a dipeptidase. The authors hypothesized that these peptidases might be involved in the breakdown of proteins from the spermatophore. Perhaps this could assist sperm motility. Since ACE activity is also high in the spermatheca, it is possible that ACE mediates proteolysis of energy sources in the spermatheca. Female accessory glands of the sand fly, *P. papatasi* contain significant lipase activity. A major protein component of their secretion is a lipase-like protein (*P. papatasi* lipase – *Phpa*-LIP) that shares a sequence similarity with mammalian lipases, including the presence of conserved catalytic residues (Rosetto *et al*., 2003a). Perhaps *Phpa*-LIP

might function in the utilization of male derived lipids that are transferred to females during mating, as suggested for *Drosophila* (Smith *et al.*, 1994). This, in turn, could provide an energy source for sperm, as well as, potentially, modifying the sperm to increase their fertilizing efficiency. Thus, by regulating the ionic environment and providing energy sources, female gonadal glands may assist in keeping sperm viable and motile when in storage.

1.5.3.3. Modulating Sperm–Egg Interaction

In addition to promoting the movement of gametes to sites where they could meet (Section 1.5.3.1), female gonadal gland secretions could promote sperm–egg interaction by modifying the sperm or egg to facilitate the penetration of the egg by the sperm. Three examples are consistent with such action.

Studies on the housefly *M. domestica* suggest that female accessory glands' products may modify the egg to allow more efficient penetration by sperm (Leopold and Degrugil, 1973). If the accessory glands are removed from mated female houseflies, the females' fertility is reduced to less than 1% of normal. Eggs deposited by these females rarely contain sperm, suggesting that female accessory gland secretions were needed either to allow sperm to reach the site of egg penetration or to penetrate the eggs. Subsequent work indicated that the secretion is involved in the disintegration of the micropylar cap of the egg (Degrugillier and Leopold, 1976; Leopold *et al.*, 1978; Degrugillier and Grosz, 1981), which could facilitate sperm entry. This dissolution of the micropylar cap is likely the result of hydrolytic enzymes produced in the female's accessory gland. Esterases, amidase, and trypsin-like activity have been found in this tissue (Leopold, 1981; Judd *et al.*, 1983). These enzymes also could potentially play a role in activating proteins on the sperm surface.

Female accessory gland products, such as two β-*N*-acetylhexosaminidase isoenzymes (HEX1 and HEX2) reported in *C. capitata*, have also been suggested to modify proteins on the micropyle's surface, perhaps to improve the binding of sperm to the micropyle (Marchini, 1989). Specific lectin binding sites are seen on the *C. capitata* egg's micropylar cap, at different stages in the egg production process (Dallai *et al.*, 1986). A lectin, *Dolichos biflorus* agglutinin, that is specific for terminal *N*-acetylhexosaminyl residues reacts with mature eggs obtained from ovaries but not with deposited eggs. It is possible, but not proven, that enzymes made by the female gonadal glands could mediate this change in micropylar surface sugars, perhaps to control the ability of sperm to penetrate the eggs.

1.5.3.4. Egg Protection – Physical Barriers

Secretions from female gonadal glands contribute to protective structures that enclose the eggs deposited by certain types of female insect and/or assist in inserting or attaching the eggs to the substratum.

Examples of protective structures include the egg-cases (oothecae) that enclose cockroach eggs and protect them from desiccation, predation, and parasitization. Depending on the biology of the cockroach species, females deposit egg-cases soon after they are produced, carry the egg cases protruding from their genitalia for a while prior to deposition, or carry the cases around until the neonate larvae hatch (Stay and Roth, 1962; Willis and Brunet, 1966; Adiyodi, 1968; Gillott, 1988). Egg cases are composed of structural proteins synthesized in the female accessory glands. When newly-formed, these egg-cases consist of a sticky substance, which hardens rapidly, and then tans (see **Chapter 4.4**). Female accessory glands of several cockroaches (e.g., *Periplaneta australasiae*, *B. discoidalis*) contain 3,4-dihydroxybenzoic acid (DOBA), which is thought to participate in the tanning process (Kramer *et al.*, 1991; Hopkins *et al.*, 1999). In *Nauphoeta cinerea*, a viviparous cockroach, two protein fractions from the left female accessory gland comprise the major structural proteins of the egg case. Their appearance and accumulation correlates with the time of ootheca formation. These proteins accumulate in females with maturing ovaries, and are absent, or present only in trace amounts, in post-ovulating females (Adiyodi, 1968). Reproductive cues also appear to regulate protein production in the left accessory gland of other cockroaches and in two cases, JH, or at least the CA, have been shown to stimulate the production of oothecal protein (*B. germanica*, Burns *et al.*, 1991; *P. americana*, Willis and Brunet, 1966).

Locust and mantid female accessory glands produce a foamy secretion that forms a protective sheath encasing the egg batches deposited by the female in the soil. Egg batches of the desert locust *S. gregaria* are embedded in an egg pod that is capped by hardened foam. The foam is derived from viscous proteinaceous material produced by the female accessory glands and lateral oviducts (Szopa, 1981). Components that induce tanning of the foam are produced in female gonadal glands. For example, the median glandular pouch, positioned near the posterior opening of *S. gregaria* oviduct, may produce a component involved in the frothing of the proteinaceous material (Szopa,

1981). Similarly, the female accessory (Comstock-Kellog glands) of the grasshopper *Romalea guttata* produce material that tans the eggs and hardens the froth in which the eggs are embedded (Eisner *et al*., 1966). Lastly, the female accessory glands/calyx of the grasshopper *M. saguinipes* produce catechols that participate in the process of tanning. DOBA and its precursor 3,4-dihydroxyphenylalanine (DOPA), respectively, are the major and minor catechols produced in the female accessory glands of females with fully developed eggs. Young females with undeveloped eggs or females that had recently oviposited have little or no DOBA (Hopkins *et al*., 1999).

In many insect species, ovipositing females attach their eggs to the substratum. Female accessory glands of these insects secrete adhesive material, including adhesive proteins and also, at least in *Chrysomya putoria* flies, polysaccharides, mucoproteins, and glycoproteins (Tirone and Avancini, 1997). The adhesive pastes the eggs, egg case, or ootheca to the substratum. The importance of the role of the accessory glands in producing this glue can be seen in the saturniid moth *Antheraea mylitta*. If accessory glands are removed from female moths just after mating, the moths deposit only "loose" eggs (e.g., not pasted to the substratum) (Chaudhuri and Sinha, 1994). The egg deposition rate in these moths is faster, presumably because there are no viscous glue proteins to slow the passage of loose eggs through the oviducts.

1.5.3.5. Egg Protection – Antimicrobial Molecules

As noted earlier (Section 1.5.2.3), during copulation and thereafter in the mated female reproductive tract, deposited eggs and stored sperm are potentially subject to microbial attack and, in the case of deposited eggs, also to attack by other organisms. In addition to antibacterial/antimicrobial/protective agents donated by the male in seminal fluid, molecules made in female gonadal glands can protect the female reproductive tract, gametes, or deposited eggs from infection or parasitization. Some of these molecules appear to be made constitutively in females, providing continual protection for a reproductive tract that is topologically open to the exterior.

For example, *C. capitata* female accessory glands produce at least four antibacterial peptides called ceratotoxins. Although eight distinct ceratotoxins gene sequences have been identified in *C. capitata* or in *C. rosa* (Marchini *et al*., 1995; Rosetto *et al*., 1996, 1999, 2003b), only four mature peptides (ceratotoxin A, B, C, and D) have been isolated in *C. capitata* (Rosetto *et al*., 1997). Ceratotoxins A, B, and C are effective against Gram-negative and Gram-positive bacteria and have lytic activity against human erythrocytes (Marchini *et al*., 1993; Rosetto *et al*., 1996, 2000). Ceratotoxins have sequence and structural similarities to other insect antimicrobial peptides such as cecropins and melittin (Boman and Hultmark, 1987), and to the amphibian defense peptide magainins and dermaseptin (Bevins and Zasloff, 1990; Nicolas and Mor, 1995). The production of ceratotoxins specifically expressed in glands of sexually mature females is enhanced by mating and modulated by JH (Marchini *et al*., 1995; Rosetto *et al*., 1996). Since ceratotoxins were found on the surface of deposited eggs, Marchini *et al*. (1991, 1997) hypothesize that the antimicrobial material delivered by the egg to the egg deposition site can block the attack of microbial agents and thus create an environment that increases the survival of eggs and larvae. Several antimicrobial peptides are also produced in *Drosophila* female reproductive tract tissues – cecropin, defensin, and drosomycin in the sperm-storage organs, and drosocin in the oviduct and ovarian calyx of *Drosophila* (Charlet *et al*., 1996; Tzou *et al*., 2002). However, to date none have been reported to be produced in female accessory glands.

1.5.3.6. Chemical Marking

Molecules produced by female gonadal glands can be placed onto the substratum, not only onto the surface of the deposited egg as in the case discussed in the above section. Once on the substratum these molecules can potentially exert effects, such as oviposition-deterrence or host-marking, phenomena seen in many phytophagous insects, e.g., the cigarette beetle, *Lasioderma serricorne* (Kohno *et al*., 1986; Imai *et al*., 1990); the cabbage seed weevil, *Ceutorhynchus assimilis* (Ferguson and Williams, 1991; Mudd *et al*., 1997); the orange-tip butterfly, *Anthocharis cardamines* (Dempster, 1992); and many tephritid flies (Straw, 1989). It has been suggested that such marking and subsequent avoidance of egg deposition sites by subsequent females can increase the survival rate of eggs deposited by a female (Anbutsu and Togashi, 2001).

In the Japanese pine sawyer, *Monochamus alternatus*, the spermathecal gland of females produces a jelly-like secretion that is deposited on oviposition sites. This secretion contains semiochemicals that deter conspecific females from depositing eggs at the same site (Anbutsu and Togashi, 2001). Similar mechanisms may be used by other insects, including some for which the semiochemicals originate from other reproductive organs, e.g., ovaries (Prokopy

et al., 1976; Zimmerman, 1979); or female accessory glands (Schoonhoven, 1990).

1.5.3.7. Behavior Modification

Female gonadal gland secretions can participate in mediating the post-mating changes that are triggered, when females receive substances from male gonadal glands. For example, a sensory bristle at the distal end of the spermathecal duct in female grasshoppers (*Gomphocerus rufus*) appears to function in signaling females to be nonreceptive. This signaling is triggered by mating, and specifically by secretions that the females receive from their mates' accessory glands' white secretory tubule 1 (WS), and that enter the female's spermathecal duct (Hartmann and Loher, 1996). Hartmann and Loher (1999) hypothesized that the WS proteins function as carriers of small molecules that are thus brought into the spermathecal lumen. Female-provided proteases, secreted from the spermathecal gland cells (Hartmann and Loher, 1999), are then hypothesized to liberate these small molecules, which then stimulate chemoreceptors on the sensory bristle, thus conferring female nonreceptive behavior.

1.5.3.7.1. Caste-dependent plasticity In the honeybee, *A. mellifera*, secretions of female's Dufours gland differ in composition between the queen and her cohort worker bees (Katzav-Gozansky *et al.*, 1997). A fertile queen's Dufours gland produces a blend of esters (queen pheromone) that control worker bee behavior (Katzav-Gozansky *et al.*, 2003; see **Chapter 3.13**). In a colony with a fertile queen, worker bees do not produce these esters; instead they produce the corresponding alcohols, although their Dufours glands can be stimulated to produce esters when incubated *in vitro* (Katzav-Gozansky *et al.*, 2000). However, if a queen is removed from a colony, stimulating ovary maturation in workers, the workers' Dufours glands now begin to produce esters *in vivo*. Thus, it appears that a gonadal gland of fertile queens produces esters that regulate the caste and ester production by workers in her colony. In the queen's physical or functional absence, workers can co-opt her social status and her gonadal glands' ability to produce these esters.

1.5.3.7.2. Transgenerational behavioral modification Locusts can exhibit a continuum of types of behaviors, ranging from being extremely solitary to being extremely gregarious. The accessory glands of ovipositing locust females produce a factor that affects the subsequent behavior of her offspring. This factor is a part of the froth enveloping the egg pod and composing the foam plug above the deposited egg batch. Ligation of the distal accessory glands of crowded females demonstrated that in the absence of the glandular material, the locust nymph behavior significantly shifted towards solitary behavior, compared to conspecific nymphs from control-treated females (Hägele *et al.*, 2000). The intensity of the phase characteristics of locusts is due not only to the population density of the current generation, but is intensified by the phase history of previous generations (see Uvarov, 1966). Depending on parental population density, isolated larvae are more typically solitary in morphometrics, coloration, and behavior and, conversely, crowded larvae are more typically gregarious. Enhanced cumulative (trans-generational) gregarious characteristics are attributed to a small (<3 kDa), hydrophilic labile "gregarizing" factor which is produced at the time of egg deposition and which predisposes neonate nymphs to attain characteristics of the gregarious phase (McCaffery and Simpson, 1998). The presence of three ketones: (*Z*)-6-octen-2-one, (*E*,*E*)-3,5-octadien-2-one, and (*E*,*Z*)-3,5-octadien-2-one – in the foam and in gregarious female accessory glands – has been reported (Malual *et al.*, 2001). They may be part of the repertoire of maternal effects resulting in gregarious locusts, but this requires further study.

1.5.3.8. Supporting Larval Development

In viviparous species of some Diptera, progeny larvae develop within the uterus of the female. Products of the female's accessory glands, called "milk glands" in these species, enhance female fertility by supporting the development of these larvae, apparently by furnishing the larvae with nutrients or other components needed for development. For example, in the tsetse flies *Glossina morsitans morsitans*, pregnant females develop an extensive tubular network of milk glands to mediate the transfer of nutrients to larvae developing within their uterus (Yan *et al.*, 2002). Chitinase (see **Chapter 4.3**) is among the proteins produced by these milk glands and can be detected in intrauterine larvae and pupae, although no mRNA for chitinase can be detected in these intrauterine larvae (Yan *et al.*, 2002), suggesting that they obtained their chitinase from their mother's milk glands. A major protein of the mother's milk glands is synthesized as her larvae molt to the second instar and protein levels decline rapidly as the larva matures. Osir *et al.* (1991) hypothesized that this protein could provide essential amino acids needed for the larva to form a puparium. Finally, Cmelik *et al.* (1969) found that the midgut of developing larvae also contained proteins, peptides, and free amino acids and lipids

(phospholipids, cholesterol, and triglycerides) derived from the mother's milk glands. It was not clear whether the lipids had been made in the milk glands or were derived from the fat body and later sequestered in the milk glands (see also Gillott, 1988).

1.5.3.9. Anti-Immune Molecules

Eggs deposited by parasitic wasps within lepidopteran larvae are accompanied by molecules and macromolecular complexes that co-opt the host's physiology to allow development of the parasitoid. Female gonadal glands of the parasitoid are the source of some of the host-modifiers that are injected into the host along with the parasitoid's eggs.

For example, secretions from the oviduct of the scelionid egg parasitoid *Telenomus heliothidi* appear to regulate, and halt, host development (Strand *et al*., 1980), perhaps by stimulating production of a family of growth blocking peptides (GBP) that possess diverse biological activities associated with immunological defense mechanisms and response to stress (Aizawa *et al*., 2001). Finally, Chelonine wasps usurp development and reproductive control of their immunologically compromised lepidopteran hosts by down-regulating host JH production, thereby inducing precocious metamorphosis and preventing reproductive maturation. These responses may be due to a variety of effectors introduced into the host larvae during deposition of the endoparasite egg that are reinforced subsequently by the parasitoid larva (Jones, 1996). For example, polydnaviruses (e.g., from *Cotesia congregata*) interfere with the host larval immune response against the deposited egg (see **Chapter 6.10**). These viruses replicate in the adult parasitoid's ovarian calyx cells (Savary *et al*., 1999). Their bioactivity is attributed to specific immunoevasive proteins (IEPs) in the virion surface coat. IEPs, which are encoded in the parasitoids genome, and are transcribed, translated, and secreted in the lateral oviduct (Tanaka *et al*., 2002). Thus, the parasite has evolved to take advantage of the synthetic capacity of gonadal glands and the ability of gonadal gland secretions to accompany the egg. Another ichneumonid endoparasitoid, *Venturia canescens*, inhibits permissive host hemolymph defense melanization against the parasitoid egg, by injecting a serpin from the ovarian calyx fluid (Beck *et al*., 2000).

1.5.4. Male/Female Interactions and Evolutionary Considerations

1.5.4.1. Male–Female Interactions at the Cellular and Molecular Level

Apart from whole-organism interaction between male and female insects, reproduction involves interaction between male- and female-derived molecules and cells. Perhaps the best known of these are the interactions between sperm and oocyte, which result in fertilization (see **Chapter 1.6**). Although in many organisms this interaction also activates the oocyte to support embryo development, in at least some insects sperm penetration is not necessary to activate oocytes, e.g., in *Pimpla turionellae* (Went and Krause, 1974) and other haplodiploid hymenopterans whose unfertilized eggs develop into males as well as in *D. melanogaster* (Doane, 1960; Mahowald *et al*., 1983; Page and Orr-Weaver, 1997; Heifetz *et al*., 2001b). Male–female cellular and molecular interactions include also those between female reproductive tract cells or molecules, and male-derived molecules or sperm cells. These interactions, many of which were discussed in previous sections of the chapter, modulate sperm storage, maintenance, and utilization, and regulate female reproductive physiology and behavior.

A final area of interaction is one that the authors hypothesize – a give-and-take between male and female that results in "priming" the female's reproductive tract for efficient fertility. It is suggested that efficient fertilization requires an oviduct environment, or "ecology," that best supports the final maturation of the egg and the sperm and promotes their union. In mammals, the female reproductive tract undergoes hormonally mediated cyclic modifications in morphology and secretion, resulting in an optimal environment for gametes and fertilization during the preovulatory period (e.g., Salamonsen *et al*., 1994). It is proposed that an analogous change might occur in insects, though in this case the hormonal triggers could be mating, sperm, or gonadal gene products from the male. This model is based on experiments in *Drosophila*. Virgin female *Drosophila* make, ovulate, and deposit eggs at a low rate; receipt of gonadal gland secretions from their mates stimulates the rate of these processes. Heifetz and Wolfner (2004) have shown that mating, and in some cases male accessory gland secretions, modulate the release of vesicle contents from peptidergic nerve termini innervating parts of the female reproductive tract. That these vesicles were ready for release before mating indicates that the female was physiologically "poised," and awaiting only mating signals to progress into an advanced reproductive state. Thus, it is proposed that mating, sperm, and male gonadal secretions trigger a final maturation of the female's reproductive capacity, switching her from a "virgin" physiological state to a "mated" physiological state (**Figure 2**). In *Drosophila*, such a sensitizing event presumably can occur in any mating, but for insects such as those moths that

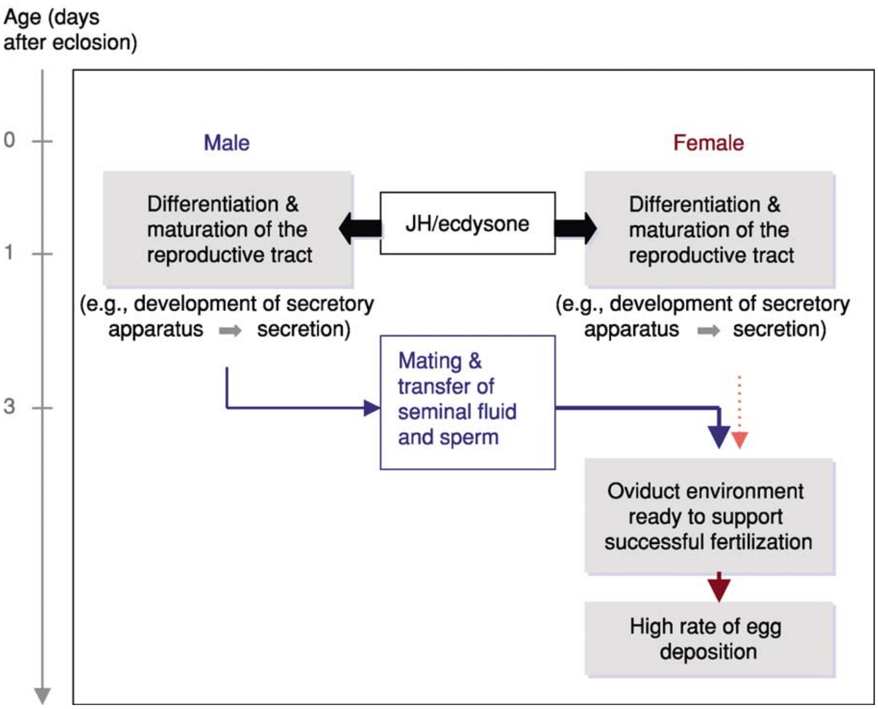


Figure 2 Model for conversion of female reproductive tract from virgin to mated state by male-donated effectors. See text for details.

mate only once in their lifetime, this sensitization only occurs once and its effects would then be "fixed" cellularly and/or molecularly.

1.5.4.2. Evolutionary Considerations

That male gonadal products trigger post-mating changes in the female would seem, on first consideration, to be advantageous to both the male and the female. For example, it would seem to be in both the female's and the male's interests to tie increased egg production to mating: the female would thus allocate resources to egg production only when sperm are present to fertilize her eggs, and the male would have more progeny, more rapidly. Although the male provides physiological modifiers that can modulate the female's reproductive capacity, the female is not a passive player in the reproductive interaction. Eberhard (1996) recounts many examples in which the female exerts control over the reproductive fate of her mate's gametes. In an example consistent with the *D. melanogaster* female genotype, the female influences the outcome of sperm competition (Clark and Begun, 1998). One could imagine that through natural selection, females have evolved strategies to "exploit" the male as a source of timed critical regulators of female physiology. For example, having the male provide the molecules that up-regulate egg production provides a fail-safe switch to couple changes in resource allocation to mating.

Yet, all may not be as cooperative as implied by part of the previous paragraph. Several experiments and theories posit that the interactions between males and females also have the potential to reflect opposing interests (e.g., Rice, 1996; Holland and Rice, 1999; Rice and Chippindale, 2001; review: Chapman *et al*., 2003a). For example, it may be to the male's advantage to induce his mate to produce more eggs after mating than her long-term well-being can tolerate. This could drive the evolution of female-resistance to the male inducers of increased egg-production, keeping the effects to tolerable levels for females. This might in turn select for males who can make more potent inducers, driving additional changes in the inducing molecules, whereas, theoretically, strict monogamy, whether by choice or enforced experimentally, should lead to evolutionary harmony of sexual interaction. Deviation from this state could increase the potential for conflict between the interests of the male and of the female, that could reinforce inter-sexual antagonistic coevolution (Hosken *et al*., 2001). Another potential example of conflicts between males and females is the "cost-of-mating," discussed in a separate section below. Finally there can be other pressures driving the evolution of molecules in reproductive tracts, such as co-evolution of protective molecules with pathogen types that are introduced during mating.

An unusually high proportion of *Drosophila* male accessory gland proteins and the genes that encode them show evolutionary dynamics that are consistent with these models (Coulthart and Singh, 1988; Aguadé *et al*., 1992; Cirera and Aguadé, 1997, 1998a, 1998b; Tsaur and Wu, 1997; Aguadé, 1998, 1999; Tsaur *et al*., 1998, 2001; Ravi Ram and Ramesh, 1999, 2001; Begun *et al*., 2000; Swanson *et al*., 2001). For example *Drm*-Acp26Aa, the ovulation hormone described previously, is the most rapidly-evolving gene identified to date in *Drosophila* (Tsaur and Wu, 1997; Aguadé, 1998; Tsaur *et al*., 1998, 2001). In another example, the percent of predicted *Drosophila* male accessory gland proteins that show elevated rates of nonsynonymous amino acid substitutions is ten-fold higher than for control, nonreproductive genes (Swanson *et al*., 2001). Evolutionary modulation also has occurred in terms of the expression of the enzyme glucose dehydrogenase. This enzyme is expressed in the ejaculatory ducts of some *Drosophila* species but not others, and its expression there correlates with the morphology of the ejaculatory duct (Cavener, 1985).

One outcome of sexual conflict can be redundancy or degeneracy, the latter being the presence of totally different molecules serving the same function (Edelman and Gally, 2001). As males evolve multiple effectors to compensate for those to which females have become resistant, redundancy and degeneracy can also shield essential female processes from complete cessation if one of their male effectors changes so much that its effectiveness is lost. Because of redundant or degenerate effectors, loss of one effector of an essential reproductive processes could decrease fertility levels but not cause sterility. This is in fact what is observed in *D. melanogaster*: all knockouts/knockdowns of specific male accessory gland proteins retained some residual fertility (Herndon and Wolfner, 1995; Neubaum and Wolfner, 1999a; Heifetz *et al*., 2000; Chapman *et al*., 2003b; Liu and Kubli, 2003). That this is likely due to redundant/degenerate effectors is perhaps best illustrated for the sex peptide *Drm*-SP. Females mated to males that lack this molecule still increase their egg production a bit after mating (Chapman *et al*., 2003b; Liu and Kubli, 2003). This likely reflects the continued availability of *Drm*-Dup99B (Saudan *et al*., 2002) and Acp26Aa (Herndon and Wolfner, 1995; Heifetz *et al*., 2000), as well as potentially of other modulators of the egg production process in the seminal fluid of SP-deficient males. Another way to shield complex multi-step processes from cessation, if their sole male-provided regulator became too divergent to function, is to have them regulated by multiple effectors that act at separate steps. This could also allow "fine-tuning" at several levels to allow the process to be at optimal efficiency (Heifetz *et al*., 2000; Bloch Qazi *et al*., 2003). In mated female *D. melanogaster*, several reproductive processes are indeed regulated at different steps by male gonadal gland products. For example, SP stimulates oogenesis (Soller *et al*., 1997, 1999) whereas Acp26Aa increases ovulation rate (Heifetz *et al*., 2000; Chapman *et al*., 2001). Thus, these two molecules could act synergistically to increase egg-production.

The picture may be still more complicated than this, however, because in species that mate multiple times there can be competition among males. For example, in *Drosophila* females that mate more than once, sperm from the first mating can be competed, or displaced, by sperm from a subsequent mating. Females can play a role in this, by choosing sperm from a preferred mate (i.e., sperm preference; Eberhard, 1996; Clark and Begun, 1998), but there also appears to be a role for male gonadal secretions in the competition between sperm of different males (Harshman and Prout, 1994; Clark *et al*., 1995; Price, 1997; Chapman *et al*., 2000). Thus, the possibility exists for evolutionary pressures to drive changes in seminal proteins that mediate male–male interactions within the females with which they had mated.

1.5.4.2.1. Cost-of-Mating Mating of animals can impose a cost on their longevity. In some cases, the longevity of both sexes is decreased because there are trade-offs in resource allocation between up-regulated reproductive functions and, for example, immunity, and these trade-offs can reduce the overall fitness of a mated animal (Rolff and Siva-Jothy, 2002). But of particular relevance to the topic of this article is the observation that mated female *D. melanogaster* and *C. capitata* die sooner than equivalent females who have not mated (Partridge *et al*., 1987; Fowler and Partridge, 1989; Partridge and Fowler, 1990; Chapman *et al*., 1995, 1998). While some cost is extracted on *D. melanogaster* females simply by exposure to males, perhaps reflecting behavior-related "costs" analogous to those that shorten the lives of mated male insects (e.g., *Drosophila* (Cordts and Partridge, 1996); versus "horned" and "unhorned" dung beetles *Onthophagus binodis* (Kotiaho and Simmons, 2003)), two components of the cost-of-mating to female *D. melanogaster*, relate to processes linked directly or indirectly to seminal proteins.

The first is that mating results in increased egg production as noted above, due in part to transfer of male gonadal products that stimulate

egg production in females. This expenditure depresses the lifespan of the mated female: energy expended to make eggs cannot also be used to sustain life. In the Mediterranean fruit fly, *C. capitata* and in the adzuki bean beetle, *Callosobruchus chinensis*, mating and egg production are also seen to impose independent costs (Chapman *et al.*, 1998; Yanagi and Miyatake, 2003).

The second effect of male-derived proteins on female longevity is seen, when experiments are conducted so as to remove the contribution of egg production to longevity (Chapman *et al.*, 1995). In this case, a significant effect of products of the male's accessory gland is seen to modulate the longevity of mated females. Whether males have evolved to extract a cost-of-mating from their mates (Johnstone and Keller, 2000), thus potentially limiting their mates' potential for future matings with competitor males, or whether the cost-of-mating is an unintended consequence of a deleterious by-product of an essential reproductive activity provided by males (Lung *et al.*, 2002), is an open and pressing question.

The molecular identity of the gonadal gland products that contribute to the cost of mating is as yet unknown. In *D. melanogaster*, genetic or RNAi experiments indicate that the ovulation hormone *Drm*-Acp26Aa, and the sperm storage protein *Drm*-Acp36DE do not contribute to this effect (Neubaum and Wolfner, 1999a; Chapman *et al.*, 2000, 2001). A candidate contributor to the cost of mating in this insect is the trypsin inhibitor Acp62F. This protein, derived from the male's accessory glands, enters the circulation of the mated female. It is toxic to flies, upon ectopic expression (Lung *et al.*, 2002). Perhaps after entry into the female's circulation Acp62F inhibits essential proteolytic cascades, causing a deleterious effect on the female. However, whether its toxicity is in fact the cause of the cost of mating remains to be demonstrated.

1.5.5. Future Perspectives and Practical Considerations

The analysis of insect reproduction in general, and of gonadal glands and their products in particular, is of broad significance in basic biology – not simply reproductive biology – and has practical applications. The vast diversity in the structures and functions of gonadal glands, and in the ways that different insects exploit these to their own ends, provides windows into the control and plasticity of physiology and behavior. That gonadal gland products from the male cause physiological changes in their mates provides tractable entry points (and zero-time-points) from which to study and modulate hormonal processes in the female reproductive tract, an advantage over studying physiology in a steady state. In another example, the fact that some gonadal gene products mediate cell targeting (e.g., sperm migration into storage), cell interaction (e.g., egg movement down the reproductive tract) and other basic processes, means that their elucidation may provide generally-applicable paradigms.

Studies of insect reproductive strategies and the molecular interactions that modulate them may provide information for evaluation of evolutionary models, and for the study of metabolic and regulatory pathways. For example, perhaps there are conserved pathways that regulate physiological events in female insects – but their proximal triggers have evolved to become divergent. Conversely, despite diversity in mating strategies and in pre- or post-mating behavior, some gonadal gene products cross-react across a range of insects (e.g., *Drm*-SP in *D. melanogaster* and *H. armigera*). In these cases, homologous bioactive peptides and proteins may act to similar ends and even by similar means, but within the context of markedly differing reproductive strategies of the different insect species. For example, although tephritid females show changes in sensory behavior post-mating, such as orienting to fruit for egg deposition rather than to leks for mate-choice, whereas noctuid moths show a cessation of pheromone production after mating, suggesting a change in pheromone biosynthetic pathways, it is possible that homologous effectors from their mates' gonadal glands could trigger the changes that led to these responses. Moreover, the outcomes are similar: in both cases, the net result is that priorities are affected and subsequent mating is deferred. Finally, as noted earlier, a significant number of *Drosophila* gonadal gene products show signs of having been subject to positive selection. Indeed, some are therefore a rich source of novel protein sequences and structures, and experiments to determine their function will assist in understanding these dynamics. As more genomic sequences are determined and analytic methodologies improve, it will be interesting to test the generality of this conclusion. There is evidence already for the rapid evolution of reproductive proteins in a number of systems (Swanson and Vacquier, 2003).

For insects in which the molecules produced by gonadal glands are known, or in the process of becoming known, the challenge and excitement will be to identify their specific functions and how those are carried out. In other insects, the functions of gonadal glands and their products are a mystery awaiting elucidation. In either case, ultimately, the

identification of the functions of insect gonadal glands will be important for practical reasons as well as their intrinsic interest. Insects are extremely prolific, and they include species that are very important agricultural helpers (e.g., honeybees) or pests (e.g., locusts or armyworms) and also play important roles as vectors of several devastating human diseases (e.g., some mosquitoes). Thus, under some circumstances, it can be important to find ecologically-rational, nonchemical methods of controlling the reproduction of particular insects. With the important role that gonadal glands and their products play in insect reproduction, it seems likely that they will be among the most important areas of investigation to exploit in order to attain their use in biological control.

Acknowledgments

We thank M. Bloch Qazi, Y. Gottlieb, H. Hagedorn, J. Mueller, K. Ravi Ram, and A. Wong for helpful suggestions. We appreciate support from NIH (grant HD38921 to MFW) and BARD (grant IS-3492-03 to YH), as well as ISF (grant 486/01-17 to SWA) and the Israel Ministry of Agriculture (grant 824-0100-03 to YH and SWA).

The authors wish to dedicate this chapter to the memory of Dr David Applebaum and Ms. Nava Applebaum.

References

Adiyodi, K.G., **1968**. Left colleterial proteins in the viviparous cockroach *Nauphoeta cinerea*. *J. Insect Physiol. 14*, 309–316.

Adiyodi, K.G., Adiyodi, R.G., **1975**. Morphology and cytology of the accessory sex glands in invertebrates. *Int. Rev. Cytol. 43*, 353–398.

Aguadé, M., **1998**. Different forces drive the evolution of the *Acp26Aa* and *Acp26Ab* accessory gland genes in the *Drosophila melanogaster* species complex. *Genetics 150*, 1079–1089.

Aguadé, M., **1999**. Positive selection drives the evolution of the Acp29AB accessory gland protein in *Drosophila*. *Genetics 152*, 543–551.

Aguadé, M., Miyashita, N., Langley, C., **1992**. Polymorphism and divergence in the *Mst26A* male accessory gland gene region in *Drosophila*. *Genetics 132*, 755–770.

Aigaki, T., Fleischmann, I., Chen, P.S., Kubli, E., **1991**. Ectopic expression of sex peptide alters reproductive behavior of female *D. melanogaster*. *Neuron 7*, 557–563.

Aizawa, T., Hayakawa, Y., Ohnishi, A., Fujitani, N., Clark, K.D., *et al.*, **2001**. Structure and activity of the insect cytokine growth-blocking peptide. Essential regions for mitogenic and hemocyte-stimulating activities are separate. *J. Biol. Chem. 276*, 31813–31818.

Anbutsu, H., Togashi, K., **2001**. Oviposition deterrent by female reproductive gland secretion in Japanese pine sawyer, *Monochamus alternatus*. *J. Chem. Ecol. 27*, 1151–1161.

Anderson, R.C., **1945**. A study of the factors affecting fertility of lozenge females of *Drosophila melanogaster*. *Genetics 30*, 280–296.

Andersson, J., Borg-Karlson, A.K., Wiklund, C., **2000**. Sexual cooperation and conflict in butterflies: a male-transferred anti-aphrodisiac reduces harassment of recently mated females. *Proc. R. Soc. Lond. B Biol. Sci. 267*, 1271–1275.

Andersson, J., Borg-Karlson, A.K., Wiklund, C., **2003**. Antiaphrodisiacs in pierid butterflies: a theme with variation! *J. Chem. Ecol. 29*, 1489–1499.

Ando, T., Kasuga, K., Yajima, Y., Katoaka, H., Suzuki, A., **1996**. Termination of sex pheromone production in mated females of the silkworm moth. *Arch. Insect Biochem. Physiol. 31*, 207–218.

Arms, K., Feeny, P., Lederhouse, R.C., **1974**. Sodium: stimulus for puddling behavior by tiger swallowtail butterflies, *Papilio glaucus*. *Science 185*, 372–374.

Arnqvist, G., Nillson, T., **2001**. The evolution of polyandry: multiple mating and female fitness in insects. *Anim. Behav. 60*, 145–164.

Baer, B., Maile, R., Schmid-Hempel, P., Morgan, E.D., Jones, G.R., **2000**. Chemistry of a mating plug in bumblebees. *J. Chem. Ecol. 26*, 1869–1875.

Bairati, A., Perotti, M.E., **1970**. Occurence of a compact plug in the genital tract of *Drosophila* females after mating. *Drosophila Inf. Serv. 45*, 67–68.

Bartelt, R.J., Schaner, A.M., Jackson, L.L., **1985**. *cis*-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol. 11*, 1747–1756.

Bastock, M., Manning, A., **1955**. The courtship of *Drosophila melanogaster*. *Behavior 8*, 85–111.

Baumann, H., **1974a**. Biological effects of paragonial substances PS-1 and PS-2, in females of *Drosophila funebris*. *J. Insect Physiol. 20*, 2347–2362.

Baumann, H., **1974b**. The isolation, partial characterization, and biosynthesis of paragonial substances, PS-1 and PS-2 of *Drosophila funebris*. *J. Insect Physiol. 20*, 2181–2194.

Baumann, H., Wilson, K.J., Chen, P.S., Humbel, R.E., **1975**. The amino-acid sequence of a peptide (PS-1) from *Drosophila funebris*: a paragonial peptide from males which reduces the receptivity of the female. *Eur. J. Biochem. 52*, 521–529.

Beck, M., Theopold, U., Schmidt, O., **2000**. Evidence for serine protease inhibitor activity in the ovarian calyx fluid of the endoparasitoid *Venturia canescens*. *J. Insect Physiol. 46*, 1275–1283.

Begun, D.J., Whitley, P., Todd, B.L., Waldrip-Dail, H.M., Clark, A.G., **2000**. Molecular population genetics of male accessory gland proteins in *Drosophila*. *Genetics 156*, 1879–1888.

Bertram, M., Akerkar, G.A., Ard, R.L., Gonzalez, C., Wolfner, M.F., **1992**. Cell type specific gene expression in the *Drosophila melanogaster* male accessory gland. *Mech. Devel. 38*, 33–40.

Bertram, M.J., Neubaum, D.M., Wolfner, M.F., **1996**. Localization of the *Drosophila* accessory gland protein Acp36DE in the mated female suggests a role in sperm storage. *Insect Biochem. Mol. Biol. 26*, 971–980.

Bevins, C.L., Zasloff, M., **1990**. Peptides from frog skin. *Annu. Rev. Biochem. 59*, 395–414.

Bhatnagar, R.D., Musgrave, A.J., **1971**. Aspects of histophysiology of the spermathecal gland of *Sitophilus granarius* (L.) (Coleoptera). *Can. J. Zool. 49*, 275–277.

Black, P.N., Landers, M.H., Happ, G.M., **1982**. Cytodifferentiation in the accessory glands of *Tenebrio molitor.* VIII. Crossed immunoelectrophoretic analysis of terminal differentiation in the postecdysial tubular accessory glands. *Devel. Biol. 94*, 106–115.

Bloch Qazi, M.C., Heifetz, Y., Wolfner, M.F., **2003**. The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster. Devel. Biol. 256*, 195–211.

Bloch Qazi, M.C., Wolfner, M.F., **2003**. An early role for the *Drosophila melanogaster* male seminal protein Acp36DE in female sperm storage. *J. Exp. Biol. 206*, 3521–3528.

Blomquist, G.J., Tillman, J.A., Reed, J.R., Peide Gu, P., Vanderwel, D., *et al.*, **1995**. Regulation of enzymatic activity involved in sex pheromone production in the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol. 25*, 751–757.

Blum, M.S., Bumgarner, J.E., Taber, S., **1967**. Composition and possible significance of fatty acids in lipid classes in honey bee semen. *J. Insect Physiol. 13*, 1301–1308.

Blum, M.S., Hermann, H.R., **1978**. Venons and venom apparatuses of the Formicidae. In: Bettini, S. (Ed.), Arthropod Venoms. Springer, Berlin, pp. 801–894.

Boman, H.G., Hultmark, D., **1987**. Cell-free immunity in insects. *Annu. Rev. Microbiol. 41*, 103–126.

Borovsky, D., Carlson, D.A., Hancock, R.G., Rembold, H., Van Handel, E., **1994**. *De novo* biosynthesis of Juvenile hormone III and I by the accessory glands of the male mosquito. *Insect Biochem. Mol. Biol. 24*, 437–444.

Boulétreau-Merle, J., **1974**. Stimulation de l'ovogenèse par la copulation chez les femelles de *Drosophila melanogaster* privée de leur complexe endocrine rétrocérebrale. *J. Insect Physiol. 20*, 2035–2041.

Braun, R.P., Wyatt, G.R., **1995**. Growth of the male accessory gland in adult locusts: Roles of juvenile hormone, JH esterase, and JH binding proteins. *Arch. Insect Biochem. Physiol. 30*, 383–400.

Brown, A.E., Bugeon, L., Crisanti, A., Catteruccia, F., **2003**. Stable and heritable gene silencing in the malaria vector *Anopheles stephensi*. *Nucleic Acids Res. 31*, 85.

Brown, M.J.F., Baer, B., Schmid-Hempel, R., Schmid-Hempel, P., **2002**. Dynamics of multiple mating in the bumble bee *Bombus hypnorum*. *Insectes Soc. 49*, 315–319.

Bullini, L., Coluzzi, M., Bianchi-Bullini, A.P., **1976**. Biochemical variants in the study of multiple insemination in *Culex pipiens* L. (Diptera: Culicidae). *Bull. Ent. Res. 65*, 683–685.

Burns, E.L., Chiang, A.S., Gadot, M., Schal, C., **1991**. Juvenile hormone regulation of the left colleterial gland in intact and ovariectomized *Blattella germanica* (L.) (Dictyoptera: Blattellidae). *J. Insect Physiol. 37*, 401–405.

Butterworth, F.M., **1969**. Lipids of *Drosophila*: a newly detected lipid in the male. *Science 163*, 1356–1357.

Callahan, P.S., Cascio, T., **1963**. Histology of the reproductive tract and transmission of sperm in corn earworm, *Heliothis zea*. *Ann. Entomol. Soc. America 56*, 535–556.

Carlisle, J.A., Loughton, B.G., **1979**. Adipokinetic hormone inhibits protein synthesis in locusts. *Nature 282*, 420–421.

Cavener, D.R., **1985**. Coevolution of the glucose dehydrogenase gene and the ejaculatory duct in the genus *Drosophila*. *Mol. Biol. Evol. 2*, 141–149.

Cavener, D.R., **1987**. Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Res. 15*, 1353–1361.

Cavener, D.R., MacIntyre, R.J., **1983**. Biphasic expression and function of glucose dehydrogenase in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 80*, 6286–6288.

Chapman, K.B., Wolfner, M.F., **1988**. Determination of male-specific gene expression in *Drosophila* accessory glands. *Devel. Biol. 126*, 195–202.

Chapman, T., **2001**. Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity 87*, 511–521.

Chapman, T., Arnqvist, G., Bangham, J., Rowe, L., **2003a**. Sexual conflict trends. *Ecol. Evol. 18*, 41–47.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., *et al.*, **2003b**. The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. *Proc. Natl Acad. Sci. USA 100*, 9923–9928.

Chapman, T., Herndon, L.A., Heifetz, Y., Partridge, L., Wolfner, M.F., **2001**. The Acp26Aa seminal fluid protein is a modulator of early egg-hatchability in *Drosophila melanogaster. Proc. R. Soc. Lond. 268*, 1647–1654.

Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F., Partridge, L., **1995**. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature 373*, 241–244.

Chapman, T., Miyatake, T., Smith, H.K., Partridge, L., **1998**. Interactions of mating, egg production and death rates in females of the Mediterranean fruit fly, *Ceratitis capitata*. *Proc. R. Soc. Lond. B 265*, 1879–1894.

Chapman, T., Neubaum, D.M., Wolfner, M.F., Partridge, L., **2000**. The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster. Proc. R. Soc. Lond. 267*, 1097–1105.

Charlet, M., Lagueux, M., Reichhart, J.M., Hoffmann, D., Braun, A., *et al.*, **1996**. Cloning of the gene encoding the

antibacterial peptide drosocin involved in *Drosophila* immunity. Expression studies during the immune response. *Eur. J. Biochem. 241*, 699–706.

Chaudhuri, A., Sinha, A.K., **1994**. Colleterectomy and its impact on some reproductive behaviour of the tropical Tasar silk moth, *Antheraea mylitta* (Drury) (Lepidoptera: Saturniidae). *Invertebr. Reprod. Devel. 26*, 145–152.

Chen, P.S., **1984**. The functional morphology and biochemistry of insect male accessory glands and their secretions. *Ann. Rev. Entomol. 29*, 233–255.

Chen, P.S., **1991**. Biochemistry and molecular regulation of the male accessory gland secretions in *Drosophila* (Diptera). *Ann. Soc. ent. Fr. 27*, 231–244.

Chen, P.S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M., *et al.*, **1988**. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster. Cell 54*, 291–298.

Christiansen, A.E., Keisman, E.L., Ahmad, S.M., Baker, B.S., **2002**. Sex comes in from the cold: the integration of sex and pattern. *Trends Genet. 18*, 510–516.

Cirera, S., Aguade, M., **1997**. Evolutionary history of the sex-peptide (Acp70A) gene region in *Drosophila melanogaster. Genetics 147*, 189–197.

Cirera, S., Aguade, M., **1998a**. Molecular evolution of a duplication: the sex-peptide (Acp70A) gene region of *Drosophila subobscura* and *Drosophila madeirensis. Mol. Biol. Evol. 15*, 988–996.

Cirera, S., Aguade, M., **1998b**. The sex-peptide gene (Acp70A) is duplicated in *Drosophila subobscura. Gene 210*, 247–254.

Clark, A.G., Aguadé, M., Prout, T., Harshman, L.G., Langley, C.H., **1995**. Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster. Genetics 139*, 189–201.

Clark, A.G., Begun, D.J., **1998**. Female genotypes affect sperm displacement in *Drosophila. Genetics 149*, 1487–1493.

Cmelik, S.H.W., Bursell, E., Slack, E., **1969**. Composition of the gut contents of third instar tsetse larvae (*Glossina morsitans* Westwood). *Comp. Biochem. Physiol. 29*, 447–453.

Coleman, S., Drahn, B., Petersen, G., Stolorov, J., Kraus, K., **1995**. A *Drosophila* male accessory gland protein that is a member of the serpin superfamily of proteinase inhibitors is transferred to females during mating. *Insect Biochem. Mol. Biol. 25*, 203–207.

Cordts, R., Partridge, L., **1996**. Courtship reduces longevity of male *Drosophila melanogaster. Anim. Behav. 52*, 269–278.

Coulthart, M.B., Singh, R.S., **1988**. Differing amounts of genetic polymorphism in testes and male accessory glands of *Drosophila melanogaster* and *Drosophila simulans. Biochem. Genet. 26*, 153–164.

Cox-Foster, D.L., Schonbaum, C.P., Murtha, M.T., Cavener, D.R., **1990**. Developmental expression of the glucose dehydrogenase gene in *Drosophila melanogaster. Genetics 124*, 873–880.

D'Rozario, A.M., **1942**. On the development and homologies of the genitalia and their ducts in Hymenoptera. *Trans. Roy. Entomol. Soc. Lond. 92*, 363–415.

Dallai, R., Marchini, D., Callaini, G., Fanciulli, P.P., Del Bene, G., *et al.*, **1986**. The egg structure of the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae). *Redia 69*, 313–327.

Davey, K.G., Webster, G.F., **1967**. Structure and secretion of spermatheca of *Rhodnius prolixus* Stal – a histochemical study. *Can. J. Zool. 45*, 653.

Degrugillier, M.E., Grosz, S.G., **1981**. Effects of female accessory gland ablation on fertility of screwworms (*Cochliomyia hominivorax*), stable flies (*Stomoxys calcitrans*) and face flies (*Musca autumnalis*). *Ann. Entomol. Soc. 74*, 217–221.

Degrugillier, M.E., Leopold, R.A., **1976**. Ultrastructure of sperm penetration of house fly eggs. *J. Ultrastruct. Res. 56*, 312–325.

Delisle, J., Picimbon, J., Simard, J., **2000**. Regulation of pheromone inhibition in mated females of *Choristoneura fumiferana* and *C. rosaceana. J. Insect. Physiol. 46*, 913–921.

Dempster, J.P., **1992**. Evidence of an oviposition-deterring pheromone in the orange-tip butterfly, *Anthocharis cardamines* (L). *Ecol. Entomol. 17*, 83–85.

DiBenedetto, A.J., Harada, H.A., Wolfner, M.F., **1990**. Structure, cell-specific expression, and mating-induced regulation of a *Drosophila melanogaster* male accessory gland gene. *Devel. Biol. 139*, 134–148.

DiBenedetto, A.J., Lakich, D.M., Kruger, W.D., Belote, J.M., Baker, B.S., *et al.*, **1987**. Sequences expressed sex-specifically in *Drosophila melanogaster* adults. *Devel. Biol. 119*, 242–251.

Ding, Z., Haussmann, I., Ottiger, M., Kubli, E., **2003**. Sex-peptides bind to two molecularly different targets in *Drosophila melanogaster* females. *J. Neurobiol. 55*, 372–384.

Doane, W.W., **1960**. Completion of meiosis in uninseminated eggs of *Drosophila melanogaster. Science 132*, 677–678.

Dussourd, D.E., Harvis, C.A., Meinwald, J., Eisner, T., **1991**. Pheromonal advertisement of a nuptial gift by a male moth (*Utetheisa ornatrix*). *Proc. Natl Acad. Sci. USA 88*, 9224–9227.

Dussourd, D.E., Ubik, K., Harvis, C., Resch, J., Meinwald, J., *et al.*, **1988**. Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix. Proc. Natl Acad. Sci. USA 85*, 5992–5996.

Duvoisin, N., Baer, B., Schmid-Hempel, P., **1999**. Sperm transfer and male competition in the bumblebee *Bombus terrestris. Anim. Behav. 58*, 743–749.

Eberhard, W.G., **1996**. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, NJ.

Edelman, G.M., Gally, J.A., **2001**. Degeneracy and complexity in biological systems. *Proc. Natl Acad. Sci. USA 98*, 13763–13768.

Eisner, T., Eisner, M., Rossini, C., Iyengar, V.K., Roach, B.L., *et al.*, **2000**. Chemical defense against predation in an insect egg. *Proc. Natl Acad. Sci. USA 97*, 1634–1639.

Eisner, T., Shepherd, J., Happ, G.M., **1966**. Tanning of grasshopper eggs by an exocrine secretion. *Science 152*, 95–97.

Eisner, T., Smedley, S.R., Young, D.K., Eisner, M., Roach, B., *et al.*, **1996**. Chemical basis of courtship in a beetle (*Neopyrochroa flabellata*): Cantharidin as "nuptial gift" *Proc. Natl Acad. Sci. USA 93*, 6499–6503.

Ekbote, U.V., Weaver, R.J., Isaac, R.E., **2003**. Angiotensin I-converting enzyme (ACE) activity of the tomato moth, *Lacanobia oleracea*: changes in levels of activity during development and after copulation suggest roles during metamorphosis and reproduction. *Insect Biochem. Mol. Biol. 33*, 989–998.

Eliyahu, D., Nagalakshmi, V., Applebaum, S.W., Kubli, E., Choffat, Y., *et al.*, **2003**. Inhibition of pheromone biosynthesis in *Helicoverpa armigera* by pheromonostatic peptides. *J. Insect. Physiol. 49*, 569–574.

Engebretson, J.A., Mason, W.H., **1980**. Transfer of 65Zn at mating in *Heliothis virescens*. *Environ. Entomol. 9*, 119–121.

Fan, Y., Rafaeli, A., Gileadi, C., Applebaum, S.W., **1999a**. Juvenile hormone induction of pheromone gland PBAN-responsiveness in *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol. 29*, 635–641.

Fan, Y., Rafaeli, A., Gileadi, C., Kubli, E., Applebaum, S.W., **1999b**. *Drosophila melanogaster* sex peptide stimulates juvenile hormone synthesis and depresses sex pheromone production in *Helicoverpa armigera*. *J. Insect. Physiol. 45*, 127–133.

Fan, Y., Rafaeli, A., Moshitzky, P., Kubli, E., Choffat, Y., *et al.*, **2000**. Common functional elements of *Drosophila melanogaster* seminal peptides involved in reproduction of *Drosophila melanogaster* and *Helicoverpa armigera* females. *Insect. Biochem. Mol. Biol. 30*, 805–812.

Feinsod, F.M., Spielman, A., **1980**. Nutrient-mediated juvenile hormone secretion in mosquitoes. *J. Insect. Physiol. 26*, 113–117.

Feng, Y., Schiff, N.M., Cavener, D.R., **1991**. Organ-specific patterns of gene expression in the reproductive tract of *Drosophila* are regulated by the sex-determination genes. *Devel. Biol. 146*, 451–460.

Ferenz, H.-J., Seidelmann, K., **2003**. Pheromones in relation to aggregation and reproduction in desert locusts. *Physiol. Entomol. 28*, 11–18.

Ferguson, A.W., Williams, I.H., **1991**. Deposition and longevity of oviposition-deterring pheromone in the cabbage seed weevil. *Physiol. Entomol. 16*, 27–33.

Fernandez, N.M., Klowden, M.J., **1995**. Male accessory gland substances modify the host-seeking behavior of gravid *Aedes aegypti* mosquitoes. *J. Insect Physiol. 41*, 965–970.

Ferrandon, D., Jung, A.C., Criqui, M., Lemaitre, B., Uttenweiler-Joseph, S., *et al.*, **1998**. A drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J. 17*, 1217–1227.

Filosi, M., Perotti, M.E., **1975**. Fine structure of the spermatheca of *Drosophila melanogaster. J. Submicr. Cytol. 7*, 259–270.

Foster, S.P., **1993**. Neural inactivation of sex pheromone production in mated lightbrown apple moths, *Epiphyas postvittana* (Walker). *J. Insect Physiol. 39*, 267–273.

Fowler, K., Partridge, L., **1989**. A cost of mating in female fruitflies. *Nature 338*, 760–761.

Friedel, T., Gillot, C., **1976**. Male accessory gland substance of *Melanoplus sanguinipes*: an oviposition stimulant under the control of the corpora allata. *J. Insect Physiol. 22*, 489–495.

Fuchs, M.S., Hiss, E.A., **1970**. The partial purification and separation of the protein components of matrone from *Aedes aegypti*. *J. Insect Physiol. 16*, 931–939.

Fuyama, Y., **1983**. Species specificity of paragonial substances as an isolating mechanism in *Drosophila*. *Experientia 39*, 190–192.

Garcia-Bellido, A., **1964**. Das sekret der paragonien als stimulus der fekundität bei weibchen von *Drosophila melanogaster. Z. Naturforsch. 19b*, 491–495.

Gessner, B., Gessner, K., **1976**. Inorganic ions in spermathecal fluid and their transport across the spermathecal membrane of the queen bee, *Apis mellifera*. *J. Insect Physiol. 22*, 1469–1474.

Giebultowicz, J.M., Raina, A.K., Uebel, E.C., Ridgway, R.L., **1991**. Two-step regulation of sex pheromone decline in mated gypsy moth females. *Arch. Insect Biochem. Physiol. 16*, 95–105.

Gilbert, D.G., **1981**. Ejaculate esterase and initial sperm use by female *Drosophila melanogaster. J. Insect Physiol. 27*, 641–650.

Gilbert, D.G., Richmond, R.C., Sheehan, K.B., **1981**. Studies of esterase 6 in *Drosophila melanogaster.* V. Progeny production and sperm use in females inseminated by males having active or null alelles. *Evolution 35*, 21–37.

Gillott, C., **1988**. Arthropoda – Insecta. In: Adiyodi, K.G., Adiyodi, R.G. (Eds.), Reproductive Biology of Invertebrates, Vol. III, Accessory Sex Glands. Wiley, Chichester, UK, pp. 319–471.

Gillott, C., **2003**. Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annu. Rev. Entomol. 48*, 163–184.

Grimnes, K.A., Bricker, C.S., Happ, G.M., **1986**. Ordered flow of secretion from accessory glands to the specific layers of the spermatophore of mealworm beetles *Tenebrio moliter*; demonstration with a monoclonal antibody. *J. Exp. Zool. 240*, 275–286.

Gromko, M.H., Gilbert, D.G., Richmond, R.C., **1984**. Sperm transfer and use in the multiple mating system of *Drosophila*. In: Smith, R.L. (Ed.), Sperm Competition and the Evolution of Mating Systems. Academic Press, NY, pp. 371–426.

Hägele, B.F., Oag, V., Bouaichi, A., McCaffery, A.R., Simpson, S.S., **2000**. The role of female accessory glands in maternal inheritance of phase in the desert locust *Schistocerca gregaria*. *J. Insect Physiol. 46*, 275–280.

Hall, J.C., **1994**. The mating of a fly. *Science 264*, 1702–1714.

Hamdaoui, A., Schoofs, L., Wataleb, S.L.V.B., Verhaert, P., Waelkens, E., *et al.*, **1997**. Purification of a novel, heat-stable serine protease inhibitor protein from ovaries of the desert locust, *Schistocerca gregaria*. *Biochem. Biophys. Res. Commun. 238*, 357–360.

Hamdaoui, A., Wataleb, S., Devreese, B., Chiou, S.-J., Vanden Broeck, J., *et al.*, **1998**. Purification and characterization of a group of five novel peptide serine protease inhibitors from ovaries of the desert locust, *Schistocerca gregaria*. *FEBS Lett. 422*, 74–78.

Happ, G.M., Yuncker, C., Huffmire, S.A., **1977**. Cytodifferentiation in the accessory glands of *Tenebrio molitor* II. Patterns of leucine incorporation in the tubular glands of post-ecdysial adult males. *J. Exp. Zool. 200*, 223–236.

Hare, J.F., Eisner, T., **1993**. Pyrrolizidine alkaloid deters ant predators of *Utetheisa ornatrix* eggs: effects of alkaloid concentration, oxidation state, and prior exposure of ants to alkaloid-laden prey. *Oecologia 96*, 9–18.

Harshman, L.G., Prout, T., **1994**. Sperm displacement without sperm transfer in *Drosophila melanogaster*. *Evolution 48*, 758–766.

Hartmann, R., Loher, W., **1996**. Control mechanisms of the behavior 'secondary defense' in the grasshopper *Gomphocerus rufus* L. (Gomphocerinae: Orthoptera). *J. Comp. Physiol. [A] 178*, 329–336.

Hartmann, R., Loher, W., **1999**. Post-mating effects in the grasshopper, *Gomphocerus rufus* L. mediated by the spermatheca. *J. Comp. Physiol. A 184*, 325–332.

He, S., Lin, Y.L., Liu, Y.X., **1999**. Functionally inactive protein C inhibitor in seminal plasma may be associated with infertility. *Mol. Hum. Reprod. 5*, 513–519.

Heifetz, Y., Lung, O., Frongillo, E.A., Wolfner, M.F., **2000**. The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Curr. Biol. 10*, 99–102.

Heifetz, Y., Tram, U., Wolfner, M.F., **2001a**. Male contributions to egg production: the role of accessory gland products and sperm in *Drosophila melanogaster*. *Proc. R. Soc. Lond. B: Biol. Sci. 268*, 175–180.

Heifetz, Y., Vandenberg, L.N., Cohn, H.I., Wolfner, M.F. Defining the active regions of ovulin, a *Drosophila* ovulation hormone donated to females during mating. In preparation.

Heifetz, Y., Wolfner, M.F., **2004**. Seminal fluid and mating mediate changes in nerve termini innervating the *Drosophila* reproductive tract. *Proc. Natl Acad. Sci. USA 101*, 6261–6266.

Heifetz, Y., Yu, J., Wolfner, M.F., **2001b**. Ovulation triggers activation of *Drosophila* oocytes. *Devel. Biol. 234*, 416–424.

Herndon, L.A., Chapman, T., Kalb, J.M., Lewin, S.M., Partridge, L., *et al.*, **1997**. Mating and hormonal triggers regulate accessory gland gene expression in male *Drosophila*. *J. Insect Physiol. 43*, 1117–1123.

Herndon, L.A., Wolfner, M.F., **1995**. A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg-laying in females for one day following mating. *Proc. Natl Acad. Sci. USA 92*, 10114–10118.

Hihara, F., **1981**. Effects of the male accessory gland secretion on oviposition and remating in females of *Drosophila melanogaster*. *Zool. Mag. 90*, 303–316.

Hirohashi, N., Vacquier, V.D., **2003**. Store-operated calcium channels trigger exocytosis of the sea urchin sperm acrosomal vesicle. *Biochem. Biophys. Res. Commun. 304*, 285–292.

Hiss, E.A., Fuchs, M.S., **1972**. The effect of matrone on oviposition in the mosquito, *Aedes aegypti*. *J. Insect Physiol. 18*, 2217–2227.

Holland, B., Rice, W.R., **1999**. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl Acad. Sci. USA 96*, 5083–5088.

Hopkins, T.L., Starkey, S.R., Xu, R., Merritt, M.E., Schaefer, J., *et al.*, **1999**. Catechols involved in sclerotization of cuticle and egg pods of the grasshopper, *Melanoplus sanguinipes*, and their interactions with cuticular proteins. *Arch. Insect Biochem. Physiol. 40*, 119–128.

Hosken, D.J., Garner, T.W., Ward, P.I., **2001**. Sexual conflict selects for male and female reproductive characters. *Curr. Biol. 11*, 489–493.

Hosken, D.J., Ward, P.I., **1999**. Female accessory reproductive gland activity in the yellow dung fly *Scathophaga stercoraria* (L.). *J. Insect Physiol. 45*, 809–814.

Iida, K., Cavener, D.R., **2004**. Glucose dehydrogenase is required for normal sperm storage and utilization in female *Drosophila melanogaster*. *J. Exp. Biol. 207*, 675–681.

Imai, T., Kodama, H., Chuman, T., Kohno, M., **1990**. Female-produced oviposition deterrents of the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *J. Chem. Ecol. 16*, 1237–1247.

Imamura, M., Haino-Fukushima, K., Aigaki, T., Fuyama, Y., **1998**. Ovulation stimulating substances in *Drosophila biarmipes* males: their origin, genetic variation in the response of females, and molecular characterization. *Insect Biochem. Mol. Biol. 28*, 365–372.

Ismail, S.M., Gillott, C., **1994**. A high affinity, juvenile hormone-binding protein in the long hyaline tubules of male *Melanoplus sanguinipes*. *Insect Biochem. Mol. Biol. 24*, 739–745.

James, D.G., **2003**. Field evaluation of herbivore-induced plant volatiles as attractants for beneficial insects: methyl salicylate and the green lacewing, *Chrysopa nigricornis*. *J. Chem. Ecol. 29*, 1601–1609.

Jang, E.B., **2002**. Physiology of mating behavior in Mediterranean Fruit Fly (Diptera: Tephritidae): chemoreception and male accessory gland fluids in female post-mating behavior. *Florida Entomol. 85*, 89–93.

Jin, Z.-Y., Gong, H., **2001**. Male accessory gland derived factors can stimulate oogenesis and enhance oviposition in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Arch. Insect Biochem. Physiol. 46*, 175–185.

Johnstone, R.A., Keller, L., **2000**. How males can gain by harming their mates: sexual conflict, seminal toxins, and the cost of mating. *Am. Nat. 156*, 368–377.

Jones, D., **1996**. Biochemical interaction between chelonine wasps and their lepidopteran hosts: after a decade of research – the parasite is in control. *Insect Biochem. Mol. Biol. 26*, 981–996.

Judd, S.R., Leopold, R.A., Turner, R.B., **1983**. Characteristics of aminopeptidase activity of female housefly reproductive accessory glands. *Int. J. Invert. Reprod. 6*, 93–98.

Jurenka, R.A., Fabrias, G., Ramaswamy, S., Roelofs, W.L., **1993**. Control of pheromone biosynthesis in mated redbanded leafroller moths. *Arch. Insect Biochem. Physiol. 24*, 129–137.

Kalb, J., DiBenedetto, A.J., Wolfner, M.F., **1993**. Probing the function of *Drosophila* melanogaster accessory glands by directed cell ablation. *Proc. Natl Acad. Sci. USA 90*, 8093–8097.

Kaldany, R.-R.J., Nambu, J.R., Scheller, R.H., **1985**. Neuropeptides in identified Aplysia neurons. *Annu. Rev. Neurosci. 8*, 431–455.

Katzav-Gozansky, T., Soroker, V., Francke, W., Hefetz, A., **2003**. Honeybee egg-laying workers mimic a queen signal. *Insectes Soc. 50*, 20–23.

Katzav-Gozansky, T., Soroker, V., Hefetz, A., **1997**. Plasticity of caste-specific Dufour's gland secretion in the honey bee (*Apis mellifera* L.). *Naturwissenschaften 84*, 238–241.

Katzav-Gozansky, T., Soroker, V., Hefetz, A., **2000**. Plasticity in caste-related exocrine secretion biosynthesis in the honey bee (*Apis mellifera*). *J. Insect Physiol. 46*, 993–998.

Keisman, E.L., Baker, B.S., **2001**. The *Drosophila* sex determination hierarchy modulates wingless and decapentaplegic signaling to deploy dachshund sex-specifically in the genital imaginal disc. *Development 128*, 1643–1656.

Keisman, E.L., Christiansen, A.E., Baker, B.S., **2001**. The sex determination gene doublesex regulates the A/P organizer to direct sex-specific patterns of growth in the *Drosophila* genital imaginal disc. *Devel. Cell 1*, 215–225.

Keplinger, B.L., Guo, X., Quine, J., Feng, Y., Cavener, D.R., **2001**. Complex organization of promoter and enhancer elements regulate the tissue- and developmental stage-specific expression of the *Drosophila melanogaster Gld* gene. *Genetics 157*, 699–716.

Keplinger, B.L., Rabetoy, A.L., Cavener, R., **1996**. A somatic reproduction organ enhancer complex activates expression in both the developing and the mature *Drosophila* reproductive tract. *Devel. Biol. 180*, 311–323.

Kingan, T.G., Bodnar, W.M., Raina, A.K., Shabanowitz, J., Hunt, D.F., **1995**. The loss of female sex pheromone after mating in the corn earworm moth *Helicoverpa zea*: identification of a male pheromonostatic peptide. *Proc. Natl Acad. Sci. USA 92*, 5082–5086.

Kingan, T.G., Thomas-Laemont, P.A., Raina, A.K., **1993**. Male accessory gland factors elicit change from "virgin" to "mated" behaviour in the female corn earworm moth *Helicoverpa zea*. *J. Exp. Biol. 183*, 61–76.

Klowden, M.J., Chambers, G.M., **1991**. Male accessory gland substances activate egg development in nutritionally stressed *Aedes aegypti* mosquitoes. *J. Insect Physiol. 37*, 721–726.

Kohno, M., Mochizuki, K., Chuman, T., Ohnishi, A., **1986**. Pheromone-like substances affecting the oviposition behavior of the female cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *Appl. Entomol. Zool. 21*, 15–20.

Kondoh, M., **2001**. Co-evolution of nuptial gift and female multiple mating resulting in diverse breeding systems. *Evol. Ecol. Res. 3*, 75–89.

Kotiaho, J.S., Simmons, L.W., **2003**. Longevity cost for males, but no longevity cost for females in the male-dimorphic dung beetle *Onthophagus binodis*. *J. Insect Physiol. 49*, 817–822.

Kramer, K.J., Christensen, A.M., Morgan, T.D., Schaefer, J., Czapla, T.H., *et al.*, **1991**. Analysis of cockroach oothecae and exuviae by solid-state 13C-NMR spectroscopy. *Insect Biochem. Mol. Biol. 21*, 149–156.

Kressin, M., Sommer, U., Schnorr, B., **1996**. The spermathecal epithelium of the queen bee (*Apis mellifera*): morphology, age-dependent changes and cell contacts. *Anat. Histol. Embryol. 25*, 31–35.

Kubli, E., **1992**. My favorite molecule: the sex peptide. *Bioessays 14*, 779–784.

Kubli, E., **2003**. Sex-peptides: seminal peptides of the *Drosophila* male. *Cell. Mol. Life Sci. 60*, 1689–1704.

Lai-Fook, J., **1991**. Absorption of phosphorus from the spermataphore through the cuticle of the bursa copularix of the butterfly, *Calpodes ehtlius*. *Tissue Cell 23*, 247–259.

Lange, A.B., Loughton, B.G., **1985**. An oviposition-stimulating factor in the male accessory reproductive gland of the locust, *Locusta migratoria*. *Gen. Comp. Endocrinol. 57*, 208–215.

Leahy, M.G., **1973**. Oviposition of virgin *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) after implant of the male accessory gland complex. *J. Entomol. (A) 48*, 69–78.

Lee, J.J., Klowden, M.J., **1999**. A male accessory gland protein that modulates female mosquito (Diptera: Culicidae) host-seeking behavior. *J. Am. Mosq. Control Assoc. 15*, 4–7.

Leopold, R.A. (Ed.), **1981**. Accessory reproductive gland involvement with the sperm–egg interaction in muscoid flies. Elsevier/North-Holland, New York.

Leopold, R.A., Degrugil, M.E., **1973**. Sperm penetration of housefly eggs – evidence for involvement of a female accessory secretion. *Science 181*, 555–557.

Leopold, R.A., Meola, S.M., Degrugillier, M.E., **1978**. The egg fertilization site within the housefly *Musca domestica* Diptera Muscidae. *Int. J. Ins. Morphol. Embryol. 7*, 111–120.

Liu, H., Kubli, E., **2003**. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 100*, 9929–9933.

Lococo, D., Huebner, E., **1980**. The ultrastructure of the female accessory gland, the cement gland, in the insect *Rhodnius prolixus*. *Tissue Cell 12*, 557–580.

Ludwig, M.Z., Uspensky, I.I., Ivanov, A.I., Kopantseva, M.R., Dianov, C.M., *et al.*, **1991**. Genetic control and expression of the major ejaculatory bulb protein PEB-me in *Drosophila melanogaster. Biochem. Genet. 29*, 215–240.

Lung, O., Kuo, L., Wolfner, M.F., **2001a**. *Drosophila* males transfer antibacterial proteins from their accessory gland and ejaculatory duct to their mates. *J. Insect Physiol. 47*, 617–622.

Lung, O., Kuo, L., Wolfner, M.F., **2001b**. Identification of and characterization of the major *D. melanogaster* mating plug protein. *Insect Biochem. Mol. Biol. 31*, 543–551.

Lung, O., Tram, U., Finnerty, C., Eipper-Mains, M., Kalb, J.M., *et al.*, **2002**. The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics 160*, 211–224.

Lung, O., Wolfner, M.F., **1999**. *Drosophila* seminal fluid proteins enter the circulatory system through the walls of the posterior vagina. *Insect Biochem. Mol. Biol. 29*, 1043–1052.

Mahowald, A.P., Goralski, T.J., Caulton, J.H., **1983**. In vitro activation of *Drosophila* eggs. *Devel. Biol. 98*, 437–445.

Malual, A.G., Hassanali, A., Torto, B., Assad, Y.O.U., Njagi, P.G.N., **2001**. The nature of the gregarization signal responsible for maternal transfer of phase to the offspring in the desert locust *Schistocerca gregaria. J. Chem. Ecol. 27*, 1423–1435.

Mane, S.D., Tompkins, L., Richmond, R.C., **1983**. Male esterase-6 catalyzes the synthesis of a sex pheromone in *Drosophila melanogaster* females. *Science 222*, 419–421.

Manning, A., **1962**. A sperm factor affecting the receptivity of *Drosophila* melanogaster females. *Nature 194*, 252–253.

Manning, A., **1967**. The control of sexual receptivity in female *Drosophila*. *Anim. Behav. 15*, 239–250.

Marchini, D., **1989**. *b-N*-Acetylhexosaminidases in the secretion of the female reproductive accessory glands of *Ceratitis capitata* (Diptera). *Insect Biochem. 19*, 549–555.

Marchini, D., Bernini, L.F., Marri, L., Giordano, P.C., Dallai, R., **1991**. The female reproductive accessory glands of the medfly *Ceratitis capitata*: antibacterial activity of the secretion fluid. *Insect Biochem. Mol. Biol. 21*, 597–605.

Marchini, D., Giordano, P.C., Amons, R., Bernini, L.F., Dallai, R., **1993**. Purification and primary structure of ceratotoxin A and B, two antibacterial peptides from the female reproductive accessory glands of the medfly *Ceratitis capitata* (Insecta: Diptera). *Insect Biochem. Mol. Biol. 23*, 591–598.

Marchini, D., Manetti, A.G., Rosetto, M., Bernini, L.F., Telford, J.L., *et al.*, **1995**. cDNA sequence and expression of the ceratotoxin gene encoding an antibacterial sex-specific peptide from the medfly *Ceratitis capitata* (diptera). *J. Biol. Chem. 270*, 6199–6204.

Marchini, D., Marri, L., Rosetto, M., Manetti, A.G., Dallai, R., **1997**. Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata. Biochem. Biophys. Res. Commun. 240*, 657–663.

Markow, T.A., Coppola, A., Watts, T.D., **2001**. How *Drosophila* males make eggs: it is elemental. *Proc. R. Soc. Lond. B Biol. Sci. 268*, 1527–1532.

McCaffery, A., Simpson, S., **1998**. A gregarizing factor present in the egg pod foam of the desert locust *Schistocerca gregaria*. *J. Exp. Biol. 201*, 347–363.

Meikle, D.B., Sheehan, K.B., Phillis, D.M., Richmond, R.C., **1990**. Localization and longevity of seminal-fluid esterase 6 in mated female *Drosophila melanogaster. J. Insect Physiol. 14*, 1159–1168.

Merle, J., **1968**. Fonctionnement ovarien et rêçeptivité sexuelle de *Drosophila melanogaster* après implantation de fragments de l'appareil génital male. *J. Insect Physiol. 14*, 1159–1168.

Monsma, S.A., Harada, H.A., Wolfner, M.F., **1990**. Synthesis of two *Drosophila* male accessory gland proteins and their fate after transfer to the female during mating. *Devel. Biol. 142*, 465–475.

Monsma, S.A., Wolfner, M.F., **1988**. Structure and expression of a *Drosophila* male accessory gland gene whose product resembles a peptide pheromone precursor. *Genes Devel. 2*, 1063–1073.

Moore, H., Dvorakova, K., Jenkins, N., Breed, W., **2002**. Exceptional sperm cooperation in the wood mouse. *Nature 418*, 174–177.

Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klauser, S., *et al.*, **1996**. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol. 32*, 363–374.

Mudd, A., Ferguson, A.W., Blight, M.M., Williams, I.H., Scubla, P., *et al.*, **1997**. Extraction, isolation, and composition of oviposition-deterring secretion of cabbage seed weevil *Ceutorhynchus assimilis*. *J. Chem. Ecol. 23*, 2227–2240.

Mueller, J.L., Ripoll, D.R., Aquadro, C.F., Wolfner, M.F., **2004**. Comparative structural modeling and interference of conserved protein classes in *Drosophila* seminal fluid. *Proc. Natl Sci. USA* (in press).

Murer, V., Spetz, J.F., Hengst, U., Altrogge, L.M., deAgostini, A., *et al.*, **2001**. Male fertility defects in mice lacking the serine protease inhibitor protease nexin-1. *Proc. Natl Acad. Sci. USA 98*, 3029–3033.

Nakayama, S., Kaiser, K., Aigaki, T., **1997**. Ectopic expression of sex peptide in a variety of tissues in *Drosophila* females using the P[GAL4] enhancer-trap system. *Mol. Gen. Genet. 254*, 449–455.

Neubaum, D.M., Wolfner, M.F., **1999a**. *Drosophila* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. *Genetics 153*, 845–857.

Neubaum, D.M., Wolfner, M.F., **1999b**. Wise, winsome or wierd: mechanisms of sperm storage in female animals. *Curr. Top. Devel. Biol. 41*, 67–97.

Nicolas, P., Mor, A., **1995**. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annu. Rev. Microbiol. 49*, 277–304.

Ninkovic, V., Ahmed, E., Glinwood, R., Pettersson, J., **2003**. Effects of two types of semiochemical on population development of the bird cherry oat aphid *Rhopalosiphum padi* in a barley crop. *Agric. For. Entomol. 5*, 27–34.

Njagi, P.G.N., Torto, B., **2002**. Evidence for a compound in Comstock-Kellog glands modulating premating behavior in male desert locust, *Schistocerca gregaria*. *J. Chem. Ecol. 28*, 1065–1074.

Ohashi, Y.Y., Hamo-Fukushima, K., Fuyama, Y., **1991**. Purification and characterization of an ovulation stimulating substance from the male accessory glands of *Drosophila suzukii*. *Insect Biochem. 21*, 413–419.

Osir, E.O., Kotengo, M., Chaudhury, M.F., Otieno, L.H., **1991**. Structural studies on the major milk gland protein of the tsetse fly, *Glossina morsitans morsitans*. *Comp. Biochem. Physiol. B 99*, 803–809.

Ottiger, M., Soller, M., Stocker, R.F., Kubli, E., **2000**. Binding sites of *Drosophila melanogaster* sex peptide pheromones. *J. Neurobiol. 44*, 57–71.

Packer, L., **2003**. Comparative morphology of the skeletal parts of the sting apparatus of bees (Hymenoptera: Apoidea). *Zool. J. Linnean Soc. 138*, 1–38.

Paemen, L., Tips, A., Schoofs, L., Proost, P., Van Damme, J., *et al.*, **1991**. Lom-AG-myotropin: a novel myotropic peptide from the male accessory glands of *Locusta migratoria*. *Peptides 12*, 7–10.

Page, A.W., Orr-Weaver, T.L., **1997**. Activation of the meiotic divisions in *Drosophila* oocytes. *Devel. Biol. 183*, 195–207.

Park, M., Wolfner, M.F., **1995**. Male and female cooperate in the processing of a *Drosophila melanogaster* seminal fluid protein. *Devel. Biol. 171*, 694–702.

Parker, G.A., **1970**. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev. 45*, 525–567.

Partridge, L., Fowler, K., **1990**. Non-mating costs of exposure to males in female *Drosophila melanogaster*. *J. Insect Physiol. 36*, 419–425.

Partridge, L., Green, A., Fowler, K., **1987**. Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *J. Insect Physiol. 10*, 745–749.

Paterson, Z.A., Weaver, R.J., **1997**. Characterization and temporal aspects of hemolymph juvenile hormone esterase in the cockroach *Periplaneta americana*. *J. Insect Physiol. 43*, 521–532.

Patterson, J.T., Stone, W.S., **1952**. Evolution in the Genus *Drosophila*. Macmillan, New York.

Pickford, R., Ewen, A.B., Gillott, C., **1969**. Male accessory gland substance: an egg-laying stimulant in *Melanoplus sanguinipes* (F). *Can. J. Zool. 47*, 1199–1203.

Polak, M., Starmer, W.T., Barker, J.S.F., **1998**. A mating plug and male mate choice in *Drosophila hibisci* Bock. *Anim. Behav. 56*, 919–926.

Price, C.S., **1997**. Conspecific sperm precedence in *Drosophila*. *Nature 388*, 663–666.

Prokopy, R.J., Reissig, W.H., Moericke, V., **1976**. Marking pheromones deterring repeated oviposition in Rhagoletis flies. *Entomol. Exp. Appl. 20*, 170–178.

Rafaeli, A., **2002**. Neuroendocrine control of pheromone biosynthesis in moths. *Int. Rev. Cytol. 213*, 49–92.

Rafaeli, A., Gileadi, C., **2000**. Inhibition of pheromone biosynthesis and mating in the stored product moth, *Plodia interpunctella*. In: Jin Zuxvn, Liang Quan, Liang Yongsheng, Tan Xianchang, Guan Lianghua (Eds.), Proc. 7th International Working Conference on Stored Product Protection, Beijing, China, Vol. 2. pp. 1269–1274.

Raina, A.K., **1989**. Male-induced termination of sex pheromone production and receptivity in mated females of *Heliothis zea*. *J. Insect Physiol. 35*, 821–826.

Ramaswamy, S.B., Mbata, G.N., Cohen, N.E., **1990**. Necessity of juvenile hormone for choriogenesis in the moth, *Heliothis virescens* (Noctuidae). *Invertebr. Reprod. Devel. 17*, 57–63.

Ramaswamy, S.B., Qiu, Y., Park, Y.I., **1996**. Neuronal control of post-coital pheromone production in the moth *Heliothis virescens*. *J. Exp. Zool. 274*, 255–263.

Ravi Ram, K., Ramesh, S.R., **1999**. Male accessory gland secretory proteins in nasuta subgroup of *Drosophila*: nature and SDS-PAGE patterns. *Indian J. Exp. Biol. 37*, 767–773.

Ravi Ram, K., Ramesh, S.R., **2001**. Male accessory gland secretory proteins in a few members of the *Drosophila nasuta* subgroup. *Biochem. Genet. 39*, 99–115.

Rice, W.R., **1996**. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature 381*, 232–234.

Rice, W.R., Chippindale, A.K., **2001**. Sexual recombination and the power of natural selection. *Science 294*, 555–559.

Riddiford, L.M., **1993**. Hormones and *Drosophila* development. In: Bate, M., Martinez, A.A. (Eds.), The Development of *Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 899–939.

Rolff, J., Siva-Jothy, M.T., **2002**. Copulation corrupts immunity: a mechanism for a cost of mating in insects. *Proc. Natl Acad. Sci. USA 99*, 9916–9918.

Rooney, J., Lewis, S.M., **1999**. Differential allocation of male-derived nutrients in two lampyrid beetles with contrasting life-history characteristics. *Behav. Ecol. 10*, 97–104.

Rosetto, M., Belardinelli, M., Fausto, A.M., Marchini, D., Bongiorno, G., *et al.*, **2003a**. A mammalian-like lipase gene is expressed in the female reproductive accessory glands of the sand fly *Phlebotomus papatasi* (Diptera, Psychodidae). *Insect Mol. Biol. 12*, 501–508.

Rosetto, M., De Filippis, T., Mandrioli, M., Zacharopoulou, A., Gourzi, P., *et al.*, **2000**. Ceratotoxins: female-specific X-linked genes from the medfly, *Ceratitis capitata*. *Genome 43*, 707–711.

Rosetto, M., De Filippis, T., Manetti, A.G., Marchini, D., Baldari, C.T., *et al.*, **1997**. The genes encoding the antibacterial sex-specific peptides ceratotoxins are clustered in the genome of the medfly *Ceratitis capitata*. *Insect Biochem. Mol. Biol. 27*, 1039–1046.

Rosetto, M., De Filippis, T., Manetti, A.G., Marchini, D., Dallai, R., **1999**. FST, a female-specific transcript from the medfly *Ceratitis capitata* (Diptera: Tephritidae). *Insect Mol. Biol. 8*, 545–550.

Rosetto, M., Manetti, A.G., Giordano, P.C., Marri, L., Amons, R., *et al.*, **1996**. Molecular characterization of ceratotoxin C, a novel antibacterial female-specific peptide of the ceratotoxin family from the medfly *Ceratitis capitata*. *Eur. J. Biochem. 241*, 330–337.

Rosetto, M., Marchini, D., De Filippis, T., Ciolfi, S., Frati, F., *et al.*, **2003b**. The ceratotoxin gene family in the medfly *Ceratitis capitata* and the Natal fruit fly *Ceratitis rosa* (Diptera: Tephritidae). *Heredity 90*, 382–389.

Rossini, C., Gonzalez, A., Eisner, T., **2001**. Fate of an alkaloidal nuptial gift in the moth *Utetheisa ornatrix*: systemic allocation for defense of self by the receiving female. *J. Insect Physiol. 47*, 639–647.

Rothman, B.S., Hawke, D.H., Brown, R.O., Lee, T.D., Dehghan, A.A., *et al.*, **1986**. Isolation and primary structure of the califins, three biologically active egg-laying hormone-like peptides from the atrial gland of *Aplysia californica*. *J. Biol. Chem. 261*, 1616–1623.

Salamonsen, L.A., Hampton, A.L., Suzuki, R., Nagase, H., **1994**. Modulation of production of matrix metalloproteinases from ovine endometrial cells by ovine trophoblast interferon. *J. Reprod. Fertil. 102*, 155–162.

Salmon, A.B., Marx, D.B., Harshman, L.G., **2001**. A cost of reproduction in *Drosophila melanogaster*: stress susceptibility. *Evolution 55*, 1600–1608.

Samakovlis, C., Kylsten, P., Kimbrell, D.A., Engstrom, A., Hultmark, D., **1991**. The andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster. EMBO J. 10*, 163–169.

Satyanarayana, K., Bhaskaran, G., Dahm, K.H., Meola, R., **1992**. Regulation of vitellogenin synthesis by juvenile hormone in the corn earworm, *Helicoverpa zea*. *Invertebr. Reprod. Devel. 21*, 169–178.

Saudan, P., Hauck, K., Soller, M., Choffat, Y., Ottiger, M., *et al.*, **2002**. Ductus ejaculatorius peptide 99B (DUP99B), a novel *Drosophila melanogaster* sex-peptide pheromone. *Eur. J. Biochem. 269*, 989–997.

Sauter, A., Brown, J.F., Baer, B., Schmid-Hempel, P., **2001**. Males of social insects can prevent queens from multiple mating. *Proc. R. Soc. Lond. B 268*, 1149–1454.

Savary, S., Drezen, J.M., Tan, F., Beckage, N.E., Periquet, G., **1999**. The excision of polydnavirus sequences from the genome of the wasp *Cotesia congregata* (Braconidae, Microgastrinae) is developmentally regulated but not strictly restricted to the ovaries of the adult. *Insect Mol. Biol. 8*, 319–327.

Schäfer, U., **1986**. Genes for male-specific transcripts in *Drosophila* melanogaster. *Mol. Gen. Genet. 202*, 219–225.

Scheller, R.H., Jackson, J.F., McAllister, L.B., Schwartz, J.H., Kandel, E.R., *et al.*, **1982**. A family of genes that codes for ELH, a neuropeptide eliciting a stereotyped pattern of behavior in Aplysia. *Cell 28*, 707–719.

Schmidt, T., Choffat, Y., Klauser, S., Kubli, E., **1993**. The *Drosophila melanogaster* sex-peptide: a molecular analysis of structure-function relationships. *J. Insect Physiol. 39*, 361–368.

Schmidt, T., Stumm-Zollinger, E., Chen, P.S., **1989**. A male accessory gland peptide with protease inhibitory activity in *Drosophila funebris*. *J. Biol. Chem. 264*, 9745–9749.

Schoonhoven, L.M., **1990**. Host-marking pheromones in Lepidoptera, with special reference to two Pieris spp. *J. Chem. Ecol. 16*, 3043–3052.

Seidelmann, K., Ferenz, H.-J., **2002**. Courtship inhibition pheromone in desert locusts, *Schistocerca gregaria*. *J. Insect Physiol. 48*, 991–996.

Seidelmann, K., Luber, K., Ferenz, H.-J., **2000**. Analysis of release and role of benzyl cyanide in male desert locusts, *Schistocerca gregaria*. *J. Chem. Ecol. 26*, 1897–1910.

Sendi, J.J., Srivastava, K.P., Singh, M.R.K., **1993**. Post-embryonic study on the development of the female efferent genital system in the lemon-butterfly, *Papilio demoleus* L. (Lepidoptera). *J. Res. Lepid. 32*, 162–169.

Seth, R.K., Kaur, J.J., Rao, D.K., Reynolds, S.E., **2002**. Sperm transfer during mating, movement of sperm in the female reproductive tract, and sperm precedence in the common cutworm *Spodoptera litura*. *Physiol. Entomol. 27*, 1–14.

Shirk, P.D., Bahaskaran, G., Roller, H., **1980**. The transfer of juvenile hormone from male to female during mating in the *Cecropia* silkmoth. *Experientia 36*, 682–683.

Simonet, G., Claeys, I., Broeck, J.V., **2002**. Structural and functional properties of a novel serine protease inhibiting peptide family in arthropods. *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 132*, 247–255.

Smedley, S.R., Eisner, T., **1995**. Sodium uptake by puddling in a moth. *Science 270*, 1816–1818.

Smedley, S.R., Eisner, T., **1996**. Sodium: a male moth's gift to its offspring. *Proc. Natl Acad. Sci. USA 93*, 809–813.

Smith, G.M., Rothwell, K., Wood, S.L., Yeaman, S.J., Bownes, M., **1994**. Specificity and localization of lipolytic activity in adult *Drosophila melanogaster. Biochem. J. 304*, 775–779.

Soller, M., Bownes, M., Kubli, E., **1997**. Mating and sex peptide stimulate the accumulation of yolk in oocytes of *Drosophila melanogaster. Eur. J. Biochem. 243*, 732–738.

Soller, M., Bownes, M., Kubli, E., **1999**. Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol. 208*, 337–351.

Spradling, A.C., **1993**. Developmental genetics of oogenesis. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. I. Cold Spring Harbor Press, NY, pp. 1–70.

Stay, B., Roth, L., **1962**. The colleterial glands of cockroaches. *Ann. Ent. Soc. 55*, 124–130.

Strand, M.R., Ratner, S., Vinson, S.B., **1980**. Maternally induced host regulation by the egg parasitoid *Telenomus heliothidis*. *Physiol. Entomol. 8*, 469–475.

Straw, N.A., **1989**. Evidence for an oviposition-deterring pheromone in *Tephritis bardanae* (Schrank) (Diptera: Tephritidae). *Oecologia 78*, 121–130.

Sturm, R., **2002**. Development of the accessory glands in the genital tract of female *Teleogryllus commodus* WALKER (Insecta, Orthoptera). *Arthropod Struct. Devel. 31*, 231–241.

Swanson, W.J., **2003**. Sex peptide and the sperm effect in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 100*, 9643–9644.

Swanson, W.J., Clark, A.G., Waldrip-Dail, H.M., Wolfner, M.F., Aquadro, C.F., **2001**. Evolutionary EST analysis identifies rapidly evolving male reproductive proteins in *Drosophila*. *Proc. Natl Acad. Sci. USA 98*, 7375–7379.

Swanson, W.J., Vacquier, V.D., **2002**. The rapid evolution of reproductive proteins. *Nat. Rev. Ganet. 3*, 137–144.

Szopa, T.M., **1981**. The role of the accessory reporoductive glands and genital ducts in egg pod formation in female *Schistocerca gregaria*. *J. Insect Physiol. 27*, 23–29.

Tanaka, K., Matsumoto, H., Hayakawa, Y., **2002**. Detailed characterization of polydnavirus immunoevasive proteins in an endoparasitoid wasp. *Eur. J. Biochem. 269*, 2557–2566.

Tirone, G., Avancini, R.M.P., **1997**. Development of female accessory glands of Chrysomya putoria (Wiedemann) (Diptera: Calliphoridae) during oogenesis. *Int. J. Insect Morphol. Embryol. 26*, 1–7.

Tram, U., Wolfner, M.F., **1999**. Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster. Genetics 153*, 837–844.

Tsaur, S.C., Ting, C.T., Wu, C.-I., **1998**. Positive selection driving the evolution of a gene of male reproduction, *Acp26Aa*, of *Drosophila*: II. Divergence versus polymorphism. *Mol. Biol. Evol. 15*, 1040–1046.

Tsaur, S.C., Ting, C.T., Wu, C.I., **2001**. Sex in *Drosophila mauritiana*: a very high level of amino acid polymorphism in a male reproductive protein gene, *Acp26Aa*. *Mol. Biol. Evol. 18*, 22–26.

Tsaur, S.C., Wu, C.-I., **1997**. Positive selection and the molecular evolution of a gene of male reproduction, *Acp26Aa* of *Drosophila*. *Mol. Biol. Evol. 14*, 544–549.

Tzou, P., De Gregorio, E., Lemaitre, B., **2002**. How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. *Curr. Opin. Microbiol. 5*, 102–110.

Uvarov, B., **1966**. Grasshoppers and Locusts, A Handbook of General Acridology. Cambridge University Press, Cambridge, UK.

Vahed, K., **1998**. The function of nuptial feeding in insects: a review of empirical studies. *Biol. Revs. 73*, 43–78.

Vilaplana, L., Piulachs, M.-D., Belles, X., **1996**. The conglobate gland of *Blatella germanica* (L.) (Dictyoptera, Blatellidae). Maturation, juvenile hormone dependency and changes during spermatophore formation. *Invertebr. Reprod. Devel. 29*, 167–172.

Visconti, P.E., Westbrook, V.A., Chertihin, O., Demarco, I., Sleight, S., *et al.*, **2002**. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J. Reprod. Immunol. 53*, 133–150.

Weller, S.J., Jacobson, N.L., Conner, W.E., **1999**. The evolution of chemical defences and mating systems in tiger moths (Lepidoptera: Arctiidae). *Biol. J. Linn. Soc. 68*, 557–578.

Went, D.F., Krause, G., **1974**. Egg activation in *Pimpla turionellae* (Hym.). *Naturwissenschaften 61*, 407–408.

Wertheim, B., Vet, L.E.M., Dicke, M., **2002**. Behavioural plasticity in support of a benefit for aggregation pheromone use in *Drosophila melanogaster. Ent. Exp. Appl. 103*, 61–71.

Whalen, M., Wilson, T.G., **1986**. Variation and genomic localization of genes encoding *Drosophila melanogaster* male accessory gland proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Genetics 114*, 77–92.

Will, M.W., Sakaluk, S.K., **1994**. Courtship feeding in decorated crickets: is the spermatophylax a sham? *Anim. Behav. 48*, 1309–1315.

Williams, R.W., Hagan, N.K., Berger, A., Despommier, D.D., **1978**. An improved assay technique for matrone, a mosquito pheromone, and its application in ultrafiltration experiments. *J. Insect Physiol. 24*, 127–136.

Willis, J.H., Brunet, P.C., **1966**. The hormonal control of colleterial gland secretion. *J. Exp. Biol. 44*, 363–378.

Wilson, T.G., DeMoor, S., Lei, J., **2003**. Juvenile hormone involvement in *Drosophila* melanogaster male reproduction as suggested by the Methoprene-tolerant (27) mutant phenotype. *Insect Biochem. Mol. Biol. 33*, 1167–1175.

Wolfner, M.F., **1997**. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem. Mol. Biol. 27*, 179–192.

Wolfner, M.F., **2002**. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. *Heredity 88*, 85–93.

Wolfner, M.F., Harada, H.A., Bertram, M.J., Stelick, T.J., Kraus, K.W., *et al.*, **1997**. New genes for accessory gland proteins in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 27*, 825–834.

Xue, L., Noll, M., **2000**. *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. *Proc. Natl Acad. Sci. USA 97*, 3272–3275.

Yan, J., Cheng, Q., Narashimhan, S., Li, C.-B., Aksoy, S., **2002**. Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol. 32*, 979–989.

Yanagi, S.-I., Miyatake, T., **2003**. Costs of mating and egg production in female *Callosobruchus chinensis*. *J. Insect Physiol. 49*, 823–827.

Yanagimachi, R., Bhattacharyya, A., **1988**. Acrosome-reacted guinea pig spermatozoa become fusion competent in the presence of extracellular potassium ions. *J. Exp. Zool. 248*, 354–360.

Yi, S.-X., Gillott, C., **1999**. Purification and characterization of an oviposition-stimulating protein of the long hyaline tubules in the male migratory grasshopper, *Melanoplus sanguinipes*. *J. Insect. Physiol. 45*, 143–150.

Yi, S.-X., Gillott, C., **2000**. Effects of tissue extracts on oviduct contraction in the migratory grasshopper, *Melanoplus sanguinipes*. *J. Insect Physiol. 46*, 519–525.

Zeng, F., Shu, S., Park, Y.I., Ramaswamy, S.B., **1997**. Vitellogenin and egg production in the moth, *Heliothis virescens*. *Arch. Insect Biochem. Physiol. 34*, 287–300.

Zimmerman, M., **1979**. Oviposition behavior and the existence of an oviposition-deterring pheromone in Hylemya. *Environ. Entomol. 8*, 277–279.

1.6 Molecular Genetics of Insect Fertilization

B Loppin and T L Karr, University of Bath, Bath, UK

1.6.1. Insect Fertilization – A Brief Overview

There is no doubt that fertilization is a central and key event in the life histories of all sexually reproducing organisms. It is also equally clear that we have little understanding of the evolution of sex or its maintenance throughout the over 600 million years of its existence. The selective forces that led to the origin of sex are shrouded in mystery and complexity, and indeed many consider sex to be, "…the Queen of problems in evolutionary biology" (Bell, 1982). The evolution of sex and related issues continues to dominate current evolutionary thought (Bell, 1982; Hamilton, 1999; Ridley, 1994; West-Eberhard, 2003). There exists a strange duality in our study and intellectualization of this topic: sex is a universal trait founded deep in evolutionary history while at the same time one of the most variable. This paradoxical duality challenges our ability to study, and indeed even to know how to study, those aspects of sex that can shed light on these important questions. Therefore, it is to be expected that deeper understanding of fertilization at the cellular, molecular, and genetic levels will not only provide much needed information about the process *per se*, but should also help to shed light on the much bigger issues fundamental to the evolution of sex noted above.

Fertilization has been studied in both mammalian and marine invertebrates for decades and the vast majority of information has come from these systems (Vacquier *et al.*, 1997; Sutovsky and Schatten, 1998; Vacquier, 1998; Jonakova *et al.*, 2000; Mori *et al.*, 2000; Swanson *et al.*, 2001, 2003; Galindo *et al.*, 2003; Payne *et al.*, 2003). We now know much about some of the molecules involved in egg activation, sperm capacitation, sperm-egg recognition, and membrane fusion. Although these can all be considered "universal" aspects of fertilization, there is a bewildering array of variations on these themes, which presumably reflect the unique selective constraints and evolutionary history of the individual lineages under study. Perhaps one of the most important results to come from these studies is the demonstration that some of the molecules known to be directly involved in the fertilization process are under strong directional (positive) selection, suggesting an important role in speciation (Vacquier, 1998; Swanson and Vacquier, 2002). Unfortunately, these systems are limited in part because they lack the genetics needed to validate and extend deeper into the pathways involved. In this regard *Drosophila* should be the ideal model system for both genetic and molecular studies of fertilization, although progress in this area has been overshadowed by other advances, for example axial

patterning (see **Chapters 1.2** and **1.8**) and gene regulation, for which *Drosophila* is so well known.

The number of reviews written on the subject of insect fertilization over the recent past is relatively small compared to its mammalian and noninsect invertebrate counterparts. For example, according to a cursory web-based literature search over the past 10 years, there have appeared no fewer than 25 reviews focused on mammalian and non-insect invertebrate groups compared to just four for insects. There are many reasons for this discrepancy. Most are practical in nature, as mammalian and echinoderm systems are easily studied in the laboratory and we, therefore, know correspondingly more about fertilization in these groups. Also, insects are generally, when it comes to fertilization, much more secretive about their sexual activities. Fertilization is often internal and well hidden from prying eyes (the opaque and tough chitinous exoskeleton (see **Chapter 4.4**) being one example of the obstacles thrown up), and *in vitro* fertilization systems have not yet been developed. Thus, biologists interested in insect fertilization have historically focused on cytological descriptions of fixed embryos during early events following sperm entrance (Sander, 1985; Karr, 1996; Fitch *et al*., 1998).

Fortunately, this is now changing through genetic studies that have either directly or indirectly identified genes involved in various aspects of *Drosophila* fertilization. In this chapter, we describe these studies and, where applicable, compare and contrast them to the mammalian and noninsect systems. As mutants that disrupt or abolish crucial key steps in the fertilization process are further identified and characterized, *Drosophila* will continue to become a more useful tool for the study of this important developmental process. For example, both maternal- and paternal-effect lethal mutations have been identified and provided key new insights into the early events of fertilization, while at the same time providing future directions for research.

Interestingly, one of the driving forces for renewed interest in *Drosophila* fertilization comes from studies of *Wolbachia*, an intracellular microbe that lives in insect gonadal tissues and can disrupt the fertilization process. This intriguing microbe infects an astonishing diversity of arthropods and crustaceans and, in doing so, causes an equally astonishing effect on host reproductive biology (Stouthamer *et al*., 1999). These effects are often targeted in the male, and of particular interest to fertilization biologists, *Wolbachia*-infected animals express a form of fertilization failure termed cytoplasmic incompatability (CI) that results in developmental defects following sperm entrance. Further work on the cellular basis of CI should provide insights into basic mechanisms of fertilization.

Finally, as a chapter focused on the molecular genetics of fertilization, this review will necessarily be limited to *D. melanogaster*, and hopefully will be illustrative of this group of dipterans. However, we understand dipterans make up only a small fraction of the Insecta and, therefore, realize that although this review represents a comprehensive review of molecular genetics in *D. melanogaster*, it should not be taken as representative of Insecta in general. Having said that, the final sections deal with fertilization in other insects, where new information relevant to specific problems in fertilization biology has come to light in recent years.

1.6.2. Some Aspects of Fertilization in *Drosophila* are Fundamentally Different from Mammalian and Echinoderm Systems

It is a curious happenstance of scientific history that fertilization was first studied extensively in marine invertebrates such as the now famous purple sea urchin (*Stronglocentrotus purpuratus*). This was the organism of choice for developmental biologists because of the easy access to both gamete types and the ability to perform large-scale *in vitro* fertilization experiments. These pioneering studies led to our current view of fertilization (e.g., see Gilbert, 1988). However, there are three ways, in which fertilization differs between these groups and insects: (1) syngamy, defined as the fusion between gamete plasma membranes, does not occur. This remarkable finding was clearly demonstrated by Perotti almost 30 years ago (Perotti, 1975). It is entirely unclear how this seemingly crude method of entry is initiated, facilitated, or regulated. It is also unclear how the sperm membranes are broken down following entrance; and (2) insects undergo a gonomeric division that has been well-described in a number of insect groups. Gonomeric division is characterized by a delay in fusion of the parental chromosomes; instead, they remain separate during the first mitotic division, and parental chromosome sets fuse only after telophase; and (3) unlike most other taxa, insects in general, and *Drosophila* in particular, make much longer sperm (Pitnick and Markow, 1994; Pitnick *et al*., 1995; see also **Chapter 1.4**). Sperm gigantism is a conundrum of developmental biology with as yet no answer, although some hypotheses have been put forward (Karr, 1991, 1996; Karr and Pitnick, 1996; Pitnick and Karr, 1998).

What is clear is that for many species, including many mammalian, avian, and marine invertebrates, total sperm entrance is a common occurrence. This fact has been known for some time but either under-appreciated or ignored. Thus, a total understanding of fertilization must include the process of sperm entry into the egg, including the sperm flagellum and paternal mitochondria (or its derivative). The fate of these giant structures in the egg has some important implications for sexual organisms (Pitnick and Karr, 1998) but is beyond the scope of this review.

1.6.3. Essential Features of *Drosophila* Fertilization

As a key step of developmental biology, fertilization has surprisingly received very little attention from geneticists until recently. This is now changing in part, because this very first step in development is also providing crucial clues for the understanding of many essential aspects of modern biology, such as epigenetics, chromatin assembly/remodeling, and cell cycle regulation. For example, extensive genetic screens in the nematode *Caenorhabditis elegans* have identified genes essential for the formation of the zygote (Ward and Miwa, 1978; Achanzar and Ward, 1997; Royal *et al*., 1997; Bowerman, 1998; Singson *et al*., 1998; Miller *et al*., 2001; Singson, 2001; Xu and Sternberg, 2003). Interesting advances have also been achieved in mammals, with the first few mutants affecting zygote formation identified in mice (Christians *et al*., 2000; Tong *et al*., 2000; Wu *et al*., 2003a, 2003b). It will, therefore, be of great interest to insect developmental biologists to compare and contrast these findings with similar ongoing work in *Drosophila*. In the following sections of this chapter, we will review basic processes associated with fertilization, and present recent advances in the molecular genetics of fertilization in *Drosophila*.

The large majority of drosophiliids studied are strict bisexually-reproducing species, which means that fertilization of the egg is absolutely required for embryonic development to occur. However, this is not always the case in the genus *Drosophila*. Several species can partially reproduce by parthenogenesis, and at least one species (*D. mangabeirai*) has been described to strictly reproduce parthenogenetically (Murdy, 1959).

1.6.3.1. Cytological Aspects of *Drosophila* Fertilization and Zygote Formation

In *D. melanogaster*, fertilization occurs in the uterus, shortly after egg ovulation. A relatively small number of sperm are stored in two different organs in the female genital tract, the sperm receptacle and the spermathecae (see **Chapter 1.5**). Eggs are usually deposited shortly after fertilization but embryonic development can also begin before egg deposition. The formation of the diploid zygote occurs very rapidly in this species, and it takes only about 17 min from sperm entry to the end of the first zygotic division. In comparison, the same event lasts about an hour in the wasp *Nasonia vitripennis* (Tram and Sullivan, 2002). The following syncytial nuclear divisions of the early *Drosophila* embryo are among the fastest mitoses described in metazoa, the first cycles lasting less than 10 min.

The cytological events that take place in the egg at fertilization have been described with great accuracy by Sonnenblick (Sonnenblick, 1950) and are presented in diagramatic form in **Figure 1**. The mature *Drosophila* oocyte is arrested in metaphase of the first meiotic division (**Figure 1a**, sperm entrance). The metaphase I spindle is located near the egg surface, in an antero-dorsal region. Ovulation is the signal for female meiosis to resume and is concomitant with sperm entry. The second meiotic division is easily recognized by the presence of two anastral spindles associated in tandem, orthogonally to the surface of the egg (**Figure 1b**). In insects, the four meiotic products remain in the egg: the innermost nucleus is determined to become the female pronucleus whereas the three other nuclei form the polar bodies. The polar bodies eventually fuse into a triploid nucleus that remains blocked in a pseudo-metaphase state at the egg periphery and that does not participate in the development of the embryo (star-like structures in **Figure 1e–h**). The mature sperm nucleus in *D. melanogaster* is a highly elongated, 9 μm long, needle-like structure that contains extremely compacted paternal chromatin that must rapidly decondense in the egg cytoplasm to form the male pronucleus.

During the second meiotic division, the sperm nucleus already appears as a round, yet still condensed, nucleus that migrates towards a more centrally located zone of the egg (**Figure 1b**). During and after migration, the sperm pronucleus decondenses its chromatin and reassembles a nuclear envelope from egg components. A pair of centrioles that is located at the base of the flagellum in the basal body is delivered in the egg cytoplasm after the sperm cell membrane breakdown (**Figure 1c**). This is a crucial component because it contributes the first centrosome of the diploid embryo. A large aster, called the sperm aster, develops from the sperm-derived MicroTubule Organizing Center (MTOC) and radiates to the egg periphery (**Figure 1c**). During

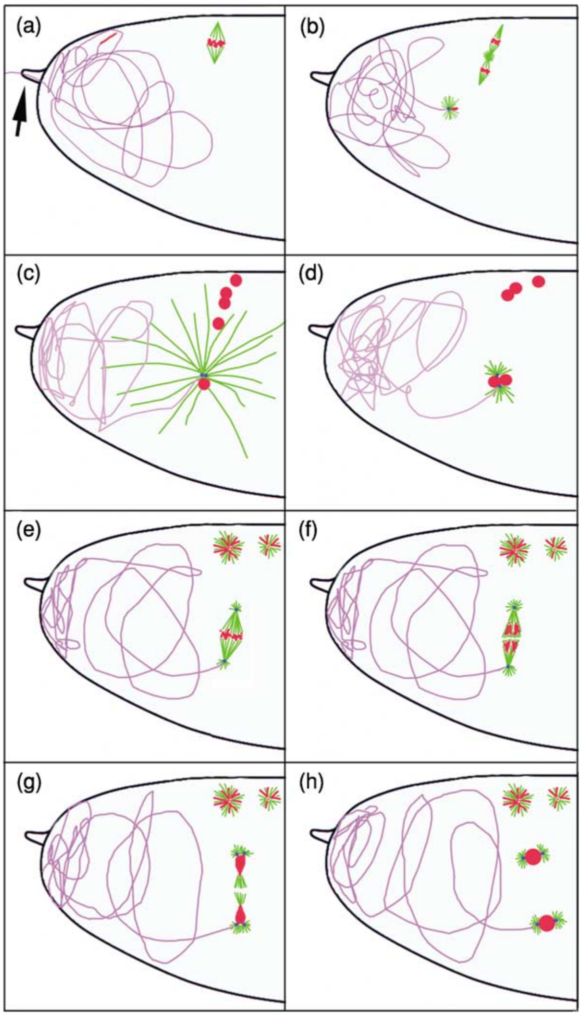


Figure 1 Fertilization in *Drosophila*. (a) Sperm entry. The spermatozoon enters the egg through the micropyle and its flagellum coils in the anterior part of the egg cytoplasm. The sperm nucleus is still very condensed at this stage. The anastral female meiotic spindle is close to the antero-dorsal periphery of the egg. Female meiosis is arrested in metaphase of the first division until egg activation, which is concomitant with sperm entry. (b) Meiosis II. The two meiotic spindles are organized in tandem around a central aster, orthogonally to the egg surface. The sperm nucleus has migrated towards a more central position. A new nuclear envelope has formed around its chromatin that has begun to decondense. A centrosome has formed around the pair of centrioles that are derived from the sperm basal body. (c) Pronuclear formation. Female meiosis produces four haploid nuclei that remain in the egg cytoplasm. The innermost nucleus is determined to become the female pronucleus whereas the three other nuclei remain at the periphery to form the polar bodies. The sperm centrosome duplicates to form the MTOC at the origin of the sperm aster. (d) The female pronucleus migrates towards the male pronucleus, along the microtubules of the giant sperm aster. At the end of its migration, the female pronucleus apposes to the fully decondensed male pronucleus. Pronuclear fusion does not occur at this stage. The sperm aster is progressively replaced by two MTOC that organize the first mitotic spindle. (e) Metaphase of the first zygotic division. Each set of parental chromosomes occupies one half of the gonomeric spindle. The sperm tail remains associated with one of the sperm-derived centrosomes. Chromosomes of the polar bodies have also recondensed into star-like configurations. Usually, the two central meiotic products fuse into a diploid polar body. Frequently, a single triploid polar body is eventually observed at the egg periphery in older embryos. (f) Anaphase. Paternally-derived and maternally-derived sister chromatids remain separated during their migration to the spindle poles. (g) Telophase. Parental genomes fuse only at the end of telophase. Centrosomes duplicate at this stage to prepare next mitosis. (h) Interphase of the second nuclear cycle. Each diploid nucleus is associated with a pair of centrosomes. The sperm tail remains attached to one of them.

its migration, the female pronucleus uses the microtubules from the sperm aster to migrate inwards to its male counterpart. Both pronuclei are eventually found apposed in a central position of the egg. It is important to note that the pronuclei do not fuse in insects (at least in those species where this process has been documented). Instead, they replicate their DNA and proceed into a gonomeric division (metaphase, Figure 1e), where both parental sets of chromosomes divide independently within a

common spindle (anaphase, **Figure 1f**). Each set of chromosomes occupies one half of the gonomeric spindle until telophase (**Figure 1g**) when parental genomes eventually fuse to form the first two diploid nuclei (**Figure 1h**). The gonomeric division is a crucial advantage for the analysis of fertilization mutant phenotypes, because it allows one to distinguish mutants that affect one set of parental chromosomes without affecting the division of the other (see below).

During the time used for female meiosis to resume, a single spermatozoon enters the egg through the micropyle and delivers its nucleus into the egg cytoplasm. Polyspermy, the entrance of more than one sperm, has been observed rarely in *D. melanogaster* (<1%) with higher rates observed in some crosses of *D. pseudoobscura* (Snook and Karr, 1998). Polyspermy in these cases results in participation of only a single male pronucleus in zygote formation.

Drosophila spermatozoa are characterized by a gigantic flagellum that can reach the amazing size of several centimetres in some species (Joly *et al*., 1995; Pitnick *et al*., 1995). A survey of the genus revealed tremendous variation in sperm length over the entire spectrum of species within this clade (Pitnick, 1996). For example, *D. melanogaster* sperm are about the size of the adult male (~1.8 mm), while sperm in members of the *virilis* subgroup, e.g., *D. virilis, D. montana, D. littoralis*, are considerably longer and many times their body length (5–8 mm) (see also **Chapter 1.4**). Incredibly, in almost all cases observed, the entire male gamete completely enters the egg cytoplasm. This rather fantastic observation was first seen in sectioned eggs (Hildreth and Lucchesi, 1963) and was later described in whole-mount eggs using sperm-specific antibodies (Karr, 1991), and later shown (Karr and Pitnick, 1996) to be a regular phenomenon in numerous species (**Figure 2**). To achieve this tour de force, the flagellum is coiled into several loops in the anterior part of the egg. During early development, the sperm flagellum remains in the anterior part of the embryo and is partially degraded. What remains of this structure is eventually internalized in the embryonic gut before being eliminated with the larval wastes (Pitnick and Karr, 1998).

What is the purpose of such extraordinary long sperm? This question, first raised over a decade ago (Karr, 1991), has as yet no clear answer. One possibility is that the "sperm-derived structure" has some as yet unrecognized postfertilization function. However, studies of the patterns of sperm tail entrance during fertilization in *D. hydei* and *D. bifurca* (where only a small fraction of the sperm enters the egg), clearly demonstrated that the complete sperm entrance is not an absolute requirement for fertilization (Karr and Pitnick, 1996). Indeed, comparative phylogenetic analysis of the patterns of fertilization in these groups demonstrated that over 35 mm of sperm length evolution occurred in the male that did involve a postfertilization function (Karr and Pitnick, 1996). The fact still remains, though, that several millimeters of sperm flagellum enter the egg upon fertilization in all cases studied, and the function, if any, of the resulting sperm-derived structure has not been resolved.

While direct postfertilization functional studies of sperm have yet to be conducted, some clues about the evolutionary significance of sperm-female co-evolution have recently emerged from laboratory selection studies. These studies selected on both female receptacle and sperm length in *D. melanogaster* and resulted in two clear classes of populations which differed significantly (Miller and Pitnick, 2002). Miller and Pitnick went on to show that females could discriminate between sperm in competition experiments, showing convincingly a role for sperm length in reproductive fitness. However, the ability of the female to discriminate among sperm of differing lengths does not directly answer the question as to why the entire structure enters the egg. Future functional studies are needed to provide a more complete overall picture of sperm utilization by females, the evolution of sperm gigantism and fertilization. As described further below, a genetics approach has proven extremely successful in identifying genes involved in early stages of fertilization.

1.6.3.1.1. Sperm heteromorphism in *Drosophila* – an evolutionary enigma It is a common occurrence for some groups of insects to produce more than one class of sperm. Termed polymegaly, or sperm heteromorphism, this phenomenon has best been described in the Lepidoptera where there is production of two distinct sperm types, a eupyrene type that contains a haploid genome and an apyrene type that contains no DNA but otherwise is normal. Apyrene are often motile and can be produced in large quantities. The significance and function of apyrene sperm is not known (see Section 1.6.6.4.2 for further discussion of lepidopterans).

Although rare in *Drosophila*, the *obscura* subgroup is distinguished by the presence of two sperm classes, short and long (review: Snook, 1997). Unlike lepidopterans, *Drosophila* of both short and long sperm types in this group produce DNA-containing sperm and therefore both should be fertilization competent. However, it has been shown quite convincingly that only the longer

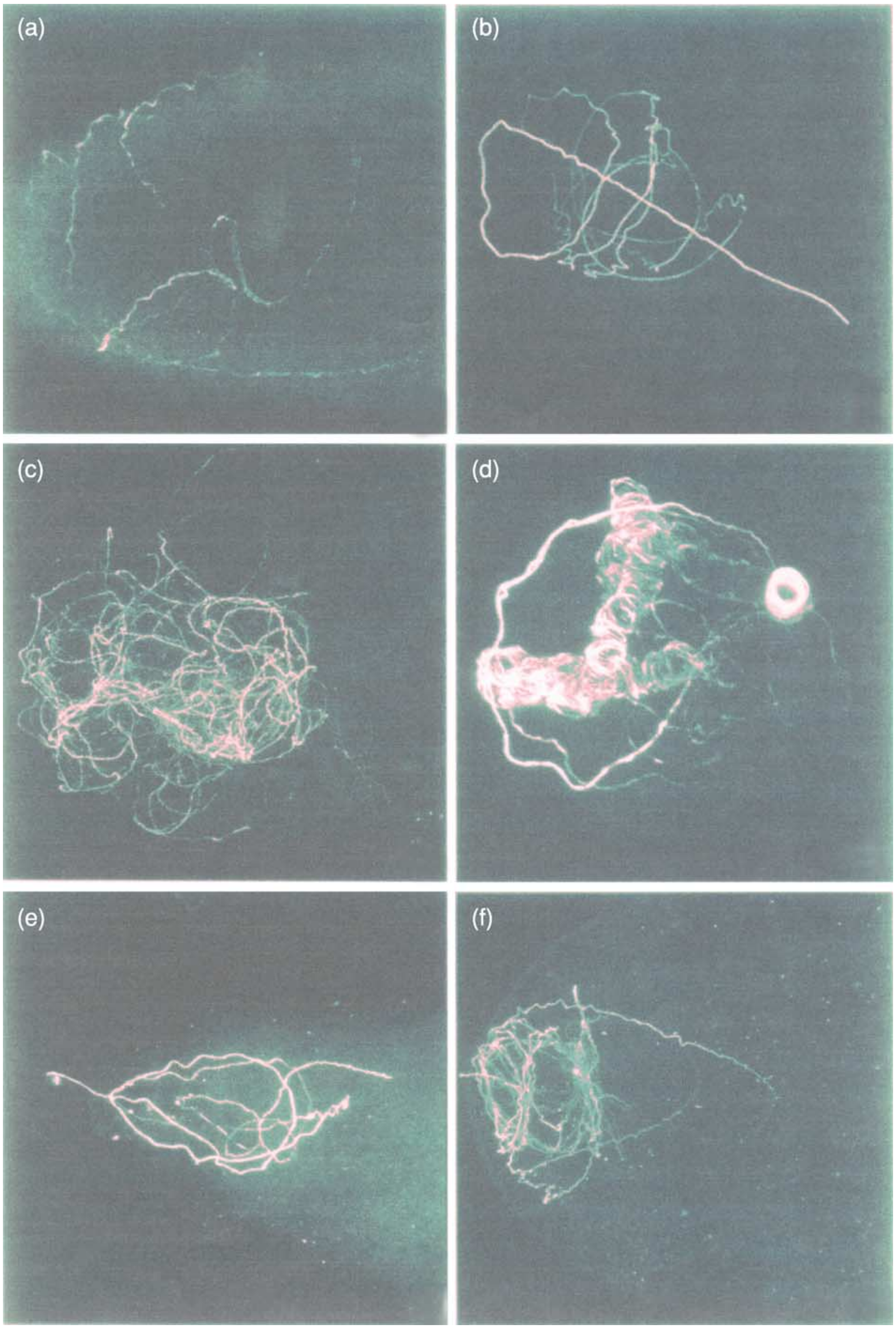


Figure 2 Sperm entry in *Drosophila*. Fertilization in six species of *Drosophila* observed using an anti-sperm polyclonal antibody. (a) *D. busckii*. (b) *D. simulans*. (c) *D. ezoana*. (d) *D. pachea*. (e) *D. bifurca*. (f) *D. wassermani*.

sperm types are capable of fertilization, as determined by direct measurement of sperm in the egg (Snook *et al*., 1994; Snook and Karr, 1998). Thus, the existence and maintenance of multipe sperm types remains an outstanding developmental and evolutionary conundrum. However, it is quite clear that the answer will lie in understanding why short sperm do not, or cannot, enter the egg.

1.6.4. Genetic Analysis: Fertilization Functions Identified by Parental Effect Mutations

In *Drosophila*, formation of the zygote and early embryonic development take place without any transcriptional activity and, thus, are essentially dependent on maternal gene products (mRNA and

proteins) delivered in the oocyte at the end of oogenesis. In comparison with the egg, the volume of the sperm cell is negligible and it contains virtually no cytoplasm. It is, thus, not expected to substantially contribute to the pool of gene products necessary for the formation of the zygote. It should be mentioned that it is unknown whether or not the gigantic flagellum, which could represents a potential source of paternal products, has any function for the zygote. However, aside from its obvious contribution of the paternal genome complement, the sperm delivers a centriole pair that is required for the assembly of the first MTOC of the zygote. Thus, a mutation that affects any step between fertilization and the formation of the zygote must exert its phenotype through a strict parental effect. These mutations can be either maternal or paternal effect mutations, and they usually induce embryonic death. Practically, most of them behave as female or male sterile mutations, in the sense that they do not produce viable progeny. However, developmental arrest can occur at various stages depending on the nature of the defects that affect the formation of the zygote. In some cases, the spermatozoon is not activated and the egg forms only polar bodies, just like an unfertilized egg. In other cases, the mutation can compromise the ability of one set of parental chromosomes to integrate into the zygote nuclei or to achieve the first nuclear cycle. Because of the gonomeric nature of the first division, the resulting embryos develop with the set of chromosomes that are not affected by the mutation. In practice, embryos develop as haploid or aneuploid embryos. Whereas aneuploid development arrests after a few nuclear divisions, haploid *Drosophila* embryos reach late embryogenesis but all die shortly before hatching. The fact that haploid development is lethal in *Drosophila* allows screening for parental effect embryonic lethal mutations.

In the sections below the various groups of characterized mutants depending on the biological process they affect during fertilization and/or the formation of the diploid zygote will be reviewed.

1.6.4.1. Acrosome Reaction/Sperm Activation

The processes by which the sperm locates, recognizes, and enters the egg have been described in details in several models such as echinoderms and mammals (Trounson and Bongso, 1996; Wolfsberg and White, 1996; Benoff, 1997; Vacquier, 1998; Wassarman, 1999; Serrano and Garcia-Suarez, 2001; Evans, 2002). Although ultrastructural studies have reported that insects, like higher animals, do possess an acrosome at the tip of their sperm, the molecular basis of sperm-egg recognition in insects is largely unknown. Insights have recently come with the identification of several *Drosophila* mutants that apparently affect sperm-egg interaction.

1.6.4.1.1. *casanova* In animals, sperm-egg recognition usually relies on the interaction of carbohydrate residues on the surface of the egg's vitelline membrane with complementary molecules on the plasma membrane of the sperm. In *Drosophila*, biochemical analysis have identified two distinct glycosidases at the surface of male gametes: β-*N*-acetylglucosaminidase and α-D-mannosidase (Cattaneo *et al*., 1997, 2002; Pasini *et al*., 1999; Perotti *et al*., 2001). *casanova (csn)* is a *Drosophila* male sterile mutant with a unique phenotype: *casanova* mutant males produce motile sperm that are stored in females but that fail to enter wild-type eggs (Perotti *et al*., 2001). Ultrastructural analysis of *casanova* sperm revealed that β-*N*-acetylglucosaminidase is absent from the spermatozoa membrane overlying the acrosome, where it is normally present. Although the *csn* gene has not been identified yet and probably does not encode this enzyme, this study suggests that a molecular sperm-egg recognition system (involving at least β-*N*-acetylglucosaminidase and its substrate β-*N*-acetylglucosamine) exists in insects, like in higher animals.

1.6.4.1.2. *sneaky* and *misfire* These two mutants are paternal effect lethal genes that display almost identical phenotypes (Fitch *et al*., 1998; Ohsako *et al*., 2003). *sneaky (snky)* and *misfire (mfr)* mutant males produce motile sperm that are able to enter the eggs but the sperm nucleus remains intact at the periphery of the egg cytoplasm (**Figure 3**). Cytological analysis of mutant sperm after fertilization suggests that the sperm activation defect, observed in both cases, is a consequence of the inability of these sperm to remove their plasma membrane after fertilization. Without plasma membrane breakdown, the sperm nucleus is not delivered to the egg cytoplasm and is thus unable to decondense its DNA. Furthermore, the paternal centrioles are not available for the egg and this prevents the migration of the female pronucleus and the potential division of maternal chromosomes. The molecular identification of *snky* and *mfr* should allow a better understanding of this crucial mechanism of sperm plasma membrane breakdown at fertilization.

1.6.4.2. Female pronucleus formation/migration Several mutants have been described that affect either the formation or the migration of the female pronucleus towards its male counterpart. In fact,

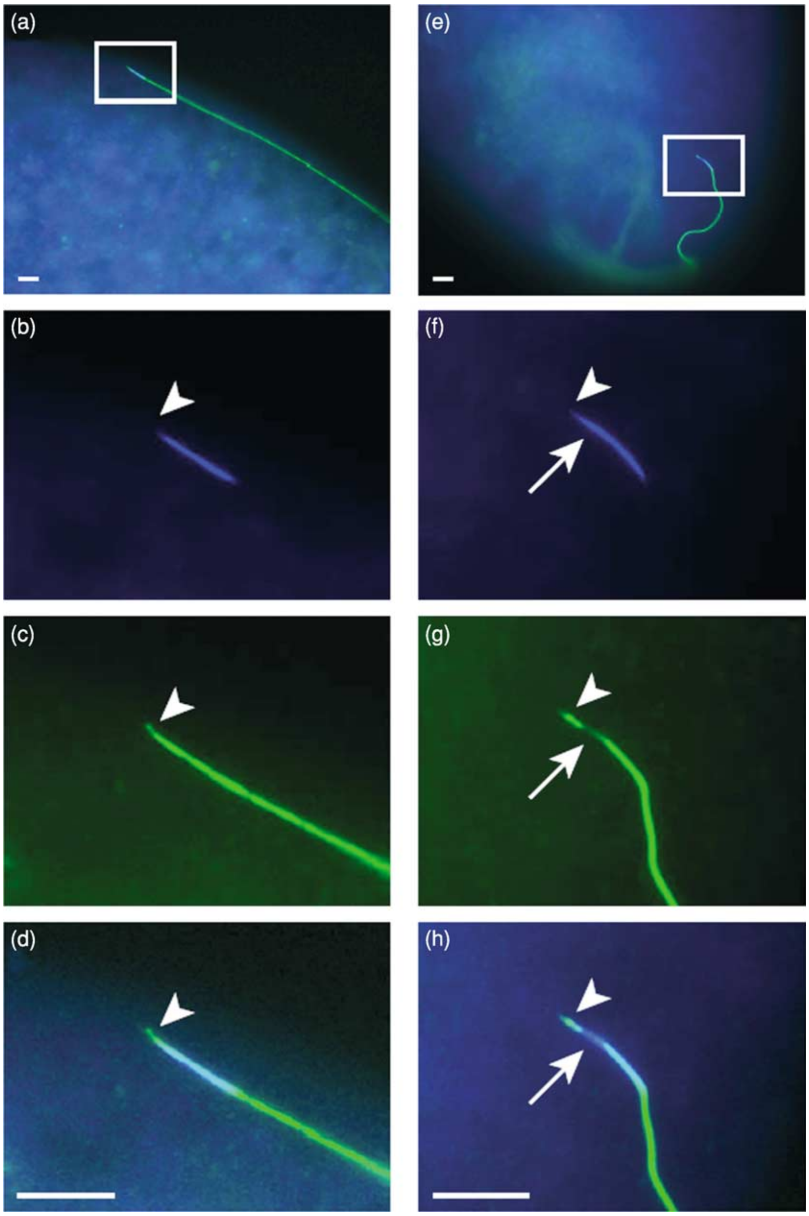


Figure 3 *misfire* (*mfr*) sperm in inseminated eggs. (a, high) and (e, low) magnification views of fertilization by *misfire* sperm (green; visualized using a sperm antibody), and DNA (blue; visualized by the DNA-specific dye, DAPI); (b, f), high magnification views of misfire sperm DNA (boxed regions in (a) and (e) showing needle-like morphology; (c, g) anti-sperm staining of (b, f) using a monoclonal anti-sperm antibody DROP 1.1; (d, h) the area of *mfr* sperm-inseminated eggs surrounded by the squares shown in panels (a) and (e), respectively. Arrowheads indicate the acrosome region; arrows indicate less stained region by DROP 1.1. Scale bar = 10 μm. (Reproduced with permission from Ohsako, T., Hirai, K., Yamamoto, M.T., **2003**. The *Drosophila* misfire gene has an essential role in sperm activation during fertilization. *Genes Genet. Sys. 78*, 253–266.)

all these mutants are allelic to genes involved in female meiosis and some of them are presented in this section.

1.6.4.2.1. α-*Tubulin 67C* and *polo* In some maternal effect alleles of α-*Tubulin 67C* and *polo*, female meiosis is abnormal and the female pronucleus is not properly formed (Komma *et al*., 1991; Riparbelli and Callaini, 1996). In the absence of a well-defined female pronucleus, the first spindle is assembled around paternal chromosomes. However, occasionally paternal chromosomes can undergo mitotic divisions and haploid androgenetic embryos (that invariably die) are observed. Presumably, because *polo* and α-*Tub 67C* are required for the assembly of normal mitotic spindles, the division of the male pronucleus is frequently impaired.

1.6.4.2.2. *KLP3A* and *wispy* *KLP3A* encodes a kinesin-like protein that is required both for male and female meiosis (Williams *et al*., 1995). In eggs laid by females homozygous for a strong allele of *KLP3A*, the meiosis is apparently normal and produces four haploid nuclei. However, none of these meiotic products migrate towards the male pronucleus, despite the presence of a sperm aster (Williams *et al*., 1997). Although an apparently normal spindle assembles around the paternal chromosomes in *KLP3A* eggs, the first mitotic division is invariably arrested in metaphase. *KLP3A* is, thus, required both for the female pronucleus to migrate but also to assemble functional mitotic spindles.

The phenotype of eggs from *wispy (wisp)* females is very similar to the KLP3A phenotype but in this case, the sperm aster is reduced and the spindle associated with the male pronucleus is abnormal with frequent dissociation of centrosomes. It has been shown that centrosomal proteins are reduced or absent in the centrosomes of *wispy* embryos. The *wispy* gene product is, thus, probably involved in the regulation of microtubule structures (Brent *et al*., 2000).

1.6.4.3. Sperm Chromatin Processing Before and After Fertilization

Spermiogenesis is a long and very complex process where the haploid spermatids produced by meiosis II differentiate into mature spermatozoa (see **Chapter 1.4**). Two major events occur during spermiogenesis that lasts several days in *Drosophila*. One obvious event is the formation of the flagellum by a process that involves the coordinated extension of the axoneme with the surrounding cell membrane and mitochondria derivatives. The other major event is the dramatic condensation of the spermatid nuclei. This is generally achieved (in mammals for example) by the partial or complete replacement of somatic histones (H2A, H2B, H3, and H4) with male germline specific transition proteins, sperm specific histones and/or protamines. In *Drosophila*, somatic histones are eliminated during spermatid condensation but the final molecular composition of the sperm chromatin in this species is still unknown.

The DNA in the *Drosophila* sperm nucleus is extremely compacted but it must decondense very rapidly once delivered in the egg cytoplasm at fertilization. The formation of the male pronucleus, in general, represents a very interesting situation for the study of chromatin assembly and remodeling, and many biochemical studies using various model organisms (including *Drosophila*) have focused on the purification of egg molecules capable of decondensing sperm nuclei *in vitro*. A classical example is nucleoplasmin, a protein abundant in amphibian eggs that has been shown to function as a histone chaperone *in vitro* (Philpott *et al*., 1991).

Drosophila is a particularly suitable organism to identify genes involved in the processing of sperm chromatin in the egg cytoplasm at fertilization. Because of the gonomeric nature of the first division, any defect in sperm chromatin processing is expected to induce haploid gynogenetic development of at least a fraction of the embryos. The following examples illustrate *Drosophila* as a promising model for the understanding of this fundamental aspect of fertilization.

1.6.4.3.1. *sésame* *sésame (ssm)* was originally obtained in a screen for X-linked female sterile recessive mutations. Homozygous *ssm*^{185b} females are completely sterile, independent of the genotype of the males used in the crosses. *sésame* is an embryonic lethal maternal effect mutation: mutant females produce normal eggs but the embryos that develop die just before hatching. These embryos die not directly because of the mutation but because of the fact they are haploid, containing only maternally-derived chromosomes. This phenotype is indicative of a defect of paternal chromosomes to integrate in the zygote nuclei and, thus, suggests a problem at fertilization. Haploid gynogenetic development of embryos is a phenotype that is found in two other parental effect mutants that will be described below. In eggs produced by *sésame* females, the male pronucleus is severely delayed in decondensation compared to wild-type (**Figure 4a** and **b**). The gonomeric spindle is organized only around the female pronucleus and the female chromosomes divide normally (**Figure 4c**). In contrast, the male pronucleus appears abnormally condensed and usually remains excluded from the first spindle (Loppin *et al*., 2000).

Interestingly, in eggs from *ssm* females, maternally provided core histones are not deposited in the chromatin of the male pronucleus, which suggests that the paternal DNA remains associated with sperm-specific chromosomal proteins in this mutant context (Loppin *et al*., 2001a). The *sésame* function is, thus, required for the assembly of nucleosomes in the male pronucleus. The *ssm* mutation, with its unique phenotype, represents the first genetic demonstration that the chromatin assembly in the male pronucleus is dependent on a maternal factor. *ssm* has been recently shown to be a point mutation in the *Hira* gene, which encodes a conserved member of the WD-repeat protein family (Loppin *et al*, unpublished data). In *Xenopus*, HIRA is critical for the assembly of nucleosomes independent of DNA replication (Ray-Gallet *et al*., 2002), a crucial

function as the male pronucleus must assemble chromatin prior to DNA replication. In *Drosophila*, *Hira* expression is not restricted to the female germline, and the *ssm*[185b] allele probably only affects the function of *Hira* at fertilization. New alleles should allow one to determine if *Hira* has a more general role in the assembly of chromatin independent of DNA replication.

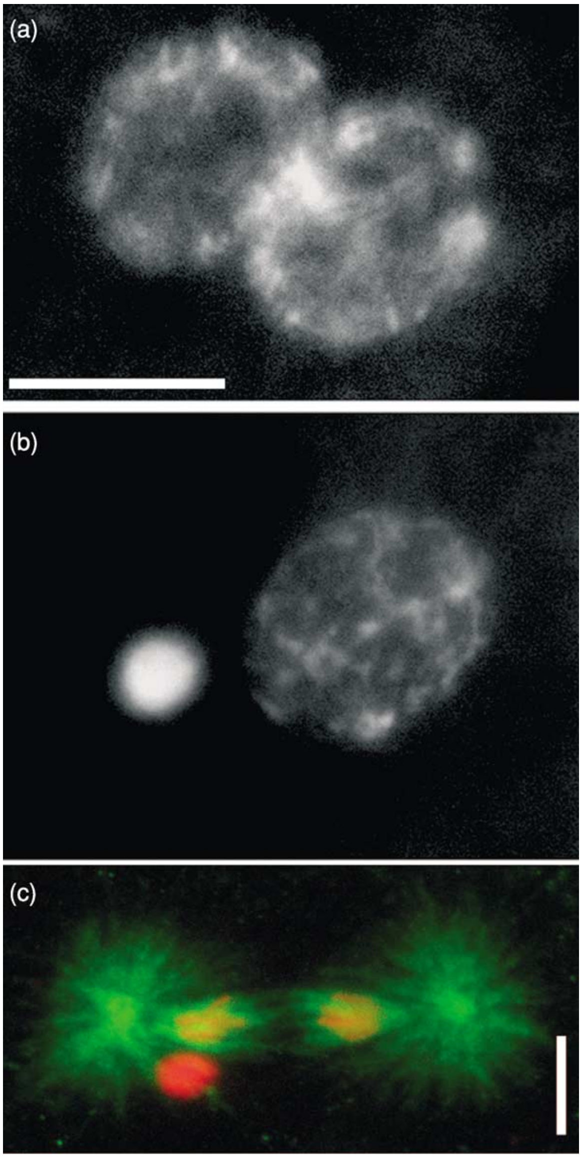


Figure 4 The *sésame* phenotype. Confocal images of fixed eggs stained with a DNA fluorescent dye. (a) Apposed pronuclei in a wild-type fertilized egg. (b) Pronuclei at the same stage in a fertilized egg laid by a ssm female. The small, bright nucleus on the left is the male pronucleus. (c) Anaphase of the first division in an embryo from a *ssm* female. The condensed male pronucleus (red, DNA) is excluded from the gonomeric spindle that contains maternally-derived sister chromatids (yellow embedded in spindle microtubules colored green). Scale bar = 10 μm.

1.6.4.3.2. *maternal haploid* *maternal haploid (mh)* is, like *sésame*, another maternal effect embryonic lethal recessive mutation. Historically, *mh* was the first mutant described to produce haploid embryos (Santamaria and Gans, 1980). Early haploid embryos do not present any major phenotype, with the exception that cellularization occurs after an extra round of nuclear division (Edgar *et al.*, 1986). Haploid embryos die later in embryogenesis and cuticle preparations of dead embryos reveal head involution defects and/or dorsal open phenotypes in all reported cases (Fuyama, 1984; Loppin *et al.*, 2000, 2001b).

Fertilization in eggs produced by *mh* females was more recently analyzed in order to understand the mechanism of paternal chromosome elimination from the developing embryos (Loppin *et al.*, 2001b). In contrast to *ssm* eggs, the male pronucleus in *mh* eggs fully decondenses and apposes to the female pronucleus, and both pronuclei are found within the gonomeric spindle. However, paternal sister chromatids are unable to separate at anaphase of the first zygotic mitosis (**Figure 5**). When female sister chromatids have fully separated during anaphase, the paternal chromatin lags behind on the metaphase plate. Paternal DNA, at least partially,

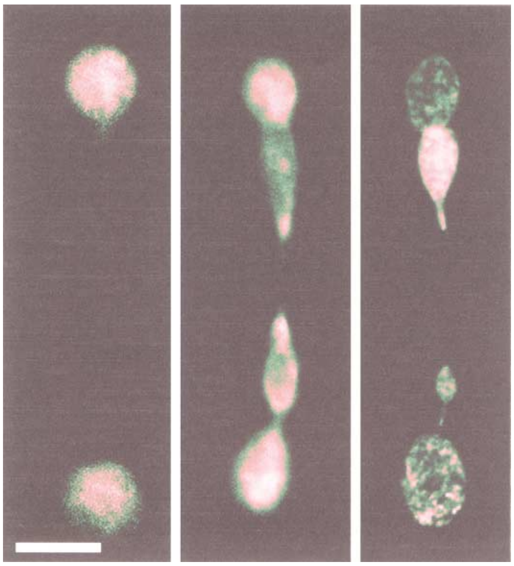


Figure 5 The *maternal haploid* phenotype. Confocal images of early embryos stained with a DNA fluorescent dye. Left panel: Late telophase of the first zygotic division in a wild-type egg. Middle panel: An embryo at the same stage from a *mh* female. The lagging paternal chromatin has formed a bridge between the two daughter nuclei containing the maternal chromatin. Right panel: An embryo from a *mh* female at the beginning of the second nuclear cycle. The haploid daughter nuclei are in interphase. Uneven segregation of paternal chromatin is clearly seen in this embryo. Scale bar = 10 μm.

replicates in *mh* eggs, as revealed by observation of a pericentromeric chromatin marker, but sister chromatids do not properly separate at the onset of anaphase. In telophase, the lagging paternal chromatin eventually forms a chromatin bridge and is torn apart by the traction of the spindle. The unequal segregation of paternal chromatin at the first division is responsible for the aneuploid development of a majority of the embryos, which usually die after a few nuclear divisions. Some of them (about 25%) manage to escape this early lethal phenotype as haploid gynogenetic embryos and die before hatching.

The *mh* phenotype demonstrates a maternal function that specifically controls the processing of paternal chromatin at fertilization. It is not clear whether *mh* directly affects the condensation of paternal chromosomes at the first division or the separation of sister chromatids. Alternatively, it is possible that *mh* is involved in sperm chromatin remodeling but with an associated mutant phenotype only detectable at the first mitosis. It is interesting to note that very similar effects on paternal chromosomes at the first division are observed for the paternal effect mutant *ms(3)K81* described below.

1.6.4.3.3. *ms(3)K81* *ms(3)K81* (*K81*) is the third mutant that produces haploid gynogenetic embryos but, in contrast to *sésame* and *maternal haploid*, *K81* is a strict paternal effect embryonic lethal mutant. Homozygous *K81* males do not present any apparent spermatogenesis defects: they produce motile sperm that fertilize eggs but the resulting embryos die. Similar to *mh*, most embryos from *K81* fathers (~90%) die after a few nuclear cycles but very few (<1 in 10^5) hatch as haploid gynogenetic embryos (Fuyama, 1984). The original *K81* allele (*K81*^{1}) was collected in the field in Japan but new deletion alleles were subsequently recovered (Yasuda *et al*., 1995). At the cytological level, the *K81* phenotype at fertilization is undistinguishable from the *mh* phenotype: paternal chromosomes are integrated in the gonomeric spindle but only maternal chromosomes divide correctly. It is interesting to note that the very same phenotype can be obtained from a maternal (*mh*) and a paternal effect (*K81*) mutant (**Figure 6**). It is reasonable to think that the *K81* phenotype is most probably due to a defect in sperm chromatin organization.

We have recently identified the gene responsible for the *K81* phenotype (B. Loppin, P. Couble and T. Karr, unpublished data). It is a small gene that contains a single exon of 555 bp with no intron that is specifically expressed in the male germline. Surprisingly enough, we have not found any ortholog of *K81* in the available database, even in the recently sequenced *D. pseudoobscura* and *Anopheles gambiae* genomes. *K81* has only been found in the closest relatives of *D. melanogaster* and the gene appears to rapidly evolve within the *melanogaster* subgroup.

K81 is, thus, a gene that is absolutely required for male fertility but that is paradoxally restricted to a few species. It is now established that many male-specific genes involved in spermatogenesis are among the most rapidly evolving genes (Vacquier *et al*., 1997; Ting *et al*., 1998; Tsaur *et al*., 2001; Swanson and Vacquier, 2002). The *K81* mutant phenotype is unique and suggests a defect in sperm chromatin organization or an aberrant epigenetic marking of the paternal genome during spermatogenesis.

1.6.4.3.4. *paternal loss* and *Horka* *paternal loss (pal)* is another strict paternal effect mutant that induces the loss of paternal chromosomes in a proportion of their progeny. Paternal chromosomes are lost at different frequencies depending on the chromosome, the fourth being the most frequently lost (~17%) whereas the Y is lost in less than 1% of embryos (Baker, 1975). It has been suggested that *pal* could encode a component of paternal chromosome centromeres (Baker, 1975). The *pal* gene product is a small basic protein of 121 amino acids that lacks significant homology with known proteins (Fitch *et al*., 1998), similar to the K81 protein (see above). The PAL protein localizes to spermatid and sperm nuclei and is, thus, probably a sperm chromosomal protein. The *pal* loss-of-function phenotype is not accompanied by any detectable defect of spermatid condensation, but its absence is critical for the stability of paternal chromosomes after male pronuclear formation (Fitch *et al*., 1998). An intriguing aspect of the *pal* phenotype is that paternal chromosome loss can occur as late as the syncytial blastoderm stage. The defect on the paternal chromosome is, thus, probably maintained through successive mitotic divisions by an epigenetic mechanism whose nature remains to be determined.

Fs(3)Horka (Hor) was originally isolated as a dominant female sterile mutant that induces meiotic defects and early embryonic development arrest (Szabad *et al*., 1995). In eggs laid by heterozygous females, the male and female pronuclei approach together but they do not divide. *Horka* also induces equational nondisjunction during spermatogenesis and possesses a dominant paternal effect that, similarly to *pal*, induces paternal chromosome loss during early embryonic development, with the exception of the Y chromosome. This suggests

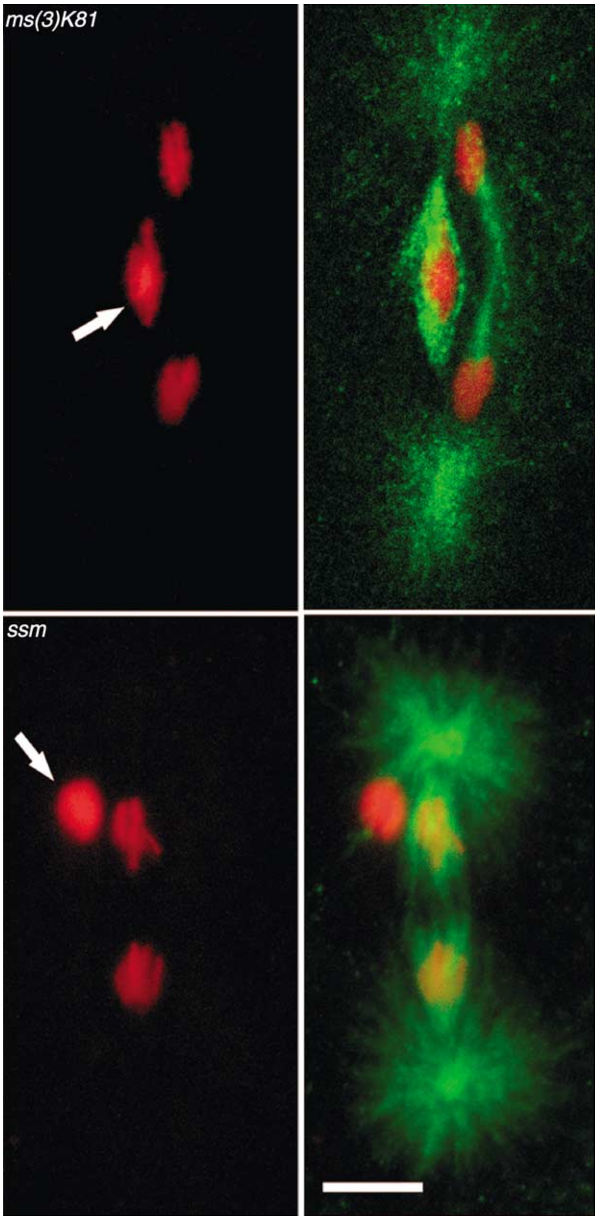


Figure 6 Comparison of mitotic phenotypes observed in *ms(3)K81* and *sesame* (*ssm*) mutants using anti-tubulin antibodies to label microtubules (green) and a DNA-specific flurochrome to label DNA (red). (Upper panels) Mitotic defect resulting from fertilization by *ms(3)K81* sperm displaying highly condensed paternal DNA of the male pronucleus (arrow). (Lower panels) *ssm* eggs fertilized by wildtype sperm displaying highly condensed maternal DNA of the female pronucleus (arrow). (Adapted from Loppin, B., Docquier, M., Bonneton, F., Couble, P., **2000**. The maternal effect mutation sesame affects the formation of the male pronucleus in *Drosophila melanogaster*. *Devel. Biol.* *222*, 392–404.)

that *Horka* could be required for normal function of the centromere, and the identification of the *Horka* gene product should allow testing of this hypothesis.

1.6.4.4. Cell Cycle Regulation

In this last section several mutants that do not exactly affect fertilization are grouped but instead control some aspects of the early mitotic cycles that take place immediately after pronuclear apposition. Since the functions affected by these mutations are critical for the completion of the cell cycle, defects are observed as early as the pronuclear stage.

1.6.4.4.1. *giant nuclei, plutonium*, and *pan gu* These three mutations are maternal effect embryonic mutations that affect the coupling of DNA replication and mitosis. *giant nuclei* (*gnu*), *plutonium* (*plu*), and *pan gu* (*png*) (Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991) all induce, through maternal effect, the formation of giant nuclei in unfertilized eggs or early embryos. The formation of giant nuclei results from the successive rounds of DNA replication that take place without mitosis. In unfertilized eggs, the female meiotic products all become giant, whereas in fertilized eggs this mutation also affects the male pronucleus. It has been recently shown that these three gene products coordinate the rapid succession of S-M phases in the early embryos by ensuring adequate Cyclin B protein level (Lee *et al.*, 2001, 2003). *png* encodes a serine/threonine protein kinase (Fenger *et al.*, 2000), and *plu* and *gnu* encode small, novel proteins (Elfring *et al.*, 1997; Renault *et al.*, 2003). PNG, Plu, and GNU constitute a novel protein kinase complex that specifically regulates S-M cell cycles.

1.6.4.4.2. *fs(1)Ya (Ya)* *fs(1)Ya* (*Young arrest*) is a maternal effect mutation that induces an arrest of development immediately after meiosis (Lopez and Wolfner, 1997). In arrested eggs, the pronuclei are close to each other, but their chromatin looks abnormally condensed and a normal spindle is not formed (Lin and Wolfner, 1991). *Ya* encodes a nuclear lamina protein that localizes to the nuclear lamina of both male and female pronuclei, as well as polar bodies. Interestingly, it has been shown recently that the YA protein is also able to bind chromatin through its interaction with DNA and histone H2B (Yu and Wolfner, 2002). This suggests that YA could regulate the condensation state of nuclei through a direct interaction between nuclear lamina and chromatin. It also raises the possibility that YA regulates the transition from meiosis to mitosis.

1.6.5. *Wolbachia*-Mediated Cytoplasmic Incompatability

Wolbachia are Gram-negative bacteria that are extraordinarily wide-spread in the animal kingdom,

particularly invertebrates. Estimated to infect 20–75% of all insects (now estimated over one million species), they also infect numerous noninsect invertebrates including spiders, mites, and various nematode species that cause diseases in humans (Werren, *et al*., 1995a, 1995b; Jeyaprakash and Hoy, 2000). *Wolbachia* are obligate intracellular microbes that cannot be cultured *in vitro* and, therefore, relatively little is known about their life-history traits, evolutionary history, and molecular biology. The unique biology of *Wolbachia* has attracted a growing number of researchers interested in questions ranging from the evolutionary implications of infection to the use of this agent for pest and disease control.

Wolbachia are closely related to the *Rickettsia*, microbes that cause numerous diseases of mammals, including humans. Molecular phylogenies based upon 16S rDNA and other gene sequences indicate a close relationship among these taxa and places the genus *Wolbachia* as a sister taxon to *Ehrlichia*, and within the clade linking *Ehrlichia*, *Wolbachia*, and *Rickettsia*. However, mapping of associated biological traits onto the phylogeny reveals that *Wolbachia* have secondarily specialized in manipulation of host reproductive biology and simultaneously lost their mammalian pathogenicity. *Wolbachia* is unique among members of this clade in that it does not cause mammalian disease and specifically targets and manipulates host reproductive processes, and this, consequently, represents an important evolutionary novelty.

The enormous diversity of species infected by *Wolbachia* and the manipulation of specific life history characters reflects a long history of coevolution within the host. As an obligate endosymbiont, *Wolbachia* must survive in an intracellular environment that presents two challenges: first, the bacteria must somehow manipulate the host immune response in a manner that renders that response ineffective (or even beneficial) and, second, replication of the bacteria must be integrated with the host environment such that replication and proliferation take place in the appropriate cellular environment. The phenomenal success of this organism in colonizing diverse arthropod lineages is testimony to its ability to solve these linked problems.

The astonishing ability of *Wolbachia* to manipulate host reproductive biology to foster their own propagation – feminization and parthenogenesis, androcidal effects, and cytoplasmic incompatibility – reflects extensive, high level integration of the parasite's biology with that of its host. Many members of the Hymenoptera (wasps, bees) undergo haplo-diploid development: fertilized eggs develop as diploid females, unfertilized eggs develop as haploid males (see **Chapter 3.13**). *Wolbachia* also induce parthenogenesis by "gamete duplication" that results in a diploid eggs. Among isopods, *Wolbachia* infection suppresses the development of an androgenic gland necessary for masculine differentiation. Androcidal, or male-killing, *Wolbachia* infections occur in Lepidoptera and Coleoptera, and, more recently, has been found in *Drosophila*. Thus, despite displaying a general facility to infect a wide variety of arthropods, *Wolbachia* also has the ability to control complex components of host development. This suggests that the various *Wolbachia* strains have sufficient history in current host species to have evolved mechanisms, which target these very diverse reproductive biology systems in their specific hosts.

1.6.5.1. Cytoplasmic Incompatibility: Sperm-Mediated Egg Lethality

Cytoplasmic Incompatibility (CI) was first recognized by the failure of certain intra- and interstrain crosses between and among various insect taxa (review: Stouthamer *et al*., 1999). In the most general case, uninfected females mated to infected males fail to produce viable progeny. In all cases so far studied, CI is manifested as the failure of eggs to hatch. Interestingly these eggs are fertilized, negating the trivial explanation that incompatible crosses result from fertilization failure. A schematic view of this is shown in **Figure 7** along with images of fertilization in both wild-type and CI crosses. The ramificatons of this process are clear – infected females win out over uninfected females 2:1 and, eventually, all other factors being equal, will completely replace the uninfected population.

CI results in early egg lethality following sperm entry (Callaini *et al*., 1996, 1997; Lassy and Karr, 1996). The earliest observed defects in CI crosses are chromosomal abnormalities during the initial zygotic division following fertilization. Developmental anomalies at later stages of development also occur, presumably as a result of this very early defect (**Figure 7**). Neither the nature of these differences nor their origins during development are yet known. However, it is clear that the developmental defects observed must have arisen earlier in the male during spermatogenesis for the following reasons: (1) an incompatible cross occurs between an infected male and an uninfected female and, therefore, the female has a less significant role in the expression of CI; and (2) mature sperm from infected males are devoid of *Wolbachia* (Snook *et al*., 2000). However, because the testes, where sperm are produced, harbor the majority of *Wolbachia*, sperm develop in an

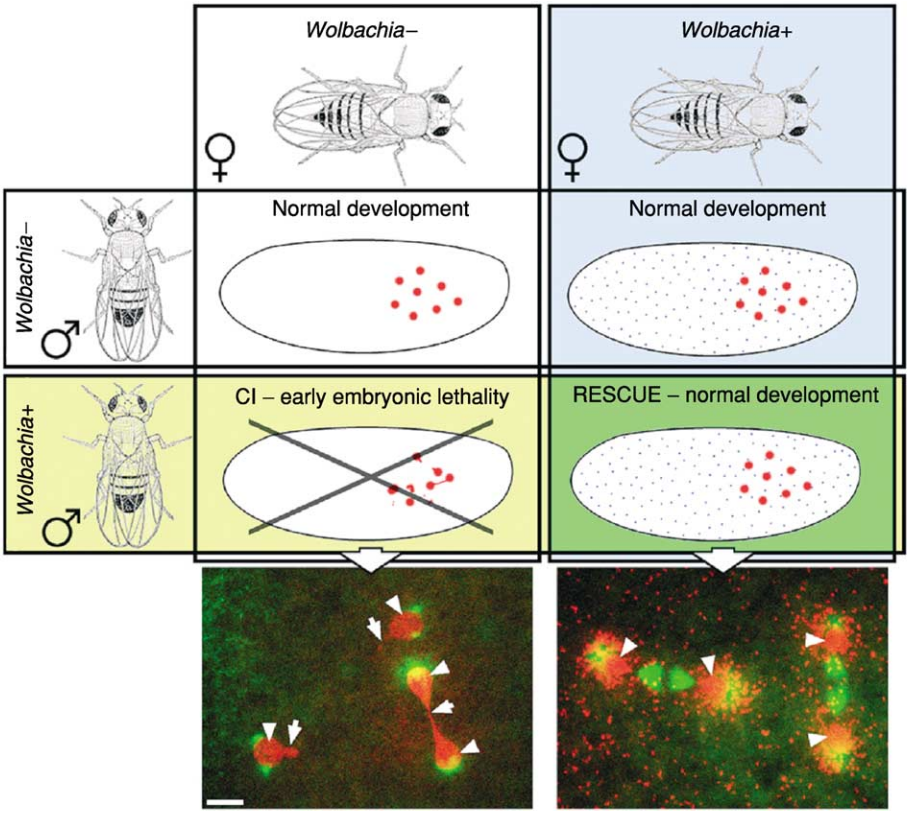


Figure 7 Cytoplasmic incompatibility induced by *Wolbachia*. There are four different mating combinations between infected and uninfected males and females. Infected females (blue) produce infected offspring that develop normally regardless of paternal infection status. Uninfected males mate successfully with both infected and uninfected females. However, infected males (yellow) mated to uninfected females produce some embryos with early embryonic lethality, characterized by defects in early mitotic divisions (CI, lower left) most often observed as defects in chromosome segregation during late telophase (nuclei, arrowhead). These defects are rescued when the same infected males are mated to infected females (Rescue, lower right). *Wolbachia* are seen as small punctuate dots, with high concentrations associated with astral microtubules. Red = DNA, Green = tubulin. Scale bar = 10 mm.

infected cellular environment and, therefore, *Wolbachia* must exert their effect(s) on sperm during spermatogenesis.

1.6.5.2. Models for CI

How do these curious microbes affect sperm function at such a crucial stage of development? Currently no single model yet explains CI. Three aspects of the biology of *Wolbachia*-induced incompatibility complicate attempts to produce a coherent picture: (1) sperm are altered, or modified so that they cannot, or can only poorly function when fertilizing uninfected eggs; (2) *Wolbachia* somehow "rescue" the sperm defect (i.e., crosses between infected males and females infected with the same strain are viable); and (3) distinct strains of *Wolbachia* can cause bidirectional CI. Thus, any complete model for CI must account for all these phenomena.

Work from a number of laboratories suggests that *Wolbachia* acts to alter sperm chromatin, resulting in a delay in or blockage of the proper formation of a male pronucleus (Callaini *et al.*, 1996, 1997; Lassy and Karr, 1996) (see **Figure 6**). As the early events of fertilization occur very rapidly, small perturbations in the kinetics of chromatin remodelling that must occur during this time, could result in failure of the two pronuclei to properly fuse, "diploidize", and form a viable zygote. Support for this model has come from cytological studies showing aberrant paternal chromosomes that fail to properly align along the first mitotic spindle. Such kinetic models are consistent with the data so far, but as yet do not explain how *Wolbachia* in the egg rescue these "kinetic" defects or how different strains cause different kinetic effects. These first molecular clues to the CI mechanism are consistent with a "kinetic" model of CI that suggested *Wolbachia* affect the relative timing of the events following sperm entry (Tram and Sullivan, 2002) (see below). Although these studies have clearly delineated the

spatio-temporal aspects of the expression of CI, more work is needed to identify the molecular components involved and the specific cellular pathways effected.

1.6.6. Fertilization in Non-Drosophiliids

Fertilization in a variety of non-Drosophiliid species has been studied and, for the most part, the basic overall picture of fertilization is similar to that found in *Drosophila* (Kawamura, 2001). In fact, for obligate sexual species, the "gonomeric" fertilization pattern appears to be universal, accepting the fact that exceptions will always be found when defining patterns and principles for such a diverse group of animals. A few examples of fertilization, zygote formation, and early development in a wide range of insects spanning numerous orders of the Insecta are provided below.

1.6.6.1. Fertilization in *Athalia rosae*

The turnip sawfly, *Athalia rosae*, is a hymenopteran unique in fertilization biology for being the only insect to date, in which *in vitro* fertilization (IVF) has been achieved (Sawa and Oishi, 1989a, 1989b; Oishi *et al*., 1993; Hatakeyama *et al*., 2000). These pioneering studies unfortunately have not led to similar successes in other insect systems, particularly *Drosophila*, where an efficient *in vitro* system would provide not only a wealth of research possibilities, but could eventually, in conjuction with cryopreservation of sperm, become a useful alternative, or complement to the stock centers. Nonetheless, the sawfly system paves the way for future studies of *in vitro* fertilization in insects, a worthy goal with high payoff for both basic and applied research.

Athalia eggs can only be successfully fertilized using intra-cytoplasmic sperm injection (ICSI), which is a practical limitation for cell biologists interested in exploiting such systems in order to study the basic cellular and molecular mechanisms of fertilization. For example, a robust IVF system could be used to acquire large numbers of synchronized eggs, a requirement for biochemical studies of kinase signalling cascades during zygote formation.

1.6.6.2. Fertilization in *Sciara coprophila*

While the major thrust in fertilization research of insects has understandably focused on *Drosophila*, fertilization and early development of the fungal gnat, *Sciara coprophila*, has recently been examined at the cell biological and molecular levels (de Saint Phalle and Sullivan, 1996; de Saint Phalle and Sullivan, 1998). In *S. coprophila*, both sexual and parthenogenetic development can be studied in the same animal, as females can be induced to lay eggs with or without mating. Early events during these stages were documented using antibody staining of fixed eggs (**Figure 8**), and molecular markers to follow the fate of chromosomes following fertilization. Parthenogenesis occurs at low frequency and unfertilized embryos initiate development without centrosomes. Thus, comparison with fertilized eggs of spindle assembly and mitosis in the presence and absence of centrosomes could be performed (de Saint Phalle and Sullivan, 1998). In both cases, although functional mitotic spindles are formed that successfully proceed through anaphase and telophase, forming two daughter nuclei separated by a midbody, significant differences were observed (**Figure 8**). The spindles assembled without centrosomes in unfertilized eggs are anastral, and it is likely that their microtubules are nucleated at or near the chromosomes. An uneven distribution of nuclei suggested a role for centrosomes. Although mitotic spindles undergo anaphase B and successfully segregate sister chromosomes, without centrosomes, the distance between the daughter nuclei in the next interphase is greatly reduced. This suggests that centrosomes are required in order to maintain nuclear spacing during the telophase to interphase transition.

Similar to *Drosophila*, nuclei in fertilized centrosome-bearing embryos maintain an even distribution as they divide and migrate to the cortex. In embryos lacking centrosomes, nuclei collide and form large, irregularly shaped DNA-containing clusters, and they never successfully migrate to the cortex. Presumably, this phenotype is a result of failure to form astral microtubules in parthenogenetic embryos lacking centrosomes. Following an indeterminate number of mitoses, the eggs invariably die due to aneuploidy and DNA clumping, suggesting that functional centrosomes are essential for viability in this organism.

1.6.6.3. Centrosome Inheritance in Haplo-Diploid Development

Sex determination in hymenopterans is haplodiploid, which usually means that haploid males develop from unfertilized eggs and diploid females are produced following fertilization. Because the sperm is thought to provide the basal body that becomes the centrosome and microtubule organizing centers (MTOCs), it has been somewhat of a mystery as to when, where, and how these crucial cell organelles form during haploid development. Although this issue was investigated in the *Sciara*

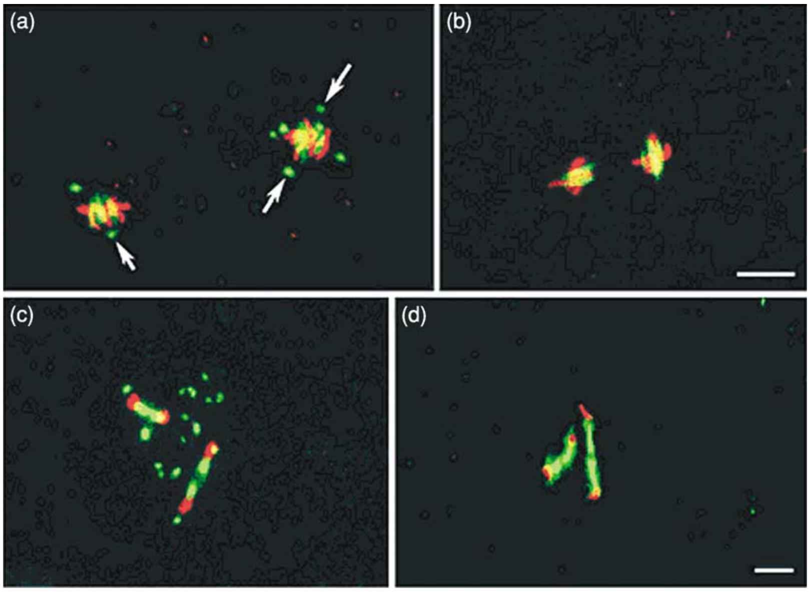


Figure 8 Unfertilized embryos do not possess MTOCs. Nuclear cycle 2 embryos are stained for their DNA (red) and microtubules (green). (a) In a fertilized embryo, two of the MTOCs (arrows) are associated with spindle poles while the others surround the metaphase plate. (b) In the equivalently staged unfertilized embryo, no MTOCs are present, and by counting the chromosome arms it can be seen that the nuclei are haploid. This may be why unfertilized nuclei seem smaller. (c and d) Telophase of nuclear cycle 2 in fertilized and unfertilized embryos, respectively. While both exhibit robust midbodies, no MTOCs are present in the unfertilized embryo. Scale bar = 10 µm.

system (see above), these studies suffered from a lack of direct observation, instead relying on fixed material that can only give a static view of the process as described below.

1.6.6.3.1. Real-time visualization of fertilization and centrosome inheritance A significant breakthrough in our understanding of haploid development in hymenopterans has come from real-time observations using microinjection of fluorescently labeled tubulin into living eggs. Coinjection of a DNA-specific fluorescent dye allowed contemporaneous visualization of both DNA and microtubule dynamics during the very early stages of zygote formation. Thus, fertilization and zygote formation in both haploid and diploid eggs were examined in *Nasonia vitripennis* as shown in Figure 9.

1.6.6.3.2. Mechanism of CI in *Nasonia*: The "slow-motion" model An intriguing variation on this theme is the effect of *Wolbachia* on the system: uninfected eggs that would normally develop as haploid males, instead develop as diploid females. In fact, studies of fertilization in hymenopterans were prompted, in part, by the unusual effect of *Wolbachia* on sex determination as mentioned above, and on understanding the origin and mechanism of early development in the absence of fertilization by sperm. These studies have led to a deeper understanding of centrosome formation and inheritance in both fertilized and unfertilized eggs in general, and lent support to a kinetic model of CI. Although beyond the scope of this review, the model basically proposes that *Wolbachia* act on sperm in such a manner as to delay sperm nuclear envelope breakdown, thus preventing the proper course of events needed for formation of a proper gonomeric spindle. This provocative model will hopefully spawn additional studies designed to test this, and other models.

1.6.6.4. Fertilization in Coleoptera, Lepidoptera, and Orthoptera

Fertilization in a coleopteran, *Aleochara bilineata*, a parasitic beetle, a lepidopteran, *Bombyx mori* (common silkworm), and an orthopteran, *Gryllus bimaculata* (common cricket), has been described and these cases are used here to illustrate the broad basic features of fertilization in these groups. It is clear that although these taxa represent very diverse groups that diverged over 300 million years ago, they maintain common features of fertilization, which may explain, atleast in part, the unprecedented success of insects.

1.6.6.4.1. *Aleochara bilineata* Coleopterans comprise the largest order of insects, with at least

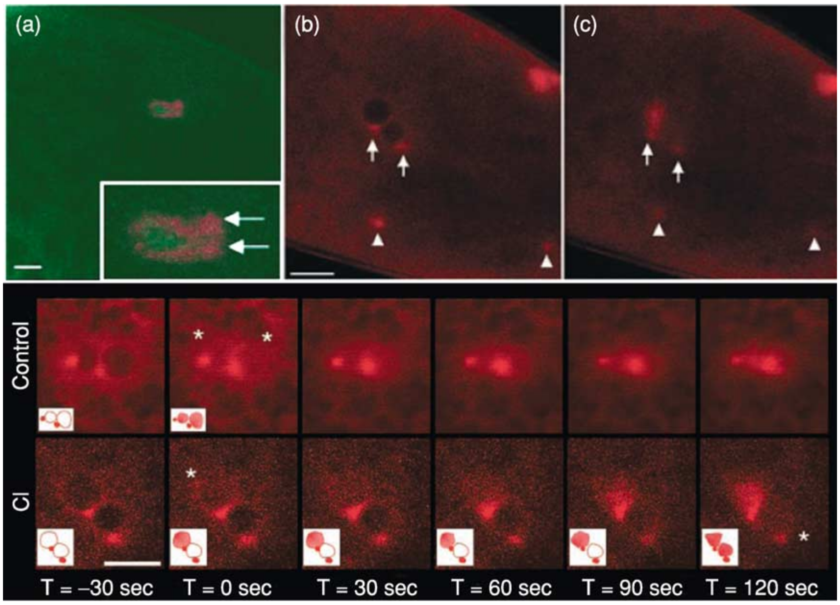


Figure 9 CI in *Nasonia vitripennis* (panels (a), (b), (c)). (a) CI embryo injected with rhodamine-tubulin (red; labeling centrosomes and nuclear periphery) and Oligreen (green; labeling DNA) showed that sperm from a *Wolbachia*-infected male is rod-shaped and associated with two paternally derived centrosomes (arrows, inset). Scale bar = 5–8 μm. (b) In CI embryo, injected with rhodamine-tubulin, pronuclei were apposed and centrosome separation (arrows) occurred normally. Maternally derived centrosomes (arrowheads) were present in the cytoplasm. (c) In same embryo as in (b), T = +510s, paternally derived centrosomes (arrows) set up mitotic spindle; maternally derived asters degenerate. (Lower panels) Real-time visualization of fertilization and centrosome inheritance in *N. vitripennis*. Relative timing of nuclear-envelope breakdown (NEB) was disrupted in CI embryos. NEB was assessed by time-lapsed confocal microscopy of 0- to 1-h embryos injected with rhodamine-tubulin. When the nuclear envelope was intact, the nucleus appeared as a black circle surrounded by a ring of red (rhodamine-tubulin) (T = 30 s). At NEB (indicated by ∗), rhodamine-tubulin invaded the nucleus (control, T = 0 s; CI, T = 0 s and T = 120 s). (Insets) Schematized interpretation of NEB. (From Tram, U., Sullivan, W., **2002**. Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science 296*, 1124–1126.)

350 000 species described and a world-wide distribution. The staphiliniid beetles have been heavily studied as a model system for sexual selection and sperm competition (Gack and Peschke, 1994; Benken *et al*., 1999). Although distantly related to *Drosophila*, *A. bilineata* males also produce relatively large sperm (∼1 mm in length). Interestingly, the entire sperm enters the egg and is restricted to a single quadrant, presumably where the sperm enters (**Figure 10**; K. Peschke and T. Karr, personal communication). This supports the idea that total sperm entrance occurs across a very wide spectrum of insects, and it will be of interest to determine if this pattern of sperm usage occurs in additional members of this highly speciose group of insects.

1.6.6.4.2. *Bombyx mori* The common silkworm has been extensively studied over the past century for its obvious commercial value. Fertilization (**Figure 11**) has been described (Kawamura, 1978, 1979) and recently reviewed in the context of fertilization in other systems such as the sea urchin and *Ascaris* models (Kawamura, 2001). Pronuclear formation, migration, and spindle formation is as described in *Drosophila*, and provides perhaps one of the best examples of gonomeric spindle structure ever recorded (Kawamura, 1978).

As in most lepidopterans, *Bombyx* males produce two sperm types – eupyrene, containing a C-complement of DNA, and apyrene, devoid of DNA. The existence and persistence of these two sperm morphs is an outstanding problem in developmental and evolutionary biology that has yet to be resolved. However, recent work has shown a role for apyrene sperm in double matings of females to apyrene-only males and males that are sterile but contain eupyrene sperm. This is the first direct demonstration for a role of apyrene sperm in assisting fertilization.

1.6.6.4.3. *Gryllus bimaculata* Orthopterans, including crickets and grasshoppers, are represented by more than 20 000 species with a world-wide distribution, and include historically difficult pests such as the locust. The cricket, *Gryllus bimaculata* has been for many years a favorite for the study of sperm competition and related life-history traits (Simmons, 1987; Simmons and Siva-Jothy, 1998;

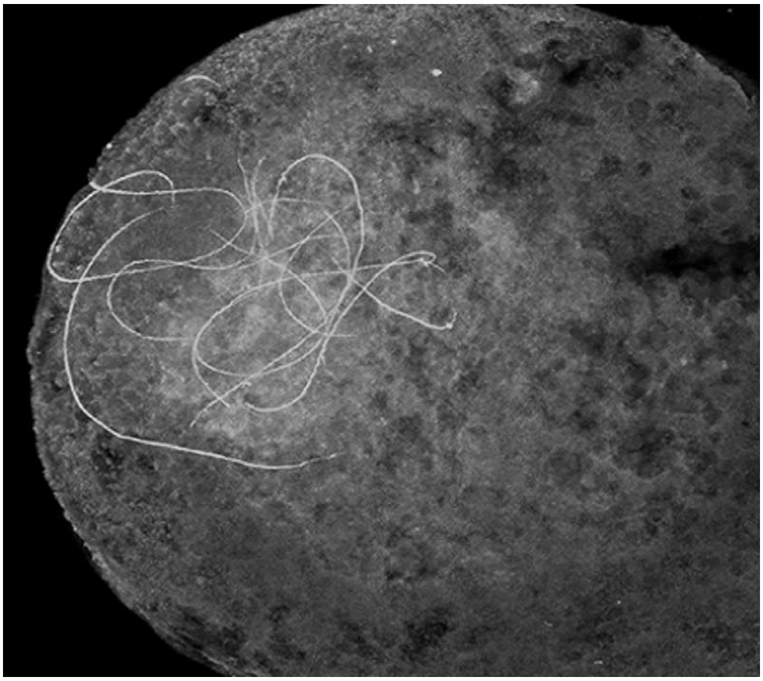


Figure 10 *Aleochara bilineata* fertilization. Indirect immunofluorescence using an anti-sperm polyclonal antibody shows the approximately 1 mm long sperm (thin bright coil on left side) inside a roughly spherical egg (T. Karr and K. Petschke, unpublished data).

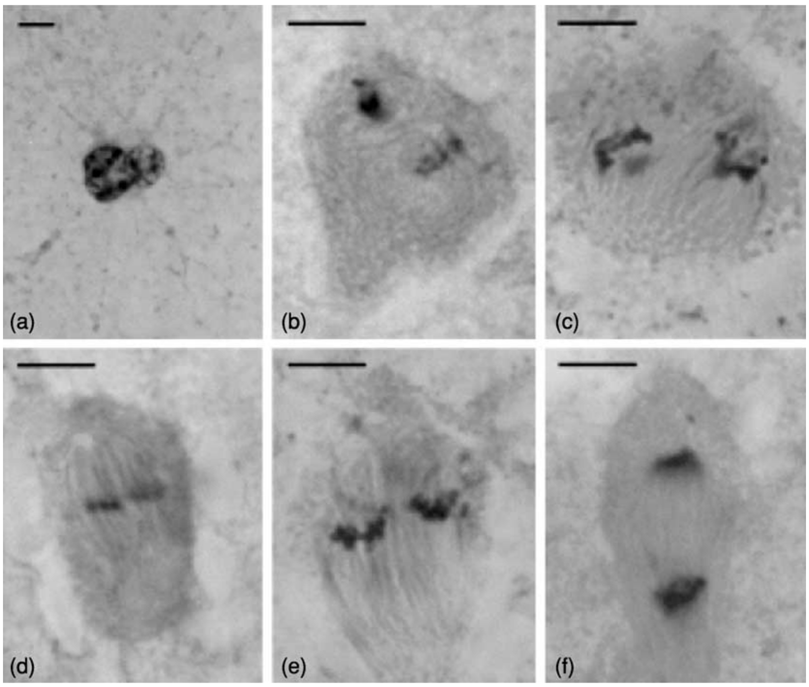


Figure 11 *Bombyx mori* fertilization. The first cleavage mitosis in the silkworm. (a) Union of male and female pronuclei. (b) Prophase. Two small spindles in the paternal and maternal pronuclei take rectangular positions. Two serial sections are superimposed. (c) Late prometaphase. Two independent spindles from the two pronuclei assume a parallel position. (d) Metaphase. Paternal and maternal chromosome groups are separate on a spindle. (e) Early anaphase. Two chromosome groups are still separate. (f) Telophase of the first cleavage. Chromosome groups are fused on the spindle poles (karyogamy). (a) Scale bar = 20 μm; (b–f) scale bar = 10 μm. (From Kawamura, N., **1978**. The early embryonic mitosis in normal and cooled eggs of the silkworm, *Bombyx mori*. *J. Morphol.* *158*, 57–72; reprinted by permission of John Wiley & Sons Inc.).

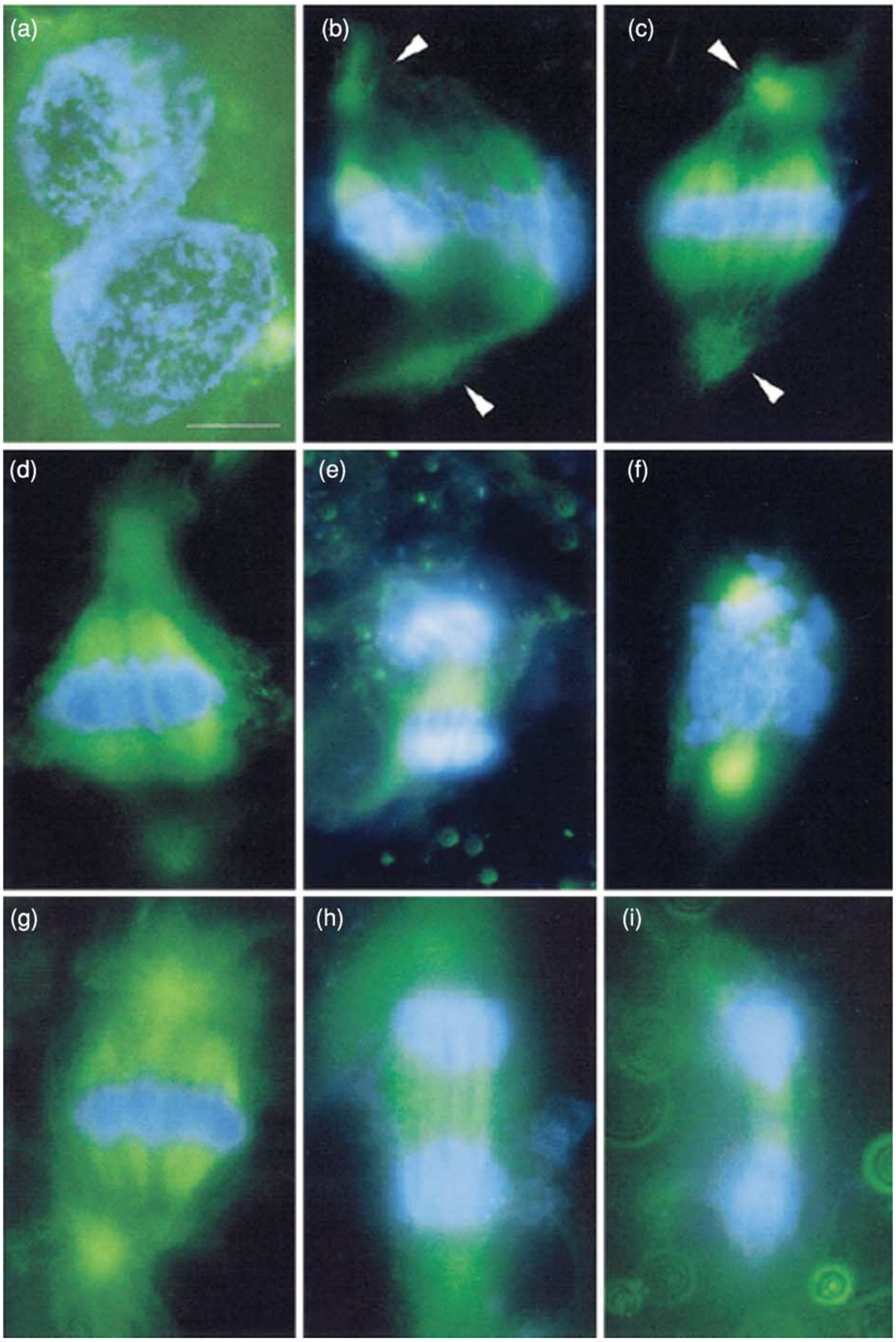


Figure 12 *Gryllus bimaculatus* fertilization. The first (a–e) and second (f–i) cleavage mitosis displayed by immunostaining for tubulin (green signal) and by Hoechst staining (blue signal) in squash preparations respectively. (a) Union of female and male pronuclei. (b) Prophase of the first cleavage mitosis. A spindle is formed in each of the parent pronuclei. Note the tubulin bundles (arrowheads) protruding from each of the spindle poles toward the opposite direction. (c) Metaphase. Spindles from parent pronuclei are still separate, while an astral center (arrowhead) is formed at each of the spindle poles. (d) Early anaphase. Astral centers become indistinct. (e) Telophase of the first cleavage mitosis. Parent chromosome groups are fused at the spindle poles. Astral centers disappear. (f) Prophase of the second cleavage mitosis. Strong signal of MTOC appears at two sides of the nucleus. (g) Metaphase. Asters radiate from both spindle poles. (h) Anaphase. Asters and MTOCs are becoming indistinct. (i) Telophase. Strong signal of microtubules disappears. Scale bar = 10 μm. (Reprinted with permission from *J. Morphology*.)

Tregenza and Wedell, 1998, 2002). It is, therefore, of great interest to molecular ecologists and animal behaviorists to understand the basis for sperm competition, and clearly sperm function during fertilization could play a role. Although we know little about fertilization in orthopterans, fertilization in *Gryllus* (**Figure 12**) was recently described (Sato and Sato, 2002). *Gryllus* eggs are similar in shape to *Drosophila* but usually 3–4 times longer. Polyspermic fertilization occurs in this species, with as many as 3–4 sperm found immediately adjacent to the micropyle, the point of sperm entrance. A single sperm (from this peripheral position) is "chosen" and moves into the interior region of the egg, forming a microtubule-rich "fertilization island", where the sperm nucleus decondenses and presumably replicates its DNA. The newly formed male and female pronuclei then move together and form the gonomeric spindle. Thus, many features of *Gryllus* fertilization are similar if not identical to *Drosophila*. One interesting difference is the presence of polyspermy, which is a regular occurrence in many insect species. However, polyspermy in *Drosophila* rarely occurs with some notable exceptions (Snook and Karr, 1998). Comparative work in polyspermic species such as *Gryllus* should prove fruitful in understanding the basis of polyspermy and its evolution.

1.6.7. Future Directions

Classical genetic analyses of *Drosophila* fertilization will continue to make significant contributions to the general field of fertilization biology and, in particular, to provide a more global view of this important and central process. However, as with any general approach to scientific investigation, genetic analyses will benefit greatly from additonal avenues of research that both complement and extend genetic approaches. For example, the recent microarray and bioinformatics approaches are currently being used to define the testis transcriptome (Andrews *et al*., 2000; Oliver, 2002a, 2002b; Oliver and Leblanc, 2003; Parisi *et al*., 2003). One area that is especially well-suited to do this is the proteomics of sperm, an area that is only now being investigated intensely (Graner *et al*., 1994; Feder *et al*., 1999). The idea is that knowledge of the complete sperm proteome will identify proteins with potential paternal effects. In theory, any protein delivered to the sperm could be a potential gene of paternal effect. To this end, we have recently initiated a whole cell mass spectrometry study of *D. melanogaster* sperm, and have identified more than 350 putative sperm proteins. An initial evolutionary analysis of the sperm proteome identified more than 190 orthologous genes in *D. pseudoobscura*, suggesting that these genes are conserved between these distantly related flies. Thus, a new data set now exists for future genetic and functional studies of fertilization in *Drosophila*.

References

Achanzar, W.E., Ward, S., **1997**. A nematode gene required for sperm vesicle fusion. *J. Cell. Sci. 110* (Pt 9), 1073–1081.

Andrews, J., Bouffard, G.G., Cheadle, C., Lu, J., Becker, K.G., *et al*., **2000**. Gene discovery using computational and microarray analysis of transcription in the *Drosophila melanogaster* testis. *Genome Res. 10*, 2030–2043.

Baker, B.S., **1975**. Paternal loss (pal): A meiotic mutant in *Drosophila melanogaster* causing loss of paternal chromosomes. *Genetics 80*, 267–296.

Bell, G., **1982**. The Masterpiece of Nature: the Evolution and Genetics of Sexuality. University of California Press, Berkeley, CA.

Benken, T., Knaak, A., Gack, C., Eberle, M., Peschke, K., **1999**. Variation of sperm precendence in the rove beetle *Aleochara curtula* (Coleoptera: Staphylinidae). *Behaviour 136*, 1065–1077.

Benoff, S., **1997**. Carbohydrates and fertilization: an overview. *Mol. Human Reprod. 3*, 599–637.

Bloch Qazi, M.C., Heifetz, Y., Wolfner, M.F., **2003**. The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster. Devel. Biol. 256*, 195–211.

Bowerman, B., **1998**. Maternal control of pattern formation in early *Caenorhabditis elegans* embryos. *Curr. Top. Devel. Biol. 39*, 73–117.

Brent, A.F., MacQueen, A., Hazelrigg, T., **2000**. The *Drosophila wispy* gene is required for RNA localization and other microtubule-based events of meiosis and early embryogenisis. *Genetics 154*, 1649–1662.

Callaini, G., Dallai, R., Riparbelli, M.G., **1997**. *Wolbachia*-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans. J. Cell Sci. 110* (Pt 2), 271–280.

Callaini, G., Riparbelli, M.G., Giordano, R., Dallai, R., **1996**. Mitotic defects associated with cytoplasmic incompatibility in *Drosophila simulans. J. Inverteb. Pathol. 67*, 55–64.

Cattaneo, F., Ogiso, M., Hoshi, M., Perotti, M.E., Pasini, M.E., **2002**. Purification and characterization of the plasma membrane glycosidases of *Drosophila melanogaster* spermatozoa. *Insect Biochem. Mol. Biol. 32*, 929–941.

Cattaneo, F., Pasini, M.E., Perotti, M.E., **1997**. Glycosidases are present on the surface of *Drosophila melanogaster* spermatozoa. *Mol. Reprod. Devel. 48*, 276–281.

Christians, E., Davis, A.A., Thomas, S.D., Benjamin, I.J., **2000**. Maternal effect of Hsf1 on reproductive success. *Nature 407*, 693–694.

de Saint Phalle, B., Sullivan, W., **1996**. Incomplete sister chromatid separation is the mechanism of programmed chromosome elimination during early *Sciara coprophila* embryogenesis. *Development 122*, 3775–3784.

de Saint Phalle, B., Sullivan, W., **1998**. Spindle assembly and mitosis without centrosomes in parthenogenetic *Sciara* embryos. *J. Cell Biol. 141*, 1383–1391.

Edgar, B.A., Kiehle, C.P., Schubiger, G., **1986**. Cell cycle control by the nucleo-cytoplasmic ratio in early *Drosophila* development. *Cell 44*, 365–372.

Elfring, L.K., Axton, J.M., Fenger, D.D., Page, A.W., Carminati, J.L., *et al.*, **1997**. *Drosophila* PLUTONIUM protein is a specialized cell cycle regulator required at the onset of embryogenesis. *Mol. Biol. Cell. 8*, 583–593.

Evans, J.P., **2002**. The molecular basis of sperm-oocyte membrane interactions during mammalian fertilization. *Hum. Reprod. Update 8*, 297–311.

Feder, M.E., Karr, T.L., Yang, W., Hoekstra, J.M., James, A.C., **1999**. Interaction of *Drosophila* and its endosymbiont *Wolbachia*: natural heat shock and the overcoming of sexual incompatibility. *Am. Zool. 39*, 363–373.

Fenger, D.D., Carminati, J.L., Burney-Sigman, D.L., Kashevsky, H., Dines, J.L., *et al.*, **2000**. PAN GU: a protein kinase that inhibits S phase and promotes mitosis in early *Drosophila* development. *Development 127*, 4763–4774.

Fitch, K.R., Yasuda, G.K., Owens, K.N., Wakimoto, B.T., **1998**. Paternal effects in *Drosophila*: implications for mechanisms of early development. *Curr. Top. Devel. Biol. 38*, 1–34.

Freeman, M., Glover, D.M., **1987**. The gnu mutation of *Drosophila* causes inappropriate DNA synthesis in unfertilized eggs. *Genes Devel. 1*, 924–930.

Fuyama, Y., **1984**. Gynogenesis in *Drosophila melanogaster. Jpn J. Genet. 59*, 91–96.

Gack, C., Peschke, K., **1994**. Spermathecal morphology, sperm transfer and a novel mechanism of sperm displacement in the rove beetle, *Aleochara curtula* (Coleoptera, Staphylinidae). *Zoomorphology 114*, 227–237.

Galindo, B.E., Vacquier, V.D., Swanson, W.J., **2003**. Positive selection in the egg receptor for abalone sperm lysin. *Proc. Natl Acad. Sci. USA 100*, 4639–4643.

Gilbert, S., **1988**. Developmental Biology. Sinauer Associates, Sunderland, MA.

Graner, M., Stupka, K., Karr, T.L., **1994**. Biochemical and cytological characterization of DROP-1: A widely distributed proteoglycan in *Drosophila*. *Insect Biochem. Mol. Biol. 24*, 557–567.

Hamilton, W., **1999**. Narrow Roads of Geneland: The Collected Papers of W.D. Hamilton. Vol. 2. Oxford University Press, New York.

Han, Y.G., Kwok, B.H., Kernan, M.J., **2003**. Intraflagellar transport is required in *Drosophila* to differentiate sensory cilia but not sperm. *Curr Biol. 13*, 1679–1686.

Harris, H.L., Braig, H.R., **2003**. Sperm chromatin remodelling and *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila*. *Biochem. Cell. Biol. 81*, 229–240.

Hatakeyama, M., Nishimori, Y., Oishi, K., **2000**. Participation of testicular spermatids in development upon intracytoplasmic injection into eggs of the sawfly, *Athalia rosae* (Insecta, Hymenoptera). *J. Exp. Zool. 286*, 181–192.

Hildreth, P.E., Lucchesi, J.C., **1963**. Fertilization in *Drosophila* I. Evidence for the regular occurence of monospermy. *Devel. Biol. 6*, 262–278.

Jeyaprakash, A., Hoy, M.A., **2000**. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol. 9*, 393–405.

Joly, D., Bressac, C., Lachaise, D., **1995**. Disentangling giant sperm. *Nature 377*, 202.

Jonakova, V.V., Manaskova, P., Kraus, M., Liberda, J., Ticha, M., **2000**. Sperm surface proteins in mammalian fertilization. *Mol. Reprod. Devel. 56*, 275–277.

Karr, T., **1991**. Intracellular sperm/egg interactions in *Drosophila*: A three-dimensional structural analysis of a paternal product in the developing egg. *Mech. Devel. 34*, 101–112.

Karr, T.L., **1996**. Paternal investment and intracellular sperm-egg interactions during and following fertilization in *Drosophila*. *Curr. Top. Devel. Biol. 34*, 89–115.

Karr, T.L., Pitnick, S., **1996**. The ins and outs of fertilization. *Nature 379*, 405–406.

Kawamura, N., **1978**. The early embryonic mitosis in normal and cooled eggs of the silkworm, *Bombyx mori. J. Morphol. 158*, 57–72.

Kawamura, N., **1979**. Cytological studies on the mosaic silkworms induced by low temperature treatment. *Chromosoma 74*, 179–188.

Kawamura, N., **2001**. Fertilization and the first cleavage mitosis in insects. *Devel. Growth Differ. 43*, 343–349.

Komma, D.J., Horne, A.S., Endow, S.A., **1991**. Separation of meiotic and mitotic effects of claret non-disjunctional on chromosome segregation in *Drosophila*. *EMBO J. 10*, 419–424.

Lassy, C.W., Karr, T.L., **1996**. Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. *Mech. Devel. 57*, 47–58.

Lee, L.A., Elfring, L.K., Bosco, G., Orr-Weaver, T.L., **2001**. A genetic screen for suppressors and enhancers of the *Drosophila* PAN GU cell cycle kinase identifies cyclin B as a target. *Genetics 158*, 1545–1556.

Lee, L.A., Van Hoewyk, D., Orr-Weaver, T.L., **2003**. The *Drosophila* cell cycle kinase PAN GU forms an active complex with PLUTONIUM and GNU to regulate embryonic divisions. *Genes Devel. 17*, 2979–2991.

Lin, H.F., Wolfner, M.F., **1991**. The *Drosophila* maternal-effect gene fs(1)Ya encodes a cell cycle-dependent nuclear envelope component required for embryonic mitosis. *Cell 64*, 49–62.

Lopez, J.M., Wolfner, M.F., **1997**. The developmentally regulated *Drosophila* embryonic nuclear lamina protein 'Young Arrest' (fs(1)Ya) is capable of associating with chromatin. *J. Cell. Sci. 110* (Pt 5), 643–651.

Loppin, B., Berger, F., Couble, P., **2001a**. The *Drosophila* maternal gene sesame is required for sperm chromatin remodeling at fertilization. *Chromosoma 110*, 430–440.

Loppin, B., Berger, F., Couble, P., **2001b**. Paternal chromosome incorporation into the zygote nucleus is controlled by maternal haploid in *Drosophila*. *Devel. Biol. 231*, 383–396.

Loppin, B., Docquier, M., Bonneton, F., Couble, P., **2000**. The maternal effect mutation sesame affects the formation of the male pronucleus in *Drosophila melanogaster*. *Devel. Biol. 222*, 392–404.

Miller, M.A., Nguyen, V.Q., Lee, M.H., Kosinski, M., Schedl, T., *et al.*, **2001**. A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science 291*, 2144–2147.

Miller, G.T., Pitnick, S., **2002**. Sperm-female coevolution in *Drosophila*. *Science 298*, 1230–1233.

Miller, G.T., Starmer, W.T., Pitnick, S., **2003**. Quantitative genetic analysis of among-population variation in sperm and female sperm-storage organ length in *Drosophila mojavensis*. *Genet. Res. 81*, 213–220.

Moore P.J., Moore A.J., **2003**. Developmental flexibility and the effect of social environment on fertility and fecundity in parthenogenetic reproduction. *Evol. Devel. 5*, 163–168.

Mori, T., Guo, M.W., Sato, E., Baba, T., Takasaki, S., *et al.*, **2000**. Molecular and immunological approaches to mammalian fertilization. *J. Reprod. Immunol. 47*, 139–158.

Murdy, W.H.A.C.H.L., **1959**. Parthenogenesis in *Drosophila mangabeirai*. *Malog. Am. Nat. 93*, 355–363.

Ohsako, T., Hirai, K., Yamamoto, M.T., **2003**. The *Drosophila* misfire gene has an essential role in sperm activation during fertilization. *Genes Genet. Sys. 78*, 253–266.

Oishi, K., Sawa, M., Hatakeyama, M., Kageyama, Y., **1993**. Genetics and biology of the sawfly, *Athalia rosae* (Hymenoptera). *Rev. Genetica 88*, 119–127.

Oliver, B., **2002a**. *Drosophila melanogaster*, two years into the post-genomic era. *Genome Biol. 3*, REPORTS 4021.

Oliver, B., **2002b**. Fly factory. *Genome Res. 12*, 1017–1018.

Oliver, B., Leblanc, B., **2003**. How many genes in a genome? *Genome Biol. 5*, 204.

Parisi, M., Nuttall, R., Naiman, D., Bouffard, G., Malley, J., *et al.*, **2003**. Paucity of genes on the *Drosophila* X chromosome showing male-biased expression. *Science 299*, 697–700.

Pasini, M.E., Cattaneo, F., Pinto, M.R., De Santis, R., Perotti, M.E., **1999**. Plasma membrane association and preliminary characterization of *Drosophila* sperm surface glycosidases. *Mol. Reprod. Devel. 52*, 166–713.

Payne, C., Rawe, V., Ramalho-Santos, J., Simerly, C., Schatten, G., **2003**. Preferentially localized dynein and perinuclear dynactin associate with nuclear pore complex proteins to mediate genomic union during mammalian fertilization. *J. Cell. Sci. 116*, 4727–4738.

Perotti, M.E., **1975**. Ultrastructural aspects of fertilization in *Drosophila*. In: Afzelius, B. (Ed.), The Functional Anatomy of the Spermatozoon. Pergamon, New York, pp. 57–68.

Perotti, M.E., Cattaneo, F., Pasini, M.E., Verni, F., Hackstein, J.H., **2001**. Male sterile mutant casanova gives clues to mechanisms of sperm-egg interactions in *Drosophila melanogaster*. *Mol. Reprod. Devel. 60*, 248–259.

Philpott, A., Leno, G.H., Laskey, R.A., **1991**. Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin. *Cell 65*, 569–578.

Pitnick, S., **1996**. Investment in testes and the cost of making long sperm in *Drosophila*. *Am. Nat. 148*, 57–80.

Pitnick, S., Karr, T.L., **1998**. Paternal products and by-products in *Drosophila* development. *Proc. R. Soc. Lond. B 265*, 821–826.

Pitnick, S., Markow, T.A., **1994**. Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. *Am. Nat. 143*, 785–819.

Pitnick, S., Spicer, G.S., Markow, T.A., **1995**. How long is a giant sperm? *Nature 375*, 109.

Ray-Gallet, D., Quivy, J.P., Scamps, C., Martini, E.M., Lipinski, M., *et al.*, **2002**. HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. *Mol. Cell. 9*, 1091–1100.

Renault, A.D., Zhang, X.H., Alphey, L.S., Frenz, L.M., Glover, D.M., *et al.*, **2003**. giant nuclei is essential in the cell cycle transition from meiosis to mitosis. *Development 130*, 2997–3005.

Ridley, M., **1994**. Red Queen: Sex and the Evolution of Human Nature. Macmillan Publishing Co., New York.

Riparbelli, M.G., Callaini, G., **1996**. Meiotic spindle organization in fertilized *Drosophila* oocyte: presence of centrosomal components in the meiotic apparatus. *J. Cell Sci. 109*, 911–918.

Roth, S., Reinhardt, K., **2003**. Facultative sperm storage in response to nutritional status in a female insect. *Proc. R. Soc. Lond. B Biol. Sci. 270*(Suppl 1), S54–S56.

Royal, D.C., Royal, M.A., Wessels, D., L'Hernault, S., Soll, D.R., **1997**. Quantitative analysis of *Caenorhabditis elegans* sperm motility and how it is affected by mutants spe11 and unc54. *Cell Motil. Cytoskel. 37*, 98–110.

Sahara, K., Takemura, Y., **2003**. Application of artificial insemination technique to eupyrene and/or apyrene sperm in *Bombyx mori*. *J. Exp. Zool. Part A Comp. Exp. Biol. 297*, 196–200.

Sander, K., **1985**. Fertilization and Egg Activation in Insects. Academic Press, London, pp. 409–430.

Santamaria, P., Gans, M., **1980**. Chimaeras of *Drosophila melanogaster* obtained by injection of haploid nuclei. *Nature 287*, 143–144.

Sato, M., Sato, H.T., **2002**. Fertilization, syngamy, and early embryonic development in the cricket *Gryllus bimaculatus*. *J. Morph. 254*, 266–271.

Sawa, M., Oishi, K., **1989a**. Delayed sperm injection and fertilization in parthenogenetically activated insect egg (*Athalia rosae*, Hymenoptera). *Roux's Arch. Devel. Biol. 198*, 242–244.

Sawa, M., Oishi, K., **1989b**. Studies on the sawfly, *Athalia rosae* (Insecta, Hymenoptera, Tenthredinidae). III. Fertilization by sperm injection. *Zool. Sci. 6*, *557–563*.

Serrano, H., Garcia-Suarez, D., **2001**. Molecular aspects of mammalian fertilization. *Asian J. Androl. 3*, 243–249.

Shamanski, F.L., Orr-Weaver, T.L., **1991**. The *Drosophila* plutonium and pan gu genes regulate entry into S phase at fertilization. *Cell 66*, 1289–1300.

Simmons, L.W., **1987**. Sperm competition as a mechanism of female choice in the field cricket, *Gryllus bimaculatus*. *Behav. Ecol. Sociobiol. 21*, 197–202.

Simmons, L.W., Siva-Jothy, M., **1998**. Sperm competition in insects: mechanisms and the potential for selection. In: Birkhead, T.R., Møller, A.P. (Eds.), Sperm Competition and Sexual Selection. Academic Press, London.

Singson, A., **2001**. Every sperm is sacred: fertilization in *Caenorhabditis elegans*. *Devel. Biol. 230*, 101–109.

Singson, A., Mercer, K.B., L'Hernault, S.W., **1998**. The C*elegans* spe-9 gene encodes a sperm transmembrane protein that contains EGF-like repeats and is required for fertilization. *Cell 93*, 71–79.

Snook, R.R., **1997**. Is the production of multiple sperm types adaptive? *Evolution 51*, 797–808.

Snook, R.R., Cleland, S.Y., Wolfner, M.F., Karr, T.L., **2000**. Offsetting effects of *Wolbachia* infection and heat shock on sperm production in *Drosophila simulans*: analyses of fecundity, fertility and accessory gland proteins. *Genetics 155*, 167–178.

Snook, R.R., Karr, T.L., **1998**. Only long sperm are fertilization-competent in six sperm-heteromorphic *Drosophila* species. *Curr. Biol. 8*, 291–294.

Snook, R.R., Markow, T.A., Karr, T.L., **1994**. Functional nonequivalence of sperm in *Drosophila pseudoobscura*. *Proc. Natl Acad. Sci. USA 91*, 11222–11226.

Sonnenblick, B.P., **1950**. In: The early embryology of *Drosophila melanogaster.* Wiley, New York, pp. 62–167.

Stouthamer, R., Breeuwer, J.A., Hurst, G.D., **1999**. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol. 53*, 71–102.

Sutovsky, P., Schatten, G., **1998**. Ultrastructural aspects of mammalian fertilization: new discoveries and inspirations from the work of Daniel Szollosi. *Reprod. Nutr. Devel. 38*, 629–641.

Swanson, W.J., Aquadro, C.F., Vacquier, V.D., **2001**. Polymorphism in abalone fertilization proteins is consistent with the neutral evolution of the egg's receptor for lysin (VERL) and positive Darwinian selection of sperm lysin. *Mol. Biol. Evol. 18*, 376–383.

Swanson, W.J., Nielsen, R., Yang, Q., **2003**. Pervasive adaptive evolution in mammalian fertilization proteins. *Mol. Biol. Evol. 20*, 18–20.

Swanson, W.J., Vacquier, V.D., **2002**. The rapid evolution of reproductive proteins. *Nat. Rev. Genet. 3*, 137–144.

Szabad, J., Mathe, E., Puro, J., **1995**. Horka, a dominant mutation of *Drosophila*, induces nondisjunction and, through paternal effect, chromosome loss and genetic mosaics. *Genetics 139*, 1585–1599.

Ting, C.T., Tsaur, S.C., Wu, M.L., Wu, C.I., **1998**. A rapidly evolving homeobox at the site of a hybrid sterility gene. *Science 282*, 1501–1504.

Tong, Z.B., Gold, L., Pfeifer, K.E., Dorward, H., Lee, E., *et al.*, **2000**. Mater, a maternal effect gene required for early embryonic development in mice. *Nat. Genet. 26*, 267–268.

Tram, U., Sullivan, W., **2002**. Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science 296*, 1124–1126.

Tregenza, T., Wedell, N., **1998**. Benefits of multiple mates in the cricket *Gryllus bimaculatus*. *Evolution 52*, 1726–1730.

Tregenza, T., Wedell, N., **2002**. Polyandrous females avoid costs of inbreeding. *Nature 415*, 71–73.

Trounson, A., Bongso, A., **1996**. Fertilization and development in humans. *Curr. Top. Devel. Biol. 32*, 59–101.

Tsaur, S.C., Ting, C.T., Wu, C.I., **2001**. Sex in *Drosophila mauritiana*: a very high level of amino acid polymorphism in a male reproductive protein gene, Acp26Aa. *Mol. Biol. Evol. 18*, 22–26.

Vacquier, V.D., **1998**. Evolution of gamete recognition proteins. *Science 281*, 1995–1998.

Vacquier, V.D., Swanson, W.J., Lee, Y.H., **1997**. Positive Darwinian selection on two homologous fertilization proteins: what is the selective pressure driving their divergence? *J. Mol. Evol. 44* (Suppl 1), S15–S22.

Vahed, K., **2003**. Structure of spermatodoses in shield-back bushcrickets (Tettigoniidae, Tettigoniinae). *J. Morphol. 257*, 45–52.

Wagner, W.E., Jr., Harper, C.J., **2003**. Female life span and fertility are increased by the ejaculates of preferred males. *Evol. Int. J. Org. Evol. 57*, 2054–2066.

Ward, S., Miwa, J., **1978**. Characterization of temperature-sensitive, fertilization-defective mutants of the nematode *Caenorhabditis elegans*. *Genetics 88*, 285–303.

Wassarman, P.M., **1999**. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell 96*, 175–183.

Werren, J.H., Windsor, D.M., Guo, L., **1995a**. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B Biol. Sci. 262*, 197–204.

Werren, J.H., Zhang, W., Guo, L.R., **1995b**. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc. R. Soc. Lond. B Biol. Sci. 261*, 55–63.

West-Eberhard, M.J., **2003**. Developmental Plasticity and Evolution. Oxford University Press, New York.

Williams, B.C., Dernburg, A.F., Puro, J., Nokkala, S., Goldberg, M.L., **1997**. The *Drosophila* kinesin-like protein KLP3A is required for proper behavior of male

and female pronuclei at fertilization. *Development 124*, 2365–2376.

Williams, B.C., Riedy, M.F., Williams, E.V., Gatti, M., Goldberg, M.L., **1995**. The *Drosophila* kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. *J. Cell Biol. 129*, 709–723.

Wolfsberg, T.G., White, J.M., **1996**. ADAMs in fertilization and development. *Devel. Biol. 180*, 389–401.

Wu, X., Viveiros, M.M., Eppig, J.J., Bai, Y., Fitzpatrick, S.L., *et al*., **2003a**. Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat. Genet. 33*, 187–191.

Wu, X., Wang, P., Brown, C.A., Zilinski, C.A., Matzuk, M.M., **2003b**. Zygote arrest 1 (Zar1) is an evolutionarily conserved gene expressed in vertebrate ovaries. *Biol. Reprod. 69*, 861–867.

Wu, C.I., Xu, E.Y., **2003**. Sexual antagonism and X inactivation–the SAXI hypothesis. *Trends Genet. 19*, 243–247.

Xu, X.Z., Sternberg, P.W., **2003**. A *Celegans* sperm TRP protein required for sperm-egg interactions during fertilization. *Cell 114*, 285–297.

Yasuda, G.K., Schubiger, G., Wakimoto, B.T., **1995**. Genetic characterization of ms (3) K81, a paternal effect gene of *Drosophila melanogaster. Genetics 140*, 219–229.

Yu, J., Wolfner, M.F., **2002**. The *Drosophila* nuclear lamina protein YA binds to DNA and histone H2B with four domains. *Mol. Biol. Cell 13*, 558–569.

1.7 Dosage Compensation

J C Lucchesi, Emory University,
Atlanta, GA, USA

1.7.1. Introduction

Insects exhibit a veritable plethora of mechanisms for determining the sex of their developing embryos (reviews: Marin and Baker, 1998; Normark, 2003) (see also **Chapter 1.1**). The majority of these mechanisms can be grouped into three broad categories. Most prevalent in all orders of insects – except Thysanoptera and Hemiptera – is diplodiploidy: males and females inherit one haploid genome from each of their two parents, and the maternal and paternal members of each homologous chromosome pair are transmitted with equal probability to the next generation. Some insects exhibit haplodiploidy whereby males transmit only their mother's genome to their offspring. In Thysanoptera, Hymenoptera, some Hemiptera, and some Coleoptera, males develop from unfertilized eggs; in other species of Hemiptera and Coleoptera, and in some species of Diptera, males develop from fertilized eggs but the paternal genome is eliminated or inactivated in the soma during early development and only the maternal genome is transmitted through the sperm. The third major category of sex-determining mechanisms is parthenogenesis. In the most common form, unfertilized eggs that produce females and males are never present; diploidy of female embryos is assured either by the mitotic origin of the eggs or by some postmeiotic event that leads to a doubling of the haploid complement. More rarely, females mate with males of closely related species but the sperm pronucleus never fuses with that of the egg; alternatively, fusion of the pronuclei occurs and the paternal genome is active in the soma but is eliminated during oogenesis. To further complicate matters, some insects alternate between two of the systems just outlined, either on a regular basis or as a consequence of some exogenous factors. Of interest is the fact that diplodiploidy and haplodiploidy never alternate although either of these mechanisms can alternate with parthenogenesis.

In the majority of diplodiploid species, the difference between the two sexes is based on the presence of special chromosomes that are called the "sex chromosomes." All of the other chromosomes that are present in the genetic material are called "autosomes" to distinguish them from the sex chromosomes. The difference in the sex chromosomes present in males and females is not the same in all species: in dipteran species, for example, females have two sex chromosomes that are identical in shape and genetic content called the X chromosomes; males have only one X chromosome and, instead of a second X, they have a sex chromosome that is different in shape and genetic content called the Y chromosome. In some Orthoptera, the only sex chromosome present in males is the X chromosome. In most Lepidoptera, males have two identical sex chromosomes and it is the females that have only one of these chromosomes and another sex chromosome that is different. To emphasize this point, the sex chromosome that is found in both sexes in these particular species is called the Z chromosome and the other sex chromosome, found only in females, is called the W chromosome. In any species, the sex that produces two different types of sex cells with respect to their sex chromosome content is said to be "heterogametic"; the other sex is termed "homogametic."

The sex chromosomes are responsible for determining the sex of an individual through the function of some of the genes that they carry (for a review see **Chapter 1.1**). Of course, there must exist a difference in these sex-determining genes between the

fertilized eggs that will give rise to females and those that will give rise to males. So, the sex chromosomes, by virtue of the fact that they are different in the two sexes, must be responsible for the necessary difference in sex-determining genes. In organisms where the only sex chromosome present in the male is one X chromosome, it is not the presence or absence of a particular gene that is the key factor in determining the sex of the fertilized egg. The difference is in the level of the product of one or more genes that are present on the X chromosome: females with two X chromosomes have twice the number of these genes and twice the amount of gene product that is found in males with a single X chromosome and only one dose of genes. It is this difference in the amount of gene products of specific genes on the X chromosome that determines whether a fertilized egg will develop into a male or a female. Surprisingly, the difference in the level of product made by genes on the X chromosome in males and females is responsible for sex determination even in some organisms where the males have both an X and a Y chromosome.

It is generally believed that all of the sex chromosome types that are found in modern species were derived from the more primitive situation of sex determination by a simple difference in the alleles of a gene present on a pair of identical chromosomes. In most diplodiploid species, one member of the sex chromosome pair is very different from the other, in shape and with respect to the genes that it carries. The changes that, over evolutionary time, eventually result in different sex chromosomes can be limited to a small segment of the chromosome or they can affect the entire chromosome. Ever since the discovery of sex chromosomes and their correlation with sex determination, the causes of these changes have been the object of considerable speculation (see, e.g., Traut and Marec, 1996; Charlesworth, 2002). Although they perform an essential genetic function, sex chromosomes also present a very serious problem to the species where they are found. It is appropriate for the Y or W chromosomes to contain genetic information that is present nowhere else in the genome and that determines that a fertilized egg will develop into an adult of a particular sex. It is also appropriate for the X (or Z) chromosome to contain sex-determining genes leading to different levels of products in XX and XY or X individuals or in ZZ or WZ individuals. But many of the numerous genes present on the pair of ancestral sex chromosomes that had nothing to do with sex determination are still located on the modern X or Z; therefore, these genes are present in two doses in XX or ZZ embryos and in a single dose in X, XY, or WZ embryos. Since the products of these genes are equally important to the development and well-being of both sexes, a difference in their level in XX and X or XY (or ZZ and WZ) individuals could lead to an imbalance in the level of gene products between sex-linked and autosomal genes whose relative contributions to particular developmental or metabolic pathways would be different in males and females. This, in turn, could result in differential selection between the sexes. As it happens, a number of insect species have avoided this problem through the evolution of a special regulatory mechanism called "dosage compensation" that renders the level of most of these gene products equal in the two sexes.

1.7.2. Dosage Compensation in *Drosophila*

1.7.2.1. Historical Perspective

The phenomenon of dosage compensation was discovered in *Drosophila melanogaster* by Herman J. Muller. His seminal observation, first reported in 1932 and later fleshed out in his famous Harvey Lecture of 1950, consisted of a comparison of eye pigment levels in males and females that carried various doses of an X-linked hypomorphic mutation (Muller, 1950). Muller noted that, although the level of pigmentation was proportional to the dosage of the hypomorphic alleles in each sex, males with one dose and females with two doses had exactly the same level of pigment (**Figure 1**). He concluded that there must exist a regulatory mechanism that compensates for the difference in dosage of X-linked genes between males and females; although he had discovered it using a mutant allele, he perceived that this regulation must surely apply to all or most wild-type genes present on the X chromosome.

Some 25 years after Muller's discovery, the famous evolutionist Theodosius Dobzhansky noted that the polytenic X chromosome in salivary glands of male larvae is wider and more diffuse than each X chromosome in the glands of females (Dobzhansky, 1957). The significance of this observation was provided by George Rudkin (Rudkin, 1964) who determined that the DNA content of the male X is equal to that of each X in females, leading to the hypothesis that the difference in morphology of this chromosome in the two sexes reflects a difference in levels of activity. This hypothesis was substantiated by A.S. Mukherjee in Wolfgang Beermann's laboratory using what, at the time, was a state-of-the-art molecular technique: transcription autoradiography (Mukherjee and Beermann, 1965). This technique

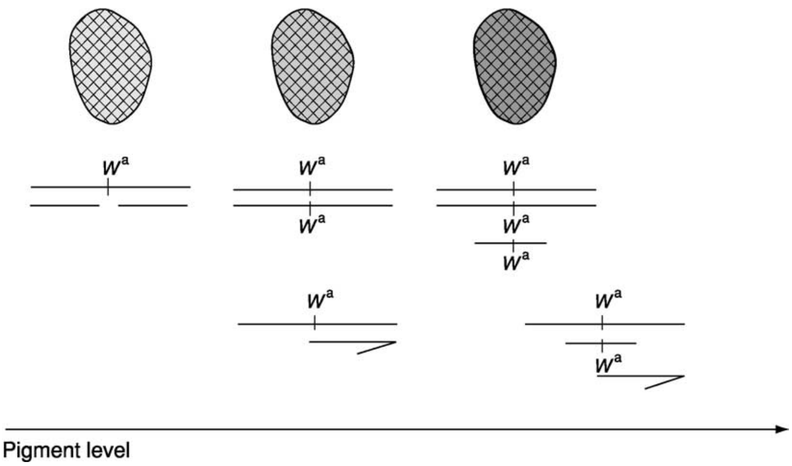


Figure 1 Phenotypic response to different genomic doses of the eye-color hypomorphic mutation white-apricot (w^a) in *Drosophila*. Different levels of pigmentation are exhibited in proportion to gene dosage within each sex, but females with two doses of the gene (upper row) have identical pigment levels as males with a single dose (lower row). The extra dose of w^a is present on a small duplication. (After Muller, H.J., **1950**. Evidence of the precision of genetic adaptation. *Harvey Lect. Ser. 43*, 165–229.)

consists of placing the salivary glands of male and female larvae in a medium containing the labeled RNA precursor [^{3}H]-uridine for a few minutes, spreading the chromosomes and covering them with a photographic film. Following a period of incubation, the film is developed and the silver grains overlying the X chromosome and an autosomal region used for control are counted. The similarity in the relative number of grains in males and females was taken as evidence that the single X chromosome in males synthesizes RNA with an efficiency similar to that of the two X chromosomes in females (Table 1).

Fifteen years later, John Belote was able to induce and recover a number of autosomal mutations that were lethal in males but had no effect on the viability of females (Belote and Lucchesi, 1980a). Using transcription autoradiography, he showed that these mutations resulted in a significant reduction in the level of transcription of the X chromosome in males (Belote and Lucchesi, 1980b). Belote's mutations could be ascribed to two separate genes – *mle* (maleless) and *msl3* (male-specific lethal on the third) – that had been discovered in natural populations by Japanese investigators (Fukunaga *et al.*, 1975; Uchida *et al.*, 1981), and to two novel genes that he named *msl1* and *msl2* (male-specific lethal one and two, respectively). Belote's work not only resulted in the identification of four different gene products involved in the execution of dosage compensation but also in the demonstration that dosage compensation in *Drosophila* is achieved by enhancing the transcription of X-linked genes in males rather than by reducing the transcription of these genes in females. All of these results

Table 1 RNA synthesis along the X chromosome in males and females

Nuclei[a]	*X autosomal segment 1*[b]	*X autosomal segment 2*
Female	8.78	1.94
Male	9.15	1.98
Female	9.29	1.85
Male	9.55	1.75

[a]Two sets of results are presented: in the top set, nuclei with high levels of labeling were selected and used for determining the average grain count on the X chromosome relative to the autosomal fragments; in the bottom set, the same calculation was performed with nuclei with low levels of labeling. No significant differences can be seen between the two types of nuclei.
[b]Autosomal segments 1 and 2 are two different regions of chromosome 3L; the level of RNA synthesis by these autosomal regions is expected to be similar in males and females.
Adapted from Mukherjee, A.S., Beermann, W., **1965**. Synthesis of ribonucleic acid by the X-chromosomes of *Drosophila melanogaster* and the problem of dosage compensation. *Nature 207*, 785–786.

emphasized a remarkable aspect of the mechanism that had been inferred since Muller's original observation: dosage compensation consists of a fairly precise twofold enhancement in gene expression.

The next landmark in the study of compensation was contributed by Thomas Skripsky (Lucchesi and Skripsky, 1981) who used the phenotypic effect of mutant interactions to discover a direct relationship between *Sxl* (Sex lethal), the master regulatory gene of female differentiation (Cline, 1979) (see also **Chapter 1.1**), and the absence of dosage compensation in females. In 1991 the first of the dosage

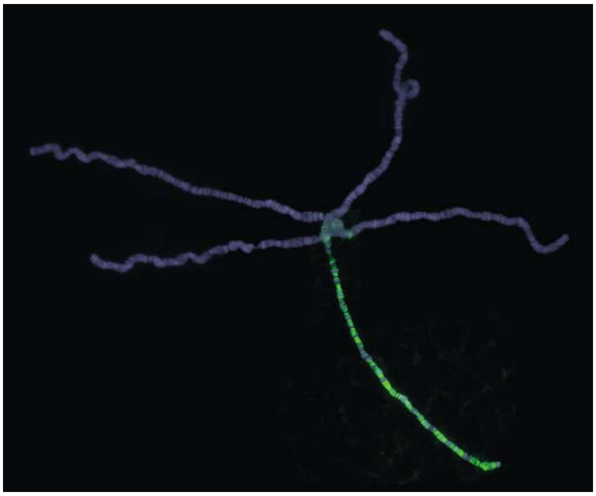


Figure 2 Polytene chromosomes from a salivary gland of a male *Drosophila* third instar larva. The chromosomes were exposed to a mouse antiserum against MSL3, then to a goat fluorescein isothyocyanate (FITC)-labeled antiserum against mouse immunoglobulin. Following immunostaining, the chromosomes were counterstained with 4,6-diamino-2-phenoleindole (DAPI). MSL3 (green fluorescence) is present at hundreds of sites along the X chromosome and is absent from the autosomes (blue fluorescence). (From a preparation made by Dr G.L. Sass in the author's laboratory.)

compensation genes (*mle*) was cloned by Mitzi Kuroda who showed by cytoimmunofluorescence that its product, as expected, "painted" the X chromosome in males (Kuroda *et al.*, 1991). Since that time, all of the known factors responsible for dosage compensation have been shown to have the same cytological distribution (**Figure 2**).

1.7.2.2. Recent Molecular Studies

Today, we have learned a great deal about the molecular basis of the mechanism of dosage compensation in *Drosophila*. The enhancement of transcription in *D. melanogaster* and, most probably, in other members of the genus is the responsibility of a multiprotein complex that represents a unique example of a novel type of chromatin remodeling machines. It is important at this point to remember that DNA does not occur in free form in the nuclei of eukaryotic cells; rather it is wrapped around octets of histones – the nucleosomes – and organized into a coiled chromatin fiber that is unfavorable to gene expression. Almost invariably, in order to allow transcription to initiate and proceed, the association of DNA with nucleosomes must be modified. This is achieved by the action of multiprotein complexes that use the energy liberated by the hydrolysis of ATP to reposition nucleosomes along the DNA, thereby allowing the transcription machinery to access more easily promoters and their regulatory

Table 2 Components of the MSL (male specific lethal) complex

Constituents	*Location*	*Features*
Proteins		
MSL1	Chromosome 2	Contains an acidic-rich region and a coiled-coil domain
MSL2	Chromosome 2	Contains a ring finger and a Mtn-like domain (synthesis repressed by the sex-lethal protein)
MSL3	Chromosome 3	Contains two noncanonical chromodomains
MLE	Chromosome 2	Member of the DEAD/H RNA helicase family
MOF	X chromosome	Histone acetyltransferase; member of the chromo-MYST family
RNAs		
roX1	X chromosome	Nuclear noncoding RNA, 4.1 kb
roX2	X chromosome	Nuclear noncoding RNA, 0.6 kb

DEAD/H, Asp-Glu-Ala-Asp (or His) box, one of eight conserved sequence motifs in RNA helicases; MYST, MOZ implicated in acute myeloid leukemia, YBF2/SAS 3 and SAS 2 involved in transcriptional silencing in yeast, and Tip60 involved in HIV Tat interactions.

sequences. It can also be achieved by the action of complexes that covalently modify histone tails and alter their affinity for DNA by targeting specific enzymes such as histone acetyltransferases or methyltransferases (reviews: Becker and Horz, 2002; Peterson, 2002).

The complex responsible for dosage compensation in *D. melanogaster* consists of at least five proteins (**Table 2**). It is referred to as the MSL complex because of the male-specific lethality of loss-of-function mutations of its protein components; similar complexes are most likely to exist for the same purpose in other members of the genus (Bone and Kuroda, 1996; Marin *et al.*, 1996; Steinemann *et al.*, 1996). In *D. melanogaster*, the complex includes both a histone acetyltransferase, males absent on the first (MOF) (Hilfiker *et al.*, 1997), and an ATPase, MLE, an ATP-dependent DNA/RNA helicase (Lee *et al.*, 1997). The presence of a sixth protein – a tandem kinase (JIL1) – has been deduced because it is enriched on the X chromosome in salivary glands of male larvae (Jin *et al.*, 1999, 2000) but it is not clear at this time whether JIL1 is an integral subunit of the complex or whether its presence is enhanced on the X chromosome in males because it is involved in the process of transcription and the relative level of transcription of the X chromosome is greater due to the process of compensation.

The MSL complex also contains at least one of the two noncoding RNAs, roX1 and roX2 (RNA on the X one and two, respectively). The existence of these RNAs was first reported independently by Victoria Meller and by Hubert Amreim (Amrein and Axel, 1997; Meller *et al.,* 1997). Both investigators had been searching for male-specific transcripts in the mushroom bodies of *Drosophila* in order to better understand sexual behavior; the roX RNAs were not only present in the brains but also in other somatic tissues of males and their distribution on polytene chromosomes of salivary glands of male larvae was limited to the X, closely following that of the MSL complex. Under normal circumstances, the roX RNAs are essential for the assembly of the complex and they are rapidly degraded if they do not associate with some of the proteins of the MSL complex (Meller *et al.,* 2000). Surprisingly, although they are completely different in size and sequence (**Table 2**), the two RNAs can substitute for each other in that males that can transcribe only one of them compensate normally and are fully viable (Meller and Rattner, 2002).

The complex is absent in females because in this sex, *Sxl* prevents its formation by inhibiting the translation of the *msl2* message (Bashaw and Baker, 1997; Kelley *et al.,* 1997; also see **Chapter 1.1**); the absence of MSL2, in turn, leads to a significant reduction in the level of MSL1 and, without these components, the complex does not form. Formation of the complex can be forced in females by the expression of a *msl2* transgene modified to allow translation of its message; in such females, the complex accesses both X chromosomes, enhances the transcription of most X-linked genes creating an imbalance with autosomal gene products and a drastic reduction in viability. It should be noted that the Y chromosome plays no role in the presence of the complex in males. This is evidenced by the observations that males lacking a Y chromosome and XXY females are fully viable; the former have normal dosage compensation while the latter do not.

In males, the complex associates exclusively with the X chromosome at hundreds of sites and its presence is correlated with the presence of a specific isoform of histone H4, monoacetylated at lysine 16. This was first observed by Bryan Turner (Bone *et al.,* 1994) who noted that this isoform is found only on the X chromosome in males. Using antisera against MSL1 or HA-tagged MSL2 (HA is a polypeptide sequence from the influenza A virus haemagglutinin) to purify the MSL complex from S2 cells, a commonly used cell culture line, Edwin Smith and Antonio Pannuti showed that this partially purified complex specifically monoacetylates H4 at lysine 16 on isolated *Drosophila* nucleosomes (Smith *et al.,* 2000). They also showed that this acetylation is absent when the complex is isolated from S2 cells that express a mutant MOF protein (the histone acetyl transferase of the complex). This work showed that MOF is uniquely responsible for the presence of H4Ac16 on the male X chromosome.

What are the factors and parameters that target the MSL complex to its many sites of action on the X chromosome and exclude it, under normal circumstances, from accessing autosomal sites? Since the roX RNAs are immediately degraded unless they associate with the proteins of the complex, and are necessary for its assembly, this assembly occurs at or near the site of transcription of the *roX* genes, and these genes are on the X chromosome. If an *roX* gene is relocated to an autosomal ectopic site, the MSL complex is found at this site (Kelley *et al.,* 1999), where it enhances the transcription of a reporter gene (Henry *et al.,* 2001). Along the X chromosome there are 30–40 sites for which the complex has a particular affinity; these sites – so-called "entry sites" – are defined as points along the X chromosome where inactive (with a mutant MLE or MOF subunits) or even partial complexes (consisting only of MSL1 and MSL2 subunits) can bind (Lyman *et al.,* 1997; Demakova *et al.,* 2003). Two of these sites are associated with the two *roX* genes and a third site has been identified from the 18D10 region of the X chromosome (Kelley *et al.,* 1999; Oh *et al.,* 2004). Although clearly special in nature, these sites are probably not exclusively responsible for the spreading of the complex along the X chromosomes.

In order to spread along the remainder of the X chromosome, the complex needs both ATPase/helicase and histone acetyltransferase activities; complexes with full-length but inactive enzymatic subunits access only the entry sites and are found, unbound, in the nucleoplasm (Gu *et al.,* 2000). In wild-type males, the distribution of the complex along the X chromosome in salivary glands of male larvae is not uniform and there are regions where its presence is reduced or where it is absent altogether (**Figure 2**). The explanation for this observation was provided by Georgette Sass who demonstrated that the distribution of the complex reflects the transcriptional activity of the X chromosome in a particular tissue where the complex accesses activated genes (Sass *et al.,* 2003). In these genes, it does not specifically target the promoter; rather it acetylates H4 at lysine 16 throughout the length of transcriptional units and beyond. This was shown to be the case by Edwin Smith who used chromatin immunoprecipitation to map this acetylation on genes known to be dosage compensated (Smith *et al.,* 2001).

This and other observations have led to the working hypothesis that the role of the acetylation mediated by the MSL complex is to facilitate transcription elongation, i.e., that the primary mechanistic target of the complex is to facilitate the processivity of the activated transcription complex. Of course, in order to result in a twofold increase in the steady-state level of transcripts, enhanced elongation must be coupled with enhanced reinitiation. The presence of the MSL complex along activated loci and its proposed specific effect on the elongation phase of transcription remind us of the association of the yeast and human Elongator complexes with activated RNA polymerase II (Otero *et al.,* 1999; Hawkes *et al.,* 2002; Kim *et al.,* 2002). Although these complexes acetylate histone H3 and, to a lesser extent, histone H4, it is not clear whether these covalent modifications are responsible for their specific role in facilitating transcription or whether they are responsible for maintaining a global level of acetylation throughout activated regions of the genome (Winkler *et al.,* 2002).

Before ending this section, it should be noted that although significant progress has been achieved in dissecting the molecular mechanisms responsible for dosage compensation, no progress has been made beyond the realm of speculation in determining what limits the transcriptional enhancement to a more or less precise twofold level.

1.7.3. Dosage Compensation in Other Insects

1.7.3.1. Haplodiploid and Parthenogenetic Species

Dosage compensation is a regulatory mechanism designed to cancel the potentially negative consequences of sex chromosome heteromorphism that is characteristic of diplodiploid species (an extreme form of which is the absence, in the heterogametic sex, of a homolog of the X or Z chromosomes). The problem that such a situation presents derives from the imbalance in the level of gene products between sex-linked and autosomal genes whose relative contributions to particular developmental or metabolic pathways would be different in males and females. Such an imbalance does not exist in haploid genomes where the ratio of sex-linked to autosomal genes is the same as in diploids. Therefore, while haploidy does generate its own particular problems relating, for example, to smaller cell size, the question of dosage compensation is not relevant to paternal-genome-loss species or parthenogenetic species with the exception of those cases where the paternal genome is retained in an active form in somatic cells and is lost only during oogenesis. In such cases, given the presence of two X chromosomes in some of the embryos and of a single X in others, the possibility of a mechanism for dosage compensation exists. Unfortunately there is no information on the level of sex-linked gene activity in these forms.

1.7.3.2. Diplodiploid Species

To establish the occurrence of dosage compensation in an organism, it is customary to identify an X-linked or Z-linked gene with a quantifiable product and to determine that the level of this product is the same in males and females. As an alternative, the time-tested technique of transcription autoradiography can be used to demonstrate that the incorporation of [^{3}H]-uridine along the X or Z chromosomes is comparable in the two sexes. Using this second methodology, the existence of dosage compensation in *Rhynchosciara* and in *Sciara* (dipteran species) was established (Casartelli and Santos, 1969; da Cunha *et al.,* 1994). In *Sciara*, Lucas Sánchez and his collaborators determined that antisera against all five protein components of the *Drosophila* MSL complex failed to stain specifically the X and associated with all chromosomes in salivary glands of male larvae, in spite of the fact that the respective *Sciara* cDNAs hybridized to single bands in *Drosophila* genomic Southern blots (Ruiz *et al.,* 2000). These observations suggest that distant genera in the same order may have subsumed different, albeit functionally related, proteins during the evolution of their dosage compensation mechanism.

Due to the usual absence or paucity of genetic information no other investigations of this type appear to have been carried out on any other insects. Instead, several workers have attempted to gain some insight into the nature of the mechanism responsible for dosage compensation in particular species without first having established that equalization of gene products between the sexes does, in fact, occur. The hypothesis tested has been, invariably, that dosage compensation would occur by the somatic inactivation of one of the two X or Z chromosomes (as occurs in all mammals). The basic approach has been to determine by a variety of cytological techniques if one of the two X or Z chromosomes in the somatic cells of XX or ZZ individuals is sensitive to particular clastogenic agents such as 5-bromodeoxyuridine, which induces aberrations in heterochromatic (inactive) chromosomes or [^{3}H]-uridine, which induces aberrations predominantly in euchromatic (active) chromosomes.

An additional test has been to determine whether one of the X or Z chromosomes is late-replicating, suggesting that it may be facultatively heterochromatic and, therefore, inactive. Using these methods, one of the two X chromosomes of *Grillotalpa fossor* and of *Acheta domesticus*, two orthopteran species, were judged to be facultatively heterochromatized and, presumably, inactivated in female somatic cells (Arora and Rao, 1979; Rao and Ali, 1982).

In *Musca domestica*, another dipteran species, the situation is complicated by the fact that, although in most populations the females are XX and the males XY, there exist instances where both sexual chromosome types are present in each sex (Nöthiger and Steinmann-Zwicky, 1985; Schmidt *et al.*, 1997). In some of these exceptional populations, the locus encoding the dominant male-determining factor M, normally Y-linked, has been translocated to an autosome. In others, the master regulatory gene of female differentiation F – normally repressed by the M factor for male differentiation – is represented by a constitutive allele. If a dosage compensation mechanism similar to the one of *Drosophila* and/or *Sciara* has evolved in *Musca* to trump the problem of X-linked dosage differences in normal populations, how can this mechanism become sex-independent in the exceptional populations, in order to allow the survival of XX and XY individuals in both sexes? The answer to this riddle is, in all likelihood, that dosage compensation is not necessary, because few, if any, genes at all are present on the X chromosome.

Another complex situation appears to prevail in *Lucilia*. In the pupal trichogen cells of this dipteran species, the autosomes exhibit the banded appearance typical of *Drosophila* polytene chromosomes, while each of the two X chromosomes in females and the single X in males are diffuse, granular masses with a small, condensed region. In contrast, the Y chromosome is predominantly condensed with a small, loosely organized segment. In XXY males, a single granular X chromosome and an additional banded element are present in trichogen cells, leading to the conclusion that the formation of such an element represents a mechanism by which the extra X-linked genes can be silenced (Bedo, 1982).

Perhaps even more surprising is the conclusion that a *prima facie* case for the absence of dosage compensation can be made in major insect groups in spite of the presence of highly heteromorphic sex chromosomes. This appears to be the case in some Lepidoptera where the specific activity of the Z-linked enzyme 6-phosphogluconate dehydrogenase is twice as high in *Heliconius melpomene* and *H. erato* ZZ males than in ZW females (Johnson and Turner, 1979). More recently, Japanese investigators measured the levels of a Z chromosome-linked transcript and an unrelated muscle protein in *Bombyx* and found them to be two times higher in males than in females (Suzuki *et al.*, 1998, 1999). These observations spell out the major caveat that the presence of heteromorphic sex chromosomes does not, of itself, imply the existence of a dosage compensation mechanism. Such a mechanism may well exist in species of current interest such as *Anopheles, Aedes, Lucilia, Ceratitis,* and others, but its presence must be evidenced by molecular substantiation.

1.7.4. Envoi

In the last decade or so, molecular genetic studies of the phenomenon of dosage compensation have provided unique and amazing windows into fundamental mechanisms of transcriptional regulation in mammals, where the facultative heterochromatization of one of the two X chromosomes in females involves the spread of noncoding RNAs as a harbinger of covalent modifications of both DNA and histones (Lee, 2003). Similar studies in *Caenorhabditis elegans* have revealed that the limited downregulation of both X chromosomes in hermaphrodites involves a subset of proteins and factors that are normally engaged in chromosome condensation during cell division (Meyer, 2000). Finally, of course, in *Drosophila* the equalization of X-linked gene products in males and females relies on a complex that may be the prototype of a novel family of chromatin remodeling complexes. Therefore, there is little doubt that, given the seemingly unending variety of developmental systems found in insects, similar studies of dosage compensation in selected model species will reveal novel and unpredictable regulatory mechanisms of gene function.

References

Amrein, H., Axel, R., **1997**. Genes expressed in neurons of adult male *Drosophila*. *Cell 88*, 459–469.

Arora, P., Rao, S.R., **1979**. Insect sex chromosomes. IV. DNA replication in the chromosomes of *Gryllotalpa fossor*. *Cytobios 26*, 45–55.

Bashaw, G.J., Baker, B.S., **1997**. The regulation of the *Drosophila msl-2* gene reveals a function for Sex-lethal in translational control. *Cell 89*, 789–798.

Becker, P.B., Horz, W., **2002**. ATP-dependent nucleosome remodeling. *Annu. Rev. Biochem. 71*, 247–273.

Bedo, D.G., **1982**. Differential sex chromosome replication and dosage compensation in polytene trichogen cells of *Lucilia cuprina* (Diptera: Calliphoridae). *Chromosoma 87*, 21–32.

Belote, J.M., Lucchesi, J.C., **1980a**. Male-specific lethal mutations of *Drosophila melanogaster. Genetics 96*, 165–186.

Belote, J.M., Lucchesi, J.C., **1980b**. Control of X chromosome transcription by the *maleless* gene in *Drosophila*. *Nature 285*, 573–575.

Bone, J.R., Kuroda, M.I., **1996**. Dosage compensation regulatory proteins and the evolution of sex chromosomes in *Drosophila*. *Genetics 144*, 705–713.

Bone, J.R., Lavender, J., Richman, R., Palmer, M.J., Turner, B.M., *et al.*, **1994**. Acetylated histone H4 on the male X chromosome is associated with dosage compensation in *Drosophila*. *Genes Devel. 8*, 96–104.

Casartelli, C.B.R., Santos, E.P., **1969**. DNA and RNA synthesis in *Rhynchosiara angelae* and the problem of dosage compensation. *Caryologia 22*, 203–211.

Charlesworth, B., **2002**. The evolution of chromosomal sex determination. *Novartis Found. Symp. 244*, 207–219.

Cline, T.W., **1979**. A male-specific lethal mutation in *Drosophila melanogaster* that transforms sex. *Devel. Biol. 72*, 266–275.

da Cunha, P.R., Granadino, B., Perondini, A.L., Sanchez, L., **1994**. Dosage compensation in sciarids is achieved by hypertranscription of the single X chromosome in males. *Genetics 138*, 787–790.

Demakova, O.V., Kotlikova, I.V., Gordadze, P.R., Alekseyenko, A.A., Kuroda, M.I., *et al.*, **2003**. The MSL complex levels are critical for its correct targeting to the chromosomes in *Drosophila melanogaster. Chromosoma 112*, 103–115.

Dobzhansky, T., **1957**. The X-chromosome in the larval salivary glands of hybrids *Drosophila insularis* × *Drosophila tropicalis*. *Chromosoma 8*, 691–698.

Fukunaga, A., Tanaka, A., Oishi, K., **1975**. *Maleless*, a recessive autosomal mutant of *Drosophila melanogaster* that specifically kills male zygotes. *Genetics 81*, 135–141.

Gu, W., Wei, X., Pannuti, A., Lucchesi, J.C., **2000**. Targeting the chromatin-remodeling MSL complex of *Drosophila* to its sites of action on the X chromosome requires both acetyl transferase and ATPase activities. *EMBO J. 19*, 5202–5211.

Hawkes, N., Otero, G., Winkler, G.S., Marshall, N., Dahmus, M.E., *et al.*, **2002**. Purification and characterization of the human elongator complex. *J. Biol. Chem. 277*, 3047–3052.

Henry, R.A., Tews, B., Li, X., Scott, M.J., **2001**. Recruitment of the male-specific lethal (MSLl) dosage compensation complex to an autosomally integrated roX chromatin entry site correlates with an increased expression of an adjacent reporter gene in male *Drosophila*. *J. Biol. Chem. 276*, 31953–31958.

Hilfiker, A., Hilfiker-Kleiner, D., Pannuti, A., Lucchesi, J.C., **1997**. *Mof*, a putative acetyl transferase gene related to the *tip60* and *moz* human genes and to the *sas* genes of yeast, is required for dosage compensation in *Drosophila*. *EMBO J. 16*, 2054–2060.

Jin, Y., Wang, Y., Johansen, J., Johansen, K.M., **2000**. JIL-1, a chromosomal kinase implicated in regulation of chromatin structure, associates with the male specific lethal (MSL) dosage compensation complex. *J. Cell Biol. 149*, 1005–1010.

Jin, Y., Wang, Y., Walker, D.L., Dong, H., Conley, C., *et al.*, **1999**. JIL-1: a novel chromosomal tandem kinase implicated in transcriptional regulation in *Drosophila*. *Mol. Cell. 4*, 129–135.

Johnson, M.S., Turner, R.G., **1979**. Absence of dosage compensation for a sex-linked enzyme in butterflies (*Heliconius*). *Heredity 43*, 71–77.

Kelley, R.L., Meller, V.H., Gordadze, P.R., Roman, G., Davis, R.L., *et al.*, **1999**. Epigenetic spreading of the *Drosophila* dosage compensation complex from roX RNA genes into flanking chromatin. *Cell 98*, 513–522.

Kelley, R.L., Wang, J., Bell, L., Kuroda, M.I., **1997**. Sex lethal controls dosage compensation in *Drosophila* by a non-splicing mechanism. *Nature 387*, 195–199.

Kim, J.H., Lane, W.S., Reinberg, D., **2002**. Human Elongator facilitates RNA polymerase II transcription through chromatin. *Proc. Natl Acad. Sci. USA 99*, 1241–1246.

Kuroda, M.I., Kernan, M.J., Kreber, R., Ganetzky, B., Baker, B.S., **1991**. The maleless protein associates with the X chromosome to regulate dosage compensation in *Drosophila*. *Cell 66*, 935–947.

Lee, C.G., Chang, K.A., Kuroda, M.I., Hurwitz, J., **1997**. The NTPase/helicase activities of *Drosophila maleless*, an essential factor in dosage compensation. *EMBO J. 16*, 2671–2681.

Lee, J.T., **2003**. Functional intergenic transcription: a case study of the X-inactivation centre. *Phil. Trans. R. Soc. Lond. B 358*, 1417–1423.

Lucchesi, J.C., Skripsky, T., **1981**. The link between dosage compensation and sex differentiation in *Drosophila melanogaster. Chromosoma 82*, 217–227.

Lyman, L.M., Copps, K., Rastelli, L.R., Kelley, L., Kuroda, M.I., **1997**. *Drosophila* male-specific lethal-2 protein: structure/function analysis and dependence on MSL-1 for chromosome association. *Genetics 147*, 1743–1753.

Marin, I., Baker, B.S., **1998**. The evolutionary dynamics of sex determination. *Science 281*, 1990–1994.

Marin, I., Franke, A., Bashaw, G.J., Baker, B.S., **1996**. The dosage compensation system of *Drosophila* is coopted by newly evolved X chromosomes. *Nature 383*, 160–163.

Meller, V.H., Gordadze, P.R., Park, Y., Chu, X., Stuckenholz, C., *et al.*, **2000**. Ordered assembly of roX RNAs into MSL complexes on the dosage-compensated X chromosome in *Drosophila*. *Curr. Biol. 10*, 136–143.

Meller, V.H., Rattner, B.P., **2002**. The roX genes encode redundant male-specific lethal transcripts required for targeting of the MSL complex. *EMBO J. 21*, 1084–1091.

Meller, V.H., Wu, K.H., Roman, G., Kuroda, M.I., Davis, R.L., **1997**. RoX1 RNA paints the X chromosome of

male *Drosophila* and is regulated by the dosage compensation system. *Cell 88*, 445–457.

Meyer, B.J., **2000**. Sex in the worm: counting and compensating X-chromosome dose. *Trends Genet. 16*, 247–253.

Mukherjee, A.S., Beermann, W., **1965**. Synthesis of ribonucleic acid by the X-chromosomes of *Drosophila melanogaster* and the problem of dosage compensation. *Nature 207*, 785–786.

Muller, H.J., **1950**. Evidence of the precision of genetic adaptation. *Harvey Lect. Ser. 43*, 165–229.

Normark, B.B., **2003**. The evolution of alternative genetic systems in insects. *Annu. Rev. Entomol. 48*, 397–423.

Nöthiger, R., Steinmann-Zwicky, M., **1985**. A single principle for sex determination in insects. *Cold Spring Harb. Symp. Quant. Biol. 50*, 615–621.

Oh, H., Bone, J.R., Kuroda, M.I., **2004**. Multiple classes of MSL binding sites target dosage compensation to the X chromosome of *Drosophila*. *Curr. Biol. 14*, 481–487.

Otero, G., Fellows, J., Li, Y., de Bizemont, T., Dirac, A.M., *et al.*, **1999**. Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. *Mol. Cell. 3*, 109–118.

Peterson, C.L., **2002**. Chromatin remodeling: nucleosomes bulging at the seams. *Curr. Biol. 12*, R245–R247.

Rao, S.R., Ali, S., **1982**. Insect sex chromosomes. VI. A presumptive hyperactivation of the male X chromosome in *Acheta domesticus* (L.). *Chromosoma 86*, 325–339.

Rudkin, G.T., **1964**. The proteins of polytene chromosomes. In: Bonner, J., Ts'o, P. (Eds.), The Nucleohistones. Holden Day, San Francisco, pp. 184–192.

Ruiz, M.F., Esteban, M.R., Donoro, C., Goday, C., Sanchez, L., **2000**. Evolution of dosage compensation in Diptera: the gene *maleless* implements dosage compensation in *Drosophila* (Brachycera suborder) but its homolog in *Sciara* (Nematocera suborder) appears to play no role in dosage compensation. *Genetics 156*, 1853–1865.

Sass, G.L., Pannuti, A., Lucchesi, J.C., **2003**. Male-specific lethal complex of *Drosophila* targets activated regions of the X chromosome for chromatin remodeling. *Proc. Natl Acad. Sci. USA 100*, 8287–8291.

Schmidt, R., Hediger, M., Roth, S., Nöthiger, R., Dubendorfer, A., **1997**. The Y-chromosomal and autosomal male-determining M factors of *Musca domestica* are equivalent. *Genetics 147*, 271–280.

Smith, E.R., Allis, C.D., Lucchesi, J.C., **2001**. Linking global histone acetylation to the transcription enhancement of X-chromosomal genes in *Drosophila* males. *J. Biol. Chem. 276*, 31483–31486.

Smith, E.R., Pannuti, A., Gu, W., Steurnagel, A., Cook, R.G., *et al.*, **2000**. The *Drosophila* MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation. *Mol. Cell. Biol. 20*, 312–318.

Steinemann, M., Steinemann, S., Turner, B.M., **1996**. Evolution of dosage compensation. *Chromosome Res. 4*, 185–190.

Suzuki, M.G., Shimada, T., Kobayashi, M., **1998**. Absence of dosage compensation at the transcription level of a sex-linked gene in a female heterogametic insect, *Bombyx mori*. *Heredity 81*, 275–283.

Suzuki, M.G., Shimada, T., Kobayashi, M., **1999**. Bm kettin, homologue of the *Drosophila* kettin gene, is located on the Z chromosome in *Bombyx mori* and is not dosage compensated. *Heredity 82*, 170–179.

Traut, W., Marec, F., **1996**. Sex chromatin in Lepidoptera. *Q. Rev. Biol. 71*, 239–256.

Uchida, S., Uenoyama, T., Oishi, K., **1981**. Studies on the sex-specific lethals of *Drosophila melanogaster.* III. A third chromosome male-specific lethal mutant. *Jap. J. Genet. 56*, 523–527.

Winkler, G.S., Kristjuhan, A., Erdjument-Bromage, H., Tempst, P., Svejstrup, J.Q., **2002**. Elongator is a histone H3 and H4 acetyltransferase important for normal histone acetylation levels *in vivo*. *Proc. Natl Acad. Sci. USA 99*, 3517–3522.

1.8 Insect Homeotic Complex Genes and Development, Lessons from *Drosophila* and Beyond

L K Robertson and J W Mahaffey, North Carolina State University, Raleigh, NC, USA

1.8.1. The History and Early Genetics of the *Homeotic Complex* Genes

1.8.1.1. Introduction

The word homeotic has its origins in the Greek word *homoios*, meaning "same" or "similar." William Bateson coined the term "homeosis" in 1894 to describe a mutation that transforms one structure to the likeness of another, particularly with respect to repeated systems such as segments, petals/sepals/stamens, vertebrae, etc. In *Drosophila* genetics, the term is used to describe a mutation that causes one segment to lose its normal identity and take on the appearance of another segment. Two well-known



Figure 1 *Drosophila* homeotic transformation. *Drosophila melanogaster* hemizygous for three regulatory mutations in the *Ultrabithorax* region of the *Bithorax Complex*: *anterobithorax* (*abx*), *bithorax*^{3} (*bx*^{3}), and *postbithorax* (*pbx*). The third thoracic segment is completely transformed to a second thoracic identity. The haltere, the dorsal appendage normally present on the third thoracic segment, is transformed to a wing, and the dorsal third thoracic cuticle develops the characteristics of the second dorsal thoracic cuticle. (Reproduced with permission from E.B. Lewis.)

examples are shown in **Figures 1** and **6c**. Pictured are a loss of function *Ultrabithorax* (*Ubx*) mutant, in which the halteres (balancing organs found on the third thoracic segment) are transformed to second thoracic wings, and a gain of function *Antennapedia* (*Antp*) mutant, in which the antennae are transformed to legs.

There are many homeotic mutations scattered throughout the *Drosophila* genome, but two regions on the right arm of the third chromosome are unique. These two clusters of homeotic genes, the *Antennapedia Complex* (*ANT-C*) and the *Bithorax Complex* (*BX-C*), are referred to jointly as the *Homeotic Complex* (*HOM-C*). *HOM-C* genes direct regional specification of the anterior–posterior body axis in insects as well as in other animals (in which they are called the *HOX* genes). The organization and expression patterns of these genes are collinear; in general, a gene's domain of expression along the anterior–posterior body axis correlates with its genomic position within the complexes (**Figure 2**). This collinearity is also conserved across phyla.

1.8.1.2. Identification of the *Drosophila Bithorax* and *Antennapedia Complexes*

The first homeotic mutation identified in *Drosophila* was *bithorax*^{1} (*bx*), isolated in Thomas Hunt Morgan's laboratory in 1915 by C.B. Bridges. This mutation affects the third thoracic segment, transforming part of the haltere towards wing. Other homeotic mutations were isolated in the following years, but it was not until the work of Edward B. Lewis that the *HOM-C* was recognized as a complex of genes required for proper anterior–posterior patterning.

Lewis began his work on genes within the *BX-C* in 1946 because these genes appeared to be organized in a cluster of duplicated and diverged genes, rather than as a series of alleles of the same gene (Lewis, 1998) – the mutant loci within the cluster were functionally related and separable by recombination, while maintaining their mutant phenotypes. The isolation of a deficiency (*Df-P9*) deleting the entire *BX-C* allowed Lewis to characterize the functions of each mutation along the *BX-C* individually and together.

Lewis' seminal review article (Lewis, 1978), describes his view on the organization and developmental function of the *BX-C*. Important was his observation that individual mutations within the complex cause transformations within specific, and sometimes exclusive, body regions. For example, the *bx* mutation effects only the anterior of the third thoracic segment, while the *bithoraxoid* (*bxd*) mutation transforms the posterior portions of the third thoracic segment and the first abdominal segment towards a posterior second thoracic identity. Based on adult and larval phenotypes of *BX-C* mutations, Lewis proposed that at least eight genes existed within the complex, which "seem(s) ... to control much of the diversification of the organism's thoracic and abdominal segments." He further suggested that these genes act "indirectly by repressing or activating other sets of genes which then directly determine the specific structures and functions that characterize a given segment." Lewis also put forth the idea that such genetic changes explained evolutionary changes in body pattern – such as the change in insects from four wings, as in lepidopterans, to the two wings found in dipterans, through the development of what Lewis called "a haltere promoting gene." Similarly, Lewis suggested that the reduction in insect leg number, from an early millipede-like ancestor, might be explained by the development of a "leg-suppressing gene." Finally, Lewis also identified mutations that defined the *Polycomb* (*Pc*) gene as encoding a regulator of the *BX-C* genes. *Polycomb* is the founding member of a group of genes that are involved in maintaining proper homeotic gene expression patterns, and is further discussed below (see Section 1.8.3.2).

In 1980, using deficiencies and complementation analysis, Thomas C. Kaufman and colleagues identified a second cluster of homeotic genes on the right arm of the third chromosome (Kaufman *et al.*, 1980) (**Figure 2**). Like the *BX-C*, the genes in this cluster had the ability to cause homeotic transformations of one segment type to another, but this cluster of genes controlled the development of the head and anterior thorax, and was named the

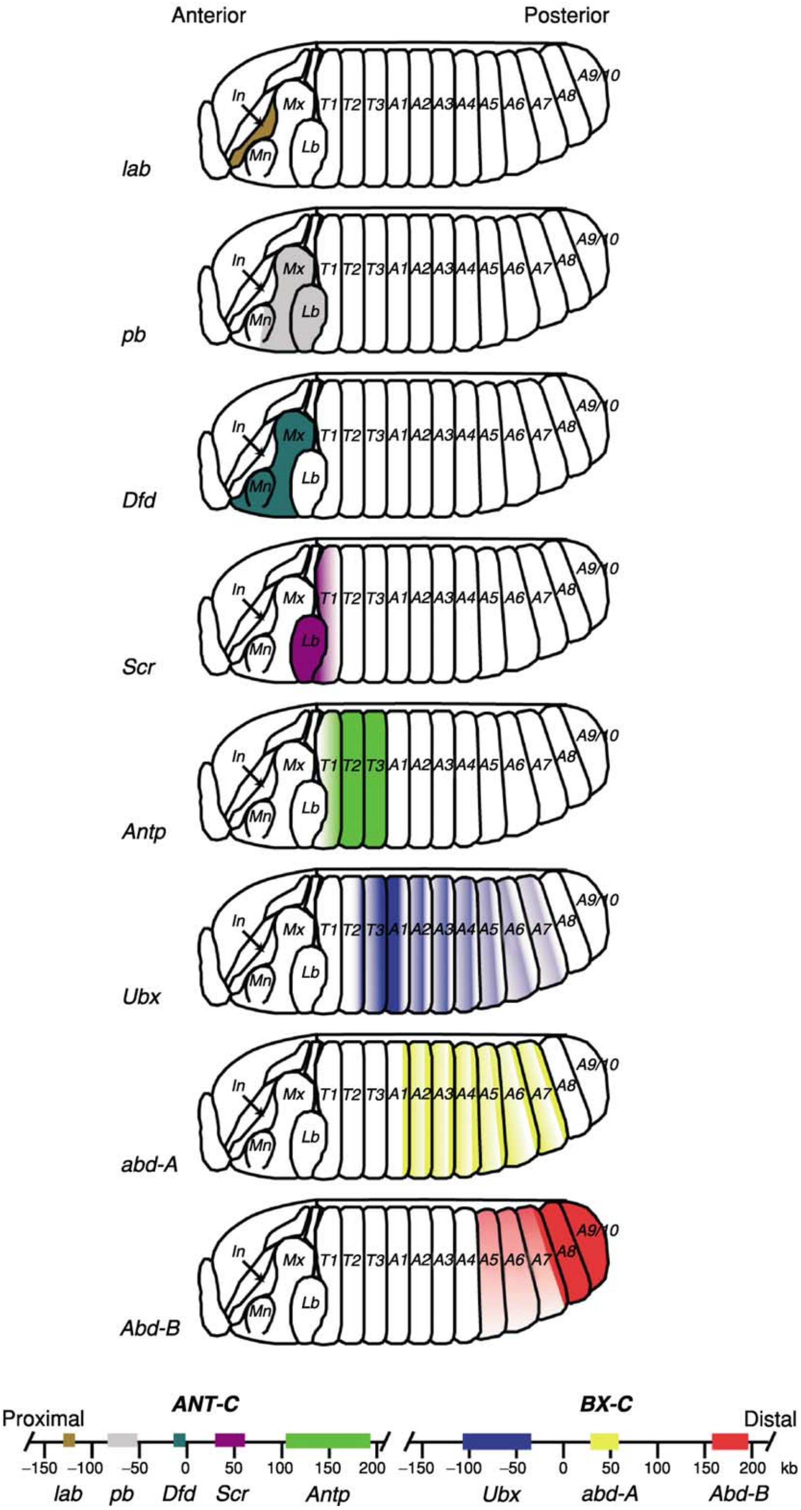


Figure 2 Regional HOM-C gene expression in *Drosophila* embryos. A simplified representation of the *HOM-C* genes' epidermal expression domains. Each gene's relative position within the *ANT-C* or *BX-C* is also shown. The collinearity of genomic location and expression along the embryonic axis is evident. The variation in the shading intensity approximates variations in the level of gene expression. Numbers along the complex are in kilobases. In, intercalary; Mn, mandibular; Mx, maxillary; Lb, labial; T#, thoracic segments; A#, abdominal segments. See text for references.

Antennapedia Complex (*ANT-C*) after its first characterized gene.

A number of diverse homeotic transformations correspond to mutations within the *ANT-C*, and many of the initially recovered mutations lead to viable, transformed adults. For example, one deficiency causes the reduction of male sex combs on the first thoracic leg, indicating that genes within this region are required for first thoracic leg identity. A gain-of-function mutation transforms the second and third thoracic legs to a first thoracic identity as evidenced by the presence of ectopic sex combs on the male second and third thoracic legs (Kaufman *et al*., 1980). Saturating X-ray and ethyl methanesulfonate (EMS) mutatagenesis allowed more precise characterization of the complex and the beginnings of the developmental mapping of the *ANT-C* genes (Lewis *et al*., 1980a, 1980b; Kaufman *et al*., 1990). The analogy to the *BX-C* suggested that larval recessive lethal mutations, resulting in homeotically altered larvae, ought to be recoverable from the *ANT-C*, and this proved to be the case (Wakimoto and Kaufman, 1981; Wakimoto *et al*., 1984; Sato *et al*., 1985).

Although genetic analyses demonstrated a requirement for the homeotic genes for the establishment of the correct body pattern of the fly, how homeotic genes accomplished this was also on the minds of these pioneering researchers. Both Lewis and García-Bellido predicted correctly that the homeotic genes encoded proteins that controlled the expression of other genes (García-Bellido, 1977; Lewis, 1978). García-Bellido referred to the homeotic genes as "selector" genes that encoded developmental switches sending the expressing cells down one or another developmental pathway. He proposed that "realizator" genes were the receivers of this message and that these encoded the products constructing specific segmental identities.

1.8.1.3. Cloning and Characterization of the *Antennapedia* and *Bithorax Complexes*

The *BX-C* and *ANT-C* regions were cloned at about the same time in different laboratories. The Hogness laboratory initiated a chromosome walk in the *Ultrabithorax* (*Ubx*) region and cloned the *BX-C* in 1983 (Bender *et al*., 1983). Both Kaufman's group and Walter Gehring's laboratory cloned the *ANT-C* complex, also in 1983 (Scott *et al*., 1983; Garber *et al*., 1983). The next significant discovery, in 1984, was that of the homeobox. This 180 bp sequence was found to be present in all *HOM-C* genes (McGinnis *et al*., 1984; Scott *et al*., 1985) and supported Lewis's original idea that the *BX-C* might represent a group of duplicated and diverged genes. The *ANT-C* was found to contain one homeobox for each homeotic gene that was characterized, and for a few that were not located within homeotic loci. The *BX-C* was, however, found to have only three homeobox sequences – significantly fewer than the eight genes predicted to reside in this region (Regulski *et al*., 1985). This discovery, along with further genetic analysis demonstrating the existence of only three lethal complementation groups in the *BX-C*, confirmed that only three genes reside within this region (Sánchez-Herrero *et al*., 1985). The remaining "genes" proved to be *cis*-regulatory elements that control subdomains of *BX-C* gene expression (see Section 1.8.3.3).

One major difference between the *ANT-C* and the *BX-C* is the presence of intervening, nonhomeotic genes within the *ANT-C* (**Figure 3**). The *fushi tarazu* (*ftz*), *bicoid* (*bcd*), *zerknüllt* (*zen*), and *zerknüllt-2* (*z-2*) genes are all required during early embryogenesis, and all of these genes contain a homeobox. Interestingly, *bicoid*, *zerknüllt*, and *zerknüllt-2* are thought to have arisen through the duplication and divergence of a single ancestral *HOM-C* gene (Falciani *et al*., 1996; Stauber *et al*., 1999). Also present in the *ANT-C* is a cluster of cuticle synthesis genes, which are repeated elsewhere in the genome and do not appear to be required for development. Finally, *amalgam* (*ama*), encoding a protein involved in cell adhesion, is also present in the *ANT-C*.

In the years following, a vast amount of study has examined *HOM-C* gene function, regulation, and structure in detail. The complexes are conserved in virtually all animals, as are the axial patterning functions and collinear relationship between expression patterns and genomic arrangement. Studies have further characterized mutant phenotypes, expression, function, and the genomic structure of the *HOM-C* genes in *Drosophila* and other insects. In 1995, Edward Lewis, along with Eric Wieschaus and Christiane Nüsslein-Volhard, were awarded the Nobel Prize for Physiology or Medicine for their discoveries concerning the genetic control of early embryonic development. We will, in subsequent pages, attempt to present a comprehensive summary of *HOM-C* gene study in insects and will add results from other organisms where appropriate.

1.8.2. *HOM-C* Gene Function, Cytology, Expression, and Mutant Phenotypes

1.8.2.1. Introduction

Without doubt, *Drosophila melanogaster* is the most studied organism with regard to the *HOM-C* genes. These transcription factors were initially identified

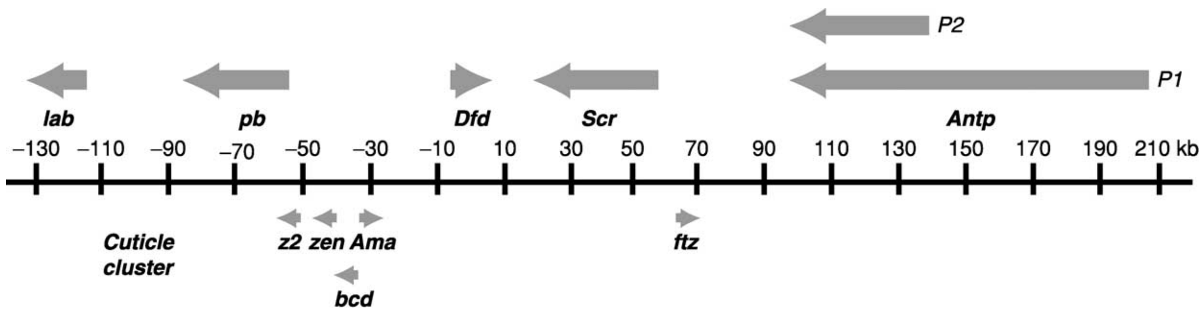


Figure 3 The molecular organization of the *Drosophila Antennapedia Complex* (*ANT-C*) locus. This portion of the *Drosophila melanogaster Homeotic Complex* (*HOM-C*) differs from the *Bithorax Complex* (*BX-C*) region by the presence of a number of genes not considered to have homeotic function, *per se*. A cluster of 16 cuticle genes lies between the *labial* (*lab*) and *proboscipedia* (*pb*) loci. The region between *pb* and *Deformed* (*Dfd*) is intervened by *zen 2* (*z2*), *zen* (*zen*), *bicoid* (*bcd*), and *amalgam* (*ama*). Finally, *fushi tarazu* (*ftz*), lies within the *Sex combs reduced* (*Scr*) regulatory region. The horizontal line represents approximately 340 kb of the *ANT-C* region marked in kilobases. The divisions derive from the extension of a chromosomal walk initiated by Scott *et al.* (1983) with the *Dfd* locus at the zero point. Arrows above and below the horizontal lines designate the genomic regions contributing to each gene's transcript(s) and the relative direction of transcription. The regions under the control of the P1 and P2 promoters of *Antennapedia* (*Antp*) are indicated separately.

and characterized in the fruit fly, and much of what is known of their regulation and function has come from this work. Characterization of *HOM-C* gene expression, function, and molecular organization also extends to a number of other animals, including nondrosophilid insect species and noninsect arthropods. Better-characterized insects include the apterygote insect *Thermobia domestica* (firebrat), *Schistocerca americana* (grasshopper), *Oncopeltus fasciatus* (milkweed bug), *Tribolium castaneum* (red flour beetle), *Bombyx mori* (silk moth), *Precis coenia* (butterfly), and *Apis mellifera* (honey bee). Less studied insects include *Ctenocephalides felis* (flea), *Aedes albopictus* and *Anopheles gambiae* (two mosquito species), *Schistocerca gregaria* (locust), *Acheta domesticus* (cricket), *Folsomia candida* (basal terrestrial apterygote), and *Neodiprion abietis* (sawfly).

In most animal species other than *Drosophila*, the *HOM-C* genes are clustered together in a single complex, differing from the split complexes observed in several *Drosophila* species (Beeman, 1987; Ferrier and Akam, 1996; Davenport *et al.*, 2000; Powers *et al.*, 2000; Cook, 2001). This split appears to have occurred within the *Drosophila* lineage, as different *Drosophila* species have split the complex in different locations (Von Allmen *et al.*, 1996; Negre *et al.*, 2003). This implies that the *Drosophila* ancestor likely had a single complex and the single insect *HOM-C* is the ancestral state. The size of the characterized clusters varies among insect species. For example, the portion of the *Tribolium* complex corresponding to the *Drosophila ANT-C* is approximately 279 kb, while the same region in *Drosophila* includes approximately 439 kb (Brown *et al.*, 2002a).

The *HOM-C* genes of the *Drosophila ANT-C* are *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*). The *HOM-C* genes of the *Drosophila BX-C* are *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*). Each *HOM-C* gene is expressed in a regional manner, and **Figure 2** illustrates the generalized expression patterns of the *HOM-C* genes in a mid-staged *Drosophila* embryo. The maximum morphological and molecular similarity between insects is observed during the embryonic germ band stage, which may represent an evolutionarily constrained (phylotypic) developmental stage (Patel, 1994).

The characterization of each *HOM-C* gene in *D. melanogaster* is first reviewed in detail, followed by a discussion of studies in other insects with emphasis on their differences from flies. We will begin with the gene governing the anteriormost development and then move posteriorly, demonstrating the collinearity of gene order and anterior–posterior body axis.

Though often depicted with rather simple expression patterns, the *HOM-C* genes can be quite dynamic, varying in both the level and the extent of expression within each respective domain. Expression boundaries can be segmental, parasegmental, or a combination of the two, and, in some cases, the expression boundaries do not coincide with segmental or parasegmental boundaries.

Each segment can be thought of as being composed of an anterior and a posterior compartment (**Figure 4**). A parasegment has the same width as a segment, but the anterior of a parasegment is the posterior compartment of a segment, and the

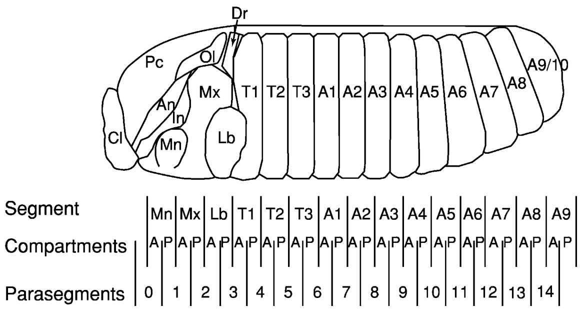


Figure 4 Segments, parasegments, and compartments. Gene expression boundaries in the developing *Drosophila* embryo can be described as segmental, parasegmental, a combination of the two, or sometimes expression boundaries coincide with neither. Parasegment (0–14) and segment boundaries are shown in relation to one another and to a germ band contracted *Drosophila* embryo. The anterior (A) and posterior (P) compartments of each segment are indicated. Parasegment boundaries are shifted anteriorly one compartment from the segmental boundaries. The anteriormost head segments are not labeled in this diagram. Cl, clypeolabrum; Ol, optic lobe; Dr, dorsal ridge; An, antennal; In, intercalary; Mn, mandibular; Mx, maxillary; Lb, labial; T#, thoracic segments; A#, abdominal segments.

posterior portion of a parasegment is the anterior compartment of the following segment. Therefore, a parasegment is similar to a segment, but it is shifted in register by one segmental compartment (Martinez-Arias and Lawrence, 1985).

The distinction between segments and parasegments is important with regard to *HOM-C* expression in the visceral mesoderm and the central nervous system (CNS). In the visceral mesoderm, the expression of most *HOM-C* genes is shifted posteriorly approximately the width of one parasegment, as compared to the ectoderm. The domains of *HOM-C* gene expression can overlap in the ectoderm, but their expression domains in the visceral mesoderm are mutually exclusive (Tremml and Bienz, 1989). As noted in the discussion below, *HOM-C* gene expression within the CNS is also frequently shifted, although by only half of a segment, such that the expression boundaries are parasegmental. The *Dfd* gene is an exception, with its expression boundaries shifting anteriorly in both the visceral mesoderm and the CNS.

1.8.2.2. The *HOM-C* Genes of the *ANT-C*

The genes of the *Drosophila ANT-C* (Figures 2 and 3) are expressed primarily in the head and thorax of the developing embryo and effect the development of corresponding regions in the adult.

1.8.2.2.1. *labial* (*lab*) As the most anteriorly expressed and most proximal (with regards to its chromosomal position) member of the *ANT-C*, *lab* is required for proper development of the intercalary head segment (Mlodzik *et al.*, 1988; Diederich *et al.*, 1989). *lab* is also required for cell fate specification in the tritocerebral neuromere (Hirth *et al.*, 1998) and for copper cell differentiation in the gut endothelium (Hoppler and Bienz, 1995). The *lab* gene spans approximately 17 kb and contains three exons, with the homeobox divided between the second and third exon. The *lab* transcript is ~3.0 kb and encodes a 67.5 kDa protein of 629 amino acids.

Transcripts of *lab* are first detected at 2 h after egg laying (AEL), with the peak of embryonic expression occurring at 6–8 h AEL (Merrill *et al.*, 1989). Expression remains constant through 16–20 h AEL, but decreases before hatching. A low level of expression can be detected throughout pupal development, and no expression is detectable in adult flies.

Temperature sensitive mutants were used to ascertain the temporal requirement for *lab* expression. These experiments have shown that the requirement for *lab* extends from 3–4 h to 16–18 h of embryogenesis, with the period from 6 to 14 h being the most critical for viability to the adult stage.

The distribution of the Lab protein has been examined in embryos and in larval imaginal discs (Diederich *et al.*, 1989; Mahaffey *et al.*, 1989). In the embryo, Lab initially accumulates during germ band extension, with two regions of expression observed. An anterior, ectodermal stripe of expression is present along the ventrolateral border of the procephalon, extending to the anterior border of the postoral segments. This region corresponds to the intercalary segment, which contains the stomadium. A more posterior domain of expression is found in the midgut, where the anterior and posterior midgut primordia fuse. Despite the gene's name, there is no detectable accumulation of Lab in the gnathal lobes (mandibular, maxillary, labial), but expression is evident in the dorsal ridge and in the CNS in the supra/subesophageal ganglia at the base of both brain hemispheres. Lab protein also accumulates lateral to the ventral nerve cord (VNC) and in individual epidermal cells that are precursors to certain sensory organs. These include cells that correspond to the labral sense organ, the black dot organs, and the sense organs of the larval tail. The Lab protein also accumulates in the eye–antennal disc in third instar larvae.

Like other homeotic genes that specify head segment identities, *lab* mutants have no obvious homeosis in the larval head. Embryos homozygous for null mutations in *lab* have grossly disrupted mouthparts and die before hatching, after failing to undergo proper head involution (Merrill *et al.*, 1989). The mandibular and maxillary lobes, which

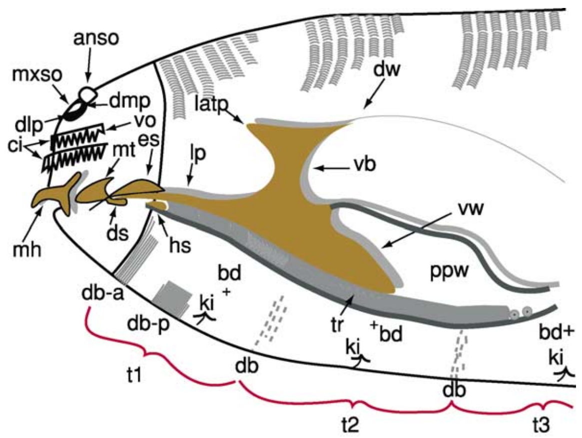


Figure 5 Structures of the *Drosophila* larval head. The cutilcular and cephalopharyngeal structures of the larval head and first thoracic segments. Abbreviations, followed by the segmental origin: dw, dorsal wing, acron; latp, latticed piece (dorsal bridge), acron; vb, vertical bridge, acron; es, epipharyngeal sclerite, labral; mt, median tooth, labral; anso, antennal sense organ, antennal; ppw, posterior pharyngeal wall, intercalary; lp, lateral process, mandibular; vw, ventral wing, mandibular; tr, T-ribs, mandibular; mh, mouth hook, mandibular (tip)/maxillary (base); mxso, maxillary sense organ, maxillary; dmp, dorsal–medial papilla of the mxso, antennal; dlp, dorsal–lateral papilla of the mxso, mandibular; ci, maxillary cirri, maxillary; vo, ventral organ, maxillary; ds, dental sclerite, maxillary; hs, hypostomal sclerite (H-piece), labial; db, denticle belt, thoracic; db-a, first thoracic anterior belt, thoracic; db-p, first thoracic posterior belt, thoracic; ki, Keilin's organs, thoracic; bd, ventral kolbchen (black dot organs), thoracic. (Data from Jürgens, G., Lehman, R., Scharding, M., Nüsslein-Volhard, C., **1986**. Segmental organization of the head in the embryo of *Drosophila melanogaster*. *Roux's Arch. Devel. Biol. 195*, 359–377 and FlyBase Consortium **2003**. The FlyBase database of the *Drosophila* genome projects and community literature. *Nucl. Acids Res. 31*, 172–175. http://flybase.org/.)

normally fuse, fail to do so (see **Figure 5** for a diagram of the *Drosophila* larval head structures). Further, the dorsal pouch, which gives rise to the dorsal portion of the larval head, develops abnormally. This disruption of head involution leaves the clypeolabrum, maxillary and mandibular lobes, and pharynx protruding from the anterior of the embryo, so that the larval cephalopharyngeal structures are not assembled properly. The lateral processes, the H-piece bridge, and the ventral arms of the cephalopharyngeal skeleton are absent. The median tooth is broadened, and amorphous sclerotized material is present in the head. In severe mutants, the T-ribs and pharynx are affected.

Interestingly, many of the affected tissues do not express *labial* during embryonic development, and thus may suffer effects secondary to *labial* loss. Also characteristic of *lab* mutants is the appearance of sclerotized material between the anal pads in the tail region. Despite the expression of *lab* in the midgut and peripheral nervous system during larval development, these tissues appear to develop normally in the absence of *lab* (Merrill *et al*., 1989).

Analysis of adult phenotypes demonstrates the homeotic nature of *lab* mutants (Merrill *et al*., 1989). Though there are variable effects on the head (one or both of the maxillary palps are missing, the ventral margin of the eye is disrupted, and lumps of tissue with bristles protrude from the surrounding cuticle), the homeosis is obvious because thoracic-like bristles appear in posterior regions of the head, and a thoracic spiracle develops near the center of the postgena. (Labeled diagrams of the adult *Drosophila* head are available at http://www.flybase.org/anatomy/image-browser.html)

The expression of *lab* has been also examined in *Thermobia* and *Tribolium*. While expression in the intercalary segment mirrors that of the fly, *lab* is not detected in the midgut or dorsal ridge of the basal insect *Thermobia* (Peterson *et al*., 1999; Nie *et al*., 2001). Unfortunately, it is not clear whether this is due to differences in expression or to technical difficulties in the mRNA localization at later stages of development. Further, the exact role of *lab* in the intercalary segment is not well defined, as there are no common appendages arising from the intercalary segment, and it is only in the higher dipterans that *lab* would have a role in head involution (Hughes and Kaufman, 2002).

1.8.2.2.2. *proboscipedia* (*pb*) The *pb* gene is required for proper development of the adult maxillary and labial appendages but, interestingly, has no role in the development of the larva, even though it is expressed in the embryonic maxillary and labial segments (Pultz *et al*., 1988). Perhaps this is reflection of a more ancient role, where appendages are found in the maxillary and labial segments of the larva, or perhaps this expression is necessary to prepare the tissues for the adult developmental program. The *pb* gene is approximately 34 kb and contains nine exons. Alternative splicing yields four different *pb* mRNA forms, each differing immediately upstream of the homeobox and encoding a different protein isoform (Cribbs *et al*., 1992). Like *lab* and *Abd-B*, the homeobox of *pb* is split between two exons, a characteristic also observed in homologs of these genes in other animals.

Embryonic expression is first evident when the germ band is extended, immediately prior to segmentation, 5.5–6 h AEL. The Pb protein is first detected in the presumptive mesoderm behind the stomodeum. As the germ band contracts, Pb protein is detected under the mandibular lobe and in the ectoderm of the maxillary and labial lobes, gradually increasing as the gnathal lobes develop. Protein

accumulation is later evident in the ventral mandibular segment, though not in the ventral regions of the maxillary or labial segments. It overlaps partially with Dfd and Scr in these regions (Mahaffey *et al*., 1989). Pb accumulates within several cells of each neuromere in the CNS (Pultz *et al*., 1988; Mahaffey *et al*., 1989). Expression of *pb* is also evident during the late larval and pupal stages in the labial disc and a small region of the eye–antennal disc.

In complete loss of function *pb* mutants, there is no observed embryonic phenotype, which makes *pb* unique among the *ANT-C* genes of *Drosophila*. Its function is required only for proper development of the adult labial and maxillary structures (Bridges and Dobzhansky, 1933; Pultz *et al*., 1988). In strong loss-of-function alleles, the adult labial palps are transformed toward prothoracic legs, though weaker alleles transform the labial palps towards antennae. The fate of the maxillary palps in *pb* mutants is unclear; they are reduced and may be transformed toward antennae (Villee, 1944; Pultz *et al*., 1988).

The expression of the *pb* gene has been characterized in several other insects, and its function in milkweed bugs and beetles has been studied using RNA interference (RNAi) and traditional genetics, respectively. In general, the expression of *pb* is similar to that of the fly, but there is a potentially significant difference in *Oncopeltus* (Rogers and Kaufman, 1997; Rogers *et al*., 2002). Here, there is no expression of *pb* in the limb buds of the maxillary segment, but *pb* is expressed in the primordia of the maxillary plate and in the labial appendage. Rogers *et al*. (2002) suggest that this difference in *pb* expression may be correlated with, or even be the cause of, a change in the mouthpart structures from a mandibulate precursor to a stylate–haustellate type. They suggest that a change in *HOM-C* expression may cause a homeotic alteration of the maxillary appendage away from an appendage similar to that of the labial segment. If true, this would be quite interesting, as it would demonstrate that body plans could tolerate changes in *HOM-C* gene expression to yield differing developmental outcomes. RNAi reduction of *Oncopeltus pb* transforms the distal labium to first thoracic legs, similar to what occurs in the fly.

Unlike *Drosophila*, mutations in *maxillopedia* (*mxp*) (the *Tribolium* homolog of *pb*) have an embryonic effect (Beeman *et al*., 1989; Shippy *et al*., 2000). Null alleles are recessive larval lethals and cause the transformation of maxillary and labial palps toward legs. Only the distal portion of the each palp (telopodite) is transformed to leg. The proximal portion of the appendage (the coxopodite and a ventral projection called the endite lobe) remains untransformed. Normally, the labial coxopodites fuse at the ventral midline; *mxp* mutants retain the fused coxopodites, but the telopodites are transformed to legs. The degree of transformation is variable – the homeotic legs may be warped, shortened, or may resemble normal palps with only a tarsal-like claw.

RNAi has been used to investigate the role of several *Tribolium HOM-C* genes, and with *mxp*, RNAi generates phenotypes similar to null larvae. Dominant gain-of-function *mxp* mutations also exist in *Tribolium*, with phenotypes that are interpreted to be a transformation of the legs and antennae toward maxillary or labial palps. In addition, the mutant larvae have altered head capsules. Thus, the phenotype of *mxp* mutants supports the theory that an ancestral *pb*-like *HOM-C/HOX* gene had embryonic functions, which were later lost in *Drosophila*. Similar phenotypic effects are observed in *A. albopictus pb*-like mutants (Quinn and Craig, 1971), but the mutation has not been localized to the mosquito *pb* locus.

1.8.2.2.3. *Deformed* (*Dfd*) *Deformed* gene expression is required for proper development of the *Drosophila* mandibular and maxillary segments (Wakimoto and Kaufman, 1984), as well as proper cell fate specification within the embryonic brain (Hirth *et al*., 2001). The first allele was recovered in 1923 as a homozygous viable, dominant mutation reducing the anterior and ventral eye margins, and also disrupting the vibrissae (head bristles on the lower sides of the adult head capsule near the mouthparts). Later work by Wakimoto and Kaufman (1984) identified the requirement for *Dfd* in the specification of the embryonic head. The *Dfd* gene encompasses about 11 kb, having five exons, which produce a single transcript of 2.8 kb and a protein of 586 amino acids (Regulski *et al*., 1987).

Deformed is the first *HOM-C* gene to be expressed, with its protein first detectable at the cellular blastoderm stage (Mahaffey *et al*., 1989). Expression continues throughout the embryonic, larval, and pupal stages of development and is also detected in adult flies. Early embryonic expression of *Dfd* encircles the embryo, encompassing the cephalic furrow. Later accumulation is detected, transiently, in the primordia of the hypopharyngial lobes, and in the developing mandibular and maxillary segments. Expression in the CNS and mesoderm is offset anteriorly, so that mesodermal and neural expression is parasegmental. Deformed (Dfd) protein expression is also present in cells between

the developing dorsal ridge and the optic lobe. By late germ band contraction, Dfd is strongly expressed in cells within the frontal sac that will contribute to the larval eye–antennal discs. Larval expression is localized to the eye–antennal disc in the peripodial membrane and the regions destined to become the maxillary palps. The Dfd protein is also detected in the basalmost portion of the labial discs (Martinez-Arias *et al*., 1987; Diederich *et al*., 1991).

Deformed gene activity is required at two critical developmental stages: from 3 to 10 h of embryonic development and later, during larval–pupal development (Merrill *et al*., 1987). Like the other head *HOM-C* genes, *Dfd* mutants exhibit no obvious larval homeotic transformations. Null mutations in *Dfd* disrupt larval head development, causing defects in the cephalopharyngeal skeleton and in sensory structures deriving from the maxillary and mandibular segments (Merrill *et al*., 1987; Regulski *et al*., 1987). The mandibular and maxillary lobes fail to fuse and fail to internalize during head involution (a movie depicting *Drosophila* head involution is available at http://flybase.bio.indiana.edu/images/lk/Animation). Lost or reduced cephalopharyngeal structures include the mouth hooks, H-piece, maxillary cirri, maxillary sense organs, and portions of the antennal–maxillary sensory complex. In the adult, lack of Dfd causes head defects, including disruption of the dorsal and ventral postorbital bristles, changes in morphology of the postgena (including ectopic bristles), loss or reduction of the maxillary palps, the inability to appropriately extend the proboscis, and inclusion of thoracic-like bristles on the posterior head, thus suggesting a transformation to a thoracic-like identity (Merrill *et al*., 1987; Regulski *et al*., 1987).

The *Dfd* gene has been cloned from, and its expression examined in, several insects. In general, expression is similar to that in flies, being present in the mandibular and maxillary segments. Loss-of-function analysis using both genetic and RNAi techniques was undertaken in *Tribolium* and *Oncopeltus*, respectively (Brown *et al*., 1999; Hughes and Kaufman, 2000). Here, a difference from *Drosophila* may occur. Loss of *Dfd* in *Tribolium* transforms the maxillary appendages to antennae and eliminates the endites from the mandibular coxopodites (Figure 6b) (Brown *et al*., 2000). In *Oncopeltus*, depletion of *Dfd* results in the transformation of both the maxillary and the mandibular segments towards antenna (Hughes and Kaufman, 2000), and it has been suggested that this may represent a "ground state" for gnathal segment identity (Stuart *et al*., 1991). The differences observed between *Drosophila Dfd* mutants and loss of *Dfd* in other insects may, however, simply reflect the fact that there are no larval appendages in *Drosophila*. Accordingly, the phenotypes of flies and other insects may still represent equivalent developmental alterations, though one must be

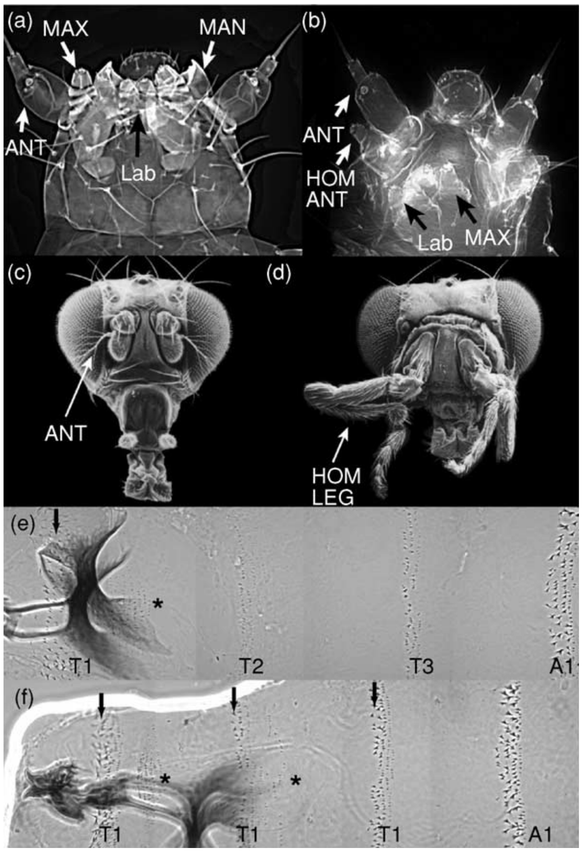


Figure 6 Phenotypic consequences of insect *HOM-C* mutations. Both loss-of-function mutations (b, f) and gain-of-function mutations (d) in the *HOM-C* genes can have dramatic phenotypic effects in *Tribolium* and *Drosophila*. Fluorescent, deconvoluted images of a wild-type (a) and a *TcDfd*[1]/*TcDfd*[1] mutant *Tribolium* larva (b). Normal antennae (ANT), mandibles (MN), maxillary palps (MAX), and labials palps (Lab) are visible in the wild-type larva. In a larva lacking *TcDfd* function (b), the mandibles are transformed to homeotic antennae (HOM ANT). A scanning electron micrograph of a wild-type (c) and a mutant (ectopic *Antp* expression) adult *Drosophila* head (d). The ectopic activation of *Antp* in the antennae (ANT) causes transformation to a second thoracic leg (HOM LEG). In a wild-type first instar *Drosophila* larva (e), the three thoracic segments differentiate characteristic denticle patterns. The first thoracic denticles include a main belt (marked with an arrow), and a second, small patch of denticles called the ''beard'' (marked by an asterisk). The morphology of the first thoracic denticles differs from those of the second and third segments, with the latter being smaller and finer. In the cuticle of an end-stage larva lacking *Antp* (f), the second and third thoracic segments are transformed towards a first thoracic identity. Note the appearance of a beard in the second thoracic denticle belt (marked by an asterisk), and the morphological change of the second and third thoracic denticles to a first thoracic type (arrows). (*Tribolium* images courtesy of S.J. Brown; *Drosophila* head images courtesy of FlyBase (FlyBase Consortium, 2003) http://www.flybase.org)

careful in interpreting any phenotype as the ground state, as it is likely that some patterning genes are still present to influence appendage development.

1.8.2.2.4. ***Sex combs reduced*** **(*Scr*)** Spanning about 100 kb, *Scr* encodes at least four separate transcripts derived from alternative splicing of four exons (Andrew, 1995). A large portion of the *Scr* regulatory region is separated from the coding region by the *ftz* segmentation gene (**Figure 3**). Mutations effecting embryonic function are 5′ to the *ftz* gene, closer to the *Scr* transcription unit, while semilethal lesions map 3′ of the *ftz* gene, towards *Antp* (Pattatucci *et al.*, 1991; Pattatucci and Kaufman, 1991). The *Scr* protein product is 417 amino acids with a molecular weight of 44 kDa (Andrew, 1995).

The *Scr* gene is particularly interesting in that it controls segment identity in two different tagma of the embryo: the labial gnathal segment and the adjoining first thoracic trunk segment. Its transcripts are first detected as gastrulation begins, 2:50 to 3:10 h AEL, and continue to be present throughout the remainder of embryogenesis (Martinez-Arias *et al.*, 1987). Although lagging by 2–3 h behind the expression of RNA, protein accumulation follows the spatial pattern of accumulation of RNA transcripts, being found throughout the labial lobes and the anterior half of the first thoracic segment (Mahaffey and Kaufman, 1987). In addition, a few cells in the dorsal ridge also accumulate the Scr protein. As the germ band begins to contract, Scr protein accumulates in the midgut visceral mesoderm (Tremml and Bienz, 1989; Reuter and Scott, 1990). The Scr protein is also required for development of the salivary glands, and accumulates in the salivary gland primordia prior to invagination. However, Scr is entirely absent after invagination of the developing glands. The *Scr* transcripts and protein accumulate in third instar larval first thoracic discs (leg and humeral) and in the labial discs (Mahaffey and Kaufman, 1987). In larvae, the Scr protein is localized to the VNC and in the majority of the labial discs, the first thoracic leg discs, and the dorsal region of the first thoracic humeral discs (Mahaffey and Kaufman, 1987; Glicksman and Brower, 1988). Protein accumulation is also weakly detectable in the second and third thoracic leg discs, but not in the dorsal counterparts of these (wing and haltere). In the adult, the protein is detected in the CNS, in the subesophageal region only.

In the embryo, the homeotic nature of *Scr* appears only in the first thoracic segment, which, in null *Scr* mutants, is transformed to a second thoracic identity. The posterior denticle belt (beard) is lost and the remaining anterior denticles assume a second thoracic morphology. Also, in embryos lacking Scr, the labial lobes fail to undergo head involution, leaving the normally internalized labial sense organs external. Although it was initially reported that the labial segment was transformed to a maxillary identity, closer examination indicated that this was not the case (Pederson *et al.*, 1996). Thus, as with other head homeotic genes, there is no larval homeotic transformation of the labial segment corresponding to the absence of Scr. Loss of *Scr* eliminates structures generated by the labial segment: the salivary glands and the bridge of the H-piece (Panzer *et al.*, 1992). Expression of *Scr* is further required in the visceral mesoderm for the formation of the gastric caeca (Reuter and Scott, 1990.). In the adult, loss of *Scr* disrupts patterning of the first thoracic discs (leg and humeral) (Pattatucci *et al.*, 1991). In the transformed first thoracic segment of adults, the leg develops with a second thoracic identity, as evidenced by the bristle pattern and the reduction of sex comb teeth in males. Occasionally, a bit of wing-like appendage appears in the dorsal first thoracic segment (Struhl, 1982; Kaufman, 1990).

Along with *pb*, *Scr* is also required for adult proboscis development (Percival-Smith *et al.*, 1997; Rogers *et al.*, 1997; Abzhanov *et al.*, 2001). In null clones of *Scr*, the labial palps transform toward a maxillary palp identity. Dominant gain-of-function mutants also exist that affect adult structures. These cause a partial transformation of the second and/or third thoracic legs toward a first thoracic identity (Pattatucci and Kaufman, 1991).

Although labial expression of *Scr* appears conserved in other insects, indicating that this gene has a conserved role in development of this segment, its expression and role in the first thoracic region is somewhat variable. In *Oncopeltus*, *Acheta*, and *Thermobia*, *Scr* expression is limited to the leg spot domains in the first thoracic segment, in contrast to its expression in the entire anterior first thoracic compartment in *Drosophila*, *Tribolium*, and *Apis* (Rogers *et al.*, 1997; Walldorf *et al.*, 2000; Curtis *et al.*, 2001). It appears, therefore, that the role of *Scr* in the first thoracic segment has expanded in higher insects.

More controversial, however, is the dorsal patch of *Scr* expression in “lower” insects. In flies, expression of *Scr* in dorsal first thoracic tissue prevents development of ectopic wing structures. This has also been proposed to be the role of the dorsal expression in *Oncopeltus* and *Acheta*. However, a similar dorsal region of expression is found in *Thermobia*, and, as a more basal apterygote, *Thermobia*

is not likely to require wing suppression (Rogers *et al*., 1997).

RNAi has been used to remove *Scr* function from *Oncopeltus*, and genetic analysis has been used to study the role of the *Scr* homolog *Cephalothorax* (*Cx*), in *Tribolium*. In *Oncopeltus*, the depletion of *Scr* results in transformation of the normally fused labium to two small leglike structures (Hughes and Kaufman, 2000). In the first thoracic leg, the bristles of the comb are altered to resemble those found on more posterior legs. In *Tribolium*, there are two classes of mutations in the *Cx* gene (Beeman *et al*., 1989; Curtis *et al*., 2001). RNAi was used to determine which is likely to be the null phenotype, and this is a transformation of the labium to antennae and a fusion of the first thoracic segment to the head. Interestingly, the labium to antennae transformation is observed in other insects only when both *pb* and *Scr* are removed. The second phenotypic class of *Cx* is more complicated, causing the labium to acquire a maxillary identity, and a duplication of the first thoracic segment. The reason for this transformation is unclear.

1.8.2.2.5. *Antennapedia* (*Antp*) The namesake of the *Drosophila ANT-C* (Kaufman *et al*., 1980), *Antp*, is required for second thoracic identity and leg development. The *Antp* gene includes a total of eight exons, with two promoters (P1 and P2) producing four different sized transcripts (Bermingham and Scott, 1988). The genomic region controlled by P1 is 103 kb while that of P2 is 36 kb. The *Antp* open reading frame, approximately 1.1 kb in length, is found within the last four exons, which encode a protein of about 43 kDa (Schneuwly *et al*., 1986). The homeodomain is encoded within the last exon. Multiple translation start sites and alternative splicing lead to at least four protein isoforms, but these vary only slightly, by up to 17 codons (Carroll *et al*., 1986; Bermingham and Scott, 1988).

In the embryo, *Antp* expression overlaps with *Scr* in the first thoracic epidermis. Though transcripts are detected earlier (Levine *et al*., 1983), the Antp protein is not detectable until germ band extension (Carroll *et al*., 1986). At this time, the protein is weakly detected in the in the anterior ventrolateral portion of the presumptive thoracic region. Initial expression is parasegmental, extending from parasegment 3 to parasegment 6. Expression is dynamic and quickly resolves to include the posterior first thoracic segment and all of the second and third thoracic ectoderm. Expression in the visceral mesoderm begins during late germ band contraction, accumulating posterior to Scr, although not directly abutting it, and coinciding with parasegments 5 and 6. CNS expression initiates during early neurogenesis, with protein accumulating in the VNC from parasegments 4 through 12 (Carroll *et al*., 1986; Wirz *et al*., 1986). As the VNC contracts, Antp expression intensifies in the thoracic region, while the abdominal segments maintain only weak expression (Carroll *et al*., 1986). Bermingham *et al*. (1990) constructed transcript-specific probes and demonstrated that the two promoters are differentially expressed during embryogenesis and have complex (both spatial and temporal) and differing expression patterns.

Transcript and protein accumulation have also been examined at the larval stages, and *Antp* transcripts persist in the ventral neuromere of the second thoracic segment and in the anterior spiracle (Levine *et al*., 1983; Wirz *et al*., 1986). Later, in third instar larvae, Antp accumulates in the humeral imaginal discs, in the wing blade portion of the wing imaginal discs, in portions of the haltere discs and, to some extent, in all of the leg discs (posterior compartment of the first thoracic leg discs and anterior compartment of the second and third thoracic leg discs).

Interestingly, there is differential expression of the P1 and P2 transcripts in the imaginal discs. The P1 transcripts localize to the anterior margins of the second thoracic wing and leg discs, while the P2 transcripts are evenly distributed in these tissues. Both transcripts are detected in the first and third thoracic leg discs (Jorgenson and Garber, 1987). Somatic clonal analysis reveals different developmental requirements for each *Antp* transcript (Abbott and Kaufman, 1986). Proper development of the adult dorsal thorax requires P1 promoter function, while the P2 transcript is required for appropriate leg and embryonic patterning.

There are three classes of dominant gain-of-function mutations within *Antp*, which often result from chromosomal rearrangements causing *Antp* expression outside its normal expression domain. Best known is a mutation responsible for the transformation of the adult antennae toward a second thoracic leg (**Figure 6d**). However, other mutations also transform dorsal head structures toward dorsal thoracic structures (Scott *et al*., 1983), and the *Scr* class transforms the second and third thoracic legs toward a first thoracic identity.

Most dominant *Antp* alleles are also recessive embryonic lethals when hemizygous or heterozygous with an *Antp* null allele. The thoracic cuticle of embryos lacking all *Antp* function is transformed to a more anterior identity (Wakimoto *et al*., 1984). Sclerotic plates resembling mouthpart material are occasionally present in the posterior of the

first thoracic segment, and the denticle belts of the second and third thoracic segments are transformed to resemble those of the first thoracic segment (**Figure 6f**). The fact that sclerotized structures form in the posterior of the first thoracic segment indicates a transformation towards a gnathal identity, while the more posterior thoracic segments adopt a first thoracic identity.

Clonal analysis was used to assess the adult requirement of *Antp* (Struhl, 1981). When clones are induced in the second thoracic leg discs, proximal and medial portions of the second thoracic legs are transformed into the corresponding portions of the antenna. The distal tarsus develops normally, however.

Hayward *et al.* (1995) compared the expression of *Drosophila Antp* to that of the grasshopper and found that, while the pattern of expression is conserved, the initial accumulation is quite different. The anterior limit in the grasshopper is segmental, corresponding to the labial/first thoracic border and, therefore, potentially would be overlapping with Scr, if Scr expression in the grasshopper occurs as in *Drosophila*. Additionally, in the grasshopper, early epithelial thoracic and CNS expression is not as widely modulated as in *Drosophila*, although the expression patterns become more similar later in development with the exception of the modulation that is observed in *Drosophila*.

Expression at the germ band stage is quite similar in all insects examined. The *Tribolium* homolog of *Antp* is called *prothoraxless* (*ptl*); Beeman *et al.*, 1989, 1993), and larvae homozygous for this null mutation bear legs that are transformed to antennae. The dorsal thorax is also significantly reduced. This differs from the effects observed in *Antp* mutants in *Drosophila*, which have a more limited leg transformation. An *Antp* mutant (*Nc*) has been also recovered in *Bombyx*, which results in the transformation of the first thoracic legs to antennae (Nagata *et al.*, 1996). Less extensive changes are also observed in the second thoracic legs, and the silk glands fail to develop properly.

1.8.2.3. The *HOM-C* Genes of the *BX-C*

The genes of the *Drosophila BX-C* govern pattern formation in the posterior thoracic and abdominal regions (**Figure 2**). The *BX-C* spans approximately 350 kb (Martin *et al.*, 1995) and is separated from the *ANT-C* by approximately 7.5 Mb (Martinez and Amemiya, 2002) (**Figure 7**).

1.8.2.3.1. *Ultrabithorax* (*Ubx*) The most proximal gene of the *Drosophila BX-C* is *Ultrabithorax* (*Ubx*), which functions as a major determinant of segment identity in the thoracic and abdominal region by initiating abdominal development and repressing appendage development (Castelli-Gair and Akam, 1995). The *Ubx* gene spans a genomic region of approximately 76 kb (O'Conner *et al.*, 1988) and produces two transcripts, 3.2 and 4.6 kb in size, which vary at the 3′ end due to alternate transcription termination signals. There are four exons including two microexons. The *Ubx* gene encodes at least five different protein variants, but the largest and smallest differ by only 43 amino acids.

Ultrabithorax transcripts are first detected in the syncytial blastoderm (Akam and Marinez-Arias, 1985) and their abundance increases as cellularization proceeds. By gastrulation, a region of strong accumulation includes the primordia of the third thoracic and first abdominal segments, or parasegment 6, with weaker pair-rule-like expression in parasegments 8, 10, and 12. After germ band extension, *Ubx*

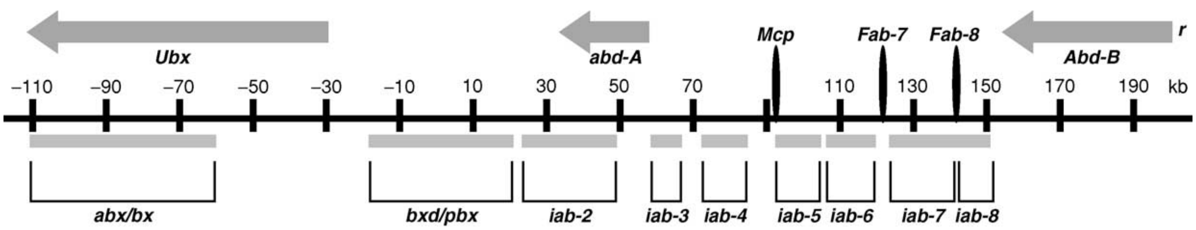


Figure 7 The organization and *cis*-regulatory control regions of the *Bithorax Complex*. The horizontal line, represents approximately 320 kb of the *BX-C* region. The transcription units are marked above the horizontal line for each *BX-C* gene product: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*). The *BX-C cis*-regulatory regions are indicated below the horizontal line. The *abx/bx* (*anterobithorax/bithorax*) and *bxd/px* (*bithoraxoid/postbithorax*) regions are involved in *Ubx* regulation. The *infra abdominal control* (*iab*) regions regulate the expression of *abd-A* and *Abd-B*. Three chromatin insulator regions are also shown – *Mcp* (*Miscadastral pigmentation*), *Fab-7* (*Frontoabdominal*), and *Fab-8* – which prevent interactions between neighboring *cis*-regulatory regions. The zero point represents the point of initiation of a chromosome walk by Bender *et al.* (1983). The genomic regions Contributing to the *m* and *r* transcripts of *Abd-B* are also shown.

transcripts are present in parasegments 6 through 12; however, there is greater accumulation in the posterior portion of each parasegment. This is complementary to the expression of *abd-A* in these parasegments (see Section 1.8.2.3.2). Expression in the somatic and visceral mesoderm (parasegment 7, immediately adjacent to *Antp*) (Tremml and Bienz, 1989) begins as the germ band contracts. In the CNS, *Ubx* transcripts are present in the neuromeres, from parasegments 5 through 12 (Akam and Marinez-Arias, 1985). Although lagging transcription by 1–2 h, protein distribution generally follows the patterns of transcript accumulation (White and Wilcox, 1984; Beachy *et al*., 1985).

In larvae, Ubx protein accumulates weakly in the wing and second leg discs, but more strongly in the haltere and third leg imaginal discs (Beachy *et al*., 1985). Pupal expression is limited to the anterior compartment of the first abdominal segment and does not overlap with expression of other *BX-C* genes (Kopp and Duncan, 2002).

Homozygous null *Ubx* individuals die as larvae, with transformation of the third thoracic and first abdominal segments to a second thoracic identity (Lewis, 1978). Individuals hemizygous for viable combinations of *Ubx* alleles give rise to the famous "four-winged fly," as the haltere is transformed to wing (**Figure 1**). In addition to ectopic wings, a wide band of notal tissue forms in the third thoracic segment, and third thoracic legs and thoracic pits can appear on the first abdominal segment. Gain-of-function alleles ectopically expressing *Ubx* exhibit a transformation of the adult second thoracic segment toward a third thoracic identity, causing a partial wing to haltere transformation (Bender *et al*., 1983). Interestingly, there appears to be a temporal component to Ubx function, as null clones induced at different times in development have different effects. Clones induced earlier than 8 h AEL transform the posterior compartments of the second and third legs towards a first thoracic leg identity, while clones induced later than 8 h AEL have normal second legs, but transform dorsal and ventral appendages of the third thoracic segment (haltere and third leg) towards the corresponding appendage of the second thoracic segment (wing and second leg) (Morata and Kerridge, 1981; Struhl, 1982).

Ubx expression and function have been examined in other insects, and there are some differences when compared with *Drosophila*. In the lepidopterans *P. coenia* and *Manduca sexta*, there are circular clearings within the Ubx and Abd-A domains, where the larval prolegs will form (Suzuki and Palopoli, 2001), supporting a role for Ubx in limb suppression. Interestingly, even though sawfly (*Neodiprion*) larvae also develop prolegs, Ubx and Abd-A proteins are detected throughout the developing prolegs, perhaps implying a convergent mechanism for proleg development. This is discussed further, when we consider *HOM-C* target genes. In *Tribolium*, *Ultrathorax* (*Utx*; the *Tribolium* homolog of *Ubx*) expression is initially detected in parasegments 4 and 5, but this expression later resolves to parasegment 5, more similar to the fly (Bennett *et al*., 1999). Genetic analysis uncovered a haploinsufficient *Utx* phenotype, demonstrating that the gene is required for proper development of the adult elytra. Heterozygous adults have warped, blistered elytra that fail to meet at the dorsal midline. Homozygosity is lethal and, similar to flies, the first abdominal and third thoracic segments are transformed to a second thoracic identity.

1.8.2.3.2. *abdominal-A* (*abd-A*) The *abd-A* gene spans a genomic region of approximately 60 kb, with the transcribed region consisting of at least eight exons (Karch *et al*., 1990). This gene is required for the promotion of abdominal development and the suppression of thoracic development in parasegments 7 through 9, for the patterning of parasegments 10 through 13, and the specification of the heart cell fate (Busturia *et al*., 1989; Lovato *et al*., 2002) (see **Chapter 2.6**). The gene encodes a single protein, which is 330 amino acids in length (Cumberledge *et al*., 1990).

Abd-A protein accumulates simultaneously in the first through eighth abdominal segments and in the amnioserosa at about 4 h AEL, during germ band extension, although the mRNA can be detected earlier (Macías *et al*., 1990). The anterior expression border coincides with parasegment 7, but the posterior border of expression appears to be segmental, laterally, extending through the seventh abdominal segment. Ventrally, the posterior expression boundary does not coincide with a compartment border but ends within the posterior region of parasegment 13. There is a gradient of Abd-A accumulation within each parasegment, such that the highest amount of protein occurs at the anterior border of each parasegment fading to nearly complete absence at the posterior border. This pattern is complementary to Ubx in parasegments 7–12, with Ubx being strongest where Abd-A is absent. Maximum Abd-A accumulation occurs in the segmental grooves, but is restricted to only the anterior side of the seventh and eighth segmental grooves. Accumulation of Abd-A is also strong around the tracheal pits.

In the visceral mesoderm, Abd-A is evident in cells abutting those cells expressing Ubx, and extends posteriorly to the end of the visceral midgut mesoderm (Tremml and Bienz, 1989; Karch *et al.*, 1990). In the CNS, Abd-A accumulation reflects the pattern in the epidermis (Karch *et al.*, 1990; Macías *et al.*, 1990). Strong expression is evident in the dorsal vessel, in the cells that will form the posterior portion of the heart (Lovato *et al.*, 2002). Somatic cells of the developing gonad also stain weakly in late embryos (Karch *et al.*, 1990). In pupae, Abd-A accumulates in parasegments 7–12, gradually increasing in amount from anterior to posterior. Its domain is adjacent and nonoverlapping to that of Ubx, in contrast to the overlapping domains observed during embryonic development (Kopp and Duncan, 2002).

Defining homeosis within the abdominal segments is difficult, due to the morphological similarities between these segments, in both the larva and the adult (Mathog, 1991). Null alleles of *adb-A* are embryonic lethals, with abdominal parasegments 7 through 9 completely transformed and parasegments 10 through 13 weakly transformed to a parasegment 6 identity. Hemizygous viable alleles allow the characterization of *abd-A* reduction in adult flies, where the second, third, and fourth abdominal segments are weakly transformed toward the first abdominal segment, with the second abdominal segment being most affected. In addition to mutations disrupting the coding region of the gene or affecting the entire *abd-A* locus, some mutations disrupt *cis*-regulatory elements and affect expression of *abd-A* in specific parasegments (Busturia *et al.*, 1989; Karch *et al.*, 1990). Dominant gain-of-function mutations often result from changes in the *infra abdominal control* (*iab*) regulatory regions, leading to ectopic expression of *abd-A* (Busturia *et al.*, 1989) and affecting adult development (**Figure 7**). For example, one allele, *abd-A*$^{iab2\text{-}S3}$, causes a partial transformation of the first abdominal segment towards a more posterior, second abdominal identity. Other regulatory mutations cause reciprocal transformations of the first abdominal segment towards the second, and the second abdominal segment towards the first (e.g., *abd-A*$^{iab2\text{-}uab1}$). These mutations allow ectopic expression of Abd-A in the developing first abdominal segment, while at the same time reducing Abd-A expression in the developing second abdominal segment. Both of these later mutations only affect imaginally derived tissues. A third gain-of-function allele, *abd-A*$^{iab3\text{-}277}$, causes a divergent transformation, with the first abdominal and third through fifth abdominal segments being all transformed to a second abdominal identity.

While the ninth and tenth abdominal segments in *Drosophila* are quite reduced, many lower insects have ten full abdominal segments. Expression of *abd-A* homologs reflects this anatomical difference. In *Tribolium*, *Abdominal* (*A*) (the homolog of *abd-A*) has a broader pattern of posterior abdominal expression, reflecting the increased number of abdominal segments (Stuart *et al.*, 1993; Shippy *et al.*, 1998). In *A* mutants, all abdominal segments develop pleuropodia in their anterior compartment (normally only found in the first abdominal segment) and protrusions with a posterior third thoracic leg identity in their posterior compartment. In *Bombyx*, loss of the *abd-A* homologue (E^{Ca}) is thought to cause a transformation of the second through seventh abdominal segments to a more anterior abdominal identity (Ueno *et al.*, 1992). Larval prolegs are normally present on the third through sixth abdominal segments, but not on the first, second, and seventh segments. In E^{Ca} mutants, the prolegs are lost from the third through the sixth abdominal segments. Although the exact identity of the transformed segments is not clear, one expects an anterior transformation to a first abdominal identity, by analogy to the role of *adb-A* in *Drosophila*. Another mutation in *Bombyx*, E^N, removes both the *Ubx* and *abd-A* homologs. Larvae homozygous for this allele develop thoracic-type legs in the first through the seventh abdominal segments with a mixed leg/proleg identity in the eighth abdominal segment. This transformation to a more anterior identity supports the proposed anterior transformation in the E^{Ca} mutants.

1.8.2.3.3. *Abdominal* B (*Abd-B*) *Abd-B* governs the development of the most posterior abdominal segments of *Drosophila*, and limits the size of the *Drosophila* abdomen by repressing *abd-A* function. The *Abd-B* gene spans approximately 100 kb, containing at least eight exons with multiple promoters. The homeobox is encoded by exons seven and eight, and the position of the intronic interruption is the same as in *lab* and *pb*. Five transcripts are produced, ranging in length from 3.3 to 7.8 kb, with each transcript sharing four common exons (Kuziora and McGinnis, 1988a). These transcripts are expressed in specific spatial and temporal patterns.

The *Abd-B* gene encodes two distinct homeoproteins of 54 and 36 kDa (Celniker *et al.*, 1989), referred to as the “m” and “r” forms, respectively (**Figure 7**). The m form has a morphogenetic role in parasegments 10–13, specifying identity in the posterior abdominal segments. The r form has a regulatory function. It suppresses the m protein’s morphogenic role in parasegment 14 and possibly

limits abdominal segmentation (Casanova *et al.*, 1986; Boulet *et al.*, 1991; Kuhn *et al.*, 1995). The r protein form lacks a glutamine-rich M- or opa-repeat (Wharton *et al.*, 1985), which is a transcription activation domain that is found in the m form. The 7.8, 3.7, and 3.3 kb transcripts encode the r protein and accumulate in parasegments 14 and 15, while the 4.3 and 4.7 kb transcripts encode the m protein and accumulate in parasegments 10 through 15 (Celniker *et al.*, 1989; Zavortink and Sakonju, 1989).

The Abd-B proteins are first detectable in the posterior ectoderm of parasegments 13 through 15 of early germ band extended embryos. Relative to the detection of the transcripts, the protein is delayed by approximately 1.5–2 h (Celniker *et al.*, 1989; Delorenzi and Bienz, 1990). As embryogenesis continues, Abd-B expression spreads anteriorly into portions of parasegments 11 and 12. There is a stepwise increase in protein concentration towards the posterior region of the embryo. During early germ band retraction, visceral mesodermal expression is first detected laterally in parasegments 12 through 14 of the mesoderm (Tremml and Bienz, 1989). Expression in the VNC is also evident during this period, and follows the pattern of the epidermis (Celniker *et al.*, 1989; Delorenzi and Bienz, 1990). Following head involution, protein accumulation fades and is not detectable again until the third instar larval stage (in the genital discs). Expression returns to the abdominal segments in pupae, and is found at progressively higher levels in the fifth through seventh abdominal segments (Kopp and Duncan, 2002).

Although the antibody used to detect Abd-B protein recognizes both the m and r forms, *iab-8* mutants are m^-, so protein accumulation in these embryos allows inference of the r form's expression pattern in parasegments 14 and 15 and in the mesoderm of parasegment 14. The anterior margin of the r protein is unchanging during development, always coinciding with the border of parasegments 13 and 14. This differs from the shifting anterior margin observed with this antibody in wild-type embryos, when both forms of the protein are present and detected. It is likely that all of the Abd-B protein detected anteriorly to parasegment 14 corresponds to the m protein. Embryos lacking the r form have a wild-type protein accumulation pattern, reflecting normal m protein in parasegments 10 through 13, and ectopic m protein in parasegment 14 that is due to its derepression by the lack of the r function (Delorenzi and Bienz, 1990).

Loss of *Abd-B* function is lethal and causes the transformation of all abdominal segments posterior to the fifth abdominal segment to a fourth or fifth abdominal cuticle pattern (Sánchez-Herrero *et al.*, 1985). Exact identification is difficult, again, due to similarity between these segments. Also, the larval posterior spiracles are reduced or eliminated. Some homozygous lethal alleles produce a few escapers, allowing the adult effects of *Abd-B* loss to be directly assessed. In adults, the fifth through eighth abdominal segments are transformed to a fourth abdominal identity. The dominant haploinsufficient effects of *Abd-B* are weak transformations of the sixth and seventh abdominal tergites which resemble the fourth or fifth abdominal type, and adult infertility.

Absence of Abd-B in the genital disc results in a transformation of the male and female genitalia to leg, with claws, tarsi, and/or tibia (Estrada and Sánchez-Herrero, 2001). Less frequently, there was a transformation towards antennae, producing second and/or third antennal segments and arista.

In *Drosophila Abd-B* mutants, a full ninth abdominal segment develops concomitant with posterior expansion of *abd-A* expression (Casanova *et al.*, 1986; Karch *et al.*, 1990). This, and comparisons with other insects, has led to the proposal that *Abd-B* sets the limit on the number of abdominal segments to be formed by repressing *abd-A* activity (Hughes and Kaufman, 2002). The *Tribolium Abd-B* homolog, *extra urogomphi* (*eu*), has a more limited expression than *Abd-B*, corresponding only to the posteriormost segments (the tenth and eleventh abdominal segments), whereas *Drosophila Abd-B* is found in approximately five abdominal segments. However, the results from *Thermobia* and *Schistocerca* may contradict this model. *Schistocerca* has a limited *Abd-B* expression domain initially (only the eleventh abdominal segment), but later expression expands anteriorly through the posterior eighth abdominal segment (Kelsh *et al.*, 1993). In *Thermobia*, the *Abd-B* homolog (*TdAbd-B*) is initially expressed posterior to the growth zone, behind the forming posterior abdominal segments (Peterson *et al.*, 1999), but extends anteriorly into the eighth abdominal segment after germ band extension.

The anterior expression in *Thermobia* and *Schistocerca* is perhaps contrary to the model predicting that extended *Abd-B* expression limits the number of abdominal segments, except that this expansion occurs after *abd-A* has been expressed in these segments. Interestingly, the domains of *Abd-B* homolog expression in other insects is similar to the expression pattern of the r-type *Drosophila* protein, while the expression domains coinciding with the *Drosophila* m-type protein appear to be absent.

Also of interest is the difference between *TdAbd-B* expression from that of other *Thermobia HOX* genes, in the limbs that develop in each gene's expression domain. The *Thermobia* homologs of *pb, Dfd, Scr, Antp*, and *Ubx* are expressed throughout the limbs developing in their segments, while *TdAbd-B* is only expressed in the cercal appendage primordia, and not in the appendages themselves as they develop (Peterson *et al*., 1999). A similar lack of expression is noted in the cercal appendages of *Schistocerca* (Kelsh *et al*., 1993). Peterson *et al.* (1999) propose that this may indicate a fundamental difference in the nature of terminal appendages versus anterior appendages.

A final interesting note is the association of *Abd-B* with genital development, a role conserved in other insects, spiders (Damen and Tautz, 1999), and even vertebrates, suggesting a general role in establishing the genitalia (Akam *et al*., 1988).

1.8.2.4. *HOX* Expression in Related Arthropods

HOM-C/HOX genes expression has been examined in several noninsect arthropods. Expression has been examined in crustaceans, chelicerates (spiders and mites), and myriapods (centipedes) and significant variations were found from the expression patterns observed in insects. These have been reviewed by Hughes and Kaufman (2002), who also discuss the evolutionary implications of the variability of these genes in arthropod body patterning.

1.8.3. Regulation of *HOM-C* Gene Function

1.8.3.1. Introduction

The *HOM-C* genes' clustered genomic structure and collinear expression are rather uniquely conserved across phyla. This raises the question: why are the *HOM-C/HOX* genes found in such highly conserved arrangements, and is this a requirement for their expression and function? Studies in vertebrates reveal shared *HOX* regulatory regions and indicate that changes in the arrangement of the complex can impact the expression of multiple *HOX* genes (Gould *et al*., 1997; Sharpe *et al*., 1998). The fact that splits in the *HOM-C/HOX* cluster have only been observed (thus far) in *Drosophila* and in the nematode *Caenorhabditis elegans* (Ruvkun and Hobert, 1998), suggests a strong evolutionary constraint for the ordered *HOM-C/HOX* cluster, and perhaps a loosening of this constraint in these two organisms, in which shared *HOM-C* gene regulatory regions have not been identified. The conservation of ordered, clustered genomic organization may be required for the temporal and spatial collinearity observed in *HOM-C* expression (van der Hoeven *et al*., 1996; Kondo and Duboule, 1999), but the mechanism underlying this requirement has yet to be determined (review: Kmita and Duboule, 2003). In spite of the existing uncertainties, however, some of the mechanisms of *HOM-C* gene regulation in *Drosophila* are understood.

As seen by their mutant phenotypes, the HOM-C proteins are powerful transcription factors, directing the downstream expression of other genes in specifying developmental pathways. Loss or misexpression of a *HOM-C* gene is sufficient to direct cells, and even whole body regions, towards dramatically different developmental fates. Frequently, these changes in expression are lethal and accompanied by gross morphological defects, and even when viable adults are produced, fitness may be greatly effected. Therefore, strict spatial and temporal control of the *HOM-C* selector genes is critical to normal body patterning. Interestingly, and perhaps predictably, alterations in *HOM-C* gene expression appear to play a role in the differential body patterning between some insect species.

The transcriptional control of the *Drosophila HOM-C* genes can be exercised at two levels, related to (1) the initiation or establishment of the active state, and (2) the maintenance of this state. This is true whether we are discussing activation or repression. Initiation or establishment of *HOM-C* gene activity is generally a short term phenomenon and, where mapped, involves local regulatory elements within close proximity to the promoters (Bienz and Müller, 1995). *Trans*-acting factors that initiate *HOM-C* expression include gap and pair rule segmentation gene products. The maintenance phase is long term and may involve DNA elements that are a large distance away from the promoter. Two protein groups, the Polycomb and the Trithorax groups, have opposing effects on the long-term regulation of *HOM-C* transcription. Self-regulation by the *HOM-C* genes and cross-regulation between *HOM-C* genes are also important to the regulation of *HOM-C* gene activity.

A summary of the regulatory factors discussed in this chapter is shown in **Table 1**.

1.8.3.2. Initiation and Establishment of *HOM-C* Gene Expression

In the *Drosophila* embryo, the establishment of early *HOM-C* expression occurs during the syncytial or early cellular blastoderm stage and is controlled by the early upstream gap and segmentation gene products. Several studies have demonstrated regulation of *HOM-C* gene expression by the gap

Table 1 Summary of the HOM-C gene regulators discussed in the text[a]

Regulator	*HOM-C gene(s)*	*Reference*
Involved in activation		
bicoid (*bcd*)	*Dfd*	McGinnis *et al.* (1990)
decapentaplegic (*dpp*)	*lab, Ubx*	Staehling-Hampton *et al.* (1994), Eresh *et al.* (1997)
engrailed	*Dfd, Scr*	Jack *et al.* (1988), Pelaz *et al.* (1993)
even-skipped (*eve*)	*Dfd, Ubx*	Ingham *et al.* (1986), Jack *et al.* (1988)
fushi tarazu (*ftz*)	*Scr, Antp, Ubx*	Riley *et al.* (1987), Martinez-Arias and White (1988), Qian *et al.* (1993)
giant	*Scr, Antp, Abd-B*	Reinitz and Levine (1990), Wu *et al.* (2001)
hunchback (*hb*)	*Scr, Antp*	White and Lehmann (1986), Riley *et al.* (1987), Harding and Levine (1988), Shimell *et al.* (1994), Casares and Sanchez-Herrero (1995), Wu *et al.* (2001)
Krüppel (*Kr*)	*Antp, Ubx*	White and Lehmann (1986), Riley *et al.* (1987), Harding and Levine (1988), Wu *et al.* (2001)
odd-paired (*opa*)	*Dfd*	Jack *et al.* (1988)
paired (*prd*)	*Dfd*	Jack *et al.* (1988)
phosphatase 2A (dPP2A, B')	*Scr*	Berry and Gehring (2000)
runt	*Dfd*	Jack *et al.* (1988)
serine/threonine kinase casein kinase II (CKII)	*Antp*	Jaffe *et al.* (1997)
tailless (*tll*)	*Dfd, Ubx, Abd-B*	Reinitz and Levine (1990), Casares and Sanchez-Herrero (1995)
wingless (*wg*) (low levels)	*lab*	Hoppler and Bienz (1995)
Involved in repression		
engrailed (*en*)	*Ubx*	Qian *et al.* (1993)
fushi tarazu (*ftz*)	*Dfd*	Jack and McGinnis (1990)
hairy	*Dfd*	Jack and McGinnis (1990)
hunchback (*hb*)	*Ubx, abd-A*	White and Lehmann (1986), Qian *et al.* (1993), Casares and Sanchez-Herrero (1995), Shimell *et al.* (2000)
knirps (*kni*)	*Antp, Ubx, Abd-B*	White and Lehmann (1986), Harding and Levine (1988), Casares and Sanchez-Herrero (1995)
Krüppel (*Kr*)	*Ubx, abd-A, Abd-B*	White and Lehmann (1986), Harding and Levine (1988), Shimell *et al.* (1994), Casares and Sanchez-Herrero (1995), Shimell *et al.* (2000)
odd-skipped (*odd*)	*Dfd*	Jack and McGinnis (1990)
tailless (*tll*)	*Ubx, abd-A*	Qian *et al.* (1993), Casares and Sanchez-Herrero (1995)
wingless (*wg*) (high levels)	*lab*	Hoppler and Bienz (1995)
Cross-regulation and Autoregulation		
Repression		
abd-A	*lab, pb, Dfd, Scr, Antp, Ubx*	Karch *et al.* (1990); Appel and Sakonju (1993), Miller *et al.* (2001b)
Abd-B	*lab, pb, Dfd, Scr, Antp, Ubx, abd-A*	Karch *et al.* (1990), Pelaz *et al.* (1993), Miller *et al.* (2001b)
Antp	*lab, pb, Dfd, Scr*	Riley *et al.* (1987); Gonzalez-Reyes *et al.* (1992), Pelaz *et al.* (1993), Miller *et al.* (2001b)
Scr	*lab, Dfd, pb* (in the embryonic mesoderm)	Gonzalez-Reyes *et al.* (1992), Miller *et al.* (2001b)
Ubx	*lab, pb, Dfd, Scr, Antp*	Struhl (1982), Appel and Sakonju (1993), Miller *et al.* (2001b)
Activation		
Antp	*Scr* (in the embryonic mesoderm)	Reuter and Scott (1990)
Dfd	*pb*	Rusch and Kaufman (2000)
pb	*Scr* (larval labial disc)	Abzhanov *et al.* (2001)
Scr	*pb*	Rusch and Kaufman (2000)
Autoregulation		
Dfd	*Dfd*	Kuziora and McGinnis (1988a), Bergson and McGinnis (1990), Regulski *et al.* (1991), Gonzalez-Reyes *et al.* (1992), Zeng *et al.* (1994), Lou *et al.* (1995)
Lab	*lab*	Lou *et al.* (1995)
Ubx	*Ubx*	Bienz and Tremml (1988)

Continued

Table 1 Continued

Regulator	*HOM-C gene(s)*	*Reference*
Involved in maintenance		
Polycomb complexes		Simon (1995), Simon and Tamkun (2002)
PRC1 (inhibits nucleosome remodeling) includes:		
Polycomb (Pc)		
polyhomeotic (ph)		
Posterior sexcombs (Psc)		
ESC-E(Z) (histone deacetylase activity) includes:		
extra sex combs (esc)		
enhancer of zeste (e(z))		
Trithorax complexes		Ingham (1998), Simon and Tamkun (2002)
TAC1 (histone modification) includes:		
Trithorax (*trx*)		
Calbindin 53E (*Cbp53E*)		
BRM (ATP dependent nucleosome alterations) includes:		
brahma (brm)		
moria (mor)		
Snf5-related 1 (Snr1)		
eyelid/osa (*osa*)		

[a]Note that *Drosophila* gene names follow a standard nomenclature: genes are generally named for the mutant phenotype, and dominant mutations are capitalized whereas recessive mutations are lowercase and are shown in italics. Protein names are generally capitalized and not italicized.

gene products. For example, Gap proteins regulate the expression of the *abd-A* and *Abd-B* genes through the *infra-abdominal* (*iab*) regions (**Figure 7**), which represent *cis*-regulatory regions of the *abd-A* and *Abd-B* genes (Casares and Sánchez-Herrero, 1995). The number following the *iab* abbreviation reflects the abdominal segment in which each region has its most prominent regulatory effect. For example, the *iab-5*, *iab-6*, *iab-7*, and *iab-8* *cis*-regulatory regions control *Abd-B* expression in segments 5, 6, 7, and 8, respectively. The *iab* regions, which do not produce protein-coding mRNAs, are transcribed initially at the blastoderm stage, and the anterior limits of their expression are collinear with the chromosomal location of each region (Sánchez-Herrero and Akam, 1989; Cumberledge *et al*., 1990). When fused to *lac-Z* reporter genes, the *iab* regions direct expression in precise and distinct domains (Simon *et al*., 1990; Galloni *et al*., 1993; McCall *et al*., 1994; Zhou and Levine, 1999; Barges *et al*., 2000). Their function appears to be the integration of positional information from the Gap proteins and the transmission of this information into specific regional *abd-A* and *Abd-B* activation domains. It has been proposed that the transcription of these regions "opens" the *abd-A* and *Abd-B* regions to allow activation (Gyurkovics *et al*., 1990).

The protein products of the gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), *tailless* (*tll*), and *knirps* (*kni*) control early *abd-A* and *Abd-B* expression through the selective regulation of the *iab* regions (Casares and Sánchez-Herrero, 1995; Shimell *et al*., 2000). Loss of either Hb or Kr allows anterior expansion of *iab-2* (via Kr), *iab 3–4* (via Kr and Hb) and *iab 5–6* (via Kr) transcription, and causes a concomitant anterior expansion of *abd-A* and *Abd-B* expression domains (Shimell *et al*., 1994). In this manner, Kr and Hb define the anterior limits of initial *abd-A* and *Abd-B* expression. The Tll protein similarly limits the posterior expression of *abd-A*; loss of Tll allows *abd-A* expression to occur almost to the end of the germ band (Casares and Sánchez-Herrero, 1995). Further, Tll and Kni regulate transcription in the *iab-8* domain and have opposing effects upon *Abd-B* activity. The loss of Kni causes anterior expansion of *Abd-B* expression through an expansion of *iab-8* transcription, while the loss of Tll eliminates *Abd-B* expression. Differing gradients of gap gene products along the anterior–posterior axis may drive the differing spatial regulation of the *iab* regions. The transcription of the *iab* regions is further regulated by chromatin insulator regions (review: Müller, 2000), which intervene between the *iab* elements (**Figure 7**). The loss of a chromatin insulator region allows transcription to occur across *iab* regions, and can result in homeotic transformation of one abdominal segment to another (Mihaly *et al*., 1997, 1998; Drewell *et al*., 2002).

Gap gene products also affect the expression of other *HOM-C* genes. The *cis*-regulatory regions of *Ubx* – *abx/bx* (*anterobithorax/bithorax*) and *bxd/pbx* (*bithoraxoid/postbithorax*) (**Figure 7**) – also require gap gene products for early regulation, although it does not appear that this involves the

transcription of noncoding RNAs (Qian *et al.*, 1993). Additionally, the loss of Hb affects the expression of other *HOM-C* genes functioning within its domain (*Scr*, *Antp*, and *Ubx*) (White and Lehmann, 1986; Riley *et al.*, 1987; Harding and Levine, 1988).

The control of the *ANT-C* gene *Dfd* by early patterning genes has also been investigated (Jack *et al.*, 1988). Early *Dfd* activity is not significantly affected by changes in the zygotic gap genes, but mutations in the pair-rule genes do affect *Dfd* expression. Mutations in *hairy* (*h*), *runt* (*run*), *even-skipped* (*eve*), *fushi tarazu* (*ftz*), *paired* (*prd*), *odd-paired* (*opa*), *odd-skipped* (*odd*), and *engrailed* (*en*), all affect *Dfd* expression. Mutations in *odd* and *ftz* cause a spatial expansion of *Dfd* expression, indicating a negative regulation of *Dfd* by the respective gene products. The remaining mutants result in contracted *Dfd* expression by varying degrees, indicating a role in the early activation of *Dfd*.

These results support a hierarchical mode of regulation, where Gap proteins regulate pair-rule genes, and pair-rule products regulate *HOM-C* genes. Other work, however, suggests a combinatorial role for early patterning genes in *HOM-C* gene regulation. In separate studies investigating the expression patterns of *Scr, Antp* P2, and *Ubx*, Ftz was shown to be required for the initial activation of these *HOM-C* genes (temporal control), while the gap gene products control their spatial domains of expression (Ingham and Martinez-Arías, 1986; Riley *et al.*, 1987; Martinez-Arías and White, 1988). More recent work demonstrates a more direct role for the gap protein Hb in the activation of *Antp*. The authors proposed that this may also be true for the Gap proteins Giant (Gt) and Kr, in terms of the activation of *Scr* and *Ubx*, respectively (Wu *et al.*, 2001). It must be noted, however, that the large-scale morphological defects in Gap gene mutants can confound the assessment of their effects on *HOM-C* expression.

1.8.3.3. Maintenance of *HOM-C* Gene Regulation

The maintenance of *Drosophila HOM-C* gene expression or repression is required beyond the early stages of embryogenesis, after the early patterning gene products are no longer available. Because cells continue to express the *HOM-C* genes in spatially restricted patterns throughout development, the cells must "remember" their *HOM-C* transcription state to avoid inappropriate activation or loss of selector gene function. This long-term retention of *HOM-C* transcription state is controlled by two large groups of *trans*-acting proteins, the Polycomb Group (PcG) (review: Simon, 1995) which maintains the *HOM-C* genes in the repressed state, and the Trithorax (Trx) Group (review: Ingham, 1998) which maintains the genes in the active state. Both the Trx group and PcG group proteins are widely conserved between *Drosophila* and mammals (Müller *et al.*, 1995; Simon, 1995).

The PcG proteins function as long-term repressors of *HOM-C* function (Bienz and Müller, 1995; Jacobs and van Lohuizen, 1999). These proteins form multimeric complexes, which are vital for the maintainance of the spatial boundaries of *HOM-C* gene expression (Franke *et al.*, 1991; Simon *et al.*, 1992). They are expressed throughout the embryo (e.g., Franke *et al.*, 1991; Paro and Zink, 1992; Lonie *et al.*, 1994; Martin and Adler, 1993) and, therefore, cannot on their own, define spatial expression of the *HOM-C* genes (Simon *et al.*, 1995). Rather, these protein complexes maintain the expression state initiated by the earlier segmentation proteins by binding PcG response elements (PREs) found within the *HOM-C* gene regions (Zink and Paro, 1989; Castelli-Gair and Garcia-Bellido, 1990; Simon *et al.*, 1995; Gindhart and Kaufman, 1995; Simon, 1995). In *PcG* mutants, the initial expression of the *HOM-C* genes is normal; however, as early segmentation gene products begin to decay, ectopic expression of the *HOM-C* genes becomes apparent (Wedeen *et al.*, 1986). This leads to homeotic transformations similar to those observed in *ANT-C* and *BX-C* gain of function mutants (e.g., Lewis, 1978; Duncan, 1982; Dura *et al.*, 1985; Choi *et al.*, 2000).

The PcG proteins are thought to mediate repression through the formation of higher-order chromatin structures associated with histone methylation (Cao *et al.*, 2002). The PREs do not have a specific recognizable consensus DNA sequence (Jacobs and van Lohuizen, 1999), and the PcG proteins are likely to be recruited to the PREs by either the repressive Gap or pair-rule proteins, or another DNA binding intermediary.

The Trithorax (Trx) group proteins serve a reciprocal role to the PcG proteins and maintain the active transcriptional state of the *ANT-C* and the *BX-C* genes (Breen and Harte, 1993; Kennison, 1993; Gindhart and Kaufman, 1995). Trithorax (Trx), the namesake and best-characterized member of the group (Ingham and Whittle, 1980), accumulates in a spatially modulated pattern, beginning with pair-rule-like stripes in the posterior of the embryo. This may regulate early *BX-C* transcription (Sedkov *et al.*, 1994), as *trx* mutants have altered *BX-C* gene expression during germ band extension. However, *ANT-C* gene expression is unaltered until the late stages of development.

There are alleles of *trx* that affect late *ANT-C* gene expression with no effect upon *BX-C* expression,

indicating the generation of multiple products, with multiple roles, from the *trx* locus. Null mutants of *trx* mimic loss-of-function mutations in the *ANT-C* and *BX-C* clusters, sometimes producing flies with six dorsal thoracic appendages of wing-like identity, the phenotype for which the gene and the group are named (Ingham, 1998).

Like the case of the PcG proteins, it is suspected that the Trx group proteins may not bind DNA directly, but act through other proteins with DNA binding activity. Interestingly, analysis of PcG and Trx protein localization on salivary gland polytene chromosomes demonstrates that these proteins bind many of the chromosomal regions that contain *ANT-C* and *BX-C*. Thus, interaction between these opposing regulatory proteins at the same chromosomal sites may be important to their function (Kuzin *et al*., 1994; Chinwalla *et al*., 1995). However, it is also important to note that although these protein complexes function to maintain the initial *HOM-C* gene activity state in a particular region, the *HOM-C* genes are by no means "locked in" to a particular activity state for the duration of development. This is evident by the dynamic patterns of expression observed for individual *HOM-C* genes during development (discussed earlier in this chapter).

1.8.3.4. Regulation between the HOM-C Proteins: Auto- and Cross-Regulation

The earliest recognized *Drosophila HOM-C* gene regulation was from proteins encoded within the complexes themselves. The *HOM-C* genes cross regulate one another's activity, and the expression of several of the *HOM-C* genes is partially directed by autoregulatory loops. Several of the HOM-C proteins function to limit the expression of *HOM-C* genes expressed in adjoining regions. For example, Abd-B represses both *Ubx* and *abd-A* to exclude their expression in the posterior abdomen (Macías *et al*., 1990). Abd-A limits the expression of *Ubx*, giving the two BX-C proteins complementary expression patterns in the trunk (Karch *et al*., 1990). Similarly, a high level of Antp in the second thoracic segment, and BX-C proteins in the remaining trunk segments, are required to limit the posterior boundary of *Scr* expression (Riley *et al*., 1987). In the absence of the *BX-C* genes, *Scr* expands its domain posteriorly to include epidermal expression in the posterior compartments of the thoracic and abdominal segments (Pelaz *et al*., 1993). Interestingly, this expansion is dependent upon moderate levels of ectopic Antp (normally repressed by the BX-C proteins), which, in the absence of the BX-C proteins, activates *Scr* in the context of the posterior trunk segments.

In two recent studies, Miller *et al*. (2001a, 2001b) utilized the UAS-Gal4 system (an inducible expression system which makes use of the yeast Gal4 transcriptional activator and its binding site, the Upstream Activation Sequence; Brand and Perrimon, 1993) to investigate the embryonic cross-regulatory relationships of the *HOM-C* genes. They separately focused on cross-regulation in the epidermis, the CNS, the visceral mesoderm, and the somatic mesoderm. In the epidermis, cross-regulation generally follows a hierarchy, whereby the more posterior proteins can repress the activation of the anterior genes, but not vice versa. For example, ectopic expression of Lab is unable to repress the activity of more posteriorly expressed *HOM-C* genes, while ectopic Abd-A protein results in reduced expression of all *HOM-C* genes expressed more anteriorly.

The *HOM-C* genes demonstrate tissue-specific cross-regulatory activities. For example, ectopic Scr and ectopic Dfd activate *pb* in the ectoderm, which is consistent with the normal role for these proteins in activating *pb* expression in the embryo (Rusch and Kaufman, 2000). In the mesoderm, however, ectopic Scr represses the expression of *pb* (Miller *et al*., 2001a). The hierarchy of cross-regulation in the CNS also differs from that in the epidermis. Ectopic Ubx and Abd-A are only able to repress two HOM-C genes (*Scr*, *lab*) in the CNS, whereas Ubx and Abd-A have greater cross-regulatory effects in the epidermis. Miller *et al*. (2001a) conclude that no simple model can describe comprehensively the regulatory relationships between the *HOM-C* genes. Their interactions in the CNS and mesoderm are more complex, and signaling cascades likely contribute to this complexity. Assessment of the relationship between cell autonomous regulation and signal transduction led these authors to propose a new hierarchical relationship in the mesoderm, called "autonomic dominance," in which the extrinsic determination of cell fate via signaling can be overridden by the expression of HOM-C proteins.

The integration of signaling and *HOM-C* gene regulation is illustrated by the induction of HOM-C signaling in the endoderm via signals from the overlying visceral mesoderm. This pathway links the regulation of two *HOM-C* genes indirectly through signaling and feedback loops (Bienz, 1997) (**Figure 8**). Ultrabithorax and Extradenticle (Exd; expressed in the visceral mesoderm) direct this complex pathway by activating the signaling molecules Decapentaplegic (Dpp) and Wingless (Wg). These molecules signal to cells in the endoderm, eventually resulting in the activation of *lab* expression and specification of endodermal cell fate (Immerglück *et al*., 1990; Reuter *et al*., 1990; Staehling-Hampton *et al*.,

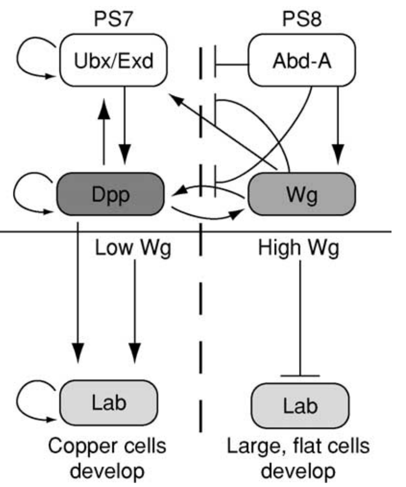


Figure 8 The integration of signaling and *HOM-C* gene regulation in endoderm induction and copper cell specification. Parasegments are approximately six to eight cells wide at the time of endoderm induction. Positive interactions are denoted with arrows, and repressive ones are denoted with bars. In parasegment 7, Ubx/Exd directly induces *dpp* expression in the visceral mesoderm overlying the presumptive endoderm. Dpp signaling results in a band of *Wg* expression in the visceral mesoderm of parasegment 8. The levels of Wg signaling modulate the position of *labial* expression in the endoderm. High levels of Wg in parasegment 8 repress the activation of *labial* and allow the development of large, flat endodermal cells. In parasegment 7, signaling input from Dpp and low levels of Wg activate *labial* and the subsequent specification of crescent-shaped endodermal copper cells. Both Wg and Abd-A repress *Ubx* transcription in parasegment 8, contributing to the spatial limitation of copper cell differentiation to the endoderm of parasegment 7. Numerous autoregulatory and feedback loops act in this pathway: both Dpp and Wg act synergistically to maintain *Ubx* expression; *Ubx*, *dpp* and *lab*, all demonstrate autoactivation; and Ubx feeds back on Dpp to maintain *dpp* expression in the midgut.

1994; Sun *et al*., 1995; Eresh *et al*., 1997; Grieder *et al*., 1997). The concentration of Wg directs the activity state of *lab* in these cells. Low Wg levels promote *lab* activation and endodermal copper cell differentiation. High levels of Wg, on the other hand, repress *lab* activation and, thus, repress the differentiation of copper cells (Hoppler and Bienz, 1995). This regulation may be mediated through the Wg signal transducers, such as *disheveled* (*dsh*), *armadillo* (*arm*), and *shaggy* (*sgg*), and clearly illustrates a link between differential regulation of a *HOM-C* gene and a specific terminal cell fate.

Autoregulation can also direct the activity of the *HOM-C* genes. Some of the earliest characterized HOM-C protein binding sites were autoregulatory elements. Several regionally specific autoregulatory elements have been characterized in *Dfd, Ubx,* and *lab* (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988b; Bergson and McGinnis, 1990; Chouinard and Kaufman, 1991; Regulski *et al*., 1991). These elements may be located upstream of the promoter or within intronic regions. For example, a CNS-specific autoregulatory element of *Dfd* maps within the large *Dfd* intron (Lou *et al*., 1995), while an epidermal enhancer resides upstream of the *Dfd* promoter (Bergson and McGinnis, 1990; Zeng *et al*., 1994).

1.8.3.5. Posterior Prevalence–Functional Hierarchies

Posterior prevalence, also called functional dominance and phenotypic suppression, refers to the phenomenon whereby a posteriorly expressed protein can override the function of a more anteriorly functioning protein, when these are simultaneously expressed in the same cells. For example, the ubiquitous ectopic expression of Scr, under the control of a heat shock promoter, is lethal and results in the transformation of the embryonic maxillary segment towards a labial identity, as evidence by the partial loss of mouthpart structures and the formation of ectopic salivary glands (Zeng *et al*., 1993). The Scr protein overrides the normal patterning directed by Dfd in the mandibular and maxillary lobes. This could be due to a higher level of Scr than Dfd in these regions. However, there is no phenotypic effect in the trunk posterior to the second thoracic segment. Ectopic Scr appears unable to supersede the functions of endogenous Ubx, Abd-A, and Abd-B, even though these proteins are probably less abundant. Similarly, the ubiquitous ectopic expression of Ubx causes the transformation of segments anterior to the first abdominal, including the development of abdominal type denticles in the head and in the thorax (González-Reyes and Morata, 1990). However, segments posterior to the first abdominal are not altered. Therefore, like Scr, Ubx is unable to override Abd-A and Abd-B function in the epidermis.

There are, however, violations to the posterior prevalence model. For example, the ectopic expression of Dfd is able to induce ectopic mouth hooks in the labial segment, alter the denticle patterning of the second thoracic segment to resemble the first thoracic segment, and produce ectopic sensory cirri and sclerotized mouthpart-like material in the trunk segments (Kuziora and McGinnis, 1988a), thus apparently altering the patterns directed by the endogenous HOM-C proteins in these cells.

1.8.3.6. Regulation of HOM-C Protein Function by Phosphorylation

Not all *HOM-C* gene regulation occurs at the level of transcription; HOM-C protein function is also

modulated posttranslationally. The N-terminal portion of the homeodomain is important for the specificity of homeodomain function (see Section 1.8.4), and this region of the Scr protein is a target for a regulatory subunit of the serine–threonine specific protein phosphatase 2A (dPP2A, B′) (Berry and Gehring, 2000). The phosphorylation state of two key residues in the N-terminal portion of the homeodomain (Thr6 and Ser7) is critical to Scr function. These residues can be phosphorylated by cAMP-dependent kinase A (PKA), and dephosphorylated by dPP2A in cultured cells. Ectopic expression of an Scr protein construct mimicking constitutive dephosphorylation (with Thr6 and Ser7 altered to alanine), reproduces the embryonic phenotype induced by ectopic expression of the wild-type protein (loss of some mouthpart structures, ectopic salivary gland formation, and transformation of the second and third thoracic segments towards a first thoracic identity). Conversely, an ectopically expressed Scr protein construct, in which Thr6 and Ser7 are altered to mimic constitutive phosphorylation (by substitution with aspartic acid) is unable to reproduce the embryonic ectopic Scr phenotype and has a reduced ability to bind a target DNA sequence *in vitro*. Further, dPP2A,B′ appears to be required for endogenous Scr function, as embryos lacking dPP2A,B′ fail to form salivary glands.

A second example of HOM-C protein functional regulation by phosphorylation involves Antp. Phosphorylation by the serine–threonine kinase casein kinase II (CKII) is implicated in preventing spatially inappropriate Antp function during *Drosophila* embryogenesis (Jaffe *et al*., 1997). Together, these results suggest that posttranslational modifications may control the functional state of a HOM-C protein.

1.8.3.7. Regulation of *HOM-C* Genes in Other Insects

Because *Drosophila* is a long germ insect, patterning its trunk segments from a continuous existing group of cells, aspects of its *HOM-C* gene regulation might be expected to differ from intermediate or short germ insects, which form their trunk segments sequentially from a continuously dividing growth zone. Homologs to many of the *Drosophila* early segmentation genes have been identified, in other insects, and their expression patterns examined. In *Tribolium*, homologs to each class of the *Drosophila* segmentation genes have been identified, and the expression of most was shown to be similar to that observed in *Drosophila* (Sommer and Tautz, 1993; Brown *et al*., 1994; Nagy and Carroll, 1994; Patel *et al*., 1994; Wolff *et al*., 1995; Brown *et al*., 1997). Yet, their function may not always be the same. For example, in *Tribolium*, deletion of *Tcftz* has no effect on segmentation (Stuart *et al*., 1991). Similarly, the homologs of the pair-rule genes *eve* and *ftz* appear to have no pair-rule function in *Schistocerca* (Akam and Dawes, 1992; Patel *et al*., 1992). Differences in the roles of these genes may also reflect differences in their ability to regulate *HOM-C* gene expression, but this remains to be investigated.

One proposed regulator of *HOM-C* gene activity in other insects is the *Tribolium* gene, *jaws*. Mutants in this gene bear a phenotype similar to one resulting from the overexpression of *mxp*, the *Tribolium* homolog of *pb* (Sulston and Anderson, 1998). Embryos lacking *jaws* have a homeotic transformation of the thoracic and anterior abdominal segments into four alternating maxillary and labial segments, and also fail to form a number of posterior abdominal segments (Sulston and Anderson, 1996). The *mxp* gene is ectopically expressed in each transformed segment, with Ultrathorax (the homolog to Ubx) and Abdominal (the homolog to Abd-A) being virtually absent in the few remaining trunk segments. The *jaws* gene is not located within the *Tribolium HOM-C*, and thus is a candidate negative regulator of *HOM-C* gene activity. Sulston and Anderson postulate that *jaws* may represent a member of the *Tribolium Polycomb* group.

Cross-regulation of *HOM-C* genes also is observed in other insects. *Tribolium* shares regulatory relationships between *pb* and *Scr* similar to those present in *Drosophila*. In the *Drosophila* embryo, *Scr* and *Dfd* positively regulate *pb* expression in the gnathal segments (Rusch and Kaufman, 2000), while in the distal region of the larval labial disc, *pb* positively regulates the activity of *Scr* (Abzhanov *et al*., 2001). In *Tribolium*, development of the larval labial appendage requires *Cx* (the *Scr* homolog) to properly activate early and midstage embryonic expression of *mxp* (the *pb* homolog) (DeCamillis *et al*., 2001), and MXP positively regulates *Cx* expression in the distal gnathal appendage fields in later embryonic development (DeCamillis and ffrench-Constant, 2003). However, this does not hold true in all insects. Similar patters of *Scr* and *pb* expression are observed in the milkweed bug, but RNAi experimentation indicates no apparent regulation of *Scr* by *pb* in gnathal appendage buds, or cross-regulation of *pb* by *Scr* (Hughes and Kaufman, 2000). It is clear therefore, that there may be differences in *HOM-C* regulation, and this is an area where additional information from other insects is needed.

1.8.4. The Homeobox and the Homeodomain: Identification, Structure, and Function

1.8.4.1. Identification and Characterization of the Homeobox and Homeodomain

Following the cloning of the *ANT-C* and *BX-C*, two groups identified a strongly conserved DNA sequence in several genes within the two complexes, which was called the H-repeat (McGinnis *et al.* 1984b; Scott and Weiner, 1985). Further testing pinpointed their genomic locations to regions within the *BX-C* (between the *bxd/pxd* and *iab-2* mutations) and the *ANT-C* (in the region of *pb*, *Dfd*, and *zen*). The H-repeat was named the "homeobox," and a second conserved repeated sequence, the M-repeat, was also identified. The M-repeat encodes a poly-glutamine repeat that had been previously identified in genes outside of the *HOM-C*, and is alternatively called the opa-repeat (Wharton *et al.*, 1985). Sequence analysis revealed strong conservation of predicted amino acid sequence between the gene regions. Using the homeobox sequences from *Antp* and *Ubx*, Regulski and collaborators mapped homeobox sequences to each *ANT-C* gene cloned at the time (*ftz, Antp, Dfd*, and *Scr*) and to three regions within the *BX-C* (*Ubx, abd-A*, and *Abd-B*) (Regulski *et al.*, 1985). They concluded, "all of the copies of homeobox homology that are well conserved with respect to both *Antp* and *Ubx* copies, map within genes of the *ANT-C* or *BX-C*." Later work showed that both *pb* and *lab* are also homeobox and M-repeat containing genes (Mlodzik *et al.*, 1988).

The homeobox is a nucleotide sequence of 180 well-conserved base pairs, encoding a 60 amino acid motif called the homeodomain (Figure 9) (McGinnis *et al.*, 1984c). Within the homeobox, nucleotide sequence similarities among the *HOM-C* genes are as high as 75–80%, and many of the nucleotide differences are silent. For example, *Antp* and *Ubx* share 79% nucleotide identity within the homeobox, but share 89% amino acid identity in the homeodomain. Outside of the 180 bp region, the sequence similarity among the *HOM-C* genes is limited to small regions, or is nonexistent.

Evolutionary conservation of the homeobox was initially reported in the genomes of three other segmented invertebrates (*D. hydei*, the beetle *Tenebrio molitor*, and the earthworm *Lumbricus* sp.) and three vertebrates (chicken, frog *Xenopus laevis*, and mouse) (Carrasco *et al.*, 1984; McGinnis *et al.*, 1984a, 1984b). The homeobox sequences from the *Drosophila Antp* and *Ubx* genes were used in cross-species genomic surveys to identify homeodomain-containing fragments in these animals, and the similarity within the homeodomains was found to be quite high. This conservation across phyla was reinforced by results from subsequent studies, with *HOM-C* homologs found in the genomes of every animal searched. In fact, remarkable conservation is even noted between the *Drosophila HOM-C* genes and their mammalian counterparts despite at least 600 million years of evolutionary separation. For

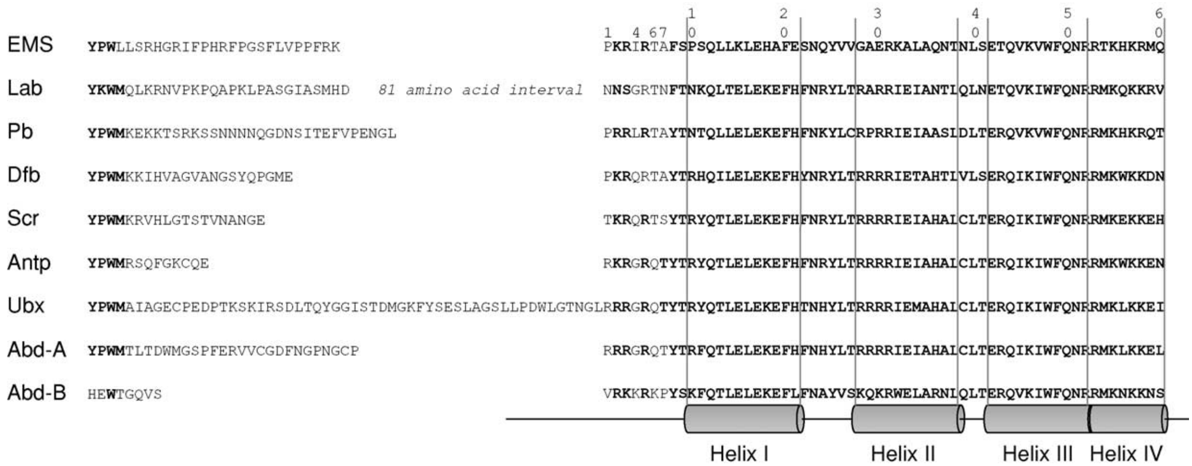


Figure 9 The HOM-C protein homeodomain and YPWM motif. The amino acid sequence of each HOM-C protein and EMS homeodomain, plus a number of residues N-terminal to the homeodomain are aligned to show sequence conservation. Some of the amino acid sequence between the LABIAL homeodomain and YPWM motif is omitted, as 81 amino acids intervene the two conserved domains. Abd-B does not exhibit the conserved YPWM motif, but a conserved tryptophan (W) residue just upstream of the homeodomain may represent the remainder of that motif, and is thus aligned as such. The secondary structure of the homeodomain is shown below the amino acid sequence. Note that α-helices III and IV abut and frequently the homeodomain is considered to have only three α-helices. N-terminal residues key for functional specificity are highlighted and discussed further in the text.

example, *Dfd*, and a mammalian homolog *HOXD-4* (*HOX-4.2*), are conserved within the homeodomain at 55 of 60 residues. *HOXD-4* expression in *Drosophila* is able to activate a *Dfd* responsive reporter in embryos, and produces adult phenotypes similar to those caused by a dominant gain-of-function *Dfd* allele (McGinnis *et al.*, 1990). This level of cross-phylum conservation within the homeobox sequence led Jonathan Slack (1984) to wonder if this conserved sequence could prove to be "the biological equivalent of the slab of basalt found in 1799 near the Egyptian town of Rosetta ... [which] provided the most important clue in the decipherment of the hieroglyphics," and touched off a profusion of study into the homeobox, the homeodomain, and *HOM-C* gene function.

The homeodomain-containing genes of all organisms are classified based on amino acid sequence similarity in and around the homeodomain (Bürglin, 1994). Six of the eight *HOM-C* genes are grouped together as part of the "Antennapedia class" (*pb, Dfd, Scr, Antp, Ubx, abd-A*), and share at least 60% sequence similarity within the homeodomain. The *labial* gene is in a class bearing its name, and its homeodomain is 55–67% similar to members of the *Antp* class. Just upstream from the homeodomain, the *Antp* and *labial* classes share a short conserved motif (the hexapeptide, or YPWM motif), making these classes members of the Hexapeptide superclass of homeobox genes. The Abd-B homeodomain is no more than 55% similar to any other HOM-C homeodomain, diverging significantly in the first two helices of the homeodomain (Celniker *et al.* 1989). The third helix, however, is quite similar, differing from the corresponding *Ubx* region by only a single amino acid. The Abd-B homeodomain does not contain the hexapeptide motif, but does retain a conserved tryptophan residue (W) just upstream of the homeodomain. In the mouse, this residue appears capable of mediating protein interactions similar to those mediated by the hexapeptide motif (Shen *et al.*, 1997). Because its homeodomain sequence diverges significantly from the other HOM-C homeodomains, *Abd-B* is classified separately, in the *Abd-B* class (Bürglin, 1994).

In addition to the *HOM-C* genes, there are numerous other *Drosophila* homeodomain-containing genes. Several are well studied and have provided much of the basis for our understanding of homeodomain structure and function. Particularly well-characterized examples include the segmentation genes *engrailed* (*en*) (Desplan *et al.*, 1985, 1988; Ohkuma *et al.*, 1990; Kissinger *et al.*, 1990; Jaynes and O'Farrell, 1991; Ades and Sauer, 1994; Clarke *et al.*, 1994; Bourbon *et al.*, 1995) and *ftz* (Nelson and Laughon, 1990; Percival-Smith *et al.*, 1990; Florence *et al.*, 1991; Walter *et al.*, 1994), and the maternal factor *bicoid* (*bcd*) (Hanes and Brent, 1989; Treisman *et al.*, 1989; Hanes *et al.*, 1994). All of the HOM-C proteins, and other homeodomain-containing proteins including Even-skipped (Eve), Ftz, and En, have a conserved glutamine (Q) residue at position 50 of the homeodomain and are referred to as the Q50 homeoproteins (Biggin and McGinnis, 1997). Thus, a generalized HOM-C protein can be described as having the following structure: a long N-terminal segment containing the conserved M-repeat/opa-repeat region, a central region, the homeodomain, a short C-terminal region, and a trailer sequence (Desplan *et al.*, 1988). Because the general properties of the homeodomains are quite similar, pertinent data from other homeodomain proteins is useful to fully characterize HOM-C homeodomain structure and function.

1.8.4.2. The HOM-C Homeodomains Bind DNA at a Core Consensus Sequence

The homeodomain sequence was recognized as having features similar to a known transcription factor, the yeast Mat α protein, and to prokaryotic helix–turn–helix repressor proteins (Laughon *et al.*, 1984). It was therefore predicted that the HOM-C proteins might function as DNA binding factors, with the homeodomain as the likely binding motif. Studies undertaken to characterize homeodomain function included genetic interactions, transcription assays, DNA–protein cross-linking assays, immunoprecipitation, and DNA footprinting experiments. The results of this work confirmed the predicted function of the homeodomain as a DNA binding motif and that of the HOM-C proteins as transcriptional regulators (e.g., see Desplan *et al.*, 1985; Müller *et al.*, 1988; Samson *et al.*, 1989; Krasnow *et al.*, 1989; Johnson and Krasnow, 1990, respectively). In fact, it is now known that the homeodomain is one of the most common DNA binding motifs found in eukaryotic transcriptional regulators, second only to the C2H2 zinc finger class (Tupler *et al.*, 2001).

Numerous *in vitro* studies with the HOM-C homeodomains resulted in the characterization of a core consensus DNA recognition sequence. An early study of HOM-C protein/DNA interactions used DNAse protection to characterize the Antp homeodomain (ANTP-HD) recognition sequence. This peptide exhibited specific DNA binding affinity for the sequence 5′-TAATG-3′ (Müller *et al.*, 1988; Affolter *et al.*, 1990; Ekker *et al.*, 1994). The ANTP-HD was also found to form a more stable complex with DNA than did the similarly structured prokaryotic HLH proteins. The extended recognition

helix, formed by helices three and four, may contribute to this increased stabilization. Other studies of HOM-C/DNA interactions involve partial or full-length Dfd, Antp, Ubx, and Abd-B proteins (Desplan *et al.*, 1988; Ekker *et al.*, 1991, 1992, 1994; Wilson *et al.*, 1996). Although Abd-B appears to bind preferentially to a slightly different core sequence of 5′-TTAT-3′ (Ekker *et al.*, 1994), the Q50 homeodomains consistently recognize a consensus core sequence of 5′-TAAT-3′ within characterized recognition sites of 5–9 bp (**Figure 10**).

Structural and genetic studies indicate that the nucleotides flanking the core DNA sequence are important for homeodomain recognition, with one upstream base and two downstream bases being of particular importance (Desplan *et al.*, 1988; Hanes and Brent, 1991). Genetic analysis also indicates the importance of bases several positions downstream of the 5′-TAAT-3′ to homeodomain recognition specificity. Interestingly, *in vitro* studies revealed that a single homeodomain peptide can bind different DNA sequences with similar affinities, and that even divergent homeodomain proteins appear to recognize very similar DNA sequences (Desplan *et al.*, 1988; Hoey and Levine, 1988; Walter *et al.*, 1994; Walter and Biggin, 1996). Ubx and Antp have been shown, *in vitro*, to recognize identical consensus DNA sequences (Ekker *et al.*, 1994). Thus, although the HOM-C proteins recognize and bind specific DNA sequences, it appears that they all recognize the same core sequence, suggesting that there must be additional *in vivo* mechanisms involved in directing their biological patterning specificities.



Figure 10 A schematic view of the homeodomain/DNA complex. The three helices of the homeodomain are shown as purple cylinders and numbered (I, II, III). The DNA helix is represented by ribbons and bars. The bar colors denote base identity: green represents adenine (A), red represents thymine (T), blue represents guanine (G), and yellow represents cytosine (C). The recognition helix (helix III) of the HOM-C homeodomain binds directly to DNA in the major groove, recognizing a core consensus sequence of 5′-TAAT-3′. The C-terminal region of this helix is less stable, and is sometimes referred to as helix IV (Qian *et al.*, 1989). The flexible N-terminal arm makes contact with DNA in the minor groove.

1.8.4.3. Functional Specificity of the HOM-C Homeodomain

Because of the strong amino acid conservation and apparent similarities in target site recognition, several studies have attempted to dissect HOM-C protein homeodomains to identify specific residues critical to their biological function. Several groups approached this problem by constructing chimeric HOM-C proteins (swapping various regions of one HOM-C protein for another), then expressing the chimeras in *Drosophila* embryos and adults to assay developmental outcomes and the effects on target gene expression. Although these types of studies have involved the majority of the HOM-C proteins, we summarize, below, the results of selected swapping studies involving the Ubx homeodomain inserted into the Dfd protein (Kuziora and McGinnis, 1989; Dessain *et al.*, 1992; Lin and McGinnis, 1992), and the Scr homeodomain inserted into the Antp protein (Gibson *et al.*, 1990; Furukubo-Tokunaga *et al.*, 1993).

Substitution of the Ubx homeodomain into an otherwise normal Dfd protein is sufficient to introduce Ubx target regulation, and override normal Dfd activity. Wild-type Dfd can autoregulate its own expression in some cells, binding to defined autoregulatory elements to maintain its transcription (Kuziora and McGinnis, 1988b; Lou *et al.*, 1995). The ectopic activation of wild-type Dfd results in the development of ectopic mouthpart material and maxillary cirri in the trunk segments, and the autoactivation of Dfd outside of its normal domain. Activation of a chimeric heat shock- inducible *Dfd* construct, encoding the Ubx homeodomain in place of the Dfd homeodomain, results in a transformation of the larval head segments toward a thoracic identity, as also results from ectopic Ubx expression (González-Reyes and Morata, 1990). The ectopic activation of *Antp* P1, a known *Ubx* target (Beachy *et al.*, 1988), and the concomitant loss of *Dfd* autoregulation are also observed (Kuziora and McGinnis, 1989). Thus, the Dfd/Ubx chimera, with only the homeodomain region changed, alters the protein function to resemble a wild-type Ubx protein construct.

Further studies mapped the functional specificity of the Ubx homeodomain using a series of chimeric proteins, in which different portions of the Ubx homeodomain were substituted into an otherwise normal Dfd protein construct (Lin and McGinnis, 1992). Each construct was tested for its ability to activate the *Dfd* autoregulatory element and the *Antp* P1 promoter. Constructs retaining the Ubx N-terminal homeodomain region also retain the larval head to thoracic transformation produced by the original Dfd/Ubx construct, and activate ectopic *Antp* P1 transcription. Further, substituting only a few N-terminal Ubx homeodomain residues (numbers 0–9) (**Figure 9**) is sufficient to alter ectopic Dfd function and allow initiation of ectopic *Antp* P1 transcription. A chimera containing the Ubx homeodomain with the N-terminal Dfd homeodomain region fails to activate ectopic *Antp* P1 transcription and loses the ability to induce a head to thorax transformation. Both the N-terminal and C-terminal homeodomain regions are required for full Dfd autoactivation: no autoactivation is possible without the N-terminal Dfd region, and only weak autoactivation is possible with the C-terminal portion absent. In summary, these studies demonstrate that the homeodomain is capable of directing HOM-C protein function, and specifically, the N-terminal homeodomain region alone is sufficient to alter HOM-C protein function. The C-terminal flanking region also contributes to functional specificity.

Others took a similar approach in comparing two ANT-C proteins, Antp and Scr. Gibson *et al.* (1990) used the degree of transformation and target gene transcript accumulation to assess the functional specificity of a series of ectopically expressed, chimeric proteins. The Scr homeodomain was substituted into an otherwise normal Antp protein construct and the chimera was expressed in embryos and adults, utilizing an inducible heat shock promoter. This work determined that functional specificity lies in residues both within and adjacent to the homeodomain, and corroborated results obtained with the Dfd and Ubx studies.

Extending the Scr/Antp study, Furukubo-Tokunaga *et al.* (1993) used the chimeric construct to assess the functional requirement of regions outside of the homeodomain, and to more specifically investigate residues within the homeodomain that confer functional specificity. Interchange of the entire protein region N-terminal to the homeodomain seems only to affect the extent of transformation, or "potentiation," but does not alter the overall function from that of the original chimeric protein. Positions 1, 4, 6, and 7 of the homeodomain (**Figure 9**) are the residues that distinguish the wild-type Antp and Scr homeodomains and prove critical to the functional specificity of the chimeric proteins. By substituting the Antp-specific homeodomain residues into the chimeric Scr/Antp protein (wild-type Antp with the Scr homeodomain), a functional Scr protein is reverted to a functional Antp protein. Thus, substitution of these four amino acids alone is sufficient to determine the functional difference between Antp and Scr. Therefore, functional specificity between the homeodomains of two different ANT-C proteins appears to be determined by only a few critical amino acids within the N-terminal portion of the homeodomain, while residues within the large region N-terminal to the homeodomain direct only the extent of the protein's functional effects. Similar studies involving Ubx and Antp, and Dfd and Abd-B produced similar results (Kuziora and McGinnis, 1991; Chan and Mann, 1993; Zhu and Kuziora, 1996).

As discussed above (see Section 1.8.3.6), the phosphorylation state of two N-terminal homeodomain residues (6 and 7) is critical to Scr function. At position 6 of the homeodomain, Scr has a threonine residue, while Antp has a nonphosphorylatable glutamine residue. The phosphorylation state of this N-terminal homeodomain residue may, at least in part, explain some of the differing functional specificities of the Scr and Antp homeodomains.

1.8.4.4. Homeodomain Structure and the Physical Interaction with DNA

The homeodomain structure is highly conserved, as evidenced by strong sequence conservation and three-dimensional structural analysis. The Antp homeodomain was the first HOM-C homeodomain to be structurally resolved, and is the best characterized with regard to the three-dimensional structure. The structure of the Antp protein, both alone and bound to DNA, was resolved by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, revealing detailed information concerning the *in vitro* physical contacts within the protein–DNA complex. These studies are consistent with the structural characterization of other homeodomain–DNA interactions (Fraenkel *et al*., 1998; Hovde *et al*., 2001). Because of strong sequence similarity between the HOM-C homeodomains, the data and conclusions from these studies can be reasonably applied to the remaining *HOM-C* genes and probably other Q50 homeodomain proteins, as well.

NMR resolution of the Antp homeodomain tertiary structure reveals that the homeodomain forms a globular domain, consisting of four helices (**Figure 9**), three well-defined alpha helices and one less organized fourth helix directly abutting the

third (Billeter *et al.*, 1990). The C-terminal end of the third helix, or recognition helix, is somewhat less stable and, therefore, occasionally characterized as a fourth helix, although the homeodomain is most commonly described as having only three helices. An extended N-terminal arm is evident, upstream of the first helix (Qian *et al.*, 1989).

A number of NMR and X-ray crystallography studies have provided the structural details of homeodomain–DNA interactions (Otting, *et al.*, 1990; Hirsch and Aggarwal, 1995; Wilson *et al.*, 1995; Tucker-Kellogg *et al.*, 1997; Fraenkel *et al.*, 1998; Fraenkel and Pabo, 1998; Hovde *et al.*, 2001). A schematic of the homeodomain–DNA interaction is shown in **Figure 10**. Contact between the homeodomain and DNA occurs at the recognition helix and at amino acid residues flanking helix one. The DNA is contacted within a 7 nucleotide region on both sides of the core binding sequence and all but one contact sites are within the major groove. Overall, contacts are made by the homeodomain across two major grooves of the DNA, with the N-terminal arm (containing four amino acids important for functional specificity, and discussed above) contacting the minor groove.

Further support for the importance of the N-terminal homeodomain residues is provided by the structural study of a truncated Antp peptide lacking the first six amino acids of the homeodomain (Qian *et al.*, 1994). Although its three-dimensional structure is virtually identical to that of the intact homeodomain, the K_d for the truncated peptide bound to DNA was found to be approximately 10-fold less than that of the intact Antp peptide. Thus, the N-terminal homeodomain residues contribute significantly to DNA binding affinity and stability, and perhaps contribute to the motif's ability to stably bind and activate target DNA sequences. Interestingly, inclusion of the conserved hexapeptide (YPWM) motif does not add to the stability of the homeodomain–DNA interaction, nor does it increase the DNA binding specificity (Qian *et al.*, 1992). These results support genetic and later structural studies demonstrating that this highly conserved motif functions in protein–protein interactions, rather than being involved in DNA sequence recognition. The YPWM motif and its function are discussed further in the section that addresses the cooperative interactions between HOM-C proteins and other factors.

In summary, the protein products of the HOM-C genes are DNA binding factors recognizing a consensus sequence with a canonical 5′-TAAT-3′ at its core. It is difficult to reconcile how the HOM-C proteins direct developmental pathways leading to distinct and varying cell morphologies when their target recognition sequences are so similar. For example, it is reasonable to assume that the genetic cascade initiated by one HOM-C protein to pattern a gnathal segment is quite different than the cascade initiated by a different HOM-C protein to pattern an abdominal segment. Yet, we see identical amino acid residues within the recognition (third) helix and the ability of multiple homeodomains to recognize identical sequences. Chimeric studies identify a few N-terminal homeodomain residues as important to developmental function, as transformations in the larval cuticle are observed when these critical residues are altered. Larval denticle patterning may, however, provide limited criteria for assessing functional specificity. Several gnathal HOM-C proteins (Dfd, Scr, and Lab), when ectopically expressed, are sufficient to transform the second and third thoracic denticle belts to a first thoracic identity (Kuziora and McGinnis, 1988b; Zeng *et al.*, 1993; T.C. Kaufman, personal communication), though in normal development, only Scr patterns the first thoracic segment. Thus, the larval denticle pattern, alone, may prove insufficient to evidence altered HOM-C functional specificities. Further, the biological specificity of isoforms of the same HOM-C protein (i.e., Ubx) may not result from differences in target DNA sequence recognition, as the homeodomains are unchanged between isoforms. This seeming incongruence between the specificity of HOM-C protein function and the apparent lack of specificity in DNA target recognition is frequently referred to as the "HOX paradox." It seems likely then, that functional specificity results from spatial and/or temporal differences in expression and differential protein interactions. The N-terminal region of the homeodomain and the highly variable regions outside of the conserved portion of the homeodomain may guide cofactor interactions to direct the developmental specificity brought about by the homeotic selector genes.

1.8.5. Cooperative Interactions between HOM-C Proteins and Other Factors

1.8.5.1. A Case for Cofactors

The results from DNA binding studies in *Drosophila*, investigating the specificity of HOM-C protein target recognition, do not appear to provide the degree of specificity required for developmentally distinct cell fates. As described above (see Section 1.8.4.3), Antp and Ubx recognize identical preferential binding sequences and have overlapping expression

domains, yet have very different biological functions (e.g., *Antp* patterns the wing, where *Ubx* patterns the haltere), implying the need for additional mechanisms or factors to direct their target recognition and resulting biological specificity.

Virtually all Q50 homeodomains bind, with varying affinity, to a consensus core sequence of 5′-TAAT-3′ (Gehring *et al*., 1994), and this motif would be expected to occur, on average, once in approximately every 256 bp of DNA in the *Drosophila* genome. Given the estimates of protein concentrations within nuclei, the homeodomain containing proteins Eve and Ftz (Q50 homeodomains) are estimated to bind to high and medium affinity sites at a density between one monomer per 4 kb of DNA (for weakly bound genes) to five monomers per 1 kb of DNA (for strongly bound genes) (Biggin and McGinnis, 1997). One would expect only a portion of these potential binding sites to be required for spatial and temporal regulation of specific target genes. Thus, the simple presence of a HOM-C protein, bound to DNA, is unlikely to bestow target status to a gene. This is nicely illustrated by studies utilizing the HOM-C protein Dfd and a known Dfd autoregulatory sequence.

Multiples of a small Dfd binding fragment, derived from a Dfd autoactivation enhancer, is sufficient to drive β-galactosidase reporter expression in the maxillary segment of *Drosophila* embryos. However, the loss of an imperfect inverted repeat sequence removes the maxillary reporter expression (Zeng *et al*., 1994), suggesting that other proteins must also bind to this fragment to allow activation of transcription. Further, the Dfd protein binds strongly, *in vitro*, to a tandem repeat of its consensus recognition site, but can only significantly activate transcription of a reporter construct containing this site, when it is accompanied by the constitutive VP16 activation domain (Li *et al*., 1999a). Thus, in spite of strong binding, Dfd alone cannot activate the reporter without the addition of an activation sequence, further indicating a requirement for additional factors. Additionally, the Dfd-VP16 fusion activates ectopic expression of *Scr in vivo*, whereas the wild-type Dfd protein does not regulate *Scr*. This demonstrates that the HOM-C proteins likely bind *in vivo* to sites that do not represent their normal target gene promoters or enhancers.

The presence of cooperatively binding proteins, or cofactors, provides a resolution to the conflict between the lack of binding specificity and developmental precision. Biggin and McGinnis (1997) described two potential models of HOM-C/Co-factor interaction, the co-selective binding model and the widespread binding model. The co-selective model proposes that a cofactor is required to direct HOM-C proteins to the appropriate target gene regulatory sites, while the widespread binding model predicts that HOM-C proteins alone are able to bind to many recognition sites throughout the genome, but are only able to activate transcription in conjunction with appropriate, available cofactors. Cooperative transcriptional control mechanisms, such as those the HOM-C proteins appear to require, are not novel and have been described in other organisms. The yeast homeodomain transcription factors MAT a1 and MAT α2 form cooperative heterodimers, which bind DNA to repress the transcription of haploid-specific mating type genes (Goutte and Johnson, 1993; Mak and Johnson, 1993). The mammalian octamer transcription factors (OCT) also contain a POU class homeodomain, which recognizes an octamer sequence motif. Activation of RNA polymerase II mediated transcription at the octamer binding site requires the heterodimerization of OCT factors (Bürglin, 1994). Yeast also provides an example of a homeodomain/zinc finger interaction between the PHO2 and SWI5 proteins during regulation of the HO endonuclease gene (Brazas and Stillman, 1993).

HOM-C gene cofactors would be expected to have properties fullfilling several genetic and biochemical criteria:

- absence of the cofactors would duplicate full or partial HOM-C loss-of-function phenotypes;
- a cofactor would act in parallel to the HOM-C protein, neither functioning as an upstream regulator, nor as a downstream target (although some regulatory "cross-talk" between the two may be expected);
- HOM-C target gene expression would be reduced or absent in the absence of the cofactor; and
- the cofactor may increase the DNA target specificity and/or the DNA binding affinity of its HOM-C partner.

Several *Drosophila* gene products have been described, which meet many, or all, of these criteria. The homeodomain encoding gene *extradenticle* (*exd*), initially described by Peifer and Weischaus (1990), is the best-characterized HOM-C cofactor. Other genes proposed to encode HOM-C cofactors include *teashirt*, (*tsh*) *disconnected* (*disco*), and the partially redundant *disco-related* (*disco-r*), whose proteins include a zinc finger DNA binding motif; *cap'n'collar* (*cnc*), a member of the bZip family; and two genes encoding putative novel transcription regulators, *lines* (*lin*) and *apontic* (*apt*).

1.8.5.2. Extradenticle is a HOM-C Protein Cofactor

The *extradenticle* gene maps cytogenetically to position 14A5 on the X-chromosome and encodes a 378 amino acid protein required for proper embryonic and adult patterning. The *exd* gene is homologous to the *pbx* group of vertebrate genes (Rauskolb *et al*., 1993) and its protein product is a member of the Pbx class of homeodomain proteins (Bürglin, 1994), which share 95% similarity within their homeodomains and 71% similarity across their entire sequences. The Exd homeodomain diverges from those of the *HOM-C* genes, having three extra residues between the first and second α-helix. Atypical homeodomains of this type are referred to as three amino acid loop extension (TALE) homeodomains. A second conserved domain is the PBC-A domain, through which Exd interacts with a second homeodomain-containing protein, Homothorax (Hth) (Ryoo *et al*., 1999). This interaction is discussed in detail below.

Exd is expressed and packaged into the egg during oogenesis (Rauskolb *et al*., 1993). Loss of maternal and zygotic *exd* expression disrupts embryonic development, even more so than does the loss of zygotic *exd* alone. Maternal Exd protein is uniformly distributed and cytoplasmically localized in early embryogenesis and persists until germ band extension (Aspland and White, 1997). Zygotic transcription initiates thereafter, with transcripts initially found uniformly throughout the embryo, and then being modulated thereafter (Rauskolb *et al*., 1995). The initiation of zygotic *exd* expression is unaltered in *HOM-C* mutants, and conversely, the loss of *exd* expression does not affect the expression of the *HOM-C* genes, indicating that these genes act in parallel developmental pathways (Peifer and Wieschaus, 1990; Rauskolb *et al*., 1995). By germ band contraction, expression of *exd* is lost in abdominal segments 7–10, the remaining Exd protein becomes localized to the nucleus, and this nuclear localization is spatially regulated (Aspland and White, 1997). Expression is also evident in the somatic and visceral mesoderm, the CNS and brain, and the larval imaginal discs (Rauskolb *et al*., 1993; Aspland and White, 1997).

Embryos lacking zygotic Exd function die during late embryogenesis and have defects in overall pattern formation (Peifer and Wieschaus, 1990). Development of some head structures is disrupted, and the thoracic segments are altered, with the second thoracic segment adopting a first thoracic-like denticle pattern, and the third thoracic segment showing characteristics of both the first thoracic and second abdominal segments. The identities of the abdominal segments are transformed to those two to three segments more posterior. For example, the first abdominal segment resembles the third or fourth. No effect is detected in the most posterior segments. Weaker alleles are postembryonic lethals, often forming pharate adults with cuticular defects, and clonal analysis demonstrates that lack of Exd also causes transformation of adult structures (González-Crespo and Morata, 1995; Rauskolb *et al*., 1995). Null *exd* clones result in homeotic transformation of adult head structures, legs, and abdominal segments, resembling the phenotypes observed in some *HOM-C* mutants. Antenna and arista are transformed to leg, while the head capsule is altered to resemble the dorsal thorax or notum. Abdominal clones in the first through fourth segments transform towards the fifth or six abdominal segments. Ubiquitous overexpression of *exd* has no affect on segment identities (Rauskolb *et al*., 1995). Because *exd* mutants appeared to have alterations in HOM-C target specificity, Peifer and Wieschaus proposed that Exd acts as a HOM-C cofactor.

Exd homologs have been identified in the cricket *Gryllus bimaculatus*, the cricket *A. domesticus*, the pine sawfly *Diprion similis*, and the grasshopper *S. americana* (Abzhanov and Kaufman, 2000; Jockusch *et al*., 2000; Suzuki and Palopoli, 2001; Inoue *et al*., 2002). These studies examined Exd expression as it is involved in leg development, and found that like *Drosophila*, nuclear Exd is present in the proximal developing leg. There are currently no published data assessing the function of Exd with the HOM-C proteins of other insects. However, the highly conserved nature of the interacting regions of these proteins across several animal taxa (Rauskolb *et al*., 1993; Pöpperl *et al*., 1995; Liu and Fire, 2000) leads us to expect that the protein serves a similarly conserved role across insect taxa. This, however, remains to be demonstrated.

To explore the interaction and contribution of the HOM-C proteins and Exd to development, embryos mutant for *exd* were compared to embryos singly mutant for various *HOM-C* genes and doubly mutant for both a *HOM-C* gene and *exd*. Double mutants differed from either single mutant, indicating that the *HOM-C* genes continued to be active within their normal domains in the absence of *exd*, although they were unable to specify segmental identity properly (Peifer and Wieschaus, 1990). For example, single *exd* mutants exhibit a transformation of the first abdominal segment to the third, the second abdominal segment to the fourth, and the third abdominal segment to the fifth. An *abd-A*

mutant exhibits a transformation of these same segments to an A1 identity. The double *exd;abd-A* mutant transforms all of these segments to an A3 identity. These results further indicate that *exd* functions in a parallel pathway to the *HOM-C* genes and is required for the normal specification of segment identity by the *HOM-C* genes examined.

Consistent with the expectation of some cross-regulation between the HOM-C proteins and their cofactors, late stage *exd* expression is modulated by HOM-C proteins (Rauskolb *et al*., 1993). Embryos deficient for the *BX-C* genes have increased posterior expression of *exd* late in embryogenesis, indicating the BX-C proteins downregulate *exd* expression in the posterior abdomen. Further, Ubx, Abd-A, and Abd-B modulate the subcellular localization of Exd protein, albeit to different extents (Azpiazu and Morata, 1998).

Genetic and biochemical studies support a cooperative transcriptional activation by a HOM-C/Exd heterodimer. The *HOM-C* genes *Ubx*, *abd-A*, and *Antp* are required for normal midgut development and directly modulate the expression of genes within this developing tissue, including *wingless* (*wg*), *teashirt* (*tsh*), and *decapentaplegic* (*dpp*) (Immerglück *et al*., 1990; Reuter *et al*., 1990). The regulation of *wg* expression within the visceral mesoderm of parasegment 8 is controlled by Abd-A, and Antp positively regulates *tsh* within the visceral mesoderm of parasegments 5 and 6. Expression of *wg* and *tsh* is lost in these regions when embryos lack Exd, although there is no alteration of the expression of either Abd-A or Antp (Rauskolb and Wieschaus, 1994). The requirement of Exd for proper HOM-C protein function is illustrated again with Ubx. The *dpp* gene, a member of the transforming growth factor (TGF)-β-encoding gene super family, is a direct target of Ubx *in vivo* and is also required for midgut morphogenesis (Capovilla *et al*., 1994; Chan *et al*., 1994). Ubx directly activates the transcription of a *dpp* minimal enhancer construct in parasegment 7 within the visceral mesoderm, and this region-specific activation requires Exd (Rauskolb and Wieschaus, 1994; Sun *et al*., 1995). Even the ectopic expression of Ubx in embryos lacking Exd fails to activate the *dpp* enhancer element.

Exd is also required for epidermal function of the *HOM-C* gene products. Removing both maternal and zygotic Exd appears to abolish Dfd function, as the mandibular and maxillary derived larval structures are eliminated (Peifer and Wieschaus, 1990). Further, some Dfd response elements require Exd to emulate normal expression *in vivo* (Pinsonneault *et al*., 1997). Unlike Antp, Abd-A, and Ubx, however, Dfd expression is severely reduced when both maternal and zygotic Exd are removed. Although initially normal, Dfd protein accumulation dramatically decreases during the germ band stage, the time at which Dfd begins to maintain its own transcription through autoactivation. Therefore, it appears that Exd is necessary for Dfd autoactivation. Similarly, Lab requires Exd for maintenance of its own expression *via* an autoactivation circuit (Chan *et al*., 1996), and Ubx requires Exd for transcription maintenance in the haltere imaginal discs (Azpiazu and Morata, 1998). It is suggested that Exd may not only cooperatively activate transcription by the HOM-C proteins, but may also alleviate the repressive action of some bound HOM-C factors (Pinsonneault *et al*., 1997; Li *et al*., 1999b) or even promote the repressive properties of HOM-C proteins (Ryoo and Mann, 1999; Merabet *et al*., 2003).

The evidence supporting Exd/HOM-C interactions is not only based on results from genetic studies. *In vitro* binding studies demonstrate that the Exd and HOM-C proteins interact directly and can bind DNA cooperatively with greater affinity than the HOM-C proteins alone (van Dijk and Murre, 1994; Chan and Mann, 1996; Pinsonneault *et al*., 1997; Ryoo and Mann, 1999). Exd can also selectively enhance the specific affinity of one HOM-C protein relative to others for binding to a particular target region. Scr and Exd bind a response element derived from the Scr target gene *fork head* (*fkh*) cooperatively and specifically (Ryoo and Mann, 1999). The Exd/Scr heterodimer binds effectively to this element *in vitro*, and both proteins are required for the activatation of a reporter construct carrying this element *in vivo*. Other Exd/HOM-C heterodimers fail to activate this element *in vivo* and fail to bind to it with high affinity *in vitro*, while Exd is unable to bind the element alone. Two half-sites are required for the Exd/HOM-C heterodimer to bind DNA, one recognized by the HOM-C protein homeodomain, and one recognized by the Exd homeodomain (van Dijk and Murre, 1994). Although these sites vary slightly for different heterodimers, a consensus sequence of 5′-ATNNATCA-3′ has been described (Mann and Chan, 1996).

The ability of the Exd mammalian homologue Pbx to bind cooperatively with mammalian HOX proteins and some of their *Drosophila* homologs *in vitro* and *in vivo*, illustrates the level of conservation of the Exd/PXB family (Chan *et al*., 1994; van Dijk and Murre, 1994; Chang *et al*., 1995; Phelan *et al*., 1995; Pöpperl *et al*., 1995; Rauskolb and Wieschaus, 1994; Sun *et al*., 1995; Chang *et al*.,

1997; Azpiazu and Morata, 1998). This strong conservation across phyla, combined with the similar interactions between the yeast factors MATa1 and MATα2, suggests that the Exd/HOM-C(HOX) interaction could be quite ancient, dating back to the earliest origins of multicellularity.

1.8.5.3. The DNA Bound Exd/Ubx Crystal Structure

Chan and Mann (1996) used a series of point mutations to characterize the Exd/Lab/DNA interaction, and proposed a structural model for HOM-C/Exd interactions, much of which was later confirmed by structural resolution. X-ray crystallography was used to resolve the crystal structure of a DNA-bound Ubx/Exd heterodimer (Passner *et al*., 1999).

The Ubx and Exd homeodomains bind opposite faces of the DNA molecule, aligned in a head to tail orientation in close proximity (**Figure 11a**). As indicated by *in vitro* and *in vivo* data, the HOM-C hexapeptide or YPWM motif and the Exd homeodomain are the sites of protein–protein interaction. Able to form a reverse turn, the YPWM motif extends, via a flexible linker, to contact Exd within a hydrophobic pocket (**Figure 11b**). This pocket is formed, in part, by the additional three residues between alpha helices 1 and 2 of the Exd homeodomain. The tryptophan (W) residue is significant to Exd/Ubx binding, mediating hydrophobic contacts within the Exd hydrophobic pocket. The contacts made by the tyrosine (Y), proline (P), and methionine (M) residues are less extensive and serve to stabilize the binding of the tryptophan (W) residue. The proximity of the two proteins results in highly overlapping binding sites, with more than half of the Exd contacts to DNA occurring within the Ubx recognition site. The binding of the Exd/Ubx dimer to DNA induces very little conformational change in the proteins, however the minor groove of the bound DNA region broadens significantly, when compared to the crystal structure of Antp bound to DNA complex (Fraenkel and Pabo, 1998). This broadening may represent a "preconfiguring" of the DNA by Exd to allow Ubx recruitment to its target site. A similar mechanism of cooperative binding has been suggested for Oct1 (Clemm and Pabo, 1996).

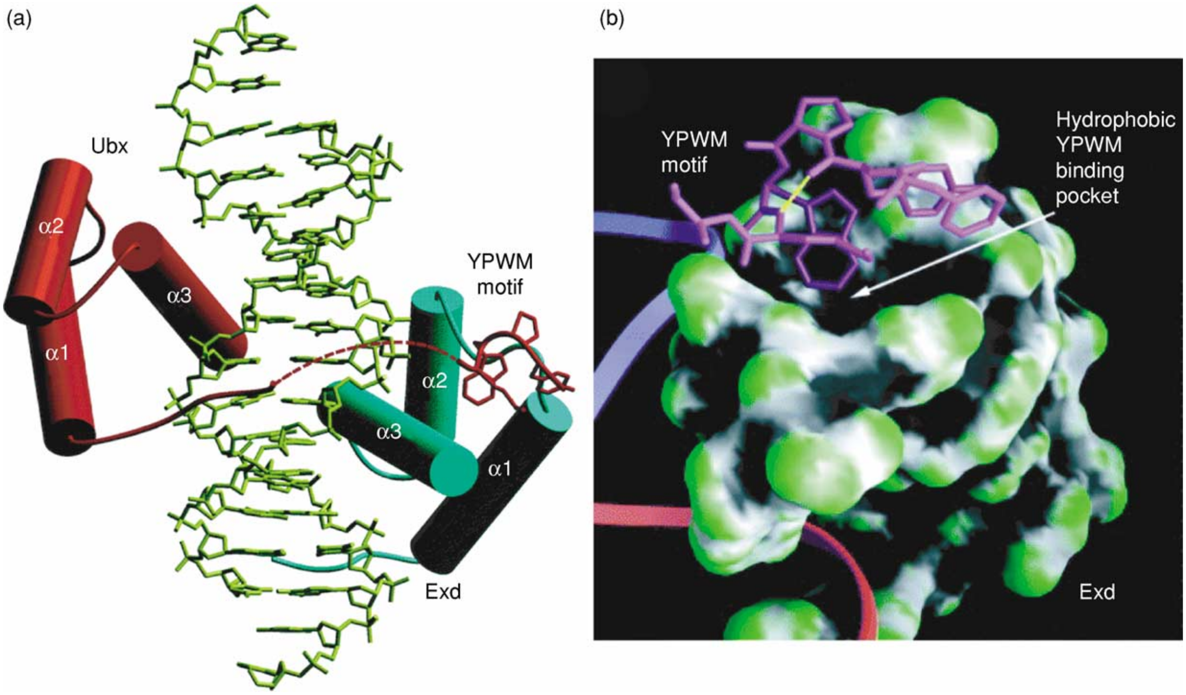


Figure 11 The Ubx/Exd/DNA ternary complex. (a) The Ubx/Exd complex binding DNA and (b) Ubx/Exd protein–protein interaction. (a) The diagram shows the Ubx (red) and Exd (green) homeodomains binding cooperatively on opposite faces of the DNA (green). The flexible linker between the Ubx homeodomain and its YPWM motif is represented by a dashed red line. (b) The YPWM motif (magenta) of Ubx forms a reverse curve to reach into a hydrophobic pocket on the Exd homeodomain. The yellow line indicates a hydrogen bond between the first and fourth residues of YPWM. The colored surface of Exd illustrates its curvature: convex patches are shaded green and concave patches are shaded gray. The DNA backbone (purple and red) is shown as a reference. (Adapted with permission from Nature Publishing Group from Passner, J.M., Ryoo, H.D., Shen, L., Mann, R.S., Aggarwal, A. **1999**. Structure of a DNA-bound Ultrabithorax–Extradenticle homeodomain complex. *Nature 397*, 714–719; http://www.nature.com/nature/)

1.8.5.4. The YPWM (Hexapeptide) Motif and Exd/HOM-C Heterodimer Formation

Variously called the hexapeptide motif, the pentapeptide motif, the tetrapeptide motif, and the YPWM motif, this short, highly conserved region N-terminal to the HOM-C homeodomain is integral to the Exd/HOM-C interaction (Chan and Mann, 1996) (**Figure 9**). Yeast two-hybrid, mutational, and structural analyses reveal that the region of interaction between Pbx/Exd and its HOX/HOM-C partners is the conserved YPWM motif (Chang *et al*., 1995; Johnson *et al*., 1995; Phelan *et al*., 1995). NMR studies involving the highly conserved vertebrate Pbx homeodomain, describe a "lock and key" interaction, with the YPWM motif of the HOX protein (key) inserting into the hydrophobic pocket (lock) of the Pbx homeodomain (Sprules *et al*., 2003).

Despite the apparent genetic and structural importance of the YPWM motif for Exd/HOM-C interaction and DNA binding, however, there are also examples of its dispensability. Abd-B, which lacks this conserved motif, fails to cooperatively interact *in vitro* with Exd (van Dijk and Murre, 1994), although a murine Abd-B homolog does appear to be able to interact with Pbx, despite the lack of the YPWM sequence (Izpisua-Belmonte *et al*., 1991). Recent work with Abd-A demonstrates that the YPWM motif is neither required for Exd recruitment, nor for DNA binding and target selection (Merabet *et al*., 2003). Elimination of the YPWM motif, or a second, short conserved linker-region sequence, does not affect Abd-A's epidermal patterning functions, its binding site selection on a *dpp* response element, or its ability to interact with Exd and Hth *in vitro*. The sequence was, however, proven to be required for correct regulation of the Abd-A target genes *wg* and *dpp* within the visceral mesoderm, where the YPWM motif confers both target activating and target repressing properties to Abd-A, in a gene-specific manner. The *wg* and *dpp* genes are differentially affected by the loss of the YPWM motif, demonstrating the importance of this motif in the control of Abd-A *trans*-regulatory functions.

1.8.5.5. Homothorax Forms a Trimer with Exd and HOM-C Proteins

In order for Exd to function as a coregulator of the HOM-C proteins, it must move from the cytoplasm into the nucleus. Interestingly, Exd has no nuclear localization signal (NLS), but relies on yet another homeodomain-containing protein, Homothorax (Hth), to translocate to the nucleus (Rieckhof *et al*., 1997; Ryoo *et al*., 1999). This relationship is also conserved among the vertebrate Pbx proteins, which require interaction with Hth homologs, the MEIS (named after the founding member, myeloid ecotropic viral integration site 1 or MEIS1; Moscow *et al*., 1995) proteins, for nuclear localization (Knoepfler *et al*., 1997). The absence of *hth* in embryos results in posterior-directed transformations, just as observed with embryos lacking *exd*. Hth has a conserved N-terminal domain, the HM (*hth/meis*) motif, which binds Exd at the PBC-A domain, allowing the formation of a trimeric Hth/Exd/HOM-C (Ryoo *et al*., 1999). This binding structure leaves the Hth homeodomain available to interact with DNA, perhaps further increasing the specificity of the Exd/HOM-C interaction. A trimeric Hth/Exd/Lab complex was shown to bind DNA at an endodermal *lab* autoregulatory enhancer (*lab48/95*). Hth is required for the *in vivo* function of a *lac-Z* reporter construct containing this enhancer, while ectopic expression of a form of Hth lacking the homeodomain acts in a dominant negative fashion, nearly abolishing reporter activity (Ryoo *et al*., 1999). DNA footprinting analysis of an Hth/Exd/Lab/DNA complex indicates that Hth binds approximately 12 bases away from the overlapping Exd/Lab binding site, and that high-affinity Hth binding requires the Exd/Lab binding as well. Lab and Hth do not bind DNA cooperatively in the absence of Exd.

1.8.5.6. Extradenticle Is Insufficient to Fully Explain HOM-C Function

An interesting series of studies illustrates the difficulties in interpreting and correlating *in vitro* binding results with *in vivo* function, particularly when examining minimal response elements. The *HOXB-1* gene is a vertebrate homolog of the *Drosophila lab* gene. A well-characterized 20 bp *HOXB-1* element (derived from the 5′ *HOXB-1* promoter) is bound cooperatively and specifically by Exd and Lab and is able to recapitulate endogenous *lab* expression patterns in *Drosophila* embryos (Pöpperl *et al*., 1995; Chan *et al*., 1996). The *in vivo* function of this reporter requires both Exd and Lab. The consensus Exd/HOM-C recognition site has been described as 5′-ATNNATCA-3′ (Mann and Chan, 1996). A change in the two central base pairs (the NN "specificity residues") of the consensus Exd/HOM-C binding site, from GG to TA, alters the *in vitro* specificity from Exd/Lab, to Exd/Dfd and results in strong *in vivo* reporter expression in a Dfd-like pattern (Chan *et al*., 1997). At first glance, it appears that a simple change in a few base pairs is sufficient to direct *in vivo* HOM-C specificity, but

further studies have shown, that, although Exd/Ubx also binds the altered 20 bp *HOXB-1* response element *in vitro*, it is unable to activate this element *in vivo* (Chan and Mann, 1996; Chan *et al.*, 1997). Additional, difficult to interpret results involve a 26 bp element from the regulatory region of the *Drosophila* gene *Distal-less* (*Dll*), an element negatively regulated by Ubx and Abd-A, and bound by both HOM-C proteins at the same site (White *et al.*, 2000). This element was altered to resemble the known Exd/Dfd consensus sequence, and placed into a reporter construct. Interestingly, while the altered *Dll* element continued to be negatively regulated by Ubx and Abd-A, it was strongly activated by Scr and only weakly activated by Dfd. Further, Li *et al.* (1999a) demonstrated that an Exd/Lab responsive element could be altered to a Dfd responsive element by the addition of a naturally derived 21 bp region that included neither an Exd/HOM-C binding site nor a Dfd binding site. Results such as these serve to reinforce the complexity of the Exd/HOM-C protein mechanism of target recognition. Obviously, a simple consensus sequence with two variable nucleotides cannot fully explain the complex action of these genes.

Although Exd certainly functions as a coregulator with HOM-C proteins, it is clear that Exd alone does not define HOM-C target specificity. Not all Dfd response elements require Exd for *in vivo* function, suggesting there are multiple regulatory mechanisms at work for a single HOM-C protein (Pederson *et al.*, 2000). *In vitro* binding studies demonstrate the ability for multiple Exd/HOM-C heterodimers to bind similar sequences (i.e., with Ubx and Abd-A), suggesting that Exd alone cannot explain the functional specificity of the HOM-C proteins (van Dijk and Murre, 1994). Abd-B appears necessary to repress *exd* activation, rather than utilize it as a cofactor, and Pb represses *exd* expression during the development of the adult proboscis (Abzhanov *et al.*, 2001). Further, in the case of Scr, which is expressed in both the head (labial segment) and the trunk (first thoracic segment), coregulation with Exd is insufficient to explain this HOM-C protein's ability to differentially pattern segments from two different tagmata, suggesting that other cooperative factors must be at work. In fact, cooperative interactions between HOM-C proteins and a cofactor may not be limited to Exd. Several other DNA binding proteins appear to coregulate HOM-C function, but in a more region-specific manner than the coregulation observed with Exd. Direct interaction between these factors and a HOM-C protein, however, has yet to be demonstrated.

1.8.5.7. Zinc Finger HOM-C Protein Cofactors

Several other *Drosophila* proteins are proposed to function as HOM-C cofactors, including the C2H2 zinc finger proteins encoded by *tsh*, *disco*, and the partially redundant *disco-r* genes, and perhaps the *buttonhead* (*btd*) gene. Of these, *tsh*, located on the left arm of chromosome 2, is well characterized in *Drosophila*, while some information is also available from other insects. The *tsh* gene was initially identified in an enhancer trap screen for embryonic segmentation genes (Fasano *et al.*, 1991). Embryos homozygous for null alleles of *tsh* die before hatching, and have a homeotic alteration of the ventral trunk segments to a gnathal- or head-like structure. The denticle belts are reduced, laterally narrowed, disorganized, and lack segment-specific characteristics. Small patches of sclerotized material, reminiscent of the cephalopharyngeal skeleton, are present in the ventral cuticle, and the anal opening is also sclerotized. The expression of *tsh* overlaps expression of *Scr*, *Antp*, *Ubx*, *abd-A*, and *Abd-B*, but is not required for transcriptional activation of these genes (Röder *et al.*, 1992). Conversely, initiation and early regulation of *tsh* is independent of the HOM-C proteins, although Antp, Ubx, and Abd-A modulate *tsh* expression later, following germ band extension (Mathies *et al.*, 1994; McCormick *et al.*, 1995). The expression of *Scr* is somewhat affected by the loss of *tsh*, exhibiting ectopic activation in the ventral prothoracic segment (Fasano *et al.*, 1991). Röder *et al.* (1992) concluded that *tsh* acts on the same hierarchical level as the *HOM-C* genes, with the two functioning together to promote proper trunk development while suppressing head identity. The *tsh* mutant phenotype and its expression in all of the trunk segments except for the most posterior abdominal, has led to its characterization as a trunk tagma specifier.

Further work by de Zulueta *et al.* (1994), supports a cooperative mechanism between *tsh* and the *HOM-C* genes of the trunk. The role of Tsh during development is highlighted by its connection with the *HOM-C* gene *Scr.* As discussed previously (see Section 1.8.2.2.4), Scr controls identity in the labial gnathal segment and in the adjoining first thoracic trunk segment. Part of this dual role is specified by *tsh*. Scr is unable to produce a first thoracic identity in the absence of *tsh*, as evidenced by the failure of *tsh* null embryos to differentiate normal first thoracic denticle belts. Ectopic activation of *tsh* in the labial segment transforms that segment toward a prothoracic identity with first thoracic-type denticles. Tsh is also required for repression of salivary gland formation in the trunk segments in embryos

ectopically expressing Scr, again demonstrating that Tsh helps to distinguish the gnathal versus the trunk roles of Scr (Andrew *et al.*, 1994).

Embryos null for the partially redundant genes *disco* and *disco-r* exhibit gnathal patterning defects quite similar to those observed in embryos lacking Dfd and Scr (Mahaffey *et al.*, 2001). These genes map to the 14B1 and 14A9 regions, respectively, of the X chromosome and encode paired zinc finger-containing proteins. Although *disco* had been previously characterized based upon neural and circadian rhythm phenotypes (Steller *et al.*, 1987; Dushay *et al.*, 1989; Helig *et al.*, 1991), its embryonic patterning role remained unrecognized until the creation of a deficiency removing both *disco* and *disco-r*; either gene alone is sufficient for normal embryonic gnathal patterning (Mahaffey *et al.*, 2001). The expression of *disco* and *disco-r* overlaps that of *Dfd* and *Scr* in the mandibular, maxillary, and labial lobes, and the former are also expressed in the larval limb primordia of the first through third thoracic segments and the clypeolabrum. In addition, *disco* is expressed in the optic lobe, where it is required for normal migration of Bolwig's nerve, and in the proctodeum, where it overlaps with *Abd-B* expression. Loss of *disco* and *disco-r* has no effect on the expression of *Dfd* or *Scr*, and the converse loss of *Dfd* and *Scr* has no significant effect on the expression of the *disco* or *disco-r* genes (Mahaffey *et al.*, 2001), further demonstrating that *disco/disco-r* null embryos exhibit reduced expression of three known Dfd target genes–*1.28*, *Serrate* (*Ser*), and *Dll*–and supporting the role of these two proteins as HOM-C cofactors.

The *btd* gene also encodes a zinc finger transcription factor required for embryonic patterning (Wimmer *et al.*, 1993). Mutations in *btd* disrupt development of the anterior head segments, including the antennal, intercalary and mandibular segments. The Btd protein interacts with and functions as a cofactor with the HOM-C-like protein, Empty Spiracles (EMS) (Dalton *et al.*, 1989), and overlaps the expression of Lab and perhaps Dfd. *ems* is a *HOM-C* like gene required for pattering the *Drosophila* larval head and brain, and for the development of the filzkörper material in the posterior spiracles (Dalton *et al.*, 1989; Jones and McGinnis, 1993). Although commonly referred to as a head gap gene (Cohen and Jürgens, 1990; Walldorf and Gehring, 1992), *ems* encodes a *HOM-C* type homeodomain and a somewhat diverged upstream YPWM motif (**Figure 9**). It has been suggested that *ems* may represent a "lost" *HOM-C* gene, separated from the *HOM-C* cluster, but retaining *HOM-C* like patterning functions (Macías and Morata, 1996). Interestingly, ectopic *ems* expression causes little disruption to development, only transforming the mandibular segment toward an intercalary identity. Ectopic *btd* expression, on the other hand, is more detrimental, causing segmentation defects. However, ectopic expression of *ems* with *btd* appears to transform the trunk segments toward an intercalary identity, demonstrating that the region of Btd accumulation defines where EMS can function, much as would be expected for a cofactor (Schöck *et al.*, 2000). This property is similar to the observations of HOM-C proteins with Tsh and Disco.

There are few studies with homologs of these zinc finger cofactors in other insects, yet this could be a very interesting and informative venture. Unlike the *HOM-C* genes, there appear to be differences in these putative zinc finger cofactors between *Drosophila* and other insects. Somewhat surprisingly, all of the zinc finger genes mentioned above have been duplicated in the *Drosophila* genome (the *Drosophila* homolog of mammalian SP1 (Kadonaga *et al.*, 1987); *tsh* is related to *tiptop*, and *btd* is related to *D-Sp1*), and *disco*'s duplicate, *disco-r* has already been mentioned above. Only *disco* and *disco-r* have been shown to be functionally redundant for their role with HOM-C proteins. The *D-Sp1* gene can substitute for *btd* in the mandibular segment (Schöck *et al.*, 2000), but little is know about the role of *tiptop*.

With several insect genome projects well under way, it is clear now that these duplications are not found in all insects. In the genomes of *A. gambiae* and *A. mellifera* only homologs of *disco-r* and *tiptop* are found. Further, a search of Genbank reveals several homologs of *tsh* cloned from other insects, and although many of these are only partial sequences, they appear to be more closely related to *tiptop* than to *teashirt*. This generates several questions. Since only *disco* and *disco-r* appear to be redundant, what are the roles of the paralogous genes in *Drosophila*, and further, what roles will be found for the genes in other insects? For example, will *tiptop* from other insects retain the roles of *tsh* and *tiptop* in *Drosophila*? Additionally, it will be important to determine which roles are ancestral and which are derived, whether or not the cofactor role for these genes is particular to *Drosophila* or whether it extends to other insects as well.

Recently, Robertson *et al.* (2004) proposed a model in which the C2H2 zinc finger transcription factors described above play a larger role during *Drosophila* development and HOM-C function. In this model, these transcription factors contribute to the regionalization of the embryo: Disco establishing the gnathal region and Tsh the trunk (**Figure 12**). Btd may have a similar role in the anterior head, as we

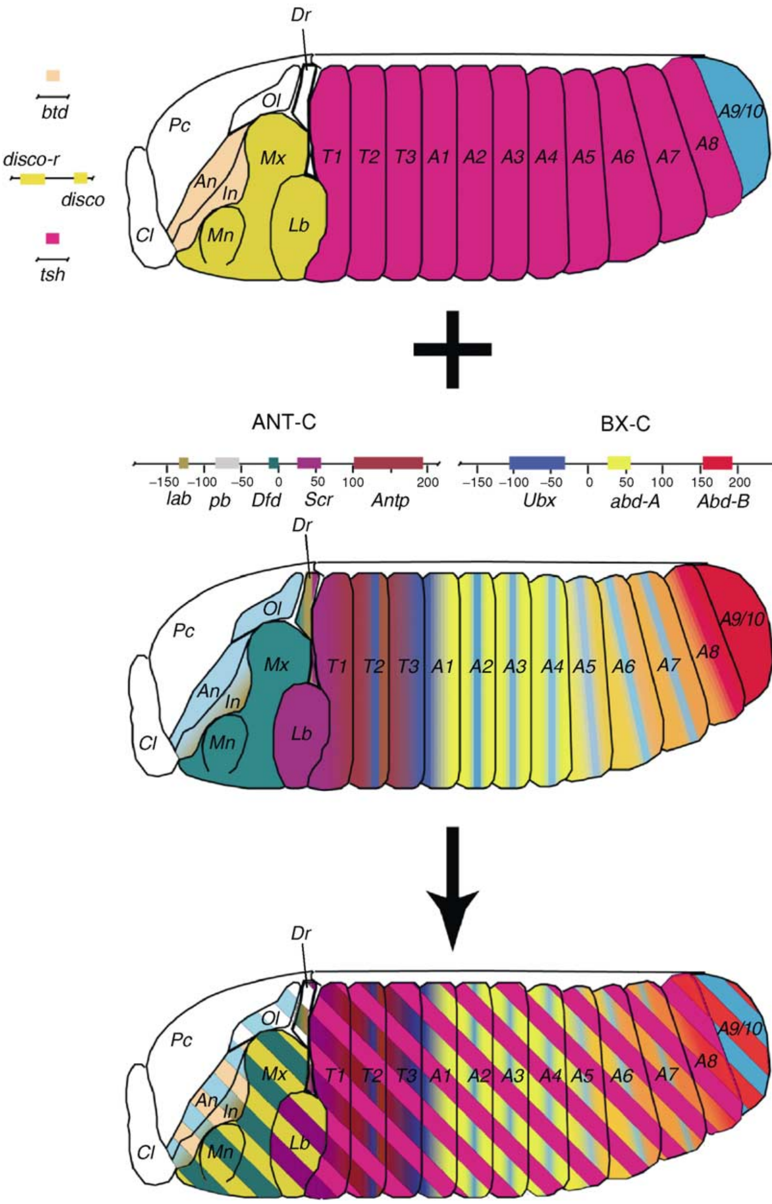


Figure 12 A model for regional specification and establishment of HOM-C protein function. The regionally expressed zinc finger transcription factors (upper image) participate in the regionalization of the *Drosophila* embryo. Some details of the expression patterns and function were intentionally left out for clarity. The anterior head region is postulated to be controlled by *btd*, the gnathal region by *disco* and *disco-r*, and the trunk region by *tsh*. At present, the identity of a similar posterior abdominal factor is not known, although there are several candidates. A gene map and diagram representing the domains of *HOM-C* gene expression and control are shown in the middle. These expression patterns are described in the text. Finally, in the lower panel, the two gene expression patterns are merged and the zinc finger expression patterns are represented by the angular views through the HOM-C expression pattern. Unique combinations of C2H2 zinc finger transcription factors and HOM-C proteins in each segment specify individual segment identities.

have suggested in Figure 12. These authors have shown that this is an interactive network, where the Tsh protein can repress expression of the *disco* genes, restricting *disco* and *disco-r* expression to the gnathal region. In the absence of Tsh, *disco* and *disco-r* are ectopically expressed in the trunk segments, which appears to be responsible for the trunk to gnathal transformation seen in homozygous *tsh* mutant embryos. In addition to this regionalization role, these zinc finger proteins participate with the HOM-C proteins during the establishment of the anterior–posterior axial pattern of

the *Drosophila* embryo. Robertson *et al.* (2004) show that *disco* expression establishes the regions where cells can respond to Dfd and Scr proteins. When embryos lack *disco* and *disco-r*, they do not respond to ectopic activation of these *HOM-C* genes, neither by activating gnathal HOM-C target genes nor by producing ectopic mouthpart structures, as do embryos expressing Disco (either endogenously or ectopically).

The picture now emerging is one in which the C2H2 zinc finger proteins help regionalize the embryo and define domains, in which specific HOM-C proteins can function. This does leave several important questions that need to be resolved. For example, are these zinc finger proteins biochemical cofactors for the HOM-C proteins? In addition, it is not known if the zinc finger proteins have the same HOM-C partners during adult development as they do during embryogenesis, and further, it is not clear whether or not this mechanism is functioning in other insects (see below). Indeed, this may be a crucial question, since there are indications that the relative expression of these zinc finger genes may be conserved in vertebrates, suggesting that function may be conserved as well. In either case, this opens a new avenue of study for the function of insect HOM-C proteins.

Although little is currently known regarding the role of related zinc finger proteins in other insects, expression of a homolog of *tsh/tiptop* (*Tdtsh*) has been examined in *Thermobia*, and some information may be inferred regarding *Tribolium*. Expression analysis for *Tdtsh* reveals a pattern altered from that observed in the fly, with *Tdtsh* being expressed primarily in the thoracic and ventral gnathal segments, with only weak expression visible in the abdominal segments (Peterson *et al.*, 1999). Peterson *et al.* (1999) suggest that *Tdtsh* may specify the thoracic tagmata in *Thermobia*, rather than the larger trunk region as proposed for the *Drosophila* larva. The gnathal expression in *Thermobia* embryos may reflect *tsh*'s role in adult *Drosophila* head patterning, where it is required to suppress the activity of *Antp* (Bhojwani *et al.*, 1997). *Tribolium* embryos lacking the *HOM-C* genes differentiate antennae in each thoracic and abdominal segment (Peterson *et al.*, 1999, Brown *et al.*, 2002b), indicating that *tsh* may not act to specify the trunk tagmata as in *Drosophila*. It is unclear, however, whether the trunk to head transformation observed in *Tribolium* is limited to the appendages, or applies to an entire segment. Thus, *tsh* may retain a role as a HOM-C cofactor in a more basal insect species, but further work is needed to determine if this is the case.

1.8.5.8. Other Potential HOM-C Protein Cofactors

The proteins encoded by *cap 'n'collar* (*cnc*), *lines* (*lin*), and *apontic* (*apt*) have also been suggested as putative HOM-C protein cofactors. The *cnc* gene, located on the right arm of chromosome 3, encodes a member of the bZip protein family and produces three protein isoforms (A, B, and C) ranging in size from 533 to 1296 amino acids (McGinnis *et al.*, 1998). Its activity is required for normal patterning of the embryonic mandibular and labral segments. Loss of *cnc* function causes a transformation of the mandibular lobe towards to a maxillary identity, demonstrated by the formation of ectopic mandibular mouth hooks and cirri (Mohler *et al.*, 1995). The CNC isoform B (CNC-B) localizes to the mandibular and labral segments, overlapping with Dfd expression in the mandibular segment. In contrast to the role of *disco* and *disco-r*, which appear necessary for Dfd activation of target genes, normal CNC-B function diminishes the activity of Dfd response elements by antagonizing the *Dfd* autoregulatory pathway. Mutants of *cnc* have persistent Dfd accumulation in the anterior mandibular lobe (where Dfd protein levels decrease in wild-type embryos during germ band contraction), and the overexpression of *cnc* during embryonic development produces a phenotype very similar to that of null *Dfd* embryos (McGinnis *et al.*, 1998). Taken together, these results support the cospecification of mandibular identity by CNC and Dfd. The gnathal pattern of *cnc* expression is very similar in *Thermobia* and *Oncopeltus*, thus the *cnc* homologs in these insects may be functioning in a similar way to *Drosophila* (Rogers *et al.*, 2002).

The putative HOM-C cofactor encoded by *apontic* (*apt*) was identified in a second chromosome screen for modifiers of Dfd function (Gellon *et al.*, 1997). Embryos mutant for *apt* share some phenotypic characteristics with both *Dfd* and *Scr* mutant embryos, including the extreme reduction of the dorsal pouch and the loss of the dorsal bridge. Other cephalopharyngeal structures lost in *Dfd* and *Scr* mutant embryos are, however, retained in *apt* mutants. Zygotic a*pt* transcripts localize to the dorsal acron, clypeo-labrum, and ventral and anteriolateral gnathal lobes. A segmentally repeated pattern is observed in the trunk epidermis and CNS. Because loss of *apt* has no apparent effect on *Dfd* and *Scr* transcription, and these *HOM-C* genes do not affect the transcription of *apt*, these genes likely function in parallel to pattern portions of the gnathal region. Like *disco* and *disco-r*, *apt* does not

affect the *Dfd* autoregulatory loop, and thus may directly coregulate some Dfd target genes.

Another region of embryonic *apt* expression includes the developing dorsal vessel and heart, and *apt* is required for appropriate heart morphogenesis and function (Su *et al*., 1999) (see **Chapter 2.6**). The homeotic proteins Antp, Ubx, and Abd-A determine the anterior–posterior portioning of the dorsal vessel into the aorta and heart, and Abd-B represses cardiogenesis (Lo *et al*., 2002; Lovato *et al*., 2002). An interesting question is whether APT might also function in concert with one or more of these HOM-C proteins in the development of the larval circulatory system.

In the eighth abdominal segment, identity is primarily controlled by the *HOM-C* gene *Abd-B*, and requires the activity of the Abd-B target genes, *empty spiracles* (*ems*), *spalt major* (*salm*), and *cut* (*ct*). The segment polarity gene *lines* (*lin*) (Volk *et al*., 1994; Bokor and DiNardo, 1996) is also required for the proper patterning of the posteriormost abdomen and may function as a cofactor for Abd-B in this domain. Expression of Abd-B is normal in *lin* mutants (Castelli-Gair, 1998), but the posteriormost segment fails to develop normally and lacks posterior spiracles, mirroring a phenotypic effect of the loss of Abd-B function (Sánchez-Herrero *et al*., 1985). The LIN protein appears to function cooperatively with Abd-B in the transcriptional activation of an *ems* reporter construct and both *salm* and *ct*. Another function of Abd-B is the repression of the more anterior *BX-C* genes, *Ubx* and *abd-A* (Struhl and White, 1985; Macías *et al*., 1990). Gene *lin* mutants have no effect upon the expression of these *HOM-C* genes, indicating Abd-B does not require LIN for this repressive function. Current information indicates that LIN only coregulates Abd-B targets, as there is no evidence of alteration in Abd-A, Ubx, or Dfd downstream target expression. As Abd-B appears not to require Exd as a cofactor, *lin* is currently the only gene known to produce a putative Abd-B transcriptional coregulator.

1.8.5.9. HOM-C Protein Function in the Absence of Cofactors: Cooperative Binding

There is much evidence to support that HOM-C proteins function while interacting with cofactors, but is there evidence that target gene regulation can occur without cofactors? Some *Drosophila* promoter and enhancer regions contain clustered recognition sites for a single transcription factor (for examples, see Laughon *et al*., 1988; Yang *et al*., 2001; Markstein *et al*., 2002), and *in vitro* studies demonstrate that homeodomain proteins can bind cooperatively to clustered sites (Desplan *et al*., 1988). Such clustering may provide the means for spatially and temporally specific gene activation based on the concentration of a given factor(s). A mechanism such as this may provide some explanation for the biological specificity directed by the *HOM-C* genes, in spite of their overlapping DNA binding specificities. Indeed, this type of transcriptional regulation was shown to be true for other early-acting patterning genes (reviews: Pankratz and Jaeckle, 1990; Small and Levine, 1991; St Johnston and Nüsslein-Volhard, 1991). Numerous studies demonstrate HOM-C protein binding to multiple, clustered recognition sites. Multiple binding sites were required for a measurable response in a yeast two-hybrid screen for transcriptional activation, even when using high-affinity HOM-C protein recognition sites (Ekker *et al*., 1992). Known Ubx target sequences contain multiple 5′-TAAT-3′ repeats, and the Ubx protein can protect regions of DNA up to 87 kb (Beachy *et al*., 1988), although the minimum consensus sequence for Ubx binding is only 9 bp (Ekker *et al*., 1992). Further, Ubx binding is increasingly stabilized by cooperative interactions resulting from clustered binding (Beachy *et al*., 1993). Interacting sites are not, however, always closely clustered. They may lie as much as 200 bp apart, with their interaction involving a DNA looping mechanism. An example of cooperative binding, important to development, occurs during haltere/wing development. Ubx represses *spalt major* (*salm*), a gene involved in wing patterning, to promote haltere formation, and it does so in the absence of either Exd or Hth through a number of Ubx monomer binding sites (Galant *et al*., 2002). Taken together, these studies provide evidence for HOM-C target regulation in the absence of known cofactors. Concentration-dependent HOM-C target activation may provide an additional mechanism to guide developmental specificity, whether acting alone or in concert with currently unrecognized factors.

For the HOM-C proteins to direct the specification of segment identities, it is apparent that additional factors or mechanisms are required beyond the simple binding of a HOM-C protein to a recognition site. How might cofactors contribute to target gene selection? We mentioned above two models proposed by Biggin and McGinnis (1997) for the role of cofactors during HOM-C protein target regulation. Although there is evidence for both models, the widespread binding model appears to have the most support. But it should be pointed out that this is not likely to be a simple process involving one, two, or even three factors. More likely, the process of target gene selection involves multiple factors,

with perhaps some, like the gap gene products, being expressed prior to the HOM-C proteins. Furthermore, when thinking of cofactor function, one might expect direct interaction with the HOM-C proteins, but this need not be the case. Some proteins functioning as cofactors may not even be DNA binding proteins, but may serve as "linkers" required to stabilize interactions between the basal transcription machinery and the HOM-C proteins. Clearly, this is an area where much more investigation is needed, and because there are differences between different insect species, perhaps a comparative study can yield some of the sought-after answers.

1.8.6. Targets of the HOM-C Proteins and Functional Specificity

Adam Wilkins (1986), challenges:

> Isn't it a rather large leap from transcription-regulation, the assumed forte of homeo box-containing genes, to explaining the large-scale interaction and movements of cells that comprise morphogenesis and pattern formation? ... the rush to pin so much on the homeo box ... reveals the eagerness, even the desperation of the molecular-developmental biologists to find universal or, at least, general principles of genetic control in development.

The *HOM-C* genes are of great interest because they drive developmental specificity, both throughout an individual organism and across phyla. Those studying animal development and evolution desire to understand the mechanisms allowing this specification to take place. Graba *et al.* (1997) state:

> The goal is to identify functions that are required to translate the positional information delivered by HOX proteins into diversified morphogenetic programmes.

Although characterization of HOM-C binding and the interactions of possible cofactors certainly contribute to understanding HOM-C function, the fact that they are so conserved and yet specify pattern in such different animal body plans is perplexing. Deciphering how this is accomplished in divergent phyla is the ultimate task. An understanding of downstream targets and how they are integrated to yield different body plans is crucial to the understanding the plasticity of development.

García-Bellido has described a model by which homeotic genes might function. As "selector" genes, HOM-C products would control developmental pathways by regulating the action of "realizator" genes. The "realizator" gene products would directly define and control "morphogenic cell properties" (such as mitosis, cell recognition, cell adhesion properties, and cell death), to produce specific cell types and morphologies (García-Bellido, 1977). In such a way, these target, "realizator" genes could allow individual, regionally expressed *HOM-C* genes to direct developmentally specific characteristics through a sequence of steps similar to those of a metabolic pathway.

Despite over 20 years of intensive research into the nature and function of the *HOM-C* genes, identification of target genes has proven illusive. In a 1997 review, Graba *et al.* (1997) identify only 19 known HOM-C protein targets. Since that time, at least 13 additional HOM-C target genes have been identified, and a compilation of known HOM-C target genes appears in **Table 2**. On the basis of functional characterization, these genes fall into four categories. The first one corresponds to García-Bellido's "realizator" genes, being directly involved in modulating cellular processes. A second category contains genes whose products are involved in signal transduction. There are a few target genes whose functions are unknown, and finally, the majority of known HOM-C target genes are, themselves, transcription factors. Based on the number of developmental processes likely to be modulated by the *HOM-C* genes, and experimental results from molecular studies, Akam (1998) proposes that these "versatile generalists" might direct as many as 1000 downstream targets, each.

1.8.6.1. The Search for Target Genes

There are characteristics inherent to the *HOM-C* genes that make the identification and characterization of target genes difficult. As discussed previously, the HOM-C proteins have similar *in vitro* binding properties, recognizing a consensus core sequence of 5′-TAAT-3′. This can prove problematic when attempting *in vitro* screens for target promoter sequences, as promiscuous binding to sites lacking *in vivo* significance can occur. Several of the *HOM-C* genes have overlapping expression domains, and target gene regulation may require input from multiple *HOM-C* genes. In such a case, the removal of one HOM-C protein may not significantly effect target gene action. A single target gene also may be regulated in adjacent regions by different HOM-C proteins, as is illustrated by the activation of *tsh* activity in the anterior midgut by Antp and in the central midgut by Ubx and Abd-A (Mathies *et al.*, 1994). Cross-regulation between the *HOM-C* genes also muddies the waters of target gene identification. It can be unclear whether an observed effect results directly from the loss of one HOM-C protein, or indirectly because of the ability of the *HOM-C* genes to cross-regulate one another. The

control regions of potential target genes can be quite large, often up to 100 kb, making systematic evaluation of potential regulatory sites challenging, and also making it difficult to ascertain if an observed HOM-C protein's effect is direct or indirect. HOM-C protein action can be non cell autonomous, acting through signaling cascades and leading to effects outside of the HOM-C protein's expression domain, and in adjacent germ layers. Finally, some *HOM-C* genes have received more attention, and thus may appear more amenable to the search for target genes. Genes under the control of Antp, Ubx and/or Abd-A make up the majority of the characterized target genes, while the more anterior HOM-C proteins (LB, Pb, Dfd, Scr*)* have far fewer or no characterized targets outside of the *HOM-C* genes themselves.

Many of the identified HOM-C target genes were previously characterized genes whose expression or function gave cause to suspect HOM-C protein control. There have been, however, a number of *in vitro* and *in vivo* screening techniques that have been employed to identify other HOM-C protein targets, with varying degrees of success (for examples, see Gould *et al*., 1990; Gould and White, 1992; Graba *et al*., 1992; Mahaffey *et al*., 1993; Feinstein *et al*., 1995; Mastick *et al*., 1995; Merabet *et al*., 2002; Marchetti *et al*., 2003). Common *in vitro* screening techniques include protein–DNA interaction screens in yeast and immunoprecipitation of bound DNA fragments with HOM-C protein antibodies. *In vivo* techniques include the use of enhancer trapping and deficiency or mutagenesis screens. Other screening techniques combine elements of both, such as subtractive hybridization and chromatin immunoprecipitation. Each approach has both advantages and drawbacks. *In vivo* studies can be quite time consuming, and sometimes produce target genes that are difficult to characterize further as to their function and the directness of HOM-C protein regulation.

Yeast reporter assays can be useful in screening a large number of genomic fragments quickly and efficiently, but nonphysiological conditions, alterations in chromatin structure, and the lack of other interacting proteins can lead to imprecise results. In one such study, Mastick *et al.* (1995) sought to identify genes directly regulated by the Ubx protein. Fifteen per cent of the genome was searched, and based on fortuitous sequence alone, at least 500 interacting sites were expected. In fact, only 53 genomic fragments were recovered, and of those, only three appeared to be associated with Ubx target regulation.

Chromatin immunoprecipitation, when utilizing DNA–protein complexes cross-linked *in vivo* at physiological conditions, can be useful in identifying novel targets of direct HOM-C regulation, but requires an antibody against the HOM-C protein of interest, and results can be affected by cloning efficiencies. Also, if true, the widespread binding model would predict the recovery of a large number of DNA sites to which the HOM-C proteins bind, but many will have no endogenous regulatory effect.

Subtractive hybridization allows the sorting of transcripts that differ between normal individuals and individuals with altered HOM-C function, although hybridization conditions and cDNA synthesis can strongly influence results. Further, if target gene activation requires several HOM-C proteins, it may not be identified by such an approach.

Marchetti *et al.* took a cytological approach to identifying targets of the *BX-C* genes through immunostaining of polytene chromosomes taken from larval fat bodies (Marchetti *et al*., 2003). Although not as endoreplicated as the salivary gland polytene chromosomes, adequate BX-C protein binding was detected to allow the mapping of more than 300 binding sites. Each site bound one or more of the BX-C proteins, and several of these sites corresponded to known BX-C target genes. Differential patterns of BX-C binding were observed when polytene chromosomes from the fat bodies and salivary glands were compared, suggesting tissue-specific activity. As the *BX-C* genes are normally expressed in the fat bodies, this approach may be more likely to identify genuine binding targets, when compared to studies utilizing Ubx overexpression in the salivary glands (Botas and Auwers, 1996).

One remaining strategy that has not yet provided additional HOM-C targets is that of whole genome microarray analysis. This technique has been used to identify candidate genes involved in dorsal–ventral patterning, circadian rhythms, and immune response (Irving *et al*., 2001; McDonald and Rosbash, 2001; Stathopoulos *et al*., 2001), and may hold the same promise for further HOM-C target discovery.

1.8.6.2. Possible Modes of HOM-C Target Activation

The HOM-C proteins could regulate, directly, a vast number of individual target genes in combinations required for regional specification. Alternately, the HOM-C proteins could function primarily through the regulation of a few downstream transcription factors to activate regional, developmentally specific genetic cascades. In most cases, the line of control between the *HOM-C* genes and downstream targets is unclear. Of the target genes summarized in **Table 2**, only eight have been demonstrated to be directly regulated by a HOM-C protein. Of those

Table 2 *Drosophila* HOM-C target genes

Target gene	*HOM-C regulator*	*Molecular type*[b]	*Biological function*[b]	*Identification method*[c]	*References*
1.28 (1.28)[a]	Dfd	Unknown	Unknown	ET	Mahaffey *et al.* (1993)
apterous(ap)[a]	Antp	Lim-homeodomain transcription factor	Wing patterning, neuronal fasciculation, myogenesis	PC	Capovilla *et al.* (2001)
belt (belt)	Antp, Ubx	Unknown	Unknown function	SH	Feinstein *et al.* (1995)
β3-tubulin (βtub60D)[a]	Ubx	Cytoskeletal protein	Visceral mesoderm differentiation	PC	Hinz *et al.* (1992)
blistered (bs)	Ubx	MADS-box transcription factor	Wing vein patterning, tracheal patterning	PC	Weatherbee *et al.* (1998)
canoe (cno)	Ubx, Abd-A	GLGF/DHR motif	Actin binding protein dorsal closure	SH	Feinstein *et al.* (1995)
centrosomin (cnn)	Antp, Ubx	Microtubule binding, myosin AtPase	Midgut morphogenesis, CNS development	CIP	Megraw *et al.* (1999)
Connectin (Con)[a]	Ubx	Cell adhesion	Neuronal fasciculation, synaptic target attraction	CIP	Gould and White (1992)
dachshund (dac)	Pb, Abd-B	Novel transcription factor	Eye disc development	PC	Estrada and Sánchez-Herrero (2001), Abzhanov *et al.* (2001)
dlarp	Scr, Ubx	Novel protein of unknown function	Unknown function	CIP	Chauvet *et al.* (2000)
decapentaplegic (dpp)[a]	Antp, Ubx	TGF-β receptor ligand	Embryonic, imaginal axial patterning, midgut morphogenesis	PC	Capovilla *et al.* (1994), Sun *et al.* (1995)
Distal-less (Dll)[a]	Pb, Dfd, Scr, Ubx, Abd-A, Abd-B	Homeodomain transcription factor	Appendage development, sensory structures	PC	Estrada and Sánchez-Herrero (2001), Abzhanov *et al.* (2001), Gebelein *et al.* (2002), O'Hara *et al.* (1993), Castelli-Gair and Akam (1995), Vachon *et al.* (1992)
Dwnt-4 (Wnt4)[a]	Antp, Ubx, Abd-A	Signaling molecule	Epidermal, visceral mesoderm development	CIP	Graba *et al.* (1995)
empty spiracles (ems)	Abd-B	Homeodomain transcription factor	Head, brain, and tracheal development	PC	Jones and McGinnis (1993)
extradenticle (exd)	Pb, Ubx, Abd-A, Abd-B	Homeodomain transcription factor	Embryonic and imaginal patterning	PC	Abzhanov *et al.* (2001), Azpiazu and Morata (1998)

fork head (fkh)	Scr	''Forkhead'' domain transcription factor	Salivary gland development	PC	Ryoo and Mann (1999), Panzer *et al.* (1992)
homothorax (hth)	Scr, Antp, Abd-A, Abd-B	Homeodomain-HM transcription factor	Embryonic and imaginal patterning	PC	Estrada and Sánchez-Herrero (2001), Yao *et al.* (1999)
modulo (mod)	Scr, Ubx	DNA/RNA binding	Protein position effect variegation	PC	Graba *et al.* (1994), Alexandre *et al.* (1996)
nervy (nvy)	Antp, Ubx, Abd-A	Zinc finger transcription factor	Unknown function	SH	Feinstein *et al.* (1995)
nessy (nes)	Antp, Ubx, Abd-A	Transmembrane protein	Unknown function	CIP	Maurel-Zaffran *et al.* (1999)
odd-paired (odd)	Antp, Ubx, Abd-A	C2H2 zinc finger transcription factor	Midgut morphogenesis	PC	Cimbora and Sakonju (1995)
paired (prd)	Dfd	Homeodomain transcription factor	Sense organ development, segmentation	PC	Li *et al.* (1999a)
pointed (pnt)	Abd-A	Type II TGF-β receptor protein	Epidermal, CNS, and tracheal patterning	ET	Pribyl *et al.* (1988)
reaper (rpr)[a]	Dfd, Abd-B	Apoptosis activating factor	Programmed cell death	PC	Lohmann *et al.* (2002)
retrotransposon 412	Abd-A	Transposable element	Gonadal mesoderm	CIP	Brookman *et al.* (1992)
scabrous (sca)[a]	Ubx, Abd-A, Abd-B	Fibrinogen family signaling protein	Eye morphogenesis, neurogenesis	CIP	Graba *et al.* (1992)
Serrate (Ser)	Dfd, Ubx, Abd-A	Signaling protein	Embryonic pattering	PC	Wiellette and McGinnis (1999)
spalt (salm)	Pb, Scr, Antp	C2H2 zinc finger transcription factor	Trachea, appendage, oenocyte patterning	ET	Wagner-Bernholz *et al.* (1991), Galant *et al.* (2002), Abzhanov *et al.* (2001)
spalt related (salr)	Ubx	C2H2 zinc finger transcription factor	Haltere patterning	PC	Weatherbee *et al.* (1998)
teashirt (tsh)	Antp, Ubx, Abd-A	C2H2 zinc finger transcription factor	Midgut morphogenesis	ET	McCormick *et al.* (1995), Mathies *et al.* (1994)
T48	Ubx, Abd-A	Transmembrane protein	Unknown	CIP	Strutt and White (1994)
unplugged (unpg)	Ubx, Abd-A	Homeodomain transcription factor	CNS and tracheal development	ET	Chiang *et al.* (1995)
wingless (wg)	Ubx, Abd-A	Signaling molecule	Embryonic and imaginal patterning	PC	Weatherbee *et al.* (1998)
vestigial (vg)	Ubx	Novel transcription factor	Wing/haltere patterning	PC	Weatherbee *et al.* (1998)

[a]Evidence supports a direct regulation by the HOM-C protein(s).

[b]Functions as indicated in the reference and/or FlyBase (FlyBase Consortium (2003)).

[c]ET, enhancer trap; SH subtractive hybridization (Hedrick *et al.*, 1984; Davis, 1986); CIP, chromatin immunoprecipitation (Gould *et al.*, 1990; Graba *et al.*, 1992); YRA, yeast reporter assay (Mastick *et al.*, 1995); PC, previously characterized gene.

with characterized direct interactions, most are either transcription factors or signaling molecules, and not the "realizator" genes envisioned by García-Bellido (1977). Based on the known functions of target genes currently characterized, it appears that some combination of these two modes of action is likely at work.

1.8.6.3. The Concentration of a HOM-C Protein Can Direct Target Activation

The concentration of a HOM-C protein can influence its downstream effects, as illustrated by phenotypic differences between null and hypomorphic alleles. For example, null *pb* mutants have a strong transformation of mouthparts to leg, while a hypomorphic *pb* allele results in the transformation of mouthparts to antenna or a combination of leg and antenna (Kaufman, 1978; Pultz *et al*., 1988). Using a transgenic heat shock-inducible *pb* construct, Cribbs *et al*. (1995) further demonstrated that a defined threshold concentration of Pb is required to overcome the dominant antennae to leg phenotype of an *Antp* mutant.

Ubx provides a second example of concentration dependent action. Ubx is expressed, endogenously, at different levels in parasegments 5 and 6, with the higher concentration in parasegment 6. Increased expression of Ubx in parasegment 5 can alter the identity of that region towards the identity of parasegment 6, as evidenced in the larval denticle belts and abdominal imaginal histoblast cells (Smolik-Utlaut, 1990; Frayne and Sato, 1991).

Because variation in HOM-C protein concentration can alter the morphological outcome, it is likely that activation of some specific target genes is concentration dependent, possibly acting via cooperative binding at multiple, clustered recognition sites (see Section 1.8.5.9). Varying the number of HOM-C monomer binding sites in target promoters and/or enhancers could define, in part, a concentration dependency for gene regulation. What is apparent is that the simple presence or absence of a HOM-C protein, at least in some cases, is insufficient to control differing morphological specificity.

1.8.6.4. Beyond Targets: HOM-C Protein Regulation of Developmental Processes

To understand the mechanisms by which the HOM-C proteins control body patterning, it is essential to look beyond single target genes, and attempt to discern their function in the context of a larger developmental process. Insect appendage development provides such an opportunity (see **Chapter 1.9**), as the adult insect has appendages on different segments, arising from both dorsal and ventral body regions, and specified by different *HOM-C* genes. The ability of a *HOM-C* gene product to specify the patterning of entire appendages is evidenced by the famous homeotic phenotypes that led to their discovery. Morata (2001) presents a model for appendage specification in which the regional expression of HOM-C proteins provides segment or regional specific identity (i.e., antennae vs. leg), while the signaling molecules provide the dorsal/ventral pattern cues (i.e., wing/haltere vs. leg). In Morata's model, *pb* acts to specify the proboscis, *Scr* to specify the first thoracic leg, *Antp* to specify the second thoracic leg, and *Ubx* to specify the third thoracic leg, while also repressing wing formation in favor of haltere development.

The HOM-C proteins can also act regionally, across segments, in appendage specification. For example, Antp is required to repress antennal development in all leg discs, and does so by repressing the expression of *hth*. This repression is required for the appropriate patterning of the distal and medial leg regions, as one *hth* function is that of an antennal selector gene (Casares and Mann, 2001). Ubx blocks the entire appendage development program in the anterior *Drosophila* abdomen via the direct repression of the leg selector gene *Dll* (Vachon *et al*., 1991). This requirement is demonstrated by the appearance of abdominal legs in some Ubx mutants (Lewis, 1978).

Ubx also has multiple levels of hierarchical control in specifying the third thoracic haltere. Weatherbee *et al*. (1998) investigated the control exerted by *Ubx* activity in the haltere over known wing patterning genes, while attempting to better define the developmental processes differentiating the haltere and the wing. Not surprisingly, they found that Ubx represses wing-specific genes; however, control beyond that initial level of repression was also observed. Ectopic expression of *vestigial* (*vg*) (required for wing formation and repressed by Ubx in the haltere) does not create ectopic wing tissue in the haltere, suggesting that Ubx is continuing to repress genes downstream of *vg*. Furthermore, normal haltere patterning requires Ubx function throughout larval development, indicating that Ubx does not simply function as an upstream initiator of the haltere developmental cascade.

We have discussed the probability of a large number of target genes for each HOM-C protein, and given examples in which a number of target genes are known to affect a single developmental process. Studies on the *Drosophila* larval oenocyte provide a well-characterized example of a how a single HOM-C protein can direct the fate of a single cell. The larval oenocyte is a secretory cell derived from the dorsal ectoderm, and is found in clusters of

about six cells in the first through seventh abdominal segments. Oenocyte precursor cells delaminate from the dorsolateral ectoderm at the extended germ band stage, and differentiate in response to induction by C1 precursor cells, which also give rise to chordotonal sensory organs (see **Chapter 1.11**). A simplified diagram of this pathway is depicted in Figure 13. Although the C1 precursor cells and the ectodermal oenocyte precursor cells, prepatterned by Spalt major (Salm) are present in both the thoracic and abdominal segments, epidermal growth factor receptor (EGFR) activity is present only around the abdominal C1 cells, limiting oenocyte formation to these segments. The *spitz* (*spi*) gene product, encoding an EGFR ligand, is secreted by the abdominal C1 cells, and received by the oenocyte precursor cells, leading to the oenocyte specific gate (Elstob *et al.*, 2001; Rusten *et al.*, 2001). The *rhomboid* (*rho*) gene encodes a processing factor for the Spi ligand, modifying a membrane bound form of Spi (mSpi) to a secreted form (sSpi). Although *rho* is initially activated by *atonal* (*ato*) in the C1 cells, a burst of *abd-A* and *exd* expression in the abdominal segments is required to maintain *rho* transcription and allow the processing of membrane bound Spi in the Golgi apparatus (by Rho)

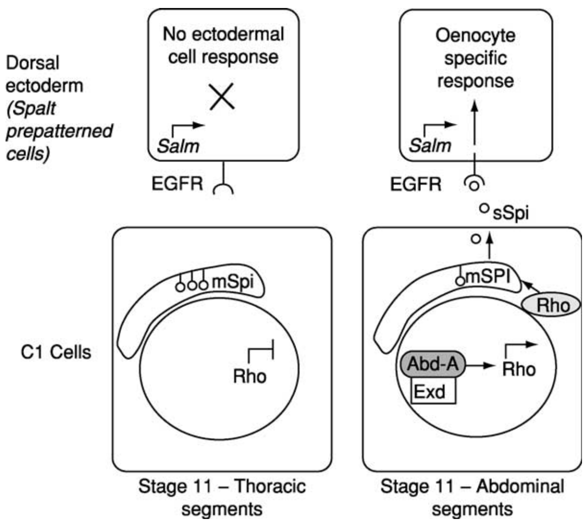


Figure 13 *Drosophila* Abd-A regulates a single principal target, *rho*, to direct larval oenocyte differentiation. The *spalt major* gene (*salm*) is transcribed in the dorsal embryonic ectoderm throughout the thoracic and abdominal segments. Rho is a processing factor, which acts in the Golgi apparatus to modify the membrane-bound form of the Spitz ligand (mSpi) to the secreted form (sSpi). In the abdominal segments at the germ band extended stage (Stage 11), a burst of *abd-A* and *exd* expression is required for *rho* transcription, allowing the production of sSpi and resulting in oenocyte-specific epidermal growth factor receptor (EGFR) output. Because Abd-A is not expressed in the thoracic cells and *rho* activity is absent, sSpi is not present to activate the oenocyte differentiation pathway.

to the secreted form (Brodu *et al.*, 2002). The processed Spi is then secreted, can activate EGFR and induce the *salm*-prepatterned ectodermal cells to form oenocytes. As oenocytes are found in a wide variety of both long and short germ insects, this system may provide a nice model for comparative insect studies of HOM-C target regulation.

Comparative studies of developmental processes in other insects are critical to the further understanding *HOM-C* gene function. The ability of the *HOM-C* genes to direct developmental processes is well documented in other insects (for examples, see Beeman *et al.*, 1989, 1993; Nagata *et al.*, 1996). Identifying the *Drosophila HOM-C* gene homologs, and their downstream targets directing these developmental processes, is beginning to provide interesting data regarding homeotic gene control of development. The direction of gnathal appendage development by the *HOM-C* genes provides an example.

In *Drosophila*, the *HOM-C* genes *Scr* and *pb* are required for appropriate patterning of the proboscis, and this specification requires suppression or down-regulation of antennal and leg-specific genes in the labial disc, such as *exd, dachshund (dac)*, *salm*, and *Dll*. For example, *pb* limits the expression of *Dll* to a compact crescent-shaped domain within the developing labial appendage (Abzhanov *et al.*, 2001). The loss of *pb* causes an increase and an expansion of *Dll* expression, leading to the leg or partial antennal transformations observed in *pb* mutants. The loss of both *pb* and *Scr* causes a transformation of the proboscis to the "ground state" of antenna. Studies undertaken in *Tribolium* indicate that these genes function similarly to direct proboscis development as well. The loss of *mxp* (*pb* homolog) in the labial appendage bud causes a transformation of the labial palp to leg, and double mutants for *Cx* (*Scr* homolog) and *mxp*, develop antenna in place of the palps, similar to the fly. Unlike *Drosophila*, however, *Dll* expression in the *Tribolium* labial appendage is not significantly altered in *mxp* mutants (DeCamillis *et al.*, 2001). It will be interesting to see the effects of *mxp* and *Cx* loss on other leg/antennal patterning genes.

Appropriate *Dll* expression is required for the distal development of all *Drosophila* appendages, and *Dll* is also expressed in the developing appendages of other insects. Its pattern of expression is well conserved, as observed in *Tribolium*, *Thermobia*, *Acheta*, *Oncopeltus*, *Precis*, and *Neodiprion*, but there do appear to be some differences in both expression and regulation (Weatherbee *et al.*, 1999; Beermann *et al.*, 2001; Suzuki and Palopoli, 2001; Rogers *et al.*, 2002). Like in *Drosophila*, *Tribolium* requires the repression of *Dll* to suppress the

appendage development pathway, but rather than utilizing Utx (Ubx homolog) and A (Abd-A homolog) for this function, A alone prevents *Dll* expression in the *Tribolium* larval abdomen (Lewis *et al.*, 2000). Utx, instead, appears to modulate patterning of a first abdominal appendage, the pleuropodia. Lepidopteran larvae, which have abdominal prolegs not found in *Drosophila* or *Tribolium* larvae, express *Dll* in the abdominal regions, in which these legs will form. Correspondingly, there are "holes" in the *BX-C* gene expression domains in the abdomen, allowing *Dll* expression and indicating that the lepidopteran *BX-C* genes may have a limb-repressing role. Studies in *Neodiprion* larvae reveal the conservation of BX-C control of *Dll* expression, even though the developmental process is altered (Suzuki and Palopoli, 2001). In contrast to lepidopteran larvae, *Neodiprion* larvae lack abdominal *Dll* expression even though these larvae develop abdominal prolegs. Concomitantly, the expression of the *BX-C* genes is found throughout the abdomen, including the developing prolegs. This reflects a conservation of the BX-C/*Dll* regulatory relationship, but a possibly convergent *Neodiprion* developmental pathway for abdominal prolegs, when compared to Lepidoptera. *Dll* is also a target of Ubx in the lepidopteran hindwing, which derives from the third thoracic segment. In contrast to the highly modified haltere of *Drosophila*, butterflies have well developed hindwings. As might be expected, several of the wing-specific genes that are repressed by Ubx in the *Drosophila* haltere remain under Ubx control in the butterfly, but are differentially regulated to allow hindwing development and patterning (Weatherbee *et al.*, 1999). These examples illustrate how differences in HOM-C gene target regulation may correlate to different developmental outcomes between insect species.

The HOM-C proteins can, in some developmental processes, directly regulate a large number of target genes to dictate regional specification. In other processes, however, far fewer targets may be required, with the primary targets being other transcription factors or signaling molecules. Specification of body regions, such as the wing or midgut, appears to be complex and involve a large number of downstream factors. In contrast, specifying the identity of only a few cells, as illustrated by the oenocyte case, can require only a single HOM-C responding gene.

1.8.7. Looking Forward

Our further understanding of how *HOM-C* gene function directs axial patterning requires a thorough understanding of the biological processes guided by the *HOM-C* genes, the target genes responding to HOM-C protein regulation, and comparative studies involving nondrosophilid insects. A three-step approach begins with the identification of additional *HOM-C* guided developmental processes. These may be as large and complex as neural development (see **Chapters 1.10** and **2.3**), or may be as specific as the patterning of a single sensory cell (see **Chapters 1.12** and **1.11**), or even the regulation of a single gene. For example, recent studies into the process of larval salivary gland development (see **Chapter 2.10**), are providing insights into how Scr function establishes cell fate and tissue morphology (Abrams *et al.*, 2003; Haberman *et al.*, 2003). Next, characterizing the manner in which the HOM-C proteins then direct specific processes through downstream targets may shed light on the various mechanisms by which they govern pattern formation. Dfd, for example, was recently shown to directly regulate expression of the proapoptotic gene, *reaper*, in sculpting the *Drosophila* embryonic gnathal lobes through the initiation of programmed cell death (Lohmann *et al.*, 2002; see also **Chapter 2.5**).

Finally, comparisons of developmental processes and genetic cascades between insect species and noninsect arthropods are critical for addressing long-standing questions regarding the role of *HOM-C/HOX* genes in both development and evolution. The success of RNAi is now allowing the characterization of gene function in a growing number of nonmodel insects (Brown *et al.*, 1999; Denell and Shippy, 2001) such as *Thermobia* and *Oncopeltus*, without the generation of mutant strains. Recent studies by Galant and Carroll (2002) and Ronshaugen *et al.* (2002) correlate functional changes in Ubx, between insect and other arthropods, to the transition, in arthropods, from legs on every segment to legs limited to only the thoracic segments. Studies such as these will certainly continue and grow to include a wide variety of insects and other arthropods. When expanded to include downstream target genes, this work should provide valuable insights correlating changes in *HOM-C* directed genetic cascades to differing morphologies or developmental outcomes.

Acknowledgments

Although the authors have attempted to be as comprehensive as possible, due to space constraints, they regret they were unable to cite all relevant literature on this topic and offer sincere apologies to those authors whose work they are unable to include.

References

Abbott, M.K., Kaufman, R.C., **1986**. The relationship between the functional complexity and the molecular organization of the *Antennapedia* locus of *Drosophila melanogaster. Genetics 114*, 919–942.

Abrams, E.W., Vining, M.S., Andrew, D.J., **2003**. Constructing an organ: the *Drosophila* salivary gland as a model for tube formation. *Trends Cell Biol. 13*, 247–254.

Abzhanov, A., Holtzman, S., Kaufman, T.C., **2001**. The *Drosophila* proboscis is specified by two Hox genes, *proboscipedia* and *Sex combs reduced*, via repression of leg and antennal appendage genes. *Development 128*, 2803–2814.

Abzhanov, A., Kaufman, T.C., **2000**. Homologs of *Drosophila* appendage genes in the patterning of arthropod limbs. *Devel. Biol. 227*, 673–689.

Ades, S.E., Sauer, R.T., **1994**. Differential DNA-binding specificity of the engrailed homeodomain: the role of residue 50. *Biochemistry 33*, 9187–9194.

Affolter, M., Percival-Smith, A., Müller, M., Leupin, W., Gehring, W.J., **1990**. DNA binding properties of the purified *Antennapedia* homeodomain. *Proc. Natl Acad. Sci. USA 87*, 4093–4097.

Akam, M., **1998**. Hox genes: from master genes to micromanagers. *Curr. Biol. 8*, R676–R678.

Akam, M., Dawes, R., **1992**. More than one way to slice an egg. *Curr. Biol.* 2, 395–398.

Akam, M., Dawson, I., Tear, G., **1988**. Homeotic genes and the control of segment diversity. *Development 104*, 123–134.

Akam, M.E., Martinez-Arias, A., **1985**. The distribution of *Ultrabithorax* transcripts on *Drosophila* embryos. *EMBO J. 4*, 1689–1700.

Alexandre, E., Graba, Y., Fasano, L., Gallet, A., Perrin, L., *et al.*, **1996**. The *Drosophila* Teashirt Homeotic protein is a DNA-binding protein and *modulo*, a HOM-C regulated modifier of variegation, is a likely candidate for being a direct target gene. *Mech. Devel. 59*, 191–204.

Andrew, D.J., **1995**. The *Sex combs reduced* gene of *Drosophila melanogaster* has multiple transcripts. *Gene 152*, 149–155.

Andrew, D.J., Horner, M.A., Petitt, M.G., Smolik, S.M., Scott, M.P., **1994**. Setting limits on homeotic gene function: restraint of *Sex combs reduced* activity by *teashirt* and other homeotic genes. *EMBO J. 13*, 1132–1144.

Appel, B., Sakonju, S., **1993**. Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in *Drosophila* embryos. *EMBO J. 12*, 1099–1109.

Aspland, S.E., White, R.A.H., **1997**. Nucleocytoplasmic localization of *extradenticle* protein is spatially regulated throughout development in *Drosophila*. *Development 124*, 741–747.

Azpiazu, N., Morata, G., **1998**. Functional and regulatory interactions between Hox and extradenticle genes. *Genes Devel. 12*, 261–273.

Barges, S., Mihaly, J., Galloni, M., Hagstrom, K., Muller, M., *et al.*, **2000**. The *Fab-8* boundary defines the distal limit of the bithorax complex *iab-7* domain and insulates *iab-7* from initiation elements and a PRE in the adjacent *iab-8* domain. *Development 127*, 779–790.

Bateson, W., **1894**. Materials for the Study of Variation. Macmillan, New York.

Beachy, P.A., Helfand, S.L., Hogness, D.S., **1985**. Segmental distribution of bithorax complex proteins during *Drosophila* development. *Nature 313*, 545–551.

Beachy, P.A., Krasnow, M.A., Gavis, E.R., Hogness, D.S., **1988**. An *Ultrabithorax* protein binds sequences near its own and the *Antennapedia* P1 promoters. *Cell 55*, 1069–1081.

Beachy, P.A., Varkey, J., Young, K.E., von Kessler, D.P., Sun, B.I., *et al.*, **1993**. Cooperative binding of an *Ultrabithorax* homeodomain protein to nearby and distant DNA sites. *Mol. Cell. Biol. 13*, 6941–6956.

Beeman, R.W., **1987**. A homoeotic gene cluster in the red flour beetle. *Nature 327*, 247–249.

Beeman, R.W., Stuart, J.J., Brown, S.J., Denell, R.E., **1993**. Structure and function of the homeotic gene complex (*HOM-C*) in the beetle, *Tribolium castaneum*. *BioEssays 15*, 439–444.

Beeman, R.W., Stuart, J.J., Hass, M.S., Denell, R.E., **1989**. Genetic analysis of the homeotic gene complex (*HOM-C*) in the beetle *Tribolium castaneum*. *Devel. Biol. 133*, 196–209.

Beermann, A., Jay, D., Beeman, R., Hulskamp, M., Tautz, D., *et al.*, **2001**. The *Short antennae* gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila* Distal-less protein. *Development 128*, 287–297.

Bender, W., Akam, M., Karch, F., Beachy, P.A., Peifer, M., *et al.*, **1983**. Molecular genetics of the *Bithorax Complex* in *Drosophila melanogaster. Science 221*, 23–29.

Bennett, R.L., Brown, S.J., Denell, R.E., **1999**. Molecular and genetic analysis of the *Tribolium Ultrabithorax* ortholog, *Ultrathorax*. *Devel. Genes Evol. 209*, 608–619.

Bergson, C., McGinnis, W., **1990**. An autoregulatory enhancer element of the *Drosophila* homeotic gene *Deformed*. *EMBO J. 9*, 4287–4297.

Bermingham, J.R., Jr., Martinez-Arias, A., Petitt, M.G., Scott, M.P., **1990**. Different patterns of transcription from the two *Antennapedia* promoters during *Drosophila* embryogenesis. *Development 109*, 553–566.

Bermingham, J.R., Jr., Scott, M.P., **1988**. Developmentally regulated alternative splicing of transcripts from the *Drosophila* homeotic gene *Antennapedia* can produce four different proteins. *EMBO J. 7*, 3211–3222.

Berry, M., Gehring, W., **2000**. Phosphorylation status of the Scr homeodomain determines its functional activity: essential role for protein phosphatase 2A,B′. *EMBO J. 19*, 2946–2957.

Bhojwani, J., Shashidhara, L.S., Sinha, P., **1997**. Requirement of *teashirt* (*tsh*) function during cell fate specification in developing head structure in *Drosophila*. *Devel. Genes Evol. 207*, 137–146.

Bienz, M., **1997**. Endoderm induction in *Drosophila*: the nuclear targets of the inducting signals. *Curr. Opin. Genet. Devel. 7*, 683–688.

Bienz, M., Müller, J., **1995**. Transcriptional silencing of homeotic genes in *Drosophila*. *BioEssays 17*, 775–784.

Bienz, M., Tremml, G., **1988**. Domain of *Ultrabithorax* expression in *Drosophila* visceral mesoderm from autoregulation and exclusion. *Nature 333*, 576–578.

Biggin, M.D., McGinnis, W., **1997**. Regulation of segmentation and segmental identity by *Drosophila* homeoproteins: the role of DNA binding in functional activity and specificity. *Development 124*, 4425–4433.

Billeter, M., Qian, Y., Otting, G., Muller, M., Gehring, W.J., *et al.*, **1990**. Determination of the three-dimensional structure of the *Antennapedia* homeodomain from *Drosophila* in solution by 1H nuclear magnetic resonance spectroscopy. *J. Mol. Biol. 214*, 183–197.

Bokor, P., DiNardo, S., **1996**. The roles of *hedgehog*, *wingless* and *lines* in patterning the dorsal epidermis in *Drosophila*. *Development 122*, 1083–1092.

Botas, J., Auwers, L., **1996**. Chromosomal binding sites of Ultrabithorax homeotic proteins. *Mech. Devel. 56*, 129–138.

Boulet, A.M., Lloyd, A., *et al.*, **1991**. Molecular definition of the morphogenetic and regulatory functions and the *cis*-regulatory elements of the *Drosophila* Abd-B homeotic gene. *Development 111*, 393–405.

Bourbon, H.M., Martin-Blanco, E., Rosen, D., Kornberg, T.B., **1995**. Phosphorylation of the *Drosophila* engrailed protein at a site outside its homeodomain enhances DNA binding. *J. Biol. Chem. 270*, 11130–11139.

Brand, A.H., Perrimon, N., **1993**. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development 118*, 401–415.

Brazas, R.M., Stillman, D.J., **1993**. Identification and purification of a protein that binds DNA cooperatively with the yeast SWI5 protein. *Mol. Cell. Biol. 13*, 5524–5537.

Breen, T.R., Harte, P.J., **1993**. *trithorax* regulates multiple homeotic genes in the *Bithorax* and *Antennapedia* complexes and exerts different tissue-specific, parasegment-specific and promoter specific effects on each. *Development 117*, 119–134.

Bridges, C., Dobzhansky, T., **1933**. The mutant "*proboscipedia*" in *Drosophila melanogaster*: a case of hereditary homöosis. *Roux's Arch. Devel. Biol. 127*, 575–590.

Brodu, V., Elstob, P.R., Gould, A.P., **2002**. *abdominal* A specifies one cell type in *Drosophila* by regulating one principal target gene. *Development 129*, 2957–2963.

Brookman, J.J., Toosy, A.T., Shashidhara, L.S., White, R.A., **1992**. The 412 retrotransposon and the development of gonadal mesoderm in *Drosophila*. *Development 116*, 1185–1192.

Brown, S.J., DeCamillis, M., Gonzalez-Charneco, K., Denell, M., Beeman, R., *et al.*, **2000**. Implications of the *Tribolium Deformed* mutant phenotype for the evolution of Hox gene function. *Proc. Natl Acad. Sci. USA 97*, 4510–4514.

Brown, S.J., Fellers, J.P., Shippy, T.D., Richardson, E.A., Maxwell, M., *et al.*, **2002a**. Sequence of the *Tribolium castaneum* Homeotic Complex: the region corresponding to the *Drosophila melanogaster* Antennapedia Complex. *Genetics 160*, 1067–1074.

Brown, S.J., Hilgenfeld, R.B., Denell, R.E., **1994**. The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. *Proc. Natl Acad. Sci. USA 91*, 12922–12926.

Brown, S.J., Holtzman, S., Kaufman, T., Denell, R.E., **1999**. Characterization of the *Tribolium Deformed* ortholog and its ability to directly regulate *Deformed* target genes in the rescue of a *Drosophila Deformed* null mutant. *Devel. Genes Evol. 209*, 389–398.

Brown, S.J., Parrish, J.K., Beeman, R.W., Denell, R.E., **1997**. Molecular characterization and embryonic expression of the *even-skipped* ortholog of *Tribolium castaneum*. *Mech. Devel. 61*, 165–173.

Brown, S.J., Shippy, T.D., Beeman, R.W., Denell, R.E., **2002b**. *Tribolium* Hox genes repress antennal development in the gnathos and trunk. *Mol. Phylogenet. Evol. 24*, 384–387.

Bürglin, T.R., **1994**. A comprehensive classification of homeobox genes. In: Duboule, D. (Ed.), Guidebook to Homeobox Genes. Oxford University Press, New York, pp. 27–71.

Busturia, A., Casonova, J., Sánchez-Herrero, E., González, R., Morata, G., **1989**. Genetic structure of the *abd-A* gene of *Drosophila*. *Development 107*, 575–583.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., *et al.*, **2002**. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science 298*, 1039–1043.

Capovilla, M., Brandt, M., Botas, J., **1994**. Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. *Cell 76*, 461–475.

Capovilla, M., Kambris, Z., Botas, J., **2001**. Direct regulation of the muscle-identity gene *apterous* by a Hox protein in the somatic mesoderm. *Development 128*, 1221–1230.

Carrasco, A.E., McGinnis, W., Gehring, W.J., Robertis, E.M., **1984**. Cloning of an *X. laevis* gene expressed during early embryogenesis that codes for a protein domain homologous to *Drosophila* homeotic genes. *Cell 37*, 409–414.

Carroll, S.B., Laymon, R.A., McCutcheon, M.A., Riley, P.D., Scott, M.P., **1986**. The localization and regulation of Antennapedia protein expression in *Drosophila* embryos. *Cell 47*, 113–122.

Casanova, J., Sánchez-Herro, E., Morata, G., **1986**. Identification and characterization of a parasegment specific regulatory element of the *Abdominal-B* gene of *Drosophila*. *Cell 47*, 627–636.

Casares, F., Mann, R.S., **2001**. The ground state of the ventral appendage in *Drosophila*. *Science 293*, 1477–1480.

Casares, F., Sánchez-Herrero, E., **1995**. Regulation of the *infraabdominal* regions of the bithorax complex

of *Drosophila* by gap genes. *Development 121*, 1855–1866.

Castelli-Gair, J., **1998**. The *lines* gene of *Drosophila* is required for specific functions of the Abdominal-B Hox protein. *Development 125*, 1269–1274.

Castelli-Gair, J., Akam, M., **1995**. How the Hox gene *Ultrabithorax* specifies two different segments: the significance of spatial and temporal regulation within metameres. *Development 121*, 2973–2982.

Castelli-Gair, J.E., Garcia-Bellido, A., **1990**. Interactions of *Polycomb* and *trithorax* with *cis* regulatory regions of *Ultrabithorax* during the development of *Drosophila melanogaster. EMBO J. 9*, 4267–4275.

Celniker, S.E., Keelan, D.J., Lewis, E.B., **1989**. The molecular genetics of the bithorax complex of *Drosophila*: characterization of the products of the *Abdominal-B* domain. *Genes Devel. 3*, 1424–1436.

Chan, S., Jaffe, L., Capovilla, M., Botas, J., Amann, R.S., **1994**. The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with Extradenticle, another homeoprotein. *Cell 78*, 603–615.

Chan, S., Mann, R.S., **1993**. The segment identity functions of Ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. *Genes Devel. 7*, 796–811.

Chan, S., Mann, R.S., **1996**. A structural model for a homeotic protein–Extradenticle–DNA complex accounts for the choice of HOX protein in the heterodimer. *Proc. Natl Acad. Sci. USA 93*, 5223–5228.

Chan, S., Pöpper, H., Krumlauf, R., Mann, R.S., **1996**. An Extradenticle induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J. 15*, 2476–2487.

Chan, S., Ryoo, H.D., Gould, A., Krumlauf, R., Mann, R.S., **1997**. Switching the *in vivo* specificity of a minimal Hox-responsive element. *Development 124*, 2007–2014.

Chang, C., Shen, W., Rozenfeld, S., Lawrence, J.H., Largman, C., *et al.*, **1995**. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Devel. 9*, 663–674.

Chang, C.P., Brocchieri, L., Shen, W.F., Largman, C., Cleary, M.L., *et al.*, **1997**. Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. *Mol. Cell. Biol. 16*, 1734–1745.

Chauvet, S., Maurel-Zaffran, C., Miassod, R., Jullien, N., Pradel, J., *et al.*, **2000**. *dlarp*, a new candidate Hox target in *Drosophila* whose orthologue in mouse is expressed at sites of epithelium/mesenchymal interactions. *Devel. Dyn. 218*, 401–413.

Chiang, C., Young, K.E., Beachy, P.A., **1995**. Control of *Drosophila* tracheal branching by the novel homeodomain gene *unplugged*, a regulatory target for genes of the bithorax complex. *Development 121*, 3901–3912.

Chinwalla, V., Jane, W., Harte, P., **1995**. The *Drosophila* trithorax protein binds to specific chromosomal sites and is co-localized with polycomb at many sites. *EMBO J. 14*, 2056–2065.

Choi, S.H., Oh, C.T., Kim, S.H., Kim, Y.T., Jeon, S.H., **2000**. Effects of Polycomb group mutations on the expression of Ultrabithorax in the *Drosophila* visceral mesoderm. *Mol. Cells. 10*, 156–161.

Chouinard, S., Kaufman, T.C., **1991**. Control of expression of the homeotic *labial* (*lab*) locus of *Drosophila melanogaster*: evidence for both positive and negative autogenous regulation. *Development 113*, 1267–1280.

Cimbora, D.M., Sakonju, S., **1995**. *Drosophila* midgut morphogenesis requires the function of the segmentation gene *odd-paired*. *Devel. Biol. 169*, 580–595.

Clarke, N.D., Kissinger, C.R., Desjarlais, J., Gilliland, G.L., Pabo, C.O., **1994**. Structural studies of the engrailed homeodomain. *Protein Sci. 3*, 1779–1787.

Clemm, J.D., Pabo, C.O., **1996**. Oct1 POU domain interactions: cooperative binding of isolated subdomains and effects of covalent linkage. *Genes Devel. 10*, 27–36.

Cohen, S.M., Jürgens, G., **1990**. Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature 346*, 482–485.

Cook, C.E., Smith, M.L., Telford, M.J., Bastianello, A., Akam, M., **2001**. Hox genes and the phylogeny of the arthropods. *Curr. Biol. 11*, 759–763.

Cribbs, D.L., Benassayag, C., Randazzo, F.M., Kaufman, T.C., **1995**. Levels of homeotic protein function can determine developmental identity: evidence from low-level expression of the *Drosophila* homeotic gene *proboscipedia* under Hsp70 control. *EMBO J. 14*, 767–778.

Cribbs, D.L., Pultz, M.A., Jonhson, D., Mazzulla, M., Kaufman, T.C., **1992**. Structural complexity and evolutionary conservation of the *Drosophila* homeotic gene *proboscipedia*. *EMBO J. 11*, 1437–1449.

Cumberledge, S., Zaratzian, A., Sakonju, S., **1990**. Characterization of two RNAs transcribed from the *cis*-regulatory region of the *abd-A* domain within the *Drosophila* bithorax complex. *Proc. Natl Acad. Sci. USA 87*, 3259–3263.

Curtis, C.D., Brisson, J.A., DeCamillis, M., Shippy, T.D., Brown, S.J., *et al.*, **2001**. Molecular characterization of *Cephalothorax*, the *Tribolium* ortholog of *Sex combs reduced*. *Genesis 30*, 12–20.

Dalton, D., Chadwick, R., McGinnis, W., **1989**. Expression and embryonic function of *empty spiracles*: a *Drosophila* homeobox gene with two patterning functions on the anterior–posterior axis of the embryo. *Genes Devel. 3*, 1940–1956.

Damen, W.G., Tautz, D., **1999**. Abdominal-B expression in a spider suggests a general role for Abdominal-B in specifying the genital structure. *J. Exp. Zool. 285*, 85–91.

Davis, M.M., **1986**. Subtractive cDNA hybridization and the T-cell receptor genes. In: Weir, D.M. (Ed.), Handbook of Experimental Immunology, vol. 2, Cellular Immunology. Blackwell Scientific Publications, Palo Alto, CA, pp. 76.1–76.13.

Davenport, M.P., Blass, C., Eggleston, P., **2000**. Characterization of the Hox gene cluster in the malaria vector mosquito, *Anopheles gambiae*. *Evol. Devel. 2*, 326–339.

de Zulueta, P., Alexandre, E., Jacq, B., Kerridge, S., **1994**. Homeotic complex and *teashirt* genes co-operate to establish trunk segmental identities in *Drosophila*. *Development 120*, 2278–2296.

DeCamillis, M.A., ffrench-Constant, R., **2003**. *Proboscipedia* represses distal signaling in the embryonic gnathal limb fields of *Tribolium castaneum*. *Devel. Genes Evol. 213*, 55–64.

DeCamillis, M.A., Lewis, D.L., Brown, S.J., Beeman, R.W., Denell, R.E., **2001**. Interactions of the *Tribolium Sex combs reduced* and *proboscipedia* orthologs in embryonic labial development. *Genetics 159*, 1643–1648.

Delorenzi, M., Bienz, M., **1990**. Expression of *Abdominal-B* homeoproteins in *Drosophila* embryos. *Development 108*, 323–329.

Denell, R.E., Shippy, T., **2001**. Comparative insect developmental genetics: phenotypes without mutants. *BioEssays 23*, 379–382.

Desplan, C., Theis, J., O'Farrell, P.H., **1985**. The *Drosophila* developmental gene, *engrailed*, encodes a sequence-specific DNA binding activity. *Nature 318*, 630–635.

Desplan, C., Theis, J., O'Farrell, P.H., **1988**. The sequence specificity of homeodomain–DNA interaction. *Cell 54*, 1081–1090.

Dessain, S., Gross, C.T., Kuziora, M.A., McGinnis, W., **1992**. *Antp*-type homeodomains have distinct DNA binding specificities that correlate with their different regulatory functions in embryos. *EMBO J. 11*, 991–1002.

Diederich, R.J., Merrill, V.K.L., Pultz, M.A., Kaufman, T.C., **1989**. Isolation, structure, and expression of *labial*, a homeotic gene of the *Antennapedia Complex* involved in *Drosophila* head development. *Genes Devel. 3*, 399–414.

Diederich, R.J., Pattatucci, A.M., Kaufman, T.C., **1991**. Developmental and evolutionary implications of *labial, Deformed* and *engrailed* expression in the *Drosophila* head. *Development 113*, 273–281.

Drewell, R.A., Bae, E., Burr, J., Lewis, E.B., **2002**. Transcription defines the embryonic domains of *cis*-regulatory activity at the *Drosophila Bithorax Complex*. *Proc. Natl Acad. Sci. USA 99*, 16853–16858.

Duncan, I.M., **1982**. *Polycomblike*: a gene that appears to be required for the normal expression of the *Bithorax* and *Antennapedia* gene complexes of *Drosophila melanogaster. Genetics 102*, 49–70.

Dura, J.M., Brock, H.W., Santamaria, P., **1985**. *Polyhomeotic*: a gene of *Drosophila melanogaster* required for correct expression of segmental identity. *Mol. Gen. Genet. 198*, 213–220.

Dushay, M.S., Rosbash, M., Hall, J.C., **1989**. The disconnected visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. *J. Biol. Rhythms 4*, 1–27.

Ekker, S.C., Jackson, D.G., von Kessler, D.P., Sun, B.I., Young, K.E., *et al.*, **1994**. The degree of variation in DNA sequence recognition among four *Drosophila* homeotic proteins. *EMBO J. 13*, 3551–3560.

Ekker, S.C., von Kessler, D.P., Beachy, P.A., **1992**. Differential DNA sequence recognition is a determinant of specificity in homeotic gene action. *EMBO J. 11*, 4059–4072.

Ekker, S.C., Young, K.E., von Kessler, D.P., Beachy, P.A., **1991**. Optimal DNA sequence recognition by the *Ultrabithorax* homeodomain of *Drosophila*. *EMBO J. 10*, 1179–1186.

Elstob, P.R., Brodu, V., Gould, A.P., **2001**. *spalt*-dependent switching between two cell fates that are induced by the *Drosophila* EGF receptor. *Development 128*, 723–732.

Eresh, S., Riese, J., Jackson, D.B., Bohmann, D., Bienz, M., **1997**. A CREB-binding site as a target for *decapentaplegic* signaling during *Drosophila* endoderm development. *EMBO J. 16*, 2014–2022.

Estrada, B., Sánchez-Herrero, E., **2001**. The Hox gene *Abdominal-B* antagonizes appendage development in the genital disc of *Drosophila*. *Development 128*, 331–339.

Falciani, F., Hausdorf, B., Schroder, R., Akam, M., Tautz, D., *et al.*, **1996**. Class 3 Hox genes in insects and the origin of zen. *Proc. Natl Acad. Sci. USA 93*, 8479–8484.

Fasano, L., Röder, L., Coré, N., Alexandre, E., Vola, C., *et al.*, **1991**. The gene *teashirt* is required for the development of *Drosophila* embryonic truck segments and encodes a protein with widely spaced zinc finger motifs. *Cell 64*, 63–79.

Feinstein, P.G., Kornfeld, K., Hogness, D., Mann, R.S., **1995**. Identification of homeotic target genes in *Drosophila melanogaster* including *nervy*, a proto-oncogene homologue. *Genetics 140*, 573–586.

Ferrier, D.E., Akam, M., **1996**. Organization of the Hox gene cluster in the grasshopper, *Schistocerca gregaria*. *Proc. Natl Acad. Sci. USA 93*, 13024–13029.

Florence, B., Handrow, R., Laughon, A., **1991**. DNA-binding specificity of the fushi tarazu homeodomain. *Mol. Cell. Biol. 11*, 3613–3623.

FlyBase Consortium, **2003**. The FlyBase database of the *Drosophila* genome projects and community literature. *Nucl. Acids Res. 31*, 172–175. http://flybase.org/.

Fraenkel, E., Pabo, C.O., **1998**. Comparison of X-ray and NMR structures for the Antennapedia homeodomain–DNA complex. *Nature Struct. Biol. 5*, 692–696.

Fraenkel, E., Rould, M.A., Chambers, K.A., Pabo, C.O., **1998**. *Engrailed* homeodomain–DNA complex at 2.2 Å resolution: a detailed view of the interface and comparison with other engrailed structures. *J. Mol. Biol. 27*, 351–361.

Franke, A., DeCamillis, M., Zink, D., Cheng, N., Brock, H.W., *et al.*, **1991**. *Polycomb* and *polyhomeotic* are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster. EMBO J. 11*, 2941–2950.

Frayne, E.G., Sato, R., **1991**. The *Ultrabithorax* gene of *Drosophila* and the specification of abdominal histoblasts. *Devel. Biol. 146*, 265–277.

Furukubo-Tokunaga, K., Flister, S., Gehring, W.J., **1993**. Functional specificity of the *Antennapedia* homeodomain. *Proc. Natl Acad. Sci. USA 90*, 6360–6364.

Galant, R., Walsh, C.M., Carroll, S.B., **2002**. Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. *Development 129*, 3115–3126.

Galloni, M., Gyurkovics, H., Shedl, P., Karch, F., **1993**. The bluetail transposon: evidence for independent *cis*-regulatory domains and domain boundaries in the bithorax complex. *EMBO J. 12*, 1087–1097.

Garber, R.I., Kuroiwa, A., Gehring, W.J., **1983**. Genomic and cDNA clones of the homeotic locus *Antennapedia* in *Drosophila*. *EMBO J. 2*, 2027–2036.

García-Bellido, A., **1977**. Homoeotic and atavic mutations in insects. *Am. Zool. 17*, 613–629.

Gebelein, B., Culi, J., Ryoo, H.D., Zhang, W., Mann, R.S., **2002**. Specificity of *Distalless* repression and limb primordia development by abdominal Hox proteins. *Devel. Cell 3*, 487–498.

Gehring, W.J., Affolter, M., Bürglin, T., **1994**. Homeodomain proteins. *Annu. Rev. Biochem. 63*, 487–526.

Gellon, G., Harding, K.W., McGinnis, N., Martin, M.M., McGinnis, W., **1997**. A genetic screen for modifiers of *Deformed* homeotic function identifies novel genes required for head development. *Development 124*, 3321–3331.

Gibson, G., Schier, A., LeMotte, P., Gehring, W.J., **1990**. The specificities of *Sex combs reduced* and *Antennapedia* that includes the homeodomain. *Cell 62*, 1087–1103.

Gindhart, J.G., Kaufman, T.C., **1995**. Identification of *Polycomb* and *trithorax* group responsive elements in the regulatory region of the *Drosophila* homeotic gene *Sex combs reduced*. *Genetics 139*, 797–814.

Glicksman, M.A., Brower, D.L., **1988**. Expression of the Sex combs reduced protein in *Drosophila* larvae. *Devel. Biol. 127*, 113–118.

González-Crespo, S., Morata, G., **1995**. Control of *Drosophila* adult pattern by *extradenticle*. *Development 121*, 2117–2125.

González-Reyes, A., Macías, A., Morata, G., **1992**. Autocatalysis and phenotypic expression of *Drosophila* homeotic gene Deformed: its dependence on polarity and homeotic gene function. *Development 116*, 1059–1068.

González-Reyes, A., Morata, G., **1990**. The developmental effect of overexpressing a UBX product in *Drosophila* embryos is dependent on its interactions with other homeotic products. *Cell 61*, 515–522.

Gould, A.P., Brookman, J.J., Strutt, D.I., White, R.A.H., **1990**. Targets of homeotic gene control in *Drosophila*. *Nature 348*, 308–312.

Gould, A.P., Morrison, A., Sproat, G., White, R.A., Krumlauf, R., **1997**. Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping Hox expression patterns. *Genes Devel. 11*, 900–913.

Gould, A.P., White, R.A.H., **1992**. *Connectin*, a target of homeotic gene control in *Drosophila*. *Development 116*, 1163–1174.

Goutte, C., Johnson, A.D., **1993**. Yeast a1 and alpha 2 homeodomain proteins form a DNA-binding activity with properties distinct from those of either protein. *J. Mol. Biol. 233*, 359–371.

Graba, Y., Aragnol, D., Laurenti, P., Garzino, V., Charmot, D., *et al.*, **1992**. Homeotic control in *Drosophila*: the *scabrous* gene is an *in vivo* target of Ultrabithorax proteins. *EMBO J. 11*, 3375–3384.

Graba, Y., Aragnol, D., Pradel, J., **1997**. *Drosophila* Hox complex downstream targets and the function of homeotic genes. *BioEssays 19*, 379–388.

Graba, Y., Gieseler, K., Aragnol, D., Laurenti, P., Mariol, M.C., *et al.*, **1995**. *DWnt-4*, a novel *Drosophila Wnt* gene acts downstream of homeotic complex genes in the visceral mesoderm. *Development 121*, 209–218.

Graba, Y., Laurenti, P., Perrin, L., Aragnol, D., Pradel, J., **1994**. The modifier of variegation *modulo* gene acts downstream of dorsoventral and HOM-C genes and is required for morphogenesis in *Drosophila*. *Devel. Biol. 166*, 704–715.

Grieder, N.C., Marty, T., Ryoo, H.D., Mann, R.S., Affolter, M., **1997**. Synergistic activation of a *Drosophila* enhancer by HOM/EXD and DPP signaling. *EMBO J. 16*, 7402–7410.

Gyurkovics, H., Gausz, J., Kummer, J., Karch, F., **1990**. A new homeotic mutation in the *Drosophila* bithorax complex removes a boundary separating two domains of regulation. *EMBO J. 9*, 2579–2585.

Haberman, A.S., Isaac, D.D., Andrew, D.J., **2003**. Specification of cell fates within the salivary gland primordium. *Devel. Biol. 258*, 443–453.

Hanes, S.D., Brent, R., **1989**. DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell 57*, 1275–1283.

Hanes, S.D., Brent, R., **1991**. A genetic model for interaction of the homeodomain recognition helix with DNA. *Science 251*, 426–430.

Hanes, S.D., Riddihough, G., Ish-Horowicz, D., Brent, R., **1994**. Specific DNA recognition and intersite spacing are critical for action of the bicoid morphogen. *Mol. Cell. Biol. 14*, 3364–3375.

Harding, K., Levine, M., **1988**. Gap genes define the limits of *Antennapedia* and *Bithorax* gene expression during early development in *Drosophila*. *EMBO J. 7*, 205–214.

Hayward, D.C., Patel, N.H., Rehm, E.J., Goodman, C.S., Ball, E.E., **1995**. Sequence and expression of grasshopper *Antennapedia*: comparison to *Drosophila*. *Devel. Biol. 172*, 452–465.

Hedrick, S.M., Cohen, D.I., Nielsen, E.A., Davis, M.M., **1984**. Isolation of cDNA clones encoding T cell-specific membrane associated proteins. *Nature 308*, 149–153.

Heilig, J.S., Freeman, M., Laverty, T., Lee, K.J., Campos, A.R., *et al.*, **1991**. Isolation and characterization of the *disconnected* gene of *Drosophila melanogaster. EMBO J. 10*, 809–815.

Hinz, U., Wolk, A., Renkawitz-Pohl, R., **1992**. Ultrabithorax is a regulator of β3 tubulin expression in the *Drosophila* visceral mesoderm. *Development 116*, 543–554.

Hirsch, J.A., Aggarwal, A.K., **1995**. Structure of the even-skipped homeodomain complexed to AT-rich DNA: new perspectives on homeodomain specificity. *EMBO J. 14*, 6280–6291.

Hirth, F., Hartmann, B., Reichert, H., **1998**. Homeotic gene action in embryonic brain development of *Drosophila*. *Development 125*, 1579–1589.

Hirth, F., Loop, T., Egger, B., Miller, D.F.B., Kaufman, T., *et al.*, **2001**. Functional equivalence of Hox gene products in the specification of the tritocerebrum during embryonic brain development of *Drosophila*. *Development 128*, 4781–4788.

Hoey, T., Levine, M., **1988**. Divergent homeo box proteins recognize similar DNA sequences in *Drosophila*. *Nature 332*, 858–861.

Hoppler, S., Bienz, M., **1995**. Two different thresholds of wingless signaling with distinct developmental consequences in the *Drosophila* midgut. *EMBO J. 14*, 5016–5026.

Hovde, S., Abate-Shen, C., Geiger, J.H., **2001**. Crystal structure of the Msx-1 homeodomain/DNA complex. *Biochemistry 40*, 12013–12021.

Hughes, C., Kaufman, T.C., **2000**. RNAi analysis of *Deformed, proboscipedia* and *Sex combs reduced* in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the hemipteran head. *Development 127*, 3683–3694.

Hughes, C., Kaufman, T.C., **2002**. Hox genes and the evolution of the arthropod body plan. *Evol. Devel. 4*, 459–499.

Immerglück, A.K., Lawrence, P.A., Bienz, M., **1990**. Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell 62*, 261–268.

Ingham, P., Whittle, R., **1980**. *Trithorax* : a new homoeotic mutation of *Drosophila melanogaster* causing transformations of abdominal and thoracic imaginal segments. *Mol. Gen. Genet. 179*, 607–614.

Ingham, P.W., **1998**. *trithorax* and the regulation of homeotic gene expression in *Drosophila*: a historical perspective. *Int. J. Devel. Biol. 42*, 423–429.

Ingham, P.W., Martinez-Arias, A., **1986**. The correct activation of *Antennapedia* and *Bithorax* complex genes requires the *fushi tarazu* gene. *Nature 324*, 592–597.

Inoue, Y., Mito, T., Miyawaki, K., Matsushima, K., Shinmyo, Y., *et al.*, **2002**. Correlation of expression patterns of *homothorax*, *dachshund*, and *Distal-less* with the proximodistal segmentation of the cricket leg bud. *Mech. Devel. 113*, 141–148.

Irving, P., Troxler, L., Heuer, T.S., Belvin, M., Kopczynski, C., *et al.*, **2001**. A genome-wide analysis of immune responses in *Drosophila*. *Proc. Natl. Acad. Sci. USA 98*, 15119–15124.

Izpisua-Belmonte, J.C., Falkenstein, J.H., Dolle, P., Renucci, A., Dubuole, D., **1991**. Murine genes related to the *Drosophila Abd-B* homeotic gene are sequentially expressed during development of the posterior part of the body. *EMBO J. 10*, 2279–2289.

Jack, T., McGinnis, W., **1990**. Establishment of the Deformed expression stripe requires the combinatorial action of coordinate, gap and pair-rule proteins. *EMBO J. 9*, 1187–1198.

Jack, T., Regulski, M., McGinnis, W., **1988**. Pair-rule segmentation genes regulate the expression of the homeotic selector gene, *Deformed*. *Genes Devel. 2*, 635–651.

Jacobs, J.J.L., van Lohuizen, M., **1999**. Cellular memory of transcriptional states by Polycomb-group proteins. *Cell Devel. Biol. 10*, 227–235.

Jaffe, L., Ryoo, H.D., Mann, R.S., **1997**. A role for phosphorylation by casein kinase II in modulating Antennapedia activity in *Drosophila*. *Genes Devel. 11*, 1327–1340.

Jaynes, J.B., O'Farrell, P.H., **1991**. Active repression of transcription by the engrailed homeodomain protein. *EMBO J. 10*, 1427–1433.

Jockusch, E.L., Nulsen, C., Newfeld, S.J., Nagy, L.M., **2000**. Leg development in flies versus grasshoppers: differences in dpp expression do not lead to differences in the expression of downstream components of the leg patterning pathway. *Development 127*, 1617–1626.

Johnson, F.B., Krasnow, M.A., **1990**. Stimulation of transcription by an Ultrabithorax protein *in vitro*. *Genes Devel. 4*, 1044–1052.

Johnson, F.B., Parker, E., Krasnow, M.A., **1995**. Extradenticle protein is a selective cofactor for the *Drosophila* homeotics: role of the homeodomain and YPWM amino acid motif in the interaction. *Proc. Natl Acad. Sci. USA 92*, 739–743.

Jones, B., McGinnis, W., **1993**. The regulation of empty spiracles by Abdominal-B mediates an abdominal segment identity function. *Genes Devel. 7*, 229–240.

Jorgenson, E.M., Garber, R.L., **1987**. Function and misfunction of the two promoters of the *Drosophila Antennapedia* genes. *Genes Devel. 1*, 544–555.

Jürgens, G., Lehman, R., Scharding, M., Nüsslein-Volhard, C., **1986**. Segmental organization of the head in the embryo of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 195*, 359–377.

Kadonaga, J., Carner, K., Masiarz, F., Tjian, R., **1987**. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell 51*, 1079–1090.

Karch, F., Bender, W., Weiffenbach, B., **1990**. *abd-A* expression in *Drosophila* embryos. *Genes Devel. 4*, 1573–1587.

Kaufman, T.C., **1978**. Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: isolation and characterization of four new alleles of the *proboscipedia* (*pb*) locus. *Genetics 90*, 579–586.

Kaufman, T.C., Lewis, R., Wakimoto, B., **1980**. Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: the homoeotic gene complex in polytene chromosome interval 84 A–B. *Genetics 94*, 115–133.

Kaufman, T.C., Seeger, M.A., Olsen, G., **1990**. Molecular and genetic organization of the *Antennapedia* gene complex of *Drosophila melanogaster. Adv. Genet. 27*, 309–362.

Kelsh, R., Dawson, I., Akam, M., **1993**. An analysis of Abdominal-B expression in the locust *Schistocerca gregaria*. *Development 117*, 293–305.

Kennison, J.A., **1993**. Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. *Trends Genet. 9*, 75–79.

Kissinger, C.R., Liu, B.S., Martin-Blanco, E., Kornberg, T.B., Pabo, C.O., **1990**. Crystal structure of an engrailed homeodomain-DNA complex at 2.8 A resolution: a framework for understanding homeodomain-DNA interactions. *Cell 63*, 579–590.

Kmita, M., Duboule, D., **2003**. Organizing axes in time and space: 25 years of collinear tinkering. *Science 301*, 331–333.

Knoepfler, P. S., Calvo, K. R., Chen, H., Antonarakis, S. E., Kamps, M. P., **1997**. Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. *Proc. Natl Acad. Sci. USA 94*, 14553–14558.

Kondo, T., Duboule, D., **1999**. Breaking collinearity in the mouse HoxD complex. *Cell 97*, 407–417.

Kopp, A., Duncan, I., **2002**. Anteroposterior patterning in adult abdominal segments of *Drosophila*. *Devel. Biol. 242*, 15–30.

Krasnow, M.A., Saffman, E.E., Kornfeld, K., Hogness, D.S., **1989**. Transcriptional activation and repression by *Ultrabithorax* proteins in cultured *Drosophila* cells. *Cell 57*, 1031–1043.

Kuhn, D.T., Turenchalk, G., Mack, J.A., Packert, G., Kornberg, T.B., **1995**. Analysis of the genes involved in organizing the tail segments of the *Drosophila melanogaster* embryo. *Mech. Devel. 53*, 3–13.

Kuzin, B., Tillib, S., Sedkov, Y., Mizrokhi, L., Mazo, A., **1994**. The *Drosophila trithorax* gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene *forkhead*. *Genes Devel. 8*, 2478–2490.

Kuziora, M.A., McGinnis, W., **1988a**. Different transcripts of the *Drosophila Abd-B* gene correlate with distinct genetic sub-functions. *EMBO J. 7*, 3233–3244.

Kuziora, M.A., McGinnis, W., **1988b**. Autoregulation of a *Drosophila* homeotic selector gene. *Cell 55*, 477–485.

Kuziora, M.A., McGinnis, W., **1989**. A homeodomain substitution changes the regulatory specificity of the *Deformed* protein in *Drosophila* embryos. *Cell 59*, 563–571.

Kuziora, M.A., McGinnis, W., **1991**. Altering the regulatory targets of the *Deformed* protein in *Drosophila* embryos by substituting the *Abdominal-B* homeodomain. *Mech. Devel. 33*, 83–94.

Laughon, A., Scott, M.P., **1984**. Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA binding proteins. *Nature 310*, 25–31.

Laughon, A., Howell, W., Scott, M.P., **1988**. The interaction of proteins encoded by *Drosophila* homeotic and segmentation genes with specific DNA sequences. *Development 104* (Suppl.), 75–83.

Levine, M., Hafen, E., Garber, R.L., Gehring, W.J., **1983**. Spatial distribution of *Antennapedia* transcripts during *Drosophila* development. *EMBO J. 2*, 2037–2046.

Lewis, D.L., DeCamillis, M., Bennett, R.L., **2000**. Distinct roles of the homeotic genes *Ubx* and *abd-A* in beetle embryonic abdominal appendage development. *Proc. Natl Acad. Sci. USA 97*, 4504–4509.

Lewis, E.B., **1978**. A gene complex controlling segmentation in *Drosophila*. *Nature 276*, 565–570.

Lewis, E.B., **1998**. The bithorax complex: the first fifty years. *Int. J. Devel. Biol. 42*, 403–415.

Lewis, R.A., Kaufman, T.C., Denell, R.E., Tallerico, P., **1980a**. Genetic analysis of the *Antennapedia* gene complex (*ANT-C*) and adjacent chromosomal regions of *Drosophila melanogaster.* 1. Polytene chromosome segments 84B-D. *Genetics 95*, 367–381.

Lewis, R.A., Wakimoto, B.T., Denell, R.E., Kaufman, T.C., **1980b**. Genetic analysis of the *Antennapedia* gene complex (*ANT-C*) and adjacent chromosomal regions of *Drosophila melanogaster* 2. Polytene chromosome segments 84A-84B1, 2. *Genetics 95*, 383–397.

Li, X., Murre, C., McGinnis, W., **1999a**. Activity regulation of a Hox protein and a role for the homeodomain in inhibiting transcriptional activation. *EMBO J. 18*, 198–211.

Li, X., Veraksa, A., McGinnis, W., **1999b**. A sequence motif distinct from Hox binding sites controls the specificity of a Hox response element. *Development 126*, 5581–5589.

Lin, L., McGinnis, W., **1992**. Mapping functional specificity in the Dfd and Ubx homeo domains. *Genes Devel. 6*, 1071–1081.

Liu, J., Fire, A., **2000**. Overlapping roles of two Hox genes and the *exd* ortholog *ceh-20* in diversification of the *C. elegans* postembryonic mesoderm. *Development 127*, 5179–5190.

Lo, P.C.H., Skeath, J.B., Gajewski, K., Schulz, R.A., Frasch, M., **2002**. Homeotic genes autonomously specify the anteroposterior subdividsion of the *Drosophila* dorsal vessel into aorta and heart. *Devel. Biol. 251*, 307–319.

Lohmann, I., McGinnis, N., Bodmer, M., McGinnis, W., **2002**. The *Drosophila* Hox gene *Deformed* sculpts head morphology via direct regulation of the apoptosis activator *reaper*. *Cell 110*, 457–466.

Lonie, A., D'Andrea, R., Paro, R., Saint, R., **1994**. Molecular characterization of the *Polycomblike* gene of *Drosophila melanogaster*, a *trans*-acting negative regulator of homeotic gene expression. *Development 120*, 2629–2636.

Lou, L., Bergson, C., McGinnis, W., **1995**. *Deformed* expression in the *Drosophila* central nervous system is controlled by an autoactivated intronic enhancer. *Nucl. Acids Res. 23*, 3481–3487.

Lovato, T.L., Nguyen, T.P., Molina, M.R., Cripps, R.M., **2002**. The Hox gene *abdominal-A* specifies heart cell fate in the *Drosophila* dorsal vessel. *Development 129*, 5019–5027.

Macías, A., Casanova, J., Morata, G., **1990**. Expression and regulation of the *abd-A* gene of *Drosophila*. *Development 110*, 1197–1207.

Macías, A., Morata, G., **1996**. Functional hierarchy and phenotypic suppression among *Drosophila* homeotic genes: the *labial* and *empty spiracles* genes. *EMBO J. 15*, 334–343.

Mahaffey, J.W., Diederich, R.J., Kaufman, T.C., **1989**. Novel patterns of homeotic protein accumulation in the head of the *Drosophila* embryo. *Development 105*, 167–174.

Mahaffey, J.W., Griswold, C.M., Cao, Q., **2001**. The *Drosophila* genes *disconnected* and *disco-related* are redundant with respect to larval head development and accumulation of mRNAs from *Deformed* target genes. *Genetics 157*, 225–236.

Mahaffey, J.W., Jones, D.F., Hickel, J., Griswold, C.M., **1993**. Identification and characterization of a gene activated by the Deformed homeoprotein. *Development 118*, 203–214.

Mahaffey, J.W., Kaufman, T.C., **1987**. Distribution of the *Sex combs reduced* gene products in *Drosophila melanogaster. Genetics 117*, 51–60.

Mak, A., Johnson, A.D., **1993**. The carboxy-terminal tail of the homeo domain protein alpha 2 is required for function with a second homeo domain protein. *Genes Devel. 7*, 1862–1870.

Mann, R.S., Chan, S., **1996**. Extra specificity from extradenticle: the partnership between HOX and PBX;EXD homeodomain proteins. *Trends Genet. 12*, 258–262.

Marchetti, M., Fanti, L., Berioco, M., Pimpinelli, S., **2003**. Differential expression of the *Drosophila BX-C* in polytene chromosomes in cells of the larval fat bodies: a cytological approach to identifying *in vivo* targets of the homeotic Ubx, Abd-A and Abd-B proteins. *Development 130*, 3683–3689.

Markstein, M., Markstein, P., Markstein, V., Levine, M.S., **2002**. Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA 99*, 763–768.

Martin, C.H., Mayeda, C.A., Davis, C.A., Ericsson, C.L., Knafels, J.D., *et al.*, **1995**. Complete sequence of the bithorax complex of *Drosophila*. *Proc. Natl Acad. Sci. USA 92*, 8398–8402.

Martin, E.C., Adler, P.N., **1993**. The Polycomb group gene Posterior Sex Combs encodes a chromosomal protein. *Development 117*, 641–655.

Martinez, P., Amemiya, C.T., **2002**. Genomics of the HOX gene cluster. *Comp. Biochem. Physiol. B 133*, 571–580.

Martinez-Arias, A., Ingham, P.W., Scott, M.P., Akam, M.E., **1987**. The spatial and temporal deployment of *Dfd* and *Scr* transcripts throughout development of *Drosophila*. *Development 100*, 673–683.

Martinez-Arias, A., Lawrence, P.A., **1985**. Parasegments and compartments in the *Drosophila* embryo. *Nature 313*, 639–642.

Martinez-Arias, A., White, R.A.H., **1988**. *Ultrabithorax* and *engrailed* expression in *Drosophila* embryos mutant for segmentation genes of the pair-rule class. *Development 102*, 325–338.

Mastick, G.S., McKay, R., Oligino, T., Donovan, K., Lopez, A.J., **1995**. Identification of target genes regulated by homeotic proteins in *Drosophila melanogaster* through genetic selection of Ultrabithorax protein binding sites in yeast. *Genetics 139*, 349–363.

Mathies, L.D., Kerridge, S., Scott, M.P., **1994**. Role of the *teashirt* gene in *Drosophila* midgut morphogenesis: secreted proteins mediate the action of homeotic genes. *Development 120*, 2799–2809.

Mathog, D.R., **1991**. Suppression of abdominal legs in *Drosophila melanogaster. Roux's Arch Devel. Biol. 199*, 449–457.

Maurel-Zaffran, C., Chauvet, S., Jullien, N., Miassod, R., Pradel, J., *et al.*, **1999**. *nessy*, an evolutionary conserved gene controlled by Hox proteins during *Drosophila* embryogenesis. *Mech. Devel. 86*, 159–163.

McCall, K., O'Connor, M. B., Bender, W., **1994**. Enhancer traps in the *Drosophila* bithorax complex mark parasegmental domains. *Genetics 138*, 389–399.

McCormick, A., Coré, N., Kerridge, S., Scott, M.P., **1995**. Homeotic response elements are tightly linked to tissue-specific elements in a transcriptional enhancer of the *teashirt* gene. *Development 121*, 2799–2812.

McDonald, M.J., Rosbash, M., **2001**. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell 107*, 567–578.

McGinnis, N., Kuziora, M.A., McGinnis, W., **1990**. Human *Hox-4.2* and *Drosophila Deformed* encode similar regulatory specificities in *Drosophila* embryos and larvae. *Cell 63*, 969–976.

McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., Gehring, W.J., **1984**. A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and bithorax complexes. *Nature 308*, 428–433.

McGinnis, N., Ragnhildstveit, E., Veraksa, A., McGinnis, W., **1998**. A cap'n'collar protein isoform contains a selective Hox repressor function. *Development 125*, 4553–4564.

McGinnis, W., Garber, R.L., Wirz, J., Kuroiwa, A., Gehring, W.J., **1984b**. A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell 37*, 403–408.

McGinnis, W., Hart, C.P., Gehring, W.J., Ruddle, F.H., **1984a**. Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of *Drosophila*. *Cell 38*, 675–680.

McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., Gehring, W.J., **1984c**. A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes. *Nature 308*, 428–433.

Megraw, T.L., Li, K., Kao, L.R., Kaufman, T.C., **1999**. The centrosomin protein is required for centrosome assembly and function during cleavage in *Drosophila*. *Development 126*, 2829–2839.

Merabet, S., Catala, F., Pradel, J., Graba, Y., **2002**. A green fluorescent protein reporter genetic screen that identifies modifiers of Hox gene function in the *Drosophila* embryo. *Genetics 162*, 189–202.

Merabet, S., Kambris, Z., Capovilla, M., Bérenger, H., Pradel, J., *et al.*, **2003**. The hexapeptide and linker regions of the AbdA Hox protein regulate its activation and repressive functions. *Devel. Cell 4*, 761–768.

Merrill, V.K.L., Diederich, R.J., Turner, F.R., Kaufman, T.C., **1989**. A genetic and developmental analysis of

mutations in *labial*, a gene necessary for proper head formation in *Drosophila melanogaster. Devel. Biol. 135*, 376–391.

Merrill, V.K.L., Turner, F.R., Kaufman, T.C., **1987**. A genetic and developmental analysis of mutations in the *Deformed* locus in *Drosophila melanogaster. Devel. Biol. 122*, 379–395.

Mihaly, J., Hogga, I., Barges, S., Galloni, M., Mishra, R.K., *et al.*, **1998**. Chromatin domain boundaries in the Bithorax complex. *Cell. Mol. Life Sci. 54*, 60–70.

Mihaly, J., Hogga, I., Gausz, J., Gyurkovics, H., Karch, F., **1997**. *In situ* detection of the *Fab-7* region of the bithorax complex into a chromatin domain boundary and a *Polycomb*-response element. *Development 124*, 1809–1820.

Miller, D.F.B., Holtzman, S.L., Kalkbrenner, A.L., Kaufman, T., **2001a**. Homeotic complex (Hox) gene regulation and homeosis in the mesoderm of the *Drosophila melanogaster* embryo: the roles of signal transduction and cell autonomous regulation. *Mech. Devel. 102*, 17–32.

Miller, D.F.B., Rogers, B.T., Kalkbrenner, A.L., Hamilton, B., Holtzman, S.L., *et al.*, **2001b**. Cross-regulation of Hox genes in the *Drosophila melanogaster* embryo. *Mech. Devel. 102*, 3–16.

Mlodzik, M., Fjose, A., Gehring, W.J., **1988**. Molecular structure and spatial expression of a homeobox gene from the labial region of the Antennapedia-complex. *EMBO J. 7*, 2569–2578.

Mohler, J., Mahaffey, J.W., Deutsch, E., Vani, K., **1995**. Control of *Drosophila* head segment identity by the bZip homeotic gene *cnc*. *Development 121*, 237–247.

Morata, G., **2001**. How *Drosophila* appendages develop. *Nature Rev. Mol. Cell. Biol. 2*, 89–97.

Morata, G., Kerridge, S., **1981**. Sequential functions of the bithorax complex of *Drosophila*. *Nature 290*, 778–781.

Moskow, J., Bullrich, F., Huebner, K., Daar, I., Buchberg, A., **1995**. Meis1, a PBX1-related homeobox gene involved in myeloid leukemia in BXH-2 mice. *Mol. Cell. Biol. 15*, 5434–5443.

Müller, J., **2000**. Transcriptional control: the benefits of selective insulation. *Curr. Biol. 10*, R241–R244.

Müller, J., Gaunt, S., Lawrence, P., **1995**. Function of the Polycomb protein is conserved in mice and flies. *Development 121*, 2847–2852.

Müller, M., Affolter, M., Leupin, W., Otting, G., Wüthrich, K., *et al.*, **1988**. Isolation and sequence-specific DNA binding of the Antennapedia homeodomain. *EMBO J. 7*, 4299–4304.

Nagata, R., Suzuki, Y., Ueno, K., Kokubo, H., Xu, X., *et al.*, **1996**. Developmental expression of the *Bombyx* Antennapedia homologue and homeotic changes in the Nc mutant. *Genes Cells 1*, 555–568.

Nagy, L.M., Carroll, S., **1994**. Conservation of *wingless* patterning functions in the short germ embryos of *Tribolium castaneum*. *Nature 367*, 460–463.

Negre, B., Ranz, J.M., Casals, F., Caceres, M., Ruiz, A., **2003**. A new split of the *Hox* gene complex in *Drosophila*: relocation and evolution of the gene *labial*. *Mol. Biol. Evol. 20*, 2042–2054.

Nelson, H.B., Laughon, A., **1990**. The DNA binding specificity of the *Drosophila* fushi tarazu protein: a possible role for DNA bending in homeodomain recognition. *New Biol. 2*, 171–178.

Nie, W., Stronach, B., Panganiban, G., Shippy, T., Brown, S., *et al.*, **2001**. Molecular characterization of *Tclabial* and the 3′ end of the *Tribolium* homeotic complex. *Devel. Genes Evol. 211*, 244–251.

O'Conner, M.B., Binary, R., Perkins, L.A., Bender, W., **1988**. Alternative RNA products from the *Ultrabithorax* domain of the bithorax complex. *EMBO J. 7*, 435–445.

O'Hara, E., Cohen, B., Cohen, S.M., McGinnis, W., **1993**. *Distal-less* is a downstream gene of *Deformed* required for ventral maxillary identity. *Development 117*, 847–856.

Ohkuma, Y., Horikoshi, M., Roeder, R.G., Desplan, C., **1990**. Binding site-dependent direct activation and repression of *in vitro* transcription by *Drosophila* homeodomain proteins. *Cell 61*, 475–484.

Otting, G., Qian, Y.Q., Billeter, M., Müller, M., Affolter, M., *et al.*, **1990**. Protein–DNA contacts in the structure of a homeodomain–DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J. 9*, 3085–3092.

Pankratz, M.J., Jaeckle, H., **1990**. Making stripes in the *Drosophila* embryo. *Trends Genet. 6*, 287–292.

Panzer, S., Weigel, D., Beckendorf, S.K., **1992**. Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes. *Development 114*, 49–57.

Paro, R., Zink, B., **1992**. The *Polycomb* gene is differentially regulated during oogenesis and embryogenesis of *Drosophila melanogaster. Mech. Devel. 40*, 37–46.

Passner, J.M., Ryoo, H.D., Shen, L., Mann, R.S., Aggarwal, A., **1999**. Structure of a DNA-bound Ultrabithorax–Extradenticle homeodomain complex. *Nature 397*, 714–719.

Patel, N.H., **1994**. Developmental evolution: insights from studies of insect segmentation. *Science 266*, 581–590.

Patel, N.H., Ball, E.E., Goodman, C.S., **1992**. Changing role of *even-skipped* during the evolution of insect pattern formation. *Nature 357*, 339–342.

Patel, N.H., Condron, B.G., Zinn, K., **1994**. Pair-rule expression patterns of *even-skipped* are found in both short and long-germ beetles. *Nature 367*, 429–434.

Pattatucci, A.M., Kaufman, T.C., **1991**. The homeotic gene *Sex combs reduced* of *Drosophila melanogaster* is differentially regulated in the embryonic and imaginal stages of development. *Genetics 129*, 443–461.

Pattatucci, A.M., Otteson, D.C., Kaufman, T.C., **1991**. A functional and structural analysis of the *Sex combs reduced* locus of *Drosophila melanogaster. Genetics 129*, 423–441.

Pederson, J.A., LaFollette, J.W., Gross, V. A., McGinnis, W., Mahaffey, J.W., **2000**. Regulation by homeoproteins: a comparison of deformed-responsive elements. *Genetics 156*, 677–686.

Pederson, J.D., Kiehart, D.P., Mahaffey, J.W., **1996**. The role of HOM-C genes in segmental transformations: reexamination of the *Drosophila Sex combs reduced* embryonic rhenotype. *Devel. Biol. 180*, 131–142.

Peifer, M., Wieschaus, E., **1990**. Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes Devel. 4*, 1209–1223.

Pelaz, S., Urquía, N., Morata, G., **1993**. Normal and ectopic domains of the homeotic gene *Sex combs reduced* of *Drosophila*. *Development 117*, 917–923.

Percival-Smith, A., Müller, M., Affolter, M., Gehringm, W.J., **1990**. The interaction with DNA of wild-type and mutant *fushi tarazu* homeodomains. *EMBO J. 9*, 3967–3974.

Percival-Smith, A., Weber, J., Gilfoyle, E., Wilson, P., **1997**. Genetic characterization of the role of the two HOX proteins, Proboscipedia and Sex Combs Reduced, in determination of adult antennal tarsal, maxillary palp, and proboscis identities in *Drosophila melanogaster*. *Development 124*, 5049–5062.

Peterson, M.D., Rogers, B.T., Popadić, A., Kaufman, T.C., **1999**. The embryonic expression pattern of *labial*, posterior homeotic complex genes and the *teashirt* homologue in an apterygote insect. *Devel. Genes Evol. 209*, 77–90.

Phelan, M.L., Rambaldi, I., Featherstone, M.S., **1995**. Cooperative interactions between Hox and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol. 15*, 3989–3997.

Pinsonneault, J., Florence, B., Vaessin, H., McGinnis, W., **1997**. A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. *EMBO J. 16*, 2032–2042.

Pöpperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., *et al.*, **1995**. Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell 81*, 1031–1042.

Powers, T.P., Hogan, J., Ke, Z., Dymbrowski, K., Wang, X., *et al.*, **2000**. Characterization of the Hox cluster from the mosquito *Anopheles gambiae* (Diptera: Culicidae). *Evol. Devel. 2*, 311–325.

Pribyl, L.G., Watson, D.K., McWilliams, M.J., Ascione, R., Papas, T.S., **1988**. The *Drosophila ets-2* gene: molecular structure, chromosomal location, and developmental expression. *Devel. Biol. 127*, 45–53.

Pultz, M.A., Diederich, R.J., Cribbs, D.L., Kaufman, T.C., **1988**. The *proboscipedia* locus of the Antennapedia Complex: a molecular and genetic analysis. *Genes Devel. 2*, 901–920.

Qian, S., Capovilla, M., Pirrotta, V., **1993**. Molecular mechanisms of pattern formation by the BRE enhancer of the *Ubx* gene. *EMBO J. 12*, 3865–3877.

Qian, Y.Q., Billeter, M., Otting, G., Müller, M., Gehring, W.J., *et al.*, **1989**. The structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell 59*, 573–580.

Qian, Y.Q., Otting, G., Furukubo-Tokunaga, K., Affolter, M., Gehring, W.J., *et al.*, **1992**. NMR structure determination reveals that the homeodomain is connected through a flexible linker to the main body in the *Drosophila* Antennapedia protein. *Proc. Natl Acad. Sci. USA 89*, 10738–10742.

Qian, Y.Q., Resendez-Perez, D., Gehring, W.H., Wüthrich, K., **1994**. The des(1–6)Antennapedia homeodomain: comparison of the NMR solution structure and the DNA-binding affinity with the intact Antennapedia homeodomain. *Proc. Natl Acad. Sci. USA 91*, 4091–4095.

Quinn, T.C., Craig, G.B., Jr, **1971**. Phenogenetics of the homeotic mutant *proboscipedia* in *Aedes albopictus*. *J. Hered. 62*, 3–12.

Rauskolb, C., Peifer, M., Wieschaus, E., **1993**. *extradenticle*, a regulator of homeotic gene activity, is a homologue of the homeobox-containing human protooncogene *pbx1*. *Cell 74*, 1101–1112.

Rauskolb, C., Smith, K.M., Peifer, M., Wieschaus, E., **1995**. *extradenticle* determines segmental identities throughout *Drosophila* development. *Development 121*, 2117–2125.

Rauskolb, C., Wieschaus, E., **1994**. Coordinate regulation of downstream genes by extradenticle and the homeotic selector proteins. *EMBO J. 13*, 3561–3569.

Regulski, M., Dessain, S., McGinnis, N., McGinnis, W., **1991**. High-affinity binding sites for the *Deformed* protein are required for the function of an autoregulatory enhancer of the *Deformed* gene. *Genes Devel. 5*, 278–286.

Regulski, M., Harding, K., Kostriken, R., Karch, F., Levine, M., *et al.*, **1985**. Homeo box genes of the Antennapedia and Bithorax complexes of *Drosophila*. *Cell 43*, 71–80.

Regulski, M., McGinnis, N., Chadwick, R., McGinnis, W., **1987**. Developmental and molecular analysis of *Deformed*, a homeotic gene controlling *Drosophila* head development. *EMBO J. 6*, 767–777.

Reinitz, J., Levine, M., **1990**. Control of the initiation of homeotic gene expression by the gap genes giant and tailless in *Drosophila*. *Devel. Biol. 140*, 57–72.

Reuter, R., Panganiban, G.E.F., Hoffman, F.M., Scott, M.P., **1990**. Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development 110*, 1031–1040.

Reuter, R., Scott, M.P., **1990**. Expression and function of the homoeotic genes *Antennapedia* and *Sex combs reduced* in the embryonic midgut of *Drosophila*. *Development 109*, 289–303.

Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M., Mann, R.S., **1997**. Nuclear translocation of *extradenticle* requires *homothorax*, which encodes *an extradenticle*-related homeodomain protein. *Cell 91*, 171–183.

Riley, P.D., Carroll, S.B., Scott, M.P., **1987**. The expression and regulation of Sex combs reduced protein in *Drosophila* embryos. *Genes Devel. 1*, 716–730.

Robertson, L.K., Bowling, D.B., Mahaffey, J.P., Imiolczyk, B., Mahaffey, J.W., **2004**. An interactive network of zinc-finger proteins contributes to regionalization of the *Drosophila* embryo and establishes the domains of HOM-C protein function. *Development 131*, 2781–2789.

Röder, L., Vola, C., Kerridge, S., **1992**. The role of the *teashirt* gene in trunk segmental identity in *Drosophila*. *Development 115*, 1017–1033.

Rogers, B.T., Kaufman, T.C., **1997**. Structure of the insect head in ontogeny and phylogeny: a view from *Drosophila*. *Int. Rev. Cytol. 74*, 1–84.

Rogers, B.T., Peterson, M.D., Kaufman, T.C., **1997**. Evolution of the insect body plan as revealed by the *Sex combs reduced* expression pattern. *Development 124*, 149–157.

Rogers, B.T., Peterson, M.D., Kaufman, T.C., **2002**. The development and evolution of insect mouthparts as revealed by the expression patterns of gnathocephalic genes. *Evol. Devel. 4*, 96–110.

Ronshaugen, M., McGinnis, N., McGinnis, W., **2002**. Nature. Hox protein mutation and macroevolution of the insect body plan. *Nature 415*, 914–917.

Rusch, D.B., Kaufman, T.C., **2000**. Regulation of *proboscipedia* in *Drosophila* by homeotic selector genes. *Genetics 156*, 183–194.

Rusten, T. E., Cantera, R., Urban, J., Technau, G., Kafatos, F. C., *et al.*, **2001**. *Spalt* restricts EGFR mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila*. *Development 128*, 711–722.

Ruvkun, G., Hobert, O., **1998**. The taxonomy of developmental control in *Caenorhabditis elegans*. *Science 282*, 2033–2041.

Ryoo, H.D., Mann, R.S., **1999**. The control of trunk Hox specificity and activity by Extradenticle. *Genes Devel. 13*, 1704–1716.

Ryoo, H.D., Marty, T., Casares, F., Affolter, M., Mann, R.S., **1999**. Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development 126*, 5137–5148.

Samson, M., Jackson-Grusby, L., Brent, R., **1989**. Gene activation and DNA binding by *Drosophila* Ubx and Abd-A proteins. *Cell 57*, 1045–1052.

Sánchez-Herrero, E., Akam, M., **1989**. Spatially ordered transcription of regulatory DNA in the bithorax complex of *Drosophila*. *Development 107*, 321–329.

Sánchez-Herrero, E., Vernós, R.M., Morata, G., **1985**. Genetic organization of *Drosophila* bithorax complex. *Nature 313*, 108–113.

Sato, R., Hayes, P.H., Denell, R.E., **1985**. Homeosis in *Drosophila*: roles and spatial patterns of expression of the *Antennapedia* and *Sex combs reduced* loci in embryogenesis. *Devel. Biol. 111*, 171–192.

Schneuwly, S., Kuroiwa, A., Baumgartner, P., Gehring, W.J., **1986**. Structural organization and sequence of the homeotic gene *Antennapedia* of *Drosophila melanogaster. EMBO J. 5*, 733–739.

Schöck, F., Reischl, J., Wimmer, E., Taubert, H., Purnell, B.A., *et al.*, **2000**. Phenotypic suppression of *empty spiracles* is prevented by *buttonhead*. *Nature 405*, 351–354.

Scott, M.P., Weiner, A.J., **1985**. Structural relationships among genes that control development: sequence homology between the *Antennapedia*, *Ultrabithorax* and *fushi tarazu* loci of *Drosophila*. *Proc. Natl Acad. Sci. USA 81*, 4115–4119.

Scott, M.P., Weiner, A.J., Hazelrigg, T.I., Polisky, B.A., Pirrotta, V., *et al.*, **1983**. The molecular organization of the *Antennapedia* locus of *Drosophila*. *Cell 35*, 763–776.

Sedkov, Y., Tillib, S., Mizrokhi, L., Mazo, A., **1994**. The bithorax complex is regulated by *trithorax* earlier during *Drosophila* embryogenesis than is the Antennapedia complex, correlating with a bithorax like expression pattern of distinct early *trithorax* transcripts. *Development 120*, 1907–1917.

Sharpe, J., Nonchev, S., Gould, A., Whiting, J., Krumlauf, R., **1998**. Selectivity, sharing and competitive interactions in the regulation of *Hoxb* genes. *EMBO J. 17*, 1788–1798.

Shen, W.F., Rozenfeld, S., Lawrence, H.J., Largman, C., **1997**. The Abd-B-like Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. *J. Biol. Chem. 272*, 8198–8206.

Shimell, M.J., Peterson, A.J., Burr, J., Simon, J.A., O'Connor, M.B., **2000**. Functional analysis of repressor binding sites in the *iab-2* regulatory region of the *abdominal-A* homeotic gene. *Devel. Biol. 218*, 38–52.

Shimell, M.J., Simon, J., Bender, W., O'Connor, M.B., **1994**. Enhancer point mutation results in a homeotic transformation in *Drosophila*. *Science 264*, 968–971.

Shippy, T.D., Brown, S.J., Denell, R.E., **1998**. Molecular characterization of the *Tribolium abdominal-A* ortholog and implications for the products of the *Drosophila* gene. *Devel. Genes Evol. 207*, 446–452.

Shippy, T.D., Guo, J., Brown, S.J., Beeman, R.W., Denell, R.E., **2000**. Analysis of *maxillopedia* expression pattern and larval cuticular phenotype in wild-type and mutant *Tribolium*. *Genetics 155*, 721–731.

Simon, J., **1995**. Locking in stable states of gene expression: transcriptional control during *Drosophila* development. *Curr. Opin. Cell. Biol. 7*, 376–385.

Simon, J., Chiang, A., Bender, W., **1992**. Ten different *Polycomb* group genes are required for spatial control of the *abdA* and *AbdB* homeotic products. *Development 114*, 493–505.

Simon J., Chiang A., Bender W., Shimell M.J., O'Conner M., **1995**. Elements of the *Drosophila* Bithorax complex that mediate repression by *Polycomb* group products. *Devel. Biol.* 158, 131–144.

Simon, J., Peifer, M., Bender, W., O'Connor, M., **1990**. Regulatory elements of the Bithorax complex that control expression along the anterior–posterior axis. *EMBO J. 9*, 3945–3956.

Simon, J. A., Tamkun, J. W., **2002**. Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. *Curr. Opin. Genet. Devel. 12*, 210–218.

Slack, J., **1984**. A Rosetta stone for pattern formation in animals? *Nature 310*, 364–365.

Small, S., Levine, M., **1991**. The initiation of pair-rule stripes in the *Drosophila* blastoderm. *Curr. Opin. Genet. Devel. 1*, 255–260.

Smolik-Utlaut, S.M., **1990**. Dosage requirements of *Ultrabithorax* and *bithoraxoid* in the determination of segment identity in *Drosophila melanogaster. Genetics 124*, 357–366.

Sommer, R.J., Tautz, D., **1993**. Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature 361*, 448–450.

Sprules, T., Green, N., Featherstone, M., Gehring, K., **2003**. Lock and key binding of the HOX YPWM peptide to the PBX Homeodomain. *J. Biol. Chem. 278*, 1053–1058.

St. Johnston, D., Nüsslein-Volhard, C., **1991**. The origin of pattern and polarity in the *Drosophila* embryo. *Cell 68*, 201–219.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., Bate, M., **1994**. *dpp* induces mesodermal gene expression in *Drosophila*. *Nature 372*, 783–786.

Stathopoulos, A., Van Drenth, M., Erives, A., Markstein, M., Levine, M., **2001**. Whole-genome analysis of dorsal–ventral patterning in the *Drosophila* embryo. *Cell 111*, 687–701.

Stauber, M., Jackle, H., Schmidt-Ott, U., **1999**. The anterior determinant bicoid of *Drosophila* is a derived *Hox* class 3 gene. *Proc. Natl Acad. Sci. USA 96*, 3786–3789.

Steller, H., Fischbach, K.F., Rubin, G.M., **1987**. *Disconnected*: a locus required for neuronal pathway formation in the visual system of *Drosophila*. *Cell 50*, 1139–1153.

Struhl, G., **1981**. A homoeotic mutation transforming leg to antenna in *Drosophila*. *Nature 13*, 635–638.

Struhl, G., **1982**. Genes controlling segmental specification in the *Drosophila* thorax. *Proc. Natl Acad. Sci. USA 79*, 7380–7384.

Struhl, G., White, R.A.H., **1985**. Regulation of the *Ultrabithorax* gene of *Drosophila* by the other bithorax complex genes. *Cell 43*, 507–519.

Strutt, D.I., White, R.A.H., **1994**. Characterisation of T48, a target of homeotic gene regulation in *Drosophila* embryogenesis. *Mech. Devel. 46*, 27–39.

Stuart, J.J., Brown, S.J., Beeman, R.W., Denell, R.E., **1991**. A deficiency of the homeotic complex of the beetle *Tribolium*. *Nature 350*, 72–74.

Stuart, J.J., Brown, S.J., Beeman, R.W., Denell, R.E., **1993**. The *Tribolium* homeotic gene *Abdominal* is homologous to *abdominal-A* of the *Drosophila* bithorax complex. *Development 117*, 233–243.

Su, M., Venkatesh, T.V., Wu, X., Golden, K., Bodmer, R., **1999**. The pioneer gene, *apontic*, is required for morphogenesis and function of the *Drosophila* heart. *Mech. Devel. 80*, 125–132.

Sulston, I.A., Anderson, K.V., **1996**. Embryonic patterning mutants in *Tribolium castaneum*. *Development 122*, 805–814.

Sulston, I.A., Anderson, K.V., **1998**. Altered patterns of gene expression in *Tribolium* segmentation mutants. *Devel. Genet. 23*, 56–64.

Sun, B., Hursh, D.A., Jackson, D., Beachy, P.A., **1995**. Ultrabithorax protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. *EMBO J. 14*, 520–535.

Suzuki, Y., Palopoli, M.F., **2001**. Evolution of insect abdominal appendages: are prolegs homologous or convergent traits? *Devel. Genes Evol. 211*, 486–492.

Treisman, J., Gönczy, P., Vashishtha, M., Harris, E., Desplan, C., **1989**. A single amino acid can determine the DNA binding specificity of homeodomain proteins. *Cell 59*, 553–562.

Tremml, G., Bienz, M., **1989**. Homeotic gene expression in the visceral mesoderm of *Drosophila* embryos. *EMBO J. 8*, 2677–2685.

Tucker-Kellogg, L., Rould, M.A., Chambers, K.A., Ades, S.E., Sauer, R.T., *et al.*, **1997**. *Engrailed* (Gln50 → Lys) homeodomain-DNA complex at 1.9 Å resolution: structural basis for enhanced affinity and altered specificity. *Structure 5*, 1047–1054.

Tupler, R., Perini, G., Green, M.R., **2001**. Expressing the human genome. *Nature 409*, 832–833.

Ueno, K., Hui, C.C., Fukuta, M., Suzuki, Y., **1992**. Molecular analysis of the deletion mutants in the E homeotic complex of the silkworm *Bombyx mori*. *Development 114*, 555–563.

Vachon, G., Cohen, B., Preifle, C., McGuffin, M.E., Botas, J., *et al.*, **1992**. Homeotic genes of the bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene *Distalless*. *Cell 71*, 437–450.

van der Hoeven, F., Zakany, J., Duboule, D., **1996**. Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. *Cell 85*, 1025–1035.

van Dijk, M.A., Murre, C., **1994**. *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell 78*, 617–624.

Villee, C., **1944**. Phenogenetic studies of the homoeotic mutants of *Drosophila melanogaster.* 2. The effects of temperature on the expression of *proboscipedia*. *J. Exp. Zool. 96*, 85–102.

Volk, T., VijayRaghavan, K., **1994**. A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo. *Development 120*, 59–70.

Von Allmen, G., Hogga, I., Spierer, A., Karch, F., **1996**. Splits in fruit fly *Hox* gene complexes. *Nature 380*, 116.

Wagner-Bernholz, J.T., Wilson, C., Gibson, G., Schuh, R., Gehring, W.J., **1991**. Identification of target genes of the homeotic gene *Antennapedia* by enhancer activity. *Genes Devel. 5*, 2467–2480.

Wakimoto, B., Kaufman, T.C., **1981**. Analysis of larval segmentation in lethal genotypes associated with the *Antennapedia* gene complex in *Drosophila melanogaster*. *Devel. Biol. 81*, 51–64.

Wakimoto, B.T., Turner, F.R., Kaufman, T.C., **1984**. Defects in embryogenesis in mutants associated with the *Antennapedia* gene complex of *Drosophila melanogaster*. *Devel. Biol. 102*, 147–172.

Walldorf, U., Binner, P., Fleig, R., **2000**. Hox genes in the honeybee *Apis mellifera*. *Devel. Genes Evol. 210*, 483–492.

Walldorf, U., Gehring, W.J., **1992**. *Empty spiracles*, a gap gene containing a homeobox involved in *Drosophila* head development. *EMBO J. 11*, 2247–2259.

Walter, J., Biggin, M.D., **1996**. DNA binding specificity of two homeodomain proteins *in vitro* and in *Drosophila* embryos. *Proc. Natl Acad. Sci. USA 93*, 2680–2685.

Walter, J., Dever, C.A., Biggin, M.D., **1994**. Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in *Drosophila* embryos. *Genes Devel. 8*, 1678–1692.

Weatherbee, S.D., Halder, G., Kim, J., Hudson, A., Carroll, S., **1998**. Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere. *Genes Devel. 12*, 1474–1482.

Weatherbee, S.D., Nijhout, H.F., Grunert, L.W., Halder, G., Galant, R., *et al.*, **1999**. Ultrabithorax function in butterfly wings and the evolution of insect wing patterns. *Curr. Biol. 9*, 109–115.

Wedeen, C., Harding, K., Levine, M., **1986**. Spatial regulation of *Antennapedia* and *bithorax* gene expression by the *Polycomb* locus in *Drosophila*. *Cell 44*, 739–748.

Wharton, K.A., Yedvobnick, B., Finnerty, V.G., Artavanis-Tsakonas, S., **1985**. opa: a novel family of transcribed repeats shared by the *Notch* locus and other developmentally regulated loci in *D. melanogaster*. *Cell 40*, 55–62.

White, R.A., Wilcox, M., **1984**. Protein products of the bithorax complex in *Drosophila*. *Cell 39*, 163–171.

White, R.A.H., Aspland, S.E., Brookman, J.J., Clayton, L., Sproat, G., **2000**. The design and analysis of a homeotic response element. *Mech. Devel. 91*, 217–226.

White, R.A.H., Lehmann, R., **1986**. A gap gene, *hunchback*, regulates the spatial expression of *Ultrabithorax*. *Cell 47*, 311–321.

Wiellette, E.L., McGinnis, W., **1999**. *Hox* genes differentially regulate *Serrate* to generate segment-specific structures. *Development 126*, 1985–1995.

Wilkins, A.S., **1986**. Homeo box fever, extrapolation and developmental biology. *BioEssays 4*, 147–148.

Wilson, D.S., Guenther, B., Desplan, C., Kuriyan, J., **1995**. High resolution crystal structure of a paired (Pax) class cooperative homeodomain dimer on DNA. *Cell 82*, 709–719.

Wilson, D.S., Sheng, G., Jun, S., Desplan, C., **1996**. Conservation and diversification in homeodomain-DNA interactions: a comparative genetic analysis. *PNAS 93*, 6886–6891.

Wimmer, E.A., Jäckle, H., Pfeifle, C., Cohen, S.M., **1993**. A *Drosophila* homologue of human Sp1 is a head-specific segmentation gene. *Nature 366*, 690–694.

Wirz, J., Fessler, L.I., Gehring, W.J., **1986**. Localization of the *Antennapedia* protein in *Drosophila* embryos and imaginal discs. *EMBO J. 5*, 3327–3334.

Wolff, C., Sommer, R., Schroder, R., Glaser, G., Tautz, D., **1995**. Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*. *Development 121*, 4227–4236.

Wu, X., Vasisht, V., Kosman, D., Reinitz, J., Small, S., **2001**. Thoracic patterning by the *Drosophila* gap gene *hunchback*. *Devel. Biol. 237*, 79–92.

Yang, D., Lu, H., Hong, Y., Jinks, T.M., Estes, P.A., *et al.*, **2001**. Interpretation of X chromosome dose at *Sex-lethal* requires non-E-box sites for the basic helix–loop–helix proteins SISB and Daughterless. *Mol. Cell. Biol. 21*, 1581–1592.

Yao, L., Liaw, G., Pai, C., Sun, Y., **1999**. A common mechanism for antenna-to-leg transformation in *Drosophila*: suppression of *homothorax* transcription by four HOM-C genes. *Devel. Biol. 211*, 268–276.

Zavortink, M., Sakonju, S., **1989**. The morphogenetic and regulatory functions of the *Drosophila Abdominal-B* gene are encoded in overlapping RNAs transcribed from separate promoters. *Genes Devel. 3*, 1969–1981.

Zeng, C., Pinsonneault, J., Gellon, G., McGinnis, N., McGinnis, W., **1994**. Deformed protein binding sites and cofactor binding sites are required for the function of a small segment-specific regulatory element in *Drosophila* embryos. *EMBO J. 13*, 2362–2377.

Zeng, W., Andrew, D.J., Mathies, L.D., Horner, M.A., Scott, M.P., **1993**. Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity. *Development 118*, 339–352.

Zhou, J., Levine, M., **1999**. A novel *cis*-regulatory element, the PTS, mediates an anti-insulator activity in the *Drosophila* embryo. *Cell 99*, 567–575.

Zhu, A., Kuziora, M.A., **1996**. Functional domains in the Deformed protein. *Development 122*, 1577–1587.

Zink, B., Paro, R., **1989**. *In vivo* binding pattern of a *trans*-regulator of homeotic genes in *Drosophila melanogaster*. *Nature 337*, 468–471.

Relevant Website

http://flybase.org – The FlyBase database of the *Drosophila* genome

1.9 *Drosophila* Limb Development

U Weihe, M Milán, and S M Cohen, European Molecular Biology Laboratory, Heidelberg, Germany

1.9.1. *Drosophila* Limb Development as a Model System

A central question in developmental biology is how growth and patterning of tissues are controlled at a molecular and genetic level. *Drosophila melanogaster* has become an important model system to approach this problem because of its suitability for genetic and molecular manipulations and its well-described developmental biology. Systematic genetic screens for loss-of-function mutations (Nüsslein-Volhard and Wieschaus, 1980), gain-of-function phenotypes (Rorth, 1996; Rorth *et al.*, 1998), and the detection of gene expression patterns by enhancer trapping (Bellen *et al.*, 1989; Calleja *et al.*, 1996) have revealed many of the genes involved in developmentally important processes (for a recent review, see **Chapter 1.8**). In addition, the completion of the genomic sequencing project for *Drosophila melanogaster* (Adams *et al.*, 2000) and, recently, for *D. pseudoobscura,* makes it possible to employ reverse genetic approaches such as RNA-mediated interference (Kennerdell and Carthew, 1998) and targeted gene disruption (Rong and Golic, 2000; Rong *et al.*, 2002) as well as genome-wide expression analyses (Furlong *et al.*, 2001; Jasper *et al.*, 2001; Arbeitman *et al.*, 2002; Klebes *et al.*, 2002; Butler *et al.*, 2003) to address a wide variety of questions concerning the developmental biology of the fruitfly and related insects.

The appendages of *Drosophila* have proven to be ideal models for the investigation of limb development. Like the appendages of vertebrates, they develop as outgrowths of the body wall (although these outgrowths are initially topologically inside the fly embryo/larva). Early in embryonic development, imaginal cells (i.e., cells that contribute to the adult animal, the "imago") segregate from their neighbors by invagination from the ectoderm (Bate and Martinez-Arias, 1991). Later, these clusters of cells separate into ventral and dorsal appendage precursors (Wieschaus and Gehring, 1976; Cohen *et al.*, 1991, 1993; Averof and Cohen, 1997) and form saclike structures called imaginal discs (**Figure 1**). Although each disc is a continuous epithelial monolayer, opposite sides of the sac acquire different properties. Most structures of the adult limb and of the neighboring body wall are derived from one side of the sac, which forms a highly folded pseudostratified epithelium (review: Cohen, 1993). It is in this epithelium that the major patterning events take place. The other side of the sac forms a squamous epithelium called the peripodial membrane, which has recently been reported to contribute to some aspects of patterning and growth

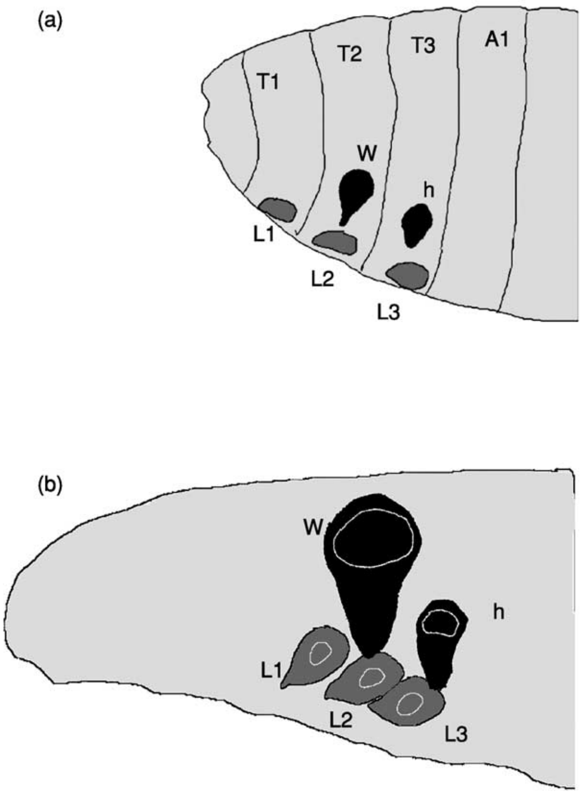


Figure 1 Specification of imaginal discs. (a) Drawing of the anterior half of a stage 17 *Drosophila* embryo (lateral view). Primordia of the imaginal discs are shown in dark gray (ventral appendages) or black (dorsal appendages). T1–3, thoracic segments 1-3; A1, abdominal segment 1; w, wing disc; h, haltere disc; L1–3 – first, second, and third leg discs. (b) Drawing of a third instar larva, showing the different imaginal discs.

control in the pseudostratified epithelial layer (Cho *et al*., 2000; Gibson and Schubiger, 2000, 2001; Ramirez-Weber and Kornberg, 2000).

Imaginal discs grow considerably during larval stages by exponential cell proliferation (García-Bellido and Merriam, 1971a; Madhavan and Schneiderman, 1977). The wing imaginal disc consists of ~50 cells at the end of embryogenesis and grows to ~50 000 cells at pupariation. Patterning and organ size regulation are, in principle, disc-autonomous processes even though disc growth requires the input of growth factors and hormones from nonimaginal tissues (review: Bryant and Simpson, 1984). *Drosophila* as a higher dipteran insect undergoes complete metamorphosis. During this process the primordium extends along its proximal–distal axis by turning inside out through the lumen (so that the appendages come to lie topologically outside the body wall after disc eversion). Actual differentiation of many adult structures (such as wing margin sensory organs, etc.) does not begin until metamorphosis. However, the structures of the adult organ reflect patterning and growth processes that occur during development.

1.9.2. Developmental Subdivisions of the Limb Primordia

The establishment of the three major axes of the limbs takes place during imaginal disc development. The primordia receive the patterning cues needed to generate an anterior–posterior, dorsal–ventral, and proximal–distal axes (**Figure 2**). Diffusible signals termed morphogens (Turing, 1952) provide cells within the epithelium with positional information (Wolpert, 1969). Axis formation involves a variety of different responses to this positional information. Changes in gene expression control cell identity, with concomitant effects on growth and differentiation.

The establishment of the limb axes depends on subdivision of their primordia into distinct functional units. The first subdivision occurs in embryogenesis in the epithelial cell layer from which the discs derive and so is inherited by the nascent disc primordia (Cohen *et al*., 1993). Subsequent subdivisions arise *de novo* in the imaginal discs during larval stages. Segregation of these cell populations seems to involve differences in cell affinity (reviews: Dahmann and Basler, 1999; McNeill, 2000; Irvine and Rauskolb, 2001). Two mechanistically distinct modes of subdivision have been described:

1. *Heritable subdivisions*. These subdivisions are based on the establishment of a heritable pattern of transcription factor expression that confers a specific identity on a field of cells. The first such subdivision was detected in the *Drosophila* wing between anterior (A) and posterior (P) "compartments" (García-Bellido *et al*., 1973, 1976). An analogous AP subdivision exists in the leg imaginal disc (Steiner, 1976). The wing disc is further subdivided into dorsal (D) and ventral (V) compartments (Diaz-Benjumea and Cohen, 1993; Blair *et al*., 1994; García-Bellido *et al*., 1976) (**Figure 2c** and **d**).
2. *Nonheritable subdivisions*. As an alternative to the stable, heritable mechanism, cell populations can also be subdivided by cell interactions. Continuous input of positional information can instruct cells about their position within a tissue and specify responses that separate cell populations. These cues can be transmitted either through local interactions with neighboring cells or through the reception of long-range morphogen gradient cues (reviews: Strigini and Cohen, 1999; Gurdon and Bourillot, 2001; Freeman and Gurdon, 2002). Cells are able to move

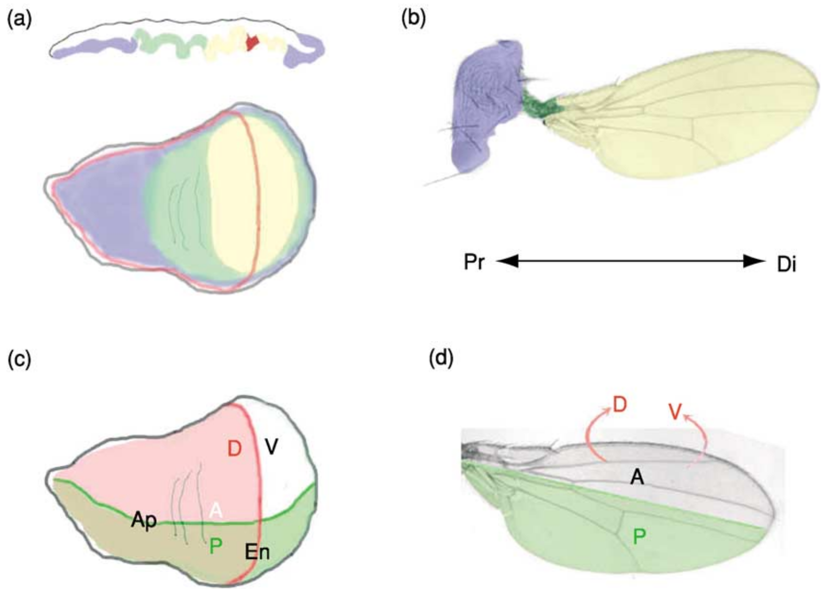


Figure 2 Organization of the axes in the wing imaginal disc. (a) Drawing of a wing imaginal disc, lateral view (upper panel) and apical view (lower panel). In the lateral view the peripodial membrane is depicted as a thin line, over the pseudostratified epithelium, proximal (Pr) structures pointing to the left and distal (Di) ones to the right. The colors represent the different adult structures that develop from the wing imaginal disc. Blue, notum; green, hinge region; yellow, wing pouch; the red line represents the dorsal–ventral boundary. (b) Picture of an adult wing. Color code as in (a). (c) Organization of the anterior–posterior (AP) and dorsal–ventral (DV) boundary within the imaginal disc. The red line represents the DV lineage restriction boundary. Apterous (Ap) is expressed in the D compartment (pink). The green line represents the AP compartment boundary; Engrailed (En) expression in the P compartment is shown in green. (d) Adult wing showing the wing's AP and DV axes.

between subdivisions, changing their expression profiles according to the position-dependent cues they receive (Campbell and Tomlinson, 1998; Weigmann and Cohen, 1999; Jungbluth *et al.*, 2001) (**Figure 2a** and **b**).

1.9.2.1. Heritable Subdivisions: Compartments

The first clues to understanding compartmentalization came from using mitotic recombination to genetically mark cells and their descendants in the *Drosophila* wing. Patches of cells homozygous for a recessive, cell-autonomous marker (e.g., cuticle pigmentation, shape of wing hairs) in an otherwise heterozygous background occupy random positions and usually have irregular borders. Clones of cells abutting the border between A and P compartments (slightly anterior to vein four in the adult wing) (**Figure 2d**) show smooth borders and remain within their compartment of origin, suggesting that cells cannot cross this "compartment boundary," even if they are given a relative growth advantage (Morata and Ripoll, 1975) (**Figure 3**). Molecular markers (e.g., green fluorescent protein (GFP) and lacZ) (Xu and Rubin, 1993) have made it possible to investigate in great detail how the compartment boundary is established and maintained (Blair and Ralston, 1997; Rodriguez and Basler, 1997; reviews: Blair, 1995; Brook *et al.*, 1996).

According to the definition of compartments, these comprise three features:

1. Compartments are subdomains of a tissue that are separated by a boundary of cell lineage restriction.
2. Compartment boundaries show organizing activity that patterns the surrounding tissue.
3. Compartments divide tissues in terms of cell fate/identity.

1.9.2.1.1. Selector genes The establishment of compartments as units of lineage restriction led to the idea that the activity of certain genes "selects" these units for a specific fate (García-Bellido, 1975). The original definition of a "selector gene" includes the following properties:

1. The functional domain is limited by a lineage restriction boundary (compartment boundary). Selector genes confer cell affinity differences that prevent mixing of cells from different compartments.
2. Selector genes have an instructive developmental role.

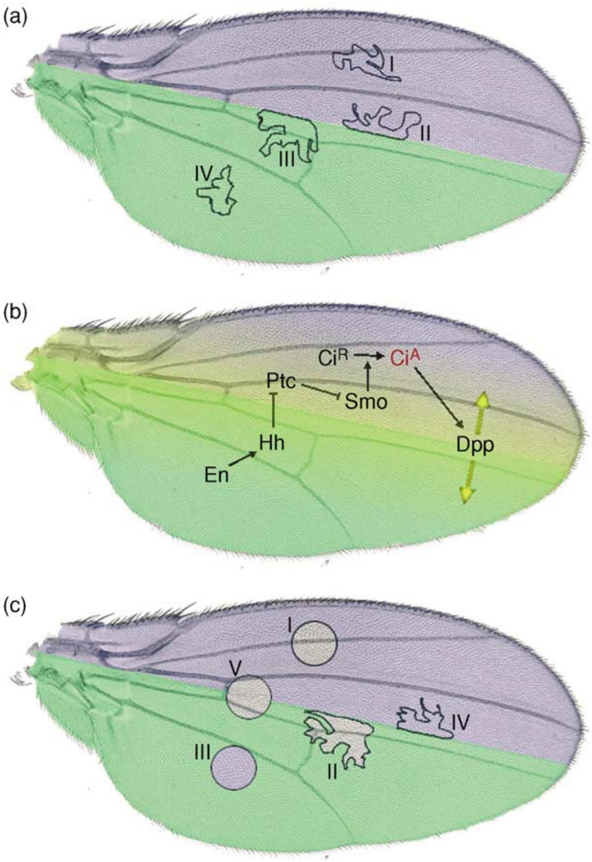


Figure 3 AP compartment formation in the *Drosophila* wing. Anterior cells are shaded blue, posterior cells are shaded green. (a) Clones of wild-type cells have irregular borders and occupy random positions within a compartment (I and IV) but never cross the AP compartment boundary. They show smooth edges when they happen to contact it (II and III). (b) Simplified schematic view of Hedgehog (Hh)-mediated signaling across the AP boundary. Hh signaling ultimately results in conversion of Cubitus interrupts (Ci) from its repressor form (Ci^R) to a transcriptional activator Ci^A. Ci^A activates the long-range morphogen Decapentaplegic (Dpp) (yellow) which spreads from the AP boundary to further pattern the wing. (c) Scheme to show the influence of Engrailed (En) and Hh pathway mutations on clone shape and localization. Posterior clones that lack En (or ectopically express Ci) either sort into the anterior compartment (IV) or round up (III). Anterior clones lacking Smoothened (Smo) (or Ci) form round clones within the anterior compartment (I) or sort into the posterior (II). Clones of cells mutant for both En and Ci have different adhesive properties from both A and P cells and can form round clones at the AP boundary (V). For other protein definitions, see text and appendix. (Modified from Irvine, K.D., Rauskolb, C. **2001**. Boundaries in development: formation and function. *Annu. Rev. Cell Devel. Biol. 17*, 189–214.)

3. Several selector genes can act in a combinatorial mode.
4. Selector genes act strictly autonomously to specify compartment-specific properties (e.g., cell fate).

The term "selector gene" is often misused to describe genes that confer regional identity in a manner not linked to lineage-restricted compartments (reviews: Mann and Morata, 2000; Mann and Carroll, 2002). In this review, the use of the term selector gene is limited to only those factors that fulfill all the criteria mentioned above. Other genes that specify subdivisions but do not fulfill these criteria will be referred to as "selector-like" or "regional identity" genes. This distinction is considered to be important because of the underlying mechanistic differences between the two modes of regionalization. Compartment boundaries defined by selector genes serve as the sources of organizing signals, which is not usually the case for noncompartmental subdivisions.

1.9.2.1.1.1. Engrailed/Invected: posterior compartment selector genes During segmentation in the embryo, P cells are programmed to express the homeodomain transcription factors Engrailed (En) and Invected (Inv) (Kornberg *et al.*, 1985) (for a list of all genes and proteins discussed in this chapter, see appendix). Even though the signals that lead to the induction of En and Inv are transient, their expression becomes heritably transmitted and is inherited by the nascent imaginal disc primordia when they form in the embryo (Cohen *et al.*, 1993). This is likely to depend on the epigenetic mechanisms based on chromatin remodeling by Polycomb (Pc) and Trithorax (Trx) group proteins (Ingham, 1981; Busturia and Morata, 1988; see **Chapter 1.8**). The En/Inv complex controls all aspects of posterior fate in the wing and leg (**Figure 2c and d**). Cells mutant for *en* show some anterior character (García-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Lawrence and Struhl, 1982). However, P cells mutant for both *en/inv* are indistinguishable from A cells (Kornberg, 1981; Tabata *et al.*, 1995; Zecca *et al.*, 1995; Blair and Ralston, 1997; Lawrence *et al.*, 1999a) (**Figure 3**). Inv alone mainly confers P identity in terms of differentiation (Simmonds *et al.*, 1995). En was shown to work as a transcriptional repressor (Jaynes and O'Farrell, 1988, 1991; Han *et al.*, 1989; Ohkuma *et al.*, 1990). The En repressor domain very strongly represses promoters activated by a variety of different activator proteins (Han and Manley, 1993; Smith and Jaynes, 1996). En/Inv are highly conserved and fulfill similar roles in other invertebrate species (Patel *et al.*, 1989).

1.9.2.1.1.2. Apterous: dorsal compartment selector gene During larval development, the wing imaginal disc is further subdivided by a DV compartment boundary (García-Bellido *et al.*, 1976). The gene responsible for the specification of all aspects of

dorsal cell developement is the transcription factor Apterous (Ap) (Blair, 1993; Diaz-Benjumea and Cohen, 1993). The gene *apterous* (*ap*) conforms to the classic definition of a selector gene. Ap activity generates an affinity difference between dorsal and ventral cells and thereby produces the DV lineage boundary. Ap selects between alternative cell fates (D and V) in an autonomous manner and acts in a combinatorial mode with the posterior selector genes *en* and *inve* (**Figure 2c** and **d**).

Early in larval development, the wing imaginal disc is patterned by two opposing signaling pathways, the Wingless (Wg) and epidermal growth factor (EGF) pathways. Hedgehog (Hh) induces Wg in the V–A domain, where it is required to specify the primordium of the wing blade (as opposed to the body wall) (Williams *et al*., 1993; Ng *et al*., 1996; Klein and Martinez-Arias, 1998) (**Figure 4a**). The *vein* (*vn*)-dependent epidermal growth factor receptor (EGFR) signaling has a dual role in early wing disc development (Wang *et al*., 2000; Zecca and Struhl, 2002a, 2002b). First, it instructs cells to adopt body wall fate by antagonizing wing fate and activating notum-specifying genes. The mutually antagonistic repression of the Wg and EGFR signaling pathways therefore separates the wing from the body wall field. Second, EGRF signaling directs cells to become part of the dorsal compartment by inducing the dorsal selector gene *ap* (**Figure 4a**). Thus, the antagonism of EGFR and Wg signaling also controls wing blade development through the initiation of DV boundary formation (**Figure 4b** and **c**).

1.9.2.1.1.1.1. Specification of dorsal cell fate through Ap Ap specifies dorsal cell fate. Removal of Ap activity causes complete transformation from D to V identity (Diaz-Benjumea and Cohen, 1993; Blair *et al*., 1994). D cells differ from V cells in the adult with respect to a set of morphological markers

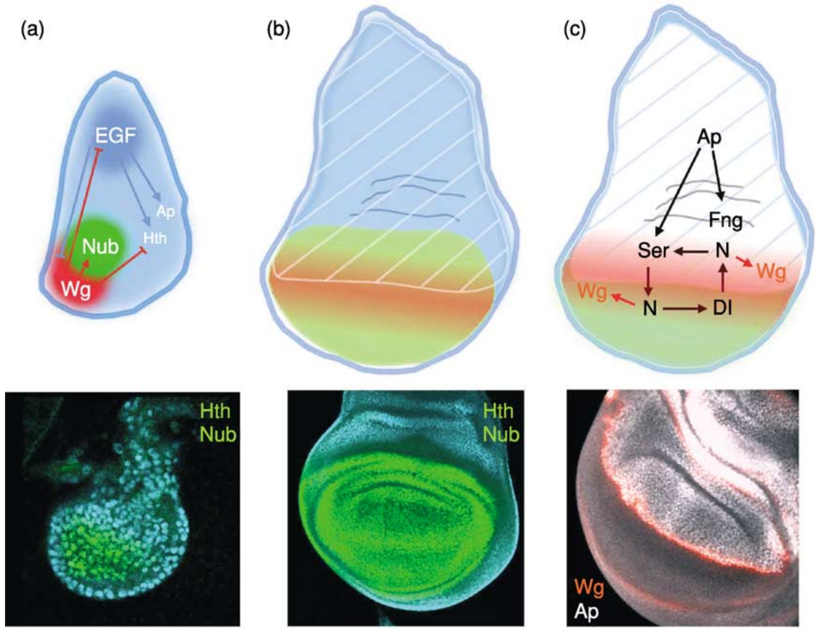


Figure 4 Establishment of the proximal–distal and dorsal–ventral boundaries. (a) (upper panel) The Wingless (Wg) and the epidermal growth factor receptor (EGFR) signaling pathways antagonize each other. Wg specifies the wing pouch through the activation of Nubbin (Nub) and Vestigial (Vg, not shown) and the repression of Vein (Vn, the ligand for the EGFR; not shown), Homothorax (Hth), Apterous (Ap), Teashirt (Tsh, not shown). Vn induces the formation of notum structures and is necessary for the activation of Ap, Hth, and Tsh, and represses the expression of Vg. Both activities together are responsible for establishment of the dorsal–ventral and proximal–distal axes. (lower panel) Single confocal section of a late second instar wing imaginal disc labeled with antibodies against Nub (green), and Hth (cyan). Nub marks the future wing pouch, while Hth marks hinge and body wall tissue. (b) (upper panel) Drawing of late third instar wing disc. The wing pouch is marked in green (Nub), notum tissue is depicted in cyan (Hth). Ap (crosshatched in white) is exclusively expressed in dorsal cells and establishes the dorsal–ventral boundary. Wg (red) is expressed at the dorsal–ventral boundary and spreads along the dorsal–ventral axis into the wing pouch. (lower panel) Late third instar wing disc labeled with anti-Nub (green) and anti-Hth (cyan) antibodies, showing the noncompartmental subdivision in notum and wing tissue. (c) (upper panel) Ap (white) induces the dorsal–ventral compartment boundary through the activation of Fringe (Fng) and Serrate (Ser) in the dorsal compartment and the repression of Delta (Dl). This leads to the activation of Notch (N) signaling at the dorsal–ventral boundary and eventually to the expression of Wg (red). (lower panel) Late third instar wing disc labeled with anti-Ap (white) and anti-Wg (red) antibodies, showing dorsal–ventral compartmentalization and dorsal–ventral organizer.

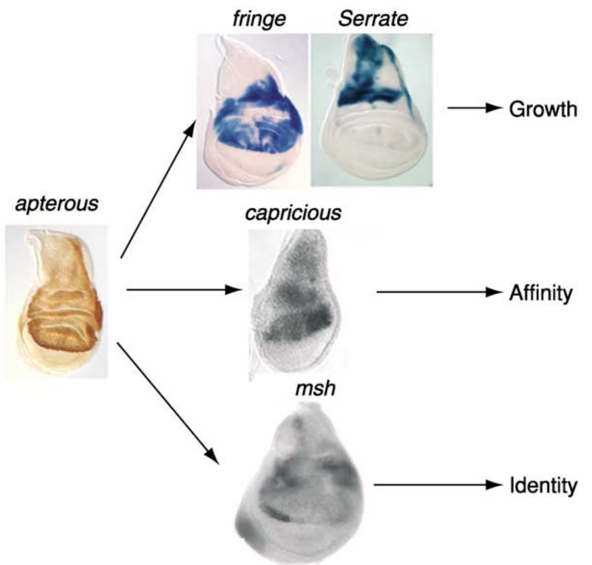


Figure 5 Summary of Apterous functions during wing development. Dorsal–ventral subdivision of the wing imaginal disc depends on the activity of Apterous (Ap) in the dorsal compartment, where it is expressed (*in situ* hybridization with a probe against *apterous*). Ap is responsible for (1) the induction of the dorsal–ventral organizer along the dorsal–ventral compartment boundary through Fringe and Serrate (X-Gal staining of enhancer trap lines inserted at the respective loci); (2) the establishment of a lineage restriction boundary between dorsal and ventral cells through induction of the affinity molecules Capricious (X-Gal staining of an enhancer trap line inserted at the *caps* locus) and Tartan (not shown) in early wing discs; and (3) dorsal specification of dorsal cell fate through Msh (*in situ* hybridization with a probe against *msh*).

such as sensory organs (see **Chapter 1.11**) and wing vein structure. It has been claimed that D cell differentiation can take place in the absence of Ap activity (Klein *et al.*, 1998). However, more careful analysis showed that the *ap* mutants used in that study were only partial loss-of-function mutations, and had enough residual Ap activity to confer dorsal cell fate (Milan and Cohen, 1999a, 2003; O'Keefe and Thomas, 2001). The *ap* confers dorsal identity by controlling expression of the homeobox gene *muscle segment homeobox (msh)* (Milan *et al.*, 2001b) (**Figure 5**). *msh* is expressed in dorsal cells in the embryonic neuroectoderm and muscle precursors (D'Alessio and Frasch, 1996; Isshiki *et al.*, 1997; Lu *et al.*, 2000). In the wing disc, *msh* is expressed in D cells where it is both necessary and sufficient to direct differentiation of dorsal cell characteristics (Milan *et al.*, 2001b). A summary of all known Apterous functions is shown in **Figure 5**.

1.9.2.1.1.1.2. Regulation of Ap activity Ap is expressed in the wing and haltere imaginal discs exclusively in D cells (Cohen *et al.*, 1992). Tight spatial and temporal regulation turned out to be essential for proper Ap activity. Ap belongs to the family of lin-11, islet-1, mec-3 (LIM)-homeodomain factors, which shares two tandemly repeated LIM domains and a homeodomain (review: Hobert and Westphal, 2000). LIM-domains are cysteine-rich zinc-finger domains that mediate protein–protein interactions (Jurata *et al.*, 1998). For LIM-homeodomain transcription factors to function, they require a class of proteins known as LIM-domain binding (LDB) or nuclear LIM interactor (NLI) (Agulnick *et al.*, 1996; Jurata *et al.*, 1996; Morcillo *et al.*, 1997; Fernandez-Funez *et al.*, 1998; review: Bach, 2000). In *Drosophila* wing development, Ap activity depends strictly on dLDB/Chip (Fernandez-Funez *et al.*, 1998). A dimer of dLDB/Chip has been shown to bridge two Ap molecules to form a tetrameric complex active *in vivo* (Milan and Cohen, 1999b; van Meyel *et al.*, 1999; Rincon-Limas *et al.*, 2000). The levels of Ap activity are further controlled both spatially and temporally during development (Milan and Cohen, 2000b). Ap regulates its own activity by inducing the expression of its target gene *Beadex/dLMO* (Milan *et al.*, 1998; Shoresch *et al.*, 1998; Zeng *et al.*, 1998). *dLMO* encodes a member of the LIM-domain only (LMO) family and contains two LIM domains highly similar to those of Ap. dLMO is able to downregulate Ap activity by binding to the LIM-interaction domains of dLDB/Chip, competing with Ap itself. Through this mechanism, Ap is inactivated (Milan *et al.*, 1998; Milan and Cohen, 1999b; van Meyel *et al.*, 1999). This limits the time window during which Ap is highly active in the dorsal compartment and during which DV boundary formation can take place. The limitation of Ap activity is essential because some of the genes regulated by Ap are used in a different developmental context later. Late in development, Serrate (Ser) and Delta (Dl) are expressed along the future veins and at both sides of the DV compartment boundary, where they contribute to the establishment of a feedback loop essential for the restriction of Notch (N) activity along veins and the DV boundary (**Figure 4c**). Continuously, high activity of Ap is not compatible with these later functions (Milan *et al.*, 1998; Milan and Cohen, 2000b; Weihe *et al.*, 2001).

The model that dLMO acts as a competitive inhibitor of Ap suggests that dLMO should displace Ap from Chip. In fact, Ap protein is destabilized in cells expressing dLMO. Interestingly, Ap appears to be destabilized in situations where it is unable to bind to DNA in active tetrameric complexes with Chip (Weihe *et al.*, 2001). dLMO competes effectively with Ap for binding to Chip, whereas

Ap competes poorly with dLMO apparently due to an intrinsic difference in the affinities of the LIM domains of Ap and dLMO. This means that dLMO is a potent inhibitor of Ap activity because it binds Chip more effectively than Ap (Weihe *et al.*, 2001).

dLDB/Chip is also involved in the activation of the *achaete–scute* (*ac-sc*) proneural complex in association with the GATA factor Pannier (Pnr). This process is important for sensory bristle patterning in the thorax (see **Chapter 1.11**). Pnr antagonizes Ap function, presumably by competitively binding to Chip. The correct stoichiometry of these three proteins is crucial for both proneural prepatterning and subdivision of the thorax (Ramain *et al.*, 2000).

1.9.2.1.2. Mechanisms of boundary formation

1.9.2.1.2.1. Affinity-based cell separation Because of the signaling interactions that ensue when A and P or D and V cells are confronted (see Section 1.9.2.1.3.2), a well-defined straight boundary between compartments is essential. Crossing of cells into the other compartments would lead to the establishment of an ectopic organizer within the wing pouch, resulting in severe morphological defects. How are cells from different compartments kept separate? Differences in cell adhesion certainly can contribute to this process. Sorting out of cell populations can be guided by both the amount and types of adhesion proteins that cells express (Steinberg and Takeichi, 1994).

One well-studied example of differential adhesion mediating morphogenesis is the sorting of the oocyte to the posterior of the ovary in *Drosophila* (see **Chapter 1.2**). The oocyte and the posterior follicle cells express higher levels of *Drosophila* EGF-domain containing cadherin (DE-cadherins), which are adhesion molecules, and catenins, which anchor the cadherins to the cytoskeleton and are necessary for strong adhesion. Loss of either cadherins or catenins leads to mislocalization of the oocyte within the ovary (Godt and Tepass, 1998; Gonzalez-Reyes and St. Johnston, 1998; review: **Chapter 1.2**). However, cadherins have not been shown to be responsible for boundary formation in the discs. Clones of cells overexpressing cadherins do sort out from surrounding cells in the discs (Dahmann and Basler, 2000), showing that the expression of adhesion molecules can, in principle, separate cell populations in imaginal discs. Affinity molecules involved in cell separation at the AP axis have not been identified yet.

Theoretically, the prospective wing margin cells (zone of nonproliferating cells (ZNCs)) could act as a physical barrier to prevent mixing of D and V compartment cells (O'Brochta and Bryant, 1985). This model predicts that the DV boundary is in fact two boundaries, one on the dorsal and one on the ventral side of the ZNC. It also requires that cells from outside the ZNC are prevented from entering it. Neither requirement was fulfilled however, when clone distribution was analyzed in discs, so the barrier model cannot explain DV boundary formation (Blair, 1993). Thus, Dorsal cells mutant for *ap* fail to respect the DV boundary and tend to sort out into the V compartment (analogous to posterior cells mutant for *en*) (**Figure 3c**). Therefore, it was hypothesized that Ap could directly regulate the expression of molecules that modify cell adhesion or affinity (Lawrence and Struhl, 1996). Indeed, *ap* controls the expression of two integrin-type adhesion molecules, PS1 and PS2. PS1 expression is activated in the D compartment by Ap activity and PS2 is restricted to V cells by repression through Ap (Blair *et al.*, 1994). However, PS1 and PS2 proved not to be involved in DV compartment formation but rather to regulate the attachment of the D and V wing blades during pupal stages (Brower and Jaffe, 1989; Blair *et al.*, 1994).

Evidence that other Ap-dependent cell interactions contribute to boundary formation has been presented (Milan *et al.*, 2001a). The *capricious (caps)* and *tartan* (*trn*) genes are targets of Ap (**Figure 5**). The genes *caps* and *trn* encode transmembrane proteins with extracellular leucine-rich repeats (LRRs) and are expressed in the D compartment during boundary formation. Caps and Tartan confer affinity for D cells, as assessed by sorting out behavior, and contribute to boundary formation, as assessed by sensitized genetic assays. However, removing both genes does not eliminate the DV boundary, indicating that other *ap* target genes may also confer D cell-affinity properties.

Taken together, the results suggest that *ap* is responsible for the specification of all aspects of D cell development in the *Drosophila* wing and works as a classical selector gene (**Figure 5**).

1.9.2.1.2.2. Signaling-based cell separation There are several examples of cell sorting processes that are clearly influenced by cellular signaling, where the mechanism may not be dependent on differences in cellular affinities per se. Rhombomeres in the vertebrate hindbrain are separated by a cell lineage restriction. Ephrin tyrosine kinase receptors and their membrane-bound Ephrin ligands are expressed in complementary rhombomeres. Both receptors and ligand are able to transduce signals. Bidirectional signaling occurs at the interface of their expression domains, causing the restriction of cell intermingling

between hindbrain segments. Unidirectional signaling is not sufficient to establish the cell lineage restriction, indicating that the ligand–receptor interaction is not sufficient to confer a cell–cell affinity difference strong enough to separate cells from different segments (Mellitzer *et al*., 1999; Xu *et al*., 1999). Many adhesion proteins form regulated connections with the cytoskeleton and participate in contact-mediated signaling (reviews: Hynes, 1999; Vleminckx and Kemler, 1999). Signaling-based repulsion or deadhesion in this case could promote segregation of cell populations. If signaling promotes reorganization of the cytoskeleton, cell interactions might be destabilized (Brückner and Klein, 1998). Repeated cycles of deadhesion and readhesion could lead to sorting out behavior.

This example is not meant to argue that cell affinity factors do not play a role in these cell sorting processes. However, they do suggest that alternative mechanisms based on signaling are possible. In fact, asymmetric signaling from P to A cells seems to affect AP boundary formation in the *Drosophila* wing. Anterior clones of cells lacking the transmembrane protein Smoothened (Smo), which is essential for *hh* signal transduction, can associate with posterior cells even though they lack En and were of anterior origin (**Figure 3c**). However, these clones do not mix perfectly with P cells either, suggesting that a contribution of *hh* signaling and En-mediated cell affinity differences, both are crucial for proper sorting of cells into the correct compartment (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence *et al*., 1999b; Dahmann and Basler, 2000).

Signaling between dorsal and ventral cells contributes to D–V boundary formation. For example, Fringe-expressing clones (which have "dorsal" Notch signaling properties) are able to cross the boundary from V to D if they happen to contact it (Micchelli and Blair, 1999; Rauskolb and Irvine, 1999). However, it has been shown that Notch activation along the DV boundary in the absence of Ap is insufficient to support DV boundary formation (Milan and Cohen, 1999a). Notch activation at the DV boundary is symmetric (in contrast to the inherently asymmetric signaling from P to A cells) (**Figure 4c**). It is therefore not obvious how symmetric Notch activation could generate an affinity border. Notch signaling is required, but is not sufficient to generate a boundary without an additional Ap-dependent input. The authors envisage a permissive role for Notch activity in the DV boundary formation, with some other Ap-dependent activity providing the instructive determinant (Milan and Cohen, 2003).

1.9.2.1.3. Compartment boundaries as organizing centers Cell interactions play a critical role during development. Pioneering experiments in the frog have shown that specific groups of cells can behave as organizing centers that control the developmental fates of nearby cells in a nonautonomous manner (Spemann and Mangold, 1924). Transplantation experiments with dorsal cells from the amphibian blastopore lip showed that these cells were able to induce a complete secondary body axis when located ectopically in ventral tissue. Many developmental organizers have been found since, e.g., the notochord (inducing the floorplate) (Placzek *et al*., 1990; Yamada *et al*., 1991), the isthmic organizer between the midbrain and hindbrain (reviews: Joyner *et al*., 2000; Simeone, 2000) and the AP and DV compartment boundaries in the fly wing and leg (Diaz-Benjumea and Cohen, 1993; Struhl and Basler, 1993; Basler and Struhl, 1994) (**Figure 2c**).

In the context of compartments, formation of organizers can be separated into three steps: (1) differential cell fate specification in adjacent compartments, (2) leading to asymmetric signaling between the two cell populations, and (3) production of a symmetric organizing signal originating at the compartment boundary (Meinhardt, 1983; Struhl and Basler, 1993; Basler and Struhl, 1994; Diaz-Benjumea *et al*., 1994; Tabata and Kornberg, 1994; review: Brook *et al*., 1996).

1.9.2.1.3.1. The A–P organizer Soon after the discovery that A and P cells are separated by a lineage restriction boundary, the suggestion was made that this boundary might serve as an organizing center in the developing appendages (Crick and Lawrence, 1975). Molecular analyses revealed that specialized cells are established along the AP axis as a consequence of asymmetric signaling by the diffusible protein Hedgehog (Hh) from P to A cells (Basler and Struhl, 1994; Tabata and Kornberg, 1994) (**Figure 3b**). En generates this asymmetry by inducing expression of Hh in the posterior compartment (Tabata *et al*., 1992; Zecca *et al*., 1995) and at the same time repressing the expression of the essential downstream component Cubitus interruptus (Ci) (Eaton and Kornberg, 1990; Schwartz *et al*., 1995) (**Figure 3b**). Ci is a transcription factor that is converted to a repressor form in the absence of Hh signaling (Aza-Blanc *et al*., 1997; Méthot and Basler, 1999). Thus, only A cells that receive the Hh signal across the compartment boundary will respond by stabilization of Ci. Hh signaling leads to the expression of the secreted signaling molecule Decapentaplegic (Dpp) at the AP boundary of wing discs and of Dpp and Wg in leg discs (Struhl and Basler, 1993;

Basler and Struhl, 1994; Diaz-Benjumea *et al.*, 1994). Dpp and Wg act as symmetric long-range morphogens in wing and leg imaginal discs (Brook and Cohen, 1996; Jiang and Struhl, 1996; Lecuit *et al.*, 1996; Nellen *et al.*, 1996; Lecuit and Cohen, 1997; Entchev *et al.*, 2000; Teleman and Cohen, 2000). By this mechanism the originally asymmetric subdivision of the limb primordium eventually leads to the establishment of a symmetric organizing gradient of the morphogen (**Figure 3b**).

1.9.2.1.3.2. The DV organizer Ap expression establishes the D–V lineage restriction boundary in the wing disc (Diaz-Benjumea and Cohen, 1993; Blair *et al.*, 1994). Short-range interactions between D and V cells lead to the activation of the Notch signal transduction pathway (Diaz-Benjumea and Cohen, 1995; Kim *et al.*, 1995; Rulifson and Blair, 1995; de Celis *et al.*, 1996b; Doherty *et al.*, 1996). Notch is activated symmetrically on both sides of the DV boundary (**Figure 4c**). Ap plays an essential role in this process by inducing expression of Serrate and Fringe in dorsal cells (Irvine and Wieschaus, 1994; Speicher *et al.*, 1994; Couso *et al.*, 1995) (**Figure 5**). Serrate and the ventrally expressed Delta encode transmembrane proteins that act as ligands for the Notch receptor protein (Fleming *et al.*, 1990; Rebay *et al.*, 1991; Doherty *et al.*, 1996). Fringe modulates the sensitivity of Notch to its ligands such that Fringe-expressing cells are sensitive to Delta and refractory to Serrate (Fleming *et al.*, 1997; Johnston *et al.*, 1997; Panin *et al.*, 1997). Fringe acts in the Golgi apparatus as a glycosyltransferase enzyme that modifies the EGF modules of Notch and enhances the ability of Notch to bind to Delta (Brückner *et al.*, 2000; Moloney, 2000; Panin *et al.*, 2002). Consequently, Sersignals to V cells and Delta signals to D cells across the DV boundary. Notch is activated symmetrically and induces Wg and Vestigial (Vg) (through its boundary enhancer) on both sides of the boundary (**Figure 4c**). Wg spreads along the DV axis of the wing and further organizes growth and patterning of the wing disc (see Section 1.9.3.3) by inducing and repressing its target genes *vg* (through the quadrant enhancer), *ac–sc, Dll, fz2, and notum* in a concentration-dependent manner (Blair, 1992; Phillips and Whittle, 1993; Couso *et al.*, 1994; Zecca *et al.*, 1996; Neumann and Cohen, 1997; Cadigan *et al.*, 1998; Giraldez *et al.*, 2002) (**Figure 7**).

1.9.2.2. Nonheritable Subdivisions of Limb Primordia

Developmental boundaries exist between compartments that separate cell lineages and also between fields of cells that are subdivided less strictly. The term "territory" has been suggested for subdivisions that are not defined by lineage boundaries but are dynamically maintained by continuous signaling (Theisen *et al.*, 1996). As long as the patterning mechanisms that initiate these subdivisions continue to operate, they can define distinct domains of gene expression.

For example, local cell–cell interactions can stabilize domains of gene expression (e.g., feedback loops with neighboring cells). In these cases, the maintenance of abutting domains of gene expression will be based on position instead of cell lineage. The critical distinction is that cells in the former scenario can move between territories and change their identity accordingly. A number of noncompartmental body subdivisions in *Drosophila* are associated with specific expression of developmental genes (Williams *et al.*, 1993; Calleja *et al.*, 1996). Here the separation of the limb primordia into proximal and distal structures as one example is considered (**Figure 2a** and **b**). The wing and – more obviously so – the leg are subdivided along their proximal-distal (PD) axes into segments of morphologically distinct identity. The wing hinge, costa, and wing blade can be considered as separate "segments" (**Figure 2b**), whereas the leg is subdivided into coxa, trochanter, femur, tibia, and five tarsi (**Figure 6c**). Several genes are expressed in PD domains of the wing and leg primordia that will give rise to the distinct morphological structures in the adult described above.

1.9.2.2.1. PD patterning in the *Drosophila* leg The homeodomain proteins Homothorax (Hth) and Extradenticle (Exd) and the Zn-finger protein Teashirt (Tsh) play important roles in defining the proximal territories in legs and wing discs. In the leg, the PD axis is set up under the control of the activities of Dpp and Wg (Diaz-Benjumea *et al.*, 1994; Campbell and Tomlinson, 1995; Lecuit and Cohen, 1997) (**Figure 6a**). Hh expression in P cells induces Dpp in the dorsal anterior half and Wg in the ventral anterior half of the leg disc (Struhl and Basler, 1993; Diaz-Benjumea *et al.*, 1994). Their combined activities specify a distal region expressing Distal-less (Dll) (Cohen *et al.*, 1989; Cohen, 1990; Diaz-Benjumea *et al.*, 1994) (**Figure 6b**), in part by repressing Hth and Tsh expressions, which are both required to specify proximal fates (Abu-Shaar and Mann, 1998; Gonzalez-Crespo *et al.*, 1998; Wu and Cohen, 1999). Hth fulfills a dual role in this context: first, it suppresses the action of the Wg and Dpp pathways and, second, it activates the expression of proximal genes such as *tsh* (Abu-Shaar and

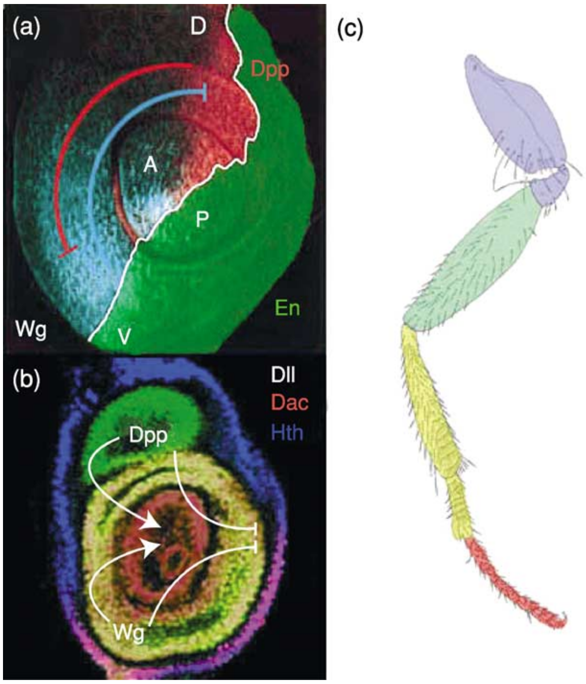


Figure 6 Proximal–distal axis formation during leg development. (a, b) Single confocal images for a third instar leg imaginal disc. (a) Wg protein (cyan) and *dpp-lacZ* (red) are activated in the anterior compartment in the ventral (V) and dorsal (D) regions respectively, by Hh signaling from the posterior compartment (green). The white line denotes the anterior–posterior (AP) boundary. Wg and Dpp repress each other's expression. (b) Wg and Dpp cooperate to activate the expression of Distalless (Dll) and Dachshund (Dac) and repress the expression of Hth. (c) Drawing of an adult leg. The colored structures correspond to the different domains of gene expression in (b).

Mann, 1998; Azpiazu and Morata, 2002). Further evidence indicates that Hth but not Tsh is essential for formation of the affinity difference between proximal and distal leg structures (Wu and Cohen, 2000) (**Figure 6b**).

The expression domain of Hth and Tsh initially forms a sharp border with more distal cells, which express Dll and later Dachshund (Dac) (Lecuit and Cohen, 1997) (**Figure 6b**). The expression domains appear to be stable but no evidence of a lineage restriction boundary can be found. Indeed, lineage tracing experiments revealed that cells born in the proximal Tsh-expressing region contribute substantially to distal territories in the leg (Weigmann and Cohen, 1999). These cells must have moved from the proximal territory into the distal territory, changing from expression of the proximal genes Tsh and Hth to expression of the distal genes Dac and Dll. These effects can be explained in terms of coupling of growth and patterning of the leg along its PD axis (Wu and Cohen, 1999, 2000). Dll-expressing cells were also shown to be able to move proximally, provided they are given a relative growth advantage (Weigmann and Cohen, 1999). Interestingly, Dll-expressing cells are able to maintain their distal identity even if Wg and Dpp signaling are inhibited, suggesting that other mechanisms besides the input of these two signals contribute to stabilization of cell fate decisions in the leg (Castelli-Gair and Akam, 1995; Lecuit and Cohen, 1997).

During the third instar, the leg disc is subdivided into presumptive segments. A number of genes expressed in rings in the leg disc prefigure segmentation of the leg (Cohen, 1993). Activation of Notch in cells at the distal end of each forming segment is clearly required for the establishment of segment boundaries, but it remains unclear how the activation of Notch is controlled by the expression of genes along the PD axis (de Celis *et al.*, 1998; Bishop *et al.*, 1999; Rauskolb and Irvine, 1999). Notch activation along the segment boundaries has a non-autonomous effect on proliferation and survival of surrounding cells (de Celis *et al.*, 1998; Kerber *et al.*, 2001). Still later, the presumptive tarsal region of the leg is further subdivided to produce the individual tarsal segments. Recently it has been shown that a gradient of EGFR activity mediated by the distal expression of its ligand Vein (Vn) and the membrane protein Rhomboid (Rho) is necessary for the patterning of these most distal elements (Campbell, 2002; Galindo *et al.*, 2002). At present it is not known how expression of the known Wg and Dpp target genes correlates with the induction of segmentation (**Figure 6**).

1.9.2.2.2. PD patterning in the *Drosophila* wing

A similar subdivision between P and D structures takes place in the wing primordium. The notum, wing hinge, costa, and wing blade are easily distinguishable morphological units (**Figure 2b**). First, the future wing field and the body wall are separated by the mutually exclusive action of Wg and EGFR signaling, respectively (see Section 1.9.2.1.1.2). Wg signaling activates transcription of the Pit, Oct, Unc (POU)-domain transcription factor *nubbin* (*nub*) and *vestigial* (*vg*) in the wing field and restricts expression of *homothorax* (*hth*), *teashirt* (*tsh*), and *vein* (*vn*) (an EGFR ligand) to the body wall (see **Figure 4a**). Later, the wing field is further subdivided into domains that will give rise to the wing blade, costa and the hinge articulating the wing with the body wall in the adult animal.

In the wing pouch, Wg and Dpp activate *vg*, which – together with *scalloped* (*sd*) – is required for growth and cell survival of wing blade cells

(Simpson and Schneiderman, 1975; Williams *et al.*, 1994; Kim *et al.*, 1996, 1997b; Halder *et al.*, 1998; Simmonds *et al.*, 1998; Liu *et al.*, 2000). Misexpression and loss-of-function analyses of Vg and Sd revealed that Vg- and Sd-dependent cell affinity differences contribute to the separation of the wing blade from the wing hinge and to a gradient of cell affinities along the PD axis of the wing (Liu *et al.*, 2000). Since Vg expression requires the continuous input of Wg and Dpp signals, the precursor cells of hinge and blade are not strictly separated by cell lineage.

The hinge is a flexible region that is required for wing flapping and for the movement of extension and flexion over the abdomen (Snodgrass, 1935). It originates from rings of tissue surrounding the wing pouch. Wg is required for costa and hinge development (Neumann and Cohen, 1996a). The regulation of Wg expression in the costa and hinge is complex and only partially understood to date. So far only the factors influencing Wg expression in the inner ring (costa) have been analyzed in detail. The *rotund (rn)* gene is a member of the Krüppel family of zinc-finger transcription factors (see **Chapter 1.8**). Loss-of-function mutations of *rn* lead to a loss of hinge structures among other phenotypes (Del Alamo Rodriguez *et al.*, 2002; St. Pierre *et al.*, 2002). Similarly, the POU-domain transcription factor Nubbin is essential for costa and hinge formation (Ng *et al.*, 1995; Del Alamo Rodriguez *et al.*, 2002). Both *rn* and *nub* are required for Wg expression in the inner ring in a cell-autonomous fashion. Vg expression in the wing pouch is essential for the activation of Wg, Rn, and Nub in the inner ring, indicating that a signal emanating from the Vg-expressing cells in the pouch contributes to patterning in the hinge. The nature of this signal remains to be determined. Interestingly, Wg and Vg coexpression is incompatible with hinge fate (Del Alamo Rodriguez *et al.*, 2002). Maintenance of Wg expression in the hinge is independent of Vg but instead requires Hth. As opposed to the wing blade, Wg activates Hth in the hinge (Azpiazu and Morata, 2000), thereby mediating *wg* autoregulation. In collaboration with Tsh this proximal expression of Hth represses Vg (Casares and Mann, 2000), thereby establishing the new hinge domain.

The notum part of the wing disc will give rise to the thorax (**Figure 2a** and **b**; blue shading). Hth expression is required for notum formation. Vg is required to repress the expression of Hth in distal wing tissue (i.e., the prospective wing blade), thus separating notum and pouch tissues. As in the distal portion of the leg, both Wg and Dpp signaling are responsible for this effect (Azpiazu and Morata, 2000). Hth and Tsh are the only markers of proximal tissue known to be common in wing and leg primordia. Nub and Dll are required for distal specification in the wing and leg, respectively. Recently, it was found that the *elbow/no ocelli* (*el/noc*) gene complex is necessary for distal specification in both wings and legs (Weihe *et al.*, 2004), providing evidence for a common molecular basis of wing and leg appendage specification.

1.9.2.2.3. Formation of vein and intervein regions Wing veins are the overt morphological readout of the AP patterning system in the adult wing (Sturtevant and Bier, 1995; de Celis, 1998; Bier, 2000; review: Milan and Cohen, 2000a). The establishment of veins and intervein regions depends on both the Hh and Dpp signaling pathways and represents a secondary subdivision of the A and P compartments. The short-range Hh gradient positions vein three (L3) (Mullor *et al.*, 1997; Strigini and Cohen, 1997) and induces expression of the EGFR ligand Vein (Vn), which is in turn required for the establishment of vein four (L4) in nearby P cells (García-Bellido *et al.*, 1994). Hh signaling also controls cell proliferation in this intervein territory (Duman-Scheel *et al.*, 2002).

The veins correspond to four longitudinal stripes of cells, where several genes of the EGFR (*argos, veinlet*) and Notch signaling pathways (*Delta*) are expressed. Intervein territories are characterized by the expression of Blistered (Bs), the *Drosophila* homolog of the serum response factor (SRF) transcription factor. Bs activity prevents the formation of vein tissue (Fristrom *et al.*, 1994). The Notch target gene $E(spl)m^{\beta}$ is also expressed in the intervein territories and excluded from the developing veins (review: de Celis, 1998). Dpp signaling along the AP axis leads to the activation of its target genes *spalt-major* (*salm*) and *spalt-related* (*slr*) in a broad medial domain of the wing disc (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). The juxtaposition of Spalt-expressing and nonexpressing cells results in the generation of vein L2 and mutations in both genes interfere with formation of vein L5 (de Celis *et al.*, 1996a; Sturtevant *et al.*, 1997).

Differentiation of the veins occurs during the pupal stages through the action of the transcription factor Ventral veinless (Vvl) (de Celis *et al.*, 1995; de Celis and Barrio, 2000). At this stage, it is possible to distinguish a central stripe where EGFR signaling is active, and two adjacent stripes where Notch signaling is active. The cells where EGFR signaling is active will differentiate as veins in the adult wing (Gabay *et al.*, 1997). Activation of Notch in the anterior and posterior lateral stripes limits the width of the vein-forming stripe.

Interestingly, clones of genetically marked cells tend to grow within a particular intervein territory without crossing the presumptive veins (Gonzalez-Gaitan *et al*., 1994). However, if given a growth advantage over surrounding tissue, clones can cross veins, indicating that veins do not constitute lineage boundaries (Baonza and García-Bellido, 1999). Therefore, intervein regions do not qualify as compartments but are subdivisions separated by differences in gene expression. Differences in cell affinity help to maintain these subdivisions. It has been shown that Hh signaling directly influences cell affinity in the intervein territory between veins 3 and 4 (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Dahmann and Basler, 2000). Activity of Spalt is involved in mediating differential cell affinity between veins 2 and 5 (Milan *et al*., 2001a).

1.9.2.2.4. Notum subdivision by Pannier and the Iroquois complex *pannier* and the genes of the *iroquois* complex (*iro*-C) are expressed in a mutually exclusive patterns in the mesothoracic part of the wing imaginal disc. Pannier confers adhesion properties that prevent medial cells from mixing with lateral cells that express Iro-C. This subdivision does not produce a cell lineage boundary (Calleja *et al*., 1996). The Iro-C complex (consisting of three related homeodomain proteins; Mirror (Mirr), Araucan (Ara), and Caupolican (Caup)) is required to specify lateral thoracic body wall (Gomez-Skarmeta and Modolell, 1996; McNeill *et al*., 1997; Cavodeassi *et al*., 2001). Early in development, Iro-C is expressed in the entire thoracic body wall due to the activity of Dpp (Cavodeassi *et al*., 2002). Cells mutant for *iro-C* transform the lateral body wall into proximal hinge structures (Diez del Corral *et al*., 1999), suggesting that the genes of the Iro-C complex fulfill selector-like roles for body wall development. Interestingly, the Iro-C complex is also required for DV specification in the developing eye and head (Dominguez and de Celis, 1998; Cavodeassi *et al*., 2000; Pichaud and Casares, 2000).

1.9.3. Morphogen Gradients and Pattern Formation

Compartment boundaries are the sources of morphogens. Morphogens are signaling molecules that are produced from a localized source forming long-range concentration gradients that pattern a field of cells (**Figure 7a**). Cells interpret their position as a function of the amount of signal they receive, thus obtaining "positional information" (Wolpert, 1989, 1996). Signaling molecules have to fulfill two stringent criteria to qualify as morphogens: (1) their effect must be exerted in a concentration-dependent manner and (2) they must act directly on target cells at a distance from the source (i.e., not through a secondary relay mechanism). When these criteria are met, the local concentration can be interpreted as a measure of distance from the source of the signal (**Figure 7a**). The three signaling proteins that qualify as morphogens in *Drosophila* wing development are Hedgehog (Hh), Decapentaplegic (Dpp), and Wingless (Wg) (e.g., Wg is shown in **Figure 7**).

It is generally accepted that cells interpret the gradient by eliciting differential transcriptional responses depending on the concentration of morphogen they are exposed to. This requires cells to make decisions depending on different threshold levels of signaling pathway activity. The concept implies that a single event – namely the production of a secreted molecule at a localized source – can lead to the formation of several different cell types in a correct spatial relationship to each other (**Figure 7**). This represents a highly efficient way of generating complex patterns in previously uncommitted cells (review: Gurdon and Bourillot, 2001).

Different modes of morphogen movement/transport have been invoked to explain long-range gradient formation: extracellular diffusion of the secreted molecule (van den Heuvel *et al*., 1989 (discussed in Lander *et al*., 2002); McDowell *et al*., 1997; Pfeiffer *et al*., 2000; Strigini and Cohen, 2000; Teleman and Cohen, 2000; Teleman *et al*., 2001), cycles of receptor-mediated endocytosis and resecretion (planar transcytosis) (Entchev *et al*., 2000; Entchev and Gonzalez-Gaitan, 2002), membranous exosomes (argosomes) (Greco *et al*., 2001), and cytoplasmic extensions (cytonemes) (Ramirez-Weber and Kornberg, 1999). Although there is no compelling evidence to date that mechanisms other than diffusion contribute productively to gradient formation, it is certainly plausible that these mechanisms could do so. Cytonemes and argosomes exist, but have not yet been shown to be required to mediate ligand movement or to transduce signals. Evidence presented in favor of endocytosis and resecretion as a mechanism of ligand transport has been questioned (Lander *et al*., 2002). Further experimental evidence is needed to support or refute these possibilities.

Several factors have been implicated in shaping morphogen gradients: posttranslational modification of the ligand can influence diffusibility (e.g., acylation of Hh and Wg (Ingham, 2000, 2001)). Regulation of receptor levels can influence ligand

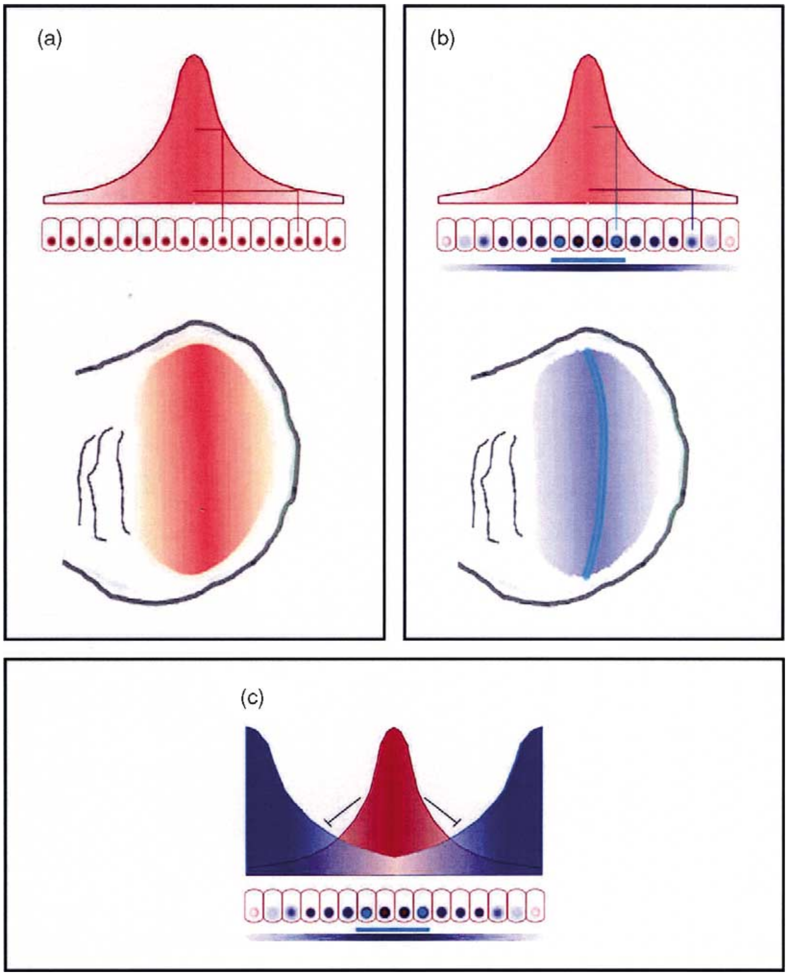


Figure 7 Example of a morphogen gradient: Wingless (Wg). (a) Drawing of a section of the wing pouch epithelium (upper panel) and of the wing pouch in an apical view (lower panel). The cells in the center express Wg which generates a gradient across the adjacent tissue. Cells at different distances receive different levels of Wg. (b) Schematic drawing of the Wg gradient and target gene activation in the epithelium in response to Wg (upper panel). The Wg gradient activates the target genes *hindsight* (cyan) and *distalless* (blue) at different threshold levels. The expression domains of Hindsight and Distalless are depicted in the same colors in the lower panel. (c) Wg downregulates its own receptor Drosophila Frizzled 2 (DFz2; blue), shaping its gradient and rendering the cells at a distance from the source of Wg more sensitive.

movement (Chen and Struhl, 1996; Cadigan *et al*., 1998; Lecuit and Cohen, 1998) (**Figure 7c**). Cell surface heparan-sulfate proteoglycans, and secreted enzymes that modify them, can modulate ligand movement (The *et al*., 1999; Baeg *et al*., 2001; Giraldez *et al*., 2002; review: Nybakken and Perrimon, 2002). Secreted ligand binding proteins can influence movement or stability (Bouwmeester *et al*., 1996; Leyns *et al*., 1997; Wang *et al*., 1997; Ashe and Levine, 1999; Ross *et al*., 2001). In addition, cellular responses to these ligands can also be modulated, contributing to shaping the activity gradients rather than the ligand gradients per se (Tsuneizumi *et al*., 1997; Teleman and Cohen, 2000; Zeng *et al*., 2000). These factors will be considered in more detail in the following sections.

1.9.3.1. Hedgehog

1.9.3.1.1. Hedgehog function The *hedgehog* (*hh*) mutant and gene were originally characterized in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980; Lee *et al*., 1992; Mohler and Vani, 1992; Tabata *et al*., 1992). Hh proteins are widely conserved within the animal kingdom. Hh signaling is involved in diverse developmental processes, and when misregulated, has been implicated in disease (review: Chuang and Kornberg, 2000) (summarized in **Figure 3b**). Loss of Hh signaling in wing discs impedes growth and patterning in both A and P compartments and gives rise to severely reduced wings (Basler and Struhl, 1994). Hh acts directly to pattern the central region of the wing (Mullor *et al*.,

1997; Strigini and Cohen, 1997). The long-range effects of reduced Hh are secondary consequences due to loss of Dpp expression along the AP compartment boundary in the wing and loss of Dpp and Wg in the leg. Ectopic expression of Hh in A cells at a distance from the AP boundary or activation of signaling by removing Hh pathway inhibitors such as patched (Ptc), protein kinase A (PKA), Costal-2 (Cos-2), or Slimb (Slmb) causes dramatic reorganization of anterior pattern. Ectopic expression of Hh can lead to complete mirror image duplication of anterior structures. This shows the importance of localized Hh signaling during wing development (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Jiang and Struhl, 1995, 1998; Lepage *et al.*, 1995; Li *et al.*, 1995; Pan and Rubin, 1995; Sisson *et al.*, 1997; Theodosiou *et al.*, 1998).

Asymmetry is critical to the function of the Hh patterning system. P cells, which express Hh, are not capable of transducing the Hh signal. They do not express the transcription factor Ci (due to repression by En). Anterior cells that do not receive Hh, process the full-length activator Ci protein (Ci^{155} or Ci^{A}) into a transcriptional repressor form Ci^{75} or Ci^{R} (Aza-Blanc *et al.*, 1997). Cells close to the boundary that receive Hh input stabilize Ci^{155}, leading to the induction of Hh target genes. The distance from the Hh source determines which Hh target genes are activated (**Figure 3b**). Hh can therefore be defined as a morphogen (Strigini and Cohen, 1997). Hh regulates *dpp*, *wg, ptc, en*, and *collier (synonym: knot)* expression in a concentration-dependent manner (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; Chen and Struhl, 1996; Mullor *et al.*, 1997; Strigini and Cohen, 1997; Vervoort *et al.*, 1999).

1.9.3.1.2. Hedgehog production Hh protein is produced as a precursor that undergoes autoproteolytic intramolecular cleavage by an intein-like mechanism (Chang *et al.*, 1994; Lee *et al.*, 1994; Porter *et al.*, 1995). This generates a 20 kDa N terminal (Hh-N) and a 25 kDa C terminal fragment (Hh-C). The enzymatic activity required for the cleavage reaction is contained within the C terminal domain (Lee *et al.*, 1994; Porter *et al.*, 1995), while the N terminal domain accounts for all known signaling activity (Porter *et al.*, 1995). During the cleavage reaction, a cholesterol moiety is covalently attached to the C terminal part of Hh-N (Porter *et al.*, 1995). Cholesterol modification may be responsible for the observed tight cell association of Hh-N to Hh producing cells. The range over which Hh-N can move and signal is greatly extended when the cholesterol modification is prevented (Porter *et al.*, 1996; Burke *et al.*, 1999). In addition, Hh is palmitoylated at its N terminus. This lipid modification is also essential for Hh function. In the absence of the acyltransferase Skinny Hedgehog (Ski), Hh lacks the N terminal palmitate residue and cannot fulfill its embryonic and larval patterning activities (Chamoun *et al.*, 2001; Lee and Treisman, 2001).

1.9.3.1.3. Hedgehog movement How do these posttranslational modifications impact on Hh movement? The cholesterol and palmitate moieties presumably bind Hh to the cell surface and might be expected to preclude diffusion. Yet Hh protein and Hh activity can be detected many cell diameters away from the cells where it is produced (Tabata and Kornberg, 1994; Mullor *et al.*, 1997; Strigini and Cohen, 1997, 1999; Chuang and Kornberg, 2000). The release of cholesterol-modified Hh-N from the producing cells depends on the activity of the protein Dispatched (Disp) in Hh-producing cells (Burke *et al.*, 1999; Caspary *et al.*, 2002; Ma *et al.*, 2002; Pfeiffer *et al.*, 2002). The *disp* mutant cells appear to produce, process, and modify Hh normally but are unable to release Hh-N and instead accumulate the protein to high levels. Intriguingly, *disp* is predicted to encode a sterol-sensing domain (SSD)-containing 12-pass transmembrane protein with sequence homology to the Hh-binding receptor protein Ptc. Ptc has been shown to limit Hh movement, in part, by targeting Hh for internalization and degradation (Chen and Struhl, 1996; Burke *et al.*, 1999; Denef *et al.*, 2000; Incardona *et al.*, 2000, 2002). Thus, both the sending and the receiving cells require the activity of an SSD-containing protein to regulate the movement of Hh-N. Receiving cells need Ptc to sequester cholesterol modified Hh-N while sending cells need Disp to release it. How exactly Disp works in Hh release remains to be determined. It is also not known how Hh moves across tissues. This may be a case where exovesicle (argosome)-mediated movement may prove to be important.

Once Hh is released, its ability to move is influenced by heparan sulfate proteoglycans (HSPGs) (Bellaiche *et al.*, 1998; Selleck, 2000). The *tout-velu* (*ttv*) gene encodes an enzyme needed for HSPG biosynthesis and its activity is needed to allow movement of cholesterol-modified Hh-N in A cells (The *et al.*, 1999; Toyoda *et al.*, 2000). HSPGs are highly O-glycosylated proteins found abundantly at the cell surface and the extracellular matrix. HSPGs interact with a variety of extracellular proteins such as growth factors, proteases, protease inhibitors, and adhesion molecules. Through these interactions, they participate in many events

during cell adhesion, migration, proliferation, and differentiation. Biochemical analyses have indicated that Hh is a heparin-binding protein (Lee *et al*., 1994). How exactly Ttv and HSPGs affect the distribution of Hh-N is still unknown. Hh-N may require HSPGs for its stability in the extracellular space. Alternatively, binding of Hh-N to HSPGs could promote its release from producing cells, prevent its reinsertion into the membrane or its sequestration by Ptc. One could speculate that transport of lipid-modified Hh depends on a yet unidentified carrier protein able to mask the lipid moiety. The possibility of a connection between such a protein and the HSPGs remains to be explored.

1.9.3.1.4. Direct patterning activity of Hedgehog Although the largest morphological changes observed in wings lacking or ectopically activating Hh signaling are caused by changes in the expression of the Hh target gene Dpp, Hh also has a more direct effect on patterning. Hh acts directly to pattern the central region of the wing, more specifically the region between the veins 3 and 4 (Mullor *et al*., 1997; Strigini and Cohen, 1997). This short-range patterning activity of Hh is at least in part mediated through the local induction of En and the transcription factor Collier (Col) (Nestoras *et al*., 1997; Vervoort *et al*., 1999). Another important function of Hh, mediated through a Ci-dependent but thus far unidentified target gene, is preventing A and P cells from mixing, thus maintaining the AP compartment boundary (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Dahmann and Basler, 2000) (**Figure 3a**).

1.9.3.2. Decapentaplegic

Decapentaplegic (Dpp) is a member of the bone morphogenetic protein (BMP) class of transforming growth factor-β (TGF-β) signaling molecules (Hoffmann and Goodman, 1987; Irish and Gelbart, 1987; Padgett *et al*., 1987; reviews: Padgett *et al*., 1998; Raftery and Sutherland, 1999; Massague and Chen, 2000). Many different biological processes are mediated by TGF-β ligands. These include the regulation of growth, differentiation, and cell fate determination. Defects in TGF-β signaling components have been implicated in a number of heritable disorders (review: Massague *et al*., 2000).

1.9.3.2.1. Dpp signaling In *Drosophila*, three BMP-like factors are known: Dpp, Glass bottom boat (Gbb), and Screw (Scw). Dpp seems to be the most important factor for limb development. TGF-β factors initiate signaling by assembling receptor complexes that activate Smad transcription factors. The ligand brings together members from two families of receptor serine/threonine kinases, known as the type I and type II receptors (reviews: Raftery and Sutherland, 1999; Massague and Chen, 2000; Massague *et al*., 2000). The principal Dpp receptors in *Drosophila* are Thickveins (Tkv) and Punt (Pnt), type I and II, respectively (Brummel *et al*., 1994; Letsou *et al*., 1995; Ruberte *et al*., 1995). Saxophone may also cooperate with Pnt to mediate Dpp signaling. The only known function of the type II receptor (Pnt) is to activate the type I receptor (Tkv). Tkv propagates the signal by phosphorylating the *Drosophila* Smad, Mad (Mothers against Dpp) (Newfeld *et al*., 1996; Wiersdorff *et al*., 1996). In addition to Mad, which belongs to the class of receptor-regulated Smads (R-Smads), Dpp signaling requires the action of the co-Smad Medea (Med) (Das *et al*., 1998; Hudson *et al*., 1998; Wisotzkey *et al*., 1998). Upon phosphorylation of Mad by Tkv, it associates with Medea and translocates into the nucleus, where the complex directly binds to DNA and regulates transcription (e.g., Kim *et al*., 1997a; review: Raftery and Sutherland, 1999). However, activation of the Dpp pathway at high levels induces the expression of an inhibitory Smad (Dad), which acts to reduce Dpp signaling activity in these cells, thereby limiting the maximal extent of pathway activation (Tsuneizumi *et al*., 1997). The transcription factor Brinker (Brk) is another antagonist that modulates cellular responsiveness to Dpp signaling. Brinker represses expression of Dpp target genes in the wing disc (Campbell and Tomlinson, 1999; Jazwinska *et al*., 1999; Minami *et al*., 1999). Interestingly, *brinker* expression is negatively regulated by Dpp, providing another level of feedback regulation.

1.9.3.2.2. Regulation of Dpp gradient formation Dpp is the main organizer of patterning along the AP axis in the wing imaginal disc. Dpp is produced by A cells adjacent the AP compartment boundary in the *Drosophila* wing (**Figure 3b**) and forms a long-range gradient that activates several target genes in a concentration-dependent manner (Lecuit *et al*., 1996; Nellen *et al*., 1996; Lecuit and Cohen, 1998; Entchev *et al*., 2000; Teleman and Cohen, 2000; Entchev and Gonzalez-Gaitan, 2002). Whether free diffusion alone can explain Dpp gradient formation remains unclear. Receptor-mediated endocytosis contributes to shaping the Dpp gradient by targeting some Dpp for degradation (Entchev *et al*., 2000; Teleman and Cohen, 2000). Downregulation of the receptor protein Tkv in response to Dpp signaling is needed to allow long-range movement of the ligand (Lecuit

and Cohen, 1998; Tanimoto *et al.*, 2000). Tkv downregulation is mediated by *mtv*, itself a target gene of En and Hh (Funakoshi *et al.*, 2001). Receptor-mediated endocytosis has also been proposed to be required for Dpp transport (Entchev *et al.*, 2000), though this proposal has been challenged (Lander *et al.*, 2002).

Different levels of Dpp signaling activity lead to further, noncompartmental, subdivision along the AP axis in the *Drosophila* wing disc. The *spalt-major* (*salm*) and *spalt-related* (*slr*) genes are expressed in broad domains centered on the AP boundary in response to Dpp signaling (de Celis *et al.*, 1996a; Lecuit *et al.*, 1996), subsequently positioning specialized cells to form the wing veins (de Celis *et al.*, 1996a; Sturtevant *et al.*, 1997). Salm and Slr regulate the expression domains of Caps and Tartan and confer a medial–lateral affinity difference that contributes to regionalization along the AP axis (Milan *et al.*, 2001a). The *optomotor blind* gene is expressed in a broader domain under Dpp control and is required for cell survival in the center of the wing disc (Grimm and Pflugfelder, 1996; Lecuit *et al.*, 1996).

1.9.3.3. Wingless

Wingless (Wg) is a member of the Wnt family of secreted glycoproteins (Rijsewijk *et al.*, 1987) and fulfills a number of roles during development. Wg forms an extracellular gradient in the wing imaginal disc (Strigini and Cohen, 2000) and activates several target genes in a concentration-dependent manner to organize the DV axis of the wing (Zecca *et al.*, 1996; Neumann and Cohen, 1997) and leg (Struhl and Basler, 1993; Brook and Cohen, 1996; Jiang and Struhl, 1996) (**Figures 4b, c, and 7**) and the PD axis in the leg (Lecuit and Cohen, 1997) (**Figure 6a**). Hence it can be defined as a morphogen. Here, the aspects of its production, secretion, and travel in the imaginal discs will be discussed. The Wg signal transduction pathway has been reviewed elsewhere (Wodarz and Nusse, 1998).

1.9.3.3.1. Wg production Wg secretion requires the activity of the gene *porcupine (por)*. Cells mutant for *por* show normal expression of the Wg protein but fail to secrete it. *por* encodes a multipass transmembrane protein with similarity to the acyltransferase ski, suggesting that Porcupine might acylate Wg (van den Heuvel *et al.*, 1993; Kadowaki *et al.*, 1996; Chamoun *et al.*, 2001; Lee and Treisman, 2001). In embryos, localized apical secretion of Wg has been shown to be important for its function (Simmonds *et al.*, 2001). It is not known whether polarized secretion is important in the imaginal discs.

1.9.3.3.2. Wg movement Diffusion of extracellular Wg protein has been shown to be sufficient for gradient formation in the wing disc (Strigini and Cohen, 2000). Mechanisms involving endocytosis/resecretion and argosomes have also been proposed to explain Wg movement. Wg secretion requires dynamin activity; however, once secreted, Wg movement does not require dynamin-mediated endocytosis/exocytosis (Strigini and Cohen, 2000). Indeeed, *dynamin* mutant cells accumulate higher than normal levels of extracellular Wg protein, suggesting that endocytosis plays an important role in shaping the gradient by removing Wg (Strigini and Cohen, 2000).

Evidence for vesicle-based transport of Wg has been presented for the embryo. Progeny of Wg-producing cells move away from the segment boundary in the embryo, allowing delayed secretion of Wg by cells at a distance from the compartment boundary where Wg mRNA is expressed (Pfeiffer *et al.*, 2000). For this reason, a membrane-tethered form of Wg is sufficient to confer a normal range of Wg signaling in the embryo. This mechanism might operate in conjunction with diffusion of secreted Wg. Endocytosis of Wg promotes its degradation in lysosomes (Dubois *et al.*, 2001). However, resecretion of Wg has also been shown to occur, raising the possibility that this mechanism may also contribute significantly to Wg movement in the embryo (Moline *et al.*, 1999; Pfeiffer *et al.*, 2002).

Another mechanism involving vesicle-mediated transport has been proposed based on the observation of Wg-containing exovesicles, called argosomes (Greco *et al.*, 2001). Argosomes were shown to move through the wing disc epithelium, showing a high degree of colocalization with Wg (review: Vincent and Magee, 2002). Despite the lack of direct evidence that these Wg-containing vesicles are required for gradient formation, it is certainly plausible that argosomes contribute to Wg movement. If Wg proves to be acylated, like Hh, argosomes may provide the means to move the lipid-modified ligand.

HSPGs also play an important role in Wg movement and signaling. HSPGs bind Wg at the cell surface and are required for cells to be Wg-responsive (Reichsman *et al.*, 1996). Genetic analysis of components of the biosynthetic pathway of proteoglycans such as Fringe connection (Frc) (Selva *et al.*, 2001), Sugarless (Sgl) (Binari *et al.*, 1997; Hacker *et al.*, 1997; Haerry *et al.*, 1997), Sulfateless (Sfl) (Lin *et al.*, 1999; Baeg *et al.*, 2001), Notum

(Gerlitz and Basler, 2002; Giraldez *et al*., 2002), and the glypican core proteins Dally and Dally-like (Dlp) (Lin *et al*., 1999; Tsuda *et al*., 1999; Baeg *et al*., 2001) revealed that they influence Wg signaling and/or movement (reviews: Bernfield *et al*., 1999; Lin and Perrimon, 2000; Perrimon and Bernfield, 2000; Selleck, 2001). It is, therefore, possible that HSPGs act as low-affinity receptors to stabilize Wg or, perhaps, as coreceptors to facilitate interaction with the signaling receptors Frizzled (Fz) and Drosophila Frizzled 2 (DFz2). Interestingly, most mutations that affect proteoglycan biosynthesis also affect other signaling molecules. Mutations in *dally* genetically interact with *dpp* (Jackson *et al*., 1997), while *sgl* and *sfl* also affect fibroblast growth factor (FGF) signaling (Lin *et al*., 1999).

The *Drosophila* Wg receptor DFz2 is downregulated in response to Wg signaling (Cadigan *et al*., 1998). This reduces the sensitivity of cells exposed to high levels of Wg and allows Wg to travel farther (**Figure 7c**). Response to Wg activity is also modulated by Wg-dependent induction of Naked (Nkd), an EF hand containing protein that serves as a signaling antagonist (Rousset *et al*., 2001, 2002; Zeng *et al*., 2000). Wg also regulates the expression of another secreted inhibitor, Notum (Giraldez *et al*., 2002). These mechanisms collaborate to fine-tune different morphogen gradients. This allows the formation of a reproducible pattern of differentiation and a constant shape and size during animal development.

1.9.4. Growth Control in *Drosophila* Limbs

The size and shape of the adult appendages are determined by the rates of cell division, growth, and death in the imaginal discs (reviews: Bryant and Simpson, 1984; Day and Lawrence, 2000). How is the final and constant size of the discs controlled during development?

1.9.4.1. Extrinsic Regulation of Size

Growth of the imaginal discs is influenced by the nutritional state of the larva. This does not influence body pattern per se, only tissue size (Johnston *et al*., 1999; Britton *et al*., 2002). One mediator of nutrition-dependent size control is the Insulin/PI3-kinase pathway (Chen *et al*., 1996; Leevers *et al*., 1996; Montagne *et al*., 1999; Weinkove *et al*., 1999; Britton *et al*., 2002; Ikeya *et al*., 2002; Oldham *et al*., 2002; reviews: Edgar, 1999; Oldham *et al*., 2000). In addition to insulin, PI3-kinase signaling can be activated by other growth factors and cytokines. PI3-kinase signaling influences the protein biosynthetic capacity of cells by activation of S6-kinase (S6K, the kinase that phosphorylates 40S ribosomal protein S6) (Montagne *et al*., 1999; Thomas, 2002). Activation of target of rapamycin (TOR) signaling is also dependent on nutritional conditions and also directly influences S6K through a parallel, insulin-independent pathway (reviews: Gingras *et al*., 2001; Oldham and Hafen, 2003). TOR-dependent activation of S6K results in direct activation of translation initiation factors and upregulation of a subset of ribosomal RNAs (Jefferies *et al*., 1997; Dennis *et al*., 1999; Meyuhas and Hornstein, 2000).

Thus, nutrition-dependent growth control by different pathways seems to be integrated by the regulation of S6K. These two pathways seem to be linked through the action of the *tuberous sclerosis complex* (*tsc*) genes. Tsc1 and Tsc2 form a complex that inhibits TOR, preventing growth-dependent protein synthesis. In the presence of growth factors, PI3-kinase signaling results in the phosphorylation of Tsc1 and Tsc2. This relieves inhibition of TOR and stimulates protein synthesis (Inoki *et al*., 2002; Manning *et al*., 2002; review: Marygold and Leevers, 2002).

1.9.4.2. Intrinsic Regulation of Size

During development, growth and patterning appear to be tightly linked. By and large patterning appears to determine growth and not vice versa (review: Day and Lawrence, 2000) but there are clear exceptions (Teleman and Cohen, 2000). One view is that size is specified by morphogen gradients emanating from the compartment boundaries (Lawrence and Struhl, 1996; Serrano and O'Farrell, 1997). Clearly, Wg and Dpp signaling are capable of inducing growth and cell cycle progression in a context-dependent manner (Nellen *et al*., 1996; Neumann and Cohen, 1996b, 1997; Martin-Castellanos and Edgar, 2002). However, morphogens cannot simply act as mitogens because they persist within the limb primordium, even after the organ has reached its final size, and proliferation rates do not correlate simply with proximity to the signaling centers.

The polar coordinate model (French *et al*., 1976; Bryant and Simpson, 1984) proposed an explanation by suggesting that growth stops once the set of positional values produced by the morphogen gradients is completed: juxtaposition of cells with disparate positional values triggering growth and intercalation. ("Intercalation" is a term used to indicate the insertion or introduction of anything among others; in this case introduction of structures of

intermediate positional value.) Experimentally induced discontinuities in positional values would be expected to result in intercalary growth. This was certainly the case when discontinuities were generated by surgical removal of a fragment of an imaginal disc leading to regeneration. However, when positional discontinuities were produced by genetic mosaics, no interacalary growth or patterning was observed. For example, clones of cells expressing activated components of morphogen signaling pathways (such as the activated form of the Dpp receptor Tkv) do not trigger intercalary cell proliferation or patterning whereas clones expressing the secreted signaling proteins do so (Lecuit *et al*., 1996; Nellen *et al*., 1996). Overexpression of Dpp in patterns expected to abolish the gradients also fail to block growth. Thus, there is compelling evidence against the idea that growth is driven by intercalation of missing positional values.

Detailed analyses of cell proliferation patterns and cell lineage analysis using X-ray induced clones during larval development have shown no correlation between growth, division, and the distance of a cell from the compartment boundaries (Gonzalez-Gaitan *et al*., 1994; Milan *et al*., 1996). Groups of neighboring cells divide synchronously with respect to their cell cycle stage; these groups are located at random positions throughout the wing anlage. The evidence currently available suggests that morphogen gradients are read out at quite low spatial resolution. Noncompartmental subdivisions of both wings and legs (e.g., wing interveins or leg segments) behave as semiautonomous units of growth control (review: Milan and Cohen, 2000a). Thus, elimination of a vein or a leg joint causes a reduction in size of the two adjacent intervein or leg segments territories to that of a single one (de Celis *et al*., 1996a; de Celis, 1998). There is no strong effect on the size of surrounding territories. These observations are difficult to reconcile with a model in which the morphogen gradients directly control final wing or leg size. Rather, they suggest that continuous subdivision of the limb primordia into smaller territories may be an important intermediate step in the growth regulation process.

An alternative to the polar coordinate model, the Entelechia model, has been proposed by Antonio García-Bellido (García-Bellido and de Celis, 1992; García-Bellido and García-Bellido, 1998). According to this model, cell proliferation is driven by differences in the activity of certain genes ("Martial genes"). Although the nature of these genes is not defined, it is assumed that the level of their activity can be communicated to neighboring cells, so that differences in level can trigger division of nearby cells. It is assumed in the model that the level of Martial genes is increased in the daughter cells, thus leading to a propagation of a wave of cell division across the tissue. An initial asymmetry in the activity levels of Martial genes is needed to trigger the process. This is envisaged to result from differences in cells on opposite sides of compartment boundaries. Cell proliferation stops when the differences between cells fall below detection thresholds, thus reaching the Entelechia condition ("Entelechia": Aristotelian term for completion or perfection). This self-assembly model explains how short-range cell interactions control final organ size, independently of morphogen gradients. Although logically consistent, this model is quite abstract. It is not evident what sort of signals could propagate across the epithelium. The basic premise that proliferation in one group of cells should drive a wave of proliferation that travels across the tissue is, in principle, testable using genetic mosaics.

Yet there is evidence that molecules involved in patterning such as Notch, Wg, and Dpp can drive cell proliferation. Overactivation of Dpp signaling causes accelerated wing cell growth and cell cycle progression in a coordinated way (Martin-Castellanos and Edgar, 2002). Experimental removal of Dpp, alternatively, leads to undergrowth (Burke and Basler, 1996; Kim *et al*., 1996). It has been suggested that Dpp acts as a survival factor and that its loss eventually results in removal of tissue from the epithelium by apoptosis (Moreno *et al*., 2002). Thus, while patterning molecules have been shown to influence cell proliferation, their role is more likely to be of permissive than of instructive nature.

1.9.5. Evolutionary Conservation of Limb Development

Vertebrate limb development has also been extensively studied because it provides an excellent experimental model to study the cellular and molecular mechanisms that regulate pattern formation during embryogenesis. A combination of classical embryological studies, ectopic expression analyses in the chick embryo, and gene disruption in the mouse has contributed to understanding how growth and patterning are controlled in the vertebrate limb bud (reviews: Johnson and Tabin, 1997; Schwabe *et al*., 1998; Capdevila and Izpisua Belmonte, 2001; Tickle and Munsterberg, 2001). There is ample evidence now that similar genes are involved in both vertebrate and invertebrate appendage formations. Does that mean that vertebrate and invertebrate limbs are homologous structures? Since molecular

tools have become available, a more rigorous examination of the evolutionary conservation of limb development is possible. In this section, a brief review is done on the mechanisms underlying vertebrate appendage development and compare them with invertebrate limb development, as exemplified by *Drosophila*.

The limb primordium (limb bud) is composed of mesenchymal cells derived from the somatic portion of the lateral plate mesoderm (LPM) and is covered by a layer of ectoderm (**Figure 8**). It appears at specific locations along the body axis of the developing embryo and will grow out of the body wall to form the limb. Interactions between the mesenchyme and the ectoderm coordinate growth and patterning of the primordium through the activity of organizing centers such as the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA).

1.9.5.1. Initiation of Limb Budding

The mechanisms that allocate the limb fields within the lateral plate mesoderm are still a matter of debate. It has been suggested that the establishment of overlapping domains of *Hox* gene expression prepatterns the embryonic flank at specific positions for limb induction. This proposal is based on Hox expression patterns; no functional studies have been performed to date (Oliver *et al.*, 1990; Rancourt *et al.*, 1995).

The onset of limb bud formation is marked by a bulge in the lateral wall of the embryo. It results from the continuous proliferation of cells in the prospective limb-forming region and a concomitant decrease in cell proliferation elsewhere along the length of the LPM (Searls and Janners, 1971). Signaling mechanisms that mediate directional transfer of information between different tissues such as the intermediate mesoderm (IM), the LPM, and the surface ectoderm are involved in initiation of the limb bud. It is not entirely clear, to date, which factors are responsible for induction of limb outgrowth. One mesenchymal signal that is essential for limb outgrowth is the product of the *Fgf10* gene (**Figure 8a** and **b**). Fgf10 is a member of the FGF family of secreted proteins that signal by binding to high-affinity cell surface receptors (FGFRs), thereby activating intracellular signaling pathways including the RAS-MAPK pathway. Fgf10 is initially widely expressed in the LPM but at the stage of limb initiation is restricted to the prospective limb regions. Both loss-of-function and gain-of-function analyses have shown that Fgf10 is a key factor in limb bud initiation (Ohuchi *et al.*, 1997; Min *et al.*, 1998; Sekine *et al.*, 1999). What induces Fgf10 in the LPM is unclear, though. Fgf8 expressed in the intermediate mesoderm was originally proposed to mediate limb outgrowth by inducing Fgf10 (e.g., Cohn *et al.*, 1995). The juxtaposition of IM and LPM was considered to be essential for this process, since ablation of the mesonephritic mesoderm by laser microsurgery abolished limb development (Geduspan and Solursh, 1992). However, genetic evidence for a role of Fgf8 in the IM for limb field formation is lacking. Recently it has been reported that Wnt-2b and Wnt-8c (like Wg members of the Wnt family of secreted signaling molecules) control Fgf10 expression in the chick forelimb and hindlimb, respectively (Kawakami *et al.*, 2001; Ng *et al.*, 2002; review: Martin, 2001) (**Figure 8a**). Whether or not this signal emanates from the IM and if other axial structures like the embryonic organizer (Hensen's node) or the somites are required for limb field initiation remains unclear.

1.9.5.2. Induction of the AER and Limb Outgrowth

Shortly after the limb bud first becomes visible, changes in cell shape within the surface ectoderm result in the appearance of a ridge that runs along the distal margin of each limb bud (**Figure 8c**). Once this AER has formed, the limb bud elongates along its PD axis, flattens along its DV axis, and becomes asymmetric along its AP axis. AER formation is a critical event because it produces signals that organize limb development (review: Capdevila and Izpisua Belmonte, 2001). The integrity of the AER is essential for continued cell proliferation after initiation of limb budding (Todt and Fallon, 1984). Surgical removal of the AER results in limb truncation at any time during development.

Several genes expressed in the surface ectoderm already show a DV asymmetry prior to AER induction, e.g., Radical fringe (Rfng) and En-1 (one of the vertebrate *engrailed* homologs). Rfng is expressed in the dorsal ectoderm (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997), whereas En-1 is expressed exclusively in ventral cells (Davis and Joyner, 1988). The AER forms right at the interface between the Rfng expressing and nonexpressing cells, En-1 ensuring repression of Rfng ventrally. Through this mechanism, a sharp boundary is maintained. Ectopic expression of En-1 in dorsal cells was reported to repress Rfng in the chick embryo and to induce ectopic AERs and outgrowths, showing that the juxtaposition of Rfng positive and negative cells is able to set up the AER (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). However, *Rfng*$^{-/-}$ mice show normal limb development (Moran *et al.*, 1999b). Functional redundancy with

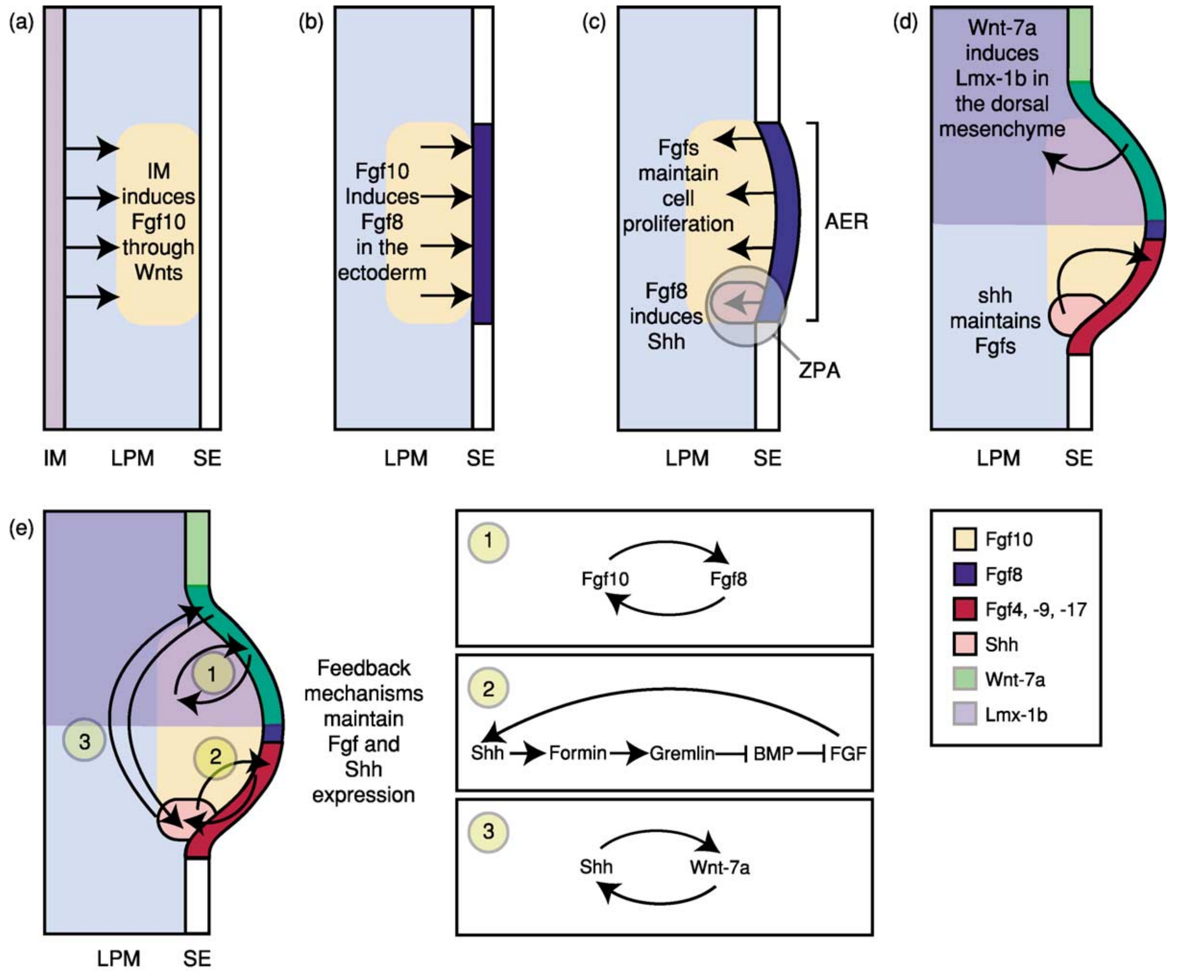


Figure 8 Vertebrate limb development. (a) Signaling from the intermediate mesoderm (IM) involving members of the Wnt family induces expression of Fgf10 in the lateral plate mesoderm (LPM). (b) Lateral plate Fgf10 induces Fgf8 expression in the surface ectoderm (SE), establishing the apical ectodermal ridge (AER). (c) Fgf signaling from the AER maintains cell proliferation in the underlying mesenchyme. Fgf8 signaling from the AER induces the expression of Sonic hedgehog (Shh) in the ventral mesenchyme, establishing the zone of polarizing activity (ZPA). (d) Shh signaling maintains Fgf signaling in the AER. Wnt-7a signaling induces the expression of Lmx-1b in the dorsal mesenchyme, where it is essential for dorsal specification. (e) Three feedback mechanisms maintain coordinate activity of the organization of three body axes. Fgf10 and Fgf8 maintain cell proliferation in the mesoderm, ensuring proper development of the proximal–distal axis. Fgf signaling from the AER maintains Shh in the ZPA. Wnt signaling from the dorsal ectoderm is required for Shh maintenance in the ZPA. The ZPA is the integration point for axis coordination in the vertebrate limb. Details of the autoregulatory loops 1–3 are shown at right.

other Rfng homologs (Lunatic Fringe and Manic Fringe) is unlikely, because expression of neither of them was detectable in the limb primordium (Johnston *et al*., 1997; Moran *et al*., 1999a). Whether these apparently contradictory results can be explained in terms of differences between the mouse and the chicken remains to be determined.

Fgf10 signaling from the LPM to the somatic ectoderm is essential for AER formation. It has been proposed that Fgf10 may be the mesenchymal mediator of the limb induction signal and thus function as the endogenous inducer of Fgf8 expression in the overlying ectoderm (Ohuchi *et al*., 1997) (**Figure 8b**). Fgf8 signaling from the AER is, in turn, essential for normal limb development (Lewandoski *et al*., 2000; Moon and Capecchi, 2000). Maintenance of Fgf10 expression in the limb bud mesoderm and Fgf8 expression in the AER is most likely dependent on a mutual positive feedback mechanism (**Figure 8c** and **d**). Recently it has been demonstrated that Wnt-3a is required for this Fgf8/Fgf10 loop to be sustained (Kawakami *et al*., 2001; Barrow *et al*., 2003). Interestingly, ablation of the AER can be rescued by artificial expression of Fgf2, Fgf4, or Fgf8 (Niswander *et al*., 1993). These factors are also capable of inducing an ectopic AER and any of them can sustain the AER's morphogenetic functions. Their redundancy can be explained by the

fact that they signal through the same FGFR (Fgf1c) in the mesoderm. Hindlimb buds of mice lacking both Fgf4 and Fgf8 do not develop a proper limb. However, since limb buds initially form, they are not required for AER formation but are essential for the expression of numerous genes in the underlying mesenchyme (e.g., Sonic hedgehog (Shh)) (Sun *et al.*, 2002). Taken together, Fgfs mediate the function of the AER in terms of limb outgrowth. They stimulate cell proliferation in the underlying mesoderm and are required to maintain Shh expression (see Section 1.9.5.4).

1.9.5.3. DV Patterning

The actual patterning of the outgrowing limb bud is tightly linked to the positioning of the AER itself (see Section 1.9.5.2). Many structures of the fully grown vertebrate limb show DV asymmetry, like tendons, bones, and muscles. Early transplantation experiments suggested that signaling from the dorsal non-ridge ectoderm is responsible for the establishment of DV asymmetry in the limb (MacCabe *et al.*, 1974). One of the signaling molecules responsible for dorsal patterning is Wnt-7a, which induces the LIM-homeodomain transcription factor Lmx-1b in the dorsal mesenchyme. Lmx-1b then specifies dorsal identities in the limb (Parr and McMahon, 1995; Riddle *et al.*, 1995; Vogel *et al.*, 1995; Chen *et al.*, 1998; review: Chen and Johnson, 1999) (**Figure 8d**). En-1 acts as a ventralizing factor and is required for repression of Wnt-7a in the ventral ectoderm (Loomis *et al.*, 1998). BMPs are necessary and sufficient to regulate the ventral expression of En-1 and are therefore required for DV patterning of the limb (Ahn *et al.*, 2001; Pizette *et al.*, 2001).

1.9.5.4. The ZPA as the Organizer of AP Polarity

In addition to DV polarity, the vertebrate limb obviously has an AP asymmetry axis as evident in muscles, bones, and tendons. Growth and patterning along the AP axis is tightly linked with development of the PD axis. This process is controlled by an interplay between the AP organizer (the ZPA) and the AER.

The ZPA is a group of cells in the posterior mesenchyme of the limb bud and its effects have been shown to be mediated by Shh (Riddle *et al.*, 1993; Chang *et al.*, 1994; Lopez-Martinez *et al.*, 1995; (**Figure 8c and d**). Shh is a homolog of the *Drosophila* secreted signaling molecule Hedgehog (see Section 1.9.3.1). While Shh is not required to establish initial AP polarity, it is absolutely essential for growth and patterning of intermediate and distal elements of the limb, and is the only factor known to date to mediate the effects of the ZPA. Shh expression is dependent on retinoic acid (RA) signaling (Helms *et al.*, 1996) and also requires AER signals, which restrict it to distal regions (Niswander *et al.*, 1994). In turn, Shh is also required for AER maintenance. Continuous Fgf signaling in the AER (which positively feeds back on Shh maintenance) depends on the limitation of BMP signaling from the underlying mesoderm (Pizette and Niswander, 1999). This effect has been proposed to be mediated by Shh through the BMP antagonist Gremlin (Capdevila *et al.*, 1999; Merino *et al.*, 1999; Zuniga *et al.*, 1999). The specification and maintenance of the AP and PD axes are tightly linked through this mechanism.

Furthermore, Shh expression is dependent on Wnt-7a signaling from the dorsal ectoderm (Yang and Niswander, 1995). Since Wnt-7a is also required for the establishment of dorsal fate (Parr and McMahon, 1995) (see Section 1.9.5.3), dorsal signals contribute to the regulation of AP patterning. The formation of all three axes of the limb is therefore tightly connected (**Figure 8**).

1.9.5.5. Proximal Patterning of the Vertebrate Limb

Interestingly, proximal patterning in the vertebrate limb seems to be dependent on similar factors to those in *Drosophila*. The *Pbx1* and *Meis* genes (homologs of *Exd* and *Hth*, respectively) are restricted to proximal parts of the limb bud. Activity of the *Meis* genes is both necessary and sufficient to confer proximal fate, similar to the action of *Hth* in *Drosophila* (Capdevila *et al.*, 1999; Mercader *et al.*, 1999, 2000).

1.9.5.6. Compartments in the Vertebrate Limb

The vertebrate limb bud ectoderm is subdivided into compartments (review: Tickle and Munsterberg, 2001). This subdivision occurs between dorsal and ventral cells even though there is some debate about the exact position of the lineage restriction boundary. All studies agree that the boundary of the ventral compartment lies at the midpoint of the AER. Surprisingly, in some experiments, dorsal cells seem to be able to cross this boundary to fill the entire ridge in the chick (but do not move into ventral ectoderm) (Altabef *et al.*, 1997). How this result can be reconciled with the existence of the ventral lineage restriction boundary remains unclear. In the mouse, the compartment boundary within the AER is transient. Additionally, a second lineage boundary between dorsal ectoderm and the AER exists (Kimmel *et al.*, 2000).

The midpoint of the AER corresponds exactly to the En-1 expression domain. However, *En-1* is not a ventral selector gene because the lineage boundary remains intact in *En-1*$^{-/-}$ mice (Loomis *et al.*, 1996). Also, misexpression of En-1 does not perturb the boundary (Altabef *et al.*, 2000). The mechanism responsible for the establishment of compartmental subdivision in the limb bud ectoderm therefore remains to be identified. For this reason, it has not been possible to experimentally eliminate boundary formation to investigate whether the existence of the boundary is essential for AER formation.

1.9.5.7. Similarities and Differences between *Drosophila* and Vertebrate Limb Development

Both *Drosophila* and vertebrate limbs are outgrowths of the body wall. In principle, they serve the animal to fulfill similar tasks. It is obvious that some elements of the regulatory circuitry and some of the same signaling pathways are used for limb generation in different organisms. This observation raises the question of whether *Drosophila* and vertebrate limbs are truly homologous structures or whether they are merely constructed according to a different plan, but using the same toolbox. The consideration of a few examples leads us to favor the latter view.

One cause for concern is raised by the idea that a DV body axis inversion has taken place between vertebrates and invertebrates (Arendt and Nubler-Jung, 1994; 1997; Holley *et al.*, 1995; De Robertis and Sasai, 1996). Should this be the case, then dorsal and ventral should be reversed in the limbs as well. In *Drosophila*, Ap is required for establishment of the dorsal compartment. It has been suggested that two LIM-homeodomain proteins, Lhx2 and Lmx1, perform Ap-related functions in vertebrate development (Rodriguez-Esteban *et al.*, 1998; Rincon-Limas *et al.*, 1999). Lmx1 confers dorsal identity in the vertebrate limb mesenchyme but is not involved in limb outgrowth (Parr and McMahon, 1995; Riddle *et al.*, 1995; Vogel *et al.*, 1995; Chen *et al.*, 1998). If these were homologous processes, Lmx1 is expected to be ventral in the vertebrate limb. In fact, Lmx1 is not very closely related to Ap, and is much more similar to the *Drosophila* LIM-homeodomain factors Lim1 and Lim3, neither of which seems to be involved in wing development (Hobert and Westphal, 2000).

The function of Lhx2 in the vertebrate limb remains even more elusive. Lhx2 is expressed in distal mesoderm in the limb bud (Rincon-Limas *et al.*, 1999; Lu *et al.*, 2000) and has the capacity to induce Rfng in the interlimb but not in the limb field. However, no AER is induced and mice lacking functional Lhx2 do not show a limb phenotype. It was proposed that Lhx2 performs a growth function (Porter *et al.*, 1997; Rodriguez-Esteban *et al.*, 1998). One could speculate that functional redundancy with another very closely related LIM-homeodomain factor, Lhx9, could explain these observations. Lhx9 is expressed in the forelimb and hindlimb mesenchyme but no further functional data is available (Retaux *et al.*, 1999). It will, therefore, depend on careful loss-of-function analyses of Lhx2 and Lhx9 to determine whether the multiple functions of Ap in *Drosophila* wing development are performed by these proteins in the vertebrate limb.

The expression pattern of Rfng in the dorsal ectoderm (see Section 1.9.5.2) is reminiscent of the dorsal pattern of expression of Fng in the *Drosophila* wing imaginal disc. However, in contrast to *Drosophila*, there is no data that supports the role of Rfng in DV patterning of the vertebrate limb (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). Moreover, Lhx2 and Rfng are not coexpressed under wild-type circumstances, with Lhx2 being expressed only in more distal tissues in the limb bud. Therefore, the inducibility of Rfng in the interlimb region by Lhx2 is not proof for a functional conservation between Lhx2/Rfng and Ap/Fng, respectively. Furthermore, the role of Rfng in the establishment of DV patterning and the AER remains unclear (see Section 1.9.5.2), and a functional conservation between Fng and Rfng, in terms of limb organization, appears unlikely.

Like in the *Drosophila* wing, En-1 plays an important role in vertebrate development. However, while in the *Drosophila* wing it performs the role of a selector gene for posterior patterning, in the vertebrate limb bud it acts in ventral ectoderm and, therefore, performs quite a different role than in *Drosophila*. Nonetheless Hh and Shh are both implicated in sending signals from posterior cells.

It is not obvious how one could make an argument for a homologous developmental regulatory network. Nevertheless, the same "genetic toolbox" is used in *Drosophila* limb development and in the formation of the vertebrate limb. Therefore, it seems more plausible that limbs in different organisms are not homologous as appendage structures, but are likely to have a common origin in evolution as body wall outgrow (see Panganiban *et al.*, 1997).

Appendix Genes and proteins involved in limb development

Abbreviation	*Gene or protein name*	*Function*
ac-sc	*achaete-scute complex*	Proneural genes
ap	*apterous*	Dorsal selector gene
ara	*araucan*	Part of the Iroquois complex; important for notum patterning
argos	*argos*	Component of the EGFR signal transduction pathway
bs	*blistered*	*Drosophila* homolog of the serum response factor (SRF)
BMP	Bone morphogenetic protein	Class of secreted factors important in development of fly (Dpp) and vertebrate development (e.g., BMP2, 4); belong to the transforming growth factor-β (TGF-β) family
brk	*brinker*	Antagonist of Dpp signaling
Bx	*Beadex*; synonym for *dLMO*	Antagonist of Apterous activity
caps	*capricious*	Leucine-rich-repeat (LRR) transmembrane protein involved in dorsal–ventral affinity regulation
caup	*caupolican*	Part of the Iroquois Complex; important for notum patterning
Chp	*Chip (dLDB)*	Cofactor required for Apterous activity
Ci	*Cubitus interruptus*	Transcription factor essential for Hh signal transduction
col (kn)	*collier (knot)*	*hh* target gene, required for specification of veins three and four in the wing
cos-2	*costal-2*	Component of the Hedgehog signal transduction pathway
dac	*dachshund*	Target gene of Wg and Dpp in the *Drosophila* leg
dad	*daughters against dpp*	Inhibitory Smad
dally	*dally*	Glypican core protein, related to Dally-like
dlp	*dally-like*	Glypican core protein, related to Dally
DFz2 (fz2)	*Drosophila Frizzled 2*	Wg receptor for the canonical Wg pathway
disp	*dispatched*	Transmembrane protein of the Patched (Ptc) family; required for release of cholesterol-modified Hedgehog (Hh)
Dl	*Delta*	Notch ligand
dLDB	*Drosophila LIM-domain binding*	Cofactor required for Apterous activity
Dll	*Distal-less*	Wg target gene required for distal specification of *Drosophila* legs
dLMO	*Drosophila LIM-domain only*	Antagonist of Apterous activity
Dpp	*Decapentaplegic*	TGFβ homolog; important for anterior–posterior patterning in *Drosophila* limbs
Ddyn	*dynamin*	Protein involved in formation and release of endocytic vescicles
E(spl)	*Enhancer of split*	*Notch* target gene
EGF	Epidermal growth factor	Ligand for the EGF receptor
EGFR	Epidermal growth factor receptor	Receptor tyrosine kinase
el	*elbow*	Zinc-finger transcriptional repressor, required for distal specification of both wings and legs; binds to No ocelli (Noc)
en	*engrailed*	Posterior selector gene
En-1	Engrailed 1	Vertebrate homolog of the transcriptional repressor Engrailed
Eph		Transmembrane receptor; able to signal bidirectionally
Ephrin		Ligand for the Eph receptor; able to signal bidirectionally
exd	*extradenticle*	A binding partner of Hth
FGF	Fibroblast growth factor	Class of secreted factors important in many processes of vertebrate development (e.g., Fgf8, Fgf70)
FGFR	Fibroblast growth factor receptor	Class of receptor tyrosine kinases binding to the secreted proteins of the Fgf class (with different specificities)
fng	*fringe*	Glycosyltransferase required for Notch–Delta interaction
formin	*formio*	In the context of limb development, the *ld* mutants are gain-of-function mutants that cause misexpression of *gremlin* and inhibit BMP activity. Formins were misidentified as the *ld* gene product
frc	*fringe connection*	Enzyme required for heparan-sulfate proteoglycan biosynthesis
fz	*frizzled*	Wg receptor for the planar polarity pathway
gbb	*glass bottom boat*	BMP-like factor in *Drosophila*
Gremlin		BMP antagonist
hh	*hedgehog*	Morphogen important for anterior–posterior patterning in the *Drosophila* wing
hindsight	*hindsight*	Transcription factor, allelic to *pebble* (*peb*)
hth	*homothorax*	Homeodomain transcription factor; required for proximal specification of both wings and legs
inv	*invected*	Posterior selector gene, closely related to *engrailed*
Iro-C	*Iroquois Complex*	Gene complex consisting of *ara*, *caup*, and *mirr*; important for notum patterning
Lfng	Lunatic fringe	Member of the Fringe family of glycosyltransferases in vertebrates
Lhx2		Vertebrate LIM homeodomain protein
Lhx9		Vertebrate LIM homeodomain protein
Lim1		Transcription factor
Lim3		Transcription factor
Lmx1		Vertebrate LIM homeodomain protein

Continued

Appendix Continued

Abbreviation	*Gene or protein name*	*Function*
Mad	*Mothers against Dpp*	*Drosophila* SMAD, signal transducer of the Dpp pathway
med	*medea*	*Drosophila* co-SMAD
Meis		Vertebrate homolog of *hth*
Mfng	Manic fringe	Member of the Fringe family of glycosyltransferases in vertebrates
mirr	*mirror*	Part of the Iroquois Complex; important for notum patterning
msh	*muscle segment homeobox*	Transcription factor mediating dorsal wing identity
mtv	*master of Thickveins*	Downregulates the Dpp receptor Thickveins
N	*Notch*	Transmembrane receptor
nkd	*naked*	EF-hand containing protein; Wingless-antagonist
noc	*no ocelli*	Zinc-finger transcriptional repressor, required for distal specification of both wings and legs; binds to Elbow (El)
notum	*notum*	Negative regulator of *wingless*
nub	*nubbin*	POU-domain transcription factor; essential for wing and hinge development
omb	*optomotor blind*	Target gene of the Dpp pathway
Pbx1	postbithorax 1	Vertebrate homolog of *Exd*
Pc	*Polycomb*	Involved in chromatin based gene silencing
PI3K	phosphatidylinositol-3-kinase	Phosphorylates phosphatidylinositol at position 3; important mediator of insulin-dependent growth signals
PKA	Protein kinase A	Negative regulator of *hh* signaling; promotes Ci processing
pnr	*pannier*	Encoding transcription factor required for notum patterning
pnt	*punt*	Type II Dpp receptor in *Drosophila*
por	*porcupine*	Required for secretion of Wingless (Wg)
PS1	Position-specific antigen 1	Integrin-type adhesion molecule
PS2	Position-specific antigen 2	Integrin-type adhesion molecule
ptc	*patched*	Hh-receptor; transmembrane protein
Rfng	Radical fringe	Member of the Fringe family of glycosyltransferases in vertebrates
rho	*rhomboid*	Membrane protein promoting the cleavage of the membrane-anchored TGFalpha-like growth factor Spitz
rn	*rotund*	Zinc-finger transcription factor of the Krüppel family; important for hinge formation
S6K	S6 kinase saxophone	Phosphorylates the small ribosomal protein 6
scw	*screw*	BMP-like factor in *Drosophila*
sd	*scalloped*	Transcription factor required for wing development; Sd binds to Vestigial (Vg)
Ser	*Serrate*	Notch ligand
sfl	*sulfateless*	Enzyme required for heparan-sulfate proteoglycan biosynthesis
sgl	*sugarless*	Enzyme required for heparan-sulfate proteoglycan biosynthesis
Shh	Sonic hedgehog	Vertebrate homolog of the morphogen Hedgehog
ski	*skinny hedgehog*	Acyl transferase
slmb	*slimb*	Negative regulator of Hh signaling
smo	*smoothened*	Transmembrane protein; Hh pathway component
salm	*spalt-major*	Target gene of the Dpp pathway, required for vein formation in the *Drosophila* wing; highly related to and found in a complex with *spalt-related*
slr	*spalt-related*	Target gene of the Dpp pathway, required for vein formation in the wing
TGF-β	Transforming growth factor-β	Class of extracellular growth factors
tkv	*thickveins*	Type II Dpp receptor in *Drosophila*
TOR	*target of rapamycin*	Activity reflects nutritional conditions and leads to the activation of S6 kinase (S6K)
trn	*tartan*	Leucine-rich-repeat (LRR) protein involved in dorsal–ventral affinity regulation
Tsc1	*tuberous sclerosis complex 1*	Forms a complex with Tsc2; inhibits target of rapamycin (TOR) when dephosphorylated
Tsc2 (gig)	*tuberous sclerosis complex 2 (gigas)*	Forms a complex with Tsc1; inhibits target of rapamycin (TOR) when dephosphorylated
tsh	*teashirt*	Required for proximal specification of both wings and legs
ttv	*tout-velu*	Enzyme required for heparan-sulfate proteoglycan biosynthesis
trx	*trithorax*	Counteracts the silencing effects of *Pc* on chromatin to maintain gene activity
ve	*veinlet*	Same as *rhomboid*
vg	*vestigial*	Transcription factor required for wing development; Vg binds to Scalloped (Sd)
vn	*vein*	EGFR ligand
vvl	*ventral veins lacking*	Homeobox transcription factor
wg	*wingless*	Secreted signaling molecule
Wnt	Wingless-related MMTV integration site I	Secreted signaling molecules

References

Abu-Shaar, M., Mann, R., **1998**. Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development 125*, 3821–3830.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Agulnick, A.D., Taira, M., Breen, J.J., Tanaka, T., Dawid, I.B., *et al.*, **1996**. Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins. *Nature 384*, 270–272.

Ahn, K., Mishina, Y., Hanks, M.C., Behringer, R.R., Crenshaw, E.B., III, **2001**. BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal–ventral patterning of the limb. *Development 128*, 4449–4461.

Altabef, M., Clarke, J.D., Tickle, C., **1997**. Dorso–ventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb. *Development 124*, 4547–4556.

Altabef, M., Logan, C., Tickle, C., Lumsden, A., **2000**. Engrailed-1 misexpression in chick embryos prevents apical ridge formation but preserves segregation of dorsal and ventral ectodermal compartments. *Devel. Biol. 222*, 307–316.

Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., *et al.*, **2002**. Gene expression during the life cycle of *Drosophila melanogaster. Science 297*, 2270–2275.

Arendt, D., Nubler-Jung, K., **1994**. Inversion of dorsoventral axis? *Nature 371*, 26.

Arendt, D., Nubler-Jung, K., **1997**. Dorsal or ventral: similarities in fate maps and gastrulation patterns in annelids, anthropods and chordates. *Mech. Devel. 61*, 7–21.

Ashe, H.L., Levine, M., **1999**. Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature 398*, 427–431.

Averof, M., Cohen, S.M., **1997**. The evolutionary origin of insects wings from ancient respiratory appendages. *Nature 385*, 627–630.

Aza-Blanc, P., Ramírez-Weber, F.-A., Laget, M.-P., Schwartz, C., Kornberg, T.B., **1997**. Proteolysis that is inhibited by Hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell 89*, 1043–1053.

Azpiazu, N., Morata, G., **2000**. Function and regulation of homothorax in the wing imaginal disc of *Drosophila*. *Development 127*, 2685–2693.

Azpiazu, N., Morata, G., **2002**. Distinct functions of homothorax in leg development in *Drosophila*. *Mech. Devel. 119*, 55–67.

Bach, I., **2000**. The LIM domain: regulation by association. *Mech. Devel. 91*, 5–17.

Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., Perrimon, N., **2001**. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development 128*, 87–94.

Baonza, A., Garcia-Bellido, A., **1999**. Dual role of *extramacrochaetae* in cell proliferation and cell differentiation during wing morphogenesis in *Drosophila*. *Mech. Devel. 80*, 133–146.

Barrow, J.R., Thomas, K.R., Boussadia-Zahui, O., Moore, R., Kemler, R., *et al.*, **2003**. Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Devel. 17*, 394–409.

Basler, K., Struhl, G., **1994**. Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature 368*, 208–214.

Bate, M., Martinez-Arias, A., **1991**. The embryonic origin of imaginal discs in *Drosophila*. *Development 112*, 755–761.

Bellaiche, Y., The, I., Perrimon, N., **1998**. *Tout-velu* is a *Drosophila* homologue of the putative tumour suppressor *EXT-1* and is needed for Hh diffusion. *Nature 394*, 85–88.

Bellen, H.J., O'Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R., *et al.*, **1989**. *P*-element-enhancer detection: a versatile method to study development in *Drosophila*. *Genes Devel. 3*, 1288–1300.

Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., *et al.*, **1999**. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem. 68*, 729–777.

Bier, E., **2000**. Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Devel. 10*, 393–398.

Binari, R.C., Staveley, B.E., Johnson, W.A., Godavarti, R., Sasisekharan, R., *et al.*, **1997**. Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. *Development 124*, 2623–2632.

Bishop, S.A., Klein, T., Martinez-Arias, A., Couso, J.P., **1999**. Composite signalling from Serrate and Delta establishes leg segments in *Drosophila* through Notch. *Development 126*, 2993–3003.

Blair, S.S., **1992**. *shaggy (zeste-white 3)* and the formation of supernumerary Bristle precursors in the developing wing blade of *Drosophila*. *Devel. Biol. 152*, 263–278.

Blair, S.S., **1993**. Mechanisms of compartment formation: evidence that non-proliferating cells do not play a critical role in defining the D/V lineage restriction in the developing wing of *Drosophila*. *Development 119*, 339–351.

Blair, S.S., **1995**. Compartments and appendage development in *Drosophila*. *BioEssays 17*, 299–309.

Blair, S.S., Brower, D.L., Thomas, J.B., Zavortink, M., **1994**. The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development 120*, 1805–1815.

Blair, S.S., Ralston, A., **1997**. Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior–posterior lineage restriction in the developing wing of *Drosophila*. *Development 124*, 4053–4063.

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B., DeRobertis, E., **1996**. Cereberus is a head-inducing

secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature 382*, 595–601.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., Edgar, B.A., **2002**. *Drosophila's* insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Devel. Cell 2*, 239–249.

Brook, W.J., Cohen, S.M., **1996**. Antagonistic interactions between Wingless and Decapentaplegic responsible for dorsal–ventral pattern in the *Drosophila* leg. *Science 273*, 1373–1377.

Brook, W.J., Diaz-Benjumea, F.J., Cohen, S.M., **1996**. Organizing spatial pattern in limb development. *Annu. Rev. Cell Devel. Biol. 12*, 161–180.

Brower, D.L., Jaffe, S.M., **1989**. Requirements for integrins during *Drosophila* wing development. *Nature 342*, 285–287.

Brückner, K., Klein, R., **1998**. Signaling by Eph receptors and their ephrin ligands. *Curr. Opin. Neurobiol. 8*, 375–382.

Brückner, K., Perez, L., Clausen, H., Cohen, S., **2000**. Glycosyltransferase activity of Fringe modulates Notch–Delta interactions. *Nature 406*, 411–415.

Brummel, T.J., Twombly, V., Marques, G., Wrana, J.L., Newfeld, S.J., *et al.*, **1994**. Characterization and relationship of Dpp receptors encoded by *saxophone* and *thick veins* genes in *Drosophila*. *Cell 78*, 251–261.

Bryant, P.J., Simpson, P., **1984**. Intrinsic and extrinsic control of growth in developing organs. *Q. Rev. Biol. 59*, 387–415.

Burke, R., Basler, K., **1996**. Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development 122*, 2261–2269.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.A., *et al.*, **1999**. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell 99*, 803–815.

Busturia, A., Morata, G., **1988**. Ectopic expression of homeotic genes caused by the elimination of the *Polycomb* gene in *Drosophila* imaginal epidermis. *Development 104*, 713–720.

Butler, M.J., Jacobsen, T.L., Cain, D.M., Jarman, M.G., Hubank, M., *et al.*, **2003**. Discovery of genes with highly restricted expression patterns in the *Drosophila* wing disc using DNA oligonucleotide microarrays. *Development 130*, 659–670.

Cadigan, K.M., Fish, M.P., Rulifson, E.J., Nusse, R., **1998**. Wingless repression of *Drosophila frizzled 2* expression shapes the Wingless morphogen gradient in the wing. *Cell 93*, 767–777.

Calleja, M., Moreno, E., Pelaz, S., Morata, G., **1996**. Visualization of gene expression in living adult *Drosophila*. *Science 274*, 252–255.

Campbell, G., **2002**. Distalization of the *Drosophila* leg by graded EGF-receptor activity. *Nature 418*, 781–785.

Campbell, G., Tomlinson, A., **1995**. Initiation of the proximodistal axis in insect legs. *Development 121*, 619–628.

Campbell, G., Tomlinson, A., **1998**. The roles of homeobox genes *aristaless* and *Distal-less* in patterning legs and wings of *Drosophila*. *Development 125*, 4483–4493.

Campbell, G., Tomlinson, A., **1999**. Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by brinker. *Cell 96*, 553–562.

Capdevila, J., Guerrero, I., **1994**. Targeted expression of the signalling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J. 13*, 4459–4468.

Capdevila, J., Izpisua Belmonte, J.C., **2001**. Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Devel. Biol. 17*, 87–132.

Capdevila, J., Tsukui, T., Rodriguez-Esteban, C., Zappavigna, V., Izpisua Belmonte, J.C., **1999**. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell 4*, 839–849.

Casares, F., Mann, R.S., **2000**. A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in *Drosophila*. *Development 127*, 1499–1508.

Caspary, T., Garcia-Garcia, M.J., Huangfu, D., Eggenschwiler, J.T., Wyler, M.R., *et al.*, **2002**. Mouse dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. *Curr. Biol. 12*, 1628–1632.

Castelli-Gair, J., Akam, M., **1995**. How the Hox gene *Ultrabithorax* specifies two different segments: the significance of spatial and temporal regulation within metameres. *Development 121*, 2973–2982.

Cavodeassi, F., Modolell, J., Campuzano, S., **2000**. The *Iroquois* homeobox genes function as dorsal selectors in the *Drosophila* head. *Development 127*, 1921–1929.

Cavodeassi, F., Modolell, J., Gomez-Skarmeta, J.L., **2001**. The *Iroquois* family of genes: from body building to neural patterning. *Development 128*, 2847–2855.

Cavodeassi, F., Rodriguez, I., Modolell, J., **2002**. Dpp signalling is a key effector of the wing–body wall subdivision of the *Drosophila* mesothorax. *Development 129*, 3815–3823.

Chamoun, Z., Mann, R.K., Nellen, D., von Kessler, D.P., Bellotto, M., *et al.*, **2001**. Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science 293*, 2080–2084.

Chang, D.T., Lopez, A., von Kessler, D.P., Chiang, C., Simandl, B.K., *et al.*, **1994**. Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development 120*, 3339–3353.

Chen, C., Jack, J., Garofalo, R.S., **1996**. The *Drosophila* insulin receptor is required for normal growth. *Endocrinology. 137*, 846–856.

Chen, H., Johnson, R.L., **1999**. Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. *Cell Tissue Res. 296*, 67–73.

Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K.C., *et al.*, **1998**. Limb and kidney defects in *Lmx1b*

mutant mice suggest an involvement of *LMX1B* in human nail–patella syndrome. *Nature Genet. 19*, 51–55.

Chen, Y., Struhl, G., **1996**. Dual roles for Patched in sequestering and transducing Hedgehog. *Cell 87*, 553–563.

Cho, K.O., Chern, J., Izaddoost, S., Choi, K.W., **2000**. Novel signaling from the peripodial membrane is essential for eye disc patterning in *Drosophila*. *Cell 103*, 331–342.

Chuang, P.T., Kornberg, T.B., **2000**. On the range of hedgehog signaling. *Curr. Opin. Genet. Devel. 10*, 515–522.

Cohen, B., McGuffin, M.E., Pfeifle, C., Segal, D., Cohen, S.M., **1992**. *apterous:* a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Devel. 6*, 715–729.

Cohen, B., Simcox, A.A., Cohen, S.M., **1993**. Allocation of the thoracic imaginal disc primordia in the *Drosophila* embryo. *Development 117*, 597–608.

Cohen, B., Wimmer, E.A., Cohen, S.M., **1991**. Early development of leg and wing primordia in the *Drosophila* embryo. *Mech. Devel. 33*, 229–240.

Cohen, S.M., **1990**. Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. *Nature 343*, 173–177.

Cohen, S.M., **1993**. Imaginal disc development. In: Martinez-Arias, A., Bate, M. (Eds.), *Drosophila* Development, vol. 2. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 747–841.

Cohen, S.M., Brönner, G., Küttner, F., Jürgens, G., Jäckle, H., **1989**. *Distal-less* encodes a homoeodomain protein required for limb development in *Drosophila*. *Nature 338*, 432–434.

Cohn, M.J., Izpisua-Belmonte, J.C., Abud, H., Heath, J.K., Tickle, C., **1995**. Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell 80*, 739–746.

Couso, J.P., Bishop, S., Martinez-Arias, A., **1994**. The wingless signalling pathway and the patterning of the wing margin in *Drosophila*. *Development 120*, 621–636.

Couso, J.P., Knust, E., Martinez-Arias, A., **1995**. *Serrate* and *wingless* cooperate to induce vestigial gene expression and wing formation in *Drosophila*. *Curr. Biol. 5*, 1437–1448.

Crick, F.H., Lawrence, P.A., **1975**. Compartments and polyclones in insect development. *Science 189*, 340–347.

Dahmann, C., Basler, K., **1999**. Compartment boundaries: at the edge of development. *Trends Genet. 15*, 320–326.

Dahmann, C., Basler, K., **2000**. Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the *Drosophila* A/P boundary. *Cell 100*, 411–422.

D'Alessio, M., Frasch, M., **1996**. msh may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Devel. 58*, 217–231.

Das, P., Maduzia, L.L., Wang, H., Finelli, A.L., Cho, S.H., *et al.*, **1998**. The *Drosophila* gene *Medea* demonstrates the requirement for different classes of Smads in dpp signaling. *Development 125*, 1519–1528.

Davis, C.A., Joyner, A.L., **1988**. Expression patterns of the homeobox-containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development. *Genes Devel. 2*, 1736–1744.

Day, S.J., Lawrence, P.A., **2000**. Measuring dimensions: the regulation of size and shape. *Development 127*, 2977–2987.

de Celis, J.F., **1998**. Positioning and differentiation of veins in the *Drosophila* wing. *Int. J. Devel. Biol. 42*, 335–343.

de Celis, J.F., Barrio, R., **2000**. Function of the *spalt/spalt*-related gene complex in positioning the veins in the *Drosophila* wing. *Mech. Devel. 91*, 31–41.

de Celis, J.F., Barrio, R., Kafatos, F.C., **1996a**. A gene complex acting downstream of *dpp* in *Drosophila* wing morphogenesis. *Nature 381*, 421–424.

de Celis, J.F., García-Bellido, A., Bray, S.J., **1996b**. Activation and function of *Notch* at the dorsal–ventral boundary of the wing imaginal disc. *Development 122*, 359–369.

de Celis, J.F., Llimargas, M., Casanova, J., **1995**. *Ventral veinless*, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila melanogaster. Development 121*, 3405–3416.

de Celis, J.F., Tyler, D.M., de Celis, J., Bray, S., **1998**. Notch signaling mediates segmentation of the *Drosophila* leg. *Development 125*, 4617–4626.

Del Alamo Rodriguez, D., Terriente, J., Galindo, M.I., Couso, J.P., Diaz-Benjumea, F.J., **2002**. Different mechanisms initiate and maintain wingless expression in the *Drosophila* wing hinge. *Development 129*, 3995–4004.

Denef, N., Neubueser, D., Perez, L., Cohen, S.M., **2000**. Hedgehog induces opposite changes in turnover and subcellular localization of Patched and Smoothened. *Cell 102*, 521–531.

Dennis, P.B., Fumagalli, S., Thomas, G., **1999**. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Opin. Genet. Devel. 9*, 49–54.

De Robertis, E.M., Sasai, Y., **1996**. A common plan for dorsoventral patterning in Bilateria. *Nature 380*, 37–40.

Diaz-Benjumea, F.J., Cohen, S.M., **1993**. Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. *Cell 75*, 741–752.

Diaz-Benjumea, F.J., Cohen, S.M., **1995**. *Serrate* signals through *Notch* to establish a *Wingless*-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. *Development 121*, 4215–4225.

Diaz-Benjumea, F.J., Cohen, B., Cohen, S.M., **1994**. Cell interactions between compartments establishes the

proximal–distal axis of *Drosophila* legs. *Nature 372*, 175–179.

Diez del Corral, R., Aroca, P., Gomez-Skarmeta, J.L., Cavodeassi, F., Modolell, J., **1999**. The Iroquois homeodomain proteins are required to specify body wall identity in *Drosophila*. *Genes Devel. 13*, 1754–1761.

Doherty, D., Fenger, G., Younger-Shepherd, S., Jan, L.-Y., Jan, Y.-N., **1996**. Dorsal and ventral cells respond differently to the *Notch* ligands *Delta* and *Serrate* during *Drosophila* wing development. *Genes Devel. 10*, 421–434.

Dominguez, M., de Celis, J.F., **1998**. A dorsal/ventral boundary established by *Notch* controls growth and polarity in the *Drosophila* eye. *Nature 396*, 276–278.

Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E., Vincent, J.P., **2001**. Regulated endocytic routing modulates wingless signaling in *Drosophila* embryos. *Cell 105*, 613–624.

Duman-Scheel, M., Weng, L., Xin, S., Du, W., **2002**. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature 417*, 299–304.

Eaton, S., Kornberg, T., **1990**. Repression of ci-D in posterior compartments of *Drosophila* by *engrailed*. *Genes Devel. 4*, 1068–1077.

Edgar, B.A., **1999**. From small flies come big discoveries about size control. *Nature Cell Biol. 1*, E191–E193.

Entchev, E.V., Gonzalez-Gaitan, M.A., **2002**. Morphogen gradient formation and vesicular trafficking. *Traffic 3*, 98–109.

Entchev, E.V., Schwabedissen, A., Gonzalez-Gaitan, M., **2000**. Gradient formation of the TGF-beta homolog Dpp. *Cell 103*, 981–991.

Fernandez-Funez, P., Lu, C.H., Rincon-Limas, D.E., García-Bellido, A., Botas, J., **1998**. The relative expression amounts of apterous and its co-factor dLdb/Chip are critical for dorso-ventral compartmentalization in the *Drosophila* wing. *EMBO J. 17*, 6846–6853.

Fleming, R.J., Gu, Y., Hukriede, N.A., **1997**. *Serrate*-mediated activation of *Notch* is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development 124*, 2973–2981.

Fleming, R.J., Scottgale, T.N., Diederich, R.J., Artavanis-Tsakonas, S., **1990**. The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster. Genes Devel. 4*, 2188–2201.

Freeman, M., Gurdon, J.B., **2002**. Regulatory principles of developmental signaling. *Annu. Rev. Cell Devel. Biol. 18*, 515–539.

French, V., Bryant, P.J., Bryant, S.V., **1976**. Pattern regulation in epimorphic fields. *Science 193*, 969–981.

Fristrom, D., Gotwals, P., Eaton, S., Kornberg, T.B., Sturtevant, M., *et al.*, **1994**. *Blistered*: a gene required for vein/intervein formation in wings of *Drosophila*. *Development 120*, 2661–2671.

Funakoshi, Y., Minami, M., Tabata, T., **2001**. *mtv* shapes the activity gradient of the Dpp morphogen through regulation of *thickveins*. *Development 128*, 67–74.

Furlong, E.E., Andersen, E.C., Null, B., White, K.P., Scott, M.P., **2001**. Patterns of gene expression during *Drosophila* mesoderm development. *Science 293*, 1629–1633.

Gabay, L., Seger, R., Shilo, B.Z., **1997**. *In situ* activation pattern of *Drosophila* EGF receptor pathway during development. *Science 277*, 1103–1106.

Galindo, M.I., Bishop, S.A., Greig, S., Couso, J.P., **2002**. Leg patterning driven by proximal–distal interactions and EGFR signaling. *Science 297*, 256–259.

García-Bellido, A., **1975**. Genetic control of wing disc development in *Drosophila*. *Ciba Found. Symp.* 161–182.

García-Bellido, A., Cortes, F., Milan, M., **1994**. Cell interactions in the control of size in *Drosophila* wings. *Proc. Natl Acad. Sci. USA 91*, 10222–10226.

García-Bellido, A., de Celis, J.F., **1992**. Developmental genetics of the venation pattern of *Drosophila*. *Annu. Rev. Genet. 26*, 277–304.

García-Bellido, A., Merriam, J.R., **1971a**. Parameters of the wing imaginal disc development of *Drosophila melanogaster. Devel. Biol. 24*, 61–87.

García-Bellido, A., Ripoll, P., Morata, G., **1973**. Developmental compartmentalisation of the wing disc of *Drosophila*. *Nature New Biol. 245*, 251–253.

García-Bellido, A., Ripoll, P., Morata, G., **1976**. Developmental compartmentalization in the dorsal mesothoracic disc of *Drosophila*. *Devel. Biol. 48*, 132–147.

García-Bellido, A., Santamaria, P., **1972**. Developmental analysis of the wing disc in the mutant *engrailed* of *Drosophila melanogaster. Genetics. 72*, 87–104.

García-Bellido, A.C., García-Bellido, A., **1998**. Cell proliferation in the attainment of constant sizes and shapes: the Entelechia model. *Int. J. Devel. Biol. 42*, 353–362.

Geduspan, J.S., Solursh, M., **1992**. A growth-promoting influence from the mesonephros during limb outgrowth. *Devel. Biol. 151*, 242–250.

Gerlitz, O., Basler, K., **2002**. Wingful, an extracellular feedback inhibitor of Wingless. *Genes Devel. 16*, 1055–1059.

Gibson, M.C., Schubiger, G., **2000**. Peripodial cells regulate proliferation and patterning of *Drosophila* imaginal discs. *Cell 103*, 343–350.

Gibson, M.C., Schubiger, G., **2001**. *Drosophila* peripodial cells, more than meets the eye? *BioEssays 23*, 691–697.

Gingras, A.C., Raught, B., Sonenberg, N., **2001**. Regulation of translation initiation by FRAP/mTOR. *Genes Devel. 15*, 807–826.

Giraldez, A.J., Copley, R.R., Cohen, S.M., **2002**. HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Devel. Cell 2*, 667–676.

Godt, D., Tepass, U., **1998**. *Drosophila* oocyte localization is mediated by differential cadherin-based adhesion. *Nature 395*, 387–391.

Gomez-Skarmeta, J.L., Modolell, J., **1996**. *araucan* and *caupolican* provide a link between compartment subdivisions and patterning of sensory organs and veins in the *Drosophila* wing. *Genes Devel. 10*, 2935–2945.
Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., Martinez, A.C., Mann, R.S., *et al.*, **1998**. Antagonism between extradenticle function and Hedgehog signalling in the developing limb. *Nature 394*, 196–200.
Gonzalez-Gaitan, M., Capdevila, M.P., García-Bellido, A., **1994**. Cell proliferation patterns in the wing imaginal disc of *Drosophila*. *Mech. Devel. 46*, 183–200.
Gonzalez-Reyes, A., St Johnston, D., **1998**. The *Drosophila* AP axis is polarised by the cadherin-mediated positioning of the oocyte. *Development 125*, 3635–3644.
Greco, V., Hannus, M., Eaton, S., **2001**. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell 106*, 633–645.
Grimm, S., Pflugfelder, G., **1996**. Control of the gene *optomotor-blind* in *Drosophila* wing development by *decapentaplegic* and *wingless*. *Science 271*, 1601–1604.
Gurdon, J.B., Bourillot, P.Y., **2001**. Morphogen gradient interpretation. *Nature 413*, 797–803.
Hacker, U., Lin, X., Perrimon, N., **1997**. The *Drosophila sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development 124*, 3565–3573.
Haerry, T.E., Heslip, T.R., Marsh, J.L., O'Connor, M.B., **1997**. Defects in glucuronate biosynthesis disrupt Wingless signaling in *Drosophila*. *Development 124*, 3055–3064.
Halder, G., Polaczyk, P., Kraus, M.E., Hudson, A., Kim, J., *et al.*, **1998**. The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in *Drosophila*. *Genes Devel. 12*, 3900–3909.
Han, K., Levine, M.S., Manley, J.L., **1989**. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell 56*, 573–583.
Han, K., Manley, J.L., **1993**. Functional domains of the *Drosophila* Engrailed protein. *EMBO J. 12*, 2723–2733.
Helms, J.A., Kim, C.H., Eichele, G., Thaller, C., **1996**. Retinoic acid signaling is required during early chick limb development. *Development 122*, 1385–1394.
Hobert, O., Westphal, H., **2000**. Functions of LIM-homeobox genes. *Trends Genet. 16*, 75–83.
Hoffmann, F.M., Goodman, W., **1987**. Identification in transgenic animals of the *Drosophila* decapentaplegic sequences required for embryonic dorsal pattern formation. *Genes Devel. 1*, 615–625.
Holley, S.A., Jackson, P.D., Sasai, Y., Lu, B., De Robertis, E.M., *et al.*, **1995**. A conserved system for dorsal–ventral patterning in insects and vertebrates involving sog and chordin. *Natural 376*, 249–253.
Hudson, J.B., Podos, S.D., Keith, K., Simpson, S.L., Ferguson, E.L., **1998**. The *Drosophila Medea* gene is required downstream of *dpp* and encodes a functional homolog of human Smad4. *Development 125*, 1407–1420.
Hynes, R.O., **1999**. Cell adhesion: old and new questions. *Trends Cell Biol. 9*, M33–M37.
Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E., **2002**. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr. Biol. 12*, 1293–1300.
Incardona, J.P., Gruenberg, J., Roelink, H., **2002**. Sonic hedgehog induces the segregation of patched and smoothened in endosomes. *Curr. Biol. 12*, 983–995.
Incardona, J.P., Lee, J.H., Robertson, C.P., Enga, K., Kapur, R.P., *et al.*, **2000**. Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. *Proc. Natl Acad. Sci. USA 97*, 12044–12049.
Ingham, P.W., **1981**. *Trithorax*: a new homeotic mutation of *Drosophila melanogaster*. II. The role of Trx^+ after embryogenesis. *Roux's Arch. Devel. Biol. 190*, 365–369.
Ingham, P.W., **2000**. How cholesterol modulates the signal. *Curr. Biol. 10*, R180–R183.
Ingham, P.W., **2001**. Hedgehog signaling: a tale of two lipids. *Science 294*, 1879–1881.
Inoki, K., Li, Y., Zhu, T., Wu, J., Guan, K.L., **2002**. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature Cell Biol. 4*, 648–657.
Irish, V.F., Gelbart, W.M., **1987**. The *decapentaplegic* gene is required for dorsal–ventral patterning of the *Drosophila* embryo. *Genes Devel. 1*, 868–879.
Irvine, K.D., Rauskolb, C., **2001**. Boundaries in development: formation and function. *Annu. Rev. Cell Devel. Biol. 17*, 189–214.
Irvine, K.D., Wieschaus, E., **1994**. *fringe*, a boundary specific signalling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell 79*, 595–606.
Isshiki, T., Takeichi, M., Nose, A., **1997**. The role of the *msh* homeobox gene during *Drosophila* neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development 124*, 3099–3109.
Jackson, S.M., Nakato, H., Sugiura, M., Jannuzi, A., Oakes, R., *et al.*, **1997**. *dally*, a *Drosophila* glypican, controls cellular responses to the TGF-β-related morphogen, Dpp. *Development 124*, 4113–4120.
Jasper, H., Benes, V., Schwager, C., Sauer, S., Clauder-Munster, S., *et al.*, **2001**. The genomic response of the *Drosophila* embryo to JNK signaling. *Devel. Cell 1*, 579–586.
Jaynes, J.B., O'Farrell, P.H., **1988**. Activation and repression of transcription by homoeodomain-containing proteins that bind a common site. *Nature 336*, 744–749.
Jaynes, J.B., O'Farrell, P.H., **1991**. Active repression of transcription by the engrailed homeodomain protein. *EMBO J. 10*, 1427–1433.
Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S., Rushlow, C., **1999**. The *Drosophila* gene *brinker* reveals a novel mechanism of *Dpp* target gene regulation. *Cell 96*, 563–573.
Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., *et al.*, **1997**. Rapamycin suppresses

5′TOP mRNA translation through inhibition of p70s6k. *EMBO J. 16*, 3693–3704.

Jiang, J., Struhl, G., **1995**. Protein kinase A and Hedgehog signaling in *Drosophila* limb development. *Cell 80*, 563–572.

Jiang, J., Struhl, G., **1996**. Complementary and mutually exclusive activities of Decapentaplegic and Wingless organize axial pattern during *Drosophila* limb development. *Cell 86*, 401–409.

Jiang, J., Struhl, G., **1998**. Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature 391*, 493–496.

Johnson, R.L., Tabin, C.J., **1997**. Molecular models for vertebrate limb development. *Cell 90*, 979–990.

Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N., Gallant, P., **1999**. *Drosophila myc* regulates cellular growth during development. *Cell 98*, 779–790.

Johnston, S.H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K.D., *et al.*, **1997**. A family of mammalian *Fringe* genes implicated in boundary determination and the Notch pathway. *Development 124*, 2245–2254.

Joyner, A.L., Liu, A., Millet, S., **2000**. Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr. Opin. Cell Biol. 12*, 736–741.

Jungbluth, S., Larsen, C., Wizenmann, A., Lumsden, A., **2001**. Cell mixing between the embryonic midbrain and hindbrain. *Curr. Biol. 11*, 204–207.

Jurata, L.W., Kenny, D.A., Gill, G.N., **1996**. Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. *Proc. Natl Acad. Sci. USA 93*, 11693–11698.

Jurata, L.W., Pfaff, S.L., Gill, G.N., **1998**. The nuclear LIM domain interactor NLI mediates homo- and heterodimerization of LIM domain transcription factors. *J. Biol. Chem. 273*, 3152–3157.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., Perrimon, N., **1996**. The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Devel. 10*, 3116–3128.

Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez-Esteban, C., *et al.*, **2001**. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell 104*, 891–900.

Kennerdell, J.R., Carthew, R.W., **1998**. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the Wingless pathway. *Cell 95*, 1017–1026.

Kerber, B., Monge, I., Mueller, M., Mitchell, P.J., Cohen, S.M., **2001**. The AP-2 transcription factor is required for joint formation and cell survival in *Drosophila* leg development. *Development 128*, 1231–1238.

Kim, J., Irvine, K.D., Carroll, S.B., **1995**. Cell recognition, signal induction and symmetrical gene activation at the dorsal/ventral boundary of the developing *Drosophila* wing. *Cell 82*, 795–802.

Kim, J., Johnson, K., Chen, H.J., Carroll, S., Laughon, A., **1997a**. *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. *Nature 388*, 304–308.

Kim, J., Magee, J., Carroll, S.B., **1997b**. Intercompartmental signaling and the regulation of vestigial expression at the dorsoventral boundary of the developing *Drosophila* wing. *Cold Spring Harb. Symp. Quant. Biol. 62*, 283–291.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., *et al.*, **1996**. Integration of positional signals and regulation of wing formation by *Drosophila vestigial* gene. *Nature 382*, 133–138.

Kimmel, R.A., Turnbull, D.H., Blanquet, V., Wurst, W., Loomis, C.A., *et al.*, **2000**. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Devel. 14*, 1377–1389.

Klebes, A., Biehs, B., Cifuentes, F., Kornberg, T.B., **2002**. Expression profiling of *Drosophila* imaginal discs. *Genome Biol. 3*, RESEARCH0038.

Klein, T., Couso, J.P., Martinez-Arias, A., **1998**. Wing development and specification of dorsal cell fates in the absence of apterous in Drosophila. *Curr. Biol. 8*, 417–420.

Klein, T., Martinez-Arias, A., **1998**. Different spatial and temporal interactions between *Notch*, *wingless*, and *vestigial* specify proximal and distal pattern elements of the wing in *Drosophila*. *Devel. Biol. 194*, 196–212.

Kornberg, T., **1981**. Compartments in the abdomen of *Drosophila* and the role of the *engrailed* locus. *Devel. Biol. 86*, 363–372.

Kornberg, T., Siden, I., O'Farrell, P., Simon, M., **1985**. The *engrailed* locus of *Drosophila*: *in situ* localization of transcripts reveals compartment-specific expression. *Cell 40*, 45–53.

Lander, A.D., Nie, Q., Wan, F.Y., **2002**. Do morphogen gradients arise by diffusion? *Devel. Cell 2*, 785–796.

Laufer, E., Dahn, R., Orozco, O.E., Yeo, C.Y., Pisenti, J., *et al.*, **1997**. Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature 386*, 366–373.

Lawrence, P.A., Casal, J., Struhl, G., **1999a**. *hedgehog* and *engrailed*: pattern formation and polarity in the *Drosophila* abdomen. *Development 126*, 2431–2439.

Lawrence, P.A., Casal, J., Struhl, G., **1999b**. The Hedgehog morphogen and gradients of cell affinity in the abdomen of *Drosophila*. *Development 126*, 2441–2449.

Lawrence, P.A., Struhl, G., **1982**. Further studies on the *engrailed* phenotype in *Drosophila*. *EMBO J. 7*, 827–833.

Lawrence, P.A., Struhl, G., **1996**. Morphogens, compartments and pattern: lessons from *Drosophila*? *Cell 85*, 951–961.

Lecuit, T., Brook, W.J., Ng, M., Calleja, M., Sun, H., *et al.*, **1996**. Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature 381*, 387–393.

Lecuit, T., Cohen, S.M., **1997**. Proximal–distal axis formation in the *Drosophila* leg. *Nature 388*, 139–145.

Lecuit, T., Cohen, S.M., **1998**. Dpp receptor levels contribute to shaping the Dpp morphogen gradient in the *Drosophila* wing imaginal disc. *Development 125*, 4901–4907.

Lee, J.D., Treisman, J.E., **2001**. Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein. *Curr. Biol. 11*, 1147–1152.

Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., *et al.*, **1994**. Autoproteolysis in *hedgehog* protein biogenesis. *Science 266*, 1528–1537.

Lee, J.J., von Kessler, D.P., Parks, S., Beachy, P.A., **1992**. Secretion and localized transcript suggest a role in positional signalling for products of the segmentation gene *hedgehog*. *Cell 71*, 33–50.

Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., Waterfield, M.D., **1996**. The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J. 15*, 6584–6594.

Lepage, T., Cohen, S.M., Diaz-Benjumea, F.J., Parkhurst, S.M., **1995**. Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning. *Nature 373*, 711–715.

Letsou, A., Arora, K., Wrana, J.L., Simin, K., Twombly, V., *et al.*, **1995**. *Drosophila* Dpp signaling is mediated by the *punt* gene product: a dual ligand-binding type II receptor of the TGF-β receptor family. *Cell 80*, 899–908.

Lewandoski, M., Sun, X., Martin, G.R., **2000**. Fgf8 signalling from the AER is essential for normal limb development. *Nat. Genet. 26*, 460–463.

Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S., De Robertis, E.M., **1997**. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell 88*, 747–756.

Li, W., Ohlmeyer, J.T., Lane, M.E., Kalderon, D., **1995**. Function of protein kinase A in Hedgehog signal transduction and *Drosophila* imaginal disc development. *Cell 80*, 553–562.

Lin, X., Buff, E.M., Perrimon, N., Michelson, A.M., **1999**. Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. *Development 126*, 3715–3723.

Lin, X., Perrimon, N., **2000**. Role of heparan sulfate proteoglycans in cell–cell signaling in *Drosophila. Matrix Biol. 19*, 303–307.

Liu, X., Grammont, M., Irvine, K.D., **2000**. Roles for *scalloped* and *vestigial* in regulating cell affinity and interactions between the wing blade and the wing hinge. *Devel. Biol. 228*, 287–303.

Loomis, C.A., Harris, E., Michaud, J., Wurst, W., Hanks, M., *et al.*, **1996**. The mouse *Engrailed-1* gene and ventral limb patterning. *Nature 382*, 360–363.

Loomis, C.A., Kimmel, R.A., Tong, C.X., Michaud, J., Joyner, A.L., **1998**. Analysis of the genetic pathway leading to formation of ectopic apical ectodermal ridges in mouse *Engrailed-1* mutant limbs. *Development 125*, 1137–1148.

Lopez-Martinez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., *et al.*, **1995**. Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Curr. Biol. 5*, 791–796.

Lu, C.H., Rincon-Limas, D.E., Botas, J., **2000**. Conserved overlapping and reciprocal expression of msh/Msx1 and apterous/Lhx2 in Drosophila and mice. *Mech. Devel. 99*, 177–181.

Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., *et al.*, **2002**. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. *Cell 111*, 63–75.

MacCabe, J.A., Errick, J., Saunders, J.W., Jr., **1974**. Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Devel. Biol. 39*, 69–82.

Madhavan, M.M., Schneiderman, H.A., **1977**. Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila* melanogaster. *Roux's Arch. Devel. Biol. 183*, 269–305.

Mann, R.S., Carroll, S.B., **2002**. Molecular mechanisms of selector gene function and evolution. *Curr. Opin. Genet. Devel. 12*, 592–600.

Mann, R.S., Morata, G., **2000**. The developmental and molecular biology of genes that subdivide the body of *Drosophila*. *Annu. Rev. Cell Devel. Biol. 16*, 243–271.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., Cantley, L.C., **2002**. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3–kinase/akt pathway. *Mol. Cell 10*, 151–162.

Martin, G., **2001**. Making a vertebrate limb: new players enter from the wings. *BioEssays 23*, 865–868.

Martin-Castellanos, C., Edgar, B.A., **2002**. A characterization of the effects of Dpp signaling on cell growth and proliferation in the *Drosophila* wing. *Development 129*, 1003–1013.

Marygold, S.J., Leevers, S.J., **2002**. Growth signaling: TSC takes its place. *Curr. Biol. 12*, R785–R787.

Massague, J., Blain, S.W., Lo, R.S., **2000**. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell 103*, 295–309.

Massague, J., Chen, Y.G., **2000**. Controlling TGF-beta signaling. *Genes Devel. 14*, 627–644.

McDowell, N., Zorn, A.M., Crease, D.J., Gurdon, J.B., **1997**. Activin has direct long-range signalling activity and can form a concentration gradient by diffusion. *Curr. Biol. 7*, 671–681.

McNeill, H., **2000**. Sticking together and sorting things out: adhesion as a force in development. *Nature Rev. Genet. 1*, 100–108.

McNeill, H., Yang, C.H., Brodsky, M., Ungos, J., Simon, M.A., **1997**. *mirror* encodes a novel PBX-class homeoprotein that functions in the definition of the

dorsal–ventral border in the *Drosophila* eye. *Genes Devel. 11*, 1073–1082.

Meinhardt, H., **1983**. Cell determination boundaries as organizing regions for secondary embryonic fields. *Devel. Biol. 96*, 375–385.

Mellitzer, G., Xu, Q., Wilkinson, D.G., **1999**. Eph receptors and ephrins restrict cell intermingling and communication. *Nature 400*, 77–81.

Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., *et al.*, **1999**. Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature 402*, 425–429.

Mercader, N., Leonardo, E., Piedra, M.E., Martinez, A.C., Ros, M.A., *et al.*, **2000**. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development 127*, 3961–3970.

Merino, R., Rodriguez-Leon, J., Macias, D., Ganan, Y., Economides, A. N., *et al.*, **1999**. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development 126*, 5515–5522.

Méthot, N., Basler, K., **1999**. Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell 96*, 819–831.

Meyuhas, O., Hornstein, E., **2000**. Translational Control of TOP mRNAs. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Micchelli, C.A., Blair, S.S., **1999**. Dorsoventral lineage restriction in wing imaginal discs requires Notch. *Nature 401*, 473–476.

Milan, M., Campuzano, S., García-Bellído, A., **1996**. Cell-cycling and patterned cell proliferation in the wing primordium of *Drosophila*. *Proc. Natl Acad. Sci. USA 93*, 640–645.

Milan, M., Cohen, S.M., **1999a**. Notch signaling is not sufficient to define the affinity boundary between dorsal and ventral compartments. *Mol. Cell 4*, 1073–1078.

Milan, M., Cohen, S.M., **1999b**. Regulation of LIM homeodomain activity *in vivo*: a tetramer of dLDB and apterous confers activity and capacity for regulation by dLMO. *Mol. Cell 4*, 267–273.

Milan, M., Cohen, S.M., **2000a**. Subdividing cell populations in the developing limbs of *Drosophila*: do wing veins and leg segments define units of growth control? *Devel. Biol. 217*, 1–9.

Milan, M., Cohen, S.M., **2000b**. Temporal regulation of *apterous* activity during development of the *Drosophila* wing. *Development 127*, 3069–3078.

Milan, M., Cohen, S.M., **2003**. A re-evaluation of the contributions of Apterous and Notch to the dorsoventral lineage restriction boundary in the *Drosophila* wing. *Development 130*, 553–562.

Milan, M., Diaz-Benjumea, F.J., Cohen, S.M., **1998**. *Beadex* encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. *Genes Devel. 12*, 2912–2920.

Milan, M., Weihe, U., Perez, L., Cohen, S.M., **2001a**. The LRR proteins capricious and Tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. *Cell 106*, 785–794.

Milan, M., Weihe, U., Tiong, S., Bender, W., Cohen, S.M., **2001b**. *msh* specifies dorsal cell fate in the *Drosophila* wing. *Development 128*, 3263–3268.

Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., *et al.*, **1998**. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to *Drosophila branchless*. *Genes Devel. 12*, 3156–3161.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H., Tabata, T., **1999**. *brinker* is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. *Nature 398*, 242–246.

Mohler, J., Vani, K., **1992**. Molecular organisation and embryonic expression of the *hedgehog* gene involved in cell–cell communication in segmental patterning of *Drosophila*. *Development 115*, 957–971.

Moline, M.M., Southern, C., Bejsovec, A., **1999**. Directionality of Wingless protein transport influences epidermal patterning in the *Drosophila* embryo. *Development 126*, 4375–4384.

Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., *et al.*, **2000**. Fringe is a glycosyltransferase that modifies Notch. *Nature 406*, 369–375.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., *et al.*, **1999**. *Drosophila* S6 kinase: a regulator of cell size. *Science 285*, 2126–2129.

Moon, A.M., Capecchi, M.R., **2000**. Fgf8 is required for outgrowth and patterning of the limbs. *Nature Genet. 26*, 455–459.

Moran, J.L., Johnston, S.H., Rauskolb, C., Bhalerao, J., Bowcock, A.M., *et al.*, **1999a**. Genomic structure, mapping, and expression analysis of the mammalian *Lunatic*, *Manic*, and *Radical fringe* genes. *Mamm. Genome. 10*, 535–541.

Moran, J.L., Levorse, J.M., Vogt, T.F., **1999b**. Limbs move beyond the radical fringe. *Nature 399*, 742–743.

Morata, G., Lawrence, P.A., **1975**. Control of compartment development by the engrailed gene in *Drosophila*. *Nature 255*, 614–617.

Morata, G., Ripoll, P., **1975**. Minutes: mutants of *Drosophila* autonomously affecting cell division rate. *Devel. Biol. 42*, 211–221.

Morcillo, P., Rosen, C., Baylies, M.K., Dorsett, D., **1997**. Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in *Drosophila*. *Genes Devel. 11*, 2729–2740.

Moreno, E., Basler, K., Morata, G., **2002**. Cells compete for decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. *Nature 416*, 755–759.

Mullor, J.L., Calleja, M., Capdevila, J., Guerrero, I., **1997**. Hedgehog activity, independent of Decapentaplegic,

participates in wing disc patterning. *Development 124*, 1227–1237.

Nellen, D., Burke, R., Struhl, G., Basler, K., **1996**. Direct and long-range action of a Dpp morphogen gradient. *Cell 85*, 357–368.

Nestoras, K., Lee, H., Mohler, J., **1997**. Role of *knot* (*kn*) in wing patterning in *Drosophila*. *Genetics 147*, 1203–1212.

Neumann, C.J., Cohen, S.M., **1996a**. Distinct mitogenic and cell fate specification functions of wingless in different regions of the wing development. *Development 122*, 1781–1789.

Neumann, C.J., Cohen, S.M., **1996b**. A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing. *Development 122*, 3477–3485.

Neumann, C.J., Cohen, S.M., **1997**. Long-range action of Wingless organizes the dorsal–ventral axis of the *Drosophila* wing. *Development 124*, 871–880.

Newfeld, S.J., Chartoff, E.H., Graff, J.M., Melton, D.A., Gelbart, W.M., **1996**. *Mothers against dpp* encodes a conserved cyotplasmic protein required in DPP/TGF-β responsive cells. *Development 122*, 2099–2108.

Ng, J.K., Kawakami, Y., Buscher, D., Raya, A., Itoh, T., *et al.*, **2002**. The limb identity gene *Tbx5* promotes limb initiation by interacting with Wnt2b and Fgf10. *Development 129*, 5161–5170.

Ng, M., Diaz-Benjumea, F.J., Cohen, S.M., **1995**. *nubbin* encodes a POU-domain protein required for proximal–distal patterning in the *Drosophila* wing. *Development 121*, 589–599.

Ng, M., Diaz-Benjumea, F.J., Vincent, J.P., Wu, J., Cohen, S.M., **1996**. Specification of the wing by localized expression of wingless protein. *Nature 381*, 316–318.

Niswander, L., Jeffrey, S., Martin, G.R., Tickle, C., **1994**. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature 371*, 609–612.

Niswander, L., Tickle, C., Vogel, A., Booth, I., Martin, G.R., **1993**. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell 75*, 579–587.

Nüsslein-Volhard, C., Wieschaus, E., **1980**. Mutations affecting segment number and polarity in *Drosophila*. *Nature 287*, 795–801.

Nybakken, K., Perrimon, N., **2002**. Heparan sulfate proteoglycan modulation of developmental signaling in *Drosophila*. *Biochim. Biophys. Acta 1573*, 280–291.

O'Brochta, D.A., Bryant, P.J., **1985**. A zone of non-proliferating cells at a lineage restriction boundary in *Drosophila*. *Nature 313*, 138–141.

Ohkuma, Y., Horikoshi, M., Roeder, R.G., Desplan, C., **1990**. Binding site dependent direct activation and repression of *in vitro* transcription by *Drosophila* homeodomain proteins. *Cell 61*, 475–484.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., *et al.*, **1997**. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development 124*, 2235–2244.

O'Keefe, D.D., Thomas, J.B., **2001**. *Drosophila* wing development in the absence of dorsal identity. *Development 128*, 703–710.

Oldham, S., Bohni, R., Stocker, H., Brogiolo, W., Hafen, E., **2000**. Genetic control of size in *Drosophila*. *Phil. Trans. Roy. Soc. B 355*, 945–952.

Oldham, S., Hafen, E., **2003**. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol. 13*, 79–85.

Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., *et al.*, **2002**. The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. *Development 129*, 4103–4109.

Oliver, G., De Robertis, E.M., Wolpert, L., Tickle, C., **1990**. Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J. 9*, 3093–3099.

Padgett, R.W., Das, P., Krishna, S., **1998**. TGF-beta signaling, Smads, and tumor suppressors. *BioEssays 20*, 382–390.

Padgett, R.W., St. Johnston, R.D., Gelbart, W.M., **1987**. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature 325*, 81–84.

Pan, D.J., Rubin, G., **1995**. Protein kinase and hedgehog act antagonistically in regulating *decapentaplegic* transcription in *Drosophila* imaginal discs. *Cell 80*, 543–552.

Panganiban, G., Irvine, S.M., Lowe, C., Roehl, H., Corley, L.S., *et al.*, **1997**. The origin and evolution of animal-appendages. *Proc. Natl Acad. Sci. USA 94*, 5162–5166.

Panin, V.M., Papayannopoulos, V., Wilson, R., Irvine, K.D., **1997**. Fringe modulates Notch–ligand interactions. *Nature 387*, 908–913.

Panin, V.M., Shao, L., Lei, L., Moloney, D.J., Irvine, K.D., *et al.*, **2002**. Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *J. Biol. Chem. 277*, 29945–29952.

Parr, B.A., McMahon, A.P., **1995**. Dorsalizing signal Wnt-7a required for normal polarity of D–V and A–P axes of mouse limb. *Nature 374*, 350–353.

Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M. C., *et al.*, **1989**. Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell 58*, 955–968.

Perrimon, N., Bernfield, M., **2000**. Specificities of heparan sulphate proteoglycans in developmental processes. *Nature 404*, 725–728.

Pfeiffer, S., Alexandre, C., Calleja, M., Vincent, J.P., **2000**. The progeny of wingless-expressing cells deliver the signal at a distance in *Drosophila* embryos. *Curr. Biol. 10*, 321–324.

Pfeiffer, S., Ricardo, S., Manneville, J.B., Alexandre, C., Vincent, J.P., **2002**. Producing cells retain and recycle Wingless in *Drosophila* embryos. *Curr. Biol. 12*, 957–962.

Phillips, R., Whittle, J.R.S., **1993**. *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development 118*, 427–438.

Pichaud, F., Casares, F., **2000**. *homothorax* and *iroquois-C* genes are required for the establishment of territories within the developing eye disc. *Mech. Devel. 96*, 15–25.

Pizette, S., Abate-Shen, C., Niswander, L., **2001**. BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development 128*, 4463–4474.

Pizette, S., Niswander, L., **1999**. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. *Development 126*, 883–894.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T.M., Dodd, J., **1990**. Mesodermal control of neural cell identity: floorplate induction by the notochord. *Science 250*, 985–988.

Porter, F.D., Drago, J., Xu, Y., Cheema, S.S., Wassif, C., *et al.*, **1997**. *Lhx2*, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development 124*, 2935–2944.

Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., *et al.*, **1995**. The product of *hedgehog* autoproteolytic cleavage active in local and long-range signalling. *Nature 374*, 363–366.

Porter, J.A., Young, K.E., Beachy, P.A., **1996**. Cholesterol modification of hedgehog signaling proteins in animal development. *Science 274*, 255–259.

Raftery, L.A., Sutherland, D.J., **1999**. TGF-beta family signal transduction in *Drosophila* development: from Mad to Smads. *Devel. Biol. 210*, 251–268.

Ramain, P., Khechumian, R., Khechumian, K., Arbogast, N., Ackermann, C., *et al.*, **2000**. Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning. *Mol. Cell 6*, 781–790.

Ramirez-Weber, F.A., Kornberg, T.B., **1999**. Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. *Cell 97*, 599–607.

Ramirez-Weber, F.A., Kornberg, T.B., **2000**. Signaling reaches to new dimensions in *Drosophila* imaginal discs. *Cell 103*, 189–192.

Rancourt, D.E., Tsuzuki, T., Capecchi, M.R., **1995**. Genetic interaction between *hoxb-5* and *hoxb-6* is revealed by nonallelic noncomplementation. *Genes Devel. 9*, 108–122.

Rauskolb, C., Irvine, K.D., **1999**. Notch-mediated segmentation and growth control of the *Drosophila* leg. *Devel. Biol. 210*, 339–350.

Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., *et al.*, **1991**. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell 67*, 687–699.

Reichsman, F., Smith, L., Cumberledge, S., **1996**. Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J. Cell Biol. 135*, 819–827.

Retaux, S., Rogard, M., Bach, I., Failli, V., Besson, M.J., **1999**. *Lhx9*: a novel LIM-homeodomain gene expressed in the developing forebrain. *J. Neurosci. 19*, 783–793.

Riddle, R.D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T.M., *et al.*, **1995**. Induction of the LIM homeobox gene *Lmx1* by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell 83*, 631–640.

Riddle, R.D., Johnson, R.L., Laufer, E., Tabin, C., **1993**. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell 75*, 1401–1416.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., *et al.*, **1987**. The *Drosophila* homologue of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell 50*, 649–657.

Rincon-Limas, D.E., Lu, C.H., Canal, I., Botas, J., **2000**. The level of DLDB/CHIP controls the activity of the LIM homeodomain protein Apterous: evidence for a functional tetramer complex *in vivo*. *EMBO J. 19*, 2602–2614.

Rincon-Limas, D.E., Lu, C.H., Canal, I., Calleja, M., Rodriguez-Esteban, C., *et al.*, **1999**. Conservation of the expression and function of *apterous* orthologs in *Drosophila* and mammals. *Proc. Natl Acad. Sci. USA 96*, 2165–2170.

Rodriguez, I., Basler, K., **1997**. Control of compartmental affinity boundaries by hedgehog. *Nature 389*, 614–618.

Rodriguez-Esteban, C., Schwabe, J.W., De La Pena, J., Foys, B., Eshelman, B., *et al.*, **1997**. Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature 386*, 360–366.

Rodriguez-Esteban, C., Schwabe, J.W., De La Pena, J., Rincon-Limas, D.E., Magallon, J., *et al.*, **1998**. *Lhx2*, a vertebrate homologue of *apterous*, regulates vertebrate limb outgrowth. *Development 125*, 3925–3934.

Rong, Y.S., Golic, K.G., **2000**. Gene targeting by homologous recombination in *Drosophila*. *Science 288*, 2013–2018.

Rong, Y.S., Titen, S.W., Xie, H.B., Golic, M.M., Bastiani, M., *et al.*, **2002**. Targeted mutagenesis by homologous recombination in *D. melanogaster. Genes Devel. 16*, 1568–1581.

Rorth, P., **1996**. A modular misexpression screen in *Drosophila* detecting tissue specific phenotypes. *Proc. Natl Acad. Sci. USA 93*, 12418–12422.

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., *et al.*, **1998**. Systematic gain-of-function genetics in *Drosophila*. *Development 125*, 1049–1057.

Ross, J.J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., *et al.*, **2001**. Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature 410*, 479–483.

Rousset, R., Mack, J.A., Wharton, K.A., Jr., Axelrod, J.D., Cadigan, K.M., *et al.*, **2001**. Naked cuticle targets dishevelled to antagonize Wnt signal transduction. *Genes Devel. 15*, 658–671.

Rousset, R., Wharton, K.A., Jr., Zimmermann, G., Scott, M.P., **2002**. Zinc-dependent interaction between *dishevelled* and the *Drosophila* Wnt antagonist *naked cuticle*. *J. Biol. Chem. 277*, 49019–49026.

Ruberte, E., Marty, T., Nellen, D., Affolter, M., Basler, K., **1995**. An absolute requirement for both the type II and type I receptors, *punt* and *thickveins*, for Dpp signaling *in vivo*. *Cell 80*, 889–897.

Rulifson, E.J., Blair, S.S., **1995**. *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. *Development 121*, 2813–2824.

Schwabe, J.W., Rodriguez-Esteban, C., Izpisua Belmonte, J.-C., **1998**. Limbs are moving: where are they going? *Trends Genet. 14*, 229–235.

Schwartz, C., Locke, J., Nishida, C., Kornberg, T.B., **1995**. Analysis of *cubitus interruptus* regulation in *Drosophila* embryos and imaginal discs. *Development 121*, 1625–1635.

Searls, R.L., Janners, M.Y., **1971**. The initiation of limb bud outgrowth in the embryonic chick. *Devel. Biol. 24*, 198–213.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., *et al.*, **1999**. Fgf10 is essential for limb and lung formation. *Nature Genet. 21*, 138–141.

Selleck, S.B., **2000**. Proteoglycans and pattern formation: sugar biochemistry meets developmental genetics. *Trends Genet. 16*, 206–212.

Selleck, S.B., **2001**. Genetic dissection of proteoglycan function in *Drosophila* and *C. elegans*. *Semin. Cell Devel. Biol. 12*, 127–134.

Selva, E.M., Hong, K., Baeg, G.H., Beverley, S.M., Turco, S.J., *et al.*, **2001**. Dual role of the fringe connection gene in both heparan sulphate and fringe-dependent signalling events. *Nature Cell Biol. 3*, 809–815.

Serrano, N., O'Farrell, P., **1997**. Limb morphogenesis: connections between patterning and growth. *Curr. Biol. 7*, 186–195.

Shoresch, M., Orgad, S., Schmueli, O., Werczberrger, R., Gelbaum, D., *et al.*, **1998**. Overexpression *Beadex* mutations and loss of function *heldup-a* mutations in *Drosophila* affect the 3′ regulatory and coding components, respectively, of the *Dlmo* gene. *Genetics. 150*, 283–299.

Simeone, A., **2000**. Positioning the isthmic organizer where Otx2 and Gbx2 meet. *Trends Genet. 16*, 237–240.

Simmonds, A.J., Brook, W.J., Cohen, S.M., Bell, J.B., **1995**. Distinguishable functions for *engrailed* and *invected* in anterior–posterior patterning in the *Drosophila* wing. *Nature 376*, 424–427.

Simmonds, A.J., dosSantos, G., Livne-Bar, I., Krause, H.M., **2001**. Apical localization of *wingless* transcripts is required for wingless signaling. *Cell 105*, 197–207.

Simmonds, A.J., Liu, X., Soanes, K.H., Krause, H.M., Irvine, K.D., *et al.*, **1998**. Molecular interactions between *Vestigial* and *Scalloped* promote wing formation in *Drosophila*. *Genes Devel. 12*, 3815–3820.

Simpson, P., Schneiderman, H.A., **1975**. Isolation of temperature sensitive mutations blocking clone development in *Drosophila melanogaster*, and the effects of a temperature sensitive cell lethal mutation on pattern formation in imaginal discs. *Roux's Arch. Devel. Biol. 178*, 247–275.

Sisson, J.C., Ho, K.S., Suyama, K., Scott, M.P., **1997**. Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway. *Cell 90*, 235–245.

Smith, S.T., Jaynes, J.B., **1996**. A conserved region of Engrailed, shared among all en-, gsc-, NK1-, NK2- and msh-class homeoproteins, mediates active transcriptional repression *in vivo*. *Development 122*, 3141–3150.

Snodgrass, R.E., **1935**. Principles of Insect Morphology. McGraw Hill, New York.

Speicher, S.A., Thomas, U., Hinz, U., Knust, E., **1994**. The *Serrate* locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. *Development 120*, 535–544.

Spemann, H., Mangold, H., **1924**. Über Induktion von Embryonenanlagen durch Implantation artfremder Organisatoren. *Arch. Mikrosk. Anat. Entw. Mech. 100*, 599–638.

Steinberg, M.S., Takeichi, M., **1994**. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proc. Natl Acad. Sci USA 91*, 206–209.

Steiner, E., **1976**. Establishment of compartments in the developing leg imaginal discs of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 180*, 9–30.

St. Pierre, S.E., Galindo, M.I., Couso, J.P., Thor, S., **2002**. Control of *Drosophila* imaginal disc development by rotund and roughened eye: differentially expressed transcripts of the same gene encoding functionally distinct zinc finger proteins. *Development 129*, 1273–1281.

Strigini, M., Cohen, S.M., **1997**. A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development 124*, 4697–4705.

Strigini, M., Cohen, S.M., **1999**. Formation of morphogen gradients in the *Drosophila* wing. *Semin. Cell Devel. Biol. 10*, 335–344.

Strigini, M., Cohen, S.M., **2000**. Wingless gradient formation in the *Drosophila* wing. *Curr. Biol. 10*, 293–300.

Struhl, G., Basler, K., **1993**. Organizing activity of *wingless* protein in *Drosophila*. *Cell 72*, 527–540.

Sturtevant, M.A., Biehs, B., Marin, E., Bier, E.A., **1997**. The *spalt* gene links the A/P compartment boundary to a linear adult structure in the *Drosophila* wing. *Development 124*, 21–32.

Sturtevant, M.A., Bier, E., **1995**. Analysis of the genetic hierarchy guiding wing vein development in *Drosophila*. *Development 121*, 785–801.

Sun, X., Mariani, F.V., Martin, G.R., **2002**. Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature 418*, 501–508.

Tabata, T., Eaton, S., Kornberg, T.B., **1992**. The *Drosophila hedgehog* gene is expressed specifically in posterior

compartment cells and is a target of *engrailed* regulation. *Genes Devel. 6*, 2635–2645.

Tabata, T., Kornberg, T., **1994**. Hedgehog is a signalling protein with a key role in patterning *Drosophila* imaginal discs. *Cell 76*, 89–102.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z., Kornberg, T.B., **1995**. Creating a *Drosophila* wing *de novo*: the role of *engrailed* and the compartment border hypothesis. *Development 121*, 3359–3369.

Tanimoto, H., Itoh, S., ten Dijke, P., Tabata, T., **2000**. Hedgehog creates a gradient of Dpp activity in *Drosophila* wing imaginal discs. *Mol. Cell 5*, 59–71.

Teleman, A.A., Cohen, S.M., **2000**. Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell 103*, 971–980.

Teleman, A.A., Strigini, M., Cohen, S.M., **2001**. Shaping morphogen gradients. *Cell 105*, 559–562.

The, I., Bellaiche, Y., Perrimon, N., **1999**. Hedgehog movement is regulated through *tout velu* dependent synthesis of a heparan sulfate proteoglycan. *Mol. Cell 4*, 633–639.

Theisen, H., Haerry, T.E., O'Connor, M.B., Marsh, J.L., **1996**. Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. *Development 122*, 3939–3948.

Theodosiou, N.A., Zahng, S., Wang, W.Y., Xu, T., **1998**. *slimb* coordinates *wg* and *dpp* expression in the dorsal–ventral and anterior–posterior axes during limb development. *Development 125*, 3411–3416.

Thomas, G., **2002**. The S6 kinase signaling pathway in the control of development and growth. *Biol. Res. 35*, 305–313.

Tickle, C., Munsterberg, A., **2001**. Vertebrate limb development: the early stages in chick and mouse. *Curr. Opin. Genet. Devel. 11*, 476–481.

Todt, W.L., Fallon, J.F., **1984**. Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. Exp. Morphol. 80*, 21–41.

Toyoda, H., Kinoshita-Toyoda, A., Selleck, S.B., **2000**. Structural analysis of glycosaminoglycans in *Drosophila* and *Caenorhabditis elegans* and demonstration that *tout-velu*, a *Drosophila* gene related to EXT tumor suppressors, affects heparan sulfate *in vivo*. *J. Biol. Chem. 275*, 2269–2275.

Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., *et al.*, **1999**. The cell-surface proteoglycan Dally regulates Wingless signalling in *Drosophila*. *Nature 400*, 276–280.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., *et al.*, **1997**. *Daughters against dpp* modulates *dpp* organizing activity in *Drosophila* wing development. *Nature 389*, 627–631.

Turing, A., **1952**. The chemical basis of morphogenesis. *Phil. Trans. Roy. Soc. 237*, 37–72.

van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N., Nusse, R., **1993**. Mutations in the segment polarity genes *wingless* and *porcupine* impair secretion of the wingless protein. *EMBO J. 12*, 5293–5302.

van den Heuvel, M., Nusse, R., Johnston, P., Lawrence, P.A., **1989**. Distribution of the wingless gene product in *Drosophila* embryos: a protein involved in cell–cell communication. *Cell 59*, 739–749.

van Meyel, D.J., O'Keefe, D.D., Jurata, L.W., Thor, S., Gill, G.N., *et al.*, **1999**. Chip and apterous physically interact to form a functional complex during *Drosophila* development. *Mol. Cell 4*, 259–265.

Vervoort, M., Crozatier, M., Valle, D., Vincent, A., **1999**. The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the *Drosophila* wing. *Curr. Biol. 17*, 632–639.

Vincent, J.P., Magee, T., **2002**. Argosomes: membrane fragments on the run. *Trends Cell Biol. 12*, 57–60.

Vleminckx, K., Kemler, R., **1999**. Cadherins and tissue formation: integrating adhesion and signaling. *BioEssays 21*, 211–220.

Vogel, A., Rodriguez, C., Warnken, W., Izpisua Belmonte, J.-C., **1995**. Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature 378*, 716–720.

Wang, S., Krinks, M., Lin, K., Luyten, F.P., Moos, M.J., **1997**. Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell 88*, 757–766.

Wang, S.H., Simcox, A., Campbell, G., **2000**. Dual role for *Drosophila* epidermal growth factor receptor signaling in early wing disc development. *Genes Devel. 14*, 2271–2276.

Weigmann, K., Cohen, S.M., **1999**. Lineage tracing cells born in different domains along the PD axis of the developing Drosophila leg. *Development 126*, 3823–3830.

Weihe, U., Dorfman, R., Wernet, M.F., Cohen, S.M., Milan, M., **2004**. Proximodistal subdivision of *Drosophila* legs and wings: the *elbow–no ocelli* gene complex. *Development 131* (4), 761–774.

Weihe, U., Milan, M., Cohen, S.M., **2001**. Regulation of Apterous activity in *Drosophila* wing development. *Development 128*, 4615–4622.

Weinkove, D., Neufeld, T.P., Twardzik, T., Waterfield, M.D., Leevers, S.J., **1999**. Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor. *Curr. Biol. 9*, 1019–1029. (Erratum: *Curr. Biol. 9,* R867.)

Wiersdorff, V., Lecuit, T., Cohen, S.M., Mlodzik, M., **1996**. *Mad* acts downstream of the Dpp receptors and reveals differential requirement for *dpp* signaling between initiation and propagation of morphogenesis in the *Drosophila* eye. *Development 122*, 2153–2162.

Wieschaus, E., Gehring, W., **1976**. Clonal analysis of primordial disc cells in the early embryo of *Drosophila melanogaster. Devel. Biol. 50*, 249–263.

Williams, J.A., Paddock, S.W., Carroll, S.B., **1993**. Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. *Development 117*, 571–584.

Williams, J.A., Paddock, S.W., Vorwerk, K., Carroll, S.B., **1994**. Organization of wing formation and induction

of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature 368*, 299–305.

Wisotzkey, R.G., Mehra, A., Sutherland, D.J., Dobens, L.L., Liu, X., *et al.*, **1998**. *Medea* is a *Drosophila Smad4* homolog that is differentially required to potentiate Dpp responses. *Development 125*, 1433–1445.

Wodarz, A., Nusse, R., **1998**. Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Devel. Biol. 14*, 59–88.

Wolpert, L., **1969**. Positional information and the spatial pattern of cellular differentiation. *J. Theoret. Biol. 25*, 1–47.

Wolpert, L., **1989**. Positional information revisited. *Development 107*, 3–12.

Wolpert, L., **1996**. One hundred years of positional information. *Trends Genet. 12*, 359–364.

Wu, J., Cohen, S.M., **1999**. Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development 126*, 109–117.

Wu, J., Cohen, S.M., **2000**. Proximal distal axis formation in the *Drosophila* leg: distinct functions of *teashirt* and *homothorax* in the proximal leg. *Mech. Devel. 94*, 47–56.

Xu, Q., Mellitzer, G., Robinson, V., Wilkinson, D.G., **1999**. *In vivo* cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature 399*, 267–271.

Xu, T., Rubin, G.M., **1993**. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development 117*, 1223–1237.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J., Jessell, T.M., **1991**. Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell 64*, 635–647.

Yang, Y., Niswander, L., **1995**. Interaction between the signalling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell 80*, 939–947.

Zecca, M., Basler, K., Struhl, G., **1995**. Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. *Development 121*, 2265–2278.

Zecca, M., Basler, K., Struhl, G., **1996**. Direct and long-range action of a Wingless morphogen gradient. *Cell 87*, 833–844.

Zecca, M., Struhl, G., **2002a**. Control of growth and patterning of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. *Development 129*, 1369–1376.

Zecca, M., Struhl, G., **2002b**. Subdivision of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. *Development 129*, 1357–1368.

Zeng, C., Justice, N.J., Abdelilah, S., Chan, Y.M., Jan, L.Y., *et al.*, **1998**. The *Drosophila* LIM-only gene, *dLMO*, is mutated in *Beadex* alleles and might represent an evolutionarily conserved function in appendage development. *Proc. Natl Acad. Sci. USA 95*, 10637–10642.

Zeng, W., Wharton, K.A., Jr., Mack, J.A., Wang, K., Gadbaw, M., *et al.*, **2000**. *naked cuticle* encodes an inducible antagonist of Wnt signalling. *Nature 403*, 789–795.

Zuniga, A., Haramis, A.P., McMahon, A.P., Zeller, R., **1999**. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature 401*, 598–602.

1.10 Early Embryonic Development: Neurogenesis (CNS)

L Soustelle and A Giangrande,
Institut de Génétique et Biologie Moléculaire
et Cellulaire-CNRS, Strasbourg, France

1.10.1. Introduction

The relatively simple organization of insect embryos provides a useful model for investigating the mechanisms that generate and pattern complex nervous systems. Certain insects, such as the grasshopper, have very large embryos and easily accessible cells. This has allowed the analysis of cellular events as well as the manipulation of their nervous systems during development (Zinn and Condron, 1994; Sanchez *et al*., 1995). For a number of years, *Drosophila* has lagged behind because of its small size, compactness, and rapid development. The generation of sophisticated genetic tools, however, has made it possible to overcome these limitations. Neural precursors and their progeny have been identified in fixed and living animals, through the use of monoclonal antibodies, transgenic lines, and cellular dyes (see below). Analyzing the phenotype of gain-of-function and loss-of-function mutations of genes affecting this process has resulted in the identification of the molecular pathways that lead to nervous system differentiation. Finally, the evolutionary conservation amongst insects has made it possible to use the knowledge gained in the grasshopper in order to understand fly neural development (Anderson *et al*., 1980; Stent and Weisblat, 1985; Boyan and Ball, 1993). The combined use of these approaches has shed light on the basic cellular and molecular mechanisms underlying insect neurogenesis.

Flies display a central nervous system (CNS) that includes the ventral nerve cord (VNC) and the brain, as well as a peripheral nervous system (PNS) (reviews: Campos-Ortega, 1993; Goodman and Doe, 1993) (see **Chapters 1.11**, **1.12**, and **2.3–2.5**). PNS and CNS share most cellular and molecular developmental pathways. For the sake of simplicity, this chapter will mainly focus on the development of the VNC.

During embryogenesis, distinct neuronal cell types including motorneurons, sensory neurons, interneurons, and different types of glia are produced. The generation of neural diversity is a multistep process that requires different types of signals

(reviews: McConnell, 1995; Matsuzaki, 2000). On the one hand, neuralizing and inhibitory factors control the neural competence of large territories and the differentiation of neural precursors. On the other hand, positional cues control the type of neural precursors and, thereby, the type of neurons and glia that populate the nervous system.

1.10.2 Neural Differentiation

1.10.2.1 Neural Tissue Formation

The first step in nervous system development involves the acquisition of the neural competence. During embryonic development, two ventral territories acquire such competence, a wide one called the neuroectoderm, and a discrete one, the mesectoderm (reviews: Campos-Ortega, 1993; Goodman and Doe, 1993). The fly neuroectoderm is a heterogeneous tissue, in the sense that it gives rise to neurons, glia, and epidermis. Such heterogeneity, which is achieved via delamination of neural precursors, is a peculiarity of insect neurogenesis, since in vertebrates, the entire neural anlage invaginates, forms the neural tube, and produces neurons and glia (Doe and Smouse, 1990; Fraser, 1991; Salzberg and Bellen, 1996; Arendt and Nubler-Jung, 1999).

The mesectoderm separates the neuroectoderm from the mesoderm and is composed of two rows of VNC cells, one on each side of the embryo. Upon gastrulation, mesectodermal cells come together and align to form a single row of cells called the midline cells (review: Jacobs, 2000). The fly midline corresponds to the vertebrate floor plate and plays a role in axonal guidance.

1.10.2.1.1. The neuroectoderm In wild-type *Drosophila* embryos, the neuroectoderm becomes morphologically manifest during the initial phase of germ-band elongation. Positional information provided solely by the mother is sufficient to subdivide the embryo into three domains (ventral, lateral, and dorsal) corresponding to distinct fates (mesoderm, neuroectoderm, and nonneural ectoderm) (reviews: Rusch and Levine, 1996; Bier, 1997; Stathopoulos and Levine, 2002) (see also **Chapter 1.2**).

The Dorsal (Dl; NF-κB related) protein is distributed throughout the cytoplasm of the growing oocytes and unfertilized eggs (Steward, 1987). After fertilization, it is released from the cytoplasm and enters the nuclei in the ventral region, whereas it stays in the cytoplasm in the dorsal region (Roth *et al.*, 1989; Steward, 1989; Gay and Keith, 1990). Nuclear Dorsal is present at high levels in the ventral presumptive mesodermal cells, at intermediate levels in lateral cells comprising the neuroectoderm, and absent from cells giving rise to nonneural ectoderm (**Figure 1**) (Roth *et al.*, 1989; Steward, 1989; Govind and Steward, 1991; review: Bier, 1997). Once in the nucleus, Dorsal controls gene expression in a concentration dependent fashion. It functions as an activator of genes expressed in ventral and lateral regions of the embryo, but it also acts as a repressor of genes expressed in the dorsal region.

In ventral cells, peak levels of Dorsal activate the mesoderm-determining genes *twist* (*twi*) and *snail* (*sna*) (Jiang *et al.*, 1991; Kosman *et al.*, 1991; Rao *et al.*, 1991). Intermediate levels of Dorsal in the lateral embryonic regions activate a set of genes that initiate the differentiation of neuroectoderm (**Figure 1**). At least seven genes appear to be activated in the neuroectoderm including *rhomboid (rho)*

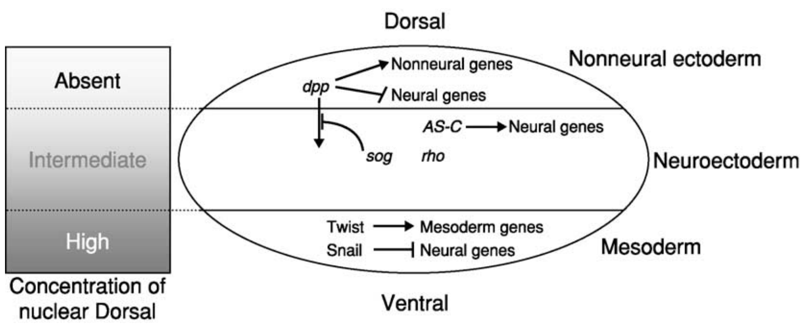


Figure 1 Dorsoventral patterning in the *Drosophila* embryo. Schematic representation of an embryo at blastoderm stage, lateral view. The molecular pathways induced by the gradient of nuclear Dorsal protein subdivide the embryo into three regions along the dorsoventral axis. High levels of nuclear Dorsal activate the expression of *twist* (*twi*) and *snail* (*sna*), which specify mesoderm identity, while intermediate levels induce *short of gastrulation* (*sog*), the *Achaete–Scute Complex* (*AS-C*), and *rhomboid* (*rho*) expression, which specify neuroectoderm identity. Absence of nuclear Dorsal activates *decapentaplegic* (*dpp*) expression. Sog prevents Dpp from signaling to the neuroectoderm and permits the expression of neural genes. (Modified from Bier, E., **1997**. Anti-neural-inhibition: a conserved mechanism for neural induction. *Cell 89*, 681–684.)

(Bier *et al*., 1990), *short gastrulation* (*sog*) (François *et al*., 1994), *lethal of scute* [*l(1)SC*] (a proneural gene belonging to the *achaete–scute* complex or *AS-C*) (Martin-Bermudo *et al*., 1995), the *m7* and *m8* genes of *the enhancer of split complex* [*E(spl)*] (Knust *et al*., 1987a), and *single-minded* (*sim*) (Kasai *et al*., 1992). Cells of the dorsal nonneural ectoderm, which contain no nuclear Dorsal, express several genes including *decapentaplegic (dpp), zerknullt (zen)*, and *tolloid (tld)* (Ip *et al*., 1991; Ray *et al*., 1991; Shimell *et al*., 1991; Huang *et al*., 1993) (**Figure 1**). In ventral and lateral cells, Dorsal represses the expression of these genes (Ray *et al*., 1991).

In vertebrates, neural tissue formation is controlled by the interplay of two types of molecules that display antagonistic functions: bone morphogenetic proteins (BMPs) and Chordin (reviews: Bier, 1997; Mehler *et al*., 1997). While BMP4 blocks the ability of ectoderm to adopt the neural fate (review: Hemmati-Brivanlou and Melton, 1997), Chordin inhibits BMP4 signaling, thereby allowing neural differentiation (Sasai *et al*., 1995; Hama and Weinstein, 2001). In *Drosophila*, *short gastrulation* (a Chordin ortholog) and *decapentaplegic* (a BMP4 ortholog) play similar roles in defining the domain of the ectoderm that will become the neuroectoderm (François and Bier, 1995; Holley *et al*., 1995; Biehs *et al*., 1996; reviews: Ferguson, 1996; Bier, 1997). Dpp is expressed in dorsal nonneural cells (Irish and Gelbart, 1987; St Johnston and Gelbart, 1987), whereas Sog is an extracellular protein expressed in the presumptive neuroectoderm (François *et al*., 1994). Both Dpp and Sog likely diffuse from their site of production into adjacent territories. In the early blastoderm embryo, Sog prevents Dpp signaling from invading the neuroectoderm (François *et al*., 1994; Biehs *et al*., 1996) (**Figure 1**). Dpp signaling functions both to maintain expression of dorsally acting genes (e.g., *zen*) and to suppress expression of genes, the so-called proneural genes, which induce the differentiation of neural precursors (Ray *et al*., 1991; Skeath *et al*., 1992; Biehs *et al*., 1996) (**Figure 1**). Complete loss of Dpp activity results in total transformation of dorsal ectoderm into neuroectoderm: both the amnioserosa (the dorsalmost region of the embryo) and the dorsal epidermis are lost in these mutant embryos (St Johnston and Gelbart, 1987). Genetic evidence supports the view that Sog opposes Dpp activity, as reducing the gene dose of *sog* rescues lethality resulting from weak mutations in the Dpp pathway (Biehs *et al*., 1996).

1.10.2.1.2. The mesectoderm In *Drosophila* embryos, the mesectoderm corresponds to a single row of cells abutting the mesoderm. Mesectoderm identity is induced by Sim, since midline cells fail to differentiate in its absence (Crews *et al*., 1988; Thomas *et al*., 1988; Nambu *et al*., 1990). The *sim* gene acts as a master gene for midline identity, as shown by the observation that its ectopic expression leads to the transformation of most lateral CNS cells into midline cells (Nambu *et al*., 1991). The *sim* gene encodes a bHLH-PAS (basic helix–loop–helix-Per-Arnt-Sim) transcription factor that heterodimerizes with the Tango (Tgo) protein (another bHLH-PAS transcription factor) to activate the expression of midline-specific genes (Sonnenfeld *et al*., 1997; Ward *et al*., 1998).

Genetic and molecular evidence indicate that *sim* expression is repressed by Snail and Suppressor of Hairless (Su(H)) in the mesoderm and the neuroectoderm, respectively (Kasai *et al*., 1992; Morel and Schweisguth, 2000). On the other hand, *sim* expression is activated by Dorsal and Twist in the cells that are located between these two territories (Kosman *et al*., 1991; Leptin, 1991; Kasai *et al*., 1992) (**Figure 2**).

The molecular mechanisms defining the mesectoderm as a single row of *sim*-expressing cells along the dorsoventral axis start being elucidated. Results from transplantation experiments indicate that cell–cell signaling between mesodermal and nonmesodermal cells is required for the early expression of *sim* (Leptin and Roth, 1994). Moreover, mesectoderm specification also involves Notch (N) activity, confirming a role of cell signaling in the regulation of *sim* expression (Menne and Klambt, 1994; Martin-Bermudo *et al*., 1995).

The current view of the Notch pathway calls for intracellular processing of Notch and formation of a complex between its intracellular domain and the Su(H) transcription factor (reviews: Jarriault *et al*., 1995; Artavanis-Tsakonas *et al*., 1999). The role of this complex is to activate the transcription of Notch responsive genes (Kidd *et al*., 1998a; Lecourtois and Schweisguth, 1998; Schroeter *et al*., 1998; Struhl and Adachi, 1998). However, recent data show that Su(H) can also act alone (Bray and Furriols, 2001). Indeed, Su(H) plays two roles: with the help of Notch, it upregulates *sim* expression in the mesectoderm, whereas it prevents *sim* expression in the neuroectoderm independently of Notch (Morel and Schweisguth, 2000).

1.10.2.2. Neural Precursor Formation

Within the neuroectoderm, also called ventral neurogenic region, nervous system development requires the activity of two classes of genes that control neural versus epidermal fate (**Figure 3**). Proneural genes promote whereas neurogenic genes inhibit neural

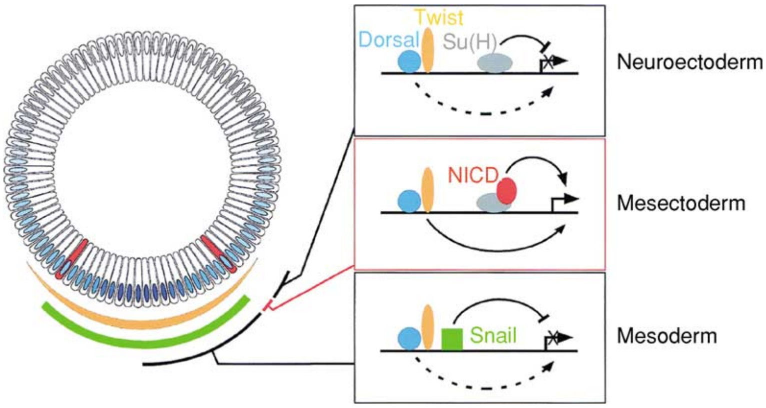


Figure 2 Molecular model for mesectoderm specification. Schematic representation of a cross-section of a fly embryo at blastoderm stage (left). In ventral nuclei, the combination of nuclear Dorsal (blue), Twist (orange), and Snail (green) transcription factors results in repression of *single-minded* (arrow at lower right). At more dorsal positions, lack of the Snail repressor as well as activation of the Notch pathway (the Notch intracellular domain (NICD) is shown in red) via the Suppressor of Hairless (Su(H)) partner and transcription factor (gray), activates mesectoderm specific genes (arrow at middle right). More dorsally, the combination of low/absent nuclear Dorsal and Su(H) dependent repression, in the absence of Notch signaling, leads to repression of *single-minded* (arrow at upper right). (Modified from Morel, V., Schweisguth, F., **2000**. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. *Genes Devel. 14,* 377–388.)

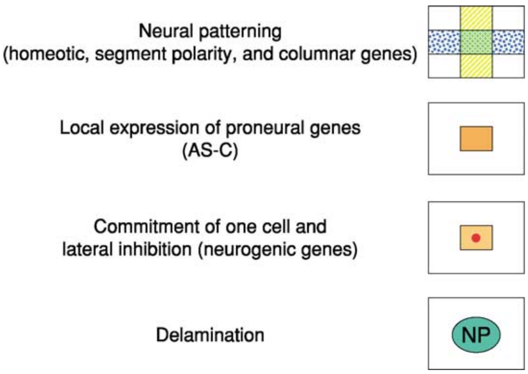


Figure 3 The neural pathway. The action of homeotic and segment polarity genes (yellow color in the first square) as well as columnar genes (blue color in the first square) subdivides the neuroectoderm in different regions along the AP and DV axis, respectively, and defines the position at which proneural clusters will be formed (green color in the first square). The expression of the *AS-C* in a cell cluster (orange rectangle) endows each cell of the cluster with the potential to become neural precursor. Progressively, due to differences in Delta expression within the cluster, the expression of the *AS-C* becomes restricted to a single cell at a stereotyped position. In all the other cells, AS-C expression progressively fades away (pale orange). The cell expressing AS-C at high levels corresponds to the presumptive neural precursor (red circle), while the adjacent ones are submitted to lateral inhibition and adopt the epidermal fate. Finally, the neural precursor (NP) emerges and delaminates from the ectoderm at a specific position, which defines the progeny of the lineage.

precursor formation, respectively (reviews: Skeath *et al.*, 1994; Hassan and Vaessin, 1996; Modolell and Campuzano, 1998; Rooke and Xu, 1998). These two gene classes act at the level of neural precursor singling out cells for delamination through a cell communication process called lateral inhibition (review: Rooke and Xu, 1998). Three steps can be recognized in this process. First, groups of cells within the neurogenic region acquire a neural competence; second, single cells within these groups become neural precursors; and third, their singling out prevents adjacent cells from taking the same fate, and leads such cells to become epidermis (review: Skeath and Carroll, 1994) (**Figures 3** and **4**).

1.10.2.2.1. Proneural genes promote neural precursor formation The classical example of proneural genes is provided by the *achaete–scute* complex (reviews: Campuzano *et al.*, 1985; Alonso and Cabrera, 1988; Campos-Ortega, 1998). Genes belonging to this complex, such as *achaete (ac), scute (sc), lethal of scute, asense (ase)*, encode bHLH transcription factors that are initially expressed in groups of equivalent cells (the proneural clusters) (Cabrera *et al.*, 1987; Jimenez and Campos-Ortega, 1990; Martin-Bermudo *et al.*, 1991; review: Skeath and Carroll, 1994). The expression of the *AS-C* endows each cell of the cluster with the potential to become neural precursor. Progressively, the expression of *AS-C* genes becomes restricted to a single cell at a stereotyped position within the cluster. This cell corresponds to the presumptive neural precursor that eventually delaminates from the neuroectoderm (Cabrera, 1990; Cubas *et al.*, 1991; Martin-Bermudo *et al.*, 1991; Skeath and Carroll, 1992) (**Figures 3** and **4**).

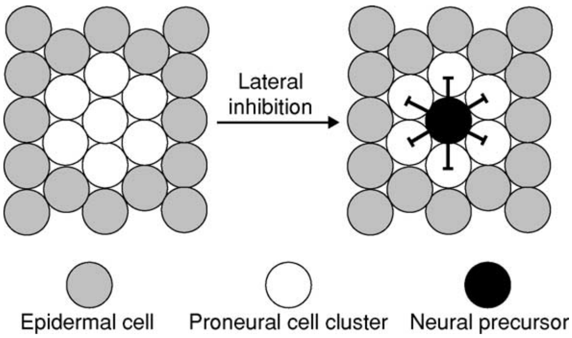


Figure 4 Lateral inhibition. Lateral inhibition is required for the selection of a single neural precursor cell during development. From a homogeneous population of epidermal cells (gray circles at left), a group of cells (the proneural cluster; white circles at left) acquires neural competence via the expression of proneural genes. Within the proneural cluster, one cell adopts the neural fate (black circle at right) due to high levels of Delta expression and thereby AS-C. This activates Notch signaling in the adjacent cells of the cluster, which inhibits AS-C expression in these cells (white circles at right). As a consequence, such cells are prevented from adopting the neural fate (black lines).

In *Drosophila*, loss-of-function analyses show that *achaete* and *scute* are required for the generation of most embryonic and adult external sense organs (Ghysen and Richelle, 1979; Jimenez and Campos-Ortega, 1990; Goriely *et al.*, 1991) (see **Chapters 1.11** and **1.12**). Similarly, gain-of-function analyses show that the *AS-C* genes are sufficient to induce the development of ectopic sensory organs at the expense of epidermis (Romani *et al.*, 1989; Rodriguez *et al.*, 1990; Dominguez and Campuzano, 1993). In the CNS, mutations in the *AS-C* lead to neural hypoplasia due to a strong reduction of the proneural cluster and the neural precursor number (Cabrera *et al.*, 1987; Dambly-Chaudière and Ghysen, 1987; Jimenez and Campos-Ortega, 1990; Cubas *et al.*, 1991; Martin-Bermudo *et al.*, 1991). As for the PNS, additional copies of *AS-C* genes cause neural hyperplasia, indicating that these genes play an instructive role in the induction of the neural fate (Jimenez and Campos-Ortega, 1990; Huang *et al.*, 1994).

Neural determination is also controlled by bHLH genes during vertebrate CNS development (reviews: Brunet and Ghysen, 1999; Bertrand *et al.*, 2002). Two gene families have been identified as being necessary for neural development: the MASH family, closely related to the *achaete–scute* genes, as well as a broad family that includes Math, Neurogenin, and NeuroD members, which are related to other bHLH *Drosophila* genes, such as *target of Poxn* (*tap*) and *atonal* (*ato*) (Jarman *et al.*, 1993; Gautier *et al.*, 1997; Kageyama *et al.*, 1997). Together with ectopic expression experiments in the ectoderm of *Xenopus* embryos, these data show that bHLH genes are necessary and sufficient to promote neurogenesis at the expense of the ectodermal fate, confirming their proneural potential (Lee *et al.*, 1995; Kim *et al.*, 1997; review: Lee, 1997).

It is interesting to note that members of the *AS-C* and *atonal* families account for all proneural activity in the fly PNS, but not in the CNS, where generation of some neural precursors does not require any of the known proneural genes. Potential bHLH candidates identified in the *Drosophila* genome are not expressed as expected for proneural factors, indicating that another family of proteins likely controls proneural activity in the fly CNS, and likely in vertebrates (Jimenez and Campos-Ortega, 1990; Moore *et al.*, 2000; Peyrefitte *et al.*, 2001). A future challenge will be to identify such proneural factors.

1.10.2.2.2. Neurogenic genes antagonize neural precursor formation Within the proneural cluster, selection of the neural precursor cell is mediated by the neurogenic genes (**Figures 3–5**). Loss-of-function mutations for such genes are characterized by nervous system hyperplasia indicating that they repress the neural fate. Neurogenic genes include those encoding the Notch receptor and its ligand Delta, as well as other genes that work in the Notch–Delta pathway (see below) (reviews: Artavanis-Tsakonas *et al.*, 1999; Baron *et al.*, 2002; Bertrand *et al.*, 2002). This pathway is involved in the process called lateral inhibition: the activation of Notch by Delta leads to receptor proteolysis (review: Chan and Jan, 1998), releasing the intracellular domain of Notch (NICD) into the cytoplasm. Then, NICD enters the nucleus and forms a complex with the Suppressor of Hairless transcription factor (**Figure 5**). Together, NICD and Su(H) activate specific targets (Bailey and Posakony, 1995) including genes of the *Enhancer of split Complex* (*E(spl)-C*), which encode transcription factors that inhibit the expression of proneural genes (Tata and Hartley, 1995; review: Bray, 1997). Since proneural genes activate Delta expression, cells in which the Notch pathway is activated eventually switch Delta expression off and adopt the epidermal fate (Kunisch *et al.*, 1994) (**Figure 5**).

The current view on the singling out of a single precursor proposes that, within the proneural cluster, all cells express proneural and neurogenic genes at similar levels. Subsequently, via mechanisms that are not understood yet, one cell of the cluster expresses more Delta than the adjacent cells. This effect is then amplified by lateral inhibition: activation of the Notch pathway in cells surrounding the presumptive neural precursor inhibits

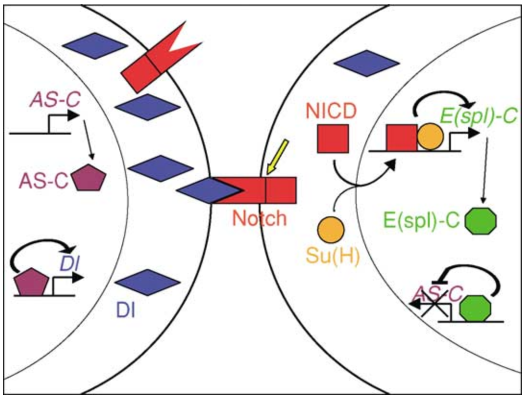


Figure 5 The Notch pathway in the proneural cluster. Thick and thin black lines indicate the cell and the nucleus membranes, respectively. Thick arrows and the T-bar indicate transcription activation and repression, respectively. Proneural genes of the *AS-C* (purple) activate Delta (Dl, blue) expression in the proneural cluster. Subsequently, one cell of the cluster (to the left) expresses more Delta than the adjacent cells (one of them is represented to the right), as simplified in the schematic drawing. Lateral inhibition entails a positive feedback loop that reinforces and maintains the initial differences between neural precursor (high Delta, cell to the left) and the other cells of the proneural cluster (low Delta, to the right). The activation of Notch (red) by Delta leads to receptor proteolysis (yellow arrow), releasing the intracellular domain of Notch (NICD, red square) into the cytoplasm. Then, NICD is transferred to the nucleus, where, together with the Suppressor of Hairless protein (Su(H), orange), it activates target genes, mainly represented by the *Enhancer of split Complex* (*E(spl)-C*, green). These target genes encode transcription factors that inhibit the expression of proneural genes (*AS-C*).

the differentiation of other neural precursors (**Figures 4** and **5**). Thus, a slight unbalance of Delta expression within the proneural cluster is sufficient to trigger a signaling pathway that allows the increase and the maintenance of AS-C and Delta expression in one cell, the neural precursor.

Mutations associated with nervous system hyperplasia permitted the identification of several other neurogenic genes, including *Enhancer of split*, *neuralized* (*neur*), *mastermind* (*mam*), *big brain* (*bib*), *almondex* (*amx*), and *neurotic* (*O-fucosyltransferase* (*O-fut1*)) (Shannon, 1972; Knust *et al.*, 1987b; Yedvobnick *et al.*, 1988; Rao *et al.*, 1990; Boulianne *et al.*, 1991; Sasamura *et al.*, 2003). All these genes modulate the Notch pathway at different steps. For example, *neurotic* encodes an O-fucosyltransferase protein that modulates the Notch–Delta interaction, and *neuralized* encodes a ubiquitin ligase that upregulates endocytosis of Delta (Boulianne *et al.*, 1991; Sasamura *et al.*, 2003). Therefore, complex gene interactions modulating the Notch pathway are involved in neurogenesis, indicating that cell–cell interactions are absolutely required to promote neural differentiation within the neuroectoderm.

1.10.2.3. Fly Neural Precursors and Lineages

1.10.2.3.1. Neuroblast lineage analysis The *Drosophila* VNC consists of a sequence of repeated units called neuromeres, each divided into two hemineuromeres separated by the midline. The singling out of the neural precursor through the interaction of neurogenic and proneural genes leads to cell delamination into a subectodermal proliferative zone. This process is achieved in five spatiotemporally distinct waves (S1–S5) to form an invariant and roughly orthogonal pattern of 30 neural precursors per hemineuromere during germ band extension (Doe, 1992) (**Figure 6**).

Three classes of neural precursors can be distinguished by the type of progeny (Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999) (**Figure 7**). Neural precursors that give rise only to neurons or glia are called neuroblasts (NBs) or glioblasts (GBs), respectively. A third type of precursor, called neuroglioblasts (NGB), produces neurons as well as glia. The expression profile of homeotic, segment polarity and columnar genes provides a panel of specific markers for each type of neural precursor cells (Doe, 1992) (**Figure 6**). The combination of stereotyped position and gene expression is sufficient to specifically identify most neural lineages at each stage of neurogenesis. Not only is the pattern of neural precursors in each neuromere invariant, but each precursor also produces an invariant population of neurons and glia. This last feature has been revealed using lineage tracers such as 1,1′-dioctaldecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and horseradish peroxidase (HRP) that allow single precursors to be followed during development (see below) (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999). The simple organization as well as the stereotyped behaviors of the neural lineages have made it possible to draw a precise map of the fly embryonic nervous system

1.10.2.3.2. Types of neural precursors

1.10.2.3.2.1. The neuroblast Each hemineuromere of the ventral cord contains 23 distinct NBs, some of them specific to abdominal or thoracic segments (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999; review: Van De Bor and Giangrande, 2002). After delamination, neural precursors initiate a series of asymmetric self-renewing divisions, each mitotic event generating another NB and a small cell, the ganglion mother cell (GMC), which divides once and produces two neurons (**Figure 7a**). Normarski microscopy was used to examine the morphological differentiation of a single NB in *Drosophila,* and *camera lucida*

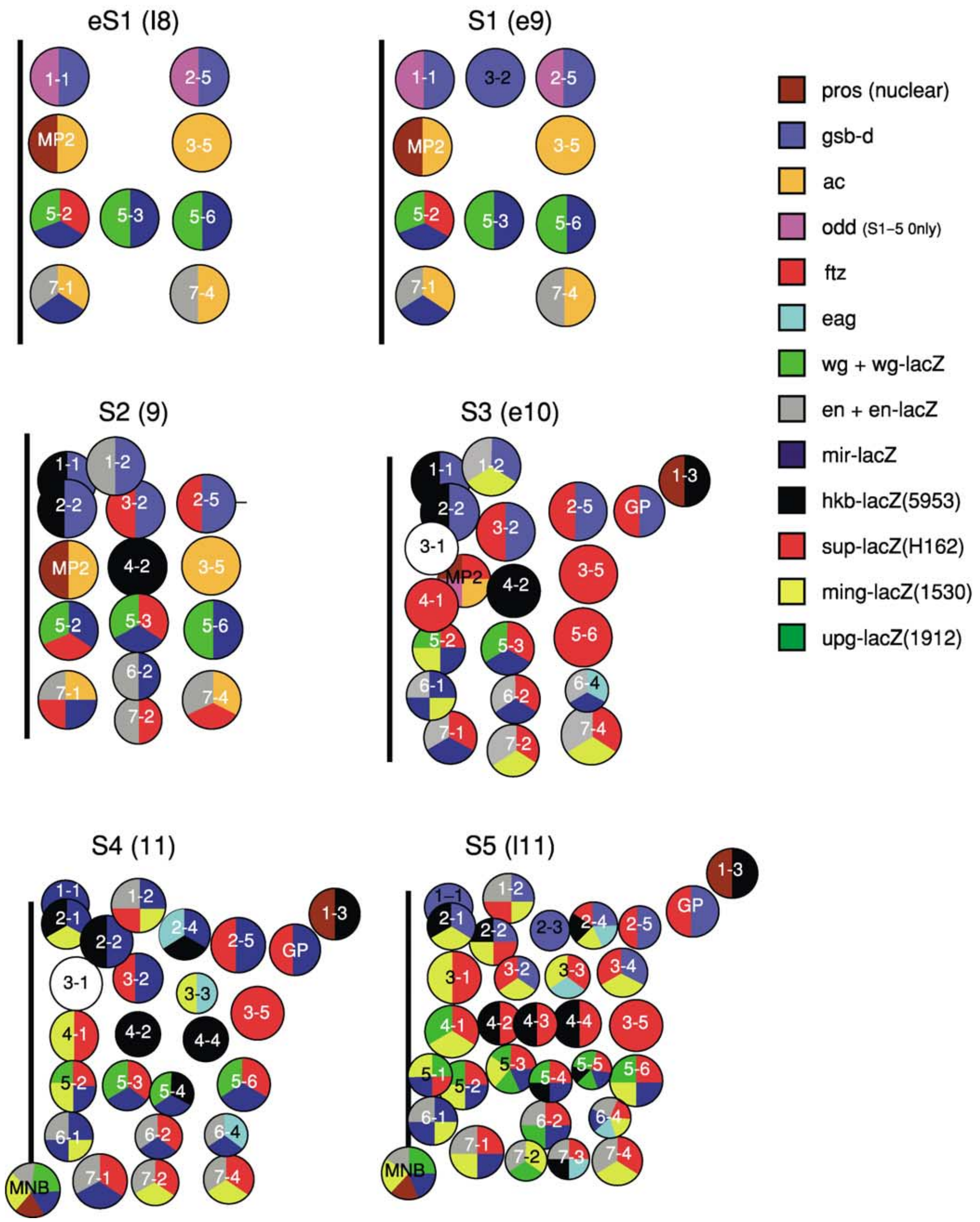


Figure 6 Identification of neuroblasts by the expression of specific molecular markers. Panels S1 to S5 indicate the five spatiotemporally distinct waves (stages are according to Campos-Ortega and Hartenstein, 1985). Numbers in brackets indicate embryonic stages, and e and l stand for early and late, respectively. In each panel, the vertical line indicates the midline. Neural precursors are distributed in seven rows (indicated by the first number in each circle) along the anteroposterior axis and five columns (indicated by the second number in each circle) along the dorsoventral axis. Each precursor is named according to its position along the axes and expresses a specific combination of molecular markers. The color coding illustrates the specific expression of the markers described at right; ac, en, wg, gsb-d, and odd represent protein markers; pros represents nuclear protein localization; eag corresponds to a mRNA pattern; wg-lacZ, en-lacZ, mir-lacZ, hkb-lacZ, svp-lacZ, ming-lacZ and upg-lacZ represent β-galactosidase staining patterns of enhancer trap lines inserted into these genes. GP, MNB, and MP2 indicate the glial precursor, median neuroblast, and midline precursor 2, respectively. The 3-1 precursor (white circle) shown in S3 and S4 does not express any of these markers. Gene abbreviations: pros (nuclear), *prospero*; gsb-d, *gooseberry-distal*; ac, *achaete*; odd, *odd-skipped*; ftz, *fushi-tarazu*; eag, *eagle*; wg and wg-lacZ, *wingless* and *wingless*-lacZ; en and en-lacZ, *engrailed* and *engrailed*-lacZ; mir-lacZ, *mirror* and *mirror*-lacZ; hkb-lacZ, *huckebein* and *huckebein*-lacZ; svp-lacZ, *seven-up* and *seven-up*-lacZ; ming-lacZ, *ming*-lacZ = *castor*-lacZ; upg-lacZ, *unplugged*-lacZ. (Modified from Hyper Neuroblast Map at http://www.neuro.uoregon.edu.)

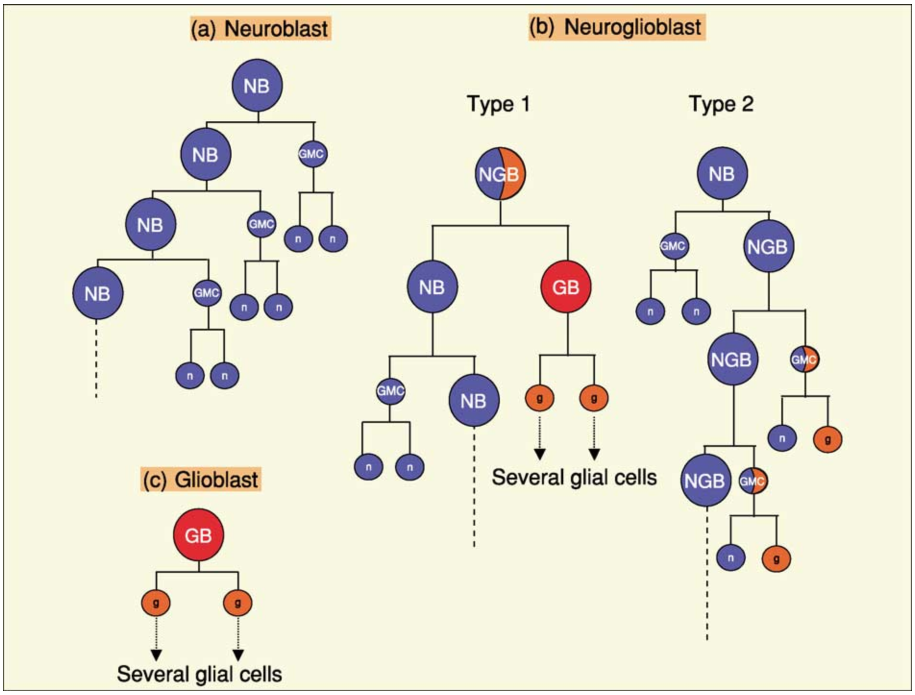


Figure 7 Types of neural precursors. Neuronal and glial potential/identities are shown in blue and red, respectively. (a) Typically, the neuroblast (NB) divides once to give rise to another NB and a small ganglion mother cell (GMC). Subsequently, the GMC divides only once to produce two neurons (n). (b) Type 1 neuroglioblast (NGB) produces restricted precursors with neuronal (NB) or glial (GB) potentials. Type 2 neuroglioblasts generate ganglion mother cells (GMCs) that produce two neurons or a glial cell (g) and a neuron. (c) Glioblasts (GB) only produce glial cells. (Modified from Van De Bor, V., Giangrande, A. **2002**. *glide/gcm*: at the crossroads between neurons and glia. *Curr. Opin. Genet. Devel. 12*, 465–472.)

tracings were used to establish the spatial relationship between NBs and GMCs (Doe, 1992). NBs form by the delamination and enlargement of individual ectodermal cells within the neurogenic region. Ectodermal cells have their nuclei localized at the ventral cell surface. When the NB forms, its nucleus moves dorsally. Soon after, this cell begins enlarging and, ultimately, delaminates from the ventral ectoderm to form a neural precursor (Doe, 1992). During NB division, a small GMC buds from its dorsal or, sometimes, lateral surface. GMCs can be molecularly identified by the presence of the Prospero (Pro) transcription factor in their nucleus (see below). This self-renewing, stem cell-like division of the NB allows the maintenance of the precursor pool and the generation of intermediate precursors, the GMCs, which have a limited proliferative potential.

1.10.2.3.2.2. The neuroglioblast (type 1 and 2) Ten NGB lineages arise from the neuroectoderm, four of them showing differences between abdominal and thoracic neuromeres (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999). By lineage analysis combined with the use of molecular markers, it has been shown that at least two types of NGBs exist in *Drosophila* (Udolph *et al.*, 1993; Bernardoni *et al.*, 1999; Ragone *et al.*, 2001; review: Van De Bor and Giangrande, 2002) (**Figure 7b**). Type 1 NGB produces glial cells just after delamination upon production of a neuroblast and a glioblast, pure precursors with neuronal and glial potentials, respectively. The 6-4 lineage in the thorax (6-4T) represents an example of type 1 NGB (**Figures 7b** and **8b**; see also below). Type 2 NGB represents a variation on theme of a pure neuroblast (**Figure 8b**). It delaminates and initially produces neurons like a neuroblast. Subsequently, however, it produces GMCs that divide once and generate a neuron and a glial cell. In this case, there is no glial precursor as such. The lineage tree of type 2 NGB has been deduced through the use of mutants, genetic markers, and clonal analysis, all of which indicate that glial cells are direct siblings to neurons in this lineage and that each glial cell/neuron pair arises by asymmetric division (Udolph *et al.*, 2001). The 1-1 lineage in the abdomen represents an example of this class of NGBs (see below).

1.10.2.3.2.3. The glioblast Two GB lineages arise from the neuroectoderm (Bossing and Technau,

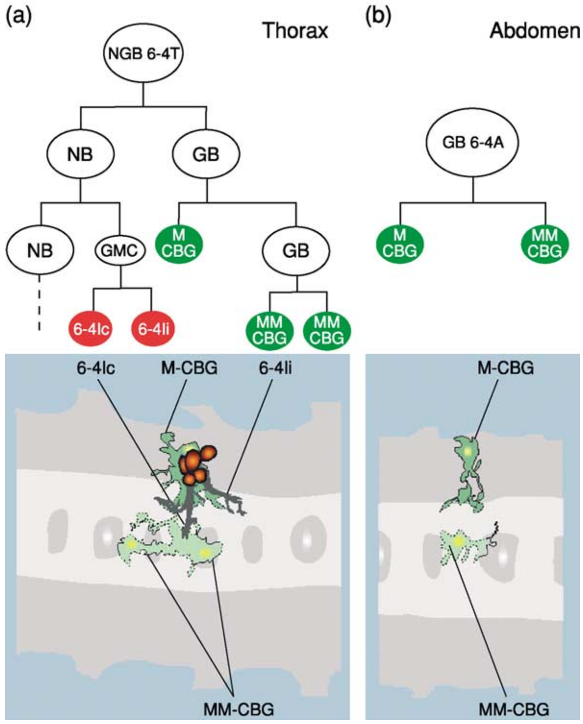


Figure 8 Drawings represent the 6-4 lineage as identified by DiI injection. The 6-4 cell produces different progenies in the thorax (a) and in the abdomen (b). (a) In the thorax, the 6-4 lineage (NGB 6-4T) gives rise to neurons (red circles) and glia (green circles). The dashed line indicates that the NB issued from the first division continues to proliferate and produces neurons, as shown by the presence of five neurons in the lower panel. (b) In the abdomen, the 6-4 lineage only produces glia (GB 6-4A). In the lower panels, cell lineages derived from 6-4 are shown in a dorsal view with anterior to the left. The cortex region and the neuropil of the ventral nerve cord are shown in gray and white, respectively. The medial dorsoventral channels, which mark the neuromere boundaries, are shown in light ovals. Clusters of neuronal cell bodies are red and their fiber projections dark gray. Glial cells are shown in green (dark and light green indicate a mediodorsal and a ventral position, respectively) and glial cell nuclei are shown in yellow. GB, glioblast; GMC, ganglion mother cell; NB, neuroblast; NGB, neuroglioblast; M-CBG, medial cell body glia; MM-CBG, medialmost cell body glia. Ic and Ii indicate interneurons (symbols are according to Bossing *et al.*, 1996).

1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999; review: Van De Bor and Giangrande, 2002). One of them is the abdominal counterpart of the thoracic 6-4 lineage (see below) and is called GB6-4A, while the other corresponds to the longitudinal glioblast. These precursors only generate glial cells (**Figure 7c**).

1.10.2.3.2.4. Midline Through the use of genetic approaches, including enhancer trap lines, in which a *LacZ* reporter gene is expressed in a lineage restricted manner, it was shown that midline neural precursors occupy a stereotyped position along the anteroposterior axis within a segment. The differentiated progeny of each precursor was then determined by morphological criteria by placing DiI on early progenitors (Bossing and Technau, 1994).

Four NB lineages arise from the posterior part of the neuromere including the midline precursors (MP), the unpaired median intermediate (UMI) precursor, the precursors of ventral unpaired median (VUM) neurons, and the median neuroblast (MNB). The precise lineage tree of all these precursors is not yet fully understood (review: Jacobs, 2000).

A pool of 10–12 midline glia (MG) precursors is localized in the anterior part of the neuromere. From this pool of precursors, only three survive by the end of embryogenesis – the anterior, the medial, and the posterior MG precursor – the others undergoing apoptosis (see below) (Klambt and Goodman, 1991; Sonnenfeld and Jacobs, 1995; Dong and Jacobs, 1997; Zhou *et al.*, 1997b).

So far, no NGB have been found in the *Drosophila* mesectoderm. Interestingly, in other insect species such as *Schistocerca americana*, a NGB differentiates at the position normally occupied by the MNB in *Drosophila* (Condron *et al.*, 1994; Condron and Zinn, 1994).

1.10.3. Neural Patterning

A total of 16 abdominal, six thoracic, and six gnathal hemineuromeres make up the VNC. At the end of neurogenesis, each neuromere contains around 400 neurons and 60 glial cells (Bossing and Technau, 1994; Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999). The type of neurons and glial cells in these neuromeres depends on the identity of neural precursors, which we will refer to as NBs for the sake of simplicity, irrespective of whether they generate neurons or glia. Thus, it is critical to dissect the genetic regulatory mechanisms that specify the identity of individual NBs.

Studies carried out in the last 10 years indicate that many of the genes that pattern the embryo along the main axes (anteroposterior or AP, dorsoventral or DV; see **Chapters 1.2**, **1.8**, and **1.9**, also function in neurogenesis (review: Skeath, 1999). The activities of these genes create a Cartesian coordinate system that gives a unique fate to individual precursors depending on their position (review: Skeath and Thor, 2003). The invariant pattern of gene expression and progeny suggests that autonomous cues control the identity of each precursor. This is further confirmed by the observation that precursor cells keep their identity even when

cultured as isolated cells (Huff *et al.*, 1989; Broadus and Doe, 1997). One piece of evidence indicates, however, that neural precursors also display some plastic features: when late-born NBs are transplanted into early stage embryos, they acquire an early identity (Udolph *et al.*, 1995). Thus, heterochronic transplantation experiments show that neuroblast specification occurs under the control of stage-specific inductive signals that act in the neuroectoderm. Therefore, autonomous as well as nonautonomous processes appear to control precursor identity during nervous system development.

1.10.3.1. Spatial Cues Control Neuroblast Differentiation

Positional cues along the AP and DV axes allocate proper identities to different neuromeres as well as to different regions within each neuromere (**Figure 3**). Different classes of genes control the establishment of these coordinates: homeotic transcription factors define the AP identity of the different neuromeres (reviews: McGinnis and Krumlauf, 1992; Gehring, 1993; **Chapter 1.8**), whereas segment polarity and columnar genes, respectively, define the AP and the DV identities within the neuromere (review: Skeath, 1999).

1.10.3.1.1. Homeotic genes specify neuroblast identity along the AP axis The AP and DV axes of the early *Drosophila* embryo are established by two key maternal morphogens: Bicoid (Bcd) and Dorsal (Dl), respectively (Steward, 1987; Berleth *et al.*, 1988; **Chapter 1.2**). Bicoid is expressed in a broad concentration gradient along the AP axis, with peak levels present at the anterior pole, while Dorsal is expressed in a gradient along the DV axis with peak levels along the ventral surface (Ip *et al.*, 1992; **Chapter 1.2**). Both morphogens establish overlapping patterns of transcriptional activators and repressors in the early embryo, leading to expression of specific genes, including homeotic genes, which define territories along AP and DV axes (Ip *et al.*, 1992; **Chapters 1.2** and **1.8**).

The different behaviors of neural lineages between thoracic and abdominal neuromeres are explained by the expression profile of homeotic genes, which are expressed in specific domains along the rostrocaudal axis of the developing nervous system. In the fly CNS, the 1-1 lineage produces both neurons and glia in the abdomen, whereas it only produces neurons in the thorax (Udolph *et al.*, 1993; Prokop and Technau, 1994). Ultrabithorax (Ubx) and Abdominal-A (Abd-A) are not expressed in the two first thoracic segments (**Figure 9a**). It has been shown that Ubx or Abd-A misexpression in this region is sufficient to induce abdominal development of the complete 1-1 lineage

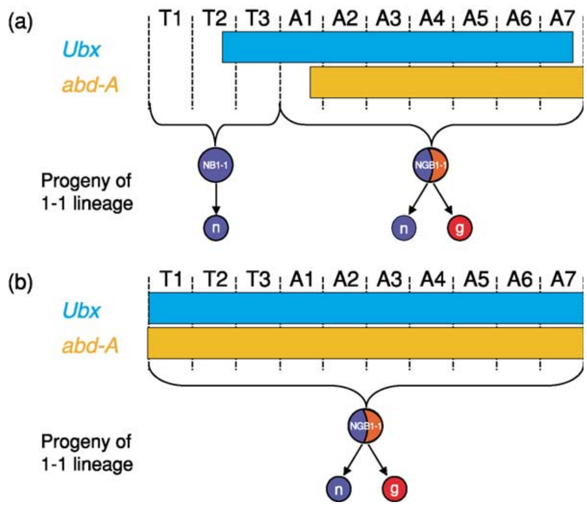


Figure 9 Homeotic genes control anteroposterior NB identity. Schematic representation of *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) gene expression domains in thoracic (T1 to T3) and abdominal (A1 to A7) neuromeres. Light blue and orange rectangles correspond to *Ubx* and *abd-A* expression domains, respectively. Anterior is to the left. Dashed vertical lines represent the boundaries of each neuromere. Blue and red indicate neuronal and glial potential/identity, respectively. (a) In wild-type embryos, *Ubx* (light blue) is expressed from T2 to A7 neuromeres whereas *abd-A* (orange) is expressed from A1 to A7. The 1-1 lineage produces both neurons (n) and glia (g) in the abdomen (NGB1-1, blue/red circle), whereas it produces only neurons in the thorax (NB1-1, blue circle). (b) When *Ubx* or *abd-A* are overexpressed in all neuromeres, the thoracic 1-1 lineage gives rise to neurons and glial cells, like in the abdomen.

and to override thoracic commitment, showing that activity of the homeotic genes *Ubx* and *abd-A* is required for abdominal specification (Prokop and Technau, 1994; **Chapter 1.8**) (**Figure 9b**).

Homeotic genes are conserved throughout evolution in their DNA binding domain (reviews: McGinnis and Krumlauf, 1992; Gehring, 1993; **Chapter 1.8**). The relative AP order of expression of *Hox* genes (the vertebrate orthologs of insect homeotic genes) in the developing vertebrate CNS is similar to the AP order of expression of homeotic genes in the *Drosophila* CNS, indicating that the role of these genes in patterning the nervous system is conserved during evolution (reviews: Boncinelli *et al.*, 1993; Krumlauf *et al.*, 1993). This is confirmed by cross-phylum gene exchange experiments showing that *Drosophila* homeotic genes can replace their vertebrate orthologs and vice versa.

While it is not clear whether *Hox* genes also control DV patterning in *Drosophila*, it has been recently shown that they do play a role in this process during vertebrate CNS development. In the mouse hindbrain, *Hoxa2* and *Hoxb2* are expressed in a pattern that defines complementary distribution along the DV axis, suggesting that these genes play a

role in DV patterning and neurogenesis (Davenne *et al.*, 1999). *Hoxa2* and double-mutant mice show DV differences in the distribution and the differentiation of early neurons, indicating a change in precursor cell identity (Davenne *et al.*, 1999). Given that nervous system development and definition of distinct neural patterns require a crosstalk between AP and DV patterning, these data suggest that, at least in vertebrates, homeotic genes integrate AP and DV positional information.

1.10.3.1.2. Segment polarity genes specify AP neuroblast differentiation and identity within the neuromere Most segment polarity genes were originally identified in the genetic screen carried out by Nüsslein-Volhard and Wieschaus (1980). The role of these genes is to subdivide each segment into an identical pattern of parallel rows. Segment polarity gene activity dissects the neuroectoderm into five transverse rows per hemineuromere and ensures that NBs that develop in different rows acquire different identities (Bhat, 1999).

Segment polarity genes include those encoding the secreted proteins Wingless (Wg) and Hedgehog (Hh), the transmembrane receptor Patched (Ptc) as well as the transcription factors Gooseberry (Gsb), Engrailed (En), and Invected (Inv) (Nüsslein-Volhard and Wieschaus, 1980; Baumgartner *et al.*, 1987; Baker, 1988; Ingham *et al.*, 1991; Mohler and Vani, 1992; Gustavson *et al.*, 1996). The expression profile of these genes subdivides the neuromere along the AP axis and spatially regulates the expression of the proneural genes *achaete* and *scute* (reviews: Skeath and Carroll, 1992, 1994). In the absence of one or more of these gene activities, a subset of proneural clusters and NBs fails to form. For example, *wg* is expressed in neuroectodermal cells that will give rise to row 5 proneural clusters, and its secreted product is also detected in adjacent rows 4 and 6 (**Figure 10**). Absence of *wg* results in a decrease of proneural cluster number in rows 4 and 6 indicating a failure in the formation of such clusters, whereas row 5 proneural clusters develop normally (Chu-LaGraff and Doe, 1993). Moreover, proneural clusters and NBs that develop in rows 4 and 6 acquire identity incorrect for their position. This indicates that *wg* promotes proneural cluster formation in rows 4 and 6 and permits them to acquire the proper identity (Chu-LaGraff and Doe, 1993) (**Figure 10**). These genes continue to be expressed in NBs, and often in their progeny, GMCs and neurons, suggesting that they play a role at later developmental stages.

Positional cues defining cell identity within the nervous system have also been identified in mesectodermal cells. In each neuromere, a single row of cells constitutes the midline cell population (review: Jacobs, 2000). The anterior midline cells produce midline glia, and the posterior ones midline neurons. Hedgehog (Hh) signaling to mesectodermal cells in the posterior domain of the neuromere is important for the determination of posterior neuronal identities. Loss of Hh results in extra midline glia (MG), because anterior lineages are duplicated when posterior identities are not specified (Hummel *et al.*, 1999a, 1999b). Conversely, expansion of Hh signaling reduces the MG number (review: Jacobs, 2000). Therefore, the Hh signaling pathway functions to determine neurons in the posterior neuromere, and is antagonistic to signals that contribute to MG identity in the anterior neuromere. Embryos mutants for *wg* and *ptc* have no anterior midline cells (including MG), indicating that *wg* and *ptc* contribute to the determination of the anterior midline identity (Zhou *et al.*, 1997a, 1997b).

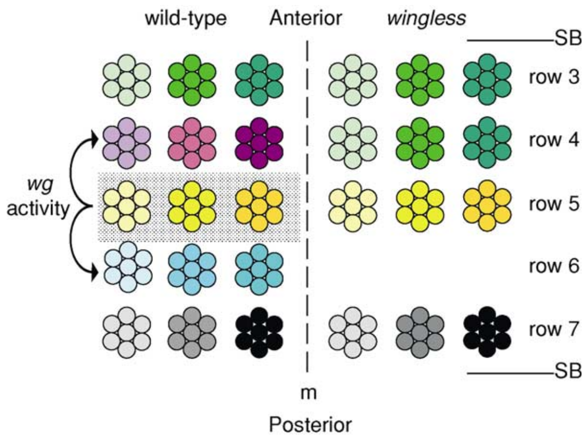


Figure 10 Segment polarity genes control NB differentiation and anteroposterior identity within the neuromere. Schematic representation of proneural clusters present within a hemineuromere of wild-type (left) or *wingless* (*wg*) (right) mutant embryos. The two hemineuromeres are separated by the midline (m, dashed line). Anterior is to the top. At this stage, five rows of clusters are present along the AP axis, called rows 3 to 7. The segment boundaries (SB) are shown by two horizontal lines. Color coding identifies specific proneural clusters along the AP and DV axes. In the wild-type embryo, Wg is expressed in row 5 (dotted pattern) and acts on adjacent rows 4 and 6 (black arrows). In the *wg* mutant embryo, proneural clusters in row 5 are still present whereas the adjacent ones are missing (row 6, absent circles) or misspecified (row 4, green circles). Row 4 switching to row 3 identity was assessed by the expression of cell specific markers in NB and GMC progeny.

1.10.3.1.3. Columnar genes specify DV neuroblast differentiation and identity within the neuromere Segment polarity gene action does not explain how NBs of the same row but different columns acquire different DV identities within the same segment.

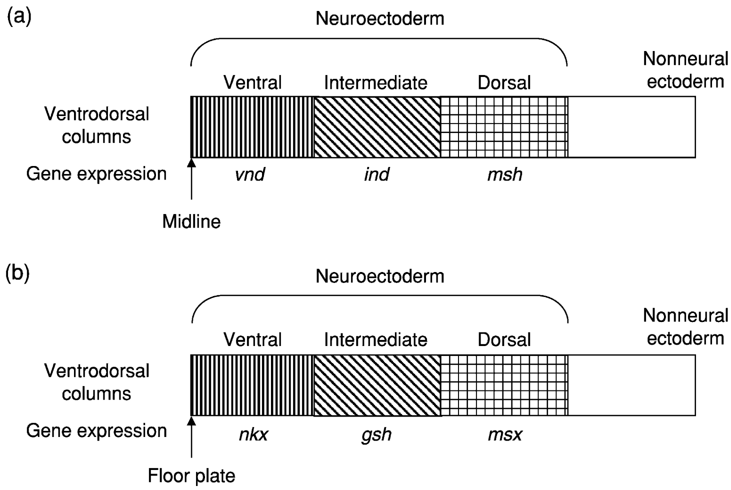


Figure 11 Evolutionary conservation of dorsoventral patterning within the neuroectoderm. Schematic representation of columnar gene expression domains in a hemineuromere of the (a) fly and (b) vertebrate neuroectoderm. Profiles of gene expression subdivide neuroectoderm into three ventrodorsal columns: *vnd/nkx*, *ind/gsh*, and *msh/msx* genes are expressed and control cell identity within ventral (vertical lines), intermediate (oblique lines), and dorsal (square motif) domains. Non neural ectoderm is indicated in white. The ventralmost position corresponds to the midline in flies and floor plate in vertebrates. (Modified from Cornell, R.A., Ohlen, T.V., **2000**. Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* 10, 63–71.)

Four factors control this process: the three transcription factors ventral nerve cord defective (Vnd) (Chu *et al.*, 1998; McDonald *et al.*, 1998), intermediate nerve cord defective (Ind) (Weiss *et al.*, 1998), and muscle segment homeobox (Msh) (Isshiki *et al.*, 1997), as well as the *Drosophila* epidermal growth factor (EGF) receptor (DER), a receptor tyrosine kinase (Livneh *et al.*, 1985; Skeath, 1998). These factors play a decisive role in dividing the neuroectoderm into three separate longitudinal regions: *vnd* is expressed in the ventral (medial) column (Mellerick and Nirenberg, 1995), *ind* is expressed in the intermediate column (Weiss *et al.*, 1998), and *msh* is expressed in the dorsal (lateral) column (D'Alessio and Frasch, 1996) (**Figure 11**). DER is expressed and required in the intermediate column to promote NB formation, and in the ventral column to specify NB identities (Yagi *et al.*, 1998). The NBs born in the dorsal column develop normally in DER mutant embryos.

Absence of columnar genes leads to defects in NB formation and to misspecification of NB identity (review: Skeath, 1999). For example, *vnd* functions in the ventral column to promote the formation of ventral identity precursors and to repress those with intermediate identity. Absence of *vnd* induces ventral cells to acquire intermediate cell identity, and *ind* expression expands into the ventral and intermediate columns (Chu *et al.*, 1998; McDonald *et al.*, 1998; Weiss *et al.*, 1998). Conversely, ubiquitous overexpression of *vnd* induces intermediate and some dorsal cells to acquire ventral identity (McDonald *et al.*, 1998). These results show that *vnd* specifies ventral column identity and prevents ventral precursors from adopting an intermediate column identity by repressing *ind*.

Elements of DV neural patterning have been evolutionarily conserved. Indeed, ortholog genes play the same role in vertebrates: the *nkx/vnd*, *Gsh-1,2/ind*, and *msx/msh* genes are expressed and control cell fates within ventral, intermediate, and dorsal domains, respectively, in the neural tube (review: Cornell and Ohlen, 2000) (**Figure 11**). It is interesting to note that upstream or downstream pathways to this "three column expression pattern" are not conserved during evolution, suggesting that this specific process plays a pivotal role in neuronal specification (review: Cornell and Ohlen, 2000).

It is also worth noting that, apart from mesectoderm-derived glia precursors, most glial precursors are localized in the dorsal part of the neuroectoderm, suggesting a role of columnar genes in the establishment of the gliogenic potential (Bossing *et al.*, 1996; Schmidt *et al.*, 1997, 1999).

A number of transcription factors such as Pannier (Pnr) and the products of the Iroquois Complex (Iro-C) have been shown to link positional cues and proneural cluster formation in the fly adult PNS (Ramain *et al.*, 1993; Leyns *et al.*, 1996; Garcia-Garcia *et al.*, 1999; Calleja *et al.*, 2000; Cavodeassi *et al.*, 2000). These transcription factors control the expression of the proneural genes in response to

extracellular molecules that assign spatial identity. Although it will be interesting to determine the role of these genes in CNS patterning, the embryonic profile of expression of some of them suggests that distinct molecular pathways may be used in adult PNS and embryonic CNS patterning.

1.10.3.2. Temporal Cues Control Neuroblast Differentiation

CNS development requires precise spatial as well as temporal cues. Indeed, fly neural precursors divide a fixed number of times and produce cells of distinct type at each division. For example, the first division of the 1-1 lineage always produces a GMC that gives rise to the aCC motorneuron and the pCC interneuron (in thorax and abdomen), whereas later divisions produce other types of motorneurons and interneurons in the thorax, and other types of interneurons as well as glia in the abdomen (Udolph *et al.*, 1993; Prokop and Technau, 1994). Thus, a temporal genetic program controls neural precursor differentiation.

Cell lineage, ablation, transplantation, *in vitro* culture, and genetic studies in *Drosophila* and others insects indicate that GMC birth order is the primary determinant of temporal identity (Doe and Goodman, 1985a, 1985b; Furst and Mahowald, 1985; Prokop and Technau, 1994; Weigmann and Lehner, 1995; Schmidt *et al.*, 1999). Recent studies have indeed shown that *Drosophila* NBs undergo temporally ordered changes in gene expression (review: Zhong, 2003). This suggests that, as NBs divide, they become temporally restricted in the type of cells that they can generate.

Good candidates to regulate this process are the transcription factors encoded by the *hunchback* (*hb*), *Krüppel* (*Kr*), *POU domain protein* (*pdm*), *castor* (*cas*), and *grainyhead* (*grh*) genes (Brody and Odenwald, 2000, 2002) (**Figure 12**). The *hunchback* and *Krüppel* genes are expressed in early-born neurons, *pdm* is expressed in middle-born neurons, while *castor* and *grainyhead* are expressed in late-born neurons. The *hb, Kr, pdm, cas* and *grh* genes are sequentially expressed in NBs, with the GMC/progeny maintaining this transcription factor profile at their birth.

This creates a layered structure, with each layer differing from the adjacent one by the expression of a specific transcription factor (**Figure 12a**). Expression of *hb* and *Kr* is necessary and sufficient to induce an early-born cell fate in multiple lineages (**Figure 12c**) (Isshiki *et al.*, 2001). Thus, *hb* and *Kr*

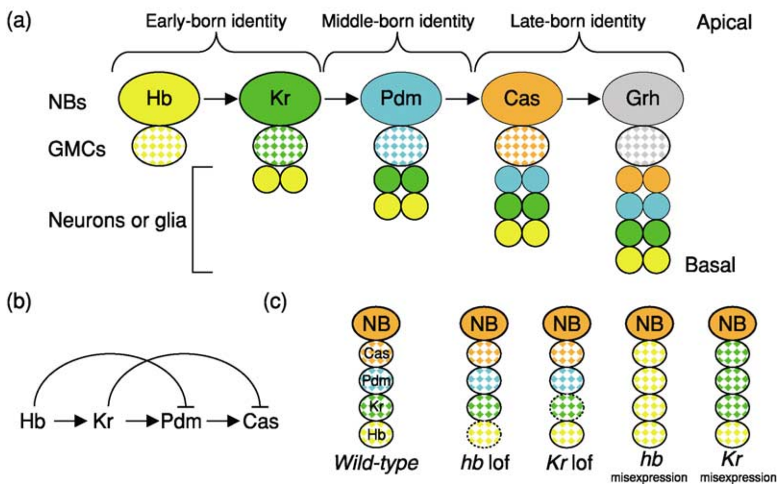


Figure 12 Temporal identity in NB lineages. (a) Schematic representation of a NB lineage: the neuroblast (NB), the ganglion mother cell (GMC), and its neuronal/glial progeny are represented from apical to basal. Color coding indicates specific gene expression and subdivides the lineage into adjacent layers according to birth date. During each temporal window of gene expression, GMCs are marked by the continuous presence of the temporal factor that is expressed in the NB at its birth. These transcription factors are also detected in postmitotic cells (neurons or glia). (b) Cross-regulation amongst ''temporal'' transcription factors. Loss-of-function and gain-of-function studies indicate cross-regulatory interactions between ''temporal'' transcription factors. Arrows and T-bars indicate activation and repression, respectively. These interactions ensure the sequential progression of temporal states during lineage development. (c) Schematic representation showing the temporal sequence of gene expression in wild-type and mutant GMCs. In wild-type, ''temporal'' genes are expressed in the NBs (large ovals) as well as in their progeny, the GMCs (lozenge-patterned small ovals). In loss-of-function (lof) *Hb* or *Kr* mutant embryos, the first or the second born identities are lost in the NB progeny, respectively. Conversely, forced continuous expression of Hb or Kr leads to the transformation of late-born identity to first or second born identity in the GMCs, respectively. Dashed circles represent abnormal GMC development (GMC transformed to a later born identity or dying GMC).

specify "early" temporal identity in *Drosophila* neural precursors.

These data indicate that loss of a temporal factor results in the alteration of neural identity in the layer in which this factor is usually expressed, and may lead to supernumerary cells expressing the transcription factor of the adjacent layer. These transcription factors, therefore, regulate the temporal identity (early versus late) of the NB and its progeny rather than cell fate (neuronal versus glial).

The molecular mechanisms underlying this temporal regulation are based on the fact that the transcription factor expressed in state 1 activates transcription factor in state 2 and represses transcription factor in state 3 (**Figure 12b**). For example, overexpression of Hb activates Kr and represses Pdm and Cas, whereas overexpression of Kr activates Pdm and represses Cas but has no effect on Hb expression leading to the model that each gene can activate the next gene in the pathway and repress the "next +1" gene (Brody and Odenwald, 2000; Isshiki *et al.*, 2001) (**Figure 12b**). Thus, temporal factors are responsible for upregulation of the next state, but also insulate the genetic program of adjacent temporal windows.

1.10.3.3. Molecular Control of Neuroblast Apoptosis, Quiescence, and "Reactivation"

The finding that temporal cues specify the identity of NB progeny raises an important question concerning the proliferative potential of neural stem cells. Fly NBs are submitted to a sharp proliferative control throughout development. Indeed, the number of cell divisions in the embryonic CNS depends on the type of NB. In most cases, the number of the progeny depends on the time of delamination, early-born NBs producing more neurons than late-born NBs (Bossing *et al.*, 1996, Schmidt *et al.*, 1997, 1999).

Moreover, the fly adult nervous system develops through extensive reorganization of the embryonic nervous system (review: Truman *et al.*, 1993) (see **Chapters 2.4** and **2.5**). A crucial pathway controlling this process is cell death by apoptosis in the sense that, once the embryonic nervous system has differentiated, some NBs undergo programmed cell death (Truman *et al.*, 1992) (see **Chapter 2.5**). For example, although each embryonic hemineuromere contains 30 NBs, their number decreases at later developmental stages. In the thorax of the larva, each neuromere retains about 23 NBs, while in the abdomen only three remain (Truman and Bate, 1988). Cell death genes of the *reaper* family account for this proapoptotic activity (Peterson *et al.*, 2002) (see **Chapter 2.5**).

Together with cell death, cell proliferation constitutes the other important pathway that determines the number of progeny and thereby shapes the adult CNS (Truman and Bate, 1988). While some NBs die, others stay quiescent and later resume proliferation ("NB reactivation") to build up the adult nervous system. The NBs that are reactivated at larval stages cease to proliferate completely after a specific number of divisions, and eventually die by apoptosis. The homeotic gene *abd-A* determines the final number of progeny that each precursor generates in the larval VNC (Bello *et al.*, 2003). Indeed, thoracic and abdominal NBs show extensive differences in the extent of proliferation. Larval thoracic NBs resume division earlier and divide for longer periods of time than abdominal NBs. A burst of expression of *abd-A* regulates the time at which cell death occurs in the larval life and, therefore, schedules the end of neural proliferation (Bello *et al.*, 2003). This shows that homeotic genes link positional information to neural proliferation. The cell-autonomous requirement of Abd-A implies that an intracellular signaling cascade controls this process. It would be interesting to determine whether the "temporal" factors play a role in controlling such death programs.

Recent studies have also shed some light on a molecular mechanism controlling neural stem cell proliferation in the brain. The Even-skipped (Eve) homeodomain containing transcription repressor acts nonautonomously to stimulate cell division of quiescent optic lobe NBs (Park *et al.*, 2001). Genetic studies have shown that Eve interacts with Terribly reduced optic lobes (Troll) and, indeed, mutations in this gene arrest NB proliferation (Voigt *et al.*, 2002). The vertebrate ortholog of *troll* encodes an extracellular matrix molecule that acts as a coreceptor for fibroblast growth factor 2 (FGF2) signaling, calling for a role of cell–cell interactions in the control of neural stem cell proliferation. Interestingly, mutations in these genes do not affect the proliferation potential of NBs that give rise to the embryonic CNS and are not programmed to die.

Building up the nervous system relies on the sequential activation of specific molecular and cellular pathways. While the maternal contribution defines the neurogenic territory, zygotic neuralizing factors and patterning genes allow the differentiation of distinct neural precursors at the right place and time. In addition, most genes work in a combinatorial fashion. The superimposition of homeotic, columnar, and segment polarity gene expression creates, within each neuromere, a Cartesian coordinate system, in which each precursor displays a unique combination of gene activity. Finally, each step must integrate with the

others, as shown by the observation that patterning genes activate proneural genes and that temporal factors control each other's activity. This intricate network of pathways guarantees the fine-tuning that is necessary for the establishment of a very complex array of neural precursors.

1.10.4. Neuroblasts and Asymmetric Divisions

1.10.4.1. Neuroblast Lineages and Asymmetric Divisions

Positional and proneural cues induce NB patterning and formation, respectively, and endow cells with specific identities in the absence of cell division. NB delamination triggers a second phase of cell specification, in which cells of the nervous system acquire distinct identities upon divisions that are asymmetric.

Asymmetric cell divisions play a crucial role in the establishment of cell diversity during development (Matsuzaki, 2000; Doe and Bowerman, 2001; Knoblich, 2001; Segal and Bloom, 2001). Asymmetry may be directed by extrinsic cues via cell–cell interaction between the progeny of a precursor cell. Alternatively, it may originate through the unequal segregation of intracellular determinants that direct distinct identities in each of the daughter cells. Although these represent extreme situations, in many instances both processes are required in the same division.

Asymmetric divisions of NBs and GMCs play crucial roles in specifying neural cell types and neuronal/glial identities, respectively (review: Fuerstenberg *et al.*, 1998). NB division is asymmetric in both size and identity of daughter cells (review: Doe *et al.*, 1998). Typically, NBs divide perpendicularly to the neuroepithelial layer and produce a GMC, from the basal side of the parent NB. While NBs undergo this type of asymmetric division repeatedly, GMCs divide only once along the same axis as the NB and produce two distinct cells that do not divide any more (review: Fuerstenberg *et al.*, 1998). NB and GMC divisions are characterized by stereotyped localization of fate determinants.

1.10.4.2. Role of Prospero/Numb in the Induction of the GMC Fate

The asymmetric divisions in *Drosophila* neural precursors have begun to be elucidated by the discovery of the asymmetric segregation of two key proteins, Prospero (Pros) and Numb, which are synthesized in NBs and asymmetrically segregate to the GMC (Doe *et al.*, 1991; Rhyu *et al.*, 1994; Knoblich *et al.*, 1995).

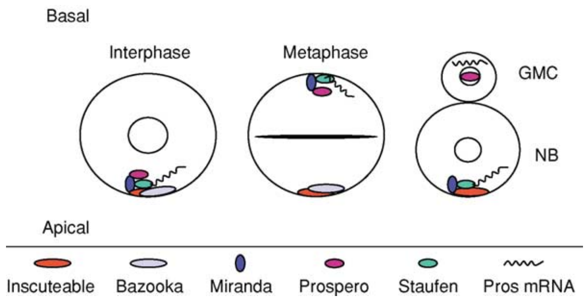


Figure 13 Distribution of cell fate determinants during the NB mitotic cycle. At late interphase, Miranda, Prospero (protein and mRNA), and Staufen are concentrated at the apical cortex and colocalize with Inscuteable and Bazooka. At metaphase, Miranda, Prospero (protein/mRNA), and Staufen move to the basal cortex whereas Inscuteable and Bazooka remain at the apical cortex. During telophase, most members of the basal complex are incorporated into the newly forming GMC. Then, Prospero translocates into the nucleus to activate GMC specific genes. Prospero and all the members of the basal complex (except Bazooka) are synthesized *de novo* in the precursor as the new mitotic cycle begins.

The *prospero* (*pros*) gene encodes a transcription factor that is expressed in all embryonic and larval NBs (Vaessin *et al.*, 1991). It is localized at the NB cortex in a highly cell cycle-dependent fashion (Hirata *et al.*, 1995; Knoblich *et al.*, 1995; Spana and Doe, 1995). At late interphase, Pros resides at the apical NB cortex in a diffuse crescent (**Figure 13**). As the NB enters mitosis, Pros is transported to the opposite side where it forms a tight crescent centered over the basal centrosome (**Figure 13**). As the GMC buds from the NB during anaphase, Pros is tightly associated with the basal cell cortex and ultimately segregates into the GMC, where it translocates into the nucleus (**Figure 13**). Nuclear Pros activates GMC-specific gene expression and represses NB-specific gene expression (Doe *et al.*, 1991; Hirata *et al.*, 1995; Knoblich *et al.*, 1995) (**Figure 13**). The complex regulation of subcellular Pros localization in the NB has two consequences: on one hand, it makes it possible to create progeny with distinct identity from a multipotent precursor and, on the other hand, it keeps the fate determinant inactive in this precursor.

It has been shown that *pros* mRNA is also asymmetrically localized in the NB in a cell cycle-dependent manner, and segregates to the GMC following mitosis (Li *et al.*, 1997; Broadus *et al.*, 1998) (**Figure 13**). In embryos lacking the RNA binding protein Staufen (Stau), the majority of NBs fail to localize *pros* mRNA (but not protein) apically at interphase or basally at mitosis, indicating that Stau controls *pros* mRNA localization independently of the Pros protein (Li *et al.*, 1997; Broadus *et al.*,

1998). In *staufen* (*stau*) mutants, the GMC fate is not affected, indicating that segregation of *pros* RNA is not sufficient to trigger GMC identity (Broadus *et al.*, 1998). However, loss of localization of *pros* mRNA or Stau alters GMC development in embryos that contain reduced levels of Pros protein, suggesting that *pros* mRNA and protein localization act redundantly to specify the GMC identity (Broadus *et al.*, 1998). This double control at the *pros* mRNA and protein levels reflects the requirement for fine-tuning in the building up the nervous system.

The *numb* gene encodes a membrane-associated protein (Uemura *et al.*, 1989) that shows the same cell cycle-dependent basal localization as seen for Pros (Rhyu *et al.*, 1994). Numb localizes normally in *pros* mutants, and Pros segregates normally in *numb* mutant embryos, indicating that the two proteins are independently localized (Knoblich *et al.*, 1995; Spana and Doe, 1995). Asymmetric localization of Numb initially requires the Partner of Numb (Pon) protein, which directly binds and colocalizes with Numb in dividing neural precursor cells (Lu *et al.*, 1998). However, Numb appears to be still attached to the cell cortex in the absence of Pon, indicating that other components likely play a role in Numb localization (Lu *et al.*, 1998). It has been recently shown that *jumeaux* (*jumu*), a winged-helix transcription factor encoding gene, is necessary to correctly localize and segregate the Pon–Numb complex (Cheah *et al.*, 2000), suggesting that unknown target genes activated by Jumeaux play a crucial role in this process.

It is unclear why Numb needs to be asymmetrically distributed during NB division, since there is no reported function of *numb* in the GMC. Nevertheless, Numb does play an important role in the specification of sibling cell identity (reviews: Buescher *et al.*, 1998; Cayouette and Raff, 2002). In most cases, the two daughter cells, A and B, produced after each GMC division are distinct. It has been shown that Numb antagonizes Notch signaling, probably by direct interaction with the Notch intracellular domain (NICD) (Spana and Doe, 1995; Guo *et al.*, 1996). *Notch* loss-of-function mutations lead to the production of two similar daughter cells (A/A) whereas *numb* mutations lead to a reciprocal phenotype (B/B) (Buescher *et al.*, 1998; Lear *et al.*, 1999). Thus, asymmetric segregation of Numb biases Notch signaling between daughter cells to create distinct cell identities. This underlines the role of both autonomous and nonautonomous pathways in the establishment of cell diversity and the importance of crosstalk between these molecular pathways in asymmetric divisions.

1.10.4.3. Genes Involved in Asymmetric Cell Division

The establishment of different identities via asymmetric division requires the integration of two processes: segregation of cell fate determinants and correct orientation of the mitotic spindle, i.e., orientation parallel to the polar distribution of these determinants.

Inscuteable protein (Insc) is necessary for the coordination of orientation of the mitotic spindle with protein localization (Kraut and Campos-Ortega, 1996; Kraut *et al.*, 1996; Tio *et al.*, 1999). Insc is localized at the apical cortex of the NB and is first detected in the apical endfoot that remains associated with the epithelial surface in the delaminating NB (**Figure 13**). The apical crescent of Insc persists in the NB through metaphase. After metaphase, it appears to be delocalized or degraded (Kraut *et al.*, 1996) (**Figure 13**). In *inscuteable* (*insc*) mutant embryos, the orientation of the mitotic spindle is random with respect to the apicobasal axis (Kraut and Campos-Ortega, 1996). Moreover, Pros and Numb are either homogeneously localized at the cortex or localized in crescents whose positions are randomized with respect to the surrounding tissue during mitosis (Kraut *et al.*, 1996). Insc appears to independently regulate both spindle orientation and basal localization of Pros and Numb during mitosis. Once Insc localization is established in the NB, all known molecular and cellular asymmetries assayed to date are positioned with respect to Insc.

1.10.4.3.1. How is Insc localization regulated? Previous work has identified Bazooka (Baz), a PSO95/DLG/ZO-1 (PDZ) domain containing protein that binds Insc (Kraut and Campos-Ortega, 1996; Ikeshima-Kataoka *et al.*, 1997; Shen *et al.*, 1997; Kuchinke *et al.*, 1998) (**Figure 13**). Removal of both maternal and zygotic Bazooka products results in the uniform distribution of Insc in the NB cytoplasm and causes defects in the distribution of cell fate determinants and in spindle orientation similar to those seen in *Insc* mutant embryos (Schober *et al.*, 1999; Wodarz *et al.*, 1999). Indeed, when NBs delaminate from the epithelium, Bazooka colocalizes with Inscuteable and recruits it to the apical cortex in NBs.

The *bazooka* (*baz*) gene has been shown to be required for the polarity of epithelial cells, in which its protein is localized at the apical cortex (Muller and Wieschaus, 1996). This supports the idea that NBs inherit the apicobasal polarity from the epithelium. Two proteins, Par-6 and atypical protein kinase C (aPKC), colocalize with Bazooka

and form a complex at the apical cortex of NBs. The *par-6* gene encodes a PDZ containing protein (Petronczki and Knoblich, 2001) and *aPKC* encodes a serine/threonine kinase (Wodarz *et al*., 2000). In *par-6* or *aPKC* mutant embryos, defects are seen in the apical localization of Bazooka and Insc in NBs, as well as in the orientation of the spindle (Wodarz *et al*., 2000; Petronczki and Knoblich, 2001). These results indicate that a functional complex between Bazooka, Par-6, and aPKC plays a role in polarizing NBs to localize Inscuteable properly following delamination.

Strikingly, *insc* mutations show a relatively low penetrant phenotype (Kraut and Campos-Ortega, 1996; Kraut *et al*., 1996). Since no Insc homologs exist in flies, it is likely that additional proteins are necessary to coordinate mitotic spindle organization and fate determinant localization. Using cell specific markers, it will be interesting to determine whether Insc affects specific NBs or whether it affects all NBs but is only required in some divisions within the neuroblast lineage (early versus late, for example).

Recently, it has been shown that asymmetrical localization of Insc requires the presence of Partner of Inscuteable (Pins), a protein with multiple repeats of the tetratricopeptide motif as well as motifs implicated in binding of Gα proteins (Schaefer *et al*., 2000; Yu *et al*., 2000). Pins colocalizes with Insc at the apical cell cortex in interphase and mitotic neuroblasts, and loss of Pins results in the same phenotype as seen in *insc* mutant embryos (Bellaiche *et al*., 2001). Moreover, the G protein α subunit Gαi binds and localizes Pins at the cortex, and the effects of loss of Gαi or Pins are very similar, supporting the idea that Pins/Gαi act together to mediate neuroblast asymmetric division (Schaefer *et al*., 2000). In general, the three subunits that compose the G protein (α, β, and γ) localize together at the proximity of seven transmembrane domain receptors also known as G protein-coupled receptors (GPCRs). Signal transduction signaling involves GPCR activation and dissociation of the three subunits, which triggers an intracellular molecular cascade (Offermanns, 2003) (see **Chapter 5.5**). Interestingly, binding of Pins to Gαi can cause the release of the Gβ subunit from Gαi, suggesting that Gαi may function during asymmetric cell division via a receptor-independent mechanism (review: Knust, 2001). This suggests that an intracellular process may regulate G protein activation.

These data demonstrate that Pins and Insc are dependent on each other for asymmetric localization, and suggest that a receptor-independent G protein signaling is required to mediate and coordinate basal protein localization with mitotic spindle orientation (reviews: Schaefer *et al*., 2000; Knust, 2001; Schaefer *et al*., 2001).

1.10.4.3.2. How is Pros localization regulated? Starting from the identification of Pros partners, it has been possible to dissect the molecular cascade triggering cell diversity in the nervous system. Miranda (Mira), a coiled-coil protein, was identified in a two-hybrid screen using the asymmetric localization domain of Pros (Ikeshima-Kataoka *et al*., 1997; Shen *et al*., 1997). *Miranda* mutations result in a failure to correctly localize Pros during or after NB division resulting in its nuclear localization in both NB and GMC (Ikeshima-Kataoka *et al*., 1997). The search for mutations that alter Mira localization in metaphase NBs lead to the identification of *lethal giant larvae* (*lgl*), *disc large* (*dlg*), and *scribble* (*sbb*), three tumor-suppressor encoding genes (Albertson and Doe, 2003). In embryos mutant for any of these genes, Mira is mislocalized (Albertson and Doe, 2003). Surprisingly, Insc localization and spindle orientation are correct, indicating that these proteins act specifically to polarize Mira by a pathway that does not involve Insc (Albertson and Doe, 2003). Therefore, although Mira is required for Pros localization to the cortex, it has no role in the establishment of an asymmetric division (**Figure 13**).

In conclusion, the asymmetric division of NBs requires multiple pathways that regulate independently the different aspects of subcellular localization of cell fate determinants.

1.10.5. Glial Differentiation in the Fly CNS

Embryonic *Drosophila* glial cells can be classified into three major categories depending on position, morphology and gene expression: surface-associated, cortex-associated, and neuropil-associated glia (Ito *et al*., 1995; Hartenstein *et al*., 1998). Nevertheless, considering the origin and molecular details of the developmental program, CNS glial cells can be subdivided into two populations: lateral glia (LG) and midline glia (MG) (reviews: Giangrande, 1996; Granderath and Klambt, 1999; Jacobs, 2000; Van De Bor and Giangrande, 2002). LG originate from the neuroectoderm and are located throughout the CNS, whereas MG originate from the mesectoderm and are located at the midline. Some 60 LG and six MG are present in each neuromere. All LG differentiation is under the control of *glial cell deficient/glial cell missing* (*glide/gcm*) locus (review: Van De Bor and Giangrande, 2002), whereas MG differentiation requires *single-minded* (*sim*) (Crews

et al., 1992). In the absence of LG, MG development is normal and vice versa, showing that these two developmental programs are completely independent.

1.10.5.1. Midline Glia Differentiation

As seen before, MG determination is under the control of the Sim/Tgo heterodimer, which likely activates the expression of DER (Jacobs, 2000). DER is specifically expressed and required in MG cells, as shown by the finding that DER hypomorph embryos lack all MG at the end of embryogenesis (Zak *et al.*, 1990; Scholz *et al.*, 1997; Stemerdink and Jacobs, 1997). The main activating ligand for DER at the midline is Spitz (Spi) (Schweitzer *et al.*, 1995b), a fly ortholog of vertebrate transforming growth factor-α (TGF-α) (Rutledge *et al.*, 1992). Moreover, Rhomboid (Rho; a seven transmembrane protein) and Star (a transmembrane protein) are primary regulators of Spitz signaling (Sturtevant *et al.*, 1993; Guichard *et al.*, 1999). Indeed loss-of-function of *spitz, rho*, or *star* results in a decrease in MG number (Sonnenfeld and Jacobs, 1994; Lanoue and Jacobs, 1999; Pickup and Banerjee, 1999). Rho and Star are both expressed throughout the mesectoderm before being restricted to MG. Star is required for the relocalization of Spi from the endoplasmic reticulum to the Golgi apparatus (**Figure 14**). There, Rho cleaves Spi in its intramembrane domain, producing a soluble form of Spi that is secreted and activates DER (Lee *et al.*, 2001; Urban *et al.*, 2001). Ligand binding to DER activates the Ras pathway leading to phosphorylation and thereby activation of the transcription factor Pointed P2 (PntP2) (Jacobs, 2000). In the absence of *pntP2*, embryos display a fused commissures phenotype, which is characteristic of MG differentiation defects (see below) (Klambt, 1993). This indicates that PntP2 is required for MG development.

During embryonic development, some of the MG undergo programmed cell death (Sonnenfeld and Jacobs, 1995; **Chapter 2.5**). This process is controlled by *argos*, a direct target of PntP2 that is expressed in a subpopulation of MG (Scholz *et al.*, 1997). The *argos* gene encodes a secreted protein acting as an antagonist of DER signaling by interfering with Spi-mediated dimerization of DER (Schweitzer *et al.*, 1995a; Jin *et al.*, 2000). Ubiquitous expression of *argos* results in the shutdown of DER expression (Schweitzer *et al.*, 1995a), suggesting that *argos*-expressing cells repress expression of DER in other MG, which eventually die. However, the way in which cells that secrete Argos are protected from an autocrine repression of DER

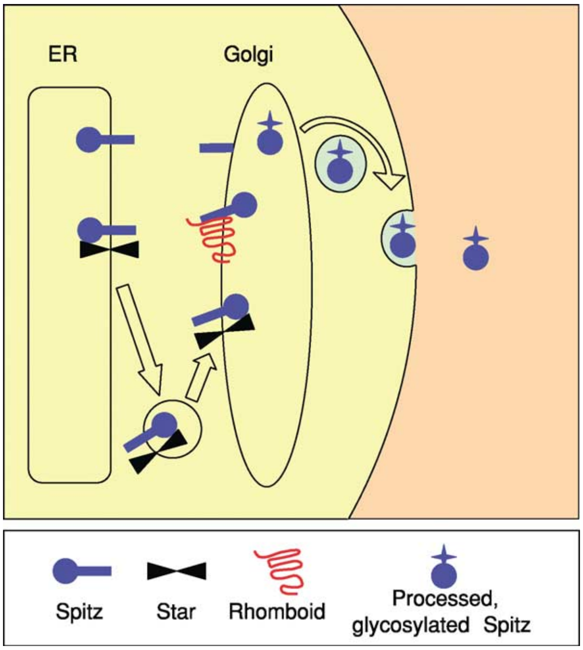


Figure 14 Mechanism of Spitz processing. Spitz (blue) is retained in the endoplasmic reticulum (ER) until Star (black) promotes its relocalization into the Golgi apparatus. There, Rhomboid (red) cleaves the Spitz protein, which is subsequently glycosylated (blue circle with spine) and secreted. (Modified from Lee, J.R., Urban, S., Garvey, C.F., Freeman, M. **2001**. Regulated intracellular ligand transport and proteolysis control egf signal activation in *Drosophila*. *Cell 107*, 161–171.)

signaling is poorly understood. The existence of a programmed cell death pathway in the midline population clearly indicates that the acquisition of the proper number of MG involves and requires the balance between two developmental pathways, cell differentiation and death.

1.10.5.2. Lateral Glia Differentiation

1.10.5.2.1. The *glide/gcm* glial promoting factor As described above, LG arise from pure (glioblasts) and mixed precursors (neuroglioblasts), all expressing and depending for function on a single locus, irrespective of precursor type (review: Van De Bor and Giangrande, 2002). This locus contains the *glide/gcm* and *glide2* genes, which encode related transcription factors of a novel type (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002) (**Figure 15a**). The *gcm* DNA-binding motif is conserved throughout evolution and provides a signature for the *gcm* gene family (Akiyama *et al.*, 1996; Schreiber *et al.*, 1997; Miller *et al.*, 1998; Kammerer and Giangrande, 2001). In embryos lacking *glide/gcm*, most LG are absent (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996). Moreover, ectopic expression of *glide/gcm* is sufficient to

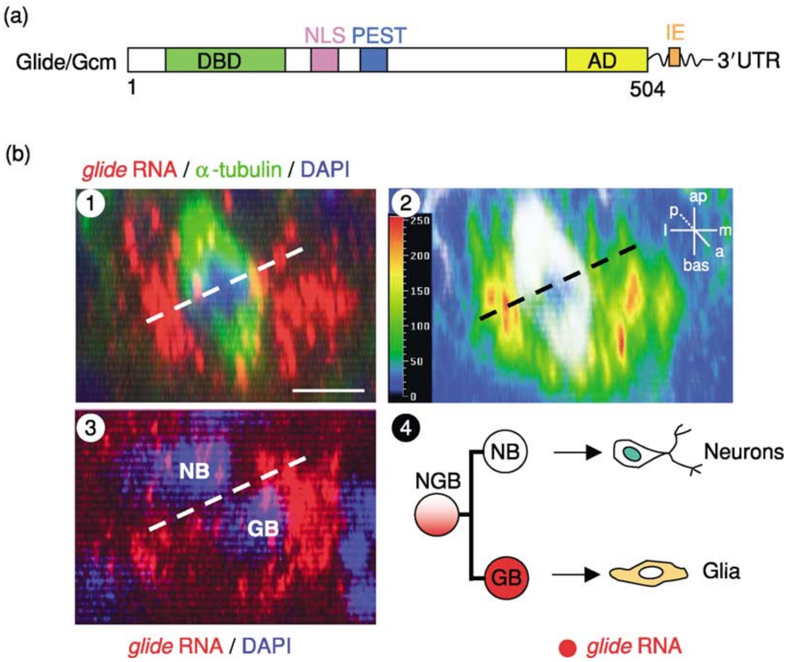


Figure 15 The role of *glide/gcm-glide2* in the determination of the glial fate. (a) Predicted Glide/Gcm mRNA structure with the encoded protein domains shown in blocks. DBD, DNA-binding domain; NLS, nuclear localization signal; PEST, rapid turnover signature; AD, activation domain; IE, instability element; UTR, untranslated region. (b) The NGB 6-4T lineage: α-tubulin labels the mitotic spindle 4′-6-diamidino-2-phenylindole (DAPI) the chromatin. (1, 2) Early NGB 6-4T anaphase: *glide* RNA is asymmetrically localized at the basoanterior pole of the cell. Dotted line is located in the middle of chromatin labeling. Scale bar = 4 μm. In (2), α-tubulin labeling (white) is superimposed to the quantification data relative to *glide* RNA. Color coding at left represents different levels of RNA accumulation: the lowest (0) is represented in blue, the highest (250) in red. ap, apical; bas, basal; a, anterior; p, posterior; l, lateral; m, medial. (3) The NGB 6-4T daughter cells, NB and GB, soon after division. Note that *glide* RNA is preferentially inherited by the GB. NB, neuroblast; GB, glioblast. (4) Schematic of *glide* mode of action during NGB 6-4T division. NGB, neuroglioblast. (Modified from Van De Bor, V., Giangrande, A. **2002**. *glide/gcm*: at the crossroads between neurons and glia. *Curr. Opin. Genet. Devel. 12*, 465–472.)

promote the glial fate within and outside the nervous system (Jones *et al*., 1995; Vincent *et al*., 1996; Akiyama-Oda *et al*., 1998; Bernardoni *et al*., 1998). Expression of *glide2* is also sufficient to promote the glial fate, but its absence has no effect on glia determination, suggesting that it plays a minor role during gliogenesis (Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). In the absence of Glide/Gcm-Glide2, glial cells are absent because they are all transformed into neurons. This suggests that the *glide/gcm* locus activates the glial program and represses the neuronal one (Hosoya *et al*., 1995; Jones *et al*., 1995; Vincent *et al*., 1996; Giesen *et al*., 1997; Bernardoni *et al*., 1998, 1999; Miller *et al*., 1999; Van De Bor *et al*., 2000; Kammerer and Giangrande, 2001). Thus, these genes trigger the glial cell fate by controlling the fate choice between glia and neurons in multipotent precursors (reviews: Wegner and Riethmacher, 2001; Van De Bor and Giangrande, 2002) (**Figure 15b**).

The role of *glide/gcm* has been extensively investigated in two lineages, the 6-4T and the 6-4A, examples of type 1 NGB and pure GB, respectively (Akiyama-Oda *et al*., 1999; Bernardoni *et al*., 1999; Ragone *et al*., 2001). During division of the 6-4A, *glide/gcm* mRNA is equally distributed in the two daughter cells leading them towards a glial fate. In multipotent precursors, the fate choice relies on a *glide/gcm* posttranscriptional control. The *glide/gcm* RNA forms a cytoplasmic gradient in the NGB, which leads to one daughter cell inheriting more *glide/gcm* transcripts than the other one (**Figures 7 and 15**). This cell (the GB) produces glia, whereas the other (the NB) produces neurons. Overexpression of *glide/gcm* in both 6-4T progeny cells results in expression of glial markers in both of them. The observation that daughters inheriting different amounts of the same cell fate determinant end up adopting distinct fates demonstrates the role of quantitative regulation in the establishment of cell diversity upon asymmetric division. This is further confirmed by the fact that *glide/gcm* is able to positively autoregulate its own expression (Miller *et al*., 1998). Although the NGB provides another example of asymmetric

division, the behavior of *glide/gcm* transcripts suggests that novel molecular mechanisms are necessary for the induction of the glial fate.

1.10.5.2.2. The *glide/gcm* targets execute the glial differentiation program Once *glide/gcm* has been activated, the glial differentiation program takes place. Because *glide/gcm* expression is transient, terminal glial differentiation is likely under the control of genes acting downstream of it. Some targets, such as *reverse polarity* (*repo*), *pointed P1* (*pntP1*), and *locomotion defects* (*loco-c1*), have been shown to execute the glial differentiation program, whereas others, e.g., *tramtrack69* (*ttk69*), inhibit the neuronal pathway. Other identified target genes are likely involved in the late differentiation steps of specific glial subtypes (Egger *et al.*, 2002; Freeman *et al.*, 2003).

The first identified target of *glide/gcm* is *reverse polarity* (*repo*), which encodes a paired-like homeodomain transcription factor expressed in all LG but not in MG (Campbell *et al.*, 1994; Xiong *et al.*, 1994; Halter *et al.*, 1995). Its promoter contains 11 Glide/Gcm consensus binding sequences, and Glide/Gcm is necessary and sufficient to induce *repo* expression *in vivo*, suggesting that *repo* represents a direct target of Glide/Gcm (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Akiyama *et al.*, 1996; Vincent *et al.*, 1996; Bernardoni *et al.*, 1998). In *repo* mutant embryos, early gliogenesis occurs normally but late glial markers are not expressed, suggesting that Repo is required for terminal glia differentiation (Campbell *et al.*, 1994; Xiong *et al.*, 1994; Halter *et al.*, 1995; Yuasa *et al.*, 2003).

The *pointed* gene encodes two transcription factor isoforms containing an ETS DNA binding domain (Klambt, 1993; Klaes *et al.*, 1994). The ETS domain is a DNA binding domain found in a family of proteins to the vertebrate *ets* oncogene. While Pointed P2 (PntP2) is only present in MG (see above), Pointed P1 (PntP1) is specifically expressed in longitudinal glia (Klambt, 1993). In the absence of PntP1, longitudinal glia fail to develop normally leading to defects in connective formation (Klaes *et al.*, 1994). Thus, PntP1 is involved in terminal differentiation of longitudinal glia (Klaes *et al.*, 1994; Granderath *et al.*, 2000).

Interestingly, ectopic expression of either Repo or PntP1 leads to the activation of some glial-specific markers in few cells, whereas combined ectopic expression has a synergistic effect (Yuasa *et al.*, 2003). Repo and PntP1 do not affect each other's expression, and this suggests that their genes are independent targets of Glide/Gcm that participate in glial specification, i.e., the differentiation of a specific type of glial cell (Yuasa *et al.*, 2003).

As mentioned above, gliogenesis needs both activation of glial-specific genes and repression of neuronal-genes. The *tramtrack* (*ttk*) gene encodes a transcription factor of the zinc finger family (Harrison and Travers, 1990). One of the two Ttk isoforms, Ttk69, constitutes the only protein expressed in all glial cells (LG and MG) and participates in neural repression (Giesen *et al.*, 1997; Badenhorst, 2001). Ectopic expression experiments and analysis of its expression profile suggest that Ttk69 acts by repressing the neuronal fate rather than the early steps of neurogenesis (Badenhorst *et al.*, 1996). This is also confirmed by the observation that Ttk69 does not repress the expression of proneural genes (Badenhorst, 2001). In this process, Ttk69 seems to cooperate with Repo, although the molecular nature of this interaction is not yet understood (Yuasa *et al.*, 2003). Ttk69 also plays a role in glial proliferation, as *ttk* loss-of-function mutants display an increase in LG number, while gain-of-function mutants show the opposite phenotype (Badenhorst, 2001).

Taken together, these results suggest that lateral gliogenesis is dependent on a single locus (*glide/gcm–glide2*), which triggers the different pathways executing glial cell differentiation. Glial specification depends on cell-specific factors that allocate distinct identities to the different glial lineages. Such factors act at early stages, by controlling the spatially and temporally regulated transcription of the *glide/gcm* locus, but are also activated at later stages by interacting with the *repo* and *pntP1* genes (Klaes *et al.*, 1994; Ragone *et al.*, 2003; Yuasa *et al.*, 2003). These factors likely include those that dictate positional cues in the neural precursors, since they are expressed throughout the neural lineage up to postmitotic cells.

Thus, *Drosophila* represents an ideal model for achieving an in-depth understanding of the molecular pathways controlling glia differentiation. Moreover, elucidating the *glide/gcm* mode of action during neuron–glia fate decision process will afford a better understanding of the mechanisms that control multipotent stem cell decisions during development.

1.10.6. Neuronal–Glial Cell Interactions Control the Development of the Nervous System

Neurons and glial cells constitute the major components of the nervous system. Although they play distinct roles, their continuous interactions are a gage of appropriate development and function of such a complex tissue. Neuron–glia interactions

play important instructive roles during cell differentiation, migration, and axonal navigation, as well as in the regulation of cell survival (review: Laming *et al.*, 2000; see also **Chapter 2.3**).

Most glia arise from precursors that also produce neurons, the control between the two fates depending on the cell-autonomous cue Glide/Gcm (see Section 1.10.5.2.2). Upstream of this choice, however, a nonautonomous pathway must be activated, at least in some precursors. In the CNS as well as in the PNS, the Notch signaling cascade controls the expression of *glide/gcm* and thereby the activation of the glial fate (Udolph *et al.*, 2001; Van De Bor and Giangrande, 2001; Umesono *et al.*, 2002). The requirement of this pathway, which is conserved throughout evolution (review: Artavanis-Tsakonas *et al.*, 1999), clearly shows that both intrinsic and extrinsic cues act on the same fate decision and participate the establishment of cell diversity.

After neuronal differentiation, polarized extensions grow toward specific targets through the use of distinct cellular and molecular cues. The fly midline represents a classical model for the study of neuron–glia interactions controlling axonal guidance (reviews: Tear, 1999; Kaprielian *et al.*, 2001). Bilaterally symmetric animals are able to transmit information between the left and right sides of their body in order to integrate sensory input and coordinate motor output. Thus, many neurons in the CNS project so-called commissural axons across the midline. Interestingly, these axons are never observed to recross the midline (**Chapter 2.3**). However, some neurons project axons that remain on the ipsilateral side of the CNS, without ever crossing the midline (reviews: Tear, 1999; Kaprielian *et al.*, 2001; **Chapter 2.3**). Many, if not most, of the fly navigational cues are provided by midline glia and their precursors (review: Lemke, 2001). Thus, Netrin (Net) chemoattractants, which are expressed in midline glia, attract axons, which express the Netrin receptor Frazzled (Fra) and allow them to cross the midline (review: Livesey, 1999). Disruption of the *netrin* (*net*) or *frazzled* (*fra*) gene leads to a reduction in the number of commissural axons tracts (Harris *et al.*, 1996; Kolodziej *et al.*, 1996; Mitchell *et al.*, 1996). However, neither mutation leads to a complete loss of all commissural axon connections (Kolodziej *et al.*, 1996; Hummel *et al.*, 1999a, 1999b), suggesting that other genes are also involved in this process. It was shown that two other mutations, *schizo* (*siz*) and *weniger* (*weg*), induce a reduced commissure phenotype (Hummel *et al.*, 1999a, 1999b). *Schizo* and *weg* mutant embryos show a more severe phenotype than that observed in *fra* embryos; however, commissural axons are still not affected. Genetic analyses indicate that the *net/fra* and *siz/weg* genes act in parallel pathways (Hummel *et al.*, 1999a, 1999b). Therefore, *siz* and *weg* encode new, yet undefined, components guiding commissural growth cones towards the midline (Hummel *et al.*, 1999a, 1999b). The molecular characterization of these genes will likely identify new molecules, which also play a role in axon guidance during vertebrate CNS development.

While Netrins are essential for at least some commissural axons to grow across the midline, the choice of a contralateral versus ipsilateral pathway is dictated by the extracellular matrix protein Slit (Sli), which is secreted by midline glial cells and found associated with the surface of axons (Rothberg *et al.*, 1988, 1990). Sli repels axons that express the Roundabout (Robo) proteins (Kidd *et al.*, 1999). Repulsion prevents ipsilaterally projecting axons from entering the midline and contralaterally projecting axons from re-entering the midline once they have crossed it (Kidd *et al.*, 1998c). Three *robo* genes, (*robo1, 2, 3*) have been identified in *Drosophila*, all encoding proteins belonging to the IG-CAM family (Seeger *et al.*, 1993; Kidd *et al.*, 1998c; Rajagopalan *et al.*, 2000; Simpson *et al.*, 2000). Loss of the three proteins mimics the commissure-collapsed phenotype observed in *slit* mutant embryos (Rajagopalan *et al.*, 2000). Axons extending toward or across the midline express very low levels of Robo proteins; in contrast, crossed segments of commissural axons as well as ipsilaterally projecting axons express high levels of Robo proteins (**Figure 16**). This suggests that Robo proteins normally prevent midline crossing and function as receptors for the Slit inhibitory ligand, which is localized at the midline (Brose *et al.*, 1999; Kidd *et al.*, 1999).

The transmembrane protein Commissureless (Comm) is normally required for axons to cross the midline, and behaves as a powerful negative regulator of Robo, as was evidenced by its mutant phenotype (Seeger *et al.*, 1993; Tear *et al.*, 1996; Kidd *et al.*, 1998c) (**Figure 16**). Tissue culture experiments have shown that coexpression of Comm and Robo alters Robo's subcellular localization (Kidd *et al.*, 1998b). In the absence of Comm, Robo protein accumulates at the cell surface; however, when both proteins are present, Robo colocalizes with Comm to intracellular compartments, which are probably late endosomes (**Figure 16**). Moreover, Comm needs to interact with Robo to affect Robo's localization (Keleman *et al.*, 2002). These findings suggest that Comm prevents Robo from reaching the cell surface by binding and targeting it directly to endosomes. One model proposes that this depends on Comm ubiquitination by the DNedd4 ubiquitin

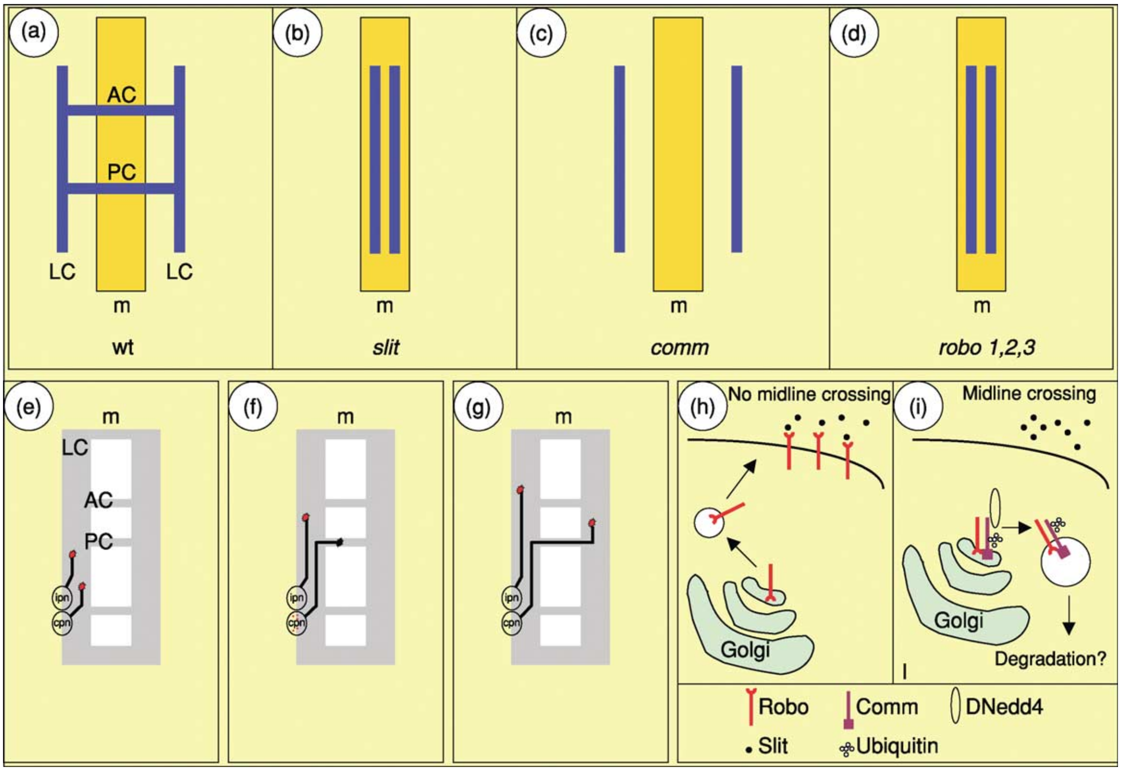


Figure 16 The role of the *slit–robo–comm* pathway in axon guidance at the fly midline. (a) In wild-type embryos, the ventral cord displays two longitudinal connectives (LC) running parallel on each side of the midline (m), an asymmetric structure that is crossed by the anterior and the posterior commissures (AC and PC, respectively). (b) In *slit* mutant embryos, all axons enter the midline but never leave it, resulting in a collapsed commissure phenotype. (c) An opposite phenotype is seen in *comm* mutant embryos in which axons never cross the midline. (d) Gene *robo1, 2, 3* triple mutant embryos show the same phenotype as that observed in *slit* embryos, i.e., collapsed commissures. (e) Initially, both growth cones of ipsilateral (ipn) and contralateral (cpn) axons express Robo (in red). (f) Comm is specifically expressed in the cpn while they are crossing the midline. This results in a loss of Robo at the cpn growth cone membrane. (g) Once cpn axons have crossed the midline, Comm expression is downregulated and Robo is present again at the cpn growth cone membrane. This allows repulsion by Slit, which prevents a new cross. The ipn axon never expresses Comm and never crosses the midline. (h) Neurons only expressing Robo (constitutively in ipn; before and after but not during midline crossing in cpn), accumulate it at the membrane. Axons can then respond to the Slit repellent signal and do not cross the midline. (i) Neurons expressing Comm and Robo do not accumulate Robo at the membrane and, as a consequence, are unable to respond to Slit and cross the midline. In these axons, Slit/Robo signaling is blocked by a mechanism involving ubiquitination of Comm by DNedd4. This ubiquitination step facilitates the sorting of Comm and Robo in late endosomes. Once the cpn has crossed the midline, Comm expression is downregulated and Robo is present again at the membrane.

ligase, which sequesters Robo away from the cell membrane, its site of action (Myat *et al*., 2002) (**Figure 16**). These studies clearly identify neuron–glia interactions as privileged cell–cell communication for the establishment of correct axonal trajectories. The fact that these cellular and molecular pathways are conserved throughout evolution (Keleman *et al*., 2002) emphasizes the importance of such interactions.

In the mature embryo, glial–neuronal interactions are pivotal for the homeostasis of the nervous system, and this is achieved through their influence on the control of glial and neuronal survival (Hidalgo *et al*., 2001; Bergmann *et al*., 2002). The importance of such interactions is highlighted by the finding that neurons send signals that control glial cell numbers, and glial cells send signals that control neuronal cell numbers. Two different members of the EGF family, Vein (Vn) and Spi, were shown to function as gliatrophins in *Drosophila*. Thus, it was demonstrated that the neuregulin homolog Vein maintains survival of a subpopulation of longitudinal glia (Hidalgo *et al*., 2001), while the TGF-α homolog Spi maintains survival of midline glia (Bergmann *et al*., 2002). In both cases, the trophic ligands are secreted by adjacent axons at concentrations that are sufficient for the survival of only a subset of the target glial population.

Conversely, mutations in genes specifically expressed in glia affect neuronal survival. The *repo* gene is expressed and required in all fly glial cells (Halter *et al*., 1995). In flies homozygous for a viable

allele of *repo*, neurodegeneration is observed in the eye, showing that alteration of glial cell function leads to neuronal death (Xiong and Montell, 1995). Similar results were described in other glia-affecting mutants like *drop-dead* (*drd*) or *swiss-cheese* (*sws*) (Buchanan and Benzer, 1993; Kretzschmar *et al.*, 1997). These two genes are expressed by glial cells and in mutant flies homozygous for each of these genes, glial cells differentiate abnormally leading to neuronal death. Finally, in *glide/gcm* embryos, where almost all glia are missing, neuronal apoptosis occurs during development (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Vincent *et al.*, 1996).

Once neurons and glia have differentiated, they move to reach their final destination within the CNS and the PNS (review: Hatten, 1999) (see **Chapter 2.3**). Indeed, cell migration is a hallmark of nervous system development and is responsible for the generation of the layered structure of the vertebrate CNS (reviews: Tsai and Miller, 2002; Honda *et al.*, 2003). The migratory process entails several steps: cells have to initiate migration, find the direction of movement, and stop migrating as they reach the final destination. The complex and timed process of migration has been mostly analyzed in glial cells of the PNS, which displays a simpler organization than the CNS (Sepp *et al.*, 2000; Tsai and Miller, 2002; Sepp and Auld, 2003). These studies have demonstrated that, in some tissues, glial cells use axons as a migratory substratum (wing-associated glia) whereas in others they do not (eye-associated glia) (Giangrande *et al.*, 1993; Choi and Benzer, 1994; Giangrande, 1994; Rangarajan *et al.*, 1999; Van De Bor *et al.*, 2000). The different glial migratory behaviors observed so far underscore the diversity of developmental strategies used in time and space to make cells reach their final destination.

The study of a dynamic process such as migration has often been limited by two factors. On one hand, most commonly used techniques for labeling cell types rely on the fixation of samples (Ono *et al.*, 1997; Affolter and Shilo, 2000; Sugimoto *et al.*, 2001); on the other hand, real-time analyses have been realized in cell culture systems and/or sliced tissues, due to the nervous system complexity. The recent development of green fluorescent protein (GFP)-based tools has opened new perspectives for the analysis of dynamic processes in whole animals and in real time (review: Yu *et al.*, 2003). This non-invasive approach will certainly provide new and important information on the role of neuron–glia interactions in the control of glial cell migration.

Although neurons and glia have very distinct roles, these data clearly demonstrate that the continuous interaction between these two cell types plays a fundamental role in neural development, and that neurons or glial cells must be considered as a whole, in order to understand their differentiation and role.

1.10.7. Evolutionary Aspects of Insect CNS Development

Much of what was initially learned on embryonic nervous system development comes from studies on large insects. Indeed, neural precursors were first identified in grasshopper embryos (Wheeler, 1891), in which it is possible to predict the identity of individual neural precursor with 100% certainty solely on the basis of its stereotyped size, morphology, and position in the neurogenic region (Raper *et al.*, 1984; Taghert and Goodman, 1984; Doe and Goodman, 1985a). By comparison, the early and most of the late-forming neural precursors can be unequivocally identified in *Drosophila* by their morphology, while the use of molecular markers is necessary for the identification of the others (Doe, 1992).

Morphological analysis of neural precursor lineages shows that subtle differences exist between *Drosophila* and the grasshopper. In *Drosophila*, the ectodermal cells adjacent to the neural precursors extend processes around the precursor, which are withdrawn soon after the latter begin to divide. In the grasshopper, these cells differentiate into “sheath cells,” which maintain processes that wrap around the GMCs and the neurons/glial cells produced from a single neural precuror (Doe and Goodman, 1985a) (**Figure 17**). As a consequence, the progeny of individual *Drosophila* neural precursor are not “packaged” together in a line, instead GMCs and neurons from adjacent neural precursors intermingle in the CNS (Doe, 1992). In addition, the grasshopper has one cell type that is not present in *Drosophila*, the cap cell, which is attached to the ventral surface of every neural precursor and is required for its asymmetric divisions (Kawamura and Carlson, 1962) (**Figure 17**).

1.10.7.1. Neural Precursor Identity and Positional Cues

With respect to position and timing of formation, pattern of gene expression and cell lineage, the development of neural precursors in *Drosophila* is similar to that observed in the larger grasshopper embryo (Bate, 1976; Doe and Goodman, 1985a; Doe, 1992). Because of this, neural precursor names in *Drosophila* have been chosen in order to indicate functional homology with those of the grasshopper (Goodman *et al.*, 1984; Doe, 1992; Jia *et al.*, 2002). However, this does not imply that each *Drosophila* neural precursor has a grasshopper

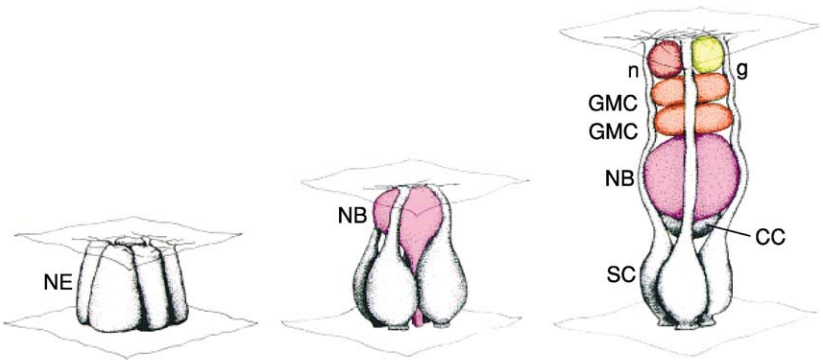


Figure 17 *Camera lucida* illustration of the delamination and the differentiation process of a neural precursor in the grasshopper. One neural precursor or neuroblast (NB) (pink) forms from a cluster of neuroectodermal cells (NE); the neuroblast enlarges and delaminates toward the interior of the embryo. Successive asymmetric divisions of the neural precursor give rise to ganglion mother cells (GMCs, orange), which divide once to produce sibling neurons or a neuron (n) and a glial cell (g) (red and yellow, respectively). In the grasshopper, sheath cells (SC) enwrap the progeny of each neural precursor. The grasshopper has one cell type not present in *Drosophila*, the cap cell (CC), which is attached to the ventral surface of every neural precursor. Ventral is down. (Modified from Doe, C.Q., Fuerstenberg, S., Peng, C.Y., **1998**. Neural stem cells: from fly to vertebrates. *J. Neurobiol. 36*, 111–127.)

equivalent. Both organisms have about 30 neural precursors per hemisegment, including a glial precursor that generates longitudinal glia and a MP2 precursor that produces the dMP2 and vMP2 neurons. Both in *Drosophila* and the grasshopper, neural precursors form in waves, with each new population of precursors arranged roughly in columns and rows (Doe and Goodman, 1985a; Doe, 1992).

Cell heterotopic transplantation experiments have shown that neural precursors are formed in the neuroectoderm before delamination (Prokop and Technau, 1994; Udolph *et al*., 1995), thus suggesting that positional information plays an essential role in neural precursor formation. Heterotopic transplantation between different Drosophilidae (*Drosophila melanogaster*, *D. virilis*, *D. hydei*, *D. pseudoobscura*, *D. latifasciaformis*, *D. buscii*) leads to results comparable to those obtained from intraspecific transplantations. Therefore, positional cues are conserved amongst these different species (Becker and Technau, 1990).

1.10.7.2. Neural Precursor Formation and Cell–Cell Interactions

The observation that the decision to adopt an epidermal or a neural fate is mediated by cell–cell interactions was first described in the grasshopper. Indeed, laser cell ablation experiments in this insect have shown that, while more cells than one in a specific region have the potential to form a neural precursor, the developing neural precursor inhibits its adjacent cells from developing as neural precursors (Taghert *et al*., 1984; Doe and Goodman, 1985a, 1985b). Thus, while under normal circumstances these adjacent cells develop as epidermoblasts, they adopt the neural fate when the neural precursor is killed. This demonstrates that the presumptive epidermoblasts are prevented by the neural precursor from adopting the neural fate. This process has been called lateral inhibition, and genetic studies in *Drosophila* have identified the Notch pathway as a major signaling process involved in cell fate choice (see Section 1.10.2.2.2).

1.10.7.2.1. Invariant neural precursor cell lineage The analysis of several identified grasshopper neural precursors has shown that each neural lineage is invariant and generates a specific set of neurons and/or glia (Bate and Grunewald, 1981; Goodman and Spitzer, 1979; Taghert *et al*., 1984), and this has allowed the establishment of a neural precursor map (Bate, 1976; Doe and Goodman, 1985a, 1985b). Subsequently, a similar map was also established in *Drosophila* (see Section 1.10.2.3). Some of the lineages in these two insects show similarities; thus, for example, both in the grasshopper and in *Drosophila*, the first GMC from NB1-1 always divides to produce the aCC and pCC motorneurons (Goodman and Spitzer, 1979; Udolph *et al*., 1993).

While embryonic thoracic and abdominal segments of hemimetabolous insects have more neural precursors present in the thoracic segments than in the abdominal ones (Bate, 1976; Doe and Goodman, 1985a; Booker and Truman, 1987), in the holometabolous *Drosophila* embryo, the neural precursor patterns in the thoracic and abdominal segments look very much the same (Doe, 1992). This implies that hemi- and holometabolous insects employ distinct mechanisms in order to produce segmental cell diversity: in the first case, cell diversity is triggered

by the production of segment-specific neural precursors, whereas in the second one, the difference in the number and/or the type of neuron and glial cell between thoracic and abdominal segments is a consequence of segment-specific neural precursor lineages. In both cases, these segmental differences are due to the action of homeotic genes that control differentiation of the neurogenic region in hemimetabolous insects and segment-specific lineage in holometabolous insects (see Section 1.10.3.1.1).

1.10.7.3. Conservation of Proneural Activity during Evolution

In general, the mechanisms involved in insect neurogenesis appear to be well conserved during evolution. How could one explain such conservation?

In *Drosophila*, the proneural genes, including the genes of the *Achaete–Scute Complex* (*ac*, *sc*, *l'sc* and *ase*), confer neural potential to cells and/or specify neuronal subtypes (Brunet and Ghysen, 1999; Chan and Jan, 1999). Because *AS-C* members are involved in the initiation of nervous system development in invertebrates and vertebrates (Section 1.10.2.2.1), the study of these genes has allowed the understanding of the evolution of development of the arthropod nervous system (Skaer *et al*., 2002).

Phylogenetic trees constructed by amino acid sequence comparison of the products encoded by the *achaete–scute* orthologs strongly suggest that the four *Drosophila* genes have arisen from three independent duplication events during insect evolution (Skaer *et al*., 2002). In contrast, *Tribolium castaneum* (Coleoptera) and *Anopheles gambiae* (an, "early" dipteran) contain a single proneural *ac-sc* like gene (Wulbeck and Simpson, 2002; Wheeler *et al*., 2003), whereas *Ceratitis*, a more recently evolved dipteran, contains two proneural *ac–sc* like genes (Wulbeck and Simpson, 2000). Thus, a duplication of an ancestral proneural *ac–sc* like gene has occurred within the dipteran lineage prior to the apperance of *Ceratitis*.

The *ase* gene is the only member that is expressed only in the neural precursors (Brand *et al*., 1993; Jarman *et al*., 1993). Within the complex, the *ase* orthologs form a group that is clearly separated from the other genes, suggesting that the duplication of an ancestral gene has given rise to *ase* and to a gene from which *l'sc*, *sc*, and *ac* evolved subsequently. Actually, it appears that the last common ancestor of arthropods contained a single prototypical *ac–sc* like gene that carried out both proneural and *ase* functions (Skaer *et al*., 2002). The duplication giving rise to *ac* and *sc* is the most recent event. Additional evidence for this is the observation that the *ac* and *sc* genes of *Drosophila* share *cis*-regulatory elements, are almost always coexpressed, and their products are functionally redundant (Rodriguez *et al*., 1990; Martinez and Modolell, 1991; Skeath and Caroll, 1991; Cubas and Modolell, 1992; Gomez-Skarmeta *et al*., 1995). After duplication, the new genes have acquired individual expression patterns but, in *Drosophila*, their products can compensate for one another, showing that a functional conservation has been maintained during evolution.

While *ac*, *sc*, and *l'sc* have the potential to induce neural precursor formation, only *ac* or *sc* can promote a correct gene expression profile and cell division pattern of the MP2 precursor (Parras *et al*., 1996; Skeath and Doe, 1996), suggesting that *ac* and *sc* control both the formation and invidual fate specification of neural precursors. Recently, it was shown that the single *ac–sc* like ortholog identified in the red flour beetle *T. castaneum* has conserved its proneural function, but is unable to allocate specific identity to neural precursors (Wheeler *et al*., 2003). This suggests that the ability of *ac* and *sc* to control specification of neural precursors represents a recent evolutionary specialization within Diptera.

1.10.8. Conclusion and Perspectives

The nervous system represents the most complex tissue in multicellular organisms. The sophisticated network of neurons and glia generated by neural stem cells allows organisms to respond and adapt to the numerous internal and external stimuli they encounter constantly during life. Characterizing the molecular and the cellular cues underlying the differentiation of the nervous system has a dual goal. First, it should allow us to understand how complex tissues are built during development and preserved during ontogenesis. Second, it should also have implications in the development of targeted therapeutic strategies for brain cancer and neurodegenerative diseases. The evolutionary conservation of the developmental strategies makes it likely that the use of a simple genetic model like *Drosophila* will help us to unravel the molecular and cellular basis of vertebrate neural development.

One of the most challenging issues for the next generation of drosophilists will be to follow the different steps that lead to nervous system differentiation *in vivo*, and to combine real-time observations with genetic dissection approaches. The ultimate goal will be to integrate the inter- and intracellular signaling pathways that coordinate

the establishment of the highly sophisticated neural network.

Acknowledgments

Work in our laboratory has been supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Hôspital Universitaire de Strasbourg, the Association pour la Recherche contre le Cancer, Ministère de la Recherche (ACI développement), European Leucodistrophy Association and by EU grant (contact QLG3-CT-2000-01224). L.S. was supported by an ARC fellowship.

References

Affolter, M., Shilo, B.Z., **2000**. Genetic control of branching morphogenesis during *Drosophila* tracheal development. *Curr. Opin. Cell. Biol. 12*, 731–735.

Akiyama, Y., Hosoya, T., Poole, A.M., Hotta, Y., **1996**. The gcm-motif: a novel DNA-binding motif conserved in *Drosophila* and mammals. *Proc. Natl Acad. Sci. USA 93*, 14912–14916.

Akiyama-Oda, Y., Hosoya, T., Hotta, Y., **1998**. Alteration of cell fate by ectopic expression of *Drosophila* glial cells missing in non-neural cells. *Devel. Genes Evol. 208*, 578–585.

Akiyama-Oda, Y., Hosoya, T., Hotta, Y., **1999**. Asymmetric cell division of thoracic neuroblast 6–4 to bifurcate glial and neuronal lineage in *Drosophila*. *Development 126*, 1967–1974.

Albertson, R., Doe, C.Q., **2003**. Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. *Nat. Cell Biol. 5*, 166–170.

Alfonso, T.B., Jones, B.W., **2002**. gcm2 promotes glial cell differentiation and is required with glial cells missing for macrophage development in *Drosophila*. *Devel. Biol. 248*, 369–383.

Alonso, M.C., Cabrera, C.V., **1988**. The achaete–scute gene complex of *Drosophila melanogaster* comprises four homologous genes. *EMBO J. 7*, 2585–2591.

Anderson, H., Edwards, J.S., Palka, J., **1980**. Developmental neurobiology of invertebrates. *Annu. Rev. Neurosci. 3*, 97–139.

Arendt, D., Nubler-Jung, K., **1999**. Comparison of early nerve cord development in insects and vertebrates. *Development 126*, 2309–2325.

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., **1999**. Notch signaling: cell fate control and signal integration in development. *Science 284*, 770–776.

Badenhorst, P., **2001**. Tramtrack controls glial number and identity in the *Drosophila* embryonic CNS. *Development 128*, 4093–4101.

Badenhorst, P., Harrison, S., Travers, A., **1996**. End of the line? Tramtrack and cell fate determination in *Drosophila*. *Genes Cells 1*, 707–716.

Bailey, A.M., Posakony, J.W., **1995**. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Devel. 9*, 2609–2622.

Baker, N.E., **1988**. Localization of transcripts from the wingless gene in whole *Drosophila* embryos. *Development 103*, 289–298.

Baron, M., Aslam, H., Flasza, M., Fostier, M., Higgs, J.E., *et al.*Multiple levels of Notch signal regulation (review). *Mol. Membr. Biol. 19*, 27–38.

Bate, C.M., **1976**. Embryogenesis of an insect nervous system. 1. A map of the thoracic and abdominal neuroblasts in *Locusta migratoria*. *J. Embryol. Exp. Morphol. 35*, 107–123.

Bate, C.M., Grunewald, E.B., **1981**. Embryogenesis of an insect nervous system. 2. A second class of neuron precursor cells and the origin of the intersegmental connectives. *J. Embryol. Exp. Morphol. 61*, 317–330.

Baumgartner, S., Bopp, D., Burri, M., Noll, M., **1987**. Structure of two genes at the *gooseberry* locus related to the paired gene and their spatial expression during *Drosophila* embryogenesis. *Genes Devel. 1*, 1247–1267.

Becker, T., Technau, G.M., **1990**. Single cell transplantation reveals interspecific cell communication in *Drosophila* chimeras. *Development 109*, 821–832.

Bellaiche, Y., Radovic, A., Woods, D.F., Hough, C.D., Parmentier, M.L., *et al.*, **2001**. The Partner of Inscuteable/Discs-large complex is required to establish planar polarity during asymmetric cell division in *Drosophila*. *Cell 106*, 355–366.

Bello, B.C., Hirth, F., Gould, A.P., **2003**. A pulse of the *Drosophila* Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron 37*, 209–219.

Bergmann, A., Tugentman, M., Shilo, B.Z., Steller, H., **2002**. Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling. *Devel. Cell 2*, 159–170.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., *et al.*, **1988**. The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J. 7*, 1749–1756.

Bernardoni, R., Kammerer, M., Vonesch, J.L., Giangrande, A., **1999**. Gliogenesis depends on glide/gcm through asymmetric division of neuroglioblasts. *Devel. Biol. 216*, 265–275.

Bernardoni, R., Miller, A.A., Giangrande, A., **1998**. Glial differentiation does not require a neural ground state. *Development 125*, 3189–3200.

Bertrand, N., Castro, D.S., Guillemot, F., **2002**. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci. 3*, 517–530.

Bhat, K.M., **1999**. Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. *BioEssays 21*, 472–485.

Biehs, B., Francois, V., Bier, E., **1996**. The *Drosophila* short gastrulation gene prevents Dpp from

autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Devel. 10*, 2922–2934.

Bier, E., **1997**. Anti-neural-inhibition: a conserved mechanism for neural induction. *Cell 89*, 681–684.

Bier, E., Jan, L.Y., Jan, Y.N., **1990**. *rhomboid*, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster. Genes Devel. 4*, 190–203.

Boncinelli, E., Simeone, A., Acampora, D., Gulisano, M., **1993**. Homeobox genes in the developing central nervous system. *Ann. Genet. 36*, 30–37.

Booker, R., Truman, J.W., **1987**. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. 1. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol. 255*, 548–559.

Bossing, T., Technau, G.M., **1994**. The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labelling. *Development 120*, 1895–1906.

Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., **1996**. The embryonic central nervous system lineages of *Drosophila melanogaster.* 1. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Devel. Biol. 179*, 41–64.

Boulianne, G.L., de la Concha, A., Campos-Ortega, J.A., Jan, L.Y., Jan, Y.N., **1991**. The *Drosophila* neurogenic gene neuralized encodes a novel protein and is expressed in precursors of larval and adult neurons. *EMBO J. 10*, 2975–2983.

Boyan, G.S., Ball, E.E., **1993**. The grasshopper, *Drosophila* and neuronal homology (advantages of the insect nervous system for the neuroscientist). *Prog. Neurobiol. 41*, 657–682.

Brand, M., Jarman, A.P., Jan, L.Y., Jan, Y.N., **1993**. *asense* is a *Drosophila* neural precursor gene and is capable of initiating sense organ formation. *Development 119*, 1–17.

Bray, S.J., **1997**. Expression and function of Enhancer of split bHLH proteins during *Drosophila* neurogenesis. *Perspect. Devel. Neurobiol. 4*, 313–323.

Bray, S., Furriols, M., **2001**. Notch pathway: making sense of suppressor of hairless. *Curr. Biol. 11*, R217–R221.

Broadus, J., Doe, C.Q., **1997**. Extrinsic cues, intrinsic cues and microfilaments regulate asymmetric protein localization in *Drosophila* neuroblasts. *Curr. Biol. 7*, 827–835.

Broadus, J., Fuerstenberg, S., Doe, C.Q., **1998**. Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. *Nature 391*, 792–795.

Brody, T., Odenwald, W.F., **2000**. Programmed transformations in neuroblast gene expression during *Drosophila* CNS lineage development. *Devel. Biol. 226*, 34–44.

Brody, T., Odenwald, W.F., **2002**. Cellular diversity in the developing nervous system: a temporal view from *Drosophila*. *Development 129*, 3763–3770.

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., *et al.*, **1999**. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell 96*, 795–806.

Brunet, J.F., Ghysen, A., **1999**. Deconstructing cell determination: proneural genes and neuronal identity. *BioEssays 21*, 313–318.

Buchanan, R.L., Benzer, S., **1993**. Defective glia in the *Drosophila* brain degeneration mutant *drop-dead*. *Neuron 10*, 839–850.

Buescher, M., Yeo, S.L., Udolph, G., Zavortink, M., Yang, X., *et al.*, **1998**. Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. *Genes Devel. 12*, 1858–1870.

Cabrera, C.V., **1990**. Lateral inhibition and cell fate during neurogenesis in *Drosophila*: the interactions between scute, Notch and Delta. *Development 110*, 733–742.

Cabrera, C.V., Martinez-Arias, A., Bate, M., **1987**. The expression of three members of the *achaete–scute* gene complex correlates with neuroblast segregation in *Drosophila*. *Cell 50*, 425–433.

Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., *et al.*, **2000**. Generation of medial and lateral dorsal body domains by the pannier gene of *Drosophila*. *Development 127*, 3971–3980.

Campbell, G., Goring, H., Lin, T., Spana, E., Andersson, S., *et al.*, **1994**. RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development 120*, 2957–2966.

Campos-Ortega, J.A., **1993**. Early neurogenesis in *Drosophila melanogaster.* In: Bate, C.M., Martinez-Arias, A. (Eds.), The Development of *Drosophila*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1091–1129.

Campos-Ortega, J.A., **1998**. The genetics of the *Drosophila achaete–scute* gene complex: a historical appraisal. *Int. J. Devel. Biol. 42*, 291–297.

Campos-Ortega, J.A., Hartenstein, V., **1985**. The Embryonic Development of *Drosophila melanogaster.* Springer, New York.

Campuzano, S., Carramolino, L., Cabrera, C.V., Ruiz-Gomez, M., Villares, R., *et al.*, **1985**. Molecular genetics of the *achaete–scute* gene complex of *D. melanogaster. Cell 40*, 327–338.

Cavodeassi, F., Modolell, J., Campuzano, S., **2000**. The Iroquois homeobox genes function as dorsal selectors in the *Drosophila* head. *Development 127*, 1921–1929.

Cayouette, M., Raff, M., **2002**. Asymmetric segregation of Numb: a mechanism for neural specification from *Drosophila* to mammals. *Nat. Neurosci. 5*, 1265–1269.

Chan, Y.M., Jan, Y.N., **1998**. Roles for proteolysis and trafficking in Notch maturation and signal transduction. *Cell 94*, 423–426.

Chan, Y.M., Jan, Y.N., **1999**. Conservation of neurogenic genes and mechanisms. *Curr. Opin. Neurobiol. 9*, 582–588.

Cheah, P.Y., Chia, W., Yang, X., **2000**. Jumeaux, a novel *Drosophila* winged-helix family protein, is required for generating asymmetric sibling neuronal cell fates. *Development 127*, 3325–3335.

Choi, K.W., Benzer, S., **1994**. Migration of glia along photoreceptor axons in the developing *Drosophila* eye. *Neuron 12*, 423–431.

Chu, H., Parras, C., White, K., Jimenez, F., **1998**. Formation and specification of ventral neuroblasts is controlled by vnd in *Drosophila* neurogenesis. *Genes Devel. 12*, 3613–3624.

Chu-LaGraff, Q., Doe, C.Q., **1993**. Neuroblast specification and formation regulated by wingless in the *Drosophila* CNS. *Science 261*, 1594–1597.

Condron, B.G., Patel, N.H., Zinn, K., **1994**. Engrailed controls glial/neuronal cell fate decisions at the midline of the central nervous system. *Neuron 13*, 541–554.

Condron, B.G., Zinn, K., **1994**. The grasshopper median neuroblast is a multipotent progenitor cell that generates glia and neurons in distinct temporal phases. *J. Neurosci. 14*, 5766–5777.

Cornell, R.A., Ohlen, T.V., **2000**. Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol. 10*, 63–71.

Crews, S., Franks, R., Hu, S., Matthews, B., Nambu, J., **1992**. *Drosophila single-minded* gene and the molecular genetics of CNS midline development. *J. Exp. Zool. 261*, 234–244.

Crews, S.T., Thomas, J.B., Goodman, C.S., **1988**. The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell 52*, 143–151.

Cubas, P., de Celis, J.F., Campuzano, S., Modolell, J., **1991**. Proneural clusters of achaete–scute expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Devel. 5*, 996–1008.

Cubas, P., Modolell, J., **1992**. The *extramacrochaetae* gene provides information for sensory organ patterning. *EMBO J. 11*, 3385–3393.

D'Alessio, M., Frasch, M., **1996**. msh may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Devel. 58*, 217–231.

Dambly-Chaudière, C., Ghysen, A., **1987**. Independent subpatterns of sense organs require independent genes of the *achaete–scute* complex in *Drosophila* larvae. *Genes Devel. 1*, 297–306.

Davenne, M., Maconochie, M.K., Neun, R., Pattyn, A., Chambon, P., *et al.*, **1999**. Hoxa2 and Hoxb2 control dorsoventral patterns of neuronal development in the rostral hindbrain. *Neuron 22*, 677–691.

Doe, C.Q., **1992**. Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development 116*, 855–863.

Doe, C.Q., Bowerman, B., **2001**. Asymmetric cell division: fly neuroblast meets worm zygote. *Curr. Opin. Cell Biol. 13*, 68–75.

Doe, C.Q., Chu-LaGraff, Q., Wright, D.M., Scott, M.P., **1991**. The *prospero* gene specifies cell fates in the *Drosophila* central nervous system. *Cell 65*, 451–464.

Doe, C.Q., Fuerstenberg, S., Peng, C.Y., **1998**. Neural stem cells: from fly to vertebrates. *J. Neurobiol. 36*, 111–127.

Doe, C.Q., Goodman, C.S., **1985a**. Early events in insect neurogenesis. 1. Development and segmental differences in the pattern of neuronal precursor cells. *Devel. Biol. 111*, 193–205.

Doe, C.Q., Goodman, C.S., **1985b**. Early events in insect neurogenesis. 2. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Devel. Biol. 111*, 206–219.

Doe, C.Q., Smouse, D.T., **1990**. The origins of cell diversity in the insect central nervous system. *Semin. Cell Biol. 1*, 211–218.

Dominguez, M., Campuzano, S., **1993**. *asense*, a member of the *Drosophila achaete–scute* complex, is a proneural and neural differentiation gene. *EMBO J. 12*, 2049–2060.

Dong, R., Jacobs, J.R., **1997**. Origin and differentiation of supernumerary midline glia in *Drosophila* embryos deficient for apoptosis. *Devel. Biol. 190*, 165–177.

Egger, B., Leemans, R., Loop, T., Kammermeier, L., Fan, Y., *et al.*, **2002**. Gliogenesis in *Drosophila*: genome-wide analysis of downstream genes of glial cells missing in the embryonic nervous system. *Development 129*, 3295–3309.

Ferguson, E.L., **1996**. Conservation of dorsal–ventral patterning in arthropods and chordates. *Curr. Opin. Genet. Devel. 6*, 424–431.

François, V., Bier, E., **1995**. *Xenopus* chordin and *Drosophila* short gastrulation genes encode homologous proteins functioning in dorsal–ventral axis formation. *Cell 80*, 19–20.

François, V., Solloway, M., O'Neill, J.W., Emery, J., Bier, E., **1994**. Dorsal–ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes Devel. 8*, 2602–2616.

Fraser, S.E., **1991**. Pattern formation in the vertebrate nervous system. *Curr. Opin. Genet. Devel. 1*, 217–220.

Freeman, M.R., Delrow, J., Kim, J., Johnson, E., Doe, C.Q., **2003**. Unwrapping glial biology: gcm target genes regulating glial development, diversification, and function. *Neuron 38*, 567–580.

Fuerstenberg, S., Broadus, J., Doe, C.Q., **1998**. Asymmetry and cell fate in the *Drosophila* embryonic CNS. *Int. J. Devel. Biol. 42*, 379–383.

Furst, A., Mahowald, A.P., **1985**. Differentiation of primary embryonic neuroblasts in purified neural cell cultures from *Drosophila*. *Devel. Biol. 109*, 184–192.

Garcia-Garcia, M.J., Ramain, P., Simpson, P., Modolell, J., **1999**. Different contributions of pannier and wingless to the patterning of the dorsal mesothorax of *Drosophila*. *Development 126*, 3523–3532.

Gautier, P., Ledent, V., Massaer, M., Dambly-Chaudiere, C., Ghysen, A., **1997**. tap, a *Drosophila* bHLH gene expressed in chemosensory organs. *Gene 191*, 15–21.

Gay, N.J., Keith, F.J., **1990**. Formation of a gradient of the *Drosophila* dorsal morphogen by differential nuclear localisation. *BioEssays 12*, 181–182.

Gehring, W.J., **1993**. Exploring the homeobox. *Gene 135*, 215–221.

Ghysen, A., Richelle, J., **1979**. Determination of sensory bristles and pattern formation in Drosophila. 2. The *achaete–scute* locus. *Devel. Biol. 70*, 438–452.

Giangrande, A., **1994**. Glia in the fly wing are clonally related to epithelial cells and use the nerve as a pathway for migration. *Development 120*, 523–534.

Giangrande, A., **1996**. Development and organization of glial cells in *Drosophila melanogaster. Int. J. Devel. Biol. 40*, 917–927.

Giangrande, A., Murray, M.A., Palka, J., **1993**. Development and organization of glial cells in the peripheral nervous system of *Drosophila melanogaster. Development 117*, 895–904.

Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A., *et al.*, **1997**. Glial development in the *Drosophila* CNS requires concomitant activation of glial and repression of neuronal differentiation genes. *Development 124*, 2307–2316.

Gomez-Skarmeta, J.L., Rodriguez, I., Martinez, C., Culi, J., Ferres-Marco, D., *et al.*, **1995**. *Cis*-regulation of *achaete* and *scute*: shared *enhancer*-like elements drive their coexpression in proneural clusters of the imaginal discs. *Genes Devel. 9*, 1869–1882.

Goodman, C.S., Spitzer, N.C., **1979**. Embryonic development of identified neurones: differentiation from neuroblast to neurone. *Nature 280*, 208–214.

Goodman, C.S., Bastiani, M.J., Doe, C.Q., du Lac, S., Helfand, S.L., *et al.*, **1984**. Cell recognition during neuronal development. *Science 225*, 1271–1279.

Goodman, C.S., Doe, C.Q., **1993**. Embryonic development of the *Drosophila* central nervous system. In: Bate, C.M., Martinez-Arias, A. (Eds.), The Development of *Drosophila*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1131–1206.

Goriely, A., Dumont, N., Dambly-Chaudiere, C., Ghysen, A., **1991**. The determination of sense organs in Drosophila: effect of the neurogenic mutations in the embryo. *Development 113*, 1395–1404.

Govind, S., Steward, R., **1991**. Dorsoventral pattern formation in *Drosophila*: signal transduction and nuclear targeting. *Trends Genet. 7*, 119–125.

Granderath, S., Bunse, I., Klambt, C., **2000**. *gcm* and *pointed* synergistically control glial transcription of the *Drosophila* gene *loco*. *Mech. Devel. 91*, 197–208.

Granderath, S., Klambt, C., **1999**. Glia development in the embryonic CNS of *Drosophila*. *Curr. Opin. Neurobiol. 9*, 531–536.

Guichard, A., Biehs, B., Sturtevant, M.A., Wickline, L., Chacko, J., *et al.*, **1999**. Rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during *Drosophila* wing vein development. *Development 126*, 2663–2676.

Guo, M., Jan, L.Y., Jan, Y.N., **1996**. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron 17*, 27–41.

Gustavson, E., Goldsborough, A.S., Ali, Z., Kornberg, T.B., **1996**. The *Drosophila engrailed* and *invected* genes: partners in regulation, expression and function. *Genetics 142*, 893–906.

Halter, D.A., Urban, J., Rickert, C., Ner, S.S., Ito, K., *et al.*, **1995**. The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster. Development 121*, 317–332.

Hama, J., Weinstein, D.C., **2001**. Is Chordin a morphogen? *BioEssays 23*, 121–124.

Harris, R., Sabatelli, L.M., Seeger, M.A., **1996**. Guidance cues at the *Drosophila* CNS midline: identification and characterization of two *Drosophila* Netrin/UNC-6 homologs. *Neuron 17*, 217–228.

Harrison, S.D., Travers, A.A., **1990**. The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the ftz transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J. 9*, 207–216.

Hartenstein, V., Nassif, C., Lekven, A., **1998**. Embryonic development of the *Drosophila* brain. 2. Pattern of glial cells. *J. Comp. Neurol. 402*, 32–47.

Hassan, B., Vaessin, H., **1996**. Regulatory interactions during early neurogenesis in *Drosophila*. *Devel. Genet. 18*, 18–27.

Hatten, M.E., **1999**. Central nervous system neuronal migration. *Annu. Rev. Neurosci. 22*, 511–539.

Hemmati-Brivanlou, A., Melton, D., **1997**. Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell 88*, 13–17.

Hidalgo, A., Kinrade, E.F., Georgiou, M., **2001**. The *Drosophila* neuregulin Vein maintains glial survival during axon guidance in the CNS. *Devel. Cell 1*, 679–690.

Hirata, J., Nakagoshi, H., Nabeshima, Y., Matsuzaki, F., **1995**. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature 377*, 627–630.

Holley, S.A., Jackson, P.D., Sasai, Y., Lu, B., De Robertis, E.M., *et al.*, **1995**. A conserved system for dorsal–ventral patterning in insects and vertebrates involving *sog* and *chordin*. *Nature 376*, 249–253.

Honda, T., Tabata, H., Nakajima, K., **2003**. Cellular and molecular mechanisms of neuronal migration in neocortical development. *Semin. Cell Devel. Biol. 14*, 169–174.

Hosoya, T., Takizawa, K., Nitta, K., Hotta, Y., **1995**. *glial cells missing*: a binary switch between neuronal and glial determination in *Drosophila*. *Cell 82*, 1025–1036.

Huang, F., Dambly-Chaudière, C., Ghysen, A., **1994**. Position-reading and the emergence of sense organ precursors in *Drosophila*. *Prog. Neurobiol. 42*, 293–297.

Huang, J.D., Schwyter, D.H., Shirokawa, J.M., Courey, A.J., **1993**. The interplay between multiple enhancer and silencer elements defines the pattern of *decapentaplegic* expression. *Genes Devel. 7*, 694–704.

Huff, R., Furst, A., Mahowald, A.P., **1989**. *Drosophila* embryonic neuroblasts in culture: autonomous differentiation of specific neurotransmitters. *Devel. Biol. 134*, 146–157.

Hummel, T., Schimmelpfeng, K., Klambt, C., **1999a**. Commissure formation in the embryonic CNS of *Drosophila*: II. Functions of the different midline cells. *Development 126*, 771–779.

Hummel, T., Schimmelpfeng, K., Klambt, C., **1999b**. Commissure formation in the embryonic CNS of *Drosophila*: I. Identification of the required gene functions. *Devel. Biol. 209*, 381–398.

Ikeshima-Kataoka, H., Skeath, J.B., Nabeshima, Y., Doe, C.Q., Matsuzaki, F., **1997**. Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions. *Nature 390*, 625–629.

Ingham, P.W., Taylor, A.M., Nakano, Y., **1991**. Role of the *Drosophila patched* gene in positional signaling. *Nature 353*, 184–187.

Ip, Y.T., Kraut, R., Levine, M., Rushlow, C.A., **1991**. The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell 64*, 439–446.

Ip, Y.T., Levine, M., Small, S.J., **1992**. The bicoid and dorsal morphogens use a similar strategy to make stripes in the *Drosophila* embryo. *J. Cell Sci. 16* (Suppl.), 33–38.

Irish, V.F., Gelbart, W.M., **1987**. The *decapentaplegic* gene is required for dorsal–ventral patterning of the *Drosophila* embryo. *Genes Devel. 1*, 868–879.

Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., **2001**. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell 106*, 511–521.

Isshiki, T., Takeichi, M., Nose, A., **1997**. The role of the *msh* homeobox gene during *Drosophila* neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development 124*, 3099–3109.

Ito, K., Urban, J., Techanu, G.M., **1995**. Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Devel. Biol. 204*, 284–307.

Jacobs, J.R., **2000**. The midline glia of *Drosophila*: a molecular genetic model for the developmental functions of glia. *Prog. Neurobiol. 62*, 475–508.

Jarman, A.P., Brand, M., Jan, L.Y., Jan, Y.N., **1993**. The regulation and function of the helix–loop–helix gene, *asense*, in *Drosophila* neural precursors. *Development 119*, 19–29.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., *et al.*, **1995**. Signaling downstream of activated mammalian Notch. *Nature 377*, 355–358.

Jia, X.X., Siegler, M.V., **2002**. Midline lineages in grasshopper produce neuronal siblings with asymmetric expression of Engrailed. *Development 129*, 5181–5193.

Jiang, J., Kosman, D., Ip, Y.T., Levine, M., **1991**. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. *Genes Devel. 5*, 1881–1891.

Jimenez, F., Campos-Ortega, J.A., **1990**. Defective neuroblast commitment in mutants of the *achaete–scute* complex and adjacent genes of *D. melanogaster. Neuron 5*, 81–89.

Jin, M.H., Sawamoto, K., Ito, M., Okano, H., **2000**. The interaction between the *Drosophila* secreted protein Argos and the epidermal growth factor receptor inhibits dimerization of the receptor and binding of secreted Spitz to the receptor. *Mol. Cell Biol. 20*, 2098–2107.

Jones, B.W., Fetter, R.D., Tear, G., Goodman, C.S., **1995**. *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell 82*, 1013–1023.

Kageyama, R., Ishibashi, M., Takebayashi, K., Tomita, K., **1997**. bHLH transcription factors and mammalian neuronal differentiation. *Int. J. Biochem. Cell Biol. 29*, 1389–1399.

Kammerer, M., Giangrande, A., **2001**. Glide2, a second glial promoting factor in *Drosophila melanogaster. EMBO J. 20*, 4664–4673.

Kaprielian, Z., Runko, E., Imondi, R., **2001**. Axon guidance at the midline choice point. *Devel. Dyn. 221*, 154–181.

Kasai, Y., Nambu, J.R., Lieberman, P.M., Crews, S.T., **1992**. Dorsal–ventral patterning in *Drosophila*: DNA binding of snail protein to the single-minded gene. *Proc. Natl Acad. Sci. USA 89*, 3414–3418.

Kawamura, K., Carlson, J.G., **1962**. Studies on cytokinesis in neuroblasts of the grasshopper, *Chortophaga viridifasciata* (De Geer). 3. Factors determining the location of the cleavage furrow. *Exp. Cell Res. 26*, 411–423.

Keleman, K., Rajagopalan, S., Cleppien, D., Teis, D., Paiha, K., *et al.*, **2002**. Comm sorts Robo to control axon guidance at the *Drosophila* midline. *Cell 110*, 415–427.

Kidd, S., Lieber, T., Young, M.W., **1998a**. Ligand-induced cleavage and regulation of nuclear entry of Notch in *Drosophila melanogaster* embryos. *Genes Devel. 12*, 3728–3740.

Kidd, T., Bland, K.S., Goodman, C.S., **1999**. Slit is the midline repellent for the Robo receptor in *Drosophila*. *Cell 96*, 785–794.

Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., *et al.*, **1998c**. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell 92*, 205–215.

Kidd, T., Russell, C., Goodman, C.S., Tear, G., **1998b**. Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. *Neuron 20*, 25–33.

Kim, P., Helms, A.W., Johnson, J.E., Zimmerman, K., **1997**. XATH-1, a vertebrate homolog of *Drosophila* atonal, induces a neuronal differentiation within ectodermal progenitors. *Devel. Biol. 187*, 1–12.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H., Klambt, C., **1994**. The Ets transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. *Cell 78*, 149–160.

Klambt, C., **1993**. The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development 117*, 163–176.

Klambt, C., Goodman, C.S., **1991**. The diversity and pattern of glia during axon pathway formation in the *Drosophila* embryo. *Glia 4*, 205–213.

Knoblich, J.A., **2001**. Asymmetric cell division during animal development. *Nat. Rev. Mol. Cell Biol. 2*, 11–20.

Knoblich, J.A., Jan, L.Y., Jan, Y.N., **1995**. Asymmetric segregation of Numb and Prospero during cell division. *Nature 377*, 624–627.

Knust, E., **2001**. G protein signaling and asymmetric cell division. *Cell 107*, 125–128.

Knust, E., Bremer, K.A., Vassin, H., Ziemer, A., Tepass, U., *et al*., **1987a**. The enhancer of *split* locus and neurogenesis in *Drosophila melanogaster. Devel. Biol. 122*, 262–273.

Knust, E., Dietrich, U., Tepass, U., Bremer, K.A., Weigel, D., *et al*., **1987b**. EGF homologous sequences encoded in the genome of *Drosophila melanogaster*, and their relation to neurogenic genes. *EMBO J. 6*, 761–766.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., *et al*., **1996**. *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell 87*, 197–204.

Kosman, D., Ip, Y.T., Levine, M., Arora, K., **1991**. Establishment of the mesoderm–neuroectoderm boundary in the *Drosophila* embryo. *Science 254*, 118–122.

Kraut, R., Campos-Ortega, J.A., **1996**. *inscuteable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeleton adaptor protein. *Devel. Biol. 174*, 65–81.

Kraut, R., Chia, W., Jan, L.Y., Jan, Y.N., Knoblich, J.A., **1996**. Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. *Nature 383*, 50–55.

Kretzschmar, D., Hasan, G., Sharma, S., Heisenberg, M., Benzer, S., **1997**. The *swiss cheese* mutant causes glial hyperwrapping and brain degeneration in *Drosophila*. *J. Neurosci. 17*, 7425–7432.

Krumlauf, R., Marshall, H., Studer, M., Nonchev, S., Sham, M.H., *et al*., **1993**. Hox homeobox genes and regionalization of the nervous system. *J. Neurobiol. 24*, 1328–1340.

Kuchinke, U., Grawe, F., Knust, E., **1998**. Control of spindle orientation in *Drosophila* by the Par-3-related PDZ-domain protein Bazooka. *Curr. Biol. 8*, 1357–1365.

Kunisch, M., Haenlin, M., Campos-Ortega, J.A., **1994**. Lateral inhibition mediated by the *Drosophila* neurogenic gene *delta* is enhanced by proneural proteins. *Proc. Natl Acad. Sci. USA 91*, 10139–10143.

Laming, P.R., Kimelberg, H., Robinson, S., Salm, A., Hawrylak, N., *et al*., **2000**. Neuronal–glial interactions and behaviour. *Neurosci. Biobehav. Rev. 24*, 295–340.

Lanoue, B.R., Jacobs, J.R., **1999**. Rhomboid function in the midline of the *Drosophila* CNS. *Devel. Genet. 25*, 321–330.

Lear, B.C., Skeath, J.B., Patel, N.H., **1999**. Neural cell fate in *rca1* and *cycA* mutants: the roles of intrinsic and extrinsic factors in asymmetric division in the *Drosophila* central nervous system. *Mech. Devel. 88*, 207–219.

Lecourtois, M., Schweisguth, F., **1998**. Indirect evidence for Delta-dependent intracellular processing of notch in *Drosophila* embryos. *Curr. Biol. 8*, 771–774.

Lee, J.E., **1997**. Basic helix–loop–helix genes in neural development. *Curr. Opin. Neurobiol. 7*, 13–20.

Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N., *et al*., **1995**. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix–loop–helix protein. *Science 268*, 836–844.

Lee, J.R., Urban, S., Garvey, C.F., Freeman, M., **2001**. Regulated intracellular ligand transport and proteolysis control egf signal activation in *Drosophila*. *Cell 107*, 161–171.

Lemke, G., **2001**. Glial control of neuronal development. *Annu. Rev. Neurosci. 24*, 87–105.

Leptin, M., **1991**. *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. *Genes Devel. 5*, 1568–1576.

Leptin, M., Roth, S., **1994**. Autonomy and non-autonomy in *Drosophila* mesoderm determination and morphogenesis. *Development 120*, 853–859.

Leyns, L., Gomez-Skarmeta, J.L., Dambly-Chaudière, C., **1996**. *iroquois*: a prepattern gene that controls the formation of bristles on the thorax of *Drosophila*. *Mech. Devel. 59*, 63–72.

Li, P., Yang, X., Wasser, M., Cai, Y., Chia, W., **1997**. Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during *Drosophila* neuroblast cell divisions. *Cell 90*, 437–447.

Livesey, F.J., **1999**. Netrins and netrin receptors. *Cell. Mol. Life Sci. 56*, 62–68.

Livneh, E., Glazer, L., Segal, D., Schlessinger, J., Shilo, B.Z., **1985**. The *Drosophila* EGF receptor gene homolog: conservation of both hormone binding and kinase domains. *Cell 40*, 599–607.

Lu, B., Rothenberg, M., Jan, L.Y., Jan, Y.N., **1998**. Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in *Drosophila* neural and muscle progenitors. *Cell 95*, 225–235.

Martin-Bermudo, M.D., Carmena, A., Jimenez, F., **1995**. Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. *Development 121*, 219–224.

Martin-Bermudo, M.D., Martinez, C., Rodriguez, A., Jimenez, F., **1991**. Distribution and function of the lethal of *scute* gene product during early neurogenesis in *Drosophila*. *Development 113*, 445–454.

Martinez, C., Modolell, J., **1991**. Cross-regulatory interactions between the proneural *achaete* and *scute* genes of *Drosophila*. *Science 251*, 1485–1487.

Matsuzaki, F., **2000**. Asymmetric division of *Drosophila* neural stem cells: a basis for neural diversity. *Curr. Opin. Neurobiol. 10*, 38–44.

McConnell, S.K., **1995**. Strategies for the generation of neuronal diversity in the developing central nervous system. *J. Neurosci. 15*, 6987–6998.

McDonald, J.A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C.Q., *et al.*, **1998**. Dorsoventral patterning in the *Drosophila* central nervous system: the *vnd* homeobox gene specifies ventral column identity. *Genes Devel. 12*, 3603–3612.

McGinnis, W., Krumlauf, R., **1992**. Homeobox genes and axial patterning. *Cell 68*, 283–302.

Mehler, M.F., Mabie, P.C., Zhang, D., Kessler, J.A., **1997**. Bone morphogenetic proteins in the nervous system. *Trends Neurosci. 20*, 309–317.

Mellerick, D.M., Nirenberg, M., **1995**. Dorsal–ventral patterning genes restrict NK-2 homeobox gene expression to the ventral half of the central nervous system of *Drosophila* embryos. *Devel. Biol. 171*, 306–316.

Menne, T.V., Klambt, C., **1994**. The formation of commissures in the *Drosophila* CNS depends on the midline cells and on the Notch gene. *Development 120*, 123–133.

Miller, A.A., Bernardoni, R., Giangrande, A., **1998**. Positive autoregulation of the glial promoting factor glide/gcm. *EMBO J. 17*, 6316–6326.

Miller, A.A., Bernardoni, R., Hindelang, C., Kammerer, M., Sorrentino, S., *et al.*, **1999**. Role and mechanism of action of glial cell deficient/glial cell missing (*glide/gcm*), the fly glial promoting factor. *Adv. Exp. Med. Biol. 468*, 33–46.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., *et al.*, **1996**. Genetic analysis of Netrin genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron 17*, 203–215.

Modolell, J., Campuzano, S., **1998**. The *achaete–scute* complex as an integrating device. *Int. J. Devel. Biol. 42*, 275–282.

Mohler, J., Vani, K., **1992**. Molecular organization and embryonic expression of the hedgehog gene involved in cell–cell communication in segmental patterning of *Drosophila*. *Development 115*, 957–971.

Moore, A.W., Barbel, S., Jan, L.Y., Jan, Y.N., **2000**. A genomewide survey of basic helix–loop–helix factors in *Drosophila*. *Proc. Natl Acad. Sci. USA 97*, 10436–10441.

Morel, V., Schweisguth, F., **2000**. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. *Genes Devel. 14*, 377–388.

Muller, H.A., Wieschaus, E., **1996**. *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J. Cell Biol. 134*, 149–163.

Myat, A., Henry, P., McCabe, V., Flintoft, L., Rotin, D., *et al.*, **2002**. *Drosophila* Nedd4, a ubiquitin ligase, is recruited by Commissureless to control cell surface levels of the roundabout receptor. *Neuron 35*, 447–459.

Nambu, J.R., Franks, R.G., Hu, S., Crews, S.T., **1990**. The *single-minded* gene of *Drosophila* is required for the expression of genes important for the development of CNS midline cells. *Cell 63*, 63–75.

Nambu, J.R., Lewis, J.O., Wharton, K.A., Jr., Crews, S.T., **1991**. The *Drosophila single-minded* gene encodes a helix–loop–helix protein that acts as a master regulator of CNS midline development. *Cell 67*, 1157–1167.

Nusslein-Volhard, C., Wieschaus, E., **1980**. Mutations affecting segment number and polarity in *Drosophila*. *Nature 287*, 795–801.

Offermanns, S., **2003**. G-proteins as transducers in transmembrane signaling. *Prog. Biophys. Mol. Biol. 83*, 101–130.

Ono, K., Yasui, Y., Rutishauser, U., Miller, R.H., **1997**. Focal ventricular origin and migration of oligodendrocyte precursors into the chick optic nerve. *Neuron 19*, 283–292.

Park, Y., Fujioka, M., Kobayashi, M., Jaynes, J.B., Datta, S., **2001**. *even skipped* is required to produce a *trans*-acting signal for larval neuroblast proliferation that can be mimicked by ecdysone. *Development 128*, 1899–1909.

Parras, C., Garcia-Alonso, L.A., Rodriguez, I., Jimenez, F., **1996**. Control of neural precursor specification by proneural proteins in the CNS of *Drosophila*. *EMBO J. 15*, 6394–6399.

Peterson, C., Carney, G.E., Taylor, B.J., White, K., **2002**. *reaper* is required for neuroblast apoptosis during *Drosophila* development. *Development 129*, 1467–1476.

Petronczki, M., Knoblich, J.A., **2001**. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. *Nat. Cell Biol. 3*, 43–49.

Peyrefitte, S., Kahn, D., Haenlin, M., **2001**. New members of the *Drosophila* Myc transcription factor subfamily revealed by a genome-wide examination for basic *helix–loop–helix* genes. *Mech. Devel. 104*, 99–104.

Pickup, A.T., Banerjee, U., **1999**. The role of *star* in the production of an activated ligand for the EGF receptor signaling pathway. *Devel. Biol. 205*, 254–259.

Prokop, A., Technau, G.M., **1994**. Early tagma-specific commitment of *Drosophila* CNS progenitor NB1–1. *Development 120*, 2567–2578.

Ragone, G., Bernardoni, R., Giangrande, A., **2001**. A novel mode of asymmetric division identifies the fly neuroglioblast 6– 4t. *Devel. Biol. 235*, 74–85.

Ragone, G., Van De Bor, V., Sorrentino, S., Kammerer, M., Galy, A., *et al.*, **2003**. Transcriptional regulation of glial cell specification. *Devel. Biol. 255*, 138–150.

Rajagopalan, S., Vivancos, V., Nicolas, E., Dickson, B.J., **2000**. Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the *Drosophila* CNS. *Cell 103*, 1033–1045.

Ramain, P., Heitzler, P., Haenlin, M., Simpson, P., **1993**. *pannier*, a negative regulator of *achaete* and *scute* in *Drosophila*, encodes a zinc finger protein with

homology to the vertebrate transcription factor GATA-1. *Development 119*, 1277–1291.

Rangarajan, R., Gong, Q., Gaul, U., **1999**. Migration and function of glia in the developing *Drosophila* eye. *Development 126*, 3285–3292.

Rao, Y., Jan, L.Y., Jan, Y.N., **1990**. Similarity of the product of the *Drosophila* neurogenic gene *big brain* to transmembrane channel proteins. *Nature 345*, 163–167.

Rao, Y., Vaessin, H., Jan, L.Y., Jan, Y.N., **1991**. Neuroectoderm in *Drosophila* embryos is dependent on the mesoderm for positioning but not for formation. *Genes Devel. 5*, 1577–1588.

Raper, J.A., Bastiani, M.J., Goodman, C.S., **1984**. Pathfinding by neuronal growth cones in grasshopper embryos. 4. The effects of ablating the A and P axons upon the behavior of the G growth cone. *J. Neurosci. 4*, 2329–2345.

Ray, R.P., Arora, K., Nusslein-Volhard, C., Gelbart, W.M., **1991**. The control of cell fate along the dorsal–ventral axis of the *Drosophila* embryo. *Development 113*, 35–54.

Rhyu, M.S., Jan, L.Y., Jan, Y.N., **1994**. Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell 76*, 477–491.

Rodriguez, I., Hernandez, R., Modolell, J., Ruiz-Gomez, M., **1990**. Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J. 9*, 3583–3592.

Romani, S., Campuzano, S., Macagno, E.R., Modolell, J., **1989**. Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Devel. 3*, 997–1007.

Rooke, J.E., Xu, T., **1998**. Positive and negative signals between interacting cells for establishing neural fate. *BioEssays 20*, 209–214.

Roth, S., Stein, D., Nusslein-Volhard, C., **1989**. A gradient of nuclear localization of the Dorsal protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell 59*, 1189–1202.

Rothberg, J.M., Hartley, D.A., Walther, Z., Artavanis-Tsakonas, S., **1988**. *slit*: an EGF-homologous locus of *D. melanogaster* involved in the development of the embryonic central nervous system. *Cell 55*, 1047–1059.

Rothberg, J.M., Jacobs, J.R., Goodman, C.S., Artavanis-Tsakonas, S., **1990**. *Slit*: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. *Genes Devel. 4*, 2169–2187.

Rusch, J., Levine, M., **1996**. Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. *Curr. Opin. Genet. Devel. 6*, 416–423.

Rutledge, B.J., Zhang, K., Bier, E., Jan, Y.N., Perrimon, N., **1992**. The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal–ventral axis formation and neurogenesis. *Genes Devel. 6*, 1503–1517.

Salzberg, A., Bellen, H.J., **1996**. Invertebrate versus vertebrate neurogenesis: variations on the same theme? *Devel. Genet. 18*, 1–10.

Sanchez, D., Ganfornina, M.D., Bastiani, M.J., **1995**. Contributions of an orthopteran to the understanding of neuronal pathfinding. *Immunol. Cell Biol. 73*, 565–574.

Sasai, Y., Lu, B., Steinbeisser, H., De Robertis, E.M., **1995**. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature 376*, 333–336.

Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H.O., *et al.*, **2003**. *neurotic*, a novel maternal neurogenic gene, encodes an *O*-fucosyltransferase that is essential for Notch–Delta interactions. *Development 130*, 4785–4795.

Schaefer, M., Petronczki, M., Dorner, D., Forte, M., Knoblich, J.A., **2001**. Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. *Cell 107*, 183–194.

Schaefer, M., Shevchenko, A., Knoblich, J.A., **2000**. A protein complex containing Inscuteable and the Gα-binding protein Pins orients asymmetric cell divisions in *Drosophila*. *Curr. Biol. 10*, 353–362.

Schmidt, A., Chiba, A., Doe, C.Q., **1999**. Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development 126*, 4653–4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., *et al.*, **1997**. The embryonic central nervous system lineages of *Drosophila melanogaster*. 2. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Devel. Biol. 189*, 186–204.

Schober, M., Schaefer, M., Knoblich, J.A., **1999**. Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature 402*, 548–551.

Scholz, H., Sadlowski, E., Klaes, A., Klambt, C., **1997**. Control of midline glia development in the embryonic *Drosophila* CNS. *Mech. Devel. 62*, 79–91 (Corrected and republished in *Mech. Devel.* (1997) *64*, 137–51).

Schreiber, J., Sock, E., Wegner, M., **1997**. The regulator of early gliogenesis glial cells missing is a transcription factor with a novel type of DNA-binding domain. *Proc. Natl Acad. Sci. USA 94*, 4739–4744.

Schroeter, E.H., Kisslinger, J.A., Kopan, R., **1998**. Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. *Nature 393*, 382–386.

Schweitzer, R., Howes, R., Smith, R., Shilo, B.Z., Freeman, M., **1995a**. Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature 376*, 699–702.

Schweitzer, R., Shaharabany, M., Seger, R., Shilo, B.Z., **1995b**. Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes Devel. 9*, 1518–1529.

Seeger, M., Tear, G., Ferres-Marco, D., Goodman, C.S., **1993**. Mutations affecting growth cone guidance in

Drosophila: genes necessary for guidance toward or away from the midline. *Neuron 10*, 409–426.

Segal, M., Bloom, K., **2001**. Control of spindle polarity and orientation in *Saccharomyces cerevisiae*. *Trends Cell Biol. 11*, 160–166.

Sepp, K.J., Auld, V.J., **2003**. RhoA and Rac1 GTPases mediate the dynamic rearrangement of actin in peripheral glia. *Development 130*, 1825–1835.

Sepp, K.J., Schulte, J., Auld, V.J., **2000**. Developmental dynamics of peripheral glia in *Drosophila melanogaster. Glia 30*, 122–133.

Shannon, M.P., **1972**. Characterization of the female-sterile mutant almondex of *Drosophila melanogaster. Genetica 43*, 244–256.

Shen, C.P., Jan, L.Y., Jan, Y.N., **1997**. Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. *Cell 90*, 449–458.

Shimell, M.J., Ferguson, E.L., Childs, S.R., O'Connor, M.B., **1991**. The *Drosophila* dorsal–ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. *Cell 67*, 469–481.

Simpson, J.H., Kidd, T., Bland, K.S., Goodman, C.S., **2000**. Short-range and long-range guidance by *slit* and its Robo receptors Robo and Robo2 play distinct roles in midline guidance. *Neuron 28*, 753–766.

Skaer, N., Pistillo, D., Gibert, J.M., Lio, P., Wulbeck, C., *et al.*, **2002**. Gene duplication at the *achaete–scute* complex and morphological complexity of the peripheral nervous system in Diptera. *Trends Genet. 18*, 399–405.

Skeath, J.B., **1998**. The *Drosophila* EGF receptor controls the formation and specification of neuroblasts along the dorsal–ventral axis of the *Drosophila* embryo. *Development 125*, 3301–3312.

Skeath, J.B., **1999**. At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the *Drosophila* embryonic central nervous system. *BioEssays 21*, 922–931.

Skeath, J.B., Carroll, S.B., **1991**. Regulation of *achaete–scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Devel. 5*, 984–995.

Skeath, J.B., Carroll, S.B., **1992**. Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. *Development 114*, 939–946.

Skeath, J.B., Carroll, S.B., **1994**. The *achaete–scute* complex: generation of cellular pattern and fate within the *Drosophila* nervous system. *FASEB J. 8*, 714–721.

Skeath, J.B., Doe, C.Q., **1996**. The *achaete–scute* complex proneural genes contribute to neural precursor specification in the *Drosophila* CNS. *Curr. Biol. 6*, 1146–1152.

Skeath, J.B., Panganiban, G.F., Carroll, S.B., **1994**. The ventral nervous system defective gene controls proneural gene expression at two distinct steps during neuroblast formation in *Drosophila*. *Development 120*, 1517–1524.

Skeath, J.B., Panganiban, G., Selegue, J., Carroll, S.B., **1992**. Gene regulation in two dimensions: the proneural *achaete* and *scute* genes are controlled by combinations of axis-patterning genes through a common intergenic control region. *Genes Devel. 6*, 2606–2619.

Skeath, J.B., Thor, S., **2003**. Genetic control of *Drosophila* nerve cord development. *Curr. Opin. Neurobiol. 13*, 8–15.

Sonnenfeld, M.J., Jacobs, J.R., **1994**. Mesectodermal cell fate analysis in *Drosophila* midline mutants. *Mech. Devel. 46*, 3–13.

Sonnenfeld, M.J., Jacobs, J.R., **1995**. Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development 121*, 569–578.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., *et al.*, **1997**. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian *Arnt* and controls CNS midline and tracheal development. *Development 124*, 4571–4582.

Spana, E.P., Doe, C.Q., **1995**. The Prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development 121*, 3187–3195.

St Johnston, R.D., Gelbart, W.M., **1987**. Decapentaplegic transcripts are localized along the dorsal–ventral axis of the *Drosophila* embryo. *EMBO J. 6*, 2785–2791.

Stathopoulos, A., Levine, M., **2002**. Dorsal gradient networks in the *Drosophila* embryo. *Devel. Biol. 246*, 57–67.

Stemerdink, C., Jacobs, J.R., **1997**. Argos and Spitz group genes function to regulate midline glial cell number in *Drosophila* embryos. *Development 124*, 3787–3796.

Stent, G.S., Weisblat, D.A., **1985**. Cell lineage in the development of invertebrate nervous systems. *Annu. Rev. Neurosci. 8*, 45–70.

Steward, R., **1987**. *dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, *c-rel*. *Science 238*, 692–694.

Steward, R., **1989**. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell 59*, 1179–1188.

Struhl, G., Adachi, A., **1998**. Nuclear access and action of notch *in vivo*. *Cell 93*, 649–660.

Sturtevant, M.A., Roark, M., Bier, E., **1993**. The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Devel. 7*, 961–973.

Sugimoto, Y., Taniguchi, M., Yagi, T., Akagi, Y., Nojyo, Y., *et al.*, **2001**. Guidance of glial precursor cell migration by secreted cues in the developing optic nerve. *Development 128*, 3321–3330.

Taghert, P.H., Goodman, C.S., **1984**. Cell determination and differentiation of identified serotonin-immunoreactive neurons in the grasshopper embryo. *J. Neurosci. 4*, 989–1000.

Tata, F., Hartley, D.A., **1995**. Inhibition of cell fate in *Drosophila* by *Enhancer of split* genes. *Mech. Devel. 51*, 305–315.

Tear, G., **1999**. Axon guidance at the central nervous system midline. *Cell. Mol. Life Sci. 55*, 1365–1376.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C.S., *et al.*, **1996**. *commissureless* controls growth cone guidance across the CNS midline in *Drosophila* and encodes a novel membrane protein. *Neuron 16*, 501–514.

Thomas, J.B., Crews, S.T., Goodman, C.S., **1988**. Molecular genetics of the *single-minded* locus: a gene involved in the development of the *Drosophila* nervous system. *Cell 52*, 133–141.

Tio, M., Zavortink, M., Yang, X., Chia, W., **1999**. A functional analysis of *inscuteable* and its roles during *Drosophila* asymmetric cell divisions. *J. Cell Sci. 112*, 1541–1551.

Truman, J.W., Bate, M., **1988**. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster. Devel. Biol. 125*, 145–157.

Truman, J.W., Thorn, R.S., Robinow, S., **1992**. Programmed neuronal death in insect development. *J. Neurobiol. 23*, 1295–1311.

Truman, J.W., Taylor, B., Awad, A.T., **1993**. Formation of the adult nervous system. In: Bate, C.M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1245–1277.

Tsai, H.H., Miller, R.H., **2002**. Glial cell migration directed by axon guidance cues. *Trends Neurosci. 25*, 173–176.

Udolph, G., Luer, K., Bossing, T., Technau, G.M., **1995**. Commitment of CNS progenitors along the dorsoventral axis of *Drosophila* neuroectoderm. *Science 269*, 1278–1281.

Udolph, G., Prokop, A., Bossing, T., Technau, G.M., **1993**. A common precursor for glia and neurons in the embryonic CNS of *Drosophila* gives rise to segment-specific lineage variants. *Development 118*, 765–775.

Udolph, G., Rath, P., Chia, W., **2001**. A requirement for Notch in the genesis of a subset of glial cells in the *Drosophila* embryonic central nervous system which arise through asymmetric divisions. *Development 128*, 1457–1466.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., **1989**. *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell 58*, 349–360.

Umesono, Y., Hiromi, Y., Hotta, Y., **2002**. Context-dependent utilization of Notch activity in *Drosophila* glial determination. *Development 129*, 2391–2399.

Urban, S., Lee, J.R., Freeman, M., **2001**. *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell 107*, 173–182.

Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L.Y., *et al.*, **1991**. *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell 67*, 941–953.

Van De Bor, V., Giangrande, A., **2001**. Notch signaling represses the glial fate in fly PNS. *Development 128*, 1381–1390.

Van De Bor, V., Giangrande, A., **2002**. *glide/gcm*: at the crossroads between neurons and glia. *Curr. Opin. Genet. Devel. 12*, 465–472.

Van De Bor, V., Walther, R., Giangrande, A., **2000**. Some fly sensory organs are gliogenic and require *glide/gcm* in a precursor that divides symmetrically and produces glial cells. *Development 127*, 3735–3743.

Vincent, S., Vonesch, J.L., Giangrande, A., **1996**. Glide directs glial fate commitment and cell fate switch between neurones and glia. *Development 122*, 131–139.

Voigt, A., Pflanz, R., Schafer, U., Jackle, H., **2002**. Perlecan participates in proliferation activation of quiescent *Drosophila* neuroblasts. *Devel. Dynam. 224*, 403–412.

Ward, M.P., Mosher, J.T., Crews, S.T., **1998**. Regulation of bHLH-PAS protein subcellular localization during *Drosophila* embryogenesis. *Development 125*, 1599–1608.

Weigmann, K., Lehner, C.F., **1995**. Cell fate specification by even-skipped expression in the *Drosophila* nervous system is coupled to cell cycle progression. *Development 121*, 3713–3721.

Wegner, M., Riethmacher, D., **2001**. Chronicles of a switch hunt: *gcm* genes in development. *Trends Genet. 17*, 286–290.

Weiss, J.B., Von Ohlen, T., Mellerick, D.M., Dressler, G., Doe, C.Q., *et al.*, **1998**. Dorsoventral patterning in the *Drosophila* central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Devel. 12*, 3591–3602.

Wheeler, S.R., Carrico, M.L., Wilson, B.A., Brown, S.J., Skeath, J.B., **2003**. The expression and function of the *achaete–scute* genes in *Tribolium castaneum* reveals conservation and variation in neural pattern formation and cell fate specification. *Development 130*, 4373–4381.

Wheeler, W.M., **1891**. Neuroblasts in the arthropod embryo. *J. Morphol. 4*, 337–343.

Wodarz, A., Ramrath, A., Kuchinke, U., Knust, E., **1999**. Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature 402*, 544–547.

Wodarz, A., Ramrath, A., Grimm, A., Knust, E., **2000**. *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol. 150*, 1361–1374.

Wulbeck, C., Simpson, P., **2000**. Expression of *achaete–scute* homologues in discrete proneural clusters on the developing notum of the medfly *Ceratitis capitata*, suggests a common origin for the stereotyped bristle patterns of higher Diptera. *Development 127*, 1411–1420.

Wulbeck, C., Simpson, P., **2002**. The expression of *pannier* and *achaete–scute* homologues in a mosquito suggests an ancient role of *pannier* as a selector gene in the regulation of the dorsal body pattern. *Development 129*, 3861–3871.

Xiong, W.C., Montell, C., **1995**. Defective glia induce neuronal apoptosis in the *repo* visual system of *Drosophila*. *Neuron 14*, 581–590.

Xiong, W.C., Okano, H., Patel, N.H., Blendy, J.A., Montell, C., **1994**. *repo* encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. *Genes Devel. 8*, 981–994.

Yagi, Y., Suzuki, T., Hayashi, S., **1998**. Interaction between *Drosophila* EGF receptor and *vnd* determines

three dorsoventral domains of the neuroectoderm. *Development 125*, 3625–3633.

Yedvobnick, B., Smoller, D., Young, P., Mills, D., **1988**. Molecular analysis of the neurogenic locus mastermind of *Drosophila melanogaster. Genetics 118*, 483–497.

Yu, F., Morin, X., Cai, Y., Yang, X., Chia, W., **2000**. Analysis of partner of *inscuteable*, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in *inscuteable* apical localization. *Cell 100*, 399–409.

Yu, Y.A., Oberg, K., Wang, G., Szalay, A.A., **2003**. Visualization of molecular and cellular events with green fluorescent proteins in developing embryos: a review. *Luminescence 18*, 1–18.

Yuasa, Y., Okabe, M., Yoshikawa, S., Tabuchi, K., Xiong, W.C., *et al*., **2003**. *Drosophila* homeodomain protein Repo controls glial differentiation by cooperating with ETS and BTB transcription factors. *Development 130*, 2419–2428.

Zak, N.B., Wides, R.J., Schejter, E.D., Raz, E., Shilo, B.Z., **1990**. Localization of the DER/flb protein in embryos: implications on the faint little ball lethal phenotype. *Development 109*, 865–874.

Zinn, K., Condron, B.G., **1994**. Cell fate decisions in the grasshopper central nervous system. *Curr. Opin. Cell Biol. 6*, 783–787.

Zhong, W., **2003**. Diversifying neural cells through order of birth and asymmetry of division. *Neuron 37*, 11–14.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L.M., Steller, H., *et al*., **1997a**. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc. Natl Acad. Sci. USA 94*, 5131–5136.

Zhou, L., Xiao, H., Nambu, J.R., **1997b**. CNS midline to mesoderm signaling in *Drosophila*. *Mech. Devel. 67*, 59–68.

1.11 Development of Insect Sensilla

V Hartenstein, University of California, Los Angeles, CA, USA

1.11.1. Structure of Insect Sensilla

1.11.1.1. General Remarks

The sensory system of insects (see also **Chapter 1.12**) is comprised of a large number and great diversity of small organs, called sensilla, which form part of the epidermis. The central component of each sensillum is one or more sensory neurons, which respond to a specific stimulus. The sensory neuron(s) is embedded in a stimulus-receiving apparatus formed by modified epidermal cells, summarily called accessory cells. Stimulation of the sensory neurons results in an action potential that is transmitted via the axon towards the central nervous system (CNS). The special morphology and molecular composition of sensory neurons and accessory cells adapt sensilla towards the reception of a wide range of stimuli, including mechanical stimuli (touch, pressure, vibration, stretching), chemical stimuli (olfactory, gustatory), temperature, and humidity. Most sensilla have contact to the external surface; some sensillum types, in particular stretch receptors, are attached to the inner surface of the body wall or internal organs. The complex morphology of mature insect sensilla has been surveyed in many comprehensive reviews (e.g., Keil and Steinbrecht, 1984; Zacharuk, 1985; Barth *et al.*, 1986; Keil, 1997, 1998; Eguchi and Tominaga, 1999) and can only be touched upon briefly in this chapter whose focus is development.

Despite their great diversity, sensilla are built according to only two main schemes (**Figure 1**). The large majority of sensilla (type I) contain bipolar sensory neurons surrounded by a pair of inner and outer accessory cells. The inner accessory cells form a sheath around the dendrite and, variably, the

A list of all genes and proteins discussed in this chapter is provided in the Appendix.

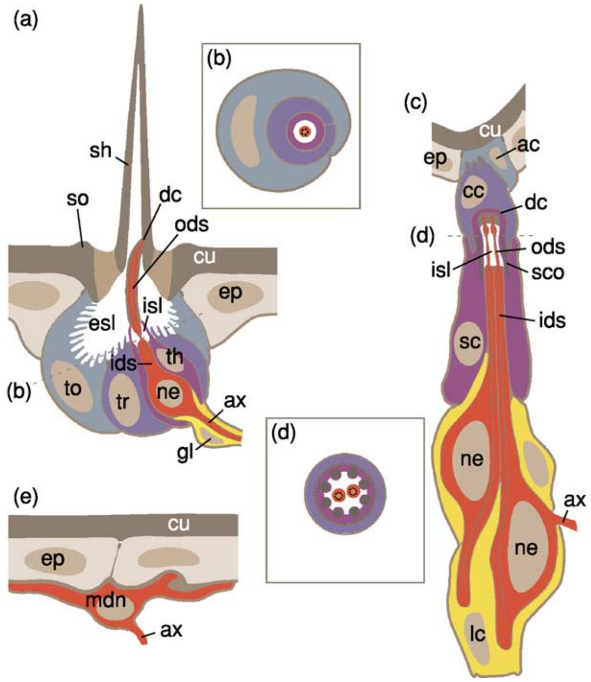


Figure 1 Structure of mature insect sensilla. Schematic sections of the three main classes of sensilla. In this and all following schematics each sensillum cell is consistently rendered in its own color. (a, b) External sensillum. (c, d) Chordotonal organ. (a) and (c) show longitudinal sections; transverse sections at the level indicated by dotted lines are depicted in (b) and (d), respectively. (e) Multidendritic neuron. ac, attachment cell; ax, axon; cc, cap cell; cu, cuticle; dc, dendritic cap; ep, epidermal cell; esl, external sensillum lymph space; gl, glial cell; ids, inner dendritic segment; isl, internal sensillum lymph space; lc, ligament cell; mdn, multidendritic neuron; ne, bipolar sensory neuron; ods, outer dendritic segment (modified cilium); sc, scolopale cell; sco, scolopale; so, sensillum socket; th, thecogen cell; to, tormogen cell; tr, trichogen cell.

soma/proximal axon of the sensory neuron(s). The outer accessory cells are arranged concentrically around the inner ones (Figure 1b). They form processes that, in case of sensilla situated at the surface (external sensilla or ES organs), secrete cuticle in the shape of hairs or bristles (Figure 1a). Outer accessory cells of subepidermal stretch receptors, called chordotonal organs, form ligaments that attach the sensory neuron(s) to the bodywall (Figure 1c and d).

The second major type of sensilla (type II) consists of subepidermally located multipolar (multidendritic) neurons which are either "naked" (Figure 1e) or are associated with variable (and largely unexplored) types of accessory cells. As will be presented in more detail below, evidence has been accumulated in *Drosophila* that type I and type II sensilla form part of the same lineage.

1.11.1.2. External Sensilla

External sensilla possess an external cuticular shaft of various size and shape, and a socket or joint that anchors the process in the surrounding epidermis (Figures 1a and 2a). The shaft and socket are formed by the outer accessory cells, called trichogen and tormogen cell, respectively. Trichogen and tormogen cells are specialized epidermal cells that, when viewed from their apical surface, are folded in the shape of a doughnut around an inner cylinder that consists of the sensory neuron and inner accessory cells (Figure 1b). These latter cells form the part of an external sensillum that cannot be seen at the surface. Their cell bodies (somata) are sunken beneath the level of neighboring epidermal cells and of the outer accessory cells (Figures 1a and 2e).

The somata of sensory neurons are spindle-shaped, bipolar cells (type I sensory neurons) whose dendrites extend apically through the opening provided by the trichogen cell (Figure 1a). The proximal part of the dendrite close to the soma, called inner dendritic segment, ultrastructurally resembles the soma itself (Figures 1a and 2f). The inner segment ends level with the apical surface of the surrounding trichogen cell. Beyond this level, the dendrite continues as the outer segment, which constitutes a modified cilium (Figures 1a and 2e). Like a typical cilium, the outer segment of a sensory dendrite contains an axoneme, a circular array of microtubules (Figure 2e). These microtubules emanate from a basal body which is located at the narrow transition between the inner and outer dendritic segment (Figures 1a and 2f). Basally, bundles of microtubules and microfilaments, the so-called rootlet filaments (Figure 2f), anchor the basal body to the cytoskeleton of the sensory neuron. Apically, the outer dendritic segments show variable specializations related to stimulus transduction. In mechanoreceptive sensilla, outer segments end as an enlarged bulb, called the tubular body, which contains stacks of microtubules (Figure 2c and f). Depending on sensillum modality, the outer segment of sensory dendrites extends into the lumen of the sensory process (Figure 2d) or terminates at its base (Figures 1a and 2c; see below).

The inner accessory cells form glia-like sheaths around the sensory neurons. One cell, called the thecogen cell, is located apically of the neuron, that is, between the neuronal soma and the trichogen cell. The thecogen cell forms a thin, cylindric sheath around the dendrite (Figures 1a, b, and 2e, f). This process secretes an extracellular matrix, the dendritic cap, around the tip of the outer dendritic segment (Figures 1a and 2c, f). The second, more basal inner accessory cell, which can be missing in many

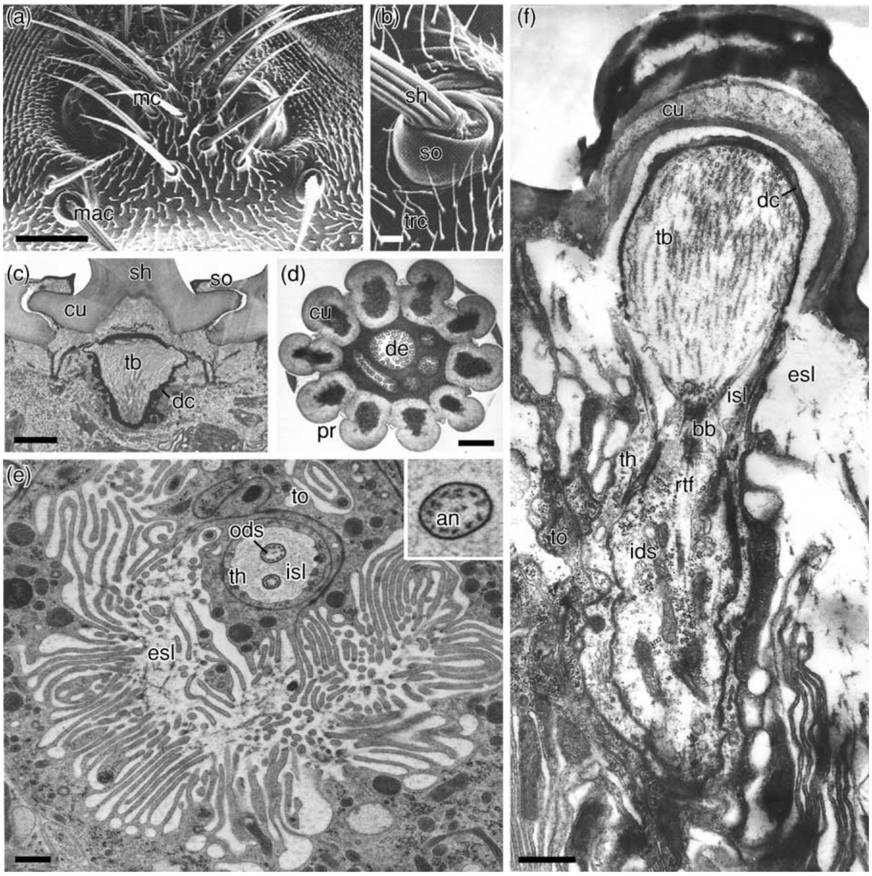


Figure 2 Structure of mature insect external sensilla. (a) Scanning electron micrograph of dorsal head capsule of *Drosophila*, showing arrays of small mechanoreceptive bristles (mc, microchaetes) and large bristles (mac, macrochaetes). (b) Higher magnification of basal part of macrochaete with bristle shaft (sh) and socket (so). Sensory bristles are surrounded by small, non-innervated trichomes (trc) formed by epidermal cells. (c, d) Transmission electron micrographs (TEMs) of apical cross-sections of cricket mechanosensory sensillum (c) and chemosensory sensillum (d). In (c), the tip of outer dendritic segment forms a bulbar thickening, the tubular body (tb) that is surrounded by the dendritic cap (dc). The tubular body terminates underneath the cuticle of the sensillum shaft (sh). Dendrites of chemosensory neurons (de in (d)) extend into the lumen of the shaft, which connects to the outside via numerous pores (pr). (e) TEM of basal cross-section of mechanosensory sensillum, showing the internal sensillum lymph space (isl), microvilli-lined external sensillum lymph space (esl), thecogen cell (th), and ciliary outer dendritic segments (ods). Inset shows ciliary dendrite with axoneme (an) at higher magnification. (f) TEM of longitudinal section of *Drosophila* campaniform sensillum, showing dome-shaped cuticular sensillum shaft (cu), tubular body (tb), dendritic cap (dc), basal body (bb), rootlet fiber (rtf), and inner dendritic segment (ids) surrounded by thecogen cell (th). Scale bar = 10 μm (a); 1 μm (b–f). ((a, b): Reproduced with permission from Bang, A.G., Posakony, J.W., **1992**. The *Drosophila* gene *Hairless* encodes a novel basic protein that controls alternative cell fates in adult sensory organ development. *Genes Devel. 6*, 1752–1769; © Cold spring Harbor Laboratory Press. ©: Reproduced from Keil, T.A., **1997**. Functional morphology of insect mechanoreceptors. *Microsc. Res. Tech. 39*, 506–531; © John Wiley & Sons, Inc. (d, e): Reproduced with permission from Keil, T.A., Steinbrecht, R.A.,**1984**. Mechanosensitive and olfactory sensilla of insects. In: King, R.C., Akai, H. (Eds.), Insect Ultrastructure, vol. 2. Plenum, New York, pp. 477–516.)

external sensilla, represents a *bona fide* glial cell (on the basis of its expressing glial specific markers; Lai and Orgogozo, 2004) that ensheaths part of the sensory soma and axon (**Figure 1a**).

Two cavities filled with hemolymph of special ionic composition surround the dendrite and are crucial for the generation of a receptor potential that takes place in the dendrite (Thurm and Kueppers, 1980). The first cavity, called the external sensillum lymph space (ESL), is formed in the interior of the bristle, where the apical membranes of the trichogen and tormogen cells retreat from the cuticle (**Figures 1a** and **2e**). The ESL is exterior to the thecogen cell and the dendritic sheath. A second, smaller cavity, the inner sensillum lymph space (ISL), forms in between thecogen and sensory dendrite, near the junction of the inner and outer dendritic segments (**Figures 1a** and **2e, f**).

External sensilla present many different morphologies adapted to their modality. Structurally, one distinguishes between hairs (sensilla trichoidea; **Figure 3a** and **b**), bristles (sensilla chaetica; **Figure 3c**),

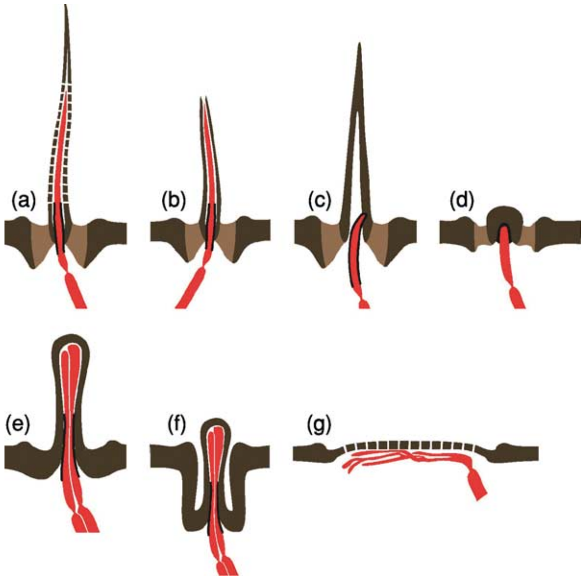


Figure 3 Morphological classification of external sensilla. (a) Multiporous trichoid sensillum (olfactory); (b) uniporous trichoid sensillum (gustatory); (c) aporous bristle (mechanosensory); (d) campaniform sensillum (external stretch receptor); (e) basiconical sensillum (hyogro/thermosensory); (f) coeloconical sensillum (hygro/thermosensory); and (g) hair plate (chemosensory).

domes (sensilla campaniformia; Figure 3d), cones (sensilla basiconica; Figure 3e), sunken cones (sensilla ceoloconica; Figure 3f), and plates (Figure 3g).

Sensilla campaniformia act as external stretch receptors, sensing deformations of the surrounding cuticle. In these sensilla, the dendritic tip is inserted into the apex of the cuticular process, such that cuticular stretching will lead to pressure on the outer dendritic segment, which elicits the receptor potential. Hairs and bristles act as receptors for touch and air flow. Here, the dendritic tip is attached to the base of the hair or bristle and reacts to deflection of these structures (Figure 3c). Cones and sunken cones (Figure 3e and f) are often receptors of temperature and humidity (Altner *et al.*, 1981). The outer dendritic segments fill the lumen of the cone (Figure 3e and f) and are compressed by dilation or contraction of the cone cuticle that occurs in response to changes in heat and moisture.

Both bristle/hair-shaped and cone-shaped sensilla can also act as chemoreceptors. Morphologically, chemoreceptors can be identified by pores in the cuticle which admit the chemical stimuli. Typically, contact chemoreceptors (gustatory) are characterized by a single opening at the tip of the process (uniporous; Figure 3b), whereas olfactory chemoreceptors have multiple pores all along the shaft of the process (Altner, 1977; see **Chapter 1.12**; Figure 3a; see also Figure 2d). In many sensilla, multiple neurons with different sensory modalities are clustered together. In view of the enormous diversity of insect morphology and ecology, this rough classification covers only the major classes of sensilla.

1.11.1.3. Chordotonal Organs

Chordotonal organs are subepidermal stretch receptors, which nevertheless share numerous structural and developmental characteristics with external sensilla (Moulins, 1976). Chordotonal organs typically consist of multiple units called scolopidia. Each scolopidium has a bipolar sensory neuron with a short apical dendrite and a basal axon (Figure 1c). Outer dendritic segments of chordotonal neurons differ from their counterparts in external sensilla in several aspects. The former are typically shorter and straighter (stiffer) than the latter, and have a conspicuous subapical "swelling" called the ciliary dilation (Figure 4a). The anchoring rootlet filaments extending from the basal body at the junction between inner and outer dendritic segment are longer and more massive than their counterparts in external sensilla (Figure 4c and d). The dendrite is encapsulated by the scolopale cell, homologous to the thecogen cell of external sensilla (Figures 1c, d and 4a–c). The scolopale cell forms the dendritic cap around the tip of the dendrite (Figure 4a); further basally, it produces the scolopale, a conspicuous circular array of intracellular, tubulin-rich rods surrounding the dendrite (Figure 4a–c). Between scolopale and dendrite the inner receptor lymph space opens. Basally, the sensory neuron is embedded in the ligament cell which attaches at the epidermis (Figure 4d). The ligament cell, which on the basis of recent findings can be homologized to the glia cell of an external sensillum (Jones *et al.*, 1995; Lai and Orgogozo, 2004; see below), is missing in some chordotonal organs.

The outer accessory cells of a chordotonal organ are represented by the cap cell and the attachment cell which connect the scolopale and the dendrite enclosed in it to the epidermis (Figures 1c and 4a). In this manner, the scolopidium is anchored at two points to the epidermis. Stretching of the epidermis will cause a lengthening of the scolopale, which compresses the dendrite and elicits a receptor potential (Moulins, 1976; McIver, 1985).

1.11.1.4. Type II Multidendritic Sensilla

Besides the sensilla that are innervated by bipolar neurons, the insect sensory system comprises another set of multidendritic subepidermal neurons which act as receptors for pressure and stretch (Finlayson, 1976; Frye, 2001). A subset of these sensory neurons is

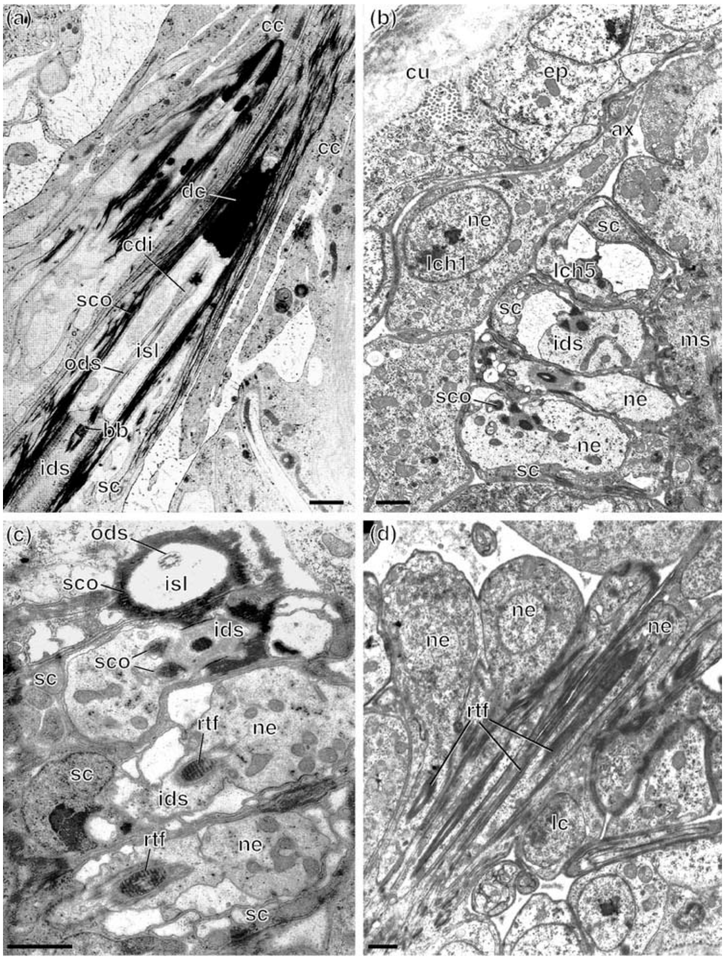


Figure 4 Structure of the mature chordotonal organ. TEMs of sections of chordotonal organs in the cricket (a) and the *Drosophila* larva (b–d). (a) Longitudinal section of apices of two scolopidia, showing inner dendritic segment (ids) and outer dendritic segment (ods). Surrounding the dendrite is the scolopale cell (sc), which forms the scolopale rods (sco) and, at the tip of the dendrite, the dendritic cap (dc). The scolopale cell is inserted at the cap cell (cc). Note the characteristic subapical ciliary dilation (cdi) of the dendrite. (b) Transverse section of lateral pentascolopidial (lch5) and monoscolopidial (lch1) chordotonal organs, which occupy the narrow niche between epidermis (ep) and somatic musculature (ms). The lch5 is sectioned at a level at which the neuronal cell body (ne) continues as the inner dendritic segment (ids). Note the tips of scolopale rods (sco) surrounding dendrite. The lch1 is sectioned at a more basal level where the soma produces an anteriorly directed axon (ax). (c) Tilted transverse section of lch5, showing profiles of scolopidia at different apico-basal levels. The most anterior scolopidium (top) is sectioned furthest apically, showing outer dendritic segment (ods) and scolopale (sco). At this apical level, the scolopale rods have merged into a ring-like structure. The adjacent scolopidium is sectioned at a more basal level near the transition of inner (ids) and outer dendritic segment. The scolopale (sco) is broken up into individual rods. Continuing basally (downwards in the panel) one sees the inner dendritic segment (ids) containing the massive rootlet filaments (rtf). (d) Longitudinal section of base of lch5, showing sensory cell bodies (ne) surrounded by sheaths formed by the ligament cells (lc). Note the thick rootlet filaments (rtf) which continue all the way from the dendrite downwards into the basal extension of the neuronal cell bodies. Scale bar = 1 μm. ((a): Reproduced with permission from Keil, T.A., **1997**. Functional morphology of insect mechanoreceptors. *Microsc. Res. Tech. 39*, 506–531; © John Wiley & Sons, Inc.)

accompanied by specialized ligament cells by which they stretch out alongside muscles; examples are the wing hinge stretch receptors and pleural stretch receptors. Most multidendritic neurons are naked, extending complex dendritic arbors at the basal surface of the epidermis (Figure 5a). These neurons, which have been identified in soft-bodied insects (i.e., mostly larval stages of holometabolans) are called dendritic arbor (*da*) neurons. In *Manduca* and *Drosophila*, four classes (α, β, γ, and δ) with different dendritic complexity, axonal termination, and physiological properties were defined (Grueber *et al*., 2001, 2002; Figure 5b). Members of one class completely tile the epidermis. Thus, dendritic branches of a given *da* neuron form a dense mesh in a given domain of the epidermis. Another *da* neuron of the same class innervates a domain that is contiguous, yet nonoverlapping with the first one. In this manner, the entire epidermis is completely covered with the innervation domains of the *da* neurons of a given class.

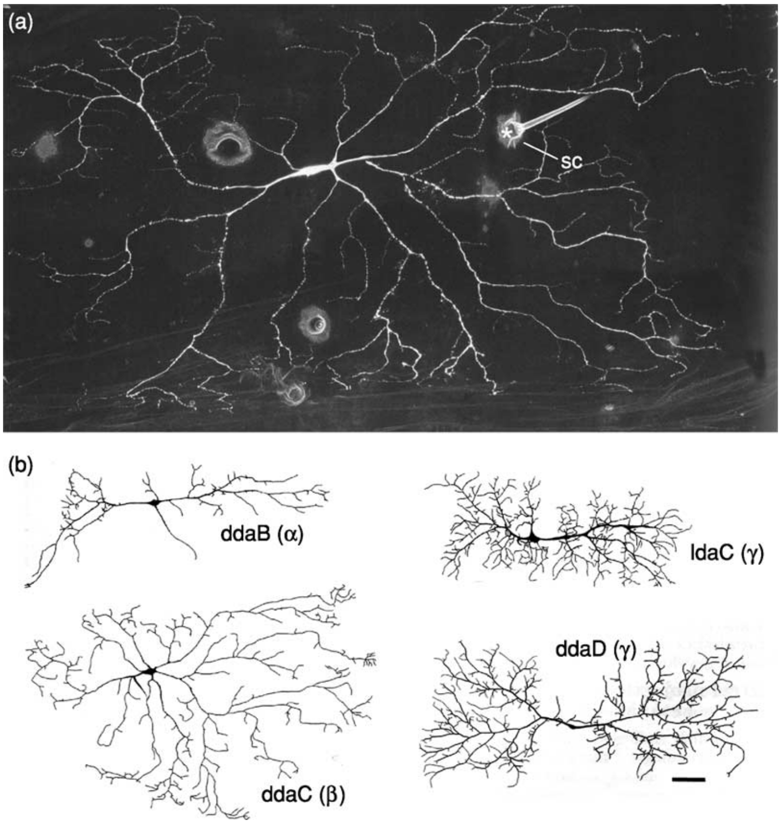


Figure 5 Multidendritic (Type II) neurons. (a) Dye filling of subepidermal multidendritic neuron (β class) in *Manduca*. (b) Line drawings of multidendritic neurons (ddaB, ddaC, ddaD, ldaC; For nomenclature, see **Figure 7a**) that represent the four morphologically defined classes (Grueber *et al.*, 2001). Neurons of class α have a small dendritic arbor with a few branches. Class β neurons are small, but possess highly branched dendrites. Class γ is large and possesses low branch density; δ is large and has high branch density. Scale bar = 100 μm. ((a, b): Reproduced from Grueber, W.B., Graubard, K., Truman, J.W., **2001**. Tiling of the body wall by multidendritic sensory neurons in *Manduca Sexta*. *J. Comp. Neurol. 440*, 271–283.)

1.11.1.5. Pattern of Sensilla

The distribution of sensilla of different structures and modalities is extremely diverse among insects, and only a few generalizations will be made here. Bristle and hair-shaped mechanoreceptors can occur everywhere on the trunk, head, and appendages. These mechanoreceptors typically form large arrays of more or less evenly spaced elements, such as those shown for the dorsal head of *Drosophila* (**Figure 2a**) and the trunk of juvenile *Acheta* (**Figure 6a**). External stretch receptors (campaniform sensilla) form tight clusters on some appendages (e.g., halteres in Dipterans; **Figure 6b**), but also occur as scattered individual sensilla on the trunk.

Chordotonal organs and certain types of type II stretch receptors form clusters laterally and ventrolaterally. In the thorax and head, these lateral chordotonal organs form stretch receptors at the base of the appendages. Large arrays of chordotonal organs in the antenna and (in some insect groups) the thorax have adopted an auditory function. In these cases, a large area of surrounding cuticle and inner tracheal sacs is incorporated into a complex, multichambered structure, called Johnston's organ in the antenna, and the tympanal organ in the leg of many insects, that receives and amplifies sound waves (reviews: Yager, 1999; Caldwell and Eberl, 2002).

Chemoreceptors are found on the appendages and in the mouth cavity (see **Chapter 1.12**). Olfactory bristles and cones are clustered on the antenna and maxillary palp; gustatory hairs occur in the pharynx, but also on the wings and legs. Thermo- and hygroreceptory cones have been described on the antenna of some insects (**Figure 6c**), but also occur on the trunk of various insect larvae (**Figure 6d**).

An impression of the variability of sensillum number and distribution can be gained from **Figure 6e** which compares the pattern of external sensilla found in the abdomen of different insect groups (Green and Hartenstein, 1997). The only feature most of these patterns have in common is that they form a rather narrow vertical array located in the center or somewhat posteriorly to the center of each segment. (As will be discussed in a later section, this

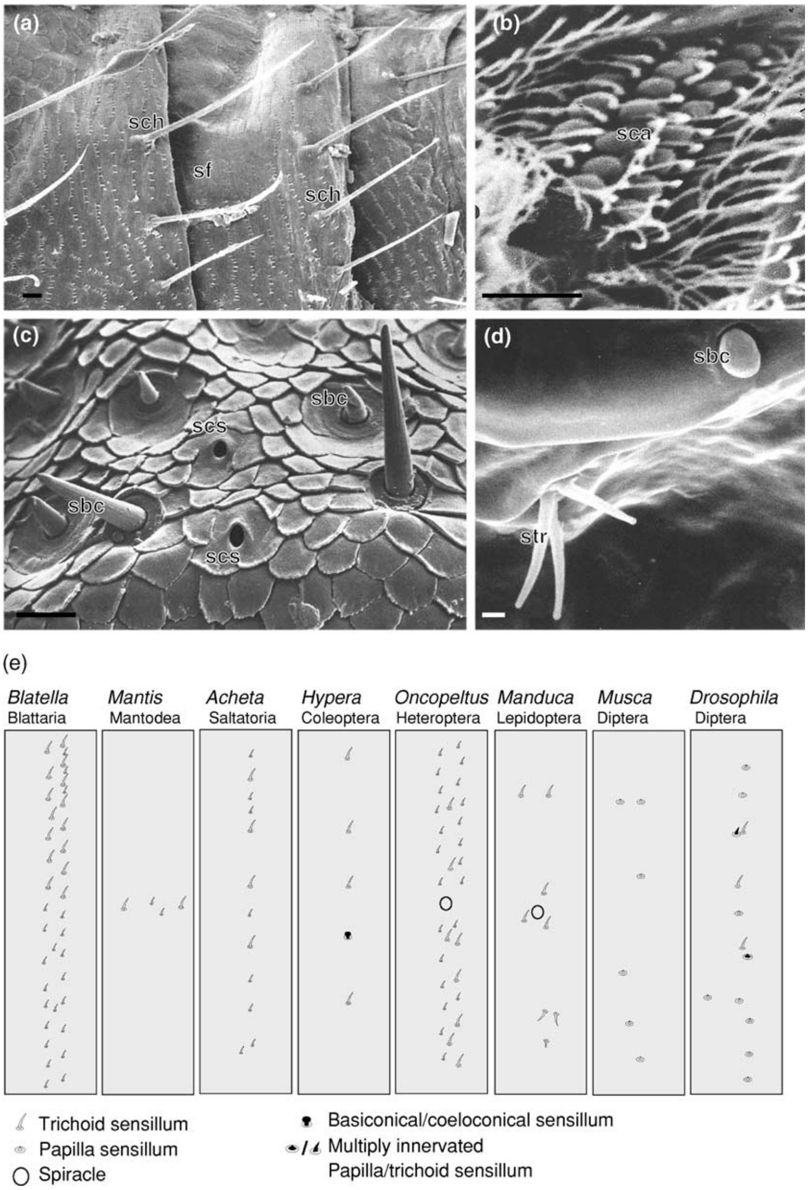


Figure 6 Pattern of external sensilla in different insect groups. (a–d) represent SEMs of parts of the bodywall. (a) Linear arrays of bristles (sensilla chaetica, sch) in the abdomen of juvenile *Acheta*. sf, segment boundary. (b) Campaniform sensilla (sca) on the base of the haltere of adult *Drosophila*. (c) Basiconical (sbc) and coeloconical (scs) sensilla on the antenna of *Locusta*. (d) Basiconical sensillum (sbc) and trichoid sensilla (str) on the ventral thorax of larval *Drosophila*. (e) Schematic depiction of sensillum pattern in eight different insect species whose names and taxa are given at the top of panel. Each rectangle represents one abdominal hemisegment, with the dorsal midline at the top and the ventral midline at the bottom. Different types of sensilla are indicated by different graphic symbols, as shown in key at the bottom of figure. Scale bar = 10 μm (a–c); 1 μm (d). ((a, d, e): Green, P.J., Hartenstein, V., **1997**. Structure and spatial pattern of the sensilla of the body segments of insect larvae. *Microsc. Res. Tech. 39*, 470–478; © Wiley. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (c): Reproduced with permission from Altner, H., Routil, C., Loftus, R., **1981**. The structure of bimodal chemo-, thermo-, and hygroreceptive sensilla on the artenna of *Locusta migratoria. Cell Tissue Res. 215*, 289–308; © Springer-Verlag.)

location, which is also found in the *Drosophila* larva, can be explained in terms of the Wingless morphogen gradient.) However, the types of sensilla vary radically. In *Blatella* and *Oncopeltus* one encounters several rows of trichoid sensilla; in most dipteran larvae, interspersed trichoid sensilla, papilla sensilla (which could be external stretch receptors, similar to campaniform sensilla) and a few conical sensilla form one row; in some dipterans, only papilla sensilla or conical sensilla are found.

Despite the diversity of sensillum patterns in the mature animal (be it larval or adult), there seems to be a high degree of similarity between the patterns in which sensilla assemble in early embryos. This

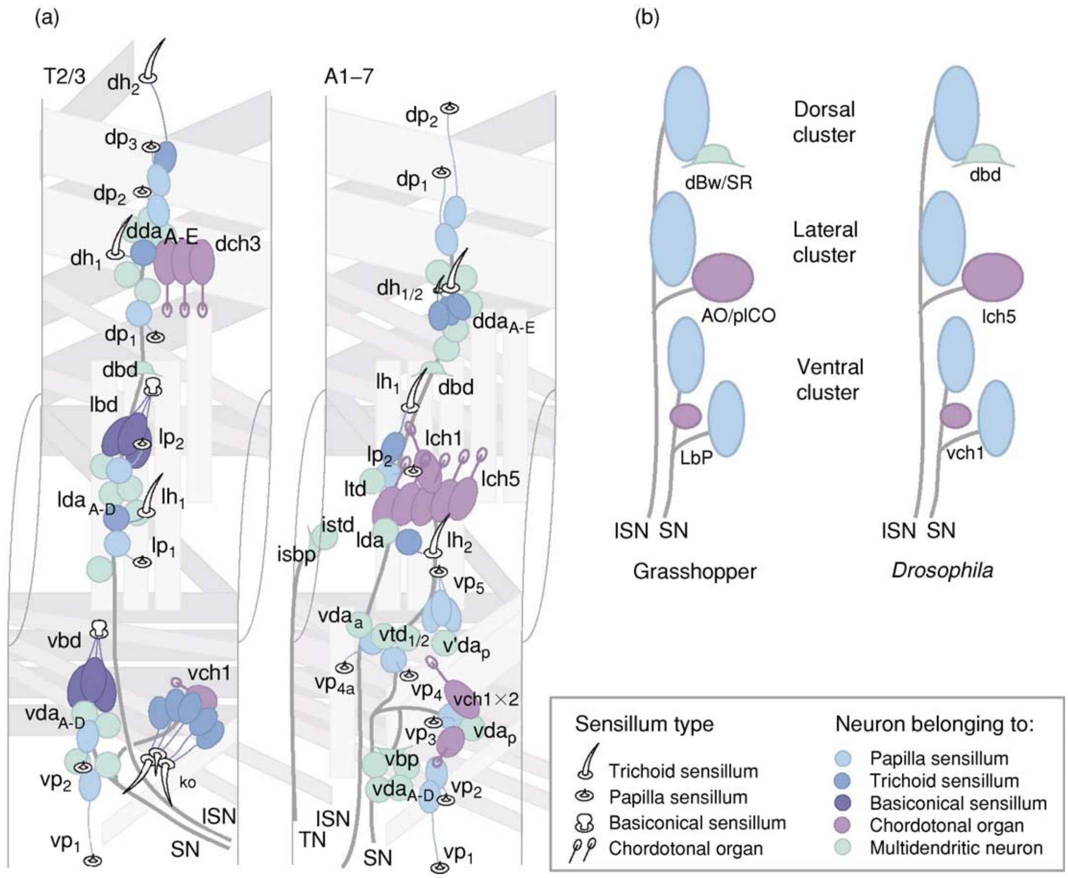


Figure 7 Pattern of sensilla in embryonic *Drosophila* and grasshopper. (a) Schematic drawing of a thoracic (T2/3) and abdominal (A1–7) hemisegment in which sensilla and muscle fibers are depicted. For both hemisegments, the dorsal midline is at the top, the ventral midline at the bottom, anterior is to the left. The location of sensilla is indicated by graphic symbols (see key at bottom right of figure). Somatic muscle fibers are shown in gray. For details on the pattern see text. (b) Simplified pattern of sensillum precursors in embryonic grasshopper and *Drosophila*. Colored profiles show sensory neurons belonging to external sensilla (blue), chordotonal organs (violet), and multidendritic neurons (green). In both species one sees a dorsal, lateral, and ventral cluster of sensillum precursors. Dorsal and lateral sensilla are inervated by the intersegmental nerve (ISN), ventral ones by the segmental nerve (SN). The elements of the sensillum pattern identified by color labeling can be homologized. AO/plCO, lateral chordotonal organ (grasshopper); dbd, dorsal bidenritic neuron (stretch receptor); dBw/SR, wing hinge receptor (grasshopper); dch3, dorsal triscolopidial chordotonal organ; $dda_{A\text{-}E}$, dorsal multidenritic neurons; $dh_{1\text{-}2}$, dorsal hairs (trichoid sensilla); $dp_{1\text{-}3}$, dorsal papilla sensilla; isbp, istd intersegmental multidendritic neurons; ISN, intersegmental nerve; lbd, lateral basiconical sensillum; LbP, ventral chordotonal organ (grasshopper); lch1, lateral monoscolopidial chordotonal organ; lch5, lateral pentascolopidial chordotonal organ; $lda_{A\text{–}D}$, ltd lateral multidendritic neurons; lh_1, lateral hair (trichoid sensillum); $lp_{1\text{-}2}$, lateral papilla sensilla; SN, segmental nerve; TN, transverse nerve; vbd, ventral basiconical sensillum; vch1, ventral monocolopidial chordotonal organs; $vda_{A\text{–}D}$, vbp, vda_a, vda_p, vtd1-2, $v'da_p$, ventral multidendritic neurons; $vp_{1\text{-}5}$, ventral papilla sensilla. ((a) Adapted from Campos-Ortega, J.A., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila melanogaster*, 2nd edn. Springer, Berlin; (b) Adapted from Meier, T., Chabaud, F., Reichert, H. **1991**. Homologous patterns in the embryonic development of the peripheral nervous system in the grasshopper *Schistocerca gregaria* and the fly *Drosophila melanogaster*. *Development 112*, 241–253.)

conclusion can be drawn from the study of Meier *et al.* (1991) who compared the embryonic formation of the peripheral nervous system in the grasshopper and *Drosophila*.

The late embryonic sensillum pattern of *Drosophila*, which will be referred to repeatedly in later sections, is shown in detail in **Figure 7a**. Sensilla of different types form a dorsal, lateral, and ventral cluster (Campos-Ortega and Hartenstein, 1985; Ghysen *et al.*, 1986; Bodmer and Jan, 1987). Dorsal and lateral sensilla project their axons via the intersegmental nerve, whereas ventral sensilla project through the more posterior segmental nerve.

In the abdominal segments, the dorsal cluster comprises two closely spaced trichoid sensilla (dh_1 and dh_2) and two papilla sensilla (dp_1 and dp_2), as well as six multidendritic type II sensory neurons ($dda_{A\text{-}E}$, dbd). The lateral cluster contains two hairs (lh_1, lh_2), one papilla sensillum (lp_2), two multidendritic neurons (ltd, lda), and two chordotonal organs (lch5 with five scolopidia, and lch1 with one scoplopidium). The ventral cluster has

six papilla sensilla (vc1-3, vc4 and vc4a, vc5), ten multidendritic neurons ($vda_{A\text{-}D}$, vda_p, $v'da_p$, vda_a, vtd1-2, vbp), and two chordotonal organs (vch1).

The thoracic pattern of sensilla is similar, with several notable exceptions. The lateral chordotonal organ has shifted towards dorsally (dch3); a multiply innervated conical sensillum, not found anywhere in the abdomen, forms part of the lateral and ventral thoracic cluster (lbd and vbd, respectively); and a star-shaped array of three hair sensilla, the so-called Keilin's organ, is found in the ventral cluster.

If one compares the *Drosophila* embryonic pattern to that in grasshopper embryos, the similarities are striking (**Figure 7b**). In both, embryonic sensilla form three clusters, of which the dorsal and lateral ones project through the intersegmental nerve, and the ventral one through the segmental nerve (Meier *et al*., 1991). A prominent type II stretch receptor (dbd in *Drosophila*, dBw or wing hinge receptor in grasshopper) forms part of the early dorsal cluster and is one of the pioneers of the intersegmental nerve. Laterally chordotonal organs in early clusters of both species; and a ventral chordotonal organ (LbP in grasshopper, vch1 in *Drosophila*). Great similarities also exist in the pattern of multidendritic *da* neurons (subepidermal stretch receptors) between the lepidopteran *Manduca* and *Drosophila* (Grueber *et al*., 2001, 2002).

1.11.1.6. Sensillum Development: Overview

Insect sensilla have attracted the attention of developmental biologists since the early days of experimental biology. It was recognized that, in many sensilla, all cells originate from a single cell, the sensillum progenitor cell (SOP; **Figure 8**), which divides in a stereotyped pattern. SOPs can be easily observed because of their size and staining properties, and they became a classical paradigm for the study of cell fate determination. It was (rightly) assumed that intrinsic factors, expressed by the SOP and forwarded in a specific pattern to its progeny, must act as determinants of the different sensillum cell fates. This assumption has turned out to be correct, and several specific molecules involved in sensillum fate determination have been identified. Insight has also been gained into the cell biological mechanism by which these determinants are assigned to the proper cells during SOP mitosis. Finally, begun to understand the connection between cell fate determinants, which are transcription factors acting in the nucleus of a cell, and the way in which these molecules effect changes in cell structure during sensillum differentiation.

The following sections of this chapter attempt to provide an overview of sensillum development and the mechanisms guiding it. First, it will be seen how SOPs of different types of sensilla are specified, and what mechanisms are responsible for the sensillum pattern. Next, the SOP mitoses generating the different SOPs will be followed. The main focus of attention during this phase in sensillum development rests on the mechanism controlling the mitotic spindle and thereby influences theexpression of sensillum cell type specific determinants in the proper cells. Furthermore, cell–cell signaling events among the sensillum cell precursors, representing the second source of information guiding sensillum cell fate, will be considered. Next, the morphological aspects of sensillum differentiation, consisting of stereotyped changes in cell shape and position, will be analyzed. The last section will look beyond insect sensilla and address the evolutionary connections between these organs and sensory structures in other animal groups.

1.11.2. The Specification of SOPs

1.11.2.1. General Characteristics of SOPs

SOPs are derived from the embryonic ectoderm and, in holometabolous insects such as *Drosophila*, from the imaginal discs that form the adult body (see also **Chapter 1.10**). Structurally, SOPs can be distinguished from their epidermal neighbors by their larger size, more basal nucleus, and protracted proliferation that results in the production of sensillum lineages (Cubas *et al*., 1991; Skeath and Carroll, 1991; Hartenstein *et al*., 1994; LeBorgne *et al*., 2002; **Figure 8a** and **b**). The pattern of SOPs largely reflects the later pattern of sensilla they give rise to; following SOP division, SOP cells remain at the location where they were born and differentiate. Only the position of somata of neurons and inner accessory cells occasionally shifts away from the location where these cells were initially placed into the ectoderm, due to the lengthening of the dendrite and dendritic sheath.

1.11.2.2. Proneural Clusters and Proneural Genes

SOPs are selected from the ectoderm in a two-step mechanism (**Figure 8c**). First, relatively large clusters of contiguous ectodermal cells, called proneural clusters, become competent to develop as SOP. In some cases, such as the large bristles (macrochaetes) on the *Drosophila* notum (**Figure 8a** and **b**), each sensillum is represented by a proneural cluster (Cubas *et al*., 1991). More typically, arrays of closely spaced sensilla, such as the microchaetes on the notum, or the sensilla on the antenna, legs, and wing margin, come from stripe-like proneural domains (**Figure 8b**).

Proneural clusters can be visualized by the expression of a family of transcription factors called proneural genes (Campos-Ortega, 1997; Hinz, 1997; Modolell, 1997; Caldwell and Eberl, 2002; Skaer

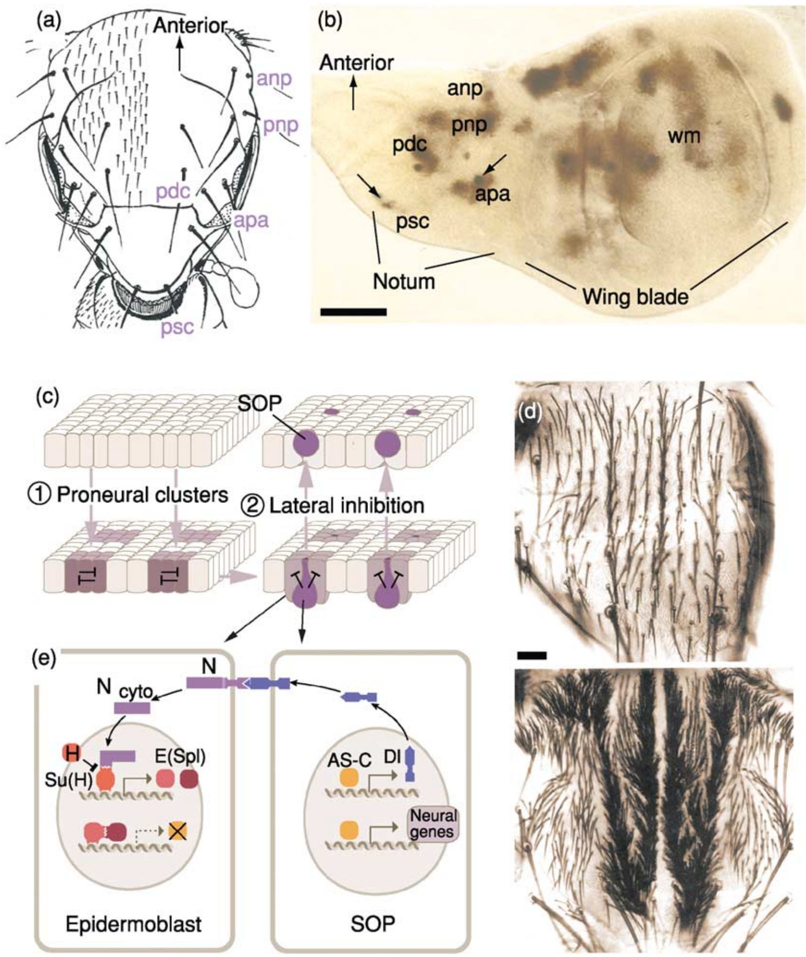


Figure 8 Genetic control of sensillum patterning. (a) Schematic drawing of *Drosophila* notum (dorsal thorax) indicating invariant pattern of macrochaetes. Some of the macrochaetes are identified individually. anp, anterior notopleural; apa, anterior postalar; pdc, posterior dorsocentral; pnp, posterior notopleural; psc, posterior scutellar. (b) Expression of the proneural gene *scute* (blue) in wing imaginal disc. SOPs are double-labeled (brown) by expression of a reporter gene construct driven by the *neuralized* promoter. In the proximal region of the disc which gives rise to the notum, *scute* is expressed in proneural clusters which can be assigned to individual macrochaetes. Arrows indicate SOPs which have emerged from some of the clusters. In the distal region, one large stripe of *scute* expression coincides in position with the prospective wing margin (wm) and foreshadows the appearance of several rows of microchaetes. (c) Two-step model of SOP specification. During step 1, small groups of contiguous cells (proneural clusters) express a given proneural gene and thereby become committed towards a neural fate. In step 2, lateral inhibition within proneural clusters results in the specification of only one SOP, whereas the remainder of the cells within a proneural cluster become epidermoblasts. (d) Pattern of bristles on the notum of wild-type *Drosophila* (upper panel). Removing *Notch* function at the time interval when lateral inhibition of notum SOPs occurs (8–12 h after puparium formation) causes a strong hyperplasia of bristles. (e) The Notch–Delta signaling pathway. For details see text. Scale bar = 50 μm (b), 10 μm (d). ((a) Adapted from Ferris, G.F., **1965**. External morphology of the adult. In: Demerec, M. (Ed.), Biology of *Drosophila*. Hafner, New York. (b): Reproduced with permission from Cubas, P., de Celis, J.F., Campuzano, S., Modolell, J., **1991**. Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Devel. 5*, 996–1008; © Cold spring Harbor, Laboratory press. (d): Reprinted with permission from Hartenstein, V., Posakony, J.W., **1990**. A dual function of the *Notch* gene in *Drosophila* sensillum development. *Devel. Biol. 142*, 13–30; © Elsevier.)

et al., 2002) (see **Chapter 1.10**) (**Figure 8b**). Proneural genes are essential for the development of SOPs. In the absence of these genes, SOPs will not form. In *Drosophila*, several different proneural genes have been identified to date. All of them belong to the family of basic helix-loop-helix (bHLH) proteins (see **Chapter 1.8**). Three of them, *achaete* (*ac*), *scute* (*sc*), and *lethal of scute* (*l'sc*) form part of a large gene complex, the Achaete-Scute complex (AS-C), which also encodes other transcription factors involved at later steps of sensillum development. These three genes are expressed in and required for proneural clusters that give rise to mechano-sensory external sensilla and most type II multidendritic neurons. *atonal* (*ato*) determines proneural clusters for chordotonal organs, chemoreceptors, and photoreceptors (Jarman *et al.*, 1993,

1995) (see **Chapter 1.12**), and *amos* (Goulding *et al.*, 2000) is expressed in chemoreceptors and some type II sensilla.

1.11.2.3. Neurogenic Genes and Lateral Inhibition

During a second step, the number of SOPs formed in each proneural cluster is reduced by a mechanism called lateral inhibition (**Figure 8c**). Thus, proneural genes activate a signaling pathway in the cells in which they are expressed, the Notch/Delta (N/Dl) pathway, which inhibits proneural gene expression in all but a few cells (Muskavitch, 1994; Campos-Ortega, 1995; Artavanis-Tsakonas *et al.*, 1999). Genes encoding elements of the lateral inhibition pathway, such as the gene *Notch* that encodes the receptor, *Delta* (encoding the Notch ligand), or *Enhancer of split complex* (*E(spl)*; encoding transcription factors inhibiting proneural genes) are called neurogenic genes, because, in their absence, a large excess of SOPs develops. Thus, in normal development, cells which turn down proneural genes due to N/Dl signaling will join the cells outside the proneural clusters and develop as epidermal cells. If the N/Dl signaling pathway is deficient, all cells of the proneural cluster become SOPs, resulting in many supernumerary sensilla and holes in the epidermis (**Figure 8d**).

As seen in the later section, N/Dl signaling is also a key mechanism distinguishing between the different sensillum cell fates, and the elements of this pathway should therefore be introduced in some detail (**Figure 8e**). Upon binding to a signaling protein (e.g., Delta), the cytoplasmic domain of the Notch receptor is cleaved and translocates to the nucleus where it activates target genes, notably the transcription factors encoded by the Enhancer of split Complex. These proteins promote the development of epidermal cells, as opposed to SOPs, by inhibiting the proneural genes. E(spl) proteins belong to the group of Hairy-related proteins, a distinct subfamily of bHLH proteins that generally operate as DNA-binding transcriptional repressors. They act in opposition to bHLH transcriptional activator proteins (such as the proneural proteins); together, the activator and repressor genes that encode these proteins have co-evolved as a regulatory gene "cassette" involved in many cell fate decisions, including the formation of sensory organs.

The activation of *E(spl)* requires a number of additional nuclear proteins that form a complex with Notch and, in a context-dependent manner, either activate or inhibit Notch function. The DNA-binding transcription factor Suppressor of Hairless (Su(H)) functions as an activator when Notch signaling is active, but can act as a repressor in the absence of signaling. Hairless (H) antagonizes N signaling by inhibiting DNA binding of Su(H) (Lecourtois and Schweisguth, 1997; Artavanis-Tsakonas *et al.*, 1999; Bray and Furriols, 2001).

1.11.2.4. SOP Induction

For a number of sensilla which occur in a tight cluster, such as campaniform sensilla on the appendages or the lateral chordotonal organs of the *Drosophila* embryo, SOPs are determined in a specific sequence, and early SOPs send out signals that induce later ones. The induction of SOPs has been studied for the embryonic chordotonal organ lch5, which comprises five scolopidia aligned in a row. Only the SOPs for three scolopidia express and require the proneural gene *atonal*. The SOPs of the other two scolopidia are induced by the Transforming Growth Factor α(TGFα)-like signaling molecule Spitz (Spi), which activates the MAP kinase (MAPK) signaling pathway (Lage *et al.*, 1997). This signaling pathway mediates a number of inductive events in the developing nervous system of *Drosophila*, notably the specification of photoreceptors and accessory cells in the compound eye.

1.11.2.5. SOP Identity Genes

Although initially indistinguishable, SOPs become different from each other as they undergo mitosis, giving rise to different types of sensilla. This implies that, from an early stage onward, they express determinants responsible for the different SOP fates. First among these determinants are the proneural genes which, after SOPs have become selected from the proneural clusters, are enriched in the SOPs. As described above, the expression of five proneural genes, *ac*, *sc*, *l'sc*, *ato*, and *amo*, accounts for the determination of three fundamentally different classes of sensilla, namely mechanosensory external sensilla, chordotonal organs, and chemosensory external sensilla. Loss of any of these genes causes the corresponding class of sensilla to be absent. In addition to the proneural genes, several transcription factors expressed in SOPs slightly later were identified. Deserving of particular mention among these are *cut* (*ct*) and *pox-neuro* (*poxn*), both encoding proteins containing a homeodomain. *cut* is expressed in SOPs of all external sensilla, *poxn* in SOPs of chemoreceptors and thermo/hygroreceptors (Bodmer *et al.*, 1987; Blochlinger *et al.*, 1990; Dambly-Chaudiere *et al.*, 1992; Vervoort *et al.*, 1995; Awasaki and Kimura, 1997). Loss of *ct* causes the transformation of external sensilla into

chordotonal organs; loss of *poxn* results in a transformation of chemoreceptors into mechanoreceptors. In the case of *ct* it was shown that this gene suppresses the expression of *atonal* in external sensilla, explaining the loss of *ct* function phenotype as the result of an expansion of the *ato* expressing domain. Much remains to be learned about the interplay between proneural and SOP identity genes.

1.11.2.6. Morphogen Gradients and Coordinate Genes

The expression pattern of proneural genes roughly determines the pattern of sensilla on the bodywall of an insect. That leads to the question of how the proneural gene expression pattern itself is controlled. Although our knowledge concerning this issue is still very preliminary, a general mechanism involving morphogen gradients (see also **Chapters 1.8** and **1.9**) and coordinate genes is at the root of the process (**Figure 9**). Morphogens are envisioned as diffusible molecules that are released from one or more discrete sources. As a result, the concentration of these morphogens is high near the source, and decreases with increasing distance from the source (**Figure 9a**).

Morphogens act as signals that control gene expression in a concentration-dependent manner. Thus, cells receiving a high concentration of the morphogen express genes that are different from those expressed by cells encountering a low morphogen concentration. In this manner, the concentration of the morphogen within its dorsal-to-ventral gradient is thought to provide cells in a developmental field with the positional information that directs their differentiation at a given position. Assuming a two-dimensional field, such as the ectodermal domain that will give rise to one segment of the insect body, a minimum of two morphogens would be required to determine the anterior–posterior (ap) and dorsal–ventral (dv) coordinates that constitute the positional information of a given cell (**Figure 9b**).

Morphogens act on patterning by controlling the pattern of expression of a diverse group of genes, which can best be described as coordinate genes. These genes will appear in domains shaped like stripes, oriented either longitudinally (if the corresponding gene responds to the dorsoventral morphogen gradient) or transversally (if it is affected by the anteroposterior gradient). The coordinate genes in turn will control determinants (such as the proneural genes) for specific structures that are destined to develop in the corresponding domains (**Figure 9a**).

The role of morphogens and coordinate genes in the context of sensillum patterning has been studied in most detail in the *Drosophila* embryonic ectoderm and imaginal discs. A molecule fitting the description of a dorsoventral morphogen is Decapentaplegic (Dpp), a member of the TGF family of growth factors, which is expressed at the dorsal margin of the ectoderm (**Figure 9d**). In the imaginal discs, Dpp is expressed at the most proximal parts, which give rise to the dorsal epidermis of the adult body (e.g., the notum in the case of the mesothoracic wing disc; see **Chapter 1.9**). Lowering the Dpp concentration results in the lack of dorsal structures (dorsal sensilla and other epidermal specializations) and the encroachment of ventral structures into dorsal territories (Tomoyasu *et al.*, 1998; deCelis *et al.*, 1999). Two of the coordinate genes dependent on Dpp are *pannier* (*pnr*; a transcription factor of the GATA family), and *iroquois* (*iro*; a homeodomain protein; Gomez-Skarmeta *et al.*, 1996; Leyns *et al.*, 1996; Garcia-Garcia *et al.*, 1999). *Pnr* requires a high Dpp level and is therefore expressed right next to the dorsal midline (**Figure 9e and f**); Iro appears laterally of Pnr (**Figure 9e**). Both Pnr and Iro are required for the upregulation of the proneural gene *sc* in the proneural domains that fall within their respective stripes of expression (**Figure 9i–l**).

Two other secreted signals, Wingless (Wg) and Hedgehog (Hh), play a role in patterning sensilla by affecting proneural gene expression. In the embryo, both are expressed in transverse stripes flanking the segment boundaries, thereby forming gradients along the ap axis (**Figure 9c**). In the wing imaginal disc, Wg is expressed along the margin, and Hh in a longitudinal stripe along the middle of the wing blade. Although the molecular details of how Wg or Hh interact with proneural genes are still unknown, they are both required for the expression of these genes (Phillips and Whittle, 1993; Garcia-Garcia *et al.*, 1999; Phillips *et al.*, 1999). In embryos lacking Wg, proneural genes are not expressed and no sensilla form in any of the trunk segments (**Figure 9h**). Likewise, Wg loss during wing development causes the absence of proneural genes along the wing margin.

1.11.3. Control of SOP Division and Sensillum Cell Fate

1.11.3.1. The Canonical SOP Lineage

Recent studies using high resolution markers were able to visualize division events that had previously been overlooked. These led to the satisfying conclusion that there exist a few, if not only one, fundamental SOP lineages that can account for the different types of sensilla, including external sensilla, chordotonal organs, and type II multidendritic sensilla (Lai and Orgogozo, 2004).

In most cases, all individual cells of a sensillum are born in three consecutive divisions. The SOP,

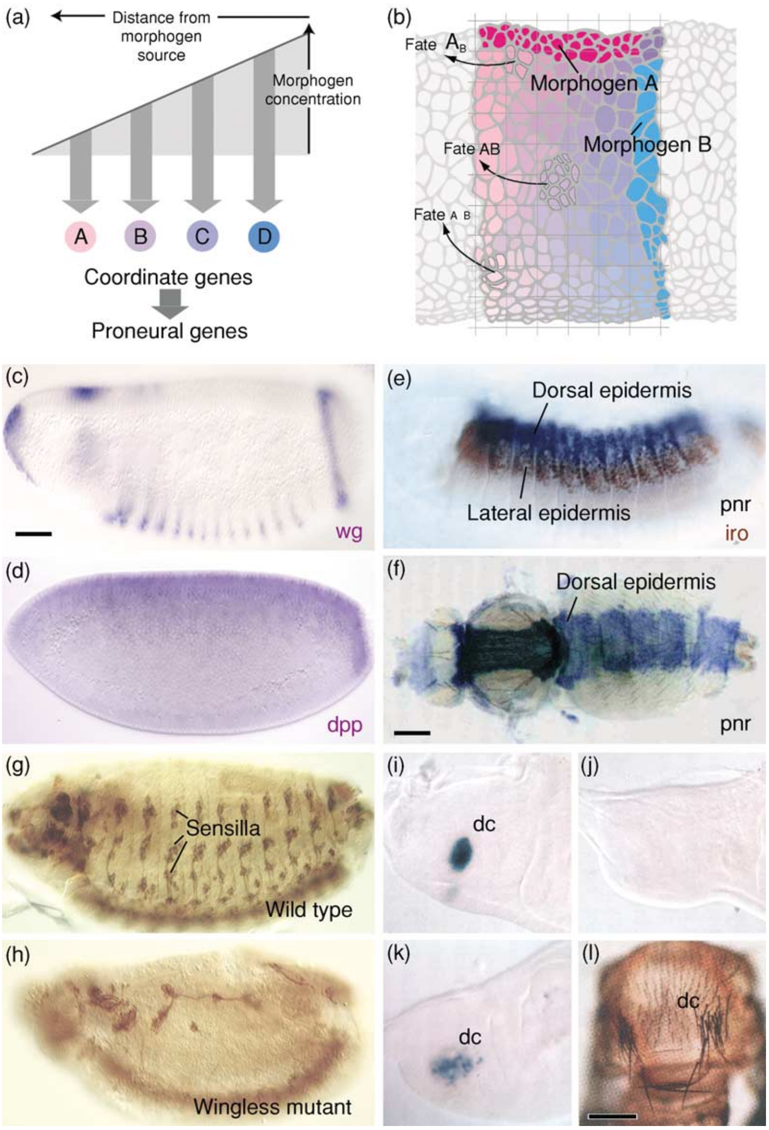


Figure 9 Morphogen gradients control proneural genes via coordinate genes. (a) Schematic diagram illustrating the concept of concentration-dependent activation of coordinate genes by morphogen. (b) Schematic diagram of a developmental field exemplified by a *Drosophila* embryonic segment. Ectoderm cells are indicated by gray outlines. Two morphogens, A (red) and B (blue) are produced along the dorsal and posterior segment boundary, respectively. Their localized secretion causes a graded concentration across the segment. Like a point in a Cartesian coordinate system, each cell in the field is characterized by a certain concentration AB of the two morphogens and adjusts its fate accordingly. (c) Expression of the morphogen gene *wingless* in segmentally reiterated stripes in the early *Drosophila* embryo. (d) Expression of the morphogen gene *dpp* along the dorsal midline of the early *Drosophila* embryo. (e) Expression of the coordinate genes *pannier* (blue) and *iroquois* (brown) in the *Drosophila* embryo. (f) Expression of *pannier* in the dorsal epidermis of the adult fly. (g) Late *Drosophila* wild-type embryo in which sensilla were labeled with the antibody Mab22C10. (h) Late *wingless* mutant embryo. Sensilla are all but absent. (i) *Scute* expressing proneural cluster of the dorsocentral bristles (dc) in the *Drosophila* wing imaginal disc. (j) In a *pannier* mutant, *scute* is not expressed in the dc cluster. (k) Overexpression of *pannier* causes the expansion of the dc cluster. (l) *pannier* overexpression causes an increase in the number of dc bristles. Scale bar = 50 μm (c, d, e, g–k); 200 μm (f); 100 μm (l). ((e, f): Reproduced with permission from Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., *et al.*, **2000**. Generation of medial and Lateral dorsal body domains by the *pannier* gene of *Drosophila. Development 127*, 3971–3980; © The Company of Biologists Ltd. (i–l) Garcia-Garcia, M.J., Ramain, P., Simpson, P., Modolell, J., **1999**. Different contributions of *pannier* and *wingless* to the patterning of the dorsal mesothorax of *Drosophila*. *Development 126*, 3523–3532.)

also called the primary precusor cell or pI, divides into two pII daughters called pIIa and pIIb (Figure 10a1). PIIa divides once more and gives rise to the outer accessory cells, the tormogen, and trichogen cell for the case of an external sensillum, and the cap cell and attachment cell for the case of a chordotonal organ (Figure 10a4, b and c). PIIb gives rise to the sensory neuron(s) and inner accessory

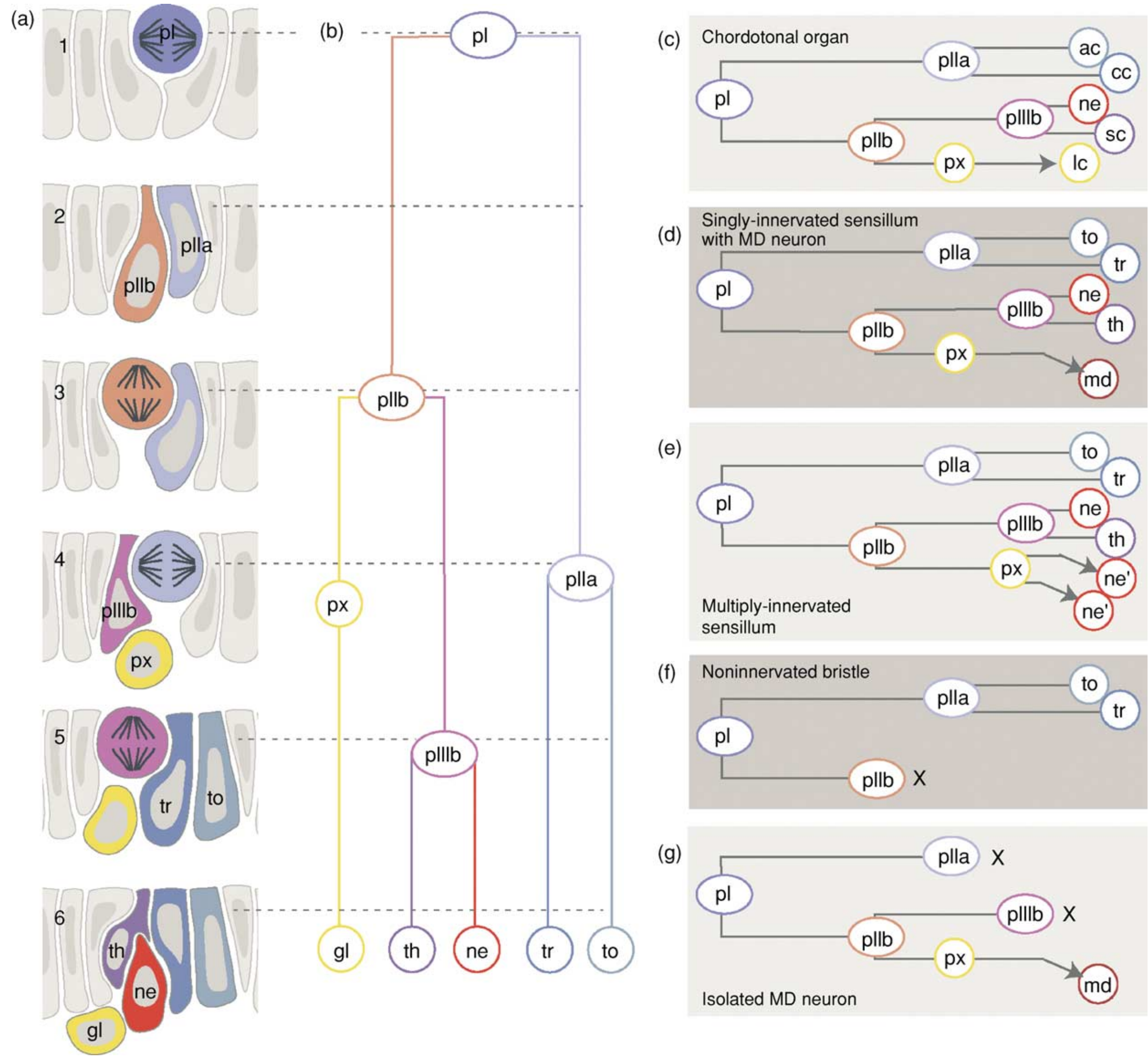


Figure 10 The SOP lineage. (a) Series of schematic sections of the *Drosophila* microchaete SOP. The initial division of this cell (a1) takes place with a horizontal mitotic spindle. The daughter cells resulting from this division are the two secondary precursors, pIIa and pIIb, which lie beside each other in the epithelium of the pupal notum (a2). Subsequently pIIb divides with a vertical spindle (a3), causing one of its daughters, pX, to be located subepidermally (a4), whereas the other daughter, pIIIb, remains within the epithelium. Panel a4 also depicts the horizontal division of pIIa which produces two postmitotic cells, the presumptive tormogen cell (to) and the trichogen cell (tr; a5). PIIIb undergoes its final division with another vertical spindle (a5), forming an epithelial thecogen cell (th) and a subepidermal neuron (ne; a6). (b) Tree diagram of the canonical microchaete SOP lineage. Hatched lines spanning between panels a and b match the division events shown graphically in (a) to the lineage tree shown in (b). (c–g) Lineages of SOPs that produce a variety of different sensilla (indicated at the top or bottom of each panel). Note that all of these lineages represent variations on them represented by the canonical lineage shown in (a) and (b). For details see text. ((a) Adapted from LeBorgne, R., Bellaiche, Y., Schweisguth, F., **2002**. *Drosophila* E-cadherin regulates the orientation of asymmetric cell division in the sensory organ lineage. *Curr. Biol. 12*, 95–104. (c) Adapted from Lai, E.C., Orgogozo, V., **2004**. A hidden program in *Drosophila* peripheral neurogenesis revealed: fundamental principles underlying sensory organ diversity. *Devel. Biol. 269*, 1–17.)

cells in two consecutive divisions. The first division produces precursor pIIIb, which divides into sensory neuron and thecogen cell (external sensilla) or scolopale cell (chordotonal organs), and a postmitotic cell which can adopt a variety of different fates (pX; Figure 10a4 and b). These fates include that of a glial cell (many external sensilla; Figure 10b), ligament cell (many chordotonal organs; Figure 10c), or a multidendritic type II sensory neuron (Figure 10d). In cases of multiply innervated (chemosensory) sensilla, pX undergoes additional rounds of divisions giving rise to the extra neurons (Figure 10e).

Shortly after the SOP has gone through this stereotyped sequence of mitoses, SOPs are located at

characteristic locations relative to the epidermal epithelium. In external sensilla, the tormogen, trichogen, and thecogen cell form an integral part of the epidermis, and only the neuron(s) and glial cell are subepithelial (**Figure 10a6**). In chordotonal organs, all cells are subepidermal; the same is the case for multidendritic neurons.

The cell type-specific location is at least in part the result of the orientation of the mitotic spindle during the SOP division. In external sensilla, division of pI is typically in the plane of the epithelium, so that both pIIa and pIIb remain side by side in the epithelial layer (**Figure 10a1** and **a2**). Similarly, pIIa divides horizontally and forms the two epithelial outer accessory cells (**Figure 10a4** and **a5**). The relative position of the daughter cells resulting from these two divisions is not random. For example, in the michrochaetes of the *Drosophila* thorax, pIIb is always located anterior to pII, and the trichogen cell is anterior to the tormogen cell. The position of these cells seems to play an important role in later sensillum differentiation. Thus, the bristle shafts of microchaetes are all directed posteriorly (**Figure 8d**). Experiments that alter the mitotic spindle of pI and/or pIIa (see below) result in misoriented microchaetes.

Unlike pIIa, which divides horizontally and with a spindle oriented like that one of pI, pIIb typically switches spindle orientation, such that the pX daughter (to become glial cell or multidendritic neuron) is pushed subepidermally, and pIIIb remains intraepithelial (**Figure 10a3**). PIIIb (in those few cases where it has been followed in detail) also seems to divide vertically, with the thecogen cell remaining intraepithelial, and the neuron subepidermal (**Figure 10a5** and **a6**).

Spindle orientation has not been investigated in sufficient detail for chordotonal lineages. It has been reported that for most of the embryonic chordotonal organs in *Drosophila*, the primary pI delaminates and divides in a subepidermal location (Younossi-Hartenstein and Hartenstein, 1997). This would explain the subepidermal location of the accessory cells of a chordotonal organ, whose counterparts in external sensilla form an integral part of the epidermis.

SOP lineage patterns that deviate from the above canonical pattern have been described for some sensilla. For example, olfactory sensilla in numerous insect orders possess three outer accessory cells, whereby the socket is formed by an outer and inner tormogen cell, rather than a single tormogen cell (Keil, 1992). (see also **Chapter 1.12**). In this case it was observed that the outer tormogen cell derives from an additional mitosis which precedes the pI division. In other words, a cell "p0" divides into the outer tormogen cell and a second daughter, which subsequently divides like the classical pI. A similar p0 division, resulting in an outer accessory cell and a pI-like progenitor, was seen in chordotonal organs of *Periplaneta* (Bloechl and Selzer, 1988) and in *Limnophilus* (Roensch, 1954). In evaluating this and numerous other division SOP lineage patterns, it should be borne in mind that, in the absence of injected or molecular markers, the fate of individual cells of the SOP lineage prior to their differentiation is very difficult to follow. Even in *Drosophila*, the pIIb division was overlooked by all authors prior to the studies by Manning and Doe (1999) and Reddy and Rodrigues (1999), even though some limited molecular labeling was available from the outset of such studies. It seems appropriate to feel fairly confident about the canonical lineage for most sensilla in *Drosophila*, but, also, to reinvestigate lineage patterns that have been reported to be different in other systems as molecular markers become available and the need arises.

Apoptosis (see **Chapter 2.5**) plays an essential role in several SOP lineages and reduces the number of cells that make it into the mature sensilla. In the *Drosophila* microchaete lineage, most pX cells undergo programmed cell death (Fichelson and Gho, 2003), and only a few cells survive to form glial cells around the sensory axons. In mature mechanoreceptive external sensilla, trichogen cells are frequently absent; these cells, after having deposited the cuticle-forming bristle shaft, undergo apotosis. Apoptosis also removes a fraction of sensory neurons in multiply chemosensory neurons in the lepidopteran *Pieris* (Lee and Altner, 1985). Apoptosis is also critical in the formation of noninnervated hairs, such as those found along the posterior margin of the fly wing, or scales in lepidopteran wings. These noninnervated epidermal appendages derive from progenitors that divide according to the canonical pattern into a pIIa and pIIb, followed by apoptosis of pIIb (**Figure 10f**). Finally, some of the *Drosophila* larval type II multidendritic neurons develop from SOP lineages in which all but the pX cell are removed by apoptosis (**Figure 10g**; Lai and Orgogozo, 2004).

In the canonical SOP lineage described above, sensilla derive from a single pI that divides into a pIIa and pIIb daughter cell. However, sensilla have been described, where "switching" of pIIa cells and pIIb cells occurs. Cases in point are the dense arrays of sensilla on the *Drosophila* wing margin and antenna (Reddy *et al.*, 1997). Here, SOPs also divide into secondary precursors with the characteristics of pIIa (producing tormogen and trichogen cell) and pIIb (producing neurons and thecogen cell). However, the pIIa of one SOP can join the pIIb of a different (adjacent) SOP to become part of one

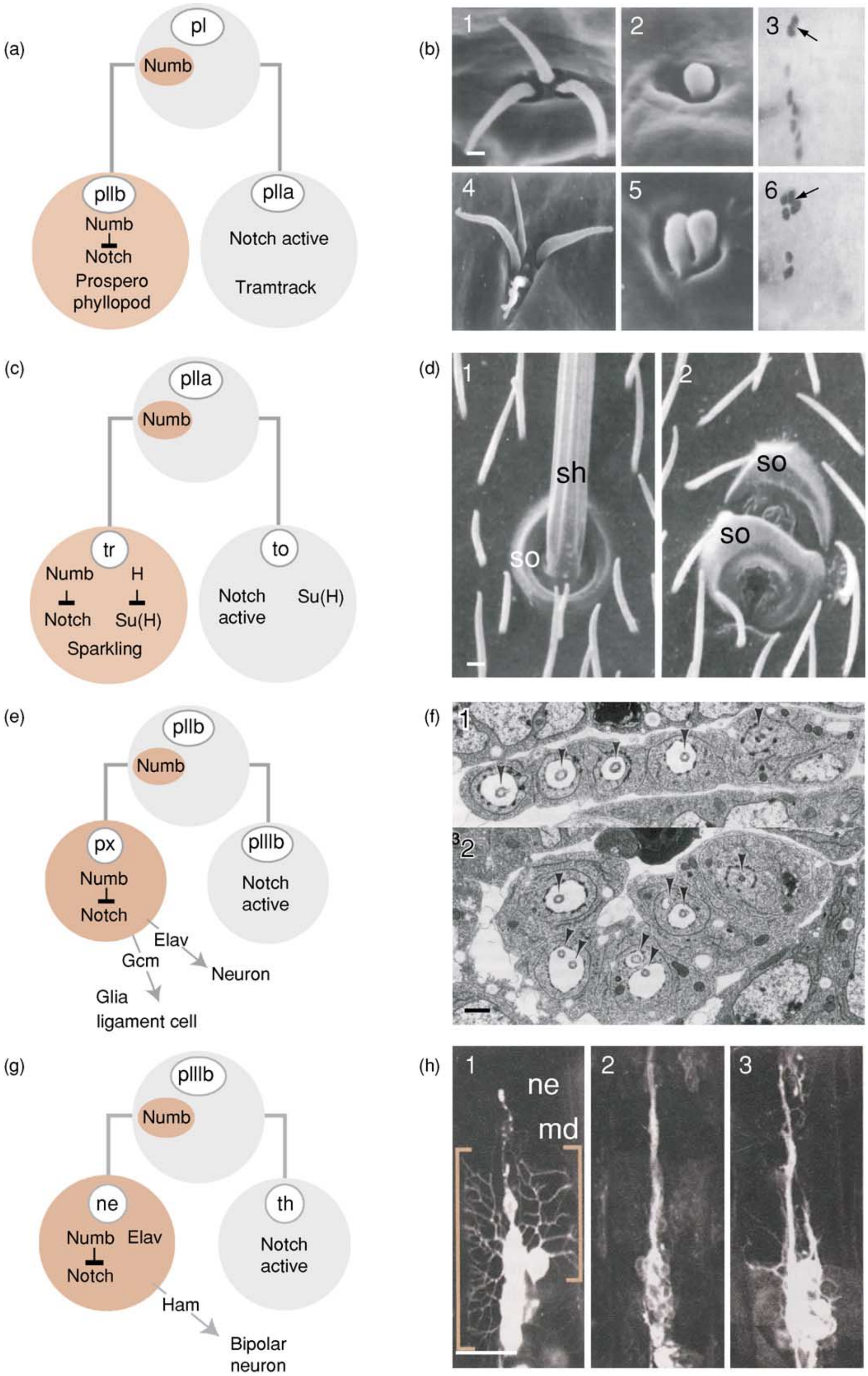


Figure 11 Distribution of fate determinants and Notch signaling in the SOP lineage. (a) During the pI mitosis, Numb, an inhibitor of Notch signaling, is distributed to pIIb. (b) Loss of Numb in pI causes transformation of pIIb into pIIa, with a concomitant doubling of outer accessory cells and absence of neuron/thecogen cell. Examples of this transformation are the Keilin's organ which, in wild type (b1) has three shafts/sockets and, in *numb* mutant (b4), has five to six shafts/sockets. Likewise, duplication of the shaft occurs in the basiconical sensillum vbd (b2): wild type; (b5): *numb* mutant). (b5) shows molecular marker Cut expressed in two outer

individual sensillum. Thus, cell–cell interaction must occur between pII cells, which make sure that one pIIa always becomes conjoined with one pIIb. Observations such as this one support the idea that at an earlier stage in evolution, sensory neurons probably employed signals that recruited neighboring cells into an accessory cell fate (Lai and Orgogozo, 2004; see below).

1.11.3.2. Intrinsic Determinants, the Notch Pathway, and Sensillum Cell Fate

A considerable number of proteins expressed in various parts of the SOP lineage have been identified. Most of these "sensillum cell determinants" have proven essential for the fate of the cell in which they are expressed, in that their absence causes loss or malformation of the corresponding cell. However, little is as yet known about the way in which these intrinsic factors control sensillum cell differentiation. It seems clear that their effect has to be quite indirect in most cases, since the proteins found in specific sensillum cells are also expressed in several other cell types, such as the cells of the compound eye. The other generalization that can be made is that most of the sensillum cell determinants are controlled by the N/Dl signaling pathway, or are themselves part of the mechanism controlling this pathway. This finding underlines the notion that sensillum cell fate determination is the outcome of intricately linked intrinsic control mechanisms and cell–cell signaling (reviews: Posakony, 1994; Cayouette and Raff, 2002; Lai and Orgogozo, 2004; see also **Chapter 1.10**).

1.11.3.2.1. The pI division – pIIa and pIIb Asymmetric expression of sensillum fate determinants originates with the first division of pI into pIIa and pIIb (**Figure 11a**). A crucial factor in generating this asymmetry is the protein Numb. In pI, the Numb protein becomes localized to one side of the cytocortex, so that when this cell divides, only one daughter "inherits" Numb (**Figure 11a**) (Uemura *et al*., 1989; Rhyu *et al*., 1994; Guo *et al*., 1996; Frise *et al*., 1996; **Chapter 1.10**). The cell containing Numb becomes pIIb. The role of Numb in pIIb is to disrupt the N signaling pathway, by preventing N from translocating to the nucleus. Thus, upon activation of the N receptor in both pIIa and pIIb, the signal can get through only in pIIa. If *numb* is deleted, N can act in both pII cells, which changes the fate of pIIb to that of pIIa, and causes a duplication of outer accessory cells and loss of neuron/thecogen cell (**Figure 11b**).

Inhibition of N in pIIb causes several subsequent transcriptional regulators important for the neural fate to be expressed in this cell. Among these is Prospero (Pros), a transcription factor which appears in pIIb and both of its daughters, the neuron and thecogen cell. Subsequently, Prospero is downregulated in the neuron but maintained in the thecogen cell (Manning and Doe, 1999; Reddy and Rodrigues, 1999). Loss of *prospero* causes the same phenotype as that following loss of *numb*, i.e., the conversion of pIIb into pIIa, thus producing two trichogen and two tormogen cells, and no neuron/thecogen cell. Ectopic expression of *pros* (or *numb*) results in the duplication of neuron/thecogen cell, and loss of tormogen/trichogen cell. A second protein with the same distribution and effect as Prospero is Phyllopod (Phyll), a signal transducer in the Ras pathway (Pi *et al*., 2001).

A factor that is activated, rather than inhibited, by the N pathway is Tramtrack (Ttk), a member of the BTB/POZ domain family of transcription factors (Guo *et al*., 1995; Okabe *et al*., 2001; **Figure 11a**). Ttk accumulates in pIIa, and absence of this protein causes the transformation of pIIa into pIIb, with the

accessory cells of the sensillum dc2 (arrow); in *numb* mutant (b6) these cells have increased to four. (c) Following the pIIa mitosis, determination of the trichogen cell (tr) requires Numb, Hairless (H), and the Pax-2 homolog, Sparkling/Shaven. (d) In loss of *hairless* function (d2), the trichogen cell is transformed into a tormogen cell, causing the appearance of a double socket (2xso), instead of a shaft (sh) and socket as in wild type (d1). (e) During the pIIb division, Numb is inherited by pX. This cell can then give rise to a neural or glial/ligament cell, depending on the expression of Elav or Gcm, respectively. (f) Loss of *gcm* causes transformation of ligament cell into supernumerary neuron in *Drosophila* larval chordotonal organs. Wild-type chordotonal organ (f1) posses one dendrite per scolopidium (arrowheads); in many scolopidia of *gcm* mutant embryos (f2) one finds two dendrites (arrowheads). (g) Following pIIIb mitosis, Numb is distributed to the neuron. Realization of the neural fate requires Elav; the distinction between bipolar and multidendritic neuron depends on the expression of the transcription factor Hamlet (Ham). (h) Dorsal sensory neurons in wild-type embryo (h1) represent a mixture of bipolar (ne) and multidendritic (md; brackets) neurons. In embryos overexpressing Ham under the control of different promoters (h2, h3), multidendritic neurons are transformed into bipolar neurons. Scale bar = 1 μm (b, d, f); 10 μm (h). (b): Reprinted with permission from Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., **1989**. *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell 58*, 349–360; © Elsevier. (d) Bang, A.G., Hartenstein, V., Posakony, J.W., **1991**. *Hairless* is required for the development of adult sensory organ precursor cells in *Drosophila*. *Development 111*, 89–104; (c): Reprinted with permission from Jones, B.W., Fetter, R.D., Tear, G., Goodman, C.S., **1995**. *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell 82*, 1013–1023; © Elsevier. (h): Reprinted with permission from Moore, A.W., Jan, L.Y., Jan, Y.N., **2002**. *hamlet*, a binary genetic switch between single- and multiple-dendrite neuron morphology. *Science 297*, 1355–1358; © AAAS. Permission from AAAS is required for all other uses.)

ensuing loss of outer accessory cells and duplication of neurons/thecogen cells.

1.11.3.2.2. The pIIa division – trichogen and tormogen cell Just as in the original pI, pIIa starts out with an activated N signaling pathway (**Figure 11c**). As the division into trichogen and tormogen cell occurs, N becomes attenuated in the tormogen cell. Again, Numb is an essential player in inhibiting N signaling. Numb appears in pIIa shortly before its mitosis and localizes to its anterior pole, whereby it ends up in the trichogen cell. Two nuclear proteins, Hairless (H) and Suppressor of Hairless (Su(H)), which form a complex with the cleaved N fragment when it enters the nucleus and binds to target genes, are also an integral part of the asymmetry generating mechanism (Bang *et al*., 1991, 1995; Bang and Posakony, 1992; Schweisguth and Posakony, 1992, 1994; Bailey and Posakony, 1995; Barolo *et al*., 2000). H, a repressor of N, accumulates in both trichogen and tormogen cells. Su(H) becomes restricted to the tormogen cell. Loss of H results in the transformation of trichogen into tormogen cell ("double-socket"; **Figure 11d**). Overexpression of H, or loss of Su(H) or N, causes the opposite phenotype, a duplication of trichogen cell and loss of socket.

The silencing of *N* in the trichogen cell results in the upregulation of the paired-homeobox (Pax) gene *sparkling/shaven* (*sv*; Kavaler *et al*., 1999). Mutations in this gene have an effect on trichogen cell differentiation. Thus, shafts of hairs and bristles formed by this cell are reduced in length or absent.

As outlined above, it has been suggested that the pIIa cell of chordotonal organs divides into the two accessory cells called cap cell and attachment cell (Lai and Orgogozo, 2004). Although it is tempting to speculate that a N-dependent mechanism operates in this asymmetric division, just as it does in the pIIa of external sensilla, no genetic details are known about the chordotonal pIIa.

1.11.3.2.3. The pIIb division – pIIIb and pX Asymmetric localization of Numb causes the inhibition of N signaling in pX. In some external sensilla, pX differentiates as a glial cell; in other cases, it becomes a multidendritic type II sensory neuron (Brewster and Bodmer, 1995, 1996; Orgogozo *et al*., 2001). Glial development is prompted by the expression of *glial cell missing* (*gcm*), a transcription factor that is also pivotal in the specification of glial cells in the CNS (Jones *et al*., 1995; **Chapter 1.10**; **Figure 11e**). By contrast, multidendritic neuronal development is initiated by the expression of Elav, an RNA-binding protein found in all *Drosophila* neurons. It has been recently speculated that neuronal development constitutes the phylogenetically more ancient pathway of pX (Lai and Orgogozo, 2004). Thus, elimination of *gcm*, in cases where pX expresses a glial fate, causes the transformation of this cell into an Elav-positive neuron. In some chordotonal organs, where pX differentiates into a nonneuronal ligament cell that attaches the neuron to the epidermis, loss of *gcm* also causes transformation of the ligament cell into a (bipolar) neuron (Jones *et al*., 1995; **Figure 10f**). It is noteworthy in this context that some chordotonal organs, notably the auditory Johnston's organ in the fly antenna, possess scolopidia which lack a ligament cell, but are innervated by two bipolar sensory neurons each. It is likely that in these scolopidia, one neuron of each pair develops from the pX cell, while the other neuron is born from the asymmetric division of pIIIb (Lai and Orgogozo, 2004).

It has been shown, for several types of chemosensory external sensilla, that the additional bipolar sensory neurons found in these structures are produced by extra rounds of division of the pX cell. In these cases, the gene *poxn*, which is encountered in the previous section as the sensillum identity gene, is responsible for the stimulation of mitosis in pX.

1.11.3.2.4. The pIIIb division – neuron and thecogen/scolopale cell PIIIb divides into a Numb-positive, N-negative, and Elav positive neuronal precursor, and a Numb-negative, N-positive thecogen cell (Wang *et al*., 1997; **Figure 11g**). Manipulation of the N pathway during this asymmetric division results in the expected cell fate changes, just as in the division of pI, pIIa, and pIIb.

BarH1 and BarH2 represent a complex of two homeobox genes which, in the embryo, are specifically expressed in the neuron and thecogen cell (Higashijima *et al*., 1992). Interestingly, these genes do not seem to be involved in the cells in which they are expressed, but excert a noncell autonomous effect on the outer accessory cells. Thus, loss of BarH1/H2 causes the lengthening of the trichogen process, thereby "transforming" papilla sensilla into trichoid sensilla.

A unique cell fate determining gene called *hamlet* (*ham*) specifies the fate of cells derived from pIIIb in embryonic SOP lineages, giving rise to external sensilla and multidendritic neurons (Moore *et al*., 2002; **Figure 11g**). The most notable phenotype of loss of *ham* consists of an increase in multidendritic neurons at the expense of bipolar neurons innervating external sensilla. Further detailed analysis revealed that the products of pIIIb were a multidendritic neuron with its typical large and branched

dendritic arbor, and a trichogen-like cell. Conversely, expression of *ham* in multidendritic neurons normally derived from pIIIb converted these cells into bipolar neurons (**Figure 11h**).

1.11.3.3. The Control of SOP Mitosis

It has been seen in the previous section that the localization of Numb and its subsequent effect on N signaling is mainly responsible for distributing cell fate to the proper cells of the SOP lineage. How is the asymmetric localization of Numb itself controlled?

At the center of this mechanism is a membrane-associated protein complex which has recently been studied in great detail, because it also controls asymmetric divisions in the stem cells of the CNS (neuroblasts), and because it seems to be highly conserved in all bilaterian animals. This complex, among several other elements, contains the proteins Inscuteable (Insc) and Bazooka (Baz; **Figure 12a**; Campos-Ortega, 1997; Wodarz *et al*., 1999; Jan and Jan, 2001; Knoblich, 2001; Chia and Yang, 2002; Lai and Orgogozo, 2004; see **Chapter 1.10**). Located apically in the neuroblasts, as well as the neurectoderm from which neuroblasts delaminate, the Insc complex anchors the centrioles in a vertical orientation, which results in a vertical mitotic spindle. Along with spindle orientation, the basal localization of Numb also depends on the Insc complex. The apically localized Insc complex also controls the vertical spindle and basal localization of Numb in pIIb and pIIIb of the external sensillum SOP lineage (**Figure 12d**), both of which are intra-epithelial cells, which as seen above, give off one daughter basally.

The regulation of the mitotic spindle and Numb localization in pI, which divides horizontally in the plane of the epithelium, requires elements of the Insc complex, in addition to other factors (**Figure 12b**; Orgogozo *et al*., 2001; Roegiers *et al*., 2001). As in neuroblasts and pIIb/IIIB, the asymmetry in Numb

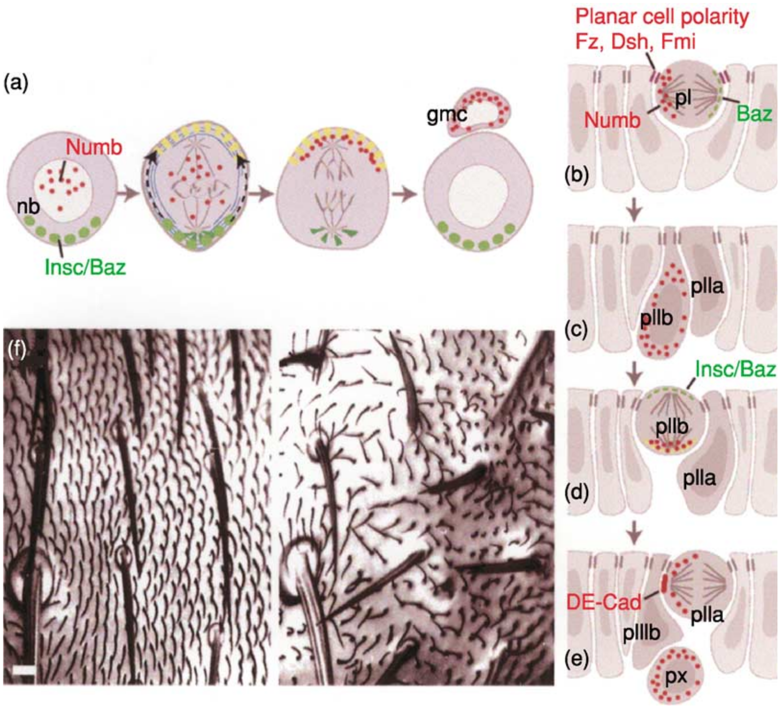


Figure 12 Control of spindle orientation during SOP division. (a) Schematic diagram of neuroblast division. The Inscuteable/Bazooka (Insc/Baz) protein complex is located apically in dividing neuroblasts (nb) and anchors the mitotic spindle (sp) in a vertical position. The same complex, in conjunction with the basally concentrated protein Miranda (yellow), is responsible for asymmetric localization of Numb at the basal side of the neuroblast. After neuroblast division (right panel), Numb becomes localized exclusively in one of the daughter cells, the ganglion mother cell (gmc). (b–e) Schematic drawings of dividing microchaete SOP (see **Figure 10**). Spindle orientation and Numb localization in pI (b, c) is controlled by the Inscuteable complex and the planar cell polarity pathway. Mutations for any member of this pathway (Fz, Dsh, Fmi) result in randomized mitotic spindle orientations, followed by abnormally oriented microchaetes. This condition is illustrated in panel f, where the left half shows wild-type microchaete pattern, and the right half demonstrates the bristle pattern in a mutation of *frizzled*. (d) Vertical spindle orientation in dividing pIIb is controlled by the Insc/Baz complex. (e) Asymmetric division of pIIa does not depend on Insc/Baz, but on the cell adhesion molecule DE-Cadherin (DE-Cad). At most stages, this protein is localized around the apical pole of epithelial cells. At a later stage, when pIIa divides, a discrete spot of DE-Cad appears at a more basal position. Scale bar = 5 µm. ((f): Reprinted with permission from Tree, D.R., Ma, D., Axelrod, J.D., **2002**. A three-tiered mechanism for regulation of planar cell polarity. *Semin. Cell Devel. Biol. 13*, 217–224; © Elsevier.)

localization (accumulated anteriorly in pI) depends on Baz. Eliminating this protein causes randomized spindles and Numb accumulation.

It could recently be demonstrated that molecules that form part of the so-called planar cell polarity (PCP) pathway also play a role in orienting the mitosis of pI of *Drosophila* thoracic microchaetes (**Figure 12b**; Adler and Taylor, 2001; Tree *et al.*, 2002). The PCP pathway involves the membrane receptor Frizzled (Fz) and the signal transducer Dishevelled (Dsh), which is localized to the zonula adherens and becomes hyperphosphorylated as a result of binding to Fz. Other molecules, including the cadherin Flamingo (Fmi), play a role in the proper localization of the Fz/Dsh complex in the membrane (Lu *et al.*, 1999), and effectors downstream of this complex include the *Drosophila* rhoA GTPase (DrhoA), which could directly act on the actin cytoskeleton and thereby exert an effect on cell shape and cell orientation. The PCP pathway controls the orientation of epidermal hairs (trichomes) and directed cell movement, for example, in *Drosophila* compound eye development and vertebrate gastrulation. Impairment of this pathway also causes randomization of the mitotic spindle in pI, followed by abnormal orientation of the microchaete bristles (**Figure 12f**).

In pIIa, the progenitor of the trichogen and tormogen cell, spindle orientation and Numb localization involve a mechanism that does not require the Insc complex. PIIa divides horizontally, and the orientation of the spindle matches that of its mother cell pI, i.e., it is aligned along the ap axis. Even if the pI spindle is randomized (by interference with Insc or Baz), the pIIa spindle lines up with the previous pI spindle. The main factor that has been identified so far in controlling the pIIa spindle is the adhesion molecule *Drosophila* E-Cadherin (DE-Cad; LeBorgne *et al.*, 2002). DE-Cad is concentrated in the zonula adherens of epithelial cells, including pI, pIIa, and pIIb. In pII, it becomes localized in an asymmetric fashion, in that a "patch" of high DE-Cad concentration accumulates at the pIIa/pIIb interface (**Figure 12e**). The asymmetric DE-Cad expression is crucial for pIIa division. Reducing DE-Cad function prior to pIIa division randomizes the mitotic spindle and leads to mislocalization of Numb.

1.11.4. Sensillum Morphogenesis and Differentiation

1.11.4.1. Formation of External Sensilla

Following the division of the SOP of an external sensillum, the presumptive outer accessory cells and thecogen cell form integral parts of the

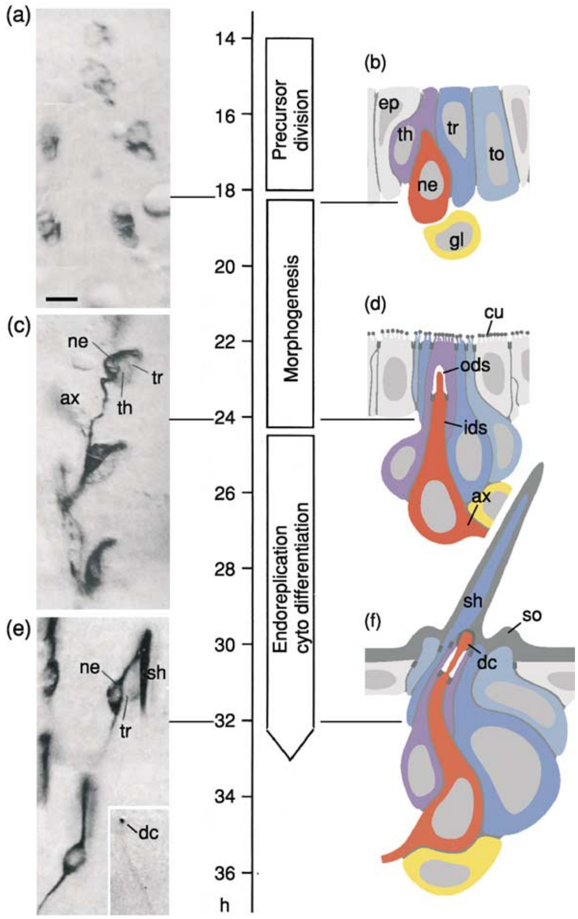


Figure 13 Morphogenesis of external sensilla. Left panels (a, c, e) show photographs of *Drosophila* notum microchaetes, labeled with the antibody Mab22C10, which stains sensory neurons and some of the accessory cells, at three different stages of pupal development. The time line in the center of the figure indicates hours after puparium formation, and provides the time frame for the three major phases of microchaete development (SOP proliferation, sensillum morphogenesis, sensillum differentiation). The right panels (b, d, f) show schematic sections of developing microchaete at stages corresponding to those illustrated by the photographs at the left. (a, b) Shortly after SOP division, accessory cells (to, tormogen cell; tr, trichogen cell, th, thecogen cell) lie in the plane of the epidermal epithelium (ep); neuron (ne) and glial cell (gl) are subepidermal. The 22C10 antibody labels triplets of cells, which correspond to the neuron, thecogen, and trichogen cell. (c, d) During sensillum morphogenesis, the accessory cells form concentric sheaths around the extending sensory dendrite (ids, inner dendritic segment; ods, outer dendritic segment), whilst their cell bodies shift basally. Neurons grow out axons (ax). (e, f) Neuron and accessory cell differentiation. Both trichogen cell and tormogen cell undergo several rounds of endoreplication, resulting in an increase of the size of these cells. At the same time, the shaft (sh) and socket (so) form as apical extensions of the trichogen and tormogen cell, respectively. The dendritic cap (dc) is secreted by the thecogen cell around the tip of the dendrite. Scale bar = 5 μm (a, c, e). ((a, c, e) From Hartenstein, V., Posakony, J.W., **1989**. The development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development 107*, 389–405.)

epidermis, while the precursor of the sensory neuron(s) and the pX cell (which will become either glia or neuron, or undergoes apoptosis) are subepidermal (Figure 13b and c). Subsequently these cells undergo a series of morphogenetic changes in shape and position, which leads up to the architecture of the mature sensillum. Sensillum morphogenesis has been described at the ultrastructural level in several insect groups (Blattodea: Seidl and Altner, 1990; Orthoptera: Ameismeier, 1987; Gnatzy,

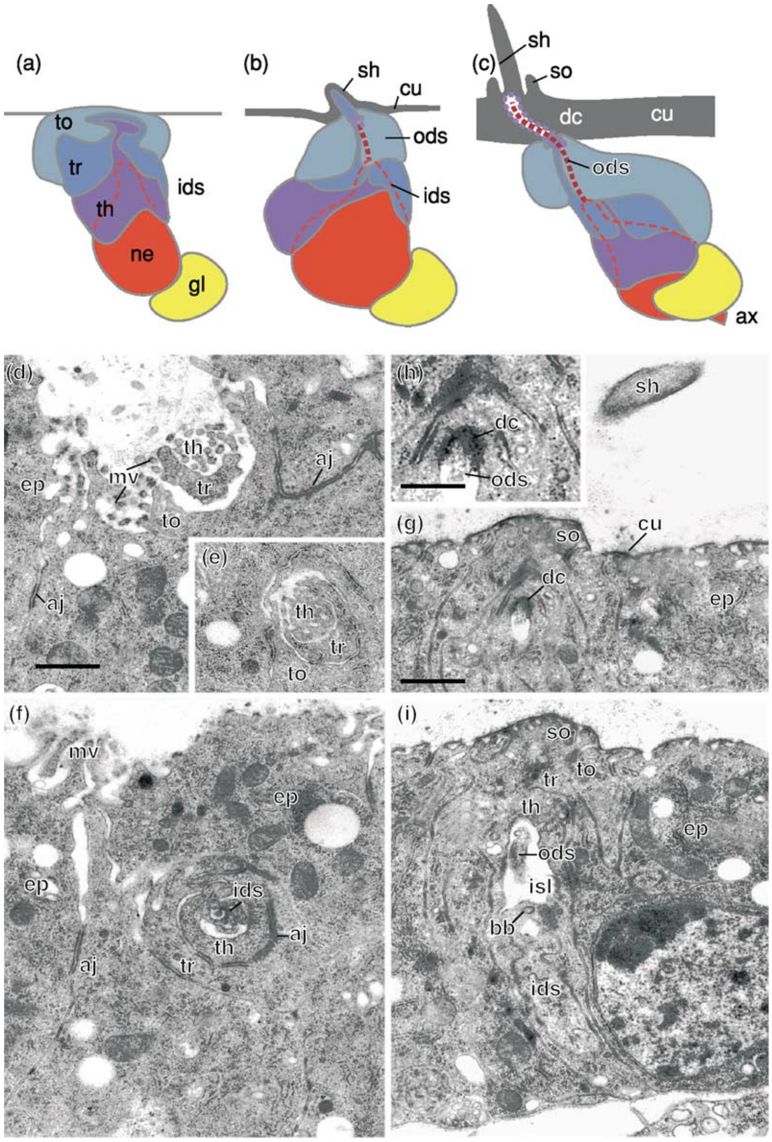


Figure 14 Morphogenesis of external sensilla. (a–c): Semi-schematic drawings of a *Drosophila* trichoid sensillum at embryonic stage 13 (50% of development; (a)), 15 (60% of development; (b)), and 16 (70% of development; (c)), showing sensillum cells (gl, glial cell; ne, neuron; th, thecogen cell; to, tormogen cell; tr, trichogen cell) in different colors. (d–f) TEMs of transverse sections of nascent trichoid sensillum at embryonic stage 13. Sections correspond to different levels along the apico-basal axis of the sensillum, with d showing the most apical level. Note the concentric arrangement of the thecogen cell (th), trichogen cell (tr), and tormogen cell (to) at apical levels (d, e). Microvilli (mv) and conspicuous adherens junctions (aj) characterize the sub-apical membrane of accessory cells and surrounding epidermal cells (ep). The sensory neuron has started to form inner dendritic segment (ids in (f)) which pushes into the thecogen cell from below, but does not yet reach the apical surface of the embryo (note the absence of dendrite in planes shown in (d) and (e)). (g–i) Near longitudinal sections of trichoid sensillum at embryonic stage 16. The sensory dendrite has lengthened and forms a ciliary outer segment (ods) apically. The dendritic cap (dc) is formed between the thecogen cell and the tip of the dendrite (g); (h) shows a magnified view of tip of dendrite with dendritic cap). The tormogen and trichogen cell have formed a sensillum socket (so) and shaft (sh), respectively. Cuticle (cu) is deposited around the socket and shaft, and at the tips of microvilli protruding from epidermal cells (ep). Scalebar = 0.5 μm (d–f, h); 1 μm (g, i). ((a–c) Adapted from Hartenstein, V., **1988**. Development of the *Drosophila* larval sensory organs: spatiotemporal pattern of sensory neurones, peripheral axonal pathways, and sensilla differentiation. *Development 102*, 869–886.)

1978, 1980; Edwards and Chen, 1979; Schmidt and Berg, 1994; Coleoptera: Ernst, 1972; Lepidoptera: Keil, 1992; Hymenoptera: Schmidt and Kuhbandner, 1983; Martini, 1986; Diptera: Hansen and Hansen-Delkeskamp, 1983; Kuhbandner, 1984; 1985; De Kramer and Van der Molen, 1984; Hartenstein, 1988; Hartenstein and Posakony, 1989).

1.11.4.1.1. Formation of the inner dendritic segment and dendritic sheath The first phase in sensillum morphogenesis is embedded in more generalized morphogenetic movements that shape the epidermis surrounding the sensillum. Thus, during a time interval that precedes the deposition of cuticle, epidermal cells, and accessory cells of external sensilla form horizontal, sleeve-like processes that, to a variable extent, insert in between and wrap around neighboring cells. Whereas these processes are transient in regular epidermal cells, they remain in place in the case of trichogen and tormogen cells, resulting in the mesaxonal sheaths formed by these cells around the thecogen cell (**Figures 13d** and **14a, d**). During the same time period, the sensory neuron forms the inner dendritic segment at its apical pole (**Figures 13c, d** and **14b, f**). While pushing upward, the dendrite pierces the cytoplasm of the thecogen cell from a basal to apical direction. This movement results in the cylindrical dendritic sheath formed by the thecogen cell around the dendrite (**Figure 14f**).

Formation of the trichogen and tormogen mesaxon around the thecogen cell follows an apical to basal gradient. Thus, at an early stage of sensillum morphogenesis, a complete mesaxon is only present at the apical level of the zonula adherens (**Figure 14a**); further basally, the tormogen and trichogen sheaths are only partial. Toward the later stages, the width of the mesaxons increases, until most of the length of the dendrite is enclosed by these sheaths (**Figure 14b** and **c**).

The lengthening of the dendrite, and concomitant widening of the accessory sheaths surrounding it, is paralleled by a basal shift of the cell bodies of all sensillum cells. In most mature sensilla, the cell bodies of neuron and thecogen cells are beneath the level of the basal surface of the epidermis, and can be even removed for some distance in the horizontal plane, from the location where the dendrite and its sheath are inserted in the epidermis (**Figure 13e** and **f**). The cell bodies of the tormogen and trichogen cells also shift below the level of the surrounding epidermis (**Figures 13d, f** and **14a–c, i**).

1.11.4.1.2. Elaboration of the apical sensillum apparatus This second phase in morphogenesis is unique to sensillum cells. Thus, whereas surrounding epidermal cells maintain a flat apical membrane that starts to secrete cuticle, the sensory dendrite and its surrounding accessory cell processes grow apically (**Figures 13e, f** and **14b, c, g**). The trichogen cell forms the sensillum shaft that differs in shape and length depending on the type of sensillum (e.g., basiconcal, campaniform, or trichoid sensilla). Apically directed growth of the tormogen cell produces the socket of the sensillum. As the shaft and socket processes approach their mature length, they start to secrete cuticle.

At the same time as the shaft and socket of the sensillum are formed, the sensory neuron extends the outer dendritic segment, which constitutes a modified cilium (**Figures 13f** and **14c, g–i**). The basal body, derived from the centriole that had organized the mitotic spindle of the SOPs, acts as the nucleation center for the array of microtubules forming the axoneme of the cilium. Interestingly, the cilium does not start its outgrowth from the apical membrane of the sensory neuron. Instead, the basal body sinks several microns deep beneath the membrane. A small membraneous vesicle forms above the basal body, and the nascent cilium pushes into the lumen of the vesicle. Subsequently, the vesicle shifts apically and fuses with the apical cell membrane, thereby moving the base of the cilium into the apical membrane. The same mechanism of ciliogenesis is typical for ciliated cells in vertebrates (Keil and Steinbrecht, 1997).

As the outer dendritic segment of the sensory neuron elongates beyond the apical surface of the thecogen cell, it protrudes into the interior of the trichogen shaft. In the case of the many chemosensory sensilla, the dendrite(s) reach all the way to the tip of the shaft; in mechanoreceptors, the dendritic tips terminate at a more basal level (see **Figure 2b** and **c**). The dendritic tip of many mechanosensitive sensilla ends in a characteristic thickening, the tubular body. Like the outer dendritic segment as a whole, the tubular body is filled with microtubules which, in conjunction with the membrane of the tubular body, are the pivotal elements in mechanotransduction.

At the time when cuticle is deposited around the socket and shaft processes of the outer accessory cells, the thecogen cell secretes a dense extracellular material, the dendritic cap (**Figures 13e** and **14g, h**). The dendritic cap forms the "glue" that connects the dendrite to the cuticle of the sensillum shaft; as discussed below, mutations in which this connection is severed result in failure of mechanotransduction.

During molting, the apical processes of the trichogen and tormogen cell retract and grow out anew before the next cuticle layer is deposited. At the

same time, the outer dendritic segments, surrounded by the thecogen sheath, remain at their full length. They can now be seen to protrude beyond the level of the epidermis, leaving the enclosing outer accessory cells through a pore called the molting pore (Gnatzy, 1978). After shaft and socket have reformed, the dendrite is again taken into the interior of these processes.

1.11.4.1.3. Axonogenesis and glial sheath The basal membrane of the sensory neuron lengthens to protrude as the axon. The axon, tipped by a growth cone, extends towards the CNS, typically using the basement membrane underlying the epidermis or surrounding nearby muscles as a substrate to grow on. Sensory axons become surrounded by a perineural glial sheath. Close to the sensory cell body, this sheath is elaborated by the glial cell derived from pX (**Figure 14c**). At more proximal levels, additional glial cells (peripheral glia), unrelated to the SOP lineages, surround sensory nerves. The sheath formed by the pX glial cell occasionally enwraps the sensory cell body as well, such as in the case of the *Drosophila* embryonic basiconical sensilla (Hartenstein, 1988). In most sensilla, the sensory cell body lacks a glial sheath.

1.11.4.1.4. Molecular aspects of bristle shaft differentiation Very little is known about the molecular mechanisms underlying the complex series of early morphogenetic processes that shape the outer accessory cells. It is reasonable to assume that finely tuned changes in adhesion molecule expression and/or distribution account for the gradual formation of the mesaxonal sheath processes produced by the trichogen and tormogen cell; however, the genetic analysis of representatives of the well known classes of adhesion molecules, such as the cadherins, which have been identified to date, has not yet produced any phenotype that would shed light on mesaxon formation and early accessory cell morphogenesis in general. The picture becomes clearer at later stages, when accessory cells form their apical processes, i.e., the sensillum socket and shaft. These events are driven by a dynamic rearrangement of microfilaments and microtubules. Our understanding of this process, which in many respects may serve as a model system for cell elongation in general, has recently improved with the molecular-genetic analysis of *Drosophila* thoracic microchaetes (Tilney *et al*., 1995, 1996, 2000a, 2000b).

The trichogen cell of microchaetes starts forming an apical shaft at around 32 h of pupal development (**Figure 13e**). Shortly after their appearance, shafts reach their final diameter of approximately 4 µm; from then onward, they grow over a time period of 15 h to a final length of about 70 µm. Mature microchaetes are roughly cone-shaped and have a rifled surface (**Figure 15c**). Rifles are caused by 7–11 tight bundles of microfilaments arranged at regular intervals around the periphery of the shaft (**Figure 15a**). These bundles co-vary in thickness with the diameter of the shaft; near the base of the microchaete, bundles are thicker (and contain more microfilaments) than near the tip (**Figure 15d**). Microtubules, rather than forming bundles, are evenly distributed throughout the cytoplasm of the microchaete shaft (**Figure 15a**). Applying drugs that specifically disrupt actin and tubulin turnover to organ cultures of developing microchaetes (Turner and Adler, 1998; Tilney *et al*., 2000b) showed that actin polymerization is essential for bristle elongation, but that microtubules play little role in this process.

Microchaete growth occurs preferentially at the tip by directed polymerization of actin (**Figure 15d**, phase 1). Soon after their formation, microfilaments are tied together into bundles. Following an initial phase of thin, loosely-packed bundles (seen near the tip of the bristle; phase 2 in **Figure 15d**) microfilaments are forced into a maximally cross-linked, hexagonal array, found at more proximal levels of the bristle (**Figure 15d**, phase 3; see high magnification of microfilament bundle in **Figure 15b**). Two different actin binding proteins, Forked (F) and Singed (Sn) (the latter constituting the *Drosophila* homolog of the Fascin protein that is crucial for microfilament bundling in vertebrate cells), are responsible for these two steps in bundling the microfilaments of the microchaete. Loss of either of these proteins causes stunted bristle growth, as well as irregular shapes and branching of the bristle (**Figure 15g and j**). Forked protein is localized at the tip of the growing bristle, where the loose bundling of freshly polymerized actin takes place. In mutations of *forked,* the number of microfilaments that are recruited into the bundles is drastically reduced, resulting in thin bundles; alternatively, the cross-linking step is normal, so that filaments are tightly packed and regularly spaced (**Figure 15f**). Singed is found along the length of the bristle, supporting the notion that this protein is incorporated into the bundles as they mature, which causes the transition from the initial loose packing of filaments into the hexagonal arrays. Bristles of *singed* mutants have reduced microfilaments with loosely packed, irregularly spaced filaments (**Figure 15i**).

Besides the cross-linking proteins Singed and Forked, numerous other proteins, known to play a role in controlling microfilament polymerization

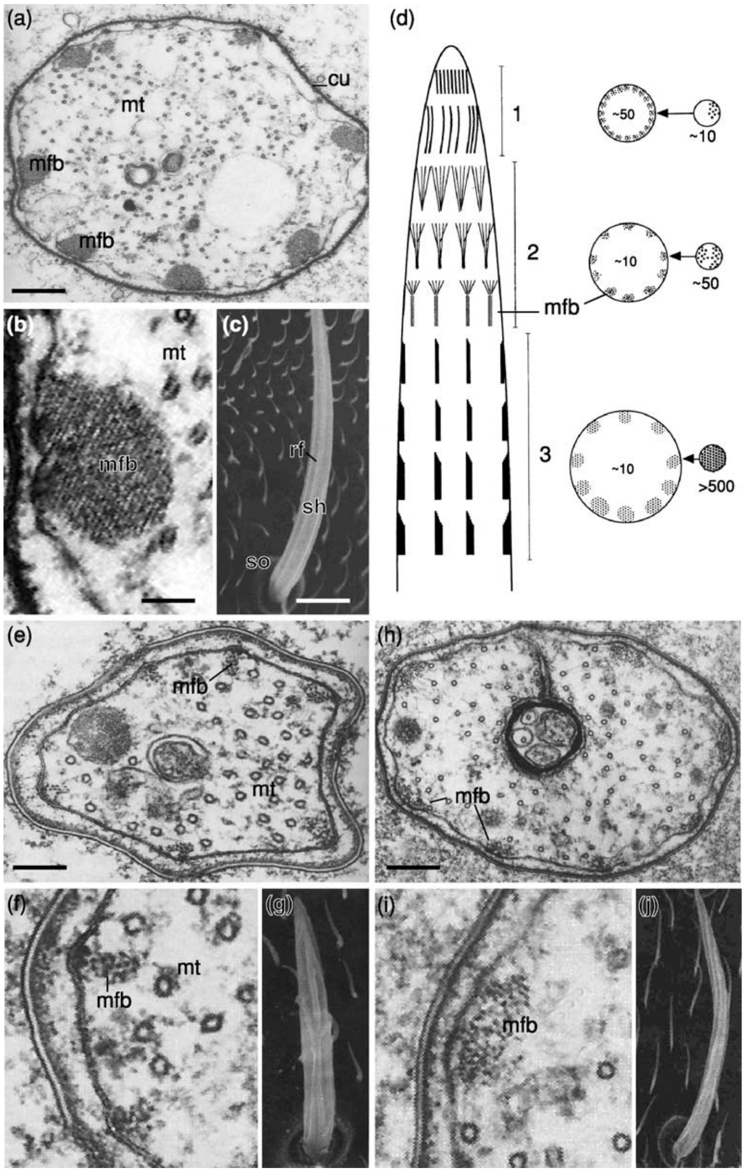


Figure 15 Sensillum differentiation: elongation of the sensillum shaft. (a–c) TEMs (a, b) and SEM of *Drosophila* thoracic macrochaete. Cross-section of the macrochaete shaft (a) shows regularly spaced longitudinal bundles of microfilaments (mfb) and scattered microtubules (mt). (b) Shows magnified view of microfilament bundle, illustrating the ''crystalline pattern'' of hexagonally packed microfilaments. Microfilament bundles impress rifles (rf) on cuticular surface (cu) of macrochaete (a, c). (d) Schematic longitudinal section (left of panel) and consecutive cross-sections (right) of macrochaete shaft. Phases 1 (bristle tip) to 3 (bristle base) correspond to steps in microfilament bundle assembly. During phase 1 microfilaments associate into approximately 50 thin, loosely packed bundles of about 10 fibres each. In phase 2 these bundles coalesce into fewer, thicker bundles. These early phases depend on the Forked protein, which is expressed along the entire length of the bristle. During phase 3, bundles grow in thickness and microfilaments become cross-linked into hexagonal arrays. The fascin homolog Singed, expressed only at the bristle base, is responsible for the cross-linking. (e–g) TEMs and SEM of macrochaete in a *forked* mutant. Note the smaller size of bundles (e) and abnormal bristle morphology (g). Althoughy strongly reduced in size, microfilament bundles still consist of hexagonal arrays of microfilaments (f). (h–j) TEMs and SEM of macrochaete in a *singed* mutant. As in *forked*, microfilament bundles are reduced in size (h), causing abnormal bristle morphology (j); in addition, microfilaments are randomly spaced within bundles (i). Scalebar = 0.5 mm (a, h); 0.1 μm (b, f, i); 10 μm (c, g, j); 0.3 μm (e). ((a–c), (e–j): Reproduced from Tilney *et al.* 1995 by copyright permission of The Rockefeller University Press. (d): Reproduced from Tilney *et al.* 2000a by copyright permission of The Rockefeller University Press.)

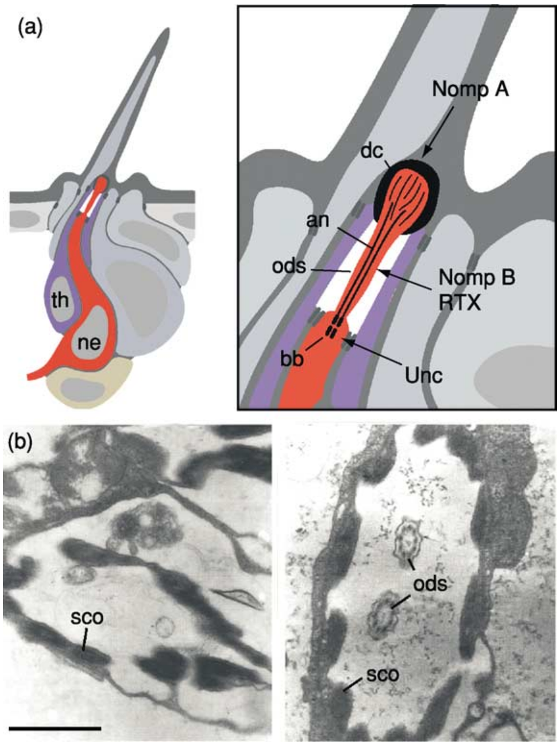


Figure 16 Differentiation of the outer dendritic segment and dendritic cap. (a) Schematic drawing of a mechanosensory sensillum, showing the outer dendritic segment (ods) and related structures (bb, basal body; an, axoneme; dc, dendritic cap). Four proteins involved in sensory dendrite formation (NompA, NompB, RFX, Unc) and their supposed point of action are indicated. (b) TEMs of cross-sections of scolopidia of Johnston's organ of *Drosophila* wild type (right panel) and a *rfx* mutant (left panel). The wild-type scolopale (sco) surrounds two dendrites (ods); no dendrites are detectable in the mutant scolopale. Scale bar = 0.5 μm. ((b): Reproduced with permission from Dubruille, R., Laurencon, A., Vandaele, C., Shishido, E., Coulon-Bublex, M., *et al.*, **2002**. *Drosophila* regulatory factor X is necessary for ciliated sensory neuron differentiation. *Development 129*, 5487–5498; © The Company of Biologists Ltd.)

and bundling, were identified as factors in microchaete growth. Notable among these are Crinkled (Ck), the *Drosophila* homolog of myosin VIIa, and members of the short GTPases (Dcdc42 and DRho; Eaton *et al.*, 1996).

1.11.4.1.5. Factors controlling sensory dendrites and dendritic sheaths Genetic screens for loss of sensory function have led to the isolation of genes important for the development and physiology of sensory neurons. To date, two aspects of sensory neuronal development have been addressed: the outgrowth of the cilium-like outer dendritic segment, and the connection of the dendritic tip to the dendritic cap formed by the thecogen cell (**Figure 16**).

Formation of the outer dendritic segment is driven by the elongation of the axoneme, the circular array of microtubules extending along the ciliary center. Axoneme elongation has been investigated in more detail in other organisms, such as the alga *Chlamydomonas* (Rosenbaum and Witman, 2002) and the nematode *Caenorhabditis elegans* (Swoboda *et al.*, 2000). These studies showed that axoneme formation requires a complex (Intra Flagellar Transport or IFT complex) of several microtubule-binding proteins that are transported along the axoneme. The *Drosophila* gene *no-mechanoreceptor-potential B* (*nompB*) has been suggested to encode one of these transported ciliary proteins (Caldwell and Eberl, 2002; **Figure 16a**). Mutants in *nompB* have shortened sensory cilia in external sensilla and chordotonal organs. Another gene discovered in the systematic screen for mutations deficient in mechanoreception, *uncoordinated* (*unc*), has been suggested to be involved in the formation of the basal body that organizes the outgrowth of the ciliary axoneme (Baker *et al.*, 2001; Caldwell and Eberl, 2002).

The expression of the IFT complex is under the control of a transcription factor of the Regulatory Factor X (RFX) family. *Drosophila rfx* has been recently identified (Dubruille *et al.*, 2002); it is expressed and required for sensory neuronal differentiation and function. Loss of *rfx* results in missing or shortened outer dendritic segments in all bipolar (type I) sensory dendrites, including those of external sensilla and chordotonal organs (**Figure 16a and b**). This developmental defect leads to loss of excitability of the sensory neurons by their appropriate stimuli.

Besides proteins involved in ciliary growth, proteins linking the cilium to the dendritic cap, which is produced by the thecogen cell, have been identified. Mutations in genes encoding such proteins also lead to loss of sensory function, supporting the notion that the tight connection between the dendrite and the dendritic cap is crucial for eliciting a receptor potential in the sensory neuron (Caldwell and Eberl, 2002). One protein required for the dendrite-to-dendritic cap linkage is NompA, a transmembrane protein produced in the thecogen cell and targeted to the dendritic cap. Mutations in the *nompA* gene cause gaps in the dendritic cap around many dendrites (**Figure 16a**; Chung *et al.*, 2001).

1.11.4.2. Chordotonal Organs

1.11.4.2.1. Formation of the *Drosophila* embryonic chordotonal organs The development of chordotonal organs has been analyzed at the ultrastructural level in *Periplaneta* (Bloechl and Selzer, 1988) and *Drosophila* (Hartenstein, 1988). In *Drosophila*,

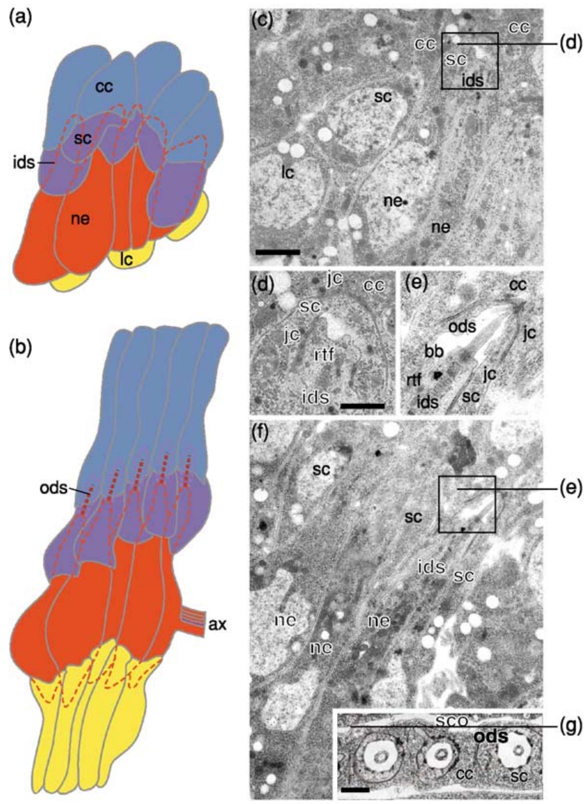


Figure 17 Morphogenesis of chordotonal organs. (a, b) Schematic drawings of a *Drosophila* lateral pentascolopidial organ (lch5) at embryonic stage 13 (a) and 16 (b), showing sensillum cells (lc, ligament cell; ne, neuron; sc, scolopale cell; cc, cap cell) in different colors. (c, d) TEMs of longitudinal section of nascent chordotonal organ at embryonic stage 13. Neurons (ne) are located basally (bottom of panel) and start growing out inner dendritic segments (ids) which push into the apically adjacent scolopale cells (sc). Magnified view of this region in (d) shows rootlet filament (rtf) in inner dendritic segment and junctional complexes (jc) between dendrite, scolopale cell, and cap cell. (e, f) Longitudinal section of the developing chordotonal organ at stage 15 of embryonic development. The main changes from the previous stage are the lengthening of the inner dendritic segment and the appearance of a short ciliary outer segment (ods) emanating from a basal body (bb). (g) Transverse section of a stage 16 chordotonal organ, showing the formation of scolopidial rods (sco) in the cytoplasm of scolopale cells. Scale bar = 0.5 µm (c, f, g); 0.2 µm (d, e). ((a, b) Adapted from Hartenstein, V., **1988**. Development of the *Drosophila* larval sensory organs: spatiotemporal pattern of sensory neurones, peripheral axonal pathways, and sensilla differentiation. *Development 102*, 869–886. (g): Reprinted with permission from Jones *et al.* 1995; © Elsevier.)

following SOP division, presumptive chordotonal cells are all located subepidermally and become arranged in the form of columns, each column representing one scolopidium (Figure 17a). In the case of the embryonic lateral chordotonal organ lch5, presumptive attachment cells are located most dorsally, attached to the basal membrane of the epidermis. Attachment cells are followed by cap cells, scolopale cells, neurons, and ligament cells (called soma sheath cells in Hartenstein (1988)). As morphogenesis begins, sensory neurons form dendrites that extend dorsally into the adjacent scolopale cells (Figure 17a, c and d). This event resembles, in many ways, the behavior of external sensillum neurons whose dendrites "pierce" the apically adjacent thecogen cell (the homolog of the scolopale cell; see above). However, dendrites of chordotonal sensory neurons do not protrude beyond the scolopale cell; rather, they remain "buried" in a conical cavity surrounded by the scolopale cell (Figure 17b, e, f). The scolopale cell itself also lengthens in the vertical axis and pushes into the cap cell. Prominent adherens junctions and septate junctions strengthening the intercellular adhesion form in the vaulted clefts in between the cap cell and scolopale cell, and scolopale cell and dendrite (Figure 17d and e).

Two cells, the attachment cell (apically) and the ligament cell (basally) (see Figure 1) suspend the neuron-scolopale-cap cell complex from the epidermis. Both of these cells usually lengthen into thin cables that are attached to the epidermis. Ligament cells seem to be absent in many chordotonal organs (e.g., Johnston's organ, ventral larval chordotonal organs in *Drosophila*), and, also, attachment cells have not been positively identified in all cases.

A structure found uniquely in chordotonal organs is the scolopale, a circular array of thick cytoskeletal bundles that form in the cytoplasm of the scolopale cell. As the interior of the scolopale cell cavitates to receive the sensory dendrite, densities ("rods" formed by closely packed actin and tubulin filaments) form at regular intervals around the inner cavity (Figure 17g). Towards the later stages, the cell membrane bordering the inner cavity of the scolopale cell recedes peripherally, so that the scolopale rods come to lie inside the cavity, surrounding the outer dendritic segment (see the TEM of the mature chordotonal organ shown in Figure 4). Both the scolopale and the dendritic cap formed by the scolopale cell around the tip of the outer dendritic segment are thought to play an essential role in mechanotransduction.

1.11.4.2.2. Chordotonal organ in *Periplaneta* The morphogenesis of a chordotonal organ in *Periplaneta*, investigated by Bloechl and Selzer (1988), varies in several early aspects from that reported for *Drosophila*. Some of these differences are informative in regard to the homologization between

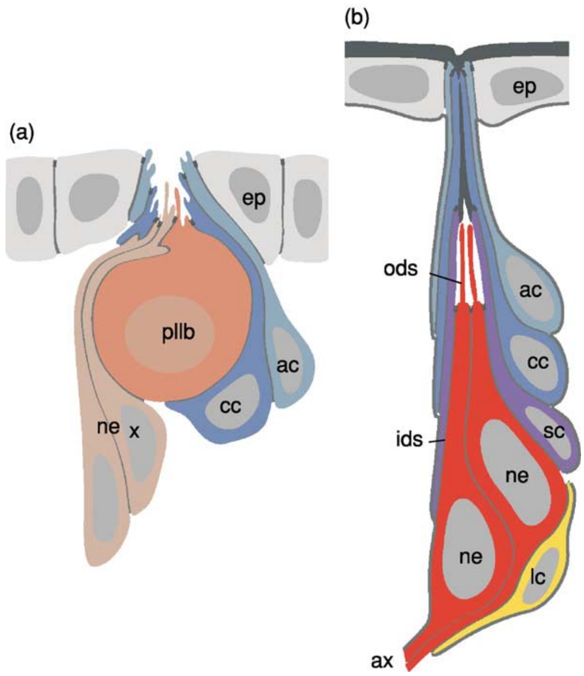


Figure 18 Development of the chordotonal organ in *Periplaneta*. Schematic longitudinal sections of nascent chordotonal organ at early (a) and late (b) embryonic stages. In a, the outer accessory cells (ac, attachment cell; cc, cap cell) are postmitotic, whereas the pIIb progenitor of neurons and scolopale cell is just dividing. All three of these cells are in contact with the apical surface. Two transient neurons (ne_x) which will later undergo apoptotic cell death are also visible at this stage. At the late stage depicted in panel b, all cells have reached their final disposition. Note that the outer accessory cells are still in contact with the apical epidermal surface. (Reproduced with permission Bloechl, R., Selzer, R., **1988**. Embryogenesis of the connective chordotonal organ in the pedicel of the American cockroach: cell lineage and morphological differentiation. *Cell Tissue Res. 252*, 669–678; © Springer–Verlag.)

the cells of a chordotonal organ and external sensilla. In *Periplaneta*, the dividing SOP daughter cells remain integrated into the epidermis. Even in the mature organ, the two outer accessory cells are in contact with the apical surface via thin cytoplasmic necks, whereby the neck of the outer one (corresponding to the attachment cell in *Drosophila*) surrounds mesaxon-like the neck of the inner one (the cap cell; Figure 18). This arrangement of the outer accessory cells in *Periplaneta* supports the homology of the attachment cell with the tormogen cell of an external sensillum, and of the cap cell with the trichogen cell.

1.11.4.2.3. Molecular aspects of chordotonal organ differentiation Given the strong similarity between cells forming external sensilla and chordotonal organs, it comes as no surprise that the majority of proteins encountered above in discussing external sensillum differentiation are involved in quite similar ways in chordotonal organ differentiation. This holds particularly true for proteins involved in the development of the sensory dendrites (e.g., Rfx, NompA, NompB; Dubruille *et al*., 2002; Caldwell and Eberl, 2002). A few genes that seem to be required exclusively in dendrite formation in chordotonal organs and not in external sensilla are *beethoven* (*btv*; a putative cadherin) and *tilB/smetana* (*smet*; Eberl *et al*., 2000; protein as yet unknown). This suggests that significant differences exist at the molecular level between chordotonal organs and external sensilla in regard to the way in which sensory dendrites form. These differences are reflected in a number of structural details that set chordotonal dendrites apart from external sensillum dendrites (see section above).

1.11.4.3. Differentiation of Type II Multidendritic Sensilla

Multidendritic (MD) sensory neurons develop from the same SOP lineages that also give rise to external sensilla. Following the division of pIIb, these cells obtain a subepidermal position. Typically, MD neurons do not become associated with non-neuronal accessory cells, but differentiate at the positions where they were born as isolated, scattered cells, attached to the basal surface of the epidermis (Bodmer and Jan, 1987; Bodmer *et al*., 1989; Brewster and Bodmer, 1995; Orgogozo *et al*., 2001; Grueber *et al*., 2002; Lai and Orgogozo, 2004). Some MD neurons are associated with internal structures, including tracheal branches, muscle fibers, or peripheral nerves (see **Chapters 2.1**, **2.3**, **2.4**, and **2.7**).

MD neuron differentiation has been followed in *Drosophila* (Grueber *et al*., 2002; Sugimura *et al*., 2003), and numerous genes involved in their morphogenesis have been identified in genetic screens (Gao *et al*., 1999, 2000). As described for other insect groups (Grueber *et al*., 2001; and discussed earlier), *Drosophila* possesses four classes of MD neurons which differ in dendritic complexity and physiological properties (see **Figure 5**). Differentiation begins in the late embryo when each neuron extends two to five primary dendrites and an axon. The first dendrites are directed dorsally; they are followed by lateral dendrites extending anteriorly and posteriorly (**Figure 19a1–4**). During the larval period, which is characterized by an enormous increase in body size, the complexity of the dendritic arbors increases. This is achieved by successive bifurcations of dendrites at their tip, as well as by outgrowth of higher order branches along the entire length of the primary dendrites (**Figure 19a5**).

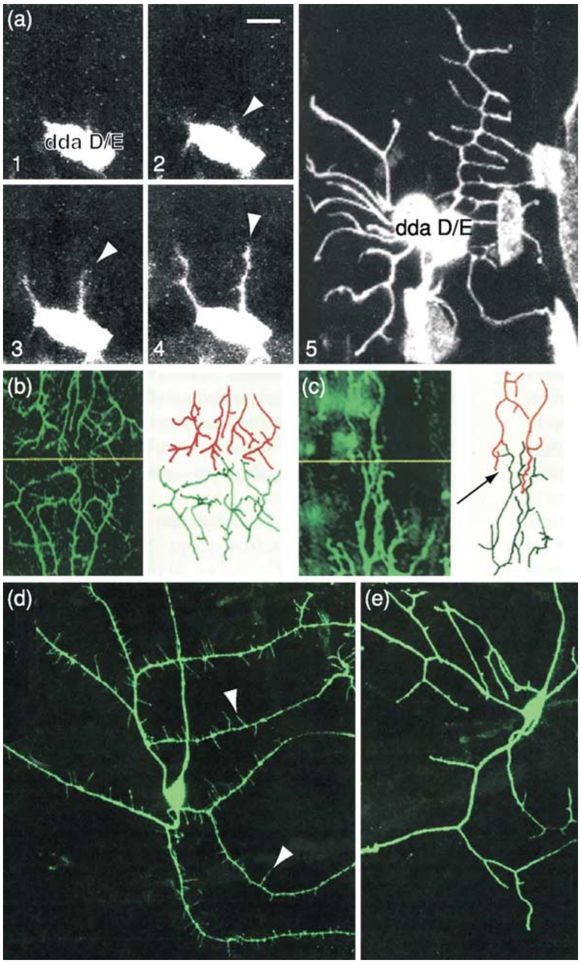


Figure 19 Development of multidendritic neurons. (a) Images of labeled ddaD/E neurons in the late embryo (a1–4) and larva (5). Note the fast outgrowth of vertical dendrites (arrowheads) in the embryo; secondary branching takes place in the larva (5). (b) Dendrites of wild-type dorsal MD neurons (labeled green) from both sides meet at the dorsal midline (yellow line) but do not overlap. The drawing shows MD dendrites of a right hemisegment in red, and of the corresponding left hemisegment in green. In *flamingo* mutant embryos (c), overlap of the dorsal MD dendrites are frequent (arrow). (d) Labeled MD neuron ddaA in a wild-type *Drosophila* larva. This neuron belongs to the γ class of MD neurons (see **Figure 5**), characterized by a wide dendritic arbor and multiple secondary branches (arrowheads). Loss of *cut* (e) causes a reduction in dendrite size and loss of secondary branches. Scalebar = 5 μm (a–e). ((a): Reproduced with permission from sugimura, K., Yamamoto, M., Niwa, R., Satoh, D., Goto, S., **2003**. District developmental modes and lesion-induced reactions of dendrites of two classes of *Drosophila* sensory neurons. *J. Neurosci. 23*, 3752–3760; © by the Society for Neuroscience. (b, c): Reprinted with permission from Gao, F.B., Kohwi, M., Brenman, J.E., Jan, L.Y., Jan, Y.N., **2000**. Control of dendritic field formation in *Drosophila*: the roles of *flamingo* and competition between homologous neurons. *Neuron 28*, 91–101; © Elsevier. (d, e): Reprinted with permission from Grueber, W.B., Jan, L.Y., Jan, Y.N., **2003**. Different levels of the homeodomain protein Cut regulate distinct dendrite branching patterns of *Drosophila* multidendritic neurons. *Cell 112*, 805–818; © Elsevier.)

Mature MD neurons of a given class have dendritic arbors that completely tile the bodywall. In *Drosophila*, a single MD neuron spans the entire antero-posterior length of the segment it is located in, but stops abruptly at the segment boundary. Dendritic arbors of dorsally-located MD neurons reach the dorsal midline, but do not cross over it, and therefore avoid any overlap with the dendrites of their contralateral counterpart (**Figure 19b**). This suggests that repulsive interactions exist between adjacent neurons. Such interactions could indeed be shown by ablating individual MD neurons, and observing that dendrites of the serial homologs of these cells in adjacent segments spread into the territory vacated by the ablated cell. An adhesion molecule of the cadherin family, Flamingo (Fmi), has been shown to be involved in restricting dendritic growth of MD neurons (Gao *et al*., 2000). Lack of Fmi causes exuberant dorsal growth and overlap of dendritic arbors of homologous MD neurons (**Figure 19c**).

Another regulator of dendritic arborization is the homeodomain protein Cut (Ct) that was discussed in the context of SOP fate specification (see above). Cut is expressed in SOPs of external sensilla and MD neurons, in which it remains detectable after differentiation has set in. The level of Cut expression is correlated with dendritic complexity, in that MD neurons with larger and more highly branched dendritic arbors express more Cut than MD neurons with simpler dendrites (Grueber *et al*., 2003). Experimental studies confirmed the hypothesis that Cut is causally related to dendrite formation: removal of this protein in individual dendritic arbor neurons caused a dramatic drop in the complexity of the corresponding dendrites (**Figure 19d** and **e**).

1.11.4.4. Axonal Arborization of Sensory Neurons

Axons of sensory neurons project towards the CNS where they arborize in the neuropil. A great wealth of information exists on the details of sensory axonal arborization patterns and the neuronal circuits in which they are integrated (see **Chapters 1.10** and **2.4**). Presenting these data in adequate detail would exceed the scope of this chapter, and only a brief survey of important general aspects of sensory projections will be given here.

As a general rule, sensory axons project to the neuromere of the segment to which the corresponding sensilla belong. Bristles of the fly wing project to the mesothoracic neuromere; taste sensilla on the labium project to the subesophageal ganglion (that contains the labial neuromere), and olfactory neurons of the antenna to the antennal neuromere

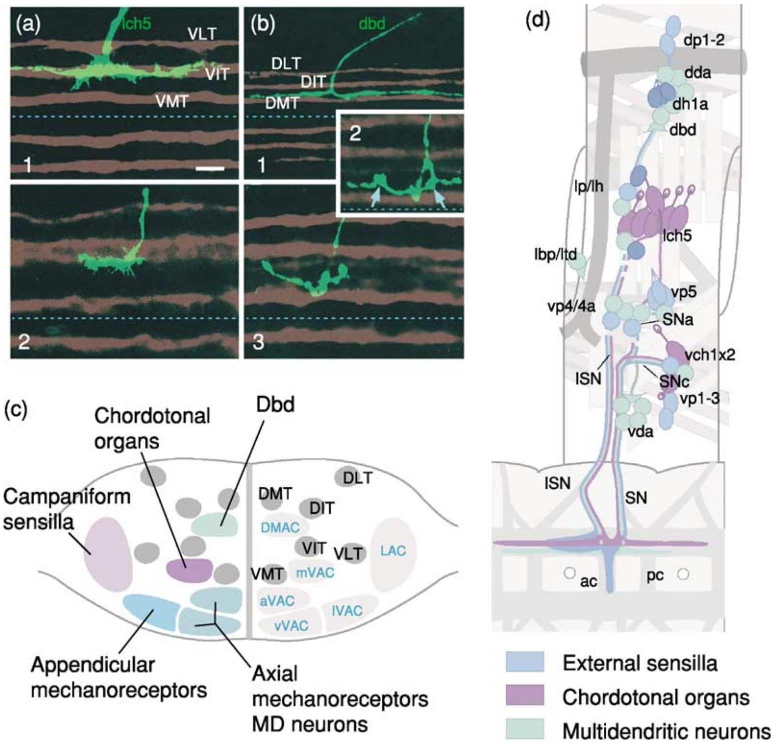


Figure 20 Sensory projections in the central nervous system (CNS). (a, b) Dorsal view of *Drosophila* late embryonic ventral nerve cord where principal longitudinal tracts are labeled with anti-Fasciclin II antibody (brown; DIT, dorso-intermediate tract; DLT, dorso-lateral tract; DMT, dorso-medial tract; VIT, ventro-intermediate tract; VLT, ventro-lateral tract; VMT, ventro-medial tract). Blue dotted line indicates midline of the CNS. In (a), the sensory neuron of the lateral pentascolopidial chordotonal organ (lch5; green) was filled with Lucifer yellow to show the axonal termination close to the VIT tract. (a1) and (a2) represent two different stages (stages 16 and 17, respectively) in development. Note that the lch5 termination relative to the VIT tract remains unchanged, whereas the longitudinal extent of projection retracts with age. In (b1–3) the dbd neuron was labeled. Terminal arborizations of this cell are found more dorsally and medially, close to the DMT tract. (c) Schematic cross-section of a generalized insect ventral nerve cord in which tracts (dark gray, black lettering) and sensory neuropils (light gray; blue lettering) are identified on the right side. On the left side site, sensory modalities are correlated with specific sensory neuropils. (d) Schematic drawing of an abdominal (a1–7) hemisegment in which sensilla and muscle fibers are depicted. The dorsal midline is at the top, the ventral midline at the bottom; anterior is to the left. Colored profiles show sensory neurons belonging to external sensilla (blue), chordotonal organs (violet), and multidendritic neurons (green). Somatic muscle fibers are shown in gray. The axonal projection of different classes of sensilla through the intersegmental nerve (ISN) and the segmental nerve (SN) and the site of termination in the ventral nerve cord are illustrated. Scalebar = 10 μm. ((a, b): Reprinted with permission from Zlatic, M., Landgraf, M., Bate, M., **2003**. Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the *Drosophila* nervous system. *Neuron 37*, 41–51; © Elsevier. (c) Adapted from Pflüger, H.J., Bräunig, P., Hustert, R., **1988**. The organization of mechanosensory neuropils in locust thoracic ganglia. *Philos. Trans. R. Soc. Lond. B 321*, 1–26; Schrader, S., Merritt, D.J., **2000**. Central projections of *Drosophila* sensory neurons in the transition from embryo to larva. *J. Comp. Neurol. 425*, 34–44; and Zlatic, M., Landgraf, M., Bate, M., **2003**. Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the *Drosophila* nervous system. *Neuron 37*, 41–51. (d) Adapted from Campos-Ortega, J.A., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila melanogaster*, 2nd edn. Springer, Berlin.)

(called the deuterocerebrum). One can go one step further in stating that the terminal arbors of most sensory neurons are restricted to the segment to which these neurons belong (**Figure 20b**). There are, however, notable exceptions such as the axons of scolopidium #4 of the *Drosophila* lch5 chordotonal, which ascend along the entire length of the ventral nerve cord (Schrader and Merritt, 2000).

Within a neuromere, sensory axonal arborizations are restricted to certain sensory modality-specific domains. These domains have been defined for several insect species (to name but a few studies: Murphey *et al*., 1985, 1989; Pflüger *et al*., 1988; Newland, 1991; Merritt and Whitington, 1995; Schrader and Merritt, 2000; Zlatic *et al*., 2003). To subdivide the neuropil, the main bundles of longitudinal axons serve as useful landmarks. There exist three rather evenly spaced sets of these bundles, a medial, an intermediate, and a lateral set (**Figure 20a–c**; Pflüger *et al*., 1988; Nassif *et al*., 2003; Zlatic *et al*., 2003). Both medial and lateral sets comprise a dorsal and a ventral bundle (DMT and VMT; DLT and VLT, respectively). The intermediate set contains a dorsal bundle (DIT) and several

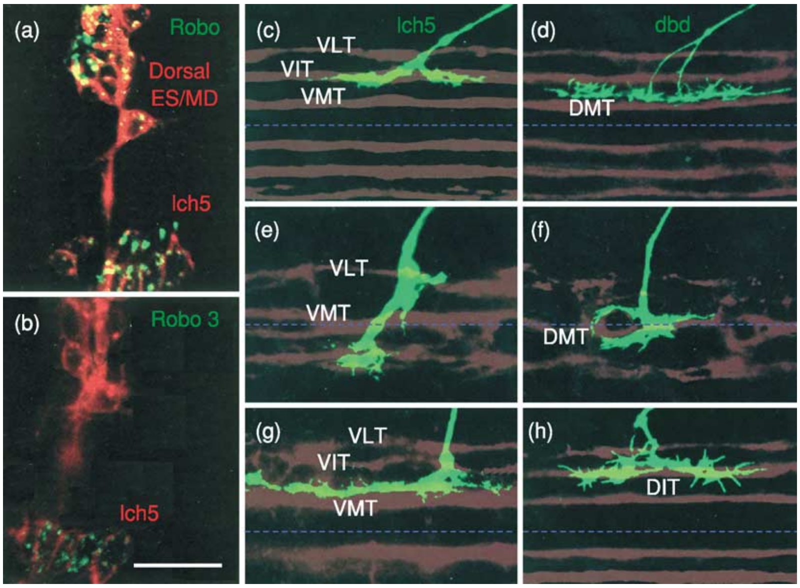


Figure 21 The role of Robo receptors in controlling sensory axonal projections. (a) Robo (green) is expressed in all *Drosophila* embryonic sensory neurons, including the multidenrtitic neurons (MD) and external sensilla (ES) of the dorsal group, and lateral chordotonal organs (lch5). (b) Expression of Robo 3 is restricted to chordotonal organs. (c, d) Termination of axon of lch5 (c) on VIT tract and axon of dbd stretch receptor (dbd) on DMT (d) in wild-type *Drosophila* ventral nerve cord. Blue dotted line indicates midline. (e, f) Mutation of Robo results in abnormal central tracts and midline crossings of chordotonal axons (e) and dbd axons (f). (g) Loss of Robo 3 causes chordotonal axon to project to a more medial position near the VMT. (h) Ectopic expression of Robo 3 in dbd forces dbd axonal termination into a more lateral position near the DIT. Scale bar = 10 μm (a–h). (Reprinted with permission from Zlatic, M., Landgraf, M., Bate, M., **2003**. Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the *Drosophila* nervous system. *Neuron 37*, 41–51; © Elsevier.)

small bundles (VIT1-3) extending along the center of the neuropil. The domain that receives sensory axons is located predominantly in the ventral half of the neuropil. Within the sensory neuropil, axons of tactile receptors occupy a domain ventral of that of proprioceptors (chordotonal organs, MD stretch receptors; Pflüger *et al*., 1988; Schrader and Merritt, 2000; Zlatic *et al*., 2003; **Figure 20c**). Tactile hairs (trichoid sensilla) and multidendritic neurons, located on the trunk, project to a ventromedial domain surrounding the VMT bundle (**Figure 20c** and **d**); trichoid sensilla on the appendages project more laterally to a neuropil domain ventral and lateral of VLT. In several instances, a pronounced somatotopic ordering of sensory projections has been identified. For example, projections of trichoid sensilla on the dorsal trunk terminate dorsally of those from ventral sensilla (Pflüger *et al*., 1988). Terminal arbors of chordotonal organs are found dorsally of tactile projections, surrounding the VIT bundles (**Figure 20a** and **c**). Some stretch receptors have terminal arbors that are even more dorsal. For example, the *Drosophila* dbd stretch receptor terminates around the DMT bundle (**Figure 20b** and **c**); external proprioceptors, such as the campaniform sensilla and hairplates located on the legs, project to a dorsolateral domain within the neuropil.

Recent findings have provided a first insight into the molecular mechanisms that control the pattern of sensory projections in the CNS. A pivotal element of this mechanism is the extracellular signal Slit, which emanates from the midline cells of the CNS and forms a medio-lateral gradient. Axons of many neurons, including the central inter- and motoneurons and peripheral sensory neurons, express receptors for Slit (Rajagopalan *et al*., 2000; Simpson *et al*., 2000; Guthrie, 2001; Wong *et al*., 2002). Three different receptors, encoded by the *roundabout* genes (*robo*, *robo 2*, *robo 3*) and having different affinities for Slit have been identified. Slit acts as a repellant, so that axons, which express Robo and thereby become sensitive to Slit, stay away from the midline (i.e., do not cross) and form longitudinal projections instead. Furthermore, the expression of Robo receptors with different affinities for Slit determines the distance between an axon and the midline. Axons expressing Robo 3 (high affinity) keep at a greater distance from the midline than axons which only have Robo.

The correlation between Robo type expression and projection pattern has been experimentally

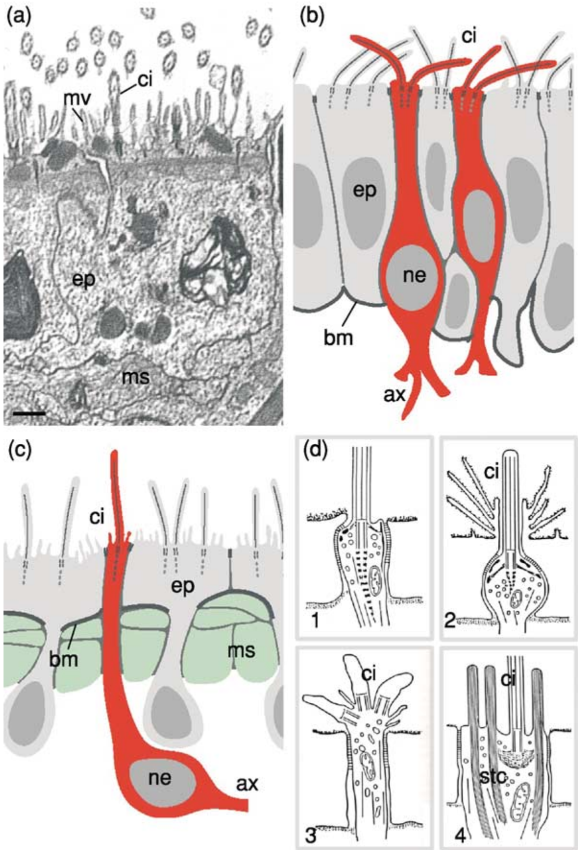


Figure 22 Sensilla in coelenterates and flatworms. (a) TEM of section of the epidermis of the flatworm *Macrostomum* sp., showing the muscle layer (ms) and epidermal cells (ep) which bear multiple cilia (ci) and microvilli (mv). (b) Schematic section of cnidarian epidermis with multiciliated epidermal cells (ep) and sensory neurons (ne). (c) Naked sensory neuron (ne) with subepidermally shifted cell body in a flatworm. (d1–4) Different types of ciliary endings of flatworm sensory neurons. (d1) Simple mechanosensory cilium. (d2) Cilium surrounded by specialized microvilli. (d3) Multiciliated ending of chemoreceptor. (d4) Mechanoreceptor with enlarged microvilli (stereocilia). bm, basement membrane; stc, stereocilia. Scale bar = 0.5 μm (a). (b) Adapted from Chia, F.S., Koss, R., **1979**. Fine structural studies of the nervous system and the apical organ in the planula larva of the sea anemone *Anthopleura elegantissima*. *J. Morphol. 160*, 275–298. (d) Adapted from Wright, K.A., **1992**. Peripheral sensilla of some lower invertebrates: the Platyhelminthes and Nematoda. *Microsc. Res. Tech. 22*, 285–297.

addressed in the *Drosophila* sensory axons (Zlatic *et al.*, 2003). Robo is expressed by all sensory neurons, and is responsible for maintaining sensory axons ipsilaterally. Loss of the gene encoding Robo causes many sensory axons to cross the midline and form bilateral projections (Figure 21e and f). Chordotonal neurons express Robo 3, beside Robo 4, and it is this high-affinity receptor for Slit that maintains the position of chordotonal sensory axons at a position more lateral than that of axons of external sensilla and MD sensilla. Mutations in Robo 3 cause chordotonal axons to terminate at a medial position (Figure 21g); vice versa, expressing Robo 3 in MD neurons forces the axons of these cells into a more lateral position (Figure 21h).

One might assume that the type of Robo receptor expressed by a sensory neuron is ultimately under the control of the SOP identity gene that is responsible for the overall morphology of the sensillum which the neuron is part of. Such control has been demonstrated to exist for Robo 3, expressed in chordotonal neurons as a target of the proneural/SOP identity gene *atonal* (Zlatic *et al.*, 2003). Expressing *atonal* in MD neurons turns on Robo 3 in these cells and changes their sensory projection to that normally seen for chordotonal neurons.

1.11.5. Phylogenetic Aspects of Sensillum Structure and Development

1.11.5.1. Coelenterates and "Lower" Bilateria

Sensory structures integrated into the epidermal covering of the body can be found in all invertebrate phyla from coelenterates onward. In coelenterates and "lower" protostomes (e.g., flatworms), such structures comprise single or clustered sensory neurons whose dendrite is a modified cilium (Chia and Koss, 1979; Wright, 1992; Kass-Simon and Hufnagel, 1992; Figure 22b and c). In evolutionary terms, such ciliated peripheral neurons can be regarded as modified epidermal cells, whom they resemble closely in their ultrastuctural appearance. Thus, the epidermis of coelenterates and flatworms consists of an epithelial layer of ciliated cells, and coordinated ciliary beating is the mechanism by which coeleneterates and flatworms move. Each epidermal cell produces one (in coelenterates) or multiple (in flatworms) cilia, which are surrounded by microvilli (Figure 22a). A similar morphology is seen in sensory neurons of lower invertebrates. However, unlike those of epidermal cells, cell bodies of sensory neurons are often sunken beneath the level of the epidermis or can even reside within the CNS. Furthemore, the ciliary dendrites can take on various shapes in accordance with their mechano- or chemoreceptive function (Figure 22d). In most sensory neurons, microvilli remain inconspicuous, although neurons with large microvillar processes resembling the stereocilia of hair cells in vertebrates (see below) have been observed (Figure 22d). Accessory cells, such as the trichogen or tormogen cell of arthropods, are rare or absent in lower invertebrates.

The special senses of lower invertebrates comprise simple eyes and statocysts (gravity receptors). Eyes

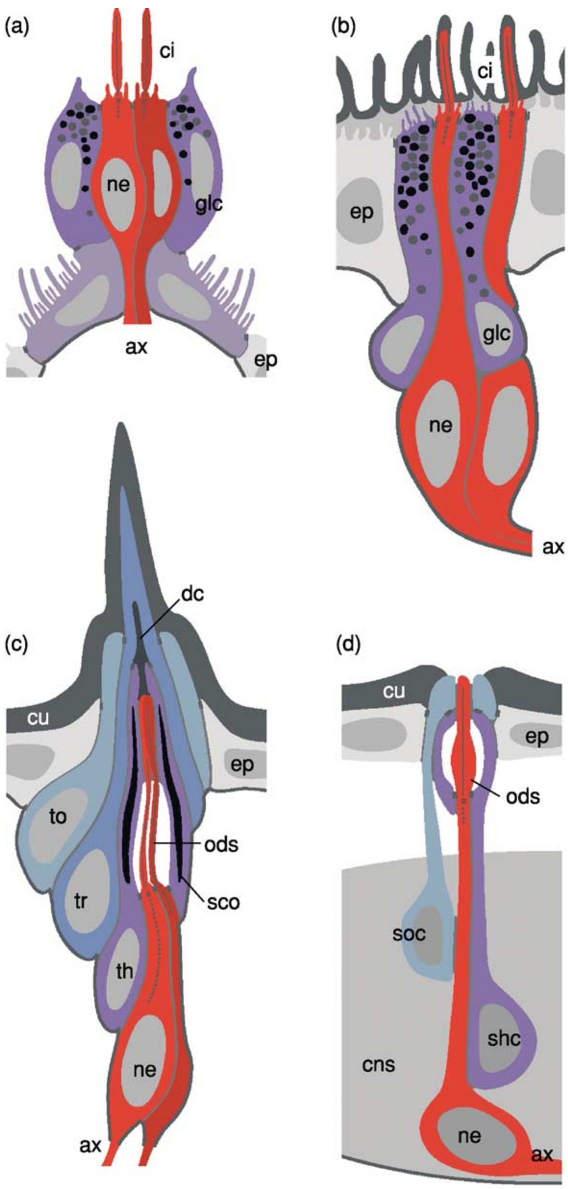


Figure 23 Sensilla in ''higher'' protostomes. Ciliated sensory neurons (ne) have aquired specialized epidermal support cells (ep) with possible glandular function (glc) in mollusks (a) and annelids (b). Crustacean external sensilla (c) closely resemble insect sensilla. As shown in this diagram, neurons (ne) and thecogen cells (th) show attributes seen only in chordotonal organs in insects (sco, scolopale). (d) The nematode sensillum typically contains ciliated neuron (ne) and two accessory cells, a socket cell (soc) and sheath cell (shc). Cell bodies of all sensillum cells are contained within the central nervous system (cns). ax, axon; ci, cilium; cu, cuticle; dc, dendritic cap; ods, outer dendritic segment (modified cilium); sco, scolopale; to, tormogen cell; tr, trichogen cell. ((a) Adapted from Moir, 1977. (b) Adapted from Dorsett, D.A., **1976**. The structure and function of proprioceptors in soft-bodied invertebrates. In: Mill, P.J. (Ed.), Structure and Function of Proprioceptors in Insects. Chapman and Hall, London, pp. 443–483. (c) Adapted from Kouyama, N., Shimozawa, T., **1982**. The structure of a hair mechanoreceptor in the antennule of crayfish (Crustacea). *Cell Tissue Res. 226*, 565–578. (d) Adapted from Wright, K.A., **1992**. Peripheral sensilla of some lower invertebrates: the Platyhelminthes and Nematoda. *Microsc. Res. Tech. 22*, 285–297.)

consist of clusters of ciliated cells in which either the cilium (ciliary photoreceptors) or the microvilli (rhabdomeric photoreceptors) are grossly enlarged to carry the photopigment. A statocyst is formed by a fluid-filled vesicle lined by an epithelium. The epithelium produces the statolith, a minuscule sphere of calcified extracellular material that moves around in the statocyst as the animal changes its position relative to gravitational pull. The epithelial lining of the statocyst contains sensory neurons. Movement of the statolith will bend the cilia of these neurons and thereby elicit a receptor potential.

1.11.5.2. "Higher" Protostomes: Annelids, Mollusks, Nematodes, Arthropods

The repertoire of sensory structures characterized for annelids and mollusks is similar to that seen in lower invertebrates (Dorsett, 1976; Bullock *et al.*, 1977). Bipolar sensory neurons with a ciliary dendrite occur singly or in clusters integrated in the epidermis of the body wall and feeding structures. Accessory cells are absent in most cases, but have been described for both mollusks and annelids, where ciliated sensory neurons are surrounded by specialized cells filled with secretory granules (**Figure 23a and b**). It is unknown whether sensory neurons, and such accessory cells form a lineage derived from a single SOP-like progenitor cell. Subepidermal sensory neurons, which are likely to serve as stretch receptors have been identified in annelids and cephalopods. These cells are multipolar, similar to insect MD neurons, and stretch out at the basal surface of the epidermis and around muscles. In polychaete worms, similarly shaped cells send branched dendrites to the internal processes of the large setae and react to deflections of the setae, not unlike the mechanosensitive trichoid sensilla in insects. Again, it is not known whether the setae-forming cell(s) and sensory neurons form part of a lineage born from single progenitor cells.

Sensilla, closely resembling those described for insects in this chapter, exist in all arthropod classes (Hallberg and Hansson, 1999). External sensilla with lineages comprising sensory neurons and inner and outer accessory cells forming sheaths around the dendrite were found in crustaceans (**Figure 23c**), spiders, and myriapods. Similarly, internal MD neurons have been found in all classes. Chordotonal organs, alternatively, seem to be present only in crustaceans and insects, which reinforces the notion that these two classes are more closely related and have further evolved from the arthropod ancestor. Comparing the morphology of external sensilla and chordotonal organs in crustaceans and insects provides several clues to the evolution of chordotonal organs. Thus, whereas in insects the

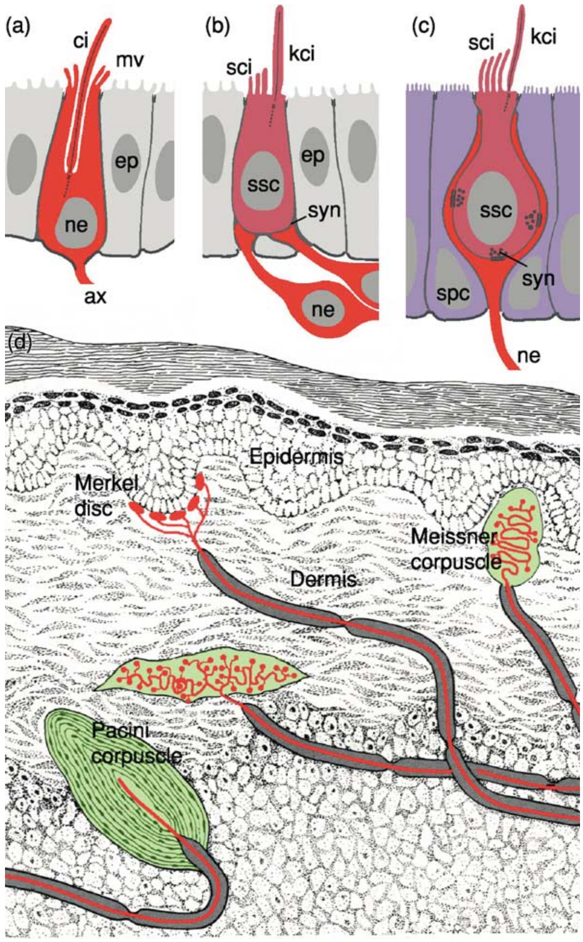


Figure 24 Sensilla in deuterostomes. (a) Primary ciliated sensory neuron (ne) in ascidian epidermis. (b) Secondary sensory cell (hair cell; ssc) in lateral line of fish, forming synaptic contact (syn) with bipolar neurons (ne). (c) Hair cell of vertebrate ear. Hair cells have apical specializations derived from microvilli (stereocilia, sci) and from cilia (kinocilia, kci). In the vertebrate inner ear, hair cells are surrounded by specialized support cells (spc). (d) Mechanoreceptors in vertebrates (green) are located in the deep layers of the skin (dermis) and develop from mesodermal cells contacted by sensory dendrites (red) of neurons located in dorsal root ganglia. ax, axon; ci, cilium; ep, epidermal cell; mv, microvillus. ((a, b) Adapted from Bone, Q., Ryan, K.P., **1978**. Cupular sense organs in *Ciona* (Tunicata: Ascidiacea). *J. Zool. Lond. 186*, 417–429. (c) Adapted from Bullock, T.H., Orkand, R., Grinnell, A., **1977**. Introduction to nervous systems. W.H. Freeman, San Francisco. (d) Adapted from Pansky, B., Delmas, J.A., **1980**. Review of Neuroscience. Macmillan Publishing, New York.)

two types of sensilla form discrete classes with several characteristic differences, this is not the case in crustacea. Here, many external sensilla with external (apical) processes possess a scolopale which is formed in the thecogen cell (Kouyama and Shimozawa, 1982; Merritt, 1997; **Figure 23c**). Vice versa, chordotonal organs of crustacea frequently possess external cuticular specializations. As discussed above, even in primitive insects the cap cells and attachment cells of chordotonal organs have processes inserted into the epidermis. From such findings, a picture emerges according to which chordotonal organs are derived from external sensilla, as in, for example, campaniform sensilla, which also serve as stretch receptors (Moulins, 1976; Merritt, 1997). Primarily, the outer accessory cells are an integral part of the epidermis and form specialized cuticle structures. The thecogen produces a dendritic cap, as well as a scolopale. Secondarily, the organ shifts underneath the epidermis, and the outer accessory cells no longer form cuticular specializations; this would bring the chordotonal organ to the level we find in cases like that described in Section 1.11.4.2.2 for *Periplaneta*. Finally, outer accessory cells move out of the epidermis altogether, producing the most "evolved" type of chordotonal organs prevalent in Diptera.

Nematodes have chemo- and mechanoreceptive sensilla composed of bipolar sensory neurons and two accessory cells called socket cell and sheath cell (**Figure 23d**; Ward, 1975; Wright, 1992). The cell bodies of neurons and accessory cells are internalized and form part of the CNS. However, as decribed for arthropods, the accessory cells form mesaxonal sheaths around the dendrite. Furthermore, as in arthropods, the ciliary outer dendritic segment is enclosed in a receptor lymph space formed by the sheath cell.

1.11.5.3. Sensilla in the Deuterostome Lineage

Peripherally located primary neurons with ciliated sensory dendrites can be found as integral part of the epidermis in all deuterostomes except vertebrates (**Figure 24a**). As in protostomes, the cell bodies of the sensory neurons are typically sunken beneath the level of the epidermis (Dorsett, 1976; Bone and Ryan, 1978). Specialized accessory cells may be present, but no lineage studies investigating the origin of sensory neurons and their relationship to accessory cells have been performed. Arrays of ciliated mechanosensitive cells which have lost their axon and are associated with secondary sensory neurons have been recently identified in the urochordate *Ciona*. These cells may represent the evolutionary ancestor of the lateral line system and inner ear of vertebrates.

With the evolution of vertebrates a dramatic change in the structure of sensory organs, in particular those of the trunk region, has taken place. Ciliated primary sensory neurons have all but disappeared from the periphery. In their stead, mesodermally-derived sensory organs located in the deep layers of the skin act as mechanoreceptors (**Figure 24d**). These structures, including among others the Pacini corpuscles and Merkel's discs, react to stretching and compression of the overlying skin. They are

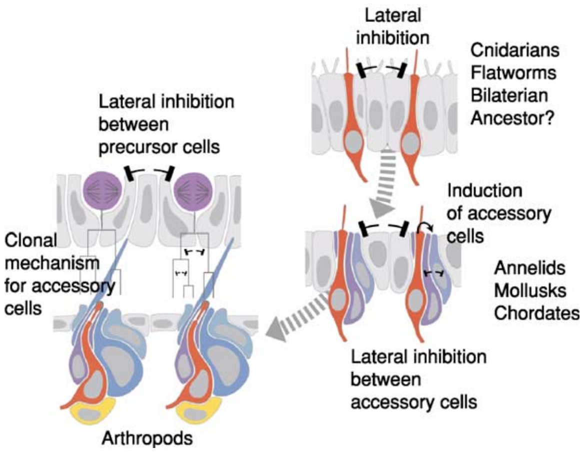


Figure 25 Phylogeny of sensilla. Three stages are proposed: naked ciliated sensory neurons; simple sensilla where sensory neurons recruit adjacent epidermal cells into specialized accessory cells; and clonally derived sensilla in which cell fates of neurons and accessory cells are specified by intrinsic determinants distributed during the asymmetric divisions of SOPs.

innervated by the dendrites of secondary sensory neurons whose cell bodies are far away from the sensory structures themselves, residing in the dorsal root ganglia near the spinal cord. Other classes of sensory neurons with "free ending" dendrites exist. Among these are neurons sensitive to temperature and tissue damage (pain). One structural correlate to the disappearance of invertebrate-type sensilla from the body wall of vertebrate is the thick protective "armor" (scales, keratin layer, hair, feathers) produced by the multilayered skin. These specializations would render the mechanoreceptive function of microscopic sensilla ineffective.

Ciliated sensory neurons with chemo- and mechanosensitive function do still exist in the vertebrate head. They comprise primary sensory neurons integrated into the mucous membranes of the nose, and the hair cells of the inner ear/lateral line system (Figure 24c). Olfactory receptors consist of primary sensory neurons with branched ciliary dendrites, surrounded by glandular support cells. The inner ear consists of a complex of liquid filled vesicles. The vesicles are lined by an epithelium that consists of sensory hair cells surrounded by support cells. The "hairs" of a hair cell consist of stereocilia, grossly enlarged microvilli filled with bundles of actin. Hair cells do not themselves produce an axon. They are innervated by the dendrites of secondary neurons whose cell bodies are clustered in the vicinity of the inner ear (Figure 24c). Movement of the liquid, induced by sudden changes of the position of the body relative to gravitational pull, or, in the case of the ear, by vibration of the tympanal membrane, leads to deflection of the stereocilia and elicits a receptor potential in the hair cells.

1.11.5.4. Conclusion: Evolution of Sensilla in the Metazoa

There is little reason to doubt that at the level of single cells (rather than complex organs) the sensory cells of the vertebrate head, which form part of the nose, inner ear, and eye, are homologous with invertebrate sensory neurons carrying out similar functions. Homology implies that the common ancestor, from which present day vertebrates and the various invertebrate taxa are derived, already possessed the structure stated to be homologous. Since even coelenterates (which branched off the animal tree before the bilaterian ancestor) possess ciliated mechano- and chemosensory neurons, it is safe to assume that the bilaterian ancestor had these cell types as well (Figure 25). Furthermore, it is reasonable to think that in the lines of ancestors leading up to the extant taxa, these cell types were continuously present. All extant taxa possess these cell types, and it is hard to envision ecological scenarios which would favor the disappearance of sensory cells that inform the organism about the chemical and mechanical aspects of its environment.

Molecular evidence supports the close phylogenetic relationship between certain sensory systems in all animals. Thus, the nasal mucosa and inner ear are derived from neurectodermal placodes (nasal placode, otic placode) that invaginate in the wake of neurulation, and give rise to the sensory receptors and support cells that make up the mature structure. Transcription factors of the bHLH family act as regulatory genes required for the fate of sensory neurons (Guillemot *et al.*, 1993; Perez *et al.*, 1999). For example, the vertebrate homolog of the *Drosophila achaete* gene is expressed and required in the olfactory neurons of the nasal mucosa, as well as the dorsal root ganglion cells innervating mechanoreceptory organs of the skin (Guillemot *et al.*, 1993). Recent findings further show how lateral inhibition mediated by N/Dl signaling underlies the separation of sensory cells and support cells in the otic placode (Eddison *et al.*, 2000). Removing the function of this pathway at the critical time results in an excess of support cells and lack of sensory neurons/hair cells, similar to the situation in *Drosophila*, where N activation is repeatedly required in those branches of the SOP lineage that lead to accessory cells.

One can thus envisage an evolutionary scenario that started out with an organism containing "naked" sensory neurons scattered over the

bodywall, resembling the situation presented by present day coelenterates or flatworms (**Figure 25**). It is possible that lateral inhibition involving the N pathway took part in controlling the number and spacing of sensory neurons. The next step was taken with the formation of accessory cells, specialized epidermal cells which protect the neuron and aid in presenting and/or transducing the stimulus. Such accessory cells are variably present in sensilla of "higher" animals. Initially, they might have been induced by signals sent out by the sensory neurons. Signaling pathways, including the EGFR pathway, still play a prominent role in the recruitment of accessory cells in the *Drosophila* compound eye, as well as the induction of SOPs in many modular sensory organs such as chordotonal organs and dense arrays of external sensilla (see section above). The N signaling mechanism was recruited into the specification of accessory cells also. Initially responsible for the pattern of sensory neurons, this pathway also controlled the number and arrangement of accessory cells surrounding each sensory neuron. Finally, in arthropod sensilla and selected chordate sensory organs, a clonal mechanism became an essential factor that was able to guarantee a precise pattern of sensillum cells.

Some recent molecular results have prompted evolutionary speculations on homologies between vertebrate and invertebrate (*Drosophila*) sensory organs that appear unrealistic in the light of existing data. An example is the comparison of the inner ear hair cells in vertebrates and chordotonal organs in *Drosophila*. Starting the debate was the finding that one of the vertebrate homologs of *atonal*, the proneural gene required for *Drosophila* chordotonal organs, some of which serve auditory functions, is also expressed and required in hair cells/sensory neurons of the inner ear (Bermingham *et al*., 1999; Chen *et al*., 2002). The question was raised by subsequent authors, prompting the question of whether specific homologies exist between inner ear and insect chordotonal organs. This question can be answered in the negative with reasonable certainty, given what we know about the structure and function of chordotonal organs and their occurrence among animals. As presented above, chordotonal organs are relative "late-comers" in evolution, lacking in myriapods and chelicerates and all non-arthropods. The recruitment of chordotonal organs into the auditory function most likely occurred even later than the origin of these organs themselves, given that most chordotonal organs are stretch receptors associated with the trunk or joints of the appendages. Thus, it is extremely unlikely that the bilaterian ancestor possessed highly specialized sensory structures resembling chordotonal organs. Alternatively, one might speculate that there exists among the "naked" bipolar sensory neurons with mechanoreceptive properties (which one finds in all bilateria) a specialized class of cells which expressed Atonal, and which became repeatedly recruited into more complex sensory organs which sensed vibration and sound. This would explain the finding that auditory organs which, as multicellular organs, evolved convergently, still express homologous genetic pathways.

Appendix List of genes and gene products discussed in this chapter

achaete-scute complex (AS-C)		gene complex including *ac, sc, l'sc*
achaete (ac)	bHLH TF	proneural gene
amos (amos)	bHLH TF	proneural gene
atonal (ato)	membrane protein (signal)	neurogenic gene
BarH1, BarH2	homeodomain TF	sensillum fate determinant
bazooka (baz)	cytoplasmic protein	asymmetric mitosis
beethoven	details unknown	
crinkled (ck)	myosin VII	microfilament function
cut (ct)	Homeodomain TF	SOP identity gene
decapentaplegic (dpp)	secreted TGF-like signal	morphogen
Delta (Dl)	membrane-bound signal	lateral inhibition
dishevelled (dsh)	cytoplasmic protein	planar cell polarity
elav	RNA binding protein	neural fate determinant
Enhancer of split [E(spl)]	HLH TF	neurogenic gene
flamingo (fmi)	cadherin-adhesion molecule	planar cell polarity
forked (f)	actin binding protein	microfilament bundling
frizzled (fz)	membrane receptor	planar cell polarity
glial cells missing (gcm)	TF	sensillum fate determinant
groucho (gro)	TF	modulates N signaling
hairless (H)	TF	inhibitor of Notch

Continued

Appendix Continued

hamlet (ham)	TF	sensillum fate determinant
hedgehog (hh)	secreted signal	morphogen
inscuteable (insc)	cytoplasmic protein	asymmetric mitosis
iroquois (iro)	homeodomain TF	coordinate gene
lethal of scute (*l'sc*)	HLH TF	proneural gene
no mechanoreceptor. potential A (nompA)	cytoplasmic protein	cilium formation
no mechanoreceptor. potential B (nompB)	membrane protein	dendritic cap
Notch (N)	membrane receptor	neurogenic gene
numb (numb)	cytoplasmic protein	inhibitor of Notch
pannier (pnr)	GATA TF	coordinate gene
phyllopod (phyl)	cytoplasmic protein	signal transducer in RAS pathway
pox-neuro (poxn)	paired box TF	SOP identity gene
prospero (pros)	TF	sensillum fate determinant
Drosophila rfx (Drfx)	TF	cilium formation
Drosophila rhoA (DrhoA)	GTPase	modulates microfilaments
roundabout (robo)	receptor	axonal pathfinding
scute (*sc*)	bHLH TF	proneural gene
shotgun (shg)	E-cadherin (DE-Cad)	adhesion, asymmetric cell division
singed (sn)	actin binding protein	microfilament bundling
Slit (Sli)	secreted signal	axonal pathfinding
sparkling/shaven (sv)	paired box TF	sensillum fate determinant
spitz (spi)	secreted TGFa-like signal	activates RAS pathway
Suppressor of Hairless (Su(H))	TF	inhibitor of Hairless
tilB/smetana (*smet*)	details unknown	
tramtrack (trk)	BTB/POZ TF	sensillum fate determinant
uncoordinated (unc)	cytoplasmic protein	axoneme formation
wingless (wg)	secreted signal	morphogen

TF = transcription factor.

Acknowledgments

This work was supported in part by NIH grant NS29367 to V.H.

References

Adler, P.N., Taylor, J., **2001**. Asymmetric cell division: plane but not simple. *Curr. Biol. 11*, R233–R236.

Altner, H., **1977**. Insektensensillen: bau und funktionsprinzipien. *Verh. Dtsch. Zool. Ges. 70*, 139–153.

Altner, H., Routil, C., Loftus, R., **1981**. The structure of bimodal chemo-, thermo-, and hygroreceptive sensilla on the antenna of *Locusta migratoria*. *Cell Tissue Res. 215*, 289–308.

Ameismeier, F., **1987**. Ultrastructure of the chemosensitive basiconic single walled wall pore sensilla on the antennae in adults and embryonic stages of *Locusta migratoria* L. (Insecta, Orthoptera). *Cell Tissue Res. 247*, 605–612.

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., **1999**. Notch signaling: cell fate control and signal integration in development. *Science 284*, 770–776.

Awasaki, T., Kimura, K., **1997**. *pox-neuro* is required for development of chemosensory bristles in *Drosophila*. *J. Neurobiol. 32*, 707–721.

Bailey, A.M., Posakony, J.W., **1995**. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Devel. 9*, 2609–2622.

Baker, J.D., Esenwa, V., Kernan, M., **2001**. Uncoordinated is a novel protein required for the organization and function of ciliogenic centrioles. *Mol. Biol. Cell 12* (Suppl.), 447.

Bang, A.G., Bailey, A.M., Posakony, J.W., **1995**. *Hairless* promotes stable commitment to the sensory organ precursor cell fate by negatively regulating the activity of the Notch signaling pathway. *Devel. Biol. 172*, 479–494.

Bang, A.G., Hartenstein, V., Posakony, J.W., **1991**. *Hairless* is required for the development of adult sensory organ precursor cells in *Drosophila*. *Development 111*, 89–104.

Bang, A.G., Posakony, J.W., **1992**. The *Drosophila* gene *Hairless* encodes a novel basic protein that controls alternative cell fates in adult sensory organ development. *Genes Devel. 6*, 1752–1769.

Barolo, S., Walker, R.G., Polyanovsky, A.D., Freschi, G., Keil, T., *et al.*, **2000**. A Notch-independent activity of Suppressor of hairless is required for normal mechanoreceptor physiology. *Cell 103*, 957–969.

Barth, F.G., **1986**. Zur organisation sensorischer systeme: die cutikularen mechanorezeptoren der arthropoden. *Verh. Dtsch. Zool. Ges. 79*, 69–90.

Bermingham, N.A., Hassan, B.A., Price, S.D., Vollrath, M.A., Ben-Arie, N., *et al.*, **1999**. *Math1*: an essential gene for the generation of inner ear hair cells. *Science 284*, 1837–1841.

Blochlinger, K., Bodmer, R., Jan, L.Y., Jan, Y.N., **1990**. Patterns of expression of Cut, a protein required for external sensory organ development in wild-type and *cut* mutant *Drosophila* embryos. *Genes Devel. 4*, 1322–1331.

Bloechl, R., Selzer, R., **1988**. Embryogenesis of the connective chordotonal organ in the pedicel of the American cockroach: cell lineage and morphological differentiation. *Cell Tissue Res. 252*, 669–678.

Bodmer, R., Barbel, S., Sheperd, S., Jack, J.W., Jan, L.Y., *et al.*, **1987**. Transformation of sensory organs by mutations of the *cut* locus of *D. melanogaster. Cell 51*, 293–307.

Bodmer, R., Carretto, R., Jan, Y.N., **1989**. Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron 3*, 21–32.

Bodmer, R., Jan, Y.N., **1987**. Morphological differentiation of the embryonic peripheral neurons in *Drosophila*. *Roux's Arch. Devel. Biol. 196*, 69–77.

Bone, Q., Ryan, K.P., **1978**. Cupular sense organs in *Ciona* (Tunicata: Ascidiacea). *J. Zool. Lond. 186*, 417–429.

Bray, S., Furriols, M., **2001**. Notch pathway: making sense of Suppressor of Hairless. *Curr. Biol. 11*, R217–R221.

Brewster, R., Bodmer, R., **1995**. Origin and specification of type II sensory neurons in *Drosophila*. *Development 121*, 2923–2936.

Brewster, R., Bodmer, R., **1996**. Cell lineage analysis of the *Drosophila* peripheral nervous system. *Devel. Genet. 18*, 50–63.

Bullock, T.H., Orkand, R., Grinnell, A., **1977**. Introduction to nervous systems. W.H. Freeman, San Francisco.

Caldwell, J.C., Eberl, D.F., **2002**. Towards a molecular understanding of *Drosophila* hearing. *J. Neurobiol. 53*, 172–189.

Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., *et al.*, **2000**. Generation of medial and lateral dorsal body domains by the *pannier* gene of *Drosophila*. *Development 127*, 3971–3980.

Campos-Ortega, J.A., **1995**. Genetic mechanisms of early neurogenesis in *Drosophila melanogaster. Mol. Neurobiol. 10*, 75–89.

Campos-Ortega, J.A., **1997**. Asymmetic division: dynastic intricacies of neuroblast division. *Curr. Biol. 7*, R726–R728.

Campos-Ortega, J.A., Hartenstein, V., **1985**. The Embryonic Development of *Drosophila melanogaster.* Springer, Berlin.

Campos-Ortega, J.A., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila melanogaster*, 2nd edn. Springer, Berlin.

Cayouette, M., Raff, M., **2002**. Asymmetric segregation of Numb: a mechanism for neural specification from *Drosophila* to mammals. *Nat. Neurosci. 5*, 1265–1269.

Chen, P., Johnson, J.E., Zoghbi, H.Y., Segil, N., **2002**. The role of *Math1* in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development 129*, 2495–2505.

Chia, F.S., Koss, R., **1979**. Fine structural studies of the nervous system and the apical organ in the planula larva of the sea anemone *Anthopleura elegantissima*. *J. Morphol. 160*, 275–298.

Chia, W., Yang, X., **2002**. Asymmetric division of *Drosophila* neural progenitors. *Curr. Opin. Genet. Devel. 12*, 459–464.

Chung, Y.D., Zhu, J., Han, Y., Kernan, M.J., **2001**. NompA encodes a PNS-specific, ZP domain protein required to connect mechanosensory dendrites to sensory structures. *Neuron 29*, 415–428.

Cubas, P., de Celis, J.F., Campuzano, S., Modolell, J., **1991**. Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Devel. 5*, 996–1008.

Dambly-Chaudiere, C., Jamet, E., Burri, M., Bopp, D., Basler, K., *et al.*, **1992**. The paired box gene *pox neuro*: a determinant of poly-innervated sense organs in *Drosophila*. *Cell 69*, 159–172.

De Celis, J.F., Barrio, R., Kafatos, F.C., **1999**. Regulation of the *spalt/spalt-related* gene complex and its function during sensory organ development in the *Drosophila* thorax. *Development 126*, 2653–2662.

De Kramer, J.J., Van der Molen, L.G., **1984**. Development of labellar taste hairs in the blowfly *Calliphora vicina* (Insecta, Diptera). *Zoomorphology 104*, 1–10.

Dorsett, D.A., **1976**. The structure and function of proprioceptors in soft-bodied invertebrates. In: Mill, P.J. (Ed.), Structure and Function of Proprioceptors in Insects. Chapman and Hall, London, pp. 443–483.

Dubruille, R., Laurencon, A., Vandaele, C., Shishido, E., Coulon-Bublex, M., *et al.*, **2002**. *Drosophila* regulatory factor X is necessary for ciliated sensory neuron differentiation. *Development 129*, 5487–5498.

Eaton, S., Wepf, R., Simons, K., **1996**. Roles for Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of *Drosophila*. *J. Cell Biol. 135*, 1277–1289.

Eberl, D.F., Hardy, R.W., Kernan, M.J., **2000**. Genetically similar transduction mechanisms for touch and hearing in *Drosophila*. *J. Neurosci. 20*, 5981–5988.

Eddison, M., Le Roux, I., Lewis, J., **2000**. Notch signaling in the development of the inner ear: lessons from *Drosophila*. *Proc. Natl Acad. Sci. USA 97*, 11692–11699.

Edwards, J.S., Chen, S.W., **1979**. Embryonic development of an insect sensory system, the abdominal cerci of *Acheta domesticus*. *Wilhelm Roux's Arch. 186*, 151–178.

Eguchi, E., Tominaga, Y., **1999**. Atlas of Arthropod Sensory Receptors: Dynamic Morphology in Relation to Function. Springer, Tokyo.

Ernst, K.D., **1972**. Die ontogenie der basikonischen riechsensillen auf der antenne von *Necrophorus* (Coleoptera). *Z. Zellforsch. 129*, 217–236.

Ferris, G.F., **1965**. External morphology of the adult. In: Demerec, M. (Ed.), Biology of *Drosophila*. Hafner, New York.

Fichelson, P., Gho, M., **2003**. The glial cell undergoes apoptosis in the microchaete lineage of *Drosophila*. *Development 130*, 123–133.

Finlayson, L.H., **1976**. Abdominal and thoracic receptors in inscets, centipedes and scorpions. In: Mill, P.J. (Ed.), Structure and Function of Proprioceptors in Insects. Chapman and Hall, London, pp. 153–211.

Frise, E., Knoblich, J.A., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., **1996**. The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell–cell interaction in sensory organ lineage. *Proc. Natl Acad. Sci. USA 93*, 11925–11932.

Frye, M.A., **2001**. Encoding properties of the wing hinge stretch receptor in the hawkmoth *Manduca sexta*. *J. Exp. Biol. 204*, 3693–3702.

Gao, F.B., Brenman, J.E., Jan, L.Y., Jan, Y.N., **1999**. Genes regulating dendritic outgrowth, branching, and routing in *Drosophila*. *Genes Devel. 13*, 2549–2561.

Gao, F.B., Kohwi, M., Brenman, J.E., Jan, L.Y., Jan, Y.N., **2000**. Control of dendritic field formation in *Drosophila*: the roles of *flamingo* and competition between homologous neurons. *Neuron 28*, 91–101.

Garcia-Garcia, M.J., Ramain, P., Simpson, P., Modolell, J., **1999**. Different contributions of *pannier* and *wingless* to the patterning of the dorsal mesothorax of *Drosophila*. *Development 126*, 3523–3532.

Ghysen, A., Dambly-Chaudiere, C., Aceves, E., Jan, L.Y., Jan, Y.N., **1986**. Sensory neurons and peripheral pathways in *Drosophila* embryos. *Roux's Arch. Devel. Biol. 195*, 281–289.

Gnatzy, W., **1978**. Development of the filiform hairs on the cerci of *Gryllus bimaculatus* deg (Saltatoria, Gryllidae). *Cell Tissue Res. 187*, 1–24.

Gnatzy, W., **1980**. Morphogenesis of mechanoreceptor and epidermal cells of crickets during the last instar, and its relation to molting-hormone level. *Cell Tissue Res. 213*, 369–391.

Gomez-Skarmeta, J.L., Diez del Corral, R., de la Calle-Mustienes, E., Ferre-Marco, D., Modolell, J., **1996**. *Araucan* and *caupolican*, two members of the novel *iroquois* complex, encode homeoproteins that control proneural and vein-forming genes. *Cell 85*, 95–105.

Green, P.J., Hartenstein, V., **1997**. Structure and spatial pattern of the sensilla of the body segments of insect larvae. *Microsc. Res. Tech. 39*, 470–478.

Grueber, W.B., Graubard, K., Truman, J.W., **2001**. Tiling of the body wall by multidendritic sensory neurons in *Manduca sexta*. *J. Comp. Neurol. 440*, 271–283.

Grueber, W.B., Jan, L.Y., Jan, Y.N., **2002**. Tiling of the *Drosophila* epidermis by multidendritic sensory neurons. *Development 129*, 2867–2878.

Grueber, W.B., Jan, L.Y., Jan, Y.N., **2003**. Different levels of the homeodomain protein Cut regulate distinct dendrite branching patterns of *Drosophila* multidendritic neurons. *Cell 112*, 805–818.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J., *et al.*, **1993**. Mammalian *achaete-scute homolog 1* is required for the early development of olfactory and autonomic neurons. *Cell 75*, 463–476.

Goulding, S.E., zur Lage, P., Jarman, A.P., **2000**. *amos*, a proneural gene for *Drosophila* olfactory sense organs that is regulated by *lozenge*. *Neuron 25*, 69–78.

Guo, M., Bier, E., Jan, L.Y., Jan, Y.N., **1995**. *tramtrack* acts downstream of *numb* to specify distinct daughter cell fates during asymmetric cell divisions in the *Drosophila* PNS. *Neuron 14*, 913–925.

Guo, M., Jan, L.Y., Jan, Y.N., **1996**. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron 17*, 27–41.

Guthrie, S., **2001**. Axon guidance: Robos make the rules. *Curr. Biol. 11*, R300–R303.

Hansen, K., Hansen-Delkeskamp, E., **1983**. The development of taste and tactile hairs in the pharat fly *Protophormia terranovae* (Diptera, Calliphoridae) and in the embryonal cricket *Acheata domestica* (Orthopteroidea, Ensifera). *Zoomorphology 102*, 241–259.

Hallberg, E., Hansson, B.S., **1999**. Arthropod sensilla: morphology and phylogenetic considerations. *Microsc. Res. Tech. 47*, 428–439.

Hartenstein, V., **1988**. Development of the *Drosophila* larval sensory organs: spatiotemporal pattern of sensory neurones, peripheral axonal pathways, and sensilla differentiation. *Development 102*, 869–886.

Hartenstein, V., Posakony, J.W., **1989**. The development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development 107*, 389–405.

Hartenstein, V., Posakony, J.W., **1990**. A dual fuction of the *Notch* gene in *Drosophila* sensillum development. *Devel. Biol. 142*, 13–30.

Hartenstein, V., Younossi-Hartenstein, A., Lekven, A., **1994**. Delamination and division in the *Drosophila* neurectoderm: spatio-temporal pattern, cytoskeletal dynamics, and common control by neurogenic and segment polarity genes. *Devel. Biol. 165*, 480–499.

Higashijima, S., Kojima, T., Michiue, T., Ishimaru, S., Emori, Y., *et al.*, **1992**. Dual *Bar* homeo box genes of *Drosophila* required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development. *Genes Devel. 6*, 50–60.

Hinz, U., **1997**. On the function of proneural genes in *Drosophila*. *Perspect Devel. Neurobiol. 4*, 273–284.

Jan, Y.N., Jan, L.Y., **2001**. Asymmetric cell division in the *Drosophila* nervous system. *Nat. Rev. Neurosci. 2*, 772–779.

Jarman, A.P., Grau, Y., Jan, L.Y., Jan, Y.N., **1993**. *atonal* is a proneural gene that directs chordotonal organ

formation in the *Drosophila* peripheral nervous system. *Cell 73*, 1307–1321.
Jarman, A.P., Sun, Y., Jan, L.Y., Jan, Y.N., **1995**. Role of the proneural gene, *atonal*, in formation of *Drosophila* chordotonal organs and photoreceptors. *Development 121*, 2019–2030.
Jones, B.W., Fetter, R.D., Tear, G., Goodman, C.S., **1995**. *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell 82*, 1013–1023.
Kass-Simon, G., Hufnagel, L.A., **1992**. Suspected chemoreceptors in coeloenterates and ctenophores. *Microsc. Res. Tech. 22*, 265–284.
Kavaler, J., Fu, W., Duan, H., Noll, M., Posakony, J.W., **1999**. An essential role for the *Drosophila* Pax2 homolog in the differentiation of adult sensory organs. *Development 126*, 2261–2672.
Keil, T.A., **1992**. Fine structure of a developing insect olfactory organ: morphogenesis of the silkmoth antenna. *Microsc. Res. Tech. 22*, 351–371.
Keil, T.A., **1997**. Functional morphology of insect mechanoreceptors. *Microsc. Res. Tech. 39*, 506–531.
Keil, T.A., **1998**. The structure of integumental mechanoreceptors. In: Harrison, F.W. (Ed.), Microscopic Anatomy of Invertebrates, vol 11B. Wiley and Liss, New York, pp. 385–404.
Keil, T.A., Steinbrecht, R.A., **1984**. Mechanosensitive and olfactory sensilla of insects. In: King, R.C., Akai, H. (Eds.), Insect Ultrastructure, vol. 2. Plenum, New York, pp. 477–516.
Keil, T.A., Steinbrecht, R.A., **1997**. Comparative morphogenesis of sensilla: a review. *Int. J. Insect Morphol. Embryol. 26*, 151–160.
Knoblich, J.A., **2001**. Asymmetric cell division during animal development. *Nat. Rev. Mol. Cell Biol. 2*, 11–20.
Kouyama, N., Shimozawa, T., **1982**. The structure of a hair mechanoreceptor in the antennule of crayfish (Crustacea). *Cell Tissue Res. 226*, 565–578.
Kuhbandner, B., **1984**. Ultrastructure and ontogeny of the hair sensilla on the funicle of *Calliphora erythrocephala* (Insecta, Diptera). I. The basiconical sensilla. *Zoomorphology 104*, 373–385.
Kuhbandner, B., **1985**. Ultrastructure and ontogeny of the double-walled sensilla on the funicle of *Calliphora erythrocephala* Meigen (Diptera: Calliphoridae). *Int. J. Insect Morphol. Embryol. 14*, 227–242.
Lage, P., Jan, Y.N., Jarman, A.P., **1997**. Requirement for EGF receptor signalling in neural recruitment during formation of *Drosophila* chordotonal sense organ clusters. *Curr. Biol. 7*, 166–175.
Lai, E.C., Orgogozo, V., **2004**. A hidden program in *Drosophila* peripheral neurogenesis revealed: fundamental principles underlying sensory organ diversity. *Devel. Biol. 269*, 1–17.
LeBorgne, R., Bellaiche, Y., Schweisguth, F., **2002**. *Drosophila* E-cadherin regulates the orientation of asymmetric cell division in the sensory organ lineage. *Curr. Biol. 12*, 95–104.
Lecourtois, M., Schweisguth, F., **1997**. Role of Suppressor of Hairless in the Delta-activated Notch signaling pathway. *Perspect. Devel. Neurobiol. 4*, 305–311.
Lee, J.K., Altner, H., **1985**. Sensory cell degeneration in the ontogeny of the chemosensitive sensilla in the labial palp-pit organ of the butterfly, *Pieris rapae* L. (Insecta, Lepidoptera). *Cell Tissue Res. 242*, 279–288.
Leyns, L., Gomez-Skarmeta, J.L., Dambly-Chaudiere, C., **1996**. *iroquois*: a prepattern gene that controls the formation of bristles on the thorax of *Drosophila*. *Mech. Devel. 59*, 63–72.
Lu, B., Usui, T., Uemura, T., Jan, L.N., Jan, Y.N., **1999**. Flamingo controls the planar polarity of sensory bristles and asymmetric division of sensory organ precursors in *Drosophila*. *Curr. Biol. 9*, 1247–1250.
Manning, L., Doe, C.Q., **1999**. Prospero distinguishes sibling cell fate without asymmetric localization in the *Drosophila* adult external sense organ lineage. *Development 126*, 2063–2071.
Martini, R., **1986**. Fine structure and development of the large sensilla basiconica on the antenna of sphecid wasps. *Tissue Cell 18*, 143–151.
McIver, S., **1985**. Mechanoreception. In: Kerkut, A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 6. Pergamon, New York, pp. 71–132.
Muskavitch, M.A., **1994**. Delta-Notch signaling and *Drosophila* cell fate choice. *Devel. Biol. 166*, 415–430.
Meier, T., Chabaud, F., Reichert, H., **1991**. Homologous patterns in the embryonic development of the peripheral nervous system in the grasshopper *Schistocercagregaria* and the fly *Drosophila melanogaster. Development 112*, 241–253.
Merritt, D.J., **1997**. Transformation of external sensilla to chordotonal sensilla in the *cut* mutant of *Drosophila* assessed by single-cell marking in the embryo and larva. *Microsc. Res. Tech. 39*, 492–505.
Merritt, D.J., Whitington, P.M., **1995**. Central projections of sensory neurons in the *Drosophila* embryo correlate with sensory modality, soma position and proneural function. *J. Neurosci. 15*, 1755–1767.
Modolell, J., **1997**. Patterning of the adult peripheral nervous system of *Drosophila*. *Perspect. Devel. Neurobiol. 4*, 285–296.
Moir, **1977**. Ultrastructural studies on the ciliated receptors of the long tentacles of the scallop *Placopecten magellanus* (Gmelin). *Cell Tissue Res. 184*, 367–380.
Moore, A.W., Jan, L.Y., Jan, Y.N., **2002**. *hamlet*, a binary genetic switch between single- and multiple-dendrite neuron morphology. *Science 297*, 1355–1358.
Moulins, M., **1976**. Ultrastructure of chordotonal organs. In: Mill, P.J. (Ed.), Structure and function of proprioceptors in insects. Chapman and Hall, London, pp. 387–426.
Murphey, R.K., Bacon, J.P., Johnson, S.E., **1985**. Ectopic neurons and the organization of insect sensory systems. *J. Comp. Physiol. A 156*, 381–389.

Murphey, R.K., Possidente, D., Pollack, G., Merritt, D.J., **1989**. Modality-specific axonal projections in the CNS of the flies *Phormia* and *Drosophila*. *J. Comp. Neurol. 290*, 185–200.

Nassif, C., Noveen, A., Hartenstein, V., **2003**. Early development of the *Drosophila* brain III. The pattern of neuropile founder tracts during the larval period. *J. Comp. Neurol. 455*, 417–434.

Newland, P.L., **1991**. Morphology and somatotopic organization of the central projections of afferents from tactile hairs on the hind leg of the locust. *J. Comp. Neurol. 312*, 493–508.

Okabe, M., Imai, T., Kurusu, M., Hiromi, Y., Okano, H., **2001**. Translational repression determines a neuronal potential in *Drosophila* asymmetric cell division. *Nature 411*, 94–98.

Orgogozo, V., Schweisguth, F., Bellaiche, Y., **2001**. Lineage, cell polarity and *inscuteable* function in the peripheral nervous system of the *Drosophila* embryo. *Development 128*, 631–643.

Pansky, B., Delmas, J.A., **1980**. Review of Neuroscience. Macmillan Publishing, New York.

Perez, S.E., Rebelo, S., Anderson, D.J., **1999**. Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development 126*, 1715–1728.

Pflüger, H.J., Bräunig, P., Hustert, R., **1988**. The organization of mechanosensory neuropiles in locust thoracic ganglia. *Philos. Trans. R. Soc. Lond. B 321*, 1–26.

Phillips, R.G., Warner, N.L., Whittle, J.R., **1999**. Wingless signaling leads to an asymmetric response to decapentaplegic-dependent signaling during sense organ patterning on the notum of *Drosophila melanogaster. Devel. Biol. 207*, 150–162.

Phillips, R.G., Whittle, J.R., **1993**. *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development 118*, 427–438.

Pi, H., Wu, H.J., Chien, C.T., **2001**. A dual function of phyllopod in *Drosophila* external sensory organ development: cell fate specification of sensory organ precursor and its progeny. *Development 128*, 2699–2710.

Posakony, J.W., **1994**. Nature versus nurture: asymmetric cell divisions in *Drosophila* bristle development. *Cell 76*, 415–418.

Rajagopalan, S., Nicolas, E., Vivancos, V., Berger, J., Dickson, B.J., **2000**. Crossing the midline: roles and regulation of Robo receptors. *Neuron 28*, 767–777.

Reddy, G.V., Gupta, B., Ray, K., Rodrigues, V., **1997**. Development of the *Drosophila* olfactory sense organs utilizes cell–cell interactions as well as lineage. *Development 124*, 703–712.

Reddy, G.V., Rodrigues, V., **1999**. Sibling cell fate in the *Drosophila* adult external sense organ lineage is specified by *prospero* function, which is regulated by Numb and Notch. *Development 126*, 2083–2092.

Rhyu, M.S., Jan, L.Y., Jan, Y.N., **1994**. Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell 76*, 477–491.

Roegiers, F., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., **2001**. Bazooka is required for localization of determinants and controlling proliferation in the sensory organ precursor cell lineage in *Drosophila*. *Proc. Natl Acad. Sci. USA 98*, 14469–14474.

Roensch, G., **1964**. Entwicklungsgeschichtliche untersuchungen zur Zelldifferenzierung am Flügel der Trichoptere *Limnophilus flavicornis* Fabr. *Z. Morphol. Ökol. Tiere 43*, 1–62.

Rosenbaum, J.L., Witman, G.B., **2002**. Intraflagellar transport. *Nat. Rev. Mol. Cell Biol. 3*, 813–825.

Schmidt, K., Berg, J., **1994**. Morphology and ontogeny of single-walled multiporous sensilla of hemimetabolous insects. *Tissue Cell 26*, 239–247.

Schmidt, K., Kuhbandner, B., **1983**. Ontogeny of the sensilla placodea on the antenna of *Aulacus striatus* Jurine (Hymenoptera: Aulacidae). *Int. J. Insect Morphol. Embryol. 12*, 43–57.

Schrader, S., Merritt, D.J., **2000**. Central projections of *Drosophila* sensory neurons in the transition from embryo to larva. *J. Comp. Neurol. 425*, 34–44.

Schweisguth, F., Posakony, J.W., **1992**. Suppressor of Hairless, the *Drosophila* homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. *Cell 69*, 1199–1212.

Schweisguth, F., Posakony, J.W., **1994**. Antagonistic activities of Suppressor of Hairless and Hairless control alternative cell fates in the *Drosophila* adult epidermis. *Development 120*, 1433–1441.

Seidl, S., Altner, H., **1990**. Ciliogenesis and differentiation of the sensilla of the ventral sensory field on the maxillary palp of the cockroach *Periplaneta americana* L. (Insecta, Blattodea). *Verh. Dtsch. Zool. Ges. 83*, 431.

Simpson, J.H., Kidd, T., Bland, K.S., Goodman, C.S., **2000**. Short-range and long-range guidance by Slit and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance. *Neuron 28*, 753–766.

Skaer, N., Pistillo, D., Gibert, J.M., Lio, P., Wulbeck, C., *et al.*, **2002**. Gene duplication at the *achaete-scute* complex and morphological complexity of the peripheral nervous system in Diptera. *Trends Genet. 18*, 399–405.

Skeath, J.B., Carroll, S.B., **1991**. Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Devel. 5*, 984–995.

Sugimura, K., Yamamoto, M., Niwa, R., Satoh, D., Goto, S., *et al.*, **2003**. Distinct developmental modes and lesion-induced reactions of dendrites of two classes of *Drosophila* sensory neurons. *J. Neurosci. 23*, 3752–3760.

Swoboda, P., Adler, H.T., Thomas, J.H., **2000**. The RFX-type transcription factor DAF–19 regulates sensory neuron cilium formation in *C. elegans*. *Mol. Cell. 5*, 411–421.

Thurm, U., Kueppers, J., **1980**. Epithelial physiology of insect sensilla. In: Locke, M., Smith, D.S. (Eds.), Insect Biology in the Future. Academic Press, New York, pp. 735–758.

Tilney, L.G., Connelly, P., Smith, S., Guild, G.M., **1996**. F-actin bundles in *Drosophila* bristles are assembled from modules composed of short filaments. *J. Cell Biol. 135*, 1291–1308.

Tilney, L.G., Connelly, P.S., Vranich, K.A., Shaw, M.K., Guild, G.M., **2000a**. Regulation of actin filament cross-linking and bundle shape in *Drosophila* bristles. *J. Cell Biol. 148*, 87–100.

Tilney, L.G., Connelly, P.S., Vranich, K.A., Shaw, M.K., Guild, G.M., **2000b**. Actin filaments and microtubules play different roles during bristle elongation in *Drosophila*. *J. Cell Sci. 113*, 1255–1265.

Tilney, L.G., Tilney, M.S., Guild, G.M., **1995**. F-actin bundles in *Drosophila* bristles. I. Two filament cross-links are involved in bundling. *J. Cell Biol. 130*, 629–638.

Tomoyasu, Y., Nakamura, M., Ueno, N., **1998**. Role of Dpp signalling in prepattern formation of the dorsocentral mechanosensory organ in *Drosophila melanogaster. Development 125*, 4215–4224.

Tree, D.R., Ma, D., Axelrod, J.D., **2002**. A three-tiered mechanism for regulation of planar cell polarity. *Semin. Cell Devel. Biol. 13*, 217–224.

Turner, C.M., Adler, P.N., **1998**. Distinct roles for the actin and microtubule cytoskeletons in the morphogenesis of epidermal hairs during wing development in *Drosophila*. *Mech. Devel. 70*, 181–192.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., **1989**. *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell 58*, 349–360.

Vervoort, M., Zink, D., Pujol, N., Victoir, K., Dumont, N., *et al.*, **1995**. Genetic determinants of sense organ identity in *Drosophila*: regulatory interactions between *cut* and *poxn*. *Development 121*, 3111–3120.

Wang, S., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., **1997**. Only a subset of the binary cell fate decisions mediated by Numb/Notch signaling in *Drosophila* sensory organ lineage requires Suppressor of Hairless. *Development 124*, 4435–4446.

Ward, S., **1975**. Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *J. Comp. Neurol. 160*, 313–337.

Wodarz, A., Ramrath, A., Kuchinke, U., Knust, E., **1999**. Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature 402*, 544–547.

Wong, K., Park, H.T., Wu, J.Y., Rao, Y., **2002**. Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr. Opin. Genet. Devel. 12*, 583–591.

Wright, K.A., **1992**. Peripheral sensilla of some lower invertebrates: the Platyhelminthes and Nematoda. *Microsc. Res. Tech. 22*, 285–297.

Yager, D.D., **1999**. Structure, development and evolution of insect auditory systems. *Microsc. Res. Tech. 47*, 380–400.

Younossi-Hartenstein, A., Hartenstein, V., **1997**. Pattern, time of birth and morphogenesis of sensillum precursors in *Drosophila*. *Microsc. Res. Tech. 39*, 479–491.

Zacharuk, R.Y., **1985**. Antennae and sensilla. In: Kerkut, A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 6. Pergamon, New York, pp. 1–69.

Zlatic, M., Landgraf, M., Bate, M., **2003**. Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the *Drosophila* nervous system. *Neuron 37*, 41–51.

1.12 The Development of the Olfactory System

G S X E Jefferis and L Luo, Stanford University,
Stanford, CA, USA

1.12.1. Introduction

1.12.1.1. Significance

Of all the sensory modalities, the olfactory system has historically been among the least studied in vertebrates. In contrast, there has been long-standing work in insects on its structure, development, and function. This stems in part from the recognition that olfaction is ethologically important for most insects, guiding them to food, mates, suitable oviposition sites, and so on. Besides this ethological interest, the development of the olfactory system should be of interest to developmental neurobiologists; olfaction is perhaps an unusual sense in which information enters the brain in a large number of parallel channels each distinct from one another. These channels, defined by the expression of different olfactory receptor molecules, serve as precise markers of neuronal identity. The olfactory system is therefore an excellent model for the formation of specific connections between identifiable components of neuronal networks. Furthermore, the vertebrate and insect olfactory systems show a common organizational logic, which suggests that insights gained with comparative facility in insects should be of interest to developmental neurobiologists at large. Finally, neurons and neuronal circuits in the olfactory system have proven excellent models of learning and memory, neuronal morphogenesis, developmental plasticity (neurite pruning during metamorphosis), and in some instances adult plasticity. All of these areas would benefit from an understanding of the underlying developmental mechanisms.

1.12.1.2. Scope of the Review

This review will emphasize the general principles of olfactory development in insects. *Drosophila melanogaster* will frequently be used as a model, reflecting in part the authors' own research interests and

the rapid progress occurring in our understanding of the developmental basis of this particular insect's sense of smell. This also reflects the practical limitations of space – a comprehensive summary of all that is known about the development of olfactory systems in all insects would occupy a volume rather than a chapter. Where there are clear gaps in our knowledge of *Drosophila*, detailed information will be drawn from other insects. In other cases, *Drosophila* findings are described in detail and comparisons and contrasts have been made with other insects, including moths and honeybee among holometabolous insects, and cockroaches and locusts among hemimetabolous insects.

In holometabolous insects, which undergo a complete metamorphosis and therefore form both a larval and an adult olfactory system, focus is made on the adult system. However, parts of the larval system persist through metamorphosis to the adult and even those structures that degenerate during metamorphosis may act as developmental cues during the formation of the adult structures. Although the development of the larval olfactory system is not described, its structure preparatory to a discussion of development of the adult olfactory system is reviewed.

Finally, this review will be written from a neurobiological perspective with an emphasis on the development of olfactory neuronal circuits over morphological development of olfactory structures.

1.12.1.3. Other Sources of Information

A valuable single source of information on the structure, function, and certain aspects of the development of the olfactory system is the volume *Insect Olfaction* (Hansson, 1999). In particular, there is an excellent review of the development of the antennal lobe, especially the glomeruli (Salecker and Malun, 1999). For the structure, function, and development of the mushroom body, a themed issue of the journal *Learning and Memory* (Mushroom Body Special Issue, 1998) is a valuable resource. However, there have been significant advances in certain areas since the publications of these sources. One point of great importance has been the identification of olfactory receptors in *Drosophila* through genome sequencing efforts. Since olfactory receptors define the channels of incoming olfactory information, they also serve as excellent markers for understanding the logic of wiring in this system. Keller and Vosshall (2003) is a good starting point for this work. Looking to functional analysis of the olfactory system, the application of optical imaging methods has allowed the parallel monitoring of a much greater fraction of the olfactory response (reviews: Galizia and Menzel, 2000; Korsching, 2002), although this new approach has yet to attain either the temporal resolution or sensitivity of traditional electrophysiology. Finally, significant advances have been made in the molecular and cellular basis of olfactory learning and memory, focusing on the mushroom body; Heisenberg (2003) provides a comprehensive and thoughtful review of this work.

1.12.2. Anatomy and Function

1.12.2.1. Olfaction: Logic and Function

1.12.2.1.1. Sensory transduction and the periphery Olfaction is a chemical sense. Olfactory receptor neurons (ORNs) respond to a particular subset of all possible odorant compounds. This receptive range can be very large in some cases and extremely specific in others. Detection depends on the stereochemical binding of an odorant molecule to an olfactory receptor (OR) protein present in the plasma membrane of ORN dendrites. Large families of putative olfactory receptors were initially identified in mammals (Buck and Axel, 1991). Among insects, ORs were first identified in *Drosophila* (Clyne *et al.*, 1999b; Gao and Chess, 1999; Vosshall *et al.*, 1999); the present size of this gene family, named *Drosophila* olfactory receptors (DORs), stands at 61, although expression cannot be detected for a large fraction of these. The published genome sequence of the mosquito *Anopheles gambiae* has permitted the identification of a family of ~79 receptors (Hill *et al.*, 2002), while a number of ORs from the moth, *Heliothis virescens,* have also been cloned (Krieger *et al.*, 2002). In addition to these families of presumed odorant receptors, a second family of presumed gustatory receptors (GRs) has been identified in *Drosophila* (Clyne *et al.*, 2000; Scott *et al.*, 2001b), and more recently *Anopheles* (Hill *et al.*, 2002). Scott *et al.* (2001b) were able to identify 56 putative GRs in the *Drosophila* genome; interestingly, three of seven GRs whose expression could be detected by *in situ* hybridization were expressed in the antenna, rather than gustatory structures. Indeed, the axons of ORNs expressing the *GR21a* gene seem to send axons to glomerulus V of the antennal lobe (Scott *et al.*, 2001b). All of these receptors (ORs and GRs) are seven transmembrane proteins, which are believed to couple to G protein-linked cascades. These G protein-linked cascades eventually cause membrane depolarization and the firing of action potentials, which travel down the ORN axon into the central nervous system (CNS).

Since olfactory receptors determine the receptive range of ORNs, what is the logic of their expression?

In mice, individual ORNs appear to express a single OR; indeed OR expression appears to be under strict control, so that any particular ORN does not even coexpress both alleles of the same OR gene (Chess *et al.*, 1994). In *Drosophila*, ORNs also seem to express only one specific DOR (Vosshall *et al.*, 2000), although control of gene expression seems to be less strict, since an example of coexpression of two OR genes at adjacent chromosomal locations has already been reported (Dobritsa *et al.*, 2003). As would be expected if a single receptor type confers the receptive range on any particular neuron, deletion of a single receptor gene can lead to the loss of olfactory responsivity in neurons that would normally express it. Conversely, ectopic expression of another OR in this mutant background could confer the receptive range of another ORN class on this cell (Dobritsa *et al.*, 2003).

In addition to those DORs that show selective expression, one divergent member of the DOR family, OR83b, is expressed in a large fraction, perhaps 80%, of all ORNs (Vosshall *et al.*, 2000). The functional significance of this observation is still unclear, although a coreceptor function remains a possibility. Interestingly, while it has not been possible to identify orthologs of the main group of DORs in other insects, OR83b is highly conserved and has been identified in eight insect species including examples of Diptera, Lepidoptera, Hymenoptera, and Coeloptera (Krieger *et al.*, 2003).

1.12.2.1.2. Central representations and processing In *Drosophila* and mice, olfactory receptor neuron axons bearing the same olfactory receptor terminate in discrete foci (glomeruli), in the first olfactory relay of the brain, the antennal lobe (AL) or olfactory bulb, respectively (*Drosophila*: Gao *et al.*, 2000; Vosshall *et al.*, 2000; mice: Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996; Wang *et al.*, 1998). The anatomical details of this arrangement are schematized in **Figure 1** and discussed in detail below (Section 1.12.2.2.2). The functional implication of this convergence, in conjunction with the broad tuning of ORs mentioned above, is that olfactory coding appears to be, at least in part, combinatorial; particular odorants activate discrete and reproducible sets of glomeruli (e.g., *Apis*: Galizia *et al.*, 1999b; *Drosophila*: Rodrigues, 1988; Fiala *et al.*, 2002; Ng *et al.*, 2002b; Wang *et al.*, 2003; *Heliothis*: Galizia *et al.*, 2000; vertebrates: Friedrich and Korsching, 1997; Rubin and Katz, 1999).

Olfactory processing within the antennal lobe remains a topic of great interest and some controversy. A substantial body of work in locusts has emphasized the significance of antennal lobe processing, especially via inhibitory interactions between glomeruli, which may generate temporally patterned responses. This temporal patterning includes fast oscillatory behavior, which has been widely observed in vertebrate and invertebrate olfactory systems, as well as slower temporal modulation of the firing rate in a spike train resulting from a particular odorant presentation (reviews: Laurent, 1997; Laurent *et al.*, 2001). In contrast, recent studies in *Drosophila*, using optical imaging of genetically encoded sensors of neuronal activity, have concluded that the information entering the lobe (via ORNs) and leaving the lobe (via projection neurons, PNs; **Figure 1**) shows relatively little sign of processing (Ng *et al.*, 2002b; Wang *et al.*, 2003). The distinction may reflect the much greater

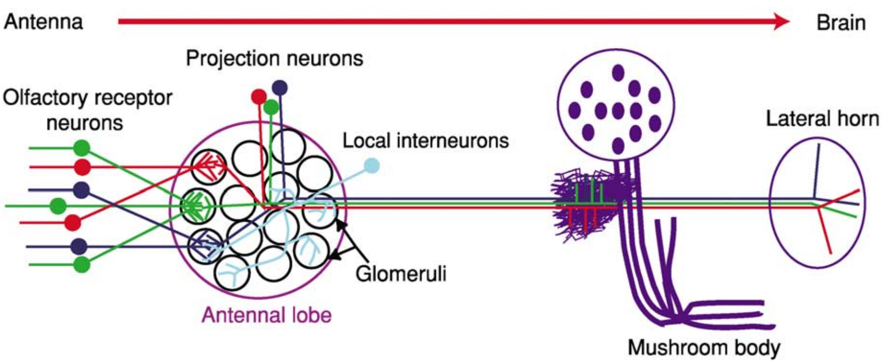


Figure 1 Schematic of olfactory system organization. Cartoon depicting the logical arrangement of components of the insect olfactory system. Olfactory receptor neurons (ORNs) project their axons to individual glomeruli according to the olfactory receptor (OR) they express. Projection Neurons (PNs) send dendrites to individual glomeruli and their axons relay this information to higher olfactory centers, the mushroom body (MB), and lateral horn (LH). Local interneurons (LNs) mediate lateral interactions between different glomeruli. (Reprinted with permission from Jefferis, G.S.X.E., Marin, E.C., Watts, R.J., Luo, L., **2002**. Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr. Opin. Neurobiol. 12*, 80–86; © Elsevier.)

sensitivity of electrophysiological methods, or could perhaps be a species specific difference. In either case, it is certain that the precise formation of neuronal connections within the antennal lobe during development will have a strong influence on the information carried by output neurons and the potential for interactions among different channels of olfactory information.

After the initial relay, olfactory information passes to higher olfactory centers, located in the protocerebrum (see below), which are presumably involved in odorant recognition and discrimination, and the synthesis of olfactory memories. One structure in particular has received a great deal of attention: the mushroom body (MB; **Figure 1**). Ablation and inactivation experiments have suggested that the MB is required for olfactory learning and memory, but not for odorant detection *per se* (de Belle and Heisenberg, 1994; Connolly *et al.*, 1996). At the molecular level, *Drosophila* MBs show enriched expression of proteins involved in cyclic AMP signaling (Nighorn *et al.*, 1991; Crittenden *et al.*, 1998), some of which are encoded by genes inactivated in a series of learning mutants (review: Roman and Davis, 2001). Indeed, this significance of cAMP in the formation of memory has been echoed in many systems from *Aplysia* to mouse. Tissue specific rescues of learning mutants have shown that MB expression of these genes can be sufficient for learning (Zars *et al.*, 2000). Finally, experiments using reversible inactivation of synaptic transmission from mushroom body neurons to target neurons, have shown that this synaptic transmission is required for retrieval of memory, but not for learning (Dubnau *et al.*, 2001; McGuire *et al.*, 2001).

The representation of olfactory information within the MB has also been investigated electrophysiologically. One recent study in the locust, *Schistocerca americana*, has argued forcefully that olfactory information is sparsened in this structure; an olfactory stimulus, which might cause a large fraction of first and second order neurons to fire a large number of action potentials, caused a small number of neurons to fire a small number of spikes in the MB (Perez-Orive *et al.*, 2002). Finally, it should be emphasized that there is a large body of literature on nonolfactory functions of MBs, which in many insects are likely to act as a multimodal integrator in addition to any specific role in olfactory learning (Mushroom Body Special Issue, 1998).

In comparison, the function, physiology, and cellular composition (see below) of the other major target of olfactory information, the lateral horn (LH; **Figure 1**), remains more mysterious (e.g., de Belle and Kanzaki, 1999). At the functional level, it has been suggested that it may be required for experience independent olfactory behavior (Heimbeck *et al.*, 2001), although the evidence is indirect. Electrophysiology of the LH is less developed than any other part of the olfactory circuit discussed so far, although Perez-Orive *et al.* (2002) have identified an inhibitory neuronal feedback from the LH to the MB, which may be responsible in part for the sparsening of the olfactory representations described above.

Understanding how the multiglomerular representation typical of an individual odorant is transformed and decoded in higher olfactory centers remains one of the key questions in the physiology of the olfactory system.

1.12.2.2. Common Anatomy

1.12.2.2.1. Periphery The major olfactory appendages in most insects are a pair of antennae mounted on the head. In *Drosophila,* there are in fact two olfactory organs (**Figure 2a**), the third antennal segment and the maxillary palp, which is mounted on the proboscis; they contain about 1200 and 120 olfactory receptor neurons per hemisphere, respectively (Singh and Nayak, 1985; Stocker, 1994, 2001). Within the ORN, the site of signal transduction is the olfactory cilium, which is a specialized dendrite containing the olfactory receptor proteins (e.g., Elmore and Smith, 2001) and their transduction apparatus along with a prominent microtubule based cytoskeleton. ORNs are mounted in olfactory sensilla, which are the primary sensory structures (**Figure 3**) (see also **Chapter 1.11**).

Besides their neuronal component, which is typically in the range of 1–5 but can range up to more than 100, sensilla contain three support cells (**Figure 3**; review: Keil, 1999; **Chapter 1.11**): (1) the innermost thecogen or sheath cell, which wraps the neuron(s) in a glial fashion; (2) the hair cuticle-secreting trichogen or (hair) shaft cell, which contains pores through which odorants can diffuse; and (3) the tormogen or socket cell, which generates the base of the hair.

Sensilla may be classified based on their morphology into a number of different types, including basiconic (short and rounded), trichoid (long, thin), and coeloconic (short, in a pit, with a double cuticular wall), which are widely recognizable in different insect species . . . (see also **Chapter 1.11**) on sensillar development). The sensillar composition of the *Drosophila* antennae has been reviewed very comprehensively by Stocker (2001) (see also **Chapter 1.11**). Numbers vary somewhat according to strain and sex, but Stocker and Gendre (1988) report approximately 240 basiconic sensilla, 110 trichoid, and 70

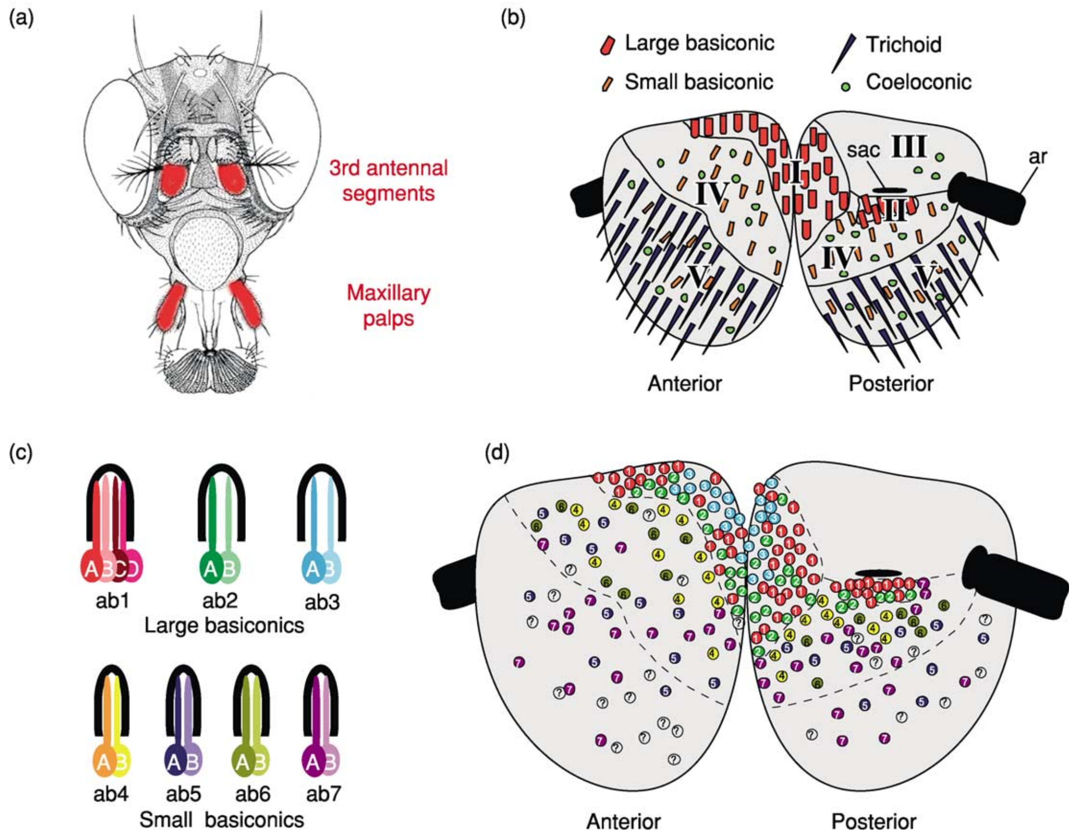


Figure 2 Location of sensory appendages and distribution of sensillar classes. (a) Frontal schematic of *Drosophila* head, showing the two pairs of sensory appendages, the third antennal segment, and the maxillary palp. (Reprinted with permission from Jefferis, G.S.X.E., Marin, E.C., Watts, R.J., Luo, L. **2002**. Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr. Opin. Neurobiol. 12*, 80–86; © Elsevier.) (b) Zonal distribution of the major classes of olfactory sensillum (indicated by roman numerals I–V after de Bruyne *et al.*, 2001). Also indicated are the sacculus (sac) and the arista (ar). (c) The basiconic sensilla can be subdivided morphologically into large and small categories. These categories have been further subdivided into functional subtypes indicated by the different color hues (de Bruyne *et al.*, 2001). Within each sensillar subtype there is a unique combination of two or four olfactory receptor neurons, each with specific olfactory response profiles; recent work has correlated most response profiles with the expression of a particular olfactory receptor (Hallem *et al.*, 2004). (d) Distribution on the antenna of the seven basiconic subtypes defined in c. There is clear spatial clustering of different sensillar subtypes. ((b–d): Reprinted with permission from de Bruyne, M., Foster, K., Carlson, J.R., **2001**. Odor coding in the *Drosophila* antenna. *Neuron 30*, 537–552; © Elsevier.)

coeloconic on female antennae; males have 40 additional trichoids and 40 fewer basiconics. Counts of the maxillary palp suggested about 60 basiconic sensilla (Singh and Nayak, 1985).

Sensillar classes are differentially distributed on the antennal surface, with some populations in relatively homogeneous zones, while other areas contain a mix of different classes (**Figure 2b**; review: Stocker, 2001). Rather remarkably, sensillar classes appear to contain defined populations of OR. For example, in *Drosophila*, de Bruyne *et al.* (2001) have mapped the olfactory responses of the antennal basiconic sensilla. They identified seven classes of basiconic sensillum, each containing a stereotyped group of two or four ORNs with particular receptive ranges (**Figure 2c**). Thus, the choice of olfactory receptor must be coupled to the development of the sensillar morphology. Furthermore, these seven different basiconic sensillar classes were clustered in different domains on the antenna (**Figure 2d**), suggesting that there may be a coarse map of ORNs expressing different ORs on the antennal surface. Consistent with this, the ORs themselves are also spatially distributed in the antenna; however these distributions are overlapping, so that there is not a precise map of receptor expression on the sensory surface (Vosshall *et al.*, 1999, 2000). Whether a similar logic exists in other insects will become clearer as more olfactory receptor repertoires are characterized.

1.12.2.2.2. Antennal lobe ORN axons terminate in the CNS in the antennal lobe (in insects) or olfactory bulb (in mammals). Although these structures

may not share an evolutionary heritage (Strausfeld and Hildebrand, 1999), the organizational features are very similar. Morphologically, the key features are the glomeruli (**Figure 1**), spherical neuropil regions usually surrounded by a glial sheath. In many insects, these glomeruli appear to be stereotyped in size, shape, and relative position, so that atlases can be generated (e.g., *Drosophila*: Laissue *et al.*, 1999; *Apis*: Galizia *et al.*, 1999a; moths: Berg *et al.*, 2002). Glomeruli are the site of synaptic contact between three key neuronal types: ORN axons, PN dendrites (**Figure 1**; see also **Figure 4b**), and the processes of local interneurons (LNs; **Figure 1**) (review: Anton and Homberg, 1999). PNs and LNs are the major analogs of vertebrate mitral cells and granule cells, respectively. In *Drosophila*, ORNs and PNs usually synapse in single glomeruli, while LNs ramify through large areas of the lobe (Stocker *et al.*, 1990) (**Figure 1**); this organization is typical of most insects, but see Section 1.12.2.3 for variations on this theme. ORNs and most PNs are believed to use acetylcholine as a transmitter (review: Homberg and Muller, 1999); a subset of PNs whose axons project via the middle antenno-cerebral tract (mACT) (see Section 1.12.2.2.3 below) have been demonstrated to be GABAergic in a number of species including *Manduca*, *Apis*, and *Periplaneta* (review: Homberg and Muller, 1999). While LNs are largely GABAergic in most species (e.g., *Manduca*: Hoskins *et al.*, 1986; honeybee: Schafer and Bicker, 1986), there has

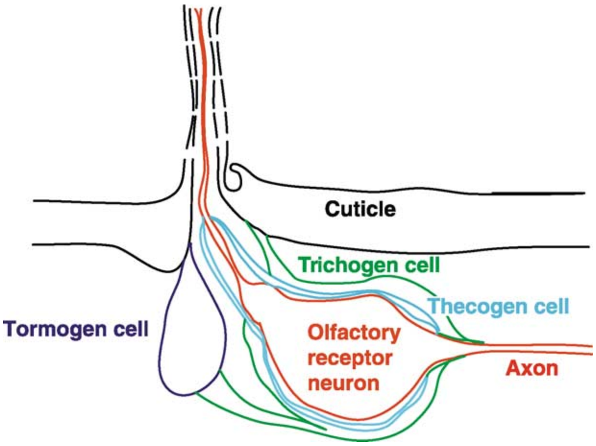


Figure 3 Schematic of the fine structure of an olfactory sensillum. A single olfactory receptor neuron is shown, although sensilla typically contain multiple neurons. The ORN is wrapped by the thecogen or sheath cell in a glial fashion. The outer pair of auxiliary cells, the trichogen (shaft) and tormogen (socket), secretes the main hair shaft and base, respectively. The ORN dendrite innervates the shaft space, where it is bathed in a sensillar lymph. Small pores in the shaft cuticle allow odorants to enter and contact the ORN.

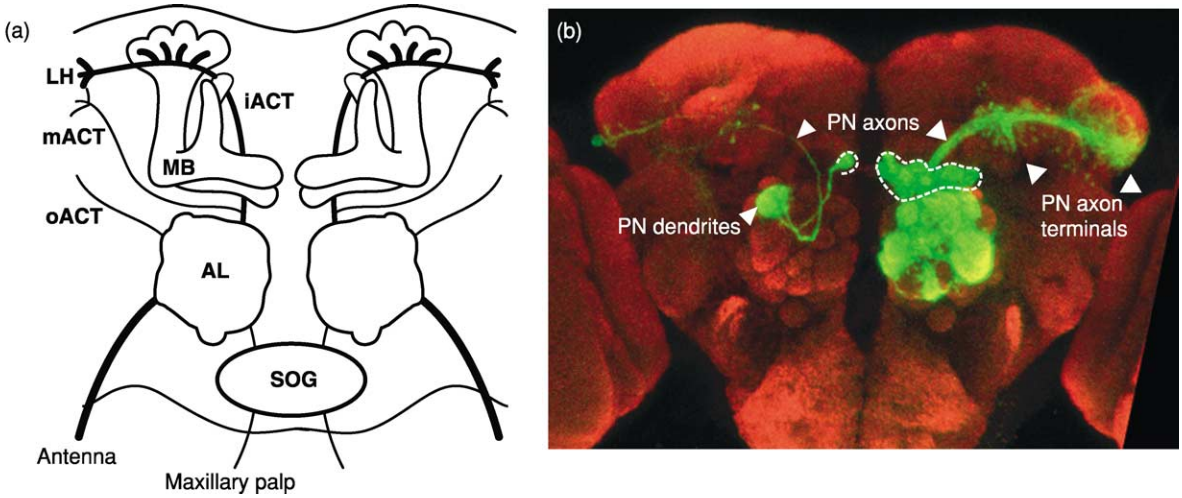


Figure 4 Schematic of the olfactory anatomy of the insect brain. (a) Frontal view of the *Drosophila* brain (the same orientation as the head in **Figure 2a**). Olfactory information arrives from the antenna, with a minor afferent from the maxillary palp taking a different route through the subesophageal ganglion (SOG), and terminates in the antennal lobe (AL) glomeruli. Projection neuron axons relay that information to the calyx of the mushroom body (MB) and/or the lateral protocerebrum via three major antenno-cerebral tracts (ACTs). Uniglomerular cholinergic PNs of the inner ACT are the most numerous and project both to the MB calyx and the lateral horn (LH) of the protocerebrum. PNs employing the middle ACT are usually GABAergic and project generally to the lateral protocerebrum. The outer ACT contains PNs that project to the inferior lateral protocerebrum. (b) Example of a labeled single cell and a group of projection neurons generated using the MARCM method (review: Lee and Luo, 2001). The single cell labeled using a membrane targeted green fluorescent protein has a cell body (dotted line) from which a neurite extends, connecting to a dendritic arbor that innervates the glomerulus DL1 and an axon that projects through the iACT to the mushroom body calyx and lateral horn. An antibody recognizing synaptic structures (red), reveals the general organization of the brain, including the glomeruli of the antennal lobe. (Reproduced with permission from Jefferis, G.S.X.E., Marin, E.C., Stocker, R.F., Luo, L., **2001**. Target neuron prespecification in the olfactory map of *Drosophila*. *Nature 414*, 204–208; © Nature Publishing Group.)

been no formal demonstration of this in *Drosophila*, although GABA receptors are present in the glomeruli (Aronstein and ffrench-Constant, 1995).

The synaptic connectivity within glomeruli has perhaps been most studied in the cockroach, *Periplaneta americana* (review: Distler and Boeckh, 1998). There are extensive synaptic connections between the three different neuronal cell types, such that PNs receive input from ORNs and LNs, while LNs are postsynaptic to ORNs, PNs, and LNs. Finally, it appears that LNs can make connections back to ORNs (review: Distler and Boeckh, 1998). The existence of these direct, indirect, and feedback pathways is likely at the origins of the oscillatory phenomena investigated most especially in the locust, *S. americana* (review: Laurent, 2002).

Besides the neurons in the AL, there are of course glia, which wrap the whole AL and send processes into it. It should be noted that the extent of glial processes within the lobe varies widely among different insects, with substantial glial presence in moths and bees (Boeckh and Tolbert, 1993; Hahnlein and Bicker, 1996), but little glia in flies and cockroaches (Stocker *et al.*, 1990; Boeckh and Tolbert, 1993). In the mature organism, glial cells are likely to have a metabolic role, for example, in neurotransmitter cycling (Hahnlein and Bicker, 1996); however, they have also been shown to be of great significance for antennal lobe development in some species (see Section 1.12.4 below; review: Oland and Tolbert, 2003).

1.12.2.2.3. Antenno-cerebral tracts Output from the antennal lobe is directed to the protocerebrum; the two major targets are the calyx (or dendritic field) of the mushroom body and the lateral horn, although there are additional terminations in the other regions of the protocerebrum. PN axons course through a number of antenno-cerebral tracts (ACT) to reach these higher olfactory centers (Kenyon, 1896) (**Figure 4a**).

The major tracts and their PN constituents can be clearly identified in a number of insect species (review: Anton and Homberg, 1999) and here the nomenclature used in *Drosophila* (Stocker *et al.*, 1990; Stocker, 1994) and *Manduca sexta* (Homberg *et al.*, 1988) is followed. The largest tract is the inner antenno-cerebral tract (iACT; **Figure 4**), through which a population of largely uniglomerular PNs send axons first to the MB, where collaterals form synapses in the calyx, and then to the LH. Two smaller tracts, the mACT (middle ACT) and oACT (outer ACT), are also widely identifiable (**Figure 4**). PNs employing these tracts seem to be more heterogeneous. The mACT PNs are frequently multiglomerular and project to the LH or through large areas of the superior or lateral protocerebrum. The oACT neurons are even more diverse, typically have multiglomerular innervation in the antennal lobe, and may terminate in the lateral protocerebrum including the LH, the superior protocerebrum, and the MB calyx, or even cross over to the contralateral hemisphere (Homberg *et al.*, 1988). Observations in *Drosophila* are consistent with these general organizational principles (Stocker *et al.*, 1990; Marin *et al.*, 2002; Wong *et al.*, 2002).

1.12.2.2.4. Mushroom body (MB) The neuronal architecture of the protocerebrum in insects is, in general, rather less understood than the deutocerebrum, the site of the antennal lobe. One clear exception is the mushroom body, an intensively studied structure first identified by Dujardin (1850). The MBs are bilaterally symmetric, densely packed neuropils. Each consists of a large number of Kenyon cells (KCs) or intrinsic MB neurons, which form a dendritic field, the calyx (often cup-shaped), and then course down the peduncle before bifurcating into two perpendicular lobes, the medial α and dorsal β lobes (**Figure 5**). There are large input synapses in the calyx; in the lobes, there are both en-passant axo-axonal synapses as well as output structures.

In *Drosophila*, there have recently been significant advances in our understanding of the fine structure of the MB. The bilobed MB structure turned out to encompass five discrete lobes, two dorsal (α, α') and three medial (β, β', γ) (Crittenden *et al.*, 1998). These different lobes could be identified by a number of different antigenic markers including the cell adhesion molecule Fasciclin II (FasII) (Crittenden *et al.*, 1998) (**Figure 5**). Cellularly, three distinct kinds of cells underlie this diversity of lobes: two forms with branched axons, $\alpha\beta$ and $\alpha'\beta'$ neurons, and unbranched γ neurons (Lee *et al.*, 1999). Further subdivisions have recently been noted by Strausfeld *et al.* (2003). Indeed an earlier study had shown that MB neurons are much more molecularly heterogeneous than previously anticipated, since enhancer trap techniques can reliably label distinct populations of MB neurons from individual to individual (Yang *et al.*, 1995).

Recently, Yasuyama and colleagues (Yasuyama *et al.*, 2002) using the electron microscope looked at the ultrastructure of the MB calyx in some detail. They concluded that synaptic organization is glomerular: cholinergic PNs terminate in a large bouton (2–7 μm diameter), which makes presynaptic contacts with a large number of postsynaptic MB neuron dendrites. Presynaptic GABAergic neurons

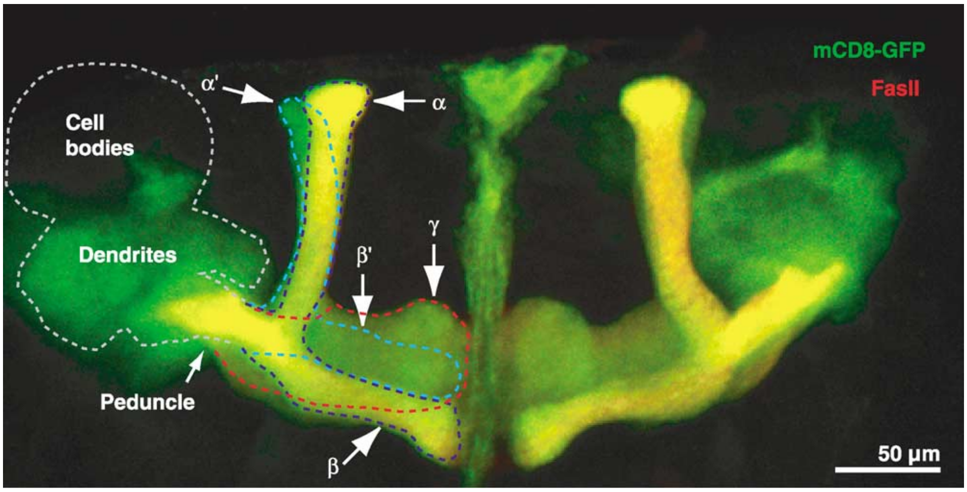


Figure 5 Structure of the *Drosophila* MBs revealed by membrane targeted GFP (mCD8-GFP, in green) driven by an enhancer trap line expressed in most MB neurons (*GAL4-OK107*). FasII (red) is strongly expressed in the α/β lobes, weakly in the γ lobe and not detectable in the α′/β′ lobes. The midline is marked with a dashed white line; dorsal is uppermost. (Reprinted with permission from Jefferis, G.S.X.E., Marin, E.C., Watts, R.J., Luo, L., **2002**. Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr. Opin. Neurobiol. 12*, 80–86; © Elsevier.)

are also associated with these glomeruli, and make contact both with MB neurons and occasionally with the cholinergic bouton itself. Extrinsic neurons, which will now be described, are candidates for this GABAergic input.

The connectivity of extrinsic neurons of the MBs is, in general, less understood (but see Ito *et al.*, 1998). This probably reflects their much more heterogeneous character and more diffuse projection patterns. At least four general classes of extrinsic neurons can be considered: input neurons, output neurons, feedback neurons, and neuromodulatory neurons. PNs are the most obvious class of input neuron, but there are others including feedback neurons originating in the lateral horn (see Section 1.12.2.2.5 below).

Extrinsic output neurons have been described with projections to the lateral protocerebrum; for example, in the honeybee, Rybak and Menzel (1998) have characterized the Pe1 neuron, which shows multimodal responses (see Section 1.12.2.3.3 below for a description of multimodal input to MBs) including transient olfactory responses dependent on learning state. GABAergic (and presumably inhibitory) feedback neurons have been described that connect the medial and dorsal branches of the MB or that connect the lobes with the calyx (Rybak and Menzel, 1993). A second class of neurons that may have feedback properties are bilateral neurons, which connect mushroom body lobes in each hemisphere.

Finally, there are neuromodulatory neurons, containing transmitters like serotonin and octopamine. The two most spectacular examples are the ventral unpaired medial (VUMx1) neuron identified in honeybees, and the dorsal paired medial (DPM) neurons present in *Drosophila*. The VUMx1 neuron is an octopaminergic neuron with a cell body at the midline of the subesophageal ganglion (SOG), which includes the termination zone of gustatory neurons. VUMx1 has bilateral dendritic arborizations within the SOG and dense axonal arborizations in the ALs, MB calyces, and LH (Hammer and Menzel, 1995). Sucrose stimulation of the proboscis causes the neuron to fire. Remarkably, depolarization of the neuron can substitute for sucrose in a classical conditioning paradigm, in which an odorant (conditioned stimulus; CS) paired with sucrose (unconditioned stimulus; US) could subsequently cause US-independent extension of the proboscis (Hammer, 1993). Firing of VUMx1 thus appears to act as a reinforcer in this associative learning.

The DPM neurons are a pair of large unilateral neurons with extensive arborizations in the mushroom body lobes. They have been identified as the highly enriched site of expression of the neuropeptide encoded by the *amnesiac* (*amn*) gene in the brain (Waddell *et al.*, 2000). In contrast to VUMx1, DPM neurons appear to play a role not in the associative learning process, but in the consolidation of memory. The *amnesiac* mutant flies, or flies in which the DPM neurons are transiently inactivated, can learn an olfactory association normally, but then have no memory whatsoever after 1 h (Waddell *et al.*, 2000). It should be noted that the anatomy of both VUMx1 and DPM neurons is consistent with their physiology. In the case of

VUMx1, relaying a gustatory signal from the SOG to diffuse contacts in higher olfactory centers may allow connections with the many potential olfactory neurons that could become active in response to the panoply of different potential CS odorants. In the case of DPMs, the memory consolidation phase is believed to occur in the intrinsic neurons of the MB.

1.12.2.2.5. Lateral protocerebrum and lateral horn The structure of the lateral protocerebrum (LPR), in general, and, in particular, that of the lateral LH, an ovoid structure in the most lateral portion of the protocerebrum, remains relatively poorly understood. Among input neurons, certain classes of PNs, already mentioned in Section 1.12.2.2.3 above, have been well described (see also **Figure 4**). Furthermore, two recent studies in *Drosophila* have provided significant new information (Marin *et al.*, 2002; Wong *et al.*, 2002). These new studies, which employed genetic single cell labeling techniques, described in great detail the axonal projection patterns of a significant fraction (perhaps one-third) of the repertoire of *Drosophila* PNs. They both conclude that PNs, whose dendrites innervate a particular glomerulus, show highly stereotyped axonal projections in the lateral horn. These axonal branching patterns are more widespread than the corresponding dendritic arborizations in individual glomeruli of the antennal lobe. These two observations indicate both a convergence and a divergence of olfactory information.

Other input neurons that innervate the lateral horn include some of the extrinsic neurons of the mushroom body (e.g., the Pe1 neuron mentioned in Section 1.12.2.2.4 above) and neuromodulatory neurons, for example, the octopaminergic VUMx1 neuron also mentioned above.

The output neurons of the lateral horn are at present rather poorly cataloged. One exception is a group of GABAergic neurons, which have been identified in the locust (Laurent and Naraghi, 1994). These form a strong connection back to the mushroom body calyx (Perez-Orive *et al.*, 2002).

A first attempt to describe the ultrastructure of the lateral horn has recently been made in *Drosophila* by Yasuyama *et al.* (2003), who found that cholinergic synapses of afferent PNs are usually presynaptic to a large number of noncholinergic profiles. Sometimes, PN terminals appeared as postsynaptic spines to large noncholinergic interneurons, which may perhaps be the GABAergic neurons described above.

1.12.2.3. Anatomical Variations

1.12.2.3.1. Antennae The antennae of insects show a huge variation in size, morphology, and other functional specializations (Schneider, 1964; Keil, 1999). Antennae may have a simple flagellar form very typical in hemimetabolans, which must pass through successive molts to reach the adult form. In holometabolans, which have the opportunity to resculpt their antennae during metamorphosis, much more elaborate structures can be observed – perhaps most dramatically, the so-called pectinate (or feather shaped) antennae of certain moths, which dramatically increase the receptive surface area. Other specializations may include antennal mobility. Ants, for example, have highly mobile antennae, which they use when following pheromone trails or other chemical cues and when identifying nestmates or prey (Holldobler and Wilson, 1990). Finally, marked intraspecies differences can also be observed, including the well-known specializations of the antennae of some male moths (especially Saturniids) for detecting female pheromones.

Antennal specializations are not limited to males: for example, female *A. stephensi* have up to three times more ORNs than their male counterparts, which is believed to reflect their requirement to seek out hosts for blood meals (McIver, 1982).

1.12.2.3.2. Antennal lobe (AL) There are large variations in the number and morphology of glomeruli in the AL. For example, *Aedes aegypti* has only 32 glomeruli (in either sex), while in locusts there may be as many as 1000 small glomeruli. This large number of glomeruli in locusts is, in fact, unusual among all insect species examined, and there is a matching difference in the morphology of ORN and PN projection patterns. Locust ORNs project their axons to multiple glomeruli (Hansson *et al.*, 1996), typically 1–3 according to Laurent (1996), while PNs project dendrites to around 10–20 glomeruli, which may be some distance apart in the lobe (Laurent and Naraghi, 1994; Anton and Hansson, 1996). Whether this pattern reflects a divergence of olfactory information from a single OR type, followed by a reconvergence of that same information in a matched population of PNs, is not clear.

Within species, there are clear examples of sexual dimorphism in the lobe. In many species, the male has a group of dramatically enlarged glomeruli near the entry point of the antennal nerve into the AL, the macroglomerular complex (MGC) in moths, which are the target of sex pheromone responsive ORNs (Hildebrand, 1995). Although it was originally assumed that these glomeruli were novel in the male and did not have homologs in the female, a recent high-resolution study of the AL in *M. sexta* (Rospars and Hildebrand, 2000) has found a similar number

of female-specific glomeruli and attempted to define homologies with the components of the MGC. Conversely, an imaginative comparative study in *D. melanogaster*, a species in which no sexual dimorphism had previously been reported, has identified two of the "ordinary" glomeruli as sexually dimorphic (Kondoh *et al.*, 2003).

A final anatomical variation of note is the existence in dipterans of a commissure joining the two antennal lobes; this tract is populated by the axons of ORNs, which, in these species, project both to an ipsilateral glomerular target and to its contralateral homolog (Stocker *et al.*, 1990). In *Drosophila*, there is also a population of local interneurons with projections across the midline to the contralateral AL, which may be cholinergic (unpublished results cited in Stocker and Rodrigues, 1999).

1.12.2.3.3. Tracts and higher centers Besides the three ACTs joining the AL to the protocerebrum (Section 1.12.2.2.3), a number of additional, smaller tracts have been identified in some insects, e.g., dACT and dmACT in *M. sexta*, which innervate the lateral horn and mushroom body calyx/lateral horn, respectively. Whether these tracts are also present in other insects remains unclear but, in general, the conservation of anatomical features at this level is quite impressive.

The MB has received a great deal of attention in many insect species, so there is a substantial amount of comparative data on its structure (review: Strausfeld *et al.*, 1998). Potential variables include the number of intrinsic MB neurons, the diversity in the incoming neuronal information, the size and structure of the calyx, and the existence of much enlarged or additional lobes. MB neuronal number varies widely among insect species – for example, *Drosophila* has an estimated 2500 neurons per side, *Musca* approximately 21 000, while honeybee drones have about 170 000 and male cockroaches (*P. americana*) upwards of 200 000 (review: Schurmann, 1987). Furthermore, this neuronal number does not vary uniformly with brain size; for example, MBs are relatively enlarged in highly social insects, such as bees, prompting Dujardin, their original discoverer, to propose them as the seat of insect intelligence (Dujardin, 1850).

Regarding morphology, Strausfeld *et al.* (1998) argue that the ancestral form of the MB may have been without a calyx; the extensive olfactory input would therefore be a later addition. Dipterans such as *Musca* and *Drosophila* have roughly ovoid calyces with few externally obvious morphological features. In contrast, other species have highly developed calyces, such as the honeybee, whose calyces are a pair of cup-shaped substructures with distinct spatial patterns of innervation for olfactory, visual, and gustatory input (Menzel *et al.*, 1994); similar findings exist for the cockroach (Nishikawa *et al.*, 1998). Anatomical studies of olfactory protocerebral regions besides the MBs are really too incomplete for comparative work to distinguish species' differences from simple lack of information.

1.12.2.3.4. Larval olfactory system Holometabolous insects with a larval form typically have a functional olfactory system by the time they hatch. The olfactory world faced by this system may be quite different from that encountered later by the adult and, consequently, the structure of the two olfactory systems might also be expected to differ. In general, larval olfactory systems have been characterized as numerically and organizationally simpler than their adult counterparts. For example, in both *Drosophila* and *Apis mellifera*, the larval antennal lobes (ALs) appear aglomerular (Masson and Arnold, 1984; Gascuel and Masson, 1991). However, in other species including the monarch butterfly, *Danaus plexippus*, and *M. sexta*, a nodular organization of the larval antennal lobe has been observed (Nordlander and Edwards, 1970; Kent and Hildebrand, 1987).

Recent studies in *Drosophila* suggest that the organizational complexity of this larval system may have been underestimated. A high resolution study by Python and Stocker (2002) finds that, in spite of the massively reduced number of incoming ORNs (approximately 21 in the larvae versus 1300 in the adult), the antennal lobe appears to have approximately 30 distinct subdivisions, reminiscent of adult glomeruli.

The cloning of a second chemosensory receptor gene family in *Drosophila*, termed gustatory receptors (GRs; although not all of these receptors appear to serve a gustatory function), had previously allowed tracing of some larval afferents to the antennal lobe (Scott *et al.*, 2001b). These authors concluded that individual classes of larval ORN terminated in discrete subregions of the antennal lobe. Python and Stocker (2002) also described a population of larval PNs labeled by the enhancer trap line GH146 (Stocker *et al.*, 1997). Single cell labeling studies have established that individual PNs have dendritic arborizations corresponding to the presumed larval glomeruli, and project to the calyx of the larval MB and LH. One novel finding is that in the larva, PNs project focally to discrete "glomerular" structures in the MB calyx, rather

than terminating at multiple sites (E.C. Marin and L. Luo, unpublished data; A. Ramaekers, N. Gendre, and R.F. Stocker, unpublished data).

Local interneurons are also present in the larval lobe, as demonstrated morphologically by examining enhancer trap lines, and immunohistochemically, by the use of anti-GABA antibodies (Python and Stocker, 2002). A single serotonin-containing neuron and octopamine-containing neurons were also detected. Overall, the structure of this particular larval olfactory system appears to be hybrid in character. Olfactory information is transduced by a dramatically reduced number of sensory neurons in the periphery, but nevertheless projects with very similar logic to the antennal lobe, before being relayed by PNs (that will persist and serve an identical function in the adult) to the MB and what appears to be a larval lateral horn.

1.12.3. Development of the Peripheral Olfactory System

1.12.3.1. Generation of Sensilla Including Olfactory Receptor Neuron Neurogenesis

In all holometabolous insects, antennae derive from imaginal discs, which exist in the larval stage as flattened pouches of undifferentiated epithelial cells set aside for later use. Here the focus is on the generation of the individual sensory units, the sensilla, from the initially unpatterned disc epithelium; for further information (see also **Chapter 1.11** on sensillar development). The formation of external sensory organs (of which olfactory sensilla are an example), and most particularly mechanosensory bristles, has been well studied in a large variety of insects. The general mode of development is the selection from cells in the epithelial field of sensory organ precursors (SOPs). Each SOP is usually selected out from one of the many proneural cell clusters in the epithelium – typically one cell within each cluster adopts the SOP fate and delaminates from the epithelium. The SOP then undergoes a small number of stereotyped asymmetric divisions to generate the different cell types within each sensillum (Jan and Jan, 1993).

Comparative studies have suggested that these lineages are rather conserved between similar sensory organs in different species (Bate, 1978), and that basic patterns of division may be reiterated with small variations in the generation of different kinds of sensory organs (Bellaiche and Schweisguth, 2001). This lineage-based generation of sensilla is in marked contrast to the generation of the photoreceptors of the compound eye, which proceeds by the sequential recruitment of neighboring cells via cell–cell interactions initiated by a founder cell (Dickson and Hafen, 1993).

There are several general themes in the developmental biology of external sensory organs. (1) How are the number, spacing, and position of SOPs controlled? Key mechanisms are the existence of prepatterns of proneural clusters (defined by the expression of bHLH transcription factors) in some epidermal fields (reviews: Simpson, 1997; Simpson *et al.*, 1999), and the employment of lateral inhibition via the Notch pathway. (2) What is the relative importance of lineage versus recruitment? (3) How are different subtypes of sensillum generated, particularly different neuronal types (for a general review of proneural genes and subtype specification, see Bertrand *et al.*, 2002)? (4) How does the SOP divide asymmetrically to generate different cell fates (review: Jan and Jan, 2001)? Some of these themes are addressed specifically in the generation of olfactory sensilla in *Drosophila*.

In *Drosophila,* the antennal imaginal discs are quite separate from the larval sensory structures, and already present as a group of 7–9 progenitor cells set aside in the embryo 2 h after egg laying (Postlethwait and Schneiderman, 1971). No further division takes place until 24 h after larval hatching (Madhavan and Schneiderman, 1977), when exponential growth proceeds through the remaining 4 days of larval life, so that there are about 2500 cells per disc at pupation (Postlethwait and Schneiderman, 1971). Around this time, differentiation of the epidermal field of the antennal disc begins and there is a halt in proliferation.

The concentric organization and development of the antennal imaginal disc has been described by Haynie and Bryant (1986). Precursor cells that will generate the third antennal segment (3AS) occupy a central location. These cells are surrounded by precursors of the second antennal segment (containing, in *Drosophila*, the auditory Johnston's organ) and, finally, at the periphery, by the first antennal segment precursors. During pupal metamorphosis, which lasts approximately 100 h in *Drosophila*, the antennal disc, like other imaginal discs, will telescope out so that the central regions become the most distal.

SOP specification and delamination occurs in three major waves (**Figure 6**) (Ray and Rodrigues, 1995; zur Lage *et al.*, 2003), which can be visualized by expression of the *senseless* (*sens*) gene (Nolo *et al.*, 2000). The first wave appears a few hours before puparium formation and consists of a well-spaced semicircle of SOPs at the periphery of the 3AS zone of the antennal disc. The second wave appears by 4 h after puparium formation (APF). It is more numerous, more centrally located, and consists of

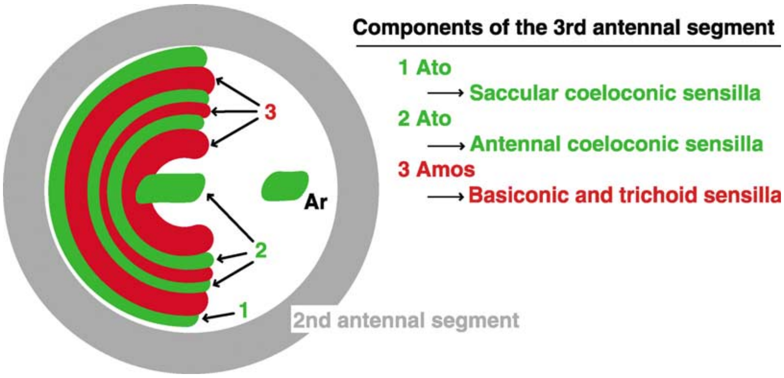


Figure 6 Three waves of sensory organ precursors (SOPs) generate the *Drosophila* olfactory sensilla. Peripherally located wave 1 SOPs express Ato, have appeared by 0 h APF, and generate the coeloconic sensilla of the sacculus. Wave 2 SOPs, which also express Ato, appear by 4 h APF, are more numerous, and arranged in three semicircular rows; these give rise to the coeloconic sensilla of the third antennal segment. Amos expressing wave 3 SOPs are much more numerous. They initially appear to intercalate between the Ato expressing semicircles, but then expand in number. These SOPs give rise to the more numerous basiconic and trichoid sensilla. An additional Ato dependent group, which gives rise to aristal sensilla (Ar), is also marked. (Redrawn with permission from zur Lage, P.I., Prentice, D.R., Holohan, E.E., Jarman, A.P., **2003**. The *Drosophila* proneural gene amos promotes olfactory sensillum formation and suppresses bristle formation. *Development 130*, 4683–4693.)

a stereotyped pattern of cells including two semicircles. Both of these waves of SOPs express the proneural gene *atonal* (*ato*), which is also required for the formation of chordotonal organs (Jarman *et al.*, 1993) and photoreceptors (Jarman *et al.*, 1994), and appear to be derived from *ato* expressing proneural clusters in the overlying epithelium (Jhaveri *et al.*, 2000a; zur Lage *et al.*, 2003). Both are absent in *atonal* mutant antennae and functionally will generate sensilla coeloconica of the sacculus (wave 1) and the main antennal surface (wave 2) (**Figure 6**) (zur Lage *et al.*, 2003). In contrast to these first two waves, *amos* expressing SOPs derived from overlying Amos positive proneural clusters, appear in a third wave first evident at 8 h APF. These SOPs initially intercalate between the wave 2 *ato*-dependent SOPs. However, by 16 h APF these *amos* SOPs are much more numerous and less obviously spatially patterned.

zur Lage *et al.* (2003) were able to identify an *amos* enhancer whose expression was maintained in SOP progeny, which turned out to be all cells in the sensilla basiconica and trichodea; this is satisfyingly consistent with the *amos* loss-of-function phenotype, which is a complete loss of sensilla basiconica and trichodea (zur Lage *et al.*, 2003).

In short, the generation of the different SOPs responsible for the three classes of olfactory sensilla has now been established with some precision and conforms, in general, to the expected norms of sensory organ development. However, the next step, which would normally be the stereotyped division of the SOPs, remains somewhat less clear. Mitotic cells are not detected in the 3AS zone of the disc until about 12 h APF and, even then, they are in the periphery of the lobe, corresponding to wave 1 cells, which delaminated at least 12 h earlier (Sen *et al.*, 2003). In the meantime, it seems that the SOPs (or, more appropriately, founder cells) recruit additional neighboring cells to form a presensillum cluster (PSC) of 2–3 cells, which can be labeled by the A101 (*neuralised; neu*) enhancer trap (Reddy *et al.*, 1997). This conclusion was supported both by BrdU labeling, with no division detected between the generation of SOPs and the formation of PSCs (Ray and Rodrigues, 1995), and by clonal analysis (Reddy *et al.*, 1997). Clones were generated at mid first larval instar, i.e., very early in antennal disc formation, and then analyzed at 48 h APF, i.e., mid-pupal stage, in principle after the final division of sensillar progenitors (see below). In all 29 sensillar clusters analyzed, clonal boundaries separated cells of the same sensillum. This unusual mode of PSC development is similar to the recruitment of neighboring cells by R8 photoreceptors, which, incidentally, are also *ato* dependent like waves 1 and 2 olfactory SOPs. Reddy *et al.* (1997) confined their studies to those SOPs generated within the first 10 h of larval development. It will be very interesting to determine if the more numerous *amos* dependent SOPs of the third wave show a similar behavior.

After the formation of the PSC, these cells finally divide at least once to generate the sensillar components (**Figure 7**). This period of division is reported by Sen *et al.* (2003) to last from 12 to 22 h APF. Sen *et al.* (2003) have identified at least three different division patterns by analysis of clones generated at 10 h APF, i.e., before division starts, and analyzed

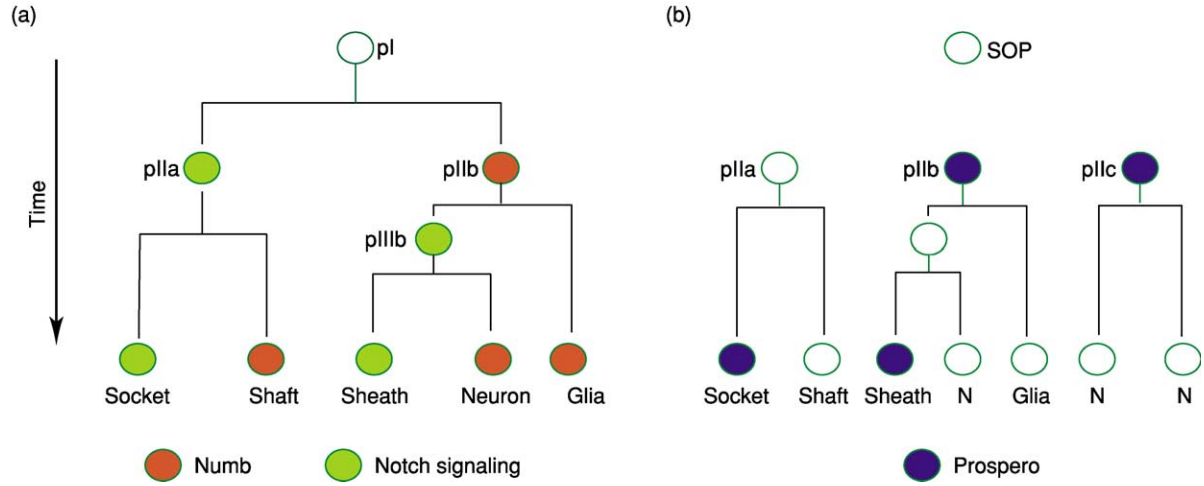


Figure 7 Lineages of the mechanosensory and olfactory sensilla in the *Drosophila* PNS. (a) Generalized lineage of the mono-innervated sensilla of the *Drosophila* PNS, illustrated for the adult microchaete lineage (review: Bellaiche and Schweisguth, 2001). Numb (red) or Notch signaling (green) show asymmetric segregation at each division. pI, pII, and pIII refer to the precursors of three successive stages of division. (b) Lineage of the olfactory sensilla proposed by Sen *et al.* (2003). Note the close correspondence between the products of the pIIa and pIIb division in this lineage and that in (a). N, neuron; SOP, sensory organ precursor.

at 36 h APF, i.e., after division ends (**Figure 7b**). Two of these patterns are directly analogous to the division pattern of the adult sensory bristle (**Figure 7a versus b**) and are named accordingly. The pIIa precursor generates the socket and shaft accessory cells, while pIIb generates a migratory glial cell and another intermediate, pIIIb, which gives rise to a neuron and the sheath (the most glial-like of the sensillar accessory cells). In fact, only the *ato*-dependent coeloconic sensilla show the full pIIb lineage, since they are gliogenic (Jhaveri *et al.*, 2000b). The corresponding PSC cells in the trichoid and basiconic lineages, which are nongliogenic, may proceed directly to the pIIIb division, or the equivalent of the glial cell could undergo apoptosis. Finally, there is a division pattern labeled pIIc by Sen *et al.*, (2003), which generates a pair of neurons.

In short, it is proposed that the generation of olfactory sensilla follows a hybrid pattern, in which the newly selected SOP recruits neighbors, rather than undergoing an initial division, but this cluster of cells then divides in a manner typical of other sensory organ lineages. It is possible that such a system allows more flexibility in the generation of sensilla with different numbers of ORNs, perhaps by the recruitment of 0, 1, or 2 cells, which will undergo the neurogenic pIIc division pattern.

1.12.3.2. Specification and Differentiation of Sensilla and ORNs

How are SOPs and their progeny specified to adopt different sensillar fates? The proneural genes *ato* and *amos* are clearly necessary for the coeloconic and basiconic/trichoid fates, respectively. Are they also sufficient to specify these alternatives? In addressing this question it should first be noted that ectopic Ato expression in other contexts leads to the formation of chordotonal organs (Jarman *et al.*, 1993), while ectopic Amos expression outside olfactory SOPs leads to formation of mechanosensory bristles (Lai, 2003; zur Lage *et al.*, 2003). Consequently, there must be additional factors within the olfactory SOPs or in other lineages that cooperate with the proneural genes. Jhaveri *et al.* (2000a) have investigated whether *ato* may be sufficient to direct coeloconic formation in the context of olfactory SOPs. By expressing Atonal in SOPs and their progeny with the A101 neu-GAL4 enhancer trap mentioned earlier, they were able to show a substantial increase in the number of coeloconic sensilla and a concomitant reduction in trichoid and basiconic numbers. The additional coeloconic sensilla were located in a domain on the antenna normally rich in basiconic sensillar, further supporting the notion of conversion. Besides differences due to these two proneural genes, there must also be mechanisms to differentiate the future trichoid and basiconic sensilla among the Amos expressing SOPs. Gupta *et al.* (1998) have suggested a role for *runt* (*run*) in this choice.

After the final division of sensillar components (described in Section 1.12.3.1 above), when do sensilla and, in particular, their constituent ORNs begin to differentiate? In *Drosophila*, various markers have been used to follow the differentiation of neurons in the peripheral nervous system (PNS)

including the monoclonal antibody mAb22C10 (Zipursky *et al*., 1984), which detects the microtubule associated protein, Futsch (Hummel *et al*., 2000). Lienhard and Stocker (1991) reported the appearance at 14 h after APF of three clusters of about 5–8 mAb22C10 positive neurons in the developing 3AS, which they labeled t1–t3. Since other molecular markers were unavailable at that time, correspondence with the more recent results obtained by zur Lage *et al*. (2003) on SOP formation is difficult. However, Lienhard and Stocker (1991) argued that t3 corresponds to a basiconic group (since it is absent in the *lozenze*^{3} (*lz*^{3}) mutant, which completely lacks basiconic sensilla), while the others are likely to be coeloconic. Thus, the *ato* dependent neurons of the first two waves described by zur Lage *et al*. (2003) probably correspond to t1–t2, while t3 may correspond to products of the first *amos* dependent SOPs. The number of mAb22C10 positive neurons in each cluster increases rapidly by 19 h APF and, in addition, further neurons appear between the clusters (likely to correspond to progeny of the wave of expansion of *amos* dependent SOPs between 8 and 16 h APF), so that by 26 h APF, the organization strongly resembles that of the adult. Ray and Rodrigues (1995) also described the development of mAb22C10 staining. They found that at 14 h APF mAb22C10 stains one or two cells in each presensillum cluster, i.e., mAb22C10 can also stain neural precursors (Hartenstein and Posakony, 1989). By 20 h APF, approximately 140 sensilla have adopted a mature mAb22C10 pattern according to Ray and Rodrigues (1995). Since there are approximately 70 coeloconic sensilla (see Section 1.12.3.1), this number should include some of the *amos* dependent basiconic or trichoid sensilla.

zur Lage *et al*. (2003) have reported that ORNs generated by *amos* dependent SOPs differentiate later than their *ato* dependent counterparts. At 24 h APF mAb22C10 labeling of neuronal processes is widespread throughout the 3AS (presumably corresponding to *ato* dependent coeloconic sensilla), while it is conspicuously absent from sensilla derived from *amos* SOPs. By 30 h APF, differentiation of the two outer cells (socket and shaft) has occurred in many of the *amos* dependent sensilla, which can therefore be identified as basiconic or trichoid. This absence of mAb22C10 staining in any sensilla derived from *amos* dependent SOPs at 24 h APF seems to contradict the number of mature mAb22C10 positive cells reported by Ray and Rodrigues (1995) at 20 h APF. There is no obvious explanation for this discrepancy, although it is possible that the amos-GAL4 enhancer generated by zur Lage *et al*. (2003) is not expressed in all *amos* dependent SOP lineages.

In attempting to connect the development of ORNs and their olfactory function, one of the key questions that must be addressed is how they come to express specific olfactory receptors. This remains an open question. For example, it is not yet clear at what stage ORNs become specified to express a particular odorant receptor (OR) type. OR expression has only been reported after 54 h APF in *Drosophila* (Clyne *et al*., 1999b), which is more than 1 day after ORN axons and projection neuron (PN) dendrites first meet. Nor is the relationship between control of sensillar type and OR expression clear. It is possible that individual SOPs are specified right from the time of their delamination to generate ORNs expressing a particular subset of receptors. The electrophysiological recordings of de Bruyne *et al*. (2001) suggest that basiconic sensilla contain only stereotyped pairings of ORNs expressing different OR types. If the recruitment hypothesis (Ray and Rodrigues, 1995; Reddy *et al*., 1997) is correct and applicable to all sensilla, then there must be a tight coupling between the fates of the daughters of the different members of the presensillum cluster. This would require that neighboring cells also have a precisely defined potential that the founder cell somehow signals instructively to indicate the exact nature of the appropriate OR choices for that sensillum. Alternatively, a choice of OR in one ORN within a sensillum could influence the OR choice of the other ORNs. Any of these possibilities would suggest rather precise intercellular signaling events, whose elucidation is awaited with much interest.

There is one additional piece of molecular data that may offer an opening into the OR/ORN specification problem. At the same time that Clyne *et al*. (1999b) were characterizing the olfactory receptor family of *Drosophila* (see Section 1.12.2.1.1), they were also working to characterize a long-standing behavioral mutant, *abnormal chemosensory jump 6* (*acj6*). The *acj6* mutant was one of a series of mutants isolated in an olfactory behavioral screen approximately 10 years earlier (McKenna *et al*., 1989). The *acj6* mutants did not respond to a subset of odorants, and an abnormal electroantennogram suggested that the defect was likely to be peripheral (Ayer and Carlson, 1991). When the gene was cloned (Clyne *et al*., 1999a), it was found to encode a POU (Pit-1/Oct-1/Unc-86) domain transcription factor homologous to the vertebrate Brn3 family, whose members are expressed in a variety of sensory neurons (Ryan and Rosenfeld, 1997). Expression of Acj6 was demonstrated in most if not all ORNs, while the mutant phenotype included the loss of

expression of an estimated one-quarter of ORs. At the physiological level, *acj6* mutant sensilla displayed at least three classes of mutant phenotype: (1) loss of olfactory responsivity; (2) transformation to another ORN type, with a different response profile, present in the same sensillum; and (3) transformation to a novel response profile. These phenotypes are consistent with a role for *acj6* in specifying subsets of ORNs; however, the broad expression pattern but more restricted function suggests that it must co-operate with other factors. Clyne *et al.* (1999a) noted that expression could first be observed in the antennal disc at about 16 h APF. Staining at this time is limited to a small number of neurons, which appear (to us) to be products of the wave 1 saccular coeloconic precursors characterized by zur Lage *et al.* (2003). Consequently, Acj6 expression appears not to be in SOPs or presensillum cluster cells, but rather specifically in the ORNs. This relatively late expression suggests that *acj6* must be late-acting and perhaps quite proximal to the choice of OR expression. The first ORN axons reach the AL at 16 h APF, so it is possible that *acj6* could help specify glomerular targets, an interesting avenue for future research.

As the ORNs begin to differentiate and send out axons, they exit the third antennal segment in three well-defined fascicles, pass through the second and first antennal segments, and then fasciculate together. It has been observed in *Drosophila* (Tissot *et al.*, 1997) and in *Bombyx mori* (Svacha, 1992) that the adult nerve appears to follow the path of persistent larval sensory neurons to the antennal lobe. Indeed, this may be a general feature of adult sensory projections (Williams and Shepherd, 2002). Having arrived at the edge of the antennal lobe, the ORN must now synapse (along with other ORNs expressing the same OR) with appropriate partner neurons in the CNS, thereby relaying olfactory information to the brain.

1.12.3.3. Variations

At this point, antennal neurogenesis has been studied rather more extensively in *Drosophila* than in other insects. However, the general periods of antennal neurogenesis can be compared in different insects as follows. In holometabolous insects, neurogenesis in the antennal disc is restricted to relatively short periods in early metamorphosis. Thus, in *Manduca*, neurogenesis occurs during the first two stages (out of 18) of pupal development, and in *Drosophila* between 12 and 22 h APF (in a 100 h period of metamorphosis). This rapid neurogenesis is possible because neurons in the PNS develop in parallel from a large number of precursors (the sensory organ precursors, SOPs) via a small number of divisions (**Figure 7**); this is in stark contrast to the CNS where neurogenesis occurs serially over extended periods from a relatively small number of asymmetrically dividing neuroblasts (Section 1.12.4.2.1 below and **Figure 10**). In hemimetabolous insects, although PNS neurogenesis is presumably mechanistically related to that in holometabolous ones, the lengths of the neurogenic phases are reversed. Peripheral neurogenesis continues throughout nymphal development, while central neurogenesis is restricted to the embryonic phases, at least for the AL neurons (Section 1.12.4.6 below).

The details of sensillar development in other holometabolan antennae are likely to follow the same general plan as in the *Drosophila* antennal disc described in the previous two sections. However, the question of sensillar lineage does remain open. For example, Keil and Steiner (1991) followed the development of olfactory sensilla in the silkmoth, *Antheraea polyphemus*, by electron microscopy studies and concluded that sensilla are monoclonal; the proposed lineages are similar to the basic plan in **Figure 7a**. Whether this monoclonal origin is a definite distinction between Lepidoptera and the very highly diversified Diptera, is an interesting comparative question. Perhaps in the near future it will be possible to compare these developmental events among different species using the molecular findings and tools that have been developed in *Drosophila*.

In *Drosophila*, a rapid and significant increase in our understanding of specification of different ORNs can be expected. One interesting feature is the existence of some spatial order in the antennal disc and, consequently, in the third antennal segment (**Figure 2**). In contrast, there is no obvious distinction among olfactory sensitivity of the 80 or so segments in a *Manduca* antenna (discussed in Tolbert and Sirianni, 1990). In hemimetabolous insects, such as cockroaches, there is successive extension of the antennal flagellum at each nymphal stage, but since the glomerular number is constant from embryogenesis, it would be expected that a diversity of different neurons must be generated at each stage. Another very interesting feature that has come to light in detailed studies of the *Drosophila* antenna, is the specific pairings of ORNs of particular olfactory sensitivities within different sensilla, which suggests a strong coupling between OR choice among ORNs within a sensillum. The authors are not aware of similar physiological studies in other insects, which would indicate if this could be a general phenomenon, but suspect this may well be the case.

1.12.4. Development of the Central Olfactory System: Antennal Lobe

The development of the antennal lobe (AL) and in particular AL glomeruli is one of the major problems in the development of the olfactory system. As the AL is the first sensory relay or processing center in the brain, deciphering its developmental logic is of key importance in understanding how olfactory information is passed to higher centers. The parallel nature of incoming olfactory information, combined with the uniglomerular projection patterns of ORNs and PNs, provides this system with great potential for study of the general problem of wiring specificity in the nervous system. In this section, the development of the major constituents of the holometabolan antennal lobe – ORNs, PNs, and local interneurons (LNs), as well as glia – and how they interact to form the mature structure are discussed.

To appreciate the wiring challenge inherent in the generation of the mature AL, it may be helpful to imagine oneself at the growth cone of a developing ORN. ORNs are generated in the antennal disc and extend axons towards the antennal lobe. As previously described (Section 1.12.2.2.2), ORNs expressing the same receptor must eventually converge on a stereotypically positioned glomerulus within the adult antennal lobe, making connections with projection neuron dendrites and local interneurons. What kind of environment will ORN growth cones encounter as they enter the developing AL and how are they programed to respond to it?

More specifically, from the point of view of wiring specificity, the following questions should be addressed: (1) are ORNs already specified to express a particular OR at the time that their axons reach the AL and if so, how precisely are they specified? (2) is the AL spatially patterned at this time? and (3) are the central partner neurons in the lobe, especially the uniglomerular PNs, already specified to form connections with ORNs bearing a specific receptor? In *Drosophila*, the respective answers to the above questions can be summarized as follows: (1) definitely crudely, probably precisely; (2) yes, but not precisely; and (3) yes, apparently with great precision.

Wiring specificity is probably best considered using molecular genetic approaches, which at present are uniquely available in *Drosophila*. This species will be the focus for much of the discussion, but it is assumed that this logical framework is likely to be generally applicable among other insect species. In particular, glomerular development has been extensively studied from the cellular perspective in *Manduca* and interactions between different cell types have been described and manipulated.

It is important to be quite precise about what is meant by a glomerulus, when it can be said to exist, and how it is experimentally detected. Functionally, a glomerulus represents the convergence of a class of ORNs expressing a particular OR. It constitutes the arrival point of a specific olfactory information channel, and information will be faithfully transferred if PN dendrites that contact these ORNs do not contact other ORNs.

Anatomically, the glomerulus consists of the axon terminals of those ORNs and the processes of partner PNs and LNs, all wrapped by a glial sheath. Experimentally, a glomerulus is usually defined morphologically as an area of increased synaptic density surrounded by an area of reduced synaptic density (presumably the glial sheath or asynaptic neurites).

This typical experimental definition may be somewhat divorced from the functional one. Consider a hypothetical situation in which the glial sheath is removed so that synaptic density is uniform throughout the AL. In this case, glomeruli would not be experimentally discernible by standard techniques, but the antennal lobe would still be highly spatially patterned. While this is clearly hypothetical, during development there may be stages in which synapses do not yet exist between ORNs and PNs and the glial border is poorly developed. ORNs might also have restricted arborizations that spatially overlapped with restricted arborizations of appropriate partner PNs. From the functional perspective, these neurons would be ready to transmit and receive information faithfully, but morphologically this order would not be apparent. Situations like this must be carefully considered before drawing final conclusions from developmental studies.

Finally, it is worth emphasizing that glomerular development may occur in several phases, such as initial formation, maturation, and maintenance. When considering the role of experimental perturbations, developmental time courses are often important to determine which of these steps is affected.

1.12.4.1. Olfactory Receptor Neuron Axons

As described above (Section 1.12.3), in holometabolous insects ORNs are generated in the antennal disc and extend axons towards the brain, typically following the course of the larval antennal nerve. Upon arrival at the AL, it has generally been argued that ORNs are the primary cell type responsible for generating glomerular organization. Results for the model system of choice, *Drosophila*, are first

discussed and then compared and contrasted with work in other insects, especially *M. sexta*.

In *Drosophila*, the development of ORN axons has been followed largely by the use of enhancer trap lines that label subsets of ORNs. This has the advantage of specific and reproducible labeling, but there is the caveat that some populations may be missed. Tissot *et al.* (1997) used two GAL4 lines, KL116 and MT14, which label mostly trichoid and large basiconic sensilla, respectively, to follow in-growth of ORNs into the AL. The ORN axons appeared to reach the lobe around 24 h APF. However, since the trichoid and basiconic sensilla derive from the *amos* dependent third wave of SOPs (described in Section 1.12.3.1 above), it seems likely that there could be some ORNs that reach the lobe sooner than this. Indeed Jhaveri *et al.* (2000b) reported that an *atonal* promoter fusion with the LacZ reporter was expressed in an early population of ORNs, perhaps derived from the *ato* dependent SOPs, which generate coeloconic sensilla; these ORN axons reached the antennal lobe around 18 h APF. Most recently, Jefferis *et al.* (2004) have taken a more unbiased approach to label incoming ORNs using a pan-neural GAL4 in combination with *eyeless*-Flp to drive mitotic recombination in antennal (and eye) disc precursors, generating large, positively marked ORN clones. These results have shown that the first ORN populations reach the antennal lobe around 16–18 h APF (**Figure 8a**).

Having reached the antennal lobe, how do ORNs interact with existing constituents of the AL and what might these be? The development of the other cellular components of the AL will be discussed in detail in the following sections, but there does seem to be a core neuropil that precedes the arrival of ORN axons. In *Drosophila*, this neuropil is strongly immunoreactive for the cell adhesion protein N-Cadherin (Ncad; **Figure 8a**) and consists, at least in part, of the dendrites of PNs (Section 1.12.4.2.2 below; Jefferis *et al.*, 2004). How do ORNs interact with this existing structure? Initially, ORN processes seem to surround the lobe (Jhaveri *et al.*, 2000b; Jefferis *et al.*, 2004) but only enter the core of the AL around 25 h APF (**Figure 8b and c**). Detailed double labeling studies of ORNs and PNs have not yet been carried out, but it may be that at these early stages, ORN axons are interacting amongst themselves rather than with other cell types.

By 30 h APF, there are more axonal processes within the central part of the antennal lobe, and by 36 h APF, at least one glomerulus, VA1, can be identified (Jhaveri *et al.*, 2000b). At 36 h APF, the glomerular organization is rather diffuse – it is clear that while different ORN types are accumulating in distinct foci, they still overlap with their neighbors. A progressive period of refinement follows, with a fairly mature organization observed by 48 h APF (Jhaveri *et al.*, 2000b). It should be recalled that this organization is achieved before the expression of olfactory receptors can be detected (Section 1.12.3.2 above). One key developmental question that remains unanswered is the mode of innervation of individual ORNs. Do they immediately project to a specific region of the lobe in which the future glomeruli will form, or do they initially have

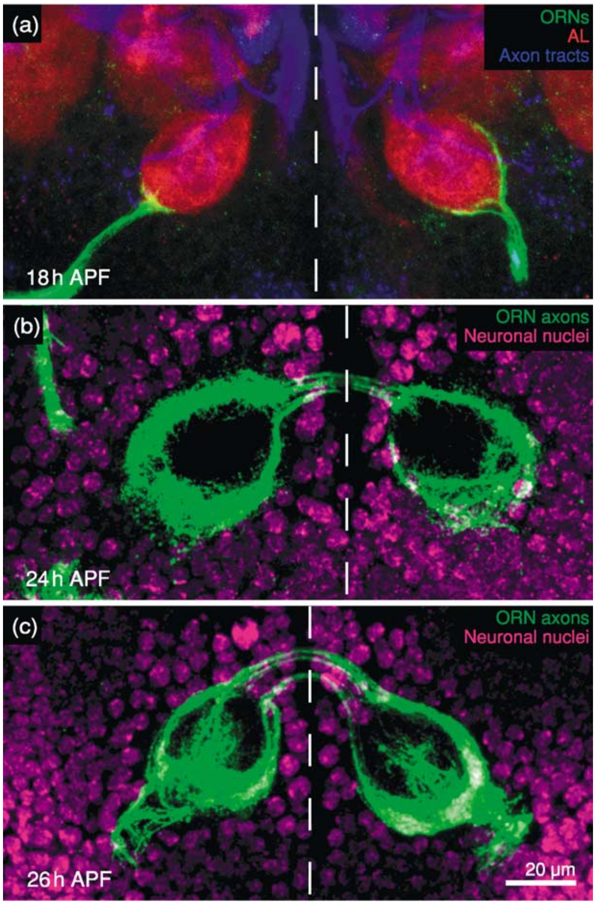


Figure 8 Early development of ORNs in *Drosophila*. The eyless promoter is used to drive mitotic recombination in the antennal disc, generating large GFP-labeled MARCM clones (green) in olfactory receptor neurons. (a) 18 h APF: ORN axons have just reached the antennal lobe (stained by anti Ncad in red) and have started to grow around the margin of the AL. FasII is used to label axon tracts as a counterstain (blue). (b) 24 h APF: ORN axons now form a thick margin around the AL, but do not yet enter the lobe. Counterstain in (b) and (c) is antiembryonic lethal abnormal visual system (Elav), which stains neuronal nuclei (magenta). (c) 26 h APF: ORN axon processes now extend deeper into the antennal lobe. (Reproduced with permission from Jefferis, G.S.X.E., Vyas, R.M., Berdnik, D., Ramaekers, A., Stocker, R.F., *et al.*, **2004**. Developmental Origin of wiring specificity in the olfactory system of *Drosophila*. *Development* 131, 117–130.)

a broad termination area, which is subsequently refined? Enhancer traps that label, from an early stage, ORNs whose axons will target to the same glomerulus should help to address this question.

Molecular genetic studies to understand the basis of axonal targeting to specific glomeruli have only recently begun in *Drosophila*. So far, the axon guidance molecule Dscam (Hummel *et al*., 2003) and the intracellular signaling components Dreadlocks (Dock) and P21-activated kinase (Pak) have been identified as being required for proper ORN axonal targeting (Ang *et al*., 2003). Dscam is a member of the immunoglobulin (Ig) domain superfamily of transmembrane proteins homologous to the human Down's syndrome cell adhesion molecule (apparently so-called largely because of its location on chromosome 21 rather than any clear functional link to the Down's syndrome). When the *Drosophila* gene was first cloned and shown to be required for axon guidance in the larval visual system, it aroused considerable interest because of its potential to encode a huge number of isoforms, an estimated 38 016, by alternative splicing, largely in the extracellular region containing the Ig domains (Schmucker *et al*., 2000). Hummel *et al*. (2003) find that ORNs and central neurons within the AL express multiple isoforms of Dscam. By making large clones of mutant *Dscam*$^{-/-}$ ORNs, it seems that Dscam is required for correct targeting of some but not all ORNs; mutant neurons converged at ectopic locations within, or occasionally outside, the antennal lobe. Interestingly, mutant maxillary palp ORNs sometimes formed several sites of convergence completely outside the AL in the subesophageal ganglion, which is, for example, a normal site of convergence for gustatory neurons. These ectopic structures had the appearance of glomeruli (by neuropil staining), and different ORN classes still seemed to segregate into different foci, suggesting that Dscam is required for guidance, or perhaps growth, but is not (uniquely) required for different ORN classes to segregate in a class-specific manner. Additional studies will be required to determine what role Dscam diversity may play in the development of different ORN classes.

In a second study, Ang *et al*. (2003) used a similar methodology to investigate the function in ORNs of Pak and Dock, a protein kinase and an adaptor protein, respectively, both previously shown to be required for downstream signaling from receptors involved in photoreceptor axon guidance (Garrity *et al*., 1996; Hing *et al*., 1999) and perhaps Dscam signaling (Schmucker *et al*., 2000). Whole animal mutants or animals with large clones of mutant ORNs (but wild-type target neurons), had antennal lobes which were significantly reduced in size and showed little glomerular organization; *pak* and *dock* function seemed to be specifically required in the ORNs since expression of recombinant wild-type Pak or Dock in ORNs rescued the phenotype of whole animal mutants. Although these genes are clearly required for ORN targeting, the phenotypes did not show any specificity for particular ORN subtypes.

Although *Dscam* mutants did show some subtype specificity, the question of what molecular mechanisms endow different ORNs with different target specificities remains very much open. In this regard, recent work in *M. sexta*, describing the expression pattern of the cell adhesion molecule FasII (Higgins *et al*., 2002) and an Ephrin (Eph) and its receptor (Kaneko and Nighorn, 2003), is most intriguing. These molecules are expressed in ORNs during glomerular formation and seem to be differentially expressed in ORNs targeting to different glomeruli (**Figure 9**). Most interestingly, Ephrins, which are usually repulsive cues, and their receptors show a complementary expression: cells expressing a high level of the receptor express a low level of the ligand. This suggests that they may be involved in axo-axonal signaling during segregation of different ORN classes into different glomeruli (Kaneko and Nighorn, 2003).

1.12.4.2. Projection Neuron Dendrites

1.12.4.2.1. Neurogenesis In *Drosophila*, Projection neurons (PNs) of the adult AL are generated during both the embryonic (G.S.X.E. Jefferis, E.C. Marin and L. Luo, unpublished data) and larval phases (Stocker *et al*., 1997; Jefferis *et al*., 2001) and must, therefore, be essentially complete before the generation of ORNs in the antennal disc, which begins around 12 h APF (see Section 1.12.3.1 above). Their generation pattern is believed to conform to the general model of neurogenesis in the *Drosophila* CNS: a neuroblast divides asymmetrically to regenerate a neuroblast along with a smaller ganglion mother cell (GMC), which divides once more to make two postmitotic neurons (**Figure 10**).

The neurogenesis of different classes of PN has been studied in detail using the *GAL4-GH146* enhancer trap line (Stocker *et al*., 1997) and the mosaic analysis with a repressible cell marker (MARCM) system for mosaic analysis (Lee and Luo, 1999; review: Lee and Luo, 2001). MARCM allows for positive labeling of a small fraction of cells using a membrane targeted GFP, in an otherwise unlabeled brain. Specific cell populations are targeted by the combination of tissue specific GAL4 enhancer trap lines and the application of a heat

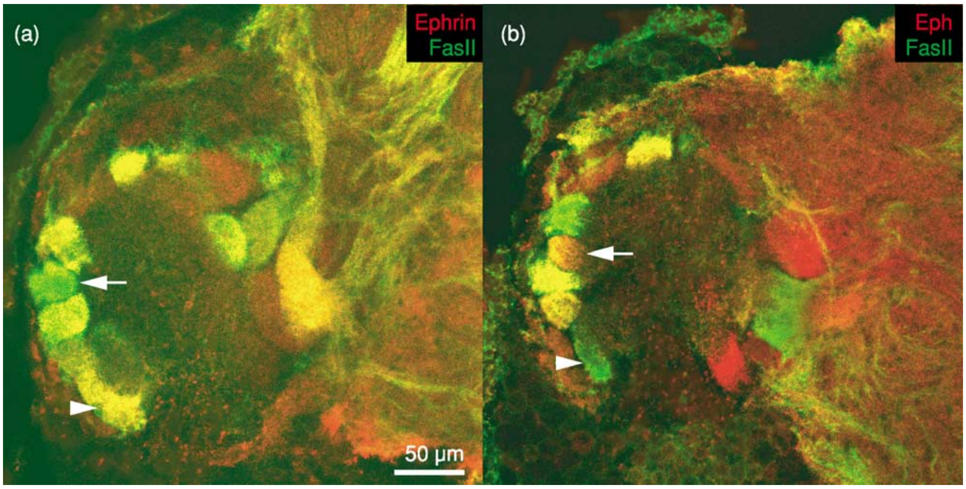


Figure 9 Mosaic expression of FasII and Ephrin/Eph receptor in *Manduca* ORNs. Frontal section through a *M. sexta* antennal lobe, with the antennal nerve entering from the right (lateral) and dorsal uppermost in the images. Samples have been double labeled at pupal stage 6 for FasII (in green) and Ephrin (in (a)) or Eph receptor (in (b)) (in red). Note the mosaic expression of these three molecules in different glomeruli. Comparison of indentifiable glomeruli suggests that ORNs expressing high levels of the repulsive Ephrin ligand tend to express low levels of the Eph receptor and vice versa (compare glomeruli identified by arrowheads or arrows, respectively). Other evidence suggests that Ephrin and Eph receptors are both expressed on ORN axons, but not on PN dendrites. (Reproduced with permission from Kaneko, M., Nighorn, A., **2003**. Inter-axonal Eph-ephrin signaling may mediate sorting of olfactory sensory axons in *Manduca sexta*. *J. Neurosci. 23*, 11523–11538; © by the society for Neuroscience.)

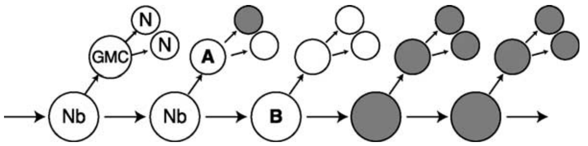


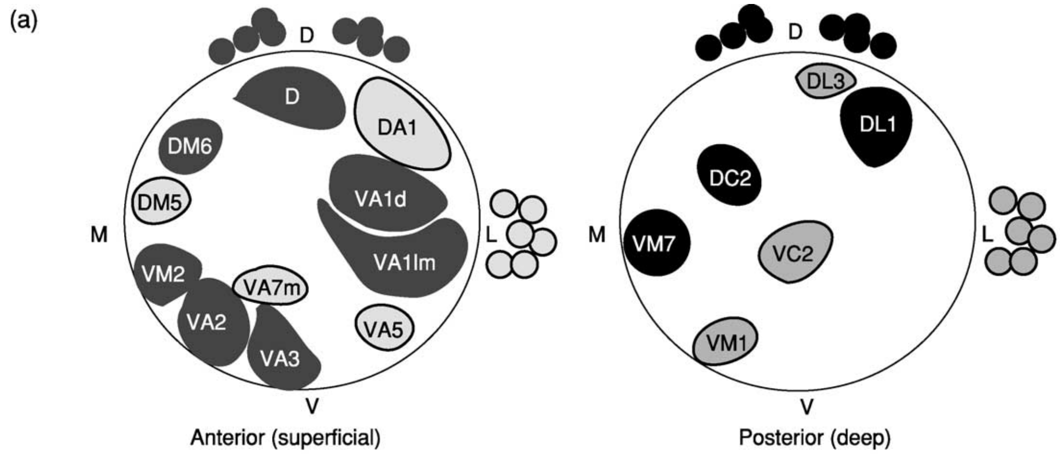
Figure 10 CNS neuroblast division pattern in *Drosophila*. In the CNS, neurons are generated by the asymmetric division of a neuroblast (Nb). At each division, the Nb divides asymmetrically to regenerate a Nb, while generating a smaller ganglion mother cell (GMC). This GMC divides once more to generate two postmitotic neurons (N). If a clonal analysis system such as MARCM is used to investigate neurogenesis, this pattern of division results in the generation of characteristic clonal units. If a clone is generated at point A, then a single labeled cell will be generated (filled gray circles). In contrast, if the clone is generated by an event at point B, then 50% of the time the Nb itself falls within the clone and all subsequent daughter cells will be labeled. Another 50% of the time the GMC falls within the clone resulting in a labeled two-cell clone (not shown).

shock pulse at a desired time in development; the heat shock drives mitotic recombination, which causes permanent labeling of a fraction of the cells dividing at that time and any progeny they may subsequently generate. Given the asymmetric division pattern of CNS neuroblasts described above (Figure 10), MARCM typically generates three kinds of labeled clones: (1) single cells (when a recombination event occurred in a dividing GMC); and, if recombination occurs in the neuroblast, either a labeled (2) two-cell or (3) a neuroblast clone, in which all of the subsequent progeny of the neuroblast fall within the clone.

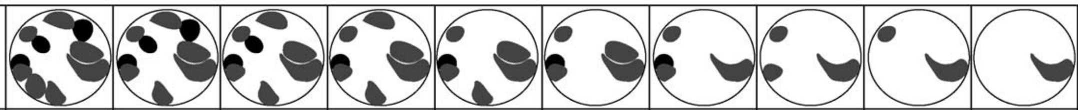
GH146 labels approximately 90 PNs, which are divided into three discrete clusters of cell bodies, positioned anterodorsal (ad), lateral (l), and ventral (v) to the antennal lobe (Stocker *et al.*, 1997; Jefferis *et al.*, 2001), with approximately 50, 35, and 6 cells in each cluster according to Jefferis *et al.* (2001). Application of the MARCM method demonstrated that these three groups of cells were born from three different identifiable neuroblasts (Nbs). While the lateral and ventral clusters seemed to consist entirely of neurons born during postembryonic development, this seems to be the case for only about 30 of the 50 adNb neurons (Jefferis *et al.*, 2001). The balance, about 20 neurons, seem to be generated by the same neuroblast during embryonic development and, therefore, to function both during larval and adult life in their respective olfactory systems (G.S.X.E. Jefferis, E.C. Marin, and L. Luo, unpublished data). An earlier study suggests that the lateral group of PNs may be produced by a single neuroblast, which divides unusually early in larval development (Stocker *et al.*, 1997); although this neuroblast may be dividing in newly hatched larvae and probably also in emrbryos, it does not seem to produce GH146 positive PNs until 24 h after larval hatching (Jefferis *et al.*, 2001).

Does any logic connect a PN's parental neuroblast and its connectivity? The adNb and lNb PNs were for the most part uniglomerular and projected axons via the inner iACT to the MB calyx and LH; vNb PNs all projected via the mACT and while some were uniglomerular, one striking example

(a)



(b)



VA2	+									
DL1	+	+								
DC2	+	+	+							
D	+	+	+	+						
VA3	+	+	+	+	+					
VA1d	+	+	+	+	+	+				
VM7	+	+	+	+	+	+	+			
VM2	+	+	+	+	+	+	+	+		
DM6	+	+	+	+	+	+	+	+	+	
VA1lm	+	+	+	+	+	+	+	+	+	+

Figure 11 Lineage and birth order predict dendritic targets of *Drosophila* PNs. (a) Stereotypic dendritic projection patterns of 2 PN lineages superimposed on cartoons of sections through the anterior and posterior antennal lobe. PNs of the anterodorsal (darker cell bodies and glomeruli) and lateral (paler cell bodies and glomeruli) lineages send their dendrites to stereotyped, intercalated but nonoverlapping glomerular targets. This indicates that the connections that any particular PN can make are limited by its parental neuroblast. D, V, L, and M indicate the dorsal, ventral, lateral, and medial aspects of the lobe, respectively. Individual glomeruli are named according to the map of Laissue *et al.* (1999). (b) Analysis of Nb clones indicates that the last-born PNs of the anterodorsal lineage will always go on to innervate a specific glomerulus, VA1lm. Neuroblast clones generated a little earlier in development have PNs, whose dendrites innervate both VA1lm and a second glomerulus DM6. Neuroblast clones are generated a little earlier yet, contain PNs innervating VA1lm, DM6, and VM2. In this manner it was possible to work all the way back to the large clones generated after larval hatching with each clone type completely containing the smaller clones that follow it. The conclusion from these observations is that PNs innervating different glomeruli must be generated in a highly stereotyped order. ((a,b): Reproduced with permission from Jefferis, G.S.X.E., Marin, E.C., Stocker, R.F., Luo, L., **2001**. Target neuron prespecification in the olfactory map of *Drosophila*. *Nature 414*, 204–208; © Nature Publishing Group.)

innervated almost all the glomeruli in the lobe (see Section 1.12.2.2.3 above for an anatomical summary). Therefore, it seems that different functional types of PN are produced by different Nbs. What other organizational features exist? One interesting feature of this initial analysis was that PNs born from the three Nbs had highly distinctive and reproducible dendritic innervation patterns in the lobe. Furthermore, the major groups, the anterodorsal and lateral lineages, innervated intercalated but nonoverlapping sets of glomeruli (**Figure 11a**). Thus, the daughter PNs of the adNb and lNb are restricted to forming dendritic connections with discrete sets of incoming ORNs expressing different sets of ORs and, therefore, different olfactory information (Jefferis *et al.*, 2001).

Are the individual PNs within a lineage further restricted in their dendritic connectivity? Apparently, yes. Analysis of single cell MARCM clones of the adNb, at different times in larval development, produced an ordered but overlapping set of birth times for PNs innervating different glomeruli. All single cell clones generated during the first 24 h of larval development had dendrites that innervated the DL1 glomerulus in the mature antennal lobe. For technical reasons, the accuracy of birth dating by the

MARCM system in this experiment was probably only about ±12 h by the end of larval development, so a second analysis was carried out. Neuroblast clones of the anterodorsal Nb generated at different times in development were analyzed. As expected, later-born neuroblast clones had fewer cells and innervated correspondingly fewer glomeruli than the larger clones generated earlier in development. However, intriguingly, the last 3–5 cells of the adNb lineage all innervated the same glomerulus, VA1lm. Furthermore, clones generated slightly earlier in development innervated this glomerulus (VA11m) and a second glomerulus, DM6. All 54 clones analyzed from a variety of developmental time-points confirmed this impression of a fixed sequence: each smaller and later-born clone was completely contained within the earlier born clones that preceded it (**Figure 11b**). In short, for the adNb at least, PNs are born in a highly stereotyped order. Since PN axonal and dendritic projection patterns are tightly coupled (as described in Section 1.12.2.2.5 above), this means that PNs pick up and deliver olfactory information in a highly stereotyped fashion according to birth order (Jefferis *et al*., 2001).

How can the birth order of a PN influence its dendritic and axonal connectivity? In the case of DL1 PNs (**Figure 11**), connections are formed as much as 5 days after birth. One possibility is that PN dendrites might differentiate sequentially according to their birth order; if different classes of ORNs also arrived sequentially at the antennal lobe, then sequential differentiation could explain class specific ORN/PN connections. However, Jefferis *et al*. (2001) reported that PNs born at different times in development have similar dendritic arborizations at the time of ORN ingrowth, making such a sequential differentiation hypothesis most unlikely. Instead, it seems that PNs are likely to possess molecular differences, which allow class specific interactions with incoming ORN axons.

How could a neuroblast generate a series of molecularly distinct PNs? One possibility is that time-varying extrinsic signals, present at or about the time of PN birth, could cause PNs to adopt different fates. A second possibility is that sequential signaling among PNs, after their birth, could lead to a coordinated set of fates. A final possibility, which the present authors favor, is that PNs generated at different epochs of neuroblast division may inherit molecular determinants that direct a distinct set of fates. This possibility is supported by the demonstration, in neuroblasts of the *Drosophila* embryo (both *in vivo* and in culture), of the cyclic expression of a set of transcription factors (review: Brody and Odenwald, 2002) and, in particular, the demonstration that these factors directly regulate the fate of GMCs/neurons born at different times (Isshiki *et al*., 2001).

In conclusion, it seems that in *Drosophila*, second order projection neurons are precisely specified to form connections with specific incoming sensory neurons, and relay that information to higher olfactory centers. It is not yet clear whether this is a general phenomenon in insect (or vertebrate) olfactory systems. However, this stereotypical wiring may be a substrate for stereotypical and innate behavioral responses to odors, which are a general feature of insects.

1.12.4.2.2. Morphogenesis In the section above, the generation of different classes of PNs in *Drosophila* have been discussed. How do these classes of PNs acquire their distinctive shape and connectivity? These issues are addressed here, first by detailing the development of PNs and then by highlighting some recent genetic studies. While there is a strong recent literature on PN development in *Drosophila*, development of PNs has also been described in other insect species including *Manduca* (Malun *et al*., 1994), which will be discussed in Section 1.12.4.5.

Developmental studies of the AL, and specifically of PNs, in *Drosophila* are a relatively recent phenomenon, but there has been good progress. In their initial characterization of the GH146 enhancer trap line, Stocker *et al*. (1997) noted that GH146-positive PNs showed signs of dendritic outgrowth at 24 h APF, shortly after the arrival of ORN axons. By 48 h APF, dendrites appeared to adopt a glomerular organization. A second study by Jhaveri and Rodrigues (2002), focused mainly on the development of ORNs, but also presented interesting results on PN development. In particular, they observed that GH146-positive PNs had dendritic arborizations in the area of the developing antennal lobe as early as 14 h APF. However, since GH146 labels both larval and adult PNs (see Section 1.12.4.2.1 above), it is not possible to conclude whether these dendrites are the remnants of the larval antennal lobe or newly developing dendrites of the adult antennal lobe. Jhaveri *et al*. (2002) also demonstrated that at 45 h APF, PNs seemed to have a glomerular organization, confirming the observation of Stocker *et al*. (1997). A more recent study by Jefferis *et al*. (2004) significantly extends these findings. The MARCM system was used to follow identified PNs with single cell resolution. Additionally, by labeling cells born at or after defined periods in development, it was possible to tease apart contributions from the degenerating larval lobe and the newly developing adult antennal lobe.

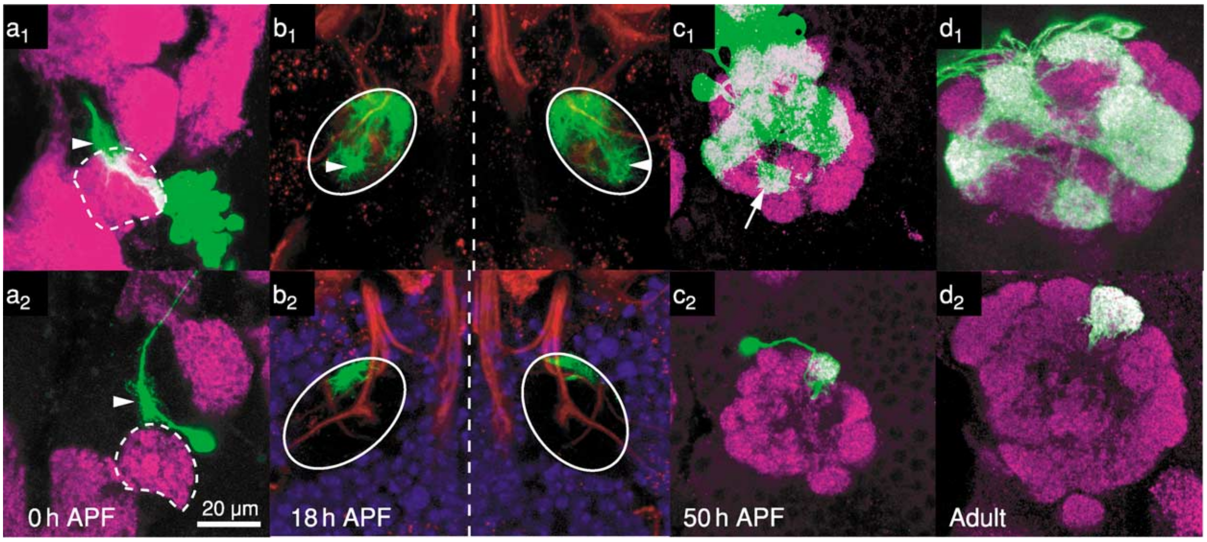


Figure 12 Development of *Drosophila* PNs. Anterodorsal neuroblast (a_1–d_1) or single cell (a_2–d_2) MARCM clones of projection neurons at different developmental stages: 0, 18, 50 h after puparium formation and adult (a, b, c and d, respectively). Counterstains are monoclonal antibody nc82 (magenta) which recognizes neuropil, FasII (red) for axon tracts, and Elav (blue) for neuronal nuclei. (a) Dotted outlines indicate larval antennal lobe, arrowheads developing PN dendrites. PN dendrites grow out from axonal processes in a location adjacent to the larval lobe; the overlap between green dendrites and magenta neuropil in (a_1) is an artifact of the maximum intensity Z projection of a stack of confocal images. (b) Solid lines delineate the adult antennal lobe, while a dotted line indicates the midline; two arrowheads indicate dendritic arborizations in similar areas of the developing antennal lobe. Note how the two single-cell clones in (b_2) (which will occupy a specific glomerulus, DL1) occupy similar regions of the developing AL. (c) Glomeruli can be detected by the neuropil marker nc82 by 50 h APF. Single cell and neuroblast clones indicate that PNs have dendritic arbors confined to appropriate and identifiable glomeruli. (d) There is continued growth of the AL during the second half of pupal development. (Reproduced with permission from Jefferis, G.S.X.E., Vyas, R.M., Berdnik, D., Ramaekers, A., Stocker, R.F., *et al.*, **2004**. Developmental origin of wiring specificity in the olfactory system of *Drosophila*. *Development 131*, 117–130.)

The first finding of this new study was that at 0 h APF, PN dendrites begin to elaborate new dendrites at a location adjacent to, but distinct from the larval lobe (**Figure 12a**). However, earlier studies had argued that the larval antennal lobe is the direct precursor of the adult antennal lobe (e.g., Stocker *et al.*, 1995); the discrepancy is probably due to the fact that these structures are small, directly adjacent, and hard to distinguish without the molecular markers now available. It will be interesting to determine if other holometabolous insects show any separation of the two structures.

The next critical point of this study (Jefferis *et al.*, 2004) was an analysis of PN dendrites at 18 h APF. As described in Section 1.12.4.1 above, ORNs first reach the antennal lobe around 16–18 h APF, and remain at the periphery of the lobe for some time thereafter. However, at this stage, PN dendrites have extended throughout the developing antennal lobe (**Figure 12b$_1$**). Most importantly, PN dendrites at this time occupied spatially stereotyped locations within the lobe. For example, early born PNs that innervate the DL1 glomerulus already had dendritic arborizations restricted to a dorsolateral corner of the developing antennal lobe (**Figure 12b$_2$**). Although PN dendrites of different classes still showed some overlap at these stages, this observation suggests that PNs are able to generate a coarse dendritic map in the absence of their presynaptic ORN partners. The implications of this early ORN independent phase of development are discussed below (Section 1.12.4.5).

Development proceeds rapidly after ORN arrival, so that glomeruli appear morphologically almost mature by 50 h APF, as determined either by PN dendrite projection patterns or by the monoclonal antibody nc82, which detects neuropil areas (**Figure 12c**; Jefferis *et al.*, 2004). Although organizationally mature, at this stage ALs are still at least 50% smaller in linear dimensions (and therefore about a quarter of the volume) than in the adult (**Figure 12d**). It is not clear whether this growth represents predominantly local increases in the arborization of the existing axons and dendrites or the ingrowth of new processes, such as local interneuron neurites or glial processes (see below).

The developmental studies described above will provide an important basis for understanding the work that is now underway in a number of laboratories into the genetic basis of wiring specificity in

the antennal lobe of *Drosophila*. The first foray into the genetics of this system was reported by Komiyama *et al.* (2003). A candidate gene approach identified two POU domain transcription factors, abnormal chemosensory jump 6 (Acj6, previously mentioned in Section 1.12.3.2 in the context of ORN differentiation) and Drifter (Dfr) expressed in the anterodorsal and lateral PN lineages, respectively. Loss-of-function analysis, using the MARCM system to generate homozygous mutant clones in heterozygous brains, indicated that each gene is required for correct dendritic targeting of a subset of PNs in their respective lineages. Furthermore, reciprocal misexpression experiments (of Acj6 in the lateral lineage or Dfr in the anterodorsal lineage) caused dendrites to target inappropriate glomeruli, including glomeruli usually targeted by the opposite lineage. However, neither Acj6 nor Dfr misexpression generated a complete switch of dendritic targeting, even when the misexpression was coupled with removal of the endogenous transcription factor. The conclusion was that *acj6* and *dfr* are probably part of a transcriptional program, which generates discrete PN fates in different neuroblast lineages, perhaps in combination with the ubiquitous neuroblast transcriptional timer mechanism hypothesized above (Section 1.12.4.2.1). Finally, it should be noted that *acj6* mutant PNs also had axonal targeting defects, which are discussed below (Section 1.12.5.1).

1.12.4.3. Local Interneurons

Compared to the recent accumulation of information on ORNs and PNs, little is known in *Drosophila* about the development of local interneurons (LNs). Stocker *et al.* (1997) followed the expression of an enhancer trap GH298, which labels about 20 multiglomerular LNs; they first observed detectable expression at about 48 h APF and, then, in only about 10 cell bodies. Whether this represents late differentiation of LNs or simply late expression of this marker is unclear. Finer developmental time courses and additional enhancer trap lines will probably soon resolve this issue.

A little more information about the development of LNs is available in *M. sexta*. Antennal lobe development in *Manduca* is characterized by a clear distinction between two neuropil layers. A central core of the AL initially contains the processes of central neurons such as PNs and LNs. When ORN axons arrive, they are restricted to a superficial layer surrounding the antennal lobe, and it is in this layer that protoglomerular accumulations form. Although LNs, as detected either by single cell fills (Oland *et al.*, 1990) or by GABA immunoreactivity (Homberg and Hildebrand, 1994), have already arborized extensively by the time ORNs arrive, they initially remain restricted to the core neuropil, and only later invade the protoglomerular layer several days after the formation of contacts between ORNs and PNs.

Stocker *et al.* (1997) also used hydroxyurea treatment at different times in development to ablate dividing neuroblasts. As mentioned in Section 1.12.4.2.1, an early-dividing lateral neuroblast seems to generate lateral PNs. Since early larval application of hydroxyurea also prevented formation of LNs, the authors suggested that the same neuroblast may generate LNs and the lateral group of PNs. A more direct test, for example, clonal analysis using drivers expressed in both PNs and LNs, will be required to confirm this suggestion.

The authors are not aware of any developmental data concerning non-GABAergic LNs in *Drosophila*. However, a serotonergic neuron has been identified in a number of insect species including *M. sexta*, in which it seems to be born embryonically, or at an early larval stage, and persist right through pupal development into the adult (Kent *et al.*, 1987). A detailed developmental study suggests that these neurons show a similar developmental pattern to the GABAergic PNs described above (Oland *et al.*, 1995), i.e., early arborization within the central core, followed by later invasion of the protoglomerular layer.

1.12.4.4. Glia

As described in Section 1.12.2.2.2 above, glial processes wrap individual glomeruli (although the extent of the glial sheath differs markedly among insects) and separate the AL from other neuropil compartments. In *Drosophila*, glia seem less likely to play a significant role in glomerular development, since their processes only invade the AL around 48 h APF, when glomerular organization is largely mature (Jhaveri *et al.*, 2000b; Jefferis *et al.*, 2004). However, there is a ring of glial cell bodies surrounding the lobe, and these cells could provide an external cue, which might be of significance, for example, when ORNs first invade the periphery of the antennal lobe (see Section 1.12.4.1 above). Additional glia are generated after ORN arrival (Jhaveri *et al.*, 2000b), although it is not clear if gliogenesis is dependent on ORNs.

Work in *Manduca* has highlighted a significant role for glia in glomerular development. Before ORN arrival, glia form a border around the AL, which is several cell bodies thick. At pupal stage 4, when ORNs first enter the AL, glial cell processes start to extend into the lobe, and further glial proliferation begins (Oland and Tolbert, 1987). The

changes in glial cell structure (but not number, which is hormonally regulated; Kirschenbaum *et al.*, 1995) are dependent on ORN axon arrival, since they do not occur in deantennated lobes (Oland and Tolbert, 1987). In developmental stages 5 and 6, ORN axons coalesce into protoglomeruli, which are then rapidly invaded by PN dendrites; as this occurs, glial cell bodies migrate and processes extend further, partitioning off the newly forming glomeruli. Glia appear to be actively involved in this process of glomerular maturation, since γ-irradiation (Oland *et al.*, 1988) or hydroxyurea (Oland and Tolbert, 1988) treatment at stages 4 and 5 (after antennal and central neurogenesis are complete) prevents the normal large expansion in glial number and seems, consequently, to perturb glomerular development. Protoglomerular accumulations of ORNs dissolve rapidly in these glial deficient conditions, except in occasional locations, where there are enough glia to form a crude septa (Baumann *et al.*, 1996); there is also a consequent absence of LN dendritic tufts.

Do glia have a very specific scaffolding role during glomerular formation or are they additionally required for ORN targeting to the correct region of the AL? It would be rather interesting to know if the diffuse ORN terminations that persist after manipulations to prevent the normal expansion of glial populations show signs of spatial patterning; the ability to label ORN classes in *Manduca* might permit such an experiment.

Why should glia have a vital scaffolding role during glomerular formation in *Manduca*, while in *Drosophila* glial cell processes only invade the AL when glomerular formation is largely complete? One viable hypothesis might be the effect of glomerular size, which is much larger in *M. sexta*, where a typical glomerulus developing at stage 5/6 can vary from 20 to as much as 50 μm in diameter; 50 μm is about the size of the whole AL in *Drosophila* at 48 h APF, when glomerular organization is essentially complete. To give a numerical comparison, approximately 5000 ORNs terminate in a mature *Manduca* glomerulus versus an average of about 25 in *Drosophila*. Perhaps, the accumulation of a much larger number of ORN terminals in a *Manduca* protoglomerulus requires an additional support structure, in addition to adhesive interactions among axons.

Besides their role in protoglomerular stabilization, a second function for glia has been proposed in *Manduca*. Oland *et al.* (1998) have demonstrated that ORN axons show a dramatic reorganization at the point of entry into the antennal lobe, and identified a substantial glial population in this region. In this sorting zone, axons appear to defasciculate and selectively refasciculate with other ORNs, which then target to the same glomerulus. Rössler *et al.* (1999a) find that the generation of the glial sorting zone depends on central gliogenesis, which appears to be induced by ORN axons (in contrast to proliferation of the AL neuropil associated glia described above), and that in its absence (using γ-irradiation as above), selective refasciculation is disrupted and ORNs frequently overshoot the lobe completely.

1.12.4.5. Cellular Interactions in AL Development

Neuronal processes of sensory and central neurons must interact to form mature glomeruli. Exactly when and where this occurs and the significance of, for example, ORN–ORN interactions versus ORN–PN interactions, all remain important questions. In *Drosophila*, the developmental studies described above (Sections 1.12.4.1 and 1.12.4.2.2) suggest that PN patterning occurs early, that ORNs initially remain restricted to the exterior of the lobe, and that glia are relatively late participants in AL development. This has led Jefferis *et al.* (2004) to hypothesize that both PNs and ORNs may have substantial autonomous patterning ability. Generation of the final mature organization of the antennal lobe would result from the interaction of these two spatial maps. Initial coarse targeting by both populations could explain how specific sets of ORNs and PNs are able to find each other in the complex environment of the developing antennal lobe.

What cellular cues might be required for these initial ORN and PN targeting steps and subsequent glomerular formation? The logic of highly specific wiring of ORNs and PNs has been uniquely demonstrated in *Drosophila*, but experiments to address the significance of interactions between different cell types in AL development have been much more extensive in other systems. In particular, a lot of work has been done in *Manduca*, where surgical manipulations including deantennation, antennal disc transplantation, and ablation of certain PN populations, have proved possible, in addition to the glial ablation studies described above.

1.12.4.5.1. Influence of ORNs on AL development Antennal denervation before ORN ingrowth results in a failure of glomerular organization, although an AL of reduced size still forms (Hildebrand *et al.*, 1979). There seems to be a threshold effect: partial removal of the developing antenna indicates that if 12 or more of the normal 80 antennal segments form, then glomerular development will proceed relatively normally. The lobe will, of course, be much smaller, given that the ORN

number is much reduced from about 300 000 to, for example, 20 000 per antenna, when 12 antennal segments remain (Tolbert and Sirianni, 1990). Around this threshold, about two-thirds of the normal number of glomeruli form. When 30 antennal segments remained, essentially all glomeruli formed (Tolbert and Sirianni, 1990).

Similar deantennation experiments have been carried out in a wide variety of insects including *Periplaneta* (Salecker and Boeckh, 1996) and *Apis* (Gascuel and Masson, 1991), with each case reporting a loss of glomerular organization. Surgery of this type would be extremely difficult in *Drosophila*, but examination of mutants of the gene *lozenge* (*lz*) provides some comparison: lz^3 mutant antennae lack all basiconic sensilla and there is a loss of the glomerulus V, a major basiconic target, apparently because of a requirement for *lz* in the antenna (Stocker and Gendre, 1988). It would appear, therefore, that ORNs are strictly required for the formation of olfactory glomeruli. There also seems to be a requirement for maturation: ORNs reach the *Manduca* AL at stage 3, glomeruli are evident by late stage 5, but deantennation until stage 7 can cause a significant regression in glomerular organization, suggesting that ORN contact is required for at least 4–6 days (Tolbert and Sirianni, 1990). Salecker and Boeckh (1996) report a similar phenomenon in *Periplaneta*.

Glomerular organization is only one end point in the development of the AL; as discussed in the introduction to Section 1.12.4, spatial patterning may exist in the absence of morphologically discernible glomeruli. The consequence of deantennation on PN development was studied in *Manduca* by Malun *et al.* (1994), who found that PNs nevertheless extend dendrites into the outer regions of the lobe. At stages 5 and 6 when glomeruli would normally be forming, PN arbors appear broader and more diffuse, but they do form a glomerular termination, apparently analogous to a single early forming glomerulus at the entry to the AL. Even at the last pupal stage (18), single-filled PNs had sparser dendritic arbors than normal, but innervated a discrete portion of the AL, not substantially different in size from a normal glomerulus. These results suggest that, at least in *Manduca*, PN dendritic development, while clearly influenced by ORNs, is less dependent on ORNs than might have been expected. It is also interesting to note that PN dendritic arbors can be maintained without ORN partners. Perhaps this stabilization is due to contact with other neurons in the AL or with glia. Similar experiments have yet to be reported in other species, with the exception of Salecker and Boeckh (1996), who reported that mass stained PNs in *Periplaneta* seem to be more severely affected by deantennation. Dendritic arborizations were severely reduced and disorganized when deantennated embryos were examined at late embryonic stages (16 days after surgery). It would be interesting to know, in this case, whether PN dendritic arborizations ever form (i.e., a potential requirement for maintenance rather than elaboration), and whether single cell labeling studies might reveal any remaining signs of order.

1.12.4.5.2. Influence of ORNs on sexually dimorphic glomeruli If ORNs are necessary for glomerular formation, to what extent can they be considered sufficient? The sexually dimorphic glomeruli of *Manduca* have provided an interesting test case. In a classic experiment, Schneiderman *et al.* (1982) developed a surgical technique to transplant the larval antenna and its attached antennal disc. When a male antennal disc was grafted into a female host before pupariation, the resultant male ORNs were able to induce the male specific MGC in the otherwise female brain. In contrast, when a female disc was transplanted into a male host, no MGC developed. Rössler *et al.* (1999b) recently revisited this experiment. They were able to confirm the presence in 18/21 cases of all three MGC glomeruli, the cumulus and the two toroids. Indeed, in some gynandromorphs, the induced MGC was morphologically indistinguishable from the wild-type. In the case of female transplants into male hosts, Rössler *et al.* (1999b) were also able to demonstrate the induction of what appear to be the two lateral female specific glomeruli (LFGs).

How are postsynaptic PNs influenced by transplanted ORNs? While the cell number in the medial cluster of PN cell bodies is sexually dimorphic, with an additional 30 cells in males (Homberg *et al.*, 1988), this is not under afferent control, since no change in PN cell number was observed in either class of gynandromorph (Rössler *et al.*, 1999b). In contrast, female PNs of the medial cluster do innervate ectopic MGC glomeruli (Rössler *et al.*, 1999b). This raises a very interesting question: to what extent can the ectopic ORNs specify central connectivity of their partner neurons?

A follow-up to the original Schneiderman experiment had shown that some female moths with transplanted male antennae could show male-like behavior in response to female pheromone (Schneiderman *et al.*, 1986). It might be revealing to trace out the axonal projection pattern of PNs in gynandromorphs, and see whether their connectivity is characteristic of host sex or antennal sex. It is

interesting to note in this context that the Rössler *et al*. (1999b) study suggests that the PNs innervating the MGC and the LFGs may be to a certain extent homologous. In both males and females, these PNs develop particularly early. Furthermore, in the case of female into male grafts, the LFGs were very strongly innervated, perhaps by the extra 30 male specific PNs, while in male into female grafts, the MGC was comparatively weakly innervated. Perhaps MGC and LFG PNs have a rather similar dendritic branching specificity – the heterotypic transplant may "work," in part because of the existence of a molecularly similar postsynaptic population, in addition to any instructive ability of ectopic ORNs. Homology of sexually dimorphic glomeruli has also been proposed by Rospars and Hildebrand (2000) in a recent high-resolution study of the structure of the *M. sexta* AL; a third female specific glomerulus was identified, and the group of three female specific glomeruli were proposed to be directly homologous to the three MGC glomeruli.

1.12.4.5.3. Influence of PNs on AL development As discussed above, ORNs seem to be strictly required for glomerular formation, although perhaps not for dendritic arborization. What role then do projection neurons PNs play in the development of other cell types? The only significant attempt to address this question so far has been in *Manduca*. In a rather impressive feat of surgery, Oland and Tolbert (1998) were able to excise a large fraction, or in some cases all, of the PNs in the medial cell cluster, which may be composed exclusively of PNs (Homberg *et al*., 1988). Even in the case in which no medial PNs could be detected, glomerular development, in the region normally innervated by these PNs, appeared largely unaffected. In particular, ORN axons still formed protoglomeruli and proceeded to a mature glomerular organization. Although it is possible that PNs derived from the second major cluster (lateral) could substitute for medial PNs in these circumstances, Oland and Tolbert (1998) argued that they would arrive later in development, after ORN arrival. Therefore, from these experiments, it would seem that PN dendrites are unlikely to act as targets for incoming ORN axons, or provide a scaffold for other central neuron dendrites.

The experiments described above suggest that PNs may not be required for the development of other cell types in the AL. However, PNs may interact with each other during development. In *Drosophila*, PN dendrites are substantially patterned at 18 h APF and appear to be the major component of the developing antennal lobe, up until this point. This suggests that dendro-dendritic interactions among PNs may play a role in patterning of the AL (Jefferis *et al*., 2004). Experimental evidence to support this comes from studies on the role of the cell adhesion protein N-cadherin in PN development (Zhu and Luo, 2004). Single-cell MARCM clones revealed that PNs homozygous for a loss-of-function *N-cadherin* mutation no longer restrict their dendrites to single glomeruli. Interestingly, labeled wild-type single PNs, in the presence of unlabeled neuroblast clones homozygous for the *N-cadherin* mutation, exhibit these same dendritic overspilling phenotypes. This indicates that the PN–PN interaction mediated by N-cadherin is essential for limiting dendritic targeting of each PN to a single glomerulus.

1.12.4.6. Development of the Hemimetabolan AL

The sections above have focused on *Drosophila* and *M. sexta*, both holometabolous insects. How does the development of hemimetabolous insects compare? In general, one would expect that by the end of embryonic development, the hemimetabolan AL would be functionally almost equivalent to the adult; sexually dimorphic functions might be a possible exception to this rule. However, the size and innervation of the antennal lobe must be very different at these two stages. At each new molt, new antennal segments are added (Rospars, 1988), so that OR number in *Periplaneta* increases from 14 000 at the first larval molt (Schaller, 1978) to more than 200 000 in the mature adult (Section 1.12.2.3.3). The glomeruli, however, do not increase in number. For example, in *Blaberus craniifer*, 109 glomeruli can be counted at all larval stages to adulthood (Rospars, 1988). While the ORN number clearly increases greatly, neurogenesis of antennal lobe neurons seems to occur solely during embryogenesis, suggesting that the number of PNs leaving the antennal lobe is likely to be fixed. This is in contrast to the MBs, where, for example, in *Periplaneta*, neurogenesis continues through successive nymphal stages leading to a massive increase in MB neuron number, paralleling the increase in ORNs (see Section 1.12.5.4.1 below).

Among hemimetabolous insects, the most detailed work on the initial development of the antennal lobe glomeruli has been reported in *Periplaneta* (Salecker and Boeckh, 1995). The sequence of events during the 31 days of embryonic development can be described as follows. At stage E6, neurogenesis is evident in the deuterocerebrum, and there are already signs of a fine neuropil. By E10, the neuropil of the future antennal lobe is surrounded

by a glial layer. This is also the stage, when the first ORN axons enter the antennal lumen and begin their journey towards the CNS. By E12, an antenno-cerebral tract (ACT) is visible, indicating that PN differentiation is proceeding rapidly. At E15, ORN axons enter the AL neuropil, by E18 irregular, darker staining structures can be seen in the periphery of the AL, and at E19, the first spherical glomeruli are apparent, with most glomeruli being evident by E22. Glial cell processes invade the lobe at E18/19, just as the first glomeruli are forming. By E26, mature glomerular structures with a glial wrapping are evident.

1.12.5. Development of Higher Olfactory Centers: Mushroom Body and Lateral Protocerebrum

1.12.5.1. Projection Neuron Axons

In *Drosophila*, embryonically born Projection neurons (PNs) appear to be the functional projection neurons in larvae. When examined in wandering third instar larvae, each embryonically born PN not only sends dendrites into the larval AL, but also its axons into the larval calyx, and beyond, into the LPR (E.C. Marin and L. Luo, unpublished data).

Axons from larva-born projection neurons can be recognized as early as 72 h APF (Jefferis *et al.*, 2004). However, even at the wandering third instar larval stage, these axons, although extending to the MB calyx region, do not extend within the calyx nor do they enter the lateral protocerebrum. This target innervation occurs in the first 50 h APF.

Among all classes of PNs, the development of the DL1 class has been studied most extensively, as it alone can be precisely identified by its birth time. In the absence of suitable class-specific enhancer trap lines, other PNs must be identified by the glomerular target of their dendrites, which are not identifiable until 50 h APF (see Section 1.12.4.3). In adults, each DL1 neuron exhibits a stereotyped branching pattern within the LH (Marin *et al.*, 2002). As soon as the axon enters the lateral horn, there is a bifurcation, giving rise to a major branch extending continuously laterally towards the lateral edge of the LH, and a dorsal branch projecting to the dorsal-medial corner of the LH. When and how does this branching pattern develop? Time-course analysis of single cell MARCM clones (Jefferis *et al.*, 2004) indicates that the lateral main branch develops first, reaching the lateral edge of the lateral horn before 24 h APF. In the next 6 h or so, the dorsal branch extends as a collateral. The DL1 axon branches in the MB calyx can be visualized at 12 h APF as sprouts, and are resolved into distinct branches at 18 h APF. In both the MB calyx and lateral horn, axon branches can be seen to exhibit numerous filopodial structures in early pupa; however, stable branch structures resembling those of adults are evident at 50 h APF, before the first OR expression can be detected. This would suggest that the establishment of PN axon connection specificity is independent of patterned sensory input.

A first step has been made in the genetic analysis of these stereotyped PN axonal projection patterns. Komiyama *et al.* (2003) found that in homozygous *acj6*$^{-/-}$ PNs of the DL1 class, the highly characteristic dorsal branch in the lateral horn is absent or greatly shortened, whereas the lateral branch is largely unaffected. Expression of an *acj6* rescue transgene, specifically in these mutant PNs, largely rescued this axonal targeting defect. In contrast, expression of Drifter (Dfr) in *acj6*$^{-/-}$ PNs qualitatively enhanced the phenotype, leading in some cases to novel axonal termination patterns. These results suggest two interesting hypotheses: (1) that the specific dendritic and axonal connectivities of PNs may be coregulated by a common transcriptional control system; and (2) that similar downstream effector molecules (such as cell surface proteins) may participate in dendritic and axonal wiring specificity in the antennal lobe and lateral horn, respectively.

1.12.5.2. Mushroom Body Neurons

1.12.5.2.1. Neurogenesis In *Drosophila*, mushroom body (MB) neurons originate from four neuroblasts per hemisphere (Ito and Hotta, 1992), which begin to divide at embryonic stage 9 (of 17) (Noveen *et al.*, 2000). Initial rounds of division generate approximately 60–80 neurons per hemisphere by stage 14 (Noveen *et al.*, 2000); this indicates that each neuroblast is dividing about once per hour. At this point, most other neuroblasts (approximately 80 in the central brain) show a clear pause in proliferation between the embryonic and larval stages (Ito and Hotta, 1992). However, the four MB neuroblasts (and one additional neuroblast located lateral to the antennal lobe) are unusual, in that they divide continuously throughout embryonic and larval development (Truman and Bate, 1988; Ito and Hotta, 1992). The total number of MB neurons generated during embryogenesis has not been accurately determined, but must be less than 300 neurons per hemisphere, since that is the number of axons that can be observed in the peduncle in the first instar larvae (Technau and Heisenberg,

1982). Furthermore, MB neuroblasts are unique in continuing to proliferate beyond a general cut-off of about 30 h APF – they only eventually terminate division at about 90 h APF, shortly before eclosion as an adult (Ito and Hotta, 1992). Indeed, in other insect species including the cricket, *Acheta domesticus*, mushroom body neurogenesis has been found to persist even into the adult (Cayre *et al.*, 1996).

What are the final products of these four neuroblasts? Further studies by Ito *et al.* (1997) showed that each neuroblast generated an apparently identical clonal unit of approximately 500 neurons in the adult, for a total of 2000 per hemisphere, which is consistent with earlier studies (e.g., Technau and Heisenberg, 1982). Each clonal unit consists exclusively of intrinsic MB neurons (Ito *et al.*, 1997) and a number of glial cells near the MB neuron cell body region (Ito *et al.*, 1995).

By a combined single cell labeling and clonal analysis technique, Lee *et al.* (1999) were able to demonstrate that during larval and pupal development, each neuroblast gives rise sequentially to the three major classes of MB neuron postulated by Crittenden *et al.* (1998) (introduced in Section 1.12.2.2.4 above). During the first 3 days of larval development, the neurons innervating the adult γ lobe are born. Neurons that will innervate the adult α' and β' lobes are born between 3 days and shortly before puparium formation (4.5–5 days after larval hatching). Finally, during pupal life, neurons that will innervate the larval α and β lobes are born (**Figure 13**).

Embryonically born MB neurons are most likely γ neurons similar to those born in early larvae. This is based on their sharing similar GAL4 lines (such as GAL4-201Y, which is γ neuron specific until late pupa) and their similar axon pruning behavior during metamorphosis (see Section 1.12.5.4.3). This sequential generation of different MB neuronal types turns out to be a general phenomenon, which has been observed in *Apis* (Farris *et al.*, 1999), *Periplaneta* (Farris and Strausfeld, 2001), and *Acheta* (Malaterre *et al.*, 2002).

It is generally thought that, in the *Drosophila* CNS, the cell division pattern is such that a neuroblast undergoes asymmetric division, generating a new neuroblast and a ganglion mother cell, which divides once more to give rise to a pair of postmitotic neurons (**Figure 10**). This scheme of cell division has rarely been rigorously proven, but in MB neuroblasts it is most certainly the case, at least for divisions shortly after larval hatching. When applying the MARCM technique in conjunction with a GAL4 line that appears to label all MB neurons (GAL4-OK107), three types of clones have been observed. Neuroblast clones and two-cell clones are generated at the same frequency, reflecting the random segregation of the GAL80 repressor to one of the two daughter cells during neuroblast division. Single cell clones, presumably generated during ganglion mother cell division, are also observed but at a different frequency (**Figure 11** and Lee *et al.*, 1999). As development proceeds, the frequency of neuroblast/two-cell clones decreases compared to single cell clones, likely reflecting changes of accessibility to Flp/FRT mediated mitotic recombination due to changes in cell cycle length. Ito *et al.* (1997) estimated that for the four MB neuroblasts to generate 500 MB neurons, they need to divide on average every 55 min, continuously from newly hatched larva to late pupa. Perhaps, because of this demand and the relative ease with

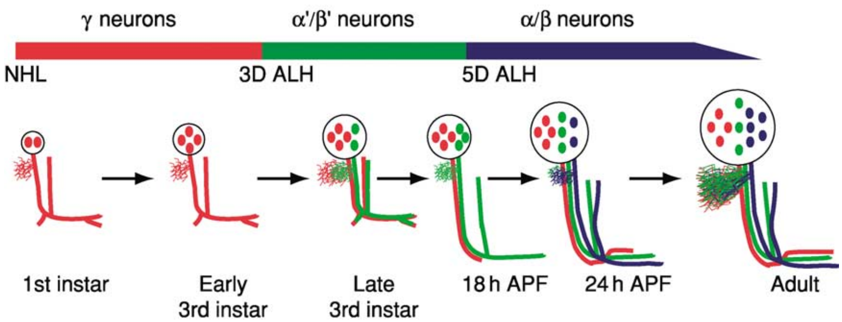


Figure 13 Development of *Drosophila* MB neurons. Each MB neuroblast divides asymmetrically over the course of embryonic to late pupal life. During that time, three distinct major classes of MB neurons are generated, which are named for the adult MB lobes that their axons innervate. Thus, γ neurons are born during embryonic and early to mid larval stages and constitute the larval MB, then α'/β' neurons are born and, finally, α/β neurons. Note that γ neurons initially extend two branches during larval stages, but subsequently prune back both the dorsal and medial branches of axons as well as all dendrites, before re-extending adult-specific dendrites and axon branches only to the medial γ lobe. NHL, newly hatched larva; ALH, after larval hatching; APF, after puparium formation. (Adapted from Lee, T., Lee, A., Luo, L., **1999**. Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development 126*, 4065–4076.)

which defects in MB neuroblast proliferation can be identified by looking at axonal projection patterns in the adult, mutations in many genes have been linked to MB proliferation, including those previously identified based on miniature MBs (Melzig *et al.*, 1998; Guan *et al.*, 2000); Rho GTPases and other actin cytoskeleton regulators (Awasaki *et al.*, 2000; Lee *et al.*, 2000b; Billuart *et al.*, 2001); microtubule motors and their regulators (Liu *et al.*, 2000); genes implicated in chromatin remodeling (Scott *et al.*, 2001a); members of the ubiquitin proteasome system (Watts *et al.*, 2003); and ~1 in 10 chromosomes treated with a moderate dose of ethyl methane sulfonate (EMS) (Reuter *et al.*, 2003). In several cases, neuroblast proliferation defects have been more rigorously proven using BrdU incorporation experiments (Lee *et al.*, 2000b; Liu *et al.*, 2000; Scott *et al.*, 2001a).

1.12.5.2.2. Morphogenesis Because of the complex morphological development of different classes of MB neurons and the ease with which MARCM analysis can be carried out, MB neurons in *Drosophila* have been used as a model system to study different aspects of neuronal morphogenesis. These studies have contributed to our general understanding of morphogenetic mechanisms that can be applied to neurons in other structures and in other organisms. Here only those studies are summarized that reveal special features of the morphogenesis of the MB neurons themselves.

Like most invertebrate CNS neurons, MB neurons in *Drosophila* are unipolar. To recap their structural features described in Section 1.12.2.2.4 above, each neuron extends a single process from the cell body. Shortly after leaving the cell body, the process gives off several collaterals, which extend and generate further branches, giving rise to the calyx or dendritic field of MB neurons. The main process continues to extend anteriorly into the peduncle and becomes the axon (**Figure 5**). Given that MB neurons come from four neuroblasts, do they give rise to four separate units, or are processes from these four neuroblasts intermingled? Zhu *et al.* (2003) found that in wandering third instar larvae, dendrites from each neuroblast clone occupy the entire calyx, indicating that dendrites from the four clonal units intermingle. However, after pruning and re-extension during metamorphosis (see Section 1.12.5.4.2), γ neuron dendrites from each neuroblast clone occupy one-quarter of the dendritic area occupied by the entire γ neuron population, and pupal born α/β neurons likewise obey their clonal boundary, so that the calyx exhibits a fourfold symmetry, with dendrites of MB neurons from one neuroblast occupying one-quarter of the field. There appear to be further divisions within each quadrant, with γ dendrites occupying the border and α/β dendrites occupying the center (Zhu *et al.*, 2003). The functional significance of these different patterns of innervation of the calyx may be revealed by detailed comparison with the termination of afferent projection neuron axons.

In contrast, axons from four clonal units are intermingled shortly before entering into the peduncles in both larvae and adult (Ito *et al.*, 1997). The sequential generation of neuronal types also has its analog in the layered organization of axons in the peduncle and lobes. Results using either a slow maturing red fluorescent protein (Verkhusha *et al.*, 2001), or birth-dating studies with the MARCM system (Kurusu *et al.*, 2002) have demonstrated that in larval lobes, newly born MB neurons project their axons into the core, while peripheral layers contain earlier born neurons. This layered structure is most easily observed in peduncles, but it is also preserved in the two larval lobes; this concentric organization gradually evolves into the adult organization with two separate dorsal lobes and three separate medial lobes.

One of the most prominent morphological features of most MB neurons is their bifurcated axonal structure, with prominent branches in a dorsal and medial lobe. In the embryo, this appears to arise by collateral formation, as Noveen *et al.* (2000) demonstrated using 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl lindocarbocyanine (DiI) labeling of embryonic MB neurons. At embryonic stage 14 (~11 h out of 22 h of embryonic development), a rudimentary peduncle is evident. By stage 16, ~3 h later, a distinctive right angle turn is evident, as axons change course forming the medial lobe. Only by late stage 17 (~ 20 h of embryonic development) is the dorsal lobe evident. Since these were mass labeling studies, it does remain possible that there is an early embryonic MB neuron population that is unbranched and innervates the medial lobe only, which is followed later in embryonic development by a class of neurons with bifurcated axons. Single cell labeling could rule out this alternative explanation.

Does axonal branching in the adult follow a similar logic? The clonal analysis studies of Lee *et al.* (1999) found no signs of asynchronous development of the two axon branches, either of larval neurons or of adult specific α′/β′ or α/β neurons. However, for technical reasons, it was not possible to visualize single cells earlier than 24 h after their birth, so it remains possible that a collateral branching event could occur within this window. A more recent study has provided a possible molecular insight

into the branching process (Wang *et al.*, 2002). A mutation with defective MB branching was isolated in a mosaic screen (in which a small number of homozygous mutant MB neurons are inspected in an animal, which is otherwise heterozygous for the mutation and probably phenotypically wild-type). In this mutant, which was identified as an allele of the Dscam axon guidance receptor (Schmucker *et al.*, 2000), unbranched γ neurons appeared unaffected, but branched α/β neurons showed more branching than wild-type. The excess branches of these single cell mutant clones also distributed themselves randomly between the α and β lobes, rather than distributing themselves evenly between the lobes. Thus, it was postulated that Dscam may mediate a repulsive interaction between sister branches and, in so doing, ensure these enter different lobes, thereby preventing multiple branch formation (Wang *et al.*, 2002). This interpretation makes most sense if α/β neuron axon branching occurs through growth cone splitting rather than collateral branching, a notion yet to be proven.

Further insight into axon branching was obtained in the analysis of Rac (related C3 botulinum toxin substrate) GTPases in MB axon development. Rac GTPases were found to be essential for axon growth, guidance, and branching (Ng *et al.*, 2002a). In neuroblast clones, where hundreds of neurons are homozygous mutant for a given combination of Rac GTPases and exhibit branching defects, it was found that all mutant axons enter either dorsal or medial lobes. They almost never distribute themselves into two populations entering two different lobes, as if the axons were making their decisions together. The existence of such a "community effect" was further supported by the finding that, often, homozygous mutant axons could cause their heterozygous (and phenotypically wild-type) neighboring axons to exhibit the same guidance or branching errors; conversely, the presence of wild-type axons could, to some extent, reduce the error rate of mutant axons.

MB neurons were used to demonstrate the notion that there are dedicated signal transduction pathways for controlling axon branch stability (Billuart *et al.*, 2001). When a negative regulator (p190RhoGAP) for an axon retraction pathway involving small GTPase RhoA is inhibited, MB axons undergo retraction. Interestingly, the dorsal lobes are preferentially retracted compared with the medial lobes; the mechanism for this selective sensitivity is unknown. The activity of p190 RhoGAP could be modulated by integrin, previously shown to be required for MB mediated memory (Grotewiel *et al.*, 1998), so the retraction pathway could be used in the structural plasticity that underlies memory (Billuart *et al.*, 2001). In this regard, it is noteworthy that the dorsal lobe has been especially implicated in mediating long-term memory (Pascual and Preat, 2001).

1.12.5.3. Lateral Protocerebrum

Given that relatively little is known about the cellular components of the LH, and that those neurons that have been identified are quite heterogeneous, it is unsurprising that our knowledge of LPR/LH development is very limited. The existing information centers essentially exclusively on the afferent antennal lobe projection neurons, which was summarized in Section 1.12.5.1 above.

1.12.5.4. Variations

1.12.5.4.1. Development of other MBs The development of the MBs has been investigated more intensively in *Drosophila* than any other insect species. However, important results have been obtained in the cricket and the cockroach (as examples of holometabolous insects) and the honeybee (where the MBs are numerically almost three orders of magnitude more complex), among others. There are many developmental similarities to *Drosophila*, including the extended period of MB neurogenesis compared to the rest of the brain (*Acheta*: Cayre *et al.*, 1996; *Apis*: Farris *et al.*, 1999; Ganeshina *et al.*, 2000); a layered birthdate organization arising from cell bodies that are pushed away from the center by newly born MB neurons (Farris *et al.*, 1999); and the development of axons (i.e., lobes) preceding dendrites (calyx) (Farris *et al.*, 1999).

Despite the similarities in MB development between these species, there are also substantial differences. Comparing, first of all, *Apis* and *Drosophila*, one striking difference is the absence in *Apis* of any embryonic development of the mushroom body. Fly larvae are born with small but functional mushroom bodies, while it is the third day (out of 8) of larval development in bees before any MB neuropil can be observed (Farris *et al.*, 1999). It has been suggested that this relative timing difference between the two species may be due to their different larval lifestyles (Farris *et al.*, 1999). Fly larvae must immediately fend for themselves and locate food sources, whilst bee larvae are fed by nurse bees within the colony. This late generation of MB neuropils correlates with the different patterns of neurogenesis in *Apis* (Farris *et al.*, 1999). At the peak of neurogenesis, around the seventh day of larval development, there are 1000 active neuroblasts in each hemisphere, evenly divided between a medial and a lateral cluster. This large number of neuroblasts (Nbs) is presumably

required to generate the huge mushroom bodies of the adult bee, which consist of some 170 000 neurons (see Section 1.12.2.3.3 above). This late population of neuroblasts originates from an earlier population of 90 or fewer neuroblasts per hemisphere (i.e., 45 per cluster), which are present in the newly hatched larva. Again, in contrast to *Drosophila*, there is initially almost no asymmetric division of these neuroblasts; rather, there are symmetric divisions that expand the neuroblast population. The vast majority of neurons in *Apis* are, therefore, generated late in development from this larger Nb population.

There are also differences in the morphogenesis of the MBs, with significant growth of the lobes only evident in bees on larval day 4. From this point on, however, growth seems to be one continuous process (Farris *et al.*, 1999) without any obvious discontinuity in the early pupal stage comparable with the pruning of the embryonic and larval born γ neurons observed in *Drosophila* (Technau and Heisenberg, 1982; Lee *et al.*, 1999). This may again reflect the absence of a significant larval requirement for higher olfactory function in bees.

The development of MBs in *Periplaneta* (a hemimetabolous insect) is more likely to represent the evolutionarily basal state. In cockroaches, as in *Drosophila*, a small MB is already fully formed by the end of embryogenesis. Lifestyle considerations would, of course, require this, since the environment of the cockroach nymph is largely that of the adult. MB neurogenesis proceeds through 9–11 nymphal instars and there is a corresponding steady integration of MB neurons into the fully functional MB. Adult cockroach MBs have an obvious laminar structure; each lamina is composed of a doublet with, for example, different neurotransmitter levels, and laminar divisions are respected by many efferent extrinsic neurons, suggesting that they may be of functional significance (Li and Strausfeld, 1997). Interestingly, these laminae appear serially over development (Strausfeld and Li, 1999), and an integration lamina at the posterior margin of the lobe is constantly regenerated at each nymphal stage by the arrival of more immature MB neurons (Farris and Strausfeld, 2001). This mode of development may allow a complex MB structure to be built up gradually, in comparison to the small number of sharper transitions seen in *Drosophila* development (Farris and Strausfeld, 2001).

1.12.5.4.2. Developmental plasticity: metamorphosis As described earlier (Section 1.12.5.2.1 above), *Drosophila* MBs are in essentially continuous development, from the mid embryo until late pupa. During this time, a substantial reorganization of the whole brain occurs, as pupal metamorphosis proceeds. Early electron micrograph studies by Technau and Heisenberg (1982) suggested that there was an abrupt 40% reduction in the number of axons in the MB peduncle during the first day of pupal development. The number of MB neuron cell bodies did not change substantially at this time, suggesting that axons were pruned, while their cell bodies remained alive. A more recent study using enhancer traps labeling different subsets of MB neurons has confirmed that large-scale reorganization takes place (Armstrong *et al.*, 1998). Furthermore, this study demonstrated that different lobes of the adult MB have different origins during metamorphosis. For example, hydroxyurea, which is toxic to dividing cells, was fed to newly hatched larvae, resulting in arrest of the five neuroblasts (Nbs) dividing at this time and, consequently, almost complete absence of the normal MB neuron populations. The small number of remaining neurons were assumed to be of embryonic origin and projected solely to the γ lobe; indeed, the vertical lobes were completely absent. Thus, it would seem that the γ lobe is specific to early-born neurons. The subsequent analysis of Lee *et al.* (1999) using the MARCM system confirmed and extended these findings with single-cell resolution. The first-born neurons, γ neurons, were found to degenerate extensively at metamorphosis, so that by 18 h APF their bifurcated axons had been pruned back to their original branch point at the end of the peduncle; this retention of the peduncle is in contrast to the earlier findings of Technau and Heisenberg (1982), who concluded that peduncular axons were also pruned. In contrast, the two later born classes (α′/β′ and α/β) showed no axonal reorganization at metamorphosis. Re-extension was rapid, with regenerated axons extending substantially by 24 h APF and extension appearing complete by 36 h APF (**Figure 13**).

What is the molecular basis of this axonal pruning? Metamorphic processes in many insects are triggered by the steroid hormone 20-hydroxyecdysone (termed hereafter ecdysone). This is released in specific pulses from the prothoracic gland, binds to a receptor protein, the ecdysone receptor (EcR), in conjunction with a coreceptor, ultraspiracle (Usp), and thereby triggers transcriptional changes associated with metamorphosis (review: Thummel, 1996) (see also **Chapter 3.1**; **Chapter 3.5**; and **Chapter 2.4**). A forward genetic screen for mutants that failed to undergo MB γ neuron remodeling, and a candidate gene approach, converged on the Usp coreceptor, indicating that this event is indeed

controlled by ecdysone (Lee *et al.*, 2000a) (see also **Chapter 2.4**). The *usp* mutant neurons failed to remodel, but retained their larval branching pattern. Since this approach used the MARCM system, in which only small clones of labeled cells were mutant, this requirement for Usp is clearly cell autonomous, i.e., it is required for a transcriptional program directly in MB neurons. Why is it specifically the γ neurons, rather than the coexisting α′/β′ neurons, that remodel? This same study also identified the ecydsone receptor subtype B1 as the relevant Usp partner, and demonstrated that it was expressed in γ neurons, but not in α′/β′ neurons.

Working further upstream, Zheng *et al*. (2003) showed that transforming growth factor beta (TGF-β) signals received by Baboon (Babo), a type 1 receptor (see Shi and Massague, 2003 for a review of TGF-β signaling), and dSmad2 its transcriptional effector, are required for high-level expression of EcR-B1 and for γ neuron remodeling; the cognate ligand is likely to be dActivin. EcR-B1 only turns on in γ neurons in mid-third instar, shortly before the ecdysone pulses that trigger the first events of metamorphosis, but after the last of the pulses that trigger molting between larval stages. It is an attractive hypothesis that a TGF-β signal might temporally restrict *EcR-B1* expression; however, the identified signaling components are not expressed with the appropriate temporal specificity. Interestingly, although Baboon, the type 1 receptor, was necessary for remodeling and *EcR-B1* expression, an ectopically expressed dominant active receptor was not sufficient to activate *EcR-B1* expression. Thus, MB neurons may have some temporal specificity, of unknown mechanism, in their ability to respond to a TGF-β signal.

The studies described above focus on signals that initiate MB γ neuron pruning. What is known about the execution phase? Watts *et al*. (2003) have described the cellular events of pruning. They conclude that axonal pruning occurs by a process of local degeneration, rather than axonal retraction, in which γ neuron axons start to fragment at 8 h APF. By 18 h APF most of the cellular debris has been cleared, and the neuron is ready to begin re-extending a new axon. What molecular events might underlie this process? Watts *et al*. found two clues. Following the expression of a number of different cellular markers in degenerating γ neurons, they noted that microtubule destabilization preceded any change in axonal morphology, suggesting that this may be an early step in pruning. They also identified the ubiquitin proteasome system as a key regulator of pruning. Overexpression in MB neurons of a yeast ubiquitin protease, UBP2, which should oppose the ubiquitination process, dramatically decreased pruning, so that γ neurons retained their larval branches in the adult. Similarly, loss-of-function mutations in the E1 ubiquitin activating enzyme Uba1 and the proteasome components Mov34 (Moloney leukemia virus-34 proviral integration homolog) and Rpn6 (regulatory particle non-ATPase6) also autonomously blocked pruning. In relation to the earlier results concerning initiation of axon pruning, Watts *et al.* also demonstrated that these mutants did not interfere with normal expression of EcR-B1 in γ neurons. It would, therefore, seem that the requirement for the ubiquitin proteasome system is downstream of the ecdysone signaling and closer to the point of execution. Intriguingly, both the requirement for ubiquitin proteasome system function and the early destabilization of microtubules have recently been demonstrated in the process of Wallerian degeneration, which occurs when mammalian axons are severed (Zhai *et al*., 2003). Thus, MB pruning may provide a powerful model system to study axon degeneration, one of the hallmarks of many neurodegenerative diseases.

In conclusion, in *Drosophila*, pruning of the axons and dendrites of the early-born γ neurons allows them to transition from their larval to adult function. Substantial progress has already been made in understanding both the cellular mechanism, which seems to be a local degenerative process, and the underlying molecular mechanisms, which include TGF-β signaling, ecdysone signaling, and the ubiquitin proteasome system. Given the widespread use of ecdysone in insects and some molecular parallels with vertebrates in the execution phase, these insights are likely to be of general relevance.

1.12.5.4.3. Adult plasticity Many insect neurobiologists, and certainly vertebrate neurobiologists, when thinking of insect nervous systems, have a tendency to emphasize the degree to which the nervous system undergoes extremely stereotyped, precisely genetically programed development. However, there have been numerous reports of quite substantial plasticity in insect brains, especially in the MBs.

Structural plasticity can be considered at different levels, from neurogenesis to remodeling of neuronal arbors, to synaptic plasticity, which presumably requires changes evident at the molecular level, if not at the level of cellular morphology. For example, during the well-studied process of olfactory learning in *Drosophila*, it is generally assumed that there must be synaptic potentiation at MB synapses (see **Chapter 3.15**). However, whether potentiation

occurs at input or output synapses is admittedly less clear. However, other forms of structural plasticity that have been observed are inherently rather more surprising.

In *Drosophila*, there have been numerous studies relating experience during development, or as an adult, to the status of the MBs. For example, Heisenberg *et al.* (1995) report that larval crowding can actually cause an increase in MB neuron number of about 20%, and a corresponding volume change in the calyx. These changes are not just confined to development, since neuropil volume changes were also reported in adult animals. For example, differences in MB calyx volume were described for pairs of flies reared together in the second week of adult life: same sex pairs had larger calyces. While these changes are reproducible, their behavioral significance is often somewhat obscure.

A series of studies in the honeybee gives an example of structural plasticity with a more obvious behavioral relevance. An initial report indicated that the volume of MB neuropil was some 15% greater in forager bees than in 1 day old adults (Withers *et al.*, 1993); somewhat curiously, the cell body volume actually decreases in these older animals, so that there is a substantial change in the ratio of MB neuropil to cell body volume. Honeybees transition through several behavioral stages from newly eclosed adult to nurse bee (remaining in the hive and caring for larvae), to foragers (leaving the hive and foraging for food). The neuropil/cell body ratio is also greater in foragers than in nurse bees, suggesting the possibility that these changes may be directly related to a bee's behavioral status (caste). However, since foragers in a normal colony are typically a week older than nurse bees, the effect of age must be separated from caste. By generating a synchronized colony, Withers *et al.* (1993) were able to confirm that these volume differences were still present in age-matched nurse and forager bees. Subsequent results have indicated that changes in neuropil volume are not dependent on neurogenesis (Fahrbach *et al.*, 1995) and have both experience-dependent and independent components (Fahrbach *et al.*, 1998). At the cellular level, a recent report suggests that the neuropil volume changes can be related to the morphology of individual MB neurons: in older animals and even in age-matched foragers compared with nurse bees, MB dendrites were longer and more highly branched, especially in regions of the calyx receiving visual input (Farris *et al.*, 2001).

In contrast to the strictly morphogenetic changes just described, in the cricket, *A. domesticus*, it has been reported that neurogenesis persists in the adult (Cayre *et al.*, 1996). This appears to be of functional significance, since the same group has more recently reported that sensory input can modulate this adult neurogenesis. Under enriched conditions, females that were reared in large cages, could hear male songs and smell environmental odors; impoverished conditions included solitary rearing in small cages in the dark. A 25% reduction in MB neurogenesis, as assayed by BrdU incorporation, was noted in females in impoverished conditions compared with those from the enriched environment (Scotto Lomassese *et al.*, 2000). This effect can still be observed in the absence of the corpora allata (their product, juvenile hormone, is known to have a neurogenic effect) (Scotto-Lomassese *et al.*, 2002). Finally, in a nice experiment, unilateral visual deprivation (by painting over one eye) led to a significant but unilateral decrease in neurogenesis (Scotto-Lomassese *et al.*, 2002). This suggests that the neurogenic effect may be a direct result of neuronal activity levels rather than being mediated, for example, by a centrally released hormone.

1.12.6. Conclusions

1.12.6.1. Among Insects

This review has focused, to a great extent, on the development of the olfactory system in *Drosophila*. To what extent are developmental mechanisms conserved among different insect species? The basic unit of the peripheral olfactory system, the sensillum, is structurally extremely conserved, and it is to be expected that the details of the developmental mechanisms will also show conservation. Even the typical differences between holometabolous and hemimetabolous insect development may not be so great, when considered at this level. Will the molecular findings of expression of a single specific olfactory receptor (OR) in different ORNs and the convergence to individual glomeruli be generalizable? Again, the authors would predict yes; the pheromone detection system of moths (see **Chapter 3.15**) gives an example, in which this seems very likely to be the case. For example, in *Agrotis segetum*, ORNs responding to two identified pheromone components project to discrete glomeruli in the MGC; an inhibitory compound is detected by ORNs projecting to a third MGC glomerulus (Hansson *et al.*, 1992). Furthermore, the findings from AL imaging studies suggest that the nature of olfactory coding is similar among different insect species (Section 1.12.2.1.2). Another specific case is the glomerulus V, which is CO_2-responsive in *Drosophila* (M. de Bruyne, unpublished data).

CO_2-responsive neurons of the labial pit organ in *M. sexta* also project to a single, distinctive ventral glomerulus in the AL. Interestingly, in *Drosophila*, the CO_2-responsive neurons are on the third antennal segment (de Bruyne *et al*., 2001). Thus, in this instance, the central target of a particular ORN class may be more conserved than its peripheral location. It should be noted that uniglomerular projections of ORN classes cannot be completely universal, given the anatomy of the locust system (Section 1.12.2.3.2).

The development of the antennal lobe and, in particular, AL glomeruli offers another interesting point of comparison. Although the antennal lobe in holometabolous and hemimetabolous insects develops at completely different times, similar principles have been noted: for example, the requirement of ORN axons for glomerular formation and the extension of glia or glial processes into the antennal lobe during glomerular formation. There are, however, likely to be numerous differences. Thus, for example, extension of glial processes into the lobe appears to occur much later in *Drosophila* than in *Manduca* and *Periplaneta*. Similarly, in *Manduca*, ORNs form protoglomeruli apparently without associating with other cell types, while in *Periplaneta*, ORN axons seem to develop in association with dendrites of central neurons. In *Drosophila*, protoglomerular accumulation of ORNs may exist, but the resolution of existing studies is not high enough to confirm or contradict this. Finally, the glial sorting zone identified at the entry to the developing *Manduca* AL has yet to be observed in other insects. Such a sorting zone may be particularly important given the rapid arrival of up to 300 000 ORNs in *Manduca*; there are only 1300 ORNs in *Drosophila*, while the AL of *Periplaneta* is the target of a similar number of ORNs as for *Manduca*, but these ORNs arrive over months of embryonic and nymphal development. If such issues do arise because of differences in the scale and intensity of development, it is possible that they might also be present in the development of vertebrate olfactory systems: *Manduca* glomeruli are quite comparable in size to rodent glomeruli, albeit about 10- or 20-fold less numerous.

At this stage, the general significance of the *Drosophila* observations on prespecification/sequential generation of different PN types (Section 1.12.4.2.1) is not certain. The authors would strongly suspect the existence of such mechanisms in other insects, both because of an expected conservation of basic developmental mechanisms and because this is an elegant way to allow the persistence of labeled olfactory lines deep into the nervous system.

In the MBs, as mentioned in Section 1.12.5.4.1, sequential generation of different cell types is also a common theme. Not that much is yet known about the functional significance of these different cell types, although different MB lobes (and consequently MB neurons born at different times) have been linked to specific memory processes in *Drosophila* (e.g., Pascual and Preat, 2001). From a comparative stance, the development of axon lobes and then dendritic calyx, and a concentric arrangement of cell bodies according to birthdate, have been identified by Farris and Strausfeld (2001) as common developmental themes among both holometabolous and hemimetabolous insects. As usual, however, the more gradual development of the latter does have consequences, for example, in the laminar organization of cockroach MBs (Section 1.12.5.4.1).

In conclusion, the insect world encompasses an enormous variety of olfactory systems. It could be argued that the structure and organization of peripheral sensory appendages (but perhaps not individual sensilla) is most variable among different species, while the logic of antennal lobe organization is very similar. While there may be substantial differences in scale and some differences in the timing of the cellular events accompanying olfactory development, mechanisms are likely to be broadly conserved and the advent of molecular studies will presumably only strengthen this impression.

1.12.6.2. Future Directions

The last few years have witnessed substantial advances in our knowledge of olfactory system development in insects, particularly *Drosophila*. The identification of olfactory receptors (ORs) (thanks to the *Drosophila* and more recently the *Anopheles* genome projects) reveals organizational principles in the peripheral olfactory system (Section 1.12.2.1). Furthermore, the development of genetic tools allowing tracing of neuronal projections in both groups and at the single cell level, have also contributed to this progress. The pace of discovery is expected to increase even more now that these initial successes have attracted a large group of scientists to research the olfactory system. In this section, as in many others, the focus is first on *Drosophila* and then on other insects.

What do we envisage as areas of special interest in olfactory system development? One ultimate goal of developmental biology is to understand the generation of an organ or system as it pertains to its function. In olfaction, this means understanding the developmental basis of odorant discrimination, recognition, and innate and learned olfactory

responses. At the first level, in the antenna and the maxillary palp, each of the 1300 ORNs probably expresses one specific member from a group of ~50 ORs, thereby creating specific channels of information processing. How is olfactory receptor expression choice made during development? How is this developmental decision related to general pattern formation in the AL? With the tools created by the cloning of OR genes, answers to these questions are in sight.

Much progress is expected in our understanding of wiring specificity will be made in the antennal lobe, the first olfactory relay. The convergent projection of ORN axons to specific glomeruli according to the OR they express, and the independent determination of PN dendritic glomerular targets according to their lineage and birth order, beg the question as to how this extraordinary wiring specificity is achieved. With the genetic tools in hand, it should be possible in the near future to decipher the logic behind how each individual ORN selects one from among ~50 glomeruli as its axonal target, how each individual PN selects one from these ~50 glomeruli as its dendritic target, and how these choices are coordinated. The logic used to achieve wiring specificity in the olfactory system could well be applied to many other parts of the brain.

Going deeper into the brain, it will be fruitful to examine how the wiring specificity of projection neuron axons in central targets is coordinated with their dendritic targets at the antennal lobe. To address this question more thoroughly, and to understand how olfactory information is further processed in higher brain centers, it is essential to identify third-order neurons with which PN axons form synapses. One major class of third order neurons has already been identified as the MB neurons; studying connection specificity between PNs and MB neurons will be important. A second class of third order neurons are the putative LH output neurons that synapse with PN axons in the lateral horn, the nature of which is still elusive. Developing new technologies to identify these neurons, as well as neurons downstream from lateral horn output neurons and mushroom body neurons, will remain a future challenge. Having identified these lateral horn neurons, the development of wiring specificity in both these higher structures should be amenable to study; comparisons of the logic used here versus in the antennal lobe will be interesting. Finally, a long-term goal must be to trace the process from sensory detection to integration and behavioral (motor) output. Olfaction can be characterized as an organizationally shallow sense – it probably only takes two synaptic levels to move from sensory stimulus to percept. Olfactory circuits should, thus, be particularly amenable to the tracing out of circuits that mediate an organism's behavioral response as well as understanding its developmental and genetic basis.

While, at least at the gross level, the olfactory system appears to be hard-wired and, as such, the substrate for innate behavior, it is entirely possible that experience will modify the strength or presence of connections at the level of individual synapses. Indeed, olfactory learning and memory is one of the most robust and best-characterized behavioral paradigms in *Drosophila*. With the basic wiring diagram characterized, it is now possible to examine how experience will change wiring. To what extent does experience dependent rewiring share mechanistic similarity with genetically specified wiring during development?

Another interesting future direction is the integration of the molecular genetics of olfactory development with olfactory physiology and behavior. Recent development of electrophysiological and optical imaging techniques starts to illuminate how olfactory information is being processed along the central pathway. These techniques could also be used to study the consequences of altered wiring using molecular genetic manipulation. Ultimately, it will be extremely interesting to study how precise genetic manipulation leads to altered physiological responses and changes in animal behavior, thus yielding insights into how genes, neurons, and circuits control behavior. Similar techniques may be applicable in studying the role of species specific genetic variation in other insects.

Lastly, an important future direction is the spread of molecular genetic approaches to other insects. The completion or near completion of genomic DNA sequencing in the silkmoth, ant and honeybee should allow identification of odorant receptor repertoires, as has been shown in *Drosophila* and *Anopheles*. Transgenic technologies using transposon or retroviral approaches have recently been demonstrated in a wide variety of insect species. Such tools could allow the labeling of specific classes of ORNs as well as study of their central projections in these insects. Furthermore, double-stranded RNA interference techniques to knockdown gene function have also become commonplace in the last few years. The availability of these techniques should broaden the experimental horizon so that the special advantages of different model systems can be fully exploited. For example, it would be of great experimental benefit to combine the surgical

manipulations or explant cultures developed in *M. sexta* with genetic manipulation or labeling of precise cell populations.

One fascinating question that may yield to this kind of multisystem approach is an understanding of how the fine organization of the olfactory system in different insects may be related. For instance, it would be extremely interesting to compare the evolution of olfactory receptor number and diversity with glomerular number. Similarly, it will be most interesting to understand how changes in the peripheral sensory array might be reflected in their partner neurons within the CNS. For example, it may be possible to determine if PNs that receive input from orthologous ORs have a consistent molecular identity and axonal projection pattern in higher centers. In short, it can be predicted that insect olfaction should provide fertile ground for investigation at many different levels, from molecules to behavior, both within individual species as well as an exciting system for comparative approaches between species.

1.12.6.3. Note Added in Proof

N-cadherin, in addition to its role in uniglomerular restriction of projection neuron dendritic targeting, also regulates the stability of a specific subset of PN axon terminal branches in the lateral horn (Zhu and Luo, 2004). N-cadherin is also required in ORNs for glomerular formation: the axons of N-cadherin deficient ORNs initially enter the developing antennal lobe but then withdraw to the surface of the antennal lobe, resulting in disruption of normal glomerular development (Hummel and Zipursky, 2004).

Two additional studies shed light on molecules and mechanisms for ORN axon targeting. Loss-of-function analyses as well as misexpression studies indicate that the axon guidance receptors of the Roundabout (Robo) family regulate the glomerular position of ORN axons in the antennal lobe. Robo2 also regulates midline crossing of ORN axons projecting to contralateral glomeruli (Jhaveri *et al.*, 2004). In a second study (Komiyama *et al.*, 2004), it was shown that the POU domain transcription factor Acj6, in addition to its role in PN dendritic targeting, also regulates axon targeting for a subset of ORN classes. Mosaic analyses reveal cooperative targeting between axon terminals of the same ORN classes and hierarchical interactions among different ORN classes. These observations support the emergent idea that the development of highly specific wiring between ORN axons and PN dendrites in the olfactory system involves substantial prepatterning of both ORN axons and PN dendrites through axon–axon and dendrite–dendrite interactions.

Acknowledgments

We thank M. de Bruyne, E.C. Marin, A. Nighorn, A. Ramaekers, and R.F. Stocker for sharing recent unpublished results. For comments on various sections and versions of this manuscript, we are grateful to D. Berdnik, E.C. Marin, A. Ramaekers, R.F. Stocker, R.J. Watts, H. Zhu and A.L. Taylor Tavares, whom we also thank for assistance with the figures. In addition to those mentioned above, we thank L. Oland and P. zur Lage for patiently fielding questions relating to their areas of expertise.

References

Ang, L.H., Kim, J., Stepensky, V., Hing, H., **2003**. Dock and Pak regulate olfactory axon pathfinding in *Drosophila*. *Development 130*, 1307–1316.

Anton, S., Hansson, B.S., **1996**. Antennal lobe interneurons in the desert locust *Schistocerca gregaria* (Forskal): processing of aggregation pheromones in adult males and females. *J. Comp. Neurol. 370*, 85–96.

Anton, S., Homberg, U., **1999**. Antennal lobe structure. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 97–124.

Armstrong, J.D., de Belle, J.S., Wang, Z., Kaiser, K., **1998**. Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in *Drosophila*. *Learn. Memory 5*, 102–114.

Aronstein, K., ffrench-Constant, R., **1995**. Immunocytochemistry of a novel GABA receptor subunit Rdl in *Drosophila melanogaster*. *Invertebr. Neurosci. 1*, 25–31.

Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., *et al.*, **2000**. The *Drosophila* trio plays an essential role in patterning of axons by regulating their directional extension. *Neuron 26*, 119–131.

Ayer, R.K., Carlson, J., **1991**. acj6: a gene affecting olfactory physiology and behavior in *Drosophila*. *PNAS 88*, 5467–5471.

Bate, C.M., **1978**. Development of sensory systems in Arthropods. In: Jacobson, M. (Ed.), Handbook of Sensory Physiology. Springer, Berlin, pp. 1–53.

Baumann, P.M., Oland, L.A., Tolbert, L.P., **1996**. Glial cells stabilize axonal protoglomeruli in the developing olfactory lobe of the moth *Manduca sexta*. *J. Comp. Neurol. 373*, 118–128.

Bellaiche, Y., Schweisguth, F., **2001**. Lineage diversity in the *Drosophila* nervous system. *Curr. Opin. Genet. Devel. 11*, 418–423.

Berg, B.G., Galizia, C.G., Brandt, R., Mustaparta, H., **2002**. Digital atlases of the antennal lobe in two species of tobacco budworm moths, the Oriental *Helicoverpa assulta* (male) and the American *Heliothis virescens* (male and female). *J. Comp. Neurol. 446*, 123–134.

Bertrand, N., Castro, D.S., Guillemot, F., **2002**. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci. 3*, 517–530.

Billuart, P., Winter, C.G., Maresh, A., Zhao, X., Luo, L., **2001**. Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. *Cell 107*, 195–207.

Boeckh, J., Tolbert, L.P., **1993**. Synaptic organization and development of the antennal lobe in insects. *Microsc. Res. Tech. 24*, 260–280.

Brody, T., Odenwald, W.F., **2002**. Cellular diversity in the developing nervous system: a temporal view from *Drosophila*. *Development 129*, 3763–3770.

Buck, L., Axel, R., **1991**. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell 65*, 175–181.

Cayre, M., Strambi, C., Charpin, P., Augier, R., Meyer, M.R., *et al.*, **1996**. Neurogenesis in adult insect mushroom bodies. *J. Comp. Neurol. 371*, 300–310.

Chess, A., Simon, I., Cedar, H., Axel, R., **1994**. Allelic inactivation regulates olfactory receptor gene expression. *Cell 78*, 823–834.

Clyne, P.J., Certel, S.J., de Bruyne, M., Zaslavsky, L., Johnson, W.A., *et al.*, **1999a**. The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factor. *Neuron 22*, 339–347.

Clyne, P.J., Warr, C.G., Carlson, J.R., **2000**. Candidate taste receptors in *Drosophila*. *Science 287*, 1830–1834.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., *et al.*, **1999b**. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron 22*, 327–338.

Connolly, J.B., Roberts, I.J.H., Armstrong, J.D., Kaiser, K., Forte, M., *et al.*, **1996**. Associative learning disrupted by impaired G_s sigaling in *Drosophila* mushroom bodies. *Science 274*, 2104–2107.

Crittenden, J.R., Sloulakis, E.M.C., Han, K.-A., Kalderon, D., Davis, R.L., **1998**. Tripartite mushroom body architecture revealed by antigenic markers. *Learn. Memory 5*, 38–51.

de Belle, J.S., Heisenberg, M., **1994**. Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science 263*, 692–695.

de Belle, J.S., Kanzaki, R., **1999**. Protocerebral olfactory processing. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 243–281.

de Bruyne, M., Foster, K., Carlson, J.R., **2001**. Odor coding in the *Drosophila* antenna. *Neuron 30*, 537–552.

Dickson, B.J., Hafen, E., **1993**. Genetic dissection of eye development in *Drosophila*. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster.* CSHL Press, New York, pp. 1327–1362.

Distler, P.G., Boeckh, J., **1998**. An improved model of the synaptic organization of insect olfactory glomeruli. *Ann. N. Y. Acad. Sci. 855*, 508–510.

Dobritsa, A.A., van der Goes van Naters, W., Warr, C.G., Steinbrecht, R.A., Carlson, J.R., **2003**. Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron 37*, 827–841.

Dubnau, J., Grady, L., Kitamoto, T., Tully, T., **2001**. Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature 411*, 476–480.

Dujardin, F., **1850**. Memoire sur le systeme nerveux des insectes. *Ann. Sci. Nat. Zool. 14*, 195–206.

Elmore, T., Smith, D. P., **2001**. Putative *Drosophila* odor receptor OR43b localizes to dendrites of olfactory neurons. *Insect Biochem. Mol. Biol. 31*, 791–798.

Fahrbach, S.E., Moore, D., Capaldi, E.A., Farris, S.M., Robinson, G.E., **1998**. Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learn. Memory 5*, 115–123.

Fahrbach, S.E., Strande, J.L., Robinson, G.E., **1995**. Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. *Neurosci. Lett. 197*, 145–148.

Farris, S.M., Robinson, G.E., Davis, R.L., Fahrbach, S.E., **1999**. Larval and pupal development of the mushroom bodies in the honey bee, Apis mellifera. *J. Comp. Neurol. 414*, 97–113.

Farris, S.M., Robinson, G.E., Fahrbach, S.E., **2001**. Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. *J. Neurosci. 21*, 6395–6404.

Farris, S.M., Strausfeld, N.J., **2001**. Development of laminar organization in the mushroom bodies of the cockroach: Kenyon cell proliferation, outgrowth, and maturation. *J. Comp. Neurol. 439*, 331–351.

Fiala, A., Spall, T., Diegelmann, S., Eisermann, B., Sachse, S., *et al.*, **2002**. Genetically expressed cameleon in *Drosophila* melanogaster is used to visualize olfactory information in projection neurons. *Curr. Biol. 12*, 1877–1884.

Friedrich, R.W., Korsching, S.I., **1997**. Combinatorial and chemotopic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. *Neuron 18*, 737–752.

Galizia, C.G., McIlwrath, S.L., Menzel, R., **1999a**. A digital three-dimensional atlas of the honeybeef antennal lobe based on optical sections acquired by confocal microscopy. *Cell Tissue Res. 295*, 383–394.

Galizia, C.G., Menzel, R., **2000**. Odour perception in honeybees: coding information in glomerular patterns. *Curr. Opin. Neurobiol. 10*, 504–510.

Galizia, C.G., Sachse, S., Mustaparta, H., **2000**. Calcium responses to pheromones and plant odours in the antennal lobe of the male and female moth *Heliothis virescens*. *J. Comp. Physiol. A 186*, 1049–1063.

Galizia, C.G., Sachse, S., Rapert, A., Menzel, R., **1999b**. The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. *Nat. Neurosci. 2*, 473–478.

Ganeshina, O., Schafer, S., Malun, D., **2000**. Proliferation and programmed cell death of neuronal precursors in the mushroom bodies of the honeybee. *J. Comp. Neurol. 417*, 349–365.

Gao, Q., Chess, A., **1999**. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. *Genomics 60*, 31–39.

Gao, Q., Yuan, B., Chess, A., **2000**. Convergent projections of *Drosophila* olfactory neurons to specific glomeruli in the antennal lobe. *Nat. Neurosci. 3*, 780–785.

Garrity, P.A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., *et al.*, **1996**. *Drosophila* photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell 85*, 639–650.

Gascuel, J., Masson, C., **1991**. Developmental study of afferented and deafferented bee antennal lobes. *J. Neurobiol. 22*, 795–810.

Grotewiel, M., Beck, C., Wu, K., Zhu, X., Davis, R., **1998**. Integrin-mediated short-term memory in *Drosophila*. *Nature 391*, 455–460.

Guan, Z., Prado, A., Melzig, J., Heisenberg, M., Nash, H.A., *et al.*, **2000**. Mushroom body defect, a gene involved in the control of neuroblast proliferation in *Drosophila*, encodes a coiled-coil protein. *Proc. Natl Acad. Sci. USA 97*, 8122–8127.

Gupta, B.P., Flores, G.V., Banerjee, U., Rodrigues, V., **1998**. Patterning an epidermal field: *Drosophila lozenge*, a member of the AML-1/Runt family of transcription factors, specifies olfactory sense organ type in a dose-dependent manner. *Devel. Biol. 203*, 400–411.

Hahnlein, I., Bicker, G., **1996**. Morphology of neuroglia in the antennal lobes and mushroom bodies of the brain of the honeybee. *J. Comp. Neurol. 367*, 235–245.

Hallem, E.A., Ho, M.G., Carlson, J.R., **2004**. The molecular basis of odor coding in the *Drosophila* antenna. *Cell 117*, 965–979.

Hammer, M., **1993**. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature 366*, 59–63.

Hammer, M., Menzel, R., **1995**. Learning and memory in the honeybee. *J. Neurosci. 15*, 1617–1630.

Hansson, B.S. (Ed.) **1999**. Insect Olfaction. Springer, Berlin.

Hansson, B.S., Ljungberg, H., Hallberg, E., Lofstedt, C., **1992**. Functional specialization of olfactory glomeruli in a moth. *Science 256*, 1313–1315.

Hansson, B.S., Ochieng, S.A., Grosmaitre, X., Anton, S., Njagi, P.G.N., **1996**. Physiological responses and central nervous projections of antennal olfactory receptor neurons in the adult desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae). *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 179*, 157–167.

Hartenstein, V., Posakony, J.W., **1989**. Development of adult sensilla on the wing and notum of *Drosophila melanogaster. Development 107*, 389–405.

Haynie, J.L., Bryant, P.J., **1986**. Development of the eye-antenna imaginal disc and morphogenesis of the adult head in *Drosophila melanogaster. J. Exp. Zool. 237*, 293–308.

Heimbeck, G., Bugnon, V., Gendre, N., Keller, A., Stocker, R.F., **2001**. A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 98*, 15336–15341.

Heisenberg, M., **2003**. Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci. 4*, 266–275.

Heisenberg, M., Heusipp, M., Wanke, C., **1995**. Structural plasticity in the *Drosophila* brain. *J. Neurosci. 15*, 1951–1960.

Higgins, M.R., Gibson, N.J., Eckholdt, P.A., Nighorn, A., Copenhaver, P.F., *et al.*, **2002**. Different isoforms of fasciclin II are expressed by a subset of developing olfactory receptor neurons and by olfactory-nerve glial cells during formation of glomeruli in the moth *Manduca sexta*. *Devel. Biol. 244*, 134–154.

Hildebrand, J.G., **1995**. Analysis of chemical signals by nervous systems. *Proc. Natl Acad. Sci. USA 92*, 67–74.

Hildebrand, J.G., Hall, L.M., Osmond, B.C., **1979**. Distribution of binding sites for 125I-labeled alpha-bungarotoxin in normal and deafferented antennal lobes of *Manduca sexta*. *Proc. Natl Acad. Sci. USA 76*, 499–503.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, **2002**. G protein-coupled receptors in *Anopheles gambiae*. *Science 298*, 176–178.

Hing, H., Xiao, J., Harden, N., Lim, L., Zipursky, S., **1999**. Pak functions downstream of Dock to regulate photoreceptor axon guidance in *Drosophila*. *Cell 97*, 853–863.

Holldobler, B., Wilson, E., **1990**. The Ants. Belknap Press of Harvard University Press, Cambridge, MA.

Homberg, U., Hildebrand, J.G., **1994**. Postembryonic development of gamma-aminobutyric acid-like immunoreactivity in the brain of the sphinx moth *Manduca sexta*. *J. Comp. Neurol. 339*, 132–149.

Homberg, U., Montague, R.A., Hildebrand, J.G., **1988**. Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta*. *Cell Tissue Res. 254*, 255–281.

Homberg, U., Muller, U., **1999**. Neuroactive substances in the antennal lobe. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 181–206.

Hoskins, S.G., Homberg, U., Kingan, T.G., Christensen, T.A., Hildebrand, J.G., **1986**. Immunocytochemistry of GABA in the antennal lobes of the sphinx moth *Manduca sexta*. *Cell Tissue Res. 244*, 243–252.

Hummel, T., Krukkert, K., Roos, J., Davis, G., Klambt, C., **2000**. *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. *Neuron 26*, 357–370.

Hummel, T., Vasconcelos, M.L., Clemens, J.C., Fishilevich, Y., Vosshall, L.B., *et al.*, **2003**. Axonal targeting of olfactory receptor neurons in *Drosophila* is controlled by Dscam. *Neuron 37*, 221–231.

Hummel, T., Zipursky, S.L., **2004**. Afferent induction of olfactory glomeruli requires N-cadherin. *Neuron 42*, 77–88.

Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., **2001**. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identify of their neuronal progeny. *Cell 106*, 511–521.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y., Yamamoto, D., **1997**. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurons and glial cells. *Development 124*, 761–771.

Ito, K., Hotta, Y., **1992**. Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster. Devel. Biol. 149*, 134–148.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D., *et al.*, **1998**. The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* meigen. *Learn. Memory 5*, 52–77.

Ito, K., Urban, J., Technau, G., **1995**. Distribution, classification and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Devel. Biol. 204*, 284–307.

Jan, Y.N., Jan, L.Y., **1993**. The peripheral nervous system. In: Martinez Arias, A. (Ed.), The development of *Drosophila melanogaster.* CSHL Press, New York.

Jan, Y.N., Jan, L.Y., **2001**. Asymmetric cell division in the *Drosophila* nervous system. *Nat. Rev. Neurosci. 2*, 772–779.

Jarman, A.P., Grau, Y., Jan, L.Y., Jan, Y.N., **1993**. Atonal is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell 73*, 1307–1321.

Jarman, A.P., Grell, E.H., Ackerman, L., Jan, L.Y., Jan, Y.N., **1994**. Atonal is the proneural gene for *Drosophila* photoreceptors. *Nature 369*, 398–400.

Jefferis, G.S.X.E., Marin, E.C., Stocker, R.F., Luo, L., **2001**. Target neuron prespecification in the olfactory map of *Drosophila*. *Nature 414*, 204–208.

Jefferis, G.S.X.E., Marin, E.C., Watts, R.J., Luo, L., **2002**. Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr. Opin. Neurobiol. 12*, 80–86.

Jefferis, G.S.X.E., Vyas, R.M., Berdnik, D., Ramaekers, A., Stocker, R.F., *et al.*, **2004**. Developmental Origin of wiring specificity in the olfactory System of *Drosophila*. *Development 131*, 117–130.

Jhaveri, D., Rodrigues, V., **2002**. Sensory neurons of the Atonal lineage pioneer the formation of glomeruli within the adult *Drosophila* olfactory lobe. *Development 129*, 1251–1260.

Jhaveri, D., Saharan, S., Sen, A., Rodrigues, V., **2004**. Positioning sensory terminals in the olfactory lobe of *Drosophila* by Robo signaling. *Development 131*, 1903–1912.

Jhaveri, D., Sen, A., Reddy, G.V., Rodrigues, V., **2000a**. Sense organ identity in the *Drosophila* antenna is specified by the expression of the proneural gene atonal. *Mech. Devel. 99*, 101–111.

Jhaveri, D., Sen, A., Rodrigues, V., **2000b**. Mechanisms underlying olfactory neuronal connectivity in *Drosophila* – the atonal lineage organizes the periphery while sensory neurons and glia pattern the olfactory lobe. *Devel. Biol. 226*, 73–87.

Kaneko, M., Nighorn, A., **2003**. Inter-axonal Eph-ephrin signaling may mediate sorting of olfactory sensory axons in *Manduca sexta*. *J. Neurosci. 23*, 11523–11538.

Keil, T.A., **1999**. Morphology and development of the peripheral olfactory organs. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 5–47.

Keil, T.A., Steiner, C., **1991**. Morphogenesis of the antenna of the male silkmoth, *Antheraea polyphemus*. III. Devlopment of olfactory sensilla and the properties of hair-forming cells. *Tissue Cell 23*, 821–851.

Keller, A., Vosshall, L.B., **2003**. Decoding olfaction in *Drosophila*. *Curr. Opin. Neurobiol. 13*, 103–110.

Kent, K.S., Hildebrand, J.G., **1987**. Cephalic sensory pathways in the central nervous system of larval *Manduca sexta* (Lepidoptera: Sphingidae). *Philos. Trans. R. Soc. Lond. B Biol. Sci. 315*, 1–36.

Kent, K.S., Hoskins, S.G., Hildebrand, J.G., **1987**. A novel serotonin-immunoreactive neuron in the antennal lobe of the sphinx moth *Manduca sexta* persists throughout postembryonic life. *J. Neurobiol. 18*, 451–465.

Kenyon, F.C., **1896**. The brain of the bee. A preliminary contribution to the morphology of the nervous system of the arthropoda. *J. Comp. Neurol. 6*, 133–210.

Kirschenbaum, S.R., Higgins, M.R., Tveten, M., Tolbert, L.P., **1995**. 20-Hydroxyecdysone stimulates proliferation of glial cells in the developing brain of the moth *Manduca sexta*. *J. Neurobiol. 28*, 234–247.

Komiyama, T., Carlson, J.R., Luo, L., **2004**. Olfactory receptor neuron axon targeting: intrinsic transcriptional control and hierarchical interactions. *Nat. Neurosci. 7*, 819–825.

Komiyama, T., Johnson, W.A., Luo, L., Jefferis, G.S., **2003**. From lineage to wiring specificity: POU domain transcription factors control precise connections of *Drosophila* olfactory projection neurons. *Cell 112*, 157–167.

Kondoh, Y., Kaneshiro, K.Y., Kimura, K., Yamamoto, D., **2003**. Evolution of sexual dimorphism in the olfactory brain of Hawaiian *Drosophila*. *Proc. R. Soc. Lond. B Biol. Sci. 270*, 1005–1013.

Korsching, S., **2002**. Olfactory maps and odor images. *Curr. Opin. Neurobiol. 12*, 387–392.

Krieger, J., Klink, O., Mohl, C., Raming, K., Breer, H., **2003**. A candidate olfactory receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 189*, 519–526.

Krieger, J., Raming, K., Dewer, Y.M., Bette, S., Conzelmann, S., *et al.*, **2002**. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci. 16*, 619–628.

Kurusu, M., Awasaki, T., Masuda-Nakagawa, L.M., Kawauchi, H., Ito, K., *et al.*, **2002**. Embryonic and larval development of the *Drosophila* mushroom bodies: concentric layer subdivisions and the role of fasciclin II. *Development 129*, 409–419.

Lai, E.C., **2003**. Drosophila tufted is a gain-of-function allele of the proneural gene amos. *Genetics 163*, 1413–1425.

Laissue, P.P., Reiter, C., Hiesinger, P.R., Halter, S., Fischback, K.F., *et al.*, **1999**. Three-dimensional reconstruction of the antennal lobe in *Drosophila melanogaster. J. Comp. Neurol. 405*, 543–552.

Laurent, G., **1996**. Dynamical representation of odors by oscillating and evolving neural assemblies. *TINS 19*, 489–496.

Laurent, G., **1997**. Olfactory processing: maps, time and codes. *Curr. Opin. Neurobiol. 7*, 547–553.

Laurent, G., **2002**. Olfactory network dynamics and the coding of multidimensional signals. *Nat. Rev. Neurosci. 3*, 884–895.

Laurent, G., Naraghi, M., **1994**. Odorant-induced oscillations in the mushroom bodies of the locust. *J. Neurosci. 14*, 2993–3004.

Laurent, G., Stopfer, M., Friedrich, R.W., Rabinovich, M.I., Volkovskii, A., *et al.*, **2001**. Odor encoding as an active, dynamical process: experiments, computation, and theory. *Annu. Rev. Neurosci. 24*, 263–297.

Lee, T., Lee, A., Luo, L., **1999**. Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development 126*, 4065–4076.

Lee, T., Luo, L., **1999**. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron 22*, 451–461.

Lee, T., Luo, L., **2001**. Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *TINS 24*, 251–254.

Lee, T., Marticke, S., Sung, C., Robinow, S., Luo, L., **2000a**. Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron 28*, 807–818.

Lee, T., Winter, C., Marticke, S.S., Lee, A., Luo, L., **2000b**. Essential roles of *Drosophila* RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron 25*, 307–316.

Li, Y., Strausfeld, N.J., **1997**. Morphology and sensory modality of mushroom body extrinsic neurons in the brain of the cockroach, *Periplaneta americana*. *J. Comp. Neurol. 387*, 631–650.

Lienhard, M.C., Stocker, R.F., **1991**. The development of the sensory neuron pattern in the antennal disc of wild-type and mutant (lz3, ssa) *Drosophila melanogaster. Development 112*, 1063–1075.

Liu, Z., Steward, R., Luo, L., **2000**. Drosophila Lis1 is required for neuroblast proliferation, dendritic elaboration and axonal transport. *Nat. Cell Biol. 2*, 776–783.

Madhavan, M.M., Schneiderman, H.A., **1977**. Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 183*, 269–305.

Malaterre, J., Strambi, C., Chiang, A.S., Aouane, A., Strambi, A., *et al.*, **2002**. Development of cricket mushroom bodies. *J. Comp. Neurol. 452*, 215–227.

Malun, D., Oland, L.A., Tolbert, L.P., **1994**. Uniglomerular projection neurons participate in early development of ofactory glomeruli in the moth *Manduca sexta*. *J. Comp. Neurol. 350*, 1–22.

Marin, E.C., Jefferis, G.S.X.E., Komiyama, T., Zhu, H., Luo, L., **2002**. Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell 109*, 243–255.

Masson, C., Arnold, G., **1984**. Ontogeny, maturation and plasticity of the olfactory system in the workerbee. *J. Insect. Physiol. 30*, 7–14.

McGuire, S.E., Le, P.T., Davis, R.L., **2001**. The role of *Drosophila* mushroom body signaling in olfactory memory. *Science 293*, 1330–1333.

McIver, S.B., **1982**. Sensilla of mosquitoes (Diptera: Culicidae). *J. Med. Entomol. 19*, 489–535.

McKenna, M., Monte, P., Helfand, S.L., Woodard, C., Carlson, J., **1989**. A simple chemosensory response in *Drosophila* and the isolation of acj mutants in which it is affected. *Proc. Natl Acad. Sci. USA 86*, 8118–8122.

Melzig, J., Rein, K.H., Schafer, U., Pfister, H., Jackle, H., *et al.*, **1998**. A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the *Drosophila* adult central nervous system. *Curr. Biol. 8*, 1223–1226.

Menzel, R., Durst, C., Erber, J., Eichmuller, S., Hammer, M., *et al.*, **1994**. The mushroom bodies in the honeybee: from molecules to behaviour. In: Schildberger, K., Elsner, N. (Eds.), Neural Basis of Behavioural Adaptations. Fischer, Stuttgart, pp. 81–102.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., *et al.*, **1996**. Visualizing an olfactory sensory map. *Cell 87*, 675–686.

Mushroom Body Special Issue, **1998**. Learning and Memory. Cold Spring Harbor Laboratory Press, New York, pp. 1–191.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., *et al.*, **2002a**. Rac GTPases control axon growth, guidance and branching. *Nature 416*, 442–447.

Ng, M., Roorda, R.D., Lima, S.Q., Zemelman, B.V., Morcillo, P., *et al.*, **2002b**. Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. *Neuron 36*, 463–474.

Nighorn, A., Healy, M.J., Davis, R.L., **1991**. The cyclic AMP phosphodiesterase encoded by the *Drosophila dunce* gene is concentrated in the mushroom body neuropil. *Neuron 6*, 455–467.

Nishikawa, M., Nishino, H., Mizunami, M., Yokohari, F., **1998**. Function-specific distribution patterns of axon terminals of input neurons in the calyces of the mushroom body of the cockroach, *Periplaneta americana*. *Neurosci. Lett. 245*, 33–36.

Nolo, R., Abbott, L.A., Bellen, H.J., **2000**. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell 102*, 349–362.

Nordlander, R., Edwards, J., **1970**. Postembryonic brain development in the monarch butterfly, *Danaus plexippus plexippus* L. III. Morphogenesis of centers other then the optic lobes. *Roux's Arch. Entwicklungsmech. Org. 164*, 247–260.

Noveen, A., Daniel, A., Hartenstein, V., **2000**. Early development of the *Drosophila* mushroom body: the roles of eyeless and dachshund. *Development 127*, 3475–3488.

Oland, L.A., Kirschenbaum, S.R., Pott, W.M., Mercer, A.R., Tolbert, L.P., **1995**. Development of an identified serotonergic neuron in the antennal lobe of the moth and effects of reduction in serotonin during construction of olfactory glomeruli. *J. Neurobiol. 28*, 248–267.

Oland, L.A., Orr, G., Tolbert, L.P., **1990**. Construction of a protoglomerular template by olfactory axons initiates the formation of olfactory glomeruli in the insect brain. *J. Neurosci. 10*, 2096–2112.

Oland, L.A., Pott, W.M., Higgins, M.R., Tolbert, L.P., **1998**. Targeted ingrowth and glial relationships of olfactory recetor axons in the primary olfactory pathway of an insect. *J. Comp. Neurol. 398*, 119–138.

Oland, L.A., Tolbert, L.P., **1987**. Glial patterns during early development of antennal lobes of *Manduca sexta*: a comparison between normal lobes and lobes deprived of antennal axons. *J. Comp. Neurol. 255*, 196–207.

Oland, L.A., Tolbert, L.P., **1988**. Effects of hydroxyurea parallel the effects of radiation in developing olfactory glomeruli in insects. *J. Comp. Neurol. 278*, 377–387.

Oland, L.A., Tolbert, L.P., **1998**. Glomerulus development in the absence of a set of mitral-like neurons in the insect olfactory lobe. *J. Neurobiol. 36*, 41–52.

Oland, L.A., Tolbert, L.P., **2003**. Key interactions between neurons and glial cells during neural development in insects. *Annu. Rev. Entomol. 48*, 89–110.

Oland, L.A., Tolbert, L.P., Mossman, K.L., **1988**. Radiation-induced reduction of the glial population during development disrupts the formation of olfactory glomeruli in an insect. *J. Neurosci. 8*, 353–367.

Pascual, A., Preat, T., **2001**. Localization of long-term memory within the *Drosophila* mushroom body. *Science 294*, 1115–1117.

Perez-Orive, J., Mazor, O., Turner, G.C., Cassenaer, S., Wilson, R.I., *et al.*, **2002**. Oscillations and sparsening of odor representations in the mushroom body. *Science 297*, 359–365.

Postlethwait, J.H., Schneiderman, H.A., **1971**. A clonal analysis of development in *Drosophila melanogaster*: morphogenesis, determination, and growth in the wild-type antenna. *Devel. Biol. 24*, 477–519.

Python, F., Stocker, R.F., **2002**. Adult-like complexity of the larval antennal lobe of *D. melanogaster* despite markedly low numbers of odorant receptor neurons. *J. Comp. Neurol. 445*, 374–387.

Ray, K., Rodrigues, V., **1995**. Cellular events during development of the olfactory sense organs in *Drosophila melanogaster. Devel. Biol. 167*, 426–438.

Reddy, G.V., Gupta, B., Ray, K., Rodrigues, V., **1997**. Development of the *Drosophila* olfactory sense organs utilizes cell-cell interactions as well as lineage. *Development 124*, 703–712.

Ressler, K.J., Sullivan, S.L., Buck, L.B., **1994**. Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell 79*, 1245–1255.

Reuter, J.E., Nardine, T.M., Penton, A., Billuart, P., Scott, E.K., *et al.*, **2003**. A mosaic genetic screen for genes necessary for *Drosophila* mushroom body neuronal morphogenesis. *Development 130*, 1203–1213.

Rodrigues, V., **1988**. Spatial coding of olfactory information in the antennal lobe of *Drosophila melanogaster. Brain Res. 453*, 299–307.

Roman, G., Davis, R.L., **2001**. Molecular biology and anatomy of *Drosophila* olfactory associative learning. *Bioessays 23*, 571–581.

Rospars, J.P., **1988**. Structure and development of the insect antennodeutocerebral system. *Int. J. Insect Morphol. Embryol. 17*, 243–294.

Rospars, J.P., Hildebrand, J.G., **2000**. Sexually dimorphic and isomorphic glomeruli in the antennal lobes of the sphinx moth *Manduca sexta*. *Chem. Senses 25*, 119–129.

Rossler, W., Oland, L.A., Higgins, M.R., Hildebrand, J.G., Tolbert, L.P., **1999a**. Development of a glia-rich axon-sorting zone in the olfactory pathway of the moth *Manduca sexta*. *J. Neurosci. 19*, 9865–9877.

Rossler, W., Randolph, P.W., Tolbert, L.P., Hildebrand, J.G., **1999b**. Axons of olfactory receptor cells of transsexually grafted antennae induce development of sexually dimorphic glomeruli in *Manduca sexta*. *J. Neurobiol. 38*, 521–541.

Rubin, B.D., Katz, L.C., **1999**. Optical imaging of odorant representations in the mammalian olfactory bulb. *Neuron 23*, 499–511.

Ryan, A.K., Rosenfeld, M.G., **1997**. POU domain family values: Flexibility, partnerships, and developmental codes. *Genes Devel. 11*, 1207–1225.

Rybak, J., Menzel, R., **1993**. Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. *J. Comp. Neurol. 334*, 444–465.

Rybak, J., Menzel, R., **1998**. Integrative properties of the Pe1 neuron, a unique mushroom body output neuron. *Learn. Memory 5*, 133–145.

Salecker, I., Boeckh, J., **1995**. Embryonic development of the antennal lobes of a hemimetabolous insect, the cockroach *Periplaneta americana*: light and electron microscopic observations. *J. Comp. Neurol. 352*, 33–54.

Salecker, I., Boeckh, J., **1996**. Influence of receptor axons on the formation of olfactory glomeruli in a hemimetabolous insect, the cockroach *Periplaneta americana*. *J. Comp. Neurol. 370*, 262–279.

Salecker, I., Malun, D., **1999**. Development of olfactory glomeruli. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 97–124.

Schafer, S., Bicker, G., **1986**. Distribution of GABA-like immunoreactivity in the brain of the honeybee. *J. Comp. Neurol. 246*, 287–300.

Schaller, D., **1978**. Antennal sensory system of *Periplaneta americana* L.: distribution and frequency of

morphologic types of sensilla and their sex-specific changes during postembryonic development. *Cell Tissue Res. 191*, 121–139.

Schmucker, D., Clemens, J.C., Shu, H., Worby, C.A., Xiao, J., *et al.*, **2000**. Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. *Cell 101*, 671–684.

Schneider, D., **1964**. Insect antennae. *Annu. Rev. Entomol. 9*, 103–122.

Schneiderman, A., Matsumoto, S., Hildebrand, J., **1982**. Trans-sexually grafted antennae influence development of sexually dimorphic neurons in moth brain. *Nature 298*, 844–846.

Schneiderman, A.M., Hildebrand, J.G., Brennan, M.M., Tumlinson, J.H., **1986**. Trans-sexually grafted antennae alter pheromone-directed behaviour in a moth. *Nature 323*, 801–803.

Schurmann, F., **1987**. The architecture of the mushroom bodies and related neuropils in the insect brain. In: Gupta, A. (Ed.), Arthropod Brain: Its Evolution, Development, Structure, and Functions. Wiley, New York, pp. 231–264.

Scott, E., Lee, T., Luo, L., **2001a**. enoki encodes a *Drosophila* putative histone acetyltransferase required for mushroom body neuroblast proliferation. *Curr. Biol. 11*, 99–104.

Scott, K., Brandy, R., Jr., Cravchik, A., Morozov, P., Rzhetsky, A., *et al.*, **2001b**. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell 104*, 661–673.

Scotto Lomassese, S., Strambi, C., Strambi, A., Charpin, P., Augier, R., *et al.*, **2000**. Influence of environmental stimulation on neurogenesis in the adult insect brain. *J. Neurobiol. 45*, 162–171.

Scotto-Lomassese, S., Strambi, C., Aouane, A., Strambi, A., Cayre, M., **2002**. Sensory inputs stimulate progenitor cell proliferation in an adult insect brain. *Curr. Biol. 12*, 1001–1005.

Sen, A., Reddy, G.V., Rodrigues, V., **2003**. Combinatorial expression of Prospero, Seven-up, and Elav identifies progenitor cell types during sense-organ differentiation in the *Drosophila* antenna. *Devel. Biol. 254*, 79–92.

Shi, Y., Massague, J., **2003**. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell 113*, 685–700.

Simpson, P., **1997**. Notch signalling in development: on equivalence groups and asymmetric developmental potential. *Curr. Opin. Genet. Devel. 7*, 537–542.

Simpson, P., Woehl, R., Usui, K., **1999**. The development and evolution of bristle patterns in Diptera. *Development 126*, 1349–1364.

Singh, R.N., Nayak, S.V., **1985**. Fine-structure and primary sensory projections of sensilla on the maxillary palp of *Drosophila-Melanogaster* Meigen (Diptera, Drosophilidae). *Int. J. Insect. Morphol. Embryol. 14*, 291–306.

Stocker, R.F., **1994**. The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Res. 275*, 3–26.

Stocker, R.F., **2001**. Drosophila as a focus in olfactory research: mapping of olfactory sensilla by fine structure, odor specificity, odorant receptor expression, and central connectivity. *Microsc. Res. Tech. 55*, 284–296.

Stocker, R.F., Gendre, N., **1988**. Peripheral and central nervous effects of lozenge3: a *Drosophila* mutant lacking basiconic antennal sensilla. *Devel. Biol. 127*, 12–24.

Stocker, R.F., Heimbeck, G., Gendre, N., de Belle, J.S., **1997**. Neuroblast ablation in *Drosophila* P[GAL4] lines reveals origins of olfactory interneurons. *J. Neurobiol. 32*, 443–452.

Stocker, R.F., Lienhard, M.C., Borst, A., Fischbach, K.-F., **1990**. Neuronal architecture of the antennal lobe in *Drosophila melanogaster. Cell Tissue Res. 262*, 9–34.

Stocker, R.F., Rodrigues, V., **1999**. Olfactory Neurogenetics. In: Hansson, S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 283–314.

Stocker, R.F., Tissot, M., Gendre, N., **1995**. Morphogenesis and cellular proliferation pattern in the developing antennal lobe of *Drosophila melanogaster. Roux's Arch. Devel. Biol. 205*, 62–72.

Strausfeld, N.J., Hansen, L., Li, Y., Gomez, R.S., Ito, K., **1998**. Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn. Memory 5*, 11–37.

Strausfeld, N.J., Hildebrand, J.G., **1999**. Olfactory systems: common design, uncommon origins? *Curr. Opin. Neurobiol. 9*, 634–639.

Strausfeld, N.J., Li, Y., **1999**. Representation of the calyces in the medial and vertical lobes of cockroach mushroom bodies. *J. Comp. Neurol. 409*, 626–646.

Strausfeld, N.J., Sinakevitch, I., Vilinsky, I., **2003**. The mushroom bodies of *Drosophila melanogaster*: An immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. *Microsc. Res. Tech. 62*, 151–169.

Svacha, P., **1992**. What are and what are not imaginal discs: reevaluation of some basic concepts (Insecta, Holometabola). *Devel. Biol. 154*, 101–117.

Technau, G., Heisenberg, M., **1982**. Neural reorganization during metamorphosis of the corpora pedunculata in *Drosophila melanogaster. Nature 295*, 405–407.

Thummel, C.S., **1996**. Files on steroids – *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet. 12*, 306–310.

Tissot, M., Gendre, N., Hawken, A., Stortkuhl, K.F., Stocker, R.F., **1997**. Larval chemosensory projections and invasion of adult afferents in the antennal lobe of *Drosophila. J. Neurobiol. 32*, 281–297.

Tolbert, L.P., Sirianni, P.A., **1990**. Requirement for olfactory axons in the induction and stabilization of olfactory glomeruli in an insect. *J. Comp. Neurol. 298*, 69–82.

Truman, J.W., Bate, M., **1988**. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster. Devel. Biol. 125*, 145–157.

Vassar, R., Chao, S.K., Sitcheran, R., Nunez, J.M., Vosshall, L.B., *et al.*, **1994**. Topographic organization of

sensory projections to the olfactory bulb. *Cell 79*, 981–991.

Verkhusha, V.V., Otsuna, H., Awasaki, T., Oda, H., Tsukita, S., *et al.*, **2001**. An enhanced mutant of red fluorescent protein DsRed for double labeling and developmental timer of neural fiber bundle formation. *J. Biol. Chem. 276*, 29621–29624.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., Axel, R., **1999**. A Spatial map of olfactory receptor expression in the *Drosophila* Antenna. *Cell 96*, 725–736.

Vosshall, L.B., Wong, A.M., Axel, R., **2000**. An olfactory sensory map in the fly brain. *Cell 102*, 147–159.

Waddell, S., Armstrong, J.D., Kitamoto, T., Kaiser, K., Quinn, W.G., **2000**. The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell 103*, 805–813.

Wang, F., Nemes, A., Mendelsohn, M., Axel, R., **1998**. Odorant receptors govern the formation of a precise topographic map. *Cell 93*, 47–60.

Wang, J., Zugates, C.T., Liang, I.H., Lee, C.H., Lee, T., **2002**. *Drosophila* Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron 33*, *559–571*.

Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., Axel, R., **2003**. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell 112*, 271–282.

Watts, R.J., Hoopfer, E.D., Luo, L., **2003**. Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron 38*, 871–885.

Williams, D.W., Shepherd, D., **2002**. Persistent larval sensory neurones are required for the normal development of the adult sensory afferent projections in *Drosophila*. *Development 129*, 617–624.

Withers, G.S., Fahrbach, S.E., Robinson, G.E., **1993**. Selective neuroanatomical plasticity and division of labour in the honeybee. *Nature 364*, 238–240.

Wong, A.M., Wang, J.W., Axel, R., **2002**. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell 109*, 229–241.

Yang, M.Y., Armstrong, J.D., Vilinsky, I., Strausfeld, N.J., Kaiser, K., **1995**. Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression pattern. *Neuron 15*, 45–54.

Yasuyama, K., Meinertzhagen, I.A., Schurmann, F.W., **2002**. Synaptic organization of the mushroom body calyx in *Drosophila melanogaster. J. Comp. Neurol. 445*, 211–226.

Yasuyama, K., Meinertzhagen, I.A., Schurmann, F.W., **2003**. Synaptic connections of cholinergic antennal lobe relay neurons innervating the lateral horn neuropile in the brain of *Drosophila melanogaster. J. Comp. Neurol. 466*, 299–315.

Zars, T., Fischer, M., Schulz, R., Heisenberg, M., **2000**. Localization of a short-term memory in *Drosophila*. *Science 288*, 672–675.

Zhai, Q., Wang, J., Kim, A., Liu, Q., Watts, R., *et al.*, **2003**. Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. *Neuron 39*, 217–225.

Zheng, X., Wang, J., Haerry, T.E., Wu, A.Y., Martin, J., *et al.*, **2003**. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell 112*, 303–315.

Zhu, H., Luo, L., **2004**. Diverse functions of N-cadherin in dendritic and axonal terminal arborization of olfactory projection neurons. *Neuron 42*, 63–75.

Zhu, S., Chiang, A.S., Lee, T., **2003**. Development of the *Drosophila* mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. *Development 130*, 2603–2610.

Zipursky, S.L., Venkatesh, T.R., Teplow, D.B., Benzer, S., **1984**. Neuronal development in the *Drosophila retina: monoclonal antibodies as molecular probes. Cell 36, 15–26*.

zur Lage, P.I., Prentice, D.R., Holohan, E.E., Jarman, A.P., **2003**. The *Drosophila* proneural gene amos promotes olfactory sensillum formation and suppresses bristle formation. *Development 130*, 4683–4693.

2.1 Myogenesis and Muscle Development

S M Abmayr, L Balagopalan, B J Galletta, and S-J Hong, Penn State University, University Park, PA, USA

2.1.1. Introduction

With recent advances in molecular genetics and cell biology, our progress in understanding the embryonic development of larval body wall muscles in the past 10 or 15 years has outpaced that of the previous four or five decades combined. One of the many insights provided by numerous studies is that fundamental cell fate decisions, at the level of changes in gene expression, occur long before the morphological consequences of myogenesis are visible. It is now clear, for example, that the mechanisms responsible for patterning the musculature and establishing the identity of each muscle fiber act very shortly after myogenic determination. These early cell fate choices dictate an almost invariant pattern of muscle attachment and innervation hours later.

While recent advances have dramatically expanded upon, clarified, or corrected earlier notions, there is no doubt that these advances were built on a solid foundation. Our understanding of embryonic myogenesis and appreciation of muscle pattern were influenced by tedious and time-consuming studies summarized in classic reviews by Poulson (1950) and Crossley (1978). Morphological studies first described the presumptive musculature as inner and outer layers of mesodermal cells that appeared to retain their relative positions in later stages (Poulson, 1950). These layers, the so-called splanchnopleura and somatopleura, were apparent during germ band retraction. The former was thought to give rise to the visceral musculature, and the latter to the somatic muscles. The somatic mesoderm was then observed to separate into dorsolateral and ventrolateral portions, with the ventrolateral portions giving rise to the musculature and the dorsolateral portions giving rise to fat body, circulatory system, and gonadal mesoderm. Later, the segmented ventrolateral domain appeared to remain in close association with the ectoderm and, upon completion of germ band shortening, to be limited to intersegmental regions where the developing syncytia establish contact with the developing apodemes. While the use of molecular probes, genetic tricks, and transgenic technology have suggested some differences, this description remains essentially correct.

Features of differentiated muscle fibers and the first naming of the individual *Drosophila* members of the larval body wall musculature were described in an extensively cited review by Crossley (1978). At the time of the initial fusion events, the multinucleated myoblasts were reported to have sparse endoplamic reticulum, many mitochondria and a large number of microtubules oriented along the longitudinal axis of the syncytia. Based on studies in other insects, these results suggested that the microtubules serve as a scaffold for the assembly of microfilament arrays (Crossley, 1978). No obvious mitoses were reported, though the possibility of limited mitotic events was not eliminated. Consistent with this was the observation that myoblasts in primary cultures undergo only one round of mitosis before differentiation into mononucleate muscle fibers (Bernstein *et al*., 1993). Myofibrils were evident in sections from embryos at 10–11 h, and the musculature was found to be complete in all of its details by 13–14 h. Coordinated muscle contraction becomes apparent by this time. Slightly later, Z bodies were observed. These structures organize into Z-lines in parallel arrays within the microfilament bundles, between 16 and 18 h. With few exceptions, the remainder of our early understanding of insect myogenesis was restricted to structural details of the fully differentiated larval muscles or, more commonly, the adult musculature (Crossley, 1978; Bernstein *et al*., 1993; see also **Chapters 2.2** and **2.3**).

In this review, our aim is to expand upon these early studies and summarize the experimental approaches, results, and concepts that form the foundation of our current understanding of embryonic myogenesis.

2.1.2. Mesoderm Specification and Subdivision

The present volume includes chapters devoted to many different aspects of embryonic development, and includes a discussion of zygotic genome expression and pattern formation (see, for example, **Chapters 1.2**, **1.8–1.10**). Thus, some of the key genes and decisions influencing the formation of the germ layers in the embryo, and their subsequent development, are covered more thoroughly elsewhere. However, as some of the genes that play critical events in the early embryo are also utilized later in myogenesis, we include a brief summary here. We have restricted this discussion to mesoderm specification and subdivision as it relates to the subsequent musculature.

2.1.2.1. Early Mesodermal Events

The larval musculature of *Drosophila melanogaster* derives from the embryonic mesoderm, which is formed by cells that are positioned ventrally in a blastoderm stage embryo. Mesoderm formation is initiated by the establishment of the dorsoventral axis of the embryo, a process that is accomplished by the maternally-provided Dorsal protein, a NF-κB-related transcription factor (see **Chapter 1.2**). A morphogenetic gradient of Dorsal is then generated by transport of the protein from the shared cytoplasm of the syncytial embryo to nuclei along the ventral side of the embryo (Bate, 1993; Belvin and Anderson, 1996). Upon subsequent cellularization, the nuclei of these ventrally-located cells contain high levels of Dorsal while those cells on the dorsal side of the embryo contain much lower levels. This concentration difference then contributes to early positional information in cells of the blastoderm stage embryo that commits them to different fates.

The nuclear localization of Dorsal results in the transcriptional activation of two zygotic genes, *twist* (*twi*) and *snail* (*sna*). Genetic studies have established that mesoderm formation is dependent on the presence of *twi*, which takes its name from the twisted appearance of embryos that attempt to gastrulate in its absence (Simpson, 1983; Grau *et al*., 1984). An abnormal ventral furrow is observed in these mutant embryos, but no mesoderm forms. By comparison, embryos mutant for *sna* exhibit no distinct furrow but do have subtle folds of cells that appear in the ventral region during gastrulation. Similar to the situation in *twi* mutants, mesoderm does not form in *sna* mutant embryos. The *twi* gene, which encodes a basic helix–loop–helix (bHLH)-containing transcription factor, is initially expressed in mesodermal cells at Stage 5 (Thisse *et al*., 1988; Leptin, 1991). This expression generally begins to decline during Stage 11 but persists in a subset of mesodermal cells that are progenitors of the muscles (Thisse *et al*., 1988; Bate, 1993; see Sections 2.1.2.2.2 and 2.1.2.3.1). By Stage 13, *twi* expression is confined to cells that include progenitors of the adult muscles and cells associated with the alary muscles (Bate *et al*., 1991).

The *sna* locus, another Dorsal target gene, encodes a zinc-finger-containing protein (Boulay, *et al*., 1987) that is also detected during Stage 5 (Kosman *et al*., 1991; Leptin, 1991). The key difference between the expression patterns of the two Dorsal target genes *twi* and *sna* is that the *sna* expression pattern exhibits sharp lateral borders that demarcate the boundary between the presumptive

mesoderm and neurogenic ectoderm. While both of these genes are required for mesoderm formation, comparison of the mutant phenotypes of embryos lacking either *twi* or *sna*, and embryos lacking both *twi* and *sna* suggest that the two genes play different roles in mesoderm development. Thus, *sna* is thought to play a predominantly permissive role by repressing nonmesodermal genes in the presumptive mesoderm. By contrast, *twi* appears to act as a transcriptional activator of downstream genes that are required for invagination, patterning, and differentiation of various mesodermal derivatives (Kosman *et al*., 1991; Leptin, 1991). It contributes, for example, to the transcriptional activation of *tinman* (*tin*) in the specification of heart and visceral muscle derivatives and, at high levels, is sufficient to specify cells as somatic muscle (Baylies and Bate, 1996; see Sections 2.1.2.2.2 and 2.1.2.3.1).

Following specification of the mesoderm, the *twi/sna*-expressing cells invaginate through the ventral furrow at gastrulation and form a tube-like structure (Leptin and Grunewald, 1990; Leptin *et al*., 1992). Mesoderm invagination is dependent on the *folded gastrulation* (*fog*) gene (Costa *et al*., 1994), which is expressed in a *twi*-dependent manner in ventral regions of the blastoderm. In *fog* mutants, the concerted apical constriction of mesodermal cells does not occur, leading to their failure to invaginate. As *fog* encodes a secreted protein, it is thought to act as a signal that coordinates apical constrictions of mesodermal cells (Costa *et al*., 1994). Shortly after invagination, as the embryonic germ band extends, mesodermal cells migrate dorsally to form a monolayer just beneath the ectoderm (Leptin and Grunewald, 1990; Leptin *et al*., 1992). This movement of mesodermal cells depends on mesodermal expression of the *Drosophila* fibroblast growth factor receptor, Heartless (Htl)/DFR1 (Shishido *et al*., 1993; Bieman *et al*., 1996 and Gisselbrecht *et al*., 1996; Shishido *et al*., 1997). In *htl/DFR1* mutant embryos, the mesoderm invaginates normally but fails to reach its dorsal destination to receive inductive signals from the ectoderm (Gisselbrecht *et al*., 1996). Understanding how Htl/DFR1 influences the dorsal migration of mesodermal cells awaits identification of the Htl/DFR1 ligand.

2.1.2.2. Segregation of the Visceral and Somatic Mesoderm

Shortly after dorsal migration of the invaginated mesoderm has occurred, the mesoderm is subdivided into units, from which the progenitors of different mesodermal tissues will arise. These tissues include the visceral and somatic musculature, the heart, and the fat body (Dunin-Borkowski *et al*., 1995; Azpiazu *et al*., 1996; Riechmann *et al*., 1997; see also **Chapters 2.6** and **2.9**). It is now clear that this subdivision is accomplished by combinatorial mechanisms that include both mesoderm-autonomous contributions as well as inductive influences from the overlying ectoderm. The overlapping signals derived from each of these mechanisms intersect in defined regions along the dorsoventral and anteroposterior axis. The developmental program of cells within each of these regions is then dictated by the combination of the signals they receive.

2.1.2.2.1. Signaling along the dorsal-ventral axis Dorsoventral patterning of the mesoderm occurs in response to secretion of the *decapentaplegic* (*dpp*) gene product, a member of the transforming growth factor-β (TGF-β) family (Padgett *et al*., 1987), from the dorsal ectoderm (Staehling-Hampton *et al*., 1994; Frasch, 1995). The Dpp signal in turn activates genes specific to the dorsal mesoderm, including *tin*, *bagpipe* (*bap*), and *Fasciclin III* (*FasIII*), that contribute to formation of the heart and visceral musculature. Ectopic expression of Dpp in ventral regions of the embryo, in either mesodermal or overlying ectodermal cells, results in the ventral expansion of the domains of *bap* and *FasIII* expression (Staehling-Hampton *et al*., 1994; Frasch, 1995). Dpp signaling also plays a role in the repression of ventral genes in the dorsal regions of the embryo. Among these is *pox-meso* (*poxm*), which encodes a paired domain-containing protein, and is normally expressed in ventrally-located mesodermal cells that contribute to the bulk of the somatic musculature (Bopp *et al*., 1989). Expression of *bap* and *FasIII* is abolished in *dpp* loss-of-function mutants, but is recovered when Dpp is provided ectopically (Frasch, 1995). In a reciprocal fashion, ectopic Dpp represses expression of *pox-meso* in the ventral mesodermal regions whereas *dpp* loss-of-function mutants exhibit an expanded domain of *pox-meso* (Staehling-Hampton *et al*., 1994). Thus, Dpp plays an inductive role in promoting proper specification of dorsal mesoderm and a repressive role in preventing these cells from being specified as ventral mesoderm.

The homeodomain-containing protein Tin, a target of Dpp, is essential for the formation of the heart, visceral musculature, and dorsal somatic musculature (Bodmer *et al*., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). The role of *tin* in cardiac development is covered in **Chapter 2.6** in this series, and will not be discussed further herein. Activation of *tin* by Dpp likely occurs through interaction of Dpp with the Dpp receptor Thickveins (Tkv; Yin and Frasch, 1998). Of note, *tin* is also a target of

the bHLH-containing Twi protein (Yin *et al.*, 1997). Specification of the visceral musculature is accomplished by the Tin-mediated activation of *bap*, another homeodomain-protein-encoding gene. Bap is expressed exclusively in the visceral mesoderm, and is essential for differentiation of the visceral musculature (Azpiazu and Frasch, 1993; see Section 2.1.4). Its expression is activated by Tin, and is absent in embryos mutant for *tin* (Azpiazu and Frasch, 1993). As expected from this cascade of expression, the visceral musculature is severely disrupted in embryos lacking *bap*. Interestingly, the presumptive visceral mesoderm can be transformed in these mutant backgrounds into body wall musculature and gonadal mesoderm (Azpiazu and Frasch, 1993).

2.1.2.2.2. Signaling along the anteroposterior axis The anteroposterior subdivision of the *Drosophila* embryo into a series of metameric units, or segments, has been extensively documented in the ectoderm. Similarly, the tissues that differentiate from the somatic and visceral mesoderm reflect a segmental organization and identity. The actual subdivision of the mesoderm into its derivatives occurs along the anterior–posterior axis within each metameric unit.

Dunin-Borokowski (Dunin-Borkowski *et al.*, 1995) examined this subdivision by marking all mesodermal cells with the mammalian cell surface protein CD2, expressed under the control of the *twi* promoter. This approach allowed cells located in different regions of the mesoderm to be followed, such that their subsequent developmental programs could be examined. As a consequence, they determined that the dorsal anterior cells invaginate and become visceral mesoderm. By contrast, both the dorsal and ventral posterior cells become somatic muscle progenitors. Their descriptions were reinforced, in this and other studies, by the examination of expression patterns of a variety of molecular markers for each of the progenitor types in mutant embryos (Dunin-Borkowski *et al.*, 1995; Azpiazu *et al.*, 1996; Riechmann *et al.*, 1997). The regional assignments of cells to different progenitor populations, including those of the visceral and somatic muscles, the heart and the fat body, were revealed by these analyses (Riechmann *et al.*, 1997; **Figure 1**).

Studies have now established that the mesoderm is partitioned along the embryonic anteroposterior axis by the action of the pair-rule genes *even-skipped* (*eve*) and *sloppy-paired* (*slp*) (Azpiazu *et al.*, 1996; Riechmann *et al.*, 1997; **Figure 1**). The *eve* locus encodes a homeodomain-containing protein, while the *slp* locus encodes a paired domain-containing protein (Macdonald *et al.*, 1986;

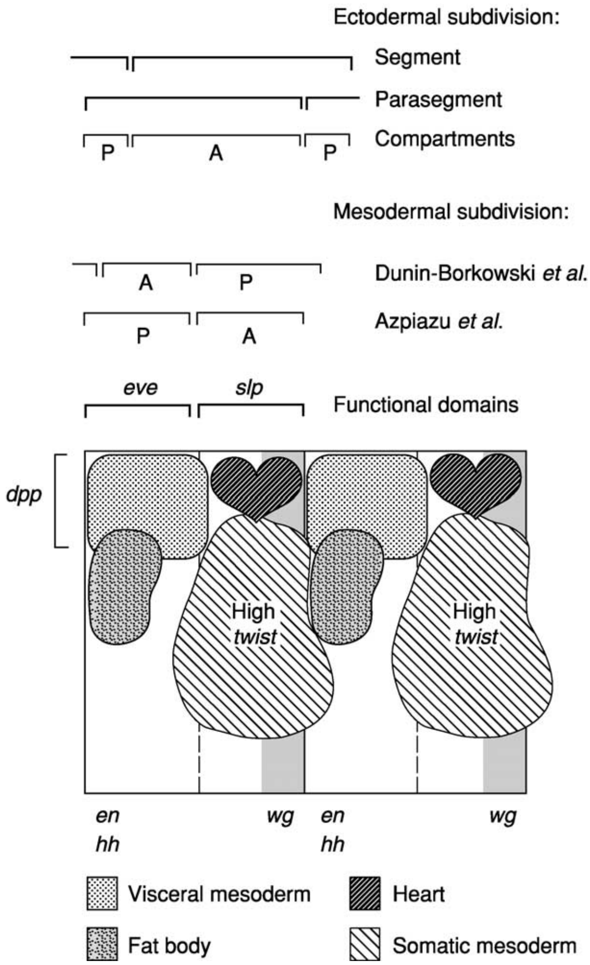


Figure 1 Positions of mesodermal subpopulations. The diagram shows the locations of mesodermal subpopulations relative to the units of subdivision used for the ectoderm (compartments and parasegments), the early mesoderm (A and P according to Azpiazu *et al.*, 1996), later mesoderm (A and P according to Dunin-Borkowski *et al.*, 1995), and the domains of *eve* and *slp* function in the mesoderm. The *slp* domain gives rise to the dorsally located heart precursors and to the majority of somatic muscles. Development of somatic muscles requires the maintenance of high levels of *twist* expression (Baylies and Bate, 1996). In the *eve* domain of each segment, the most dorsally located group of cells gives rise to the visceral musculature. The fat body primordium lies slightly more ventrally. The primordia of the visceral muscles and the fat body overlap the *engrailed* (*en*) and *hedgehog* (*hh*) expression domain, and these genes are necessary for their spatial determination (Azpiazu *et al.*, 1996). The heart primordium and the high Twist domain overlap the *wingless* (*wg*) expression domain and require Wg for their proper development (Bate and Rushton, 1993; Baylies *et al.*, 1995; Lawrence *et al.*, 1995; Wu *et al.*, 1995; Azpiazu *et al.*, 1996; Park *et al.*, 1996; Ranganayakulu *et al.*, 1996). Dpp signaling from the ectoderm induces development of visceral mesoderm, heart and dorsal somatic mesoderm (Staehling-Hampton *et al.*, 1994; Frasch, 1995; Baylies and Bate, 1996). (Reproduced with permission from Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R., Leptin, M., **1997**. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development 124*, 2915–2922; © The Company of Biologists Ltd.)

Grossniklaus *et al*., 1992). Subdivision by *eve* and *slp* generates alternating domains within each embryonic segment, from which progenitors of different mesodermal tissues derive. As DNA-binding transcriptional regulators, these two molecules then induce subsequent genes associated with the determination and differentiation of cells within each of these domains. The "*eve* domain," which gives rise to the progenitors of the visceral muscles and the fat body, is characterized by the subsequent expression of *bap* and *serpent* (*srp*), respectively. The "*slp* domain" includes presumptive somatic myoblasts that will go on to express high levels of Twi, and heart progenitors that will later express genes that include *tin* (Azpiazu *et al*., 1996; Riechmann *et al*., 1997). These different mesodermal anlagen form interdependent groups of cells within the mesoderm, such that an increase or decrease in the number of progenitor cells for one tissue results in a compensatory change in the number of one or more of the other progenitors. For example, primordia of the somatic muscles that express high levels of Twi are absent in embryos lacking *slp* (Riechmann *et al*., 1997), while those of the visceral musculature are expanded in these mutants. By comparison, embryos lacking *eve* lose cells that express *bap*, and retain cells that express high levels of Twi. Although the domain of high Twi-expressing cells is not expanded in the *eve* mutant embryos, that of the *slp*-expressing cells is increased. Together, these data support the view that *eve* and *slp* play critical roles, both negative and positive, in the specification of each of these cell types (Riechmann *et al*., 1997).

The subdivision along the anteroposterior axis is also controlled, at least in part, by segment polarity genes that include *wingless* (*wg*), *hedgehog* (*hh*), and *engrailed* (*en*). All of these genes function downstream of *eve* and *slp* (Azpiazu *et al*., 1996; Riechmann *et al*., 1997; **Figure 1**). For example, the effect of *slp* on high Twi-expressing myogenic progenitors is partly due to its effect on *wg*, a Wnt family member. Wg is expressed in regions of the *slp* ectodermal domain and plays an important role in the development of the mesodermal *slp* domain (Azpiazu *et al*., 1996). Thus, in *wg* mutants, heart progenitors are absent (Lawrence *et al*., 1995; Wu *et al*., 1995; Park *et al*., 1996) as well as some somatic muscle precursors (Bate and Rushton, 1993; Volk and VijayRaghavan, 1994; Baylies *et al*., 1995; Ranganayakulu *et al*., 1996). Conversely, the *bap*-expressing progenitors of the visceral muscles are significantly expanded in *wg* mutant embryos (Azpiazu *et al*., 1996). In addition, overexpression of *wg* and components of the *wg* signaling pathway induce supernumerary heart precursors (Lawrence *et al*., 1995; Park *et al*., 1996) at the expense of the visceral mesoderm (Park *et al*., 1996).

The *hh* gene, which encodes a secreted molecule related to vertebrate Sonic hedgehog, is expressed in the ectodermal domain of Eve-expressing cells, and promotes the further development of cells within the mesodermal *eve* domain (Azpiazu *et al*., 1996). Its expression is required, but not sufficient, for formation of the visceral muscles and fat body through the induction of *bap* and *srp* expression. In a negative regulatory loop, *wg* antagonizes the action of *hh* in the *slp* domain, preventing *bap* and *srp* expression and promoting development of the heart and somatic muscles (Azpiazu *et al*., 1996). These results suggest that *wg* and *hh* are required for the maintenance of differential gene expression that is initially activated by pair-rule genes in the *eve* and *slp* domains of the mesoderm. It should be noted that, like the requirement for secreted Dpp in the dorsoventral patterning of the mesoderm described earlier, signaling molecules responsible for inductive events along the anteroposterior axis can originate in overlying ectodermal cells as well as within the mesoderm itself (Lawrence *et al*., 1994; Baylies *et al*., 1995; Lawrence *et al*., 1995; Azpiazu *et al*., 1996; Ranganayakulu *et al*., 1996), leading to the suggestion that they might play a permissive rather than an instructive role (Lawrence *et al*., 1995).

As implied in the above discussion, an important manifestation of mesoderm subdivision, specifically related to the visceral and somatic musculature, is the differential expression of Bap and Twi. Bap expression is restricted to cells that will develop as visceral musculature and Bap serves as a marker for the subsequent differentiation of these progenitors. By contrast, Twi is initially expressed uniformly throughout the mesoderm but later modulates along the anteroposterior axis such that one observes domains with high levels of expression and domains with somewhat lower levels of expression (Dunin-Borkowski *et al*., 1995). This modulation occurs in response to *wg* and *slp*, such that high levels of Twi are present in cells within the *slp* domains and low levels in cells of the *eve* domains (Dunin-Borkowski *et al*., 1995). In subsequent studies, the perdurance of high levels of Twi in the *slp* domain was shown to be essential for somatic myogenesis and prevents the formation of other mesodermal derivatives (Baylies and Bate, 1996; see Section 2.1.2.2.1).

Lastly, additional genes appear to be expressed in broad domains in the early mesoderm and may play roles in the specification and/or subdivision of the mesoderm, including *zinc finger homeodomain 1* (*zfh1*) (Lai *et al*., 1991) and the P2 transcript of *pointed* (Klambt, 1993). The actual role of each of

these proteins has not been revealed by genetic studies, since only subtle somatic muscle defects are displayed by each of these mutant embryos. However, some data suggests that *zfh1* may play a negative role in somatic myogenesis. It encodes a zinc-finger homeobox protein that is initially expressed throughout the presumptive mesoderm, but is later downregulated (Lai *et al*., 1991). It can bind to and represses transcriptional activity from E-boxes, the canonical target sites of bHLH-containing proteins. Moreover, ectopic expression of *zfh1* beyond its normal time period prevents differentiation of somatic but not visceral muscle (Postigo *et al*., 1999).

2.1.2.3. Determination of Somatic Myoblasts

Transcription factors play a critical role in activating a program of gene expression that commits cells to a myogenic program of differentiation. Some of the key factors associated with the somatic musculature are discussed below.

2.1.2.3.1. The role of the bHLH protein Twist As described earlier, the somatic mesoderm is characterized by high levels of the bHLH transcriptional regulatory protein Twi, whereas relatively lower levels of Twi are present in the progenitors of the visceral musculature and heart. Baylies and Bate (1996) demonstrated the importance of the level of Twi in determining the developmental fates of mesodermal cells by artificially modulating this level using the GAL4/UAS system. Their studies clearly demonstrated that the fate of presumptive visceral and cardiac myoblasts could be selectively altered by high levels of Twi. Changes included decreased expression of markers associated with visceral muscle fate and increased expression of muscle myosin, both characteristic features of somatic muscle fibers. While these changes suggested that high levels of Twi commit cells to a somatic muscle fate, this interpretation was more firmly established by the demonstration that ectopic expression of Twi could drive ectodermal cells into a myogenic program of differentiation (Baylies and Bate, 1996). Thus, Twi acts early in the pathway, through which myoblasts acquire the ability to express muscle-specific structural genes.

More recent studies have explored the observation that bHLH-containing proteins function as dimers, and have addressed whether Twi functions as a homodimer or acts in concert with the bHLH-containing E-protein Daughterless (Da) as a heterodimer partner. In short, these studies demonstrated that Twi homodimers function to promote a somatic muscle phenotype in mesodermal cells, while Da-Twi heterodimers actually repress this fate, presumably functioning in other mesodermal derivatives (Castanon *et al*., 2001).

2.1.2.3.2. Selection and segregation of muscle progenitors Following the segmental subdivision of the mesoderm discussed earlier, the somatic mesoderm is further divided into regions that are competent to form muscles with specific identities (Halfon *et al*., 2000). Within these competence domains, the progenitors for particular muscles will be singled out from cells that comprise an equivalence group (Carmena *et al*., 1995). This process has been best illustrated by a series of genetic experiments in which modulation of various signaling components defines sequential events for the development of a pair of dorsal embryonic muscles (reviewed in Frasch, 1999; Baylies and Michelson, 2001).

First, ectodermal signaling from both Wg and Dpp defines a region which becomes competent for dorsal muscle development (Carmena *et al*., 1998a) (**Figure 2**). The combined activities of Wg and Dpp then result in the activation of the proneural gene *lethal of scute l(1)sc* in the cells of this competence domain (Carmena *et al*., 1995; Ruiz-Gomez and Bate, 1997; Carmena *et al*., 1998a). Through a novel mechanism, the Notch protein plays a key repressive role in restricting the size of this competence domain (Rusconi and Corbin, 1998; Brennan *et al*., 1999; Rusconi and Corbin, 1999). Once determined, the localized activation of the Ras1 pathway in this domain restricts L(1)sc to a smaller cell cluster that represents the equivalence group (Carmena *et al*., 1998a). This process is mediated by Htl/DFR1 and the *Drosophila* epidermal growth factor receptor (EGFR), two receptor tyrosine kinases (RTKs) (Buff *et al*., 1998).

All cells comprising this individual L(1)sc-expressing equivalence group are initially thought to be equipotent at this stage. However, two cell types, both of them critical for somatic myogenesis, will be derived from the cells in this population. These include the muscle progenitors, which play a key role in patterning the musculature, and fusion competent cells, which are essential to the formation of multinucleate syncytia. In each L(1)sc-expressing equivalence group, a single muscle progenitor will emerge as a consequence of lateral inhibition mediated by Notch/Delta (Dl) signaling (Corbin *et al*., 1991; Bate *et al*., 1993; Carmena *et al*., 1995; Baker and Schubiger, 1996) and the activity of Argos (Aos), a diffusible inhibitor of EGFR (Carmena *et al*., 2002). L(1)sc expression will be progressively restricted to all but one cell (Carmena *et al*., 1995). This cell will go on to serve as a muscle progenitor

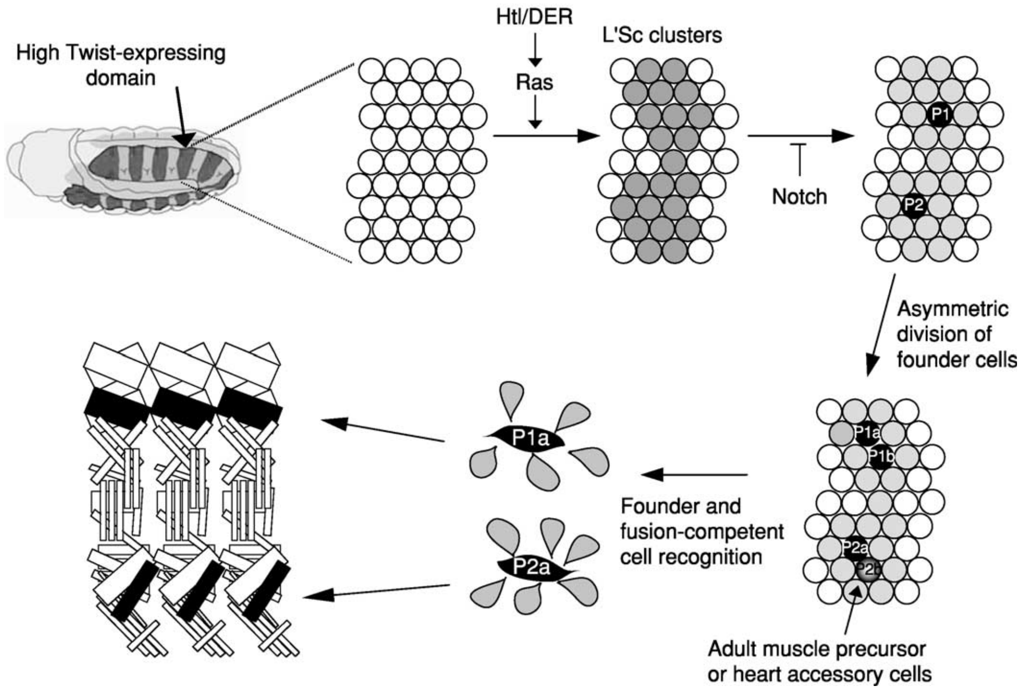


Figure 2 A schematic representation of the steps involved in *Drosophila* somatic muscle development. Twi is expressed at high (dark gray) and low (light gray) levels in segmentally repeated domains in the embryo. A variety of positional cues, in addition to localized activation of the Ras1 signaling pathway via the RTKs Htl and EGFR, result in discrete clusters of L(1)sc expressing cells in the high Twist-expressing domain. Two representative clusters are shown in gray. The process of lateral inhibition mediated by the Notch then singles out muscle progenitors (represented by P1 and P2 in black), with L(1)sc expression declining in the surrounding cells. Asymmetric division (see **Figure 3**) of the progenitor results in two founders (P1a and P1b) or a founder (P2a) and an adult muscle precursor or heart accessory cell (P2b). The other cells in the cluster become fusion-competent cells (light gray). The founder cells then fuse with surrounding fusion-competent cells to form multinucleate syncytia.

(**Figure 2**), while the rest of the cells within each group appear to become fusion-competent cells. A similar sequence of events is expected to occur in the specification of other muscle progenitors, with different combinations of signals being responsible for each equivalence group and resulting muscle progenitor. Though not actually shown for the progenitors of each muscle fiber, every equivalence group seems likely to give rise to a specific muscle progenitor with a restricted cell fate. Consistent with this hypothesis, there appear to be at least 19 distinct L(1)sc-expressing equivalence groups in each hemisegment, which arise from four regionally different cell populations within the somatic mesoderm (Carmena *et al.*, 1995). Thus, the potential exists for many distinct progenitors within the pool of equivalence groups. Of note, all muscle progenitors appear to segregate from L(1)sc-expressing equivalence groups through the action of Notch (Corbin *et al.*, 1991; Bate *et al.*, 1993; Carmena *et al.*, 1995; Baker and Schubiger, 1996). However, while many progenitors require *wg* and the activities of Htl/DFR1 and EGFR, not all of them do (Baylies *et al.*, 1995; Ranganayakulu *et al.*, 1996; Buff *et al.*, 1998; Schulz and Gajewski, 1999). Thus, perhaps a combination of signaling molecules distinguishes each equivalence group and, more importantly, the resulting muscle progenitor. Consistent with this model, the actions of Wg, Dpp and Ras have been shown to converge on a single enhancer element that integrates these positive inputs to regulate expression of the dorsal founder cell marker *eve* (Halfon *et al.*, 2000).

In contrast to the progenitor myoblasts, it is generally assumed that fusion-competent cells from different equivalence groups are all identical. Cell transplantation studies have indicated that the muscle cells are of equivalent potential and can contribute to the appropriate muscles in the region of the mesoderm into which they are tranplanted (Beer *et al.*, 1987). However, these studies examined cells approximately 8–12 min after gastrulation, which may be too early for some cell fate decisions to have been made. Thus, it seems a formal possibility that these cells may not be a homogeneous population, particularly since they arise from equivalence

groups in different regions of the mesoderm that are subjected to the influence of different signaling molecules. Future efforts may provide additional information on this topic.

After segregation of the L(1)sc-expressing muscle progenitors by various combinations of signals, each will initiate expression of a particular repertoire of nuclear proteins that mark subsets of progenitors. These proteins, the so-called muscle identity factors, play a major role in the specification and development of progenitors for each muscle fiber. This cell also divides asymmetrically to give rise to two daughters, each with a different muscle fate. The resulting founder myoblasts are the fundamental units that will then pattern the musculature, discussed in the next section.

2.1.3. Generation of Pattern in the Somatic Musculature

The somatic or body wall muscles of the *Drosophila* larva comprise a segmentally repeating pattern of 30 distinct muscle fibers per abdominal hemisegment. Each of these muscles is unique, and has properties acquired in the course of its development, through which its identity can be distinguished. These properties include size, shape, pattern of innervation, and sites of epidermal attachment. Even before such morphological distinctions are apparent, a variety of studies in the last 15 years have revealed that the pattern of muscle fibers and the identity of individual myoblasts are determined quite early in the myogenic process. This identity is established at the level of the equivalence groups and muscle progenitors described above. These cells give rise to founder myoblasts, each defined by its unique position and characteristic pattern of gene expression. The expression of particular genes provides markers for different muscle founders, and likely controls their subsequent developmental programs. Through the action of the proteins that these genes encode, founder myoblasts seed the formation of particular muscle fibers and begin to acquire features characteristic of particular muscles.

2.1.3.1. Generation of Muscle Founder Cells by Asymmetric Division

Following segregation of the muscle progenitors by a combination of signaling events, as discussed in Section 2.1.2.3 and shown in **Figure 2**, the progenitors divide to give rise to two cells with different fates. Studies following the fates of individual daughter cells have revealed six examples, in which the progenitors give rise to two sibling larval muscle founder cells (Carmena *et al*., 1995; Ruiz-Gomez and Bate, 1997; Jagla *et al*., 1998; Crozatier and Vincent, 1999). Thus, the "typical" founder cell seeds the formation of two distinct embryonic muscle fibers. Two examples have also been identified in which division of the progenitor cell gives rise to a larval founder cell and an adult muscle precursor (Carmena *et al*., 1995; Jagla *et al*., 1998). Interestingly, the adult muscle precursor maintains expression of Twi, proliferates, and goes on to form specific adult muscles (Bate *et al*., 1991; Carmena *et al*., 1995). Furthermore, one example has been found in which the progenitor gives rise to a larval founder cell and a pair of heart accessory cells (Carmena *et al*., 1998b; Park *et al*., 1998). Since the terminal fates of all daughter cells derived from progenitor cell division have not been mapped, future studies may reveal other fates for these daughter cells and provide insights into the frequency of each of the above paths.

Clearly, however, asymmetric division by muscle progenitors is a crucial step in the generation of muscle founder cells with distinct identities. In each of the progenitor cell divisions, daughter cells acquire a different cell fate as a consequence of asymmetric distribution of molecules. Insights into the identity of these molecules and possible functions in the somatic musculature came from studies on neurogenesis. Asymmetric division during development of the peripheral nervous system involves the cytoplasmic proteins Numb, Inscutable (Insc), and Prospero (Pros). In neuronal cells, Numb and Pros are provided to only one daughter cell in a mechanism that is dependent on *insc* (Rhyu *et al*., 1994; Hirata *et al*., 1995; Knoblich *et al*., 1995; Spana and Doe, 1995; Kraut and Campos-Ortega, 1996; Kraut *et al*., 1996; Spana and Doe, 1996; **Chapters 1.10, 1.11**, and **2.3**). Embryos lacking either *numb* or *insc* exhibit defects in muscle development, implicating them in the asymmetric division of muscle progenitors, shown schematically in **Figure 3** (Burchard *et al*., 1995; Uemura *et al*., 1996; Knirr *et al*., 1999). In contrast, *pros* does not appear to be expressed in the mesoderm, and mutants do not display any mesodermal defects (Broadie and Bate, 1993a). It is now clear that Numb is spatially restricted to a crescent on one side of the parent muscle progenitor, and segregates to only one daughter founder cell upon division (Ruiz-Gomez and Bate, 1997; Carmena *et al*., 1998b) (**Figure 3**). As in the nervous system, this asymmetric distribution is dependent on *insc* [previously identified as *not-enough-muscles* (*nem*); Burchard *et al*., 1995], and is localized in a reciprocal pattern to that of Numb on the opposite side of the cell (Ruiz-Gomez and Bate, 1997; Carmena *et al*., 1998b).

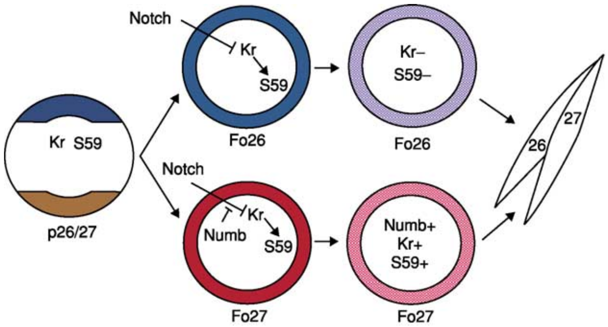


Figure 3 Sibling founder cells produced by a muscle progenitor adopt different cell fates due to asymmetric localization of cytoplasmic proteins. In this example, the progenitor to muscles 26 and 27 (p26/27) expresses the muscle identity genes *S59* and *Kr*, among others. Numb (shown in red) and Inscuteable (shown in blue) are asymmetrically distributed as crescents on opposite sides of the muscle progenitor. Upon cell division, Numb segregates only to one of the daughter cells, in which it then inhibits Notch signaling. Inactivation of the Notch pathway in this cell allows maintenance of *Kr* and *S59* expression, and defines this daughter cell as the founder to muscle 27 (f27). Activation of the Notch pathway in the sibling cell, which does not receive Numb, inhibits the expression of the muscle identity genes *Kr* and *S59*. This cell goes on in development to become the founder to muscle 26 (f26).

The *insc* gene encodes a putative cytoskeletal adaptor protein (Kraut and Campos-Ortega, 1996), and mutant embryos exhibit defects in the orientation of the mitotic spindle and localization of intrinsic factors (Burchard *et al*., 1995; Kraut *et al*., 1996; Knirr *et al*., 1997; Buescher *et al*., 1998; Jan and Jan, 1998). By comparison, *numb* encodes a membrane-associated protein that contains a PTB motif, which is essential for interaction with Notch (Uemura *et al*. 1989; Guo *et al*., 1996). As in neuronal specification, Numb inhibits the Notch signaling pathway (Frise *et al*., 1996; Ruiz-Gomez and Bate, 1997; Carmena *et al*., 1998b; **Chapters 1.10**, **1.11**, and **2.3**). The differential activation of Notch in only one daughter cell is, in turn, responsible for a different pattern of gene expression in this daughter cell. Thus, segregation of Numb switches cells from a default state, in which Notch signaling represses muscle identity genes, to an alternate state, in which the absence of Notch signaling allows the continued expression of these genes. In *insc/nem* mutant embryos, Numb fails to segregate to one side of the muscle progenitor and is distributed to both daughter founder cells (Ruiz-Gomez and Bate, 1997). Therefore, two identical muscle founder cells are present rather than two founder cells for different muscles. As a consequence, *numb* mutant embryos exhibit a defective muscle pattern, in which there is a duplication of some muscles and corresponding loss of others. A reciprocal change in the identity of the duplicated and missing muscles is observed when Numb is overexpressed (Ruiz-Gomez and Bate, 1997).

Another molecule involved in asymmetric cell division is *sanpodo* (*spdo*), which was shown to control the asymmetry of neuronal sibling fate by acting in opposition to *numb* (Dye *et al*., 1998; Skeath and Doe, 1998). Park *et al*. (1998) have demonstrated that *numb* is epistatic to both *spdo* and *Notch*. Since *spdo* encodes a tropomodulin-like protein, the authors speculate that it acts to mediate Notch signaling and transduces information from the membrane to the nucleus, possibly through association with the actin cytoskeleton.

In summary, the intrinsic factors Numb, Insc, Notch and Spdo play important roles in the generation of diversity of the larval body wall muscles, by ensuring that sibling founder cells have distinct fates. These founders go on to seed the formation of the 30 unique muscle fibers that make up the pattern of the larval musculature discussed in Section 2.1.3.2.

2.1.3.2. Grasshopper Muscle Pioneers and *Drosophila* Founder Myoblasts

Implicit in the mechanism described earlier for the segregation of founders is the notion that each founder myoblast possesses the information necessary to specify a particular muscle, and that it is able to convey this information to other cells as fusion progresses. The concept that muscle patterning might be accomplished by cells with such roles, originated with morphological studies of myogenesis in the grasshopper. Using a dye-fill approach, muscle "pioneers" were identified by their distinct morphological characteristics and behavior (Ho *et al*., 1983). These pioneers are large cells of mesodermal origin that arise early in development, when the embryonic environment is relatively simple. At this stage, the pioneers do not display features characteristic of differentiated muscles. Rather, they associate with specific sites of the ectoderm, erecting

a scaffold that delineates the territories of future muscles (Ho *et al*., 1983). Later in development, the number of nuclei in the pioneers was observed to increase, as a consequence of fusion with neighboring smaller mesodermal cells. These cells cluster around the large muscle pioneer and fuse with it to form an individual muscle (Ball and Goodman, 1985a; Xie *et al*., 1992). Most importantly, the position of every muscle in the grasshopper embryo appears to be prefigured by the presence of a mononucleate muscle pioneer in the same location (Ball and Goodman, 1985a; Ball and Goodman, 1985b; Ball *et al*., 1985b; Xie *et al*., 1992). These observations suggested that the identity of each muscle fiber might be connected to the specific features of the muscle pioneer from which it was derived.

In addition to their being morphologically distinct from the smaller mesodermal cells, the grasshopper muscle pioneers exhibit different labeling patterns with monoclonal antibodies, suggesting that these cells are also molecularly distinct (Chang *et al*., 1983; Ball *et al*., 1985a). The muscle-organizing pioneers are necessary for motor neuron growth cones to locate (or recognize) the appropriate target (Ball *et al*., 1985a). However, the most direct demonstration of their role in muscle development and muscle identity came from ablation experiments, in which the loss of a specific pioneer early in development correlated with the loss of a particular muscle fiber later. In its place, investigators observed a loose mass of small mesodermal cells that do not surround a muscle pioneer (Ball *et al*., 1985a). Thus, single pioneer cells provide an early framework for pattern formation that is retained even as the embryonic terrain becomes more complex.

The central role for muscle pioneers as pattern organizers in early muscle development seems to be a general feature of muscle assembly in arthropods and annelids (Jellies, 1990). Prior to the identification of markers for the muscle progenitors and founder cells, morphological studies suggested the presence of pioneer-like cells in the embryonic mesoderm of *D. melanogaster*. Both immunostaining with β3-tubulin and dye filling have identified segmentally repeated cells at the end of gastrulation that extend characteristic, growth cone-like processes that appeared to delineate sites of formation of future muscle fibers (Leiss *et al*., 1988; Johansen *et al*., 1989). In a methodical and thorough morphological analysis of somatic muscle formation in the *Drosophila* embryo, Bate (1990) identified distinct binucleate and trinucleate precursors in characteristic and reproducible positions as the earliest signs of muscle differentiation. All of these precursors were observed to lie exclusively in the outer layer of mesodermal cells that are in direct contact with the ectoderm. The cells immediately beneath this layer appeared to provide a population of cells that fused with the precursor and became depleted as the muscles enlarged. On the basis of these observations, Bate inferred the presence of cells reminiscent of the grasshopper pioneers that initiate the formation of bi or trinucleate precursors (Bate, 1990). These cells were termed "founder myoblasts". In contrast to the pioneers in the grasshopper, which enlarge and extend growth cones before fusion begins, muscle founders in the *Drosophila* embryo are not large enough to span the territory of the muscles they prefigure. As fusion with surrounding myoblasts proceeds, however, the syncytial precursor grows and extends growth cone-like endings toward future insertion sites on the epidermis. The remaining pool of cells that fuse with founders to form muscle precursors and mature muscle fibers was termed the "fusion-competent" population, mentioned in previous sections. Bate further proposed that the fusion-competent cells are equivalent prior to their fusion with a founder cell, but then become entrained by this founder cell to the distinct differentiation program of a unique muscle fiber (Bate, 1990). Concurrent studies corroborated the presence of distinct muscle precursors by using antisera directed against the homeodomain-containing transcription factor S59 to visualize the inferred "founder cells" (Dohrmann *et al*., 1990). In these studies, S59 expression was observed in a small subset of segmentally repeated mononucleate cells in the embryonic mesoderm that are larger than other mesodermal cells, consistent with their identification as muscle founders.

These studies imply that in *Drosophila*, as in the grasshopper, the muscle precursors and/or muscle founder cells are morphologically distinct from the fusion-competent cells, in that they are larger (Dohrmann *et al*., 1990) and extend characteristic growth cone-like processes (Leiss *et al*., 1988; Johansen *et al*., 1989). Nevertheless, these cells were observed only transiently, since fusion begins shortly after founder cell segregation. The existence of these founder myoblasts was addressed more directly using embryos, in which the process of myoblast fusion was blocked, and the muscle progenitors and founder cells were more readily observed (Rushton *et al*., 1995). This analysis utilized embryos mutant for the *myoblast city* (*mbc*) locus (Rushton *et al*., 1995; Erickson *et al*., 1997) to examine the morphology of the myoblasts late in myogenic differentiation. In the absence of fusion, the founder myoblasts were present in characteristic locations, and expressed the normal repertoire

of muscle identity genes (see Section 2.1.3.3). They appeared to differentiate as mononucleate contractile muscle fibers, with proper innervation and functional neuromuscular synapses (Rushton *et al*., 1995; Prokop *et al*., 1996). Moreover, they extended processes toward the correct muscle attachment sites. In this early study, it was concluded that the fusion-competent cells remained rounded and did not develop features associated with a mature muscle fiber, with the exception of expression of contractile proteins (Rushton *et al*., 1995). More recent phenotypic examination of *mbc* null embryos has revealed that these fusion-competent cells are actually capable of migrating to the founder myoblasts but are unable to generate syncytia (Abmayr *et al*., 2003). Thus, studies from both the grasshopper and the fruitfly indicate that the insect mesoderm is prepatterned by somatic myoblasts with distinct morphologies and individual identities, and that these pioneers or founder cells possess information associated with the identity of each particular muscle.

In the *Drosophila* embryo, segment-specific pattern of muscle precursors is apparent from their earliest emergence, prompting the question of whether homeotic genes play a role in development of segmental differences. The role of homeotic genes in the generation of pattern in the somatic musculature is discussed in Section 2.1.3.3.2 (see also **Chapter 1.8**).

2.1.3.3. Establishment of Muscle Identity

The grasshopper muscle pioneers described above are striking in that, not only do they provide a morphological illustration of the presence of two types of myoblasts, but each also has a distinct muscle-specific identity (Chang *et al*., 1983; Ball *et al*., 1985a). Indeed, features of the developing *Drosophila* musculature are reminiscent of those seen in the grasshopper, and indicate that similar cell fate decisions occur (Rushton *et al*., 1995). In this section, we turn to molecular studies describing the genes that confer and/or interpret these identities and direct unique patterns of specification or differentiation.

2.1.3.3.1. Combinatorial control of muscle identity As muscle progenitors and/or founder myoblasts segregate from the other cells of the equivalence group, they begin to express specific nuclear proteins. This unique pattern of gene expression is one of the first molecular indications, that the founder myoblasts possess distinct identities, and suggests that these identities are established at the time of cell segregation. Founder cell-specific nuclear proteins include putative transcription factors such as Nautilus (Nau) (Michelson *et al*., 1990; Paterson *et al*., 1991), S59 (Dohrmann *et al*., 1990), Apterous (Ap) (Bourgouin *et al*., 1992), Vestigial (Vg) (Williams *et al*., 1991), Muscle segment homeobox (Msh) (Lord *et al*., 1995), Ladybird (Lb) (Jagla *et al*., 1998), Kruppel (Kr) (Gaul *et al*., 1987), Eve (Frasch *et al*., 1987), and Collier (Col) (Crozatier and Vincent, 1999). Since these proteins have been useful markers for founder cells, many have been mentioned in earlier sections of this review that deal with the origins of the founder cells. We now turn to their actual roles in generating muscle diversity.

In all cases, these proteins are initially expressed in the nuclei of a subset of founder myoblasts and thereafter in all nuclei of the syncytia derived from these founders. Thus, fusion of a founder cell with surrounding fusion-competent cells results in the induction of the corresponding marker in other nuclei of the syncytium. The expression of these proteins can, in most cases, be followed into a subset of mature muscles. In one example of such a pattern of expression, staining for S59 demonstrated that its expression is turned on in the nuclei of myoblasts that fuse with the S59 expressing founder. This expression can be followed into four mature muscle fibers in each hemisegment of the embryo (Dohrmann *et al*., 1990). A second example is shown in **Figure 4**, in which Nau (Michelson *et al*., 1990; Paterson *et al*., 1991) is observed in an early embryo in the founder cell for muscle 26 [using the nomenclature of Crossley (1978) or VA1 using the nomenclature of Bate (1993)], and can later be seen in multiple nuclei within the muscle 26 (VA1) syncytium (Abmayr *et al*., 1992; Abmayr and Keller, 1998). These data suggest that information associated with distinct muscle fiber identities is contained within the founder cells and transferred to the "naïve" fusion-competent cells as they fuse with it (Dohrmann *et al*., 1990), and are consistent with the hypothesis put forth by Bate (1990) that fusion-competent cells are entrained by the founder cell to the distinct differentiation program of a unique muscle fiber.

The differential expression patterns of *nau* and the genes encoding the other proteins discussed above suggest that they act as "muscle-identity" genes and could play a pivotal role in specifying the characteristics of individual muscles. Indeed, the results of genetic studies analyzing both loss-of-function mutations and overexpression of several of these proteins are consistent with such a hypothesis. The first identified muscle identity gene, *S59*, encodes a homeodomain-containing protein that is expressed in a subset of muscles precursors and

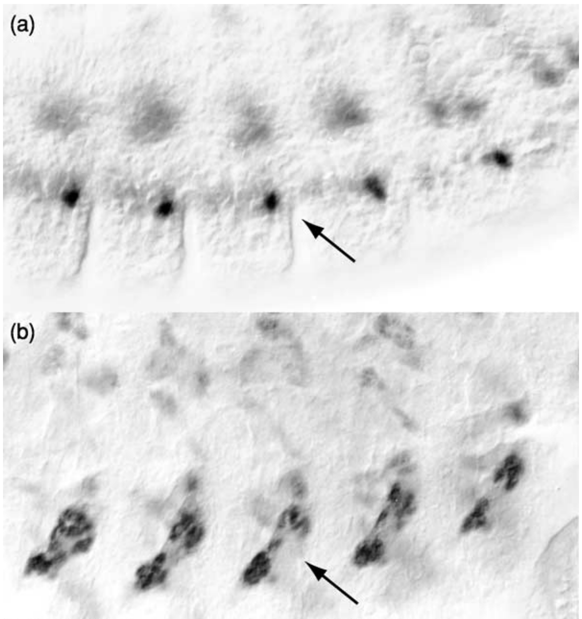


Figure 4 Fusion-competent cells take on Nau expression as they fuse with a Nau-expressing founder. Using Nau as a representative muscle identity gene, these data demonstrate that Nau is initially expressed in the nucleus of a specific founder myoblast. As multiple rounds of fusion occur, its expression can be followed in all the nuclei of the syncytia derived from this founder, supporting the model that the fusion-competent cells take on the characteristics of the founder cell with which they fuse. (a) Wild type Stage 12 embryo in which Nau expression is detected in a subset of single mesodermal muscle precursor cells. (b) Wild type Stage 15 embryo in which some cycles of fusion has occurred. As fusion progresses additional nuclei within the developing syncytia take on Nau expression. (Reprinted with permission from Abmayr, S.M., Keller, C.A., **1998**. *Drosophila* myogenesis and insights into the role of *nautilus*. In: Pedersen, R.A., Schatten, G.P. (Eds.), Current Topics in Developmental Biology. Academic Press, New York, NY, pp. 35–80; © Elsevier.)

neurons. Expression of S59 can be followed into several differentiated fibers (Dohrmann *et al.*, 1990; Carmena *et al.*, 1995), and overexpression produces alterations in the somatic muscle pattern (Knirr *et al.*, 1999). In mutant alleles of *S59*, termed *slouch* (*slou*), the development of muscles from the S59 expressing founder myoblasts is disrupted (Knirr *et al.*, 1999). The *ap* gene encodes a LIM- and homeodomain-containing protein that is also expressed in a subset of muscle precursors and neurons (Bourgouin *et al.*, 1992). Lack of *ap* function results in the loss of specific muscle fibers in the embryo, while misexpression causes muscle duplications. Kr and Lb, two homeodomain-containing proteins, are required for the formation of specific muscles, and their development is affected in embryos lacking their genes (Ruiz-Gomez and Bate, 1997; Jagla *et al.*, 1998). Conversely, Kr overexpression leads to muscle transformations (Ruiz-Gomez and Bate, 1997). Col, a COE (Col/Olf-1/EBF) transcription factor, plays a role in the formation of one specific muscle fiber (Crozatier and Vincent, 1999). Its gene is initially transcribed in the progenitors and founder cells for muscles DA3 and DO5, but expression is maintained only in the founder for DA3. While ectopic expression in the DO5 founder does not induce it to adopt a DA3 fate, *COE* mutant embryos are missing muscle DA3. Thus *COE* appears to serve as another muscle identity gene.

Msh, another homeodomain-containing protein, also marks a subset of muscle precursors (Lord *et al.*, 1995; D'Alessio and Frasch, 1996). Ectopic expression of Msh results in a disruption of the somatic muscle pattern, whereas a subset of muscles are missing in *msh* mutants (Lord *et al.*, 1995; Nose *et al.*, 1998). Interestingly, the Msh-expressing muscle progenitors and founder cells appears to segregate normally in *msh* mutants, suggesting that it is required for their subsequent differentiation (Nose *et al.*, 1998). A similar conclusion was reached in the case of Nau, the *Drosophila* MyoD homolog. In these studies, the fact that founder myoblast for muscle 3 (DA3) expresses both Nau and Vg proved to be very useful. In the same studies, embryos lacking *nau* were found to be missing a specific subset of muscle fibers, including muscle 3 (DA3) (Keller *et al.*, 1998; **Figure 5c**). Nevertheless, the founder for muscle 3 (DA3) was present, as evidenced by its expression of Vg. This cell did not differentiate into a multinucleate syncytium, however, indicating that *nau* was essential for this process. In some cases, muscles that normally express *nau* were present in the loss-of-function alleles, but their position and structure was somewhat aberrant. By comparison, ectopic expression of *nau* does not appear to affect the differentiation of muscles that normally express *nau*. Rather, the differentiation of founder myoblasts that do not normally express *nau* appears to be perturbed. These muscles are often incorrectly attached, in the wrong location, or appear to be duplications of muscles that normally express *nau*, such as muscle 5 (LO1), shown in **Figure 5d** (Keller *et al.*, 1997). Such duplications suggest that *nau* expression has altered the differentiation program of a specific muscle founder cell to an alternative plan.

It seems unlikely that a unique muscle identity gene exists for each of the 30 muscles in an abdominal hemisegment to drive each of their differentiation programs. In fact, many of these proteins are expressed in multiple, overlapping, subsets of founder myoblasts and differentiated muscle fibers. Some of these proteins may serve redundant roles

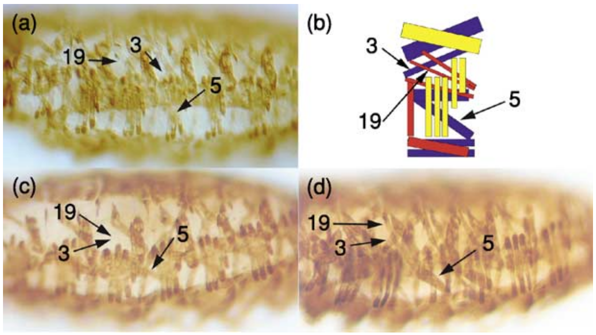


Figure 5 Loss of function and ectopic expression of muscle identity genes can alter the differentiation programs of specific subsets of somatic muscle fibers. Using the example of Nau, these data demonstrate that it plays an important role in dictating the characteristics of individual muscles. Embryos are positioned with dorsal to the top and anterior to the left. The somatic muscle pattern is visualized by immunostaining for muscle myosin. (a) Wild type embryo with somatic muscle pattern of 30 muscle fibers per hemisegment. (b) Schematic representation of a subset of the larval somatic muscle fibers. Inner, middle, and outer layers are shown in blue, red, and yellow respectively. Muscle fibers 3 and 19 have been highlighted. (c) Embryo that is mutant for *nau*. A subset of muscle fibers in the dorsolateral region of the embryo, including muscles 3 and 19, are missing. (d) Embryo in which Nau has been ectopically expressed in the mesoderm. Enlarged muscles are found in orientations reminiscent of muscle 5. (Reprinted with permission from Keller, C.A., Erickson, M.S., Abmayr, S.M., **1997**. Misexpression of *nautilus* induces myogenesis in cardioblasts and alters the pattern of somatic muscle fibers. *Dev. Biol. 181*, 197–212; and Keller, C.A., Grill, M.A., Abrnayr, S.M., **1998**. A role for *nautilus* in the differentiation of muscle precursors. *Dev. Biol. 202*, 157–171; © Elsevier.)

in subsets of the founder cells, providing alternative ways of generating a particular muscle fiber, or function in combination to specify an individual founder cell's fate. If correct, one might predict that the loss of particular genes may not necessarily result in the loss of all muscle fibers in which it is expressed. This hypothesis is consistent with the observation that mutations in many of these genes do not result in a complete absence of all fibers in which they are expressed. For example, mutations in *ap*, *nau*, and *Kr* do not affect all the muscles in which the corresponding factors are normally expressed (Bourgouin *et al*., 1992; Ruiz-Gomez *et al*., 1997; Keller *et al*., 1998). In another example, *slou* mutant embryos that lack *S59* activity exhibit various levels of disruption of muscle fibers that are derived from *S59* expressing founder cells (Knirr *et al*., 1999). These authors followed the development of three groups of muscle fibers that normally express *S59*, and found that loss of *S59* activity has different effects on individual muscle types. Loss of S59 has the strongest impact on muscles 5 (LO1) and 25 (VT1), which are absent and transformed into muscles with different identities. By comparison, absence of S59 has an intermediate effect on muscles 26 (VA1) and 27 (VA2), and only a weak effect on muscles 11 (DO3) and 18 (DT1), which are formed in *slou* mutant embryos, but with aberrant features (Knirr *et al*., 1999). Thus, muscle identity factors may function within regulatory hierarchies in each muscle, and act in a combinatorial way within these hierarchies to specify the fate of a single muscle fiber.

Studies such as those described above suggest that one or a combination of factors directs the differentiation program of a unique muscle fiber and confers on a mature muscle fiber its distinct characteristics. If such a mechanism were in place, one might anticipate that altering patterns of gene expression in muscle precursors would lead to predictable transformations in the pattern of differentiated muscles. Direct support for such a mechanism came from experiments, in which expression of a muscle identity gene characteristic of one muscle fiber was switched to that of another fiber (Ruiz-Gomez *et al*., 1997). These studies focused on a single muscle progenitor that divides to give rise to two founder cells, which form muscles 26 (VA1) and 27 (VA2). Both the muscle progenitor and, initially, the founder siblings co-express S59 and Kr. However, as development proceeds, S59 and Kr expression are lost from the founder cell for muscle 26 (VA1). By contrast, the sibling founder cell maintains expression of both genes and gives rise to muscle 27 (VA2). To analyze the role of differential *Kr* expression in the two founders, embryos that lack mesodermal Kr expression were analyzed. In these embryos, S59 expression was initiated normally in the p26/27 (VA1/2) progenitor cell, but was not maintained in the founder to muscle 27 (VA2). Consequently, muscle 27 (VA2) appears to be absent in *Kr* mutants and a muscle fiber with the characteristics of muscle 26 (VA1) is observed in its place. Conversely, expression of S59 is maintained in the founder for muscle 26 (VA1) upon ectopic expression of Kr, leading to the opposite transformation and two muscles that have the orientation of muscle 27 (VA2) (Ruiz-Gomez *et al*., 1997). This study demonstrates that Kr-mediated maintenance of expression of *S59* (and, probably, of other genes), defines specific muscle characteristics.

Other studies, on the role of these transcription factors in regulating specific programs of differentiation, suggest the existence of repressive mechanisms. For example, repression of *lb* by S59 is required in the progenitor for muscles 5 (LO1) and 25 (VT1). In *slou* mutants, which lack S59 activity, *lb* is expressed in the p5/25 (LO1/VT1) muscle progenitor, leading to a transformation of muscles 5

(LO1) and 25 (VT1) into muscles that normally express *lb* (Knirr *et al.*, 1999). A reciprocal effect is observed in embryos in which *lb* is ectopically expressed (Jagla *et al.*, 1998). Such negative interactions have also been observed between *lb* and *msh* in other progenitors (Jagla *et al.*, 1999). Finally, a regulatory role for mutual repression of identity genes has also been observed (Jagla *et al.*, 2002). In these studies, the muscle identity genes *lb*, *msh*, and *eve* act as mutual repressors. For example, presumptive *msh* muscle cells switch on *lb* or *eve* expression inappropriately in *msh* mutant embryos, indicating that *msh* normally acts as a repressor of *lb* and/or *eve* in these cells. The apparent switch in fate of the *msh*-expressing cells in *msh* mutant embryos results in the overproduction of heart cells (normally specified by *lb*) or dorsal muscles (normally specified by *eve*). By comparison, ectopic expression of *lb* represses endogenous *msh* and *eve*, leading to a re-specification of the muscle progenitors that normally express these proteins. As a consequence, an overproduction of heart cells is observed (Jagla *et al.*, 2002). In summary, reciprocal repression between transcription factors may serve as an effective mechanism to establish discrete boundaries of identity gene expression.

2.1.3.3.2. Regional muscle identity: the role of the homeotic genes Historically, loss and gain-of-function mutations in the homeotic genes were observed to cause transformation of the epidermal characteristics of one segmental unit to those of another, and mosaic analyses revealed that this effect was cell autonomous (Garcia-Bellido *et al.*, 1976). The *Drosophila* larval somatic musculature exhibits segment-specific differences from the time that the somatic muscle precursors become apparent in the mesoderm (Bate, 1990, 1993; Bate and Rushton, 1993), prompting the suggestion that homeotic genes play an autonomous role in segmental diversification of the muscle pattern. Consistent with this hypothesis, studies by Akam and Martinez-Arias (Akam *et al.*, 1985) revealed that the region of *Ultrabithorax* (*Ubx*) transcription in the embryonic mesoderm was out of register with that in the epidermis. Subsequent studies by Hooper (1986) examined the effects of homeotic mutations on the segmental pattern of larval muscle fibers. In these studies, the segmental muscle pattern of *Ubx* mutant embryos was transformed to that of more anterior segments, similar to the transformation of ectodermal derivatives. On closer examination, however, it appeared that the expression of UBx and the transformed mesodermal region were out of register in the ectoderm and mesoderm, offset by 1.5 segments (Hooper, 1986). Thus, homeotic genes are expressed in the mesoderm and regulated in a segment specific pattern, analogous to, but different from, that seen in the ectoderm. Moreover, transformations in the muscle pattern caused by homeotic mutations do not mirror the transformations seen in the epidermis. These analyses suggested an autonomous function for *Ubx* and, by extrapolation, other homeotic genes in the muscle fibers. Both *Ubx* and *abdominal-A* (*abd-A*) are expressed early in mesodermal development, before the appearance of the bi and trinucleate muscle precursors. This early expression pattern suggests that they might exert an effect on the identity of the muscle progenitors and founder myoblasts. Consistent with this hypothesis, targeted expression of Ubx and *abd-A* in the mesoderm leads to transformation of the thoracic pattern of *nau*-expressing muscle precursors and mature somatic muscles to an abdominal pattern (Michelson, 1994). The timing of expression and the effects of misexpression on muscle identity raised the possibility that the muscle identity genes discussed earlier may be targets of homeotic gene action. Indeed, such a role has been shown in regulation of the *ap* gene by Antennapedia (Antp; Capovilla *et al.*, 2001).

A notable exception to the autonomous role of homeotic genes in embryonic somatic pattern development is the second thoracic segment. In embryos that are null for *Antp*, a gene of the Antennapedia complex, the T2 and T3 somatic musculature is severely disrupted (Roy and VijayRaghavan, 1997). However, neither *Antp* nor any other known homeotic genes are expressed in the T2 mesoderm, suggesting that the observed muscle defects are due to the absence of Antp in the overlying ectoderm (Roy and VijayRaghavan, 1997). Indeed, expression of Antp exclusively in the ectoderm of *Antp* mutant embryos rescued the T2 muscle pattern, demonstrating that Antp need not be expressed in the mesoderm to influence segmental muscle pattern (Roy and VijayRaghavan, 1997). By contrast, in T3, in which Antp is normally expressed in the mesoderm, ectodermal expression is insufficient for complete rescue of the T3 muscle pattern. The authors interpret this phenotype as an indication that both ectodermal and mesodermal expression of *Antp* are required to pattern the T3 musculature. Finally, ectopic expression of Antp in the T2 mesoderm leads to a transformation of the T2 musculature to a T3-like pattern, demonstrating that these cells can be influenced by autonomous Antp. Together, these studies suggest that inductive mechanisms from the ectoderm and autonomous effects within the mesoderm influence the muscle

pattern of T2 and T3, respectively. It seems likely that a similar variety of effects of the homeotic genes on the muscle pattern of other segments is likely to be observed.

In summary, the generation of pattern in the *Drosophila* somatic musculature is not a single decision or event. It depends on a series of events involving a wide spectrum of genes that take place at different times in development to generate the complex pattern that is the larval somatic musculature. Once the pattern is crystallized, differentiation and fusion of the muscle precursors must occur to give rise to the mature larval somatic muscle pattern. This will be the subject of Section 2.1.5.

2.1.4. Determination and Differentiation of Visceral Myoblasts

The visceral, or splanchic, musculature surrounds the gut, and is responsible for contractions associated with the digestive system. The gut and overlying musculature can be divided into three different compartments that include the foregut, midgut, and hindgut. All three regions will ultimately be surrounded by circular muscles, so called because they are laid out in a network that encircles the gut (Sandborn *et al*., 1967; Goldstein and Burdette, 1971). In the midgut, a second layer of muscles is present on top of the circular muscles. These longitudinal muscles are organized into periodic linear arrays of cells that extend along the anterior–posterior axis of the gut (Campos-Ortega and Hartenstein, 1997; Georgias *et al*., 1997). In contrast to the smooth muscle of vertebrates, which serves a similar purpose to that of the insect visceral musculature, both the longitudinal and circular muscle types are striated and contain well-defined sarcomeres (Crossley, 1978). These muscles, nevertheless, have other features in common with the smooth, unstriated, musculature of vertebrates. For example, examination at the level of the electron microscope has revealed a poorly organized sarcoplasmic reticulum and transverse T tubules (Goldstein and Burdette, 1971). Additionally, while these muscles have a slow rate of contraction, they are able to contract to less than 50% of their resting length (Crossley, 1978).

The circular visceral musculature is set aside during embryonic development by the expression of *tin* and *bap*. Tin is essential for several derivatives of the dorsal mesoderm, including the circular visceral muscles, while Bap is required exclusively for these muscles (Azpiazu and Frasch, 1993; Zaffran and Frasch, 2002). Bap is a target of *tin* in the midgut (Azpiazu and Frasch, 1993; Bodmer, 1993; Yin and Frasch, 1998). It is also expressed in the precursors of the circular muscles of the foregut and hindgut, which form independently of *dpp*, and presumably the *dpp*-mediated activation of *tin* (Campos-Ortega and Hartenstein, 1997; Zaffran *et al*., 2001). Note, however, that *tin* is also a target of *twi* (Section 2.1.2.2.1), which is expressed at high levels in these cells (Baylies and Bate, 1996). The midgut circular musculature is derived from clusters of *bap*-expressing cells within the metameric repeats of the trunk mesoderm previously described, and shown in **Figure 1** (Tremml and Bienz, 1989; Azpiazu and Frasch, 1993). These cells migrate internally until they form two parallel rows of cells along the longitudinal axis. The cells then change to a more columnar shape, and become organized into a morphologically distinct layer or “palisade” (Campos-Ortega and Hartenstein, 1997). At this time, they are marked by expression of FasIII (Klapper *et al*., 2002). The cells within this layer then migrate dorsally and ventrally in a network, ultimately forming a closed tube around the gut.

In the midgut circular musculature, the combination of *tinman* (*tin*) and *bagpipe* (*bap*) influences the expression of downstream effectors of the visceral muscle differentiation program. One apparent target of these genes is *biniou* (*bin*), a member of the FoxF family of forkhead domain genes, which is activated by *dpp*, *tin*, and *bap* (Zaffran *et al*., 2001). Activation by Dpp is clearly direct, as the *bin* promoter contains a Dpp enhancer element and ectopic Dpp leads to ectopic Bin (Zaffran *et al*., 2001). Bin is expressed in both the circular and longitudinal visceral muscles, though its effect on the migration of the caudal mesoderm appears to be nonautonomous. It is necessary for the activation of differentiation genes that are expressed in the circular muscles of the trunk visceral mesoderm, including *FasIII*, *vimar*, and *brokenheart* (*bkh*) (Zaffran *et al*., 2001). Interestingly, mutation and ectopic expression of *bin* lead to reciprocal transformations between visceral and somatic muscle fates. *vimar*, another gene that is dependent on *bap* for its expression, encodes a protein with 15 tandem copies of the *armadillo* repeat (Lo and Frasch, 1998). It is expressed in both the midgut and hindgut visceral musculature slightly later than *bap*, and its expression is dependent on *bap*. The role of *vimar* in visceral muscle differentiation has yet to be defined.

The longitudinal muscles originate in the posterior region of the mesoderm, the caudal visceral mesoderm (CVM), and migrate anteriorly in two bilaterally symmetric groups of cells between the inner surface of the mesoderm and the gut (Tepass

and Hartenstein, 1994; Campos-Ortega and Hartenstein, 1997; Georgias *et al*., 1997). Ultimately, they become organized into periodic linear arrays of cells that extend longitudinally along the anterior-posterior axis of the gut. The progenitors of the longitudinal musculature are specified by a mechanism independent of that specifying the circular visceral muscles. These cells express high levels of bHLH54 (Georgias *et al*., 1997) and the homeodomain protein Zfh1 (Broihier *et al*., 1998). The bHLH54F protein was identified in a yeast two-hybrid screen for proteins that interact with the basic helix–loop–helix (bHLH) protein Daugheterless (Da). This protein is expressed transiently in the somatic mesoderm, but is expressed quite strongly in the early CVM and the migrating progenitors of the longitudinal visceral muscles. A similar pattern of expression, with respect to the visceral musculature, has been observed for *crocodile* (*croc*) and for a construct in which the *croc* promoter drives expression of β-galactosidase (Hacker *et al*., 1995). The latter construct has facilitated examination of the genetic specification of the CVM, and revealed that specification of the progenitors of the longitudinal visceral muscles is dependent on *forkhead* (*fkh*), *brachyenteron* (*byn*), and *zfh1* (Broihier *et al*., 1998; Kusch and Reuter, 1999). Of note, the circular muscles of the hindgut also originate from the caudal visceral mesoderm (Broihier *et al*., 1998; Klapper, 2000). These muscles, like the longitudinal muscles that originate in this location, are completely dependent on the presence of *brachyenteron* (Kusch and Reuter, 1999).

Another gene critical to visceral muscle development is the recently identified *jelly-belly* (*jeb*) locus, which encodes a signaling protein with an LDL receptor-repeat (Weiss *et al*., 2001). Using a yeast assay system, *jeb* was isolated in a screen for genes that were regulated transcriptionally by *tin*. As anticipated from this method of isolation, ectopic expression of *tin* results in ectopic *jeb*, indicating that *tin* is sufficient for its expression. Redundant mechanisms exist for *jeb* activation, however, since loss of *tin* has no significant impact on the presence of *jeb* transcripts. Interestingly, *jeb* is actually synthesized in the somatic mesoderm. It is expressed in the precursors of the somatic muscles of Stage 8 and Stage 9 embryos and becomes restricted to the ventral region of the somatic mesoderm by Stage 10. Jeb is then secreted and taken up by the presumptive visceral mesoderm, where it is thought to activate a signaling cascade that induces differentiation of the circular muscles. In *jeb* mutant embryos, both *myocyte enhancer factor 2* (*mef2*)-expressing and *bap*-expressing cells are clearly evident. This observation led to the conclusion that *jeb* is necessary for the differentiation, rather than specification, of visceral mesodermal precursors (Weiss *et al*., 2001; Taylor, 2002a). At the time of germ band shortening in these embryos, the *bap*-expressing precursors of the circular muscles express very reduced levels of FasIII and fail to migrate normally. It remains unclear whether the primary role of *jeb* in these cells is to direct migration, or to induce a state of differentiation that is a prerequisite for migration. Interestingly, like that described for *bin* above, the absence of *jeb* may result in a conversion of cells from visceral to somatic muscle. While experimental evidence has not definitively established that such a change occurs, the number of somatic myoblast nuclei appears to be greater than that in wild type embryos. Moreover, no significant increase in programmed cell death was observed (Weiss *et al*., 2001). If this hypothesis is correct, it is interesting that the increased number of somatic myoblasts appears to cause no defects in the somatic muscles.

An important aspect of visceral muscle differentiation that has, until recently, been unrecognized, is the ability of these cells to fuse into multinucleate syncytia before completing their differentiation program. Though numerous studies over several decades claimed that the circular and longitudinal muscles were mononucleate (Crossley, 1978; Campos-Ortega and Hartenstein, 1985; Bate, 1993; Campos-Ortega and Hartenstein, 1997), three recent studies took advantage of modern approaches and reagents to re-examine this issue. These studies revealed that the precursors of the longitudinal and hindgut circular visceral myoblasts (Klapper *et al*., 2001; San Martin and Bate, 2001) and midgut circular visceral muscles (San Martin and Bate, 2001; Klapper *et al*., 2002) are syncytial. Interestingly, these visceral myoblast fusion events utilize some of the same loci that are essential for somatic myoblast fusion (San Martin and Bate, 2001; Klapper *et al*., 2002), including *mbc* (Erickson *et al*., 1997), *sticks-and-stones* (*sns*) (Bour *et al*., 2000), and either *dumfounded/kin of irreC* (*duf/kirre*) (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001) or *irregular-optic-chiasma-C/roughest* (*irreC/rst*) (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001; see Section 2.1.6).

In fact, the circular muscles, like the somatic muscles discussed elsewhere in this review (see Section 2.1.6), are derived from founder and fusion-competent cells that express the cell adhesion molecules Duf (Ruiz-Gomez *et al*., 2000) and Sns (Bour *et al*., 2000) on their surface (San Martin and

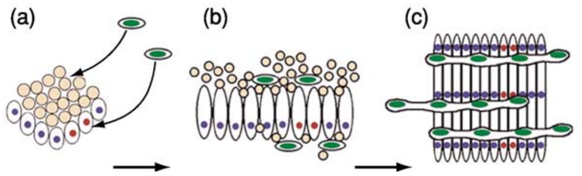


Figure 6 Diagram to illustrate the formation of longitudinal and circular visceral muscles from founders and fusion-competent cells. (a) Visceral mesoderm of late Stage 11 embryos with fusion-competent cells (orange) and associated circular founders (blue and red nuclei) together with inwardly migrating longitudinal founders (green nuclei). Local patterns of gene expression in circular founders are indicated by red and blue nuclei. (b) Stage 12 embryo showing the forming palisade of circular muscle precursors (red and blue nuclei) and associated longitudinal precursors (green nuclei) together with fusion-competent myoblasts (orange), which contribute to both kinds of muscles. (c) The completed lattice of circular and longitudinal visceral muscles, with localised patterns of gene expression as indicated. (Reprinted with permission from San Martin, B., Bate, M., **2001**. Hindgut visceral mesoderm requires an ectodermal template for normal development in *Drosophila*. *Development 128*, 233–242; © Elsevier.)

Bate, 2001; Klapper *et al.*, 2002). Examination of the founder and fusion competent cells with these markers has now revealed a clearer picture of the origins of each of visceral myoblast populations and their subsequent behavior (**Figure 6**). In short, the midgut longitudinal muscle precursors and hindgut circular muscle precursors, which are located in the posterior CVM, correspond to founder cells (Klapper *et al.*, 2001). They migrate anteriorly until they are distributed along the length of the presumptive midgut musculature and, fuse with Sns-expressing fusion competent cells (Klapper *et al.*, 2001, 2002). The midgut circular muscle founder cells, by contrast, are, essentially, the bulk of the morphologically distinct columnar cells present in the palisades of the trunk mesoderm (San Martin and Bate, 2001; Klapper *et al.*, 2002). They begin expressing β-gal driven by the enhancer trap line rP298-lacZ, which resembles Duf (Nose *et al.*, 1998) at Stage 11, and align with Sns-expressing cells. This common pool of Sns-expressing fusion-competent cells can fuse with either the longitudinal or circular founder cells, and are only loosely associated with the cells of the palisades (San Martin and Bate, 2001). These cells, not previously appreciated or described in association with the visceral musculature, are cuboidally shaped and appear to lie slightly internal to the founder cells (**Figure 6b**). Both the fusion competent cells and the circular founder cells initially express FasIII, but this expression declines in the fusion-competent cells and is detected in only the circular founder cells (San Martin and Bate, 2001; Klapper *et al.*, 2002; Chakravarti, unpublished). Fusion with Sns-expressing cells is complete by Stage 13 (Klapper *et al.*, 2002).

Interestingly, like the founder cells of the somatic musculature discussed in Section 2.1.3, the founder cells of the circular muscles do not appear to be identical, and include subsets of cells that express different founder cell markers. These markers include some of the same genes that are expressed in subsets of somatic myoblasts (San Martin and Bate, 2001), the significance of which remains to be shown. In contrast to the somatic musculature, however, only one nucleus of the syncytia, the original founder cell nucleus, expressed rP298-lacZ (Klapper *et al.*, 2002).

2.1.5. Differentiation of Founder and Fusion-Competent Somatic Myoblasts

Superimposed on or subsequent to the cell fate decisions, through which founder and fusion-competent myoblasts are specified, are the mechanisms through which these cells acquire the overt characteristics of somatic muscle. Differentiation of these cell types appears to occur in distinct steps and to be controlled by multiple gene products.

2.1.5.1. Essential Muscle-Specific Transcription Factors

In the last two decades, our understanding of the process of terminal differentiation of tissues, and the proteins that promote differentiation, has been greatly aided by the knowledge that these proteins often function as transcriptional regulators. Perhaps not surprisingly, these factors activate genes associated with a differentiated phenotype, including those that encode critical structural proteins, characteristic enzymes, and proteins that influence biosynthetic properties or morphological behavior. In this section, we discuss key transcriptional regulatory proteins that promote differentiation of somatic muscles in *Drosophila*.

2.1.5.1.1. The bHLH-containing Twist protein As described earlier, the somatic mesoderm is characterized by high levels of the bHLH transcriptional regulatory protein Twi, which acts early in the pathway, through which myoblasts acquire the ability to express muscle-specific structural genes. Studies have now shown that Twi can function as a homodimer to promote a somatic muscle phenotype in mesodermal cells. By contrast, heterodimers of Twi and the more broadly expressed bHLH protein Da can actually repress the somatic muscle fate, presumably by functioning in other mesodermal derivatives to repress the targets of Twi that promote

myogenic differentiation (Castanon *et al.*, 2001). While these results provide compelling evidence that high levels of Twi play a critical role in committing cells to a somatic muscle phenotype, the requirement for *twi* expression in mesoderm formation just a few hours earlier complicates the genetic analysis of this later role. Present studies do not specifically address whether this high level of Twi is required for the specification of both founder and fusion-competent myoblasts. In addition, studies have not addressed whether Twi might have a direct role in the transcriptional activation of genes associated with a terminal myogenic phenotype in the embryo, such as muscle myosin.

The later requirement for Twi in activating the myogenic differentiation program seems to be due at least in part to the Twi-mediated activation of *mef2* in the somatic mesoderm. *mef2* transcripts are absent in *twi* mutant embryos, and ectopic expression of Twi in ectodermal tissue induces the expression of *mef2* (Taylor *et al.*, 1995). That the effect of Twi on *mef2* is through direct transcriptional activation has been further demonstrated by the identification of a 175 bp region in the *mef2* promoter that is both necessary for *mef2* expression in the somatic mesoderm and sufficient to drive its ectopic expression in response to ectopic Twi (Cripps *et al.*, 1998). Moreover, the Twi protein binds to sequences within this enhancer (Cripps *et al.*, 1998). It is clear, however, that the sole function of high Twi is not simply to activate *mef2* in myogenic tissues. The fact that *twi* is essential for expression of Sns (B.A. Bour and S.M.A., unpublished), but *mef2* is not (Bour *et al.*, 2000), suggests the existence of other Twi targets, perhaps including such genes as *lameduck/myoblasts-incompetent/gleeful* (*lmd/minc/glee*) discussed below (see Section 2.1.5.1.2).

Another factor that suggests additional complexity in the myogenic regulatory pathway is Dmeso18E (Taylor, 2000). Its gene was identified in a rigorous subtractive hybridization-based molecular screen selecting for genes that were transcribed in wild-type 6.5–7.25 h embryos but not in embryos of the equivalent stage that were lacking *twi*. It encodes a nuclear protein with several regions of low complexity but no obvious motifs that might shed light on its function. *Dmeso18E* is expressed broadly in the mesoderm, and is observed in somatic, visceral, and cardiac cell lineages (Taylor, 2000). Expression in all of these tissues is dependent on *twi*. Intriguingly, expression is maximal in the presence of *mef2*, but is not absolutely dependent on *mef2*. This gene is therefore a potential target of *twi*, but may be modulated by *mef2* at least in the somatic musculature.

2.1.5.1.2. The GLI superfamily member lmd/minc/glee One protein responsible for complete specification and differentiation of the fusion-competent cell population is the zinc-finger containing, Gli superfamily member, Lmd/Minc/Glee (Duan *et al.*, 2001; Furlong *et al.*, 2001; Ruiz-Gomez *et al.*, 2002). Duan *et al.* identified *lmd* through their efforts to dissect the regulatory enhancer sequences of the muscle-specific transcription factor Mef2, discussed in Section 2.1.5.1.3 (Duan *et al.*, 2001). In short, these investigators utilized a *twi*-dependent *mef2* enhancer driving expression of β-galactosidase, to conduct a screen for mutations that exhibited defects in myogenic differentiation, as assayed by expression of lacZ. Their analysis revealed that embryos homozygous for mutations in *lmd* are completely lacking differentiated muscle fibers, but contain founder cells. A similar conclusion was reached by Ruiz-Gomez (Ruiz-Gomez *et al.*, 2002), who named this gene *myoblasts-incompetent* (*minc*). In both studies, the founder cells appeared to be fully differentiated, as evidenced by their expression of founder cell markers such as Kr (discussed in Section 2.1.3), and structural proteins such as muscle myosin (Duan *et al.*, 2001; Ruiz-Gomez *et al.*, 2002). These founder cells take up their normal positions, elongate, and attach to the epidermis, resulting in a muscle array reminiscent of that seen in wild type embryos. They are, nonetheless, seriously diminished in size due to the apparent lack of fusion with fusion-competent myoblasts. These observations suggested that Lmd/Minc/Glee is specifically required for determination and/or differentiation of the fusion-competent myoblasts.

Further evidence in support of this hypothesis was provided by examination of the pattern of *lmd/minc/glee* expression in Notch mutant embryos. As discussed in Section 2.1.2, Notch plays a critical role in lateral inhibition within clusters of L(1)sc-expressing myoblasts, through which founder cells are selected. In its absence, reminiscent of the interplay between neuroblasts and epidermis (Simpson, 1998), lateral inhibition does not occur and all cells take on a founder cell fate at the apparent expense of the fusion competent cells. In embryos lacking Notch, one sees a dramatic loss of Lmd/Minc/Glee-expressing cells, consistent with the model that these cells represent the diminished population of fusion-competent cells (Duan *et al.*, 2001).

As anticipated from its expression pattern in Notch mutant embryos, Lmd/Minc/Glee is expressed predominantly in the fusion-competent cells of the embryo, and is not expressed in cells coincident with founder cell markers (Duan *et al.*, 2001;

Ruiz-Gomez *et al*., 2002). Ruiz-Gomez *et al*. sought to address whether Lmd/Minc/Glee was explicitly required for specification versus differentiation of the fusion competent cells by examining expression of *twi* in these mutant embryos. In wild-type embryos, as mentioned above, expression of Twi protein is initially high in the presumptive mesoderm and declines as myoblast differentiation ensues. However, Twi persists in embryos lacking Lmd/Minc/Glee, allowing examination of the number of cells later in embryogenesis. The number of Twi-expressing cells in embryos lacking Minc was, therefore, compared to the number of Mef2-positive cells in embryos with later-acting defects in myoblast fusion. In short, the authors found much fewer *twi*-positive cells than Mef2-positive cells, suggesting that Minc is necessary for specification of fusion-competent cells, and that its absence causes a severe reduction in this population. Thus, Lmd/Minc/Glee appears to play a critical role in specification of most, if not all, of the fusion competent myoblasts, in addition to a role in their differentiation into cells that express such genes as *mef2*, *sns*, and *myosin*.

The likelihood that Lmd/Minc/Glee functions as a transcriptional regulatory protein to direct expression of genes essential for the subsequent differentiation of this population of cells, is suggested by its regulation of Mef2. Lmd/ Minc/Glee is clearly necessary for Mef2 and, either directly or indirectly, muscle myosin expression in the fusion-competent myoblasts (Duan *et al*., 2001; Ruiz-Gomez *et al*., 2002). Although Lmd/Minc/Glee does not get transported to the nucleus in all of the cells in which it is expressed, its nuclear localization is coincident with the presence of Mef2 (Duan *et al*., 2001). In addition, Lmd/Minc/Glee can activate a *mef2* enhancer that directs expression in fusion-competent cells, and sequences essential for this expression have been narrowed to a region of approximately 50 bp that binds the Lmd protein (Duan *et al*., 2001). These results demonstrate that *mef2* is a direct transcriptional target of Lmd/Minc/Glee in fusion-competent myoblasts. Other targets of Lmd are likely to include *sns*, which encodes a fusion-competent cell specific adhesion molecule (see Section 2.1.6). Expression of Sns is dependent on Lmd/Minc/Glee and is induced upon its ectopic expression (Duan *et al*., 2001; Ruiz-Gomez *et al*., 2002). While it was a formal possibility that the effect of Lmd/Minc/Glee on myogenic differentiation was solely through its induction of *mef2*, it is noteworthy that *mef2* is not essential for expression of Sns (Bour *et al*., 2000). These data suggest that Lmd/Minc/Glee must function, in part, through pathways independently of *mef2*. To test this hypothesis directly, Ruiz-Gomez *et al*. (2002) examined the ability of *mef2* to rescue the Lmd/ Minc/Glee mutant phenotype. The lack of differentiated muscle fibers in these mutant embryos indicates that Lmd/Minc/Glee is a key component of an essential myogenic regulatory pathway that functions, at least in part, independent of *mef2*.

2.1.5.1.3. The MADS box transcription factor Mef2 Mef2, the single *Drosophila* member of the Myocyte Enhancer Factor 2 (Mef2) gene family identified in vertebrates, encodes a muscle-specific MADS box (MCM1, Agamous, Deficiens, Serum Response Factor) transcription factor (Lilly *et al*., 1994; Nguyen *et al*., 1994). It is a direct target of Twi in the somatic musculature, as discussed above, and is absolutely essential for proper and complete development of somatic, cardiac, and visceral muscles (Bour *et al*., 1995; Lilly *et al*., 1995; Ranganayakulu *et al*., 1995). Embryos mutant for *mef2* are lacking differentiated, myosin-expressing cardiac cells and visceral musculature. They are also completely lacking differentiated muscle fibers. It was therefore initially compelling to consider that Mef2 might play a role, similar to that of Twi, in transcriptional regulatory mechanisms that commit cells to a myogenic program of development. However, the presence of precursors specific to the cardiac, visceral, and somatic musculature clearly indicates that these cells can be specified correctly in the absence of Mef2, and suggests a later role in differentiation. The developing mesoderm is clearly present in Stage 8 *mef2* mutant embryos, as evidenced by the pattern of cells expressing Htl/DFR1 (Lilly *et al*., 1995). Individual derivatives of this mesodermal tissue are also apparent. For example, the dorsal vessel is visible by differential interference microscopy (Bour *et al*., 1995) and expresses *tin* (Lilly *et al*., 1995). Additionally, cells committed to a visceral muscle differentiation program are present, as evidenced by expression of *FasIII* and *bap* (Lilly *et al*., 1995). Precursors of the founder myoblasts are present, as evidenced by the expression of muscle identity genes such as *nau*, *ap*, *S59*, and *eve* (Bour *et al*., 1995; Lilly *et al*., 1995; Ranganayakulu *et al*., 1995). Indeed, these marked founder cells appear to migrate correctly during germband retraction and take up their normal positions within each segment, but simply remain mononucleate and do not express proteins associated with terminal differentiation of muscle. The fusion-competent cells also appear to be specified in *mef2* mutant embryos, though the initial lack of a marker specific for this cell population prevented such an unequivocal conclusion. These myoblasts have already been specified and exhibit early signs of myogenic differentiation

that include expression of genes, such as *β3-tubulin* (Bour *et al*., 1995). While no studies have indicated that this marker is specific to the fusion-competent cell population, the mere appearance of this large number of expressing myoblasts suggested that the fusion-competent cells must be present in *mef2* mutant embryos.

In light of the above results, the major function of *mef2* in the somatic musculature appears to be to drive founder and fusion competent myoblasts to differentiate. In its absence, these myoblasts are unable to differentiate further and do not express, for example, proteins associated with the contractile apparatus such as myosin heavy chain. In fact, vertebrate Mef2 was originally identified on the basis of its ability to bind to and activate transcription from muscle specific enhancers, and a unique Mef2 recognition site in the DNA of these enhancers has been characterized. Nguyen *et al.* (1994) and Lilly (1994), have both shown that *Drosophila* Mef2 can act through this same sequence. Moreover, studies suggest that Mef2 is responsible for the activation of similar muscle-specific genes in *Drosophila*. Their products include myosin heavy chain (Bour *et al*., 1995; Lilly *et al*., 1995), α_{PS2} integrin (Ranganayakulu *et al*., 1995; Frasch and Nguyen, 1999), troponin T (Frasch and Nguyen, 1999), and muscle-specific LIM domain containing structural proteins that associate with muscle attachment sites (Stronach *et al*., 1999). The zinc finger transcription factor CF2 (Bagni *et al*., 2002) is a potential target of Mef2. This protein is first observed in developing somatic muscles at Stage 12, coincident with myoblast fusion, and later in all three muscle derivatives, somatic, visceral, and cardiac. The *muscleblind* (*mbl*) gene encodes a nuclear Cys3-His zinc finger-containing protein that participates in the organization of Z-bands and muscle attachment (Artero *et al*., 1998). Though *mbl* expression is not restricted to the musculature, it is regulated by Mef2 in these tissues. Expression of *mbl* is reduced in *mef2* mutant embryos, and ectopic Mef2 in the epidermis is sufficient to drive ectopic *mbl*. Intriguingly, Dmeso18E, a nuclear protein described above that is expressed in all three muscle types, is also dependent on Mef2 for maximal levels of transcription in the somatic musculature, but is expressed in its absence (Taylor, 2000). In the heart and visceral musculature, expression of DmesoE is completely independent of Mef2. It remains to be shown that Mef2 has a direct effect on the transcription of *Dmeso18E*, myosin heavy chain, α_{PS2} integrin and the other examples cited above, or that the expression of these genes is dependent on the presence of Mef2 binding sites.

By contrast, a requirement for Mef2 binding sites in maximal expression has been shown for the genes encoding paramyosin (Arredondo *et al*., 2001), Actin 57B (Kelly, *et al*., 2002), tropomyosin (Tm1) (Lin and Storti, 1997), and β3 tubulin (Damm *et al*., 1998). Kelly *et al*. (2002) have reported the presence of a 595 bp enhancer in *Actin 57B* that contains two essential Mef2 binding sites and is necessary for its expression in all three muscle lineages. Of note, however, this enhancer is not sufficient for maximal expression in muscle cells, nor is it responsive to ectopic Mef2. These observations led the authors to suggest the presence of additional positive and inhibitory factors that can act on this enhancer in different tissues. A similar conclusion was suggested by the analysis of *Tm1* (Lin and Storti, 1997). These authors demonstrated the presence of two enhancers in the *Tm1* gene, both of which contain Mef2 binding sites that are necessary for maximal expression. Intriguingly, these sites, like those in *Actin 57B*, are not sufficient for maximal *Tm1* expression. However, ectopic expression of Mef2 in the ectoderm does, in fact, induce *Tm1*. This result argues against inhibitory proteins in other tissues that interfere with transcription activation of these enhancers by ectopic Mef2. Rather, it favors a requirement for additional proteins that contribute to the transcriptional activation of some muscle-specific structural proteins, and suggests that some of these proteins may be present in other tissues. One potential protein involved in regulation of *Tm1* is, for example, the PAR-domain bZIP transcription factor Pdp1 (Lin *et al*., 1997b). Pdp1 is expressed in a wide variety of tissues derived from all three germ layers. It was identified, however, on the basis of its ability to interact with and activate expression of *Tm1*, and may function in collaboration with Mef2 in the regulation of this promoter. Loss-of-function alleles of *Pdp1*, however, suggest a more significant role as an essential clock gene that regulates circadian rhythm (Cyran *et al*., 2003).

A direct role for Mef2 in maximal transcription has also been suggested for the *β3-tubulin* promoter (Damm *et al*., 1998). The *β3-tubulin* gene contains an enhancer 5′ to the transcriptional initiation site that is sufficient to drive its expression in somatic myoblasts (Gasch *et al*., 1989). Critical sequences within this region are complex, but a 279 bp region containing a Mef2 consensus binding site can direct this expression (Damm *et al*., 1998). Moreover, expression driven by this sequence is completely dependent on the presence of *mef2*. Interestingly, although some discrepancy exists in the observed level of expression, β3-tubulin is clearly expressed in the somatic musculature in the absence of Mef2

(Bour *et al*., 1995; Damm *et al*., 1998). Part of this discrepancy may be a consequence of the time at which the embryos were observed. For example, the myoblasts in a *mef2* mutant embryo will ultimately be engulfed by phagocytes (Bour *et al*., 1995), a characteristic of cells that are unable to complete their differentiation program. This might account for the reduced β3-tubulin expression observed by Damm and colleagues (Damm *et al*., 1998). Additionally, it may be that β3-tubulin expression is maximal only after fusion, such that its level is lower, albeit significant, in the unfused myoblast of a *mef2* mutant embryo.

As mentioned above, ectopic expression of Mef2 in nonmuscle tissues of the embryo is capable of inducing a muscle phenotype in these tissues, including the expression of genes such as *β3-tubulin* (Damm *et al*., 1998), *Tm1* (Lin *et al*., 1997a), *nau* (Lin *et al*., 1997a), and *mbl* (Artero *et al*., 1998), but not *Actin 57B* (Kelly *et al*., 2002). In combination with results discussed above, that *nau* and β3-tubulin-expressing cells are apparent in *mef2* mutant embryos, these results appear to be inconsistent with a simple model in which Mef2 causes direct transcriptional initiation of the *nau* and *β3-tubulin* genes. Rather, the response of these genes to ectopic expression of *mef2* seems to suggest that it has committed these cells to a myogenic fate. Together, these data are consistent with several distinct mechanisms, through which *mef2* might effect myogenic gene expression. It may, for example, play an essential role in the transcriptional activation of genes such as *myosin*, for which transcripts are completely absent in *mef2* mutant embryos. It may also play an important, though more modest, role in expression of genes such as *Tm1* and *Actin 57B*, which are expressed at low levels in its absence. These data also support the existence of transcriptional regulatory proteins that have some functional redundancy with *mef2* and play a significant role in expression of genes that are not absolutely dependent on *mef2*.

In light of observations such as those described above, we suggest that *mef2* plays a primary role in the differentiation of all myoblasts, and is necessary for developing myoblasts to express proteins essential for overt characteristics of muscle. We further suggest, however, that it plays a more complex role in the expression of individual genes, with a range in which it is absolutely essential for the expression of some, and plays only a minor role in others. The recent application of microarray technology to identify genes specifically expressed in the musculature provides an enormous wealth of tools in which to address this possibility (Furlong *et al*., 2001). Interestingly, different levels of Mef2 appear to be necessary in different muscle fibers. This latter observation is derived from rescue experiments, in which it was shown that some muscle types were rescued by expression of lower levels of Mef2 while other muscles required higher levels (Gunthorpe *et al*., 1999). Thus, *mef2* may play a more diverse and complex role in myogenic differentiation than driving all cells into a simple myogenic differentiation program. *mef2* is inadequate, for example, for the complete differentiation of all myoblasts. The ability of the fusion-competent myoblasts to fuse is dependent on the cell adhesion molecule Sns (see Section 2.1.6). Sns, which serves as a marker for the fusion-competent population of myoblasts, is not dependent on *mef2* for its expression (Bour *et al*., 2000). Therefore, acquisition of a fully differentiated phenotype, in which the contractile proteins as well as fusion-associated proteins such as Sns are expressed, must invoke at least one additional protein.

Thus, both *mef2* and *lmd/minc/glee* are required for fusion-competent cells to express genes associated with overt myogenic differentiation, but only *mef2* is required in the founder cells (Taylor, 2002b). It remains to be determined whether *mef2* activation by Twi in these cells is sufficient or whether a molecule that serves a role parallel to that of Lmd/Minc/Glee is present in the founder cells. In any case, it is clear that the programs for terminal differentiation of the founder cells and the fusion-competent cells have some aspects in common, but are distinct and have important differences.

2.1.5.2. Other Aspects of Myogenic Gene Expression

In the *Drosophila* genome, a single gene encodes all of the protein isoforms of the *myosin heavy chain* gene. Hess *et al*. (1989) demonstrated that the major cis regulatory elements are located between −450 nt and 2095 nt downstream of the transcription initiation site, which includes the first two exons and intervening intron. A major level of regulation of this gene is not, however, at the level of transcription initiation but, rather, in the alternative splicing of the transcripts for different protein isoforms in different tissues and at different times (Hess and Bernstein, 1991). Zhang and Bernstein (2001) have carried out an extensive effort to correlate expression in individual muscle fibers of the embryo with utilization of particular alternatively spliced forms of the transcript. The physiological consequences of these patterns of isoform expression are now

under investigation. Presumably, the utilization of different isoforms contributes to distinct contractile activities in each muscle. Standiford *et al.* (1997, 2001) examined the sequences controlling these alternative splicing events in an effort to better understand the mechanism, through which this precise pattern is laid out, and determined that both negatively and positively acting intron sequences are present. Of the two tropomyosins expressed in muscle (Basi and Storti, 1986; Karlik and Fyrberg, 1986), *Tm1* has been the most well characterized with respect to transcriptional regulation, as described above. Though far more modest than that occurring in the myosin heavy chain gene, *Tm1* gene regulation also includes the utilization of alternative splicing to generate different protein isoforms (Basi *et al.*, 1984).

2.1.6. Myoblast Recognition and Fusion

One important aspect of myogenic differentiation in many organisms, including *D. melanogaster*, is the generation of multinucleate syncytia through the fusion of myoblasts. In vertebrates, this process cannot be initiated until the myoblasts have become competent to fuse (Abmayr *et al.*, 2003). They must then identify and adhere to their fusion partners, leading to the recruitment or activation of the fusion machinery and formation of a syncytial myotube. In *Drosophila* and the grasshopper, fusion does not occur between two equivalent myoblasts. Instead, a founder myoblast initiates the process by recruitment of a fusion-competent myoblast. Subsequent fusion events occur between the developing syncytium and more fusion-competent myoblasts, until the syncytium has attained its final cell number. In this section, we discuss molecules that have been implicated in recognition and adhesion between insect myoblasts, intracellular proteins that are essential for and promote the fusion process, and ultrastructural features at the interface of the fusing cells.

2.1.6.1. Myoblast Recognition and Adhesion

Prior to myoblast fusion in the *Drosophila* embryo, fusion-competent myoblasts must identify, migrate to, and adhere to the founder cell or developing myotube, with which they will eventually fuse. This recognition appears to be specific and directional, since founder:founder and fusion-competent:fusion-competent cell fusion has not been observed. Studies in recent years have now established that this process is driven, at least in part, by cell adhesion molecules that are expressed on the surface of myoblasts.

2.1.6.1.1. The immunoglobulin superfamily (IgSF) Genetic studies in *Drosophila* have identified three genes encoding members of the Immunoglobulin Superfamily (IgSF) that are essential for myoblast recognition and adhesion. These include *sns* (Bour *et al.*, 2000), *duf/kirre* (Ruiz-Gomez *et al.*, 2000), and *irreC/rst* (Strunkelnberg *et al.*, 2001). The *sns* locus was identified on the basis of its mutant phenotype in embryos, in which there is a complete absence of multinucleate muscle fibers and a correspondingly large number of unfused myosin-expressing myoblasts (Bour *et al.*, 2000). It is predicted to encode a protein with eight extracellular Ig domains, a single fibronectin type-III domain, a transmembrane region, and a cytoplasmic region. The *sns* transcript and protein are expressed exclusively in the fusion-competent cells of the somatic (Bour *et al.*, 2000) and visceral (San Martin and Bate, 2001; Klapper *et al.*, 2002) musculature. Sns expression is evident just prior to fusion and decreases rapidly as fusion is completed. Both biochemical and *in situ* analyses have indicated that it localizes to the plasma membrane of embryonic cells (Bour *et al.*, 2000; R. Banerjee, M. Chakravarti and S.M. Abmayr, unpublished observations). The cytoplasmic region of Sns contains potential phosphorylation sites, two proline rich regions, and stretches of homology with related proteins. The presence of these motifs may be an indication that it functions as a signaling molecule, directing events that regulate myoblast migration, recognition, and/or adhesion. This role is consistent with the mutant phenotype seen in embryos, in which the fusion-competent myoblasts do not migrate toward or adhere to the founder cells (**Figure 7**). Sns shares homology with human Nephrin, which has been implicated in the Congenital Nephrotic Syndrome (Kestila *et al.*, 1998; Lenkkeri *et al.*, 1999), *Drosophila* Hbs (Artero *et al.*, 2001; Dworak *et al.*, 2001), and a *C. elegans* transcript that has been designated C26G2.1 (S. Reynolds, W. Hanna-Rose, and S.M. Abmayr, unpublished). Of note, genetic and molecular studies have revealed that mutations in the *sns* locus (Bour *et al.*, 2000) and EMS-induced mutants in the *rolling stone* (*rost*) locus (Paululat *et al.*, 1995, 1997) are actually allelic, and map to the genetic position of *sns* (Paululat *et al.*, 1999; Bour *et al.*, 2000).

Duf was originally identified through its association with the founder cell specific enhancer trap line rP298-lacZ (Nose *et al.*, 1998; Ruiz-Gomez *et al.*, 2000; San Martin and Bate, 2001), which is expressed preferentially in the founder cells, and is not observed in the fusion competent myoblasts. It

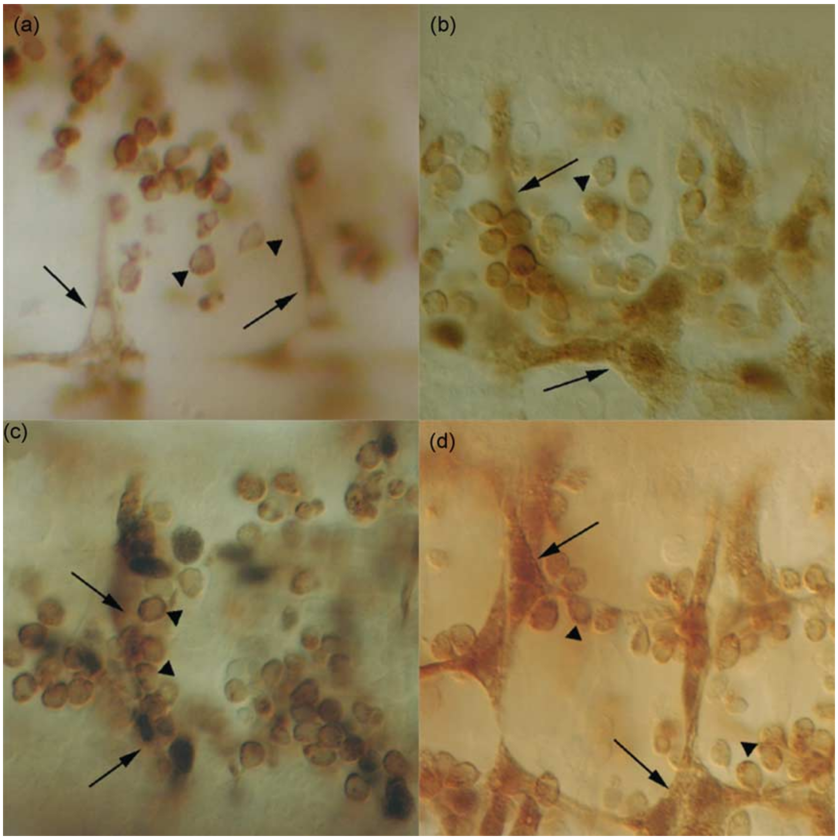


Figure 7 Stage 16 *Drosophila* embryos, in which the musculature has been visualized immunohistochemically using an antibody raised against muscle myosin heavy chain. In (b), (c), and (d), the embryos carry the *rP298lacZ* enhancer trap line, which allows identification of FO myoblast nuclei with an anti-β-galactosidase antibody. In each panel, myoblast fusion has been blocked by a different mutation. Founder (FO) myoblasts are indicated with arrows, and fusion-competent (FC) myoblasts have been indicated with arrowheads. Note that the founder myoblasts attempt to span the territory of the muscle they specify, as originally described by Rushton *et al.*, 1995. (a) Df(1)w^{67k30}/Y. This deficiency removes both the *duf/kirre* and *irreC/rst* loci. In these embryos, the FC myoblasts fail to recognize and/or migrate to their FO myoblast targets (Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001). (b) $sns^{Zf1.4}/sns^{Zf1.4}$. In these *sns* mutant embryos (Bour *et al.*, 2000) the FC myoblast have not migrated to their FO myoblast targets. (c) $mbc^{D11.2}/mbc^{D11.2}$. Unlike the embryos in (a) and (b), the FC myoblasts in embryos lacking *mbc* (Rushton *et al.*, 1995) can recognize and adhere to their FO targets, but do not fuse. (d) $blow^2/blow^2$. Like in (c) the FC myoblasts in a *blow* mutant embryo (Doberstein *et al.*, 1997) can recognize and adhere to their FO myoblast targets, but do not fuse.

is predicted to encode a single-pass membrane-spanning protein with five extracellular Ig domains and a cytoplasmic tail (Ruiz-Gomez *et al.*, 2000). Examination of embryos bearing a deficiency that removes *duf* and nearby genes revealed a complete absence of differentiated muscle fibers and a large number of unfused myosin-expressing myoblasts (Ruiz-Gomez *et al.*, 2000). Like what was observed in *sns* mutant embryos, the fusion competent myoblasts do not migrate or associate with founder cells in these embryos. Consistent with this deficiency phenotype, the *duf* transcript is expressed in a limited number of cells that give rise to the founder cells (Ruiz-Gomez *et al.*, 2000). It is not expressed in the fusion-competent myoblasts. This transcript remains detectable during fusion, but decreases after fusion is complete. Since *duf* was identified independently as a gene with significant homology to *irreC/rst*, and termed *kin of irreC* (*kirre*) (Strunkelnberg *et al.*, 2001), we refer to it as *duf/kirre*.

The *irreC/rst* gene, which also encodes a member of the IgSF, was originally identified on the basis of defects in axonal projection in the adult brain (Ramos *et al.*, 1993). The encoded protein is 45% similar to Duf/Kirre, and includes five extracellular Ig domains, a transmembrane region, and a highly homologous cytoplasmic tail (Ramos *et al.*, 1993; Strunkelnberg *et al.*, 2001). Interestingly, *duf/kirre* is located 127 kilobases (kb) away from *irreC/rst*. Thus, the original deficiency used to evaluate the *duf/kirre* loss-of-function phenotype also eliminated *irreC/rst* (Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001). Moreover, the myoblast fusion defect in these embryos can be rescued by expression of either Duf/Kirre or IrreC/Rst in the mesoderm

(Strunkelnberg *et al.*, 2001). Thus, while not originally appreciated, *duf/kirre* and *irreC/rst* actually appear to play redundant roles in the founder cells. As anticipated from this result, myoblast fusion appears to take place normally in embryos lacking only *duf/kirre*, and these embryos give rise to viable adults. While the loss of *irreC/rst* alone also gives rise to viable adults, subtle muscle defects are observed in *irreC/rst* mutant embryos. These defects include muscles that appear thin and wispy, are sometimes absent, or are present in ectopic positions (Strunkelnberg *et al.*, 2001). In comparison with *duf/kirre*, *irreC/rst* expression begins at Stage 4 and can be seen in a majority of mesodermal cells by Stage 12. Of note, IrreC/Rst is expressed in both the founder myoblasts and fusion-competent myoblasts (Strunkelnberg *et al.*, 2001). Examination of embryos mutant for *mbc*, in which myoblast fusion is blocked (Rushton *et al.*, 1995), revealed pronounced expression of IrreC/Rst in the filopodial extensions of founder myoblasts. Finally, IrreC/Rst appears to be expressed in the muscle attachment sites, or apodemes (Strunkelnberg *et al.*, 2001).

Based on their mutant phenotypes discussed above, Duf/Kirre, IrreC/Rst, and Sns all play roles in the recognition of founder cells by fusion-competent cells. Duf/Kirre and IrreC/Rst both appear to act as attractants for fusion-competent cells, since expression of either protein in the embryonic ectoderm is sufficient to target migration of fusion-competent cells (Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001). The fusion-competent cells in *duf/kirre* and *irreC/rst* mutant embryos do not migrate toward or adhere to the founder cells, similar to that seen in the *sns* mutants (**Figure** 7). However there is a difference in the morphology of the fusion competent cells between *irreC/rst*; *duf/kirre*, and *sns* mutant embryos. In the absence of *sns*, the fusion-competent cells remain round and do not appear to extend filopodia. In contrast, the fusion-competent cells in mutants lacking *duf/kirre* and *irreC/rst* extend filopodia, but these projections are randomly oriented (Ruiz-Gomez *et al.*, 2000). It is enticing to consider that Duf/Kirre and IrreC/Rst serve as attractants on the founder cells, and that Sns is the receptor for these attractants on the surface fusion competent cells. A similar mechanism for guidance cues being received by an IgSF member has been established in the nervous system (Kolodziej *et al.*, 1996; Bashaw and Goodman, 1999; Bashaw *et al.*, 2000). In these studies, Roundabout (Robo) and Frazzled (Fra), both IgSF members, receive extracellular signals and transmit this information to intracellular targets, leading to changes in the migratory behavior of axons (see **Chapters 1.10** and **1.11**).

A fourth IgSF member that is expressed in the developing musculature, and appears to play a role in myoblast fusion, is encoded by *hibris* (*hbs*) (Artero *et al.*, 2001; Dworak *et al.*, 2001). The *hbs* gene was identified in a screen for transcripts that were differentially expressed in founder cells versus fusion-competent cells (Artero *et al.*, 2001) and, simultaneously, through database searches for members of the IgSF in *Drosophila* (Dworak *et al.*, 2001). The Hbs protein is predicted to have eight or nine Ig domains, a fibronectin type-III domain, a transmembrane spanning region, and a cytoplasmic region. Like Sns, the cytoplasmic region contains potential phosphorylation sites, tyrosine residues that are conserved with Sns and/or Nephrin, and a candidate PEST sequence. Hbs is 29% identical and 44% similar to human Nephrin, and 48% identical and 63% similar to Sns (Artero *et al.*, 2001). It is expressed earlier in development than Sns, and in nonmuscle tissues that include the trachaea. This expression includes a subset of the fusion-competent cells but not the founder cells (Artero *et al.*, 2001; Dworak *et al.*, 2001). In cells that express both proteins, Sns and Hbs co-localize at discrete points on the cell surface (Artero *et al.*, 2001).

Surprisingly, *hbs* mutant embryos have subtle muscle defects in which muscles are missing or are smaller than normal, and exhibit only a modest increase in the number of unfused myoblasts (Artero *et al.*, 2001; Dworak *et al.*, 2001). These defects have no effect on survival, however, since these mutant embryos survive to become semi-fertile adults. They do, however, display defects in eye development, consistent with *hbs* expression in imaginal tissues (Artero *et al.*, 2001). Interestingly, overexpression of Hbs in the embryonic mesoderm results in muscle loss and an increase in the number of unfused myoblasts. This effect appears to be mediated through the cytoplasmic domain, since expression of this domain alone mimics the overexpression phenotype. Moreover, these unfused myoblasts are not observed upon ectopic expression of either a secreted or membrane bound extracellular domain (Artero *et al.*, 2001). Lastly, the mild myoblast fusion defect of *hbs* mutant embryos is dominantly suppressed by loss of one copy of *sns*, while *sns* mutations enhance the phenotype seen by overexpression of Hbs in the mesoderm. Together, these results have led to the suggestion that Hbs may function to antagonize the action of Sns (Artero *et al.*, 2001).

Consistent with the suggested roles for Duf/Kirre, IrreC/Rst, Sns, and Hbs in founder cell: fusion-competent cell recognition, these proteins are

capable of mediating adhesive events in cultured cells. Aggregation experiments in S2 cells indicate that Duf/Kirre and IrreC/Rst can mediate homotypic aggregation (Schneider *et al.*, 1995; Dworak *et al.*, 2001; M. Chakravarti, B.J. Galletta and S.M. Abmayr, unpublished data), whereas neither Sns nor Hbs alone is sufficient to induce aggregation. However, both Sns-expressing and Hbs-expressing cells can adhere to cells that express Duf/Kirre (Dworak *et al.*, 2001), and Sns-expressing cells can adhere to cells that express IrreC/Rst (Dworak and Sink, 2002; M. Chakravarti, B.J. Galletta and S.M. Abmayr, unpublished data). Together, the genetic and cell culture studies suggest that Duf/Kirre, IrreC/Rst, Hbs, and Sns play roles in fusion-competent/founder cell recognition and adhesion. Future studies will determine whether they play any role in later events essential to myoblast fusion.

2.1.6.1.2. Additional molecules that may function in myoblast recognition and adhesion Other members of the IgSF are expressed in the developing muscles and could also play a role in myoblast recognition and adhesion. These include *neuromusculin* (*nrm*), which is expressed in the cells of the peripheral nervous system, some cells of the central nervous system, and most embryonic muscle (Kania *et al.*, 1993). The expression of *nrm* in the muscles becomes evident by Stage 15 and increases during Stage 16. Embryos lacking Nrm complete embryogenesis and hatch, but die during the second instar larval stage. The CNS and PNS of these embryos have no obvious defects (Kania *et al.*, 1993). An analysis of the muscles of embryos homozygous for a *nrm* mutation has not been reported. Lastly, Nrm is able to mediate homotypic aggregation of S2 cells, as long as one mutates the amidation site that gives rise to the secreted form of the protein (Kania *et al.*, 1993). The *sidestep* (*side*) gene encodes another IgSF member that is expressed in the developing musculature coincident with myoblast fusion (Sink *et al.*, 2001). Gene *side* mutant embryos appear to have normal muscles. However, redundancy between these molecules and others could be masking a role in muscle fiber formation, as already observed for *duf/kirre* and *irreC/rst* (Strunkelnberg *et al.*, 2001).

Cadherins have been implicated in myoblast fusion by studies in vertebrate cell culture systems. However, cadherins have not been extensively studied during *Drosophila* myogenesis. *DE-cadherin*, or *shotgun* (*shg*), is a classic cadherin gene present in the *Drosophila* genome, and is expressed in embryonic epithelial tissues (Oda *et al.*, 1994; Tepass *et al.*, 1996; Uemura *et al.*, 1996). The protein is absent from mature muscles (Oda *et al.*, 1994), but its expression in pre-fusion myoblasts has not been examined. The effect of *shg* mutations on muscles has not been reported. Finally, many additional cell adhesion molecules have been identified in *Drosophila* (Hortsch and Goodman, 1991; Bunch and Brower, 1993), not including candidate genes identified through genome analysis. However, either these genes play no apparent role in myoblast fusion or this possibility has not yet been considered. Additionally, redundancy in function or a requirement earlier in embryogenesis may preclude identification of their role in myoblast fusion.

2.1.6.2. Intracellular Events Associated with Myoblast Fusion

It seems likely that recognition and adhesion between myoblasts, in turn, signals intracellular events that culminate in fusion between their plasma membranes. Thus, signaling components must be in place that effect intracellular changes and accomplish this purpose once two myoblasts have associated. One enticing possibility is that the cell surface molecules involved in recognition and adhesion may themselves initiate intracellular signaling events via their cytoplasmic tails. These may play a role in recruiting muscle-specific machinery to the sites of fusion, where subsequent membrane reorganization will take place. A second likelihood is that myoblast fusion will involve ubiquitously-expressed proteins that are associated with the reorganization of the cytoskeleton that underlies the fusing membranes.

2.1.6.2.1. Requirements for muscle-specific proteins The process of axon guidance (see also **Chapters 1.10** and **2.3**), in which the cytoplasmic tails of cell adhesion molecules regulate the actin cytoskeleton (Patel and Van Vactor, 2002), provides a useful framework, in which to consider how the cytoplasmic domains of myoblast-specific IgSF members might promote intracellular signaling. During axon guidance, the IgSF members Fra and Robo mediate attraction and repulsion, respectively. Fra has been shown to mediate attraction towards its ligand Netrin, while Robo mediates repulsive behavior of the axon in response to its ligand Slit (Bashaw and Goodman, 1999; Bashaw *et al.*, 2000; Wong *et al.*, 2001). The cytoplasmic domains of these proteins determine attractive or repulsive behavior, such that replacement of the Robo cytoplasmic domain with that of Fra converts it to mediating attraction in response to Slit. The cytoplasmic domain of Robo has also been implicated

in intracellular signaling because of its interaction with known signaling molecules including Abelson kinase (Abl), its substrate Enabled (Ena) (Bashaw *et al.*, 2000), and the novel family of Slit-Robo GTPase Activating Proteins (GAPs) (Wong *et al.*, 2001). These molecules establish a strong precedent for the potential of cytoplasmic tails of IgSF members to play a role in intracellular signaling.

In the context of myoblast fusion, IgSF members may also play a role in signal transduction via their cytoplasmic domains. This hypothesis is supported by the recent finding that the cytoplasmic domain of Duf/Kirre is capable of interacting biochemically with Antisocial (Ants)/Rolling Pebbles (Rols) (Chen and Olson, 2001). The *ants/rols* locus was identified in three independent genetic screens for genes affecting the development of the embryonic musculature (Chen and Olson, 2001; Menon and Chia, 2001; Rau *et al.*, 2001). Myoblast fusion does not occur in *ants/rols* mutant embryos. The predicted Ants/Rols protein contains several potential protein-protein interaction motifs including a RING finger, nine ankyrin repeats, three tetratricopeptide repeats (TPRs), and a coiled-coiled (Chen and Olson, 2001; Menon and Chia, 2001; Rau *et al.*, 2001). The presence of these motifs is consistent with the possibility that Ants/Rols directs protein-protein interactions that are part of a signal transduction cascade. Ants/Rols is expressed in the founder myoblasts at a time consistent with myoblast fusion. Moreover, Duf/Kirre and Ants/Rols are present on the myoblast membrane, and co-localize at points of cell–cell contact (Menon and Chia, 2001). A role for Ants/Rols as a signaling intermediate downstream of Duf/Kirre is also supported by experiments in which Ants/Rols can interact with the N-terminal region of Mbc when both proteins are expressed in *Drosophila* S2 cells (Chen and Olson, 2001). Lastly, a mouse ortholog of Ants, mants1, is expressed in the mesoderm of the mouse embryo and suggests that the function of Ants/Rols may be conserved (Chen and Olson, 2001).

In combination with data discussed in Section 2.1.6, these data are consistent with a mechanism in which the fusion-competent myoblasts migrate towards the founder myoblasts, and interact in a Duf/Kirre and Sns-dependent manner. This interaction may lead to the formation of specialized sites along the membrane at points of cell:cell contact. In founder cells, Ants/Rols could be recruited to these sites through interaction with the Duf/Kirre cytoplasmic tail. Finally, signaling molecules such as Mbc may be recruited through additional protein interaction domains, and modulate cytoskeletal structures that are critical to the fusion process. Potential targets of such a pathway are discussed in the next section.

2.1.6.2.2. Nonmuscle-specific signaling molecules: Mbc and DRac1 The *mbc* locus was originally identified through its embryonic loss-of-function phenotype, which is characterized by the absence of multinucleate muscle fibers and presence of large numbers of unfused myoblasts (Rushton *et al.*, 1995). It encodes a protein of somewhat novel structure with extensive homology to *C. elegans* Ced-5 (Wu and Horvitz, 1998), and human Dock180 (Hasegawa *et al.*, 1996). Together, these three proteins have been termed the CDM family of proteins. This protein superfamily now consists of at least 11 human proteins (Kashiwa *et al.*, 2000; Cote and Vuori, 2002; Meller *et al.*, 2002), including Dock180 and Dock2 (Hasegawa *et al.*, 1996; Nishihara, 1999), four *Drosophila* proteins (Cote and Vuori, 2002), including Mbc (Erickson *et al.*, 1997) and *Sponge* (Schejter *et al.*, 2003), three *Caenorhabditis elegans* proteins (Cote and Vuori, 2002), including Ced-5 (Wu and Horvitz, 1998), three *Dictyostelium discoideum* proteins (Cote and Vuori, 2002), one protein in *Saccharomyces cerevisiae* (Cote and Vuori, 2002), and one protein in *Arabidopsis thaliana* (Cote and Vuori, 2002; Qiu *et al.*, 2002). The most extensive studies have been done on the founding members of the CDM family, Mbc, Dock180, and Ced-5. All of these proteins contain a SH3 domain at their N-terminus, at least two proline-rich, putative SH3 binding domains at their C-terminus, and at least two additional blocks of sequence homology.

Mbc is not muscle specific, and is expressed in a wide variety of tissues in the developing embryo (Erickson *et al.*, 1997). Consequently, the defects seen in *mbc* mutant embryos are not limited to myoblast fusion, but include defects such as incomplete dorsal closure of the epidermis, abnormal fasciculation of the ventral nerve cord neurons, and severely impaired migration of border cells in the adult female ovary (Erickson *et al.*, 1997; Nolan *et al.*, 1998; Duchek *et al.*, 2001). The orthologs of Mbc that have been examined in other systems appear to be involved in diverse processes that include cell engulfment, cell migration, epithelial morphogenesis and migration, and oncogenic transformation (Hasegawa *et al.*, 1996; Wu and Horvitz, 1998). While these processes appear diverse at first glance, they all have in common the potential reorganization of the cytoskeleton. In fact, *mbc* mutant embryos exhibit perturbations in the actin cytoskeleton along the leading edge of the migrating epidermis during dorsal closure (Erickson *et al.*,

1997). As discussed in Section 2.1.6.2.1, the founder cell-specific proteins Duf/Kirre and Ants/Rols may serve as upstream activators of Mbc. Consistent with the biochemical interactions between their encoded proteins discussed above, *duf/kirre*, *rols*, and *mbc* mutants all are defective in myoblast fusion (Hasegawa *et al*., 1996; Erickson *et al*., 1997; Wu and Horvitz, 1998; Ruiz-Gomez *et al*., 2000; Chen and Olson, 2001; Menon and Chia, 2001; Rau *et al*., 2001). It is, therefore, enticing to suggest that Duf/Kirre may serve to recruit Mbc to discrete points on the membrane via Ants/Rols, leading to localized changes in the cytoskeleton. However, this model awaits *in vivo* testing.

In *Drosophila* embryonic myoblasts, localization of Mbc to the cell membrane may be mediated by interactions with the Ants/Rols protein (Chen and Olson, 2001). In other organisms and tissue types, however, the CDM proteins appear to be dependent on upstream proteins that include the small SH2-SH3 domain containing adaptor protein Crk (CT10 regulator of kinase) (Matsuda *et al*., 1996; Reddien and Horvitz, 2000). Crk has been proposed to function by linking phosphotyrosine signals at the membrane via its SH2 domain and with downstream effectors via its SH3 domain. This can lead to accumulation of Crk and its downstream effectors to discrete sites at the plasma membrane (Feller *et al*., 1998). The interaction between a CDM family member and Crk appears to be conserved in *Drosophila*. For example, *Dcrk* was identified in a Far-Western screen for proteins interacting with the C-terminus of Mbc (Galletta *et al*., 1999). Like Crk-II (Matsuda *et al*., 1994) and Ced-5 (Reichman *et al*., 1992), DCrk consists of one SH2 and two SH3 domains. Similar to that observed for Mbc (Erickson *et al*., 1997), it is expressed in a broad range of tissues during embryogenesis (Galletta *et al*., 1999). The *Dcrk* locus is located on the fourth chromosome, and a significant level of its transcripts appears to be provided to the embryos by the mother. Thus, analysis of the loss-of-function phenotype has not been straightforward. Of note, however, recent studies have demonstrated that the Crk binding sites are not essential for Mbc to function in embryos (L.B. and S.M.A., unpublished), suggesting that other proteins, perhaps including Ants/Rols, can serve the same function as Crk in bringing Mbc to the membrane.

In *Drosophila*, as in other organisms, the small GTPase Rac1 (Ras-related C3 botulinum toxin substrate) may function as a major target of the CDM family (Kiyokawa *et al*., 1998a, 1998b; Nolan *et al*., 1998; Albert *et al*., 2000; Reddien and Horvitz, 2000; Gumienny *et al*., 2001). Rac1 is a member of the Rho family of small GTPases, which are often associated with regulation of changes in the cytoskeleton. Expression of dominant negative or constitutively active forms of DRac1 in the mesoderm causes a loss of myoblast fusion (Luo *et al*., 1994), presumably by interfering with cytoskeletal events critical for myoblast fusion. More recently, loss-of-function alleles of *Drac1* and *Drac2* have been isolated and used to generated double mutants. These mutant embryos exhibit significant defects in myoblast fusion (Hakeda-Suzuki *et al*., 2002). In addition, DRac1 and Mbc have been linked genetically in the eye, in which loss of one copy of *mbc* suppresses the *Drac1* overexpression phenotype (Nolan *et al*., 1998). Studies in vertebrate cell culture systems have revealed a potential mechanism for the CDM protein influence on Rac1. The CDM proteins do not contain a consensus Dbl (diffuse B cell lymphoma)-homology region, which has been identified in previously reported guanine nucleotide exchange factors (GEFs). However, two groups have recently demonstrated an unconventional GEF activity on Rac1, via a region conserved in CDM proteins that has been termed Docker (Dock) or Dock Homology Region-2 (DHR-2) (Brugnera *et al*., 2002; Cote and Vuori, 2002). Both groups have shown that this region is capable of mediating GEF activity *in vitro*. Moreover, expression of the DHR-2 region of Dock180 is sufficient to increase the GTP loading of Rac1 in 293-T cells (Cote and Vuori, 2002). However, Brugnera *et al*. (2002) have shown that the GEF activity of Dock180 in 293-T cells is dependent on co-expression of the ELMO1 protein. ELMO1 is the vertebrate ortholog of *C. elegans* Ced-12, which was identified on the basis of a defect in the engulfment of cell corpses in *C. elegans*, and subsequently cloned (Wu and Horvitz, 1998; Chung *et al*., 2000; Gumienny *et al*., 2001; Wu *et al*., 2001; Zhou *et al*., 2001). The predicted sequences of the ELMO1 and Ced-12 proteins contain putative pleckstrin-homology domains and proline-rich regions. The *ced-12* gene acts genetically at the same step as *ced-5* (Reddien and Horvitz, 2000). Ced-12/ELMO also appears to interact physically with Ced-5/Dock180 and these proteins have been shown to cooperate in several cell culture assays (Conradt, 2001; Gumienny *et al*., 2001; Wu *et al*., 2001; Zhou *et al*., 2001). It is enticing to consider the possibility that ELMO proteins may function with CDM proteins in cellular processes.

2.1.6.3. Other Genes with Roles in Myoblast Fusion

In addition to the loci discussed above, several genes have been identified that appear to be involved in

myoblast fusion, but to which no specific biochemical role has been ascribed. The *blown fuse* (*blow*) locus was identified in a screen for lethal mutations defective in guidance of motoneurons (Doberstein *et al*., 1997). However, this defect is an indirect consequence of the lack of somatic muscles and resulting difficulties in axon targeting. Embryos lacking *blow* have no multinucleate syncytia, demonstrating that *blow* is essential for myoblast fusion. The *blow* transcript is restricted to myoblasts, and appears just prior to fusion. The predicted Blow protein, which contains no recognizable motifs or homology with proteins of known function, appears to reside in the cytoplasm (Doberstein *et al*., 1997). Two genes that have not been fully characterized also contribute to myoblast fusion. *Singles-bar* appears to encode a hydrophobic protein that is essential for myoblast fusion (Maeland *et al*., 1996). Although myoblast fusion does not occur in embryos mutant for *singles-bar*, the fusion-competent myoblasts cluster around the founder myoblasts (Ruiz-Gomez *et al*., 2000). This mutant phenotype suggests that *singles-bar* functions after cell recognition and adhesion in the myogenic program, possibly in membrane-associated events that are critical during the fusion process itself (Maeland *et al*., 1996).

The *rost* locus, mentioned previously because EMS-induced alleles of *rost* correspond to mutations in Sns (see Section 2.1.6.1), was originally identified through a P-element insertion line (Paululat *et al*., 1995, 1997). While complications in the characterization of this locus arose due to insertions in both the *rost* and *sns* loci (Paululat *et al*., 1999; Bour *et al*., 2000), it appears that the *bona fide rost* locus at cytological region 30 has defects in myoblast fusion. The transcript associated with this locus is expressed in the mesoderm, and a promoter-*lacZ* reporter that includes 400 bp upstream of the ATG is expressed in mature muscles (Paululat *et al*., 1997). The predicted Rost protein is hydrophobic and may span the membrane up to seven times. It is enriched in biochemical preparations of partially-purified membranes (Paululat *et al*., 1997), supporting its association with the membrane. While the true *rost* loss-of-function phenotype remains unclear, embryos expressing antisense *rost* exhibit a block in myoblast fusion (Paululat *et al*., 1997). In these embryos, the fusion-competent myoblasts appear to recognize and adhere to the founder myoblasts but do not fuse. This finding is supported by studies in which the P-element insertion in *rost* has been genetically separated from the P-element perturbation in *sns*, and appears to have independent effects on myoblast fusion (Paululat *et al*., 1999).

D-Titin, the *Drosophila* homolog of vertebrate Titin, serves as an elastic scaffold for both muscle sarcomeres and chromosomes (Gregorio *et al*., 1999; Trinick and Tskhovrebova, 1999; Machado and Andrew, 2000). D-Titin, a protein of approximately 2.0 mDa, is composed primarily of Ig and FnIII (fibronectin III) domains followed by a PEVK motif (Machado and Andrew, 2000; Zhang *et al*., 2000). D-Titin is expressed at a high level in myoblasts prior to fusion and appears to accumulate on the myoblast surface, often at points of contact between myoblast and myotube (Machado and Andrew, 2000; Zhang *et al*., 2000). Interestingly, it has been shown that recruitment of D-Titin to points of cell–cell contact in founder myoblasts is dependent on the Ants/Rols protein discussed above (Menon and Chia, 2001). Analysis of D-Titin mutant embryos has shown that it is essential not only for the integrity of the sarcomere, but also for myoblast fusion (Machado and Andrew, 2000; Zhang *et al*., 2000). Thus, it is possible that D-Titin is playing a role in either directly or indirectly organizing the actin cytoskeleton.

The involvement of a muscle structural protein in the myoblast fusion process is not unique to Titin. Recent studies from the Bernstein lab (Liu *et al*., 2003) have shown that paramyosin also plays a role in myoblast fusion and accumulates at discrete points in between fusing myoblasts.

2.1.6.4. Ultrastructure of the Adherent Myoblast Interface

In a detailed morphological analysis of fusing myoblasts at the level of the electron microscope, Doberstein *et al*. (1997) described a series of intracellular events that precede fusion. One first observes the appearance of clusters of electron dense vesicles on the cytoplasmic sides of opposed plasma membranes (**Figure 8**). These vesicles, which have not been observed in other cell types, appear to align with one another across the intervening membranes to form the so-called "paired vesicles" (Doberstein *et al*., 1997). After alignment, electron dense plaques are observed at the myoblast interface, on the cytoplasmic sides of the corresponding plasma membranes. The myoblasts align with each other and elongate, and fusion pores are occasionally observed adjacent to fusion plaques. Of note, fusion appears to occur at multiple sites (Doberstein *et al*., 1997), similar to that seen previously in the Lepidopteran *Antheraea polyphemus* (Stocker, 1974). Thus, it does not appear that myoblast fusion in *Drosophila* utilizes a simple zipper mechanism that originates from a single site. The fusion process is completed as the membrane vesiculates and is

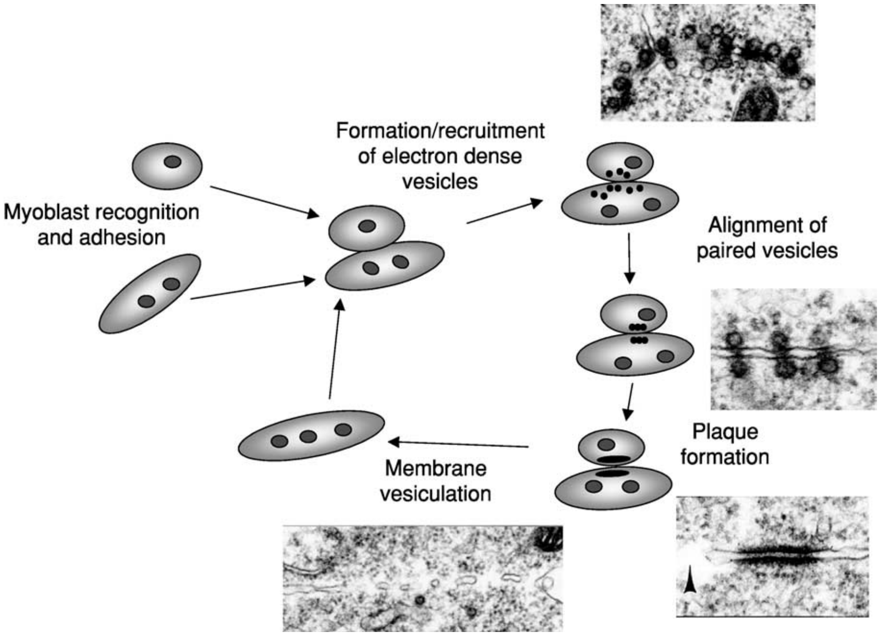


Figure 8 A schematic representation of the ultrastructure of the interface of fusing myoblasts in *Drosophila*, with corresponding electron micrographs. First, myoblasts must recognize and adhere to each other (upper left). Electron dense vesicles are then observed on both sides of the opposing plasma membranes (top). These vesicles subsequently align with each other across the plasma membranes (upper right) and form a structure, which has been termed the prefusion complex. Next, electron dense plaques are observed just under the opposing plasma membranes (lower left). Finally, the membranes vesiculate leading to cytoplasmic continuity (bottom). (Reproduced from Doberstein, S.K., Fetter, R.D., Mehta, A.Y., Goodman, C.S., **1997**. Genetic analysis of myoblast fusion: *blown fuse* is required for progression beyond the prefusion complex. *J. Cell Biol. 136*, 1249–1261; by copyright permission of The Rockefeller University Press.)

removed. Figure 8 diagrams the proposed steps of myoblast fusion and shows electron micrographs collected by Doberstein *et al.* (1997).

In an effort to extend the study of these ultrastructural features of myoblast fusion, similar morphological studies were carried out using embryos mutant for several of the loci discussed in this section. These embryos were examined at the ultrastructural level to determine the point at which the fusion process was arrested. In *blow* mutant embryos, for example, the vesicles accumulate in prefusion complexes, but no electron dense plaques are observed. Assuming that the electron dense plaques are formed from an association between vesicles and the plasma membrane, it is possible that the cytoplasmic Blow protein is involved in initiating or mediating this step. The EMS-induced *rost*^{15} mutation, which is actually allelic to *sns*, was also examined at the ultrastructural level. Surprisingly, fusion is arrested at a fairly late stage in these mutant embryos, since electron dense plaques accumulate but the plasma membrane does not break down. Recall, as discussed earlier in Section 2.1.6, that the founder and fusion-competent myoblasts do not recognize or adhere to one another in *sns* mutant embryos (Figure 7). However, since the sequence lesion in *rost*^{15} has not yet been identified, it is possible that it is a hypomorphic mutation and that some of the Sns protein is still functional. Support for this possibility is provided by the mutant phenotype of *rost*^{15} mutant embryos (Paululat *et al.*, 1995), in which myoblast fusion does not appear to be completely blocked. Few prefusion complexes were apparent in embryos lacking Mbc, suggesting that this protein may play an early role in vesicle accumulation. It is possible, for example, that Mbc-induced changes in the actin cytoskeleton are required for the recruitment of the electron dense vesicles.

Two additional genes that affect myoblast fusion have been examined at the ultrastructural level. In a separate analysis, embryos mutant for the *rost* locus were examined, and small syncytia containing 2–4 nuclei were observed (Rau *et al.*, 2001). However, fusion did not proceed further, and no prefusion complexes or electron dense plaques were observed. Finally, Doberstein (Doberstein *et al.*, 1997) also examined the ultrastructure of fusing myoblasts in embryos, in which a constitutively active form of the small GTPase Rac1 had been expressed. These embryos have prefusion complexes, electron dense vesicles, and isolated fusion pores. The plasma membranes of the myoblasts come in close apposition but do not appear to vesiculate. For these

reasons, constitutively active Rac1 is believed to block myoblast fusion at a relatively late step.

2.1.7. Maturation of Muscle Fibers

Muscle differentiation and maturation involves three critical processes: formation of the contractile apparatus, attachment of the muscle to the epidermis, and formation of neuromuscular junctions (NMJs) that control the muscle contractions (see also **Chapters 2.2** and **2.3**). These three processes are essential for the generation of force by the muscle, exertion of this force onto the body skeleton, and regulation of this force through neural connections. The contractile apparatus has been described in some detail elsewhere (Bernstein *et al*., 1993). Herein we briefly describe some aspects of muscle attachment and innervation (see also **Chapters 1.10** and **2.3**).

2.1.7.1. Epidermal Attachment

Following the earliest fusion events, the developing myotubes extend growth cone-like filopodia that migrate over the epidermis. Projections on both ends of the muscle will seek out and contact attachments sites, or apodemes, on the surface of the Epidermal Muscle Attachment (EMA) cells (Bate, 1990; Volk, 1999). These attachments must withstand the forces associated with muscle contraction. They must also, like axon guidance, assemble in a carefully orchestrated pattern.

The EMA cells are a specialized class of epidermal cells associated with muscle attachment, and some of the earliest-acting molecules required for muscle attachment are proteins that influence the differentiation program of these cells. Recent studies demonstrate that positioning of these cells is achieved through repression of the *hh* pathway through *wg* signaling at the parasegment boundary (Piepenburg *et al*., 2000). Molecules that are specifically expressed in and control differentiation of the EMA cells include Stripe (Sr), an EGR (erythrocyte growth factor receptor)-like nuclear protein that contains zinc-finger motifs and may function as a transcription factor (Lee *et al*., 1995; Frommer *et al*., 1996). Embryos homozygous for a severe mutant allele of *stripe* exhibit an aberrant somatic muscle pattern, in which the myotubes often send filopodia in incorrect directions. In late stages of embryonic development, most of the myotubes are randomly scattered, do not attach to the epidermis, and fusion is blocked (Frommer *et al*., 1996). Interestingly, ectopic expression of Stripe in other epidermal cells causes them to differentiation as muscle attachment cells and to serve as attractants for somatic myotubes (Becker *et al*., 1997; Vorbruggen *et al*., 1997).

Mutations in segment-polarity genes that cause an absence or ectopic appearance of these cells also result in disorganized muscle fibers, suggesting that the EMA cells normally guide migrating muscles to their attachment sites (Volk and VijayRaghavan, 1994). The *delilah* (*dei*) gene, which encodes a bHLH containing transcription factor (Armand *et al*., 1994), has the potential to play a role in the differentiation of these cells. The *Drosophila* epidermal growth factor receptor DER is required for the final differentiation of EMA cells and strengthening of muscle attachment, and the *Drosophila* neuregulin homolog Vein is thought to be the ligand of DER (Yarnitzky and Volk, 1995). Inductive interactions between the myotube and the attachment cells are mediated by *vein* (Yarnitzky *et al*., 1997).

Additional genes that are expressed in the EMA cells include attachment-site specific markers such as *groovin* (Volk and VijayRaghavan, 1994), *alien* (Goubeaud *et al*., 1996), and β_1*-tubulin* (Buttgereit *et al*., 1991). Transcription of β_1*-tubulin* is actually dependent on muscle insertion (Buttgereit, 1996). The *masquerade* (*mas*) gene encodes a secreted molecule that is expressed in the epidermis (Murugasu-Oei *et al*., 1995). It has the potential to function as a serine protease, though no protease activity has been detected. Embryos mutant for *masquerade* exhibit defects in muscle attachment, suggesting that its role is to stabilize this interaction. Examples of other genes that are expressed at muscle attachment sites include those encoding the *Drosophila* Sema5C (Bahri *et al*., 2001), a cell-surface glycoprotein, and Hbs, an IgSF member described in Section 2.1.6.1.1 (Artero *et al*., 2001). While no Sns protein has been detected at attachment sites, it is intriguing that its mRNA appears to be present in these cells (Bour *et al*., 2000). Phenotypic analysis of mutations in these genes has not revealed a role in the formation of epidermal attachment sites, though it remains a formal possibility that muscle attachment involves redundant mechanisms.

On the muscle side, the spectrin-related molecule Msp-300, which is expressed at the leading edge of migrating muscles, appears to play a role in the migration of the filopodia (Volk, 1992), and Msp-300 loss-of-function mutants exhibit defects in the ability of the myotube to extend toward its attachment site. This effect is amplified in genetic combination with mutations in *laminin* (Rosenberg-Hasson *et al*., 1996), suggesting an involvement of the extracellular matrix. M-spondin is among the extracellular matrix (ECM) molecules that is expressed in the developing muscle (Umemiya *et al*., 1997).

Once attachment has occurred, one remaining step in this process is to strengthen the attachments, allowing them to withstand the forces of contraction. This step includes the association of cell surface receptors specific to both the myotube and the ectoderm, and occurs rather late in the myogenic differentiation program. The position-specific integrin proteins $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$ play a key role in this aspect of muscle attachment. Whereas β_{PS} is expressed on both cell types, α_{PS1} and α_{PS2} are expressed in complementary patterns in the EMA cell membrane and muscle cell membrane, respectively (Brown, 1994). These complexes play a general role in mediating interactions between the muscle and the overlying epidermis. At Stage 16, embryos bearing mutations in the β_{PS} subunit gene, *lethal myospheroid* (*l(1)mys*), contain multinucleate syncytia that appear to form normally. By Stage 17, however, the body wall muscles appear to begin detaching from the epidermis as a consequence of contraction, and this becomes pronounced after hatching. Although they remain attached for a slightly longer time, the muscles of embryos mutant for the α_{PS2} subunit gene, *inflated*, exhibit a similar mutant phenotype (Bogaert *et al*., 1987; Brown, 1994). Tiggrin, the ligand for the α_{PS2} integrin, is required for proper muscle function as well (Bunch *et al*., 1998). These proteins, then, appear to function as anchors to stabilize and strengthen the muscle-apodeme interaction against the forces of contraction. However, the delayed response of the muscles to the absence of the α_{PS2} subunit suggests the possible contribution of other molecules to a strong attachment site. One structural protein that plays a role in the integrity of muscle attachment is β_1-tubulin. It is expressed at the apodemes, possibly to provide a rigid structure that will withstand the tensile forces of muscle contraction (Buttgereit *et al*., 1991).

Like extending axons in the nervous system, mechanisms must be in place to direct myotube elongation to the correct location. One might expect molecules associated with membrane extensions and filopodia formation to play a major role in the ability of the muscles to physically contact their attachment sites. This targeting depends on the EMA cells (Volk and VijayRaghavan, 1994), which may provide guidance cues or attractants that direct migration. The EMA cells may also provide specific recognition molecules associated with precise attachment. The accuracy of attachment is also likely to depend on molecules specific to the muscle fiber that control its precise site of insertion. The sites utilized by the myotube in order to accomplish this purpose, seem destined to be intimately associated with the identity of the muscle fiber itself, and the specific differentiation program controlled by the founder cell, from which the muscle derived. At present, the mechanisms by which differentiated myotubes identify the correct place to insert into the apodemes, or epidermal muscle attachment sites, are not clear (Volk, 1999).

Nevertheless, we have some early hints. Interestingly, the Derailed (Drl) protein is expressed on a small number of muscles and tendon cells (Callahan *et al*., 1996). In *derailed* mutants, these particular muscles fail to elongate or elongate improperly, consistent with the notion that Derailed plays a role in the precise recognition pattern that is achieved. The fact that *derailed* encodes a receptor tyrosine kinase suggests that signaling events between these two germ layers may be occurring. Possibly, this is an early indication that signaling mechanisms reminiscent of those in axon guidance may come into play in this process.

2.1.7.2. Motor Neuron Innervation

Formation of synapses between motoneurons and the muscles that they control begins after the muscle pattern is crystallized, midway through embryogenesis (see also **Chapter 2.3**). Using retrograde dye labeling in embryos at about 13 h after egg laying, two groups examined the behavior of the motoneurons during muscle contact. They demonstrated that filopodia extend from individual motoneurons to contact multiple muscle fibers (Halpern *et al*., 1991; Sink and Whittington, 1991). Ultimately these filopodia retract from adjacent muscles and undergo stereotypical branching to maximize contacts on a single muscle fiber. Confocal time lapse imaging demonstrated that the filopodia are highly dynamic, as they appear to scan along the surface of the muscle fibers (Keshishian, 1993; Keshishian and Chiba, 1993).

One of the most significant advances in our understanding of muscle innervation in the last decade is the role of the muscle cells in this process. In the mid 1980s, Lawrence and colleagues took advantage of the observation that the muscle pattern of the *Drosophila* adult differed between males and females in one segment, to examine the role of innervation in dictating muscle pattern. A now classic set of experiments utilized genetic mosaics to ask which cells controlled the development of this male-specific muscle (Lawrence and Johnston, 1984; Lawrence and Johnston, 1986), and revealed that the determinative cells did not contribute to the muscle itself and were not myoblasts. Rather, the authors suggested that the sex and segmental identity of this muscle was controlled by the neuron

that innervated it. In contrast to the hypothesis put forward by these studies, however, it is clear that motoneuron innervation does not contribute to the patterning of the larval body wall muscles. Indeed, as elaborated in Section 2.1.3, the pattern of muscle fibers is dictated by the presence of single founder cells that seed the fusion process and are specified hours before innervation occurs. Broadie and Bate sought to address this issue directly, and examined muscle development in embryos in which motoneuron development is delayed (Broadie and Bate, 1993b). Their observations revealed that, in the absence of motor nerves, myoblasts can fuse normally, form proper attachments, and develop the electrical properties of larval muscles in a normal time course. Thus, innervation appears to play no role in patterning, morphogenesis, maintenance, or physiological development of the somatic muscles in the *Drosophila* embryo (Broadie and Bate, 1993b).

By contrast, numerous studies have addressed the role of the muscle cells in the neuromuscular junction. Embryos mutant for the *twi* gene and completely lacking mesoderm, still exhibit morphologically normal presynaptic zones (Prokop *et al.*, 1996). However, the motor axons fail to defasciculate from the neuronal bundles and do not branch (Landgraf *et al.*, 1999). Embryos mutant for *mef2*, in which the myoblasts fail to differentiate, also form active presynaptic zones. In this case, the zones fail to localize at neuromuscular contacts, indicating that a *mef2*-dependent step in differentiation is necessary (Prokop *et al.*, 1996). Interestingly, a single differentiated founder cell is sufficient to provide the cue for branching morphogenesis (Landgraf *et al.*, 1999). Moreover, neither defasciculation nor axon targeting are dependent on myoblast fusion, since motoneurons of embryos mutant for *mbc* exhibit functional neuromuscular synapses (Prokop *et al.*, 1996).

Although the initial targeting of the motoneuron is broader than the final pattern of contact sites, the precision with which this pattern is achieved suggests underlying forms of recognition. Indeed, experimental studies have addressed the plasticity of motoneuron targeting by deleting or duplicating specific muscle fibers. These studies revealed that *Drosophila* motoneurons can distinguish between individual muscle fibers, and that this recognition is mediated by the growth cones (Cash *et al.*, 1992; Keshishian *et al.*, 1993). Only limited information is available, however, concerning the exact molecular mechanisms governing pathfinding and synaptic recognition. A genetic screen, designed to identify loci associated with motoneuron pathfinding and targeting, uncovered several loci, including *beaten path*, *stranded*, *short stop*, *walkabout*, and *clueless* (Van Vactor *et al.*, 1993). Not surprisingly, candidates for target recognition also include adhesion molecules that are expressed on the surface of muscle fibers and at synapses. Fasciclin III, a cell adhesion molecule in the immunoglobulin superfamily, is expressed on motoneuron RP3 and its synaptic targets, the ventral longitudinal muscles 6 and 7 (Chiba *et al.*, 1995). Similarly connectin and Toll, two cell surface glycoproteins of the leucine-rich repeat family, are expressed on a subset of neurons and the muscle fibers that they innervate (Nose *et al.*, 1992; Halfon *et al.*, 1995). The loss of one or both alleles of *Toll* results in widespread defects in muscle pattern and motoneuron number (Halfon *et al.*, 1995). In some cases, these molecules can mediate adhesion between cells in culture. Semaphorin II, a secreted protein, is expressed transiently in a single large muscle during the time of motoneuron outgrowth and synapse formation (Matthes *et al.*, 1995). Of note, mutations in several of these genes do not have significant effects on neuromuscular connections, suggesting either that the appropriate molecules have not yet been identified or, more likely, that formation of correct synaptic connections is ensured by redundancy. In either case, our understanding of how the elaborate pattern of muscle innervation by motoneurons is accomplished is only in its early stages.

2.1.8. Concluding Remarks

The last decade or so of the 20th century yielded remarkable advances in our understanding of myogenesis in the *Drosophila* embryo. Many of these advances have been made possible by new technologies that pair molecular biology with genetics or cell biology, and include *in situ* hybridization, confocal microscopy, large-scale genome-wide genetic screens, and the availability of the *D. melanogaster* genome sequence, to name a few. Indeed, progress is occuring at such a rapid pace that additional information is likely to be forthcoming as this review goes to press. The approaches that have been developed and information that is now available hold promise for the 21st century in continuing this rapid pace. Among the issues to be investigated are the combinatorial pathways, through which muscle identity genes act, the molecular details of fusion-competent cell specification, the mechanism by which the extent of fusion and resulting muscle size are determined, and the molecules regulating the specificity of muscle attachment. The next two decades have the potential to be quite exciting.

Appendix

Genes discussed in this review that have an impact on embryonic myogenesis. Some of these genes play a global role in cell fate specification, and are discussed elsewhere in this volume. Others may be expressed in nonmuscle tissues, but affect muscle development. Finally, the majority of these genes are expressed within muscle tissues and affect their specification, differentiation or function.

Appendix

Name	*Abbr.*	*Structure and/or apparent function of encoded protein*
Actin 57B	*Act57B*	Component of muscle sarcomere
alien		Nuclear corepressor of the hormone receptor superfamily
Antennapedia	*Antp*	Homeotic gene; homeodomain containing protein; controls segmental identity of muscle pattern
antisocial (*rols*)	*ants*	Ring Finger, Ankyrin repeat, TPR, coiled coil containing cytoplasmic protein; founder cell specific signaling that is essential for myoblast fusion
apterous	*ap*	LIM homeodomain containing protein; muscle identity gene
argos	*aos*	Diffusible inhibitor of DER
β3-tubulin		Expressed in somatic and visceral muscle
bagpipe	*bap*	Homeodomain encoding gene; subdivides mesoderm; essential for visceral mesoderm
beaten path	*beat*	Novel secreted protein that regulates defasciculation at motor axon choice points
bHLH54		bHLH containing protein
biniou	*bin*	FoxF family of forkhead domain containing proteins involved in visceral mesoderm development and midgut morphogenesis
blown fuse	*blow*	Novel cytoplasmic protein; essential for myoblast fusion
brachyenteron	*byn*	Involved in early specification and features of the caudal visceral mesoderm (CVM)
brokenhearted	*bkh*	Encodes the alpha subunit of the Go protein, involved in specification of precursor cells of the heart tube, the visceral muscles, and the nervous system
CF2		Zinc finger containing transcription factor
clueless (abrupt)	*ab*	Zinc finger transcription factor; myogenic marker downstream of Mef2
collier	*col*	COE transcription factor family member; muscle identity gene
Connectin	*Con*	Cell surface glycoprotein involved in neuromuscular junctions
crk		SH2-SH3 containing adapter protein
crocodile	*croc*	Fork head domain protein required for head structures
daughterless	*da*	bHLH containing E protein
decapentaplegic	*dpp*	TGFβ family member; signaling associated with subdivision of the mesoderm
delilah	*dei*	bHLH containing nuclear protein expressed in muscle attachment sites
derailed	*drl*	Axon guidance receptor
dorsal	*dl*	NF-κB related transcription factor; required for specifying D/V axi
Drac1		Small monomeric GTPase; essential for myoblast fusion
dumfounded (kirre)	*duf*	Ig Superfamily transmembrane protein expressed in muscle founders
egfr		Epidermal growth factor receptor
engrailed	*en*	Homeodomain containinging protein; muscle identity gene
even-skipped	*eve*	Homeodomain-containing pair-rule gene; muscle identity gene
Fasciclin 3	*Fas3*	Ig Superfamily cell surface protein
folded gastrulation	*fog*	Secreted protein required during *Drosophila* gastrulation
forkhead	*fkh*	Transcription factor that promotes cell shape change
frazzled	*fra*	Ig Superfamily transmembrane receptor involved in axon guidance
gleeful (lmd, minc)	*glee*	Zinc finger containing member of Gli superfamily essential for mef2 expression
groovin (short stop)	*shot*	EF-hand gene family; expressed in muscle attachment sites
heartless (DFR1)	*htl*	Fibroblast growth factor receptor; required for morphogenesis of the mesoderm and tracheal system
hedgehog	*hh*	Secreted signaling factor
hibris	*hbs*	Ig Superfamily transmembrane protein expressed in muscles
inflated	*if*	α_{PS2} integrin subunit associated with cell:cell and cell:matrix adhesion; associated with muscle attachment
inscutable (nem)	*insc*	Cytoplasmic protein associated with asymmetric division (of founder cells)
irregular-optic chiasma C (roughest)	*rst*	Ig Superfamily transmembrane protein; substitutes for duf/kirre in myoblast fusion

Continued

Appendix Continued

Name	*Abbr.*	*Structure and/or apparent function of encoded gene*
jelly belly	*jeb*	LDL receptor repeat containing secreted protein associated with fusion of visceral myoblasts
kin of irre (*duf*)	*kirre*	Ig Superfamily transmembrane protein expressed in founder myoblasts; redundant function with IrreD/rst in fusion of these myoblasts
kruppel	*kr*	Homeodomain containing protein; muscle identity gene
ladybird	*lb*	Homeodomain containing protein;
lameduck (minc, glee)	*lmd*	Zinc finger containing member of Gli superfamily essential for mef2 expression
lethal of scute	*l(1)sc*	bHLH containing transcription factor; expressed in myoblast clusters
masquerade	*mas*	Protease associated with plasma membrane
meso18E		Novel mef2 regulated nuclear protein
msp-300		Spectrin-related molecule expressed in sarcomere and muscle attachment sites
M-spondin	*mspo*	Extracellular matrix protein
muscle segment homeobox	*msh*	Homeodomain containing transcription factor
muscleblind	*mbl*	Nuclear Cys-His zinc finger containing protein associated with muscle attachment
myoblast city	*mbc*	SH3 and DH domain containing cytoplasmic protein essential for myoblast fusion
myoblasts-incompetent (lmd, glee)	*minc*	Zinc finger containing member of Gli superfamily essential for myoblast specification
myocyte enhancer factor 2	*mef2*	MADS box containing transcription factor essential for myoblast morphogenesis
myosin heavy chain	*mhc*	Component of muscle sarcomere
myospheroid	*mys*	B_{PS} integrin subunit associated with muscle attachment
nautilus	*nau*	bHLH containing MRF family member; muscle identity gene
neuromusculin	*nrm*	Ig Superfamily transmembrane protein associated with larval muscles
not-enough-muscles (insc)	*nem*	Cytoplasmic protein associated with asymmetric division (of founder cells)
numb		Cytoplasmic/plasma membrane associated protein asymmetrically distributed in muscle progenitors
Paramyosin	*Prm*	Muscle structural protein
PAR-domain protein 1	*Pdp1*	PAR-domain bZIP transcription factor that regulates Mef2
Pox-meso	*Poxm*	Paired-domain containing protein expressed in the somatic mesoderm
rolling pebbles (ants)	*rols*	Ring Finger, Ankyrin repeat, TPR, coiled coil containing cytoplasmic protein essential for myoblast fusion
rolling stone	*rost*	Novel protein associated with myoblast fusion
roughest(irreC)	*rst*	Ig Superfamily transmembrane protein; substitutes for duf/kirre in myoblast fusion
S59/slouch	*slou*	Homeodomain-containing protein; muscle identity gene
sallimus (D-titin)	*sls*	Filamentous protein that is a component of muscle sarcomeres
sanpodo	*spdo*	Tropomodulin-like protein associated with asymmetric division of muscle progenitors
serpent	*srp*	GATA-like zinc-finger transcription factor expressed in midgut primordia
short stop(Groovin)	*shot*	Actin binding protein involved in axon guidance
shotgun	*shg*	DE-cadherin cell adhesion molecule
sidestep	*side*	Ig Superfamily transmembrane protein involved in targeting axons to muscle
singles-bar		Involved in myoblast fusion
snail	*sna*	Zinc-finger containing transcription factor essential for mesoderm formation
sticks-and-stones	*sns*	Ig Superfamily transmembrane receptor expressed in fusion competent cells and essential for myoblast fusion
stripe	*sr*	Zinc-finger containing nuclear protein involved in muscle attachment
tinman	*tin*	Homeodomain containing protein involved in heart development
Toll	*Tl*	Cell surface glycoprotein
Tropomyosin 1	*Tm1*	Component of sarcomere
upheld	*up*	Troponin T; component of sarcomere
twist	*twi*	bHLH-containing transcription factor essential for mesoderm specification; promotes myogenic differentiation
vestigial	*vg*	Novel nuclear protein; muscle identity gene
wingless	*wg*	Wnt-family member
zfh1		Zinc finger homeodomain protein expressed in pericardial cells and fat body; perturbations in muscle development
vimar		15 tandem armadillo repeats; expressed in visceral musculature

References

Abmayr, S.M., Balagopalan, L., Galletta, B.J., Hong, S.J., **2003**. Cell and molecular biology of myoblast fusion. *Int. Rev. Cytol. 225*, 33–89.

Abmayr, S.M., Keller, C.A., **1998**. *Drosophila* myogenesis and insights into the role of *nautilus*. In: Pedersen, R.A., Schatten, G.P. (Eds.), Current Topics in Developmental Biology. Academic Press, New York, NY, pp. 35–80.

Abmayr, S.M., Michelson, A.M., Corbin, V., Young, M.W., Maniatis, T., **1992**. *nautilus*, a *Drosophila* member of the myogenic regulatory gene family. In: Blau, H.M., Kelly, A.M. (Eds.), Neuromuscular Development and Disease. Raven Press, New York, NY, pp. 1–16.

Akam, M.E., Martinez-Arias, A., Weinzierl, R., Wilde, C.D., **1985**. Function and expression of ultrabithorax in the *Drosophila* embryo. *Cold Spring Harb. Symp. Quant. Biol. 50*, 195–200.

Albert, M.L., Kim, J.I., Birge, R.B., **2000**. alphavbeta5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nat. Cell Biol. 2*, 899–905.

Armand, P., Knapp, A.C., Hirsh, A.J., Wieschaus, E.F., Cole, M.D., **1994**. A novel basic helix–loop–helix protein is expressed in muscle attachment sites of the *Drosophila* epidermis. *Mol. Cell. Biol. 14*, 4145–4154.

Arredondo, J.J., Ferreres, R.M., Maroto, M., Cripps, R.M., Marco, R., *et al.*, **2001**. Control of *Drosophila* paramyosin/miniparamyosin gene expression. Differential regulatory mechanisms for muscle-specific transcription. *J. Biol. Chem. 276*, 8278–8287.

Artero, R., Prokop, A., Paricio, N., Begemann, G., Pueyo, I., *et al.*, **1998**. The muscleblind gene participates in the organization of Z-bands and epidermal attachments of *Drosophila* muscles and is regulated by Dmef2. *Devel. Biol. 195*, 131–143.

Artero, R.D., Castanon, I., Baylies, M.K., **2001**. The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. *Development 128*, 4251–4264.

Azpiazu, N., Frasch, M., **1993**. *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Devel. 7*, 1325–1340.

Azpiazu, N., Lawrence, P.A., Vincent, J.P., Frasch, M., **1996**. Segmentation and specification of the *Drosophila* mesoderm. *Genes Devel. 10*, 3183–3194.

Bagni, C., Bray, S., Gogos, J.A., Kafatos, F.C., Hsu, T., **2002**. The *Drosophila* zinc finger transcription factor CF2 is a myogenic marker downstream of MEF2 during muscle development. *Mech. Devel. 117*, 265–268.

Bahri, S.M., Chia, W., Yang, X., **2001**. Characterization and mutant analysis of the *Drosophila* sema 5c gene. *Devel. Dyn. 221*, 322–330.

Baker, R., Schubiger, G., **1996**. Autonomous and nonautonomous Notch functions for embryonic muscle and epidermis in *Drosophila*. *Development 122*, 617–626.

Ball, E.E., Goodman, C.S., **1985a**. Muscle development in the grasshopper embryo. II. Syncytial origin of the extensor tibiae muscle pioneers. *Devel. Biol. 111*, 399–416.

Ball, E.E., Goodman, C.S., **1985b**. Muscle development in the grasshopper embryo. III. Sequential origin of the flexor tibiae muscle pioneers. *Devel. Biol. 111*, 417–424.

Ball, E.E., Ho, R.K., Goodman, C.S., **1985a**. Development of neuromuscular specificity in the grasshopper embryo: guidance of motoneuron growth cones by muscle pioneers. *J. Neurosci. 5*, 1808–1819.

Ball, E.E., Ho, R.K., Goodman, C.S., **1985b**. Muscle development in the grasshopper embryo. I. Muscles, nerves, and apodemes in the metathoracic leg. *Devel. Biol. 111*, 383–398.

Bashaw, G.J., Goodman, C.S., **1999**. Chimeric axon guidance receptors: the cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. *Cell 97*, 917–926.

Bashaw, G.J., Kidd, T., Murray, D., Pawson, T., Goodman, C.S., **2000**. Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell 101*, 703–715.

Basi, G.S., Boardman, M., Storti, R.V., **1984**. Alternative splicing of a *Drosophila* tropomyosin gene generates muscle tropomyosin isoforms with different carboxy-terminal ends. *Mol. Cell. Biol. 4*, 2828–2836.

Basi, G.S., Storti, R.V., **1986**. Structure and DNA sequence of the Tropomyosin I gene from *Drosophila melanogaster. J. Biol. Chem. 261*, 817–827.

Bate, M., **1990**. The embryonic development of larval muscles in *Drosophila*. *Development 110*, 791–804.

Bate, M., **1993**. The mesoderm and its derivatives. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1013–1090.

Bate, M., Rushton, E., **1993**. Myogenesis and muscle patterning in *Drosophila*. *C. R. Acad. Sci. Paris 316*, 1055–1061.

Bate, M., Rushton, E., Currie, D.A., **1991**. Cells with persistent *twist* expression are the embryonic percursors of adult muscles in *Drosophila*. *Development 113*, 79–89.

Bate, M., Rushton, E., Frasch, M., **1993**. A dual requirement for neurogenic genes in *Drosophila* myogenesis. *Development* (Supplement), 149–161.

Baylies, M.K., Bate, M., **1996**. *twist*: A myogenic switch in *Drosophila*. *Science 272*, 1481–1484.

Baylies, M.K., Martinez-Arias, A., Bate, M., **1995**. *wingless* is required for the formation of a subset of muscle founder cells during *Drosophila* embryogenesis. *Development 121*, 3829–3837.

Baylies, M.K., Michelson, A.M., **2001**. Invertebrate myogenesis: looking back to the future of muscle development. *Curr. Opin. Genet. Devel. 11*, 431–439.

Becker, S., Pasca, G., Strumpf, D., Min, L., Volk, T., **1997**. Reciprocal signaling between *Drosophila* epidermal muscle attachment cells and their corresponding muscles. *Development 124*, 2615–2622.

Beer, J., Technau, G.M., Campos-Ortega, J.A., **1987**. Lineage analysis of transplanted individual cells in

embryos of *Drosophila melanogaster.* IV. Commitment and proliferative capabilities of mesodermal cells. *Roux's Arch. Devel. Biol. 196*, 222–230.

Belvin, M.P., Anderson, K.V., **1996**. A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. *Annu. Rev. Cell. Devel. Biol. 12*, 393–416.

Bernstein, S.I., O'Donnell, P.T., Cripps, R.M., **1993**. Molecular genetic analysis of muscle development, structure and function in *Drosophila*. *Int. Rev. Cytol. 143*, 63–152.

Bieman, M., Shilo, B.Z., Volk, T., **1996**. Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Devel. 10*, 2993–3002.

Bodmer, R., **1993**. The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development 118*, 719–729.

Bodmer, R., Jan, L.Y., Jan, Y.N., **1990**. A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation of *Drosophila*. *Development 110*, 661–669.

Bogaert, T., Brown, N., Wilcox, M., **1987**. The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell 51*, 929–940.

Bopp, D., Jamet, E., Baumgartner, S., Burri, M., Noll, M., **1989**. Isolation of two tissue-specific *Drosophila* paired box genes, *Pox meso* and *Pox neuro*. *EMBO J. 8*, 3447–3457.

Boulay, J.L., Dennefeld, C., Alberga, A., **1987**. The *Drosophila* developmental gene *snail* encodes a protein with nucleic acid binding fingers. *Nature 330*, 395–398.

Bour, B.A., Chakravarti, M., West, J.M., Abmayr, S.M., **2000**. *Drosophila* Sns, a member of the Immunoglobulin Superfamily that is essential for myoblast fusion. *Genes Devel. 14*, 1498–1511.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., *et al.*, **1995**. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes Devel. 9*, 730–741.

Bourgouin, C., Lundgren, S.E., Thomas, J.B., **1992**. *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. *Neuron 9*, 549–561.

Brennan, K., Baylies, M., Martinez-Arias, A., **1999**. Repression by Notch is required before Wingless signalling during muscle progenitor cell development in *Drosophila*. *Curr. Biol. 9*, 707–710.

Broadie, K., Bate, M., **1993a**. Innervation directs receptor synthesis and localization in *Drosophila* embryo synaptogenesis. *Nature 361*, 350–353.

Broadie, K., Bate, M., **1993b**. Muscle development is independent of innervation during *Drosophila* embryogenesis. *Development 119*, 533–543.

Broihier, H.T., Moore, L.A., Van Doren, M., Newman, S., Lehmann, R., **1998**. zfh–1 is required for germ cell migration and gonadal mesoderm development in *Drosophila*. *Development 125*, 655–666.

Brown, N.H., **1994**. Null mutations in the αps2 and βps integrin subunit genes have distinct phenotypes. *Development 120*, 1221–1231.

Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S.F., *et al.*, **2002**. Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. *Natl Cell Biol. 4*, 574–582.

Buescher, M., Yeo, S.L., Udolph, G., Zavortink, M., Yang, X., *et al.*, **1998**. Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are nonstochastic and require inscuteable-mediated asymmetry of ganglion mother cells. *Genes Devel. 12*, 1858–1870.

Buff, E., Carmena, A., Gisselbrecht, S., Jimenez, F., Michelson, A.M., **1998**. Signaling by the epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development 125*, 2075–2086.

Bunch, T.A., Brower, D.L., **1993**. *Drosophila* cell adhesion molecules. *Curr. Top. Devel. Biol. 28*, 81–123.

Bunch, T.A., Graner, M.W., Fessler, L.I., Fessler, J.H., Schneider, K.D., *et al.*, **1998**. The PS2 integrin ligand tiggrin is required for proper muscle function in *Drosophila*. *Development 125*, 1679–1689.

Burchard, S., Paululat, A., Hunz, U., Renkawitz-Pohl, R., **1995**. The mutant *not enough muscles (nem)* reveals reduction of the *Drosophila* embryonic muscle pattern. *J. Cell Sci. 108*, 1443–1454.

Buttgereit, D., **1996**. Transcription of the beta 1 tubulin (beta Tub56D) gene in apodemes is strictly dependent on muscle insertion during embryogenesis in *Drosophila melanogaster. Eur. J. Cell. Biol. 71*, 183–191.

Buttgereit, D., Leiss, D., Michiels, F., Renkawitz-Pohl, R., **1991**. During *Drosophila* embryogenesis the β1 tubulin gene is specifically expressed in the nervous system and the apodemes. *Mech. Devel. 33*, 107–118.

Callahan, C.A., Bonkovsky, J.L., Scully, A.L., Thomas, J.B., **1996**. *derailed* is required for muscle attachment site selection in *Drosophila*. *Development 122*, 2761–2767.

Campos-Ortega, J.A., Hartenstein, V., **1985**. The embryonic development of *Drosophila melanogaster.* Springer-Verlag, Berlin.

Campos-Ortega, J.A., Hartenstein, V., **1997**. The embryonic development of *Drosophila melanogaster.* Springer-Verlag, Berlin.

Capovilla, M., Kambris, Z., Botas, J., **2001**. Direct regulation of the muscle-identity gene apterous by a Hox protein in the somatic mesoderm. *Development 128*, 1221–1230.

Carmena, A., Bate, M., Jimenez, F., **1995**. *lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. *Genes Devel. 9*, 2373–2383.

Carmena, A., Buff, E., Halfon, M.S., Gisselbrecht, S., Jimenez, F., *et al.*, **2002**. Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the *Drosophila* embryonic mesoderm. *Devel. Biol. 244*, 226–242.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F., Michelson, A.M., **1998a**. Combinatorial signaling codes for progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Devel. 12*, 3910–3922.

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenez, F., Chia, W., **1998b**. *inscutable* and *numb* mediate asymmetric muscle cell progenitor cell divisions during *Drosophila* myogenesis. *Genes Devel. 12*, 304–315.

Cash, S., Chiba, A., Keshishian, H., **1992**. Alternate neuromuscular target selection following the loss of single muscle fibers in *Drosophila*. *J. Neurosci. 12*, 2051–2064.

Castanon, I., Von Stetina, S., Kass, J., Baylies, M.K., **2001**. Dimerization partners determine the activity of the Twist bHLH protein during *Drosophila* mesoderm development. *Development 128*, 3145–3159.

Chang, S., Ho, R.K., Goodman, C.S., **1983**. Selective groups of neuronal and mesodermal cells recognized early in grasshopper embryogenesis by a monoclonal antibody. *Brain Res. 285*, 297–304.

Chen, E.H., Olson, E.N., **2001**. Antisocial, an intracellular adaptor protein, is required for myoblast fusion in *Drosophila*. *Devel. Cell 1*, 705–715.

Chiba, A., Snow, P., Keshishian, H., Hotta, Y., **1995**. Fasciclin III as a synaptic target recognition molecule in *Drosophila*. *Nature 374*, 166–168.

Chung, S., Gumienny, T.L., Hengartner, M.O., Driscoll, M., **2000**. A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in C. elegans. *Nat. Cell Biol. 2*, 931–937.

Conradt, B., **2001**. Cell engulfment, no sooner ced than done. *Devel. Cell 1*, 445–447.

Corbin, V., Michelson, A.M., Abmayr, S.M., Neel, V., Alcamo, E., *et al.*, **1991**. A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell 67*, 311–323.

Costa, M., Wilson, E.T., Wieschaus, E., **1994**. A putative cell signal encoded by the folded gastrulation gene coordinates cell shape changes during *Drosophila* gastrulation. *Cell 76*, 1075–1089.

Cote, J.F., Vuori, K., **2002**. Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. *J. Cell Sci. 115*, 4901–4913.

Cripps, R.M., Black, B.L., Zhao, B., Lien, C.L., Schulz, R.A., *et al.*, **1998**. The myogenic regulatory gene *Mef2* is a direct target for transcriptional activation by Twist during *Drosophila* myogenesis. *Genes Devel. 12*, 422–434.

Crossley, A.C., **1978**. The morphology and development of the *Drosophila* muscular system. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila* 2b. Academic Press, London, pp. 499–560.

Crozatier, M., Vincent, A., **1999**. Requirement for the *Drosophila* COE transcription factor Collier in formation of an embryonic muscle. Transcriptional response to Notch signaling. *Development 126*, 1495–1504.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R., *et al.*, **2003**. vrille, Pdp1, and dClock form a second feedback loop in the *Drosophila* circadian clock. *Cell 112*, 329–341.

D'Alessio, M., Frasch, M., **1996**. *msh* may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Devel. 58*, 217–231.

Damm, C., Wolk, A., Buttgereit, D., Loher, K., Wagner, E., *et al.*, **1998**. Independent regulatory elements in the upstream region of the *Drosophila* beta 3 tubulin gene (beta Tub60D) guide expression in the dorsal vessel and the somatic muscles. *Devel. Biol. 199*, 138–149.

Doberstein, S.K., Fetter, R.D., Mehta, A.Y., Goodman, C.S., **1997**. Genetic analysis of myoblast fusion: *blown fuse* is required for progression beyond the prefusion complex. *J. Cell Biol. 136*, 1249–1261.

Dohrmann, C., Azpiazu, N., Frasch, M., **1990**. A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Devel. 4*, 2098–2111.

Duan, H., Skeath, J.B., Nguyen, H.T., **2001**. *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. *Development 128*, 4489–4500.

Duchek, P., Somogyi, K., Jekely, G., Beccari, S., Rorth, P., **2001**. Guidance of cell migration by the *Drosophila* PDGF/VEGF receptor. *Cell 107*, 17–26.

Dunin-Borkowski, O.M., Brown, N.H., Bate, M., **1995**. Anterior–posterior subdivision and the diversification of the mesoderm in *Drosophila*. *Development 121*, 4183–4193.

Dworak, H.A., Charles, M.A., Pellerano, L.B., Sink, H., **2001**. Characterization of *Drosophila hibris*, a gene related to human nephrin. *Development 128*, 4265–4276.

Dworak, H.A., Sink, H., **2002**. Myoblast fusion in *Drosophila*. *Bioessays 24*, 591–601.

Dye, C.A., Lee, J.-K., Atkinson, R.C., Brewster, R., Han, P.-L., *et al.*, **1998**. The *Drosophila sanpodo* gene controls sibling cell fate and encodes a tropomodulin homolog, an acitn/tropomyosing associated protein. *Development 125*, 1845–1856.

Erickson, M.R.S., Galletta, B.J., Abmayr, S.M., **1997**. *Drosophila myoblast city* encodes a conserved protein that is essential for myoblast fusion, dorsal closure and cytoskeletal organization. *J. Cell Biol. 138*, 589–603.

Feller, S.M., Posern, G., Voss, J., Kardinal, C., Sakkab, D., *et al.*, **1998**. Physiological signals and oncogenesis mediated through Crk family adapter proteins. *J. Cell Physiol. 177*, 535–552.

Frasch, M., **1995**. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature 374*, 464–467.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H., Levine, M., **1987**. Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J. 6*, 749–759.

Frasch, M., Nguyen, H.T., **1999**. Genetic control of mesoderm patterning and differentiation during *Drosophila* embryogenesis. *Adv. Devel. Biochem. 5*, 1–47.

Frise, E., Knoblich, J.A., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., **1996**. The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell–cell interaction in sensory organ lineage. *Proc. Natl Acad. Sci. 93*, 11925–11932.

Frommer, G., Vorbruggen, G., Pasca, G., Jackle, H., Volk, T., **1996**. Epidermal egr-like zinc finger protein of *Drosophila* participates in myotube guidance. *EMBO J. 15*, 1642–1649.

Furlong, E.E., Andersen, E.C., Null, B., White, K.P., Scott, M.P., **2001**. Patterns of gene expression during *Drosophila* mesoderm development. *Science 293*, 1629–1633.

Galletta, B.J., Niu, X.-P., Erickson, M.R.S., Abmayr, S.M., **1999**. Identification of a *Drosophila* homologue to vertebrate Crk by interaction with MBC. *Gene 228*, 243–252.

Garcia-Bellido, A., Ripoll, P., Morata, G., **1976**. Developmental compartmentalization in the dorsal mesothoracic disc of *Drosophila*. *Devel. Biol. 48*, 132–147.

Gasch, A., Hinz, U., Renkawitz-Pohl, R., **1989**. Intron and upstream sequences regulate expression of the *Drosophila* beta 3-tubulin gene in the visceral and somatic musculature, respectively. *Proc. Natl Acad. Sci. 86*, 3215–3218.

Gaul, U., Seifert, E., Schuh, R., Jackle, H., **1987**. Analysis of *Kruppel* protein distribution during early *Drosophila* development reveals posttranscriptional regulation. *Cell 50*, 639–647.

Georgias, C., Wassar, M., Hinz, U., **1997**. A basic-helix–loop–helix protein expressed in precursors of *Drosophila* longitudinal visceral muscles. *Mech. Devel. 69*, 115–124.

Gisselbrecht, S., Skeath, J.B., Doe, C.Q., Michelson, A.M., **1996**. *heartless* encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Devel. 10*, 3003–3017.

Goldstein, M.A., Burdette, W.J., **1971**. Striated visceral muscle of *Drosophila melanogaster. J. Morphol. 134*, 315–334.

Goubeaud, A., Knirr, S., Renkawitz-Pohl, R., Paululat, A., **1996**. The *Drosophila* gene *alien* is expressed in the muscle attachment sites during embryogenesis and encodes a protein highly conserved between plants, *Drosophila* and vertebrates. *Mech. Devel. 57*, 59–68.

Grau, Y., Carteret, C., Simpson, P., **1984**. Mutations and chromosomal rearrangements affecting the expression of snail, a gene involved in embryonic patterning in *Drosophila melanogaster. Genetics 108*, 347–360.

Gregorio, C.C., Granzier, H., Sorimachi, H., Labeit, S., **1999**. Muscle assembly: a titanic achievement? *Curr. Opin. Cell Biol. 11*, 18–25.

Grossniklaus, U., Pearson, R.K., Gehring, W.J., **1992**. The *Drosophila* sloppy paired locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. *Genes Devel. 6*, 1030–1051.

Gumienny, T.L., Brugnera, E., Tosello-Trampont, A.C., Kinchen, J.M., Haney, L.B., *et al.*, **2001**. CED–12/ ELMO, a novel member of the CrkII/ Dock180/Rac pathway, is required for phagocytosis and cell migration. *Cell 107*, 27–41.

Gunthorpe, D., Beatty, K.E., Taylor, M.V., **1999**. Different levels, but not different isoforms, of the Drosophila transcription factor DMEF2 affect distinct aspects of muscle differentiation. *Devel. Biol. 215*, 130–145.

Guo, M., Jan, L.Y., Jan, Y.N., **1996**. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron 17*, 27–41.

Hacker, U., Kaufmann, E., Hartmann, C., Jurgens, G., Knochel, W., *et al.*, **1995**. The *Drosophila* fork head domain protein crocodile is required for the establishment of head structures. *EMBO J. 14*, 5306–5317.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., *et al.*, **2002**. Rac function and regulation during *Drosophila* development. *Nature 416*, 438–442.

Halfon, M.S., Carmena, A., Gisselbrecht, S., Sackerson, C.M., Jimenez, F., *et al.*, **2000**. Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell 103*, 63–74.

Halfon, M.S., Hashimoto, C., Keshishian, H., **1995**. The *Drosophila* toll gene functions zygotically and is necessary for proper motoneuron and muscle development. *Devel. Biol. 169*, 151–167.

Halpern, M.E., Chiba, A., Johansen, J., Keshishian, H., **1991**. Growth cone behavior underlying the development of stereotypic synaptic connections in *Drosophila* embryos. *J. Neuro. Sci. 11*, 3227–3238.

Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., *et al.*, **1996**. DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. *Mol. Cell. Biol. 16*, 1770–1776.

Hess, N., Kronert, W.A., Bernstein, S.I., **1989**. Transcriptional and post-transcriptional regulation of *Drosophila* myosin heavy chain gene expression. In: Kedes, L.H., Stockdale, F.H. (Eds.), Cellular and Molecular Biology of Muscle Development. Alan R. Liss, Inc., New York, NY, pp. 621–631.

Hess, N.K., Bernstein, S.I., **1991**. Developmentally regulated alternative splicing of *Drosophila* Myosin heavy chain transcripts: *in vivo* analysis of an unusual 3′ splice site. *Devel. Biol. 146*, 339–344.

Hirata, J., Nakagoshi, H., Nabeshima, Y., Matsuzaki, F., **1995**. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature 377*, 627–630.

Ho, R.K., Ball, E.B., Goodman, C.S., **1983**. Muscle pioneers: large mesodermal cells that erect a scaffold for developing muscles and motoneurones in grasshopper embryos. *Nature 301*, 66–69.

Hooper, J., **1986**. Homeotic gene function in the muscles of *Drosophila* larvae. *EMBO J. 5*, 2321–2329.

Hortsch, M., Goodman, C.S., **1991**. Cell and substrate adhesion molecules in *Drosophila*. *Annu. Rev. Cell Biol. 7*, 505–557.

Jagla, T., Bellard, F., Lutz, Y., Dretzen, G., Bellard, M., *et al.*, **1998**. *ladybird* determines cell fate decisions during diversification of *Drosophila* somatic muscles. *Development 125*, 3699–3708.

Jagla, T., Bellard, F., Vonesch, J.L., Bellard, M., Dastugue, B., *et al.*, **1999**. Plasticity within the lateral somatic mesoderm of *Drosophila* embryos. *Int. J. Devel. Biol. 43*, 571–573.

Jagla, T., Bidet, Y., Da Ponte, J.P., Dastugue, B., Jagla, K., **2002**. Cross-repressive interactions of identity genes are essential for proper specification of cardiac and muscular fates in *Drosophila*. *Development 129*, 1037–1047.

Jan, Y.N., Jan, L.L., **1998**. Asymmetric cell division. *Nature 392*, 775–778.

Johansen, J., Halpern, M.E., Keshishian, H., **1989**. Axonal guidance and the development of muscle fiber-specific innervation in *Drosophila* embryos. *J. Neurosci. 9*, 4318–4332.

Jellies, J., **1990**. Muscle assembly in simple systems. *Trends Neurosci. 13*, 126–131.

Kania, A., Han, P.L., Kim, Y.T., Bellen, H., **1993**. Neuromusculin, a *Drosophila* gene expressed in peripheral neuronal precursors and muscles, encodes a cell adhesion molecule. *Neuron 11*, 673–687.

Karlik, C.C., Fyrberg, E.A., **1986**. Two *Drosophila melanogaster* tropomyosin genes: structural and functional aspects. *Mol. Cell. Biol. 6*, 1965–1973.

Kashiwa, A., Yoshida, H., Lee, S., Paladino, T., Liu, Y., *et al.*, **2000**. Isolation and characterization of novel presenilin binding protein. *J. Neurochem. 75*, 109–116.

Keller, C.A., Erickson, M.S., Abmayr, S.M., **1997**. Misexpression of *nautilus* induces myogenesis in cardioblasts and alters the pattern of somatic muscle fibers. *Devel. Biol. 181*, 197–212.

Keller, C.A., Grill, M.A., Abmayr, S.M., **1998**. A role for *nautilus* in the differentiation of muscle precursors. *Devel. Biol. 202*, 157–171.

Kelly, K.K., Meadows, S.M., Cripps, R.M., **2002**. Drosophila MEF2 is a direct regulator of Actin57B transcription in cardiac, skeletal, and visceral muscle lineages. *Mech. Devel. 110*, 39–50.

Keshishian, H., **1993**. Making the right connections. *Nature 361*, 299–300.

Keshishian, H., Chiba, A., **1993**. Neuromuscular development in *Drosophila*: insights from single neurons and single genes. *Trends Neurosci. 16*, 278–283.

Keshishian, H., Chiba, A., Chang, T.N., Halfon, M.S., Harkins, E.W., *et al.*, **1993**. Cellular mechanisms governing synaptic development in *Drosophila melanogaster. J. Neurobiol. 24*, 757–787.

Kestila, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., *et al.*, **1998**. Positionally cloned gene for a novel glomerular protein-Nephrin-is mutated in congenital nephrotic syndrome. *Mol. Cell 1*, 572–582.

Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., *et al.*, **1998a**. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Devel. 12*, 3331–3336.

Kiyokawa, E., Hashimoto, Y., Kurata, T., Sugimura, H., Matsuda, M., **1998b**. Evidence that DOCK180 up-regulates signals from the CrkII-p130Cas complex. *J. Biol. Chem. 273*, 24479–24484.

Klambt, C., **1993**. The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development 117*, 163–176.

Klapper, R., **2000**. The longitudinal visceral musculature of *Drosophila melanogaster* persists through metamorphosis. *Mech. Devel. 95*, 47–54.

Klapper, R., Heuser, S., Strasser, T., Janning, W., **2001**. A new approach reveals syncytia within the visceral musculature of *Drosophila melanogaster. Development 128*, 2517–2524.

Klapper, R., Stute, C., Schomaker, O., Strasser, T., Janning, W., *et al.*, **2002**. The formation of syncytia within the visceral musculature of the *Drosophila* midgut is dependent on duf, sns and mbc. *Mech. Devel. 110*, 85–96.

Knirr, S., Azpiazu, N., Frasch, M., **1999**. The role of the NK-homeobox gene *slouch* (S59) in somatic muscle patterning. *Development 126*, 4525–4535.

Knirr, S., Breuer, S., Paululat, A., Renkawitz-Pohl, R., **1997**. Somatic mesoderm differentiation and the development of a subset of pericardial cells depend on the not enough muscles (nem) locus, which contains the inscuteable gene and the intron located gene, skittles. *Mech. Devel. 67*, 69–81.

Knoblich, J.A., Jan, L.Y., Jan, Y.N., **1995**. Asymmetric segregation of Numb and Prospero during cell division. *Nature 377*, 624–627.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., *et al.*, **1996**. *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell 87*, 197–204.

Kosman, D., Ip, Y.T., Levine, M., Arora, K., **1991**. Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. *Science 254*, 118–122.

Kraut, R., Campos-Ortega, J.A., **1996**. *inscutable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeletal adaptor protein. *Devel. Biol. 174*, 65–81.

Kraut, R., Chia, W., Jan, L.Y., Jan, Y.N., Knoblich, J.A., **1996**. Role of inscuteable in orienting asymmetric cell divisions in *Drosophila*. *Nature 383*, 50–55.

Kusch, T., Reuter, R., **1999**. Functions for *Drosophila* brachyenteron and forkhead in mesoderm specification and cell signalling. *Development 126*, 3991–4003.

Lai, Z., Fortini, M.E., Rubin, G.M., **1991**. The embryonic expression patterns of *zfh–1* and *zfh–2*, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. *Mech. Devel. 34*, 123–134.

Landgraf, M., Baylies, M., Bate, M., **1999**. Muscle founder cells regulate defasciculation and targeting of motor axons in the *Drosophila* embryo. *Curr. Biol. 9*, 589–592.

Lawrence, P.A., Bodmer, R., Vincent, J.P., **1995**. Segmental patterning of heart precursors in *Drosophila*. *Development 121*, 4303–4308.

Lawrence, P.A., Johnston, P., **1984**. The genetic specification of pattern in a *Drosophila* muscle. *Cell 36*, 775–782.

Lawrence, P.A., Johnston, P., **1986**. The muscle pattern of a segment of *Drosophila* may be determined by neurons and not by contributing myoblasts. *Cell 45*, 505–513.

Lawrence, P.A., Johnston, P., Vincent, J.P., **1994**. Wingless can bring about a mesoderm-to-ectoderm induction in *Drosophila* embryos. *Development 120*, 3355–3359.

Lee, J.C., VijayRaghavan, K., Celniker, S.E., Tanouye, M.A., **1995**. Identification of a *Drosophila* muscle development gene with structural homology to mammalian early growth response transcription factors. *Proc. Natl Acad. Sci. 92*, 10344–10348.

Leiss, D., Hinz, U., Gasch, A., Mertz, R., Renkawitz-Pohl, R., **1988**. Beta 3 tubulin expression characterizes the differentiating mesodermal germ layer during *Drosophila* embryogenesis. *Development 104*, 525–531.

Lenkkeri, U., Mannikko, M., McCready, P., Lamerdin, J., Gribouval, O., *et al*., **1999**. Structure of the gene for congenital nephrotic syndrome of the Finnish type (NPHS1) and characterization of mutations. *Am. J. Hum. Genet. 64*, 51–61.

Leptin, M., **1991**. *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. *Genes Devel. 5*, 1568–1576.

Leptin, M., Casal, J., Grunewald, B., Reuter, R., **1992**. Mechanisms of early *Drosophila* mesoderm formation. *Development* (Suppl.), 23–31.

Leptin, M., Grunewald, B., **1990**. Cell shape changes during gastrulation in *Drosophila*. *Development 110*, 73–84.

Lilly, B., Galewsky, S., Firulli, A.B., Schulz, R.A., Olson, E.N., **1994**. D-MEF2: A MADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during *Drosophila* embryogenesis. *Proc. Natl Acad. Sci. 91*, 5662–5666.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., *et al*., **1995**. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science 267*, 688–693.

Lin, M.H., Bour, B.A., Abmayr, S.M., Storti, R.V., **1997a**. Ectopic expression of MEF2 in the epidermis induces epidermal expression of muscle genes and abnormal muscle development in *Drosophila*. *Devel. Biol. 182*, 240–255.

Lin, S.C., Lin, M.H., Horvath, P., Reddy, K.L., Storti, R.V., **1997b**. PDP1, a novel *Drosophila* PAR domain bZIP transcription factor expressed in developing mesoderm, endoderm and ectoderm, is a transcriptional regulator of somatic muscle genes. *Development 124*, 4685–4696.

Lin, S.C., Storti, R.V., **1997**. Developmental regulation of the *Drosophila* Tropomyosin I (Tm1) gene is controlled by a muscle activator enhancer region that contains multiple cis-elements and binding sites for multiple proteins. *Devel. Genet. 20*, 297–306.

Liu, H., Mardahl-Dumesnil, M., Sweeney, S.T., O'Kane, C.J., Bernstein, S.I., **2003**. *Drosophila* paramyosin is important for myoblast fusion and essential for myofibril formation. *J. Cell Biol. 160*, 899–908.

Lo, P.C., Frasch, M., **1998**. bagpipe-Dependent expression of vimar, a novel Armadillo-repeats gene, in *Drosophila* visceral mesoderm. *Mech. Devel. 72*, 65–75.

Lord, P.C.W., Lin, M., Hales, K.H., Storti, R.V., **1995**. Normal expression and the effects of ectopic expression of the *Drosophila muscle segment homeobox (msh)* gene suggest a role in differentiation and patterning of embryonic muscles. *Devel. Biol. 171*, 627–640.

Luo, L., Liao, Y.J., Jan, L.Y., Jan, Y.N., **1994**. Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Devel. 8*, 1787–1802.

Macdonald, P.M., Ingham, P., Struhl, G., **1986**. Isolation, structure, and expression of even-skipped: a second pair-rule gene of *Drosophila* containing a homeo box. *Cell 47*, 721–734.

Machado, C., Andrew, D.J., **2000**. D-Titin: a giant protein with dual roles in chromosomes and muscles. *J. Cell. Biol. 151*, 639–652.

Maeland, A.D., Bloor, J.W., Brown, N.H., **1996**. Characterization of *singles bar* a new mutant in *Drosophila melanogaster* required for muscle development. *Mol. Cell. Biol. 7*, 39a.

Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., *et al*., **1994**. Crk protein binds to two guanine nucleotide-releasing proteins for the ras family and modulates nerve growth factor-induced activation of ras in PC12 cells. *Mol. Cell. Biol. 14*, 5495–5500.

Matsuda, M., Ota, S., Tanimura, R., Nakamura, H., Matuoka, K., *et al*., **1996**. Interaction between the amino-terminal SH3 domain of Crk and its natural target proteins. *J. Biol. Chem. 271*, 14468–14472.

Matthes, D.J., Sink, H., Kolodkin, A.L., Goodman, C.S., **1995**. Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell 81*, 631–639.

Meller, N., Irani-Tehrani, M., Kiosses, W.B., Del Pozo, M.A., Schwartz, M.A., **2002**. Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. *Nat. Cell Biol. 4*, 639–647.

Menon, S.D., Chia, W., **2001**. *Drosophila* rolling pebbles: a multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast attractant Dumbfounded. *Devel. Cell. 1*, 691–703.

Michelson, A.M., **1994**. Muscle pattern diversification in *Drosophila* is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development 120*, 755–768.

Michelson, A.M., Abmayr, S.M., Bate, M., Martinez-Arias, A., Maniatis, T., **1990**. Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes Devel. 4*, 2086–2097.

Murugasu-Oei, B., Rodrigues, V., Yang, X., Chia, W., **1995**. Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the *Drosophila* embryo. *Genes Devel. 9*, 139–154.

Nguyen, H.T., Bodmer, R., Abmayr, S.M., McDermott, J.C., Spoerel, N.A., *et al.*, **1994**. *D-mef2*: a new *Drosophila* mesoderm-specific MADS box-containing gene with a bi-modal expression profile during embryogenesis. *Proc. Natl Acad. Sci. 91*, 7520–7524.

Nishihara, H., **1999**. Analysis of hematopoietic cell specific protein, M-DOCK. *Hokkaido Igaku Zasshi 74*, 157–166.

Nolan, K.M., Barrett, K., Lu, Y.K.-Q.H., Vincent, S., Settleman, J., **1998**. Myoblast city, the *Drosophila* homolog of DOCK180/CED–5, is required in a Rac signaling pathway utilized for multiple developmental processes. *Genes Devel. 12*, 3337–3342.

Nose, A., Isshiki, T., Takeichi, M., **1998**. Regional specification of muscle progenitors in *Drosophila*: the role of the *msh* homeobox gene. *Development 125*, 215–223.

Nose, A., Mahajan, V.B., Goodman, C.S., **1992**. Connectin: a homophilic cell adhesion molecule on a subset of muscles and the motoneurons that innervate them in *Drosophila*. *Cell 70*, 553–567.

Oda, H., Uemura, T., Harada, Y., Iwai, Y., Takeichi, M., **1994**. A *Drosophila* homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Devel. Biol. 165*, 716–726.

Padgett, R.W., St. Johnson, R.D., Gelbart, W.M., **1987**. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-beta family. *Nature 325*, 81–84.

Park, M., Wu, X., Golden, K., Axelrod, J.D., Bodmer, R., **1996**. The wingless signaling pathway is directly involved in *Drosophila* heart development. *Devel. Biol. 177*, 104–116.

Park, M., Yaich, L.E., Bodmer, R., **1998**. Mesodermal cell fate decisions in *Drosophila* are under the control of the lineage genes *numb*, *Notch*, and *sanpodo*. *Mech. Devel. 75*, 117–126.

Patel, B.N., Van Vactor, D.L., **2002**. Axon guidance: the cytoplasmic tail. *Curr. Opin. Cell Biol. 14*, 221–229.

Paterson, B.M., Walldorf, U., Eldridge, J., Dubendorfer, A., Frasch, M., *et al.*, **1991**. The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Natl Acad. Sci. 88*, 3782–3786.

Paululat, A., Burchard, S., Renkawitz-Pohl, R., **1995**. Fusion from myoblasts to myotubes is dependent on the *rolling stone* gene (*rost*) of *Drosophila*. *Development 121*, 2611–2620.

Paululat, A., Goubeaud, A., Damm, C., Knirr, S., Burchard, S., *et al.*, **1997**. The mesodermal expression of *rolling stone* (*rost*) is essential for myoblast fusion in *Drosophila* and encodes a potential transmembrane protein. *J. Cell Biol. 138*, 337–348.

Paululat, A., Holz, A., Renkawitz-Pohl, R., **1999**. Essential genes for myoblast fusion in *Drosophila* embryogenesis. *Mech. Devel. 83*, 17–26.

Piepenburg, O., Vorbruggen, G., Jackle, H., **2000**. Drosophila segment borders result from unilateral repression of hedgehog activity by wingless signaling. *Mol. Cell 6*, 203–209.

Postigo, A.A., Ward, E., Skeath, J.B., Dean, D.C., **1999**. zfh–1, the *Drosophila* homologue of ZEB, is a transcriptional repressor that regulates somatic myogenesis. *Mol. Cell. Biol. 19*, 7255–7263.

Poulson, D.F., **1950**. Histogenesis, organogenesis, and differentiation in the embryo of *Drosophila melanogaster* meigen. In: Demerec, M. (Ed.), Biology of *Drosophila*. John Wiley and sons, Inc., New York, NY, pp. 168–274.

Prokop, A., Landgraf, M., Rushton, E., Broadie, K., Bate, M., **1996**. Presynaptic development at the *Drosophila* neuromuscular junction: Assembly and localization of presynaptic active zones. *Neuron 17*, 617–626.

Qiu, J.L., Jilk, R., Marks, M.D., Szymanski, D.B., **2002**. The Arabidopsis SPIKE1 gene is required for normal cell shape control and tissue development. *Plant Cell 14*, 101–118.

Ramos, R.G., Igloi, G.L., Lichte, B., Baumann, U., Maier, D., *et al.*, **1993**. The irregular chiasm C-roughest locus of *Drosophila*, which affects axonal projections and programmed cell death, encodes a novel immunoglobulin-like protein. *Genes Devel. 7*, 2533–2547.

Ranganayakulu, G., Schulz, R.A., Olson, E.N., **1996**. Wingless signaling induces *nautilus* expression in the ventral mesoderm of the *Drosophila* embryo. *Devel. Biol. 176*, 143–148.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J.D., Olson, E.N., *et al.*, **1995**. A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in *Drosophila*. *Devel. Biol. 171*, 169–181.

Rau, A., Buttgereit, D., Holz, A., Fetter, R., Doberstein, S.K., *et al.*, **2001**. rolling pebbles (rols) is required in *Drosophila* muscle precursors for recruitment of myoblasts for fusion. *Development 128*, 5061–5073.

Reddien, P.W., Horvitz, H.R., **2000**. CED–2/CrkII and CED–10/Rac control phagocytosis and cell migration in C. elegans. *Nat. Cell Biol. 2*, 131–136.

Reichman, C.T., Mayer, B.J., Keshav, S., Hanafusa, H., **1992**. The product of the cellular *crk* gene consists primarily of SH2 and SH3 regions. *Cell Growth Diff. 3*, 451–460.

Rhyu, M.S., Jan, L.Y., Jan, Y.N., **1994**. Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell 76*, 477–491.

Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R., Leptin, M., **1997**. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development 124*, 2915–2922.

Rosenberg-Hasson, Y., Renert-Pasca, M., Volk, T., **1996**. A *Drosophila* Dystrophin-related protein, MSP–300, is required for embryonic muscle morphogenesis. *Mech. Devel. 65*, 83–94.

Roy, S., VijayRaghavan, K., **1997**. Homeotic genes and the regulation of myoblast migration, fusion, and fibre-specific gene expression during adult myogenesis in *Drosophila*. *Development 124*, 3333–3341.

Ruiz-Gomez, M., Bate, M., **1997**. Segregation of myogenic lineages in *Drosophila* requires Numb. *Development 124*, 4857–4866.

Ruiz-Gomez, M., Coutts, N., Price, A., Taylor, M.V., Bate, M., **2000**. *Drosophila* Dumbfounded: a myoblast attractant essential for fusion. *Cell 102*, 189–198.

Ruiz-Gomez, M., Coutts, N., Suster, M.L., Landgraf, M., Bate, M., **2002**. myoblasts incompetent encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*. *Development 129*, 133–141.

Ruiz-Gomez, M., Romani, S., Hartmana, C., Jackle, H., Bate, M., **1997**. Specific muscle identities are regulated by *Kruppel* during *Drosophila* embryogenesis. *Development 124*, 3407–3414.

Rusconi, J., Corbin, V., **1998**. Evidence for a novel Notch pathway required for muscle precursor selection in *Drosophila*. *Mech. Devel. 79*, 39–50.

Rusconi, J., Corbin, V., **1999**. A widespread and early requirement for a novel Notch function during *Drosophila* embryogenesis. *Devel. Biol. 215*, 388–398.

Rushton, E., Drysdale, R., Abmayr, S.M., Michelson, A.M., Bate, M., **1995**. Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development 121*, 1979–1988.

San Martin, B., Bate, M., **2001**. Hindgut visceral mesoderm requires an ectodermal template for normal development in *Drosophila*. *Development 128*, 233–242.

Sandborn, E.B., Duclos, S., Messier, P.E., Roberge, J.J., **1967**. Atypical intestinal striated muscle in *Drosophila melanogaster*. *J. Ultrastruct. Res. 18*, 695–702.

Schejter, E.D., Cohen, Y., Abramson, I., **2003**. The *Drosophila* CDM-family homolog sponge mediatis instructive interactions between cytoskeletal elements in the syncytial blastoderm embryo. *A. Dros. Res. Conf. 44*, 225c.

Schneider, T., Reiter, C., Eule, E., Bader, B., Lichte, B., *et al.*, **1995**. Restricted expression of the irreC-rst protein is required for normal axonal projections of columnar visual neurons. *Neuron 15*, 259–271.

Schulz, R.A., Gajewski, K., **1999**. Ventral neuroblasts and the heartless FGF receptor are required for muscle founder cell specification in *Drosophila*. *Oncogene 18*, 6818–6823.

Shishido, E., Higashijima, S.-I., Emori, Y., Saigo, K., **1993**. Two FGF-receptor homologues of *Drosophila*: one is expressed in mesodermal primordium in early embryos. *Development 117*, 751–761.

Shishido, E., Ono, N., Kojima, T., Saigo, K., **1997**. Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development 124*, 2119–2128.

Simpson, P., **1983**. Maternal-zygotic gene interactions during formation of the dorsoventral pattern in *Drosophila* embryos. *Genetics 106*, 615–632.

Simpson, P., **1998**. Introduction: Notch signaling and choice of cell fates in development. *Semin. Cell. Devel. Biol. 9*, 581–582.

Sink, H., Rehm, E.J., Richstone, L., Bulls, Y.M., Goodman, C.S., **2001**. sidestep encodes a target-derived attractant essential for motor axon guidance in *Drosophila*. *Cell 105*, 57–67.

Sink, M., Whitington, P.M., **1991**. Early ablation of target muscles modulates the arborisation pattern of an identified embryonic *Drosophila* motor axon. *Development 113*, 701–770.

Skeath, J., Doe, C.Q., **1998**. Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the *Drosophila* CNS. *Development 125*, 1857–1865.

Spana, E.P., Doe, C.Q., **1995**. The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development 121*, 3187–3195.

Spana, E.P., Doe, C.Q., **1996**. Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron 17*, 21–26.

Staehling-Hampton, K., Hoffman, F.M., Baylies, M.K., Rushton, E., Bate, M., **1994**. *dpp* induces mesodermal gene expression in *Drosophila*. *Nature 372*, 783–786.

Standiford, D.M., Davis, M.B., Sun, W., Emerson, C.P., Jr., **1997**. Splice-junction elements and intronic sequences regulate alternative splicing of the *Drosophila* myosin heavy chain gene transcript. *Genetics 147*, 725–741.

Standiford, D.M., Sun, W.T., Davis, M.B., Emerson, C.P., Jr., **2001**. Positive and negative intronic regulatory elements control muscle-specific alternative exon splicing of *Drosophila* myosin heavy chain transcripts. *Genetics 157*, 259–71.

Stocker, R.F., **1974**. Electron microscopic observations on the fusion of myoblasts in Antheraea polyphemus (Lepidoptera). *Experientia 30*, 896–898.

Stronach, B.E., Renfranz, P.J., Lilly, B., Beckerle, M.C., **1999**. Muscle LIM proteins are associated with muscle sarcomeres and require dMEF2 for their expression during *Drosophila* myogenesis. *Mol. Cell. Biol. 10*, 2329–2342.

Strunkelnberg, M., Bonengel, B., Moda, L.M., Hertenstein, A., de Couet, H.G., *et al.*, **2001**. rst and its paralogue kirre act redundantly during embryonic muscle development in *Drosophila*. *Development 128*, 4229–4239.

Taylor, M.V., **2000**. A novel *Drosophila*, mef2-regulated muscle gene isolated in a subtractive hybridization-based molecular screen using small amounts of zygotic mutant RNA. *Devel. Biol. 220*, 37–52.

Taylor, M.V., **2002a**. *Drosophila* development: novel signal elicits visceral response. *Curr. Biol. 12*, R102–R104.

Taylor, M.V., **2002b**. Muscle differentiation: how two cells become one. *Curr. Biol. 12*, R224–R228.

Taylor, M.V., Beatty, K.E., Hunter, H.K., Baylies, M.K., **1995**. *Drosophila* MEF2 is regulated by *twist* and is expressed in both the primordia and differentiated cells of the embyronic somatic, visceral and heart musculature. *Mech. Devel. 50*, 29–41.

Tepass, U., Gruszynski-DeFeo, E., Haag, T.A., Omatyar, L., Torok, T., *et al.*, **1996**. shotgun encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Devel. 10*, 672–685.

Tepass, U., Hartenstein, V., **1994**. The development of cellular junctions in the *Drosophila* embryo. *Devel. Biol. 161*, 563–596.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., Perrin-Schmitt, F., **1988**. Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J. 7*, 2175–2183.

Tremml, G., Bienz, M., **1989**. Homeotic gene expression in the visceral mesoderm of *Drosophila* embryos. *EMBO J. 8*, 2677–2685.

Trinick, J., Tskhovrebova, L., **1999**. Titin: a molecular control freak. *Trends Cell Biol. 9*, 377–380.

Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kotaoka, Y., *et al.*, **1996**. Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. *Genes Devel. 10*, 659–671.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., **1989**. numb, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell 58*, 349–360.

Umemiya, T., Takeichi, M., Nose, A., **1997**. M-spondin, a novel ECM protein highly homologous to vertebrate F-spondin, is localized at the muscle attachment sites in the *Drosophila* embryo. *Devel. Biol. 186*, 165–176.

Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R., Goodman, C.S., **1993**. Genes that control neuromuscular specificity in *Drosophila*. *Cell 73*, 1137–1153.

Volk, T., **1992**. A new member of the spectrin superfamily may participate in the formation of embryonic muscle attachments in *Drosophila*. *Development 116*, 721–730.

Volk, T., **1999**. Singling out *Drosophila* tendon cells: a dialogue between two distinct cell types. *Trends Genet. 15*, 448–453.

Volk, T., VijayRaghavan, K., **1994**. A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo. *Development 120*, 59–70.

Vorbruggen, G., Constien, R., Zilian, O., Wimmer, E.A., Dowe, G., *et al.*, **1997**. Embryonic expression and characterization of a Ptx1 homolog in *Drosophila*. *Mech. Devel. 68*, 139–147.

Weiss, J.B., Suyama, K.L., Lee, H.H., Scott, M.P., **2001**. Jelly belly: a *Drosophila* LDL receptor repeat-containing signal required for mesoderm migration and differentiation. *Cell 107*, 387–398.

Williams, J.A., Bell, J.B., Carroll, S.B., **1991**. Control of *Drosophila* wing and haltere development by the nuclear *vestigial* gene product. *Genes Devel. 5*, 2481–2495.

Wong, K., Ren, X.R., Huang, Y.Z., Xie, Y., Liu, G., *et al.*, **2001**. Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. *Cell 107*, 209–221.

Wu, X., Golden, K., Bodmer, R., **1995**. Heart development in *Drosophila* requires the segment polarity gene *wingless*. *Devel. Biol. 169*, 619–628.

Wu, Y.C., Horvitz, H.R., **1998**. C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. *Nature 392*, 501–504.

Wu, Y.C., Tsai, M.C., Cheng, L.C., Chou, C.J., Weng, N.Y., **2001**. C. elegans CED–12 acts in the conserved crkII/DOCK180/Rac pathway to control cell migration and cell corpse engulfment. *Devel. Cell 1*, 491–502.

Xie, F., Meier, T., Reichart, H., **1992**. Embryonic development of muscle patterns in the body wall of the grasshopper. *Roux's Arch. Devel. Biol. 201*, 301–311.

Yarnitzky, T., Min, L., Volk, T., **1997**. The *Drosophila* neuregulin homolog Vein mediates inductive interactions between myotubes and their epidermal attachment cells. *Genes Devel. 11*, 2691–2700.

Yarnitzky, T., Volk, T., **1995**. Laminin is required for heart, somatic muscles, and gut development in the *Drosophila* embryo. *Devel. Biol. 169*, 609–618.

Yin, Z., Frasch, M., **1998**. Regulation and function of tinman during dorsal mesoderm induction and heart specification in *Drosophila*. *Devel. Genet. 22*, 187–200.

Yin, Z., Xu, X.L., Frasch, M., **1997**. Regulation of the twist target gene tinman by modular cis-regulatory elements during early mesoderm development. *Development 124*, 4971–4982.

Zaffran, S., Frasch, M., **2002**. The beta 3 tubulin gene is a direct target of bagpipe and biniou in the visceral mesoderm of *Drosophila*. *Mech. Devel. 114*, 85–93.

Zaffran, S., Kuchler, A., Lee, H.H., Frasch, M., **2001**. biniou (FoxF), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in *Drosophila*. *Genes Devel. 15*, 2900–2915.

Zhang, S., Bernstein, S.I., **2001**. Spatially and temporally regulated expression of myosin heavy chain alternative exons during *Drosophila* embryogenesis. *Mech. Devel. 101*, 35–45.

Zhang, Y., Featherstone, D., Davis, W., Rushton, E., Broadie, K., **2000**. *Drosophila* D-titin is required for myoblast fusion and skeletal muscle striation. *J. Cell. Sci. 113*, 3103–3115.

Zhou, Z., Caron, E., Hartwieg, E., Hall, A., Horvitz, H.R., **2001**. The C. elegans PH domain protein CED-12 regulates cytoskeletal reorganization via a Rho/Rac GTPase signaling pathway. *Devel. Cell. 1*, 477–489.

2.2 The Development of the Flight and Leg Muscle

J O Vigoreaux, University of Vermont, Burlington, VT, USA
D M Swank, San Diego State University, San Diego, CA, USA

2.2.1. Introduction

2.2.1.1. Scope of the Review

This review will examine the cellular and molecular events that transpire during the development of the adult leg, jump, and flight muscles of insects. All adult muscles are striated and, like their counterparts in vertebrates (skeletal muscle), consist of three principal cellular components: mitochondria, myofibrils, and membrane systems (sarcoplasmic reticulum (SR) and T tubules). The functional properties of each muscle arise from the interplay among these three components (Lindstedt *et al.*, 1998; Rome and Lindstedt, 1998). There is very little understanding of the developmental and regulatory mechanisms that establish the relative proportions of these functional units in the muscle cell (see Section 2.2.3.3.3). In contrast, major advances have been made in identifying early events in cell determination and the process by which structural proteins assemble into ordered myofibrils. These topics will be the subject of this review. Given the preeminent position of the fruit fly *Drosophila melanogaster* as a model organism in biological research, this species will serve as the focal point of this review. For comparison, the important contributions from other insect groups are also discussed. For additional information regarding myogenesis and muscle development, the reader is referred to **Chapter 2.1** in this encyclopedia.

2.2.1.2. Muscle Type Definitions

Insect flight muscles are classified functionally as direct (DFM) or indirect (IFM), physiologically as asynchronous or synchronous, and morphologically as tubular, close-packed, or fibrillar. The leg and jump muscles are invariably tubular and synchronous. The following are brief definitions of these and other terms that will be used throughout this chapter (for additional details see: Tregear, 1977; Crossley, 1978; Squire, 1986; Bernstein *et al.*, 1993; Dudley, 2000; Josephson *et al.*, 2000).

- Indirect flight muscles (IFM): Power-producing muscles that move the wings indirectly by deformation of the thoracic exoskeleton (Crossley, 1978). The IFM consist of two perpendiculary oriented, antagonistic muscles: the dorsolongitudinal muscles (DLM) and dorsoventral muscles (DVM) (**Figure 1a**). The DLM extend nearly

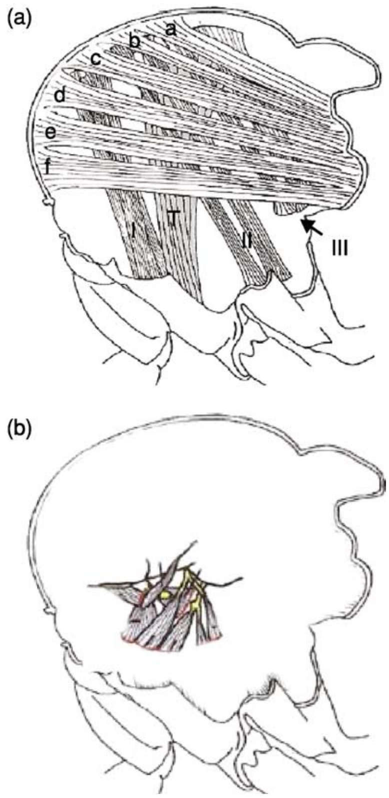


Figure 1 Major thoracic muscles in *Drosophila*. (a) Schematic diagram of a midsagittal section showing the right hemithorax of an adult fly. The six dorsolongitudinal muscle (DLM) fibers are labeled a–f and the three dorsoventral muscles (DVM) are labeled in roman numerals. DVM I consists of three fibers, and DVM II and III of two fibers each. The jump muscle (TDT) is labeled T. Anterior is to the left and dorsal at the top. (b) As (a) but with indirect flight muscles (IFM) removed to view the flight control muscles (DFM). (Modified from Rivlin, P.K., Schneiderman, A.M., Booker, R., **2000**. Imaginal pioneers prefigure the formation of adult thoracic muscles in *Drosophila melanogaster*. *Devel. Biol.* *222*, 450–459.)

parallel to the long body axis while the DVM extend from the tergum to the sternum. DLM function as wing depressors (downstroke) and their contraction stretches the DVM, which in turn contract to elevate the wings (upstroke) and stretch the DLM.

- Direct flight muscles (DFM): Muscles that act directly on the wing base. The power-producing muscles of primitive insects (e.g., Odonata) are directly attached to the base of the wings and their contractions bring about "direct" flapping of the wings. These muscles are also involved in rotation of the wings. In some insects (e.g., Orthoptera), the mesothoracic DVM are also used in walking. In insects with IFM (e.g., Diptera, Hymenoptera, and Coleoptera), the "power" DVM (asynchronous and fibrillar) and the "steering" DFM (synchronous and tubular) are distinct muscle groups; the latter group produces low power and functions only as control muscles (**Figures 1b** and **5**).
- Fibrillar: Large diameter fibers from which well-defined, large diameter and circular myofibrils can be easily dissociated. Found only in the flight muscles of Coleoptera, Hymenoptera, Diptera, Strepsiptera, Hemiptera, and Thysanoptera, and some Homoptera and Psocoptera (Cullen, 1974; Dudley, 2000). The term fibrillar is synonymous with asynchronous and myogenic (Pringle, 1977; Josephson *et al*., 2000).
- Tubular: Nuclei are located in a central position surrounded by columns of rectangular myofibrils. These muscles typically have a high proportion of sarcoplasmic reticulum (~20% of total volume) and low mitochondrial content (~5% volume). They are found commonly in leg muscles but also in some flight muscles (e.g., Odonata). The highly specialized jump muscle (tergal depressor of trochanter or TDT) is a mixed fiber-type tubular muscle that differs histochemically from other tubular muscles and from the IFM (Deak, 1977a; O'Donnell *et al*., 1989). It is the largest of all the tubular muscles in the adult and its primary function is to initiate flight (**Figure 1a**).
- Close packed: Characterized by small myofibrils evenly interspaced with mitochondria. These muscles are found in higher Orthoptera, Trichoptera, and Lepidoptera.
- Asynchronous: Oscillatory muscles in which the frequency of contraction is decoupled from the frequency of activating neuronal impulses. Asynchronous muscles thus attain much higher contraction rates than synchronous muscles and produce higher wing beat frequencies. This type of muscle is common among small insects (flies and bees) and some bugs (see fibrillar).
- Synchronous: Muscles in which the frequency of nervous stimulation and contraction are congruent. Synchronous flight muscles are nonfibrillar and phylogenetically ancestral to asynchronous muscles. They are found in insects with primitive flight muscles and larger wings such as locusts, moths, and butterflies.

2.2.2. Fate Specification and Cell Determination

2.2.2.1. General Features Established by the Early Studies

The general principles established by the early studies on this topic were previously reviewed in depth (Nuesch, 1985; see also **Chapter 2.1**). The developmental mechanisms of insect flight and leg muscle

can be broadly classified depending on whether the insect goes through a pupal transition to reach adulthood (holometabolous), or lacks a pupal transition (hemimetabolous) (Teutsch-Felber, 1970). In hemimetabolous insects, such as the cockroach *Periplaneta americana*, the complete set of adult muscles is already present in the larval form and most muscles (except flight and genital) are in full use. Larval muscles do not degenerate; instead, they are all taken over into the adult form. The flight muscles do not undergo much of a transition. Flight muscles are often present at hatching, remain small until the last larval stage, and reach their full size only before or even after the last molt. The little change that does occur takes place in the last larval stage, and involves growth by fiber splitting and an increase in the number of myofibrils. The leg muscles grow in pace with the rest of the body.

This scenario is in sharp contrast to adult muscle development during pupation of holometabolous insect (Nuesch, 1985; **Chapter 2.1**). In these insects, most of the larval muscles degenerate and new muscles develop, depending on the morphology of the adult. Larval muscles may or may not contribute to the adult muscle set; the muscles that do not contribute are removed by phagocytes (Hufnagel, 1918; Nuesch and Bienz-Isler, 1972; **Chapters 2.1** and **2.3**). Contributing larval muscles have been called "transformation muscles" but are now referred to as "larval templates," "persistent larval fibers," or "larval precursors." A typical example where larval tonic muscles are replaced by the adult phasic DLM is *Antheraea* and other Lepidoptera (Hufnagel, 1918; Nuesch, 1985; **Chapter 2.1**). During the prepupal stage the larval fibers structurally degenerate. In the pupal stage the degenerated larval fibers associate with multiplying mononucleated myoblasts to form the adult muscle anlage. Adult muscle development is initiated by fusion of myoblasts with the degenerated larval fibers. Fusion and cell membrane breakdown of larval fibers appears to be initiated by contact with the myoblasts (Stocker, 1974). The larval fibers separate from one another, grow, and elongate by fusing with more myoblasts, and finally divide into the individual adult fibers. In this insect, contractile proteins such as myosin and actin appear on day 8 of adult development (Bienz-Isler, 1968).

Similar mechanisms have been described for the IFM of the blowflies *Calliphora* and *Lucilia* (Peristianis and Gregory, 1971; Crossley, 1972). However, the primitive dipteran *Simulium*, and the wax moth, *Galleria mellonella*, use a *de novo* formation mechanism, at least for the DVM, that does not involve larval muscle. Instead, myoblasts proliferate during metamorphosis and fuse to form the adult flight muscles (Hinton, 1959, Sahota and Beckel, 1967).

Leg muscles generally also use a *de novo* mechanism. Muscle pioneers (MPs) in the grasshopper leg muscle were the first identified as organizing cells in myogenesis (Ho *et al.*, 1983, Ball and Goodman, 1985). An MP cell serves as a focus for smaller migrating mesodermal cells that collect nearby and fuse with it. The large MP then cleaves into parallel muscle fibers. Thus, a single MP delineates a single bundle of muscle fibers.

Motor nerves have also been well investigated in terms of their role in adult muscle formation (Nuesch, 1985; **Chapters 2.3** and **2.5**). The role of the nervous system in controlling flight muscle development and maintenance was examined by surgically severing neural connections feeding the muscles (e.g., removal of thoracic ganglia), just before or during larval/pupal development (Nuesch, 1968). Studies in *Antheraea* showed that denervation early in development resulted in a more pronounced muscle defect than denervation late in development. Denervation, however, delayed development but did not prevent it (Basler, 1969). In saturniid moths, denervation affected the anlagen, whereas in hemimetabolous insects (crickets, cockroaches) it affected only the differentiated, active muscles. The main affect of denervation appeared to be on nuclear division (Eigenmann, 1965). Despite the presence of clear differences among species on how nerve may exert its effects, all early studies suggested that nerves have an important function in the development and maintenance of muscles. Furthermore, although the mechanisms by which nerves influence muscle developmental decisions remained undefined, several studies pointed to a possible role in the provision of trophic factors (Nuesch, 1985; **Chapter 2.5**).

The motor nerves themselves are remodeled during the pupal transition (see **Chapters 2.3–2.5**). In the saturniid *Antheraea* and other Lepidoptera, larval motor nerves degenerate at their contact points with the larval muscles, from which new nerve sprouts will grow into the adult anlagen. During this time, the motor nerves undergo transformation from larval slow to adult fast nerve cells (Nuesch, 1955, 1985; Heinertz, 1976).

Hormones play a prominent role in adult muscle development. Thus, 20-hydroxyecdysone (20E; the molting hormone), for example, triggers profound metamorphic changes by inducing larval muscles to break down, stimulating imaginal discs to grow and differentiate, and reprogramming certain larval muscles to become adult muscles (Truman, 1973; **Chapters 2.4** and **2.5**). In contrast, juvenile hormone (JH) is responsible for the maintenance of existing

structures (Wilde and DeBoer, 1961). This was demonstrated in *Rhodnius prolixus*, where injection of JH temporarily prevents the breakdown of larval muscles during pupation (Lockshin and Williams, 1964).

The developing epidermis was also shown to influence muscle development, primarily the orientation of the muscle fibers. Thus, moving pieces of the epidermis of *Galleria mellonella* by 180° relative to the muscle development site, caused the DLM to reorient in order to maintain the same epidermis attachment sites (Sahota and Beckel, 1967). Furthermore, in some *Drosophila* homeotic mutants lacking wings, the duplication of cuticle structures was accompanied by considerable rearrangement of the thoracic muscles, but the muscles themselves were not duplicated (Deak, 1978).

In summary, by 1985, nerves, hormones, and epidermal influences had been implicated in muscle development, but their precise role remained largely undefined. Moreover, studies on genetic regulation were sparse. Research that has addressed some of these deficiencies are discussed in the following sections.

2.2.2.2. Precursor Cells

In holometabolous insects, adult muscle precursor cells are determined before metamorphosis. Precursors for both the flight and leg muscles arise from the mesoderm (see **Chapter 2.1**). Adult muscle precursors originate as sister cells of embryonic founders (reviews: Becker and Bernstein, 1994; Baylies *et al*., 1998; Frasch, 1999; Consoulas *et al*., 2000; see also **Chapter 2.1**) (**Figure 2**). Embryonic muscle founders and adult muscle precursors appear at well-defined locations during the final mitotic division of somatic mesodermal cells, and differ from the surrounding myoblasts by their specific expression of regulatory genes (often referred to as "muscle identity genes") such as *twist* (*twi*), a helix–loop–helix transcription factor (**Table 1**). In vertebrates, Twist functions by directly inhibiting the ability of myogenic regulators like myocyte enhancer factor-2 (Mef-2) to bind to their target genes to activate transcription. Expression of *twist* in *Drosophila* ectoderm drives the cells into myogenesis (Baylies and Bate, 1996) (see also **Chapter 2.1**).

Larval mesoderm cells that will become embryonic and larval muscles gradually lose expression of *twist*, fuse and differentiate into various embryonic muscle types. Myoblasts that will become adult muscle cells persist in *twist* expression, postpone differentiation and proliferate actively during the larval stage (Bate *et al*., 1991; Fernandes *et al*., 1991) (**Figure 2**). Broadie and Bate (1993) demonstrated, by hydroxyurea ablation of *twist*-expressing cells in the late embryo, that larval mesodermal lineages expressing high

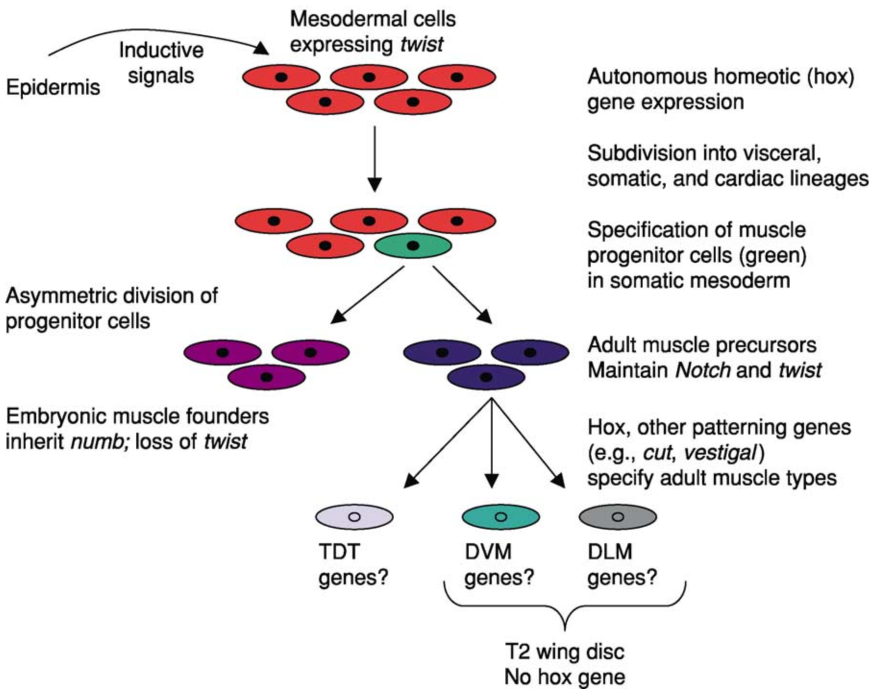


Figure 2 Specification of adult muscle precursors during embryogenesis of *D. melanogaster*. Major morphological divisions, autonomous gene expression, and inductive cues are shown. Genes known to be expressed in myoblasts that contribute to each of the three major muscle groups in the adult thorax are also listed.

Table 1 Genes involved in flight and leg muscle development

Gene[a]	*Description*	*Primary function(s)*	*Reference*
twist (*twi*)	Helix–loop–helix transcription factor	Expression prevents differentiation of adult mesoderm during embryogenesis; regulates *D-mef2* expression.	Baylies and Bate (1996), Cripps and Olson (1998)
Notch (*N*)	Transmembrane receptor	Maintains undifferentiated state, likely through regulation of *twist* expression	Anant *et al.* (1998)
numb (*nb*)	Membrane-associated protein	Blocks N-mediated repression of differentiation	Ruiz Gomez and Bate (1997)
erect wing (*ewg*)	Transcription factor	Required for IFM formation, perhaps involved in fusion of myoblasts to IFM	DeSimone *et al.* (1996), Roy and VijayRaghavan (1999)
Drosophila myocyte enhancer factor 2 (*D-mef2*)	MADS domain transcription factor	Activates transcription of muscle genes during differentiation	Ranganayakulu *et al.* (1995)
cut	Transcription factor	Expressed in myoblast that will become DFM	Sudarsan *et al.* (2001)
vestigal (*va*)	Transcription factor	Expressed in myoblast that will become IFM	Sudarsan *et al.* (2001)
scalloped (*sd*)	Transcription factor	Expressed in adult myoblasts	Roy and VijayRaghavan (1999)
heartless (*htl*)	Fibroblast growth factor receptor	Expressed in adult myoblasts	Roy and VijayRaghavan (1999)
Antennapedia (*Antp*)	Homeodomain transcription factor	Expressed in haltere imaginal discs	Roy and VijayRaghavan (1999), Roy *et al.* (1997)
Sex combs reduced (*Scr*)	Homeodomain transcription factor	Expressed in myoblasts associated with ventral leg imaginal discs	Roy and VijayRaghavan (1999), Roy *et al.* (1997)
Ultrabithorax (*Ubx*)	Homeodomain transcription factor	Expressed in abdominal muscles and T3 epidermis	Roy and VijayRaghavan (1999), Rivlin *et al.* (2001)
Octopod (*Octo*)	Homeodomain transcription factor (*Manduca*)	Mutation causes additional set of legs with poorly developed homeotic muscles	Miles and Booker (1993)
dumbfounded (*duf*)	Immunoglobin superfamily	Required for DFM myoblast aggregation and fusion	Ruiz Gomez *et al.* (2000)
apterous (*ap*)	Homeodomain transcription factor	Expressed in DFM founder cells	Kozopas and Nusse (2002)

[a]Genes listed in order of appearance in this chapter.
MADS, MCM1-Agamous-Deficiens-SRF; DFM, direct flight muscles; IFM, indirect flight muscles.

levels of *twist* give rise to specific subsets of muscle fibers within the adult legs. Each primordium showed a unique fate and, after ablation, could not be replaced by neighboring cells. The authors concluded that fate restriction of myoblast pools in early development defined the elements of the final adult muscle pattern. This could be due to genetic determination within the mesoderm, and/or to spatial isolation of otherwise equivalent cells.

Persistent *twist* expression in the mesoderm appears to require *Notch* (*N*) gene expression. *Notch* expression is maintained in mesodermal sister cells that do not express the *numb* (*nb*) gene after asymmetric division of parent mesoderm cells (Ruiz Gomez and Bate, 1997; Anant *et al.*, 1998; review: Roy and VijayRaghavan, 1999) (**Figure 2**). Twist is a regulator of various transcription factors. For example, *erect wing* (*ewg*), which encodes a DNA binding protein, is expressed in myoblasts at the earliest stages of IFM differentiation, 10 h after puparium formation (APF) as *twist* expression declines (DeSimone and White, 1993; DeSimone *et al.*, 1996). This correlation suggests a negative regulation of *ewg* by Twist. The *Drosophila* homolog of the vertebrate *myocyte-specific enhancer factor 2* (*D-mef2*), a MADS domain transcription factor that acts as a transactivator of muscle specific differentiation genes, is likely also to be regulated by Twist (MADS is an acronym formed from the initials of the first four discovered members of this gene family: MCM1, Agamous, Deficiens, and SRF). The focal point of *D-mef2* function is myoblast differentiation; this protein is expressed in adepithelial cells (precursors of adult muscle) that are attached to the wing, haltere, and leg imaginal discs in the third larval stage (review: Roy and VijayRaghavan, 1999).

In the third instar larva, the expression of genes encoding other transcription factors, i.e., *cut, vestigial* (*vg*), *scalloped* (*sd*), and *heartless* (*htl*), is correlated with *twist* expression in myoblasts (Roy and VijayRaghavan, 1999). Sudarsan *et al.* (2001) investigated the contribution of two of these genes, *cut* and *vestigial*, to the diversification of myoblasts in third instar wing discs. Vestigial and low levels of Cut are expressed in myoblasts that will contribute to IFM. In contrast, high levels of Cut but not Vestigial is required in myoblasts for formation of DFM. Vestigial and Cut expression is stabilized by a mutually repressive feedback loop (Sudarsan *et al.*, 2001).

By the pupal stage of *Drosophila*, adult thoracic muscle precursors have proliferated to produce a pool of *twist*-expressing imaginal myoblasts that are associated with wing and leg imaginal discs in the thoracic segments (Bate *et al.*, 1991; Fernandes *et al.*, 1991) (**Figure 3**). Smaller, separate pools of myoblasts are associated with peripheral nerves in the thorax and presumably contribute to the generation of the muscles that the specific nerves will innervate. Myoblasts on the wing disc are not genetically restricted to become flight muscle, however. When myoblasts from third instar larval wing discs are transplanted into mature host larvae, they contribute to muscles in both the abdomen and thorax of the adult host (Roy and VijayRaghavan, 1999). Thus, the earlier presumption that all myoblasts from *Drosophila* wing discs give rise to flight muscle and all myoblasts from the leg disc give rise to leg muscles (Roy and VijayRaghavan, 1999), appears to be an overgeneralization. This is discussed further below (see Section 2.2.2.5.2).

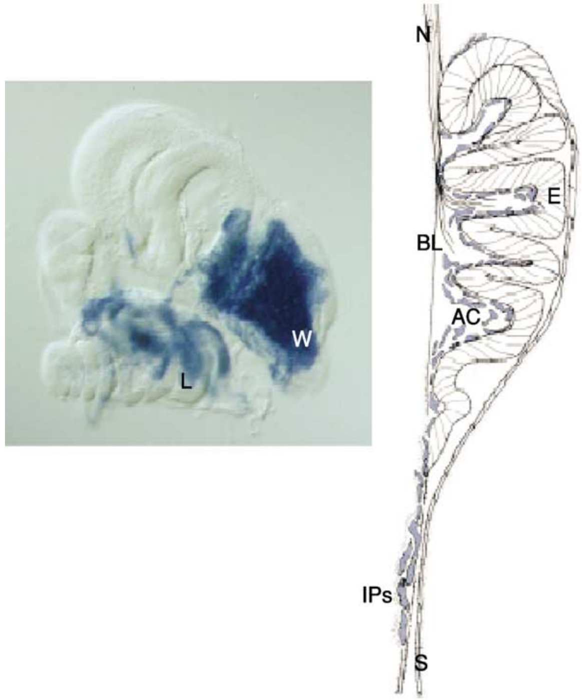


Figure 3 Myoblasts and muscle pioneers in *Drosophila* imaginal discs. The light micrograph on the left shows a wing disc (W) and a leg disc (L). The areas stained in blue contain *twist*-expressing cells as evidenced by the expression of a *twist*-lacZ transgene. The drawing on the right is a sagittal view of a second thoracic (T2) leg disc. The adepithelial cells (AC, light blue) are the imaginal myoblasts that reside in the cavity formed by the disc epithelium (E) and the basal lamina (BL). A morphologically distinct class of imaginal myoblasts is found associated with the stalk (S) that attaches the disc to the epidermis. This group of cells represents the imaginal pioneers (IPs). N, nerve. (Reprinted with permission from Rivlin, P.K., Schneiderman, A.M., Booker, R., **2000**. Imaginal pioneers prefigure the formation of adult thoracic muscles in *Drosophila melanogaster*. *Devel. Biol. 222*, 450–459; © Elsevier.)

2.2.2.3. Patterning

How are the *twist*-expressing cells partitioned into the proper segments so that some will give rise to flight muscle and some to jump muscle? This occurs primarily through the action of epidermal signals and Hox genes.

2.2.2.3.1. Role of the epidermis The epidermis plays an important role in myoblast migration to muscle development sites. VijayRaghavan *et al.* (1996) followed the paths of imaginal myoblast migration from wing and leg discs. Wing or leg discs were transplanted onto the thorax or abdomen of pupal hosts. In these animals, the myoblasts from both discs migrated over the host epidermis when transplanted onto pupal thorax, but no migration occurred when the transplantation site was on the abdomen. The resulting flies had ectopic wings or legs. The migration path cues from the epidermis may be due to expression of Hox genes (Roy and VijayRaghavan, 1997). Epidermal cells also contribute to the formation of muscle attachment sites (see Section 2.2.3.3.2).

2.2.2.3.2. Role of Hox genes Homeotic selector (Hox) genes are widely known from their mutations in *Drosophila*, which result in gross duplications or transformations of body segments (see **Chapter 1.8**). A dramatic example is the "four winged" fly, which results from the absence of expression of *Ultrabithorax* (*Ubx*) in the third thoracic segment (T3), where the haltere and leg discs reside (Fernandes *et al.*, 1994). Hox genes influence, directly or indirectly, the segment-specific diversification of the mesoderm into exact muscle patterns (review: Roy and VijayRaghavan, 1999). For example, expression of *Antennapedia* (*Antp*) in mesoderm is specific to

myoblasts that are associated with the haltere disc in the T3 segment. In contrast, myoblasts on the wing disc in the second thoracic segment (T2) do not express any Hox genes (Roy *et al.*, 1997). Thus, any influences of Hox genes must occur indirectly through inductive cues from the ectoderm (Rivlin *et al.*, 2001).

Initial studies of Hox gene expression in the "four winged" fly of *Bithorax-Complex* (*BX-C*) mutants suggested that *Ubx* expression has little or no influence on adult thoracic muscle patterning (Fernandes *et al.*, 1994; Roy *et al.*, 1997). Even though T3 is transformed in such a way that the epidermis looks like the second segment, the haltere disc myoblasts associated with the transformed segment continue to express *Antp* and do not transform into IFM-like muscles (Roy and VijayRaghavan, 1999). However, a recent investigation of *Ubx* regulatory mutations (*anterobithorax, bithorax, postbithorax*, and *bithoraxoid*) acting on the adult T3 musculature suggests an indirect role for *Ubx* in thoracic muscle patterning (Rivlin *et al.*, 2001). Almost all mutant allele combinations tested by Rivlin *et al.* (2001) resulted in homeotic IFM and DFM in the transformed T3 segment. While the amount and/or quality of homeotic IFM increased in the transformed segment, the amount of homeotic DFM did not vary significantly as the severity of the ectodermal transformation increased. Because *Ubx* is not expressed in adult T3 mesoderm, the results suggest that inductive cues play a major role in the patterning of adult thoracic muscles.

Hox genes also play a role in muscle patterning along the dorsal–ventral axis within a segment. *Sex combs reduced* (*Scr*) is exclusively expressed in myoblasts associated with ventral leg imaginal discs in all thoracic segments (Glicksman and Brower, 1988a). Misexpression of *Scr* in wing disc-associated precursors of dorsal muscles (IFM and DFM) disrupts the development of these muscles (Glicksman and Brower, 1988b; Roy and VijayRaghavan, 1999).

Myoblasts expressing a specific homeotic selector gene can fuse with myoblats expressing a different homeotic selector gene (i.e., different segmental identities). For example, myoblasts transplanted from the thorax to the abdomen readily fuse with resident myoblasts and are incorporated into abdominal muscles (Roy and VijayRaghavan, 1997). However, once fused, myoblasts from Hox mutants often cannot carry out their original differentiation program. Thus, misexpression of *Ubx* in the developing IFM causes suppression of a thoracic specific actin gene, *Act88F.* Conversely, loss of function of *Ubx* in abdominal muscles results in the ectopic transcription of *Act88F* (Roy and VijayRaghavan, 1999).

Hox genes are likely to be conserved in all insects. *Ubx/abdominal-A* genes have been studied in the grasshopper embryos (Kelsh *et al.*, 1994), and the homeotic mutation *Octopod* (*Octo*) has been studied in *Manduca* (Miles and Booker, 1993). *Octopod* transformed the ventral epidermis of the first abdominal segment into a T3. This resulted in the presence of thoracic-type legs attached to the abdominal segment with the legs containing homeotic leg muscles.

2.2.2.4. Degeneration of Larval Muscles

During metamorphosis of holometabolous insects, most embryonic muscles degenerate and are replaced by adult specific muscles (see **Chapter 2.5**). In *Drosophila*, histolysis of the embryonic muscles begins with the elimination of most muscles in the head and thoracic segments soon after the onset of the white prepupal stage (Fernandes *et al.*, 1991). The signal for this programmed cell death comes from the anterior region, presumably the head. The timing of the signal suggests that the eclosion hormone may be involved in this process (Kimura and Truman, 1990).

Rheuben (1992) described the ultrastructural changes in the larval mesothoracic muscle associated with the early stages of degeneration in *Manduca*. The muscle fibers decreased in cross-sectional area but increased in apparent surface area. Large numbers of electron-dense granules or droplets are formed and extruded from the muscle cytoplasm. The thick basal lamina is specifically removed, and this may be essential for subsequent fusion of myoblasts to the residual larval myofibrils. Contractile elements degenerate, tracheoles withdraw, and mitochondria appear to degenerate (Rheuben, 1992; Sonea and Rheuben, 1992).

However, not all larval muscles degenerate in holometabolous insects. Some adult muscles differentiate using remnants of the larval fibers as templates. An example of this is the DLM discussed below (see Section 2.2.2.5).

2.2.2.5. Formation of the Indirect Flight Muscles

In dipterans, the IFM are composed of two opposing sets of muscle fibers, the DLM and DVM that attach directly to the thoracic cuticle (**Figure 1**). These muscles have generally been assumed to have identical physiological characteristics and the same protein isoform profile (Lebart-Pedebas, 1990). This, however, has not been rigorously tested and prominent exceptions have been reported. In *Drosophila*, these muscles have different origins and are regulated by different developmental mechanisms.

2.2.2.5.1. The dorsolongitudinal flight muscle (DLM) The DLM formation in the *Drosophila* musculature occurs by transformation of pre-existing larval muscles into adult fibers. Larval scaffolds are employed for its generation instead of founder cells (used in embryonic muscle patterning) (see **Chapter 2.1**). Three mesothoracic muscles of the second thoracic segment of the larval body wall survive histolysis and undergo radical transformations (Fernandes *et al*., 1991). The persistent larval fibers "dedifferentiate" 10 h APF. Starting at 6 h APF, a series of vacuoles appear and the larval fibers show a decrease in birefringence. At 10 h APF, the fibers are highly vacuoled and distorted in shape. By 12 h APF, myofibril organization has degenerated and fibers have started to elongate (**Table 2**).

The primary source of myoblasts for fusion to the larval scaffolds is the imaginal wing disc (**Figure 3**). The myoblasts are located between the folds of the wing imaginal disc, and are termed adepithelial cells (ACs) (Bate *et al*., 1991). In contrast, myoblasts that will become leg and TDT muscles, as well as some that will contribute to the DVM, arise from mesothoracic leg discs (Bate *et al*., 1991).

Just after pupation, the wing discs evert and release myoblasts. The myoblasts migrate to the larval templates, possibly using the flight muscle motor nerves as guides (Fernandes *et al*., 1991) (**Figure 4**). However, these are not necessarily the first myoblasts to arrive on site; myoblasts associated with peripheral larval thoracic motor nerves may be another source of DLM components. By 14 h APF, *twist*-expressing myoblasts have surrounded the persistent larval muscles. At 14–18 h APF, the three larval fibers split longitudinally thus generating six fibers per half thorax (**Table 2**). Myoblasts fuse and the templates grow in size. Fernandes *et al*. (1991) suggested that larval templates have a similar function as the muscle pioneers in grasshopper embryos (Ho *et al*., 1983), i.e., persistent larval muscles partition the initial pool of myoblasts among themselves.

To test this and other possible roles of the persistent larval muscle fibers (MFs), Farrell *et al*. (1996) used lasers to ablate one, two, or all three larval fibers (MF9, MF10, and MF19). Surprisingly, in the absence of larval muscle fibers, the DLM still developed. Thus, the larval fibers are not required to initiate myoblast fusion, specify total DLM fiber volume, or locate the DLM in the thorax. However, the larval fibers are required to establish the correct number of adult fibers. For example, if only one fiber is ablated (e.g., MF9), development of the other two fibers proceeds normally, but the resulting IFM has a variable number of fibers (from four to six or more) (Farrell *et al*., 1996; Fernandes and Keshishian, 1996; Roy and VijayRaghavan, 1999). It has been postulated that the appropriate number of fibers is achieved by controlling the partitioning of myoblasts between the larval scaffolds.

Larval muscles are also not required for establishing DLM identity. *De novo* fibers that form after ablation express the correct actin gene (*Act88F*), and have normal innervations and normal insertion sites (Fernandes and Keshishian, 1996). Thus, larval scaffolds are not true muscle organizers in the same sense of muscle founders in embryogenesis.

By 20 h APF (**Table 2**), there are six clearly visible developing DLM template fibers (Fernandes *et al*., 1991). Three rows of nuclei are visible within each fiber. By 24 h APF, an additional row of nuclei has appeared. Fusion of myoblasts with the developing DLM is completed by 28 h APF. By 36 h APF, the developing IFM are approximately one-third of their final size (Fernandes *et al*., 1991).

Roy and VijayRaghavan (1998) showed that the splitting of the larval templates is concomitant with invasion by imaginal myoblasts and the onset of differentiation. In their study, blocking of myoblast proliferation by ectopic expression of a cell cycle inhibitor protein (the human cyclin dependent kinase inhibitor p21) prevented myoblast association with the persistent larval fibers. As a result, larval templates failed to split, suggesting that splitting is not an autonomous property of the persistent larval muscles. Instead, it requires interactions with myoblasts (Roy and VijayRaghavan, 1998). Nerves are not crucial for this, as laser ablation of the flight muscle motor nerve does not affect the development of the DLM (Fernandes and Keshishian, 1998) (see Section 2.2.2.9.1).

As mentioned earlier, *ewg* is expressed in myoblasts during their fusion to DLM larval scaffolds, and may act as an important genetic regulator in DLM splitting and differentiation. Loss of *ewg* function results in embryonic lethality. Viable alleles of *ewg* (hypoexpressors) cause severe abnormalities in IFM, including failure of founder DLM to split, and early degeneration of DLM and some DVM fibers. These observations suggest a crucial role for *ewg* in proper initiation of the IFM-specific differentiation program, most importantly influencing differentiation events downstream of fusion (DeSimone *et al*., 1996; Fernandes and Keshishian, 1996).

As indicated above (see Section 2.2.2.2), *ewg* may be regulated by *twist*. The influence of *twist* on IFM development has been examined through the use of temperature sensitive alleles (Anant *et al*., 1998; Cripps *et al*., 1998; Cripps and Olson, 1998). When mutant pupae are switched to nonpermissive

Table 2 Sequence of events during IFM development and myofibril assembly in *Drosophila*

Molecular events	*Time*[a]	*Muscle morphology*	*Myofibril assembly*
twist-expressing myoblast in wing/leg imaginal disc	0	Larval body wall muscle intact	
stripe-expressing epidermal cells in notal region of wing disc			
stripe-expressing cells form clusters in the thorax	8	Three larval muscles (9, 10, 19) that persist to give rise to DLM are surrounded by myoblasts	
		Muscles become highly vacuoled and distorted in shape	
twist-expressing myoblasts and PS-2α expressing myoblasts surround persistent larval muscles	12	Adult-specific nerve outgrowth over elongating larval muscle templates and future DVM site	No remnants of larval muscle myofibrils
Act88F gene expression begins in DLM and DVM	16	Larval templates split longitudinally as wing disc-associated myoblasts fuse into them	
Clusters of *stripe*-expressing cells increase in size		Higher-order nerve branches arise	
No PS integrin expression		DVM visible	
No tubulin expression		Tendon cells contact myotubes	
One IP nucleus per DVM fiber	20	Muscle splitting complete and adult neuromuscular pattern is formed	
Muscle ends and their connecting epidermal processes express integrin		Filopodial extensions from DLM make contact with EMA	
		Occasional phagocytes engulfing unfused myoblasts	
Clusters of *stripe*-expressing cells at ends connecting muscle to epidermis	24	Epidermal tendon cell processes connecting DVM to dorsal muscle attachment are evident	Abundant microtubules, few stress fiber-like structures
Mhc expression begins	30	Myoblast fusion nearly complete	
No *twist* expression	38	Modified terminal Z band formed at MTJ	Thick and thin filaments appear
			Formation of individual myofibrils (310 sarcomeres ~1.7 μm long, three or four thick filaments across) within microtubule envelopes
		Muscles approx. half final size	
	48		Uniform length increases of thick and thin filaments
			Thin filaments grow from pointed end in the presence of 43 kDa TMOD
			Addition of new myofilaments to periphery of myofibril
			Regular Z bands and M lines detected
Expression of accessory proteins (e.g., FLN, A225)	60		

Continued

Table 2 Continued

Molecular events	*Time*[a]	*Muscle morphology*	*Myofibril assembly*
Peak in protein synthesis	68		
	75	Evidence of contraction	
			No muscle cell microtubules
	85		
	100	Eclosion	Sarcomeres 85–95% final length, 60–70% final width
Changes in phosphorylation of FLN, RLC, TnT, PM			
Tmod 43–45 kDa isoform switch			Tendon guide cell columns retract
			Formation of tonofibrils and insertion into cuticle
	105	Final adult structure	310 sarcomeres 3.2 μm long, 35 thick filaments across

[a]Time (h) after puparium formation (APF): approximate times at 25 °C.

DLM, dorsolongitudinal muscles; DVM, dorsoventral muscles; IP, imaginal pioneer; EMA, epidermal muscle attachment; MTJ, myotendon junction; TMOD, tropomodulin; FLN, flightin; RLC, myosin regulatory light chain; TnT, troponin T; PM, paramyosin.

Data obtained from the following references: Fernandes, J., Bate, M., VijayRaghavan, K., **1991**. Development of the indirect flight muscles of *Drosophila*. *Development 113*, 67–77; Reedy, M.C., Beall, C., **1993a**. Ultrastructure of developing flight muscle in *Drosophila* 1. Assembly of myofibrils. *Devel. Biol. 160*, 443–465; Reedy, M.C., Beall, C., **1993b**. Ultrastructure of developing flight muscle in *Drosophila* 2. Formation of the myotendon junction. *Devel. Biol. 160*, 466–479; Vigoreaux, J.O., Perry, L.M., **1994**. Multiple isoelectric variants of *flightin* in *Drosophila* stretch-activated muscles are generated by temporally regulated phosphorylations. *J. Muscle Res. Cell Motil. 15*, 607–616; Fernandes, J.J., Celniker, S.E., VijayRaghavan, K., **1996**. Development of the indirect flight muscle attachment sites in *Drosophila*: role of the PS integrins and the stripe gene. *Devel. Biol. 176*, 166–184; Maroto, M., Arredondo, J., Goulding, D., Marco, R., Bullard, B., *et al.*, **1996**. *Drosophila* paramyosin/miniparamyosin gene products show a large diversity in quantity, localization, and isoform pattern: a possible role in muscle maturation and function. *J. Cell Biol. 134*, 81–92; Domingo, A., Gonzalez-Jurado, J., Maroto, M., Diaz, C., Vinos, J., *et al.*, **1998**. Troponin-T is a calcium-binding protein in insect muscle: *in vivo* phosphorylation, muscle-specific isoforms and developmental profile in *Drosophila melanogaster*. *J. Muscle Res. Cell Motil. 19*, 393–403; Fernandes, J.J., Keshishian, H., **1998**. Nerve–muscle interactions during flight muscle development in *Drosophila*. *Development 125*, 1769–1779; Roy, S., VijayRaghavan, K. **1999**. Muscle pattern diversification in *Drosophila*: the story of imaginal myogenesis. *BioEssays 21*, 486–498; Mardahl-Dumesnil, M., Fowler, V.M. **2001**. Thin filaments elongate from their pointed ends during myofibril assembly in *Drosophila* indirect flight muscle. *J. Cell Biol. 155*, 1043–1053.

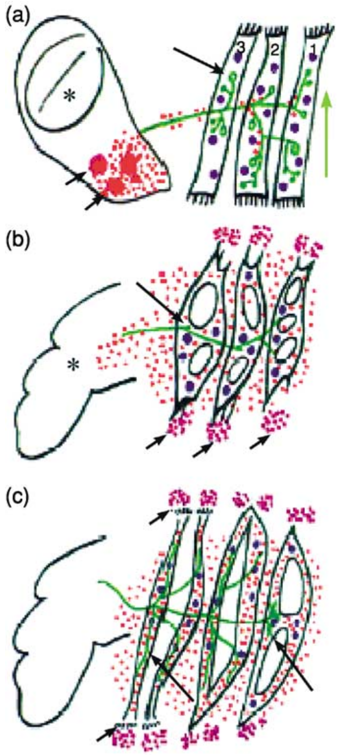


Figure 4 Early events in the development of *Drosophila* DLM. (a) Diagram showing a wing imaginal disc (asterisk) and three dorsal oblique muscles (numbered 1, 2, 3) in a late instar larva. Adepithelial cells (myoblasts) are shown in red and the larval muscle nuclei are shown in blue. Large red dots in the notal epithelium of the wing disc represent groups of *stripe*-expressing cells that will become the attachment sites for the DLM (purple dots in b and c). In addition to the disc, myoblasts are also seen associated with motor nerves (green) migrating towards the larval muscles. A synaptic bouton is indicated by the black arrow. The dorsal midline is indicated by the green arrow. (b) By 6–10 h APF, the disc has everted and the myoblasts are seen migrating over the heavily vacuolated remnants of the three larval muscles. The large arrow points towards a regressing motor neuron. (c) Longitudinal splitting of larval fibers is evident by 16 h APF. The templates will grow as myoblasts continue to fuse with them. New motor neuron connections are made over the splitting muscles (large arrows). (Reproduced with permission from Roy, S., VijayRaghavan, K., **1998**. Patterning muscles using organizers: larval muscle templates and adult myoblasts actively interact to pattern the dorsal longitudinal flight muscles of *Drosophila*. *J. Cell Biol.* *141*, 1135–1145; © The Rockefeller University Press.)

temperatures, *twist* expression levels decrease causing a reduction in the number (from six pairs to only three) and volume of the DLM fibers. DFM, DVM, and jump muscles are not affected by these mutations even though contributing myoblasts to these muscles normally express *twist*. Cripps *et al.* (1998) showed that although *twist* is expressed in precursors of adult muscles throughout the larval and early pupal stages, *twist* is only required for DLM patterning during the late larval stage, just prior to the time the three larval scaffolds split to form the six templates. No splitting of DLM larval template fibers was observed in *twist* mutants. The authors speculate that this function of *twist* is mediated by *D-mef2*, since, in *D-mef2* hypomorphic mutants, splitting is also disrupted. In addition, hypomorphic mutations in *D-mef2* cause a reduction in DLM size and disrupt the patterning of DLM fibers (Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995). This observation supports the notion that *D-mef2* expression is dependent on *twist*.

Notch (N) also appears to be involved in the *twist* pathway, as a phenotype, similar to that described in *twist* mutants, is observed with N temperature sensitive alleles, i.e., reduced levels of N result in failure of DLM to divide (Anant *et al.*, 1998). A premature reduction in *twist* expression is observed when N is decreased. Evidence that N is a crucial signal for maintenance of *twist* expression comes from overexpression studies of N, which results in persistent *twist* gene expression, failure to complete differentiation (as evidenced by lower myosin and actin levels), and loss of DLM muscles in the adult (Anant *et al.*, 1998). No effect of N overexpression is observed in the DFM.

Similar to *Drosophila*, the hawk moth *Manduca sexta* uses pre-existing larval muscles as templates for DLM formation. The DLM arises from an anlage containing remnants of specific larval dorsal body wall muscles and extrinsic myoblasts. Using histology techniques, Duch *et al.* (2000) showed that larval template fibers degenerate and motor terminals retract from muscle fibers 2 days before pupation. Five larval fiber bundles are incorporated into the anlage, for adult DLM development, instead of being lost during the pupal transformation. Myoblasts accumulate and proliferate near the retracted motor nerve endings that persist around the degenerating larval template fibers. At 2 days APF, the anlage splits into five bundles, each innervated by one motor neuron. By day 7 APF, striations are observed and motor neurons reach the attachment sites. A key difference from *Drosophila* is that myoblasts do not originate from wing discs. Thus, ablation of the wing disc prevents formation of the wing but has no effect on DLM development. Instead, myoblasts arrive by several pathways to accumulate around the tufts of nerve terminals. A similar phenomenon is observed for myoblast accumulation in the *Manduca* leg muscle (see below). For this case, it has been suggested that a signaling molecule may be released from nerve terminal endings to attract the myoblasts (Duch *et al.*, 2000).

Cifuentes-Diaz (1989) studied DLM formation in *Pieris brassicae* (Lepidoptera) at the electron

microscopy (EM) and light microscopy level. As in *Manduca*, the DLM arise from two symmetrical pairs of dorsal mesothoracic larval muscles. Myoblasts migrate to the median region of these templates, along nerves and tracheae. Myoblasts proliferate and penetrate the larval fibers, and the fibers break into fragments. The developing anlage eventually re-forms into the five adult fibers. A major conclusion of the study was that myoblasts arise from exogenous sources, with few or none arising from degeneration of larval fibers.

The maturation of the adult IFM in the Colorado beetle, *Leptinotarsa decemlineata* is also different from that observed in *Drosophila*, specifically in terms of lack of imaginal characteristics (Smit and Velzing, 1986). In the prepupal period, the larval muscles (muscle *obliquus lateralis dorsalis*) dedifferentiate and fragment. The fragments contain very few myofibrils, and the sarcoplasmic reticulum and T-tubular system (see Section 2.2.1.1) is greatly reduced. Myoblasts develop from satellite cells, penetrate the muscle fiber, and fuse with the larval cell fragments. New myofibrils that have the characteristic adult filament lattice pattern appear after 2–5 days.

Novicki (1989) investigated the control of growth and ultrastructure of the cricket *Teleogryllus oceanicus* DLM flight muscle. The flight musculature in this hemimetabolous insect is initially patterned during embryogenesis. The analysis revealed that within 8 days from the start of the last nymphal instar, the DLM increased 15-fold in mass and acquired interfibrillar tracheoles, and the percentage volume occupied by mitochondria quadrupled. However, the myofibril and sarcoplasmic reticulum content decreased. An investigation into the neural and hormonal control of this process concluded that growth and remodeling are dependent on both innervation and JH, but maturation is more dependent on JH (Novicki, 1989).

Similarly, in the fifth larval instar of the hemimetabolous desert locust *Schistocerca gregaria*, the conversion of juvenile flight muscle into the high-energy-consuming adult form requires that mitochondria proliferate more rapidly than all the other cellular components, and requires the development of tracheoles (Wang *et al.*, 1993) (see Section 2.2.3.3.3).

An unusual case of DLM "development" is the flightless grasshopper, *Barytettix psolus* (Arbas and Tolbert, 1986). In this species, DLM are present and innervated during nymphal life, but these muscles are absent in adults. Despite the absence of muscles, however, the motor nerve persists in the adults.

2.2.2.5.2. The dorsoventral flight muscle (DVM) In contrast to DLM formation, DVM in *Drosophila* are formed by *de novo* fusion of imaginal myoblasts. At least some of these myoblasts arise from a pool separate from the one supplying myoblasts for the DLM. Rivlin *et al.* (2000) have provided the most recent analysis of how DLM arise in *Drosophila* by tracking the progression of *twist*-expressing cells from different imaginal disc pools. Prior to the third instar larval stage, a pool of *twist*-expressing myoblasts exists in the wing and the leg imaginal discs. All myoblasts and their nuclei are uniform in size. By the start of the third instar stage, *twist*-expressing cells containing nuclei that are twice the size (9–10 μm) of other cells are found in the stalk region of the T2 leg disc (**Figure 3**). There are up to five of these larger size cells that contain larger nuclei. These cells have been termed "imaginal pioneers" (IPs), as they appear to play a very similar role to muscle pioneers in embryogenesis, and share several morphological similarities, including the presence of microtubules (Rivlin *et al.*, 2000). In contrast, other *twist*-expressing cells have no microtubules. Upon leg disc eversion, the IPs take up the position of the adult fibers. They elongate to >75 μm by extending growth cones ventrally that anchor to the epidermis. Each DVM fiber is prefigured by a single IP. At 24 h APF, each DVM fiber appears as a syncytium of nuclei surrounded by individual myoblasts.

Earlier papers had reported that all myoblasts for the *Drosophila* DVM originate from the wing discs (e.g., Lawrence, 1982). Rivlin *et al.* (2000) stated that both discs provide myoblasts for the DVM, but only the leg disc provides DVM IPs. Fernandes *et al.* (1991) reported that myoblasts from leg discs take two paths, one group forming the leg muscles and the other group migrating dorsally to contribute to muscles in the ventral thorax. Perhaps a better general statement is that, in the thorax, the dorsal muscles arise from wing disc myoblasts and the ventral ones from the leg disc myoblasts. Since DVM are vertically aligned, myoblasts are contributed from both discs.

Lebart-Pedebas (1990) investigated similar DVM developmental questions in the primitive dipteran *Chironomus*. In contrast to *Drosophila*, the *Chiroyomus* DVM develop during the last larval instar, prior to metamorphosis. They are organized into two groups, one consisting of 10 fibrillar DVM and a second group of four that is split into two groups on either side of the TDT. The group of 10 develops from myoblasts fusing with larval muscle. The larval muscles dedifferentiate (lose myofibrils), but the large nuclei of the larval muscle are maintained in columnar-shaped fibers with which the

myoblasts fuse. The larval muscle has an unusual hollow shape with nerves innervating its inner surface. The muscle is connected to the imaginal leg disc, so that the hollow cavity communicates with the ACs present in the disc. However, no migration of myoblasts is observed from the imaginal disc toward the larval muscle or vice versa, suggesting that the DVM develop from an anlage that is independent of the imaginal disc. The author proposed that myoblasts migrate with hemolymph to arrive at the anlage. Thus, in this species, both the DVM and DLM are formed using larval templates.

How does the artificial *de novo* fusion of DLM myoblasts resulting from laser ablation of larval templates compare to cases where DVM development does not rely on larval templates? Fernandes and Keshishian (1996) found that, at 14 h APF, myoblasts that would have fused with the ablated larval scaffold, instead clustered (four to six cells) in the region of the ablated fiber and lined up end-to-end, similar to the way in which DVM myoblasts line up. The subsequent fusion process was very similar to DVM fusion. Is one mode of flight muscle formation evolutionarily ancestral to the other? Rivlin *et al.* (2000) made the point that *Drosophila* DVM can have a variable number of fibers. This is not seen in DLM unless the larval scaffolds are ablated. Thus, perhaps the scaffold mechanism has evolved as a way of ensuring proper fiber number. Whether this is, in fact, the case, has yet to be demonstrated.

2.2.2.6. Direct Flight Muscle Formation

In *Drosophila*, DFM (steering muscles) develop from founder cells in the region of the wing imaginal disc (Ghazi *et al*., 2000; Kozopas and Nusse, 2002) (**Figure 5**). At about 36 h APF, clusters of *dumbfounded* (*duf*)-expressing cells gather and prefigure individual muscles. Duf, a fusion-promoting adhesion molecule in embryos, is required for the aggregation and fusion of DFM founder cells (Kozopas and Nusse, 2002). The *apterous* (*ap*) gene is also expressed in the small clusters of founder cells that will lead to the formation of the DFM myotubes (Ghazi *et al*., 2000). The *duf*- and *ap*-expressing cells are adjacent to cells expressing *Drosophila Wnt oncogene analog 2* (*D-Wnt-2*) that likely give rise to DFM attachment sites (see Section 2.2.3.3.2).

Kozopas and Nusse (2002) proposed that each adult DFM uses a group of *duf*-expressing cells to act as "cofounders." Cofounders may pass muscle identity to feeder cells by activating genes encoding transcription factors. Each cofounder cell helps seed the syncytial growth of the muscle that will take on the shared identity of the entire founder group.

2.2.2.7. Jump Muscle

The jump muscle (TDT) of *Drosophila* is formed adjacent to the DVM (see Section 2.2.1.2). It is composed of 30 fibers that extend from the trocanteral segment of the leg to the dorsal notum (**Figure 1**). The TDT arises *de novo* and does not use larval scaffolds (Rivlin *et al*., 2000). Rivlin *et al.* (2000) found that, as in DVM, there are two separate pools of myoblasts: one pool, the IPs, serve a muscle founder cell role, while the other pool (feeder cells) gives rise to myoblasts that will fuse with IPs. The IPs arise from myoblasts on the leg imaginal disc. Most myoblast feeder cells have small nuclei (2–3 μm) and are seen on the outermost edge of the developing TDT. At 4 h APF, 12 or 13 TDT IPs line up parallel to one another, take up the position of the adult fiber, elongate, and extend dorsoventrally. The number of IPs stays constant during the rest of development. At 20–24 h APF, there is an array of

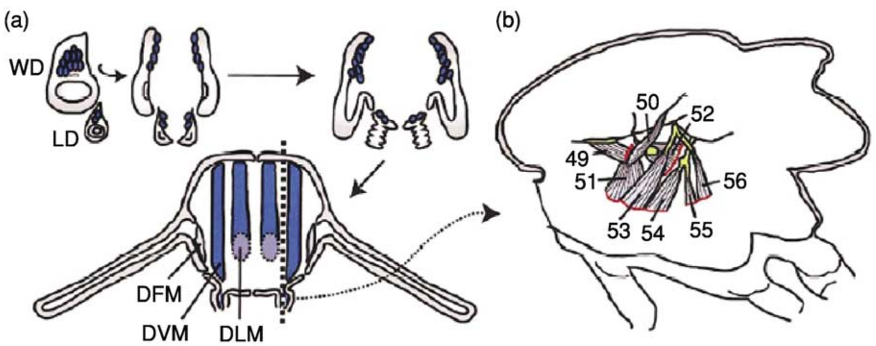


Figure 5 *Drosophila* direct flight muscles (DFM) arise from wing disc-associated myoblasts. (a) Myoblasts associated with the wing disc (WD) and the leg disc (LD) are shown in blue. The DFM develop in the region of the wing hinge. (b) Sagittal view of an adult thorax showing the DFM. The dorsal attachment site of each muscle is shown in yellow, the ventral attachment site is shown in red. Muscles are numbered according to Miller (1950). DLM, dorsolongitudinal muscles; DVM, dorsoventgal muscles. (Reprinted with permission from Kozopas, K.M., Nusse, R., **2002**. Direct flight muscles in *Drosophila* develop from cells with characteristics of founders and depend on DWnt-2 for their correct patterning. *Devel. Biol. 243*, 312–325; © Elsevier.)

thin fibers surrounding a core of myoblasts. The authors concluded that TDT IPs are very similar to the muscle pioneers of the grasshopper embryo.

2.2.2.8. Leg Muscle

During metamorphosis, the larval thoracic legs of *Manduca* are replaced by a new set of adult legs (Consoulas and Levine, 1997). Unlike *Drosophila*, *Manduca* has no true leg imaginal discs. Consoulas and Levine (1997) investigated leg development using various stains to identify proliferating and differentiating cells. Larval leg muscles are among the first muscles to degenerate during the larval-to-pupal transition, possibly by apoptosis. There was no evidence of surviving larval muscles or nuclei, and it is unlikely that myoblasts arising from degenerating larval muscle contribute to adult leg formation. Instead, myoblasts that are formed in the larvae undergo rapid proliferation at the late larval stage, and aggregate to form adult muscle anlage at specific production sites. These new myoblasts are associated with the peripheral nerves and imaginal tracheae that may guide their migration to development sites. As the larval muscles degenerate, the new myoblasts form spindle-shaped cells that proliferate, fuse, and differentiate into myotubes early in the pupal stage. The study concluded that most, if not all, of the imaginal muscles are generated *de novo* without the involvement of larval elements.

2.2.2.9. Inductive Mechanisms

2.2.2.9.1. Nervous system The influence of nerves on adult myogenesis (see **Chapter 2.3**) is in stark contrast to embryogenesis where the formation of larval muscle is independent of innervation (Broadie and Bate, 1993; Consoulas *et al*., 2000; **Chapter 2.1**). Fernandes and Keshishian (1998) examined the interactions between differentiating nerves and the two sets of flight muscles during *Drosophila* pupal metamorphosis. They eliminated synaptic interactions during formation of the IFM by cutting motor nerves at different locations using laser ablation. Denervation was found to cause an overall decrease in the size of the myoblast pool. DVM formation was dramatically inhibited. Fusion of myoblasts was not completely abolished, but denervation prevented the complete formation of the mature fiber. One possibility suggested by the authors is that IPs likely do not form. The adult muscles that use larval muscle fibers as myoblast fusion targets (DLM) still developed, but the fibers were smaller, probably due to fewer myoblasts fusing or to a delay in myoblast fusion. The results suggested that both nerves and larval scaffolds influence fusion. However, when the larval muscle precursors were ablated in conjunction with nerve severance, the DLM showed an absolute dependence on the motor neurons for their formation. The authors concluded that the size of the myoblast pool and early events in fiber formation depend on the presence of the nerve, which perhaps supplies a nerve-derived proliferation factor.

Manduca has been especially useful for the study of nerve–muscle interactions because most motor neurons that innervate the newly formed adult muscles are respecified larval motor neurons, and adult muscle development occurs in constant proximity of the motor neuron endings (Kent and Levine, 1988). Bayline *et al*. (2001) found that *Manduca* DLM failed to develop following complete denervation because myoblasts failed to accumulate in the DLM anlage. Each of the five fiber bundles is innervated by a separate motor neuron. When four of the five neurons were severed, the nerve to the fifth muscle, MN5, remained specific to it and did not rescue the other four fibers. Myoblast accumulation, but not myonuclear proliferation, increased around the MN5 terminals producing a hypertrophied adult DLM5 (Bayline *et al*., 2001). Therefore, motor neurons compete for uncommitted myoblasts. Myoblast accumulation in the DLM anlage requires innervation, and individual muscle fiber bundles require specific innervations for their development.

A nerve–muscle co-culture system was used to investigate critical nerve–muscle interactions during development in *M. sexta* (Luedeman and Levine, 1996). Muscle precursor cells derived from the developing thoracic legs of early pupae were cultured in the presence of neurons. Interestingly, contractile myotubes did not form when muscle precursors cells were cultured alone. Live neurons were also needed, but, as suggested by the lack of an effect by tetrodotoxin, these did not need to be electrically active. Bromodeoxyuridine (BrdU) studies suggested that the co-cultured neurons promoted the proliferation of myogenic cells. Steroid hormone (20E), on the other hand, also enhanced BrdU incorporation into nuclei of myogenic cells, even in the absence of neurons. The results suggest that both neurons and ecdysteroids (see Section 2.2.2.9.2) play an important regulatory role in adult muscle development, in part by enhancing the proliferation of myogenic cells.

Consoulas and Levine (1997) also investigated the influence of nerves on *Manduca* leg muscle precursors. Adult legs form from myoblasts that originate in production sites located within the legs, and migrate to the sites of muscle formation where they accumulate, proliferate, and fuse to form muscle

fibers. Cutting of the larval leg nerves prior to metamorphosis resulted in the myoblasts failing to accumulate in the appropriate regions of the leg. The myoblasts did not die, but remained in the denervated legs as dispersed cells or as aggregates in inappropriate regions. When the legs were denervated after the myoblasts had aggregated, myoblast proliferation was reduced but differentiation continued (**Figure 6**). Thus, motor nerves are essential for determining both the accumulation of myoblasts into the correct areas of muscle development and the level of myoblast proliferation (Consoulas and Levine, 1997).

2.2.2.9.2. Hormones Hormones exert control over most developmental processes during metamorphosis. Ecdysone (20E) and JH are the most studied hormones, primarily in *M. sexta* (Weeks and Truman, 1986a, 1986b; review: Truman and Riddiford, 2002; **Chapters 3.5** and **3.7**). 20E is a steroid hormone released from the prothoracic gland in *Manduca* and the ring gland in *Drosophila* prior to larval molts and metamorphosis (Hegstrom *et al.*, 1998; **Chapters 3.2** and **3.3**). It interacts with at least two isoforms of ecdysone receptors (EcR), EcR-A and EcR-B1, in *Manduca*, or three isoforms, EcR-A, EcR-B1, and EcR-B2, in *Drosophila* (see **Chapter 3.5**).

During the last larval instar in *Manduca*, a small peak of ecdysteroids commits the larva to pupal differentiation (Riddiford, 1976). This peak is followed by a larger, prepupal peak that promotes molting to the pupal stage. After pupal ecdysis, there is a large release of steroids, the adult peak, which regulates the development of the adult moth. The fall of ecdysteroid titer later in adult development leads to reduced growth of the flight muscle. Shortly before eclosion, the flight muscle becomes unresponsive to these hormones (Tischler *et al.*, 1989). Other muscles respond differently to the peaks, in ways that are partially dependent on the receptor isoform expressed in the muscle.

In the *Manduca* muscle, the selection of EcR isoforms during metamorphosis is regulated by the motor neurons innervating the muscle. The subsequent release of ecysteroids determines the nature of the response in the muscle. For example, Hegstrom *et al.* (1998) followed the remodeling of the larval dorsal external oblique 1 (DEO1) muscle, which consists of five fibers, over the course of metamorphosis. Four of these fibers degenerate, while the remaining one participates in regrowth (nuclear proliferation) of the dorsal external five (DE5) muscle of the adult. Degeneration of the four fibers correlates with expression of only EcR-A in them just prior to pupal ecdysis followed by low levels of both EcR-A and EcR-B1 shortly after pupation. The fifth fiber shows upregulation of EcR-B1 3 days after pupal ecdysis. It is also the only one that stays innervated. Thus, the fate of the muscle DEO1 is determined by interactions between rising and falling ecdysteroid titers, the pattern of specific EcR isoform expression, and the interactions with other cells in the local environment (Hegstrom and Truman, 1996; Hegstrom *et al.*, 1998). In *Drosophila*, larval muscle cells express mostly EcR-B1, while imaginal discs and the proliferation zones express EcR-A.

20E regulates the function of *Broad-Complex* (*BR-C*) (as well as other ecdysone responsive genes) (Restifo and White, 1992). The *BR-C* locus encodes a family of zinc finger containing transcription factors (review: Riddiford *et al.*, 2003). Mutations in these genes, which belong to *reduced bristles on palpus* (*rbp*) class, have differential effects on flight muscle development (see Section 2.2.3.3.2).

2.2.3. Muscle Differentiation

2.2.3.1. General Features Established by the Early Studies

Research in flight muscle development in Diptera dates back over 100 years. A major area of study in the early and mid twentieth century focused on the description of ultrastructural changes that take place in the developing flight muscle cell using light microscopy and EM (e.g., Gregory *et al.*, 1968; Bursell, 1973; Valvassori *et al.*, 1978). EM analyses of the *Drosophila* IFM revealed a pattern of myofibril growth from the center, increasing in diameter by addition of peripheral myofilaments (Shafiq, 1963; Auber, 1969). Studies on *Calliphora* DLM, showed that no larval muscle myofibrils persisted in the adult muscle; instead, residual microtubules appeared to function as scaffolds for the assembly of new myofibrils (Crossley, 1972). The longitudinal growth of the DLM was proposed to occur by a mechanism whereby new sarcomeres were added by splitting of the Z bands, perhaps driven by tension from the guide (tendon) cells (Auber, 1969; Houlihan and Newton, 1979; review: Crossley, 1978). This was followed by a period where muscle length increased by lengthening of sarcomeres, a process that continued for several hours after eclosion and was believed to be driven by stretching of the muscle in response to increased hemolymph pressure (Houlihan and Breckenridge, 1981). An alternative model of muscle growth, based on studies in the blowfly *Lucilia cuprina* flight muscle,

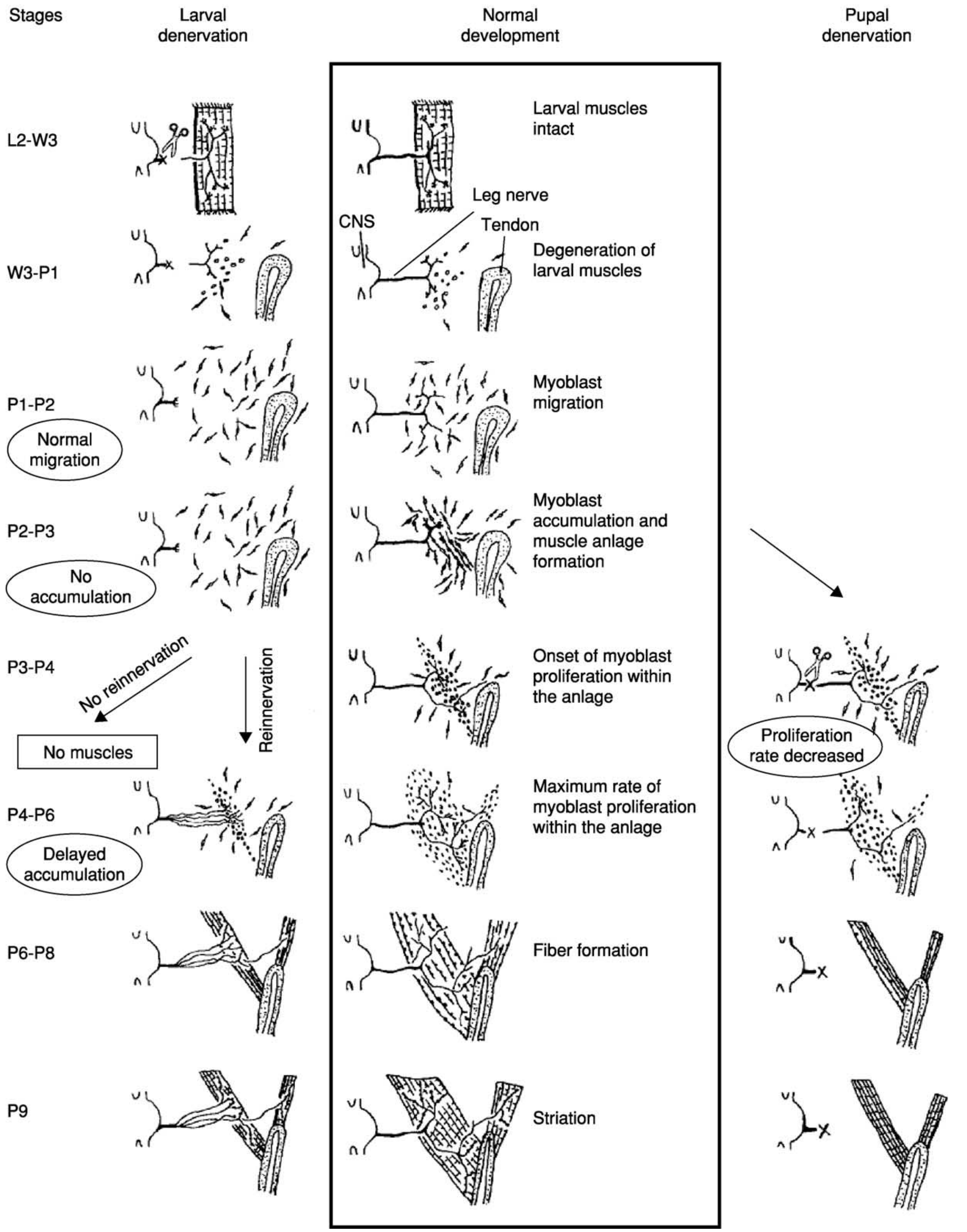


Figure 6 Development of adult leg muscle in *Manduca*. The association between nerves and developing muscles is depicted in the central panel. The effect of denervation prior to metamorphosis is shown on the left panel, and the effect after metamorphosis is shown on the right panel. Muscles do not form when denervation is performed in the larval stage because myoblasts fail to aggregate and proliferate in the areas of adult muscle formation associated with the developing tendon. Denervation in the pupal stage reduces the rate of myoblast proliferation and results in the formation of smaller muscles. (Consoulas, C., Levine, R.B. **1997**. Accumulation and proliferation of adult leg muscle precursors in *Manduca* are dependent on innervation. *J. Neurobiol. 32*, 531–553; © John Wiley & Sons, Inc., Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

stipulated addition of sarcomeres at the terminal ends of the myofibril (Peristianis and Gregory, 1971). Electron-dense bodies (similar to the "Z bodies" described in *Drosophila* by Shafiq (1963)) were found in close association with microtubules near the sarcolemma. These bodies appear to split while retaining the attached myofibrils that could serve as "seeds" for new myofibrils.

A significant development in the early 1970s was the implementation of genetic screens to identify flightless *Drosophila* mutants. It was not until the advent of recombinant DNA technology in the 1980s that many of the mutant gene products were identified as contractile proteins (e.g., actin, myosin, and troponin T) and DNA binding proteins (e.g., *erect wing* and *stripe*) (Koana and Hotta, 1978; Deak *et al*., 1982; reviews: Bernstein *et al*., 1993; Vigoreaux, 2001). This combined molecular–genetic approach fueled a major wave of research into muscle development and function that has continued until this date.

2.2.3.2. Expression of Muscle Structural Protein Genes

The myofibril is a structural complex of 20 or so proteins organized into separate but interacting filament systems and cross-linking structures (see Vigoreaux (2001) for a list of *Drosophila* IFM myofibrillar proteins) (**Table 3**). Coordinating the expression and assembly of contractile proteins can be achieved at multiple levels of regulation. In *Drosophila*, the interaction between *trans* regulatory factors and *cis* regulatory DNA sequences has been examined for a handful of structural protein genes.

2.2.3.2.1. Transcriptional regulation In *Drosophila*, actin is encoded by six genes that display temporal and tissue specific patterns of expression (Fyrberg *et al*., 1983). Two of the genes, *Act79B* and *Act88F*, have been shown by *in situ* hybridization and by fusion of DNA regulatory sequences to reporter genes (e.g., encoding β-galactosidase or green fluorescent protein (GFP)) to be expressed primarily in adult muscles. *Act88F* encodes the main actin isoform of the IFM and *Act79B* encodes the main isoform of tubular muscles found in the leg and the thorax (Sanchez *et al*., 1983; Courchesne-Smith and Tobin, 1989). *Cis* regulatory sequences that confer tissue specificity to *Act79B* are located within 4 kb of the 5′ flanking DNA. Tissue-specific expression of *Act88F* is also provided by sequences close to the point of transcription initiation (Geyer and Fyrberg, 1986; Hiromi *et al*., 1986). Wild-type levels of *Act88F* mRNA accumulation during flight muscle development are obtained when ~1 kb immediately 5′ of the transcription initiation site is present; derivatives retaining 600 or fewer base pairs of flanking DNA do not accumulate detectable levels of mRNA.

The specific regulatory sequences have not been delineated but it is intriguing that the *Act88F* promoter lacks D-Mef2 binding sites (Geyer and Fyrberg, 1986). D-Mef2 is a known transactivator of muscle-specific differentiation genes whose expression persists in adult muscle fibers (Bour *et al*., 1995; Roy and VijayRaghavan, 1999), and many of the genes expressed in differentiated adult muscles (e.g., those encoding myosin heavy chain and troponin T) have one or more pairs of D-Mef2 binding sites (Arbeitman *et al*., 2002). A small number of *D-mef2 trans* heterozygote mutant adults have defects in their IFM and fail to fly (Ranganayakulu *et al*., 1995). Moreover, it is known that misexpression of *D-mef2* in nonmesodermal tissue results in transcription of contractile protein genes (Lin *et al*., 1997). Thus, this transcription factor is likely to be required for normal expression of some IFM genes.

In addition to the IFM, *Act88F* is expressed at very low levels in other muscles, including muscles in the leg (Roy and VijayRaghavan, 1997; Nongthomba *et al*., 2001). The same promoter construct (~1.8 kb of 5′ flanking DNA) drives *Act88F* expression in IFM and the other muscles. It remains to be established whether the same or different *cis* regulatory elements are functional in individual muscle groups that express *Act88F*.

Homologs of the *Act79B* (*ActD1*) and *Act88F* (*ActE2*) genes in the closely related *Drosophila virilis* species show expression patterns similar to those described for *D. melanogaster* (Lovato *et al*., 2001). In *D. virilis*, *ActD1* is predominantly expressed in the jump muscle and in some muscles in the thorax and leg. *ActE2* is predominantly expressed in the IFM and in some tubular muscles of the leg (Lovato *et al*., 2001). *ActE1*, the homolog of *D. melanogaster Act87E*, is expressed in the tubular muscles of the ventral thorax and leg, and at low levels in the TDT, suggesting that some tubular muscles express more than one actin isoform. Comparison of the 5′ flanking sequences between *D. virilis* and *D. melanogaster* should facilitate identification of *cis* regulatory elements that confer tissue and temporal specificity. One such comparison conducted for the *troponin T* (*TnT*) genes identified several conserved sequences in the region immediately upstream of the transcription initiation sites (Benoist *et al*., 1998).

Like actin, troponin C (TnC) is also encoded by a multigene family in *D. melanogaster*. The expression of the four *TnC* genes is developmentally regulated and one gene (*TnC41C*, renamed *DmTnC1*) is expressed exclusively in adult muscles (Fyrberg

Table 3 Genes encoding structural proteins of *Drosophila* adult leg and flight muscle myofibrils

Structure/gene	*Description*	*Properties*	*References*
Connecting filament			
sallimus	Encodes several isoforms of large modular proteins including kettin and a titin-like protein	Component of connecting filaments in IFM that impart passive stiffness; possible role in the assembly of the terminal Z disc in IFM	Hakeda *et al.* (2000), Machado and Andrew (2000), Kolmerer *et al.* (2000)
bent	Encodes different size variants of projectin that also differ in their sarcomeric distribution in leg and IFM	Component of connecting filaments in IFM that impart passive stiffness; role in development not known	Vigoreaux *et al.* (1991), Ayme-Southgate *et al.* (1995), Fyrberg *et al.* (1992)
Thick filaments			
Myosin heavy chain	Encodes myosin heavy chain and myosin rod protein	Alternative splicing generates different isoforms in leg and IFM; transcription from intronic promoter generates shorter myosin rod protein in DFM; myosin heavy chain essential for thick filament assembly	Swank *et al.* (2000), Standiford *et al.* (1997)
Paramyosin	Encodes paramyosin and mini-paramyosin	Transcription from intronic promoter generates shorter mini-paramyosin. Phosporylation may modulate role in myofilament assembly	Becker *et al.* (1992), Vinos *et al.* (1992)
Stretchin-Mlck	Alternative splicing generates variants with and without myosin light chain kinase domain.	At least one isoform (myostrandin/A225) expressed in IFM	Champagne *et al.* (2000), Patel and Saide (2003)
Myosin light chain 2	Encodes myosin regulatory light chain	Wild-type MLC-2 stoichiometry is required for normal IFM myofibril assembly and function	Warmke *et al.* (1992)
Myosin light chain 1	Encodes myosin essential (alkali) light chain	IFM-specific isoform generated by alternative splicing	Falkenthal *et al.* (1987)
flightin	Encodes flightin strictly in IFM	IFM devoid of flightin assembles longer than normal thick filaments	Reedy *et al.* (2000)
Thin filaments			
sanpodo	Encodes tropomodulin	Involved in thin filament length determination in IFM	Mardahl-Dumesnil and Fowler (2001)
Actin 79B	Encodes a tubular muscle actin	Likely critical for thin filament assembly in leg but not formally tested	Courchesne-Smith and Tobin (1989)
Actin 88F	Encodes IFM actin and arthrin	Essential for thin filament and Z band assembly in IFM	Hiromi and Hotta (1985), Beall *et al.* (1989)
upheld	Encodes troponin T	Required for myofibril integrity	Fyrberg *et al.* (1990a)
wings up A	Encodes troponin I	Alternative splicing generates multiple isoforms in IFM and leg muscles. Absence in IFM prevents sarcomere formation	Beall and Fyrberg (1991)
Troponin C 41F	Encodes major IFM isoform. Separate genes encode minor IFM isoform and other muscle isoforms	No role in development known	Qiu *et al.* (2003)
Tropomyosin 1	Encodes standard muscle and cytoplasmic tropomyosin and two IFM-specific troponin H isoforms	TnH haploidy does not affect myofibril assembly	Kreuz *et al.* (1996)
Tropomyosin 2	Encodes standard muscle tropomyosin expressed in IFM, TDT, and other muscles	Absence results in structurally weaker myofibrils in IFM	Kreuz *et al.* (1996)
Glutathione-S-*transferase S1*	Encodes glutathione-*S*-transferase	Expressed in IFM but not other muscles. No role in development known	Clayton *et al.* (1998)
Z bands			
α actinin	Encodes different α-actinin size isoforms by alternative splicing	Same isoform expressed in leg and IFM; role in Z band and terminal insertion sites	Fyrberg *et al.* (1990b)
Unknown	Encodes Z(210)/zetalin	Expression limited to IFM and large cells of the jump muscle	Vigoreaux *et al.* (1991)

IFM, indirect flight muscles; DFM, direct flight muscles; TDT, tergal depressor of trochanter.

et al., 1994; Qiu *et al*., 2003) (**Table 3**). Two isoforms are expressed in the IFM; the major isoform (encoded by *DmTnC4*) differs from all others by having one instead of two calcium binding sites. This isoform is also expressed at high levels in the asynchronous IFM of the waterbug *Lethocerus* and the mosquito *Anopheles gambiae* (Qiu *et al*., 2003).

All muscle myosin heavy chains (MHC) in *D. melanogaster* are encoded by a single gene that undergoes alternative splicing of RNA transcripts to generate tissue-specific isoforms (reviews: Bernstein *et al*., 1993; Swank *et al*., 2000). A *Mhc* transgene containing 9 kb of flanking DNA at the 5′ and 3′ end is sufficient to dictate expression of this gene in all muscles and correct all defects engendered by *Mhc* amorphic alleles (Cripps *et al*., 1994). Nevertheless, as little as 400 base pairs of upstream sequence is required for transcription if the first intron is included. The first intron also contains a controlling element that is involved in IFM expression (Hess *et al*., 1989; Swank *et al*., 2000). Similarly, the first intron of the *Act88F* gene contains DNA sequences involved in temporal regulation (Fyrberg *et al*., 1998).

There are two genes encoding tropomyosin (TM) in *D. melanogaster*: the *TmI* gene (also called *Tm2*) encodes standard muscle TM, and *TmII* (also called *Tm1*) encodes muscle and cytoplasmic TM and the functionally distinct, IFM-specific TM fusion protein known as troponin H (TnH) (Karlik and Fyrberg, 1986; Hanke and Storti, 1988) (**Table 3**). IFM and TDT controlling elements are found in the proximal muscle enhancer located within the first intron of the *TMI* gene (Schultz *et al*., 1991; Lin and Storti, 1997). Putative Mef2 binding sites and a basal level muscle activator region adjoining this enhancer element are required for high level expression in TDT and IFM. Transcriptional regulatory elements also reside within the first intron of the *TmII* gene (Bernstein *et al*., 1993; Meredith and Storti, 1993). In fact, many contractile protein genes in *Drosophila* have introns close to the transcription initiation site, but the extent to which intronic sequences dictate tissue and developmental regulation has not been fully examined.

Like *TmII*, there are several other genes in *Drosophila* that encode more than one type of protein through the use of alternative promoters and/or alternative splicing (**Table 3**). Myosin rod protein (MRP) mRNA, for example, is transcribed from a promoter located within intron 12 of the *Mhc* gene (Standiford *et al*., 1997). MRP shares the same sequence as the MHC except for the first 77 amino acids (Standiford *et al*., 1997). The tissue distribution of MRP is distinct from that of MHC suggesting that the two promoters are regulated independently. Miniparamyosin (mPM) mRNA, on the other hand, is transcribed from a promoter within intron 7 of the paramyosin (*Pm*) gene (Maroto *et al*., 1995). Both proteins are expressed in adult tubular and flight muscles albeit at different ratios depending on the muscle type (see Section 2.2.3.3.3). The *mPm* and *Pm* promoters are regulated independently (Becker *et al*., 1992; Maroto *et al*., 1995; Arredondo *et al*., 2001a). Arredondo *et al*. (2001a) have shown that a Mef2 site in the *Pm* promoter appears not to be important for high expression in adult muscles but to be required for tissue specificity. The *mPm* promoter lacks consensus binding sites for known muscle transcription factors (Becker *et al*., 1992; Maroto *et al*., 1995; Arredondo *et al*., 2001a). Comparison of the upstream sequence of the *mPm* transcription unit between *D. melanogaster* and *D. virilis* revealed three conserved regions in sequence and position, at least one of which regulates *mPm* transcription in adult muscles (Arredondo *et al*., 2001a). Nevertheless, these studies did not uncover *cis*-acting elements that regulate IFM expression within the 4 kb upstream of the transcription start site.

A *D. melanogaster* gene at 62C (known as *sallimus*, *kettin*, or *D-titin*) encodes titin and several isoforms of the related protein kettin (Kolmerer *et al*., 2000; Zhang *et al*., 2000). The two proteins share the same N-terminal sequence but kettin is about one-third shorter than titin. The mechanism that gives rise to these different proteins, presumably alternative splicing or differential termination, has not been elucidated.

Unfortunately, progress in the understanding of the transcriptional regulation of individual genes has not translated into an understanding of the mechanisms controlling myofibril assembly. The *Act88F* promoter is activated very early in pupal development, approximately 16 h APF (Fernandes *et al*., 1991), or 10 h before activation of *Mhc* or any other known myofibrillar protein gene examined to date (**Table 2**). It is not yet known how the rates of actin expression correlate with the rates of actin polymerization *in vivo* but the early activation of actin gene transcription suggests that thin filament assembly may be a critical event in the initiation of myofibrillogenesis. On the other hand, studies of the thin filament pointed end capping protein tropomodulin (TMOD) are more consistent with the view that thin filament assembly is subservant to thick filament assembly (see Section 2.2.3.3.1).

Only a few studies have examined the transcriptional activation of contractile protein genes during

development of a particular muscle type. Western blot analysis of developing IFM showed that the IFM-specific protein flightin (FLN) is first detected about 60 h APF, well after thick filaments have begun to assemble and align transversely with interdigitating thin filaments (Vigoreaux *et al.*, 1993). At this time, another thick filament accessory protein, A225, and the Z band protein Z(210) are first detected (Patel and Saide, 2003; Vigoreaux, unpublished data). The transcript encoding myosin essential (alkali) light chain (ELC) is absent in early pupal stages but detected in mid-pupa (~48 h APF) onward (Falkenthal *et al.*, 1984). Finally, Northern blot analysis of total animal RNA showed that *Pm* and *mPm* gene transcripts, which are expressed in several muscles of the adult, are absent 12–14 h APF but present at 75 h APF (Becker *et al.*, 1992). In lepidopteran tubular (synchronous) muscle, PM appears before MHC (Lehmann, 1984). Additional studies that examine the temporal accumulation of other contractile proteins or their transcripts are needed in order to uncover the broad rules that govern the transcriptional control of myofibril assembly.

Genomic approaches are currently employed for the study of muscle development, but the benefits from this type of global approach have yet to fully materialize. White *et al.* (1999) used DNA microarrays to analyze the expression of genes during metamorphosis in *Drosophila* and found that many of the genes encoding contractile proteins are not activated until more than 12 h APF. This study relied on whole-animal RNA and did not differentiate among the adult muscle types (White *et al.*, 1999). A follow-up microarray study identified a subset enriched for genes expressed in terminally differentiated muscle (Arbeitman *et al.*, 2002). The peak expression pattern of these genes at late pupal stages was preceded by a peak in the expression of *D-mef2*, which in turn, was preceded by peak expression of *twist*. Thus, the study showed the potential of the microarray approach to reveal a muscle regulatory hierarchy.

Other large-scale functional genomic approaches such as insertional mutagenesis and enhancer detection systems that are operational in a variety of insects could also prove highly beneficial for the study of muscle development (Horn *et al.*, 2003). Finally, experimental approaches that rely on genomic or proteomic platforms applied to individual tissues (e.g., IFM) are likely to be most informative in deciphering how the assembly of contractile proteins is orchestrated during muscle development.

In summary, there is a dearth of information regarding the role of transcriptional activation as a control mechanism for coordinating the *in vivo* assembly of contractile proteins. In this regard, continuing and future studies with transgenic *Drosophila* should be informative. For example, expression of a *flightin* (*fln*) transgene regulated by the *Act88F* promoter does not appear to alter the timing of Flightin (FLN) expression or interfere with myofibril assembly (Barton *et al.*, 2002). Thus, other, posttranscriptional regulatory mechanisms must be considered.

2.2.3.2.2. Posttranscriptional regulation Five of the 19 exons of the *Drosophila Mhc* gene (exons 3, 7, 9, 11, and 15) are alternatively spliced to produce multiple forms of the protein in different muscles (reviews: Bernstein *et al.*, 1993; Swank *et al.*, 2000). Standiford *et al.* (2001) identified three conserved intronic elements that function in combination with nonconsensus splice donor sequences to control splicing of the IFM-specific exon 11e. One element acts as a splice repressor while the other two behave as splice enhancers (Standiford *et al.*, 2001).

The significance of alternative splicing is highlighted by studies of mutations that interfere with the tissue-specific splicing of an alternative exon. One example is Mhc^{10}, a mutation that blocks the splicing of alternative exon 15a and compromises the assembly of thick filaments in IFM and TDT but not in muscles that do not use this exon (Collier *et al.*, 1990). Differences in the mechanisms of tissue-specific splicing are also evident by mutations such as Mhc^{11}. This mutation affects the 5′ splice site of exon 9a by blocking the expression of correctly spliced transcripts containing this exon in the IFM and TDT (Kronert *et al.*, 1991). While the IFM produces an aberrantly spliced transcript (containing exons 9a, 9b, and 9c), which does not produce a protein, the TDT substitutes exon 9b for 9a, resulting in normal levels of myosin and a functional jump muscle.

Drosophila ELC transcripts are also alternatively spliced in a tissue-specific fashion (Falkenthal *et al.*, 1987). IFM is the only tissue in the adult that accumulates an alternatively spliced mRNA that lacks exon 5, resulting in a protein with a unique C-terminal tail of 14 amino acids. The choice between splicing pathways involves the use of a nonconsensus 3′ splice junction in larvae and in the tubular muscles of adults, whereas in the IFM only consensus splicing sequences are utilized. The *TmI* gene of *Drosophila* also relies on 3′ alternative splicing to produce two different TM polypeptides that differ in their C-terminal 27 amino acids. One of the transcripts encodes flight and jump muscle TM (Basi *et al.*, 1984; Basi and Storti, 1986). The *Drosophila TnT* gene also undergoes tissue-specific

splicing resulting in the generation of one variant that is unique to the IFM and TDT (Benoist *et al.*, 1998).

While most examples of alternative splicing have been shown to generate tissue-specific isoforms, there are cases in which differential splicing occurs in the same tissue. One such case involves the *Drosophila TmII* gene whose alternative splicing at the 3′ end generates the IFM-specific proteins TnH-33 and TnH-34. Three kettin isoforms, possibly arising from alternative splicing, have been also detected in *Drosophila* IFM myofibrils (Kulke *et al.*, 2001), while different size variants are also evident in *Lethocerus* flight and leg muscle (Lakey *et al.*, 1990). Finally, different patterns of alternative splicing of TnT in the flight muscle have been shown to occur in separate populations of dragonflies (Marden *et al.*, 2001). These differences impact flight muscle contractile performance and are likely to manifest complex ecological behaviors. The functional role, if any, of kettin, TnH, and TnT isoforms on myofibril development is not known.

To summarize, the muscle-specific expression of contractile protein genes is under complex regulation. The regulatory factors (transcription factors, splicing factors) responsible for tissue specificity have not been identified. Other types of posttranscriptional control mechanisms (e.g., translational control, regulated proteolysis) are possible but evidence in support of these mechanisms has not been uncovered.

2.2.3.2.3. Protein modifications and muscle-specific variants Many myofibrillar proteins are posttranslationally modified, but the potential role of these modifications in development is largely unexplored. Dramatic changes in IFM protein phosphorylation occur during the pupal to adult transition in *Drosophila*. Myosin regulatory light chain (RLC) and FLN have been shown to undergo multiple phosphorylations during the final stages of IFM development, at or near eclosion (Takano-Ohmuro *et al.*, 1990; Vigoreaux and Perry, 1994). Replacement of two RLC serines that are phosphorylated by myosin light chain kinase (MLCK) with alanines had no effect on myofibrillogenesis (Tohtong *et al.*, 1995; Dickinson *et al.*, 1997). Furthermore, heterozygotes for a deficiency that deletes the *mlck* gene fly normally suggesting that phosphorylation of the two RLC serine residues has no role in development (Tohtong *et al.*, 1997).

In contrast to RLC and FLN, mPM and PM undergo dephosphorylation during the transition from late stage pupa to flying adult (Maroto *et al.*, 1996). Overexpression of mPM does not affect its phosporylation profile (Arredondo *et al.*, 2001b). Changes in TnT phosphorylation have been documented to occur during the final stages of *Drosophila* flight muscle development (Benoist *et al.*, 1998; Domingo *et al.*, 1998). In the honeybee *Apis mellifera*, different molecular weight TnT bands are detected on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of thorax proteins extracted from bees 1 day (nonflyers) and 5 days (flyers) after hatching (Domingo *et al.*, 1998). However, the functional significance of the above changes in the phosphorylation status of these proteins remains unclear.

To date, the only IFM protein kinases that have been identified are MLCK (Tohtong *et al.*, 1997) and projectin (Maroto *et al.*, 1992; Ayme-Southgate *et al.*, 1995). Neither protein kinase has been shown to be directly involved in muscle development. In vertebrates, titin kinase (Mayans *et al.*, 1998) and titin phosphorylation (Gautel *et al.*, 1993) have been shown to play a role in myofibrillogenesis. In *Drosophila*, however, the *titin* gene lacks kinase encoding sequences (Zhang *et al.*, 2000).

Studies *in vitro* have shown that the phosphorylation status of projectin affects the type of filaments formed by purified myosin and paramyosin. When autophosphorylated projectin from flight muscle of the locust *Locusta migratoria* is added to myosin and paramyosin, subfilament-like structures 7–10 nm in diameter are preferentially formed, instead of the 20–50 nm filaments that are formed in the presence of unphosphorylated projectin (Fahrmann *et al.*, 2002). On the other hand, heterozygotes of the *Drosophila* projectin mutant *bent*$^{\mathrm{Dominant}}$, which express ~20% truncated projectin missing the kinase domain, do not exhibit any muscle defects (Moore *et al.*, 1999).

Other protein modificiations, such as ubiquitination of actin to generate arthrin, occur during myofibril assembly (Ball *et al.*, 1987). The stoichiometry of arthrin to actin in *Lethocerus* IFM (1 : 6) suggests that there is one arthrin molecule for each TM–TN complex. The appearance of arthrin after TM and TN during *Drosophila* development is consistent with the TM–TN complex determining which actin subunit is targeted for conjugation with ubiquitin. *Drosophila* actin is also N-terminally processed by the product of the *modification* (*mod*) gene which is likely responsible for removing the *N*-acetyl-cysteine residue. Fly strains that are *mod*$^{-}$ have flight defects but their flight muscles are structurally normal, suggesting that absence of this posttranslational modification has no effect on actin polymerization or assembly (Schmitz *et al.*, 2000).

2.2.3.3. Ultrastructure of the Developing Muscle

2.2.3.3.1. Myofibril assembly The ultrastructural changes that take place during myofibril formation have been described for *Drosophila* DLM. Using EM, Reedy and Beall showed that the number of sarcomeres in each myofibril is determined early in development and remains constant throughout (Reedy and Beall, 1993a). This is in contrast to earlier studies in *Calliphora*, which showed the number of sarcomeres to be increasing during the early pupal stages (Houlihan and Newton, 1979). In the latter case, initial myofibrils (~42 h APF) consist of ~310 sarcomeres with uniform length (~1.7 µm) and width (three or four thick filaments across). During the next 60 h, the length and width of sarcomeres (and thick and thin filaments) increases gradually and synchronously to a final length of ~3.2 µm and width of 32–35 thick filaments (**Table 2**). This is also in contrast to DLM development in *Pieris brassicae*, where lengthening of thick filaments does not occur (Cifuentes-Diaz, 1989).

Similar to the *Calliphora* and *Lucilia* flight muscle (Crossley, 1978), *Drosophila* myofibrils form within temporary scaffolds of microtubules that are disassembled shortly before eclosion but before the muscle reaches its final dimensions (Reedy and Beall, 1993a). Microtubules also are seen during myofibril assembly of the jump muscle, but they tend to associate more with the membrane of the SR than with the myofibril. The study by Reedy and Beall (1993a) did not reveal any evidence for sarcomere splitting at the Z band (as had been described for *Calliphora* by Houlihan and Newton, 1979), addition of new sarcomeres at the terminal ends, stress fiber-like assemblies, scattered or out-of-register myofilaments, or bundles of unincorporated thick or thin filaments. Instead, it has shown that there is consistent regularity in sarcomere dimensions throughout development, unlike in *Calliphora* where different length sarcomeres are found in the terminal and medial regions of the growing myofibril (Houlihan and Newton, 1979). Studies in *P. brassicae* also failed to provide evidence of Z band splitting as a mechanism for adding new sarcomeres (Cifuentes-Diaz, 1989).

It is not obvious from these studies how sarcomere assembly is initiated, only that thick and thin filaments appear simultaneously in interdigitating arrays. Nerves contact the periphery of the myotube at about the same time, but the influence of nerves on the initiation of myofibril assembly is not yet established. In *Manduca*, striations are detected on day 7 after pupation, coincident with the arrival of growing motor neurons at their attachment sites in muscle fibers (Duch *et al.*, 2000).

There is increasing evidence that accessory proteins are intimately involved in determining the precise and final length of thick and thin filaments in *Drosophila* IFM. TMOD, encoded by the *sanpodo* gene in *Drosophila*, participates in thin filament length determination, while the myosin rod binding protein FLN is required for proper thick filament length determination (Reedy *et al.*, 2000; Mardahl-Dumesnil and Fowler, 2001).

During IFM development, a 43 kDa isoform of TMOD acts as a dynamic cap allowing continuous elongation from the pointed end. Normally, a 45 kDa isoform of TMOD, which appears to be specific for the IFM, replaces the 43 kDa in the adult, after assembly is completed. When TMOD is transiently overexpressed during the pupal stages through the use of a heat shock inducible promoter, the 43kDa isoform behaves as a permanent cap, instead of a dynamic one, preventing core thin filaments from growing, even after TMOD expression returns to normal levels. Furthemore, the switch from the 43 kDa to the 45 kDa isoform fails to take place in pupae that have been heat shocked. The length of core thin filaments is established at the time the heat shock is applied, and the most deleterious effects are seen when heat shock induction is applied during mid-to-late myofibril assembly (day 3 late – day 4 early APF). While the growth of core thin filaments is arrested, sarcomere elongation, addition of peripheral thin filaments, and thick filament length are unaffected by overexpression of TMOD, and the resulting sarcomeres in adult IFM are of normal length (Mardahl-Dumesnil and Fowler, 2001).

The length of thick filaments is determined, at least in part, by the expression of FLN. In late stage pupa of the *fln* null, fln^0, thick filaments and sarcomeres appear, on average, longer than normal (Reedy *et al.*, 2000). Very long (~10 µm) thick filaments are also evident in the IFM of very young fln^0 adults. The longer than normal sarcomeres observed in fln^0 pupae are in contrast with the normal length sarcomeres observed in TMOD overexpressing flies. Together, the studies of fln^0 and TMOD overexpression suggest that thick filament length may be the primary determinant for sarcomere length in *Drosophila* IFM. Since thin filaments appear to grow longer in fln^0, it is possible that the length of thin filaments is established through coordinated interactions with the thick filament, perhaps by the myosin head or other thick-to-thin filament cross-connecting structures (Reedy *et al.*, 2000; Mardahl-Dumesnil and Fowler, 2001).

The gradual and incremental changes in thin and thick filament length during myofibril assembly in

Drosophila IFM are not consistent with the "molecular ruler" hypothesis that has been proposed for vertebrate skeletal muscles (Whiting *et al*., 1989; Gregorio *et al*., 1999). These latter muscles contain titin, a gigantic protein that spans one half of a sarcomere (from Z line to M line) and associates with numerous myofibrillar proteins (Gregorio *et al*., 1999). Evidence that titin is involved in vertebrate thick filament and sarcomere assembly comes from studies in which the myofibroblastic cell line BHK-21-Bi, which expresses a truncated titin, fails to assemble sarcomeres and lacks thick filaments (van der Ven *et al*., 2000). Although a titin/kettin-encoding gene has been identified in *Drosophila*, the existence of titin in the IFM remains controversial (Machado *et al*., 1998; Kulke *et al*., 2001). Mutations in the *Drosophila titin* gene result in defective myoblast fusion and abnormal myofilament organization in embryonic muscles (Machado and Andrew, 2000; Zhang *et al*., 2000), but the role of this protein in adult muscle development has not been explored.

The parallel increase in the length of thick and thin filaments suggests that interactions between myofilaments, whether via the myosin head, accessory cross-linking proteins, or auxiliary proteins, play a prominent role in establishing filament and sarcomere length. This is supported by the studies of contractile protein mutants described in the following sections. Thick filaments that assemble in the absence of thin filaments (i.e., null mutant for IFM actin) show hexagonal packing only near the M line but are otherwise disorganized (Beall *et al*., 1989). Likewise, in the absence of thick filaments (i.e., null mutant for IFM MHC), reasonably well-ordered thin filaments are observed that are anchored aperiodically within Z disc-type assemblies (Beall *et al*., 1989; O'Donnell *et al*., 1989; Bernstein *et al*., 1993). The broad picture that emerges from these studies is that thick filaments and thin filaments assemble simultaneously but independently of one another (Reedy and Beall, 1993a). However, the ordered interdigitation of precise length myofilaments into regular sarcomeric assemblies requires the presence of the myosin motor domain and possibly other protein components that interconnect thick filaments to thin filaments (Fyrberg and Beall, 1990).

Cripps *et al*. (1999) engineered a transgenic "headless" myosin (i.e., a myosin whose N-terminus is the RLC binding site) that was expressed in the IFM via the *Act88F* promoter. Some aspects of myofibril assembly (e.g., double hexagonal interdigitation of thick and thin filaments) were not perturbed but the overall sarcomere assembly was highly disrupted. The defects were more pronounced with a headless construct expressing the embryonic hinge region and C-terminus than with a headless construct expressing the corresponding adult hinge region and C-terminus (Cripps *et al*., 1999). The observed defects implicate the myosin head in myofibril assembly but leave open the question as to the precise mechanism by which the head exerts its effect. One possibility is that the presence of the head domain is required simply for proper spacing and/or alignment of thick and thin filaments. A second, not mutually exclusive, possibility is that contractile forces are required for some aspects of myofibril assembly (see Section 2.2.3.3.1.4). A mutant "dead-head" myosin, i.e., a myosin with normal motor structure that is biochemically incapacitated, may help to distinguish between these two possibilities. In conclusion, it remains an open question how myofilaments intercommunicate to achieve synchronous and incremental elongation throughout IFM development. Studies to date implicate the myosin motor domain and, possibly, accessory proteins such as FLN and TMOD.

The genetic analysis in *Drosophila* has contributed greatly to our understanding of muscle development. The following sections will summarize the key findings that have emerged from studies of different classes of muscle mutants. Two broad conclusions from these studies are: (1) certain defects are not uncovered by ultrastructural analysis of the developing muscle but reveal themselves after the mature muscle has engaged in contractile activity; and (2) the broad characterization of many of these mutants' effects has yet to uncover the mechanisms by which the mutations affect myofibril assembly.

2.2.3.3.1.1. Effect of alterations in protein stoichiometries It is not surprising that all loss-of-function mutant homozygotes of contractile proteins examined to date have an effect on muscle structure. These studies indicate that each protein plays a fundamental role in myofibril assembly that is distinct from its role in contractile mechanics (Vigoreaux, 2001).

IFM from actin null homozygotes lack thin filaments and fail to assemble Z bands, but contain well-aligned thick filaments (Beall *et al*., 1989; Fyrberg and Beall, 1990). Some missense mutations in actin, e.g., $Act88F^{V339I}$, likely cause protein instability as no actin or thin filaments are present in the muscle and no distinct muscle structure is formed (Drummond *et al*., 1991). Like the actin null $Act88F^{KM88}$, this mutant also interferes with the accumulation of other proteins (see below).

Analogously, IFM from *Mhc* null homozygotes (*Mhc*^{7}) lack thick filaments but show continuous arrays of thin filaments anchored aperiodically within Z discs.

Mhc^{7} and *Act88F*^{KM88} have broad effects on the proteome: at least 10 additional proteins are missing or reduced in the IFM of *Mhc*^{7} (Mogami and Hotta, 1981; Chun and Falkenthal, 1988). One of these proteins, RLC, has been shown to be synthesized and degraded, suggesting that the absence of MHC triggers proteolysis of thick filament-associated proteins. Alternatively, a mechanism of regulated proteolysis may operate normally in the IFM to eliminate unassembled RLC. These results demonstrate that thick and thin filaments can assemble independently of each other but, as discussed below, the formation of native sarcomeres of regular length and width is strictly dependent on proper stoichiometry of the two filament types. The results also demonstrate that the expression (or accumulation) of subsets of contractile proteins is highly interdependent.

Many contractile protein genes are haploinsufficient for muscle development. Heterozygote loss-of-function mutants have defective myofibrillar structures (review: Bernstein *et al*., 1993). For example, *Mhc* null heterozygotes produce reduced levels of protein, have myofibrillar defects, and are flightless (Mogami *et al*., 1986). The most common defect seen in mutant heterozygotes is out-of-register peripheral myofilaments surrounding a well-ordered core of hexagonally arranged thick and thin filaments (**Figure 7**). The consistency of this phenotype, regardless of the mutant gene, suggests that assembly starts at the center and radiates out, and that actomyosin interactions are important for establishing and/or maintaining sarcomeric order during assembly (Reedy and Beall, 1993a). There are few mutants, such as *Act88F*^{G245D}, that cause myofibrillar degeneration preferentially in the center of the myofibril (Sakai *et al*., 1990; Naimi *et al*., 2001). Developmental defects also tend to be more pronounced in the IFM than in the leg or jump muscle. Certain loss-of-function mutant heterozygotes (e.g., FLN and kettin) show normal or nearly normal muscle structure in the pharate adult (Vigoreaux *et al*., 1998; Hakeda *et al*., 2000). The ultrastructural defects that are evident in the IFM of mutant *ket* and *fln* heterozygotes appear after eclosion and are likely precipitated by contractile activity.

Gene copy number can also be manipulated by transgenic approaches. Transgenic flies with excess (three or four) copies of the *Mhc* gene (with its own 5′ and 3′ flanking sequences) were shown to overexpress MHC and exhibit ultrastructural defects, namely excess thick filaments at the periphery of the myofibrils (Cripps *et al*., 1994). The presence of three or four copies of the *Mhc* gene increased MHC levels by 1.5- and 2-fold, respectively, and resulted in decreased flight performance. EM revealed concomitant defects in the IFM, with excess thick filaments at the periphery of a well-formed myofibril core. Homyk and Emerson (1988) found that increasing the *Mhc* gene copy to three using a chromosomal duplication had no effect on flight performance or wing posture (and presumably muscle structure as well). Nevertheless, the duplication does affect muscle function when placed in a genetic background deficient for TnT (Homyk and Emerson, 1988). In the case of actin, increasing the gene copy number to four does not appear to affect flight performance dramatically (Hiromi *et al*., 1986). The latter study needs to be extended, however, to establish if higher levels of actin are present in the IFM and to determine the nature of possible myofibrillar defects.

Tropomyosin is also essential for normal muscle structure. The IFM of the *TmI* hypomorph *Ifm(3)3* shows various sarcomeric defects including Z bands that are irregular in size and shape, and disordered arrays of peripheral myofilaments. Sarcomeres are also, on average, shorter than in the wild-type (2.7 μm versus 3.4 μm) (Karlik and Fyrberg, 1985; Miller *et al*., 1993). This mutant also shows defects in jump muscle ultrastructure manifested by disorganized and variably shaped myofibrils. However, it is not known if these structural anomalies reflect developmental defects or induced atrophy from muscle use, since only adult flies were examined. Kreuz *et al*. (1996) used a deficiency which deletes both *TmI* and *TmII* and found that haploidy of *TmI* causes myofibrillar disruptions in the IFM as well as flightless behavior, while haploidy of only *TmII* causes neither. As for *Ifm(3)3*, it is not known if the deficiency brings about errors in development or not (Kreuz *et al*., 1996). Another caveat of the study of Kreuz *et al*. is that protein expression levels were not measured. Thus, while reduced *TmI* expression is the most likely explanation for the observed haploid effect, it is possible that other factors (e.g., alterations in the expression or assembly of interacting proteins) could account for the defects.

Sarcomeres are not present in the IFM of *TnT* mutants that eliminate (*upheld*^{2}) or greatly reduce (*upheld*^{3}) expression of TnT in this muscle. Instead, the IFM displays loosely organized thick filament skeins and randomly scattered I–Z–I brushes (Fyrberg *et al*., 1990a). Menke and Peterson (1989) showed that early in IFM development, the overall

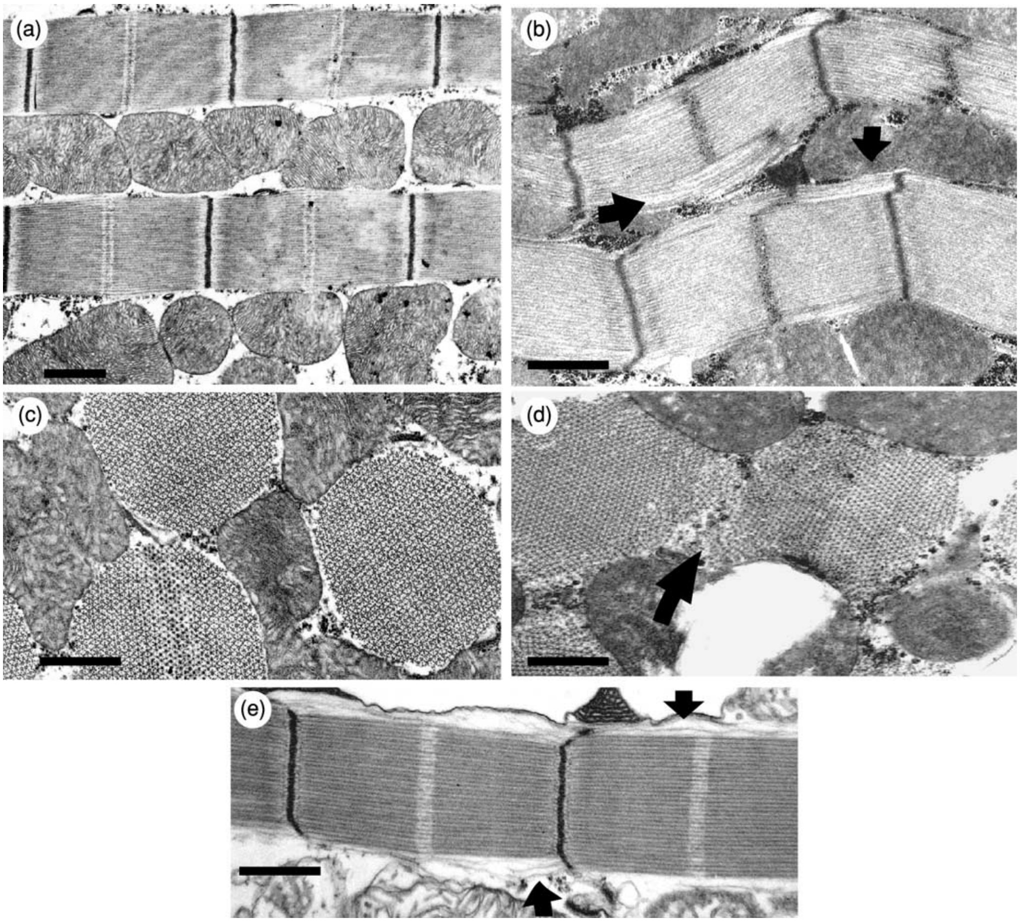


Figure 7 Myofibrillar defects in the indirect flight muscles (IFM) of the *flightin* mutant heterozygote *Df(3L)fln*[1]. Longitudinal (a, b, and e) and transverse (c, d) sections of *Df(3L)fln*[1] heterozygotes reveal disruptions in the periphery of the sarcomere (arrows) amid a well-preserved central core. Peripheral myofilaments are loosely organized resulting in sarcomere width inhomogeneities and disruptions in the double hexagonal arrays of thick and thin filaments that are evident in the myofibrillar core as well as in myofibrils from wild-type IFM (a, c). Note also that mutant myofibrils do not show the regular cylindrical shape characteristic of wild-type myofibrils. (e) Treatment of *Df(3L)fln*[1] fibers with a detergent ''skinning'' solution removes the majority of the loosely associated peripheral myofilaments. A few remaining myofilaments wander freely along the myofibrillar surface (arrows). Scale bar = 1 μm. For further details, see Vigoreaux *et al.* (1998).

pattern of protein expression in *upheld*[2] is similar to that of wild-type, but the mutant IFM begins to express two heat shock proteins at approximately 61 h APF, perhaps in response to the presence of unassembled or misfolded proteins. Similarly, mutants that fail to express one or more IFM TnI isoforms (*heldup*[3], *heldup*[4], *heldup*[5]) lack sarcomeres despite the presence of thick and thin filaments (Deak *et al.*, 1982; Beall and Fyrberg, 1991). The fact that myofibrils are absent in newly eclosed mutant adults is indicative of developmental defects and not syndromes associated with contractile activity (Beall and Fyrberg, 1991).

The IFM normally expresses very low levels of mPm. In contrast, transgenic lines in which the *mPm* gene is regulated by the *Act88F* promoter, express three to five times more mPm than wild-type flies (Arredondo *et al.*, 2001b). The higher mPm levels have little or no effect on myofibril assembly, mPm localization, thick filament diameter, or expression of other myofibrillar proteins. Nevertheless, subtle developmental defects are evident and flies display moderate flight impairments. More importantly, transgenic *Act88F–mPM* flies, which express higher levels of mPM, show more developmental defects and age-dependent degeneration of the IFM than transgenic flies in which the *mPm* gene is regulated by its own promoter (Arredondo *et al.*, 2001b). Thus, reprogramming the transcriptional activation of the *mPm* gene results in small developmental defects which are later magnified in the working muscle.

The higher levels of mPm expression cannot be explained by higher transcriptional activity of the

Act88F promoter since an *Act88F* promoter–headless myosin chimeric transgene does not result in overexpression of the rod construct over the wild-type MHC (Cripps *et al.*, 1999). Interestingly, IFM myofibrils in transgenic *Act88F* promoter-fln^{+} have narrower diameters (i.e., less thick filaments) than in the wild-type suggesting that premature activation of *fln* transcription has an effect on thick filament number (Barton *et al.*, 2002). This contrasts the situation with fln^{0} where the absence of flightin results in thick filaments that have longer than normal length (Reedy *et al.*, 2000).

Mutations in Z band-associated proteins interfere with normal myofibril assembly. Rare homozygous escapers of the weak hypomorphic *kettin* allele ket^{5} show Z bands that do not intersect the entire width of the sarcomere, resulting in peripheral myofilaments that are out of register (Hakeda *et al.*, 2000). Mutants in α-actinin that affect RNA splicing ($fliA^{3}$ and $fliA^{4}$) also show myofibrillar defects. The mutant $fliA^{3}$ expresses very low levels of α-actinin in the IFM and tubular leg muscles, mostly the slower migrating supercontractile muscle isoform (Roulier *et al.*, 1992). In newly eclosed adults, IFM sarcomeres are relatively normal except for Z bands that appear thin and punctate (Roulier *et al.*, 1992). In older adults, however, the Z disc disintegrates causing thick and thin filaments to loose their lateral registration. This is in contrast to $fliA^{4}$, in which α-actinin expression is similarly reduced, but no age-dependent degeneration is observed. One interpretation of these results is that the nature of the α-actinin isoform expressed in $fliA^{3}$, rather than a reduction in its quantity, is responsible for the muscle degeneration (Roulier *et al.*, 1992) (see Section 2.2.3.3.1.3).

In light of the studies summarized above, the muscle defects seen in contractile protein gene aneuploids can be ascribed to quantitative imbalances. While this has been demonstrated in some mutants (e.g., MHC and RLC; Warmke *et al.*, 1992; Cripps *et al.*, 1994), it is not universally true and other mechanisms must be invoked to explain defects in muscle development of IFM mutants. Nevertheless, there is strong evidence that correct stoichiometry of the major contractile proteins is important for normal IFM development (Beall *et al.*, 1989; Cripps *et al.*, 1994). Alterations in the actin : myosin protein ratio result in abnormal assembly, as evidenced by unintegrated peripheral myofilaments and occassional fraying of the hexagonal lattice, and nonfunctional muscle that leads to flight impairment. The *Act88F* null heterozygote and the *Mhc* null heterozygote display more myofibrillar defects than the *Act88F* : *Mhc* double null heterozygote. One interpretation of these results is that filament imbalances, rather than an absolute deficit of one myofilament type, is the underlying cause of the myofibrillar defects (Beall *et al.*, 1989).

In summary, more detailed developmental analyses of the above mutants are needed to establish the stage at which myofibril assembly is impaired and the molecular processes that are perturbed by each mutation. The regulatory mechanisms that coordinate contractile protein synthesis and assembly remain largely unexplored.

2.2.3.3.1.2. Effects of mutant proteins In addition to loss-of-function mutants, the study of flight muscle development has been greatly aided by mutations that result in amino acid changes in contractile proteins. Analysis of these mutants has provided insights into the sequence of developmental events that lead to the formation of the myofibril. Furthermore, the study of genetic interactions, mutations in separate genes that either suppress or enhance the mutant phenotype of a separate locus, are also providing valuable insights into the protein interactions that are fundamental for myofibril assembly (Homyk and Emerson, 1988; Prado *et al.*, 1995; Kronert *et al.*, 1999; Naimi *et al.*, 2001; Nongthomba *et al.*, 2003).

Mutations in the genes for actin and MHC are among the most frequently recovered in genetic screens that rely on a flight test to select mutants. In contrast, screens that rely on visual inspection of IFM morphology have helped uncover recessive loci that are involved in early and late stages of adult myogenesis (Nongthomba and Ramachandra, 1999). Results from these studies have been discussed in detail in several reviews (Drummond *et al.*, 1991; Bernstein *et al.*, 1993; Sheterline *et al.*, 1998; Swank *et al.*, 2000; Vigoreaux, 2001) and, for this reason the discussion is limited to the general trends rather than to an in-depth analysis of each mutant strain.

Different missense mutations in *Act88F* have been described that interfere with actin's interaction with other myofibrillar proteins. For example, the two mutants $Act88F^{E334K}$ and $Act88F^{E93K}$ form thin filaments but lack Z bands (Drummond *et al.*, 1991; Sparrow *et al.*, 1991). The absence of α-actinin and other high molecular weight proteins in the IFM of $Act88F^{E93K}$ suggests that this mutation interferes with normal assembly of the Z band (Sparrow *et al.*, 1991). This effect is probably independent of the mutation's effect on tropomyosin binding and actomyosin kinetics (Bing *et al.*, 1998; Razzaq *et al.*, 1999; review: Vigoreaux, 2001). Another actin mutant that causes defects in Z band assembly is

Act88F^{G6AA7T}, in which amino acids 6 and 7 (glycine and alanine, respectively) are converted to alanine and threonine. In this mutant, multiple level Z bands that fail to cross the entire width of the myofibril are found. As a result, unanchored peripheral thin filaments of opposite polarities are present within the same half sarcomere. This is evidenced by the formation of reverse rigor chevrons by the myosin cross-bridge (Reedy *et al*., 1989).

Another group of actin mutations (*Act88F*^{R28C}, *Act88F*^{E334Q}, and *Act88F*^{G268D}) result in abnormal fiber morphology in the developing pupa (Nongthomba *et al*., 2003). In some cases, fibers detach from the cuticle and this is followed by partial or complete hypercontraction during or after eclosion. The hypercontraction phenotype suggests a role for contractile forces in muscle development (see Section 2.2.3.3.1.4). In contrast, the mutant *Act88F*^{R95C}, which maps to the myosin S1 binding site, has no efffect on the initial myofibril development but leads to highly disrupted and hypercontracted fibers after eclosion (An and Mogami, 1996; Nongthomba *et al*., 2003).

Like *Act88F*, many *Mhc* alleles have been recovered from screens of flightless flies. Several missense mutations have been characterized that have little or no noticeable effect on muscle development but render the muscle susceptible to breakdown in the adult. An interesting example is *Mhc*^{13}, a point mutation in the light meromyosin (LMM) region of the myosin rod that changes a glutamic acid for a lysine. The structure of the developing myofibril is relatively unaffected but the mutation interferes with the normal accumulation of FLN in the IFM (Kronert *et al*., 1995). The absence of FLN leads to hypercontraction and rapid degeneration of the myofibril subsequent to eclosion. Though the mutation is in a constitutive exon that is expressed in every muscle, the phenotype is manifested primarily in the IFM suggesting that the absence of FLN, rather than an intrinsic defect in the myosin filament, compromises muscle structure. Other studies, showing that binding of FLN to myosin rod is abolished by the *Mhc*^{13} mutation (Ayer and Vigoreaux, 2003) and that *fln*^{0} manifests a phenotype similar to *Mhc*^{13} (Reedy *et al*., 2000), provide further evidence that the myosin rod–flightin interaction is essential for the establishment of structurally sound thick filaments.

Mhc^{9} is a missense mutation in the adult-specific alternative exon 9a (used in IFM and TDT) that results in a glutamic acid 482 to lysine change in a region of the myosin motor close to the actin binding site (Bernstein and Milligan, 1997). In IFM, the mutation behaves like the null allele *Mhc*^{7}, i.e., it prevents the accumulation of myosin and of the same subset of proteins that appear missing or reduced in *Mhc*^{7} (O'Donnell *et al*., 1989). Thick filaments and myofibrils are absent. Abnormal structure and reduced number of thick filaments are also seen in the four small anterior cells of the TDT. The mutation most likely affects protein stability, as evidenced by normal accumulation of *Mhc* RNA in the IFM and TDT. A similar mutation in *Caenorhabditis elegans* myosin abolishes ATPase activity, raising the possibility that abnormal interaction with actin may lead to protein instability in the developing muscle (Kronert *et al*., 1994). However, the fact that the mutant myosin fails to accumulate in IFM that lacks thin filaments, argues against this possibility and leaves open the question of how the myosin motor domain participates in myofibril assembly (Kronert *et al*., 1994; Cripps *et al*., 1999).

A caveat of many of the studies summarized above is that muscle structure was examined only in mature adults, while there is little information on muscle development *per se*. Detailed developmental analysis of each mutant strain is necessary to establish if and when each mutation interferes with myofibrillar assembly. An important distinction to be made is whether a particular mutation disrupts assembly or permits normal assembly only to give rise to a compromised myofibril with insufficient structural integrity. This is particularly true for actin and myosin mutants, where there is a broad repertoire of mutants, but very few have been examined developmentally (Drummond *et al*., 1991; Bernstein *et al*., 1993; An and Mogami, 1996; Sheterline *et al*., 1998; Swank *et al*., 2000).

2.2.3.3.1.3. Effect of isoform substitutions Unlike the case with vertebrate muscles, there is little evidence of isoform switching during development of flight or leg muscle in *Drosophila* (Caplan *et al*., 1983; Swank *et al*., 2000). As mentioned above (see Section 2.2.3.3.1), a 45 kDa TMOD isoform replaces a 43 kDa isoform well after the majority of myofibril assembly is completed (Mardahl-Dumesnil and Fowler, 2001). Maturational changes in TnT isoforms also occur in the flight muscle of dragonfly, affecting the temperature-dependent Ca^{2+} sensitivity of muscle activation. As a result, mature adult muscle is able to generate more force per unit area than newly emerged adult muscle (Fitzhugh and Marden, 1997).

There is ample evidence that distinct muscle types express different isoforms of contractile proteins, but the extent to which these differences influence developmental decisions is far less clear. One way of

approaching this question is to use transgenic techniques to express a protein isoform distinct from the one normally expressed in the muscle. In the case of actin, isoforms have been shown not to be functionally interchangeable, as demonstrated by the expression of a human (β)-cytoplasmic actin in the IFM of the actin null *Act88F*^{KM88} (Brault *et al.*, 1999). This actin, which differs from IFM actin in only 15 residues, can assemble into thin filaments to which endogenous myosin cross-bridges attach. Overall sarcomeric organization is nevertheless deficient, perhaps due to the inability of the cytoplasmic actin to interact with one or more actin-binding proteins in the IFM (Brault *et al.*, 1999). Similarly, Fyrberg *et al.* (1998) found that converting the flight muscle actin-encoding gene to one specifying a nonmuscle *Drosophila* isoform, a change entailing a total of 18 amino acid replacements, resulted in disruption of IFM structure and function. Interestingly, the majority of single isoform-specific amino acid replacements did not perturb myofibril assembly. However, when portions of the *Drosophila Act88F* actin gene were substituted with corresponding regions of genes encoding other isoforms, most constructs engendered myofibrillar defects, whose severity correlated with the number of replacements within the chimeric genes (Fyrberg *et al.*, 1998). These studies showed that, while most individual amino acid changes are tolerated, replacements of multiple amino acids are not. It is not known when or how these isoform substitutions perturb myofibrillogenesis.

In contrast to actin, *Drosophila* MHC isoforms are functionally interchangeable for development but not for physiology or, in some cases, muscle stability. Substitution of individual adult-specific alternative exons with their embryonic counterparts has no effect on IFM development (Swank *et al.*, 2001, 2003). Similarly, a mutation that results in the substitution of exon 9a for exon 9b has no effect on the assembly properties of myosin in the TDT (Kronert *et al.*, 1991). Wells *et al.* (1996) created transgenic *Drosophila* that expressed a single embryonic MHC isoform in all muscles. This isoform permitted normal development of IFM and TDT, indicating that the type of MHC isoform expressed in a muscle does not dictate the structural qualities of the sarcomere. However, transformed flies are flightless and show reduced jumping ability. In addition, the IFM myofibrillar structure deteriorates in aging adults, but not in the TDT. The aging effect on IFM ultrastructure is partly ameliorated by substituting the C-terminus of the embryonic MHC transgene with the adult C-terminus, suggesting that this region of the molecule is important for stability of IFM myofibrils (Wells *et al.*, 1996; Swank *et al.*, 2000).

Tropomyosin isoforms are also functionally interchangeable. In *Drosophila*, two isoforms that differ at their C-termini are encoded by the *TmI* gene: Ifm-TmI is expressed in IFM and TDT, while Scm-TmI is expressed in other muscles of the larva and adult (Miller *et al.*, 1993). Miller *et al.* (1993) demonstrated that the IFM and TDT tolerate isoform substitutions by using a transgene encoding the Scm-TmI to correct the *Ifm(3)3*-induced defects in both muscle types (see Section 2.2.3.3.1.1).

2.2.3.3.1.4. Role of mechanical forces The development of vertebrate striated muscle has been shown to be influenced by mechanical forces (Simpson *et al.*, 1994). Flow-induced forces imparted by the circulating blood are also essential for vertebrate heart development (Taber, 1998). There is some evidence, albeit indirect, that externally applied and internally generated forces are important epigenetic factors in flight muscle development. For example, the DLM of the cricket *T. oceanicus* contracts during the nymphal instars in the absence of neural activity and before it becomes functional for flight (Ready and Josephson, 1982).

Longitudinal growth of the flight and jump muscles of the blowfly *Calliphora vomitoria* occurs in response to stretch at the time of emergence from the puparium. Houlihan and Breckenridge (1981) proposed that the expansion of the newly eclosed blowfly from air intake and increased hemolymph pressure brings about lengthening of the sarcomere. Changes in sarcomere length are also associated with increased actomyosin ATPase activity (Houlihan and Breckenridge, 1981). The number of sarcomeres along a myofibril remains constant, in contrast to pupal development, where muscle growth is believed to occur through increased number of sarcomeres by Z band splitting (Houlihan and Newton, 1979) (but see Section 2.2.3.1). The increased sarcomere length must be accompanied by elongation of thick and thin filaments, but this has not been shown conclusively. Cutting of the tendon guide cells appears to halt sarcomere elongation and induces muscle shortening, consistent with the idea that muscle grows in response to tensile forces (Houlihan and Newton, 1979). Force transmission at the muscle–tendon junction may also explain why certain α-actinin mutations in *Drosophila* result in more disruption of terminal Z bands than of internal sarcomeric Z bands (Fyrberg *et al.*, 1990b). The molecular mechanisms by which muscle senses and responds to mechanical stress are not understood.

Studies of *Drosophila* IFM mutants are providing increasing evidence that contractile forces play a determining role in myofibril assembly. In the mutant *heldup*^{2}, a single amino acid substitution in TnI (alanine 116 for valine) causes hypercontraction of the IFM that begins to manifest approximately 78 h APF (Naimi *et al.*, 2001). The muscle condition progressively worsens resulting in complete loss of sarcomere structure just 2 days after eclosion (Beall and Fyrberg, 1991). The assembly defects engendered by *heldup*^{2} are likely to result from unregulated contractions, as evidenced by the following observations. The condition is ameliorated in *heldup*^{2};*Mhc*^{7} double mutants, in which thick filaments are absent (Beall and Fyrberg, 1991). A screen for genetic suppressors of the wings up phenotype of *heldup*^{2} uncovered an intragenic mutation, a mutation in TM, and four mutations in the MHC motor domain (Prado *et al.*, 1995; Kronert *et al.*, 1999; Naimi *et al.*, 2001). The mechanism(s) by which the second site mutations suppress muscle hypercontraction has not been established, but the results of these studies implicate unregulated, Ca^{2+}-activated actomyosin-generated tension as the underlying cause of the *heldup*^{2} developmental defect. More recently, Nongthomba *et al.* (2003) isolated additional *Mhc* missense alleles that act as suppressors of *heldup*^{2} and *upheld*^{101}, a TnT point mutation that shows similar characteristics to *heldup*^{2} (Fyrberg *et al.*, 1990a). The finding that the IFM hypercontraction phenotype of *heldup*^{2} and *upheld*^{101} can also be suppressed by the transgenic "headless" myosin (lacking the motor domain) provides further evidence that contractile forces are generated during myofibril assembly (Nongthomba *et al.*, 2003). How these forces impact muscle development remains to be established.

Mutations in the regulatory subunit of the *Drosophila* protein phosphatase calcineurin (*canB2*) also result in a hypercontracted phenotype, in which the bulk of the IFM fiber mass collapses towards the posterior end of the thorax (Gajewski *et al.*, 2003). The mutation preferentially affects the DLM, with little or no effect on the DVM or the jump muscle. Like *heldup*^{2}, developing IFM fibers in *canB2* mutant pupae begin to shorten irreversibly during day 3 of pupal development. Calcineurin is known to function in cell signaling pathways in a variety of cell types, including vertebrate cardiac and skeletal muscle, but its role in IFM development has not been elucidated.

2.2.3.3.2. Formation of the myotendon junction Epidermal cells that will contribute to the formation of the myotendon junction (MTJ) in *Drosophila* IFM are defined as early as the third larval instar by expression of the transcription factor Stripe (Fernandes *et al.*, 1996). These cells form four clusters located in the notal region of the disc epithelium in close association with myoblasts (**Figure 4**). The target genes for Stripe have not been identified but genes that encode structural proteins are likely candidates (Roy and VijayRaghavan, 1999). Mutants in the *stripe* gene preferentially affect the DLM, while mutants in the *vertical wing* gene affect DVM, DLM, and TDT in decreasing order of severity (Deak, 1977b; de la Pompa *et al.*, 1989). The function of *vertical wing* is not known but fate mapping experiments suggest its primary site of action is not the muscle (Deak, 1977b). The product of *erect wing* (*ewg*) is a transcription factor that is expressed in imaginal progenitor myoblasts (DeSimone *et al.*, 1996).

The attachment sites for both ends of the DLM and the dorsal ends of the DVM and TDT arise from the wing imaginal disc, while the attachment sites for the ventral ends of the DVM and the TDT arise from the leg imaginal disc. Another difference between the two flight muscles is that imaginal myoblasts that fuse to form DVM do not express integrin (PS-2α), while those that fuse with larval remnants to form DLM do express this protein (Fernandes *et al.*, 1996). The expression of PS-2α is likely to be regulated by D-Mef2 and mutants of the latter show abnormal pattening and reduced size DLM (Ranganayakulu *et al.*, 1995).

Once the muscle contacts the epidermis, different integrins are detected at the muscle and epidermal ends (**Table 2**). PS-2α first appears in myoblasts that surround the persistent larval templates during metamorphosis, but the expression ceases after a few hours (Fernandes *et al.*, 1996). It then reappears after the initial steps in muscle development are completed (i.e., approximately 24 h APF), a timing that is more consistent with a role in the stabilization and maturation of the muscle–epidermal contacts (Fernandes *et al.*, 1996). Mutations in the *struthio* gene, which encodes a putative RNA binding protein, cause flight impairments that most likely arise from defects in epidermal muscle attachment sites (Lo and Frasch, 1997). Moreover, TDT development is curtailed by a mutation (*mys*^{nj42}) in the PS-β integrin gene, suggesting that a different integrin is involved in the formation of the MTJ for this muscle (Wilcox *et al.*, 1989).

The ends of developing IFM fibers project filopodium-like extensions that reach towards epidermal cells that express PS-1α (Roy and VijayRaghavan, 1999). The first processes that connect muscle cells to the epidermis are epidermal in origin, at least for

the anterior ends of DLM (Fernandes *et al.*, 1996). Using ultrathin section electron microscopy, Reedy and Beall (1993b) provided a detailed description of the ultrastructural events that result in formation of the flight muscle MTJ. Extensions of tendon guide cells first contact developing myotubes shortly before myofibrils begin to asssemble. Focal dense plaques at the membrane junctions thicken and enlarge to form a modified terminal Z band that links the myofibril to the membrane via a dense feltwork. Abundant microtubules play a prominent role on both sides of the junction. Muscle microtubules form transient "sleeves" surrounding developing myofibrils, while tendon cell microtubules merge with specific dense sites on the junctional membrane and later link the tendon cell basal membrane to its apical (cuticular) membrane (Reedy and Beall, 1993b). Mutations in *α-actinin* disrupt myofibrillar attachments at the terminal Z band (Fyrberg *et al.*, 1990b). Mutations in *kettin* also have been shown to disrupt this structure. In $ket^{14}/+$ mutant heterozygotes, the terminal Z band is reduced in size, and some myofilaments appear to bypass this structure and contact the cell membrane directly (Hakeda *et al.*, 2000).

Differentiation of the DVM MTJ is under the control of BRC-Z1, a zinc finger transcription factor encoded by the *Broad Complex* (*BRC*) ecdysone responsive gene (Sandstrom and Restifo, 1999). *BRC* mutants of the *reduced bristles on palps* (*rbp*) complementation group, which encode BRC-Z1, have few intact DVM fibers that are often attached to incorrect locations on the epidermis (Sandstrom *et al.*, 1997). The mutations also affect the attachment of the DLM and TDT demonstrating that rbp^+ function is required for the formation and maintenance of the MTJ. Using mosaic flies whose thoracic muscles and epidermis were of different genotypes, Sandstrom and Restifo (1999) demonstrated that BRC-Z1 acts in the dorsal epidermis, not in the muscle fiber, to establish the correct location and formation of the MTJ.

The attachment sites for the DFM are formed by *stripe*-expressing epidermal cells in the wing hinge primordium. Unlike the IFM, additional *stripe*-independent mechanisms that rely on *D-Wnt-2* are involved in the formation of muscle attachment sites (Kozopas and Nusse, 2002). Regions of *D-Wnt-2* expression in the wing hinge primordia are adjacent to regions of *stripe* expression; however, mutants in *D-Wnt-2* that affect patterning of DFM do not interfere with *stripe* expression.

2.2.3.3.3. Functional specialization of muscle types Ultrastructural and biochemical changes of the flight muscle accompany the transition to adulthood and the subsequent maturational progression. Among the most dramatic changes are in the activity of metabolic enzymes and on the size, ultrastructure, and number of mitochondria (Scaraffia *et al.*, 1997). Changes in flight muscle size and ultrastructure during adult maturation are well documented for a variety of insect taxa (review: Marden, 2000). For example, the cross-sectional area of the mitochondria increases during adult maturation in dragonfly, in response to aerodynamic demands and ecological challenges. For the desert locust *S. gregaria*, mitochondrial growth during flight muscle development occurs independently of JH or ecdysteroid hormone titers (Wang *et al.*, 1993). In contrast, flight muscle hypertrophy is under juvenile and ecdysteroid hormone control, but neural factors also play a role in some species (Marden, 2000). JH also influences flight muscle degeneration in insects that no longer have a need to fly (e.g., in crickets initiating reproductive activity after migratory flight).

Differences among muscle types are clearly reflected in the structure and composition of the myofibril. Some generalities in sarcomere design are well known, e.g., asynchronous IFM have a lower thin-to-thick filament ratio than synchronous flight muscle (3 : 1 versus 5 : 1 or higher) (Squire, 1986; Josephson *et al.*, 2000). A major strategy for achieving functional specialization is differential expression of contractile protein isoforms that impart fiber-specific physiological properties. For example, maximum velocity of muscle shortening (V_{max}) in the vertebrate muscle is in large part determined by the particular myosin isoform expressed in that tissue (Lowey *et al.*, 1993). Thus, it comes as no surprise that *Drosophila* IFM myosin has among the fastest *in vitro* motility velocity ever recorded for a striated muscle myosin (Swank *et al.*, 2001). Surprisingly, there is no correlation between unloaded shortening velocity (V_{max}) and wing beat frequency among the various insects tested (Marden, 2000).

The muscle may also modulate the relative expression of proteins to achieve distinct biomechanical properties. The amount of Pm varies over a broad range among insect muscle types, and differences in Pm content are often associated with thick filaments that differ in form and size (Beinbrech *et al.*, 1985; Maroto *et al.*, 1996). Differences in metabolic enzyme activity also have been documented during flight muscle development (Deak, 1977a) and among populations (Zera *et al.*, 1985). In the latter case, expression of polymorphic loci encoding metabolic enzymes is thought to directly influence flight performance (e.g., flight duration

and aerodynamic power output) (Barnes and Laurie-Ahlberg, 1986).

A second strategy for achieving functional specialization is the recruitment of protein isoforms to perform distinct roles. For example, two different molecular weight variants of projectin are expressed in the IFM and tubular muscles of *Drosophila* (Vigoreaux *et al.*, 1991). These two isoforms, which are likely generated by alternative splicing, also differ in their sarcomeric distribution; I band in the IFM and A band in tubular muscles (Vigoreaux *et al.*, 1991; Daley *et al.*, 1998). These differences in protein structure and sarcomere distribution suggest that IFM and tubular projectins perform different functions. However, this has yet to be demonstrated. Another example is *Drosophila* mPm, which is found all through the A band in the TDT but restricted to the M line and the ends of thick filaments in IFM. It is unclear what, if any, are the functional consequences of this difference in distribution. Unlike *Drosophila*, *Lethocerus* mPm is distributed along the entire length of the thick filament in both IFM and TDT (Maroto *et al.*, 1996). In addition, the distribution of the flight muscle specific isoform of glycerol-3-phosphate dehydrogenase (GPDH-1) is restricted to the sarcomere M line and Z band. A different GPDH isoform (GPDH-3) that differs by the absence of a C-terminal tripeptide, does not show the sarcomeric distribution of GPDH-1 when expressed transgenically in the IFM of a *Gpdh* null mutant (Wojtas *et al.*, 1997). Furthermore, transgenic expression of GPDH-3 also affects the localization of two additional glycolytic enzymes. These results indicate that the IFM isoform of GPDH may have the added role of organizing glycolytic complexes in the myofibril.

A third strategy is the expression of novel proteins that are restricted to specific muscle types. Examples include the flight muscle-specific proteins FLN (Vigoreaux *et al.*, 1993), TnH (Bullard *et al.*, 1988), arthrin (Bullard *et al.*, 1985), and zeelin 2 (Ferguson *et al.*, 1994). Arthrin is found only in asynchronous IFM of most Diptera and Hemiptera but is not found in the IFM of Hymenoptera or Coleoptera (Peckham *et al.*, 1992). TnH is found in both synchronous and asynchronous flight muscle (Peckham *et al.*, 1992). The species distribution of zeelin 2 and FLN has not been examined. A distinct form of TnC is predominant in the IFM (Qiu *et al.*, 2003). The Z band protein Z(210) also shows a limited muscle distribution, found only in the large cells of the TDT and in the IFM (Vigoreaux *et al.*, 1991). The expression of MRP in tubular muscles (some DFM and leg muscles) but not in IFM very likely contributes to the distinct sarcomere assemblies and function of these two muscle types. MRP is randomly integrated along the thick filament, resulting in irregular patches of smooth and cross-bridge bearing thick filaments, and disordered thick and thin filament arrays (Standiford *et al.*, 1997). Another thick filament protein, MP20, is expressed in tubular muscles but not IFM (Ayme-Southgate *et al.*, 1989). The availability of mutants for some of these proteins should help to elucidate their roles in the developmental histories and functional properties of fibrillar and tubular muscles.

The two classes of insect flight muscle, synchronous and asynchronous, differ in many physiological properties including their frequency of contraction, mechanical power output, and efficiency of energy utilization (Josephson *et al.*, 2001). While many factors contribute to these differences, one of the primary determinants is the amount of space allocated to each of the three major structural units of the muscle cell: mitochondria, myofibrils, and membrane systems. In the asynchronous IFM, large rows of mitochondria lie alongside abundant myofibrils, while the sarcoplasmic reticulum is very scarce. The synchronous flight muscle has a much higher proportion of sarcoplasmic reticulum, myofibrils of smaller diameter, and fewer mitochondria (Josephson and Young, 1987; Josephson *et al.*, 2000). In the migratory locust *Locusta migratoria* mitochondria enlarge up to 30-fold during maturation of the flight muscle (Sogl *et al.*, 2000). The jump muscle, on the other hand, has few mitochondria (5% of muscle volume) but an extensive sarcoplasmic reticulum (20% of muscle volume) (Schouest *et al.*, 1986). Flight muscle is also characterized by abundant interfibrillar tracheoles. Studies on *Locusta* and other species have shown that tracheolization precedes genesis of mitochondria and coincides with the peak of lactate dehydrogenase activity (Beenakkers *et al.*, 1985). Anaerobic metabolism ceases once interfibrillar tracheoles are fully developed, and adult specific metabolic patterns take over.

It remains to be established how space allocation decisions are made during muscle development. Understanding the genetic control of these processes represents a significant challenge given the importance of cellular allocation in dictating the eventual functional capacity of the muscle.

2.2.4. Summary

The mechanism by which contractile proteins assemble into a sarcomeric lattice remains ill defined. Substantial headway has been made into understanding particulars but great challenges remain.

Cell culture and *in vitro* approaches have been instrumental in understanding myofibrillogenesis of vertebrate skeletal and cardiac muscle but their usefulness for insect systems has been limited. Instead, the combination of genetics and cell imaging techniques has proven instrumental for the understanding the sequence of events *in vivo*. In this respect, *D. melanogaster* has been and will continue to be a valuable model system for the study of myofibrillogenesis.

References

An, H., Mogami, K., **1996**. Isolation of 88F actin mutants of *Drosophila melanogaster* and possible alterations in the mutant actin structures. *J. Mol. Biol. 260*, 492–505.

Anant, S., Roy, S., VijayRaghavan, K., **1998**. Twist and Notch negatively regulate adult muscle differentiation in *Drosophila*. *Development 125*, 1361–1369.

Arbas, E.A., Tolbert, L.P., **1986**. Presynaptic terminals persist following degeneration of "flight" muscle during development of a flightless grasshopper. *J. Neurobiol. 17*, 627–636.

Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., *et al.*, **2002**. Gene expression during the life cycle of *Drosophila melanogaster. Science 297*, 2270–2275.

Arredondo, J.J., Marco Ferreres, R., Maroto, M., Cripps, R.M., Marco, R., *et al.*, **2001a**. Control of *Drosophila* paramyosin/miniparamyosin gene expression: differential regulatory mechanisms for muscle-specific transcription. *J. Biol. Chem. 276*, 8278–8287.

Arredondo, J.J., Mardahl-Dumesnil, M., Cripps, R.M., Cervera, M., Bernstein, S.I., **2001b**. Overexpression of miniparamyosin causes muscle dysfunction and age-dependant myofibril degeneration in the indirect flight muscles of *Drosophila melanogaster. J. Muscle Res. Cell Motil. 22*, 287–299.

Auber, J., **1969**. La myofibrillogenèse du muscle strié. 1. Insectes. *J. Microsc. (Paris) 8*, 197–233.

Ayer, G., Vigoreaux, J.O., **2003**. Flightin is a myosin rod binding protein. *Cell Biochem. Biophys. 38*, 41–54.

Ayme-Southgate, A., Lasko, P., French, C., Pardue, M.L., **1989**. Characterization of the gene for mp20: a *Drosophila* muscle protein that is not found in asynchronous oscillatory flight muscle. *J. Cell Biol. 108*, 521–531.

Ayme-Southgate, A., Southgate, R., Saide, J., Benian, G.M., Pardue, M.L., **1995**. Both synchronous and asynchronous muscle isoforms of projectin (the *Drosophila* bent locus product) contain functional kinase domain. *J. Cell Biol. 128*, 393–403.

Ball, E.E., Goodman, C.S., **1985**. Muscle development in the grasshopper embryo. 1. Muscles, nerves, and abdomens in the metathoracic leg. *Devel. Biol. 111*, 383–398.

Ball, E.E., Karlik, C.C., Beall, C.J., Saville, D.L., Sparrow, J.C., *et al.*, **1987**. Arthrin, a myofibrillar protein of insect flight muscle, is an actin-ubiquitin conjugate. *Cell 51*, 221–228.

Barnes, P.T., Laurie-Ahlberg, C.C., **1986**. Genetic variability of flight metabolism in *Drosophila melanogaster.* 3. Effects of Gpdh allozymes and environmental temperature on power output. *Genetics 112*, 267–294.

Barton, B.E., Ayer, G., Cajigas, I.J., Maughan, D., Vigoreaux, J.O., **2002**. Defects in flight muscle ultrastructure and function in transgenic *Drosophila* with mutations of phosphorylation sites in flightin. *Mol. Biol. Cell 13S*, 319a.

Basi, G.S., Boardman, M., Storti, R.V., **1984**. Alternative splicing of a *Drosophila* tropomyosin gene generates muscle tropomyosin isoforms with different carboxy-terminal ends. *Mol. Cell Biol. 4*, 2828–2836.

Basi, G.S., Storti, R.V., **1986**. Structure and DNA sequence of the tropomyosin I gene from *Drosophila melanogaster. J. Biol. Chem. 261*, 817–827.

Basler, W., **1969**. Untersuchung der Nervenwirkung bei *Antheraea pernyi* Guer. (Lep.) unter besonderer Berucksichtigung der dorsolongitudinalen Flugmuskeln. *Rev. Suisse Zool. 76*, 297–362.

Bate, M., Rushton, E., Currie, D., **1991**. Cells with persistent *twist* expression are the embryonic precursors of adult muscles in *Drosophila*. *Development 113*, 79–89.

Baylies, M.K., Bate, M., **1996**. *twist*: a myogenic switch in *Drosophila*. *Science 272*, 1481–1484.

Baylies, M.K., Bate, M., Ruiz Gomez, M., **1998**. Myogenesis: a view from *Drosophila*. *Cell 93*, 921–927.

Bayline, R.J., Duch, C., Levine, R.B., **2001**. Nerve–muscle interactions regulate motor terminal growth and myoblast distribution during muscle development. *Devel. Biol. 231*, 348–363.

Beall, C.J., Fyrberg, E., **1991**. Muscle abnormalities in *Drosophila melanogaster heldup* mutants are caused by missing or aberrant troponin-I isoforms. *J. Cell Biol. 114*, 941–951.

Beall, C.J., Sepanski, M.A., Fyrberg, E.A., **1989**. Genetic dissection of *Drosophila* myofibril formation: effects of actin and myosin heavy chain null alleles. *Genes Devel. 3*, 131–140.

Becker, K.D., Bernstein, S.I., **1994**. Genetic and transgenic approaches to dissecting muscle development and contractility using the *Drosophila* model system. *Trends Cardiovasc. Med. 4*, 243–250.

Becker, K.D., O'Donnell, P.T., Heitz, J.M., Vito, M., Bernstein, S.I., **1992**. Analysis of *Drosophila* paramyosin: identification of a novel isoform which is restricted to a subset of adult muscles. *J. Cell Biol. 116*, 669–681.

Beenakkers, A.M.T., Van der Horst, D.J., Van Marrewijk, W.J.A., **1985**. Biochemical processes directed to flight muscle metabolism. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 451–486.

Beinbrech, G., Meller, U., Sasse, W., **1985**. Paramyosin content and thick filament structure in insect muscles. *Cell Tissue Res. 241*, 607–614.

Benoist, P., Mas, J.A., Marco, R., Cervera, M., **1998**. Differential muscle-type expression of the *Drosophila* troponin T gene: a 3-base pair microexon is involved in visceral and adult hypodermic muscle specification. *J. Biol. Chem. 273*, 7538–7546.

Bernstein, S.I., Milligan, R.A., **1997**. Fine tuning a molecular motor: the location of alternative domains in the *Drosophila* myosin head. *J. Mol. Biol. 271*, 1–6.

Bernstein, S.I., O'Donnell, P.T., Cripps, R.M., **1993**. Molecular genetic analysis of muscle development, structure and function in *Drosophila*. *Int. Rev. Cytol. 143*, 63–152.

Bienz-Isler, G., **1968**. Elektronenmikroskopische Untersuchungen über die imaginable Strucker der dorsolongitudinalen Flugmuskeln von *Antheraea pernyi* Guer. (Lepidoptera). *Acta Anat. 70*, 416–433.

Bing, W., Razzaq, A., Sparrow, J., Marston, S., **1998**. Tropomyosin and troponin regulation of wild type and E93K mutant actin filaments from *Drosophila* flight muscle. *J. Biol. Chem. 273*, 15016–15021.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., *et al.*, **1995**. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes Devel. 9*, 730–741.

Brault, V., Reedy, M.C., Sauder, U., Kammerer, R.A., Aebi, U., *et al.*, **1999**. Substitution of flight muscle-specific actin by human (beta)-cytoplasmic actin in the indirect flight muscle of *Drosophila*. *J. Cell Sci. 112*, 3627–3639.

Broadie, K., Bate, M., **1993**. Muscle development is independent of innervation during *Drosophila* embryogenesis. *Development 119*, 533–543.

Bullard, B., Bell, J., Craig, R., Leonard, K., **1985**. Arthrin: a new actin-like protein in insect flight muscle. *J. Mol. Biol. 182*, 443–454.

Bullard, B., Leonard, K., Larkins, A., Butcher, G., Karlik, C., *et al.*, **1988**. Troponin of asynchronous flight muscle. *J. Mol. Biol. 204*, 621–637.

Bursell, E., **1973**. Development of mitochondrial and contractile components of the flight muscle in adult tsetse flies, *Glossina morsitans*. *J. Insect Physiol. 19*, 1079–1086.

Caplan, A.I., Fiszman, M.Y., Eppenberger, H.M., **1983**. Molecular and cell isoforms during development. *Science 221*, 921–927.

Champagne, M.B., Edwards, K.A., Erickson, H.P., Kiehart, D.P., **2000**. *Drosophila* stretchin-MLCK is a novel member of the titin/myosin light chain kinase family. *J. Mol. Biol. 300*, 759–777.

Chun, M., Falkenthal, S., **1988**. Ifm(2)2 is a myosin heavy chain allele that disrupts myofibrillar assembly only in the indirect flight muscle of *Drosophila melanogaster*. *J. Cell Biol. 107*, 2613–2621.

Cifuentes-Diaz, C., **1989**. Mode of formation of the flight muscles in a butterfly *Pieris brassicae*. *Tissue Cell 21*, 875–889.

Clayton, J.D., Cripps, R.M., Sparrow, J.C., Bullard, B., **1998**. Interaction of troponin-H and glutathione *S*-transferase-2 in the indirect flight muscles of *Drosophila melanogaster*. *J. Muscle Res. Cell Motil. 19*, 117–127.

Collier, V.L., Kronert, W.A., O'Donnell, P.T., Edwards, K.A., Bernstein, S.I., **1990**. Alternative myosin hinge regions are utilized in a tissue-specific fashion that correlates with muscle contraction speed. *Genes Devel. 4*, 885–895.

Consoulas, C., Duch, C., Bayline, R.J., Levine, R.B., **2000**. Behavioral transformations during metamorphosis: remodeling of neural and motor systems. *Brain Res. Bull. 53*, 571–583.

Consoulas, C., Levine, R.B., **1997**. Accumulation and proliferation of adult leg muscle precursors in *Manduca* are dependent on innervation. *J. Neurobiol. 32*, 531–553.

Courchesne-Smith, C.L., Tobin, S.L., **1989**. Tissue-specific expression of the 79B actin gene during *Drosophila* development. *Devel. Biol. 133*, 313–321.

Cripps, R.M., Becker, K.D., Mardahl, M., Kronert, W.A., Hodges, D., *et al.*, **1994**. Transformation of *Drosophila melanogaster* with the wild-type myosin heavy-chain gene: rescue of mutant phenotypes and analysis of defects caused by overexpression. *J. Cell Biol. 126*, 689–699.

Cripps, R.M., Black, B.L., Zhao, B., Lien, C.L., Schulz, R.A., *et al.*, **1998**. The myogenic regulatory gene Mef2 is a direct target for transcriptional activation by Twist during *Drosophila* myogenesis. *Genes Devel. 12*, 422–434.

Cripps, R.M., Olson, E.N., **1998**. Twist is required for muscle template splitting during adult *Drosophila* myogenesis. *Devel. Biol. 203*, 106–115.

Cripps, R.M., Suggs, J.A., Bernstein, S.I., **1999**. Assembly of thick filaments and myofibrils occurs in the absence of the myosin head. *EMBO J. 18*, 1793–1804.

Crossley, A.C., **1972**. Ultrastructural changes during transition of larval to adult intersegmental muscle at metamorphosisin the blowfly *Calliphora erythrocephala*. 2. The formation of adult muscle. *J. Embryol. Exp. Morphol. 27*, 75–101.

Crossley, A.C., **1978**. The morphology and development of the *Drosophila* muscular system. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*. Academic Press, London, pp. 499–560.

Cullen, M.J., **1974**. The distribution of asynchronous muscle in insects with special reference to the Hemiptera: an electron microscope study. *J. Entomol. 49A*, 17–41.

Daley, J., Southgate, R., Ayme-Southgate, A., **1998**. Structure of the *Drosophila* projectin protein: isoforms and implication for projectin filament assembly. *J. Mol. Biol. 279*, 201–210.

Deak, I.I., **1977a**. A histochemical study of the muscles of *Drosophila melanogaster*. *J. Morphol. 153*, 307–316.

Deak, I.I., **1977b**. Mutations of *Drosophila melanogaster* that affect muscles. *J. Embryol. Exp. Morphol. 40*, 35–63.

Deak, I.I., **1978**. Thoracic duplications in the mutant *wingless* of *Drosophila* and their effect on muscles and nerves. *Devel. Biol. 66*, 422–441.

Deak, I.I., Bellamy, P.R., Bienz, M., Dubuis, Y., Fenner, E., *et al.*, **1982**. Mutations affecting the indirect flight muscles of *Drosophila melanogaster. J. Embryol. Exp. Morphol. 69*, 61–81.

de la Pompa, J.L., Garcia, J.R., Ferrus, A., **1989**. Genetic analysis of muscle development in *Drosophila melanogaster. Devel. Biol. 131*, 439–454.

DeSimone, S., Coelho, C., Roy, S., VijayRaghavan, K., White, K., **1996**. ERECT WING, the *Drosophila* member of a family of DNA binding proteins is required in imaginal myoblasts for flight muscle development. *Development 122*, 31–39.

DeSimone, S.M., White, K., **1993**. The *Drosophila erect wing* gene, which is important for both neuronal and muscle development, encodes a protein which is similar to the sea urchin P3A2 DNA binding protein. *Mol. Cell Biol. 13*, 3641–3649.

Dickinson, M.H., Hyatt, C.J., Lehmann, F.-O., Moore, J.R., Reedy, M.C., *et al.*, **1997**. Phosphorylation-dependent power output of transgenic flies: an integrated study. *Biophys. J. 73*, 3122–3134.

Domingo, A., Gonzalez-Jurado, J., Maroto, M., Diaz, C., Vinos, J., *et al.*, **1998**. Troponin-T is a calcium-binding protein in insect muscle: *in vivo* phosphorylation, muscle-specific isoforms and developmental profile in *Drosophila melanogaster. J. Muscle Res. Cell Motil. 19*, 393–403.

Drummond, D.R., Hennessey, E.S., Sparrow, J.C., **1991**. Characterisation of missense mutations in the *Act88F* gene of *Drosophila melanogaster. Mol. Gen. Genet. 226*, 70–80.

Duch, C., Bayline, R.J., Levine, R.B., **2000**. Postembryonic development of the dorsal longitudinal flight muscle and its innervation in *Manduca sexta*. *J. Comp. Neurol. 422*, 1–17.

Dudley, R., **2000**. The biomechanics of insect flight. Princeton University Press, Princeton, NJ.

Eigenmann, R., **1965**. Untersuchungen über die Entwicklung der dorsolongitudinalen Flugmuskeln von *Antheraea pernyi* Guer. (Lepidoptera). *Rev. Suisse Zool. 72*, 789–840.

Fahrmann, M., Fonk, I., Beinbrech, G., **2002**. The kinase activity of the giant protein projectin of the flight muscle of *Locusta migratoria*. *Insect Biochem. Mol. Biol. 32*, 1401–1407.

Falkenthal, S., Graham, M., Wilkinson, J., **1987**. The indirect flight muscle of *Drosophila* accumulates a unique myosin alkali light chain isoform. *Devel. Biol. 121*, 263–272.

Falkenthal, S., Parker, V.P., Mattox, W.W., Davidson, N., **1984**. *Drosophila melanogaster* has only one myosin alkali light-chain gene which encodes a protein with considerable amino acid sequence homology to chicken myosin alkali light chains. *Mol. Cell Biol. 4*, 956–965.

Farrell, E.R., Fernandes, J., Keshishian, H., **1996**. Muscle organisers in *Drosophila*: the role of persistent larval fibres in adult flight muscle development. *Devel. Biol. 176*, 220–229.

Ferguson, C., Lakey, A., Hutchings, A., Butcher, G.W., Leonard, K.R., *et al.*, **1994**. Cytoskeletal proteins of insect muscle: location of zeelins in *Lethocerus* flight and leg muscle. *J. Cell Sci. 107*, 1115–1129.

Fernandes, J., Bate, M., VijayRaghavan, K., **1991**. Development of the indirect flight muscles of *Drosophila*. *Development 113*, 67–77.

Fernandes, J., Celniker, S.E., Lewis, E.B., VijayRaghavan, K., **1994**. Muscle development in the four-winged *Drosophila* and the role of the *Ultrabithorax* gene. *Curr. Biol. 4*, 957–964.

Fernandes, J.J., Celniker, S.E., VijayRaghavan, K., **1996**. Development of the indirect flight muscle attachment sites in *Drosophila*: role of the PS integrins and the stripe gene. *Devel. Biol. 176*, 166–184.

Fernandes, J.J., Keshishian, H., **1996**. Patterning the dorsal longitudinal flight muscles (DLM) of *Drosophila*: insights from the ablation of larval scaffolds. *Development 122*, 3755–3763.

Fernandes, J.J., Keshishian, H., **1998**. Nerve–muscle interactions during flight muscle development in *Drosophila*. *Development 125*, 1769–1779.

Fitzhugh, G., Marden, J., **1997**. Maturational changes in troponin T expression, Ca^{2+}-sensitivity and twitch contraction kinetics in dragonfly flight muscle. *J. Exp. Biol. 200*, 1473–1482.

Frasch, M., **1999**. Controls in patterning and diversification of somatic muscles during *Drosophila* embryogenesis. *Curr. Opin. Genet. Devel. 9*, 522–529.

Fyrberg, C.C., Labeit, S., Bullard, B., Leonard, K., Fyrberg, E., **1992**. *Drosophila* projectin: relatedness to titin and twitchin and correlation with lethal (4) 102 CDa and bent-dominant mutants. *Proc. Roy. Soc. London B 249*, 33–40.

Fyrberg, E., Beall, C., **1990**. Genetic approaches to myofibril form and function in *Drosophila*. *Trends Genet. 6*, 126–131.

Fyrberg, E., Fyrberg, C.C., Beall, C., Saville, D.L., **1990a**. *Drosophila melanogaster* troponin-T mutations engender three distinct syndromes of myofibrillar abnormalities. *J. Mol. Biol. 216*, 657–675.

Fyrberg, E.A., Fyrberg, C.C., Biggs, J.R., Saville, D., Beall, C.J., *et al.*, **1998**. Functional nonequivalence of *Drosophila* actin isoforms. *Biochem. Genet. 36*, 271–287.

Fyrberg, E., Kelly, M., Ball, E., Fyrberg, C., Reedy, M.C., **1990b**. Molecular genetics of *Drosophila* alpha-actinin: mutant alleles disrupt Z disc integrity and muscle insertions. *J. Cell Biol. 110*, 1999–2011.

Fyrberg, E.A., Mahaffey, J.W., Bond, B.J., Davidson, N., **1983**. Transcripts of the six *Drosophila* actin genes accumulate in a stage- and tissue-specific manner. *Cell 33*, 115–123.

Fyrberg, C., Parker, H., Hutchison, B., Fyrberg, E., **1994**. *Drosophila melanogaster* genes encoding three troponin-C isoforms and a calmodulin-related protein. *Biochem. Genet. 32*, 119–135.

Gajewski, K., Wang, J., Molkentin, J.D., Chen, E.H., Olson, E.N., *et al.*, **2003**. Requirement of the

calcineurin subunit gene canB2 for indirect flight muscle formation in *Drosophila*. *Proc. Natl Acad. Sci. USA 100*, 1040–1045.
Gautel, M., Leonard, K., Labeit, S., **1993**. Phosphorylation of KSP motifs in the C-terminal region of titin in differentiating myoblasts. *EMBO J. 12*, 3827–3834.
Geyer, P.K., Fyrberg, E.A., **1986**. 5′-flanking sequence required for regulated expression of a muscle-specific *Drosophila melanogaster* actin gene. *Mol. Cell Biol. 6*, 3388–3396.
Ghazi, A., Anant, S., VijayRaghavan, K., **2000**. Apterous mediates development of direct flight muscles autonomously and indirect flight muscles through epidermal cues. *Development 127*, 5309–5318.
Glicksman, M.A., Brower, D.L., **1988a**. Expression of the *Sex combs reduced* protein in *Drosophila* larvae. *Devel. Biol. 127*, 113–118.
Glicksman, M.A., Brower, D.L., **1988b**. Misregulation of homeotic gene expression in *Drosophila* larvae resulting from mutations at the *extra sex combs* locus. *Devel. Biol. 126*, 219–227.
Gregorio, C.C., Granzier, H., Sorimachi, H., Labeit, S., **1999**. Muscle assembly: a titanic achievement? *Curr. Opin. Cell Biol. 11*, 18–25.
Gregory, D.W., Lennie, R.W., Birt, L.M., **1968**. An electron-microscopic study of flight muscle development in the blowfly *Lucilia cuprina*. *J. Roy. Microsc. Soc. 88*, 151–175.
Hakeda, S., Endo, S., Saigo, K., **2000**. Requirements of Kettin, a giant muscle protein highly conserved in overall structure in evolution, for normal muscle function, viability, and flight activity of *Drosophila*. *J. Cell Biol. 148*, 101–114.
Hanke, P.D., Storti, R.V., **1988**. The *Drosophila melanogaster* tropomyosin II gene produces multiple proteins by use of alternative tissue-specific promoters and alternative splicing. *Mol. Cell Biol. 8*, 3591–3602.
Hegstrom, C.D., Riddiford, L.M., Truman, J.W., **1998**. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth, *Manduca sexta*. *J. Neurosci. 18*, 1786–1794.
Hegstrom, C.D., Truman, J.W., **1996**. Steroid control of muscle remodeling during metamorphosis in *Manduca sexta*. *J. Neurobiol. 29*, 535–550.
Heinertz, R., **1976**. Untersuchungen and thorakalen Nervensystem von *Antheraea polyphemus* Guer. (Lepidoptera) unter besonderer Berucksichtigung der Metamorphose. *Rev. Suisse Zool. 83*, 215–242.
Hess, N., Kronert, W.A., Bernstein, S.I., **1989**. Transcriptional and post-transcriptional regulation of *Drosophila* myosin heavy chain gene expression. In: Kedes, L., Stockdale, F. (Eds.), Cellular and Molecular Biology of Muscle Development. A.R. Liss, New York, pp. 621–631.
Hinton, H.E., **1959**. How the indirect flight muscles of the insects grow. *Sci. Progr. 186*, 321–333.
Hiromi, Y., Hotta, Y., **1985**. Actin gene mutations in *Drosophila*: heat shock activation in the indirect flight muscles. *EMBO J. 4*, 1681–1687.
Hiromi, Y., Okamoto, H., Gehring, W.J., Hotta, Y., **1986**. Germline transformation with *Drosophila* mutant actin genes induces constitutive expression of heat shock genes. *Cell 44*, 293–301.
Ho, R.K., Ball, E.E., Goodman, C.S., **1983**. Muscle pioneers: large mesodermal cells that erect a scaffold for developing muscles and motoneurones in grasshopper embryos. *Nature 301*, 66–69.
Homyk, T., Emerson, C.P., **1988**. Functional interactions between unlinked muscle genes within haploinsufficient regions of the *Drosophila* genome. *Genetics 119*, 105–121.
Horn, C., Offen, N., Nystedt, S., Hacker, U., Wimmer, E.A., **2003**. *piggyBac*-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. *Genetics 163*, 647–661.
Houlihan, D.F., Breckenridge, L., **1981**. Stretch-induced growth of blowfly muscle. *J. Insect Physiol. 27*, 521–525.
Houlihan, D.F., Newton, J.R.L., **1979**. Sarcomere formation and longitudinal growth in the developing flight muscles of *Calliphora*. *J. Insect Physiol. 25*, 879–893.
Hufnagel, A., **1918**. Recherches histologiques sur la métamorphose d'un Lépidoptère (*Hyponomeuta padella L.*). *Arch. Zool. Exp. 57*, 47–202.
Josephson, R.K., Malamud, J.G., Stokes, D.R., **2000**. Asynchronous muscle: a primer. *J. Exp. Biol. 203*, 2713–2722.
Josephson, R.K., Malamud, J.G., Stokes, D.R., **2001**. The efficiency of an asynchronous flight muscle from a beetle. *J. Exp. Biol. 204*, 4125–4139.
Josephson, R.K., Young, D., **1987**. Fiber ultrastructure and contraction kinetics in insect fast muscles. *Am. Zool. 27*, 991–1000.
Karlik, C.C., Fyrberg, E.A., **1985**. An insertion within a variably spliced *Drosophila* tropomyosin gene blocks accumulation of only one encoded isoform. *Cell 41*, 57–66.
Karlik, C.C., Fyrberg, E.A., **1986**. Two *Drosophila melanogaster* tropomyosin genes: structural and functional aspects. *Mol. Cell Biol. 6*, 1965–1973.
Kelsh, R., Weinzierl, R.O., White, R.A., Akam, M., **1994**. Homeotic gene expression in the locust *Schistocerca*: an antibody that detects conserved epitopes in Ultrabithorax and abdominal-A proteins. *Devel. Genet. 15*, 19–31.
Kent, K.S., Levine, R.B., **1988**. Neural control of leg movements in a metamorphic insect: sensory and motor elements of the larval thoracic legs in *Manduca sexta*. *J. Comp. Neurol. 271*, 559–576.
Kimura, K.I., Truman, J.W., **1990**. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster*. *J. Neurosci. 12*, 403–411.
Koana, T., Hotta, Y., **1978**. Isolation and characterization of flightless mutants in *Drosophila melanogaster*. *J. Embryol. Exp. Morphol. 45*, 123–143.
Kolmerer, B., Clayton, J., Benes, V., Allen, T., Ferguson, C., *et al.*, **2000**. Sequence and expression of the kettin

gene in *Drosophila melanogaster* and *Caenorhabditis elegans*. *J. Mol. Biol. 296*, 435–448.

Kozopas, K.M., Nusse, R., **2002**. Direct flight muscles in *Drosophila* develop from cells with characteristics of founders and depend on DWnt-2 for their correct patterning. *Devel. Biol. 243*, 312–325.

Kreuz, A.J., Simcox, A., Maughan, D., **1996**. Alterations in flight muscle ultrastructure and function in *Drosophila* tropomyosin mutants. *J. Cell Biol. 135*, 673–687.

Kronert, W.A., Acebes, A., Ferrus, A., Bernstein, S.I., **1999**. Specific myosin heavy chains mutations suppress troponin I defects in *Drosophila* muscles. *J. Cell Biol. 144*, 989–1000.

Kronert, W.A., Edwards, K.A., Roche, E.S., Wells, L., Bernstein, S.I., **1991**. Muscle-specific accumulation of *Drosophila* myosin heavy chains: a splicing mutation in an alternative exon results in an isoform substitution. *EMBO J. 10*, 2479–2488.

Kronert, W.A., O'Donnell, P.T., Bernstein, S.I., **1994**. A charge change in an evolutionarily-conserved region of the myosin globular head prevents myosin and thick filament accumulation in *Drosophila*. *J. Mol. Biol. 236*, 697–702.

Kronert, W.A., O'Donnell, P.T., Fieck, A., Lawn, A., Vigoreaux, J.O., *et al.*, **1995**. Defects in the *Drosophila* myosin rod permit sarcomere assembly but cause flight muscle degeneration. *J. Mol. Biol. 249*, 111–125.

Kulke, M., Neagoe, C., Kolmerer, B., Minajeva, A., Hinssen, H., *et al.*, **2001**. Kettin, a major source of myofibrillar stiffness in *Drosophila* indirect flight muscle. *J. Cell Biol. 154*, 1045–1057.

Lakey, A., Ferguson, C., Labeit, S., Reedy, M., Larkins, A., *et al.*, **1990**. Identification and localization of high molecular weight proteins in insect flight and leg muscle. *EMBO J. 9*, 3459–3467.

Lawrence, P.A., **1982**. Cell lineage of the thoracic muscles of *Drosophila*. *Cell 29*, 493–503.

Lebart-Pedebas, M.C., **1990**. Origin and development of the dorso-ventral flight muscles in Chironomus (Diptera; Nematocera). *Tissue and Cell 22*, 199–211.

Lehmann, K., **1984**. The developmental profile of paramyosin and other myofibrillar proteins of larval and imaginal thoracic muscles of Lepidoptera. *J. Insect Physiol. 30*, 217–223.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., *et al.*, **1995**. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science 267*, 688–693.

Lin, M.H., Bour, B.A., Abmayr, S.M., Storti, R.V., **1997**. Ectopic expression of MEF2 in the epidermis induces epidermal expression of muscle genes and abnormal muscle development in *Drosophila*. *Devel. Biol. 182*, 240–255.

Lin, S.C., Storti, R.V., **1997**. Developmental regulation of the *Drosophila* Tropomyosin I (TmI) gene is controlled by a muscle activator enhancer region that contains multiple *cis*-elements and binding sites for multiple proteins. *Devel. Genet. 20*, 297–306.

Lindstedt, S.L., McGlothlin, T., Percy, E., Pifer, J., **1998**. Task-specific design of skeletal muscle: balancing muscle structural composition. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 120*, 35–40.

Lo, P.C., Frasch, M., **1997**. A novel KH-domain protein mediates cell adhesion processes in *Drosophila*. *Devel. Biol. 190*, 241–256.

Lockshin, R.A., Williams, C.M., **1964**. Programmed cell death. 2. Endocrine potentiation of the breakdown of the intersegmental muscles of silkworms. *J. Insect Physiol. 10*, 643–649.

Lovato, T.L., Meadows, S.M., Baker, P.W., Sparrow, J.C., Cripps, R.M., **2001**. Characterization of muscle actin genes in *Drosophila virilis* reveals significant molecular complexity in skeletal muscle types. *Insect Mol. Biol. 10*, 333–340.

Lowey, S., Waller, G.S., Trybus, K.M., **1993**. Function of skeletal muscle myosin heavy and light chain isoforms by an *in vitro* motility assay. *J. Biol. Chem. 268*, 20414–20418.

Luedeman, R., Levine, R.B., **1996**. Neurons and ecdysteroids promote the proliferation of myogenic cells cultured from the developing adult legs of *Manduca sexta*. *Devel. Biol. 173*, 51–68.

Machado, C., Andrew, D.J., **2000**. D-titin: a giant protein with dual roles in chromosomes and muscles. *J. Cell Biol. 151*, 639–652.

Machado, C., Sunkel, C.E., Andrew, D.J., **1998**. Human autoantibodies reveal titin as a chromosomal protein. *J. Cell Biol. 141*, 321–334.

Mardahl-Dumesnil, M., Fowler, V.M., **2001**. Thin filaments elongate from their pointed ends during myofibril assembly in *Drosophila* indirect flight muscle. *J. Cell Biol. 155*, 1043–1053.

Marden, J.H., **2000**. Variability in the size, composition, and function of insect flight muscles. *Annu. Rev. Physiol. 62*, 157–178.

Marden, J.H., Fitzhugh, G.H., Girgenrath, M., Wolf, M.R., Girgenrath, S., **2001**. Alternative splicing, muscle contraction and intraspecific variation: associations between troponin T transcripts, Ca(2+) sensitivity and the force and power output of dragonfly flight muscles during oscillatory contraction. *J. Exp. Biol. 204*, 3457–3470.

Maroto, M., Arredondo, J., Goulding, D., Marco, R., Bullard, B., *et al.*, **1996**. *Drosophila* paramyosin/miniparamyosin gene products show a large diversity in quantity, localization, and isoform pattern: a possible role in muscle maturation and function. *J. Cell Biol. 134*, 81–92.

Maroto, M., Arredondo, J.J., San Roman, M., Marco, R., Cervera, M., **1995**. Analysis of the paramyosin/miniparamyosin gene: miniparamyosin is an independently transcribed, distinct paramyosin isoform, widely distributed in invertebrates. *J. Biol. Chem. 270*, 4375–4382.

Maroto, M., Vinos, J., Marco, R., Cervera, M., **1992**. Autophosphorylating protein kinase activity in titin-like arthropod projectin. *J. Mol. Biol. 224*, 287–291.

Mayans, O., van der Ven, P.F., Wilm, M., Mues, A., Young, P., *et al.*, **1998**. Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature 395*, 863–869.

Menke, S.D., Peterson, N.S., **1989**. Protein Synthesis during flight muscle development in normal and WupB indirect flight muscles of *Drosophila melanogaster. Insect Biochem. 19*, 723–729.

Meredith, J., Storti, R.V., **1993**. Developmental regulation of the *Drosophila* tropomyosin II gene in different muscles is controlled by muscle-type-specific intron enhancer elements and distal and proximal promoter control elements. *Devel. Biol. 159*, 500–512.

Miles, C.I., Booker, R., **1993**. *Octopod*, a homeotic mutation of the moth *Manduca sexta*, affects of development of both mesodermal and ectodermal structures. *Devel. Biol. 155*, 147–160.

Miller, A., **1950**. The internal anatomy and histology of the imago of *Drosophila melanogaster.* In: Demerec, M. (Ed.), Biology of *Drosophila*. Wiley, New York, pp. 420–534.

Miller, R.C., Schaaf, R., Maughan, D.W., Tansey, T.R., **1993**. A non-flight muscle isoform of *Drosophila* tropomyosin rescues an indirect flight muscle tropomyosin mutant. *J. Muscle Res. Cell Motil. 14*, 85–98.

Mogami, K., Hotta, Y., **1981**. Isolation of *Drosophila* flightless mutants which affect myofibrillar proteins of indirect flight muscle. *Mol. Gen. Genet. 183*, 409–417.

Mogami, K., O'Donnell, P.T., Bernstein, S.I., Wright, T.R., Emerson, C.P., Jr., **1986**. Mutations of the *Drosophila* myosin heavy-chain gene: effects on transcription, myosin accumulation, and muscle function. *Proc. Natl Acad. Sci. USA 83*, 1393–1397.

Moore, J.R., Vigoreaux, J.O., Maughan, D.W., **1999**. The *Drosophila* projectin mutant, *bentD*, has reduced stretch activation and altered indirect flight muscle kinetics. *J. Muscle Res. Cell Motil. 20*, 797–806.

Naimi, B., Harrison, A., Cummins, M., Nongthomba, U., Clark, S., *et al.*, **2001**. A tropomyosin-2 mutation suppresses a Troponin I myopathy in *Drosophila*. *Mol. Biol. Cell 12*, 1529–1539.

Nongthomba, U., Cummins, M., Clark, S., Vigoreaux, J.O., Sparrow, J.C., **2003**. Suppression of the muscle hypercontraction phenotype by mutations in the myosin heavy chain gene of *Drosophila melanogaster. Genetics 164*, 209–222.

Nongthomba, U., Pasalodos-Sanchez, S., Clark, S., Clayton, J.D., Sparrow, J.C., **2001**. Expression and function of the *Drosophila* ACT88F actin isoform is not restricted to the indirect flight muscles. *J. Muscle Res. Cell Motil. 22*, 111–119.

Nongthomba, U., Ramachandra, N.B., **1999**. A direct screen identifies new flight muscle mutants on the *Drosophila* second chromosome. *Genetics 153*, 261–274.

Novicki, A., **1989**. Rapid postembryonic development of a cricket flight muscle. *J. Exp. Biol. 250*, 253–272.

Nuesch, H., **1955**. Segmentierung und Muskelinnervation bei *Telea polyphemus* (Lep.). *Rev. Suisse Zool. 62*, 211–218.

Nuesch, H., **1968**. The role of the nervous system in insect morphogenesis and regeneration. *Annu. Rev. Entomol. 13*, 27–44.

Nuesch, H., **1985**. Control of muscle development. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 2. Pergamon Press, Oxford, pp. 425–452.

Nuesch, H., Bienz-Isler, G., **1972**. Die Entwicklung denervierter Imaginalmuskeln bei *Antheraea polyphemus* (Lep.) und die Frage der entwicklungsfordernden Nervenwirkung. *Zool. Jb. Anat. 89*, 333–355.

O'Donnell, P.T., Collier, V.L., Mogami, K., Bernstein, S.I., **1989**. Ultrastructural and molecular analyses of homozygous-viable *Drosophila melanogaster* muscle mutants indicate there is a complex pattern of myosin heavy-chain isoform distribution. *Genes Devel. 3*, 1233–1246.

Patel, S.R., Saide, J.D., **2003**. A(225), an A-band associated protein of *Drosophila* IFM, has a myosin dependent and a myosin independent isoform. *Biophys. J. 84*, 564a.

Peckham, M., Cripps, R., White, D., Bullard, B., **1992**. Mechanics and protein content of insect flight muscles. *J. Exp. Biol. 168*, 57–76.

Peristianis, G.C., Gregory, D.W., **1971**. Early stages of flight muscle development in the blowfly *Lucilia cuprina*: a light and electron microscope study. *J. Insect Physiol. 17*, 1005–1022.

Prado, A., Canal, I., Barbas, J.A., Molloy, J., Ferrus, A., **1995**. Functional recovery of troponin I in a *Drosophila heldup* mutant after a second site mutation. *Mol. Biol. Cell 6*, 1433–1441.

Pringle, J.W.S., **1977**. The mechanical characteristics of insect fibrillar muscle. In: Tregear, R.T. (Ed.), Insect Flight Muscle. North Holland, Amsterdam, pp. 177–196.

Qiu, F., Lakey, A., Agianian, B., Hutchings, A., Butcher, G.W., *et al.*, **2003**. Troponin C in different insect muscle types: identification of an isoform in *Lethocerus*, *Drosophila* and *Anopheles* that is specific to asynchronous flight muscle in the adult insect. *Biochem. J. 371*, 811–821.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J.D., Olson, E.N., *et al.*, **1995**. A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in *Drosophila*. *Devel. Biol. 171*, 169–181.

Razzaq, A., Schmitz, S., Veigel, C., Molloy, J.E., Geeves, M.A., *et al.*, **1999**. Actin residue glu (93) is identified as an amino acid affecting myosin binding. *J. Biol. Chem. 274*, 28321–28328.

Ready, N.E., Josephson, R.K., **1982**. Flight muscle development in a hemimetabolous insect. *J. Exp. Zool. 220*, 49–56.

Reedy, M.C., Beall, C., **1993a**. Ultrastructure of developing flight muscle in *Drosophila*. 1. Assembly of myofibrils. *Devel. Biol. 160*, 443–465.

Reedy, M.C., Beall, C., **1993b**. Ultrastructure of developing flight muscle in *Drosophila*. 2. Formation of the myotendon junction. *Devel. Biol. 160*, 466–479.

Reedy, M.C., Beall, C., Fyrberg, E., **1989**. Formation of reverse rigor chevrons by myosin heads. *Nature 339*, 481–483.

Reedy, M.C., Bullard, B., Vigoreaux, J.O., **2000**. Flightin is essential for thick filament assembly and sarcomere stability in *Drosophila* flight muscles. *J. Cell Biol. 151*, 1483–1499.

Restifo, L.L., White, K., **1992**. Mutations in a steroid hormone regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle, and gut. *Roux's Arch. Devel. Biol. 201*, 221–234.

Rheuben, M.B., **1992**. Degenerative changes in the muscle fibers of *Manduca sexta* during metamorphosis. *J. Exp. Biol. 167*, 91–117.

Riddiford, L.M., **1976**. Hormonal control of insect epidermal cell commitment *in vitro*. *Nature 259*, 115–117.

Riddiford, L.M., Hiruma, K., Zhou, X., Nelson, C.A., **2003**. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster. Insect Biochem. Mol. Biol. 33*, 1327–1338.

Rivlin, P.K., Gong, A., Schneiderman, A.M., Booker, R., **2001**. The role of Ultrabithorax in the patterning of adult thoracic muscles in *Drosophila melanogaster. Devel. Genes Evol. 211*, 55–66.

Rivlin, P.K., Schneiderman, A.M., Booker, R., **2000**. Imaginal pioneers prefigure the formation of adult thoracic muscles in *Drosophila melanogaster. Devel. Biol. 222*, 450–459.

Rome, L.C., Lindstedt, S.L., **1998**. The quest for speed: muscles built for high-frequency contractions. *News Physiol. Sci. 13*, 261–268.

Roulier, E.M., Fyrberg, C., Fyrberg, E., **1992**. Perturbations of *Drosophila* α-actinin cause muscle paralysis, weakness, and atrophy but do not confer obvious nonmuscle phenotypes. *J. Cell Biol. 116*, 911–922.

Roy, S., Shashidhara, L.S., VijayRaghavan, K., **1997**. Muscles in the *Drosophila* second thoracic segment are patterned independently of autonomous homeotic gene function. *Curr. Biol. 7*, 222–227.

Roy, S., VijayRaghavan, K., **1997**. Homeotic genes and the regulation of myoblast migration, fusion, and fibre-specific gene expression during adult myogenesis in *Drosophila*. *Development 124*, 3333–3341.

Roy, S., VijayRaghavan, K., **1998**. Patterning muscles using organizers: larval muscle templates and adult myoblasts actively interact to pattern the dorsal longitudinal flight muscles of *Drosophila*. *J. Cell Biol. 141*, 1135–1145.

Roy, S., VijayRaghavan, K., **1999**. Muscle pattern diversification in *Drosophila*: the story of imaginal myogenesis. *BioEssays 21*, 486–498.

Ruiz Gomez, M., Bate, M., **1997**. Segregation of myogenic lineages in *Drosophila* requires numb. *Development 124*, 4857–4866.

Ruiz Gomez, M., Coutts, N., Price, A., Taylor, M.V., Bate, M., **2000**. *Drosophila dumbfounded*: a myoblast attractant essential for fusion. *Cell 102*, 189–198.

Sahota, T.S., Beckel, W.E., **1967**. Orientation determination of developing flight muscles in *Galleria mellonella*. *Canad. J. Zool. 45*, 407–420.

Sakai, Y., Okamoto, H., Mogami, K., Yamada, T., Hotta, Y., **1990**. Actin with tumor-related mutation is antimorphic in *Drosophila* muscle: two distinct modes of myofibrillar disruption by antimorphic actins. *J. Biochem. (Tokyo) 107*, 499–505.

Sanchez, F., Tobin, S.L., Rdest, U., Zulauf, E., McCarthy, B.J., **1983**. Two *Drosophila* actin genes in detail: gene structure, protein structure and transcription during development. *J. Mol. Biol. 163*, 533–551.

Sandstrom, D.J., Bayer, C.A., Fristrom, J.W., Restifo, L.L., **1997**. Broad-complex transcription factors regulate thoracic muscle attachment in *Drosophila*. *Devel. Biol. 181*, 168–185.

Sandstrom, D.J., Restifo, L.L., **1999**. Epidermal tendon cells require Broad Complex function for correct attachment of the indirect flight muscles in *Drosophila melanogaster. J. Cell Sci. 112*, 4051–4065.

Scaraffia, P.Y., Remedi, M.S., Maldonado, C., Aoki, A., de Burgos, N.M.G., **1997**. Enzymatic and ultrastructural changes in thoracic muscles of triatomine insects during the last stages of metamorphosis. *Comp. Biochem. Physiol. A, Physiol. 116*, 173–179.

Schmitz, S., Clayton, J., Nongthomba, U., Prinz, H., Veigel, C., *et al.*, **2000**. *Drosophila* ACT88F indirect flight muscle-specific actin is not N-terminally acetylated: a mutation in N-terminal processing affects actin function. *J. Mol. Biol. 295*, 1201–1210.

Schouest, L.P., Jr., Anderson, M., Miller, T.A., **1986**. The ultrastructure and physiology of the tergotrochanteral depressor muscle of the housefly, *Musca domestica*. *J. Exp. Zool. 239*, 147–158.

Schultz, J.R., Tansey, T., Gremke, L., Storti, R.V., **1991**. A muscle-specific intron enhancer required for rescue of indirect flight muscle and jump muscle function regulates *Drosophila tropomyosin* I gene expression. *Mol. Cell Biol. 11*, 1901–1911.

Shafiq, S.A., **1963**. Electron microscopic studies on the indirect flight muscles of *Drosophila melanogaster.* 2. Differentiation of myofibrils. *J. Cell Biol. 17*, 363–374.

Sheterline, P., Clayton, J., Sparrow, J., **1998**. Actin. Oxford University Press, Oxford.

Simpson, D.G., Carver, W., Borg, T.K., Terracio, L., **1994**. Role of mechanical stimulation in the establishment and maintenance of muscle cell differentiation. *Int. Rev. Cytol. 150*, 69–94.

Smit, W.A., Velzing, E.H., **1986**. Fusion of myocytes with larval muscle remnants during flight muscle development in the Colorado beetle. *Tissue Cell 18*, 469–478.

Sogl, B., Gellissen, G., Wiesner, R.J., **2000**. Biogenesis of giant mitochondria during insect flight muscle development in the locust, *Locusta migratoria* (L.): transcription, translation and copy number of mitochondrial DNA. *Eur. J. Biochem. 267*, 11–17.

Sonea, I.M., Rheuben, M.B., **1992**. Degenerative changes in the function of neuromuscular junctions of *Manduca sexta* during metamorphosis. *J. Exp. Biol. 167*, 61–89.

Sparrow, J., Reedy, M., Ball, E., Kyrtatas, V., Molloy, J., *et al.*, **1991**. Functional and ultrastructural effects of a missense mutation in the indirect flight muscle-specific actin gene of *Drosophila melanogaster. J. Mol. Biol. 222*, 963–982.

Squire, J.M., **1986**. Muscle: Design, Diversity, and Disease. Benjamin/Cummings, Menlo Park, CA.

Standiford, D.M., Davis, M.B., Miedema, K., Franzini-Armstrong, C., Emerson, C.P., Jr., **1997**. Myosin rod protein: a novel thick filament component of *Drosophila* muscle. *J. Mol. Biol. 265*, 40–55.

Standiford, D.M., Sun, W.T., Davis, M.B., Emerson, C.P., Jr., **2001**. Positive and negative intronic regulatory elements control muscle-specific alternative exon splicing of *Drosophila* myosin heavy chain transcripts. *Genetics 157*, 259–271.

Stocker, R.F., **1974**. Elektronenmikroskopische Beobaentungen über die fusion myogener Zellen bei *Antheraea polyphemus* (Lepidoptera). *Experientia 30*, 896–898.

Sudarsan, V., Anant, S., Guptan, P., VijayRaghavan, K., Skaer, H., **2001**. Myoblast diversification and ectodermal signaling in *Drosophila*. *Devel. Cell 1*, 829–839.

Swank, D.M., Bartoo, M.L., Knowles, A.F., Iliffe, C., Bernstein, S.I., *et al.*, **2001**. Alternative exon-encoded regions of *Drosophila* myosin heavy chain modulate ATPase rates and actin sliding velocity. *J. Biol. Chem. 276*, 15117–15124.

Swank, D.M., Knowles, A.F., Kronert, W.A., Suggs, J.A., Morrill, G.E., *et al.*, **2003**. Variable N-terminal regions of muscle myosin heavy chain modulate ATPase rate and actin sliding velocity. *J. Biol. Chem. 278*, 17475–17482.

Swank, D.M., Wells, L., Kronert, W.A., Morrill, G.E., Bernstein, S.I., **2000**. Determining structure/function relationships for sarcomeric myosin heavy chain by genetic and transgenic manipulation of *Drosophila*. *Microsc. Res. Tech. 50*, 430–442.

Taber, L.A., **1998**. Mechanical aspects of cardiac development. *Prog. Biophys. Mol. Biol. 69*, 237–255.

Takano-Ohmuro, H., Takahashi, S., Hirose, G., Maruyama, K., **1990**. Phosphorylated and dephosphorylated myosin light chains of *Drosophila* fly and larva. *Comp. Biochem. Physiol. B 95*, 171–177.

Teutsch-Felber, D., **1970**. Experimentelle und histologische Untersuchungen an der Thoraxmuskulatur von *Periplaneta americana* L. *Rev. Suisse Zool. 77*, 481–523.

Tischler, M.E., Cook, P., Hodsden, S., McCready, S., Wu, M., **1989**. Ecdysteroids influence growth of the dorsolongitudinal flight muscle in the tobacco hornworm (*Manduca sexta*). *J. Insect Physiol. 35*, 1017–1022.

Tohtong, R., Rodriguez, D., Maughan, D., Simcox, A., **1997**. Analysis of cDNAs encoding *Drosophila melanogaster* myosin light chain kinase. *J. Muscle Res. Cell Motil. 18*, 43–56.

Tohtong, R., Yamashita, H., Graham, M., Haeberle, J., Simcox, A., *et al.*, **1995**. Impairment of muscle function caused by mutations of phosphorylation sites in myosin regulatory light chain. *Nature 374*, 650–655.

Tregear, R.T., **1977**. Insect Flight Muscle. North Holland, Amsterdam.

Truman, J.W., **1973**. How moths “turn on”: a study of the action of hormones on the nervous system. *Am. Sci. 61*, 700–706.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Valvassori, R., De Eguileor, M., Lanzavecchia, G., **1978**. Flight muscle differentiation in nymphs of a dragonfly *Anax imperator. Tissue Cell 10*, 167–178.

van der Ven, P.F., Bartsch, J.W., Gautel, M., Jockusch, H., Furst, D.O., **2000**. A functional knock-out of titin results in defective myofibril assembly. *J. Cell Sci. 113*, 1405–1414.

Vigoreaux, J.O., **2001**. Genetics of the *Drosophila* flight muscle myofibril: a window into the biology of complex systems. *BioEssays 23*, 1047–1063.

Vigoreaux, J.O., Hernandez, C., Moore, J., Ayer, G., Maughan, D., **1998**. A genetic deficiency that spans the *flightin* gene of *Drosophila melanogaster* affects the ultrastructure and function of the flight muscles. *J. Exp. Biol. 201*, 2033–2044.

Vigoreaux, J.O., Perry, L.M., **1994**. Multiple isoelectric variants of *flightin* in *Drosophila* stretch-activated muscles are generated by temporally regulated phosphorylations. *J. Muscle Res. Cell Motil. 15*, 607–616.

Vigoreaux, J.O., Saide, J.D., Pardue, M.L., **1991**. Structurally different *Drosophila* striated muscles utilize distinct variants of Z band-associated proteins. *J. Muscle Res. Cell Motil. 12*, 340–354.

Vigoreaux, J.O., Saide, J.D., Valgeirsdottir, K., Pardue, M.L., **1993**. Flightin, a novel myofibrillar protein of *Drosophila* stretch-activated muscles. *J. Cell Biol. 121*, 587–598.

VijayRaghavan, K., Gendre, N., Stocker, R.F., **1996**. Transplanted wing and leg imaginal discs in *Drosophila melanogaster* demonstrate interactions between epidermis and myoblasts in muscle formation. *Devel. Genes Evol. 206*, 46–53.

Vinos, J., Marato, M., Garesse, R., Marco, R., Cervera, M., **1992**. *Drosophila melanogaster* paramyosin: developmental pattern, mapping, and properties deduced from its complete coding sequence. *Mol. Gen. Genet. 231*, 385–394.

Wang, Z., Chen, X., Haunerland, N.H., **1993**. Flight muscle development in juvenile and adult forms of the desert locust, *Schistocerca gregaria*. *J. Insect Physiol. 39*, 325–333.

Warmke, J., Yamakawa, M., Molloy, J., Falkenthal, S., Maughan, D., **1992**. Myosin light chain-2 mutation affects flight, wing beat frequency and indirect flight muscle contraction kinetics in *Drosophila*. *J. Cell Biol. 119*, 1523–1539.

Weeks, J.C., Truman, J.W., **1986a**. Hormonally mediated reprogramming of muscles and motoneurones during the larval–pupal transformation of the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 125*, 1–13.

Weeks, J.C., Truman, J.W., **1986b**. Steroid control of neuron and muscle development during the metamorphosis of an insect. *J. Neurobiol. 17*, 249–267.

Wells, L., Edwards, K.A., Bernstein, S.I., **1996**. Myosin heavy chain isoforms regulate muscle function but not myofibril assembly. *EMBO J. 15*, 4454–4459.

White, K.P., Rifkin, S.A., Hurban, P., Hogness, D.S., **1999**. Microarray analysis of *Drosophila* development during metamorphosis. *Science 286*, 2179–2184.

Whiting, A., Wardale, J., Trinick, J., **1989**. Does titin regulate the length of muscle thick filaments? *J. Mol. Biol. 205*, 263–268.

Wilcox, M., DiAntonio, A., Leptin, M., **1989**. The function of PS integrins in *Drosophila* wing morphogenesis. *Development 107*, 891–897.

Wilde, J., DeBoer, J.A., **1961**. Physiology of diapause in the adult Colorado beetle. 2. Diapause as a case of pseudo-allatectomy. *J. Insect Phsyiol. 6*, 152–161.

Wojtas, K., Slepecky, N., von Kalm, L., Sullivan, D., **1997**. Flight muscle function in *Drosophila* requires colocalization of glycolytic enzymes. *Mol. Biol. Cell 8*, 1665–1675.

Zera, A.J., Koehn, R.K., Hall, J.G., **1985**. Allozymes and biochemical adaptation. In: Kerlat, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Biochemistry and Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 633–674.

Zhang, Y., Featherstone, D., Davis, W., Rushton, E., Broadie, K., **2000**. *Drosophila* D-titin is required for myoblast fusion and skeletal muscle striation. *J. Cell Sci. 113*, 3103–3115.

2.3 Functional Development of the Neuromusculature

D E Featherstone, University of Illinois at Chicago, Chicago, IL, USA
K S Broadie, Vanderbilt University, Nashville, TN, USA

2.3.1. Introduction and Overview

The neuromusculature drives movement. It is composed of three functionally intertwined cell types: (1) beautifully patterned striated body muscles; (2) architecturally elaborate motor neurons that precisely innervate these muscles; and (3) peripheral glia that maintain and modulate motor neuron transmission. For the last century or more, numerous insect species have been exploited to study the basis of neuromuscular development and function. The primary attractions of insect systems include large individually identifiable neurons, simple neuromuscular architecture, and easy propagation and manipulation of insect species in the laboratory. A well-refined maps of insect neural circuits and detailed physiological insight into the bases of insect neuromuscular development and function are provided. Until approximately 20 years ago, most

insect physiologists focused on species such as grasshoppers, crickets, cockroaches, or moths, which have particularly large, experimentally tractable cells. In the last two decades, increasingly, the vast majority of studies in insect physiology and neuromuscular development have shifted to the tiny fruit fly, *Drosophila melanogaster*. The reason, of course, is that *Drosophila* has emerged as a premier genetic model system. This amenability to genetic manipulation, coupled with the increasing molecular focus of physiology and development, has led to the fruit fly's dominance as the insect model for studies in development, physiology, and behavior, despite the considerable disadvantage of small size compared to larger insect species. Although other insect species still dominate fields including ecology, toxicology, and endocrinology, modern insect development and physiology is, for the most part, now synonymous with *Drosophila* development and physiology. Thus, an accurate review of advances in insect neuromuscular development focuses on *Drosophila*, and this chapter will focus strongly on details of the *Drosophila* neuromusculature.

The neuromusculature has been most intensively investigated in mammals, but, in general, neuromuscular function and development is very similar in insects and mammals. There are, however, some important differences. The most obvious difference is that in insects, muscle contraction is triggered by the excitatory neurotransmitter glutamate. The vertebrate neuromuscular junction (NMJ) uses acetylcholine. However, glutamate is the primary excitatory synapse type in the vertebrate central nervous system (CNS). Thus, in addition to being a general model of the insect NMJ, the *Drosophila* NMJ has also served as a leading genetic model of glutamatergic synapses. *Drosophila* studies have concentrated on glutamatergic "type I" NMJ synapses on embryonic and larval muscles, which drive muscle contraction. There has been much less focus on "type II" or "type III" synapses, which serve primarily modulatory functions. Therefore, unless specified otherwise, this chapter will discuss *Drosophila* type I NMJ synapses. The focus of this chapter is on the acquisition and maturation of physiological properties (e.g., functional development) of these NMJs in the embryo and larva. This chapter does not discuss the development that occurs in pupae during metamorphosis (see **Chapters 2.2 2.4**, and **2.5**).

This chapter begins with an overview of the components and function of the mature larval *Drosophila* neuromuscular system (see Section 2.3.1). The cell types of the neuromusculature, the physiological properties of these cells, and patterns of neuromuscular connectivity are discussed (see Sections 2.3.1.1 and 2.3.1.2), and then summarize the functional properties of NMJ transmission (see Sections 2.3.1.3 and 2.3.1.4). The bulk of the chapter is then devoted to the functional development of this neuromuscular system. First, the origin and morphological development of the *Drosophila* neuromusculature is given, describing the specification of cellular identities and the maturation of neuromuscular patterning and connectivity (see Sections 2.3.2–2.3.4). This information is not intended to be exhaustive, but rather to provide references for the reader to accurately place functional maturation within the wider context of overall neuromuscular development. More comprehensive descriptions of neuromuscular structural and functional development can be obtained in **Chapters 1.10** (neurogenesis), **2.1**, and **2.2** (myogenesis), **2.4**, and **2.5**. Second, the functional development of the NMJ synapse is focused. The maturation of neurotransmitter release (see Section 2.3.5), induction of postsynaptic specializations and the appearance of postsynaptic receptor fields (see Sections 2.3.6–2.3.7), the interaction between pre- and postsynaptic cells (see Section 2.3.7), developmental changes in functional plasticity (see Section 2.3.8), and the effects of hormones on NMJ function (see Section 2.3.9) are discussed. The chapter is concluded by considering the most pressing unanswered questions and suggesting fruitful directions for investigation in the future.

2.3.1.1. Types of Cells

2.3.1.1.1. Motor neurons Compared to vertebrates, insects have a relatively simple neuromusculature composed of relatively a few cells (see **Figures 1 and 2**). Rather than a motor neuron pool (hundreds of cells) innervating a multicellular muscle (thousands of myotubes), as in mammals, insects are characterized by a single motor neuron innervating a single myotube. Moreover, as they are bilaterally symmetrical segmented animals, the basic unit of the insect neuromusculature is a single hemisegment, which is simply reiterated a number of times depending on the length of the animal. Each hemisegment is nearly identical, with a few segment specific modifications overlying a common cellular plan. Thus, in the *Drosophila* embryo/larva body, thoracic segments T1–T3 are highly similar (although each is unique) and the abdominal segments A2–A7 are identical (A1 and A8 have slight variations). Insect neurobiologists work with identified, named neurons. In most insect species, neurons are readily discernible via basic imaging techniques, and can be individually identified in living preparations based

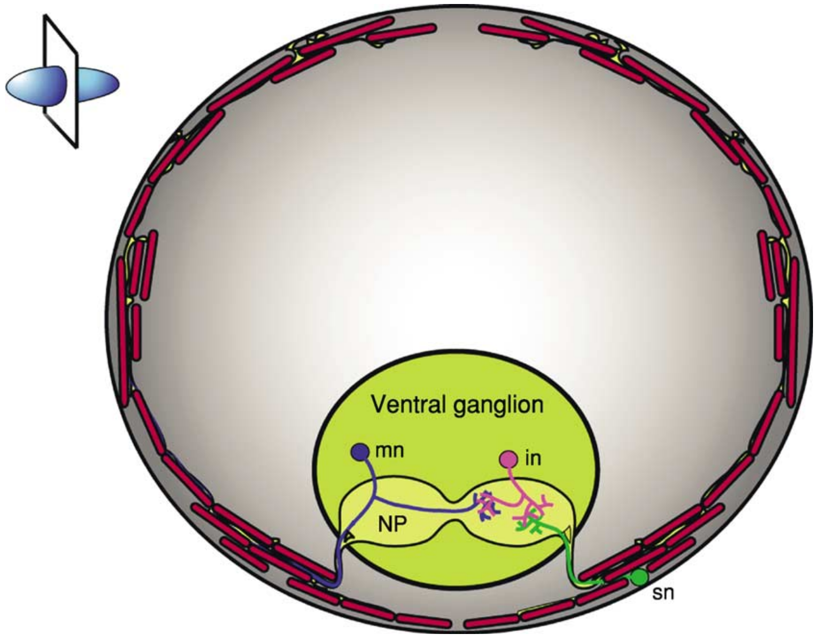


Figure 1 Diagrammatic representation of the *Drosophila* embryonic/larval neuromusculature, viewed in cross-section. One of each general type of neuron is shown: motor neuron (mn), interneuron (in), and sensory neuron (sn). The cell bodies of motor neurons and interneurons lie within the cortex of the ventral ganglion. The cell bodies of peripheral sensory cells are found near the cuticle in the periphery. Motor neurons send axons either contralaterally or (as shown) ipsilaterally along the ventral nerve and into the peripheral musculature, where the axons (yellow) synapse onto one (or sometimes more, depending on the neuron) appropriate muscle target(s) (red rectangles). Note that the most distal muscle targets are also most dorsal, since axons grow along the periphery from the ventral side of the animal. Simultaneously, sensory neurons project their axons from the periphery back toward the CNS along ventral nerves. Although the motor and sensory axons are shown traversing different nerves in this diagram for clarity, each nerve actually contains multiple motor axons going out and multiple sensory axons going in. Interneurons lie entirely within the ventral ganglion. Neuron–neuron synapses occur within the neuropil (NP), the area of the ventral ganglion containing most axonal and dendritic tracts, but no cell bodies.

on cell body size, location, and axonal projection. For example, drosophilists commonly visualize a single raw prawn 3 (RP3) motor neuron innervating a specific pair of ventral, longitudinal muscle cells (muscles 6 and 7; Broadie *et al*., 1993). The application of cell-filling techniques and antibodies further increases the number of identified, named neurons. Due to their large size, clear axonal projections, and known function, motor neurons have received the most focused analysis and are best characterized. In the large grasshopper embryo, all identifiable motor neurons have been described. Motor neuron cell identities have been highly conserved among diverse insect species, allowing studies from different insect species to cross-fertilize and inform parallel studies in other species (Thomas *et al*., 1984). Knowledge gleaned from the grasshopper, in particular, provided the vital framework for subsequent studies in *Drosophila*.

In the *Drosophila* embryo/larva, all motor neurons in the identical A2–A7 abdominal segments have been named and mapped. A combination of transgenic markers and antibodies also assign unique molecular identities to many of these cells. Motor neurons share two general organizational properties. First, the neuronal structure is simple, with either a unipolar structure or a weakly bipolar structure. Typically, motor neuron axonal projections are either ipsilateral or contralateral, with a limited dendritic tree branching from a single process near the soma. Second, motor neurons innervating muscles of similar position (dorsal versus ventral) and orientation are often clustered, with overlapping dendritic trees that converge in the same region of the neuropil. Even in cases where neurons innervating similar muscles have soma positions that are quite different, the cells share a common domain of dendritic arborization within the CNS (Landgraf *et al*., 1997). Each hemisegment has 30 muscles and each muscle is (with a few exceptions) innervated in a 1 : 1 arrangement by one of 30 “major” motor neurons. These motor neurons display distinctive, enlarged “type I” NMJ boutons that drive muscle contraction. In addition to these 30 major motor neurons, there are four motor neurons that each innervate a large field of muscles and have characteristically smaller NMJ boutons. These

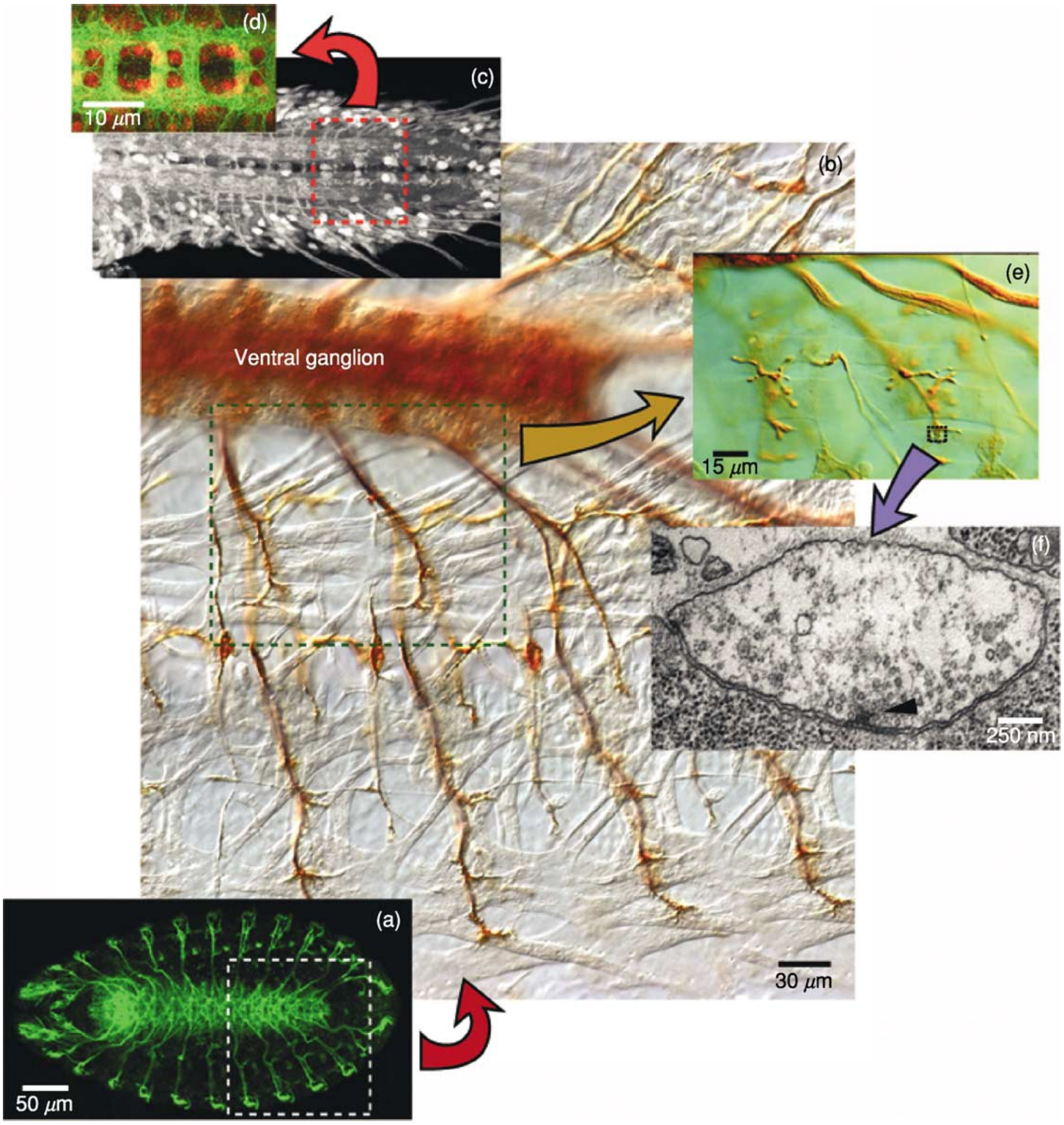


Figure 2 Microscopic views of the *Drosophila* embryonic neuromuscular anatomy. (a) Confocal image of an intact (whole mount) embryo viewed from above (anterior is left). Anti-FasII antibodies have been used to visualize the motor nerves (green). Note the segmentally repeated peripheral nerves extending from the ventral ganglion out toward the body wall neuromusculature. (b) An enlarged view of a section of the embryonic peripheral neuromusculature. As in (a), axons and growth cones (brown) have been visualized using antibodies that recognize the cell adhesion molecule FasII. In each hemisegment, a group of axons extends from the ventral ganglion, where they first encounter the ventral longitudinal muscles (two hemisegments of which are enclosed by the dashed green box). Some axons continue on toward more distal, dorsal muscles (the dorsal midline is near the bottom of the image). The embryo in (b) has been split dorsally and laid flat; in the intact animal, the axons extending toward the bottom of the image (and the muscles they innervate) would curve up out of the page and back toward the ventral ganglion, such that the bottom of the image (which shows the dorsal midline) would overlap the midline of the ventral ganglion. (c) A close-up of the ventral ganglion, showing a subset of neurons visualized using green fluorescent protein (GFP) expressed specifically in cholinergic neurons (GAL4-Cha;UAS-GFP). Note the two parallel areas devoid of cell bodies; these are the neuropile. (d) A more detailed image of approximately 2.5 neuromeres of ventral ganglion, showing axons (green) and cell bodies (red). The overall appearance of a ''ladder'' is formed by segmentally repeated lateral and commissural axon tracts. (e) A detailed view of a portion of two neighboring hemisegments showing NMJs (brown) on ventral longitudinal muscles 7 and 6. The edge of the ventral ganglion is visible in the upper left corner. (f) An electron micrograph cross-section of a stage 17 embryonic neuromuscular bouton. The arrow indicates a presynaptic T-bar, surrounded by synaptic vesicles.

motor neurons are believed to mediate modulatory functions. Thus, in total, there are 34 identified motor neurons per abdominal hemisegment.

Until recently, relatively little was known about the functional properties of *Drosophila* motor neurons *in situ*. Recent physiological assays have focused on a small, medial dorsal cluster of motor neurons with both ipsi- and contralateral axons terminating in type I glutamatergic muscle terminals (Hoang and Chiba, 2001; Rohrbough and Broadie, 2002). These cells have resting potentials of −50 to −60 mV and fire repetitive action potentials (AP) during a depolarizing current injection (Rohrbough and Broadie, 2002), consistent with the sharp bursts of action potentials that drive peristaltic movement *in vivo* (Broadie and Bate, 1993e; Cattaert and Birman, 2001). The majority of motor neurons exhibit adaptive AP firing patterns, characterized by a variable decrease, but not termination, in spike frequency that is likely specific to each cell type (Rohrbough *et al*., 2003). A few motor neurons exhibit tonic firing at constant frequency during prolonged depolarization. Similar adaptive and tonic AP firing characteristics have been previously described in cultured *Drosophila* neurons (Saito and Wu, 1991; O'Dowd, 1995; Zhao and Wu, 1997; Schmidt *et al*., 2000). *In situ*, the heterogeneity of AP firing properties among specific motor neurons is likely regulated by intrinsic variables such as different patterns of impinging synaptic input.

A considerable amount is known about the voltage-gated ion channels that mediate electrical signaling in *Drosophila* motor neurons (Wu and Ganetzky, 1992; see also **Chapters 2.6** and **5.1**). The primary voltage-gated Na^+ channel is encoded by the *paralytic* (*para*) gene, although at least one other voltage-gated Na^+ channel is also present (Loughney *et al*., 1989). The other primary inward current is carried by Ca^{2+} via several distinct classes of voltage-gated Ca^{2+} channels. The gene *cacophony* (*cac*) encodes the L-type voltage-gated Ca^{2+} channel in *Drosophila* (Kulkarni and Hall, 1987). Null mutations in the *Dmca1A* gene result in embryonic lethality; partial loss-of-function mutations reduce the L-type Ca^{2+} current (Smith *et al*., 1996). Presynaptic N-type Ca^{2+} channels regulate neurotransmitter release (Rieckhof *et al*., 2003).

The hyperpolarizing outward K^+ current can be divided into at least four components: two voltage-gated K^+ currents (I_A, I_K) and two calcium-gated K^+ currents (I_{CF}, I_{CS}). The *Shaker* (*Sh*; encodes I_A channel) and *ether-a-gogo* (*eag*; encodes a subunit of multiple K^+ channels) genes were identified based on hyperactivity of mutants under anesthesia (Ganetzky and Wu, 1983; Ganetzky *et al*., 1999). Double *eag;Sh* mutants show a highly elevated frequency of action potentials in motor neurons and heightened neurotransmission at the NMJ. In cell body recordings, the principal I_A current is not carried by *Sh* channels but rather by channels encoded by the *Shaker cognate l* (*Shal*) gene, which exhibits different kinetics (Tsunoda and Salkoff, 1995a). Null *Shaker* mutants do not display altered I_A currents in records from embryonic motor neuron cell bodies (Rohrbough *et al*., 2003). Thus, it has been suggested that *Shal* encoded I_A dominates in neuronal cell bodies and/or axons, whereas *Shaker* encoded I_A dominates in synaptic terminals (Tsunoda and Salkoff, 1995b). The neuronal I_K current is carried by at least two channels; the larger component mediated by a slowly inactivating channel encoded by *Shaker cognate b* (*Shab*) and the smaller component mediated by a noninactivating channel encoded by *Shaker cognate w* (*Shaw*) (Solc and Aldrich, 1988; Tsunoda and Salkoff, 1995a). The I_{CF} current is encoded by the *slowpoke* (*slo*) locus and *slo* mutants completely lack the Ca^{2+} activated K^+ current (Elkins *et al*., 1986; Elkins and Ganetzky, 1990). The channel or channels carrying the I_{CS} current have not yet been identified (Chopra *et al*., 2000).

Acetylcholine (ACh) and GABA are the primary excitatory and inhibitory neurotransmitters, respectively, in the insect CNS (see **Chapters 5.4** and **5.9**). This conclusion is based primarily on neurotransmitter and receptor expression studies, electrophysiology, and (in *Drosophila*) transgenic reporters specific to transmitters – green fluorescent protein coupled to, for example, choline acetyltransferase expression (Schuster *et al*., 1993; Jonas *et al*., 1994; Aronstein *et al*., 1996; Yasuyama and Salvaterra, 1999). Glutamatergic cells and glutamate receptors (both NMDA and non-NMDA types; see also Section 2.3.1.3) are also expressed throughout the CNS (Littleton and Ganetzky, 2000), but less is known about their circuit function. Larval motor neurons *in situ* respond to iontophoretic application of ACh, GABA, and glutamate (Rohrbough and Broadie, 2002). As expected, ACh is strongly excitatory, generating sustained depolarizations and usually driving bursts of action potentials. ACh responses are mediated by nicotinic ACh receptors as evidenced by their reversible blockade by d-tubocurare (dtC; Rohrbough and Broadie, 2002). GABA and glutamate cause inhibitory responses mediated primarily by Cl^- channel currents. Glutamate-gated currents are particularly prolonged and act to strongly suppress AP firing. Both inhibitory transmitters display similar reversal potentials near normal resting potential (∼−55 mV), and

are reversibly blocked by the Cl^- channel blocker picrotoxin (Rohrbough and Broadie, 2002). Thus, motor neurons *in situ* express functional receptors appropriate for excitatory cholinergic input and inhibitory GABAergic and glutamatergic input.

Motor neurons display several distinct types of endogenous synaptic activity, ranging from fast, unitary events to sustained excitatory currents and potentials. Both endogenous and evoked events require external Ca^{2+} and are reversibly blocked by dtC application, demonstrating cholinergic synaptic input. These cells display unitary spontaneous excitatory synaptic currents (sepscs; 5–50 pA) closely resembling fast cholinergic sepscs reported in *Drosophila* cultured neurons (Lee and O'Dowd, 1999; Lee and O'Dowd, 2000; Lee *et al*., 2003). Most striking, however, are endogenous events with much larger amplitudes and longer durations termed spontaneous rhythmic currents (SRCs) or potentials (SRPs) that appear to be a type of periodic endogenous excitatory motor output, supporting bursts of AP firing (Rohrbough and Broadie, 2002). Interestingly, blockade of NMDA-type glutamate receptors abolishes centrally generated endogenous rhythmic output at the NMJ (Cattaert and Birman, 2001). Thus, while cholinergic input provides the major excitatory synaptic drive to motor neurons, additional forms of central transmission, including glutamatergic input, likely shape the frequency and pattern of motor rhythms controlling coordinated movement.

Compared to our knowledge of motor neurons residing in the comparatively simple and invariant central abdominal segments, relatively little is known about the physiology, identity, or connectivity of cells upstream of the motor neurons (Lohr *et al*., 2002; Landgraf *et al*., 2003; Taghert and Veenstra, 2003). There are an estimated 200 neurons per hemisegment neuromere. Apart from the few identified neurosecretory cells (Taghert and Veenstra, 2003), which also send projections into the periphery, the vast bulk of these cells are interneurons, both local and projection subtypes. Almost all of these interneurons remain unnamed and their functions remain uncharacterized. Interneurons functionally upstream of motor neurons have cell bodies scattered throughout the central and lateral CNS neuropil and numerous varicosities (presumed synaptic boutons) overlapping spatially with the dendritic arbors of motor neurons (Rohrbough *et al*., 2000; Rohrbough and Broadie, 2002). Paired synaptic recording and cell labeling experiments indicate that fast synaptic responses in motor neurons are efficiently evoked when stimulation is applied to the neuropil adjacent to the motor neuron's primary dendrites (Rohrbough and Broadie, 2002; Rohrbough *et al*., 2003), suggesting that cholinergic inputs are formed by ascending or descending longitudinal axons.

2.3.1.1.2. Peripheral glia As in other animals, glia are an abundant cell type in the insect CNS and peripheral nervous system (PNS). Just how abundant, is a matter of debate, as estimates range widely and little effort has been made to catalog glial subtypes. In the *Drosophila* CNS, glial cells adopt morphologies resembling vertebrate astrocytes, yet essentially nothing is known about their mature functions. In the periphery, defined glial cells are found where peripheral nerves exit the CNS, and at regular intervals along the peripheral nerves (Auld *et al*., 1995; Sepp *et al*., 2000). These glia associate tightly with both motor and sensory axons, and have extensive cytoplasmic processes that fully ensheath peripheral nerves. The number of insulating peripheral glial cells per nerve seems to be somewhat variable (8–10 per hemisegment in *Drosophila*) and the exact cell body positions are not well mapped. Therefore, there is no general nomenclature or numbering scheme for *Drosophila* peripheral glia, unlike the situation for both motor neurons and muscles. In addition to *bona fide* peripheral glia, there are other cell types closely associated with the peripheral nerves. For example, *Drosophila* also contains nerve associated cells that are characterized by persistent larval expression of the transcription factor Twist. These persistent Twist (PT) cells represent the mesodermal anlage of the adult muscle that divide during larval life to form the adult myoblasts (Bate *et al*., 1991; Broadie and Bate, 1991).

In many animals, peripheral glia have been shown to be essential for action potential conduction, and thus neuromuscular transmission. Similarly, *Drosophila* peripheral glia tightly wrap around all exposed peripheral nerves, enclosing afferent and efferent axons in a common glial sheath that excludes the hemolymph (Auld *et al*., 1995). Adjacent glial processes are joined by a specialized intercellular junction called a pleated septate junction (pSJ), which acts as a diffusion barrier similar to the tight junctions of vertebrates (Schulte *et al*., 2003). Although less highly specialized than the myelin sheath of mammalian Schwann cells, this insect glial sheath seems to serve comparable functions. Mutation of any of three *Drosophila* genes encoding glial proteins (Gliatactin, Neurexin IV, and Axotactin) causes blockade of motor nerve action potential propagation and, therefore, paralysis (Auld *et al*., 1995; Baumgartner *et al*., 1996;

Yuan and Ganetzky, 1999). The *gliatactin* and *neurexin* IV mutants manifest very subtle disruptions in the glial sheath surrounding the motor nerves, and the conduction blockade can be rescued by reducing [K^+] in the external bath, indicating that the glial sheath is essential for insulating the peripheral nerves from the high [K^+] of the insect hemolymph. In contrast, Axotactin appears to be part of a novel glia–neuron signaling pathway required for proper electrical signaling in neuronal axons (Yuan and Ganetzky, 1999). The functional roles of the glia in neuronal signaling remain to be elucidated. Nuclei of glial sheath cells lie on the outside of the motor nerve just proximal to the site where nerves enter the body wall musculature. This nerve entry point on the muscle is the location where the glial sheath covering the motor nerve terminates; insect NMJs are not covered in any sort of "capping" glia, unlike vertebrate NMJs.

2.3.1.1.3. Muscle *Drosophila* muscle is similar to mammalian muscle: syncytial (multinucleate; arising from myoblast fusion) and beautifully striated with actin–myosin contractile units (sarcomeres). However, the insect muscle can be very different at the level of multicellular organization. For example, in the *Drosophila* embryo/larva, a given muscle is composed of a single syncytial cell, whereas in mammals, each muscle is composed of a large aggregate of syncytial muscle cells (myotubes). In the adult *Drosophila* thorax, however, bundles of muscle cells do become the basic contractile unit, paralleling the situation in mammals (see also **Chapter 2.2**). The insect muscle attaches directly to the epidermis via specialized tendon cells distributed so that individual muscles span longitudinal, transverse, or oblique domains. Each segment has a highly specific, invariant pattern of muscles, each of a certain size and orientation, typically arranged in a number of discrete layers (Bate, 1990; **Chapter 2.2**). Muscle cells vary greatly in size, depending on the number of myoblasts that fuse to give rise to the mature myotube. In the *Drosophila* embryo/larva, each abdominal hemisegment A2–A7 contains exactly 30 muscles, arranged in three comparably sized layers (~10 muscles/layer; Bate, 1990; **Chapter 2.2**). Each *Drosophila* muscle has a distinguishing name, although two different naming schemes are routinely used (Bate, 1993).

In *Drosophila*, larval muscles are geometrically simple (typically rectangular) and nearly isoelectric throughout; their passive electrical properties have been well characterized (Jan and Jan, 1976b; Wu and Ganetzky, 1992; Singh and Wu, 1999). Muscles are weakly electrically coupled within and between segments (Ueda and Kidokoro, 1996). In contrast to vertebrate muscle, insect muscle lacks voltage-gated Na^+ channels, and Ca^{2+} influx is required for insect muscle contraction (Aidley, 1975; Jan and Jan, 1976b; Singh and Wu, 1999). In total, five prominent voltage-activated currents are present in mature larval muscle; the voltage-gated calcium current (I_{Ca}), two voltage-gated potassium currents (I_A, I_K), and two calcium-gated potassium currents (I_{CF}, I_{CS}) (Wu and Ganetzky, 1992; Singh and Wu, 1999).

The voltage-gated Ca^{2+} channels are present in two subtypes: one with the pharmacological and biophysical properties of vertebrate L-type Ca^{2+} channels (high voltage activation; maximal at −10 to 0 mV threshold, inactivate slowly) and the other with the characteristics of the vertebrate T-type Ca^{2+} channels (low voltage activation threshold) (Gielow *et al.*, 1995). In *Drosophila*, two Ca^{2+} channel α subunit genes, *Dmca1A* and *Dmca1D*, encode channels with six transmembrane domains, both similar to the vertebrate voltage-gated Ca^{2+} channels (Zheng *et al.*, 1995; Smith *et al.*, 1996). Definitive genetic analyses of the function of these gene products in muscle has not yet been done.

Of the two voltage-gated K^+ currents, I_A is rapidly activated and rapidly inactivated, whereas I_K is slowly activated and shows little inactivation during sustained depolarization. I_A is selectively eliminated in *Sh* mutants; the *Drosophila Sh* gene was the first cloned K^+ channel α subunit, a six transmembrane voltage-gated channel (Kamb *et al.*, 1987; Kamb *et al.*, 1988). Thus, the molecular identity of the principal I_A is different in neurons and muscle; Shaker I_A is only detectable in a subpopulation of neurons, and even in these neurons does not constitute the major I_A current (Baker and Salkoff, 1990; Tsunoda and Salkoff, 1995a). In contrast, the *shaw* and *shab* genes encoding I_K channels in neurons are also responsible for the I_K current present in muscle (Tsunoda and Salkoff, 1995a). Mutations in the *Shab* gene reduce I_K without affecting I_A.

Of the two Ca^{2+}-activated K^+ currents, I_{CF} is rapidly activated downstream of Ca^{2+} channel opening and inactivates, whereas I_{CS} activates slowly and shows little inactivation. Mutations of the *slowpoke* (*slo*) gene eliminates I_{CF} in muscle (Elkins *et al.*, 1986; Wu and Ganetzky, 1992). The channel carrying I_{CS} has not yet been identified (Chopra *et al.*, 2000). Additional genes for K^+ channel subunits have been identified in *Drosophila* muscle, including *eag*, a structural K^+ channel subunit, and *Hyperkinetic* (*Hk*), an auxiliary, membrane associated β subunit (Yao and Wu, 1999). Mutations in *eag* alter all four K^+ currents in the muscle, reducing all but eliminating none, and it has been

suggested that *eag* acts as a modulatory subunit in several different K^+ channels (Zhong and Wu, 1993). In contrast, mutations in *Hk* alter the kinetics of the I_A current alone, without affecting any of the others (Yao and Wu, 1999; Wang *et al.*, 2000).

In addition to these voltage- and calcium-gated channels, a mechanically gated, stretch-activated large conductance K^+ channel is present in *Drosophila* muscle (Zagotta *et al.*, 1988; Gorczyca and Wu, 1991). The molecular identity and exact functional significance of these channels remains unknown.

Synaptic transmission at the NMJ is mediated by ionotropic glutamate receptors (GluRs), which are L-glutamate gated cation channels expressed in all muscles (see **Chapter 5.9**). Electrophysiological evidence for other receptors exists (see later sections), but the molecular identity of these proteins remains unknown. The exact subunit composition of any *Drosophila* channel, either ligand or voltage-gated, and the functional consequences of channel modulation also remain largely unknown.

2.3.1.2. Overview of Neuromuscular Connectivity

2.3.1.2.1. Patterns of motor innervation Many insects contain segmental ganglia. In *Drosophila*, however, segmental ganglia are fused into one ventral nerve cord (VNC), although segmental units (neuromeres) can be recognized by landmarks including segmental nerves. Each neuromere consists of a nearly identical set of cortical neuronal somata in characteristic positions, enclosing a soma-free zone (neuropil) dense with synaptic connections. The neuropil is scaffolded by a highly recognizable "ladder" of two lateral axon tracts (longitudinal connectives) connected across the midline by an anterior and posterior axon tract (transverse commissures). Synaptic contacts within the CNS are restricted to the neuropil excluding the neuronal soma. Most motor axons converge at lateral exits located on the side of each hemisegment neuromere. Two major axon tracts converge on these exits: (1) the anterior intersegmental nerve (ISN) tract and (2) the posterior segmental nerve (SN) tract. A small number of axons also exit the CNS through a dorsal exit point in each segment (see **Figures 1 and 2**).

In the periphery, five major nerves extend from each lateral CNS exit point. First, the ISN carries the motor innervation to the most dorsal muscle set. Second, multiple SN branches innervate the lateral and ventral muscles: the A branch (SNa) innervates the lateral muscle group, the B branch (SNb) innervates the next most lateral/ventral muscle group, the C branch (SNc) innervates the internal ventral muscles, and the D branch (SNd) innervates the most ventral, external muscle group. From the dorsal CNS exit extends the transverse nerve (TN) that runs along the anterior segmental border. The TN carries just a few motor axons together with the processes of neurosecretory cells (Taghert and Veenstra, 2003).

2.3.1.2.2. Morphological and functional classes of NMJ In *Drosophila*, motor neurons innervating larval muscle have been classified as types I, II, and III (see **Chapters 2.1** and **2.2**). Most motor neurons (~32/hemisegment) are type I. Type I neurons use glutamate as their primary neurotransmitter and are the principal mediators of muscle excitability and contraction. Type I terminals are usually restricted to one muscle, contain thick, variably branched arbors, and typically extend terminal branches for only a relatively short distance from the entry point. Type I NMJ varicosities, or boutons (**Figure 2f**), are deeply embedded in the muscle membrane, and are characterized presynaptically by dense pools of small, clear synaptic vesicles (SVs) clustered around T-bars marking glutamate release sites (active zones) and postsynaptically by an extensive field of elaborate muscle membrane foldings called the subsynaptic reticulum (SSR). The SSR is an expanded area of postsynaptic receptivity that contains the GluRs and dense concentrations of other proteins involved in postsynaptic glutamatergic transmission. Morphologically, type I neurons come in two subdivisions: (1) type Ib neurons possess big (2–5 μm) boutons that innervate muscles on a 1 : 1 basis and (2) type Is neurons possess small (1–3 μm) boutons that innervate several adjacent muscles. Type Is NMJ boutons have a greatly reduced SSR relative to type Ib. These two neurons drive physiologically different patterns of transmission, varying in excitation threshold, excitatory junctional current (EJC) amplitude, and functional plasticity properties (Kurdyak *et al.*, 1994). It has been proposed that type Ib and Is NMJ terminals are analogous to the well-characterized crustacean tonic and phasic NMJs, respectively.

There are only two type II motor neurons per hemisegment, which innervate a large number of adjacent muscles in the dorsal and ventral muscle fields. Compared to type I boutons, type II NMJ terminals are characterized by much longer, much thinner, branched arborizations studded with small (<1 μm) synaptic boutons. Type II terminals are glutamatergic and contain small, clear SVs, but they predominantly contain larger, elliptical dense core vesicles (DCV) holding a variety of neuromodulators (e.g., biogenic amine (octopamine) and small peptide (proctolin) transmitters; Taghert and

Veenstra, 2003). Type II terminals do not appear to activate ionotropic receptors in the muscle, but likely work through G protein-coupled receptors, most of which have not been characterized. Hence, type II motor neurons are proposed to be modulatory regulators of NMJ synaptic and/or muscle function, although their exact roles are not at all well understood.

Type III neuron terminals are similar to type II terminals, but contain larger DCVs harboring a different array of modulators (e.g., Insulin; Taghert and Veenstra, 2003). Type III neurons, like type II, are believed to mediate modulatory functions in the neuromusculature. At an ultrastructural level, the release sites of type III boutons are distinctive, and it is possible that these terminals are largely neurohemal, releasing peptide hormones to the entire body musculature. Types II and III NMJ terminals reside in shallow grooves in the muscle surface and are not associated with a postsynaptic SSR.

Each identified muscle cell is innervated by a specific combination of types I, II, and III motor neurons. All muscles possess type Ib innervation, in a 1 : 1 relation, but different muscles are variably innervated by type Is and type II neurons. Type III innervation is usually restricted to the centrally located muscle 12. Each motor neuron contacts each muscle at a highly characteristic nerve entry point. The NMJ structure of each muscle is highly conserved from segment to segment and from animal to animal, so much so that any given NMJ is instantly recognizable to anyone familiar with the *Drosophila* neuromuscular anatomy. However, the terminals are not invariant and may differ in the number/length of the branches and the number of synaptic boutons. Indeed, the NMJ shows a high degree of structural plasticity that is sensitive to the relative mobility and density of the animals during postembryonic development (Sigrist *et al*., 2003). Type II terminals are particularly liable to structural modification as a consequence of environmental conditions.

Several chemical classes of NMJ neurotransmission exist in different insect species. NMJs are commonly glutamatergic and excitatory, but some insects also have direct inhibitory inputs on the muscle mediated by GABA or glutamate (see also **Chapters 5.4** and **5.9**). *Drosophila* embryos and larvae have only excitatory glutamatergic NMJs with no inhibitory GABAergic or glutamatergic receptors (Featherstone *et al*., 2000). In addition to these classic, fast neurotransmitters, there are a number of other neuromodulators capable of mediating both ionotropic and metabotropic muscle responses (Taghert and Veenstra, 2003). These include neuropeptides such as pituitary adenyl cyclase activating polypeptide (PACAP)-like peptide, which (probably indirectly) gates and modulates ionic currents (Zhong, 1996) and biogenic amines, such as octopamine and tyramine (Nishikawa and Kidokoro, 1999; Nagaya *et al*., 2002), which modulate the efficacy of synaptic transmission (presumably via G protein-coupled receptors). The number of these other transmitters, potentially quite large, and the scope of their physiological functions, remains largely unknown.

2.3.1.3. Overview of NMJ Neurotransmission

The presynaptic mechanisms of neurotransmitter release have been particularly highly conserved between invertebrate and vertebrate species. In *Drosophila*, presynaptic depolarizations are typically triggered by short bursts (10–20 Hz) of action potentials, resulting in Ca^{2+} influx through N-type voltage-gated Ca^{2+} channels. Uniform, electron-lucent 30–40 nm SVs containing glutamate virtually fill the synaptic bouton, but can be functionally differentiated into three discrete pools including: (1) a reserve pool in the bouton interior removed from active cycling except under conditions of high demand; (2) a cycling pool clustered near release sites; and (3) a readily releasable pool (RRP) of vesicles morphologically docked at release sites, available for imminent release (Rodesch and Broadie, 2000). The SV release sites contain a distinctive electron-dense T-bar. In *Drosophila* and other dipterans, T-bars appear in cross-section as a bar-like density approximately 100–200 nm wide, oriented parallel to the cell membrane and supported by a “stem” approximately 50 nm in height projecting into the cytosol (Lane, 1985; Jia *et al*., 1993; Prokop, 1999). The true shape of a T-bar, however, is apparently more similar to a “table” when considered in three dimensions: three or four electron-dense “legs” or “prongs” (Rheuben *et al*., 1999) support each bar; the density typically appears as a “T” because only one “leg” is typically visible in cross-section (Atwood *et al*., 1993). The appearance of the presynaptic density varies among insect species, suggesting that its function is not dependent on a particular shape. In *Manduca*, for example, the presynaptic density appears simply as a narrow 2 nm bar. In longitudinal sections, T-bars can extend for hundreds of nm (Lane, 1985).

The T-bar is surrounded by clear SVs that are typically 30–40 nm in *Drosophila*. Because the T-bar/presynaptic density is presumably the site of synaptic vesicle fusion (or near it), each T-bar traditionally identifies an “active zone.” However, the exact role of the T-bar (or presynaptic density in

any organism) in synaptic vesicle cycling is not known. Omega-shaped membrane profiles indicative of exo/endocytosis are not always adjacent to T-bars (Lane, 1985). Omegas that are present occur some distance away and are coated, suggesting that they are endocytic. Verstreken *et al*. (2002) argued that basal release requires only partial (kiss and run) SV fusion and showed electron micrographs where the few existing vesicles that are interacting with the membrane are not clustered around T-bars. Haghigi *et al*. (2003) showed micrographs containing apparently functional active zones with clustered vesicles, but no T-bars. If vesicles mark sites of neurotransmitter release, then T-bar components are clearly not prerequisites for neurotransmitter release. The molecular composition of the T-bar is also unknown. In *Drosophila*, no molecules are yet known to be restricted to the active zone, and no mutants have been identified that eliminate the T-bar structure.

Many proteins involved in SV trafficking, fusion, and endocytosis have been exhaustively assayed in *Drosophila*. Examples include: (1) synaptotagmin, the proposed Ca^{2+} sensor for exocytosis (Littleton *et al*., 1993b; Broadie *et al*., 1994; DiAntonio and Schwarz, 1994); (2) multiple components of the soluble NSF attachment protein receptor (SNARE) complex, proposed to mediate SV fusion (Broadie *et al*., 1995; Schulze *et al*., 1995; Fergestad *et al*., 2001); (3) regulators of the SNARE complex such as Uncoordinated 13 (Unc-13) and Ras opposite (Rop)/Unc-18 (Harrison *et al*., 1994; Schulze *et al*., 1994; Aravamudan *et al*., 1999; Aravamudan and Broadie, 2003); (4) regulators of Clathrin-mediated endocytosis such as α-Adaptin and Endophilin (Gonzalez-Gaitan and Jackle, 1997; Fergestad *et al*., 1999; Fergestad and Broadie, 2001; Verstreken *et al*., 2002); and (5) mediators of SV endocytosis and recycling such as the GTPase Shibire/Dynamin (Koenig and Ikeda, 1989; van der Bliek and Meyerowitz, 1991). To a very high degree, the molecular machinery of neurotransmitter release and SV cycling is highly conserved between *Drosophila* and other species, including mammals. Many reviewers have considered the conserved nature of this machinery of neurotransmitter release in detail (Keshishian *et al*., 1996; Lloyd *et al*., 2000; Broadie and Richmond, 2002).

Postsynaptic composition and function varies according to the nature of the neurotransmitter. In *Drosophila*, muscle contraction is generated by a burst of EJCs (5–10 Hz) at the glutamatergic NMJ. Current influx through glutamate receptors is primarily carried by Na^{+} and, to a lesser extent, Ca^{2+} (Jan and Jan, 1976a). The highly folded SSR is believed to greatly increase the density of voltage-gated Ca^{2+} channels near the NMJ. Insect muscles do not contain voltage-gated Na^{+} channels. Since the presynaptic arbor can cover a considerable length of muscle area, it is likely that the synaptic response is widespread in order to mediate near-synchronous muscle contraction. In *Drosophila*, Ca^{2+} based muscle action potentials can be recorded, but it is not generally thought that larval muscles depend on regenerative potentials *in vivo* (Broadie and Bate, 1993d). In larger insects, there is clear evidence of muscle action potentials.

The nature of the postsynaptic glutamate receptor field at the *Drosophila* NMJ has been most thoroughly investigated. There are at least three functional classes of GluRs in *Drosophila* muscle including: (1) extrasynaptic ionotropic receptors; (2) synaptic ionotropic receptors; and (3) metabotropic receptors (Chang *et al*., 1994; Nishikawa and Kidokoro, 1995; Chang and Kidokoro, 1996; Parmentier *et al*., 1996; Ciani *et al*., 1997; Petersen *et al*., 1997; Zhang *et al*., 1999).

Ionotropic GluRs are multimeric L-glutamate-gated cation pores that are each composed of four subunits (Madden, 2002). Three different subunits are known to be expressed in embryonic/larval NMJs: GluRIIA, GluRIIB, and GluRIII (Petersen *et al*., 1997; DiAntonio, personal communication). GluRIII is a required subunit, and GluRIIA and GluRIIB appear to compete for assembly with GluRIII. Thus, native receptors in *Drosophila* appear to be multimers of GluRIIA/GluRIII, or GluRIIB/GluRIII (DiAntonio, personal communication).

The A and B subunits appear to have arisen through a fairly recent genetic duplication event, since the genes are adjacent in the genome, show a high level of sequence identity, and the genetic removal of either shows that neither alone is required for NMJ function and viability (Petersen *et al*., 1997). Removal of either GluRIIA or GluRIIB alters single channel current amplitude, however, suggesting that the A and B subunits confer different biophysical properties (Petersen *et al*., 1997). If both A and B subunits are eliminated, the mutant is embryonic lethal and apparently lacks all NMJ transmission. Thus, the presence of either GluRIIA or GluRIIB, but not both, along with GluRIII, is essential for function.

Mammalian GluRs were originally classified pharmacologically, based on relative sensitivity to the agonists amino-3-hydroxy-*S*-methylisoxazole-4-propionate (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA). GluRIIA, IIB, and III amino acid

sequences demonstrate that *Drosophila* NMJ GluRs have highest sequence similarity to mammalian kainate receptors. Insect muscle glutamate receptors, however, are activated by quisqualate (Usherwood, 1994), as are mammalian AMPA receptors. Furthermore, heterologously expressed GluRIIA receptors are relatively insensitive to both AMPA and kainate (Schuster *et al.*, 1991). Thus, the ionotropic glutamate receptors at insect NMJs can safely be considered "non-NMDA type," but further distinctions based on mammalian receptor pharmacology are probably meaningless.

The only metabotropic glutamate receptor identified so far at the fly NMJ is encoded by the *DmGluRA* gene, which is expressed both in the CNS neuropil and at the NMJ (Parmentier *et al.*, 1996; Mohrmann and Broadie, unpublished data). Null mutants in the *DmGluRA* gene are viable, and the receptor appears to play rather subtle functions in the regulation of NMJ structure and functional plasticity (Parmentier *et al.*, 1996; Mohrmann and Broadie, unpublished data).

Other than the actual GluRs, only a few other components of the postsynaptic density are known. These proteins are, almost exclusively, present on both sides of the synaptic cleft and include: (1) transmembrane receptors such as members of the position specific (PS) integrin family (3α and 1β subunit; Beumer *et al.*, 1999, 2002) and homophilic neural cell adhesion molecules (NCAMs) Fasciclins (II and III) (Schuster *et al.*, 1996a; Davis *et al.*, 1997; Zito *et al.*, 1997); (2) membrane associated PDZ-domain anchoring proteins such as Discs Large (Dlg, a PSD-95 homolog) and the associated Scribbler protein (Budnik *et al.*, 1996; Roche *et al.*, 2002); and (3) signaling complex molecules such as Ca^{2+}/Calmodulin-dependent kinase II (CamKII), Rho-type guanine nucleotide exchange factor, and protease activated kinase (PAK) (Griffith, 1997; Beumer *et al.*, 2002; Roche *et al.*, 2002; Kazama *et al.*, 2003). Based on much longer lists compiled for other species, the majority of the components of the postsynaptic density in insects have yet to be elucidated.

2.3.1.4. Modulation of NMJ Neurotransmission

The *Drosophila* NMJ is plastic in both its structural and functional properties. The degree of electrical activity modulates the terminal size and architectural complexity of the NMJ. Hyperactivity through mutation of the K^+ channel genes *eag* and *Sh* results in a striking increase in synaptic size and complexity (Budnik *et al.*, 1990). This morphological change is causally linked to a change in the expression of the homophilic NCAM Fasciclin II (Davis *et al.*, 1996a). In addition, however, many other signaling molecules mutate to alter the degree of structural complexity including CamKII, Integrins, and many other proteins (Zhong and Shanley, 1995; Budnik, 1996; Griffith, 1997; Rohrbough *et al.*, 1999, 2000, 2003). It is likely that only a subset of these proteins are mechanistically linked to activity-dependent plasticity, whereas others simply mediate synaptic growth.

To date, only short-term (up to minutes) functional plasticity has been revealed at the *Drosophila* NMJ. It may be that longer forms of functional plasticity are not present at the insect NMJ, although practical limitations in recording configurations have simply prevented the undertaking of long-term assays. In addition, plastic features are only apparent at low $[Ca^{2+}]$, which may not reflect the normal physiological condition (Broadie, 2000). A likely explanation for this restriction is that in higher $[Ca^{2+}]$, synaptic depression caused by depletion of resources (e.g., SVs) tends to dominate over any manifestation of facilitation (Broadie *et al.*, 1997; Broadie, 2000).

Four forms of synaptic modulation are routinely assayed at the *Drosophila* NMJ. In order of duration, these are: (1) paired-pulse facilitation (PPF); (2) short-term facilitation (STF); (3) augmentation; and (4) post-tetanic potentiation (PTP). All four forms of plasticity result in elevated EJC sizes following specific patterns of stimulation. PPF is manifest on the millisecond time scale, when one stimulation is followed after a short interval by a second stimulation. Significant PPF begins at a ~100 ms interval and increases rapidly at shorter intervals. STF occurs during short, high frequency stimulation trains on the second time scale. Significant Ca^{2+} dependent facilitation usually begins with ~2 Hz stimulation trains, and increases rapidly at higher frequencies. Augmentation is a similar form of maintained facilitation that occurs during prolonged, high frequency stimulation trains. Augmentation increases with the frequency of the stimulus train and will persist for the period of stimulus. The longest term modulation, PTP, follows a prolonged, high-frequency stimulus train (5–10 Hz for >30 s) but persists after a return to a basal frequency (0.5 Hz) of stimulation. PTP represents a prolonged, low-level (25–50%) potentiation that lasts for the duration of the recording (>20 min). PTP at the *Drosophila* NMJ occurs via cAMP dependent signaling (Zhong and Wu, 1991, 1993; Renden and Broadie, 2003).

2.3.2. Development of Motor Neuron Function

2.3.2.1. Motor Neuron Morphogenesis

2.3.2.1.1. Early neurogenesis In insects, neurons are produced by progenitor cells called neuroblasts that arise from a specialized neurogenic region called the ventral neuroectoderm (for relevant reviews, see also **Chapters 1.10–1.12**). Neuroblasts are stem cells that divide repeatedly to form a specific, limited lineage of progeny. In *Drosophila*, the neuroectoderm first becomes distinguishable soon after gastrulation during the very early stages of germ-band extension (see Figure 3). Following gastrulation, a regularly spaced array of neuroblasts develop in the ventral domain of each embryonic hemisegment, interspersed among cells forming the ventral epidermis (epidermoblasts). These two sets of progenitors segregate based on a competitive interaction called lateral inhibition. The genes mediating this inhibition mechanism are called neurogenic genes, since they allow all progenitor cells to acquire a neural fate. The lateral inhibition hypothesis proposes that all cells within the

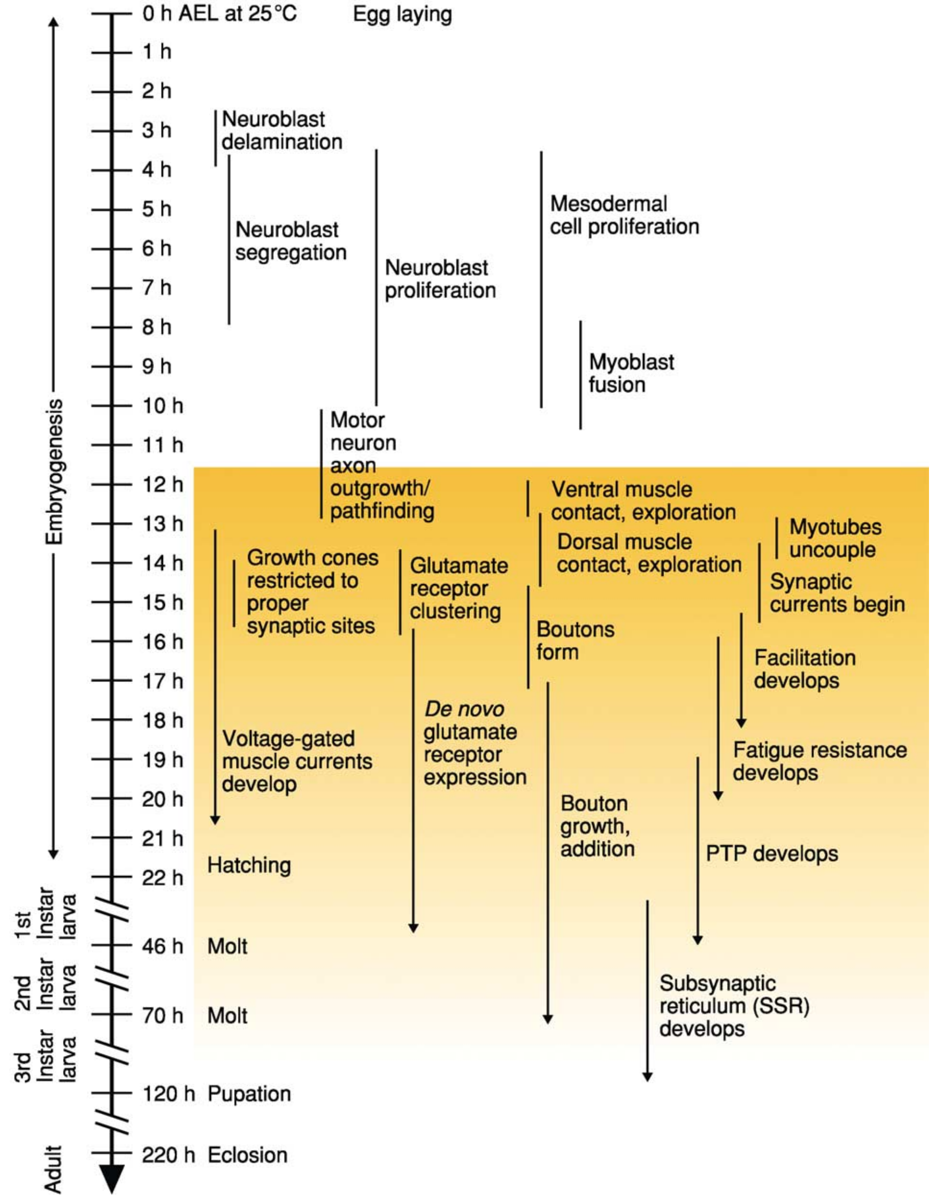


Figure 3 A timeline of *Drosophila* NMJ development. The time scale on the left represents hours after egg laying (AEL) at 25 °C. Under these conditions, embryogenesis takes approximately 22 h. Embryonic/larval NMJ synaptogenesis occurs during the period delimited by the golden color.

neuroectoderm acquire the competence to develop as neuronal progenitors (a competence group), but within each group stochastic interactions single out only a single cell to develop as a specific neuroblast. This neuroblast subsequently inhibits all the surrounding cells within the group, forcing them to assume a secondary epidermal fate. The selected neuroblasts segregate individually into the interior of the embryos ("delaminate") prior to initiating rounds of stem cell division.

In *Drosophila*, neuroblasts delaminate in three distinct phases, eventually forming a monolayer of neural stem cells between the ectoderm and the underlying mesoderm (30 neuroblasts/hemisegment). The surprisingly regular neuroblast array has allowed the production of spatial maps with individually defined, named neuroblasts, increasingly accurately defined based on the expression of specific molecular markers (Bossing *et al*., 1996; Schmid *et al*., 1999). A well-defined coordinate system of positional information (anterior–posterior, dorsal–ventral) controls the identity of neuroblasts. In *Drosophila*, each individual neuroblast can be specifically identified based on the combination of genes it expresses and, later, the characteristic lineage it gives rise to. Soon after leaving the ectodermal layer, neuroblasts become more spherical and begin to divide in a series of asymmetric divisions to generate ganglion mother cells (GMCs), which then divide symmetrically to produce two neurons. Most neuroblasts perform multiple rounds of division, with most mitotic activity ending at a period corresponding to the completion of germ-band retraction, although rare divisions may continue in late embryonic stages. In *Drosophila*, these same neuroblasts reappear, after a period of quiescence, in early postembryonic stages, to again begin producing neurons that will form the adult nervous system (Prokop and Technau, 1991).

2.3.2.1.2. Establishing motor neuron identity Cell ablation studies indicate that neuroblasts are initially quite labile in terms of fate. Elimination of a neuroblast can be compensated for by the reversal of an epidermal fate decision and the emergence of a new neuroblast. In *Drosophila* and grasshopper embryos, neuroblast ablation and transplantation experiments, together with genetic studies, have shown that GMC birth order is the primary determinant of identity (Doe and Goodman, 1985; Furst and Mahowald, 1985; Prokop and Technau, 1994; Schmid *et al*., 1999). Neuroblasts grown in single-cell isolated cultures produce defined neuronal lineages (Huff *et al*., 1989; Schmidt *et al*., 2000), suggesting that neuronal identity is largely derived from intrinsic determinants. Single identified neuroblasts grown in culture give rise to progeny that express molecular markers and functional properties expected for that lineage (Luer and Technau, 1992; Schmidt *et al*., 2000), suggesting that at least some aspects of neuronal differentiation are cell autonomous. The identity of neurons arising from early neuroblast divisions is controlled, at least largely, by intrinsic fate determinants that are asymmetrically distributed to the daughter cells. A hierarchy of transcription factors, which first function during segmentation, later regulate temporal identity in the nervous system, including *hunchback* (*hb*), *castor* (*cas*), and *Kruppel* (*Kr*) (Gaul *et al*., 1987; Cui and Doe, 1992; Mellerick *et al*., 1992; Isshiki *et al*., 2001). These transcription factors are sequentially expressed in neuroblasts, with GMCs maintaining the expression profile present at birth. This transcription factor expression specifies temporal identity and thus defines neuronal cell fate (Isshiki *et al*., 2001). Although the neuronal type is dependent on the GMC, the two daughter cells also acquire different fates. Cell ablation studies in the grasshopper clearly show that cell interactions between the siblings determine their respective fates (Kuwada and Goodman, 1985).

In a *Drosophila* hemisegment neuromere, the 30 named neuroblasts generate ~200 neurons including a small number (34) of motor neurons, a large number of interneurons (>150), and a few neurosecretory cells (<10). These same neuroblasts generate a large number of glial cells. Comprehensive cell lineage studies have defined the clone of neurons and glia produced by each *Drosophila* neuroblast (Schmid *et al*., 1999). These studies have identified the founding neuroblasts for all the cell types of the embryonic CNS: motor neurons, local and projection interneurons, neurosecretory cells, and glia. An important observation is that neuroblasts at similar dorsoventral positions usually generate similar cell lineages, including the same motor neuron subtype. Lineage analysis and connectivity mapping have been completed on the 31 motor neurons identified in the *Drosophila* embryo (Landgraf *et al*., 1997). Most (26 of 31) motor neurons derive from just a few neuroblasts in the ventral neuroectoderm. Although neuroblasts give rise to more than one type of motor neuron, structurally similar motor neurons generally derive from a common neuroblast and innervate operationally related muscles. This suggests a developmental mechanism linking the progeny of a specific neuroblast with a specific subset of muscles, which likely mediates formation of nerve–muscle partnerships. Despite this general principle, neurons deriving

from different neuroblast lineages can also innervate operationally related muscles, so this rule is not invariable.

2.3.2.1.3. Axonogenesis and pathfinding Growth cones lead extending axonal processes toward muscle targets. The small Rho GTPases are vital for regulation of the actin cytoskeleton dynamics that mediate growth cone extension during axonal pathfinding. In *Drosophila*, Cdc42 is involved in overall neuron morphogenesis, Rac1 is involved in axonal outgrowth and steering, and RhoA is essential for dendritic but not axonal growth (Luo *et al*., 1994; Lee *et al*., 2000; Ng *et al*., 2002). Growth cones use a complex cocktail of secreted cues and cell surface markers on epithelial cells, mesodermal cells, glial cells, and other neurons in their guidance. In the CNS, glial cells provide one of the most important guidepost functions for pioneer neurons. In the periphery, motor axons are guided by a complex combination of attractive and repulsive cues to initially select the appropriate axon tracts, then to select the appropriate point to defasciculate from this tract, and finally to select the correct target muscle within a domain of overlapping muscles. Among the best characterized of the attractive signals are members of the NCAM Immunoglobulin (IgG) superfamily (Tessier-Lavigne and Goodman, 1996), especially the Fasciclins (Bastiani *et al*., 1987; Patel *et al*., 1987; Bieber *et al*., 1989). Among the best characterized repulsive signals, are both secreted and membrane bound forms of the Semaphorin family (Kolodkin *et al*., 1993; Winberg *et al*., 1998). Many axons extend selectively along the surface of pioneer axons (Bate, 1976), forming axon bundles or fascicles, a process called selective fasciculation that requires NCAMs (Hortsch and Goodman, 1991; Tessier-Lavigne and Goodman, 1996). In addition, growth cones are guided to targets via diffusible, secreted ligands. Growth cones can also be guided by contact-dependent and diffusible repulsive signals that establish inhibitory domains.

Much of the knowledge of motor neuron guidepost cells and attractive growth substrates comes from work on the large grasshopper embryo (Hortsch and Goodman, 1991). Both glial cells and the epidermis have been shown to provide permissive, guiding substrates for pioneer axons (Bastiani *et al*., 1986; O'Connor *et al*., 1990). In both grasshoppers and *Drosophila*, the anterior corner cell (aCC) motor neuron pioneers the ISN, which carries the axons innervating the dorsal muscle field (Thomas *et al*., 1984). Later-growing axons depend on fasciculation to these pioneer axonal tracts, coupled to appropriate turning responses at distinctive choice points. These choice points are often associated with glia (Bastiani *et al*., 1986; Doe *et al*., 1986).

The initial efforts to identify the guiding molecules depended on monoclonal antibody staining screens in both the grasshopper and *Drosophila* (Hortsch and Goodman, 1991). This approach identified the surface glycoproteins Fasciclin I–IV and Neuroglian, which are each expressed on a distinctive, but overlapping set of growth cones, fascicles, and guidepost glial cells (Bastiani *et al*., 1987; Patel *et al*., 1987; Bieber *et al*., 1989; Kolodkin *et al*., 1992). Three of these proteins are members of the IgG superfamily related to vertebrate NCAM (Tessier-Lavigne and Goodman, 1996). *In vitro* aggregation studies show that these proteins function as homophilic receptors. The dynamic expression of these proteins on axons and guidepost glia is highly conserved across insect species, with similar patterns on identified motor neurons in both the grasshopper and *Drosophila* (Thomas *et al*., 1984).

In the *Drosophila* embryo, motor neurons, and the muscles they target, often express the same transcription factor prior to any contact. This paired specification is believed to drive the expression of matched homophilic receptors that will guide motor neuron pathfinding and target selection. For example, motor neurons targeting the dorsal muscles express the homeobox transcription factor *even-skipped* (*eve*), and this expression is both necessary and sufficient to guide these axons via the ISN to dorsal muscles (Landgraf *et al*., 1999b). Similarly, motor neurons targeting the ventral muscle field express the Lim-1/Isl-1/Mec-3 (LIM) homeobox transcription factor *islet*, which is sufficient to specify ventral motor axon projection (Thor and Thomas, 1997). Thus, these two genes constitute a bimodal switch regulating motor axon pathfinding to either ventral or dorsal muscle targets. The Eve transcription factor regulates the expression of NCAMs that promote adhesion to the ISN and so direct motor axons into the dorsal muscle field (Landgraf *et al*., 1999b). Misexpression of *eve* in normally ventrally projecting motor neurons will reroute them to the dorsal muscle field, bypassing their normal ventral targets. Thus, regulation of selective fasciculation is sufficient to direct motor axons towards the correct muscle group in the periphery.

What controls when motor axons leave the nerve fascicle and select specific muscle targets? One mechanism is to decrease the adhesion between motor axons, either by decreasing their NCAM expression or locally introducing repulsion. In *Drosophila*, the *beaten path* (*beat*) gene encodes

a secreted IgG superfamily protein, expressed in motor axons coincident with the period of defasciculation. In *beat* mutants, motor axons fail to defasciculate and travel past their intended muscle (Fambrough and Goodman, 1996). Moreover, misexpression of the anti-adhesive Beat protein in the dorsal-projecting *eve* expressing cells will cause them to prematurely defasciculate into the ventral muscle field (Landgraf *et al*., 1999b). Thus, it appears that Beat decreases the attractiveness of motor axons for other motor axons and directs the motor axon nerve departure choice point. A complementary mechanism that triggers defasciculation is increased adhesion to an alternative substrate, specifically muscle progenitors and target muscles. In mutants lacking muscle targets, motor axons extend into peripheral nerves, but do not defasciculate at the proper choice points (Prokop *et al*., 1996). Thus, muscles, and in particular muscle progenitors in the process of muscle formation, act to trigger proper defasciculation of motor axons (Landgraf *et al*., 1999b). One molecule that increases the adhesiveness of muscle is the *Drosophila sidestep* (*side*) gene, which encodes a novel cell surface protein (Sink *et al*., 2001). The Side protein is expressed on muscles during motor axon defasciculation and *side* mutant axons ignore their appropriate exit points and continue to extend along the motor nerves (Sink *et al*., 2001). Ectopic Side expression results in continued contact by motor axon growth cones and inappropriate pathfinding decisions. Thus, Side functions as an attractant, presumably as a ligand for an unknown receptor on growth cones. The coordinate control of appropriate adhesion and repulsion guides *Drosophila* motor axons along their peripheral routes.

2.3.2.1.4. Dendrite formation in the CNS At the same time that motor axons are pathfinding towards muscle outputs in the periphery, the dendrites of the motor neurons are similarly developing highly individual morphologies within the CNS neuropil. Compared to our knowledge of axon guidance, much less is known about the mechanisms responsible for directed outgrowth of dendrites. Very recent work has begun to examine dendrite formation in the *Drosophila* embryo and examine the partitioning of the neuropil into functionally distinct regions of dendritic arborization. The architecture of the neuropil has been mapped based on modality-specific sensory projections (Schrader and Merritt, 2000) and the scaffolding revealed by Fasciclin II expressing axonal tracts (Landgraf *et al*., 2003). Another class of reliable neuropil landmark is the constant topography of neuropeptidergic projections including Serotonin, Insulin-, FMRF-, Allatostatin-, Corazonin-, Substance-P-, and Leucokinin-1-positive neurons (Nassel *et al*., 1990; Gorczyca *et al*., 1993; Nichols *et al*., 1999; Landgraf *et al*., 2003; Taghert and Veenstra, 2003). Particularly powerful genetic mosaic strategies have been used to map the arrangement of output synapses on individual neurites in the neuropil (Lohr *et al*., 2002). These studies indicate three distinctive neuropil compartments: (1) compartments that contain primary transport pathways mostly lacking output synapses; (2) compartments that contain output presynaptic terminals; and (3) compartments that are postsynaptic. In *Drosophila*, as in other insects, the dorsal neuropil contains the arbors of efferent neurons that show little overlap with sensory projections, indicating that direct connection between sensory and motor neurons rarely, if ever, occur. Individual efferent neurons differentiate dendritic arbors in distinctive patterns (Landgraf *et al*., 1997). An important defining principle is that motor neurons that innervate muscles of similar position and orientation are often clustered and have overlapping dendritic trees.

Guidance strategies in the CNS neuropil have only recently begun to be analyzed, therefore the state of our understanding is primitive compared to motor axon targeting in the periphery. A recent surprising finding is that projections in the neuropil are controlled, at least in part, by guidance cues provided from the CNS midline rather than the target cells with which they will form synapses. In *Drosophila*, the Slit protein secreted by midline glia acts as a repellent to position axon fascicles in the neuropil at specific distances from the midline. The precise distance depends on differential combinations of the axonal Slit receptors, called "Roundabout (Robo) receptors" (Simpson *et al*., 2000). The position of dendritic arbors also depends on expression of Robo receptors responding to the same midline-derived Slit repellent (Zlatic *et al*., 2003). Robo has likewise been shown to regulate dendritic patterning and synaptic connectivity in the giant fiber system of adult *Drosophila* (Godenschwege *et al*., 2002). A recent study on cell-autonomous guidance signals showed that both Frazzled and Robo receptors on dendrites respond to diffusible signals from the midline to select their field of arborization (Furrer *et al*., 2003). It is probable that a combination of such diffusible morphogen signals and contact-dependent target signals (as yet unknown) together control dendritic pathfinding and target recognition in the CNS.

Motor neurons innervating similar muscles are often, but not necessarily, related by lineage or

position. Interestingly, however, in instances where no such developmental linkage exists, the motor neurons still share a common domain of dendritic arborization within the neuropil (Landgraf *et al.*, 1997). Thus, a general rule appears to be that muscles related by function (position and orientation) are innervated by motor neurons with a common domain of dendritic arborization, irrespective of whether the neuronal somata share a common precursor/position or not. A feature of these developing dendrites is that they express the same homophilic adhesion molecules as the axons, proteins known to mediate selective adhesion during fasciculation and target recognition. For example, motor neuron dendrites expressing the homophilic cell adhesion molecule connectin project together to the same region lateral to the anterior commissure (Nose *et al.*, 1992, 1997; Landgraf *et al.*, 2003). These parallels support the hypothesis that connectin-mediated adhesion may be another mechanism governing dendritic branching in the neuropil.

2.3.2.1.5. Target recognition and selection Identified motor neuron projections to specific target muscles were mapped first in the grasshopper, and later in *Drosophila* (Ball *et al.*, 1985; Sink and Whitington, 1991). In the grasshopper, motor neuron growth cones come into contact with muscle pioneers (see below) and show highly selective affinities for their specific targets. In *Drosophila*, similar muscle progenitors are the source of cue(s) that trigger motor axon defasciculation, targeting axons to restricted muscle fields (Landgraf *et al.*, 1999a). Subsequent to defasciculation, motor growth cones explore the forming muscle cells within the target domain, initially quite widely. But increasingly, filopodia are restricted to only a specific target myotube. When such a specific muscle target is ablated in the grasshopper, the motor axon growth cone that would have innervated it, instead continues its exploration to a more distal region (Ball *et al.*, 1985). In *Drosophila*, a combination of surgical and genetic manipulations have allowed specific muscle targets to be either duplicated or deleted in order to test targeting specificity. When ventral longitudinal muscles 6/7 are removed, for example, their motor neuron, RP3, grows past the normal target area and eventually innervates incorrect muscles in a variable pattern (Sink and Whitington, 1991). The motor growth cone terminates and differentiates into a synapse in the absence of its normal target, but only after a period of delay and continued searching. Similar observations are seen when other specific muscles are ablated (Cash *et al.*, 1992; Chiba *et al.*, 1993). Perhaps more interestingly, if a muscle target is duplicated, both muscles are innervated equally by the correct motor neuron. These observations show an extreme high level of specificity in motor axon target recognition (see also **Chapters 2.1** and **2.2**).

Motor neuron axons are guided to their proper muscles by a hierarchy of cues, starting with a neuronal transcription factor code that underlies their delivery to particular regions of the muscle field, and continuing with muscle target-specific transcription factor codes that drive the expression of defining cell surface receptors (Thor *et al.*, 1999; Odden *et al.*, 2002; see also **Chapters 2.1** and **2.2**). Different muscles express unique combinations of cell surface glycoproteins that mark them and, in at least some cases, clearly delineate the future site of motor neuron innervation in a subdomain of the myotube surface. For example, the homophilic NCAM Fasciclin III is expressed both on the RP3 growth cone and muscles 6/7, transiently at the time of target recognition (Halpern *et al.*, 1991), but is downregulated as synaptogenesis proceeds. In mutants where innervation is blocked or delayed, the myotubes still clearly express Fasciclin III in a restricted domain corresponding to the normal site of the NMJ (Broadie and Bate, 1993b). Likewise, the leucine-rich repeat (LRR) CAMs Connectin and Toll are also expressed on a small subset of muscles and the growth cones that innervate these muscles. Following target recognition, Toll is localized to synaptic sites on the muscle and downregulated (Nose *et al.*, 1992; Broadie and Bate, 1993b). These examples suggest that each muscle simply expresses a specific code of surface CAMs that allow selective adhesion with growth cones carrying a complementary set of CAMs. However, the mechanism is not simple. For example, genetic ablation of Fasciclin III, which prefigures the muscle's synaptic site prior to growth cone contact and is expressed by the growth cone during contact, does not prevent NMJ formation (Chiba *et al.*, 1995). Rather, target recognition appears, at most, only slightly impaired, consistent with the vague idea that multiple redundant recognition cues direct the first stages of synaptogenesis. Consistently, ectopic expression of these recognition factors uniformly causes more severe perturbation of neuromuscular connectivity (Chiba *et al.*, 1995). These embryonic connectivity studies have, in general, only detected a single axon innervating any muscle. However, most muscles are known to have multiple innervations in the mature larva (Keshishian *et al.*, 1996). Much of this later stage innervation is derived from the widespread terminals of ventral unpaired median (VUM) neurons, the likely source of octopaminergic type II NMJs of

larval muscles (Monastirioti *et al.*, 1995; Taghert and Veenstra, 2003). Nothing is yet known about the mechanism driving this later stage of neuromuscular target recognition and synaptogenesis.

2.3.2.2. Development of Motor Neuron Electrical Properties

Most of what we know about the electrical properties of developing insect neurons comes from studies in acutely dissociated cell cultures. In *Drosophila*, neurons developing *in vitro* from cells harvested at gastrulation, as well as cleavage arrested neuroblasts that give rise to "giant neurons" in culture, have been extensively used for defining the development of excitability and the function of genes encoding ion channels and their regulators (Solc *et al.*, 1987; O'Dowd and Aldrich, 1988; Saito and Wu, 1991; O'Dowd, 1995; Tsunoda and Salkoff, 1995a). However, after nearly two decades of *Drosophila* culture studies, spontaneous synaptic transmission between neurons *in vitro* has only recently been recorded electrophysiologically (Lee and O'Dowd, 1999, 2000), and evoked neuronal transmission has never been recorded.

This progress in fruit flies contrasts sharply with early strides made using other species. For example, limited cell migration and axon outgrowth were observed in cultured mosquito larva ganglia in 1937 (Pfeiffer, 1937) and, since then, researchers using the cockroach and other species have had great success propagating neuromuscular tissues in culture, both as explants and individual cells. The morphology, ultrastructure, and electrophysiology of these preparations (including that of NMJs formed *in vitro*) are relatively well described (Beadle and Hicks, 1985; Thomas *et al.*, 1987; Levine and Weeks, 1996). In contrast to this long history of *in vitro* studies, *in vivo* motor neuron electrophysiology in *Drosophila* has been limited to just a few recent studies, all concentrated on an accessible cluster of five motor neurons (aCC and RP1–4), and one interneuron (pCC), located in a superficial, dorsal position within the embryonic VNC (Rohrbough *et al.*, 2003). In the embryo, these identified neurons are accessible to patch-clamp recording from the cell body following removal of the overlying neurolemma with protease (Baines and Bate, 1998; Baines *et al.*, 1999).

In developing motor neurons of the *Drosophila* VNC, the first voltage-gated currents appear during mid-embryogenesis (stage 16, 13–14 h at 25 °C; ~65% embryonic development; Baines and Bate, 1998). During later embryogenesis (mid-stage 16 onwards; 70% development), motor neurons display a sequential appearance of voltage-gated ionic currents, including several outward K^+ currents, an inward Ca^{2+} current, and an inward Na^+ current (Baines and Bate, 1998; Baines *et al.*, 2001). Northern analyses show that *shaw* and *shab* transcripts encoding the I_K K^+ channels are expressed simultaneously in developing motor neurons from stage 13 (9 h at 25 °C; ~45% development; Tsunoda and Salkoff, 1995a). Consistently, the first detectable current to appear in cell body recordings is the delayed outward K^+ current (I_K). Subsequently, the calcium current (I_{Ca}) is detectable at 15 h (~70% development), the sodium current (I_{Na}) at 16 h (~75% development), and the rapidly inactivating potassium current (I_A) at 17 h (~80% development). The developmental sequence of current appearance is somewhat distinct from that seen in both *Drosophila* embryonic and pupal muscle (see below; also, Salkoff, 1985; Broadie and Bate, 1993d). Action potentials are first recorded at 17 h (~80% development), closely coincident with the appearance of I_A. By 85% development, *Drosophila* motor neurons manifest the ability to fire repetitive action potentials (Baines *et al.*, 2001).

Drosophila motor neurons exhibit multiple ligand gated receptors, although only a small subset of these have been documented *in situ*. The first ligand gated current to emerge is an ACh-gated inward cation current (I_{ACh}) during mid-embryogenesis (stage 16, 13 h at 25 °C; 62% development), at the same time that the first voltage-gated K^+ current is detectable (Baines and Bate, 1998). Picrotoxin-sensitive GABA receptors are present in cultured embryonic motor neurons (Lee and O'Dowd, 1999; Lee *et al.*, 2003), and are known to be present in *in situ* motor neurons during late embryogenesis (Rohrbough and Broadie, unpublished data), but the appearance and development of the GABA current has not been reported. Interestingly, the dorsal unpaired median (DUM) neurons of the grasshopper embryos respond to ACh (and GABA) very considerably earlier in embryogenesis (40% development), coincident with the initiation of axon outgrowth (Goodman and Spitzer, 1979). Thus, different species differ considerably in the timing of their neuronal electrical development, both in absolute terms and relative to the stage of neuronal morphological maturation.

Inhibitory GluRs are also present in *Drosophila* motor neurons, and a *Drosophila* homolog of this receptor family, expressed in oocytes, mediates a glutamate-gated chloride current (Cully *et al.*, 1996). The inhibitory glutamate-gated current is present in mature embryonic motor neurons during late embryogenesis (Rohrbough and Broadie, unpublished data), but its development has not been charted.

Endogenous activity in *Drosophila* motor neurons is first detected at 15 h (~71% development) in the form of very small (peak amplitude, 5 pA) spontaneous events that probably result from the opening of a small number of ion channels. Over the next hour, larger events (peak amplitude, 25 pA) first appear that represent synaptic currents, the first indication of synaptic transmission. The timing of the onset of synaptic function in *Drosophila* (70–75% development) is comparable to the appearance of transmission in DUM motor neurons in grasshoppers (75% development) but delayed relative to synaptic development between cercal afferents and central giant interneurons in cockroaches (55% development; Goodman and Spitzer, 1979; Blagburn *et al*., 1996). The peripheral neuromuscular junction in *Drosophila* first becomes functional just slightly early (65–70% development; Broadie and Bate, 1993e), corresponding to the time that motor neurons first display their full complement of ion channels.

2.3.2.3. Synaptogenesis in the CNS

Despite its molecular and genetic advantages, the small size of *Drosophila* has greatly hampered studies in the CNS of this species. Progress in understanding fruit fly CNS function is all very recent, and will be reviewed here. However, there is an extensive body of literature describing synaptic transmission between other insect species' neurons *in vivo* (Levine, 1984; Callec, 1985; Weeks *et al*., 1997). Most of this work focuses on mature synapses; relatively little is known about the development of CNS function.

In *Drosophila* cultured embryonic neurons, pharmacological analyses reveal that the majority of fast excitatory synaptic currents are cholinergic and mediated by nicotinic AChRs (Lee and O'Dowd, 1999, 2000). Less common inhibitory synaptic currents, mediated by picrotoxin sensitive GABA gated chloride receptors, are observed in a subset of cultured neurons. In *Drosophila* motor neurons *in situ*, the appearance of electrical currents and ligand gated neurotransmitter channels is followed closely by the onset of endogenous synaptic activity (Baines and Bate, 1998). The inputs onto these motor neurons are predominantly excitatory, cholinergic interneurons (Baines *et al*., 1999, 2001; Rohrbough and Broadie, 2002). *In situ*, both *Drosophila* motor neurons (aCC and RP2) and interneurons (pCC) exhibit infrequent fast, spontaneous events after 16 h (~75% of development; Baines and Bate, 1998), and then subsequently sustain inward currents supporting APs after 19–20 h (Baines *et al*., 1999, 2001). Both of these types of endogenous activity require external Ca^{2+}, and are reduced/eliminated by mutations that block AP firing, deplete SV supplies, or specifically eliminate cholinergic transmission, thus indicating that they represent cholinergic synaptic input (Baines and Bate, 1998; Baines *et al*., 1999, 2001). By 18–19 h at 25 °C (85–90% of development), synaptic input to motor neurons is present in the form of both fast AP mediated synaptic currents and periodic, sustained (up to 1 s duration) episodes of excitatory input occurring at least several times per minute (Baines *et al*., 1999, 2001). This sustained, "rhythmic" excitatory activity requires cholinergic synaptic input, since it is eliminated by conditionally blocking ACh synthesis in cholinergic interneurons (Baines *et al*., 2001). Sustained bouts of cholinergic input activate large excitatory potentials accompanied by bursts of AP firing in the motor neuron (Rohrbough and Broadie, 2002; Rohrbough *et al*., 2003). The output of this centrally generated motor activity is manifest at the NMJ as periodic bursts of high-frequency glutamatergic transmission to drive the peristaltic muscle contraction underlying coordinated locomotion (Broadie and Bate, 1993a; Baines *et al*., 2001; Cattaert and Birman, 2001).

In *Drosophila*, motor neurons develop in the absence of CNS synaptic transmission without any apparent change in synaptic connectivity (Baines *et al*., 2001). Although it is difficult to be certain that central synapses do not form in inappropriate areas under blockade conditions, it seems probable that there is no requirement for synaptic activity in establishing central synapses. In the absence of synaptic transmission, however, motor neurons do develop slightly abnormal basal electrical properties, including greater voltage activated Na^+ and K^+ currents, which result in altered neuronal excitability (Baines *et al*., 2001). The increased currents appear to be due, at least in part, to upregulated expression of *para* (I_{Na}) and *slowpoke* ($I_{K(Ca)}$)(Rohrbough *et al*., 2003). When neurotransmission is blocked, the number of action potentials fired by a depolarizing voltage ramp (−60 to +60 mV) is increased, and the amplitude of the initial action potential is significantly larger. The modulation of neuronal electrical properties is not permanent, but rather can be reversed by restoration of synaptic function. This finding suggests that synaptic transmission has at least some role in regulating the development of electrical properties. Somewhat surprisingly, eliminating synaptic transmission selectively in motor neurons, rather than globally as above, does alter synapse formation onto these cells (Baines *et al*., 1999). Ultrastructural analyses reveal fewer presynaptic input sites and a lower frequency of endogenous synaptic events onto synaptically silenced *Drosophila* motor neurons (Baines *et al*., 1999).

These results suggest that synaptic output by the postsynaptic motor neuron regulates synaptogenesis by interneurons onto these cells. Why might pan-neuronal transmission blockade cause different effects than selective blockade only in a few motor neurons? Perhaps CNS competition between neurons is a decisive factor in regulating circuit formation in the *Drosophila* embryo. A similar mechanism in mammals is central to synapse assembly in systems ranging from retinotectal projections to motor terminals.

In addition to its role in axon fasciculation (above), the homophilic NCAM Fasciclin II operates to stabilize synaptic connections and mediate activity dependent plasticity in the postembryonic NMJ (Schuster *et al.*, 1996a, 1996b; Davis *et al.*, 1997; Davis and Goodman, 1998). At the NMJ, Fasciclin II is expressed in pre- and postsynaptic cells from early stages of synaptogenesis, although it is not required for synapse formation (Schuster *et al.*, 1996a, 1996b). In contrast, overexpression of Fasciclin II in embryonic muscle can initiate ectopic synapse formation, suggesting that postsynaptic Fasciclin II may be limiting during synaptogenesis (Davis *et al.*, 1997; Davis and Goodman, 1998). Based on these findings, the role of Fasciclin II in CNS synaptogenesis was examined, and it was shown that Fasciclin II acts as an important determinant of central synapse regulation (Baines *et al.*, 2002). As observed at the NMJ, synaptogenesis by identified neurons in the embryonic CNS does not require Fasciclin II, but the postembryonic elaboration of these synaptic connections is impaired in the absence of Fasciclin II. In addition, just like the NMJ, central synapses are highly sensitive to alterations in the balance of Fasciclin II between pre- and postsynaptic cells. Increasing Fasciclin II in either cell during embryogenesis disrupts synaptic connectivity, causing defects comparable to the targeted blockade of synaptic transmission (Baines *et al.*, 2002). In contrast to the findings at the NMJ, however, and to work on the Fasciclin II homolog in the mammalian hippocampus, elevated Fasciclin II reduces the number of functional synapses in the *Drosophila* CNS. Thus, in flies, Fasciclin II is not required for synaptogenesis but can regulate the elaboration of synaptic contacts, especially during postembryonic development, in a synapse-type specific manner.

2.3.3. Development of Glial Function

2.3.3.1. Central Glia and CNS Scaffolding

Compared to neurons, relatively little has been revealed so far about the functional development of glial cells in insects, although their importance for insect development has long been recognized (Anderson *et al.*, 1980; Oland and Tolbert, 2003). Many classes of glial cells (glia) have been identified in insects. Two major classes include: (1) glia that surround or enclose neuronal cell bodies and (2) glia that first prefigure and later wrap around axon tracts (Jacobs and Goodman, 1989). In the CNS, a prominent set of glia exist at the midline. One subset of midline glia act as the source of signals that attract (Netrin) and repulse (Slit) motor axons from crossing the midline, and thus determine ipsilateral versus contralateral motor axon projections. The Slit signal working on related Roundabout receptors (Robo I, II, and III) also determines the lateral distance traveled prior to axonal turning into discrete fascicles. As discussed above, Slit signaling also plays a role in establishing dendritic domains relative to the CNS midline. A separate subset of midline glia mediate contact-dependent guidance and, later, insulation functions.

In *Drosophila*, six midline glia per hemisegment are characterized by unique gene expression patterns, and mutation of these genes reveal critical axonal guidance functions (Klambt *et al.*, 1991; Hidalgo, 2003). These six glia first prefigure the future path of the axons, just before axons are extended. These cells continue to divide and, in later stages, enwrap the longitudinal axonal connectives in the CNS. Many other glia are not associated with axons but rather reside in the cell body layers in direct contact with the neuronal soma. The role of these glial cells is not well established. The mesodermally derived belt glia extend circumferentially around the CNS, generating a tough enclosing sheath that acts as a blood–brain barrier to chemically isolate the CNS from the rest of the body.

Recent work in *Drosophila* has begun to systematically reveal genes specific to glial cell functional development. A genomic screen utilizing *glial cells missing* (*gcm*, a gene encoding a protein that determines whether cells assume glial or neuronal fates, Jones *et al.*, 1995; see also below) mutants, has identified >80 new *Drosophila* genes with expression restricted to glia (Freeman *et al.*, 2003). These genes show expression patterns varying from pan-glial, through peripheral/CNS specific, to select subpopulations of glia. This work shows that a midline-derived netrin signal orients glia cell migration via a repulsive netrin receptor (dUnc-5), and *draper*, one of the newly identified genes, mediates glial wrapping (Freeman *et al.*, 2003; see also below). Importantly, many (>80%) of the identified genes have close homologs expressed in corresponding sets of mammalian glia, indicating conserved molecular bases of glial function.

2.3.3.2. Peripheral Glia and Motor Neuron Pathfinding

Peripheral glia arise from the neural crest in vertebrates and the lateral edge of the CNS in insects. Peripheral glia in *Drosophila* are molecularly distinct from CNS glia. Both express unique proteins, such as gliotactin in the peripheral glia (Auld *et al.*, 1995), and wrapper in the central glia (Noordermeer *et al.*, 1998). In *Drosophila*, each hemisegment neuromere has two peripheral nerve roots, by which motor axons exit and sensory axons enter the CNS. Several classes of glial cells are associated with these peripheral nerves at different points. First, there are a small number of glial cells associated with the segmental (middle of neuromere) and intersegmental (neuromere boundary) nerve roots, where they diverge from the main CNS axonal tracts. These cells are present prior to axon extension, and ablation of this glial class in the grasshopper prevents formation of the peripheral nerve (Bastiani *et al.*, 1986). Second, the so-called "exit glia" are located just outside the CNS, where the segmental and intersegmental neurons diverge into peripheral axonal pathways. In addition, there are several glial cells that associate with peripheral axonal pathways, and enwrap the axons once the paths have been established.

Most peripheral glia are born in the CNS near the lateral exit points for the peripheral nerves. Lineage analyses in *Drosophila* have shown that approximately 8–10 (some variation) peripheral glia per hemisegment arise specifically from neuroblasts 1–3 and 2–5 (Schmid *et al.*, 1999; Sepp *et al.*, 2001). At least one further glial cell is born in the periphery, associated with the ISN, and remains in its birth place through later development (Hidalgo, 2003). During mid-embryogenesis, the centrally born glia migrate over great distances to their final peripheral locations. These cells migrate as a continuous chain of glia, with the pioneering glial cells actively exploring the environment using Actin-based filopodia (Sepp and Auld, 2003). The small GTPases RhoA and Rac1 mediate the Actin cytoskeletal rearrangements required for this migration (Sepp and Auld, 2003).

In the CNS, the immature glia seem to act as intermediate guidepost cells for pioneer motor axons migrating into the periphery. Peripheral glia prefigure axon paths through the CNS/periphery transition zone through which axons migrate into and out of the CNS. When peripheral glia are ablated early, via targeted expression of the cell death genes *grim* and *ced-3*, motor axon trajectories are initially perturbed, although later largely corrected (Sepp *et al.*, 2001). This suggests that peripheral glia do act as early guideposts for motor axons, but that other cues in the periphery are sufficient to guide these axons to their correct target muscles. Sensory axons entering the CNS are also disrupted, but these pathfinding errors are not corrected (Sepp *et al.*, 2001). Following this early guidance, motor axon growth cones extend past the immature glia and begin to pathfind into the developing muscle field. At this point, these motor axons appear to now serve as the guidance substrate for the migration of glial cells into the periphery (Sepp *et al.*, 2000). For example, in *Drosophila*, the aCC neuron pioneers the ISN during stage 12 (~40% development). Once it has passed the 8–10 glia at the CNS exit point, the glia begin to follow the aCC axon into the periphery (Sepp *et al.*, 2000). Importantly, the glial growth cones never appear to extend past the aCC pioneer growth cone, suggesting that the aCC is acting as the substrate for glial cell migration. During stages 13–15 (47–60% development), the peripheral glia first follow the outgrowing motor axons through the ventral field, and then the mixed motor/sensory axons, through the dorsal field (Sepp *et al.*, 2000).

In *Drosophila*, genetic mutations that impair glial differentiation result in profound defects in peripheral nerves. For example, mutations in the homeobox transcription factor *gcm* gene disrupt the specification of most glia and cause severe axonal defasciculation and pathfinding errors (Jones *et al.*, 1995). However, these mutations affect all glial cells, not just the peripheral glia selectively, so the cause of these defects is uncertain. Ablation or genetic disruption of peripheral glia results in defasciculated axon tracts (Sepp *et al.*, 2001), but it has yet to be genetically proven that peripheral glia mediate axonal pathfinding. Indeed, the developmental profile given above suggests the opposite: in peripheral axons, first motor axons ventrally and then sensory axons dorsally provide guidance for the peripheral glia to reach their mature destinations.

2.3.3.3. Glial Wrapping of Peripheral Axons

During migration from the CNS to their final peripheral destinations, glial cells already begin to extend processes that enwrap surrounding motor and sensory axons. Although considerable glial wrapping occurs during the last several hours of *Drosophila* embryogenesis, glia do not entirely wrap motor axons in the embryo, and the terminal ends of motor axons remain unsheathed at hatching (Auld *et al.*, 1995; Sepp *et al.*, 2000). This suggests that peripheral glia are not directly involved in the generation of neuromuscular contacts or the establishment

of functional NMJs. During postembryonic development, peripheral glia continue to extend cytoplasmic process, until axonal wrapping is complete and glial processes extend completely to the NMJ (Sepp *et al.*, 2000). The glial ensheathment surrounds all the axons on the nerve fascicle in a single bundle, insulating a common axonal compartment from the surrounding hemolymph. This process occurs gradually over the first and second instar stages, and yet must be remarkably rapid to keep up with the impressive rate of larval growth. All wrapping in larvae appears to be due to extension of existing glial processes, since further glial division does not appear to occur in postembryonic stages (Sepp *et al.*, 2000). By the third instar, glial processes extend completely to the NMJ. In both type I and type II NMJs, glial processes are usually observed extending to the first bouton on the synapse, although never beyond this point (Sepp *et al.*, 2000).

Glial cells regulate neural activity by insulating neurons and controlling the axonal microenvironment. Peripheral glia help maintain the appropriate ionic environment to facilitate AP propagation in motor axons. In insects, for example, peripheral glia shield axons from the high K^+ concentration in the hemolymph, which would be predicted to largely abolish neuronal excitability. Little is known about the molecular mechanisms underlying glial wrapping of axons. However, a *Drosophila* mutant named *fray* has revealed a novel class of serine/threonine kinase in peripheral glia required for axon ensheathment (Leiserson *et al.*, 2000). The *fray* mutants do not ensheath peripheral axons properly, leading to axonal defasciculation and early larval death (Leiserson *et al.*, 2000). In normal embryos, the tight glial barrier is maintained by septate junctions between glial cells, which serve the function of vertebrate tight junctions to maintain a selective-diffusion barrier. Tight junctions are absent in insects. *Drosophila* has two types of septate junction (SJ), smooth (sSJs) and pleated (pSJs) (Campos-Ortega and Hartenstein, 1997). There are five characterized SJ associated proteins in *Drosophila*, all with clear roles in SJ formation: Gliotactin (Gli), Neurexin IV (Nrx), Discs large (Dlg), Scribble (Scrib), and Coracle (Cora). Mutation of any of these genes disrupts SJ formation (Woods and Bryant, 1993; Auld *et al.*, 1995; Baumgartner *et al.*, 1996; Bilder *et al.*, 2003). Gli is a cholinesterase-like molecule (noncatalytically active) that is a member of a class of adhesion proteins termed Electrotactins (Auld *et al.*, 1995; Schulte *et al.*, 2003). Nrx is a transmembrane protein, primarily characterized as a synapse associated protein (Baumgartner *et al.*, 1996). Neurexins were first identified as synaptic receptors for Latrotoxin, the component of black widow spider venom that triggers massive exocytosis, and are thought to be involved in presynaptic differentiation in vertebrates (Scheiffele *et al.*, 2000). Dlg and Scrib are membrane associated PSD-95/Dlg/ZO-1 (PDZ) domain containing proteins that act as multiprotein complex scaffolds (Woods and Bryant, 1993; Bilder, 2001; Bilder *et al.*, 2003). Cora is a Band 4.1 homolog with a four point One/Ezrin/Vadixin/Moesin protein–protein interaction domain that associates Band 4.1 protein attaches the cell cytoskeleton to transmembrane signaling proteins.

Gliotactin is expressed throughout the processes of peripheral glia in late embryos and larvae and is required for the formation of the peripheral blood–nerve barrier (Auld *et al.*, 1995). In *gli* mutants, peripheral glia develop normally at the light microscope level, but ultrastructurally they contain small gaps in the glia–glia cell interfaces, compromising the peripheral blood–nerve barrier. This can be shown experimentally by exposing a nerve to an electron-dense dye, which is completely excluded from the axonal compartment in normal embryos but penetrates the impaired glial sheath of the mutants (Auld *et al.*, 1995). In *gli* mutants, the high $[K^+]$ hemolymph also contacts motor axons, action potentials fail to propagate, and the embryos are paralyzed. Low $[K^+]$ saline rescues both action potential propagation and movement (Auld *et al.*, 1995). Furthermore, in *gli* mutants, Nrx, Dlg, and Cora expression is strongly perturbed. In contrast to the other SJ mutants, SJ are present in *gli* mutants, but are morphologically immature (Schulte *et al.*, 2003).

Neurexin IV (Nrx) localizes to septate junctions in peripheral and subperineural glial junctions (Baumgartner *et al.*, 1996). As in *gli* mutants, *nrx* mutants maintain peripheral glial cells that look normal at the light microscope level. However, ultrastructural examination reveals that *nrx* mutants lack the glial–glial SJs that are required to maintain the tight blood–nerve–brain barrier. As a result, *nrx* mutants, like *gli* mutants, lack action potentials and are paralyzed (Baumgartner *et al.*, 1996). Loss of Nrx causes the mislocalization of Cora, the *Drosophila* protein 4.1 homolog, and eliminates ultrastructurally defined SJs.

Another member of the Neurexin protein superfamily is encoded by the *Drosophila axotactin* (*axo*) gene (Yuan and Ganetzky, 1999). Unlike the transmembrane proteins described above, Axo is secreted by glia and subsequently localized to axonal tracts. Mutations in *axo* are temperature sensitive; at 37 °C, action potentials are blocked (Yuan and Ganetzky, 1999). However, the blood–nerve–brain barrier is

intact in *axo* mutants. This suggests that Axo is a component of a glial–neuronal signaling pathway that acutely regulates the electrical properties of target axons.

To summarize, these results demonstrate that insect glial cells are vital for the acute maintenance of neuronal signaling. Defined glial functions include: (1) glial wrapping to insulate motor axons from the ionic composition of the hemolymph and (2) glial-neuronal communication required to develop or maintain neuronal signaling properties.

2.3.4. Development of Muscle Function

2.3.4.1. Muscle Morphogenesis and Patterning

2.3.4.1.1. Early myogenesis Fate-mapping techniques place the primordial cells of mesodermal structures, prominently including the somatic muscles, in the midventral regions of the blastoderm embryo. These cells will become mesoderm, but they do not appear to possess any finer fate restriction at this stage. Thus, zygotic gene expression in the blastoderm defines the mesodermal anlage, but later information is required to specify precise developmental pathways, such as those leasing to development of fat body, heart, blood, somatic, and visceral muscle (see also **Chapters 2.1**, **2.2**, **2.6**, and **2.9**). During gastrulation, presumptive mesodermal cells move into the interior of the embryo along the invaginating ventral furrow. This embryonically defined germ layer corresponds with a distinctive domain of gene expression (e.g., *twist* and *snail*). Twist (basic HLH transcription factor) and Snail (zinc finger transcription factor) are required for the formation of the mesoderm as mutants in both genes fail to form any mesodermal derivatives. Twist activates many mesoderm specific genes, whereas Snail suppresses the expression of nonmesodermal genes (Leptin, 1991; Casal and Leptin, 1996). Different domains of Twist expression (high and low) are apparent in the mesoderm following gastrulation, and the somatic (body wall) muscles are derived from only the high [Twist] domains. Twist drives the transcription of the *Drosophila* homolog of myocyte enhancer factor (dMef2), an essential regulator of the myogenic pathway from the mesoderm (Baylies and Bate, 1996; Artero *et al*., 1998).

In extended germ band embryos, such as *Drosophila*, the patterning of the mesoderm takes place beginning at the period between gastrulation and germ band retraction. This period is characterized by migration, proliferation (cell division), and restructuring of the mesodermal progenitor cells. Invaginating cells appear to be committed to mesodermal fates at the time of gastrulation, but their specific fates within the mesoderm are still labile at this time. Numerous waves of mitosis occur in the mesoderm (at least four rounds of division in *Drosophila*), with the final divisions continuing during late germ band retraction and soon after shortening is completed. The exact number of cell divisions seems to vary widely between different progenitor cells. Mesodermal cells begin to acquire specific differentiated fates just prior to or at the time of germ band retraction. Cell transplantation studies indicate that the fate of a mesodermal cell is dictated by its position within the invaginated mesoderm and by its final position relative to the embryonic ectoderm.

Following migration, mesodermal cells form a thin sheet covering the inner surface of the developing ectoderm. Classic experiments performed in the 1940s to 1950s on beetle and lacewing embryos indicated that mesodermal fates are dependent on the adjacent ectoderm. These studies suggested that early mesodermal cells are equivalent until fate restrictions are dictated by the ectodermal cells they come in contact with. Genetic studies in *Drosophila* have revealed two important ectodermal signals: Decapentaplegic (Dpp), a transforming growth factor-β protein, and Wingless (Wg), a Wnt signaling protein. Dpp acts as an inductive signal to divide myoblasts into dorsal and ventral domains, whereas Wg appears to regulate Twist levels and is required for the specification of the majority of muscles (Staehling-Hampton *et al*., 1994; Baylies *et al*., 1995; Riechmann *et al*., 1997).

The formation of somatic muscles is first detectable during germ band retraction. The somatic mesoderm gives rise to myoblasts that have the capacity to fuse with appropriate neighboring cells. Nascent syncytia, formed when myoblasts begin to fuse, first appear ventrally, immediately adjacent to the developing CNS, at the onset of germ band retraction (Bate, 1990). As germ band retraction continues, myoblasts fuse with syncytia in lateral and dorsal locations. Each developing syncytial myotube continues to enlarge via fusion with neighboring myoblasts, so that the syncytia rapidly become more prominent while the pool of unfused myoblasts dwindles. Myoblast fusion continues for several hours, with the size of individual myotubes dictated by the number of fusion events (Bate, 1990). For any given muscle, the number of nuclei is tightly controlled but is not invariant. While fusion continues, and following its completion, the enlarging myotube extends growth cone-like processes that reach over the surface of the epidermis and probe for attachment sites (Bate, 1990). By ~60% embryonic development in *Drosophila*, muscle assembly

appears complete: fusion events stop and muscles are attached to the epidermis to form the specific muscle field pattern. The end of muscle assembly coincides with the arrival of motor neuron growth cones exploring the surface of the myotubes in preparation of forming NMJs. Innervation plays no part in establishing or maintaining the muscle pattern (Broadie and Bate, 1993c).

Myoblast fusion is a multiple-step process involving initial cell recognition/adhesion, formation of a prefusion complex, plaque formation, and, finally, plasma membrane fusion driving to myotube formation. This cellular process appears well conserved between *Drosophila* and vertebrates. Genetic analyses in *Drosophila* have identified a growing number of genes specifically required for myoblast fusion. The *rolling stone* (*rost*) gene, for example, encodes an adhesive transmembrane protein involved in the early stages of myoblast recognition/adhesion and development of the prefusion complex (Frasch and Leptin, 2000). The *myoblast city* (*mbc*) gene encodes a homolog of human DOCK180, and functions as an upstream regulator of the Rho family GTPase Rac1 (Rushton *et al*., 1995; Frasch and Leptin, 2000). The *blown fuse* (*blow*) gene encodes a cytoplasmic protein required for progression between intercellular recognition/adhesion and formation of the prefusion complex (Doberstein *et al*., 1997). The *titin* gene encodes an enormous elastic protein known to regulate cytoskeletal scaffolds. During myoblast fusion, Titin organizes cytoskeletal elements required for formation of membrane plaques between prefused cells, and later controls organization of the contractile cytoskeleton (Zhang *et al*., 2000).

2.3.4.1.2. Establishing muscle identity *Drosophila* syncytial muscles form a precise pattern. Each muscle has a unique identity defined by its size (number of nuclei), shape (arrangement of epidermal attachment sites), and pattern of innervation (Bate *et al*., 1999). This identity is presumably determined by transcription factor cascades that trigger expression of slightly different sets of genes, such that each muscle cell displays a specific number of myoblast fusion events and a set of cell surface proteins. Depending on the cell surface proteins they express, myoblasts recognize the appropriate attachment sites on the epidermis and the appropriate innervating neurons extending from the CNS. Consistent with this hypothesis, a small number of spatially restricted transcription factors have been identified (Michelson *et al*., 1990; Riechmann *et al*., 1997; Carmena *et al*., 1998) that define small subsets of myotubes from the onset of myogenesis. In several cases, the expression of these transcription factors is restricted to a single myoblast, but expression extends to subsequent nuclei following fusion. These observations suggest that unique cells may be specified based on position, provided an identity based on expression of one or more distinguishing transcription factors, and endowed with the power to recruit neighboring myoblasts through fusion and subsequent influence of gene expression (see also **Chapters 2.1** and **2.2**).

The founder cell hypothesis for establishing muscle identity was proposed by Michael Bate in 1990 (Bate, 1990). In this model, the essential step in crystallizing the muscle pattern is a process that selects a unique "founder cell" for each muscle in every segment. In *Drosophila*, 30 founders are specified for the 30 muscles in an abdominal hemisegment. The founder cells have the ability to initiate fusion with the surrounding cloud of myoblasts and are, thus, termed "fusion-competent" cells. These cells are proposed to be naive or equivalent myoblasts that are subsequently entrained to a particular muscle identity only following fusion with a founder cell myoblast. Muscle founder cells express combinations of transcriptional regulators that confer a specific identity, such as Krüppel (Kr) and S59/Slouch (Slou) (Ruiz-Gomez *et al*., 1997; Baylies and Michelson, 2001). The *dumbfounded* (*duf/kire*) gene is expressed only in founder cells, and its product acts as an attractant or adhesive required to initiate fusion with fusion-competent cells (Ruiz-Gomez *et al*., 2000). Ectopic expression of *dumbfounded* causes inappropriate cellular fusion, indicating that this protein alone confers the ability in founder cells to initiate fusion. The expression of genes such as *sticks and stones* (*sns*) and *myoblasts incompetent* (*minc*) is restricted to fusion-competent cells, suggesting they are a selected population of cells determined by distinctive regulatory mechanisms (Ruiz-Gomez *et al*., 2002). In the absence of these genes, myoblasts fail to fuse and remain as an arrested population of mesodermal cells (see also **Chapters 2.1** and **2.2**).

Although derived primarily from observations in *Drosophila*, this founder cell hypothesis was influenced by earlier work in grasshopper embryos. Cell ablation studies in the grasshopper have shown that muscle progenitors, called "muscle pioneers," are uniquely identifiable cells containing fate restriction to become specific muscles (Ho *et al*., 1983; Ball *et al*., 1985). In the grasshopper, muscle pioneers are distinctive large cells that extend growth cones to define the mature muscle placement, and subsequently fuse with neighboring myoblasts to form the syncytial myotubes. Ablation studies have shown

that removal of a pioneer results in the loss of the muscle, despite the fact that the bulk of the fusion-competent myoblasts remain in place (Ball and Goodman, 1985a, 1985b). In *Drosophila myoblast city* mutants that block fusion, founder cells act similarly to grasshopper pioneer cells in spanning the area of the future muscle and otherwise acquiring the mature muscle properties (Rushton *et al.*, 1995; Prokop *et al.*, 1996). Thus, the founder cell hypothesis proposes that unique cells, founder cells in *Drosophila* and muscle pioneers in the grasshopper, seed the muscle pattern and are irreplaceable for the specification of muscle identity. In *Drosophila*, the adult muscles appear seeded in a similar way. When *twist* expression declines rapidly following the onset of germ band retraction, it leaves behind a small subset of *twist* expressing cells as isolated single cells associated with the developing PNS (Bate *et al.*, 1991). These single cells divide starting in early larval stages to form the progenitors for specific subsets of adult muscles (Broadie and Bate, 1991; see also **Chapter 2.2**).

There are considerable parallels between the specification of muscle founder cells and neuroblast specification in the developing nervous system. As in the nervous system, the earliest indication of muscle specification is the appearance of "competence domains" expressing the proneural gene *lethal of scute* (*l(1)sc*) (Carmena *et al.*, 1995, 1998). As in the nervous system, each muscle competence group gives rise to a single progenitor (the founder cell), which specifies a specific muscle in the mesoderm. Furthermore, as in the nervous system, the Notch signaling pathway blocks all other cells from adopting the founder cell fate (lateral inhibition mechanism). In neurogenic mutants, the number of founder cells in the mesoderm, defined by patterns of gene expression, is dramatically increased. Rather than discrete individual cells, clusters of presumptive founder cells are observed at similar positions in the developing mesoderm (Ruiz-Gomez *et al.*, 1997). Thus, as in the nervous system, neurogenic genes appear to act in a lateral interference mechanism to select a specific muscle founder cell from a group of competent cells.

2.3.4.1.3. Development of muscle integrity Prior to recognition of the muscle target by the motor axon growth cone, *Drosophila* myoblast fusion is complete and the newly formed syncytial myotubes have finalized their attachments to the epidermis, generating the mature muscle pattern. Consistent with this timing, the arrest of motor axon outgrowth does not alter the development of the muscle pattern (Broadie and Bate, 1993c). However, at this stage (12–13 h at 25 °C; ~60% embryonic development), myotubes are extensively electrically and dye-coupled together into large multifiber networks, but lack individual integrity (Broadie and Bate, 1993e). Myotubes are interconnected to their neighbors via extensive cytoplasmic bridges, surrounded by thin, patchy basal laminae (Rheuben *et al.*, 1999). Coupling occurs between myotubes of different positional classes (longitudinal, transverse, oblique) within a hemisegment, as well as, strangely, across segmental borders. It is not clear how cytosolic bridges span the segmental boundaries. The purpose of the extensive connectedness of developing myotubes is not known either, but it may aid in intercellular communication or help to maintain synchronous myogenesis within and between segments.

During early stage 16 (13–14 h at 25 °C; 62–65% development), *Drosophila* myotubes abruptly and synchronously uncouple (Broadie and Bate, 1993e). This uncoupling coincides with the contacts between myotubes and motor neuron partners. Uncoupling also appears to coincide with the onset of electrogenesis in the myotubes (Broadie and Bate, 1993d). However, because it is difficult to effectively voltage-clamp the coupled myotubes, it is formally possible that currents may be initiated at earlier stages. In the absence of innervation, myotubes uncouple at the correct time and to the correct extent, and the appearance and maturation of muscle electrical properties is not detectably perturbed (Broadie and Bate, 1993d). Therefore, uncoupling is not due to instruction from the motor neuron.

After uncoupling, detectable dye transfer is restricted to <10% of dye injected myotubes, a figure that remains relatively constant for the remainder of embryonic and larval development (Ueda and Kidokoro, 1996). Although relatively strong electrical coupling between myotubes persists through maturity, tight voltage-clamp of individual myotubes is now readily accomplished. At maturity, the intersegmental coupling coefficients between muscles 6 and 7 remain high (range of 0.33–0.43), although coupling with other muscles within the segment is much lower or undetectable (Ueda and Kidokoro, 1996). Coupling of adjacent muscle 6 cells between segments (intrasegmental coupling) is still reliably detected (coupling coefficient of 0.16). The purpose of muscle electrical coupling is unknown, but it may be to aid the coordination of muscle contraction during locomotion, particularly between muscles acting like a single functional unit, such as the ventral longitudinal muscles 6 and 7.

In the *Drosophila* neuromusculature, as in the neuromusculature of other insects (Walther, 1981), the myotubes appear to actively engage in guiding

motor axons to the correct target area of innervation (Bate, 1990). Myotubes extend dynamic Actin-based processes towards the filopodia of the motor axon prior to contact. The resemblance of the two motile projections has led to the term "myopodia" to describe the myotube processes (Ritzenthaler *et al*., 2000). Following the completion of myoblast fusion, myopodia appear to extend randomly from the myotube, but as they contact and court the motor axon filopodia, they become progressively restricted to the developing site of innervation. This restriction to the innervation site is due to interaction with the neuronal growth cone, as myopodia clustering does not occur in the absence of motor neuron outgrowth (Ritzenthaler *et al*., 2000). It is unclear whether restriction of myopodia to the innervation site is due to instruction from the motor axon growth cone or reflects mutual interactions between the synaptic partners. However, genetic manipulation of the expression of the myotube proteins α-Integrin (PSII) and Toll, which regulate synaptic adhesiveness, reduces the frequency of myopodia clustering during innervation (Ritzenthaler and Chiba, 2003). It was, therefore, suggested that myopodia clustering is due to the adhesion between a subset of myopodia and filopodia of the innervating growth cone, which maintains these processes while the others are eliminated. Although these observations show that myopodia join filopodia in a dynamic dance during target recognition, there is as yet no indication of the role myopodia play in this process, or even whether they are required for neuromuscular synaptogenesis.

2.3.4.2. Development of Muscle Electrical Properties

The first intracellular recordings from insect muscle (and functional characterization of insect neuromuscular transmission) was published in 1953 by Castillo, Hoyle, and Machne (Castillo *et al*., 1953). Since then, insect muscles have been the subject of many detailed electrophysiological studies, and a large body of literature exists that describes these findings (Ashcroft, 1982; Aidley, 1985). Few of these studies have sought to understand development of neuromuscular transmission, however, or sought to trace changes in electrical properties over time (Saito and Kawai, 1987; Rose *et al*., 2001; Keyser *et al*., 2003). The relevant findings from *Drosophila* are reviewed below.

As mentioned earlier (Section 2.3.1.1.3), five voltage-gated currents are present in the larval muscles; an inward Ca^{2+} current (I_{Ca}), two outward K^+ currents (I_A, I_K), and two outward Ca^{2+} dependent K^+ currents (I_{CF}, I_{CS}) (Singh and Wu, 1999). These currents mature in a rapid sequential order and are first detectable at the time that the myotubes uncouple during early stage 16 (~62% embryonic development), when muscles can first be reliably voltage-clamped (Broadie and Bate, 1993a, 1993d). Apparent already when the myotubes uncouple, is a small inward, voltage-gated I_{Ca}. Initially, this is the only current apparent in voltage step recordings, indicating that it is the first voltage-gated current present in embryonic muscle (Broadie and Bate, 1993c, 1993d). A stretch-activated, outward K^+ current is also recordable as soon as myotubes uncouple, and this persists throughout later development. Within 1 h of myotube uncoupling, at ~14 h (~66% development), outward voltage-gated K^+ currents appear in the myotubes. Two distinct classes of K^+ current appear at the same time: (1) a delayed, noninactivating K current (I_K) and (2) a rapid-onset, rapidly inactivating A current (I_A) (Broadie and Bate, 1993d). Finally, much later in embryogenesis, during mid-late stage 17 (~18 h at 25 °C; ~85% development), calcium-dependent K^+ currents appear. Again, two distinct classes of current are first detectable at or close to the same time: (1) a rapid, inactivating I_{CF} and (2) a delayed noninactivating I_{CS} (Broadie and Bate, 1993e).

The developmental profile of these different myotube currents is specific for each class of current and very different between currents. I_{Ca} is already quite robust and easily detectable when the myotubes uncouple, and its amplitude increases gradually but continuously throughout embryogenesis; at hatching, the I_{Ca} current peak amplitude has approximately doubled (Broadie and Bate, 1993c, 1993d). In contrast, the amplitude of the K^+ currents increases much more rapidly soon after their appearance. The I_K current climbs quickly in the first 2 h following myotube uncoupling, then levels off to a relatively gradual increase for the rest of embryogenesis (Broadie and Bate, 1993d). The I_A current climbs much more rapidly showing a dramatic increase in amplitude soon after myotube uncoupling at ~18 h (~85% development). At this point, I_A has peaked and shows no further increase through hatching (Broadie and Bate, 1993d). The late appearing calcium-gated K^+ currents are small relative to the other currents, and display a gradual rise in amplitude during the last hours prior to hatching. As a result of these developmental profiles, the voltage-gated K^+ currents dominate the myotube current profile throughout embryonic myogenesis, with the I_A current having the largest amplitude and the I_K current having the second largest. In whole cell recordings, the inward I_{Ca} current is masked by these outward K^+ currents

but, when the K^+ currents are removed, the I_{Ca} is revealed to be robust and have the third greatest amplitude. The calcium-gated K^+ currents contribute only marginally to the myotube current through the end of embryogenesis. Development of these currents in postembryonic muscles has not been reported.

The timing and order of appearance of currents in myotubes differs from motor neurons. In general, electrogenesis in neurons is delayed, albeit relatively slightly, relative to myotubes. In both cases, I_{Ca} and I_K are the first currents to appear, nearly synchronously, but the order of their very earliest detection is reversed in neurons relative to myotubes. In neurons, the next current to appear is I_{Na}, which is not present in insect muscles. Finally, I_A appears markedly later in neurons than in muscles. In neurons, the appearance of I_A coincides with the first action potentials (see above). The Ca^{2+} dependent K^+ currents have not been reported in embryonic neurons, either because they are not yet present or are not easily detectable. In embryonic muscles, the Ca^{2+} dependent K^+ currents are the last to appear (just prior to hatching), and remain very small and relatively hard to detect.

The properties of currents in embryonic myotubes appear similar to currents in mature larval muscle. The notable exception is the embryonic I_A current. During early myogenesis, prior to 14 h at 25 °C (<65% development), the I_A current has a midpoint of steady-state inactivation 40 mV more negative than in the mature embryo or larva (Broadie and Bate, 1993d). An I_A current with this inactivation gate should be entirely inactivated at the normal embryonic myotube resting potential of ~ -60 mV. Experimentally, a prolonged, negative voltage prepulse of ~ -100 mV is required to completely activate the I_A current in the early myotube (Broadie and Bate, 1993d). Therefore, the prominent I_A current reported here is predicted to be completely nonfunctional under normal developmental conditions. As myogenesis proceeds, however, a developmental change can be observed, as the I_A steady-state inactivation curve develops a biphasic character (Broadie and Bate, 1993d). This change suggests that a low-voltage inactivation I_A channel is present early and replaced later by a high-voltage inactivation I_A channel. The I_A current at all developmental stages is completely eliminated in *Shaker* (Sh^{KS133}) mutants, suggesting that the Shaker I_A channel may be differentially regulated during development relative to the mature muscle (Broadie and Bate, 1993d). The mechanism and significance of this developmental shift in I_A channel properties remains unclear.

2.3.4.3. Development of Muscle Contractile Properties and Patterned Movement

The development of neuromuscular behavior in *Drosophila* has recently been quantitatively assayed using videomicroscopy to monitor peristaltic muscle contraction waves during the last quarter of embryogenesis, starting shortly after the completion of the muscle pattern and innervation through hatching (Suster and Bate, 2002). The muscle contractile machinery driving this movement closely resembles that of the vertebrate muscle: the basic contractile unit is the sarcomere, a Myosin thick filament surrounded by a regular array of Actin thin filaments (Zhang *et al*., 2000). Myosin and Actin are both expressed first in myoblasts during the process of fusion to form myotubes (~10 h at 25 °C; 40–50% development). If fusion is prevented, Myosin expression is clearly maintained in the unfused myoblasts (Rushton *et al*., 1995), indicating that the synthesis of contractile machinery is independent of myotube formation. Assembly of the contractile apparatus is a lengthy process, with the muscles first assembling recognizable sarcomeres at the end of stage 15 (~12.5 h at 25 °C; ~60% development). The first weak endogenous movements are observed at around 14–15 h (66–71% development), and electrical stimulation of the motor nerve first leads to a detectable muscle twitch during this same period (Broadie and Bate, 1993e). The embryo first begins to move vigorously during the period of 16–18 h (76–86% development), at which time clear body length peristaltic waves are first observed.

Embryonic movements include vigorous, repeated forward and backward waves of peristalsis (Suster and Bate, 2002). When NMJ neurotransmission is blocked with transgenic toxin expression or in a range of mutants (*syntaxin, synaptobrevin, dunc-13*), peristalsis is eliminated, indicating that it is completely dependent on neural input (Broadie *et al*., 1995; Sweeney *et al*., 1995; Aravamudan *et al*., 1999). In contrast, local body contraction of one or a few segments persists, as do quite vigorous head movements, driven by the myogenic activity of the pharyngeal muscles. Thus, embryonic peristalsis, similar to other repetitive movements, depends on central pattern generating (CPG) circuits that functionally mature during the last quarter of embryogenesis (Suster and Bate, 2002). Interestingly, removal of sensory inputs during development has shown that these are not essential for the differentiation of the CPG or maturation of behaviorally normal movement (Suster and Bate, 2002). By 18–19 h (85–90% development), the embryos seem

to have perfected larval locomotory movements. Within the egg shell, both forward and reverse locomotory movements are evident, involving coordinated waves of muscle contraction along all the body segments (Suster and Bate, 2002). Following this apparent entrainment period, the embryo becomes markedly inactive during the last few hours of embryogenesis. Quite abruptly, the hatching movement program is then engaged, initially involving vigorous head movements to puncture the egg shell and then forward peristaltic waves to drive the new larva free.

2.3.5. Development of NMJ Presynaptic Function

Once a motor neuron growth cone contacts an appropriate muscle target, the filopodia-ringed vacuolated sheet that characterizes the "seeking" axon terminus transforms itself into a branched presynaptic terminal with swellings (or "boutons") specialized for neurotransmitter release (Rheuben *et al*., 1999). Functional presynaptic terminals normally differentiate after contact with postsynaptic muscle, but the muscle targets do not need to be appropriate, or even present, for formation of relatively normal presynaptic structures (Prokop *et al*., 1996). In the grasshopper *Barrytettix*, for example, presynaptic terminals normally persist in adulthood opposite inappropriate targets, such as glia and basal lamina, after previously innervated nymph muscles degenerate (Arbas and Tolbert, 1986). Thus, presynaptic development, once triggered by unknown signals, appears to proceed to completion without any target derived signal. Without a differentiated postsynaptic target, however, presynaptic specializations are oriented randomly in the presynaptic membrane rather than exclusively toward the postsynaptic muscle target (Prokop *et al*., 1996). For example, *Drosophila twist* mutants do not form myoblasts (see above) and, thus, motor terminals are completely deprived of target cells. Nevertheless, fully differentiated presynaptic terminals form, except that presynaptic densities, with associated clusters of SVs, are oriented randomly. Fully formed but improperly oriented presynaptic specializations are also seen in *mef-2* mutants, which are capable of producing only undifferentiated myoblasts (see above; Prokop *et al*., 1996; Prokop, 1999). It is as if axons know "when" and approximately "where" to form a presynaptic terminal (presumably via regional cues during outgrowth) but, without interaction from a differentiated postsynaptic partner, they do not know exactly which way to point it.

As with other aspects of embryonic body wall development, differentiation of presynaptic nerve terminals occurs in a ventral to dorsal "wave," as axons growing outward from the ventral ganglion around the periphery of the cylindrical embryo meet increasingly distant and more dorsal muscles (Broadie *et al*., 1993; see **Figure 1**). This wave of development occurs throughout the latter half of embryogenesis, but differentiation of individual terminals occurs much more rapidly. In *Drosophila*, where embryogenesis lasts ~21 h at 25 °C, individual terminals mature from seeking growth cones to functional presynaptic structures in a few (2–3) hours (Broadie *et al*., 1993; Yoshihara *et al*., 1997). By the end of embryogenesis, each muscle is functionally innervated, but new boutons continue to be added, grow and differentiate throughout larval development.

2.3.5.1. Functional Development of the Presynaptic Terminal

The basic processes of presynaptic function appear to be highly conserved across phyla. Thus, insect nerve terminals are structurally and functionally very similar to those described in other animals, including the extensively described terminals of frog, mouse, and rat. Development of presynaptic function is difficult to track because neurotransmitter release is typically measured using activation of postsynaptic receptors as an assay. Nevertheless, all evidence suggests that at least some functional synaptic vesicle release machinery assembles prior to muscle target contact (Fletcher *et al*., 1994). For example, experiments in *Drosophila*, using voltage-clamped "sniffer cells" to detect neurotransmitter release from growing neuronal axons in culture, show that presynaptic terminals release neurotransmitters prior to contacting their postsynaptic target (Yao *et al*., 2000). Spontaneous and evoked activation of postsynaptic GluRs is also immediately detected after the nascent presynaptic terminal contacts target muscle, suggesting that presynaptic terminals are already functional before synaptogenesis *per se* (Broadie and Bate, 1993b, 1993e).

A more direct assay for presynaptic function is the uptake and release of lipophilic fluorescent dyes such as FM1-43, which stains and destains functional terminals during synaptic vesicle endocytosis and fusion, respectively (Cochilla *et al*., 1999). Chick growth cones in culture stain with FM1-43 (Diefenbach *et al*., 1999), but this dye uptake and release may be due to extensive membrane turnover caused by neurite growth and reorganization rather than synaptic vesicle cycling. *Drosophila* embryonic motor terminals also cycle FM1-43 efficiently

(Fergestad and Broadie, 2001), but their ability to do so prior to target contact has not been examined.

Other hallmarks of presynaptic differentiation include immunocytochemical detection of vesicle proteins such as Synaptotagmin, Synaptobrevin, and Cysteine string protein (CSP) (Parfitt *et al.*, 1995; Wu and Bellen, 1997), which appear as distinct puncta in differentiated presynaptic terminals (Featherstone *et al.*, 2001). Functionally mature synaptic terminals also concentrate neurotransmitters in their cytosol, a necessary prerequisite for pumping it up a steep concentration gradient into SVs. Glutamate immunoreactivity, localization of synaptic proteins such as Synaptotagmin, Synaptobrevin and CSP, clustered SVs, and presynaptic dense bodies (T-bars) are all visible in growth cones and during the earliest stages of presynaptic terminal differentiation, again supporting the idea that the presynaptic specializations are functional before target contact (Rheuben and Kammer, 1981; Broadie and Bate, 1993e; Broadie, 1996).

Spectrins are cytoskeletal membrane matrix proteins expressed ubiquitously in *Drosophila*, but concentrated at NMJs (Featherstone *et al.*, 2001). Spectrins are also prominent cytoskeletal components of mammalian synapses (Phillips *et al.*, 2001). Mutations that eliminate alpha and beta Spectrin in *Drosophila* show that both proteins are required for localization of most presynaptic proteins, including CSP, Synaptotagmin, Synapsin, and Syntaxin (Featherstone *et al.*, 2001), but neither nerve terminal morphology nor synaptic vesicle number or distribution are affected. Because of the mislocalization of presynaptic proteins, synaptic transmission is reduced to approximately 25% of normal. These results suggest that synaptic proteins are organized (probably indirectly) by a Spectrin scaffold during synaptogenesis.

2.3.5.2. Maturation of Neurotransmitter Release

In the NMJ of the *Drosophila* ventral longitudinal muscle 6/7, evoked EJCs increase from about 2000 pA in late-stage embryos to approximately 400 000 pA in third instar larvae, a 200-fold increase (Broadie and Bate, 1993e; Rohrbough *et al.*, 1999; Broadie, 2000). This increase is probably necessary in order to compensate for the large developmental drop in muscle input resistance (Lnenicka and Keshishian, 2000), which otherwise would lead to insufficient postsynaptic depolarization in larvae. This dramatic increase in synaptic efficacy could be accounted for by several factors: (1) an increase in the number of presynaptic boutons; (2) an increase in the number of active zones within each bouton; and/or (3) an increase in the number of postsynaptic GluRs (Featherstone *et al.*, 2002; Reiff *et al.*, 2002). The embryonic muscle 6/7 NMJs contain approximately 10 boutons. In third instar larvae, there are 50–100 boutons. Embryonic boutons typically contain 6–8 presynaptic active zones (Rheuben *et al.*, 1999), while third instar larvae boutons possess two to three times that number (Atwood *et al.*, 1993; Renger *et al.*, 2000). Therefore, the number of synapses (active zones) at each NMJ increases 10- to 30-fold over the course of late embryonic and larval development. Each active zone also increases in size, from about 200 nm in embryos (Prokop *et al.*, 1996; Prokop, 1999) to about 500 nm in third instar larvae (Atwood *et al.*, 1993; Rheuben *et al.*, 1999; Renger *et al.*, 2000). Consistent with this presynaptic change seen ultrastructurally, postsynaptic glutamate receptor clusters imaged confocally also increase in size approximately threefold between the end of embryogenesis and the end of third instar stage (D.E. Featherstone, unpublished data). In other words, embryonic through larval development of each NMJ involves all three changes mentioned above, i.e., addition of boutons, addition of active zones within each bouton, and an increase in size of each active zone. Nevertheless, these pre- and postsynaptic morphological differences account for only about half of the increase in EJC size measured during larval development. Thus during larval development, individual active zones also undergo functional changes that increase neurotransmitter release. Such changes could be due to increased presynaptic calcium channel influx (Yoshihara *et al.*, 2000; Reiff *et al.*, 2002), facilitated SNARE-mediated vesicle fusion, perhaps due to increased calcium sensitivity (Littleton *et al.*, 1993a, 1999), or more efficient vesicle cycling (Kuromi and Kidokoro, 1999).

Neurotransmitter release is highly dependent on extracellular calcium, which invades the presynaptic terminal when voltage-gated calcium channels open during presynaptic depolarization. Comparison of different electrophysiological studies suggests that the calcium dependence of NMJ neurotransmitter release approximately doubles between late embryos and third instar larva (Broadie, 1999). Thus, more release is achieved due to increased sensitivity to calcium. In *Drosophila*, the terminal membrane area increases many fold during development (from about 3 μm^2 in embryos to about 100 μm^2 in third instar larvae), potentially increasing the number of routes for calcium influx. Unfortunately, developmental studies of calcium channel density or calcium influx in terminals have not been performed. One explanation for the increased calcium sensitivity could be a developmental shift in the

expression of Synaptotagmin family members, which confer different calcium dependence to release (Littleton *et al.*, 1999). Several Synaptotagmin genes are indeed expressed in *Drosophila*, but a developmental shift in their expression at the NMJ has not yet been reported. From embryonic synaptogenesis through larval stages, each active zone also becomes associated with an increasing number of SVs. In the embryonic NMJs, for example, T-bars are typically surrounded by 5–10 vesicles, with few or no vesicles in the surrounding cytosol. In third instar larvae, T-bars are often surrounded by 2–3 dozen vesicles, with hundreds more in the nearby cytosol. The increased availability of SVs during development probably results in the fusion of more vesicles per stimulus at each active zone, as well as in fatigue resistance due to a larger number of reserve vesicles. Thus, the efficacy of individual active zones also increases during development, although the molecular mechanisms underlying these presynaptic changes remain largely unexplored.

2.3.5.3. Maturation of Mature Synaptic Properties

Early in synaptogenesis, when the motor nerve terminal first contacts the postsynaptic muscle (13.5 h; 60% embryonic development at 25 °C), endogenous currents at the nascent NMJ can be observed, although evoked transmission is easily fatigued and variable (Broadie and Bate, 1993e). Before 15 h of embryogenesis (68% development), when NMJs have just formed, the synapse fatigues rapidly and completely even with relatively low frequency (<5 Hz) stimulation (Broadie and Bate, 1993e). Between 16 and 18 h (72–82% development), NMJ transmission is more consistent, and patterned synaptic activity is observed such that higher stimulation frequencies (>5 Hz) are needed to induce fatigue, from which the terminal recovers within a few seconds (Broadie and Bate, 1993e). After 18 h, the NMJ maintains reliable transmission with little fatigue at stimulation frequencies up to about 10 Hz (Broadie and Bate, 1993e). Reliability and fatigue resistance increase throughout larval development. Thus, third instar *Drosophila* larvae are capable of maintaining transmission (albeit with marked depression) at very high stimulation frequencies (up to 50–100 Hz) with almost no failures (Rohrbough *et al.*, 1999; Featherstone, personal communications). These functional changes during development can be attributed again to several factors, including the addition of presynaptic boutons, the addition of active zones within those boutons, an increase in active zone size, an increase in functional efficacy of each active zone, and an enlargement of the postsynaptic receptor field.

Late-stage embryonic and larval NMJs also show increasing facility for activity-dependent synaptic plasticity, wherein synaptic properties change following specific patterns of stimulation (described in more detail in Sections 2.3.1.4 and 2.3.8). These changes may reflect maturation of intracellular signaling pathways and mechanisms for modulation (see discussion below).

2.3.5.4. Differentiation of Synaptic Vesicle Pools and Cycling

Most (>70%) SVs in embryonic motor terminals cluster around the active zone; relatively few vesicles are distributed throughout the center of the bouton, despite the large amount of space available (Prokop *et al.*, 1996; Prokop, 1999). In larval terminals, however, bouton centers are often filled with vesicles, and vesicles are stacked many rows deep around active zones (Atwood *et al.*, 1993; Rheuben *et al.*, 1999; Renger *et al.*, 2000). Electron microscopy, in combination with electrophysiological and mutant analyses, has shown that the vesicles immediately adjacent to active zones are sufficient for basal neuromuscular transmission. Furthermore, ultrastructural and dye studies suggest that, typically, only a minority of presynaptic vesicles are usually involved in basal transmission. Locust terminals, for example, only show, on average, a 20% decrease in vesicle density after stimulation (Titmus, 1981). In *Drosophila*, high bath potassium, which depolarizes cells and triggers release, causes release of about half of the total number of SVs (Kuromi and Kidokoro, 1998).

Only under very high frequency stimulation or, especially, in conditions where endocytosis is inhibited, are the majority of vesicles recruited (Kuromi and Kidokoro, 1998, 1999, 2000, 2002). These reluctantly recruited "reserve" vesicles are the morphologically "central" vesicles not associated with active zones. Under conditions of high vesicle demand, such as high-frequency stimulation or impaired endocytosis, these reserve vesicles supplement and/or replace vesicles from the primary pool. In embryos, the reserve pool is absent – there are only vesicles immediately around T-bars, and the centers of boutons are devoid of SVs. Consistent with this, embryos display markedly lower fatigue resistance (Broadie and Bate, 1993e), because they have no reserve vesicle pool (Prokop, 1999; Fergestad and Broadie, 2001). Because embryos only contain vesicles around their T-bars, we can also infer that nascent vesicles, which all embryos have, are first targeted to active zones and, when these areas are sufficiently occupied, a reserve pool begins to develop. In mutant larvae that develop without

Endocytosis (e.g., *Drosophila* Endophilin mutants), the cycling pool is greatly reduced and the reserve pool is completely absent (Verstreken *et al*., 2002), again arguing that vesicles are first targeted to active zones and then, as this area fills, to the "reserve pool." Because Cytochalasin D, a selective disruptor of filamentous Actin, eliminates the reserve pool, it is thought that actin is responsible for incorporating or maintaining SVs in the reserve pool (Kuromi and Kidokoro, 1998). CyclicAMP signaling mutations also disrupt reserve vesicle recruitment (Kuromi and Kidokoro, 2000), demonstrating a need for this pathway in building and/or maintaining the reserve vesicle pool. In *Drosophila*, cAMP levels are increased by cytosolic calcium (Kuromi and Kidokoro, 2002), suggesting that calcium probably regulates vesicle pool size in insects as it does in mammals.

Taken together, these observations suggest that the increased fatigue resistance seen in late larval NMJs, compared to embryonic NMJs, is due to gradual accumulation of a reserve vesicle pool, which forms after the primary vesicle pool.

2.3.6. Development of NMJ Postsynaptic Function

2.3.6.1. The Muscle Membrane Prior to Innervation

Prior to innervation, the *Drosophila* body wall muscle membranes express cell adhesion molecules (CAMs) such as FasIII, FasII, Connectin, and Toll, which appear to mediate motor axon guidance and/or target recognition. FasII and Connectin are initially expressed throughout the muscle membrane, but are localized to NMJs during the initial stages of NMJ formation. FasIII, on the other hand, is initially loosely localized only near the normal site of muscle innervation, as if to mark the site, and then disappears after the start of NMJ development. Consistent with the idea that these CAMs mediate cell–cell "stickiness," overexpression of FasII results in ectopic synapses due to "trapping" of growth cones that normally explore several potential targets before selecting the proper postsynaptic cell. Similarly, misexpression of FasIII causes inappropriate synapses between axons that normally innervate FasIII-expressing muscles and the muscles that misexpress FasIII (Chiba *et al*., 1995; Suzuki *et al*., 2000). Toll, on the other hand, is repulsive to neurons that do not ordinarily innervate Toll-expressing muscles (Rose *et al*., 1997; Suzuki *et al*., 2000). Since each motor neuron innervates a unique muscle (or set of muscles), there is presumably a unique molecular cell adhesion "code" that allows matching between appropriate pre- and postsynaptic partners (Chiba and Rose, 1998). The CAM interactions between undecided partners are apparently aided by mobile cellular protuberances on both the pre-(growth cone filopodia) and postsynaptic sides. Decades ago, it was observed that the embryonic insect muscle appears "ruffled" at the normal site of innervation (Walther, 1981). Close examination has revealed that the membrane ruffling is due to small muscle membrane projections reminiscent of the axonal growth cone filopodia. These projections, termed "myopodia," are extended randomly from the muscle surface before innervation, but cluster at the site of innervation during contact by the appropriate motor neuron growth cone (Ritzenthaler and Chiba, 2003).

Fast excitatory signaling at insect NMJs occurs via postsynaptic ionotropic GluRs (Usherwood, 1977). In *Drosophila* NMJs prior to innervation, functional receptors are scattered in small numbers throughout the postsynaptic muscle membrane, such that iontophoresis of glutamate opens only one or two channels regardless of application site (Broadie and Bate, 1993b). Discs Large (Dlg) is the *Drosophila* homolog of mammalian PSD-95, a MAGUK (membrane-associated guanylate kinase) protein that is prominent at glutamatergic synapses. Similar to GluRs, Dlg is diffusely expressed initially, but localized and upregulated at synapses after innervation (Thomas *et al*., 2000).

2.3.6.2. Development of Postsynaptic Glutamate Receptors

Once the proper axon and muscle contact each other, they form a functional, albeit weak, synapse within minutes (Broadie and Bate, 1993b, 1993e). Consistent with this, postsynaptic glutamate receptors are distributed at low levels throughout the muscle membrane prior to innervation. Under normal circumstances, receptors outside the area of muscle contact disappear within an hour, while the number of synaptic receptors simultaneously increases (Broadie and Bate, 1993b; Saitoe *et al*., 1997), presumably as preexisting receptors cluster at the site of innervation (however, the possibility that new synaptic receptors are inserted while extrasynaptic receptors are degraded, has not been excluded). In *Drosophila*, EJCs, measured from barely innervated ventral muscle 6 at 13.5 h (60% of embryonic development), increase from single channel size (about 12 pA at −60 mV) to approximately 700 pA by 15 h (68% development). After this initial phase of postsynaptic development, EJC size gradually triples (to about 2000 pA) by the end of embryogenesis (22 h) (Broadie and Bate, 1993b),

as new receptors are synthesized and inserted. Glutamate receptor upregulation in *Drosophila* fails if presynaptic electrical activity is completely blocked using either Tetrodotoxin (TTX) or genetic ablation of voltage-gated sodium channels (Broadie and Bate, 1993a; Saitoe *et al*., 1997). Incomplete blockage or increased activity have relatively minor effects on postsynaptic development (Broadie and Bate, 1993a) (see **Figure 4**).

Without innervation, GluRs are neither localized nor upregulated (Broadie and Bate, 1993b). Furthermore, clustering and upregulation of receptors occurs at ectopic synapses. Thus, contact by the presynaptic nerve terminal is both necessary and

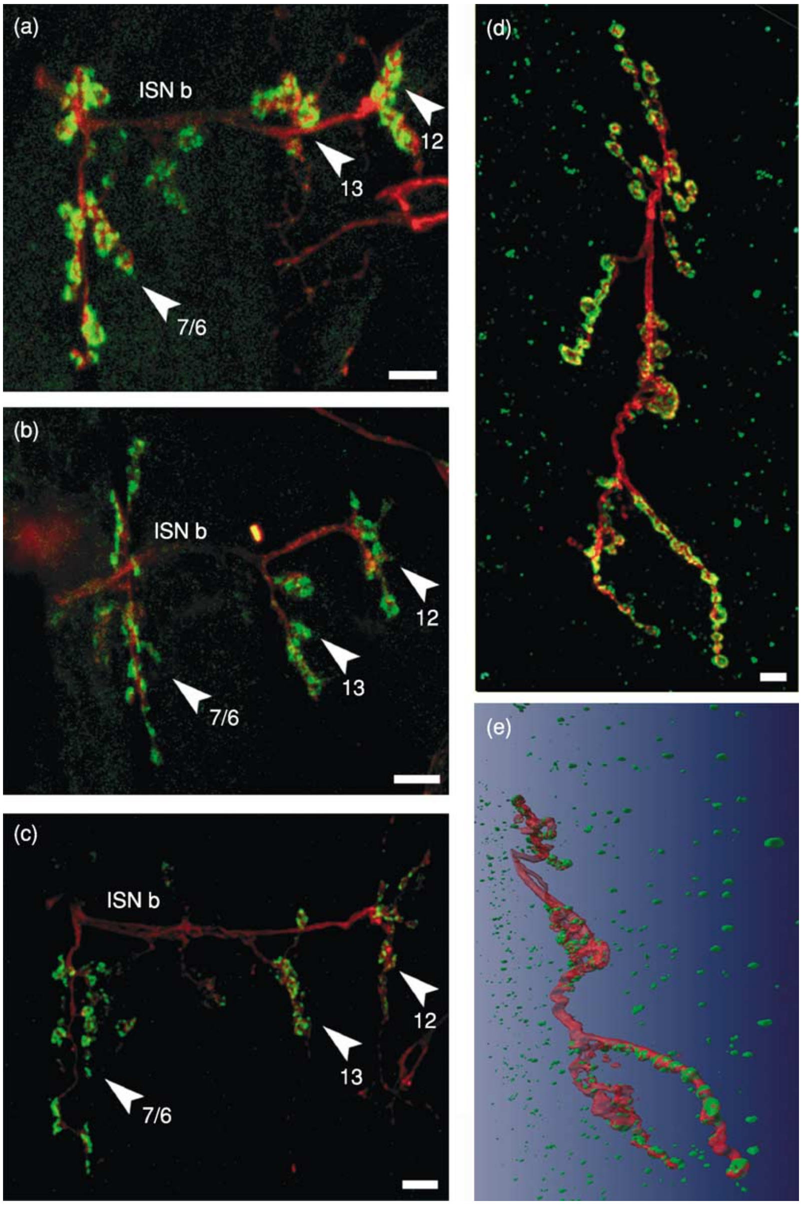


Figure 4 Confocal micrographs showing the development of *Drosophila* ventral longitudinal embryonic/larval NMJs, visualized using antibodies that recognize neuronal tissue (HRP, red) and glutamate receptor subunit GluRIIA (green). (a–c) Motor axons from the intersegmental nerve (ISNb) branch b form NMJs on ventral longitudinal muscles 7, 6, 13, and 12, in very late-stage (22 h, 100% embryonic development) embryos (a), first instar larvae (b), and second instar larvae (c). (d) The 6/7 NMJ in a third instar larva. Scale bar: 10 μm in all panels. Note the slight change in scale bar length between images. Over development, NMJ arbors grow larger and branchier, and glutamate receptor cluster size increases about threefold in area. Note also the large number of extrasynaptic glutamate receptor clusters visible in third instar muscle (d). Panel (e) shows a three-dimensional isosurface rendering of the same NMJ presented in panel (d), rotated slightly to show the edge of the confocal section visible near the upper left, where the axons extend to meet the bottom side of the muscles (which is the side facing away from the interior of the larvae).

sufficient for postsynaptic development. Similar to GluRs, Dlg is diffusely expressed initially, but localized and upregulated at synapses after innervation (Thomas *et al*., 2000). Like GluRs, Dlg is neither synaptically localized nor upregulated in the absence of innervation (D.E. Featherstone, unpublished data). The signal from nerve to muscle that triggers postsynaptic development, however, is unknown. In mammalian NMJs, the secreted proteoglycan Agrin triggers receptor clustering and upregulation of postsynaptic ACh receptors via a muscle specific receptor tyrosine kinase (MuSK) (Sanes and Lichtman, 1999). Glutamatergic synapses, including insect NMJs, however, appear to use a different signal for postsynaptic induction (Colledge and Froehner, 1998). No obvious Agrin homolog is encoded by the *Drosophila* genome, and several kinases similar to MuSK, such as Neurospecific receptor kinase (Nrk), Heartless (Htl), and RAR-related orphan receptor (Ror), appear to be expressed in neurons (where Ach receptors are expressed), but not muscle. The Wnt protein encoded by the *wg* gene has recently been shown to concentrate in developing larval NMJs, where it appears to be secreted by the presynaptic terminal. In *wg* mutants, or larvae with mutations in the TGF β type II receptor encoded by the gene *wishful thinking* (*wit*), NMJs remain underdeveloped (Packard *et al*., 2002). Thus, Wg signaling via Wit has been proposed to be an essential intercellular signal necessary for coordination of pre- and postsynaptic differentiation (Packard *et al*., 2003).

Wnts are known to participate in many other cell–cell interactions during development, and have been implicated in presynaptic differentiation in mammalian synapses (Salinas, 1999). However, although Wnt signaling appears to regulate larval NMJ development, it does not seem to be required for synaptogenesis, and it remains to be established whether Wnts are secreted by insect motor neuron growth cones prior to target contact (which would be required for the initial induction signal). The Spectrin cytoskeleton is required for proper localization of Dlg but not glutamate receptors (Featherstone *et al*., 2001), suggesting that although clustering and upregulation of both these postsynaptic proteins depends on a nerve-derived signal, the mechanisms by which they localize diverge thereafter.

Postsynaptic glutamate receptor (GluR) mRNA is translated at NMJs and the translation initiation factor eIF4E as well as the poly(A)-binding protein PABP, which are required for translation, are localized in the postsynaptic muscle near NMJs. Their genetic disruption alters synaptic development and GluR function (Sigrist *et al*., 2000). Consistent with local translation, Sigrist *et al*. (2000) also showed that GluR mRNA is localized near NMJs. This result, however, differs from earlier examinations which showed that GluR mRNA is widely distributed in embryonic and larval muscle fibers (Currie *et al*., 1995). Induction of GluR fields by the presynaptic nerve could trigger transcription of GluR mRNA, local translation of pre-existing mRNAs, or both. Preliminary evidence (Q. Sheng and D.E. Featherstone, unpublished data) suggests that innervation dependent upregulation of glutamate receptors during embryogenesis does not involve transcription, since mRNA levels remain unchanged while receptor protein accumulates.

Once induced, postsynaptic GluRs in *Drosophila* form distinct clusters under presynaptic terminals (Featherstone *et al*., 2002). Between late embryogenesis and third instar larval stage, the GluR cluster size area increases threefold (from about 0.5 μm^2 to about 1.5 μm^2; D.E. Featherstone, unpublished data). This size increase matches almost perfectly the postsynaptic density diameter measured by electron microscopy (Rheuben *et al*., 1999) and the increases in miniature junction currents recorded electrophysiologically (which increase from about 150 pA in embryos to 500 pA in third instars) (Rohrbough *et al*., 1999; Featherstone and Broadie, 2000; Featherstone *et al*., 2001). Because changes in the receptor cluster area mirror the functional (mEJC size) alterations so closely, receptor density within each cluster probably does not change during development. The number of receptor clusters per bouton also does not increase dramatically during larval development, but the bouton number does; therefore, the number of clusters at each NMJ also increases.

In addition to synaptic GluRs, extrajunctional GluRs have been found electrophysiologically and microscopically in the muscle membrane of all insects examined (Cull-Candy, 1976; Usherwood, 1994; Nishikawa and Kidokoro, 1995). There are two types of extrajunctional GluRs: D and H, which, when activated, depolarize and hyperpolarize the muscle membrane, respectively (Cull-Candy, 1976). The H receptor current appears to be carried by Avermectin-sensitive glutamate-gated chloride channels (Cully *et al*., 1996) and may be specific to adult muscle (Saito and Kawai, 1985). Excitatory D receptors are expressed during larval development, but the function of these nonsynaptic receptors is not understood. One hypothesis is that these extrajunctional D receptors represent "synaptic" receptors moving to/from the postsynaptic density. This is thought to be the case in embryonic muscle, where the disappearance of extrajunctional

receptors immediately following innervation is coincident with the appearance of junctional receptors (Broadie and Bate, 1993a; Saitoe *et al*., 1997). As the postembryonic muscle matures, however, extrajunctional receptors return to prominence (in *Drosophila*, during second instar stage; see **Figure 4**).

Extrajunctional receptors differ from junctional receptors in fundamental functional ways (Usherwood, 1994; Nishikawa and Kidokoro, 1995). For example, extrajunctional receptors have lower conductance, less rectification, and decreased calcium permeability compared to junctional ones (Usherwood, 1994; Nishikawa and Kidokoro, 1995). In the *Drosophila* muscle, this permeability difference is large enough to shift the receptor current reversal potential from near zero (for extrajunctional receptors) to positive (about 12 mV) values (Nishikawa and Kidokoro, 1995). Extrajunctional receptors also desensitize much more rapidly (in ~15 ms; Heckmann and Dudel, 1997) compared to junctional receptors (Nishikawa and Kidokoro, 1995). If extrajunctional receptors represent a pool of synaptic receptors held in "reserve" or trafficked to/from the synapse, then the differences in functional properties must represent a form of regulation determined by differential localization.

Although GABA-gated currents have also been demonstrated in some insect muscle (typically adult muscle), they are not present in the *Drosophila* embryonic/larval body wall muscle (Broadie, 1999; Featherstone and Broadie, 2000). Glutamic acid decarboxylase (GAD), the enzyme responsible for synthesizing the neurotransmitter GABA is, however, expressed in embryonic and larval motor nerve terminals, where it regulates cytosolic glutamate levels and (indirectly) postsynaptic GluR numbers (see Section 2.3.6.3). The presence of GAD protein in embryonic/larval motor neurons without apparent GABAergic transmission may also suggest that these motor neurons become GABAergic during metamorphosis. However, this possibility has not been investigated and no circumstantial evidence exists for it. Essentially nothing is known about the development of GABAergic transmission in insects, including whether insect GABA receptors are localized using mechanisms similar to those employed by vertebrates (Kardos, 1999; Sassoe-Pognetto and Fritschy, 2000; Fritschy and Brunig, 2003). The *Drosophila* protein Cinnamon (Cin), previously identified as a molybdenum cofactor, represents a fly homolog of Gephyrin, which is a mammalian protein important for glycine and GABA receptor field development (Kamdar *et al*., 1994; Wittle *et al*., 1999). Cinnamon's role in insect synaptic development or inhibitory neurotransmission has not yet been explored.

2.3.6.3. Molecular Maturation of Postsynaptic Signaling

As mentioned earlier, *Drosophila* third instar larval muscles contain large aggregates of the translation initiation factors eIF4E and poly(A)-binding protein (PABP) in or near the SSR of NMJs (Sigrist *et al*., 2000). According to Sigrist *et al*. (2000), GluR mRNA is localized near these postsynaptic translation aggregates (but others claim that GluR mRNA is unlocalized; Currie *et al*., 1995). When synaptic activity or cAMP levels are upregulated using K^+ channel (*eag,sh*) or cAMP-phosphodiesterase (*dunce*) mutants, respectively, the number of postsynaptic translation aggregates increases, the amount of postsynaptic glutamate receptors goes up (without a corresponding rise in mRNA), and EJC size increases (Sigrist *et al*., 2000). Increased larval locomotion also upregulates the number of postsynaptic translation aggregates and receptors (Sigrist *et al*., 2003). These activity-dependent changes can be rapidly reversed by using a temperature sensitive mutation in a voltage-gated sodium channel to induce paralysis and thus decrease NMJ activity (Sigrist *et al*., 2003). Taken together, these data suggest that *Drosophila* NMJ glutamate receptors are synthesized in or near the SSR by translation factors that are somehow regulated by synaptic activity. Postsynaptic synthesis probably allows faster synaptic changes; indeed, Sigrist *et al*. (2003) measured significant changes in synaptic strength after 2 h.

Synthesis and insertion of new receptors is not the only mechanism by which *Drosophila* NMJs control glutamate receptor number. The number of NMJ postsynaptic glutamate receptors at any time after initial induction represents a balance between receptor addition and receptor downregulation. In *Drosophila*, the presynaptic neuron slows the addition of postsynaptic receptors by secreting a signal that triggers receptor downregulation. Surprisingly, this signal is glutamate itself, released via an as yet unknown nonvesicular mechanism. In a genetic screen for mutations that affect postsynaptic development, Featherstone *et al*. (2000) paradoxically discovered that GAD enzyme levels in glutamatergic terminals regulate the number of postsynaptic glutamate receptors. Since *Drosophila* embryonic/larval NMJs are not GABAergic (Jan and Jan, 1976a; Broadie and Bate, 1993e; Featherstone *et al*., 2000), it was hypothesized that GAD's primary function in the NMJ was to degrade glutamate, and that glutamate caused downregulation of its postsynaptic receptors. By genetically manipulating the concentration of presynaptic cytosolic glutamate,

Featherstone *et al.* (2002) showed that the number of postsynaptic glutamate receptors is inversely correlated with presynaptic glutamate levels, consistent with the hypothesis that extracellular glutamate released from presynaptic nerve terminals causes downregulation of postsynaptic glutamate receptors. Thus, the nerve terminal first induces the clustering and upregulation of glutamate receptors (see Section 2.3.6.2), and then gradually puts a brake on the process using glutamate. Interestingly, glutamate-triggered downregulation of glutamate receptors is not blocked by mutations that eliminate synaptic vesicle fusion (Broadie *et al.*, 1995; Sweeney *et al.*, 1995; Aravamudan *et al.*, 1999; Featherstone *et al.*, 2002), demonstrating that glutamate-triggered receptor downregulation is primarily mediated by nonvesicular glutamate.

Even after excitatory glutamatergic transmission at insect NMJs was widely accepted, mammalian physiologists were still questioning whether glutamate could serve as a transmitter in the vertebrate CNS (Nicholls and Sihra, 1986). One of the main arguments against glutamate was the fact that extracellular glutamate levels in the vertebrate nervous system seemed too high. Extracellular glutamate also triggers downregulation of glutamate receptors in cultured mammalian neurons (Lissin *et al.*, 1999), and nonvesicular neurotransmitter release through various antiporters, neurotransmitter pumps, gap junctions and other channels, has recently been demonstrated in mammalian cells (Attwell *et al.*, 1993; Baker *et al.*, 2002; Demarque *et al.*, 2002; Duan *et al.*, 2003; Ye *et al.*, 2003). Regulation of glutamate receptors by nonvesicular glutamate may thus be a conserved mechanism by which presynaptic terminals balance presynaptic properties and postsynaptic sensitivity. However, the mechanisms by which nonvesicular glutamate is released and the methods that the muscle cell uses for receptor downregulation remain to be elucidated. It also remains unknown whether receptors containing specific subunits are preferentially downregulated, so that receptor properties are "tuned" depending on innervation.

2.3.7. Postembryonic Maturation of the NMJ

Although a functional NMJ is fully established (e.g., functional pre- and postsynaptic elements are in place) by the end of embryogenesis, the synapse continues to grow and elaborate dramatically during the larval stages (Walther, 1981). Almost none of this postembryonic development occurs without extensive input from the synaptic partner, and all of it appears to be regulated by complex and incompletely described cascades of intracellular messengers.

2.3.7.1. Elaboration of Synaptic Architecture

Between hatching and late larval stages, the size and structural elaboration of NMJs increase dramatically. New synaptic boutons and axonal branches are added to make, in general, a larger, "bushier" presynaptic arbor. Time-lapse studies suggest that there is no specific growth zone for presynaptic elaboration (Zito *et al.*, 1999). Rather, new terminals or branches are added, apparently randomly, either by splitting of pre-established boutons, the formation of new boutons on pre-established branches, or bouton formation at the end of terminal branches (Zito *et al.*, 1999). NMJ growth and subsequent morphology depends strongly on synaptic activity. As described above (see Section 2.3.6.3), NMJ locomotory activity in larvae induces accumulation of postsynaptic translation aggregates, increased production of postsynaptic glutamate receptors, and increased EJC size within 2 h. On a longer time scale (12–24 h), increased NMJ activity also induces dramatic synaptic growth (Budnik *et al.*, 1990; Sigrist *et al.*, 2000; Sigrist *et al.*, 2003). How are NMJ activity and morphology linked? Increased activity, as well as genetic manipulation of cAMP levels, are associated with a large (~50%) reduction in pre- and postsynaptic FasII at the NMJ and increased NMJ size (Davis *et al.*, 1996b; Schuster *et al.*, 1996b; Cheung *et al.*, 1999; Sigrist *et al.*, 2000, 2003). Reductions in FasII are both necessary and sufficient for NMJ growth (Schuster *et al.*, 1996b), but neither the FasII level changes nor the subsequent alterations in NMJ size affect synaptic function (Davis *et al.*, 1996b). Activity-dependent changes in synaptic function associated with NMJ growth require cAMP signaling via the cAMP response element binding protein CREB (Davis *et al.*, 1996b), in addition to the faster changes in glutamate receptor synthesis described above (see Section 2.3.6.3). Thus, NMJ activity induces cAMP levels and FasII downregulation, which upregulate synaptic function and growth, respectively. FasII levels may also be coupled to synaptic activity via presynaptic calcium released from intra- or extracellular stores, and triggered perhaps by phospholipase C activation, since changes in these processes also affect synaptic growth (Hannan and Zhong, 1999).

Because FasII is a cell adhesion molecule, it was suggested that synaptic growth is regulated by a careful balance between adhesion (which makes the neuron "grip" the muscle) and repulsion (which allows the neuron to grow over the muscle surface). Consistent with this, integrins are also expressed at

NMJs and required for normal morphology and function of *Drosophila* NMJs (Beumer *et al.*, 1999, 2002; Rohrbough *et al.*, 2000). Beta PS integrins appear to act through CamKII to regulate Dlg and FasII (Beumer *et al.*, 2002). Dlg is itself regulated by CamKII and required for FasII localization and NMJ growth (Budnik *et al.*, 1996; Thomas *et al.*, 1997; Koh *et al.*, 1999).

In addition to a careful balance in cell adhesion, NMJ growth also depends on the microtubule cytoskeleton and control of its assembly (Hummel *et al.*, 2000; Roos *et al.*, 2000; Zhang *et al.*, 2001; Eaton *et al.*, 2002; Pennetta *et al.*, 2002). *Drosophila* NMJ boutons contain microtubule loops oriented parallel to the postsynaptic muscle membrane (Roos *et al.*, 2000). During formation of new boutons, these cytoskeletal loops are rearranged. The *Drosophila* Futsch protein is a MAP1B homolog required for the microtubule rearrangements and thus for new bouton formation (Roos *et al.*, 2000). Futsch protein levels are controlled by the fragile X related protein (FXR), a mRNA binding protein and translational repressor (Zhang *et al.*, 2001). In *fxr* mutants, Futsch levels are increased and NMJs are overgrown, similar to the synaptic overgrowth found in human victims of fragile X mental retardation (Zhang *et al.*, 2001).

2.3.7.2. Formation of the SSR

During *Drosophila* embryogenesis, there is no SSR at the NMJ. At about the time of hatching, postsynaptic muscle cells produce short arm-like membrane extensions around type I presynaptic terminals, which also begin to sink into a growing dip in the postsynaptic membrane. By late larval stages, the presynaptic type I terminal is typically buried within layers of elaborately infolded muscle membrane (Rheuben *et al.*, 1999). The SSR forms in only a subset of NMJs. Specifically, it is most elaborate in type Ib, smaller in type Is, and undetectable in types II and III boutons (Lane, 1985; Rheuben *et al.*, 1999). Presumably, type I terminals possess a specific secreted or transmembrane "tag" that allows muscles to recognize them as such and build an SSR. No candidates for this presynaptic tag have been proposed. On the postsynaptic side, however, it has been shown that Dlg is critical for SSR formation (Lahey *et al.*, 1994; Budnik *et al.*, 1996; Guan *et al.*, 1996).

The insect SSR, like the membrane folds at mammalian NMJs, is thought to allow a higher density of membrane proteins in the region of the presynaptic terminal. The SSR may also be important for control of the fluid microenvironment around the NMJ, since insects have been shown to maintain fluid compositions around their nervous system that are dramatically different from the composition found elsewhere in the relatively open blood system (Treherne, 1985; see also Section 2.3.3). When the SSR is insufficiently developed due to mutations that affect levels of Dlg, the number of postsynaptic glutamate receptors is also decreased (Parnas *et al.*, 2001). This change is probably due simply to a reduction in available postsynaptic membrane, since loss of Dlg does not affect glutamate receptor numbers or localization prior to formation of the SSR (D.E. Featherstone, unpublished data).

2.3.7.3. Developmental "Feedback" between Pre- and Postsynaptic Cells

After initial synapse formation, the morphology and function of NMJs continue to change during larval development. Typically, postsynaptic muscle volume increases several tenfold, and presynaptic arbor area increases several fold. To maintain coordinated movement, it is important that muscle depolarization following nerve activity remain relatively invariant throughout this dramatic growth. To ensure this, presynaptic neurotransmitter release is developmentally regulated based on levels of postsynaptic depolarization. For example, *Drosophila* mutants with decreased muscle depolarization (due to manipulation of voltage-gated potassium channels or glutamate receptors in the muscle) develop larval presynaptic terminals with double the normal number of T-bars and more neurotransmitter release (Haghighi *et al.*, 2003). This increase in neurotransmitter release increases transmission "throughput" and corrects the deficit in muscle depolarization. Because the apparent "goal" of these changes is to maintain the size of the postsynaptic depolarization, the term "synaptic homeostasis" has been used (Paradis *et al.*, 2001). Although the retrograde (muscle to neuron) signal that mediates synaptic homeostasis remains unidentified, CamKII is required postsynaptically (presumably to "sense" depolarization levels), and a bone morphogenetic protein (BMP) type II receptor is required presynaptically (presumably to receive the retrograde signal) (Haghighi *et al.*, 2003). Alterations in glutamate receptor number do not cause compensatory changes in embryonic NMJs, suggesting that homeostatic changes occur during larval development (Broadie and Bate, 1993a; Featherstone *et al.*, 2000, 2002).

Paradoxically, manipulations that increase postsynaptic glutamate receptor numbers in larvae (such as overexpression of the receptors themselves, overexpression of postsynaptic translation aggregate proteins, or increased NMJ activity; Sigrist *et al.*, 2000, 2002, 2003) increase the density of

presynaptic active zones and number of boutons (Sigrist *et al*., 2002). Synaptic homeostasis predicts that more receptors would cause a reduction of NMJ size, as the presynaptic cell tries to compensate for increased postsynaptic sensitivity.

2.3.8. Development of Activity-Dependent Synaptic Plasticity

2.3.8.1. Development of Activity-Dependent Functional Plasticity

Two types of developmental plasticity have been recognized at the *Drosophila* NMJ: "morphological plasticity" and "functional plasticity." Morphological plasticity involves changes in NMJ size (bouton number, terminal length, or branching), which may or may not alter the size of postsynaptic currents. Functional plasticity involves changes in the size of postsynaptic EJCs, which may or may not involve alterations in NMJ size. As described in previous sections, NMJ activity levels can have dramatic effects on both NMJ growth (morphological plasticity) and EJC size (functional plasticity). Because morphological changes typically take a relatively long time (many hours in *Drosophila*) to manifest, activity-dependent morphological plasticity is usually assayed in third instar NMJs following chronic genetic manipulations. Activity-dependent functional plasticity, however, can be observed following both chronic (days) and acute (milliseconds–minutes) manipulations. The latter type of functional plasticity is considered "short-term functional plasticity." As described in Section 2.3.1.4, several types of short-term functional plasticity can be observed in *Drosophila* NMJs: (1) paired-pulse facilitation (PPF); (2) short-term facilitation (STF); (3) augmentation; and (4) posttetanic potentiation (PTP). All forms of short-term plasticity first develop late in embryogenesis (Broadie *et al*., 1997), but are much more robust and reproducible in third instar larvae (Zhong *et al*., 1992; Rohrbough *et al*., 2000). Mutations that affect learning and memory also affect short-term plasticity in the fly NMJ, suggesting that learning and memory utilizes processes shared by very different synapses. Proteins shown to be required for both learning and memory and NMJ plasticity in *Drosophila* include integrins (Rohrbough *et al*., 2000), 14-3-3 proteins (Broadie *et al*., 1997), and molecules involved in cAMP signaling (Zhong *et al*., 1992). Frequenin is a novel *Drosophila* neuronal synaptic calcium binding protein that facilitates frequency dependent facilitation in NMJs (Pongs *et al*., 1993). The mammalian homolog of Frequenin, called Neuronal calcium sensor-1 (NCS-1), has been shown to be both necessary and sufficient for paired pulse facilitation in glutamatergic synapses (Sippy *et al*., 2003). Plasticity might improve during larval development as signaling pathways mature (e.g., proteins like Frequenin accumulate at the synapse), or the variable and easily fatigued nature of transmission at younger stages might just make activity-dependent plasticity more difficult to trigger and measure.

2.3.9. Distant Signals: Hormonal Effects on NMJ Development and Function

The embryonic/larval insect PNS and body wall musculature is, as in most invertebrates, bathed by a relatively open circulatory system. Thus, the NMJ is readily accessible to any of the many substances produced by neurosecretory cells throughout the animal. Any bioactive substance transported from relatively distant cells is a hormone, but since synaptic transmission is really a short-distance form of endocrine signaling specialized for speed and specificity, the difference between presynaptic effects and hormonal effects can degenerate into semantics. For example, in addition to motor inputs utilizing "fast" neurotransmitters (e.g., glutamate or GABA), the larval body wall musculature also contains terminals that secrete peptides and amines such as PACAP, Proctolin, Leucokinin, Insulin, and Octopamine (Lane, 1985; Hannan and Zhong, 1999). There are receptors for some of these substances on neighboring muscle, but active zones for these "modulatory" terminals are often oriented away from the muscle, toward the body cavity (Lane, 1985; Orchard and Loughton, 1985; Prokop, 1999). Thus, although the target(s) of these substances may include muscle cells immediately adjacent to the terminal, distant muscle cells and other tissues may also be affected (Orchard and Loughton, 1985). As a result, the peptides and amines could be considered both as transmitters and hormones. The literature covering insect endocrinology is extensive, since almost every aspect of postembryonic insect development is regulated by hormones. Here, just a very few examples of how hormones affect NMJ growth and function are considered.

2.3.9.1. Evidence for Hormonal Regulation of NMJ Development

Almost all aspects of insect postembryonic development depend strongly on hormones. Development of the NMJ is no exception. Ecdysone is a hormone predominantly associated with developmental

changes during metamorphosis, which peaks in concentration during late larval stages just prior to pupation **Chapters 2.4**, **3.3**, **3.5**, and **3.10**). In *Drosophila* mutants where ecdysone is eliminated, the strength of NMJ transmission in the early third instar larva is unchanged, but is increased slightly (~20%) in the late third instar larva (Li and Cooper, 2001). Consistent with this, late (but not early) third instar ecdysoneless mutants also show NMJs with longer branches and more synaptic boutons (Li and Cooper, 2001). Thus, ecdysone appears to slow larval NMJ growth prior to pupation.

Calcium activated protein for secretion (CAPS) is a highly conserved protein that is required for fusion of dense cored vesicles containing neuropeptides and appears to be necessary for release of all peptide hormones. Genetic elimination of CAPS (and thus all peptide hormone secretion) in *Drosophila* results in reduced movement and embryonic/early larval lethality. Mutant embryonic NMJs have transmission strength reduced to approximately one-half the normal, with no obvious changes in synaptic morphology. Selective expression of CAPS only in presynaptic neurons does not correct the NMJ defects, demonstrating that the reduction in transmission is entirely due to loss of one or more (unidentified) peptide hormones originating in other cells (Renden *et al*., 2001). It is possible that, were CAPS mutants to live beyond the early first instar stage, more dramatic effects on NMJ development and function might be observed due to loss of peptidergic output. The specific neuropeptides(s) responsible for deficient NMJ development in *dCAPS* mutants remains unidentified.

2.3.9.2. Modulation: Short-Term Hormonal Regulation of NMJ Function

Many hormones have dramatic effects on behavior and several studies have demonstrated short-term modulation of NMJ function in larvae. This modulation can result in increases or decreases in NMJ transmission. For example, the mysosuppressing FLRFamide has been identified in the nervous systems of many insect species, including moths, cockroaches, locusts, flesh flies, and fruit flies (Nachman *et al*., 1996). Myosuppressins cause potent inhibition of muscle contraction. Direct application of FLRF or analogs to the mealworm NMJ results in reduced synaptic transmission, suggesting that one of the normal targets of myosuppressins is the NMJ (Nachman *et al*., 1996). FLRFamide analogs also affect other insect synapses (Nachman *et al*., 1996). FMRFamides are another class of neuropeptides found in many insect species. FMRFamide-related peptides increase larval muscle contraction. Like FLRFamide, the target of FMRFamide is the NMJ. Direct application of 0.1 μM FMRFamide in *Drosophila* increases postsynaptic currents to approximately 150% of normal (Hewes *et al*., 1998). Presumably, any hormone that constitutively affects NMJ function will eventually trigger activity-dependent developmental changes such as homeostatic upregulation of transmitter release or alterations in synapse morphology.

2.3.10. Unanswered Questions

The purpose of this chapter is twofold: (1) to summarize the current state of knowledge about functional neuromuscular development in insects (especially *Drosophila*); and (2) to highlight some of the many remaining havens of ignorance concerning this topic. Our focus here has been largely restricted to *Drosophila*, as it is almost exclusively in fruit flies that the genetic and molecular bases of neuromuscular development have begun to be dissected. Despite the rapid and remarkable progress made recently using flies, many questions remain regarding insect neuromuscular development and functional maturation of synapses. The following list of unanswered questions is, of course, not exhaustive. Rather, it highlights areas that, in our opinion, represent the highest priorities for investigation.

First, there is a general deficit in truly *developmental* studies. *Drosophila* mutant screens designed to study NMJ proteins usually try to isolate either conditional (i.e., temperature sensitive) mutants, or morphological mutants. Conditional mutant screens intentionally seek to avoid "developmental artifacts." Morphological mutants infer developmental problems, but the progression of the phenotype (which is typically measured in third instar larvae just prior to pupation) during NMJ development is almost never explored. Thus, we are typically left knowing that something at some time went wrong, but we do not know exactly what or when or where. This is inadequate.

Drosophila neurobiologists have been baffled by the difficulty of perturbing motor neuron–muscle connectivity with traditional loss-of-function mutants. Mutations in most genes with accepted roles in axon outgrowth and target recognition typically display relatively low penetrance (e.g., the phenotypes are spotty). Low penetrance is typically blamed on redundancy, where each pair of pre- and postsynaptic targets recognizes each other using multiple cues. Redundancy seems to be required by the fact that motor neuron growth cones follow

amazingly invariant routes, after which they unerringly innervate a specific section of a particular target and form a distinctly shaped presynaptic arbor. Wild-type axons are able to innervate their targets with almost perfect reliability, even despite developmental delays. Without redundancy, it is hard to believe that reliable innervation results from a long series of chancy guidance and target selection "choices" involving transiently expressed cues. For example, even if the error rate at any particular decision point were only 0.01, almost one quarter of the NMJs would show an aberration after only two dozen "decisions." But most developmental biologists also accept the theory that connectivity is determined through the use of combinatorial "codes," with growth cones and their postsynaptic targets each displaying a unique set of molecules that allow them to recognize each other. The presence of unique combinatorial codes and redundancy do not seem compatible, however. The already staggering number of unique codes required for connectivity in the nervous system becomes even more unmanageable if redundancy is involved. Accordingly, we believe that developmental biologists are still missing something fundamental in our concepts of pathfinding and target selection.

Following pathfinding and target recognition, we still know almost nothing about the signals and mechanisms involved in the conversion of a growth cone into a synapse. Although a few candidate molecules have been implicated in this conversion process (e.g., the neural tetraspanin Late Bloomer (Lbm; Kopczynski *et al*., 1996), we have no clear models on how such molecules function. The few early reports (see, for example Fradkin, 2002) have mostly not been followed up. Thus, the interface between neuromuscular target recognition and the initiation of synaptogenesis seems unduly neglected, despite the detailed characterization of the process at the level of cell morphology (Yoshihara *et al*., 1997). What role does the postsynaptic cell play in signaling this transition? What is the nature of the intercellular signals? How do such signals regulate the synthesis and localization of the diverse new proteins needed for synaptic function? Once pre- and postsynaptic cells recognize their partner as "appropriate," exactly how do they initially build a synapse? Studies on mammalian neurons indicate that active zones are preassembled in large "Piccolo-Bassoon transport vesicles" (PTVs; Piccolo and Bassoon are protein components of these vesicles with undefined function) (Shapira *et al*., 2003). Active zones are inserted preformed when one of these vesicles fuses with the cytoplasmic membrane. Since proper orientation of active zones requires a properly differentiated postsynaptic partner, these PTVs must somehow be targeted to the appropriate location using transsynaptic cues from the postsynaptic cell, or the active zones must quickly migrate to the correct location after random insertion, and then anchored opposite the postsynaptic cell. Clearly homologous genes encoding the PTV proteins Piccolo and Bassoon have not been identified in the *Drosophila* genome, however, and PTVs have not been shown to exist in insects.

On the postsynaptic side of the NMJ, there is an extremely short list of molecular players. Both biochemical and genetic approaches need to be employed to gain perspective on the number of molecules composing the postsynaptic density (PSD). One obvious priority is the identification of the junctional and extrajunctional GluR channel subunits. Two decades ago, the study of glutamate receptors was essentially synonymous with the study of insect (or at least arthropod) glutamate receptors. Since then, however, it is embarrassing how little progress has been made with regard to insect GluRs in the face of the great strides accomplished by mammalian glutamatergic synapse aficionados. Without knowing the insect receptor subunits, we cannot determine how each is synthesized, how they assemble, and how the receptors are localized. We also need the complete list of subunits in order to ask whether there is a developmental shift in receptor composition, comparable to the situation with AChRs at the vertebrate NMJ. We know even less about the classes, localization, or function of other types of receptors, such as metabotropic GluRs, peptide and nonpeptide receptors, which control a range of downstream signaling cascades.

There are also large gaps in our lists of presynaptic proteins. For example, no molecules have yet been identified as restricted components of the active zone. The molecular nature of the T-bar remains unknown. What is the function of the T-bar? Is it required for targeted SV exocytosis or any other aspect of presynaptic function? Similarly, are there distinctive "active zones" of other presynaptic functions (e.g., SV endocytosis)? Multiple Ca^{2+} and K^{+} channels play crucial roles in the development of efficacy and plasticity at the NMJ on both sides of the cleft. How do these channels get specified and targeted to the correct membrane domain? Do PDZ domain proteins adequately explain how channels become correctly associated with other membrane and membrane-associated proteins to generate localized assemblies required for efficient synaptic transmission? If so, what is the full complement of PDZ domain proteins in

the pre- and postsynaptic cells, and how do they interact to compartmentalize functional domains? Although the presynaptic terminal has been defined in terms of active and periactive zones, the true functional nature of this division remains unknown. Is the periactive zone a functional compartment or is it merely the zone defined as being outside of the active zones? The questions regarding the nature of such functional domains and the ways in which they are established during synaptogenesis are particularly important.

In *Drosophila* there is now plenty of evidence for the existence of multiple transsynaptic signals traveling in both anterograde and retrograde directions. In the embryo, for example, an anterograde signal establishes the GluR field by inducing both GluR clustering and *de novo* GluR synthesis (Broadie and Bate, 1993b), while a retrograde signal dictates the placement of presynaptic active zones opposite their muscle targets (Prokop *et al*., 1996). In the larva, modification of postsynaptic GluR function leads to a compensating change in presynaptic neurotransmitter release (Davis *et al*., 1998; DiAntonio *et al*., 1999; Haghighi *et al*., 2003). What are the signals and receptor mechanisms in these diverse signaling situations? Identification of the transsynaptic signals mediating synaptogenesis and matching presynaptic input to postsynaptic responsiveness is a major challenge. Is there a functional analog of Agrin at glutamatergic synapses? Is even the concept of a secreted signaling mechanism correct? Alternate transmembrane signaling mechanisms (e.g., Neuroligin-Neurexin signaling) have been compellingly proposed for the induction of presynaptic differentiation, but, so far, genetic support for such models has not been forthcoming and these mechanisms remain unexplored in insects.

The primary focus of this chapter has been the maturation of the type I glutamatergic NMJ in *Drosophila*. Essentially nothing is known about the functional development of other classes of synapse. How, when, and why do the different types of NMJ bouton become morphologically, functionally, and molecularly distinct? Are different mechanisms involved in modulatory synapse formation, compared to fast excitatory (glutamatergic) synapses? In *Drosophila* we have a single candidate protein, dCAPS (Renden *et al*., 2001), which appears to be required for all peptidergic transmission. Are there other molecules specifically required for DCV formation and SV fusion? How are these molecules targeted to specific active zones? The investigation of modulatory synapse formation and function represents a major growth area.

In recent years, the first attempts have been made to examine CNS function and functional development in *Drosophila*. These advances are crucial to any true, integrated understanding of neural circuit formation and function utilizing the *Drosophila* genetic system. Very preliminary work to date has focused on three tasks. First, the functional properties of mature neurons and the nature of CNS synaptic communication have begun to be described (Baines and Bate, 1998; Baines *et al*., 1999; Rohrbough and Broadie, 2002; Baines, 2003; Rohrbough *et al*., 2003). A crucial need is to define circuits in which synaptic transmission can be driven, recorded, and modulated. Second, the wiring diagram of connectivity in the CNS has begun to be elucidated (Godenschwege *et al*., 2002; Lohr *et al*., 2002; Marin *et al*., 2002; Komiyama *et al*., 2003; Landgraf *et al*., 2003; Rohrbough *et al*., 2003; Scott *et al*., 2003). This work involves the definition of the functional compartments in the CNS, the mapping of the location and nature of the synaptic connections and, ultimately, the definition of neural circuits. The third, least advanced area is charting the normal development of function within CNS circuits (Baines and Bate, 1998; Allen *et al*., 1999; Baines *et al*., 1999). As with the NMJ, a firm grasp of the normal developmental processes is a basic prerequisite to any attempt to unravel mutant phenotypes. These early studies need to be supported and encouraged. The *Drosophila* NMJ is inherently important as a model for understanding the insect NMJ, as well as a more general model of the glutamatergic synapse. But with regard to understanding the development of general synaptic properties, the NMJ is but a foot in the door. It is time for *Drosophila* neurobiologists to grapple with the complexity of functional development and plasticity within the CNS.

Acknowledgments

The authors would like to thank Matthias Landgraf, Jeffrey Rohrbough, Kale Bodily, and Warren Davis for the images shown in Figure 2. We also appreciate the input from many other scientists who shared unpublished results, insights, and discussion.

References

Aidley, D.J., **1975**. Excitation-contraction coupling and mechanical properties. In: Usherwood, P.N.R. (Ed.), Insect Muscle. Academic Press, New York, pp. 337–356.

Allen, M.J., Shan, X., Caruccio, P., Froggett, S.J., Moffat, K.G., *et al*., **1999**. Targeted expression of truncated glued disrupts giant fiber synapse formation in *Drosophila*. *J. Neurosci. 19*, 9374–9384.

Anderson, H., Edwards, J.S., Palka, J., **1980**. Developmental neurobiology of invertebrates. *Annu. Rev. Neurosci. 3*, 97–139.

Aravamudan, B., Broadie, K., **2003**. Synaptic *Drosophila* UNC-13 is regulated by antagonistic G-protein pathways via a proteasome-dependent degradation mechanism. *J. Neurobiol. 54*, 417–438.

Aravamudan, B., Fergestad, T., Davis, W.S., Rodesch, C.K., Broadie, K., **1999**. *Drosophila* UNC-13 is essential for synaptic transmission. *Nat. Neurosci. 2*, 965–971.

Arbas, E.A., Tolbert, L.P., **1986**. Presynaptic terminals persist following degeneration of "flight" muscle during development of a flightless grasshopper. *J. Neurobiol. 17*, 627–636.

Aronstein, K., Auld, V., french-Constant, R., **1996**. Distribution of two GABA receptor-like subunits in the *Drosophila* CNS. *Invert. Neurosci. 2*, 115–120.

Artero, R., Prokop, A., Paricio, N., Begemann, G., Pueyo, I., *et al.*, **1998**. The muscleblind gene participates in the organization of Z-bands and epidermal attachments of *Drosophila* muscles and is regulated by Dmef2. *Devel. Biol. 195*, 131–143.

Ashcroft, F.M., **1982**. Electrical properties of insect muscle. In: Podesta, R.B. (Ed.), Membrane Physiology of Invertebrates. Dekker, New York, pp. 567–604.

Attwell, D., Barbour, B., Szatkowski, M., **1993**. Nonvesicular release of neurotransmitter. *Neuron 11*, 401–407.

Atwood, H.L., Govind, C.K., Wu, C.F., **1993**. Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. *J. Neurobiol. 24*, 1008–1024.

Auld, V.J., Fetter, R.D., Broadie, K., Goodman, C.S., **1995**. Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in *Drosophila*. *Cell 81*, 757–767.

Baines, R.A., **2003**. Postsynaptic protein kinase A reduces neuronal excitability in response to increased synaptic excitation in the *Drosophila* CNS. *J. Neurosci. 23*, 8664–8672.

Baines, R.A., Bate, M., **1998**. Electrophysiological development of central neurons in the *Drosophila* embryo. *J. Neurosci. 18*, 4673–4683.

Baines, R.A., Robinson, S.G., Fujioka, M., Jaynes, J.B., Bate, M., **1999**. Postsynaptic expression of tetanus toxin light chain blocks synaptogenesis in *Drosophila*. *Curr. Biol. 9*, 1267–1270.

Baines, R.A., Seugnet, L., Thompson, A., Salvaterra, P.M., Bate, M., **2002**. Regulation of synaptic connectivity: levels of Fasciclin II influence synaptic growth in the *Drosophila* CNS. *J. Neurosci. 22*, 6587–6595.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., Bate, M., **2001**. Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. *J. Neurosci. 21*, 1523–1531.

Baker, D.A., Xi, Z.X., Shen, H., Swanson, C.J., Kalivas, P.W., **2002**. The origin and neuronal function of *in vivo* nonsynaptic glutamate. *J. Neurosci. 22*, 9134–9141.

Baker, K., Salkoff, L., **1990**. The *Drosophila* Shaker gene codes for a distinctive K^+ current in a subset of neurons. *Neuron 4*, 129–140.

Ball, E.E., Goodman, C.S., **1985a**. Muscle development in the grasshopper embryo. II. Syncytial origin of the extensor tibiae muscle pioneers. *Devel. Biol. 111*, 399–416.

Ball, E.E., Goodman, C.S., **1985b**. Muscle development in the grasshopper embryo. III. Sequential origin of the flexor tibiae muscle pioneers. *Devel. Biol. 111*, 417–424.

Ball, E.E., Ho, R.K., Goodman, C.S., **1985**. Muscle development in the grasshopper embryo. I. Muscles, nerves, and apodemes in the metathoracic leg. *Devel. Biol. 111*, 383–398.

Bastiani, M.J., du Lac, S., Goodman, C.S., **1986**. Guidance of neuronal growth cones in the grasshopper embryo. I. Recognition of a specific axonal pathway by the pCC neuron. *J. Neurosci. 6*, 3518–3531.

Bastiani, M.J., Harrelson, A.L., Snow, P.M., Goodman, C.S., **1987**. Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell 48*, 745–755.

Bate, C.M., **1976**. Pioneer neurones in an insect embryo. *Nature 260*, 54–56.

Bate, M., **1990**. The embryonic development of larval muscles in Drosophila. *Development 110*, 791–804.

Bate, M., Landgraf, M., Ruiz Gomez Bate, M., **1999**. Development of larval body wall muscles. *Int. Rev. Neurobiol. 43*, 25–44.

Bate, M., Rushton, E., Currie, D.A., **1991**. Cells with persistent twist expression are the embryonic precursors of adult muscles in *Drosophila*. *Development 113*, 79–89.

Bate, M, Arias, A.M. (Eds.) **1993**. The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, New York.

Baumgartner, S., Littleton, J.T., Broadie, K., Bhat, M.A., Harbecke, R., *et al.*, **1996**. A *Drosophila* neurexin is required for septate junction and blood–nerve barrier formation and function. *Cell 87*, 1059–1068.

Baylies, M.K., Bate, M., **1996**. twist: a myogenic switch in *Drosophila*. *Science 272*, 1449–1450.

Baylies, M.K., Martinez Arias, A., Bate, M., **1995**. wingless is required for the formation of a subset of muscle founder cells during *Drosophila* embryogenesis. *Development 121*, 3829–3837.

Baylies, M.K., Michelson, A.M., **2001**. Invertebrate myogenesis: looking back to the future of muscle development. *Curr. Opin. Genet. Devel. 11*, 431–439.

Beadle, D.J., Hicks, D., **1985**. Insect nerve culture. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Nervous System: Structure and Function. pp. 181–212.

Beumer, K., Matthies, H.J., Bradshaw, A., Broadie, K., **2002**. Integrins regulate DLG/FAS2 via a CaM kinase II-dependent pathway to mediate synapse elaboration and stabilization during postembryonic development. *Development 129*, 3381–3391.

Beumer, K.J., Rohrbough, J., Prokop, A., Broadie, K., **1999**. A role for PS integrins in morphological growth

and synaptic function at the postembryonic neuromuscular junction of *Drosophila*. *Development 126*, 5833–5846.

Bieber, A.J., Snow, P.M., Hortsch, M., Patel, N.H., Jacobs, J.R., *et al*., **1989**. *Drosophila* neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell 59*, 447–460.

Bilder, D., **2001**. PDZ proteins and polarity: functions from the fly. *Trends Genet. 17*, 511–519.

Bilder, D., Schober, M., Perrimon, N., **2003**. Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat. Cell Biol. 5*, 53–58.

Blagburn, J.M., Sosa, M.A., Blanco, R.E., **1996**. Specificity of identified central synapses in the embryonic cockroach: appropriate connections form before the onset of spontaneous afferent activity. *J. Comp. Neurol. 373*, 511–528.

Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., **1996**. The embryonic central nervous system lineages of *Drosophila melanogaster.* I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Devel. Biol. 179*, 41–64.

Broadie, K.S., **1996**. Regulation of the synaptic vesicle cycle in *Drosophila*. *Biochem. Soc. Trans. 24*, 639–645.

Broadie, K.S., **1999**. Development of electrical properties and synaptic transmission at the embryonic neuromuscular junction. *Int. Rev. Neurobiol. 43*, 45–67.

Broadie, K.S., **2000**. Electrophysiological approaches to the neuromusculature. In: Sullivan, W., Ashburner, M., Hawley, R.S. (Eds.), Drosophila Protocols. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 273–295.

Broadie, K., Bate, M., **1991**. Development of adult muscles in *Drosophila*: ablation of identified muscle precursor cells. *Development 113*, 103–118.

Broadie, K., Bate, M., **1993a**. Activity-dependent development of the neuromuscular synapse during *Drosophila* embryogenesis. *Neuron 11*, 607–619.

Broadie, K., Bate, M., **1993b**. Innervation directs receptor synthesis and localization in *Drosophila* embryo synaptogenesis. *Nature 361*, 350–353.

Broadie, K., Bate, M., **1993c**. Muscle development is independent of innervation during *Drosophila* embryogenesis. *Development 119*, 533–543.

Broadie, K.S., Bate, M., **1993d**. Development of larval muscle properties in the embryonic myotubes of *Drosophila melanogaster. J. Neurosci. 13*, 167–180.

Broadie, K.S., Bate, M., **1993e**. Development of the embryonic neuromuscular synapse of *Drosophila melanogaster. J. Neurosci. 13*, 144–166.

Broadie, K., Bellen, H.J., DiAntonio, A., Littleton, J.T., Schwarz, T.L., **1994**. Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. *Proc. Natl Acad. Sci. USA 91*, 10727–10731.

Broadie, K., Prokop, A., Bellen, H.J., O'Kane, C.J., Schulze, K.L., *et al*., **1995**. Syntaxin and synaptobrevin function downstream of vesicle docking in *Drosophila*. *Neuron 15*, 663–673.

Broadie, K.S., Richmond, J.E., **2002**. Establishing and sculpting the synapse in *Drosophila* and *C. elegans*. *Curr. Opin. Neurobiol. 12*, 491–498.

Broadie, K., Rushton, E., Skoulakis, E.M., Davis, R.L., **1997**. Leonardo, a *Drosophila* 14-3-3 protein involved in learning, regulates presynaptic function. *Neuron 19*, 391–402.

Broadie, K., Sink, H., Van Vactor, D., Fambrough, D., Whitington, P.M., *et al*., **1993**. From growth cone to synapse: the life history of the RP3 motor neuron. *Devel. Suppl.* 227–238.

Budnik, V., **1996**. Synapse maturation and structural plasticity at *Drosophila* neuromuscular junctions. *Curr. Opin. Neurobiol. 6*, 858–867.

Budnik, V., Koh, Y.H., Guan, B., Hartmann, B., Hough, C., *et al*., **1996**. Regulation of synapse structure and function by the *Drosophila* tumor suppressor gene dlg. *Neuron 17*, 627–640.

Budnik, V., Zhong, Y., Wu, C.F., **1990**. Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J. Neurosci. 10*, 3754–3768.

Callec, J.J., **1985**. Synaptic transmission in the central nervous system. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Nervous System: Structure and Function. Pergamon, New York, pp. 139–180.

Campos-Ortega, J.A., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila melanogaster.* Springer, Berlin.

Carmena, A., Bate, M., Jimenez, F., **1995**. Lethal of scute, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. *Genes Devel. 9*, 2373–2383.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F., Michelson, A.M., **1998**. Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Devel. 12*, 3910–3922.

Casal, J., Leptin, M., **1996**. Identification of novel genes in *Drosophila* reveals the complex regulation of early gene activity in the mesoderm. *Proc. Natl Acad. Sci. USA 93*, 10327–10332.

Cash, S., Chiba, A., Keshishian, H., **1992**. Alternate neuromuscular target selection following the loss of single muscle fibers in *Drosophila*. *J. Neurosci. 12*, 2051–2064.

Castillo, J., Hoyle, G., Machne, X., **1953**. Neuromuscular transmission in a locust. *J. Physiol. 121*, 539–547.

Cattaert, D., Birman, S., **2001**. Blockade of the central generator of locomotor rhythm by noncompetitive NMDA receptor antagonists in *Drosophila* larvae. *J. Neurobiol. 48*, 58–73.

Chang, H., Ciani, S., Kidokoro, Y., **1994**. Ion permeation properties of the glutamate receptor channel in cultured embryonic *Drosophila* myotubes. *J. Physiol. 476*, 1–16.

Chang, H., Kidokoro, Y., **1996**. Kinetic properties of glutamate receptor channels in cultured embryonic *Drosophila* myotubes. *Jpn. J. Physiol. 46*, 249–264.

Cheung, U.S., Shayan, A.J., Boulianne, G.L., Atwood, H.L., **1999**. *Drosophila* larval neuromuscular

junction's responses to reduction of cAMP in the nervous system. *J. Neurobiol. 40*, 1–13.
Chiba, A., Hing, H., Cash, S., Keshishian, H., **1993**. Growth cone choices of *Drosophila* motoneurons in response to muscle fiber mismatch. *J. Neurosci. 13*, 714–732.
Chiba, A., Rose, D., **1998**. "Painting" the target: how local molecular cues define synaptic relationships. *Bioessays 20*, 941–948.
Chiba, A., Snow, P., Keshishian, H., Hotta, Y., **1995**. Fasciclin III as a synaptic target recognition molecule in *Drosophila*. *Nature 374*, 166–168.
Chopra, M., Gu, G.G., Singh, S., **2000**. Mutations affecting the delayed rectifier potassium current in *Drosophila*. *J. Neurogenet. 14*, 107–123.
Ciani, S., Nishikawa, K., Kidokoro, Y., **1997**. Permeation of organic cations and ammonium through the glutamate receptor channel in *Drosophila* larval muscle. *Jpn. J. Physiol. 47*, 189–198.
Cochilla, A.J., Angleson, J.K., Betz, W.J., **1999**. Monitoring secretory membrane with FM1-43 fluorescence. *Annu. Rev. Neurosci. 22*, 1–10.
Colledge, M., Froehner, S.C., **1998**. Signals mediating ion channel clustering at the neuromuscular junction. *Curr. Opin. Neurobiol. 8*, 357–363.
Cui, X., Doe, C.Q., **1992**. ming is expressed in neuroblast sublineages and regulates gene expression in the *Drosophila* central nervous system. *Development 116*, 943–952.
Cull-Candy, S.G., **1976**. Two types of extrajunctional L-glutamate receptors in locust muscle fibres. *J. Physiol. 255*, 449–464.
Cully, D.F., Paress, P.S., Liu, K.K., Schaeffer, J.M., Arena, J.P., **1996**. Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. *J. Biol. Chem. 271*, 20187–20191.
Currie, D.A., Truman, J.W., Burden, S.J., **1995**. *Drosophila* glutamate receptor RNA expression in embryonic and larval muscle fibers. *Devel. Dyn. 203*, 311–316.
Davis, G.W., DiAntonio, A., *et al.*, **1998**. Postsynaptic PKA controls quantal size and reveals a retrograde signal that regulates presynaptic transmitter release in Drosophila. *Neuron 20*(2), 305–315.
Davis, G.W., Goodman, C.S., **1998**. Synapse-specific control of synaptic efficacy at the terminals of a single neuron. *Nature 392*, 82–86.
Davis, G.W., Schuster, C.M., Goodman, C.S., **1996a**. Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron 17*, 669–679.
Davis, G.W., Schuster, C.M., Goodman, C.S., **1996b**. Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity [see comments]. *Neuron 17*, 669–679.
Davis, G.W., Schuster, C.M., Goodman, C.S., **1997**. Genetic analysis of the mechanisms controlling target selection: target-derived Fasciclin II regulates the pattern of synapse formation. *Neuron 19*, 561–573.
Demarque, M., Represa, A., Becq, H., Khalilov, I., Ben-Ari, Y., *et al.*, **2002**. Paracrine intercellular communication by a Ca^{2+}- and SNARE-independent release of GABA and glutamate prior to synapse formation. *Neuron 36*, 1051–1061.
DiAntonio, A., Patersen, S.A., *et al.*, **1999**. Glutamate receptor expression regulates quantal size and quantal content at the Drosophila neuromuscular junction. *J. Neurosci. 19*(8), 3023–3032.
DiAntonio, A., Schwarz, T.L., **1994**. The effect on synaptic physiology of synaptotagmin mutations in *Drosophila*. *Neuron 12*, 909–920.
Diefenbach, T.J., Guthrie, P.B., Stier, H., Billips, B., Kater, S.B., **1999**. Membrane recycling in the neuronal growth cone revealed by FM1-43 labeling. *J. Neurosci. 19*(21), 9436–9444.
Doberstein, S.K., Fetter, R.D., Mehta, A.Y., Goodman, C.S., **1997**. Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. *J. Cell Biol. 136*, 1249–1261.
Doe, C.Q., Bastiani, M.J., Goodman, C.S., **1986**. Guidance of neuronal growth cones in the grasshopper embryo. IV. Temporal delay experiments. *J. Neurosci. 6*, 3552–3563.
Doe, C.Q., Goodman, C.S., **1985**. Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Devel. Biol. 111*, 206–219.
Duan, S., Anderson, C.M., Keung, E.C., Chen, Y., Swanson, R.A., **2003**. P2X7 receptor-mediated release of excitatory amino acids from astrocytes. *J. Neurosci. 23*, 1320–1328.
Eaton, B.A., Fetter, R.D., Davis, G.W., **2002**. Dynactin is necessary for synapse stabilization. *Neuron 34*, 729–741.
Elkins, T., Ganetzky, B., **1990**. Conduction in the giant nerve fiber pathway in temperature-sensitive paralytic mutants of *Drosophila*. *J. Neurogenet. 6*, 207–219.
Elkins, T., Ganetzky, B., Wu, C.F., **1986**. A *Drosophila* mutation that eliminates a calcium-dependent potassium current. *Proc. Natl Acad. Sci. USA 83*, 8415–8419.
Fambrough, D., Goodman, C.S., **1996**. The *Drosophila* beaten path gene encodes a novel secreted protein that regulates defasciculation at motor axon choice points. *Cell 87*, 1049–1058.
Featherstone, D.E., Broadie, K., **2000**. Surprises from *Drosophila*: genetic mechanisms of synaptic development and plasticity. *Brain Res. Bull. 53*, 501–511.
Featherstone, D.E., Davis, W.S., Dubreuil, R.R., Broadie, K., **2001**. *Drosophila* alpha- and beta-spectrin mutations disrupt presynaptic neurotransmitter release. *J. Neurosci. 21*, 4215–4224.
Featherstone, D.E., Rushton, E., Broadie, K., **2002**. Developmental regulation of glutamate receptor field size by nonvesicular glutamate release. *Nat. Neurosci. 5*, 141–146.

Featherstone, D.E., Rushton, E.M., Hilderbrand-Chae, M., Phillips, A.M., Jackson, F.R., *et al.*, **2000**. Presynaptic glutamic acid decarboxylase is required for induction of the postsynaptic receptor field at a glutamatergic synapse. *Neuron 27*, 71–84.

Fehon, R.G., Dawson, I.A., Artavanis-Tsakonas, S., **1994**. A *Drosophila* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. *Development 120*, 545–557.

Fergestad, T., Broadie, K., **2001**. Interaction of stoned and synaptotagmin in synaptic vesicle endocytosis. *J. Neurosci. 21*, 1218–1227.

Fergestad, T., Davis, W.S., Broadie, K., **1999**. The stoned proteins regulate synaptic vesicle recycling in the presynaptic terminal. *J. Neurosci. 19*, 5847–5860.

Fergestad, T., Wu, M.N., Schulze, K.L., Lloyd, T.E., Bellen, H.J., *et al.*, **2001**. Targeted mutations in the syntaxis H3 domain specifically disrupt SNARE complex function is synaptic transmission. *J. Neurosci. 21*(23), 9142–9150.

Fletcher, T.L., De Camilli, P., Banker, G., **1994**. Synaptogenesis in hippocampal cultures: evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. *J. Neurosci. 14*, 6695–6706.

Fradkin, L.G., Kamphorst, J.T., *et al.*, **2002**. Genomewide analysis of the Drosophila tetraspanins reveals a subset with similar function in the formation of theembryonic sysnapse. *Proc. Natl Acad. Sci. USA 99*(21), 13663–13668.

Frasch, M., Leptin, M., **2000**. Mergers and acquisitions: unequal partnerships in *Drosophila* myoblast fusion. *Cell 102*, 127–129.

Freeman, M.R., Delrow, J., Kim, J., Johnson, E., Doe, C.Q., **2003**. Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. *Neuron 38*, 567–580.

Fritschy, J.M., Brunig, I., **2003**. Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. *Pharmacol. Ther. 98*, 299–323.

Furrer, M.P., Kim, S., Wolf, B., Chiba, A., **2003**. Robo and Frazzled/DCC mediate dendritic guidance at the CNS midline. *Nat. Neurosci. 6*, 223–230.

Furst, A., Mahowald, A.P., **1985**. Differentiation of primary embryonic neuroblasts in purified neural cell cultures from *Drosophila*. *Devel. Biol. 109*, 184–192.

Ganetzky, B., Robertson, G.A., Wilson, G.F., Trudeau, M.C., Titus, S.A., **1999**. The eag family of K^+ channels in *Drosophila* and mammals. *Ann. N. Y. Acad. Sci. 868*, 356–369.

Ganetzky, B., Wu, C.F., **1983**. Neurogenetic analysis of potassium currents in *Drosophila*: synergistic effects on neuromuscular transmission in double mutants. *J. Neurogenet. 1*, 17–28.

Gaul, U., Seifert, E., Schuh, R., Jackle, H., **1987**. Analysis of Kruppel protein distribution during early *Drosophila* development reveals posttranscriptional regulation. *Cell 50*, 539–547.

Gielow, M.L., Gu, G.G., Singh, S., **1995**. Resolution and pharmacological analysis of the voltage-dependent calcium channels of *Drosophila* larval muscles. *J. Neurosci. 15*, 6085–6093.

Godenschwege, T.A., Simpson, J.H., Shan, X., Bashaw, G.J., Goodman, C.S., *et al.*, **2002**. Ectopic expression in the giant fiber system of *Drosophila* reveals distinct roles for roundabout (Robo), Robo2, and Robo3 in dendritic guidance and synaptic connectivity. *J. Neurosci. 22*, 3117–3129.

Gonzalez-Gaitan, M., Jackle, H., **1997**. Role of *Drosophila* alpha-adaptin in presynaptic vesicle recycling. *Cell 88*, 767–776.

Goodman, C.S., Spitzer, N.C., **1979**. Embryonic development of identified neurones: differentiation from neuroblast to neurone. *Nature 280*, 208–214.

Gorczyca, M., Augart, C., Budnik, V., **1993**. Insulin-like receptor and insulin-like peptide are localized at neuromuscular junctions in *Drosophila*. *J. Neurosci. 13*, 3692–3704.

Gorczyca, M.G., Wu, C.F., **1991**. Single-channel K^+ currents in *Drosophila* muscle and their pharmacological block. *J. Membr. Biol. 121*, 237–248.

Griffith, L.C., **1997**. *Drosophila melanogaster* as a model system for the study of the function of calcium/calmodulin-dependent protein kinase II in synaptic plasticity. *Invert. Neurosci. 3*, 93–102.

Guan, B., Hartmann, B., Kho, Y.H., Gorczyca, M., Budnik, V., **1996**. The *Drosophila* tumor suppressor gene, dlg, is involved in structural plasticity at a glutamatergic synapse. *Curr. Biol. 6*, 695–706.

Haghighi, A.P., McCabe, B.D., Fetter, R.D., Palmer, J.E., Hom, S., *et al.*, **2003**. Retrograde control of synaptic transmission by postsynaptic CaMKII at the *Drosophila* neuromuscular junction. *Neuron 39*, 255–267.

Halpern, M.E., Chiba, A., Johansen, J., Keshishian, H., **1991**. Growth cone behavior underlying the development of stereotypic synaptic connections in *Drosophila* embryos. *J. Neurosci. 11*, 3227–3238.

Hannan, F., Zhong, Y., **1999**. Second messenger systems underlying plasticity at the neuromuscular junction. *Int. Rev. Neurobiol. 43*, 119–138.

Harrison, S.D., Broadie, K., van de Goor, J., Rubin, G.M., **1994**. Mutations in the *Drosophila* Rop gene suggest a function in general secretion and synaptic transmission. *Neuron 13*, 555–566.

Heckmann, M., Dudel, J., **1997**. Desensitization and resensitization kinetics of glutamate receptor channels from *Drosophila* larval muscle. *Biophys. J. 72*, 2160–2169.

Hewes, R.S., Snowdeal, E.C., III, Saitoe, M., Taghert, P.H., **1998**. Functional redundancy of FMRFamide-related peptides at the *Drosophila* larval neuromuscular junction. *J. Neurosci. 18*, 7138–7151.

Hidalgo, A., **2003**. Neuron-glia interactions during axon guidance in *Drosophila*. *Biochem. Soc. Trans. 31*, 50–55.

Ho, R., Ball, E.E., Goodman, C.S., **1983**. Muscle pioneers: large mesodermal cells that erect a scaffold for developing muscles and motoneurones in grasshopper embryos. *Nature 301*, 66–69.

Hoang, B., Chiba, A., **2001**. Single-cell analysis of *Drosophila* larval neuromuscular synapses. *Devel. Biol. 229*, 55–70.

Hortsch, M., Goodman, C.S., **1991**. Cell and substrate adhesion molecules in *Drosophila*. *Annu. Rev. Cell Biol. 7*, 505–557.

Huff, R., Furst, A., Mahowald, A.P., **1989**. *Drosophila* embryonic neuroblasts in culture: autonomous differentiation of specific neurotransmitters. *Devel. Biol. 134*, 146–157.

Hummel, T., Krukkert, K., Roos, J., Davis, G., Klambt, C., **2000**. *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. *Neuron 26*, 357–370.

Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., **2001**. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell 106*, 511–521.

Jacobs, J.R., Goodman, C.S., **1989**. Embryonic development of axon pathways in the *Drosophila* CNS. I. A glial scaffold appears before the first growth cones. *J. Neurosci. 9*, 2402–2411.

Jan, L.Y., Jan, Y.N., **1976a**. L-Glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. *J. Physiol.* (*Lond.*) *262*, 215–236.

Jan, L.Y., Jan, Y.N., **1976b**. Properties of the larval neuromuscular junction in *Drosophila melanogaster. J. Physiol.* (*Lond.*) *262*, 189–214.

Jia, X.X., Gorczyca, M., Budnik, V., **1993**. Ultrastructure of neuromuscular junctions in *Drosophila*: comparison of wild type and mutants with increased excitability. *J. Neurobiol. 24*, 1025–1044.

Jonas, P.E., Phannavong, B., Schuster, R., Schroder, C., Gundelfinger, E.D., **1994**. Expression of the ligand-binding nicotinic acetylcholine receptor subunit D alpha 2 in the *Drosophila* central nervous system. *J. Neurobiol. 25*, 1494–1508.

Jones, B.W., Fetter, R.D., Tear, G., Goodman, C.S., **1995**. Glial cells missing: a genetic switch that controls glial versus neuronal fate. *Cell 82*, 1013–1023.

Kamb, A., Iverson, L.E., Tanouye, M.A., **1987**. Molecular characterization of Shaker, a *Drosophila* gene that encodes a potassium channel. *Cell 50*, 405–413.

Kamb, A., Tseng-Crank, J., Tanouye, M.A., **1988**. Multiple products of the *Drosophila* Shaker gene may contribute to potassium channel diversity. *Neuron 1*, 421–430.

Kamdar, K.P., Shelton, M.E., Finnerty, V., **1994**. The *Drosophila* molybdenum cofactor gene cinnamon is homologous to three *Escherichia coli* cofactor proteins and to the rat protein gephyrin. *Genetics 137*, 791–801.

Kardos, J., **1999**. Recent advances in GABA research. *Neurochem. Int. 34*, 353–358.

Kazama, H., Morimoto-Tanifuji, T., Nose, A., **2003**. Postsynaptic activation of calcium/calmodulin-dependent protein kinase II promotes coordinated pre- and postsynaptic maturation of *Drosophila* neuromuscular junctions. *Neuroscience 117*, 615–625.

Keshishian, H., Broadie, K., Chiba, A., Bate, M., **1996**. The drosophila neuromuscular junction: a model system for studying synaptic development and function. *Annu. Rev. Neurosci. 19*, 545–575.

Keyser, M.R., Anson, B.D., Titus, S.A., Ganetzky, B., Witten, J.L., **2003**. Molecular characterization, functional expression, and developmental profile of an ether a-go-go K^+ channel in the tobacco hornworm *Manduca sexta*. *J. Neurobiol. 55*, 73–85.

Klambt, C., Jacobs, J.R., Goodman, C.S., **1991**. The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell 64*, 801–815.

Koenig, J.H., Ikeda, K., **1989**. Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. *J. Neurosci. 9*, 3844–3860.

Koh, Y.H., Popova, E., Thomas, U., Griffith, L.C., Budnik, V., **1999**. Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation. *Cell 98*, 353–363.

Kolodkin, A.L., Matthes, D.J., Goodman, C.S., **1993**. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell 75*, 1389–1399.

Kolodkin, A.L., Matthes, D.J., O'Connor, T.P., Patel, N.H., Admon, A., *et al.*, **1992**. Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron 9*, 831–845.

Komiyama, T., Johnson, W.A., Luo, L., Jefferis, G.S., **2003**. From lineage to wiring specificity. POU domain transcription factors control precise connections of *Drosophila* olfactory projection neurons. *Cell 112*, 157–167.

Kopczynski, C.C., Davis, G.W., *et al.*, **1996**. A neural tetraspanin, encoded by late bloomer, that facilitates synapse formation. *Science 271*(*5257*), 1867–1870.

Kulkarni, S.J., Hall, J.C., **1987**. Behavioral and cytogenetic analysis of the cacophony courtship song mutant and interacting genetic variants in *Drosophila* melanogaster. *Genetics 115*, 461–475.

Kurdyak, P., Atwood, H.L., Stewart, B.A., Wu, C.F., **1994**. Differential physiology and morphology of motor axons to ventral longitudinal muscles in larval *Drosophila*. *J. Comp. Neurol. 350*, 463–472.

Kuromi, H., Kidokoro, Y., **1998**. Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive *Drosophila* mutant, shibire. *Neuron 20*, 917–925.

Kuromi, H., Kidokoro, Y., **1999**. The optically determined size of exo/endo cycling vesicle pool correlates with the quantal content at the neuromuscular junction of *Drosophila* larvae. *J. Neurosci. 19*, 1557–1565.

Kuromi, H., Kidokoro, Y., **2000**. Tetanic stimulation recruits vesicles from reserve pool via a cAMP-mediated process in *Drosophila* synapses. *Neuron 27*, 133–143.

Kuromi, H., Kidokoro, Y., **2002**. Selective replenishment of two vesicle pools depends on the source of Ca^{2+} at the *Drosophila* synapse. *Neuron 35*, 333–343.

Kuwada, J.Y., Goodman, C.S., **1985**. Neuronal determination during embryonic development of the grasshopper nervous system. *Devel. Biol. 110*, 114–126.

Lahey, T., Gorczyca, M., Jia, X.X., Budnik, V., **1994**. The *Drosophila* tumor suppressor gene dlg is required for normal synaptic bouton structure. *Neuron 13*, 823–835.

Landgraf, M., Baylies, M., Bate, M., **1999a**. Muscle founder cells regulate defasciculation and targeting of motor axons in the *Drosophila* embryo. *Curr. Biol. 9*, 589–592.

Landgraf, M., Bossing, T., Technau, G.M., Bate, M., **1997**. The origin, location, and projections of the embryonic abdominal motorneurons of *Drosophila*. *J. Neurosci. 17*, 9642–9655.

Landgraf, M., Roy, S., Prokop, A., VijayRaghavan, K., Bate, M., **1999b**. even-skipped determines the dorsal growth of motor axons in *Drosophila*. *Neuron 22*, 43–52.

Landgraf, M., Sanchez-Soriano, N., Technau, G.M., Urban, J., Prokop, A., **2003**. Charting the *Drosophila* neuropile: a strategy for the standardised characterisation of genetically amenable neurites. *Devel. Biol. 260*, 207–225.

Lane, N.J., **1985**. Structure and components of the nervous system. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Nervous System: Structure and Motor Function. Pergamon, Oxford, pp. 1–49.

Lee, D., O'Dowd, D.K., **1999**. Fast excitatory synaptic transmission mediated by nicotinic acetylcholine receptors in *Drosophila* neurons. *J. Neurosci. 19*, 5311–5321.

Lee, D., O'Dowd, D.K., **2000a**. cAMP-dependent plasticity at excitatory cholinergic synapses in *Drosophila* neurons: alterations in the memory mutant dunce. *J. Neurosci. 20*, 2104–2111.

Lee, D., O'Dowd, D.K., **2000b**. cAMP-dependent plasticity at excitatory cholinergic synapses in *Drosophila* neurons: alterations in the memory mutant dunce. *J. Neurosci. 20*, 2104–2111.

Lee, D., Su, H., O'Dowd, D.K., **2003**. GABA receptors containing Rdl subunits mediate fast inhibitory synaptic transmission in *Drosophila* neurons. *J. Neurosci. 23*, 4625–4634.

Lee, T., Winter, C., Marticke, S.S., Lee, A., Luo, L., **2000**. Essential roles of *Drosophila* RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron 25*, 307–316.

Leiserson, W.M., Harkins, E.W., Keshishian, H., **2000**. Fray, a *Drosophila* serine/threonine kinase homologous to mammalian PASK, is required for axonal ensheathment. *Neuron 28*, 793–806.

Leptin, M., **1991**. twist and snail as positive and negative regulators during *Drosophila* mesoderm development. *Genes Dev. 5*, 1568–1576.

Levine, R.B., **1984**. Changes in neuronal circuits during insect metamorphosis. *J. Exp. Biol. 112*, 27–44.

Levine, R.B., Weeks, J.C., **1996**. Cell culture approaches to understanding the actions of steroid hormones on the insect nervous system. *Devel. Neurosci. 18*, 73–86.

Li, H., Cooper, R.L., **2001**. Effects of the ecdysoneless mutant on synaptic efficacy and structure at the neuromuscular junction in *Drosophila* larvae during normal and prolonged development. *Neuroscience 106*, 193–200.

Lissin, D.V., Carroll, R.C., Nicoll, R.A., Malenka, R.C., von Zastrow, M., **1999**. Rapid, activation-induced redistribution of ionotropic glutamate receptors in cultured hippocampal neurons. [Published erratum appears in J. Neurosci. 1999; 19 (8), 3275.] *J. Neurosci. 19*, 1263–1272.

Littleton, J.T., Bellen, H.J., Perin, M.S., **1993a**. Expression of synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse. *Development 118*, 1077–1088.

Littleton, J.T., Ganetzky, B., **2000**. Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron 26*, 35–43.

Littleton, J.T., Serano, T.L., Rubin, G.M., Ganetzky, B., Chapman, E.R., **1999**. Synaptic function modulated by changes in the ratio of synaptotagmin I and IV. *Nature 400*, 757–760.

Littleton, J.T., Stern, M., Schulze, K., Perin, M., Bellen, H.J., **1993b**. Mutational analysis of *Drosophila* synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release [see comments]. *Cell 74*, 1125–1134.

Lloyd, T.E., Verstreken, P., Ostrin, E.J., Phillippi, A., Lichtarge, O., Bellen, H.J., **2000**. A genome-wide search for synaptic vesicle cycle proteins in *Drosophila*. *Neuron 26*, 45–50.

Lnenicka, G.A., Keshishian, H., **2000**. Identified motor terminals in *Drosophila* larvae show distinct differences in morphology and physiology. *J. Neurobiol. 43*, 186–197.

Lohr, R., Godenschwege, T., Buchner, E., Prokop, A., **2002**. Compartmentalization of central neurons in *Drosophila*: a new strategy of mosaic analysis reveals localization of presynaptic sites to specific segments of neurites. *J. Neurosci. 22*, 10357–10367.

Loughney, K., Kreber, R., Ganetzky, B., **1989**. Molecular analysis of the para locus, a sodium channel gene in *Drosophila*. *Cell 58*, 1143–1154.

Luer, K., Technau, G.M., **1992**. Primary culture of single ectodermal precursors of *Drosophila* reveals a dorsoventral prepattern of intrinsic neurogenic and epidermogenic capabilities at the early gastrula stage. *Development 116*, 377–385.

Luo, L., Liao, Y.J., Jan, L.Y., Jan, Y.N., **1994**. Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Devel. 8*, 1787–1802.

Madden, D.R., **2002**. The structure and function of glutamate receptor ion channels. *Nat. Rev. Neurosci. 3*, 91–101.

Marin, E.C., Jefferis, G.S., Komiyama, T., Zhu, H., Luo, L., **2002**. Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell 109*, 243–255.

Mellerick, D.M., Kassis, J.A., Zhang, S.D., Odenwald, W.F., **1992**. castor encodes a novel zinc finger protein required for the development of a subset of CNS neurons in *Drosophila*. *Neuron 9*, 789–803.

Michelson, A.M., Abmayr, S.M., Bate, M., Arias, A.M., Maniatis, T., **1990**. Expression of a MyoD family

member prefigures muscle pattern in *Drosophila* embryos. *Genes Devel. 4*, 2086–2097.

Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., *et al.*, **1995**. Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster. J. Comp. Neurol. 356*, 275–287.

Nachman, R.J., Olender, E.H., Roberts, V.A., Holman, G.M., Yamamoto, D., **1996**. A nonpeptidal peptidomimetic agonist of the insect FLRFamide myosuppressin family. *Peptides 17*, 313–320.

Nagaya, Y., Kutsukake, M., Chigusa, S.I., Komatsu, A., **2002**. A trace amine, tyramine, functions as a neuromodulator in *Drosophila melanogaster. Neurosci. Lett. 329*, 324–328.

Nassel, D.R., Lundquist, T., Hoog, A., Grimelius, L., **1990**. Substance P-like immunoreactive neurons in the nervous system of *Drosophila*. *Brain Res. 507*, 225–233.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., *et al.*, **2002**. Rac GTPases control axon growth, guidance and branching. *Nature 416*, 442–447.

Nicholls, D.G., Sihra, T.S., **1986**. Synaptosomes possess an exocytotic pool of glutamate. *Nature 321*, 772–773.

Nichols, R., McCormick, J.B., Lim, I.A., **1999**. Structure, function, and expression of *Drosophila melanogaster* FMRFamide-related peptides. *Ann. N.Y. Acad. Sci. 897*, 264–272.

Nishikawa, K., Kidokoro, Y., **1995**. Junctional and extrajunctional glutamate receptor channels in *Drosophila* embryos and larvae. *J. Neurosci. 15*, 7905–7915.

Nishikawa, K., Kidokoro, Y., **1999**. Octopamine inhibits synaptic transmission at the larval neuromuscular junction in *Drosophila melanogaster. Brain Res. 837*, 67–74.

Noordermeer, J.N., Kopczynski, C.C., Fetter, R.D., Bland, K.S., Chen, W.Y., *et al.*, **1998**. Wrapper, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheath commissural axons in *Drosophila*. *Neuron 21*, 991–1001.

Nose, A., Mahajan, V.B., Goodman, C.S., **1992**. Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in *Drosophila*. *Cell 70*, 553–567.

Nose, A., Umeda, T., Takeichi, M., **1997**. Neuromuscular target recognition by a homophilic interaction of connectin cell adhesion molecules in *Drosophila*. *Development 124*, 1433–1441.

O'Connor, T.P., Duerr, J.S., Bentley, D., **1990**. Pioneer growth cone steering decisions mediated by single filopodial contacts in situ. *J. Neurosci. 10*, 3935–3946.

Odden, J.P., Holbrook, S., Doe, C.Q., **2002**. *Drosophila* HB9 is expressed in a subset of motoneurons and interneurons, where it regulates gene expression and axon pathfinding. *J. Neurosci. 22*, 9143–9149.

O'Dowd, D.K., **1995**. Voltage-gated currents and firing properties of embryonic *Drosophila* neurons grown in a chemically defined medium. *J. Neurobiol. 27*, 113–126.

O'Dowd, D.K., Aldrich, R.W., **1988**. Voltage-clamp analysis of sodium channels in wild-type and mutant *Drosophila* neurons. *J. Neurosci. 8*, 3633–3643.

Oland, L.A., Tolbert, L.P., **2003**. Key interactions between neurons and glial cells during neural development in insects. *Annu. Rev. Entomol. 48*, 89–110.

Orchard, I., Loughton, B.G., **1985**. Neurosecretion. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Endocrinology I. Pergamon, New York, pp. 61–108.

Packard, M., Koo, E.S., Gorczyca, M., Sharpe, J., Cumberledge, S., *et al.*, **2002**. The *Drosophila* Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell 111*, 319–330.

Packard, M., Mathew, D., Budnik, V., **2003**. Wnts and TGF beta in synaptogenesis: old friends signalling at new places. *Nat. Rev. Neurosci. 4*, 113–120.

Paradis, S., Sweeney, S.T., Davis, G.W., **2001**. Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. *Neuron 30*, 737–749.

Parfitt, K., Reist, N., Li, J., Burgess, R., Deitcher, D., *et al.*, **1995**. *Drosophila* genetics and the functions of synaptic proteins. *Cold Spring Harb. Symp. Quant. Biol. 60*, 371–377.

Parmentier, M.L., Pin, J.P., Bockaert, J., Grau, Y., **1996**. Cloning and functional expression of a *Drosophila* metabotropic glutamate receptor expressed in the embryonic CNS. *J. Neurosci. 16*, 6687–6694.

Parnas, D., Haghighi, A.P., Fetter, R.D., Kim, S.W., Goodman, C.S., **2001**. Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. *Neuron 32*, 415–424.

Patel, N.H., Snow, P.M., Goodman, C.S., **1987**. Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell 48*, 975–988.

Pennetta, G., Hiesinger, P., Fabian-Fine, R., Meinertzhagen, I., Bellen, H., **2002**. *Drosophila* VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron 35*, 291–306.

Petersen, S.A., Fetter, R.D., Noordermeer, J.N., Goodman, C.S., DiAntonio, A., **1997**. Genetic analysis of glutamate receptors in *Drosophila* reveals a retrograde signal regulating presynaptic transmitter release. *Neuron 19*, 1237–1248.

Pfeiffer, H.H., **1937**. Versuche nuit ganglionkulturen aus corethra larven. *Archiv. Exp. Zellforschung 20*, 225–229.

Phillips, G.R., Huang, J.K., Wang, Y., Tanaka, H., Shapiro, L., *et al.*, **2001**. The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron 32*, 63–77.

Pongs, O., Lindemeier, J., Zhu, X.R., Theil, T., Engelkamp, D., *et al.*, **1993**. Frequenin – a novel calcium-binding protein that modulates synaptic efficacy in the *Drosophila* nervous system. *Neuron 11*, 15–28.

Prokop, A., **1999**. Integrating bits and pieces: synapse structure and formation in *Drosophila* embryos. *Cell Tissue Res. 297*, 169–186.

Prokop, A., Landgraf, M., Rushton, E., Broadie, K., Bate, M., **1996**. Presynaptic development at the *Drosophila* neuromuscular junction: assembly and localization of presynaptic active zones. *Neuron 17*, 617–626.

Prokop, A., Technau, G.M., **1991**. The origin of postembryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster*. *Development 111*, 79–88.

Prokop, A., Technau, G.M., **1994**. Early tagma-specific commitment of *Drosophila* CNS progenitor NB1-1. *Development 120*, 2567–2578.

Reiff, D.F., Thiel, P.R., Schuster, C.M., **2002**. Differential regulation of active zone density during long-term strengthening of *Drosophila* neuromuscular junctions. *J. Neurosci. 22*, 9399–9409.

Renden, R., Berwin, B., Chin, C.T., Kreber, R., Ganetzky, B., *et al.*, **2001**. *Drosophila* CAPS is an essential gene required for the regulation of dense core vesicle release and synaptic vesicle fusion. *Neuron 31*, 421–437.

Renden, R.B., Broadie, K., **2003**. Mutation and activation of Galpha s similarly alters pre- and postsynaptic mechanisms modulating neurotransmission. *J. Neurophysiol. 89*, 2620–2638.

Renger, J.J., Ueda, A., Atwood, H.L., Govind, C.K., Wu, C.F., **2000**. Role of cAMP cascade in synaptic stability and plasticity: ultrastructural and physiological analyses of individual synaptic boutons in *Drosophila* memory mutants. *J. Neurosci. 20*, 3980–3992.

Rheuben, M.B., Kammer, A.E., **1981**. Membrane structures and physiology of an immature synapse. *J. Neurocytol. 10*, 557–575.

Rheuben, M.B., Yoshihara, M., Kidokoro, Y., **1999**. Ultrastructural correlates of neuromuscular junction development. *Int. Rev. Neurobiol. 43*, 69–92.

Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R., Leptin, M., **1997**. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development 124*, 2915–2922.

Rieckhof, G.E., Yoshihara, M., Guan, Z., Littleton, J.T., **2003**. Presynaptic N-type calcium channels regulate synaptic growth. *J. Biol. Chem. 278*(42), 41099–41108.

Ritzenthaler, S., Chiba, A., **2003**. Myopodia (postsynaptic filopodia) participate in synaptic target recognition. *J. Neurobiol. 55*, 31–40.

Ritzenthaler, S., Suzuki, E., Chiba, A., **2000**. Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. *Nat. Neurosci. 3*, 1012–1017.

Roche, J.P., Packard, M.C., Moeckel-Cole, S., Budnik, V., **2002**. Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble. *J. Neurosci. 22*, 6471–6479.

Rodesch, C.K., Broadie, K., **2000**. Genetic studies in *Drosophila*: vesicle pools and cytoskeleton-based regulation of synaptic transmission. *Neuroreport 11*, R45–53.

Rohrbough, J., Broadie, K., **2002**. Electrophysiological analysis of synaptic transmission in central neurons of *Drosophila* larvae. *J. Neurophysiol. 88*(2), 847–860.

Rohrbough, J., Grotewiel, M.S., Davis, R.L., Broadie, K., **2000**. Integrin-mediated regulation of synaptic morphology, transmission, and plasticity. *J. Neurosci. 20*, 6868–6878.

Rohrbough, J., O'Dowd, D.K., Baines, R.A., Broadie, K., **2003**. Cellular bases of behavioral plasticity: establishing and modifying synaptic circuits in the *Drosophila* genetic system. *J. Neurobiol. 54*, 254–271.

Rohrbough, J., Pinto, S., Mihalek, R.M., Tully, T., Broadie, K., **1999**. latheo, a *Drosophila* gene involved in learning, regulates functional synaptic plasticity. *Neuron 23*, 55–70.

Roos, J., Hummel, T., Ng, N., Klambt, C., Davis, G.W., **2000**. *Drosophila* Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. *Neuron 26*, 371–382.

Rose, D., Zhu, X., Kose, H., Hoang, B., Cho, J., *et al.*, **1997**. Toll, a muscle cell surface molecule, locally inhibits synaptic initiation of the RP3 motoneuron growth cone in *Drosophila*. *Development 124*, 1561–1571.

Rose, U., Ferber, M., Hustert, R., **2001**. Maturation of muscle properties and its hormonal control in an adult insect. *J. Exp. Biol. 204*, 3531–3545.

Ruiz-Gomez, M., Coutts, N., Price, A., Taylor, M.V., Bate, M., **2000**. *Drosophila* dumbfounded: a myoblast attractant essential for fusion. *Cell 102*, 189–198.

Ruiz-Gomez, M., Coutts, N., Suster, M.L., Landgraf, M., Bate, M., **2002**. myoblasts incompetent encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*. *Development 129*, 133–141.

Ruiz-Gomez, M., Romani, S., Hartmann, C., Jackle, H., Bate, M., **1997**. Specific muscle identities are regulated by Kruppel during *Drosophila* embryogenesis. *Development 124*, 3407–3414.

Rushton, E., Drysdale, R., Abmayr, S.M., Michelson, A.M., Bate, M., **1995**. Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development 121*, 1979–1988.

Saito, M., Kawai, N., **1985**. Developmental changes in the glutamate receptor at the insect neuromuscular synapse. *Brain Res. 350*, 97–102.

Saito, M., Kawai, N., **1987**. Patch clamp study of single glutamate channels during development in insect muscle. *Devel. Biol. 121*, 90–96.

Saito, M., Wu, C.F., **1991**. Expression of ion channels and mutational effects in giant *Drosophila* neurons differentiated from cell division-arrested embryonic neuroblasts. *J. Neurosci. 11*, 2135–2150.

Saitoe, M., Tanaka, S., Takata, K., Kidokoro, Y., **1997**. Neural activity affects distribution of glutamate receptors during neuromuscular junction formation in *Drosophila* embryos. *Devel. Biol. 184*, 48–60.

Salinas, P.C., **1999**. Wnt factors in axonal remodelling and synaptogenesis. *Biochem. Soc. Symp. 65*, 101–109.

Salkoff, L., **1985**. Development of ion channels in the flight muscles of *Drosophila*. *J. Physiol.* (*Paris*) *80*, 275–282.

Sanes, J.R., Lichtman, J.W., **1999**. Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci. 22*, 389–442.

Sassoe-Pognetto, M., Fritschy, J.M., **2000**. Mini-review: gephyrin, a major postsynaptic protein of GABAergic synapses. *Eur. J. Neurosci. 12*, 2205–2210.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., Serafini, T., **2000**. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell 101*, 657–669.

Schmid, A., Chiba, A., Doe, C.Q., **1999**. Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development 126*, 4653–4689.

Schmidt, H., Luer, K., Hevers, W., Technau, G.M., **2000**. Ionic currents of *Drosophila* embryonic neurons derived from selectively cultured CNS midline precursors. *J. Neurobiol. 44*, 392–413.

Schrader, S., Merritt, D.J., **2000**. Central projections of *Drosophila* sensory neurons in the transition from embryo to larva. *J. Comp. Neurol. 435*, 34–44.

Schulte, J., Tepass, U., Auld, V.J., **2003**. Gliotactin, a novel marker of tricellular junctions, is necessary for septate junction development in *Drosophila*. *J. Cell Biol. 161*, 991–1000.

Schulze, K.L., Broadie, K., Perin, M.S., Bellen, H.J., **1995**. Genetic and electrophysiological studies of *Drosophila* syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell 80*, 311–320.

Schulze, K.L., Littleton, J.T., Salzberg, A., Halachmi, N., Stern, M., *et al.*, **1994**. rop, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. *Neuron 13*, 1099–1108.

Schuster, C.M., Davis, G.W., Fetter, R.D., Goodman, C.S., **1996a**. Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth [see comments]. *Neuron 17*, 641–654.

Schuster, C.M., Davis, G.W., Fetter, R.D., Goodman, C.S., **1996b**. Genetic dissection of structural and functional components of synaptic plasticity. II. Fasciclin II controls presynaptic structural plasticity [see comments]. *Neuron 17*, 655–667.

Schuster, C.M., Ultsch, A., Schloss, P., Cox, J.A., Schmitt, B., *et al.*, **1991**. Molecular cloning of an invertebrate glutamate receptor subunit expressed in *Drosophila* muscle. *Science 254*, 112–114.

Schuster, C.M., Ultsch, A., Schmitt, B., Betz, H., **1993**. Molecular analysis of *Drosophila* glutamate receptors. *EXS 63*, 234–240.

Scott, E.K., Reuter, J.E., Luo, L., **2003**. Dendritic development of *Drosophila* high order visual system neurons is independent of sensory experience. *BMC Neurosci. 4*, 14.

Sepp, K.J., Auld, V.J., **2003**. Reciprocal interactions between neurons and glia are required for *Drosophila* peripheral nervous system development. *J. Neurosci. 23*, 8221–8230.

Sepp, K.J., Schulte, J., Auld, V.J., **2000**. Developmental dynamics of peripheral glia in *Drosophila melanogaster. Glia 30*, 122–133.

Sepp, K.J., Schulte, J., Auld, V.J., **2001**. Peripheral glia direct axon guidance across the CNS/PNS transition zone. *Devel. Biol. 238*, 47–63.

Shapira, M., Zhai, R.G., Dresbach, T., Bresler, T., Torres, V.I., *et al.*, **2003**. Unitary assembly of presynaptic active zones from Piccolo–Bassoon transport vesicles. *Neuron 38*, 237–252.

Sigrist, S.J., Reiff, D.F., Thiel, P.R., Steinert, J.R., Schuster, C.M., **2003**. Experience-dependent strengthening of *Drosophila* neuromuscular junctions. *J. Neurosci. 23*, 6546–6556.

Sigrist, S.J., Thiel, P.R., Reiff, D.F., Lachance, P.E., Lasko, P., *et al.*, **2000**. Postsynaptic translation affects the efficacy and morphology of neuromuscular junctions. *Nature 405*, 1062–1065.

Sigrist, S.J., Thiel, P.R., Reiff, D.F., Schuster, C.M., **2002**. The postsynaptic glutamate receptor subunit DGluR-IIA mediates long-term plasticity in *Drosophila*. *J. Neurosci. 22*, 7362–7372.

Simpson, J.H., Bland, K.S., Fetter, R.D., Goodman, C.S., **2000**. Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position. *Cell 103*, 1019–1032.

Singh, S., Wu, C.F., **1999**. Ionic currents in larval muscles of *Drosophila*. *Int. Rev. Neurobiol. 43*, 191–220.

Sink, H., Rehm, E.J., Richstone, L., Bulls, Y.M., Goodman, C.S., **2001**. sidestep encodes a target-derived attractant essential for motor axon guidance in *Drosophila*. *Cell 105*, 57–67.

Sink, H., Whitington, P.M., **1991**. Pathfinding in the central nervous system and periphery by identified embryonic *Drosophila* motor axons. *Development 112*, 307–316.

Sippy, T., Cruz-Martin, A., Jeromin, A., Schweizer, F.E., **2003**. Acute changes in short-term plasticity at synapses with elevated levels of neuronal calcium sensor-1. *Nat. Neurosci. 6*, 1031–1038.

Smith, L.A., Wang, X., Peixoto, A.A., Neumann, E.K., Hall, L.M., *et al.*, **1996**. A *Drosophila* calcium channel alpha1 subunit gene maps to a genetic locus associated with behavioral and visual defects. *J. Neurosci. 16*(24), 7868–7879.

Solc, C.K., Aldrich, R.W., **1988**. Voltage-gated potassium channels in larval CNS neurons of *Drosophila*. *J. Neurosci. 8*, 2556–2570.

Solc, C.K., Zagotta, W.N., Aldrich, R.W., **1987**. Single-channel and genetic analyses reveal two distinct A-type potassium channels in *Drosophila*. *Science 236*, 1094–1098.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., Bate, M., **1994**. dpp induces mesodermal gene expression in *Drosophila*. *Nature 372*, 783–786.

Suster, M.L., Bate, M., **2002**. Embryonic assembly of a central pattern generator without sensory input. *Nature 416*, 174–178.

Suzuki, E., Rose, D., Chiba, A., **2000**. The ultrastructural interactions of identified pre- and postsynaptic cells during synaptic target recognition in *Drosophila* embryos. *J. Neurobiol. 42*, 448–459.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., O'Kane, C.J., **1995**. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates

synaptic transmission and causes behavioral defects. *Neuron 14*, 341–351.

Taghert, P.H., Veenstra, J.A., **2003**. *Drosophila* neuropeptide signaling. *Adv. Genet. 49*, 1–65.

Tessier-Lavigne, M., Goodman, C.S., **1996**. The molecular biology of axon guidance. *Science 274*, 1123–1133.

Thomas, J.B., Bastiani, M.J., Bate, M., Goodman, C.S., **1984**. From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature 310*, 203–207.

Thomas, U., Ebitsch, S., Gorczyca, M., Koh, Y.H., Hough, C.D., *et al*., **2000**. Synaptic targeting and localization of discs-large is a stepwise process controlled by different domains of the protein. *Curr. Biol. 10*, 1108–1117.

Thomas, U., Kim, E., Kuhlendahl, S., Koh, Y.H., Gundelfinger, E.D., *et al*., **1997**. Synaptic clustering of the cell adhesion molecule fasciclin II by discs-large and its role in the regulation of presynaptic structure. *Neuron 19*, 787–799.

Thomas, W.E., Jordan, F.L., Townsel, J.G., **1987**. The status of the study of invertebrate neurons in tissue culture – phylum Arthropoda. *Comp. Biochem. Physiol. A 87*, 215–222.

Thor, S., Andersson, S.G., Tomlinson, A., Thomas, J.B., **1999**. A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature 397*, 76–80.

Thor, S., Thomas, J.B., **1997**. The *Drosophila* islet gene governs axon pathfinding and neurotransmitter identity. *Neuron 18*, 397–409.

Titmus, M.J., **1981**. Ultrastructure of identified fast excitatory, slow excitatory and inhibitory neuromuscular junctions in the locust. *J. Neurocytol. 10*, 363–385.

Treherne, J.E., **1985**. Blood-brain barrier. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Nervous System: Structure and Motor Function. Pergamon, New York, pp. 115–138.

Tsunoda, S., Salkoff, L., **1995a**. Genetic analysis of *Drosophila* neurons: Shal, Shaw, and Shab encode most embryonic potassium currents. *J. Neurosci. 15*, 1741–1754.

Tsunoda, S., Salkoff, L., **1995b**. The major delayed rectifier in both *Drosophila* neurons and muscle is encoded by Shab. *J. Neurosci. 15*, 5209–5221.

Ueda, A., Kidokoro, Y., **1996**. Longitudinal body wall muscles are electrically coupled across the segmental boundary in the third instar larva of *Drosophila melanogaster. Invert. Neurosci. 1*, 315–322.

Usherwood, P.N., **1977**. Glutamatergic synapses in invertebrates [proceedings]. *Biochem. Soc. Trans. 5*, 845–849.

Usherwood, P.N.R., **1994**. Insect glutamate receptors. In: Evans, P.D. (Ed.), Advances in Insect Physiology. Academic Press, New York, pp. 309–341.

van der Bliek, A.M., Meyerowitz, E.M., **1991**. Dynamin-like protein encoded by the *Drosophila* shibire gene associated with vesicular traffic. *Nature 351*, 411–414.

Verstreken, P., Kjaerulff, O., Lloyd, T.E., Atkinson, R., Zhou, Y., *et al*., **2002**. Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell 109*, 101–112.

Walther, C., **1981**. Synaptic terminals from an identified motoneuron in locust muscle: comparison between first instar larva and adult. *Neurosci. Lett. 27*, 237–242.

Wang, J.W., Humphreys, J.M., Phillips, J.P., Hilliker, A.J., Wu, C.F., **2000**. A novel leg-shaking *Drosophila* mutant defective in a voltage-gated K(+) current and hypersensitive to reactive oxygen species. *J. Neurosci. 20*, 5958–5964.

Weeks, J.C., Jacobs, G.A., Pierce, J.T., Sandstrom, D.J., Streichert, L.C., *et al*., **1997**. Neural mechanisms of behavioral plasticity: metamorphosis and learning in *Manduca sexta*. *Brain Behav. Evol. 50* (Suppl. 1), 69–80.

Winberg, M.L., Mitchell, K.J., Goodman, C.S., **1998**. Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell 93*, 581–591.

Wittle, A.E., Kamdar, K.P., Finnerty, V., **1999**. The *Drosophila* cinnamon gene is functionally homologous to *Arabidopsis* cnx1 and has a similar expression pattern to the mammalian gephyrin gene. *Mol. Gen. Genet. 261*, 672–680.

Woods, D.F., Bryant, P.J., **1993**. ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate and synaptic cell junctions. *Mech. Devel. 44*, 85–89.

Wu, C.F., Ganetzky, B., **1992**. Neurogenetic studies of ion channels in *Drosophila*. *Ion Channels 3*, 261–314.

Wu, M.N., Bellen, H.J., **1997**. Genetic dissection of synaptic transmission in *Drosophila*. *Curr. Opin. Neurobiol. 7*, 624–630.

Yao, W.D., Rusch, J., Poo, M., Wu, C.F., **2000**. Spontaneous acetylcholine secretion from developing growth cones of *Drosophila* central neurons in culture: effects of cAMP-pathway mutations. *J. Neurosci. 20*, 2626–2637.

Yao, W.D., Wu, C.F., **1999**. Auxiliary hyperkinetic beta subunit of K^+ channels: regulation of firing properties and K^+ currents in *Drosophila* neurons. *J. Neurophysiol. 81*, 2472–2484.

Yasuyama, K., Salvaterra, P.M., **1999**. Localization of choline acetyltransferase-expressing neurons in *Drosophila* nervous system. *Microsc. Res. Tech. 45*, 65–79.

Ye, Z.C., Wyeth, M.S., Baltan-Tekkok, S., Ransom, B.R., **2003**. Functional hemichannels in astrocytes: a novel mechanism of glutamate release. *J. Neurosci. 23*, 3588–3596.

Yoshihara, M., Rheuben, M.B., Kidokoro, Y., **1997**. Transition from growth cone to functional motor nerve terminal in *Drosophila* embryos. *J. Neurosci. 17*, 8408–8426.

Yoshihara, M., Suzuki, K., Kidokoro, Y., **2000**. Two independent pathways mediated by cAMP and protein kinase A enhance spontaneous transmitter release at Drosophila neuromuscular junctions. *J. Neurosci. 20*, 8315–8322.

Yuan, L.L., Ganetzky, B., **1999**. A glial-neuronal signaling pathway revealed by mutations in a neurexin-related protein. *Science 283*, 1343–1345.

Zagotta, W.N., Brainard, M.S., Aldrich, R.W., **1988**. Single-channel analysis of four distinct classes of potassium channels in *Drosophila* muscle. *J. Neurosci. 8*, 4765–4779.

Zhang, D., Kuromi, H., Kidokoro, Y., **1999**. Activation of metabotropic glutamate receptors enhances synaptic transmission at the *Drosophila* neuromuscular junction. *Neuropharmacology 38*, 645–657.

Zhang, Y.Q., Bailey, A.M., Matthies, H.J., Renden, R.B., Smith, M.A., *et al.*, **2001**. *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell 107*, 591–603.

Zhang, Y., Featherstone, D., Davis, W., Rushton, E., Broadie, K., **2000**. *Drosophila* D-titin is required for myoblast fusion and skeletal muscle striation. *J. Cell Sci. 113*, 3103–3115.

Zhao, M.L., Wu, C.F., **1997**. Alterations in frequency coding and activity dependence of excitability in cultured neurons of *Drosophila* memory mutants. *J. Neurosci. 17*, 2187–2199.

Zheng, W., Feng, G., Ren, D., Eberl, D.F., Hannan, F., *et al.*, **1995**. Cloning and characterization of a calcium channel alpha 1 subunit from *Drosophila* melanogaster with similarity to the rat brain type D isoform. *J. Neurosci. 15*, 1132–1143.

Zhong, Y., **1996**. Genetic dissection of signal transduction mechanisms underlying PACAP-like neuropeptide transmission in *Drosophila*: synergy of cAMP and Ras/Raf pathways. *Ann. N. Y. Acad. Sci. 805*, 67–79, discussion 79–80.

Zhong, Y., Budnik, V., Wu, C.F., **1992**. Synaptic plasticity in Drosophila memory and hyperexcitable mutants: role of cAMP cascade. *J. Neurosci. 12*, 644–651.

Zhong, Y., Shanley, J., **1995**. Altered nerve terminal arborization and synaptic transmission in *Drosophila* mutants of cell adhesion molecule fasciclin I. *J. Neurosci. 15*, 6679–6687.

Zhong, Y., Wu, C.F., **1991**. Altered synaptic plasticity in *Drosophila* memory mutants with a defective cyclic AMP cascade. *Science 251*, 198–201.

Zhong, Y., Wu, C.F., **1993**. Differential modulation of potassium currents by cAMP and its long-term and short-term effects: dunce and rutabaga mutants of *Drosophila*. *J. Neurogenet. 9*, 15–27.

Zito, K., Fetter, R.D., Goodman, C.S., Isacoff, E.Y., **1997**. Synaptic clustering of Fascilin II and Shaker: essential targeting sequences and role of Dlg. *Neuron 19*, 1007–1016.

Zito, K., Parnas, D., Fetter, R.D., Isacoff, E.Y., Goodman, C.S., **1999**. Watching a synapse grow: noninvasive confocal imaging of synaptic growth in *Drosophila*. *Neuron 22*, 719–729.

Zlatic, M., Landgraf, M., Bate, M., **2003**. Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the *Drosophila* nervous system. *Neuron 37*, 41–51.

2.4 Hormonal Control of the Form and Function of the Nervous System

J W Truman, University of Washington, Seattle, WA, USA

2.4.1. Introduction

Nervous systems are notable for the diversity of cell types they contain and for the specificity of connections made between these cells. This complexity arises from a diversity of local signaling systems beginning with those that select cells to become the stem cells that generate neurons and glia, progressing through a blend of short-range and long-range attraction and repulsion factors involved in axon guidance (e.g., Tessier-Lavigne and Goodman, 1996; **Chapters 1.10** and **2.3**), and ending with systems that mediate target recognition and synapse stability (see **Chapters 1.10–1.12, 2.1, 2.3**, and **2.6**). Besides this plethora of local signaling systems, the nervous system also makes use of "global" cues provided by the endocrine system. At first sight, the widespread presence of a hormone in the blood would seem to be unsuitable for the individualized responses that are the hallmark of central nervous system (CNS) development. Hormones, though, have essential roles in coordinating development across the organism and in linking environmental information with developmental programs.

The most obvious role of hormones is associated with their wide distribution within the organism, which enables them to maintain developmental coordination between distant regions of the CNS and between the CNS and the periphery. In establishing this coordination, they often serve as "gatekeepers" via hormone-sensitive mechanisms that control the ability of neurons to transition from one phase of development to the next. In this regard, hormones provide us with important experimental tools to identify key transition points in neuronal development, and lead us to the switch genes that oversee these transitions. Global signaling, though, can come into conflict with the need for individualized timing differences in the nervous systems so that neural circuits can be assembled in their appropriate sequence. The diversification of hormone receptors and temporal regulation of expression of receptor isoforms provides a mechanism by which individual cellular responses can be adjusted

in time despite the global landscape of hormone titers.

Attempt is made to take a comparative and evolutionary perspective in writing this review. Most of the complex hormone–neuron interactions that are studied in insects are in the context of metamorphosis in the holometabolous orders. In ancestral forms, though, most of these processes occur during embryogenesis and may, or may not, have been under hormonal control. In this regard, the actions of hormones in embryos of both basal and derived insects need to be better understood before we can understand how ancestral embryonic systems were modified to produce the larval stage, whether hormones had a role in these modifications, and how the endocrine system "captured" these developmental pathways and put them under hormonal control. Consequently, the author has started with the embryo in the discussion of these systems, and has tried to highlight where we have serious gaps in knowledge.

Because of limitations in length, the scope of this review has been narrowed to systems for which some knowledge of the cellular or molecular changes is available, and the review is confined to hormonal effects that fall within the developmental realm. Other issues such as the regulation of the complex behavioral tasks at ecdysis (see **Chapter 3.1**) and the complex polyphenisms shown by social insects (see **Chapter 3.13**) have been covered in detail in this volume. Also, this treatment has an extreme "neurocentric" focus. Glial cells have a crucial role in development of the CNS, but they have largely been omitted from this review. An excellent review of the role of glial cells in neuronal development has been written recently by Oland and Tolbert (2003; see also **Chapters 1.10** and **2.3**).

2.4.2. Patterns of Development of the Insect Nervous System

Although insects vary in their early embryonic development in terms of how body segments arise (i.e., short versus long germ-band insects), they are remarkably similar by the time they start to build their CNS at the extended germ band stage (e.g., Thomas *et al.*, 1984; see **Chapters 1.10**, **1.11**, and **2.3**). Studies on grasshopper embryos provided initial insights into how the nervous system is constructed. The cellular composition of the central ganglia is determined by an interplay between neurogenesis and programmed cell death (see **Chapter 2.5**). The neurons arise from large neuronal stem cells, the neuroblasts (NBs), which enlarge and move inward from the ventral neuroectoderm. As seen in **Figure 1**, each NB undergoes repeated asymmetric divisions, producing a series of ganglion mother cells (GMCs), each of which then divides once to produce two daughter neurons. Although the existence of NBs and their pattern of division had been known for over 100 years, a milestone in the field was the publication by Bate (1976a) showing that NBs are arranged in stereotyped arrays that were invariant from individual to individual, and that each NB could be identified uniquely based on its position in this array (see **Chapter 1.10**). The NB arrays are almost identical from segment to segment but those in the thorax typically generate more neurons than their abdominal counterparts (Shepherd and Bate, 1990). The NBs were first identified in terms of position alone but later studies in *Drosophila* showed that each NB expresses a unique combination of molecular markers (Doe, 1992) and the same NB typically shows similar markers in diverse species (Broadus *et al.*, 1995).

Two aspects of neurogenesis are important for comparative studies of the CNS. First, the types of neurons arising from a particular NB are unique to that stem cell, and the destruction of the stem cell results in the failure of these neurons to appear (e.g., Taghert *et al.*, 1984; **Chapter 1.10**). Therefore, the neurons in a hemiganglion arise in 31 autonomous clones, each being a product of a single NB; **Chapter 1.10**. Second, the arrays of NBs have been extremely stable through evolution. The set found in embryos of very basal insects, such as silverfish (Truman and Ball, 1998), is identical to that in grasshoppers and is very similar to that found

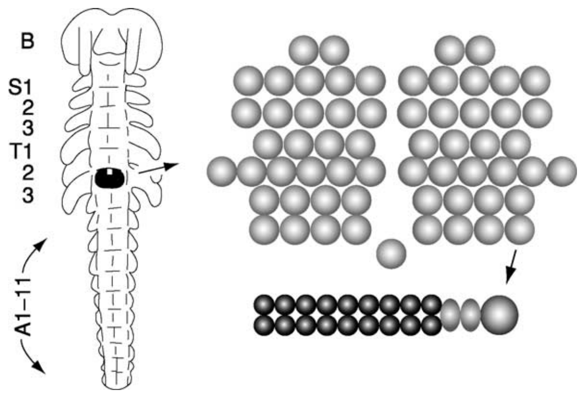


Figure 1 Drawing of a grasshopper embryo showing the segmental arrangement of the neuroblasts (NB) that produce the neurons of the CNS. Asymmetrical divisions by the NB produces a sequence of ganglion mother cells (GMC), each of which then divides to produce two daughter neurons.

in advanced insects such as *Drosophila* (Doe, 1992). Also, a given NB appears to produce similar types of neurons in diverse groups of insects (Thomas *et al*., 1984; Witten and Truman, 1998). This conservation allows a level of comparative analysis that is unrivaled by any other cells or tissues in insects. Most work on neuronal lineages has dealt with the relatively simple segmental ganglia, but rapid strides are being made in establishing the lineage relationships in the brain and in lineage comparisons between species (e.g., Urbach and Technau, 2003).

Within a lineage, the identity of a neuron depends on the birth order of the GMC from which it arises (Kuwada and Goodman, 1985). Brody and Odenwald (2000, 2002) showed that as they divide, the NBs progress through a stereotyped program of gene expression, including *hunchback* (*hb*), *Krüppel* (*Kr*), *POU domain protein* (*pdm*), and *caster* (*cas*) – a set of genes that were used earlier to establish regions of the body (see **Chapter 1.8**). Each GMC retains the expression of the gene activated at the time of its birth and passes it on to its progeny. Studies by Isshiki *et al*. (2001), using mutation and gain-of-function approaches, showed that these genes do indeed provide the information by which a neuron is informed of the birth-order of its GMC, and, hence, determine its identity.

Cell death also plays a prominent role in sculpting segmental differences within the CNS. For example, the leg motoneurons are generated in both thoracic and abdominal lineages, but they degenerate in the abdominal segments (Whitington *et al*., 1982). Also, Thompson and Siegler (1993) showed that the difference in the number of neurons in the thoracic versus the A1 version of the median NB lineage is due primarily to differential neuronal death (see also **Chapter 2.5**).

The first-born neurons serve a pioneer role as they establish the major pathways within the CNS and out into the periphery (Bate, 1976b; **Chapter 1.10**). The progressive events of axon pathfinding, target recognition, and synaptogenesis then bring about the orderly construction of neural circuits (see **Chapter 2.3**).

2.4.2.1. The Hemimetabolous Pattern of CNS Development

Our understanding of nervous system development in hemimetabolous insects is based primarily on studies of orthopteroid insects such as crickets, grasshoppers, and cockroaches, in which the body form of the nymph differs little from that of the adult except that it lacks functional wings and genitalia. Unfortunately, we have little metamorphic data for the CNS from groups like dragonflies and mayflies, in which the nymphal and adult forms are more disparate. In orthopteroid orders, the NBs generate their entire lineage of neurons prior to hatching and; consequently, the nymph hatches with a full set of central neurons. The only regions known to add neurons after hatching are the mushroom bodies and the optic lobes. The mushroom bodies are associated with various types of learning, and certain species continue to add mushroom body neurons even in the adult (e.g., in the cricket *Acheta domesticus*; Cayre *et al*., 1996). The neurogenesis in the optic lobes provides the new interneurons needed to deal with the rows of ommatidia that are added during nymphal molts.

At the time of hatching, neurons already have a dendritic shape characteristic of the adult cell (e.g., Shankland and Goodman, 1982; Boyan, 1983; Kutsch and Heckmann, 1995) although they are only a fraction of their adult size (see **Chapters 1.10** and **2.3**). This similarity is not surprising for neurons having similar roles in the nymph and the adult, but it was not expected for neurons involved in unique adult behaviors such as flight (**Figure 2**; Kutsch and Heckmann, 1995) and reproduction. Not only do these neurons have their adult morphology, but the adult behavioral circuits, have already been assembled by hatching, even though the insect may not show the behavior until weeks or months later, when it finally attains its adult morphology. For example, a newly hatched locust shows the typical flight posture when suspended without tarsal contact, and generates rhythmic alternating bursts by the motoneurons that will supply the elevators and depressor muscles, even though these muscles are undeveloped in the early nymph and there are no wings to move (Stevenson and Kutsch, 1988). Similarly, pharmacological treatment of the terminal ganglia of female grasshoppers can already elicit an "adult" oviposition motor pattern from the embryonic ganglion (Thompson, 1993).

Although the CNS is stable in terms of the number of interneurons, additional sensory neurons are added to the periphery at each molt. Consequently, as the nymph grows, a constant number of interneurons are faced with an increasing number of sensory neurons competing for synaptic space. A well studied example involves the medial giant interneuron (MGI) that receives direct synaptic contact from the filiform hairs on the cercus in the house cricket, *A. domesticus*. As the number of filiform hairs increases with each nymphal molt, the dendrites of MGI must provide additional synaptic space to accommodate the expanding sensory input. An interesting issue with this growth, though,

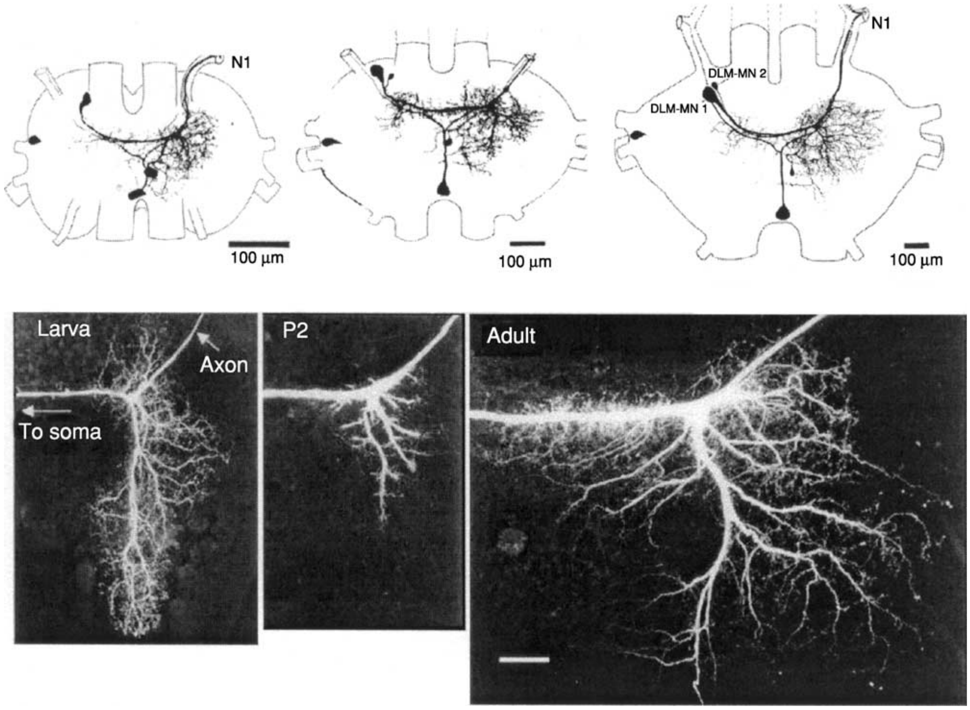


Figure 2 Postembryonic development of flight motoneurons in a hemimetabolous insect (top) *Schistocerca gregaria* and a Holometabolous insect (bottom), the moth *Manduca sexta*. The grasshopper neurons in the late embryo (left), 3rd instar nymph (middle), and adult (right) are revealed by backfilling with cobalt ions from the nerve leading to the site of the dorsal longitudinal flight muscles. The major cells filled include a large dorsal unpaired midline neuron that is neuromodulatory and one of the large motoneurons to the flight muscles (DLM-MN1). The overall structure of this neuron changes very little during postembryonic life. (Modified from Kutsch, W., Hechmann, R., **1995**. Homologous structures, exemplified by motoneurons of Mandibulata. In: Breidbach, O., Kutsch, W. (Eds.), The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach. Birkhauser Verlag, Basel, pp. 221–248.) Photomicrographs show the dendritic arbor of the moth motoneuron that is the homolog of DLM-MN1. The neuron has a well-developed dendritic arbor in the larva. The arbor is pruned back in the pupa and an extensive adult-specific arbor then forms during adult differentiation. (From Duch, C., Bayline, R.J., Levine, R.B., **2000**. Postembryonic development of the dorsal longitudinal flight muscle and its innervation in *Manduca sexta*. *J. Comp. Neurol. 422*, 1–17.)

is that a sensory hair's transduction properties change as it grows longer: short hairs are sensitive to wind acceleration but as they grow longer they become more sensitive to wind velocity. The MGI is sensitive to stimulus acceleration and newly born, short hairs make strong connections onto this interneuron. With successive molts, as the hair grows longer, the strength of synaptic connections that its neuron makes on the MGI wanes but the same neuron strengthens its synapses onto velocity-sensitive interneurons such as IN 10-3 (Chiba *et al.*, 1988). Hence, some sensory neurons show striking plasticity during growth and remodel their synapses to accommodate the changing properties of their sensory apparatus (see **Chapters 1.10** and **1.11**).

At the time of metamorphosis, there is a qualitative change in the sensory system, with the addition of new sensilla associated with adult-specific structures, like wings and genitalia. Also, to a lesser extent, there is a loss of sensilla that function only in the nymph.

2.4.2.2. The Holometabolous Pattern of CNS Development

The evolution of the holometabolous larva has been accompanied by a number of innovations in CNS development including proliferative and developmental arrests to accommodate the delayed production of the adult form (Figure 3). Also, embryonic neurons do not immediately assume an adult shape but acquire a novel morphology and connectivity to accommodate the larval body.

A fundamental change that occurred during embryogenesis is the truncation of neurogenesis. Rather than generating their entire lineage of neurons during embryogenesis, the NBs produce an initial set of neurons and then arrest. Their early progeny still pioneer the tracts and commisures to provide the basic architecture of the neuropils (Thomas *et al.*, 1984), and most then function in regulating the behavior of the larva. The majority of their progeny, though, are produced well after hatching.

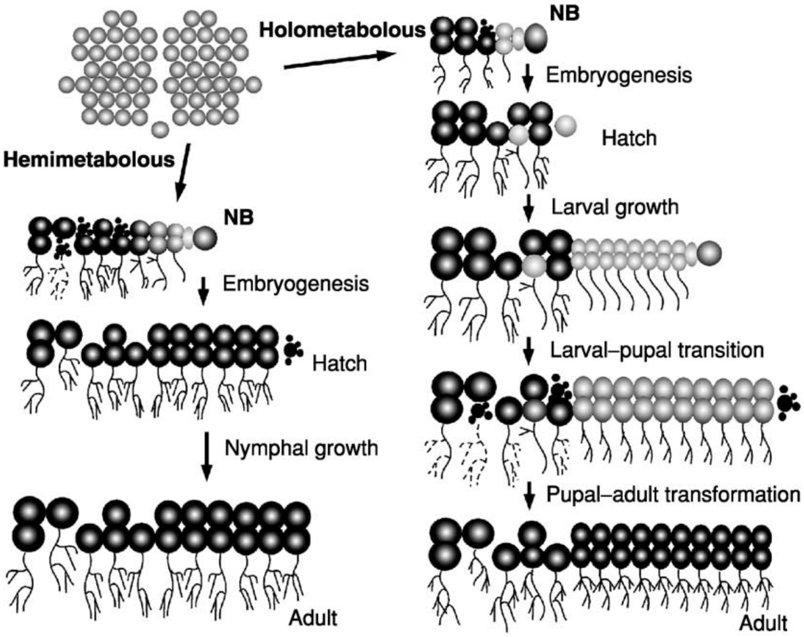


Figure 3 Summary of the changes in the development of neuronal lineages that accommodated the shift from a hemimetabolous to a holometabolous pattern of development. In the ancestral, hemitabolous condition each neuroblast (NB) generated its entire lineage of neurons during embryogenesis, and, during the process, some of the progeny were removed by programmed cell death. With the evolution of the holometabolous larva, the neuroblasts generate only the early portion of their lineage. Some of these progeny arrest as immature neurons (light colored progeny) and cell death is prevented in some of the neurons that normally died in ancestral forms. Neurogenesis resumes during larval life but the newly-born neurons are stockpiled at an early stage in their development. The onset of metamorphosis then stimulates the development of the arrested neurons as well as cell death and dendritic and axonal pruning in the larval neurons. Adult-specific arbors are then elaborated during the pupal–adult transformation.

The extent of cell death may also have been modified to accommodate metamorphosis. In grasshoppers, a thoracic ganglion has about fivefold more neurons than its abdominal counterpart. This disparity arises during embryogenesis, in part from abdominal NBs undergoing fewer divisions than thoracic ones (Shepherd and Bate, 1990) but also from enhanced cell death in the abdominal lineages (Thompson and Siegler, 1993). By contrast, larvae of holometabolous insects have become "abdominalized" as the abdomen has taken over most or all of the larva's locomotor function, and, accordingly, their abdominal ganglia may have twice as many neurons as in the corresponding ganglia in a grasshopper. In lepidopteran caterpillars, for example, each abdominal ganglion has over 1000 neurons, only a few hundred less than seen in a thoracic ganglion in these larvae. The embryonic neurogenic periods are similar for the thorax and abdomen in caterpillars, which suggests that the extent of embryonic neuronal death does not differ dramatically between the two regions. This numerology presents the interesting possibility that the larval CNS may have acquired the extra neurons that it needed simply by maintaining neurons that normally had died in their hemimetabolous ancestors. These evolutionarily "un-dead" neurons then serve as functional neurons, but then finally undergo their ancestral fate of degeneration as metamorphosis begins and the adult form is established.

A neuron's form and pattern of connectivity in the larva often differs markedly from that seen in the adult (**Figure 2**; Levine and Truman, 1985; Duch and Levine, 2000). Most larval neurons are not just partially differentiated adult cells. They are fully differentiated neurons that have specializations adapted to the larval body plan. Their specializations can include their electrical properties (Duch and Levine, 2000) and transmitters (Tublitz and Slywester, 1990), as well as their morphology and synaptic connections. The difference between a neuron's larval and adult morphology varies widely from cell to cell and likely relates to the divergence in the cell's function between the two stages.

Importantly, not all neurons born during embryogenesis acquire a mature phenotype (see **Chapters 2.2**

and **2.3**). The leg motoneurons in *Drosophila*, for example, are born during embryogenesis but their muscle targets do not appear until metamorphosis. In the larva these neurons are found with a sparse dendritic arbor and an axon that extends into the growing leg imaginal discs. Similarly, the larval form of the indirect flight muscle motoneuron MN5 possess an axon but has no dendrites or peripheral targets (Consoulas *et al*., 2002). It is not known, however, if these "immature" neurons contribute to the functioning of the larval CNS despite their immature appearance.

During larval growth, the arrested embryonic NBs reactivate and generate the remainder of their lineage. The neurons born during this second, postembryonic bout of neurogenesis extend an axon to an initial target but then arrest. They are then stockpiled in this immature condition until the start of metamorphosis (**Figure 4**).

Hence, the larva enters metamorphosis with a CNS composed of larval neurons and arrested, immature neurons of both embryonic and postembryonic origins. Some of the dismantling of the larval nervous system comes about through neuronal death, especially in the abdomen, as the ancestral ratios of thoracic to abdominal neurons are finally achieved. Many larval neurons, though, are remodeled. Their larval dendritic and axonal arbors are pruned back and new, adult-specific arbors grow to achieve the shape and connectivity appropriate for the adult stage (**Figure 2**). While larval neurons are starting their remodeling, the immature neurons initiate their adult outgrowth. These diverse processes are orchestrated by the circulating ecdysteroid titers.

2.4.3. Developmental Hormones and Their Receptors

The developmental hormones in insects are the sesquiterpene juvenile hormones (JHs; see **Chapter 3.7**), and the ecdysteroids, the steroid molting hormones (Gilbert *et al*., 2002; see **Chapters 3.3** and **3.5**). The major circulating ecdysteroids are ecdysone (E), which is released from the prothoracic glands, and 20 hydroxyecdysone (20E), an active metabolite of E made by peripheral tissues. The pattern of hormone secretion in hemimetabolous insects is relatively simple (**Figure 5a**). Periodic surges of ecdysteroids drive the molting from one nymphal stage to the next. The presence of JH maintains the nymphal form, but JH titers decline after the formation of the last nymphal stage, allowing the differentiation of adult characters, when the insect is next challenged with ecdysteroid. JH typically reappears in the adult to regulate aspects of reproduction.

The holometabolous life history is associated with a more complex pattern of hormone secretion (**Figure 5b**). Ecdysteroids still cause molting during larval stages and JH is required to maintain the larval form, its so-called "status quo" function (Riddiford, 1994). The decline in JH at the start of the last larval stage then allows the preparation for metamorphosis. During the last larval stage, small surges of steroid occur, which are too small to induce

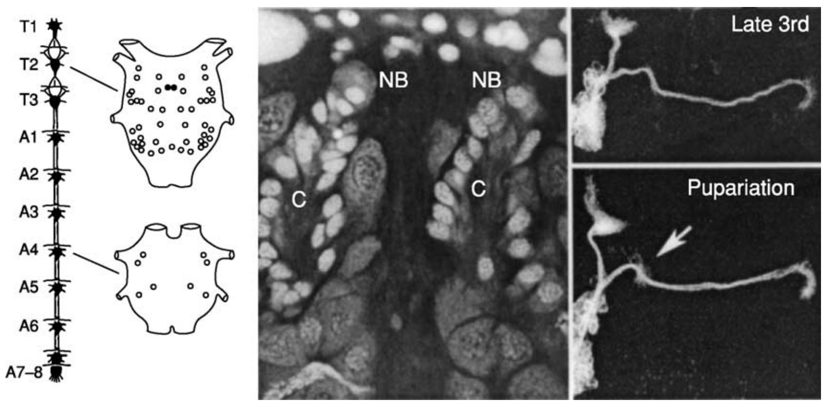


Figure 4 Postembryonic neurogenesis in larvae of holometabolous insects. (Left) Drawing of the ventral nervous system of a *Manduca* larva showing the location of the reactivated embryonic neuroblasts. A thoracic ganglion has about 20 more neuroblast pairs than does an abdominal ganglion. The locations of the paired ''F'' neuroblasts are indicated by the black circles. (Middle) A confocal section showing a dorsal view of a thoracic ganglion stained with propidium iodide showing DNA and RNA. The section shows the 2 ''F'' neuroblasts (NB) and their respective clusters (C) of arrested, adult-specific neurons. (Right) Dorsal views of *Drosophila* thoracic neuromeres showing the postembryonic progeny of a single neuroblast that expresses green fluorescent protein (GFP). The immature neurons extend axons either to the next anterior neuromere or across the midline to a contralateral site. The cells only begin to sprout dendrites (arrow) when metamorphosis begins at pupariation.

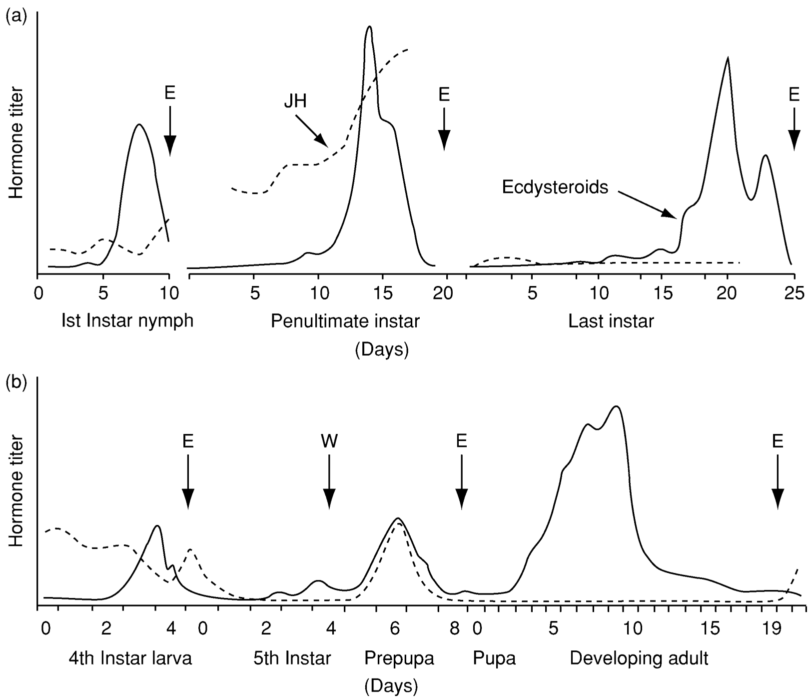


Figure 5 Juvenile hormone (dotted line) and ecdysteroid (solid line) titers in (a) a hemimetabolous insect, the cockroach *Nauphoeta cinerea* and (b) a holometabolous insect, the moth *Manduca sexta*. E, ecdysis; H, hatching; W, wandering. (Titers based on Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., *et al.*, **1985**. Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. *Experientia 41*, 913–917; and Riddiford, L.M., **1994**. Cellular and molecular actions of juvenile hormone. I. General considerations and permetamorphic actions. *Adv. Insect Physiol. 24*, 213–274 (review).)

molting but nevertheless direct premetamorphic programs in tissues (Wolfgang and Riddiford, 1986). The largest of these small peaks, the commitment peak, terminates larval feeding, causes wandering behavior, and commits larval tissues to pupal differentiation (Riddiford, 1994). The latter is significant because it renders larval tissues insensitive to subsequent exposure to JH. A major ecdysteroid peak then causes the formation of the pupal stage. This peak is accompanied by the reappearance of JH which acts on selected imaginal tissues to prevent their premature adult differentiation (Williams, 1961; Kiguchi and Riddiford, 1978; Champlin *et al.*, 1999). In higher flies, like *Drosophila*, this large ecdysteroid peak also causes the larval cuticle to transform into a protective case, the puparium, inside of which the true pupal stage forms. JH disappears prior to pupal ecdysis and a prolonged surge of ecdysteroids then drives adult differentiation. In *Manduca*, the major circulating steroid at the beginning of adult differentiation is E but 20E then becomes prominent as metamorphosis progresses (Warren and Gilbert, 1986). As with hemimetabolous insects, JH then returns in the adult, typically in association with some aspect of reproductive control (see **Chapters 1.3** and **3.9**).

Although the overall patterns of hormone secretion are similar in moths and flies, there are major differences in how their tissues respond to this hormonal landscape. The most striking differences are seen in the response of tissues to JH. Application of exogenous JH during the last larval stage of *Manduca* causes the larva to molt to a supernumerary larval stage rather than to pupa. Also, the application of JH to the pupa, at the outset of adult differentiation, results in a second pupal stage, although some adult features may also be present (Riddiford and Ajami, 1973). In *Drosophila*, by contrast, treatment of the last larval stage with JH does not result in an extra larval instar, and an apparently normal puparium and pupa are formed. During subsequent adult differentiation, though, the abdominal epidermis makes a second pupal cuticle, while the imaginal discs in the head and thorax make normal adult structures (Postlethwait, 1974; Riddiford and Ashburner, 1991). JH also suppresses aspects of adult differentiation of internal tissues including the CNS (Restifo and Wilson, 1998; Williams and

Truman, 2004). Hence, in higher flies, JH sensitivity is lost during the larval pupal transition and appears only in selected tissues during the differentiation to the adult.

The changing sensitivity of tissues to hormonal signals could be encoded at the level of their receptors. In the case of JH, though, the issue is unclear because a JH receptor has yet to be unequivocally demonstrated (see **Chapter 3.7**). Indeed, multiple receptor systems might be involved in mediating the action of this versatile hormone (Wheeler and Nijhout, 2003). By contrast, the receptors that mediate ecdysteroid action have been known for over a decade (Koelle *et al.*, 1991; see **Chapter 3.5**), and receptor distributions direct the time and nature of a cell's response to this steroid.

The action of ecdysteroids is mediated through a heterodimer of two members of the nuclear receptor family, the Ecdysone Receptor (EcR) and Ultraspiracle (Usp) (see **Chapter 3.5**; Riddiford *et al.*, 2000 for reviews). Like other family members, EcR has a DNA binding domain consisting of two Zn fingers, and a ligand binding domain that carries an activation function (the AF2 domain) that mediates ligand-dependent activation. The *Ecr* gene encodes different protein isoforms that share a common DNA and ligand-binding domain, but differ in their N-termini. *Drosophila* has three isoforms, EcR-B1, -B2, and -A, whereas other Diptera and Lepidoptera have only two isoforms, EcR-B1 and -A. Cell transfection experiments have shown that the A/B regions of EcR-B1 and -B2 have potent AF1 activation domains, whereas this region of EcR-A appears to have a repressive function (Mouillet *et al.*, 2001; Hu *et al.*, 2003).

The EcR/Usp complex binds to specific DNA response elements. The unliganded receptor binds corepressors and mediates local transcriptional silencing. With binding of ligand, coactivators are assembled allowing transcriptional activation. Transcriptional activation can be mediated through two regions of the receptor – through the AF1 site in the A/B region (causing "ligand-independent" activation) and the AF2 site in the LBD. The coactivator and corepressor complexes include a number of components, many of which participate in multiple signaling pathways. They may interact with the core promoter and/or locally modify the chromatin (e.g., through acetylation or deacetylation of histones) to facilitate or suppress transcription (review: Kraus and Wong, 2002).

EcR mutant and receptor misexpression studies indicate that the receptor isoforms have both unique and overlapping functions (Bender *et al.*, 1998; Schubiger *et al.*, 1998; Li and Bender, 2000; Cherbas *et al.*, 2003). Since EcR-A and EcR-B1 show dramatic differences in their distribution in both time and space, their expression and action is a key to understanding how different neurons may show differing responses to the same hormonal signal.

2.4.4. Hormones and the Generation of the Larval CNS

The specializations that brought about the larval CNS include the truncation of neurogenesis, a suppression of neuronal death, and an alteration in the growth patterns of embryonic neurons. The roles that ecdysteroids and JH may have had in these events, though, are poorly understood. During embryogenesis, holometabolous insects show a precocious appearance of JH relative to that seen in embryos from hemimetabolous groups. This early appearance of JH is thought to be associated with the truncation of growth and premature differentiation that was necessary to generate the novel larval stage (Truman and Riddiford, 2000; Erezyilmaz *et al.*, 2004). At this point, though, the linkage between key developmental switches in the CNS and the hormone titers stands only as a correlation.

A striking feature of the neurogenesis in embryos of both *Drosophila* and Lepidoptera is its abrupt arrest that occurs at about 50% of embryogenesis (e.g., **Figure 6**). This arrest contrasts with the pattern seen in ametabolous and hemimetabolous insects, in which some neuroblasts continue proliferation until almost hatching (Shepherd and Bate, 1990; Truman and Ball, 1998), and is consistent with there being an extrinsic signal responsible for the arrest. The arrest occurs at the time that the rising embryonic ecdysteroid titer causes the production of the first larval cuticle. Therefore, this embryonic ecdysteroid surge may be responsible for the arrest, but experimental data are needed to support this speculation.

2.4.5. Nervous System Changes during Larval or Nymphal Growth

2.4.5.1. Somatic Sensory Systems

An initial set of sensory neurons differentiate during embryogenesis in both larvae and nymphs. These include proprioceptors, such as stretch receptors and chorditonal organs, that monitor body wall stretch and joint position, and external sensory organs with cuticular hairs and having mechano- or chemoreceptive functions (see **Chapters 1.11, 1.12**, and **3.15**). This initial set of receptors is highly stereotyped and homologous cells can be found between *Schistocerca gregaria*, *Drosophila* (Meier

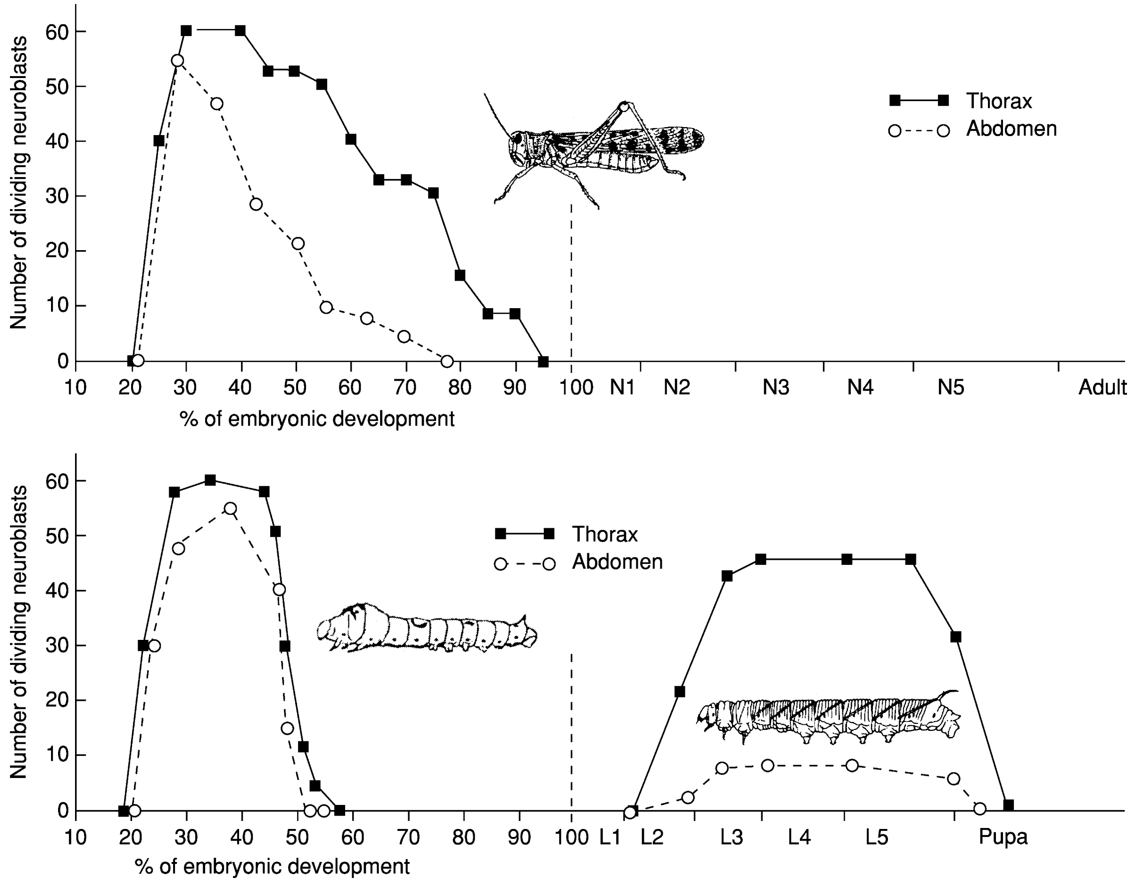


Figure 6 Comparison of the duration of neurogenesis in a hemimetabolous insect, the grasshopper *Schistocerca gregaria*, and in holometabolous lepidopteran larvae. (The grasshopper data are based on Shepherd, D., Bate, C.M., **1990**. Spatial and temporal patterns of neurogenesis in the embryo of the locust (*Schistocerca gregaria*). *J. Comp. Neurol. 319*, 436–453, while the lepidopteran data are based on Booker, R., Truman, J.W., **1987a**. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol. 255*, 548–559 and J.W. Truman, unpublished data.)

et al., 1991) and *Manduca sexta* (Grueber and Truman, 1999). Typically, the number of proprioceptors usually remains unchanged during the growth of the larva or nymph, but the sensory hairs show dramatic increases in numbers through successive instars (**Figure 7**). The larvae of higher Diptera, though, are exceptional in that they add no new sensory neurons after hatching (see **Chapter 1.11**).

The positions of the first hairs that arise during embryogenesis are very stereotyped, but during the postembryonic period interactions between the epidermal cells and existing sensory hairs determine the placement of new sensory hairs. The studies by Wigglesworth (1940) on the spacing of new sensory hairs in *Rhodnius* provided the first experimental demonstration of lateral inhibition. He showed that existing bristles suppressed the formation of a new sensory organ precursor (SOP) in their immediately vicinity, thereby insuring that new bristles were placed in spaces that lacked them.

The production of new bristles is coordinated with the molt cycle. In *Rhodnius*, the quartet of cells that comprise the sensory organ (SO) appears 6 days after the insect has taken a blood meal, and is associated with a general mitotic peak at the start of a molt (Wigglesworth, 1953). The sensory neuron extends an axon towards the CNS, and the trichogen and tormogen cells that form the cuticular hair and socket, enlarge soon thereafter. The new sensillum appears to be anatomically functional by the time the molt is completed.

Although *Rhodnius* undergoes the steps from the establishment of the SOP to the finished sensory organ within a single molt, the process extends over two molts in large rapidly growing larvae, such as *M. sexta* (Grueber and Truman, 1999). In larvae of *Manduca* each SOP produces five cells including two neurons. One neuron is a typical mechanoreceptor neuron associated with the new bristle but the other is a dendritic arborization (da) sensory

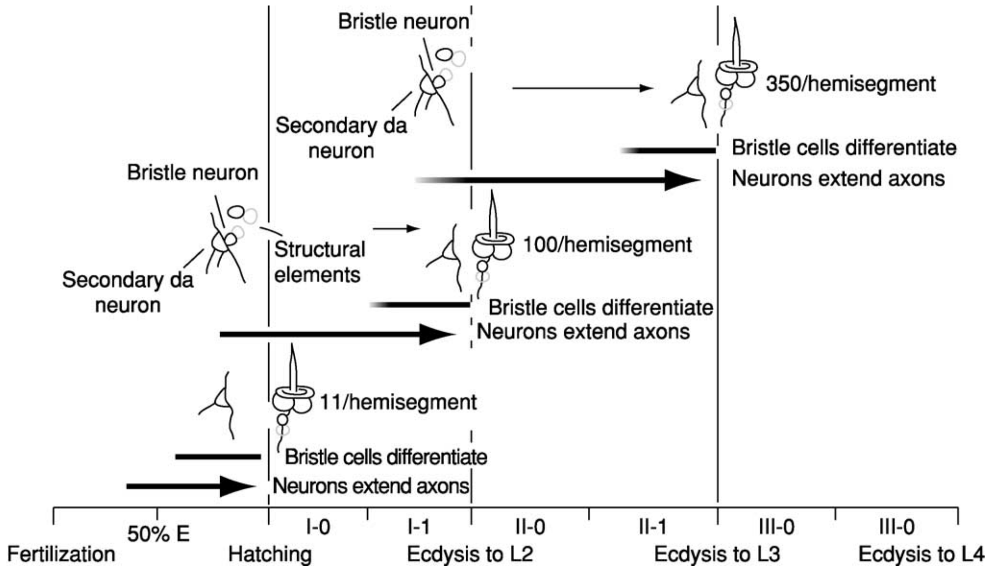


Figure 7 The timing of the birth and differentiation of sensory bristles on the second abdominal segment of *Manduca sexta* caterpillars. The larva hatches with 11 sensory bristles and 12 dendritic arborization (da) sensory neurons. Two waves of sensory neurons are then added, the first prior to hatching and the second during the molt to the second instar. The neurons immediately extend axons to the CNS but the sensory hair does not differentiate until the succeeding molt. No sensory hairs are added after the second wave. (Based on data from Grueber, W.B., Truman, J.W., **1999**. Development and organization of a nitric-oxide-sensitive peripheral neural plexus in larvae of the moth, *Manduca sexta*. *J. Comp. Neurol. 404*, 127–141.)

neuron whose dendrites ramify under the soft cuticle of the larva. At this point, it is not known whether the dense network of processes from the latter is involved in detecting cuticular stretch or in sensing noxious, damaging stimuli. During the first molt the SOP is established and undergoes the divisions to produce the two neurons and three support cells. The cuticular structures made by the trichogen and tormogen then appear at the next molt, in response to the next molting surge of ecdysone (Figure 7). This delay may be necessary in large insects to allow axons from the bristle to grow into the CNS and make connections with their central targets.

2.4.5.2. Growth of the Eyes

In many nymphs new ommatidia are added to the eye at each molt. In *S. gregaria*, for example, the number of ommatidia increases from about 2500 in the newly hatched nymph to over 9000 in the adult. The new ommatidia arise from a region along the anterior margin of the eye, which is divided into proliferation and differentiation zones (Anderson, 1978). The proliferation zone shows a basal level of cell division that probably produces photoreceptor neurons at a constant rate, since outgrowing axons from new photoreceptors are evident at all times during the intermolt and molting periods. At the start of the molt there is an increase in mitoses in the proliferation zone and also a spike of proliferation in the differentiation zone, where the ommatidia are being assembled. The divisions in the differentiation zone, likely produce support cells, such as those that make the crystalline cone and the screening pigments. Except for responses to circulating factors, the growth of the eye is under autonomous control, as illustrated by normal patterns of proliferation being observed in eyes transplanted to the surface of the prothorax (Anderson, 1978).

Unlike nymphs, the eyes of larvae consist of only a small set of separated facets, the stemmata. These likely arose from the most posterior set of ommatidia in the eyes of the ancestral larva. The stemmata, though, add neither facets nor more photoreceptors as the larva progresses through its larval instars. The compound eye of the adult appears only at metamorphosis and forms immediately anterior to the stemmata.

2.4.5.3. New Central Neurons. Regulation of Neurogenesis

In hemimetabolous insects, postembryonic neurogenesis in the CNS is confined to the mushroom bodies and the optic lobes. There is no information as to whether neurogenesis in the mushroom bodies is influenced by ecdysone and molting. In the optic lobe of *Schistocerca*, the production of new neurons for the lamina and the medulla, the outer two optic neuropils, occurs at a constant rate throughout the instar, without any modulation during the time of the molts (Anderson, 1978). This pattern of constant

interneuron production matches the pattern of constant addition of photoreceptor axons in the periphery. While the eye does not need the optic lobes to make new ommatidia, the optic lobes do need the eyes to some extent. In *Schistocerca* the surgical removal of the anterior proliferation and differentiation zones result in no new photoreceptor axons growing into the optic lobe. Despite the lack of ingrowing axons, there appears to be no difference in the rate of division of NB or GMCs for either the lamina or medulla. There is, though, a dramatic effect of cell survival as one sees enhanced death of the lamina ganglion cells (Anderson, 1978). In *Manduca* and *Drosophila*, the relationship between photoreceptor axons and lamina neurons differs from that seen above, because ingrowing axons influence proliferation as well as cell survival (see Section 2.4.8.4).

Postembryonic neurogenesis in the ventral CNS has been studied primarily in *Manduca* (Booker and Truman, 1987a) and *Drosophila* (White and Kankel, 1978; Truman and Bate, 1988), but also occurs in larvae of Coleoptera, Hymenoptera, and Neuroptera (J.W. Truman, unpublished data). Hence, it is a ubiquitous feature of the holometabolous life style. In highly derived larvae, like those of *Manduca* and *Drosophila*, neurogenesis begins early in larval life and concludes shortly after pupal ecdysis (**Figure 4**). The factors responsible for the reactivation of the arrested embryonic neuroblasts are best understood in *Drosophila*. The need for nutritional cues was shown by experiments, in which newly hatched larvae were maintained on a glucose diet that lacked amino acids. On such a diet, the energetic requirements of the larva are met by the sugar but growth is not possible. Under these conditions neuroblasts that are already cycling, such as those of the mushroom bodies, continued to divide, but arrested neuroblasts could not reenter the cell cycle (Britton and Edgar, 1998).

The control over NB activity may not be cell autonomous and may involve the surrounding glia. Mutations in the gene *anachronism* (*ana*), that encodes a cell-surface protein, results in the precocious onset of postembryonic neurogenesis (Ebens *et al*., 1993). Interestingly, *ana* is not expressed in the NB but, rather, it is expressed in the surrounding glia, suggesting that these cells may actively inhibit NB reactivation. The suppression imposed by *ana* appears to be antagonized by the expression of the *trol* gene (*terribly reduced optic lobes*) (Caldwell and Datta, 1998) which acts through cyclin E to allow the transition of the stem cells from G1 into the S phase. The activation of proliferation may also involve the action of ecdysteroids in the early larvae (Datta, 1995; Park *et al*., 2001).

2.4.6. Role of Hormones in the Metamorphosis of Hemimetabolous Nervous Systems

The metamorphic changes seen in hemimetabolous insects are most evident in the peripheral nervous system, because there is an obvious acquisition of adult-specific sensory neurons and, to a lesser extent, the loss of nymph-specific sensilla. An example of the latter is seen in *Rhodnius prolixus*, in which most of the sensory hairs are lost on the dorsum of the adult abdomen since it is covered by the wings. Interestingly, although the cuticular hairs are lost, the sensory neurons that supplied them do not die and persist in the adult (Wigglesworth, 1953). Presumably, they cease to serve as functional sensory neurons since their transducing hairs are no longer present. The topical application of JH prevents both the loss of these nymphal sensory hairs and the appearance of adult-specific sensilla. Another example of JH preventing the appearance of adult-specific sensilla is seen in cockroaches in which JH treatment of the last nymphal stage suppresses the appearance of pheromone-sensitive hairs on the antennae (Schaffer and Sanchez, 1973).

The metamorphic changes in the CNS of hemimetabolous insects are subtle and do not involve massive neuronal death or neuronal remodeling. There may be, however, important functional changes that occur during the molt to the adult (Olberg, 1986). This is seen most strikingly in the visual system of dragonflies. In the adult, there are eight descending visual interneurons that carry wide-field information from the optic lobes down to the thoracic ganglia. These interneurons are also present in the nymph, but only one is functional and the others are electrically silent. They finally become electrically active during the molt to the adult. The one functional interneuron in the nymph receives information from the whole visual field of the nymph, but after metamorphosis those ommatidia now comprise the extreme posterior border of the adult eye, and the interneuron is now responsive only to stimuli and the periphery of the visual field.

2.4.7. Hormones and the Metamorphosis of Holometabolous Nervous Systems

2.4.7.1. Death of Larval Neurons

Most studies on the metamorphic fates of larval neurons have focused on the motor system (see **Chapter 2.5**). The fates of motoneurons, though, need to be considered in the context of their target

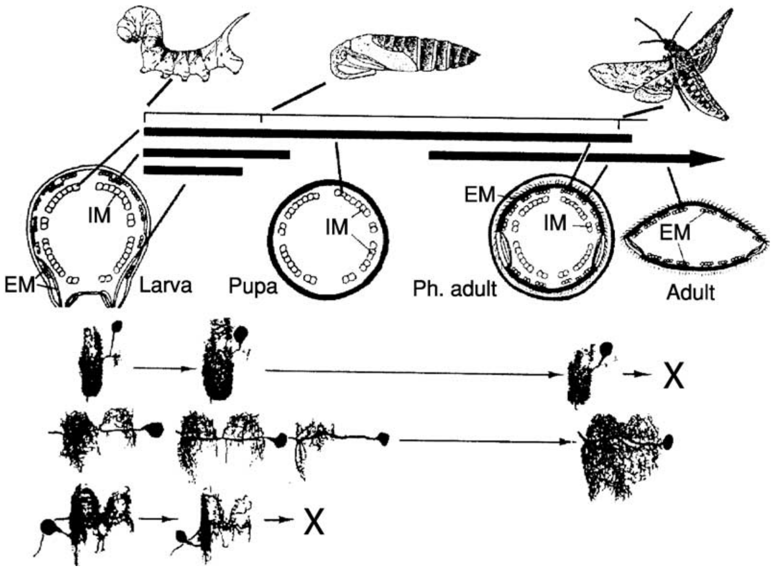


Figure 8 Summary of the fates of larval neurons and muscles during metamorphosis in *Manduca sexta*. (Top) Time-line showing the three major muscle fates during metamorphosis. The cross-section views show the positions of abdominal internal (IM) and external (EM) muscle groups in the larva, pupa, pharate adult (Ph. adult), and adult. The bars show the time when functional muscle is present. Many internal muscles persist through metamorphosis but then die after adult emergence. One group of external muscles die in the prepupa while others degenerate1–2 days after pupal ecdysis. The remains of some of these muscles are used for templates for the growth of adult muscles. (Bottom) Examples of the changes in morphology and survival of motoneurons associated with muscles having the three kinds of fates. (Neuron data from Weeks, J.C., Truman, J.W., **1985**. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J. Neurosci. 5*, 2290–2300; and Levine, R.B., Truman, J.W., **1985**. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J. Neurosci. 5*, 2424–2431.)

muscles (**Figure 8**; reviews: Consoulas *et al*., 2000a; **Chapters 2.1** and **2.3**). In both *Manduca* and *Drosophila* the larval musculature is dismantled in three steps. An initial bout of degeneration occurs prior to pupal ecdysis, and includes most of the musculature of the head and thorax, and selected muscles in the abdomen. The early loss of the head and thoracic musculature provides space for the growth and expansion of the imaginal discs that will produce parts of the adult head and thorax. A second period of muscle death occurs early in pupal life and involves muscles that were necessary for pupal ecdysis. In *Manduca*, the pupa is then left with a set of internal longitudinal muscles in segments A3 to A6, and these mediate the limited behavior of the pupa. *Drosophila* similarly has a set of “persistent larval muscles” but these lose their contractile machinery through the middle of metamorphosis. The adult muscles begin forming after pupal ecdysis. Some are formed from templates provided by the remains of the larval muscles, while others are formed *de novo* as groups of myoblasts aggregate to found adult muscles. In *Manduca*, the muscles of the head and thorax (except for the indirect flight muscles) are formed *de novo*, whereas most of the abdominal musculature is formed on larval templates. In *Drosophila*, by contrast, the vast majority of the adult muscles arise *de novo*, with the indirect flight muscles being one of the few muscles organized on a larval template (Fernandes and Keshishian, 1998). In both species, at the end of adult development, the abdomen has both persisting larval muscles and the new adult-specific musculature. The larval muscles, along with a few specialized ecdysial muscles, then degenerate a few hours after emergence leaving the animal with its adult-specific musculature.

2.4.7.1.1. Death during the larval–pupal transition Although larval muscles die in three waves, the neurons that innervate them only die at two times (**Figure 8**) (see **Chapter 2.5**). In both *Drosophila* and *Manduca*, most larval neurons die during the initial period that occurs shortly after pupal ecdysis. The most extensive cell loss is seen in the abdominal CNS, reflecting a reduction in the behavioral importance of this region of the body. With the vermiform body of the larva, the abdominal CNS controlled a complex musculature and had the major responsibility for locomotion. At metamorphosis the latter functions are given over to the thorax, and the abdomen becomes

severely reduced both in its musculature and its behavioral capacity.

The most detailed studies of neuronal death during this first period are from *Manduca* and examine the fates of PPR and APR, abdominal motoneurons that control the retraction of the larval prolegs. Two endocrine conditions are needed for the death of PPR: the lack of JH during the commitment peak of ecdysteroids, and the subsequent high 20E titers of the prepupal peak (Weeks and Truman, 1985). Although the death of PPR follows that of its muscle, the neuron's death is not induced by the loss of its target. This conclusion is based on experiments showing that PPR continues to show its appropriate 20E-dependent fate even when its muscle is surgically removed.

An elegant set of *in vitro* experiments using the motoneuron APR confirmed that the degeneration response was not caused by the death of its target. This larval motoneuron undergoes programmed cell death but in a segment-specific context: those in segments A5 and A6 degenerate whereas those in segments A3 and A4 survive (Sandstrom and Weeks, 1998). Neurons taken from larvae and maintained without 20E *in vitro* survive regardless of their segmental origin. When exposed to 20E, however, some undergo programmed cell death, but only if they were removed from segments in which these cells would normally die (Streichert *et al.*, 1997) Thus, the hormone-induced death of these neurons is intrinsically programmed and does not depend on interactions either within the ganglion or with their muscle targets.

2.4.7.1.2. Death during the pupal–adult transition The last wave of neuron and muscle death occurs shortly after adult ecdysis, and primarily involves the ecdysial muscles that will not be needed again in the adult. Post-ecdysial muscle death appears to be a universal feature of adult ecdysis in higher insects, but the loss of their motoneurons is not. In the moth *Hyalophora cecropia*, for example, the intersegmental muscles degenerate but their motoneurons survive through the remainder of adult life (S. Fahrbach, personal communication; see **Chapter 2.5**). The hormonal signals that control the death of these muscles and their motoneurons are varied. In all cases, the decline in ecdysteroids at the end of adult differentiation is an essential component of this control, and late injections with 20E delay the degeneration response. In some species, such as the moth *Antheraea polyphemus*, a peptide hormone provides the final trigger for muscle degeneration (Schwartz and Truman, 1982). In *Manduca*, by contrast, the withdrawal of ecdysteroids alone appears to be sufficient for muscle death.

As with the muscles, the death of neurons in both *Manduca* and *Drosophila* requires a decline in ecdysteroids, and injection of 20E just before adult ecdysis prevents the death (Truman and Schwartz, 1984). The control over the degeneration is complicated, though, because the doomed neurons are involved in both ecdysis and post-ecdysis behaviors, such as wing inflation, and these behaviors can be separated in time if the insect is not given an appropriate site to inflate its wings. Accordingly, in *Manduca*, behavioral manipulations, such as forcing newly emerged moths to dig through soil, result in some neurons dying on schedule but delay the death of others (Truman, 1983). Nerve cord transection experiments in *Manduca* showed that one of the last neurons to die, MN12, requires a descending signal via the nerve cord in order to degenerate (Fahrbach and Truman, 1987). A similar situation appears to hold for some of the doomed neurons in *Drosophila* since decapitation of the newly emerged fly prevents the death of a subset of the cells (Kimura and Truman, 1990; Robinow *et al.*, 1997). Hence, the death of some of the late dying neurons requires both the absence of 20E and a descending signal from the head.

The death of neurons in *Drosophila* is immediately preceded by the appearance of transcripts for the cell death genes, *reaper* and *grim* (Robinow *et al.*, 1997). Focusing on *reaper* expression, these authors showed that the injection of 20E, less than an hour before the transcripts normally appear, blocked both the appearance of *reaper* and the onset of neuronal death. This timing is consistent with the hypothesis that 20E directly suppresses the transcription of these cell death genes. Interestingly, the decapitation of newly emerged flies, which prevents the death of a subset of doomed cells, does not prevent *reaper* transcripts from appearing in these cells (Robinow *et al.*, 1997). Apparently the descending signal acts downstream of *reaper* transcription, perhaps at the level of translation of this protein.

2.4.7.2. Remodeling of Larval Neurons

Figure 9 summarizes the main developmental phases, through which a neuron progresses as it changes from its larval to its adult form. The first changes appear at the larval–pupal transition during the "pruning phase," as neurons rapidly remove axonal and dendritic branches. For the axonal branches of mushroom body neurons and the dendrites of

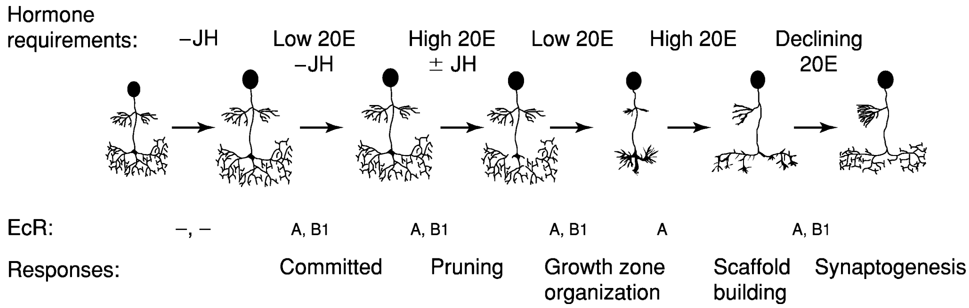


Figure 9 Summary of the developmental changes that occur during the life of a larval neuron during larval growth and metamorphosis. The EcR isoform data were obtained from *Drosophila* and the hormonal requirements are based on studies on *Manduca sexta*. The one process whose endocrine requirements are known to be different in moths and flies is during commitment: JH treatment cannot prevent *Drosophila* neurons from subsequent pruning.

dendritic arborization (da) sensory neurons, pruning occurs as branches are pinched off and undergo fragmentation (Watts *et al.*, 2003; D.W. Williams and J.W. Truman, unpublished data). This process is mediated through the ubiquitin-proteosome pathway (Watts *et al.*, 2003) similar to Wallerian degeneration seen in severed vertebrate axons. Fragmentation is not the only mechanism employed during pruning, though, since time-lapse movies show that the loss of the axonal arbors of the Tv neurosecretory neurons occurs by retraction of the axonal branches (H. Brown and J.W. Truman, unpublished data) more like the more gradual retraction of processes seen during synapse elimination at vertebrate neuromuscular junctions (e.g., Walsh and Lichtman, 2003).

Pruning is followed by a "growth zone organization phase," as the cell prepares for its adult outgrowth. One part of the arbor may show retraction bulbs as pruned stumps are retracted, while another part is enlarging and organizing into a growth cone. This is also a time when soma size increases, a change that is especially obvious for the flight motoneurons (Duch *et al.*, 2000).

The growth cones begin to extend and branch during the "scaffold building phase." *In vivo* imaging of the outgrowth of dendritic arbors from the da sensory neurons show that, as the arbor expands, branch retraction also occurs as the cell establishes the basic "foot-print" of the arbor (Williams and Truman, 2004). Studies on *Manduca* motoneurons show that the outgrowth phase is marked by changes in Ca^{2+} currents in the cell (Duch and Levine, 2000). During the early phase of elongation, Ca^{2+} currents are small and confined to distal ends of extending branches. In the later phases, the cell shows Ca^{2+} spikes that provide Ca^{2+} transients throughout the cell. Eventually, these Ca^{2+} spikes are masked by the appearance of strong K^{+} conductances.

The "synaptogenesis" phase then involves the formation and maturation of synaptic contacts. This phase occupies the last half of adult differentiation.

2.4.7.2.1. The pruning phase and its hormonal control The pruning of larval dendritic and axonal branches in both *Manduca* and *Drosophila* is associated with the large peak of ecdysteroid that causes the transition from the larval to the pupal stage. Ligation and hormone replacement experiments in *Manduca* demonstrated that the pruning of the proleg motoneuron PPR requires two discrete endocrine cues. 20E, acting in the absence of JH at the wandering peak, is necessary to "commit" the neuron to prune, when it is next exposed to high levels of ecdysteroid. The following high 20E titers of the prepupal peak then cause the pruning (Weeks and Truman, 1985). In the case of PPR, dendritic pruning is followed a few days later by the death of the cell. These two responses, though, are triggered separately, since the length of steroid exposure needed to induce pruning is less than that needed to cause cell death (Weeks *et al.*, 1992). Although appropriately, timed treatment with JH mimics can prevent axon and dendritic pruning in *Manduca*, JH is not able to suppress the pruning response in *Drosophila* neurons (Williams and Truman, 2004).

The demonstration that dendritic pruning removes larval-specific synaptic contacts comes from work on the proleg withdrawal reflex in *Manduca* caterpillars. When the plantal hairs on the end of the proleg contact a foreign object, the firing of their sensory neurons (PH-SNs) causes a reflexive withdrawal of the proleg. The PH-SNs make monosynaptic contacts with PPR, the motoneuron that innervates the proleg retractor muscles. Early in metamorphosis, the proleg retraction reflex weakens. This occurs at the time that the proleg retractor muscles are degenerating, but there are also changes

to the synapse between the PH-SNs and PPR. Stimulation of the sensory nerve carrying the axons of the PH-SNs show a marked weakening of synaptic strength associated with the pruning of dendrites of the motoneuron (Jacobs and Weeks, 1990). Localized treatment of a proleg with JH mimics can produce a pupa that carries a larval proleg with its normal complement of plantal hairs. Even though these JH-treated sensory neurons maintain their larval morphology, PPR still prunes back its dendrites with the associated drop in synaptic efficacy. These results argue that the dendritic pruning is a cell autonomous response rather than one that is elicited by changes in presynaptic partners.

Similar attempts to use ecdysteroid-induced mosaics to examine axonal pruning in motoneurons were not as clear cut (Hegstrom and Truman, 1996). In *Manduca*, permanent larval abdomens were established by ligation, and then 20E was applied locally to the epidermis over a muscle to cause local degeneration of the muscle. The muscles showed a mixed early metamorphic response with some fibers regressing and others remaining contractile. The motoneuron arbor withdrew endplates from the regressing fibers but maintained endplates on fibers that were intact. Thus, the regression of the muscle is sufficient to cause the withdrawal of endplates. The unresolved issue, though, is whether target regression is *necessary* for synapse removal to occur. In other words, would suitably hormone primed motoneuron still withdraw contact from a healthy target muscle? Manipulation of steroid receptors, as described in Section 2.4.9.1, suggests that the target response is not necessary for the neuron to prune back dendrites and axons. The loss of endplates described above may be a homeostatic response of the neuron to target degeneration, and may not be relevant to the changes seen during metamorphosis.

2.4.7.2.2. Hormonal control of arbor outgrowth The outgrowth of pruned axonal arbors involves organization of the outgrowth zone followed by scaffold production and synaptogenesis (**Figures 9 and 10**). The ecdysteroid requirements for some of these events have been addressed primarily in *Manduca*. The growth zone organization phase occurs during the first few days of adult differentiation, when the ecdysteroid titer is dominated by ecdysone with relatively low levels of 20E. The concentration of 20E begins its rapid rise about day P + 7 to 8, and correlates with the shift to the rapid branch extension that underlies scaffold building (**Figure 10**). Synaptogenesis begins on about day P + 12 as the 20E titer begins to fall (Truman and Reiss, 1995).

The organization of the growth zone requires the initiation of the adult peak of ecdysteroids, as shown by diapausing pupae, which lack 20E and maintain their neurons in a post-pruning state for months. A growth zone only begins to form, when these pupae are provided with 20E (Truman and

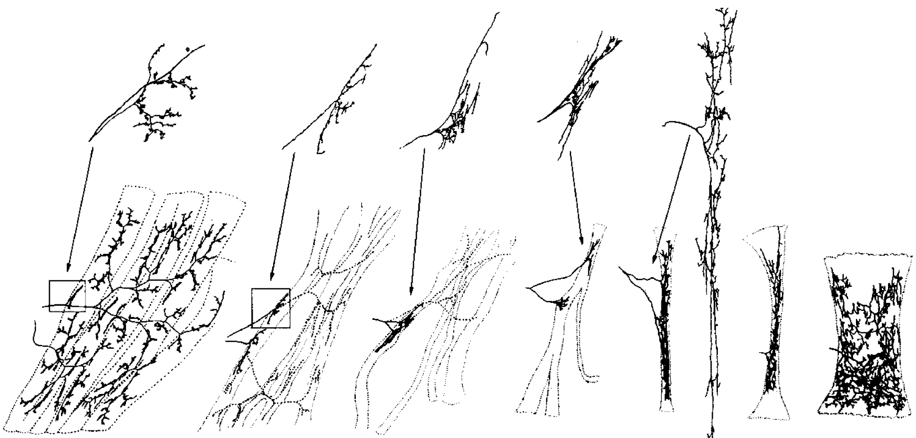


Figure 10 *Camera lucida* drawings showing the remodeling of the axonal arbor of motoneuron MN-12 through the pupal–adult transition (indicated as percentage of development) of *Manduca sexta*. The target muscle and its remains are shown in dotted outline. The arbor in the boxed area is shown at higher magnification above. Between 5 and 15% of development, the axonal arbor undergoes a massive collapse, leaving only two attachment sites on the remains of the first muscle fiber. These contact sites grow in size and complexity through 30% and then rapidly extend and branch over the proliferating muscle. Adult muscle differentiation and adult endplates are then elaborated between 60 and 95% of development. (Data from Truman, J.W., Reiss, S.E., **1995**. Neuromuscular metamorphosis in the moth *Manduca sexta*: hormonal regulation of synapses loss and remodeling. *J. Neurosci. 15*, 4815–4826; © by the Society for Neuroscience.)

Reiss, 1995). The concentration of steroid provided, though, is critical, as demonstrated by injecting pupae with a high dose of 20E early in adult development, thereby mimicking the rise in 20E that occurs on day P + 8. The early 20E causes the premature extension of the axonal arbor over its muscle target (Truman and Riddiford, 2002). Hence, E or low levels of 20E are associated with growth zone organization, whereas high levels of 20E shift the neuron into branch extension and scaffold formation.

A number of *in vitro* studies with isolated, identified neurons show that neurite outgrowth is a cell-autonomous response that can be elicited by 20E (review: Levine and Weeks, 1996). The earliest study utilized *Manduca* leg motoneurons (Prugh *et al.*, 1992), which were labeled *in vivo* by injecting a retrograde tracer into the leg of the caterpillar. Thoracic ganglia were then dissociated and the leg motoneurons grown in low density cultures. In the absence of 20E, these motoneurons extended an axon and showed long-term survival. When cultured with 1 μg 20E ml^{-1}, however, they showed extensive branching growth that mimicked their response to 20E *in vivo*. The 20E treatment had a striking effect on the form and the cytoskeletal composition of the growth cones of these neurons (Matheson and Levine, 1999). Growth cones from 20E-exposed neurons were larger and had an increased number of microtubule-based branches, presumably leading to enhanced formation and retention of higher order branches. Interestingly, if the same neurons were explanted from larval rather than pupal nervous systems, they showed no sprouting but a slight reduction in branching when challenged with steroid. Therefore, the stage specificity of the nature of the response that the cell shows *in vivo*, in terms of steroid-induced pruning versus sprouting, is preserved when the neurons are treated in isolation.

Most *in vitro* studies of identified neurons in both *Manduca* and *Drosophila* (e.g., Prugh *et al.*, 1992; Kraft *et al.*, 1998; Matheson and Levine, 1999) have used a concentration of 20E of 1 μg ml^{-1}. The study by McGraw *et al.* (1998), though, examined the role of different concentrations of 20E on the pattern of neurite outgrowth from identified neurosecretory cells (**Figure 11**). 20E levels in the 100 ng ml^{-1} range were ineffective in supporting the growth of secondary and tertiary branches, and concentrations over 1 μg ml^{-1} were needed for this higher-order branching. Interestingly, the neurons grew the most elaborate arbors if they were first exposed to 100 ng ml^{-1} for 5 days before the shift to the high steroid concentration. Possibly, the low dosage of steroid was having an effect similar to growth zone organization seen *in vivo*, which then resulted in a more extensive arbor, when the cell was shifted to the high hormone conditions.

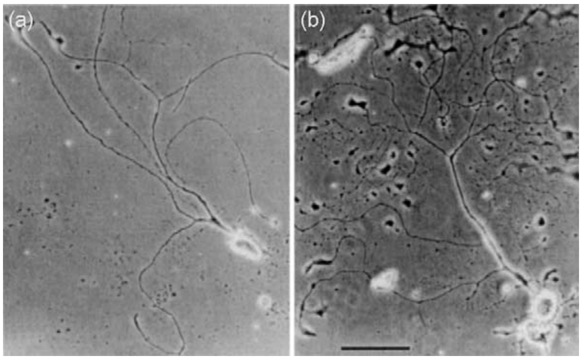


Figure 11 Photomicrographs of single lateral neurosecretory cells (LNCs) *in vitro*. (a) A single LNC in culture exposed to 100 ng 20E ml^{-1} for the entire 10-day period of growth. (b) A single LNC exposed to 100 ng 20E ml^{-1} for the first 5 days *in vitro* and then shifted to 1.5 μg 20E ml^{-1} for days 6–10. (From McGraw, H.F., Prier, K.R.S., Wiley, J.C., Tublitz, N.J., **1998**. Steroid-regulated morphological plasticity in a set of identified peptidergic neurons in the moth, *Manduca sexta*. *J. Exp. Biol. 201*, 2981–2992.)

The ecdysteroid requirements for the last phase, that of synaptogenesis, have not been examined. As with other events that occur during the latter half of adult differentiation (Schwartz and Truman, 1983), it is likely that the withdrawal of 20E may be essential for these events to go to completion.

JH is notable during the outgrowth phase only by its absence. In *Manduca*, the experimental application of JH mimics prior to the ecdysteroid rise results in a severe suppression of outgrowth, and neurons remain in a stunted, pupal-like form (Truman and Reiss, 1995). This ability of early JH treatment to suppress adult differentiation of these motoneurons is not surprising and is consistent with JH causing the repetition of a pupal molt. The more intriguing results are seen with later applications of JH after the neuron has been committed to its adult differentiation. For *Manduca* motoneurons, late treatment with JH has a quantitative effect and results in neurons with reduced adult arbors. The manner by which JH exerts quantitative control over arbor growth is discussed for the remodeling of sensory neurons in Section 2.4.8.2.

One question concerning the quantitative effects of JH on neuronal form is whether the effect of JH is directly on the neuron or indirect through effects on its targets. This is especially pertinent for the motor arbors, since the JH treatments reduce the size of both the target muscle and the arbor of the neuron that grows over it. Local application

of JH in *Manduca*, though, showed that local treatment to the muscle caused the expected reduction of muscle growth but did not suppress the normal elaboration of the motoneuron's axonal arbor (Truman and Reiss, 1995). Hence, the reduced arbor size seen after systemic treatment with JH is likely due to a direct action on the neuron itself.

2.4.7.3. Differentiation of Adult-Specific Neurons

2.4.7.3.1. Differentiation of arrested interneurons At the outset of metamorphosis, the larva possesses two classes of arrested interneurons: a small number of neurons born during embryogenesis and a much larger set born later during larval growth. Studies on tangential visual interneurons in *Drosophila* suggest that these two sets of neurons may differ in their developmental time-tables during metamorphosis (Taghert *et al.*, 2000). The OL2-A and OL3 classes of visual interneurons express the neuropeptide dFMRFamide in the adult. The large OL2-A neurons are born during embryogenesis and already show some morphological differentiation at the start of metamorphosis. Based on a *dFMRFa* reporter, the OL2-A neurons start transcribing the *dFMRFa* gene at the beginning of metamorphosis and start expressing the dFMRFamide peptide soon thereafter. The numerous OL3 neurons are generated postembryonically. They begin transcribing the *dFMRFa* gene slightly later than the OL2-A neurons but delay peptide expression until just before adult ecdysis. At this time, it is not known if the OL neurons present a unique case or if this difference in developmental trajectory is consistent for adult-specific neurons that have embryonic versus postembryonic origins.

Studies in *Manduca* have examined the hormonal requirements needed to initiate differentiation of the arrested imaginal neurons (Booker and Truman, 1987b). Resumption of development occurs during wandering, as the neurons show enlargement of their somata and dendritic and axonal sprouts appear (e.g., **Figure 4**). The *Manduca* studies focus on the abdominal lineages and deal only with cell survival and soma size. In the days following wandering, some of the immature neurons within a cluster degenerate, while others show soma enlargement. When larval abdomens were isolated either before (day $5+2$) or after (W 0) the wandering pulse of ecdysteroids, both neuronal loss and soma growth were prevented. Infusion of 20E into such abdomens restored the normal pattern of cell loss and soma growth. The effect of JH treatment on cell loss was complex, but treatment with a JH mimic suppressed the growth-promoting effects of 20E, irrespective of whether it was given before or after the wandering peak of ecdysteroids. Therefore, unlike remodeling larval neurons, these adult-specific neurons do not show "commitment" in response to the wandering peak of ecdysteroids. Their early outgrowth is responsive to the JH that normally appears during the prepupal peak. This action of JH may prevent these neurons from showing precocious outgrowth in response to the prepupal peak of ecdysteroids.

2.4.7.3.2. Visual interneurons of the optic lobes Although the majority of postembryonic neurons likely deal with new adult sensory information, the sensory neurons play no role in their production. Indeed, neurogenesis in the CNS is completed well before sensory axons reach the CNS. An exception to this generalization, though, occurs in the developing visual system. Rows of ommatidia are added progressively from the posterior to the anterior margin of the eye disc, and the outer layers of the optic lobe, the lamina and medulla, undergo a similar sequential production of visual interneurons. Neuroblasts in an outer proliferation zone (OPZ) generate the neurons for both of these regions. In early larval stages, symmetrical cell divisions within the OPZ expand the population of NBs. In the last larval stage, though, the NBs switch to asymmetrical divisions that produce GMCs, which then make neurons. In *Manduca*, this shift to neurogenesis requires the decline in JH (Monsma and Booker, 1996), and medulla neurons start being born early in the last larval stage. Lamina neurons, however, only appear after wandering, as the the first retinal afferents reach the optic lobe. In both *Manduca* (Monsma and Booker, 1996) and *Drosophila* (Selleck *et al.*, 1992), removal of the afferents prevents the birth of lamina neurons, although medulla neurons continue to be made. Studies on *Drosophila* show that the lamina GMCs undergo a G1 arrest and degenerate, unless they are contacted by the afferent axons, which supply Hedgehog, which drives them through the G1 blockage (Huang and Kunes, 1996).

The birth of lamina neurons has a unique requirement for afferent axons, but all regions of the optic lobe are sensitive to ecdysteroids. The ecdysteroid requirement is absolute in *Manduca* (Champlin and Truman, 1998a). In the absence of E or 20E, the cells in the outer proliferation zone arrest in the G2 phase of the cell cycle. A pulse of 20E, as short as 1.5–2 h, is sufficient to remove the block and send cells into mitosis, but they then arrest at the next G2 phase if no steroid is present. Therefore, during each cell cycle there is a single checkpoint, when steroid concentrations are assessed, and sustained proliferation therefore requires the tonic presence of E or a moderate level of 20E. The control point

is likely the transcription of the *Manduca* homolog of *string/cdc2 phosphatase*. This gene is a prominent regulator of the transition from G2 to M phase (Edgar and O'Farrell, 1989). In G2-arrested neuroblasts *string* transcription is directly and rapidly induced by exposure to 20E (D. Champlin and J.W. Truman, unpublished data).

Besides acting via transcriptional control, ecdysteroids also influence neurogenesis through a rapid response pathway that likely does not involve the nucleus. The neuroblasts in the OPZ respond to threshold 20E concentrations in an all-or-none fashion – either all of the cells are cycling or none of them (**Figure 12a**; Champlin and Truman, 2000). This all-or-none coordination within a lobe is mediated through local suppression of proliferation caused by nitric oxide (NO). Inhibitors of NO production, such as L-NAME, abolish this coordination and lower the levels of steroid needed to drive proliferation. Under these conditions, though, one now sees a graded response to the concentration of ecdysteroids (**Figure 12b** and **c**). The source of NO in the OPZ appears to be the NBs themselves. Treatment with moderate levels of 20E suppresses NO production within 15 min and is independent of RNA synthesis. The effect of NO appears to be downstream of *string* transcription because treatment with 20E in the presence of NO results in the rapid induction of *string* transcripts but the NBs still remain blocked in G2 (D. Champlin and J.W. Truman, unpublished data). Hence, 20E provides a stimulatory signal to NBs to promote neurogenesis and also acts to remove a local inhibition from the neighboring NBs. While ecdysteroids promote neurogenesis at moderate concentrations in *Manduca*, high levels of 20E terminate neurogenesis by causing the death of the NBs (Champlin and Truman, 1998a).

Drosophila differs from *Manduca* in that there is not an absolute requirement for E or 20E to support neurogenesis in the OL. Moderate levels of 20E do enhance proliferation and, as in *Manduca*, high levels of 20E terminate it. In the absence of 20E, neurogenesis is extended beyond its normal time and the brain overproduces optic lobe neurons (T. Awad and J.W. Truman, unpublished data).

2.4.8. Metamorphosis of the Sensory System

The transformation from a sedentary larva to an active, flying adult requires a profound change in how the insect senses its world. Most of the larval sensory system is directed towards proximal stimuli,

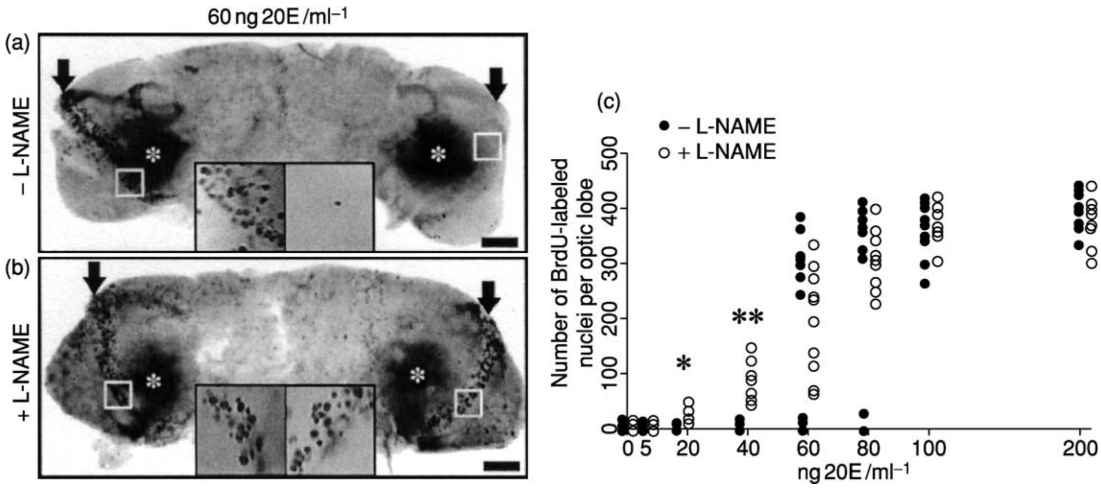


Figure 12 Ecdysteroid-dependent proliferation in the presence and absence of an inhibitor of nitric oxide synthase (NOS). Brains were collected 1 day after pupal ecdysis, desheathed, and cultured for 24 h in various concentrations of 20E. They were then pulsed with Bromodeoxyuridine (BrdU) for 2 h, and the number of cells incorporating BudR was then determined by immunocytochemistry. (a, b) Photomicrographs of brains cultured with 60 ng 20E ml^{-1}. In (b) 1 mM L-NAME was included from the start of the culture. BudR-labeled nuclei are black; arrows point to the outer proliferation zone. ∗ show pigment spots present in each optic lobe. The insets are higher magnification views of the boxed areas in each optic lobe. The brain in (a) illustrates the all-or-none character of the proliferation response seen at threshold concentrations of 20E, with one hemisphere inactive while the other is in full proliferation. With the NOS inhibitor (b), both lobes show similar, moderate levels of proliferation to the same concentration of 20E. (c) The number of cells that labeled with BudR after various treatments. Each point is from a single optic lobe incubated at the indicated 20E concentration with or without L-NAME. The low concentration data sets differ by ∗, $P < 0.05$, and ∗∗, $P < 0.005$. (Data from Champlin, D.T., Truman, J.W., **2000**. Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. *Development 127*, 3543–3551.)

depending heavily on mechanoreception and having poorly developed visual and olfactory capacities. The adult has elaborate antennae and compound eyes, adapted for perceiving stimuli at distances, and batteries of new external sense organs associated with the adult legs, wings, and genitalia. The extent of carryover of larval sensory receptors into the adult stage varies. In *Drosophila*, all of the larval external sensory neurons degenerate, and adult external sensory neurons arise during the differentiation of imaginal discs and histoblast nests. The few larval sensory neurons that persist through metamorphosis are proprioceptors and some of the da sensory neurons. The axons from these persisting cells provide pathways that are utilized by the axons from adult-specific neurons, as they navigate towards the CNS (Usui-Ishihara *et al*., 2000; Williams and Shepherd, 2002).

In contrast to *Drosophila, Manduca* shows a greater percentage of larval sensory neurons that are carried through metamorphosis. The transition between the larval and adult sensory systems has been best described for the thoracic legs. As the larval leg undergoes degeneration and replacement by the adult leg, most of the larval sensory neurons degenerate but some of the neurons that supplied the external sensilla and the chorditonal organs persist through metamorphosis (Consoulas, 2000). Of special interest are the neurons of larval femoral chordotonal organ. While about half of the original 13 neurons that supplied the larval organ degenerate, the remainder serve to organize the adult structure. They are joined by 45–60 new neurons to make up the chorditonal organs of the adult leg (Consoulas *et al*., 2000b). While most studies of the hormonal control over the metamorphosis of the peripheral nervous system have focused on differentiation of adult-specific neurons, there is some information on remodeling sensory systems.

2.4.8.1. Remodeling of Larval Neurons: Pupal-Specific Modifications

The pupa stage has little behavioral relevance for the vast majority of central neurons, but this is not true for the sensory system. Pupae display some specialized behaviors, and a small set of peripheral and central neurons are dedicated to maintain these behaviors as the rest of the nervous system undergoes its metamorphic upheaval. In *Manduca*, these pupal-specific behaviors include abdominal respiratory movements and a pupal-specific defensive reflex, the gin-trap reflex. The neural basis of the latter is best understood (Bate, 1973).

Abdominal segments A4 to A6 of the pupa bear sharp-sided cuticular cavities, the gin traps, that protect the pupa from small soil arthropods. Stimulation of the sensory hairs in the trap excites an intersegmental interneuron that then excites the motoneurons to the ipsilateral, internal longitudinal muscles causing the rapid closure of the trap, crushing whatever is inside. The sensory hairs within the gin trap are recruited from the larval sensory hairs along the anterior border of the segment. In their transition to their pupal state, the hairs acquire a new morphology, and the sensory neurons expand their axonal projections within the CNS (**Figure 13**).

The studies on the metamorphosis of the gin-trap system was the first use of JH-induced mosaics to dissect developmental interactions within the CNS (Levine *et al*., 1986). Local application of JH to larval epidermis, fated to form the gin-trap, resulted in larval cuticle being deposited in that region ,while the remainder of the animal became pupal. A normal gin-trap formed on the control side, and its sensory neurons showed their normal axonal expansion and could drive the pupal reflex (**Figure 13a**). On the treated side, by contrast, the projection pattern of the sensory neurons remained larval, and stimulation of the cells could not evoke the closure reflex.

The pupal-specific sprouting of the sensory neuron requires both an exposure to 20E as well as the absence of JH. The 20E requirement was shown by permanently arresting the development of the larval abdomen by ligating the abdomen after the wandering stage. Under these conditions, the neurons destined for the gin trap permanently retain their larval morphology. Topical application of 20E in undecane to the presumptive gin trap region caused a local pupal molt in the underlying cells, while the remainder of the abdomen, including the CNS, remained arrested. The gin-trap sensory neurons showed their pupal sprouting, despite doing so in a CNS that was permanently larval (**Figure 13b**; Levine, 1989). These pupal neurons, though, could not initiate a gin-trap reflex, presumably because the interneuron involved in the reflex still was in its larval form. Together, these two sets of experiments show that the growth of the sensory arbor is not induced by the state of the CNS, but rather depends on the hormonal conditions experienced in the periphery, where the cell body resides.

2.4.8.2. Adult-Specific Remodeling of Sensory Neurons

The da sensory neurons extend complex dendritic arbors over the epidermis of the larva. At metamorphosis neurons like ddaE have their dendrites severely pruned back, the cell bodies migrate dorsally and then elaborate new dendritic trees (Williams and Truman, 2004). During their remodeling, these

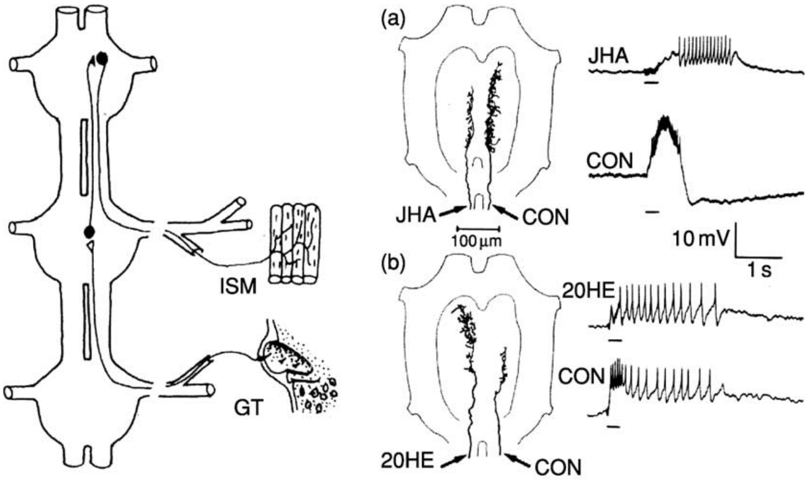


Figure 13 Hormonal control over the larval–pupal tranformation of the gin-trap system. (Left) Drawing showing the relationship between the sensory hairs in the gin-trap (GT) and the motoneurons to intersegmental muscles (ISM) that mediate the trap closure. (Right) Motoneuron responses to stimulation of the trap sensory neurons in heterochronic mosaic animals. (a) The A4 ganglion from a mosaic pupa, in which the presumptive gin-trap region of the left fifth segment was treated topically with a JH mimic. The drawing shows that single sensory neurons from the JH treated region retained their sparse larval arbors, whereas the neuron from the control side grew the expanded terminal arbor characteristic of the pupal stage. To the right of the drawing are shown the responses of ipsilateral, pupal ISM motoneurons to stimulation of treated and control sensory neurons. The control neurons evoked the abrupt excitation characteristic of the closure response, whereas the treated neurons evoked a low-level, sustained, larval-like response. (b) The A4 ganglion from a mosaic larva. After abdominal ligation early in the 5th larval stage, the presumptive gin-trap region on one side of the fifth segment was treated with 20E. The contralateral control neurons maintained their larval pattern of branching but the 20E-treated cells grew pupal-like arbors. In both cases, however, the stimulation of the sensory neurons evoked larval-like responses from the ipsilateral motoneurons. ((Right) Data from Levine, R.B., Weeks, J.C., **1990**. Hormonally mediated changes in simple reflex circuits during metamorphosis in *Manduca*. *J. Neurobiol. 21*, 1022–1036.)

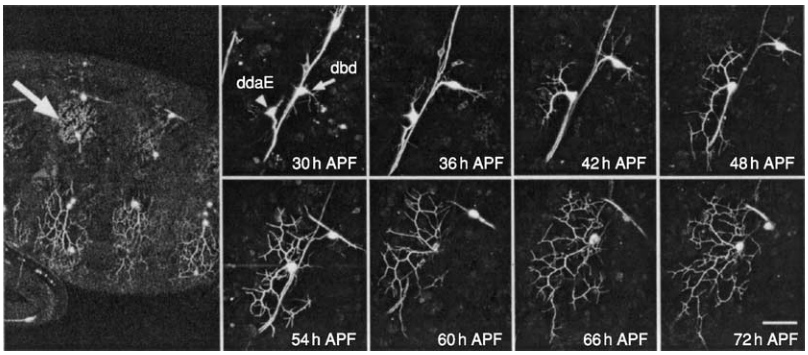


Figure 14 Adult outgrowth of the dendrites of the da sensory neuron ddaE. (Left) Low-power confocal image of the lateral aspect of the abdomen of a live developing adult of *Drosphila* at about 70 h APF (after puparium formation). The da neurons are expressing a membrane-targeted green fluorescent protein (GFP); arrow shows one of the segmentally-repeated ddaE's. (Right) Confocal images of the same set of neurons followed through metamorphosis from 32 to 72 h APF showing the time-course of adult dendrite outgrowth by ddaE. Dbd is a sensory neuron that produces a dorsal stretch receptor. (From Williams, D.W., Truman, J.W., **2004**. Mechanisms of dendritic elaboration of sensory neurons in *Drosophila*: insights from in vivo time-lapse. *J. Neurosci. 24*, 1541–1550; © by the Society for Neuroscience.)

sensory neurons progress through similar phases to those seen for central neurons, except that cell body migration occurs as new outgrowth zones are being established. Also, the dendrites of these primary sensory neurons do not receive synaptic inputs.

The growth of the adult dendrites by the da neurons occurs during the surge of 20E that causes adult differentiation. As evident from the time-lapse images in Figure 14, the dendrites show a rapid extension that is also accompanied by occasional branch retractions and the formation of interstitial branches. After the overall form and extent of the arbor is established, the tree then fills in with higher order twigs (Williams and Truman, 2004). The

manner by which 20E directs this outgrowth has not been examined, but the effects of JH are notable. As with other tissues in *Drosophila*, JH treatment does not suppress the larval–pupal transition of these cells – i.e., they prune back normally irrespective of the presence or absence of JH. JH treatment, though, does impair the adult outgrowth of these cells, and they remain in a pupal-like form. Time-lapse studies suggest that the failure of these JH-treated cells to extend branches may be due to the persistence of the strong retraction programs that were part of the pruning process (Williams and Truman, 2004). The effect of JH treatment on the maintenance of retraction programs is more evident for animals treated with JH mimics at about 24 h APF. As with late treatment of motoneurons (see Section 2.4.7.2.2), da sensory neurons in such animals grow an adult arbor but it is reduced in extent and complexity. These neurons show a normal outgrowth program as measured by the rate of birth of new dendritic branches. The retraction programs, however, are maintained beyond their normal time, thereby reducing the rate of branch extension and the number of branches that finally persist. The manner by which JH prevents the inactivation of retraction programs is unknown.

2.4.8.3. Control Over Birth of Adult Sensory Neurons

Studies with cultured wing imaginal discs from wandering larvae show that the 20E of the pupariation peak is required for the birth of the sensory neurons on the anterior margin of the wing of *Drosophila*. The effect of this hormone, though, is to relieve a repression imposed by the ecdysone receptor complex (Schubiger and Truman, 2000). This relationship first became evident from experiments, in which clones lacking Usp were induced in wing imaginal discs (Schubiger and Truman, 2000). Neuronal birth and axonal outgrowth occurred earlier within such clones as compared to surrounding, wild-type tissues. Similar advancement in development with the lack Usp was also seen for the ecdysone-dependent movement of the morphogenetic furrow in the eye disc (Zelhof *et al*., 1997; Ghbeish *et al*., 2001). Importantly, when wing discs containing such clones were transferred into culture lacking 20E, they showed the expected arrested development except for the patches that lacked Usp. The latter cells progressed into neurogenesis and axonal outgrowth despite the lack of steroid (**Figure 15**). More recent experiments using RNAi techniques to remove EcR (Roignant *et al*., 2003) similarly showed that the lack of EcR also brings about the precocious

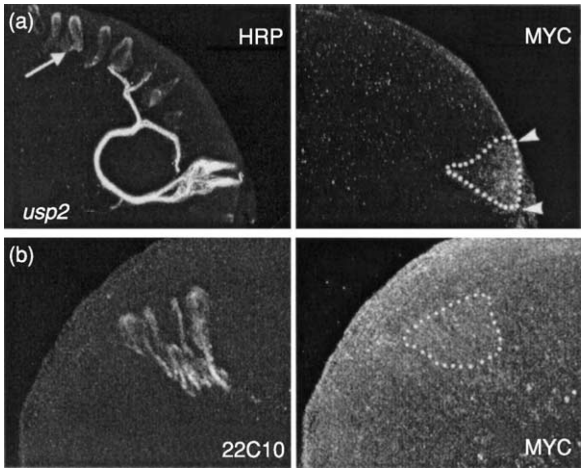


Figure 15 The effect of the lack of Usp on the early birth and differentiation of sensory neurons in the wing disc of *Drosophila*. The wing discs bore clonal patches of tissue that lacked a functional *usp* gene and were marked by expression of the MYC protein (right, enclosed by dotted area). Neuronal differentiation was determined by immunostaining for a horseradish peroxidase (HRP) epitope or with the monoclonal antibody 22C10 (left). (a) *In vivo* at pupariation, the sensory neurons along the anterior wing margin (arrow) are just beginning axonal outgrowth and expression of the HRP epitope, but within the *usp* null clone the neurons are well advanced in their development with extensive axonal outgrowth. (b) Wing discs from early wandering larvae that were maintained *in vitro* in the absence of 20E. Metamorphic development failed to occur in the wild-type tissue, but cells within the *usp* null clone showed normal neurogenesis and axonal outgrowth despite the lack of steroid. (Data from Schubiger, M., Truman, J.W., **2000**. The RXR-ortholog Usp suppresses early metamorphic processes in the absence of ecdysteroids. *Development 127*, 1151–1159.)

birth and axonal outgrowth by wing sensory neurons (M. Schubiger and J.W. Truman, unpublished data). Consequently, in the imaginal discs, the EcR/Usp complex functions as a potent repressor of genes involved in the selection and differentiation of sensory neurons. The major role of ecdysteroids is to relieve this repression, thereby allowing development to progress.

Although ecdysteroids are clearly involved in controlling the birth of adult sensory neurons, relatively little is known about how the steroid titer impacts their subsequent differentiation. One case in which we have some insights, though, is in the development of chemosensory sensilla on the antenna of *M. sexta* (Vogt *et al*., 1993; see **Chapters 1.12** and **3.15**). The final maturation of the chemosensory sensilla occurs during the last 10% of adult development with the appearance of odorant binding proteins (OBPs) and odorant degrading enzymes. In cultured antennae, OBP expression could be induced prematurely if 20E was absent from the culture medium. The addition of 20E then

blocked the premature expression of the OBPs. Hence, declining levels of ecdysteroid may have an important function in coordinating the terminal maturation of the sensory organs.

2.4.8.4. Sensory Systems: The Compound Eye

The building of the compound eye is one of the spectacular achievements of metamorphosis. The organization of the eye imaginal disc begins at the posterior border of the disc and progresses anteriorly as the morphogenetic furrow moves across the disc. In *Manduca* the hormonal control over the organization of the eye disc is relatively simple because the establishment of the furrow occurs after wandering, when metamorphosis has commenced. In *Drosophila*, by contrast, the establishment and early movement of the furrow is advanced to the middle of the last larval stage. The role of hormones in controlling this early patterning of the disc is unclear, but once wandering has begun, the furrow movement then appears to require ecdysteroids (Brennan *et al*., 2001).

The analysis of the role of ecdysteroids in the formation and patterning of the compound eye is most detailed for *Manduca* (**Figure 16**; Champlin and Truman, 1998a). In this insect, the eye imaginal disc does not form until well into the last larval stage. The earlier larval instars have a crescent-shaped eye primordium immediately anterior of the stemmata, but during the larval molts these cells make head capsule cuticle, as do their neighbors. Their developmental potential becomes apparent early in the last larval stage, when they enlarge, detach from the overlying cuticle, and begin proliferating to form the eye imaginal disc. The growing disc rapidly enlarges until the time of wandering, at which time it becomes dependent on ecdysteroid to support further proliferation. The divisions in this latter phase are directed towards the patterning of the eye disc and support the movement of a morphogenetic furrow. In the wake of the furrow, cells differentiate into the photoreceptors and the cone and pigment cells that comprise the ommatidia. The progression of the furrow begins the day after wandering and is completed about 10 days later, well into adult differentiation.

In vitro experiments show that both E and 20E support proliferation and movement of the furrow (Champlin and Truman, 1998b). The levels of

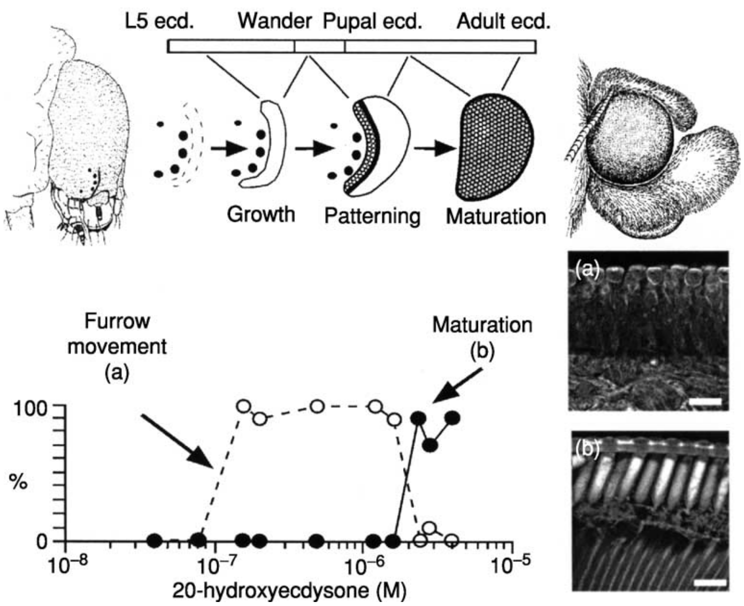


Figure 16 Eye development in *Manduca sexta*. (Top) Time-line of development: at the start of the last larval stage, the crescent-shaped eye primordium (dashed line) lies just anterior to the larval stemmata. These cells detach and begin proliferating by 2 days into the last instar and form the eye imaginal disc. After the larva stops feeding and begins the wandering stage, low levels of ecdysteroid support the movement of the morphogenetic furrow to organize the ommatidia units that then mature in response to high levels of 20E. (Bottom) Summary of the response of the pupal eye imaginal discs to treatment with 20E *in vitro*. (Left) The dose–response relationship showing the 20E concentrations needed to evoke mitosis and movement of the morphogenetic furrow (open circles), and the cellular maturation of the ommatidial units (filled circles). (Right) Confocal optical sections of the region posterior to the furrow. In response to low levels of 20E (a), the ommatidial cell types differentiate and organize into ommatidial clusters. High concentrations of 20E (b) induce terminal maturation as manifest by the synthesis of the screening pigments, cuticular lens, crystalline cones, and elongated rhabdomeres. Scale bars = 25 μm. (Data from Champlin, D.T., Truman, J.W., **1998b**. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. *Development 125*, 2009–2018.)

20E needed to support proliferation are lower than E, but the latter levels are still in the physiological range seen during early adult differentiation (Warren and Gilbert, 1986). As discussed for the optic lobes above (see Section 2.4.7.3.2), proliferation and patterning of the eye disc requires the tonic presence of steroid but levels must be maintained in a range between 60 and 1000 ng 20E ml^{-1}. Levels above 1000 ng ml^{-1} permanently suppress proliferation and furrow movement and cause the cells behind the furrow to undergo maturation and make rhabdoms, crystalline cones, screening pigments etc. (**Figure 16**). The eye disc cells in front of the furrow, by contrast, are undetermined and are induced to make a naked, adult cuticle (Champlin and Truman, 1998b). The exposure to high levels of 20E initiates a differentiative program that can continue even if the steroid is subsequently withdrawn.

Once the imaginal disc has started ecdysteroid-dependent patterning, the tissue can be shifted into the terminal differentiation at any time by raising the 20E titer above 1000 ng ml^{-1}. Hence, if high levels of 20E are experimentally supplied precociously, then a miniature eye forms (Champlin and Truman, 1998b). One complication with this relationship is that the patterning eye disc is actually exposed to levels of 20E above 1000 ng ml^{-1} twice; first during the larval pupal transformation and then at about day 8 of adult development. Importantly, though, JH is also present during this first exposure to high levels of 20E. If JH is experimentally removed by allatectomy of the larva, then the high 20E levels of the larval–pupal molt do indeed promote early eye differentiation, and the pupa forms with a miniature adult eye (Kiguchi and Riddiford, 1978; Champlin and Truman, 1998b). *In vitro* experiments on the differentiation of the ventral diaphragm muscle in *Manduca* show that JH does not interfere with responses to moderate levels of 20E but suppresses the new responses that occur when shifting to high levels of 20E (Champlin *et al.*, 1999).

Experiments, in which Usp function was removed from the patches of cells in the eye imaginal disc of *Drosophila*, also illustrate the complex control of the differentiation of this structure (Zelhof *et al.*, 1997; Ghbeish *et al.*, 2001). In clones of cells that lack *usp*, the furrow shows an advancement over that seen in surrounding wild-type tissue. This result is similar to that seen for the wing sensory neurons (see Section 2.4.8.3) and argues that ecdysteroids regulate furrow progression by removing the repression imposed by EcR/Usp. Later on, Usp also has a role in hormone-dependent activation (Ghbeish *et al.*, 2001).

2.4.9. Steroid Receptors and the Coordination of the Metamorphic Development

2.4.9.1. Relationship of Receptor Expression and Cellular Responses

Indications that steroid receptor levels vary through time in the CNS came with autoradiogarphic studies of the binding of radiolabeled ponasterone. Bidmon *et al.* (1991) reported that very few brain neurons showed ponasterone accumulation early in the last larval stage but that the number increased dramatically shortly after wandering, and then declined as the ecdysteroid peak declined. Substantial ponasterone binding then reappeared after pupal ecdysis. The variation in ponasterone binding at certain times could be correlated with different steroid-dependent fates. For example, at the end of metamorphosis neurons fated to die showed much higher levels of ponasterone binding than did those that would persist in the adult stage (Fahrbach and Truman, 1989).

Studies of receptor distributions became easier with the identification of the EcR gene (Koelle *et al.*, 1991) and with the production of antibodies to detect the receptor protein. The story became complex, though, with the discovery that there were three isoforms of the receptor (Talbot *et al.*, 1993) and that these were expressed in a complex pattern through metamorphosis (**Figure 17**; Truman *et al.*, 1994; see **Chapter 3.5**). The expression patterns of only EcR-A and EcR-B1 are known in detail. That of EcR-B2 is generally assumed to be similar to EcR-B1, but this remains to be established. The pattern of EcR-B1 and -A expression mirrors the diverse origins and cellular fates of the neurons in the CNS. Little EcR expression is detected in neurons through the early larval instars. Also, when EcRs that carry dominant negative mutations (Cherbas *et al.*, 2003) are expressed in larval neurons, they produce no obvious effects on the morphology or functioning of the neurons (J.W. Truman, L. Cherbas, and P. Cherbas, unpublished data). This suggests that the ecdysteroid signaling pathway has little function in most larval neurons prior to metamorphosis.

Detectable EcR first appears in *Drosophila* neurons in the middle of the last larval instar in preparation for metamorphosis. As summarized in **Figures 9 and 17**, each of the phases in metamorphic remodeling of larval neurons is associated with a different combination of EcR isoforms. EcR-B1 is at its highest levels in larval neurons and in the γ-neurons of the mushroom bodies just before both sets of cells begin pruning back their larval processes (Truman *et al.*, 1994; Lee *et al.*, 2000). All other

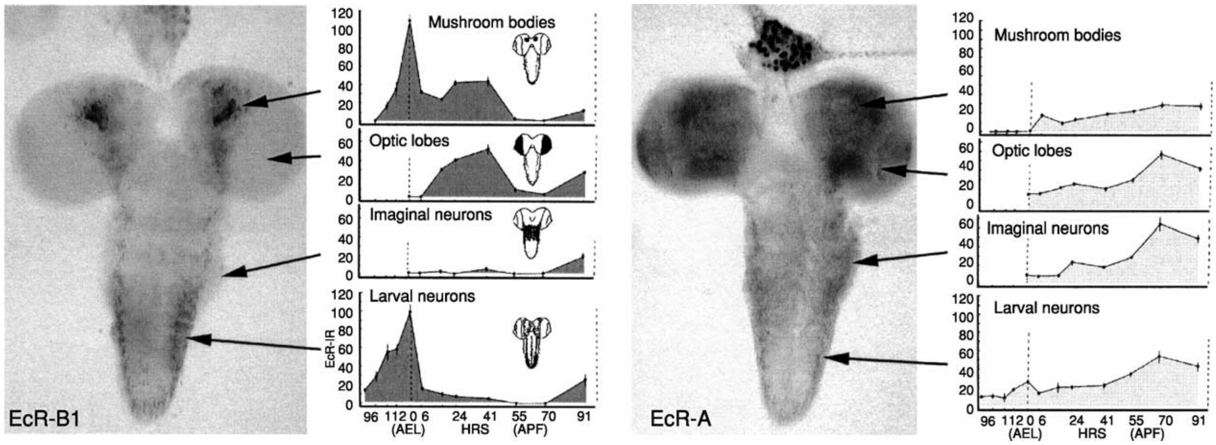


Figure 17 Temporal and spatial variation in the expression of the EcR-B1 (Left) and EcR-A (Right) through metamorphosis. The photomicrograph shows the distribution of both isoforms in the brain lobes and ventral nervous system at the time of puparium formation; the endocrine ring gland is located in front of the brain at the top of the picture. The graphs show the isoform-specific immunoreactivity in various types of neurons as determined by confocal microscopy. Major peaks of ecdysteroid occur at pupariation, and about 24–40 h after puparium formation (APF). Hours 96–112 AEL (after egg-laying) are during the 3rd larval instar. (Modified from Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., **1994**. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development 120*, 219–234.)

postembryonically derived neurons, as well as the arrested embryonic neurons (J.W. Truman and D.D. Williams, unpublished data), lack this early EcR-B1 expression. The importance of the EcR-B isoforms to the pruning response was established using *Drosophila* mutants that lacked one or both of the EcR-B isoforms. Most larvae homozygous for such mutations die during larval life but a few survive to the start of metamorphosis (Bender *et al.*, 1998), at which time tissues within the larva desynchronize their development; the larval tissues arrest their development but imaginal tissues continue into metamorphosis. Within the CNS, arrested imaginal neurons begin adult differentiation, but larval neurons (Schubiger *et al.*, 1998) and the γ neurons of the mushroom bodies (Lee *et al.*, 2000) do not prune back their larval processes. Since EcR-B mutants show this early metamorphic arrest, it was possible that the lack of pruning was not the direct result of a lack of EcR-B in the neurons in question. Subsequent experiments expressed each of the EcR isoforms in specific neurons in arrested EcR-B mutants. Both axonal pruning in the mushroom body neurons (Lee *et al.*, 2000) and dendritic pruning in the Tv neurosecretory cells (Schubiger *et al.*, 2003) were induced in arrested mutant larvae, when EcR was expressed in the respective neurons. Importantly, EcR-A was relatively ineffective, EcR-B1 was moderately effective, and EcR-B2 was very effective in causing pruning of the larval branches (Figure 18). Hence, the proper receptor isoform(s) is essential for mediating the steroid-induced pruning of these cells. A question remains, though, as to whether the pruning decision is a "binary" one or whether variation in the levels of EcR-B isoforms determines how much of a neuron's arbor will be removed.

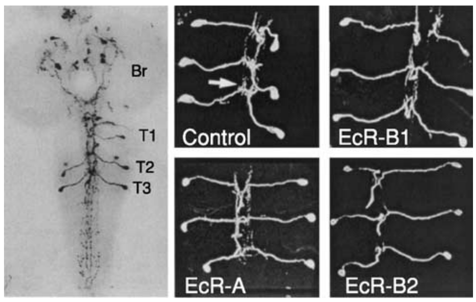


Figure 18 EcR isoform requirements for the pruning of dendritic arbors by the Tv neurosecretory neurons. (Left) Photomicrograph showing the immunostaining of the larval nervous system of *Drosophila* with an antibody against the molluscan neuropeptide SCP_B. The Tv neurosecretory cells in the thoracic neuromeres (T1, T2, T3) show prominent immunostaining (one cell of the Tv pair in T1 is missing). (Right) Confocal images showing the Tv neurons in an EcR-B mutant. The cells were visualized by using the UAS/GAL4 system to express a membrane directed GFP in only the Tv neurons. The fine dendrites at the midline (arrow) are the larval dendrites that fail to be removed in the mutant (Control). For the remaining examples, each of the indicated EcR isoforms was expressed in the Tv neurons in an EcR-B mutant background. Full pruning occurred with expression of the EcR-B2 isoform. (Data from Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., **2003**. Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Devel. 120*, 909–918.)

Pruning is the only phase of neuronal development in which EcR isoform requirements have been rigorously established by loss-of-function and gain-of-function experiments. Correlation of isoform

presence with other developmental phases, however, suggests that the EcR-B isoforms may have functions beyond pruning. For example, EcR-B1 persists in remodeling neurons as they organize their growth zone, but then disappears with the beginning of axon elongation and scaffold formation. Perhaps the presence of EcR-B during this phase maintains some branch retraction programs that help in organizing a growth zone, and also prevents the premature extension of the neuron to its final targets.

The neurons of the optic lobes are unique because they do not prune but they show prominent EcR-B1 expression. This expression begins relatively late, starting at 10 h APF, when the last wave of photoreceptor axons arrive in the OL, and lasting until about 45 h APF, when the gradients of development across the optic lobes are finally complete (Meinertzhagen and Hansen, 1993). This EcR-B1 expression in the OL may have a function similar to that proposed above for organization of the new growth zone in remodeling larval neurons, i.e., it may maintain the growth cones of the interneurons in a plastic state until all of neurons across the optic lobe have developed to the state that they can finalize their synaptic partners.

Both remodeling larval neurons and adult-specific neurons express only EcR-A at the time when they extend branches over their target or through the CNS. They all express EcR-B1, however, as they enter into the synaptogenesis phase. The functional roles of these receptor isoforms during this last phase have not been examined experimentally.

A striking aspect of EcR expression is seen in the neurons that will die at the end of metamorphosis. These neurons begin showing high levels of EcR-A early in adult differentiation (18 h APF) and these levels stay elevated until the neurons degenerate at 6–10 h after adult emergence (Robinow *et al.*, 1993). As described above (see Section 2.4.7.1.2), the disappearance of 20E at the end of metamorphosis is essential to induce *reaper* and to bring about cell death. Since the A/B region of EcR-A appears to have a repressive function (Mouillet *et al.*, 2001), it may be that the protein that represses *reaper* may be EcR-A itself. This repressive function would only be relieved when the steroid titers finally fell at the end of the molt.

2.4.9.2. Regulation of EcR Isoform Expression

EcR is a direct response gene and treatment with 20E enhances the production of EcR transcripts (see **Chapter 3.5**). At least at the start of metamorphosis, the B promoter appears more sensitive than the A promoter, so that rising steroid titers initially favor EcR-B1 expression but then switch to EcR-A when titers are high enough (e.g., Hiruma *et al.*, 1997). In the developing eye of *Manduca*, for example, levels of 20E that shift the eye from proliferation and patterning to final differentiation also causes a shift in expression from EcR-B1 to EcR-A (Champlin and Truman, 1998b).

The steroid regulation of EcR expression is likely shared by many tissues, but the selection of EcR-isoforms may also depend on cell–cell interactions. For example, in the remodeling musculature of *Manduca*, myoblasts at the site of nerve contact show EcR-B1 expression and elevated mitotic activity. Denervation of the remodeling muscle results in both the rapid loss of EcR-B expression and the suppression of proliferation (Hegstrom *et al.*, 1998). These results suggest that the neuron controls the selection of receptors expressed by the underlying myoblasts. Such interactions also take place in the CNS. Mutations in the TGF-β/activin receptor suppress pruning in the γ-neurons of the mushroom bodies and also blocks the appearance of EcR-B1 in these cells. Importantly, in mutant animals, the selective expression of EcR-B1 in the γ-neurons partially restores the ability of these cells to prune (Zheng *et al.*, 2003).

Acknowledgments

I am grateful to Darren Williams, Margrit Schubiger, Heather Brown, and Lynn Riddiford for many fruitful discussions during the preparation of this review. Unpublished results were from research supported by NIH (NS 13079 and NS 29971).

References

Anderson, H., **1978**. Postembryonic development of the visual system of the locust, *Schistocerca gregaria*. II. An experimental investigation of the formation of the retina–lamina projection. *J. Embryol. Exp. Morphol. 46*, 147–170.

Bate, C.M., **1973**. The mechanism of the pupal gin-trap. I. Segmental gradients and the connections of triggering sensilla. *J. Exp. Biol. 59*, 95–107.

Bate, C.M., **1976a**. Embryogenesis of an insect nervous system. I. A map of the thoracic and abdominal neuroblasts in *Locusta migratoria*. *J. Embryol. Exp. Morphol. 35*, 107–123.

Bate, C.M., **1976b**. Pioneer neurones in an insect embryo. *Nature 260*, 54–56.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., Hogness, D.S., **1998**. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell 91*, 777–788.

Bidmon, H.J., Granger, N.A., Cherbas, P., Maroy, P., Stumpf, W.E., **1991**. Ecdysteroid receptors in the

central nervous system of *Manduca sexta*: their changes in distribution and quantity during larval–pupal development. *J. Comp. Neurol. 310*, 337–355.

Booker, R., Truman, J.W., **1987a**. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol. 255*, 548–559.

Booker, R., Truman, J.W., **1987b**. Postembryonic neurogenesis in the CNS of the tobacco Hornworm, *Manduca sexta*. II. Hormonal control of imaginal nest cell degeneration and differentiation during metamorphosis. *J. Neurosci. 7*, 4107–4114.

Boyan, G.S., **1983**. Postembryonic development in the auditory system of locusts. *J. Comp. Physiol. A 151*, 499–513.

Brennan, C.A., Li, T.R., Bender, M., Hsiung, F., Moses, K., **2001**. Broad-complex, but not ecdysone receptor, is required for progression of the morphogenetic furrow in the *Drosophila* eye. *Development 128*, 1–11.

Britton, J.S., Edgar, B.A., **1998**. Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development 125*, 2149–2158.

Broadus, J., Skeath, J.B., Spana, E.P., Bossing, T., Technau, G., *et al.*, **1995**. New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. *Mech. Devel. 53*, 393–402.

Brody, T., Odenwald, W.F., **2000**. Programmed transformations in neuroblast gene expression during *Drosophila* CNS lineage development. *Devel. Biol. 226*, 34–44.

Brody, T., Odenwald, W.F., **2002**. Cellular diversity in the developing nervous system: a temporal view from *Drosophila*. *Development 129*, 3763–3770.

Caldwell, M., Data, S., **1998**. Expression of cyclin E or DP/E2F rescues the G1 arrest of *trol* mutant neuroblasts in the *Drosophila* larval central nervous system. *Mech. Devel. 79*, 121–131.

Cayre, M., Strambi, C., Charpin, P., Augier, R., Meyer, M.R., *et al.*, **1996**. Neurogenesis in adult insect mushroom bodies. *J. Comp. Neurol. 371*, 300–310.

Champlin, D.T., Truman, J.W., **1998a**. Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. *Development 125*, 269–277.

Champlin, D.T., Truman, J.W., **1998b**. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. *Development 125*, 2009–2018.

Champlin, D.T., Truman, J.W., **2000**. Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. *Development 127*, 3543–3551.

Champlin, D.T., Reiss, S.E., Truman, J.W., **1999**. Hormonal control of ventral diaphragm myogenesis during metamorphosis of the moth, *Manduca sexta*. *Devel. Genes Evol. 209*, 265–274.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., **2003**. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development 130*, 271–284.

Chiba, A., Shepherd, D., Murphey, R.K., **1988**. Synaptic rearrangement during postembryonic development in the cricket. *Science 240*, 901–905.

Consoulas, C., **2000**. Remodeling of the leg sensory system during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol. 419*, 154–174.

Consoulas, C., Duch, C., Bayline, R.J., Levine, R.B., **2000a**. Behavioral transformations during metamorphosis: remodeling of neural and motor systems. *Brain Res. Bull. 53*, 571–583.

Consoulas, C., Rose, U., Levine, R.B., **2000b**. Remodeling of the femoral chordotonal organ during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol. 426*, 391–405.

Consoulas, C., Restifo, L.L., Levine, R.B., **2002**. Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. *J. Neurosci. 22*, 4906–4917.

Datta, S., **1995**. Activation of neuroblast proliferation in explant cultures of the *Drosophila* CNS. *Brain Res. 818*, 77–83.

Doe, C.Q., **1992**. Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development 116*, 855–863.

Duch, C., Bayline, R.J., Levine, R.B., **2000**. Postembryonic development of the dorsal longitudinal flight muscle and its innervation in *Manduca sexta*. *J. Comp. Neurol. 422*, 1–17.

Duch, C., Levine, R.B., **2000**. Remodeling of membrane properties and dendritic architecture accompanies the postembryonic conversion of a slow into a fast motoneuron. *J. Neurosci. 20*, 6950–6961.

Ebens, A.J., Garren, H., Cheyette, B.N., Zipursky, S.L., **1993**. The *Drosophila* anachronism locus: a glycoprotein secreted by glia inhibits neuroblast proliferation. *Cell 74*, 15–27.

Edgar, B.A., O'Farrell, P.H., **1989**. Genetic control of cell division patterns in the *Drosophila* embryo. *Cell 57*, 177–187.

Erezyilmaz, D.F., Riddiford, L.M., Truman, J.W., **2004**. Juvenile hormone acts at embryonic molts and induces the nymphal cuticle in the direct-developing cricket. *Devel. Genes Evol. 214*, 313–323.

Fahrbach, S.E., Truman, J.W., **1987**. Possible interactions of a steroid hormone and neural inputs in controlling the death of an identified neuron in the moth *Manduca sexta*. *J. Neurobiol. 18*, 497–508.

Fahrbach, S.E., Truman, J.W., **1989**. Autoradiographic identification of ecdysteroid-binding cells in the nervous system of the moth *Manduca sexta*. *J. Neurobiol. 20*, 681–702.

Fernandes, J.J., Keshishian, H., **1998**. Nerve–muscle interactions during flight muscle development in *Drosophila*. *Development 125*, 1769–1779.

Ghbeish, N., Tsai, C.C., Schubiger, M., Zhou, J.Y., Evans, R.M., *et al.*, **2001**. The dual role of ultraspiracle, the *Drosophila* retinoid X receptor, in the ecdysone response. *Proc. Natl Acad. Sci. USA 98*, 3867–3872.

Gilbert, L.I., Rybczynski, R., Warren, J.T., **2002**. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol. 47*, 883–916.

Grueber, W.B., Truman, J.W., **1999**. Development and organization of a nitric-oxide-sensitive peripheral neural plexus in larvae of the moth, *Manduca sexta. J. Comp. Neurol. 404*, 127–141.

Hegstrom, C.D., Truman, J.W., **1996**. Synapse loss and axon retraction in response to local muscle degeneration. *J. Neurobiol. 31*, 175–188.

Hegstrom, C., Riddiford, L.M., Truman, J.W., **1998**. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth *Manduca sexta. J. Neurosci. 18*, 1786–1794.

Hiruma, K., Bocking, D., Lafont, R., Riddiford, L.M., **1997**. Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta. Gen. Comp. Endocrinol. 107*, 84–97.

Hu, X., Cherbas, L., Cherbas, P., **2003**. Transcription activation by the ecdysone receptor (EcR/Usp): identification of activation functions. *Mol. Endocrinol. 17*, 716–731.

Huang, Z., Kunes, S., **1996**. Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. *Cell 86*, 411–422.

Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., **2001**. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell 106*, 511–521.

Jacobs, G.A., Weeks, J.C., **1990**. Postsynaptic changes at a sensory-to-motoneuron synapse contribute to the developmental loss of a reflex behavior during insect metamorphosis. *J. Neurosci. 10*, 1341–1356.

Kiguchi, K., Riddiford, L.M., **1978**. The role of juvenile hormone in pupal development of the tobacco hornworm, *Manduca sexta. J. Insect Physiol. 24*, 673–680.

Kimura, K.I., Truman, J.W., **1990**. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster. J. Neurosci. 10*, 403–411.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.

Kraft, R.N., Levine, R.B., Restifo, L.L., **1998**. The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. *J. Neurosci. 18*, 8886–8899.

Kraus, W.L., Wong, J., **2002**. Nuclear receptor-dependent transcription with chromatin. Is it all about enzymes? *Eur. J. Biochem. 269*, 2275–2283.

Kuwada, J.Y., Goodman, C.S., **1985**. Neuronal determination during embryonic development of the grasshopper nervous system. *Devel. Biol. 110*, 114–126.

Kutsch, W., Hechmann, R., **1995**. Homologous structures, exemplified by motoneurons of Mandibulata. In: Breidbach, O., Kutsch, W. (Eds.), The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach. Birkhauser Verlag, Basel, pp. 221–248.

Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., *et al.*, **1985**. Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea. Experientia 41*, 913–917.

Lee, T., Marticke, S., Sung, C., Robinow, S., Luo, L., **2000**. Cell-autonomous requirement of the Usp/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila. Neuron 28*, 807–818.

Levine, R.B., **1989**. Expansion of the central arborizations of persistent sensory neurons during insect metamorphosis: the role of the steroid hormone, 20-hydroxyecdysone. *J. Neurosci. 9*, 1045–1054.

Levine, R.B., Truman, J.W., **1985**. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta. J. Neurosci. 5*, 2424–2431.

Levine, R.B., Truman, J.W., Linn, D., Bate, C.M., **1986**. Endocrine regulation of the form and function of axonal arbors during insect metamorphosis. *J. Neurosci. 6*, 293–299.

Levine, R.B., Weeks, J.C., **1990**. Hormonally mediated changes in simple reflex circuits during metamorphosis in *Manduca. J. Neurobiol. 21*, 1022–1036.

Levine, R.B., Weeks, J.C., **1996**. Cell culture approaches to understanding the actions of steroid hormones on the insect nervous system. *Devel. Neurosci. 18*, 73–86.

Li, T., Bender, M., **2000**. A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in *Drosophila. Development 127*, 2897–2905.

Matheson, S.F., Levine, R.B., **1999**. Steroid hormone enhancement of neurite outgrowth in identified insect motor neurons involves specific effects on growth cone form and function. *J. Neurobiol. 38*, 27–45.

McGraw, H.F., Prier, K.R.S., Wiley, J.C., Tublitz, N.J., **1998**. Steroid-regulated morphological plasticity in a set of identified peptidergic neurons in the moth, *Manduca sexta. J. Exp. Biol. 201*, 2981–2992.

Meinertzhagen, I.A., Hanson, T.E., **1993**. The development of the optic lobe. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster.* Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 1363–1491.

Meier, T., Chabaud, F., Reichert, H., **1991**. Homologous patterns in the embryonic development of the peripheral nervous system in the grasshopper *Schistocerca gregaria* and the fly *Drosophila melanogaster. Development 112*, 241–253.

Monsma, S.A., Booker, R., **1996**. Genesis of the adult retina and outer optic lobes of the moth, *Manduca sexta*. II. Effects of differentiation and developmental hormone manipulation. *J. Comp. Neurol. 367*, 21–35.

Mouillet, J.F., Henrich, V.C., Lezzi, M., Vogtli, M., **2001**. Differential control of gene activity by isoforms A, B1 and B2 of the *Drosophila* ecdysone receptor. *Eur. J. Biochem. 268*, 1811–1819.

Oland, L.A., Tolbert, L.P., **2003**. Key interactions between neurons and glial cells during neural development in insects. *Annu. Rev. Entomol. 48*, 89–110.

Olberg, R.M., **1986**. Metamorphosis of identified visual interneurons which steer flight in the dragonfly. *Soc. Neurosci. Abst. 12*, 927.

Park, Y., Fujioka, M., Kobayashi, M., Jaynes, J.B., Datta, S., **2001**. *even skipped* is required to produce a *trans*-acting signal for larval neuroblast proliferation that can be mimicked by ecdysone. *Development 128*, 1899–1909.

Pfluger, H.J., Witten, J.L., Levine, R.B., **1993**. Fate of abdominal ventral unpaired median cells during metamorphosis of the hawkmoth, *Manduca sexta*. *J. Comp. Neurol. 335*, 508–522.

Postlethwait, J.H., **1974**. Juvenile hormone and the adult development of *Drosophila*. *Biol. Bull.* (*Woods Hole, Mass.*) *147*, 119–135.

Prugh, J., Della Croce, K., Levine, R.B., **1992**. Effects of the steroid hormone, 20-hydroxyecdysone, on the growth of neurites by identified insect motoneurons *in vitro*. *Devel. Biol. 154*, 331–347.

Restifo, L.L., Wilson, T.G., **1998**. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible broad complex transcription factors. *Devel. Genetics* 22, 141–159.

Riddiford, L.M., **1994**. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Adv. Insect Physiol. 24*, 213–274.

Riddiford, L.M., Ajami, A.M., **1973**. Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol. 19*, 749–762.

Riddiford, L.M., Ashburner, M., **1991**. Effects of juvenile hormone mimics on larval development and metamorphosis of *Drosophila melanogaster. Gen. Comp. Endocrinol. 82*, 172–183.

Riddiford, L.M., Cherbas, P., Truman, J.W., **2000**. Ecdysone receptors and their biological actions. *Vitamins Hormones 60*, 1–73.

Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., **1993**. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor. *Development 119*, 1251–1259.

Robinow, S., Draizen, T.A., Truman, J.W., **1997**. Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. *Devel. Biol. 190*, 206–213.

Roignant, J.Y., Carre, C., Mugat, B., Szymczak, D., Lepesant, J.A., *et al*., **2003**. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA 9*, 299–308.

Sandstrom, D.J., Weeks, J.C., **1998**. Segment-specific retention of a larval neuromuscular system and its role in a new, rhythmic, pupal motor *pattern* in *Manduca sexta*. *J. Comp. Physiol. [A] 183*, 283–302.

Schaffer, R., Sanchez, T.V., **1973**. Antennal sensory system of the cockroach *Periplaneta americana*: postembryonic development and morphology of sense organs. *J. Comp. Neurol. 149*, 335–354.

Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., **2003**. Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Devel. 120*, 909–918.

Schubiger, M., Truman, J.W., **2000**. The RXR-ortholog Usp suppresses early metamorphic processes in the absence of ecdysteroids. *Development 127*, 1151–1159.

Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., Bender, M., **1998**. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development 125*, 2053–2062.

Schwartz, L.M., Truman, J.W., **1982**. Peptide and steroid regulation of muscle degeneration in an insect. *Science 215*, 1420–1421.

Schwartz, L.M., Truman, J.W., **1983**. Hormonal control of rates of metamorphic development in the tobacco hornworm *Manduca sexta*. *Devel. Biol. 99*, 103–14.

Selleck, S., Gonzalez, C., Glover, D.M., White, K., **1992**. Regulation of the G1-S transition in postembryonic neuronal precursors by axon ingrowth. *Nature 355*, 253–255.

Shankland, M., Goodman, C.S., **1982**. Development of the dendritic branching pattern of the medial giant interneuron in the grasshopper embryo. *Devel. Biol. 92*, 489–506.

Shepherd, D., Bate, C.M., **1990**. Spatial and temporal patterns of neurogenesis in the embryo of the locust (*Schistocerca gregaria*). *J. Comp. Neurol. 319*, 436–453.

Stevenson, P.A., Kutsch, W., **1988**. Demostration of functional connectivity of the flight motor system in all stages of the locust. *J. Comp. Physiol. A 162*, 247–259.

Streichert, L.C., Pierce, J.T., Nelson, J.A., Weeks, J.C., **1997**. Steroid hormones act directly to trigger segment-specific programmed cell death of identified motoneurons *in vitro*. *Devel. Biol. 183*, 95–107.

Taghert, P.H., Doe, C.Q., Goodman, C.S., **1984**. Cell determination and regulation during development of neuroblasts and neurones in grasshopper embryo. *Nature 307*, 163–165.

Taghert, P.H., Roberts, M.E., Renn, S.C., Jacobs, P.S., **2000**. Metamorphosis of tangential visual system neurons in *Drosophila*. *Devel. Biol. 222*, 471–485.

Talbot, W.S., Swyryd, E.A., Hogness, D.S., **1993**. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell 73*, 1323–1337.

Tessier-Lavigne, M., Goodman, C.S., **1996**. The molecular biology of axon guidance. *Science 274*, 1123–1133.

Thomas, J.B., Bastiani, M.J., Bate, M., Goodman, C.S., **1984**. From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature 310*, 203–207.

Thompson, K.J., **1993**. Embryonic neural activity and serial homology underlying grasshopper oviposition. *Soc. Neurosci. Abst. 19*, 348.

Thompson, K.J., Siegler, M.V., **1993**. Development of segment specificity in identified lineages of the grasshopper CNS. *J. Neurosci. 13*, 3309–3318.

Truman, J.W., **1983**. Programmed cell death in the nervous system of an adult insect. *J. Comp. Neurol. 216*, 445–452.

Truman, J.W., Ball, E.E., **1998**. Patterns of embryonic neurogenesis in a primitive wingless insect, the silverfish, *Ctenolepisma longicaudata*: comparison with those seen in flying insects. *Devel. Genes Evol. 208*, 357–368.

Truman, J.W., Bate, M., **1988**. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster. Devel. Biol. 125*, 145–157.

Truman, J.W., Reiss, S.E., **1995**. Neuromuscular metamorphosis in the moth *Manduca sexta*: hormonal regulation of synapses loss and remodeling. *J. Neurosci. 15*, 4815–4826.

Truman, J.W., Riddiford, L.M., **2000**. The origins of insect metamorphosis. *Nature 401*, 447–452.

Truman, J.W., Riddiford, L.M., **2002**. Insect developmental hormones and their mechanism of action. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain and Behavior, vol. 2. Academic Press, San Diego, pp. 841–873.

Truman, J.W., Schwartz, L.M., **1984**. Steroid regulation of neuronal death in the moth nervous system. *J. Neurosci. 4*, 274–280.

Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., **1994**. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development 120*, 219–234.

Tublitz, N.J., Sylwester, A.W., **1990**. Postembryonic alteration of transmitter phenotype in individually identified peptidergic neurons. *J. Neurosci. 10*, 161–168.

Urbach, R., Technau, G.M., **2003**. Early steps in building the insect brain: neuropil formation and segmental patterning in the developing brain of different insect species. *Arthropod Struct. Devel. 32*, 103–123.

Usui-Ishihara, A., Simpson, P., Usui, K., **2000**. Larval multidendrite neurons survive metamorphosis and participate in the formation of imaginal sensory axonal pathways in the notum of *Drosophila*. *Devel. Biol. 225*, 357–369.

Vogt, R.G., Rybczynski, R., Cruz, M., Lerner, M.R., **1993**. Ecdysteroid regulation of olfactory protein expression in the developing antenna of the tobacco hawk moth, *Manduca sexta*. *J. Neurobiol. 24*, 581–597.

Walsh, M.K., Lichtman, J.W., **2003**. *In vivo* time-lapse imaging of synaptic takeover associated with naturally occurring synapse elimination. *Neuron 37*, 67–73.

Warren, J.T., Gilbert, L.I., **1986**. Ecdysone metabolism and distribution during the pupal–adult development of *Manduca sexta*. *Insect Biochem. 16*, 65–82.

Watts, R.J., Hoopfer, E.D., Luo, L., **2003**. Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron 38*, 871–885.

Weeks, J.C., Truman, J.W., **1985**. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J. Neurosci. 5*, 2290–2300.

Weeks, J.C., Roberts, W.M., Trimble, D.L., **1992**. Hormonal regulation and segmental specificity of motoneuron phenotype during metamorphosis of the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 149*, 185–196.

Wheeler, D.E., Nijhout, H.F., **2003**. A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. *Bioessays 25*, 994–1001.

White, K., Kankel, D.R., **1978**. Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster. Devel. Biol. 65*, 296–321.

Whitington, P.M., Bate, M., Seifert, E., Ridge, K., Goodman, C.S., **1982**. Survival and differentiation of identified embryonic neurons in the absence of their target muscles. *Science 215*, 973–975.

Wigglesworth, V.B., **1940**. Local and general factors in the development of 'pattern' in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol. 17*, 180–200.

Wigglesworth, V.B., **1953**. The origin of sensory neurons in an insect, *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci. 94*, 93–112.

Williams, C.M., **1961**. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia Silkworm. *Biol. Bull.* (*Woods Hole, Mass.*) *116*, 323–338.

Williams, D.W., Shepherd, D., **2002**. Persistent larval sensory neurones are required for the normal development of the adult sensory afferent projections in *Drosophila*. *Development 129*, 617–624.

Williams, D.W., Truman, J.W., **2004**. Mechanisms of dendritic elaboration of sensory neurons in *Drosophila*: insights from in vivo time-lapse. *J. Neurosci. 24*, 1541–1550.

Witten, J.L., Truman, J.W., **1998**. Distribution of GABA-like immunoreactive neurons in insects suggests lineage homology. *J. Comp. Neurol. 398*, 515–528.

Wolfgang, W.J., Riddiford, L.M., **1986**. Larval cuticular morphogenesis in the tobacco hornworm, *Manduca sexta*, and its hormonal regulation. *Devel. Biol. 113*, 305–316.

Zelhof, A.C., Ghbeish, N., Tsai, C., Evans, R.M., McKeown, M., **1997**. A role for ultraspiracle, the *Drosophila* RXR, in morphogenetic furrow movement and photoreceptor cluster formation. *Development 124*, 2499–2506.

Zheng, X., Wang, J., Haerry, T.E., Wu, A.Y., Martin, J., *et al.*, **2003**. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell 112*, 303–315.

2.5 Programmed Cell Death in Insect Neuromuscular Systems during Metamorphosis

S E Fahrbach, Wake Forest University, Winston-Salem, NC, USA
J R Nambu and L M Schwartz, University of Massachusetts, Amherst, MA, USA

2.5.1. Introduction

Programmed cell death (PCD) is a fundamental component of the postembryonic development of the neuromuscular systems of holometabolous insects (see **Chapters 1.10–1.12**, **2.2**, and **2.3**). Nerve and muscle cells born during embryogenesis are deleted in a segment- and cell-specific fashion at both the larval–pupal and pupal–adult transitions. Although such deaths presumably occur in all insects with complete metamorphosis, almost all of the information on this topic has been obtained from the study of two species, the fruit fly *Drosophila melanogaster* and the tobacco hawkmoth *Manduca sexta*. The sophistication of the genetic tools available for manipulating and analyzing *Drosophila* has allowed many of the key regulatory events in cell death to be identified in this model. In addition, the success of large-scale genetic screens for embryonic pattern formation as well as detailed analysis of Hox gene clusters (see **Chapter 1.8**) has provided tremendous insight into the mechanisms underlying specification of cell populations, elaboration of body axes, and organogenesis. These insights into early development provide a strong base for our understanding of metamorphosis. The small size of fruit flies, however, limits the utility of *Drosophila* for physiological and biochemical studies involving hormone manipulations, tissue transplantation, and surgical interventions. The lepidopteran *M. sexta* is an alternative model that is uniquely suited for these sorts of experimental approaches and serves as the insect equivalent of the laboratory white rat (Fahrbach, 1997).

Most investigations have focused on the regulation of PCD by hormonal signals or the molecular mechanisms of developmental cell death (see

Chapter 2.4). In the first sections of this chapter, the history of this field is reviewed, and then an overview of the *Manduca* and *Drosophila* models is presented. The following sections describe the endocrine control of cell death, and the molecular, physiological, and genetic mechanisms that bring about the controlled loss of these cells. A final section presents unresolved issues. An unusual feature of this chapter will be the parallel presentation of information on neuronal and muscle death during metamorphosis. The aim of this chapter is to highlight the features of PCD common to both tissues without obscuring tissue-specific features.

2.5.2. PCD, Apoptosis, Autophagy, or Necrosis?

It is now widely recognized that cell death is a normal part of development in all multicellular and some single-celled organisms (Ameisen, 2002). The capacity to remove selected cells during development provides organisms with a plastic response to many developmental contingencies. In vertebrates, PCD has been demonstrated to:

1. Match the sizes of interacting populations of cells, such as oligodendrocytes, to the axons they myelinate (Barres *et al*., 1992).
2. Remove deleterious cells, such as self-reactive T cells (Smith *et al*., 1989).
3. Sculpt the body, such as in the loss of interdigital cells in the fetal hand (Zuzarte-Luis and Hurle, 2002).
4. Remove "obsolete" cells, such as the tail of the tadpole (Yoshizato, 1996).

In insects, the best-characterized examples of PCD take place not only during embryogenesis, but also during metamorphosis in holometabolous insects, when larval tissues are destroyed to allow the formation of new structures in the adult. PCD during insect development, therefore, provides specific examples of a widespread phenomenon rather than being unique to Arthropoda.

2.5.2.1. Apoptosis

In both vertebrates and insects, dying cells can display one of several distinct morphologies. The most widely displayed and best-characterized morphology associated with both developmental and pathological cell death is apoptosis, a term that was coined by Kerr, Currie, and Wyllie (Kerr *et al*., 1972). As introduced, apoptosis was a purely morphological description that implied neither an underlying mechanism nor a specific developmental context. During the process of apoptosis, cells shrink and display dramatic plasma membrane zeosis, during which numerous protuberances or "blebs" are formed. Time-lapse photographs of cells undergoing apoptosis in culture look like drops of water skittering on a hot skillet. Under normal conditions *in vivo*, these cells are rapidly phagocytosed either by neighboring cells or by macrophage-like phagocytes (Platt *et al*., 1998; Savill and Fadok, 2000; Fadok and Chimini, 2001; Henson *et al*., 2001; Franc, 2002; Geske *et al*., 2002).

The nucleus of apoptotic cells condenses, and the chromatin in these cells becomes electron dense and marginated along the inner aspect of the nuclear envelope. These morphological changes are the physical manifestation of a massive cleavage of genomic DNA that occurs when endogenous nucleases become activated and cleave the linker DNA between individual nucleosomes (Wyllie *et al*., 1984; Enari *et al*., 1998). The fragmentation of the genome can be visualized when DNA is extracted and fractionated by size in agarose (Eastman, 1995).

It is sometimes difficult to detect these "apoptotic ladders" because dying cells are usually intermingled with healthy ones. In addition, as tissues are homogenized during DNA isolation, it is impossible to determine which subpopulation of cells within a tissue contributed to the degraded DNA. An alternative strategy for detecting cells with fragmented genomic DNA is to employ *in situ* labeling techniques on tissue sections such as terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling, commonly referred to as the TUNEL method (Gavrieli *et al*., 1992; Ben-Sasson *et al*., 1995).

2.5.2.2. Autophagy

While apoptosis is the morphology that is most commonly observed during PCD, particularly during embryogenesis, it is not the only one. Other suicidal cell deaths are designated as autophagic or type II degeneration (Clarke, 1990; Nixon and Cataldo, 1993; Bursch, 2001). Cells undergoing autophagy lack, in general, an initial condensation of chromatin and are instead characterized by the formation of autophagic bodies within the cytoplasm. Unlike apoptosis, there are no definitive markers visible using light microscopy that define autophagic cell death. This has limited the analysis of autophagy during development.

2.5.2.3. PCD is an Inclusive Term

The term PCD encompasses both apoptotic and autophagic cell deaths that occur as a normal part of development. The term PCD is used in this

chapter, unless the death of a uniquely identifiable cell at a particular developmental stage has been clearly defined as being either apoptotic or autophagic based on ultrastructural criteria. The distinction is more than semantic, as the evidence argues strongly that there are multiple molecular mechanisms of nonpathological cell deaths (Schwartz *et al.*, 1993; Bursch *et al.*, 2000; Jones and Schwartz, 2001; Thummel, 2001). Occasional confusion has arisen in the literature because autophagy and apoptosis can sometimes share features, such as caspase activation and positive TUNEL staining (Ben-Sasson *et al.*, 1995; Kinch *et al.*, 2003); also, relatively few studies have investigated the ultrastructure of dying insect cells, which is required for the detection of autophagic bodies.

2.5.2.4. Necrosis

While apoptosis and autophagy are carefully orchestrated developmental decisions, all cells can be induced to die by necrosis, a passive form of cell death that occurs in the absence of normal physiological responses. Necrosis can be induced in any cell following external insult such as heat, salt, abrasion, toxin exposure, etc. (Dive *et al.*, 1992; Kroemer *et al.*, 1998). Necrosis typically follows direct or indirect disruption of plasma membrane integrity, which in turn results in an influx of ions and water and subsequent cell swelling and lysis. In vertebrates, which have an adaptive immune response, necrosis provides a valuable warning to the immune system that focal injury has occurred, because the cellular constituents that are liberated during necrosis are highly inflammatory (MacDonald and Stoodley, 1998; Fadok *et al.*, 2001). In fact, endogenous responses to necrosis are responsible for much of the secondary tissue damage that accompanies injury.

2.5.3. Historical Overview and Current Trends

The first morphological description of PCD during insect metamorphosis of which we are aware was a description of the death of intersegmental muscles (ISMs) provided in 1936 by Kuwana working with the silkmoth *Bombyx mori* (Kuwana, 1936). The phenomenon of ISM death was largely unknown in the West, until it was independently described by Finlayson (1956). With the exception of a modest number of descriptive papers documenting examples of cell death in other insect taxa, little was published about cell loss during arthropod development for most of the subsequent decade. (A compendium of the early studies of cell death in insects can be found in Glücksmann's (1951) famous but now largely forgotten review of the field of cell death.) A multifaceted analysis of PCD based on analysis of the ISMs was initiated in the mid-1960s by Richard Lockshin and Caroll Williams (Lockshin and Williams, 1964, 1965a, 1965b, 1965c). In fact, it was in these early reports that the term "programmed cell death" was first introduced into the literature (Lockshin, 1963).

The first detailed description of neuronal death during insect metamorphosis, published in 1974, was a description of the postembryonic changes in neuronal populations observed in the abdominal ganglia of *M. sexta* (Taylor and Truman, 1974). James Truman and his colleagues exploited their ability to count the motoneurons in individual ganglia by backfilling the cut ends of peripheral nerves with cobalt; they reported that motoneurons were lost from the abdominal ganglia at both the larval–pupal and pupal–adult transitions. Many of these motoneurons were later identified as specific individuals, permitting the precisely regulated nature of metamorphic neuron death to be established (Truman, 1983; Levine and Truman, 1985).

Studies by Schwartz and Truman subsequently demonstrated that the changes associated with ISM development and death in *Manduca* at the end of metamorphosis are controlled by hormonal signals, particularly by the steroid hormone 20-hydroxyecdysone (20E) (Schwartz and Truman, 1982, 1983) (see **Chapter 2.4**). This principle of steroid control of nerve and muscle fate at the metamorphic transitions was extended to the death of abdominal neurons that occurs after adult eclosion in *Manduca* (Truman and Schwartz, 1984) and to the death of *Manduca* proleg motoneurons and their associated muscles at pupation (Weeks and Truman, 1985; Weeks, 1987, 1999). Studies of changes in the pattern of gene expression in dying ISMs pioneered investigations of the molecular mechanisms of PCD in insects (Schwartz *et al.*, 1990a).

Descriptive studies of the death of neurons and muscles after adult eclosion in *D. melanogaster* (Kimura and Truman, 1990) foreshadowed the initiation in the 1990s of an intensive genetic analysis of PCD in fruit flies (reviews: McCall and Steller, 1997; Wing and Nambu, 1998a; Abrams, 1999; Bangs and White, 2000; Vernooy *et al.*, 2000). Efforts have focused primarily on the elucidation of the intracellular machinery of cellular suicide and on extracellular signals that trigger death in specific target cells. Two important current trends are the use of genomic approaches to identify genes associated with different forms of PCD, and the

development of *Drosophila* models of human neurodegenerative disease (Mutsuddi and Nambu, 1998; Gorski and Marra, 2002; Muqit and Feany, 2002; Driscoll and Gerstbrein, 2003; Lee *et al*., 2003). The underpinning of this work is the growing consensus within the field of cell death studies that mechanisms of cell death are broadly conserved across phylogeny (Yuan *et al*., 1993; Steller, 1995; Jacobson *et al*., 1997; Tittel and Steller, 2000; Vernooy *et al*., 2000) (see Section 2.5.5.4.1). Studies of muscle and nerve cell death in insects are, therefore, well positioned to address specific questions concerning metamorphosis, basic questions in cell biology, and topics of direct relevance to human health.

2.5.3.1. Earlier Reviews of PCD Relevant to Insects

The following previously published reviews provide essential background on PCD of insect nerve and muscle cells during metamorphosis: Truman *et al.* (1990, 1992), Schwartz (1992), and Weeks (1999). PCD in other insect tissues during development is reviewed in other chapters in this volume (see **Chapters 2.9** and **2.10**). Reviews covering the discovery of the first genes associated with PCD in *D. melanogaster* are found in McCall and Steller (1997), Wing and Nambu (1998a), Abrams (1999), Bangs and White (2000), and Vernooy *et al.* (2000). Autophagic and apoptotic cell deaths during *Drosophila* metamorphosis are compared in Thummel (2001); and a recent review of *Drosophila* as a model system for the study of PCD is found in Richardson and Kumar (2002). Overviews of PCD during animal development in general are provided in Milligan and Schwartz (1997), Jacobson *et al.* (1997), Meier *et al*. (2000), and Baehrecke (2002).

2.5.4. The *Manduca* Model

2.5.4.1. Choice of *Manduca sexta* as a Model System for the Study of PCD of Nerve and Muscle Cells

Insect endocrinologists and neurobiologists have long favored *M. sexta* (Lepidoptera: Sphingidae) because of its large size and ease of rearing in culture on artificial diet (Bell and Joachim, 1976; Arnett, 1993; Fahrbach, 1997). This species has a facultative rather than an obligatory diapause and, therefore, all life stages can be produced in the laboratory at any time of year. Development from egg to adult requires roughly 40 days. There are five successive larval stages (each referred to as an instar, so that the final larval stage is the fifth instar) and a single pupal stage. The adult lives approximately 10 days. The relatively large size of this insect in all life stages permits extensive surgical and endocrine manipulations. The central nervous system (CNS) consists of a dorsal brain and a ventral nerve cord. The abdominal portion of the ventral nerve cord retains its segmental organization of discrete thoracic and abdominal ganglia throughout the postembryonic period, a feature that facilitates anatomical, electrophysiological, and functional analyses.

The thoracic and abdominal musculature of *M. sexta* has also been fully described (Eaton, 1988). Importantly, many aspects of neuromuscular metamorphosis first described in *Manduca* have now also been identified in *Drosophila* (e.g., Kimura and Truman, 1990; Truman *et al*., 1994), despite evidence that the insect orders of Lepidoptera and Diptera have been evolving independently for at least 200 and, possibly, 300 million years (Hoy, 1994). This suggests that many aspects of neuromuscular metamorphosis, including PCD, are ancient in origin and highly conserved. This relationship permits phenomena first observed in *Manduca* to be subjected to indirect molecular genetic analysis by shifting to *Drosophila* for follow-up studies (e.g., Robinow *et al*., 1993). Conversely, orthologs of genes first identified in *Drosophila* can be cloned in *Manduca* and their developmental expression patterns determined (e.g., Nagy *et al*., 1991; Kraft and Jäckle, 1994).

The striking changes in the organization of the nervous and muscular systems that accompany lepidopteran metamorphosis result from a combination of postembryonic cell proliferation, modification of structures formed initially in the embryo, and PCD. While cell death is typically restricted to small populations of cells in the CNS, wholesale loss of entire bundles of muscle fibers in the periphery is more usual (Schwartz, 1992; Bayline *et al*., 1998). In fact, the coordinated loss of the ISMs following adult eclosion in *Manduca* offers a nearly ideal system for the study of PCD, because these cells are exceptionally large, easily accessible throughout the entire process of degeneration, and uncontaminated by persisting muscle fibers (**Figure 1**).

The ISMs of *Manduca* are composed of giant, syncytial fibers approximately 5 mm long and 1 mm in diameter. These embryonically derived cells are used by the larva for locomotion and by the pupa for defensive and respiratory behaviors. The ISMs also perform the major abdominal movements required for the eclosion behavior of the adult moth. The ISMs begin to atrophy at 3 days prior to adult eclosion, which results in a loss of 40% of muscle mass, although the ability to contract is maintained during this early phase (Schwartz and

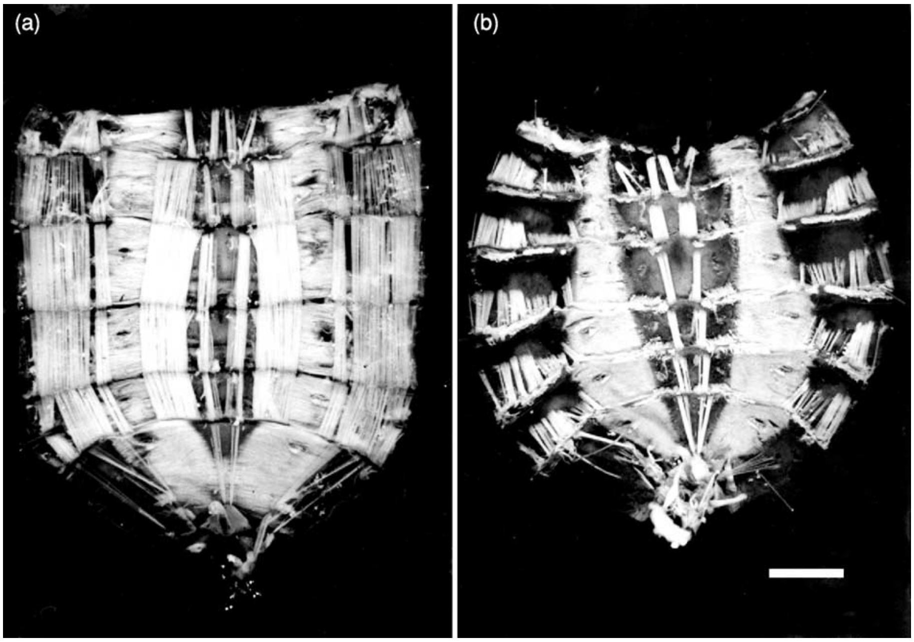


Figure 1 Loss of the intersegmental muscles (ISMs) following adult eclosion in *Manduca sexta*. These photographs display the abdominal musculature from animals (a) before and (b) 36 h after adult eclosion. The ISMs are the large vertical muscle fibers seen prior to eclosion. The small oblique muscles present after eclosion are external muscles that form during adult development and persist for the life span of the adult. Scale bar = 5 mm. (Adapted from Schwartz, L.M. **1992**. Insect muscle as a model for programmed cell death. *J. Neurobiol. 23*, 1312–1326.)

Ruff, 2002). Once the ISMs have participated in eclosion, they are no longer required for any adult behaviors and die during the subsequent 30 h (Schwartz, 1992). As described below (see Section 2.5.4.5), studies of the ISM death of *Manduca* have provided numerous insights into the cellular and molecular mechanisms of PCD.

2.5.4.2. Hormonal Regulation of Metamorphosis

As in all insects, metamorphosis in *Manduca* is regulated by two categories of developmental hormones, ecdysteroids and juvenoids (Truman and Riddiford, 2002; Nijhout, 1994) (see **Chapters 3.1**, **3.5**, and **3.7**). The ecdysteroids (see **Chapters 3.3** and **3.4**) are steroid hormones that exert their primary actions through members of the nuclear hormone receptor superfamily of proteins (Robinson-Rechavi *et al*., 2003) (see **Chapters 3.5** and **3.6**). The juvenile hormones are terpinoids (Nijhout, 1994) (see **Chapter 3.7**). At present, the cellular mode of action of juvenile hormones in metamorphosis remains to be defined and possibly involves more than one signaling pathway (Gilbert *et al*., 2000; Jones and Jones, 2000). Experiments on the hormonal regulation of neuromuscular metamorphosis in *Manduca* have clearly established that the changes in cell populations and connectivity that occur during neuromuscular metamorphosis are controlled by the direct actions of ecdysteroids and juvenile hormones on neurons, glia, and muscle (Bennett and Truman, 1985; Streichert *et al*., 1997) (see **Chapter 2.4**). Receptors for ecdysteroids have been localized to neuronal, glial, and muscle cell nuclei in *Manduca* (Bidmon and Koolman, 1989; Fahrbach and Truman, 1989; Bidmon and Sliter, 1990; Bidmon *et al*., 1992; Hegstrom *et al*., 1998; Fahrbach, 1992) (see **Chapter 2.4**).

2.5.4.3. PCD of Neurons during Metamorphosis

2.5.4.3.1. Background and overview Neuronal death during metamorphosis in *Manduca* involves motoneurons, interneurons, and identified peptidergic neurons. Most studies have focused on the death of motoneurons not only because of the greater ease of identification of specific neurons across individuals, but also because the highly visible degeneration of muscles during postembryonic development often suggests that innervating motoneurons will be lost, so that investigators in effect "know where to look." The presence of sexually dimorphic structures in the adult (see **Chapter 1.1**), such as the oviduct, also often suggests where PCD will be found in the CNS (Giebultowicz and Truman, 1984; Thorn and Truman, 1989). Dying peptidergic neurons can be identified after the detection of stage-specific patterns of antibody staining (Ewer *et al*., 1998). Cell counts and the presence of small pycnotic (shrunken) profiles often provide the only

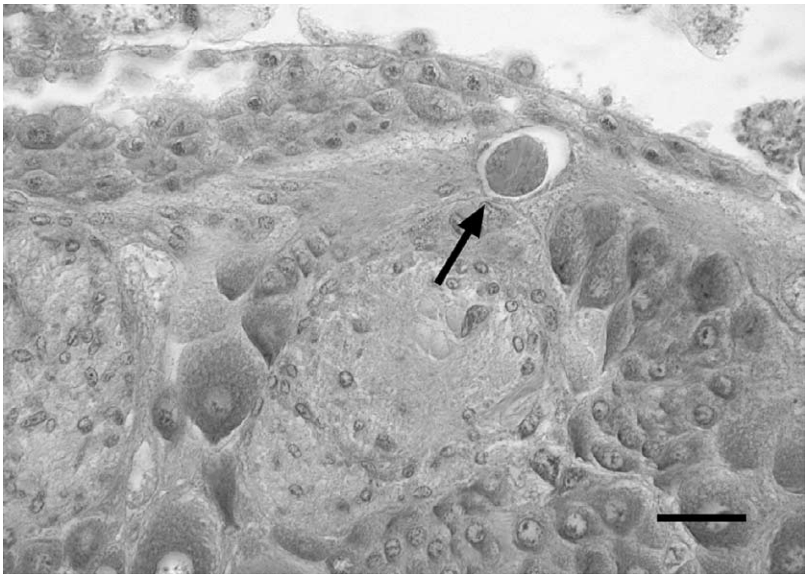


Figure 2 Transverse section (8 μm) through the first thoracic (prothoracic or T1) ganglion of the ventral nerve cord of *Manduca sexta* fixed in alcoholic Bouins 72 h after pupal ecdysis. After fixation, the ganglion was dehydrated, embedded in a paraffin-based medium, sectioned, and stained with hematoxylin and eosin. The arrow points to the shrunken, condensed profile of a motoneuron that has undergone programmed cell death (PCD). Because of the absence of phagocytosis in the ventral nerve cord during metamorphosis, the persisting fragments of such neurons are easily detected in insect nervous tissue prepared using routine histological techniques. Scale bar = 30 μm.

anatomical clues that interneurons have died, although the impact of such deaths on the function of neural circuits must be profound (Truman, 1983). The observation that the corpses of many dying neurons are not phagocytosed and therefore persist in the ganglia facilitates the identification and analysis of uniquely identified cells or populations well after the initiation of the PCD program.

Patterns of neuronal death during metamorphosis in *Manduca* have been mostly fully described for the motoneurons of the abdominal ganglia, although histological surveys of thoracic ganglia reveal that the PCD of neurons is also found in this tissue at the metamorphic transitions (S.E. Fahrbach, unpublished data) (**Figure 2**). At the larval–pupal transition, the best-studied examples of neuronal death are the proleg motoneurons (Weeks and Truman, 1985; Weeks, 1999). Studies of neuronal death associated with adult eclosion have focused primarily on the death of motoneurons (including the death of proleg motoneurons that persist after pupation) in the unfused abdominal ganglia, A3 through A5. In addition to the deaths of fully differentiated motoneurons and peptidergic interneurons, deaths of undifferentiated neurons occur in the imaginal nests of the segmental ganglia, both as the larvae feed and at the onset of metamorphosis (Giebultowicz and Truman, 1984; Booker and Truman, 1987a; Thorn and Truman, 1989; Booker *et al*., 1996). The PCD of neuroblasts that are active during postembryonic life terminates proliferation in specific lineages in both the segmental ganglia and the brain: in *Manduca*, the death of neuroblasts has been most comprehensively studied in the segmental ganglia (Booker and Truman, 1987a; Booker *et al*., 1996).

The emphasis on PCD of neurons at the larval–pupal and pupal–adult transitions in *Manduca* should not obscure the fact that the majority of larval neurons survive metamorphosis and become integral components of the adult nervous system, where they interact with other neurons derived from embryonic and postembryonic neurogenesis.

2.5.4.3.2. PCD of abdominal motoneurons during metamorphosis in *M. sexta* With rare exceptions (see Section 2.5.3.1), the abdominal motoneurons of *Manduca* are born during embryonic life. At both the larval–pupal and the pupal–adult metamorphic transitions, some of the larval motoneurons lose their muscle targets as a consequence of changes in the organization of the skeletal musculature (Levine and Truman, 1985). At the larval–pupal transition, some of these now targetless motoneurons die. Examples of motoneurons that undergo PCD at this time include some of the motoneurons that innervate muscles associated with the five pairs of abdominal prolegs, the locomotory appendages of the caterpillar that disappear at pupation (Weeks and Truman, 1985). In some segments however, the proleg motoneurons survive this round of PCD,

and after a period of dendritic regression, acquire new muscle targets in the developing adult (Weeks and Ernst-Utzschneider, 1989; Weeks *et al*., 1992). Targetless motoneurons, can therefore, either undergo PCD or become respecified. Experimental evidence suggests that the death of these motoneurons is triggered by endocrine cues independent of contact with target muscles (Bennett and Truman, 1985; Weeks and Truman, 1985). Abdominal motoneurons that lose their muscle targets after adult eclosion all die within 48 h of emergence, as the short (approximately 10 day) life of the adult apparently does not provide sufficient opportunity for respecification. It should be noted that unpublished observations from studies on the giant silkmoth *Antheraea pernyi* (J.W. Truman) and *Hyalophora cecropia* (S.E. Fahrbach) suggest that, in these species, motoneurons persist well after the death of the ISMs and, therefore, neither respecify nor die. This suggests that these motoneurons do not play out a hormonally triggered program of PCD and that, in contrast to many vertebrate motoneurons, their survival is not dependent on target-derived trophic signals. Comparative studies on a broader range of insect taxa are needed to complete our understanding of neuronal PCD following adult eclosion.

2.5.4.3.3. Sex-specific PCD of abdominal motoneurons during metamorphosis The populations of abdominal motoneurons are the same in male and female larvae, but during metamorphosis the genital segments undergo sex-specific morphological changes that are accompanied by a wave of sex-specific PCD in the ganglia that innervate these segments (Giebultowicz and Truman, 1984; Thorn and Truman, 1989). This is an example of equal opportunity sexual differentiation, as some motoneurons persist in males but degenerate in females, while others show the opposite pattern (Thorn and Truman, 1989). There is also a difference in the timing of sex-specific PCD relative to pupation, with most neurons in females dying during the first 2 days following pupation, while most neurons in males die during the third to the sixth day after pupation (Thorn and Truman, 1994a).

A particularly interesting example of sex-specific neuronal death in *Manduca* is seen in the case of some of the imaginal midline neurons (IMNs), which are highly unusual motoneurons, because they are born postembryonically during the fourth (penultimate) larval instar (Thorn and Truman, 1994a). These motoneurons innervate visceral rather than skeletal musculature, and some of them can be tracked during the larval–pupal transition, because they are immunoreactive with an antibody directed against molluscan small cardioactive peptide b (Thorn and Truman, 1994b). IMNs that innervate the sperm duct in males are absent from the terminal ganglia of females, while IMNs that innervate the oviduct in females are absent from the terminal ganglia of males. There is some evidence that contact with an appropriate target enhances the survival of the IMNs that innervate the sperm duct in males, another quite atypical aspect of their physiology (Thorn and Truman, 1994b).

2.5.4.3.4. PCD of identified peptidergic neurons during metamorphosis Two identified interneurons (INs) that contain crustacean cardioactive peptide (CCAP), cell 27 and IN 704, have been shown to undergo PCD within 36 h of adult eclosion (Ewer *et al*., 1998). Both cell 27 and IN 704 display increases in cGMP immunoreactivity during larval ecdyses, and cell 27 also shows this response at pupal ecdysis and at adult eclosion (Ewer *et al*., 1994; Ewer and Truman, 1997). Because application of CCAP to the isolated CNS can trigger the motor patterns of ecdysis, this set of neurons appears to be central to the control of molting behavior (Gammie and Truman, 1997; Mesce and Fahrbach, 2002). The death of peptidergic neurons involved in the control of ecdysis behavior, after the moth's molting career is finished, supports the hypothesis that obsolescent neurons are actively eliminated from the *Manduca* nervous system.

2.5.4.3.5. PCD in the brain during metamorphosis Detailed studies of specific regions of the *Manduca* brain have clearly demonstrated that newly generated neurons die during adult development. PCD is a prominent feature of the development of both the medulla and lamina cortices in the optic lobes (Monsma and Booker, 1996a). Both developmental hormones and retinal afferents appear to regulate this process (Monsma and Booker, 1996b). The extent of PCD in other regions of the developing *Manduca* brain is largely unstudied. A general role for ingrowing sensory afferents in the regulation of neuronal survival is suggested by the sex differences that arise in populations of antennal lobe interneurons, as a result of sexual dimorphisms in the antennae (Schneiderman *et al*., 1982).

2.5.4.3.6. PCD of imaginal nest cells during metamorphosis Postembryonic neurogenesis is a major source of adult populations of neurons in holometabolous insects, yet a curious aspect of this phenomenon is that many neurons born during the postembryonic period die before they can differentiate. New neurons, which accumulate in nests

around their parent neuroblast, die in a nest- and segment-specific pattern during the feeding larval stages and the onset of metamorphosis (Booker *et al.*, 1996). Studies using bromodeoxyuridine as a marker for mitosis have produced estimates that between a third and 70% of imaginal neurons die within 24 h of their birth (Booker *et al.*, 1996). There is some evidence that the death of imaginal neurons and their associated neuroblasts in *Manduca* is regulated by developmental hormones (Booker and Truman, 1987b).

2.5.4.4. Regulation of PCD in the Nervous System during Metamorphosis

Neuronal death during insect metamorphosis has been described as hormone dependent, segment specific, and target independent (Truman *et al.*, 1992). Considerable experimental evidence supports each of these claims, although, in some special cases, a role for cell–cell interactions is implicit in the data.

The ecdysteroids are important regulators of PCD in the nervous system, acting to couple neuronal death with other metamorphic changes. It is important to note that the ecdysteroid cue for triggering metamorphic neuronal death can be either a rising or a falling titer, depending upon developmental stage. For example, the decline in circulating levels of 20E that occurs at the end of adult development prior to adult eclosion is the cue for the death of abdominal motoneurons at this time, and treatment with exogenous ecdysteroid at this time blocks the initiation of PCD (Truman and Schwartz, 1984). By contrast, it is the prepupal rise in circulating ecdysteroids that is responsible for the larval–pupal transition death of the motoneurons in abdominal ganglia A5 and A6, which innervate the accessory plant retractor muscle of the prolegs (Weeks and Ernst-Utzschneider, 1989; Weeks *et al.*, 1992; Zee and Weeks, 2001). The response of these proleg motoneurons to the steroid signal, however, is segment specific. Homologous neurons in abdominal ganglia A3 and A4 persist through the pupal stage and adult development, but then undergo PCD within 24 h of adult eclosion (Zee and Weeks, 2001). These responses to 20E, as well as the segment specificity of the response at different stages in development, are retained when individual proleg motoneurons are cultured *in vitro*, providing strong evidence for the cell-autonomous, target-independent nature of these PCDs (Streichert *et al.*, 1997; Hoffman and Weeks, 1998).

It is to be assumed that ecdysteroids exert their control on the timing of PCD by means of transcriptional regulation, but the target genes for steroid hormone action have not been identified in *Manduca*. In addition, the tissues of *Manduca* and other insects express several different isoforms of the ecdysone receptor, which may have varying relationships to cell fate. In *Drosophila*, the expression of the A isoform of the ecdysone receptor (EcR-A) has been directly correlated with the occurrence of post-eclosion neuronal death in the CNS (Robinow *et al.*, 1993). Whether or not a similar relationship prevails in *Manduca* neurons remains to be determined, although autoradiographic evidence has demonstrated that *Manduca* motoneurons fated to die at the start of adult life display nuclear concentration of radiolabeled ecdysteroids (Fahrbach and Truman, 1989).

Evidence that other signals may fine-tune the timing of the death of neurons during metamorphosis comes from several sources. Adult *M. sexta* emerge from their pupal cuticle in an underground pupation chamber, and must then dig to the surface before inflating their wings. Adult moths forced to continue digging hours beyond the time this behavior would normally cease, exhibited delayed death of abdominal motoneurons (Truman, 1983). In addition, transection of the ventral nerve cord, prior to adult eclosion, blocks the death of specific motoneurons after adult eclosion in ganglia posterior to the point of transection, even in moths in which the levels of 20E undergo their normal decline (Fahrbach and Truman, 1987).

A well-studied example of a spared abdominal motoneuron is MN-12 (**Figure 3**). Subsequent to ventral nerve cord transection, this supernumerary member of the adult abdominal ganglion maintains both its normal central arborizations and electrophysiological properties, implying that the cell death program has been completely blocked in the absence of a descending signal (Fahrbach *et al.*, 1995; DeLorme and Mesce, 1999). Treatment of cultured abdominal ganglia with extracts prepared from ventral nerve cord restores the normal pattern of cell death to MN-12, but the active factor in the extracts remains to be identified (Choi and Fahrbach, 1995). Other examples of motoneuron death, such as the death of the accessory planta refractors (APRs) at the larval–pupal transition, however, are unaffected by cutting of the connectives prior to the normal time of death (Weeks and Davidson, 1994). This suggests that the phenomenon of interganglionic cell death signaling affects only a limited set of cells.

Because of the scattered, episodic nature of neuronal death during metamorphosis, and the unavailability of transgenic *Manduca* for analysis, relatively little is known about the molecular mechanisms of neuronal death in this species. Hormone-dependent neuronal death is blocked by treatment with inhibitors of transcription or translation,

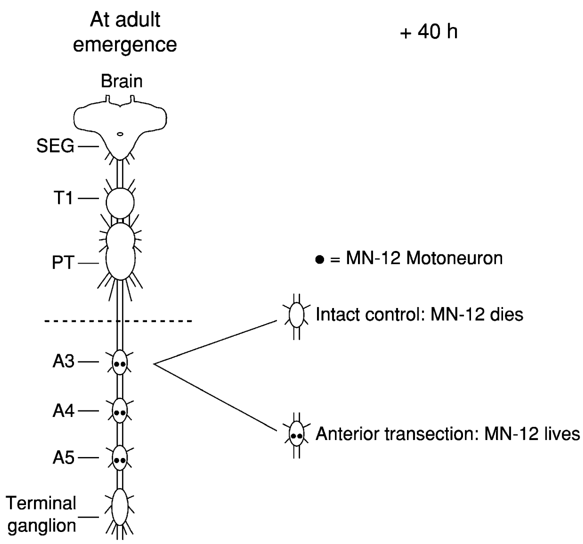


Figure 3 Schematic diagram of the central nervous system of an adult *Manduca sexta* moth. The MN-12 motoneurons in the abdominal ganglia normally undergo programmed cell death (PCD) by 40 h after adult emergence (eclosion), but are spared this fate if connections to the anterior nervous system are severed just posterior to the pterothoracic ganglion (Fahrbach and Truman, 1987). The dashed line indicates the point of anterior nerve cord transection. A3, A4, and A5, abdominal ganglia; PT, pterothoracic ganglion; SEG, subesophageal ganglion; T1, prothoracic ganglion.

suggesting the existence of an active gene-mediated cell death program (Weeks *et al.*, 1993; Fahrbach *et al.*, 1994; Ewer *et al.*, 1998; Hoffman and Weeks, 1998). In support of the hypothesis that PCD of *Manduca* neurons requires protein synthesis, a two-dimensional polyacrylamide gel electrophoresis analysis of protein patterns in the *Manduca* ventral nerve cord revealed changes associated with programmed neuronal death at adult eclosion (Montemayor *et al.*, 1990). Mitochondrial involvement in the death of APRs has been shown by studies in which inhibition of caspase activity blocked the death of cultured proleg motoneurons (Hoffman and Weeks, 2001). Ultrastructural studies of dying *Manduca* neurons are uncommon, but studies of the motoneurons that innervate the ISMs and of APR motoneurons indicate that neuron death in *Manduca* during metamorphosis is autophagic rather than apoptotic (Stocker *et al.*, 1978; Kinch *et al.*, 2003).

Immunostaining studies, in which the distribution of several death-associated gene products (initially identified from a screen of dying moth muscle; see Section 2.5.5.4.3) was examined in the segmental ganglia, failed to provide a reliable correlation of enhanced ubiquitin- or multicatalytic proteinase-immunoreactivity within dying neurons (Fahrbach and Schwartz, 1994; Hashimoto *et al.*, 1996), despite an association of these gene products with PCD in insect skeletal muscles (Jones *et al.*, 1995; Haas *et al.*, 1995). This discrepancy in gene expression during PCD in neurons and muscles remains unexplained. By contrast, one gene product, expressed by both dying neurons and degenerating muscles in *Manduca*, is apolipophorin III, a finding that suggests that this molecule has functions in addition to its role in lipid transport (Sun *et al.*, 1995).

2.5.4.5. PCD of Muscles during Metamorphosis

The ISMs of *Manduca* (**Figure 1**) are the major abdominal muscles of the larva, pupa, and pharate adult. The ISMs are divided into three separate pairs of bilaterally symmetric bundles, each of which attaches to the cuticle at the intersegmental boundaries. These muscles initially form in the embryo and span eight of the abdominal segments in the larva. The ISMs provide the major propulsive force for both hatching and subsequent larval locomotion. Following pupation, the muscles in the first two and last two abdominal segments die and rapidly disappear. The muscles in the middle four segments persist throughout metamorphosis and are used for the defensive and respiratory movements of the pupa. Following adult eclosion, these remaining ISMs undergo PCD and are lost during the subsequent 30 h. While the molecular basis for this segmental fate determination has not been examined, presumably it is established early in embryogenesis due to the actions of gap, pair-rule, and segment-polarity genes (Bejsovsec and Wieschaus, 1993; DiNardo *et al.*, 1994; French, 2001; Sanson, 2001).

The nuclear changes that accompany ISM death display none of the features of apoptosis (Schwartz *et al.*, 1993) (**Figure 4**). The chromatin does not become electron dense but remains dispersed throughout the nucleoplasm. In addition, agarose gel electrophoresis of ISM genomic DNA fails to reveal apoptotic ladders, although activation of DNases may occur as a very late event, after membrane integrity has been lost. Ultrastructurally, there is an increase in autophagic vesicles, and these cells are throught to die by autophagy (Lockshin and Beaulaton, 1974, 1979).

Following PCD in most organisms, the cell corpse is phagocytosed by neighboring cells or circulating macrophage-like cells (see Section 2.5.2.1). A classic example of this phenomenon is found in amphibian metamorphosis, where the massive tail musculature is lost during the short transition from larva to adult (Weber, 1964; Watanabe and Sassaki, 1974). During this process, the muscle fibers become

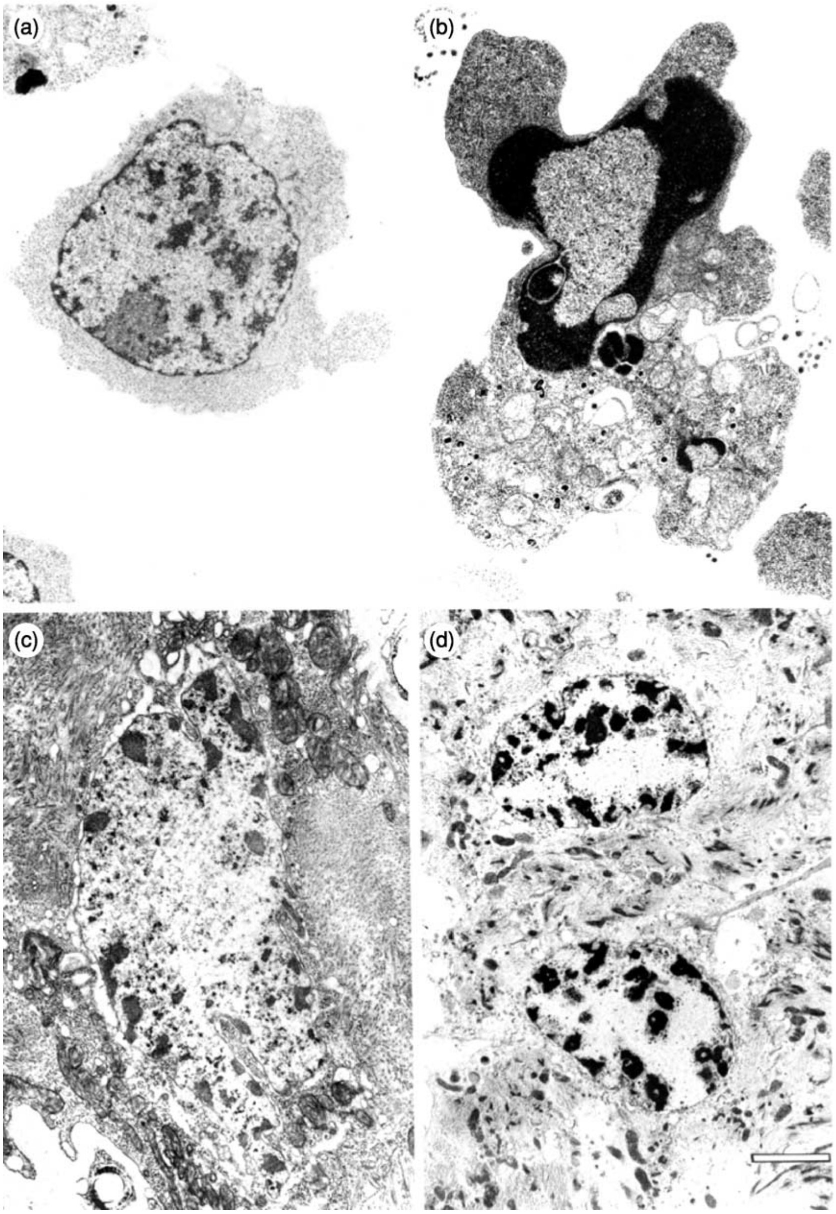


Figure 4 Transmission electron micrographs comparing the morphology of mouse lymphocyte apoptosis (a and b) with the autophagic death of the ISMs of *Manduca* (c and d). The mouse T cell hybrid cell line D011.10 was induced to die with the glucocorticoid dexamethasone, and examined either at time zero (a) or 16 h after exposure (b). These cells die by apoptosis and display many of the classical features of the program, including membrane blebbing and the deposition of electron-dense chromatin along the inner surface of the nuclear envelope. These images contrast those of normal *Manduca* ISMs (in a day 16 pupa; c) and dying ISMs (at 18 h posteclosion; d). During autophagy, the nuclei round up and the chromatin remains dispersed in the nucleoplasm.

decorated with macrophages that, in turn, contain identifiable remnants of skeletal muscle debris (Metchnikoff, 1892; Nishikawa *et al*., 1998).

While dying muscles in insects are often phagocytosed by hemocytes (Crossley, 1968), this is not universally the case (Jones *et al*., 1978). In particular, the death of the ISMs, following adult eclosion in moths, does not attract macrophage-like cells or rely on phagocytosis for resolution (Beaulaton and Lockshin, 1977). Although this cannot be accounted for solely by trivial reasons, such as an absence of circulating macrophages capable of phagocytosing foreign bodies (Jones and Schwartz, 2001), estimates of ISM volumes and hemocyte numbers in adult *Manduca* suggest that animals would require at least an order of magnitude greater number of phagocytic cells than has been shown to reside in the hemolymph (Jones and Schwartz,

2001). While the ISMs of adult animals are not phagocytosed, some of the ISMs that die following pupation in *Manduca* appear to behave differently. Rheuben examined the death of mesothoracic muscles in pupae, and observed an intimate association between phagocytic hemocytes and the sarcolemma (Rheuben, 1992). The phagocytes were well spaced along the fibers and appeared to specifically degrade the basal lamina. One difference between the ISMs and the mesothoracic fibers is that the latter are not completely degraded during development. Instead, they act as scaffolds for myoblasts that remodel the fibers during the formation of adult muscle fibers. Phagocytes may play a more significant role in facilitating tissue remodeling, as opposed to cell death.

2.5.4.5.1. Endocrine control of ISM death The timing of ISM death must be precisely coordinated with other metamorphic events or the animal might suffer disastrous consequences. For example, premature loss of the ISMs in moths would leave the animal trapped within the pupal cuticle and locked in either a cocoon or an underground chamber. Delays in ISM death might also have deleterious consequences, possibly depriving the adult of essential nutrients required for gametogenesis.

As described above (see Section 2.5.4.4), the titer of ecdysteroids serves as an endogenous developmental time reference, that can be used by the different organs of the pupa to coordinate developmental decisions. Sequential declines in the ecdysteroid titer serve to coordinate developmental changes in the tissues, including the timing of ISM death (Schwartz and Truman, 1983).

Early reports identified the cessation of motor neuron activity as the proximal trigger for ISM death (Lockshin and Williams, 1965b). This hypothesis was subsequently proven incorrect, when it was found that normal timing of ISM death in *A. polyphemus* was not altered either by silencing motoneuron activity with the highly specific sodium channel blocker tetrodotoxin or by the removal of the entire ventral nerve cord (Schwartz and Truman, 1983, 1984b). Instead, it was shown that the peptide eclosion hormone (EH) acts to trigger ISM death in this species (Schwartz and Truman, 1984b; 1984a). The EH acts via cGMP, and the description of its role in ISM death represented the first study demonstrating that cGMP met all of Earl Sutherland's requirements for identifying a second messenger for a hormone action (Sutherland, 1972; Schwartz and Truman, 1984a). The capacity of EH to act on the ISMs is itself under the control of circulating ecdysteroids, as a decline in circulating 20E is required to determine both the timing of EH release and the developmental capacity of the muscles to respond to this trigger (Truman, 1984; Schwartz and Truman, 1984b).

2.5.4.5.2. Physiology of ISM death The size of the ISMs and the coordinated nature of the developmental changes that take place in this tissue during metamorphosis facilitate the examination of the physiological changes that accompany naturally occurring muscle atrophy and death. Under laboratory conditions, metamorphosis in *Manduca* takes 18 days, with adult eclosion taking place late on day 18. On day 15 of adult development, the mass of the ISMs begins to decline and, during the subsequent 3 days, ISMs lose 40% of their mass. This preeclosion program of atrophy is nonpathological, and the muscles retain virtually all of their normal physiological responses, including force/cross-sectional area and sensitivity to calcium ions in skinned fiber preparations. This suggests that the reduction in muscle mass, observed during the atrophy phase, reflects a generalized enhancement of protein turnover rather than a selective destruction of specific contractile proteins. Ultrastructural studies by Lockshin and Beaulaton (1979) have shown that entire contractile bundles are lost during this phase.

In *Manduca*, the ISMs begin the death program coincident with adult eclosion, late on day 18 after pupation, with the muscles beginning to lose mass at a rate of approximately $4\%\,h^{-1}$ (Schwartz and Truman, 1982). Concurrently, there is a slow, progressive depolarization of the fibers at a rate of $\sim 2.5\,mV\,h^{-1}$ (Runion and Pipa, 1970; Schwartz and Ruff, 2002). By 24 h posteclosion, reliable resting potentials can no longer be recorded. The retention of a resting potential during the active phase of ISM death verifies that the cells are not undergoing a necrotic process. While there is little change in the organization of the contractile apparatus during the atrophy phase, the postemergence period is marked by profound sarcomere disruption (Lockshin and Beaulaton, 1979). Whole filaments disappear rapidly, with a preferential loss of thick filaments relative to thin filaments (Beaulaton and Lockshin, 1977). During this same period, mitochondria are lost, autophagic vacuoles form, and the T tubule system swells.

Not surprisingly, there are physiological consequences that accompany these dramatic changes in ISM structure. The fibers rapidly weaken, even when force is normalized to cross-sectional area. This is true for twitches, and for tetanus and caffeine-induced contractions (Schwartz and Ruff, 2002). There are also defects in the ability of the

contractile apparatus to respond to free calcium in either intact muscles or skinned fiber preparations.

2.5.4.5.3. Patterns of gene expression during PCD of ISMs While ISM atrophy appears to involve an increase in protein catabolism over synthesis (Haas *et al.*, 1995), there is incontrovertible evidence that the posteclosion death of these cells involves the implementation of a new developmental program. Insights into the molecular mechanisms of ISM death were provided in 1969, when Lockshin reported that the death of silkmoth ISMs is blocked by inhibitors of RNA or protein synthesis (Lockshin, 1969). These results are similar to those reported for PCD in other insect tissues, including the nervous system (see Section 2.5.4.4), as well as other taxa such as amphibians (Weber, 1965). Taken together, these data provided some of the earliest evidence that the ability of cells to die required *de novo* gene expression.

To confirm and extend this hypothesis, Schwartz and colleagues made cDNA libraries from condemned, day 18 ISM RNA, and screened them for differentially expressed genes. While the vast majority of ISM genes were found to be constitutively expressed, a small number were observed to be either repressed or induced when the muscles became committed to die. The cloning of these new sequences from day 18 ISMs represented the first published report of death-associated gene expression changes from any organism (Schwartz *et al.*, 1990a). Subsequent characterization of these sequences has provided new information about the molecular mechanisms of PCD.

Among the genes that are repressed, when the ISMs become committed to die, are actin and myosin heavy chain (Schwartz *et al.*, 1993) (**Figure 5**). These transcripts are among the most abundant in the muscle throughout metamorphosis, but they begin to disappear late on the day before adult eclosion (day 17), coincident with the commitment to die. By the time of adult eclosion, they are almost undetectable on Northern blots. A single injection of 25 μg of 20E on day 17 is sufficient to delay both the ISM death on day 18 and the loss of these transcripts, thus linking this change in gene expression to the commitment of the muscles to die.

Given that most muscle genes are constitutively expressed during ISM atrophy and death, there must be some advantage to reducing selectively the

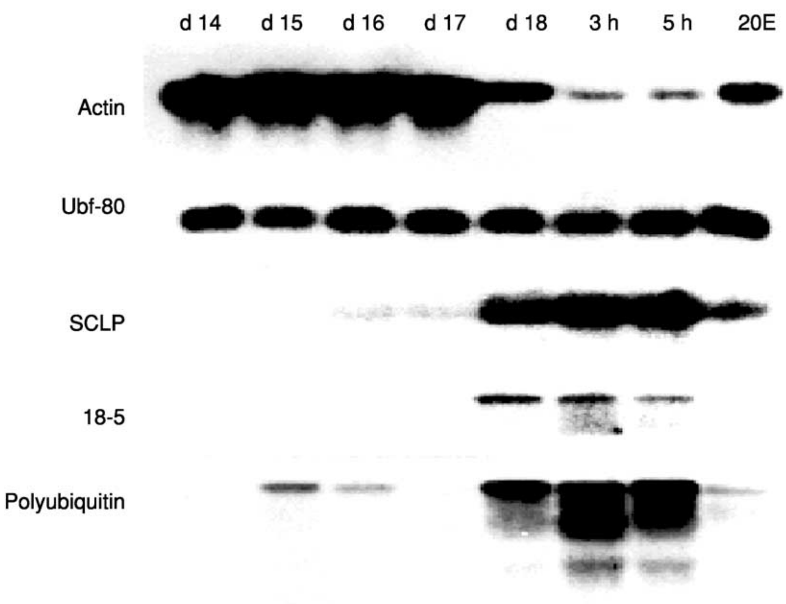


Figure 5 Changes in the patterns of gene expression during the atrophy and death of the intersegmental muscles in *Manduca*. RNA was isolated from the ISMs at the time indicated and fractionated by size in agarose. After transfer to a nylon membrane, the Northern blots were hybridized with different cDNAs isolated from *Manduca*. The expression of some genes, like actin and myosin heavy chain (not shown), is repressed when the ISMs become committed to die on day 18. When animals are injected with 20-hydroxyecdysone (20E) on day 17 of pupal–adult development and examined 5 h after the normal time of eclosion, the loss of these transcripts is reduced. The majority of ISM genes are constitutively expressed throughout the period of ISM development examined, as shown here for the *ubiquitin fusion*-80 (*Ubf-80*) gene. A final class of genes is induced when the ISMs become committed to die, including: *SCLP* (small cytoplasmic leucine-rich repeat protein), *polyubiquitin*, and the as yet uncharacterized *18-5* gene. Pretreatment with 20E delays the accumulation of these induced transcripts. d, day of pupal–adult development; h, hours – postadult eclosion.

abundance of actin and myosin heavy chain mRNA. One possible explanation is that it allows the muscles to change, in a dramatic fashion, their developmental program from one of homeostasis to another of death, by allowing newly expressed transcripts to accumulate and effectively compete for the cellular translational machinery.

One mechanism for reducing transcript abundance is transcriptional repression. A complementary mechanism is to enhance transcript degradation. In fact, it was found that there is a transient increase in endogenous ISM RNAse activity on day 17, which facilitates the removal of all transcripts prior to the induction of new gene expression (Cascone and Schwartz, 2001). This coordinated control of transcription and degradation may allow the muscles to shift developmental programs extremely rapidly. It has yet to be determined whether a similar strategy is used by other organisms during development.

In addition to transcript loss, several newly expressed death-associated cDNAs have been cloned and characterized from the ISMs (Schwartz *et al*., 1990a). While the majority of these cDNAs encoded previously uncharacterized genes, the first one to be sequenced was shown to be the product of the polyubiquitin gene (Schwartz *et al*., 1990b). Ubiquitin is a 76 amino acid peptide that is the most highly conserved protein across phylogeny. At the protein level, insect and human ubiquitins are identical (review: Rechsteiner, 1988). The posttranslational covalent attachment of ubiquitin to selected lysine residues on substrate proteins serves as a molecular tag, to help target proteins to specific fates within the cell (Murphey and Godenschwege, 2002; Schwartz and Hochstrasser, 2003). The addition of single ubiquitin moieties serves to target proteins to specific subcellular locations, while the addition of multiple head-to-tail ubiquitin chains promotes binding to the 26S proteasome. This multisubunit protease then releases the ubiquitin, unfolds the substrate and rapidly degrades it to small peptides.

In *Manduca* ISMs, expression of polyubiquitin mRNA increases transiently on days 15 and 16 of adult development, and then accumulates to prodigious levels on day 18, the day of adult eclosion. The early expression of ubiquitin is associated with the increased turnover of contractile proteins that accompanies the atrophy program that begins on day 15 and a transient increase in ubiquitin-dependent proteolysis (Haas *et al*., 1995). On day 18, there is a coordinated exponential increase in the expression of the entire ubiquitin-proteasome pathway, including ubiquitin conjugation, ubiquitin-dependent proteolysis, and proteasome subunit expression. In fact, it has been found that there is a coordinated exponential increase in the expression of both 20S and 26S proteasome subunit proteins, at both the mRNA and protein levels (Schwartz *et al*., 1990b; Dawson *et al*., 1995; Haas *et al*., 1995; Jones *et al*., 1995; Takayanagi *et al*., 1996; Low *et al*., 1997). Placed in a broader context, this enhancement in ubiquitin-dependent proteolysis is presumably adaptive, because the ISMs are not phagocytosed and, therefore, the liberation of cellular constituents requires a cell-autonomous mechanism.

Other previously described transcripts were also identified in the condemned ISMs, but their relationship to PCD remains unknown. For example, the abundance of apolipoprotein III (apoLp-III) is dramatically induced, at both the RNA and protein levels, in both the ISMs and in certain dying interneurons (Sun *et al*., 1995). ApoLp-III is synthesized predominantly in the fat body and normally associates with lipophorin in the hemolymph to facilitate lipid transport. The role of apolipoprotein in PCD is mysterious, particularly since the ISMs do not express lipophorin.

The majority of cDNAs isolated in the ISM screen encoded novel proteins of unknown function. For example, one of these cDNAs encodes a small protein that is composed of multiple leucine-rich repeat protein–protein interaction motifs (Kuelzer *et al*., 1999). Termed *SCLP* (small cytoplasmic leucine-rich repeat protein), this gene is dramatically induced at both the RNA and protein levels. Exploiting a paradigm for the study of genes initially isolated in moths, transgenic flies were generated that express *Manduca SCLP* under the control of the Gal4 protein upstream activating sequence (UAS) using the *P* element system that allows gene expression to be targeted to specific tissues in the fly. Ectopic expression of *SCLP* in a variety of tissues failed to reveal an overt phenotype, suggesting that overexpression of *SCLP* is nonpathological (Kuelzer *et al*., 1999). Studies employing loss-of-function mutations will be needed to define the role of *SCLP* in development and death.

In contrast to *SCLP*, other novel death-associated transcripts from *Manduca* ISMs have been shown to play key roles in PCD. The characterization of two such genes has provided insights into the development and death of muscle in mammals as well as insects. The first of these genes is *DALP* (death-associated LIM-only protein). In contrast to the genes described above, *DALP* is induced on day 17, well in advance of the other death-associated cDNAs from *Manduca* ISMs (Hu *et al*., 1999). Expression of the DALP protein is restricted to the ISMs and is not detected in the flight muscle, fat body, Malpighian tubules, ovary/eggs or male

sexual accessory gland. The most significant structural feature of the DALP protein is the presence of one perfect and two imperfect LIM domains, structural motifs that consist of paired zinc fingers, which participate in protein–protein interactions (Leon and Roth, 2000; Laity *et al*., 2001). While many LIM proteins also possess a homeodomain, DALP belongs to the subfamily of LIM-only proteins (LMO) that lack this DNA binding motif.

As with SCLP, the function of *Manduca* DALP was explored using transgenic flies (Hu *et al*., 1999). Ectopic expression of *Manduca DALP* in the abdominal intersegmental muscles of fly pupae resulted in the disorganization of the contractile apparatus and subsequent muscle atrophy (**Figure 6**). Targeted mutations in the LIM domain blocked muscle atrophy, suggesting that the observed effects of ectopic *DALP* expression were dependent on expression of the intact functional protein.

Further insights into the function of DALP were gained by examining the effects of expressing *Manduca DALP* in the mouse myoblast C_2C_{12} cell line (Hu *et al*., 1999). This muscle satellite cell line has been extensively used as a model for examining skeletal muscle differentiation in mammals. C_2C_{12} cells can be maintained as a stable, nontransformed line that ceases cycling and differentiates into multinucleated myotubes following incubation in a low serum differentiation medium (Yaffe and Saxel, 1977). Expression of *DALP* was able to block the ability of C_2C_{12} cells to differentiate into myotubes by blocking the induction of MyoD, a basic helix–loop–helix muscle transcription factor required for differentiation. These effects of DALP were overcome by cotransfecting the cells with an expression vector driving ectopic MyoD. In addition to blocking differentiation, DALP greatly enhanced the probability of cell death. Identical data were obtained when C_2C_{12} cells were forced to express ectopic *Hic-5* (hydrogen peroxide-inducible clone-5), the mammalian ortholog of *DALP*. Taken together, these data demonstrate that DALP and Hic-5 are phylogenetically conserved proteins that function as negative regulators of muscle differentiation and survival in both insect and mammalian cells. These results also validate the view that general conclusions about PCD and differentiation may be drawn from the study of insect metamorphosis.

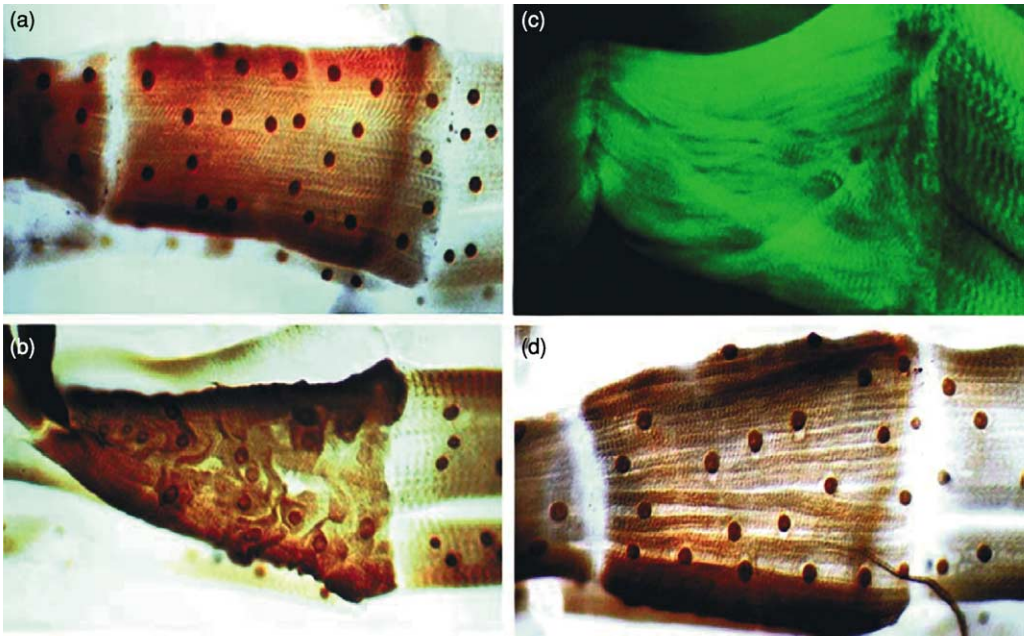


Figure 6 The effects of ectopic *DALP* expression in *Drosophila* skeletal muscle. (a) Bacterial β-galactosidase was expressed in *Drosophila* ISMs using the Gal4/UAS expression system. Expression (nuclear as well as cytoplasmic) was monitored via anti-β-galactosidase immunocytochemistry. (b) The same Gal-4 enhancer trap line was used to coexpress β-galactosidase and moth *DALP* in the ISMs. *DALP* expression was independently shown to parallel that of β-galactosidase, monitored in this panel by immunohistochemistry. (c) The organization of the contractile apparatus was visualized using fluorescein isothiocyanate (FITC)-labeled phalloidin in separate animals expressing ectopic *DALP*. In this panel, the disorganization of the contractile apparatus is evidenced by the loss of the sarcomeres that are obvious in the two fibers at right. (d) The WHPEHF motif was deleted from *DALP* and coexpressed with *β-galactosidase* in the ISMs. Muscles retained normal appearance and were contractile. Note that the triangular muscle in these assays is muscle 31 and is about 260 μm long. (Reproduced from Hu, Y., Cascone, P., Cheng, L., Sun, D., Nambu, J.R., Schwartz, L.M. **1999**. Lepidopteran DALP, and its mammalian ortholog Hic-5, function as negative regulators of muscle differentiation. *Proc. Natl Acad. Sci. USA 96*, 10218–10223.)

The last gene of the moth ISMs to be cloned and analyzed to date encodes a protein named Acheron (Ach; after the river that must be crossed to reach to the realm of the dead in Greek mythology). Acheron is undetectable in ISMs until early on day 18, when it begins to accumulate to high levels (Wang, Valavanis, Sun, and Schwartz, unpublished data). This increase in *Acheron* expression on day 18 can be blocked with a single intrathoracic injection of 25 μg of 20E on day 17. *Acheron* expression is not restricted to the ISMs, however, because Northern blot analysis revealed low levels of expression in the fat body and flight muscle but not in Malphigian tubules, ovaries, or the male sexual accessory gland. Because the ovary is composed predominantly of unfertilized oocytes, these data suggest that *Acheron* is not a maternal transcript.

Database analysis revealed a human expressed sequence tag (EST) that shares 59% identity and 68% similarity over 86 amino acids with *Manduca Acheron* (Wang, Valavanis, Sun, and Schwartz, unpublished data). Using this sequence as a probe, full-length human and mouse homologs of Acheron were cloned. It was found that they display about 31% identity and 40% similarity to *Manduca Acheron*.

Mammalian *Acheron* expression has also been studied in the mouse C_2C_{12} line (Wang, Valavanis, Sun, and Schwartz, unpublished data). It appears that *Acheron* acts near the apex of the genetic hierarchy that regulates myoblast survival and differentiation, because it is required for myotube formation and, when its activity is blocked with either antisense or a dominant-negative form of the protein, differentiation is blocked. Acheron was also found to block the expression of the anti-apoptotic protein Bcl-2 (B cell lymphoma 2), which, in turn, enhances the death of cells. These data suggest that Acheron, like DALP/Hic-5, acts to control key developmental decisions in muscle cells, including the initiation of PCD.

2.5.5. The *Drosophila* Model

2.5.5.1. Choice of *Drosophila melanogaster* as a Model System for the Study of PCD of Nerve and Muscle Cells

The successful application of genetics to define the molecular controls of PCD in the nematode *Caenorhabditis elegans* provided impetus to apply this approach to the study of PCD in the fruit fly, *D. melanogaster* (Diptera: Drosophilidae). Kimura and Truman (1990) extended earlier observations of muscle death during *Drosophila* metamorphosis by systematically documenting the accompanying post-eclosion neuronal death in the fused ventral ganglia. Injection of living flies with a toluidine blue solution revealed numerous examples of dying neurons in the dorsal and lateral regions of the abdominal and metathoracic neuromeres (Kimura and Truman, 1990). These investigators also mapped changes in both the head and abdominal musculature, before and after eclosion, by using polarized light microscopy to monitor the loss of birefringent muscle fibers. Several studies described below (see Section 2.5.5.3.1) have made profitable use of these baseline observations to explore ecdysteroid regulation and molecular mechanisms of PCD in the nervous system of adult flies (Robinow *et al*., 1993, 1997; Draizen *et al*., 1999).

However, the first identification of novel cell death genes in *Drosophila* was not based on the study of metamorphosis, but instead resulted from a screen for aberrant patterns of cell death in embryos carrying homozygous chromosomal deletions, carried out in the laboratory of Hermann Steller (White *et al*., 1994). Embryo staining with the vital dye acridine orange was used to compare wild-type embryos with 129 different deletion strains. Chromosomal deletions that remove a small region at 75C on chromosome 3L, such as Df(3L)H99, were found to exhibit a blockade of both developmental and X-irradiation induced apoptosis. Subsequent studies led to the identification of four related proapoptotic genes that reside within a 300 kb interval in this region, *reaper* (*rpr*), *head involution defective (hid)*, *grim*, and *sickle (skl)* (White *et al*., 1994; Grether *et al*., 1995; Chen *et al*., 1996; Christich *et al*., 2002; Srinivasula *et al*., 2002; Wing *et al*., 2002) (**Figure 7**). These linked genes are all transcribed in the same direction and are expressed predominantly in doomed and dying cells. As will be described below, proper regulation of cell survival in *Drosophila* involves precise transcriptional control of this gene complex.

Reaper, Grim, and Sickle are all small proteins (65, 138, and 108 amino acids respectively), while Hid is substantially larger at 410 amino acids. Each of these proteins possesses a related 14 amino acid region at the N-terminus, the RHG (Reaper, Hid, Grim) motif or IBM (inhibitor of apoptosis (IAP)-binding-motif) that has potent proapoptotic activities. The RHG and IBM motifs of Reaper and Grim are very similar: they share 71% identity, and three of the four amino acid differences are conservative substitutions. Surprisingly, despite this similarity, *in vivo* studies have shown that these domains possess distinct death-inducing activities (Wing *et al*., 1998b). Reaper, Grim, and Sickle also share a

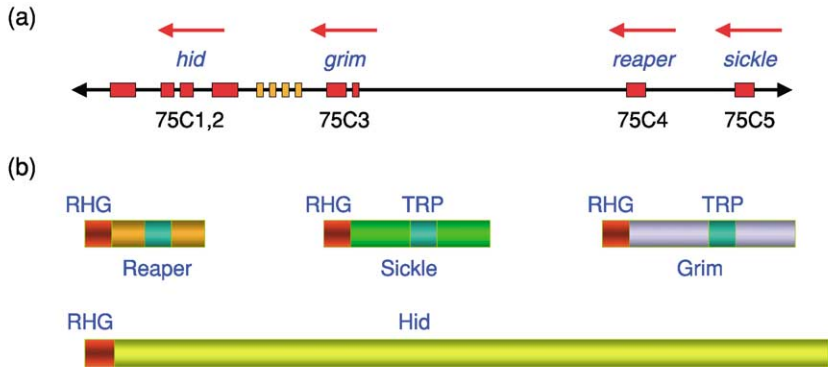


Figure 7 The *grim-reaper* genes encode related proapoptotic proteins. (a) The *hid, grim*, *reaper*, and *sickle* genes all reside within a 300 kb interval in the 75C region of chromosome 3L of *Drosophila melanogaster*. The four genes are transcribed in the same direction (arrows above genes) and are expressed predominantly in doomed and dying cells. No genes are predicted to reside between *grim* and *reaper*, or *reaper* and *sickle*, while four unrelated genes (orange blocks) reside between *hid* and *grim*. (b) *reaper*, *sickle*, and *grim* encode small proteins that contain a related N-terminal RHG motif/IBM as well as a C-terminal Trp block/GH3 domain. Hid is a substantially larger protein that also contains an RHG motif but does not exhibit strong sequence similarity to the Trp block/GH3 domain.

second region of sequence similarity, the 15 amino acid Trp block or Grim Helix 1, 2, and 3 (GH3) domain (**Figure 7**), which also has proapoptotic functions (Wing *et al*., 2001; Claveria *et al*., 2002). While Hid possesses four regions that resemble a Trp block/GH3 domain, the level of similarity is much lower. Together, the *reaper*, *hid*, *grim*, and *sickle* genes should be considered a genetic complex that controls apoptosis.

The considerable insights into the molecular mechanisms of PCD gained by further studies of this complex during *Drosophila* embryonic development and metamorphosis are described in detail below (see Section 2.5.5.4). In addition, it should be noted that targeted ectopic expression of these genes in cells that are normally fated to live, provides a valuable tool for producing cell-specific lesions of the CNS (McNabb *et al*., 1997; Renn *et al*., 1999; Rulifson *et al*., 2002; Park *et al*., 2003).

2.5.5.2. Hormonal Regulation of Metamorphosis in *Drosophila melanogaster*

Hormonal regulation of metamorphosis in *Drosophila* is similar to that described for other insects, with postembryonic development wholly dependent upon exposure of tissues to coordinated pulses of 20E, which, in turn, produce a coordinated cascade of gene expression (Riddiford, 1993; Truman and Riddiford, 2002). Cloning of the *Drosophila* ecdysone receptor (*EcR*) gene permitted the undertaking of studies of expression patterns of the receptor in tissues, including neurons and muscles (Koelle *et al*., 1991; Robinow *et al*., 1993; Talbot *et al*., 1993; Truman *et al*., 1994). Consistent with the results from *Manduca*, these studies have reinforced the view that ecdysteroid regulation of PCD in insects is typically a result of direct action of the steroid on the cells that are fated to die (Robinow *et al*., 1993).

2.5.5.3. PCD of Neurons during Early Development and Metamorphosis

In *Drosophila*, there are four periods of cell death in the nervous system: during mid to late embryogenesis, in late third instar larvae, in pupae during metamorphosis, and in the newly eclosed adult. The cell death that occurs during these stages permits the generation of a functional larval and adult nervous system by eliminating cells that are produced in excess, such as the embryonic midline glia, or cells with transient functions, such as larval abdominal ganglion neuroblasts (Kimura and Truman, 1990; Truman *et al*., 1993; Sonnenfeld and Jacobs, 1995a; Zhou *et al*., 1995). As in mammalian development, the extent of cell death in the developing *Drosophila* nervous system is profound: approximately two-thirds of all cells born within the embryonic CNS die before larval hatching (Abrams *et al*., 1993; White *et al*., 1994) (**Figure 8**).

The great extent of cell death in the *Drosophila* embryos suggests that this process is critical for early nervous system development. In this regard, mutations in several genes that are important for cell death result in hypertrophy of the embryonic, larval, or adult CNS, and these mutants can exhibit stage-specific lethality or sterility (Grether *et al*., 1995; Song *et al*. 1997; Zhou *et al*., 1997; Peterson *et al*., 2002). Interestingly, the removal of dead cells within the CNS is also apparently important for nervous system development, as mutants lacking functional macrophages exhibit disruptions in the normal architecture of the embryonic axon scaffold (Sears *et al*., 2003).

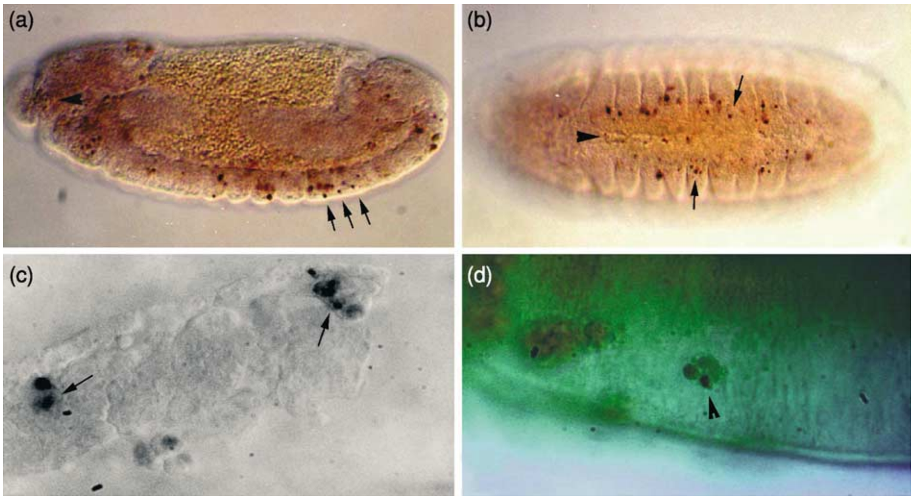


Figure 8 Detection of dying cells in *Drosophila* embryos. (a) Sagittal view of a stage 12 wild-type embryo analyzed via *in situ* nick translation to detect fragmented chromatin in dying cells. Note the prominent cell death in the retracting germ band (arrows). Significant cell death is also detected in the cephalic region of the embryo (arrowhead). Anterior is left and dorsal is up. (b) Ventral view of a stage 12 embryo labeled via *in situ* nick translation. Note the asymmetric pattern of dying cells in the neuroectoderm (arrows) on both sides of the CNS midline (arrowhead). Anterior is to left. (c) Section of tissue showing multiple labeled apoptotic bodies within phagocytic macrophages (arrows). (d) Ventral region of stage 16 embryo indicating an apoptotic body (arrowhead) engulfed by a circulating macrophage.

In contrast to the situation in the embryo, the proportion of neurons that die during the postembryonic period is much lower, and the widespread degeneration of larval tissues, characteristic of metamorphosis in flies, does not extend to the CNS. As in the *Manduca* model described above (see Section 2.5.4.3.1), most neurons of the larva persist rather than die, and the adult nervous system is, therefore, a composite structure of persisting neurons, born during embryogenesis, and adult-specific neurons. After pupariation, however, many dying neurons are observed in the ventral CNS in both thoracic and abdominal neuromeres (Truman *et al.*, 1993). The identity of most of these neurons has not been determined, with the exception of several motoneurons identified by retrograde fills of the T2 mesothoracic nerve (Consoulas *et al.*, 2002). The population of neurons that die at this metamorphic transition likely includes motoneurons that have lost their muscle targets, and interneurons in circuits for larval-specific behaviors. In addition, PCD of neuroblasts after pupariation marks the termination of postembryonic neurogenesis in the ventral nervous system (Truman and Bate, 1988; Truman *et al.*, 1993).

The death of neurons after adult eclosion has received more in-depth study than the neuronal deaths associated with pupariation (Kimura and Truman, 1990). Neurons dying at this time presumably include the motoneurons that innervate the abdominal muscles used during ecdysis and wing expansion (Truman *et al.*, 1993). Many features of posteclosion neuronal death in *Drosophila* mirror phenomena previously is described in the *Manduca* model. For example, neuronal death is delayed in newly eclosed flies that are forced to continue ecdysis behavior beyond the normal period (Kimura and Truman, 1990), as is the death of motoneurons in the abdominal ganglia of moths forced to dig after eclosion (Truman, 1983). Decapitation of flies at the time of eclosion results in inhibition of neuronal death in the ventral nervous system, similar to the sparing of MN-12 in *Manduca* by nerve cord transection (Kimura and Truman, 1990) (see Section 2.5.4.4).

Another major feature shared by the *Manduca* and *Drosophila* models of posteclosion neuronal death is its regulation by ecdysteroids. Treatment of both newly emerged flies and moths with ecdysteroids delays the PCD of neurons: only in the fly, however, can the molecular basis of this phenomenon be readily investigated (e.g., Baehrecke, 2000). In fact, the powerful molecular genetic approaches available in *Drosophila* make it possible to explore in detail the specific mechanisms that regulate cell survival in the developing nervous system. In addition to the continuing study of the posteclosion neuronal deaths initially described by Kimura and Truman (1990), this issue has been addressed using several distinct cellular systems, including the embryonic CNS midline, as well as embryonic and larval neuroblasts. In the following sections these

examples will be considered and the specific contributions that each has made toward the understanding of cell death regulation will be discussed.

2.5.5.3.1. Role of EcR in posteclosion neuronal death The *EcR* gene of *Drosophila* encodes three ecdysone receptor subunits: EcR-A, EcR-B1, and EcR-B2 (Talbot *et al.*, 1993). These receptor subunits share common DNA and ligand-binding domains, but have different N-terminal regions. EcR-A and EcR-B1 can be distinguished by monoclonal antibodies. The use of these antibodies to examine the expression of EcR isoforms in the metamorphosing *Drosophila* CNS revealed that a high level of expression of EcR-A is correlated with posteclosion PCD (Robinow *et al.*, 1993). Nearly 300 neurons, whose neuronal status was confirmed by double labeling with antibodies against EcR-A and ELAV (embryonic lethal, abnormal vision, a neuron-specific protein) in the ventral ganglia, displayed high levels of EcR-A-immunoreactivity. With rare exceptions, all EcR-A/ELAV-immunopositive neurons were absent from the ganglion 24 h after adult eclosion. Confocal microscopy of EcR-A-immunopositive neurons during day 1 of adult life revealed that these cells underwent PCD during this time. This study implies that EcR-A expression during the period of ecdysteroid decline, which accompanies the end of metamorphosis, is critical to the decision for these neurons to commit suicide.

Because nuclear hormone receptors such as EcR act as transcription factors, an important follow-up to the aforementioned studies is to determine the identity of genes that are regulated by ecdysteroids via the EcR-A. Obvious candidates are the *reaper*, *grim*, and *hid* genes, previously shown to be involved in the apoptotic pathway, now known to be indirect regulators of caspase activity by means of their ability to bind to inhibitors of apoptosis proteins (IAPs) (review: Hay, 2000) (see Section 2.5.5.4.1). When newly emerged adult flies were injected with 20E within 20 min of eclosion, the presence of the steroid blocked both PCD and the accumulation of *reaper* transcripts, normally seen in EcR-A-immunopositive neurons at this time (Robinow *et al.*, 1997). Neurons destined for death at this time were also demonstrated to accumulate *grim* but not *hid* transcripts.

The relationship of EcR-A to other examples of PCD in *Drosophila* and in insects, generally, remains to be determined. It is known that EcR-A and EcR-B1 have different tissue distributions during *Drosophila* metamorphosis (Talbot *et al.*, 1993), and that not all cells that express EcR-A will undergo PCD. However, at present there is no model to explain the tissue-specific distributions of the different isoforms. Expression of individual isoforms in a line of dominant-negative EcR mutants showed that EcR-A can support metamorphosis in numerous tissues, including the fat body, eye discs, salivary glands, and wing discs, but revealed no links that could be interpreted in relationship to PCD (Cherbas *et al.*, 2003).

2.5.5.4. Molecular Mechanisms of Neuronal Death

Most of the insights into the genetics of PCD in *Drosophila* have come from studies of embryonic development rather than metamorphosis. The key insights from studies on embryos, as they form the foundation for understanding control of postembryonic PCD are presented here.

2.5.5.4.1. The *Drosophila* cell death "machinery" Flies utilize a conserved cell death machinery that includes caspases, a Ced (cell death abnormal)-4/Apaf (apoptosome associated factor)-1 ortholog, and Ced-9/Bcl-2 protein family members (Tittel and Steller, 2000; Vernooy *et al.*, 2000) (**Table 1**). These proteins all have critical functions in apoptosis that were initially defined by studies in *C. elegans* (Horvitz *et al.*, 1994; Liu and Hengartner, 1999). In addition, however, several novel cell death regulators and cell survival mechanisms have been identified in flies.

Caspases are specialized members of the cysteine protease family that cleave aspartate or glutamate residues within enzymatic or structural substrate proteins to promote the dismantling of a cell (Hengartner, 2000). In *Drosophila*, there are seven caspases (**Table 1**), including three long prodomain initiator caspases, such as Dronc (*Drosophila* nedd2-like caspase; similar to caspase-9), and four

Table 1 *Drosophila* cell death regulators

Family	*Protein*
Caspases	Dronc
	Dcp-1
	Drice
	Dredd
	Strica/Dream
	Decay
	Damm
Caspase inhibitor	DIAP1/Thread
	DIAP2
Bcl-2 family	Drob-1/Debcl/Dborg-1/Dbok
	Buffy/Dborg-2
IAP inhibitors	Reaper
	Hid
	Grim
	Sickle
	Jafrac2
Apaf-1/Ced 4 family	Dark/HAC-1/Dapaf-1

short prodomain effector caspases, such as drICE (similar to caspase-3) and DCP-1 (death caspase1; similar to caspase-7). Loss-of-function mutations in *dcp-1* result in larval lethality and formation of melanotic tumors (Song *et al.*, 1997). The *Drosophila* Ced-4/Apaf-1 homolog is known as Dark (Drosophila apaf-1 related Killer)/HAC (homolog of apaf-1 and Ced-4)-1/Dapaf-1 and, like its worm and mammalian counterparts, associates with and promotes activation of long prodomain caspases (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999; Zhou *et al.*, 1999). Dark is expressed in dying cells and, interestingly, resembles its mammalian but not worm counterpart in that it contains WD repeats that can bind cytochrome *c*, and *dark* mutants exhibit decreased cell death in the embryonic CNS, hypertrophy of the larval CNS, and the formation of melanotic tumors.

Flies possess two Bcl-2 family members, drob (*Drosophila* ortholog of the Bcl-2 family)-1/debcl (death executioner Bcl-2 homolog)/Dborg (*Drosophila* Bcl-2 ortholog)-1/dbok and Buffy/Dborg-2, which both possess Bcl-2 homology (BH) domains BH1, BH2, and BH3 (Brachmann *et al.*, 2000; Colussi *et al.*, 2000; Igaki *et al.*, 2000; Zhang *et al.*, 2000). Both of these proteins resemble the proapoptotic mammalian Bok protein and act to promote apoptosis. Thus, unlike *C. elegans* and mammals, *Drosophila* does not appear to possess antiapoptotic Bcl-2 family members. This suggests that, both in terms of molecular and cellular complexity, *Drosophila* may prove to be a very useful system for bridging the gap that exists between the relatively simple nematode *C. elegans* and the more complex mammals. For example, in *C. elegans*, cell death eliminates approximately 10% of all cells, is governed largely by cell lineage, and is not required for organismal viability or fertility (Horvitz *et al.*, 1994; Liu and Hengartner, 1999). In contrast, in flies and mammals a much larger proportion of cells die, their survival is often governed by extrinsic signals, and normal progression of cell death programs is required for viability.

2.5.5.4.2. Role of IAP family proteins in PCD The Grim-Reaper proteins, initially identified in the first molecular genetic studies of PCD in *Drosophila*, all act upstream of caspases and their proapoptotic activities are blocked by the baculovirus caspase inhibitor, p35 (Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996; Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002). Because the Grim-Reaper proteins do not share extensive homology to other proteins of known function, the mechanism through which they promote cell death was not immediately apparent. Elucidation of their actions was greatly advanced by the identification and analysis of DIAP1 and DIAP2, *Drosophila* members of the IAP family (Hay *et al.*, 1995). IAPs were first identified from the *Cydia pomonella* granulosis virus (Crook *et al.*, 1993); since then, a large number of viral and cellular IAPs have been identified that are potent death inhibitors (Deveraux and Reed, 1999; Miller, 1999; Hay, 2000). IAPs all contain one or more copies of a 70 amino acid baculovirus IAP repeat (BIR) domain, typically located in the central or N-terminal region of the protein. In addition, they also contain a 50 amino acid RING (Really Interesting New Gene) domain, generally situated towards the C-terminal region of the protein. The sequences of the BIR and RING domains resemble zinc fingers, and both serve as protein–protein interaction domains. The BIR domains and associated linker regions are required to block caspase activities, and association between the linker domain and caspases is strengthened by the BIR domain (Huang *et al.*, 2001). The RING domain possesses ubiquitin E3 ligase activity that can target bound caspases for polyubiquitination and degradation via the 26S proteasome (Suzuki *et al.*, 2001b; Wilson *et al.*, 2002). In addition, IAPs bound to caspases are also targeted themselves for proteasome-dependent proteolysis via the N-end rule pathway of protein turnover (Ditzel *et al.*, 2003). The turnover of caspases via the ubiquitin–proteasome pathway affords an efficient mechanism for the IAPs to reduce caspase levels, and the interplay between caspases and IAPs is a key determinant of cell survival. The levels of IAPs themselves are also under rigid regulation, as proapoptotic stimuli can result in IAP autoubiquitination and proteasome-dependent degradation, thus promoting cell death (Yang *et al.*, 2000; Yoo *et al.*, 2002). In *Drosophila*, DIAPs play a particularly critical role in cell survival; loss of *diap1* results in early embryonic lethality associated with massive ectopic cell death (Wang *et al.*, 1999; Goyal *et al.*, 2000; Lisi *et al.*, 2000).

Significantly, the BIR domains of DIAP1 bind both caspases and the Grim-Reaper proteins. In particular, the BIR2 domain of DIAP1 associates with the RHG motif/IBM of the Grim-Reaper proteins. Hid, Grim, and, presumably, Reaper utilize a conserved 7 amino acid stretch of the RHG motif/IBM (amino acids 2–8) to directly contact residues within the BIR2 domain (Wu *et al.*, 2001). Sickle also binds the BIR2 domain, but its RHG motif/IBM differs from that of Reaper, Hid, and Grim and only the first four residues make direct contacts (Srinivasula *et al.*, 2002). This association with Grim-Reaper proteins promotes cell death by

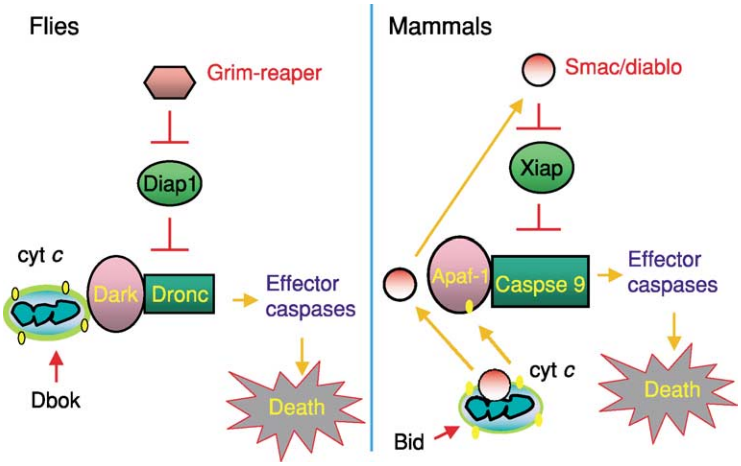


Figure 9 IAP inhibition is a conserved mechanism of regulation of apoptosis. In *Drosophila*, expression of the *grim-reaper* genes is activated in doomed cells, and the corresponding proteins bind to DIAP1 and block its ability to inhibit caspases. This results in active caspases that degrade cellular proteins to mediate apoptosis. In mammals, proapoptotic stimuli induce the release of Smac/Diablo as well as other proapoptotic factors, including cytochrome *c*, from the mitochondria. Cytoplasmic Smac/Diablo binds XIAP and represses its caspase-inhibitory actions, thereby promoting apoptosis. In both flies and mammals, the orthologous Dark and Apaf-1 proteins as well as proapoptotic members of the Ced-9/Bcl-2 family promote activation of initiator caspases.

blocking the ability of DIAP1 to bind and repress caspases. Thus, cell survival in *Drosophila* is controlled by a "double-inhibition" mechanism (**Figure 9**). In surviving cells, DIAP1 binds and inhibits caspase activities. In contrast, in cells signaled to die, the Grim-Reaper proteins are expressed and compete with caspases for DIAP1 binding. The displacement of caspases from DIAP1 results in proteolytically active molecules that promote cell death. Cell survival decisions are, therefore, determined to a large extent by the interactions between DIAP1, Grim-Reaper proteins, and caspases. Not surprisingly, the relative levels of these proteins within a cell are critical, and the effect of ectopic expression of these genes is highly dosage sensitive.

IAP inhibition is a conserved mechanism for regulating cell survival (**Figure 9**; review: Vaux and Silke, 2003). Thus, in mammals Smac (second mitochondria-derived activator of caspase)/Diablo is a mitochondrial protein that is released along with cytochrome *c* in dying cells (Du *et al*., 2000; Verhagen *et al*., 2000). Cytoplasmic Smac/Diablo associates with XIAP to prevent its binding and inhibition of caspase-9 (Chai *et al*., 2000; Liu *et al*., 2000). Strikingly, the binding of Smac/Diablo to IAPs is mediated through an N-terminal tetrapeptide sequence that is conserved in the Grim-Reaper RHG motif, indicating conserved modes of action for the fly and mammalian proteins (Chai *et al*., 2000; Srinivasula *et al*., 2000, 2002). Another mammalian IAP-inhibitor protein is the mitochondrial serine protease, Omi/HtrA2 (high temperature requirement protein A2), which also contains the conserved BIR-binding tetrapeptide at its N-terminus (Suzuki *et al*., 2001a; Hegde *et al*., 2002; Martins *et al*., 2002; van Loo *et al*., 2002; Verhagen *et al*., 2002). These similarities indicate that analysis of IAP inhibition mechanisms will provide insights into cell survival pathways in a wide range of species.

2.5.5.4.3. Evolutionary scenarios for the *grim-reaper* gene complex The small Reaper, Grim, and Sickle proteins are clearly ancestrally related, and the much larger Hid protein is also likely to share a common progenitor with them. Why are four related proteins needed to control DIAP1 inhibition and apoptosis, and how similar are the functions of these proteins? What proapoptotic activities, in addition to IAP inhibition, do the Grim-Reaper proteins share? Gene complexes can provide enhanced capabilities for a cell to finely control specific biological processes by providing gene products that exhibit overlapping yet distinct expression patterns and functions. Thus, multiple Hox proteins, for example, act together to permit elaboration of profound as well as subtle distinctions in cell and tissue types, and morphogenesis, along a major body axis (see **Chapter 1.8**). Presumably, the existence of the *reaper*, *hid*, *grim*, and *sickle* genes enables cells to control apoptosis with the exquisite specificity and efficiency that are required for developmental and physiological processes. The Grim-Reaper proteins do exhibit differences in their cell death-inducing abilities in developing tissues (Chen *et al*., 1996; Wing *et al*., 1998b, 2002), suggesting that they may have distinct abilities to interact with

DIAPs and promote apoptosis. In addition, gene expression studies as well as analysis of loss-of-function and gain-of-function mutants have indicated that these genes exhibit overlapping yet distinct patterns of expression and nonredundant cell killing activities (Grether *et al*., 1995; Robinow *et al*., 1997; Zhou *et al*., 1997; Wing *et al*., 1998b, 2002; Draizen *et al*., 1999). Presumably, each individual Grim-Reaper protein provides a unique capability to promote apoptosis and, together, these proteins permit a more complex control of apoptosis patterns than would be afforded by a single protein.

Interestingly, while the *grim-reaper* genes are essential cell death activators in *Drosophila*, *bona fide* structural homologs have so far been identified only in other *Drosophila* species. Thus, a *reaper* ortholog was described in *D. simulans* (White *et al*., 1994), and each *grim-reaper* gene is conserved in *D. pseudoobscura*. However, clear cut *grim-reaper* orthologs have not been identified from any other species, including the mosquito *Anopheles gambiae*. Therefore, the proapoptotic properties of Grim-Reaper proteins may have evolved only during divergence within the insect lineage. Finally, while the RHG motif/IBM has well-defined functions in IAP inhibition, the activities of the conserved Trp block/GH3 domain are less clear. The Trp block/GH3 domain appears to be important for mitochondrial actions of Grim and Reaper (Claveria *et al*., 2002; Olson *et al*., 2003), and may also be involved in interactions with other apoptosis regulators, such as Scythe, a large protein with a ubiquitin-like domain at its N-terminus (Thress *et al*., 1998). Alternatively, the Trp block/GH3 domain could be important for Reaper and Grim to act as general translational inhibitors (Holley *et al*., 2002; Yoo *et al*., 2002), and may promote cell death by favoring the accumulation of long-lived caspases versus less stable IAPs. Interestingly, the Trp block/GH3 domain, which shares sequence similarity to a nonstructural protein from some insect bunyaviruses (J.R. Nambu unpublished data), may also act to repress translation and promote cell death (Bridgen *et al*., 2001).

2.5.5.4.4. Regulation of embryonic glial cell survival One important mechanism for regulating the survival of neurons and glia within the developing vertebrate nervous system involves the actions of trophic factors (Raff *et al*., 1993). These prosurvival molecules are synthesized in restricted amounts by target tissues, and permit winnowing of innervating cells to ensure appropriate matching of interacting cell populations. Both neuron and glial cell types can either provide or respond to trophic signaling.

In *Drosophila*, interactions between neurons and glia are also important for cell survival (Booth *et al*., 2000; Kinrade *et al*., 2001), and recent studies have revealed that trophic mechanisms regulate cell survival in the developing CNS. Specifically, members of the epidermal growth factor (EGF) family of proteins have been shown to act as neuron-derived glial survival factors (Hidalgo *et al*., 2001; Bergmann *et al*., 2002).

Approximately 10% of all cells within the *Drosophila* nervous system are glia, and these cells provide critical functions in neuronal homeostasis, axonogenesis, and removal of cellular debris (Jones, 2001; Klambt *et al*., 2001). Among the best-characterized glial cells are the embryonic longitudinal and midline glia of the CNS. In both these lineages, cells undergo extensive apoptosis during embryogenesis (Sonnenfeld and Jacobs, 1995a; Zhou *et al*., 1995; Kinrade *et al*., 2001) (**Figure 10**). The selective survival of subsets of these cell populations is governed by trophic actions of EGF-related ligands and activation of the Ras/MAP (rat sarcoma/mitogen activated protein) kinase pathway via the EGF receptor homolog (EGFR).

During embryogenesis, a single, laterally positioned glioblast precursor gives rise to approximately 10 longitudinal glia in each hemisegment of the ventral nerve cord. These glial cells play a critical role in the formation of the longitudinal axon bundles; they migrate medially, contact pioneer longitudinal axons, and ultimately ensheathe the longitudinal nerve bundles (Hidalgo and Booth, 2000) (see **Chapter 1.10**). Coinciding with the onset of axon/glial contact, many of the glia undergo apoptosis (Kinrade *et al*., 2001), suggesting that, as the glia contact the axons, they become dependent upon them for survival. Consistent with this notion, ablation of pioneer and other neurons results in a decrease in longitudinal glia (Hidalgo *et al*., 2001). Thus, apoptosis determines the final numbers of longitudinal glia during embryogenesis, and axon-derived factors are required for longitudinal glial survival. One of these factors is Vein (Vn), a *Drosophila* neuregulin homolog that contains both an IgG domain and EGF domain (Schnepp *et al*., 1996). The *vein* gene is expressed in a small subset of neurons within the embryonic CNS, including the midline precursor 2 (MP2) pioneer neurons as well as the Ventral Unpaired Median (VUM) neurons of the CNS midline (Hidalgo *et al*., 2001) (see **Chapters 1.10** and **2.3**). Gene *vein* mutants exhibit ectopic apoptosis of longitudinal glial cells, and ectopic apoptosis also results from RNAi-mediated loss of *vein* function in either all neurons or the MP2 neurons (Hidalgo *et al*., 2001).

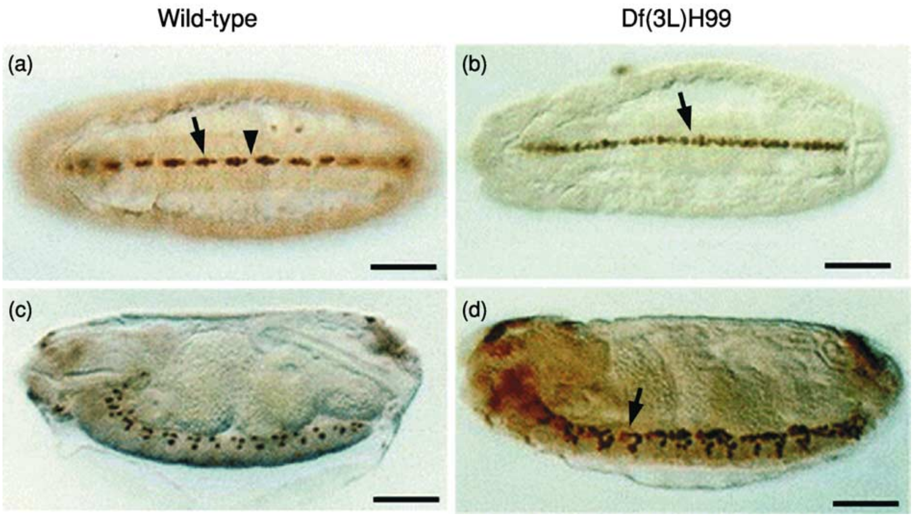


Figure 10 The embryonic CNS midline glia undergo developmental apoptosis. Embryos carrying a P[1.0slit-lacZ] transgene were immunostained with anti-β-galactosidase to detect the CNS midline glia. (a, c) In wild-type embryos (stages 15 and 16, respectively), there are approximately three midline glia per segment within the ventral nerve cord (arrow in a). The arrowhead in (a) points to the separation between two adjacent segmental clusters of midline glia that are eliminated with advanced development (c). (b, d) In Df(3L)H99 mutant embryos (stages 15 and 16, respectively), which lack *reaper*, *hid*, and *grim*, there is a blockade of apoptosis and accumulation of excess midline glia (arrow in b). The rescued midline glial cells accumulate at the dorsal surface of the ventral nerve cord (arrow in d).

Vein is a secreted ligand for the *Drosophila* EGFR receptor homolog (EGFR), and EGFR-mediated activation of the Ras/MAP kinase pathway is essential for longitduinal glial cell survival (Hidalgo *et al.*, 2001). The EGFR is transiently expressed in a subset of longitudinal glia, suggesting that Vein is secreted from axons to promote survival of longitudinal glial cells. Thus, Vein functions similarly to vertebrate neuregulins by providing axon-derived trophic support for developing glial cells via activation of receptor tyrosine kinase signaling pathways.

Another EGF family member, the transforming growth factor alpha (TGF-α) homolog Spitz (Spi), acts as a trophic factor to promote survival of the CNS midline glia (Bergmann *et al.*, 2002).

The midline glia are essential for proper formation of the axon scaffold, as migrating midline glia contact, separate, and ultimately ensheathe the anterior and posterior axon commisures (Klambt *et al.*, 1991). They synthesize several axon guidance factors such as D-Netrin (Net), Commissureless (Comm), and Slit (Sli) that control the crossing and recrossing of commisural axons (Jacobs, 2000; Kaprielian *et al.*, 2000).

Ultrastructural and genetic analyses indicate that the midline glia undergo apoptosis, which reduces their number from an initial set of nine cells to three cells in each segment of the mature ventral nerve cord (Sonnenfeld and Jacobs, 1995a; Zhou *et al.*, 1995). This apoptosis is dependent upon caspases and the actions of multiple Grim-Reaper proteins; loss of *reaper*, *hid*, and *grim* blocks all midline glial cell death and results in the survival of the nine midline glia per segment. The loss of *hid* and *grim* results in approximately seven to eight midline glia per segment, while the loss of *hid* alone results in six midline glia (Zhou *et al.*, 1997; Bergmann *et al.*, 2002). Thus, *hid* is required for the death of three midline glia and *reaper* and *grim* are together essential for the death of the other three midline glia.

Hid activities in the midline glia are regulated by proteins in the EGF signaling pathway, such as Spitz and EGFR (Bergmann *et al.*, 1998, 2002; Kurada and White, 1998). Both Spitz and EGFR are required for midline glial differentiation and survival, which, in turn, are essential for proper elaboration of the anterior and posterior commissures (Klambt *et al.*, 1991; Dong and Jacobs, 1997; Bergmann *et al.*, 2002). The loss of midline glia in *spitz* mutants is rescued in *spitz*; *hid* double mutants, implying that the proapoptotic functions of Hid are normally opposed by the prosurvival functions of Spitz.

The midline glia are normally in close contact with commissural axons, suggesting that glial–axon contact may be important for midline glial cell survival. Consistent with this notion, in *commissureless* mutants where the commissures fail to form, the isolated midline glial cells that fail to contact axons undergo apoptosis (Sonnenfeld and Jacobs, 1995a). The Spitz protein appears to be the axon-derived trophic factor required for midline

glial survival (Bergmann *et al*., 2002). The loss of midline glia in *spitz* mutants is rescued by targeted expression of the transmembrane Spitz precusor protein in commissural neurons, and the precise number of surviving midline glia can be modulated by controlling the levels of Spitz activity (Bergmann *et al*., 2002). The model that has emerged is that axon-derived Spitz protein signals the midline glia via EGFR, resulting in activation of the Ras/MAP kinase pathway. Hid activity is subsequently down-regulated via phosphorylation, which permits midline glial survival. Taken together, the actions of Vein in the longitudinal glia and Spitz in the midline glia indicate that trophic survival mechanisms are utilized to match the sizes of interacting populations of neurons and glia in invertebrates as well as vertebrates.

2.5.5.4.5. Regulation of cell survival by cell intrinsic mechanisms in sensory neuron lineages In *C. elegans*, cell lineage controls the pattern of cell survival, and the identity of each doomed cell can be determined by location, birth date, and gene expression patterns (Horvitz *et al*., 1994; Liu and Hengartner, 1999). In flies, stereotyped lineages within the developing CNS also undergo cell death (Bossing *et al*., 1996; Schmidt *et al*., 1997), and the question of how cell lineage controls cell survival decisions has been addressed in the developing peripheral nervous system (**Figure 11**). In particular, the ventral multidendritic sensory neuron 1a (vmd1a), which expresses the Cut (Ct) protein, is present in a cluster of five multidendritic (md) neurons in the ventral region of abdominal segments A1–A7

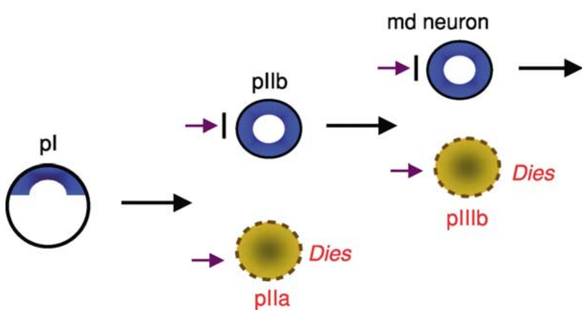


Figure 11 A model for the control of cell survival in the *Drosophila* vmdl sensory neuron lineage. The pI precursor cell exhibits asymmetric distribution of the Numb protein (blue). Upon division (black arrow) of pI, Numb becomes partitioned into the pIIb but not the pIIa daughter cell. Numb inhibits (black bar) the proapoptotic effects of Notch signaling (purple arrows) in the pIIb cell, which survives and divides to give rise to a Numb-positive multidendritic (md) neuron and a pIIIb daughter cell. As a result of Notch signaling that occurs in Numb-negative pIIa and pIIIb cells, the apoptosis activators Reaper and Grim are expressed (orange), resulting in caspase activation and apoptosis (brown and dashed line).

(Orgogozo *et al*., 2002). The vmd1a neuron is produced by two asymmetric divisions of a single sensory organ primary precursor cell (pI). The pI cell divides to generate a pIIa and pIIb progeny, one of which, pIIa, undergoes apoptosis. The pIIb cell divides again to generate the md neuron and a pIIIb cell, which also dies. Interestingly, the Numb protein, which is a cell fate determinant (Hirata *et al*., 1995; Knobloich *et al*., 1995; Spana *et al*., 1995), segregates asymmetrically into the daughter cells during both of these divisions. In both cases, Numb is detected in the surviving cell and is not present in the daughter cell that undergoes apoptosis. In addition, during the asymmetric division of the pIIb cell, the Prospero (Pros) protein also segregates into the surviving daughter cell. In contrast, the cell death activators Reaper and Grim, but not Hid, are expressed in the pIIa and pIIIb cells, which die. In embryos where cell death is blocked, an ectopic sensory (es) organ is detected, which contains four Cut-expressing cells. These include an ectopic shaft/socket and neuron/sheath cell pairs that are similar to the md–es lineage, where the pIIa and PIIIb cells do not die. Thus, in the absence of cell death, the vmd1a lineage is transformed into an md–es lineage. Cell death is, therefore, used to refine the sensory organ structures by eliminating cells that would otherwise give rise to additional cell types and morphological structures.

Mutants of the *numb* gene exhibit ectopic *reaper* or *grim* gene transcription in the progeny of the vmd1a pI daughter cells, and both daughter cells undergo apoptosis without giving rise to any Cut-expressing progeny. In contrast, ectopic Numb expression in the pI daughter cells results in duplication of md neuron and pIIIb cells. The Numb protein contains a zinc finger and a phosphotyrosine binding domain. Numb directly associates with Notch (N) and interferes with Notch signaling (Guo *et al*., 1996; Spana and Doe, 1996). This suggests that the death of cells in the vmd1a lineage is promoted by Notch, and that Numb acts to block Notch proapoptotic signaling (**Figure 11**). Consistent with this notion, ectopic expression of activated Notch, Nintra (Notch intracellular), was shown to promote the death of the pIIb cell when expressed during the period of pI cell division. Ectopic expression of *reaper* and *grim* was also detected, indicating that the effects of Nintra were similar to those observed for *numb* mutants. Therefore, in the vmd1a lineage, the distribution of Numb protein is critical for determining the survival of progeny cells. Daughter cells that inherit Numb are able to repress Notch-mediated activation of *reaper* and *grim* transcription, and subsequent apoptosis (**Figure 11**). This

appears to be a conserved mechanism of cell death during neural differentiation, as asymmetric distribution of the Numb protein also governs survival of the progeny from the embryonic neuroblast NB7-3 by inhibiting the proapoptotic actions of Notch signaling (Lundell *et al*., 2003).

2.5.5.4.6. Control of neuron number by neuroblast apoptosis In the *Drosophila* embryonic CNS, approximately 30 neuroblasts are formed in each segment that divide asymmetrically to generate a smaller ganglion mother cell, which divides once to give rise to two post-mitotic neurons (Goodman and Doe, 1993) (see **Chapters 1.10**, **1.12**, and **2.3**). The neurons that form during embryogenesis give rise to the larval nervous system. Generation of the adult nervous system requires the proliferation and differentiation of larval neuroblasts (see **Chapters 1.11** and **2.3**). Strikingly, the number of larval neuroblasts varies dramatically along the anterior–posterior axis. Thus, thoracic segments retain about 23 of the 30 neuroblasts generated during embryogenesis, while the abdominal segments retain only three embryonic neuroblasts (Truman *et al*., 1993). Abdominal and thoracic neuroblasts also differ in their proliferative properties, as thoracic neuroblasts divide for 4 days and produce approximately 100 cells, while abdominal neuroblasts divide for only 30 h and generate 4–12 cells.

The rapid cessation of abdominal neuroblast cell division is brought about by their elimination via apoptosis midway through the third larval instar. In contrast, the thoracic neuroblasts do not undergo a stage-specific cell death. Abdominal neuroblast apoptosis requires the actions of *reaper*, *hid*, and *grim* and, in Df(3L)H99 mutants, there is an accumulation of ectopic abdominal neurons (Bello *et al*., 2003). Interestingly, the rescued neuroblasts continue to proliferate and give rise to ectopic neural progeny. Reaper appears to be a key player in this process as *reaper* mutants also result in a survival of normally doomed abdominal neuroblasts, and *reaper* mutant flies exhibit a hypertrophied abdominal ganglia and male infertility (Peterson *et al*., 2002). Thus, the programmed death of abdominal neuroblasts directly controls their reproductive capacity and number of progeny.

The segment-specific differences in neuroblast survival are controlled by the homeotic selector protein, Abdominal A (AbdA), which, along with Ultrabithorax (Ubx) and AbdB, govern abdominal segment identities (Lewis, 1978; Sanchez-Herrero *et al*., 1985). Loss of AbdA function results in increased numbers of abdominal neuroblast progeny, and the mutant abdominal neuroblasts also survive longer than the wild type (Bello *et al*., 2003). In addition, ectopic expression of AbdA is sufficient to induce premature cell death of thoracic neuroblasts. This death requires the *grim-reaper* genes and is blocked in a Df(3L)H99 mutant background. Interestingly, expression of either the Antennapedia (Antp) or Ubx homeodomain proteins also induces thoracic neuroblast apoptosis, implying that a very precise control of homeodomain protein expression is necessary for appropriate cell survival patterns. Indeed, while postmitotic progeny of thoracic neuroblasts do express Ubx or Antp, the neuroblasts themselves do not.

The death of the three abdominal neuroblasts occurs in a highly synchronized fashion, and this timing appears to be the direct result of a very tight burst of AbdA expression specifically within the neuroblasts, but not in the ganglion mother cells or neurons, during the third larval instar (Bello *et al*., 2003). Thus, the timing of AbdA expression is a critical determinant of cell survival. Indeed, precocious AbdA expression results in a smaller number of neural progeny and early death of the neuroblasts.

One important issue that remains unresolved is how the transient and specific expression of AbdA is controlled in abdominal neuroblasts. Is there a transient extrinsic signal that cues the abdominal neuroblasts to activate AbdA expression, or do the neuroblasts possess an intrinsic counting mechanism that permits them to keep track of the number of divisions they undergo? Elucidation of these questions will provide a molecular mechanism to directly link Hox gene actions and segment-specific differences in the nervous system.

2.5.5.4.7. Removal of dying cells is important for proper organization of the embryonic CNS The role of phagocytic cells in PCD is described above (see Section 2.5.2.1). In *Drosophila*, specialized macrophages differentiate from hemocyte precursors that are derived from head mesoderm tissue (Tepass *et al*., 1994). Macrophages migrate throughout the body cavity, along several stereotypic routes, and rely on guidance cues that include PVR (PDGF and VEGF receptor-related), a member of the platelet-derived and vascular endothelial growth factor (PDGF/VEGF) receptor tyrosine kinase family (Cho *et al*., 2002). While macrophages are excluded from the CNS itself, they engulf dying cells that are expelled from the nervous system (Sonnenfeld and Jacobs, 1995b).

Because of their intimate association with the CNS, it was of interest to determine if macrophage functions might be important for nervous

system development. Mutations that disrupt macrophage development, migration, or engulfment abilities produce mutants exhibiting disruptions in the normal architecture of the axon scaffold (Sears *et al.*, 2003). Specifically, the junctures between the longitudinal axon connectives and anterior and posterior commissures in each segment exhibit reduced separation and a rounded appearance. In addition, many CNS glia were misplaced and accumulated along the ventral midline. Thus, the efficient removal of dead and dying cells from the CNS is important for the elaboration of the axon scaffold and the glial cell positions within the CNS. In contrast, mutants that lack macrophages still exhibit essentially normal patterns of cell death, indicating that, although macrophages sculpt the CNS, they do not have a major role in promoting apoptosis (Tepass *et al.*, 1994; Sears *et al.*, 2003).

2.5.5.5. PCD of Muscles during Metamorphosis

As is the case in *Manduca*, fruit flies undergo extensive death of abdominal muscles after adult eclosion (Miller, 1950); the muscles that die at this time appear to be larval muscles that persisted during metamorphosis (Kimura and Truman, 1990). These persisting larval muscles are a very small subset of all larval muscles, the majority of which undergo PCD at the onset of metamorphosis (Currie and Bate, 1991). In the abdomen, only a few muscles survive; although some of the persisting muscles continue to function during pupal life and adult emergence, others persist only to provide a template for adult myogenesis (Broadie and Bate, 1991; Bate, 1993) (see **Chapters 2.1–2.3**). Some of these new muscles, including the male-specific muscle, may be dependent upon innervation for differentiation and survival (Bate, 1993). Phagocytosis of degenerated (also referred to as histolyzed) muscle is a feature of the larval–pupal transition in *Drosophila* (Crossley, 1978; Bate, 1993), but phagocytic cells do not appear to be associated with dying muscles after adult eclosion, as has also been reported for *Manduca* ISMs (Kimura and Truman, 1990; Jones and Schwartz, 2001).

In contrast to the situation in *Manduca*, PCD of muscles during metamorphosis in *Drosophila* has not been a focus of research, and at present it is not possible to say more than that, most likely, the typical complement of cell death genes is expressed in dying muscle in a steroid-regulated manner. As described above (see Section 2.5.4.5.3), muscle abnormalities have been observed in transgenic *Drosophila* created to study genes identified in a screen of dying moth ISMs. Interest in the study of PCD of muscle in *Drosophila* may be stimulated by the development of fly muscle models for human diseases, such as spinal muscular atrophy (Chan *et al.*, 2003) and Parkinson's disease (Greene *et al.*, 2003). The latter studies are particularly interesting, because flies, with loss of function mutations in the *parkin* (*park*) gene, which encodes a ubiquitin E3 ligase, are associated with mitochondrial abnormalities: the involvement of ubiquitin regulation of proteolysis and the mitochondrial abnormalities suggests an important relationship between pathology and PCD in normal development (Haas *et al.*, 1995).

2.5.6. Future Directions

Many unanswered questions remain concerning PCD of the insect nerve and muscle during metamorphosis. A partial list includes the following:

- Are all deaths of neurons during insect metamorphosis controlled by ecdysteroids?
- What deaths of insect neurons during metamorphosis are apoptotic, if any? Why do different cell populations (or the same cell populations studied at different stages of development) display different forms of PCD?
- What is the relationship between apoptosis in the insect embryo and PCD during metamorphosis?
- Faced with evidence that some genes associated with death in insect muscles are not associated with death in insect neurons, can we conclude that there are tissue-specific death genes?
- What is the source of the segment specificity of PCD at the metamorphic transitions? Can we generalize some of the mechanisms that account for segment specificity in neuroblast apoptosis to postembryonic cell deaths?
- Why is phagocytosis not evident in many examples of metamorphic PCD?
- Since death-associated transcripts accumulate in the ISMs hours before their proteins can be detected or death begins, what molecular event triggers translation?

Answers to these questions are likely to expand our understanding of developmental PCD over a broad taxonomic range.

References

Abrams, J.M., **1999**. An emerging blueprint for apoptosis in *Drosophila*. *Trends Cell Biol.* *9*, 435–440.

Abrams, J.M., White, K., Fessler, L.I., Steller, H., **1993**. Programmed cell death during *Drosophila* embryogenesis. *Development* *117*, 29–43.

Ameisen, J.C., **2002**. On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. *Cell Death Differ.* *9*, 367–393.

Arnett, R.H.J., **1993**. American Insects: A Handbook of the Insects of America North of Mexico. Sandhill Crane Press, Gainesville, FL.

Baehrecke, E.H., **2000**. Steroid regulation of programmed cell death during *Drosophila* development. *Cell Death Differ. 7*, 1057–1062.

Baehrecke, E.H., **2002**. How death shapes life during development. *Nature Rev. Mol. Cell Biol. 3*, 779–787.

Bangs, P., White, K., **2000**. Regulation and execution of apoptosis during *Drosophila* development. *Devel. Dynam. 218*, 68–79.

Baker, R.T., Board, P.G., **1987**. The human ubiquitin gene family: structure of a gene and pseudogenes from the Ub B subfamily. *Nucl. Acids Res. 15*, 443–463.

Barres, B.A., Hart, I.K., Coles, H.S., Burne, J.F., Voyvodic, J.T., *et al.*, **1992**. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell 70*, 31–46.

Bate, M., **1993**. The mesoderm and its derivatives. In: Bate, M., Arias, A.M. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1013–1090.

Bayline, R.J., Khoo, A.B., Booker, R., **1998**. Innervation regulates the metamorphic fates of larval abdominal muscles in the moth, *Manduca sexta*. *Devel. Genes Evol. 208*, 369–381.

Beaulaton, J., Lockshin, R.A., **1977**. Ultrastructural study of the normal degeneration of the intersegmental muscles of *Antheraea polyphemus* and *Manduca sexta* (Insecta, Lepidoptera) with particular reference to autophagy. *J. Morphol. 154*, 39–58.

Bejsovsec, A., Wieschaus, E., **1993**. Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development 119*, 501–517.

Bell, R.A., Joachim, F.G., **1976**. Techniques for rearing laboratory cultures of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. America 69*, 365–373.

Bello, B.C., Hirth, F., Gould, A.P., **2003**. A pulse of the *Drosophila* Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron 37*, 209–219.

Ben-Sasson, A.A., Sherman, Y., Gavrieli, Y., **1995**. Identification of dying cells: *in situ* staining. In: Schwartz, L.M., Osborne, B.A. (Eds.), Cell Death. Academic Press, New York, pp. 29–39.

Bennett, K.L., Truman, J.W., **1985**. Steroid-dependent survival of identifiable neurons in cultured ganglia of the moth *Manduca sexta*. *Science 229*, 58–60.

Bergmann, A., Agapite, J., McCall, K., Steller, H., **1998**. The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. *Cell 95*, 331–341.

Bergmann, A., Tugentman, M., Shilo, B.Z., Steller, H., **2002**. Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling. *Devel. Cell 2*, 159–170.

Bidmon, H.-J., Koolman, J., **1989**. Ecdysteroid receptors located in the central nervous system of an insect. *Experientia 45*, 106–109.

Bidmon, H.-J., Sliter, T.J., **1990**. The ecdysteroid receptor. *Invertebr. Reprod. Devel. 18*, 13–27.

Bidmon, H.-J., Stumpf, W.E., Granger, N.A., **1992**. Ecdysteroid receptors in the neuroendocrine–endocrine axis of a moth. *Experientia 48*, 42–47.

Booker, R., Babashak, J., Kim, J.B., **1996**. Postembryonic neurogenesis in the central nervous system of the tobacco hornworm, *Manduca sexta*. 3. Spatial and temporal patterns of proliferation. *J. Neurobiol. 29*, 233–248.

Booker, R., Truman, J.W., **1987a**. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. 1. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol. 255*, 548–559.

Booker, R., Truman, J.W., **1987b**. Postembryonic neurogenesis in the CNS of the tobacco hornworm, *Manduca sexta*. 2. Hormonal control of imaginal nest cell differentiation during metamorphosis. *J. Neurosci. 7*, 4107–4114.

Booth, G.E., Kinrade, E.F., Hidalgo, A., **2000**. Glia maintain follower neuron survival during *Drosophila* CNS development. *Development 127*, 237–244.

Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., **1996**. The embryonic central nervous system lineages of *Drosophila melanogaster*. 1. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Devel. Biol. 179*, 41–64.

Brachmann, C.B., Jassim, O.W., Wachsmuth, B.D., Cagan, R.L., **2000**. The *Drosophila* bcl-2 family member dBorg-1 functions in the apoptotic response to UV-irradiation. *Curr. Biol. 10*, 547–550.

Bridgen, A., Weber, F., Fazakerley, J.K., Elliott, R.M., **2001**. *Bunyamwera bunyavirus* nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc. Natl Acad. Sci. USA 98*, 664–669.

Broadie, K.S., Bate, M., **1991**. Development of adult muscles in *Drosophila*: ablation of identified muscle precursor cells. *Development 113*, 103–118.

Bursch, W., **2001**. The autophagosomal–lysosomal compartment in programmed cell death. *Cell Death Differ. 8*, 569–581.

Bursch, W., Ellinger, A., Gerner, C., Frohwein, U., Schulte-Hermann, R., **2000**. Programmed cell death (PCD): apoptosis, autophagic PCD, or others? *Ann. New York Acad. Sci. 926*, 1–12.

Cascone, P.J., Schwartz, L.M., **2001**. Role of the 3′ UTR in regulating the stability and translatability of death-associated mRNAs in moth skeletal muscle. *Devel. Genes Evol. 211*, 397–405.

Chai, J., Du, C., Wu, J.W., Kyin, S., Wang, X., *et al.*, **2000**. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature 406*, 855–862.

Chan, Y.B., Miguel-Aliaga, I., Franks, C., Thomas, N., Trulzsch, B., *et al.*, **2003**. Neuromuscular defects in a *Drosophila* survival motor neuron gene mutant. *Hum. Mol. Genet. 12*, 1367–1376.

Chen, P., Nordstrom, W., Gish, B., Abrams, J.M., **1996**. *grim*, a novel cell death gene in *Drosophila*. *Genes Devel. 10*, 1773–1882.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., **2003**. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development 130*, 271–284.

Cho, N.K., Keyes, L., Johnson, E., Heller, J., Ryner, L., *et al.*, **2002**. Developmental control of blood cell migration by the *Drosophila* VEGF pathway. *Cell 108*, 865–876.

Choi, M.K., Fahrbach, S.E., **1995**. Evidence for an endogenous neurocidin in the *Manduca sexta* ventral nerve cord. *Arch. Insect Biochem. Physiol. 28*, 273–289.

Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S.I., *et al.*, **2002**. The damage-responsive *Drosophila* gene *sickle* encodes a novel IAP binding protein similar to but distinct from *reaper*, *grim*, and *hid*. *Curr. Biol. 12*, 137–140.

Ciechanover, A., **1998**. The ubiquitin–proteasome pathway: on protein death and cell life. *EMBO J. 17*, 7151–7160.

Clarke, P.G.H., **1990**. Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol. 181*, 195–213.

Claveria, C., Caminero, E., Martinez, A.C., Campuzano, S., Torres, M., **2002**. GH3, a novel proapoptotic domain in *Drosophila grim*, promotes a mitochondrial death pathway. *EMBO J. 21*, 3327–3336.

Colussi, P.A., Quinn, L.M., Huang, D.C., Coombe, M., Read, S.H., *et al.*, **2000**. Debcl, a proapoptotic Bcl-2 homologue, is a component of the *Drosophila melanogaster* cell death machinery. *J. Cell Biol. 148*, 703–714.

Consoulas, C., Restifo, L.L., Levine, R.B., **2002**. Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. *J. Neurosci. 22*, 4906–4917.

Crook, N.E., Clem, R.J., Miller, L.K., **1993**. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol. 67*, 2168–2174.

Crossley, A.C., **1968**. The fine structure and mechanism of breakdown of larval intersegmental muscles in the blowfly *Calliphora erythrocephala*. *J. Insect Physiol. 14*, 1389–1407.

Crossley, A.C., **1978**. The morphology and development of the *Drosophila* muscular system. In: Ashburner, M., Wright, T. (Eds.), The Genetics and Biology of *Drosophila*. Academic Press, New York, pp. 499–560.

Currie, D., Bate, M., **1991**. Development of adult abdominal muscles in *Drosophila*: adult myoblasts express *twist* and are associated with nerves. *Development 113*, 91–102.

Dawson, S.P., Arnold, J.E., Mayer, N.J., Reynolds, S.E., Billett, M.A., *et al.*, **1995**. Developmental changes of the 26S proteasome in abdominal intersegmental muscles of *Manduca sexta* during programmed cell death. *J. Biol. Chem. 270*, 1850–1858.

DeLorme, A.W., Mesce, K.A., **1999**. Programmed cell death of an identified motoneuron examined *in vivo*: electrophysiological and morphological correlates. *J. Neurobiol. 39*, 307–322.

Deveraux, Q.L., Reed, J.C., **1999**. IAP family proteins: suppressors of apoptosis. *Genes Devel. 13*, 239–252.

DiNardo, S., Heemskerk, J., Dougan, S., O'Farrell, P.H., **1994**. The making of a maggot: patterning the *Drosophila* embryonic epidermis. *Curr. Opin. Genet. Devel. 4*, 529–534.

Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., *et al.*, **2003**. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nature Cell Biol. 5*, 467–473.

Dive, C., Gregory, C.D., Phipps, D.J., Evans, D.L., Milner, A.E., *et al.*, **1992**. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim. Biophys. Acta 1133*, 275–285.

Dong, R., Jacobs, J.R., **1997**. Origin and differentiation of supernumerary midline glia in *Drosophila* embryos deficient for apoptosis. *Devel. Biol. 190*, 165–177.

Draizen, T.A., Ewer, J., Robinow, S., **1999**. Genetic and hormonal regulation of the death of peptidergic neurons in the *Drosophila* central nervous system. *J. Neurobiol. 38*, 455–465.

Driscoll, M., Gerstbrein, B., **2003**. Dying for a cause: invertebrate genetics takes on human neurodegeneration. *Nature Rev. Genet. 4*, 181–194.

Du, C., Fang, M., Li, Y., Li, L., Wang, X., **2000**. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. *Cell 102*, 33–42.

Eastman, A., **1995**. Assays for DNA fragmentation, endonucleases, and intracellular pH and Ca2+ associated with apoptosis. In: Schwartz, L.M., Osborne, B.A. (Eds.), Cell Death. Academic Press, New York, pp. 41–55.

Eaton, J.L., **1988**. Lepidopteran Anatomy. John Wiley, New York.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., *et al.*, **1998**. A caspase activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature 391*, 43–50.

Ewer, J., De Vente, J., Truman, J.W., **1994**. Neuropeptide induction of cyclic GMP increases in the insect CNS: resolution at the level of single identifiable neurons. *J. Neurosci. 14*, 7704–7712.

Ewer, J., Truman, J.W., **1997**. Invariant association of ecdysis with increases in cyclic 3′,5′-guanosine monophosphate immunoreactivity in a small network of peptidergic neurons in the hornworm, *Manduca sexta*. *J. Comp. Physiol. A 181*, 319–330.

Ewer, J., Wang, C.M., Klukas, K.A., Mesce, K.A., Truman, J.W., *et al.*, **1998**. Programmed cell death of identified peptidergic neurons involved in ecdysis behavior in the moth, *Manduca sexta*. *J. Neurobiol. 37*, 265–280.

Fadok, V.A., Bratton, D.L., Guthrie, L., Henson, P.M., **2001**. Differential effects of apoptostic versus lysed cells on macrophage production of cytokines: role of proteases. *J. Immunol. 166*, 6847–6854.

Fadok, V.A., Chimini, G., **2001**. The phagocytosis of apoptotic cells. *Semin. Immunol. 13*, 365–372.

Fahrbach, S.E., **1992**. Developmental regulation of ecdysteroid receptors in the nervous system of *Manduca sexta*. *J. Exp. Zool. 261*, 245–253.

Fahrbach, S.E., **1997**. The regulation of neuronal death during insect metamorphosis. *BioScience 47*, 77–85.

Fahrbach, S.E., Choi, M.K., Truman, J.W., **1994**. Inhibitory effects of actinomycin D and cycloheximide on neuronal death in adult *Manduca sexta*. *J. Neurobiol. 25*, 59–69.

Fahrbach, S.E., DeLorme, A.W., Klukas, K.A., Mesce, K.A., **1995**. A motoneuron spared from steroid-activated developmental death by removal of descending neural inputs exhibits stable electrophysiological properties and morphology. *J. Neurobiol. 26*, 511–522.

Fahrbach, S.E., Schwartz, L.M., **1994**. Localization of immunoreactive ubiquitin in the nervous system of the *Manduca sexta* moth. *J. Comp. Neurol. 343*, 464–482.

Fahrbach, S.E., Truman, J.W., **1987**. Possible interactions of a steroid hormone and neural inputs in controlling the death of an identified neuron in the moth *Manduca sexta*. *J. Neurobiol. 18*, 497–508.

Fahrbach, S.E., Truman, J.W., **1989**. Autoradiographic identification of ecdysteroid-binding cells in the nervous system of the moth *Manduca sexta*. *J. Neurobiol. 20*, 681–702.

Fichelson, P., Gho, M., **2003**. The glial cell undergoes apoptosis in the microchaete lineage of *Drosophila*. *Development 130*, 123–133.

Finlayson, L.H., **1956**. Normal and induced degeneration of abdominal muscles during metamorphosis in the Lepidoptera. *Q. J. Microsc. Sci. 97*, 215–233.

Franc, N.C., **2002**. Phagocytosis of apoptotic cells in mammals, *Caenorhabditis elegans*, and *Drosophila melanogaster*: molecular mechanisms and physiological consequences. *Frontiers Biosci. 1*, 1298–1313.

French, V., **2001**. Insect segmentation: genes, stripes and segments in "Hoppers." *Curr. Biol. 11*, R910–R913.

Gammie, S.C., Truman, J.W., **1997**. Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. *J. Neurosci. 17*, 4389–4397.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., **1992**. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol. 119*, 493–501.

Geske, F.J., Monks, J., Lehman, L., Fadok, V.A., **2002**. The role of the macrophage in apoptosis: hunter, gatherer, and regulator. *Int. J. Hematol. 76*, 16–26.

Giebultowicz, J.M., Truman, J.W., **1984**. Sexual differentiation in the terminal ganglion of the moth *Manduca sexta*: role of sex-specific neuronal death. *J. Comp. Neurol. 226*, 87–95.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Glücksmann, A., **1951**. Cell deaths in normal vertebrate ontogeny. *Biol. Rev. 26*, 59–86.

Goodman, C.S., Doe, C.Q., **1993**. Embryonic development of the *Drosophila* central nervous system. In: Bate, M., Arias, A.M. (Eds.), The Development of *Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1131–1206.

Gorski, S., Marra, M., **2002**. Programmed cell death takes flight: genetic and genomic approaches to gene discovery in *Drosophila*. *Physiol. Genom. 9*, 59–69.

Goyal, L., McCall, K., Agapite, J., Hartwieg, E., Steller, H., **2000**. Inhibition of apoptosis by *Drosophila reaper*, *hid*, and *grim* through inhibition of IAP function. *EMBO J. 19*, 589–597.

Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M.B., *et al.*, **2003**. Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* parkin mutants. *Proc. Natl Acad. Sci. USA 100*, 4078–4083.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., Steller, H., **1995**. The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Devel. 9*, 1694–1708.

Guo, M., Jan, L.Y., Jan, Y.N., **1996**. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron 17*, 27–41.

Haas, A.L., Baboshina, O., Williams, B., Schwartz, L.M., **1995**. Coordinated induction of the ubiquitin pathway accompanies the developmentally programmed death of insect skeletal muscle. *J. Biol. Chem. 270*, 9407–9412.

Hashimoto, M.K., Mykles, D.L., Schwartz, L.M., Fahrbach, S.E., **1996**. Imaginal cell-specific accumulation of the multicatalytic proteinase complex (proteasome) during post-embryonic development in the tobacco hornworm, *Manduca sexta*. *J. Comp. Neurol. 365*, 329–341.

Hay, B.A., **2000**. Understanding IAP function and regulation: a view from *Drosophila*. *Cell Death Differ. 7*, 1045–1056.

Hay, B.A., Wassarman, D.A., Rubin, G.M., **1995**. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell 83*, 1253–1262.

Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., *et al.*, **2002**. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J. Biol. Chem. 277*, 432–438.

Hegstrom, C.D., Riddiford, L.M., Truman, J.W., **1998**. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth, *Manduca sexta*. *J. Neurosci. 18*, 1786–1794.

Hengartner, M.O., **2000**. The biochemistry of apoptosis. *Nature 407*, 770–776.

Henson, P.M., Bratton, D.L., Fadok, V.A., **2001**. Apoptotic cell removal. *Curr. Biol. 11*, R795–R805.

Hershko, A., Ciechanover, A., **1998**. The ubiquitin system. *Annu. Rev. Biochem. 67*, 425–479.

Hidalgo, A., Booth, G.E., **2000**. Glia dictate pioneer axon trajectories in the *Drosophila* embryonic CNS. *Development 127*, 393–402.

Hidalgo, A., Kinrade, F., Georgiou, M., **2001**. The *Drosophila* neuregulin *vein* maintains glial survival during axon guidance in the CNS. *Devel. Cell 1*, 679–690.

Hirata, J., Nakagoshi, H., Nabeshima, Y., Matsuzaki, F., **1995**. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature 377*, 627–630.

Hoffman, K.L., Weeks, J.C., **1998**. Programmed cell death of an identified motoneuron *in vitro*: temporal requirements for steroid exposure and protein synthesis. *J. Neurobiol. 35*, 300–322.

Hoffman, K.L., Weeks, J.C., **2001**. Steroid-mediated programmed cell death of motoneurons: required role of caspases and mitochondrial events. *Devel. Biol. 229*, 517–536.

Holley, C.L., Olson, M.R., Colon-Ramos, D.A., Kornbluth, S., **2002**. *Reaper* eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nature Cell Biol. 4*, 439–444.

Horvitz, H.R., Shaham, S., Hengartner, M.O., **1994**. The genetics of programmed cell death in the nematode *Caenorhabditis elegans*. *Cold Spring Harbor Symp. Quant. Biol. 59*, 377–385.

Hoy, M.A., **1994**. Insect Molecular Genetics. Academic Press, New York.

Hu, Y., Cascone, P., Cheng, L., Sun, D., Nambu, J.R., *et al.*, **1999**. Lepidopteran DALP, and its mammalian ortholog Hic-5, function as negative regulators of muscle differentiation. *Proc. Natl Acad. Sci. USA 96*, 10218–10223.

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., *et al.*, **2001**. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell 104*, 781–790.

Igaki, T., Kanuka, H., Inohara, N., Sawamoto, K., Nunez, G., *et al.*, **2000**. Drob-1, a *Drosophila* member of the Bcl-2/CED-9 family that promotes cell death. *Proc. Natl Acad. Sci. USA 97*, 662–667.

Jacobs, J.R., **2000**. The midline glia of *Drosophila*: a molecular genetic model for the developmental functions of glia. *Progr. Neurobiol. 62*, 475–508.

Jacobson, M.D., Weil, M., Raff, M.C., **1997**. Programmed cell death in animal development. *Cell 88*, 347–354.

Jones, B.W., **2001**. Glial cell development in the *Drosophila* embryo. *BioEssays 23*, 877–887.

Jones, G., Jones, D., **2000**. Considerations on the structural evidence of a ligand-binding function of *ultraspiracle*, an insect homolog of vertebrate RXR. *Insect Biochem. Mol. Biol. 30*, 671–679.

Jones, M.E.E., Schwartz, L.M., **2001**. Not all muscles meet the same fate when they die. *Cell Biol. Int. 25*, 539–545.

Jones, M.E.E., Haire, M.F., Kloetzel, P.M., Mykles, D.L., Schwartz, L.M., **1995**. Changes in the structure and function of the multicatalytic proteinase (proteasome) during programmed cell death in the intersegmental muscles of the hawkmoth, *Manduca sexta*. *Devel. Biol. 169*, 437–447.

Jones, R.G., Davis, W.L., Hung, A.C., Vinson, S.B., **1978**. Insemination-induced histolysis of the flight musculature in fire ants (*Solenopsis* spp.). *Am. J. Anat. 151*, 603–610.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H., *et al.*, **1999**. Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Mol. Cell 4*, 757–769.

Kaprielian, Z., Imondi, R., Runko, E., **2000**. Axon guidance at the midline of the developing CNS. *Anat. Rec. 261*, 176–197.

Kerr, J.F.R., Wyllie, A.H., Currie, A.R., **1972**. Apoptosis: a basic biological process with wide-ranging implications in tissue kinetics. *Br. J. Cancer 26*, 239–257.

Kimura, K.-I., Truman, J.W., **1990**. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster*. *J. Neurosci. 10*, 403–411.

Kinch, G., Hoffman, K.L., Rodrigues, E.M., Zee, M.C., Weeks, J.C., **2003**. Steroid-triggered programmed cell death of a motoneuron is autophagic and involves structural changes in mitochondria. *J. Comp. Neurol. 457*, 384–403.

Kinrade, E.F., Brates, T., Tear, G., Hidalgo, A., **2001**. *Roundabout* signalling, cell contact and trophic support confine longitudinal glia and axons in the *Drosophila* CNS. *Development 128*, 207–216.

Klambt, C., Hummel, T., Granderath, S., Schimmelpfeng, K., **2001**. Glial cell development in *Drosophila*. *Int. J. Devel. Neurosci. 19*, 373–378.

Klambt, C., Jacobs, J.R., Goodman, C.S., **1991**. The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell 64*, 801–815.

Knoblich, J.A., Jan, L.Y., Jan, Y.N., **1995**. Asymmetric segregation of Numb and Prospero during cell division. *Nature 377*, 624–627.

Koelle, M.R., Talbot, W.S., Seagraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.

Kraft, R., Jäckle, H., **1994**. *Drosophila* mode of metamerization in the embryogenesis of the lepidopteran insect, *Manduca sexta*. *Proc. Natl Acad. Sci. USA 91*, 6634–6638.

Kroemer, G., Dallaporta, B., Resche-Rigon, M., **1998**. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu. Rev. Physiol. 60*, 619–642.

Kuelzer, F., Kuah, K., Bishoff, S.J., Cheng, L., Nambu, J., *et al.*, **1999**. SCLP (small cytoplasmic leucine-rich repeat protein) encodes a novel protein that is dramatically up-regulated during the programmed death of moth skeletal muscle. *J. Neurobiol. 41*, 482–494.

Kurada, P., White, K., **1998**. Ras promotes cell survival in *Drosophila* by downregulating Hid expression. *Cell 95*, 319–329.

Kuwana, **1936**. Degeneration of muscles in the silkworm moth. *Zool. Mag. Tokyo 48*, 881–884.

Laity, J.H., Lee, B.M., Wright, P.E., **2001**. Zinc finger proteins: new insights into structural and functional diversity. *Curr. Opin. Struct. Biol. 11*, 39–46.

Lee, C.-Y., Clough, E.A., Yellon, P., Teslovich, T.M., Stephan, D.A., *et al.*, **2003**. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*. *Curr. Biol. 13*, 350–357.

Lee, C.Y., Baehrecke, E.H., **2001**. Steroid regulation of autophagic programmed cell death during development. *Development 128*, 1443–1455.

Lehmann, M., Jiang, C., Ip, Y.T., Thummel, C.S., **2002**. AP-1, but not NF-kappa B, is required for efficient steroid-triggered cell death in *Drosophila*. *Cell Death Differ. 9*, 581–590.

Leon, O., Roth, M., **2000**. Zinc fingers: DNA binding and protein–protein interactions. *Biol. Res. 33*, 21–30.

Levine, R.B., Truman, J.W., **1985**. Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. *J. Neurosci. 5*, 2424–2431.

Lewis, E.B., **1978**. A gene complex controlling segmentation in *Drosophila*. *Nature 276*, 565–570.

Lisi, S., Mazzon, I., White, K., **2000**. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. *Genetics 154*, 669–678.

Liu, Q.A., Hengartner, M.O., **1999**. The molecular mechanism of programmed cell death in *C. elegans*. *Ann. New York Acad. Sci. 887*, 92–104.

Liu, Z., Sun, C., Olejniczak, E.T., Meadows, R.P., Betz, S.F., *et al.*, **2000**. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature 408*, 1004–1008.

Lockshin, R., **1963**. Programmed Cell Death in an Insect. Harvard University Press, Cambridge, MA.

Lockshin, R.A., **1969**. Programmed cell death: activation of lysis by a mechanism involving the synthesis of protein. *J. Insect Physiol. 15*, 1505–1516.

Lockshin, R.A., Beaulaton, J., **1974**. Programmed cell death: cytochemical evidence for lysosomes during the normal breakdown of the intersegmental muscles. *J. Ultrastruct. Res. 46*, 43–62.

Lockshin, R.A., Beaulaton, J., **1979**. Programmed cell death: electrophysiological and ultrastructural correlations in metamorphosing muscles of lepidopteran insects. *Tissue and Cell 11*, 803–819.

Lockshin, R.A., Williams, C.A., **1964**. Programmed cell death. 2. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. *J. Insect Physiol. 10*, 643–649.

Lockshin, R.A., Williams, C.A., **1965a**. Programmed cell death. 1. Histology and cytology of the breakdown of the intersegmental muscles in saturniid moths. *J. Insect Physiol. 11*, 123–133.

Lockshin, R.A., Williams, C.A., **1965b**. Programmed cell death. 3. Neural control of the breakdown of the intersegmental muscles of silkmoths. *J. Insect Physiol. 11*, 601–610.

Lockshin, R.A., Williams, C.M., **1965c**. Programmed cell death. 4. The influence of drugs on the breakdown of the intersegmental muscles of silkmoths. *J. Insect Physiol. 11*, 803–809.

Low, P., Bussell, K., Dawson, S.P., Billett, M.A., Mayer, R.J., *et al.*, **1997**. Expression of a 26S proteasome ATPase subunit, MS73, in muscles that undergo developmentally programmed cell death, and its control by ecdysteroid hormones in the insect *Manduca sexta*. *FEBS Lett. 400*, 345–349.

Lundell, M.J., Lee, H.K., Perez, E., Chadwell, L., **2003**. The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of *Drosophila*. *Development 130*, 4109–4121.

MacDonald, R.L., Stoodley, M., **1998**. Pathophysiology of cerebral ischemia. *Neurol. Med. Chir. Tokyo 38*, 1–11.

McCall, K., Steller, H., **1997**. Facing death in the fly: genetic analysis of apoptosis in *Drosophila*. *Trends Genet. 13*, 222–226.

McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., *et al.*, **1997**. Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron 19*, 813–823.

Martins, L.M., **2002**. The serine protease Omi/HtrA2: a second mammalian protein with a Reaper-like function. *Cell Death Differ. 9*, 699–701.

Meier, P., Finch, A., Evan, G., **2000**. Apoptosis in development. *Nature 407*, 796–801.

Mesce, K.A., Fahrbach, S.E., **2002**. Integration of endocrine signals that regulate insect ecdysis. *Front. Neuroendocrinol. 23*, 179–199.

Metchnikoff, E., **1892**. La phagocytose musculaire. 1. Atrophie des muscles pendant la transformation des batéaciens. *Ann. Inst. Pasteur 6*, 1–12.

Miller, A., **1950**. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: Demerec, M. (Ed.), Biology of *Drosophila*. John Wiley, New York, pp. 420–534.

Miller, L.K., **1999**. An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol. 9*, 323–328.

Milligan, C.M., Schwartz, L.M., **1997**. Programmed cell death during animal development. *Br. Med. Bull. 53*, 570–590.

Monsma, S.A., Booker, R., **1996a**. Genesis of the adult retina and outer optic lobes of the moth, *Manduca sexta*. 1. Patterns of proliferation and cell death. *J. Comp. Neurol. 367*, 10–20.

Monsma, S.A., Booker, R., **1996b**. Genesis of the adult retina and outer optic lobes of the moth, *Manduca sexta*. 2. Effects of deafferentation and developmental hormone manipulation. *J. Comp. Neurol. 367*, 21–35.

Montemayor, M.E., Fahrbach, S.E., Giometti, C.S., Roy, E.J., **1990**. Characterization of a protein that appears in the nervous system of the moth *Manduca sexta* coincident with neuronal death. *FEBS Lett. 276*, 219–222.

Muqit, M.M., Feany, M.B., **2002**. Modelling neurodegenerative diseases in *Drosophila*: a fruitful approach? *Nature Rev. Neurosci. 3*, 237–243.

Murphey, R.K., Godenschwege, T.A., **2002**. New roles for ubiquitin in the assembly and function of neuronal circuits. *Neuron 36*, 5–8.

Mutsuddi, M., Nambu, J.R., **1998**. Neural disease: *Drosophila* degenerates for a good cause. *Curr. Biol. 8*, R809–R811.

Myer, A., Schwartz, L.M., **1996**. Allelic variation in the polyubiquitin gene in the tobacco hawkmoth *Manduca sexta* and its regulation by heat shock and programmed cell death. *Insect Biochem. Mol. Biol. 26*, 1037–1046.

Nagy, L.M., Booker, R., Riddiford, L.M., **1991**. Isolation and embryonic expression of an abdominal-A-like gene

from the lepidopteran, *Manduca sexta*. *Development 112*, 119–129.

Nijhout, H.F., **1994**. Insect Hormones. Princeton University Press, Princeton, NJ.

Nishikawa, A., Murata, E., Akita, M., Kaneko, K., Moriya, O., *et al*., **1998**. Role of macrophages in programmed cell death and remodeling of tail and body muscle of *Xenopus laevis* during metamorphosis. *Histochem. Cell Biol. 109*, 11–17.

Nixon, R.A., Cataldo, A.M., **1993**. The lysosomal system in neuronal cell death: a review. *Ann. New York Acad. Sci. 679*, 87–109.

Olson, M.R., Holley, C.L., Gan, E.C., Colon-Ramos, D.A., Kaplan, B., *et al*., **2003**. A GH3-like domain in reaper required for mitochondrial localization and induction of IAP degradation. *J. Biol. Chem. 278*, 44758–44768.

Orgogozo, V., Schweisguth, F., Bellaiche, Y., **2002**. Binary cell death decision regulated by unequal partitioning of Numb at mitosis. *Development 129*, 4677–4684.

Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., Ewer, J., **2003**. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and timing of ecdysis behavior. *Development 130*, 2645–2656.

Peterson, C., Carney, G.E., Taylor, B.J., White, K., **2002**. *reaper* is required for neuroblast apoptosis during *Drosophila* development. *Development 129*, 1467–1476.

Platt, N., da Silva, R.P., Gordon, S., **1998**. Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol. 8*, 365–372.

Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., *et al*., **1993**. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science 262*, 695–700.

Rechsteiner, M., **1988**. Ubiquitin. Plenum Press, New York.

Renn, S.C.P., Park, J.H., Rosbash, M., Hall, J.C., Taghert, P.H., **1999**. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell 99*, 781–802.

Rheuben, M.B., **1992**. Degenerative changes in the muscle fibers of *Manduca sexta* during metamorphosis. *J. Exp. Biol. 167*, 91–117.

Richardson, H., Kumar, S., **2002**. Death to flies: *Drosophila* as a model system to study programmed cell death. *J. Immunol. Methods 265*, 21–38.

Riddiford, L.M., **1993**. Hormones and *Drosophila* development. In: Bate, M., Arias, A.M. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 899–939.

Robinow, S., Draizen, T.A., Truman, J.W., **1997**. Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. *Devel. Biol. 190*, 206–213.

Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., **1993**. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development 119*, 1251–1259.

Robinson-Rechavi, M., Garcia, H., Laudet, V., **2003**. The nuclear receptor superfamily. *J. Cell Sci. 116*, 585–586.

Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., *et al*., **1999**. *Dark* is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nature Cell Biol. 1*, 272–279.

Rulifson, E.J., Kim, S.K., Nusse, R., **2002**. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science 296*, 1118–1120.

Runion, H.I., Pipa, R.L., **1970**. Electrophysiological and endocrinological correlates during the metamorphic degeneration of a muscle fibre in *Galleria mellonella* (L.) (Lepidoptera). *J. Exp. Biol. 53*, 9–24.

Sanchez-Herrero, E., Vernos, I., Marco, R., Morata, G., **1985**. Genetic organization of *Drosophila* bithorax complex. *Nature 313*, 108–113.

Sanson, B., **2001**. Generating patterns from fields of cells: examples from *Drosophila* segmentation. *EMBO Rep. 2*, 1083–1088.

Savill, J., Fadok, V., **2000**. Corpse clearance defines the meaning of cell death. *Nature 407*, 784–788.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., *et al*., **1997**. The embryonic central nervous system lineages of *Drosophila melanogaster*. 2. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Devel. Biol. 189*, 186–204.

Schneiderman, A.M., Matsumoto, S.G., Hildebrand, J.G., **1982**. Trans-sexually grafted antennae influence development of sexually dimorphic neurons in moth brain. *Nature 298*, 844–846.

Schnepp, B., Grumbling, G., Donaldson, T., Simcox, A., **1996**. *Vein* is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins. *Genes Devel. 10*, 2302–2313.

Schwartz, D.C., Hochstrasser, M., **2003**. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. *Trends Biochem. Sci. 28*, 321–328.

Schwartz, L.M., **1992**. Insect muscle as a model for programmed cell death. *J. Neurobiol. 23*, 1312–1326.

Schwartz, L.M., Kosz, L., Kay, B.K., **1990a**. Gene activation is required for developmentally programmed cell death. *Proc. Natl Acad. Sci. USA 87*, 6594–6598.

Schwartz, L.M., Myer, A., Kosz, L., Engelstein, M., Maier, C., **1990b**. Activation of polyubiquitin gene expression during developmentally programmed cell death. *Neuron 5*, 411–419.

Schwartz, L.M., Ruff, R.L., **2002**. Changes in contractile properties of skeletal muscle during developmentally programmed atrophy and death. *Am. J. Physiol. Cell Physiol. 282*, C1270–C1277.

Schwartz, L.M., Smith, S.W., Jones, M.E.E., Osborne, B.A., **1993**. Do all programmed cell deaths occur via apoptosis? *Proc. Natl Acad. Sci. USA 90*, 980–984.

Schwartz, L.M., Truman, J.W., **1982**. Peptide and steroid regulation of muscle degeneration in an insect. *Science 215*, 1420–1421.

Schwartz, L.M., Truman, J.W., **1983**. Hormonal control of rates of metamorphic development in the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 99*, 103–114.

Schwartz, L.M., Truman, J.W., **1984a**. Cyclic GMP may serve as a second messenger in peptide-induced muscle degeneration in an insect. *Proc. Natl Acad. Sci. USA 81*, 6718–6722.

Schwartz, L.M., Truman, J.W., **1984b**. Hormonal control of muscle atrophy and degeneration in the moth *Antheraea polyphemus*. *J. Exp. Biol. 111*, 13–30.

Sears, H.C., Kennedy, C.J., Garrity, P.A., **2003**. Macrophage-mediated corpse engulfment is required for normal *Drosophila* CNS morphogenesis. *Development 130*, 3557–3565.

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., Owen, J.J., **1989**. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature 337*, 181–184.

Song, Z., McCall, K., Steller, H., **1997**. DCP-1, a *Drosophila* cell death protease essential for development. *Science 275*, 536–540.

Sonnenfeld, M.J., Jacobs, J.R., **1995a**. Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development 121*, 569–578.

Sonnenfeld, M.J., Jacobs, J.R., **1995b**. Macrophages and glia participate in the removal of apoptotic neurons from the *Drosophila* embryonic nervous system. *J. Comp. Neurol. 359*, 644–652.

Spana, E.P., Doe, C.Q., **1996**. Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron 17*, 21–26.

Spana, E.P., Kopczynski, C., Goodman, C.S., Doe, C.Q., **1995**. Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. *Development 121*, 3489–3494.

Srinivasula, S.M., Datta, P., Fan, X.J., Fernandes-Alnemri, T., Huang, Z., *et al.*, **2000**. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J. Biol. Chem. 275*, 36152–36157.

Srinivasula, S.M., Datta, P., Kobayashi, M., Wu, J.W., Fujioka, M., *et al.*, **2002**. *sickle*, a novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-inhibitory protein. *Curr. Biol. 12*, 125–130.

Steller, H., **1995**. Mechanisms and genes of cellular suicide. *Science 267*, 1445–1449.

Steller, H., Grether, M.E., **1994**. Programmed cell death in *Drosophila*. *Neuron 13*, 1269–1274.

Stocker, R.F., Edwards, J.S., Truman, J.W., **1978**. Fine structure of degenerating moth abdominal motorneurons after eclosion. *Cell Tissue Res. 191*, 317–331.

Streichert, L.C., Pierce, J.T., Weeks, J.C., **1997**. Steroid hormones act directly to trigger segment-specific programmed cell death of identified neurons *in vitro*. *Devel. Biol. 183*, 95–107.

Sun, D., Ziegler, R., Milligan, C.E., Fahrbach, S.E., Schwartz, L.M., **1995**. Apoliphorin III is dramatically up-regulated during the programmed death of insect skeletal muscle and neurons. *J. Neurobiol. 26*, 119–129.

Sutherland, E.W., **1972**. Studies on the mechanism of hormone action. *Science 177*, 401–408.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., *et al.*, **2001a**. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell 8*, 613–621.

Suzuki, Y., Nakabayashi, Y., Takahashi, R., **2001b**. Ubiquitin–protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc. Natl Acad. Sci. USA 98*, 8662–8667.

Swindle, J., Ajioka, J., Eisen, H., Sanwal, B., Jacquemot, C., *et al.*, **1988**. The genomic organization of the ubiquitin genes of *Trypanosoma cruzi*. *EMBO J. 7*, 1121–1127.

Takayanagi, K., Dawson, S., Reynolds, S.E., Mayer, R.J., **1996**. Specific developmental changes in the regulatory subunits of the 26S proteasome in intersegmental muscles preceding eclosion in *Manduca sexta*. *Biochem. Biophys. Res. Commun. 228*, 517–523.

Talbot, W.S., Swyryd, E.A., Hogness, D.S., **1993**. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell 73*, 1323–1337.

Taylor, H.M., Truman, J.W., **1974**. Metamorphosis of the abdominal ganglia of the tobacco hornworm, *Manduca sexta*: changes in population of identified motor neurons. *J. Comp. Physiol. 90*, 367–388.

Tenev, T., Zachariou, A., Wilson, R., Paul, A., Meier, P., **2002**. Jafrac2 is an IAP antagonist that promotes cell death by liberating Dronc from DIAP1. *EMBO J. 21*, 5118–5229.

Tepass, U., Fessler, L.I., Aziz, A., Hartenstein, V., **1994**. Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*. *Development 120*, 1829–1837.

Thorn, R.S., Truman, J.W., **1989**. Sex-specific neuronal respecification during the metamorphosis of the genital segments of the tobacco hornworm moth *Manduca sexta*. *J. Comp. Neurol. 284*, 489–503.

Thorn, R.S., Truman, J.W., **1994a**. Sexual differentiation in the central nervous system of the moth *Manduca sexta*. 1. Sex and segment-specificity in production, differentiation, and survival of the imaginal midline neurons. *J. Neurobiol. 25*, 1039–1044.

Thorn, R.S., Truman, J.W., **1994b**. Sexual differentiation in the central nervous system of the moth, *Manduca sexta*. 2. Target dependence for the survival of the imaginal midline neurons. *J. Neurobiol. 10*, 1054–1066.

Thress, K., Henzel, W., Shillinglaw, W., Kornbluth, S., **1998**. Scythe: a novel reaper-binding apoptotic regulator. *EMBO J. 17*, 6135–6143.

Tittel, J.N., Steller, H., **2000**. A comparison of programmed cell death between species. *Genome Biol. 1*(3), REVIEWS0003.

Thummel, C.S., **2001**. Steroid-triggered death by autophagy. *BioEssays 23*, 677–682.

Truman, J.W., **1983**. Programmed cell death in the nervous system of an adult insect. *J. Comp. Neurol. 216*, 445–452.

Truman, J.W., **1984**. Ecdysteroids regulate the release and action of eclosion hormone in the moth *Manduca sexta*. In: Hoffman, J., Porchet, M. (Eds.), Biosynthesis, Metabolism, and Mode of Action of Invertebrate Hormones. Springer, New York, pp. 136–144.

Truman, J.W., Bate, M., **1988**. Spatial and temporal patterns of neurogenesis in the CNS of *Drosophila melanogaster. Devel. Biol. 125*, 146–157.

Truman, J.W., Fahrbach, S.E., Kimura, K.-I., **1990**. Hormones and programmed cell death: insights from invertebrate studies. In: Coleman, P., Higgins, G., Phelps, C. (Eds.), Progress in Brain Research. Elsevier, Amsterdam, pp. 25–35.

Truman, J.W., Riddiford, L.M., **2002**. Insect developmental hormones and their mechanism of action. In: Pfafff, D.W. (Ed.), Hormones, Brain and Behavior. Academic Press, New York, pp. 841–873.

Truman, J.W., Schwartz, L.M., **1984**. Steroid regulation of neuronal death in the moth nervous system. *J. Neurosci. 4*, 274–280.

Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., **1994**. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development 120*, 219–234.

Truman, J.W., Taylor, B.J., Awad, T.A., **1993**. Formation of the adult nervous system. In: Bate, M., Arias, A.M. (Eds.), The Development of *Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1245–1275.

Truman, J.W., Thorn, R.S., Robinow, S., **1992**. Programmed neuronal death in insect development. *J. Neurobiol. 23*, 1295–1311.

van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S.M., Rodriguez, I., *et al.*, **2002**. The serine protease Omi/HtrA2 is released from mitochondria during apoptosis: Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death Differ. 9*, 20–26.

Vaux, D.L., Silke, J., **2003**. Mammalian mitochondrial IAP binding proteins. *Biochem. Biophys. Res. Commun. 304*, 499–504.

Verhagen, A.M., Coulson, E.J., Vaux, D.L., **2001**. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol. 2*(7), REVIEWS3009.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., *et al.*, **2000**. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell 102*, 43–53.

Verhagen, A.M., Silke, J., Ekert, P.G., Pakusch, M., Kaufmann, H., *et al.*, **2002**. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J. Biol. Chem. 277*, 445–454.

Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J., *et al.*, **2000**. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol. 150*, 69–76.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A., Hay, B.A., **1999**. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell 98*, 453–463.

Watanabe, K., Sassaki, F., **1974**. Ultrastructural changes in regressing tail muscles of anuran tadpoles during metamorphosis. *Cell Tissue Res. 155*, 321–336.

Weber, R., **1964**. Ultrastructural changes in regressing tail muscles of *Xenopus* larvae at metamorphosis. *J. Cell Biol. 68*, 251–306.

Weber, R., **1965**. Inhibitory effect of actinomycin D on tail atrophy in *Xenopus* larvae at metamorphosis. *Experientia 21*, 665–666.

Weeks, J.C., **1987**. Time course of hormonal independence for developmental events in neurons and other cell types during insect metamorphosis. *Devel. Biol. 124*, 163–176.

Weeks, J.C., **1999**. Steroid hormones, dendritic remodeling and neuronal death: insights from insect metamorphosis. *Brain Behav. Evol. 54*, 51–60.

Weeks, J.C., Davidson, S.K., **1994**. Influence of interganglionic interactions on steroid-mediated dendritic reorganization and death of proleg motor neurons during metamorphosis in *Manduca sexta*. *J. Neurobiol. 25*, 535–554.

Weeks, J.C., Davidson, S.K., Debu, B.H.G., **1993**. Effects of a protein synthesis inhibitor on the hormonally mediated regression and death of motoneurons in the tobacco hornworm, *Manduca sexta*. *J. Neurobiol. 24*, 125–140.

Weeks, J.C., Ernst-Utzschneider, K., **1989**. Respecification of larval proleg motoneurons during metamorphosis of the tobacco hornworm, *Manduca sexta*: segmental dependence and hormonal regulation. *J. Neurobiol. 20*, 569–592.

Weeks, J.C., Roberts, W.M., Trimble, D.L., **1992**. Hormonal regulation and segmental specificity of motoneuron phenotype during metamorphosis of the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 149*, 185–196.

Weeks, J.C., Truman, J.W., **1985**. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. *J. Neurosci. 5*, 2290–2300.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., *et al.*, **1994**. Genetic control of programmed cell death in *Drosophila*. *Science 264*, 677–683.

White, K., Tahaoglu, E., Steller, H., **1996**. Cell killing by the *Drosophila* gene *reaper*. *Science 271*, 805–807.

Wiborg, O., Pederson, M.S., Wind, A., Berglund, L.E., Marcker, K.A., *et al.*, **1985**. The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences. *EMBO J. 4*, 755–759.

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., *et al.*, **2002**. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nature Cell Biol. 4*, 445–450.

Wing, J.P., Karres, J.S., Ogdahl, J.L., Zhou, L., Schwartz, L.M., *et al.*, **2002**. *Drosophila sickle* is a novel grim-reaper cell death activator. *Curr. Biol. 12*, 131–135.

Wing, J.P., Nambu, J.R., **1998a**. Apoptosis in *Drosophila*. In: Potten, C.S., Booth, C., Wilson, J.W. (Eds.), Apoptosis Regulatory Genes. Chapman and Hall, London, pp. 205–241.

Wing, J.P., Schwartz, L.M., Nambu, J.R., **2001**. The RHG motifs of *Drosophila* Reaper and Grim are important for their distinct cell death-inducing abilities. *Mech. Devel. 102*, 193–203.

Wing, J.P., Zhou, L., Schwartz, L.M., Nambu, J.R., **1998b**. Distinct cell killing properties of the *Drosophila reaper*, *head involution defective*, and *grim* genes. *Cell Death Differ. 5*, 930–939.

Wu, J.W., Cocina, A.E., Chai, J., Hay, B.A., Shi, Y., **2001**. Structural analysis of a functional DIAP1 fragment bound to Grim and Hid peptides. *Mol. Cell 8*, 95–104.

Wyllie, A.H., Morris, R.G., Smith, A.L., Dunlop, D., **1984**. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol. 142*, 67–77.

Yaffe, D., Saxel, O., **1977**. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature 270*, 725–727.

Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M., Ashwell, J.D., **2000**. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science 288*, 874–877.

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., *et al.*, **2002**. *hid*, *rpr* and *grim* negatively regulate DIAP1 levels through distinct mechanisms. *Nature Cell Biol. 4*, 416–424.

Yoshizato, K., **1996**. Cell death and histolysis in amphibian tail during metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, New York, pp. 647–671.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., Horvitz, H.R., **1993**. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell 75*, 641–652.

Zee, M.C., Weeks, J.C., **2001**. Developmental change in the steroid hormone signal for cell-autonomous, segment-specific programmed cell death of a motoneuron. *Devel. Biol. 235*, 45–61.

Zhang, H., Huang, Q., Ke, N., Matsuyama, S., Hammock, B., *et al.*, **2000**. *Drosophila* pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *J. Biol. Chem. 275*, 27303–27306.

Zhou, L., Hashimi, H., Schwartz, L.M., Nambu, J.R., **1995**. Programmed cell death in the *Drosophila* central nervous system midline. *Curr. Biol. 5*, 784–790.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L.M., Steller, H., *et al.*, **1997**. Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc. Natl Acad. Sci. USA 94*, 5131–5136.

Zhou, L., Song, Z., Tittel, J., Steller, H., **1999**. HAC-1, a *Drosophila* homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. *Mol. Cell 4*, 745–755.

Zuzarte-Luis, V., Hurle, J.M., **2002**. Programmed cell death in the developing limb. *Int. J. Devel. Biol. 46*, 871–876.

2.6 Heart Development and Function

R Bodmer and R J Wessells,
The Burnham Institute, La Jolla, CA, USA
E C Johnson, Wake Forest University,
Winston-Salem, NC, USA
H Dowse, University of Maine, Orono, ME, USA

2.6.1. Introduction

The heart in vertebrates is the central organ of a functional organism. In insects, the functional importance of a heart is less evident, since they do not have an elaborate vascular system and transport of oxygen is carried out by the highly branched tracheal system. Nevertheless, the molecular components and basic control mechanisms of how the heart develops and how it functions are strikingly similar in vertebrates, chordates, and insects (Bodmer, 1995; Harvey, 1996; Bodmer and Venkatesh, 1998; Cripps and Olson, 2002; Zaffran and Frasch, 2002; Holland *et al*., 2003). Therefore, it is likely that an ancestral "heart" existed before the split of insects and chordates during evolution. Even nematodes, which do not have a heart, employ some of the same key genes that normally control formation of the cardiac progenitors (Haun *et al*., 1998). These genes control the formation of the pharyngeal muscle, which is another mesodermally derived organ with recurrent waves of contraction and relaxation. Therefore, it may be that the control of rhythmical movements, such as that of the pharynx and the heart, has a common evolutionary origin. Heart research with evolutionary distant insects may shed more light on these relationships. The availability of the whole genome sequence from the dipteran *Drosophila* and the mosquito *Anopheles* will greatly facilitate such efforts.

In Section 2.6.2, the molecular genetic basis of heart development in *Drosophila*, the only insect in which this process has been studied in detail and at the molecular level, is reviewed. Because of the conservation of the basic mechanisms that regulate the specification of the heart, many of the insights gained from *Drosophila* will undoubtedly also be applicable to other insects, and hopefully spur research in suitable (i.e., larger) species, where studying embryological events may be advantageous.

In Section 2.6.3, the functional properties of the insect heart by drawing on information from a variety of insects have been discussed. As in all hearts, the physiological control of the heart beat in frequency and vigor is of most critical importance, and thus the cardiac pacemaker is most extensively discussed. Recent advances in *Drosophila* begin to shed light on the molecular control of the pacemaker and other properties of heart function.

Finally, in Section 2.6.4 recent studies in heart function during the aging process are reviewed. Again, conserved molecular mechanisms have emerged that regulate lifespan from nematodes to mammals (Tatar *et al*., 2003). In *Drosophila*, and probably in other insects as well as in vertebrates, genes controlling the rate of aging also seem to operate specifically in determining the efficacy of heart function. Thus, as insects have been the key to understanding global genetic mechanisms of development, they may provide us again with the opportunity

of gaining mechanistic insights into the relationship of aging and (heart) organ function.

2.6.2. Heart Development

2.6.2.1. Embryology of Heart Formation

As in other insects, the heart of *Drosophila* is a linear tubular structure that forms at the dorsal midline of the embryo (**Figure 1**). In an open circulatory system, the heart pumps the hemolymph through the larval and adult body cavity (Miller, 1950; Rizki, 1978). Studies in *Drosophila* and another dipteran, *Calliphora*, suggest that during metamorphosis, the heart persists but is substantially remodeled (Jensen, 1973; Curtis *et al*., 1999; Molina and Cripps, 2001). In larvae, a simple valve is located in the middle of the heart (Rizki, 1978), which separates the narrower anterior portion, the aorta, from the wider posterior part, the heart proper and cardiac pacemaker, which are located in the posterior abdominal segments (see Section 2.6.4). This posterior larval heart also contains three segmental pairs of inlet valves, the ostia, which express and require the COUP transcription factor encoded by *seven-up* (*svp*) (Rizki 1978; Gajewski *et al*., 2000; Lo and Frasch, 2001, Molina and Cripps, 2001; Ponzielli *et al*., 2002). After remodeling to the adult heart during metamorphosis, which results in histolysis of the heart portion, four new pairs of *svp* expressing ostia differentiate in the anterior portion of the abdomen (Molina and Cripps, 2001). The larval heart in *Drosophila* consists of two major cell types: the inner two rows are contractile muscle cells (myocardial cells), expressing the muscle MADS-box transcription factor Dmef2, flanked on each side by rows of pericardial cells (**Figure 1**). The myocardial cells are crescent shaped and face each other, thereby forming a central cavity, the lumen of the heart (**Figure 1**). The heart is attached to the epidermis by small skeletal muscles, the alary muscles, which suspend it along the dorsal midline. In contrast to insects, the vertebrate heart is a more complex, asymmetrically looped organ containing multiple valves, chambers, and tissue layers (see Rosenthal and Harvey, 1999).

2.6.2.1.1. Mesodermal movements and subdivision The knowledge on the embryology, let alone genetics, of heart formation in insects other than *Drosophila* is scarce (e.g., Seidel *et al*., 1940), thus the focus is on the fruit fly, *Drosophila melanogaster* ("Vinegar fly"). During gastrulation of *Drosophila* embryos, the ventral third portion of the blastoderm embryo, the presumptive mesoderm, invaginates in a furrow along the ventral midline and initially localizes internally as a ventral mass of cells. This mesodermal mass then flattens and spreads dorsally by cell migration to form a morphologically uniform monolayer of cells in close contact with the ectoderm (Leptin and Grunewald, 1990). At the same time, the long germ band folds over and extends anteriorly, so that the posterior end of the embryo comes in contact with the head region (**Figure 2a**). When the mesodermal layer reaches the dorsal edge of the ectoderm, the first subdivision occurs into dorsal mesoderm and ventral mesoderm, as defined by marker gene expression (see below). After that, the mesoderm undergoes the first discernible morphological subdivisions. The dorsal mesoderm segregates into two layers: the outer, dorsal somatic mesoderm giving rise mostly to skeletal muscles, and the inner, visceral mesoderm, which assumes the form of a distinct and continuous epithelial layer (Borkowski *et al*., 1995). Then, the progenitors of the heart emerge from the dorsal edge of this outer mesodermal layer, termed the cardiac mesoderm (**Figure 2**; Bodmer *et al*., 1990). At that time (early to mid stage 11), the first cardiac

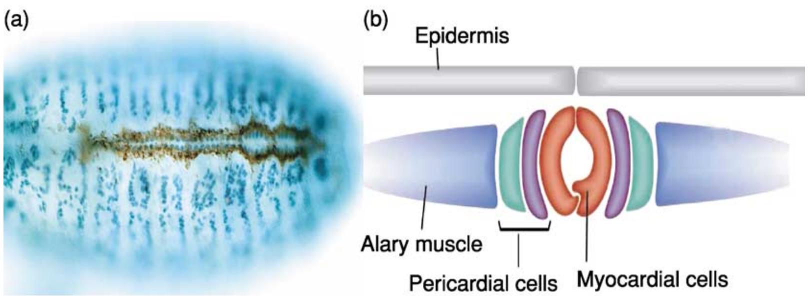


Figure 1 (a) Muscle nuclei for the muscle differentiation transcription factor Dmef2 labeled in blue; pericardial cells for a collagen encoded by the *pericardin* gene labeled in brown. Note the stereotyped juxtaposition of myocardial cells in between the pericardial cells. Image kindly supplied by Ming-Tsan Su. (b) Cross-section through the heart illustrating its simple cellular arrangement. Note that two crescent shaped myocardial cells from either side of the dorsal midline outline the heart lumen. (Diagram kindly supplied by Krista Golden.)

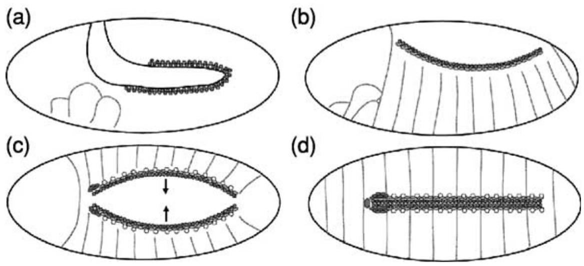


Figure 2 Stages of cardiac mesoderm and heart tube assembly. (a) Side view of heart field at late stage 11 germ band extension. (b) Side view of heart progenitors at stage 13 germ band retraction. (c) Dorsal view of bilateral rows of cells in the process of migration towards the midline (arrows). (d) Dorsal view of the heart after assembly. (Diagrams kindly supplied by Krista Golden.)

specific marker genes are expressed in the presumptive heart field (see below). The fat body and gonadal mesoderm are also specified from the dorsal mesoderm (Azpiazu *et al*., 1996; Riechmann *et al*., 1997; **Chapters 1.2** and **2.9**).

2.6.2.1.2. Heart morphogenesis Initially, the bilaterally symmetrical heart primodia are only distinguishable by marker gene expression. After stage 12, the germ band retracts again (Campos-Ortega and Hartenstein, 1997), leaving the dorsal side of the embryo open with a thin sheath of extraembryonic tissue, the amnioserosa, covering the yolk sac. After germ band retraction, the two rows of cardiac mesoderm move towards the dorsal midline (**Figure 2c**) by following the ectoderm, which precedes the mesodermal movements during this process of dorsal closure (Reed *et al*., 2001). Prior to the joining of the bilateral symmetrical primordium into a heart tube beneath the dorsal midline, the myocardial progenitors take on an epithelial character with distinct basal (ventral) and apical (dorsal) polarity (Rugendorff *et al*., 1994; Chartier *et al*., 2002). Cell shape and subcellular changes of the prospective myocardial cells during cardiac morphogenesis have not yet been studied in detail, and the molecular–genetic basis of orderly alignment of myocardial cells and subsequent formation of the lumen remains largely unexplored.

It has been proposed that the process of heart tube assembly is reminiscent of the assembly of blood vessels in vertebrates (Rugendorff *et al*., 1994). In an alternative model, it has been suggested that the embryological origin and the overall process of heart tube formation from bilateral mesodermal primordia most distal to the start of gastrulation is equivalent to that of vertebrates, given the inversion of dorsal–ventral axis (Bodmer, 1995). Moreover, many of the molecular determinants of cardiogenesis have conserved roles in the animal kingdom (see below; reviews: Harvey, 1996; Bodmer and Venkatesh, 1998; Lockwood *et al*., 2001; Cripps and Olson, 2002; Zaffran and Frasch, 2002). One can imagine that these two models are not mutually exclusive and may have many elements in common, since both blood vessels and hearts form a lumen and are able to contract. Insects lack a closed circulatory system, which in vertebrates is accomplished by a seamless endothelial connection between the vertebrate specific endocardium and blood vessels (Fishman and Chien, 1997); and the insect hearts remain linear, unlike their vertebrate counterparts. Thus, it is conceivable that hearts and blood vessels share a common ancestor, which may still be manifest in the insect heart. Moreover, other rhythmically contracting organs such as the pharynx have been proposed to share a common ancestor with hearts, based on interchangeable homologous molecular determinants that function not only in the vertebrate and insect heart (see below), but are also required to build the nematode's pharynx (Haun *et al*., 1998).

2.6.2.1.3. Cardiac cell types and lineages Although the mechanisms and detailed events of cardiac morphogenesis, cell polarity changes, and lumen formation remain to be elucidated, the lineages that contribute to the cell types that form the heart tube and surrounding pericardial cells have been studied in some detail (**Figure 3**; Carmena *et al*., 1998; Park *et al*., 1998; Ward and Skeath, 2000; Alvarez *et al*., 2003; Han and Bodmer, 2003). Within the emerging cardiac anlagen at the dorsal mesodermal edge (**Figure 2a**), sets of progenitors express distinct combinations of transcription factors, which are thought to be involved in conferring the appropriate differentiation pathway to these cardiac precursors (see below; Ward and Skeath, 2000; Han *et al*., 2002; Jagla *et al*., 2002; Klinedinst and Bodmer, 2003). These progenitors also undergo defined and segmentally repeated patterns of lineage divisions, which generate a diversity of cardiac cell types (Ward and Skeath, 2000; Alvarez *et al*., 2003; Han and Bodmer, 2003). As outlined below, some of the heart precursors divide symmetrically, whereas others undergo stereotyped asymmetric cell divisions.

2.6.2.1.3.1. Cell-type specific markers The cardiac progenitors are first distinguishable from other mesodermal cells at mid stage 11, when most if not all express a NK-type homeobox transcription factor encoded by the *tinman* (tin) gene, as a two to three cell-wide chain of dorsal mesodermal cells

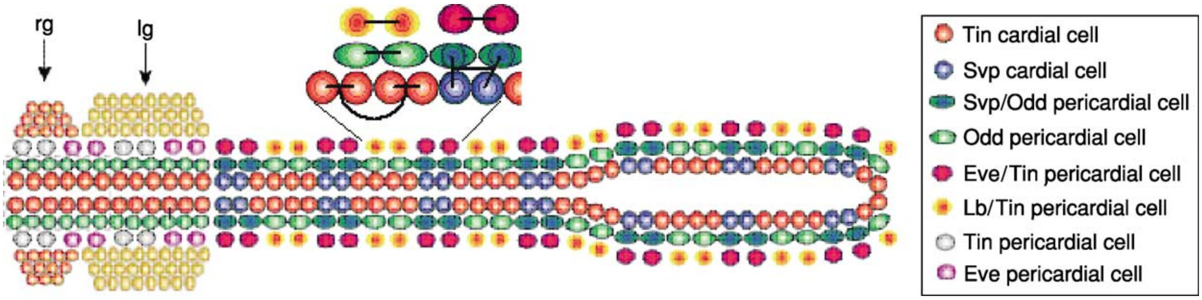


Figure 3 Diagrammatic representation of myocardial and pericardial cell types associated with the heart. rg, ring gland; lg, lymph gland. Enlarged half segment diagram (upper middle): back bars connecting cells indicate lineage relationships. Bar of arc connecting two pairs of siblings indicate higher order lineage relationships. (Diagram kindly supplied by Krista Golden.)

spanning from the third thoracic segment (T3) to the eighth abdominal segment (A8) (**Figure 2**; Bodmer *et al.*, 1990). At that time or shortly before or after, subsets of these progenitors or various combinations of their progeny begin to express additional marker genes, which has led to a detailed map of all heart cells (**Figure 3**): the segmentation homeobox gene *even-skipped* (*eve*) is expressed by two pericardial cells with large nuclei within each hemisegment (Frasch *et al.*, 1987); the homeobox gene *ladybird* (*lb*; Jagla *et al.*, 1997) and the COUP steroid receptor encoded by *seven-up* (*svp*; Mlodzik *et al.*, 1990) are expressed in two myocardial and two pericardial cells that do not overlap with the *eve* cells or with each other (see Han *et al.*, 2002); the T-box genes encoded by *dorsal cross* (*doc*) are also expressed in the *svp* myocardial cells (Reim *et al.*, 2003); the zinc finger gene *odd-skipped* (*odd*) is expressed in four pericardial cells, two of which overlap with the *svp* pericardial cells (Ward and Skeath, 2000); and *tinman* is expressed in four myocardial and at least four pericardial cells, two of which also express *lb* but not *svp* or *odd* (Alvarez *et al.*, 2003; Han and Bodmer, 2003). In the two anterior-most segments of the heart (T3/A1), all six myocardial cells express *tinman*, but not *svp*, and the pericardial cells are more numerous, many of which form the lymph gland, which becomes the larval blood forming organ (Rizki, 1978).

Additional markers of the myocardial cells are the ABC family gene *Dsur* (Nasonkin *et al.*, 1999; Lo and Frasch, 2001; T. Akasaka and R. Bodmer, unpublished data) and the T-box gene *neuromancer* (also known as H15; Griffin *et al.*, 2000; L. Qian and R. Bodmer, unpublished data). The basic helix–loop–helix motif encoding the *dHand* gene is expressed in all myocardial and pericardial cells (Moore *et al.*, 2000; Kolsch and Paululat, 2002). The myocardial cells share many of their muscle characteristics with other skeletal muscle cells, for example, expression of the muscle differentiation gene *Dmef2*; a MADS-box transcription factor (**Figure 1**; Lilly *et al.*, 1994; 1995; Nguyen *et al.*, 1994; Bour *et al.*, 1995), myosin heavy chain (Zhang and Bernstein, 2001), and beta-3-tubulin (Damm *et al.*, 1998).

Two sets of asymmetric cell division have been described in the cardiac mesoderm (**Figure 3**; Ward and Skeath, 2000; Alvarez *et al.*, 2003; Han and Bodmer, 2003): (1) the progenitors of the Svp lineage divide asymmetrically to generate Svp myocardial cells (SMC) and Svp-Odd pericardial cells (SOPC) (Ward and Skeath, 2000); (2) the progenitors of the mesodermal Eve lineage generate Eve pericardial cells (EPC) and muscle founders DA1 and DO2 (Park *et al.*, 1998; Halfon *et al.*, 2000; Carmena *et al.*, 2002; Han *et al.*, 2002; see **Chapter 2.1**). These asymmetric cell divisions are similar in the sense that they both distinguish between myogenic and nonmyogenic sibling cell fates. All other heart associated cell fates in the main portion of the heart (A2–8) appear to be generated by symmetric cell divisions, including the *tinman-lb* (TLMC) and the *tinman*-only (TMC) myocardial cells, the non-*svp* OPC, and the *tinman*-only pericardial cells (Alvarez *et al.*, 2003; Han and Bodmer, 2003). In T3/A1, all myocardial cells may have pericardial siblings but none express *svp*, and the exact lineage relations have yet to be determined.

2.6.2.1.3.2. Lineage tracing Three types of methods have been used to determine the lineages described above: (1) observation of cell division patterns (Carmena *et al.*, 1998, 2002; Halfon *et al.*, 2000); (2) clonal analysis using the site-specific Flp/FRT recombination system from yeast (Golic and Lindquist, 1989; Ward and Skeath, 2000; Alvarez *et al.*, 2003); and (3) analysis of cell cycle mutants to arrest early or late cell divisions during precursor lineages (Han and Bodmer, 2003). Flp/FRT

based clonal analysis is the most reliable method but also the most time-consuming when done at a sufficiently low clonal density. Clones have been generated at stage 8 (before mesodermal subdivisions) by heatshock induction of the yeast Flp recombinase in transgenic flies. This causes random recombination between two Flp recognition elements (FRTs), which results in excision of the region in between the FRTs and thus in reporter gene expression, such as *lacZ*, in the recombinant cell and all its progeny (Struhl and Basler, 1993). The patterns of co-labeled cells at low levels of induction then allow the determination of lineage relationships.

The asymmetric nature of the Eve lineages has first been suspected based on observation of the nuclear staining pattern of the Eve protein in the developing cardiac mesoderm (Carmena *et al.*, 1998), which was later confirmed by clonal analysis (Alvarez *et al.*, 2003). Two promuscular *eve* expressing clusters are specified in each half segment, out of which two progenitors emerge that undergo asymmetric cell divisions. In each segmental half, one Eve progenitor generates the two EPCs (Eve/Tinman positive) and a body wall muscle founder (DO2). The other Eve progenitor gives rise to a muscle founder (DA1) and an unidentified sibling. The final division of the immediate precursor of the EPCs is symmetrical. The *svp* expressing myocardial and pericardial cells are also generated again by an asymmetric lineage, as are the heart muscle cells in the anterior most myocardium (T3/A1). In contrast, the remaining myocardial and pericardial cells of the abdomen are apparently generated by symmetric lineages, each of which gives rise to two indistinguishable myocardial or pericardial progeny. In conclusion, all the lineages that participate in the embryonic heart have been elucidated (illustrated in **Figure 3**).

2.6.2.1.3.3. Cell cycle mutants Similar conclusions to those described in the previous section were reached by studying cell cycle mutants (Han and Bodmer, 2003). These mutants have been shown to arrest cell divisions at different stages and cell cycle number within the ectoderm. It is believed that most cells in the embryo undergo three partially synchronous rounds of cell division after blastoderm, in the mesoderm slightly earlier than in the ectoderm (Foe, 1989; Campos-Ortega and Hartenstein, 1997). The last round of global cell division, mitosis 16, is blocked in *cyclinA* (*cycA*) or *rca1* mutants (Knoblich and Lehner, 1993; Dong *et al.*, 1997), but *cyclinB* (*cycB*) mutants have no obvious effect. The *cycA* and *cycB* double mutants, however, act synergistically and arrest cell division at the G2/M transition of cycle 15 (Knoblich and Lehner, 1993). In embryos mutant for *string* (*stg*) (*cdc25* in yeast), cell division is arrested after blastoderm and does not enter metaphase of mitosis 14 (Foe, 1989; Edgar and O'Farrell, 1989, 1990). Additional genes are required for cells to exit the cell cycle, including the CDK inhibitor *dacapo* (*dap*) and *fizzy-related* (*fzr*), which negatively regulates cyclin levels. Loss of either gene causes cell division progression through an extra cycle, and overexpression inhibits mitosis and results in cell division arrest (Lane *et al.*, 1996; Sigrist and Lehner, 1997).

It is expected that the cell cycle has to be coordinated within the cardiac progenitors for correct specification of the progeny. A recent study shows that the heart precursors continue to differentiate in cell cycle mutants and in embryos in which cell cycle inhibitors are overexpressed (**Figure 4**; Han and Bodmer, 2003). As anticipated, the cell fate of symmetrically dividing progenitors of either myocardial- or pericardial-only lineages is not altered by premature arrest of cell division at cycle

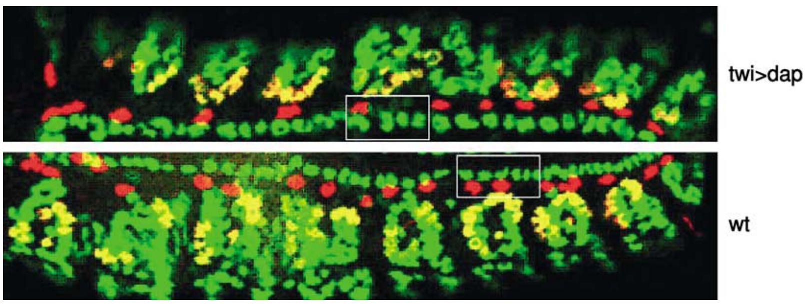


Figure 4 Premature cell cycle arrest changes the pattern of cardiac cell types revealing lineage relationships. Confocal images of the heart stained with antibodies against Dmef2 (green) and Eve (red). Bottom image shows the wild-type pattern of six myocardial cells (green) and two EPCs (red) and a double-labeled DA1 syncytial body wall muscle per half segment. Top image is an embryo in which the cell cycle inhibitor dacapo (dap) has been overexpressed in the mesoderm inhibiting the last division (cycle 16). As a consequence there is only one EPC and four myocardial cells per half segment (rectangle in top panel), instead of two and six, respectively (rectangle in bottom panel) (see text, **Figure 3**, and Han and Bodmer, 2003, for details). (Images kindly supplied by Zhe Han.)

16. By contrast, the progenitors of the asymmetric Eve and Svp lineages always adopt a myogenic, as opposed to the alternative pericardial, fate and are prevented from completing their last asymmetric divisions.

Interestingly, when cell division is halted earlier at cycle 15, as in *cycA*;*cycB* double mutants, only two of the normally six myocardial cells are formed in the A2–A7 segments: one expresses *tinman* and the other *svp*. This suggests that in each hemisegment two higher order myocardial progenitors are initially specified: one that divides twice symmetrically giving rise to the *tinman* expressing myocardial cells, whereas the other is likely to first divide symmetrically and then asymmetrically giving rise to the two *svp* myocardial and the two *svp* pericardial cells (**Figure 3**; Han and Bodmer, 2003). Taken together, there are probably 5–6 progenitors in each hemisegment that give rise to 14–16 heart associated cells (6 myocardial and 8–10 pericardial cells; see **Figure 3**).

2.6.2.1.3.4. Genetic control of asymmetric cardiac lineages As in the nervous system and other organs, a mechanism for generating cell type diversity within the heart field is by specifying alternative cell fates during asymmetric cell division. This involves intracellular determinants, which are localized asymmetrically in the predivisional cell, and which segregate to only one of the two daughter cells. In the nervous system of *Drosophila*, the membrane associated product of the gene *numb* has been shown to be necessary and sufficient for determining alternative daughter cell fates during asymmetric cell divisions (Uemura *et al*., 1989; Rhyu *et al*., 1994; Brewster and Bodmer, 1995; Spana *et al*., 1995; Guo *et al*., 1996; Spana and Doe, 1996; reviews: Vervoort *et al*., 1997; Lu *et al*., 1998; **Chapter 2.1**). In the daughter cell that inherits the Numb protein, the function of a transmembrane receptor protein encoded by *Notch* and a four-pass transmembrane protein encoded by *sanpodo* (*spdo*) is inhibited. As a consequence, the Numb-containing cell assumes a fate that is different from its sibling, in which Notch activity is unperturbed (Dye *et al*., 1998; Skeath and Doe, 1998; O'Connor-Giles and Skeath, 2003).

In the heart, the asymmetric *eve* and *svp* lineages are also under the control of *numb*, *Notch*, and *spdo* (Park *et al*., 1998; Ward and Skeath, 2000; Jagla *et al*., 2002; Alvarez *et al*., 2003; Han and Bodmer, 2003; **Chapter 2.1**). As in the nervous system, asymmetrically segregating Numb protein in the *eve* and *svp* lineages antagonizes *Notch* and *spdo* activity and causes distinction from the fate of its siblings. This mechanism is not limited to insect lineages since *numb* homologs have been identified also in vertebrates and shown to be localized asymmetrically during ventricular stem cell divisions in the developing brain (Verdi *et al*., 1996; Zhong *et al*., 1996, 1997). However, in vertebrates *numb* may function in maintaining the stem cell population of neural progenitors rather than participating in choosing specific pathways of differentiation (Petersen *et al*., 2002).

In contrast to the fly's asymmetric cardiac lineages, repression or constitutive activation of the *Notch* pathway has no apparent effect on the symmetric myocardial- or pericardial-only lineages (Han and Bodmer, 2003). For example, *numb* loss-of-function or ectopic activation of *Notch* results in twice the number of pericardial SOPC at the expense of the myocardial SMC, but the number of TMC is unaltered. Conversely, overexpression of *numb* causes the opposite phenotype in the *svp* lineage, again without affecting the TMC lineage. Thus, *Notch* signaling dramatically affects cell fate in asymmetric lineages by preventing a myogenic fate, but it is incapable of altering the myogenic fate of symmetrically dividing progenitors, even when its activated form is forcefully expressed in this lineage. Thus, inhibition of *Notch* activity is critical for specifying a myogenic cell fate only in asymmetric lineages; and a similar *Notch* responsive system is absent in symmetric lineages. Apparently, this switch from *Notch* insensitive to *Notch* responsive can occur from cell division to cell division, as in the case of the higher order *svp* progenitor, which first divides symmetrically then asymmetrically, or in the case of the EPC progenitor, which first divides asymmetrically and then symmetrically. It is conceivable that a common way to achieve increased cellular diversity in multicellular organisms is by switching to a *numb*/*Notch* responsive mode during evolution.

2.6.2.2. Genetics of Mesoderm Formation

2.6.2.2.1. *twist* and *heartless*: mesoderm and mesodermal movements As the formation of the heart depends on the prior formation of the mesoderm, mesoderm formation itself is directly linked to the determination of the dorsal–ventral axis of the embryo, and this has been studied in considerable detail. The dorsal–ventral axis depends on maternally active genes, which initiate the specification of this axis during oogenesis when the critical molecular coordinates are set up (review: St. Johnston and Nusslein-Volhard, 1992; **Chapter 1.2**). The executing morphogen of the dorsal–ventral axis is a Rel-related maternal transcription factor, encoded by

the gene *dorsal*, whose product accumulates in a ventrally increasing nuclear gradient at blastoderm stage prior to gastrulation. As a consequence of high levels of nuclear Dorsal protein, the first zygotic mesoderm-specific genes are activated in the ventral third of the blastoderm embryo: *twist*, which codes for a basic helix–loop–helix protein (Thisse *et al.*, 1988), and *snail*, which codes for a zinc finger protein (Boulay *et al.*, 1987). Both *twist* and *snail* are critical for specifying mesoderm, since in the absence of either gene function, the presumptive mesoderm does not form and gastrulation is blocked (Simpson, 1983; Nusslein-Volhard *et al.*, 1984; Alberga *et al.*, 1987; Thisse *et al.*, 1987). However, the ways in which they act are different: *twist* acts as a positive regulator of mesoderm-specific differentiation, while *snail*'s role in promoting mesoderm formation is by repressing neuroectodermal gene expression within the mesodermal anlagen (Kosman *et al.*, 1991; Leptin, 1991).

Before gastrulation starts, *twist* turns on a number of target genes throughout the mesodermal primordia of the trunk region which includes: *tinman*, a homeodomain containing nuclear protein (Bodmer *et al.*, 1990); *zfh1*, a nuclear protein containing a homeodomain and several zinc fingers (Lai *et al.*, 1991; Su *et al.*, 1999); *heartless* (as known as DFR1), a membrane protein of the FGF receptor family (Shishido *et al.*, 1993, 1997; Gisselbrecht *et al.*, 1996; Beiman *et al.*, 1996); *Drac1*, a GTPase (Luo *et al.*, 1994); *pointed P2*, an ETS transcription factor (Klambt, 1993); and *Dmef2*, the *Drosophila* homolog of the MEF2 family of MADS-box containing nuclear proteins (Nguyen *et al.*, 1994, Lilly *et al.*, 1994, 1995; Bour *et al.*, 1995). These early *twist* dependent transcription factors are candidates for executing the "mesodermal program," for example, by providing (transcriptional) context information for mesoderm-specific differentiation (see Section 2.6.4).

After gastrulation, the invaginated mesodermal mass flattens into a monolayer and spreads along the ectodermal body wall dorsally. Formation of this uniform cell layer closely apposed to the ectoderm is due to an active migratory process, which requires *heartless* function (Beiman *et al.*, 1996; Gisselbrecht *et al.*, 1996; Shishido *et al.*, 1997). In the corresponding mutants, the invaginated mesoderm barely flattens and for the most part is unable to reach the dorsal ectoderm; thus, heart and visceral muscle formation is severely reduced. As discussed below, secreted signals emanating from the dorsal ectoderm are crucial for inducing cardiogenic differentiation in the underlying mesoderm. Since these signals seem to act only over a short distance, they are unable to influence specification of the distant mesodermal mass in *heartless* mutants (see Bodmer and Frasch, 1999).

2.6.2.2.2. *tinman* and *dpp*: subdivision of the mesoderm Because the early Twist targets are expressed throughout the mesoderm, the patterning information that specifies the heart at the dorsal mesodermal margin must involve additional positional information. This information is provided by the ectoderm and is interpreted in a mesoderm specific fashion. In contrast to *heartless*' role in cell migration, the transcription factor Tinman is crucial for regionalization of the mesodermal monolayer, as a prerequisite of the development of (dorsal) mesoderm derived organs.

tinman is first expressed uniformly in the trunk presumptive mesoderm, and this expression is directly dependent on *twist*, the determinant of all mesoderm (Bodmer *et al.*, 1990; Yin *et al.*, 1997). After dorsal migration during gastrulation, *tinman* expression is limited to the dorsal portion of the mesoderm. In *tinman* mutants, derivatives of the dorsal mesoderm do not form, including the heart, the visceral, and many dorsal somatic body wall muscles (Azpiazu and Frasch, 1993; Bodmer, 1993). In contrast, a majority of ventral muscles and fat body form normally (for exceptions see Azpiazu and Frasch, 1993; Gorczyca *et al.*, 1994). Thus, specification of "dorsal mesoderm" is the first pivotal step in the process of mesodermal subdivision, prior to the specification of individual cell types or organs.

Before the mesoderm subdivides, *tinman* (and *Dmef2*) is restricted to the presumptive dorsal mesoderm (Bodmer *et al.*, 1990; Nguyen *et al.*, 1994). Since *tinman* mutants primarily affect dorsal mesoderm derived cell fates, it is likely that the dorsally restricted expression of *tinman* provides the key distinction between dorsal and ventral mesodermal cell fates, supported by the fact that absolutely no heart, visceral, and dorsal-most muscle progenitors are specified (Bodmer, 1993; Azpiazu and Frasch, 1993).

Since *tinman* is first expressed in all trunk mesoderm, it is unlikely to be the only determinant of dorsal mesoderm, and additional patterning mechanisms must operate to restrict its expression dorsally. Indeed, the dorsal ectoderm is closely apposed to the underlying mesoderm and, thus, is a likely source of this information, as is also suggested by the *heartless* phenotype of incomplete mesodermal migration (see above), and experiments in which gastrulation movements are blocked altogether (Baker and Schubiger, 1995). Earlier experiments in other insects have also put forward the proposition that heart formation depends on signals from

the overlaying ectoderm (Seidel *et al.*, 1940). In a set of elegant experiments, it was shown that the product of *decapentaplegic* (*dpp*), a secreted factor of the TGF-β superfamily and morphogen of the embryonic dorsal–ventral axis (Spencer *et al.*, 1982; Ferguson and Anderson, 1992; Francois *et al.*, 1994), acts as the key inductive signal, not only for dorsal ectodermal patterning, but also across germlayers for patterning the underlying dorsal mesoderm, which includes maintaining *tinman* expression (Staehling-Hampton *et al.*, 1994; Frasch, 1995). Like *tinman* mutants, embryos mutant for *dpp* lack all heart and visceral mesoderm formation, and *tinman* expression fades and does not become restricted to the dorsal mesoderm. Conversely, when the *dpp* pathway activity is expanded to more ventral regions, the *tinman* expression domain is also expanded ventrally (Frasch, 1995). Thus, *dpp* endows the dorsal mesoderm with the competence to form heart and other dorsal mesodermal cell types, in part via the direct regulation of *tinman* (Xu *et al.*, 1998; review: Bodmer and Frasch, 1999). *tinman* continues to be expressed in the visceral mesoderm for a short period of time as it segregates internally, and becomes prominently and exclusively expressed in cardiac progenitors of the embryonic trunk (**Figure 2a**). This heart-specific expression of *tinman* remains through adulthood (Molina and Cripps, 2001). Individual enhancer elements of the *tinman* gene have been identified that direct expression of the three early phases of *tinman* expression: the entire early mesoderm, the dorsal mesoderm, and cardiac mesoderm at the dorsal mesodermal edge (Yin *et al.*, 1997; Xu *et al.*, 1998, Venkatesh *et al.*, 2000).

2.6.2.3. Genetics of Heart Formation

Although the inductive *dpp* signal from the ectoderm apparently suffices to maintain *tinman* expression in the dorsal mesoderm and to specify the corresponding fates, the distinction of cardiac progenitors from other dorsal mesodermal derivatives and restriction of *tinman* expression to the cardiogenic region at the dorsal mesodermal edge requires further patterning information. Genetic evidence suggests that, within the context of the mesoderm the necessary and to some extent also sufficient cardiac patterning information is provided by the dynamic expression patterns of *dpp* and another secreted factor from the ectoderm, encoded by the secreted glycoprotein product of *wingless*, the original Wnt of *Drosophila*, which is expressed in segmental dorsal–ventral stripes oriented orthogonally to the broad anterior–posterior band of *dpp* (**Figure 5**; Lockwood and Bodmer, 2002). A model is presented in which the patterning information of these global positioning cues is interpreted mesoderm-specifically, and the crucial cardiogenic context for correct interpretation is given by Tinman, as well as by another transcription factor restricted within the mesoderm to the cardiogenic region, the zinc finger GATA factor encoded by the *pannier* gene (Lockwood *et al.*, 2001; Lockwood and Bodmer, 2002; Klinedinst and Bodmer, 2003).

2.6.2.3.1. Inductive factors: *wingless* and *dpp* Not only *dpp* but also *wingless* is absolutely necessary for cardiac mesoderm formation, but unlike *dpp*, *wingless* is not required for initiation of the visceral mesoderm (Wu *et al.*, 1995). Most, if not all, known components of the canocical *wingless* pathway participate in transducing the cardiogenic Wingless (Wg) signal (Park *et al.*, 1996). Interestingly, in vertebrates it is the noncanonical Wnt signaling that is required for promoting cardiogenesis (Pandur *et al.*, 2002). *wingless* is expressed until embryonic stage 10 in continuous, segmentally repeated ectodermal stripes, which later become interrupted in the lateral regions. The cardiac requirement parallels the *wingless* pattern of expression: it is required at stage 10 (Wu *et al.*, 1995), and again later at mid stage 11 (Jagla *et al.*, 1997). *dpp* also has two phases of expression: first in a broad dorsal ectodermal band until stage 9/10, then a brief period of no expression, and lastly by early stage 11 in a narrow band along the dorsal edge, immediately above the cardiac progenitors, as well as in a band along the ventrolateral region. A specific requirement for the late pattern of *dpp* expression has not yet been demonstrated rigorously, because, unlike in the case for *wingless*, there are no steeply temperature-sensitive mutants available for *dpp*. Interestingly, *tinman* expression, beginning with the restriction to dorsal mesoderm, closely follows the *dpp* pattern in the ectoderm. In contrast, *wingless* expression parallels *tinman* expression much less, but does overlap with it (**Figure 5**).

Given that *wingless* and *dpp* are expressed in dynamic patterns, and are used manifold during development for partitioning the embryo and for specifying tissues, the question arises how is their patterning information computed in order to provide the precision of spatial positioning of the cardiac precursors within the embryo? This led to the hypothesis that it is the convergence of both *wingless*- and *dpp*-dependent signals that is necessary to maintain *tinman* expression in the cardiac progenitors and to specify cardiac-specific cell fates, and that the correct cardiogenic interpretation of

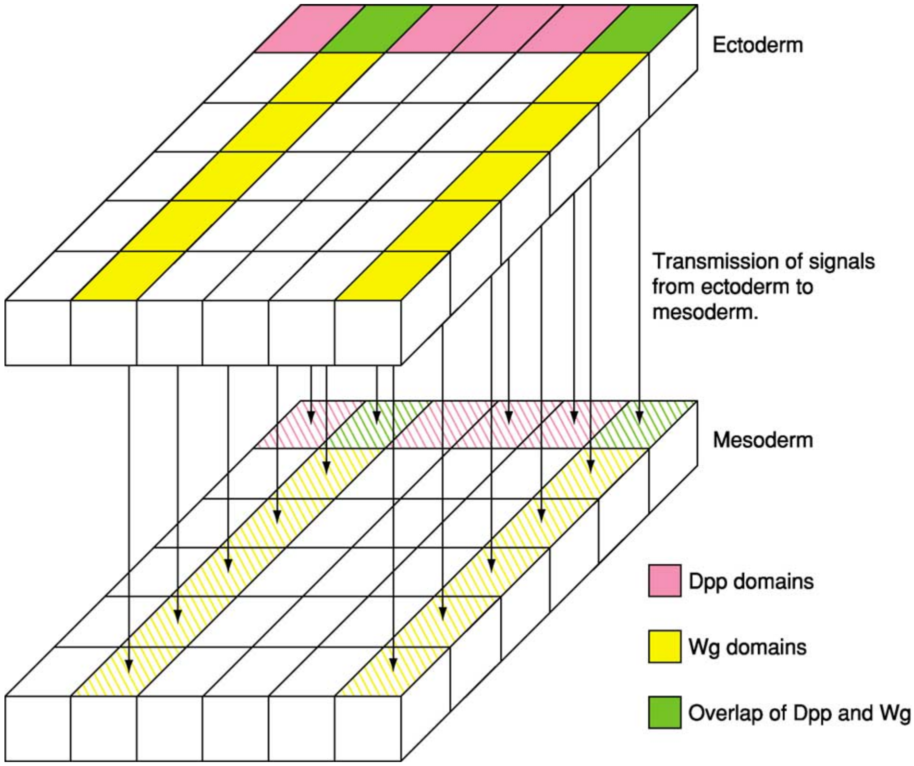


Figure 5 Diagrammatic representation of the convergence of the secreted signals Dpp and Wg from the ectoderm on the cardiogenic region in the underlying mesoderm. (Diagram kindly supplied by Wendy K. Lockwood.)

this convergence is provided by the presence of *tinman* in the dorsal mesoderm (Lockwood *et al.*, 2001; Lockwood and Bodmer, 2002). Thus, the heart is specified by *tinman*-expressing mesodermal cells in dorsal segmented clusters, where these cells are also exposed to signaling by *wingless* and *dpp*, both at early (stage 9/10), as well as later (stage 11) stages (**Figure 5**).

To test this hypothesis, *wingless* and/or *dpp* were misregulated by uniform overexpression in the mesoderm, with the expectation that wherever this misregulation resulted in an early and late coincidence of all three factors (Tinman, Wingless, and Dpp), cardiac specific differentiation would be initiated. For example, uniform *wingless* does not cause ectopic heart formation since no persistent trifold coincidence is generated. In contrast, uniform *dpp* does generate heart progenitors ventrally, since *tinman* is also expanded ventrally by ectopic *dpp* (see Frasch, 1995), and the late *wingless* is present in a dorsal as well as ventral stripe. Thus, the trifold dorsal and ventral overlap induces a ventral in addition to a dorsal heart (see Lockwood and Bodmer, 2002, for details).

2.6.2.3.2. Transcription factors: *tinman* and *pannier* The information content of the Wingless and Dpp morphogens alone, however, cannot account for the tissue specificity of cardiac differentiation, since they overlap often within the embryo causing a variety of outputs. Indeed, the pattern of *tinman* expression is also dynamic, first in all trunk mesoderm, then in dorsal mesoderm and finally along the dorsal mesodermal edge coincident with the cardiac progenitors, the latter pattern being dependent on both morphogens. It is possible that Wingless and Dpp are merely required to position and maintain *tinman* appropriately, and it is *tinman* that then acts autonomously in initiating cardiogenesis. If this were the case, one would expect that simply overexpressing *tinman* at the time when the heart progenitors are specified would be sufficient to create ectopic heart induction. However, uniform expression of *tinman* does not cause ectopic cardiogenesis beyond the dorsal edge of the mesoderm (Lockwood and Bodmer, 2002).

If the trifold overlap of *wingless*, *dpp*, and *tinman* is indeed instructive in initiating cardiogenesis, then it may be possible to generate cardiac fates ectopically, for example, at nonmesodermal intersects of *wingless* and *dpp*. Indeed, overexpression of *tinman* in the embryonic imaginal disc primordia, an obligatory ectodermal intersection of *wingless* and *dpp*, results in induction of cardiac specific markers

(Lockwood and Bodmer, 2002). Therefore, *wingless* and *dpp* are not only required to correctly position cardiogenic context information, such as *tinman*, within the mesoderm, but their signaling activities are directly participating in heart specification.

The necessary and sufficient roles of these three factors provide a minimal genetic framework for cardiac induction. The next question is how ectodermal Wingless and Dpp transmit their signal to the underlying mesoderm? Are the signaling pathways acting directly on the relevant enhancers of *tinman* and other genes expressed early in the heart or are there intermediates? Interestingly, the enhancer responsible for Eve expression in a subset of cardiac progenitors directly depends on the Wingless and *dpp* signaling pathways (Halfon *et al*., 2000; Knirr and Frasch, 2001; Han *et al*., 2002). In contrast, the cardiac specific enhancer of *tinman* (Venkatesh *et al*., 2000) may not be directly regulated by Wingless, but rather depend on the intermediary Forkhead transcription factor encoded by *sloppy paired* (*slp*), which is directly induced by the *wingless* pathway in corresponding stripes within the mesoderm (Lee and Frasch, 2000).

Another question is how the *dpp* signal is converted into cardiac specific gene activation. Addressing this question is complicated by the fact that *dpp* is the principal zygotic organizer of dorsal–ventral patterning and because of its role in dorsal closure of the embryo, which indirectly affects cardiac morphogenesis. One of the ectodermal targets of the Dpp morphogen is the GATA factor Pannier (Ramain *et al*., 1993; Winick *et al*., 1993; Ashe *et al*., 2000; Herranz and Morata, 2001), which is also expressed in the heart forming region of the mesoderm at the time when *tinman* is restricted to the cardiac primordia (mid to late stage 11; Gajewski *et al*., 1999; Klinedinst and Bodmer, 2003). This raises the possibility that Pannier may be a mediator of the ectodermal Dpp signal within the mesoderm to correctly specify and position the heart to the dorsal mesodermal margin.

In *pannier* mutants, formation of the *tinman* expressing cardiac mesoderm at mid stage 11 is severely reduced (Klinedinst and Bodmer, 2003). To determine the tissue specific requirement of *pannier*, two complementary sets of experiments were carried out: (1) a dominant-negative construct containing the N-terminal zinc fingers fused to the Engrailed repressor domain was expressed in the mesoderm or the ectoderm; and (2) in rescue experiments *pannier* cDNA was expressed in either germlayer of *pannier* mutants. It was found that *pannier* is primarily required within the mesoderm for heart formation, suggesting that this gene indeed participates in specifying the heart forming region alongside *tinman* (Klinedinst and Bodmer, 2003). Interestingly, however, ectodermal *pannier* is also needed for heart formation, at least in part by maintaining *dpp* expression after stage 11/12. It is not yet known if *pannier* is activated directly by the *dpp* pathway, as *slp* is by the *wingless* pathway (Lee and Frasch, 2000), or indirectly via another mediator.

Not all the heart lineages are equally affected in *pannier* mutants. For example, the *ladybird early* (*lbe*) and *svp* lineages are more severely reduced than the *eve* lineage, suggesting that *pannier* may also take a direct part in the specification of certain lineages. In agreement with this idea, GATA consensus sites were found in the cardiac enhancer of *tinman* but not in the mesodermal *eve* enhancer (Han *et al*., 2002; J. Liu and R. Bodmer, unpublished data). *pannier* is also a likely direct target of Tinman (Gajewski *et al*., 2001), suggesting that both of these transcription factors participate in maintaining each others expression. One can envision the following sequence of events during cardiogenesis: at stage 9/10 Dpp maintains *tinman* expression in the dorsal mesoderm and *pannier* expression in the dorsal ectoderm (Xu *et al*., 1998; Ashe *et al*., 2000). At early to mid stage 11, Tinman and Dpp/Wingless initiate Pannier in the cardiogenic region, and Pannier and Dpp/Wingless maintain Tinman expression in the heart field. Later, Tinman and Pannier maintain each other, and ectodermal Pannier maintains late Dpp expression. In conclusion, Pannier may have a pivotal role in cardiac specification as Tinman has. Another target of Dpp, the *dorsal cross* (*doc*) genes encoding T-box transcription factors, are required for repressing *wingless* in the lateral ectoderm (Reim *et al*., 2003), thus likely contributing to the dorsal restriction of *tinman* and *pannier*. Interestingly, the dorsally restricted strips of *wingless* expression at stage 11 depend on *pannier* as well (S.L. Klinedinst and R. Bodmer, unpublished data). Thus, the two Dpp targets, *doc* and *pannier*, contribute in different ways, both directly and indirectly, to the specification and positioning of the heart.

Overexpression experiments suggest that *tinman* and *pannier* may act synergistically, or in parallel, in specifying cardiac development (Klinedinst and Bodmer, 2003): forced expression of *tinman* has little capacity in promoting ectopic heart formation, and pan-mesodermal *pannier* only transiently induces *tinman* in ventrolateral regions. However, when both are expressed throughout the mesoderm simultaneously, a robust and sustained initiation of cardiac marker genes occurs, for example, of the basic helix–loop–helix transcription factor encoded

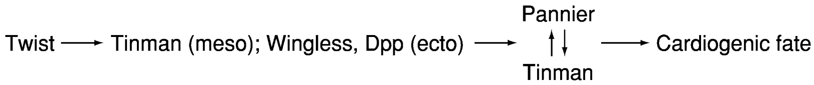


Figure 6 Genetic cascade of heart specification.

by *dHand* and of the ABC-class transmembrane protein encoded by *Dsur* (Klinedinst and Bodmer, 2003). This is consistent with *in vitro* experiments using vertebrate cell lines, in which it was shown that the vertebrate homologs of these genes activate target genes synergistically (e.g., Sepulveda *et al.*, 1998, 2002; Lee *et al.*, 1998). In conclusion, it may be that once *tinman* and *pannier* are activated in the prospective heart region, they may no longer require the parallel function of *wingless* and *dpp*, but now may function autonomously, analogous to homeotic selector genes, in specifying a cardiac fate (**Figure 6**).

There is a third transcription factor that is confined to the cardiac mesoderm at late stage 11, the time of heart progenitor formation: the tandem T-box factors encoded by *neuromancer I* (also known as *H15*; Griffin *et al.*, 2000) and its neighbor *neuromancer II* (L. Qian and R. Bodmer, unpublished data). Not only is this gene pair expressed at the right time in the right place, but also deletion mutants dramatically reduce heart progenitor formation and mesodermal overexpression transiently induces ectopic *tinman* expression, reminiscent of *pannier*, thus consistent with a central role in cardiac specification (L. Qian and R. Bodmer, unpublished data). Moreover, *in vitro* protein binding and transactivation studies in vertebrates also suggest a synergistic relationship between these three transcription factors (Garg *et al.*, 2003; Stennard *et al.*, 2003). Thus, it will be interesting to see if the combination of *tinman*, *pannier*, and *neuromancer* is not only necessary but also sufficient in promoting cardiogenesis.

2.6.2.4. Genetics of Cardiac Cell-Type Specification

2.6.2.4.1. *even-skipped* and *ladybird* Although more work is required to understand the details of cardiac initiation, the principal components of the genetic framework that generates cardiac competence along the dorsal mesodermal margin are now identified: inductive patterning information provided by *wingless* and *dpp*, and mesodermal context information provided and integrated by *tinman* and *pannier* (and perhaps *neuromancer*). The next question that needs to be addressed is how individual cell fates are specified within discrete locations along the heart primordium. It is important to

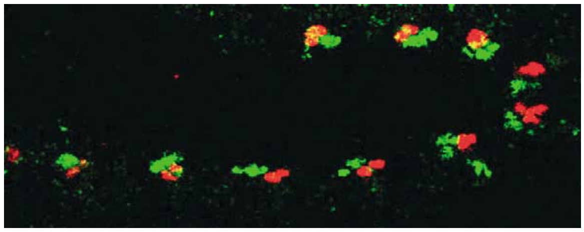


Figure 7 Side view of a late-stage 11/12 embryo double-labeled for Eve (red) and Lbe (green) protein in cell clusters within the cardiac mesoderm (compare with **Figure 2a**). Anterior is to the left. (Confocal image kindly supplied by Jiandong Liu.)

keep in mind that the specification of distinct cell types may occur concurrently or in parallel with the overall determination of "cardiac mesoderm." Between early stage 11 and stage 12, at least four groups of heart progenitors emerge within the prospective cardiogenic region as segmental clusters arranged in a stereotyped positional and temporal order, expressing either *eve*, *lbe*, *svp*, or *odd* (Frasch *et al.*, 1987; Jagla *et al.*, 1997, 2002; Ward and Skeath, 2000; Han *et al.*, 2002).

The first clusters to emerge within a segmental unit are the *eve* cells at stage 10/early stage 11 immediately underneath the ectodermal *wingless* stripes. This is at the time when *tinman* is still expressed in the broad dorsal mesoderm domain. This expression is before *pannier* and *tinman* are activated in the cardiogenic mesoderm at mid stage 11. Consistent with this is the finding that *tinman* is absolutely required for initiating mesodermal *eve* expression, whereas in *pannier* mutants the *eve* clusters are only moderately affected, unlike the other later-emerging clusters (Klinedinst and Bodmer, 2003). The specification of the cell types expressing mesodermal *eve* and the regulation of this expression has been extensively studied (see also next section: Carmena *et al.*, 1998; Buff *et al.*, 1998; Su *et al.*, 1999; Halfon *et al.*, 2000; Carmena *et al.*, 2002; Han *et al.*, 2002; Bidet *et al.*, 2003). The two *eve* progenitor cells in each hemisegment (**Figure 7**) appear to be selected from larger "promuscular clusters" by *Notch*-dependent lateral inhibition. These promuscular clusters, in addition to requiring *dpp* and *wingless* signaling, also require *ras*-mediated input from EGF and FGF receptor tyrosine kinases. Moreover, after the progenitors have divided, correct differentiation of pericardial EPCs requires an additional layer of context information, encoded by the early pan-mesodermal

gene *zfh-1*, which at this stage is expressed in all pericardial cells (Lai *et al*., 1991; Su *et al*., 1999).

Eve is not only a marker for the heart-associated cells it is expressed in, but is also required autonomously within these cell for their differentiation (Su *et al*., 1999; Han *et al*., 2002; Jagla *et al*., 2002; M. Fujioka, R.J. Wessells, Z. Han, R. Bodmer, and J.B. Jaynes, unpublished data). To separate the role of *eve* during segmentation from its role in the mesoderm, the enhancer responsible for mesodermal *eve* expression (see below) was selectively eliminated in a genomic rescue construct (Fujioka *et al*., 1999). Flies that are null mutant for *eve* but contain this "*eve* meso minus" rescue construct as a transgene are viable and fertile, and most cardiac cell types and the linear heart tube appear to form normally, except they lack all progeny of the mesodermal *eve* progenitors, the EPCs, and the DA1/DO2 muscles. Interestingly, these flies exhibit dramatically compromised heart function and lifespan, suggesting for the first time a specific role for at least one set of pericardial cells in insects (M. Fujioka, R.J. Wessells, Z. Han, R. Bodmer, and J.B. Jaynes, unpublished data).

Immediately anterior to the *eve* clusters emerge the *lbe* expressing progenitors at late stage 11 (**Figure 7**; Jagla *et al*., 1997). In deficiency embryos that delete the two tandem *ladybird* genes (*lbe* and *ladybird late*, *lbl)* the *eve* clusters are enlarged anteriorly; and mesodermal overexpression of *lbe* suppresses *eve* (Han *et al*., 2002; Jagla *et al*., 2002;). Conversely, "*eve* meso minus" rescue embryos have expanded *lbe* expression; and mesodermal overexpression of *eve* eliminates *lbe* in the cardiogenic region. Therefore, the distinction of the *eve* versus *lbe* clusters is maintained by a cross-repressive relationship between these two genes in the cardiogenic region. As discussed below, Lbe directly represses *eve* transcription in the cells anteriorly to the *eve* clusters (Han *et al*., 2002).

The anterior-most myocardial cells expressing *lbe* at the tip of the heart, the outflow track, are contacted by a group of ectodermal *lbe*-expressing cells, the heart-anchoring cells (HANCs; Zikova *et al*., 2003). The function of *lbe* apparently is necessary for this contact and for outflow track morphogenesis. Interestingly, when *lbe* expression is blocked only in the myocardial cells by ectopic *eve* expression in all mesoderm or specifically in the *tinman* expressing myocardial cells, the stereotyped morphological arrangement of the cardiac outflow region is also disrupted (Zikova *et al*., 2003). These findings suggest that the role of the HANC cells in fly heart morphogenesis is reminiscent of the role the neural crest plays in vertebrates, which is also in patterning the cardiac outflow track (Kelly and Buckingham, 2002).

The *svp* expressing cell clusters arise at late stage 11/12 near the segmental boundaries. *svp* is first coexpressed with *tinman*, but then suppresses it in the *svp* cells (Gajewski *et al*., 2000; Molina and Cripps, 2001; Lo and Frasch, 2001). Unlike *lbe* and *eve*, however, gain- or loss-of-*svp*-function does not influence cardiac *eve* or *lbe* expression. Later in embryogenesis, *wingless* is expressed in the ostia forming *svp* myocardial cells of the posterior heart segments A6–A8. In *svp* mutants, *wingless* is no longer expressed in the heart and the function of the ostia as inlet valves for the hemolymph into the heart seems to be compromised (Lo *et al*., 2002; Ponzielli *et al*., 2002).

There are two sets of *odd* expressing cells within the forming heart (Ward and Skeath, 2000): one co-expresses *svp* (see above) and the other will give rise to a set of pericardial-only cells. The function of *odd* in the heart has not yet been determined. There are probably additional *tinman*-expressing cells within the heart-forming region that are not yet accounted for by specific marker gene expression, such as two *tinman*-only myocardial cells (also expressing the muscle-specific marker *Dmef2*) and two *tinman*-only pericardial cells (also expressing the pan-pericardial marker *zfh1*) in each half segment.

2.6.2.4.2. Combinatorial transcriptional control: the *eve* mesodermal enhancer How is the information that specifies a certain population of (cardiac) cells integrated at the transcriptional level that allows the precise spatial positioning of the corresponding cell types within the embryo? For example, how is the highly stereotyped and restricted pattern of *eve* expression in the mesoderm along the dorsal edge of the mesoderm achieved? The identification of an enhancer element that is necessary and sufficient for faithful expression confined to the mesodermal *eve* clusters has provided a model to answer these questions (Fujioka *et al*., 1999; Halfon *et al*., 2000; Knirr and Frasch, 2001; Han *et al*., 2002). Studying this enhancer not only provided insight into how the positional information that specifies the location of the heart within the mesoderm is integrated transcriptionally, but also how subpatterns of gene expression are achieved within the heart field (Han *et al*., 2002). First, how *eve* is activated within the cardiac mesoderm, and then how it is confined to a small subset of heart progenitors is described.

Within a few hundred base pairs of genomic region 6 kb 3′ to the *eve* coding region, multiple consensus or *in vitro* binding sites have been

identified for all the transcription factors that have been shown to be required genetically for *eve* expression in the heart forming region. Among them are sites for Pangolin/dTCF (*wingless* pathway), Mad (*dpp* pathway), Pointed (ETS factor, *ras* pathway), Twist, and Tinman (Halfon *et al*., 2000; Knirr and Frasch, 2001; Han *et al*., 2002). The consensus appears to be that there are multiple sites for each transcription factor, which act overall in an additive fashion within and in between classes of sites. For example, mutation of progressively more Tinman sites eventually eliminates enhancer-driven reporter gene expression in transgenic flies. Similarly, mutation of only a few sites of two different classes, each set alone not having much influence on expression, also eliminates expression. Thus, the basic combinatorial genetic network that is in control of positioning cardiogenic competence to the dorsal edge of the mesoderm also acts combinatorially at a single enhancer. Of note, however, is the finding that a crucial cardiac determinant, *pannier*, apparently only acts indirectly here, via *tinman* most likely, since this enhancer apparently lacks GATA consensus binding sites and mesodermal *eve* expression is less affected in *pannier* mutants than cardiac *tinman* is (Klinedinst and Bodmer, 2003; Z. Han, M. Liu, and R. Bodmer, unpublished data). Moreover, mesodermal *eve* apparently is directly regulated by the *wingless* pathway, even though cardiac restricted *tinman* may not be. Thus, there are important differences in the details of how each gene's expression in the cardiogenic region is regulated.

Although the above activator inputs explain well why mesodermal *eve* is expressed within the heart field of the embryo, they are not sufficient to elucidate how *eve* transcription is confined to a rather small cluster of heart associated cells within each half segment (**Figure** 7). Since *lbe* acts genetically as a repressor of *eve*, it may do so by direct interaction with the mesodermal *eve* enhancer. Indeed, Lbe protein binds to the enhancer *in vitro* and mutating the corresponding sites in the enhancer leads to a dramatic expansion of reporter gene expression to fill the entire cardiogenic region (Han *et al*., 2002). Moreover, overexpression of *lbe* is no longer able to suppress the cardiac-wide reporter gene expression driven by this mutant enhancer, thus further supporting a direct regulation by Lbe. While Lbe represses *eve* anteriorly, it remains to be elucidated how *eve* is restricted posteriorly (see **Figure** 7). Whatever the outcome, the mechanism of regulation must include the same sites that Lbe binds to.

Studying gene regulation by taking enhancer regions out of the normal gene context and examining their ability to drive reporter gene expression in a spatially and temporally restricted fashion in the developing embryo has much enhanced our understanding of the mechanisms involved. To validate the findings, however, it has not been practical to manipulate enhancers within the context of the endogenous gene. An exception is the case of the mesodermal *eve* enhancer: the lack of *eve* cluster formation in "meso minus" *eve* rescue flies (see Section 2.6.2.4.1) demonstrates that this enhancer is not only sufficient to drive mesodermal *eve* expression faithfully, but despite its location far downstream of the coding region, it is absolutely required within the context of the entire *eve* gene (Fujioka *et al*., 1999; Han *et al*., 2002). Interestingly, mesodermal *eve* expression is also abolished when only a portion of the enhancer is deleted in the rescue construct, leaving intact an adjacent region, which is sufficient to drive expression faithfully when assayed in isolation. Thus, within the context of the entire gene, the regulatory requirements may be more complex and stringent than is apparent from studying isolated enhancers.

2.6.2.4.3. *pointed*, *msh*, and *hedgehog* Although a basic genetic framework is now available of how the heart is positioned within the mesoderm and how individual cell fates are generated, additional gene functions are likely to be involved in fine-tuning the spatial precision or exact number and relative position of individual cardiac progenitors. Two new gene functions have recently been identified that when mutated cause a dramatic increase in the myocardial cell population of the heart: the ETS gene *pointed* and the *muscle-specific homeobox* (*msh*) gene (Jagla *et al*., 2002; Alvarez *et al*., 2003). It is likely that in mutants of these genes, more heart progenitors are recruited from the dorsal mesoderm, perhaps at the expense of dorsal skeletal muscles.

In *pointed-P2* isoform mutants, it is primarily the *svp* myocardial cells that are increased in number at the probable expense of skeletal muscles and/or pericardial-only progenitors (Alvarez *et al*., 2003). Conversely, pan-mesodermal *pointed-P2* expression can rescue *pointed* null mutants, but does not diminish the normal complement of myocardial cells, suggesting that by itself *pointed-P2* is a permissive cue to restrict myocardial progenitors to the normal number. As suggested by Alvarez *et al*. (2003), this function of *pointed-P2* in determining myocardial cell number may be in addition to its function as an effector of the *ras* pathway, and thus independent of EGF or FGF signaling.

In the case of *msh* mutants, a set of dorsal skeletal muscle precursors, which normally express *msh*,

seem to be redirected towards a myocardial fate, reminiscent of *pointed* mutants (Jagla *et al*., 2002). In contrast to the *pointed* phenotype, however, loss-of-*msh* may also increase rather than decrease the pericardial cell number, suggesting that *msh* and *pointed*'s cardiogenic function differ to some extent. Because *Dmef2*-positive myocardial cells and *odd*-positive myocardial cells are increased in number in *msh* mutants, it might be that this is largely due to an increase in the *svp* expressing progenitors (see lineage in **Figure 3**), although the *tinman*-positive myocardial cells are also increased. In contrast to *pointed-P2*, overexpression of *msh* diminishes myocardial and pericardial cell numbers, affecting *lbe* expression more severely than *eve*. It has been proposed that *lbe*, *eve*, and *msh* are opposing each other's expression and function in a cross-regulatory network to specify spatially restricted cardiac (and skeletal) cell fates (Jagla *et al*., 2002).

A question that has not yet been resolved is how individual cell clusters are positioned relative to one another within the cardiogenic region. Even though both the *eve* and the *lbe* clusters require *dpp*, *wingless*, *tinman*, and *pannier* expression, and repress each other, why is *eve* always posterior to *lbe* within each segmental unit (**Figure 7**)? Clearly, additional patterning information must come into play. One such source may be the striped expression of *hedgehog*, which is just posterior to the *wingless* stripes and required for maintaining its expression. Consequently, *hedgehog* is also required for heart formation altogether, as is *wingless* (Wu *et al*., 1995; Park *et al*., 1996). In a genetic situation in which *hedgehog* is eliminated but *wingless* is maintained ectopically by forced expression in the mesoderm or in the normal *wingless* domain, no *eve* cell clusters form and the *lbe* clusters are expanded within the cardiogenic region (J. Liu, L. Qian, and R. Bodmer, unpublished data). The converse phenotype of expanded *eve* and reduced *lbe* clusters is observed when *hedgehog* is overexpressed, which is reminiscent of *ras* pathway activation (Buff *et al*., 1998; J. Liu, L. Qian, and R. Bodmer, unpublished data). Indeed, manipulation of *hedgehog* alters the expression level of *rhomboid* in the dorsal mesoderm, a protease that regulates EGF receptor-mediated *ras* activation. Taken together, these findings suggest that striped *hedgehog* signaling positions mesodermal *eve* expression nearby in the underlying mesoderm, via augmenting *ras* signaling. Further away from the *hedgehog* stripes then emerge *lbe* clusters, which do not require *hedgehog*. The mutual repression between *eve* and *lbe* then maintains the stereotyped anterior–posterior arrangement of these cell fates within each segment. In conclusion, some of the basic genetic principles by which individual heart progenitors and their progeny are specified, positioned, and distinguished from their neighbors are now understood.

2.6.2.5. Homeotic Control in Anterior–Posterior Regionalization of the Heart

Although before the formation of the heart tube, the anterior portion of the heart (A2–A5), the aorta, appears to be indistinguishable from the posterior portion (A6–A8) (**Figure 2**), at later stages the posterior heart is wider (**Figures 1 and 3**), contains the main pacemaker activity (see Section 2.6.3), and the *svp* myocardial cells express *wingless* and differentiate as ostia, the hemolymph inlet valves (Molina and Cripps, 2001). Several recent studies have elucidated the genetic control of this broad regional distinction between anterior and posterior heart by the *hox* genes of the Bithorax Complex (Lo *et al*., 2002; Lovato *et al*., 2002; Ponzielli *et al*., 2002; review: Lo and Frasch, 2003; **Chapter 1.8**). Of these genes, *abdominal-A* (*abd-A*) seems to be the principal player that discriminates between the aorta and the posterior heart by acting as the homeotic selector gene of the posterior heart fate.

The pattern of *hox* expression in the forming heart is arranged in a sequential anterior–posterior pattern, similar to their cuticular patterning role in the overlaying ectoderm (see **Chapter 1.8**), although the segmental register seems to be shifted posteriorly by 2–4 segments. In the anterior aorta (T3–A1), *Antennapedia* (*Antp*) is expressed primarily in the A1 region. No other *hox* gene is known to be expressed in the anterior-most region of the heart outflow track (T3). *Utrabithorax* (*Ubx*) is expressed at high levels in the posterior aorta (A2–A5) and at lower levels more posteriorly. *abd-A* expression is confined to the heart proper (A6–A8). In the posterior-most four myocardial cells (in A8) *Abdominal-B* (*Abd-B*) is highly expressed. In order to determine the *hox* gene function in cardiac specification, three markers were used in addition to morphological criteria: *troponinC-akin1* (*tina1*) is expressed at high levels in posterior heart and not in the aorta (Lovato *et al*., 2002), while *wingless* and a truncated heart enhancer of *tinman* are expressed in the ostia-forming *svp* myocardial cells of the heart (Lo *et al*., 2002).

In null mutants of *abd-A*, the posterior heart markers *tina1* and *wingless* are no longer expressed and the morphology of the posterior heart is narrower like that of the aorta. Moreover, *Ubx* is now expressed at higher levels in the posterior heart. Conversely, in embryos in which *abd-A* is expressed uniformly in the mesoderm, the anterior portion of

the heart (T3–A5) is now wider in diameter expressing *tina1* as does the posterior heart, *Ubx* expression is reduced, and in each segment double pairs of *svp*- and *wingless*-expressing myocardial cells form an apparently functional ostia. Interestingly, even the outflow track segments (T3–A1), which normally derive from lineages that are distinct from those in posterior segments (Alvarez *et al*., 2003; Han and Bodmer, 2003), are transformed towards the posterior heart identity. When a weaker, later onset but heart-specific driver is used, a similar phenotype is observed in the region of the posterior aorta but not of the outflow track, suggesting that the posterior aorta is either more susceptible to *abd-A* or for a longer time period than the anterior-most segments. A temporal difference in outflow track versus aorta specification is consistent with the finding that the last divisions of the outflow track lineages takes place before those of the more posterior segments (Han and Bodmer, 2003). It will be interesting to see if an anterior *hox* gene, such as *Antp*, participates in endowing the outflow track primordium with its distinct progenitor lineages. Taken together, these findings suggest that the *hox* genes, at least in the case of *abdA*, function autonomously within the heart progenitors to promote correct regional identities of differentiation.

Since *abd-A* is expressed and required in the posterior heart, *Ubx* may have a similar role as a homeotic selector gene in specifying a "posterior aorta" fate, as would be expected according to the "posterior prevalence" model of *hox* gene function, in which the anterior expression domain is functionally most important. Indeed, loss-of-*Ubx*-function does not alter posterior heart differentiation, and *abd-A* is slightly elevated in the aorta. However, the aorta of *Ubx* mutants does not undergo a dramatic change in its developmental program of the myocardial cell specification, except for some abnormalities in epithelial polarity (Ponzielli *et al*., 2002), since *tina1* and *svp* appear to be expressed normally. No significant additional changes are observed in the aorta of *Ubx,abd-A* double mutants, but insufficient landmarks have been monitored to decide upon a possible transformation towards an outflow track fate. In contrast to the heart tube itself, however, the primordia of the larval blood-forming organs, the lymph glands (**Figure 3**), which are pericardial specializations in the A1 region of the heart, are expanded along the entire posterior aorta in *Ubx* mutants (Mastick *et al*., 1995).

Embryos with pan-mesodermal overexpression of *Ubx* have defects in ostia formation of the posterior heart and in overall morphology, but *abd-A* is expressed normally. Unexpectedly, this gain-of-*Ubx*-function situation results in anterior expansion of the posterior heart marker *tina1* throughout the aorta, and an additional pair of ostia develops in the posterior-most segment of the aorta (A5). This is reminiscent of the *abd-A* overexpression phenotype (see above), suggesting that in this over expression assay, *Ubx* can partially substitute for its relative *abd-A*. In contrast, *abd-A*'s posterior relative *Abd-B*, which is only present in a few posterior-most myocardial cells after the heart tube has formed, completely suppresses cardiogenesis when overexpressed throughout the mesoderm. Conversely, in *Abd-B* mutants, additional heart progenitors develop more posteriorly, resulting in a "longer" heart (Lo *et al*., 2002). Thus, *abd-A* and possibly other *hox* genes act as positive regulators, whereas *Abd-B* acts as a negative regulator of heart development.

2.6.3. Heart Function

2.6.3.1. The Cardiac Pacemaker

The regular, rhythmic beat of the heart is generated by a membrane-bound oscillator consisting of an ensemble of ion channels. This is true whether the oscillation arises in a nerve ganglion (neurogenic) or the muscle (myogenic) of the heart itself. The horseshoe crab, *Limulus*, has for a long time been the type specimen for the neurogenic heart, and it was assumed that the majority of arthropods possessed a similar mechanism (Prosser and Brown, 1961). This conclusion for insects was largely based on research done on the cockroach heart, which is heavily innervated and seemed initially to be neurogenic (Miller, 1985). Nonetheless, all insect hearts thus far investigated, including that of the cockroach, have cardiac pacemakers integral to the myocardium, although extensive control by neural systems is widespread (Miller, 1985).

2.6.3.1.1. Vertebrate hearts Vertebrate myogenic cardiac pacemakers have been intensely studied over a long period of time. Given the observed conservation of ion channel-based systems across phyla (Hille, 2001), it is reasonable to start with vertebrate pacemakers as models, giving a solid basis from which to consider insect and, in particular, *Drosophila* pacemaking mechanisms. The underlying biophysics of the oscillatory behavior may vary in detail, but the overall similarities are striking and insect research has already carried over into understanding vertebrate mechanisms, with implications for human health.

In the lowest vertebrates, initiation of heartbeat starts in the weakly muscled *sinus venosus* (SV),

which receives the systemic blood (Torrey and Feduccia, 1979; Kardong, 2002). Tissue from all chambers of the heart retain a spontaneous beat upon dissection, but the SV, as the first chamber through which blood must pass, has a beat that is measurably faster than those downstream (review: Patten, 1949). In the intact heart, the beat from this chamber triggers a peristalsis-like wave of contractions that passes from chamber to chamber, thus entraining or driving the oscillations in the other, slower-beating, chambers (Adolph, 1967). The SV, then, constitutes the pacemaker of these hearts. The SV has been lost as a separate chamber in the mammalian heart, but is retained functionally as the sinoatrial (SA) node in the atrium near the point where the *vena cava* enters (Kardong, 2002). This nodal tissue is histologically quite distinct from other cardiac tissue, and retains its role as the dominant pacemaker (Irisawa *et al*., 1993). Infarct or other damage to the SA node results in serious pacemaking issues. There are other nodes and tissues that differ distinctly from the rank and file cardiac muscle cells, but the SA node is the primary area where the beat originates. Purkinje fiber cells serve a nerve-like function in transmitting the beat information through the entire heart, for example, and ionic currents in these cells have also been studied thoroughly. But, given that they do not constitute a spontaneous pacemaking system, nor are homologous structures found in insects, this rich topic further will not be discussed.

The ionic currents, the population of ion channels responsible for them, and their electrical and chemical interactions constitute the pacemaker. These are still not completely understood, nor have all the channels been identified molecularly. Essentially, however, the basic components are known across classes. In other excitable cells, like neurons, there is a negative resting potential of around -70 mV (Alberts *et al*., 2002). When the system is disturbed, for example, by some depolarizing input, an action potential is generated consisting of a wave of depolarization caused by an inward sodium current (Hille, 2001). This traveling wave passes down the axon. The membrane quickly repolarizes to its resting potential and enters into a refractory period, which prevents retriggering by the receding wave.

Cardiac pacemakers, however, have no true resting state as they never hold still (Baumgarten and Fozzard, 1992). By convention, the "resting potential" of true pacemaking cells is often taken to be the most negative potential the system reaches in its cycle, but this is not even a "metastable" state and the cell is at this potential only as it passes through it unless held by voltage clamp. It is possible to find a clamping voltage in pacemaker cells in which little current is elicited, and this may be taken as a resting potential as well (Irisawa *et al*., 1993).

In the simplest case, one may construct a useful "Gedankenschrittmacher," or "thought pacemaker" in a generic heart consisting of a collection of interacting ion channels. Beginning at an arbitrary phase in the cycle, assume an outward cation current that hyperpolarizes the cell. This might be the end of the repolarizing current. Next, add a channel that responds to hyperpolarization in this potential range by opening to a specific cation (or mixture of cations) that is more concentrated extracellularly. The result of this inward cation current must perforce be a rise in intracellular voltage towards some triggering potential. This crucial current is defined to be the pacemaker current. Once voltage reaches this threshold, a second inwardly moving cation current very rapidly depolarizes the cell in a characteristic "action potential" not dissimilar to the axonal spike. The difference lies in the species of ions contributing to the currents. There follows a likewise similar repolarization phase brought about by an outwardly directed cation current. This repolarization sets the system up for the next cycle and may actually contribute to the current starting it. Studies on vertebrates have yielded a considerable amount of information, which yields models that are variations on this simplistic one. The specifics vary across the classes, and even within them, but models such as the one above generate testable hypotheses and serve as a framework to be fleshed out with data.

Owing to the difficulties in studying the electrophysiology of single cardiac myocytes, especially those in the pacemaker, a great deal of the early work was done using microelectrodes in tissues other than the SV or atrioventricular (AV) node. An important distinction must be made between the electrophysiology of the true pacemakers and that of other downstream myocardial cells. The trigger to beat starts in the pacemaker and is distributed to the rest of the heart through tissue specifically dedicated to this task. The signal is termed the cardiac action potential, and can be studied conveniently in tissues such as the Purkinje fibers or even cells of the ventricle. The cells can be distinguished histologically and, critically, the profiles of the ionic currents are different in several crucial aspects. True pacemaker cells have four distinguishing characteristics: (1) they have spontaneous activity; (2) there is no inward rectifying K^+ current; (3) they are insensitive to tetrodotoxin (TTX); and (4) they are sensitive to Ca^{2+} channel blockers (Irisawa *et al*., 1993).

The final event in the process is excitation contraction (EC) coupling, the process by which the internally transported cardiac action potential causes the actual power stroke in the appropriate contractile portion of the myocardium (Bers, 2001). The complexity of the vertebrate EC system is beyond the scope of this work. Fortunately, the problem is considerably more tractable in insects given the straightforward topology of their hearts.

There are two major classes of ionic currents in SA nodal cells depending on whether they are time dependent or independent (Irisawa *et al.*, 1993). Time dependent currents are mediated by gated ion channels carrying Na^+ (I_{Na}), Ca^{2+} ($I_{Ca,L}$ and $I_{Ca,T}$), K^+ (I_K and $I_{Ca,K}$), and a mixture (I_f or, variously, I_h) (Hille, 2001). The nomenclature varies among authors. Ion pumps create the two time invariant currents; one exchanges three inward traveling Na^+ ions for a single Ca^{2+} yielding a net inward cation current (I_{NaCa}), and the other exchanges two inward traveling K^+ ions for three outward moving Na^+ ions (I_{NaK}) (Irisawa *et al.*, 1993). There is another potentially important background depolarizing Na^+ "leak" current, I_{bNa} (Giles *et al.*, 1982).

2.6.3.1.1.1. I_{Na} The role of sodium channels (see **Chapter 5.1**) in pacemaking is problematic, given that true pacemaker cells are TTX insensitive, whether vertebrate or invertebrate (Irisawa *et al.*, 1993). There are reports, however, that some individual SA node cells have a portion of their action potential sensitive to TTX (Denyer and Brown, 1987). One of the central pacemaking currents may be a mixed one, passing through a channel that is not completely selective. The role of Na^+ in other myocardial tissues in vertebrates is central, but this topic will not be considered further.

2.6.3.1.1.2. I_{Ca} Two distinct Ca^{2+} currents, carried by different channels, contribute to the cardiac action potential. The larger is termed L type, owing to its current being relatively Long-lived and Large ($I_{Ca,L}$) and the other Tiny and Transient (I_{Ca},T; Hille, 2001). The channels may be separated on the basis of pharmacology as well as kinetics. L-type channels are sensitive to 1,4-dihydropyridines (DHP; nifedipine, for example). The therapeutic calcium channel blockers verapamil and diltiazem selectively block L-type conductances (Hille, 2001). One view of the cooperative role of these two types of Ca^{2+} channels in pacemaker cells is that $I_{Ca,T}$ seems to be active for a short period of time during the first part of the action potential or major spike of rising voltage, carrying the membrane to the voltage range at which $I_{Ca,L}$ activates and does the heavy lifting in the spike (Hagiwara *et al.*, 1988).

In addition to the flow of Ca^{2+} down its gradient from the relatively more concentrated extracellular regions, there is recent evidence that calcium plays another, qualitatively different role in triggering the pacemaker (Lipsius *et al.*, 2001). The observation that ryanodine causes a lowering of diastolic depolarizing current in the SA node (Rigg and Terrar, 1996; Li *et al.*, 1997; Rigg *et al.*, 2000; Terrar and Rigg, 2000) implicated this release in SA node pacemaking. Ryanodine selectively inhibits release of calcium from the sarcoplasmic reticulum (Rousseau *et al.*, 1987). Release appears to be triggered by $I_{Ca,T}$ based on the low-voltage range (lower than L-type) in which the calcium release is seen and the inhibition of the observed release when T-type channels are blocked specifically by Ni^{2+} (Hagiwara *et al.*, 1988; Hüser *et al.*, 2000). The contribution of this release is thought to be stimulation of the net inward current of the sodium/calcium exchanger ($I_{Na–Ca}$), further depolarizing the plasma membrane, perhaps sufficiently for depolarization. The effect is more pronounced in latent, as opposed to working, pacemaker cells of the SA (Hüser *et al.*, 2000).

2.6.3.1.1.3. I_K K^+ plays a central role in the repolarization of the membrane after the calcium spike in the same manner as seen in neurons (Gintant *et al.*, 1992). In the heart, there are two major subcategories of channels, with the distinction based on differing activation modes. The first consists of voltage-activated gating. As the potential rises above threshold, the channels open permitting outward flow of K^+ along its concentration gradient. The second category consists of channels gated on the cytosolic side by Ca^{2+} sensing. Given that the cardiac action potential results from a calcium spike, this is a crucial step. The inrush of calcium would trigger the repolarization in consort with the voltage-gated K^+ channels. These calcium-activated potassium channels (CaKs) appear to play a central role in the early stages of the repolarization (Gintant *et al.*, 1992). The current responsible for this early phase of repolarization has been commonly referred to as the transient outward potassium current (I_{to}) (Gintant *et al.*, 1992). A second role for K^+ flux is discussed below with regard to the pacemaker currents.

2.6.3.1.1.4. I_f I_f is the "funny current," also called the hyperpolarization-activated current, or I_h (*inter alia*). It has been identified in some but not all pacemaker cells, and its role remains somewhat controversial (DiFrancesco, 1993). One early hypothesis for

the nature of the "pacemaker current" was that decay of the outward potassium current at the end of repolarization, against a constant inward leak current (for example, a background Na^+ current), could result in the gradually rising net inward current that ultimately starts the two-stage depolarization of the Ca^{2+} spike (DiFrancesco, 1993; Irisawa *et al*., 1993). However, several workers have reported reliable recordings of a separate slow depolarizing current, most likely carried by a combination of both Ca^{2+} and Na^+, that fulfils the definition of a true pacemaker current. It is activated when the membrane is hyperpolarized, and depolarizes the cell to the point where T-type Ca^{2+} channels open to start the spike of the action potential (Van Ginneken and Giles, 1991; Moroni *et al*., 2001).

2.6.3.1.2. Insect hearts Insect hearts are simple tubular structures that beat peristaltically to circulate hemolymph. The beat consists of a contraction phase (systole), followed by relaxation (diastole), and in some insects, there is a rest period (diastasis) preceding the next systole (Jones, 1977; Rizki, 1978). The pacemaker is myogenic, but there has been confusion on this issue. *Limulus* has been used as the perfect example of a neurogenic arthropod heart, and unfortunately, was taken to be the model for all arthropods (Prosser and Brown, 1961; Wiggelsworth, 1965). For some time, insect hearts were assumed to be generally neurogenic (Miller, 1985). A popular insect that was used to tackle the problem of myogenicity versus neurogenicity was the cockroach, *Periplaneta americana*, but this proved an unfortunate choice as its heart is heavily innervated (Alexandrowicz, 1926; McIndoo, 1945; Miller, 1969). It was initially thought to be neurogenic, owing largely to the presence of this nervous tissue, but also to pharmacological evidence (Jones, 1977). Krijgsman and Krijgsman-Berger (1951) prepared isolated, perfused *P. americana* hearts that retained their closely associated putative pacemaker ganglia and exposed them to a wide array of substances with known effects on vertebrate cardiac preparations. Caffeine, which stimulates the vertebrate heart, inhibited that of the cockroach, while digitalin, which has no effect on the vertebrate heart, was found to be acceleratory. Similarly, acetylcholine (ACh) slows the vertebrate heart but accelerates the heart rate in their insect tests. Given these responses, it was concluded that the pacemakers of vertebrates and cockroaches must be fundamentally different, and that of the latter (and by extension, that of other insects) must reside in the associated ganglia.

Insect hearts, however, have been found to be myogenic. Evidence of myogenicity is straightforward and unequivocal and comes from a number of experimental approaches (Miller, 1985). If practicable, demonstration of continued heartbeat after surgical ablation of any innervation that might contribute to pacemaking is telling. This was successfully done in *P. americana* despite its extensively innervated heart (Miller, 1969). The rate of isolated cockroach hearts continues to respond to exogenously applied cardioactive neurotransmitters like Ach and norepinephrine, indicating that not only is the pacemaker myogenic, but that some control is possible in the absence of innervation (Miller, 1969). Early work on the moths *Hyalophora cecropia* (Sanger and McCann, 1968a, 1968b) and *Manduca sexta* (Tublitz, 1988) reported a complete lack of cardiac innervation. Given that neural control of reversal of heartbeat in *Manduca* has been reported (Dulcis *et al*., 2001), clearly some innervation must exist in that animal, and this has been delineated using modern neuronal tracing techniques (Davis *et al*., 2001). However, evidence of neuronal control is not evidence of neurogenicity, and continued normal beat in the near or complete absence of intact cardiac neuronal structures strongly supports myogenicity (McCann, 1970). In *Drosophila*, which is intermediate in the extent of its innervation (Rizki, 1978; Dulcis and Levine, 2003), surgical removal of all cardiac nerves allows the beat to continue unchanged (Miller, 1950), but "twigs" of remaining axons cannot be ruled out as the source of the beat, rendering surgical evidence not entirely conclusive in this species (Rizki, 1978).

A second line of investigation utilizes pharmacology. The substance procaine is a powerful inhibitor of nerve function (Taylor, 1959; Hille, 1966), but perfusion of the cockroach heart with this substance does not affect the beat (Maloeuf, 1935) and this should have settled the matter at the time. The specific and highly effective blocker of Na^+ channels, TTX (Narahashi *et al*., 1964), is also without effect on the cockroach heart (Smith, 1969), providing a very powerful argument against neurogenicity in this heavily innervated heart. These results were repeated recently in *Drosophila*, thereby reinforcing Rizki's (1978) earlier interpretation of myogenicity in this animal (Dowse *et al*., 1995; Gu and Singh, 1995).

Manipulation of ion concentration in the perfusates of isolated hearts has been informative concerning potential pacemaking mechanisms. Various moths have been useful in these studies. The hemolymph of Lepidoptera is low in Na^+ (Carrington and Tenney, 1959; McCann, 1963) and the animals

are large and easily manipulated. McCann (1963) studied the electrophysiology of *Telea polyphemus* while varying the concentration of Na^+, K^+, Ca^{2+}, and Mg^{2+} bathing the isolated hearts. Even in the complete absence of Na^+, there was no interference with normal beating. In contrast, heart rates of both *T. polyphemus* (McCann, 1963) and *H. cecropia* (McCann, 1971), were shown to be sensitive to changes in Ca^{2+} concentration, leading to the conclusion that the myocardial action potential depends on a calcium spike rather than the typical Na^+ based neuronal action potential (McCann, 1971).

Differential effects of ACh and epinephrine have long been regarded as diagnostic in discerning myogenic versus neurogenic pacemakers, but this has turned out to be unreliable (McCann, 1970). The widely held belief was that epinephrine would accelerate both myogenic and neurogenic pacemakers, but that acetylcholine would accelerate and decelerate the neurogenic and myogenic activity, respectively. In insects, this breaks down. ACh accelerates some insect hearts, e.g., that of *Drosophila* (Johnson *et al.*, 1997, 2002), while decelerating that of *Manduca* (McCann, 1969). The effects of ACh in *Drosophila* are particularly provocative. It requires two orders of magnitude higher concentration of ACh than norepinephrine to achieve the same minimal threshold response (Johnson *et al.*, 1997), and ACh is without effect even at very high concentrations in flies bearing the temperature-sensitive *paralytic* (*para^{ts}*) mutation (Suzuki *et al.*, 1971) (see below for details on this mutation) held above the restrictive temperature, which eliminates Na^+ based action potentials. This implies that the effect of ACh is mediated through the nervous system (Johnson *et al.*, 2002). Co-injection of TTX also negates any effect of ACh (E.C. Johnson, unpublished data). Effects of norepinephrine are unaltered by the *para^{ts}* mutation (Johnson *et al.*, 2002).

In *Drosophila*, genetics offers a direct molecular approach in the form of ion channel gene mutants. Dowse *et al.* (1995) tested the mutant *para^{ts}*, which encodes a sodium channel (Loughney *et al.*, 1989). At restrictive temperatures, all sodium-dependent action potentials cease owing to a reversible change in a subunit of the essential conducting channel (Suzuki *et al.*, 1971). Adult flies pass out at this temperature, but revive when cooled back to permissive levels (Suzuki *et al.*, 1971). The heart, however, is unaffected in these animals at the restrictive temperature. This observation, along with that of the TTX application, strongly supports the argument for a myogenic origin of the beat in this organism (Dowse *et al.*, 1995).

Electrophysiology has been employed to characterize pacemaker types. The well-known neurogenic heart of *Limulus* is fairly representative of this type of pacemaking system (Prosser, 1973). Bursts in the cardiac pacemaking ganglia precede systole and subside during diastole. This makes sense, given that the ganglia are signaling the myocardium to contract and coordinating the beat process. In the cockroach, however, three things may be recorded depending on timing and conditions. First, the heart may be seen to beat when there is no activity in the ganglia whatsoever. Second, the activity in the ganglia may be intense, but not coordinated with the beat in any meaningful way. Third, there may be overt synchrony, but this will occur during diastole, not systole. The interpretation of this is that the ganglia are actually responding to the heart's autonomous beat by a burst of activity resulting from a stretching of the nerve tissue by the physical movement of the myocardium (Prosser, 1973; Jones, 1977; Miller, 1985). Recordings of electrical activity in the insect myocardium itself are similar to classical myogenic action potentials in the vertebrate heart. Rather than the clustering of spikes seen prior to systole in the pacemaking ganglion typical of *Limulus*, insect hearts show a slow rising pacemaker potential leading to a single, relatively long-lived action potential spike (McCann, 1963; Rizki, 1978; Miller, 1985).

This typical pattern can be seen in insects across all levels of cardiac innervation. The highly innervated heart of *P. americana* still produces a depolarizing pacemaker potential leading to a long-lived action potential, followed by a refractory phase (Miller, 1985). The least innervated heart commonly studied is that of *H. cecropia* (Sanger and McCann, 1968a, 1968b; McCann and Sanger, 1969). Recordings from single myocardial cells in *H. cecropia* yield the significant observation that individual cells could produce a range of action potential profiles depending on the depolarization imposed on them (McCann and Sanger, 1969). In an extensive study of the mealworm beetle, *Tenebrio molitor*, recordings from the myocardium yield typical myogenic patterns (Markou and Theophilidis, 2000). Johnson *et al.* (2001) demonstrated simple two-electrode electrocardiograms of *Drosophila*. Though these do not have the resolution of individual cell recordings, they clearly indicate myogenicity, lacking any sharp bursts correlated with contraction as is found in the neurogenic condition. A useful preparation for monitoring the isolated heart of *Drosophila* has been reported by Papaefthimiou and Theophilidis (2001) with the same pattern being evident.

2.6.3.1.2.1. Pacemaker location The hearts of vertebrates are arranged in a hierarchy, with the SA node cells, or at least a subpopulation thereof, setting the beat for the rest of the myocardium

(Irisawa *et al.*, 1993). This appears not to be the case in insects. Distinctive nodal tissue remains unreported in histological studies of insect hearts (see, for example, Sanger and McCann, 1968a or Curtis *et al.*, 1999). Functional approaches suggest that all of the heart proper may function to generate a beat in many insects. In *T. polyphemus*, all heart regions tested, under appropriate circumstances, demonstrate the classic pacemaker potential that confers automaticity (McCann, 1963). This implies that in the moth, the myocardium generally can act as a pacemaker, and that such activity is not localized to a specific node-like region. In *T. molitor*, when the heart is sectioned, each segment retains intrinsic rhythmicity in isolation. However, the *Tenebrio* aorta, unlike that of *Manduca*, contains no self-sustaining pacemaker function (Markou and Theophilidis, 2000).

Despite this evidence of anatomical and histological homogeneity of pacemaking, there are reports that suggest that some specialization exists. As detailed below, injection of the L-type calcium blockers verapamil or diltiazem appears to stop contractions in the myocardium in general, while leaving beat in a caudal section of the heart unaffected (Johnson *et al.*, 1998). This implies that there is a different population of ion channels in the two regions and that the most caudal region may serve as a "master pacemaker."

Functional localization of discrete pacemaker regions is also suggested by a peculiarity of insect hearts, namely the observation that the direction of the peristaltic wave that constitutes diastole can reverse direction (Jones, 1977). This has been noted across a wide range of insects. The implication is that there must be two distinct pacemaker regions that can alternate dominance and must be coordinated. Jones (1977) mentions a remarkable example of beats initiating at both ends of the heart simultaneously, with the resultant waves meeting in the middle and canceling each other out. This process is likely to be under neural control at some level, to account for the coordination of pacemakers. Reversal is often seen to result from some external cue, such as behavior (Kuwasawa *et al.*, 1999). One common thread may be alterations in gas exchange patterns as the hemolymph flow changes direction (Wasserthal, 1981, 1996). Birchard and Arendse (2001) applied an allometric analysis to relate respiration with cardiovascular function. Despite the obvious lack of oxygen-carrying pigments in insect hemolymph, measurements of critical heart parameters like rate and cardiac output scaled linearly with oxygen consumption, with nearly the same exponent, providing further circumstantial evidence of a functional relationship (Birchard and Arendse, 2001). Neural control of pacemaker dominance has been well documented in several instances, with *M. sexta* being a major contributor in these studies. Dulcis *et al.* (2001) used electrocardiograms to document changes in heart function in larvae, pupae, and adults of this moth. Their findings in larvae corroborated earlier work in that only anterograde pacemaking occurred (Platt and Reynolds, 1985; Smits *et al.*, 2000). In early pupae, however, they saw reversals, with rapid anterograde pulsing alternating with slow retrograde. In all cases, the pupal heart rate was slower than that found in the adult. By treating the preparations with TTX, they were able to show both that the beat was myogenic, (clearly no surprise) but the elimination of Na^+ dependent action potentials eliminated the alternation between anterograde and retrograde, leaving only the slow retrograde. Surgical section of the nerves to the posterior portion of the heart mimicked the effects of TTX, leaving only the anterior pacemaker active. Stimulation of this nerve could be used to control the reversals as well (Dulcis *et al.*, 2001). Thus, although the entire myocardium may be generally capable of pacemaking, discrete regions may be dominant.

2.6.3.1.2.2. Electrogenesis of the pacemaking signal Given that the origin of heartbeat in insects is myogenic, a major concern is the electrogenesis of the pacemaker and action potentials in the myocardium. The ultimate elucidation will culminate with the identification and biophysical characterization of the ion channels involved, the genes encoding them, and the specific splice variants represented in the pacemaker tissue. A great deal of general cardiac physiology has been previously done in a range of insects, most notably *Periplaneta, Manduca,* and *Hyalophora*, which gives a good basic understanding of the ion species involved and their conduction kinetics (McCann, 1970; Miller, 1985). Determining which ion species are involved in the cardiac pacemaker and action potentials is a good place to start. This can be done by straightforward manipulation of concentration in the perfusate bathing isolated hearts. One obvious possibility is that the pacemaker is Na^+ dependent, given the ubiquitous nature of sodium inward currents in action potentials in the nervous system. The work described above to determine whether the pacemaker is neurogenic clearly eliminates this possibility. Insect pacemakers turn out not to be Na^+ dependent (McCann, 1963, 1969; McCann and Sanger, 1969; Miller, 1985), and this matches precisely the findings in mammals (Irisawa *et al.*, 1993).

Based on extensive work on the vertebrate heart, a prime candidate is Ca^{2+} (Irisawa *et al.*, 1993). McCann (1971) demonstrated Ca^{2+} spikes in *H. cecropia* by showing a relationship between cardiac action potential levels and Ca^{2+} concentration. With calcium-free perfusate, the action potential quickly drops to zero. In further support of this, replacement of Ca^{2+} with Mn^{2+} in the perfusate also quickly abolished excitability in the cells. K^+ is a second possible candidate. McCann (1963), working with *in situ* hearts of *T. polyphemus* that had been decapitated and had the mesothoracic ganglion removed, determined that varying the concentration of K^+ in the perfusate could affect heart rate.

In order to establish the ionic mechanism of the insect pacemaker, a test organism that can facilitate such studies is required. Given the clear advantages of working with *D. melanogaster* in unraveling cellular and molecular mechanisms, it is surprising that work on its heart only began in earnest in the mid 1990s. In 1978, Rizki published a thorough monograph, covering the anatomy, histology, composition of the hemolymph, and physiology in *Drosophila*. In this work, the question of myogenic versus neurogenic arose; however, Rizki did not attempt to settle it, but simply noted that the beat was probably myogenic, given that physical isolation of the heart did not cause it to cease beating, but that the process of reversal suggested some neural control. He also reported on the temperature dependence of the *Drosophila* heartbeat, but did not speculate on its cause (Rizki, 1978).

Drosophila cardiac physiology was not investigated further substantively until the work of White *et al.* (1992). Given the linearly dose-dependent effect of deuterium oxide ("heavy water" or D_2O) in slowing the circadian clock (Edmunds, 1988), these investigators looked at its effects on heart rate and their interactions with temperature. The choice was made to observe only very early stage pupae for this (and later studies from this laboratory cited here), owing to the ease with which heartbeat could be monitored with a noninvasive optical system (Dowse *et al.*, 1995; Johnson *et al.*, 1997, 1998). Flies were raised on medium containing various concentrations of D_2O and the rate was measured over a range of temperatures. In this work, the data were analyzed using current digital signal analysis techniques, including maximum entropy spectral analysis (MESA) (Burg, 1967; Ulrych and Bishop, 1975) and signal-to-noise ratio (SNR) programs specifically adapted by one of the authors for biological time series (Dowse and Ringo, 1989; review: Levine *et al.*, 2002). This allowed precise estimates of heartbeat frequency and regularity. The authors discovered that D_2O decreased heart rate in a dose-dependent fashion, but noticed a curious interaction between temperature and deuterium concentration. There was a flattening of the slope relating heart rate to temperature as the concentration of D_2O was raised, suggesting that the heart was not as sensitive to changes in temperature when deuterated. Unfortunately, owing to the multiple effects of heavy water on cellular processes, no firm conclusions could be drawn regarding the mechanism of the pacemaker or its control (White *et al.*, 1992).

Immediately, further studies appeared that applied a combination of genetics and pharmacology to unraveling the ionic basis of the pacemaker and its control. Dowse *et al.* (1995) began by searching for mutants that affected heart rate or regularity from among a large group of candidates including those displaying neurological and behavioral phenotypes. Among these were lesions in genes encoding ion changes. The pupal preparation described in White *et al.* (1992) was employed here, with the addition of a new method of quantifying rhythmicity. The height of the third peak of the autocorrelation function was taken as the "rhythmicity index" (RI). The decay envelope of this function is a reliable estimator of long-range regularity in a rhythmic time series (Chatfield, 1980; Levine *et al.*, 2002). Among these were lesions in genes encoding ion channels. The first *bona fide* heart temperature-sensitive mutant to surface was *no action potential* (*nap^{ts}*) (Wu *et al.*, 1978; Dowse *et al.*, 1995). This had originally been thought to encode a Na^+ channel, but turned out to be a regulatory gene controlling, among other things, the gene *$para^{ts}$* via a splicing catastrophe (Suzuki *et al.*, 1971; Wu *et al.*, 1978; Ganetzky *et al.*, 1984; Jackson *et al.*, 1984; Loughney *et al.*, 1989; Reenan *et al.*, 2000). In testing *$para^{ts}$* itself, it turned out to have no effect on heart rate or regularity (Dowse *et al.*, 1995). This finding further established the myogenicity of the *Drosophila* heart, but also begged the question of what exactly *nap^{ts}* was doing to the heart (Dowse *et al.*, 1995). The hearts of mutant flies were arrhythmic compared to wild-type at all temperatures, and this irregularity increased with temperature. A curious fact emerged, and that was that *nap^{ts}* hearts were less able to track increases in temperature, barely increasing frequency at all over more than a 10 °C range. This is most curious, as heart rate in poikilotherms is closely tied to temperature, and the physical chemistry of the oscillating system demands an alteration in rate. Yet no change occurs.

K^+ channels have been firmly established as components of the *Drosophila* pacemaker. Tetraethylammonium (TEA), a general inhibitor of K^+

channel function (Gho and Mallart, 1986), slows the heart (Gu and Singh, 1995; Johnson *et al.*, 1998), but this substance affects all four known subgroups of K^+ currents in larval and adult muscle. There are two Ca^+ activated currents, fast (I_{CF}) and one slow (I_{CS}), and two voltage activated currents, fast (I_A) and slow (I_K) (Singh and Wu, 1989). These currents and the responsible channels can be separated by combining pharmacology and genetics (Singh and Wu, 1989). To do so for the heart pacemaker, an initial genetic screen yielded three channels as candidate pacemaker components, all of which pass K^+ ions (Johnson *et al.*, 1998). The most profound K^+ channel mutation affecting the heart is *slowpoke* (*slo*) (Elkins *et al.*, 1986). Flies bearing this mutation have almost nonexistent heartbeat, and such rate as may be discerned does not increase with temperature as was found with nap^{ts}. *slo* encodes the pore forming subunit of a Ca^{2+} activated K^+ (CaK) channel (Atkinson *et al.*, 1991) and interferes specifically with I_{CF} (Elkins *et al.*, 1986). Such channels have been implicated in repolarization in the vertebrate heart pacemaker (see above). To cross-check this finding, pupae were injected with charybdotoxin (CTX), a specific blocker of CaKs, and the result was exactly as was seen in the mutants, i.e., extreme interference with heart function (Johnson *et al.*, 1998).

Two other mutants turned up as candidates. The first, *ether-a-go-go* (*eag*), encoding a voltage-gated K^+ channel (Wu *et al.*, 1983; Warmke *et al.*, 1991; Zhong and Wu, 1991), affects all four currents and makes the heart beat slightly more slowly and irregularly than in the wild-type (Johnson *et al.*, 1998). The final candidate, *Shaker* (*Sh*), encodes another voltage-gated K^+ channel (Salkoff and Wyman, 1981; Singh and Wu, 1989) and turns out to be extremely interesting, in that it renders heartbeat more rapid and rhythmic than in the wild-type (Johnson *et al.*, 1998). Other K^+ channel blockers had minimal effect (Johnson *et al.*, 1998).

Chief among the missing components, based on the vertebrate model, are voltage-gated Ca^{2+} channels. Manipulation of Ca^{2+} concentrations in insect hearts (see above) demands their participation. Gu and Singh (1995) found that the L-type calcium channel blockers verapamil and diltiazem affected heart rate in their preparations. Johnson *et al.* (1998), using an optical monitoring system on intact pupae, as well as semi-isolated perfused pupal heart preparations, found that the two blockers had no effect on heart pacemaker rate or rhythmicity. However, the interesting observation was made that while the posterior region of the heart continued to function normally, the rest of the myocardium did not (see above). This leaves open the provocative possibility that either EC coupling is disrupted, or that L-type channels are necessary for the action potential in the bulk of the heart muscle, while there is a separate true pacemaker located posteriorly that is responsible for generation of the beat. The rest of the heart may be able to beat on autonomously, but requires this unique pacemaker tissue for normal heart rate and rhythmicity. This would be analogous to the SA nodal tissue in mammals. No histologically unique tissue could be discerned in this area (Curtis *et al.*, 1999).

Participation of a non-L-type calcium channel was discovered pharmacologically using a toxin from a piscivorous snail, ω-conotoxin VIIC (ω-CgTx), which targets precisely a non-L-type voltage-gated K^+ channel (Olivera *et al.*, 1994). This toxin renders the *Drosophila* pupal heart almost totally inactive at very low doses (Johnson *et al.*, 1998). Pharmacology, however, does not easily deliver the targeted channel molecularly. Thus, genetics remains as the path to the final piece in the puzzle.

Two Ca^{2+} channels currently under study are likely candidates for playing a role in heart function. The first is an L-type channel, and thus is a likely contributor to the action potential in the myocardium generally. It is encoded by the *calcium channel alpha1D* subunit (*DmCa1D*; Eberl *et al.*, 1998), and is DHP sensitive, hence predictably L-type in its kinetics (Eberl *et al.*, 1998), it is a bona fide heart mutant. Flies homozygous for the allele *AR66* live until the early pupal stage, and may be tested for heart function in the usual manner, though they die before eclosion. Such flies have an aberrant heartbeat (E.C. Johnson and H. Dowse, unpublished data). The second is a DHP-insensitive Ca^{2+} channel named DmCa1A, the α1 subunit of which is encoded by the gene *cacophony* (*cac*) (Schilcher, 1976, 1977; Smith *et al.*, 1996). Two *cac* alleles, cac^S (for *cac-song defective*), originally uncovered by Schilcher (1976, 1977) and cac^{ts2} (*temperature sensitive 2*) (Dellinger *et al.*, 2000), affect the pulse component in the *Drosophila* mating song, causing several extra oscillations in each pulse (Schilcher, 1977; Kulkarni and Hall, 1987). The *cac* alleles both affect the heart in an unusual way, but one that makes sense in light of what is observed in the song pulses. The heart beats more rapidly and more regularly than wild-type, especially at high temperatures (V. McGowan and H. Dowse, unpublished data).

The results of this genetic and pharmacological identification of essential pacemaker ion channels suggested a simple model for the *Drosophila* pacemaker (Johnson *et al.*, 1998). A leaky cation channel would cause slow depolarization, i.e., the

pacemaker current, enough to trigger a Ca^{2+} spike, the main outward current of the action potential. This inrush of Ca^{2+} would trigger a calcium-activated K^+ channel (e.g., *slo*?), causing delayed repolarization of the membrane and return to the "resting potential." Such a model is not dissimilar to one proposed for generation of the pulse in *Drosophila* song involving the same ensemble of ion channels (Peixoto and Hall, 1998). Without the leak pacemaker current to confer automaticity, an oscillator constituted in this way has been delineated in the vertebrate ear. Oscillatory interactions between voltage-gated Ca^{2+} channels and Ca^{2+}-gated K^+ channels underlie the resonant response to sound in the vertebrate hair cell (Art and Fettiplace, 1987; Hudspeth and Lewis, 1988a, 1988b), and frequency response has been attributed to the differential expression of Ca^{2+}-gated K^+ channel isoforms along the length of the cochlea (Rosenblatt *et al.*, 1997; Black, 1998; Jones *et al.*, 1999). It is hypothesized that this gradation is the substrate for frequency resolution atleast lower in lower vertebrate hearing (Rosenblatt *et al.*, 1997; Black, 1998; Jones *et al.*, 1999).

The Johnson *et al.* (1998) model has received experimental support from Markou and Theophilidis (2000) working with *T. molitor*. These workers depleted Ca^{2+} in the perfusate of isolated hearts and found that not only did the heart stop beating, but it also entered a state of depolarization. As soon as Ca^{2+} was added to the perfusate, the heart cells immediately repolarized and regained oscillatory function. The interpretation was that Ca^{2+} was needed to trigger the K^+ current through a CaK channel enabling repolarization.

Mathematical modeling of this simple scheme confirms that these components are capable of generating autonomous oscillations (S. Crook, unpublished data). **Figure 8** illustrates the currents required by the model, with an outward leak current (I_{lk}) that serves as the pacemaker current needed for automaticity, an inward calcium-spike (I_C), and the repolarizing outward potassium current triggered by rising intracellular calcium concentration (I_{K-Ca}).

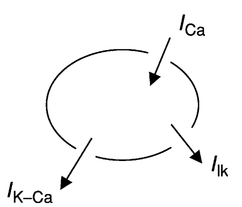


Figure 8 Diagrammatic summary of the currents needed for the *Drosophila* pacemaker model delineated by Johnson *et al.* (1998). See text for details.

A Hodgkin Huxley-type model was implemented in Simulink (S. Crook, unpublished data).

The following equations describe the ionic currents; the parameters denoted by g represent the maximal conductances for each current, which are determined by the density of ion channels, while the parameters denoted by E represent the reversal potentials for each current:

$$I_{K-Ca} = g_{K-Ca} r(v - E_K)$$
$$I_{Ca} = g_{Ca} qp(v - E_{Ca})$$
$$I_{lk} = g_{lk}(v - E_{lk})$$

From these current relationships, the following equations were derived to create the model pacemaker:

$$\frac{dv}{dt} = -I_{K-Ca} - I_{Ca} - I_{lk} \quad [1]$$

$$\frac{dc}{dt} = -\alpha I_{Ca} - \beta c \quad [2]$$

$$\frac{dq}{dt} = \frac{q_\infty(v) - q}{\tau_q(v)} \quad [3]$$

$$\frac{dp}{dt} = \frac{p_\infty(v) - p}{\tau_p(v)} \quad [4]$$

$$\frac{dr}{dt} = \frac{r_\infty(c) - r}{\tau_r(c)} \quad [5]$$

The following are state variables in the system: v, membrane potential; c, the concentration of intracellular Ca^{2+}; q, p, gating variables representing the voltage dependent activation and inactivation (respectively) of the Ca^{2+} channel; and r, the Ca^{2+} dependent activation of the K^+ channel.

The model equations include a current balance equation (eqn [1]) describing membrane potential change over time and how the changes depend on ionic currents including the leak current. Equation [2] describes the change in $[Ca^{2+}]$ with time. Ca^{2+} is removed by membrane based pumps with a rate proportional to intracellular $[Ca^{2+}]$. Equations [3]–[5] describe the change of the gating variables in time as voltage- and calcium-gated channels activate and inactivate. In eqs [3]–[5], the curves denoted by "∞" represent the steady-state activation or inactivation curves for each gating variable and the τ curves represent time constants for reaching those steady-state values as voltage or Ca^{2+} varies. The simulated model voltage trace shown in **Figure 9** shows that membrane potential in the system is spontaneously oscillatory.

Internal release of calcium from ryanodine receptors has also been implicated in heart function,

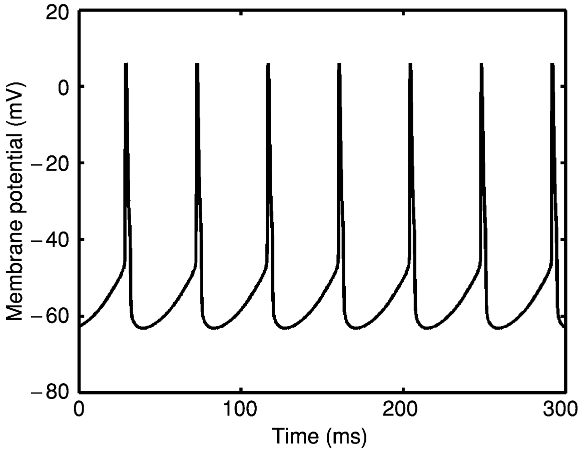


Figure 9 Output of the model demonstrating self-sustained pacemaker-like oscillatory behavior.

potentially in the pacemaker. The *Ryr[16]* mutation slows heartbeat substantially in all three larval stages (Sullivan *et al.*, 2000), and injection of ryanodine is also effective in slowing heart rate in larvae (Sullivan *et al.*, 2000) and pupae (E.C. Johnson and H. Dowse, unpublished data). Coupled with this, interference with normal levels of cytoplasmic Ca^{2+} by lowering sequestration rates into the sarcoplasmic reticulum also causes what appears to be pacemaking problems. A mutation that effects a decrement in function in the *s*arcoplasmic/*e*ndoplasmic *r*eticulum *ca*lcium ATPase (SERCA) causes severe disruption of heart function (M. Ramaswami, S. Subhabrata, and H. Dowse, unpublished data). One allele, *kumbhakarna* (*kum[170]*), causes almost total cessation of heartbeat after a 41 °C heatshock.

2.6.3.1.3. Broader applicability of the insect findings Elucidation of the pacemaker ion channels in insects may have a direct bearing on matters of human health. The gene *eag* was initially discovered in *Drosophila* (Catsch, 1944; Kaplan and Trout, 1968). The cDNA from this gene was used to detect a closely related channel in mammals: human ether a go go related gene (HERG) (Warmke and Ganetzky, 1994). HERG was ultimately shown to be a homolog of the *Drosophila* gene *seizure* (Jackson *et al.*, 1984; Wang *et al.*, 1997). Mutations in HERG result in a cardiac arrhythmicity in humans, chromosome 7-linked long QT (LQT2) syndrome (Keating and Sanguinetti, 1996; Sanguinetti *et al.*, 1996). The HERG channel passes a rapidly-activating, delayed-rectifier current (I_{Kr}), and in individuals carrying the mutation, there is a delay in cardiac repolarization (Curran *et al.*, 1995; Sanguinetti *et al.*, 1995; Sanguinetti *et al.*, 1996). Provocatively, *eag*, causes a congenital defect in the *Drosophila* heart (Johnson *et al.*, 1998).

2.6.3.2. Physiological Basis of Modulation

A requirement of cardiac pacemaking is that it must be flexible, as it needs to adjust cardiac output to meet metabolic demands over a large range of different environmental and behavioral conditions. In insects, multiple functions are associated with heartbeat and while respiration in insects is largely an independent process, the delivery of nutrients and hormones depends upon a functional cardiovascular system. In a number of these poikilothermic animals, heartbeat contributes to the process of thermoregulation (Heinrich, 1970, 1971). Additionally, in the holometabolous insects, the process of adult wing inflation involved in ecdysis behaviors rely upon the mechanical actions of the heart pumping hemolymph (Baker *et al.*, 1999; Park *et al.*, 2003).

The cardiac pacemaker of insects is responsive to changes in temperature, electrochemical gradients, and transmitter titers and alters output to meet different physiological demands. Ultimately, changes in heart rate are mediated through alterations of transmembrane potentials, such as the ones outlined above (Trautwein and Osterrieder, 1986). Some stimuli may mediate these modifications on pacemaker potentials directly, e.g., diffusible transmitters acting upon ionotropic receptors. Other stimuli do so indirectly, through networks of intracellular signaling pathways leading to biochemical modifications of pacemaker components. As discussed above, the pacemaker consists of an ensemble of ion channels, coordinating the rhythmic contractions of the heart through the generation of oscillations in transmembrane potential. Therefore, it follows that any stimulus capable of changing pacemaker output must somehow act upon these electrochemical oscillations. Acceleratory and inhibitory stimuli might alter pacemaker currents through either the addition of novel ionic currents that are normally quiescent, or through the modification of existing currents underlying pacemaker activity.

The authors could extrapolate what has been discovered about the ionic pacemaker to make general assumptions regarding modulatory mechanisms. Acceleratory stimuli must affect these currents predictably; specifically, they must shorten the length of the repolarization or rest phase of pacemaker tissue. By definition, inhibitory stimuli actions must do the opposite. This suggests that different transmitters acting upon the pacemaker in a similar fashion may share common signaling pathways.

2.6.3.2.1. Signaling pathways Ion channels typically contain multiple consensus sites for various modifying proteins, including various kinases and phosphatases, linking them as potential targets of intracellular signaling pathways. For example, the *slo* channel is phosphorylated by the cAMP dependent kinase (PKA), as well as Src tyrosine kinases (Wang *et al*., 1999; Zhou *et al*., 2002); these changes culminate in a decrement in channel activity (Zhou *et al*., 2002). Likewise, another candidate pacemaker channel, encoded by the *eag* gene, is a substrate for calmodulin-CAM-kinase II phosphorylation and this biochemical modification causes a reduction in *eag* current amplitude. In whole cell recordings of the *Drosophila* retina, the biogenic amine serotonin affects the potential at which *Shaker* channels open, and stimulation of G proteins mirrors this effect (Hevers and Hardie, 1995). Calcium channel activity in *Drosophila* is modulated by the activity of PKA and PKC (Gu and Singh, 1997). Unfortunately, technical constraints make it difficult to directly connect a transmitter to an altered pacemaker current. However, pharmacological and genetic approaches have been utilized to implicate signaling pathways that mediate cardiac modulation. Manipulations of G proteins, cAMP, cGMP, and protein kinases through genetic variants and/or pharmacological agents have illuminated some common elements of excitatory and inhibitory modulatory networks.

In *Drosophila*, at least one member of each representative G protein α subunit is present (Gs, Gq, Gi, and Go), as well as β and γ subunits (Wolfgang *et al*., 1990; Quan *et al*., 1993; Talluri *et al*., 1995). Pharmacological agents that discriminate between these G protein subtypes (see **Chapter 5.5**) have been used to assess possible involvement of specific G proteins in cardiac modulation. Cholera toxin, which activates Gs pathways, produces no significant change in heartbeat, while in contrast, pertussis toxin, which blocks receptor-Gi/o protein interactions, significantly increases basal heart rate (Johnson *et al*., 2002). Inhibitory stimuli operate through a pertussis-sensitive G protein subtype and a number of neuropeptides and transmitters have been shown to slow the heart (reviewed below). These effects are potentially mediated through the α subtypes. A candidate gene, $G_{o\alpha}47A$, is expressed in cardiac tissue during development and regulates formation of the cardiac epithelia (Guillen *et al*., 1990; Fremion *et al*., 1999).

In *Drosophila*, two mutations have been identified as essential components of cAMP signaling, *dunce* (*dnc*) and *rutabaga* (*rut*), which encode cAMP phosphodiesterase and adenylate cyclase, respectively (Chen *et al*., 1986; Davis and Davidson, 1986; Levin *et al*., 1992). Both *dnc* and *rut* pupal hearts responded normally to an increasing temperature paradigm (Dowse *et al*., 1995). These mutants also have wild-type responses to cardioacceleratory transmitters (Johnson *et al*., 2002). These results, together with the demonstrated lack of effect with applications of forskolin, imply that the cAMP signaling system is either not involved in or plays a minor role in cardiac signaling (Johnson *et al*., 2002). However, the possibility remains that both *dnc* and *rut* gene expression is limited to other tissues, and other cyclases and phosphodiesterases are expressed in the heart. Consonant with this alternative, injection of a cAMP analog increases heart rate, producing modest but significant differences in heart rate (Johnson *et al*., 2002).

In *Drosophila*, injections of a cGMP analog significantly increase heart rate in a dose-dependent fashion suggesting a role for cGMP signaling in cardiac modulation (Johnson *et al*., 2002). cGMP modulation has been demonstrated as a cardiac mediator in the heart of *M. sexta* as evinced by experiments dealing with the cardioacceleratory neuropeptide, CAP_{2B}. This peptide liberates cGMP, which subsequently elevates the concentrations of free calcium (Tublitz, 1988; Tublitz *et al*., 1992; Davies *et al*., 1995). Further demonstration of the role that this cyclic nucleotide plays a role in cardiac modulation stems from experiments on a mutant of cGMP-dependent protein kinase (PKG). In *Drosophila*, multiple genes encode isoforms of PKG, and one was identified due to its effects on feeding behavior and called *foraging* (Osbourne *et al*., 1997). Two alleles occur in natural populations, *rover* and *sitter*, with *rover* alleles being wild-type and fully dominant (deBelle *et al*., 1989). The *sitter* allele is a hypomorph of *foraging*, producing less PKG, and these variants exhibit tachycardia (Johnson *et al*., 2002). While this result may not be in the predicted direction, it further implicates a cGMP-dependent pathway in modulating heartbeat. A possible explanation for the acceleration of heart rate in a hypomorph of PKG is that the targets of PKG are part of a feedback mechanism. The increase in intracellular calcium, caused by cGMP, might directly alter cardiac action potentials through the activation of the calcium-activated potassium channel, *slowpoke*. However, cGMP could be acting directly on other pacemaker ion channels like *eag*, as these channels are gated by this cyclic nucleotide (Bruggemann *et al*., 1993), or indirectly through the activation of PKG and its subsequent phosphorylation of target molecules. Therefore, the modulatory action of cGMP could occur at a number of different levels.

The release of intracellular calcium is a feature of pacemaker modulation, specifically of a calmodulin-CAM kinase II (CAMKII)-dependent signaling pathway. Flies bearing a single copy of *l(4)16*, which encodes CAM kinase II (Griffith *et al.*, 1993), eliminated the cardioacceleratory action of serotonin (Johnson *et al.*, 2002). Heart rate in these flies is stimulated by catecholamines, suggesting different mechanisms of action for these different agents. These genetic results were recapitulated by pharmacological experiments; a specific inhibitor of CAMKII abolished the cardioacceleratory effects of serotonin but not norepinephrine (Johnson *et al.*, 2002). The fact that ryanodine, which blocks the release of calcium from intracellular stores, nearly eliminates heartbeat (H. Dowse and E.C. Johnson, unpublished data), also argues that intracellular calcium is required for pacemaker function. The many peptide and transmitter receptors, which liberate calcium via a phospholipase C (PLC) dependent pathway, are indicative of a signaling role of intracellular calcium.

2.6.3.2.2. Small molecule transmitters The biogenic amines are common transmitters and hormones with multiple roles in different physiological, developmental, and behavioral processes. The wealth of classical genetic information and the advances in molecular genetic techniques in *Drosophila* have facilitated the identification of numerous transmitter and modulator substances and their respective downstream elements (Restifo and White, 1990; Buchner, 1991; Monastirioti, 1999). In *Drosophila*, biochemical analysis indicates the presence of the biogenic amines and acetylcholine (Watson *et al.*, 1993), and genes that encode critical enzymes for the synthesis of these transmitters have been identified, allowing for manipulations and assessment of biogenic amine signaling.

Injections of neurotransmitters have identified specific agents that are cardioactive. In *Drosophila*, pupal heart rate increases in response to application of serotonin, dopamine, octopamine, norepinephrine, and acetylcholine, in that order of potency (Johnson *et al.*, 1997). GABA modestly affects the heart of *Drosophila*, decreasing heart rate (Zornik *et al.*, 1999). Glutamate and glycine, common excitatory amino acid transmitters, are without effect on the *Drosophila* pacemaker (Johnson *et al.*, 1997; Zornik *et al.*, 1999). In *Periplaneta*, studies have demonstrated nearly the same pharmacological profile as in *Drosophila*, with serotonin being a powerful cardioacceleratory agent, followed in potency by octopamine, dopamine, and norepinephrine (Miller and Metcalf, 1968). Similar actions of these transmitters have been reported in *Manduca* (Prier *et al.*, 1994), and *Periplaneta* (Collins and Miller, 1977), suggesting conservation of cardiac aminergic signaling across insect orders.

2.6.3.2.3. Synthetic pathways The *pale* (*ple*) gene, which encodes the enzyme tyrosine hydroxylase (TH) (Neckameyer and White, 1993), catalyzes the rate-limiting conversion of tyrosine to L-dopa (Neckameyer and Quinn, 1989). This enzyme shows nearly 50% homology to vertebrate TH and is alternatively spliced to form two transcripts of 3.2 and 3.65 kb, with the latter being adult-specific (Neckameyer and White, 1993). Mutants in the *pale* gene are devoid of catecholamines, but not 5′-hydroxy-tryptamine (5-HT) (Budnik and White, 1987). A temperature-sensitive mutant, ple^3, significantly reduces heart rate and rhythms (E.C. Johnson and H. Dowse, unpublished data).

A major enzyme *en route* to dopamine and serotonin synthesis is dopa decarboxylase (Prosser and Brown, 1961; Tempel *et al.*, 1984). The *Drosophila* gene encoding the dopa decarboxylase (Ddc) enzyme (Hirsch and Davidson, 1981) is functionally similar to those of other species (Black and Smarelli, 1986). Raising temperature sensitive mutants for this gene at restrictive temperatures for 24–48 h causes the natural titers of these transmitters to fall to zero (Livingstone and Tempel, 1983). Heart rates and rhythms are also significantly decreased in Ddc^{ts1} mutants (Johnson *et al.*, 1997; Ashton *et al.*, 2001).

Another relevant enzyme that catalyzes the synthesis of octopamine is tyramine β-hydroxylase, which can be used as a marker for the identification of octopaminergic neurons. A null mutation in the tyramine β-hydroxylase gene results in an octopamine-less fly, but heartbeat has not been examined in this interesting genetic and physiological background (Monastirioti *et al.*, 1996). In an anatomical study using an antibody against this enzyme, a number of cardiac neurons in *Drosophila* were identified that are octopaminergic (Siegmund and Korge, 2001). Specifically, neurons arising from the *pars intercerebralis* that innervate the aortic wall are octopaminergic. These results not only indicate a cardioactive role for the biogenic amines, but also suggest a background role of neurotransmitters for normal pacemaker activity (Johnson *et al.*, 1997). This may also be the case in the cockroach, *Periplaneta*, in which the perfusion of hemolymph onto isolated hearts accelerates heart rate (Metcalf *et al.*, 1964). It is plausible that the decrement in heart rate exhibited by *Ddc* and *ple* mutants is caused by the loss of excitatory transmitters so that the effects

of inhibitory transmitters, unchanged by these mutations, are visible. Therefore, the relative levels of excitatory and inhibitory transmitters contribute to determining basal heart rate. Ashton *et al.* (2001) screened for aberrant cardiac phenotypes resulting from haploinsufficiency and found a number of loci exhibiting tachycardia and bradycardia. These loci included genes encoding receptors for serotonin as well as dopamine.

2.6.3.2.4. Transmitter receptors Small molecule transmitters can bind to multiple classes of receptor proteins, namely, G protein-coupled receptors (GPCRS: see **Chapter 5.5**) and ionotropic receptors. The annotated genome of *Drosophila* has identified the complement of proteins belonging to the GPCR superfamily (Adams *et al.*, 2000; Brody and Cravchik, 2000). A genomic analysis indicates the presence of multiple subtypes of receptors for each transmitter (Brody and Cravchik, 2000; Blenau and Baumann, 2001). Specifically, there are ~21 genes predicted to encode GPCRs for the biogenic amines (Brody and Cravchik, 2000), with multiple types for serotonin (Tierney, 2001), dopamine (Hearn *et al.*, 2002; Kim *et al.*, 2003), octopamine (Reale *et al.*, 1997; Chatwin *et al.*, 2003), tyramine (Saudou *et al.*, 1990) and acetylcholine (Harrison *et al.*, 1995), in addition to ~10 orphans that are phylogenetically similar to other amine receptors. Likewise, the presence of ligand gated ion channels have been identified in *Drosophila*, and function as receptors for histamine (Zheng *et al.*, 2002), GABA (ffrench-Constant *et al.*, 1992; **Chapter 5.4**), glutamate (Ultsch *et al.*, 1992), and acetylcholine (Chamaon *et al.*, 2002). However, precise definition of the ligands and the distribution of these receptors is presently lacking. Currently, the identification and characterization of all of these receptor(s) involved in cardioregulation is pending.

Despite these obstacles, the results from a pharmacological study in *Drosophila* can be applied to identify candidate receptors. Injections of serotonergic receptor agonists and antagonists suggest that a receptor with similar pharmacological properties similar to the mammalian 5-HT_2 receptor is involved in cardiac signaling (Johnson *et al.*, 2002). Such a receptor has been cloned in *Drosophila*, shares pharmacological similarities and interacts with the same downstream effectors to the homologous mammalian 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors (Colas *et al.*, 1995).

2.6.3.2.5. Cardiac innervation A question to consider with studies on cardiomodulatory substances is upon what tissue is a given molecule exerting its affect? It is generally accepted that the origin of heartbeat in insects is myogenic and subject to neuronal modulation. For instance, some insects display heartbeat reversal, in which the "normal" posterograde conduction of beat is replaced by an anterograde mode of heartbeat (see above). This reversal of heartbeat mode is under neural control, and in the lepidopteran, *Agrius convolvuli,* it was found that the transmitter octopamine accelerated heartbeat in the anterograde heartbeat, and facilitated the reversal of posterograde to anterograde beat (Matsushita *et al.*, 2002). In *Manduca*, cyclical patterns of heartbeat mode are observed during metamorphosis, and, as TTX disrupts this physiological cycle, heartbeat reversal is under neuronal control (Dulcis *et al.*, 2001). Furthermore, systematic dissection of nerves resulted in the location of a precise nerve (DN8) that innervates the caudal end of the heart, and is responsible for this heartbeat reversal behavior (Dulcis *et al.*, 2001).

The site of action must be determined for cardiac transmitters, as information regarding anatomical targets for cardiac modulators is necessary to establish a complete picture of pacemaker modulation. In the cockroach, denervation of the heart abolishes the cardioactivity of acetylcholine, while other transmitters, including serotonin, were still effective (Metcalf *et al.*, 1964). The conclusion from these observations is that transmitters like serotonin are active on the heart proper, whereas acetylcholine is active at neuronal targets. A similar situation is present in *Drosophila*, in which a sodium channel mutation was used to assess likely anatomical sites of actions of cardioactive transmitters. When various transmitters were injected into the *para* mutant above the restrictive temperature, which eliminates neuronal signaling, the cardioacceleratory action of acetylcholine was found to be abolished (see above), while other transmitters including serotonin remained active (Johnson *et al.*, 2002). These results were similar to those obtained with coinjections of TTX with candidate transmitters (Johnson *et al.*, 2002). The above results, of course, do not preclude neuronal targets of serotonin, dopamine, octopamine, and norepinephrine, but imply that these transmitters are acting on the myocardium directly.

There is a fair amount of variability in innervation patterns in insects, which range from extensive, as exemplified in *Periplaneta*, to absent as in *Hyalophora* (Sanger and McCann, 1968a, 1968b; for relevant reviews see **Chapters 1.10**, **2.3**, and **2.4**). Perhaps, this variation in degree of innervation underlies differing identities and actions of peptide transmitters. Typically, most insects have a variable number of paired segmental nerves that innervate the heart, and these arise from

the ventral nerve cord. Some insects, most conspicuously the cockroaches, contain a pair of lateral heart nerves, which also emanate from the ventral nerve cord. Additionally, there may be nerves that innervate the anterior and posterior portions of the dorsal vessel and aorta (Siegmund and Korge, 2001). An interesting finding is that cardiac innervation differs as a function of development. In *Manduca,* cardiac innervation is absent in larval forms, but is present in the adult (Davis *et al.*, 2001). The same applies to *Drosophila*, where there are many ultrastructural differences between pupal and adult hearts (Curtis *et al.*, 1999), including the innervation of the adult heart (Dulcis and Levine, 2003).

2.6.3.2.6. Peptide transmitters and receptors Modulation of cellular activity by peptides has long been recognized in common signaling pathways as these transmitters can alter gene expression, enzymatic reactions, and electrical activity in target tissues. The large number of peptides reported to affect heartbeat in insects begs two questions. Are these effects specifically targeted to the heart? Is there a peptide or peptides dedicated as modulators of heart rate? It is possible that the cardioacceleratory effect of any given peptide is a mechanism to ensure its own circulation. Therefore, these effects may not be in specific response to a change in metabolic demand or to fulfill a functional physiological requirement, but rather a secondary effect to circulate a signal. Although this is an attractive hypothesis, it cannot be applied to inhibitory factors. Nonetheless, there is a clear need to modulate heartbeat in insects, and while a single, well-defined control system may not emerge, it is hard to deny the importance of peptide transmitters in cardiac regulation.

Small peptide hormones almost exclusively bind to GPCRs, and the complete annotation of the genomes of *Drosophila* (Adams *et al.*, 2000) and *Anopheles* has facilitated a detailed analysis of GPCRs (Holt *et al.*, 2002; Brody and Cravchik, 2000; Hewes and Taghert, 2001; Hill *et al.*, 2002). Therefore, identification of candidate receptors for cardioactive peptides will advance our knowledge of insect cardioregulation, and make it possible to pinpoint precise anatomical loci of peptide actions as well as to identify intracellular signaling components and to link these to altered heart function.

2.6.3.2.6.1. Crustacean cardioactive peptide Perhaps the best candidate for a dedicated regulator of heart function (if one exists) is the aptly named crustacean cardioactive peptide (CCAP), which was first isolated from the pericardial organs of the crab (Stangier *et al.*, 1988). Subsequent to its discovery, it has been isolated in a number of insect species, including *Locusta migratoria* (Truman *et al.*, 1996), *M. sexta* (Cheung *et al.*, 1992; Lehman *et al.*, 1993), and *T. molitor* (Furuya *et al.*, 1993). Remarkably, the primary sequence of this neuropeptide is invariant over large phylogenetic distances, and genes encoding this peptide have been identified in *Drosophila*, *Anopheles,* and *Manduca* (Hewes and Taghert, 2001; Loi *et al.*, 2001; Riehle *et al.*, 2002).

CCAP is a multifunctional transmitter and has different physiological roles; it has been implicated in the ecdysis behavioral program (Truman *et al.*, 1996; Baker *et al.*, 1999; Gammie and Truman, 1999; Park *et al.*, 2003), and the release of adipokinetic hormone (AKH) (Veelaert *et al.*, 1997; Harthoorn *et al.*, 2002), in addition to its established role as a myotropic and cardioactive agent. Ablation of CCAP-expressing neurons in *Drosophila* leads to deficits in the circadian rhythms of eclosion (see **Chapters 3.11** and **4.11**) as well as defects in the ecdysis program (Park *et al.*, 2003; **Chapter 3.1**). The role in ecdysis is of particular interest as it has been proposed that the mechanical forces required for cuticular shedding (see **Chapters 3.1**, **4.2–4.4**) and wing inflation derive from hemostatic pressure originating from heart activity (Park *et al.*, 2003). The targeted ablation of CCAP neurons in *Drosophila* leads to several phenotypes that may be explained by the loss of cardiac regulation. These animals displayed lengthened larval molts and increased mortality at the pupal stages, and animals surviving to adulthood have a characteristic phenotype of uninflated wings (Park *et al.*, 2003). Consistent with the notion of cardioactivity being an integral component of ecdysis, other factors involved in the ecdysis developmental program (see **Chapter 3.1**), namely ecdysis triggering hormone (ETH) (Park *et al.*, 1999) and eclosion hormone (EH) (Horodyski *et al.*, 1989), also increase heart rate (Baker *et al.*, 1999). The release of AKH via CCAP action may also be relevant in pacemaker activity. This peptide is involved in the mobilization of stored energy reserves from the fat body, and in a variety of insects, this peptide has cardioacceleratory effects. Perhaps, the cardioacceleratory effects of CCAP emanate from the action of other peptides, including ETH, EH, or AKH.

Consonant with its various actions in different insects, the anatomical distribution of CCAP varies from species to species. It is found in the central nervous system (CNS), in neuroendocrine cells, and in the *corpora cardiaca*, suggesting dual roles as both

a synaptic and hormonal modulator as follows. CCAP has been localized in several cardiac nerves in *Manduca,* which innervate the aorta, the alary musculature, and the caudal end of the adult heart (Davis *et al*., 2001). Likewise, in *Drosophila*, a number of cardiac innervating neurons express this peptide (Dulcis and Levine, 2003). The observation that this neuropeptide is cardioactive in *Drosophila* (Nichols *et al*., 1999b) at developmental stages that lack cardiac innervation suggests that the action of CCAP is direct on the pacemaker and/or the myocardium itself. Additionally, the neurons innervating the adult heart are labeled with antisera derived against CCAP, and suggest potential direct effects (Dulcis and Levine, 2003). Experiments on the neuronal control of heartbeat reversal in *Manduca* also support a direct action of this peptide, as CCAP accelerates heartbeat independently of mode (anterograde or retrograde) of heartbeat (Dulcis *et al*., 2001).

Recently, a GPCR sharing sequence similarity with the mammalian vasopressin receptors (Hewes and Taghert, 2001) was shown to respond specifically to this neuropeptide (Park *et al*., 2002; Cazzamali *et al*., 2002). The receptor encoded by the *CG6111* gene signals through increases in intracellular calcium in a heterologous expression system (Park *et al*., 2002). If this finding is paralleled in native tissues, a reasonable assumption given the relatively few subtypes of *Drosophila* G proteins, it is tempting to link CCAP activity to the activation of *slo* channels in the pacemaker. Increased free calcium could shift the kinetics of the channel to pass more current, thereby repolarizing the pacemaker, a feature invoked as a critical component of accelerating pacemaker activity. Alternatively, rises in free calcium potentially could activate components of the PLC pathway leading to the stimulation of PKC, which could phosphorylate pacemaker-relevant channels. With the repertoire of molecular genetic techniques available in *Drosophila*, manipulations of this receptor *in vivo* should provide insights into the mechanisms of CCAP modulation of heartbeat.

2.6.3.2.6.2. FMRFamides and Myosuppressins The FMRFamide related peptides (FaRPs) are a group of extended N-terminal peptides with a similar C-terminus, which were first identified in mollusks and include the FMRFamides, myosuppressins, and sulfakinins (Nässel, 2002; Taghert and Veenstra, 2003). The *FMRF* (*CG2346*) gene encodes a large propeptide that undergoes posttranslational processing to yield active peptides; eight potential peptides are predicted from analysis of the amino acid sequence, based on consensus sites for proteolytic cleavage and amidation (Hewes *et al*., 1998). Dromyosuppressin (DMS), encoded by a separate gene, *CG6440* (Nichols, 1992), has the primary sequence of TDVDHFLRFamide and is the sole *Drosophila* member of the FLRFamide subfamily.

Several of the individual peptides produced from the processing of the FMRFamide gene are cardioactive in *Drosophila* and slow basal heart rate (Nichols *et al*., 1999a; Johnson *et al*., 2000). Similarly, DMS significantly lowers heart rate in *Drosophila* (Johnson *et al*., 2000), contrasting the effects this peptide has on the larval neuromuscular junction (NMJ) (Hewes *et al*., 1998). The peptide is present in the cardiac recurrent nerve in a related Dipteran (Richer *et al*., 2000) and is suggestive of receptor expression in the heart. Similarly, FLRFamide is cardioinhibitory in the locust (Schoofs *et al*., 1993) and its presence is widespread, and it is generally agreed that these are inhibitory peptides. However, the inhibitory effects of the FMRFamides in *Drosophila* are not a common feature of peptidergic regulation of the cardiac pacemaker, as these peptides are cardioacceleratory in a majority of insects. Lastly, the sulfakinins are also cardioactive, accelerating heart rate at subnanomolar concentrations in the cockroach *Periplaneta* (Predel *et al*., 1999), but reports of cardioactivity in other insects are presently lacking.

A number of GPCRs have been identified as candidate receptors for the FMRFamides and myosuppressins. The receptor encoded by *CG2114* displays greater sensitivity to the peptide emanating from the processing of the FMRFamide gene (Meeusen *et al*., 2002; Cazzamali and Grimmelkhjuzen, 2002). This receptor is putatively linked to Gq pathways, increasing intracellular calcium levels. Therefore, this receptor, if its actions are direct, would probably be involved in initiating a cardioacceleratory pathway (McGaw *et al*., 1995). However, a pair of linked receptor paralogs encoded by *CG8985* and *CG13803* show greater sensitivity to the DMS peptide, but are also activated by the FMRFamides in the nanomolar range (Egerod *et al*., 2003b; Johnson *et al*., 2003b). Likewise, the *CG2114* receptor does respond to DMS, but is more sensitive to the FMRFamides (Meeusen *et al*., 2002; Cazzamali and Grimmelkhjuzen, 2002). All three of these receptors are distantly related to the thyroptropin releasing hormone receptor family (Hewes and Taghert, 2001) and given that these receptors do respond to related peptides, the actions of any one peptide is problematic and studies aimed at determining receptor expression are required. Furthermore, the *CG8985*/*CG13803*-encoded receptor pair is apparently coupled to Gi proteins, as these peptides cause a significant decrease in the

forskolin-stimulated increase of cAMP levels (Johnson *et al*., 2003b) and these receptors presumably could produce antagonistic actions to the *CG2114*-encoded receptor. The receptors encoded by *CG6851* and *CG6887*, which are related to the mammalian CCK receptors (Hewes and Taghert, 2001), respond specifically to two of the three predicted sulfakinin peptides (Kubiak *et al*., 2002).

The degree of relatedness of these peptides and the demonstrated pharmacological overlap of the receptors makes it difficult to pinpoint a singular mode of action for these RFamides, and future studies with these putative receptors will be important for refining the roles of these peptides as cardiac modulators. Nevertheless, the physiological activity and anatomical evidence of these peptides argue for an important role in cardiac regulation.

2.6.3.2.6.3. Proctolin The pentapeptide proctolin (RYLPT) was the first insect peptide to be isolated on the basis of its potent stimulation of hindgut contractions in *Periplaneta* (Brown and Starratt, 1975). Consequently, its distribution and actions throughout many orders of insects have come under intense scrutiny. In addition to its potent effects on hindgut contractions, proctolin increases heart rate in several insects, including *Tenebrio*, *Periplaneta*, *Locusta*, and *Drosophila* (Anderson *et al*., 1988; Sliwowska *et al*., 2001). Proctolin also affects the accessory pulsatile organs of the cockroach (Hertel and Pass, 2002).

Second messenger activation and ligand binding demonstrates that the GPCR encoded by the gene *CG6986* responds specifically to proctolin (Johnson *et al*., 2003a, 2003b; Egerod *et al*., 2003a). Proctolin liberates intracellular calcium with subnanomolar sensitivity in heterologous cells in accord with reports of proctolin action *in vivo* (Wegener and Nässel, 2000; Johnson *et al*., 2003a). An antibody against a portion of the receptor was used to determine anatomical expression, which confirmed its action as a cardioactive peptide. The receptor is expressed in pericardial cells, in neurons innervating the anterior portion of the adult heart, and in neurons terminating at the anterior aorta in the larvae (Johnson *et al*., 2003a). Expression of the receptor in neurosecretory cells indicates a possible role as a hormonal modulator, perhaps regulating the release of other cardioactive factors (Johnson *et al*., 2003a). This lends credence to the report of an inhibitory action of proctolin in *Drosophila* (Zornik *et al*., 1999), which contradicts a previous study in *Drosophila* (Anderson *et al*., 1988). Possible explanations to reconcile these differences could be the presence of multiple proctolin receptors and/or differential sites of action. The strong proctolin receptor expression in pericardial cells is difficult to interpret with regard to cardiac physiology and could suggest secondary effects. Identification of a candidate proctolin receptor, irrespective of species, will facilitate the cloning and characterization of proctolin receptors from other insects, like *Periplaneta* and *Locusta*. This will be invaluable considering the abundance of physiological studies on proctolin activity in these insects to address phlyogenetic differences in proctolin activity and potential for multiple receptors. Proctolin cardioactivity appears not to be a uniform feature in insects, as the peptide has no effect in *Anopheles* (Messer and Brown, 1995), and a receptor ortholog is absent (Johnson *et al*., 2003a).

2.6.3.2.6.4. The kinins The pyrokinins are a group of peptides that are characterized by their FXPRLamide sequences at the C-terminus. CAP_{2B}, a cardioacceleratory peptide has been isolated in *Manduca* (Huesmann *et al*., 1995) and *Drosophila* (Davies *et al*., 1995). In *Manduca*, this peptide is cardioacceleratory (Tublitz, 1988) and acts synergistically with the biogenic amines, namely serotonin and octopamine (Prier *et al*., 1994). Additionally, this peptide factor is associated with increased heart rates during flight in *Manduca* (Tublitz, 1989). In *Drosophila*, the peptide affects diuresis in the Malphigian tubules, and no cardiac actions have been reported. In *Drosophila*, a GPCR specific for this peptide has been isolated (Park *et al*., 2002). The *CG14575* gene encodes a GPCR related to the neurotensin ancestral group (Hewes and Taghert, 2001), and is activated by submicromolar concentrations of the peptide (Iversen *et al*., 2002; Park *et al*., 2002). This receptor is linked to increases in free calcium as demonstrated *in vitro* (Park *et al*., 2002), consistent with the actions of this peptide described *in vivo* (Tublitz, 1988; Davies *et al*., 1995). Other pyrokinin peptides in *Drosophila,* which are encoded by the *hugin* gene, are cardioacceleratory (Meng *et al*., 2002), and these peptides bind to the receptor paralogs, encoded by *CG8784* and *CG8795* (Park *et al*., 2002).

The tachykinins are a family of neuropeptides that are identified by a FXGXR at the C-terminal sequence. The tachykinins are multifunctional peptides with effects on the heart (Sliwowska *et al*., 2001) and other visceral muscles in a variety of insects (Nässel, 2002). Several peptides are produced from a tachykinin precursor and have similar EC_{50} values as determined by hindgut contractions (Siviter *et al*., 2000). Receptors have been identified that recognize these peptides: *CG6515* and *CG7887*

(Li *et al.*, 1991; Monnier *et al.*, 1995; Torfs *et al.*, 2001; Johnson *et al.*, 2003b). The *CG7887* receptor couples to multiple signaling pathways, and receptor orthologs have been identified in another dipteran, the stable fly (Torfs *et al.*, 2001).

The leucokinins are unrelated groups of peptides with pronounced effects on the fluid secretion by the stellate cells of Malpighian tubules (Coast, 1996, 2001; **Chapter 2.8**). There are limited reports on this peptide as a potential cardioactive agent, and they include anatomical evidence that this peptide is present in the lateral heart nerves in the cockroach *Leucophaea* (Winther *et al.*, 1996). In three different dipterans, including *Drosophila*, leucokinin immunoreactivity is present in transverse cardiac neurons (Cantera and Nässel, 1992). A receptor for this peptide was first identified in the snail *Lymnaea* (Cox *et al.*, 1997), and the *Drosophila* ortholog, *CG10626*, is a functional leucokinin receptor (Radford *et al.*, 2002).

2.6.3.2.6.5. Allatostatins and allatotropins The allatostatins are a group of unrelated peptides that inhibit the production and release of juvenile hormone from the *corpora allata* (see **Chapter 3.7**). Three different families of allatostatins have been isolated from different species: the allatostatin A family (Ast-A), characterized by a C-terminal sequence of FXFGLamide), isolated from the cockroach, *Diploptera* (Woodhead *et al.*, 1989, Pratt *et al.*, 1991); the allatostatin B family (with a diagnostic C-terminal GW-amide sequence), isolated from the cricket *Gryllus* (Lorenz *et al.*, 1995), and allatostatin C, isolated from *Manduca* (Kramer *et al.*, 1991). To date, only a single member of this peptide family has been identified in *Manduca* and *Drosophila* (Price *et al.*, 2002). These peptides differ in their allatostatic properties, and in their presence in different insects. For instance, all three types have been identified in *Drosophila* (Williamson *et al.*, 2001a, 2001b), although none appear to be truly allatostatic in these dipterans (Price *et al.*, 2002).

Allatostatin A inhibits heart rate in the cockroach *Blatella* (Vilaplana *et al.*, 1999) and this result is strengthened by immunological evidence that the cardiac lateral nerves in another cockroach, *Periplaneta*, express this peptide (Ude and Agricola, 1995). These cardiac nerves are absent in other insect orders, notably Diptera and Lepidoptera (Sanger and McCann, 1968a, 1968b; Davis *et al.*, 2001), and the potential cardiac activity of these peptides in these insects has not been reported, despite their presence (Hewes and Taghert, 2001; Vanden Broek, 2001). An interesting feature is the colocalization of Ast-A and FMRF immunoreactivity (Ude and Agricola, 1995). Whether this suggests redundancy or integration of these peptides as cardiac factors will require additional studies.

A similar scenario emerges with the cricket-like allatostatins (allatostatin B or myoinhibitory peptide, MIP). Immunoreactivity suggests the presence of the peptide in nerves innervating the locust heart (Schoofs *et al.*, 1996), although there is a dearth of corresponding physiological studies with this peptide providing additional evidence of cardioactivity. In *Manduca*, there is a colocalization of this peptide with CCAP (Davis *et al.*, 2003), and it is unknown if this suggests a role of this peptide in ecdysis and/or heartbeat. This peptide is released by the epiproctodeal gland in *Manduca* (Davis *et al.*, 2003), suggesting a hormonal function. Whether this peptide is a *bona fide* regulator of heartbeat and whether cardiac regulation by Ast-B is a widespread insect phenomenon remains to be seen.

The *Manduca*-like allatostatin, allatostatin C, is cardioactive in *Drosophila*, where application of this peptide inhibits heart rate (Price *et al.*, 2002). The activity of this peptide is presumed to be hormonal in nature; antisera to this peptide recognize numerous endocrine cells in the *Drosophila* adult midgut. It remains to be seen if this peptide is a cardiac modulator in other insect species.

Notably, GPCRs for each of these peptides have been identified in *Drosophila*: *CG2872* and *CG10001* respond to Ast-A (Birgul *et al.*, 1999; Larsen *et al.*, 2001; Lenz *et al.*, 2001); *CG14484* responds to Ast-B (Johnson *et al.*, 2003b); and *CG7285* and *CG13702* to Ast-C (Kreienkamp *et al.*, 2002; Johnson *et al.*, 2003b). The receptors for the Ast-A peptides belong to the galanin-like family of receptors, the Ast-B receptor belongs to the bombesin-like receptors, and the Ast-C receptors belong to the somatostatin-like receptors (Hewes and Taghert, 2001). With the exception of the Ast-A receptors, a few conclusions concerning these receptors and their subsequent mechanisms of cardioaction can be reached, as it is presently unknown through which second messenger systems these receptors are signaling and in what tissues they are found. The two Ast-A receptors, encoded by *CG2872* and *CG10001*, couple to Gi/o proteins in mammalian cells, as they were shown to signal through calcium in a pertussis-sensitive fashion (Larsen *et al.*, 2001). These results should serve as a hypothetical framework to address the *in vivo* action, signaling, and distribution of these various allatostatin receptors and their involvement in the cardiac system.

Allatotropin is a peptide that was first isolated in *Manduca* and mediates the release of JH from the

corpus allatum (Veenstra *et al.*, 1994; **Chapter 3.7**). It accelerates the heart rate in *Manduca* (Veenstra *et al.*, 1994), in another lepidopteran, the army worm, *Pseudaletia* (Koladich *et al.*, 2002), and in the cockroaches *Periplaneta* and *Leucophaea* (Rudwall *et al.*, 2000). A candidate gene encoding this peptide is conspicuously absent from the *Drosophila* genome (Nässel, 2002; Taghert and Veenstra, 2003), although antisera against this peptide labels various neurons (Zitnan *et al.*, 1993).

The distribution of this peptide, evaluated through immunocytochemistry, is in a number of cardiac relevant neurons, specifically in the cockroach lateral and transverse nerves. Additionally, this peptide is colocalized with serotonin in both the cockroach and army worm (Rudwall *et al.*, 2000; Koladich *et al.*, 2002), and there is evidence that these factors are acting synergistically on the army worm heart (Koladich *et al.*, 2002).

2.6.3.2.6.6. Corazonin and adipokinetic hormone (AKH) Corazonin was first isolated in the cockroach, *Periplaneta*, based on its potent cardioactivity (Veenstra, 1994). The peptide is widely distributed throughout many insect orders and is associated with a number of different behaviors. For instance, corazonin is implicated as the causative factor of the gregarious-associated pigmentation of cuticle in locusts (Tawfik *et al.*, 1999). Its functional role in *Drosophila* and many insects is unknown, and perhaps corazonin is a common cardioactive factor.

A GPCR (*CG10698*) for this peptide has been identified in *Drosophila* and activation of this receptor increases intracellular calcium levels (Park *et al.*, 2002; Johnson *et al.*, 2003b). The identification of a *Drosophila* corazonin receptor will determine if corazonin is involved in cardioregulation in *Drosophila* and/or whether it could be useful in identifying the cockroach ortholog for *CG10698*.

AKH is a neurohormone released by the *corpus cardiacum* to regulate hemolymph carbohydrate concentrations (review: Gade and Auerswald, 2003). Given its direct role in modulating mobilization of sugars, it is perhaps not surprising that it has been implicated as a cardioactive factor in *Drosophila* (Noyes *et al.*, 1995) as well as other insects. In *Drosophila*, a candidate GPCR, encoded by the *CG11325* gene, has been identified for this neuropeptide (Park *et al.*, 2002; Staubli *et al.*, 2002) and should help facilitate definition of cardiac relevant roles.

2.6.3.2.6.7. PACAP-like peptides The *amnesiac* mutation (Quinn *et al.*, 1979) affects learning and memory storage (Tully and Quinn, 1985) and ethanol sensitivity (Moore *et al.*, 1998), and produces a reduced heart rate and an associated cardiac arrhythmia (Johnson *et al.*, 1997). This gene has been cloned and found to encode the neuropeptide homologous to vertebrate pituitary adenylate cyclase activating peptide (PACAP) (Feany and Quinn, 1995), although the precise amino acid sequence of the peptide(s) remains unclear. This mutation also reduces oscillations of intracellular calcium in the *Drosophila* CNS (Rosay *et al.*, 2001). Further work is needed to map out the cardiac effects of this peptide.

2.6.3.2.7. Regulation of receptor activity If the effects of any transmitter are the consequence of its receptor(s) activation, then it also follows that factors that regulate receptor activity or expression may be critical in mediating or modulating cardiac effects. In mammals, GPCRs are downregulated by the concerted action of the arrestins and G protein-coupled receptor kinases (GRKs) (Kohout and Lefkowitz, 2003). Ligand binding places the receptor in a conformation that is recognized by GRKs and subsequently phosphorylated. This phosphorylation leads to the recruitment of cytoplasmic arrestins to the membrane, where GPCR–G protein interactions are blocked. The complex is then internalized, resulting in a reduction in the pool of receptors capable of being activated by ligand. GPCR regulation in insects is likely to be fundamentally similar to this, as one nonsensory arrestin (Roman *et al.*, 2000) and two GRKs (Cassill *et al.*, 1991) have been identified in the *Drosophila* genome. Additionally, *Drosophila* GPCRs have displayed similar properties in arrestin associations and these elements have led to the identification of several *Drosophila* GPCRs (Johnson *et al.*, 2003b). However, the impact that receptor regulation has on the cardiac system has not been evaluated fully. There is evidence to suggest that pacemaker function relies upon normal receptor regulation. In *Drosophila*, a mutation that disrupts receptor downregulation has profound effects on heartbeat; manifest as cardiac arrhythmia and the inability to respond normally to increasing temperature and cardioacceleratory transmitters (Johnson *et al.*, 2001). Electrocardiogram analysis of flies bearing the *shibire* (*shi*) mutation suggests that the cardiac pacemaker is being driven to rates that the myocardium cannot accommodate (Johnson *et al.*, 2001). The *shi* gene encodes dynamin (Chen *et al.*, 1991; Van der Bliek and Meyerowitz, 1991), which functions as a molecular motor (Shpetner and Vallee, 1992). Dynamin is essential for vesicle formation by forming a ring structure and excising clathrin coated pits (Hinshaw

and Schmid, 1995). Thus, dynamin is essential for receptor downregulation through the endocytotic machinery. This raises the possibility that the heart defects are caused by the loss of neurotransmitter receptor recycling due to impaired endocytosis in the pericardial cells or in the myocardium or both.

The *shi* phenotype is suppressed by another "regulatory" gene, *nap,* which is an allele of the *maleless* gene (Kernan *et al*., 1991). The *nap* mutation produces bradycardia and arrhythmia (Dowse *et al*., 1995). This gene encodes a RNA–DNA helicase and produces errors in the splicing of certain transcripts (Palladino *et al*., 2000; Reenan *et al*., 2000); therefore, the effects of this mutation are indirect. These results imply that the quantity of active channels and receptors represent a state variable of the cardiac oscillator.

2.6.3.2.8. Modulation of heartbeat by temperature One of the functional roles that heartbeat fulfills is that of thermoregulation, as initially described in the elegant studies of Heinrich on bumblebees. Clearly, poikilotherms must account for the wide range of environmental conditions. Heart rate is positively correlated with temperature and with increases across the physiological range (White *et al*., 1992). And while the observation that heartbeat is sensitive to temperature change is beyond dispute, the mechanisms that facilitate such changes are largely unresolved.

It could be that the physical chemistry of the system is responsible for altering heart rates with fluctuating temperatures. This hypothesis has some supporting experimental results; deuterium, which slows heartbeat, also decreases the temperature sensitivity of the system (White *et al*., 1992). However, work on the *nap* mutation has shown that this mutant is practically unresponsive over a wide range of temperatures (Dowse *et al*., 1995). These results would implicate that the magnitude of such change has to be mediated by a specific detection system. The pioneering work on cardiac function as a thermoregulatory mechanism by Heinrich also implicates a neuronal component of the modulation by temperature (Heinrich, 1970, 1976). However, flies carrying the *paralytic* mutation have normal heartbeat over a wide range of temperatures (18–42 °C) (Dowse *et al*., 1995) and given that this mutation disrupts neuronal function above 29 °C (Suzuki *et al*., 1971), this result suggests a lack of involvement of the nervous system.

The response to temperature in Ddc^{ts1} mutants is normal, suggesting that the mechanism by which temperature exerts its effects on heart rate is not mediated by an increase in neurotransmitter concentrations or efficacy (Johnson *et al*., 1997). Perhaps other transmitters are involved and are not perturbed in this mutant. To speculate wildly, the demonstration that Transient receptor protential (Trpc) channels can be gated by temperature could represent a direct mechanistic link of temperature modulation on the cardiac system.

2.6.4. Aging Heart

2.6.4.1. Insects as Models for Aging

2.6.4.1.1. Models of aging The process of growing old has fascinated biologists and natural philosophers for centuries. In recent years, genetic and biochemical studies using insect model organisms have begun to outline some of the mechanisms that underlie the aging process. Excitingly, and perhaps surprisingly, these mechanisms appear to be employed to control senescence in a broad spectrum of organisms, including yeast, nematodes, mice, and humans (Finch and Ruvkun, 2001; Partridge and Gems, 2002; Tatar *et al*., 2003). This section will briefly review recent work on the mechanisms of aging using insect models, then discuss some of the limitations of the insect model systems. Thus, it is concluded with a discussion of new techniques designed to address these limitations by making use of the insect heart as a model to identify factors that control aging at the level of an individual organ.

Two major hypotheses have been proposed to explain how organismal senescence occurs. The first, the "rate of living" hypothesis, as proposed by Daniel Pearl (Pearl, 1928), is that each individual has a genetically determined sum of vitality, and that each individual lifespan is controlled by the rate at which this sum of vitality is used up. The second, which we will refer to as the "genetic plan" hypothesis (Helfland and Rogina, 2003), asserts that changing patterns of gene expression over the lifespan of the animal leads to differential production of factors, including hormones, which tend to promote or prevent gradual deterioration of organ systems, until the eventual failure of at least one essential organ becomes the cause of mortality. Evidence from insect model systems has been used to support each of these ideas.

2.6.4.1.2. Oxidative stress The rate of living hypothesis can be explained molecularly by the "oxidative stress" hypothesis, which states that the underlying cause of senescence is accumulated damage caused by reactive oxygen species (ROS) to lipids, proteins, and DNA. ROS are generated as a byproduct of essential biochemical reactions during cellular respiration, but can accumulate to

damaging levels if produced more rapidly than antioxidant proteins, such as Cu,Zn-superoxide dismutase (SOD), and catalase can remove them. Thus, an organism with an increased activity level may be expected to accumulate higher ROS levels, thus accumulating more damage to cellular macromolecules and thereby undergoing an earlier senescence (Stadtman, 1992; Sohal, 2002).

By comparing the rates of mitochondrial ROS generation in dipteran species with widely varying lifespans, it has been observed that an inverse relationship exists between lifespan and rate of ROS generation (Sohal *et al*., 1995). In houseflies, lowering the rate of muscle activity (by preventing the animals from flying) leads both to a lower level of ROS accumulation in aging individuals and to a corresponding increase in lifespan (Agarwal and Sohal, 1994). Additional anecdotal evidence abounds in various insect species for an inverse relationship between metabolic rate and lifespan, e.g., worker honeybees that emerge in summer during the foraging season have shorter lifespans than worker bees that overwinter as nurses (Amdam and Omholt, 2002).

Conversely, when exposed to hyperoxic conditions, houseflies exhibit increased levels of ROS generation and protein modification, as well as a decreased lifespan (Sohal *et al*., 1993). Interestingly, the amount of oxygen consumption by houseflies during an average lifespan is the same under different metabolic conditions, while the rate of oxygen consumption varies inversely with the length of life (Miquel *et al*., 1976; Sohal, 1982).

Alternatively, attempts to increase the lifespan of fruit flies by overexpression of genes encoding antioxidant proteins have produced mixed results. Ubiquitous overexpression of Cu,Zn-SOD, Mn-SOD, catalase, or thioredoxin reductase gives no appreciable extension of lifespan, despite providing some degree of increased stress resistance (Seto *et al*., 1990; Reveillaud *et al*., 1991; Orr and Sohal, 1993; Mockett *et al*., 2003). Flies carrying an extra transgenic copy of Cu, Zn-SOD, and catalase, however, did live about one-third longer than controls (Orr and Sohal, 1994). More recently, inducible overexpression of Cu,Zn-SOD conferred a lifespan increase in some lines but not others (Sun and Tower, 1999), with the degree of lifespan extension being greatest in lines in which control flies had a relatively short lifespan. However, overexpression of the human Cu,Zn-SOD in fruit fly motor neurons is capable of extending lifespan by 41% (Parkes *et al*., 1998) and, remarkably, Cu,Zn-SOD overexpression in motoneurons can rescue the shortened lifespan of *Cu, Zn-SOD* mutants (Parkes *et al*., 1998). In summary, rate of production of ROS appears to have a powerful correlation with lifespan. However, overexpression of antioxidants seems to extend lifespan best in genetic backgrounds or tissues where antioxidant activity may already be low, while conferring little additional benefit to genetic backgrounds that are not already compromised for lifespan. This correlates well with studies showing little benefit to overexpression of oxidants in mice (Huang *et al*., 2000) or administration of antioxidants to mice or humans (Kohn, 1971; Kim *et al*., 1993; Albanes *et al*., 1995; Omenn *et al*., 1996).

2.6.3.1.3. Genetic programs of aging: insulin, juvenile hormone, and ecdysone Emerging evidence from insect models, especially the fruit fly *D. melanogaster*, supports the idea there may be one or several genetically regulated programs of aging that link the influences of metabolism, oxidative stress, and numerous signaling pathways. Compelling evidence for the existence of an intricate genetic control of aging comes from single gene mutations that cause an increase in lifespan. Examples include the putative G protein-coupled receptor *methusela* (*meth*) (Lin *et al*., 1998), the metabolite transporter *I'm not dead yet* (*Indy*) (Rogina *et al*., 2000), ecdysone receptor (*EcR*) (Simon *et al*., 2003), the insulin receptor substrate *chico* (*ch*) (Clancy *et al*., 2001), and certain alleles of the insulin receptor (*InR*) itself (Tatar *et al*., 2001).

While it is plausible that some, or even all, genetic mutations that affect lifespan may do so solely by affecting metabolic rates, there is a growing body of evidence suggesting that lifespan may be regulated by a programmed response to varying environmental conditions that involves release of neuropeptides, which themselves guide the release of hormones, and can regulate lifespan in a manner that can sometimes be separable from effects on fecundity or metabolic rate (Tatar *et al*., 2003).

A key hormone for regulation of aging in fruit flies (Tatar *et al*., 2003) is the sesquiterpenoid juvenile hormone (JH; see **Chapter 3.7**). Reduction in Insulin Receptor signaling by mutation in *InR* or *chico* leads to a drastic reduction in JH levels, while the extended lifespan conferred by such mutations can be rescued by treatment with the JH analog methoprene (Clancy *et al*., 2001; Tatar *et al*., 2001; Tu *et al*., 2002). Reduction in JH itself can also affect lifespan (Tatar and Yin, 2001).

The role of JH in the regulation of lifespan and fertility appears to be conserved for a variety of insects. For example, honeybee workers have varying levels of vitellogenin depending on whether they are short-lived summer bees or long-lived winter bees (Amdam and Omholt, 2002). Since JH

promotes vitellogenesis by regulating the production of vitellogenin in flies (Wyatt and Davey, 1996; see **Chapters 1.3** and **3.9**), these results may again reflect the importance of JH in lifespan regulation. The decision for a Monarch butterfly to be reproductively active or to be a longer lived migratory butterfly also appears to be regulated by JH titers (Herman, 1981; Tatar and Yin, 2001). Longevity can also be doubled in several species of grasshoppers by removal of the *corpora allata*, the site of JH synthesis. Suggestively, the longevity phenotype of animals undergoing such allatectomies is independent of the amount of muscle activity of the animals (Pener, 1972). Indeed, since the levels of JH production seem to influence diapause behavior in many insects, one could view the effects of reduced JH levels on the rate of aging as a less drastic version of diapause (Tatar and Yin, 2001).

Another line of evidence for genetically programed aging comes from microarray experiments, in which global gene regulation can be examined in genetically identical fruit flies of different ages. Such experiments have demonstrated that transcription profiles vary widely and reproducibly in fruit flies as a function of age as well as level of food intake (Pletcher *et al*., 2002). More targeted experiments using gene-specific reporter constructs or observing temporal changes in gene expression in particular tissues have also supported the idea that gene expression during aging is a dynamic, regulated process rather than a simple consequence of deterioration in global homeostasis (Rogina and Helfland 1995; Wheeler *et al*., 1995; Rogina *et al*., 1998; see also **Chapter 3.10**).

Additional recent evidence has suggested that perhaps the rate of living hypothesis and the genetic plan hypothesis are not necessarily in opposition. For example, fruit flies carrying mutations in the gene encoding the ecdysone receptor (*EcR*) have an increased lifespan, despite having normal fecundity (Simon *et al*., 2003). The ecdysone receptor is capable of forming a complex with ultraspiracle (Usp), which has been postulated as a receptor for JH (Xu *et al*., 2002). In turn, the EcR/Usp complex appears to interact with stress induced molecular chaperones as well as multiple histone deacetylases (HDACs) (Tsai *et al*., 1999; Arbeitmann and Hogness, 2000; **Chapter 3.5**), including Rpd3, mutations in which affect lifespan in both yeast and nematodes (Guarente and Kenyon, 2000). Interestingly, general chemical inhibition of HDACs in fruit flies can also result in increased lifespan with no apparent reduction in physical activity (Kang *et al*., 2002). Among the genes induced when HDAC activity is inhibited are those encoding superoxide dismutases, mitchondrial electron transport proteins, and molecular chaperones involved in stress response (Kang *et al*., 2002).

Thus, one can begin to sketch an interrelated chain of events (**Figure 10**), in which nutritional

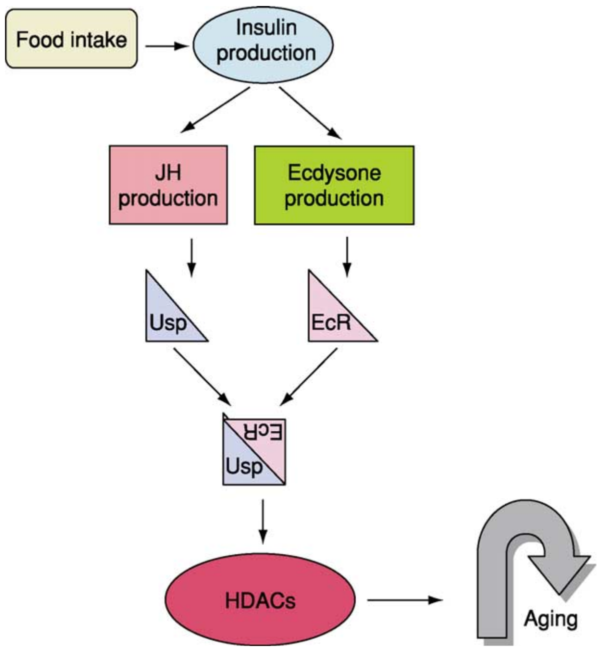


Figure 10 A cartoon depicting a simplified version of the events connecting food intake to the rate of aging. Food intake increases insulin signaling, which increases production of the secondary hormones ecdysone and juvenile hormone, which signal through their putative receptors, ecdysone receptor and ultraspiracle, respectively. These receptors are capable of heterodimerizing and regulating the activity of various histone deacetylases, which globally alter gene expression to drive the aging process.

and other environmental cues lead to the regulation of insulin production. This in turn leads to production of secondary hormones such as ecdysone and JH (see **Chapters 3.3** and **3.7**), which then induce transcriptional changes resulting in changes in gene activity necessary for stress response and defense against damage from ROS. Indeed, the damage produced by excess ROS itself seems to be nonrandom, preferentially targeting specific proteins for carbonylation (Yan *et al*., 1997; Yan and Sohal, 1998; Das *et al*., 2001), while leaving the vast majority of enzymes catalytically active throughout the aging process (Kanungo, 1980; Rothstein *et al*., 1980). This seems to be true not only in fruit fly mitochondria, but also in houseflies (Zahavi and Tahori, 1965; Yan and Sohal, 2000) and in rodent plasma (Jana *et al*., 2002).

As the above summary suggests, insect model systems have proven to be effective models for basic research into the mechanisms of aging. The fruit fly *D. melanogaster* has offered particular advantages (Helfland and Rogina, 2003) due to its quick reproductive cycle and nearly immediate reproductive capacity (justifying classification as adults for the purposes of aging research), the postmitotic nature of the majority of its tissues, and, most importantly, the many tools available for use in a widely studied genetic model organism.

2.6.4.2. The Insect Heart as a Model for Aging

2.6.4.2.1. Cardiac performance: towards a model for organ-specific aging A general shortcoming of any aging model thus far has been the lack of defining criteria during the aging process, such as stage-specific landmarks, before death. Thus, limitations still exist even to the utility of fruit flies in aging research. Despite the availability of large-scale genetic screens for mutations affecting aging, the complicated nature of lifespan as an output phenotype makes it difficult to reliably screen for mutations that reduce lifespan. As a result, the mutations identified in genetic screens to date have all been such as to extend lifespan (e.g., Lin *et al*., 1998; Rogina *et al*., 2000). Additionally, the long-term usage of the fruit fly as a laboratory animal has resulted in a selection process favoring high early life fecundity and short lifespan (Promislow and Tatar, 1998; Sgro and Partridge, 1999). As a result, some mutations that extend the lifespan of laboratory strains of fruit flies do not similarly extend the lifespan of fly strains with a greater inherent longevity (Sun and Tower, 1999; Leips and McKay, 2000; Rogina *et al*., 2000; Linnen *et al*., 2001; Spencer *et al*., 2003).

One way to combat these limitations would be to identify some sort of staged molecular or physiological clock, by which one could identify the stage of the aging process the animal was undergoing, independently of chronological time or lifespan. This has been attempted by taking advantage of age-specific gene expression profiles to mark the physiological “age” of individuals or cultured lines. In support of this idea, age-specific gene expression has been shown to vary proportionately in flies aged at different temperatures, where their “rate of living” is altered (Rogina and Helfland, 1995).

Another way to approach this issue would be to study “aging” of a specific organ system, and use the physiological deterioration of the tissue as a readout of the physiological age of the animal, irrespective of its metabolic rate or lifespan. Studies measuring the heart rate and cardiac performance under external electrical stress have strongly implicated the fly heart (dorsal vessel) as a useful readout in this context (**Figure 11**, Paternostro *et al*., 2001; Wessells and Bodmer, 2004). The heart performance has been observed to decline with age, and screens using heart performance at varying ages have identified loci that can regulate the rate of this decline (Wessells and Bodmer, 2004). Using this readout has allowed the isolation of mutants, which seemingly hasten the aging process, as suggested by the performance parameter changes, as well as mutants that retard this process. By testing cardiac performance at progressively older ages of various mutant lines, it has been observed that the correlation between lifespan and cardiac performance at advanced ages is strong. Interestingly, however, these parameters are separable in some cases (R.J. Wessells and R. Bodmer, unpublished data), suggesting that aging might not only be a global process, which affects the organism as a whole, but that individual organs may also be subjected to aging autonomously.

2.6.4.2.2. Mutations that affect cardiac performance with age In initial steps to characterizing the mechanisms of cardiac aging in the fruit fly, it has been observed that flies lacking Chico, an insulin receptor substrate, are not only long lived (Clancy *et al*., 2001; Tu *et al*., 2002) but also have slowed deterioration of cardiac performance, as measured both by resting heart rate and by the failure rate of hearts subjected to external electrical stress (R.J. Wessells, M. Tatar, and R. Bodmer, unpublished data). This slowed rate of cardiac deterioration is dependent on the reduction in JH levels that is a consequence of impairment of the insulin receptor signaling pathway (Tatar *et al*., 2001), since treatment of *chico* null flies with the JH analog methoprene restores normal lifespan. Similar results have also been obtained with certain

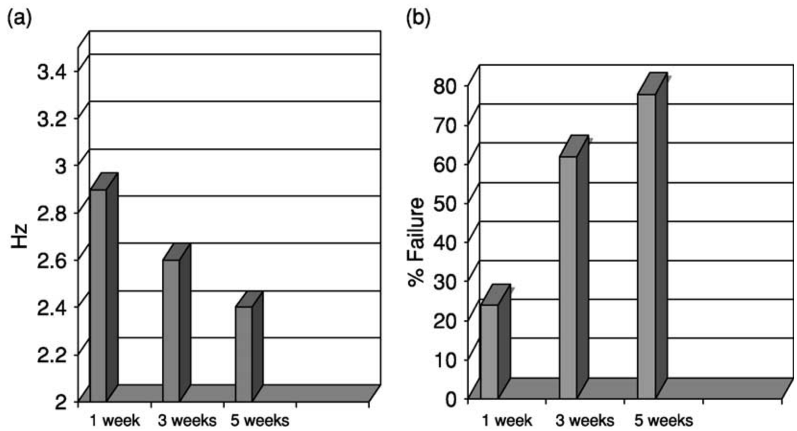


Figure 11 The *Drosophila* heart changes its physiological characteristics with age. (a) The decline in heart rate in flies between 1 and 5 weeks of age. (b) The increase with age of the percentage of flies exhibiting cardiac arrest or fibrillation (% failure) in response to electrical pacing (for methods see Wessells and Bodmer, 2004). Both parameters are consistent across several wild-type genotypes.

allelic combinations of the insulin receptor itself (R.J. Wessells, M. Tatar, and R. Bodmer, unpublished data).

The fact that cardiac performance at advanced ages can serve as an analytical readout for the effect of various mutations on aging at the organismal level, raises the question to what extent the cardiac aging process involves actual deterioration of the organ itself, and to what extent does the deterioration of cardiac performance simply reflect a gradual loss of metabolic homeostasis throughout the organism? Overexpression of the insulin receptor specifically in the heart leads to prematurely decreased cardiac performance in young flies as measured by response to external electrical pacing. Conversely, overexpression of inhibitors of the insulin receptor signaling leads to an improvement in cardiac performance in aged flies, suggesting that there is indeed an organ specific component to the aging process.

Another important question that can be addressed by examining cardiac performance at different stages of the lifecycle is the relationship of cardiac performance to lifespan. For example, when cardiac performance at advanced ages is improved by blocking insulin signaling in myocardial cells, the lifespan is not extended (R.J. Wessells, M. Tatar, and R. Bodmer, unpublished data). In contrast, mutations that cause a reduced heart rate throughout the life cycle result in a dramatically diminished lifespan (M. Fujioka, R.J. Wessells, R. Bodmer, and J.B. Jaynes, unpublished data). This supports a model in which gradual deterioration proceeds in all organs during aging, and failure of any organ system may lead to death. Interestingly, mutations that reduce heart rate do not necessarily result in diminished response to external electrical pacing, suggesting that stress response and resting heart performance are separable phenomena, both of which may contribute to lifespan.

Large-scale genetic screens are ongoing in multiple laboratories using age-dependent decline in cardiac performance to identify new loci that affect age related changes of the heart. It is expected that candidate genes will be identified that affect the global aging process as well as candidates that affect heart performance specifically. In addition to providing insight into cardiac physiology and aging in insect systems, such studies should produce many exciting therapeutic possibilities to be tested in mammalian systems. Such pioneering research in insects may eventually lead to new techniques for intervention to prevent or slow cardiac deterioration in the aging human population.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome of *Drosophila melanogaster*. *Science 287*, 2185–2195.

Adolph, E., **1967**. The heart's pacemaker. *Scient. Am. 216*(3), 32–37.

Agarwal, S., Sohal, R.S., **1994**. DNA oxidative damage and life expectancy in houseflies. *Proc. Natl. Acad. Sci. USA 91*(25), 12332–12335.

Albanes, D., Heinonen, O.P., Huttunen, J.K., Taylor, P.R., Virtamo, J., *et al.*, **1995**. Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study. *Am. J. Clin. Nutr. 2*(Suppl 6), 1427S–1430S.

Alberga, A., Boulay, J.L., Dennefeld, C., **1987**. The snail gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germlayers. *Development 111*, 983–992.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., *et al.*, **2002**. Molecular Biology of the Cell, 4th edn. Garland, New York.

Alexandrowicz, J., **1926**. The innervation of the heart of the cockroach (*Periplaneta orientalis*). *J. Comp. Neurol. 41*, 291–310.

Alvarez, A.D., Shi, W., Wilson, B.A., Skeath, J.B., **2003**. Pannier and pointed P2 act sequentially to regulate *Drosophila* heart development. *Development 130*, 3015–3026.

Amdam, G.V., Omholt, S.W., **2002**. The regulatory anatomy of honeybee lifespan. *J. Theor. Biol. 216*(2), 209–228.

Anderson, M.S., Halpern, M.E., Keshishian, H., **1988**. Identification of the neuropeptide transmitter proctolin in *Drosophila* larvae: Characterization of muscle fiber-specific neuromuscular endings. *J. Neurosci. 8*, 242–255.

Arbeitmann, M.N., Hogness, D.S., **2000**. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell 101*(1), 67–77.

Art, J.J., Fettiplace, R., **1987**. Variation of membrane properties in hair cells isolated from the turtle cochlea. *J. Physiol. 385*, 207–242.

Ashe, H.L., Mannervik, M., Levine, M., **2000**. Dpp signaling thresholds in the dorsal ectoderm of the *Drosophila embryo*. *Development 127*, 3305–3312.

Ashton, K., Wagoner, A.P., Carrillo, R., Gibson, G., **2001**. Quantitative trait loci for the monoamine-related traits heart rate and headless behavior in *Drosophila melanogaster*. *Genetics 157*, 283–294.

Atkinson, N.S., Robertson, G., Ganetzky, B., **1991**. A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science 253*, 551–555.

Azpiazu, N., Frasch, M., **1993**. Tinman and bagpipe: two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Devel. 7*, 1325–1340.

Azpiazu, N., Lawrence, P.A., Vincent, J.-P., Frasch, M., **1996**. Segmentation and specification of the *Drosophila* mesoderm. *Genes. Devel. 10*, 3183–3194.

Baker, J.D., McNabb, S.L., Truman, J.W., **1999**. The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster. J. Exp. Biol. 202*, 3037–3048.

Baker, R., Schubiger, G., **1995**. Ectoderm induces muscle-specific gene expression in *Drosophila embryos*. *Development 121*, 1387–1398.

Baumgarten, C., Fozzard, H., **1992**. Cardiac resting and pacemaker potentials. In: Fozzard, H.A., *et al.* (Eds.), The Heart and Cardiovascular System, 2nd edn. Raven Press, New York, pp. 963–1001.

Beiman, M., Shilo, B.Z., Volk, T., **1996**. Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Devel. 10*, 2993–3002.

Bers, D.M., **2001**. Excitation-Contraction Coupling and Cardiac Contractile Force. Kluwer Academic Publishers Group, Dordecht.

Bidet, Y., Jagla, T., Da Ponte, J.P., Dastugue, B., Jagla, K., **2003**. Modifiers of muscle and heart cell fate specification identified by gain-of-function screen in *Drosophila*. *Mech. Devel. 120*, 991–1007.

Birchard, G.F., Arendse, A., **2001**. An allometric analysis of oxygen consumption rate and cardiovascular function in the cockroach, *Balberus discoidalis*. *Comp. Biochem. Physiol. A 129*, 339–344.

Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., **1999**. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J. 18*, 5892–5900.

Black, D., **1998**. Splicing in the inner ear: A familiar tune, but what are the instruments? *Neuron 20*, 165–168.

Black, B.C., Smarelli, J., **1986**. A kinetic analysis of *Drosophila melanogaster* dopa decarboxylase. *Biochim. Biophys. Acta 870*, 31–40.

Blenau, W., Baumann, A., **2001**. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect. Biochem. Physiol. 48*, 13–38.

Bodmer, R., **1993**. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development 118*, 719–729.

Bodmer, R., **1995**. Heart development in *Drosophila* and its relationship to vertebrate systems. *Trends Cardiovasc. Med. 5*, 21–28.

Bodmer, R., Frasch, M., **1999**. Genetic determination of *Drosophila* heart development. In: Rosenthal, N., Harvey, R. (Eds.), Heart Development. Academic Press, San Diego, pp. 65–90.

Bodmer, R., Jan, L.Y., Jan, Y.N., **1990**. A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of *Drosophila*. *Development 110*, 661–669.

Bodmer, R., Venkatesh, T.V., **1998**. Heart development in *Drosophila* and vertebrates: conservation of molecular mechanisms. *Devel. Genet. 22*, 181–186.

Borkowski, O.M., Brown, N.H., Bate, M., **1995**. Anterior-posterior subdivision and the diversification of the mesoderm in *Drosophila*. *Development 121*, 4183–4193.

Boulay, J.L., Dennefeld, C., Alberga, A., **1987**. The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers. *Nature 330*, 395–398.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M., Nguyen, H.T., **1995**. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes Devel. 9*, 730–741.

Brewster, R., Bodmer, R., **1995**. Origin and specification of type II sensory neurons in *Drosophila*. *Development 121*, 2923–2936.

Brody, T., Cravchik, A., **2000**. *Drosophila melanogaster* G protein-coupled receptors. *J. Cell Biol. 150*, F83–F88.

Brown, B.E., Starratt, A.N., **1975**. Isolation of proctolin, a myotropic peptide, from *Periplaneta americana*. *J. Insect Physiol. 21*, 1879–1881.

Bruggemann, A., Pardo, L., Stuhmer, W., Pongs, O., **1993**. *ether a go-go* encodes a voltage-gated channel permeable to K^+ and Ca^+ and modulated by cAMP. *Nature 365*, 445–448.

Buchner, E., **1991**. Genes expressed in the adult brain of *Drosophila* and effects of their mutations on behavior: a survey of transmitter- and second messenger-related genes. *J. Neurogenet. 7*, 153–192.

Budnik, V., White, K., **1987**. Genetic dissection of dopamine and serotonin synthesis in the nervous system of *Drosophila melanogaster. J. Neurogenet. 4*, 309–314.

Buff, E., Carmena, A., Gisselbrecht, S., Jimenez, F., Michelson, A.M., **1998**. Signalling by the *Drosophila* epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development 125*, 2075–2086.

Burg, J.P., **1967**. Maximum entropy spectral analysis. In: Childers, G. (Ed.), Modern Spectral Analysis. IEEE Press, New York, pp. 34–41.

Campos-Ortega, J.A., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila Melanogaster*, 2nd edn. Springer, New York.

Cantera, R., Nässel, D.R., **1992**. Segmental peptidergic innervation of abdominal targets in larval and adult dipteran insects revealed with an antiserum against Leucokinin I. *Cell Tissue Res. 269*, 459–471.

Carmena, A., Buff, E., Halfon, M.S., Gisselbrecht, S., Jimenez, F., *et al.*, **2002**. Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the *Drosophila* embryonic mesoderm. *Devel. Biol. 244*, 226–242.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F., Michelson, A.M., **1998**. Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Devel. 12*, 3910–3922.

Carrington, C., Tenney, S., **1959**. Chemical constituents of hemolymph and tissue in *T. polyphemus*, Cram. *J. Insect Physiol. 3*, 402.

Cassill, J.A., Whitney, M., Joazeiro, C.A., Becker, A., Zuker, C.S., **1991**. Isolation of *Drosophila* genes encoding G protein-coupled receptor kinases. *Proc. Natl Acad. Sci. USA 88*, 11067–11070.

Catsch, A., **1944**. Eine erbliche Störung des Bewegungsmechansmus bei *Drosophila melanogaster. Z. Ind. Abstr. Vererb. 82*, 64–66.

Cazzamali, G., Grimmelikhuijzen, C.J., **2002**. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 12073–12078.

Cazzamali, G., Saxild, N., Grimmelikhuijzen, C.J., **2002**. Molecular cloning and functional expression of a *Drosophila* corazonin receptor. *Biochem. Biophys. Res. Commun. 298*, 31–36.

Chamaon, K., Smalla, K.H., Thomas, U., Gundelfinger, E.D., **2002**. Nicotinic acetylcholine receptors of *Drosophila*: three subunits encoded by genomically linked genes can co-assemble into the same receptor complex. *J. Neurochem. 80*, 149–157.

Chartier, A., Zaffran, S., Astier, M., Semeriva, M., Gratecos, D., **2002**. Pericardin, a *Drosophila* type IV collagen-like protein is involved in the morphogenesis and maintenance of the heart epithelium during dorsal ectoderm closure. *Development 129*, 3241–3253.

Chatfield, C., **1980**. The Analysis of Time Series. Chapman and Hall, London.

Chatwin, H.M., Rudling, J.E., Patel, D., Reale, V., Evans, P.D., **2003**. Site-directed mutagenesis studies on the *Drosophila* octopamine/tyramine receptor. *Insect Biochem. Mol. Biol. 32*, 173–182.

Chen, C.-N., Denome, S., Davis, R.L., **1986**. Molecular analysis of cDNA clones and the corresponding genomic coding sequence of the *Drosophila dunce*$^+$ gene, the structural gene for cAMP phosphodiesterase. *Proc. Natl Acad. Sci. USA 83*, 9313–9317.

Chen, M.S., Obar, R.A., Schroeder, C.C., Austin, T.W., Poodry, C.A., *et al.*, **1991**. Multiple forms of dynamin are encoded by *shibire*, a *Drosophila* gene involved in endocytosis. *Nature 351*, 583–586.

Cheung, C.C., Loi, P.K., Sylwester, A.W., Lee, T.D., Tublitz, N.J., **1992**. Primary structure of a cardioactive neuropeptide from the tobacco hawkmoth, *Manduca sexta*. *FEBS Lett. 313*, 165–168.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., *et al.*, **2001**. Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. *Science 292*(5514), 104–106.

Coast, G.M., **1996**. Neuropeptides implicated in the control of diuresis in insects. *Peptides 17*, 327–336.

Coast, G.M., **2001**. Diuresis in the housefly (*Musca domestica*) and its control by neuropeptides. *Peptides 22*, 153–160.

Colas, J.-F., Launay, J.-M., Kellermann, O., Rosay, P., Maroteaux, L., **1995**. *Drosophila* 5-HT_2 serotonin receptor: coexpression with *fushi-tarazu* during segmentation. *Proc. Natl Acad. Sci. USA 92*, 5441–5445.

Collins, C., Miller, T., **1977**. Studies on the action of biogenic amines on the cockroach heart. *J. Exp. Biol. 67*, 1–15.

Cox, K.J., Tensen, C.P., Van der Schors, R.C., Li, K.W., van Heerikhuizen, H., *et al.*, **1997**. Cloning, characterization, and expression of a G-protein-coupled receptor from *Lymnaea stagnalis* and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. *J. Neurosci. 17*, 1197–1205.

Cripps, R.M., Olson, E.N., **2002**. Control of cardiac development by an evolutionarily conserved transcriptional network. *Devel. Biol. 246*, 14–28.

Curran, M., Splawski, I., Timothy, K., Vincent, G., Green, E., *et al.*, **1995**. A molecular basis for cardiac arrhythmia: *HERG* mutations cause long QT syndrome. *Cell 80*, 795–803.

Curtis, N., Ringo, J., Dowse, H., **1999**. Morphology of the pupal heart, adult heart, and associated tissues in the fruit fly (*Drosophila melanogaster*). *J. Morphol. 240*, 225–235.

Damm, C., Wolk, A., Buttgereit, D., Loher, K., Wagner, E., *et al.*, **1998**. Independent regulatory elements in the upstream region of the *Drosophila* beta 3 tubulin gene (beta Tub60D) guide expression in the dorsal vessel and the somatic muscles. *Devel. Biol. 199*, 138–149.

Das, N., Levine, R.L., Orr, W.C., Sohal, R.S., **2001**. Selectivity of protein oxidative damage during aging in *Drosophila melanogaster. Biochem. J. 360*(Pt 1), 209–216.

Davies, S., Huesmann, G., Maddrell, S., O'Donnel, M., Saker, N., *et al.*, **1995**. CAP2b, a cardioacceleratory peptide, is present in *Drosophila* and stimulates fluid secretion via cGMP. *Am. J. Physiol. 269*, R1321–R1326.

Davis, R.L., Davidson, N., **1986**. Isolation of the *Drosophila melanogaster dunce* chromosomal region and recombination mapping of *dunce* sequence with restriction polymorphisms as genetic markers. *Mol. Cell Biol. 4*, 358–367.

Davis, N., Dulcis, D., Hildebrand, J., **2001**. Innervation of the heart and aorta of *Manduca sexta. J. Comp. Neurol. 440*, 245–260.

Davis, N.T., Blackburn, M.B., Golubeva, E.G., Hildebrand, J.G., **2003**. Localization of myoinhibitory peptide immunoreactivity in *Manduca sexta* and *Bombyx mori*, with indications that the peptide has a role in molting and ecdysis. *J. Exp. Biol. 206*, 1449–1460.

deBelle, J., Hilkner, A., Soklowski, M., **1989**. Genetic localization of *foraging (for)*: a major gene for larval behavior in *Drosophila melanogaster. Genetics 123*, 157–163.

Dellinger, B., Felling, R., Ordway, R.W., **2000**. Genetic modifiers of the *Drosophila* NSF mutant, *comatose*, include a temperature-sensitive paralytic allele of the calcium channel α1-subunit gene, *cacophony. Genetics 155*, 203–211.

Denyer, J., Brown, H., **1987**. A method for isolating rabbit sinoatrial node cells which maintains their natural shape. *Jpn J. Physiol. 37*, 963–965.

DiFrancesco, D., **1993**. Pacemaker mechanisms in cardiac tissue. *Annu. Rev. Physiol. 55*, 455–472.

Dong, X., Zavitz, K.H., Thomas, B.J., Lin, M., Campbell, S., *et al.*, **1997**. Control of G1 in the developing Drosophila eye: rca1 regulates Cyclin A. *Genes Devel. 11*, 94–105.

Dowse, H., Ringo, J., **1989**. The search for hidden periodicities in biological time series revisited. *J. Theoret. Biol. 139*, 487–515.

Dowse, H.B., Ringo, J., Power, J., Johnson, E., Kinney, K., *et al.*, **1995**. A congenital heart defect in *Drosophila* caused by an action potential mutation. *J. Neurogenet. 10*, 153–168.

Dulcis, D., Davis, N., Hildebrand, J., **2001**. Neuronal control of heart reversal in the Hawkmoth *Manduca Sexta. J. Comp. Physiol. A 187*, 837–849.

Dulcis, D., Levine, R., **2003**. Innervation of the heart of the adult fruit fly, *Drosophila melanogaster. J. Comp. Neurol. 465*, 560–578.

Dye, C.A., Lee, J.K., Atkinson, R.C., Brewster, R., Han, P.L., *et al.*, **1998**. The *Drosophila* sanpodo gene controls sibling cell fate and encodes a tropomodulin homolog, an actin/tropomyosin-associated protein. *Development 125*, 1845–1856.

Eberl, D., Ren, D., Feng, G., Lorenz, L., VanVactor, D., *et al.*, **1998**. Genetic and developmental characterization of *Dmca1D*, a calcium channel α_1 subunit gene in *Drosophila melanogaster. Genetics 148*, 1159–1169.

Edgar, B.A., O'Farrell, P.H., **1989**. Genetic control of cell division patterns in the *Drosophila embryo*. *Cell 57*, 177–187.

Edgar, B.A., O'Farrell, P.H., **1990**. The three postblastoderm cell cycles of *Drosophila embryogenesis* are regulated in G2 by string. *Cell 62*, 469–480.

Edmunds, L.N., Jr., **1988**. Cellular and Molecular Bases of Biological Clocks. Springer, New York.

Egerod, K., Reynisson, E., Hauser, F., Williamson, M., Cazzamali, G., *et al.*, **2003a**. Molecular identification of the first insect proctolin receptor. *Biochem. Biophys. Res. Commun. 306*, 437–442.

Egerod, K., Reynisson, E., Hauser, F., Cazzamali, G., Williamson, M., *et al.*, **2003b**. Molecular cloning and functional expression of the first two specific insect myosuppressin receptors. *Proc. Natl Acad. Sci. USA 100*, 9808–9813.

Elkins, T., Ganetzky, B., Wu, C.-F., **1986**. A *Drosophila* mutation that eliminates a calcium-dependent potassium current. *Proc. Natl Acad. Sci. USA 83*, 8415–8419.

Ewer, J., Truman, J.W., **1996**. Increases in cyclic 3′, 5′-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide-immunoreactive insect neuronal network. *J. Comp. Neurol. 370*, 330–341.

Feany, M., Quinn, W., **1995**. A neuropeptide gene defined by the *Drosophila* memory mutant *amnesiac*. *Science 268*, 869–873.

Ferguson, E.L., Anderson, K.V., **1992**. Localized enhancement and repression of the activity of the TGF-beta family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the *Drosophila embryo*. *Development 114*, 583–597.

ffrench-Constant, R.H., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J., Roush, R.T., **1992**. Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: an invertebrate gamma-aminobutyric acid subtype A receptor locus. *Proc. Natl Acad. Sci. USA 88*, 7209–7213.

Finch, C.E., Ruvkun, G., **2001**. The genetics of aging. *Annu. Rev. Genom. Hum. Genet. 2*, 435–462.

Fishman, M.C., Chien, K.R., **1997**. Fashioning the vertebrate heart: earliest embryonic decisions. *Development 124*, 2099–2117.

Foe, V.E., **1989**. Mitotic domains reveal early commitment of cells in *Drosophila embryos*. *Development 107*, 1–22.

Francois, V., Solloway, M., O'Neill, J.W., Emery, J., Bier, E., **1994**. Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes. Devel. 8*, 2602–2616.

Frasch, M., **1995**. Induction of visceral and cardiac mesoderm by ectodermal Dpp in early *Drosophila embryo*. *Nature 374*, 464–467.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H., Levine, M., **1987**. Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J. 6*, 749–759.

Fremion, F., Astier, M., Zaffran, S., Guillen, A., Homburger, V., Semeriva, M., **1999**. The heterotrimeric protein Go is required for the formation of heart epithelium in *Drosophila*. *J. Cell Biol. 145*, 1063–1076.

Fujioka, M., Emi-Sarker, Y., Yusibova, G.L., Goto, T., Jaynes, J.B., **1999**. Analysis of an even-skipped rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development 126*, 2527–2538.

Furuya, K., Liao, S., Reynolds, S.E., Ota, R.B., Hackett, M., *et al.*, **1993**. Isolation and identification of a cardioactive peptide from *Tenebrio molitor* and *Spodoptera eridania*. *Biol. Chem. Hoppe Seyler 374*, 1065–1074.

Gade, G., Auerswald, L., **2003**. Mode of action of neuropeptides from the adipokinetic hormone family. *Gen. Comp. Endocrinol. 132*, 10–20.

Gajewski, K., Choi, C.Y., Kim, Y., Schulz, R.A., **2000**. Genetically distinct cardial cells within the *Drosophila* heart. *Genesis 28*, 36–43.

Gajewski, K., Fossett, N., Molkentin, J.D., Schulz, R.A., **1999**. The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. *Development 126*, 5679–5688.

Gajewski, K., Zhang, Q., Choi, C.Y., Fossett, N., Dang, A., *et al.*, **2001**. Pannier is a transcriptional target and partner of tinman during *Drosophila* cardiogenesis. *Devel. Biol. 233*, 425–436.

Gammie, S.C., Truman, J.W., **1999**. Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behavior. *J. Exp. Biol. 202*, 343–352.

Ganetzky, B., **1984**. Genetic studies of membrane excitability in *Drosophila*: lethal interaction between two temperature-sensitive paralytic mutations. *Genetics 108*, 897–911.

Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., *et al.*, **2003**. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature 424*, 443–447.

Giles, W., Lederer, W., Eisner, D., **1982**. Sinus pacemaker activity in the heart. In: Carpenter, D.O. (Ed.), Cellular Pacemakers. Wiley, New York, pp. 91–127.

Gintant, G., Cohen, I., Datyner, N., Kline, R., **1992**. Time-dependent outward currents in the heart. In: Fozzard, H.A., *et al.* (Eds.), The Heart and Cardiovascular System, 2nd edn. Raven, New York, pp. 1121–1169.

Gisselbrecht, S., Skeath, J.B., Doe, C.Q., Michelson, A.M., **1996**. Heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila embryo*. *Genes Devel. 10*, 3003–3017.

Gho, M., Mallart, A., **1986**. Two distinct calcium-activated potassium currents in larval muscle fibers of *Drosophila melanogaster*. *Pfluger's Arch. 407*, 526–533.

Golic, K.G., Lindquist, S., **1989**. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell 59*, 499–509.

Gorczyca, M.G., Phillis, R.W., Budnik, V., **1994**. The role of tinman, a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in *Drosophila*. *Development 120*, 2143–2152.

Griffin, K.J., Stoller, J., Gibson, M., Chen, S., Yelon, D., *et al.*, **2000**. A conserved role for H15-related T-box transcription factors in zebrafish and *Drosophila* heart formation. *Devel. Biol. 218*, 235–247.

Griffith, L., Verselis, L., Aitken, K., Kyriacou, C., Danho, W., *et al.*, **1993**. Inhibition of calcium/calmodulin-dependent protein kinase in *Drosophila* disrupts behavioral plasticity. *Neuron 10*, 501–509.

Guarente, L., Kenyon, C., **2000**. Genetic pathways that regulate ageing in model organisms. *Nature 408*(6809), 255–262.

Gu, G.-G., Singh, S., **1995**. Pharmacological analysis of heartbeat in *Drosophila*. *J. Neurobiol. 28*, 269–280.

Gu, G.-G., Singh, S., **1997**. Modulation of the dihydropyridine-sensitive calcium channels in *Drosophila* by a phospholipase C-mediated pathway. *J. Neurobiol. 33*, 265–275.

Guillen, A., Jallon, J.M., Fehrentz, J.A., Pantaloni, C., Bockaert, J., *et al.*, **1990**. A Go-like protein in *Drosophila melanogaster* and its expression in memory mutants. *EMBO J. 9*, 1449–1455.

Guo, M., Jan, L.Y., Jan, Y.N., **1996**. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron 17*, 27–41.

Hagiwara, N., Irisawa, H., Kameyama, M., **1988**. Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J. Physiol. 395*, 233–253.

Halfon, M.S., Carmena, A., Gisselbrecht, S., Sackerson, C.M., Jimenez, F., *et al.*, **2000**. Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell 103*, 63–74.

Han, Z., Fujioka, M., Su, M.-T., Liu, M., Jaynes, J.B., *et al.*, **2002**. Transcriptional integration of competence modulated by mutual repression generates cell-type specificity within the cardiogenic mesoderm. *Devel. Biol. 252*, 225–240.

Han, Z., Bodmer, R., **2003**. Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the *Drosophila* heart, with or without cell division. *Development 130*, 3039–3051.

Harrison, J.B., Chen, H.H., Blake, A.D., Huskisson, N.S., Barker, P., *et al.*, **1995**. Localization in the nervous

system of *Drosophila melanogaster* of a C-terminus anti-peptide antibody to a cloned *Drosophila* muscarinic acetylcholine receptor. *J Neuroendocrinol. 7*, 347–352.

Harthoorn, L.F., Oudejans, R.C., Diederen, J.H., Van der Horst, D.J., **2002**. Coherence between biosynthesis and secretion of insect adipokinetic hormones. *Peptides 23*, 629–634.

Harvey, R.P., **1996**. NK-2 homeobox genes and heart development. *Devel. Biol. 178*, 203–216.

Haun, C., Alexander, J., Stainier, D.Y., Okkema, P.G., **1998**. Rescue of Caenorhabditis elegans pharyngeal development by a vertebrate heart specification gene. *Proc. Natl Acad. Sci. USA 95*, 5072–5075.

Hearn, M.G., Ren, Y., McBride, E.W., Reveillaud, I., Beinborn, M., *et al.*, **2002**. A *Drosophila* dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. *Proc. Natl Acad. Sci. USA 99*, 14554–14559.

Heinrich, B., **1970**. Nervous control of the heart during thoracic temperature regulation in a sphinx moth. *Science 169*, 606–607.

Heinrich, B., **1971**. Temperature regulation of the sphinx moth, *Manduca sexta*. II. Regulation of heat loss by control of blood circulation. *J. Exp. Biol. 54*, 153–166.

Heinrich, B., **1976**. Heat exchange in relation to blood flow between thorax and abdomen in bumblebees. *J. Exp. Biol. 64*, 561–585.

Helfland, S.L., Rogina, B., **2003**. Molecular genetics of aging in the fly: is this the end of the beginning? *Bioessays 25*(2), 134–141.

Herman, W.S., **1981**. Studies on the adult reproductive diapause of the monarch butterflies, *Danaus plexippus*. *Biol. Bull. 160*, 89–106.

Herranz, H., Morata, G., **2001**. The functions of pannier during *Drosophila* embryogenesis. *Development 128*, 4837–4846.

Hertel, W., Pass, G., **2002**. An evolutionary treatment of the morphology and physiology of circulatory organs in insects. *Comp. Biochem. Physiol. A Mol. Integr. Physiol. 133*, 555–575.

Hevers, W., Hardie, R.C., **1995**. Serotonin modulates the voltage dependence of delayed rectifier and *Shaker* potassium channels in *Drosophila* photoreceptors. *Neuron 14*, 845–856.

Hewes, R.S., Snowdeal, E.C., III, Saitoe, M., Taghert, P., **1998**. Functional redundancy of FMRFamide-related peptides at the *Drosophila* larval neuromuscular junction. *J. Neurosci. 18*, 7138–7151.

Hewes, R., Taghert, P.H., **2001**. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res. 11*, 1126–1142.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, **2002**. G protein-coupled receptors in *Anopheles gambiae*. *Science 298*, 176–178.

Hille, B., **1966**. Common mode of action of three agents that decrease the transient change in sodium permeability in nerves. *Nature 210*, 1220–1222.

Hille, B., **2001**. Ion Channels of Excitable Membranes. Sinauer, Sunderland, MA.

Hinshaw, J.E., Schmid, S.L., **1995**. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature 374*, 190–192.

Hirsch, J., Davidson, N., **1981**. Isolation and characterization of the *dopa decarboxylase* gene of *Drosophila melanogaster. Mol. Cell Biol. 1*, 475–485.

Holland, N.D., Venkatesh, T.V., Holland, L.Z., Jacobs, D.K., Bodmer, R., **2003**. AmphiNk2-tin, an amphioxus homeobox gene expressed in myocardial progenitors: insights into evolution of the vertebrate heart. *Devel. Biol. 255*, 128–137.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Horodyski, F.M., Riddiford, L.M., Truman, J.W., **1989**. Isolation and expression of the eclosion hormone gene from the tobacco hornworm, *Manduca sexta*. *Proc. Natl Acad. Sci. USA 86*, 8123–8127.

Huang, T.T., Carlson, E.J., Gillespie, A.M., Shi, Y., Epstein, C.J., **2000**. Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. *J. Gerontol. A Biol. Sci. Med. Sci. 55*(1), B5–B9.

Hudspeth, A.J., Lewis, R.S., **1988a**. Kinetic analysis of voltage- and ion-dependent conductances in saccular hair cells of the bull-frog, *Rana catesbeiana*. *J. Physiol. 400*, 237–274.

Hudspeth, A.J., Lewis, R., **1988b**. A model for electrical resonance and frequency tuning in saccular hair cells of the bull-frog, *Rana catesbeiana*. *J. Physiol. 400*, 275–297.

Huesmann, G.R., Cheung, C.C., Loi, P.K., Lee, T.D., Swiderek, K.M., Tublitz, N., **1995**. Amino acid sequence of CAP2b, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*. *FEBS Lett. 371*, 311–314.

Hüser, J., Blatter, L., Lipsius, S., **2000**. Intracellular Ca^{2+} release contributes to automaticity in cat atrial pacemaker cells. *J. Physiol. 524*, 415–422.

Irisawa, H., Brown, H., Giles, W., **1993**. Cardiac pacemaking in the sinoatrial node. *Physiol. Rev. 73*, 197–227.

Iversen, A., Cazzamali, G., Williamson, M., Hauser, F., Grimmelikhuijzen, C.J., **2002**. Molecular identification of the first insect ecdysis triggering hormone receptors. *Biochem. Biophys. Res. Commun. 299*, 924–931.

Jackson, F., Wilson, S., Strichartz, G., Hall, L., **1984**. Two types of mutants affecting voltage-sensitive sodium channels in *Drosophila melanogaster. Nature 308*, 189–191.

Jagla, K., Jagla, T., Heitzler, P., Dretzen, G., Bellard, F., *et al.*, **1997**. Ladybird, a tandem of homeobox genes that maintain late wingless expression in terminal and dorsal epidermis of the *Drosophila embryo*. *Development 124*, 91–100.

Jagla, T., Bidet, Y., Da Ponte, J.P., Dastugue, B., Jagla, K., **2002**. Cross-repressive interactions of identity genes are essential for proper specification of cardiac and

muscular fates in *Drosophila*. *Development 129*, 1037–1047.

Jana, C.K., Das, N., Sohal, R.S., **2002**. Specificity of age-related carbonylation of plasma proteins in the mouse and rat. *Arch. Biochem. Biophys. 397*(2), 433–439.

Jensen, P.V., **1973**. Structure and metamorphosis of the larval heart of *Calliphora erythrocephala*. *K. Dansk. Vidensk. Selsk. Biol. Skrift. 20*, 2–19.

Johnson, E., Ringo, J., Dowse, H., **1997**. Modulation of *Drosophila* heartbeat by neurotransmitters. *J. Comp. Physiol. B. 167*, 89–97.

Johnson, E., Ringo, J., Bray, N., Dowse, H., **1998**. Genetic and pharmacological identification of ion channels central to *Drosophila's* cardiac pacemaker. *J. Neurogenet. 12*, 1–24.

Johnson, E., Ringo, J., Dowse, H., **2000**. Native and heterologous peptides are cardioactive in *Drosophila*. *J. Insect. Physiol. 46*, 1229–1236.

Johnson, E., Ringo, J., Dowse, H., **2001**. Dynamin, encoded by *shibire,* is central for cardiac function. *J. Exp. Zool. 289*, 81–89.

Johnson, E., Sherry, T., Ringo, J., Dowse, H., **2002**. Modulation of the cardiac pacemaker of *Drosophila*: cellular mechanisms. *J. Comp. Physiol. B. 172*, 227–636.

Johnson, E.C., Garczynski, S.F., Park, D., Crim, J.W., Nässel, D.R., Taghert, P.H., **2003a**. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 100*, 6198–6203.

Johnson, E.C., Bohn, L.M., Barak, L.S., Birse, R.T., Nässel, D.R., Caron, M.G., Taghert, P.H., **2003b**. Identification of *Drosophila* neuropeptide receptors by GPCR-βarrestin interactions. *J. Biol. Chem. 278*, 52172–52178.

Jones, E., Gray-Keller, M., Fettiplace, R., **1999**. The role of Ca^{2+}-activated K^{+} channel spliced variants in the tonotopic organization of the turtle cochlea. *J. Physiol. 518*, 653–665.

Jones, J.C., **1977**. The Circulatory System of Insects. Charles C. Thomas, Springfield, IL.

Kang, H.L., Benzer, S., Min, K.T., **2002**. Life extension in *Drosophila* by feeding a drug. *Proc. Natl Acad. Sci. USA 99*(2), 838–843.

Kanungo, M.S., **1980**. Biochemistry of Aging. Academic Press, London.

Kardong, K.V., **2002**. Vertebrates: Comparative Anatomy, Function, Evolution, 3rd edn. McGraw-Hill, New York.

Kaplan, W., Trout, W., **1968**. The behavior of four neurological mutants of *Drosophila*. *Genetics 61*, 399–409.

Keating, M.T., Sanguinetti, M., **1996**. Molecular genetic insights into cardiovascular disease. *Science 272*, 681–685.

Kelly, R.G., Buckingham, M.E., **2002**. The anterior heart-forming field: voyage to the arterial pole of the heart. *Trends Genet. 18*, 210–216.

Kernan, M., Kuroda, M., Kreber, R., Baker, B., Ganetzky, B., **1991**. *nap^{ts}*, a mutation affecting sodium channel activity in *Drosophila*, is an allele of *mle*, a regulator of dosage compensation. *Cell 66*, 949–959.

Kim, I., Williamson, D.F., Byers, T., Koplan, J.P., **1993**. Vitamin and mineral supplement use and mortality in a US cohort. *Am. J. Public Health 83*(4), 546–550.

Kim, Y.C., Lee, H.G., Seong, C.S., Han, K.A., **2003**. Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of *Drosophila melanogaster*. *Gene Expr. Patterns 3*, 237–245.

Klambt, C., **1993**. The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development 117*, 163–176.

Klinedinst, S.L., Bodmer, R., **2003**. GATA-factor Pannier is required to establish competence for heart progenitor formation. *Development 130*, 3027–3038.

Knirr, S., Frasch, M., **2001**. Molecular integration of inductive and mesoderm-intrinsic inputs governs even-skipped enhancer activity in a subset of pericardial and dorsal muscle progenitors. *Devel. Biol. 238*, 13–26.

Knoblich, J.A., Lehner, C.F., **1993**. Synergistic action of *Drosophila* cyclins A and B during the G2-M transition. *EMBO J. 12*, 65–74.

Kohn, R.R., **1971**. Effect of antioxidants on life-span of C57BL mice. *J. Gerontol. 26*, 378–380.

Kohout, T.A., Lefkowitz, R.J., **2003**. Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol. Pharmacol. 63*, 9–18.

Koladich, P.M., Cusson, M., Bendena, W.G., Tobe, S.S., McNeil, J.N., **2002**. Cardioacceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth, *Pseudaletia unipuncta*. *Peptides 23*, 645–651.

Kolsch, V., Paululat, A., **2002**. The highly conserved cardiogenic bHLH factor Hand is specifically expressed in circular visceral muscle progenitor cells and in all cell types of the dorsal vessel during *Drosophila* embryogenesis. *Devel. Genes Evol. 212*, 473–485.

Kosman, D., Ip, Y.T., Levine, M., Arora, K., **1991**. Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. *Science 254*, 118–122.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., *et al.*, **1991**. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. *Proc. Natl Acad. Sci. USA 88*, 9458–9462.

Kreienkamp, H.J., Larusson, H.J., Witte, I., Roeder, T., Birgul, N., *et al.*, **2002**. Functional annotation of two orphan G-protein-coupled receptors, *Drostar1* and -2, from *Drosophila melanogaster* and their ligands by reverse pharmacology. *J. Biol. Chem. 277*, 39937–39943.

Krijgsman, B., Krijgsman-Berger, N., **1951**. Physiological investigations into the heart function of arthropods. The heart of *Periplaneta Americana*. *Bull. Entomol. Res. 42*, 143–155.

Kubiak, T.M., Larsen, M.J., Burton, K.J., Bannow, C.A., Martin, R.A., *et al.*, **2002**. Cloning and functional expression of the first *Drosophila melanogaster* sulfakinin receptor DSK-R1. *Biochem. Biophys. Res. Commun. 291*, 313–320.

Kulkarni, S.J., Hall, J.C., **1987**. Behavioral and cytogenetic analysis of the *cacophony* courtship song mutant and interacting genetic variants in *Drosophila melanogaster. Genetics 115*, 461–475.

Kuwasawa, K., Ai, H., Matsushita, T., **1999**. Cardiac reflexes and their neural pathways in lepidopterous insects. *Comp. Biochem. Physiol. 124A*, 581–586.

Lai, Z.C., Fortini, M.E., Rubin, G.M., **1991**. The embryonic expression patterns of zfh-1 and zfh-2, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. *Mech. Devel. 34*, 123–134.

Lane, M.E., Sauer, K., Wallace, K., Jan, Y.N., Lehner, C.F., *et al.*, **1996**. Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development. *Cell 87*, 1225–1235.

Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., *et al.*, **2001**. Type A allatostatins from *Drosophila melanogaster* and *Diplotera puncata* activate two *Drosophila* allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. *Biochem. Biophys. Res. Commun. 286*, 895–901.

Lee, H.H., Frasch, M., **2000**. Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target sloppy paired. *Development 127*, 5497–5508.

Lee, Y., Shioi, T., Kasahara, H., Jobe, S.M., Wiese, R.J., *et al.*, **1998**. The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol. Cell. Biol. 18*, 3120–3129.

Lehman, H.K., Murgiuc, C.M., Miller, T.A., Lee, T.D., Hildebrand, J.G., **1993**. Crustacean cardioactive peptide in the sphinx moth, *Manduca sexta*. *Peptides 14*, 735–741.

Leips, J., MacKay, T.F., **2000**. Quantitative trait loci for life span in *Drosophila melanogaster*: interactions with genetic background and larval density. *Genetics 155*(4), 1773–1788.

Lenz, C., Williamson, M., Hansen, G.N., Grimmelikhuijzen, C.J., **2001**. Identification of four *Drosophila* allatostatins as the cognate ligands for the *Drosophila* orphan receptor DAR-2. *Biochem. Biophys. Res. Commun. 286*, 1117–1122.

Leptin, M., **1991**. Twist and snail as positive and negative regulators during *Drosophila* mesoderm development. *Genes Devel. 5*, 1568–1576.

Leptin, M., Grunewald, B., **1990**. Cell shape changes during gastrulation in *Drosophila*. *Development 110*, 73–84.

Levin, L., Han, P., Hwang, P., Feinstein, P., Davis, R., *et al.*, **1992**. The *Drosophila* learning and memory mutant *rutabaga* encodes a Ca^{2+}/calmodulin-responsive adenylyl cyclase. *Cell 68*, 479–489.

Levine, J., Funes, P., Dowse, H., Hall, J., **2002**. Signal analysis of behavioral and molecular cycles. *Biomed. Central. Neurosci. 3*, 1.

Li, X.J., Wolfgang, W., Wu, Y.N., North, R.A., Forte, M., **1991**. Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides. *EMBO J. 10*, 3221–3229.

Li, J., Qu, J., Nathan, R.D., **1997**. Ionic basis of ryanodine's negative chronotropic effect on pacemaker cells isolated from the sinoatrial node. *Am. J. Physiol. 227*, H2481–H2489.

Lilly, B., Galewsky, S., Firulli, A.B., Schulz, R.A., Olson, E.N., **1994**. D-MEF2: a MADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during *Drosophila* embryogenesis. *Proc. Natl Acad. Sci. USA 91*, 5662–5666.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., *et al.*, **1995**. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science 267*, 688–693.

Lin, Y.J., Seroude, L., Benzer, S., **1998**. Extended life-span and stress resistance in the *Drosophila* mutant methuselah. *Science 282*(5390), 943–946.

Linnen, C., Tatar, M., Promislow, D., **2001**. Cultural artifacts: a comparison of senescence in natural, laboratory-adapted and artificially selected lines of *Drosophila melanogaster. Evol. Ecol. Res. 3*, 877–888.

Lipsius, S., Hüser, J., Blatter, L., **2001**. Intracellular Ca^{2+} release sparks atrial pacemaker activity. *News Physiol. Sci. 16*, 101–106.

Livingstone, M., Tempel, B., **1983**. Genetic dissection of monoamine neurotransmitter synthesis in *Drosophila*. *Nature 303*, 67–70.

Lo, P.C., Frasch, M., **2001**. A role for the COUP-TF-related gene seven-up in the diversification of cardioblast identities in the dorsal vessel of *Drosophila*. *Mech. Devel. 104*, 49–60.

Lo, P.C., Frasch, M., **2003**. Establishing A-P polarity in the embryonic heart tube: a conserved unction of SOX genes in *Drosophila* and vertebrates? *Trends Cardiovasc. Med 13*, 182–187.

Lo, P.C., Skeath, J.B., Gajewski, K., Schulz, R.A., Frasch, M., **2002**. Homeotic genes autonomously specify the anteroposterior subdivision of the *Drosophila* dorsal vessel into aorta and heart. *Devel. Biol. 251*, 307–319.

Lockwood, W.K., Bodmer, R., **2002**. The patterns of wingless, decapentaplegic, and tinman position the *Drosophila* heart. *Mech. Devel. 114*, 13–26.

Lockwood, W.K., Liu, M., Su, M.-T., Bodmer, R., **2001**. A genetic model for cardiac pattern formation and cell fate determination. In: Haddad, G., Xu, T. (Eds.), Genetic Models In Cardiorespiratory Biology, Lung Biology Series. New York, pp. 179–201.

Loi, P.K., Emmal, S.A., Park, Y., Tublitz, N.J., **2001**. Identification, sequence and expression of a crustacean cardioactive peptide (CCAP) gene in the moth *Manduca sexta*. *J. Exp. Biol. 204*, 2803–2816.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., **1995**. Identification of two allatostatins from the cricket, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae): additional members of a family of neuropeptides inhibiting juvenile hormone biosynthesis. *Regul. Pept. 57*, 227–236.

Loughney, K., Kreber, R., Ganetzky, B., **1989**. Molecular analysis of the *para* locus, a sodium channel gene in *Drosophila*. *Cell 58*, 1143–1154.

Lovato, T.L., Nguyen, T.P., Molina, M.R., Cripps, R.M., **2002**. The Hox gene abdominal-A specifies heart cell fate in the *Drosophila* dorsal vessel. *Development 129*, 5019–5027.

Lu, B., Rothenberg, M., Jan, L.Y., Jan, Y.N., **1998**. Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in *Drosophila* neural and muscle progenitors. *Cell 95*, 225–235.

Luo, L., Liao, Y.J., Jan, L.Y., Jan, Y.N., **1994**. Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Devel. 8*, 1787–1802.

Maloeuf, N., **1935**. The myogenic automatism of the contraction of the heart of insects. *Ann. Ent. Soc. Am. 28*, 322–337.

Markou, T., Theophilidis, G., **2000**. The pacemaker activity generating the intrinsic mygenic contraction of the dorsal vessel of *Tenebrio molitor* (Coleoptera). *J. Exp. Biol. 203*, 3471–3483.

Mastick, G.S., McKay, R., Oligino, T., Donovan, K., Lopez, A.J., **1995**. Identification of target genes regulated by homeotic proteins in *Drosophila melanogaster* through genetic selection of Ultrabithorax protein-binding sites in yeast. *Genetics 139*, 349–363.

Matsushita, T., Kuwasawa, K., Uchimura, K., Ai, H., Kurokawa, M., **2002**. Biogenic amines evoke heartbeat reversal in larvae of the sweet potato hornworm, *Agrius convolvuli*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol. 133*, 625–636.

McCann, F.V., **1963**. Electrophysiology of an insect heart. *J. Gen. Physiol. 46*, 803–821.

McCann, F.V., **1969**. The insect heart as a model of electrophysiological studies. In: Kerkut, G.A. (Ed.), Experiments in Physiology and Biochemistry, vol. 2. Academic Press, New York, pp. 60–88.

McCann, F., Sanger, J., **1969**. Ultrastructure and function in an insect heart. *Experientia Suppl. 15*, 29–46.

McCann, F., **1970**. Physiology of insect hearts. *Annu. Rev. Entomol. 15*, 173–200.

McCann, F., **1971**. Calcium action potentials in insect myocardial fibers. *Comp. Biochem. Physiol. 40A*, 353–357.

McGaw, I.J., Wilkens, J.L., McMahon, B.R., Airriess, C.N., **1995**. Crustacean cardioexcitatory peptides may inhibit the heart *in vivo*. *J. Exp. Biol. 198*, 2547–2550.

McIndoo, N., **1945**. Innervation of insect hearts. *J. Comp. Neurol. 83*, 141–155.

Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., *et al.*, **2002**. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 15363–15368.

Meng, X., Wahlstrom, G., Immonen, T., Kolmer, M., Tirronen, M., *et al.*, **2002**. The *Drosophila hugin* gene codes for myostimulatory and ecdysis-modifying neuropeptides. *Mech. Devel. 117*, 5–13.

Messer, A.C., Brown, M.R., **1995**. Non-linear dynamics of neurochemical modulation of mosquito oviduct and hindgut contractions. *J. Exp. Biol. 198*, 2325–2336.

Metcalf, R., Winton, M., Fukoto, **1964**. The effects of cholinergic substances upon the isolated heart of *Periplaneta Americana*. *J. Insect. Physiol. 10*, 353–361.

Miller, A., **1950**. The internal anatomy and histology of the imago of *Drosophila melanogaster.* In: Demerec, M. (Ed.), Biology of *Drosophila*. Wiley, New York, pp. 420–534.

Miller, T., **1969**. Initiation of activity in the cockroach heart. *Experientia Suppl. 15*, 206–218.

Miller, T., **1985**. Structure and physiology of the circulatory system. In: Kerkut, G., Gilbert, M. (Eds.), Comprehensive Insect Physiology, vol. 3. Pergamon, Oxford, pp. 289–353.

Miller, T., Metcalf, R., **1968**. Site of action of pharmacologically active compounds on the heart of *Periplaneta americana*. *J. Insect Physiol. 14*, 383–394.

Miquel, J., Lundgren, P.R., Bensch, K.G., Atlan, H., **1976**. Effects of temperature on the life span, vitality and fine structure of *Drosophila melanogaster. Mech. Ageing Devel. 5*(*5*), 347–370.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., Rubin, G.M., **1990**. The *Drosophila* seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell 60*, 211–224.

Mockett, R.J., Bayne, A.C., Kwong, L.K., Orr, W.C., Sohal, R.S., **2003**. Ectopic expression of catalase in *Drosophila* mitochondria increases stress resistance but not longevity. *Free Radic. Biol. Med. 34*(2), 207–217.

Molina, M.R., Cripps, R.M., **2001**. Ostia, the inflow tracts of the *Drosophila* heart, develop from a genetically distinct subset of cardial cells. *Mech. Devel. 109*, 51–59.

Monastirioti, M., Linn, C.E., Jr., White, K., **1996**. Characterization of *Drosophila* tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci. 16*, 3900–3911.

Monastirioti, M., **1999**. Biogenic amine systems in the fruit fly *Drosophila melanogaster. Microsc. Res. Tech. 45*, 106–121.

Monnier, D., Colas, J.F., Rosay, P., Hen, R., Borrelli, E., *et al.*, **1995**. NKD, a developmentally regulated tachykinin receptor in *Drosophila*. *J. Biol. Chem. 267*, 1298–1302.

Moore, A.W., Barbel, S., Jan, L.Y., Jan, Y.N., **2000**. A genomewide survey of basic helix-loop-helix factors in *Drosophila*. *Proc. Natl. Acad. Sci. USA 97*, 10436–10441.

Moore, M.S., DeZazzo, J., Luk, A.Y., Tully, T., Singh, C.M., *et al.*, **1998**. Ethanol intoxication in *Drosophila*: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell 93*, 997–1007.

Moroni, A., Gorza, L., Beltrame, M., Gravante, B., Vaccari, T., *et al.*, **2001**. Hyperpolarization-activated cyclic nucleotide-gated channel 1 is a molecular determinant of the cardiac pacemaker current I_f. *J. Biol. Chem. 276*, 29233–29241.

Nässel, D.R., **2002**. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol. 68*, 1–84.

Narahashi, T., Moore, J., Scott, W.R., **1964**. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol. 47, 965–974.*

Nasonkin, I., Alikasifoglu, A., Ambrose, C., Cahill, P., Cheng, M., *et al.*, **1999**. A novel sulfonylurea receptor family member expressed in the embryonic *Drosophila* dorsal vessel and tracheal system. *J. Biol. Chem. 274*, 29420–29425.

Neckameyer, W.S., Quinn, W.G., **1989**. Isolation and characterization of the gene for *Drosophila* tyrosine hydroxylase. *Neuron 2*, 1167–1175.

Neckameyer, W.S., White, K., **1993**. *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. *J. Neurogenet. 8*, 189–199.

Nguyen, H.T., Bodmer, R., Abmayr, S.M., McDermott, J.C., Spoerel, N.A., **1994**. Dmef2: a *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. *Proc. Natl Acad. Sci. USA 91, 7520–7524.*

Nichols, R., **1992**. Isolation and structural characterization of *Drosophila* TDVDHVFLRFamide and FMRFamide-containing neural peptides. *J. Mol. Neurosci. 3*, 213–218.

Nichols, R., McCormick, J., Cohen, M., Howe, E., Jean, C., *et al.*, **1999a**. Differential processing of neuropeptides influences *Drosophila* heart rate. *J. Neurogenet. 13*, 89–104.

Nichols, R., Kaminski, S., Walling, E., Zornik, E., **1999b**. Regulating the activity of a cardioacceleratory peptide. *Peptides 20*, 1153–1158.

Noyes, B.E., Katz, F.N., Schaffer, M.N., **1995**. Identification and expression of the *Drosophila* adipokinetic hormone gene. *Mol. Cell. Endocrinol. 109*, 133–141.

Nusslein-Volhard, C., Wieschaus, E., Kluding, H., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* 1. Zygotic loci on the second chromosome. Roux's Arch. Devel. Biol. *183, 267–282.*

O'Connor-Giles, K.M., Skeath, J.B., **2003**. Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in *Drosophila*. *Devel. Cell 5*, 231–243.

Olivera, B., Miljanich, G., Ramachandran, G., Adams, M., **1994**. Calcium channel diversity and neurotransmitter release: The ω-Conotoxins and ω-Agatoxins. *Annu. Rev. Biochem. 63*, 823–867.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Blames, J., Cullen, M.R., *et al.*, **1996**. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *J. Natl Cancer Inst. 88*(21), 1550–1559.

Orr, W.C., Sohal, R.S., **1993**. Effects of Cu-Zn superoxide dismutase overexpression of life span and resistance to oxidative stress in transgenic *Drosophila melanogaster. Arch. Biochem. Biophys. 301*(1), 34–40.

Orr, W.C., Sohal, R.S., **1994**. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster. Science 263*, 1128–1130.

Osbourne, K., Robichon, A., Burgess, E., Butland, S., Shaw, R., *et al.*, **1997**. Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science 277*, 834–836.

Palladino, M.J., Keegan, L.P., O'Connell, M.A., Reenan, R.A., **2000**. A-to-I pre-mRNA editing in *Drosophila* is primarily involved in adult nervous system function and integrity. *Cell 102*, 437–449.

Pandur, P., Lasche, M., Eisenberg, L.M., Kuhl, M., **2002**. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature 418*, 636–641.

Papaefthimiou, C., Theophilidis, G., **2001**. An in vitro method for recording the electrical activity of the isolated heart of the adult *Drosophila melanogaster. In Vitro Cell. Devel. Biol. Animal 37*, 445–449.

Park, M., Wu, X., Golden, K., Axelrod, J.D., Bodmer, R., **1996**. The wingless signaling pathway is directly involved in *Drosophila* heart development. *Devel. Biol. 177*, 104–116.

Park, M., Yaich, L.E., Bodmer, R., **1998**. Mesodermal cell fate decisions in *Drosophila* are under the control of the lineage genes numb, Notch, and sanpodo. *Mech. Devel. 75*, 117–126.

Park, Y., Zitnan, D., Gill, S.S., Adams, M.E., **1999**. Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster. FEBS Lett. 463*, 133–138.

Park, Y., Kim, Y.J., Adams, M.E., **2002**. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc. Natl Acad. Sci. USA 99*, 11423–11428.

Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., Ewer, J., **2003**. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. *Development 130*, 2645–2656.

Parkes, T.L., Kirby, K., Phillips, J.P., Boulianne, G.L., **1998**. Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons. *Nat. Genet. 19*(2), 171–174.

Partridge, L., Gems, D., **2002**. Mechanisms of ageing: public or private? *Nat. Rev. Genet. 3*(3), 165–175.

Paternostro, G., Vignola, C., Bartsch, D.U., Omens, J.H., McCulloch, A.D., *et al.*, **2001**. Age-associated cardiac dysfunction in *Drosophila melanogaster. Circ. Res. 88*(10), 1053–1058.

Patten, B., **1949**. Initiation and early changes in the character of the heart beat in vertebrate embryos. *Physiol. Rev. 28*, 31–47.

Pearl, D., **1928**. The Rate Of Living. Alfred A. Knopf, New York.

Peixoto, A.A., Hall, J.C., **1998**. Analysis of temperature-sensitive mutants reveals new genes involved in courtship song of *Drosophila*. *Genetics 148*, 827–838.

Pener, M.P., **1972**. The corpus allatum in adult acridids: the inter-relation of its functions and possible correlations with the life cycle. In: Hemming, C.F., Taylor, T.H.C. (Eds.), Proceedings of the International Study Conference on the Current and Future Problems of Acridology. Centre for Overseas Pest Research, London, pp. 135–147.

Petersen, P.H., Zou, K., Hwang, J.K., Jan, Y.N., Zhong, W., **2002**. Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. *Nature 419*, 929–934.

Platt, N., Reynolds, S.E., **1985**. Cardioactive peptides from the CNS of a caterpillar, the tobacco hornworm, *Manduca Sexta*. *J. Exp. Biol. 114*, 397–414.

Pletcher, S.D., Macdonald, S.J., Marguerie, R., Certa, U., Stearns, S.C., *et al*., **2002**. Genome-wide. transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr. Biol. 12*(9), 712–723

Ponzielli, R., Astier, M., Chartier, A., Gallet, A., Therond, P., *et al*., **2002**. Heart tube patterning in *Drosophila* requires integration of axial and segmental information provided by the Bithorax Complex genes and hedgehog signaling. *Development 129*, 4509–4521.

Pratt, G.E., Farnsworth, D.E., Fok, K.F., Siegel, N.R., McCormack, A.L., *et al*., **1991**. Identity of a second type of allatostatin from cockroach brains: an octadecapeptide amide with a tyrosine-rich address sequence. *Proc. Natl Acad. Sci. USA 88*, 2412–2416.

Predel, R., Brandt, W., Kellner, R., Rapus, J., Nachman, R.J., *et al*., **1999**. Post-translational modifications of the insect sulfakinins: sulfation, pyroglutate-formation and O-methylation of glutamic acid. *Eur. J. Biochem. 263*, 552–560.

Price, M.D., Merte, J., Nichols, R., Koladich, P.M., Tobe, S.S., *et al*., **2002**. *Drosophila melanogaster* flatline encodes a myotropin orthologue to *Manduca sexta* allatostatin. *Peptides 23*, 787–794.

Prier, K.R., Beckman, O.H., Tublitz, N.J., **1994**. Modulating a modulator: biogenic amines at subthreshold levels potentiate peptide-mediated cardioexcitation of the heart of the tobacco hawkmoth *Manduca sexta*. *J. Exp. Biol. 197*, 377–391.

Promislow, D.E., Tatar, M., **1998**. Mutation and senescence: where genetics and demography meet. *Genetica 102–103*(1–6), 299–314.

Prosser, C.L., **1973**. Circulation of body fluids. In: Prosser, C.L. (Ed.), Comparative Animal Physiology, 3rd edn. Saunders, Philadelphia, pp. 835–856.

Prosser, C., Brown, F., Jr., **1961**. Comparative Animal Physiology, 2nd edn. Little and Brown, New York.

Quan, F., Wolfgang, W.J., Forte, M., **1993**. A *Drosophila* G-protein alpha subunit, Gf alpha, expressed in a spatially and temporally restricted pattern during *Drosophila* development. *Proc. Natl Acad. Sci. USA 90*, 4236–4240.

Quinn, W.G., Sziber, P.P., Booker, R., **1979**. The *Drosophila* memory mutant *amnesiac*. *Nature 277*, 212–214.

Radford, J.C., Davies, S.A., Dow, J.A., **2002**. Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem. 277*, 38810–38817.

Ramain, P., Heitzler, P., Haenlin, M., Simpson, P., **1993**. Pannier, a negative regulator of achaete and scute in *Drosophila*, encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. *Development 119*, 1277–1291.

Reale, V., Hannan, F., Midgley, J.M., Evans, P.D., **1997**. The expression of a cloned *Drosophila* octopamine/tyramine receptor in *Xenopus* oocytes. *Brain Res. 769*, 309–320.

Reed, B.H., Wilk, R., Lipshitz, H.D., **2001**. Downregulation of Jun kinase signaling in the amnioserosa is essential for dorsal closure of the *Drosophila* embryo. *Curr. Biol. 11*, 1098–1108.

Reenan, R.A., Hanrahan, C.J., Ganetzky, B., **2000**. The mle^{napts} RNA helicase mutation in *Drosophila* results in a splicing catastrophe of the *para* Na^+ channel transcript in a region of RNA editing. *Neuron 25*, 139–149.

Reim, I., Lee, H.H., Frasch, M., **2003**. The T-box-encoding Dorsocross genes function in amnioserosa development and the patterning of the dorsolateral germ band downstream of Dpp. *Development 130*, 3187–3204.

Restifo, L., White, K., **1990**. Molecular and genetic approaches to neurotransmitter and neuromodulator systems in *Drosophila*. *Adv. Insect Physiol. 22*, 115–219.

Reveillaud, I., Niedzwiecki, A., Bensch, K.G., Fleming, J.E., **1991**. Expression of bovine superoxide dismutase in *Drosophila melanogaster* augments resistance of oxidative stress. *Mol. Cell. Biol. 11*(2), 632–640.

Rhyu, M.S., Jan, L.Y., Jan, Y.N., **1994**. Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell 76*, 477–491.

Richer, S., Stoffolano, J.G., Jr., Yin, C.M., Nichols, R., **2000**. Innervation of dromyosuppressin (DMS) immunoreactive processes and effect of DMS and benzethonium chloride on the *Phormia regina* (Meigen) crop. *J. Comp. Neurol. 421*, 136–142.

Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R., Leptin, M., **1997**. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development 124*, 2915–2922.

Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., **2002**. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science 298*, 172–175.

Rigg, L., Heath, B., Cui, Y., Terrar, D., **2000**. Localisation and functional significance of ryanodine receptors during β-adrenoceptor stimulation in the guinea-pig sinoatrial node. *Cardiovasc. Res. 48*, 254–264.

Rigg, L., Terrar, D., **1996**. Possible role of calcium release from the sarcoplasmic reticulum in pacemaking in guinea-pig sino-atrial node. *Exp. Physiol. 81*, 877–880.

Rizki, T., **1978**. The circulatory system and associated cells and tissues. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*. Academic Press, London, pp. 1839–1845.

Rogina, B., Helfland, S.L., **1995**. Regulation of gene expression is linked to life span in adult *Drosophila*. *Genetics 141*(3), 1043–1048.

Rogina, B., Reenan, R.A., Nilsen, S.P., Helfland, S.L., **2000**. Extended life-span conferred by cotransporter gene mutations in *Drosophila*. *Science 290*(5499), 2137–2140.

Rogina, B., Vaupel, J.W., Partridge, L., Helfland, S.L., **1998**. Regulation of gene expression is preserved in aging *Drosophila melanogaster*. *Curr. Biol. 8*(8), 475–478.

Roman, G., He, J., Davis, R.L., **2000**. *kurtz*, a novel nonvisual arrestin, is an essential neural gene in *Drosophila*. *Genetics 155*, 1281–1295.

Rosay, P., Armstrong, J.D., Wang, Z., Kaiser, K., **2001**. Synchronized neural activity in the *Drosophila* memory centers and its modulation by *amnesiac*. *Neuron 30*, 759–770.

Rosenblatt, K., Sun, Z., Heller, S., Hudspeth, A., **1997**. Distribution of Ca^{2+}-activated K^+ channels along the tonotopic gradient of the chicken's cochlea. *Neuron 19*, 1061–1075.

Rosenthal, N., Harvey, R.P., **1999**. In: Rosenthal, N., Harvey, R. (Eds.), Heart Development. Academic Press, San Diego, pp. 65–90.

Rothstein, M., Coppens, M., Sharma, H.K., **1980**. Effect of aging on enolase from rat muscle, liver and heart. *Biochim. Biophys. Acta 614*(2), 591–600.

Rousseau, E., Smith, J.S., Meissner, G., **1987**. Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *Am. J. Physiol. 253*, C364–C368.

Rudwall, A.J., Sliwowska, J., Nässel, D.R., **2000**. Allatotropin-like neuropeptide in the cockroach abdominal nervous system: myotropic actions, sexually dimorphic distribution and colocalization with serotonin. *J. Comp. Neurol. 428*, 159–173.

Rugendorff, A., Younossi-Hartenstein, A., Hartenstein, V., **1994**. Embryonic origin and differentiation of the *Drosophilia* heart. *Roux's Arch. Devel. Biol. 203*, 266–280.

Salkoff, L., Wyman, R., **1981**. Genetic modification of potassium channel mutants in *Drosophila Shaker* mutants. *Nature 293*, 228–230.

Sanger, J.W., McCann, F.V., **1968a**. Ultrastructure of the myocardium of the moth, *Hyalophora cecropia*. *J. Insect Physiol. 14*, 1105–1111.

Sanger, J.W., McCann, F.V., **1968b**. Ultrastructure of the alary muscles and their attachment to the heart wall. *J. Insect Physiol. 14*, 1539–1544.

Saudou, F., Amlaiky, N., Plassat, J-L., Borelli, E., Hen, R., **1990**. Cloning and characterization of a *Drosophila* tyramine receptor. *EMBO J. 9*, 3611–3617.

Sanguinetti, M., Curran, M., Spector, P., Keating, M., **1996**. Spectrum of HERG K^+-channel dysfunction in an inherited cardiac arrhythmia. *Proc. Natl Acad. Sci. USA 93*, 2208–2212.

Sanguinetti, M., Jiang, C., Curran, M., Keeting, M., **1995**. A mechanistic link between an inherited and an acquired cardiac arrythmia: *HERG* encodes the I_{kr} potassium channel. *Cell 81*, 299–307.

Schilcher, F.V., **1976**. The behavior of *cacophony*, a courtship song mutant in *Drosophila melanogaster*. *Behav. Biol. 17*, 187–196.

Schilcher, F., **1977**. A mutation which changes courtship song in *Drosophila melanogaster*. *Behav. Genet. 7*, 251–259.

Schoofs, L., Holman, G.M., Paemen, L., Veelaert, D., Amelinckx, M., *et al.*, **1993**. Isolation, identification, and synthesis of PDVDHFLRFamide (SchistoFLRFamide) in *Locusta migratoria* and its association with the male accessory glands, the salivary glands, the heart, and the oviduct. *Peptides 14*, 409–421.

Schoofs, L., Veelaert, D., Broeck, J.V., De Loof, A., **1996**. Immunocytochemical distribution of locustamyoinhibiting peptide (Lom-MIP) in the nervous system of *Locusta migratoria*. *Regul. Pept. 63*, 171–179.

Seidel, F., Bock, E., Krause, G., **1940**. Die organisation des insekteneies. *Naturwissenschaften 28*, 434–447.

Sepulveda, J.L., Belaguli, N., Nigam, V., Chen, C., Nemer, M., *et al.*, **1998**. GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol. Cell. Biol. 18*, 3405–3415.

Sepulveda, J.L., Vlahopoulos, S., Iyer, D., Belaguli, N., Schwartz, R.J., **2002**. Combinatorial expression of GATA4, Nkx2–5, and serum response factor directs early cardiac gene activity. *J. Biol. Chem. 277*, 25775–25782.

Seto, N.O., Hayashi, S., Tener, G.M., **1990**. Overexpression of Cu-Zn superoxide dismutase in *Drosophila* does not affect life-span. *Proc. Natl Acad. Sci. USA 87*(11), 4270–4274.

Sgro, C.M., Partridge, L., **1999**. A delayed wave of death from reproduction in *Drosophila*. *Science 286*(5449), 2521–2524.

Shishido, E., Higashijima, S.-I., Emori, Y., Saigo, K., **1993**. Two FGF-receptor homologues of *Drosophila*: one is expressed in mesodermal primordium in early embryos. *Development 117*, 751–761.

Shishido, E., Ono, N., Kojima, T., Saigo, K., **1997**. Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development 124*, 2119–2128.

Shpetner, H.S., Vallee, R.B., **1992**. Dynamin is a GTPase stimulated to high levels of activity by microtubules. *Nature 355*, 733–735.

Siegmund, T., Korge, G., **2001**. Innervation of the ring gland of *Drosophila melanogaster*. *J. Comp. Neurol. 431*, 481–491.

Sigrist, S.J., Lehner, C.F., **1997**. *Drosophila* fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell 90*, 671–681.

Simon, A.F., Shih, C., Mack, A., Benzer, S., **2003**. Steroid control of longevity in *Drosophila melanogaster*. *Science 299*(5611), 1407–1410.

Simpson, P., **1983**. Maternal-zygotic gene interactions involving the dorsal-ventral axis in *Drosophila* embryos. *Genetics 105*, 615–632.

Singh, S., Wu, C.-F., **1989**. Complete separation of four potassium currents in *Drosophila*. *Neuron 2*, 1325–1329.

Siviter, R.J., Coast, G.M., Winther, A.M., Nachman, R.J., Taylor, C.A., *et al.*, **2000**. Expression and functional characterization of a *Drosophila* neuropeptide precursor with homology to mammalian preprotachykinin A. *J. Biol. Chem. 275*, 23273–23280.

Skeath, J.B., Doe, C.Q., **1998**. Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the *Drosophila* CNS. *Development 125*, 1857–1865.

Sliwowska, J., Rosinski, G., Nässel, D.R., **2001**. Cardioacceleratory action of tachykinin-related neuropeptides and proctolin in two coleopteran insect species. *Peptides 22*, 209–217.

Smith, N., **1969**. Observations on the neural rhythmicity in the cockroach. *Experientia (Suppl.) 15*, 200–205.

Smith, L.A., Wang, X., Peixoto, A., Neumann, E., Hall, L., *et al.*, **1996**. A *Drosophila* calcium channel α-1 subunit gene maps to a genetic locus associated with behavioral and visual defects. *J. Neurosci. 16*, 7868–7879.

Smits, A.W., Burggren, W., Oliveras, D., **2000**. Developmental changes in *in vivo* cardiac performance in the moth *Manduca Sexta*. *J. Exp. Biol. 203*, 369–378.

Sohal, R.S., **1982**. Oxygen consumption and life span in the adult male housefly, *musca* domestica. *Aging 5*, 21–24.

Sohal, R.S., **2002**. Oxidative stress hypothesis of aging. *Free Radic. Biol. Med. 33*(5), 573–574.

Sohal, R.S., Agarwal, S., Dubey, A., Orr, W.C., **1993**. Protein oxidative damage is associated with life expectancy of houseflies. *Proc. Natl Acad. Sci. USA 90*(15), 7255–7259.

Sohal, R.S., Sohal, B.H., Orr, W.C., **1995**. Mitochondrial superoxide and hydrogen peroxide generation, protein oxidative damage, and longevity in different species of flies. *Free Radic. Biol. Med. 19*(4), 499–504.

Spana, E.P., Doe, C.Q., **1996**. Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron 17*, 21–26.

Spana, E.P., Kopczynski, C., Goodman, C.S., Doe, C.Q., **1995**. Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. *Development 121*, 3489–3494.

Spencer, C.C., Howell, C.E., Wright, A.R., Promislow, D.E., **2003**. Testing an ‘aging gene’ in long-lived *Drosophila* strains: increased longevity depends on sex and genetic background. *Aging Cell 2*, 123–130.

Spencer, F.A., Hoffmann, F.M., Gelbart, W.M., **1982**. Decapentaplegic: a gene complex affecting morphogenesis in *Drosophila melanogaster. Cell 28*, 451–461.

St. Johnston, D., Nusslein-Volhard, C., **1992**. The origin of pattern and polarity in the *Drosophila* embryo. *Cell 68*, 201–219.

Stadtman, E.R., **1992**. Protein oxidation and aging. *Science 257*(5074), 1220–1224.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., Bate, M., **1994**. dpp induces mesodermal gene expression in *Drosophila*. *Nature 372*, 783–786.

Stangier, J., Hilbich, C., Dircksen, H., Keller, R., **1988**. Distribution of a novel cardioactive neuropeptide (CCAP) in the nervous system of the shore crab *Carcinus maenas*. *Peptides 9*, 795–800.

Staubli, F., Jorgensen, T.J., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99*, 3446–3451.

Stennard, F.A., Costa, M.W., Elliott, D.A., Rankin, S., Haast, S.J., *et al.*, **2003**. Cardiac T-box factor Tbx20 directly interacts with Nkx2–5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Devel. Biol. 262*, 206–224.

Struhl, G., Basler, K., **1993**. Organizing activity wingless protein in *Drosophila*. *Cell 72*, 527–540.

Su, M.T., Fujioka, M., Goto, T., Bodmer, R., **1999**. The *Drosophila* homeobox genes zfh-1 and even-skipped are required for cardiac-specific differentiation of a numb-dependent lineage decision. *Development 126*, 3241–3251.

Sullivan, K., Scott, K., Zuker, C., Rubin, G., **2000**. The ryanodine receptor is essential for larval development in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 97*, 5492–5497.

Sun, J., Tower, J., **1999**. FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster* flies. *Mol. Cell. Biol. 19*(1), 216–228.

Suzuki, D.T., Grigliatti, T., Williamson, R., **1971**. Temperature-sensitive mutations in *Drosophila melanogaster*, VII. *A mutation (para^{ts})* causing a reversible adult paralysis. *Proc. Natl Acad. Sci. USA 68*, 890–893.

Taghert, P.H., Veenstra, J.A., **2003**. *Drosophila* neuropeptide signaling. *Adv. Genet. 49*, 1–65.

Talluri, S., Bhatt, A., Smith, D.P., **1995**. Identification of a *Drosophila* G protein alpha subunit (dGq alpha-3) expressed in chemosensory cells and central neurons. *Proc. Natl Acad. Sci. USA 92*, 11475–11479.

Tatar, M., Bartke, A., Antebi, A., **2003**. The endocrine regulation of aging by insulin-like signals. *Science 299*, 1346–1351.

Tatar, M., Kopelman, A., Epstein, D., Tu, M-P., Yin, C.M., *et al.*, **2001**. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science 292*(5514), 107–110.

Tatar, M., Yin, C., **2001**. Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. *Exp. Gerontol. 36*(4–6), 723–738.

Tawfik, A.I., Tanaka, S., De Loof, A., Schoofs, L., Baggerman, G., *et al.*, **1999**. Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant. *Proc. Natl Acad. Sci. USA 96*, 7083–7087.

Taylor, R.E., **1959**. Effects of procaine on electrical properties of squid axon membrane. *Am. J. Physiol. 196*, 1071–1078.

Tempel, B.L., Livingstone, M.S., Quinn, W.G., **1984**. Mutations in the dopa decarboxylase gene affect learning in *Drosophila. Proc. Natl Acad. Sci. USA 81*, 3577–3581.

Terrar, D., Rigg, L., **2000**. What determines the initiation of the heartbeat? *J. Physiol. 524*, 316.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., Perrin-Schmitt, F., **1988**. Sequence of the twist gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J. 7*, 2175–2183.

Thisse, B., Stoetzel, C., Messal, M.E., Perrin-Schmitt, F., **1987**. Genes of the *Drosophila* dorsal group control the specific expression of the zygotic gene twist in presumptive mesodermal cells. *Genes Devel. 1*, 709–715.

Tierney, A.J., **2001**. Structure and function of invertebrate 5-HT receptors: a review. *Comp. Biochem. Physiol. A Mol. Integr. Physiol. 128*, 791–804.

Torfs, H., Oonk, H.B., Broeck, J.V., Poels, J., Van Poyer, W., *et al.*, **2001**. Pharmacological characterization of STKR, an insect G protein-coupled receptor for tachykinin-like peptides. *Arch. Insect. Biochem. Physiol. 48*, 39–49.

Torrey, T.W., Feduccia, A., **1979**. Morphogenesis of the Vertebrates. Wiley, New York.

Trautwein, W., Osterreider, W., **1986**. In: Nathan, R.D. (Ed.), Cardiac Muscle: the Regulation of Excitation and Contraction. Academic Press, Orlando, pp. 87–128.

Truman, J., Ewer, J., Ball, E., **1996**. Dynamics of cyclic GMP levels in identified neurones during ecdysis behaviour in the locust *Locusta migratoria*. *J. Exp. Biol. 199*, 749–758.

Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M., Evans, R.M., **1999**. SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Mol. Cell 4*(2), 175–186.

Tublitz, N., Allen, A., Cheung, C., Edwards, K., Kimble, D., *et al.*, **1992**. Insect cardioactive peptides: regulation of hindgut activity by cardioacceleratory peptide 2 (CAP2) during wandering behaviour in *Manduca sexta* larvae. *J. Exp. Biol. 165*, 241–277.

Tublitz, N., **1988**. Insect cardioactive neuropeptides: peptidergic modulation of the intrinsic rhythm of an insect heart is mediated by inositol 1,4,5-trisphosphate. *J. Neurosci. 8*, 4394–4399.

Tublitz, N., **1989**. Insect cardioactive peptides: neurohormonal regulation of cardiac activity by two cardioacceleratory peptides during flight in the tobacco hawkmoth, *Manduca sexta*. *J. Exp. Biol. 142*, 31–48.

Tully, T., Quinn, W.G., **1985**. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol. A 157*, 263–277.

Ude, J., Agricola, H., **1995**. FMRFamide-like and allatostatin-like immunoreactivity in the lateral heart nerve of *Periplaneta americana*: colocalization at the electron microscopic level. *Cell Tissue Res. 282*, 69–80.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., **1989**. Numb, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell 58*, 349–360.

Ulrych, T., Bishop, T., **1975**. Maximum entropy spectral analysis and autoregressive decomposition. *Rev. Geophys. Space Phys. 13*, 183–200.

Ultsch, A., Schuster, C.M., Laube, B., Schloss, P., Schmitt, B., *et al.*, **1992**. Glutamate receptors of *Drosophila melanogaster* cloning of a kainate-selective subunit expressed in the central nervous system. *Proc. Natl Acad. Sci. USA 89*, 10484–10488.

Van der Bliek, A., Meyerowitz, E.M., **1991**. Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature 351*, 411–414.

Vanden Broeck, J., **2001**. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster. Peptides 22*, 241–254.

Van Ginneken, A., Giles, W., **1991**. Voltage clamp measurement of the hyperpolarization-activated inward current I_f in single cells from rabbit sino-atrial node. *J. Physiol. 434*, 57–83.

Veelaert, D., Passier, P., Devreese, B., Vanden Broeck, J., Van Beeumen, J., *et al.*, **1997**. Isolation and characterization of an adipokinetic hormone release-inducing factor in locusts: the crustacean cardioactive peptide. *Endocrinology 138*, 138–142.

Veenstra, J., **1994**. Isolation and structure of the *Drosophila* corazonin gene. *Biochem. Biophys. Res. Commun. 204*, 292–296.

Veenstra, J.A., Lehman, H.K., Davis, N.T., **1994**. Allatotropin is a cardioacceleratory peptide in *Manduca sexta*. *J. Exp. Biol. 188*, 347–354.

Venkatesh, T.V., Park, M., Ocorr, K.A., Nemacheck, J.A., Golden, K., *et al.*, **2000**. Cardiac enhancer activity of the homeobox gene tinman depends on CREB consensus binding sites in *Drosophila*. *Genesis 26*, 55–66.

Verdi, J.M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., *et al.*, **1996**. Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies cell fate. *Curr. Biol. 6*, 1134–1145.

Vervoort, M., Dambly-Chaudiere, C., Ghysen, A., **1997**. Cell fate determination in *Drosophila*. *Curr. Opin. Neurobiol. 7*, 21–28.

Vilaplana, L., Maestro, J.L., Piulachs, M., Belles, X., **1999**. Modulation of cardiac rhythm by allatostatins in the cockroach *Blattella germanica*. *J. Insect Physiol. 45*, 1057–1064.

Wang, X., Reynolds, E., Déak, P., Hall, L., **1997**. The *seizure* locus encodes the *Drosophila* homolog of the HERG potassium channel. *J. Neurosci. 17*, 882–890.

Wang, J., Zhou, Y., Wen, H., Levitan, I.B., **1999**. Simultaneous binding of two protein kinases to a calcium-dependent potassium channel. *J. Neurosci. 19*, RC4.

Ward, E.J., Skeath, J.B., **2000**. Characterization of a novel subset of cardiac cells and their progenitors in the *Drosophila* embryo. *Development 127*, 4959–4969.

Warmke, J., Drysdale, R., Ganetzky, B., **1991**. A distinct potassium channel polypeptide encoded by the *Drosophila eag* locus. *Science 252*, 1560–1562.

Warmke, J., Ganetzky, B., **1994**. A family of potassium channel genes related to eag in *Drosophila* and in mammals. *Proc. Natl Acad. Sci. USA 91*, 3438–3442.

Wasserthal, L.T., **1981**. Oscillating haemolymph 'circulation' and discontinuous tracheal ventilation in the giant

silk moth *Attacus atlas* L. *J. Comp. Physiol. B 145*, 1–15.
Wasserthal, L.T., **1996**. Interaction of circulation and tracheal ventilation in holometabolous insects. *Adv. Insect Physiol. 26*, 297–351.
Watson, D.G., Zhou, P., Midgley, J.M., Milligan, C.D., Kaiser, K., **1993**. The determination of biogenic amines in four strains of the fruit fly *Drosophila melanogaster. J. Pharm. Biomed. Anal. 11*, 1145–1149.
Wegener, C., Nässel, D.R., **2000**. Peptide-induced Ca(2+) movements in a tonic insect muscle: effects of proctolin and periviscerokinin. *J. Neurophysiol. 84*, 3056–3066.
Wessells, R., Bodmer, R., **2004**. Screening assays for heart function mutants in *Drosophila*. *BioTechniques 37*, 2–7.
Wheeler, J.C., Bieschke, E.T., Tower, J., **1995**. Muscle-specific expression of *Drosophila* hsp70 in response to aging and oxidative stress. *Proc. Natl Acad. Sci. USA 92*(22), 10408–10412.
White, L., Ringo, J., Dowse, H., **1992**. Effects of deuterium oxide and temperature on heart rate in *Drosophila melanogaster. J. Comp. Physiol. B. 162*, 278–283.
Wiggelsworth, V., **1965**. Principles of Insect Physiology, 6th Edn. Methuen, New York.
Williamson, M., Lenz, C., Winther, A.M., Nässel, D.R., Grimmelikhuijzen, C.J., **2001a**. Molecular cloning, genomic organization, and expression of a C-type (*Manduca sexta*-type) allatostatin preprohormone from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 282*, 124–130.
Williamson, M., Lenz, C., Winther, A.M., Nässel, D.R., Grimmelikhuijzen, C.J., *et al.*, **2001b**. Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 281*, 544–550.
Winick, J., Abel, T., Leonard, M.W., Michelson, A.M., Chardon-Loriaux, I., *et al.*, **1993**. A GATA family transcription factor is expressed along the embryonic dorsoventral axis in *Drosophila melanogaster. Development 119*, 1055–1065.
Winther, A.M., Lundquist, C.T., Nassel, D.R., **1996**. Multiple members of the leucokinin neuropeptide family are present in cerebral and abdominal neurohemal organs in the cockroach *Leucophaea maderae. J. Neuroendocrinol. 8*, 785–792.
Wolfgang, W.J., Quan, F., Goldsmith, P., Unson, C., Spiegel, A., *et al.*, **1990**. Immunolocalization of G protein alpha-subunits in the *Drosophila* CNS. *J. Neurosci. 10*, 1014–1024.
Woodhead, A.P., Stay, B., Seidel, S.L., Khan, M.A., Tobe, S.S., **1989**. Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA 86*, 5997–6001.
Wu, C.-F., Ganetzky, B., Haugland, B., **1983**. Potassium currents in *Drosophila*: different components affected by mutations of two genes. *Science 220*, 1076–1078.
Wu, C.-F., Ganetzky, B., Jan, Y., Jan, L., Benzer, S., **1978**. A *Drosophila* mutant with a temperature-sensitive block in nerve conduction. *Proc. Natl Acad. Sci. USA 75*, 4047–4051.
Wu, X., Golden, K., Bodmer, R., **1995**. Heart development in *Drosophila* requires the segment polarity gene wingless. *Devel. Biol. 169*, 619–628.
Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. In: Evans, P.D. (Ed.), Advances Insect Physiology. Academic Press, San Diego, pp. 1–155.
Xu, X., Yin, Z., Hudson, J.B., Ferguson, E.L., Frasch, M., **1998**. Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. *Genes Devel. 12*, 2354–2370.
Xu, Y., Fang, F., Chu, Y., Jones, D., Jones, G., **2002**. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle. *Eur. J. Biochem. 269*(24), 6026–6036.
Yan, L.J., Levine, R.L., Sohal, R.S., **1997**. Oxidative damage during aging targets mitochondrial aconitase. *Proc. Natl Acad. Sci. USA 94*(21), 11168–11172.
Yan, L.J., Sohal, R.S., **1998**. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc. Natl Acad. Sci. USA 95*(22), 12896–12901.
Yan, L.J., Sohal, R.S., **2000**. Effects of aging and hyperoxia on oxidative damage to cytochrome c in the housefly, Musca domestica. *Free Radic. Biol. Med. 29*(1), 90–97.
Yin, Z., Xu, X.-L., Frasch, M., **1997**. Regulation of the Twist target gene tinman by modular cis-regulatory elements during early mesoderm development. *Development 124*, 4971–4982.
Zaffran, S., Frasch, M., **2002**. Early signals in cardiac development. *Circ. Res. 91*, 457–469.
Zahavi, M., Tahori, A.S., **1965**. Citric acid accumulation with age in houseflies and other Diptera. *J. Insect. Physiol. 11*, 811–816.
Zhang, S., Bernstein, S.I., **2001**. Spatially and temporally regulated expression of myosin heavy chain alternative exons during *Drosophila* embryogenesis. *Mech. Devel. 101*, 35–45.
contribution>Zheng, Y., Hirschberg, B., Yuan, J., Wang, A.P., Hunt, D.C., *et al.*, **2002**. Identification of two novel *Drosophila melanogaster* histamine-gated chloride channel subunits expressed in the eye*J. Biol. Chem. 277*, 2000–2005.
Zhong, W., Feder, J.N., Jiang, M.M., Jan, L.Y., Jan, Y.N., **1996**. Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. *Neuron 17*, 43–53.
Zhong, W., Jiang, M.M., Weinmaster, G., Jan, L.Y., Jan, Y.N., **1997**. Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. *Development 124*, 1887–1897.
Zhong, Y., Wu, C.-F., **1991**. Alteration of four identified K^+ currents in *Drosophila* muscle by mutation in *eag*. *Science 252*, 1562–1564.

Zhou, Y., Wang, J., Wen, H., Kucherovsky, O., Levitan, I.B., **2002**. Modulation of *Drosophila* slowpoke calcium-dependent potassium channel activity by bound protein kinase a catalytic subunit. *J. Neurosci. 22*, 3855–3862.

Zikova, M., Da Ponte, J.P., Dastugue, B., Jagla, K., **2003**. Patterning of the cardiac outflow region in *Drosophila*. *Proc. Natl Acad. Sci. USA 100*, 12189–12194.

Zitnan, D., Sehnal, F., Bryant, P.J., **1993**. Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed *Drosophila*. *Devel. Biol. 156*, 117–135.

Zornik, E., Paisley, K., Nichols, R., **1999**. Neural transmitters and a peptide modulate *Drosophila* heart rate. *Peptides 20*, 45–51.

2.7 Tracheal System Development and Morphogenesis

A E Uv, University of Gothenburg, Gothenburg, Sweden
C Samakovlis, Stockholm University, Stockholm, Sweden

2.7.1. Introduction

Multicellular organisms have evolved specialized tubular organs to transport gases and liquids throughout the body. In vertebrates, such organs include the lung, the vascular system, and the kidney. Respiration in insects is mainly facilitated by an extensive tubular network, the trachea. A common feature of these tissues is their branched tubular structure, where epithelial cells are used as building-blocks. Morphogenesis of elaborate tubular networks has received much attention over the last decade, as it involves intriguing developmental questions: How do cells create tubes? How do new branches arise? What determines the direction of branch growth? How are the specific branch lengths and diameters determined? The respiratory organ of the fruit fly *Drosophila melanogaster* is an appreciated and thoroughly studied model organ for insect tracheal development and general branching morphogenesis. Here we aim to describe the main results from this research, which begins to highlight the basic molecular mechanisms leading to the development of a branched tubular organ.

The insect trachea is an air-filled network of tubes that spans the entire organism (**Figure 1a**). Air enters the network through specialized openings, called spiracles, and becomes distributed to all branches of the network, ending in narrow capillary-like blind-ended tubes, which facilitate gas exchange with target tissues. Tracheal tubes are void of contractile muscle cells, and simply consist of a single-layered epithelium with the apical cell surface facing the lumen. In order to optimize air supply, the tracheal branches must extend into all tissues in the animal, and individual tubes have to attain specific dimensions. This is achieved by a two-step developmental process: first, a stereotyped larval tracheal network forms during embryogenesis, controlled by a strict genetic branching program (**Figure 1b** and c). Later, during larval growth, the network expands by the enlargement of the preexisting branches and ramification of the blind-ended capillaries that infiltrate new targets to satisfy the metabolic demands.

During metamorphosis, the tracheal branches become reorganized, where most cells of the larval trachea are histolyzed and a new tracheal system is formed from imaginal tracheoblasts (Manning and Krasnow, 1993). As most information on tracheogenesis has come from studies of larval trachea

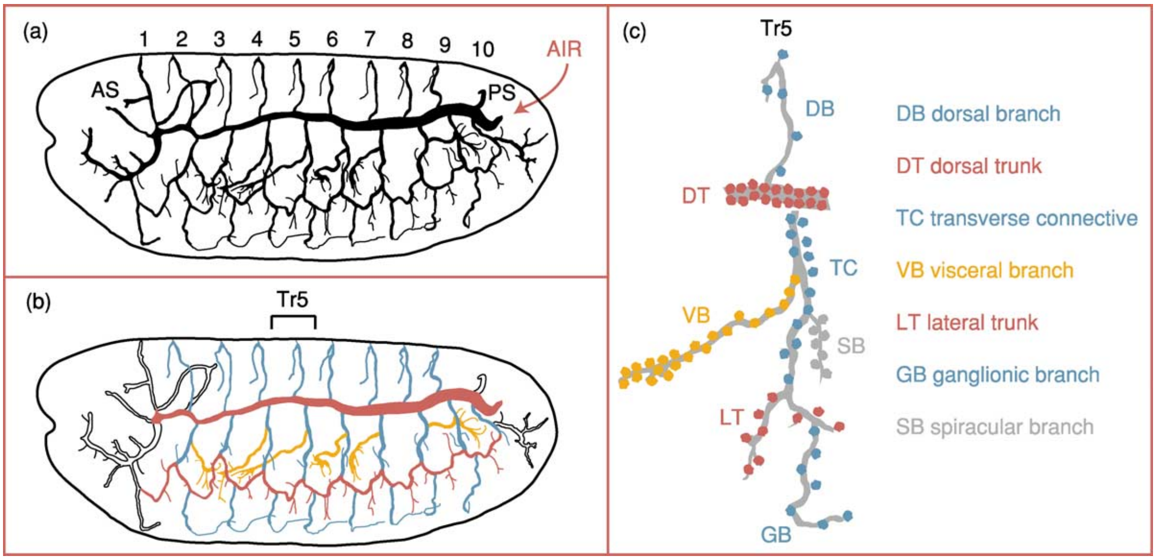


Figure 1 The *Drosophila* tracheal system. At late embryonic stage 16, the tracheal system is a fully branched network that will fulfill the respiratory demands of the newly hatched larvae. (a) Lateral view of the embryonic tracheal system (lumen) where dorsal is up and anterior is to the left. During larval life, air enters the posterior spiracles (PS) and becomes distributed to the gradually narrower branches, to reach the blind-ended capillaries for gas exchange with target tissues. The network is composed of 20 independently developed and fused tracheal metameres, 10 on each side the embryo (Tr1–Tr10), which are indicated by the numbers. (b) The main branches in Tr2–Tr9 are indicated by different colors of branch lumens. (c) The nomenclature for the main branches of a typical metamere (Tr5). Each metamere is composed of ~80 cells and displays a stereotyped branch patterning with characteristic numbers and positions of cells in each branch (circles represent nuclei, whose colors match those of the branch definitions in (b)). (Reproduced from Samakovlis, C., Manning, G., Steneberg, P., Hacohen, N., Cantera, R., *et al.*, **1996b**. Genetic control of epithelial tube fusion during *Drosophila* tracheal development. *Development 122*, 3531–3536.)

during embryogenesis, we focus our discussion on these aspects of tracheal development.

2.7.2. Histological Description of the Larval Tracheal System

The fully developed larval trachea consists of a continuous single-layered squamous epithelium of ~1600 cells, with the apical cell surface facing the lumen and the basal surface facing the internal tissues. The luminal surface is lined by a cuticle, which is secreted by the tracheocytes and is thought to function as a barrier against dehydration and invading microorganisms. Both the tracheal epithelium and its apical lining are continuous with the epidermis and the epidermal cuticle through the spiracular branches. A basal lamina surrounding the outer tube surface is also secreted, at least in part, by the tracheal cells.

Each tracheal cell is tightly connected to its neighboring cells, via intercellular junctions, along their lateral surfaces (Tepass and Hartenstein, 1994). The "zonula adherens" (apical junction) is located near the apical (luminal) surface, whereas pleated septate junctions are found at the lateral cell surface providing a diffusion barrier across the epithelium (**Figure 2**). Although gap junctions are only sporadically detected in the tracheal epithelium by electron microscopy (Tepass and Hartenstein,1994), recent experiments show that several gap junction proteins (innexins) are persistently expressed in the tracheal cells (Stebbings *et al.*, 2002).

How do epithelial cells create tubular structures? Studies of the larval trachea have revealed four different tubular designs, each of them associated with a distinct cellular architecture and a certain range in diameter and length (Samakovlis *et al.*, 1996a; Beitel and Krasnow, 2000) (**Figure 2**). Type I tubes (represented by the dorsal trunk) are composed of flattened, wedge-shaped cells connected by intercellular epithelial cell junctions. In a cross-section of the dorsal trunk, two to four cells encircle the lumen and provide a tube diameter of 4–5 μm. The second tube type (type II) is also multicellular, but narrower (~1 μm in diameter), and is represented by the dorsal, visceral, and ganglionic branches. Type II branches are formed by cells that are rolled up and sealed by autocellular junctions. Each tube-shaped cell is connected to its proximal and distal neighbors by intercellular junctions. Type III tubes are represented by the bicellular fusion-branches (anastomoses) that connect the individual metameres to a network. These tubes consist of a

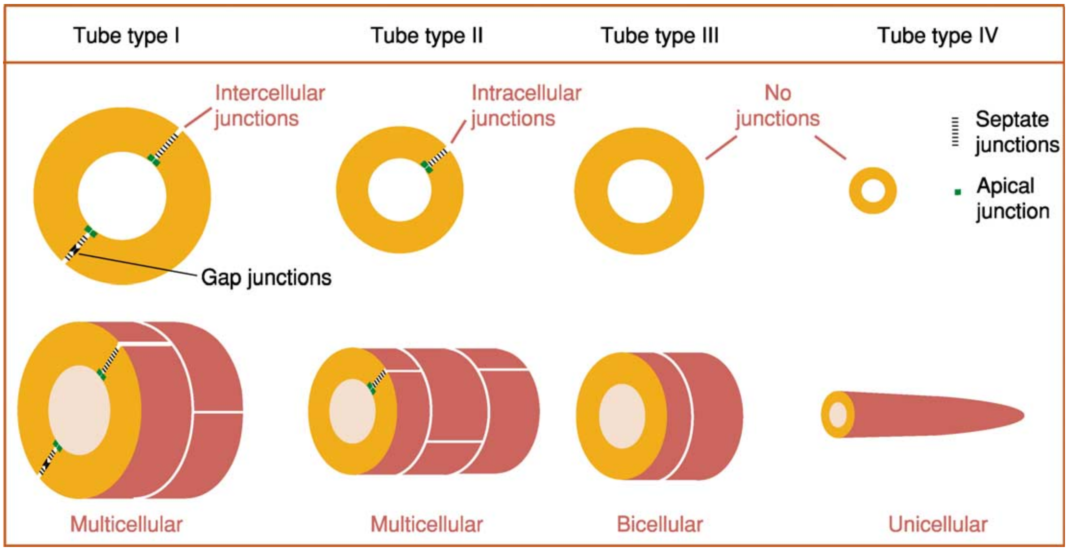


Figure 2 Cellular architecture of tracheal branches. Four structurally distinct tracheal tube types, termed I, II, III, and IV, have been described in the tracheal system. Top panels illustrate cross-sections of the different tube types, and their cellular architectures are drawn below. Type I: the main airways (4–5 μm diameter) are multicellular branches built from wedge-shaped cells surrounding the lumen circumference. Type II: air distribution to dorsal and ventral regions proceeds via narrower (∼1 μm) multicellular branches in which single tube-shaped cells with autocellular junctions encircle the lumen. The cells lie in a row and form intercellular junctions to proximal and distal cells within the branch. Type III: bicellular fusion branches (anastomoses) connect the individual metameres and are made by two cells. Each fusion cell is doughnut-shaped with no autocellular junctions and forms intercellular junctions with its fusion partner (and with the following cell in the primary branch). The type III lumen diameter is the same as that of the primary branches they connect, and varies between 0.5 and 5 μm. Type IV: terminal branches mediate gas exchange with target tissues and are made up of seamless (without autocellular junctions) tubular protrusions (<0.5 μm diameter) from single cells. Their large surface area and thin walls facilitate gas diffusion.

pair of doughnut-shaped cells, connected by intercellular junctions. In contrast to the cells of the narrow multicellular type II branches that fold over themselves, type III tubes are seamless, without intracellular junctions, and their diameter varies from 5 to 0.5 μm, depending on which of the primary branches they connect. Type IV tubes are represented by the blind-ended tracheoles. These branches are extended, hollow protrusions emanating from a single terminal cell, and become increasingly narrower along their length (less than 0.5 μm). A single terminal cell can form up to three capillaries during embryogenesis and many more during larval life, depending on the tissue to which it is attached to (see Section 2.7.3.3). For comparison to other tubular organs, the first tube type is found in lungs, blood vessels, and many glandular organs, whereas seamless tubes and tubes with autocellular junctions have been found in the vertebrate vasculature (Wolff and Bar, 1972) and the *Caenorhabditis elegans* excretory cell (Buechner, 2002).

2.7.3. Embryonic Tracheal Development: Morphogenetic Events

The metameric structure of the embryonic trachea (**Figure 1**) reflects its developmental origin, as it originates from 20 independent units with similar branching patterns, 10 on each side of the embryo (Samakovlis *et al*., 1996a). Each tracheal metameric unit (named Tr1–Tr10) is initially an epithelial pocket formed by invagination of epidermal cells during stage 10, and contains approximately 80 cells. The cells are oriented with their apical membrane lining the lumen and their basal cell surface facing the developing embryonic tissues. Over the next 12 h (until the end of embryogenesis) and without further cell division, the cells in each of these pockets undergo a synchronized program of branch outgrowth and branch fusion to generate a continuous tubular system (Samakovlis *et al*., 1996a). The branching process involves most aspects of epithelial morphogenesis, with extensive but highly regulated changes in cell shape and cell movements, and is divided into the following phases: invagination, primary branch formation, secondary branch formation, terminal branch formation, and branch fusion. Throughout these phases, the developing tracheal epithelium maintains its apical basal polarity and barrier function, and secretes luminal components. Just before larval hatching, the filamentous material filling the tubes during embryogenesis is cleared, and the tracheal system becomes functional as air fills the system and the posterior spiracles open up.

In this section, the different phases of embryonic tracheogenesis are discussed, focusing on tracheal branch morphology and the cellular events that underlie formation of the different branches. The different developmental stages are illustrated by tracheal luminal staining (**Figure 3**) and by green fluorescent protein fluorescence (**Figure 4**).

2.7.3.1. Invagination of Tracheal Precursor Cells

During stage 10, the 20 tracheal primordia can be recognized in the thoracic and abdominal segments T2–A8 as ectodermal placodes, 10 on each lateral side of the embryo (Campos-Ortega and Hartenstein, 1985). Each placode is a slightly thickened region of the epithelium with a shallow central depression, composed of tightly packed columnar cells. During stages 10 and early 11, the tracheal precursor cells undergo their 15th and 16th cell division cycles. Cell division cycle 15 occurs just before invagination, and cell cycle 16 occurs while the cells invaginate towards the underlying mesoderm to form the 20 tracheal pits. After the 16th cell division cycle, there are about 80 cells in each tracheal metamere 2 to 9 (Tr2–Tr9), and approximately 150 cells in Tr1 and 50 cells in Tr10. No further cell divisions are observed in the tracheal system until the onset of metamorphosis (Samakovlis *et al.*, 1996a).

As the tracheal precursor cells invaginate, the tracheal pits close but remain connected to the epidermis through the cells of the spiracular branch (SB). During stage 11, each pocket begins to form six buds, which will give rise to the primary branches. In the following sections, we are only discussing the events taking place in Tr2–Tr9. For a description of the first and tenth tracheal metameres, the reader is refered to a previous review (Manning and Krasnow, 1993).

2.7.3.2. Primary Branching

By mid stage 12, six primary branches have formed within each tracheal metamere. The six primary branches are termed dorsal trunk anterior (DTa), dorsal trunk posterior (DTp), dorsal branch (DB), visceral branch (VB), lateral trunk anterior (LTa), and lateral trunk posterior/ganglionic branch (LTp/GB) (**Figure 3**). Each branch appears as a finger-like extension, which grows away from the site of invagination in a stereotyped manner that is similar for all metameres. All primary branches start as type I tubes, and maintain their tubular structure and integrity as they grow. Their outgrowth is initiated by the extension of broad cellular protrusions from the tip cells in each bud. Subsequently, the cell nucleus moves in the same direction, and the apical surface enlarges to promote lumen extension. In this way, three branches grow out from the dorsal region of each pocket to form DTa, a short DTp and DB, one from the central region of the pit forming the VB, and two from the ventral region to form the LTa and LTp/GB. A few cells within the central region of the pit form the transverse connective (TC), which connects the dorsal and ventrolateral parts of the tracheal metamere. The spiracular branch (SB), which is constituted by cells that have remained near the site of invagination, closes during stage 12 (Campos-Ortega and Hartenstein, 1985), and the branch collapses. Thus, the SB has no respiratory function in the *Drosophila* larvae, but it opens at each molt to expel the tracheal cuticle.

The six primary branches continue their extension toward their targets throughout stages 12 and 13 (**Figure 3**). The DB grows towards the dorsal midline, following mesodermal grooves just underneath the epidermis. The DTa and DTp grow in the anterior and posterior directions, respectively, and traverse fields of mesodermal cells, following a groove as they migrate (Franch-Marro and Casanova, 2000). Visceral branches grow inwards to target the gut: VB2 (Tr2) grows towards the junction between foregut and midgut, VB4–VB7 (Tr4–Tr7) extend inwards and anteriorly along the lateral surface of the midgut, and VB8 (Tr8) bifurcates into an anterior and posterior branch on the surface of the gut (Tr3 and Tr9 do not form visceral branches). The LTa extends in the anteroventral direction towards the LTp of the preceding tracheal metamere, and the GB, which now can be discriminated from the LTp, grows ventrally towards the central nervous system (CNS).

During stages 14 and 15, three of the primary branches, DB, GB, and VB, continue to extend (**Figure 3**), developing into narrow type II primary branches. This conversion in the structure of the tubes involves substantial changes in cell contacts and shapes, and results in the repositioning of cells that initially face each other, into a single row around the lumen. The cellular rearrangements involved in the process have not yet been described in detail, but they resemble the intercalation of cells in epithelial sheets.

2.7.3.3. Secondary Branching

As primary branches navigate towards their targets, a distinct number of cells, typically located at the tip of each primary branch, sprout off and form smaller unicellular branches (Samakovlis *et al.*, 1996a). Approximately 23 sprouts emerge during stage 14 within each hemisegment. Twenty of these will grow towards target tissues and form terminal branches,

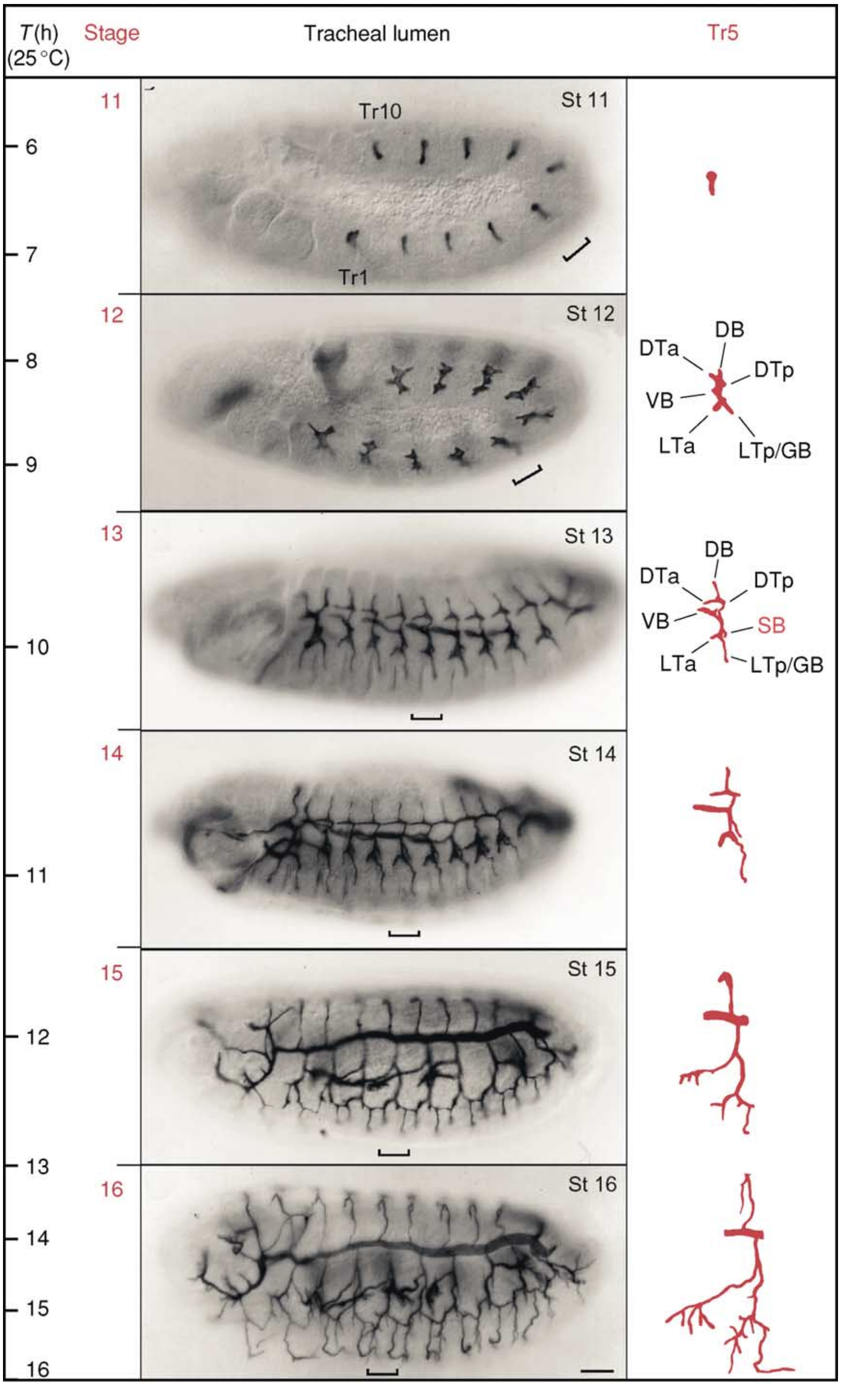


Figure 3 Overview of embryonic tracheal development. The left side of the tracheal system from stage 11 to stage 16, visualized by staining the lumen with the antiserum TL-1 (stages 11–14) and mAb2A12 (stages 15 and 16), is shown in the central panel, where dorsal is up and anterior is to the left. The age of each embryo can be deduced from the left axis in terms of hours after egg laying (AEL) at 25 °C. In the right panel, the lumen of a representative metamere (Tr5) for the relevant stage is drawn, and lines point to the six developing primary branches, as well as transverse connective (TC), which forms between the dorsal and lateral trunk. At stage 11, the lumina of Tr1 to Tr10 are seen as individual sacks, elongated in the dorsoventral orientation. These begin to bud, forming six rudimentary primary branches, which, during stage 12, become evident as protrusions that extend in distinct orientations. During stage 13, primary branches continue to grow, and the fusions between DTa and DTp begin. At stage 14, the dorsal trunk fusions are completed to form a continuous trunk, and the remaining primary branches ensue their elongation. During this stage, secondary branches also begin to sprout. At stage 15, the LTa and LTp have met and fused to form the lateral trunk, and the dorsal and ganglionc branches continue their extension toward the dorsal and ventral midline. The fusion events along the dorsal midline, to connect the left and right sides of the tracheal network, occur during stage 16. At the end of this stage, all fusion events are completed and terminal branches appear as long luminal protrusions from the primary branches. DB, dorsal branch; DTa, dorsal trunk anterior; DTp, dorsal trunk posterior; GB, ganglionic branch; LTa, Lateral trunk anterior; LTp, lateral trunk posterior; SB, spiracular branch; VB, visceral branch.

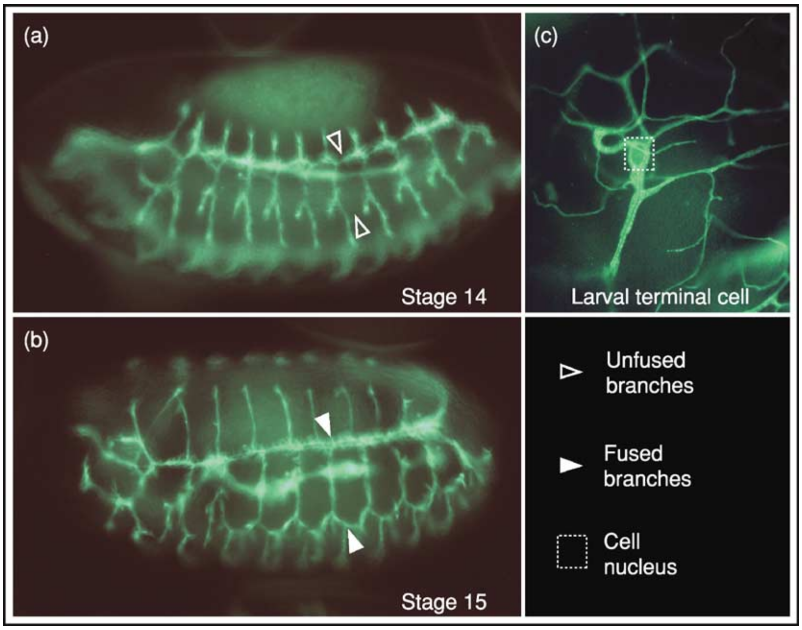


Figure 4 The tracheal system visualized by green fluorescent protein (GFP) fluorescence. GFP expressed in the developing trachea using the UAS-GAL4 system can be used to follow the cellular behavior during tracheal development, and visualization of the larval tracheal network. (a) and (b) A GFP reporter associated to microtubuli (Tau GFP) in the trachea of an early stage 14 embryo (a) when the dorsal trunk fusions are just initiated (upper open arrowhead) in a posterior to anterior gradient. The most posterior fusion event is already completed, but lateral trunk fusion events are not yet initiated (lower open arrowhead). (b) At stage 15, 2 h later, the dorsal and lateral trunk fusions are completed (filled arrowheads). Anterior is to the left, dorsal is up. (c) A single ramified terminal cell in a third instar larvae visualized by a cytoplasmic GFP marker. The nucleus is boxed, and the thin cellular extensions are type IV tubes that extend into surrounding tissues for gas exchange.

and three will contact corresponding sprouts from neighboring tracheal metameres to form branch fusion anastomoses. In addition, two fusion cells are located at the tip of the DTa and DTp, respectively, but these do not generate extended secondary branch sprouts. The position and number of unicellular sprouts within each primary branch is illustrated in Figure 5a.

The initial outgrowth of these branches has been termed secondary branching, but it is not entirely clear how these branches form and into which tube class they belong. In doublelabeling experiments, using antibodies against a cytoplasmic marker for tracheal cells and a septate junction protein, it appears that these branches are type II branches in their proximal region (i.e., the cell is folded over itself and sealed with autocellular junctions), but "seamless" type III tubes at their distal end (lacking autocellular junctions) (Samakovlis *et al.*, 1996b). More recent analysis of the roots of the new branches, however, indicates that the apparent autocellular junctions in the proximal region of the sprout actually belong to the adjacent stalk cell of the primary branch. This following cell in the stalk of the primary branch seems to penetrate the proximal end of the unicellular sprouts to provide an initial lumen, whereas the unicellular sprout itself forms a seamless tube that grows on top of the stalk cell lumen (Uv *et al.*, 2003). At the tips of DB, VB, and LTa, where two or more unicellular branches are followed by a single stalk cell, the stalk cell is predicted to form a bi- or trifurcated lumen to supply each unicellular sprout with a proximal lumen.

2.7.3.4. Branch Fusion

Branch fusion is a prerequisite for the function of many tubular networks. During mammalian angiogenesis, for example, newly sprouted capillaries fuse to each other or to preexisting vessels to facilitate blood circulation. In *Drosophila*, branch fusion connects the primary branches of the 20 independent hemisegments to a continuous network. Two cells, one at the tip of each connecting branch, undergo a complex process of partner recognition and intracellular tube formation to link adjacent branches by type III tubes (Samakovlis *et al.*, 1996b).

Within each hemisegment there are five fusion cells, one at the tip of each DTa, DTp, DB, LTa, and LTp (Figure 5a). During stage 13, the DTa and DTp from neighboring metameres meet endon and fuse to form the two dorsal trunks, one along each side of the embryo. The dorsal trunk fusion events

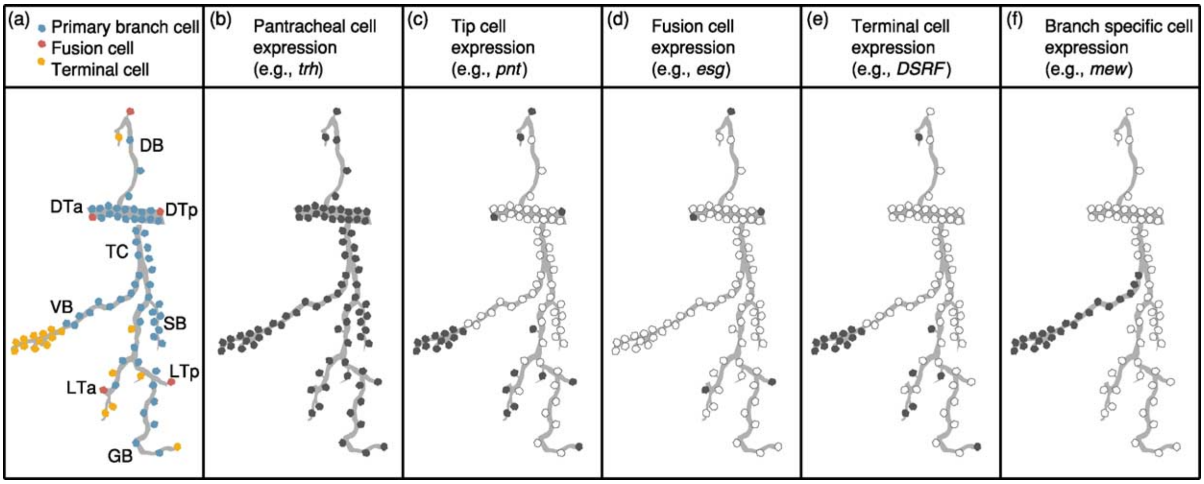


Figure 5 Distinct cell identities and gene expression patterns within each metamere. All drawings illustrate one typical tracheal metamere, lateral views with anterior to the left. (a) Multicellular primary branches of type I and II branches (blue nuclei) form the basic framework. Unicellular branches that sprout off from these form either terminal branches (type IV tubes, yellow nuclei) that target extratracheal tissues for oxygen delivery, or fusion branches (type III tubes, red nuclei) that connect the primary branches of neighboring metameres through luminal branch fusion. (b–f) Expression of different tracheal gene classes is indicated by filled nuclei. (b) Pan-tracheal genes, e.g., *trachealess*, are expressed in all tracheal cells. (c) Pantip genes, such as ''*pointed*,'' are expressed in the cells that form unicellular sprouts (fusion and terminal branches). (d) Fusion genes are specifically expressed in the five fusion cells; *escargot* is the earliest known fusion gene. (e) Terminal branch genes, such as *DSRF*, are expressed in all cells that will form terminal branches. (f) Expression of branch-specific genes is confined to the cells of one or a few branches, and *multiple edematous wings* (*mew*), encoding an αPS1 integrin, is an example that is specifically expressed in the visceral branch.

generally occur in a gradient across the embryo, with the posterior metameres fusing first (**Figure 4a** and **b**). Each of the dorsal trunk fusion cells migrates over a relatively short distance, before it meets its partner from the adjacent metamere to form a specialized connecting branch (type III tube) with the same diameter as the dorsal trunk.

The lateral trunk fusion events generate the continuous lateral trunk and are mediated by the fusion cell lying at the tip of LTa and a fusion cell that sprouts off LTp/GB in the preceding hemisegment. These anastomoses also form in a temporal sequence starting from the posterior metameres during stage 14, and ending in the most anterior ones during early stage 16. Finally, the fusion cells lying at the tip of each DB link the left and right sides of the trachea together at the dorsal midline, generating the 10 dorsal anastomoses. Occasionally, some DB branches are found unfused at hatching. In wild-type embryos, the number of unfused dorsal branches may vary between 0% and 5%. In addition, the GBs of Tr1, Tr2, and Tr3 form ventral anastomoses during stage 17 by connecting the three most anterior GBs with the GBs of the contralateral metameres.

Fusion branches are tubes with the same luminal diameter as the branches they connect, yet the strategy for generating such tubes must differ from that employed during primary branching, as fusion cells are doughnut-shaped and lack autocellular junctions. The fusion event begins as the fusion cells extend broad cytoplasmic processes towards each other. After the two fusion cells have recognized each other and established an initial contact point, they move closer to each other broadening their contact surface, and simultaneously forming a new lumen continuous with the primary branch lumen (Samakovlis *et al.*, 1996b). Lumen growth within the fusion cell appears to involve the assembly and fusion of luminal vesicles, thus producing a junctionless unicellular tube.

2.7.3.5. Terminal Branching

Most secondary sprouts do not form fusion anastomoses but instead develop into terminal branches (type IV). They extend away from primary branches to target distinct cells and tissues for gas exchange. These blind-ended capillaries are liquid-filled at the end and become closely associated with the plasma membranes of target cells to allow gas diffusion. Despite that terminal branches are generated from single cells, they may extend more than 50 μm during embryogenesis and undergo extensive ramification during larval life (**Figure 4c**) (Wigglesworth, 1983; Guillemin *et al.*, 1996; Samakovlis *et al.*, 1996a).

The initial sprouting of terminal cells in the interim of stage 14 (**Figure 3**) appears analogous to that of fusion cells. The cells extend broad cytoplasmic processes away from the primary branch, move their nuclei along, and acquire an initial branch lumen

that may be provided by the neighboring stalk in the primary branch. The succeeding terminal branch growth, which accounts for more than 90% of the embryonic branch length, is, however, an entirely intracellular process. It entails extension of long and narrow cytoplasmic protrusions that shortly afterwards become invaded by a lumen (Guillemin *et al*., 1996). Terminal branches are void of autocellular junctions, and their lumen apparently grows by fusion of luminal vacuoles in a proximal to distal orientation. During terminal branch extension, the terminal cell nucleus remains nearly in the same position. Thus, terminal branch growth may be reminiscent of the growth of neuronal axons, except that the cellular protrusions become hollow.

During embryogenesis, terminal branches follow prefixed paths towards a variety of targets, including the epidermis, different muscles, the digestive system, and the CNS, and may follow elaborate routes to reach their targets. Extension of terminal branches within the CNS, for example, transpires along a complex track around each hemisegment of the ventral nerve cord (Englund *et al*., 1999). Initially, it grows along the intersegmental nerve towards the nerve cord (see **Chapters 1.10** and **2.3**). At the CNS entry point, the terminal cell switches substrate to follow the segmental nerve to the ventral midline. During subsequent branch navigation into the nerve cord, the single ganglionic branch terminal cell turns at least five times along its journey to the target in order to penetrate this tissue.

The mechanisms underlying the local control of organotypic growth pattern in the vascular system of mouse skin appear to be analogous. Like the cells of the ganglionic branches, endothelial cells follow axons and glia, and these contacts determine both arterial differentiation and branching pattern (Mukouyama *et al*., 2002).

2.7.4. Embryonic Tracheal Development: Molecular Mechanisms

Each tracheal branch within a metamere (**Figure 1b**) has fixed cell numbers (**Figure 1c**) and characteristic tubular architectures (**Figure 2**), and grows on stereotyped tracks. Not surprisingly, the cells of the developing trachea display a prominent molecular diversity. This became first evident from the analysis of expression patterns of more than 100 tracheal enhancer trap markers (Samakovlis *et al*., 1996a). Apart from the general tracheal marker genes expressed in all tracheal cells, the expression of a characteristic group of tracheal marker genes is associated with the formation of each tube type, I–IV (**Figure 2**), and likely represents genetically distinct designs for tube construction. In addition, a number of markers display a regional or branch-specific expression within each metamere, or temporally regulated expression patterns during tracheal development that may relate to pathfinding, maturation, or tube size control of the individual branches (examples are shown in **Figure 5**). The molecular analysis of genes involved in tracheal development has revealed important roles for attractant and repellent signals, differentiation factors, and determinants for tube formation and dimensions, in tracheal development. These will be discussed below, and a table summarizing the genes involved in each process is presented within the relevant section.

2.7.4.1. Tracheal Cell Fate Specification

Tracheal development starts with the specification of the tracheal precursors in the ectoderm (**Table 1**). This relies on the combined action of two transcription factors, Trachealess (Trh), a basic helix–loop–helix (bHLH)-PAS domain protein, and Ventral veinless (Vvl, also termed Drifter), a POU-domain containing DNA binding protein (de Celis *et al*., 1995; Boube *et al*., 2000).

Trachealess expression identifies the clusters of epidermal tracheal precursor cells at stage 10 and, in *trh* mutants, the tracheal precursors fail to undergo the cytoskeletal rearrangements necessary for their invagination, and lack expression of many downstream genes necessary for branching (*btl, sms, rho, tdf,* and *peb*) (Isaac and Andrew, 1996; Wilk *et al*., 1996; Boube *et al*., 2000) (**Tables 3 and 8**). The PAS domain of Trh binds to the dARNT/HIFβ (*Drosophila* aryl hydrocarbon receptor nuclear translocator/hypoxia induced factor β) homolog Tango (Tgo), a ubiquitous protein with a more prominent tracheal expression (Ohshiro and Saigo, 1997; Sonnenfeld *et al*., 1997; Zelzer *et al*., 1997). The tracheal phenotypes of *tgo* zygotic mutants are weak, presumably due to the abundant maternal contribution of this gene product. These phenotypes become exaggerated in *trh* heterozygous embryos and, together with protein and DNA binding studies, indicate that Trh acts as a heterodimer with Tgo and is required for several steps during tracheal development.

Ectopic expression of *trh* is not sufficient to induce tracheal cell identity and placode formation throughout the ectoderm. This activity also requires the spatially restricted cofactor Vvl (Anderson *et al*., 1995; de Celis *et al*., 1995). In *vvl* mutants, tracheal cells invaginate only partially and fail to initiate migration and branching. Vvl is also required for the activation of an overlapping set of genes that are

activated by Trh (including *btl, sms, rho*, and *tkv*) (Anderson *et al*., 1996; Llimargas and Casanova, 1997) (**Tables 2 and 3**). The combined action of Vvl and Trh/Tgo is sufficient to induce ectopic activation of some (e.g., *btl*) but not all tracheal downstream genes in the epidermis. These results support a model where tracheal cell fates are induced by the cooperation of several factors rather than by the activation of a single tracheal master gene (Boube *et al*., 2000).

The expression domains of *trh* and *vvl* in the embryonic trunk appear to be controlled by the patterning genes that specify embryonic segmentation. The JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway is required for activation of *trh* expression. Decapentaplegic (Dpp; transforming growth factor (TGF)-β homolog) controls the *trh* expression boundaries along the dorsal–ventral axis, and Wingless (Wg; Wnt) is required for its correct expression along the anterior–posterior axis (de Celis *et al*., 1995; Wilk *et al*., 1996; Brown *et al*., 2001). The restriction of tracheal placodes to the central segments of the embryo, (T2–A8) depends on Spalt major (Salm), a negative regulator of tracheal cell differentiation. Salm is expressed in the anterior and posterior regions of the embryo, where it represses *trh*, *vvl*, and several other tracheal genes (Kuhnlein and Schuh, 1996; Boube *et al*., 2000). In *salm* mutants, an additional placode is acquired, which forms and invaginates anteriorly to the first tracheal pit.

An additional protein that affects *trh* expression is the zinc finger transcription factor Jing (Sedaghat *et al*., 2002). Jing expression in the trachea begins at the placode stage and persists throughout tracheogenesis and in *jing* mutants, fewer epidermal cells express *trh* and some of the tracheal precursors undergo apoptosis.

In summary, tracheal cell identity is determined by the cooperative action of at least two factors, Trh and Vvl. Their expression coordinates are set by embryonic patterning genes and maintained in the trachea through positive autoregulation. Trh and Vvl then activate genes necessary for placode invagination (like *rho*) and branching (like *btl*) (**Table 3**).

Already at stage 10, while the tracheal placode cells are specified, each tracheal placode is subdivided into dorsal and ventral subdomains. This regionalization is brought about by the selective expression of *salm* in the dorsal half of the placode (Franch-Marro and Casanova, 2002). Salm is necessary to repress the expression of genes like *unplugged* (*unpg*) (**Table 6**), which are normally only expressed in the ventral part of the trachea, namely the lateral trunk and ganglionic branches. In *salm* mutants, the dorsal trunk and dorsal branches develop like a mirror image of the lateral trunk and ganglionic branches. This is the second phase of Salm requirement in tracheal development, independent of its function in restricting Trh and Vvl expression in the placodes of the embryonic trunk. The regional requirement of Salm indicates that the tracheal placode is subdivided into domains with different cell fates prior to the activation of cell signaling pathways that allocate cells to different primary branches and direct their migration (see Sections 2.7.4.2 and 2.7.4.3.1).

2.7.4.2. Allocation of Cells to Different Primary Branches

Cell migration, tube formation, and elongation are the central cellular processes giving rise to the six primary tracheal branches, yet different primary branches present distinct tubular designs (type I in the dorsal trunk and transverse connective versus type II in the DB and the GB) (**Table 2**). Moreover, each of the branches attains a characteristic length and diameter and is composed by a fixed number of cells (Samakovlis *et al*., 1996a) (**Figure 1b**). The morphological diversity between the branches within a metamere is accompanied by regional and branch-specific gene expression patterns in each metamere.

The regional compartmentalization of cells in each tracheal pit requires the localized expression of Dpp (Vincent *et al*., 1997), Rhomboid (Rho; an intramembrane protease that generates active epidermal growth factor, EGF) (Wappner *et al*., 1997), Wingless (Wg) (Chihara and Hayashi, 2000; Llimargas, 2000), and Hedgehog (Hh) (Glazer and Shilo, 2001). Dpp is expressed in ectodermal stripes dorsal and ventral to the tracheal placode, setting up regional identities in the dorsal and ventral parts of the placode, that will give rise to DB, LTa, and LTp/GB (**Figure 6a**). Rho and Wg establish a central domain that gives rise to DT and VB (**Figure 6a**), whereas Hh, deriving from the cells just anterior to the placode, induces gene expression changes along the anterior–posterior axis.

The activation of these signaling pathways promotes a regional differentiation of the tracheal cells that is manifested by branch-specific expression of transcription factors. Decapentaplegic signaling is required in the DB, LTa and LTp/GB, and induces the expression of the transcription factors Knirps (Kni) and Knirps related (Knrl) in the responding tracheal cells (Chen *et al*., 1998) (**Figure 6a**). Selective inactivation of the Dpp signaling pathway does not impair oriented migration of the tracheal cells, but prevents the formation of type II tubes of the

Table 1 Genes required for tracheal cell fate specification[a]

Gene	CG number	Product	Expression	Phenotype	Function	References
decapentaplegic (dpp)	CG9885	TGF-β family member	In ectodermal stripes dorsal and ventral to the tracheal placode	Expansion of *dpp* expression domain recruits extra cells to the placode	Embryonic patterning gene, controls *trh* expression and positioning of placode along the DV axis; Dpp binds and activates its receptors Punt and Thickveins	Wilk *et al.* (1996)
domeless (dome) = master of marelle (mom)	CG14226	Transmembrane interleukin receptor homolog	Maternal, zygotic expression in tracheal pits	No tracheae in zygotic null mutants without maternal contribution	Receptor of the JAK–STAT signaling pathway required to initiate *trh* expression	Chen *et al.* (2002)
Epidermal growth factor receptor (Egfr) = DER = flb	CG10079	EGF receptor tyrosine kinase	Ubiquitously expressed	Impaired tracheal cell invagination	Receptor for activated Spitz (see *rho*)	Wappner *et al.* (1997), Llimargas and Casanova (1999)
hedgehog (hh)	CG4637	Patched ligand with a sonic hedgehog domain	Segment polarity expression	Reduced number of cells express *trh*, some cells fail to invaginate	Segment polarity gene; tracheal placode cell fate specification and invagination	Glazer and Shilo (2001)
jing	CG9403	Zinc finger transcription factor	Tracheal placode	Reduced number of placode cells, some undergo apoptosis: no VB, disrupted TC and DT	Assigns appropriate number of cells to the placodes	Sedaghat *et al.* (2002)
protein kinase B = Akt	CG4006	Protein kinase	Broad	Reduced *trh* expression and thus *btl* transcription	Activates and induces nuclear translocation of Trh by phosphorylation	Jin *et al.* (2001)
rhomboid (rho)	CG1004	Protease, 7 transmembrane domains	Tracheal placode	Impaired tracheal cell invagination	Cleaves and activates the EGF-like protein Spitz; required for complete invagination of tracheal placode	Llimargas and Casanova (1999)
spalt major (salm)	CG6464	Zinc finger transcription factor	Tracheal placode	Extratracheal placodes develop in terminal segments; DB and DT adopt ventral branch fates	Represses tracheal placode formation in terminal segments (by repressing *trh*, *vvl*, and other genes); specifies dorsal and ventral domains of the placode	Kuhnlein and Schuh (1996), Franch-Marro and Casanova (2002)

spitz (spi)	CG10334	EGF receptor ligand, TGF-α-like precursor	Ubiquitously expressed	DT and VB are missing or incomplete	Ligand for EGF receptor; cleaved and activated by Rho (see *rho*)	Wappner *et al.* (1997)
stat92E = *marelle (mrl)*	CG4257	Transcription factor with a SH2-domain	Maternal contribution	No tracheae in zygotic null mutants without maternal contribution	Acts downstream of Domeless in the JAK–STAT signaling pathway; required to initiate *trh* expression	Brown *et al.* (2001), Chen *et al.* (2002)
tango (tgo) = *dARNT* = *HIF*β	CG11987	bHLH-PAS domain heterodimerizes with Trh	Broadly embryonic expression	Variable tracheal defects	Specifies tracheal precursor cells in the ectoderm together with Trh; regulates expression of *rho*, *btl*, *sms*, *tdf*, and *peb*	Ohshiro and Saigo (1997), Sonnenfeld *et al.* (1997), Zelzer *et al.* (1997)
trachealess (trh)	CG6883	bHLH-PAS domain heterodimerizes with Tango	In all tracheal cells from stage 10 until end of embryogenesis	No tracheae; placodes fail to invaginate	Specifies tracheal precursor cells in the ectoderm together with Vvl; regulates expression of *rho*, *btl*, *sms*, *tdf*, and *peb*	Isaac and Andrew (1996), Wilk *et al.* (1996), Boube *et al.* (2000)
ventral veinless (vvl)/drifter	CG10037	POU domain transcription factor	Tracheal placode	Abnormal invagination; no primary budding	Specification of tracheal cell fate, together with Trh/Tgo; regulates expression of *rho*, *btl*, *stumps*, and *tkv*	Anderson *et al.* (1995, 1996), de Celis *et al.* (1995), Llimargas and Casanova (1997), Boube *et al.* (2000)
wingless (wg) = *wnt1*	CG4889	Wnt-1 family member	Segment polarity expression	Some tracheal cells fail to invaginate	Segment polarity gene, tracheal placode cell fate specification; sets dorsal and anterior–posterior limits on *trh* and *vvl* expression; required for invagination of some tracheal cells; ligand for Frizeled signaling pathway	Chihara and Hayashi (2000), Llimargas (2000)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in tracheal cell fate specification, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

bHLH, basic helix–loop–helix; DB, dorsal branch; DT, dorsal trunk; DV, dorsoventral; EGF, epidermal growth factor; JAK–STAT, Janus kinase – signal transducer and activator of transcription; POU (see www.expasy.org/prositi); TC, transverse connective; TGF, transforming growth factor; VB, visceral branch.

Table 2 Genes involved in allocation of cells to different primary branches[a]

Gene	CG number	Product	Expression	Phenotype	Function	References
armadillo (arm)	CG11579	β-Catenin	Ubiquitously expressed	Arm(XM19): compromises *wg* signaling; gives a phenotype in DT (missing or reduced)	Downstream component of Wg signaling; required for branch specification	Chihara and Hayashi (2000), Llimargas (2000)
decapentaplegic (dpp)	CG9885	TGF-β family member	Expression in ectodermal stripes dorsal to, and two clusters ventral to the tracheal placode	Tracheal cells fail to migrate along the DV axis	Required for migration of tracheal branches in the DV axis (in DB, LTa, and LTp /GB), activates *kni* and *knrl* in these branches; Dpp binds and activates its receptors Pnt and Tkv	Vincent *et al.* (1997)
elbow B (elB)	CG4220	Zinc finger transcription factor	Pantracheal at stage 11, confined to DB and LT at stage 12	Delayed and abnormal migration of LT and DB cells	Necessary for correct LT and DB cell identity; suppresses VB and DT fate in these cells, probably heterodimerizes with Noc	Dorfman *et al.* (2002a)
Epidermal growth factor receptor (EGFR) = DER = flb	CG10079	EGF receptor tyrosine kinase	Ubiquitously expressed	DT and VB are missing or incomplete	Receptor for activated Spi (see *rho*)	Wappner *et al.* (1997), Llimargas and Casanova (1999)
hedgehog (hh)	CG4637	Patched ligand with a sonic hedgehog domain	Segmental stripes; punctuate pattern overlapping anterior pit cells	Impaired migration of all tracheal branches to different extent	Dorsal branch identity gene; Hh is required for expression of anterior tracheal markers	Glazer and Shilo (2001)
knirps (kni)	CG4717	Zinc finger transcription factor	Placode (stage 11), DB, LT, GB, and VB	DB fails to form, cells remain in DT	DB identity gene; represses *salm* expression	Chen *et al.* (1998)
knirps-related (knrl)	CG4761	Zinc finger transcription factor	Placode (stage 11), dorsalmost tracheal cells (future DB)	DB fails to form, cells remain in DT	DB identity gene; represses *salm* expression	Chen *et al.* (1998)
Mothers against dpp (Mad)	CG12399	Transcription factor		Similar phenotype as when blocking Dpp signaling pathway	Activated by Tkv and Put, induces expression of *kni* and *knrl*	Newfeld *et al.* (1997), Chen *et al.* (1998), Raftery and Sutherland (1999)
no ocelli (noc)	CG4491	Zinc finger transcription factor	Tracheal pits from stage 11; ubiquitous from stage 13	Delayed and abnormal migration of LT and DB cells	Necessary for correct LT and DB cell identity; suppresses VB and DT fate in these cells, probably heterodimerizes with Elb	Dorfman *et al.* (2002a)

punt (put)	CG7904	Type II TGF-β receptor family, serine-threonine kinase		(See *dpp*)	Dpp-receptor; activation of Put leads to phosphorylation of Mad, which induces expression of *kni* and *knrl* (see *dpp*)	Affolter *et al.* (1994b), Ruberte *et al.* (1995), Vincent *et al.* (1997)
rhomboid (rho)	CG1004	Protease, 7 transmembrane domains	Central domain of tracheal placode	Affects the direction of tracheal migration; DT and VB are not formed	Cleaves and activates the EGF-like protein Spi; assigns DT and VB cell fate (central domain of placodes); promotes *salm* expression	Wappner *et al.* (1997)
spalt major (salm)	CG6464	Zinc finger transcription factor	Future DT	No DT; DT cells migrate dorsally together with DB	Activated by Wg/Wnt and EGF signaling, repressed by Kni and Knrl; Salm is necessary for AP cell migration	Llimargas (2000), Kuhnlein and Schuh (1996), Boube *et al.* (2000)
spitz (spi)	CG10334	EGF receptor ligand, produced as inactive transmembrane precursor	Ubiquitously expressed	DT and VB are missing or incomplete	Cleaved and activated by Rho (see *rho*)	Wappner *et al.* (1997), Llimargas and Casanova (1999)
star (S)	CG4385	Type II single transmembrane domain protein	Ubiquitously expressed	DT and VB are missing or incomplete	Required for Spi activation, probably transports inactive Spi from endoplasmic reticulum to Rho-containing organelles	Wappner *et al.* (1997), Tsruya *et al.* (2002)
thickveins (tkv)	CG14026	Type I TGF-β receptor family, serine-threonine kinase		(See *dpp*)	Dpp-receptor; activation of Tkv leads to phosphorylation of Mad, which induces expression of *kni* and *knrl* (see *dpp*)	Affolter *et al.* (1994b), Ruberte *et al.* (1995), Vincent *et al.* (1997)
wingless (wg) = *wnt1*	CG4889	Wnt-1 family member	Two ectodermal patches dorsal/ lateral to the placode	DT is partially lost or fails to migrate	Wg is required for differentiation of DT (vs. VB) through activation of *spalt*; ligand for Frizeled signaling pathway	Chihara and Hayashi (2000), Llimargas (2000)
Wnt oncogene analog 2 (Wnt2)	CG1916	Wnt family member	Dorsal to the placode at stages 10 and 11	No defects in *wnt2* mutants, but *wg*:*wnt2* mutants display enhanced *wg* phenotype	Ligand for Frizeled signaling pathway	Llimargas and Lawrence (2001)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in allocation of cells to different primary branches, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

AP, anteroposterior; DB, dorsal branch; DT, dorsal trunk; EGF, epidermal growth factor; GB, ganglionic branch; LT, lateral trunk; LTa, lateral trunk anterior; LTp, lateral trunk posterior; TGF, transforming growth factor; VB, visceral branch.

Table 3 Genes involved in primary branching[a]

Gene	CG number	Product	Expression	Phenotype	Function	References
anterior open (aop) = AOP/ Yan = yan	CG3166	ETS domain transcriptional repressor	Begins at stage 11; dynamic expression in subset of tracheal cells		Repressor of *btl* expression; Aop is inactivated in the presence of Bnl–Btl signaling in growing tracheal branches	Ohshiro *et al.* (2002)
armadillo (arm)	CG11579	β-Catenin	Ubiquitously expressed	Arm(XM19): compromises Wg signaling, GB misrouted	Downstream component of Wg signaling; required for GB guidance probably through the canonical Wg–Wnt signaling pathway	Llimargas (2000)
branchless (bnl)	CG4608	Homolog of mammalian FGF	Prior to and during primary branching in cell clusters surrounding each sac	No primary branching	Bnl is a chemoattractant, which activates Btl in tracheal cells, guiding their migration	Sutherland *et al.* (1996)
breathless (btl)	CG6714	Homolog of FGF receptor, a receptor tyrosine kinase	Activated in tracheal cells from stage 10	No primary branching	The only known receptor for Bnl; Bnl–Btl signal transduction activates the Ras/MAPK pathway; activated by Trh/Tango and Vvl	Glazer and Shilo (1991), Klambt *et al.* (1992), Reichman-Fried and Shilo (1995)
cdc42	CG12530	Ras GTPase superfamily		No branch extension	May act downstream of Bnl to promote cytoplasmic cell extensions during branch growth	Wolf *et al.* (2002)
corkscrew (csw)	CG3954	Nonreceptor protein tyrosine phosphatase		Severe interruption of primary branch outgrowth	Component of RTK signaling pathway	Perkins *et al.* (1996)
crumbs (crb)	CG6383	Transmembrane protein with EGF, RGD, and laminin A repeats	In trachea from early stage 12 onwards	Affects branch outgrowth and fusion	Required for epithelial polarity and integrity	Tepass *et al.* (1990)
decapentaplegic (dpp)	CG9885	TGF-β family member	Ectodermal patch dorsal to the tracheal pocket	DB cells fail to migrate dorsally	Not required for initial Bnl-induced migration, but to maintain DB extension; Dpp binds and activates its receptors Pnt and Tkv	Ribeiro *et al.* (2002)
Heparan sulfate 6-*O-sulfotransferase (dHS6st)*	CG17188	Enzyme catalyzing heparan sulfate proteoglycan biosynthesis	Selectively in tracheal cells from stages 10 to 12	No primary branching	Required for synthesis of HSPGs, which function as coreceptors mediating formation of active FGF/FGFR signaling complex	Kamimura *et al.* (2001)
l(1) tracheae lacking (l(1)tl)				No main trunks		Manning and Krasnow (1993)
leak (lea) = roundabout 2	CG5481	Slit receptor	All cells at stage 11, DT, DB, and VB at stage 13, DB1 and DB2 at stage 16	Late DB and GB migration disrupted	Mediates tracheal attractant function of Slit	Englund *et al.* (2002)

multiprotein bridging factor 1 (mbf1)	CG4143	Transcription cofactor with a HTH domain	Broad	Mutants become haploinsufficient for *tdf* (tracheal breaks)	Transcriptional coactivator for Tdf	Liu *et al.* (2003b)
protein kinase B = Akt	CG4006	Protein kinase	Broad		May induce cytoskeletal rearrangements; phosphorylates Trh	Jin *et al.* (2001)
Rac1	CG2248	Ras GTPase superfamily	Ubiquitous in all cells, associated with plasma membranes	Increased levels and more lateral distribution of cadherin/catenin complexes; broken DT, misguided DB, no terminal branches	Regulates the amount of E-cad deposited at apical junctions; Rac-activation may be part of Bnl-induced cell motility	Chihara *et al.* (2003)
Rac2	CG8556	Ras GTPase superfamily	(See Rac1)	(See Rac1)	(See Rac1)	Chihara *et al.* (2003)
raw	CG12437	No conserved domains	Broad in epithelial cells	Reduced primary branching	Required in tubular epithelia for cells to assume specialized shapes; possible role for Raw in organizing the actin cytoskeleton	Jack and Myette (1997), Blake *et al.* (1999)
RhoA	CG8416	Ras GTPase superfamily		Dominant negative RhoA; inhibits lumen formation	Necessary for lumen formation; regulation of the cytoskeleton during cell migration; may function upstream of Shot	Lee and Kolodziej (2002)
ribbon (rib)	CG7230	BTB transcription factor with a Pipsqueak DNA-binding domain	All tracheal cells	No primary branching	Required for primary branch lumen growth and cell body movement	Bradley and Andrew (2001), Shim *et al.* (2001)
rolled (rl) = MAPK	CG12559	MAP kinase, protein serine-threonine kinase	Activated MAPK is detected in leading cells of primary branches		Activated by Bnl–Btl signaling; is thought to mediate part of Bnl-induced branch migration	Gabay *et al.* (1997)
slit (sli)	CG8355	Extracellular matrix protein, Robo and Robo2 ligand	In the CNS midline and many other tissues surrounding growing branches	Late DB and GB migration is disrupted	Cell migration signal; activates Robo receptors expressed in tracheal cells to guide DB and GB branch migration	Englund *et al.* (2002)
stripe (sr)	CG7847	Zinc finger transcription factor	Dynamic expression in ectodermal cell clusters during primary branch growth	Delayed migration of DT, LTa, and GB cells	Acts nonautonomously to guide branch growth possibly by providing cues for branch migration	Dorfman *et al.* (2002b)
stumps (sms)/dof/ heartbroken (hbr)	CG18485	Novel intracellular protein, similar to PI3-kinase adaptor		No primary branching	Promotes FGF-directed cell migration; probably an adaptor between FGFR and downstream effectors	Michelson *et al.* (1998), Vincent *et al.* (1998), Imam *et al.* (1999), Dossenbach *et al.* (2001)

Continued

Table 3 Continued

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
sugarless (sgl)	CG10072	Enzyme catalyzing heparan sulfate proteoglycan biosynthesis		No primary branching	Required for synthesis of HSPGs, which function as coreceptors mediating formation of active FGF/FGFR signaling complex	Lin *et al.* (1999)
sulfateless (sfl)	CG8339	Enzyme catalyzing heparan sulfate proteoglycan biosynthesis		No primary branching	Required for synthesis of HSPGs, which function as coreceptors mediating formation of active FGF/FGFR signaling complex	Lin *et al.* (1999)
trachea broken (tbr)				Broken or missing parts of DT with compensatory branching around breaks		Manning and Krasnow (1993)
trachea defective (tdf) = *apontic (apt)*	CG5393	Transcription factor, a bZip protein with a Myb-type DNA binding domain	All tracheal cells from stage 10	Inhibits tracheal cell migration; lack of both maternal and zygotic contribution leads to lack of pit formation	Invagination and branch growth; expression is Trh dependent	Eulenberg and Schuh (1997)

[a]The expression pattern, phenotype and function described for any gene in this table only refers to its role in primary branching, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles or RNAi. CNS, central nervous system; DB, dorsal branch; DT, dorsal trunk; ETS, erythroblast transformation specific; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GB, ganglionic branch; GTPase, guanosine triphosphatase; HSPGs, heparan sulfate proteoglycans; LTa, lateral trunk anterior; MAP, mitogen activated protein; VB, visceral branch; BTB, HTH, Myb, RGD (see www.expasy.org/prositi).

DBs (Ribeiro *et al.*, 2002). Activation of EGF by Rho expression in a central subset of tracheal cells is required, first, for tracheal cell invagination (Llimargas and Casanova, 1999), and then for proper development of the DT and VB. The distinction of DT and VB cells is provided by Wg signaling, which induces DT cell fates. Both Wg and EGF receptor (EGFR) signaling appear to promote

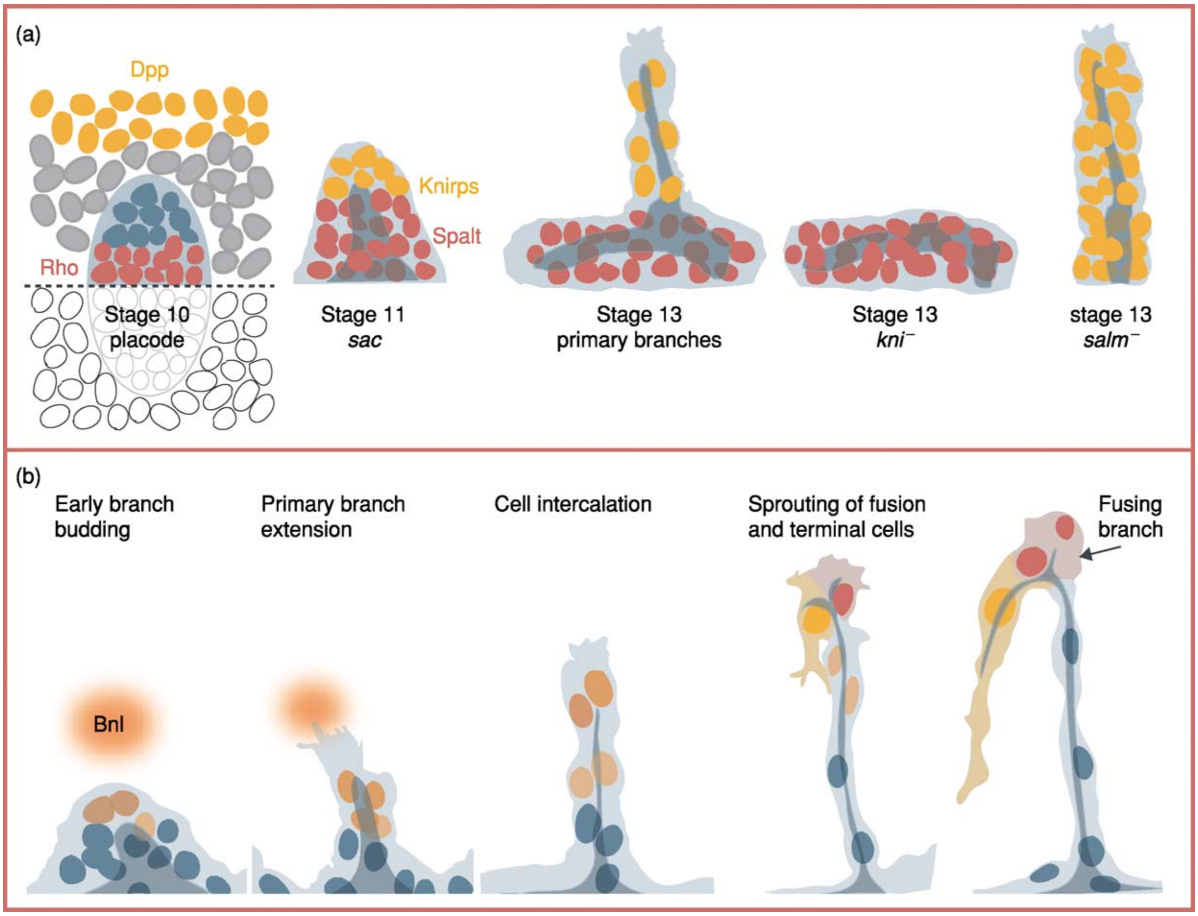


Figure 6 Specification and cellular dynamics of the developing dorsal branch. (a) Model of dorsal branch specification by Spalt, Decapentaplegic (Dpp), and Rhomboid (Rho, a protease that generates active epidermal growth factor (EGF)). Early expression of Spalt subdivides the placode (oval in stage 10) into a dorsal (upper) and a ventral (lower; not colored in this figure) domain. At stage 10, Dpp (yellow) is expressed in the dorsal ectoderm (gray nuclei depict other ectodermal cells). Dpp signals to the dorsal part of the placode, the presumptive DB, DTa, and DTp, to induce expression of the transcription factors Knirps (Kni) and Knirps related (Knrl) in the responding tracheal cells (yellow in stages 11 and 13). In turn, Kni and Knrl repress *salm* (red nuclei) in their domain of expression. Activation of *EGF* by *Rho* expression (red) in a central domain of tracheal placode cells at stage 10 is first required for tracheal cell invagination, and then for proper development of the DT and the VB and activation of *salm* expression. The differentiation between DT and VB cells is mediated by Wg signaling (not shown), which induces DT cell fates. In the absence of Kni and Knrl (stage 13: kni$^-$), the DB does not form and the cells integrate into the DT. In *spalt* mutants (stage 13: salm$^-$), the DT does not form and the cells integrate into DB. (b) Cell dynamics of DB development. The DB arises from cellular rearrangements that generate a dorsal bud within each epithelial sack. First panel: cells at the tip of the bud (brown) respond to Branchless (Bnl; orange), and this leads to subsequent branch extension. Six cells in each hemisegment are destined to follow Bnl-induced migration in the dorsal orientation. Second panel: broad cytoplasmic protrusions emanate from the leading cells towards the Bnl chemoattractant, followed by cellular movements and lumen growth, generating a type I tube. Third panel: the tip cells express the pantip marker Pnt (brown nuclei) in response to the Bnl signal. Dorsal branch elongation proceeds, as the four proximal cells in the branch change their positions from facing each other around the lumen to lying in a row. Fourth panel: the two tip cells differentiate into one terminal cell (yellow) and one fusion cell (red). Intercalation of the four proximal cells to produce a type II tube is completed, and the two tip cells begin to form unicellular branches by extending broad cellular processes and moving their cell bodies away from the primary branch. The dorsal branch lumen bifurcates to provide the initial lumen bud within each of the two tip cells. Towards the end of embryogenesis, the fusion cell has formed a hollow tube and contacted its partner in the contralateral metamere to connect the two at the dorsal midline by a type III tube (two red nuclei). The terminal cell has formed a long subcellular tube extension (type IV tube) and continues to grow towards its target. Fifth panel: the final stalk of the dorsal branch consists of only three cells, because the most proximal cell retracts back into the dorsal trunk.

activation of the transcription factor Salm, and Salm is repressed by Kni and Knrl (Chen *et al.*, 1998) (**Figure 6a**). Additional transcription factors like Unplugged (Unpg), Elbow (Elb), and No ocelli (Noc) are also required for the outgrowth of a subset of branches, but their connection to the emerging scheme is unclear (Chiang *et al.*, 1995; Dorfman *et al.*, 2002a).

The role of the regional placode specification in primary branch migration is beginning to be revealed. Inhibition of Dpp signaling in tracheal cells does not affect Bnl-induced outgrowth and filopodial activities in the tip cells of the DB (see Section 2.7.4.3.1). Instead, the cells fail to migrate the full distance and eventually retract back into the dorsal trunk (Ribeiro *et al.*, 2002). Furthermore, the spatial expression of Kni, Knrl, and Salm is required for branch-specific expression of PS1 integrin in VB cells, which is necessary for correct migration of these cells along the midgut (Boube *et al.*, 2001). The regional differentiation may, therefore, aid oriented branch growth towards the localized sources of Bnl, by preparing the cells for migration along distinct substrates. It may also determine the characteristic dimensions and structure of each tube.

2.7.4.3. Branch Formation and Guidance

Following the establishment of regional and branch-specific identities within the tracheal placode, the tracheal cells begin an extraordinary morphogenetic process to form the elaborate tracheal network. These tubulogenic events involve oriented cell migration, and a hierarchic genetic program that determines cell fate choices induced by signals from the extra tracheal environment.

2.7.4.3.1. Primary branches: the formation and growth of multicellular tubes What determines where new branches form and their direction of growth? For the budding and oriented outgrowth of primary branches, these answers lie in a signal transduction pathway initiated by Branchless (Bnl), a homolog of the secreted fibroblast growth factor (FGF) family (**Table 3**). At stage 10, just before primary branching commences, all tracheal cells in each metamere begin to express the tyrosine kinase receptor Breathless (Btl), a homolog of mammalian FGF receptors (Glazer and Shilo, 1991; Klambt *et al.*, 1992). Bnl, the Btl ligand, is expressed in cell clusters surrounding each individual tracheal metamere (Sutherland *et al.*, 1996). Every cluster of Bnl-expressing cells will activate Btl in the nearby tracheal cells, which respond by oriented cell migration towards the signaling source, and the formation of a primary branch (**Figure 6b**). Hence, a typical metamere receives Bnl from six surrounding cell clusters at stages 11–12. The expression of *bnl* is dynamically regulated and turned off when the respective primary branch reaches the cluster (**Figure 6b**). For some branches, the Bnl source reappears at a new location further along the migratory track, promoting further extension of the branch.

Embryos mutant for *bnl* or *btl* do not form primary branches, and their embryonic trachea consists of 20 unconnected elongated sacks of tracheal cells. Localized ectopic expression of Bnl in epidermal cells can redirect primary branch growth towards its new site of expression in the epidermal clusters (Sutherland *et al.*, 1996). Thus, Bnl is a chemoattractant that promotes and guides primary branch growth; its temporal and spatial expression establishes the pattern of primary branching.

What determines the expression pattern of Bnl? It is likely that the *bnl* promoter contains complex control elements that allow independent transcription in cell clusters positioned at several distinct anterior–posterior and dorsal–ventral coordinates within each segment. The dorsal patch of *bnl* expression, for example, is absent in mutants for *dpp*, demonstrating an independent control of *bnl* expression in this cluster (Vincent *et al.*, 1997).

Breathless expression is also regulated in primary branches. After its onset in all tracheal cells at stage 10, its expression declines in DT and TC during stage 12 and, at late stage 13, it is restricted almost completely to growing branches such as the DB, VB, LTa, and LTp (Ohshiro *et al.*, 2002). It appears that Btl expression during stages 12 and 13 is regulated by a positive feedback loop: Bnl–Btl signaling activates Rolled (Rl), mitogen activated protein kinase (MAPK) (see below), which in turn destabilizes Anterior-open (Aop), a repressor of Btl expression, and thus activates Btl expression. In this way a continuous supply of Btl receptor to membranes of the extending tracheal cells is ensured.

Additional molecules that act in the Bnl–Btl signal transduction pathway, either upstream or downstream of *btl* activation, have been identified. The intracellular protein Downstream-of-FGF receptor (FGFR) (Dof; also known as Stumps and Heartbroken) is specifically required for FGF signal transduction in *Drosophila*, and *dof* mutants show a tracheal phenotype similar to that of *btl* and *bnl* (Michelson *et al.*, 1998; Vincent *et al.*, 1998; Imam *et al.*, 1999; Dossenbach *et al.*, 2001). The *dof* gene encodes a novel protein that is expressed in the developing trachea and is enriched at the plasma membrane. As it contains potential docking sites for several intracellular signaling proteins, Dof may function as an adapter that couples FGFR signaling to

downstream effectors in cell migration. Mutations in either of the two genes *sugarless* (*sgl*) and *sulfateless* (*sfl*), which encode enzymes that synthesize heparan sulfate proteoglycans (HSPGs), also block activation of primary branch outgrowth (Lin *et al.*, 1999). The HSPGs function as coreceptors mediating formation of active FGF–FGFR signaling complexes (Pellegrini, 2001) and, in *sgl* and *sfl* mutants, MAPK activation in the trachea is blocked, but can be re-established by overexpression of the Bnl ligand.

A third gene involved in HSPG biosynthesis is *heparan sulfate* 6-O-*sulfotransferase* (*dHS6ST*). The dHS6ST protein belongs to a class of enzymes that locally modify HSPGs, and is selectively expressed in tracheal cells from stage 10 to 12 (Kamimura *et al.*, 2001). Inhibition of dHS6ST activity by RNA interference (RNAi) disrupts primary branch outgrowth and blocks MAPK activation, suggesting that a particular HSPG structure is required for Bnl signaling.

The molecular mechanisms downstream of Btl activation and their direct link to primary branch growth (**Figure 6b**) are poorly understood, but the first clues are now emerging. The initial extension of broad cellular protrusions, containing filamentous actin, requires active Bnl–FGF signaling (Ribeiro *et al.*, 2002). As active MAPK is present in the leading cells of primary branches (Gabay *et al.*, 1997), but reduced or absent in mutants for *bnl*, *btl*, and *dof*, part of the Bnl-induced branch migrations is most likely conveyed through activation of MAPK (Vincent *et al.*, 1998) (**Table 4**). In support of this, ectopic tracheal expression of activated forms of Ras and Raf partially rescues the primary branch growth defects in *btl* mutants. Furthermore, mutants for *corkscrew* (*csw*), which encodes a nonreceptor protein tyrosine phosphatase that can activate Ras, show severe defects in primary branch outgrowth (Reichman-Fried *et al.*, 1994; Perkins *et al.*, 1996).

The initial dynamic filopodial extensions towards the Bnl source also appear to involve the Rho family guanosine triphosphatases (RhoA, Cdc42, Rac1, and Rac2), which are instrumental in the regulation of the cytoskeleton during cell migration. Overexpression of constitutively active Cdc42 in the trachea induces filopodial extensions, whereas expression of a dominant negative form of Cdc42 impedes branch extension (Wolf *et al.*, 2002). Further cell movement and growth of apical cell surface during primary branching seem to require transcriptional regulation. The putative transcription factor, Ribbon (Rib), is expressed in all tracheal cells (Bradley and Andrew, 2001; Shim *et al.*, 2001) and, in *rib* mutants, filopodia extend toward the Bnl source, but cell body movement and lumen growth are aborted. The *rib* gene may be directly activated by the Bnl–Btl signal transduction pathway (perhaps via MAPK), and Rib may translate the Bnl signal into cell movement and apical membrane growth. Alternatively, it may act in parallel to the Bnl–FGF-induced branch growth, to accommodate these aspects of branch extension once the cells have received the Bnl signal.

Another putative transcription factor, Trachea defective (Tdf), is also required for primary branch outgrowth. The Tdf protein is expressed in the developing trachea, from the placode stage, in a *trh*-dependent manner and, in zygotic *tdf* mutants, primary branching is impeded, but the formation of tracheal placodes, invagination of tracheal cells, and generation of tracheal sacs occur normally (Eulenberg and Schuh, 1997). Removal of maternal Tdf, however, prevents the invagination of the tracheal precursors from the epidermis, indicating that Tdf is required for tracheal cell rearrangements underlying both invagination and branch growth.

Primary branches grow toward, along, and across different cell types to reach their targets. Is the chemoattractant activity of Bnl sufficient to specify the migratory paths taken by different branches and the extent of branch elongation? Primary branch elongation may in part be influenced by the nature of Bnl expression, since localized Bnl sources cease to reappear at new locations further along the migratory track during primary branch growth (Sutherland *et al.*, 1996).

Accumulating evidence from the analysis of mutants affecting the outgrowth of primary branches also indicates that the branches require branch-specific migratory cues in addition to the localized sources of Bnl. Such a guiding function maybe provided by the dynamic expression of Slit (Sli), a phylogenetically conserved cell migration signal, in many of the tissues surrounding the developing trachea. Mutations in *slit* and one of its three fly receptors, *roundabout 2* (*robo2*), hamper the outgrowth of the dorsal, ganglionic, and visceral primary branches (Englund *et al.*, 2002). Ectopic Slit is sufficient to redirect and attract new primary branches towards its ectopic site of expression, and this phenotype is dependent on the expression of the right combination of Robo and Robo2 receptors in the receiving tracheal branches. However, the long-range attractant function of Slit is not sufficient to induce primary branching in the absence of functional Bnl signaling, suggesting a sequential requirement in the activation of different signaling pathways during the migration of primary branches.

Another prerequisite for primary branch migration is cell adhesion to appropriate migration

Table 4 Genes required for secondary branching[a]

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
anterior open (aop) = AOP/ Yan = yan	CG3166	ETS domain transcriptional repressor	Stage 11 on, dynamic expression in subset of tracheal cells		Repressor of late *btl* expression; Aop is inactivated in the presence of Bnl–Btl signaling in growing tracheal branches	Ohshiro *et al.* (2002)
branchless (bnl)	CG4608	Homolog of mammalian FGF	Ceases as primary branches reach the *bnl* source	No induction of secondary branch genes	Controls secondary branch cell specification by inducing expression of secondary branch gene expression; induces expression of *sty*	Sutherland *et al.* (1996)
breathless (btl)	CG6714	Homolog of FGF receptor, a receptor tyrosine kinase	Upregulated in tip cells by positive feedback loop	No induction of secondary branch genes	Bnl receptor (see *bnl*)	Reichman-Fried and Shilo (1995), Lee *et al.* (1996)
pointed (pnt)	CG17077	ETS domain transcription factor	First in primary branch tip cells, becomes restricted to secondary branch cells, then to terminal cells	No secondary branching	Secondary branch gene, induced by Bnl; maintains *btl* expression, induces expression of *sty* and *bs*, inhibits *esg* expression	Samakovlis *et al.* (1996a), Hacohen *et al.* (1998), Ohshiro *et al.* (2002)
rolled (rl) = MAPK	CG12559	MAP kinase, protein serine-threonine kinase	Activated MAP kinase is detected in tip cells (primary branch)		Activated by Bnl–Btl signaling; destabilizes Aop and activates Pnt, thus upregulating *btl* expression	Gabay *et al.* (1997)
sprouty (sty)	CG1921	Novel protein with cystein-rich motif; localizes to cell membrane	Secondary branch cells	Extra terminal branches due to overactivation of the Bnl signaling pathway in stalk cells	Intracellular inhibitor of Ras/MAP kinase pathway, and acts as branching inhibitor in the trachea; reported to act cell nonautonomously; induced by the Bnl pathway	Hacohen *et al.* (1998)

[a]The expression pattern, phenotype and function described for any gene in this table only refers to its role in secondary branching, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles or RNAi. ETS, erythroblast transformation specific; FGF, fibroblast growth factor; MAP, mitogen activated protein.

substrates, which is necessary for dorsal trunk formation. Despite the fact that the extension of DTa and DTp is relatively short and straight (towards a localized source of Bnl between the two buds), their correct migration and subsequent branch fusion require a single cell of mesodermal origin, the bridge cell, which is located at the position where the two branches meet (Wolf and Schuh, 2000). Bridge cells are distinguished by their selective expression of the transcription factor Hunchback (Hb), and, in *hb* mutants, bridge cells undergo apoptosis resulting in DT fusion defects (**Table 5**). As ectopic expression of *hb* in additional cells, close to the bridge cells, interferes with DT formation, Hb appears to be required not only for bridge cell viability, but also for its function in providing an adhesion-dependent guiding post for branch migration. Thus, despite the key role of the Bnl chemoattractant in primary branching, there is a requirement for additional signals that regulate the cellular dynamics of the tracheal epithelium and its interactions with surrounding tissues.

2.7.4.3.2. Sprouting of unicellular branches Within the interim of stage 14, 23 of the 80 cells in the typical tracheal metamere form unicellular sprouts that emanate from the primary branches (**Table 4**). The cells that will form these secondary branch sprouts are molecularly recognized at stage 13 due to their selective expression of pantip marker genes (Samakovlis *et al*., 1996a) (see also **Figure 5**). Pantip gene expression is induced by the same Bnl signal that patterns the primary branches (**Figure 6b**). Most pantip genes are activated already during stage 12 in the tip cells of primary branch buds. These are the cells closest to the Bnl signaling source and, during stage 13, pantip gene expression becomes defined to the exact number of cells that will produce the unicellular secondary sprouts. Several pantip genes are not induced in *bnl* and *btl* mutants, and ubiquitous ectopic Bnl expression activates the expression of secondary and terminal branch genes throughout the tracheal system, causing a hypersprouting phenotype (Lee *et al*., 1996; Sutherland *et al*., 1996). Thus, the potential to activate pantip genes and form unicellular sprouts appears to be a common feature of all tracheal cells, but is selected and restricted by the localized *bnl* expression within each hemisegment.

How is the expression of secondary branch genes restricted to a precise number of cells at stage 13? As Bnl signaling promotes degradation of Aop, which in turn represses *btl* expression (Ohshiro *et al*., 2002), this positive feedback loop restricts the number of cells that perceive and can respond to the Bnl signal during stages 12 and 13. The Bnl signaling also activates expression of genes that limit secondary branching. Sprouty (Spry), an evolutionary conserved protein with a novel cysteine-rich motif, is induced by Bnl and antagonizes Btl and other receptor tyrosine kinase (RTK) signaling pathways (Hacohen *et al*., 1998; Casci *et al*., 1999; Kramer *et al*., 1999; Reich *et al*., 1999). Tracheal *spry* expression is detected in primary branches, as they begin to bud, and is subsequently maintained at high levels in the tip of branches, i.e., in the cells that form secondary branches. In the trachea, Spry acts on cell nonautonomously to stop neighboring cells from expressing secondary branch genes and, in *spry* mutants, more cells form secondary sprouts due to ectopic activation of the Bnl signaling pathway in them. The Spry protein localizes to the cell membrane and appears to interact with canonical members of the RTK pathway upstream of MAPK. However, the mechanism of its cell nonautonomous action in the trachea remains unclear.

One well-studied secondary branch target of the Bnl/Btl signaling pathway is the gene encoding the erythroblast transformation specific (ETS) family transcription factor Pointed (Pnt). In *pnt* mutants, the framework of primary branches develops normally, but no secondary sprouts form. The Pnt protein plays an essential role in the selection and identity of cells that will undergo sprouting: it is required to maintain the expression of Btl in primary branch tip cells (Ohshiro *et al*., 2002), and also induces the expression of *spry* (Hacohen *et al*., 1998). In addition, it promotes the next stage of branching by activating transcription of (*DSRF Drosophila serum response factor* homolog) (**Table 6**) and repressing the expression of the fusion branch gene *escargot* (*esg*) (Samakovlis *et al*., 1996a) (**Table 5**) (see Sections 2.7.4.3.3 and 2.7.4.3.4). As no secondary branches are observed in *pnt* mutants, it is possible that Pnt also regulates cellular components required for the controlled cell shape changes that occur during secondary sprouting.

The selected secondary branch cells are further differentiated into fusion cells or terminal cells by intricate cellular cross talk, involving the reiterated use of Bnl, Dpp, Wg, and the activation of Notch (N) signaling (Ikeya and Hayashi, 1999; Llimargas, 1999, 2000; Steneberg *et al*., 1999; Chihara and Hayashi, 2000) (**Table 5**). Localized expression of *bnl* and *wg* in nearby tissues activates expression of the fusion gene *esg* in individual cells at the ends of primary branches, as well as the expression of *Delta* (*Dl*) (**Table 5**). In the dorsal branch, Dpp signaling is also involved in the activation of *esg*. Delta activates *Notch* in the neighboring cells,

Table 5 Genes required for branch fusion[a]

Gene	CG number	Product	Expression	Phenotype	Function	References
armadillo (arm)	CG11579	β-Catenin	Ubiquitously expressed	No branch fusions	Downstream component of Wg signaling; required for branch fusion and *esg* expression, probably through Wg–Wnt signaling pathway	Llimargas (2000), Chihara and Hayashi (2000)
branchless (bnl)	CG4608	Homolog of mammalian FGF	Ceases as primary branches reach the *bnl* source	No induction of secondary branch genes	Promotes fusion gene expression	Ikeya and Hayashi (1999)
decapentaplegic (dpp)	CG9885	TGF-β family member	In cell cluster dorsal to DB	No DB fusion	Promotes fusion gene expression in DB	Steneberg *et al.* (1999)
Delta (Dl)	CG3619	Transmembrane protein with EGF repeats and cell adhesion function	Fusion cells	Various fusion defects	Fusion gene; activates Notch in neighboring cells, thereby preventing these from becoming fusion cells	Ikeya and Hayashi (1999), Llimargas (1999), Steneberg *et al.* (1999)
dysfusion (dys)	CG12561	HLH-PAS transcription factor	Fusion cells	Impaired fusion of DB, LT, and GB	Heterodimerizes with Tgo; required for fusion of DB, LT, and GB; activated by Esg.	Jiang and Crews (2003)
escargot (esg)	CG3758	Zinc finger transcription factor	All fusion cells	DB and LT fusion events are disrupted	Induces expression of later fusion genes such as *shg*, *mbo*, *hdc*, and *dys* in DB, LTa, and LTp; represses the expression of the terminal branch gene *bs*	Samakovlis *et al.* (1996b), Tanaka-Matakatsu *et al.* (1996)
fear-of-intimacy (foi)	CG6817	Transmembrane protein, zinc transporter domains	Broad	LT fusion disrupted	May be important for cell–cell contact or cell shape changes	Van Doren *et al.* (2003)
headcase (hdc)	CG15532	Novel cytoplasmic protein	Fusion cells in DB, LTa, and LTp	Extra terminal branches near fusion cells in DB, LTa, and LTp	Represses terminal branching; acts cell nonautonomously	Steneberg *et al.* (1998)

hunchback (hb)	CG9786	Zinc finger transcription factor	Bridge cells	Impaired DT pathfinding and loss of DT fusion	Required for bridge cell viability and expression of guidance cues for DT	Wolf *et al*. (2002)
members only (mbo)	CG6819	Nucleoporin	Upregulated in fusion cells	Disruption in DB fusion	Required for DB fusion; upregulated by Esg	Uv *et al*. (2000)
Notch (N)	CG3936	Transmembrane protein with EGF repeats and cell adhesion function	Upregulated in tip cells	Various fusion defects	Activated Notch represses fusion gene expression including *esg*	Ikeya and Hayashi (1999), Llimargas (1999), Steneberg *et al*. (1999), Chihara and Hayashi (2000)
short stop (shot)	CG18076	Actin and microtubule binding plakin	Ubiquitously expressed	Disrupted fusion	Required for remodeling of initial E-cadherin contact between fusion cells, formation of the associated F-actin structure, and lumen formation	Lee and Kolodziej (2002)
shotgun (shg)	CG3722	DE-cadherin	Upregulated in all fusion cells before fusion	Interrupts all fusion events	Required for initial fusion cell contact and subsequent cell rearrangement during fusion; upregulated by Esg in DB, LTa, and LTp	Tanaka-Matakatsu *et al*. (1996)
wingless (wg) = *wnt1*	CG4889	Wnt-1-family member		Loss or partial loss of branch fusion	Promotes fusion gene expression; ligand for Frizeled signaling pathway	Chihara and Hayashi (2000), Llimargas (2000)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in branch fusion, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

DB, dorsal branch; DT, dorsal trunk; EGF, epidermal growth factor; FGF, fibroblast growth factor; GB, ganglionic branch; HLH, helix–loop–helix; LT, lateral trunk; LTa, lateral trunk anterior; LTp, lateral trunk posterior; TGF, transforming growth factor.

Table 6 Genes required for terminal branching[a]

Gene	CG number	Product	Expression	Phenotype	Function	References
adrift (aft)	CG5032	Novel nuclear protein	Terminal cells	Misguidance of GB terminal cell into the CNS	*aft* is induced by Bnl; required for GB terminal cell to switch migration substrate from intersegmental to segmental nerve and entry into the CNS	Englund *et al.* (1999)
blistered (bs) = pruned = DSRF	CG3411	Drosophila serum response factor, a MADS domain transcription factor	Terminal cells, just before terminal branching begins	No terminal branch formation	Regulator of terminal branching; forms a transcription complex together with Elk-1; *bs* expression is activated by Bnl signaling	Affolter *et al.* (1994b), Guillemin *et al.* (1996)
inflated (if) = αPS2	CG9623	αPS2-integrin, heterodimerizes with βPS integrin (Mys)	Visceral mesoderm	VB fail to migrate along the visceral mesoderm	Mediates adhesion between VB and visceral mesoderm	Boube *et al.* (2001)
Lamin (lam) = misguided	CG6944	Nuclear lamin	Broad	Terminal branches misguided and sometimes stalled	Component of the nuclear lamina, required for polarized outgrowth of terminal branches	Guillemin *et al.* (2001)
laminin A	CG10236	Component of basal lamina		Gaps in DT	Probable ligand for integrins	Stark *et al.* (1997)
multiple edematous wings (mew) = αPS1	CG1771	αPS1-integrin, heterodimerizes with βPS integrin (Mys)	Visceral branches (stages 11–13)	VB fail to migrate along the visceral mesoderm	Mediates adhesion between VB and visceral mesoderm; *mew* is regulated by the branch-specific Salm, Kni, and Knrl	Boube *et al.* (2001)
myospheroid (mys) = βPS	CG1560	βPS integrin, heterodimerizes with αPS-integrins	Maternal contribution	Similar to *mew*, but more extreme: some VBs detach from mesoderm, gaps in the DT and defects in DB	Mediates adhesion between VB and visceral mesoderm	Boube *et al.* (2001)
roundabout (robo)	CG13521	Slit receptor	From stage 11, in DT at stage 13	Misguidance of GB terminal cell within the CNS	Required in tracheal cells for perceiving the repellent Slit signal, thereby preventing the GB terminal cells from crossing the CNS midline	Englund *et al.* (2002)
scab (scb) = αPS3	CG8095	α-Integrin, forms heterodimer with βPS integrin (Mys)	In tracheal pits and later in a subset of tracheal branches	Gaps in DT	Might be involved in migration and fusion of tracheal cells	Stark *et al.* (1997)
slit (sli)	CG8355	Calcium-binding EGF-like domain, EGF-laminin domain	CNS midline	Misguidance of GB terminal cell within the CNS	Robo-ligand; prevents terminal branch cells in GB from crossing the CNS midline	Englund *et al.* (2002)
synaptobrevin (syb)	CG12210	Vesicular transmembrane protein (v-SNARE activity)		Larval mutant clones produce lumenless terminal branches	Mediates vesicle fusion, essential for lumen growth	Jarecki *et al.* (1999)
unplugged (unpg)	CG1650	HTH transcription factor with a homeobox domain	Ganglionic branches	Misrouted GB	Required for GB entry into the CNS	Chiang *et al.* (1995)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in terminal branching, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

CNS, central nervous system; DB, dorsal branch; DT, dorsal trunk; EGF, epidermal growth factor; GB, ganglionic branch; HTH, MADS (see www.expasy.org/prosite); VB, visceral branch.

which, in turn, represses fusion cell fate by repressing expression of *esg*. This lateral inhibition mechanism singles out one cell at the tip of each branch, the one with the highest initial expression of *esg* and *Dl*, to become the fusion cell. Esg in turn represses the expression of the terminal branch gene *bs-DSRF*, which thus becomes restricted to the terminal cells. In addition, Esg activates the expression of *headcase* (*hdc*) (**Table 5**), which encodes a cytoplasmic protein acting cell nonautonomously to prevent further stalk cells from undergoing terminal branching (Steneberg *et al.*, 1998). Fusion cells will now form the specialized anastomoses that interconnect the hemisegments to a network (tube type III), whereas terminal cells will continue their journey towards target organs, which they penetrate by the formation of extraordinary capillary tubules (tube type IV).

2.7.4.3.3. Branch fusion The five fusion cells in each hemisegment (see also **Figure 5**) cease branching, and instead contact the corresponding fusion cell in the adjacent or the contralateral metamere (**Table 5**). The two contacting fusion cells then undergo a synchronized process that leads to the formation of an intracellular lumen in each cell and to lumen fusion that connects the two metameres (see Section 2.7.3.4).

The earliest fusion cell-specific protein identified is the zinc finger transcription factor *esg*, which begins to be expressed about 2 h before the initiation of the fusion event (Samakovlis *et al.*, 1996b). The Esg protein is expressed in all five fusion cells, and is required for three of the five fusion events to occur (for the DB, LTa, and LTp), but is redundant for DT fusion, despite its expression in DTa and DTp fusion cells. Because ectopic expression of *esg* in all tracheal cells can suppress terminal branching and promote extra fusion events, Esg appears to act as a key regulator of the fusion cell fate in DB, LTa, and LTp. Indeed, some of the later fusion genes, including *shotgun* (*shg; DE-cadherin*), *members only* (*mbo*), and *hdc*, are not expressed in these fusion cells in *esg* mutants (Samakovlis *et al.*, 1996b; Tanaka-Matakatsu *et al.*, 1996; Steneberg *et al.*, 1998).

The first molecularly recognized step of the fusion event is the deposition of DE-cadherin (DE-cad, Dcad2) at the contact point between the two fusion partners (Tanaka-Matakatsu *et al.*, 1996). The DE-cad protein is an epidermal transmembrane adhesion protein localized at the apical adherence junctions of epithelial cells (Tepass *et al.*, 1996) and is transcriptionally upregulated in fusion cells by Esg. DE-cad interacts with Armadillo (Arm; β-catenin) and Dα-catenin, which, in turn, bind actin filaments linking the plasma membrane to the cytoskeleton. The new deposition of DE-cad at the contact between fusion cells is also associated with Dα-catenin and Filamentous actin (F-actin). In addition, Short stop (Shot), a cytoskeleton associated plakin that interacts both with actin and microtubules, follows the DE-cad distribution throughout the fusion process and is required for DE-cad accumulation at the fusion cell interface (Lee and Kolodziej, 2002). As fusion advances, DE-cad and its associated proteins organize themselves into a line in the proximal–distal orientation. DE-cad then assembles into a circle perpendicular to the presumptive lumen at the interface between the two fusion cells, whereas F-actin remains concentrated in the line, which now spans both fusion cells from one initial lumen bud to the other. Membrane vesicles are expected to accumulate and fuse along this line to facilitate the growth of the intracellular lumen. As the two growing lumina approach the fusion cell interface, the fusion cells contract, displacing their cell bodies cortically, and the F-actin track disassembles to reappear together with microtubular staining at the new adherence ring. The adherence ring expands to the same diameter as that of the connecting primary branches, and lumen fusion between the two fusion cells generates a bicellular anastomosis. Thus, DE-cad redistribution and cytoskeletal modulation are instrumental steps in branch fusion. Reduction of the DE-cad amounts in fusion cells arrests the fusion events at one of the two stages: either before the initial cell contacts are made, or before the expansion of the DE-cad ring, illustrating the requirement for increased amounts of DE-cad at these two stages of the fusion event (Tanaka-Matakatsu *et al.*, 1996). The increasingly detailed description of the fusion process raises many questions on the molecular regulation of the formation and expansion of the DE-cad ring, the polarized growth of the intracellular lumen, and the mechanisms that determine plasma membrane fusion to allow connection of the growing intracellular tubules.

The requirement for increased amounts of DE-cad in the DT fusion cells is not strictly dependent on Esg, since DT fusion is unaffected in *esg* mutants, suggesting that these cells may use a different regulator for branch fusion. Another molecular difference between the fusion cells in DTs and the fusion cells of the DBs and LTs is reflected by the expression of Headcase (Hdc), which occurs in all fusion cells except those of the DT (Steneberg *et al.*, 1998). Whether this difference reflects a distinct mechanism for the actual fusion event in the DT branches, or just a different regulatory mechanism determining fusion cell fate, is not clear.

2.7.4.3.4. Terminal branches Terminal branches are extended unicellular sprouts that target distinct cells and tissues to facilitate gas exchange (see **Table 6**). What triggers the growth of these cells towards different targets and how do single cells form these exquisitely ramified intracellular tubes?

Terminal branch growth selectively requires Blistered (Bs; also called Pruned and DSRF) expression as, in *bs* mutant embryos, terminal cells undergo the initial sprouting from primary branches, but fail to develop the intracellular tube distal to the nucleus (Affolter *et al.*, 1994a; Guillemin *et al.*, 1996). The *bs* gene encodes the *Drosophila* homolog of mammalian serum response factor (SRF) (Montagne *et al.*, 1996), a MADS domain transcription factor. SRF, together with Elk-1, forms part of a transcription complex whose activity is regulated by RTK signaling and the Ras–MAPK pathway (Treisman, 1994). Expression of constitutively active DSRF in tracheal cells induces excessive branch outgrowth, with the terminal branches becoming abnormally extended and branched, and additional terminal branches forming from nearby stalk cells (Guillemin *et al.*, 1996). The molecular targets of DSRF that mediate this unique tube growth remain unknown.

During embryogenesis, terminal branches follow prefixed paths towards a variety of targets. During outgrowth of terminal branches, there is no prominent *bnl* expression in the surrounding tissues. Rather, the code for terminal branch migration appears to be the branches' sensing of tissue- or cell-specific guidance cues that are provided within the immediate migratory environment. Extension of the ganglionic branch terminal cell, for example, is guided by a number of signals that are also followed by neuronal axons (see **Chapters 1.10** and **2.3**). At the CNS entry point, the terminal cell switches its substrate from the intersegmental nerve to follow the segmental nerve to the ventral midline, a process requiring the homeodomain protein Unplugged (Unpg) (Chiang *et al.*, 1995) and the nuclear protein Adrift (Aft) in the terminal cell (Englund *et al.*, 1999). The subsequent branch navigation into the nerve cord is determined by specific cellular contacts made by the terminal cells, and signals emanating from distinct glial cell populations. The CNS provides a rich source of attractant and repellent signals for terminal cell migration, since the single ganglionic branch terminal cell that penetrates this tissue turns at least five times along its journey to the target. Slit signaling is reemployed by the GB terminal cells and, this time, it acts to repel both terminal cells and axons from crossing the midline (Englund *et al.*, 2002).

Another studied example of terminal branch pathfinding is the targeting of the gut, carried out by the terminal cells located at the tip of the visceral branch. α-PS1 integrin is selectively expressed in visceral branch cells and is required for their migration along the visceral mesoderm, which expresses the α-PS2 integrin ligand. Ectopic tracheal expression of PS1 integrin results in misguidance, highlighting again the importance of localized expression of distinct guidance receptors and adhesion molecules in different groups of terminal cells (Boube *et al.*, 2001). How is then such cell- and branch-specific expression achieved? PS1 integrin expression is activated and restricted to visceral branches by Kni, Knrl, and Salm (**Table 2**), which are induced by the early signaling pathways that subdivide the tracheal placode (see Section 2.7.4.2). Aft, alternatively, is expressed in the tip cells of all growing primary branches, and later becomes restricted to terminal cells and is activated by the Bnl signaling pathway (Englund *et al.*, 1999). Thus, at least two independent genetic hierarchies intersect to direct unique combinations of tracheal cell-specific expression of guidance receptors, allowing terminal cells to respond to cues in their immediate environment.

2.7.5. Control of Branch Size

The transport efficiency of tubular organs is dependent on the coordination of tube length and diameter of the branches of the network. In the trachea, air enters into the DT and becomes distributed to gradually narrower branches towards target tissues. Morphometric analysis of the diameter and length of primary branches during embryogenesis indicates that both dimensions are under strict genetic control: branch sizes are invariable from embryo to embryo, expand at certain developmental intervals, and show consistent variations between the different metameres (e.g., the DT diameter becomes gradually narrower in the more anterior metameres) (Beitel and Krasnow, 2000) (see also **Table 7**).

Tube dimensions can be influenced by at least three parameters: cell number, cell size, and cell shape. How are these parameters co-coordinated during tracheogenesis to give rise to distinct tube dimensions within the network? In the following discussion, type I branches are considered, as most studies regarding tube size involve analyses of these branches.

During embryogenesis, the developing dorsal trunk diameter increases three- to fivefold between stages 14 and 16, although the number of DT cells remains constant. In addition, the tube grows by

Table 7 Genes required for correct tube dimensions[a]

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
bulbous (bulb)	49C1-50D1	Not known		Similar to *nrv2*	Tube size control	Beitel and Krasnow (2000)
convoluted (conv)		Not known		Similar to *nrv2*	Tube size control; not required for paracellular diffusion barrier	Beitel and Krasnow (2000), Paul *et al.* (2003)
coracle (cora)	CG11949	Cytoskeleton protein binding	Broad	Same as *nrv2*	Tube size control; septate junction component; required for paracellular diffusion barrier	Paul *et al.* (2003)
cystic (cyst)	II	Not known		Dilations and constrictions in tube diameter, little or no effect on tube length	Tube size control; required for paracellular diffusion barrier	Beitel and Krasnow (2000), Paul *et al.* (2003)
dacapo (dap)	CG1772	Cyclin-dependent protein kinase inhibition activity	Broad	DT branch does not expand to normal diameter	Cell cycle regulator; in *dap* mutants, all cells divide an extra time, but total cell mass is constant	Beitel and Krasnow (2000)
fasciclin 2 (fas2)	CG3665	Cell adhesion protein with immunoglobulin and MHC domain, fibronectin type 3	All tracheal cells until stage 15	Convoluted tracheae with constrictions and dilations	Homophilic cell adhesion molecule localized to the lateral surface of tracheal cells	Hemphala *et al.* (2003)
gliotactin (gli)	CG3903	Serine esterase activity		Similar to *nrv2*	Tube size control; required for paracellular diffusion barrier	Paul *et al.* (2003)
gnarled (gnar)	85D11-F16	Not known		Similar to *nrv2*	Tube size control	Beitel and Krasnow (2000)
grainyhead (grh)	CG5058	Transcription factor Elf-1/NTF-1	All tracheal cells	Convoluted tracheae	Restricts branch elongation; appears to control apical membrane growth; post translationally regulated by Bnl	Hemphala *et al.* (2003)
l(1) tracheae enlarged (l(1)te)	1-0.3			Greatly enlarged main tracheae; PS sometimes nonfunctional		Manning and Krasnow (1993)
l(1) tracheae ramified (l(1)tr)	1-56			Thick main tubes and numerous side branches		Manning and Krasnow (1993)
l(1) tracheae stretched (l(1)trs)	1-8.0			Thin, stretched tracheae		Manning and Krasnow (1993)
megatrachea = pickel (pck)	2A2-B4	Not known		Too long tubes, little or no diameter effects	Tube size control; required for paracellular diffusion barrier	Beitel and Krasnow (2000), Paul *et al.* (2003)
Na pump α subunit (Atpα)	CG5670	α-Subunit of Na^{+}/K^{+} ATPase	All tracheal cells	Convoluted tracheae with constrictions and dilations	Tube size control; required for proper localization of Cora, Fasciclin 3, Nrx and Disc large to septate junctions; required for paracellular diffusion barrier	Hemphala *et al.* (2003), Paul *et al.* (2003)

Continued

Table 7 Continued

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
nervana 2 (nrv2) = ectatic (ecta)	CG9261	β-Subunit of Na^+/K^+ ATPase	Not known, required at placode stage	Convoluted tracheae with constrictions and dilations	Tube size control; required for proper localization of Cora, Fasciclin 3, Nrx, and Disc large to septate junctions; required for paracellular diffusion barrier	Paul *et al.* (2003)
neurexin (nrx)	CG6827	Transmembrane receptor	Broad	Same as *nrv2*	Tube size control; required for paracellular diffusion barrier	Paul *et al.* (2003)
neuroglian (nrg)	CG1634	Immunoglobulin superfamily cell adhesion molecule	Stage 15; posterior spiracles, transiently	Similar to *nrv2*	Tube size control; required for paracellular diffusion barrier	Paul *et al.* (2003)
pebbled (peb) = hindsight	CG12212	Zinc finger transcription factor	All tracheal cells	Collapsed or dilated tubes during expansion phase; subsequent fragmentation may be due to lack of taenidia	Regulates maintenance of the tracheal epithelium and assembly of taenidia; not required for paracellular diffusion barrier	Wilk *et al.* (2000)
sinuous (sinu)	CG10624	Not known		Similar to *nrv2*	Tube size control	Beitel and Krasnow (2000)
stranded-at-second (sas)	CG2507	Plasma membrane protein with fibronectin type III domains	Strongly in trachea at stage 15 and 16	Convoluted tracheae	Localizes at the apical membrane together with Crb	Schonbaum *et al.* (1992), Wodarz *et al.* (1995)
varicose (vari)	39D3-40B1-	Not known		Similar to *nrv2*	Tube size determinant; required for paracellular diffusion barrier	Beitel and Krasnow (2000), Paul *et al.* (2003)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in tube size control, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

DT, dorsal trunk; MHC (major histocompatibility complex); NTF, neurogenic binding element transcription factor; PS, posterior spiracle.

approximately 20% in length. This observation suggests that the dimensions of type I tubes are not simply set by cell number. Furthermore, genetic manipulation of the number of tracheal cells indicates that cell number does not correlate with changes in tube dimensions (Beitel and Krasnow, 2000). In *cyclin A* mutants, cell division cycle 16 is converted to an endocycle resulting in half the normal number of cells with twice the normal ploidy and size (Sprenger *et al.*, 1997). In these mutants, however, both the diameter and length of the DT are the same as in wild-type embryos. In embryos mutant for *dacapo (dap),* where many cells undergo an extra round of division without proportionate cell growth (Lane *et al.*, 1996), the dorsal trunks do not become larger than in the wild-type. Their length is the same and the diameter is actually smaller. Although these experiments do not disengage total cell mass (number × size) from tube dimensions, they illustrate that if the cell numbers are altered, the cells somehow compensate for this by altering their cell shape to achieve the correct dimensions. Thus, the mechanisms that control tube dimensions in multicellular type I branches are not intrinsically programmed in individual cells, but rather appear to rely on a global control mechanism.

As tracheal branches grow, their luminal surface is kept relatively smooth, whereas the basal surface appears bulky, suggesting that tube diameter may be regulated by adjusting the luminal cell circumferance rather than the basal outer diameter of the cells. Moreover, during width expansion, it is the inner, luminal diameter that increases the most, whereas the outer one increases only a little if at all. According to measurements of the developing Tr2 DT, the inner diameter increases fourfold (from 0.6 to 2.4 μm) from stages 14 to 16 and the tube elongates by 20%, whereas its outer diameter remains constant (approximately 8 μm) during this period (Beitel and Krasnow, 2000). As this can be achieved without significant changes in total cell mass, the expansion may depend entirely on cell shape changes. Thus, at the level of individual cells, three major cellular events appear essential for tube expansion:

1. Growth of tracheal cell membranes, particularly the apical cell membrane, to accommodate the growing luminal surface.
2. Rearrangements along the lateral cell surfaces to decrease the distance between the apical and basal surface.
3. Cytoskeletal alterations necessary to change cell shape.

A number of mutants have been identified that affect tube dimensions without influencing branch patterning. In two of these mutants, *ribbon* (*rib*) Bradley and Andrew, 2001; Shim *et al.*, 2001) (**Table 3**) and *synaptobrevin* (*syb*) (Jarecki *et al.*, 1999) (**Table 6**), tube growth is inhibited; in one, *grainyhead* (*grh*) (Hemphala *et al.*, 2003) (**Table 7**), the tubes become too long; and in the remaining ones, the tubes are both elongated and also form cysts (local dilations and constrictions in diameter) (Beitel and Krasnow, 2000; Hemphala *et al.*, 2003; Paul *et al.*, 2003). Mutations in *ribbon* (see Section 2.7.4.3.1) and *synaptobrevin* (a vesicular transmembrane protein that mediates vesicle fusion), as well as expression of dominant negative *RhoA* (Lee and Kolodziej, 2002) (**Table 3**), impair or prevent lumen formation, but allow branch budding to proceed, suggesting they are required for apical membrane growth, at least during branch extension. The transcription factor Grainyhead (Grh), on the other hand, appears to specifically prevent excess apical (luminal) membrane growth, and its activity restricts branch extension. The *grh* mutants develop elongated and convoluted tubes towards the end of tracheogenesis, and tracheal cells accumulate excess apical membrane. Tracheal overexpression of Grh produces a phenotype reminiscent of *rib* loss of function, with arrests in lumen growth, but only partial inhibition of tracheal cell movements, generating short branches that lack a lumen. As Grh activity is posttranslationally modulated by Bnl, these results suggest a direct function of Bnl in the control of apical membrane growth (Hemphala *et al.*, 2003).

Mutations for *fasciclin2* (*fas2*), *Atpα* (encoding a Na-K ATPase α-subunit), *nervana2* (*nrv2*; encoding an Na/K ATPase β-subunit), *coracle* (*cora*), *neurexin* (*nrx*), *gliotactin* (*gli*), and *neuroglian* (*nrg*) (**Table 7**) cause the tracheal tubes to become convoluted (too long) and display diameter constrictions and dilations along their lengths (Hemphala *et al.*, 2003; Paul *et al.*, 2003). The proteins encoded by these genes all localize to the lateral cell surface of tracheal cells: Cora and Nrx are septate junction components, and Gli and Nrg are needed for the blood–brain barrier formed by septate junctions. Thus, structures or events at the lateral cell surface also appear to be essential for defining or maintaining tube dimensions.

In summary, the analyses of tracheal tube size genes carried out to date confirm the importance of apical cell membrane growth and lateral surface modulations in controlling tube size. However, no fundamental biological principle for the regulated control of tube size has been discovered. How are the subcellular events coordinated within a single cell and, perhaps more importantly, how are these

events synchronized between all cells in the network to produce correct tube dimensions? The mechanisms ensuring coordinated tube growth may rely on long-range signals from extratracheal sources, tracheal cell-communication, or molecules released into the lumen.

2.7.6. Cuticle and Apical Extracellular Matrix

The cuticle is a key structural component of the trachea (**Table 8**). The insect cuticle is composed of fibrils of chitin, a β1-4 linked polymer of *N*-acetyl-D-glucosamine, embedded in a matrix of proteins and lipids (Cohen, 2001) that provides a formidable barrier to the environment. Although the tracheal cuticle is continuous with the epidermal cuticle, its morphology differs as it forms prominent ridges, called the taenidial folds, that project into the lumen (Manning and Krasnow, 1993; Tepass and Hartenstein, 1994). The taenidial folds appear to run a helical course along the luminal surface, and are thought to provide elasticity to the structure of tracheal tubes, which allows them to expand and contract during the movements of the larva.

The tracheal cuticle is composed of morphologically distinct layers. The most apical epicuticle is composed of two electron-dense leaflets and covers the layer of procuticle, which is made by chitin and its accossiated proteins. The structure and thickness of the cuticular layer varies in different branches.

The tracheal cuticular lining is secreted in the late embryo, beginning at stage 15 or 16 (Campos-Ortega and Hartenstein, 1985; Tepass and Hartenstein, 1994). By this stage, a continuous tracheal network has formed, but the tracheal lumen is still filled with liquid and electron-dense filamentous material (**Figure 7**). The cuticle appears as irregularly spaced electron-dense areas along the apical membrane associated with granular material being deposited in the lumen. Within 1–2 h after secretion begins, a cuticular bilayer (presumably corresponding to the inner and outer epicuticle) has formed (**Figure 7**). Taenidial folds become visible and the cuticle is mature by the end of stage 17 (**Figure 7**).

A zinc finger domain transcription factor encoded by the *pebbled* (*peb*, also called *hindsight*) gene (Wilk *et al*., 2000), and an extracellular cell adhesion protein, Pollux (Plx) (Zhang *et al*., 1996) (**Table 8**) are required for the assembly of the taenidia and the organization and attachment of the apical extracellular matrix to the epithelium. The regular patterning of taenidia is generated by an unknown mechanism, which may involve the regular spacing of apical, microvillar-like protrusions of the underlying tracheal epithelium.

The synthesis of the chitin chains, catalyzed by chitin synthases, appears to occur intracellularly. Analysis of the sheep blowfly (*Lucilia cuprina*) chitin synthase suggests an integral membrane protein with the catalytic domain located on the cytosolic face of the plasma membrane (Tellam *et al*., 2000). Thus, the polymers must be extruded across the membrane, perhaps through a pore formed by the 15–20 predicted transmembrane domains of the chitin synthase. Two *Drosophila* genes encoding chitin synthases have been identified (Gagou *et al*., 2002) and at least one of these, *krotzkopf verkehrt* (*kkv*) (Ostrowski *et al*., 2002) (**Table 8**), is expressed in the developing embryonic trachea. Surprisingly, *kkv* begins to be expressed in all tracheal cells already during stage 13, much earlier than the reported cuticle secretion. Perhaps Kkv is produced as a zymogen that is first activated during stage 15; alternatively, chitin secretion starts long before the appearance of a cuticular layer in transmission electron microscopy (TEM) preparations.

The transcription factor Grainy Head (Grh) (**Table 8**), which is expressed in all tracheal cells throughout embryogenesis, also appears to be involved in cuticle formation, since one of its target genes is *dopadecarboxylase* (*ddc*) (**Table 8**), encoding an enzyme that is necessary for hardening and darkening of the cuticle (Bray and Kafatos, 1991). Ddc is expressed late in embryogenesis (stage 17) and is thus involved in the terminal stage of the cuticle synthesis program.

Apart from the apical cuticle, tracheal cells, as well as epidermal cells, secrete an intraluminal matrix, evident from electron microscopy studies (Tepass and Hartenstein, 1994). This appears already at stage 11 and can be seen until late stage 15, when the cuticle becomes evident. The molecular composition or function of this matrix are not known. It could contain chitin and molecules recognized by orphan antibodies (like the 2A12 antigen) that stain the tracheal lumen and the large protein Dumpy, which may form large intraluminal fibrils (Wilkin *et al*., 2000). Such a matrix could provide anchoring for proteins that not only facilitate cross-linking and structural support of the matrix, but also influence the behavior of the underlying tracheal epithelium.

2.7.7. Gas Filling of the Lumen

From the time the tracheal system begins to form until late in embryogenesis, it is filled with liquid and electron-dense material. The liquid initially

Table 8 Genes required for cuticle formation[a]

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
dumpy (dp)	CG15637	Large extracellular matrix protein	In tracheal cells from pit stage to at least stage 12	Structural defect in cuticle; trachea fails to fill with gas	Predicted membrane-anchored fiber that is cross-linked within the cuticle to provide strong anchor for the underlying tissue	Wilkin *et al.* (2000)
grainyhead (grh)	CG5058	Transcription factor Elf-1/NTF-1	All tracheal cells	Thin and flimsy cuticle	Activates *dopadecarboxylate*, which is needed for coloring and hardening of the cuticle	Bray and Kafatos (1991)
krotzkopf verkehrt (kkv)	CG2666	Chitin synthase	In all tracheal cells beginning at stages 11–12		Catalyzes the synthesis of chitin chains, the main component of cuticle	BDGP
Matrix metalloproteinase 1 (Mmp1)	CG4859	Metalloendopeptidase activity	Larval trachea	Larvae exhibit short and sometimes broken DT and other branches; uneven DT diameter	Required for growth of the larval trachea and release of cuticle, may be due to inability of cells to detach from the cuticle	Page-McCaw *et al.* (2003)
pebbled (peb) = hindsight	CG12212	Zinc finger transcription factor	All tracheal cells	Taenidia do not form on the luminal surface of tracheal cells	Regulates assembly of taenidia, may be indirect due to requirement for tracheal epithelium integrity	Wilk *et al.* (2000)
pollux (plx)	CG1093	Extracellular adhesion protein	In primary branches from stage 14	Fluid-congested trachea in 1st instar larvae	Required for contact between tracheal epithelium and extracellular matrix	

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in cuticle formation, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

BDGP, Berkeley *Drosophila* genome project; DT, dorsal trunk.

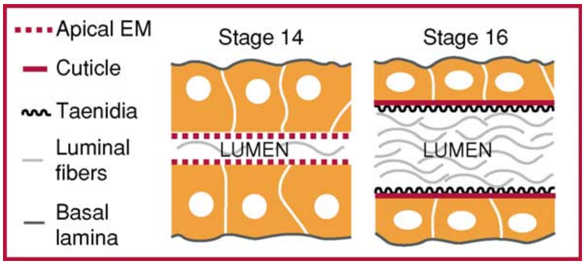


Figure 7 Changes in tube shape and extra cellular components during dorsal trunk development. Drawings of longitudinal sections of the dorsal trunk at stage 14 (left) and stage 16 (right). The dorsal trunk expands about threefold in diameter from stage 14 to stage 16, and is actively secreting apical luminal components during this time period. At stage 14, an apical extracellular matrix (EM) covers the apical cell surface, and filamentous material begins to accumulate in the lumen. At stage 16, a cuticle with the characteristic taenidia is lining the apical cell surface, and the lumen is filled with fibrillar material. This becomes cleared during stage 17 before the larva hatches. A basal lamina is associated with the outer cell surface throughout tracheogenesis.

derives from fluid in the perivitelline space that surrounds the embryo. About 2 h before hatching, this liquid is absorbed and the tracheal system gradually fills with gas. Gas filling occurs before the spiracles open, and can occur when the egg is surrounded by oil, indicating that the gas must derive from nearby tissue or from liquid in the trachea (Manning and Krasnow, 1993).

Lumen clearance appears to be an active process mediated by the tracheal epithelium, where the liquid is absorbed into the tissues. In mammals, the conversion from liquid- to air-filled tubules in the lung occurs at birth, when the epithelium actively absorbs salt and hence liquid. This process requires the activity of the epithelial Na^+ channel (ENaC), which contributes to salt and liquid absorption. Liu *et al*. (2003a) showed that related *Drosophila* degenerin DEG/ENaC family members are expressed in the embryonic tracheal system. These have been termed Pickpocket (Ppk) 4, –7, –10, –11, –12, –14, –19, and –28 (**Table 9**), and may function to clear liquid and generate an air-filled tubule system. By *in situ* hybridization, their expression appeared in late-stage embryos after tracheal tube formation, with individual *ppk* genes showing distinct temporal and branch-specific expression patterns. Moreover, when RNA interference was used to silence two of these genes, *ppk4* and *ppk11*, the larvae failed to clear tracheal liquid. Thus, the related *Drosophila* Ppk proteins and mammalian ENaC proteins may share a physiological function in clearing liquid from the airways.

A second gene that is required for lumen clearance and gas filling without affecting the overall development of the tracheal network is *congested-like trachea (colt)* (Hartenstein *et al*., 1997) (**Table 9**). It encodes a putative mitochondrial carrier protein but its function in the process remains unknown.

2.7.8. Larval Growth of the Tracheal System

The stereotyped migration of terminal branches ensures air supply to the different tissues of newly hatched larvae. Larval life, however, is a period of extensive organismal growth with a continuous need for enhanced oxygen supply. This is accommodated both by the size expansion of the existing tracheal tubes (Beitel and Krasnow, 2000) and by terminal branch ramification.

2.7.8.1. Growth in Branch Dimensions

As new terminal branches ramify during insect larval growth, the pre-existing proximal branches expand in diameter, so that their cross-sectional area is roughly the same as the combined cross-sectional area of its more distal branches, in order to maintain sufficient gas flow. The cellular basis for larval branch expansion in *Drosophila* depends on enlargements in tracheal cell size, rather than cell division, while in other insects it may employ both cell growth and division (Manning and Krasnow, 1993).

In 1958, Locke showed that an increase in lumen diameter of proximal branches in *Rhodnius* is observed, when the larva is subjected to limited oxygen conditions. The signal to increase luminal size is unknown, but Locke's studies suggested that it derives from the terminal branches and propagates retrograde toward the larger, more proximal branches, without diversion to side-branches. In *Drosophila*, the dorsal trunk diameter expands 40-fold from embryonic stage 12 until late third instar larvae, but larval tube growth (at least in the case of DT and LT) occurs within discrete periods (Beitel and Krasnow, 2000). The diameters are constant throughout the first larval period, but increase abruptly at each larval molt, when the cuticle is shed and replaced by the newly deposited cuticle. Growth in tube length, however, steadily increases throughout larval life and oxygen physiology does not appear to influence tube expansion. A comparison of the DT and TC branch size between larvae reared in normal (21%) or low (5%) oxygen (a condition that stimulates terminal branching) showed that tube diameters were not significantly changed by hypoxia, and there was only a small reduction in DT and TC length, which paralleled the reduction in overall body size seen under the hypoxic condition. The

Table 9 Genes required for gas filling[a]

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
congested-like trachea (colt)	CG3057	Mitochondrial carrier protein (carnitine/acyl-carnitine)		Collapsed trachea	Tracheal gas filling	Hartenstein *et al.* (1997)
cut (ct)	CG11387	Homeodomain transcription factor	Cells surrounding anterior–posterior spiracles, some PNS cells around main branches	Incomplete gas filling		Blochlinger *et al.* (1990)
l(3)86Fg	86F6-87A2			Uninflated tracheae		Manning and Krasnow (1993)
Nach = pickpocket 4 (ppk4)	CG8178	Sodium channel	Similar to *ppk11*	Liquid clearance defects	Probably involved in Na^+ absorption to help clear liquid from trachea	Liu *et al.* (2003a)
pickpocket 11 (ppk11)	CG4110	Sodium channel	From stages 15 to 17 beginning in DT, spreading to narrower branches	Liquid clearance defects	Probably involved in Na^+ absorption to help clear liquid from trachea	Liu *et al.* (2003a)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in tracheal development, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).
DT, dorsal trunk; PNS, peripheral nervous system.

Table 10 Genes required for hypoxic response[a]

Gene	*CG number*	*Product*	*Expression*	*Phenotype*	*Function*	*References*
branchless (bnl)	CG4608	Homolog of mammalian FGF	In tracheal target tissues	In hypermorphs, hypoxic tissues fail to attract tracheal branches	Induced by hypoxia to induce and attract new capillaries	Jarecki *et al.* (1999)
breathless (btl)	CG6714	Homolog of FGF receptor, a receptor tyrosine kinase		Dominant negative Btl inhibits terminal branching	Receptor for Bnl	Jarecki *et al.* (1999)
HIF prolyl hydroxylase (Hph)	CG1114	Prolyl hydroxylase	Ubiquitous, upregulated in hypoxia during embryogenesis	Embryonic Sima protein stabilized in normoxia, with subsequent activation of hypoxic reporter genes	In normoxia Hph modifies Sima so it is recognized by E3 ubiquitin–ligase complex, leading to Sima degradation by the proteasome	Bruick and McKnight (2001), Lavista-Llanos *et al.* (2002)
similar (sima) = HIF-1α	CG7951	bHLH-PAS domain, α-subunit of HIF transcription factor	In hypoxia, strong expression in trachea at stages 15–16, but not in normoxia		Regulator of hypoxic response; under hypoxic conditions, Sima protein levels are increased and Sima translocates to the nucleus to activate hypoxia-responsive genes	Lavista-Llanos *et al.* (2002)
tango (tgo) = dARNT = HIF-1β	CG11987	bHLH-PAS domain, β-subunit of HIF transcription factor	Broadly expressed	Fails to induce hypoxic reporter gene expression	Regulator of hypoxic response	Lavista-Llanos *et al.* (2002)
von Hippel–Lindau (Vhl)	CG13221	vHL domain	DT at stage 15, then in smaller branches	DT breaks and ectopic branching	Part of E3 ubiquitin–ligase complex involved in Sima degradation; appears to halt cell movements at branch tips	Adryan *et al.* (2000), Aso *et al.* (2000)

[a]The expression pattern, phenotype, and function described for any gene in this table only refers to its role in hypoxia, as does the chosen reference(s). The phenotype of genes with multiple functions during development may have been concluded from studies using hypomorphic or temperature sensitive alleles, expression of dominant negative alleles, or RNA interference (RNAi).

bHLH, basic helix–loop–helix; DT, dorsal trunk; FGF, fibroblast growth factor; HIF, hypoxia induced factor.

conclusion from these experiments was that differences in oxygen physiology and general growth control have little or no effect on tube diameter in *Drosophila*, but can influence tube length, and that tube diameter and tube length may be independently regulated (Beitel and Krasnow, 2000).

2.7.8.2. Formation of New Capillaries

Each terminal branch may ramify several times during the larval period, generating complex trees of tracheoles that infiltrate the target tissue (**Figure 4c**). Such sprouting and growth of larval tracheoles is regulated by local oxygen demand in body cells, and the molecular components involved in the activation of hypoxic responses appears to be conserved between *C. elegans*, *Drosophila*, and mammals. Cells that experience hypoxia turn on the expression of Bnl, and nearby terminal branches respond by growing new tubes towards the Bnl source, where they arborize (Jarecki *et al.*, 1999). The molecular mechanism of activation of Bnl in hypoxic tissues is not yet understood. It may involve hypoxia response elements resembling the DNA binding sites of the mammalian hypoxia-inducible factor (HIF-1) in the *bnl* promoter (Nagao *et al.*, 1996). Such reporter elements can be activated in *Drosophila* by the endogenous transcription factors Similar (Sima, a HIF-α homolog) and Tango (a HIF-β homolog) (**Table 10**). Interestingly, Sima appears to be regulated posttranslationally by a prolyl hydroxylase protein encoded by the *CG1114* gene (Lavista-Llanos *et al.*, 2002) (**Table 10**).

Thus, terminal branches are guided both by developmental cues and physiological needs. Being formed by single cells, they undeniably provide a powerful system to aid the dissection of cellular mechanisms governing pathfinding and intracellular tube growth.

2.7.9. Concluding Remarks

The genetic analysis of larval tracheal development in *Drosophila* has established many of the basic principles of branch patterning and tube growth that generate a highly elaborate epithelial tubular network. Cell migration, changes in cell shape, and selective growth of apical membrane are essential events in the directed tracheal tube growth toward its targets. These processes are employed in four different schemes of tube formation to accommodate optimal airflow, branch fusion, and tissue targeting for direct gas exchange.

Many of the molecules involved in tracheal development are common to all aspects of epithelial organ morphogenesis, and the question becomes defined to the regulatory aspects of these molecules during tube formation. The development of new methodologies for visualizing subcellular structures and the continuously expanding possibilities for genetic analysis ensure that the ongoing dissection of tracheal development will provide significant contributions to the molecular understanding of luminal growth control, branch fusion, and responses to signals from the developing surrounding tissues.

An important aspect of tracheal development that remains almost completely unexplored at the molecular level is the formation of the adult respiratory system (review: Manning and Krasnow, 1993). Cell division plays an important role in the formation and growth of the pupal and adult trachea, and the study of their development is certainly going to unravel new principles in the control of branching morphogenesis. Another key issue that is only beginning to be addressed at the molecular level is how the physiology of respiration may control capillary growth and retraction. The pioneering studies by Wigglesworth and colleagues on tracheal remodeling in *Rhodnius prolixus* exposed to hypoxia or hyperoxia have shown a fascinating interplay between the air-deprived cells and the adjacent trachea. Thus, it will be of great interest to identify the molecular mechanisms underlying such dynamic cell behavior. The wealth of genetic and molecular information emerging from the analysis of embryonic tracheal development will hopefully aid the molecular dissection of these processes in *Drosophila* and other insects.

Acknowledgments

The authors are indebted to Anna Tonning for her great contribution to the tables. They thank Daniel Mattson and Pär Steneberg for allowing the use of unpublished images and help with the figures, and Johanna Hemphälä for comments on the article.

References

Adryan, B., Decker, H.J., Papas, T.S., Hsu, T., **2000**. Tracheal development and the von Hippel–Lindau tumor suppressor homolog in *Drosophila*. *Oncogene 19*, 2803–2811.

Affolter, M., Montagne, J., Walldorf, U., Groppe, J., Kloter, U., *et al.*, **1994a**. The *Drosophila* SRF homolog is expressed in a subset of tracheal cells and maps within a genomic region required for tracheal development. *Development 120*, 743–753.

Affolter, M., Nellen, D., Nussbaumer, U., Basler, K., **1994b**. Multiple requirements for the receptor serine/threonine kinase thick veins reveal novel functions of TGF beta homologs during *Drosophila* embryogenesis. *Development 120*, 3105–3117.

Anderson, M.G., Certel, S.J., Certel, K., Lee, T., Montell, D.J., *et al.*, **1996**. Function of the *Drosophila* POU domain transcription factor drifter as an upstream regulator of breathless receptor tyrosine kinase expression in developing trachea. *Development 122*, 4169–4178.

Anderson, M.G., Perkins, G.L., Chittick, P., Shrigley, R.J., Johnson, W.A., **1995**. *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Devel. 9*, 123–137.

Aso, T., Yamazaki, K., Aigaki, T., Kitajima, S., **2000**. *Drosophila* von Hippel–Lindau tumor suppressor complex possesses E3 ubiquitin ligase activity. *Biochem. Biophys. Res. Commun. 276*, 355–361.

Beitel, G.J., Krasnow, M.A., **2000**. Genetic control of epithelial tube size in the *Drosophila* tracheal system. *Development 127*, 3271–3282.

Blake, K.J., Myette, G., Jack, J., **1999**. *ribbon, raw*, and *zipper* have distinct functions in reshaping the *Drosophila* cytoskeleton. *Devel. Genes Evol. 209*, 555–559.

Blochlinger, K., Bodmer, R., Jan, L.Y., Jan, Y.N., **1990**. Patterns of expression of cut, a protein required for external sensory organ development in wild-type and *cut* mutant *Drosophila* embryos. *Genes Devel. 4*, 1322–1331.

Boube, M., Llimargas, M., Casanova, J., **2000**. Cross-regulatory interactions among tracheal genes support a co-operative model for the induction of tracheal fates in the *Drosophila* embryo. *Mech. Devel. 91*, 271–278.

Boube, M., Martin-Bermudo, M.D., Brown, N.H., Casanova, J., **2001**. Specific tracheal migration is mediated by complementary expression of cell surface proteins. *Genes Devel. 15*, 1554–1562.

Bradley, P.L., Andrew, D.J., **2001**. *ribbon* encodes a novel BTB/POZ protein required for directed cell migration in *Drosophila melanogaster. Development 128*, 3001–3015.

Bray, S.J., Kafatos, F.C., **1991**. Developmental function of Elf-1: an essential transcription factor during embryogenesis in *Drosophila*. *Genes Devel. 5*, 1672–1683.

Brown, S., Hu, N., Hombria, J.C., **2001**. Identification of the first invertebrate interleukin JAK/STAT receptor, the *Drosophila* gene *domeless*. *Curr. Biol. 11*, 1700–1705.

Bruick, R.K., McKnight, S.L., **2001**. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science 294*, 1337–1340.

Buechner, M., **2002**. Tubes and the single *C. elegans* excretory cell. *Trends Cell Biol. 12*, 479–484.

Campos-Ortega, J.A., Hartenstein, V., **1985**. The Embryonic Development of *Drosophila melanogaster.* Springer, New York.

Casci, T., Vinos, J., Freeman, M., **1999**. Sprouty, an intracellular inhibitor of Ras signaling. *Cell 96*, 655–665.

Chen, C.K., Kuhnlein, R.P., Eulenberg, K.G., Vincent, S., Affolter, M., *et al.*, **1998**. The transcription factors Knirps and Knirps Related control cell migration and branch morphogenesis during *Drosophila* tracheal development. *Development 125*, 4959–4968.

Chen, H.W., Chen, X., Oh, S.W., Marinissen, M.J., Gutkind, J.S., *et al.*, **2002**. *mom* identifies a receptor for the *Drosophila* JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family. *Genes Devel. 16*, 388–398.

Chiang, C., Young, K.E., Beachy, P.A., **1995**. Control of *Drosophila* tracheal branching by the novel homeodomain gene *unplugged*, a regulatory target for genes of the bithorax complex. *Development 121*, 3901–3912.

Chihara, T., Hayashi, S., **2000**. Control of tracheal tubulogenesis by Wingless signaling. *Development 127*, 4433–4442.

Chihara, T., Kato, K., Taniguchi, M., Ng, J., Hayashi, S., **2003**. Rac promotes epithelial cell rearrangement during tracheal tubulogenesis in *Drosophila*. *Development 130*, 1419–1428.

Cohen, E., **2001**. Chitin synthesis and inhibition: a revisit. *Pest Mgt Sci. 57*, 946–950.

de Celis, J.F., Llimargas, M., Casanova, J., **1995**. *ventral veinless*, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila melanogaster. Development 121*, 3405–3416.

Dorfman, R., Glazer, L., Weihe, U., Wernet, M.F., Shilo, B.Z., **2002a**. Elbow and Noc define a family of zinc finger proteins controlling morphogenesis of specific tracheal branches. *Development 129*, 3585–3596.

Dorfman, R., Shilo, B.Z., Volk, T., **2002b**. Stripe provides cues synergizing with branchless to direct tracheal cell migration. *Devel. Biol. 252*, 119–126.

Dossenbach, C., Rock, S., Affolter, M., **2001**. Specificity of FGF signaling in cell migration in *Drosophila*. *Development 128*, 4563–4572.

Englund, C., Steneberg, P., Falileeva, L., Xylourgidis, N., Samakovlis, C., **2002**. Attractive and repulsive functions of Slit are mediated by different receptors in the *Drosophila* trachea. *Development 129*, 4941–4951.

Englund, C., Uv, A.E., Cantera, R., Mathies, L.D., Krasnow, M.A., *et al.*, **1999**. *adrift*, a novel bnl-induced *Drosophila* gene, required for tracheal pathfinding into the CNS. *Development 126*, 1505–1514.

Eulenberg, K.G., Schuh, R., **1997**. The tracheae defective gene encodes a bZIP protein that controls tracheal cell movement during *Drosophila* embryogenesis. *EMBO J. 16*, 7156–7165.

Franch-Marro, X., Casanova, J., **2000**. The alternative migratory pathways of the *Drosophila* tracheal cells are associated with distinct subsets of mesodermal cells. *Devel. Biol. 227*, 80–90.

Franch-Marro, X., Casanova, J., **2002**. *spalt*-induced specification of distinct dorsal and ventral domains is required for *Drosophila* tracheal patterning. *Devel. Biol. 250*, 374–382.

Gabay, L., Seger, R., Shilo, B.Z., **1997**. MAP kinase *in situ* activation atlas during *Drosophila* embryogenesis. *Development 124*, 3535–3541.

Gagou, M.E., Kapsetaki, M., Turberg, A., Kafetzopoulos, D., **2002**. Stage-specific expression of the chitin synthase

DmeChSA and DmeChSB genes during the onset of *Drosophila* metamorphosis. *Insect Biochem. Mol. Biol. 32*, 141–146.

Glazer, L., Shilo, B.Z., **1991**. The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Devel. 5*, 697–705.

Glazer, L., Shilo, B.Z., **2001**. Hedgehog signaling patterns the tracheal branches. *Development 128*, 1599–1606.

Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., *et al.*, **1996**. The pruned gene encodes the *Drosophila* serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development 122*, 1353–1362.

Guillemin, K., Williams, T., Krasnow, M.A., **2001**. A nuclear lamin is required for cytoplasmic organization and egg polarity in *Drosophila*. *Nat. Cell Biol. 3*, 848–851.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., Krasnow, M.A., **1998**. *sprouty* encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell 92*, 253–263.

Hartenstein, K., Sinha, P., Mishra, A., Schenkel, H., Torok, I., *et al.*, **1997**. The congested-like tracheae gene of *Drosophila melanogaster* encodes a member of the mitochondrial carrier family required for gas-filling of the tracheal system and expansion of the wings after eclosion. *Genetics 147*, 1755–1768.

Hemphala, J., Uv, A., Cantera, R., Bray, S., Samakovlis, C., **2003**. Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling. *Development 130*, 249–258.

Ikeya, T., Hayashi, S., **1999**. Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the *Drosophila* trachea. *Development 126*, 4455–4463.

Imam, F., Sutherland, D., Huang, W., Krasnow, M.A., **1999**. *stumps*, a *Drosophila* gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics 152*, 307–318.

Isaac, D.D., Andrew, D.J., **1996**. Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product. *Genes Devel. 10*, 103–117.

Jack, J., Myette, G., **1997**. The genes *raw* and *ribbon* are required for proper shape of tubular epithelial tissues in *Drosophila*. *Genetics 147*, 243–253.

Jarecki, J., Johnson, E., Krasnow, M.A., **1999**. Oxygen regulation of airway branching in *Drosophila* is mediated by branchless FGF. *Cell 99*, 211–220.

Jiang, L., Crews, S.T., **2003**. The *Drosophila* dysfusion basic helix–loop–helix (bHLH)-PAS gene controls tracheal fusion and levels of the trachealess bHLH-PAS protein. *Mol. Cell. Biol. 23*, 5625–5637.

Jin, J., Anthopoulos, N., Wetsch, B., Binari, R.C., Isaac, D.D., *et al.*, **2001**. Regulation of *Drosophila* tracheal system development by protein kinase B. *Devel. Cell 1*, 817–827.

Kamimura, K., Fujise, M., Villa, F., Izumi, S., Habuchi, H., *et al.*, **2001**. *Drosophila* heparan sulfate 6-O-sulfotransferase (dHS6ST) gene: structure, expression, and function in the formation of the tracheal system. *J. Biol. Chem. 276*, 17014–17021.

Klambt, C., Glazer, L., Shilo, B.Z., **1992**. *breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Devel. 6*, 1668–1678.

Kramer, S., Okabe, M., Hacohen, N., Krasnow, M.A., Hiromi, Y., **1999**. Sprouty: a common antagonist of FGF and EGF signaling pathways in *Drosophila*. *Development 126*, 2515–2525.

Kuhnlein, R.P., Schuh, R., **1996**. Dual function of the region-specific homeotic gene *spalt* during *Drosophila* tracheal system development. *Development 122*, 2215–2223.

Lane, M.E., Sauer, K., Wallace, K., Jan, Y.N., Lehner, C.F., *et al.*, **1996**. Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development. *Cell 87*, 1225–1235.

Lavista-Llanos, S., Centanin, L., Irisarri, M., Russo, D.M., Gleadle, J.M., *et al.*, **2002**. Control of the hypoxic response in *Drosophila melanogaster* by the basic helix–loop–helix PAS protein similar. *Mol. Cell Biol. 22*, 6842–6853.

Lee, S., Kolodziej, P.A., **2002**. The plakin Short Stop and the RhoA GTPase are required for E-cadherin-dependent apical surface remodeling during tracheal tube fusion. *Development 129*, 1509–1520.

Lee, T., Hacohen, N., Krasnow, M., Montell, D.J., **1996**. Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the *Drosophila* tracheal system. *Genes Devel. 10*, 2912–2921.

Lin, X., Buff, E.M., Perrimon, N., Michelson, A.M., **1999**. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. *Development 126*, 3715–3723.

Liu, L., Johnson, W.A., Welsh, M.J., **2003a**. *Drosophila* DEG/ENaC pickpocket genes are expressed in the tracheal system, where they may be involved in liquid clearance. *Proc. Natl Acad. Sci. USA 100*, 2128–2133.

Liu, Q.X., Jindra, M., Ueda, H., Hiromi, Y., Hirose, S., **2003b**. *Drosophila* MBF1 is a co-activator for Tracheae Defective and contributes to the formation of tracheal and nervous systems. *Development 130*, 719–728.

Llimargas, M., **1999**. The Notch pathway helps to pattern the tips of the *Drosophila* tracheal branches by selecting cell fates. *Development 126*, 2355–2364.

Llimargas, M., **2000**. Wingless and its signalling pathway have common and separable functions during tracheal development. *Development 127*, 4407–4417.

Llimargas, M., Casanova, J., **1997**. *ventral veinless*, a POU domain transcription factor, regulates different transduction pathways required for tracheal branching in *Drosophila*. *Development 124*, 3273–3281.

Llimargas, M., Casanova, J., **1999**. EGF signalling regulates cell invagination as well as cell migration during formation of tracheal system in *Drosophila*. *Devel. Genes Evol. 209*, 174–179.

Llimargas, M., Lawrence, P.A., **2001**. Seven Wnt homologues in *Drosophila*: a case study of the developing tracheae. *Proc. Natl Acad. Sci. USA 98*, 14487–14492.

Manning, G., Krasnow, M.A., **1993**. Development of the *Drosophila* tracheal system. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. X. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 609–686.

Michelson, A.M., Gisselbrecht, S., Buff, E., Skeath, J.B., **1998**. Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. *Development 125*, 4379–4389.

Montagne, J., Groppe, J., Guillemin, K., Krasnow, M.A., Gehring, W.J., *et al*., **1996**. The *Drosophila* serum response factor gene is required for the formation of intervein tissue of the wing and is allelic to *blistered*. *Development 122*, 2589–2597.

Mukouyama, Y.S., Shin, D., Britsch, S., Taniguchi, M., Anderson, D.J., **2002**. Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell 109*, 693–705.

Nagao, M., Ebert, B.L., Ratcliffe, P.J., Pugh, C.W., **1996**. *Drosophila melanogaster* SL2 cells contain a hypoxically inducible DNA binding complex which recognises mammalian HIF-binding sites. *FEBS Lett. 387*, 161–166.

Newfeld, S.J., Mehra, A., Singer, M.A., Wrana, J.L., Attisano, L., *et al*., **1997**. *Mothers against dpp* participates in a DDP/TGF-beta responsive serine-threonine kinase signal transduction cascade. *Development 124*, 3167–3176.

Ohshiro, T., Emori, Y., Saigo, K., **2002**. Ligand-dependent activation of breathless FGF receptor gene in *Drosophila* developing trachea. *Mech. Devel. 114*, 3–11.

Ohshiro, T., Saigo, K., **1997**. Transcriptional regulation of *breathless* FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in *Drosophila* developing trachea. *Development 124*, 3975–3986.

Ostrowski, S., Dierick, H.A., Bejsovec, A., **2002**. Genetic control of cuticle formation during embryonic development of *Drosophila melanogaster. Genetics 161*, 171–182.

Page-McCaw, A., Serano, J., Sante, J.M., Rubin, G.M., **2003**. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. *Devel. Cell 4*, 95–106.

Paul, S.M., Ternet, M., Salvaterra, P.M., Beitel, G.J., **2003**. The Na^+/K^+ ATPase is required for septate junction function and epithelial tube-size control in the *Drosophila* tracheal system. *Development 130*, 4963–4974.

Pellegrini, L., **2001**. Role of heparan sulfate in fibroblast growth factor signalling: a structural view. *Curr. Opin. Struct. Biol. 11*, 629–634.

Perkins, L.A., Johnson, M.R., Melnick, M.B., Perrimon, N., **1996**. The nonreceptor protein tyrosine phosphatase corkscrew functions in multiple receptor tyrosine kinase pathways in *Drosophila*. *Devel. Biol. 180*, 63–81.

Raftery, L.A., Sutherland, D.J., **1999**. TGF-beta family signal transduction in *Drosophila* development: from Mad to Smads. *Devel. Biol. 210*, 251–268.

Reich, A., Sapir, A., Shilo, B., **1999**. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development 126*, 4139–4147.

Reichman-Fried, M., Dickson, B., Hafen, E., Shilo, B.Z., **1994**. Elucidation of the role of *breathless*, a *Drosophila* FGF receptor homolog, in tracheal cell migration. *Genes Devel. 8*, 428–439.

Reichman-Fried, M., Shilo, B.Z., **1995**. *breathless*, a *Drosophila* FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. *Mech. Devel. 52*, 265–273.

Ribeiro, C., Ebner, A., Affolter, M., **2002**. *In vivo* imaging reveals different cellular functions for FGF and Dpp signaling in tracheal branching morphogenesis. *Devel. Cell 2*, 677–683.

Ruberte, E., Marty, T., Nellen, D., Affolter, M., Basler, K., **1995**. An absolute requirement for both the type II and type I receptors, punt and thick veins, for Dpp signaling *in vivo*. *Cell 80*, 889–897.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D.C., Guillemin, K., *et al*., **1996a**. Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development 122*, 1395–1407.

Samakovlis, C., Manning, G., Steneberg, P., Hacohen, N., Cantera, R., *et al*., **1996b**. Genetic control of epithelial tube fusion during *Drosophila* tracheal development. *Development 122*, 3531–3536.

Schonbaum, C.P., Organ, E.L., Qu, S., Cavener, D.R., **1992**. The *Drosophila melanogaster stranded at second* (*sas*) gene encodes a putative epidermal cell surface receptor required for larval development. *Devel. Biol. 151*, 431–445.

Sedaghat, Y., Miranda, W.F., Sonnenfeld, M.J., **2002**. The *jing* Zn-finger transcription factor is a mediator of cellular differentiation in the *Drosophila* CNS midline and trachea. *Development 129*, 2591–2606.

Shim, K., Blake, K.J., Jack, J., Krasnow, M.A., **2001**. The *Drosophila ribbon* gene encodes a nuclear BTB domain protein that promotes epithelial migration and morphogenesis. *Development 128*, 4923–4933.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., *et al*., **1997**. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian *Arnt* and controls CNS midline and tracheal development. *Development 124*, 4571–4582.

Sprenger, F., Yakubovich, N., O'Farrell, P.H., **1997**. S-phase function of *Drosophila* cyclin A and its downregulation in G_1 phase. *Curr. Biol. 7*, 488–499.

Stark, K.A., Yee, G.H., Roote, C.E., Williams, E.L., Zusman, S., *et al*., **1997**. A novel alpha integrin subunit associates with betaPS and functions in tissue morphogenesis and movement during *Drosophila* development. *Development 124*, 4583–4594.

Stebbings, L.A., Todman, M.G., Phillips, R., Greer, C.E., Tam, J., *et al.*, **2002**. Gap junctions in *Drosophila*: developmental expression of the entire innexin gene family. *Mech. Devel. 113*, 197–205.

Steneberg, P., Englund, C., Kronhamn, J., Weaver, T.A., Samakovlis, C., **1998**. Translational readthrough in the hdc mRNA generates a novel branching inhibitor in the *Drosophila* trachea. *Genes Devel. 12*, 956–967.

Steneberg, P., Hemphala, J., Samakovlis, C., **1999**. Dpp and Notch specify the fusion cell fate in the dorsal branches of the *Drosophila* trachea. *Mech. Devel. 87*, 153–163.

Sutherland, D., Samakovlis, C., Krasnow, M.A., **1996**. *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell 87*, 1091–1101.

Tanaka-Matakatsu, M., Uemura, T., Oda, H., Takeichi, M., Hayashi, S., **1996**. Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot. *Development 122*, 3697–3705.

Tellam, R.L., Vuocolo, T., Johnson, S.E., Jarmey, J., Pearson, R.D., **2000**. Insect chitin synthase cDNA sequence, gene organization and expression. *Eur. J. Biochem. 267*, 6025–6043.

Tepass, U., Gruszynski-DeFeo, E., Haag, T.A., Omatyar, L., Torok, T., *et al.*, **1996**. *shotgun* encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Devel. 10*, 672–685.

Tepass, U., Hartenstein, V., **1994**. The development of cellular junctions in the *Drosophila* embryo. *Devel. Biol. 161*, 563–596.

Tepass, U., Theres, C., Knust, E., **1990**. *crumbs* encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell 61*, 787–799.

Treisman, R., **1994**. Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Devel. 4*, 96–101.

Tsruya, R., Schlesinger, A., Reich, A., Gabay, L., Sapir, A., *et al.*, **2002**. Intracellular trafficking by Star regulates cleavage of the *Drosophila* EGF receptor ligand Spitz. *Genes Devel. 16*, 222–234.

Uv, A., Cantera, R., Samakovlis, C., **2003**. *Drosophila* tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. *Trends Cell Biol. 13*, 301–309.

Uv, A.E., Roth, P., Xylourgidis, N., Wickberg, A., Cantera, R., *et al.*, **2000**. *members only* encodes a *Drosophila* nucleoporin required for rel protein import and immune response activation. *Genes Devel. 14*, 1945–1957.

Van Doren, M., Mathews, W.R., Samuels, M., Moore, L.A., Broihier, H.T., *et al.*, **2003**. *fear of intimacy* encodes a novel transmembrane protein required for gonad morphogenesis in *Drosophila*. *Development 130*, 2355–2364.

Vincent, S., Ruberte, E., Grieder, N.C., Chen, C.K., Haerry, T., *et al.*, **1997**. Dpp controls tracheal cell migration along the dorsoventral body axis of the *Drosophila* embryo. *Development 124*, 2741–2750.

Vincent, S., Wilson, R., Coelho, C., Affolter, M., Leptin, M., **1998**. The *Drosophila* protein Dof is specifically required for FGF signaling. *Mol. Cell 2*, 515–525.

Wappner, P., Gabay, L., Shilo, B.Z., **1997**. Interactions between the EGF receptor and Dpp pathways establish distinct cell fates in the tracheal placodes. *Development 124*, 4707–4716.

Wigglesworth, V.B., **1983**. The physiology of insect tracheoles. *Adv. Insect. Physiol. 17*, 86–148.

Wilk, R., Reed, B.H., Tepass, U., Lipshitz, H.D., **2000**. The *hindsight* gene is required for epithelial maintenance and differentiation of the tracheal system in *Drosophila*. *Devel. Biol. 219*, 183–196.

Wilk, R., Weizman, I., Shilo, B.Z., **1996**. *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Devel. 10*, 93–102.

Wilkin, M.B., Becker, M.N., Mulvey, D., Phan, I., Chao, A., *et al.*, **2000**. *Drosophila* dumpy is a gigantic extracellular protein required to maintain tension at epidermal–cuticle attachment sites. *Curr. Biol. 10*, 559–567.

Wodarz, A., Hinz, U., Engelbert, M., Knust, E., **1995**. Expression of *crumbs* confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell 82*, 67–76.

Wolf, C., Gerlach, N., Schuh, R., **2002**. *Drosophila* tracheal system formation involves FGF-dependent cell extensions contacting bridge-cells. *EMBO Rep. 3*, 563–568.

Wolf, C., Schuh, R., **2000**. Single mesodermal cells guide outgrowth of ectodermal tubular structures in *Drosophila*. *Genes Devel. 14*, 2140–2145.

Wolff, J.R., Bar, T., **1972**. ‘Seamless’ endothelia in brain capillaries during development of the rat’s cerebral cortex. *Brain Res. 41*, 17–24.

Zelzer, E., Wappner, P., Shilo, B.Z., **1997**. The PAS domain confers target gene specificity of *Drosophila* bHLH/PAS proteins. *Genes Devel. 11*, 2079–2089.

2.8 Development of the Malpighian Tubules in Insects

B Denholm and H Skaer, University of Cambridge, Cambridge, UK

2.8.1. Introduction

The Malpighian tubules were first described by the seventeenth-century anatomist Marcello Malpighi in his 1669 monograph on the silkworm, *Bombyx mori* (Malpighi, 1669). He was the first to link anatomical description of these organs with their physiological function, namely excretion. In most insects, the Malpighian tubules represent the chief excretory organ and, as such, are an important site for regulating physiological homeostasis. To this end, they may be called upon to perform a number of functions: (1) removal of the end products of metabolism; (2) removal of excess salts; (3) elimination of toxins; and (4) maintenance of the ionic and osmotic properties of the blood.

The work of Wigglesworth and Ramsay during the course of the last century established the Malpighian tubules as a model for the study of transporting epithelia (Wigglesworth, 1939; Ramsay, 1954, 1955, 1958). These early works focused on the tubules of the blood-sucking hemipteran *Rhodnius prolixus* and the stick insect *Dixippus morosus*, both large species with experimentally tractable tubules. It was here that the foundations of experimental insect physiology were established. This tradition continued throughout the latter part of the twentieth century and, with the development of an isolated tubule assay to facilitate study in other insect species, a relatively comprehensive description of Malpighian tubule physiology across several species was established.

In parallel with these physiological analyses, Malpighian tubules from various species have been examined both at the morphological and ultrastructural levels (Wigglesworth and Salpeter, 1962; Berridge and Oschman, 1972; Wessing and Eichelberg, 1978; Skaer *et al.*, 1987). Studies such as these reveal that

despite their varied morphology, insect tubules conform to a basic design: polarized epithelia tubes with regions specialized for fluid secretion, toxin clearance, and reabsorption of solutes and water. In some cases, these studies, in combination with physiological analysis, allow direct correlation between structure and physiological function (Skaer *et al.*, 1990; O'Donnell *et al.*, 1998).

In the last decade, the subject of tubule development has come into focus. This area of study has been dominated by a single insect species, namely *Drosophila melanogaster.* The use of *Drosophila* has been fruitful in revealing both cellular and molecular aspects of tubule development. These findings provide a reference point not only for studies of tubules in other species but for questions relating to the development of transporting epithelia and differentiated cell function in general. Recently, the coupling of traditional physiological assays (Dow *et al.*, 1994) with the power of *Drosophila* genetics has allowed a more detailed molecular description of physiological function to emerge.

The focus of this chapter is the development of insect Malpighian tubules, with particular emphasis given to a molecular description of development. In light of this, much of what follows is dominated by description of tubule development in *Drosophila*, because it is in this species that a comprehensive dissection of development has been performed.

The development of *Drosophila* tubules can be viewed as occurring in several, relatively discrete, steps: (1) the allocation of tubule primordial cells; (2) cell division of these primordial cells; (3) differentiation of specific cell types; (4) cell rearrangement and morphogenesis; (5) recruitment of cells to the tubules; and (6) the onset of physiological competence (**Figure 1**). These steps are dealt with separately below (see Sections 2.8.3 to 2.8.8). Despite the amount of space given over to descriptions of *Drosophila* development, comparisons with other insect systems will be made where data exist. A comprehensive description of insect Malpighian tubule physiology is not included here as this has been reviewed recently elsewhere (Dow and Davies, 2001). A brief summary of tubule physiology is provided below (see Section 2.8.2.1).

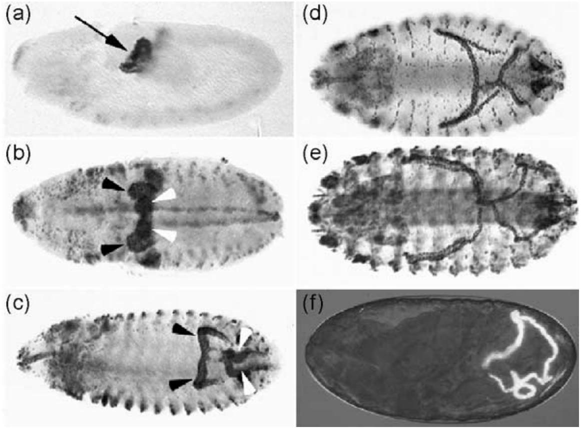


Figure 1 Embryonic development of *Drosophila* Malpighian tubules. (a–e) Embryos stained with an antibody against the transcription factor Cut, which labels tubule cells throughout embryonic development. (a) The tubules evert from the hindgut when the germband is fully extended at stage 9 (arrow). (b–d) The tubules grow by cell division during stages 9–13, as the germband retracts (b and c) and thereafter by elongation, through convergent-extension movements (d). (e) At stage 16, the tubules have adopted their stereotypical arrangement within the embryonic cavity. (f) At stage 17 the first signs of physiological activity can be seen as uric acid is deposited into the posterior tubules (revealed as a bright deposit under polarized light). Embryos are shown in lateral view (a) and in dorsal view (b–f) with anterior at left and posterior at right. Black arrowhead, anterior tubule; white arrowhead, posterior tubule.

2.8.2. Malpighian Tubule Form and Function

2.8.2.1. Insect Malpighian Tubule Function and Physiology

The function of an excretory system is to remove harmful or unnecessary substances from the body. In most animals this is achieved in the same way. First, a relatively nonselective filtration barrier filters the extracellular fluid to hold back cells and large molecules such as proteins, but permits the passage of smaller molecules, including harmful substances. Second, the filtrate passes along the lumen of a tube where retrieval of useful substances takes place and further transport of harmful substances into the lumen occurs. The Malpighian tubules are the primary organ of excretion in insects and as such conform to this fundamental design (Maddrell, 1981). Primary urine production results from transport across distal tubule cells and the resulting solvent drag increases flow into the lumen through the paracellular spaces between the tubule cells. This forms the filtration barrier through which novel toxins are cleared from the hemolymph. Importantly, this barrier is relatively nonselective allowing the passage of toxic and unwanted substances in addition to essential substances. The filtrate is then modified by active reabsorption of essential compounds and additional active transport of toxic molecules into the lumen by specific transporters.

Central to the function of the tubules is an apical vacuolar-type H^+-ATPase (Davies *et al.*, 1996; review: Dow and Davies, 2001) (**Figure 2**). This

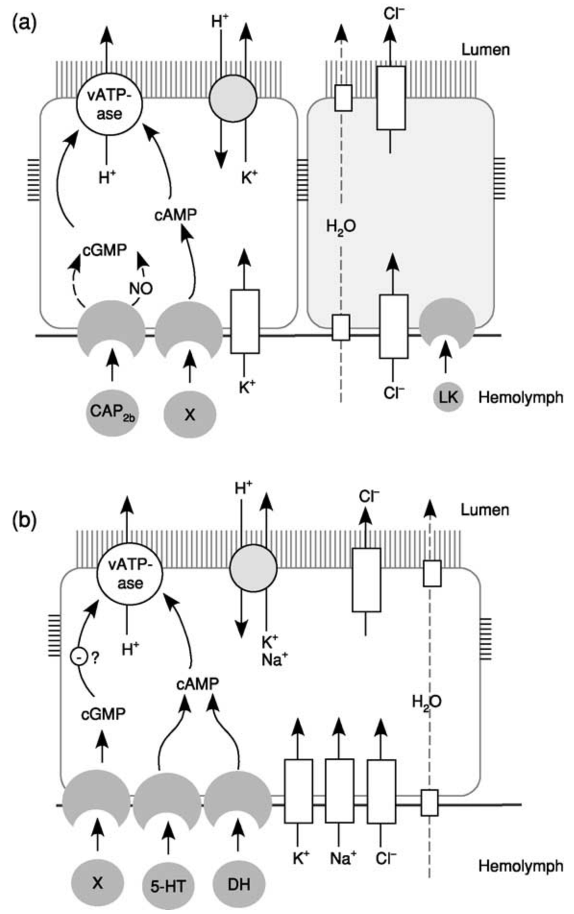


Figure 2 The transport processes involved in tubule function. Ion transport is driven by the activity of an H^+-transporting vacuolar-ATPase (V-ATPase) situated on the luminal membrane. The V-ATPase, coupled with a cation/H^+ antiporter, constitutes a cation pump that transports sodium ions, potassium ions, or both from the cytoplasm into the tubule lumen. Chloride ions move into the lumen down an electrochemical gradient through chloride-permeable channels. The rate of entry into the cell, from the hemolymph, of sodium, potassium, and chloride ions, through channels and transporters, varies between insect species and depends on the state of stimulation of the tubule. (a) In *Drosophila*, cation transport occurs across the principal cells (PCs, left) whereas chloride ion movement is across the stellate cells (StCs, right). (b) In *Rhodnius* only one secretory cell type exists, through which both cation and anion movements take place. 5-HT, 5-hydroxy tryptamine; CAP2b, cardioacceleratory peptide 2b; DH, diuretic hormone; LK, leukokinin; NO, nitric oxide. The apical cell surface is enriched with microvilli (parallel lines in lumen). Intercellular junctions are shown as cross-hatching between the cells. X indicates as yet unknown agonist(s) that stimulate the elevation of intracellular cyclic AMP levels in *Drosophila* and cyclic GMP in *Rhodnius*.

pump maintains a proton gradient across the apical membrane that ultimately drives the movement of alkaline cations from cell to lumen through apical Na^+/H^+ and/or K^+/H^+ exchangers (see **Chapter 5.1**). The pump also establishes a favorable electrical gradient for the movement of chloride ions from cell to lumen, largely by transcellular transport via chloride channels (see below). Water movement is largely a secondary consequence of active ion transport, the two processes probably being coupled by osmosis. There is some evidence in *Drosophila* that water movement is additionally facilitated by water channels of the aquaporin family (Dow *et al*., 1995) (**Figure 2**). The control of fluid transport in the tubules in response to environmental stimuli is regulated by a number of second messenger pathways including cAMP, cGMP, nitric oxide, and calcium (review: Dow and Davies, 2001). In some species, cation and anion transport occur at largely separable loci within the tubule. In *Drosophila*, cation transport occurs across the apical membrane of principal cells, whereas anion flow is through the stellate cells (O'Donnell *et al*., 1996, 1998). In addition, there is evidence to suggest that transcelluar movement of water via aquaporin water channels in *Drosophila* may also occur specifically through stellate cells (Dow *et al*., 1995, 1998). In contrast, the tubules of *Rhodnius* have only one secretory cell type, through which both cation and anion movement occurs (**Figure 2**).

2.8.2.2. Insect Tubule Anatomy

The general anatomy and cellular architecture of insect Malpighian tubules share a number of common features. They are usually simple tubular structures made up of a single layer of epithelial cells surrounding a lumen. They open into the hindgut, into which excretory products are expelled. Most tubule cells possess the typical attributes of a transporting epithelium such as a distinct apico-basal polarity, with the apical surface facing the lumen, elaboration of membrane surfaces to form microvilli and enrichment with organelles such as mitochondria (**Figure 3**).

Despite these fundamental similarities there is considerable variation in the form and number of Malpighian tubules across the insect class (**Figure 4**). Generally, the Malpighian tubules lie freely in the hemolymph of the body cavity (**Figure 4a**) but, in some species, regions of the tubule can form intimate contacts with parts of the gut. Some tenthredinid and lepidopteran larvae and many beetles have a cryptonephridial arrangement, where the distal ends of the tubules associate with the rectum, with which they are enclosed in a common membrane and muscular sheath (Ishimori, 1924) (**Figure 4b**). Such arrangements permit maximum water reabsorption and are often associated with species living in particularly dry environments. Some species possess tubules of more than one

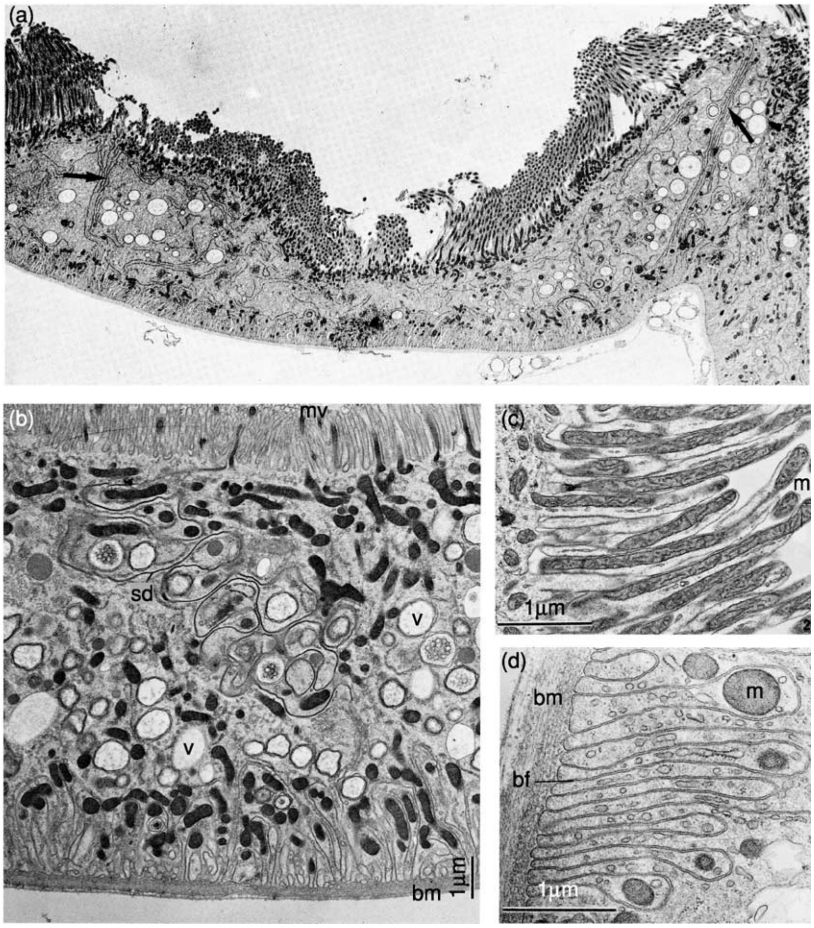


Figure 3 The secretery region of the Malpighian tubules of adult *Rhodnius prolixus* (a) and *Periplaneta americana* (b–d). The Malpighian tubules are a single cell-layered epithelium with pronounced apicobasal polarity. In both species, the apical membrane is densely populated with microvilli, and the basal membrane forms convoluted infoldings that can clearly be seen at higher magnification (c, microvilli and d, basal infoldings). Arrows in (a) show intercellular junctions; bf, basal folds; bm, basement membrane; m, mitochondrion; mv, microvilli; sd, septate desmosome; v, vacuole. Scale bar in (b–d) = 1 μm. (Reproduced with permission from Berridge, M.J., Oschman, J.L., **1972**. Transporting Epithelia. Academic Press, London.)

type; for example, in the curculionid *Apion*, there are four long tubules and two short tubules forming globular or flask-shaped glands (Holmgren, 1902). Branched tubules are also found sometimes. For example, in chrysomelids such as *Galerucella*, there are two types of tubule, two short unbranched anterior tubules, and four elongated posterior tubules, which unite on each side into a common stem, and branch on the surface of the rectum (Heymons and Lühmann, 1933) (**Figure 4c**). Finally, in some species, the tubules anastomose with each other to form closed loops, as in *Gnaptor*, *Tipula*, and cecidomyids (Gorka, 1914; Bodenheimer, 1924) or form crossings through which the lumen is continuous, as in some Homoptera (Licent, 1912). The tubules can also vary considerably in number, with some Hemiptera and various coccids possessing only two, while the locust *Schistocerca gregaria* has in excess of 200 (**Figure 4d**).

The variety in form observed in tubules of different species represents different ways of achieving adequate excretory capacity. For example, the tubules of species with high numbers of tubules are often short, whereas those from species with few tubules are often long and convoluted. As insects grow, there is a concomitant requirement for an increase in excretory capacity, and insects vary in the way that they cope with this challenge. In some species, increased capacity is achieved by cell growth, resulting from endoreplication of tubule cell DNA, without further cell division, whereas other species produce additional tubules throughout life so that, depending on the species, the adult number is from 50 to 250 (**Figure 4d**). Of the groups studied to date, Diptera, Lepidoptera, Coleoptera, and Hemiptera adopt the first strategy, while Orthoptera, Dictyoptera, and Hymenoptera adopt the second, though some ants and bees appear to

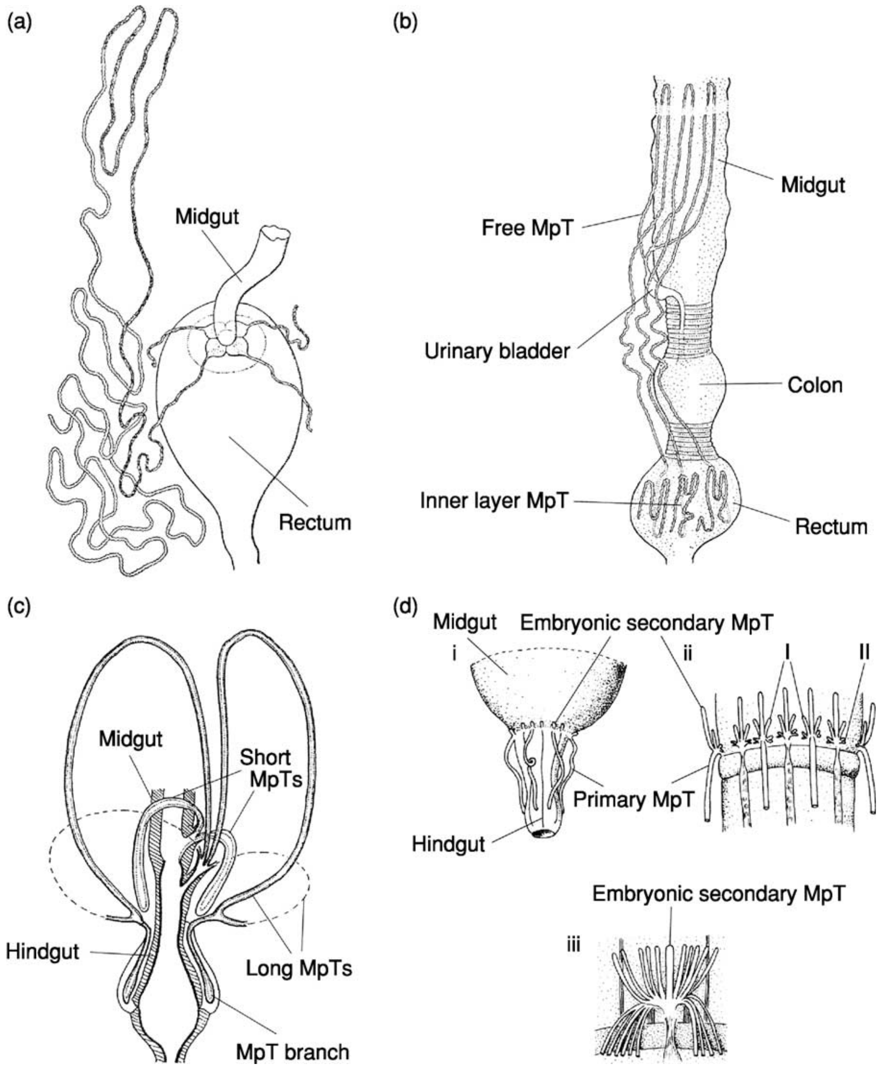


Figure 4 Diversity of Malpighian tubule form in insects. (a) *Rhodnius prolixus* has four free floating Malpighian tubules (only one tubule is shown in its entirety). (b) The Malpighian tubules of *Vanessa urticae* have a cryptonephridial arrangement, where the distal ends are intimately associated with the rectum, buried beneath a membrane and muscular sheath. (c) *Galerucella* has six Malpighian tubules, two short unbranched and four long branched tubules. The long tubules unite on each side into a common stem which then splits into three terminal branches lying in a chamber on the surface of the rectum. (d) The Malpighian tubules of (i) embryonic, (ii) second instar, and (iii) adult *Schistocerca* (a single cluster is shown), illustrating the continued production of new tubules during postembryonic development (I, first instar secondary tubules; II, second instar secondary tubules). ((a–c) Reproduced from Wigglesworth, V.B., **1939**. The Principles of Insect Physiology. Methuen, London; and (d) reproduced with permission from Savage, A.A., **1956**. The development of the Malpighian tubules of *Schistocerca gregaria* (Orthoptera). *Q. J. Microsc. Soc. 97*, 599–615.)

adopt both, retaining four tubules through embryonic and larval life but replacing them with large numbers of tubules (over 50) in the adult (review: Wigglesworth, 1939).

2.8.2.3. The Anatomy and Function of *Drosophila* Malpighian Tubules

In *Drosophila*, there are four Malpighian tubules, a longer anterior and a shorter posterior pair (Figure 5). The tubules contain two major cell types with separate developmental origins – the principal cells (PCs) and the stellate cells (StCs) (see Section 2.8.7). The anterior tubules each contain 144 ± 10 PCs and 33 ± 0.4 StCs, whereas the posterior tubules each contain 103 ± 8 PCs and 22 ± 0.4 StCs (Janning *et al.*, 1986; Skaer and Martinez-Arias, 1992; Sozen *et al.*, 1997). Each tubule pair is joined by a common ureter that enters the intestine at the junction between the hindgut and midgut. The tubules float freely in hemolymph of the body cavity.

At the ultrastructural level, all cells of the tubule have a polarized architecture. The basal region of the cell faces the hemolymph, with the membrane in this region containing numerous infoldings of

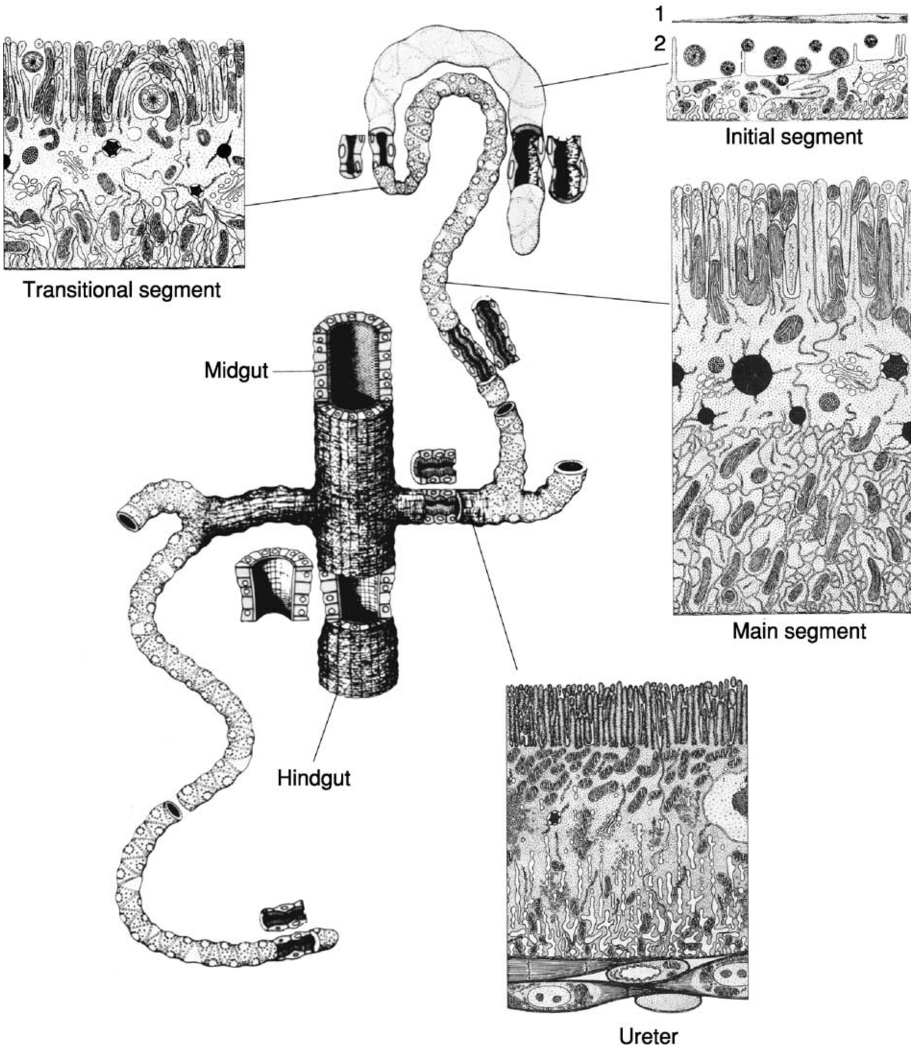


Figure 5 Representation of the Malpighian tubules of *Drosophila melanogaster*. *Drosophila* has four tubules, a longer anterior and shorter posterior pair (one tubule of each pair is depicted). Each tubule can be divided into four distinct regions based on proximodistal differences in cell structure: initial, transitional and main segments and the ureter. The principal cells (PCs) in the initial segment (2) are flattened; they have numerous basal infoldings and possess sparse, short microvilli. The more distally located cells often contain vesicles. The stellate cells (StCs) in the initial segment (1) are also flattened, but unlike the PCs (2), they do not have microvilli or basal infoldings, nor do they contain vesicles. The lumen in this region of the tubule is characteristically filled with many spherical concretions. The cells of the transitional segment are similar to those of the main segment but the microvilli often form arched structures. In the main segment, the PCs have complex basal infoldings and a dense brush border formed by long micovilli. Storage vacuoles are also present in the PCs, but not StCs. The StCs in the main segment are smaller and flatter, and their microvilli are shorter than the PCs. In addition, the StC luminal surface is far smaller than that of the PCs. The cells of the distal ureter are similar to those of the main segment. Proximally, the ureteric cells become more midgut-like, with fewer but wider basal infoldings that penetrate deep within the cell and are ensheathed in visceral mesoderm and invested with tracheoles. (From Wessing, A., Eichelberg, D., **1978**. Malpighian tubules, rectal papillae and excretion. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*, vol. 2c. Academic Press, London, pp. 1–42.)

various depths. The apical side of the cell faces the tubule lumen and possesses microvilli, the density and size of which vary, depending upon the proximodistal position of the cell along the tubule length. Mitochondria are enriched both basally, between the infoldings, and in the apical region at the base of the microvilli, into which they often penetrate. The nucleus and other organelles, such as the Golgi apparatus, endoplasmic reticulum, as well multivesicular bodies, are generally found in an intermediate region of the cell (Wessing and Eichelberg, 1978) (**Figure 5**). The cells of the mature tubules are bound by septate junctions but not *zonulae adherentes*, which are present during early development but are then disassembled towards the end of embryogenesis (Tepass and Hartenstein, 1994).

Differences in cell structure exist between the cells along the proximodistal axis of the tubule (Wessing and Eichelberg, 1978). In the anterior tubule, four structurally defined domains have been described: an initial segment at the distal end of the tubule, a short transitional segment, a main segment and a proximal segment (**Figure 5**). This structural classification has been validated by a more recent molecular analysis. Sozen *et al.* (1997) used an enhancer trap technique to reveal patterns of gene expression in the tubules of adult flies. This method showed that some genes are differentially expressed in the tubules, and that these expression domains often correspond to those regions defined by structural criteria. Furthermore, this technique uncovered previously unidentified tubule domains. For example, some enhancer trap lines revealed differences in gene expression between morphologically indistinguishable PCs, suggesting that, although superficially identical, they perform different functions.

Proximodistal differences along the tubule reflect differences in the physiological function of the cells. Primary urine is secreted into the distal region of the tubule (initial and transitional segments), where the epithelium is relatively leaky and, as it flows down the tubule, it is modified by selective reabsorbtion and further inward transport. Modification of the primary urine takes place principally in the main segment, where the apical membrane is decorated with many microvilli to maximize surface area. In this region, useful molecules, such as glucose, are selectively reabsorbed and toxic or unwanted substances are transported into the lumen.

2.8.3. Allocation of Tubule Primordial Cells

2.8.3.1. Origin of the Posterior Gut

Fate mapping experiments (Hartenstein *et al.*, 1985) have shown that the posterior gut arises from two neighboring anlagen: the endodermal posterior midgut anlage and the ectodermal proctodeum that will subsequently give rise to both the hindgut and Malpighian tubules. The posterior gut primordia are specified by the activation of the maternally supplied Torso receptor tyrosine kinase at the posterior of the embryo (St. Johnston and Nüsslein-Volhard, 1992). Signaling through this receptor results in the transcriptional activation, in overlapping posterior caps, of two zygotic transcription factors encoded by *tailless* (*tll*) and *huckebein* (*hkb*) (Weigel *et al.*, 1990). The *hkb* gene is expressed in a smaller, more posterior domain than *tll* and establishes the posterior border of the proctodeal ring (Brönner *et al.*, 1994). The Tll protein acts to establish the proctodeal ring by activating the expression of *brachyentron* (*byn*), *forkhead* (*fkh*), *bowel* (*bowl*), and *wingless* (*wg*) (Mahoney and Lengyel, 1987; Pignoni *et al.*, 1990; Weigel *et al.*, 1990; Kispert *et al.*, 1994; Diaz *et al.*, 1996; Singer *et al.*, 1996; Wang and Coulter, 1996; Wu and Lengyel, 1998). Forkhead in turn activates the expression of the zinc-finger transcription factor Krüppel (Kr) which, like Fkh, is expressed as a cap over the posterior pole of the blastoderm embryo and subsequently in the amnioproctodeal invagination during gastrulation and germband extension (Knipple *et al.*, 1985; Gaul and Jäckle, 1987; Gaul and Weigel, 1990). In addition to these fators, *caudal* (*cad*), which encodes a homeodomain transcription factor, is required for normal posterior gut development (Wu and Lengyel, 1998). The *cad* gene is expressed both maternally in a posterior to anterior gradient and, subsequently, zygotically in a stripe of cells that corresponds to the anlagen of the hindgut and the anal pads (Wu and Lengyel, 1998). In the absence of *cad* expression, terminal expression of *wg* and *fkh* is either abolished or diminished, leading to misspecification and subsequent death of the hindgut primordium (Wu and Lengyel, 1998).

2.8.3.2. Specification of the Tubules: Signaling between Gut Compartments

The tubules originate within the ectodermally derived hindgut primordium in a region adjacent to the midgut anlage (Hartenstein *et al.*, 1985). During gastrulation, the posterior gut primordia invaginate and come to lie internally within the embryo. Specification of tubule fate is first evident soon after gastrulation is complete, during early stage 9 at 4.5 h after egg laying (AEL), when *Krüppel* (*Kr*) refines from a broad domain of cells to a narrower stripe of cells that will form the tubules (see Section 2.8.3.3) (Gaul *et al.*, 1987; Gaul and Jäckle, 1987). The *fkh* gene continues to be expressed throughout the gut, indicating that other factors must regulate *Kr* after cellular blastoderm to restrict its expression. Fate mapping (Hartenstein *et al.*, 1985) reveals that tubule cells are not allocated until after cellular blastoderm, and therefore cell specification must take place some time between the onset of gastrulation and the point at which *Kr* expression refines.

Although the tubules arise from the ectodermal primordium shared with the hindgut, juxtaposition between this tissue and the endodermally derived midgut is necessary for tubule specification. If the midgut is ablated genetically in embryos

doubly mutant for *huckebein* (*hkb*) and *serpent* (*srp*), two genes required for midgut development, tubule cells are never allocated but instead, like cells in the midgut domain, become transformed into hindgut (Ainsworth *et al*., 2000; Ainsworth *et al*., unpublished data). These results are consistent with a hypothesis in which an instructive signal emanating from the midgut is required to specify tubule fate in the shared hindgut/Malpighian tubule primordium. In the absence of the midgut, and therefore the hypothesized signal, tubule fate is not specified and the transformed tissue follows a hindgut fate. The nature of this possible signal or signals is not yet known. Interestingly, a deficiency screen uncovering approximately 70% of the whole genome did not reveal any loci in which tubule specification failed completely (Harbecke and Lengyel, 1995). It is therefore possible that multiple signals acting either additively or redundantly are required for tubule fate specification.

In addition to this putative inductive signal(s) emanating from the midgut, signaling through the Wnt pathway is required at this early stage for the normal specification of tubule cells. Attenuation of either *wg*, or the pathway activators *armadillo* (*arm*; encoding the *Drosophila* β-catenin) and *dishevelled* (*dsh*) leads to a reduction in the number of tubule cells specified. In contrast, loss of the pathway inhibitors *naked* (*nkd*) or *zeste-white3* (*zw-3*)/*shaggy* (*sgg*) result in the specification of supernumerary tubule cells (Ainsworth *et al*., unpublished data). The *wg* gene is expressed in the innermost region of the proctodeum, where tubule cells arise during early stage 9 (Baker, 1988), suggesting that the Wg pathway activity could be a permissive signal for tubule cell allocation (Ainsworth *et al*., unpublished data).

2.8.3.3. Tubule Specification and Tubule Eversion

During stage 9 of embryogenesis, before any morphological differences are manifest, tubule cells can be distinguished from hindgut cells by changes in the expression pattern of Kr. At this stage, Kr expression refines from a broad domain throughout the hindgut to a narrow band of cells and then to four small clusters that will soon evert to form the tubule primordia. Previous reports have suggested that an early activity of Kr is to initiate the expression of the gene encoding the homeodomain-containing transcription factor Cut in the tubule primordium (Liu and Jack, 1992). Contrary to these data, however, it is found that, in the absence of Kr, Cut expression is initiated during stage 9, but is not subsequently maintained (Wan, 2001).

The combined activity of Kr and Cut are necessary for normal tubule eversion (Ainsworth *et al*., unpublished data). Tubule cells in embryos mutant for either *Kr* or *cut* fail to make the normal changes in cell shape, and either remain in the hindgut and proliferate in a manner typical of this tissue (in *Kr* mutants) (Gloor, 1950; Redemann *et al*., 1988; Harbecke and Janning, 1989) or form multilayered blisters that never develop into tubular structures (in *cut* mutants) (Liu and Jack, 1992). The transformation of tubule fate towards hindgut fate in *Kr* mutant embryos has led to the suggestion that *Kr* acts as a homeotic gene for tubule development (Liu and Jack, 1992). However, this transformation is incomplete as, later in development, the transformed cells secrete uric acid, a function characteristic of tubule cells (Skaer, 1993). For these reasons, other unknown factors besides *Kr* and *cut* must also be involved in directing tubule specific cell differentiation.

The tubules evert from the gut during stage 10 as four small buds, each containing approximately 20 cells. The newly formed tubule buds retain their apicobasal polarity as they evert. The genes regulated by Kr and Cut, which drive morphological rearrangement leading to tubule eversion, are not yet known but are likely to include molecules which regulate the cytoskeleton, modulate cell junctions and adhesion, and direct cell movement and cell shape changes.

The laterally positioned buds give rise to the anterior tubules, whereas the posterior tubules grow from the dorsally positioned buds (**Figure 1**). The exact details of how anterior versus posterior fate is specified are not known yet. The two pairs emerge at different positions relative to the dorsal–ventral axis and, therefore, one possibility is that anterior/posterior tubule fate may be controlled by the same factors that also pattern this axis (see **Chapter 1.2**). A second possibility is that the epidermal growth factor (EGF) pathway may be involved, because embryos with mutations in this pathway develop two, rather than four, tubules. In line with this, an EGF receptor ligand, Spitz (Spi), is secreted from the embryonic midline (Golembo *et al*., 1996). It is possible that this source of Spitz generates a morphogen gradient, to which the tubule primordial cells are exposed and specified in a concentration dependent manner.

2.8.4. The Tip Cell

2.8.4.1. The Tip Cell Lineage

Soon after the tubules bud from the gut at stage 10 of embryonic development, approximately 5 h AEL, a new cell lineage is born in each of the four tubule primordia, the tip cell lineage. This lineage arises

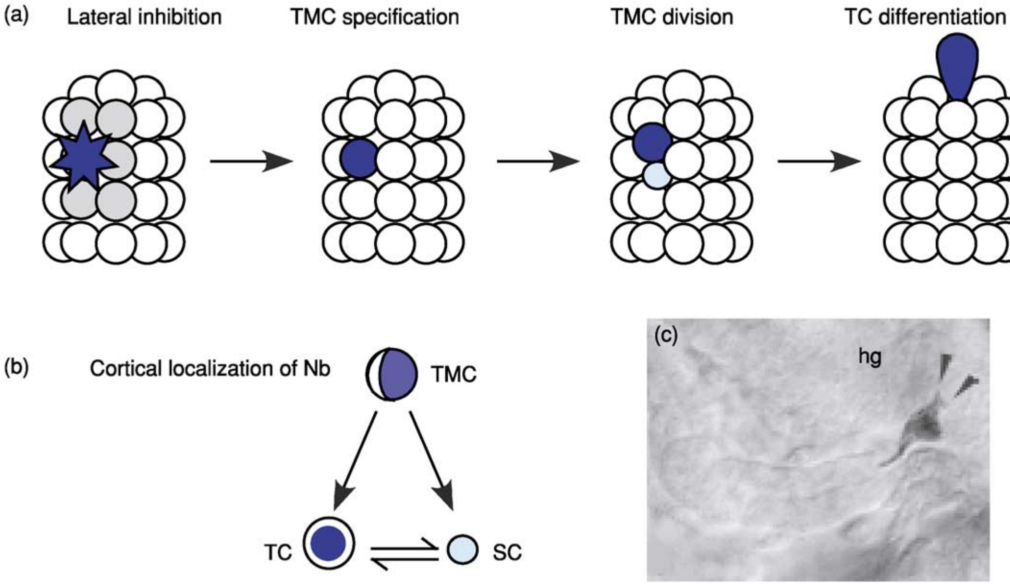


Figure 6 Specification of the tip cell lineage. (a) The tip mother cell (TMC) (dark blue) is selected from a group of proneural gene-expressing cells (gray) by a process of lateral inhibition. The TMC divides once to produce the tip cell (TC) (dark blue) and the sibling cell (SC) (pale blue). The TC differentiates, undergoing a characteristic change in morphology. (b) TC vs. SC fate is determined by asymmetric division of the TMC. The Numb (Nb) protein (white) is asymmetrically localized to one side of the TMC, and is inherited by a single daughter cell. Numb biases the competitive interaction that occurs between the two daughter cells, so that the cell that receives Nb becomes the TC. (c) A posterior MpT from a stage 15/16 *Drosophila* embryo stained with the TC-specific antibody 22C10, showing the long apical extension of the TC projecting into the tubule lumen, and the basal processes (arrowheads) that contact the nerve running along the hindgut (hg).

from the specification of the tip mother cell (TMC), a large cell morphologically distinguishable on the basis of its size relative to the other cells in the tubule (Hoch *et al*., 1994; Wan *et al*., 2000) (**Figure 6**). The TMC divides once, approximately 6.5 to 7.5 h AEL, during cell division cycle 16 (see Section 2.8.5.1), to produce two daughter cells, the tip cell (TC) and the sibling cell (SC) (**Figure 6**). The TC retains its distal position protruding from the tip of the tubule and forming a long apical extension into the tubule lumen. At this stage the TC exits the cell cycle and no longer divides. The TC and SC play at least two important roles in subsequent events during tubule development: (1) they are required for the regulation of cell division (see below and Section 2.8.5), and (2) they are required for tubule positioning as the tubules elongate (see Section 2.8.6).

The TC is required for cell proliferation in the tubule. If the TC is removed, either surgically (Skaer, 1989) or genetically (Hoch *et al*., 1994), a subset of the normal pattern of cell proliferation does not occur. TC-mediated control of cell division is described in detail below (see Section 2.8.5). TCs are not confined only to *Drosophila* embryonic tubules; they are also found in the developing Malpighian tubules of *R. prolixus*, in the locust *S. gregaria*, and in the cockroach *Periplaneta americana* (Skaer, 1989). The TCs of these other species share much in character with those of *Drosophila*: they are large, nonproliferative cells located at the distal end of each tubule. Cell ablation experiments have confirmed that the TC of *Rhodnius* is also required for normal cell division (Skaer, 1992), suggesting that TC-mediated control is a general mechanism for patterning cell division in insect tubules.

2.8.4.2. Specification of the TC Lineage

Specification of the TC lineage occurs by a mechanism reminiscent of the specification of neuroblasts in the neurogenic ectoderm (Hartenstein and Campos-Ortega, 1986; Hoch *et al*., 1994) (see **Chapter 1.10**). Before the tubules have everted, during late stage 9/early stage 10 (approximately 4.5 h AEL), expression of the proneural genes *achaete* (*ac*), *scute* (*sc*), and *lethal of scute* (*l'sc*) becomes apparent in four groups of 6–8 cells in the proctodeum (Hoch *et al*., 1994; Wan *et al*., 2000) (**Figure 6**). A little later, at the end of stage 10 (5–5.5 h AEL), proneural gene expression resolves to a single cell within each cluster, the TMC. In embryos lacking proneural gene function, the TMC is never specified, indicating that these genes, and those that they regulate, are important for TMC specification (Hoch *et al*., 1994). The TMC is singled out from

the proneural cluster by a process of lateral inhibition mediated by neurogenic components such as the ligand Delta (Dl), the receptor Notch (N), and elements of their signal transduction machinery (Brand and Campos-Ortega, 1988; Skeath and Carroll, 1992). The neurogenic gene products mediate competitive interactions between the cells in the cluster, such that one cell continues to express the proneural genes and subsequently adopts the TMC fate, while the remaining cells downregulate proneural gene transcription and adopt an alternative fate (see Section 2.8.5.3) (**Figure 6**). In the absence of neurogenic gene function, lateral inhibition fails and all cells in the cluster retain proneural gene expression and develop into TMCs.

The molecular details of the initial patterning of the proneural cluster within the tubule primordia are not known, but the process is likely to be under the control of the genes that pattern the posterior gut in the early embryo. An example of such a regulator is Wg, which is required to maintain *ac* gene expression in the cluster, and subsequently in the TMC. In embryos mutant for *wg*, *ac* expression begins to fade at 5–5.5 h AEL and is completely absent by stage 11 (6.5 h AEL) (Wan *et al.*, 2000). The requirement for a Wg signal at this stage is likely to be permissive rather than instructive, because ectopic expression of *wg*, although sufficient to maintain *ac* expression for a limited period, is not sufficient to establish supernumerary TMCs (Wan *et al.*, 2000).

The TMC undergoes one asymmetric cell division to produce two daughters; the tip cell (TC) and its sibling (SC) (**Figure 6b**). The TC versus SC cell fate distinction is again established by neurogenic gene-mediated lateral inhibition but, in this instance, the outcome is biased by the Notch inhibitor Numb (Nb) (Uemura *et al.*, 1989; Rhyu *et al.*, 1994; Berdnik *et al.*, 2002). Localization of the Nb protein in the TMC is initially symmetric but, as the cell enters mitosis, Nb localizes to a crescent on one side of the cell (**Figure 6b**). When the TMC divides, Nb segregates to a single daughter cell. The presence of Nb in one of the daughters renders that cell deaf to inhibitory signaling through Notch; it continues to express the proneural genes and subsequently adopts the TC fate. Without Nb, the SC transduces the Notch inhibitory signal, downregulates proneural gene expression, and so adopts the alternative, SC, fate. In *nb* mutant embryos, both daughters of the TMC transduce the Notch signal and two SCs form at the expense of the TC (Wan *et al.*, 2000). Conversely, ectopic expression of *nb* in both daughter cells suppresses Notch signaling and results in tubules with two TCs and no SCs (Wan *et al.*, 2000).

Similarities between the mechanisms that establish the TMC lineage and the neuroblast lineage have already been drawn. Further similarities between the TC and those of neural lineages are evident later in TC development. Towards the end of embryogenesis, the TCs begin to express a number of molecules that are typically expressed in neural cells such as *futsch* (the epitope recognized by the 22C10 antibody) (**Figure 6c**), *synaptotagmin* (*syt*), *synaptophysin* and the *Cysteine-string protein* (*Csp*) (Hoch *et al.*, 1994). In addition, during stage 16 the posterior TCs become closely associated with paired nerves that run on either side of the hindgut (Hoch *et al.*, 1994). Expression of these neuronal markers and the close association of the posterior TCs with hindgut nerves suggest the TC may have some neuronal function in tubule physiology. However, this possibility has not been examined further.

2.8.5. Proliferation of Malpighian Tubule Cells

2.8.5.1. Tubule Cell Number and the Pattern of Cell Division

There are two main cell types in the Malpighian tubules of *Drosophila*: the PCs and the StCs. The PCs arise from the division of cells derived from ectodermal cells in the proctodeum, whereas the StCs are recruited from a different tissue primordium and become incorporated into the postmitotic tubules. Control of cell division in primordial PCs is dealt with in this section, while StC recruitment is described in more detail below (see Section 2.8.7).

The number of PCs in mature Malpighian tubules is relatively constant between individuals: 484 cells, with 103 ± 8 in each posterior tubule and 144 ± 10 in each anterior tubule (Janning *et al.*, 1986). These cells are generated by division of primordial cells allocated in the proctodeum during stage 9 (see Section 2.8.3). The mature number of PCs is reached once proliferation is complete, midway through embryogenesis, at approximately stage 13.

Early divisions of the zygotic nuclei in *Drosophila* are synchronous and are not accompanied by cytokinesis until the 14th mitotic cycle. At this stage, cellularization occurs and cell division becomes spatially regulated (Foe, 1989). The first division of this type in the posterior gut (cycle 14) occurs in a domain that encompasses the hindgut and the tubule cells before they are specified (3.5 h AEL) (Foe, 1989). Once the tubule buds form, the pattern of cell division becomes distinct from that of the hindgut epithelium. In the first tubule-specific division (cycle 15), which occurs at 5.5–6 h AEL,

all cells in the tubule primordia divide synchronously. The second wave of mitosis (cycle 16, 7 h AEL) involves only a subset of tubule cells, those cells on the posterior side of the tubule, which again divide synchronously. Subsequent cycles (cycle 17 onwards) are nonsynchronous and are restricted to the distal region of the four tubules.

2.8.5.2. Regulation of Cell Proliferation

The invariant pattern of cell division described above is tightly regulated through the expression of two genes, *string* (*stg*) and *cyclin E* (*cycE*), that are limiting components of the cell cycle. The *stg* gene encodes the *Drosophila* homolog of the Cdc25 phosphatase and is involved in the regulation of the G_2/M transition of the cell cycle, whereas CycE regulates the G_1/S transition. The early synchronous divisions, up to and including cycle 13, are sustained by maternal contribution of these two gene products. However, the first cycle after cellularization (mitosis 14) and all subsequent divisions require the zygotic expression of *stg* to propel the cells though the G_2/M transition (Edgar and O'Farrell, 1990; Edgar *et al*., 1994). By cycle 17, maternal supplies of CycE are exhausted and, thus, division becomes additionally dependent on regulation of zygotic *cycE* expression to progress through the G_1/S transition of the cell cycle (Richardson *et al*., 1993; Knoblich *et al*., 1994). Cell division in the tubules is regulated by two pathways: by the Wg signaling pathway and by the *Drosophila* epidermal growth factor receptor (EGFR) pathway (Skaer and Martinez-Arias, 1992; Kerber *et al*., 1998; review: Duronio, 1999).

The *wg* gene is expressed in the proctodeum of stage 9/10 embryos and, at the time the tubule buds evert, all tubule cells express the gene. Once the tubules have completed their eversion, *wg* expression becomes restricted to cells on the posterior side of each tubule, and this pattern persists until late stage 12 (approximately 9 h AEL), with *wg* expression extending along the whole posterior length of the tubule (Skaer and Martinez-Arias, 1992). This pattern of *wg* expression covers the domain in which tubule cells divide during cycles 15 and 16. In *wg* mutant embryos the tubule cells arrest after M15 without going through the S phase of cycle 16 (Ainsworth *et al*., unpublished data). The molecular basis of Wg regulation of cycle 16 is not yet known. However, the role of the Wg pathway as a cell cycle regulator is separable from its role in the stabilization of TC fate. Thus, the Wg pathway has a dual requirement in the regulation of cell division in the tubules; in the posterior domain it permits cycle 16 progression, and secondly, stabilizes the TC, itself a source of the mitogenic signal, for cycle 17 and all subsequent divisions. In addition to these roles in regulating the mitogenic signal, the Wg pathway is required to pattern the cells that can respond to the signal. This role of the pathway is described in more detail below (see Section 2.8.5.3).

A mitogenic role for the EGF pathway in the tubules was indicated in early work showing that embryos mutant for either the EGFR or the gene encoding the ligand of the pathway, *spitz* (*spi*), possess very small tubules with a severe reduction in cell number relative to wild-type (Baumann and Skaer, 1993). More recent work has shown that EGFR pathway activity is required specifically for the late cell divisions in the tubules (Kerber *et al*., 1998). In embryos mutant for EGF pathway components, such as the EGFR or *seven-up* (*svp*), the early divisions (cycles 14–16) occur normally, but cell division arrests during cycle 17 (Kerber *et al*., 1998; Sudarsan *et al*., 2002). Previous work showed that late divisions, which are restricted to the distal region of the tubule, occur only in the presence of the TC and SC (Skaer, 1989; Hoch *et al*., 1994), thus suggesting that the TC/SC are the source of the EGF signal (**Figure 7**). This hypothesis was confirmed by the discovery that Rhomboid (Rho) and Star (S), two proteins required for the processing of Spi into its active secreted form (Lee *et al*., 2001; Urban *et al*., 2001), were expressed in both the TC and SC (Kerber *et al*., 1998; Sudarsan *et al*., 2002) (**Figure 7b**). Furthermore, the expression of *stg* and *cycE*, both of which are normally expressed in the distal region of the tubule during late divisions, is abolished in embryos mutant for either the EGFR, or for an effector gene of the pathway, *svp*. These data suggest a model whereby the EGF ligand Spi is the mitogenic signal for late tubule divisions. This signal, secreted from the TC/SC, stimulates the EGFR pathway in neighboring cells, activating Svp, which, in turn, induces the expression of *stg* and *cycE* and passage through the cell cycle (**Figure 7**).

Interestingly, the mature tubules of EGF pathway mutants have far fewer cells (between 23 and 27 cells) than would be predicted simply on the basis of cell cycle arrest at cycle 17 (wild-type tubules at the start of cycle 17 have approximately 70 cells). This discrepancy is due to the fact that the EGF pathway is additionally required to promote tubule cell survival (Sudarsan *et al*., 2002). In EGFR mutant embryos, tubule cell numbers begin to decline after cycle 16 and continue to do so until the end of embryogenesis, suggesting that cells die. If cell death is prevented in these embryos, the mature number

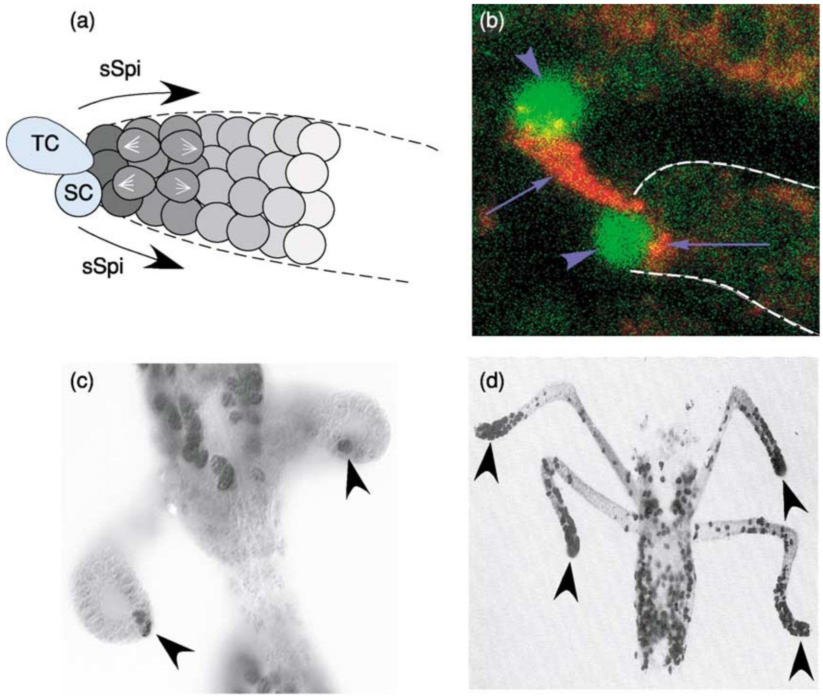


Figure 7 The tip cell (TC) and sibling cell (SC) are the sources of the mitogenic signal for late tubule cell division. (a) Schematic figure to show the TC and SC secretion of the epidermal growth factor (EGF) ligand Spitz (sSpi). A gradient of EGF pathway activity is generated in the tubule (dark gray, high activity; light gray, low fading to no activity), as sSpi diffuses from the TC and SC. Cell division occurs in response to EGF activity. (b) A confocal image showing expression of the serine-type peptidase, Rhomboid (red, arrows), specifically in the TC and SC (nuclear marker in green, arrowheads). Spi is only secreted after cleavage by Rho, and thus the TC and SC form the sole source of sSpi in the tubule. (c) Dissected tubules of a *Drosophila* embryo stained for the incorporation of BrdU to mark S phase of cycle 17; only cells in the distal region of the tubule, close to the mitogenic source, enter this cycle (arrowheads). (d) Dissected tubules from a *Rhodnius* embryo 55% through development stained for the incorporation of BrdU. Cells in S phase are predominantly in the distal region of the tubules (arrowheads) close to the TC.

of tubule cells is 74 ± 1, which corresponds exactly to the number of cells predicted on the basis of the mitogenic phenotype (Sudarsan *et al*., 2002).

2.8.5.3. Restriction of the Mitogenic Response

As the EGF ligand Spi is secreted from the TC and SC, only distal cells that are close to the source of Spi will receive the mitogenic signal and subsequently divide. Overlying this is a second level of regulation conferred by a restricted domain of competence to respond to the signal. Not all cells are capable of dividing, because they are not primed to respond. The molecular basis of this competency is the expression of two EGF pathway effector genes, *seven-up* (*svp*) and *pointed-P2* (*pnt*). Only those cells that express these two factors can transduce the EGF signal to induce the expression of *stg* and *cycE* and, thus, respond by dividing. How is this competence domain established?

Although transcription of *svp* and *pnt* is regulated in later stages by the EGF pathway itself, their initial transcription is independent of the pathway and is under control of the proneural genes and the *wg* pathway respectively. The TMC is specified during stage 10, and arises by the selection of a single cell from a group of proneural gene-expressing cells by the process of lateral inhibition (see Section 2.8.4) (**Figure 6**). A consequence of this process is that proneural gene transcription is maintained and upregulated in the TMC, while it is extinguished in the remaining cells of the cluster. Therefore, two cell populations are established with regard to proneural gene activity: a TMC, in which proneural activity is high, and a second population with lower, transient levels of activity. These levels are important, because regulation of proneural target genes occurs in a concentration dependent manner. High levels of activity in the TMC establishes the TC/SC fate and induces the expression of *rho*. In contrast, the low, transient level of activity in the other cells of the cluster is sufficient to activate the transcription of *svp* but not *rho* (**Figure 8**). Thus lateral inhibition segregates two cell fates within the proneural cluster: the primary TMC fate; and a second cell fate, for those cells competent to respond to the mitogenic signal by virtue of their expression of *svp* (Sudarsan *et al*., 2002).

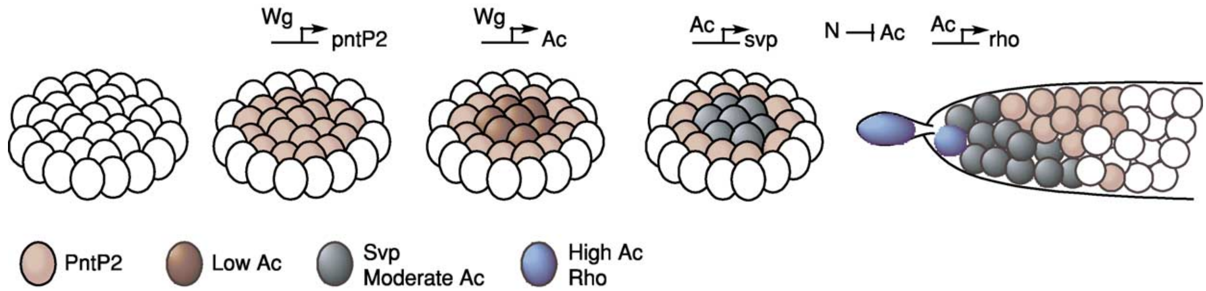


Figure 8 Model for the establishment of mitotic competence in *Drosophila* Malpighian tubules. Wg signaling activates the expression of *pntP2* in a subset of tubule cells (brown), and is required to establish proneural gene expression in the proneural cluster (represented by *ac*, dark brown). Moderate levels of Ac induce *svp* expression in the cluster (gray) but, as Ac expression increases in the TMC, a threshold for the initiation of *rho* expression is reached (blue). The TMC divides to produce the TC and SC (blue). As *ac* expression is lost from the SC by stage 12, the maintenance of *rho* expression from this time onwards is independent of Ac. The cells that originally formed the proneural cluster are competent to respond to EGF signaling by virtue of their expression of *pntP2* and *svp*. They divide in response to Spi secreted by the TC and SC.

These data also resolve a paradox relating to the phenotype of the neurogenic genes. Tubules of embryos mutant for any of the neurogenic loci have supernumerary TCs, because lateral inhibition fails, and all cells in the proneural cluster adopt a TC fate. Because the TC is the source of mitogenic signal, it might be expected that supernumerary TCs would induce more cells to divide, thereby producing tubules with many more cells than in the wild-type. However, in spite of excess signal, the tubules of neurogenic mutants have only 70 cells, the number found in tubules lacking TC/SCs. The discovery that lateral inhibition normally segregates two cell fates resolves this paradox. In neurogenic mutant embryos, all cells of the proneural cluster adopt the TC fate at the expense of the mitogenically competent cells. Therefore, despite the availability of elevated signal, no division occurs, because cells competent to do so have not been specified.

The second effector of the EGF pathway required to establish mitotic competence is *pnt*. The *pnt* gene is expressed on the posterior side of the tubules from stage 10 in a slightly wider domain than that of the proneural cluster, and is under control of the Wg pathway (**Figure 8**). It is not yet known whether this pathway directly upregulates the expression of *pnt*, but *pnt* expression is lost in *wg* mutant embryos and expands into the anterior domain of the tubule, when *wg* is expressed ectopically throughout the embryo (Sudarsan *et al*., 2002).

Thus, patterning of the later tubule cell divisions (which happens during stages 12–13) is established earlier in development, during stage 10. At this stage the progenitor of the mitogenic signal source, the TC and SC, is specified. In addition, a population of cells expressing both *svp* and *pnt* is established, and it is these cells that will respond to the mitogenic signal and subsequently divide. It is worth considering the reason why a competency domain needs to be established. Most tissues in the embryo go through three postblastodermal divisions and then cease to divide further. Therefore, the late tubule cell divisions are unusual and occur in a mitotically quiescent environment. It might be important to define the limits of division in such conditions so that, should mitogenic signaling become deregulated, the extent of the competence domain imposes an effective brake on tubule hyperplasia.

2.8.5.4. Endoreplication in the Postmitotic Tubules

Growth of the animal through larval life needs to be matched by increasing the excretory capacity of the tubules. In *Drosophila*, this is achieved not by increasing the number of tubule cells – cell division in the Malpighian tubules is complete by stage 13 of embryogenesis – but by cell growth. To permit this increase in cell size, every tubule cell undergoes several rounds of endoreplication (Smith and Orr-Weaver, 1991; Orr-Weaver, 1994). The tubules undergo seven endocytic cycles. The first endocytic cycle begins midway through stage 13, with all cells entering this cycle, and continues through larval and pupal development so that in the adult the DNA copy number is 252 (Lamb, 1982).

Endoreplication followed by cell growth also occurs in *Rhodnius* as a mechanism for increasing excretory capacity. Here, the first endocytic cycle occurs approximately 60% through embryonic development, as a wave involving every nucleus, starting with the proximal tubule cells and sweeping distally (Skaer, 1992). Unlike *Drosophila*, this first endocytic cycle is accompanied by a wave of nuclear but not cytoplasmic division, so that, by 75–80%

of development, all cells in the *Rhodnius* tubule are binucleate (Skaer, 1992). In *Rhodnius*, an endoreplicative cycle occurs at every larval molt, and this, in turn, is associated with an increase in cell size (Maddrell *et al*., 1985).

2.8.6. Morphogenesis, Cell Rearrangement, and Tubule Navigation

2.8.6.1. Tubule Morphogenesis

At stage 10, the tubules evert from the proctodeum forming four small buds, each containing approximately 20 cells. *Kr* and *cut* are expressed in the everting tubules during this time and, in the absence of either gene product, eversion does not occur normally, with tubule cells remaining in the hindgut or forming multilayered blisters on the hindgut (Gloor, 1950; Redemann *et al*., 1988; Harbecke and Janning, 1989; Liu and Jack, 1992). These phenotypes suggest that *Kr* and *cut* are likely to have a significant role in regulating factors that, in turn regulate morphogenetic events leading to eversion. A candidate for such a factor is *walrus* (*wal*), as mutations in this gene result in failure of eversion, with the tubule cells remaining as a ring in the proctodeum (Liu *et al*., 1999). The molecular nature of the *walrus* product, however, an electron transfer flavoprotein, does not immediately suggest what function it might play.

After eversion, until approximately stage 13, the main activity happening in the tubules is cell division. Relatively little cell rearrangement occurs during this phase, possibly because the mechanism by which cells rearrange and the associated modifications in cell adhesion are not compatible with cell division. Starting midway through stage 13 and continuing until stage 16, the tubules undergo a dramatic, but very stereotypical change in morphology (**Figure 1**). At stage 13 the tubules are relatively short, with approximately six to ten cells surrounding the tubule lumen (Skaer, 1993). Tubule elongation results from a series of convergent-extension movements so that, by stage 16 the length of each tubule has dramatically increased, while the diameter decreases, so that only two cells surround the lumen. In a similar manner, cell rearrangement in the tubules of *Rhodnius* begins only after cell division has ceased. As the tubules undergo convergent-extension, their length increases approximately sixfold and their diameter narrows, with the number of cells around the circumference of the lumen decreasing from 8 to 12 immediately before elongation, to just two cells afterwards (Skaer, 1992).

The process of convergent-extension is driven by organized cell rearrangement and is likely to be regulated by two classes of molecules: those providing spatial information to orchestrate the movement, and those that execute the cellular processes required for rearrangement. It would be sufficient to define a single axis to provide the spatial information required for cells to determine their position within the plane of this single cell-layered epithelium. The cells could then shift position relative to one another so that the net movement in the tissue occurs in a coherent manner. The molecular basis by which this is achieved is not known. Preliminary data, however, suggest that components of the signaling pathway that establishes planar polarity in other tissues are also required for normal convergent-extension movements in the tubules (Wan *et al*., 2001).

Mutagenesis screens for genes affecting tubule morphogenesis have identified genes required for the execution of cell rearrangement (Harbecke and Lengyel, 1995; Jack and Myette, 1999; Liu *et al*., 1999). Unsurprisingly, a number of these genes have direct roles in regulating cell shape, cell movement, and various aspects of cytoskeletal reorganization. The *zipper* (*zip*) gene which encodes the nonmuscle myosin heavy chain, is required and is necessary for the cell shape changes and movements associated with morphogenesis to occur (Young *et al*., 1993; Blake *et al*., 1999). The *ribbon* (*rib*) gene, which encodes a nuclear protein with a BTB/POZ domain, is also required for cell shape changes during tubule morphogenesis and may act by regulating the actin cytoskeleton (Jack and Myette, 1997; Blake *et al*., 1999; Shim *et al*., 2001). The *yolky/crooked neck* (*yok/crn*), *mummy* (*mmy*), *foreclosed* (*fcl*), *lines* (*lin*), and *buttonhead* (*btd*) genes all appear to be required for the elongation process, as the tubules in embryos mutant for these loci do not extend, but are instead distended and variable in diameter (Harbecke and Lengyel, 1995). However, the exact roles of these genes during elongation have not been elucidated.

In addition, members of the small Rho guanosine triphosphatase (GTPase) subfamily are required for normal elongation. Expression of either dominant negative or constitutively active versions of Rho, Rac, or Cdc42 result in defects in tubule elongation (Skaer *et al*., unpublished data). These small GTPases have been shown to have multiple roles in coordinating cell behavior during morphogenesis, including modification of the cytoskeleton and the adhesive properties of cells, junctional adhesion, and effects on gene transcription (Hall, 1998; Etienne-Manneville and Hall, 2002). Any one or a combination of these factors may cause the tubule defects observed when GTPase function is perturbed.

2.8.6.2. Organizing Tubule Arrangement within the Embryo

Throughout the elongation process, the tubules course a remarkably stereotypic path through the embryo and, by the time elongation is complete at stage 16, each tubule has taken up an invariant three-dimensional position within the body cavity (**Figure 1**). In addition, the TC of each tubule locates to an exact position within the embryo. One of the anterior TCs contacts the allary muscle of the dorsal vessel in abdominal segment A3 (Skaer *et al*., unpublished data), while the other one makes contact with the visceral mesoderm at the base of the ureter (Skaer, 1993). The two posterior TCs contact the paired nerves that run along either side of the hindgut (Hoch *et al*., 1994). The stereotypic path that the tubules take during elongation and the final destination of the distal tips critically require both the TC and the SC. In *numb* (*nb*) mutant embryos, in which two SCs but no TCs are formed, the precise configuration is lost, and the distal ends randomly locate within the embryo (Wan *et al*., 2000). A similar outcome occurs when SCs fail to be specified, a condition which can be induced experimentally by ectopic expression of *nb* in the TMC (Wan *et al*., 2000). These results indicate that both the TC and the SC have distinctive roles in ensuring the correct localization of the tubules within the embryo. It is not known if the TCs and SCs are required only to anchor the tubule tips at specific positions within the embryo or whether they have a pathfinding role in tubule navigation.

The distal tip cell (DTC) of the *Caenorhabditis elegans* gonad arm shares much in common with the TC in insect tubules. It too is a large cell located at the distal tip of a tubular epithelium, which regulates proliferation in the gonad by producing a mitogenic signal (Kimble and White, 1981; Austin *et al*., 1989; Henderson *et al*., 1994). In addition to this mitogenic role, the DTC also controls morphogenesis of the gonad arm, providing a paradigm for morphogenetic control executed at the level of a single cell. The DTC leads the outgrowth of each arm to produce the bilaterally hooked structure that characterizes the mature organ. *Cell death abnormal-5* (*ced-5*), a member of a gene family that also includes human DOCK-180 and *Drosophila myoblast city* (*mbc*) (Wu and Horwitz, 1998; Erickson *et al*., 1997), is expressed in the DTC and is required for the morphogenetic movements in the gonad. These genes encode regulators of the Rac GTPase (Klyokawa *et al*., 1998; Nolan *et al*., 1998) which has been shown to have multiple roles in controlling cell morphogenesis, for example, controlling cell protrusions required for cell movement (Hall, 1998; Ridley *et al*., 1992; Etienne-Manneville and Hall, 2002). It is possible that the TC and/or SC in *Drosophila* plays an analogous function to the DTC in controlling tubule navigation. This parallel may extend to the molecular mechanism by which morphogenesis is executed, because tubule navigation also goes awry in embryos mutant for *mbc* (Ainsworth *et al*., 2000).

A number of other factors have been implicated in morphogenetic movements in the *C. elegans* gonad including GON-1 (Blelloch and Kimble, 1999). GON-1 is a ADAMTS metalloproteinase, a proteolytic enzyme that degrades extracellular matrix components and so prepares the way for cell migration and tissue remodeling during organogenesis. The *Drosophila* genome contains a number of metalloproteinases but their function during morphogenesis has not been studied in detail. Indirect evidence for a role for metalloproteinases in tubule morphogenesis comes from studies into *papilin* (*ppn*), a large glycoprotein sharing a number of common domains with ADAMTS metalloproteinases (Kramerova *et al*., 2000). Papilin inhibits the activity of metalloproteinases *in vitro* and has been shown to cause defects in tubule morphogenesis, when ectopically expressed in the *Drosophila* embryo (Kramerova *et al*., 2000).

The data from the DTC in *C. elegans* and the TC/SC in *Drosophila* suggest that the activity of specific cells in guiding tissue morphogenesis may be a conserved feature of tubulogenesis. It will be interesting to examine the role of metalloproteinases and other molecules similarly expressed in these cells, to determine if the molecular basis of this control is also conserved. As the TC and SC have distinctive roles in tubule morphogenesis, it will also be interesting to discern the individual contribution of these two cell types.

2.8.6.3. Tubule Navigation: The Role of Other Tissues

As the tubules extend, they make contact with other tissues within the embryonic cavity, and these tissues are often necessary for proper tubule morphogenesis. It is likely that they act as substrates to provide traction for tubule movement. One example of this is the gut visceral mesoderm. In *heartless* (*htl*) mutants, in which the gut visceral muscle does not form (Beiman *et al*., 1996), the anterior tubules do not extend forward but become mislocalized within the body cavity (Skaer *et al*.,

unpublished data). In addition to this role, it is possible that these tissues have a more instructive role, by providing spatial cues to orient the tubules as they navigate.

2.8.7. Cell Recruitment to the Malpighian Tubules

2.8.7.1. Stellate Cells are Derived from a Separate Tissue Primordium

The mature tubules contain two main cell types, PCs and StCs, the latter being conspicuous in the adult by their very distinctive star-shaped morphology, from which they get their name (**Figure 9**). These two cell types perform different physiological functions in the tubules: PCs are the main channel for cation exchange, whereas StCs facilitate chloride ion and water movement (Dow *et al.*, 1995; O'Donnell *et al.*, 1996, 1998) (see Section 2.8.2.1) (**Figure 2**).

There is a total of 484 PCs (Janning *et al.*, 1986) and 110 StCs (Sozen *et al.*, 1997) in the four mature *Drosophila* tubules. The StCs are regularly interspersed along the length of the four tubules with the exception of the proximal domain, from which they are absent (**Figure 9**). Until recently, it was assumed that the StCs were derived from the same tissue primordium as the PCs, but recent work has shown that the origin of the two cell types is different. The 484 PCs are generated by division of the primordial cells derived from the ectoderm (see Section 2.8.5). In contrast, lineage tracing studies have shown that the StCs arise from the mesoderm and that they migrate to and subsequently integrate into the postmitotic tubules (Denholm *et al.*, 2003). The precise origin of the StCs is a region of posterior mesoderm that also gives rise to the caudal visceral mesoderm (CVM). The posterior mesoderm internalizes with the amnioproctodeal invagination during gastrulation and, by stage 9, lies over the hindgut. At this stage, a subset of the posterior mesoderm, the CVM, begins to migrate anteriorly. These cells subsequently provide the muscle founder cells for the longitiudinal visceral muscles of the midgut (Kusch and Reuter, 1999; Lorén *et al.*, 2001; Martin *et al.*, 2001) (**Figure 10a**). At stage 9, as the CVM begins to migrate anteriorly, the tubules evert from the hindgut and bisect this mesodermal population (**Figure 10a**). The subset of mesodermal cells on the posterior side of the tubules remain associated with the tubules and later become the StCs.

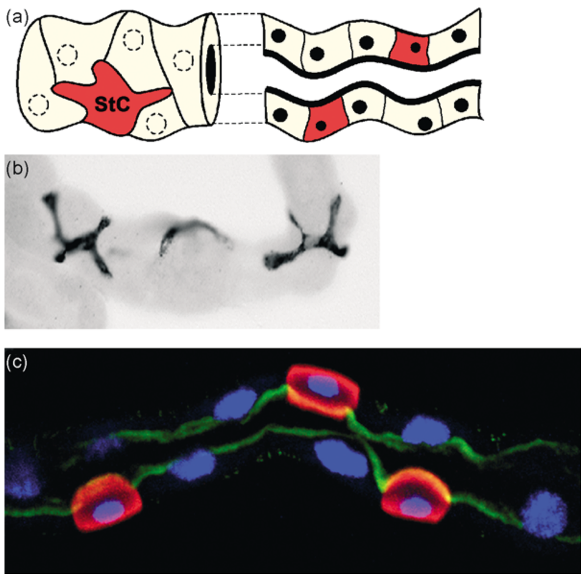


Figure 9 The cells of *Drosophila* Malpighian tubules. (a) Schematic representation of an adult Malpighian tubule. Two cell types are depicted: the principal cells (PCs) (yellow) and the stellate cells (StCs) (red). The PCs are interspersed with the StCs throughout the distal region of the tubule. (b) In the adult fly, the StCs have a star-shaped morphology from which they derive their name. In this experiment, the StCs are distinguished from PCs by a genetic label (they have succinic hydrogenase positive nuclei) used to trace lineage (see Denholm *et al.* (2003) for details). (c) The tubules from a third instar larva stained with an enhancer trap line (red) inserted in the *teashirt* (*tsh*) gene, which marks out StCs, for F-actin (green), and for DNA (blue). The StCs, but not PCs, express Tsh. Both cell types exhibit apicobasal polarity, as demonstrated by the accumulation of F-actin in the microvilli on the luminal side of the cell.

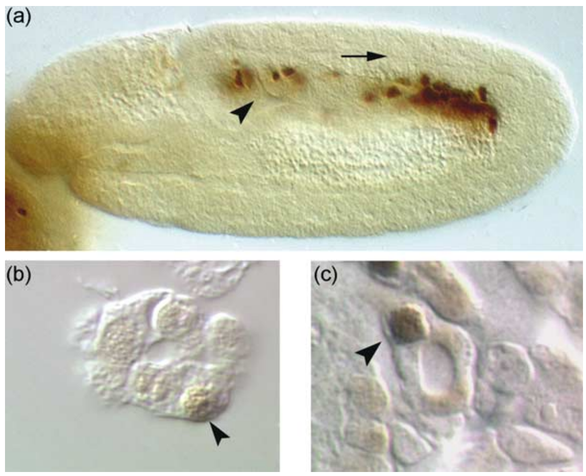


Figure 10 Stellate cells are derived from a mesodermal population and subsequently integrate into the tubule epithelium. (a) An extended germband embryo stained for an enhancer trap line that marks a subset of the posterior mesoderm. While the majority of this mesodermal population (the CVM) migrates anteriorly during stage 10–11 (arrow), a subset remains associated with the tubules as they evert from the hindgut (arrowhead). (b, c) Sections through a stage 13 embryonic tubule demonstrating a Tsh-positive StC (dark brown) in the process of integration (b) and a stage 15 embryonic tubule showing a Tsh-positive StC (dark brown) fully integrated into the tubule epithelium (c).

During the period between stages 9 and 12, the StCs adhere to the tubule epithelium and are mesenchymal in character. During stage 13, StCs start to express the transcription factor encoded by *teashirt* (*tsh*) and begin to integrate into the tubule epithelium (**Figure 10b** and **c**). Tsh is critical for this process, because StCs do not integrate in *tsh* mutant embryos (Skaer *et al*., unpublished data). During or subsequent to their integration, the StCs polarize and, by the end of embryogenesis, become epithelial in character with a defined apical membrane and a polarized cytoskeleton (Denholm *et al*., 2003). Notably, this mesenchymal to epithelial transition does not involve the activity of Crumbs (Crb), a protein required for establishing and maintaining epithelial integrity in ectodermally derived epithelia, as Crb is not expressed in the StCs.

The processes by which the StCs are specified, recruited, integrated, and subsequently polarized is still not completely understood. However, one factor that has been shown to play a role is Hibris (Hbs), a single transmembrane spanning immunoglobulin-like molecule. In *hbs* mutants, StC numbers are reduced to 68 ± 4 (cf. 110 in wild-type tubules), clearly demonstrating a role for *hbs* in StC development (Denholm *et al*., 2003). Hbs has been shown to interact with other immunoglobulin-like molecules such as Sticks and stones (Sns) and Dumbfounded (Duf) during myogenesis, where it is required for normal myoblast attraction and fusion (Artero *et al*., 2001; Dworak *et al*., 2001). Although the precise role of Hbs during StC development is not yet established, it is intriguing to speculate that similar heterotypic interactions may occur between Hbs in the StCs and other immunoglobulin-like molecules in the neighboring PCs, and that this interaction may be required in order for StC integration to be stabilized.

These data provide the first evidence that Malpighian tubules are derived from cells of a separate origin, and suggest that the development of these simple epithelial tubes may be more complex than previously thought. StCs are not just a peculiarity of *Drosophila*; they have been discovered in other Diptera including the fleshfly *Sarcophaga bullata*, in the mosquitoes *Anopheles* and *Aedes aegypti*, and in the cockroach *Periplaneta americana*. It will be interesting to determine the origin of the StCs in these species. However, it is also known that other insect species do not have StCs, an example being *Rhodnius*, which has secretory cells of a single type (see **Figure 2**). These findings raise two interesting questions: why is physiological function divided between two separate cell populations in some species but not in others; and secondly, how did a bipartite system consisting of cells from separate origins first evolve?

2.8.8. The Development of a Physiologically Competent Tissue

Unlike most other aspects of Malpighian tubule development, maturation of physiological function has not been analyzed in detail in *Drosophila*. The study of Malpighian tubule physiology has been conducted largely in bigger insect species, such as *Rhodnius*, that possess tubules more amenable to physiological studies. In addition, tubule development occurs over a longer period in these species and this makes it easier to correlate changes in cell architecture with the development of physiological competence brought about by these changes. For these reasons, the following section describes the cellular and structural changes associated with the maturation of physiological competence in *Rhodnius prolixus*.

2.8.8.1. The Onset of Physiological Activity in *Rhodnius prolixus*

The onset of physiological activity in *Rhodnius* Malpighian tubules has been analyzed in two ways: first, by measuring secretory rates and second, by examining their ability to transport specific classes of molecules (Skaer *et al*., 1990). Measurements of either urine production in freshly fed intact animals or secretion from isolated tubules demonstrate a linear increase in the rate of secretion from approximately 24 h after hatching until about 7–8 days post hatching, when the rate reaches a plateau (**Figure 11**). Embryonic tubules or tubules from animals younger than 24 h are not able to secrete fluid, suggesting that the onset of secretory capability occurs at approximately 1 day after hatching. Despite this, maximal secretory capacity is only achieved 7–8 days post hatching (**Figure 11**). It is notable in this regard, that *Rhodnius* do not usually feed immediately after eclosion but wait a number of days before taking their first blood meal.

Maturity of tubule function can also be assessed by analysis of ability to transport different classes of solutes. One such endogenous solute is uric acid, which is transported from the hemolymph by the cells of the lower tubule into the tubule lumen, where it precipitates (O'Donnell *et al*., 1983). Small amounts of uric acid begin to appear in the lumen at 3 days prior to hatching. During the subsequent 72 h, the amount of uric acid increases, so that, by the time of hatching, the whole lower third of the tubule is filled with uric acid crystals. In addition to uric acid, Malpighian tubules have been shown to transport a number of other compounds. One of

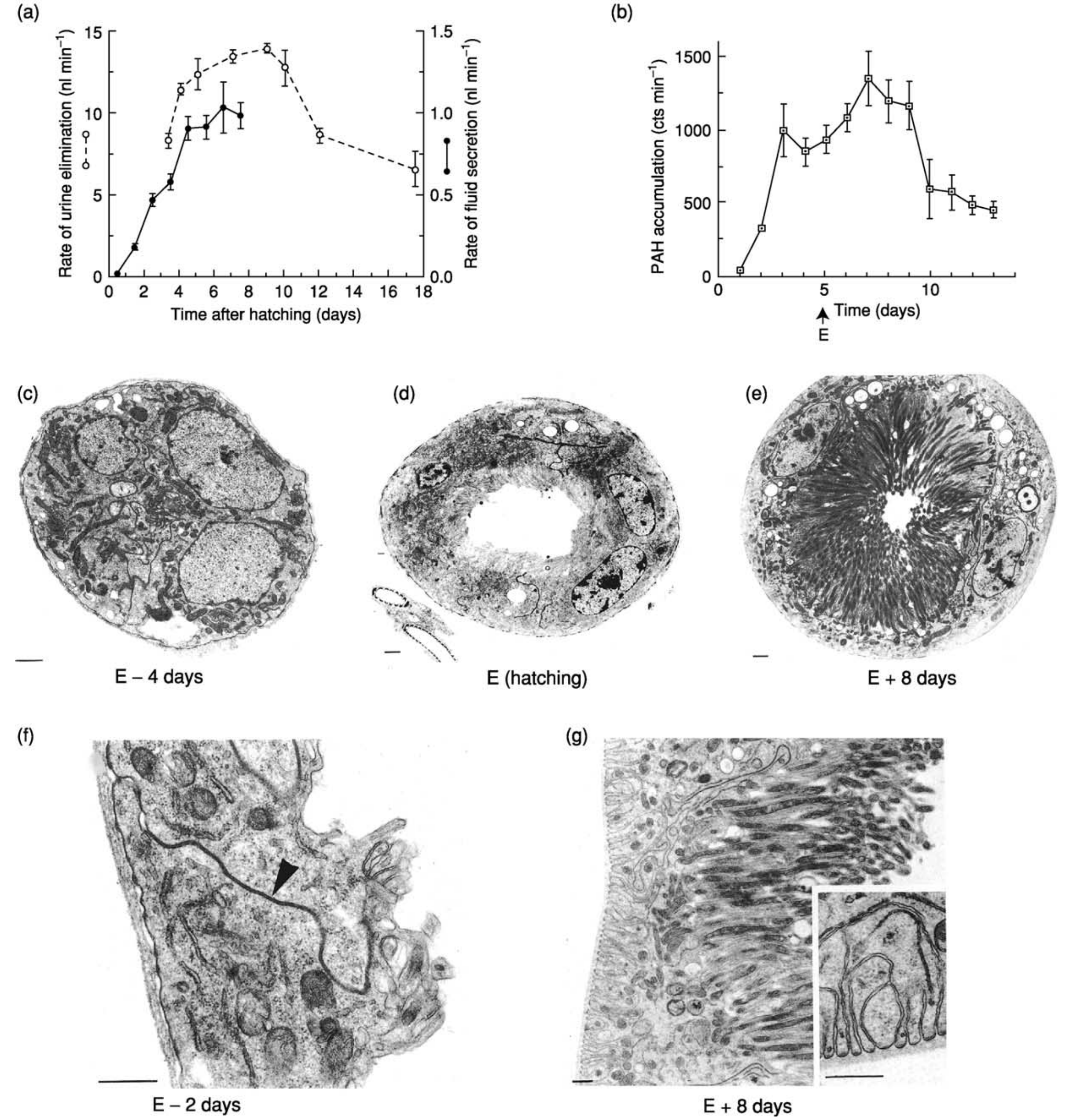


Figure 11 Physiological and structural maturation of the Malpighian tubule of *Rhodnius prolixus*. (a) Maximum rates of urine elimination by freshly fed first instar *Rhodnius* (broken line) and rates of fluid secretion by individual, isolated Malpighian tubules (solid line). Both parameters demonstrate a linear increase until approximately 7–8 days after hatching, when the rates plateau. (b) Accumulation of *p*-aminohippunic acid (PAH) in embryonic and hatchling *Rhodnius* tubules as measured after 30 min of exposure to radiolabeled PAH. Day of emergence is indicated (E). PAH transport begins at approximately 4 days before hatching and the rate of transport continues to rise until about 3 days postemergence. (c–e) Transverse sections of upper Malpighian tubule at different stages of development showing the dilation of the lumen and the elaboration of apical and basal membranes: (c) 4 days before hatching; (d) day of emergence; (e) 8 days postemergence. Scale bar = 1 μm. (f, g) Longitudinal section through an upper Malpighian tubule showing the elaboration of the basal and apical membranes through development: (f) 2 days before hatching; (g) 8 days postemergence. Inset in (g) shows the basal infoldings at higher magnification. Scale bar = 500 nm. Arrowhead in (f) indicates the intercellular junction.

these, *p*-aminohippuric acid (PAH), is transported by the cells in the upper region of the tubule (Maddrell *et al*., 1974). Transport of PAH begins at approximately 4 days prior to hatching and accumulates rapidly in the tubules throughout the remainder of embryonic development (Figure 11).

These data demonstrate that there are at least two periods of functional maturation, separable by

several days. The first period, which begins 3–4 days prior to hatching, corresponds to the ability to transport organic solutes such as uric acid and PAH. The second phase, which occurs over the period 1–8 days after hatching, is the onset of fluid transport. These different phases of functional maturation correlate with underlying changes in cell architecture. An important step for the first phase appears to be the establishment of mature septate junctions. During the earlier stages of embryogenesis, tubule cells are linked by *adherens* junctions and gap junctions, but it is not until approximately 4 days before hatching that mature septate junctions are assembled (Skaer *et al*., 1990). The likely function of septate junctions is to reduce the permeability of the paracellular pathway by increasing the effective path length and by the retention of extracellular material between adjacent cells, thereby reducing the possibility of bulk flow of fluid between the cells. Mature septate junctions have been shown to possess these properties (Skaer and Maddrell, 1987; Skaer *et al*., 1987). Concomitant with septate junction assembly, and representing an additional requirement for the first phase, is the formation of the lumen (**Figure 11**). At 4 days before hatching, there is no clear lumen in the tubules. However, 2 days later (2 days before hatching), a lumen of approximately 10 μm in diameter forms (Skaer *et al*., 1990). The lumen diameter continues to expand until at least day 8 after hatching. The formation of a lumen provides space for the deposition of the organic solutes that the tubules begin to transport at this stage. It is possible that dilation of the lumen is a passive process, as the high concentrations of solutes in the lumen are likely to draw in water by osmosis.

The second phase in the development of physiological function, the onset of fluid transport, begins soon after hatching and continues for 7–8 days. This phase is associated with a considerable reorganization of the apical and basal membrane architecture and the redistribution of cellular organelles (**Figure 11**). At 2 days before hatching, microvilli begin to appear on the apical surface. At first, they are small and sparsely distributed but, by the day of hatching, they have formed a dense border covering the entire apical surface (**Figures 3** and **11**). The microvilli continue to grow both in length and density so that, by day 8 after hatching, they almost fill the luminal space. Over a similar period, elaboration of the basal membrane occurs, first by forming simple fingerlike folds that later interdigitate to generate the convoluted basal membrane architecture of mature tubules (**Figure 11**). The density of mitochondria associated with the basal infoldings and apical microvilli also increases dramatically over this period. It is likely that specialization of the membrane, through localization of specific receptors, ion pumps, and channels, occurs during this period. These changes in cell architecture increase the surface area of the membrane in preparation for the prodigies of excretion called for by the immense size of the first feed. It has been calculated that an animal can excrete a volume of fluid equivalent to the unfed body weight in the space of 40 min (Maddrell, 1991).

2.8.8.2. Physiological Maturation in *Drosophila*

In *Drosophila*, the transport of solutes and urates, in particular, is established before the end of embryogenesis. The first evidence of physiological activity is the accumulation of uric acid in the posterior tubules beginning at stage 17 of embryogenesis (**Figure 1**). Unlike *Rhodnius*, however, *Drosophila* larvae begin to feed almost immediately after hatching, and it is necessary that the tubules are competent to transport fluid by this time.

For the reasons outlined above, the developmental changes associated with physiological maturation in *Drosophila* tubules have not been analyzed in as much detail as those of other insect species such as *Rhodnius*. However, classical morphological descriptions of the *Drosophila* tubule reveal that spatial differences in cell type and tissue architecture exist along its length (Wessing and Eichelberg, 1978) (see Section 2.8.2.3) (**Figure 5**). The tailoring of physiological assays for use in *Drosophila* (Dow *et al*., 1994) has allowed the integration of these classically defined morphological descriptions with physiological function (see Section 2.8.2.3).

2.8.8.3. Physiological Maturation in *Drosophila*: A New Perspective

To date we know relatively little about how the tubules of *Drosophila* progress to physiological maturation. The integration of classical morphological description with physiological analysis is now permitting a correlation between form and function. How these specializations occur and are regulated during development is still largely unknown. Understanding the development of physiological maturation remains one of the challenges for the future, and *Drosophila* provides a unique system in which to address this. It is one of only a few insect with a sequenced and comprehensively annotated genome. This data forms the basis from which candidate genetic approaches can be performed to uncover new molecules required for a given physiological process. Complementary to this approach is the ease with which mutations can be generated

in a chosen gene (if mutant stocks of the gene do not already exist) and their phenotype analyzed at a morphological and/or physiological level. In addition, transgenic technology delivers a whole suite of useful techniques. An enhancer trap screen has already revealed novel tubule domains with differential patterns of gene expression (Sozen *et al.*, 1997). Additional screens such as this are likely to reveal not only the factors underlying physiological function but also those required for physiological maturation. Transgenesis technologies also allow gene expression and function to be manipulated in a defined subset of cells in any given tissue of an otherwise normal animal, a luxury not normally afforded to physiologists. Furthermore, advances in immunocytochemistry and the ability to label molecules *in vivo* can reveal the cellular location of proteins or protein complexes, and can be used to visualize molecular interactions. These techniques should help us to understand the function that specific molecules play during tubule differentiation and physiological maturation.

2.8.9. Malpighian Tubule Development: Toward a Comparative Approach

Over the course of the last 50 years, we have learnt much about the cellular and molecular aspects of physiological function of the Malpighian tubules from a variety of insect species (review: Dow and Davies, 2001). Unsurprisingly, many aspects of tubule function are conserved across the insect class and, consequently, the early studies serve to inform and accelerate physiological analysis in other species. In this chapter, a description of tubule development in *Drosophila* is presented. Over the course of the past 15 years, a relatively comprehensive picture of many aspects of tubule development has been pieced together for this species. In contrast, our knowledge of tubule development in other species is, at best, rudimentary. A challenge for the future will be to determine the way in which tubule development occurs in other insect species. The knowledge we have gained from the analysis in *Drosophila* should provide a framework to facilitate this approach. The mechanism of TC-mediated control of cell proliferation in the tubules of *Rhodnius* (see Section 2.8.4.1) is an example where a comparative approach has proved fruitful. In addition, the genomic sequence of *Drosophila melanogaster*, *Drosophila pseudoobscura,* and of the mosquito *Anopheles gambiae* are now published and, with other insect genome sequence projects well under way (Kaufman *et al.*, 2002), a comparative approach will become easier.

Of particular interest will be those cases where the mode of tubule development is very different from *Drosophila*. A good example is the locust, in which additional tubules develop during each instar, with every tubule possessing a TC (Savage, 1956). This prompts a number of interesting developmental questions: is cell proliferation in the locust tubules also under the control of the TC? Does TC specification in the locust occur by the same molecular mechanism as in *Drosophila*, and if so, are all TCs specified during embryonic development and then remain quiescent until required, or are they specified *de novo* at each larval molt? Are new primary Malpighian tubule cells specified at each molt or do TCs activate a dormant stem cell population to generate new tubules? Does the locust tubule possess StCs and if so, is the mechanism of their recruitment similar to that in *Drosophila*? Furthermore, there are some insect species that lack tubules altogether, possessing instead the more ancestral excretory system of coelomoduct nephridia. Accordingly, the comparative analysis could be extended further to determine the relationship between these two organs. Questions of this nature will extend our understanding of insect excretory system development in particular, and illuminate evolutionary aspects of organogenesis in general.

References

Ainsworth, C., Wan, S., Skaer, H., **2000**. Coordinating cell fate and morphogenesis in *Drosophila* renal tubules. *Phil. Trans. R. Soc.* London *355*, 931–937.

Artero, R.D., Castanon, I., Baylies, M.K., **2001**. The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. *Development 128*, 4251–4264.

Austin, J., Maine, E., Kimble, J., **1989**. Genetics of intercellular signalling in *C. elegans*. *Development 107* (Suppl.), 53–57.

Baker, N.E., **1988**. Localization of transcripts from the *wingless* gene in whole *Drosophila* embryos. *Development 103*, 289–298.

Baumann, P., Skaer, H., **1993**. The *Drosophila* EGF receptor homologue (DER) is required for Malpighian tubule development. *Development* (Suppl.), 65–75.

Beiman, M., Shilo, B.Z., Volk, T., **1996**. Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Devel. 10*, 2993–3002.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M., Knoblich, J.A., **2002**. The endocytic protein alpha-Adaptin is required for *numb*-mediated asymmetric cell division in *Drosophila*. *Devel. Cell 3*, 221–231.

Berridge, M.J., Oschman, J.L., **1972**. Transporting Epithelia. Academic Press, London.

Blake, K.J., Myette, G., Jack, J., **1999**. The products of *ribbon* and *raw* are necessary for proper cell shape and cellular localization of nonmuscle myosin in *Drosophila*. *Devel. Biol. 203*, 177–188.

Blelloch, R., Kimble, J., **1999**. Control of organ shape by a secreted metalloprotease in the nematode *Caenorhabditis elegans*. *Nature 399*, 586–590.

Bodenheimer, F., **1924**. Beiträge zur Kenntnis der Kohlschnake (*Tipula oleracea* L.). Zur Anatomie und Oekologie der Imago. *Z. Wiss. Zool. 121*, 393–441.

Brand, M., Campos-Ortega, J.A., **1988**. Two groups of interrelated genes regulate early neurogenesis in *Drosophila melanogaster. Roux's. Arch. Devel. Biol. 198*, 275–285.

Brönner, G., Chu-LaGraff, Q., Doe, C.Q., Cohen, B., Weigel, D., *et al.*, **1994**. Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in *Drosophila*. *Nature 369*, 664–668.

Davies, S.A., Goodwin, S.F., Kelly, D.C., Wang, Z., Sozen, M.A., *et al.*, **1996**. Analysis and inactivation of *vha55*, the gene encoding the vacuolar ATPase B-subunit in *Drosophila melanogaster* reveals a larval lethal phenotype. *J. Biol. Chem. 271*, 30677–30684.

Denholm, B., Sudarsan, V., Pasalodos-Sanchez, S., Artero, R., Lawrence, P., *et al.*, **2003**. Dual origin of the renal tubules in *Drosophila*: mesodermal cells integrate and polarize to establish secretory function. *Curr. Biol. 13*, 1052–1057.

Diaz, R.J., Harbecke, R., Singer, J.B., Pignoni, F., Janning, W., *et al.*, **1996**. Graded effect of *tailless* on posterior gut development: molecular basis of an allelic series of a nuclear receptor gene. *Mech. Devel. 54*, 119–130.

Dow, J.A., Davies, S.A., **2001**. The *Drosophila melanogaster* Malpighian tubule. *Adv. Insect Physiol. 28*, 1–83.

Dow, J.A.T., Davies, S.A., Sozen, M.A., **1998**. Fluid secretion by the *Drosophila* Malpighian tubule. *Am. Zool. 38*, 450–460.

Dow, J.A.T., Kelly, D.C., Davies, S.A., Maddrell, S.H.P., Brown, D., **1995**. A novel member of the major intrinsic protein family in *Drosophila*: are aquaporins involved in insect Malpighian (renal) tubule fluid secretion? *J. Physiol. 489*, 110P–111P.

Dow, J.A., Maddrell, S.H., Gortz, A., Skaer, N.J., Brogan, S., *et al.*, **1994**. The malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol. 197*, 421–428.

Duronio, R.J., **1999**. Establishing links between developmental signaling pathways and cell-cycle regulation in *Drosophila*. *Curr. Opin. Genet. Devel. 9*, 81–88.

Dworak, H.A., Charles, M.A., Pellerano, L.B., Sink, H., **2001**. Characterization of *Drosophila hibris*, a gene related to human *nephrin*. *Development 128*, 4265–4276.

Edgar, B.A., O'Farrell, P.H., **1990**. The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by *string*. *Cell 62*, 469–480.

Edgar, B.A., Sprenger, F., Duronio, R.J., Leopold, P., O'Farrell, P.H., **1994**. Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Devel. 8*, 440–452.

Erickson, M.R., Galletta, B.J., Abmayr, S.M., **1997**. *Drosophila myoblast city* encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. *J. Cell Biol. 138*, 589–603.

Etienne-Manneville, S., Hall, A., **2002**. Rho GTPases in cell biology. *Nature 420*, 629–635.

Foe, V.E., **1989**. Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development 107*, 1–22.

Gaul, U., Jäckle, H., **1987**. Pole region-dependent repression of the *Drosophila* gap gene *Krüppel* by maternal gene products. *Cell 51*, 549–555.

Gaul, U., Seifert, E., Schuh, R., Jäckle, H., **1987**. Analysis of Krüppel protein distribution during early *Drosophila* development reveals posttranscriptional regulation. *Cell 50*, 639–647.

Gaul, U., Weigel, D., **1990**. Regulation of *Krüppel* expression in the anlage of the Malpighian tubules in the *Drosophila* embryo. *Mech. Devel. 33*, 57–67.

Gloor, H., **1950**. Schädigungsmuster eines Letalfaktors (Kr) von *Drosophila melanogaster. Arch. Julius Klaus-Stift. Vererbungsforsch. Sozialanthropol. Rassenhyg. 25*, 38–44.

Golembo, M., Raz, E., Shilo, B.Z., **1996**. The *Drosophila* embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. *Development 122*, 3363–3370.

Gorka, A., **1914**. Experimentelle und morphologische Beiträge zur Physiologie der Malpighischen Gefäße der Käfer. *Zool. Fahrb. Physiol. 34*, 233–338.

Hall, A., **1998**. Rho GTPases and the actin cytoskeleton. *Science 279*, 509–514.

Harbecke, R., Janning, W., **1989**. The segmentation gene *Krüppel* of *Drosophila melanogaster* has homeotic properties. *Genes Devel. 3*, 114–122.

Harbecke, R., Lengyel, J., **1995**. Genes controlling posterior gut development in the *Drosophila* embryo. *Roux's Arch. Devel. Biol. 204*, 308–329.

Hartenstein, V., Campos-Ortega, J.A., **1986**. The peripheral nervous system of mutants of early neurogenesis in *Drosophila*. *Roux's Arch. Devel. Biol. 195*, 210–221.

Hartenstein, V., Technau, G.M., Campos-Ortega, J.A., **1985**. Fate-mapping in wild-type *Drosophila melanogaster.* 3. A fate map of the blastoderm. *Roux's Arch. Devel. Biol. 194*, 213–216.

Henderson, S., Gao, S., Lambie, E., Kimble, J., **1994**. *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. *Development 120*, 2913–2924.

Heymons, R., Lühmann, M., **1933**. Die Vasa Malpighi von *Galerucella viburni* Payk (Coleoptera). *Zool. Anz. 102*, 78–86.

Hoch, M., Broadie, K., Jäckle, H., Skaer, H., **1994**. Sequential fates in a single cell are established by the

neurogenic cascade in the Malpighian tubules of *Drosophila*. *Development 120*, 3439–3450.

Holmgren, N., **1902**. *Anat. Anz. 22*, 225–239.

Ishimori, N., **1924**. Distribution of the Malpighian vessels in the wall of the rectum of lepidopterous larvae. *Ann. Entomol. Soc. America 17*, 75–86.

Jack, J., Myette, G., **1997**. The genes *raw* and *ribbon* are required for proper shape of tubular epithelial tissues in *Drosophila*. *Genetics 147*, 243–253.

Jack, J., Myette, G., **1999**. Mutations that alter the morphology of the Malpighian tubules in *Drosophila*. *Devel. Genes Evol. 209*, 546–554.

Janning, W., Lutz, A., Wissen, D., **1986**. Clonal analysis of the blastoderm anlage of the Malpighian tubules in *Drosophila melanogaster. Roux's Arch. Devel. Biol. 195*, 22–32.

Kaufman, T.C., Severson, D.W., Robinson, G.E., **2002**. The *Anopheles* genome and comparative insect genomics. *Science 298*, 97–98.

Kerber, B., Fellert, S., Hoch, M., **1998**. Seven-up, the *Drosophila* homolog of the COUP-TF orphan receptors, controls cell proliferation in the insect kidney. *Genes Devel. 12*, 1781–1786.

Kimble, J., White, J., **1981**. On the control of germ cell development in *Caenorhabditis elegans*. *Devel. Biol. 81*, 208–219.

Kispert, A., Herrmann, B.G., Leptin, M., Reuter, R., **1994**. Homologs of the mouse *Brachyury* gene are involved in the specification of posterior terminal structures in *Drosophila*, *Tribolium*, and *Locusta*. *Genes Devel. 8*, 2137–2150.

Klyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kutata, T., *et al.*, **1998**. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Devel. 12*, 3331–3336.

Knipple, D.C., Seifert, E., Rosenberg, U.B., Preiss, A., Jäckle, H., **1985**. Spatial and temporal patterns of *Krüppel* gene expression in early *Drosophila* embryos. *Nature 317*, 40–44.

Knoblich, J.A., Sauer, K., Jones, L., Richardson, H., Saint, R., *et al.*, **1994**. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell 77*, 107–120.

Kramerova, I.A., Kawaguchi, N., Fessler, L.I., Nelson, R.E., Chen, Y., *et al.*, **2000**. Papilin in development: a pericellular protein with a homology to the ADAMTS metalloproteinases. *Development 127*, 5475–5485.

Kusch, T., Reuter, R., **1999**. Functions for *Drosophila brachyenteron* and *forkhead* in mesoderm specification and cell signalling. *Development 126*, 3991–4003.

Lamb, M.J., **1982**. The DNA content of polytene nuclei in the midgut and Malpighian tubule cells of adult *Drosophila melanogaster. Roux's Arch. Devel. Biol. 191*, 381–384.

Lee, J.R., Urban, S., Garvey, C.F., Freeman, M., **2001**. Regulated intracellular ligand transport and proteolysis control EGF signal activation in *Drosophila*. *Cell 107*, 161–171.

Licent, P.E., **1912**. Recherches d'anatomie et de physiologie comparés sur le tube digestif des Homoptères supérieurs. *La Cellule 28*, 7–161.

Liu, S., Jack, J., **1992**. Regulatory interactions and role in cell type specification of the Malpighian tubules by *cut*, *Krüppel* and *caudal* genes of *Drosophila*. *Devel. Biol. 150*, 133–143.

Liu, X., Kiss, K., Lengyel, J.A., **1999**. Identification of genes controlling Malpighian tubule and other epithelial morphogenesis in *Drosophila melanogaster. Genetics 151*, 685–695.

Lorén, C.E., Scully, A., Grabbe, C., Edeen, P.T., Thomas, J., *et al.*, **2001**. Identification and characterization of DAlk: a novel *Drosophila melanogaster* RTK which drives ERK activation *in vivo*. *Genes Cells 6*, 531–544.

Maddrell, S.H.P., **1981**. The functional design of the insect excretory system. *J. Exp. Biol. 90*, 1–15.

Maddrell, S.H.P., **1991**. The fastest fluid-secreting cell known: the upper Malpighian tubule cell of *Rhodnius*. *Bioessays 13*, 357–362.

Maddrell, S.H.P., Gardiner, B.O., Pilcher, D.E., Reynolds, S.E., **1974**. Active transport by insect Malpighian tubules of acidic dyes and of acylamides. *J. Exp. Biol. 61*, 357–377.

Maddrell, S.H.P., Lane, N.J., Harrison, J.B., Gardiner, B.O.C., **1985**. DNA replication in binucleate cells of the Malpighian tubules of hemipteran insects. *Chromosoma 91*, 201–209.

Mahoney, P.A., Lengyel, J.A., **1987**. The zygotic segmentation mutant *tailless* alters the blastoderm fate map of the *Drosophila* embryo. *Devel. Biol. 122*, 464–470.

Malpighi, M., **1669**. Dissertatio Epistolica de Bombyce. Martyn and Allestry, London.

Martin, B.S., Ruiz-Gomez, M., Landgraf, M., Bate, M., **2001**. A distinct set of founders and fusion-competent myoblasts make visceral muscles in the *Drosophila* embryo. *Development 128*, 3331–3338.

Nolan, K., Barrett, K., Lu, Y., Hu, K., Vincent, S., *et al.*, **1998**. *Myoblast city*, a *Drosophila* homolog of DOCK180/CED-5, is required in a rac signaling pathway utilized for multiple developmental processes. *Genes Devel. 12*, 3337–3342.

O'Donnell, M.J., Dow, J.A.T., Huesmann, G.R., Tublitz, N.J., Maddrell, S.H.P., **1996**. Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster. J. Exp. Biol. 199*, 1163–1175.

O'Donnell, M.J., Maddrell, S.H.P., Gardiner, B.O.C., **1983**. Transport of uric acid by the Malpighian tubule cells of *Rhodnius* and other insects. *J. Exp. Biol. 103*, 169–184.

O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., *et al.*, **1998**. Hormonally controlled chloride movement across *Drosophila* tubules is via ion channels in stellate cells. *Am. J. Physiol. 274*, 1039–1049.

Orr-Weaver, T.L., **1994**. Developmental modification of the *Drosophila* cell cycle. *Trends Genet. 10*, 321–327.

Pignoni, F., Baldarelli, R.M., Steingrimsson, E., Diaz, R.J., Patapoutian, A., *et al.*, **1990**. The *Drosophila* gene *tailless* is expressed in the embryonic termini and is a member of the steroid receptor superfamily. *Cell 62*, 151–163.

Ramsay, J.A., **1954**. Active transport of water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. Exp. Biol. 31*, 104–113.

Ramsay, J.A., **1955**. The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. Exp. Biol. 32*, 200–216.

Ramsay, J.A., **1958**. Excretion by the Malpighian tubules of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae): amino acids, sugars and urea. *J. Exp. Biol. 35*, 871–891.

Redemann, N., Gaul, U., Jäckle, H., **1988**. Disruption of a putative Cys–zinc interaction eliminates the biological activity of the Krüppel finger protein. *Nature 332*, 90–92.

Rhyu, M.S., Jan, L.Y., Jan, Y.N., **1994**. Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell 76*, 477–491.

Richardson, H.E., O'Keefe, L.V., Reed, S.I., Saint, R., **1993**. A *Drosophila* G_1-specific *cyclin E* homolog exhibits different modes of expression during embryogenesis. *Development 119*, 673–690.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., Hall, A., **1992**. The small GTP-binding Rac regulates growth factor-induced membrane ruffling. *Cell 70*, 389–399.

Savage, A.A., **1956**. The development of the Malpighian tubules of *Schistocerca gregaria* (Orthoptera). *Q. J. Microsc. Soc. 97*, 599–615.

Shim, K., Blake, K.J., Jack, J., Krasnow, M.A., **2001**. The *Drosophila ribbon* gene encodes a nuclear BTB domain protein that promotes epithelial migration and morphogenesis. *Development 128*, 4923–4933.

Singer, J.B., Harbecke, R., Kusch, T., Reuter, R., Lengyel, J.A., **1996**. *Drosophila brachyenteron* regulates gene activity and morphogenesis in the gut. *Development 122*, 3707–3718.

Skaer, H., **1989**. Cell division in Malpighian tubule development in *Drosophila melanogaster* is regulated by a single tip cell. *Nature 342*, 566–569.

Skaer, H., **1992**. Cell proliferation and rearrangement in the development of the hemipteran, *Rhodnius prolixus*. *Devel. Biol. 150*, 372–380.

Skaer, H., **1993**. The alimentary canal. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 941–1012.

Skaer, H., Harrison, J.B., Maddrell, S.H.P., **1990**. Physiological and structural maturation of a polarised epithelium: the Malpighian tubules of a blood-sucking insect, *Rhodnius prolixus*. *J. Cell Sci. 96*, 537–547.

Skaer, H., Maddrell, S.H.P., **1987**. How are invertebrate epithelia made tight? *J. Cell Sci. 88*, 139–141.

Skaer, H., Maddrell, S.H.P., Harrison, J.B., **1987**. The permeability properties of septate junctions in Malpighian tubules of *Rhodnius*. *J. Cell Sci. 88*, 251–265.

Skaer, H., Martinez-Arias, A., **1992**. The *wingless* product is required for cell proliferation in the Malpighian tubules of *Drosophila melanogaster. Development 116*, 745–754.

Skeath, J.B., Carroll, S.B., **1992**. Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. *Development 114*, 939–946.

Smith, A.V., Orr-Weaver, T.L., **1991**. The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development 112*, 997–1008.

Sozen, M.A., Armstrong, J.D., Yang, M.-Y., Kaiser, K., Dow, J.A.T., **1997**. Functional compartments are specified to single-cell resolution in a *Drosophila* epithelium. *Proc. Natl Acad. Sci. USA 94*, 5207–5212.

St. Johnston, D., Nüsslein-Volhard, C., **1992**. The origin of pattern and polarity in the *Drosophila* embryo. *Cell 68*, 201–219.

Sudarsan, V., Pasalodos-Sanchez, S., Wan, S., Gampel, A., Skaer, H., **2002**. A genetic hierarchy establishes mitogenic signalling and mitotic competence in the renal tubules of *Drosophila*. *Development 129*, 935–944.

Tepass, U., Hartenstein, V., **1994**. The development of cellular junctions in the *Drosophila* embryo. *Devel. Biol. 161*, 563–596.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., Jan, Y.N., **1989**. *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell 58*, 249–360.

Urban, S., Lee, J.R., Freeman, M., **2001**. *Drosophila Rhomboid-1* defines a family of putative intramembrane serine proteases. *Cell 107*, 173–182.

Wan, S., **2001**. Genetic regulation of tip cell specification in the Malpighian tubules of *Drosophila*. Ph.D. thesis, University of Sheffield, UK.

Wan, S., Cato, A.-M., Skaer, H., **2000**. Multiple signalling pathways establish cell fate and cell number in *Drosophila* Malpighian tubules. *Devel. Biol. 217*, 153–165.

Wang, L., Coulter, D.E., **1996**. *bowel*, an *odd-skipped* homolog, functions in the terminal pathway during *Drosophila* embryogenesis. *EMBO J. 15*, 3182–3196.

Weigel, D., Jurgens, G., Klingler, M., Jäckle, H., **1990**. Two gap genes mediate maternal terminal pattern information in *Drosophila*. *Science 248*, 495–498.

Wessing, A., Eichelberg, D., **1978**. Malpighian tubules, rectal papillae and excretion. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*, vol. 2c. Academic Press, London, pp. 1–42.

Wigglesworth, V.B., **1939**. The Principles of Insect Physiology. Methuen, London.

Wigglesworth, V.B., Salpeter, M.M., **1962**. Histology of the Malpighian tubules of *Rhodnius prolixus* (Hemiptera). *J. Insect Physiol. 8*, 299–307.

Wu, L.H., Lengyel, J.A., **1998**. Role of *caudal* in hindgut specification and gastrulation suggests homology between *Drosophila* amnioproctodeal invagination and the vertebrate blastopore. *Development 125*, 2433–2442.

Wu, Y.C., Horwitz, H.R., **1998**. *Caenorhabditis elegans* phagocytosis and cell-migration protein CED-5 is similar to DOCK180. *Nature 392*, 501–504.

Young, P.E., Richman, A.M., Ketchum, A.S., Kiehart, D.P., **1993**. Morphogenesis in *Drosophila* required non-muscle myosin heavy chain function. *Genes Devel. 7*, 29–41.

2.9 Fat-Cell Development

D K Hoshizaki, University of Nevada,
Las Vegas, NV, USA

> The fat bodies are small and inconspicuous . . .
>
> Poulson (1950)
>
> The fat body is the annoying tissue that sticks to salivary glands
>
> Anonymous (1981)
>
> There is little to say about the development of the fat body
>
> Campos-Ortega and Hartenstein (1985)

2.9.1. Introduction

Since the first publication of this series nearly two decades ago, the fat body has emerged as a dynamic tissue involved in longevity, in coordinating insect growth with metamorphosis, and in mediating the innate immune response. Developmental studies on the insect fat body suggest intriguing cell lineage relationships between fat cells and other mesoderm derivatives and have provided insights into tissue remodeling. This chapter will discuss the genetic basis of embryonic fat-cell determination and differentiation, the origin of the adult fat body, the role of the fat body as an endocrine organ and, finally, will describe studies on tissue remodeling of the fat body during metamorphosis. Emphasis is on functional studies that address underlying mechanisms and includes descriptive studies as they relate to the understanding of these mechanisms. Because of advances in cell biology tools, the completion of the genome project, and the extensive genetics, most of our recent understanding of the fat body comes from studies in *Drosophila melanogaster* (Flybase, 2003). As such, much of this chapter deals with this organism but, where possible, studies from other insects are included. Several topics relating to the fat body are not represented in this chapter but have been discussed in recent reviews: storage proteins (Telfer and Kunkel, 1991; Wheeler and Martinez, 1995; Haunerland, 1996; Burmester and Scheller, 1999), regional expression in the fat body (Haunerland and Shirk, 1995; Harshman and James, 1998), and the innate immune response.

The insect fat body is often, but *inaccurately*, described as the insect equivalent of the mammalian liver. Foremost, the fat body and liver are derived from different germ layers; the fat body is mesodermally derived whereas the liver arises from the endoderm. Furthermore, even though the fat body shares some metabolic processes with the liver, it is not the sole tissue to carry out these functions. Alcohol dehydrogenase (Adh), a key enzyme in alcohol metabolism in the mammalian liver, is present in the fat body, but Adh is also present in the middle and anterior midgut (Fischer and Maniatis, 1986). The activity of Adh in the fat body is the critical observation that promoted the idea that the fat body is involved in detoxification and is the functional analog of the mammalian liver. However, detoxification in insects is primarily a function of the midgut where, similar to the liver, the smooth endoplasmic

reticulum is abundant and functions to modify or detoxify chemicals (Locke, 2003). In constrast, the insect fat body almost never has a smooth endoplasmic reticulum (Locke, 2003).

Molecular studies aimed at understanding the transcriptional control of *Adh* led to the notion that the tissue-specific control of *Adh* in the fat body is mediated by DNA-binding proteins also involved in liver-specific gene transcription (Sondergaard, 1993 and references therein). The *D. melanogaster B-box factor-2* (*BBF-2* also known as *Bbf1*) gene codes for a transcriptional activator of *Adh* (Abel *et al.*, 1992). BBF-2 is a member of the CREB/ATF family of transcription factors that are also active in the liver. Based upon Northern blot analysis, *BBF-2* is expressed throughout the life cycle of *D. melanogaster*, albeit at various levels (Abel *et al.*, 1992); the highest levels of *BBF-2* mRNA are seen during embryogenesis and in adult males. Northern blot analysis of early third-instar larvae also demonstrated that *BBF-2* mRNA is present in trachea, gut, and fat body. Hybridization studies to confirm the tissue-specific expression of *BBF-2* transcripts has not been reported, and *BBF-2* is not expressed in the embryonic fat body (D.K. Hoshizaki, unpublished data). Thus, *BBF-2* is not fat-cell specific nor is it co-expressed with *Adh* in the embryonic fat body.

A second *D. melanogaster* gene with homology to a liver transcriptional activator gene is *HNF-4(D)* (Zhong *et al.*, 1993). *HNF-4(D)* (also known as *Hnf4*) is a homolog of *HNF-4* (*Hepatic Nuclear Factor-4*), a steroid-hormone receptor gene encoding a mammalian transcription factor involved in the activation of a wide array of liver-specific genes (Costa *et al.*, 1989; Sladek *et al.*, 1990; Mietus-Snyder *et al.*, 1992). *HNF-4(D)* is reported to be expressed in the embryonic fat body, salivary glands, and the anterior and posterior midgut primordia. Further analysis of *HNF-4(D)*, however, demonstrated that *HNF-4(D)* is not expressed in the embryonic fat body, nor does the lack of *HNF-4(D)* affect the formation of the embryonic fat body or the expression of fat body genes (Hoshizaki *et al.*, 1994). Although the fat body and mammalian liver carry out some of the same functions, the assertion that the fat body is the insect equivalent of the mammalian liver should be made with care.

2.9.2. Origin of the Embryonic/Larval Fat Body

The embryonic fat body is derived from cells that are arranged in a metameric repeating pattern within the mesoderm. The formation of the progenitor fat cells is dependent upon the subdivision of the mesoderm and inductive events from the ectoderm. A descriptive account of the developing fat body from its various progenitor cells is presented here, and is followed by discussions of the cell patterning events that lead to the specification of the fat-cell primordia (Sections 2.9.2.1 and 2.9.2.2).

Prior to the advent of techniques to mark individual cells, histological studies suggested that fat cells arose in the mesoderm after stage 13 (Poulson, 1950; stages are those of Campos-Ortega and Hartenstein, 1985). The development of *in situ* hybridization and immunohistochemistry protocols to whole-mount embryos has greatly improved the visualization of individual cells and facilitated the description of embryonic tissues, including the fat body (Bier *et al.*, 1989; Wilson *et al.*, 1989; Hartenstein and Jan, 1992; Hoshizaki *et al.*, 1994). The embryonic fat body arises from cells identified at stage 10/11 and is composed of single layers of cells organized into a morphologically distinct tissue; the main or lateral fat body forms a wide bilateral ribbon located between the somatic body-wall muscle and the gut. Extending from the anterior region of the lateral fat body is a collar of fat cells (the ventral commissure) that spans the ventral midline. On the dorsal side of the embryo are fat cells that form a pair of projections extending from the posterior region of the lateral fat body which flank the dorsal vessel (heart) (**Figure 1**) (Hartenstein and Jan, 1992; Hoshizaki *et al.*, 1994; Riechmann *et al.*, 1998; Miller *et al.*, 2002). Upon completion of embryogenesis, the newly emerged larva has a developed fat body.

The first reconstruction of the *D. melanogaster* fat-cell lineage relied upon the expression of the P-element enhancer-trap line [29D] and the known genes, *Adh*, *Drosophila collagen IV* (*DCg1*), and *seven-up* (*svp*) (Hoshizaki *et al.*, 1994). P[29D] is expressed in both the lateral mesoderm and ectoderm. The gene associated with P[29D] has not been identified (Blackburn, 1994; D.K. Hoshizaki, unpublished data). The expression of P[29D] in nine bilateral mesodermal cell clusters in parasegment (PS) 4–12 marks the cells that give rise to the lateral fat body (**Figure 2**). The initial expression of P[29D] at stage 10/11 in mesodermal cell clusters is concurrent with the specification of the progenitors to the somatic body wall muscle (Dohrmann *et al.*, 1990; Michelson *et al.*, 1990; Paterson *et al.*, 1991). By analogy to muscle determination, the P[29D]-expressing cells mark the progenitor fat cells and fat-cell determination is assumed to take place prior to germ band retraction at stage 10/11.

In a manner similar to defining the stages of adipogenesis in the mouse (Ailhaud *et al.*, 1992), the

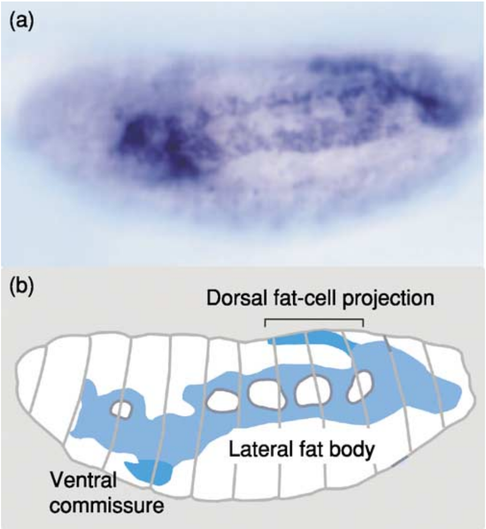


Figure 1 The fat body. (a) Lateral view of a whole-mount stage-16 embryo stained for Serpent (Srp) protein. (b) Corresponding schematic drawing highlighting the fat body domains in blue and segment boundaries in gray. The fat body in a stage-16 embryo is made up of three morphological domains: the lateral fat body, the dorsal fat-cell projections and the ventral commissure. The lateral fat body is made up of a single layer of cells that forms a bilateral ribbon spanning the lateral region of the embryo and is punctuated by stereotypic disruptions that form holes in the tissue. The dorsal fat-cell projections are fused with the posterior-dorsal region of the lateral fat body and extend in the anterior direction, flanking the dorsal vessel. The ventral commissure extends from the anterior-lateral fat body and spans the ventral midline. (Adapted with permission from Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K., **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster*. *Insect Mol. Biol.* *11*, 67–77.)

temporal expression patterns of fat-cell markers, *Adh*, *DCg1*, and *svp*, were used to define the stages of fat-cell differentiation within the developing embryo (**Figure 2**) (Hoshizaki *et al*., 1994). Both *Adh* and *DCg1* are involved in the cellular function of the fat-cell and are expressed in the developing fat body as early as stage 15. The expression of these two genes at stage 15 is used to mark the beginning of terminal fat-cell differentiation, and the fat cells present at this time represent late precursor fat cells. The *svp* gene encodes an orphan steroid hormone receptor (Mlodzik *et al*., 1990) and is expressed in the nine bilateral cell clusters starting at stage 12, before the expression of either *Adh* or *DCg1* (Hoshizaki *et al*., 1994). The early expression of *svp* marks the beginning of fat-cell differentiation in the early precursor fat cells. Loss of *svp* function does not disrupt fat body morphology but does result in the fat-cell-specific loss of *Adh* expression and a reduction in *DCg1* transcripts (Hoshizaki *et al*., 1994). Thus, Svp is likely to function as a transcriptional activator during terminal fat-cell differentiation. Because *svp* expression only overlaps with *Adh* and *DCg1* expression in the fat body, these data provide confirmation that the fat-cell lineage had been correctly identified. The identified nine bilateral cell clusters are part of the fat-cell lineage that gives rise to the lateral fat body (Hoshizaki *et al*., 1994; see below).

In addition to the aforementioned cell clusters that give rise to the lateral fat body, two other groups of cells contribute to the mature fat body. These two groups of cells, the lateral and ventral secondary fat-cell clusters, were identified based on the expression of *A-Box binding factor* gene (*Abf*), also known as *serpent* (*srp*). Abf is a member of the GATA transcription factor family that binds to positive regulatory elements in the larval promoters of all known *Adh* genes (Abel *et al*., 1993). The *Abf* gene was identified as the previously genetically defined gene *srp*, an embryonic gene necessary for germ band retraction, and hemocyte, fat-cell and gut formation (Jurgens *et al*., 1984; Rehorn *et al*., 1996; Sam *et al*., 1996) (Section 2.9.2.2).

A detailed examination of *srp* expression in early embryos confirmed the identification of the lateral fat-body lineage. This study identified the primary fat-cell clusters in PS 4–9, the lateral and ventral secondary fat cells in PS 4–12 and PS 3–11, respectively, and the dorsal fat-cell cluster in PS 13/14 (**Figure 3**) (Riechmann *et al*., 1998). Six of the nine aforementioned bilateral fat-cell clusters that are located in PS 4–9 correspond to the primary fat-cell clusters of Riechmann. The three remaining P[29D] enhancer trap-positive cell clusters in PS 10–12 are likely to correspond to the lateral secondary fat-cell clusters in PS 10–12.

The lateral and ventral secondary fat-cell clusters arise shortly after *srp* expression in the primary fat cells at stage 11 (Riechmann *et al*., 1998). The lateral secondary fat cells in PS 4–12 are composed of metameric repeating cell clusters located immediately adjacent to the position of the primary fat cell location in PS 4–9 (**Figure 3c and d**). The lack of cell markers specific for the lateral secondary fat cells makes it difficult to determine the precise contribution of these cells to the mature fat body, but it is likely that they contribute to the lateral fat body.

The nine cell clusters located in the ventral mesoderm of PS 3–11 make up the ventral secondary fat cells (**Figure 3c and d**). A detailed study of these cells has been carried out using Serpent (Srp) antibodies and a *lacZ* reporter gene driven by a ventral fat-cell enhancer from the promoter region of *srp* (Miller *et al*., 2002). The ventral secondary cell clusters in PS 3–5 give rise to the ventral commissure, which

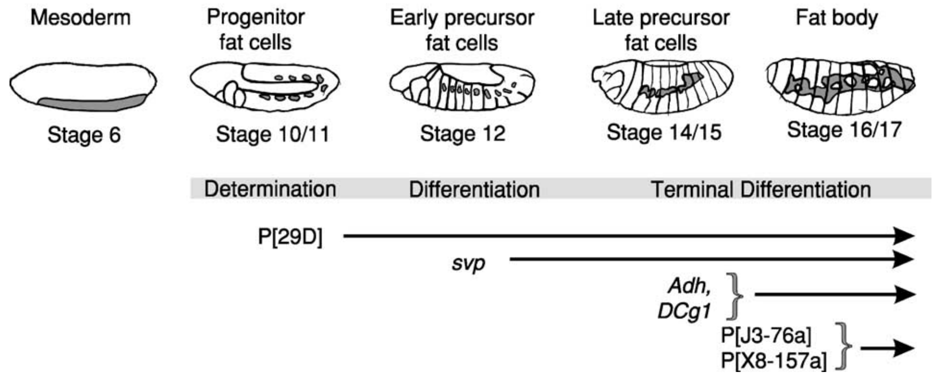


Figure 2 Summary of the fat-cell lineage of the lateral fat body and proposed stages of fat-cell development. The first identified fat-cell lineage described the development of the lateral fat body from nine bilateral clusters of mesodermal cells. The stages of fat-cell development (determination, differentiation, and terminal differentiation) were identified based on the temporal expression of the genes *Adh*, *DCg1*, and *svp* and the enhancer trap lines P[29D], P[J3-76a], and [PX8-157a]. The mesoderm arises from the ventral region of the embryo at stage 6 (shaded area in the stage-6 embryo). The nine bilateral clusters of cells (stage-10, 11 embryo) are represented by the 29D-positive cells which are first detected prior to germ-band retraction during the specification of the mesoderm. The development of the fat body from the nine bilateral clusters of cells is the result of the expansion of these cell clusters and extension of cells arising from the posterior region to form the dorsal fat body (stage-12 embryos). The *svp* gene is transiently expressed in the fat-cell lineage and is first detected at stage 12 in the nine bilateral cells marked by P[29D]. The *svp* gene is expressed before either *Adh* or *DCg1* and is a fat-cell-specific regulator of *Adh* and *DCg1* expression. The expression of *svp* in the precursor fat cells marks the beginning of fat-cell differentiation (stage-12 embryo). The Svp-positive cells at this stage mark the early precursor fat cells. Both *Adh* and *DCg1* are expressed in the developing fat body as early as stage 15 and are involved in the metabolic function of the fat-cell. The initial expression of these genes at stage 15 marks the beginning of terminal fat-cell differentiation and identifies the late precursor fat cells. Finally, the enhancer-trap lines P[J3-76a], and [PX8-157a] are active late in stage 15. (Adapted with permission from Hoshizaki, D.K., Lunz, R., Ghosh, M., Johnson, W., **1995**. Identification of fat-cell enhancer activity in *Drosophila melanogaster* using P-element enhancer traps. *Genome 38*, 497–506.)

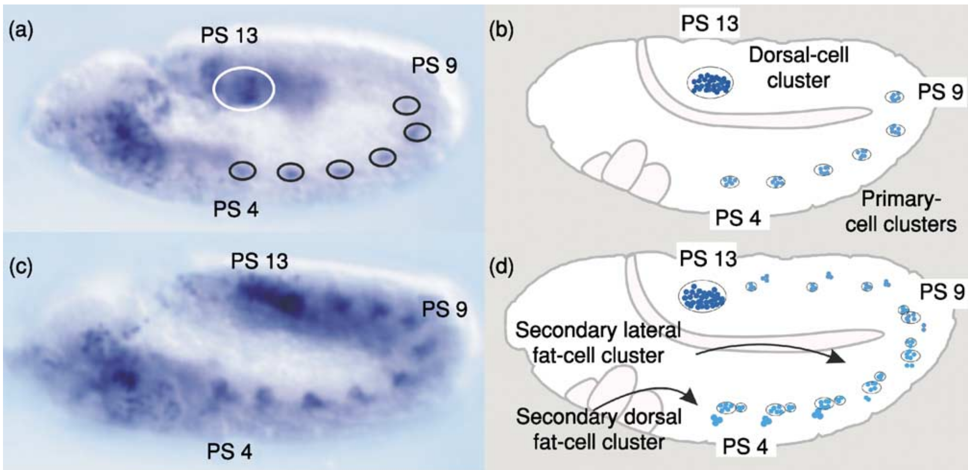


Figure 3 Identification of the precursor cells for the fat body domains. (a, c) Lateral view of a whole-mount embryo stained for Srp. (b, d) Corresponding schematic drawings highlighting precursor fat-cell clusters (blue clusters) and basic outline of the embryo (gray lines). (a, b) Stage-10 embryo. The dorsal fat-cell cluster is located in the dorsal mesoderm of PS 13/14 (large circle in (a) and (b) and the primary fat-cell clusters are located in the lateral mesoderm of PS 4–9 (smaller circles in (a) and (b). (c, d) Stage-11/12 embryo. The secondary ventral fat-cell clusters are located in the ventral mesoderm of PS 3–11 immediately ventral to the primary fat-cell clusters (uncircled blue clusters in (d). A second group of subsidiary precursor fat cells (secondary lateral fat-cell clusters) are located as small cell clusters immediately posterior to the primary cell clusters in PS 4–9 and in the equivalent position in PS 10–12 (small circled blue clusters in (d). (Adapted with permission from Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K., **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster*. *Insect Mol. Biol. 11*, 67–77.)

is composed of two fat-cell bridges (Figure 4). The anterior bridge is derived from the cells in PS 3/4 and is located more internally than the posterior bridge (Figure 4b). The posterior bridge is derived from cells in PS 5 and fuses with the ventralmost cells of the lateral fat body (Figure 4c). Bilateral horns of fat cells extend from the posterior bridge and cross the anterior bridge (Figure 4e). The remaining ventral

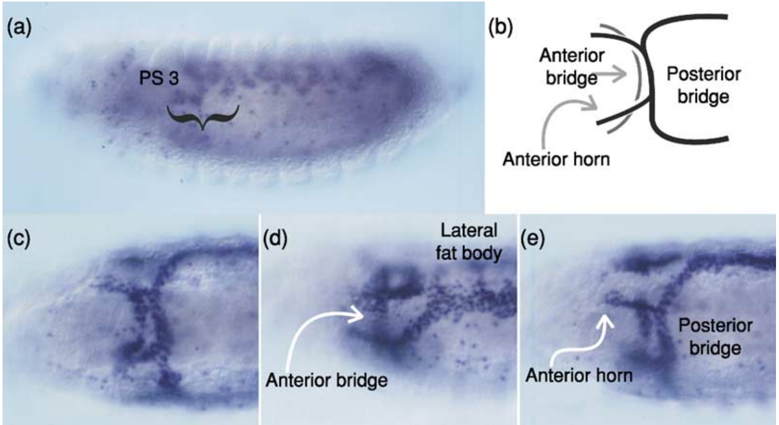


Figure 4 The ventral commissure is composed of two fat-cell bridges. (a) Ventral-lateral view of a stage-13 embryo stained for Srp highlighting the secondary fat-cell clusters in PS 3–5 (bracket). The cell clusters in PS 3 and PS 4 are dorsal to the cluster in PS 5. (b) Schematic drawing of the ventral commissure. The ventral commissure is composed of fat cells that span the ventral midline. The anterior bridge of cells extends from the secondary ventral fat-cell clusters in PS 3 and PS 4 and fuses across the ventral midline. The posterior bridge extends from cells in the bilateral ventral fat-cell cluster in PS 5 and fuses across the ventral midline. (c–d) Ventral and ventral-lateral views of stage-16 embryos stained for Srp. The posterior bridge lies in a more peripheral position within the embryo than the anterior bridge and is a continuation of the ventralmost cells of the lateral fat body. (e) Extending from the posterior bridge are two horns that extend in the anterior direction and cross over the anterior bridge (the second horn is out of the plane of focus). (Adapted with permission from Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K., **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster*. *Insect Mol. Biol. 11*, 67–77.)

secondary fat cells in PS 6–12 populate the ventral edge of the lateral fat body (Miller *et al.*, 2002).

The final group of precursor cells, the dorsal fat cells, lie in PS 13 and part of PS 14. These cells give rise to bilateral projections of fat cells and a portion of the lateral fat body. The projections emerge from the posterior of the lateral fat body and extend in the anterior direction along the dorsal side of the embryo (**Figure 1**) (Riechmann *et al.*, 1998; Miller *et al.*, 2002).

Using a *lacZ* reporter gene driven by a dorsal fat-cell enhancer from the *srp* promoter, the dorsal fat-cell clusters were traced to their final position in the fat-cell projections and the posterior-most cells of the lateral fat body (**Figure 5**) (Miller *et al.*, 2002). Briefly, the dorsal fat-cell projections arise from a bilateral cluster of cells first detected at stage 10 within the dorsal mesoderm (**Figure 5a and b**) (Riechmann *et al.*, 1998; Miller *et al.*, 2002). The dorsal fat-cell cluster spans PS 13 and part of PS 14 (Riechmann *et al.*, 1998; Miller *et al.*, 2002). During germ band retraction, the dorsal fat-cell cluster separates along the dorsoventral axis into two subgroups of cells (**Figure 5e**). Eventually the cells of the dorsal subgroup migrate anteriorly, crossing parasegmental boundaries, to form the bilateral dorsal fat-cell projections, which flank the dorsal vessel (**Figure 5g–i**) (Miller *et al.*, 2002). The cells of the ventral subgroup, together with a small cluster of ventral cells in PS 13, form the posterior-most portion of the lateral fat body (**Figure 5g–j**) (Miller *et al.*, 2002).

2.9.2.1. The Mesoderm and Fat-Cell Lineages

The mature fat body does not have an apparent metameric organization but is derived from cell clusters arranged in a repeating pattern. This observation supports the original suggestion of Rizki and Rizki (1978) that the fat body has a metameric origin. Work from several laboratories has demonstrated that the primordia of the fat body, somatic body wall, visceral and heart muscle, and gonads are derived from segmentally arranged clusters of cells that are dependent upon the subdivision of the mesoderm by pair-rule genes, upon genes involved in segment identity and, in some cases, upon induction events from the ectoderm (Miller, 2002 and references therein).

The mesoderm is derived from ectodermal cells from the ventral-third of the blastoderm and eventually is arranged into two layers, the splanchnopleura or inner layer, and the somatopleura or outer layer (Campos-Ortega and Hartenstein, 1985). Briefly, during gastrulation the presumptive mesoderm cells invaginate into the interior of the embryo to form a furrow along the ventral midline (the ventra1 furrow) and begin an ectoderm-to-mesenchyme transition (Leptin and Grunewald, 1990). Upon completion of invagination, the ventral furrow closes to form the mesodermal tube (germ band; Bate, 1993; Campos-Ortega and Hartenstein, 1997). At stage 8, cells of this internalized mass divide mitotically in the first of three postblastoderm mitoses. During this

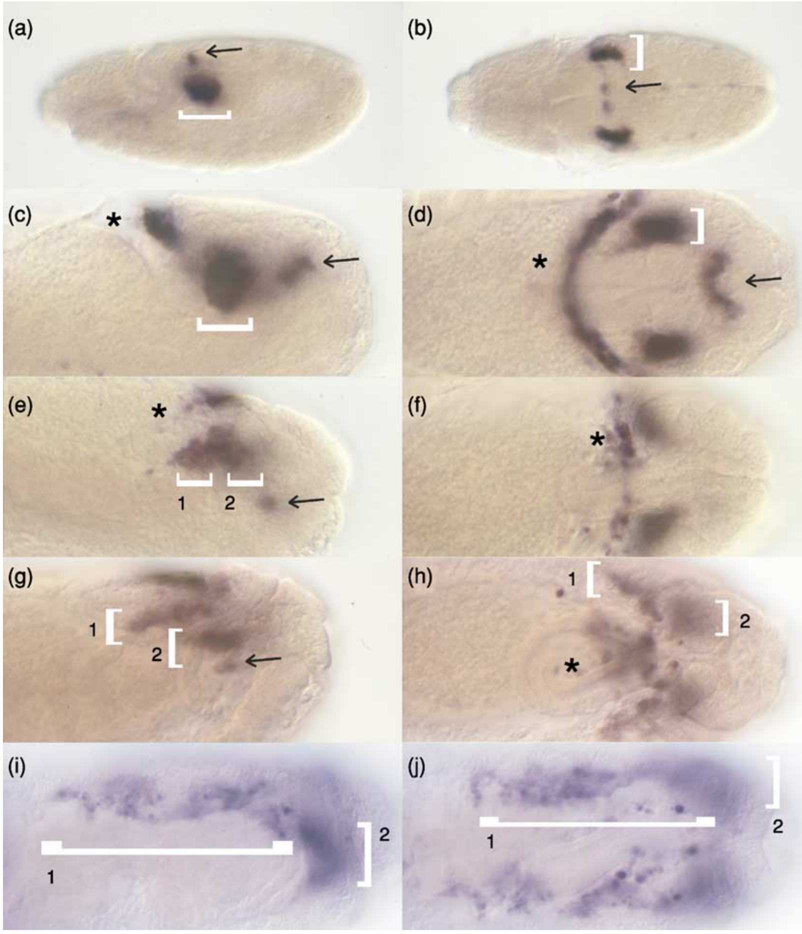


Figure 5 Development of the fat-cell projections. (a) Lateral and (b) dorsal view of a stage-11 embryo stained for β-galactosidase protein. A *lacZ* reporter gene driven by a dorsal fat-cell enhancer was used to follow the development of the dorsal fat-cell cluster. *lacZ* reporter gene activity is detected in the dorsal cell cluster of PS 13 (square bracket) and in a small bilateral ventral cell cluster (arrow). (c) Lateral and (d) dorsal view of a stage-12 embryo. As the germ band retracts, the bilateral ventral clusters of cells in PS 13 fuse across the ventral midline (arrow). *lacZ* reporter gene expression is also detected in the posterior-most amnioserosa cells [star in (c, d)] and in the dorsal fat-cell cluster [bracket in (c, d)]. (e) Lateral and (f) dorsal view of a late stage-12 embryo. As the germ band fully retracts, the dorsal fat-cell cluster begins to separate into two subgroups of cells (square brackets, 1 and 2) and the ventral cluster is now located below the dorsal cell cluster [arrow in (e) and out of the plane of focus in (f)]. *lacZ* reporter gene expression is still detected in the posterior amnioserosa cells (star). (g) Lateral and (h) dorsal view of stage-14 embryo. The dorsal fat-cell cluster has separated into distinct subgroups of cells (square brackets, 1 and 2). The ventral cell cluster is still present (arrow in g). (i) Lateral and (j) dorsal view of a stage 16 embryo. The subgroup of cells from the dorsal fat-cell cluster marked by [bracket 1 in panels (g, h)] forms the dorsal fat-cell projection [bracket 1 in (i, j)] and the second subgroup marked by bracket 2 in (g, h), together with the ventral cell cluster from PS 13 contributes to the posteriormost portion of the lateral fat body. (Reproduced with permission from Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K., **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster*. *Insect Mol. Biol.* *11*, 67–77.)

division, the cells dissociate from one another and abandon all remaining ectodermal characteristics (Bate, 1993; Campos-Ortega and Hartenstein, 1997). The mesoderm forms at stage 9, when the cells elongate and flatten to form a monolayer under the ectoderm. These cells enter the slow phase of germ band extension and undergo the second post-blastoderm mitosis, while still maintaining a monolayer (Campos-Ortega and Hartenstein, 1985; Bate, 1993; Borkowski *et al*., 1995). By stage 10, the first morphological subdivision is detected by a regular fluctuation of *twist* (*twi*) expression along the anterior/posterior axis, which prepatterns the segregation of the somatopleura and the splanchnopleura (Borkowski *et al*., 1995). Mesoderm cells in the high *twi* expression domain appear to adhere strongly to the ectoderm, whereas their immediate neighbors in the low *twi* domain tend to coalesce and segregate inward during the third postblastoderm mitosis to form the splanchnopleura (Borkowski *et al*., 1995; Campos-Ortega and Hartenstein, 1997).

It was originally maintained, based on histological analysis, that fat cells arise from the somatopleura, which also gives rise to the somatic and cardiac musculature (Campos-Ortega and Hartenstein, 1985; see also **Chapter 2.6**). However, based on the location of the fat-cell progenitors (primary fat cells), the fat body primordia is likely to arise from the splanchnopleura (Hoshizaki *et al*., 1994), which also gives rise to the circular visceral muscle precursors (Campos-Ortega and Hartenstein, 1985). This observation is of particular interest because dorsal fat-cell projections and visceral muscle may share an earlier lineage (Miller, 2002; see Section 2.9.2.4).

The early mesoderm is divided by the activity of pair-rule genes into repeating units known as parasegments (Lawrence, 1992; see also **Chapter 1.8**). These same pair-rule genes provide many of the patterning cues necessary for establishing the metameric repeating pattern of the fat-cell primordia. The *srp* gene product is the cell marker used to locate and define the initial appearance of the three groups of fat-cell primordia: the primary, secondary, and dorsal fat-cell clusters (Riechmann *et al*., 1998). As such, *srp* expression in the fat-cell primordia is thought to be important for the final steps of fat-cell specification and commitment to a fat-cell fate (Abel *et al*., 1993; Rehorn *et al*., 1996; Sam *et al*., 1996; Hayes *et al*., 2001). Each group of fat-cell precursors is assigned to a specific functional domain within a parasegment based on its spatial arrangement and genetic requirement for specific pair-rule genes (Riechmann *et al*., 1997, 1998).

The pair-rule genes *even-skipped* (*eve*) and *sloppy-paired* (*slp*) are expressed in stage-11 embryos in an alternating 14-stripe pattern that subdivides each of the parasegments into an *eve* and *slp* functional domain (**Figure 6**) (Frasch *et al*., 1987; Hacker *et al*., 1992). The Eve and Slp functional domains subdivide the parasegment into an anterior and posterior domain, respectively (**Figure 6**); these domains, however, do not correspond to anterior/posterior compartments (Riechmann *et al*., 1997 and appendix therein). The subdivision of each parasegment into these two functional domains best describes the repeating unit that reflects the origins of various mesodermal tissues (Riechmann *et al*., 1997 and

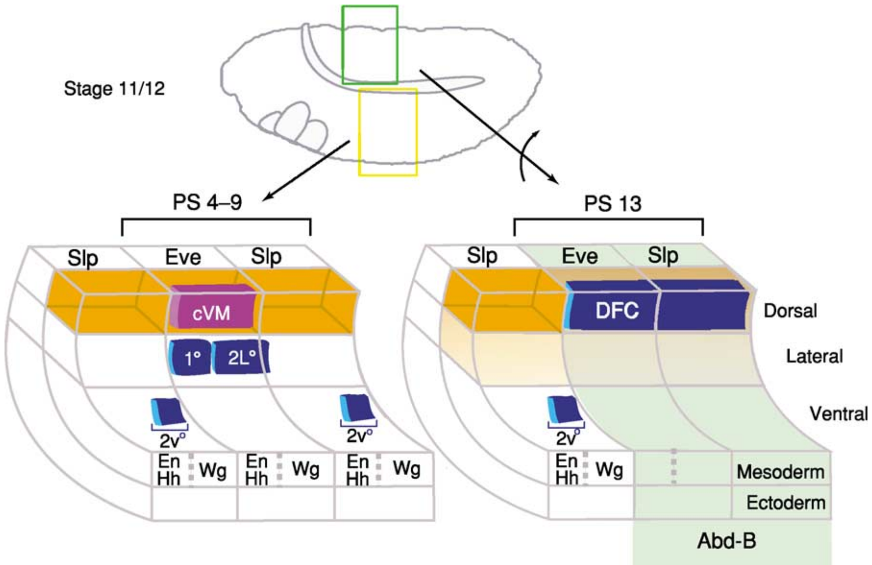


Figure 6 Schematic representation of the ectodermal and mesodermal layers from sections of a stage-11 embryo. The expression pattern of several genes along the anterior/posterior axis is responsible for subdividing the mesoderm into functional domains and subdomains within each parasegment (PS). The genes *eve* and *slp* are expressed in alternative bands in stage-11/12 embryos and functionally subdivide the parasegment (PS). In PS 4–9 the circular visceral muscle precursors (VMc), arise in the dorsal region of each of the Eve domains, while cells in the lateral region of the Eve domain produce two distinct groups of precursor fat cells: the primary fat-cell precursors (1°), which lie within the En, Hh subdomain, and the lateral secondary fat-cell precursors (L2°), which are outside the En, Hh subdomain. A third group of fat-cell precursors cells arises in the lateral region of the Slp within the Wg subdomain. In PS 13, *en* and *hh* are no longer expressed in the anterior compartment of the Eve domain. The presence of Abd-B protein in PS 13 represses precursor fat-cell formation in the lateral region, and in the dorsal region of PS 13 Abd-B simultaneously represses cVM formation and induces fat-cell formation. These precursor fat cells form the dorsal fat-cell cluster (DFC). (Based on data from Riechmann, V., Rehorn, K.P., Reuter, R., Leptin, M., **1998**. The genetic control of the distinction between fat body and gonadal mesoderm in *Drosophila*. *Development 125*, 713–723, and references therein, adapted with permission from Miller, J.M., **2002** Analysis of the cell-specific expression of the *serpent* gene and its role as a cell-fate switch in *Drosophila melanogaster*. Ph.D. thesis, University of Nevada, Las Vegas.)

appendix therein). The primary fat-cell clusters reside in the lateral mesoderm within the *eve* domain of PS 4–9 and are restricted to the anterior region of the *eve* domain by the expression of *engrailed* (*en*) and *hedgehog* (*hh*) (**Figure 6**) (Riechmann *et al.*, 1998).

Shortly after the emergence of the primary fat cells, two sets of secondary fat-cell clusters arise in a metameric pattern (**Figure 3**) (Riechmann *et al.*, 1998). The lateral secondary fat cells arise in the *wingless* (*wg*) subdomain of the Eve domain of PS 4–12 (**Figure 6**). These cell clusters are located immediately posteriorly of the position of the primary cell clusters and outside of the *en* and *hh* subdomain, and may extend into the *slp*-expression domain (Riechmann *et al.*, 1998). The third group of fat cells, the secondary ventral fat-cell clusters, are under the control of *wg* within the Slp domain, but it is likely that the reorganization of the ventral mesoderm brings about the placement of the ventral fat-cell cluster to the border of the Eve and Slp domain (Riechmann *et al.*, 1998). The *slp* gene encodes a forkhead domain transcription factor that negatively regulates *eve* activity (Hacker *et al.*, 1992). Thus, the ventral secondary fat-cell clusters depend upon positional information that is incompatible with the specification of the primary fat-cell clusters, which requires *eve* (and *en*/*hh*) activity. The final group of fat cells, the dorsal fat-cell cluster, resides in the dorsal mesoderm and spans the entire PS 13 and the Eve domain of PS 14 (**Figure 6**) (Riechmann *et al.*, 1998; Miller *et al.*, 2002).

Along the anterior/posterior axis, the presence or absence of groups of fat-cell clusters varies according to parasegment identity. It is likely that segment identity genes, such as the homeotic genes of the *Bithorax Complex,* further influence the specific nature of specified cells (Lawrence, 1985; Hooper, 1986; Bate, 1990; Bienz, 1994; Capovilla *et al.*, 1994; Michelson, 1994). Target genes for various homeotic genes have been identified such as *scabrous* (*sca*; Graba *et al.*,1992) and *decapentaplegic* (*dpp*; Immerglück *et al.,* 1990). In most cases, however, these target genes are involved in the final steps of cell or tissue differentiation and are removed from the initial cell specification steps. *srp* is involved in fat-cell specification and is an *in vitro* target of the homeotic transcription factor Ultrabithorax (Ubx) (Mastick *et al.*, 1995). *Ubx* is expressed in the mesoderm of PS 6–12 (White and Wilcox, 1984; Akam and Martinez-Arias, 1985) and is not expressed in the parasegments that give rise to the dorsal fat-cell projection (PS 13/14) or the ventral commissure (PS 3–5). Loss of *Ubx* activity has little effect on the formation of the dorsal projections, but misexpression of *Ubx* throughout the mesoderm reduces the dorsal projections to single rows of cells (**Figure 7**) (Miller *et al.*, 2002).

Ubx represses the formation of the ventral commissure (Miller *et al.*, 2002). The secondary ventral fat-cells in PS 3–5 lie outside the Ubx domain. Loss of *Ubx* activity results in strong derepression of *srp* within the secondary ventral fat-cell clusters in PS 6–12 and the formation of an ectopic fat-cell bridge, which arises from PS 6 (**Figure 7**) (Mastick *et al.*, 1995; Miller *et al.*, 2002). Furthermore, misexpression of *Ubx* throughout the mesoderm is sufficient

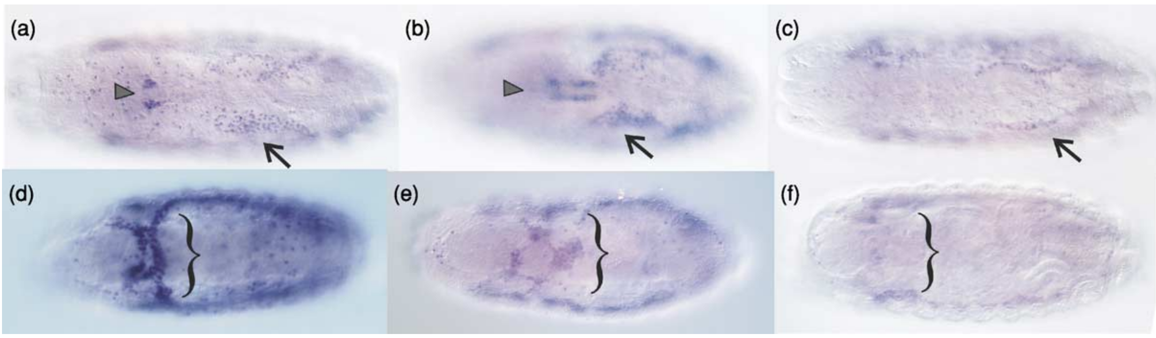


Figure 7 *Ubx* is involved in the formation of the dorsal fat-cell projection and ventral commissure. (a) Dorsal view of wild-type, (b) *Ubx* mutant, and (c) *twi-GAL4;UAS-Ubx* stage-16 embryos stained for Srp protein. (a) Wild-type dorsal fat-cell projection (arrow) and lymph glands (arrowhead). (b) Loss of *Ubx* has little or no effect on the number of cells contributing to the dorsal fat-cell projection (arrow). The lymph glands have hypertrophied (arrowhead) as previously described by Mastick *et al.* (1995). (c) In the *twi-GAL4;UAS-Ubx* embryo, *Ubx* is misexpressed throughout the mesoderm. Misexpression of *Ubx* reduces the width of the dorsal fat-cell projections (arrow) to 1–2 cells, compared to the normal 6–8-cell width, and causes the loss of the lymph glands. Ventral view of stage-16 wild-type (d), *Ubx* mutant (e), and *twi-GAL4;UAS-Ubx* (f) embryos stained for Srp protein. (d) Wild-type ventral commissure (bracket). (e) Loss of *Ubx* results in the formation of an ectopic ventral commissure (bracket) from PS 6, while (f) misexpression of *Ubx* throughout the mesoderm represses the formation of the ventral commissure. Bracket marks the loss of the ventral commissure. (Adapted with permission from Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K., **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster*. *Insect Mol. Biol.* *11*, 67–77.)

to completely suppress the formation of the endogenous ventral commissure (**Figure 7**) (Miller *et al.*, 2002). The loss- and gain-of-function data indicate that Ubx represses the formation of two fat-body domains: the ventral commissure and the dorsal projections. These data are in contrast to the *in vitro* data, which indicate *srp* is a direct target of Ubx and therefore suggest that *Ubx* might have a role in fat-cell specification through the control of *srp*. It is likely that the role of *Ubx* in the repression of the ventral commissure and the dorsal projections is the result of segment identity changes through the action of a hierarchy of genes rather than through direct activation of *srp*.

In contrast to *Ubx*, the other two genes of the Bithorax complex, *abdominal A* (*abd-A*) and *Abdominal B* (*Abd-B*), have a role in the specification of fat cells and are likely to be directly involved in the transcriptional control of *srp*. *abd-A* is expressed in PS 10–12 and represses the formation of the primary fat-cell clusters in these parasegments (Moore *et al.*, 1998). In the absence of Abd-B, primary fat-cell clusters are induced in PS 10–12 and replace the somatic gonadal precursors (Moore *et al.*, 1998; see also Section 2.9.2.3).

Abd-B's role in fat-cell specification is complicated and context dependent. The dorsal fat-cell cluster arises in PS 13/14 within the mesodermal expression domain of the morphogenic (m) isoform of the homeotic gene *Abd-B* (Akam and Martinez-Arias, 1985; Casanova *et al.*, 1986). *Abd-B* is expressed at its highest levels in PS 13 (**Figure 6**), and is present in declining levels in PS 11–12 (Delorenzi and Bienz, 1990). Loss- and gain-of-function experiments have demonstrated that high levels of *Abd-B* expression are both necessary and sufficient for formation of the dorsal fat-cell cluster *within* the dorsal mesoderm (Miller, 2002; see Section 2.9.2.4 for details). In the absence of *Abd-B* gene function, the dorsal fat-cell cluster in PS 13/14 is not specified (Riechman *et al.*, 1998; Miller, 2002; see also Section 2.9.2.4) while misexpression of *Abd-B* in parasegments anterior to PS 13 results in the induction of a stereotypic repeating pattern of dorsal fat-cell clusters in the dorsal mesoderm (**Figure 8**) (Miller, 2002; see also Section 2.9.2.4). Outside the dorsal mesoderm, in the lateral and ventral mesoderm, high levels of Abd-B repress formation of the primary and secondary fat cells in the lateral and ventral mesoderm. Thus, Abd-B induces fat-cell formation in the dorsal mesoderm and represses fat-cell formation in the lateral and ventral mesoderm. As noted, *Abd-B* is also expressed in PS 11 and 12, albeit at lower levels than in PS 13. In PS 10–12 of wild-type embryos, the secondary fat-cell clusters in the lateral mesoderm are present, but the primary fat-cell clusters are missing. In the absence of Abd-B, a putative primary fat-cell cluster appears in PS 12 (Moore *et al.*, 1998). Thus, the titer of Abd-B also appears to play a role in the induction/repression of the fat cells along the dorsal ventral axis of the mesoderm.

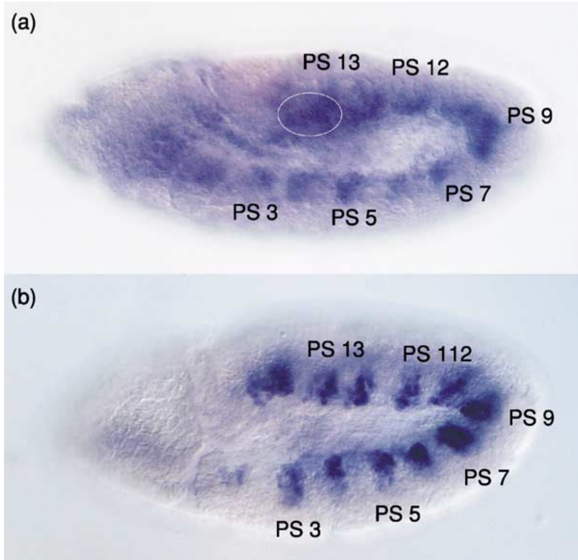


Figure 8 Misexpression of *Abd-B* in the mesoderm results in the serial duplication of the dorsal fat-cell cluster. (a) *twi-GAL4;UAS-Abd-B* embryo where *Abd-B* is misexpressed throughout the mesoderm. Embryo is stained for Srp protein, and reveals the presence of the DFC cluster in PS 13 (circle) and ectopic *srp*-expressing clusters in the posterior compartment of PS 2–12 within the dorsal mesoderm. Primary and secondary fat-cell clusters are not detected. In (b), the *twi-GAL4;UAS-Abd-B* embryo carries the dorsal fat-cell *lacZ* reporter gene and is stained for β-galactosidase protein. The expression pattern of β-galactosidase protein recapitulates the ectopic Srp-expression pattern in (a) and, thus, the ectopic *srp*-expressing cells are derived from ectopic dorsal fat-cell clusters. (Adapted with permission from Miller, J.M., **2002**. Analysis of the cell-specific expression of the *serpent* gene and its role as a cell-fate switch in *Drosophila melanogaster*. Ph.D. Thesis, University of Nevada, Las Vegas.)

In the aforementioned experiments, the presence or absence of fat-cell clusters was determined by *srp* gene expression. Thus, Abd-B is a positive regulator of *srp* in the dorsal mesoderm and a negative regulator of *srp* in the lateral/ventral mesoderm. The opposing response of *srp* to Abd-B reflects fundamental patterning differences between the dorsal and lateral/ventral mesoderm. The dorsal mesoderm is defined by *decapentaplegic* (*dpp*)-maintained *tinman* (*tin*) activity. *tin* is an NK homeodomain protein-encoding gene that is expressed in three phases during embryonic development (Kim and Nirenberg, 1989). Initially, *tin* is expressed in a pan-mesodermal fashion. In the second phase, *dpp* activity in the ectoderm induces the continued expression of *tin* in the

underlying dorsal region of the mesoderm, while *tin* expression in the ventral and lateral mesoderm declines. In this second phase, *tin* defines the dorsal mesoderm. In the third phase, *tin* expression is restricted to and marks the precursor to the heart (Azpiazu and Frasch, 1993; Bodmer, 1993; Yin and Frasch, 1998).

Moore *et al*. (1998) suggested that early *tin* expression is responsible for the specification of fat cells, based on a comparison of *tin* and *dpp* mutant phenotypes. Because the second phase of *tin* expression is dependent upon *dpp*, this comparison can distinguish between the requirement of the first or second phase of *tin* actvity on fat-cell specification. Based upon an apparent increase in fat-cell number in *dpp* mutants compared to *tin* mutants, it was concluded that the initial phase of *tin* expression is important for the specification of fat cells. However, because of the gross developmental defects associated with the loss of *dpp*, it is difficult to estimate the change in the number of fat cells (Moore *et al*., 1998).

Using a *lacZ* reporter gene driven by a dorsal fat-cell enhancer from the *srp* promoter, Miller *et al*. (2002) re-examined the role of *tin* and *dpp* in the specification of one fat-cell domain, the dorsal fat-cell projections. In embryos in which *dpp* is misexpressed throughout the mesoderm, the expression of *tin* persists in the lateral and ventral mesoderm. In these embryos, the dorsal fat-cell cluster is expanded to fill most of PS 13 by extending into the lateral and ventral regions (**Figure 9**). In contrast, in *dpp* mutants (and *tin* mutants), few, if any, dorsal fat cells are detected (**Figure 9**) and, concomitantly, the dorsal fat-cell projections (and the posterior-most cells of the lateral fat body) are absent. Thus, the specification of the dorsal fat-cell cluster depends upon the second phase of *tin* expression, which is responsible for the formation of the dorsal mesoderm (Miller *et al*., 2002). It has yet to be determined whether Tin or downstream factors intrinsic to the dorsal mesoderm are direct transcriptional activators of *srp*. To summarize, the role of *Abd-B* gene expression in the specification of the different precursor fat cells is integrated with both positional information and the concentrations of *Abd-B* protein.

2.9.2.2. Role of Serpent in Fat-Cell Specification

Key to the specification, differentiation, and maintenance of fat cells in *D. melanogaster* is the activity of *srp*. Although *srp* is expressed in derivatives of all three germ layers, within the trunk mesoderm *srp* is expressed in precursor fat cells and is the earliest identified fat-cell gene (Abel *et al*., 1993; Rehorn *et al*., 1996; Sam *et al*., 1996). Srp is a key regulator of fat-cell development. Loss of *srp* results in the inability to maintain the progenitor fat cells of the lateral fat body and the loss of all mature fat cells (Rehorn *et al*., 1996; Sam *et al*., 1996), whereas misexpression of *srp* in the mesoderm is sufficient to induce ectopic fat cells and to disrupt the specification/differentiation of the somatic and visceral muscle and the gonadal mesoderm (Hayes *et al*., 2001). These lines of evidence suggest that *srp* can operate to various degrees as a cell-type determining gene within the mesoderm.

Srp is a member of the GATA transcription factor family, and is involved in cell-fate specification and differentiation (Ramain *et al*., 1993; Winick *et al*.,

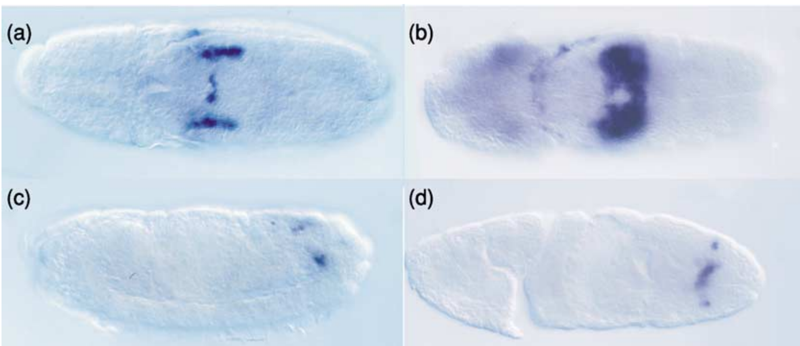


Figure 9 Specification of the dorsal cell cluster requires the formation of the dorsal mesoderm. (a) Stage-11 wild-type embryo carrying the *lacZ* dorsal fat-cell reporter gene of Miller *et al*. (2002): β-galactosidase staining marks the dorsal fat-cell cluster. (b) Stage-11 *twi-GAL4*;*UAS-dpp* embryo carrying the *lacZ* dorsal fat-cell reporter gene stained for β-galactosidase protein: *dpp* is misexpressed throughout the mesoderm and causes the ventral expansion of the dorsal fat-cell cluster to occupy most of PS 13. (c) Stage-13 *tin* and stage-12 *dpp* mutant (d) embryos carrying the *lacZ* dorsal fat-cell reporter: the dorsal fat-cell cluster consists of a few cells in both mutant backgrounds revealed by the loss of *lacZ*-expressing cells (c, d) and results in the loss of the fat-cell projections (and posterior-most cells of the lateral fat body). (Adapted with permission from Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K. **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster. Insect Mol. Biol. 11*, 67–77.)

1993; Harigae *et al.*, 1998; Gajewski *et al.*, 1999). It was initially identified as a transcriptional activator of *Adh* in fat cells (Abel *et al.*, 1993) and was subsequently shown to have a role in fat-cell determination and differentiation (Rehorn *et al.*, 1996; Sam *et al.*, 1996; Moore *et al.*, 1998; Riechmann *et al.*, 1998; Hayes *et al.*, 2001). In addition, *srp* is also involved in the differentiation and morphogenesis of the gut (Reuter, 1994; Rehorn *et al.*, 1996; Murakami *et al.*, 1999), germband retraction (Frank and Rushlow, 1996), hematopoiesis (Rehorn *et al.*, 1996; Lebestky *et al.*, 2000, 2003; Fossett *et al.*, 2001; Waltzer *et al.*, 2002), and larval fat-cell specific expression (Brodu *et al.*, 1999, 2001; Petersen *et al.*, 1999; Tingvall *et al.*, 2001).

Based on genetic studies, *srp* gene expression is also necessary for maintaining fat-cell identity in the lateral fat body (Sam *et al.*, 1996). In transcript- and protein-expressing *srp* loss-of-function mutants, *srp* gene-expressing cells are readily detected in the position of the fat-cell precursors, but later these cells appear to undergo apoptosis (Sam *et al.*, 1996). The loss of *srp* function, however, does not prevent early expression of the enhancer trap line [29D] in the progenitor fat cells that give rise to the lateral fat body (Sam *et al.*, 1996). Thus, *srp* appears *not* to be necessary for specification of the lateral fat body *per se*, but is necessary for the maintenance of progenitor fat-cell identity.

Experiments based on deregulated expression of *srp* reveal that *srp* can also induce embryonic fat-cell formation and disrupt development of other mesodermal tissues (Hayes *et al.*, 2001). Misexpression of *srp* throughout the mesoderm results in an increase in the number of fat cells and an expansion of the lateral fat body (**Figure 10**) (Hayes *et al.*, 2001).

The ability of *srp* to induce fat-cell formation is likely to be cell autonomous and to depend upon mesodermal factors. Misexpression of *srp* in the ectoderm is not sufficient to induce the fat-cell markers *Adh* or *DCg1* in the ectoderm, nor does it alter the normal development or morphology of the lateral fat body (Hayes *et al.*, 2001). The capacity of *srp* to generate fat cells is also dependent upon other factors, because premature expression of *srp* does not result in premature expression of fat-cell genes (Hayes *et al.*, 2001). It is likely that the preservation of the correct temporal sequence in fat-body development reflects the dependence of *srp* on the expression of temporally restricted mesodermal factors.

The ectopic fat cells generated by the misexpression of *srp* in the mesoderm are not derived from the endogenous fat-cell lineage (Hayes *et al.*, 2001). Early expression of the fat-cell marker P[29D] is independent of *srp* (Sam *et al.*, 1996) and, thus, P[29D] was used as a cell marker to examine the origin of the ectopic fat cells. The number and organization of the fat cells marked by P[29D] appear normal in the embryos, even though these embryos have an increased number of ectopic fat cells, which express several fat-cell markers, e.g., *Adh* and *DCg1* (Hayes *et al.*, 2001). Thus, the increase in fat cells is not due to hyper-proliferation of endogenous fat-cell lineage, although the possibility that the ectopic fat cells originate from the secondary fat-cell clusters, which are not labeled by P[29D], has not been eliminated.

The misexpression of *srp* in the mesoderm also results in the loss or disruption of the gonadal mesoderm, visceral muscle, and body-wall muscle (Hayes *et al.*, 2001). The body-wall muscles are syncytia derived from single founder muscle cells, which serve as focal points for fusion with the surrounding myoblasts (Rushton *et al.*, 1995; Knirr *et al.*, 1999; see **Chapter 2.1**). Misexpression of *srp* in the mesoderm results in the loss of both *nautilus* (*nau*)-expressing and *S59*-expressing founder muscle cells. Consistent with this loss is a dramatic decrease

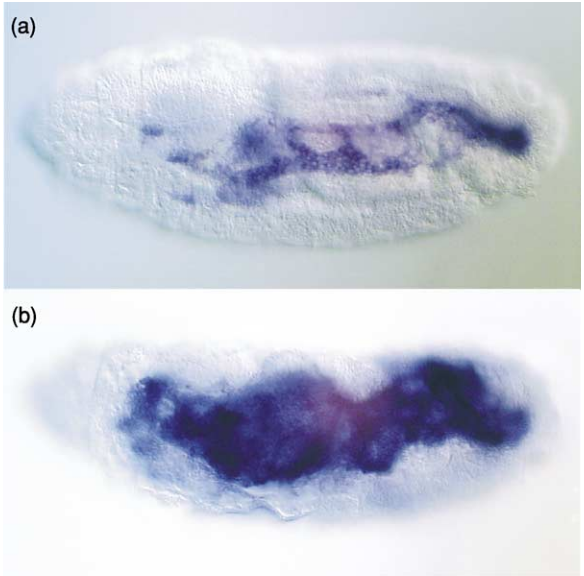


Figure 10 Misexpression of *srp* in the mesoderm leads to expansion of the fat body. (a, b) Stage-16 embryos stained for *Adh* mRNA. (a) Lateral view of a wild-type embryo. The lateral fat body consists of a ribbon-like band of cells with multiple disruptions or holes. (b) Lateral view of a *twist-GAL4;UAS-srp* embryo, where *srp* is misexpressed throughout the mesoderm. The lateral fat body is expanded in both the dorsal/ventral and anterior/posterior axis. (Reproduced with permission from Hayes, S.A., Miller, J.M., Hoshizaki, D.K., **2001**. *serpent*, a GATA-like transcription factor gene, induces fat-cell development in *Drosophila melanogaster*. *Development 128*, 1193–1200; © The Company of Biologists Ltd.)

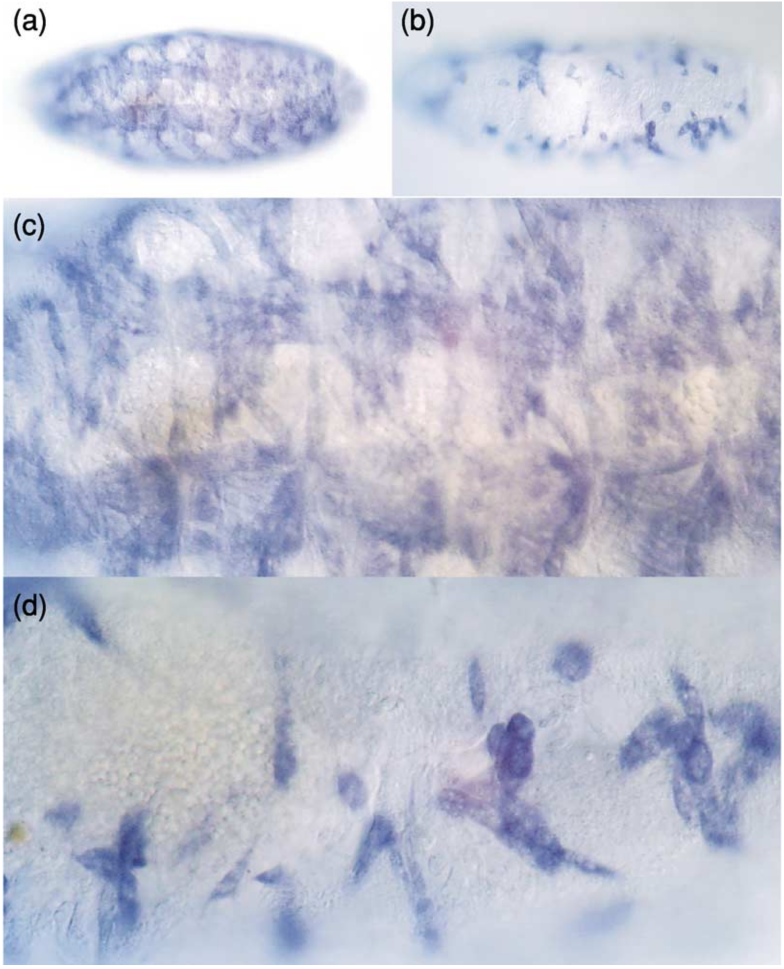


Figure 11 Misexpression of *srp* in the mesoderm leads to loss of somatic musculature. (a–d) Lateral views of stage-16 embryos stained for *Tropomyosin 1* (*Tm1*) mRNA. (a) In a wild-type embryo, *Tm1* (dark blue) is expressed in the somatic body-wall fibers. (b) In *twist-GAL4*;*UAS-srp* embryo, where *srp* is misexpressed throughout the mesoderm, most somatic muscle fibers are missing. (c, d) Higher magnifications of (a, b) respectively. (Reproduced with permission from Hayes, S.A., Miller, J.M., Hoshizaki, D.K., **2001**. *serpent*, a GATA-like transcription factor gene, induces fat-cell development in *Drosophila melanogaster*. *Development 128*, 1193–1200; © The Company of Biologists Ltd.)

in the number of muscle fibers (Figure 11). The few remaining muscle fibers are mono- and bi-nucleated and their number and location varies from embryo to embryo (Hayes *et al*., 2001). The correlation between the loss of body-wall muscle and the gain of ectopic fat cells presents an attractive possibility that the loss of founder muscle cells and myoblasts is due to the conversion of their respective primordia to a fat-cell fate. The role of *srp* in the disruption of gonads and visceral muscle formation is discussed below.

2.9.2.3. Fat Body Versus Gonads

The subdivision of the mesoderm by pair-rule genes provides many of the patterning cues necessary for establishing the fat-cell primordia. The expression of *srp* in the fat-cell primordia is important for the final steps in the commitment to a fat-cell fate and fat-cell specification. Thus, *srp* is likely to be a target of multiple transcriptional regulators that together provide unique compound positional cues associated with and according to the stereotypic location of individual fat-cell clusters. It is likely that other cell-specifying genes also respond to unique combinations of positional cues. Several lines of evidence suggest that the primary fat cells in PS 4–9 and the somatic gonadal precursors (SGPs) in PS 10–12 share a common precursor or are derived from a common pool of cells that can give rise to either cell type (Moore *et al*., 1998; Riechmann *et al*., 1998; Hayes *et al*., 2001). The SGPs give rise to the gonadal mesoderm, which makes up the somatic component of the gonads. The primary fat-cell clusters (in PS 4–12) and the SGPs (in PS 10–12) both

arise from cells in the same dorsoventral plane within the *eve* domain of their respective parasegments (**Figure 6**). These cells may represent common precursors that lie within PS 4–12 in a metameric repeating pattern. Based on genetic studies, *srp* activity in PS 4–9 directs formation of the primary fat cells, while the absence of *srp* expression in PS 10–12 allows SGP formation (Moore *et al.*, 1998; Riechmann *et al.*, 1998).

The repression of *srp* in the lateral mesoderm of PS 10–12 is mediated by *abd-A*, a homeotic gene involved in segment identity. Within the mesoderm, *abd-A* is expressed in PS 10–12 (Karch *et al.*, 1990). In *abd-A* loss-of-function mutants, the SGPs in PS 10–12 are absent, based on the lack of *zinc finger homeobox factor 1* (*zfh-1*) gene expression, and are replaced by *srp*-expressing cells (Moore *et al.*, 1998). ZFH-1 is a regulator of gonadal mesoderm development (Broihier *et al.*, 1998). In *abd-A* and *srp* double mutants, the expression pattern of *clift* (*cli*) and *412*, two SGP markers, is expanded to cells located in the equivalent position of the primary fat-cell clusters (Brookman *et al.*, 1992; Boyle *et al.*, 1997; Moore *et al.*, 1998). Thus, the apparent default fate of the common precursor is to become SGP, while *abd-A*'s role is to repress *srp* which, in turn, allows SGP formation (Moore *et al.*, 1998). The choice between primary fat cells and SGPs appears to rely upon *srp* activity that induces fat-cell formation in the common precursor, thereby preventing SGP specification.

This model predicts that forced expression of *srp* in the mesoderm should induce primary fat cells in PS 10–12 and repress SGP formation. Srp is capable of inducing fat-cell formation, as demonstrated in embryos where *srp* is misexpressed throughout the mesoderm (Hayes *et al.*, 2001). While on the surface these results favor the common precursor model, the forced expression of *srp* in the mesoderm does *not* affect the specification of the SGPs; rather, the SGPs fail to migrate and coalescence to form the gonads (**Figure 12**) (Hayes *et al.*, 2001). In wild-type embryos, *cli* is necessary for the migration and coalescence of the SGPs to form the gonads, but *not* for the

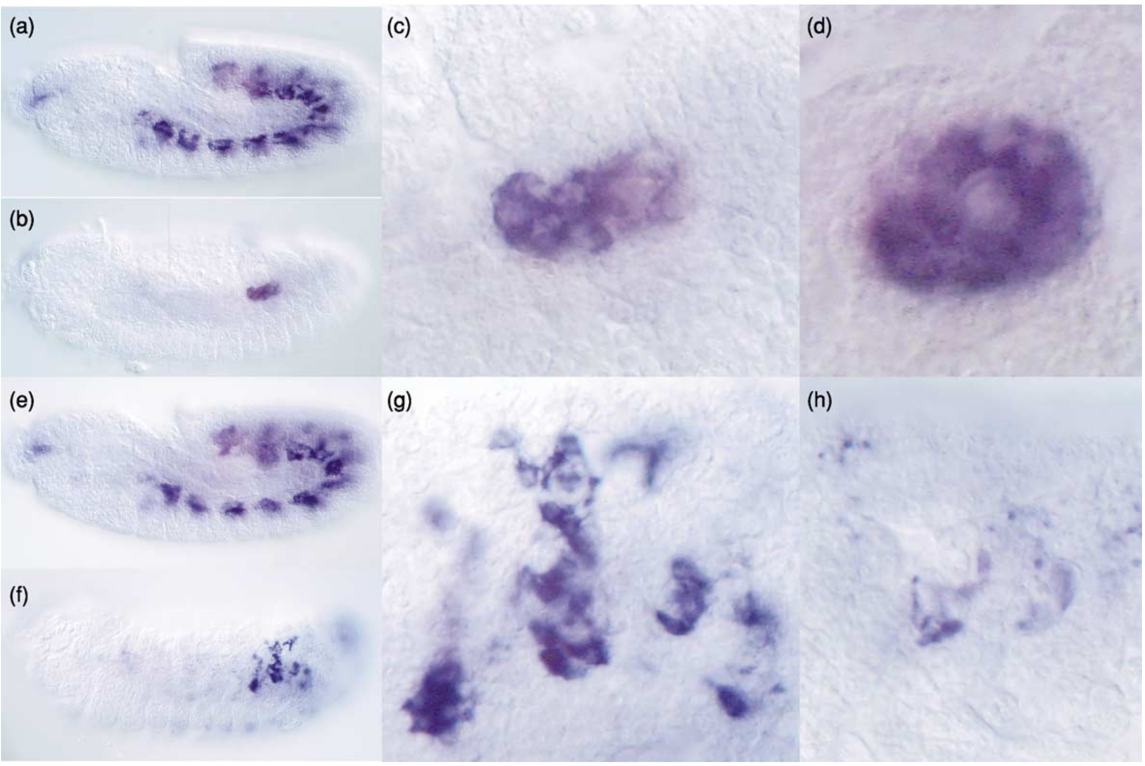


Figure 12 Misexpression of *srp* in the mesoderm leads to a disruption of the final stages of gonad formation. (a–d) Lateral view of wild type and (e, f) *twist-GAL4;UAS-srp* embryos stained for 412 mRNA. In *twist-GAL4;UAS-srp* embryos, *srp* is misexpressed throughout the mesoderm. In stage-12 wild-type embryos (a) and mutant embryos (e) *412* is expressed in clusters of cells in PS 2–12. In stage-14 wild type (b, c) and experimental (f, g) embryos *412* expression persists, in the SGPs. (c) and (g) are higher magnifications of (b) and (f), respectively. By stage 16, *412* expression marks the mature gonad in the wild-type embryo (d), while in the mutant embryos few 412-positive cells are detected (h). (Reproduced with permission from Hayes, S.A., Miller, J.M., Hoshizaki, D.K., **2001**. *serpent*, a GATA-like transcription factor gene, induces fat-cell development in *Drosophila melanogaster*. *Development 128*, 1193–1200; © The Company of Biologists Ltd.)

specification of the SGPs (Boyle *et al*., 1997). *cli* activity is also necessary for *412* expression (Boyle *et al*., 1997). In the mutant embryos where *srp* is misexpressed throughout the mesoderm, *cli* but *not 412* expression is absent in the SGPs (Hayes *et al*., 2001). This seemingly paradoxical result is best explained if the entire expression pattern of *cli* is taken into consideration. In the experimental embryos, the early expression pattern of *cli* is similar to wild type; *cli* protein is readily detected throughout the mesoderm and by early-stage 11 is lost in most mesodermal cells. At late-stage 11, *cli* expression is detected in the lateral muscle precursors as well as in the ectoderm (as seen in wild-type embryos), but no wild-type expression is observed in the SGPs (Hayes *et al*., 2001). In these same experimental embryos, *412* expression is normal; the metameric repeating expression of *412* is readily detected, and *412* undergoes the normal decline in PS 2–9 and continues to be expressed in the SGPs (PS 10–12) (Hayes *et al*., 2001). The occurrence of normal *412* expression in the experimental embryos suggests that early pan-mesoderm expression of *cli* at stage 10 is responsible for initiating *412* expression. Later in embryonic development, the *412*-expressing SGPs do not coalesce into a precursor gonad but remain scattered in the posterior region of the embryo (**Figure 12**) (Hayes *et al*., 2001). The absence of late *cli* expression in the SGPs of the mutant embryos is consistent with the inability of the SGPs to migrate and coalesce to form the gonads (Boyle *et al*., 1997).

An alternative model brought forth by Hayes *et al*. (2001) suggests that the precursors to the SGPs and the fat cells are derived from independent but closely associated groups of cells. Positional cues within each parasegment might specify which group will differentiate and be maintained. This model, in general terms, is analogous to the interaction between the mammalian Wolffian and Müllerian ducts (Hughes, 2001). In early mammalian development, before gonadal differentiation, both ducts are present. In females, the Müllerian duct develops into the oviduct while the Wolffian duct degenerates. In males, the production of Müllerian inhibition substance in the testes causes the degeneration of the Müllerian duct, while the Wolffian duct develops into the *vas deferens* (Higgins *et al*., 1989). In this alternative model, the absence of *srp* activity in PS 10–12 allows the SGPs to be specified and differentiate, while the fat-cell precursors are not maintained. Furthermore, in PS 4–12, *srp* activity allows the proliferation and maintenance of the precursor fat cells, while the SGPs do not differentiate. A role for *srp* in the maintenance of the fat-cell lineage has been previously suggested by Sam *et al*. (1996), who demonstrated that progenitor fat cells were present in *srp* mutant embryos. In the protein null mutant, srp^{6G45}, progenitor fat cells were identified by the expression of the P[29D] enhancer trap, and in the missense mutant srp^{9L06}, progenitor fat cells were detected by both P[29D] expression and immunohistochemical staining using an Srp antibody. In both mutant backgrounds the fat cells are absent by stage 15. Loss of the developing fat cells is likely to be due to programmed cell death based on the increase in apoptotic cell death in the location of the developing cells (**Figure 7** in Sam *et al*., 1996).

2.9.2.4. Fat Body Versus Visceral Muscle

The fat-cell projections arise from bilateral dorsal fat-cell clusters located within the dorsal portion of PS 13 and part of PS 14 (Riechmann *et al*., 1998; Miller *et al*., 2002). On the dorsoventral axis, the dorsal fat-cell clusters align with the precursors to the circular visceral mesoderm (cVM; **Figure 6**) in PS 3–12 (Azpiazu and Frasch, 1993; Riechmann *et al*., 1998; San Martin *et al*., 2001; Miller *et al*., 2002). Both of these cell types depend upon the formation of the dorsal mesoderm (Staehling-Hampton *et al*., 1994; Frasch, 1995; Riechmann *et al*., 1998; Miller *et al*., 2002). Recent studies on *Abd-B's* role in fat-cell specification suggest that the dorsal fat-cell projection and circular visceral musculature are derived from a pool of equivalent cells (Miller, 2002). As previously described, in the absence of *Abd-B* function, the *srp*-expressing cells that make up the dorsal fat-cell clusters are absent (Riechmann *et al*., 1998; Miller *et al*., 2002). In embryos in which *Abd-B* is misexpressed throughout the mesoderm, the primary and secondary fat cells are absent, and the only *srp*-expressing cell clusters, are serially duplicated in the dorsal mesoderm (**Figure 8**). These cells are duplicated dorsal fat-cell clusters, based on the expression of a reporter gene driven by a dorsal fat-cell enhancer (Miller, 2002). These data suggest that *Abd-B* is sufficient to activate *srp* and induce formation of a dorsal fat-cell cluster within the dorsal mesoderm.

The arrangement of the serially duplicated dorsal fat-cell clusters is reminiscent of the spatial pattern of the precursors to the cVM cells. The cVM precursors are derived from the dorsal mesoderm within the Eve domain of PS 2–12 (**Figure 6**) and are marked by *bagpipe* (*bap*) (Azpiazu and Frasch, 1993; Riechmann *et al*., 1997; San Martin *et al*., 2001). The *bap* gene is expressed in, and is necessary for, the formation of 11 cVM precursor cell clusters

(Azpiazu and Frasch, 1993). These clusters give rise to the circular visceral muscles that surround the gut and are necessary for the sterotypic constrictions that model the gut in mature embryos (Azpiazu and Frasch, 1993; San Martin *et al.*, 2001). In embryos where *Abd-B* is misexpressed throughout the mesoderm, *bap* expression is absent in the cVM (**Figure 13**) (Miller, 2002) and the gut is distended and missing its characteristic constrictions (D.K. Hoshizaki and J.M. Miller, unpublished data). This gut phenotype is similar to a loss-of-function *bap* mutant phenotype in which the circularly visceral muscles are absent. These data suggest that *Abd-B* controls a developmental switch between the dorsal fat-cell cluster and the cVM precursors.

Consistent with the common precursor model is the observation that in an *Abd-B* mutant embryo there is an ectopic cluster of cVM precursors in PS 13 (**Figure 13c**). These data suggest that *Abd-B*'s normal role is to simultaneously induce dorsal fat-cell formation and repress cVM formation in PS 13. Thus, in wild-type embryos, the absence or reduced levels of Abd-B in the anterior segments (PS 2–12), allows *bap* expression and the formation of the cVM precursors, while high levels of Abd-B in PS 13 induce *srp* expression in the dorsal mesoderm and the specification of a dorsal fat-cell cluster.

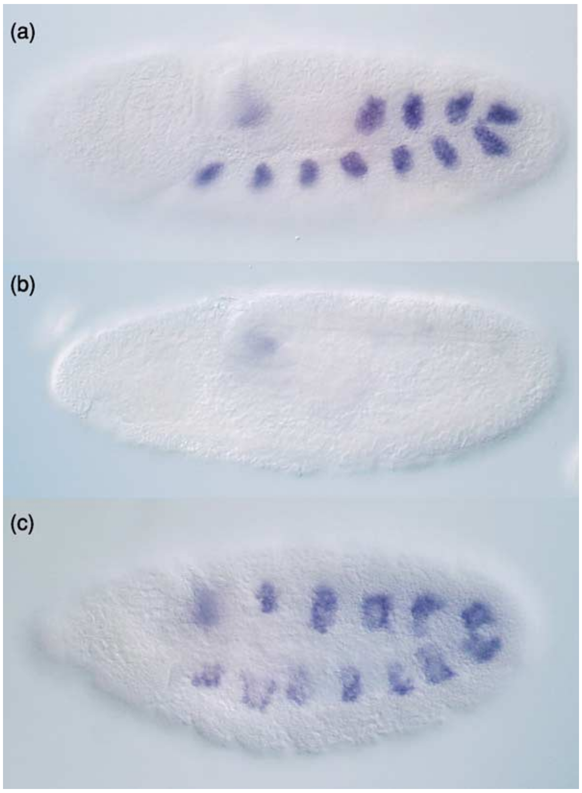


Figure 13 *Abd-B* suppresses visceral muscle formation. (a–c) Stage-11 embryos stained for Bap protein. (a) In wild-type embryos *bap* is expressed in 11 clusters in PS 2–12. (b) In *twi-GAL4*; *UAS-Abd-B* embryos, where Abd-B is expressed throughout the mesoderm, *bap*-expressing cells in the visceral muscle precursors are absent. (c) *Abd-B* mutant embryos have an ectopic *bap* cluster in PS 13. (Adapted with permission from Miller, J.M., **2002**. Analysis of the cell-specific expression of the *serpent* gene and its role as a cell-fate switch in *Drosophila melanogaster*. Ph.D. thesis, University of Nevada, Las Vegas.)

Based on misexpression experiments, *srp* is capable of repressing *bap* expression (Hayes *et al.*, 2001), and *Abd-B* may function in the dorsal mesoderm to specifically activate *srp,* which in turn inhibits cVM formation through the repression of *bap* expression. In *srp* mutants, ectopic *bap* clusters were not detected in PS 13, but this might be due to repression of cVM precursor specification by *Abd-B* (J.M. Miller and D.K. Hoshizaki, unpublished data). Because misexpression of *Abd-B* automatically leads to ectopic expression of *srp*, it cannot be determined from these experiments whether *Abd-B* can independently repress cVM formation. It is likely though that the normal absence of cVM precursors in PS 13 is due to the repression by both *Abd-B* and *srp*.

2.9.2.5. Summary

Over 25 years ago, Rizki and Rizki (1978) suggested that the larval fat body has a segmental origin. Recent studies have firmly established the segmental nature of the precursor fat cells and have identified different classes or groups of metamerically repeating cell clusters that contribute to the embryonic fat body (Abel *et al.*, 1993; Hoshizaki *et al.*, 1994; Riechmann *et al.*, 1998). The fat-cell precursors arise from unique anterior–posterior and dorsal–ventral positions within the mesoderm, which reflect the subdivisions of the mesoderm. A combination of positional information and input from different homeotic genes are involved in the establishment, maintenance, and differentiation of fat cells that populate the different regions of the fat body. The molecular mechanism underlying the commitment and specification of the different groups of mesodermal tissues relies upon *srp*, a GATA transcription factor gene involved in the specification and differentiation of fat cells (Rehorn *et al.*, 1996; Sam *et al.*, 1996; Hayes *et al.*, 2001) and, perhaps, represents a key component of the molecular machinery that determines cell-fate choices (Moore *et al.*, 1998; Riechmann *et al.*, 1998; Miller, 2002).

2.9.3. Origins of the Adult Fat Cells

The larval and adult cells are derived from two distinct groups of cells. During embryogenesis the

presumptive imaginal cells, which give rise to the adult epidermis, are segregated from the larval cells (reviews: Nöthiger, 1972; Bate and Arias, 1991). These imaginal cells reside in the larva either as discs of ectodermal cells surrounded by peripodial membrane or as clusters of cells known as histoblasts, which do not have a peripodial membrane. Imaginal cells are also integrated into their specific larval organ system, for example, the salivary gland and the gut. The imaginal cells are ectodermal in origin and the mechanism by which the mesodermal (adepithelial) cells become associated with the imaginal discs and eventually form the adult mesodermal derivatives in the head, legs, and thorax is not known. Only the adepithelial cells and the mesodermal histoblasts that give rise to muscle cells have been identified (Bate *et al*., 1991; Currie and Bate, 1991).

Transplantation studies of partial discs by Lawrence and Brower (1982) showed that the adepithelial cells associated with the presumptive notum of the dorsal mesothoracic (wing) disc are a source of adult muscle precursors. More recently, studies using *twi* as a cell marker for precursor adult muscle cells, have confirmed these observations and shown that *twi*-expressing adepithelial cells are associated with the leg, wing, haltere, and genital discs (Bate *et al*., 1991; Currie and Bate, 1991). In addition, *twi*-expressing histoblasts are located in each of the larval abdominal segments and are the precursor cells of the adult abdominal muscles (Currie and Bate, 1991).

The origin of the adult fat body is unclear. Robertson (1936) and Koch (1945) suggested that the abdominal fat body, oenocytes, and abdominal epidermis arise from histoblasts. Studies by Lawrence and Johnston (1986), however, have established that oenocytes and the abdominal epidermis share a common cell lineage, which is separate from the lineage that gives rise to the adult abdominal muscles. The lineage relationship of the fat-body cells with oenocytes, abdominal epidermis, and adult abdominal muscles was not determined owing to the lack of appropriate cell markers. It seems unlikely, though, that the adult abdominal fat cells would have a common cell lineage with the oenocytes and abdominal epidermis, because fat cells are derived from the mesoderm, while the oenocytes and abdominal epidermis are derived from the ectoderm.

Based on the analysis of two independent enhancer trap lines, X8-157a and 3-76a, the putative imaginal fat cells were identified as a subset of histoblast cells associated with the body wall of the larva, and as a subset of adepithelial cells associated with specific imaginal discs (Hoshizaki *et al*., 1995). The *lacZ* reporter gene associated with these two enhancer-trap lines exhibits activity in embryonic, larval, and adult fat cells and appears to mark all fat-cell lineages. In addition, *lacZ* activity is present in small clusters of cells (histoblasts) associated with the abdominal segments, and in adepithelial cells associated with the dorsal mesothoracic disc and the eye antennal disc (**Figure 14**). Furthermore, *lacZ* activity is present in the adepithelial cells located along the margin and does not overlap with the adepithelial cells of the adult muscle. Finally, *lacZ*-positive cells are also located in two clusters of adepithelial cells associated with the eye antennal disc.

These *lacZ*-positive adepithelial cells are associated with discs that give rise to structures containing fat cells. The dorsal mesothoracic disc gives rise to wing and thoracic structures, and eye-antennal discs give rise to head and antennal structures, all of which contain adult fat cells. In contrast, adult legs lack fat cells and, correspondingly, leg discs lack *lacZ*-positive adepithelial cells. Based on these correlations, it is likely that the *lacZ*-positive adepithelial cells and histoblasts in X8-157a and 3-76a lines represent the imaginal fat cells.

2.9.4. The Fat Body as an Endocrine Organ

The larval stage is characterized by rapid growth, which takes place mainly by an increase in cell size via endoreplication (Smith and Orr-Weaver, 1991; Royzman *et al*., 1997). The insect fat body has a central role in larval development by fueling this rapid growth through its participation in the intermediary metabolism. Results from a variety of studies suggest that the larval fat body also plays an active role in coordinating larval growth by monitoring the nutritional status of the animal and altering the feeding and other behavioral responses according to need (Section 2.9.4.3). Although most larval tissues undergo endoreplication (e.g., fat body, salivary glands, Malpighian tubules, trachea, muscle, and epidermis), the imaginal cells, which generate the adult-specific tissues, proliferate using a mitotic cell cycle. From studies in *D. melanogaster*, it is known that these mitotic tissues include imaginal discs, larval neuroblasts, the germ cells, and the abdominal histoblast nest. Each of these groups of cells exhibits a specific temporal proliferation pattern and, in most cases, proliferation is initiated after the first-instar larvae begin to feed (Nöthiger, 1972). The control of imaginal cell proliferation also appears to be dependent upon the fat body

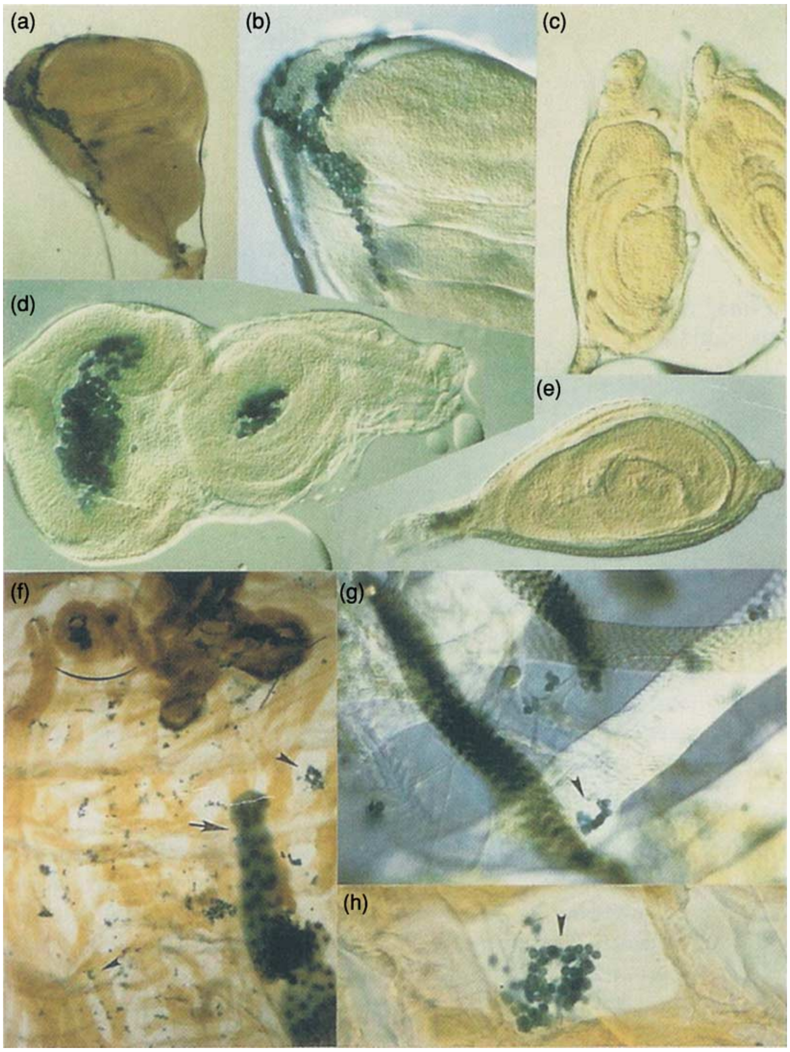


Figure 14 Identification of imaginal fat-body cells. *lacZ* activity in adepithelial cells of various imaginal discs and the fat-body cells in the enhancer-trap line X8-157a. *lacZ*-positive cells (dark staining) are detected in (a) the dorsal mesothoracic and (d) eye-antennal disc; (b) is a higher magnification of (a). *lacZ*-positive cells are not detected in the paired prothoraic discs (c) nor in the mesothoracic leg disc; (e). (f) Body wall and fat body (arrow) from dissected third-instar larva. The *lacZ*-positive adepithelial cells are associated with the body wall (arrowheads) and with specific imaginal disc, for example, eye-antennal disc (arc). (g, h) Higher magnifications of adepithelial cells associated with the body wall (arrowheads) in (f). (Reproduced with permission from Hoshizaki, D.K., Lunz, R., Ghosh, M., Johnson, W., **1995**. Identification of fat-cell enhancer activity in *Drosophila melanogaster* using P-element enhancer traps. *Genome 38*, 497–506.)

through the production of fat body mitogenic growth factors (Sections 2.9.4.1 and 2.9.4.2).

2.9.4.1. Fat Body, Cell Growth, and Differentiation

Since the initial efforts to culture insect cells and tissues, the addition of fat body or medium preconditioned with fat body was found to be an important requirement for *in vitro* cell proliferation and differentiation. The early work of Davis and Shearn (1977), for example, has shown that *in vitro* cultures of *D. melanogaster* imaginal discs require fat-body conditioned medium, insulin, and a juvenile hormone (JH) analog for their maintenance. These supplements allow for optimal growth and normal cell division. Furthermore, imaginal discs cultured with these supplements can maintain their capacity to differentiate into normal patterns of adult cuticular structures. *In vitro* cuticle deposition in cultured lepidopteran imaginal discs is also dependent upon fat-body conditioned media (and 20-hydroxyecdysone; 20E), but is inhibited by JH (Oberlander and Tomblin, 1972; Dutkowski and Oberlander, 1974; Oberlander, 1976b). The *in vitro*

differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae is also dependent upon the presence of fat-body conditioned media (Sadrud-Din *et al*., 1994, 1996). In the early 1990s Loeb and Hakim (1991) reported that 20E-stimulated fat body could induce the development of the genital imaginal disc of *Heliothis virescens in vitro*. Nine fractions from 20E-treated fat body were identified that could promote growth and development of the genital tract (Loeb, 1994). More recently, it was shown that co-culture of fat bodies from feeding larvae with quiescent central nervous systems from starved larvae can specifically induce neuroblast proliferation (Britton and Edgar, 1998). Based on a variety of tissue and cell culture studies, it is clear that the fat body is a source of secreted systemic growth factors.

2.9.4.2. Fat Body and the Production of Growth Factors

Genetic and biochemical studies have further established the role of the fat body as an endocrine organ and provided evidence that the fat body is a critical player in coordinating cellular growth with the nutritional status of the animal. The gene *minidiscs* (*mnd*) is expressed in the fat body and is responsible for a secreted factor necessary for imaginal disc proliferation (Martin *et al*., 2000). Mutations in *minidiscs* result in smaller imaginal discs and brain lobes, the fat body appears to be depleted of lipids, and these mutant animals die late in larval development (Shearn *et al*., 1971; Martin *et al*., 2000). Based on sequence analysis, *minidiscs* encodes an integral membrane protein with 12 putative membrane-spanning domains that has sequence similarities to amino acid transporter subunits from other eukaryotes (Martin *et al*., 2000). *In situ* hybridization studies have shown that *minidiscs* is ubiquitously expressed initially in the early embryo and, later, is expressed primarily in the embryonic fat body. In the larva, *minidiscs* is predominately expressed in the fat body, although weak expression is also detected in the imaginal disc and the optic lobes of the brain (Martin *et al*., 2000). Consistent with the biochemical and sequence data, transplanted *minidiscs* mutant discs grow in nonmutant hosts, suggesting that *minidiscs* dependent factors produced in the fat body are released into the hemolymph and are necessary for imaginal disc proliferation (Martin *et al*., 2000). The mechanism of *minidiscs* action on imaginal proliferation has yet to be determined, but it may coordinate imaginal disc proliferation and maturation in response to amino acid uptake and subsequent production of an imaginal disc specific growth factor(s).

Studies by Bryant and co-workers (Kawamura *et al*., 1999; Zurovec *et al*., 2002) have identified two classes of growth factors produced by the fat body. The family of imaginal disc growth factors (IDGFs) was isolated by systematically identifying extrinsic growth factors from conditioned medium (Kawamura *et al*., 1999), while the adenosine deaminase-related growth factors (ADGFs) were identified by sequence homology to insect-derived growth factors from *Sarcophaga* cells (Zurovec *et al*., 2002). The ADGFs stimulate imaginal disc proliferation and one of them, ADGF-D, is expressed primarily in the fat body and brain.

The IDGF family is expressed most strongly in the embryonic yolk cells and in the fat body of the embryo and larva, respectively. This family of factors may correspond to the mitogenic factors responsible for growth in fat-body conditioned media (Davis and Shearn, 1977; Kawamura *et al*., 1999). The IDGF proteins are structurally related to chitinases, but they have an amino acid substitution known to abrogate catalytic activity. Thus, it seems likely that these proteins evolved from chitinases that acquired a new growth-promoting function (Kawamura *et al*., 1999). Recombinant forms of IDGF1 and IDGF2 promote cell proliferation in the imaginal disc cell line C1.8 and promote cell growth and formation of pseudopodia in the presence of insulin (Kawamura *et al*., 1999). The cooperation between IDGFs and insulin in stimulating imaginal disc cell growth suggests that the IDGFs might function as cofactors to insulin and insulin-like peptides (Stocker and Hafen, 2000; Ikeya *et al*., 2002), which promote growth in *Drosophila* primary cultures (Echalier, 1997) and are necessary for proliferation of some imaginal disc cells (Cullen and Milner, 1991).

2.9.4.3. Fat Body and the Control of Feeding Behavior

It has been long established that the fat body has a central role in intermediary metabolism (Keeley, 1985; see also **Chapters 4.6** and **4.8**); however, evidence is accumulating that the fat body also plays an active role in establishing energy homeostasis through altering feeding and other behavioral responses. Based on a genetic screen to isolate mutant larvae defective in feeding, Zinke *et al*. (1999) identified *pumpless* (*ppl*), a behavioral mutant involved in suppressing food intake in response to amino acids. During the first larval stage, the *ppl* mutant larvae first accumulate food in the pharynx, and then move out of the food and begin to wander. These animals have a drastic reduction in growth and eventually die as small larvae (Zinke *et al*., 1999). Although these animals appear to have a

blockage in the pharynx, the entire larval feeding apparatus, the pharynx, esophagus, and proventriculus, appear normal (Hartenstein *et al*., 1994; Zinke *et al*., 1999). The wandering *ppl* mutants also do not appear to be starving, because starvation markers such as Lipase3 and hosphoenolpyruvate Carboxykinase (Pepck) are not elevated (Zinke *et al*., 1999). The *ppl* gene is expressed specifically in the fat body and codes for a protein, which has high homology to the vertebrate protein H subunit of the glycine cleavage system. This vertebrate enzyme complex is involved in the catabolism of glycine into ammonia, carbon dioxide, and other one-carbon molecules.

The *ppl* gene is likely to also be involved in amino acid metabolism through the catabolism of glycine and the generation of one-carbon molecules. Surprisingly, the addition of high levels of lysine, but not glycine, in the culture medium of wild-type animals phenocopies the characteristic accumulation of food in the pharynx and the growth reduction (Zinke *et al*., 1999). The mechanism by which the fat body and *ppl* may monitor changes in the quality or composition of the food source is not known. Shortly before pupariation, an ecdysone (20E) pulse signals a change in behavior and the larvae go through the transition from a feeding to a wandering nonfeeding larval stage (Riddiford, 1993). It is possible that *ppl* coordinates the nutritional status the animal (or the fat body) with this ecdysone pulse. The premature cessation of food intake and precocious initiation of wandering that occurs in the *ppl* mutant might reflect a premature activation of the wandering program.

2.9.5. Fat-Body Dissociation and Remodeling

During the last larval instar, at the onset of metamorphosis, the fat body undergoes extensive structural reorganization as its function changes and the animal undergoes the transition from larval to adult life. The fat body switches from the production and secretion of storage proteins, which serve as a source of polypeptides and amino acids for the re-architecture of the animal into the adult form (Haunerland, 1996; review: Burmester and Scheller, 1999), to the re-adsorption of these proteins from the hemolymph. The storage proteins are subsequently sequestered into large proteinaceous storage granules. During metamorphosis, these proteins are used as a source of polypeptides and amino acids for the re-architecture of the animal into its adult form (Haunerland, 1996; review in Burmester and Scheller, 1999). Early in metamorphosis the fat body undergoes a striking transformation from an organized tissue to a loose association of individual fat cells (Dean *et al*., 1985; Keeley, 1985). In the majority of insects, fat-body cells survive metamorphosis and undergo cellular reconstruction and transformation into the adult fat cells (Larsen, 1976). However, in some Hymenoptera and in the higher Diptera, the dissociated larval fat-body undergoes cytolysis in the adult and is replaced by an adult fat body, which arises from imaginal fat cells (Dean *et al*., 1985; Keeley, 1985; Hoshizaki *et al*., 1995).

Extensive studies describing fat body structure based on electron micrographs have been published for *Calpodes ethlius*, *Rhodnius prolixus*, *Periplaneta americana*, *Locusta migratoria*, and *Apis mellifera* (Locke, 1998). The last general review of fat body reorganization, however, was published over a decade ago (Rizki, 1978; Dean *et al*., 1985; Keeley, 1985). The most noted advances in our understanding of the events involved in fat-body dissociation during metamorphosis have taken place in *Sarcophaga peregrina* and *D. melanogaster.* In *S. peregrina*, the hemocytes appear to control the breakdown of the fat-body tissue into individual fat cells, while in *D. melanogaster* cellular signaling pathways have been identified that could establish fat body competence to undergo dissociation. The recent advances and the new experimental tools available in *D. melanogaster* should allow us to obtain further insights into fat-body dissociation and remodeling and are discussed below.

2.9.5.1. Fat-Body Reorganization: Morphological Changes

During metamorphosis the fat body undergoes a striking transformation that leads to its dissociation into independent cells. Fat body cells, like all insect cells with the exception of the hemocytes, are encased within a basal lamina (Ashurst, 1982). The fat body is made up of a single cell layer of cells that form sheets of tissue. The basal lamina of both the apical and basal surfaces of the fat body is exposed to the hemolymph while the lateral regions are associated with a basal lamina that is held to the plasma membrane by hemidesmosomes between adjacent fat cells (Dean *et al*., 1985). Below the basal lamina, the plasma membrane of the fat cells forms a reticular system from subsurface inward folding of the cell membranes that surround each of the fat cells (**Figure 15**) (Butterworth and Forrest, 1984; Locke, 2003). Studies on the fat body in *C. ethlius* and *R. prolixus* highlight the nature of the basal lamina and the plasma membrane reticular system that surround the fat body (review: Locke, 2003). Both the basal lamina and the entrance to the reticular system are selectively permeable and func-

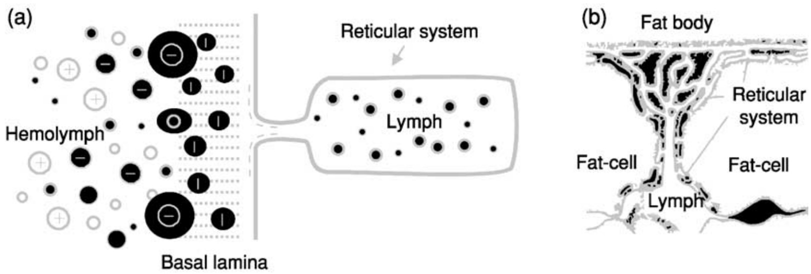


Figure 15 The reticular membrane system of the fat body. (a) The plasma membrane reticular system and intercellular lymph spaces are separated from the hemolymph by negatively charged barriers tending to exclude large positively charged particles. Both the basal lamina and reticular system entrances are negatively charged sieves (Brac, 1983). (b) Many insect cells interface with their environment through a compartment of their own creation. Fat body cells elaborate the lymph spaces between them by the extension of surface processes. They also fold their surface membrane inward to create reticular systems. (With permission, from the *Annual Review of Entomology*, Volume 48; © 2003 by Annual Reviews www.annualreviews.org.)

tion as negatively charged sieves that restrict the passage of charged molecules larger than 15 nm in diameter (Brac, 1983; Locke and Huie, 1983; Reddy and Locke, 1990). This restricted flow is likely to result in a lymph space between the basal lamina and plasma membrane that lacks positively charged and larger molecules (**Figure 15a**) (review: Locke, 2003). Whether components of the lymph and the plasma membrane reticular system play a role in basal lamina degradation is not known.

In Lepidoptera, fat-body cells lose their adhesion to each other and to the tracheoles during metamorphosis (Larsen, 1976). The mechanism of fat body breakdown is not known; however, because hemocytes associate with the fat body during metamorphosis, it is likely that the hemocytes might play a role in the degradation of the fat-body basement membrane and the release of fat cells. In *C. ethlius* the individual fat cells re-organize and become associated in nodular clumps surrounding the tracheoles. The dissociated fat cells undergo a type of programmed remodeling, where the mitochondria, microbodies, and rough endoplasmic reticulum become sequestered in organelle-specific autophagic vacuoles and are destroyed by hydrolytic enzymes (Locke and Collins, 1968; Locke, 1971, 1998). The cells, however, persist and new populations of the organelles are regenerated shortly after the eclosion of the adult (Larsen, 1976; Locke, 1998).

In *D. melanogaster* most of the larval tissues undergo autophagy and compete histolysis during metamorphosis (review: Thummel, 2001). The fat body, however, is transformed from an intact tissue to individual fat cells that are dispersed throughout the pupa. The larval fat cells persist through metamorphosis and are present in the newly eclosed adult; there are no reports of fat cells undergoing cellular remodeling (Rizki and Rizki, 1970; Rizki, 1978). The "larval" fat cells in young adults are present as single and small clumps of cells that float freely in the adult body cavity (Hoshizaki *et al*., 1995). Approximately 1000 "larval" fat cells are found in the abdomens of newly eclosed male adults (Butterworth, 1972). This is less than the 1700 fat cells estimated to be present in the adult male abdomen and the estimated number of fat body cells in male larvae (2161 ± 54 cells) (Rizki, 1969; Butterworth, 1972). Based on these observations, it is possible that a portion of fat-body cells undergo cell death during metamorphosis. To date, however, there is no direct experimental evidence for fat-cell death during metamorphosis. Approximately 90% of the "larval" fat cells present in the adult disappear within the first two days of adult life, while the remaining cells disappear by day 4. This process has been referred to as histolysis, but as suggested by Richard *et al.* (1993), it is best described as cytolysis because the fat body has already dissociated into individual cells. The "larval" fat cells are replaced by adult fat cells that are thought to arise from the adepithelial cells of the dorsal thoracic and eye-antennal discs and by the histoblasts that are associated with the body wall of the larva (Hoshizaki *et al*., 1995).

Although the mechanism of fat-cell elimination in the adult is unclear, it is thought to involve a form of programmed cell death. Early studies suggested that JH triggers the elimination of the "larval" fat cells based on the observation that the removal of the *corpus allatum* from young *Sarcophaga* adults prevented the loss of these cells (Day, 1943). The isolation of *apterous*^{4} (*ap*^{4}), a nonvitellogenic, JH-deficient mutant of *D. melanogaster* in which the "larval" fat cells in the adult do not undergo complete cytolysis, lent further support to this idea (Butterworth and King, 1965; Butterworth, 1972). In transplantation experiments, the behavior of the *ap*^{4} fat body is not cell autonomous (Butterworth

and Bodenstein, 1967; Butterworth, 1972). Head-abdomen ligation experiments mimic the ap^4 fat body mutant phenotype, and the application of the JH mimic methoprene (ZR515) to isolated abdomens and to mutant animals restores normal cytolysis and vitellogenesis (Postlethwait and Jones, 1978). These observations suggest a causal role of JH in the cytolysis of the "larval" fat cells in the adult. However, additional experiments showed that although the application of methoprene could indeed elicit the initiation of vitellogenesis, it could *not* restore histolysis in the ap^4 mutants (Richard *et al*., 1993). Furthermore, the absence of cytolysis is unique to the ap^4 mutant; other *ap* mutants exhibit cytolysis even though they also have low levels of JH (Richard *et al*., 1993). Thus, reduced levels of JH may not be causative for the loss of cytolysis of the "larval" fat cells in ap^4 mutant adult.

The deduced amino acid sequence of the Apterous (Ap) protein predicts a homeo domain and a cysteine/histidine-rich domain that make it a member of the LIM (Lin-11/Isl-1/Mec-3) homeobox family of regulatory proteins (Cohen *et al*., 1992). The *ap* gene has the capability of eliciting a number of independent biological process (review: Richard *et al*., 1993), which are not dependent upon normal JH production; one of them may be the induction of "larval" fat-cell cytolysis in the adult.

The early work of Rizki (Rizki and Rizki, 1970; Rizki, 1978) described the gross changes in fat-body dissociation, cell reorganization during metamorphosis, and the location of the dissociated fat cells within the newly emerged adults. The position of the larval fat cells within the pupa is retained in the adults and can be followed using the mutant combination *tumor (2)*^{w} (*tu*^{w}); *red cells* (*rc*) (Rizki, 1978; see also below).

Current advances in cell biology techniques have provided new tools that allow more detailed descriptions of tissue changes during metamorphosis (Ward *et al*., 2003). Thus, the development of green fluorescent protein (GFP) cell markers expressed in specific tissues has greatly improved the ability to visualize cells *in vivo*. Studies using differential interference contrast (DIC) and fluorescent microscopy in combination with GFP fat-cell markers make it possible to describe fat-body dissociation in live animals (**Figure 16**).

Several descriptions of the gross changes that occur during metamorphosis in *D. melanogaster* have been published earlier, although these accounts do not focus on the fat body (Robertson, 1936; Bodenstein, 1950; Bainbridge and Bownes, 1981; Ashburner, 1989). The early stages of metamorphosis with regard to the changes in the fat body can be summarized as follows (**Figure 16**). At 25 °C, approximately 10 h prior to pupariation, the larva leaves the food and begins the "wandering stage." This behavior is triggered by a small rise in the ecdysone (20E) titer, which begins 24 h after ecdysis to the third instar (Schwartz *et al*., 1984). During this stage, the larval fat body is recognized as sheets

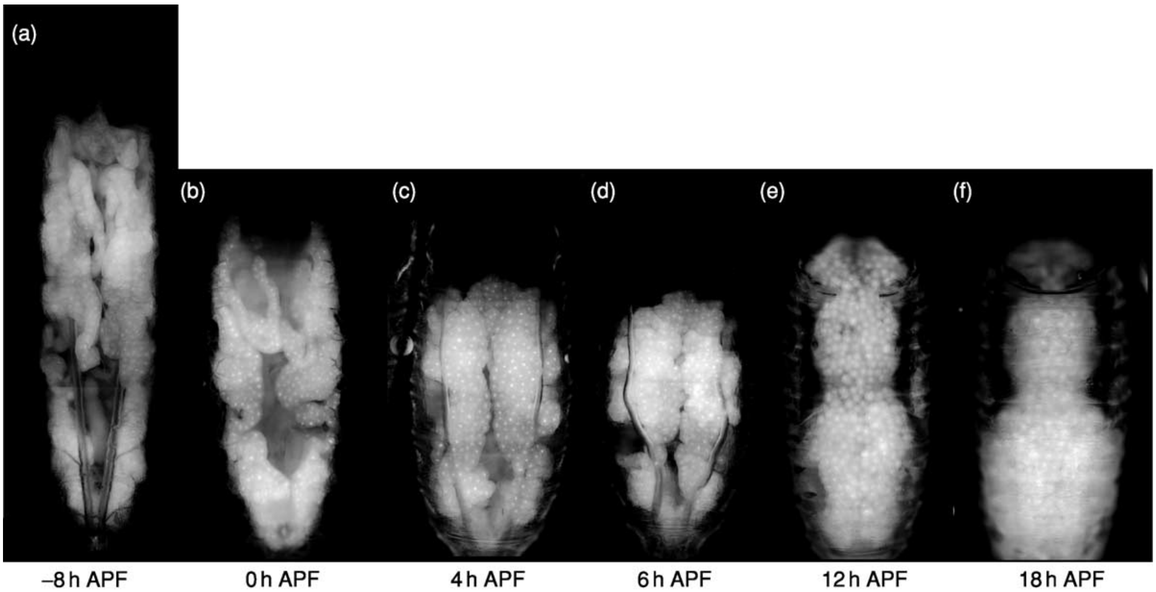


Figure 16 Third-larval instar and early pupal stages of *D. melanogaster*. Fluorescent images of fat-body cells from live animals expressing green fluorescent protein. (a) Late third-instar larva; (b) white prepupa; (c) pre-apolysis larva; (d) early prepupa; (e) early pupa; and (f) late pupa. (AFP is hours after puparium formation at 25 °C.)

of white, translucent cells that float in the hemolymph between the body wall and the midgut (Figure 16a). Pupariation is triggered by a large rise in the 20E titer in the hemolymph (Richard *et al.*, 1993). At this time, the larva shortens and becomes immobile, forming what is generally referred to as a white prepupa (wpp), a stage that lasts approximately 10 min and represents a convenient developmental marker from which to stage animals (Bodenstein, 1950). At this stage, the animal is still a larva because neither apolysis nor ecdysis have occurred (Ashburner, 1989). The fat body can be visualized through the cuticle using light or fluorescent microscopy; the gross morphology of the fat body is unchanged, and the fat body fills most of the peripheral space between the body wall and the gut (Figure 16b).

Hardening and tanning of the larval cuticle leading to the formation of the puparium cuticle occurs over the next 3.5 h. Approximately 4–6 h after puparium formation (APF), apolysis occurs and the epidermis is separated from the puparium cuticle, which now represents the outermost pupal cuticle. Between pupariation and apolysis, the animal is still a larva but has often incorrectly been referred to as a prepupa (Ashburner, 1989). During this time the fat body retracts from the anterior region of the animal (Figure 16c). Consistent with the observation of Rizki (1978), at 6 h APF, the fat-body cells from the head region are completely displaced toward the abdominal region, while the fat body still appears as an intact tissue (Figure 16d). Pupation occurs at 12 h APF and is triggered by a small rise in 20E (Handler, 1982; Sliter and Gilbert, 1992). Post-apolysis, the fat body is restricted to the abdominal region and begins to acquire a loose appearance, with the anterior cells appearing more spherical in shape, suggestive of a loss in cell–cell adhesion (Figure 16e). Pupation occurs at 12 h APF and is triggered by a small rise in 20E (Handler, 1992; Sliter and Gilbert, 1992). The muscular contractions that drive head eversion also release individual fat cells from the bulk of the fat-body cells. The anterior-most fat cells are released first and are distributed into the head region. Upon completion of head eversion, the entire fat body dissociates into individual spherical fat cells. At 18 h post pupariation the fat cells are freely packed in the open interior space of the pupa (Figure 16f).

2.9.5.2. Fat-Body Reorganization: Biochemical and Cellular Interactions

Little is known about the controlled breakdown of the fat-body basement membrane and the subsequent release of fat cells. Reports by Rizki (Rizki and Rizki, 1974a, 1974b, 1979) describe the breakdown of the basement membrane in the *D. melanogaster* mutant strain tu^{w} and the associated transformation of the spherical hemocytes to the flattened lamellocytes, which is normally seen during pupal development. Based on scanning electron micrographs, the tu^{w} fat body shows signs of dissociation in the caudal fat cells prior to the hemocyte changes. The caudal fat body of tu^{w} larvae grown at 25 °C exhibits abnormal loss of basement membrane 72 h after hatching. During the interval from 72 to 86 h, plasmatocytes and podocytes aggregate at the exposed cell surface and flatten to form layers of lamellocytes. By 93 h after hatching, the caudal fat body is encapsulated into a compact melanized mass (Rizki and Rizki, 1974a). These observations suggest that the breakdown of the basement membrane is an underlying factor in marking the site that the hemocytes will encapsulate. The particulate material that accumulates on and around the caudal fat cells may trigger the response of the hemocytes. If the metamorphosing fat body normally provides a signal that triggers the fat body-hemocytes association, then the observed premature appearance of lamellocytes in tu^{w} larvae might be due to caudal fat body taking on a "pupal" fat body status (Rizki and Rizki, 1974a). These observations suggest that in *D. melanogaster* the fat body plays a role in signaling its own dissociation.

In *S. peregrina* the dissociation of the fat body is mediated by hemocytes that associate with and degrade the basement membrane of the fat body. Key to understanding the dissociation process in *S. peregrina* was the establishment of an *in vitro* system that mimiced the dissociation of the fat body during metamorphosis (Kurata *et al.*, 1989). The prepupal hemocytes (4–6 h APF at 27 °C), but not the larval hemocytes, were shown to be capable of dissociating both larval and pupal fat body *in vitro*, and causing the release of individual fat cells (Kurata *et al.*, 1989). These results suggest that, after pupariation, the hemocytes acquire the ability to dissociate the fat body and that, at least *in vitro*, both larval and pupal fat body are susceptible to dissociation.

The dissociation of the fat body is mediated by the release of a 29 kDa Chymotrypsin-like protease from the hemocytes (Kurata *et al.*, 1990). The 29 kDa protein has been cloned and, based on sequence comparisons, was shown to correspond to Cathepsin B, a cysteine protease involved in intracellular degradation and turnover of proteins (Takahashi *et al.*, 1993). Cathepsin B is synthesized as a 344 amino acid pro-protein with a 20 amino acid putative signal sequence and a 68 amino acid pro region, both of which are cleaved to produce an

256 amino acid active enzyme (Takahashi *et al.*, 1993). The substrate specificity of *S. peregrina* Cathepsin B (S-Cathepsin B) is unique: it recognizes the peptidyl substrates Suc-Leu-Leu-Val-Tyr-MCA and Z-Phe-Arg-MCA (Suc, succinyl; MCA, methylcoumaryl-7-amide; Z carbobenzoxy), two substrates for Chymotrypsin and Cathepsin B and L (Kurata *et al.*, 1992b), but not the Chymotrypsin and Cathepsin B substrates Suc-Ala-Ala-Pro-Phe-MCA and Z-Arg-Arg-MCA, respectively (Kurata *et al.*, 1992b).

In *S. peregrina* the pupal, but not the larval, hemocytes are active in fat-body dissociation, thus it is likely that fundamental changes occur in the hemocytes during pupariation to facilitate hemocyte-fat body association and/or to activate Cathepsin B. Based on indirect immunofluorescence microscopy, Cathepsin B protein is located in heterogeneous granules in a subset of pupal hemocytes (Kurata *et al.*, 1992a). Early reports also suggest that the degranulation and subsequent release of active Cathepsin B from the hemocytes is mediated through hemocyte-fat body interactions (Kobayashi *et al.*, 1991; Kurata *et al.*, 1991).

Recognition molecule(s) carried on pupal hemocytes are proposed to stimulate hemocyte secretion of Cathepsin B upon interaction with the fat body basement membrane. A 200 kDa pupal hemocyte-specific membrane protein was reported as a candidate recognition molecule (Kobayashi *et al.*, 1991; Kurata *et al.*, 1991), but a recent study showed that this protein is myosin heavy chain derived from muscle fragments and not from pupal hemocytes (Hori *et al.*, 1997; Natori *et al.*, 1999).

2.9.5.3. Fat-Body Reorganization: Role of Ecdysone and TGF-β Signaling

The controlled breakdown of the fat body into its individual cells is a normal event during metamorphosis and is likely to be also regulated by the 20E pulse associated with pupariation formation. *In vitro*, the dissociation of the fat body occurs in a 20E dose-dependent manner (Oberlander, 1976a). This observation suggests that fat-body dissociation is cell autonomous and does not require pupal hemocytes. The first *in vivo* experimental evidence demonstrating a role for 20E in fat-body dissociation came from studies designed to distinguish the function of different ecdysone receptor (EcR) isoforms (Cherbas *et al.*, 2003). These authors developed dominant-negative mutants of EcR (EcR-DN), EcR-F645A and EcR-W600A which block ecdysone signaling. Regulated expression of *EcR-DN* was achieved through the use of the GAL4/UAS system that allows controlled expression of genes (Brand and Perrimon, 1993). Using this system, ecdysone signaling was blocked specifically in the fat body through the use of the *Larval serum protein 2* (*Lsp2*) promoter. The *Lsp2* gene is expressed specifically in the fat body early after ecdysis to the third instar, and *Lsp2* transcripts reach maximum steady-state levels at mid-third instar (Andres *et al.*, 1993). Expression of *Lsp2* is also detected in the prepupa, albeit at lower levels (Andres *et al.*, 1993). The disruption of 20E signaling in the fat body by expression of the dominant negative *EcR* prevented the dissociation of the fat body (Cherbas *et al.*, 2003). These data confirm that 20E signaling is involved in fat body dissociation during metamorphosis.

During metamorphosis only the fat body undergoes programmed tissue dissociation, while other tissues are subjected to 20E-dependent autophagy (review: Thummel, 2001). It is likely that other signaling pathways are integrated with 20E signaling to initiate the breakdown of the fat body. Genetic analysis has revealed that the fat body is extremely sensitive to the disruption of transforming growth factor-β (TGF-β) signaling pathway, which results in premature dissociation of the larval fat body.

The details of TGF-β signaling in *D. melanogaster* are well understood; its components (receptors and Smads) that transduce the TGF-β signal have been identified and many of their biochemical interactions have been characterized (**Figure 17**) (review: Massague *et al.*, 2000; Massague and Chen, 2000; Massague and Wotton, 2000). The TGF-β secretory polypeptide family controls multiple developmental processes in *D. melanogaster* through its receptors and a network of Smad transcription factors, which mediate changes in gene transcription.

Loss of TGF-β/Smad signaling, as detailed below, results in the premature dissociation of the larval fat body and loss of fat-body cells. Because TGF-β/Smad signaling mediates cellular responses through the activation or repression of target genes, it is possible that some target genes directly or indirectly regulate adhesion molecules, proteases, or regulatory components involved in the maintenance or degradation of the fat body basal lamina, or participate in the fat body-induced response of hemocytes.

In *Drosophila*, there are four TGF-β related factors, Decapentaplegic (Dpp), Glass-bottom-boat (Gbb), Screw, and Activin. The TGF-β ligands function as dimers and signal through transmembrane protein serine/threonine kinases known as type I and II receptors, which also function as homodimers. The TGF-β ligands serve to bring the two receptors together, such that the type II receptor can phosphorylate the type I receptor at its GS domain, thereby activating the type I receptor kinase. The

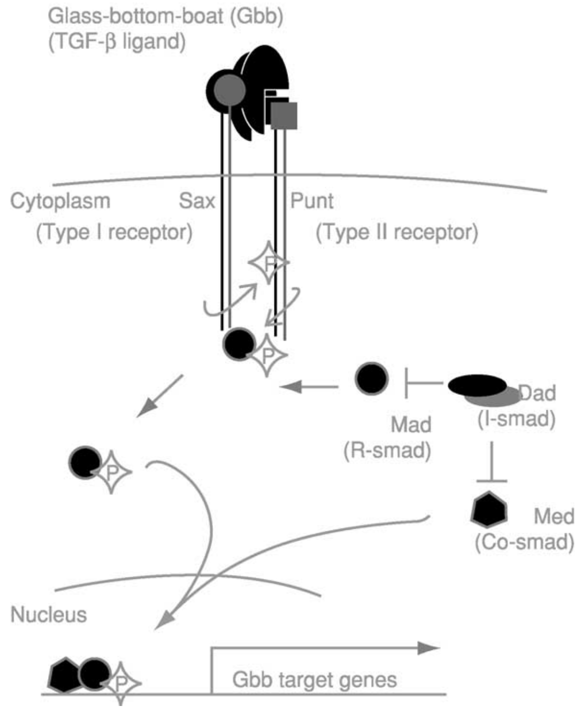


Figure 17 Schematic representation of TGF-β signaling network highlighting *Drosophila* Glass-bottom-boat/Smad signaling network. The *D. melanogaster* ligand Gbb signals through the type II receptor Punt and the type I receptor Sax. Ligand binding stimulates formation of an active receptor complex that phosphorylates Mad, which, in turn, oligomerizes with Medea (Med). The Mad/Med complex functions as a transcription factor to activate target genes.

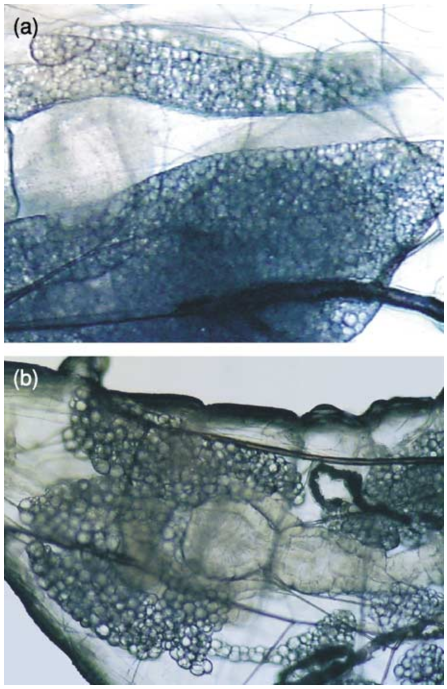


Figure 18 Loss of *sax* leads to a morphological change in the larval fat body. (a) Wild-type fat body. The fat body remains intact until metamorphosis when the fat body dissociates into individual cells. (b) Fat body from a *sax* mutant grown at 18 °C. The fat body is loosely organized; later the fat body dissociates into individual cells. The *sax* mutants do not metamorphose and the individual fat body cells are thought to eventually undergo apoptosis.

TGF-β signal is transduced from the receptor–ligand complex to the cytoplasm by the phosphorylation of Smad proteins (Mothers-against-decapentaplegic (Mad) and Medea (Med)) which, in turn, function as transcription factors (Figure 17) (Raftery and Sutherland, 1999).

2.9.5.3.1. Mutations in TGF-β signaling The TGF-β ligand, Glass-bottom-boat (Gbb) (also known as 60A) interacts synergistically with Dpp in several developmental process including wing disc patterning, embryonic mid-gut morphogenesis, and embryonic ectoderm patterning (Khalsa *et al.*, 1998; Wharton *et al.*, 1999; see also **Chapters 1.8** and **1.9**). A striking feature of the *gbb* mutant is its defect in both the quantity and quality of the fat body resulting in a transparent mutant larva, hence the name of the gene. This fat body phenotype is not detected in *dpp* mutants, and thus the Gbb signaling network that controls fat body morphology is independent of Dpp signaling (Chen *et al.*, 1998; Khalsa *et al.*, 1998; Wharton *et al.*, 1999). A detailed developmental time course describing fat body changes and loss of fat body cells in *gbb* mutants has not been reported yet.

The *Drosophila* TGF-β ligands (Gbb, Screw, Dpp, and dActivin) signal through the same type II receptor, Punt, but also use unique type I receptors. Based on genetic interaction studies, the type I receptor Saxophone (Sax) mediates Gbb signaling. In genotypes containing a single copy of a *sax* null allele, the viability of *gbb* homozygous mutants is compromised (Khalsa *et al.*, 1998). Although mutations in *sax* result in embryonic lethality, the sax^4 allele is temperature sensitive (D.K. Hoshizaki, unpublished data). Mutant sax^4 larvae grown at 18 °C develop through late-third instar, but fail to pupariate. During the third instar the fat body becomes transparent and the fat cells appear loosely associated with each other and begin to round up (Figure 18). Consistent with the *sax* participation in Gbb-signaling, the *sax* and *gbb* mutants have similar fat body defects.

The first identified substrates of the type I receptor kinase were the *Drosophila mothers-against-decapentaplegic* (*mad*) gene and the *C. elegans Sma* gene. These proteins established the Smad family of conserved signal transducers and transcription

factors (review: Raftery and Sutherland, 1999; Moustakas *et al*., 2001). In *Drosophila*, the Smad family is made up of two receptor Smads (R-Smads, Mad, and dSmad2), a single common Smad, (Co-Smad, Medea), and a single inhibitory Smad (I-Smad, Daughters-against-decapentaplegic (Dad)) (**Figure 17**) (review: Raftery and Sutherland, 1999). Both *medea* and *mad* were isolated as dominant enhancers of *dpp* mutant phenotypes (Raftery *et al*., 1995; Sekelsky *et al*., 1995). Homozygous *mad* and *medea* mutants have reduced imaginal discs, and exhibit pupal lethality and defects in midgut morphogenesis. These mutant phenotypes are consistent with Mad/Medea's role in transducing the Dpp signal. *mad* and *medea* mutants also have fat body defects that are not seen in *dpp* mutants but are similar to the *sax* fat body mutant phenotype (Raftery *et al*., 1995; Sekelsky *et al*., 1995; D.K. Hoshizaki, unpublished data). Based on expression studies using a *mad* enhancer trap, *mad* was demonstrated to be expressed in the fat body (D.K. Hoshizaki, unpublished data). This observation suggests that Gbb-signaling through Sax, Mad, and Medea takes place within the fat body cells and that loss of signaling by these factors results in the disruption of fat body structure.

2.9.5.4. Speculation

Taken together, the studies from *Sarcophaga* and *Drosophila* provide the genetic, cellular, and biochemical information that is necessary in order to build a working model for the control of tissue dissociation. In this model, ecdysone signaling induced by the ecdysone pulse that also triggers pupariation has a role in establishing fat body competence for dissociation. In the larva, maintenance of the fat body integrity is controlled by Gbb/Smad signaling which is down-regulated by ecdysone at the onset of metamorphosis. Because TGF-β signaling is known to be integrated with other signaling networks (Wotton *et al*., 1999; Attisano and Wrana, 2002; Lutz and Knaus, 2002), cross-talk between ecdysone and TGF-β signaling *within* the fat body may result in fat body acquisition of competence for dissociation. Based on data from *Drosophila*, the events that trigger dissociation are intrinsic to the fat body. However, in *S. peregrina* fat body dissociation is hemocyte-mediated and dependent on fat body-hemocyte interactions. It is likely that fat body dissociation involves both fat body intrinsic events and the interplay with the hemocytes that provide Cathepsin B. As part of the working model, an ecdysone pulse also triggers events in the hemocytes that may also be related to translation of Cathepsin B. Alternatively, interactions between hemocyte recognition proteins and specific binding sites on the fat body may activate Cathepsin B through the cleavage of the pro-enzyme and the local release of Cathepsin B from storage granules. The *D. melanogaster Cathepsin B* gene has been cloned and identified as a recessive lethal mutation (Bourbon *et al*., 2002; Flybase, 2003). Thus, it should be possible to examine the cellular regulation of Cathepsin B *in vivo* and test the specific role of the fat body and the hemocytes in the fat body dissociation events using the advanced cell biology tools and genetic approaches that are available in *D. melanogaster.*

Acknowledgments

I am grateful to the undergraduate students Nichole Bond, Jenifer Utz, and Jeffrey Wagner for technical support. Dr. Jennell Miller's artistic efforts in generating portions of Figures 1, 3, and 6 are especially appreciated and her permission to use these drawings is acknowledged. A portion of this work was supported by a Biomedical Research Infrastructure Network grant awarded to the University of Nevada.

References

Abel, T., Bhatt, R., Maniatis, T., **1992**. A *Drosophila* CREB/ATF transcriptional activator binds to both fat body- and liver-specific regulatory elements. *Genes Devel. 6*, 466–480.

Abel, T., Michelson, A.M., Maniatis, T., **1993**. A *Drosophila* GATA family member that binds to *Adh* regulatory sequences is expressed in the developing fat body. *Development 119*, 623–633.

Ailhaud, G., Grimaldi, P., Negrel, R., **1992**. Cellular and molecular aspects of adipose tissue development. *Annu. Rev. Nutr. 12*, 207–233.

Akam, M.E., Martinez-Arias, A., **1985**. The distribution of Ultrabithorax transcripts in *Drosophila* embryos. *EMBO J. 4*, 1689–1770.

Andres, A.J., Fletcher, J.C., Karim, F.D., Thummel, C.S., **1993**. Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. *Devel. Biol. 160*, 388–404.

Ashburner, M., **1989**. *Drosophila*, A laboratory handbook. Cold Spring Harbor Laboratory Press, New York.

Ashurst, D.E., **1982**. The structure and development of insect connective tissues. In: King, R.C., Aakai, H. (Eds.), Insect Ultrastructure, vol. 1. Plenum, New York.

Attisano, L., Wrana, J.L., **2002**. Signal transduction by the TGF-beta superfamily. *Science 296*, 1646–1647.

Azpiazu, N., Frasch, M., **1993**. *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Devel. 7*, 1325–1340.

Bainbridge, S.P., Bownes, M., **1981**. Staging the metamorphosis of *Drosophila melanogaster. J. Embryol. Exp. Morphol. 66*, 57–80.

Bate, M., **1990**. The embryonic development of larval muscles in *Drosophila*. *Development 110*, 791–804.

Bate, M., **1993**. The mesoderm and its derivatives. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory Press, New York, pp. 1013–1090.

Bate, M., Arias, A.M., **1991**. The embryonic origin of imaginal discs in *Drosophila*. *Development 112*, 755–761.

Bate, M., Rushton, E., Currie, D.A., **1991**. Cells with persistent *twist* expression are the embryonic precursors of adult muscles in *Drosophila*. *Development 113*, 79–89.

Bienz, M., **1994**. Homeotic genes and positional signaling in the *Drosophila* viscera. *Trends Genet. 10*, 22–26.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., *et al*., **1989**. Searching for pattern and mutation in the *Drosophila* genome with a *P-lacZ* vector. *Genes Devel. 3*, 1273–1287.

Blackburn, T.L., **1994**. The *cwr* gene encodes a leucine rich protein that may have a role in muscle patterning. MS Thesis, University of Illinois at Chicago.

Bodenstein, D., **1950**. The postembryonic development of *Drosophila*. In: Demerec, M. (Ed.), Biology of *Drosophila*. Cold Spring Harbor Laboratory Press, New York, pp. 275–375.

Bodmer, R., **1993**. The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development 118*, 719–729.

Borkowski, O.M., Brown, N.H., Bate, M., **1995**. Anterior-posterior subdivision and the diversification of the mesoderm in *Drosophila*. *Development 121*, 4183–4193.

Bourbon, H.M., Gonzy-Treboul, G., Peronnet, F., Alin, M.F., Ardourel, C., *et al*., **2002**. A P-insertion screen identifying novel X-linked essential genes in *Drosophila*. *Mech. Devel. 110*, 71–83.

Boyle, M., Bonini, N., DiNardo, S., **1997**. Expression and function of *clift* in the development of somatic gonadal precursors within the *Drosophila* mesoderm. *Development 124*, 971–982.

Brac, T., **1983**. Charges sieving by the basal lamina and the distribution of anionic sites on the external surfaces of fat body cells. *Tissue Cell 15*, 489–498.

Brand, A.H., Perrimon, N., **1993**. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development 118*, 401–415.

Britton, J.S., Edgar, B.A., **1998**. Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development 125*, 2149–2158.

Brodu, V., Mugat, B., Fichelson, P., Lepesant, J.A., Antoniewski, C., **2001**. A UAS site substitution approach to the *in vivo* dissection of promoters: interplay between the GATAb activator and the AEF-1 repressor at a *Drosophila* ecdysone response unit. *Development 128*, 2593–2602.

Brodu, V., Mugat, B., Roignant, J.Y., Lepesant, J.A., Antoniewski, C., **1999**. Dual requirement for the EcR/USP nuclear receptor and the dGATAb factor in an ecdysone response in *Drosophila melanogaster. Mol. Cell. Biol. 19*, 5732–5742.

Broihier, H.T., Moore, L.A., Van Doren, M., Newman, S., Lehmann, R., **1998**. *zfh-1* is required for germ cell migration and gonadal mesoderm development in *Drosophila*. *Development 125*, 655–666.

Brookman, J.J., Toosy, A.T., Shashidhara, L.S., White, R.A., **1992**. The *412* retrotransposon and the development of gonadal mesoderm in *Drosophila*. *Development 116*, 1185–1192.

Burmester, T., Scheller, K., **1999**. Ligands and receptors: common theme in insect storage protein transport. *Naturwissenschaften 86*, 468–474.

Butterworth, F.M., **1972**. Adipose tissue of *Drosophila melanogaster*. V. Genetic and experimental studies of an extrinsic influence on the rate of cell death in the larval fat body. *Dev. Biol. 28*, 311–325.

Butterworth, F.M., Bodenstein, D., **1967**. Adipose tissue of *Drosophila melanogaster* II. The effect of the adult internal environment on growth, protein deposition, and histolysis in the larval fat body. *J. Exp. Zool. 164*, 251–266.

Butterworth, F.M., Forrest, E.C., **1984**. Ultrastructure of the preparative phase of cell death in the larval fat body of *Drosophila melanogaster. Tissue Cell 16*, 237–250.

Butterworth, F.M., King, R.C., **1965**. The developmental genetics of *apterous* mutants of *Drosophila melanogaster. Genetics 52*, 1153–1174.

Campos-Ortega, J.A., Hartenstein, V., **1985**. The Embryonic Development of *Drosophila melanogaster*, 1st edn. Springer, Berlin.

Campos-Ortega, J.A., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila melanogaster*, 2nd edn. Springer, Berlin.

Capovilla, M., Brandt, M., Botas, J., **1994**. Direct regulation of decapentaplegic by Ultrabithorax and its role in *Drosophila* midgut morphogenesis. *Cell 76*, 461–475.

Casanova, J., Sanchez-Herrero, E., Morata, G., **1986**. Identification and characterization of a parasegment specific regulatory element of the *abdominal*-B gene of *Drosophila*. *Cell 47*, 627–636.

Chen, Y., Riese, M.J., Killinger, M.A., Hoffmann, F.M., **1998**. A genetic screen for modifiers of *Drosophila* decapentaplegic signaling identifies mutations in *punt*, *Mothers against dpp* and the *BMP-7* homologue, *60A*. *Development 125*, 1759–1768.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., **2003**. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development 130*, 271–284.

Cohen, B., McGuffin, M.E., Pfeifle, C., Segal, D., Cohen, S.M., **1992**. Apterous, a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Devel. 6*, 715–729.

Costa, R.H., Grayson, D.R., Darnell, J.E., Jr., **1989**. Multiple hepatocyte-enriched nuclear factors function in the regulation of *transthyretin* and *alpha 1-antitrypsin* genes. *Mol. Cell. Biol. 9*, 1415–1425.

Cullen, C.F., Milner, M.J., **1991**. Parameters of growth in primary cultures and cell lines established from *Drosophila* imaginal discs. *Tissue Cell 23*, 29–39.

Currie, D.A., Bate, M., **1991**. The development of adult abdominal muscles in *Drosophila*: myoblasts express *twist* and are associated with nerves. *Development 113*, 91–102.

Davis, K.T., Shearn, A., **1977**. *In Vitro* growth of imaginal disks from *Drosophila melanogaster. Science 196*, 438–440.

Day, M.F., **1943**. The function of the corpus allatum in muscoid Diptera. *Biol. Bull. 84*, 127–140.

Dean, R.L., Locke, M., Collins, J.V., **1985**. Structure of the fat body. In: Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 3. Pergamon, Oxford, UK, p. 155.

Delorenzi, M., Bienz, M., **1990**. Expression of Abdominal-B homeoproteins in *Drosophila* embryos. *Development 108*, 323–329.

Dohrmann, C., Azpiazu, N., Frasch, M., **1990**. A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Devel. 4*, 2098–2111.

Dutkowski, A.B., Oberlander, H., **1974**. Interactions between beta-ecdysone and fat body during wing disk development *in vitro*. *J. Insect Physiol. 20*, 743–749.

Echalier, G., **1997**. *Drosophila* cells in culture. Academic Press, San Diego.

Fischer, J.A., Maniatis, T., **1986**. Regulatory elements involved in *Drosophila Adh* gene expression are conserved in divergent species and separate elements mediate expression in different tissues. *EMBO J. 5*, 1275–1289.

Flybase, C., **2003**. The FlyBase database of the *Drosophila* genome projects and community literature. *Nucleic Acids Res. 31*, 172–175.

Fossett, N., Tevosian, S.G., Gajewski, K., Zhang, Q., Orkin, S.H., *et al.*, **2001**. The Friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in *Drosophila*. *Proc. Natl Acad. Sci. USA 98*, 7342–7347.

Frank, L.H., Rushlow, C., **1996**. A group of genes required for maintenance of the amnioserosa tissue in *Drosophila*. *Development 122*, 1343–1352.

Frasch, M., **1995**. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature 374*, 464–467.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H., Levine, M., **1987**. Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J. 6*, 749–759.

Gajewski, K., Fossett, N., Molkentin, J.D., Schulz, R.A., **1999**. The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. *Development 126*, 5679–5688.

Graba, Y., Aragnol, D., Laurenti, P., Garzino, V., Charmot, D., *et al.*, **1992**. Homeotic control in *Drosophila*: the scabrous gene is an *in vivo* target of Ultrabithorax proteins. *EMBO J. 11*, 3375–3384.

Hacker, U., Grossniklaus, U., Gehring, W., Jackle, H., **1992**. Developmentally regulated *Drosophila* gene family encoding the fork head domain. *Proc. Natl Acad. Sci. USA 89*, 8754–8758.

Handler, A.M., **1982**. Ecdysteroid titers during pupal and adult development in *Drosophila melanogaster. Devel. Biol. 93*, 73–82.

Harigae, H., Takahashi, S., Suwabe, N., Ohtsu, H., Gu, L., *et al.*, **1998**. Differential roles of GATA-1 and GATA-2 in growth and differentiation of mast cells. *Genes Cells 3*, 39–50.

Harshman, L.G., James, A.A., **1998**. Differential gene expression in insects: transcriptional control. *Annu. Rev. Entomol. 43*, 671–700.

Hartenstein, V., Jan, Y.N., **1992**. Studying *Drosophila* embryogenesis with *P-lacz* enhancer trap lines. *Roux's Arch. Devel. Biol. 201*, 194–220.

Hartenstein, V., Tepass, U., Gruszynski-Defeo, E., **1994**. Embryonic development of the stomatogastric nervous system in *Drosophila*. *J. Comp. Neurol. 350*, 367–381.

Haunerland, N.H., **1996**. Insect storage proteins: gene families and receptors. *Insect Biochem. Mol. Biol. 26*, 755–765.

Haunerland, N.H., Shirk, P.D., **1995**. Regional and functional differentiation in the insect fat body. *Annu. Rev. Entomol. 40*, 121–145.

Hayes, S.A., Miller, J.M., Hoshizaki, D.K., **2001**. *serpent*, a GATA-like transcription factor gene, induces fat-cell development in *Drosophila melanogaster. Development 128*, 1193–1200.

Higgins, S.J., Young, P., Cunha, G.R., **1989**. Induction of functional cytodifferentiation in the epithelium of tissue recombinants. II. Instructive induction of Wolffian duct epithelia by neonatal seminal vesicle mesenchyme. *Development 106*, 235–250.

Hooper, J., **1986**. Homeotic gene function in the muscles of *Drosophila* larvae. *EMBO J. 5*, 2321–2329.

Hori, S., Kobayashi, A., Natori, S., **1997**. Monoclonal antibodies against pupa-specific surface antigens of *Sarcophaga peregrina* (flesh fly) hemocytes. *Biochem. Biophys. Res. Commun. 236*, 497–501.

Hoshizaki, D.K., Blackburn, T., Price, C., Ghosh, M., Miles, K., *et al.*, **1994**. Embryonic fat-cell lineage in *Drosophila melanogaster. Development 120*, 2489–2499.

Hoshizaki, D.K., Lunz, R., Ghosh, M., Johnson, W., **1995**. Identification of fat-cell enhancer activity in

Drosophila melanogaster using P-element enhancer traps. *Genome 38*, 497–506.
Hughes, I.A., **2001**. Minireview: sex differentiation. *Endocrinology 142*, 3281–3287.
Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E., **2002**. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr. Biol. 12*, 1293–1300.
Jurgens, G., Wieschaus, E., Nusslein-Volhard, C., Kluding, H., **1984**. Mutations affecting the pattern of the larva cuticle in *Drosophila melanogaster. Roux's Arch. Devel. Biol. 193*, 283–295.
Karch, F., Bender, W., Weiffenbach, B., **1990**. AbdA expression in *Drosophila* embryos. *Genes Dev. 4*, 1573–1587.
Kawamura, K., Shibata, T., Saget, O., Peel, D., Bryant, P.J., **1999**. A new family of growth factors produced by the fat body and active on *Drosophila* imaginal disc cells. *Development 126*, 211–219.
Keeley, L.L., **1985**. Physiology and biochemistry of the fat body. In: Comprehensive Insect Physiology Biochemistry and Pharmacology. Pergamon, Oxford, UK, p. 211.
Khalsa, O., Yoon, J.W., Torres-Schumann, S., Wharton, K.A., **1998**. TGF-beta/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the *Drosophila* wing. *Development 125*, 2723–2734.
Kim, Y., Nirenberg, M., **1989**. *Drosophila* NK-homeobox genes. *Proc. Natl Acad. Sci. USA 86*, 7716–7720.
Knirr, S., Azpiazu, N., Frasch, M., **1999**. The role of the NK-homeobox gene *slouch (S59)* in somatic muscle patterning. *Development 126*, 4525–4535.
Kobayashi, H., Kurata, S., Natori, S., **1991**. Purification of the 200 kDa hemocyte membrane protein of *Sarcophaga peregrina* and its specific interaction with fat body. *Insect Biochem. 21*, 517–522.
Koch, J., **1945**. Die oenocyten von *Drosophila melanogaster. Rev. Suisse Zool. 52*, 415–420.
Kurata, S., Kobayashi, H., Natori, S., **1991**. Participation of a 200-kDa hemocyte membrane protein in the dissociation of the fat body at the metamorphosis of *Sarcophaga*. *Dev. Biol. 146*, 179–185.
Kurata, S., Komano, H., Natori, S., **1989**. Dissociation of *Sarcophaga peregrina* (flesh fly) fat body by pupal haemocytes *in vitro*. *J. Insect Physiol. 35*, 559–565.
Kurata, S., Saito, H., Natori, S., **1990**. Participation of hemocytes proteinase in dissociation of the fat body on pupation of *Sarcophaga peregrina* (flesh fly). *Insect Biochem. 20*, 461–465.
Kurata, S., Saito, H., Natori, S., **1992a**. The 29-kDa hemocyte proteinase dissociates fat body at metamorphosis of *Sarcophaga*. *Devel. Biol. 153*, 115–121.
Kurata, S., Saito, H., Natori, S., **1992b**. Purification of a 29-kDa hemocyte proteinase of *Sarcophaga peregrina*. *Eur. J. Biochem. 204*, 911–914.
Larsen, W.J., **1976**. Cell remodeling in the fat body of an insect. *Tissue Cell 8*, 73–92.
Lawrence, P.A., **1985**. Pattern formation in the muscles of *Drosophila*. *Cold Spring Harb. Symp. Quant. Biol. 50*, 113–117.
Lawrence, P.A., **1992**. The Making of a Fly. Blackwell Scientific Publications, Cambridge, MA.
Lawrence, P.A., Brower, D.L., **1982**. Myoblast from *Drosophila* wing disks can contribute to developing muscles throughout the fly. *Nature 295*, 55–57.
Lawrence, P.A., Johnston, P., **1986**. Observations on cell lineage of internal organs of *Drosophila*. *J. Embryol. Exp. Morphol. 91*, 251–266.
Lebestky, T., Chang, T., Hartenstein, V., Banerjee, U., **2000**. Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. *Science 288*, 146–149.
Lebestky, T., Jung, S.H., Banerjee, U., **2003**. A *serrate*-expressing signaling center controls *Drosophila* hematopoiesis. *Genes Devel. 17*, 348–353.
Leptin, M., Grunewald, B., **1990**. Cell shape changes during gastrulation in *Drosophila*. *Development 110*, 73–84.
Locke, M., **1998**. In: The Fat Body, vol. 11B. Wiley-Liss, New York, p. 641.
Locke, M., **1971**. The origin and fate of microbodies in the fat body of an insect. *J. Cell Biol. 48*, 61–78.
Locke, M., **2003**. Surface membranes, golgi complexes, and vacuolar systems. *Annu. Rev. Entomol. 48*, 1–27.
Locke, M., Collins, J.V., **1968**. Protein uptake into multivesicular bodies and storage granules in the fat body of an insect. *J. Cell Biol. 36*, 453–483.
Locke, M., Huie, P., **1983**. A function for plasma membrane reticular systems. *Tissue Cell 15*, 885–902.
Loeb, M.J., **1994**. Genital tract growth factors from a moth, the tobacco budworm, *Heliothis virescens. In Vitro Cell Devel. Biol. Anim. 30A*, 702–708.
Loeb, M.J., Hakim, R.S., **1991**. Development of genital imaginal discs of *Heliothis virescens* culture *in vitro* with 20-hydroxyecdysone and fat body or testis sheaths. *Invert. Reprod. Devel. 20*, 181–191.
Lutz, M., Knaus, P., **2002**. Integration of the TGF-beta pathway into the cellular signaling network. *Cell Signal 14*, 977.
Martin, J.F., Hersperger, E., Simcox, A., Shearn, A., **2000**. Minidiscs encodes a putative amino acid transporter subunit required non-autonomously for imaginal cell proliferation. *Mech. Devel. 92*, 155–167.
Massague, J., Blain, S.W., Lo, R.S., **2000**. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell 103*, 295–309.
Massague, J., Chen, Y.G., **2000**. Controlling TGF-beta signaling. *Genes Devel. 14*, 627–644.
Massague, J., Wotton, D., **2000**. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J. 19*, 1745–1754.
Mastick, G.S., McKay, R., Oligino, T., Donovan, K., Lopez, A.J., **1995**. Identification of target genes regulated by homeotic proteins in *Drosophila melanogaster* through genetic selection of Ultrabithorax protein-binding sites in yeast. *Genetics 139*, 349–363.

Michelson, A.M., **1994**. Muscle pattern diversification in *Drosophila* is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development 120*, 755–768.

Michelson, A.M., Abmayr, S.M., Bate, M., Arias, A.M., Maniatis, T., **1990**. Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes Devel. 4*, 2086–2097.

Mietus-Snyder, M., Sladek, F.M., Ginsburg, G.S., Kuo, C.F., Ladias, J.A., *et al.*, **1992**. Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein *CIII* gene expression in liver and intestinal cells. *Mol. Cell. Biol. 12*, 1708–1718.

Miller, J.M., **2002**. Analysis of the cell-specific expression of the *serpent* gene and its role as a cell-fate switch in *Drosophila melanogaster.* Ph.D. thesis, University of Nevada, Las Vegas.

Miller, J.M., Oligino, T., Pazdera, M., Lopez, A.J., Hoshizaki, D.K., **2002**. Identification of fat-cell enhancer regions in *Drosophila melanogaster. Insect Mol. Biol. 11*, 67–77.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., Rubin, G.M., **1990**. The *Drosophila seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell 60*, 211–224.

Moore, L.A., Broihier, H.T., Van Doren, M., Lehmann, R., **1998**. Gonadal mesoderm and fat body initially follow a common developmental path in *Drosophila*. *Development 125*, 837–844.

Moustakas, A., Souchelnytskyi, S., Heldin, C.H., **2001**. Smad regulation in TGF-beta signal transduction. *J. Cell. Sci. 114*, 4359–4369.

Murakami, R., Takashima, S., Hamaguchi, T., **1999**. Developmental genetics of the *Drosophila* gut: specification of primordia, subdivision and overt-differentiation. *Cell. Mol. Biol. 45*, 661–676 (Noisy-le-grand).

Natori, S., Shiraishi, H., Hori, S., Kobayashi, A., **1999**. The roles of *Sarcophaga* defense molecules in immunity and metamorphosis. *Devel. Comp. Immunol. 23*, 317–328.

Nöthiger, R., **1972**. The larval development of imaginal discs. In: Urspung, H., Nöthiger, R. (Eds.), Results and Problems in Cell Differentiation. The Biology of Imaginal Disks, vol. 5. Springer, New York.

Oberlander, H., **1976a**. Dissociation and reaggregation of fat body cells during insect metamorphosis. In: Invertebrate Tissue Culture: Applications in Medicine, Biology, and Agriculture. Academic Press, New York, pp. 241–246.

Oberlander, H., **1976b**. Hormonal control of growth and differentiation of insect tissues cultured *in vitro*. *In Vitro 12*, 225–235.

Oberlander, H., Tomblin, C., **1972**. Cuticle deposition in imaginal disks: effects of juvenile hormone and fat body *in vitro*. *Science 177*, 441–442.

Paterson, B.M., Walldorf, U., Eldridge, J., Dubendorfer, A., Frasch, M., *et al.*, **1991**. The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Natl Acad. Sci. USA 88*, 3782–3786.

Petersen, U.M., Kadalayil, L., Rehorn, K.P., Hoshizaki, D.K., Reuter, R., *et al.*, **1999**. *serpent* regulates *Drosophila* immunity genes in the larval fat body through an essential GATA motif. *EMBO J. 18*, 4013–4022.

Postlethwait, J.H., Jones, G.J., **1978**. Endocrine control of larval fat body histolysis in normal and mutant *Drosophila melanogaster. J. Exp. Zool. 203*, 207–214.

Poulson, D.F., **1950**. Histogenesis, organogenesis, and differentiation in the embryo of *Drosophila melanogaster.* In: Demerec, M. (Ed.), Biology of *Drosophila*. Cold Spring Harbor Laboratory Press, New York, p. 168.

Raftery, L.A., Sutherland, D.J., **1999**. TGF-beta family signal transduction in *Drosophila* development: from Mad to Smads. *Devel. Biol. 210*, 251–268.

Raftery, L.A., Twombly, V., Wharton, K., Gelbart, W.M., **1995**. Genetic screens to identify elements of the decapentaplegic signaling pathway in *Drosophila*. *Genetics 139*, 241–254.

Ramain, P., Heitzler, P., Haenlin, M., Simpson, P., **1993**. *pannier*, a negative regulator of *achaete* and *scute* in *Drosophila*, encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. *Development 119*, 1277–1291.

Reddy, J.T., Locke, M., **1990**. The size limited penetration of gold particles through insect basal laminae. *J. Insect Physiol. 36*, 397–407.

Rehorn, K.P., Thelen, H., Michelson, A.M., Reuter, R., **1996**. A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. *Development 122*, 4023–4031.

Reuter, R., **1994**. The gene *serpent* has homeotic properties and specifies endoderm versus ectoderm within the *Drosophila* gut. *Development 120*, 1123–1135.

Richard, D.S., Arnim, A.E., Gilbert, L.I., **1993**. A reappraisal of the hormonal regulation of larval fat body histolysis in female *Drosophila melanogaster. Experientia 49*, 150–156.

Riddiford, L.M., **1993**. Hormone receptors and the regulation of insect metamorphosis. *Receptor 3*, 203–209.

Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R., Leptin, M., **1997**. Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development 124*, 2915–2922.

Riechmann, V., Rehorn, K.P., Reuter, R., Leptin, M., **1998**. The genetic control of the distinction between fat body and gonadal mesoderm in *Drosophila*. *Development 125*, 713–723.

Rizki, R.M., Rizki, T.M., **1974a**. Basement membrane abnormalities in melanotic tumor formation of *Drosophila*. *Experientia 30*, 543–546.

Rizki, R.M., Rizki, T.M., **1979**. Cell interactions in the differentiation of a melanotic tumor in *Drosophila*. *Differentiation 12*, 167–178.

Rizki, T.M., **1969**. Genetics and evolution of a cell phenotypes in *Drosophila*. *Jap. J. Genet. 44*(suppl.), 51–57.

Rizki, T.M., **1978**. Fat body. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*, vol. 2b. Academic Press, New York, p. 561.

Rizki, T.M., Rizki, R.M., **1970**. The genetic basis of a cell pattern homology in *Drosophila* species. In: Hecht, M.K., Steere, W.C. (Eds.), Essays in Evolution and Genetics in Honor of Theodosius Dobzhansky. Appleton-Century-Crofts, New York, p. 289.

Rizki, T.M., Rizki, R.M., **1974b**. Topology of the caudal fat body of the *tumor-w* mutant of *Drosophila melanogaster. J. Invert. Pathol. 24*, 37–40.

Rizki, T.M., Rizki, R.M., **1978**. Larval adipose tissue of homoeotic bithorax mutants of *Drosophila*. *Devel. Biol. 65*, 476–482.

Robertson, C.W., **1936**. The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. *J. Morphol. 59*, 351–399.

Royzman, I., Whittaker, A.J., Orr-Weaver, T.L., **1997**. Mutations in *Drosophila* DP and E2F distinguish G1-S progression from an associated transcriptional program. *Genes Devel. 11*, 1999–2011.

Rushton, E., Drysdale, R., Abmayr, S.M., Michelson, A.M., Bate, M., **1995**. Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development 121*, 1979–1988.

Sadrud-Din, S., Loeb, M., Hakim, R., **1996**. *In Vitro* differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae. *J. Exp. Biol. 199*, 319–325.

Sadrud-Din, S.Y., Hakim, R.S., Loeb, M., **1994**. Proliferation and differentiation of midgut epithelial cells from *Manduca sexta*, *in vitro*. *Invert. Reprod. Devel. 26*, 197–204.

Sam, S., Leise, W., Hoshizaki, D.K., **1996**. The *serpent* gene is necessary for progression through the early stages of fat-body development. *Mech. Devel. 60*, 197–205.

San Martin, B., Ruiz-Gomez, M., Landgraf, M., Bate, M., **2001**. A distinct set of founders and fusion-competent myoblasts make visceral muscles in the *Drosophila* embryo. *Development 128*, 3331–3338.

Schwartz, M.B., Imberski, R.B., Kelly, T.J., **1984**. Analysis of metamorphosis in *Drosophila melanogaster*: characterization of *giant*, an ecdysteroid-deficient mutant. *Devel. Biol. 103*, 85–95.

Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., Gelbart, W.M., **1995**. Genetic characterization and cloning of *mothers against dpp*, a gene required for decapentaplegic function in *Drosophila melanogaster. Genetics 139*, 1347–1358.

Shearn, A., Rice, T., Garen, A., Gehring, W., **1971**. Imaginal disc abnormalities in lethal mutants of *Drosophila*. *Proc. Natl Acad. Sci. USA 68*, 2594–2598.

Sladek, F.M., Zhong, W.M., Lai, E., Darnell, J.E., Jr., **1990**. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Devel. 4*, 2353–2365.

Sliter, T.J., Gilbert, L.I., **1992**. Developmental arrest and ecdysteroid deficiency resulting from mutations at the dre4 locus of *Drosophila*. *Genetics 130*, 555–568.

Smith, A.V., Orr-Weaver, T.L., **1991**. The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny. *Development 112*, 997–1008.

Sondergaard, L., **1993**. Homology between the mammalian liver and the *Drosophila* fat body. *Trends Genet. 9*, 193.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., Bate, M., **1994**. Dpp induces mesodermal gene expression in *Drosophila*. *Nature 372*, 783–786.

Stocker, H., Hafen, E., **2000**. Genetic control of cell size. *Curr. Opin. Genet. Devel. 10*, 529–535.

Takahashi, N., Kurata, S., Natori, S., **1993**. Molecular cloning of cDNA for the 29 kDa proteinase participating in decomposition of the larval fat body during metamorphosis of *Sarcophaga peregrina* (flesh fly). *FEBS Lett. 334*, 153–157.

Telfer, W.H., Kunkel, J.G., **1991**. The function and evolution of insect storage hexamers. *Annu. Rev. Entomol. 36*, 205–228.

Thummel, C.S., **2001**. Steroid-triggered death by autophagy. *Bioessays 23*, 677–682.

Tingvall, T.O., Roos, E., Engstrom, Y., **2001**. The GATA factor Serpent is required for the onset of the humoral immune response in *Drosophila* embryos. *Proc. Natl Acad. Sci. USA 98*, 3884–3888.

Waltzer, L., Bataille, L., Peyrefitte, S., Haenlin, M., **2002**. Two isoforms of Serpent containing either one or two GATA zinc fingers have different roles in *Drosophila* haematopoiesis. *EMBO J. 21*, 5477–5486.

Ward, R.E., Reid, P., Bashirullah, A., D'Avino, P.P., Thummel, C.S., **2003**. GFP in living animals reveals dynamic developmental responses to ecdysone during *Drosophila* metamorphosis. *Devel. Biol. 256*, 389–402.

Wharton, K.A., Cook, J.M., Torres-Schumann, S., de Castro, K., Borod, E., *et al.*, **1999**. Genetic analysis of the bone morphogenetic protein-related gene, *gbb*, identifies multiple requirements during *Drosophila* development. *Genetics 152*, 629–640.

Wheeler, D.E., Martinez, T., **1995**. Storage proteins in ants (Hymenoptera: Formicidae). *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 112*, 15–19.

White, R.A., Wilcox, M., **1984**. Protein products of the bithorax complex in *Drosophila*. *Cell 39*, 163–171.

Wilson, C., Pearson, R.K., Bellen, H.J., O'Kane, C.J., Grossniklaus, U., *et al.*, **1989**. P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Devel. 3*, 1301–1313.

Winick, J., Abel, T., Leonard, M.W., Michelson, A.M., Chardon-Loriaux, I., *et al.*, **1993**. A GATA family transcription factor is expressed along the embryonic dorsoventral axis in *Drosophila melanogaster. Development 119*, 1055–1065.

Wotton, D., Lo, R.S., Lee, S., Massague, J., **1999**. A Smad transcriptional corepressor. *Cell 97*, 29–39.

Yin, Z., Frasch, M., **1998**. Regulation and function of tinman during dorsal mesoderm induction and heart specification in *Drosophila*. *Devel. Genet. 22*, 187–200.

Zhong, W., Sladek, F.M., Darnell, J.E., Jr., **1993**. The expression pattern of a *Drosophila* homolog to the mouse transcription factor HNF-4 suggests a determinative role in gut formation. *EMBO J. 12*, 537–544.

Zinke, I., Kirchner, C., Chao, L., Tetzlaff, M., Pankratz, M., **1999**. Suppression of food intake and growth by amino acids in *Drosophila*: the role of *pumpless*, a fat body expressed gene with homology to vertebrate glycine cleavage system. *Development 126*, 5275–5284.

Zurovec, M., Dolezal, T., Gazi, M., Pavlova, E., Bryant, P.J., **2002**. Adenosine deaminase-related growth factors stimulate cell proliferation in *Drosophila* by depleting extracellular adenosine. *Proc. Natl Acad. Sci. USA 99*, 4403–4408.

2.10 Salivary Gland Development and Programmed Cell Death

D J Andrew and M M Myat, Johns Hopkins University School of Medicine, Baltimore, MD, USA

2.10.1. Introduction

Insect salivary glands are instrumental in the spread of diseases that affect humans, livestock, and agricultural crops. For example, the salivary glands of the mosquito vector *Anopheles gambiae* harbor and transmit the infectious *Plasmodium falciparum*, the microorganism causing malaria (Ghosh *et al.*, 2000). Similarly, the salivary glands of the western black-legged tick *Ixodes pacificus* harbor the spirochetal bacterium *Borrelia burgdorferi*, which is responsible for Lyme disease (Crippa *et al.*, 2002). Salivary glands have also been implicated in the transmission of Dengue fever and leishmaniasis from their insect vectors to both humans and livestock. Plant viruses, such as the tomato spotted wilt virus, are also transmitted through the salivary glands of insect hosts (Kritzman *et al.*, 2002). Termite salivary glands produce the cellulolytic enzymes required to digest wood products, leading to the deterioration of many manmade structures.

Despite their critical roles both in disease and in economic enterprises, very little is known about the specification and formation of salivary glands in most insect species, the one exception being the salivary glands of *Drosophila melanogaster.* In this chapter, the early development of insect salivary glands and salivary gland histolysis during metamorphosis are reviewed. Focus is placed on the specification and morphogenesis of the *Drosophila* salivary gland, drawing parallels with silk gland development in *Bombyx mori*, which appears to use a similar regulatory hierarchy for its specification and early morphogenesis (see **Chapter 2.11**). The molecular and cellular changes associated with *Drosophila* salivary gland destruction during metamorphosis are also reviewed, and limited studies on salivary gland histolysis in other insects, which suggest a common mechanism for salivary cell death, are discussed. A brief synopsis of salivary gland physiology and its relation to human disease is also presented and discussed. The parallels in gene expression in structures as divergent as the *Drosophila* salivary gland and the *Bombyx* silk gland suggest that the cellular and molecular mechanisms underlying *Drosophila* salivary gland specification, morphogenesis, and physiological specialization are likely to be shared among the salivary glands of all insect species. A list of the genes that are involved in the specification and morphogenesis of the *Drosophila* salivary gland is given in the appendix at the end of this chapter.

2.10.2. Specification of the *Drosophila* Salivary Gland Primordia

2.10.2.1. Introduction to *Drosophila* Salivary Glands

The embryonic/larval salivary gland of *D. melanogaster* is a relatively simple tubular organ composed

of three functional cell types, the duct and secretory cells, which function during embryonic and larval stages, and the imaginal ring cells, which give rise to the adult salivary gland during metamorphosis. The secretory cells form paired elongated tubular glands that are positioned adjacent to the lateral body wall muscles of the thorax. These cells synthesize and secrete protein beginning in the early embryonic stages and continuing throughout the larval stages. Levels of protein synthesis and secretion increase dramatically just prior to metamorphosis, when the secretory glands make the glue proteins, which are required for adhesion of the larva to solid substrates prior to pupariation. The salivary duct is a Y-shaped tubular structure with two individual ducts, which connect to the secretory tubes, and a central common duct, which connects the individual ducts to the mouth. The duct cells form a conduit, which carries the secretory gland secretions to the larval mouth. The imaginal ring cells, the precursors to the adult salivary gland, form a ring of cells located between the proximal ends of the secretory tubes and the distal ends of the individual duct tubes with proximal being closest to the embryo's ventral surface.

All of the cells that give rise to the embryonic/larval and adult salivary gland derive from ventral ectodermal cells located in a posterior region of the head, a domain known as parasegment 2. Once specified, secretory gland precursor cells cease normal cell division and instead grow by an increase in individual cell size. To accommodate the increased metabolic needs of the large secretory cells, the chromosomes undergo a special form of polyploidization, known as endoreduplication (review: Edgar and Orr-Weaver, 2001). In this process, the DNA strands replicate but fail to separate, ultimately creating the giant polytene chromosomes, which have been extensively used for mapping deficiencies, chromosomal rearrangements, cloned genes, and protein binding sites. Like the salivary secretory cells, the duct cell chromosomes are also polytenized, although to a lesser extent than the secretory cells (review: Edgar and Orr-Weaver, 2001). In contrast, the imaginal ring cells remain diploid, continuing to divide during the larval stages.

Since normal mitotic cell divisions cease once the cells are specified and because the salivary gland cells do not normally undergo programmed cell death during morphogenesis, duct and secretory cell numbers remain constant throughout morphogenesis and the subsequent larval stages (Campos-Ortega and Hartenstein, 1997). This constancy in cell number greatly simplifies studies of the cellular events of invagination, tube elongation, and directed migration, which are required to form a normally shaped and properly positioned salivary gland. The salivary gland that forms during embryogenesis functions throughout the three larval stages and into the early prepupal stage. In the early prepupal stage, the *Drosophila* salivary glands undergo programmed cell death in response to ecdysone pathway activation in the absence of juvenile hormone (Jiang *et al*., 2000). The larval salivary glands are ultimately replaced by the adult salivary gland, which forms from the imaginal ring cells.

2.10.2.2. Anterior–Posterior Determinants

The *Drosophila* salivary glands derive entirely from the ventral portion of the ectoderm that spans a domain known as parasegment 2 (PS2). All cells that form the embryonic/larval salivary gland express the homeotic transcription factor gene *Sex combs reduced* (*Scr*) (see **Chapter 1.8**) and *Scr* is absolutely required for salivary gland formation (Panzer *et al*., 1992; Andrew *et al*., 1994). In the absence of *Scr* function, salivary glands fail to form, and when *Scr* is expressed in new places in the embryo, additional salivary glands form in other (para)segments (Panzer *et al*., 1992; Andrew *et al*., 1994). Formation of additional salivary glands in other domains due to ectopic expression of *Scr* is limited, however, to PS0 and PS1 due to the expression of other salivary gland-repressing genes that are expressed in posterior segments (Andrew *et al*., 1994). In the region spanning PS3–PS13, the zinc finger transcription factor gene *teashirt* (*tsh*) blocks ectopic salivary gland formation (Andrew *et al*., 1994) and, in PS14, the homeotic transcription factor gene, *Abdominal-B* (*Abd-B*) (see **Chapter 1.8**), blocks salivary gland formation (Andrew *et al*., 1994). Thus, localized expression of homeotic proteins and other transcription factors limits salivary gland formation to PS2, which approximately coincides with the labial segment, the most posterior segment of the head (**Figure 1a**).

Salivary gland formation also requires two other homeodomain-containing transcription factor genes, both of which are expressed relatively ubiquitously in the early embryo (Henderson and Andrew, 2000). Loss of maternal and zygotic function of *extradenticle* (*exd*) or zygotic loss of function of *homothorax* (*hth*) result in the complete failure of salivary gland formation, as is observed with loss of *Scr* (Henderson and Andrew, 2000). Thus, Scr, Exd, and Hth (see **Chapter 1.8**) are likely to work as a transcriptional complex controlling gene expression in the salivary gland (Ryoo and Mann, 1999; Ryoo *et al*., 1999).

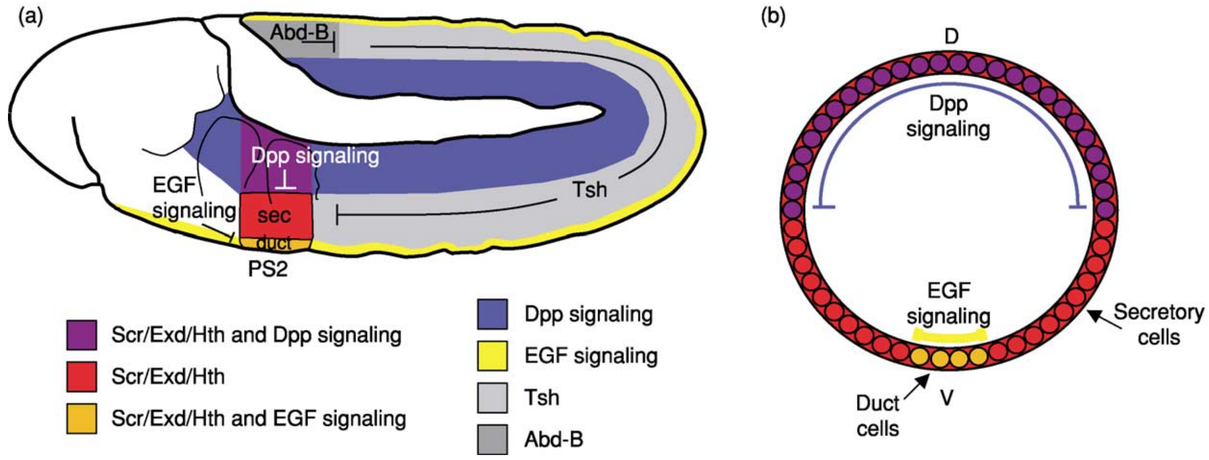


Figure 1 Genes controlling the specification, positioning, and number of salivary gland cells in *Drosophila*. (a) A schematic lateral view of a stage 11 embryo. (b) A schematic cross-section through parasegment 2 (PS2) depicting only ectodermal cells. The salivary gland fate is specified by the positive activities of Scr, which is expressed throughout the PS2 ectoderm, and the more ubiquitously expressed transcription factors, Exd and Hth (red background in (b)). Dpp signaling along the dorsal (D) region of the embryo blocks Scr/Exd/Hth activation of salivary gland gene expression in these cells (blue and purple in (a) and (b)), thus limiting salivary gland precursors to the ventral ectoderm of PS2. When *Scr* is ectopically expressed during development, Tsh and Abd-B (gray and dark gray in (a), respectively) block Scr/Exd/Hth activation of salivary gland fates in posterior regions of the embryo. Epidermal growth factor (EGF) signaling (yellow and orange in (a) and (b) in the ventral cells (V) blocks Scr/Exd/Hth activation of secretory cell-specific genes, including *fkh*. Expression of *fkh* (not shown) blocks expression of duct-specific genes in the secretory cells. Since the salivary gland precursors cease dividing once specified and do not normally undergo programmed cell death during their development, the same process that specifies the salivary gland fate also determines the number of cells in this organ. (Reproduced with permission from Bradley, P.L., Haberman, A.S., Andrew, D.J., **2001**. Organ formation is *Drosophila*: Specification and morphogenesis of the salivary gland. *BioEssays 23*, 901–911; © John Wiley & Sons, Inc.)

Expression of *Scr* and *hth* and the nuclear localization of Exd disappear shortly after the salivary gland cells have begun to internalize to form the secretory tubes (Henderson and Andrew, 2000). This change in gene expression and protein localization is due to repression of *hth* expression in the salivary gland by Scr/Exd/Hth. Shutting off *hth* expression in the salivary glands results in a loss of nuclear Exd since Exd requires Hth to block its nuclear export (Rieckhof *et al*., 1997; Pai *et al*., 1998; Abu-Shaar *et al*., 1999; Jaw *et al*., 2000). *Scr* expression disappears in the invaginating salivary gland as well, since maintenance of its transcription in the ventral ectoderm of PS2 requires *exd* and *hth* (Henderson and Andrew, 2000). Thus, Scr and its cofactors, Exd and Hth, are required to specify but not maintain a salivary gland fate, and these proteins act together to activate and repress gene expression in the salivary gland.

2.10.2.3. Dorsal–Ventral Determinants

Although all of the PS2 ectoderm initially expresses Scr and its cofactors, Exd and Hth, only the ventral ectodermal cells become salivary gland. Dorsal cells within PS2 do not form salivary glands due to activation of the Decapentaplegic (Dpp) signaling cascade (Panzer *et al*., 1992; Henderson *et al*., 1999). Expression of *dpp* is restricted to the dorsal half of the embryo (Figure 1b) through repression by the Dorsal transcription factor, which is nuclear in only ventral cells due to earlier signaling events under the control of maternally encoded gene products (Morisato and Anderson, 1995). Loss-of-function mutations in *dpp*, or in any of the genes encoding the downstream signaling components, lead to the expression of salivary gland genes throughout the PS2 ectoderm (Panzer *et al*., 1992; Henderson *et al*., 1999). Conversely, ectopic expression of *dpp* throughout the embryo, including the ventral cells of PS2, can repress expression of all tested salivary gland genes. Mutations in *brinker* (*bri*), which encodes a nuclear protein that blocks transcription of Dpp target genes and thus limits the extent of Dpp signaling (Affolter *et al*., 2001), result in smaller salivary gland primordia (Lammel and Saumweber, 2000; Torres-Vazquez *et al*., 2001).

Overall, the combination of localized expression of transcription factors, including Scr, Tsh, and Abd-B, and localized Dpp signaling limits salivary gland formation to the ventral ectodermal cells of PS2 (Figure 1b). Since these cells neither divide nor die during salivary gland formation, these factors simultaneously determine both the position and number of cells that comprise the entire embryonic/larval salivary gland.

2.10.2.4. Determinants of Cell Types within the Primordium

The distinction between duct and secretory primordia within the salivary gland is controlled by epidermal growth factor (EGF) signaling (**Figure 1**) (Panzer *et al.*, 1992; Kuo *et al.*, 1996). In *Drosophila*, most components of the EGF signaling pathway are expressed in all cells (Shilo, 2003). Localized activation of the EGF pathway is mediated by localized expression of *rhomboid* (*rho*), which encodes a resident transmembrane endoplasmic reticulum protein required for the processing of the secreted Spitz (Spi) ligand of the EGF receptor (Urban *et al.*, 2001; Tsruya *et al.*, 2002). The most ventral ectodermal cells express *rho*, leading to the subsequent release of the active ligand along the ventral ectodermal midline, which diffuses two to three cell diameters to activate the EGF receptor (EGFR). Salivary cells that receive sufficient levels of the EGF signal adopt a duct cell fate whereas cells positioned more laterally (away from the source of processed ligand) adopt a secretory cell fate. EGF signaling works, at least in part, by blocking the expression of secretory-specific genes in the salivary duct. One such gene is *fork head* (*fkh*), which itself encodes a transcription factor whose expression is normally limited to secretory cells (Kuo *et al.*, 1996). In the secretory cells, Fkh blocks expression of all duct-specific genes tested so far, including *trachealess* (*trh*), *Serrate* (*Ser*), and *dead ringer* (*dri*), three genes whose expression in the duct is independent of the others (Haberman and Andrew, 2003). The *trh* gene encodes a transcription factor expressed in duct cells and required for these cells to invaginate and form tubes (Isaac and Andrew, 1996). *Ser* encodes a Notch ligand required to specify a third cell type within the salivary gland (see below) and *dri* encodes another transcription factor whose role in the duct is not yet known (Haberman and Andrew, 2003).

Subsequent signaling between the duct and secretory cell primordia specifies the adult salivary gland cell precursors, the imaginal ring cells (Haberman and Andrew, 2003). During metamorphosis, the imaginal ring cells will replace the larval salivary glands, which undergo programmed cell death in response to ecdysone (Jiang *et al.*, 1997). Among the genes expressed in the salivary duct primordia is *Ser* (Kuo *et al.*, 1996), which encodes one of two known transmembrane ligands for Notch (Fleming *et al.*, 1990; Thomas *et al.*, 1991). Notch, a transmembrane receptor protein regulating numerous cell fate decisions, is expressed relatively ubiquitously but is transiently upregulated in the salivary gland secretory cell primordia prior to internalization (Kidd *et al.*, 1989). Although adequate markers do not yet exist to identify the imaginal cells in the early embryonic salivary gland, these cells express at least one tissue-specific gene in late embryos, known as *Tetraspanin74F* (*Tsp74F*), and are also easily distinguished in second instar larvae on the basis of their smaller nuclear size. The imaginal cells have smaller nuclei because these cells remain diploid throughout larval life and do not become polytenized as do the adjacent larval duct and secretory cells. In *Ser* mutants, expression of *Tsp74F* is absent in the region of the adult imaginal ring precursors in late embryos. Moreover, the imaginal cell population is missing in *Ser* mutant second instar larvae. These findings suggest that Ser in the duct cell primordia signals to the adjacent secretory primordia to specify the imaginal ring cell fate (Haberman and Andrew, 2003). In the absence of the Ser signal, cells adopt a default state, which is to form secretory cells. Thus, early EGF signaling distinguishes the two cell populations that form the larval salivary gland, duct versus secretory cells (**Figure 1**). Subsequently, duct-specific *Ser* expression activates the Notch pathway in the immediately adjacent secretory cells, specifying the precursors to the adult salivary gland.

Even within the apparently homogeneous duct and secretory primordia, additional subdomains of gene expression are evident (Jones *et al.*, 1998; Myat and Andrew, 2000a; Zhou *et al.*, 2001). In the duct primordia, these subdomains distinguish the more posterior individual duct precursors from the more anterior common duct precursors (Jones *et al.*, 1998). Although in the secretory portion of the gland, subdomains of gene expression do not correlate with obvious differences in cell type or tube dimensions, they do correlate with the order in which the cells internalize (Myat and Andrew, 2000a, 2002; Zhou *et al.*, 2001). The subdomains of gene expression within the salivary gland primordia reveal additional, perhaps more subtle input from both dorsal–ventral determinants, such as the EGF and Dpp signals, and from segment polarity genes, which are differentially expressed and/or activated along the anterior–posterior axes within individual parasegments.

2.10.3. Morphogenesis of the Salivary Gland

2.10.3.1. Secretory Cell Internalization and Tube Formation

The secretory primordia of the salivary gland form two ectodermal plates of columnar epithelial cells

known as the salivary gland placodes. The cells within each placode undergo a series of shape changes to internalize and form elongated tubes. Cell shape change is accompanied by basal migration of nuclei and constriction of the apical surfaces, which occurs in a regulated, sequential manner beginning with cells in the dorsal–posterior domain, then in dorsal–anterior, then ventral–anterior and, finally, the ventral–posterior domain (Myat and Andrew, 2000a) (**Figure 2**). Apical constriction of salivary gland cells requires *fkh* (Myat and Andrew, 2000b), an early salivary gland gene directly regulated by Scr and Exd (Ryoo and Mann, 1999). In the absence of *fkh* function, the nuclei move to a basal position in the cells but the cells remain approximately columnar instead of forming the pyramidal-shaped cells observed in wild-type salivary glands (Myat and Andrew, 2000b). Either as a cause or consequence of the failure to undergo this shape change, the secretory cells fail to internalize and form tubes in *fkh* mutants (**Figure 3a**). Interestingly, *fkh* is also required to prevent apoptotic cell death in the salivary gland primordia (Myat and Andrew, 2000b). In *fkh* mutants, expression of both *reaper* (*rpr*) and *head involution defective* (*hid*), two of the known positive effectors of programmed cell death (see also **Chapter 2.10**), are activated in the secretory cells prior to internalization. The secretory cells of *fkh* mutants die, exhibiting features of an apoptotic cell death. Furthermore, *fkh* mutant salivary glands can be rescued from death simply by deleting the genes encoding the positive activators of programmed cell death (**Figure 3a**).

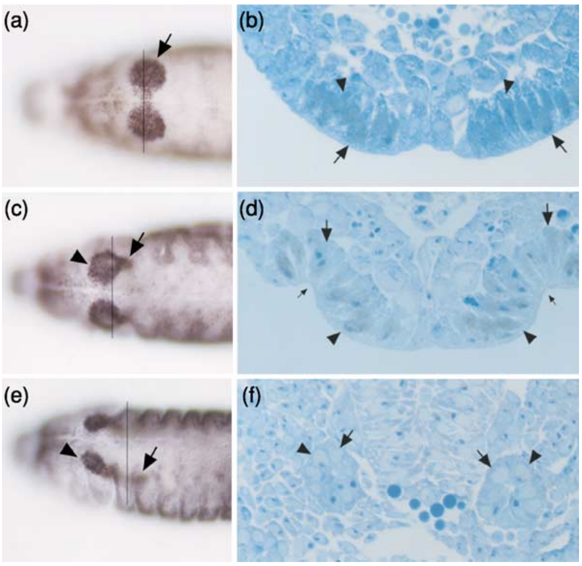


Figure 2 Morphogenesis of the secretory tubes of the *Drosophila* salivary gland. Embryos in (a), (c), and (e) were stained for dCREB-A to visualize the nuclei of secretory cells. Panels (b), (d), and (f) are representative cross-sections of invaginating salivary cells at the embryonic stages corresponding to those in (a), (c), and (e), respectively. The line through each salivary gland in (a), (c), and (e) indicates the approximate region from which the corresponding cross-sections were obtained. Prior to invagination (a), when all secretory cells are found at the ventral surface of the embryo, the cells are columnar (b) with nuclei located randomly in the apical (arrow) or basal (arrowhead) domains. When cells in a dorsal–posterior region of the placode begin to invaginate (c, arrow), an invagination pit is formed (d, small arrow) consisting of pyramidal shaped cells with nuclei coordinately located in a basal position (d, large arrow) in contrast to noninvaginating cells (d, arrowhead) that remain columnar and have randomly positioned nuclei. Towards the end of invagination, when most secretory cells are internalized (e, arrow), an elongated secretory tube is formed (e and f, arrows) consisting of a single layer of wedge-shaped cells surrounding a central lumen.

Once the secretory cells have invaginated in wild-type embryos, they generate a large amount of apical membrane elongated in the proximal–distal axis of the salivary gland tube. Apical membrane generation at this postinvagination step is critical to achieving the final shape of the gland. In mutants where either too little or too much apical membrane is produced, the shape of the secretory tubes is altered significantly (Myat and Andrew, 2000a, 2002). As a consequence of limited apical membrane growth, *huckebein* (*hkb*) mutants form short, dome-shaped glands instead of the elongated tubes that characterize the secretory tubes of wild-type embryos (**Figure 3a**). In *hairy* (*h*) mutants, excessive apical membrane is formed leading to either branched salivary glands or salivary glands with expanded lumina (**Figure 3a**). The salivary glands of *h-hkb* double mutant flies look identical to those of *hkb* mutants, indicating that *hkb* function is required for the apical expansion observed in the *hairy* mutants.

The *hkb* gene encodes a zinc finger transcription factor (Brönner *et al.*, 1994) that regulates membrane growth, at least in part, through two downstream target genes (Myat and Andrew, 2002) (**Figure 4**). One target is *crumbs* (*crb*), an apical membrane protein required for apical membrane specification and growth (Tepass *et al.*, 1990, 1996; Wodarz *et al.*, 1995; Izaddoost *et al.*, 2002; Pellikka *et al.*, 2002). The other target is *klarsicht* (*klar*), a putative regulator of dynein ATPase proposed to promote minus-end directed microtubule-dependent organelle transport (Fischer-Vize and Mosley, 1994; Welte *et al.*, 1998; Mosley-Bishop *et al.*, 1999). Loss of *klar* function results in salivary gland shapes similar to those of *hkb* mutants and overexpression of *klar* leads to the same branching and bulging phenotypes observed with *hairy* mutations (Myat and Andrew, 2002). Expression of

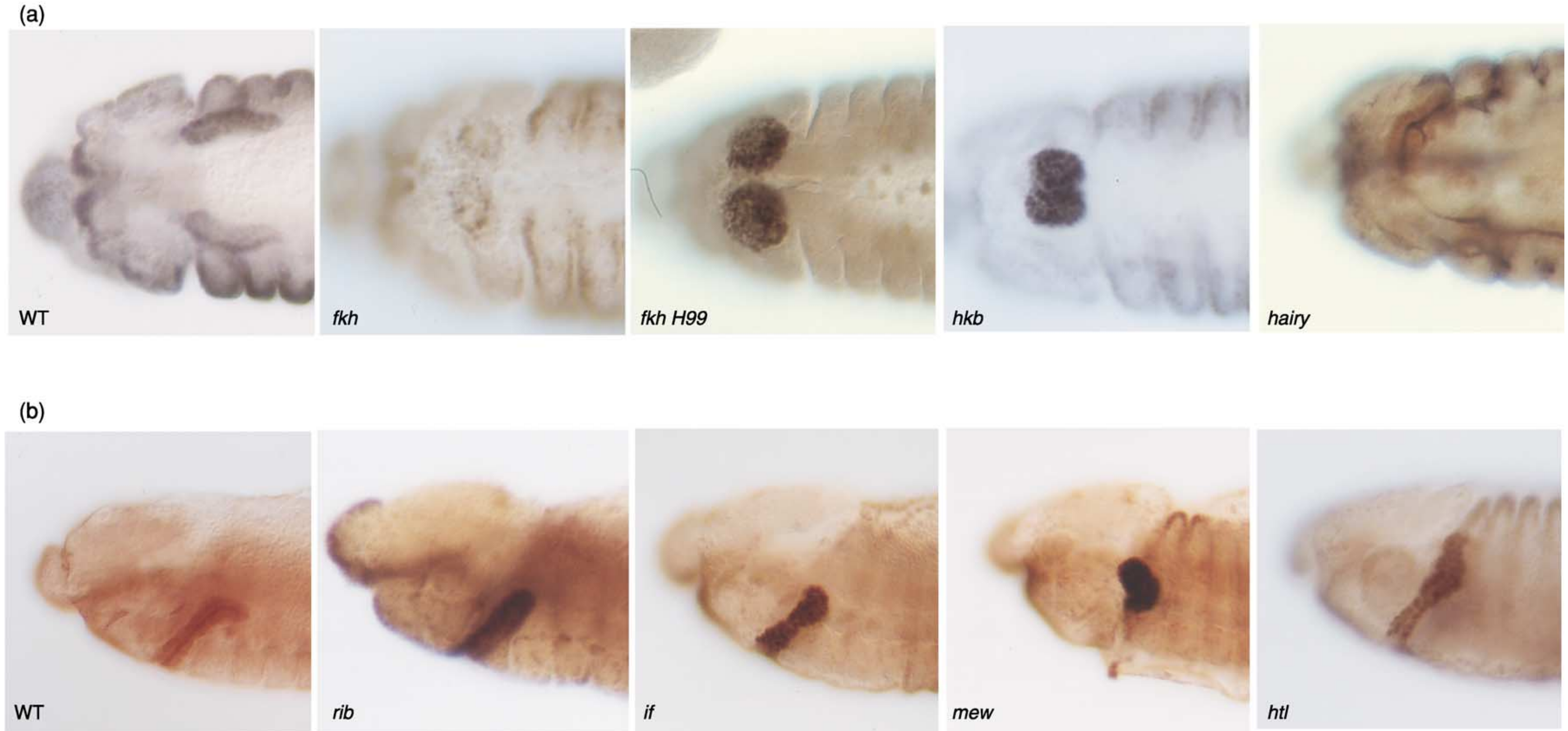


Figure 3 (a) Mutations affecting secretory cell invagination and tube elongation in the *Drosophila* salivary gland. All embryos were stained for dCREB-A except for *hairy*, which was also stained for Crumbs to visualize the salivary gland lumen. In contrast to wild-type (WT) embryos that form elongated secretory tubes, *fkh* embryos do not form secretory tubes because the secretory cells fail to invaginate and instead die by apoptosis. In *fkh H99* embryos, where apoptosis is inhibited, salivary secretory cells still fail to invaginate and remain at the ventral surface of the embryo. In *hkb* embryos, dome-shaped salivary glands are formed, and in *hairy* embryos, branched salivary glands are formed. (b) Mutations affecting migration and final placement of the Drosophila salivary glands. All embryos were stained for dCREB-A. In wild-type embryos, the salivary gland that was initially oriented dorsally turns and migrates posteriorly. In contrast, in *rib, if*, and *mew* embryos, the salivary gland fails to migrate. In *htl* embryos, where formation of the visceral mesoderm is disrupted, the salivary gland migrates further dorsally.

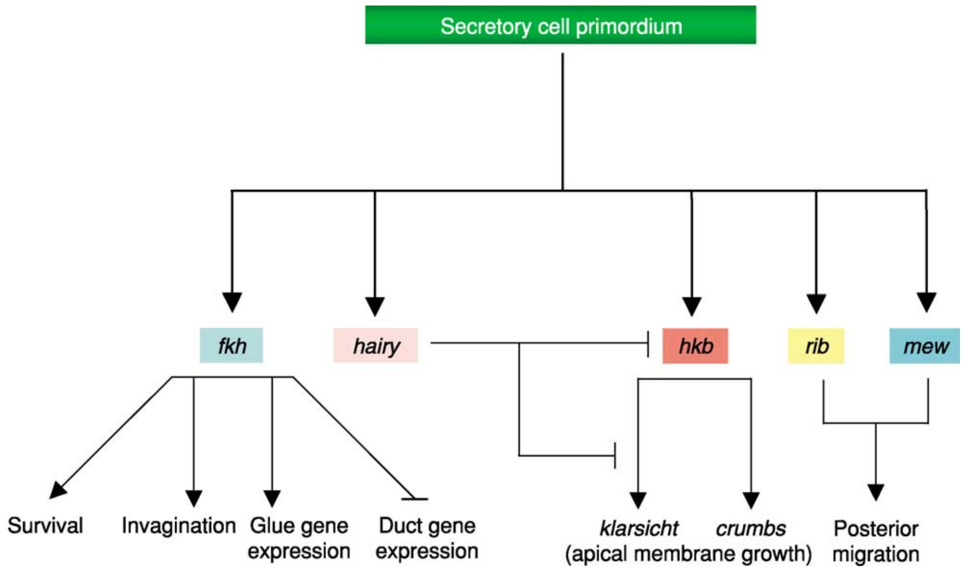


Figure 4 Regulatory network controlling formation of the secretory portion of the salivary gland. Among the earliest expressed genes in the *Drosophila* salivary gland are the transcription factor genes, *fkh, hairy, hkb*, and *rib*, as well as an α-integrin subunit gene, *mew* (also known as α*PS1*). Fkh, Hairy, and Hkb are required for normal invagination and elongation of the secretory tube, whereas Rib and Mew are required for posterior migration of the secretory tube. Fkh is also required for secretory cell survival, expression of glue protein genes in late third instar larvae, and repression of duct-specific genes in the secretory cells. Hairy represses expression of *hkb* and *klar*. Hkb activates expression of *crb* and *klar*, which are required for the generation and delivery of apical membrane, respectively.

wild-type *klar* in *hkb* mutants partially rescues tube elongation. Overexpression of *crb* throughout wild-type salivary glands causes expansion of the salivary gland lumina just like overexpression of *hkb*. In the experiments overexpressing *crb* RNA, high level accumulation of the Crb protein is observed only in cells that also express high levels of wild-type *hkb*, unless either *hkb* or *klar* are also overexpressed.

Based on the genetic interaction studies as well as gene expression patterns in *hairy* mutants, wild-type Hairy is proposed to limit the amount of both Hkb and Klar by repressing expression of both genes shortly after their expression is initiated in the salivary gland primordia (Figure 4). The transient, highly regulated expression of *hkb* and its target genes is thus critical to forming the simple, unbranched, and elongated secretory tubes observed in wild-type embryos.

2.10.3.2. Secretory Cell Migration and Positioning

Once the secretory cells have internalized and cells of the tube have contacted the layer of cells surrounding the developing gut, known as the visceral mesoderm (VM), the cells turn and migrate posteriorly. This posterior migration is required to position the secretory tubes with their long axes parallel to the anterior–posterior axis of the embryo. Posterior migration of the salivary gland requires wild-type function of the *ribbon* (*rib*) gene, integrin signaling, and an intact VM. In embryos mutant for *rib*, the secretory cells internalize normally and form tubular organs. However, the tubes fail to migrate past the point at which wild-type salivary gland cells normally initiate posterior migration (Bradley and Andrew, 2001) (**Figure 3b**). The salivary duct cells of *rib* mutants also fail to elongate and form tubes. *rib* mutants show migration defects in other tissues, including the tracheae, where migration of most branches is delayed and migration of the dorsal trunk cells, which normally occurs along the anterior–posterior body axis, fails entirely. The *rib* gene encodes a nuclear BTB/POZ protein with weak homology to the DNA-binding domains of the Pipsqueak family of transcription factors and the Rib protein contains seven consensus sites for mitogen-activated protein kinase (MAPK) phosphorylation (Bradley and Andrew, 2001; Shim *et al*., 2001). Based on its phenotypes in other tissues, including the trachea, epidermis, and Malphigian tubules (Blake *et al*., 1998; Bradley and Andrew, 2001; Shim *et al*., 2001; see also **Chapters 2.7** and **2.8**), Rib may function to integrate signals from both Wingless and MAPK pathways to mediate changes in cell shape, both during migration and in the fully formed organs.

Mutations in integrin signaling show defects in secretory tube migration very similar to those observed with mutations in *rib* (Bradley *et al*., 2003).

Salivary glands of embryos homozygous for either *multiple edematous wings* (*mew*), which encodes an integrin α-subunit expressed in the secretory cells (Brower *et al*., 1995), or *inflated* (*if*), which encodes another integrin α-subunit expressed in all mesoderm (Bogaert *et al*., 1987), completely fail to migrate past the initial point of contact between secretory cells and the VM (Bradley *et al*., 2003) (**Figure 3b**). However, perhaps because the duct tubes form normally in the integrin mutants and continue to internalize, the secretory tubes of integrin mutants often buckle forming U-shaped tubes with the tips of the tubes directed anteriorly. In the complete absence of integrin function, a situation created either by simultaneously removing function of both α-subunits expressed in the salivary gland (*mew* and *scabrous* (*sca*)) or by removing both the maternal and zygotic function of the essential β-subunit (Brown *et al*., 2000), defects similar to loss of only *mew* or *if* are observed (Bradley *et al*., 2003). The secretory cells internalize and form elongated tubes, but fail to migrate posteriorly. Thus, internalization and formation of the secretory tubes occur in the complete absence of integrin function and may be mediated entirely by cell shape change. This finding suggests that cell migration may be required only at later stages to properly position the secretory tubes within the embryo.

Mutations in genes that disrupt the physical integrity of the VM suggest a role for this tissue in both the initiation of posterior salivary gland migration and in providing a suitable migration substrate for continued posterior movement. In *heartless* (*htl*) or *heartbroken* (*hbr*) mutants, which encode one of the two known fibroblast growth factor (FGF) receptors in *Drosophila* and a downstream effector, respectively, not all of the VM precursors migrate to their final position (Bradley *et al*., 2003) (**Figure 3b**). The defect in cell migration leads to a discontinuous layer of mesodermal cells around the forming gut (Shishido *et al*., 1993; Beiman *et al*., 1996; Gisselbrecht *et al*., 1996; Michelson *et al*., 1998; Iman *et al*., 1999). Salivary gland migration in the *htl* and *hbr* mutant embryos occurs, but is frequently aberrant (Bradley *et al*., 2003). At early stages, when expression of VM markers is easily followed, the migration defects in the FGF signaling mutants correlate with breaks in the VM.

Very similar salivary gland migration defects are observed in *thick veins* (*tkv*) mutants, where breaks are also observed in the VM (Bradley *et al*., 2003). The *tkv* gene encodes one of the essential receptors for Dpp (Nellen *et al*., 1994) and Dpp signaling is required to specify a subset of VM precursors (review: Frasch, 1999).

In *tinman* (*tin*) mutants, where the VM is not properly specified (Azpiazu and Frasch, 1993) and only the most anterior portion of the VM forms, the secretory cells initiate posterior migration but migrate dorsally off course at the point where the VM ends (Bradley *et al*., 2003).

The migration defects observed with mutations in FGF signaling, Dpp signaling, and Tin indicate that an intact VM is required to form secretory tubes that are fully elongated along the anterior–posterior body axis. These phenotypes, combined with the phenotypes observed with mutations in the integrin genes, indicate that the VM functions both as a physical barrier and as a suitable substrate for migration. However, to perform these functions, it is not necessary for the VM precursor cells to differentiate fully into VM. In *biniou* (*bin*) mutants, the VM cells fail to express markers that distinguish VM from other mesoderm and many of the VM precursors appear to take on a somatic mesodermal fate (Zaffran *et al*., 2001). Nonetheless, posterior migration of secretory cells in *bin* mutants is normal (Bradley *et al*., 2003).

Studies of the salivary gland genes expressed in the early secretory tubes indicate roles for these genes in morphogenesis (**Figure 4**). Fkh, Hairy, Hkb, and their downstream target genes are required to form simple, unbranched, and elongated secretory tubes (Myat and Andrew, 2000a, 2000b, 2002). Rib and Mew are required for posterior migration of the secretory tubes, a process necessary to position the tubes along the anterior–posterior axis of the embryo (Bradley and Andrew, 2001; Bradley *et al*., 2003). Additional tissue–tissue interactions and signaling pathways are likely to be required for posteriorly directed movement and for later stages of tube positioning.

2.10.3.3. Salivary Duct Formation

Although much is known about the events controlling the formation of the secretory portion of the *Drosophila* salivary gland, less is known about the events underlying salivary duct formation. The duct forms from two to three rows of ectodermal cells flanking each side of the ventral midline of PS2. Cells in the posterior half of the duct primordium form the individual ducts, whereas cells in the anterior half form the common duct. The individual ducts internalize first, immediately following the internalization of the secretory cells (**Figure 5**). Once the individual ducts have fully internalized and formed tubes, the common ducts internalize to form the central common tube that connects the individual ducts to the larval mouth. Considerable cell rearrangements occur during formation of the salivary duct, leading to the suggestion that salivary

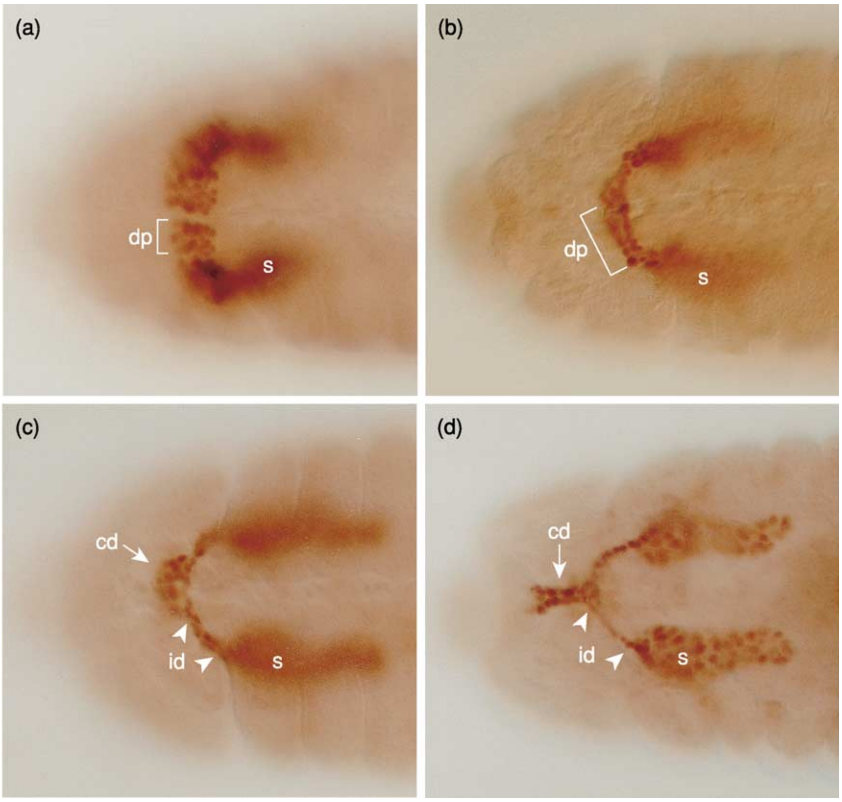


Figure 5 Morphogenesis of the salivary gland duct. *Drosophila* embryos carrying a *P* element insertion in the *trh* gene were stained with an anti-β-galactosidase antibody to visualize all salivary gland cells. All views are from the ventral surface with the secretory cells (s) typically out of focus. (a) The duct primordium (dp; one half is bracketed) remains on the embryo surface when the secretory cells have internalized. (b) During stage 12, the duct primordia become narrower along the anteroposterior axis and elongate laterally. (c) By stage 13, the individual ducts (id) have fully internalized and formed tubes. The common duct (cd) precursors begin to elongate along the anteroposterior axis. (d) The common duct tube forms by embryonic stage 14. (Reproduced with permission from Bradley, P.L., Haberman, A.S., Andrew, D.J., **2001**. Organ formation is *Drosophila*: Specification and morphogenesis of the salivary gland. *BioEssays 23*, 901–911; © John Wiley & Sons, Inc.)

ducts form by the process of convergent extension (Jones *et al*., 1998). Convergent extension is defined as a change in the shape of an organ or group of cells that is mediated by cell intercalation (Keller, 2002).

Among the genes known to be expressed and/or required in the salivary duct are several encoding putative transcription factors, including *trachealess* (*trh*), *eyegone* (*eyg*), and *dead ringer* (*dri*) (**Figure 6**). Additional duct-expressed genes include *Serrate* (*Ser*), which encodes a Notch ligand, and *breathless* (*btl*), which encodes an FGF receptor homolog. Of these genes, only a subset has been shown to be required for normal duct formation. The *trh* gene, which encodes a bHLH-PAS family transcription factor, is required for the duct cells to invaginate and form tubes (Isaac and Andrew, 1996). Much like the *trh* mutant phenotypes observed in other Trh-expressing cells (the trachea and filzkörper, posterior tubes lined with cuticular threads that function as filters for the trachea), the duct precursor cells in *trh* mutants fail to undergo any of the morphological changes of tube formation and simply remain on the embryo surface at their site of origin.

As a transcription factor, Trh must regulate tube formation through the transcriptional regulation of downstream target genes. Among the downstream target genes is *btl* (Ohiro and Saigo, 1997; Sonnenfeld *et al*., 1997; Zelzer *et al*., 1997), which is required for migration of all of the branching tubes of the tracheal system (Klämbt *et al*., 1992; Reichman-Fried *et al*., 1994) (**Figure 6**). However, despite its Trh-dependent expression in the salivary duct, the salivary duct appears normal in *btl* mutant embryos. Another reported Trh target gene is *eyg*, whose expression in late embryos is limited to the individual duct and proximal secretory cells, including the cells that normally form the imaginal ring (Jones *et al*., 1998). Due to the absence of specific loss-of-function mutations in *eyg*, functional studies on *eyg*'s role in salivary duct formation have been limited to embryos either homozygous for

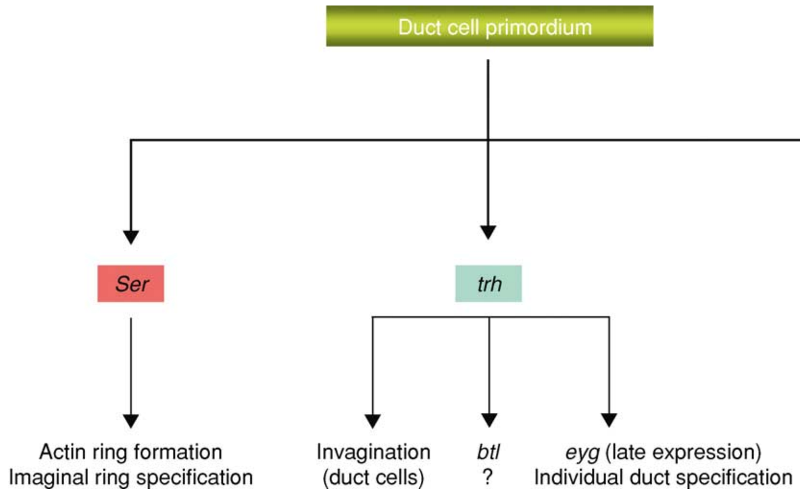


Figure 6 Regulatory network controlling the formation of the duct portion of the salivary gland. Among the earliest expressed genes in the salivary duct of *Drosophila* are two transcription factor genes, *trh* and *dri*, and the gene encoding a transmembrane Notch ligand, *Ser*. Trh is required for invagination and tube formation in the salivary duct, trachea, and filzkörper, and is required for the expression of two downstream duct genes, *btl* and *eyg*. The *eyg* gene has been implicated in specification of the individual ducts. Mutations in *btl* and *dri* do not lead to detectable defects in the salivary duct. *Ser* is required for actin ring formation on the apical surface of the salivary duct and for specification of the adult salivary gland primordia, known as the imaginal ring cells.

a small deficiency removing an estimated 20 genes, and embryos carrying the deficiency in *trans* to an inversion that maps near the *eyg* gene. Embryos of both genetic compositions show defects in duct formation that suggest a role for *eyg* in distinguishing the individual ducts from the common duct (Kuo *et al*., 1996). Similarly, a failure to observe imaginal ring cells associated with the unattached secretory portions of the glands has been interpreted to reflect an additional requirement for *eyg* in specifying this cell type. Expression of a transgene carrying the *eyg* cDNA under the control of a heat shock-inducible promoter partially rescues the individual duct phenotypes observed in homozygous mutant embryos, although the imaginal ring cells of these same animals were not examined. The assignment of a role for *eyg* in individual duct and imaginal ring specification is further complicated by the existence of a close *eyg* homolog, known as *twin of eyegone* (*toe*), which maps immediately adjacent to *eyg* on the third chromosome and is expressed in a very similar pattern in the salivary gland. Thus, it is unclear whether the salivary duct phenotypes observed in the *eyg* mutant backgrounds are solely due to loss of *eyg* function.

Two of the known duct genes, *Ser* and *dri*, are expressed in the duct cells of *trh* mutants to wild-type levels as is *trh* itself, indicating that Trh does not control expression of all duct-specific genes. The *dri* mutants have apparently normal salivary ducts, suggesting either a very subtle role for *dri* in this tissue or some redundancy in its function. Alternatively *Ser* mutants, exhibit a loss of imaginal ring cells, as discussed earlier, and lack the apical actin rings that are observed in wild-type salivary ducts of early larvae (Haberman and Andrew, 2003). These apical actin rings may be precursors of taenidial-like structures observed in the late third instar salivary ducts. In the trachea, taenidia are a series of epithelial folds thought to provide both strength and flexibility. The related structures in the salivary duct may serve a similar function.

2.10.4. Specification and Formation of the *Bombyx* Silk Gland

The silk glands of *Bombyx mori* (see **Chapter 2.11**) are thought to be evolutionarily homologous to the salivary glands of *Drosophila* since they develop from the same embryonic segment and their formation correlates with the expression of the *Bombyx* homolog of the homeotic gene *Scr* (see **Chapter 2.11**). The *Bombyx* silk gland invaginates from the ventral ectoderm of the labial segment to form a tubular structure that elongates and moves into the embryo until the distal end of the gland reaches a muscle in abdominal segment 6 (Matsunami *et al*., 1999). The posterior portions of the gland also associate tightly with the visceral branches of the trachea. Once the silk gland becomes associated with the abdominal muscle and trachea, it curls slightly as it continues to elongate. After the silk gland primordial cells have invaginated, the cells rearrange to form two orderly rows of cells throughout the length of the gland. Unlike the *Drosophila*

salivary glands, the silk gland cells continue to divide during invagination and tube elongation. Even after cell division ceases, the cells continue to enlarge. In the mature silk gland, there are approximately 250 cells in the anterior silk gland (ASG), 200 cells in the middle silk gland (MSG), and 450 cells in the posterior silk gland (PSG). Cells of the MSG and PSG are involved in the secretion of silk proteins and the ASG cells are likely to act in their transport. Based on their distinct roles and their respective gene expression patterns (see below and **Chapter 2.11**), it appears that the *Bombyx* ASG is equivalent to the *Drosophila* salivary duct whereas the MSG and PSG are equivalent to the *Drosophila* secretory glands (**Figure 7**).

Interestingly, some of the homologs of major players of *Drosophila* salivary gland development are expressed in the *Bombyx* silk gland, including *Scr*, *fkh*, and *trh*. Moreover, *Bombyx* Scr may activate *Bombyx fkh* expression as occurs in the *Drosophila* salivary gland; this regulatory interaction is, however, likely to be indirect (Kokubo *et al*., 1997) unlike in *Drosophila* (Ryoo and Mann, 1999). In the *Nc* mutant, which is a deletion of the sequences encoding the homeodomain of the *Bombyx Antennapedia* gene, *Bombyx Scr* and *fkh* expression are observed in new places. In *Nc/Nc* homozygotes, the *Bombyx Scr* gene is ectopically expressed in three posterior thoracic segments, T1, T2, and T3. In these *Nc/Nc* mutants, small invaginating structures resembling the silk glands form in T1–T3. Just as expression of the *Bombyx Scr* gene disappears in the invaginating cells of the *Nc/Nc* mutants, the *Bombyx fkh* gene is activated in the same cells, with the same timeline as is observed in the normal silk gland. These studies suggest that although *fkh* expression in the silk gland may be activated by Scr, the delayed expression of *fkh* relative to Scr indicates a role for some intermediate molecule.

The *Bombyx trh* gene is very similar to its *Drosophila* homolog, with 100% identity in the bHLH domain, 97% homology in the PAS-A domain, and 80% homology in the PAS-B domain (Matsunami *et al*., 1999). Like the *Drosophila* gene, *Bombyx trh* is initially expressed in all cells of the early silk gland, but becomes restricted to the ASG and MSG at later stages. Strong staining persists in the ASG with only weak staining in the MSG. The *Bombyx trh* gene is also, not surprisingly, expressed in the entire trachea from the primordial stages and throughout embryogenesis, as is its *Drosophila* homolog. The expression profile of the *Bombyx trh* gene suggests that it may play roles in silk gland and tracheal development that parallel the roles of its *Drosophila* homolog in the salivary gland and trachea. However, as with the *Bombyx fkh* gene, no loss-of-function studies have been done with the *Bombyx trh* gene to confirm a requirement for the gene in forming tubular structures.

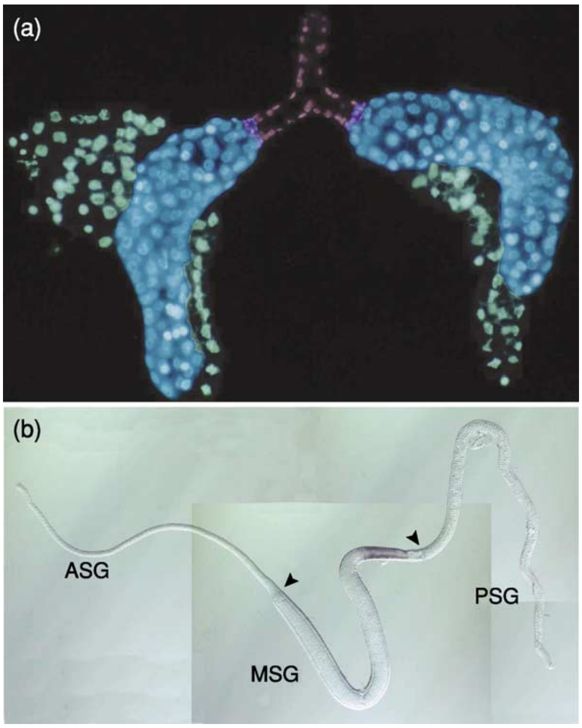


Figure 7 (a) Fluorescent image of a *Drosophila* second instar salivary gland stained with Hoechst and colorized to indicate the different cell types. Salivary secretory cells are shown in cyan, duct cells in pink, and adult precursor imaginal ring cells in purple. The attached fat body cells are shown in green. (Photograph courtesy of A. Haberman.) (b) Phase image of a *Bombyx mori* larval silk gland. The anterior silk gland (ASG) is like the *Drosophila* salivary duct, functioning as a conduit for secretions made in the middle and posterior silk glands (MSG and PSG, respectively). (Photograph courtesy of Y. Suzuki.)

2.10.5. Salivary Gland Destruction During Metamorphosis

During the normal developmental program of multicellular organisms, programmed cell death allows the removal of unwanted cells in a regulated manner (Raff, 1992). Tissue destruction by programmed cell death is an essential process since, in many tissues, more cells are produced than are necessary or the continued presence of certain cell types may be disadvantageous for the organism. As early as the mid-1930s, larval salivary glands were observed to self-destruct during metamorphosis (Robertson, 1936). In insects and other arthropods, the steroid hormone 20-hydroxyecdysone (20E), induces programmed cell death during larval molting,

resulting in the degradation of the majority of larval tissues (Truman, 1992) (see **Chapters 2.4** and **2.5**). Studies within the past two decades are beginning to reveal the cellular changes associated with salivary gland cell death and to provide a molecular understanding of the genetic regulatory pathway by which stage-specific pulses of 20E lead to programmed cell death. Comparison of cell death in *Drosophila* salivary glands and their structural homologs in other insects suggests a similar 20E-induced mechanism for tissue destruction.

2.10.5.1. Regulation of *Drosophila* Salivary Gland Histolysis

During *Drosophila* metamorphosis, two high-titer pulses of 20E occur, first at the late larval stage and subsequently at the prepupal stage, to signal the end of larval development and the initiation of puparium formation (Riddiford, 1993) (**Figure 8**). In response to 20E, larval tissues, such as the midgut and salivary glands, undergo programmed cell death and are replaced by adult tissues arising from groups of progenitor cells set aside earlier in development. While the larval midgut degenerates in response to the late larval pulse of ecdysone, the larval salivary glands survive the late larval pulse and are only destroyed in response to the prepupal pulse of 20E (Lee *et al*., 2002a). The larval salivary glands are eventually replaced by adult salivary tissue arising from the imaginal ring cells, whereas the larval midgut is replaced by midgut island cells dispersed throughout the larval gut (Robertson, 1936).

Larval salivary glands are destroyed by a cell death pathway that shares features characteristic of both apoptosis and autophagic cell death. Apoptosis is characterized by nuclear and cytoplasmic condensation, DNA fragmentation, and the subsequent clearance of corpses by phagocytes (review: Baehrecke, 2002) and usually occurs at the level of individual dying cells (Kerr *et al*., 1972). In contrast, autophagic cell death occurs by the formation of acidic intracellular vacuoles and subsequent cellular destruction without the aid of nearby phagocytes (review: Baehrecke, 2002). Autophagic cell death is more prevalent in the destruction of parts of or entire tissues (Schweichel and Merker, 1973). Dying salivary gland cells exhibit several characteristics indicative of an autophagic cell death, including large vacuoles containing intracellular structures such as mitochondria (Lane *et al*., 1972; Lee and Baehrecke, 2001) and increases in acid phosphatase activity (Jochova *et al*., 1997). However, salivary gland cell death also involves genes known to function in apoptosis. For example, expression of a caspase gene, *dronc*, increases just prior to salivary gland cell death and the inhibition of caspase activity prevents DNA fragmentation and partially inhibits the cellular changes associated with autophagic cell death (Dorstyn *et al*., 1999; Lee *et al*., 2000; Lee and Baehrecke, 2001). Furthermore, recent genomic approaches to identify genes involved in programmed cell death in the *Drosophila* larval salivary gland confirm and extend the notion that this process in the salivary gland utilizes genes known to be involved in both autophagy and apoptosis (Gorski *et al*., 2003; Lee *et al*., 2003).

Precise regulatory mechanisms are required to ensure that 20E will induce a timely cell death in

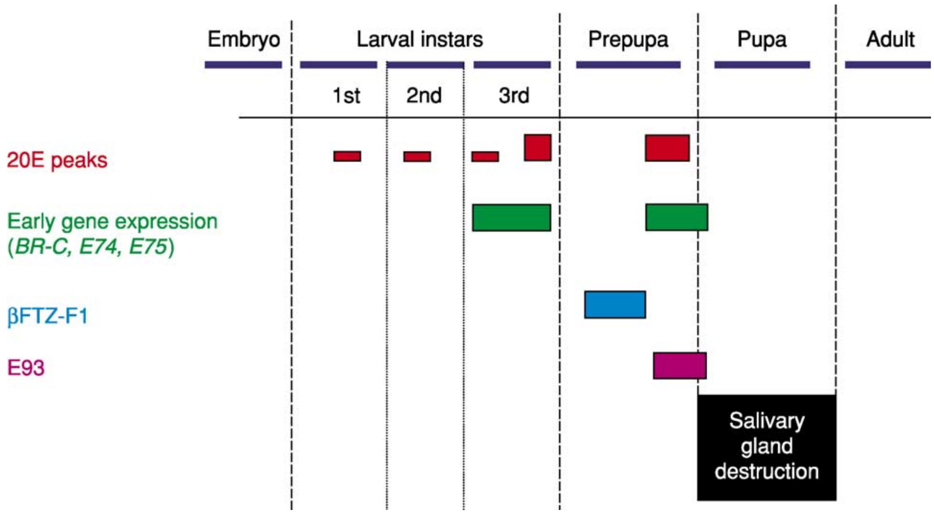


Figure 8 20E-induced gene expression patterns during *Drosophila* metamorphosis. The profile of 20E titer in relation to the expression of the early response genes, BR-C, E74, and E75, the competence factor, βFTZ-F1, and the stage-specific early gene, E93, is shown in correspondence with each of the developmental stages of the *Drosophila* life cycle.

larval tissues while simultaneously promoting development of preadult tissues. To achieve their timely destruction, salivary cells implement a two-step regulatory hierarchy involving the expression of early and late 20E-response genes (**Figure 9**). The 20E receptor is a heterodimer of two nuclear hormone receptors (Thummel, 1995), the ecdysone receptor (EcR) (Koelle *et al.*, 1991) and Ultraspiracle (Usp) (Oro *et al.*, 1990). The existence of additional related orphan receptors, however, raises the possibility that EcR and Usp may also form heterodimers with other receptor-like proteins (Thummel, 1995). The EcR complex promotes salivary gland destruction by directly controlling the expression of a set of early response genes, including the transcription factor genes βFTZ-F1, E74, E75, E93, and the genes in the Broad-Complex (BR-C) (Ashburner, 1974; Richards, 1976). The βFTZ-F1 and E75 genes encode members of the orphan nuclear receptor superfamily (Segraves and Hogness, 1990; Lavorgna *et al.*, 1993), whereas the BR-C genes encode zinc finger transcription factors (DiBello *et al.*, 1991). The E74 gene encodes two isoforms of an ETS-domain transcription factor and E93 encodes a novel nuclear protein (Burtis *et al.*, 1990; Lee *et al.*, 2000). Mutations in each of the early 20E-regulated genes prevents the timely destruction of the larval salivary glands, indicating a role for these genes in salivary gland programmed cell death. For example, mutations in βFTZ-F1 and E93 disrupt the early cellular changes associated with autophagic cell death (Lee *et al.*, 2002b), whereas mutations in the BR-C and E74A genes disrupt cell death progression (Lee and Baehrecke, 2001; Lee *et al.*, 2002b). The 20E-regulated transcription factors activate cell death in the salivary gland by regulating the expression of downstream target genes that are more directly involved in mediating the death response. Downstream targets of the early 20E-regulated genes include the apoptosis inhibitor *diap*; the caspase *dronc*; the caspase-activator *ark*; the apoptosis activators *reaper* (*rpr*) and *head involution defective* (*hid*); and the phagocyte marker *croquemort* (*crq*) (Jiang *et al.*, 1997, 2000; Franc *et al.*, 1999; Kanuka *et al.*, 1999; Lee *et al.*, 2000) (**Figure 9**). Interestingly, *crq* is expressed in the cytoplasm of degenerating salivary cells, suggesting that both phagocytes and autophagic cells may use *crq* for removal of dying cells (Lee and Baehrecke, 2001). The expression of *diap* prior to salivary gland death is activated by βFTZ-F1 and then repressed by E75A and E75B (Jiang *et al.*, 2000). This transient elevation of *diap* expression occurs prior to induction of the apoptosis activators *rpr* and *hid*. Thus, by coordinating the expression of death activators and inhibitors, the 20E-regulated transcription factors βFTZ-F1, BR-C, E74, E75, and E93 are able to precisely control the time of salivary gland destruction.

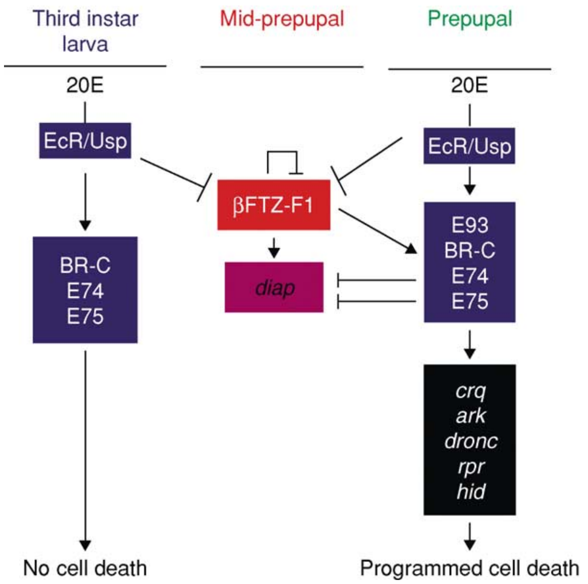


Figure 9 20E-mediated genetic regulatory network leading to salivary gland programmed cell death. At the third instar larval stage, 20E induces transcription of the early response genes, BR-C, E74, and E75, by interacting with the EcR/Usp heterodimer. However, this early induction does not lead to salivary cell death. Low 20E titer at the mid-prepupal stage induces the competence factor, βFTZ-F1, which in turn induces expression of the apoptosis inhibitor, *diap2*, thus delaying programmed cell death until the high titer pulse of ecdysone at the prepupal stage. The βFTZ-F1 protein also facilitates 20E-dependent induction of the early genes, BR-C, E74, and E75, and E93 at the prepupal stage. E93 acts as a stage-specific regulator of cell death by regulating the expression of the early reponse genes as well as the cell death genes, *rpr, hid, ark, dronc*, and *crq*, ultimately leading to programmed cell death in the salivary cells.

The BR-C, E74, and E75 genes are also expressed earlier in the salivary gland and in other tissues without activating PCD. Thus, although these genes are necessary for salivary gland cell death, they are not sufficient. In contrast, the early ecdysone-regulated gene βFTZ-F1 and the stage-specific gene E93, are both necessary and sufficient for inducing salivary gland destruction. Gene βFTZ-F1 transcription is repressed by 20E; its expression is only detected in a brief interval at the mid-prepupal stage when the hormone titers are low (Woodard *et al.*, 1994) (**Figure 8**). The expression of βFTZ-F1 at the mid-prepupal stage reinduces the expression of the late prepupal genes, Br-C, E74A, and E75A, thus acting as a competence factor that allows the expression of these genes in response to the late prepupal pulse of 20E (Lavorgna *et al.*, 1993; Woodard *et al.*, 1994; Broadus *et al.*, 1999) (**Figure 9**). βFTZ-F1

represses its own transcription and as a result ensures a short period of competence (Woodard *et al*., 1994). βFTZ-F1-mediated salivary gland destruction requires the stage-specific early response gene, E93 (Lee *et al*., 2002b). In E93 mutant salivary cells, expression of the 20E-induced early genes, BR-C, E74, and E75, and the cell death genes *rpr*, *hid*, *crq*, and *dronc* are reduced (Lee *et al*., 2000). Like βFTZ-F1, ectopic expression of E93 is sufficient to induce programmed cell death both in tissues that normally undergo hormone-regulated morphogenesis, as well as those that develop in a hormone-independent manner (Lee *et al*., 2000; Lee and Baehrecke, 2001).

Although salivary gland cell death is partly mediated by caspase activity, caspase inhibition by the baculovirus caspase inhibitor, p35, does not inhibit E93-induced cell death (Lee and Baehrecke, 2001). One possible explanation for this disparity is that additional caspase-independent mechanisms may be involved in salivary gland cell death. For example, proteolysis during programmed cell death may involve ubiquitin-mediated destruction in addition to caspases (Schwartz *et al*., 1993; Yang *et al*., 2000). Indeed, in a recent genome-wide analysis of steroid-triggered salivary gland cell death, several noncaspase proteases and related inhibitors have been identified that exhibit dynamic changes in transcription just hours prior to salivary gland destruction (Lee *et al*., 2003).

2.10.5.2. Additional Roles for the BR-C Genes

In addition to the transcriptional regulation of late gene expression during puparium formation (Crossgrove *et al*., 1996), the BR-C proteins are also required earlier at the third larval instar stage for activation of the salivary gland secretion (*Sgs*) genes (Hansson and Lambertsson, 1989). The *Sgs* genes are expressed exclusively in the salivary glands and are required specifically for the production of the glue that allows the puparium to adhere to the substratum (Lehmann, 1995). Activation of the *Sgs* genes requires the BR-C as well as the presence of binding sites for the Fkh transcription factor (Lehmann and Korge, 1996; Mach *et al*., 1996). *In vitro* and *in vivo* studies suggest that Fkh directly controls the salivary gland-specific expression of at least two of the *Sgs* genes, *Sgs-3* and *Sgs-4* (Lehmann and Korge, 1996; Mach *et al*., 1996), and possibly a third, *Sgs-1* (Roth *et al*., 1999). *Sgs* gene expression is subsequently repressed by the *2Bc* function of BR-C in response to the late-larval pulse of 20E (Crowley and Meyerowitz, 1984; Hansson and Lambertsson, 1989); however, *2Bc*-mediated repression of *Sgs* genes is indirect and occurs through Fkh, which is transcriptionally repressed by *2Bc* at puparium formation (Renault *et al*., 2001). Elimination of *fkh* expression by BR-C may also be an early step towards stage-specific destruction of this tissue in response to 20E, since Fkh is essential for salivary secretory cell survival during early embryogenesis. In the absence of *fkh* function, *reaper* and *hid* are transcriptionally upregulated in the embryonic salivary cells, and the salivary gland subsequently degenerates by apoptosis (Myat and Andrew, 2000b). The adult salivary gland will form from the imaginal ring cells that are specified during embryogenesis (Haberman and Andrew, 2003). To date, no studies have been published on the development of the adult salivary gland.

2.10.5.3. Salivary Gland Destruction in Insects other than *Drosophila*

Programmed cell death mediated by 20E in insects other than *Drosophila* also appears to occur by apoptosis and autophagic cell death. The labial glands of the tobacco hornworm, *Manduca sexta*, the anterior silk gland of the silkworm *B. mori*, and the salivary glands of the blowfly *Calliphora vomitoria*, all undergo programmed cell death in response to ecdysone. Programmed cell death during larval–pupal metamorphosis in the *Manduca* labial glands is characterized by the intracellular movement of lysosomes and fragmentation of the endoplasmic reticulum, resulting in the formation of large intracellular vacuoles (Halaby *et al*., 1994). Massive vacuolization of the cell occurs concomitantly with large-scale DNA damage and fragmentation (Jochova *et al*., 1997). These characteristics of labial gland destruction in *Manduca* suggest an autophagic as well as an apoptotic type of cell death, as occurs in the salivary glands of *Drosophila* early pupae.

In the silkworm *B. mori*, ecdysone induces programmed cell death of the anterior segment of the silk gland during the larval–pupal transition (Chinzei, 1975; Terashima *et al*., 2000). Morphological and biochemical studies of silk gland destruction demonstrate the increased presence of autophagosomes and the prevalent role that lysosomes play in the degradation of cytoplasmic organelles (Shiba *et al*., 2001). Furthermore, programmed cell death of the *Bombyx* silk gland occurs in the absence of phagocytes, as observed in the *Drosophila* salivary gland (Matsura *et al*., 1968). In a recent study, the cathepsin B- and L-like lysosomal proteinases were found to be involved in the destruction of the silk gland (Shiba *et al*., 2001). In addition to lysosomal proteinases, the ubiquitin–proteasome system may also be involved in the removal of

cytoplasmic proteins during programmed cell death in the silk gland (Ichimura *et al*., 1994).

While DNA fragmentation is often described as a hallmark of apoptotic cell death and is prevalent during *Drosophila* salivary gland degeneration (Lee and Baehrecke, 2001), studies in *Manduca* and *Calliphora* suggest that DNA fragmentation may not contribute to cell death to the same extent as it does in mammalian cells. In programmed cell death of the *Manduca* labial gland, vacuolization and cytoplasmic destruction are prominent very early, with DNA fragmentation only occurring at the end of gland destruction (Zakeri *et al*., 1993). Similarly, in the dying cells of the *Bombyx* silk gland, the degree of nuclear condensation and DNA fragmentation is variable (Terashima *et al*., 2000).

Finally, salivary gland cell death in *Calliphora* also occurs by vacuolization of the cell and the subsequent dispersal of cellular contents into the lumen; however, no evidence exists to date for a role for DNA degeneration during gland destruction (Bowen *et al*., 1993). Thus, 20E-induced glandular destruction in the insects described above appears to always include early vacuole-mediated cytoplasmic destruction, whereas the involvement of nuclear DNA fragmentation varies.

2.10.6. Salivary Gland Physiology and Disease

For blood-feeding arthropods, such as the mosquito *Anopheles* and the Ixodidae family of hard ticks, the salivary glands are essential for feeding and serve additional functions. In both mosquitoes and ticks, sexual dimorphism exists for feeding habits and correlates with salivary gland morphology. In mosquitoes, although both sexes feed on sugar solutions of fruits and flora, only the female takes blood meals. The mosquito salivary gland is a simple, relatively unbranched tubular structure comprising two lateral lobes and a single medial lobe; the female salivary gland is larger and is composed of more cell types than its male counterpart (James, 1994). Similarly, female ixodid ticks consume large quantities of blood over an extended period of time, whereas male ticks feed only occasionally and on low volumes of blood.

Ixodid tick salivary glands consist of two large lateral glands with multiple branches containing both ducts and *acini* (*alveoli*). A single large duct within each of the two glands allows access to the food channel through a broad, shallow tube called the *salivarium* (review: Sauer *et al*., 1995). Female ixodid ticks have three types of *alveoli* whereas male ticks have four, with each *alveolus* differing in morphology and in the type and number of granular and agranular cells it contains (Sauer *et al*., 1995). In newly hatched ixodid larvae, the salivary glands are extremely small and only ducts are distinguishable. At later larval stages, three types of *alveoli* are observed.

Salivary gland destruction in ixodid ticks occurs after larvae become engorged from blood feeding and is characterized by degeneration of only the *alveoli* (Sauer *et al*., 1995). The remaining salivary ducts form branching structures with small terminal *alveoli* containing undifferentiated cells. The undifferentiated *alveoli* increase in number and subsequently differentiate into the three or four types of *alveoli*, depending on gender (Sauer *et al*., 1995). During metamorphosis to adult, the salivary glands degenerate and redifferentiate through a process similar to that occurring in the larval glands (Sauer *et al*., 1995). As with the other insects described here, salivary gland degeneration in ixodid ticks is also thought to be triggered by 20E (Harris and Kaufmann, 1985).

The ixodid tick salivary gland is a multifunctional organ that secretes a cement substance, which allows the slow-feeding ticks to attach firmly to the host. The gland also secretes toxic substances and pharmacological agents that cause tissue destruction at the site of feeding, ultimately leading to trauma to the host (review: Sauer, 1977). Salivary glands of ixodid ticks also serve as osmoregulatory organs. During the off-host stage when the tick is not feeding, which comprises the majority of its life cycle, the salivary glands secrete a hygroscopic solution onto the mouthparts, which absorbs water from unsaturated air and allows the tick to remain hydrated (Sauer *et al*., 1995). In contrast, during feeding and attachment to the host, the tick concentrates the blood meal and eliminates excess fluid and ions by secreting them back into the host through the salivary glands (Sauer *et al*., 1995). Approximately one third to one half of the ingested fluid is secreted back to the host using this mechanism.

Blood-feeding arthropods serve as important vectors for diseases caused by such pathogens as viruses, bacteria, rickettsiae, and protozoa. The most common route of infection is through the salivary glands with certain pathogens replicating within the salivary cells (review: Bowman *et al*., 1997). Plasmodium parasites, which cause malaria, are transmitted by the blood-sucking mosquito, *Anopheles gambiae*. During the parasite's life cycle in the *Anopheles* vector, sporozoites invade the salivary gland epithelium by attaching to the basal lamina (Pimenta *et al*., 1994). After a transient stay in the cytoplasm, the sporozoites enter the secretory cavity

where they remain viable for the life of the mosquito (review: Ghosh *et al*., 2000). During blood feeding, a small number of sporozoites enter the vertebrate host through the salivary duct. Saliva not only serves to deliver the sporozoites but also aids in their infectivity since it contains pharmacologically active compounds capable of counteracting the host's defense mechanisms (Ghosh *et al*., 2000). Inhibition of salivary gland invasion by *Plasmodium* sporozoites may be one approach towards elimination of malaria.

2.10.7. Conclusion

Recent studies of the *Drosophila* salivary glands have revealed many of the underlying mechanisms and molecules required to form an organ and to mediate its destruction at appropriate developmental stages. Such studies provide meaningful paradigms for organ specification, formation, and homeostasis in higher organisms, including humans. Recent studies in other insect species suggest a high level of conservation of regulatory molecules controlling both the genesis and demise of salivary gland cells. This high level conservation of gene function may ultimately yield strategies for limiting the spread of the many pernicious human and animal diseases carried by insect vectors.

Acknowledgments

The authors thank the many people whose work has contributed to their understanding of insect salivary gland development, physiology, and destruction, E. Baehrecke and A. Haberman for their critical reading of the chapter, S. Karim for sharing the tick and mosquito salivary gland literature, and A. Haberman for the image of the second instar *Drosophila* salivary gland and Y. Suzuki for the image of the *B. mori* silk gland. Their current research on *Drosophila* salivary gland development is supported by NIH RO1 DE12873 and RO1 DE13899 grants (to D.J.A.), and an NIH K22 Award (to M.M.M.).

Appendix Genes involved in salivary gland specification, morphogenesis, and programmed death

Gene	*Protein*	*Role in salivary gland*
Abdominal-B (Abd-B) (see **Chapter 1.8**)	Homeodomain transcription factor expressed in posterior abdominal segments	Blocks salivary gland formation in PS14
Apaf-1-related-killer (Ark)	Caspase activator	Role in programmed larval salivary cell death during metamorphosis
βFTZ-F1	Ligand-dependent nuclear receptor transcription factor	Regulation of programmed larval salivary cell death during metamorphosis
breathless (btl)	Transmembrane receptor tyrosine kinase (FGF receptor) expressed in duct cells under control of TRH	Role in duct unknown, no visible defects in mutants
brinker	Nuclear transcription factor	Blocks transcription of Dpp target genes, mutations result in smaller salivary glands
Broad-Complex (BR-C)	BTB/POZ ranscription factors induced early and late in response to ecdysone signaling	Regulation of programmed larval salivary cell death during metamorphosis
croquemort (crq)	Plasma membrane protein; scavenger/ macrophage receptor	Role in programmed larval salivary cell death during metamorphosis
crumbs (crb)	Transmembrane protein localized to apical membranes and subapical region of epithelial cells	Specification and maintenance of apical membrane domains of salivary epithelial cells
decapentaplegic (dpp)	Secreted protein of the TGF-β family expressed in dorsal cells	Represses salivary gland formation in dorsal cells of PS2
dead ringer (dri)	ARID-box transcription factor expressed in duct cells, repressed in secretory cells by Fkh	Role in duct unknown, no visible defects in mutants
dorsal (dl)	Transcription factor with nuclear localization in ventral cells	Specification of ventral cell fates; mutants do not have salivary glands
dronc	Nedd2-like caspase	Salivary gland destruction during metamorphosis
Drosophila inhibitor of apoptosis (diap)	Inhibitor of apoptosis	Regulation of programmed larval salivary cell death during metamorphosis
E74	Transcription factor induced early and late in response to ecdysone signaling	Regulation of programmed larval salivary cell death during metamorphosis
E75	Ligand-dependent nuclear receptor transcription factor	Regulation of programmed larval salivary cell death during metamorphosis
E93	Transcription factor induced late in response to ecdysone signaling	Regulation of programmed larval salivary cell death during metamorphosis

Continued

Appendix Continued

Gene	Protein	Role in salivary gland
Ecdysone receptor (EcR)	Receptor subunit for steroid hormone 20-hydroxyecdysone	Regulation of programmed larval salivary cell death during metamorphosis
epidermal growth factor receptor (EGF-R)	Transmembrane receptor tyrosine kinase (EGF receptor)	Blocks salivary gland secretory cell fate, allowing salivary gland duct cell fate
extradenticle (exd)	Homeodomain transcription factor expressed relatively ubiquitously	Salivary gland specification
eyegone (eyg)	PAX family transcription factor expressed dynamically in subset of secretory and duct cell primordia	Individual duct specification, potential role in specification of imaginal ring cell fate
fork head (fkh)	Winged-helix transcription factor expressed in the secretory cells	Secretory cell internalization, prevention of apoptotic cell death, repression of duct gene expression in secretory cells, activation of glue gene expression in late larval glands
hairy (h)	Myc-like bHLH transcription factor expressed transiently in secretory cells	Prevention of branching and bulging by limiting apical membrane growth in secretory cells through transcriptional repression of *hkb* and *klar*
head involution defective (hid)	Inducer of programmed cell death	Positive activator of programmed cell death (autophagy) in larval salivary glands during metamorphosis
heart broken (hbr)	Cytoplasmic protein required for FGF signaling	Mesodermal migration, required to form intact visceral mesoderm for salivary gland posterior migration
heartless (htl)	Transmembrane receptor tyrosine kinase (FGF receptor)	Mesodermal migration, required to form intact visceral mesoderm for salivary gland posterior migration
homothorax (hth) (see **Chapter 1.8**)	Homeodomain transcription factor expressed relatively ubiquitously	Salivary gland specification
huckebein (hkb)	Zinc finger transcription factor expressed transiently and dynamically in early secretory cells	Apical membrane growth and tube elongation in secretory cells through transcriptional activation of *crb* and *klar*
inflated (if)	PS2 α-integrin subunit; transmembrane protein (expressed in mesoderm)	Migration of secretory cells
klarsicht (klar)	Cytoplasmic protein proposed to mediate minus-end transport of organelles along microtubules	Apical membrane growth/delivery in secretory cells
Multiple edematous wings (mew)	PS1 α-integrin subunit; transmembrane receptor (expressed in secretory cells)	Migration of secretory cells
Notch (N)	Transmembrane receptor; upon signaling, C-terminal fragment enters nucleus to regulate transcription	Prevents neurogenic fate in salivary gland precursors; later, specification of imaginal ring cells
reaper (rpr)	Cytoplasmic inducer of programmed cell death	Positive activator of programmed cell death (autophagy) in larval salivary glands during metamorphosis
rhomboid (rho)	ER localized transmembrane protease required for processing of EGF ligands, expressed in ventral cells	Blocks salivary gland secretory cell fate, allowing salivary gland duct cell fate
ribbon (rib)	Putative transcription factor with BTB/POZ domain and domain of homology to Pipsqueak DNA-binding domains	Migration of secretory cells, elongation of duct tubes
scabrous (sca)	PS3 α-integrin subunit; transmembrane receptor (expressed in secretory cells)	Mild defects in salivary gland placement at late stages
Serrate (Ser)	Transmembrane ligand for Notch, expressed in duct cells, repressed in secretory cells by Fkh	Specification of imaginal ring cell fate in salivary cells neighboring the duct precursors, required for actin-ring structures in duct
Sex combs reduced (Scr) (see **Chapter 1.8**)	Homeodomain transcription factor expressed in parasegment 2 (PS2)	Salivary gland specification
spitz (spi)	Secreted EGF ligand	Blocks salivary gland secretory cell fate, allowing salivary gland duct cell fate
teashirt (tsh)	Zinc finger transcription factor expressed in trunk and abdominal segments	Blocks salivary gland formation in PS3–13
tetraspanin 74F (tsp74F)	Transmembrane protein expressed in imaginal ring cells during embryogenesis	Function in imaginal ring cells unknown

Continued

Appendix Continued

Gene	*Protein*	*Role in salivary gland*
thick veins (tkv)	Transmembrane serine/threonine kinase, Dpp receptor	Represses salivary gland formation in dorsal cells of PS2
tinman (tin)	Homeodomain-containing nuclear transcription factor	Specification of visceral mesoderm precursors; required to form intact visceral mesoderm for salivary gland posterior migration
trachealess (trh)	bHLH-PAS transcription factor expressed transiently in all salivary cells, later restricted to duct cells by Fkh repression	Internalization of duct precursors to form the salivary duct tubes
twin of eyegone (toe)	PAX family transcription factor expressed dynamically in subset of secretory and duct cell primordia	With *eyegone* may have a role in individual duct specification and a potential role in specification of imaginal ring cell fate
Ultraspiracle (Usp)	Receptor subunit for steroid hormone 20-hydroxyecdysone	Regulation of programmed larval salivary cell death during metamorphosis

References

Abu-Shaar, M., Ryoo, H.D., Mann, R.S., **1999**. Control of nuclear localization of Extradenticle by competing nuclear import and export signals. *Genes Devel. 13*, 935–945.

Affolter, M., Marty, T., Vigano, M.A., Jazwinska, A., **2001**. Nuclear interpretation of Dpp signaling in *Drosophila*. *EMBO J. 20*, 3298–3305.

Andrew, D.J., Horner, M.A., Petitt, M.G., Smolik, S.M., Scott, M.P., **1994**. Setting limits on homeotic gene function: restraint of *Sex combs reduced* activity by *teashirt* and other homeotic genes. *EMBO J. 13*, 1132–1144.

Ashburner, M., **1974**. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster.* 2. The effects of inhibitors of protein synthesis. *Devel. Biol. 39*, 141–157.

Azpiazu, N., Frasch, M., **1993**. *Tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Devel. 7*, 1325–1340.

Baehrecke, E.H., **2002**. How death shapes life during development. *Nat. Rev. Mol. Cell Biol. 10*, 779–787.

Beiman, M., Shilo, B.Z., Volk, T., **1996**. *Heartless*, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesoderm lineases. *Genes Devel. 10*, 2993–3002.

Blake, K.J., Myette, G., Jack, J., **1998**. The products of *ribbon* and *raw* are necessary for proper cell shape and cellular localization of nonmuscle myosin in *Drosophila*. *Devel. Biol. 203*, 177–188.

Bogaert, T., Brown, N., Wilcox, M., **1987**. The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell 51*, 929–940.

Bowen, I.D., Morgan, S.M., Mullarkey, K., **1993**. Cell death in the salivary glands of metamorphosing *Calliphora vomitoria*. *Cell Biol. Int. 17*, 13–33.

Bowman, A.S., Coons, L.G., Needham, G.R., Sauer, J.R., **1997**. Tick saliva: recent advances and implications for vector competence. *Med. Vet. Entomol. 11*, 277–285.

Bradley, P.L., Haberman, A.S., Andrew, D.J., **2001**. Orgon formation is *Drosophila*: specification and morphogenesis of the salivary gland. *BioEssays 23*, 901–911.

Bradley, P.L., Andrew, D.J., **2001**. *Ribbon* encodes a novel BTB/POZ protein required for directed cell migration in *Drosophila melanogaster. Development 128*, 3001–3015.

Bradley, P.L., Myat, M.M., Comeaux, C.A., Andrew, D.J., **2003**. Posterior migration of the salivary gland requires an intact visceral mesoderm and integrin function. *Devel. Biol. 256*, 249–262.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., Woodard, C.T., **1999**. The *Drosophila* beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell 3*, 143–149.

Brönner, G., Chu-LaGraff, Q., Doe, C.Q., Cohen, B., Weiogel, D., *et al.*, **1994**. Spi/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in *Drosophila*. *Nature 369*, 664–668.

Brower, D.L., Bunch, T.A., Mukai, L., Adamson, T.E., Wehrli, M.S.L., *et al.*, **1995**. Nonequivalent requirements for PS1 and PS2 integrin at cell attachments in *Drosophila*: genetic analysis of the alpha ps1 integrin subunit. *Development 121*, 1311–1320.

Brown, N.H., Gregory, S.L., Martin-Bermudo, M.D., **2000**. Integrins as mediators of morphogenesis in *Drosophila*. *Devel. Biol. 223*, 1–16.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., Hogness, D.A., **1990**. The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell 61*, 85–99.

Campos-Ortega, J., Hartenstein, V., **1997**. The Embryonic Development of *Drosophila melanogaster.* Springer-Verlag, Berlin.

Chinzei, Y., **1975**. Biochemical evidence of DNA transport from the silk gland to the fat body of the silk worm, *Bombyx mori*. *J. Insect Physiol. 21*, 163–171.

Crippa, M., Rais, O., Gern, L., **2002**. Investigations on the mode and dynamics of transmission and infectivity of *Borrelia burgdorferi sensu stricto* and *Borrelia*

afzelii in *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis. 2*, 3–9.

Crossgrove, K., Bayer, C.A., Fristrom, J.W., Guild, G.M., **1996**. The *Drosophila* Broad-complex early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade. *Devel. Biol. 180*, 745–758.

Crowley, T.E., Meyerowitz, E.M., **1984**. Steroid regulation of RNAs transcribed from the *Drosophila* 68C polytene chromosome puff. *Devel. Biol. 102*, 110–121.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., Guild, G.M., **1991**. The *Drosophila* Broad-complex encodes a family of related proteins containing zinc fingers. *Genetics 129*, 385–397.

Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H., Kumar, S., **1999**. DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl Acad. Sci. USA 96*, 4307–4312.

Edgar, B.A., Orr-Weaver, T.L., **2001**. Endoreplication cell cycles: more for less. *Cell 105*, 297–306.

Fischer-Vize, J.A., Mosley, K.L., **1994**. *Marbles* mutants: uncoupling cell determination and nuclear migration in the developing *Drosophila* eye. *Development 120*, 2609–2618.

Fleming, R.J., Scottgale, T.N., Diederich, R.I., Artavanis-Tsakonas, S., **1990**. The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster. Genes Devel. 4*, 2188–2201.

Franc, N.C., Heitzler, P., Ezekowitz, R.A., White, K., **1999**. Requirement for *croquemort* in phagocytosis of apoptotic cells in *Drosophila*. *Science 284*, 1991–1994.

Frasch, M., **1999**. Intersecting signalling and transcriptional pathways in *Drosophila* heart specification. *Semin. Cell Devel. Biol. 10*, 61–71.

Ghosh, A., Edwards, M.J., Jacobs-Lorena, M., **2000**. The journey of the malaria parasite in the mosquito: hopes for the new century. *Parasitol. Today 16*, 196–201.

Gisselbrecht, S., Skeath, J.B., Doe, C.Q., Michelson, A.M., **1996**. *Heartless* encodes a fibroblast growth factor recptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Devel. 10*, 3003–3017.

Gorski, S.M., Chittaranjan, S., Pleasance, E.D., Freeman, J.D., Anderson, C.L., *et al.*, **2003**. A SAGE approach to discovery of genes involved in autophagic cell death. *Curr. Biol. 13*, 358–363.

Haberman, A.S., Andrew, D.J., **2003**. Specification of cell fates within the salivary gland primordium. *Devel. Biol. 258*, 443–453.

Halaby, R., Zakeri, Z., Lockshin, R.A., **1994**. Metabolic events during progammed cell death in insect labial glands. Biochem. *Cell Biol. 72*, 597–601.

Hansson, L., Lambertsson, A., **1989**. Steroid regulation of glue protein genes in *Drosophila melanogaster. Hereditas 110*, 61–67.

Harris, R.A., Kaufmann, W.R., **1985**. Ecdysteroids: possible candidates for the hormone which triggers salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*. *Experientia 41*, 740–742.

Henderson, K.D., Andrew, D.J., **2000**. Regulation and function of *Scr*, *exd*, and *hth* in the *Drosophila* salivary gland. *Devel. Biol. 217*, 362–374.

Henderson, K.D., Isaac, D.D., Andrew, D.J., **1999**. Cell fate specification in the *Drosophila* salivary gland: the integration of homeotic gene function with the DPP signaling cascade. *Devel. Biol. 205*, 10–21.

Ichimura, S., Mita, K., Numata, M., **1994**. Protein ubiquitination in the posterior silk glands of *Bombyx mori*. *Insect Biochem. Mol. Biol. 24*, 717–722.

Iman, F., Sutherland, D., Huang, W., Krasnow, M.A., **1999**. *stumps*, a *Drosophila* gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. *Genetics 152*, 307–318.

Isaac, D.D., Andrew, D.J., **1996**. Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product. *Genes Devel. 10*, 103–117.

Izaddoost, S., Nam, S.C., Bhat, M.A., Bellen, H.J., Choi, K.W., **2002**. *Drosophila Crumbs* is a positional cue in photoreceptor adherens junctions and rhabdomeres. *Nature 416*, 178–183.

James, A.A., **1994**. Molecular and biochemical analysis of the salivary glands of vector mosquitoes. *Bull. Inst. Pasteur 92*, 133–150.

Jaw, T.J., You, L.R., Knoepfler, P.S., Yao, L.C., Pai, C.Y., *et al.*, **2000**. Direct interaction of two homeoproteins, Homothorax and Extradenticle, is essential for Exd nuclear localization and function. *Mech. Devel. 91*, 279–291.

Jiang, C., Baehrecke, E.H., Thummel, C.S., **1997**. Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development 124*, 4673–4683.

Jiang, C., Lamblin, A.-F.J., Steller, H., Thummel, C.S., **2000**. A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. *Mol. Cell 5*, 445–455.

Jochova, J., Quaglino, D., Zakeri, Z., Woo, K., Sikorska, M., *et al.*, **1997**. Protein synthesis, DNA degradation, and morphological changes during programmed cell death in labial glands of *Manduca sexta*. *Devel. Genet. 21*, 249–257.

Jones, N.A., Kuo, Y.M., Sun, Y.H., Beckendorf, S.K., **1998**. The *Drosophila Pax* gene *eye gone* is required for embryonic salivary duct development. *Development 125*, 4163–4174.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H., *et al.*, **1999**. Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Mol. Cell 4*, 757–769.

Keller, R., **2002**. Shaping the vertebrate body plan by polarized embryonic cell movements. *Science 298*, 1950–1954.

Kerr, J.F., Wyllie, A.H., Currie, A.R., **1972**. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer 26*, 239–257.

Kidd, S., Baylies, M.K., Gasic, G.P., Young, M.W., **1989**. Structure and distribution of the Notch protein in developing *Drosophila*. *Genes Devel. 3*, 1113–1129.

Klämbt, C., Glazer, L., Shilo, B.-Z., **1992**. *Breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Devel. 6*, 1668–1678.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.

Kokubo, H., Ueno, K., Amanai, K., Suzuki, Y., **1997**. Involvement of the *Bombyx Scr* gene in development of the embryonic silk gland. *Devel. Biol. 186*, 46–57.

Kritzman, A., Gera, A., Raccah, B., van Lent, J.W., Peters, D., **2002**. The route of tomato spotted wilt virus inside the thrips body in relation to transmission efficiency. *Arch. Virol. 147*, 2143–2156.

Kuo, Y.M., Jones, N., Zhou, B., Panzer, S., Larson, V., *et al.*, **1996**. Salivary duct determination in *Drosophila*: roles of the EGF receptor signalling pathway and the transcription factors *fork head* and *trachealess*. *Development 122*, 1909–1917.

Lammel, U., Saumweber, H., **2000**. X-linked loci of *Drosophila melanogaster* causing defects in the morphology of the embryonic salivary glands. *Devel. Genes Evol. 210*, 525–535.

Lane, N.J., Carter, Y.R., Ashburner, M., **1972**. Puffs and salivary gland function: the fine structure of the larval and prepupal salivary glands of *Drosophila melanogaster*. *Roux's Archiv. Devel. Biol. 169*, 216–238.

Lavorgna, G., Karim, F.D., Thummel, C.S., Wu, C., **1993**. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl Acad. Sci. USA 90*, 3004–3008.

Lee, C.Y., Baehrecke, E.H., **2001**. Steroid regulation of autophagic programmed cell death during development. *Development 128*, 1443–1445.

Lee, C.Y., Clough, E.A., Yellon, P., Teslovich, T.M., Stephan, D.A., *et al.*, **2003**. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*. *Curr. Biol. 13*, 350–357.

Lee, C.Y., Cooksey, B.A.K., Baehrecke, E.H., **2002a**. Steroid regulation of midgut cell death during *Drosophila* development. *Devel. Biol. 250*, 101–111.

Lee, C.Y., Simon, C.A., Woodard, C.T., Baehrecke, E.H., **2002b**. Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Devel. Biol. 252*, 138–148.

Lee, C.Y., Wendel, D.P., Reid, P., Lam, G., Thummel, C.S., *et al.*, **2000**. E93 directs steroid-triggered programmed cell death in *Drosophila*. *Mol. Cell 6*, 433–443.

Lehmann, M., **1995**. *Drosophila* SGS genes: stage and tissue specificity of hormone responsiveness. *BioEssays 18*, 47–54.

Lehmann, M., Korge, G., **1996**. The *fork head* product directly specifies the tissue-specific hormone responsiveness of the *Drosophila Sgs-4* gene. *EMBO J. 15*, 4825–4834.

Mach, V., Ohno, K., Kokubo, H., Suzuki, Y., **1996**. The *Drosophila* Forkhead factor directly controls larval salivary gland-specific expression of the glue protein gene *Sgs3*. *Nucl. Acids Res. 24*, 2387–2394.

Matsunami, K., Kokubo, H., Ohno, K., Xu, P.-X., Ueno, K., *et al.*, **1999**. Embryonic silk gland development in *Bombyx*: molecular cloning and expression of the *Bombyx trachealess* gene. *Devel. Genes Evol. 209*, 507–514.

Matsura, S., Morimoto, T., Nagata, S., Tashiro, Y., **1968**. Studies on the posterior silk gland of the silkworm, *Bombyx mori*. 2. Cytolytic processes in posterior silk gland cells during metamorphosis from larva to pupa. *J. Cell Biol. 38*, 589–603.

Michelson, A.M., Gisselbrecht, S., Buff, E., Skeath, J.B., **1998**. Heartbroken is a specific downstream mediator of FGF receptor signaling in *Drosophila*. *Development 125*, 4379–4389.

Morisato, D., Anderson, K.V., **1995**. Signaling pathways that establish the dorsal–ventral pattern of the *Drosophila* embryo. *Annu. Rev. Genet. 29*, 371–399.

Mosley-Bishop, K.L., Li, Q., Patterson, L., Fischer, J.A., **1999**. Molecular analysis of the *klarsicht* gene and its role in nuclear migration within differentiating cells of the *Drosophila* eye. *Curr. Biol. 9*, 1211–1220.

Myat, M.M., Andrew, D.J., **2000a**. Organ shape in the *Drosophila* salivary gland is controlled by regulated, sequential internalization of the primordia. *Development 127*, 679–691.

Myat, M.M., Andrew, D.J., **2000b**. Fork head prevents apoptosis and promotes cell shape change during formation of the *Drosophila* salivary glands. *Development 127*, 4217–4226.

Myat, M.M., Andrew, D.J., **2002**. Epithelial tube morphology is determined by the polarized growth and delivery of apical membrane. *Cell 111*, 879–891.

Nellen, D., Affolter, M., Basler, K., **1994**. Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by Decapentaplegic. *Cell 78*, 225–237.

Ohiro, T., Saigo, K., **1997**. Transcriptional regulation of *breathless* FGF receptor gene by binding of TRACHEALESS/DARNT heterodimers to three central midline elements in *Drosophila* developing trachea. *Development 124*, 3975–3986.

Oro, A.E., McKeown, M., Evans, R.M., **1990**. Relationship between the product of the *Drosophila ultraspiracle* locus and vertebrate retinoid X receptor. *Nature 347*, 298–301.

Pai, C.Y., Kuo, T.S., Jaw, T.J., Kurant, E., Chen, C.T., *et al.*, **1998**. The homothorax homeoprotein activates the nuclear localization of another homeoprotein, Extradenticle, and suppresses eye development in *Drosophila*. *Genes Devel. 12*, 435–446.

Panzer, S., Weigel, D., Beckendorf, S.K., **1992**. Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes. *Development 114*, 49–57.

Pellikka, M., Tanentzapf, G., Pinto, M., Smith, C., McGlade, C.J., *et al.*, **2002**. *Crumbs*, the *Drosophila*

homologue of human CRB1/RP12, is essential for photoreceptor morphogenesis. *Nature 416*, 143–149.

Pimenta, P.F., Touray, M., Miller, L., **1994**. The journey of malaria sporozoites in the mosquito salivary gland. *J. Eukary. Microbiol. 41*, 608–624.

Raff, M.C., **1992**. Social controls on cell survival and cell death. *Nature 356*, 397–400.

Reichman-Fried, M., Dickson, B., Hafen, E., Shilo, B.-Z., **1994**. Elucidation of the role of *breathless*, a *Drosophila* FGF receptor homolog, in tracheal migration. *Genes Devel. 8*, 428–439.

Renault, N., King-Jones, K., Lehmann, M., **2001**. Downregulation of the tissue-specific transcription factor Fork head by Broad-Complex mediates a stage-specific hormone response. *Development 128*, 3729–3737.

Richards, G., **1976**. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster. 5*. The late prepupal puffs. *Devel. Biol. 54*, 264–275.

Riddiford, L.M., **1993**. Hormones and *Drosophila* development. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 899–940.

Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M., Mann, R.S., **1997**. Nuclear translocation of Extradenticle requires Homothorax, which encodes an Extradenticle-related homeodomain protein. *Cell 91*, 171–183.

Robertson, C.W., **1936**. The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. *J. Morphol. 59*, 351–399.

Roth, G.E., Wattler, S., Bornschein, H., Lehmann, M., Korge, G., **1999**. Structure and regulation of the salivary gland secretion protein gene *Sgs-1* of *Drosophila melanogaster. Genetics 153*, 753–762.

Ryoo, H.D., Mann, R.S., **1999**. The control of trunk Hox specificity and activity by Extradenticle. *Genes Devel. 13*, 1704–1716.

Ryoo, H.D., Marty, T., Casares, F., Affolter, M., Mann, R.S., **1999**. Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development 126*, 5137–5148.

Sauer, J.R., **1977**. Acarine salivary glands: physiological relationships. *J. Med. Entomol. 14*, 1–9.

Sauer, J.R., McSwain, J.L., Bowman, A.S., Essenberg, R.C., **1995**. Tick salivary gland physiology. *Annu. Rev. Entomol. 40*, 245–267.

Schwartz, L.M., Smith, S.W., Jones, M.E.E., Osborne, B.A., **1993**. Do all programmed cell deaths occur via apoptosis? *Proc. Natl Acad. Sci. USA 90*, 980–984.

Schweichel, J.-U., Merker, H.-J., **1973**. The morphology of various types of cell death in prenatal tissues. *Teratology 7*, 253–266.

Segraves, W.A., Hogness, D.A., **1990**. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Devel. 4*, 204–219.

Shiba, H., Uchida, D., Kobayashi, H., Natori, M., **2001**. Involvement of cathepsin B- and L-like proteinases in silk gland histolysis during metamorphosis of *Bombyx mori. Arch. Biochem. Biophys. 390*, 28–34.

Shilo, B.-Z., **2003**. Signaling by the *Drosophila* epidermal growth factor receptor pathway during development. *Exp. Cell Res. 284*, 140–149.

Shim, K., Blake, K.J., Jack, J., Krasnow, M.A., **2001**. The *Drosophila ribbon* gene encodes a nuclear BTB domain protein that promotes epithelial migration and morphogenesis. *Development 128*, 4923–4933.

Shishido, E., Higashijima, S., Emori, Y., Saigo, K., **1993**. Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development 124*, 2119–2128.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., *et al.*, **1997**. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian ARNT and controls CNS midline and tracheal development. *Development 124*, 4571–4582.

Tepass, U., Gruszynski-DeFeo, E., Haag, T.A., Omatyar, L., Torok, T., *et al.*, **1996**. *Shotgun* encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Devel. 10*, 672–685.

Tepass, U., Theres, C., Knust, E., **1990**. *crumbs* encodes an egf-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell 61*, 787–799.

Terashima, J., Yasuhara, N., Iwami, M., Sakurai, S., **2000**. Programmed cell death triggered by insect steroid hormone, 20-hydroxyecdysone, in the anterior silk gland of the silkworm, *Bombyx mori*. *Devel. Genes Evol. 210*, 545–558.

Thomas, U., Speicher, S.A., Knust, E., **1991**. The *Drosophila* gene *Serrate* encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. *Development 111*, 749–761.

Thummel, C.S., **1995**. From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. *Cell 83*, 871–877.

Torres-Vazquez, J., Park, S., Warrior, R., Arora, K., **2001**. The transcription factor Schnurri plays a dual role in mediating Dpp signaling during embryogenesis. *Development 128*, 1657–1670.

Truman, J.W., **1992**. The eclosion hormone system of insects. *Progr. Brain Res. 92*, 361–374.

Tsruya, R., Schlesinger, A., Reich, A., Gabay, L., Sapir, A., *et al.*, **2002**. Intracellular trafficking by star regulates cleavage of the *Drosophila* EGF receptor ligand spitz. *Genes Devel. 16*, 222–234.

Urban, S., Lee, J.R., Freeman, M., **2001**. *Drosophila* Rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell 107*, 173–182.

Welte, M.A., Gross, S.P., Postener, M., Block, S.M., Wieschaus, E., **1998**. Developmental regulation of

vesicle transport in *Drosophila* embryos: forces and kinetics. *Cell 92*, 547–557.

Wodarz, A., Hinz, U., Engelbert, M., Knust, E., **1995**. Expression of Crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell 82*, 67–76.

Woodard, C.T., Baehrecke, E.H., Thummel, C.S., **1994**. A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell 79*, 607–615.

Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M., Ashwell, J.D., **2000**. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science 288*, 874–877.

Zaffran, S., Kuchler, A., Lee, H.H., Frasch, M., **2001**. Biniou (foxf), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in *Drosophila*. *Genes Devel. 15*, 2900–2915.

Zakeri, Z., Quaglino, D., Latham, T., Lockshin, R.A., **1993**. Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB J. 7*, 470–478.

Zelzer, E., Wappner, P., Shilo, B.-Z., **1997**. The PAS domain confers target gene specificity of *Drosophila* bHLH/PAS proteins. *Genes Devel. 11*, 2079–2089.

Zhou, B., Bagri, A., Beckendorf, S.K., **2001**. Salivary gland determination in *Drosophila*: a salivary-specific, fork head enhancer integrates spatial pattern and allows fork head autoregulation. *Devel. Biol. 237*, 54–67.

Relevant Website

http://www.fruitfly.org – data on the genetics of *Drosophila*.

2.11 Silk Gland Development and Regulation of Silk Protein Genes

E Julien, M Coulon-Bublex, and A Garel,
CNRS-Université Claude Bernard, Lyon, France
C Royer and G Chavancy, INRA, Unité Nationale Séricicole, La Mulatière, France
J-C Prudhomme and P Couble, CNRS-Université Claude Bernard, Lyon, France

2.11.1. Introduction

With its many inherent merits, the silkworm *Bombyx mori* has been established as a domesticated insect exploited for sericulture and as a model system for basic research. The growing collection of the silkworm genetic stock worldwide – some 3000 genotypes – places *B. mori* second to *Drosophila* for genetic studies in insects (Nagaraju *et al.*, 2001). Amongst the biological processes investigated in *B. mori*, the study of the differentiation of the silk gland, the organ that synthesizes, secretes, and organizes the silk proteins into a thread, has attracted constant attention. The silk gland is very simply organized as a one-cell layered glandular epithelium that comprises two distinct types of secretory cells, the one that produces fibroin and the other that synthetizes sericins, the two components of silk. The high specialization of these cells provides an excellent opportunity to study the repertoire of regulatory factors that specify each of them.

Investigations on the regulation of silk protein genes have identified a series of transcription factors, which may also play key functions in the development of the silk gland. They have, moreover, suggested that chromatin-remodeling activities are very important in modifying the accessibility of the transcription factors to their target DNA sequences in the two categories of secretory cells. Thus, both qualitative and quantitative differences in regulatory gene expression probably induce the selective activation of the promoters of the different silk-encoding genes in the two categories of cells. One challenging problem that remains is to understand how these transcriptional differences are achieved during the course of development of the silk gland and how they are maintained during the impressive postembryonic growth of the organ. This review aims at summarizing our current knowledge on the development of the silk gland and the mode of production of silk proteins.

2.11.2. The Functioning of the Silk Gland

2.11.2.1. The Silk Gland Epithelium

The silk gland of *B. mori* is a tubular epithelium divided in three regions with precise cell-to-cell boundaries and a fixed number of cells (Figure 1). The posterior (PSG) and the middle (MSG) silk gland are secretory territories specialized for the massive production of the silk proteins. The three fibroin subunits, encoded by the heavy fibroin chain (*h-fib*), light fibroin (*l-fib*), and fibrohexamerin (*fhx*) (formerly referred to as *P25*) genes, are synthesized in the PSG cells whereas the sericins encoded

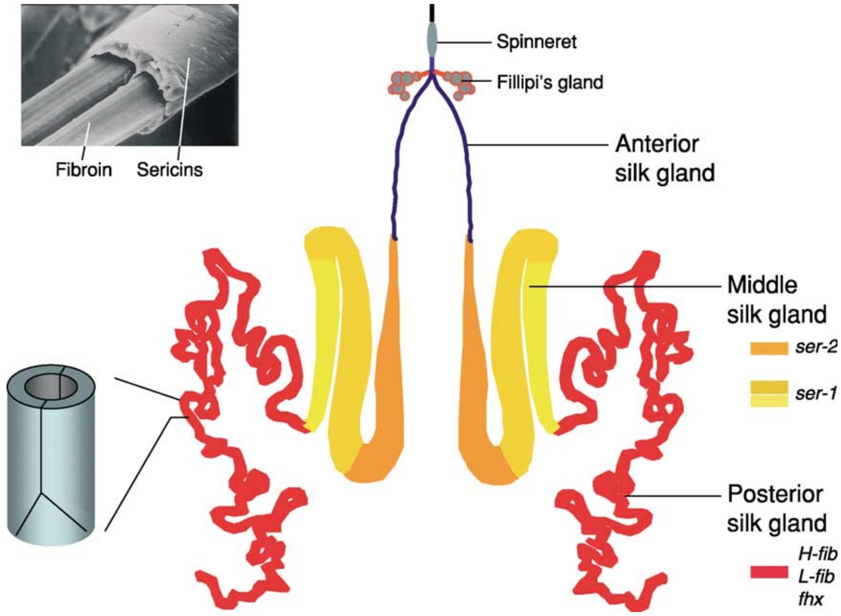


Figure 1 *Bombyx mori* silk gland organization. The silk protein encoding genes expressed in the different territories of the gland are shown by colors. The two middle silk gland (MSG) territories expressing *ser-1* correspond to two subpopulations of cells exerting distinct splicing modalities of the *ser-1* mRNA precursor (see Section 2.11.2.3). In the most anterior section, the paired ducts, to which the Fillipi's glands abduct, fuse into a single narrow canal at a short distance from the spinneret. The left-hand side shows the organization of the secretory epithelium with one layer of silk protein secreting cells surrounding a lumen and two cells facing each other. The upper left insert shows a scanning microscope picture of the two cables of insoluble fibroin spun out the right and left silk glands embedded in a matrix made of sericins. (Courtesy of Giuliano Freddi.)

by the genes *ser-1* and *ser-2* are produced in the MSG cells (Maekawa and Suzuki, 1980; Couble *et al.*, 1985, 1987; Kimura *et al.*, 1985; Horard *et al.*, 1997). Paired secretory parts are linked to the labial spinneret by long fine ducts, the anterior silk glands (ASG), to which the glands of Fillipi adduct and discharge their secretion (**Figure 1**).

Silk gland morphogenesis is completed in the embryo 24–48 h before hatching. Full differentiation is characterized by the transcription of all five silk protein-encoding genes in the embryo silk gland, and the synthesis of silk that is spun out by the newly hatched larvae (Maekawa and Suzuki, 1980; Ohta *et al.*, 1988; Matsunami *et al.*, 1998). Following differentiation, the growth of the silk gland is rhythmically controlled by the regular occurrence of endomitotic cycles during each of the five larval instars. The succession of 18 replication cycles in most of the PSG cells results in the presence of an estimated 400 000 haploid genomes per PSG cell at the end of the larval life. An additional replication cycle takes place in most of the MSG cells leading to twice the nuclear amount of DNA than observed in PSG cells (Perdrix-Gillot, 1979). The massive amplification of DNA by endomitotic cycles preserves the representation of each individual chromosome and locus (Gage and Manning, 1976).

The specializations of PSG and MSG cells remain unchanged during the spectacular growth of the organ suggesting that the relevant "guiding" information is restored after each round of DNA replication, likely through mechanisms that duplicate and maintain precisely the ordered structure of chromatin. Even though the ploidy level of fully grown silk gland cells is the highest ever reported for eukaryotic cells, silk gland development is reminiscent of many other insect tissues, whose growth is similarly associated with endomitoses.

The fifth and last intermolt is featured by a hypertrophy of the silk gland and by an increased rate of silk protein synthesis, prior to cocoon spinning, with associated cell ultrastructural changes related to the massive protein synthesis, transport, and secretion. After cocoon spinning, the silk gland initiates an apoptotic process that leads to its complete degeneration. This cell death program, mostly studied in the anterior silk gland, is likely under the action of 20-hydroxyecdysone (20E) and the BmEcR-A and -B1 isoforms of its receptor (Chinzei, 1975; Kamimura *et al.*, 1997; Terashima *et al.*, 2000).

Silk is mainly composed of fibroin and sericins, whose genes have been cloned and their transcriptional regulation studied, as described below. It also contains minor polypeptides referred to as seroins,

which do not contribute to the silk thread structure. Potent proteinase inhibitors are also present and it is speculated that their activity confers protection to the cocoon against proteolysis by microbe proteases (Grzelak *et al.*, 1988; Zurovec *et al.*, 1998b; Nirmala *et al.*, 2001; review: Fedic *et al.*, 2002). No investigation of the regulation of expression of seroin and proteinase inhibitor genes is yet available.

2.11.2.2. The Posterior Silk Gland Cells and Fibroin Production

PSG cells produce fibroin, the matter of the insoluble core of the silk thread. This complex protein is composed of three subunits: the heavy (H-fibroin) and the light fibroin chain (L-fibroin) which are linked through disulfide bonds, and fibrohexamerin (previously designated P25), which is associated with the H-fibroin/L-fibroin dimer through noncovalent bonds (Inoue *et al.*, 2000). The stoichiometry relationship accommodates a 6 : 6 : 1 relative ratio of the three subunits, as shown on the schematic representation of fibroin presented in **Figure 2**.

The 391 kDa H-fibroin subunit confers the remarkable physicochemical properties to the silk fiber. It is encoded by a 17 kb long gene, organized in one short and one very long exon separated by a 1 kb intron (Tsujimoto and Suzuki, 1979). The *h-fib* gene is located at one extremity of linkage group 25 (Doira, 1983). The complete sequencing of the gene (together with 60 kbp of the 5′ flanking domain) allowed the deduction of the sequence of the entire protein (Zhou *et al.*, 2000). H-fibroin is a 5263-residue polypeptide chain with high glycine content (46%) that exhibits a characteristic structure with 12 crystalline domains made of Gly-X repeats alternating with 11 identical 43-residue spacer sequences. The repetitive domains fold in canonical β-pleated sheet structures, and build up the protein crystal moiety of the silk thread. The 151 and 58 residues at the N- and C-termini are nonrepetitive. The C-terminal sequence carries three cysteine residues, the first one being implicated in a disulfide bond to L-fibroin, whereas the two others form an intramolecular bond (Tanaka *et al.*, 1999b) (**Figure 2**).

The *l-fib* gene is 13.5 kb long. It encompasses seven exons and maps onto linkage group 14 (Hyodo *et al.*, 1984; Kimura *et al.*, 1985). The very large first intron, which accounts for about 60% of the gene, contains multiple repeated sequences, some of them related to the *Bm1* and *BmX* transposable elements (Kikuchi *et al.*, 1992). The *l-fib* gene codes for a 25.8 kDa protein characterized by a relatively high incidence of leucine, isoleucine, valine, and acidic amino acids. It is linked to H-fibroin through a single disulfide bond that mobilizes cysteine 172, the two other cysteine residues forming an intra-chain bond (Tanaka *et al.*, 1999b) (**Figure 2**).

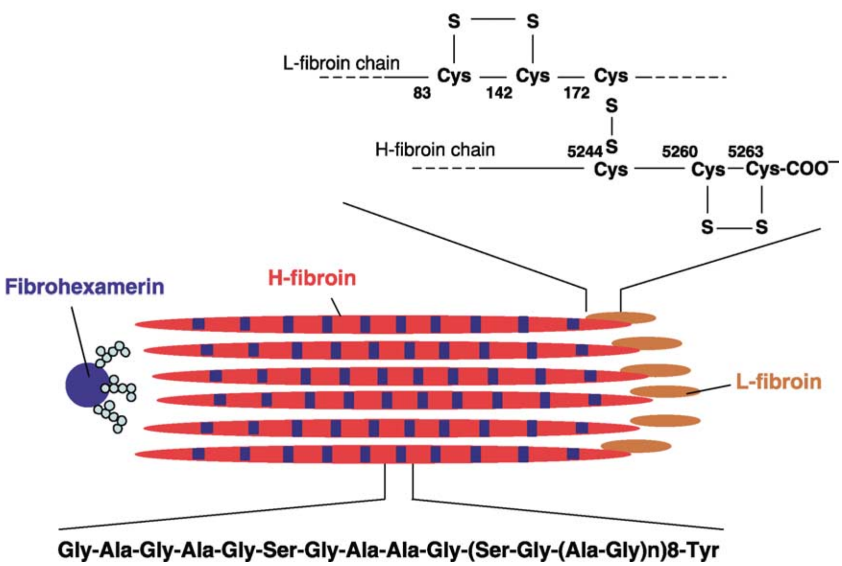


Figure 2 Molecular organization of fibroin. Schematic representation of the supramolecular organization of fibroin, the insoluble component of silk. The spatial arrangement of the six H-fibroin–L-fibroin dimers bond to one fibrohexamerin molecule is hypothetical and inferred from the data of Tanaka *et al.* (1999a, 1999b) and Inoue *et al.* (2000). H-fibroin is symbolized as a linear chain with the succession of 12 highly repeated regions (characterized by a typical glycine-rich motif) and the alternation of spacer sequences. The intra- and intermolecular disulfide bonds in the L-fibroin chain and in the C-terminus of the H-fibroin chain are shown in the upper portion of the figure. Fibrohexamerin hydrophobic interactions with H-fibroin is represented arbitrarily at the N-terminal region of the H-fibroin chains. Fibrohexamerin is displayed with its three *N*-linked oligosaccharide chains.

The L-chain and H-chain covalent bond is essential for the intracellular transport and secretion of the fibroin complex (Takei *et al.*, 1987). This was demonstrated by studying two *l-fib* mutant alleles (*Nd-s* and *Nd-s*^{D}) that display a “naked pupa” phenotype, where pupae are covered with a very thin and transparent cocoon. *Nd-s* mutants secrete only 0.3% of the normal-level fibroin producers and their silk consists mostly of sericins. In both the *Nd-s* and *Nd-s*^{D} strains, the L-fibroin and H-fibroin covalent linkage is impaired and the heavy chain is retained in the endoplasmic reticulum (Mori *et al.*, 1995); correlatively, the Golgi apparatus and fibroin secretory vesicles are poorly developed (Gamo and Sato, 1985). It is likely that the covalent bonding between H-fibroin and L-fibroin chains is required to free the membrane-anchored H-chain and allow its translocation to the Golgi vesicles. The blocking of fibroin secretion is accompanied by a severe reduction in the growth of the PSG, whereas the MSG develops normally (Takei *et al.*, 1987). This remarkable pleiotropic effect of the Nd-s and Nd-s^D mutations suggests a feed back between silk secretion and cell growth.

Fibrohexamerin is a 220 amino acid long peptide encoded by *fhx*, a 3.5 kb single-copy gene with five exons (Couble *et al.*, 1985; Chevillard *et al.*, 1986). It is linked to the H-fibroin–L-fibroin dimer through hydrophobic interactions with H-fibroin (Tanaka and Mizuno, 2001). Its function is viewed as an organizer of a supramolecular unit combining six H-fibroin–L-fibroin dimers and one molecule of fibrohexamerin. This would allow transport of the complex into the organelles and secretion of the H-fibroin chain into the gland lumen in a soluble conformation (Inoue *et al.*, 2000). *N*-linked oligosaccharides anchored at three asparagine residues of fibrohexamerin are likely to play a critical role in the interactions with H-fibroin (Tanaka *et al.*, 1999a). This role is probably ancestral, since a fibrohexamerin ortholog was identified in several lepidopteran species such as *Galleria mellonella* (Zurovec *et al.*, 1998a), *B. mandarina*, *Dendrolimus spectabilis,* and *Papilio xuthus* (Tanaka and Mizuno, 2001). By contrast, the moths of the Saturnidae secrete a fibroin molecule made by the dimerization of a H-fibroin-like protein and are apparently devoid of *l-fib* and *fhx*-like genes (Tanaka and Mizuno, 2001).

2.11.2.3. The Middle Silk Gland Cells and Sericin Production

The sericin fraction of the silk is composed of related peptides produced in MSG cells and characterized by their solubility in hot and alkaline water, a property that enables the industrial degumming of silk, and by their unusual composition, in which serine represents about one-third of all amino acids (Lucas and Rudall, 1968). Sericins serve to glue the silk threads together. Their diversity arises from frequent allelic polymorphisms of the two single-copy genes, *ser-1* and *ser-2*, and from the differential splicing of their respective precursor mRNA (Okamoto *et al.*, 1982; Michaille *et al.*, 1986, 1990a; Garel *et al.*, 1997).

The *ser-1* gene, which encodes the four major sericins of the silk thread, maps genetically on the *src-1* locus in linkage group 11 (Gamo, 1982). It is a 23 kb long gene with nine exons, from which four mature mRNA species are expressed, 10.5, 9.0, 4.0, and 2.8 kb long (Michaille *et al.*, 1986). The larger exon that spreads over 6 kb carries tandem arrays of a 114 bp sequence encoding a 38-residue long serine-rich motif.

The 12-kbp *ser-2* gene that contains four exons (Michaille *et al.*, 1990a, 1990b) is likely located at the *Src-2* locus in the same linkage group as *Src-1* (Gamo, 1982). Differential splicing of its pre-mRNA gives rise to two mature transcripts of 3.1 kb for one, and 5–6.4 kb, depending on the *ser-2* allele, for the other. Size variations are attributed to the occurrence of unequal crossovers within a region carrying multiple well-conserved 45 bp repeats.

The expression of *ser-1* and *ser-2* is not synchronous and varies both temporally and spatially during the last larval instar. Indeed, the MSG cells expressing *ser-1* are specialized for the synthesis of distinct mature mRNAs, depending on their topological location and on the stage of development (Michaille *et al.*, 1986; Couble *et al.*, 1987). The territory of expression of *ser-1* comprises the 150 most posterior MSG cells, contiguous to the PSG (**Figure 1**). This territory includes two subpopulations of cells: the 42 most posterior ones which are specialized in the expression of the shortest 2.8 kb *ser-1* mRNA during the whole instar, and the 108 more anterior ones (**Figure 1**) which realize the three other modalities of splicing in a sequential manner, leading to the successive accumulation of the 4.0, the 10.5, and the 9.0 kb mRNAs. In these last cells, the modalities of splicing of the *ser-1* primary transcript change twice during the last instar. The mechanisms that trigger these changes have not been deciphered, nor is it fully understood whether they relate to the two rounds of endoreplication that take place in MSG cells during the same period.

The expression of the other sericin gene, *ser-2*, is featured by the coaccumulation of the two corresponding mature mRNAs in about the same

proportion. In the early stages of the fifth larval instar, all MSG cells express *ser-2*, but the synthesis of the two mRNAs is progressively restricted to the 120 most anterior cells of the middle silk gland, which do not express *ser-1* (Couble *et al.*, 1987) (Figure 1).

The finely tuned regulation of expression of *ser-1* and *ser-2* leads to a characteristic deposition of the different sericins onto the fibroin stored in the lumen of the middle silk gland (Couble *et al.*, 1987), and likely provides a peculiar organization of the compacted silk proteins prior to spinning. A similar regulation of alternative splicing of the primary transcript of the two sericin genes was also observed in *G. mellonella* (Fedic *et al.*, 2002), suggesting that it may represent a common mechanism that contributes to the generation of diversity of sericins in the Lepidoptera.

2.11.3. Transcriptional Regulation of Silk Protein Genes

Many direct and indirect observations support the notion that transcription is the main step at which the expression of the five silk protein encoding genes, *h-fib*, *l-fib*, *fhx*, *ser-1*, and *ser-2* is controlled (Tsujimoto and Suzuki, 1979; Maekawa and Suzuki, 1980; Couble *et al.*, 1983; Couble *et al.*, 1985; Kimura *et al.*, 1985; Michaille *et al.*, 1989). As reviewed below, original experimental strategies, mostly dedicated to *h-fib, ser-1*, and *fhx*, were developed to identify the factors involved in PSG- and MSG-specific expression (Table 1). Efforts were made subsequently to progress on the central issue of chromatin organization and the control of access of the *trans*-regulatory factors to their target sequences.

2.11.3.1. Regulation of *h-fib*: Are Homeoproteins Involved?

Cell-free transcription assays employing silk gland nuclear extracts were used by Suzuki and coworkers to reproduce the PSG and MSG cell specificities of expression (Suzuki *et al.*, 1986; Matsuno *et al.*, 1989). Sequences immediately upstream of *h-fib*, located at −167 to −115 and at −73 to −44, were first shown to be capable of enhancing *in vitro* transcription. The enhancer effect was dependent upon silk gland-specific *trans*-acting factors, since it was observed only with PSG or MSG extracts but not with other systems derived from *B. mori* cultured cells or ovaries, *Antheraea* silk glands, or HeLa cells (Hirose *et al.*, 1985). Besides the enhancer sequences, a contribution of both the transcription initiation region around the TATA box and an intronic sequence in the regulation of the gene was also suggested (Takiya *et al.*, 1990; Takiya and Suzuki, 1993). The opposing transcriptional status of *h-fib* in PSG and MSG cells (where it is expressed and permanently repressed, respectively) could not, however, be reconstituted *in vitro*, despite many

Table 1 Characteristics of the trans-regulatory factors acting *in vitro* or *in vivo* on the different silk protein gene promoters in the posterior and the middle silk gland cells

Factors	*Gene promoter*	*Tissue incidence*	*Comments*	*Reference*
BmFA	*fhx*	Ubiquitous	Posttranslationally modified at the onset of each larval molt correlatively to *fhx* repression	Durand *et al.* (1992) Nony *et al.* (1995)
BmFKH (FMBP-2 or SGF-1 or SGFB)	*fhx h-fib ser-1*	MSG and PSG cells	*In vitro* (*ser-1* and *h-fib*) and *in vivo* (*fhx*) powerful activator; homologous to the *Drosophila* forkhead protein	Mach *et al.* (1995) Horard *et al.* (1997) Julien *et al.* (2002) Takiya *et al.* (1997)
FF1	*h-fib*	Ubiquitous	A 125 kDa putative activator that binds DNA *in vitro* with another factor FF2 to *h-fib* elements	Hui *et al.* (1990)
FMBP-1	*h-fib*	Abundant in PSG at feeding stages, and in ovarian tissues	Binds *in vitro* the upstream and intronic modulators of *h-fib.*	Takiya *et al.* (1997)
POU (Pit1/Oct-1/Unc-86)-M1 (FMBP-3 or SGF-3)	*h-fib ser-1*	Ubiquitous, but abundant in MSG cells	38 kDa factor of the POU type family of factor; possible *ser-1* activator (see Section 2.11.3.2). Binds *in vitro* to *h-fib* upstream and intron elements.	Fukuta *et al.* (1993) Takiya *et al.* (1997)
SGF-4	*h-fib ser-1*	Ubiquitous	Binds *in vitro* to homeodomain protein consensus sequences of the *h-fib* promoter	Hui *et al.* (1990)

refinements in the protocol and the use of fractionated PSG and MSG extracts (Suzuki *et al.*, 1990).

In vitro DNA-protein interactions using probes taken in the *h-fib* upstream domain (−214 to −66) and in the intron, led to the identification of putative regulatory factors, all featuring the capacity to bind very AT-rich sequence elements (Takiya *et al.*, 1990, 1997) (**Table 1**). A series of *in vitro* DNA–protein complexes were observed, among which SGF-1 (further named as BmFKH) was the first to be found to be specific for silk gland cells and to be present in both the PSG and the MSG (Suzuki and Suzuki, 1988; Hui *et al.*, 1990). The target sequences to which these complexes bind, contain frequent TTAATT or AATTAA motifs (see **Chapter 1.8**), like the core element of homeodomain binding sites, and it was hypothesized that proteins encoded by homeogenes likely activate *h-fib* (Hui and Suzuki, 1990, 1995). The observation that the *Drosophila* homeoproteins EVE and ZEN could bind *in vitro* at these same sites supported this hypothesis (Hui *et al.*, 1990), which, however, still awaits *in vivo* validation.

These studies suggested that *h-fib* expression is controlled by ubiquitous and silk gland-specific factors, that likely define both the tissue specificity and the intensity of transcription. However, they did not provide any specific explanations for the PSG restricted expression of *h-fib*. Thus, the question is still open as to whether this property simply rests upon the existence of PSG-specific factors or combination of factors, or from a specific structure of the gene in PSG cell chromatin, that is maintained by nonspecific chromatin remodeling proteins throughout larval development.

A study of the distribution of DNaseI hypersensitive sites within a 46 kb genomic region encompassing *h-fib* revealed the existence of several PSG-specific hypersensitive sites at 2 kbp and as far as 17 and 20 kbp upstream from the transcription start site (Waga and Mizuno, 1993). Interestingly, the nuclease sensitivity of these sites in PSG chromatin is greatly reduced at molting, when *h-fib* is temporarily repressed. The role of such potent regulatory complexes in specifying *h-fib* characteristics of expression remains to be established, but the presence of remote control elements may change our current view of how *h-fib* is activated in the PSG *in vivo*.

2.11.3.2. POU-M1 and the Control of the *ser-1* Promoter

The study of *ser-1* regulation was approached by *in vitro* transcription assays, but in contrast to *h-fib*, marked differences were noted when *ser-1* derived templates were introduced into MSG and PSG cell-free transcription reactions.

The MSG-preferred activation was dependent on the presence of an enhancer located between −239 and −173 (Hui and Suzuki, 1990; Matsuno *et al.*, 1990). Two elements contained in this DNA stretch were found to interact *in vitro* with distinct protein complexes.

The first element contained the target sequence for a POU-type transcription factor. The cloning of POU-M1 was achieved by screening a silk gland cDNA library with a mammalian POU cDNA probe (Fukuta *et al.*, 1993) (**Table 1**). The factor was found to be expressed in the two secretory regions of the silk gland during the fourth molt, but to be absent in the PSG and abundant in the MSG during the following intermolt. These experiments featured POU-M1 as the first isolated regulatory factor of the silk gland with a strict territory-specific incidence at the stage of intense *ser-1* transcription.

The promoter of the POU-M1 gene was also studied by *in vitro* transcription. Devoid of a TATA box, its 5′ DNA was found to carry positively and negatively acting sequences that likely restrict its expression in MSG cells *in vivo*. It also harbors a POU-M1 target sequence, the mutation of which led to an enhanced transcription *in vitro*, suggesting that POU-M1 expression is modulated by a negative autoregulatory control (Xu *et al.*, 1994).

The second sequence element, contained in the −239 and −173 upstream DNA of the *ser-1* gene, was found to be involved in the formation of the so-called SGF1 complex *in vitro*. The DNA-binding component was purified from MSG nuclei and characterized as BmFKH (**Table 1**), the *B. mori* homolog of the Forkhead protein of *Drosophila melanogaster* (Mach *et al.*, 1995). BmFKH is present in both PSG and MSG cells.

Thus, the experiments carried out by Suzuki's group led to the isolation of two factors that displayed distinctive properties. *In vitro*, the *ser-1* promoter studies tended to assign a role of positive regulators to both POU-M1 and BmFKH, and *ser-1* intense transcription was viewed for a while as the result of the combined action of these two factors (Mach *et al.*, 1995). However, as outlined below, this hypothesis is contradicted by the observation that POU-M1 accumulation follows an anteroposterior gradient in the different subparts of the MSG, which is opposite to that of *ser-1* expression. It is, therefore, likely that POU-M1 exerts a negative regulatory function on *ser-1*, and that such an effect has not been noted *in vitro* (Matsunami *et al.*, 1998). Thus, further experiments are needed to

define precisely the relationship between BmFKH and POU-M1 in the process of driving MSG-specific expression of *ser-1*.

2.11.3.3. Regulation of *fhx* Transcription

In vivo experiments were employed to identify the pertinent *cis*-regulatory sequences and their cognate *trans*-binding factors acting on the *fhx* promoter. The use of transgenic *D. melanogaster* as a possible heterologous system was tested first, assuming an evolutionary conservation of the silk gland transcriptional machinery with that of the fruit fly salivary gland (see **Chapter 2.10**). *Drosophila* transgenic lines carrying *fhx* promoter sequences fused to a reporter sequence were made. Remarkably, a transgene containing 1453 bp of *fhx* 5′ flanking DNA was found to be exclusively expressed in the anterior subpart of the larval salivary gland of the fly at all the three larval stages (Bello and Couble, 1990). The absence of expression of the transgene in other tissues strengthened the hypothesis that common-specific factors controlling the spatial and temporal activation of the *fhx* promoter were present in the larval labial gland of the fruit fly and the silkworm. Through sequential deletions, it was demonstrated that the 254 bp stretch of 5′ proximal DNA was necessary and sufficient for conferring the tissue specificity in *D. melanogaster* (Bello *et al.*, 1994). The selective expression of the *fhx-lacZ* transgene in the anterior salivary gland cells and the expression of *fhx* in the PSG cells raised the question of the precise homology between the different subparts of the two organs.

Efforts were made to set up a homologous *in vivo* functional assay in the silk gland cells themselves. This was achieved using a biolistic-aided method by which a silk gland is bombarded with DNA-coated tungsten particles and further grafted into a host larva. This protocol allowed measurements of the activity of transduced DNA constructs in a fully natural physiological environment (Horard *et al.*, 1994). With this assay, it was shown that, as in transgenic *D. melanogaster*, a very short domain of *fhx* upstream sequence is sufficient to drive intense PSG-specific expression of a *fhx-lacZ* fusion gene (Horard *et al.*, 1997). Interestingly, this short domain carries the sequence element TATTTATTTAAC to which BmFKH binds *in vitro*, suggesting that this factor could play a central role in *fhx* expression (Durand *et al.*, 1992; Nony *et al.*, 1995; Julien *et al.*, 2002). *In vivo* chromatin footprinting showed that the target site was occupied in PSG but not in MSG chromatin, and the use of anti-BmFKH antibodies did confirm that the DNA protection was dependent upon the factor (Julien *et al.*, 2002).

Thus, BmFKH (**Table 1**) appeared to be the main regulator of both *fhx* and *ser-1* in PSG and MSG cells, respectively, and this raised questions regarding the nature of the mechanisms that would constrain their spatial expression. It was hypothesized that either the status of the BmFKH factor itself or one of its partners would differ between the two categories of secretory cells, or that the access of BmFKH is limited by independent mechanisms acting on the chromatin organization of PSG or MSG expressed genes, the two propositions being not exclusive of each other.

The characterization of BmFKH in PSG and MSG cells, respectively, using partial proteinase cleavage of the factor complexed to a radioactive probe, and sequencing of the BmFKH mRNA did not reveal any differences for the factor in the two categories of cells (Julien *et al.*, 2002, and unpublished data). Attempts were also made to isolate BmFKH partner proteins by using the yeast two-hybrid technique, but this failed because all the tested BmFKH subpeptides were capable of directly transactivating yeast genes (Julien *et al.*, unpublished data), probably via interactions with general transcription factors including TBP itself, as was demonstrated for a few other factors of the wing-helix Forkhead family (Hellqvist *et al.*, 1998). Thus, the demonstration that BmFKH itself is capable of conferring specificity to *fhx* or *ser-1* promoter activity is still lacking.

The chromatin organization around the *fhx* gene was studied in both PSG and MSG cells taken at the fourth larval molt (when *fhx* is temporarily repressed in PSG cells) and at the fifth intermolt (when *fhx* is actively transcribed in PSG cells). DNAse-I partial digestion revealed the presence of two prominent PSG-specific hypersensitive sites. One of them, centered at −55 and including the BmFKH binding site, disappears when *fhx* is silenced at molting (Julien *et al.*, 2002) (**Figure 3**). The other is located at around +70 and is detected all along the fourth molt and the fifth larval instar. Contrary to that at −55, this site is independent of the transcriptional status of the gene (**Figure 3**). It is relevant to note that these two regions of *fhx* were present in the transgene construct active in *D. melanogaster*.

The significance of the +70 hypersensitive site remains to be established. It is suspected that its PSG-exclusive presence is linked to the mechanisms by which *fhx* chromatin structure is maintained in an active transcriptional configuration in PSG. Indeed, *fhx* activation involves dynamic changes of nucleosome structure during larval development (Julien *et al.*, 2002). While the *fhx* upstream DNA is characterized by a tightly compacted nucleosome

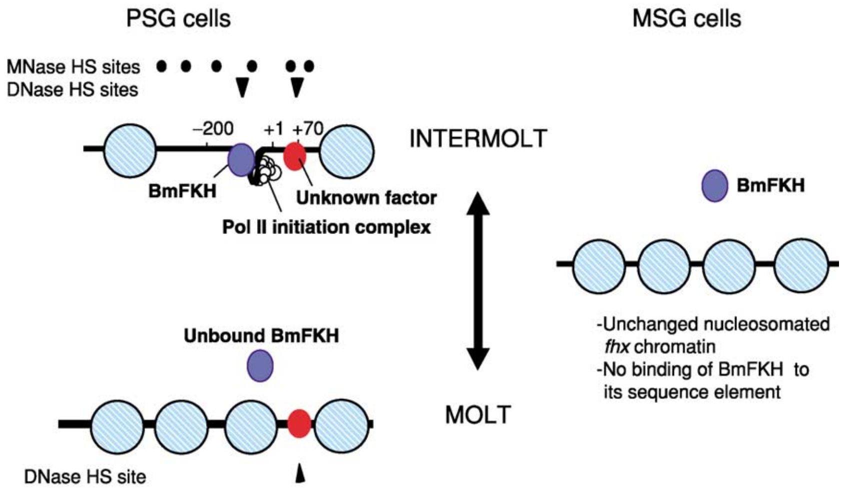


Figure 3 Functioning of the *fhx* promoter. As shown on the left, the *fhx* promoter in PSG cells is alternatively active and inactive during the larval intermolts and molts, respectively. The turning on of the gene is associated with the removal of nucleosomes in the upstream domain of the chromatin, which contains multiple MNase hypersensitive sites and two prominent DNaseI hypersensitives sites, centered at −55 and +70. This active state is also featured by the binding of BmFKH resulting in enhanced transcription. At the onset of molting, BmFKH is excluded from DNA but remains in the nucleus; correlatively, the upstream domain is rapidly nucleosomated and most of the MNase sites and the −55 DNaseI site disappear. The mechanism that prevents BmFKH from stably binding to its target sequence is likely triggered by the signal that promotes larval molts. The hypersensitive site at +70 is a permanent hallmark of *fhx* chromatin in PSG cells, and is thought to channel BmFKH toward its target sequence at the next intermolt. It is suggested that this organization of chromatin governs PSG-specific *fhx* expression. (Data from Horard, B., Julien, E., Nony, P., Garel, A., Couble, P. **1997**. Differential binding of the *Bombyx mori* silk gland specific factor SGFB to its target sequence drives posterior cell restricted expression. *Mol. Cell. Biol. 17*, 1572–1579; and Julien, E., Bordeaux, M.C., Garel, A., Couble, P., **2002**. Fork head alternative binding drives stage-specific expression in the silk gland of *Bombyx mori*. *Insect Biochem. Mol. Biol. 32*, 377–387.)

array in all silk gland cells at molting, the activation of the promoter in the PSG cells during intermolt is accompanied by the loss of nucleosome patterning and an enhancement of its accessibility to micrococcal nuclease (Julien *et al.*, 2002) (**Figure 3**). Thus, changes in the structure of chromatin in the differentiated silk gland cells are probably important for conferring the time and tissue specificity of silk protein gene expression. It is not known how these chromatin changes are orchestrated, but it is tempting to imagine that the establishment of such distinct structures in PSG and MSG cells occurs during the early silk gland organogenesis.

2.11.4. Embryonic Development of the Silk Gland

The embryogenesis of the silkworm lasts 10–12 days at 25 °C. Takami and Kitazawa (1960) recognized 30 distinct morphological stages, to which we refer in the following description of silk gland embryonic development. Pictures of the growing silk gland together with a schematic representation of its morphogenesis are shown in **Figures 4 and 5**, respectively.

The silk glands arise, at stages 18–19, from the recruitment of ectoderm cells in two lateral ventral placodes in the posterior part of the labial segment (parasegment 2) (**Figure 4a and b**). At stage 19, the tip of the presumptive silk gland invaginates from the primordium and becomes visible in the anterior first thoracic segment. As embryogenesis proceeds, the anteroposterior growth of the gland tube follows the lateroventral side of the embryo and, at the time of germ band retraction (stage 20), reaches the mesoderm of the abdominal segment 1. During germ band shortening (stages 21A and 21B), the convex ventral side of the embryo adopts a concave shape (**Figure 4c**). Simultaneously, the silk gland elongates and, at mid-course of abdominal segment 2, its extremity progresses dorsally behind the tracheal pit and grows under the visceral mesoderm toward the dorsal side of the embryo. After reaching a dorsolateral position, the extremity of the gland emerges from the visceral mesoderm and then progresses dorsally toward the posterior direction (**Figure 4c**). During dorsal closure of the embryo (stages 22–23), the future silk gland reaches the most posterior extent of its growth, the tracheal pit of the abdominal segment 6.

During the growth of the silk gland, proliferating cells were identified by 5-bromo-2-deoxyuridine (BrdU) incorporation. Mitoses are detected in only the six to eight subapical cells in contact with the blind-ended tubular extension, from stages 18 to 22, until the organ reaches the abdominal segment 6

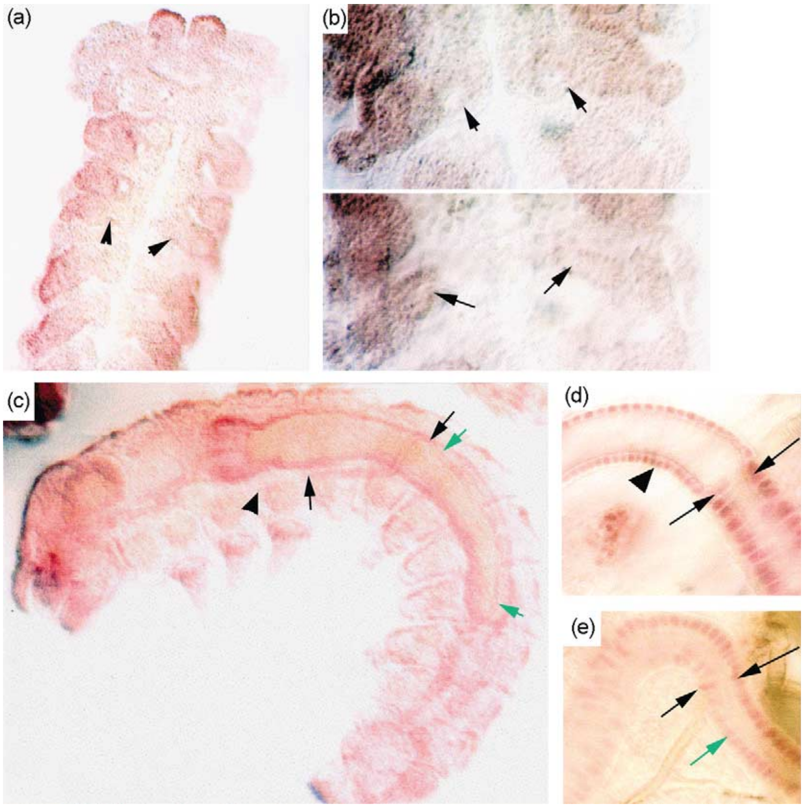


Figure 4 *Bombyx mori* silk gland embryonic development. (a) and (b) are ventral views of embryos at stage 19. In (a) arrows point to the silk gland primordia in the labial segment, enlarged in (b) to show two focal planes of the labial and anterior thoracic segments with the silk glands invaginating from the medial part of the posterior labial segment (arrows). (c) Display of an embryo at stage 24, with the silk gland extending from the labial segment to the dorsolateral abdominal segment 6; the arrowhead points to the future anterior silk gland (ASG); the future middle silk gland (MSG) stretches between the two black arrows and the growing future posterior silk gland (PSG) between the two green arrows. (d–e): Higher magnifications of the junctions between the future ASG and MSG (d) and between the middle and posterior silk glands (e) at stage 24.

(Figure 5). The mitotic activity of these few cells, the mitotic spindles of which run parallel to the long axis of the tube, was also confirmed by observation of Feulgen-stained silk glands. In this regard, silk gland tubulogenesis is comparable to that of the *D. melanogaster* Malpighian tubules (**Chapter 2.8**), which also originate from ectodermic primordia and whose cell proliferation also involves the presence of a single nondividing cell at the distal tip of the growing tubule (Skaer, 1989; Skaer and Martinez-Arias, 1992; see **Chapter 2.8**).

The successive differentiation of the silk gland in three territories by sequential anteroposterior differentiation of the future anterior, median, and posterior cells, respectively, suggests that changes in gene expression occur when and where the transition occurs between the future anterior to median, and median to posterior boundaries. During germ band extension and shortening and during dorsal closure, the interactions between the silk gland tip cells and the other tissues are modified. In particular, the close contacts with visceral mesoderm cells at stage 20 are likely involved in determining the future boundaries between specialized silk gland territories.

Following complete extension of the silk gland at stages 21C–22, a sudden and specific burst of cell divisions takes place in the future MSG at late stage 22, resulting in its extension in the posterior region of the MSG and its forward folding, within PS7 boundaries, as schematized in Figure 5. At this stage, the ASG, MSG, and PSG cells already exhibit distinctive features: the ASG cells are thin and border a large lumen, whereas the MSG cells are large with long nuclei and the PSG cells are more compact with round nuclei (Figure 4d and e). The ASG–MSG and MSG–PSG boundaries are easily visible by microscopic observation.

From late stage 22 onwards, S-phases are not followed by cell divisions, thus fixing the transition from diploidy to polyploidy between stage 23 and late 24. The first burst of DNA endoreplication affects all three territories, but in an asynchronous

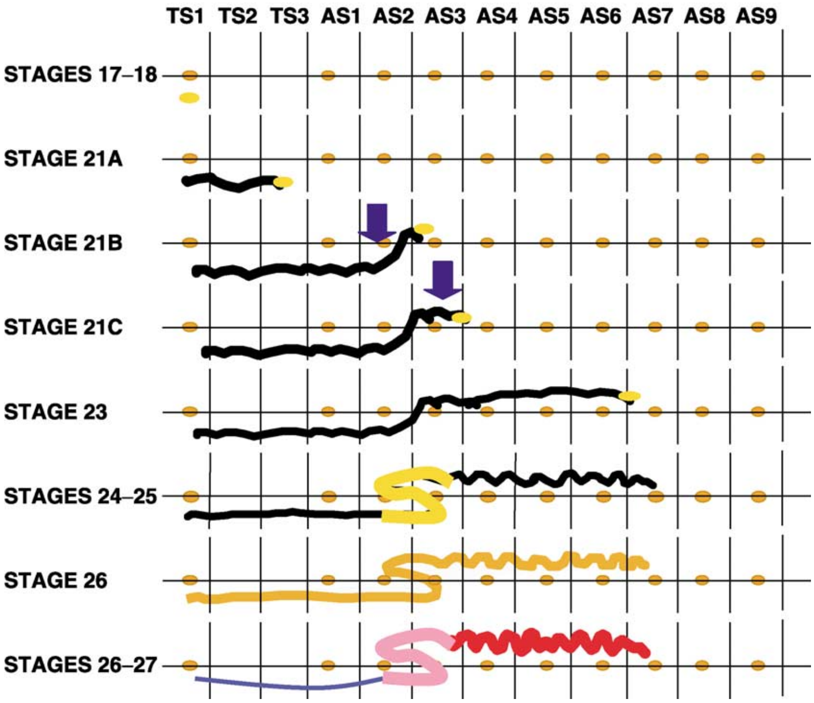


Figure 5 Sequence of morphogenesis of the silk gland during the embryonic development. The chronology of embryogenesis on the left-hand side refers to the 30 morphological stages established by Takami and Kitazawa (1960). At the top of the figure, the anteroposterior axis of the embryo is marked with the limits of the three thoracic (TS1 to TS3) and the nine abdominal (AS1 to AS9) segments. The growing future silk gland is represented as a dark line with a gray patch at the posterior tip symbolizing the six to eight subapical cells of the tube that retain the capacity to divide (see text). At stages 24–25, the cells of the future middle silk gland (MSG) undergo a round of mitoses as symbolized by the yellow line. The initiation of the first round of endomitoses (dark orange) occurs then at stage 26 in the whole silk gland. At late stage 26 (26–27), the silk gland is fully functional and the MSG and PSG cells express the genes that characterize their terminal differentiation, *ser-1* and *ser-2* for the MSG cells (in pink), and *h-fib, l-fib,* and *fhx* for the PSG cells (in red). The two blue arrows signal the stage and place at which the transitions occur between the ASG and the MSG, and between the MSG and the PSG.

manner: MSG cells are the first concerned, then PSG cells and, finally, ASG cells which replicate their chromosomes just before the embryonic molt. At this stage, the number of cells of the silk gland is fixed and is approximately 250, 200, and 500 in the ASG, MSG, and PSG territories, respectively.

2.11.4.1. Comparative Development of the *B. mori* Silk Gland and the *D. melanogaster* Salivary Gland

Genes that likely play a role in silk gland development in *B. mori* have been identified mostly by *in situ* hybridization and histochemistry studies (see also **Chapter 2.10**). Since no mutants are yet available, the involvement of these genes is, most often, inferred from the role of their cognate homologs in *D. melanogaster*, usually with reference to the development of the salivary gland (Andrew *et al.*, 2000; Panzer *et al.*, 1992), the fruit fly homolog of the silk gland.

The silkworm silk gland and the fruit fly salivary gland (see also **Chapter 2.10**) are thought to represent homologous organs, because both originate from lateral ventral placodes of the labial ectoderm. They also differentiate as tubular epithelia with one layer of polyploid secretory cells. Moreover, they exert similar functions of synthesis, secretion and export of a large amount of proteins of repeated structure: the glue proteins encoded by the *sgs* genes in the fruit fly and the silk proteins in the silkworm. The glue proteins link the puparium onto the substratum and silk constitutes the cocoon, a mechanical protection to the pupa. A functional illustration of the homology between the two tissues is the exclusive expression of a *fhx-lacZ* transgene in the salivary gland of transgenic *D. melanogaster*, which strongly suggests that the major elements of the transcription regulating machinery have been conserved through the 250 million years of divergence between the two species (Bello and Couble, 1990). Interestingly, the activity of the *fhx-lacZ* promoter closely mimics that of *fhx* in *B. mori*, by occurring at all larval instars in the fruit fly, in contrast to *sgs* gene expression, which is restricted to

only the last larval instar. The difference probably lies in the hormonal stimulation of transcription of the glue protein encoding genes, which are direct targets of the ecdysone receptor (Lehmann and Korge, 1996). In contrast, *fhx* and the other silk protein-encoding genes are expressed at all larval molts but are not directly controlled by ecdysone.

Despite common traits, the organogenesis of the *D. melanogaster* salivary gland does not follow the same pattern as that of the silk gland. The salivary gland ectodermal placode contains the final number of cells of the tissue prior to invagination, in contrast with the silk gland primordium, which is made of cells that actively divide during subsequent tubulogenesis (Andrew *et al.*, 2000). Moreover, the salivary gland duct, which is made of cuboidal cells, and the secretory part that consists of columnar cells, arise from distinct regions of the placode and are separately organized, whereas in the silk gland, the whole placode gives rise to both the duct and the secretory regions.

2.11.4.2. Genes Involved in Silk Gland and Salivary Gland Development

Efforts have been made to unravel the contribution of various genes at the early phases of silk gland development (see also **Chapter 2.10**). Kokubo *et al.* (1997a) first observed that the mRNA and the protein encoded by the *B. mori* gene *Sex combs reduced* (*BmScr*) accumulate at stage 19 in the labial segment, where ectoderm cells are committed to form the silk gland. They suggested that, similarly to the action of *Scr* in the *D. melanogaster* salivary gland (Andrew *et al.*, 2000), the homeotic *BmSrc* selector gene is a positive regulator of silk gland fates in the silkworm. This hypothesis was supported by the analysis of *Nc* homeotic mutants that lack a portion of the *Antennapedia* gene (*BmAntp*) including the DNA coding for the homeodomain and the C-terminal portion of the protein. In these mutants, *BmScr* expression expands into regions normally controlled by the wild-type *BmAntp* (Nagata *et al.*, 1996). Such ectopic expression of *BmScr* in the thoracic region induces the invagination of additional pairs of silk glands in the three thoracic segments. Thus, it is likely that the cascade of gene control initiated by *Antp* and *Scr* in the *D. melanogaster* salivary gland also occurs in the *B. mori* silk gland.

The embryonic expression of *Bmfkh* (also called *SGF-1* or *SGFB*), the *B. mori* homolog of *forkhead* (*fkh*), was also investigated, since the internalization of the salivary gland primordia and the formation of the secretory tubes are blocked in *D. melanogaster fkh* mutants. In the silkworm, *Bmfkh* mRNA is detected in the invaginating silk gland, and the protein is present at stages 23–25 in the almost fully differentiated secretory cells (MSG and PSG) but not in the ASG ones (Kokubo *et al.*, 1996). *Bmfkh* is, thus, one of the earliest genes expressed in the secretory cells, as is *fkh* in the *D. melanogaster* salivary gland. The functional dependence of *Bmfkh* upon *BmSrc* is suggested by its early expression in the invaginating ectopic silk glands in *Nc* mutants (Kokubo *et al.*, 1997b). However, it is not known whether *BmSrc* activation of *BmFkh* is direct, as is suggested in *D. melanogaster* (Andrew *et al.*, 2000), or indirect.

The protein BmFKH is a wing helix factor that plays crucial roles in the transcriptional regulation of the silk protein encoding genes. As described above (see Sections 2.11.3.2 and 2.11.3.3), BmFKH transactivates the sericin-1 and the fibrohexamerin genes, as shown by *in vitro* (Mach *et al.*, 1995) and *in vivo* (Julien *et al.*, 2002) studies, respectively. Thus, the same factor likely plays a dual role in silk gland development, being first required at the early stages of primordium invagination, tubulogenesis and establishment of secretory cell fates and, after full differentiation is reached, as a key factor in the transcriptional activation of silk protein genes. The role of BmFKH at the early steps of silk gland development has not been studied yet, but may be enlightened by the accurate dissection of the function of *D. melanogaster* Forkhead, which plays three distinct and successive roles in the salivary gland: (1) it prevents apoptosis of salivary gland precursor cells prior to internalization of the placode, (2) it promotes the cell shape changes that trigger the invagination of the future salivary gland, and (3) it directly activates the glue protein encoding genes *sgs-1*, *sgs-3*, and *sgs-4* (Lehmann and Korge, 1996; Mach *et al.*, 1996; Lehmann *et al.*, 1997; Roth *et al.*, 1999; Myat and Andrew, 2000).

Different vertebrate Forkhead-like factors have been shown to exert chromatin-modifying activities, and may be well suited to bind target genes at early development. It is possible that the binding of such factors in precursor cells mediates developmental competence, whereby selected genes are physically discriminated, thus permitting the subsequent steps of differentiation to occur efficiently (review: Zaret, 1999). The presumed conserved dual role of FKH and BmFKH suggests that glue and silk protein genes may gain the competence, at the early stages of development, to become activated after the completion of organogenesis.

Another gene expressed early in *D. melanogaster* salivary gland formation is *trachealess* (*Trh*), which is required for duct cell differentiation (Isaac and

Andrew, 1996). *Trh* encodes a basic loop–helix PAS (Per, ARNT, Sim) protein that functions as a transcription factor (Zelzer *et al*., 1997). *Trh* is initially expressed in the entire salivary gland primordia, but becomes repressed in secretory cells in a *fkh*-dependent manner, suggesting that proper expression of *fkh* and *Trh* is indeed crucial for the distinction between duct and secretory cell fates in the salivary gland (Andrew *et al*., 2000). Interestingly, the study of *B. mori Trh* (*BmTrh*) showed that it is expressed in the labial segment, where the silk gland invaginates. Its expression is maintained in the growing silk gland, but becomes restricted to the future anterior silk gland at stage 23, while *Bmfkh* expression is constrained to the secretory parts (Matsunami *et al*., 1999). Thus, the same restriction of the expression of *forkhead* and *trachealess* in specific territories of the silk and salivary glands emphasizes the conservation of genetic cascades that operate during the development of the two organs.

The expression of the *ser-1* gene has been detected from stage 26 with an increasing anteroposterior gradient pattern in the MSG (Matsunami *et al*., 1998). A *ser-1* transcriptional regulator known as SGF-3/POU-M1 was detected from stage 20 in the whole silk gland. Interestingly, its expression rapidly vanishes in PSG and, from stage 26 onwards, it is detected only in the ASG and MSG cells. In MSG cells, SGF-3/POU-M1 exhibits a decreasing anteroposterior gradient pattern suggesting that it acts as a negative regulator of *ser-1* transcription (Matsunami *et al*., 1998). It is present in many organs of the embryo and its contribution to the early differentiation of the silk gland remains to be deciphered.

Finally, a few other genes have been isolated from the silkworm, whose expression also occurs at the early stages of silk gland organogenesis and in specific subregions of the organ. The study of the *wnt-1* (*wingless*) and *Ci* (*Cubitus interruptus*) homologs revealed that these are coexpressed in the anterior portion of the MSG at late embryogenesis and throughout larval development (Dhawan and Gopinathan, 2002). Interestingly, the turning on of these genes in the anterior portion of the MSG is functionally relevant, because this region is occupied by the *ser-2* expressing cells. However, the mechanisms that spatially constrain *wnt-1* and *Ci* expressions and the relationship between activities of these two factors and *ser-2* transcription regulation are not known. Part of the answer may lie in the exclusive expression of *engrailed*, a potent suppressor of *Ci*, which is precisely expressed in the middle and posterior portion of the MSG (K.P. Gopinathan, personal communication).

2.11.5. Conclusions and Future Prospects

The long standing study of *B. mori* silk gland embryonic development and the mode of gene regulation in the MSG and PSG cells has provided a wealth of information. Two factors, BmFKH and POU-M1, have been identified and isolated, which participate in the region-specific transcription of silk protein encoding genes. These two factors were also found to be expressed at early stages of silk gland morphogenesis, before MSG and PSG cells differentiate into sericin- and fibroin-secreting cells. This raises the question of the dual roles played by these factors before and after cell differentiation during silk gland development. Such a plural function has been assigned to HNF3, a factor belonging to the Forkhead family, and to a GATA protein, for the formation of tissues from the gut endoderm (Zaret, 1999). Their role is to promote a developmental competence to target genes, such as the albumin gene in the hepatic primordium, by inducing a specific, chromatin structure that discriminates them from the nonexpressed genes.

Most of the hypotheses on how transcription is controlled in the differentiated silk gland stipulate the existence of MSG- or PSG-specific factors. However, it should be noted that, to date, this simple view has not been demonstrated, either for activators or for repressors. At the same time, the study of *fhx* regulation has revealed that PSG-specific expression depends on a PSG-specific chromatin organization of the gene. This strongly suggests that the differential accessibility of the *trans*-acting factors to their target sequences may play the central role in building up the silk gland phenotype.

Complete understanding of silk protein gene regulation will most probably be brought about by investigating the *in vivo* mechanisms that allow the embryonic silk gland cells to become committed to MSG or PSG cell fates. It can be hypothesized that the acquisition of a competence to become a MSG or a PSG cell rests upon the potentiation of the different silk protein-encoding genes by a combination of factors that are present in the immature MSG or PSG cells. Analysis of the embryonic development of the silk gland showed that BmFKH and POU-M1 are early factors that discriminate the future ASG portion from the future MSG and PSG secretory parts and the PSG from the MSG parts, respectively. Owing to its key role in activating *ser-1* and *fhx*, it can be proposed that BmFKH may be a master element in the process of potentiating silk protein encoding genes, which all have a BmFKH binding element in their immediate upstream DNA.

BmFKH alone, however, cannot determine the fate of MSG and PSG cells and likely interacts with other factors that have yet to be identified. POU-M1 may be viewed as playing such a role.

The study of the role of chromatin organization of silk protein genes will benefit from the powerful *in vivo* experimentation using transgenic silkworms (Tamura *et al*., 2000). Indeed, it is now possible to correlate the acquisition of proper chromatin structure and transcriptional competence with the presence of pertinent *cis*-acting sequences carried by modified transgenes.

The differentiation of the three subparts of the embryonic silk gland probably involves unknown genes that do not directly regulate silk protein gene expression. Their isolation and characterization will be facilitated by the sequencing of the silkworm genome and by new high-throughput procedures of transcriptome and proteome analyses. These post-genomic approaches will provide new data on the contribution of specific genes to the determination and differentiation of the silk gland.

Which of the developmental mechanisms are conserved between or are unique in the *B. mori* silk gland and the *D. melanogaster* salivary gland? Although silkworm mutants for all the genes under study are not available, the creation of phenocopies by RNA interference is feasible, as is the testing of the capacity of *B. mori* genes to correct defects in relevant *D. melanogaster* mutants. These approaches will open a new era of research, aimed at the understanding of the integration of functions in secretory cells, for which the silk gland remains one of the most remarkable models.

Acknowledgments

Research in our laboratory was partly supported by grants from the Indo-French CEFIPRA/IFCPAR. Collaborative research with Japanese scientists was supported by a CNRS-MAFF cooperation (PICS 907).

References

Andrew, D.J., Henderson, K.D., Seshaiah, P., **2000**. Salivary gland development in *Drosophila melanogaster. Mech. Devel. 92*, 5–17.

Bello, B., Couble, P., **1990**. Specific expression of a silk encoding gene of *Bombyx mori* in the anterior salivary gland of *Drosophila melanogaster. Nature 346*, 480–482.

Bello, B., Horard, B., Couble, P., **1994**. The selective expression of silk-protein-encoding genes in *Bombyx mori* silk gland. *Bull. Inst. Pasteur 92*, 81–100.

Chevillard, M., Couble, P., Prudhomme, J.C., **1986**. Complete nucleotide sequence of the gene encoding the *B. mori* silk protein P25 and predicted amino acid sequence of the protein. *Nucl. Acids Res. 14*, 6341–6342.

Chinzei, Y., **1975**. Induction of histolysis by ecdysterone *in vitro*: breakdown of anterior silk gland in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool. 10*, 136–138.

Couble, P., Chevillard, M., Moine, A., Ravel Chapuis, P., Prudhomme, J.C., **1985**. Structural organization of the P25 gene of *Bombyx mori* and comparative analysis of its flanking DNA with that of the fibroin gene. *Nucl. Acids Res. 13*, 1801–1810.

Couble, P., Michaille, J.J., Garel, A., Couble, M.L., Prudhomme, J.C., **1987**. Developmental switches of sericin mRNA splicing in individual cells of *Bombyx mori* silk gland. *Devel. Biol. 124*, 431–440.

Couble, P., Moine, A., Garel, A., Prudhomme, J.C., **1983**. Developmental variations of a non-fibroin mRNA of *Bombyx mori* silk gland encoding a low-molecular weight silk protein. *Devel. Biol. 97*, 398–407.

Dhawan, S., Gopinathan, K.P., **2002**. Molecular cloning and expression pattern of a *Cubitus interruptus* homologue from the mulberry silkworm *Bombyx mori. Mech. Devel. 118*, 203–207.

Doira, H., **1983**. Linkage maps of *B. mori*: status quo in 1983. *Sericologia 23*, 245–256.

Durand, B., Drevet, J., Couble, P., **1992**. P25 gene regulation in *Bombyx mori* silk gland: two promoter binding factors have distinct tissue and development-specificities. *Mol. Cell. Biol. 12*, 5768–5777.

Fedic, R., Zurovec, M., Sehnal, F., **2002**. The silk of Lepidoptera. *J. Insect Biotechnol. Sericol. 71*, 1–15.

Fukuta, M., Matsuno, K., Hui, C.C., Nagata, T., Takiya, S., *et al*., **1993**. Molecular cloning of a POU domain-containing factor involved in the regulation of the *Bombyx mori* sericin-1 gene. *J. Biol. Chem. 268*, 19471–19475.

Gage, L.P., Manning, R.F., **1976**. Determination of the multiplicity of the silk fibroin gene and detection of fibroin gene-related DNA in the genome of *Bombyx mori. J. Mol. Biol. 101*, 327–348.

Gamo, T., **1982**. Genetic variants of the *Bombyx mori* silkworm encoding sericin proteins of different lengths. *Biochem. Genet. 20*, 165–177.

Gamo, T., Sato, S., **1985**. Ultrastructural study of the posterior silk gland in the *Nd*, *Nd-s* and *Nd-s*^{D} mutants with a defect of fibroin synthesis. *J. Sericult. Sci. Japan 54*, 412–419.

Garel, A., Deleage, G., Prudhomme, J.C., **1997**. Structure and organization of the *Bombyx mori* Sericin 1 gene and of the sericins 1 deduced from the sequence of the Ser1B cDNA. *Insect Biochem. Mol. Biol. 27*, 469–477.

Grzelak, K., Couble, P., Garel, A., Kludkiewicz, B., Al Rouz, H., **1988**. Low molecular weight silk proteins in *Galleria mellonella*. *Insect Biochem. 18*, 223–228.

Hellqvist, M., Mahlapuu, M., Blixt, A., Enerback, S., Carlsson, P., **1998**. The human forkhead protein

FREAC-2 contains two functionally redundant activation domains and interacts with TBP and TFIIB. *J. Biol. Chem. 273*, 23335–23343.

Hirose, S., Tsuda, M., Suzuki, Y., **1985**. Enhanced transcription of fibroin gene *in vitro* on covalently closed circular templates. *J. Biol. Chem. 260*, 10557–10562.

Horard, B., Julien, E., Nony, P., Garel, A., Couble, P., **1997**. Differential binding of the *Bombyx mori* silk gland specific factor SGFB to its target sequence drives posterior cell restricted expression. *Mol. Cell. Biol. 17*, 1572–1579.

Horard, B., Mangé, A., Pélissier, B., Couble, P., **1994**. *Bombyx mori* gene promoter analysis in transplanted silk gland transformed by particle delivery system. *Insect Mol. Biol. 3*, 261–265.

Hui, C.-C., Suzuki, Y., **1990**. Homeodomain binding sites in the promoter region of three silk protein genes. *Devel. Growth Different. 32*, 263–273.

Hui, C.-C., Suzuki, Y., **1995**. Regulation of the silk protein genes and the homeobox genes in silk gland development. In: Goldsmith, M.R., Wilkins, A.S. (Eds.), Molecular Model Systems in the Lepidoptera. Cambridge University Press, New York, pp. 249–271.

Hui, C.-C., Suzuki, Y., Kikuchi, Y., Mizuno, S., **1990**. Homeodomain binding sites in the 5′ flanking region of the *Bombyx mori* silk fibroin light-chain gene. *J. Mol. Biol. 213*, 395–398.

Hyodo, A., Yamamoto, T., Ueda, H., Takei, F., Kimura, K., *et al.*, **1984**. Linkage analysis of the fibroin light chain gene in the silkworm, *Bombyx mori*. *Jap. J. Genet. 59*, 285–296.

Inoue, S., Tanaka, T., Arisaka, A., Kimura, S., Ohtomo, K., *et al.*, **2000**. Silk fibroin of *Bombyx mori* is secreted, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio. *J. Biol. Chem. 275*, 40517–40528.

Isaac, D.D, Andrew, D.J., **1996**. Tubulogenesis in *D. melanogaster*: a requirement for the *trachealess* gene product. *Genes Devel. 10*, 103–117.

Julien, E., Bordeaux, M.C., Garel, A., Couble, P., **2002**. Fork head alternative binding drives stage-specific expression in the silk gland of *Bombyx mori*. *Insect Biochem. Mol. Biol. 32*, 377–387.

Kamimura, M., Tomita, S., Kiuchi, M., Fujiwara, H., **1997**. Tissue-specific and stage-specific expression of two silkworm ecdysone receptor isoforms: ecdysteroid-dependent transcription in cultured anterior silk glands. *Eur. J. Biochem. 248*, 86–93.

Kikuchi, Y., Mori, K., Suzuki, S., Yamaguchi, K., Mizuno, S., **1992**. Structure of the *Bombyx mori* fibroin light-chain-encoding gene: upstream sequence elements common to the light and heavy chain. *Gene 110*, 151–158.

Kimura, K., Oyama, F., Ueda, H., Mizuno, S., Shimura, K., **1985**. Molecular cloning of the fibroin light chain complementary DNA and its use in the study of the expression of the light chain gene in the posterior silk gland of *Bombyx mori*. *Experientia 41*, 1167–1171.

Kokubo, H., Takiya, S., Mach, V., Suzuki, Y., **1996**. Spatial and temporal expression pattern of *Bombyx mori* fork head/SGF-1 gene in embryogenesis. *Devel. Genes Evol. 206*, 80–85.

Kokubo, H., Ueno, K., Amanai, K., Suzuki, Y., **1997a**. Involvement of the *Bombyx mori Scr* gene in development of the embryonic silk gland. *Devel. Biol. 186*, 46–57.

Kokubo, H., Xu, P.X., Xu, X., Matsunami, K., Suzuki, Y., **1997b**. Spatial and temporal expression pattern of POU-M1/SGF3 in *Bombyx mori* embryogenesis. *Devel. Genes Evol. 206*, 494–502.

Lehmann, M., Korge, G., **1996**. The fork head product directly specifies the tissue-specific hormone responsiveness of the *D. melanogaster Sgs-4* gene. *EMBO J. 18*, 4825–4834.

Lehmann, M., Wattler, S., Korge, G., **1997**. Two new regulatory elements controlling the *D. melanogaster Sgs-3* gene are potential ecdysone receptor and fork head binding site. *Mech. Devel. 62*, 15–27.

Lucas, F., Rudall, K.M., **1968**. Extracellular fibrous proteins: The silks. In: Florkin, M., Stota, E.H. (Eds.), Comprehensive Biochemistry, vol. 26B. Elsevier, Amsterdam, pp. 475–558.

Mach, V., Ohno, H., Kokubo, H., Suzuki, Y., **1996**. The *Drosophila melanogaster* fork head factor directly controls larval salivary gland-specific expression of the glue protein Sgs-3. *Nucl. Acids Res. 24*, 2387–2394.

Mach, V., Takiya, S., Ohno, K., Handa, H., Imai, T., *et al.*, **1995**. Silk gland factor-1 involved in the regulation of *Bombyx mori* sericin-1 gene contains fork head motif. *J. Biol. Chem. 270*, 9340–9346.

Maekawa, H., Suzuki, Y., **1980**. Repeated turn-off and turn-on of fibroin gene transcription during silk gland development of *Bombyx mori*. *Devel. Biol. 78*, 394–406.

Matsunami, K., Kokubo, H., Ohno, K., Suzuki, Y., **1998**. Expression pattern analysis of the SGF-3/POU-M1 in relation to the sericin-1 gene expression in the silk gland. *Devel. Growth Different. 40*, 591–597.

Matsunami, K., Kokubo, H., Ohno, K., Xu, P.X., Ueno, K., *et al.*, **1999**. Embryonic silk gland development in *Bombyx mori:* molecular cloning and expression of the *Bombyx mori trachealess* gene. *Devel. Genes Evol. 209*, 507–514.

Matsuno, K., Hui, C.C., Takiya, S., Suzuki, T., Ueno, K., *et al.*, **1989**. Transcription signals and protein binding sites for sericin gene transcription *in vitro*. *J. Biol. Chem. 264*, 18707–18713.

Matsuno, K., Takiya, S., Hui, C.C., Suzuki, T., Fukuta, M., *et al.*, **1990**. Transcriptional stimulation via SC site of *Bombyx* sericin-1 gene through an interaction with a DNA binding protein SGF3. *Nucl. Acids Res. 18*, 1853–1858.

Michaille, J.J., Couble, P., Prudhomme, J.C., Garel, A., **1986**. A single gene produces multiple sericin mRNAs in the silk gland of *Bombyx mori*. *Biochimie 68*, 1165–1173.

Michaille, J.J., Garel, A., Prudhomme, J.C., **1989**. The expression of five middle silk gland specific genes is transcriptionally regulated during the larval development of *B. mori*. *Insect Biochem. 19*, 19–27.

Michaille, J.J., Garel, A., Prudhomme, J.C., **1990a**. Cloning and characterization of the highly polymorphic *ser-2* gene of *Bombyx mori*. *Gene 86*, 177–184.

Michaille, J.J., Garel, A., Prudhomme, J.C., **1990b**. Expression of *ser-1* and *ser-2* genes in the middle silk gland of *Bombyx mori* during the fifth instar. *Sericologia 30*, 49–60.

Mori, K., Tanaka, K., Kikuchi, Y., Waga, M., Waga, S., *et al.*, **1995**. Production of a chimeric fibroin light-chain polypeptide in a fibroin secretion-deficient naked pupa mutant of the silkworm *B. mori*. *J. Mol. Biol. 251*, 217–228.

Myat, M., Andrew, D.J., **2000**. Fork head prevents apoptosis and promotes cell shape change during formation of the *D. melanogaster* salivary glands. *Development 127*, 4217–4226.

Nagaraju, J., Klimenko, V., Couble, P., **2001**. The silkworm *Bombyx mori*, a model genetic system. In: Reeves, E. (Ed.), Encyclopedia of Genetics. Fitzroy Dearborn, London, pp. 219–239.

Nagata, T., Suzuki, Y., Ueno, K., Kokubo, H., Xu, X., *et al.*, **1996**. Developmental expression of the *Bombyx mori antennapedia* homologue and homeotic changes in the *Nc* mutant. *Genes Cells 1*, 555–568.

Nirmala, X., Kodric, D., Zurovec, M., Sehnal, F., **2001**. Insect silk contains both a Kunitz-type and a unique Kazal-type proteinase inhibitor. *Eur. J. Biochem. 268*, 2064–2073.

Nony, P., Prudhomme, J.C., Couble, P., **1995**. Regulation of the P25 gene transcription in the silk gland of *Bombyx mori*. *Biol. Cell 84*, 43–52.

Ohta, S., Suzuki, Y., Hara, W., Takiya, S., Suzuki, T., **1988**. Fibroin gene transcription in the embryonic stages of the silkworm, *Bombyx mori*. *Devel. Growth Different. 30*, 293–299.

Okamoto, H., Ishikawa, E., Suzuki, Y., **1982**. Structural analysis of sericin genes: homologies with fibroin gene in the 5′ flanking nucleotide sequences. *J. Biol. Chem. 257*, 15192–15199.

Panzer, S., Weigel, D., Beckendorf, S.K., **1992**. Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes. *Development 114*, 49–57.

Perdrix-Gillot, S., **1979**. DNA synthesis and endomitoses in the giant nuclei of the silk gland of *Bombyx mori*. *Biochimie 61*, 171–204.

Roth, G.E., Wattler, S., Bornschein, H., Lehmann, M., Korge, G., **1999**. Structure and regulation of the salivary gland secretion protein gene *Sgs-1* of *Drosophila melanogaster*. *Genetics 53*, 753–762.

Skaer, H., **1989**. Cell division in Malpighian tubule development in *Drosophila melanogaster* is regulated by a single tip cell. *Nature 342*, 566–569.

Skaer, H., Martinez-Arias, A., **1992**. The *wingless* product is required for cell proliferation in the Malpighian tubules of the *Drosophila melanogaster*. *Development 116*, 745–754.

Suzuki, Y., Obara, T., Takiya, S., Hui, C.-C., Matsuno, K., *et al.*, **1990**. Differential transcription of the fibroin and sericin-1 genes in cell free extracts. *Devel. Growth Different. 32*, 179–187.

Suzuki, T., Suzuki, Y., **1988**. Interaction of composite protein complex with the fibroin enhancer sequence. *J. Biol. Chem. 263*, 5979–5986.

Suzuki, Y., Tsuda, M., Takiya, S., Hirose, S., Suzuki, E., *et al.*, **1986**. Tissue-specific transcription enhancement of the fibroin gene characterized by cell-free systems. *Proc. Natl Acad. Sci. USA 83*, 9522–9526.

Takami, S., Kitazawa, T., **1960**. Normal stages of the embryonic development in the silkworm, *Bombyx mori*. *Tech. Bull. Sericult. Exp. Station 75*, 1–31.

Takei, F., Kikuchi, A., Mizuno, S., Shimura, K., **1987**. Further evidence for importance of the subunit combination of silk fibroin in its efficient secretion from the posterior silk gland cells. *J. Cell. Biol. 105*, 175–180.

Takiya, S., Hui, C-C., Suzuki, Y., **1990**. A contribution of the core promoter and its surrounding regions to the preferential transcription of the fibroin gene in posterior silk gland extracts. *EMBO J. 9*, 489–496.

Takiya, S., Kokubo, H., Suzuki, Y., **1997**. Transcriptional regulatory elements in the upstream and intron of the fibroin gene bind three specific factors POU-M1, BmFkh and FMBP-1. *Biochem. J. 321*, 645–653.

Takiya, S., Suzuki, Y., **1993**. Role of the core promoter for the preferential transcription of fibroin gene in the posterior silk gland extract. *Devel. Growth Different. 35*, 311–321.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., *et al.*, **2000**. Germ-line transformation in the silkworm, *Bombyx mori* L using a *piggyBac* transposon-derived vector. *Nature Biotechnol. 18*, 81–84.

Tanaka, K., Inoue, S., Mizuno, S., **1999a**. Hydrophobic interaction of P25, containing Asn-linked oligosaccharide chains, with the H–L complex of silk fibroin produced by *Bombyx mori*. *Insect Biochem. Mol. Biol. 29*, 269–276.

Tanaka, K., Kajiyama, N., Ishikura, K., Waga, S., Kikuchi, A., *et al.*, **1999b**. Determination of the site of disulfide linkage between heavy and light chains of silk fibroin produced by *Bombyx mori*. *Biochim. Biophys. Acta 1432*, 92–103.

Tanaka, K., Mizuno, S., **2001**. Homologues of fibroin L-chain and P25 of *Bombyx mori* are present in *Dendrolimus spectabilis* and *Papilio xuthus* but not detectable in *Antherea yamamai*. *Insect Biochem. Mol. Biol. 31*, 665–677.

Terashima, J., Yasuhara, N., Iwami, M., Sakurai, S., **2000**. Programmed cell death triggered by insect steroid hormone, 20-hydroxyecdysone, in the anterior silk gland of the silkworm, *Bombyx mori*. *Devel. Genes Evol. 210*, 545–558.

Tsujimoto, Y., Suzuki, Y., **1979**. The DNA sequence of *Bombyx mori* fibroin gene including the 5′ flanking,

mRNA coding, entire intervening and fibroin coding regions. *Cell 18*, 591–600.

Waga, S., Mizuno, S., **1993**. Different behavior of chromatin domains encompassing fibroin heavy-chain gene in active, temporarily inactive, and permanently inactive transcriptional states in silk gland nuclei. *J. Biol. Chem. 268*, 6429–6436.

Xu, P. X., Fukuta, M., Takiya, S., Matsuno, K., Xu, P. X., *et al.*, **1994**. Promoter of the POU-M1/SGF-3 gene involved in the expression of the *Bombyx mori* silk genes. *J. Biol. Chem. 269*, 2733–2742.

Zaret, K.S., **1999**. Regulatory phases of early liver development: paradigms of organogenesis. *Nature Rev. Genet. 3*, 499–512.

Zelzer, E., Wappner, P., Shilo, B.Z., **1997**. The PAS domain confers target gene specificity of *Drosophila* bHLH/PAS proteins. *Genes Devel. 11*, 2079–2089.

Zhou, C.Z., Confalonieri, F., Medina, N., Zivanovic, Y., Esnault, C., *et al.*, **2000**. Fine organization of *Bombyx mori* fibroin heavy chain. *Nucl. Acids Res. 28*, 2413–2419.

Zurovec, M., Kodrik, D., Yang, C., Sehnal, F., Scheller, K., **1998a**. The P25 component of *Galleria* silk. *Mol. Gen. Genet. 257*, 264–270.

Zurovec, M., Yang, C., Kodrik, D., Sehnal, F., **1998b**. Identification of a novel type of silk protein and regulation of its expression. *J. Biol. Chem. 273*, 15423–15428.

3.1 Neuroendocrine Regulation of Insect Ecdysis

D Zitnan, Slovak Academy of Sciences, Bratislava, Slovakia
M E Adams, University of California, Riverside, CA, USA

3.1.1. Introduction

Insects are the dominant terrestrial life forms on earth. Their impressive evolutionary success is in no small measure a consequence of unique developmental and reproductive strategies, which have facilitated efficient resource exploitation and radiation into a wide range of ecological niches (Truman and Riddiford, 2002). Developmental strategies are most remarkable in the holometabolous insects where, as vermiform larvae, they are essentially mobile digestive systems, optimized for a wide range of food resources. The complete morphological transformation from vermiform larva to flying adult (metamorphosis) in many ways represents the birth of a new organism within the same life cycle, and enables the same species to minimize intraspecific

competition by occupying separate ecological niches. The remarkable ability of insects to change form during the life cycle, to enter dormancy at various stages of development, and to adapt to environmental changes via polyphenism, is made possible by the processes of molting and ecdysis.

Each stage of insect development is characterized by an alternation between feeding and molting, the latter culminating in ecdysis. During this intricate process, insects shed the old cuticle surrounding the external surface, and the lining of the foregut, hindgut, and tracheal tubes of the respiratory system. While ecdysis is the gateway to the next developmental stage, it also is effectively a life-threatening process, since failure to execute the sequence on schedule can result in death of the organism. Its success depends on coordinated gene expression controlled by ecdysteroids and juvenile hormones responsible for the formation of new cuticle and other tissues appropriate for the next stage. Ecdysteroids also participate in assembly of a peptide signaling cascade that regulates the ecdysis sequence at the end of the molt.

3.1.1.1. The Molt Cycle

Upon hatching from the egg, growth through successive immature stages culminates in the winged, reproductive adult, exquisitely adapted for dispersal and proliferation of new young. At the beginning of each larval instar, the animal feeds and grows until it acquires the proper size and nutritional state. The feeding stage or "intermolt" proceeds until integrative processes in the brain produce a decision to move on to the next stage, whereupon production and release of ecdysone occurs. The appearance of elevated ecdysteroid levels brings an end to the intermolt and onset of the molt associated with production of new cuticle (Figure 1). At the end of the last larval instar, holometabolous insects empty their guts and engage in wandering behavior aimed at finding an optimal location for pupation. Metamorphosis is initiated in pharate pupae (pupal development) and continues after pupation (adult development). This very complex process involves remodeling or programmed cell death of larval organs (see **Chapter 2.5**), and the proliferation of new cells and tissues specific for pupal and adult stages. In spite of these dramatic changes, it seems likely that basic mechanisms controlling larval, pupal, and adult ecdyses may be essentially similar or only slightly modified.

The critical endocrinological signal that switches the animal from feeding to molting is the elevation of ecdysteroids, including ecdysone, 20-hydroxyecdysone (20E), and other hydroxylated analogs of ecdysone (see **Chapter 3.3**), depending on the insect. Epidermal cells respond by changing shape and expressing a set of genes that prepare the animal for passage to the next stage. An early manifestation of this process is detachment of the epidermal cell layer from the existing cuticle, or apolysis, whereupon synthesis of a new cuticle layer appropriate for the next stage begins. Secretion of enzymatic molting fluid by the epidermal cells dissolves inner layers of the old cuticle so that its unsclerotized chitin and protein components can be recovered and recycled

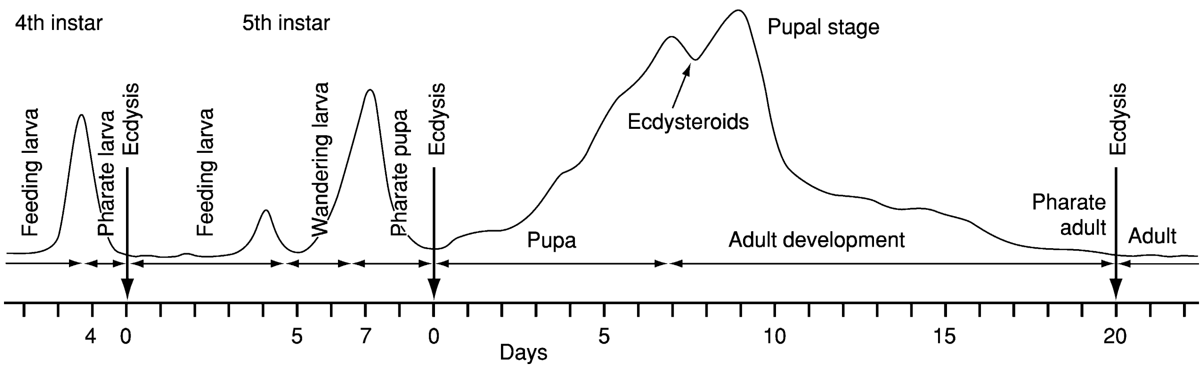


Figure 1 Ecdysteroid levels in hemolymph during development of *Manduca*. Ecdysteroid levels are low during feeding stages, but their levels increase about 30 h before ecdysis, larvae of the 4th instar stop feeding and develop a new cuticle (pharate 5th instar larvae). After ecdysteroid levels decline, pharate larvae ecdyse to 5th instar (day 0) and resume feeding on days 1–4. A small ecdysteroid peak on day 4 or 5 induces wandering stage (larvae stop feeding and move to a suitable pupation site). Large ecdysteroid peak induces pupal development on days 7–9 (pharate pupae) and after ecdysteroids decrease, animals pupate (day 0). Subsequent elevated ecdysteroid levels control adult development. (Normalized ecdysteroid levels are adapted from Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23* (3), 523–535 (4th instar larvae), Zitnanova, I., Adams, M.E., Zitnan, D., **2001**. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta. J. Exp. Biol. 204* (Pt 20), 3483–3495 (5th instar larvae); and Bollenbacher, W.E., Smith, S.L., Goodman, W., Gilbert, L.I., **1981**. Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta. Gen. Comp. Endocrinol. 44* (3), 302–306 (pupal stage).)

into new cuticle or other tissues. Similarly, the old cuticular lining of the tracheal tubes detaches from underlying epidermal cells and the gap between these layers is filled with molting fluid. This fluid is later reabsorbed from the old head capsule and space between the old and new cuticles, while the new tracheal tubes become inflated with air.

Apolysis begins the "pharate" stage, defined as the incipient new animal covered by its old shell. Thus, a pharate 5th instar larva has already produced a new cuticle, but is still wearing its old coat from 4th instar. A pharate pupa is a developing pupa still encased in its old larval cuticle. A pharate adult has developed an adult phenotype, but is still covered by its old pupal cuticle.

As described in this review, the rise and fall of ecdysteroid levels induce sequential bouts of gene expression, resulting in the production of peptides and their receptors, peptide processing enzymes, and signal transduction proteins. These events ensure the activation and proper timing of processes that underly the upcoming ecdysis sequence, which involves air swallowing, tracheal dynamics, and a series of behaviors that culminates in cuticle shedding. The order of these events and their precise timing are determined by a peptide and neurotransmitter signaling cascade.

The molt cycle can be considered according to the following sequential steps:

1. Feeding and growth, which begins shortly after ecdysis of the previous stage.
2. Initiation of the molt induced by increased ecdysteroid levels in concert with juvenile hormones (see **Chapter 3.7**) to regulate gene expression important for development of the next stage phenotype, including the formation of new cuticle and tissues and synthesis of regulatory molecules.
3. The ecdysis sequence regulated by a decline in steroid levels and secretion of peptide hormones.
4. Termination of the molt during postecdysial processes characterized by expansion, sclerotization, and melanization of the new cuticle (see **Chapters 4.2**, **4.3**, and **4.4**) important for a definitive shape of the new developmental stage.

3.1.1.2. Regulation of the Ecdysis Sequence by a Peptide Signaling Cascade

An endocrine basis for ecdysis was demonstrated over 30 years ago through a series of classic brain transplantation experiments in giant silkmoths (Truman and Riddiford, 1970). These animals display "gated" circadian eclosion rhythms, meaning that adults may eclose only during a certain time of day when the gate is "open." Once the gate is closed, adult ecdysis (eclosion) is delayed until the next day. Eclosion gating was abolished by surgical removal of the brain, but could be re-established by implantation of the loose brain into the body cavity of the animal. Even more astounding, swapping brains between two species of moths that eclose at different times of day led to recipient moths following the eclosion schedule of the donor brain. This elegantly demonstrated two things: the existence of a timing device in the brain and the fact that the brain (see **Chapters 3.11** and **4.11**) acts as an endocrine organ by secreting a hormonal factor(s) that initiates eclosion. These experiments were reminiscent of the historic Kopec experiments, where transplantation of brains in moths was used to demonstrate an endocrine basis for the molt (Kopec, 1917, 1922). Eventually, a 62 amino acid peptide was identified as eclosion hormone (EH), which induces eclosion upon injection into pharate adults (Truman, 1992). Implicit in this work is the assumption that EH both initiates ecdysis or eclosion and is responsible for the gated nature of this behavior.

Our understanding of the complexity of signaling underlying ecdysis has grown considerably in recent years. That EH is not the sole initiator of ecdysis was demonstrated through discovery of the epitracheal endocrine system and Inka cells, which secrete pre-ecdysis triggering hormone (PETH) and ecdysis triggering hormone (ETH) into the hemolymph (Zitnan *et al.*, 1996; Ewer *et al.*, 1997; Kingan *et al.*, 1997a; Zitnan *et al.*, 1999). PETH and ETH act directly on the central nerrous system (CNS) to activate specific neuronal circuits for pre-ecdysis and ecdysis (Adams and Zitnan, 1997; Zitnan *et al.*, 1999; Zitnan and Adams, 2000; Zitnan *et al.*, 2002). It is likely that PETH and ETH trigger release of numerous peptides within the CNS, including EH, crustacean cardioactive peptide (CCAP), and others to coordinate the sequence of pre-ecdysis, ecdysis, and postecdysis behaviors (Ewer *et al.*, 1997; Gammie and Truman, 1999; Zitnan and Adams, 2000). Recent evidence indicates that additional neuropeptides including corazonin, FLRFamides, and myoinhibitory peptides (MIPs) also are involved in orchestration of the ecdysis sequence (Miao *et al.*, 1998; Davis *et al.*, 2003; Kim *et al.*, 2004).

Publication of the *Drosophila* genome (Adams *et al.*, 2000) has opened a new era of opportunity for elucidation of the peptide cascade controlling ecdysis. Further studies focusing on identification of target cells and organs producing G protein-coupled receptors (GPCRs) (see **Chapter 5.5**) for ecdysis

peptides and understanding of signal transduction induced by ligand–receptor binding will provide new insights into activation of the ecdysis sequence.

3.1.1.3. Chapter Overview

In this chapter, the current knowledge of the neuroendocrine basis for ecdysis, focusing on cellular and chemical signaling mechanisms that control gene expression prior to its initiation, and its timing and proper execution is reviewed. With regard to ecdysteroid regulated gene expression, the focus is on developmental events that create the peptidergic signaling framework underlying activation and execution of the ecdysis sequence. Regarding the ecdysis sequence itself, emphasis is given to peptide hormones and neuromodulators, which coordinate steps leading to shedding of the cuticle, including swallowing behaviors, tracheal dynamics, and pre-ecdysis, ecdysis, and postecdysis behaviors. Ultimately, a truly mechanistic analysis of ecdysis will depend on understanding its molecular basis. Because emphasis is placed on the neuroendocrine basis of ecdysis, many details of ecdysis in different stages and orders of insects are not covered in depth. Most of the discussion is centered around the moths *Manduca* and *Bombyx*, and the fruit fly *Drosophila*. Where possible, immunohistochemical demonstration of homologous cellular substrates for ecdysis in different insect phyla is presented, since such information may help to guide future research in these groups. For aspects of ecdysis not covered in this chapter, the reader is referred to a recent review on ecdysis (Ewer and Reynolds, 2002).

Section 3.1.2 characterizes the peptides implicated in ecdysis control, and the cells producing them. PETH, ETH, EH, and CCAP already are strongly implicated as causal signals in ecdysis, and the authors anticipate future progress in the field by summarizing what is known about peptides recently implicated in ecdysis regulation, including MIPs, corazonin, FLRFamides and ion transport peptides (ITPs). Only in the past few years have receptors for ecdysis peptides been discovered, made possible through the publication of organismal genomes, principally *Drosophila* and human. Section 3.1.3 therefore summarizes recent discoveries of G protein-coupled receptors for ecdysis peptides. Section 3.1.4 examines the role of gene expression in creating the signaling system underlying ecdysis, and how sequential gene expression ensures the proper timing of ecdysis initiation. Section 3.1.5 describes how a combination of factors contribute to ETH release from Inka cells, and Section 3.1.6 examines the peripheral and central factors involved in orchestration of pre-ecdysis and ecdysis motor programs. Section 3.1.7 presents a mechanistic model for endocrine control of the ecdysis sequence and summarizing statements.

3.1.2. Peptidergic Signals in Ecdysis

3.1.2.1. Introduction

During the past 10 years, the number of peptides implicated in ecdysis control has increased considerably. EH was the first peptide identified as an ecdysis initiator, and it is now thought to function both as a circulatory hormone and a neuromodulator within the CNS. A well-documented circulatory role for EH is to release ETHs from Inka cells. Circulating ETH acts directly on the CNS to initiate a neuropeptide cascade that regulates sequential steps in the ecdysis sequence. Included in the downstream cascade is the release of EH both into the hemolymph and within the CNS. Thus, EH and ETH engage in a positive feedback loop to ensure complete depletion of ETH from Inka cells. ETH also initiates tracheal inflation in *Drosophila* prior to the onset of ecdysis behaviors. The ability of ETH to trigger tracheal dynamics and behaviors depends on downstream peptides released from and within the CNS.

The list of peptides involved in ecdysis regulation continues to grow. One is CCAP, released centrally to activate ecdysis motor patterns in *Manduca*. In addition to CCAP, ITPs, MIPs, corazonin, and FLRFamide-related peptides are becoming recognized as putative ecdysis signals. The following sections summarize current knowledge on the properties of these peptides, their cellular origins, and likely functions in ecdysis regulation.

It is worth mentioning that many of these peptides were discovered years ago through the use of facile bioassays such as heartbeat and gut contraction. The names crustacean cardioactive peptide, corazonin, myoinhibitory peptides, and myosuppressins reflect the biological activities of peptides discovered in this manner. However, it is increasingly recognized that these names may not necessarily represent the authentic or most important physiological functions of these peptides. It is possible if not likely that many of these peptides are central neuromodulators involved in behaviors and other physiological functions that are associated with developmental processes such as ecdysis. It also seems likely that many peptides are multifunctional, carrying out some signaling functions not only as circulatory hormones, but also as transmitters and/or neuromodulators through release at or near synapses within the CNS.

3.1.2.2. Ecdysis Triggering Hormones

3.1.2.2.1. Epitracheal glands and Inka cells The first observations of epitracheal glands were published in the early part of the last century using *Bombyx mori* (Ikeda, 1913). These glands, which appear to have both endocrine and exocrine functions, remained unnoticed for almost 80 years, until they were rediscovered on histological sections of pharate larvae of the waxmoth *Galleria mellonella* during a search for peptidergic cells using an antiserum to FMRFamide (Zitnan, 1989). Nine pairs of segmentally distributed glands were found to be closely associated with the tracheal system and spiracles. One pair of glands is attached to tracheae near each prothoracic spiracle and eight pairs of glands are located on tracheae close to each abdominal spiracle (**Figure 2a**). Only the largest cell of each epitracheal gland shows FMRFamide-like immunoreactivity (IR), while 2–3 other cells are not stained (**Figure 2b**; Zitnan, 1989). Ultrastructural studies of epitracheal glands revealed numerous electrodense droplets in the largest gland cell of pharate pupal *B. mori*, which degenerate in freshly ecdysed pupae, but their function was not determined (Akai, 1992). Further immunohistochemical studies with antisera to horseradish peroxidase (HRP) and small cardioactive peptide B (SCP_B) showed that each epitracheal gland of *M. sexta* is composed of one prominent peptidergic cell and 2–3 smaller cells. The smaller cells degenerate after pupation, leaving only nine pairs of large peptidergic cells in developing adults. The large endocrine cells show strong SCP_B-IR in pharate larvae, pupae, and adults, but peptide staining disappears after each ecdysis, indicating that these cells release their contents into the hemolymph (Zitnan *et al*., 1996). These remarkable peripheral endocrine cells were named Inka cells in honor of a beautiful fairy from the Tatra mountains.

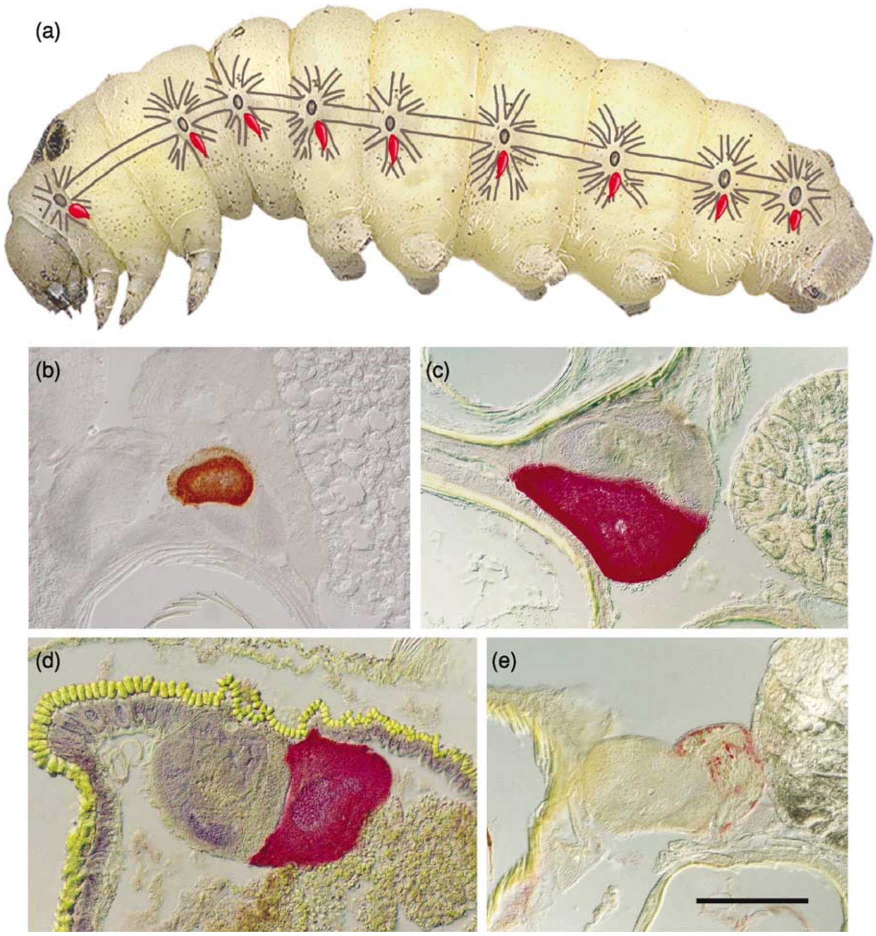


Figure 2 Distribution and peptide immunoreactivity (IR) of Inka cells in the moths *B. mori*, *G. mellonella*, and *M. sexta*. (a) Schematic drawings of Inka cells attached to the tracheal system of *Bombyx* pharate pupa. One prothoracic and eight abdominal Inka cells are attached to lateral tracheae near each spiracle. Another set of corresponding cells is located on the opposide side of the body. (b) The peptidergic nature of Inka cells was first identified with antiserum to FMRFamide in the waxmoth, *Galleria*. (c–e) Strong PETH-IR (c) and ETH-IR (d) in Inka cells of *Manduca* pharate pupae disappears at the onset of ecdysis (e). Scale bar = 150 μm.

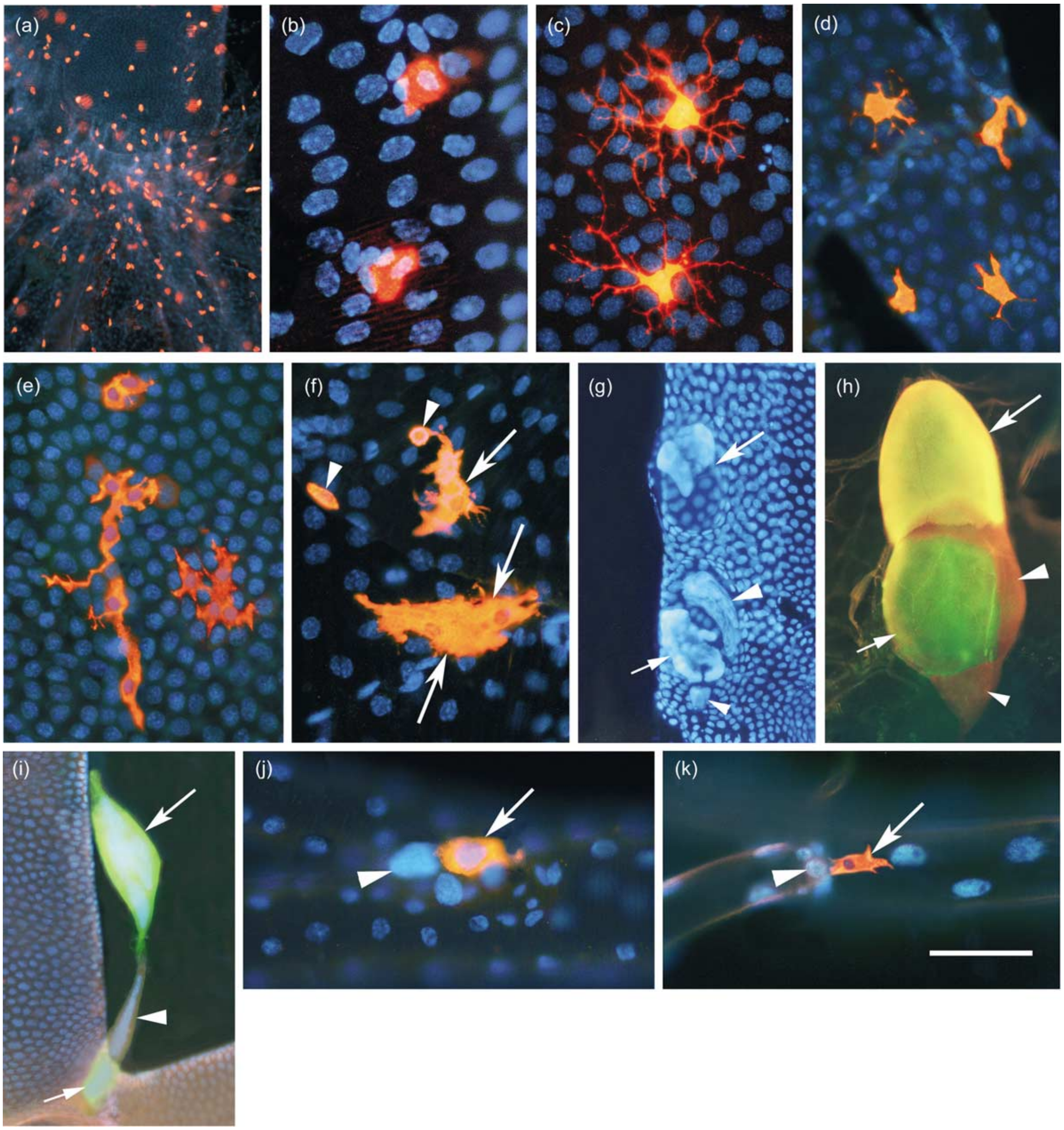


Figure 3 Variability of Inka cells in different insect species. (a–f) Immunohistochemical staining with PETH antiserum shows that all hemimetabolous and most holometabolous insects contain small Inka cells (stained orange–red or yellow), which are scattered throughout the surface of the entire tracheal system (nuclei are stained blue with diamino-phenylindole; DAPI). (a) PETH-IR (stained orange–red) in thousands of small Inka cells of the dragonfly *Sympetrum* sp. (b) Inka cells of the cockroach *N. cinerea* are simple and oval, whereas the cricket *A. domestica* (c) contains cells with prominent cytoplasmic processes. (d) Numerous Inka cells with narrow processes are distributed on narrow tracheae of the bug *Pyrrhocoris apterus*. (e) The water beetle *Laccophilus* sp. contains groups of 2–4 Inka cells with thick processes. (f) Two types of cells are present in the mealworm beetle *Tenebrio molitor*; groups of larger single or coupled Inka cells are located on major tracheae close to spiracles (arrows), whereas single small cells are distributed throughout narrower branching tracheae (arrowheads). (g–i) Large epitracheal glands containing Inka cells in *Manduca* and *Bombyx*. (g) DAPI nuclear staining shows that each *Manduca* epitracheal gland is composed of the prominent Inka cell (large arrow), narrow cell (large arrowhead), exocrine cell (small arrow), and canal cell (small arrowhead). (h,i) Double immunohistochemical staining with antisera to PETH and HRP reveals that only Inka cells of *Manduca* (h) and *Bombyx* (i) produce peptide hormones (yellow, large arrow). The exocrine cells are only stained with HRP antiserum (green, small arrow), while the other two cells are not labeled (large and small arrowheads). (j,k) PETH-IR in Inka cells (stained orange–red, arrows) of the cranefly, *Tipula* (j) and *Drosophila* (k). Note that other small cells of epitracheal glands show no PETH-IR (arrowheads). Scale bar = 200 μm in (a); 50 μm in (b–k).

Subsequent investigations demonstrated that Inka cells produce PETH and ETH, peptide hormones that activate pre-ecdysis and ecdysis motor programs in the CNS (**Figure 2c–e**).

Ultrastructural studies of epitracheal glands in *Lymantria dispar* showed that each gland contains four cells, named as follows: type I endocrine cell (Inka cell homolog), type II endocrine cell, exocrine cell, and canal cell (Klein *et al*., 1999). Inka cells release their hormonal content at ecdysis, but secretion of type II endocrine cells was not observed and their endocrine function requires confirmation by other techniques. As described in *Lymantria*, *Manduca* epitracheal glands contain an exocrine cell, which projects a duct through the canal cell into the lumen between new and old tracheae (Zitnanova *et al*., 2001). The apparent size decrease of the exocrine cell in freshly ecdysed larvae and pupae indicates that the cell's content is released into the tracheal lumen, possibly to aid shedding of the old tracheae during ecdysis.

The widespread occurrence of Inka cells in insects has been demonstrated by immunohistochemistry using an antiserum against PETH. Using this approach, Inka cells were observed to occur in 40 representatives of diverse insect orders (Zitnan *et al*., 2003). Surprisingly, two patterns of Inka cells were found. In most insect orders, hundreds or thousands of Inka cells of various sizes and shapes are scattered throughout the tracheal surface (**Figure 3a–f**). This contrasts sharply with the pattern described originally in certain holometabolous insects, where a well-defined system of 16–18 pairs of segmental epitracheal glands occurs, each with a single, prominent Inka cell associated with 2–4 other cells (**Figure 3g–k**; Zitnan *et al*., 2002, 2003). Numerous small, round Inka cells were stained in the dragonfly *Sympetrum* sp. (**Figure 3a**), and similar cells were found in the apterygote silverfish *Lepisma saccharina* and primitive aquatic insects–mayflies and damselflies. Two distinct cell types were found on different tracheae of the cockroach *Nauphoeta cinerea*; oval Inka cells were found on the major segmental tracheae (**Figure 3b**), while cells with cytoplasmic processes were stained on thin tracheae of the gonads and gut. Simple oval Inka cells also are found in the cockroach *Periplaneta americana* and the locust *Locusta migratoria*, whereas cells with prominent cytoplasmic processes are detected in cockroaches *Blabera craniifera* and *Phylodromica* sp., the cricket *Acheta domestica* (**Figure 3c**), the stonefly *Perla* sp., and bugs *Pyrhocoris apterus* (**Figure 3d**) and *Triatoma infestans*.

Inka cells of holometabolous insects also can be quite variable. Numerous, single Inka cells dispersed throughout the tracheae are found in the alderfly *Sialis* and the antlion *Myrmeleon*. Variability of Inka cells in representatives of several beetle species reflects the enormous diversity of this large insect group. For example, the water beetle *Laccophilus* sp. contains coupled elongated Inka cells with short cytoplasmic processes (**Figure 2e**), but the mealworm beetle *Tenebrio molitor* has two distinct types of Inka cells: groups of 2–6 larger cells that are attached to tracheae near each spiracle, and small cells scattered throughout the tracheal surface (**Figure 3f**). Numerous small Inka cells of various shapes are found in other beetles, but only nine pairs of large Inka cells associated with epitracheal glands are present in the Colorado potato beetle, *Leptinotarsa decemlineata* (for details see Zitnan *et al*., 2003). Similar epitracheal glands occur in most investigated hymenopterans, and all lepidopterans and dipterans. The only exception is the honeybee *Apis mellifera*, which contains a large number of small Inka cells (Zitnan *et al*., 2003). Variability of epitracheal glands containing prominent Inka cells is depicted in moths *Manduca* and *Bombyx*, the crane fly *Tipula* sp., and fruit fly, *Drosophila melanogaster* (**Figure 3g–k**).

Strong PETH-IR detected in representatives of both hemi- and holometabolous insects before ecdysis disappears after each larval, pupal, and adult ecdysis. This suggests that Inka cells release their hormonal content into the hemolymph to initiate and regulate shedding of the old cuticle. Inka cells of hemimetabolous insects appear to degenerate after adult ecdysis as indicated by permanent disappearance of PETH-IR on tracheal surfaces, whereas Inka cells in some representative holometabolous insects (beetles, moths, and dipterans) persist in adults and continue to produce peptide hormones related to PETH and ETH. Release and function of these peptides in adults has not been examined to date (Zitnan *et al*., 2003).

3.1.2.2.2. Ecdysis triggering hormones in lepidoptera A physiological function for epitracheal glands and identification of an active peptide hormone were first described in *Manduca sexta* (Zitnan *et al*., 1996). Injection of epitracheal gland extracts into *M. sexta* pharate larvae induced within 5–10 min the ecdysis behavioral sequence. Exposure of the isolated CNS *in vitro* to the same extracts produced corresponding motor bursts (see Section 3.1.5.1 for details). Saline extracts of 50 glands from four pharate pupae were separated by a single step of high-performance liquid chromatography (HPLC). Biological activity identical to that of the gland extract was found associated with a linear, 26 amino acid, C-terminally amidated peptide (mol. wt. 2940)

(**Figure 4**). This peptide was named *Manduca* ecdysis triggering hormone or Mas-ETH (Zitnan *et al*., 1996). Injection of synthetic ETH mimicked the effects of the native peptide. A similar peptide (mol. wt. 2656), with a three amino acid deletion near the N-terminus subsequently was isolated from Inka cells of pharate pupal *B. mori* and called Bom-ETH (**Figure 4**; Adams and Zitnan, 1997). Either peptide is sufficient to induce the ecdysis sequence upon injection *Manduca* or *Bombyx* pharate larvae.

Identification of the cDNA precursor and gene encoding ETH in *Manduca* revealed two additional peptides produced by Inka cells (**Figure 5**). One of these, an 11 amino acid, C-terminally amidated peptide (mol. wt. 1269), was subsequently isolated from epitracheal gland extracts by HPLC and synthesized. Since this peptide induced only pre-ecdysis behavior in *Manduca*, it was named pre-ecdysis triggering hormone (PETH; Zitnan *et al*., 1999). The third peptide, composed of 47 amino acids with a free C-terminus, was also synthesized, but showed no obvious biological function upon injection into pharate larvae. It was therefore named ETH-associated peptide or ETH-AP (mol. wt. 5658). Significant levels of partially processed precursor peptides corresponding to PETH-ETH (PE; mol. wt. 4407) and ETH-ETH-AP (EA; mol. wt. 8953), along with the entire, unprocessed precursor PETH-ETH-ETH-AP (PEA; mol. wt. 10419) were also isolated directly from Inka cells by HPLC. The ETH precursor thus contains one copy each of PETH, ETH, and ETH-AP (Zitnan *et al*., 1999). A similar precursor structure and corresponding peptides occur in *B. mori* (Zitnan *et al*., 2002).

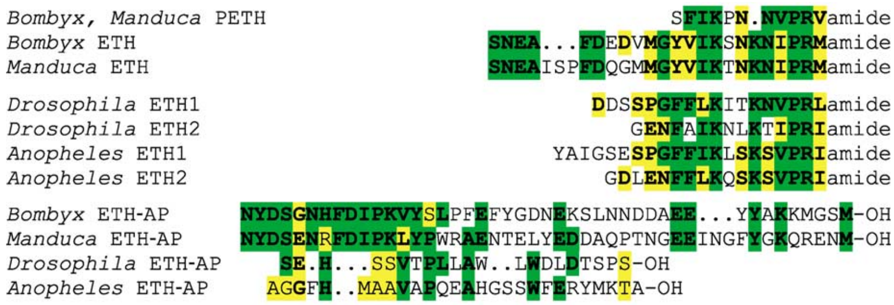


Figure 4 Amino acid sequences of PETH, ETH, and ETH-AP of moths and dipterans. Green represents sequence identity, yellow sequence similarity.

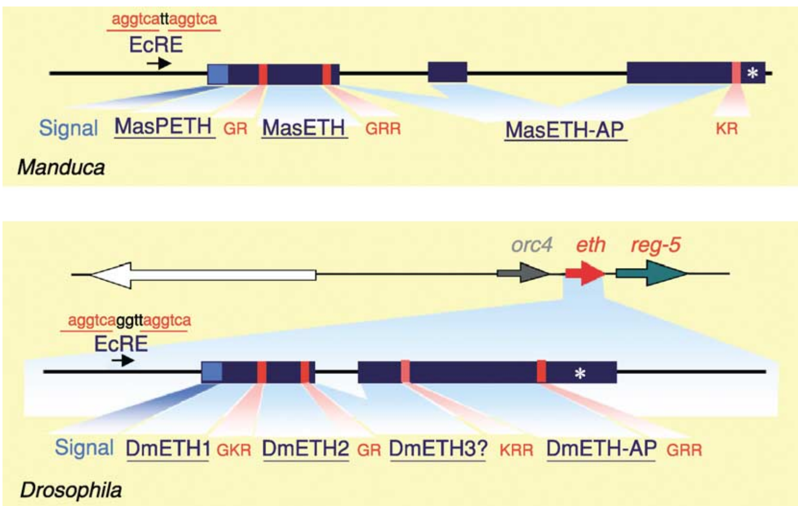


Figure 5 Similarity in the organization of *Manduca* and *Drosophila* genes encoding ETH. The *Manduca* ETH gene consists of a signal sequence, followed by one copy each of MasPETH, MasETH, and MasETH-AP. An upstream direct repeat ecdysone response element (EcRE) is a putative steroid receptor binding site for regulation of the gene. The Mas-ETH-AP sequence is interrupted by two introns. The signal sequence of *Drosophila eth* is followed by one copy each of DrmETH1 and DrmETH2. An upstream EcRE is also present in this gene. The sequence immediately downstream of DrmETH2 is designated DrmETH-AP1. Its sequence is followed by a second much larger peptide designated DrmETH-AP2. The gene *eth* occurs on the second chromosome right arm 60E1-2; see Zitnan *et al*. (1999), Park *et al*. (1999), and Park and Adams (unpublished data) for details.

Candidate regulatory elements contributing to ETH expression were revealed through identification of promoter regions in the ETH genes of moths and flies (**Figure 5**). Among the elements observed are direct repeat motifs thought to function as ecdysone response elements (EcRE). In *Manduca*, the direct repeat AGGTCATTAGGTCA and elements of Broad Complex are present in the ETH gene promoter (Zitnan *et al*., 1999). A similar sequence occurs in the *Drosophila eth* promoter: AGGTCAggttAGTTCA (Park *et al*., 1999). Such EcRE are known to bind the ecdysone receptor-ultraspiracle (EcR-USP) heterodimer in *Drosophila* and mosquitos (Vogtli *et al*., 1998; Wang *et al*., 1998) (see **Chapter 3.5**), and seem likely to function in the transcriptional regulation of ETH gene expression.

Immunohistochemical staining with antisera specific to ETH and ETH-AP show that these peptides are produced only in Inka cells of *Manduca* and *Bombyx* (Zitnan *et al*., 1999, 2002). The release of PETH and ETH into the hemolymph at each ecdysis was demonstrated by enzyme immunoassays and immunohistochemistry using antisera raised against PETH and ETH (Zitnan *et al*., 1999, 2002; Zitnanova *et al*., 2001). These studies demonstrated that during the initial 20 min of pre-ecdysis, low levels of these peptides (up to 5 nM) are present in the hemolymph, apparently in response to corazonin release (**Figure 6**; see Section 3.1.5.1). Hemolymph levels of PETH and ETH then rise sharply, reaching ~30 nM about 20 min later. This massive peptide secretion into the hemolymph correlates with elevation of 3′,5′-cyclic guanosine monophosphate (cGMP) in the Inka cell and depletion of its stores, probably in response to EH release (**Figure 6**; see Section 3.1.5.2). Initiation of ecdysis behavior only occurs after a 20 min delay, by which time peptide levels have subsided to ~15 nM (see Section 3.1.6.1.4 for details regarding this delay; Zitnan *et al*., 1999). Low levels of a partially processed precursor peptide (ETH-

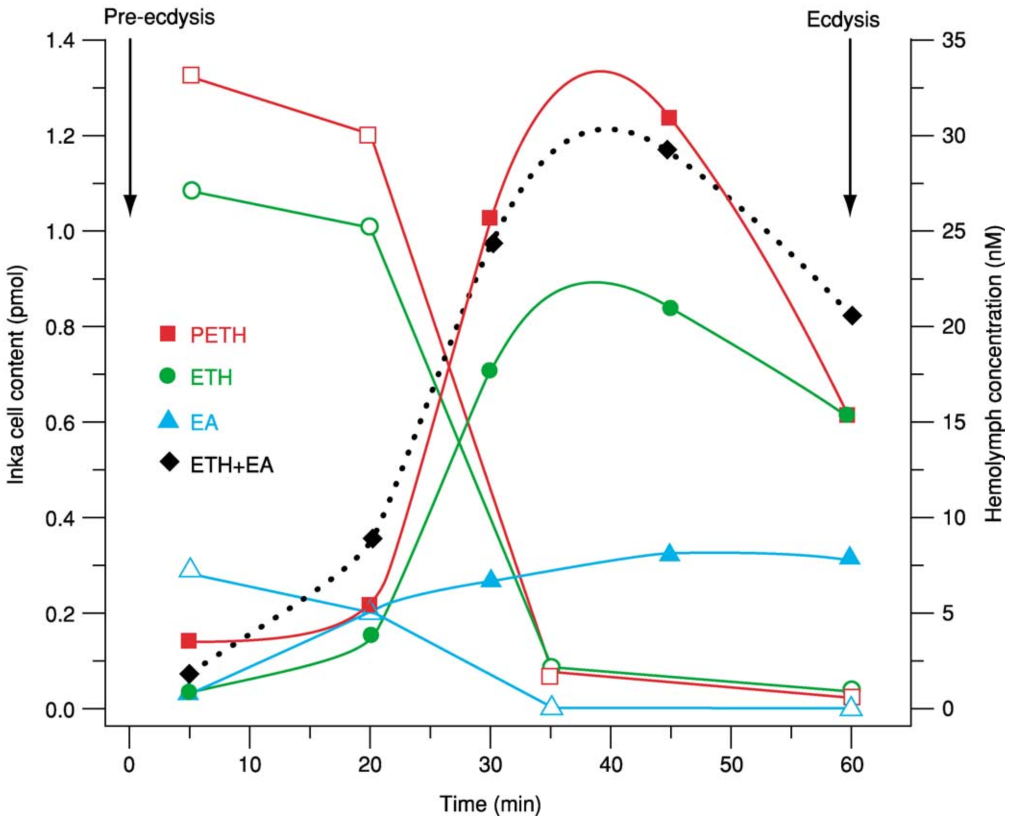


Figure 6 PETH and ETH levels in Inka cells and hemolymph during the ecdysis sequence. Low levels of PETH and ETH are present in the hemolymph (solid symbols) during the initial 20 min of pre-ecdysis. A sharp increase of *Manduca* Inka cell peptides in the hemolymph was detected about 20 min later. This is correlated with elevated cGMP levels and depletion of these peptides in Inka cells (open symbols). Hemolymph levels of PETH and ETH have decreased to about a half by the onset of ecdysis. Note that low levels of a partially processed precursor peptide ETH-ETH-AP (EA) are coreleased into the hemolymph. (Reprinted with permission from Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23* (3), 523–535; © Elsevier.)

ETH-AP) also are co-released into the bloodstream along with fully processed peptides. The physiological significance of this precursor co-release, if any, is unclear.

3.1.2.2.3. Ecdysis triggering hormones in Diptera "In silico" screening using the nucleotide sequence encoding the *Manduca* ETH precursor led to identification of the *Drosophila eth* gene (Park *et al.*, 1999). This approach later was used to identify a similar gene in the mosquito *Anopheles gambiae* (Zitnan *et al.*, 2003). The *eth* gene of both dipterans encodes two related amidated peptides, ETH1 and ETH2, and a different nonamidated peptide ETH-AP (Park *et al.*, 1999; Park *et al.*, 2002a; Zitnan *et al.*, 2003). Recently, ETH-AP1 has been isolated from tracheal extracts of *Drosophila* (Schoofs, Clynen, Spalovská-Valachova, and Zitnan, unpublished data). Sequences of all peptide hormones and organization of the ETH gene of moths and dipterans are shown in **Figures 4** and **5**.

The N-terminal of ETH1 was deduced through algorithms designed for prediction of signal peptide cleavage sites in eukaryotic cells (Nielsen *et al.*, 1997). This prediction led to the assignment of ETH1 as a 18 amino acid peptide with N-terminal amino acids DDSSP-, but an alternative sequence consisting of 23 amino acids containing the N-terminal sequence AISQADDSSP-was also considered (Park *et al.*, 1999, 2002a; Zitnan *et al.*, 2003). These peptides were both prepared by chemical synthesis and compared in bioassays for potency in triggering adult eclosion. The data showed ETH1 (N-terminus DDSSP-) was at least 100 times more potent in triggering adult eclosion than the alternative peptide (N-terminus AISQADDSSP-). Subsequent mass spectometric analysis provided confirmatory evidence for the original sequence assignments for both ETH1 and ETH2 (**Figure 4**; Hermesman and Adams, unpublished data).

The *eth* gene in *Drosophila* is expressed only in Inka cells. This was demonstrated by producing a transgenic line of flies expressing the transgene *2eth3-egfp* (Park *et al.*, 2002a). The construct contains the upstream promoter and coding regions of the *eth* gene followed by the chimeric *egfp* gene encoding enhanced green fluorescent protein (EGFP). These flies show colocalization of ETH-IR and EGFP-IR in Inka cells (**Figure 7**); no other cells in the entire organism were stained in larvae and adults. Secretory activity of Inka cells was observed as a sharp decline of cellular EGFP fluorescence just before tracheal inflation and initiation of the ecdysis behavioral sequence in larvae (Park *et al.*, 2002a). These findings confirm that the ETH gene is expressed in Inka cell specific fashion, and that the peptides are released at the appropriate time just preceding ecdysis in flies.

An antiserum to mollusk myomodulin was used to stain "peritracheal myomodulin (PM) cells" in six insect species (O'Brien and Taghert, 1998). Double labeling with myomodulin and SCP_B antibodies indicated that PM cells were Inka cell homologs. These authors speculated that myomodulin-like peptide(s) were coreleased with ETHs to control different functions at ecdysis. However, no function for myomodulin has been determined and no myomodulin-like peptide has been identified in insects. Inka cells of moths and flies also show strong immunohistochemical staining with antibodies to several other neuropeptides (e.g., FMRFamide, PBAN, vasopressin; Zitnan *et al.*, 2003). These neuropeptides share conserved amidated sequence motifs (-RXamide or -PRXamide; X = I, L, M, V) with PETH and ETH, indicating that antisera to these neuropeptides cross-react with amidated C-termini of PETH and ETH. Indeed, cross-reactivity of myomodulin and FMRFamide antisera with PETH and ETH was confirmed by specific enzyme immunossays in HPLC-isolated extracts of *Manduca* epitracheal glands and in *Drosophila* and *Musca* tracheal extracts (Zitnan *et al.*, 2003). It was further demonstrated that Inka cells of mutant larvae lacking the *eth* gene failed to react with myomodulin antiserum, while strong staining was detected in Inka cells of control *Drosophila* larvae (Canton S) producing ETH (Zitnan *et al.*, 2003). These data demonstrate that Inka cells are highly specialized for production of peptide hormones expressed by the *eth* gene, and do not produce myomodulin- or FMRFamide-related peptides in moths and flies.

3.1.2.2.4. Conservation of ETH signaling in the insecta The presence and biological activity of ETH-related peptide hormones in Inka cells from various insects was tested by injection of *Bombyx* pharate larvae with tracheal extracts from the cockroach *Nauphoeta*, cricket *Acheta*, bug *Pyrrhocoris*, beetle *Tenebrio*, fly *Drosophila*, and mosquito *Aedes* (Zitnan *et al.*, 2003). These extracts induced pre-ecdysis within a few minutes followed in most instances by ecdysis behavior in *Bombyx* larvae. Extracts from *Nauphoeta*, *Acheta*, *Drosophila*, and *Aedes* were most active in this assay. These data suggest that the ETH signaling system is conserved in diverse insect groups and perhaps all insects.

Further searches for Inka cell peptides in hemimetabolous insects resulted in detection of PETH-immunoreactive peaks in HPLC purified tracheal

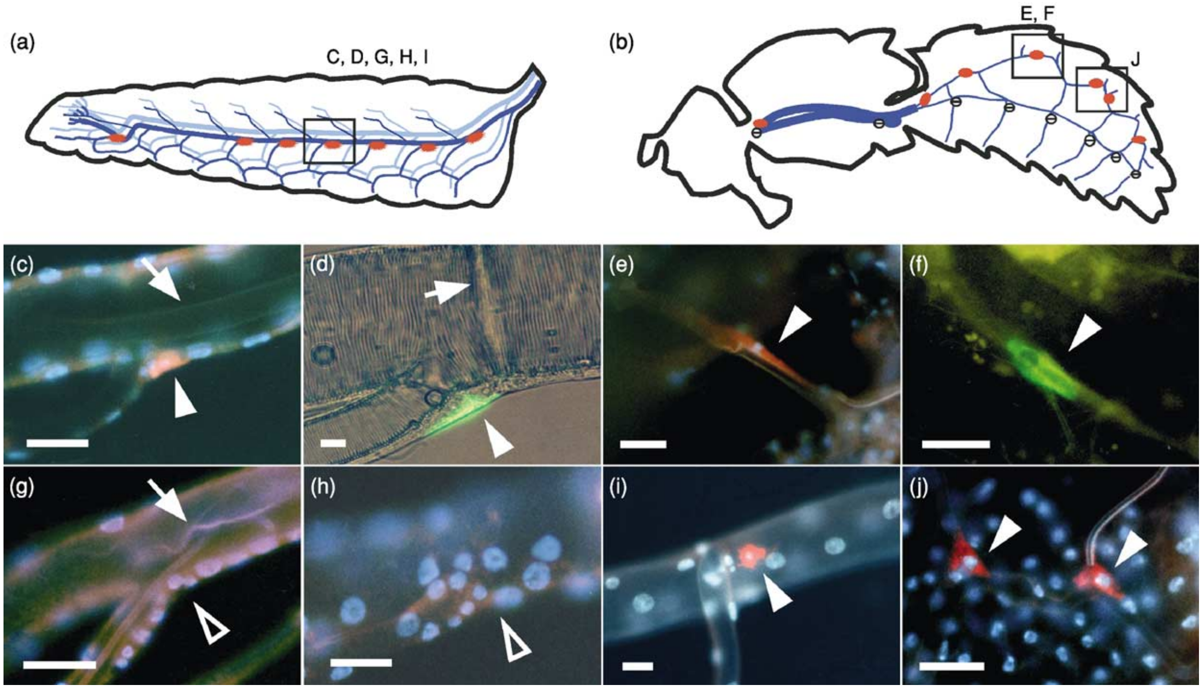


Figure 7 *Drosophila* Inka cell immunohistochemistry, EGFP expression in the *2eth3egfp* fly line, and loss of immunoreactivity in the deletion mutant *eth*[25b]. Red, blue, and green colors in photomicrographs are from Cy3, DAPI, and EGFP, respectively. Scale bar = 10 μm. (a) Diagram showing the larval tracheal system and positions of Inka cells (red). (b) Diagram showing adult tracheal system and positions of the Inka cells (red). Letters associated with boxed areas in the diagrams are shown in panels c–j below. (c) Cy3 staining in the late 1st instar using the DrmETH1 antibody. Old intima (arrow) is already separated from new intima. The Inka cell (arrowhead) is located along the main dorsal tracheal trunk at each branchpoint of the transverse connectives. (d) Expression of the *eth3-egfp* transgene in late 3rd instar. The cell expressing EGFP (arrowhead) at a node (arrow, see **Figure 1b**) is shown with low intensity transmitted light. (e) Cy3 staining in the adult stage using the DrmETH1 antibody. (f) Expression of the *eth3-egfp* transgene in late 3rd instar. (g) The *eth* deletion mutant *eth*[25b] Cy3 (late 1st instar) shows no immunohistochemical staining of the Inka cell. The collapsed old tracheal tube (arrow) is separated from new intima. The open arrowhead indicates location of the epitracheal gland in wild-type flies. (h) Depletion of ETH immunoreactivity in the Inka cell of wild-type flies (open arrowhead) immediately after ecdysis to early 2nd instar. (i) Immunoreactive Inka cell in the late 3rd instar using an antibody against MasPETH. (j) Inka cell immunoreactivity in the adult stage using the MasPETH antiserum. (Data from Park, Y., Filippov, V., Gill, S.S., Adams, M.E., **2002a**. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development 129*(2), 493–503.)

extracts of *Nauphoeta* and *Pyrrhocoris*. Four immunopositive peaks were detected in extracts of *Nauphoeta*, whereas only two peaks were found in *Pyrrhocoris* (Zitnan *et al*., 2003).

3.1.2.3. Brain Ventromedial Cells and Eclosion Hormone

The role of EH in insect ecdysis has recently been reviewed in detail (Ewer and Reynolds, 2002). Here the focus is on highlights of EH biology relevant to this chapter. The endocrine control of ecdysis was demonstrated by Truman in classic experiments on adult emergence (referred to as "eclosion" in this stage). EH, located in the brain of giant silkmoths, was shown to be released into the circulatory system from the corpora cardiaca–corpora allata complex (CC–CA; Truman and Riddiford, 1970; Truman, 1971). Further studies showed that CC–CA extracts from pharate adults triggered premature ecdysis in pharate larvae, pupae, and adults upon injection into the hemocoel (Reynolds *et al*., 1979; Copenhaver and Truman, 1982; Truman, 1985).

After initial isolation and determination of the partial N-terminal sequence of EH in *Bombyx* (Nagasawa *et al*., 1983, 1985), its complete sequence was simultaneously identified in three laboratories from CC–CA or heads of *Manduca* and *Bombyx* pharate adults (Kataoka *et al*., 1987; Kono *et al*., 1987; Marti *et al*., 1987; Nagasawa *et al*., 1987). EH of both species is a closely related 62 amino acid peptide with three internal disulfide bonds (**Figure 8**; Terzi *et al*., 1988; Kono *et al*., 1990b; Kataoka *et al*., 1992). Soon thereafter, the cDNA and gene encoding EH were described in *Manduca*, *Bombyx*, and *Drosophila* (Horodyski *et al*., 1989; Kamito *et al*., 1992; Horodyski *et al*., 1993).

Immunohistochemistry using an antiserum against *Manduca* EH combined with physiological techniques was used to identify the source of EH. These approaches led to identification of a cluster of lateral neurosecretory cells (type Ia) in the brain, which showed "eclosion hormone" biological activity and immunoreactivity in *Manduca* pharate adults (Copenhaver and Truman, 1985, 1986a, 1986b, 1986c). However, *in situ* hybridization studies did not confirm EH expression in type Ia cells, but rather in two pairs of ventromedial cells now recognized as the authentic EH neurons (Horodyski *et al*., 1989). Independent studies have recently shown that a subset of these cells (type Ia_1) produces a neuropeptide corazonin (**Figure 9a** and **b**; Wise *et al*., 2002). Unexpectedly, injection of corazonin mimics the action of EH (see Section 3.1.5.2). Another subset of the lateral cells (type Ia_2) produces ion transport peptide (ITP) (**Figure 9c**; see Section 3.1.2.4; Spalovská-Valachova, Zitnan, Cho, Park, Adams, unpublished data). Interestingly, ITP shows a striking sequence homology with EH in one part of its primary structure, and it therefore seems possible that the EH antiserum used in previous studies may have cross-reacted with conserved regions of the ITP sequence (**Figure 8**).

Subsequent immunohistochemical studies in *Manduca* showed EH is produced by two pairs of the brain ventromedial neurosecretory cells (VM cells, type V, **Figure 9a**). These neurons project axons along the entire length of the ventral nerve cord, which exit the CNS via the proctodeal nerves to make neurohemal release sites on the hindgut surface

```
Bombyx EH                SPAIASSYDAMEICIENCAQCKKMFGPWFEGSLCAESCIKARGKDIPECESFASISPFLNKL-OH
Manduca EH               NPAIATGYPPMEICIENCAQCKKMLGAWFEGPLCAESCIKFKGKLIPECEDFASIAPFLNKL-OH
Drosophila EH            FDSMGGIDFVQVCLNNCVQCKTMLGDYFQGQTCALSCLKFKGKAIPDCEDIASIAPFLNAL-OH
Bombyx ITP          S.FFTLECKGVFDAAIFARLDRICDDCFNLFREPQLYTLCRAECFTTPYFKGCMESLYLYDEKEQIDQMIDFVamide
Drosophila ITP      SNFFDLECKGIFNKTMFFRLDRICEDCYQLFRETSIHRLCKAECFGSPFFNACIEALQLHEEMDKYNEWRDTLamide
Schistocerca ITP    S.FFDIQCKGVYDKSIFARLDRICEDCYNLFREPQLHSLCRSDCFKSPYFKGCLQALLLIDEEEKFNQMVEILamide
Carcinus CHH        pQIYDTSCKGVYDRALFNDLEHVCDDCYNLYRTSYVASACRSNCYSNLVFRQCMDDLLMMDEEDQYARKVQMVamide
```

Figure 8 Comparison of EH and ITP/CHH sequences in insect and crustaceans. Green represents sequence identity, yellow sequence similarity.

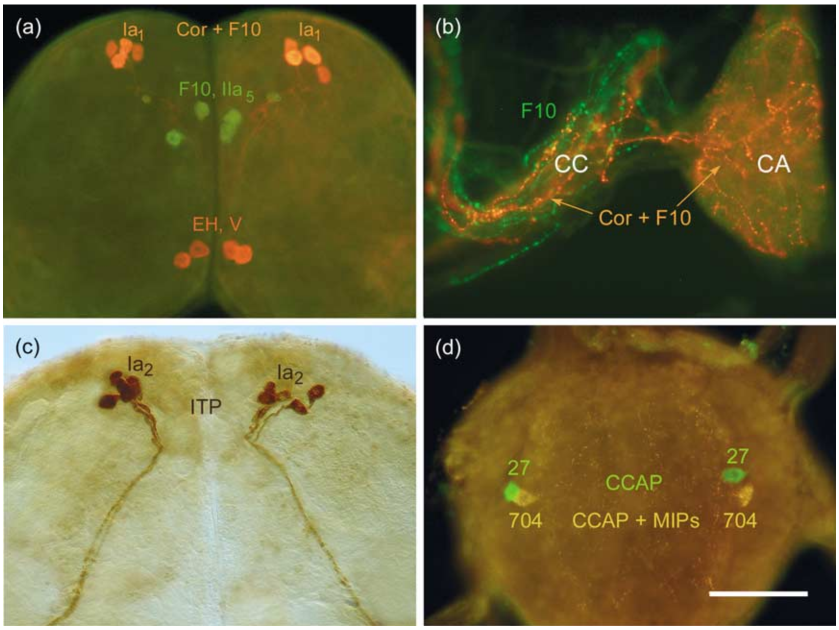


Figure 9 Peptidergic cells implicated in control of the ecdysis sequence in *Manduca*. (a) Triple immunohistochemical staining of a pharate 4th instar larva brain showing colocalization of corazonin and F10 in the ipsilateral neurosecretory cells (type Ia_1, orange color). F10 is also produced by medial neurosecretory cells (type IIa_5, green color), while EH is expressed in the ventromedial cells (type V, red color). (b) Corazonin and F10 produced by Ia_1 cells are stored in the axon terminals of entire corpora cardiaca and corpora allata complex (CC, CA; orange color, arrows), while F10 from medial cells IIa5 is mostly stored in the CC region (green color). (c) ITP-immunoreactivity in ipsilateral neurosecretory cells Ia_5. (d) Double staining with antisera to CCAP and MIP indicates that abdominal neurosecretory cells 27 produce only CCAP (green color), while interneurons 704 express both CCAP and MIPs (orange color). Scale bar = 100 μm.

(Truman and Copenhaver, 1989). During adult development they project additional axons to the CC, while axons running through the ventral nerve cord remain intact (Riddiford *et al.*, 1994). Similar pairs of VM cells have been identified in *Bombyx* with a monoclonal antibody to *Bombyx* EH (Kono *et al.*, 1990a). *In situ* hybridizations of the CNS in embryos, larvae, and developing adults, and Northern blots of different larval ganglia of the CNS confirmed that the gene encoding EH is only expressed in the brain VM cells (Horodyski *et al.*, 1989; Kamito *et al.*, 1992; Riddiford *et al.*, 1994). Similarly, *Drosophila* EH is expressed in a single pair of dorsomedial neurons projecting from the brain into the CC as well as posteriorly within the fused ventral ganglion (Horodyski *et al.*, 1993; Baker *et al.*, 1999).

Immunohistochemical staining with an antiserum raised against the C-terminus of *Manduca* EH revealed EH-immunoreactivity (EH-IR) in the CNS of diverse insect groups (**Figure 10**). These insects show EH-IR in a variable number of neurosecretory cells in the brain. For example, two pairs of ventromedial (VM) cells are present in the dragonfly *Sympetrum* sp., while a cluster of 4–10 EH-IR neurons is stained in the cockroach *Nauphoeta cinerea* and bug *Pyrrhocoris apterus*, and one pair of cells is found in the locust *L. migratoria* and cricket *A. domestica*. VM neurons of most species project nonarborizing axons posteriorly along the entire ventral nerve cord. Interestingly, axons of *Acheta* VM cells show obvious arborizations in the anterior and posterior regions of each ventral ganglion, and two additional pairs of lateral neurons are stained in these ganglia (**Figure 8c**). EH-producing neurons in most holometabolous insects examined resemble those described in *Manduca* (Truman and Copenhaver, 1989). Two pairs of VM neurons are present in alderfly *Sialis* sp., antlion *Myrmeleon* sp., mealworm beetle *T. molitor* and silkwmoth *B. mori*, while only one pair of dorsomedial neurons shows EH-IR in the fruitfly *Drosophila* (**Figure 10**).

Immunohistochemical staining and physiological experiments suggest that EH is released centrally from axons within ventral ganglia to participate in ecdysis activation (Hewes and Truman 1991; Baker *et al.*, 1999; Zitnan *et al.*, 2002), as well as peripherally to participate in the release of PETH and ETH from Inka cells and other functions (Kingan *et al.*, 1997a).

3.1.2.4. Ion Transport Peptides and Crustacean Hyperglycemic Hormones

Insect ITPs belong to a large family of peptides that are prominent in crustacean physiological functions: crustacean hyperglycemic hormones (CHHs), molt inhibiting hormones (MIHs), vitellogenesis inhibiting hormones (VIHs), and mandibular organ inhibiting hormones (MOIHs) (reviews: de Kleijn *et al.*, 1998; Van Herp, 1998; Webster, 1998; see also **Chapter 3.16**). As their names indicate, these large peptides regulate various functions including water homeostasis, energy mobilization, vitellogenesis, and biosynthesis of ecdysteroids and methyl farnesoate (Keller, 1992; Audsley *et al.*, 1992a, 1992b; Meredith *et al.*, 1996; Wainwright *et al.*, 1996; Liu *et al.*, 1997; Keller *et al.*, 1999).

Since some actions of CHH are associated with crustacean ecdysis, it is given emphasis here. CHH was originally identified in sinus gland extracts of *Carcinus maenas* as an amidated 72 amino acid peptide (Kegel *et al.*, 1989). Subsequently, numerous related peptides and corresponding precursors and genes were identified in several crustaceans (Weidemann *et al.*, 1989; Tensen *et al.*, 1991; de Kleijn *et al.*, 1994, 1995; Ohira *et al.*, 1997; Gu and Chan, 1998; Gu *et al.*, 2000).

Peptides of the CHH family control multiple functions. For example, CHH stimulates glucose and lipid release into the hemolymph, secretion of digestive enzymes from the hepatopancreas, and ion transport in the gill. This peptide also inhibits production of ecdysteroids and methyl farnesoate from endocrine glands (Keller, 1992; Van Herp, 1998; Spanings-Pierrot *et al.*, 2000). Immunohistochemistry revealed that, in several crustaceans, CHH is produced by the eyestalk neuroendocrine system composed of neurosecretory cells in the X-organ and neurohemal sinus gland (Dircksen *et al.*, 1988).

Recent studies showed that CHH immunoreactivity also is produced by a novel endocrine system comprising thousands of endocrine cells in the fore- and hindgut of *Carcinus* (Chung *et al.*, 1999; Webster *et al.*, 2000). Biochemical and molecular evidence confirmed that this gut immunoreactive peptide and its precursor sequence were identical to CHH produced by the eyestalk neurosecretory system (Chung *et al.*, 1999). Massive release of CHH from gut endocrine cells was detected during ecdysis of *Carcinus*, causing water and ion uptake necessary for swelling of the animal and rupture of the epidermal lines, followed by ecdysis of the old cuticle and the subsequent increase in size during postecdysis (Chung *et al.*, 1999). CHH-related peptides likely control water and ion balance in other arthropods as well, as described in the locust (Phillips *et al.*, 1996, 1998).

Ion transport peptide was originally identified in the storage lobe of locust CC and shown to stimulate salt and water reabsorption, and to inhibit acid

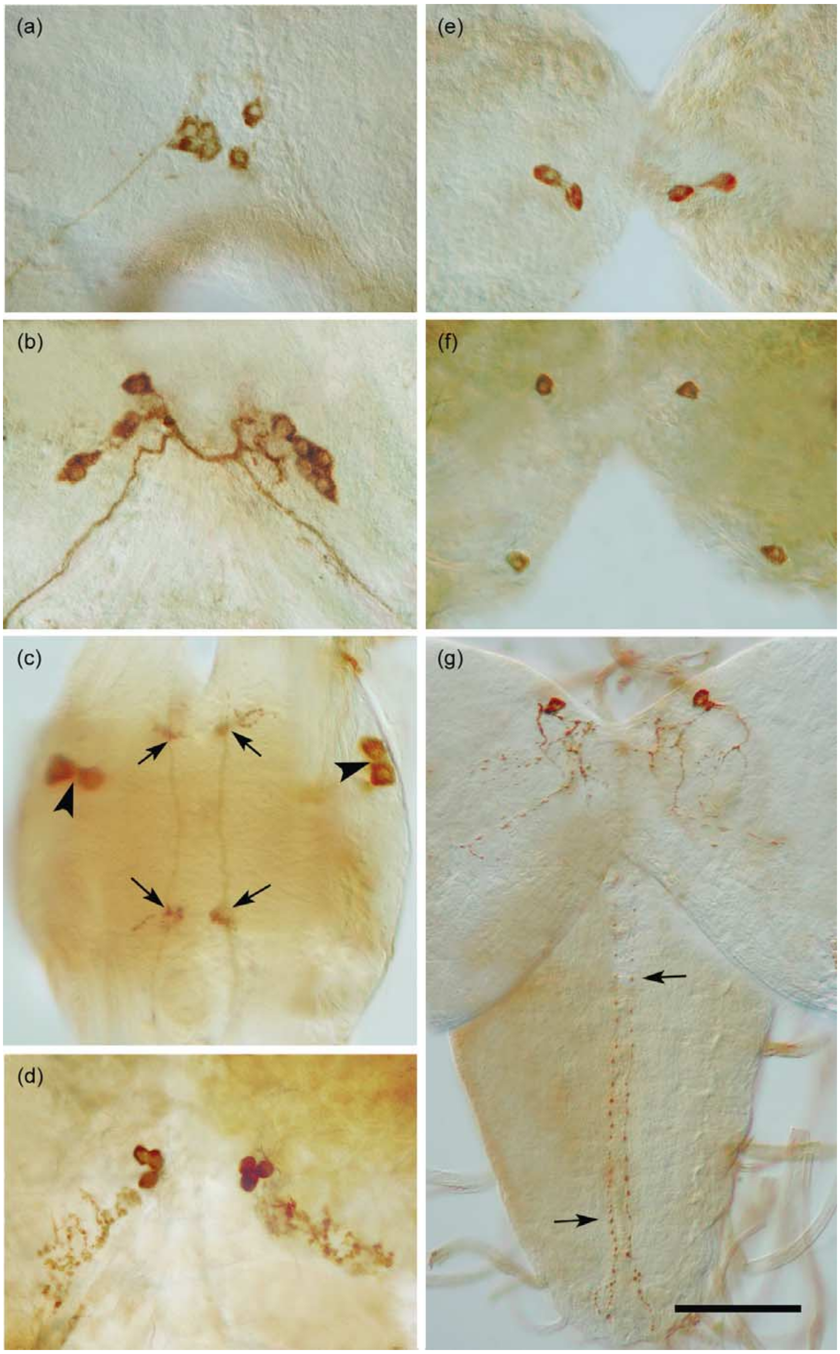


Figure 10 EH-immunoreactivity (EH-IR) in diverse insect species. Hemimetabolous insects usually show a variable number of EH-IR cells in the brain. (a, b) Two brain specimens of the cockroach *Nauphoeta* show EH-IR in a cluster of six (a) and ten (b) ventromedial (VM) cells. (c) VM cells of the cricket *Acheta* project axons throughout the entire nerve cord and make prominent arborizations in each ventral ganglion (AG4, arrows). Two pairs of additional dorsolateral neurons are stained in these ventral ganglia (AG4, arrowheads). (d) Six EH-IR neurons were detected in the brain of the bug *Pyrrhocoris*, but other specimens show 4–9 immunopositive cells. (e) Two pairs of VM cells are stained in the brain of alderfly *Sialis* and (f) antlion *Myrmeleon.* Note that a second pair of VM neurons in *Myrmeleon* is located more posteriorly. (g) Only one pair of EH producing neurons is stained in the dorsomedial brain region of the fruitfly *Drosophila*. These cells arborize in the brain and project two axons along entire ventral nerve cord (arrows). Scale bar = 100 μm in (a–e); 50 μm in (g).

secretion by the hindgut of *Schistocerca gregaria* (Audsley *et al.*, 1992a, 1992b). Western blots with antisera to *Schistocerca* ITP suggest that this peptide is quickly transported from the brain neurosecretory cells into the neurohemal CC–CA. On Western blots, a strongly stained band corresponding to ITP was detected in extracts of two CC–CA, while only a weakly labeled band was visible in extracts of 50 brains (Macins *et al.*, 1999). Using *Schistocerca* ileal bioassays and Western blots, evidence for the presence of ITP was detected in the CC or brain extracts of orthopteroid insects, including several locusts, cricket *A. domestica*, cockroach *Periplaneta americana*, and stick insect *Carausius morosus*. By the same criteria, ITP was not detected in holometabolous insects, such as the moth *Spodoptera litura* and the fly *Neobellieria bullata* (Macins *et al.*, 1999). Nevertheless, precursors encoding peptides related to ITP/CHH have been identified in both hemi- and holometabolous insects such as *Schistocerca, Locusta*, and *Bombyx* (Meredith *et al.*, 1996; Macins *et al.*, 1999; Endo *et al.*, 2000), and gene sequences encoding putative ITPs became generally accessible after publication of the *Drosophila* and *Anopheles* genomes. These precursors and genes encode an amidated 72 amino acid ITP and 1–2 longer free C-terminal ITP-like peptides (Figure 8).

ITP immunoreactivity and mRNA encoding the peptide occur in the CNS of moths. *In situ* hybridization with anti-sense riboprobes detected ITP mRNA in 5–6 pairs of large neurons in the *Bombyx* brain (Endo *et al.*, 2000). Immunohistochemical staining with CHH and ITP antisera (Figure 9c; Spalovská-Valachová and Zitnan, unpublished data) showed that these neurons are ipsilateral neurosecretory cells (type Ia$_2$). Smaller paired neurons were also stained in each ventral ganglion of *Schistocerca* and *Manduca* (Dircksen and Heyn, 1998; Spalovska-Valachova and Zitnan, unpublished data).

At the present time, definitive roles for ITP/CHH-like peptides in insect ecdysis are not well understood. But based on analogy with gut CHH in crustaceans and the demonstrated role of ITP in gut ion transport in locusts, it is speculated that blood-borne ITP may control functions associated with water reabsorption or air inflation, which are necessary preparatory steps for shedding of the old cuticle.

3.1.2.5. Crustacean Cardioactive Peptide

A neuropeptide with heart stimulatory activity, crustacean cardioactive peptide (CCAP), was originally identified from the CNS of crab *Carcinus maenas* (Stangier *et al.*, 1987). Later, identical neuropeptides were isolated from the CNS of the various insects including locust *L. migratoria*, beetle *T. molitor*, and moths *Spodoptera eridania* and *M. sexta* (Figure 11; Stangier *et al.*, 1989; Cheung *et al.*, 1992; Furuya *et al.*, 1993; Lehman *et al.*, 1993). The gene encoding a single copy of CCAP was identified in *Manduca* and *Drosophila* (Loi *et al.*, 2001).

CCAP	PFCNAFTGCamide	
Locusta MIP	AWQDLNAGWamide	
Manduca MIP1	AWQDLNSAWamide	100%
Manduca MIP2	GWQDLNSAWamide	20%
Manduca MIP3	APEKWAAFHGSWamide	20%
Manduca MIP4	GWNDMSSAWamide	20%
Manduca MIP5	GWQDMSSAWamide	50%
Manduca MIP6	AWSALHGAWamide	20%
Drosophila MIP1	AWQSLQSSWamide	
Drosophila MIP2	AWKSMNVAWamide	
Drosophila MIP3	EAQGWNKFRGAWamide	
Drosophila MIP4	EPTWNNLKGMWamide	
Drosophila MIP5	DQWQKLHGGWamide	

Figure 11 Amino acid sequences of CCAP and MIPs from different insect species. Green/turquoise represent sequence identity, yellow sequence similarity.

Immunohistochemical studies with antisera to CCAP revealed a conserved pattern of paired lateral neurons in subesophageal, thoracic, and abdominal ganglia (AG1–7) in *Ctenolepisma, Thermobia, Locusta, Gryllus, Tenebrio, Aedes, Drosophila*, and *Calliphora* (Figures 12 and 13; Breidbach and Dircksen, 1991; Dircksen *et al.*, 1991; Jahn *et al.*, 1991; Helle *et al.*, 1995; Ewer and Truman, 1996; Kostron *et al.*, 1996). These paired neurons were identified in *Manduca* as neurosecretory cells 27 (or NS-L$_1$) and interneurons 704 (IN-704) (Taghert and Truman, 1982a; Sandstrom and Weeks, 1991; Davis *et al.*, 1993; Ewer *et al.*, 1994). Homologs of Cell 27 in *Gryllus* and *Periplaneta* were described as lateral white (LW) cells (Adams and O'Shea, 1981; O'Shea and Adams, 1981), while homologs of 27 and 704 were named type I and II neurons in *Locusta* (Dircksen *et al.*, 1991; Davis *et al.*, 2003). However, for the sake of clarity, they are referred to as cells 27 and IN-704 here.

Examples of CCAP-IR neurons in various insects including silverfish *Lepisma saccharina*, cockroach *Periplaneta*, locust *Locusta*, cricket *A. domestica*, walking stick *Caraussius morosus*, bug *Pyrrhocoris apterus*, alderfly *Sialis* sp., antlion *Myrmeleon* sp., mealworm beetle *T. molitor*, and silkworm *B. mori* are shown in Figures 12 and 13. These insects show CCAP immunoreactivity (CCAP-IR) in a neuronal network closely resembling cells 27 and 704 (27/704 network) described in ventral ganglia of *Manduca* (Sandstrom and Weeks, 1991; Davis *et al.*, 1993; Ewer *et al.*, 1994; Ewer and Truman, 1996). However, the number and spatial expression of CCAP

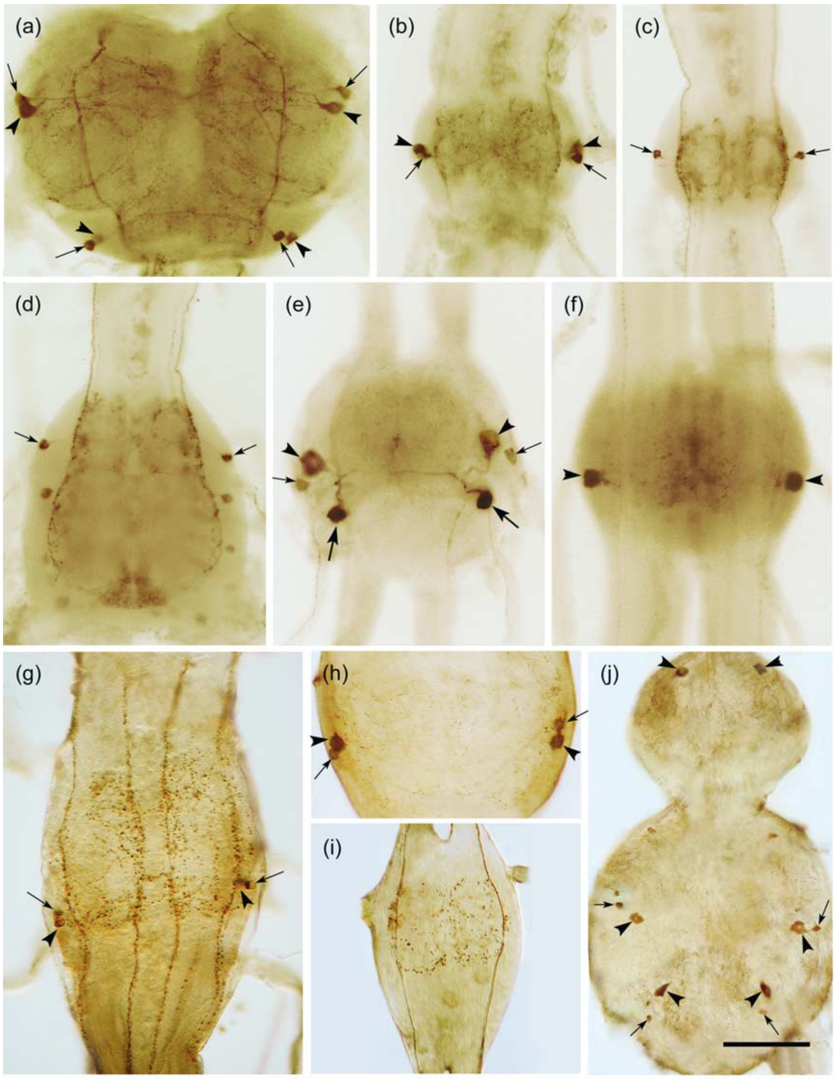


Figure 12 Variability of CCAP-IR in the CNS of hemimetabolous insects. All insects show CCAP-IR in putative homologs of neurosecretory cells 27 (arrowheads) and interneurons 704 (IN-704; small arrows). (a–d) In *Periplaneta americana* both cell types are stained in the fused TG3-AG1 (a) and anterior unfused AG4 (b), while posterior AG5 (c) and TAG (d) lack cells 27 and show only staining in IN-704 and several other small cells. (e) In addition to cells 27/704, *Locusta migratoria* shows a very strong CCAP-IR in a pair of large neurosecretory cells in AG5 (large arrows). (f) CCAP-IR in *Acheta* is restricted to a pair of cells 27 in AG5. (g) One pair of 27/704 homologs is stained in AG4 of *Lepisma*. (h, i) In *Carausius* cells 27/704 are detected in the TG2 (h), but no cell bodies are stained in AG5 (i). (j) Similarly, TG1-3 of *Pyrrhocoris* show CCAP-IR in putative 27/704 cells, but no immunopositive cells are found in the fused abdominal ganglion. Immunoreactive axons in AG of the two latter species probably originate from 27/704 cells in the SG and TG1-3. Scale bar = 100 μm.

producing cells in different ganglia is quite variable (Figures 12 and 13). Two pairs of putative cells 27 and 704 are usually stained in the SG of all hemimetabolous insects examined (Dircksen *et al.*, 1991; Ewer and Truman, 1996), while only one pair of these neurons is detected in TG1–3 (Figure 12a and h). One pair of 27/704 homologs is also stained in AG1–7 of *Lepisma* (Figure 12g), but other insects show much more variable expression of CCAP-IR in abdominal ganglia. For example, in *Periplaneta* CCAP-IR is detected in paired cells 27 and 704 of AG1–4, while cells 27 appear to be missing in AG5–7, so only paired IN-704 are stained in these posterior ganglia (Figure 12a–d). *Locusta* shows CCAP-IR in 27/704 cells in all AG1–7, plus a very strong staining is detected in another pair of large neurosecretory cells in AG1–6 (Figure 8e; Dircksen *et al.*, 1991). These cells probably also produce bursicon and show cGMP elevation during ecdysis (Truman *et al.*, 1996; see Sections 3.1.1.3 and 3.1.6.2). CCAP-IR in *Acheta* is restricted to a pair of cells 27 in each AG1–7; the dragonfly *Sympetrum* sp. shows a very

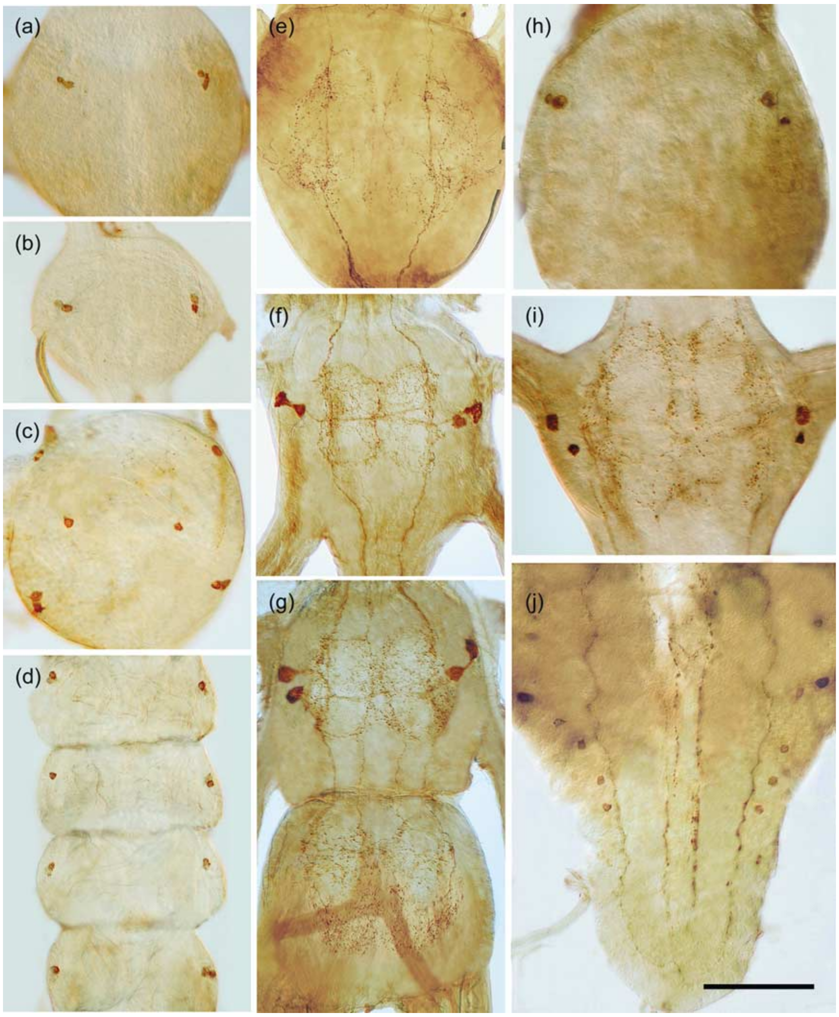


Figure 13 CCAP-IR in the ventral nerve cord of holometabolous larvae. A network of CCAP-IR neurons 27/704 is more conserved in holometabolous insects. (a, b) One pair of putative cells 27 and IN-704 is detected in each TG2 (a) and AG4 (b) in *Sialis.* (c, d) A similar distribution of CCAP-IR cells 27/704 is detected in the TG2 (c) and AG3-6 (d) of *Myrmeleon*, but some additional neurons are stained in the TG2. (e–g) In *Bombyx* larvae, cells 27/704 show strong immunoreactivity in AG4 (f) and AG7 (g), but homologous cells are not stained in TG2 (e). (h, i) A pair of cells 27/704 are detected in the TG2 (h) and AG7 (i) of *Tenebrio.* (j) *Drosophila* shows CCAP-IR in one pair of lateral cells probably homologous with cell 27 in each thoracic and first six abdominal neuromeres. Scale bar = 100 μm in (a–d); 200 μm in (e–i); 50 μm in (j).

similar staining pattern (not shown). By contrast, no cell bodies are stained in AG1–7 of *Caraussius* or fused abdominal ganglion of *Pyrrhocoris*. Strongly stained axons in abdominal ganglia of these two species probably originate from cells 27/704 detected in the SG and TG1–3 (Figure 12h–j).

A network of CCAP-producing neurons appears to be more conserved in holometabolous insects. Two pairs of putative cells 27 and IN-704 in the SG and one pair of these cells in each TG1–3 and AG1–7 show CCAP-IR in *Sialis* (Figure 13a and b), *Myrmeleon* (Figure 13c and d), *Tenebrio* (Figure 13h and i), and *Aedes* (Ewer and Truman, 1996). *Bombyx* larvae express similar staining in 27/704 cells of AG1–7 as described in other holometabolous species, but these cells lack CCAP-IR in the SG and TG1–3 (Figure 13e–g). *Drosophila* shows CCAP-IR in 3–4 pairs of lateral cells (probably homologs of cell 27) in the SG region and three pairs in TG1–3, while one pair of cells is usually stained in each of the first six abdominal neuromeres (Figure 13j; Ewer and Truman, 1996; Park *et al*., 2003b).

Using immunohistochemistry and *in situ* hybridization techniques, developmental expression of CCAP and its mRNA precursor was described in *Manduca* (Loi *et al*., 2001; Davis *et al*., 2003). In larval stages, strong CCAP-immunoreactivity is only present in one pair of cells 27/704 in each AG1–7

(Davis *et al*., 2003). Curiously, thoracic homologs of 27/704 cells do not appear to produce CCAP in *Manduca* larvae, instead these cells express IgG-like protein (Klukas *et al*., 1996). CCAP staining appears in 27/704 neurons in the pupal stage, but vanishes after adult emergence (Davis *et al*., 1993). CCAP-IR is also detected in three pairs of brain interneurons and several neurons in abdominal ganglia (e.g., a pair of motoneurons MN1, two medial neurosecretory cells NC4, and two ventral unpaired medial (VUM) neurons, as well as in the peripheral segmental neurons L1 (Davis *et al*., 2003). A very similar pattern of cell types was also shown by *in situ* hybridizations with the CCAP probe (Loi *et al*., 2001). These data suggest that most or probably all CCAP-IR cells produce genuine CCAP. Both immunohistochemistry and *in situ* hybridization techniques showed that different cell types express CCAP at specific developmental stages (Davis *et al*., 1993; Loi *et al*., 2001).

Neurons 27/704 in *Manduca* and their homologs in other diverse insects produce cGMP during each ecdysis (Ewer *et al*., 1994; Ewer and Truman, 1996), which is associated with increased excitability and CCAP release from these cells. The central release of CCAP from a network of 704 neurons in *Manduca* has been implicated as a crucial signal for the initiation and maintenance of the ecdysis motor program residing in abdominal ganglia (Gammie and Truman, 1997a; Zitnan and Adams, 2000; see Section 3.1.6.1.3). Other *in vitro* bioassays indicate that peripherally released CCAP acts as a neurohormone to stimulate heart, oviduct, and hindgut contractions in *Locusta* and *Manduca* (Cheung *et al*., 1992; Donini *et al*., 2001; Donini *et al*., 2002). CCAP in cells 27 is apparently colocalized with bursicon in *Periplaneta, Gryllus, Manduca*, and probably other insects (Kostron *et al*., 1996; Honegger *et al*., 2002). These peptides are very likely coreleased following ecdysis to control postecdysis processes; CCAP increases heart beat and blood pressure for cuticle expansion, while bursicon controls sclerotization and tanning of expanded new cuticle (see Section 3.1.6.6.2).

Using immunohistochemistry and radioimmunoassays, CCAP was detected in the CNS of several crustaceans, spiders (arachnids), and an opilionid (Dircksen and Keller, 1988; Stangier *et al*., 1988; Breidbach *et al*., 1995; reviews: Dircksen, 1994, 1998). High levels of CCAP were also detected in the hemolymph during ecdysis of decapod crustaceans, *Carcinus maenas* and *Orconectes limosus* (Phlippen *et al*., 2000). The identical structure and widespread occurrence of CCAP in a network of similar cells in various arthropods indicates a conserved function for this peptide. Experimental data provide increasing evidence that CCAP plays an important role in regulation of insect and crustacean ecdysis. However, targeted ablation of CCAP expressing neurons in *Drosophila* did not dramatically affect larval and adult ecdyses, but most animals showed severe defects during pupation and died in the pupal stage (Park *et al*., 2003b; for more details, see Section 3.1.6.3.5). The defect appears to result from an inability of the circulatory system to generate blood pressure sufficient to produce head eversion, which is necessary for successful pupation.

3.1.2.6. Myoinhibiting (Myoinhibitory) Peptides

This family of closely related neuropeptides with gut myoinhibitory activity has been identified in widely divergent insect species using various types of bioassays. They have been variously referred to as myoinhibiting peptides (MIPs) or allatostatin B peptides. The first of these was identified in cephalic ganglia of *L. migratoria*, where it was found to inhibit rhythmic contractions of the isolated hindgut and oviduct of this species and of the hindgut of the cockroach *Leucophaea maderae* (Schoofs *et al*., 1991). Another MIP inhibiting visceral muscle contractions was isolated from the CC–CA extracts of the cockroach *P. americana* (Predel, 2001). Several peptides closely related to MIPs subsequently were isolated from brains of *Gryllus bimaculatus* and *Carausius morosus*. Because they suppress juvenile hormone (JH) biosynthesis by the CA of adult crickets *in vitro*, they were named allatostatins type B. However, they failed to suppress JH production in *Carausius* (Lorenz *et al*., 1995; Lorenz *et al*., 2000). Since these peptides originally were named on the basis of gut inhibitory activity, these are referred to as MIP peptides.

In holometabolous insects, six similar MIP neuropeptides (Mas-MIP I–VI) were identified from extracts of adult *M. sexta* abdominal ganglia. The relative quantities of these peptides were conspicuously different, with ratios of 5 (MIP I):2.5 (MIP IV):1 (MIPs II, III, IV, and VI). Each peptide suppressed spontaneous contractions of the isolated hindgut (ileum) of adult *Manduca* (Blackburn *et al*., 1995, 2001). Recently, a “prothoracicostatic hormone” (PSTH) identical to Mas-MIP1 was isolated from larval brains of *B. mori* (see **Chapter 3.2**). This peptide inhibits production of ecdysteroids from prothoracic glands *in vitro* (Hua *et al*., 1999). The cDNA precursor for *Bombyx* MIP (PSTH) encodes 13 copies of seven closely related peptides (GenBank

Accession No. AB073553). Interestingly, the precursor contains five copies of MIP I (PSTH 1), two copies of MIP II and MIP V, and single copies of the other MIPs. This stoichiometry likely explains the different amounts of MIPs found in *Manduca* CNS extracts (Blackburn *et al*., 2001). Finally, a *Drosophila* cDNA precursor encoding five deduced peptides closely related to MIPs has been described (Williamson *et al*., 2001). Primary structures of MIPs from various insect species are shown in **Figure 11**.

Immunohistochemical staining has been used to detect neurons producing MIPs from several species in the cephalic ganglia of cockroaches *Leucophaea maderae* and *P. americana*, moth *Mamestra brassicae*, fly *Neobellieria bullata*, and in the entire CNS of *L. migratoria*. MIP immunoreactivity was detected in the brain medial neurosecretory cells projecting to the CC–CA complex and in a few smaller neurons in the brain and ventral nerve cord (Veelaert *et al*., 1995; Schoofs *et al*., 1996; Predel, 2001).

Using *in situ* hybridization with antisense riboprobes, MIP expression was detected in the CNS neurons of *Drosophila*. Four pairs of medial cells were revealed in the protocerebrum and a pair of lateral neurons was stained in each abdominal neuromere (Williamson *et al*., 2001). Paired neurons in the fused abdominal ganglion are probably homologs of the IN-704, which show MIP immunoreactivity in abdominal ganglia (AG1–7) of *Manduca* and *Bombyx* (Davis *et al*., 2003). Detection of MIPs in IN-704 (**Figure 9d**) is of particular interest, since they also express CCAP (Davis *et al*., 1993; Loi *et al*., 2001) an important central signal for the ecdysis motor program. Release of MIPs within CNS ganglia is indicated by decreased staining in the IN-704 and nerve arborizations after ecdysis (Davis *et al*., 2003). The role of MIPs at ecdysis is described in Section 3.1.6.1.3. In larval stages of both moths, MIP-immunoreactivity was also located in small neurons in the brain, as well as in large neurons innervating the hindgut from the terminal abdominal ganglion (TAG; Davis *et al*., 2003). None of these neurons appear to release MIPs into the hemolymph in larvae. However, adult brains show MIP staining in two pairs of the medial neurosecretory cells, which are known to produce diuretic hormone and allatostatins (Davis *et al*., 2003).

The likely source of hemolymph-borne MIPs in larvae is a pair of epiproctodeal glands, each composed of two large neurosecretory cells. Both glands are located on lateral sides of the hindgut and are part of the proctodeal nervous system (Reinecke *et al*., 1978; Zitnan *et al*., 1995a; Davis *et al*., 2003). Interestingly, gradual accumulation followed by a rapid decrease of MIP-IR was observed during the 4th larval instar. A marked decrease of MIP-IR coincides with the decline of ecdysteroid titer at the end of the instar. This suggests that MIPs may initiate the decline of ecdysteroids from prothoracic glands at that time. Indeed, MIP I (PTSH) inhibits ecdysteroid production from prothoracic glands of *B. mori in vitro* (Hua *et al*., 1999). Another possible source of circulating MIPs may come from a subset of midgut (gastric) endocrine cells, which show immunohistochemical staining in *Manduca* and *Bombyx* (Spalovská-Valachova and Zitnan, unpublished data). Expression of MIPs in these endocrine cells was confirmed by *in situ* hybridization in the *Drosophila* midgut (Williamson *et al*., 2001).

3.1.2.7. Corazonin

Corazonin was originally isolated as a potent cardioaccelerator from the CC of the cockroach *P. americana* (**Figure 14**), (Veenstra, 1989). The identical peptide was later found in other cockroaches, cricket and two moths, but surprisingly the peptide exhibited no cardioacceleratory activity in these insects (Veenstra, 1991; Hua *et al*., 2000; Predel, 2001). Another form of corazonin with a substitution at position 7 (His^{7}-corazonin) was identified

Corazonin	pQTFQYSRGWTNamide
His-corazonin	pQTFQYSHGWTNamide
Drosophila, Neobellieria	TDVDHVFLRFamide
Schistocerca, Locusta	PDVDHVFLRFamide
Locusta	ADVGHVFLRFamide
Leucophea	pQDVDHVFLRFamide
Manduca F10	pQDVVHSFLRFamide
Manduca F24	VRDYPQLLDSGMKRQDVVHSFLRFamide
Manduca F39	YAEAAGEQVPEYQALVRDYPQLLDSGMKRQDVVHSFLRFamide
Manduca F7D	DPSFLRFamide
Manduca F7G	GNSFLRFamide

Figure 14 Amino acid sequences of corazonins and FLRFamide-related peptides. Green represents sequence identity, yellow sequence similarity.

in grasshoppers, *S. americana*, *S. gregaria*, *L. migratoria*, and stick insect *C. morosus* (**Figure 14**) (Veenstra, 1991; Predel *et al*., 1999; Tawfik *et al*., 1999). The gene and cDNA encoding corazonin were described in *D. melanogaster* and in the wax moth *Galleria mellonella*, respectively. Preprocorazonin of both species contains corazonin and a longer, nonamidated peptide, corazonin precursor-related peptide of unknown function (Veenstra, 1994; Hansen *et al*., 2001).

Antisera to corazonin were used to identify corazonin producing neurons in the CNS of representatives from eight diverse insect orders (Veenstra and Davis, 1993; Cantera *et al*., 1994; Roller *et al*., 2003). In all insects except the beetle, corazonin immunoreactivity occurs in a cluster of prominent ipsilateral-projecting neurosecretory cells in the protocerebrum and in smaller cells, mostly interneurons, in the ventral nerve cord (Roller *et al*., 2003). Northern blots and *in situ* hybridizations confirmed expression of the corazonin gene in four pairs of brain lateral neurosecretory cells in *G. mellonella*, while cells in the ventral ganglia, fat body, gut, and several other organs failed to show expression of corazonin mRNA (Hansen *et al*., 2001). The brain lateral neurosecretory cells producing corazonin have been identified as Ia_1 cells originally described in *Manduca* (**Figure 9a**; Copenhaver and Truman, 1986b; Homberg *et al*., 1991; Zitnan *et al*., 1995b). Expression of corazonin in these lateral cells of moths attracted the attention of several research groups, since these cells appear to be involved in the regulation of circadian rhythms (Sauman and Reppert, 1996). Indeed, the circadian clock protein Period (Per) is colocalized with corazonin in Ia_1 cells of *Manduca* as detected by the double staining with a monoclonal antibody to Per and rabbit antiserum to corazonin (Wise *et al*., 2002). These cells appear to be involved in the regulation of circadian rhythms in the silkmoth *Antheraea pernyi* (Sauman and Reppert, 1996). *Manduca* larvae with surgically ablated type Ia_1 cells are not able to enter diapause after pupation, indicating that some of these cells may control photoperiod-induced pupal diapause (Shiga *et al*., 2003).

His^7 corazonin was isolated from the CC of two locusts as a dark pigment inducing agent controlling phase polymorphism (melanization) in an albino mutant and in normal nymphs of *L. migratoria* (Tanaka and Pener, 1994; Tawfik *et al*., 1999). But while corazonin also induces cuticle melanization in other locust species, this effect does not appear to extend beyond this group (Tanaka, 2000). Implantation of CNS ganglia, or injection of their extracts from representatives of 13 insect orders induced cuticle melanization in the albino locust, but failed to evoke melanization in donor species (Tanaka, 2000; Roller *et al*., 2003). These data indicate that most insects produce corazonin, but its role in cuticle melanization is specific for locusts (Tanaka, 2001). Injection of corazonin also prolonged the spinning period at the end of the 5th larval instar and delayed adult development in pupae of *B. mori* (Tanaka *et al*., 2002), but the functional significance of these effects is not clear.

The identification of corazonin in many different insect groups, together with its localization by immunohistochemical staining and bioassays suggest that corazonin is a highly conserved and widespread insect neuropeptide, but that its physiological function is still unclear. Recent experiments suggest that corazonin induces the release of PETH and ETH from Inka cells of moths to initiate the ecdysis behavioral sequence (Kim *et al*., 2004; see Section 3.1.5.2).

3.1.2.8. FLRFamide-Related Peptides

Since the discovery of the first tetrapeptide FMRFamide (Phe-Met-Arg-Phe-amide) in the mollusk *Macrocallista nimbosa* (Price and Greenberg, 1977), a large number of FMRFamide-related peptides (FaRPs) have been identified in various invertebrates and vertebrates (Gäde, 1997). These peptides are encoded by multiple genes and expressed in various types of cells. For example, at least four *Drosophila* genes encode N-terminally extended –RFamides, including FMRFamides, myosuppressins, sulfakinins, and neuropeptide F (NPF). These peptides are produced by specific cells of the CNS and gut (Nambu *et al*., 1988; Schneider and Taghert, 1988; O'Brien *et al*., 1991; Schneider *et al*., 1991; Nichols *et al*., 1995, 1997; Nichols and Lim, 1996; Benveniste and Taghert, 1999; Brown *et al*., 1999); for recent reviews see: Nichols *et al*., 2002; Nichols, 2003). Differential expression and effects of these peptides indicate their involvement as neuromodulators and neurotransmitters in distinct functions.

Recent studies suggest participation of *Manduca* FLRFamides in the regulation of ecdysis, and consequently our discussion will be limited to these. Quantitative analysis of three FLRFamides (F7G, F7D, and F10; **Figure 14**) originally isolated from the *Manduca* CNS (Kingan *et al*., 1990, 1996) showed that larval, pupal, and adult ecdyses are associated with decline of these peptides in ventral ganglia (Miao *et al*., 1998). Since FLRFamide-like staining was found in fine fibers innervating skeletal and visceral muscles, these authors proposed that release of these peptides could modulate muscle

performance during the ecdysis behavioral sequence. Consistent with this hypothesis, elevated levels of F7D and F10 were detected in the hemolymph 10 min after adult emergence (Kingan *et al.*, 1996). The release of F10 at ecdysis also is suggested by the considerable reduction of its precursor mRNA levels in the ventral nerve cord following each larval, pupal, and adult ecdysis (Lu *et al.*, 2002). Independent experiments suggest that F10 from the nervous system and its extended isoforms, F24 and F39, isolated from the midgut (Kingan *et al.*, 1990; Kingan *et al.*, 1997b) induce the ecdysis sequence when injected into pharate 4th or 5th instar *Manduca* larvae (Spalovská-Valachová, Kingan, Adams, Zitnan, unpublished data; see Section 3.1.5.2).

Decapeptides closely related to F10 have been isolated from the CNS of cockroaches, locusts, moths, and flies (Gäde, 1997). The gene or cDNA precursors encode only a single copy of this peptide in the cockroach *D. punctata*, fruitfly *D. melanogaster*, and moths *P. unipuncta* and *M. sexta* (Donly *et al.*, 1996; Bendena *et al.*, 1997; Vanden Broeck, 2001; Lu *et al.*, 2002). Other FaRPs therefore are encoded by different genes, which have been previously identified in *Drosophila* (Nichols *et al.*, 2002). Antisera to FMRFamide, which cross-react with all FaRPs, indicated a widespread distribution of these peptides in the CNS, enteric (stomatogastric) nervous system and midgut (gastric) endocrine cells of many insects (Copenhaver and Taghert, 1989; Homberg *et al.*, 1990; Zitnan *et al.*, 1995a; Sehnal and Zitnan, 1996).

Using *in situ* hybridization, reaction with F10 riboprobes occurs in a subset of FMRFamide-immunopositive neurons in the CNS (Lu *et al.*, 2002). Notably, this includes neurosecretory cells known as type Ia_1, IIa_5, and III (Copenhaver and Truman, 1986a; Zitnan *et al.*, 1995b). The presence of F10 in Ia_1 cells is of special interest, since double immunohistochemical staining indicates that F10 is colocalized with corazonin (**Figure 9a** and **b**) and the clock protein Per in these neurons (Wise *et al.*, 2002; see **Chapter 4.11**). Circadian expression of Per and Timeless (Tim) proteins in Ia_1 cell homologs in the moth *Antheraea pernyi* have been implicated in timing of the gated larval hatching from eggs (Sauman *et al.*, 1996), as well as adult emergence and daily activity of this moth (Sauman and Reppert, 1996; see **Chapter 4.11**). No daily rhythm in Per expression has been detected in *Manduca* (Wise *et al.*, 2002), but it is possible that Per may control the release of F10 and corazonin to initiate gated ecdysis or eclosion observed in several moths and butterflies (Truman, 1972, 1974; Zitnan, unpublished data).

Differential processing of the *Manduca* F10 protein precursor is indicated by the presence of F10 in the CNS and anterior/posterior midgut innervated by enteric and central neurons, while most F24 and F39 immunoreactivity was isolated from the middle/posterior midgut containing hundreds of immunopositive endocrine cells (Zitnan *et al.*, 1995a; Kingan *et al.*, 1997b). These results suggest that F24 and F39 are completely processed to F10 in neurons of the CNS and enteric plexus, whereas midgut endocrine cells produce the partially processed isoforms F24 and F39. However, using *in situ* hybridization, expression of mRNA encoding the precursor for F10, F24, and F39 was detected only in midgut endocrine cells, while no such F10 transcript was found in neurons of the frontal ganglion and enteric plexus of *Manduca* (Lu *et al.*, 2002). Similarly, expression of mRNA encoding F10–39 homologs was only observed in CNS neurons and midgut endocrine cells of the adult moth *P. unipuncta* (Lee *et al.*, 2002). These data suggest that FMRFamide-IR neurons in the frontal ganglion and enteric plexus produce peptide(s) different from F10. In contrast, expression of mRNA encoding the cockroach homolog of F10 (leucomyosuppressin) was observed in both central and stomatogastric (enteric) neurons as well as in endocrine cells scattered in the anterior midgut epithelium (Fuse *et al.*, 1998). In *Drosophila*, a discrete set of neurons in the CNS and two cells attached to the hindgut were also stained with a specific antibody to the F10 homolog, dromyosuppressin (McCormick and Nichols, 1993).

Closely related FLRFamide decapeptides generally are referred to as myosuppressins since they exhibit inhibitory activity on visceral muscles using *in vitro* bioassays with crop, hindgut, oviduct or heart in several insect species (Robb and Evans, 1990; Fujisawa *et al.*, 1993; Lange *et al.*, 1994; Fuse and Orchard, 1998; Duttlinger *et al.*, 2002). However, the same peptides elicit contractions of skeletal muscles (Kingan *et al.*, 1990; Robb and Evans, 1990; Lange and Cheung, 1999) and also stimulate secretion of digestive enzymes (alpha amylase and invertase) from the gut of the scallop *Pecten maximus*, cockroach *D. punctata*, and beetle *Rhynchophorus ferrugineus* (Nachman *et al.*, 1997; Fuse and Orchard, 1998). Other shorter FLRFamides or FIRFamides derived from different genes show effects opposite to those of the myosuppressins. For example, *Locust* GQERNFLRFamide, AXXRNFIRFamide, and AFIRFamide and *Manduca* heptapeptides F7G and F7D exhibit stimulatory effects on visceral muscles in the same or similar bioassays as described above (Lange *et al.*, 1994; Kingan *et al.*,

1996; Miao *et al.*, 1998). In addition, F7G and F7D were shown to inhibit ion transport through the midgut epithelium in *Manduca* (Lee *et al.*, 1998). Therefore, it is clear that FLRFamides can be both excitatory and inhibitory, depending on the cellular context.

Despite all of this information on the activities of FLRFamides, their authentic physiological functions are not well understood. The authors speculate that inhibitory actions of F10 may suppress functions not related to ecdysis, while stimulatory actions on skeletal muscles and on Inka cell secretion control specific behaviors associated with shedding of the old cuticle. Identification of specific receptors for FaRPs, determination of spatial and temporal expression patterns of these receptors and their specific ligands along with physiological experiments should help to understand the roles of these structurally related, but functionally diverse peptides.

3.1.3. Peptide Receptors in Ecdysis

3.1.3.1. Introduction

Classical approaches to receptor identification have changed dramatically in recent years with publication of entire organismal genomes. Just 20 years ago, receptor identification was accomplished strictly through biochemical approaches, and could only succeed in instances where tissue receptor density was very high. In addition, highly specific ligands often were needed to guide isolation of receptor proteins for N-terminal sequencing. The most famous example is that of the nicotinic acetylcholine receptor, present at 2% of total protein in *Torpedo* electroplax, a modified muscle used to electrocute prey. The snake toxins α-bungarotoxin and cobra toxin were useful in characterization of the receptor, from which N-terminal sequence information was obtained (Raftery *et al.*, 1980). This provided the necessary information for design of oligonucleotides subsequently employed as probes of cDNA libraries. In this manner, the first fully sequenced nicotinic receptor was elucidated (Noda *et al.*, 1983) (see **Chapter 5.3**). Similar approaches quickly led to complete sequences for other members of the ligand-gated channel superfamily as well as ion channels such as the sodium channel, L-type calcium channel, and β-adrenergic receptor, to name a few (Numa, 1989; Betz, 1990).

The identification of G protein-coupled receptors (GPCRs) for amines and peptides began with identification of the β-adrenergic receptor, a 7-transmembrane GPCR related to rhodopsin (Dixon *et al.*, 1986; Lefkowitz and Caron, 1988) (see **Chapter 5.5**). These representative templates then guided identification of new members of each class through construction of degenerate oligonucleotide primers and more recently through employment of the polymerase chain reaction (PCR). Among the first peptide GPCRs to be identified in insects were the tachykinin/neuropeptide Y/galanin-like receptors (Li *et al.*, 1991; Li *et al.*, 1992; Monnier *et al.*, 1992) and the *Manduca* diuretic hormone receptor, elucidated through expression cloning (Reagan, 1994, 1996).

Access to the *Drosophila* and human genomes (Adams *et al.*, 2000) has greatly facilitated the identification of putative peptide hormone receptors based on homology to mammalian hormone GPCRs (Brody and Cravchik, 2000; Hewes and Taghert, 2001; Vanden Broeck, 2001). Entire sets of receptor genes in each class can be recognized through sequence similarities and sorted into categories, and such insights are helping to direct the next era of functional analysis. Through genome analysis, the process of using an "orphan" ligand to probe for its unknown receptor has been reversed. A plethora of orphan receptors are now being cloned and heterologously expressed in oocytes or cell lines, where they function as sensors for libraries of ligands. In some instances, this process has been referred to as "reverse physiology," in which the expressed receptor is used to isolate unknown ligands from tissue extracts (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). This approach has been taken to isolate some of the first insect GPCR-type receptors for neuropeptides (Birgul *et al.*, 1999) (see **Chapter 5.5**). Thus, the data provided via public access to the *Drosophila*, nematode, and human genome projects combined with heterologous expression and reverse physiology approaches have greatly accelerated elucidation of ligand–receptor relationships.

Hewes and Taghert delineated 44 genes likely to encode peptide receptors in *Drosophila* (Hewes and Taghert, 2001). Strategies for "de-orphaning" these receptors have varied, with some laboratories taking a shotgun approach, randomly cloning and heterologously expressing receptors for screening of ligand libraries. These approaches presume that the highest affinity ligand will likely be the authentic physiological transmitter or hormone for that receptor, and in most cases this should turn out to be true. Others have taken a more evolutionary approach toward predicting likely receptors for particular ligands based on a theory of ligand–receptor coevolution. By using multiple databases, known ligand–receptor pairs can be used as guides for predicting the ligands of orphan receptors in other organisms.

The following sections summarize recent progress in the identification of hormone receptors likely to be involved in the ecdysis behavioral sequence, including those for ETH, CCAP, corazonin, FaRPs, and MIPs.

3.1.3.2. Discovery of the First Ecdysis-Related Receptors

Taking a bioinformatics approach to receptor identification, Park *et al*. recently identified a number of GPCRs, including receptors for CCAP and corazonin, that are likely to participate in ecdysis signaling (Park *et al*., 2002b). Ironically, these receptors were discovered in a screen for ETH receptors. A survey of the human genome was performed for peptide ligands having ETH-like -PRXamide [PRXG(KR)] C-terminal amino acid motifs. This sequence motif is characteristic of ETH and a number of other insect peptides, including pheromone biosynthesis activating neuropeptide (PBAN), cardioacceleratory peptides (CAPs), and diapause hormone. Several human peptides share the same motif, namely neuromedin U-25, vasopressin, and pancreatic polypeptide. Further analysis led to the conclusion that genes encoding neuromedin U-25 and vasopressin were most likely to have coevolved on the basis of their C-terminal amino acid sequences. Since corresponding receptors for these peptides already had been identified in the human system, it was possible to group a small number of *Drosophila* orphan GPCRs with neuromedin and vasopressin receptors by sequence homology. These receptor groupings led to a hypothesis that seven *Drosophila* GPCRs closely related to either vasopressin (CG6111, CG10698, CG11325) or neuromedin U (CG8795, CG8784, CG14575, CG9918) were likely candidates for -PRXamide ligand receptors (Figure 15).

Cloning and expression of the *Drosophila* receptors in *Xenopus* oocytes revealed that those in the neuromedin group are highly sensitive to -PRXamide peptides, including PBAN-like *hug* peptides (CG8795), CAP peptides (CG14575), and to a lesser extent, ETHs from both *Drosophila* and *Manduca*. In contrast, *Drosophila* GPCRs in the vasopressin group did not respond to -PRXamide peptides, but instead showed sensitivity to CCAP (CG6111), corazonin (CG10698), and adipokinetic hormone (AKH; CG11325) (Staubli *et al*., 2002). The involvement of CCAP receptors in ecdysis has been suggested by the ability of its ligand CCAP to elicit ecdysis motor patterns in isolated nerve cords of *Manduca* (Gammie and Truman, 1997a, 1999; Zitnan and Adams, 2000). A role for corazonin in ecdysis initiation has been shown recently in *Manduca* (Kim *et al*., 2004). Characteristics of CCAP and corazonin receptors are covered in more detail in the following sections.

Despite the fact that CG8795 responded to both *Drosophila* ETH1, it was considered unlikely to be the authentic ETH receptor. This conclusion was based on the relatively higher sensitivity of this receptor to PBAN-like *hug* peptides, and the fact that sensitivity to ETH was too low to be of physiological significance, based on blood levels of ETHs previously documented (Zitnan *et al*., 1999). Indeed, a recent account has demonstrated that the CG8795 ortholog in *Helicoverpa zea* is a likely

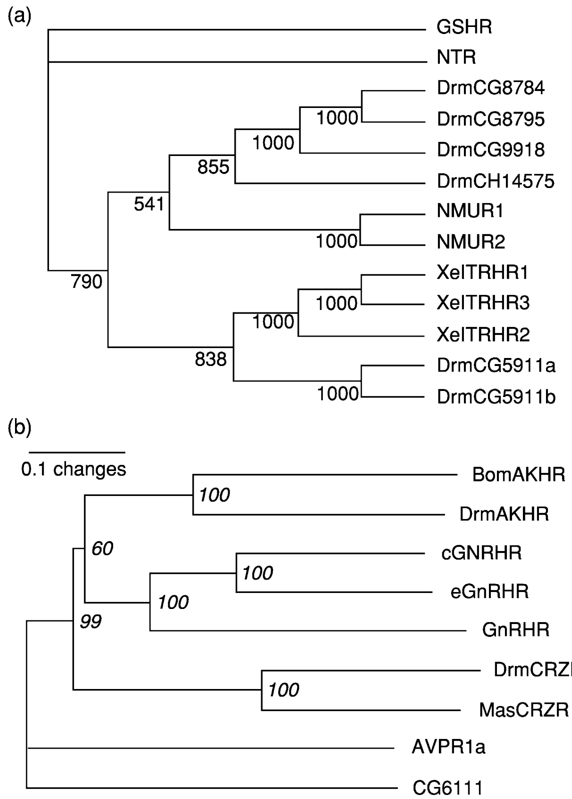


Figure 15 Evolutionary relationships of some ecdysis-related receptors. (a) Two *Drosophila* ETH receptors DrmCG5911a and DrmCG5911b are splice variants of the gene *CG5911*. These putative transcripts are grouped with thyrotropin-releasing hormone receptors (TRHR) in a distance tree with a bootstrapping value of 83.8%. (Data from Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., **2003a**. Two subtypes of ecdysis-triggering hormone receptor in *Drosophila melanogaster*. *J. Biol. Chem. 278* (20), 17710–17715). (b) Phylogenetic tree depicting genes encoding the corazonin receptors from *Manduca* (MasCRZR) and *Drosophila* (DrmCRZR CG10698) and CCAP receptor (CG6111). The number of changes between each sequence is represented by the length of each branch. The percentage of 1000 bootstrap replicates supporting each node is shown. (Data from Kim, Y.J., Spalovska-Valachova, I., Cho, K.-H., Zitnanova, I., Park, Y., *et al.*, **2004**. Corazonin receptor signaling in ecdysis initiation. *Proc. Natl Acad. Sci. USA 101*, 6704–6709.)

PBAN receptor (Choi *et al.*, 2003); Kim and Adams in preparation). This receptor shows no sensitivity at all to *Manduca* or *Drosophila* ETHs. Subsequent to this work, independent characterizations of the corazonin and CCAP receptors have appeared (Cazzamali *et al.*, 2002; Staubli *et al.*, 2002).

Interestingly, none of the GPCRs in the NMU or vasopressin receptor groups have proved to be likely ETH receptor candidates. Rather, the most likely ETH receptor is CG5911, which is distantly related to the NMU group.

3.1.3.3. Ecdysis Triggering Hormone Receptors

A *Drosophila* gene related to the vertebrate TRH receptor and more distantly to the NMU receptor group is *CG5911*. This gene, cloned and heterologously expressed in CHO cells, is highly sensitive and selective to ETHs from both *Manduca* and *Drosophila* (Iversen *et al.*, 2002; Park *et al.*, 2003a). *CG5911* encodes two receptor subtypes by alternative splicing of the 3-prime exon, which are referred to as CG5911a and CG5911b (**Figure 16a**). Preliminary indications are that CG5911a and CG5911b may play different functional roles in recruitment of the ecdysis sequence, as their relative affinities for ETHs and coupling to G proteins appear to be distinct (Park *et al.*, 2003a). Although DrmETH1 and DrmETH2 are roughly equipotent against both subtypes, MasPETH appears to be a more potent activator for CG5911b than MasETH, while the converse is true for CG5911a. At higher concentrations, related peptides having the -PRXamide sequence motif also activate CG5911b, the most active of these being SCP_B, CAP2b-1, hugγ, and HezPBAN (**Figure 16b**). At still higher concentrations, the invertebrate peptide myomodulin and vertebrate peptides NMU and thyrotropin releasing hormone (TRH) activate CG5911b. This pharmacological analysis of CG5911 supports their hypothesized evolutionary relationships with TRH receptors and the NMU receptor groups that are predicted by phylogenetic analysis.

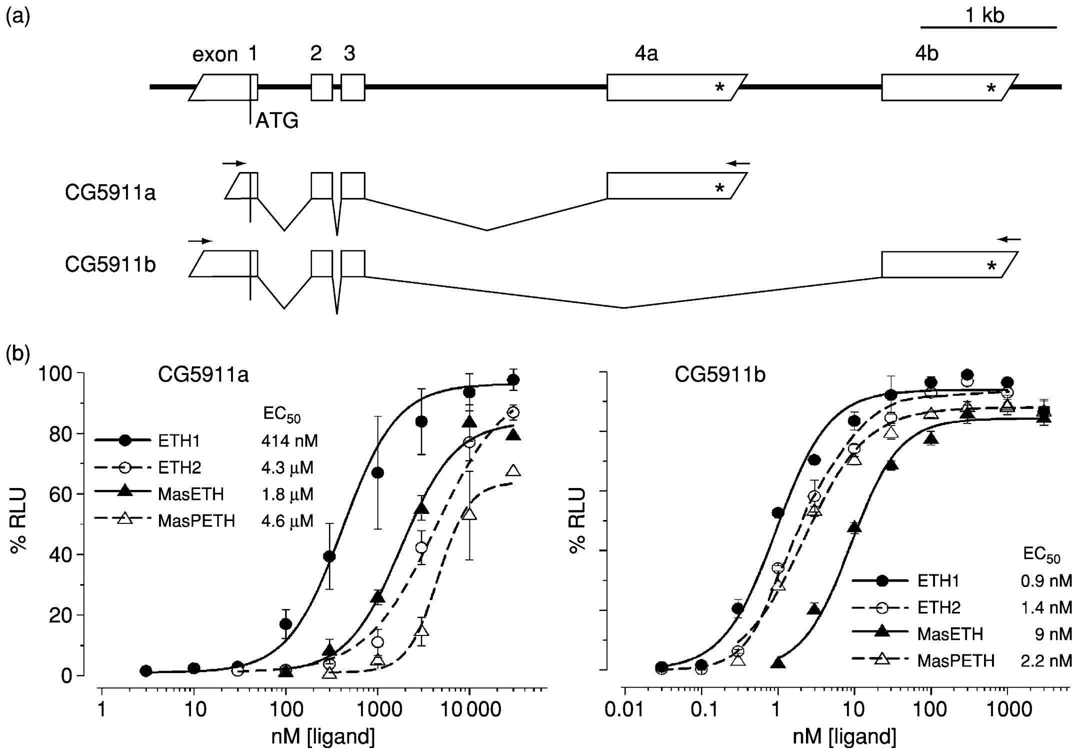


Figure 16 Organization of the ETH receptor gene and pharmacology of the ETH receptor. (a) Genomic structure of ETH receptors (CG5911a and CG5911b) arising from two alternative transcripts with different exons 4a and 4b at the 3′ end of the gene. Transcripts were confirmed experimentally by RT-PCR with the primers (arrows) located on the boundary of start and stop codons in the predicted open reading frames. Each 5′ and 3′ end of the transcript is not determined and left as a slanted rectangular of the exon boxes. (b) Pharmacology of ETH receptor subtypes A and B. Transcripts were expressed in CHO cells with the G protein Gα16 (see Park *et al.*, 2003a for details). Using this heterologous expression system, CG5911a is less sensitive to ETH peptides from *Drosophila* and *Manduca* as compared to CG5911b, perhaps due to suboptimal receptor coupling with the G protein. Note that both *Drosophila* and *Manduca* ETHs are active on both receptors. (Reproduced with permission from Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., **2003a**. Two subtypes of ecdysis-triggering hormone receptor in *Drosophila melanogaster*. *J. Biol. Chem. 278* (20), 17710–17715; © American Society of Biochemistry and Molecular Biology.)

Recent work on the cloning and expression of ETH receptors in *Manduca* indicate the presence of functional orthologs of CG5911 in the CNS (Kim, Cho, Zitnan, and Adams, in preparation). The *Manduca* receptors also occur as two subtypes through alternative splicing and exhibit pharmacological profiles that are generally consistent with those of CG5911a and CG5911b. The *Manduca* receptors show higher ligand specificity compared to their *Drosophila* counterparts. Whereas CG5911b responds to both PBAN-like *hugin* peptides, its *Manduca* ortholog is completely insensitive to PBAN or *hugin* peptides at up to 10 μM concentration. The high specificity and sensitivity of the *Manduca* receptors provides further evidence that CG5911 and its orthologs in other insects function as the physiological ETH receptors. Preliminary evidence from *in situ* hybridization work in both *Drosophila* and *Manduca* indicate the widespread occurrence of ETH receptors in central neurons located in the brain and ventral nerve cord.

3.1.3.4. Corazonin Receptors

Corazonin is a highly conserved and widespread neuropeptide in insects. Although it was initially associated with cardioacceleratory activity in the cockroach *Periplaneta* (Veenstra, 1989), such a function has not been noted in other insects. It also controls coloration changes in locusts, but again such a function has not been observed in other insects. Given its highly conserved structure across widely divergent groups of insects, it is expected to have a more universal function than has been discerned up to now. One avenue for defining such a function would be identification of cells expressing the corazonin receptor.

The *Drosophila* corazonin receptor (CG10698; DrmCRZR) occurs in the vasopressin group of GPCRs, and is related to the AKH receptor. Both receptors are thought to have evolved from a common ancestor of the vertebrate gonadotropin releasing hormone receptor (Cazzamali *et al*., 2002; Park *et al*., 2002b). Its sensitivity to corazonin is in the low nanomolar range when expressed either in *Xenopus* oocytes or CHO cells. However, studies of the *Drosophila* receptor thus far have not provided additional clues regarding the functional significance of corazonin signaling.

Recently, the DrmCRZR ortholog in *Manduca* was cloned, expressed, and localized in Inka cells of the epitracheal endocrine system as a prelude to detailed physiological studies (Kim *et al*., 2004). Corazonin receptor cDNA in *Manduca* encodes a protein of 436 amino acids with seven putative transmembrane domains. The *Manduca* corazonin receptor (Mas-CRZR) appears to bind corazonin with a higher affinity than does the *Drosophila* CRZR, with an EC_{50} value of ~200 pM when expressed in *Xenopus* oocytes and ~75 pM when expressed in CHO cells. Mas-CRZR exhibits high sensitivity and selectivity for corazonin.

Later in this chapter (Section 3.1.5.2), data showing that corazonin triggers ecdysis in *Manduca* larvae are reviewed. Following these observations, the corazonin receptor was located in Inka cells, the sole source of PETH and ETH, by Northern analyses. These findings provide further evidence implicating corazonin as an ecdysis signaling peptide upstream of PETH and ETH, and raises new questions regarding the respective roles of corazonin and EH in the peptide signaling cascade that controls this critical process in insect development.

3.1.3.5. CCAP Receptor

As mentioned in Section 3.1.3.2, the *Drosophila* gene *CG6111* encodes the likely CCAP receptor (Park *et al*., 2002b; Cazzamali *et al*., 2003). The first reported expression of this receptor was in *Xenopus* oocytes, where it responded to concentrations of CCAP above 10 nM and also showed considerable sensitivity to AKH (Park *et al*., 2002b). A different splice variant expressed in CHO cells showed much higher sensitivity and selectivity for CCAP (EC_{50} ~50 pM); no response to AKH or any other peptide was observed (Cazzamali *et al*., 2003). Differences in the potency and selectivity of these receptor isoforms may be related to the differential splicing of exon 7, or to the expression system used. A CCAP receptor ortholog with very high sequence similarity to the *Drosophila* gene was located in the *Anopheles* genome also (Cazzamali *et al*., 2003).

3.1.3.6. MIP Receptors

Using a novel expression and bioassay strategy, Taghert and colleagues deorphaned CG14484, a GPCR related to bombesin (Johnson *et al*., 2003). Instead of relying on elevation of intracellular calcium as an indication of GPCR activation, these workers utilized a β-arrestin-GFP reporter, which provides a measure of GPCR desensitization in response to ligand exposure. They showed this receptor to be sensitive to 1 μM *Drosophila* "allatostatin-B1" referred to here as *Drosophila* MIP-I (AWQSLQSSWamide).

3.1.3.7. Receptors for FMRFamide-Related Peptides

Receptors specific for several groups of FMRFamide-related peptides (FaRPs) have been recently identified in *Drosophila* and *Anopheles* (Brody and

Cravchik, 2000; Hewes and Taghert, 2001; Cazzamali and Grimmelikhuijzen, 2002; Garczynski *et al.*, 2002; Egerod *et al.*, 2003). These include receptors for peptides having C-terminal amino acid sequence motifs -FMRFa, -FLRFa, -MRFa, or -RFa, as in NPF.

Recent evidence indicates that F10, which possesses a C-terminal-FLRFa motif (**Figure 13**), can function as an ecdysis initiator upon injection into *Manduca* pharate larvae (see Section 3.1.5.1). Two *Drosophila* receptors (CG8985 and CG13803) recently cloned and expressed in CHO cells show high sensitivity and selectivity for the *Drosophila* peptide Dromyosuppressin (TDVDHVLFLRFa), the *Drosophila* homolog of F10 (Egerod *et al.*, 2003). These receptors were unresponsive to FMRFamide, *Drosophila* NPF-1 (AQRSPSLRLRFamide), and perisulfakinin (EQFDDY(SO_3H)GHMRFamide). It remains unclear how selective the receptor is for peptides ending with the –FLRFamide motif.

The FMRFamide receptor has been identified as CG2114 (Cazzamali and Grimmelikhuijzen, 2002; Meeusen *et al.*, 2002). The NPF receptor has been identified as CG1147, a member of the rhodopsin receptor family (Garczynski *et al.*, 2002). So far, neither the FMRFa nor NPF receptors and corresponding ligands have been demonstrated to take part in ecdysis signaling.

3.1.3.8. Summary

At the time of this writing, more than half of the 44 receptors predicted as peptide hormone receptors have been cloned, expressed, and associated with putative physiological ligands. The association of these receptors with high affinity ligands is the first step in defining their physiological functions.

3.1.4. Ecdysteroid Control of Gene Expression in Preparation for Ecdysis

3.1.4.1. Introduction

The transition from feeding to pharate stage during each stadium of development is induced by rising levels of ecdysteroids. This event triggers successive bouts of gene expression that have been referred to as early, early–late, and late phases, according to the appearance of sequential puffs at various positions on polytene chromosomes of *Drosophila* (Ashburner *et al.*, 1974). In this section, early and late gene expression events in *Manduca* that are involved in assembly of the peptide signaling cascade that regulates ecdysis are described. Early events that occur around the peak of ecdysteroid levels include acquisition of CNS sensitivity to ETH and increased production of ETH in Inka cells. Although Inka cells accumulate large amounts of ETH peptides during this phase, they are incapable of releasing their contents until ecdysteroids decline to low levels. Acquisition of competence to release ETH is associated with late gene expression events that occur once ecdysteroids decline to <0.1 μg ml^{-1} (Kingan and Adam, 2000).

3.1.4.2. Effects of Elevated Ecdysteroid Levels

3.1.4.2.1. Induction of CNS sensitivity to ETH In *Manduca,* only pharate stages are sensitive to PETH and ETH. Injection of these peptides into freshly ecdysed, feeding, or wandering larvae, or ecdysed pupae and adults never results in pre-ecdysis or ecdysis behaviors. Animals become sensitive and show specific behavioral responses to PETH and ETH some 1–2 days prior to ecdysis depending on the particular stage. The onset of this sensitivity has been determined for pharate 5th instar larvae and pharate pupae (Zitnan *et al.*, 1999; Zitnanova *et al.*, 2001). Pharate larvae become responsive to PETH or ETH injections at the time of peak ecdysteroid levels, which corresponds to initiation of head capsule slip (30 h prior to ecdysis). These animals respond to ETH injection with pre-ecdysis behavior only, but after the head capsule is completely slipped 2 h later, ETH injection induces the entire behavioral sequence in most animals. ETH treatment of pharate pupae elicits ecdysis behavior about 48 h prior to expected ecdysis (**Figure 17a**). These animals do not show pre-ecdysis contractions. The latency from ETH injection to the onset of ecdysis behavior is progressively shorter as animals approach the time of normal ecdysis (Zitnanova *et al.*, 2001). In *Bombyx*, sensitivity to ETH begins at 30 h prior to ecdysis of 5th instar larvae, which again corresponds to head capsule slippage, 24 h before ecdysis of nonspinning pharate pupae, and in developing adults already 6–8 days prior to eclosion (Zitnan *et al.*, 2002); Zitnan, unpublished data). These studies showed that sensitivity to PETH and ETH is correlated with the appearance of the highest ecdysteroid titers in the hemolymph (**Figure 17a**). The stage-specific differences in appearance of sensitive periods are remarkable, and reflect occurrence of the ecdysteroid peak at different times in the hemolymph during development. These results also suggest that the CNS motor program is fully competent to perform complete pre-ecdysis and ecdysis behaviors far earlier than previously indicated in experiments with EH (Copenhaver and Truman, 1982).

These aforementioned experiments lead to the hypothesis that the behavioral response to ETH is

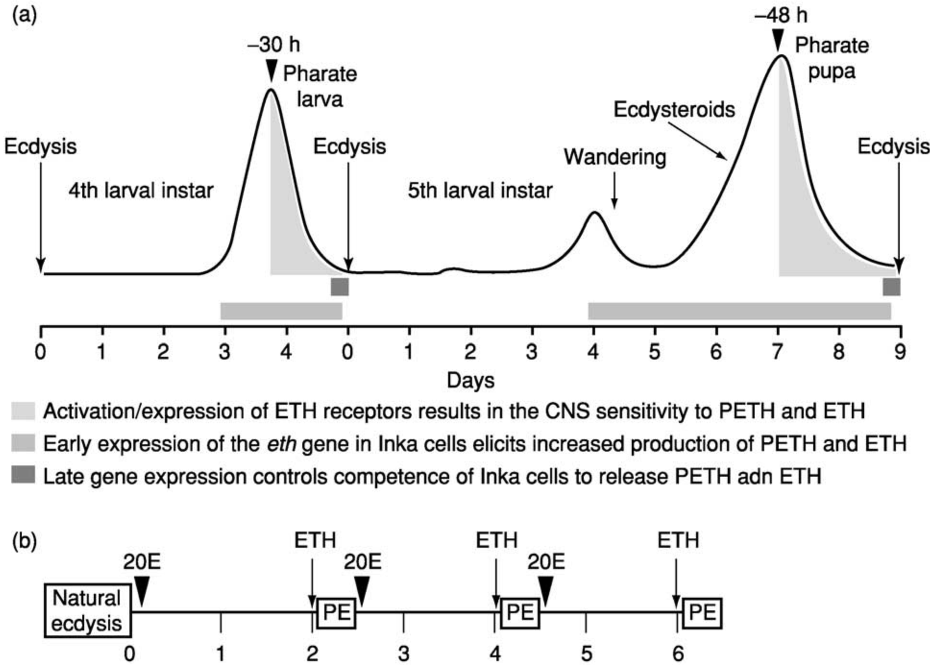


Figure 17 Ecdysteroid action on the *Manduca* CNS and Inka cells prior to larval and pupal ecdyses of *Manduca*. (a) Freshly ecdysed, feeding, or wandering larvae, which contain low ecdysteroid levels in the hemolymph are insensitive to ETH. Peak ecdysteroid levels in the hemolymph coincide with the onset of CNS sensitivity to ETH. Light grey areas illustrate time periods of CNS sensitivity to ETH. Injection of this peptide induces ecdysis sequence during this time. High ecdysteroid levels induce expression of the ETH gene, while declining ecdysteroids control secretory competence of Inka cells. (b) Injection of 20-hydroxyecdysone (20E) into isolated abdomens induces CNS sensitivity to ETH in 1–2 days. Ecdysteroid treatments (20E, arrowheads) followed by ETH injections (arrows) 2 days later induced repeated onset of pre-ecdysis behavior (PE). (Modified from Zitnanova, I., Adams, M.E., Zitnan, D., **2001**. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. *J. Exp. Biol. 204* (Pt 20), 3483–3495.)

enabled by exposure to high ecdysteroid levels. To test this, *Manduca* 5th instar larvae were examined for their ability to respond to ETH injection at various stages following treatment with ecdysone, 20-hydroxyecdysone (20E), or the ecdysteroid agonist (tebufenozide, RH-5992). The results of these experiments showed that pretreatment with steroid or steroid agonist induced ETH sensitivity in freshly ecdysed intact larvae or isolated abdomens of *Manduca* within 1–2 days (Zitnanova *et al*., 2001). ETH sensitivity was detected by injection of the peptide, which induced pre-ecdysis and in some cases ecdysis behaviors in steroid treated animals. Indeed, repeated injections of 20E followed by ETH into isolated abdomens induced the behavioral response (pre-ecdysis) three times within 6 days (Figure 17b; Zitnanova *et al*., 2001). Direct action of 20E on the CNS was demonstrated by *in vitro* experiments. The CNS dissected from freshly ecdysed or feeding larvae was incubated with physiological levels of 20E for 24–28 h. Addition of ETH into the incubation medium elicited typical ecdysis bursts in dorsal nerves of these isolated nerve cords (Zitnan *et al*., 1999; Zitnanova *et al*., 2001).

These experiments suggest that ecdysteroids act directly on the CNS to induce sensitivity to ETH. Mechanisms controlling ecdysteroid-induced CNS sensitivity to ETH are not known. However, it seems likely that high ecdysteroid concentrations cause expression of the PETH and ETH receptors in the CNS, or alternatively some critical downstream step in signal transduction pathways leading to activation of these receptors.

3.1.4.2.2. Stimulation of ETH synthesis in *Manduca* and *Bombyx* During ecdysis, Inka cells release most, if not all, of their peptide stores. Depletion of peptide stores is accompanied by a corresponding decrease in cell volume. Following ecdysis, cell volume and PETH and ETH content increase gradually during the subsequent feeding stage. A sharp increase in PETH and ETH production occurs upon appearance of ecdysteroids to signal onset of the next molt. The concomitant rise of hemolymph ecdysteroids and HPLC-purified peptides in Inka cells has been quantified by enzyme immunoassays in *Manduca* pharate 5th instar larvae. Elevation of peptide content in Inka cells was

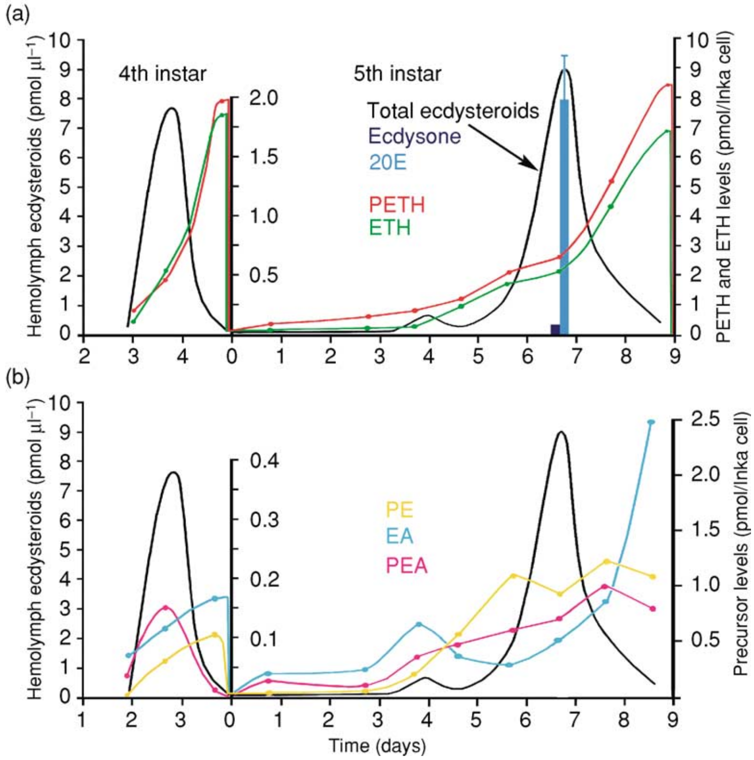


Figure 18 Ecdysteroids stimulate production of PETH, ETH, and their precursors in *Manduca* Inka cells. (a,b) Only small amounts of peptides and their precursors are present in Inka cells during low ecdysteroid levels in 4th and 5th instar larvae. Elevation in ecdysteroid levels is associated with increased production of PETH, ETH, and all precursor forms (PE, EA, PEA). Fully processed peptides and EA reached the highest levels on the last day of each instar, while levels of PE and PEA decreased prior to each ecdysis due to precursor processing. The ratio of ecdysone and 20E in the prepupal peak (5th instar) was 1:20. Each peptide determination represents the mean ± SD of 3–4 sets of 20–40 epitracheal glands. Each ecdysteroid determination represents the mean ± SD of 5–11 hemolymph samples. (Adapted from Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23* (3), 523–535, and Zitnanova I., Adams, M.E., Zitnan, D., **2001**. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. *J. Exp. Biol. 204* (Pt 20), 3483–3495.)

blocked by pretreatment with the transcription inhibitor actinomycin D, indicating the requirement for renewed gene expression (Zitnan *et al.*, 1999). Likewise, onset of the wandering stage and molt of 5th instar *Manduca* larvae involves sharp increases in the rate of PETH and ETH synthesis immediately after each of two successive ecdysteroid pulses (**Figure 18**; Zitnanova *et al.*, 2001). Interestingly, levels of peptide precursors also increased sharply following each steroid pulse, but quickly decreased as they were enzymatically processed to PETH and ETH (**Figure 18**). Consistent with this, injection of 20E or the steroid analog tebufenozide (RH5992) induced expression of the ETH gene and increased production of peptide hormones and their precursors in Inka cells (Zitnanova *et al.*, 2001). These results show that steroid pulses induce sharply increased rates of Inka cell peptide production. This increased expression activity sets the stage for ecdysis behavior at the end of the molt.

3.1.4.2.3. Stimulation of ETH synthesis in *Drosophila* Preliminary studies indicate that, consistent with events described in *Manduca* and *Bombyx*, steroids regulate *eth* expression and ETH production in *Drosophila* (Filippov, Adams, and Gill, unpublished data). ETH production was observed in a transgenic *Drosophila* line, in which an enhanced green fluorescent protein reporter (*egfp*) gene was placed under the control of the *eth* promoter (Park *et al.*, 2002a). Transformed flies exhibited expression of *egfp* only in Inka cells and paralleled expression of *eth*. Therefore, the reporter gene *in vivo* had all the necessary regulatory elements for proper gene expression. When these flies were crossed with the temperature sensitive ecdysone mutant *ecd1* (Berreur *et al.*, 1984), markedly lower expression of *egfp* occurred at the nonpermissive temperature where ecdysone production is suppressed. In particular, a fourfold decrease of reporter gene expression was observed in the absence of the

ecdysone peak that normally occurs during the third instar. These observations indicated that expression of *eth* requires ecdysone. However, attempts to rescue *eth* expression in these deficient larvae by administration of ecdysone failed, indicating that additional factors apparently are required (Filippov, Adams, and Gill, unpublished data).

Further experiments monitored expression of the ETH gene in the embryonic *Manduca* cell line GV1, which was shown to be responsive to ecdysteroid induction (Lan *et al*., 1997). Though the *eth* gene has a strict Inka cell specific expression *in vivo* (Park *et al*., 2002a), it was expressed at low-levels in this cell line. This low-level transcriptional activity of the *eth* gene provided a convenient system to test whether the *eth* promoter is responsive to ecdysteroids. Incubation of the GV1 cells with 1 μM 20E did not change the level of the *eth* gene transcription. As a positive control the expression of the *MsE75* gene, a transcriptional factor encoded by one of the early primary response genes, was enhanced by 20E. These observations indicated that the *eth* promoter in GV1 cells does not respond directly to ecdysone induction, again suggesting that other regulatory factors, possibly unique to the Inka cell, might be involved. In summary, regulation of *eth* gene expression appears to be ecdysone (20E) dependent, but a model based on a direct ecdysone regulatory mechanism (Zitnan *et al*., 1999) requires further study.

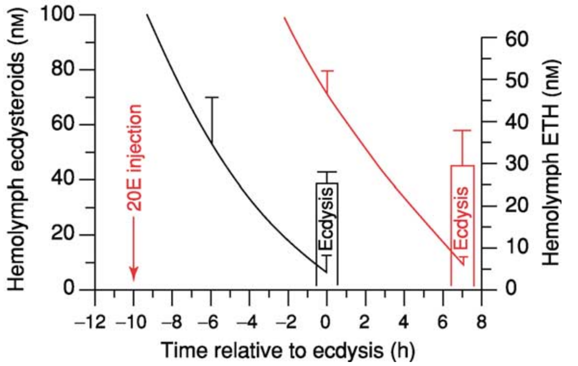


Figure 19 Ecdysteroid decline in the hemolymph is required for ETH release from *Manduca* Inka cells. In normal larvae, ecdysteroid decline to very low levels (black line) leads to ETH secretion (black bar) and onset of the ecdysis sequence at 0 h. Injection of 10 μg 20-hydroxyecdysone (20E) 10 h prior to ecdysis (red arrow) delays ETH release and ecdysis for 6–8 h (red bar). (Reprinted with permission from Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23* (3), 523–535; © Elsevier.)

3.1.4.3. Effects of Declining Ecdysteroids

3.1.4.3.1. Declining ecdysteroids enable secretory competence in Inka cells High ecdysteroid levels that initiate the molt and coordinate gene expression in preparation for the next stage also serve to inhibit onset of the ecdysis sequence. This was first demonstrated by experiments in which prolongation of high steroid levels delayed ecdysis (Slama, 1980; Truman *et al*., 1983; Curtis *et al*., 1984). However, the CNS is capable of responding to ETH by playing out the ecdysis sequence as early as 1–2 days prior to natural ecdysis, which marks the peak of ecdysteroid levels (Zitnan *et al*., 1999). It is now clear that high ecdysteroid levels inhibit ecdysis by preventing ETH release from Inka cells (Kingan and Adams, 2000).

A decline in ecdysteroid levels is necessary for ecdysis to proceed (**Figure 19**). In *Manduca*, the onset of ecdysteroid decline coincides with loss of MIP-IR from the epiproctodeal glands (Davis *et al*., 2003). When ecdysteroids drop to below 0.1 μg ml^{-1}, Inka cells become competent to release ETH. Inhibition of ecdysis by elevated ecdysteroid levels may be a failsafe method that prevents life-threatening premature ecdysis. In pharate pupae of *Manduca*, Inka cells challenged with EH acquire competence to respond with ETH release about 8 h before pupation. Injection of 20-hydroxyecdysone (20E) into precompetent insects delays this acquisition of competence, even though EH evokes accumulation of the second messenger cGMP in Inka cells. This demonstrates that the EH receptor is present in the Inka cell at an early stage, and that secretory competence involves a signaling step downstream of receptor activation. Precompetent glands transferred from a high ecdysteroid environment into steroid-free culture medium acquire competence within about 12 h, but this can be prevented by inclusion of 20E above 0.1 μg ml^{-1} (Kingan and Adams, 2000). Actinomycin D completely inhibits acquisition of competence, showing its dependence on transcriptional events. These findings indicate that declining ecdysteroids trigger late transcriptional events in Inka cells, the product of which is an ETH secretion enabling process downstream of EH receptor activation and cGMP accumulation (Kingan and Adams, 2000).

The low levels of 20E ($\leq$0.1 μg ml^{-1}) permissive for acquisition of competence correspond to those that allow expression of β-FTZ-F1, a transcription factor whose expression occurs in response to high followed by low ecdysteroid levels (Lavorgna *et al*., 1993; Hiruma and Riddiford, 2001). β-FTZ-F1 is implicated in acquisition of competence of various tissues to respond to hormonal cues, including competence of salivary glands to degenerate at

the end of the prepupal period and in the formation of normal cuticle at the end of each molt (Yamada *et al.*, 2000). Interestingly, flies that are either deficient in β-FTZ-F1 or that express it prematurely are unable to undergo successful ecdysis, and show a "double mouth hook" phenotype characteristic of ecdysis deficiency (see also Section 3.1.5.3). The timing of β-FTZ-F1 expression in *Manduca* (Hiruma and Riddiford, 2001) closely corresponds to the period during which Inka cells acquire competence to secrete ETH (Kingan and Adams, 2000), indicating that β-FTZ-F1 might be involved in this process.

3.1.4.3.2. Ecdysteroid decline and VM cell excitability The decline of ecdysteroid levels during the hours preceding ecdysis appears to be critical in the timing of EH release (Johnson *et al.*, 2003). Electrophysiological experiments have shown that the ecdysteroid decline is followed by elevated excitability of the VM neurosecretory cells that produce EH (Hewes and Truman, 1994). Intracellular recordings from these cells showed that they are not spontaneously active either during the feeding stage, when steroid levels are low, or during the early pharate stage, when steroids rise to peak levels. In contrast, subsequent ecdysteroid decline at the end of the pharate stage was followed by increased excitability of the VM neurons. Excitability of these neurons was assayed about 1 h prior to expected ecdysis. Prolongation of elevated ecdysteroid levels by injection of 20E considerably delayed the appearance of excitability in VM neurons. Likewise, treatment with the RNA synthesis inhibitor, actinomycin D (AcD) at the appropriate time (about 6 h prior to ecdysis) blocked VM activity and the ability to release EH. These experiments suggest that late gene transcription induced by ecdysteroid decline is responsible for excitability of VM cells and consequent release of EH (Hewes and Truman, 1994).

A critical factor complicating interpretation of the Hewes and Truman work on VM excitability is the discovery that circulating ETH triggers onset of the ecdysis behavioral sequence about 1 h prior to ecdysis through direct actions on the CNS (Zitnan *et al.*, 1996, 1999). One cellular target of ETH in the CNS appears to be the VM neurons, since their exposure to ETH increases excitability and causes spike broadening (Gammie and Truman, 1999). Hewes and Truman examined VM neuron excitability 0.9 h prior to ecdysis, which is precisely when ETH levels rise in the hemolymph to initiate pre-ecdysis. It is therefore possible that increased VM excitability during the hour prior to ecdysis is caused by ETH.

3.1.5. Regulation of ETH Release

3.1.5.1. Corazonin Elicits Initial Release of PETH and ETH

Recent studies have led to the surprising discovery that, besides EH, additional factors from the CNS induce and midgut induce Inka cell release of ETH. During a search for ETH activity in tracheal extracts of different insects, midgut extracts were employed as intended "negative" controls. Surprisingly, midgut extracts of *Manduca, Bombyx*, *Tenebrio*, *Aedes*, and *Drosophila* larvae induced pre-ecdysis in 20–40 min followed in some cases by ecdysis behavior when injected into *Bombyx* pharate larvae. The relatively long latencies for pre-ecdysis initiation indicated that the midgut factors might act through the release of ETH from Inka cells. Factors with similar activities were found in HPLC-purified extracts of the CNS (Spalovska-Valachova, Takac, Zitnan, unpublished data).

One of the peptides in the CNS extracts has been identified as corazonin (pQTFQYSRGWTNamide; see Section 3.1.2.7). Recent experiments show that corazonin induces pre-ecdysis and ecdysis in pharate larvae of *Manduca*, through a direct action on Inka cells, which express the corazonin receptor and respond to picomolar concentrations of corazonin by secreting PETH and ETH *in vivo* and *in vitro*. Corazonin release into the hemolymph just prior to pre-ecdysis initiation was demonstrated by a sensitive assay using a CHO cell line expressing the corazonin receptor (Kim *et al.*, 2004). These data indicate that corazonin induces initial release of PETH and ETH from Inka cells, which then act on the CNS to activate pre-ecdysis and ecdysis behaviors. Similar results were obtained with corazonin injected into *Bombyx* pharate larvae.

Additional circumstantial evidence for involvement of corazonin in ecdysis control comes from earlier studies (Copenhaver and Truman, 1986b, 1986c). These authors demonstrated "EH activity" in nine ipsilateral brain neurosecretory cells called type Ia, which transport their contents to the CC–CA. Extracts of these cells induced premature onset of ecdysis when injected into *Manduca* pharate larvae. Moreover, electrical stimulation of CC nerves (NCC 1,2) led to a marked increase of "EH activity" released into the medium (Copenhaver and Truman, 1986b, 1986c). Since a subset of type Ia cells (five pairs of type Ia_2 cells) reacted with the antiserum to EH, it was concluded that the activity originated from these neurons. However, later studies revealed that EH is only produced in the brain ventromedial neurons (VM; type V), which do not project to the CC–CA complex in larvae and

pharate pupae (Horodyski *et al.*, 1989; Kamito *et al.*, 1992; Riddiford *et al.*, 1994). Since extracts of ipsilateral cells contained both cell types (Ia_1 and Ia_2), the observed "EH activity" was very likely induced by corazonin and FLRFamide (F10), which are produced in Ia_1 cells and released through the CC–CA complex.

Determination of various FLRFamide levels in different ganglia and neurohemal organs suggest the release of these neuropeptides at ecdysis (Miao *et al.*, 1998). The authors further propose that FLRFamides may modulate contractions of skeletal and visceral muscles during pre-ecdysis and ecdysis behaviors. Recent unpublished results indeed demonstrate that injection of *Manduca* pharate larvae (−6 h) with F10 or its extended isoforms F24 and F39 induces pre-ecdysis and ecdysis within 30–40 min (Spalovská, Zitnan, Kingan and Adams, unpublished data).

Thus, in addition to EH, peptides from the CNS and midgut appear to be involved in regulation of the ecdysis behavioral sequence. These structurally diverse peptides probably control different functions during ecdysis behaviors, but have one thing in common: they act on Inka cells to ensure that their entire hormonal content is completely released during the ecdysis sequence.

3.1.5.2. Eclosion Hormone Elicits Depletion of Inka Cells

3.1.5.2.1. Timing of EH release and action on Inka cells Stereotypic pre-ecdysis and ecdysis behaviors are driven by central pattern generators intrinsic to the CNS. Induction of these behaviors was originally attributed to action of EH on the circadian gated adult eclosion (Truman and Sokolove, 1972). Thus, injection of EH initiates adult eclosion and also ecdysis in earlier instars, but only during a relatively narrow window of sensitivity that arises close to the time of natural ecdysis. (Reynolds *et al.*, 1979; Copenhaver and Truman, 1982; Weeks and Truman, 1984a, 1984b; Truman, 1985). For example, pharate pupal *Manduca* become sensitive to EH at ~8 h prior to natural ecdysis. The CNS was viewed for many years as the likely target for the hemolymph-borne EH. Evidence for this came from experiments demonstrating that semi-isolated ventral nerve cords, when treated with corpora cardiaca extracts containing EH, generate patterned motor output consistent with pre-ecdysis and ecdysis behaviors (Weeks and Truman, 1984a). However, the success of these experiments depended on the presence of an intact tracheal system, ostensibly to oxygenate the highly aerobic nervous system.

It is now recognized that the fully isolated CNS does not respond to hemolymph-borne EH, and that its inability to trigger ecdysis behaviors when injected prior to this sensitive period is due to a lack of competence in Inka cells to release ETH. Recent investigations have shown clearly that EH triggers release of the ETH from Inka cells *in vitro*, but only when they are dissected no earlier than 7–8 h prior to pupation (Kingan *et al.*, 1997a). This time corresponds precisely to *in vivo* EH sensitivity in pharate pupae. Indeed, the requirement for the tracheal system for EH action on the CNS *in vitro* is due to the obligatory presence of a segmentally distributed endocrine system of Inka cells attached to the tracheal tubes near each spiracle (Zitnan *et al.*, 1996). Hemolymph-borne EH is apparently acting on Inka cells to induce the release of PETH and ETH. The beginning of sensitivity to EH and the latencies from injection to behavioral onset in *Manduca* pharate 2nd and 5th instar larvae, pharate pupae, and pharate adults have been described (Reynolds *et al.*, 1979; Copenhaver and Truman, 1982; Truman, 1985; Ewer *et al.*, 1997). Further studies have established that EH is a potent releaser of ETH from Inka cells, and provides a mechanism for the complete depletion of ETH necessary for the transition from pre-ecdysis to ecdysis behavior (Kingan *et al.*, 1997a).

The latency between EH injection and the appearance of ecdysis depends on the stage and time of injection. For example, in the pharate larva, injection of CC–CA extracts containing EH, corazonin, and other neuropeptides 12 h prior to normal ecdysis initiates pre-ecdysis 2–3 h later. However, the onset of ecdysis behavior is not accelerated unless injections are made 3–6 h prior to normal ecdysis. Pre-ecdysis resulting from early injections is often weak and interrupted by quiet periods, and is followed by ecdysis only after several more hours (Copenhaver and Truman, 1982). If injections of extracts containing EH are made closer to the time of normal ecdysis, the latency is correspondingly reduced; injections 2–3 h before normal ecdysis show strong pre-ecdysis behavior within 15–25 min, with ecdysis following about 1 h later. Similar results were obtained with pharate pupae (Truman, 1985). The long latency from hormone injection to appearance of the behavior suggests that EH triggers a cascade of downstream events culminating in ecdysis (Truman, 1992). The longer latencies and weaker behaviors that arise when EH is injected early also suggest that this peptide persists in the hemolymph for hours after injection, but that levels remaining at the time Inka cells are capable of responding are close to the threshold for eliciting behavior.

The natural release of EH into the hemolymph during the ecdysis sequence is somewhat controversial, and difficult to pinpoint with certainty given its very low, biologically active concentration (~2–10 pM; Kingan *et al.*, 1997a). Some clues to the appearance of EH in the hemolymph are provided by the signature appearance of cGMP in Inka cells some 20 min after the onset of pre-ecdysis in *Manduca* larvae (Ewer *et al.*, 1997). Prior to this time, hemolymph levels of ETH are low, but sufficient to cause pre-ecdysis. The appearance of cGMP in Inka cells corresponds to the steep elevation of PETH and ETH in the hemolymph, known to occur upon exposure of Inka cells to EH *in vitro* (Kingan *et al.*, 1997a). These observations suggest that EH is released into the hemolymph about 20–30 min after the onset of pre-ecdysis to induce cGMP production in Inka cells and depletion of PETH and ETH. Also, concomitant loss of EH-immunoreactivity in larval proctodeal nerves, which are neurohemal organs for EH, suggests that this peptide is released at this time (Novicki and Weeks, 1996; Zitnan and Adams, 2000).

3.1.5.2.2. Signal transduction in EH-induced Inka cell secretion EH causes release of ETH from Inka cells *in vitro* at threshold concentrations of ~3–10 pM (Kingan *et al.*, 1997a). EH also elevates cGMP in Inka cells at concentrations similar to those that cause ETH release, and both cGMP and 8-Br-cGMP mimic the action of EH. The *in vitro* secretory response to EH occurs during a narrow window of development, beginning ~8 h prior to pupal ecdysis, which corresponds to the behavioral sensitivity observed previously *in vivo*. However, EH can cause cGMP elevation in epitracheal glands long before they acquire competence to release ETH, suggesting that the EH receptor and initial steps in the signal transduction cascade are in place early in development, well before the behavioral response appears (Kingan *et al.*, 1997a). The absence of a downstream step in the cascade apparently prevents secretion. During natural ecdysis, cGMP levels in Inka cells show an abrupt elevation ~30 min after onset of pre-ecdysis, well after ETH secretion has been initiated. ETH secretion therefore can be viewed as a two-step process, beginning at pre-ecdysis prior to cGMP elevation, followed by a massive release resulting from a logarithmic elevation of cGMP.

EH-induced signal transduction in Inka cells has been examined in some detail, but a full understanding of these events is incomplete at this time (Kingan *et al.*, 1997a; Kingan *et al.*, 2001; Morton and Simpson, 2002). A unique soluble, NO-insensitive guanylate cyclase, MsGC-β3, has been implicated as the likely enzyme mediating the EH-induced elevation of cGMP in target tissues (Morton and Simpson, 2002). When expressed heterologously in COS cells, MsGC-β3 exhibits significant constitutive activity that is not affected by the standard inhibitor of vertebrate soluble guanylate cyclases, ODQ (1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one; Nighorn *et al.*, 1999). The constitutive activity of MsGC-β3 and its insensitivity to NO donors fits the pharmacological profile for EH action on the Inka cell (Kingan *et al.*, 2001), as well as for a group of specialized cells in the sub-transverse nerve region (Morton and Simpson, 2002).

Assuming that MsGC-β3 is responsible for EH-induced cGMP elevation, how is the EH receptor coupled to this soluble enzyme? EH-induced cGMP increases and ETH release from Inka cells are blocked by the specific protein kinase C (PKC) inhibitors tamoxifen and calphostin C (Kingan *et al.*, 2001). While these data suggest that PKC is involved in the activation of MsGC-β3, Kingan *et al.* (2001) found that the cGMP response to EH was unaffected by staurosporine, a broad spectrum kinase inhibitor, causing some uncertainty as to whether PKC is necessary for cyclase activation.

A possible role for IP_3 was also demonstrated by measurement of intracellular calcium in Inka cells following exposure to EH (Kingan *et al.*, 2001). Cytoplasmic calcium increases within 30–120 s of Inka cell exposure to EH, and remains elevated for at least 10 min, corresponding to the time-course of secretion. Since ETH secretion can occur in the absence of extracellular calcium, cytoplasmic calcium elevation presumably originates from intracellular stores. Secretion is increased in a dose dependent manner by the Ca^{2+}-ATPase inhibitor thapsigargin, a treatment that does not elevate glandular cGMP above basal levels. The secretory response to EH is partially inhibited in glands loaded with EGTA, while cGMP levels are unaffected. These findings show that EH activates second messenger cascades in the Inka cell leading to cGMP accumulation and Ca^{2+} mobilization.

In summary, the precise mechanism of cGMP elevation in EH-stimulated Inka cells remains unclear. It could be that phospholipase C activation by EH receptors produces PKC, which in turn activates soluble guanylate cyclase. This model posits PKC activation and calcium liberation upstream of cGMP activation (Morton and Simpson, 2002). However, the inability of broad-spectrum kinase inhibitors such as staurosporine to block cGMP elevation leaves open the possibility of alternative pathways, which are not yet identified (Kingan *et al.*, 2001). The model of Kingan *et al.* (2001; **Figure 30**)

includes activation of protein kinase G (PKG) and protein phosphorylation downstream of cGMP elevation as a parallel pathway with calcium elevation, both of which contribute to ETH secretion. Considerable future work is needed to clarify how cGMP and calcium act to effect release of ETH during the ecdysis sequence.

3.1.6. Orchestration of the Ecdysis Sequence

Insect development from larva to adult involves repeated shedding of old cuticle via a set of hormonally regulated motor movements called the ecdysis sequence. The endocrinology and physiology underlying ecdysis have been studied extensively in several species of moths, which pass through various larval stages and a pupal stage prior to reaching the winged, reproductive adult stage. These include the tobacco hornworm, *Manduca* (Copenhaver and Truman, 1982; Truman, 1985; Zitnan *et al.*, 1996, 1999), giant silkmoths *Hyalophora* and *Antheraea* (Truman, 1972; Truman and Sokolove, 1972; Truman and Riddiford, 1974), and the silkworm *Bombyx* (Adams and Zitnan, 1997; Zitnan *et al.*, 2002). Recent studies have also described the ecdysis sequence in *Drosophila*, where available genetic tools have permitted studies of EH-, ETH-, and CCAP-deficient flies (McNabb *et al.*, 1997; Baker *et al.*, 1999; Park *et al.*, 1999, 2002a, 2003b).

3.1.6.1. Natural and Peptide-Induced Behaviors in *Manduca*

3.1.6.1.1. Embryonic ecdysis behavior During normal development, *Manduca* larvae undergo one embryonic and four postembryonic ecdyses. PETH and ETH production in Inka cells is detectable in embryos at about 45% of embryonic development by enzyme immunoassay and immunohistochemical staining. Peptide levels in these cells reach a peak at about 65% of development. Embryonic ecdysis at 65–70% coincides with depletion of PETH and ETH immunoreactivity in Inka cells indicating that these peptides have been released into the hemolymph. Dissected embryos respond to ETH injection by showing rhythmic contractions that result in sloughing of the embryonic cuticle. These experiments suggest that ETH controls embryonic ecdysis in *Manduca*. By contrast, larval hatching behavior is not associated with ETH release (Hermesman and Adams, unpublished data).

3.1.6.1.2. Natural and ETH-induced larval ecdysis sequence Morphological markers useful for determining the time to ecdysis onset in pharate 2nd to 5th instar *Manduca* larvae have been described (Copenhaver and Truman, 1982; Zitnanova *et al.*, 2001) and are summarized in **Table 1**. The natural ecdysis behavioral sequence in *Manduca* larvae is composed of three distinct patterns initiated in the following order: pre-ecdysis I, pre-ecdysis II, and ecdysis (Weeks and Truman, 1984a, 1984b; Miles and Weeks, 1991; Zitnan *et al.*, 1999). Observations of natural behavior indicate that larvae show occasional weak, dorso-ventral contractions of the last two abdominal segments for 2–5 min interrupted by ~5 min quiet periods within 30 min (designated as intermittent pre-ecdysis I) before launching into robust, continuous pre-ecdysis I behavior. Pre-ecdysis I is characterized by synchronous dorsoventral contractions of all abdominal segments (**Figure 20**), and lasts for 60–70 min (**Figure 21**).

About 20 min after onset of pre-ecdysis I, larvae initiate pre-ecdysis II (posterioventral and proleg contractions) and continue with both behaviors for

Table 1 Timelines of morphological markers in *Manduca sexta*

Time before ecdysis (h)	*Morphological marker*
Pharate 5th instar larva	
30	Molting fluid accumulation in the prothorax followed by slippage of the old head capsule
28	''Head capsule slip'' (HCS), slippage of the old head capsule is completed
10	''Yellow mandibles,'' yellow pigmentation of new mandibles
8	''Brown mandibles,'' darkening of brown mandibles
4–6	Resorption of molting fluid and appearance of air in the old head capsule
1	Natural pre-ecdysis
0	Natural ecdysis
Pharate pupa	
48	Yellowish dorsal thorax
24	''Yellow bars,'' light yellow bars on metathorax
10–12	''Brown bars,'' brown bars on dorsal metathorax
4.0	Resorption of molting fluid and cuticle shrinkage on ventral side of first abdominal segment
3.5	''Anterior shrink,'' distinct fold on lateral and ventral sides of first abdominal segment
2.5	''Posterior shrink,'' shrinkage of the old cuticle covering posterior abdominal segments
1.5	Tanning of the anterior dorsal region of first abdominal segment
1.0	Natural pre-ecdysis
0.5	Tanning of the anterior dorsal region of second abdominal segment
0	Natural ecdysis

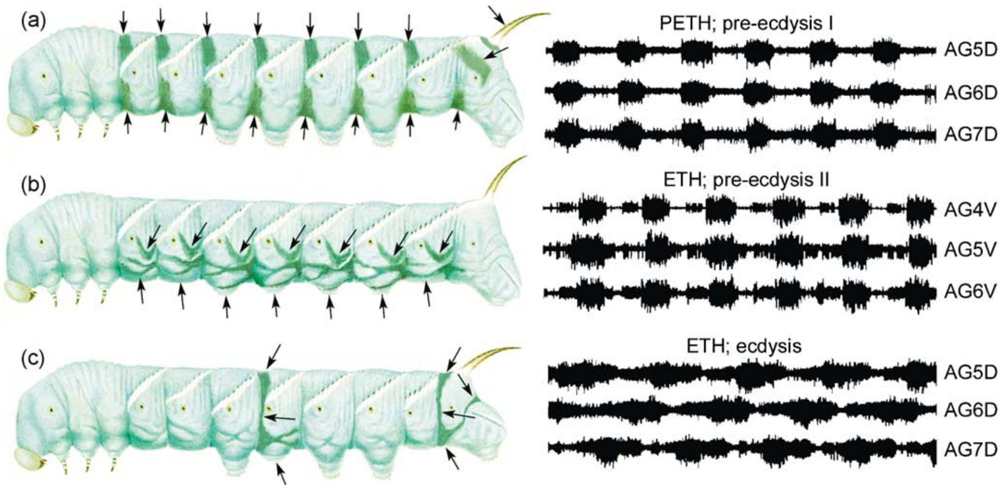


Figure 20 The ecdysis sequence of *Manduca* pharate larvae *in vivo* and *in vitro*. (a) PETH-induced pre-ecdysis I composed of synchronous dorsoventral contractions of abdominal segments (arrows; shaded areas depict muscle attachments) and corresponding synchronous bursts in dorsal nerves of abdominal ganglia 5–7 (AG5,6,7D) of the isolated nerve cord. (b) ETH-induced pre-ecdysis II characterized by posterioventral and proleg contractions (arrows) and corresponding bursts *in vitro* in ventral nerves (AG4,5,6V). (c) Ecdysis peristaltic abdominal contractions associated with proleg retractions (arrows), which result in shedding of the old cuticle and tracheae. Isolated nerve cord shows corresponding ecdysis bursts in dorsal ganglia (AG5,6,7D). (Modified from Zitnan, D., Adams, M.E., **2000**. Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioural sequence. *J. Exp. Biol. 203*, 1329–1340.)

about 40 min (Figure 21). During this time, posterioventral and proleg contractions alternate with dorsoventral contractions (Figure 20). About 1 h after initiation of pre-ecdysis I, larvae switch to ecdysis behavior, characterized by successive anteriorly directed peristaltic movements of abdominal segments and proleg retractions (Figure 20). Ecdysis movements last for about 10–12 min (Figure 21), serving first to rupture the old cuticle along the anterior midline of the thorax and subsequently to shed the cuticle with attached old tracheae.

Injection of PETH and ETH elicits pre-ecdysis and ecdysis behaviors identical to those observed under natural conditions. Likewise, exposure of the isolated CNS to these peptides elicits motor patterns closely corresponding to *in vivo* behaviors (Zitnan *et al*., 1999; Zitnan and Adams, 2000). These studies confirm that PETH and ETH activate pre-ecdysis and ecdysis behaviors organized within the CNS by central pattern generators.

In *Manduca*, there are clear differences in the overall actions of PETH and ETH. Injection of PETH into pharate larvae elicits pre-ecdysis I only (Figures 20 and 21). PETH-injected animals show pre-ecdysis I behavior for about 35–60 min and fail to advance to pre-ecdysis II or ecdysis, regardless of the dose administered (Zitnan *et al*., 1999). Corresponding pre-ecdysis I synchronous bursts in AGs are recorded *in vitro* from the isolated CNS exposed to PETH (Figure 20). Larvae injected with PETH (100–500 pmol) are unresponsive to further treatment with this peptide, but respond to ETH by initiating pre-ecdysis II followed by ecdysis (Figures 20 and 21). In contrast, naive larvae injected with ETH alone initiate pre-ecdysis I and pre-ecdysis II simultaneously, followed by ecdysis behavior. Similar results are obtained upon exposure of the isolated CNS *in vitro* (Zitnan *et al*., 1999; Figure 20). Thus, ETH can elicit the entire sequence consisting of pre-ecdysis I, pre-ecdysis II, and ecdysis.

Initiation of pre-ecdysis I and II involves direct actions of hemolymph-borne PETH and ETH on each abdominal ganglion. Pre-ecdysis I behavior is driven by a single pair of ascending interneurons whose cell bodies are located in the posterior region of the TAG, the IN-402 cells. They form monosynaptic connections with MN-2,3 in each abdominal ganglion to cause synchronous bursting (Miles and Weeks, 1991; Novicki and Weeks, 1993, 1995). Transection of the ventral nerve cord abolishes patterned bursting of MN-2,3 rostral to the cut, consistent with ascending input from these interneurons (Novicki and Weeks, 1993). In the case of PETH-injected animals exhibiting pre-ecdysis I, transection of connectives anterior to AG-7 or AG-6 leads to prolonged dorsoventral contractions in rostral abdominal segments *in vivo*. Similar results are obtained from isolated CNS following PETH exposure *in vitro*: transection of connectives leads to prolonged motor bursts in ganglia rostral to the cut (Zitnan and Adams, 2000). These findings indicate that activation of PETH receptors in each abdominal

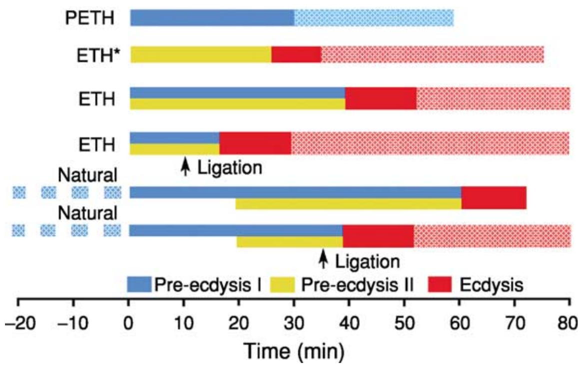


Figure 21 Timelines of natural and peptide-induced pre-ecdysis and ecdysis behaviors in *Manduca*. PETH injection into pharate 5th instar larvae induces only pre-ecdysis I behavior for about 30 min, but it can last for up to 1 h (stippled line). These larvae do not respond to further PETH treatment, but subsequent ETH injection (ETH*) induces pre-ecdysis II and ecdysis behaviors. However, naive larvae injected only with ETH initiate pre-ecdysis I and II simultaneously, followed by ecdysis movements about 40 min later. These animals cannot shed their cuticle, so ecdysis contractions last for up to 1 h. Ligation between abdominal segments 1–2 and removal of all anterior segments at 10–15 min into pre-ecdysis greatly accelerates initiation of ecdysis. The natural ecdysis sequence starts with occasional weak dorsoventral contractions in posterior abdominal segments (broken stippled squares). Continuous strong dorsoventral contractions are considered as the initiation of normal pre-ecdysis I indistinguishabe from PETH-induced behavior. About 20 min later animals initiate posteriolateral and proleg contractions (pre-ecdysis II) alternating with pre-ecdysis I compressions exactly like ETH injected larvae. Ecdysis behavior is initiated about 1 h later and lasts 12–15 min. After the cuticle is completely shed, ecdysis movements immediately stop. Ligation of larvae 35–40 min into natural pre-ecdysis accelerates ecdysis onset similarly as described above.

ganglion leads to bursting activity in interneurons and motor neurons, with the IN-402 providing the necessary input for normal synchronized rhythmic contractions (Novicki and Weeks, 1995). The IN-402 cells probably produce acetylcholine (Novicki, personal communication), but other central neurotransmitters or neuromodulators, which may control pre-ecdysis I activity are not known. IN-402 and MN2,3 retain their intrinsic properties and strength of synaptic connections in pharate pupae, indicating that weakening of pre-ecdysis in this stage is caused by other unknown components of this behavior (Novicki and Weeks, 2000).

In contrast to pre-ecdysis I, where motor neurons in each abdominal ganglion (AG) are slaves to ascending interneurons from the TAG, the pacemaker(s) for pre-ecdysis II reside in each ganglion, such that motor bursts occur independent of inputs from other ganglia. Ligation or transection of connectives between various AGs does not affect proleg and posterioventral contractions during pre-ecdysis II, and individually isolated AGs produce patterned bursting similar in duration and frequency to that recorded in intact nerve cords (Zitnan and Adams, 2000). Interneurons and neurotransmitters controlling pre-ecdysis II have not been identified, but it seems likely that the principal and accessory planta retractor motoneurons (PPRM, APRM) involved in proleg retractions during ecdysis also participate in similar contractions during pre-ecdysis II (Weeks and Truman, 1984b). The ability of ETH to elicit pre-ecdysis II contractions in isolated abdominal ganglia indicates the presence of receptors for this peptide in each ganglion as well.

3.1.6.1.3. Activation and initiation of ecdysis by central factors (cGMP, EH, CCAP, and MIPs)

Ecdysis activation in *Manduca* coincides with elevation of cyclic 3′,5′-guanosine monophosphate (cGMP) in neurosecretory cells 27 and interneurons 704 (27/704) residing in the SG, TG1–3, and AG1–7 (Ewer *et al.*, 1994, 1997). Intracellular recordings in thoracic cells 27 of *Manduca* showed that production of cGMP is associated with excitability of these neurons (Gammie and Truman, 1997b). The production of cGMP in a homologous network of 27/704 neurons was also detected at ecdyses of other unrelated insects, including silverfish, locust, cricket, mealworm beetle, and mosquito, but not in the fruitfly *Drosophila* (Ewer and Truman, 1996). These data indicate that cGMP is associated with activation of ecdysis neurons in many insects.

ETH elevates cGMP in 27/704 cells of ventral ganglia via multiple downstream pathways. In *Manduca* larvae, ETH action on the brain/SG and subsequent central release of EH within ventral ganglia apparently elicits cGMP elevation in the 27/704 cells of AG1–7 (Gammie and Truman, 1999; Zitnan and Adams, 2000). Intracellular recordings showed that either epitracheal gland extracts or synthetic ETH elicits increased electrical activity in the brain VM neurons *in vitro* within 4–13 min. Individually isolated VM somata also show increased excitability within 10 min of ETH exposure (Ewer *et al.*, 1997; Gammie and Truman, 1999). These data suggest that ETH is acting directly on VM cells to cause EH release, while hemolymph-borne EH then acts back to elicit cGMP elevation in Inka cells and ETH release in a positive feedback loop (Ewer *et al.*, 1997). Thus, VM neurons appear to be one of the primary targets for ETH. *In situ* hybridization and calcium imaging studies indicate that VM cell bodies express the ETH receptor subtype A (ETHR-A; Cho, Kim and Adams, unpublished data). However, ETH receptors are expressed in a large number of additional neurons located in the

brain and ventral ganglia and ETH action on the debrained larval nerve cord lacking VM cell bodies results in normal cGMP elevation and ecdysis bursts as observed in the control intact CNS (Zitnan and Adams, 2000). Moreover, EH action on the desheathed SG and TG1–3 causes strong cGMP elevation in all intact (non-desheathed) abdominal ganglia and, conversely, EH treatment of the desheathed abdominal ganglia results in increased cGMP production in intact SG and TG1–3 (Zitnan and Adams, 2000). These data indicate that EH probably participates with other neuropeptides or amines in the activation of the cGMP network in *Manduca*.

Central release of CCAP and MIPs from IN-704 appears to provide the necessary signaling for immediate initiation of ecdysis behavior. Immunohistochemical and *in situ* hybridization studies show that IN-704 produce CCAP and MIPs, whereas neurosecretory cells 27 produce only CCAP (**Figure 9d**; Ewer and Truman, 1996; Loi *et al*., 2001; Davis *et al*., 2003; Spalovska-Valachova and Zitnan, unpublished data). The central release of these neuropeptides during larval ecdyses of *Manduca* is indicated by decreased immunohistochemical staining with antisera to CCAP and MIP (Gammie and Truman, 1997a; Davis *et al*., 2003). Furthermore, application of synthetic CCAP on the isolated, desheathed chain of abdominal ganglia AG1–8 induces the ecdysis motor program (Gammie and Truman, 1999; Zitnan and Adams, 2000). However, CCAP only causes occasional nonpatterned bursts when applied on the entire desheathed CNS. On the other hand, application of a mixture of CCAP and MIPs induces in these nerve cords typical ecdysis bursts in just a few minutes. MIPs apparently suppress activity of pre-ecdysis neurons, while CCAP controls activity of the ecdysis network (Zitnan and Adams, unpublished data).

During pupal and adult ecdyses, only cells 27 show cGMP, elevation, indicating that CCAP and MIP release from IN-704 does not require cGMP, at least in these stages (Ewer and Truman, 1997). Genetically programmed cell death eliminates the entire 27/704 network within 36 h after adult moth eclosion (Ewer *et al*., 1998).

3.1.6.1.4. Inhibition from anterior ganglia delays ecdysis onset Ecdysis behavior in *Manduca* requires ETH activation of centers in the brain and subesophageal ganglion, from which descending inputs to the ventral nerve cord activate ecdysis neuronal circuitry (Novicki and Weeks, 1996; Zitnan and Adams, 2000). During the natural ecdysis sequence in *Manduca*, activation of ecdysis circuitry is complete about 30 min after the start of pre-ecdysis, but ecdysis is initiated some 30 min later. In ETH injected animals, activation of ecdysis circuitry is already complete 10–15 min into pre-ecdysis, well before initiation of ecdysis behavior some 30 min later. Once ecdysis circuitry is activated, the continued presence of ETH is not necessary for ecdysis initiation, indicating that ETH is a "triggering" peptide that sets in motion a sequence of events culminating in ecdysis.

The delay ($\sim$30 min) between ETH activation of the ecdysis network and initiation of behavior is controlled by a balance of excitation and inhibition from cephalic and thoracic ganglia. Evidence for this comes from experiments showing that ligations posterior to the head or thorax or brief CO_2 exposure considerably accelerates the onset of ecdysis during larval, pupal, and adult ecdyses (Zitnan and Adams, 2000; Fuse and Truman, 2002; **Figure 21**). These experiments indicate that descending inhibitory input from the SG and TG1–3 delays the central release of CCAP and MIPs from the 27/704 network in AG1–7. An independent clock in each abdominal ganglion controls the timing of disinhibition, allowing animals to switch from pre-ecdysis to ecdysis behavior.

After the old cuticle is completely shed, animals immediately stop ecdysis behavior. However, if the old cuticle cannot be shed, ecdysis contractions continue for a much longer time (up to 1–2 h). Similarly, isolated nerve cords lacking sensory connections to the cuticle show continuous ecdysis bursts for hours. These observations suggest that cessation of ecdysis behavior is controlled by sensory input from the cuticle, which probably re-activates inhibitory neurons to irreversibly stop the ecdysis motor program after shedding is completed.

Excitation and inhibition associated with initiation of ecdysis behavior appears to be related to sequential phases of cGMP elevation in different groups of central neurons. Six to eight hours prior to onset of pre-ecdysis, cGMP immunoreactivity (cGMP-IR) is detected in 27/704 neurons of the SG, TG1–3, but not in AG1–7 (Ewer and Truman, 1997; Zitnan and Adams, 2000). Following onset of pre-ecdysis, staining in the SG and TG1–3 gradually increases. At 30 min into pre-ecdysis, cGMP staining in 27/704 neurons of AG1–7 appears and increases in intensity over the next 15 min. At this time, additional neurosecretory cells ($L_{2,5}$) show weaker cGMP-IR in each AG1–7 (Zitnan and Adams, 2000; Fuse and Truman, 2002). It appears that cGMP elevation in AG1–7 neurons coincides with activation of ecdysis circuitry, since ligations and removal of the head or thorax at this time accelerates initiation of ecdysis behavior. In ETH injected

larvae, cGMP is elevated in 27/704 neurons of AG1–7 after 10–15 min. Surgery or ligations applied after this time accelerate initiation of ecdysis behavior, whereas their application before this time prevents ecdysis. Similar, but less obvious, results are obtained from measurements of behavior in ETH treated, isolated nerve cords (Zitnan and Adams, 2000).

3.1.6.1.5. Motoneurons controlling ecdysis behaviors Electrophysiological and cobalt backfilling techniques were used to identify motoneurons controlling muscle contractions during larval and pupal ecdysis behaviors of *Manduca* (Weeks and Truman, 1984a,b). These motoneurons innervate specific muscles of the body wall or prolegs and control their contractions during ecdysis behavior (Taylor and Truman, 1974; Levine and Truman, 1982, 1983; Weeks and Truman, 1984a, 1984b). For example, peristaltic contractions of intersegmental muscles are regulated by medial ISM motoneurons, which exit through dorsal nerves of the next posterior ganglion. Proleg retractions at larval ecdysis are controlled by principal and accessory planta retractor muscles innervated by the posteriolateral PPR and APR motoneurons, respectively. Interestingly, these neurons retain their activity even if prolegs degenerate at pupation (Weeks and Truman, 1984b). Dorsoventral contractions of tergopleural muscles during peristaltic movements are controlled by contralateral motoneurons 2 and 3 (MN-2 and MN-3), which also control synchronous compressions during pre-ecdysis I behavior (Weeks and Truman, 1984a). The disparate burst patterns of MN2,3 during pre-ecdysis I and ecdysis may reflect a switch in interneuron input. The IN-402 interneurons are necessary for synchronous bursts of MN2,3 during pre-ecdysis I (see Section 3.1.6.1), but interneurons controlling activity of MN2,3 during peristaltic movements are unknown. Alternatively, release of different upstream peptides could cause changes in the network properties driving these motor neurons.

3.1.6.1.6. Natural and ETH-induced pupal and adult behaviors Morphological markers useful for determination of time to pupal ecdysis have been reported in pharate pupae (Truman, 1985; Zitnanova *et al*., 2001) and are summarized in **Table 1**. The ecdysis behavioral sequence during pupation was described *in vivo* and *in vitro* (Weeks and Truman, 1984a; Truman, 1985; Zitnan *et al*., 1996). Pre-ecdysis of pharate pupae always occurs with the animal positioned ventral side down, and is visible as weak rhythmic contractions of the abdomen. Although these contractions are often difficult to discern visually, blood pressure measurements revealed clear rhythmic pulses during pre-ecdysis (Zitnan *et al*., 1996). Robust ecdysis peristaltic contractions begin approximately 1 h later in the most posterior abdominal segment and move anteriorly. After rupture of the old cuticle along the dorsal thoracic midline, animals turn ventral side up and with the aid of strong peristaltic movements the cuticle is shed. During postecdysis, the proboscis, wing pads, and legs of the fresh pupa are then expanded and sclerotized to their typical shapes.

PETH injection has either no effect or causes weak rhythmic contractions likely representing pre-ecdysis in animals injected 3–4 h prior to ecdysis. ETH treatment 3–48 h prior to natural ecdysis induces ecdysis behavior within 35–60 min; weak pre-ecdysis behavior preceding ecdysis is only observed in pharate pupae injected 3–4 h prior to natural ecdysis. Removal of the head and thorax or CO_2 treatment during pre-ecdysis accelerates the onset of ecdysis behavior as observed in larvae (Zitnan and Adams, 2000; Fuse and Truman, 2002). ETH also induces strong ecdysis bursts in dorsal nerves of the isolated nerve cord *in vitro* (Zitnan *et al*., 1996).

Adult ecdysis (eclosion) behaviors are quite different from larval and pupal ecdysis and are composed of pre-eclosion and eclosion behaviors, as well as digging from the underground pupation site (Reynolds *et al*., 1979; Mesce and Truman, 1988). Pre-eclosion behavior is initiated by occasional rotations of the abdomen, which may be missing in some animals. The onset of eclosion behavior is characterized by strong movements of wing bases to break anterior parts of the pupal cuticle while abdominal retractions and extensions help the adult to emerge from the pupal case and to escape to the surface.

Pharate adults are sensitive to ETH beginning about 24 h prior to natural eclosion. ETH injections during this time period induce occasional abdominal rotations within 3–10 min, followed by a relatively quiescent phase. Eclosion behavior is initiated 2–3 h after ETH treatment and adults emerge in 10–20 min, but are usually wet and do not completely spread their wings (Zitnan *et al*., 1996). However, animals nevertheless climb to a suitable perch and assume the posture normally associated with wing expansion. This suggests that ETH may also be responsible for posteclosion behavior. Similar to pharate larvae and pupae, decapitation or CO_2 treatment considerably accelerates eclosion onset (Mesce and Truman, 1988; Ewer *et al*., 1997; Fuse and Truman, 2002). Interestingly, adults retain the larval ecdysis motor program. Typical larval-like peristaltic abdominal movements can be induced in eclosing pharate adults by transecting connectives

between the pterothoracic and abdominal ganglia (Mesce and Truman, 1988).

Since ETH-AP is processed from the ETH precursor and released at ecdysis (Zitnan *et al*., 1999), its function was tested by injection in parallel with PETH and ETH. However, no obvious effects were observed following injections of ETH-AP into pharate larvae, pupae, or adults and the isolated larval CNS shows no specific response to ETH-AP treatment *in vitro* (Zitnan *et al*., 1999; Zitnan, unpublished observations).

3.1.6.2. Natural and Peptide-Induced Behaviors in *Bombyx*

3.1.6.2.1. Larval pre-ecdysis and ecdysis Developmental markers and behavioral sequences during larval and pupal ecdyses and adult eclosion have been described recently (Zitnan *et al*., 2002) (see Table 2). Natural larval behavior is initiated by pre-ecdysis composed of asynchronous dorsoventral, ventral, and posteriolateral body wall contractions, plus leg and proleg retractions. After about 1 h of pre-ecdysis contractions, larvae switch to anteriorly directed peristaltic movements (ecdysis behavior), which result in shedding of the old cuticle in 10–12 min (Figure 22).

Comparisons of *Manduca* pre-ecdysis and ecdysis behaviors with those in *Bombyx* revealed substantial differences. For instance, *Bombyx* pre-ecdysis I and pre-ecdysis II are not synchronized and body wall or proleg contractions can occur randomly on the left or right side of any segment (Zitnan and Adams, 2000). Ligation and transection experiments of *Bombyx* larvae *in vivo* and *in vitro* confirmed that each ganglion contains the entire circuitry for pre-ecdysis I and II, and that connections to the TAG or other distal ganglia are not necessary for generation of these motor patterns. This indicates that IN-402 necessary for synchronous pre-ecdysis I contractions in *Manduca* is probably missing or are not activated in *Bombyx*. Thus pre-ecdysis contractions in *Bombyx* and *Manduca* are quite different, while ecdysis movements are very similar in both species.

Table 2 Timelines of morphological markers in *Bombyx mori*

Time before ecdysis	*Morphological marker*
Pharate 5th instar larva	
28 h	Head capsule slip (HCS)
15–16 h	Edges of new light yellow spiracles show gray pigmentation
12 h	Edges of new spiracles turn black
5 h	Remaining lighter areas of spiracles darken to a brown–black color
Pharate pupa	
24 h	Pharate pupae stop spinning the cocoon
4 h	Shrinking of the old larval cuticle between all thoracic and abdominal segments
Pharate adult	
6–7 days	Yellow–brown pigmentation of eyes
5–6 days	Black pigmentation of eyes
1–2 days	Dark pigmentation of lines on the wings

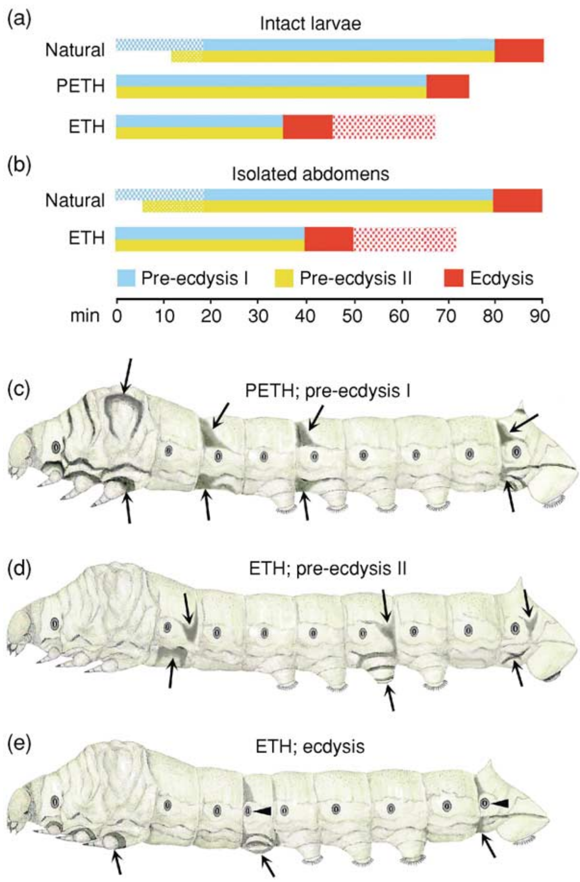


Figure 22 The ecdysis sequence of *Bombyx* pharate larvae. (a) Timelines showing duration of natural and peptide-induced pre-ecdysis and ecdysis in intact pharate larvae. Note that injection of PETH or ETH (50 pmol) induces the entire sequence and ecdysis onset is accelerated when compared with natural behavior. ETH is more effective than PETH and most larvae injected with ETH continue to show ecdysis contractions for up to 30 min (stippled line). (b) Time of the initiation and duration of natural and ETH-induced ecdysis sequences are very similar in isolated abdomens compared with intact pharate larvae of the same stage. (c) PETH-induced asynchronous dorsoventral and leg contractions in thorax and abdomen (pre-ecdysis I, arrows pointing at shaded areas indicate locations of muscle insertions and direction of muscle contractions). (d) Subsequent ETH injection induces asynchronous ventral, posteriolateral, and proleg contractions (pre-ecdysis II, shaded areas, arrows). (e) These animals then switch to anteriorly directed peristaltic contractions accompanied by proleg retractions (ecdysis, shaded areas, arrowheads). (Data from Zitnan, D., Hollar, L., Spalovska, I., Takac, P., Zitnanova, I., *et al*., **2002**. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. *J. Exp. Biol. 205* (Pt 22), 3459–3473.)

Peptide-induced behaviors in *Bombyx* can also differ substantially from those induced in *Manduca*. For example, injection of either PETH or ETH into pharate *Bombyx* larvae (10–15 h prior to ecdysis) induces both pre-ecdysis and ecdysis behaviors *in vivo*. Likewise, peptide treatments of the isolated CNS *in vitro* elicit corresponding burst patterns (Figure 23). On the other hand, injections of pharate *Bombyx* larvae 1 day prior to expected ecdysis elicit responses similar to those induced in *Manduca*: PETH elicits only pre-ecdysis I, whereas ETH induces the entire sequence. These data indicate that CNS sensitivity to PETH and ETH becomes progressively stronger as animals approach the onset of natural ecdysis behavior (Zitnan *et al*., 2002).

Peptide-induced behaviors in *Bombyx* differ from those observed in *Manduca* in another notable way. Injection of isolated abdomens with ETH elicits the entire ecdysis behavioral sequence associated with normal elevation of cGMP in abdominal ganglia, but no detectable release of EH. This means that, in *Bombyx*, descending input from the brain to abdominal ganglia is not required for activation of ecdysis circuitry as was observed in *Manduca*. Finally, although ETH-AP has no detectable behavioral action in *Manduca*, *Bombyx* larvae respond with nonsynchronized proleg and ventral contractions resembling pre-ecdysis II (Zitnan *et al*., 2002).

3.1.6.2.2. Pupal ecdysis and adult eclosion In contrast to *Manduca* (see Section 3.1.5.1), *Bombyx* pharate pupae show well-defined and pronounced pre-ecdysis behavior composed of dorsoventral, leg, and proleg contractions for about 1–1.5 h and then switch to robust ecdysis peristaltic movements (Figure 24). During anteriorly directed ecdysis contractions, larval cuticle of the head capsule and thorax is ruptured along the dorsal line, and the entire old cuticle is shed with attached larval foregut, hindgut, and tracheae in 10–12 min. As described in pharate larvae, injections of either PETH or ETH elicit the entire behavioral sequence (Figure 24). ETH-AP treatment induced weaker proleg and ventral contractions for about 1 h followed by weak and occasional ecdysis movements for up to 1 h. Isolated abdomens show natural pre-ecdysis and ecdysis at the expected time and both behaviors could be induced prematurely by ETH injection (Figure 24).

The natural eclosion behavioral sequence of pharate adults consists of three distinct phases: rotations of the abdomen, a quiescent phase, and eclosion contractions (Figure 25). During peristaltic movements of the abdomen associated with eclosion, adults partially emerge from the pupal cuticle and, after dissolving the cocoon around the head with a salivary secretion containing cocoonase, they escape and spread their wings. Injection of PETH or ETH induces in most pharate adults the entire behavioral sequence. The importance of the brain in ecdysis activation was demonstrated by surgical experiments. Decapitated or brain-extirpated pharate adults never eclose and ETH fails to induce eclosion behavior in these animals (Zitnan *et al*., 2002). In summary, these data showed that both PETH and ETH induce the entire behavioral sequence, although

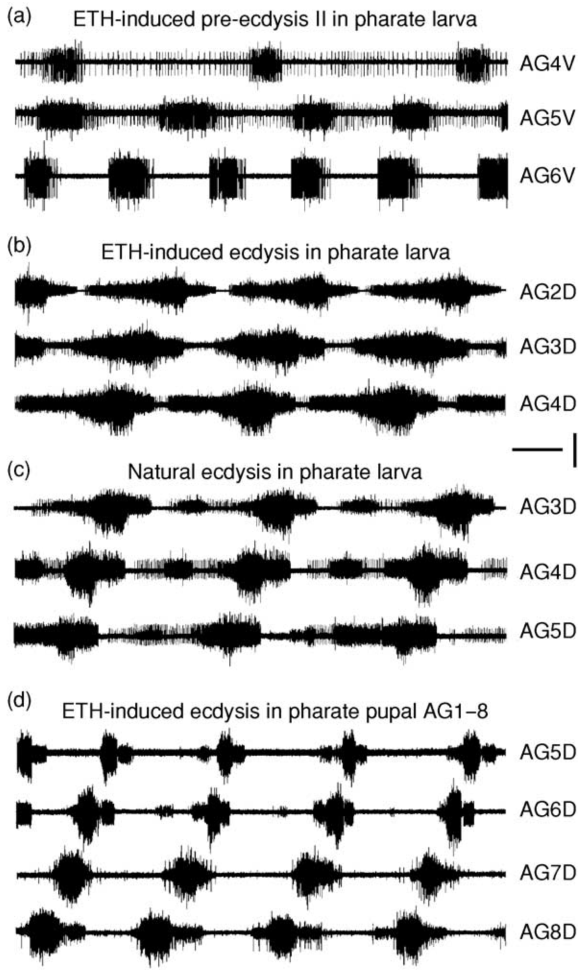


Figure 23 Pre-ecdysis and ecdysis burst patterns recorded in the isolated CNS of *Bombyx* pharate larvae and pharate pupae *in vitro*. (a, b) ETH-induced pre-ecdysis II and ecdysis in the entire CNS of pharate 5th instar larvae. (a) Note asynchronous pre-ecdysis II bursts in ventral nerves of abdominal ganglia 4–6 (AG4-6V). (b) ETH-induced ecdysis bursts in dorsal nerves of abdominal ganglia 2–5 (AG2-5D). These burst patterns are very similar to natural ecdysis bursts (c). (d) ETH-induced ecdysis bursts in dorsal nerves of the isolated chain of abdominal ganglia (AG1–8) of pharate pupa. Ecdysis burst pattens in intact CNS are very similar (not shown). Calibration bars: horizontal 5 s, vertical 10 μV. (Modified from Zitnan, D., Hollar, L., Spalovska, I., Takac, P., Zitnanova, I., *et al*., **2002**. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. *J. Exp. Biol.* *205* (Pt 22), 3459–3473.)

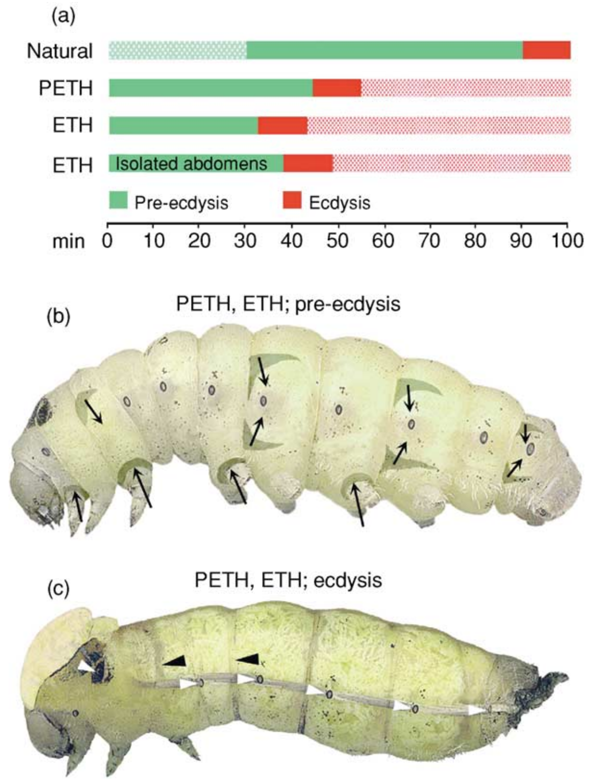


Figure 24 The ecdysis sequence of *Bombyx* pharate pupae. (a) Timelines showing duration of the natural and peptide induced ecdysis sequences. Stippled green line during the first 30 min of pre-ecdysis indicates initial weak and occasional dorsoventral contractions, which become gradually stronger. PETH or ETH injections induce the entire ecdysis sequence in intact animals and isolated abdomens. Early pharate pupae are not able to shed their old cuticle, thus ecdysis contractions last for up to 1 h (stippled red lines). (b) Shaded areas indicate dorsoventral, leg, and proleg contractions during pre-ecdysis induced by PETH or ETH injection. (c) Pre-ecdysis is later replaced by strong ecdysis peristaltic movements (black arrowheads), which result in rupture and shedding of the larval cuticle and tracheae (white arrowheads). (Data from Zitnan, D., Hollar, L., Spalovska, I., Takac, P., Zitnanova, I., *et al.*, **2002**. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. *J. Exp. Biol. 205* (Pt 22), 3459–3473.)

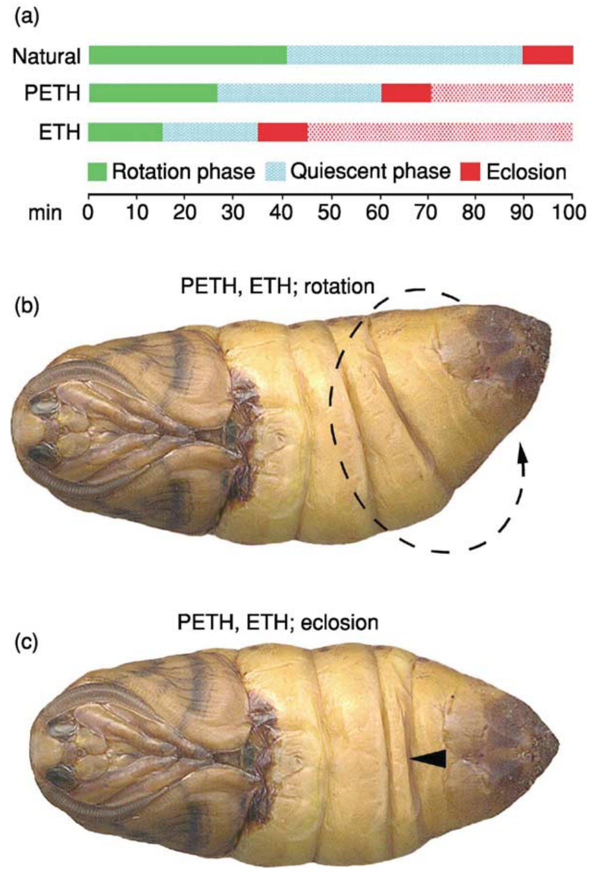


Figure 25 The eclosion sequence of *Bombyx* pharate adult. (a) Timelines showing rotation, quiescent, and eclosion behavioral phases during natural and peptide induced eclosion sequence. Animals injected with PETH or ETH show accelerated response compared to that expressed during natural eclosion. Since peptide injected animals fail to escape from the pupal cuticle, eclosion contractions last for up to 1 h. (b) Both PETH and ETH elicit vigorous rotations of the abdomen (arrow), interrupted by short quiet intervals. (c) After a prolonged quiescent phase, the animal initiates eclosion peristaltic contractions of abdominal segments (arrowhead). (Data from Zitnan, D., Hollar, L., Spalovska, I., Takac, P., Zitnanova, I., *et al.*, **2002**. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. *J. Exp. Biol. 205* (Pt 22), 3459–3473.)

latencies for the onset of ecdysis or eclosion movements by PETH were more variable and generally longer than for ETH.

3.1.6.3. Natural and Peptide-Induced Behaviors in *Drosophila*

3.1.6.3.1. Larval and pupal ecdyses The first detailed study of larval ecdysis in *Drosophila* described morphological markers and a series of stereotypic physiological and behavioral events preceding the transition from first to second instar (Park *et al.*, 2002a). About 60 min in advance of ecdysis, a new set of mouthparts for the second instar become visible adjacent to those of the first instar, leading to the appearance of "double mouth hooks." The appearance of "double vertical plates" (dVP) 30 min later was used as a reference point, or "time zero" in the ecdysis sequence (Figure 26).

Using a transgenic fly line in which the nucleotide sequence of enhanced green fluorescent protein (*egfp*) was fused to the 3-prime end of the *eth* gene, release of ETH from Inka cells was observed as a loss of fluorescence shortly after dVP. Upon ETH release, old tracheae collapse and the new second instar tracheae are inflated. This occurs 10 min after dVP. About 5 min later, pre-ecdysis begins with a series of "anterior–posterior" (A–P) contractions. Five min later this leads to another

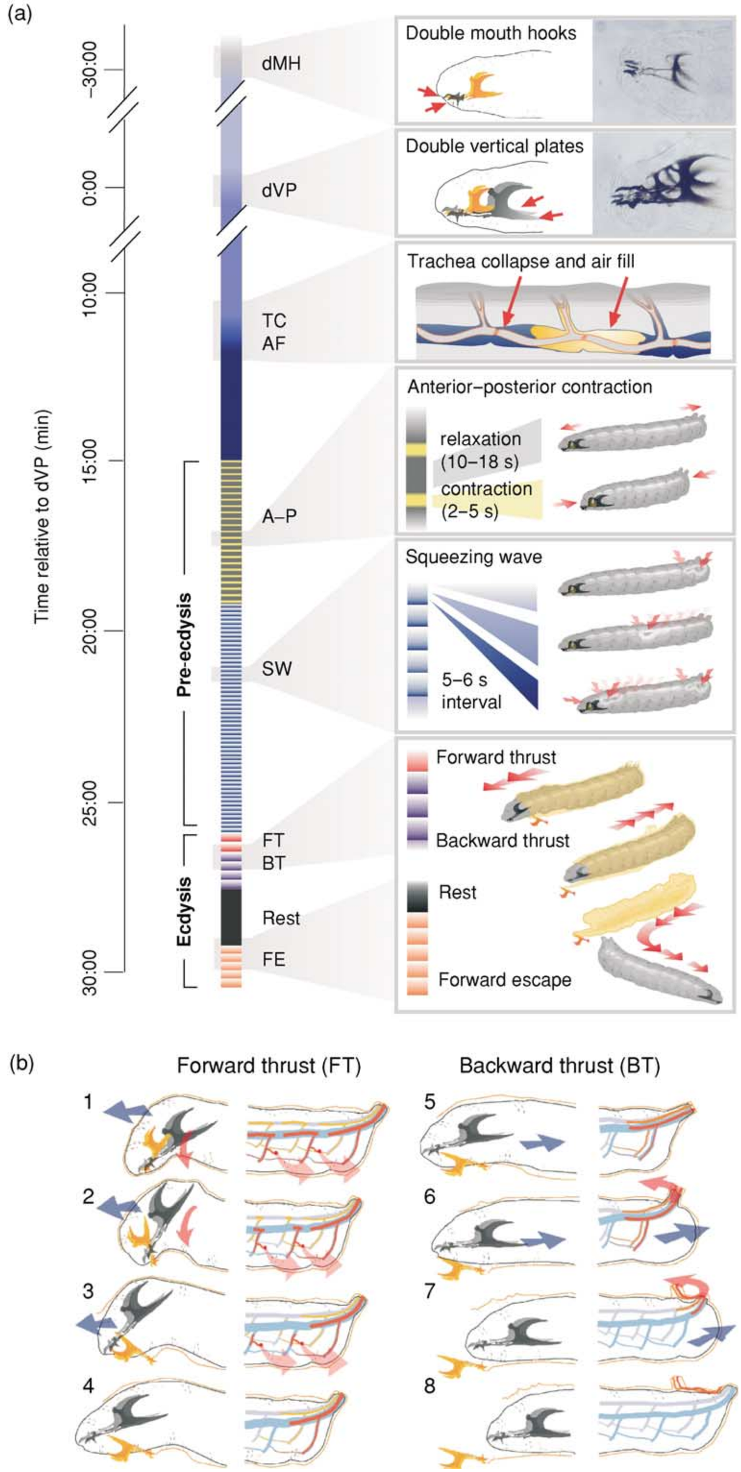


Figure 26 Timelines of the *Drosophila* ecdysis sequence. (a) About 60 min prior to ecdysis, a new set of mouth hooks for the 2nd instar larva becomes visible adjacent to those of the 1st instar ''double mouth hooks'' (dMH). The appearance of ''double vertical plates'' (dVP) 30 min later was used as ''time zero'' in the ecdysis sequence. Upon ETH release from Inka cells 10 min after dVP, old tracheae collapse and the new, 2nd instar tracheae are inflated. (a,b) About 5 min later, pre-ecdysis begins with a series of ''anterior–posterior'' (A–P) contractions followed by a different pattern of muscle contractions ''squeezing waves'' (SW). During A–P and SW pre-ecdysis movements, old and new mouthparts are separated. About 25 min after dVP, ecdysis behavior is initiated with one or two forward head thrusts (FT). These movements help to plant old mouthparts onto the substrate and to extricate old tracheae through lateral segmental spiracular pits, which are normally closed but become functional during ecdysis. Following backward thrust (BT) detaches and extricates remaining old posterior spiracles and tracheal linings out through the new spiracles. Ecdysis is completed following forward escape (FE) and turning movements. (Data from Park, Y., Filippov, V., Gill, S.S., Adams, M.E., **2002a**. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development 129* (2), 493–503.)

pattern of muscle contractions referred to as "squeezing waves" (SW). During A-P and SW pre-ecdysis behaviors, vigorous muscle contractions are observed to pull the mouthparts alternately in anterior and posterior directions. These movements may be critical to the separation of old from new mouthparts during subsequent ecdysis behavior.

Ecdysis is initiated around 20 min after dVP. It begins with one or two forward head thrusts, whereby old mouth hooks and vertical plates are detached from the new and planted onto the surface of the substrate. The forward thrust also detaches and extricates parts of the old tracheal system through lateral segmental spiracular pits, which are normally closed but become functional during ecdysis. Subsequent backward thrust detaches the old from the new posterior spiracles and pulls the remaining old tracheal linings out through the new spiracles (**Figure 26**). Ecdysis is completed after forward and turning movements help the larva to escape from the old cuticle. The sequence of events leading up to ecdysis from 2nd to 3rd instar follows a very similar pattern. The only difference is that the appearance of double mouth hooks occurs considerably sooner (104 min before dVP) as compared to the first ecdysis (30 min before dVP).

A detailed description of morphological markers and behaviors during metamorphosis was published by Bainbridge and Bownes (1981). These authors identified numerous stages from wandering larva to adult subdivided to 52 distinct events, but here the focus is on markers and behaviors associated with pupal ecdysis, which is usually described as "head eversion" behavior (Zdarek and Friedman, 1986; Ward *et al*., 2003). We consider formation of a white puparium as the reference point or time zero. A gas bubble becomes visible in the dorsal part of the abdomen 3 h later and apolysis of larval and pupal cuticles is initiated at 4–6 h. This stage is called "cryptocephalic pupa." Major lateral tracheal trunks are obscured at 6.5–8 h followed by initiation of dorsomedial abdominal contractions at about 10–12 h. The abdominal bubble disappears at 12 h, while another bubble appears in the posterior part of the puparium and gradually moves anteriorly. As the gas bubble is displaced in the anterior portion of the puparium at about 12–13 h, pupal pre-ecdysis is initiated. It is characterized by rhythmic retractions of the animal from the puparium, which last for about 9–10 min (Park *et al*., 2003b). Subsequent ecdysis behavior is indicated by peristaltic body contractions for only 1 min associated with head eversion, extension of pupal legs and wing pads posteriorly along the abdomen, and shedding of the larval tracheae. Regular weak postecdysis contractions continuing for about 2 h may assist the pupa in achieving its final shape (Park *et al*., 2003b). This stage is named "phanerocephalic pupa."

3.1.6.3.2. Adult eclosion Timelines of the eclosion sequence and morphological markers useful in predicting ecdysis events in *Drosophila* pharate adults have been described (Kimura and Truman, 1990; McNabb *et al*., 1997; Park *et al*., 1999). Resorption of molting fluid is initiated about 6–7 h before expected eclosion. The pupal cuticle then passes through a series of changes in appearance referred to as smooth (S), smooth/grainy (S/G), grainy (G), and finally white (W). Certain steps differ in duration between males and females, namely that the smooth stage lasts for ~5.5 h before females' transition to the smooth/grainy stage, whereas males show the smooth stage for only a matter of minutes before advancing to the smooth/grainy stage. Commencement of the white stage (W) occurs upon inflation of the head tracheal sacs and indicates that pre-eclosion behavior has been initiated.

Pre-eclosion starts about 1 h prior to eclosion and is composed of dorsoventral contractions of the first abdominal tergum and ptilinium extension resulting in inflation/expansion and tracheal filling of the head (W). Pre-eclosion is followed by a quiescent period, but the entire behavior may be absent in up to 24% animals (Park *et al*., 1999). Eclosion behavior consists of four consecutive events, including head expansion to open the operculum, forward head thrusts, lateral thoracic contractions, and forward peristaltic abdominal contractions helping the fly to emerge from puparium. Interestingly, neck ligation of pharate adults during pre-eclosion (extended ptilinum stage) causes immediate onset of ecdysis (Baker *et al*., 1999). This indicates that inhibitory neurons and factor(s) in the cephalic ganglia delay the initiation of ecdysis, similar to that observed in *Manduca* (Ewer *et al*., 1997; Zitnan and Adams, 2000; Fuse and Truman, 2002).

3.1.6.3.3. Role of ETH in larval and adult ecdyses Premature injection of ETH1 accelerates all the events shown in **Figure 27** including tracheal inflation, anterior–posterior contractions, squeezing waves, and ecdysis behavior. Injection of the peptide (≥0.1 fmol) into *Drosophila* larvae at the double mouth hook stage induces in 3–4 min old tracheal collapse and air inflation of new tracheae, followed by a complete set of pre-ecdysis and ecdysis behaviors. ETH2 also induces tracheal and behavioral responses, but higher concentrations are needed (≥1 fmol for tracheal dynamics; ≥10 fmol for ecdysis behavior) and pre-ecdysis behaviors are absent. After a quiescent stage

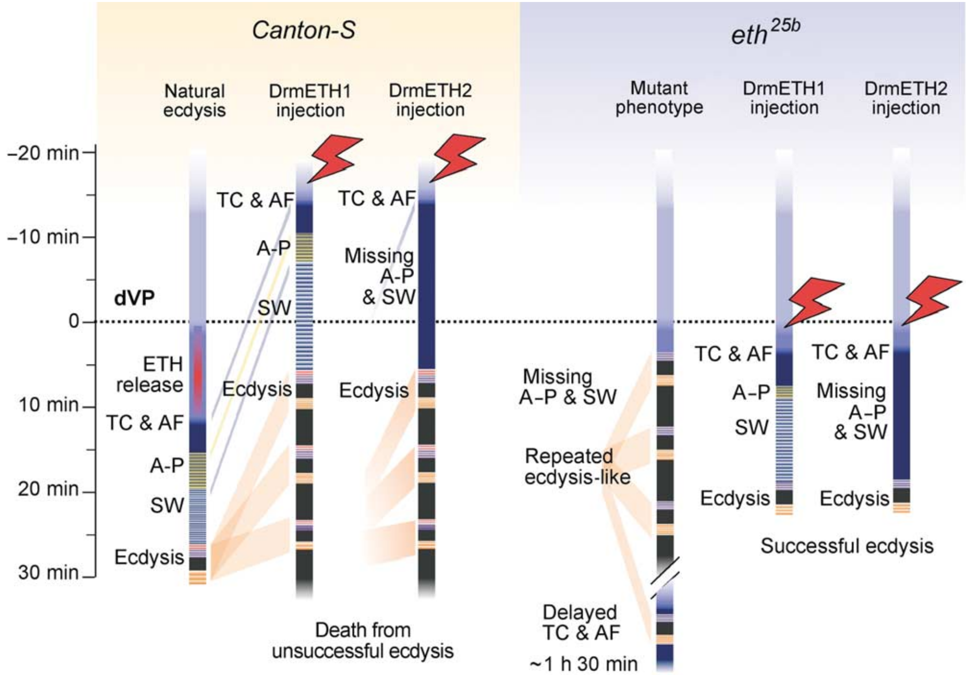


Figure 27 Time lines depicting the ecdysis sequence in wild-type Canton S and in the *eth* deletion mutant line *eth*^{25b}. During natural ecdysis, ETH release begins shortly after double vertical plates (dVP), followed by collapse of old tracheal tubes (TC) and air inflation (AF) of new, pre-ecdysis consisting of anterior–posterior (AC) contractions and squeezing waves (SW), and finally ecdysis. Premature DrmETH1 injection accelerates all events that normally follow natural ETH release, but DrmETH2 elicits only accelerated tracheal dynamics and ecdysis. When ETH1 or ETH2 is injected prematurely, ecdysis behavior ensues but is unsuccessful due to failure to separate old and new mouth hooks and vertical plates. The mutant phenotype *eth*^{25b} exhibits delayed tracheal dynamics, fails to perform pre-ecdysis and normal ecdysis behaviors, and shows high mortality (>98%) at the first larval ecdysis. Injection of ETH1 or ETH2 at the dVP stage reverses the mortality observed in this mutant phenotype. (Data from Park, Y., Filippov, V., Gill, S.S., Adams, M.E., **2002a**. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development 129* (2), 493–503.)

following tracheal inflation, animals initiate ecdysis behavior (Park *et al.*, 2002a). These studies illuminated a previously unrecognized role for ETH in eliciting tracheal dynamics, although it is likely that ETH acts through downstream signals on the tracheal system. As described in *Drosophila* larvae, injections of ETHs into pharate adults accelerate the onset of pre-eclosion and eclosion behaviors, and, again, ETH1 is more effective than ETH2 (Park *et al.*, 1999). Injection of *Manduca* ETH also induces the entire behavioral sequence (McNabb *et al.*, 1997; Baker *et al.*, 1999; Park *et al.*, 1999), but animals injected with PETH show no response.

Whether ETH is necessary for ecdysis depends on the ability to observe natural ecdysis in the absence of the peptide. To address this question, the gene *eth* encoding ETH1 and ETH2 was deleted by imprecise P-element excision (Park *et al.*, 2002a). To excise *eth*, a fly line having a P-element insertion 1427 bp downstream of the gene was obtained. The P-element was mobilized by crossing this line with the Δ2–3 transposase line. Deletion of *eth* resulted in lethal behavioral and physiological deficits. Homozygous null mutants (*eth*-) develop through embryogenesis and the first instar, but 98% die at the first larval ecdysis. These animals show considerable delays in tracheal collapse and air inflation, lack pre-ecdysis behavior, exhibit abnormal ecdysis behavior, and are unable to shed the old cuticle completely. Precisely timed injection of ETH1 restores both respiratory and behavioral defects and animals progress to the 2nd instar (**Figure 27**). The gene encoding ETH1 therefore plays an obligatory role in the regulation of ecdysis, and the absence of ETHs leads to defects in air filling of the tracheal system, unsuccessful ecdysis and lethality (Park *et al.*, 2002a).

3.1.6.3.4. Roles for EH in ecdysis There is a good deal of evidence generated from studies of EH in *Manduca* (Truman, 1992), *Bombyx* (Kono *et al.*, 1990b), and *Heliothis virescens* (Kataoka *et al.*, 1987) that EH is sufficient to trigger pre-ecdysis and ecdysis behaviors. In the first study to test for the necessity of EH in eclosion, the cell-specific promoter of the EH gene in *Drosophila* was used to drive the cell death genes *reaper* and *head involution defective* to kill EH synthesizing cells (McNabb

et al., 1997). In spite of EH cell ablation, ~50% of developing flies reached the puparium stage, and ~80% of these successfully performed eclosion.

Nevertheless, several defects in the coordination of eclosion-related events are evident in flies. First, tracheal inflation is delayed. Second, while bouts of head expansion, head thrusts, thoracic contractions, and abdominal contractions that comprise eclosion behavior are synchronized and alternate with quiescent periods in wild-type flies, they are disorganized and performed continuously with no quiescent periods in EH-KO flies. Furthermore, eclosion behaviors are weaker in the EH-KO flies and thus the entire eclosion sequence is prolonged. Finally, two-thirds of the mutant flies failed to undergo normal posteclosion expansion of wings and hardening of the adult cuticle.

These observations indicate that, despite the ability of flies to undergo larval, pupal, and adult ecdysis behaviors, they fail to perform many of the steps on schedule and in a coordinated fashion. Nevertheless, it is remarkable that almost one-half of the mutant fly population survives to the adult stage in the absence of EH. This study indicates that, while EH is involved in regulation of ecdysis and eclosion, it has a nonessential role in these processes (McNabb *et al.*, 1997).

Other interesting outcomes emerged from the study of EH-KO flies. First, based on Truman's original discoveries of EH activity in giant silkmoths, it has been assumed that EH is involved in the circadian gating of adult eclosion (Truman and Riddiford, 1974). Second, EH has been associated for many years with programed cell death of larval abdominal body wall muscles during metamorphosis (Schwartz and Truman, 1984). However, studies of EH-KO flies demonstrated both a normal free-running circadian rhythm of eclosion as well as programed cell death of intersegmental muscles (McNabb *et al.*, 1997). Therefore, other hormonal signals are likely involved in these processes.

3.1.6.3.5. Role of CCAP in ecdysis In contrast to its likely essential role in *Manduca* ecdysis behavior (Gammie and Truman, 1997a, 1999; Zitnan and Adams, 2000), CCAP does not appear to be of central importance in *Drosophila* ecdysis (Park *et al.*, 2003b). Activation of ecdysis is associated with cGMP elevation in CCAP-producing neurons of diverse insects (Ewer and Truman, 1996), but elevation of cGMP in central neurons of *Drosophila* is completely absent during larval ecdyses. During adult eclosion, cGMP is restricted to small neurons in the SG (3–5 pairs) and AG (1–2 pairs), as well as in the tracheae associated with the CNS (Ewer and Truman, 1996; Baker *et al.*, 1999). Double staining confirmed that neurons producing cGMP during adult eclosion are clearly different from the segmentally distributed CCAP expressing cells (Ewer and Truman, 1996).

To investigate the possible functional role of CCAP in *Drosophila* ecdysis, CCAP neurons were killed using a *CCAP*-GAL4 driver to cause cell-selective expression of *reaper* (Park *et al.*, 2003b). Surprisingly, larval ecdysis and adult eclosion occurred normally and survival of mutant flies through the end of the last larval instar was indistinguishable from controls. Some subtle effects included increased duration of pre-ecdysis and ecdysis. During pupal ecdysis, pre-ecdysis behavior occurred on schedule, but the majority of animals died due to failure in head eversion. Since normal head eversion is associated with CCAP release, failure of this process in CCAP-KO flies confirms its necessary role for this process. Although the precise roles of CCAP in head eversion are not known, it is likely that dysfunction in the circulatory system regulated by this peptide may be to blame. A small number of animals survived to perform relatively normal adult eclosion. Some subtle quantitative differences in the length of time involved in tracheal air filling and reduced ability to expand the body were observed. This expansion is necessary for efficient propulsion of the animal forward during peristaltic ecdysis contractions.

Overall, the major defects in CCAP-KO flies were failure of head eversion at pupal ecdysis, and failure to expand and sclerotize the wings after adult eclosion. The latter observations implicate function of CCAP neurons either in the corelease of bursicon or in the activation of downstream bursicon neurons (see Section 3.1.6.6.2).

3.1.6.4. Hatching and Ecdysis of Locust Nymphs

Behavior and neurophysiology during hatching and the first ecdysis have been decribed in nymphs of *L. migratoria* and *S. gregaria* (Bernays, 1971, 1972b, 1972c; Truman *et al.*, 1996). Three behavioral phases are observed: (1) hatching behavior of undulatory vermiform larva with legs held close to the body in order to escape from the eggshell and dig through the soil to the surface; (2) ecdysis behavior initiated upon reaching the surface to shed the embryonic cuticle; and (3) expansional behavior to assume a posture and locomotory patterns typical of a hexapod nymph (Bernays, 1971, 1972a). Immunohistochemical techniques showed that the second phase of these behaviors (ecdysis) is associated with elevation of cGMP in a set of neurons producing CCAP. Hatching and digging animals do not produce cGMP in these neurons, but cGMP appears in

the CCAP neuronal network immediately before the free larva initiates ecdysis behavior associated with air swallowing and tracheal air filling (Truman *et al.*, 1996). The cGMP immunoreactivity (cGMP-IR) reaches a peak about 5 min after initiation of ecdysis and then gradually decreases. By 20 min, cGMP-IR is considerably reduced and disappears 10 min later. If the nymphs hatch under moist cheesecloth, they do not ecdyse but continue digging behavior. These larvae do not show cGMP elevation or only traces of cGMP-IR after 10–60 min of digging. However, when ecdysing nymphs with elevated cGMP levels are covered with moist cheesecloth, they resume digging. These "digging ecdysing larvae" produce cGMP in CCAP neurons for prolonged time, ranging from 40 to 100 min. Once ecdysed nymphs assume the hopper form, covering with cheesecloth does not result in the resumption of digging behavior and cGMP elevation. Hence, sensory input from the cuticle appears to be important in the initiation as well as cessation of the ecdysis behavior and associated cGMP production in CCAP neurons.

3.1.6.5. Adult Ecdysis of Crickets and Locusts

Detailed behavioral analysis and associated muscle contractions recorded by electromyography were described during adult ecdysis of the cricket *Teleogryllus oceanicus* (Carlson and Bentley, 1977; Carlson, 1977a, 1977b). These authors divided the entire ecdysis sequence into four phases: preparatory (2 h), ecdysial (20 h), expansional (1 h), and exuvial (30 min), which are composed of 48 identified motor programs. The preparatory phase corresponds to pre-ecdysis in other insects and consists of contractions of various muscles to loosen, anchor, and split the old cuticle. This phase is considered as pre-ecdysis to be consistent with observations in other insects. Cricket adult pre-ecdysis is initiated with grooming, locomotion, and pushups, later accompanied by synchronous longitudinal contractions of the abdomen to loosen and split the old cuticle along the dorsal ecdysial line. These synchronous abdominal contractions resemble pre-ecdysis I in *Manduca*. Other motor programs are later activated, e.g., alternating contractions of coxal muscles help to anchor the claws to the substrate and antennal beating and scissoring result in loosening the antennae. The onset of ecdysis behavior is preceded by a quiescent phase for 10 min. As in other insects, the cricket ecdysis behavior is characterized by anteriorly directed bouts of abdominal contractions and air swallowing. Additional motor programs are recruited later and involve either synchronous or alternating contractions of muscles in the head, thorax, or legs to extricate the entire body from the exuvium.

The postecdysis expansional phase includes rhythmic contractions of abdominal and leg muscles, as well as air swallowing to expand the cuticle of the body and appendages (legs and wings). After the new cuticle is expanded to its definitive shape, the expansional posture is abandoned and the cricket enters the exuvial phase, which comprises the eating of its shed cuticle (exuvium) accompanied by sporadic fluttering, spreading, and refolding of wings (Carlson, 1977a).

Removal of the old cuticle prior to onset of the ecdysis sequence results in delayed initiation of behaviors by 2 h. After this delay, normal pre-ecdysis and ecdysis behaviors are initated, but the animal becomes arrested at the first half of the ecdysis motor programs. When the old cuticle is peeled at ecdysis, the expansional and exuvial phases follow after some delay. These data show that ecdysis motor programs can be initiated without sensory input in *Teleogryllus*, but that sensory information from certain structures (e.g., cerci) is necessary for initiation, termination, or reactivation of some specific programs. For example, if cerci are peeled prior to the ecdysis sequence, animals never initiate pre-ecdysis and ecdysis behaviors, but show abdominal tetanic contractions characteristic for the post-ecdysis expansional phase. By comparison, *Manduca* larvae never initiate the ecdysis sequence if the old cuticle is peeled manually, but adults usually inflate their wings and abdomens after they are removed from the pupal cuticle several hours prior to expected eclosion (Miles and Booker, 1998; Zitnan, unpublished data).

The cephalic ganglia appear to be important for inhibiting the ecdysis peristaltic program in *Teleogryllus*. Removal of the head at any time during the behavioral sequence results in the initiation of ecdysis abdominal peristaltic movements in combination with the original behavior. For example, if animals are decapitated during pre-ecdysis, they continue to show pre-ecdysis synchronous longitudinal pumping alternating with peristaltic ecdysis contractions, while decapitation at the expansional phase results in a combination of tetanic and peristaltic contractions (Carlson, 1977a). In contrast, decapitation of *Manduca* and *Drosophila* suppresses pre-ecdysis within a few minutes and accelerates ecdysis onset (see Sections 3.1.6.1.4 and 3.1.6.3.2).

The desert locust, *S. gregaria*, displays similar behaviors during adult ecdysis as described in crickets. The locust ecdysis sequence has been divided into several stages consisting of pre-emergence (stages 1, 2), emergence (stages 3, 4), expansional (stage 5), and postexpansional (stage 6) behaviors

(Hughes, 1980a). The ecdysis sequence starts after the fully grown 5th instar nymphs cease feeding and enter the pre-emergence behavior characterized by various synchronous or alternating contractions of skeletal muscles. This set of behaviors corresponds to pre-ecdysis in other animals. Emergence or ecdysis behaviors are composed of air swallowing to inflate the gut, as well as peristaltic anteriorly directed movements of the abdomen to push the animal out of the cuticle and other motor programs to extricate legs and appendages on the head. After ecdysis is completed, the new cuticle is expanded within 1 h. The last postexpansional phase is characterized by dorsoventral abdominal contractions during which the gut is deflated and tracheal sacs are filled with air (Hughes, 1980b, 1980c, 1980d).

3.1.6.6. Regulation of Processes Associated With Ecdysis

3.1.6.6.1. Control of molting fluid and air swallowing behaviors The frontal ganglion (FG) controls behaviors associated with the eclosion and feeding of adult *Manduca*. This ganglion innervates dilator and compressor muscles of a cibarial pump in the foregut that develops during metamorphosis. Motoneurons in the FG regulate contractions of the cibarial pump required for swallowing molting fluid many hours before eclosion and air swallowing necessary for wing and abdomen expansion after adult emergence. The pump is later used for feeding of emerged moths (Miles and Booker, 1998). Two types of FG motoneurons have been identified during adult feeding in the FG. These motoneurons are designated anterior and posterior dilator motoneurons (AD1 and PD1, respectively). Since activity of the cibarial pump is regulated by FG neurons during feeding as well as during molting fluid and air swallowing, AD1 and PD1 motoneurons may participate in all these behaviors. Perhaps the FG is also involved in regulation of molting fluid and air swallowing associated with larval and pupal ecdyses.

Application of EH into isolated heads of pharate adults induced within 7–105 min irregular and later rhythmic activity of the cibarial pump muscles corresponding to the air swallowing behavior. This indicates that EH may control this behavior, although natural air swallowing is initiated several hours before EH is thought to be released into the hemolymph, as evidenced by elevation of cGMP in Inka cells. By contrast, ETH injection into whole animals induced premature eclosion, but air swallowing was not initiated, suggesting that ETH may not be involved in this process (Miles and Booker, 1998). However, during normal ecdysis or eclosion ETH causes the EH release, which should induce the behavior. This may indicate that during ETH-induced eclosion, EH was not released, or EH concentrations in the hemolymph were not sufficient to induce air swallowing behavior. Also, mechanisms of EH penetration into the FG and activation of this behavior by EH are not clear and further studies are needed to determine hormonal regulation of neuronal activity in the FG.

Air swallowing behavior leading to new cuticle expansion is associated with larval and adult ecdyses of the locust *Schistocerca* (Bernays, 1971), the cricket *Teleogryllus* (Carlson and Bentley, 1977), the butterfly *Pieris brassicae* (Cottrell, 1964), and the blowfly, *Calliphora erythrocephala* (Cottrell, 1962a). Air swallowing prior to or during ecdysis behaviors is required for expanding the new exoskeleton, which is important for splitting and later shedding of the old cuticle. Freshly emerged adults need air swallowing for inflation of wings and abdomen. Generally, postecdysis air swallowing is necessary for expansion of new soft cuticle to achieve the final shape of the new developmental stage. Neurons and regulatory components controlling swallowing behavior in these insects are not known.

3.1.6.6.2. Regulation of cuticle expansion, sclerotization, and melanization Important postecdysis events include expansion, sclerotization (hardening; see **Chapter 4.4**), and melanization (darkening) of the cuticle. These processes are critical for the normal development of many structures. Some examples include expansion of the proboscis, antennae, legs, and wings in newly ecdysed *Manduca* pupae or adults. The term “tanning” has often been used to describe the combined processes of sclerotization and melanization. Tanning in blowflies could be delayed for hours by forcing newly eclosed flies to dig continuously through artificial substrate following eclosion (Fraenkel, 1935). Only when animals were allowed to break free of substrate would expansion and tanning proceed. Fraenkel also observed that mild anesthesia could abolish the delay, confirming that sensory input is important in the control of tanning. After a 30-year hiatus, a series of papers demonstrated that release of a hormonal factor was critical in the tanning process (Fraenkel and Hsiao, 1962, 1963; Cottrell, 1962a, 1962b, 1962c; Fraenkel, 1965). This factor was named “bursicon,” a term derived from the Greek words “bursa” pertaining to a hide or purse and “bursikos,” meaning a tanning process. Bursicon-like biological activity has been detected in the brain, corpora cardiaca, and ventral ganglia, and release sites are known to be both in the CC and in the transverse nerves of ventral ganglia, i.e., the

perivisceral (perisympathetic) organs (Grillot *et al.*, 1976; Taghert and Truman, 1982a; Honegger *et al.*, 2002).

Various bioassays have been devised to detect bursicon-like bioactivity. The most famous involves use of sarcophagid flies, but the assay works equally well with cyclorraphan flies (Fraenkel, 1965). Flies in the process of emergence are ligated between head and thorax. Because descending input from the brain is essential for bursicon release, no tanning occurs. Injection of tissue extracts containing bursicon into thorax or abdomen result in full tanning within 2 h. Other assays have utilized vermiform locust larvae (Bernays, 1972a), pupal *Tenebrio* (Grillot *et al.*, 1976), and wings from *Manduca* pharate adults (Truman, 1973).

The chemical nature of bursicon was deduced to be peptidic, and numerous independent investigations in different insects demonstrated it to be a large peptide (20–60 kDa) (Fraenkel, 1966; Mills and Lake, 1966; Reynolds, 1977; Taghert and Truman, 1982a). More recent studies have pinpointed its molecular weight closer to 30 kDa in *Periplaneta*, *Locusta*, *Gryllus*, *Tenebrio*, and *Calliphora* (Kostron *et al.*, 1995). The first partial sequences of bursicon have been obtained from *Periplaneta*, and antibodies have been generally useful in visualization of bursicon containing neurons (Honegger *et al.*, 2002).

Bursicon containing neurons were first identified in segmental ganglia of moths using bioassays (Taghert and Truman, 1982a). Owing to the unknown molecular nature of bursicon at the time, this work was done without immunohistochemistry, but rather by dissecting single cell bodies of neurosecretory cells displaying the Tyndall effect and assaying for bursicon bioactivity using the wing assay of Truman. This elegant work demonstrated bursicon content in neurosecretory cells 24, 25, 26, and 27 in unfused abdominal ganglia of *Manduca*. Most of these cells exhibit elevated cGMP levels during the ecdysis behavioral sequence and Cell 27 also contains CCAP (Davis *et al.*, 1993; Ewer and Truman, 1996). Furthermore, these cells project to the perisympathetic organs in the transverse nerve, a known neurohemal release site for bursicon.

Cell 27 is a paired, serially homologous neurosecretory cell in thoracic and abdominal ganglia of *Manduca*, whose homologs have been described in many other insects (**Figures 12 and 13**; Ewer and Truman, 1996; Honegger *et al.*, 2002). A network of Cell 27 homologs was described originally as “lateral A” cells, characterized by their large size, pronounced white appearance due to the Tyndall effect, and strong staining with paraldehyde-fuchsin. They are also characterized by association with large vacuole-like structures adjacent to the cell body (Gaude, 1975). More detailed descriptions of these “lateral white” (LW) cells in crickets and cockroaches showed an association with proctolin-like biological activity, but this has never been confirmed (O’Shea and Adams, 1981). Rather, work from various laboratories has provided definitive evidence for colocalization of bursicon and CCAP in these neurons (Taghert and Truman, 1982a; Adams and Phelps, 1983; Davis *et al.*, 1993). The recent availability of antisera specific for bursicon has confirmed that Cell 27 contains bursicon, and also shows intense bursicon staining in the vacuoles associated with these cell bodies (Honegger *et al.*, 2002). The presence of bursicon staining in these “vacuoles,” together with early ultrastructural work showing that the lumen of the vacuole is continuous with cisternae of the ER, indicate that the vacuole is a large storage depot for secretory product, specialized for large-scale release of hormonal substances into the hemolymph (Adams and O’Shea, 1981). In the cockroach, these cells project axons

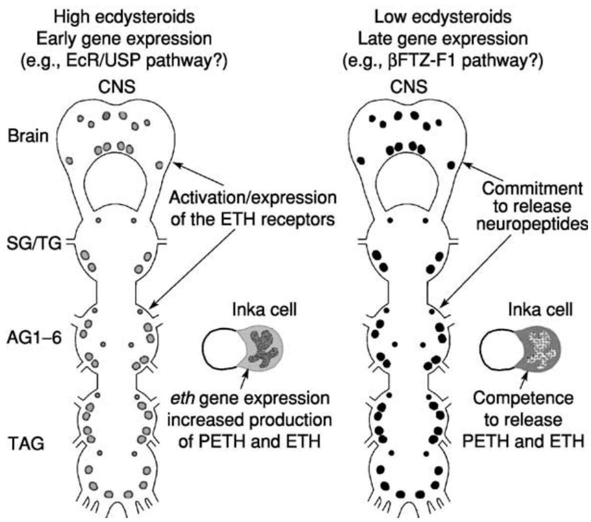


Figure 28 Model for ecdysteroid control of ecdysis in *Manduca*. High ecdysteroid levels induce CNS sensitivity to PETH and ETH. Appearance of this sensitivity indicates expression or activation of the ETH receptors (ETHR) in specific peptidergic and aminergic neurons. High ecdysteroid levels also control expression of the *eth* gene resulting in increased production of PETH, ETH, and their precursors in Inka cells. These processes are mediated by ecdysone receptor (EcR) and other ecdysteroid inducible transcription factors. At this time Inka cells are not competent to release PETH and ETH. Commitment to release CNS neuropeptides (e.g., eclosion hormone) and secretory competence of Inka cells is induced by late gene expression after ecdysteroid levels drop to very low levels at about 6–8 h prior to expected ecdysis. These processes may be mediated by βFTZ-F1 and/or other late transcription factors.

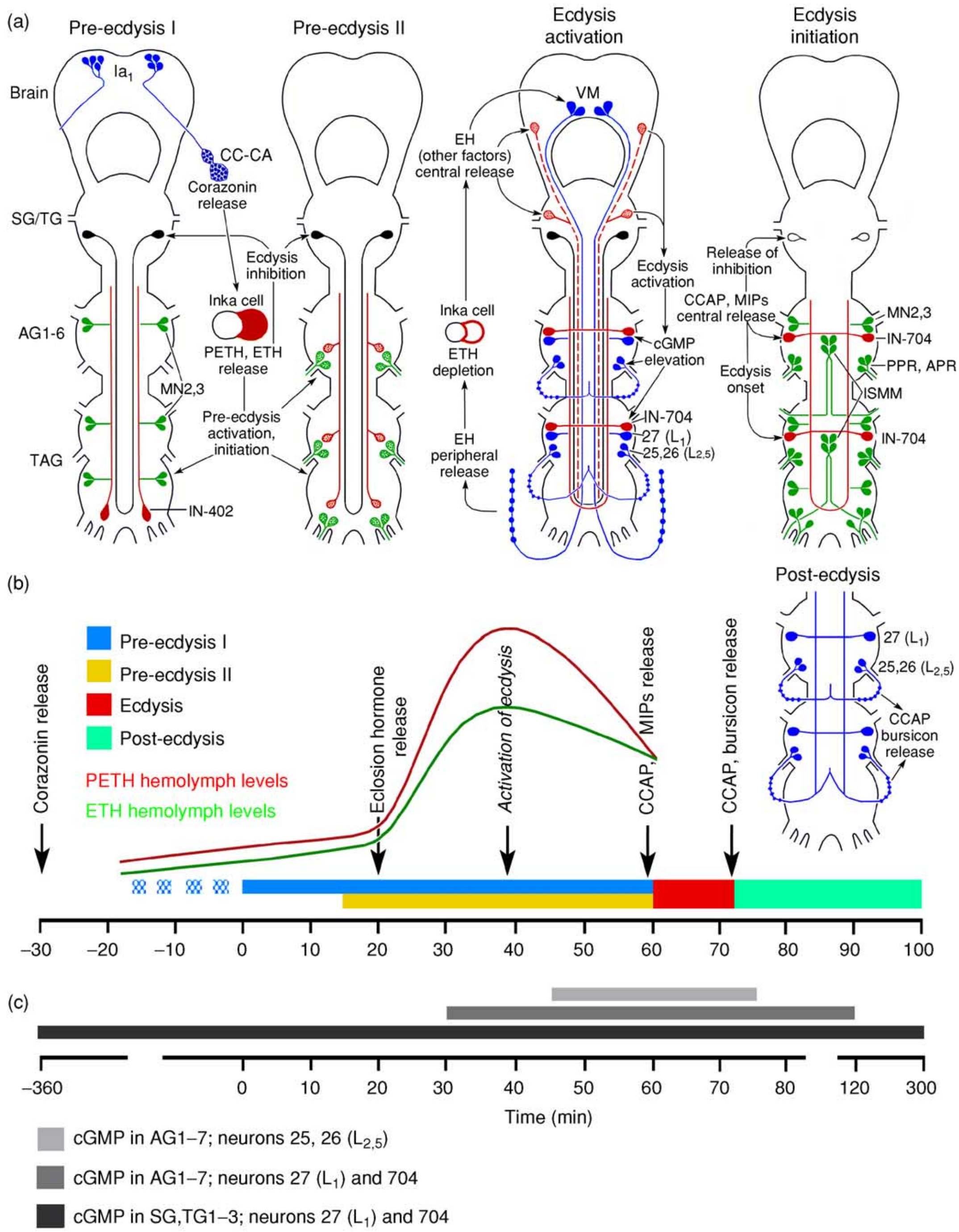


Figure 29 Model for regulation of the *Manduca* ecdysis sequence by peptide hormones. (a, b) Upon receiving sensory input indicating readiness to ecdyse, the CNS releases corazonin into the hemolymph. Corazonin induces the release of PETH and ETH from endocrine Inka cells. PETH and ETH act directly on all AG to initiate pre-ecdysis I and, after a 20 min delay, pre-ecdysis II. Interneurons IN-402 in the TAG control synchronous bursts of paired motoneurons MN-2,3 during pre-ecdysis I. Neurons controlling pre-ecdysis II are hypothetical (stippled cells). About 20 min into pre-ecdysis, EH release elicits massive release of ETH and depletion of Inka cells. Rising levels of ETH act on different targets in the brain (e.g., VM cells) and SG to activate ecdysis and post-ecdysis networks through cGMP elevation in interneurons IN-704 and cells 25–27 ($L_{1,2,5}$) already during pre-ecdysis (a–c). However, inhibitory neurons from the SG and TG1–3 delay ecdysis onset. At the appropriate time, an independent clock in each AG releases descending inhibition, leading to central release of CCAP and MIPs from IN-704 and initiation of ecdysis behavior. Motoneurons MN2,3, PPR, APR, and ISMM control ecdysis contractions. At the end of ecdysis, CCAP and bursicon are released from cells 25–27 ($L_{1,2,5}$) to control cuticle expansion, hardening, and tanning (see text for details). (a) Neurosecretory cells are blue, excitatory interneurons are red, hypothetical inhibitory interneurons are black, and motoneurons are green. (c) Note that cGMP elevation in the SG and TG is obvious several hours prior to ecdysis, while ecdysis activation is associated with cGMP elevation in AG1–7.

not only to the perisympathetic organs, but also in the heart, most likely for general liberation of bursicon into the hemolymph. The corelease of CCAP likely enhances distribution of bursicon to distant locations through its cardioacceleratory activity.

As the Fraenkel bioassay demonstrates, decapitation or ligation behind the head immediately after eclosion inhibits the tanning process. Thus, while most bursicon containing neurons reside in ventral ganglia, they depend on descending input from the cephalic ganglia for activation. Interestingly, *Drosophila* mutants in which CCAP containing neurons have been killed fail to expand or to sclerotize their wings. This indicates that bursicon release depends on activation of CCAP/bursicon-containing neurons, perhaps the Cell 27 homologs.

In order for structures in postecdysial insects to expand, plasticization of the cuticle is necessary. This occurs hours prior to ecdysis in moths. Curiously, even though bursicon is well known to control cuticle hardening, it also seems to be implicated as a plasticizing factor (Fraenkel, 1966; Mills and Lake, 1966; Reynolds, 1977; Taghert and Truman, 1982b). Unfortunately, all of these experiments have been done using tissue extracts and hence mixtures of hormones. It is unclear at the present time how a single hormone can regulate these opposite effects. Definitive assignment of bursicon functions will depend on experiments conducted with chemically pure material or on genetic experiments in which selective knockout of bursicon can be achieved.

In addition to pre-ecdysial plasticization, pre-ecdysial sclerotization of many structures also occurs well before ecdysis (Cottrell, 1962a). These include tanning of tarsal claws, parts of head capsules, and mandibles in moths, and vertical plates, mouth hooks, and sensory structures in flies. It is not clear if bursicon is involved in these pre-ecdysial tanning events; the mechanism(s) of selective tanning of these structures is unknown.

3.1.7. Model

Based on published data from various laboratories and preliminary experiments using *Manduca*, a model has been proposed for hormonal regulation of the ecdysis sequence (**Figures 28–30**). General principles of this model may apply to other insects, but there are several important differences in the regulation of ecdysis in *Bombyx* and especially in *Drosophila*, as discussed above (see Section 3.1.6.6). Successful ecdysis depends on a signaling cascade consisting of ecdysteroid and peptide hormones. Several days prior to ecdysis, high ecdysteroid levels act on Inka cells to induce expression of the ecdysteroid receptor (EcR) and the ETH gene, which results in increased production of PETH, ETH, and their precursors in Inka cells. However, high ecdysteroid levels suppress secretory competence of Inka cells to release PETH and ETH. Peak ecdysteroid levels also induce sensitivity of the CNS to Inka cell peptides. This sensitivity indicates that ecdysteroids control expression of ETH receptors (ETHR) oractivation of signal transduction in specific neurons (**Figure 28**). Specific gene expression induced by

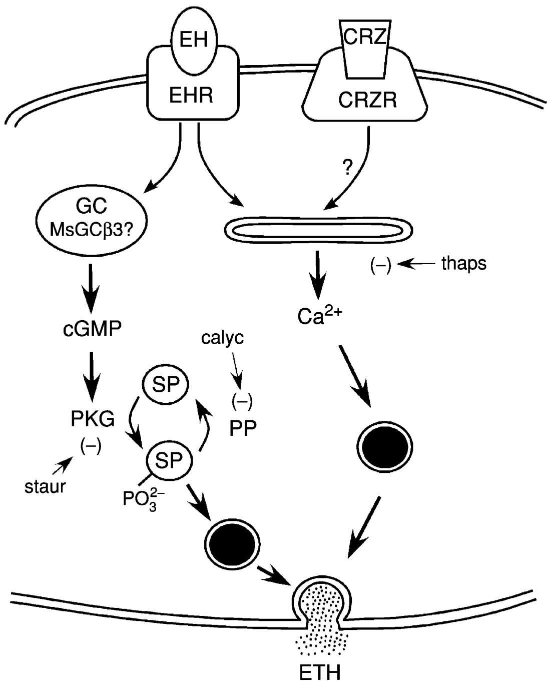


Figure 30 Model for corazonin and EH induced release of ETH from Inka cells in *Manduca*. Release of corazonin from the brain-CC–CA causes activation of its receptor (CRZR) in Inka cells resulting in secretion of PETH and ETH and initiation of pre-ecdysis (Kim *et al.*, 2004). Signal transduction mechanisms coupling receptor activity with peptide release are unknown, but this secretion may occur through liberation of calcium from intracellular stores. About 30 min into pre-ecdysis, EH action on Inka cells causes elevation of both cGMP and calcium, leading to massive depletion of ETH, whose direct action on the CNS activates the ecdysis network. EH-induced secretion occurs through parallel cGMP and calcium pathways. A soluble, NO-insensitive guanylate cyclase (MsGC-β3) mediates the action of EH to form cGMP, which in turn activates protein kinase G (PKG) (Morton and Simpson, 2002). A hypothetical substrate protein (SP) facilitates ETH release, which is augmented by the phosphatase inhibitor calyculin (calyc). The broad-spectrum kinase inhibitor staurosporine (staur) blocks ETH release, but not cGMP elevation, suggesting that its action is downstream. Release of calcium from thapsigargan-sensitive intracellular stores also contributes to release as a parallel signal transduction pathway. (Modified from Kingan, T.G., Cardullo, R.A., Adams, M.E., **2001**. Signal transduction in eclosion hormone-induced secretion of ecdysis-triggering hormone. *J. Biol. Chem. 276* (27), 25136–25142.)

peak ecdysteroid levels enables the CNS to respond to PETH and ETH and to execute the ecdysis sequence. However, these peptide hormones are released only following ecdysteroid decline several hours or days later. Declining ecdysteroid levels are required for late gene expression, which elicit secretory competence in Inka cells. Ecdysteroid decline is also required for commitment of some neurons to release central neuropeptides (e.g., EH). Expression of β-FTZ-F1 coincides with the appearance of secretory competence in Inka cells, suggesting that this transcription factor may participate in regulation of this process.

The initial secretion of PETH and ETH from Inka cells appears to be regulated by the neuropeptide corazonin (**Figures 29 and 30**). It is not known what factors control corazonin release, but it is speculated that sensory input to the brain may determine when the animal is ready to initiate the ecdysis sequence. Release of corazonin from the CC–CA causes secretion of Inka cell peptides (PETH and ETH) into the hemolymph. Other neuropeptides released from the ventral nerve cord and gut may be involved in regulation of PETH and ETH secretion. Low levels of PETH and ETH then act on abdominal ganglia to activate motor programs for pre-ecdysis I and II. In *Manduca*, PETH is a specific signal for activation of pre-ecdysis I, but ETH can activate both pre-ecdysis I and pre-ecdysis II neuronal circuitry in the absence of PETH. During pre-ecdysis I, interneurons IN-402 in the posterior TAG region have been identified to control synchronous bursts of paired motoneurons MN-2,3 in abdominal ganglia and corresponding dorsoventral contractions (Novicki and Weeks, 1995). Approximately 15–20 min later, ETH acts on additional neurons in each abdominal ganglion to induce posterioventral and proleg contractions (pre-ecdysis II). The principal modulatory neurons and their neurotransmitters that activate pre-ecdysis I and II networks within the CNS remain to be identified (**Figure 29**).

At the same time, ETH acts on the brain VM neurons and additional neurons in the tritocerebrum and SG to induce central and peripheral release of EH and other factor(s). Peripheral release of EH causes cGMP elevation and depletion of peptide hormones in Inka cells (**Figure 30**), while central release of EH and other factor(s) activates the abdominal ecdysis network. This network is composed of paired neurons 25–27 ($L_{1,2,5}$) and IN-704 producing CCAP, MIPs, and/or bursicon. Although cGMP elevation in this network is apparent already 30 min into natural pre-ecdysis, animals switch to ecdysis behavior much later. The delay from activation of the CCAP/MIP network to the onset of ecdysis behavior is controlled by putative descending inhibitory neurons from the SG and TG1–3 (see Section 3.1.6.1). The switch from pre-ecdysis to ecdysis is probably controlled by the central release of CCAP and MIPs from IN-704. MIPs suppress activities of excitatory and inhibitory neurons controlling proper execution of pre-ecdysis motor programs, while CCAP initiates the ecdysis motor program. CCAP may modulate activities of several motoneurons (MN2,3, ISM, PPR, and APR) controlling ecdysis movements (**Figure 29**; Weeks and Truman, 1984a, 1984b). After the old cuticle is shed, sensory input likely reactivates inhibitory neurons to stop ecdysis movements. Bursicon and CCAP are probably released after ecdysis from cells 25–27 ($L_{1,2,5}$) to regulate tanning and hardening of the cuticle.

Mechanisms mediating activation and initiation of different CNS motor programs by PETH and ETH are not known. The authors propose that Inka cell peptides bind to their receptors (ETHR-A and ETHR-B) expressed in a network of various peptidergic and/or aminergic neurons. The ETH receptor binding leads to a cascade of biochemical reactions controlling subsequent and precisely timed release of specific neuropeptides and neuroamines. These regulatory molecules then modulate different neuronal networks involved in pre-ecdysis and ecdysis behaviors. Thus, receptor mediated actions of PETH and ETH on different CNS ganglia are necessary for activation of specific neuronal networks, which then release central stimulatory or inhibitory factors controlling timing and performance of pre-ecdysis and ecdysis behaviors. Several of these ecdysis-specific central regulators have been identified (e.g., EH, CCAP, and MIPs), but many additional factors are expected to be essential for execution of pre-ecdysis I and pre-ecdysis II, as well as inhibition and activation of ecdysis. Identification of neurons expressing receptors for ETH, EH, CCAP, MIPs, and other "ecdysis" neuropeptides and neuroamines will provide tools for shedding more light on complex neuroendocrine interactions dedicated to shedding of the old cuticle.

Acknowledgments

We thank Y.-J. Kim, I. Spalovská-Valachova, I. Zitnanová, and L. Roller for their comments on the manuscript and technical assistance. We also thank Drs M. Blackburn, H. Dircksen, E. Marder, and Y. Tanaka for generous gifts of antisera to MIP I, CCAP, FMRFamide, and corazonin, respectively. This work was supported by grants from

the National Institute of Health (AI 40555 and GM067310) and Vedecká grantová agentúra (95/5305/800 and 2/7168/20).

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287* (5461), 2185–2195.

Adams, M.E., O'Shea, M., **1981**. Vacuolation of an identified peptidergic (proctolin-containing) neuron. *Brain Res. 230* (1–2), 439–444.

Adams, M.E., Phelps, M.N., **1983**. Co-localization of bursicon bioactivity and proctolin in identified neurons. *Soc. Neurosci. Abs. 9*, 313.

Adams, M.E., Zitnan, D., **1997**. Identification of ecdysis-triggering hormone in the silkworm *Bombyx mori. Biochem. Biophys. Res. Commun. 230* (1), 188–191.

Akai, H., **1992**. Ultrastructure of epitracheal gland during larval-pupal molt of *Bombyx mori. Cytologia 57* (2), 195–201.

Ashburner, M., Chihara, C., Meltzer, P., Richards, G., **1974**. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol. 38*, 655–662.

Audsley, N., McIntosh, C., Phillips, J.E., **1992a**. Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *J. Exp. Biol. 173*, 261–274.

Audsley, N., McIntosh, C., Phillips, J.E., **1992b**. Actions of ion-transport peptide from locust corpus cardiacum on several hindgut transport processes. *J. Exp. Biol. 173*, 275–288.

Bainbridge, S.P., Bownes, M., **1981**. Staging the metamorphosis of *Drosophila melanogaster. J. Embryol. Exp. Morph. 66*, 57–80.

Baker, J.D., McNabb, S.L., Truman, J.W., **1999**. The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster. J. Exp. Biol. 202* (Pt 21), 3037–3048.

Bendena, W.G., Donly, B.C., Fuse, M., Lee, E., Lange, A.B., *et al.*, **1997**. Molecular characterization of the inhibitory myotropic peptide leucomyosuppressin. *Peptides 18* (1), 157–163.

Benveniste, R.J., Taghert, P.H., **1999**. Cell type-specific regulatory sequences control expression of the *Drosophila* FMRF-NH2 neuropeptide gene. *J. Neurobiol. 38* (4), 507–520.

Bernays, E.A., **1971**. The vermiform larva of *Schistocerca gregaria* form and activity Insecta Orthoptera. *Z. Morphol. Tiere 70* (2), 183–200.

Bernays, E.A., **1972a**. Changes in the first instar cuticle of *Schistocerca gregaria* before and associated with hatching. *J. Insect Physiol. 18*, 897–912.

Bernays, E.A., **1972b**. The intermediate molt 1st ecdysis of *Schistocerca* Insecta Orthoptera. *Z. Morphol. Tiere 71* (2), 160–179.

Bernays, E.A., **1972c**. The muscles of newly hatched *Schistocerca gregaria* larvae and their possible functions in hatching digging and ecdysial movements Insecta Acrididae. *J. Zool. 166* (2), 141–158.

Berreur, P., Porcheron, P., Moriniere, M., Berreur-Bonnenfant, J., Belinski-Deutsch, S., *et al.*, **1984**. Ecdysteroids during the third larval instar in 1(3)ecd-1ts, a temperature-sensitive mutant of *Drosophila melanogaster. Gen. Comp. Endocrinol. 54* (1), 76–84.

Betz, H., **1990**. Ligand-gated ion channels in the brain: the amino acid receptor superfamily. *Neuron 5* (4), 383–392.

Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., **1999**. Reverse physiology in drosophila: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J. 18* (21), 5892–5900.

Blackburn, M.B., Jaffe, H., Kochansky, J., Raina, A.K., **2001**. Identification of four additional myoinhibitory peptides (MIPs) from the ventral nerve cord of *Manduca sexta. Arch. Insect Biochem. Physiol. 48* (3), 121–128.

Blackburn, M.B., Wagner, R.M., Kochansky, J.P., Harrison, D. J., Thomas-Laemont, P., *et al.*, **1995**. The identification of two myoinhibitory peptides, with sequence similarities to the galanins, isolated from the ventral nerve cord of *Manduca sexta. Regul. Pept. 57* (3), 213–219.

Bollenbacher, W.E., Smith, S.L., Goodman, W., Gilbert, L.I., **1981**. Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta. Gen. Comp. Endocrinol. 44* (3), 302–306.

Breidbach, O., Dircksen, H., **1991**. Crustacean cardioactive peptide-immunoreactive neurons in the ventral nerve cord and the brain of the meal beetle *Tenebrio molitor* during postembryonic development. *Cell Tissue Res. 265* (1), 129–144.

Breidbach, O., Dircksen, H., Wegerhoff, R., **1995**. Common general morphological pattern of peptidergic neurons in the arachnid brain: Crustacean cardioactive peptide-immunoreactive neurons in the protocerebrum of seven arachnid species. *Cell Tissue Res. 279* (1), 183–197.

Brody, T., Cravchik, A., **2000**. *Drosophila melanogaster* G protein-coupled receptors. *J. Cell Biol. 150* (2), F83–88.

Brown, M.R., Crim, J.W., Arata, R.C., Cai, H.N., Chun, C., *et al.*, **1999**. Identification of a *Drosophila* brain-gut peptide related to the neuropeptide Y family. *Peptides 20* (9), 1035–1042.

Cantera, R., Veenstra, J.A., Nassel, D.R., **1994**. Postembryonic development of corazonin-containing neurons and neurosecretory cells in the blowfly, *Phormia terraenovae. J. Comp. Neurol. 350* (4), 559–572.

Carlson, J.R., **1977a**. The imaginal ecdysis of the cricket *Teleogryllus oceanicus*. Part 1: Organization of motor programs and roles of central and sensory control. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 115* (3), 299–317.

Carlson, J.R., **1977b**. The imaginal ecdysis of the cricket *Teleogryllus oceanicus*. Part 2: The roles of identified motor units. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 115* (3), 319–336.

Carlson, J.R., Bentley, D., **1977**. Ecdysis: neural orchestration of a complex behavioral performance. *Science 195* (4282), 1006–1008.

Cazzamali, G., Grimmelikhuijzen, C.J., **2002**. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99* (19), 12073–12078.

Cazzamali, G., Hauser, F., Kobberup, S., Williamson, M., Grimmelikhuijzen, C.J., **2003**. Molecular identification of a *Drosophila* G protein-coupled receptor specific for crustacean cardioactive peptide. *Biochem. Biophys. Res. Commun. 303* (1), 146–152.

Cazzamali, G., Saxild, N., Grimmelikhuijzen, C., **2002**. Molecular cloning and functional expression of a *Drosophila* corazonin receptor. *Biochem. Biophys. Res. Commun. 298* (1), 31–36.

Cheung, C.C., Loi, P.K., Sylwester, A.W., Lee, T.D., Tublitz, N.J., **1992**. Primary structure of a cardioactive neuropeptide from the tobacco hawkmoth, *Manduca sexta*. *FEBS Lett. 313* (2), 165–168.

Choi, M.Y., Fuerst, E.J., Rafaeli, A., Jurenka, R., **2003**. Identification of a G protein-coupled receptor for pheromone biosynthesis activating neuropeptide from pheromone glands of the moth *Helicoverpa zea*. *Proc. Natl Acad. Sci. USA 100* (17), 9721–9726.

Chung, J.S., Dircksen, H., Webster, S.G., **1999**. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc. Natl Acad. Sci. USA 96* (23), 13103–13107.

Copenhaver, P.F., Taghert, P.H., **1989**. Development of the enteric nervous system in the moth. I. Diversity of cell types and the embryonic expression of FMRFamide-related neuropeptides. *Devel. Biol. 131* (1), 70–84.

Copenhaver, P.F., Truman, J.W., **1982**. The role of eclosion hormone in the larval ecdyses of *Manduca sexta*. *J. Insect Physiol. 28* (8), 695–702.

Copenhaver, P.F., Truman, J.W., **1985**. Immunocytochemical identification of the neurosecretory cells that produce the peptide eclosion hormone in the moth *Manduca sexta*. *Soc. Neurosci. Abstr. 11* (1), 326.

Copenhaver, P.F., Truman, J.W., **1986a**. Metamorphosis of the cerebral neuroendocrine system in the moth *Manduca sexta*. *J. Comp. Neurol. 249* (2), 186–204.

Copenhaver, P.F., Truman, J.W., **1986b**. Identification of the cerebral neurosecretory cells that contain eclosion hormone in the moth *Manduca sexta*. *J. Neurosci. 6* (6), 1738–1747.

Copenhaver, P.F., Truman, J.W., **1986c**. Control of neurosecretion in the moth *Manduca sexta*: physiological regulation of the eclosion hormone cells. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 158* (4), 445–455.

Cottrell, C.B., **1962a**. General observations on the imaginal ecdysis of blowflies. *Trans. R. Entomol. Soc. Lond. 114*, 317–333.

Cottrell, C.B., **1962b**. The imaginal ecdysis of blowflies. The control of cuticular hardening and darkening. *J. Exp. Biol. 39*, 395–411.

Cottrell, C.B., **1962c**. The imaginal ecdysis of blowflies. Detection of the blood-borne darkening factor and determination of some of its properties. *J. Exp. Biol. 39*, 413–430.

Cottrell, C.B., **1964**. Insect ecdysis with particular emphasis on cuticular hardening and darkening. *Adv. Insect Physiol. 2*, 175–218.

Curtis, A.T., Hori, M., Gren, J.M., Wolfgang, W.J., Hiruma, K., *et al.*, **1984**. Ecdysteroid regulation of the onset of cuticular melanization in the allatectomized and *black* mutant *Manduca sexta* larvae. *J. Insect Physiol. 30*, 597–606.

Davis, N.T., Blackburn, M.B., Golubeva, E.G., Hildebrand, J.G., **2003**. Localization of myoinhibitory peptide immunoreactivity in *Manduca sexta* and *Bombyx mori*, with indications that the peptide has a role in molting and ecdysis. *J. Exp. Biol. 206* (Pt 9), 1449–1460.

Davis, N.T., Homberg, U., Dircksen, H., Levine, R.B., Hildebrand, J.G., **1993**. Crustacean cardioactive peptide-immunoreactive neurons in the hawkmoth *Manduca sexta* and changes in their immunoreactivity during postembryonic development. *J. Comp. Neurol. 338* (4), 612–627.

de Kleijn, D.P., de Leeuw, E.P., van den Berg, M.C., Martens, G.J., van Herp, F., **1995**. Cloning and expression of two mRNAs encoding structurally different crustacean hyperglycemic hormone precursors in the lobster *Homarus americanus*. *Biochim. Biophys. Acta 1260* (1), 62–66.

de Kleijn, D.P., Janssen, K.P., Martens, G.J., Van Herp, F., **1994**. Cloning and expression of two crustacean hyperglycemic-hormone mRNAs in the eyestalk of the crayfish *Orconectes limosus*. *Eur. J. Biochem. 224* (2), 623–629.

de Kleijn, D.P., Janssen, K.P., Waddy, S.L., Hegeman, R., Lai, W.Y., *et al.*, **1998**. Expression of the crustacean hyperglycaemic hormones and the gonad-inhibiting hormone during the reproductive cycle of the female American lobster *Homarus americanus*. *J. Endocrinol. 156* (2), 291–298.

Dircksen, H., **1994**. Distribution and physiology of crustacean cardioactive peptide in arthropods. Perspectives in comparative endocrinology. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 139–148.

Dircksen, H., **1998**. Conserved crustacean cardioactive peptide (CCAP) neuronal networks and functions in arthropod evolution. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology, 65. Cambridge University Press, Cambridge, pp. 302–333.

Dircksen, H., Heyn, U., **1998**. Crustacean hyperglycemic hormone-like peptides in crab and locust peripheral intrinsic neurosecretory cells. *Ann. New York Acad. Sci. 839*, 392–394.

Dircksen, H., Keller, R., **1988**. Immunocytochemical localization of CCAP, a novel crustacean cardioactive peptide, in the nervous system of the shore crab, *Carcinus maenas*. *Cell Tissue Res. 254*, 347–360.

Dircksen, H., Mueller, A., Keller, R., **1991**. Crustacean cardioactive peptide in the nervous system of the locust *Locusta migratoria* an immunocytochemical study on the ventral nerve cord and peripheral innervation. *Cell Tissue Res. 263* (3), 439–458.

Dircksen, H., Webster, S.G., Keller, R., **1988**. Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. *Cell Tissue Res. 251*, 3–12.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., *et al.*, **1986**. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature 321* (6065), 75–79.

Donini, A., Agricola, H., Lange, A.B., **2001**. Crustacean cardioactive peptide is a modulator of oviduct contractions in *Locusta migratoria*. *J. Insect Physiol. 47* (3), 277–285.

Donini, A., Ngo, C., Lange, A.B., **2002**. Evidence for crustacean cardioactive peptide-like innervation of the gut in *Locusta migratoria*. *Peptides 23* (11), 1915–1923.

Donly, B.C., Fuse, M., Orchard, I., Tobe, S.S., Bendena, W.G., **1996**. Characterization of the gene for leucomyosuppressin and its expression in the brain of the cockroach *Diploptera punctata*. *Insect Biochem. Mol. Biol. 26* (6), 627–637.

Duttlinger, A., Berry, K., Nichols, R., **2002**. The different effects of three *Drosophila melanogaster* dFMRFamide-containing peptides on crop contractions suggest these structurally related peptides do not play redundant functions in gut. *Peptides 23* (11), 1953–1957.

Egerod, K., Reynisson, E., Hauser, F., Cazzamali, G., Williamson, M., *et al.*, **2003**. Molecular cloning and functional expression of the first two specific insect myosuppressin receptors. *Proc. Natl Acad. Sci. USA 100* (17), 9808–9813.

Endo, H., Nagasawa, H., Watanabe, T., **2000**. Isolation of a cDNA encoding a CHH-family peptide from the silkworm *Bombyx mori*. *Insect Biochem. Mol. Biol. 30* (5), 355–361.

Ewer, J., De Vente, J., Truman, J. W., **1994**. Neuropeptide induction of cyclic GMP increases in the insect CNS: resolution at the level of single identifiable neurons. *J. Neurosci. 14* (12), 7704–7712.

Ewer, J., Gammie, S.C., Truman, J.W., **1997**. Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. *J. Exp. Biol.* 869–881.

Ewer, J., Reynolds, S., **2002**. Neuropeptide control of molting in insects. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain and Behavior, vol. 3. Elsevier Science, Boston, pp. 1–92.

Ewer, J., Truman, J.W., **1996**. Increases in cyclic 3′, 5′-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide-immunoreactive insect neuronal network. *J. Comp. Neurol. 370* (3), 330–341.

Ewer, J., Truman, J.W., **1997**. Invariant association of ecdysis with increases in cyclic 3′,5′-guanosine monophosphate immunoreactivity in a small network of peptidergic neurons in the hornworm, *Manduca sexta*. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 181* (4), 319–330.

Ewer, J., Wang, C.M., Klukas, K.A., Mesce, K.A., Truman, J.W., *et al.*, **1998**. Programmed cell death of identified peptidergic neurons involved in ecdysis behavior in the moth, *Manduca sexta*. *J. Neurobiol. 37* (2), 265–280.

Fraenkel, G., **1935**. Observations and experiments on the blowfly (*Calliphora erythrocephala*) during the first day after emergence. *Proc. Zool. Soc. Lond. 87*, 893–904.

Fraenkel, G., **1965**. Bursicon, a hormone which mediates tanning of the cuticle in the adult fly and other insects. *J. Insect Physiol. 11*, 513–556.

Fraenkel, G., **1966**. Properties of bursicon: an insect protein hormone that controls cuticular tanning. *Science 151*, 91–93.

Fraenkel, G., Hsiao, C., **1962**. Hormonal and nervous control of tanning in the fly. *Science 138*, 27–29.

Fraenkel, G., Hsiao, C., **1963**. Tanning in the adult fly: A new function of neurosecretion in the brain. *Science 141*, 1057–1058.

Fujisawa, Y., Shimoda, M., Kiguchi, K., Ichikawa, T., Fujita, N., **1993**. The inhibitory effect of a neuropeptide, ManducaFLRFamide, on the midgut activity of the sphingid moth, *Agrius convolvuli*. *Zool. Sci. 10* (5), 773–777.

Furuya, K., Liao, S., Reynolds, S.E., Ota, R.B., Hackett, M., *et al.*, **1993**. Isolation and identification of a cardioactive peptide from *Tenebrio molitor* and *Spodoptera eridania*. *Biol. Chem. Hoppe Seyler 374* (12), 1065–1074.

Fuse, M., Bendena, W.G., Donly, B.C., Tobe, S.S., Orchard, I., **1998**. In situ hybridization analysis of leucomyosuppressin mRNA expression in the cockroach, *Diploptera punctata*. *J. Comp. Neurol. 395* (3), 328–341.

Fuse, M., Orchard, I., **1998**. The muscular contractions of the midgut of the cockroach, Diploptera punctata: effects of the insect neuropeptides proctolin and leucomyosuppressin. *Regul. Pept. 77* (1–3), 163–168.

Fuse, M., Truman, J.W., **2002**. Modulation of ecdysis in the moth *Manduca sexta:* the roles of the suboesophageal and thoracic ganglia. *J. Exp. Biol. 205* (Pt 8), 1047–1058.

Gäde, G., **1997**. Hormonal regulation in insects: Facts, gaps and future directions. *Physiol. Rev. 77* (4), 963–1032.

Gammie, S.C., Truman, J.W., **1997a**. Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. *J. Neurosc. 17* (11), 4389–4397.

Gammie, S.C., Truman, J.W., **1997b**. An endogenous elevation of cGMP increases the excitability of identified insect neurosecretory cells. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 180* (4), 329–337.

Gammie, S.C., Truman, J.W., **1999**. Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behavior. *J. Exp. Biol. 202* (Pt 4), 343–352.

Garczynski, S.F., Brown, M.R., Shen, P., Murray, T.F., Crim, J.W., **2002**. Characterization of a functional neuropeptide F receptor from *Drosophila melanogaster. Peptides 23* (4), 773–780.

Gaude, H., **1975**. Histological studies on the structure and function of the neuro secretory system of the house cricket *Acheta domesticus*. *Zool. Anzeiger 194* (3–4), 151–164.

Grillot, J.-P., Delachambre, J., Provansal, A., **1976**. Roles des organes perisympathetiques et dynamique de la secretion de la bursicon chez *Tenebrio molitor. J. Insect Physiol. 22*, 763–780.

Gu, P.L., Chan, S.M., **1998**. The shrimp hyperglycemic hormone-like neuropeptide is encoded by multiple copies of genes arranged in a cluster. *FEBS Lett. 441* (3), 397–403.

Gu, P.L., Yu, K.L., Chan, S.M., **2000**. Molecular characterization of an additional shrimp hyperglycemic hormone: cDNA cloning, gene organization, expression and biological assay of recombinant proteins. *FEBS Lett. 472* (1), 122–128.

Hansen, I.A., Sehnal, F., Meyer, S.R., Scheller, K., **2001**. Corazonin gene expression in the waxmoth *Galleria mellonella*. *Insect Mol. Biol. 10* (4), 341–346.

Helle, J., Dircksen, H., Eckert, M., Naessel Dick, R., Spoerhase-Eichmann, U., *et al.*, **1995**. Putative neurohemal areas in the peripheral nervous system of an insect, *Gryllus bimaculatus*, revealed by immunocytochemistry. *Cell Tissue Res. 281* (1), 43–61.

Hewes, R.S., Taghert, P.H., **2001**. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res. 11* (6), 1126–1142.

Hewes, R.S., Truman, J.W., **1991**. The roles of central and peripheral eclosion hormone release in the control of ecdysis behavior in *Manduca sexta*. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 168* (6), 697–708.

Hewes, R.S., Truman, J.W., **1994**. Steroid regulation of excitability in identified insect neurosecretory cells. *J. Neurosc. 14* (3 Pt 2), 1812–1819.

Hiruma, K., Riddiford, L.M., **2001**. Regulation of transcription factors MHR4 and betaFTZ-F1 by 20-hydroxyecdysone during a larval molt in the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 232* (1), 265–274.

Homberg, U., Davis, N.T., Hildebrand, J.G., **1991**. Peptide-immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta*. *J. Comp. Neurol. 303* (1), 35–52.

Homberg, U., Kingan, T.G., Hildebrand, J.G., **1990**. Distribution of FMRFamide-like immunoreactivity in the brain and suboesophageal ganglion of the sphinx moth *Manduca sexta* and colocalization with SCPB-, BPP-, and GABA-like immunoreactivity. *Cell Tissue Res. 259* (3), 401–419.

Honegger, H.W., Market, D., Pierce, L.A., Dewey, E.M., Kostron, B., *et al.*, **2002**. Cellular localization of bursicon using antisera against partial peptide sequences of this insect cuticle-sclerotizing neurohormone. *J. Comp. Neurol. 452* (2), 163–177.

Horodyski, F.M., Ewer, J., Riddiford, L.M., Truman, J.W., **1993**. Isolation, characterization and expression of the eclosion hormone gene of *Drosophila melanogaster. Eur. J. Biochem. 215* (2), 221–228.

Horodyski, F.M., Riddiford, L.M., Truman, J.W., **1989**. Isolation and expression of the eclosion hormone gene from the tobacco hornworm, *Manduca sexta*. *Proc. Natl Acad. Sci. USA 86* (20), 8123–8127.

Hua, Y., Ishibashi, J., Saito, H., Tawfik, A.I., Sakakibara, M., *et al.*, **2000**. Identification of [Arg7] corazonin in the silkworm, *Bombyx mori* and the cricket, *Gryllus bimaculatus*, as a factor inducing dark color in an albino strain of the locust, *Locusta migratoria*. *J. Insect Physiol. 46* (6), 853–860.

Hua, Y.J., Tanaka, Y., Nakamura, K., Sakakibara, M., Nagata, S., *et al.*, **1999**. Identification of a prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. *J. Biol. Chem. 274* (44), 31169–31173.

Hughes, T.D., **1980a**. The imaginal ecdysis of the desert locust *Schistocerca gregaria* 1. Description of the Behavior. *Physiol. Entomol. 5* (1), 47–54.

Hughes, T.D., **1980b**. The imaginal ecdysis of the desert locust *Schistocerca gregaria* 2. Motor activity underlying the preemergence and emergence behavior. *Physiol. Entomol. 5* (1), 55–72.

Hughes, T.D., **1980c**. The imaginal ecdysis of the desert locust *Schistocerca gregaria* 3. Motor activity underlying the expansional and post expansion behavior. *Physiol. Entomol. 5* (2), 141–152.

Hughes, T.D., **1980d**. The imaginal ecdysis of the desert locust *Schistocerca gregaria* 4. The Role of the Gut. *Physiol. Entomol. 5* (2), 153–164.

Ikeda, E., **1913**. Kimon Rimensen. In: Ikeda, E. (Ed.), Experimental Anatomy and Physiology of *Bombyx mori*. Meibundo, Tokyo, pp. 242–243.

Iversen, A., Cazzamali, G., Williamson, M., Hauser, F., Grimmelikhuijzen, C.J., **2002**. Molecular identification of the first insect ecdysis triggering hormone receptors. *Biochem. Biophys. Res. Commun. 299* (5), 924–931.

Jahn, G., Käuser, G., Koolman, J., **1991**. Localization of the crustacean cardioactive peptide (CCAP) in the central nervous system of larvae of the blowfly, Calliphora vicina. *Gen. Comp. Endocrinol. 82*, 287.

Johnson, E.C., Bohn, L.M., Barak, L.S., Birse, R.T., Nassel, D.R., *et al.*, **2003**. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. *J. Biol. Chem. 278* (52), 52172–52178.

Kamito, T., Tanaka, H., Sato, B., Nagasawa, H., Suzuki, A., **1992**. Nucleotide sequence of cDNA for the eclosion hormone of the silkworm, *Bombyx mori*, and the expression in a brain. *Biochem. Biophys. Res. Commun. 182* (2), 514–519.

Kataoka, H., Li, J.P., Lui, A.S., Kramer, S.J., Schooley, D.A., **1992**. Complete structure of eclosion hormone of *Manduca sexta*. Assignment of disulfide bond location. *Int. J. Pep. Protein Res. 39* (1), 29–35.

Kataoka, H., Troetschler, R.G., Kramer, S.J., Cesarin, B.J., Schooley, D.A., **1987**. Isolation and primary structure of the eclosion hormone of the tobacco hornworm, *Manduca sexta*. *Biochem. Biophys. Res. Commun. 146* (2), 746–750.

Kegel, G., Reichwein, B., Weese, S., Gaus, G., Peter-Katalinic, J., *et al.*, **1989**. Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. *FEBS Lett. 255* (1), 10–14.

Keller, R., **1992**. Crustacean neuropeptides: structures, functions and comparative aspects. *Experientia 48* (5), 439–448.

Keller, R., Kegel, G., Reichwein, B., Sedlmeier, D., Soyez, D., **1999**. Biological effects of neurohormones of the CHH/MIH/GIH peptide family in crustaceans. In: Roubos, E.W., Wendelaar Bonga, S.E., Vaudry, H., De Loof, A. (Eds.), Recent Developments in Comparative Endocrinology and Neurobiology. Shaker Publishing B.V., Maastricht, pp. 209–212.

Kim, Y.J., Spalovska-Valachova, I., Cho, K.-H., Zitnanova, I., Park, Y., *et al.*, **2004**. Corazonin receptor signaling in ecdysis initiation. *Proc. Natl Acad. Sci. USA 101*, 6704–6709.

Kimura, K.I., Truman, J.W., **1990**. Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster. J. Neurosci. 10* (2), 403–411.

Kingan, T.G., Adams, M.E., **2000**. Ecdysteroids regulate secretory competence in Inka cells. *J. Exp. Biol. 203* (19), 3011–3018.

Kingan, T.G., Cardullo, R.A., Adams, M.E., **2001**. Signal transduction in eclosion hormone-induced secretion of ecdysis-triggering hormone. *J. Biol. Chem. 276* (27), 25136–25142.

Kingan, T.G., Gray, W., Zitnan, D., Adams, M.E., **1997a**. Regulation of ecdysis-triggering hormone release by eclosion hormone. *J. Exp. Biol. 200*, 3245–3256.

Kingan, T.G., Shabanowitz, J., Hunt, D.F., Witten, J.L., **1996**. Characterization of two myotropic neuropeptides in the FMRFamide family from segmental ganglia of the moth, *Manduca sexta*: candidate neurohormones and neuromodulators. *J. Exp. Biol. 199*, 1095–1104.

Kingan, T.G., Teplow, D.B., Phillips, J.M., Riehm, J.P., Rao, K.R., *et al.*, **1990**. A new peptide in the FMRF amide family isolated from the CNS of the hawkmoth *Manduca sexta*. *Peptides 11* (4), 849–856.

Kingan, T.G., Zitnan, D., Jaffe, H., Beckage, N.E., **1997b**. Identification of neuropeptides in the midgut of parasitized insects: FLRFamides as candidate paracrines. *Mol. Cell Endocrinol. 133* (1), 19–32.

Klein, C., Kallenborn, H.G., Radlicki, C., **1999**. The 'Inka cell' and its associated cells: ultrastructure of the epitracheal glands in the gypsy moth, *Lymantria dispar. J. Insect Physiol. 45* (1), 65–73.

Klukas, K.A., Brelje, T.C., Mesce, K.A., **1996**. Novel mouse IgG-like immunoreactivity expressed by neurons in the moth *Manduca sexta*: developmental regulation and colocalization with crustacean cardioactive peptide. *Microsc. Res. Tech. 35* (3), 242–264.

Kono, T., Mizoguchi, A., Nagasawa, H., Ishizaki, H., Fugo, H., *et al.*, **1990a**. A monoclonal antibody against a synthetic carboxyl-terminal fragment of the eclosion hormone of the silkworm, *Bombyx mori*: characterization and application to immunohistochemistry and affinity chromatography. *Zool. Sci. 7*, 47–54.

Kono, T., Nagasawa, H., Isogai, A., Fugo, H., Suzuki, A., **1987**. Amino Acid Sequence of Eclosion Hormone of the Silkworm *B. mori*. *Agricult. Biol. Chem. 51* (8), 2307–2308.

Kono, T., Nagasawa, H., Kataoka, H., Isogai, A., Fugo, H., *et al.*, **1990b**. Eclosion hormone of the silkworm *Bombyx mori*. Expression in *Escherichia coli* and location of disulfide bonds. *FEBS Lett. 263* (2), 358–360.

Kopec, S., **1917**. Experiments on metamorphosis of insects. *Bull. Int. Acad. Cracovie B* 57–60.

Kopec, S., **1922**. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull. 42*, 322–342.

Kostron, B., Kaltenhauser, U., Seibel, B., Br, Auml, Unig, P., *et al.*, **1996**. Localization of bursicon in CCAP-immunoreactive cells in the thoracic ganglia of the cricket *Gryllus bimaculatus*. *J. Exp. Biol. 199* (Pt 2), 367–377.

Kostron, B., Marquardt, K., Kaltenhauser, U., Honegger, H.W., **1995**. Bursicon, the cuticle sclerotizing hormone – comparison of its molecular mass in different insects. *J. Insect Physiol. 41* (12), 1045–1053.

Lan, Q., Wu, Z., Riddiford, L.M., **1997**. Regulation of the ecdysone receptor, USP, E75 and MHR3 mRNAs by 20-hydroxyecdysone in the GV1 cell line of the tobacco hornworm, *Manduca sexta*. *Insect Mol. Biol. 6* (1), 3–10.

Lange, A.B., Cheung, I.L., **1999**. The modulation of skeletal muscle contraction by FMRFamide-related peptides of the locust. *Peptides 20* (12), 1411–1418.

Lange, A.B., Peeff, N.M., Orchard, I., **1994**. Isolation, sequence, and bioactivity of FMRFamide-related peptides from the locust ventral nerve cord. *Peptides 15* (6), 1089–1094.

Lavorgna, G., Karim, F.D., Thummel, C.S., Wu, C., **1993**. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl Acad. Sci. USA 90* (7), 3004–3008.

Lee, E., Lange, A., Orchard, I., Fuse, M., Tobe, S.S., *et al.*, **2002**. Characterization and baculovirus-directed expression of a myosuppressin encoding cDNA from the true armyworm, *Pseudaletia unipuncta*. *Peptides 23* (4), 747–756.

Lee, K.Y., Horodyski, F.M., Chamberlin, M.E., **1998**. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* LRFamides in the tobacco hornworm *Manduca sexta*. *J. Exp. Biol. 201*, 3067–3074.

Lefkowitz, R.J., Caron, M.G., **1988**. Adrenergic receptors. Models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J. Biol. Chem. 263* (11), 4993–4996.

Lehman, H.K., Murgiuc, C.M., Miller, T.A., Lee, T.D., Hildebrand, J.G., **1993**. Crustacean cardioactive peptide in the sphinx moth, *Manduca sexta*. *Peptides 14* (4), 735–741.

Levine, R.B., Truman, J.W., **1982**. Metamorphosis of the insect nervous system: changes in morphology and synaptic interactions of identified neurones. *Nature 299* (5880), 250–252.

Levine, R.B., Truman, J.W., **1983**. Peptide activation of a simple neural circuit. *Brain Res. 279* (1–2), 335–338.

Li, X.J., Wolfgang, W., Wu, Y.N., North, R.A., Forte, M., **1991**. Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides. *EMBO J. 10* (11), 3221–3229.

Li, X.J., Wu, Y.N., North, R.A., Forte, M., **1992**. Cloning, functional expression, and developmental regulation of a neuropeptide Y receptor from *Drosophila melanogaster*. *J. Biol. Chem. 267* (1), 9–12.

Liu, L., Laufer, H., Wang, Y., Hayes, T., **1997**. A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. *Biochem. Biophys. Res. Commun. 237* (3), 694–701.

Loi, P.K., Emmal, S.A., Park, Y., Tublitz, N.J., **2001**. Identification, sequence and expression of a crustacean cardioactive peptide (CCAP) gene in the moth *Manduca sexta*. *J. Exp. Biol. 204* (Pt 16), 2803–2816.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., **1995**. A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. *J. Biol. Chem. 270* (36), 21103–21108.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., Gade, G., **2000**. Identification of multiple peptides homologous to cockroach and cricket allatostatins in the stick insect *Carausius morosus*. *Insect Biochem. Mol. Biol. 30* (8–9), 711–718.

Lu, D., Lee, K.Y., Horodyski, F.M., Witten, J.L., **2002**. Molecular characterization and cell-specific expression of a *Manduca sexta* FLRFamide gene. *J. Comp. Neurol. 446* (4), 377–396.

Macins, A., Meredith, J., Zhao, Y., Brock, H.W., Phillips, J.E., **1999**. Occurrence of ion transport peptide (ITP) and ion transport-like peptide (ITP-L) in orthopteroids. *Arch. Insect Biochem. Physiol. 40* (2), 107–118.

Marti, T., Takio, K., Walsh, K.A., Terzi, G., Truman, J.W., **1987**. Microanalysis of the amino acid sequence of the eclosion hormone from the tobacco hornworm *Manduca sexta*. *FEBS Lett. 219* (2), 415–418.

McCormick, J., Nichols, R., **1993**. Spatial and temporal expression identify dromyosuppressin as a brain-gut peptide in *Drosophila melanogaster*. *J. Comp. Neurol. 338* (2), 278–288.

McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., *et al.*, **1997**. Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron 19* (4), 813–823.

Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., *et al.*, **2002**. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99* (24), 15363–15368.

Meredith, J., Ring, M., Macins, A., Marschall, J., Cheng, N.N., *et al.*, **1996**. Locust ion transport peptide (ITP): primary structure, cDNA and expression in a baculovirus system. *J. Exp. Biol. 199* (Pt 5), 1053–1061.

Mesce, K.A., Truman, J.W., **1988**. Metamorphosis of the ecdysis motor pattern in the Hawkmoth *Manduca sexta*. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 163* (3), 287–300.

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., *et al.*, **1995**. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature 377* (6549), 532–535.

Miao, Y., Waters, E.M., Witten, J.L., **1998**. Developmental and regional-specific expression of FLRFamide peptides in the tobacco hornworm, *Manduca sexta*, suggests functions at ecdysis. *J. Neurobiol. 37* (3), 469–485.

Miles, C.I., Booker, R., **1998**. The role of the frontal ganglion in the feeding and eclosion behavior of the moth *Manduca sexta*. *J. Exp. Biol. 201* (Pt 11), 1785–1798.

Miles, C.I., Weeks, J.C., **1991**. Developmental attenuation of the pre-ecdysis motor pattern in the tobacco hornworm, *Manduca sexta*. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 168* (2), 179–190.

Mills, R.R., Lake, C.R., **1966**. Hormonal control of tanning in the American cockroach. IV. Preliminary purification of the hormone. *J. Insect Physiol. 12*, 1395–1401.

Monnier, D., Colas, J.F., Rosay, P., Hen, R., Borrelli, E., *et al.*, **1992**. NKD, a developmentally regulated tachykinin receptor in *Drosophila*. *J. Biol. Chem. 267* (2), 1298–1302.

Morton, D.B., Simpson, P.J., **2002**. Cellular signaling in eclosion hormone action. *J. Insect Physiol. 48* (1), 1–13.

Nachman, R.J., Giard, W., Favrel, P., Suresh, T., Sreekumar, S., *et al.*, **1997**. Insect myosuppressins and sulfakinins stimulate release of the digestive enzyme alpha-amylase in two invertebrates: The scallop *Pecten maximus* and insect *Rhynchophorus ferrugineus*. *Ann. New York Acad. Sci. 814*, 335–338.

Nagasawa, H., Fugo, H., Takahashi, S., Kamito, T., Isogai, A., *et al.*, **1983**. Purification and properties of eclosion hormone of the silkworm *Bombyx mori*. *Agricult. Biol. Chem. 47*, 1901–1906.

Nagasawa, H., Kamito, T., Takahashi, S., Isogai, A., Fugo, H., *et al.*, **1985**. Eclosion hormone of the silkworm *Bombyx mori*. Purification and determination of

the N-terminal amino acid sequence. *Insect Biochem. 15*, 573–578.

Nagasawa, H., Mikogami, T., Kono, T., Fugo, H., Suzuki, A., **1987**. Molecular heterogeneity of eclosion hormone in adult heads of the Silkworm *Bombyx Mori*. *Agricult. Biol. Chem. 51* (6), 1741–1743.

Nambu, J.R., Murphy-Erdosh, C., Andrews, P.C., Feistner, G.J., Scheller, R.H., **1988**. Isolation and characterization of a *Drosophila* neuropeptide gene. *Neuron 1* (1), 55–61.

Nichols, R., **2003**. Signaling pathways and physiological functions of *Drosophila melanogaster* FMRFamide-related peptides. *Annu. Rev. Entomol. 48*, 485–503.

Nichols, R., Bendena, W.G., Tobe, S.S., **2002**. Myotropic peptides in *Drosophila melanogaster* and the genes that encode them. *J. Neurogenet. 16*, 1–28.

Nichols, R., Lim, I.A., **1996**. Spatial and temporal immunocytochemical analysis of drosulfakinin (Dsk) gene products in the *Drosophila melanogaster* central nervous system. *Cell Tissue Res. 283* (1), 107–116.

Nichols, R., McCormick, J., Lim, I., **1997**. Dromyosuppressin and drosulfakinin, two structurally related *Drosophila* neuropeptides, are uniquely expressed in the adult central nervous system. *Ann. New York Acad. Sci. 814*, 315–318.

Nichols, R., McCormick, J., Lim, I., Caserta, L., **1995**. Cellular expression of the *Drosophila melanogaster* FMRFamide neuropeptide gene product DPKQDFMR Famide. Evidence for differential processing of the FMRFamide polypeptide precursor. *J. Mol. Neurosci. 6* (1), 1–10.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., **1997**. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng. 10* (1), 1–6.

Nighorn, A., Byrnes, K.A., Morton, D.B., **1999**. Identification and characterization of a novel beta subunit of soluble guanylyl cyclase that is active in the absence of a second subunit and is relatively insensitive to nitric oxide. *J. Biol. Chem. 274* (4), 2525–2531.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., *et al.*, **1983**. Structural homology of *Torpedo californica* acetylcholine receptor subunits. *Nature 302* (5908), 528–532.

Novicki, A., Weeks, J.C., **1993**. Organization of the larval pre-ecdysis motor pattern in the tobacco hornworm, *Manduca sexta*. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 173* (2), 151–162.

Novicki, A., Weeks, J.C., **1995**. A single pair of interneurons controls motor neuron activity during pre-ecdysis compression behavior in larval *Manduca sexta*. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 176* (1), 45–54.

Novicki, A., Weeks, J.C., **1996**. The initiation of pre-ecdysis and ecdysis behaviors in larval *Manduca sexta*: The roles of the brain, terminal ganglion and eclosion hormone. *J. Exp. Biol. 199* (8), 1757–1769.

Novicki, A., Weeks, J.C., **2000**. Developmental attenuation of *Manduca* pre-ecdysis behavior involves neural changes upstream of motoneurons and relay interneurons. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 186* (1), 69–79.

Numa, S., **1989**. A molecular view of neurotransmitter receptors and ionic channels. In: Kaback, H.R., Kirschner, M.W., Numa, S., *et al.* (Eds.), Harvey Lectures Series, vol. 83. Alan R. Liss, New York, pp. 121–166.

O'Brien, M.A., Schneider, L.E., Taghert, P.H., **1991**. In situ hybridization analysis of the FMRFamide neuropeptide gene in *Drosophila*. II. Constancy in the cellular pattern of expression during metamorphosis. *J. Comp. Neurol. 304* (4), 623–638.

O'Brien, M.A., Taghert, P.H., **1998**. A peritracheal neuropeptide system in insects: release of myomodulin-like peptides at ecdysis. *J. Exp. Biol. 201* (Pt 2), 193–209.

O'Shea, M., Adams, M.E., **1981**. Pentapeptide (proctolin) associated with an identified neuron. *Science 213* (4507), 567–569.

Ohira, T., Watanabe, T., Nagasawa, H., Aida, K., **1997**. Cloning and sequence analysis of a cDNA encoding a crustacean hyperglycemic hormone from the Kuruma prawn *Penaeus japonicus*. *Mol. Mar. Biol. Biotechnol. 6* (1), 59–63.

Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., Ewer, J., **2003b**. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. *Development 130* (12), 2645–2656.

Park, Y., Filippov, V., Gill, S.S., Adams, M.E., **2002a**. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development 129* (2), 493–503.

Park, Y., Kim, Y.J., Adams, M.E., **2002b**. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc. Natl Acad. Sci. USA 99* (17), 11423–11428.

Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., **2003a**. Two subtypes of ecdysis-triggering hormone receptor in *Drosophila melanogaster*. *J. Biol. Chem. 278* (20), 17710–17715.

Park, Y., Zitnan, D., Gill, S.S., Adams, M.E., **1999**. Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. *FEBS Lett. 463*, 133–138.

Phillips, J.E., Meredith, J., Audsley, N., Richardson, N., Macins, A., *et al.*, **1998**. Locust ion transport peptide (ITP): A putative hormone controlling water and ionic balance in terrestrial insects. *Am. Zool. 38* (3), 461–470.

Phillips, J.E., Wiens, C., Audsley, N., Jeffs, L., Bilgen, T., *et al.*, **1996**. Nature and control of chloride transport in insect absorptive epithelia. *J. Exp. Zool. 275* (4), 292–299.

Phlippen, M.K., Webster, S.G., Chung, J.S., Dircksen, H., **2000**. Ecdysis of decapod crustaceans is associated with a dramatic release of crustacean cardioactive peptide into the haemolymph. *J. Exp. Biol. 203* (Pt 3), 521–536.

Predel, R., **2001**. Peptidergic neurohemal system of an insect: mass spectrometric morphology. *J. Comp. Neurol. 436* (3), 363–375.

Predel, R., Kellner, R., Gade, G., **1999**. Myotropic neuropeptides from the retrocerebral complex of the stick insect, *Carausius morosus* (Phasmatodea: Lonchodidae). *Eur. J. Entomol. 96* (3), 275–278.

Price, D.A., Greenberg, M.J., **1977**. The structure of a molluscan cardioexcitatory peptide. *Science 197*, 670–671.

Raftery, M.A., Hunkapiller, M.W., Strader, C.D., Hood, L.E., **1980**. Acetylcholine receptor: complex of homologous subunits. *Science 208* (4451), 1454–1456.

Reagan, J.D., **1994**. Expression cloning of an insect diuretic hormone receptor. A member of the calcitonin/secretin receptor family. *J. Biol. Chem. 269* (1), 9–12.

Reagan, J.D., **1996**. Molecular cloning and function expression of a diuretic hormone receptor from the house cricket, *Acheta domesticus*. *Insect Biochem. Mol. Biol. 26* (1), 1–6.

Reinecke, J.P., Gerst, J., O'Gara, B., Adams, T.S., **1978**. Innervation of hind gut muscle of larval *Manduca sexta* Lepidoptera Sphingidae by a peripheral multi nucleate neuro secretory neuron. *Int. J. Insect Morph. Embryol. 7* (5–6), 435–454.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., *et al.*, **1995**. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science 270* (5237), 792–794.

Reynolds, S.E., **1977**. Control of cuticle extensibility in the wings of adult *Manduca* at the time of eclosion: effects of eclosion hormone and bursicon. *J. Exp. Biol. 70*, 27–39.

Reynolds, S.E., Taghert, P.H., Truman, J.W., **1979**. Eclosion hormone and bursicon titers and the onset of hormonal responsiveness during the last day of adult development in *Manduca Sexta*. *J. Exp. Biol. 78*, 77–86.

Riddiford, L.M., Hewes, R.S., Truman, J.W., **1994**. Dynamics and metamorphosis of an identifiable peptidergic neuron in an insect. *J. Neurobiol. 25* (7), 819–830.

Robb, S., Evans, P.D., **1990**. FMRFamide-like peptides in the locust: distribution, partial characterization and bioactivity. *J. Exp. Biol. 149*, 335–360.

Roller, L., Tanaka, Y., Tanaka, S., **2003**. Corazonin and corazonin-like substances in the central nervous system of the Pterygote and Apterygote insects. *Cell Tissue Res. 312* (3), 393–406.

Sandstrom, D.J., Weeks, J.C., **1991**. Reidentification of larval interneurons in the pupal stage of the tobacco hornworm, *Manduca sexta*. *J. Comp. Neurol. 308* (2), 311–327.

Sauman, I., Reppert, S.M., **1996**. Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of Period protein regulation. *Neuron 17* (5), 889–900.

Sauman, I., Tsai, T., Roca, A.L., Reppert, S.M., **1996**. Period protein is necessary for circadian control of egg hatching behavior in the silkmoth *Antheraea pernyi*. *Neuron 17* (5), 901–909.

Schneider, L.E., O'Brien, M.A., Taghert, P.H., **1991**. *In situ* hybridization analysis of the FMRFamide neuropeptide gene in *Drosophila*. I. Restricted expression in embryonic and larval stages. *J. Comp. Neurol. 304* (4), 608–622.

Schneider, L.E., Taghert, P.H., **1988**. Isolation and characterization of a *Drosophila* gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH2 (FMRFamide). *Proc. Natl Acad. Sci. USA 85* (6), 1993–1997.

Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., De Loof, A., **1991**. Isolation, identification and synthesis of locustamyoinhibiting peptide (LOM-MIP), a novel biologically active neuropeptide from *Locusta migratoria*. *Regul. Pep. 36* (1), 111–119.

Schoofs, L., Veelaert, D., Broeck, J.V., De Loof, A., **1996**. Immunocytochemical distribution of locustamyoinhibiting peptide (Lom-MIP) in the nervous system of *Locusta migratoria*. *Regul. Pept. 63* (2–3), 171–179.

Schwartz, L.M., Truman, J.W., **1984**. Hormonal control of muscle atrophy and degeneration in the moth *Antheraea polyphemus*. *J. Exp. Biol. 111*, 13–30.

Sehnal, F., Zitnan, D., **1996**. Midgut endocrine cells. In: Lehane, M.J., Billingsley, P.F. (Eds.), The Biology of the Insect Midgut. Chapman and Hall, London, pp. 55–86.

Shiga, S., Davis, N.T., Hildebrand, J.G., **2003**. Role of neurosecretory cells in the photoperiodic induction of pupal diapause of the tobacco hornworm *Manduca sexta*. *J. Comp. Neurol. 462*, 275–285.

Slama, K., **1980**. Homeostatic function of ecdysteroids in ecdysis and oviposition. *Acta Entmol. Bohemoslovaca 77*, 145–168.

Spanings-Pierrot, C., Soyez, D., Van Herp, F., Gompel, M., Skaret, G., *et al.*, **2000**. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol. 119* (3), 340–350.

Stangier, J., Hilbich, C., Beyruether, K., Keller, R., **1987**. Unusual crustacean cardioactive peptide Ccap from pericardial organs of the shore crab *Carcinus maenas*. *Proc. Natl Acad. Sci. USA 84* (2), 575–579.

Stangier, J., Hilbich, C., Dircksen, H., Keller, R., **1988**. Distribution of a novel cardioactive neuropeptide (CCAP) in the nervous system of the shore crab *Carcinus maenas*. *Peptides 9* (4), 795–800.

Stangier, J., Hilbich, C., Keller, R., **1989**. Occurrence of crustacean cardioactive peptide Ccap in the nervous system of an insect *Locusta migratoria*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 159* (1), 5–12.

Staubli, F., Jorgensen, T.J., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99* (6), 3446–3451.

Taghert, P.H., Truman, J.W., **1982a**. Identification of the bursicon containing neurons in abdominal ganglia of the tobacco hornworm *Manduca sexta*. *J. Exp. Biol. 98*, 385–402.

Taghert, P.H., Truman, J.W., **1982b**. The distribution and molecular characteristics of the tanning hormone

bursicon in the tobacco hornworm *Manduca sexta*. *J. Exp. Biol. 98*, 373–384.

Tanaka, S., **2000**. Induction of darkening by corazonins in several species of Orthoptera and their possible presence in ten orders. *Appl. Entomol. Zool. 35*, 509–517.

Tanaka, S., **2001**. Endocrine mechanisms controlling body-color polymorphism in locusts. *Arch. Insect Biochem. Physiol. 47*, 139–149.

Tanaka, S., Pener, M.P., **1994**. A neuropeptide controlling the dark pigmentation in color polymorphism of the migratory locust. *J. Insect Physiol. 40*, 997–1005.

Tanaka, Y., Hua, Y., Roller, L., Tanaka, S., **2002**. Corazonin reduces the spinning rate in the silkworm, *Bombyx mori*. *J. Insect Physiol. 48* (7), 707–714.

Tawfik, A.I., Tanaka, S., De Loof, A., Schoofs, L., Baggerman, G., *et al.*, **1999**. Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant. *Proc. Natl Acad. Sci. USA 96* (12), 7083–7087.

Taylor, H.M., Truman, J.W., **1974**. Metamorphosis of the abdominal ganglia of the tobacco hornworm *Manduca sexta* changes in populations of identified motor neurons. *J. Comp. Physiol. 90* (4), 367–388.

Tensen, C.P., Verhoeven, A.H., Gaus, G., Janssen, K.P., Keller, R., *et al.*, **1991**. Isolation and amino acid sequence of crustacean hyperglycemic hormone precursor-related peptides. *Peptides 12* (4), 673–681.

Terzi, G., Truman, J.W., Reynolds, S.E., **1988**. Purification and characterization of eclosion hormone from the moth *Manduca sexta*. *Insect Biochem. 18* (7), 701–708.

Truman, J., Ewer, J., Ball, E., **1996**. Dynamics of cyclic GMP levels in identified neurones during ecdysis behaviour in the locust *Locusta migratoria*. *J. Exp. Biol. 199* (Pt 4), 749–758.

Truman, J.W., **1971**. Physiology of insect ecdysis Part 1: the eclosion behavior of saturniid moths and its hormonal release. *J. Exp. Biol. 54* (3), 805–814.

Truman, J.W., **1972**. Physiology of insect rhythms Part II. The Silk Moth Brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol. 81* (1), 99–114.

Truman, J.W., **1973**. Physiology of insect ecdysis. III. Relationship between the hormonal control of eclosion and of tanning in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 58*, 821–829.

Truman, J.W., **1974**. Physiology of insect rhythms Part IV. Role of the brain in the regulation of the flight rhythm of the giant silk moths. *J. Comp. Physiol. A Sens. Neur. Behav. Physiol. 95* (4), 281–296.

Truman, J.W., **1985**. Hormonal control of ecdysis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 7. Pergamon, Oxford, pp. 109–151.

Truman, J.W., **1992**. The eclosion hormone system in insects. *Progr. Brain Res. 92*, 361–373.

Truman, J.W., Copenhaver, P.F., **1989**. The larval eclosion hormone neurones in *Manduca sexta*: Identification of the brain-proctodeal neurosecretory system. *J. Exp. Biol. 147*, 457–470.

Truman, J.W., Riddiford, L.M., **1970**. Neuroendocrine control of ecdysis in silk moths. *Science 167* (3925), 1624–1626.

Truman, J.W., Riddiford, L.M., **1974**. Hormonal mechanisms underlying insect behavior. In: Treherne, J.E., Berridge, M.J. (Eds.), Advances in Insect Physiology, vol. 10. Academic Press, New York, pp. 297–352.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., **1983**. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta* (L.). *J. Insect Physiol. 29*, 895–900.

Truman, J.W., Sokolove, P.G., **1972**. Silkmoth eclosion: hormonal triggering of a centrally programmed pattern of behavior. *Science 175*, 1490–1493.

Van Herp, F., **1998**. Molecular, cytological and physiological aspects of the crustacean hyperglycemic hormone family. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology, vol. 65. Cambridge University Press, Cambridge, pp. 53–70.

Vanden Broeck, J., **2001**. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. *Peptides 22* (2), 241–254.

Veelaert, D., Schoofs, L., Tobe, S.S., Yu, C.G., Vullings, H.G., *et al.*, **1995**. Immunological evidence for an allatostatin-like neuropeptide in the central nervous system of *Schistocerca gregaria*, *Locusta migratoria* and *Neobellieria bullata*. *Cell Tissue Res. 279* (3), 601–611.

Veenstra, J.A., **1989**. Isolation and structure of corazonin, a cardioactive peptide from the American cockroach. *FEBS Lett. 250* (2), 231–234.

Veenstra, J.A., **1991**. Presence of corazonin in three insect species, and isolation and identification of [His7]corazonin from *Schistocerca americana*. *Peptides 12* (6), 1285–1289.

Veenstra, J.A., **1994**. Isolation and structure of the *Drosophila* corazonin gene. *Biochem. Biophys. Res. Commun. 204* (1), 292–296.

Veenstra, J.A., Davis, N.T., **1993**. Localization of corazonin in the nervous system of the cockroach *Periplaneta americana*. *Cell Tissue Res. 274* (1), 57–64.

Vogtli, M., Elke, C., Imhof, M.O., Lezzi, M., **1998**. High level transactivation by the ecdysone receptor complex at the core recognition motif. *Nucl. Acids Res. 26* (10), 2407–2414.

Wainwright, G., Webster, S.G., Wilkinson, M.C., Chung, J.S., Rees, H. H., **1996**. Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*. Involvement in multihormonal regulation of growth and reproduction. *J. Biol. Chem. 271* (22), 12749–12754.

Wang, S.F., Miura, K., Miksicek, R. J., Segraves, W.A., Raikhel, A.S., **1998**. DNA binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex. *J. Biol. Chem. 273* (42), 27531–27540.

Ward, R.E., Reid, P., Bashirullah, A., D'Avino, P.P., Thummel, C.S., **2003**. GFP in living animals reveals dynamic developmental responses to ecdysone during *Drosophila metamorphosis*. *Devel. Biol. 256* (2), 389–402.

Webster, S.G., **1998**. Molecular, cytological and physiological aspects of the crustacean hyperglycemic hormone family. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology, vol. 65. Cambridge University Press, Cambridge, pp. 33–52.

Webster, S.G., Dircksen, H., Chung, J.S., **2000**. Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycaemic hormone and its precursor-related peptide. *Cell Tissue Res. 300* (1), 193–205.

Weeks, J.C., Truman, J.W., **1984a**. Neural organization of peptide-activated ecdysis behaviors during the metamorphosis of *Manduca sexta*. I. Conservation of the peristalsis motor pattern at the larval-pupal transformation. *J. Comp. Physiol. A Sens., Neur. Behav. Physiol. 155*, 407–422.

Weeks, J.C., Truman, J.W., **1984b**. Neural organization of peptide-activated ecdysis behaviors during the metamorphosis of *Manduca sexta*. II. Retention of the proleg motor pattern despite the loss of prolegs at pupation. *J. Comp. Physiol. A Sens., Neur. Behav. Physiol. 155*, 423–433.

Weidemann, W., Gromoll, J., Keller, R., **1989**. Cloning and sequence analysis of cDNA for precursor of a crustacean hyperglycemic hormone. *FEBS Lett. 257* (1), 31–34.

Williamson, M., Lenz, C., Winther, A. M., Nassel, D.R., Grimmelikhuijzen, C.J., *et al.*, **2001**. Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 281* (2), 544–550.

Wise, S., Davis, N.T., Tyndale, E., Noveral, J., Folwell, M.G., *et al.*, **2002**. Neuroanatomical studies of period gene expression in the hawkmoth, *Manduca sexta*. *J. Comp. Neurol. 447* (4), 366–380.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., *et al.*, **2000**. Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster. Development 127* (23), 5083–5092.

Zdarek, J., Friedman, S., **1986**. Pupal ecdysis in flies: mechanisms of evagination of the head and expansion of the thoracic appendages. *J. Insect Physiol. 32*, 917–923.

Zitnan, D, **1989**. Regulatory Peptides and Peptidergic Organs of Insects. Ph.D. thesis. Slovak Academy of Sciences, Bratislava.

Zitnan, D., Adams, M.E., **2000**. Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioural sequence. *J. Exp. Biol. 203*, 1329–1340.

Zitnan, D., Hollar, L., Spalovska, I., Takac, P., Zitnanova, I., *et al.*, **2002**. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. *J. Exp. Biol. 205* (Pt 22), 3459–3473.

Zitnan, D., Kingan, T.G., Beckage, N. E., **1995a**. Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of *Manduca sexta*. *Insect Biochem. Mol. Biol. 25* (6), 669–678.

Zitnan, D., Kingan, T.G., Hermesman, J., Adams, M.E., **1996**. Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science 271*, 88–91.

Zitnan, D., Kingan, T.G., Kramer, S. J., Beckage, N.E., **1995b**. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J. Comp. Neurol. 356* (1), 83–100.

Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23* (3), 523–535.

Zitnan, D., Zitnanova, I., Spalovska, I., Takac, P., Park, Y., *et al.*, **2003**. Conservation of ecdysis-triggering hormone signalling in insects. *J. Exp. Biol. 206* (Pt 8), 1275–1289.

Zitnanova, I., Adams, M.E., Zitnan, D., **2001**. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. *J. Exp. Biol. 204* (Pt 20), 3483–3495.

3.2 Prothoracicotropic Hormone

R Rybczynski, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

3.2.1. General Introduction and Historical Background

The evolution of multicellular organisms possessing multiple cell types has required the coevolution of a variety of intercellular signals. These signals coordinate and control the multiplication and differentiation of a multitude of cell types from a single, initial zygotic cell. These signals also coordinate and control the interaction of these various cell types throughout the life of an organism. In vertebrates familiar to most people, i.e., reptiles, birds, and mammals, a newborn or hatched individual is generally not strikingly different from an adult and postembryonic development is relatively gradual; altricial birds and marsupials are exceptions in that individuals of these groups undergo embryonic-like development for a period of time after hatching or birth (see Truman and Riddiford, 2002). However, even in these latter vertebrate groups, developmental changes are gradual and do not involve relatively sudden and major changes in morphology and physiology. In contrast, many invertebrates, including insects, undergo striking and periodic postembryonic changes in the rate of development and in morphology and physiology. The physical growth of insects is restricted by the extracellular cuticle, which is limited in its ability to accommodate expansion. Insects solve this problem by periodic molts in which the old cuticle is partly resorbed and partly shed and a new cuticle is laid down, allowing for a further period of growth. In addition, many insects undergo molts in which significant changes in structure occur, i.e., metamorphic molts (for more thorough discussions on the evolution of metamorphosis, see Sehnal *et al*., 1996; Truman and Riddiford, 2002). Controlling and coordinating these episodes of rapid developmental change is a suite of intercellular messengers (hormones). The

polyhydroxylated steroid hormone, 20-hydroxyecdysone (20E) is a major factor regulating molting and metamorphosis. 20E is produced from ecdysone (E), which itself has some hormonal activity (see **Chapters 3.3** and **3.5**). This chapter focuses on prothoracicotropic hormone (PTTH), a brain neuropeptide hormone, and its action in regulating the physiology of the prothoracic gland. For the actual pathway of ecdysteroid synthesis, starting with cholesterol or other sterols, the reader is directed to (see **Chapter 3.3**).

It must be stated at the outset that the vast majority of data and discussion presented in this chapter come from studies of the Lepidoptera, especially the moth species *Manduca sexta* (tobacco hornworm) and *Bombyx mori* (domestic silkmoth), simply because we have much more knowledge of PTTH or PTTH-like activity from this taxon than from any other group. The first section of this chapter reviews the field of PTTH research from the 1920s to the early 1980s; for further and more detailed pictures of this time period, see reviews by Ishizaki and Suzuki (1980), Gilbert *et al.* (1981); Granger and Bollenbacher (1981); Ishizaki and Suzuki (1984) and Bollenbacher and Granger (1985), among others.

In the second and third decades of the twentieth century, the Polish biologist Stefan Kopeć first provided evidence that the brain could control metamorphosis (e.g., Kopeć, 1922). Kopeć studied the larval–pupal molt of the gypsy moth, *Lymantria dispar*, employing simple techniques like the ligation of larvae into anterior and posterior sections, and brain extirpation. His data demonstrated that the larval–pupal molt of the gypsy moth was dependent upon the brain during a critical period in the last instar larval stage. After this stage, head ligation failed to prevent pupation. Kopeć (1922) found also that this control was not dependent on intact nervous connections with regions posterior to the brain. The studies of Kopeć represent the beginning of the field of neuroendocrinology but their importance was not recognized at the time. However, later workers, notably Wigglesworth, took similar approaches with other species of insects and demonstrated again that the brain was important for larval molting at certain critical periods and that the active principle appeared to be produced in a part of the brain containing large neurosecretory cells (e.g., Wigglesworth, 1934, 1940).

In the same time period, other studies, again using simple techniques like transplantation and transection, found that the brain-derived factor was not sufficient to stimulate molting and metamorphosis. Rather, a second component produced in the thorax was necessary (e.g., Burtt, 1938; Fukuda, 1941; Williams 1947). This material originated in the prothoracic gland or ring gland and was later shown to be an ecdysteroid precursor to 20E, i.e., 3dE in Lepidoptera (Kiriishi *et al.*, 1990), or E and makisterone A in *Drosophila* (Pak and Gilbert, 1987) (see **Chapter 3.3**).

The chemical nature of PTTH was addressed in studies beginning in 1958, when Kobayashi and Kimura (1958) found that high dose of an ether extract of *B. mori* pupal brains was able to induce adult development in a *Bombyx* dauer pupa assay. This result suggested that PTTH was a lipoidal moiety, such as a sterol. In contrast, Ichikawa and Ishizaki (1961) found that a simple aqueous extract of *Bombyx* brains could elicit development in a *Samia* pupal assay, an observation not supporting a lipoidal nature for PTTH, and in a later study, provided direct evidence that *Bombyx* PTTH was a protein (Ichikawa and Ishizaki, 1963). Schneiderman and Gilbert (1964), using the *Samia* assay, explicitly tested the possibility that PTTH was cholesterol or another sterol and concluded that a sterol PTTH was highly unlikely. During the 1960s and 1970s, data accumulated from a number of studies indicated that *Bombyx* PTTH was proteinaceous in nature, but attempts to ascertain the molecular weight yielded at least two peaks of activity, one $\leq$5 kDa and a second $\geq$20 kDa (e.g., Kobayashi and Yamazaki, 1966).

In the 1970s and 1980s, a second lepidopteran, the tobacco hornworm *M. sexta*, gained favor as a model organism for the elucidation of the nature of PTTH. Initial experiments with *Manduca* utilized a pupal assay (Gibbs and Riddiford, 1977) but a system measuring ecdysteroid synthesis by prothoracic glands *in vitro* soon gained prominence (Bollenbacher *et al.*, 1979). Using the pupal assay, Kingan (1981) estimated the molecular weight of *Manduca* PTTH to be $\approx$25 kDa. A similar estimate (MW $\approx$29 kDa) resulted from assays employing the *in vitro* technique but this approach also provided evidence for a second, smaller PTTH (MW = 6–7 kDa) (Bollenbacher *et al.*, 1984).

The site of PTTH synthesis within the brain was first investigated by Wigglesworth (1940) who, by implanting various brain subregions into host animals, identified a dorsal region of the *Rhodnius* brain containing large neurosecretory cells as the source of PTTH activity. Further studies, in species such as *Hyalophora cecropia*, *Samia cynthia* and *M. sexta*, also provided evidence that PTTH was produced within regions of the dorsolateral or medial-lateral brain that contained large neurosecretory cells. This methodology was refined and ultimately culminated in the identification of the

source of PTTH as two pairs of neurosecretory cells (one pair per side) in the brain of *Manduca* (Agui *et al.*, 1979).

The corpus cardiacum (CC) of the retrocerebral complex was originally suggested to be the site of PTTH release, based on structural characteristics of the organ and the observation that axons of some cerebral neurosecretory cells terminate in the CC. However, physiological studies involving organ transplants and extracts implicated the corpus allatum (CA), the other component of the retrocerebral complex, as the site of PTTH release. *In vitro* studies utilizing isolated CCs and CAs from *Manduca* revealed that the CAs contained considerably more PTTH than CCs (Agui *et al.*, 1979, 1980). This result was also obtained when high levels of potassium were used to stimulate PTTH release from isolated CCs and CAs; potassium caused a several-fold increase in PTTH release from both organs (Carrow *et al.*, 1981) but the increase from CCs barely matched the spontaneous release from CAs. PTTH activity in the CC could be explained by the fact that axons that terminate in the CA traverse the CA and isolation of the CC would carry along any PTTH caught in transit through this organ. Additional evidence that the CAs are the neurohemal organ for the PTTH-producing neurosecretory cells was the observation that CA PTTH content correlated positively with brain PTTH content (Agui *et al.*, 1980).

The episodic nature of insect growth and morphological development reflects, *in sensu latu*, the periodic peaks of circulating molting hormone, i.e., 20E. The existence of these ecdysteroid peaks implies that peaks in PTTH synthesis and release also exist. A variety of studies tried to determine when PTTH peaks occur and what factors influence the release of PTTH into the hemolymph. Wigglesworth (1933) performed pioneering studies on the control of *Rhodnius* PTTH release. He found that distension of the intestinal tract was the most important determinant of blood meal-dependent molting. Multiple small meals were not effective but artificially increasing distension by blocking the anus with paraffin notably decreased the size of a blood meal required. A meal of saline can also trigger (but not sustain) a molt, indicating that for *Rhodnius* at least, nutritional signals are not critical (Beckel and Friend, 1964). Meal-related distension appears to be sensed by abdominal stretch or pressure receptors in insects such as *Rhodnius* (Wigglesworth, 1934) (see **Chapter 3.9**). This type of regulation is not surprising in blood-sucking insects that rapidly change the fill state of their gut but, somewhat surprisingly, it appears also to occur in some insects that are continuous rather than episodic feeders, like the milkweed bug, *Oncopeltus fasciatus* (Nijhout, 1979). In this species, subcritical weight nymphs inflated with air or saline will commence molting.

In many other insects, the physical and physiological conditions that permit and control PTTH release are subtle and partially known. In *Manduca*, it appears that a critical weight must be achieved before a molt is triggered (Nijhout, 1981); however, this weight is not a fixed quantity. Rather, the critical weight is a function of the weight and/or size at the time of the last molt. Animals that were very small or very large at the fourth to fifth larval instar molt will exhibit critical weights smaller or larger than the typical individual. Further complications revolve around the element of time. Animals that remain below critical size for a long period may eventually molt to the next stage (*Manduca*: Nijhout, 1981), or undergo stationary larval molts without growth (*Galleria mellonella:* Allegret, 1964). PTTH release may also be a "gated" phenomenon. That is, PTTH release occurs only during a specific time of the day and only in animals that meet critical criteria, like size, weight or allometric relationships between structures or physiological variables. Individuals that reach critical size within a diel cycle but after that day's gate period will not release PTTH until the next gate on the following day (see **Chapter 3.3**).

The concept of a critical size or similar threshold parameter that must be met to initiate PTTH release implies that there will be a single PTTH release per larval instar. This appears to be true through the penultimate larval instar but careful assessment of PTTH activity in *Manduca* hemolymph revealed two periods of significant levels of circulating PTTH during the last larval instar (Bollenbacher and Gilbert, 1981). In this species, three small PTTH peaks were seen about 4 days after the molt to the fifth instar while a single larger peak occurred about 2 days later. The first period of PTTH release is gated but the second appears to be neither gated nor affected by photoperiod (Bollenbacher, unpublished data cited in Bollenbacher and Granger, 1985). These two periods of higher circulating PTTH correspond to the two head critical periods (HCPs) that are found in the last larval instar of *Manduca*. HCPs represent periods of development before which the brain is necessary for progression to the next molt, as determined by ligation studies. In the fifth (last) larval instar of *Manduca*, the two periods of higher hemolymph titers of PTTH activity immediately precede and partially overlap two peaks in circulating ecdysteroids (Bollenbacher and Gilbert, 1981). The first small ecdysteroid peak

determines that the next molt will be a metamorphic one (the commitment peak: Riddiford, 1976) while the second large peak actually elicits the molt.

The existence of daily temporal gates for PTTH release suggested that a circadian clock plays a role in molting and metamorphosis (see **Chapters 3.11** and **4.11**). In fact, such a clock does appear to function in *Manduca* because shifting the photoperiod shifted the time of the gate (e.g., Truman, 1972). Furthermore, the entrainment of this circadian cycling was not dependent on retinal stimulation because larvae with cauterized ocelli still reacted to photoperiodic cues (Truman, 1972). A more overarching effect of photoperiod can be seen in the production of diapause (see **Chapter 3.12**). For instance, in *Antheraea* and *Manduca*, which exhibit pupal–diapause, short days experienced during the last larval instar result in the inhibition of PTTH release during early pupal–adult development (e.g., Williams, 1967; Bowen *et al*., 1984a); the brain PTTH content of these diapausing pupae and that of nondiapausing pupae are similar, at least at the beginning of the pupal stage. Temperature often plays an important role in breaking diapause and an elevation of temperature has been interpreted to cause the release of PTTH (Bollenbacher and Granger, 1985), with a resultant rise in circulating ecdysteroids that triggers the resumption of development. The interposition of hormonal signals between light-cycle sensing and the inhibition of PTTH release and/or synthesis can involve the juvenile hormones (JHs). In species exhibiting larval diapause, like *Diatraea grandiosella*, the persistence of relatively high JH levels controls diapause (e.g., Yin and Chippendale, 1973). Ligation experiments indicated that the head was involved in JH-related larval diapause, resulting in the suggestion that JH inhibited the release of an ecdysiotropin, i.e., PTTH (Chippendale and Yin, 1976).

In summary, by the time of the publication of *Comprehensive Insect Physiology, Biochemistry and Pharmacology* in 1985, PTTH had progressed from being an undefined brain factor controlling molting (Kopeć, 1922) to being identified as one of two brain neuropeptides that specifically regulated ecdysteroid synthesis in the prothoracic gland (e.g., Bollenbacher *et al*., 1984), and that was synthesized in a very limited set of brain neurosecretory cells (Agui *et al*., 1979). In the remainder of this chapter, the characterization and purification of PTTH will be detailed, as will the intracellular signaling pathways by which PTTH activates the prothoracic gland. On the whole, this phase of PTTH research has involved more complicated biochemical and cell biological experiments than earlier work. However, even though the methodology described has become more complicated and specialized, it is hoped that the discussions and speculations presented in this phase will remain as broad and as "biological" as those generated during that earlier, "simpler" period of PTTH research.

3.2.2. Prothoracicotropic Hormone (PTTH): Characterization, Purification, and Cloning

3.2.2.1. Purification and Cloning of the PTTHs of *Bombyx* and other Lepidoptera

Two species of Lepidoptera, *B. mori* and *M. sexta*, have emerged as the premier model species for studying the endocrinology of insect molting and metamorphosis. As described above, evidence suggested that, for these species, PTTH activity resided in proteins exhibiting two distinct molecular weights, one of 4–7 kDa and the second of 20–30 kDa. Early work with *Bombyx* brain extracts utilized a cross-species assay to determine PTTH activity. This assay, using debrained *Samia* pupae, suggested that the most effective *Bombyx* PTTH (4K-PTTH) was the smaller protein. However, as efforts to purify and further characterize *Bombyx* PTTH progressed, a *Bombyx* pupal assay was developed (Ishizaki *et al*., 1983a). This assay revealed that when *Bombyx* PTTH preparations were tested with debrained *Bombyx* pupae, *Bombyx* PTTH appeared to be the larger molecule while the small PTTH, identified with the *Samia* assay, was not effective at physiologically meaningful doses (Ishizaki *et al*., 1983a,1983b). This result was confirmed when the small and large PTTHs were tested for their ecdysteroidogenic effect *in vitro* on *Bombyx* prothoracic glands (Kiriishi *et al*., 1992).

The effort to isolate and sequence *Bombyx* PTTH shifted rapidly to the "large" PTTH form following the 1983 results; the smaller molecule, subsequently named bombyxin, is discussed briefly below (see Section 3.2.2.5). Within a few years, the sequence of the amino terminus (13 amino acids) of *Bombyx* PTTH (called 22K-PTTH in the publications of the day) was obtained after a purification effort that involved processing 500 000 adult male heads (Kataoka *et al*., 1987). The final steps of this purification involved HPLC separations of proteins and this technique suggested that PTTH might exhibit heterogeneity in sequence and/or structure. However, the nature of that heterogeneity was unknown, since only one of four potential PTTH peaks was sequenced. In addition to this limited amount of amino acid sequence, this study is significant since

it demonstrated that *Bombyx* PTTH was indeed a protein, and one that had no sequence homology to other proteins known at that time, including bombyxin. Also, significant was the finding that PTTH was highly active when injected into brainless *Bombyx* pupae. A dose of only 0.11 ng was as effective as 100–400 ng of bombyxin, thus demonstrating again that bombyxin was not the *Bombyx* PTTH (Kataoka *et al.*, 1987). A second round of *Bombyx* PTTH purification was initiated, using 1.8 million heads (derived from 1.5 tons of moths!), and this heroic effort resulted in the determination of nearly the entire PTTH amino acid sequence (104 amino acids), except for a small fragment (5 amino acids) at the C-terminus (Kataoka *et al.*, 1991). At the same time, the N-terminus sequence was used to produce a polyclonal mouse antibody against *Bombyx* PTTH, which was then successfully utilized in a cDNA expression library screening (Kawakami *et al.*, 1990). The empirically determined and deduced amino acid sequences of *Bombyx* PTTH agreed completely in the region of overlap. Furthermore, the amino acid sequence deduced from the cDNA sequence indicated that, like many neuropeptides, *Bombyx* PTTH appeared to be synthesized as a larger, precursor molecule (**Figure 1**). Two slightly smaller sequences of PTTH were also obtained during the larger PTTH purification (Kataoka *et al.*, 1991), which were missing six or seven amino acids from the N-terminal sequence obtained from the largest peptide. It is not known if this N-terminus heterogeneity was an artifact of sample storage or purification methods, or reflected a natural variation with possible biological effects on activity. A more detailed analysis of the amino acid sequence and structure of *Bombyx* PTTH and other PTTHs is provided in Section 3.2.2.3

The large number of brains required to purify PTTH has precluded attempts to purify this protein from species other than *Bombyx*, with the exception of *M. sexta*. For *Manduca*, Bollenbacher and colleagues raised a monoclonal antibody against a putative purified big PTTH (O'Brien *et al.*, 1988) and used this antibody to construct an affinity column. This immunoaffinity column was then used to attempt a purification of *Manduca* PTTH from an extract made from about 10 000 brains (Muehleisen *et al.*, 1993). Partial amino acid sequences obtained from a protein purified via this affinity column showed no sequence homology with *Bombyx* PTTH; instead, the isolated protein appeared to be a member of the cellular retinoic acid binding protein family (Muehleisen *et al.*, 1993; Mansfield *et al.*, 1998). This surprising result was in direct contrast to the finding of Dai *et al.* (1994) that an anti-*Bombyx* PTTH antibody recognized only the cells previously identified as the *Manduca* prothoracicotropes by Agui *et al.* (1979), and the observation that *Manduca* PTTH could be immune-precipitated by an anti-*Bombyx* PTTH antibody (Rybczynski *et al.*, 1996). In the latter case, the immune-precipitated *Manduca* PTTH could be recovered in a still-active state (Rybczynski *et al.*, 1996). Subsequently, the isolation of a *Manduca* PTTH cDNA, utilizing a *Bombyx* probe, and expression of the derivative recombinant *Manduca* PTTH confirmed that *Manduca* PTTH was indeed related to the *Bombyx* molecule (Gilbert *et al.*, 2000; Shionoya *et al.*, 2003). In an earlier molecular approach, Gray *et al.* (1994) used the polymerase chain reaction with *Bombyx* PTTH-derived sequences to probe *Manduca* genomic DNA and a neuronal cDNA library. This search yielded an intronless sequence differing in only two amino acids from *Bombyx* PTTH but quite different from the *Manduca* PTTH later demonstrated to exhibit bioactivity (Gilbert *et al.*, 2000); note that the gene for *Bombyx* PTTH contains five introns (Adachi-Yamada *et al.*, 1994). This unexpected result, suggesting that *Manduca* may have a second large PTTH has not been

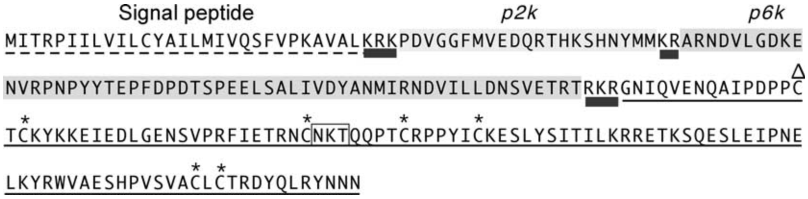


Figure 1 Amino acid sequence and main features of *Bombyx* PTTH. Dashed underlining: the putative signal peptide region; thin solid underlining: the mature, bioactive peptide; thick underlining: predicted peptide cleavage sites; light shading: putative 2-kDa peptide; dark shading: putative 6-kDa peptide; boxed amino acids: glycosylation site; ∗: cysteines involved in intramonomeric bonds; and Δ: cysteine involved in dimer formation. (Data from Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M. *et al.*, **1990**. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science 247*, 1333–1335; and Ishibashi, J., Kataoka, H., Isogai, A., Kawakami, A., Saegusa, H., *et al.*, **1994**. Assignment of disulfide bond location in prothoracicotropic hormone of the silkworm, *Bombyx mori:* a homodimeric protein. *Biochemistry, 33*, 5912–5919.)

further pursued. Evidence suggesting that the *Manduca* brain might express a small PTTH in addition to the larger peptide is discussed in Section 3.2.2.5 Additional molecular studies, again using probes derived from the *Bombyx* PTTH sequence, have yielded lepidopteran PTTH sequences from three additional species: *Samia cynthia ricini* (Ishizaki and Suzuki, 1994); *Antheraea pernyi* (Sauman and Reppert, 1996a); and *H. cecropia* (Sehnal *et al.*, 2002) (see the following section). Recombinant *Antheraea* PTTH has been expressed and shown to have bioactivity, being capable of inducing adult development in debrained pupae (Sauman and Reppert, 1996a). The bioactivity of the proteins coded for by the *Samia* and *Hyalophora* sequences has not yet been tested.

Preliminary determinations of the molecular weight of PTTH have been made recently for a few additional lepidopteran species, such as the gypsy moth *L. dispar* (Kelly *et al.*, 1992; Fescemyer *et al.*, 1995), primarily using *in vitro* assays of ecdysteroidogenesis and variously treated brain extracts. These studies revealed two molecular weight ranges for PTTH, i.e., small and large. However, the molecular weight obtained for the large *Lymantria* PTTH (11 000–12 000) varied notably from those determined for the purified and cloned forms such as *Bombyx* PTTH (native weight ≈ 25 000–30 000). This difference is probably a function of the sample treatment (acidic organic extraction versus aqueous extraction) and molecular weight analysis methods (HPLC versus SDS-PAGE), since when essentially the same extraction and HPLC methodology were applied to *Manduca* brain extracts, a similarly low molecular weight determination for “big” PTTH was obtained (11.5 kDa; Kelly *et al.*, 1996).

3.2.2.2. Characterization of the PTTH of Non-Lepidoptera

Despite the importance of the hemipteran *Rhodnius* in early studies demonstrating a brain-derived prothoracicotropic factor (e.g., Wigglesworth, 1934, 1940), relatively little work has been done on molecularly characterizing PTTH activity from species other than lepidopterans. In *Rhodnius*, Vafopoulou *et al.* (1996) found that brain extracts contained a protease-sensitive PTTH activity (see **Chapter 2.4**). Later work suggested that this activity was greater than 10 kDa in molecular weight when immunoblots of *Rhodnius* brain proteins or medium from *in vitro* incubations of brains were probed with an antibody against *Bombyx* PTTH (Vafopoulou and Steel, 2002). If nonreducing gel conditions were used, the strongest immunoreactive band was ≈68 kDa, but reducing conditions yielded only a single band at ≈17 kDa. This latter molecular weight is the same as that calculated for *Bombyx* PTTH under reducing electrophoresis conditions (Mizoguchi *et al.*, 1990). It is also very similar to the molecular weights determined for *Manduca* PTTH, again employing the *Bombyx* antibody after reducing SDS-PAGE (≈16 kDa; Rybczynski *et al.*, 1996), and for the *Antheraea* PTTH, using an *Antheraea* PTTH antibody and strong reducing conditions (≈15 kDa; Sauman and Reppert, 1996a). Also, as demonstrated earlier for *Manduca* brain extract (Rybczynski *et al.*, 1996), an anti-*Bombyx* PTTH antibody removed PTTH activity from a *Rhodnius* brain extract (Vafopoulou and Steel, 2002).

The growing use of *Drosophila melanogaster* as a model organism for the study of development, neuronal function, and many other fields has not been accompanied by a concomitant increase in our knowledge of *Drosophila* PTTH. The small size and rapid development of *Drosophila* have made it difficult to perform classic endocrinology on this species, e.g., measuring ecdysteroid titers, accumulating large quantities of brain proteins, or isolating the ring gland for *in vitro* studies of ecdysteroid synthesis. In addition, the composite nature of the ring gland, which contains multiple cell types in addition to the prothoracic gland cells, complicates the interpretation of experiments in a way that is not seen with the moth glands. Nevertheless, an *in vitro* assay for ring gland ecdysteroid synthesis has been developed (Redfern, 1983) and used to search for PTTH activity. The results of these studies have not yielded a clear or consistent picture of *Drosophila* PTTH, nor, in fact, conclusive evidence that *Drosophila* possesses a PTTH that is much like the lepidopteran molecules.

In a series of studies, Henrich and collaborators characterized PTTH activity from the central nervous system of *Drosophila* (Henrich *et al.*, 1987a, 1987b; Henrich, 1995). They found that PTTH activity was present in both the brain and in the ventral ganglion; the latter location is in contradistinction to the Lepidoptera where PTTH activity is found only in the brain and in the corpus allatum, the neurohemal organ for PTTH release. Note that in *Drosophila* and other higher flies, the ring gland, which contains the prothoracic gland, also includes the corpus allatum. Also relevant is the finding that an antibody against *Bombyx* PTTH bounds to specific cells both in the brain and in ventral ganglionic regions of *Drosophila* (Žitňan *et al.*, 1993), thus supporting the conclusion of Henrich (1995) that *Drosophila* PTTH activity was not restricted to the brain. An attempt to estimate the molecular weight

of this putative PTTH yielded equivocal results. Ultrafiltration through a 10-kDa filter resulted in PTTH activity in both the filtrate and retentate (Henrich, 1995). This might indicate the presence of two PTTH-like molecules, i.e., a big and a small PTTH, or simply the presence of a single PTTH of a molecular weight and shape that results in incompletely partitioning under the ultrafiltration protocol employed.

Two other studies yielded very different estimates of *Drosophila* PTTH molecular weight. Pak *et al.* (1992), using column chromatography, found two peaks of PTTH activity, one of ≈4 kDa and the other larger, at ≈16 kDa. In contrast, Kim *et al.* (1997), using a more complex chromatographic purification, concluded that *Drosophila* PTTH was a much larger, heavily glycosylated molecule of ≈66 kDa, with a core protein of ≈45 kDa. This group also subjected two reduced fragments of their purified PTTH to amino acid sequence analysis and found no homology with other protein sequences known at that time. However, a search of the current *Drosophila* molecular databases revealed that the sequence of these two fragments match portions of the deduced amino acid sequence encoded by *Drosophila* gene BG:*DS000180.7* (unpublished analysis). This protein is cysteine-rich, with a MW of ≈45 kDa, and contains sequence motifs that place it in the EGF protein family, thus suggesting that it is a secreted protein. Based on its sequence, the *DS000180.7* gene product has been hypothesized to be involved in cell–cell adhesion (Hynes and Zhao, 2000). As a member of the EGF family of proteins, the *DS000180.7* gene product cannot be ruled out as an intercellular signaling molecule, i.e., as a prothoracicotropic hormone, without a functional test. What is clear currently, is that this protein shows no detectable amino acid similarity to the demonstrated PTTHs of the Lepidoptera (unpublished analysis), when compared with those sequences using a standard comparison program like Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

Further clouding the interpretation of the *Drosophila* PTTH studies was the requirement for relatively large amounts of putative PTTH material that was also, relative to lepidopteran material, comparatively poor in eliciting increased ecdysteroidogenesis. Thus, in the *Drosophila* studies, Henrich and coworkers (Henrich *et al.*, 1987a, 1987b; Henrich, 1995) and Pak *et al.* (1992) needed to use approximately eight brain-ventral ganglion equivalents of extract to stimulate ecdysteroid synthesis to two to three times basal, while Kim *et al.* (1997) employed 500 ng of purified protein to achieve a similar activation. In contrast, lepidopteran PTTHs are effective at much lower doses; for instance, in *Manduca*, 0.25 brain equivalents or 0.25–0.5 ng of pure PTTH consistently results in a four to six times increase in ecdysteroidogenesis (Gilbert *et al.*, 2000) and activations of steroidogenesis of eight to ten times basal are not rare (personal observation). Further differences between putative *Drosophila* PTTH(s) and the lepidopteran PTTHs are discussed below (see Section 3.2.3.4). In addition to the work just discussed, the *Drosophila* genome project has offered a second approach to identify a PTTH candidate for this species. A search of this database with lepidopteran PTTH amino acid sequences revealed two candidate *Drosophila* sequences with intriguing similarities to the moth PTTHs and these are discussed in the next section.

Mosquitoes, such as the African malaria mosquito *Anopheles gambiae* and the yellow fever mosquito *Aedes aegypti,* are important disease vectors and have been the subject of many studies incorporating basic and applied approaches to their biology and control. In adult females of these species, a blood meal stimulates both the release of brain-derived gonadotropins (see **Chapter 3.9**), like ovary ecdysteroidogenic hormone, and of insulin-like molecules that stimulate ovarian ecdysteroid synthesis. Preadult development in mosquitoes appears to depend on ecdysteroid hormones, as in other insects; however, the morphological source of ecdysteroid production is not clear. Jenkins *et al.* (1992) provided evidence that the prothoracic gland of *A. aegypti*, which is part of a composite, ring gland-like organ, is inactive and that unknown cell types in the thorax and abdomen produce ecdysteroids. At the time of writing (2003), a genome-sequencing project is also under way for *A. aegypti* but the limited database currently available does not contain any putative PTTH homologs. However, in this context it is intriguing that the genome of *A. gambiae* might code for a PTTH-like protein. Searches of the *A. gambiae* genome database with lepidopteran and putative *Drosophila* PTTH amino acid sequences reveal two candidate gene products, which are discussed in the next section.

Antibodies against PTTH afford an additional tool to search for PTTH-like proteins and have been used in a number of studies, in addition to the immunoprecipitations of *Manduca, Drosophila*, and *Rhodnius* studies discussed above (Žitňan *et al.*, 1993; Dai *et al.*, 1994; Rybczynski *et al.*, 1996; Vafopoulou and Steel, 2002). Závodská *et al.* (2003) used polyclonal anti-*Antheraea* PTTH antibodies in an immunohistochemical survey of the central nervous systems of 12 species of insects in

10 orders: Archaeognatha, Ephemerida, Odonata, Orthoptera, Plecoptera, Hemiptera, Coleoptera, Hymenoptera, Tricoptera, and Diptera. Positive signals were seen in all species surveyed except *Locusta migratoria* (Orthoptera), with immunoreactive cells found mainly in the protocerebrum and less commonly in the subesophageal ganglion (the mayfly *Siphlonurus armatus* (Ephemerida) and the damselfly *Ischnura elegans* (Odonata)). The number of putative PTTH-containing cells ranged from two or three pairs (Archaeognatha, Hemiptera, and Hymenoptera) to five or more pairs (Plecoptera, Coleoptera, Tricoptera, and Diptera) and only in the stonefly *Perla burmeisteriana* (Plecoptera) a signal was detected in a potential neurohemal organ, the corpus cardiacum. These results are intriguing but must be interpreted with caution, pending further information such as determination of the size of the recognized protein or the immunoprecipitation of PTTH activity by the antibody. In this context, results obtained with an anti-*Manduca* PTTH antibody must serve as a cautionary tale. This monoclonal antibody recognized only a small number of cells in the CNS, including but not limited to the demonstrated prothoracicotropes (Westbrook *et al*., 1993), and apparently also bound to active PTTH (Muehleisen *et al*., 1993). Yet, the protein isolated by an immunoaffinity column constructed with this antibody proved to be an intracellular retinoid-binding protein (Mansfield *et al*., 1998) and not a secreted neurohormone.

3.2.2.3. Sequence Analyses and Comparisons of PTTHs

The amino acid sequence and structure of *Bombyx* PTTH have been characterized in a number of studies, beginning with Kataoka *et al*. (1987). **Figure 1** summarizes the major features of this protein. *Bombyx* PTTH appears to be synthesized as a 224-amino acid prohormone that is cleaved to yield an active peptide of 109 amino acids (Kawakami *et al*., 1990). The first 29 residues comprise a relatively hydrophobic region that is terminated by a trio of basic amino acids and fulfill the criteria defining a signal peptide. A second trio of basic residues immediately precedes the beginning of the mature PTTH hormone. Both these basic areas were believed to define peptide cleavage sites. Between these two sites lies a pair of basic amino acids that has been hypothesized to be an additional cleavage site (Kawakami *et al*., 1990). The presence of these three potential peptide processing sites has led to speculation that the region between the signal peptide and the mature PTTH sequence might be cleaved into two smaller peptides and that these small molecules serve a physiological function, such as modulating juvenile hormone synthesis in the corpus allatum (Kawakami *et al*., 1990; Ishizaki and Suzuki, 1992). However, there were no experimental data to support this speculation and this is still the case today.

A dimeric structure for *Bombyx* PTTH was long suspected and this was confirmed as part of the massive PTTH purification study that resulted in the determination of almost the entire amino acid sequence (Kataoka *et al*., 1991). This study also indicated that PTTH was a homodimer joined by one or more disulfide bonds and that asparagine-linked glycosylation (see **Figure 1**) was a likely cause for the disparity between the observed monomer MW of ≈17 kDa and the predicted MW of ≈12 700 (Kawakami *et al*., 1990). Further elucidation of the structure of *Bombyx* PTTH was provided by the incisive work of Ishibashi *et al*. (1994). This group used enzyme digestions of partially reduced recombinant PTTH to determine the intra- and interdimeric disulfide bonds (see **Figure 2**). This recombinant PTTH, expressed in bacteria (*Escherichia coli*) without glycosylation, had about 50% of the biological activity per nanogram of native PTTH. This result indicated that glycosylation is not necessary for biological activity although it may be required for maximum activity. *Bombyx* and other lepidopteran PTTHs (see below) do not have any significant homologs among vertebrate proteins, based on amino acid sequence (Kawakami *et al*., 1990; R. Rybczynski, unpublished BLAST analysis of vertebrate protein data bases.) However, Noguti *et al*. (1995) have shown that *Bombyx* PTTH exhibits an interesting arrangement of its intramonomeric disulfide bonds that is very much like that seen in some members of the vertebrate growth factor superfamily (βNGF, TGF-β2, and PDGF-BB). These workers suggest that PTTH is, in fact, a member of this superfamily and that PTTH and vertebrate growth factors share a common ancestor. It must be pointed out, however, that some insects, i.e., *Drosophila*, also possess genes belonging to the growth factor superfamily, such as *tolloid* and *60A*, with a significant amino acid identity to the vertebrate proteins in addition to the cysteine/disulfide bond conservation (Sampath *et al*., 1993). Furthermore, the intracellular signaling events activated by vertebrate growth factors like TGF-β, NGF, and PDGF are considerably different than those elicited by PTTH (see Section 3.2.3.4), involving receptors that are also kinases and that trigger quite different ligand-dependent intracellular processes (see Massagué, 1998; Haluska and Adjei, 2001; Huang and Reichardt,

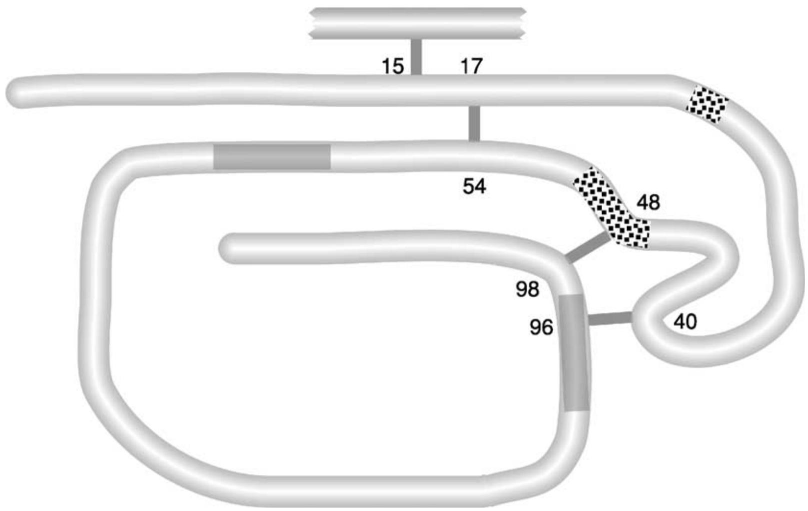


Figure 2 The structure of mature PTTHs, based on the *Bombyx* structure elucidated by Ishibashi *et al.* (1994). Dark shading indicates regions predicted to be hydrophobic in all five lepidopteran sequences while stippling indicates regions predicted to be hydrophobic in three lepidopteran sequences (Rybczynski, unpublished data). Numbers indicate the positions of the cysteines involved in intra- and intermonomeric bonds, in the mature *Bombyx* PTTH sequence.

2003). Thus, if PTTHs are members of the vertebrate growth factor superfamily, they have diverged extensively in amino acid sequence from other insect members of that superfamily. Furthermore, the coevolved events that PTTH initiate in their target cells would have to have diverged just as drastically from their vertebrate and insect counterparts. Perhaps resolution of this interesting puzzle will come from genomic studies of invertebrates more primitive than insects. In this regard, it is salient that the genome of the nematode *Caenorhabditis elegans* contains identifiable homologs to TGF proteins, e.g., *daf-7* (Inoue and Thomas, 2001), but appears to contain no gene coding for a PTTH-like protein (R. Rybczynski, unpublished data).

The amino acid sequences of the five lepidopteran PTTHs discussed above and of two dipteran candidate PTTHs are shown in **Figure 3**. The two dipteran sequences were found by searching the *D. melanogaster* and *A. gambiae* genomes with the complete *Bombyx* PTTH amino acid sequence. At present, no experimental data are available to indicate the function(s) of either of these two dipteran proteins and they are included here not only because of their general, although low, sequence identity and similarity with the lepidopteran PTTHs but, more particularly, because of the conservation of cysteine residues, i.e., it seems likely that these proteins are structurally similar to the demonstrated PTTHs. Note that due to the dynamic state of the *Anopheles* genome sequencing project at the time of writing (2003), the *Anopheles* sequence included here is undoubtedly incomplete and will be subject to revision. This may also be true of the candidate *Drosophila* sequence, although to a lesser extent.

All five lepidopteran PTTHs appear to be synthesized as prohormones that begin with a hydrophobic signal peptide sequence. The *Drosophila* protein is shown with a possible signal peptide sequence (**Figure 3**) but this region does not unequivocally meet the criteria for a cleaved signal peptide as defined by Nielsen *et al.* (1997). Following the signal peptide, these proteins exhibit only moderate sequence identity until an area preceding the mature PTTH sequence by about 35 amino acids. This cluster of greater amino acid conservation among the lepidopteran PTTHs might indicate that this region of the prohormone indeed has a function, as proposed by Ishizaki and Suzuki (1992). Note that the position of basic amino acids between the signal peptide and the mature hormone is not identical among the lepidopteran PTTHs and thus the 2 Da and 6 kDa peptide regions hypothesized for *Bombyx* (Kawakami *et al.*, 1990) do not have equivalents in *Samia*, *Antheraea*, and *Hyalophora*. A variety of proprotein convertases exist that recognize a variety of monobasic, dibasic, and tribasic amino acid patterns as well as specific sites lacking basic amino acids (see Loh *et al.*, 1984; Mains *et al.*, 1990; Seidah and Prat, 2002). Without experimental evidence, it is premature to predict where the preproPTTHs might be cleaved, in addition to the known site yielding the mature monomeric unit. The cellular location for the processing of PTTH from the longer, initial translation product to the shorter mature peptide form is not definitively

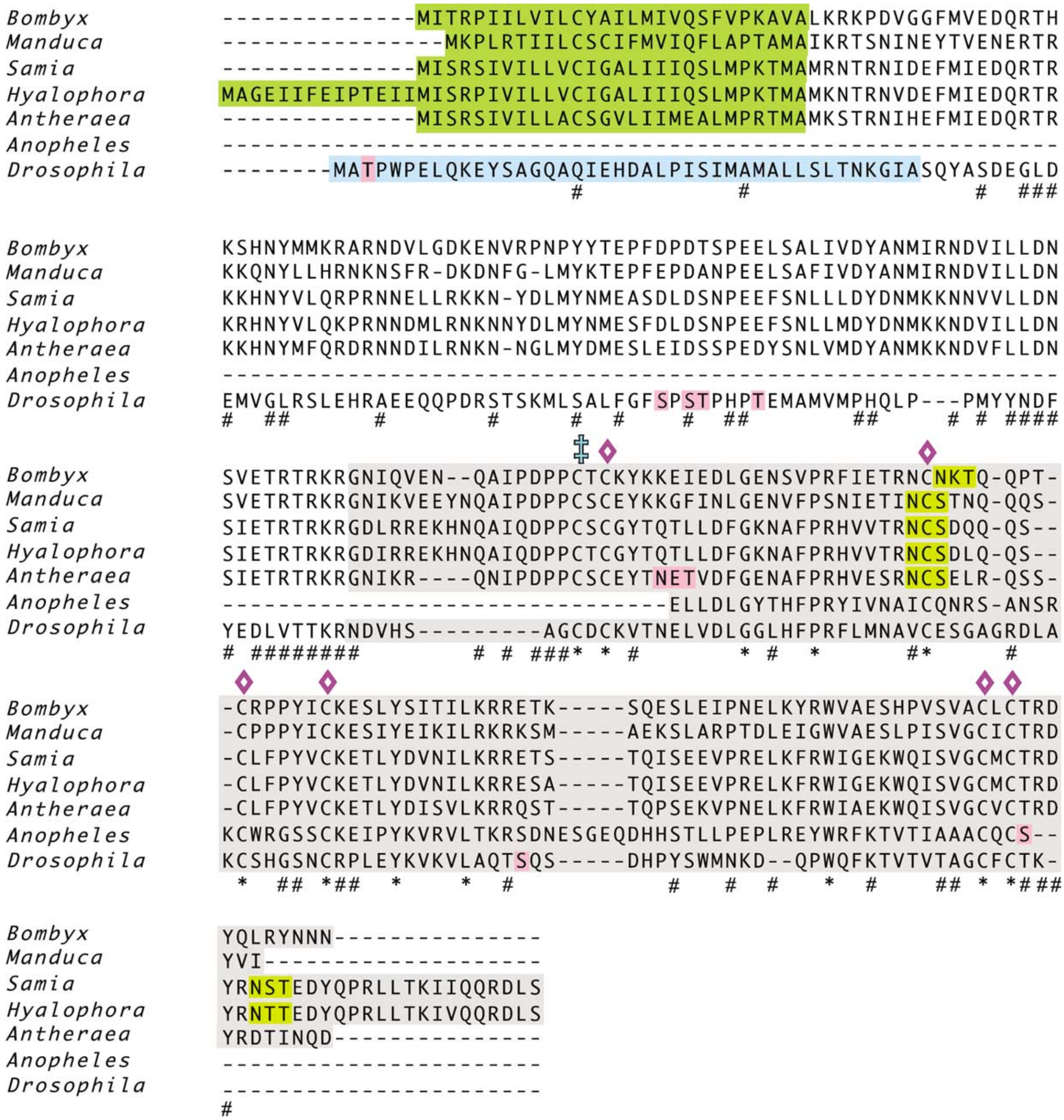


Figure 3 Sequence alignment of demonstrated and putative (*Drosophila*, *Anopheles*) PTTHs. Predicted signal peptide sequences are shaded with green. The low-probability *Drosophila* signal peptide is shaded with blue. Gray shading: the known or predicted mature PTTH sequences. #, amino acids conserved in all lepidopteran PTTHs; ∗, amino acids conserved in all PTTH sequences; ‡, cysteine participating in dimeric bonding; ◇, cysteines involved in intramonomeric bonds; yellow shading, predicted sites for *N*-linked glycosylation; magenta shading, potential sites for *O*-linked glycosylation. *N*-linked and *O*-linked glycosylation sites were predicted using the NetNGlyc 1.0 Server, the NetOGlyc 2.0 Server and the YinOYang 1.2 Server provided by the Center for Biological Sequence Analysis at the Technical University of Denmark.

known. Immunoblots of *Bombyx* and *Manduca* brain proteins revealed only the mature form, indicating that the protein is probably cleaved totally within the confines of the brain (Mizoguchi *et al.*, 1990; Rybczynski *et al.*, 1996). These observations did not reveal the intracellular site of processing, i.e., discern whether the cleavage(s) occurred completely within the soma or if cleavage occurred within axonal secretory granules, before those axons left the brain (see Loh *et al.*, 1984). It is presumed that removal of the signal sequence involves co-translational processing and takes place in the soma (see Loh *et al.*, 1984). The expected location of PTTH in neurosecretory particles has been

confirmed by immunogold electron microscopy in both *Manduca* and *Bombyx*, using the same anti-*Bombyx* PTTH antibody (Dai *et al.*, 1994, 1995).

The mature lepidopteran PTTHs begin with glycine-asparagine (GN) or glycine-aspartic acid (GD) in the lepidopteran PTTHs and the sequence from this glycine to the carboxy end has been confirmed to possess ecdysteroidogenic activity directly (*in vitro* prothoracic gland assays) in *Bombyx* (Kawakami *et al.*, 1990) and *Manduca* (Gilbert *et al.*, 2000), and indirectly (development of debrained pupae) in *Antheraea* (Sauman and Reppert, 1996a). Proteins beginning with glycines can be modified by N-terminal myristylation, yielding a hydrophobic moiety that often serves as a membrane anchor. No evidence for this or any other N-terminal or C-terminal posttranslational modifications were found during the sequencing of *Bombyx* PTTH (Kataoka *et al.*, 1991) and the derived sequences of the PTTHs do not begin or end with the amino acids most likely to be so modified, e.g., N-terminal alanines (methylation site) or serines (acetylation site) or C-terminal glycines (amidation substrate). The mature PTTH peptides all contain seven cysteines, except for the putative *Anopheles* sequence, which is preliminary and probably incomplete (Figure 3). Given the close spacing similarity among the sequences, it is likely that all PTTHs are linked by disulfide bonds and folded in the same manner as demonstrated for *Bombyx* PTTH (Ishibashi *et al.*, 1994; see Figure 2). The lepidopteran PTTHs also all contain consensus sites for *N*-linked glycosylation (Figure 3). Given a consistent disparity of 4000–5000 between molecular weights predicted by amino acid sequence versus larger molecular weights observed after SDS-PAGE, it seems likely that all five of these proteins are indeed glycosylated (for *Bombyx* compare Kawakami *et al.*, 1990 and Kataoka *et al.*, 1991; for *Manduca* compare Rybczynski *et al.*, 1996 and Shionoya *et al.*, 2003; for *Antheraea* see Sauman and Reppert, 1996a). The candidate *Drosophila* PTTH amino acid sequence does not contain a consensus site for *N*-linked glycosylation but does possess several for *O*-linked glycosylation (see Figure 3). Note that sites for *O*-linked glycosylation are difficult to predict and that only one of the potential sites in the *Drosophila* sequence falls within the putative mature peptide region. The processed (mature) PTTH sequences are, on the whole, hydrophilic proteins, with three or four short hydrophobic regions, depending on the species. Figure 2 shows a diagrammatic representation of the location of these hydrophobic regions in the lepidopteran sequences. Whether or not these hydrophobic regions play significant roles in determining monomeric or dimeric structures, or participate in receptor interactions are unanswered questions.

Table 1 summarizes the amino acid identities and similarities for pair-wise comparisons between six of the seven mature PTTH sequences presented in Figure 3; the candidate *Anopheles* sequence is omitted from the analysis due to the preliminary nature of the data. The similarities of these PTTH sequences are summarized graphically in Figure 4, using a phylogenetic tree neighbor-joining program (Saitou and Nei, 1987) applied to the mature PTTH amino acid sequences. In this analysis, the three Saturnid moths (*Antheraea*, *Hyalophora*, and *Samia*) form a clear-cut group, as do the two dipterans; *Bombyx* and *Manduca* form an outlier group in which the relationship of the two PTTH sequences do not closely match the conventional view of their taxonomic relationship. If the same analysis is performed with the prohormone rather than mature PTTH sequences, the results are essentially the same, reflecting the very similar, albeit modest, degrees of amino acid identity and similarity in the mature and prohormone sequences (see Table 1). The *Hyalophora* and *Samia* sequences are intriguingly similar and it would be very interesting to test if the PTTHs of these two species would significantly cross-activate prothoracic gland ecdysteroid synthesis. Such a comparison might

Table 1 Comparison of the amino acid identities and similarities for mature (bioactive) PTTHs

	Bombyx	Antheraea	Samia	Manduca	Hyalophora	Drosophila
Bombyx (224)[a]	X	52%[b] (75%)[c]	55% (73%)	59% (76%)	54% (73%)	22% (34%)
Antheraea (221)		X	68% (84%)	50% (68%)	71% (85%)	25% (41%)
Samia (236)			X	48% (73%)	94% (98%)	23% (36%)
Manduca (217)				X	48% (72%)	20% (39%)
Hyalophora (251)					X	24% (39%)
Drosophila (212)						X

[a]Number of amino acids in sequence.
[b]Amino acid identity.
[c]Amino acid similarity.

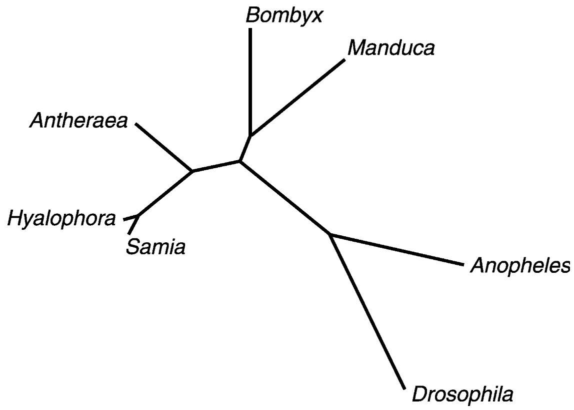


Figure 4 Graphical representation of mature PTTH amino acid sequence relationships, using a phylogenetic tree neighbor-joining program (Saitou and Nei, 1987).

enable some informed speculation as to the location of the receptor-binding regions of PTTHs.

Two additional lepidopteran PTTHs have been cloned recently from the closely related species *Heliothis virescens* (Xu and Denlinger, 2003) and *Heliocoverpa zea* (Xu *et al*., 2003). The deduced amino acid sequences of these two PTTHs revealed that the major features of lepidopteran PTTHs, as discussed above, are conserved in these two new sequences, e.g., the proteolytic site yielding the mature peptide and the number and position of the seven conserved cysteines. Comparison of the deduced amino acid sequences of the mature peptide indicated that the *Heliothis* and *Heliocoverpa* are very similar (94% identity) but the identity with other lepidopteran PTTHs is much lower (48–59%) (Xu *et al*., 2003).

3.2.2.4. Are PTTHs Species Specific?

With amino acid identities and similarities averaging around 50% and 80%, respectively, it is difficult to predict *a priori* whether PTTHs should act across species at biologically meaningful concentrations. Actual tests for cross-species bioactivity have been equivocal on this question. Agui *et al*. (1983) tested three lepidopteran pupal brain extracts with prothoracic glands from the three species *M. sexta*, *M. brassicae*, and *B. mori*. Their data suggested that conspecific PTTHs were generally most efficacious at eliciting ecdysteroid synthesis but that heterospecific PTTHs were also effective at doses (brain equivalents) from one half to about four times that of the conspecific molecule. This study was not able to take into account possible interspecific differences in endogenous PTTH levels and the brain extracts employed had not been size-selected to remove small PTTH or bombyxin (see Section 3.2.2.5). Several studies have re-examined the issue of potential *Manduca–Bombyx* PTTH cross-species activation of ecdysteroidogenesis. Gray *et al*. (1994) and Rybczynski, Mizoguchi and Gilbert (unpublished data) did not find activation of *Manduca* ecdysteroidogenesis by crude, native (size-selected) or by recombinant (pure) *Bombyx* PTTH, respectively. Additionally, *Manduca* crude PTTH was not able to activate *Bombyx* prothoracic glands in an *in vivo* dauer assay (Ishizaki quoted in Gray *et al*., 1994). Similarly, *Bombyx* crude PTTH did not stimulate development by debrained *Samia* pupae, when separated from the much smaller bombyxin molecule originally thought to be PTTH (Ishizaki *et al*., 1983b; see also Kiriishi *et al*., 1992) and *Antheraea* recombinant PTTH did not activate *Manduca* prothoracic glands *in vitro* (Rybczynski and Gilbert, unpublished data).

Yokoyama *et al*. (1996) tested brain extracts prepared from pupal day 0 brains of four species of swallowtail butterflies (*Papilio xuthus*, *P. machaon*, *P. bianor*, and *P. helenus*) and found cross-specific activation of prothoracic gland ecdysteroid synthesis *in vitro*, with similar doses for half-maximal and maximal activation being relatively independent of the species of origin. These results are somewhat in contrast to the moth cross-species studies discussed above and this difference may be a function of the evolutionary closeness of the *Papilio* species versus the evolutionary distance of the moth species.

The possibility of cross-species PTTH activity has also been examined using dipteran ring glands and brain extracts. Roberts and Gilbert (1986) found that ecdysteroid synthesis by ring glands from post-feeding larval *Sarcophaga bullata* was readily activated by extracts from *Sarcophaga* prepupal brains and that these ring glands were not activated by *Manduca* brain extract. In contrast, *Manduca* prothoracic glands showed a stage-specific response to *Sarcophaga* brain extract, with larval but not pupal glands responding positively at relatively low doses of extract, i.e., less than one brain equivalent. Henrich (1995) addressed the cross-species question with studies on the *Drosophila* ring gland. As discussed above, the activation of *Drosophila* ring gland ecdysteroidogenesis by *Drosophila* brain or ganglionic extracts is not robust. Nevertheless, in a small study, Henrich (1995) found that both small and large *Manduca* PTTH preparations were able to elicit increased steroidogenesis by the ring gland with the former possibly being more efficacious. In contrast, a preliminary study with pure recombinant *Manduca* PTTH was not able to detect stimulation of *Drosophila* ecdysteroid synthesis (Rybczynski, unpublished data).

Both *Rhodnius* and *Bombyx* have served as important experimental organisms in research aimed at understanding PTTH. Vafopoulou and Steel (1997) have explored the effect of *Bombyx* PTTH on ecdysteroid synthesis by *Rhodnius* prothoracic glands under *in vitro* conditions. They found that recombinant *Bombyx* PTTH elicited a statistically significant increase in ecdysteroidogenesis relative to controls, with effective concentrations similar to that observed in *Bombyx*–*Bombyx* experiments. This is an intriguing and surprising result, given the evolutionary distance between *Rhodnius* (Hemiptera) and *Bombyx* (Lepidoptera). A puzzling feature of the results is the very sharp optimum in concentration seen with the *Bombyx* PTTH ($8\,ng\,ml^{-1}$), with decreasing response seen at $10\,ng\,ml^{-1}$ and above, although a similar, albeit broader, optimum is also seen with *Rhodnius* brain extracts (Vafopoulou *et al*., 1996). Also of note is the approximately two-fold increase in ecdysteroid synthesis achieved with *Bombyx* PTTH-stimulated *Rhodnius* glands under the same conditions with which *Rhodnius* brain extract elicited nearly a five-fold increase (*cf*. Vafopoulou *et al*., 1996; Vafopoulou and Steel, 1997). Thus, care must be exercised in interpreting these data, especially given observations in *Manduca* that small increases in ecdysteroid synthesis can be obtained with nonspecific stimuli (Bollenbacher *et al*., 1983).

In summary, the question of PTTH species specificity remains open. The cloning, sequencing and expression of recombinant pure PTTHs will offer a more rigorous tool to investigate this topic and perhaps by doing so, gain insight into the portion of PTTH that productively interacts with PTTH receptors. If the region that contacts the receptor appears to be relatively small, then chimeric recombinant PTTHs can be used to investigate ligand-receptor interactions. At present, it seems premature to speculate on which portion(s) of the PTTH protein are necessary and sufficient for binding to the PTTH receptor and whether the area(s) that confer species-specificity are congruent with the former sites. It does seem likely that the most N-terminal region of the mature PTTH molecule is not involved in receptor binding since an antibody to this region failed to block PTTH-stimulated ecdysteroid synthesis by *Manduca* prothoracic glands (Rybczynski *et al*., 1996). If sequence divergence is any guide to areas conferring species-specificity and receptor binding, then one region of interest can be pointed out (considering only the lepidopteran species), i.e., an area between a conserved triplet of basic amino acids and a conserved serine that is located slightly past the middle of the mature peptide (see fourth of the five sets of rows in **Figure 3**). This area is also a hydrophilic region but as might be expected for a peptide hormone released into an aqueous environment, most of the mature PTTH peptide is predicted to be hydrophilic. Note that this region lies outside the common fold area that sequence-based modeling indicated that PTTH might share with members of the vertebrate growth factor superfamily (Noguti *et al*., 1995). Note also that this PTTH region is not positionally equivalent to the regions of the platelet-derived growth factor (PDGF) or brain-derived neurotrophic factor (BDNF) molecules, for example, that are believed to be crucial for receptor interaction (LaRochelle *et al*., 1992; O'Leary and Hughes, 2003); however, like the regions in these vertebrate molecules, this portion of the PTTH molecule lies in an area predicted to be within a hydrophilic area, extending out from the common fold area (Noguti *et al*., 1995).

3.2.2.5. Bombyxin and Small PTTH

The small neuropeptide bombyxin was initially characterized as *Bombyx* PTTH, using a *Samia* assay, until it was found that purified bombyxin was ineffective at stimulating ecdysteroid synthesis in *Bombyx* itself (see Section 3.2.2.1). The purification and amino acid sequencing of bombyxin, which began when it was considered to be *Bombyx* PTTH, revealed that there was actually a family of bombyxin proteins with sequence homology to the A and B chains of human insulin (see Ishizaki and Suzuki, 1994). Jhoti *et al*. (1987) modeled the three-dimensional structure of a bombyxin and found that it could assume an insulin-like tertiary structure. Despite these sequence and structural similarities, the functions of bombyxins, with nearly 40 genes in *Bombyx* (Kondo *et al*., 1996), remain practically unknown. Specific receptors in lepidopteran ovarian cells have been identified, suggesting a role in reproduction (Fullbright *et al*., 1997). Roles in ovarian and embryonic development have also been suggested (Orikasa *et al*., 1993; Tanaka *et al*., 1995). Whether or not ovarian ecdysteroid synthesis might be affected is an unaddressed question. Evidence also exists that a bombyxin may function in carbohydrate metabolism, in an insulin-like manner (Satake *et al*., 1997; Masumura *et al*., 2000). Recent work by Nijhout and Gunnert (2002) has demonstrated another role for a bombyxin in the butterfly *Precis coenia*. In this species, bombyxin II acts as a growth factor in concert with 20-hydroxyecdysone, stimulating cell division in cultured wing imaginal discs.

Finally, in regard to bombyxin, the ability of *Bombyx* bombyxin, effective at 30 pM, to stimulate adult development in debrained *Samia* pupae is still not understood (see Ishizaki and Suzuki, 1994). Nagata *et al.* (1999) used the deduced amino acid sequence of two *Samia* bombyxin homologs to synthesize the corresponding peptides. These peptides were then used in the *Samia* debrained pupae, where they proved to be effective at stimulating adult development at estimated hemolymph concentrations of 1–5 nM, a concentration at least 30 times higher than the *Bombyx* bombyxin. Given the plethora of bombyxin genes in *Bombyx*, and presumably other Lepidoptera, the possibility that one of these genes might code for a true prothoracicotropic protein cannot be ruled out. An additional factor must be kept in mind when considering bombyxins, i.e., the ability of bombyxins to activate adult development does not prove that bombyxins directly stimulate prothoracic gland ecdysteroidogenesis. In addition to the study by Kiriishi *et al.* (1992), demonstrating the relative ineffectiveness of bombyxin in stimulating ecdysteroid synthesis by isolated prothoracic glands (see Section 3.2.2.1), Vafopoulou and Steel (1997) found that *Bombyx* bombyxin was much less effective than *Bombyx* PTTH at stimulating ecdysteroid synthesis by *Rhodnius* glands *in vitro*. Other tests *in vitro* of small ecdysteroidogenic molecules have used large amounts of size-fractionated brain extracts, e.g., 50 brain equivalents per prothoracic gland, making it difficult to determine the physiological relevance of the stimulatory molecules, presumed to be bombyxins (Endo *et al.*, 1990; Fujimoto *et al.*, 1991).

What role insulin-like factors play in the acute regulation of steroidogenesis in taxa other than the Lepidoptera is also an open question. The *Drosophila* genome does not appear to contain any definite bombyxin homologs (R. Rybczynski, unpublished BLAST analysis), but it does contain seven genes that, like the bombyxins, code for proteins containing characteristic insulin motifs (Brogiolo *et al.*, 2001). These seven genes (*Drosophila* insulin-like proteins: DILP 1–7) do not show significant homology to bombyxins outside of the insulin motifs and, unlike the bombyxins, are not limited in expression to neuronal cells (Brogiolo *et al.*, 2001). It is not known if any of the DILP gene products participate in regulating ecdysteroidogenesis.

Despite the demonstration that the so-called small PTTH of *Bombyx*, now known as bombyxin, is very unlikely to be a physiologically important PTTH in this species, the question persists – is there more than one brain-derived PTTH? As discussed above (Section 3.2.2.2), size-fractionated brain extracts frequently exhibit two peaks of ecdysteroidogenic activity, with one peak at 20 000–30 000 MW and a second, smaller peak below 10 000 MW. In most cases, there are simply no data beyond approximate molecular weight and dose (brain equivalents) that can address this question. The observations indicate a much larger dose requirement for the so-called small PTTHs. Additionally, a smaller maximum response (e.g., Endo *et al.*, 1990, 1997; Yokoyama *et al.*, 1996) suggests, but does not prove, that these so-called small PTTHs may be bombyxin homologs; as noted for *Bombyx* prothoracic glands tested *in vitro* (Kiriishi *et al.*, 1992), PTTH stimulated ecdysteroidogenesis much more effectively than did bombyxin. However, there are some data from *Manduca* that suggest that small PTTH in this species may be a molecule other than a bombyxin. Bollenbacher *et al.* (1984) described a small PTTH (MW = 6–7 kDa) that has subsequently been partially characterized in regard to its developmental expression and the signal transduction events it elicits in prothoracic glands. *Manduca* small PTTH appears to be much more abundant in pupal than in larval brains, while “big” PTTH shows only a slight increase in the pupal versus larval brain (O’Brien *et al.*, 1986). Bollenbacher *et al.* (1984) reported that small PTTH was notably less effective at stimulating ecdysteroid synthesis in pupal prothoracic glands than in larval glands; however, a study by O’Brien *et al.* (1986) showed no such difference. Like big PTTH (see Section 3.2.3.4), *Manduca* small PTTH appears to stimulate ecdysteroid synthesis via Ca^{2+} influx and Ca^{2+}-dependent cAMP accumulation (Watson *et al.*, 1993; Hayes *et al.*, 1995). These observations concerning intracellular signaling suggest that *Manduca* small PTTH might not be a bombyxin homolog because, as members of the insulin peptide family, bombyxin would not be expected to stimulate either Ca^{2+} influx or Ca^{2+}-dependent cAMP (see Combettes-Souverain and Issad, 1998). However, this is an inference as authentic bombyxin-stimulated intracellular events do not appear to have been studied. It should also be pointed out that for small PTTH to have the same effects as PTTH on Ca^{2+} influx and Ca^{2+}-dependent cAMP generation is surprising and confusing, especially if small PTTH does not differentially activate larval glands (*cf.* conflicting data in Bollenbacher *et al.*, 1984 and O’Brien *et al.*, 1986). In fact, it has been hypothesized that small PTTH might be an active proteolytic fragment of PTTH (Gilbert *et al.*, 2002); if so, it is unclear whether small PTTH is generated as an artifact during PTTH extractions, or is a naturally occurring processed form.

Besides bombyxin and *Manduca* small PTTH, other, noncerebral, ecdysiotropic activities have been described, e.g., factors extracted from the proctodaea of *Manduca*, the European corn borer, *Ostrinia nubilalis*, and the gypsy moth, *L. dispar* (Gelman *et al.*, 1991; Gelman and Beckage, 1995). These data are not completely surprising, given the precedent of the vertebrate gut as an endocrine organ. However, a definitive role for such factors *in vivo* has not been established and further discussion of these candidate ecdysiotropes is outside the scope of this review.

3.2.3. Prothoracicotropic Hormone: Synthesis and Release

3.2.3.1. Sites of Synthesis and Release

Agui *et al.* (1979), using micro-dissections, demonstrated functionally that *Manduca* PTTH was present only in two pairs of dorsolateral brain neurosecretory cells. Subsequently, a number of lepidopteran PTTHs have been purified and/or cloned and antibodies raised against the derived sequences (see Section 3.2.2.1). The use of cDNA probes for *in situ* localization of these PTTH mRNAs supported the findings of Agui *et al.* (1979) in all species tested (*Bombyx*: Kawakami *et al.*, 1990; *Antheraea*: Sauman and Reppert, 1996a; and *Manduca*: Shionoya *et al.*, 2003). Similarly, the use of antibodies in immunohistochemical studies also supported the early *Manduca* work (*Bombyx*: Mizoguchi *et al.*, 1990; *Antheraea*: Sauman and Reppert, 1996a; and *Manduca*: Gilbert *et al.*, 2000) (see **Figure 5**). Of particular note was the co-localization of PTTH mRNA and protein to the same cells in *Antheraea* brain (Sauman and Reppert, 1996a). This co-localization has been assumed to be true but has only been explicitly demonstrated in *Antheraea*.



Figure 5 Immunocytochemical localization of PTTH in the pupal brain of *Manduca sexta* showing that PTTH is detectable only in the cell bodies and axons of two pairs of lateral neurosecretory cells. Scale bar = 100 μm. (Data from Gilbert, L.I., Rybczynski, R., Song, Q., Mizoguchi, A., Morreale, R., *et al.*, **2000**. Dynamic regulation of prothoracic gland ecdysteroidogenesis: *Manduca sexta* recombinant prothoracicotropic hormone and brain extracts have identical effects. *Insect Biochem. Mol. Biol. 30*, 1079–1089.)

The antibody studies discussed above were conspecific studies, e.g., an anti-*Bombyx* PTTH antibody was used to probe *Bombyx* tissues. However, as discussed in Sections 3.2.2.1 and 3.2.2.2, anti-PTTH antibodies have been used also in heterospecific immunohistochemical studies. Anti-*Bombyx* PTTH antibodies were used to probe *Manduca* (Dai *et al.*, 1994), *Samia* (Yagi *et al.*, 1995), *D. melanogaster* (Žitňan *et al.*, 1993), and *Locusta* (Goltzene *et al.*, 1992). In *Manduca* and *Samia* brains, the antibody decorated two pairs of lateral neurosecretory cells. These cells appeared to be the same as seen in *Bombyx* brain, in the conspecific probing summarized above. In *Drosophila*, Žitňan *et al.* (1993) found that the anti-*Bombyx* PTTH bound to two superior mediolateral cells that might be equivalent to the lepidopteran PTTH-positive cells. Also positive were axons and axon terminals in the neurohemal region of the ring gland, as to be expected based on the site of PTTH release determined in Lepidoptera. However, a number of additional cells in the subesophageal thoracic and abdominal ganglia were also positive, particularly in the adult. It is unknown if this last result indicates multiple sites of PTTH synthesis or a cross-reactivity to a protein besides PTTH.

PTTH immunoreactivity in *Locusta* is dependent on the antibody employed. Goltzene *et al.* (1992) found that a number of brain neurosecretory-type cells reacted positively with an anti-*Bombyx* PTTH antibody while the use of an anti-*Antheraea* PTTH antibody revealed no positive reactions (Závodská *et al.*, 2003). This same anti-*Antheraea* PTTH antiserum was used to survey a wide variety of insects (Závodská *et al.*, 2003: see Section 3.2.2.2); until we know more about the nature of PTTH in these species, it is not possible to interpret the smorgasbord of immunohistochemical patterns seen among the surveyed taxa.

Functional evidence that the corpus allatum and not the corpus cardiacum was the neurohemal organ for lepidopteran PTTH was provided by Agui *et al.* (1979, 1980) and Carrow *et al.* (1981) and immunohistochemical studies have supported this conclusion (Mizoguchi *et al.*, 1990; Dai *et al.*, 1994). If the additional immunohistochemically positive cells seen in some studies (e.g., Žitňan *et al.*, 1993, Závodská *et al.*, 2003) actually do contain PTTH,

it is presumably synthesized and released there. Further discussion of these "outlier" cells awaits confirmation that the regions of interest contain bioactive PTTH.

3.2.3.2. Control of Release: Neurotransmitters

PTTH release is apparently ultimately controlled by a number of factors, including photoperiod, size and nutritional state of the animal (see Section 3.2.1). Proximately, a number of studies have pointed out the likelihood that PTTH release is directed by cholinergic neurons synapsing with the prothoracicotropes. It must be emphasized that the data discussed in this section in the following studies do not definitively identify PTTH as the active molecule released from the brain, although this is most likely the case.

Carrow *et al.* (1981) showed that PTTH could be released by depolarizing *Manduca* (brain) neurons with a high potassium concentration in the presence of Ca^{2+}; a similar result was obtained when *Bombyx* brains were treated with high K^{+} in the presence or absence of external Ca^{2+}. Lester and Gilbert (1986, 1987) found that acetylcholine accumulation and α-bungarotoxin binding sites exhibited temporal peaks in the brain of fifth instar *Manduca* larvae and that these peaks coincided with periods of PTTH release and increased ecdysteroid synthesis. Of course, it is not likely that all the changes in the cholinergic systems of the brain that occur at these stages directly feed into the PTTH-producing neurons but these data are supportive for a role of cholinergic neurons in PTTH biology. Direct evidence that cholinergic stimulation can result in PTTH release comes from several *in vitro* studies of *Bombyx* and *Mamestra*. Agui (1989) showed that acetylcholine and cholinergic agonists caused cultured *Mamestra* brains to release PTTH into the medium while dopaminergic agents have little or no effect. Shirai *et al.* (1994) obtained similar results, using the acetylcholine agonist, carbachol, and further refined the analysis by using both muscarinic and nicotinic agents. They found that treatment with muscarine caused PTTH release from *Bombyx* brains *in vitro*, and that carbachol-induced release was blocked by the muscarinic antagonist atropine. Nicotine had no effect on PTTH release, with the composite data indicating the involvement of a muscarinic acetylcholine receptor. Later immunohistochemical studies revealed that the PTTH-producing cells of *Bombyx* do indeed express a muscarinic acetylcholine receptor (Aizono *et al.*, 1997). Shirai *et al.* (1994) further determined the effects of a calmodulin inhibitor, a phospholipase C inhibitor (PLC) and a protein kinase C (PKC) inhibitor on carbachol-induced PTTH release. All three drugs inhibited the effects of carbachol, suggesting roles for Ca^{2+}-calmodulin, PKC, and PLC in this system. A role for Ca^{2+} was more directly demonstrated, when it was found that Ca^{2+}-ionophore also caused PTTH release (Shirai *et al.*, 1995). Finally in regard to the cholinergic neurotransmission, Shirai *et al.* (1998) investigated the possible role for small G-proteins in carbachol-stimulated PTTH release. The poorly hydrolyzable GTP analog GTPγS was found to stimulate PTTH release in the presence of a PLC inhibitor; the use of the PLC inhibitor removed the positive effect that the GTPγS would have on the larger heterotrimeric G-protein directly associated with muscarinic receptors (see Hamilton *et al.*, 1995).

The experiments of Agui (1989) indicated that dopamine might have a small effect on PTTH release *in vitro*. This possibility was tested with *Bombyx* brains and it was found that dopamine and serotonin increased PTTH release, while norepinephrine had no effect (Shirai *et al.*, 1995). The effects of dopamine and serotonin were notably slower than that of carbachol, suggesting that the first two compounds might be acting at some distance from the PTTH-producing cells. It must be emphasized, though, that there are no data that demonstrate that cholinergic agonists directly act upon the prothoracicotropes.

In contrast to observations in *Bombyx* (Shirai *et al.*, 1995), serotonin had an inhibitory effect on PTTH release, as measured by prothoracic gland ecdysteroid synthesis, during short-term (3 h) co-cultures of cockroach (*Periplaneta americana*) brains and prothoracic glands (Richter *et al.*, 2000). No effect of serotonin on isolated prothoracic gland ecdysteroid synthesis was found. Serotonin is the precursor to melatonin, the latter playing an important role as neuromodulator associated with darkness in diel light/dark cycles. In contrast to the results with serotonin, Richter *et al.* (2000) found that melatonin increased PTTH release in brains and prothoracic gland co-cultures during both short-term (photophase) and long-term (12 h: scotophase) incubations. A melatonin receptor antagonist, luzindole, blocked the melatonin effect. Again, neither melatonin nor luzindole had an effect on prothoracic gland ecdysteroid synthesis in the absence of the brain. These results raise the possibility that melatonin may play a role in modulating temporal patterns of PTTH release. However, as Richter *et al.* (2000) pointed out, there was a lack of daily rhythmicity in the molting of cockroaches in their colony, suggesting that there was no rhythmicity to PTTH release either, thus indicating that an *in vivo* role for melatonin is problematic

(see Section 3.2.3.4 and **Chapter 3.11**) for further information on cycles of PTTH release).

3.2.3.3. Control of Synthesis and Release: Juvenile Hormone

Insect molts, be they simple or metamorphic, are under the general control of 20-hydroxyecdysone (20E). Of course, 20E does not act alone and there are additional hormones, like ecdysis-triggering hormone and eclosion hormone, that act downstream from 20E, playing very proximate roles in controlling ecdysis behavior (see Mesce and Fahrbach, 2002; and **Chapter 3.1**). However, juvenile hormones (JHs), which also participate in regulating molting and metamorphosis (see **Chapter 3.7**), modulate the action of 20E in a way that makes them partners with 20E rather than dependent factors (see **Chapter 3.8**). Juvenile hormones are epoxidated sesquiterpenoids produced in, and released from, the corpus allatum, which is also the site of PTTH release. Depending on the taxon and the developmental stage, a corpus allatum may synthesize one or two methylated JHs as well as JH acids (see Gilbert *et al*., 1996; Henrich *et al*., 1999; and **Chapter 3.7**).

JH is an important hormone in some aspects of insect reproduction (see Nijhout, 1994; and **Chapter 3.9**) but the relevant role of JHs, in regard to 20E and hence PTTH, is the ability of JHs to direct a molt either into a simple or into a metamorphic pathway. Basically, the presence of circulating JH during an ecdysteroid peak results in the next molt being a simple one, i.e., larva to larva, but if JH levels are very low during an ecdysteroid peak, then the ensuing molt will be a metamorphic molt, e.g., larva to pupa. In the last larval instar, of many or most insects, a small release of PTTH appears to trigger a small ecdysteroid release from the prothoracic gland during a period of very low JH titer. This small increase in ecdysteroid titer (Bollenbacher *et al*., 1981) is termed the commitment peak because it commits the animal to a metamorphic molt (Rid-Riddiford, 1976).

How JHs modulate molt quality is not well understood. A considerable number of studies have demonstrated that JH or JH analogs (JHAs) (see **Chapters 3.7** and **3.8**) have effects on ecdysteroid titers when applied topically, injected, or fed to preadult insects. In these studies involving intact larvae, pupae, debrained animals, or isolated abdomens, JHs appeared to modulate PTTH synthesis and/or release, and perhaps affected the PTTH signaling pathway in the prothoracic gland or the gland's capacity for ecdysteroidogenesis. Two periods of JH influence have been identified in such studies during the last larval instar; the influence of JH during the penultimate instar is different and is treated separately below.

In Lepidoptera, JH hemolymph titers decline dramatically in the first few days of the last larval instar (for *Manduca*, see Baker *et al*., 1987; for *Bombyx*, see Nimi and Sakurai, 1997). The JH decline is followed by a release of PTTH and the resultant small peak in the ecdysteroid titer termed the commitment peak (see Section 3.2.3.1). Application of juvenoids (JHs or JH analogs: JHAs) before the commitment peak results in a delay of development, e.g., deferral of the cessation of feeding and wandering (see Riddiford, 1994, 1996). In *Manduca*, Rountree and Bollenbacher (1986) found that prothoracic glands from early fifth instar larvae treated with JH-1 or ZR512 (a JHA) for 10 h prior to extirpation still responded to PTTH with increased ecdysteroid synthesis, although the normal developmental increase in basal ecdysteroidogenesis was inhibited. However, when the brain-retrocerebral complexes from such animals were implanted into head-ligated larvae, they exhibited a delay in activating development, relative to control complexes. This result suggested that JHs controlled the time of PTTH release at this time, although the data do not rule out an effect on PTTH content/synthesis in the complexes, as well. In a later study of *Manduca* glands, Watson and Bollenbacher (1988) did find a reduced response to PTTH *in vitro*, but this study involved animals treated with a JHA for 2 days, starting at an earlier time point. Thus, the conclusions reached may reflect the experimental paradigms as well as the animal's intrinsic biology.

In *Bombyx*, similar results have been obtained. Sakurai (1984), using the technique of brain removal, concluded that PTTH release occurred early in the fifth instar shortly after a precipitous decline in JH titer, as found for *Manduca*. Furthermore, allatatectomy late in the fourth instar shortened the phagoperiod in the fifth instar, and brain removal performed on these larvae at 48 h into the fifth instar did not block metamorphosis. Sakurai also found that JH application early in the fifth instar prolonged the larval period. The combined data suggested again that JH in the early fifth instar blocked PTTH release. Sakurai *et al*. (1989) provided evidence that JH inhibits prothoracic gland activity in *Bombyx* by showing that allatectomy of early fifth instar larvae accelerated the period of PTTH sensitivity in glands, but that this acceleration was blocked by JH treatment.

The above studies utilized either JHs, or JH analogs with JH-like chemical structures, i.e., terpenoids. However, the nonterpenoid chemical fenoxycarb, a carbamate, has also been shown to exhibit JH-like

effects when applied to larvae. Leonardi *et al.* (1996) found that single, topically applied doses of fenoxycarb, as low as 10 femtograms, were sufficient to disrupt larval–pupal ecdysis in *Bombyx*. Also studying *Bombyx*, Monconduit and Mauchamp (1998) showed that very low doses of fenoxycarb, 1 ng and below, were sufficient to induce permanent larvae when administered in the food during the phagoperiod of the last larval instar. This treatment also considerably depressed the ecdysteroid synthesis of prothoracic glands when measured *in vitro*. The glands still exhibited a qualitative response to a (crude) PTTH preparation, but the quantitative response was greatly curtailed. Dedos and Fugo (1996) found that fenoxycarb significantly and directly inhibited prothoracic gland steroidogenesis under *in vitro* conditions; however, the effective doses required in these *in vitro* experiments were much higher (0.5–1 μg/gland) than employed by Leonardi *et al.* (1996) and Monconduit and Mauchamp (1998). Thus, the possibility that fenoxycarb at these doses had effects not attributable to JH-like activities cannot be ruled out, as suggested by Mulye and Gordon (1993) and Leonardi *et al.* (1996), among others.

When exogenous JH or JH analogs are administered after the commitment peak period of last larval instars, quite different results are obtained, i.e., JHs accelerate development and shorten the period to pupation (see Sakurai and Gilbert, 1990; Smith, 1995). Brain removal or ligation after the commitment peak period does not affect this accelerative ability of JH, indicating that the brain and hence PTTH release are not the targets of JH at this time (Safranek *et al*., 1980; Gruetzmacher *et al*., 1984a). Intriguing in this regard are the observations of Sakurai and Williams (1989). They implanted prothoracic glands extirpated from feeding (day 2) fifth instar *Manduca* larvae into pupae lacking a cephalic complex. Treatment of these pupae with either 20E or the JH analog hydroprene resulted in an inhibition of steroidogenesis when the prothoracic glands were studied subsequently *in vitro*. However, if both 20E and hydroprene were administered, an increase in ecdysteroid synthesis was seen. These conditions simulate the postcommitment peak period in larvae, when JH (particularly JH acids) and 20E are both high. Thus, as Smith (1995) suggested, the prepupal ecdysteroid peak may be initiated by PTTH and maintained by JH, with the eventual decline of JH titer resulting in the decline of ecdysteroid levels that occurs shortly before larval–pupal ecdysis.

The relationship between JH and the brain-prothoracic gland axis observed in the last larval instar is not characteristic of every developmental stage. Stages prior to the last larval instar lack the commitment peak of ecdysteroids, along with the PTTH release that triggers it. In *Manduca*, the fourth (penultimate) larval instar is characterized by high to moderate JH levels throughout (see Riddiford, 1996; and **Chapter 3.7**). The fourth instar JH titer minimum is reached shortly before ecdysis to the fifth instar and ecdysteroid levels in the hemolymph begin rising while JH levels are relatively high, albeit falling. In *Bombyx*, the case is somewhat more complicated. Peaks in the JH titer occur during the early and in the middle to late portions of the penultimate instar, with the minimum between the two peaks being higher in concentration and shorter in duration than the minimum measured during the last (fifth) instar (Nimi and Sakurai, 1997).

Fain and Riddiford (1976) showed that head ligation of fourth instar *Manduca* larvae before a presumed head critical period resulted in precocious pupation; this operation isolated the sources of both PTTH and JH from the prothoracic glands. Injection of 20E immediately after ligation resulted instead in a larval molt. Delaying the 20E treatment resulted in a progressive loss of the larval response but JH injection maintained the 20E-induced larval molt. Lonard *et al.* (1996) found that application of the JH analog methoprene to fourth instar *Manduca* larvae affected neither the ecdysteroid titer *in vivo* nor the ecdysteroidogenic activity of prothoracic glands measured *in vitro*; however, allatectomy abolished the ecdysteroid peak, which could be restored by the application of methoprene. Further methoprene experiments with debrained and neck-ligated animals indicated that the restorative effect of methoprene involved the brain and was interpreted to indicate the existence of JH-dependent PTTH release.

Sakurai (1983) observed results in *Bombyx* similar to those obtained by Fain and Riddiford (1976), i.e., head ligation early in the fourth instar resulted in precocious pupation and application of methoprene shifted the postligation molts back to larval or larval–pupal intermediates. Unlike the case of JH in *Manduca*, methoprene was effective at reversing precocious pupations only when administered about 24 h after ligation, with larvae treated directly after ligation simply showing no further development. This difference between the two species may reflect a fundamental difference in the ecdysteroidogenic competence of the prothoracic gland in the time immediately following larval ecdysis (see Section 3.2.3.4). Sakurai also found that application of 20E to debrained *Bombyx*, still possessing corpora allata, resulted in larval rather than pupal molts.

These data from *Manduca* and *Bombyx* indicate that high levels of 20E in the relative absence of JHs will result in a pupal molt, even prior to the last larval instar. However, under ordinary circumstances, PTTH release and ecdysteroid synthesis take place against a significant JH titer background in stages other than the last larval instar.

The relationship between JHs and the PTTH-prothoracic gland axis during pupal–adult development has not been studied extensively, perhaps because there is no JH in early lepidopteran pharate adults, and therefore the experiments are not physiological. In general, application of exogenous juvenoids to pupae shortens the time until adult ecdysis, and the juvenoid must be administered about the time of pupal ecdysis or termination of pupal diapause (see Sehnal, 1983). Using *Bombyx*, Dedos and Fugo (1999a) injected the potent juvenoid fenoxycarb at pupal ecdysis and found that the ecdysteroids were considerably elevated above control during subsequent pupal–adult development. *In vitro* analysis of the prothoracic glands from treated animals showed elevated ecdysteroid secretion relative to controls; additionally, brain-retrocerebral complexes from fenoxycarb-treated animals secreted more PTTH than controls did, after 3 h of *in vitro* incubation; however, there was no difference from controls if the incubation was only 1 h. These data were interpreted to indicate that fenoxycarb stimulated ecdysteroid synthesis by promoting PTTH release. However, the observed delay in the fenoxycarb-facilitated increase in PTTH release *in vitro* could also be interpreted as a release from *in vivo* inhibition, once the brain-retrocerebral complexes were removed from the larvae.

Although all the studies cited above, and many others not discussed here, clearly point out the existence of cross-talk between the brain-prothoracic gland axis and juvenile hormones, considerable care must be exercised in interpreting these experiments. Ligations, extirpations, and tissue transplants have provided valuable insights into insect endocrinology over the years but these methodologies are rarely clean. For instance, allatectomy removes not only the site of juvenile hormone synthesis but also the neurohemal organ for normal PTTH release. Similarly, brain removal eliminates a source of many neuropeptides as well as a biochemical clock involved in regulating many diel cycles (see **Chapter 4.11**).

Experiments involving hormone additions to intact or nonintact animals also have to be interpreted carefully. Hormones are powerful drugs when given in large doses or at unusual times. For example, fenoxycarb-treated pupae that had elevated ecdysteroid titers also exhibited malformed rectums (Dedos and Fugo, 1999a) and whether or not this result tells us something informative about ecdysteroid titer regulation during normal adult development is open to debate. JH has often been suggested to have a negative effect on PTTH secretion in the last larval instar (see above) based on experiments in which larvae were treated with JH or JH analogs early in the instar. However, recently Mizoguchi (2001) measured the PTTH titer in JH-treated fourth and fifth instar *Bombyx* larvae, using time-resolved fluorimmunoassay. His data showed that, in fact, JH treatment had just the opposite effect, i.e., JH treatment raised the PTTH hemolymph titer! Thus, experiments in which PTTH secretion was measured *in vitro* following JH treatment of the donor larvae appear to be misleading in an attempt to understand the relationship between JH and PTTH.

Differences between species in the basic intercellular or intracellular "wiring" of their endocrinological systems may also confound experimental manipulations. An obvious example is the variation among species in prothoracic gland innervation. *Manduca* and *Bombyx* glands appear not to be innervated while prothoracic glands in other species clearly are (see Section 3.2.5.4). Brain extirpation in the latter species could have consequences on the prothoracic gland beyond the obvious removal of the source of PTTH. Less obvious but potentially important are developmental differences between species that complicate the ability to make generalizations. For instance, during the earliest portion of the last larval instar, *Bombyx* prothoracic glands are refractory to PTTH stimulation while *Manduca* glands are responsive (Sakurai, 1983). This refractoriness stems from a developmentally specific deficit in PTTH receptors or some other very early-acting component of the PTTH signal transduction cascade (Gu *et al.*, 1996; see also Section 3.2.5.1).

It must also be emphasized that direct effects of JHs on prothoracic glands have been seen rarely, indicating the probable participation of one or more additional cell types and/or hormones and/or neuronal connections in JH effects. In the rare cases where a direct effect has been demonstrated *in vitro*, the concentrations of JHs or JHAs employed are notably above physiological limits. In addition to the effect of 1 μg (≈65 μM) fenoxycarb on *Bombyx* prothoracic glands discussed above (Dedos and Fugo, 1996), Richard and Gilbert (1991) reported that JHB3 and JHIII both inhibited the secretion of ecdysteroids from *Drosophila* brain–ring gland complexes; however, millimolar concentrations were needed to elicit the effect.

3.2.3.4. PTTH Hemolymph Titer

The periodic increases in ecdysteroid synthesis and hemolymph titer that control molting and molt quality have long been interpreted to indicate that PTTH was itself released periodically into the hemolymph. However, testing this hypothesis has not been simple. Unlike ecdysone and 20E, a sensitive radioimmunoassay for PTTH has not been available until very recently. Instead, the measurement of PTTH hemolymph titers has been done primarily via *in vitro* analysis of prothoracic gland ecdysteroidogenesis using hemolymph extracts.

Bollenbacher and Gilbert (1981) determined the brain and hemolymph titers of PTTH in *Manduca* during the fourth and fifth larval instars, using an *in vitro* bioassay system and discovered little concordance between measured brain- and hemolymph-derived PTTH activities. Brain titers of PTTH rose fairly steadily during this period, punctuated by minor decreases or plateaus. A small dip in brain PTTH was seen immediately after the molt to the fifth larval instar and a larger decrease seen about 1 day prior to the pupal molt. In contrast, hemolymph PTTH activity was low throughout this period with only a few very sharp peaks. In the fourth instar, a single PTTH peak occurred late on day one, also reported later by Bollenbacher *et al.* (1987), with a second small peak spanning the molt to the fifth larval instar. During the fifth larval instar proper, four peaks were noted. Three medium-sized peaks clustered on and about the fourth day, preceding and overlapping the commitment peak of ecdysteroid synthesis. A much larger PTTH hemolymph titer was measured on day six, at the rising edge of the major larval ecdysteroid titer that precedes the metamorphic molt to pupal–adult development and closely anticipating apolysis. The PTTH titer appeared to be rising again, just before pupal ecdysis, but titers were not determined beyond this point.

Later studies of *Manduca* brain PTTH content indicated that PTTH levels in the brain increased considerably during pupal–adult development (O'Brien *et al.*, 1986) but hemolymph titers have yet to be determined at this stage for *Manduca*. These studies suggested that PTTH titers in the brain are not closely correlated with circulating levels, unlike the levels in the corpus allatum (Agui *et al.*, 1980). The PTTH titers described in the Bollenbacher and Gilbert (1981) study, while intriguing, must be considered preliminary for three reasons. First, the bioassay may miss small peaks that are not high enough to activate prothoracic glands *in vitro* but which might act *in vivo* i.e., very low levels of PTTH *in vitro* might be able to stimulate prothoracic gland steroidogenesis with a slower time course than that utilized in the *in vitro* assays. Second, very low levels of PTTH might have biologically significant effects on the prothoracic gland besides stimulating ecdysteroidogenesis. Third, if the so-called small PTTH (see Section 3.2.2.5) is secreted into the hemolymph, then an unknown fraction of the PTTH activity detected in the hemolymph might reflect this incompletely characterized protein rather than "big" PTTH.

Shirai *et al.* (1993) first addressed the topic of PTTH hemolymph titers in *B. mori*. Again, an assay was used in which fifth larval instar hemolymph extracts were tested for PTTH ecdysteroidogenic activity with prothoracic glands *in vitro*. In addition, the secretion of PTTH activity by brain–endocrine complexes was measured with the *in vitro* gland assay. Hemolymph ecdysteroid levels were also determined via radioimmunoassay. The results of this study suggested that five peaks of PTTH activity were present in the hemolymph of *Bombyx* fifth instar larvae. Two of these PTTH peaks occurred in the first half of the fifth instar, clearly before a small ecdysteroid peak on day seven or day eight to nine, depending on the particular *Bombyx* strain-cross utilized (*Shunrei* x *Shougetsu* and *Kinshu* x *Shouwa*, respectively). This small ecdysteroid peak was presumed to be the commitment peak, as seen in *Manduca*. A third PTTH peak occurred the day before the putative commitment peak while the fourth peak encompassed the time span of the commitment peak and just overlapped the beginning of the large premolt ecdysteroid peak. The fifth hemolymph peak detected by Shirai *et al.* (1993) began almost immediately after the finish of the fourth peak and overlapped with approximately the first third of the large ecdysteroid peak. These workers also found four to five peaks of PTTH activity when brain–endocrine complexes were assayed *in vitro*. These peaks anticipated the hemolymph peaks by about one day. These results generally supported the hemolymph peak data and also suggest that, at least in *Bombyx*, isolated brain–endocrine complexes might be released prematurely from negative controls that function *in vivo*. These hemolymph and *in vitro* secretion results are intriguing because they suggest that two to three releases of PTTH may not be ecdysteroidogenic, but might serve additional functions, such as "priming" the prothoracic gland to produce higher levels of ecdysteroids at a later time. This "priming" could represent a trophic effect of PTTH, in which transcription and translation of components of the ecdysteroidogenic synthetic pathway occur.

As was the case for the *Manduca* hemolymph PTTH data (Bollenbacher and Gilbert, 1981), the data obtained by Shirai *et al.* (1993) must be interpreted carefully for several reasons. First, the hemolymph and incubation medium assays measured total PTTH activity, but did not determine if the activity stems from PTTH and/or from other molecules such as small PTTH or bombyxin. Second, the peaks of PTTH activity obtained from both the hemolymph extracts and the brain-endocrine incubations were surprisingly similar in height, i.e., PTTH activity/ml, during the fifth instar, in contrast to the data obtained by Bollenbacher and Gilbert (1981). Third, because the *Bombyx* prothoracic gland is not responsive to PTTH at the beginning of the fifth instar (see Sections 3.2.3.3 and 3.2.5.1), a release of PTTH in the first day or two of the fifth larval instar of *Bombyx* would not be expected to result in a corresponding ecdysteroid peak. However, the lack of an ecdysteroid peak *in vivo* in response to the PTTH activity peak measured around days four to five is surprising, and calls into question the extraction and incubation results.

The development of a highly specific antibody against *Bombyx* PTTH (Mizoguchi *et al.*, 1990) afforded an opportunity to develop a more facile and sensitive method to determine hemolymph PTTH titers. Dai *et al.* (1995) and Mizoguchi *et al.* (2001, 2002) used this antibody in a time-resolved fluoroimmunoassay to study *Bombyx* PTTH hemolymph titers during larval–pupal and pupal–adult development. In addition, Dai *et al.* (1995) utilized this anti-PTTH antibody to assess the PTTH content of brain–retrocerebral complexes, using immunogold electron microscopy.

In the first of these three studies (Dai *et al.*, 1995), the hemolymph PTTH titer was moderately high at the beginning of the fourth instar and reached a minimum about 36 h later (scotophase of day 1, fourth instar (IV_1)). A larger peak ensued, with a slight dip in the photophase of IV_2, with a minimum again just before the molt to the fifth instar. The fifth instar PTTH titer exhibited a small but distinct peak during the first photophase on V_0 with minima 12 h later (V_1) and just at the larval–pupal molt (V_9–P_0). In between the two minima, hemolymph levels climbed fairly steadily to a plateau from V_6 through V_8, at which point a rapid decline to the molt minimum began. The parallel determinations of hemolymph ecdysteroid titer and PTTH antibody reactivity in the corpus allatum (the site of PTTH release; see Section 3.2.3.1) indicated that there was no obvious correlation between PTTH and ecdysteroid hemolymph titers, except during the fourth instar, when both of the PTTH peaks were associated with ecdysteroid peaks. The commitment and large premolt ecdysteroid increases of the fifth larval instar both occurred during the plateau period of high PTTH titer. In contrast, PTTH immunostaining in the corpus allatum was low during the early fifth instar peak (V_1) and during most of the later hemolymph plateau phase of PTTH titer; however, a striking transitory increase in PTTH immunostaining was seen on V_7, just before the rapid decline in hemolymph PTTH titer commenced.

Subsequent to these intriguing observations by Dai *et al.* (1995), it was discovered that the method used to extract the hemolymph for the PTTH fluoroimmunoassay included a hemolymph factor that contributed an unknown amount to the measured PTTH titer (Mizoguchi *et al.*, 2001). Consequently, Mizoguchi *et al.* (2001) repeated the determination of *Bombyx* hemolymph and ecdysteroid titers, using a revised hemolymph treatment protocol. This study also extended through the period of pupal–adult development and included a parallel ecdysteroid titer determination, as well as an analysis of brain PTTH content, using the time-resolved fluoroimmunoassay. Mizoguchi *et al.* (2002) provided a third determination of fifth instar and pupal–adult *Bombyx* hemolymph PTTH and ecdysteroid titers, using the revised fluoroimmunoassay, in conjunction with a study directed at discovering potential rhythmicity in PTTH secretion and the influence of diel light cycles. These two more recent studies contrast in several ways with the data obtained by Dai *et al.* (1995) and Shirai *et al.* (1993). Like Dai *et al.* (1995), Mizoguchi *et al.* (2001) found two hemolymph PTTH peaks in the fourth instar, but only the second one was accompanied by a clear-cut rise in hemolymph ecdysteroids. During the fifth instar, Mizoguchi and colleagues detected three rises and fall of hemolymph PTTH, with the instar ending in a period of rapidly increasing PTTH levels that carried on into pupal–adult development. These studies involved three different crosses of *Bombyx* races and four separate determinations (in the following section, the name of the female race in a cross is given first, and the male second). The earliest of these increases took place on V_4 and rose just slightly above background PTTH levels. Of the two crosses for which complimentary ecdysteroid titers are available, the *Kinshu* x *Showa* (Mizoguchi *et al.*, 2001) exhibited ecdysteroid peaks corresponding to all three PTTH rises (albeit the first ecdysteroid peak is very small). In the *J106* x *Daizo* hybrid (Mizoguchi *et al.*, 2002), only the second and third PTTH peaks are correlated with measurable ecdysteroid peaks, i.e., the small, presumptive commitment and the much larger premolt peaks. In

contradistinction to the associated ecdysteroid peaks, the relative heights of the second and third PTTH increases vary with the hybrid, i.e., the V_5 peak is distinctly higher than the V_7 peak in the *Kinshu* x *Showa* hybrid (Mizoguchi *et al.*, 2001) while in the *J106* x *Daizo* hybrid, the later peak (V_6) is much larger (Mizoguchi *et al.*, 2002). The immunoassay survey of fifth instar brain PTTH by Mizoguchi *et al.* (2001) revealed a period of gradual increase, starting from the first day of the instar and peaking on the fifth and sixth days, followed by a rapid decline to a minimum on the ninth day. PTTH levels then rose slightly on the ninth day and remained static through pupation on the following day.

Pupal–adult PTTH hemolymph titers were analyzed four times by Mizoguchi and colleagues and indicated one or two high peaks of concentration extending over the first half of the stage followed by a period of much lower levels and ending with a variety of levels, low, intermediate, or high, depending on the cross (Mizoguchi *et al*., 2001, 2002). The two parallel ecdysteroid titers exhibited much less variation in temporal patterning. A large, fairly symmetric peak was centered about 48 h after pupal ecdysis and ecdysteroid levels declined rapidly and evenly to very low concentrations by the time of adult ecdysis. The major difference between the two pupal–adult ecdysteroid titers was that of concentration. The *Kinshu* x *Showa* racial hybrids reached levels of more than 7 µg equivalents per ml (Mizoguchi *et al*., 2001) while the *J106* x *Daizo* hybrids peaked at only about 1.5 µg equivalents per ml (Mizoguchi *et al*., 2002).

The five immunologic determinations of *Bombyx* PTTH hemolymph titers, and the three of ecdysteroids, made by Dai *et al*., 1995 and Mizoguchi *et al.* (2001, 2002) paint a fairly similar picture of circulating PTTH levels in *Bombyx*, yet there are several important discrepancies among the results that need to be resolved. First, the small yet distinct early ecdysteroid peak seen in the fourth instar by Dai *et al*. (1995), occurring in consort with the first of the two PTTH rises, was not consistently found in other assays of fourth instar ecdysteroid titers, e.g., possibly seen by Kiguchi and Agui (1981), but not by Gu and Chow (1996) or Mizoguchi *et al*. (2001). Verifying, or disproving, the existence of this ecdysteroid increase is necessary to understand the role(s) of PTTH at this time. Second, the status of hemolymph PTTH levels at the beginning of pupal–adult life is not clear. In two of four assays, a broad peak of hemolymph PTTH was found in early pupal–adult life centered on day 2 (*Kinshu* x *Showa*: Mizoguchi *et al*., 2001; *Showa* x *Kinshu* raised at 25 °C: Mizoguchi *et al*., 2002). In a third profile (*Showa* x *Kinshu* raised at 25 °C: Mizoguchi *et al*., 2002), a peak on the second day after pupal ecdysis was followed by an approximately 2-day plateau of intermediate values, before finishing the last half of pupal–adult life with lower levels but with considerable variation, especially in the 36 h before adult ecdysis. In the fourth instance (*J106* x *Daizo*: Mizoguchi *et al*., 2002), a high, narrow peak of hemolymph PTTH occurred on the first day after ecdysis, followed by a second, broader, peak about 36 h later. A third topic of concern is the level of PTTH circulating at the end of pupal–adult development. Mizoguchi *et al.* (2001) reported an adult ecdysis peak (≈250 pg rPTTH equivalents) that was as high as that seen at the beginning of pupal–adult development, when ecdysteroid levels are also very high. In contrast, PTTH hemolymph titers in the other three pupal–adult studies were only 25% to 50% of the earlier pupal–adult peaks (Mizoguchi *et al*., 2002).

It is not possible currently to assess the reasons for the observed large temporal and concentration variations in PTTH titer. Multiple racial hybrids of *Bombyx* were used and some data came from animals raised on mulberry leaves (Shirai *et al*., 1993), rather than on an artificial diet. The length of the fifth instar ranged from 8 to 12 days while pupal–adult development varied from 8 to 11 days. The possibility that further stage-specific or hybrid-specific factors are influencing the PTTH titer data cannot be ruled out. However, since hybrid-specific differences in ecdysteroid titers were also found, especially in the peak concentrations, and ecdysteroid assays are well standardized, it seems likely that the PTTH titer variations are mainly biologically based. Given this assumption, it is possible to present an average or summary picture of PTTH and ecdysteroid hemolymph titers in *Bombyx* (**Figure 6**). Several conclusions can be drawn from these data. First, it is clear that PTTH increases do not always result in a detectable increase in hemolymph ecdysteroid concentrations and these results suggest that the gland was nearly or absolutely refractory to PTTH stimulation *in vivo* at times when a response was easily detected *in vitro*, e.g., V_6 and V_9 (Okuda *et al*., 1985). This disparity suggests that the prothoracic gland may be subject to negative controls at some stages and that this inhibition of activity is lost when the glands are placed *in vitro*. Another obvious inference stemming from these observations is that PTTH might stimulate nonsteroidogenic events at these times, perhaps in tissues other than, or in addition to, the prothoracic gland.

A second conclusion that originates from the examination of the hemolymph titers of PTTH and

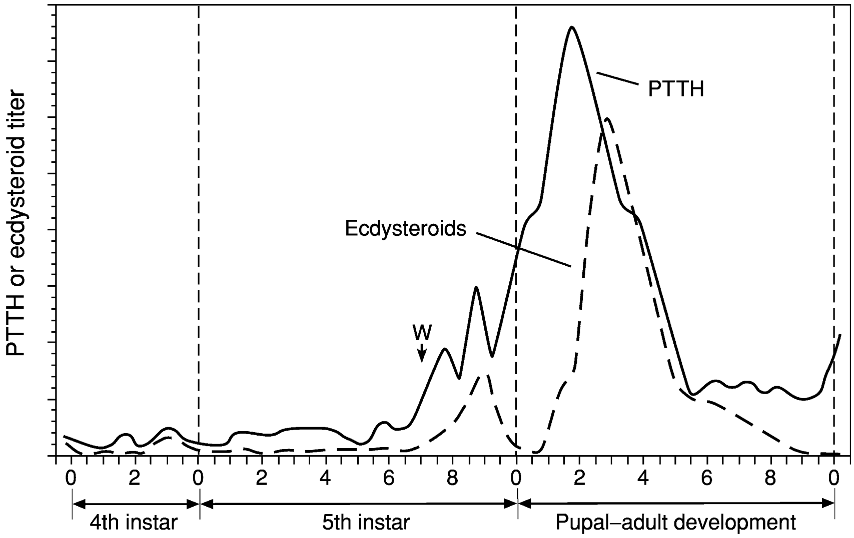


Figure 6 Typical PTTH and ecdysteroid titers in *Bombyx mori* hemolymph. Values are a composite of determinations from Dai *et al.* (1995) and Mizoguchi *et al.* (2001, 2002) and any individual, original profile may vary notably from the constructed profile shown here. W indicates wandering stage.

ecdysteroids is that there might be a negative correlation between peak concentrations of PTTH and peak concentrations of ecdysteroids during pupal–adult development. Thus, during this stage the *Kinshu* x *Showa* hybrid experienced a maximum titer of PTTH ≈270 pg rPTTH equivalents/ml while the associated ecdysteroid peak was ≈7000 ng 20E equivalents/ml (Mizoguchi *et al.*, 2001); in contrast, in the *J106* x *Daizo* hybrids, the temporally comparable peaks were ≈670 pg rPTTH/ml equivalents and ≈1500 ng 20E equivalents/ml, respectively (Mizoguchi *et al.*, 2002).

Third, while peak concentrations of PTTH are not good predictors of pupa–adult ecdysteroid levels, a suggestive two point (!) concordance does occur in the fifth instar. The maximum fifth instar PTTH titer in the *Kinshu* x *Showa* hybrid (≈90 pg rPTTH equivalents/ml) was about half that seen in the *J106* x *Daizo* hybrid, but the peak ecdysteroid level in the *Kinshu* x *Showa* hybrid was more than twice that of the *J106* x *Daizo* cross (≈1600 ng and ≈600 ng 20E equivalents/ml, respectively) (Mizoguchi *et al.*, 2001, 2002).

Fourth, in the *J106* x *Daizo* hybrids, a large temporally narrow peak of PTTH (≈880 pg rPTTH equivalents/ml) occurred immediately after larval–pupal ecdysis, which was not associated with an ecdysteroid peak and which was not seen in the other PTTH titer profiles (Mizoguchi *et al.*, 2001, 2002). The failure to see a corresponding ecdysteroid peak may not be surprising, given evidence from *in vitro* studies indicating that the prothoracic gland might be refractory to PTTH stimulation during the period of larval–pupal ecdysis (Okuda *et al.*, 1985). What this peak does suggest is that our understanding of factors involved in PTTH release and metabolism is rudimentary. One missing factor in this equation may well be juvenile hormone, the values of which were not addressed in these studies, but other hormones and factors are also likely involved.

Finally, the variation found in PTTH and ecdysteroid titers and in developmental rates, originating from methodologically similar studies (Dai *et al.*, 1995; Mizoguchi *et al.*, 2001, 2002), clearly indicate that more data are desirable from *Bombyx* and from other insects such as *Manduca*. Only then will we obtain a reliable picture of how this important piece of the insect endocrinological system functions. The open questions are many. For instance, how much does the magnitude of a PTTH peak matter? Do very high levels have unique biological effects on the prothoracic glands, or do they simply delay processes that depend on low PTTH levels for their expression or regulation? What are the targets and consequences of the orphan peaks, i.e., PTTH increases that do not elicit ecdysteroidogenesis? What is the role of PTTH in late pupal–adult development and during adult life, when prothoracic glands are generally absent due to programmed cell death?

3.2.3.5. PTTH: Rhythms and Cycles

The above discussion of PTTH hemolymph titers has concentrated on the issues of the timing and magnitude of stage-specific peaks. However, a number of studies have yielded data that showed that some PTTH peaks are "gated," that is, occur only within a narrow window within a 24 h light–dark

cycle. Some evidence also suggests that a circadian variation in PTTH titer is overlaid upon the broader sweeps of changing PTTH levels. Steel and Vafapoulou (see **Chapter 3.11**) cover the subject of circadian rhythms in detail (see also Giebultowicz, 2000; Steel and Vafapoulou, 2002; **Chapter 4.11**); only a brief treatment is given here.

Head critical period (HCP) is a term used to refer to a developmental period before which the presence of the head (brain) is necessary for later events to transpire. In the case of ecdysteroid-triggered events, the HCP has generally been used as a marker for PTTH release. Studies of the HCP for molting provided the first data indicating that an endocrine-dependent event in insect development involved circadian cycles (Truman, 1972). This work revealed that larval molting and the HCP for molt initiation in *Manduca* and *A. pernyi* were temporally gated. Since this HCP and ecdysis are ultimately controlled by PTTH, via ecdysteroid levels, these observations implicated the involvement of a circadian clock in PTTH release. Further work in *Manduca* (Truman and Riddiford, 1974) demonstrated that the PTTH release responsible for inducing wandering in the last larval instar, as revealed through HCP timing, was also temporally gated, again indicating the participation of a circadian clock. In *Samia cynthia ricini*, the HCP for gut purging was also gated (Fujishita and Ishizaki, 1982) but not every HCP is necessarily under the control of a photoperiodically controlled circadian clock. For instance in *Samia*, in contrast to the HCP for gut purging, the HCP for pupal ecdysis, which follows wandering by 2 days, was unaffected by shifts in the photoperiod conditions and appeared to be a constant (≈96 h) (Fujishita and Ishizaki, 1982). Light-entrained circadian rhythms in endocrine-dependent events are not confined to the Lepidoptera, of course, and have been studied in a number of other taxa, such as the Diptera. For instance, in *S. bullata* (Roberts, 1984) and *S. argyrostoma* (Richard *et al*., 1987) larval wandering is under strong photoperiodic control. A similar result was seen with *D. melanogaster*, if they were raised under long day (16 : 8) and low-density conditions (Roberts *et al*., 1987).

More recent work on *Rhodnius prolixus* and the recent surveys of PTTH hemolymph titers have suggested that there might be diel variations in titer, and presumably PTTH release, which are superimposed upon the larger peaks and plateaus of circulating PTTH levels. In *Bombyx*, data from the fifth larval instar and from pupal–adult development (Sakurai *et al*., 1998; Mizoguchi *et al*., 2001) suggested that there may be such a daily cycling of PTTH hemolymph levels; a similar suggestion was seen in the *in vitro* secretory activity of prothoracic glands from the last 4 days of the fifth instar (Sakurai *et al*., 1998). To examine this possibility more directly, Mizoguchi *et al*. (2001) phase shifted two groups of initially synchronous *Bombyx* larvae on the first day of the fifth instar, such that their light–dark periods were displaced by 12 h from one another. After 5 days, both groups exhibited a peak in PTTH hemolymph titer that began at the scotophase-photophase transition and peaked during the photophase, supporting the hypothesis that light–dark cycles influence some periods of PTTH release. However, the careful temporal analysis of PTTH titers afforded by this study did not indicate that every apparent PTTH peak was so influenced. In both phase-shifted populations, a second PTTH peak was observed that varied between the two groups in time of appearance (30 h versus 48 h), with one of the following peaks occurring during the scotophase and the other during the photophase. Thus, this study did not support the notion of small daily PTTH peaks.

The strongest and most direct evidence for circadian rhythms in PTTH levels and consequent ecdysteroid hemolymph levels comes from studies of the bug *R. prolixus* and the cockroach *P. americana*. In *Rhodnius*, during the last two-thirds of the final larval instar, PTTH synthesis and release appears to be controlled by a circadian clock, with peaks occurring during the scotophase (Vafopoulou and Steel, 1996; see **Chapter 3.11**). The implications of this rhythmicity for ecdysteroid synthesis are not straightforward, as studies *in vitro* revealed that the *Rhodnius* prothoracic gland exhibited circadian cycling of ecdysteroid synthesis in the absence of PTTH, although with a phase shift to peaks during the photophase (Pelc and Steel, 1997). Steroid synthesis in animals that had been either decapitated or paralyzed with tetrodotoxin, to ablate PTTH release, exhibited a rhythmicity similar to that seen *in vitro*, and the composite results indicate that PTTH is an important entraining factor for the circadian clock regulating ecdysteroid synthesis. The endogenous clock of the prothoracic gland is light entrained, specifically by a “lights-off” cue (Vafopoulou and Steel 1998, 2001). A “lights-off” cue also functions in controlling PTTH release, as shown in experiments where PTTH release was abolished under continuous light but promptly restored after transfer to dark conditions (Vafopoulou and Steel 1998, 2001). Further complexity in the control of ecdysteroidogenesis by PTTH in *Rhodnius* was indicated by the discovery that prothoracic glands also express a daily rhythm in PTTH responsiveness, i.e., PTTH receptivity is gated (Vafopoulou and Steel, 1999).

In the cockroach *P. americana*, isolated prothoracic glands did not exhibit a circadian cycling of ecdysteroid synthesis *in vitro* when they had been extirpated from stages that exhibit daily cycles of ecdysteroids in the hemolymph, i.e., the last several days of the last larval instar (Richter, 2001). However, if the *Periplaneta* glands were co-cultured with brains taken from such late instar animals, then a diel rhythm in ecdysteroidogenesis resulted, with a peak in the scotophase matching the *in vivo* hemolymph pattern (Richter, 2001). As discussed earlier (Section 3.2.3.2), melatonin dramatically increased the secretion of PTTH from brains *in vitro* during a scotophase, with lesser increases seen during a photophase (Richter, 2001). These data suggested that PTTH release might be, at least partially and at only some stages, regulated by melatoninergic neurons. This PTTH cycling would result in a circadian variation in ecdysteroid synthesis and hemolymph titer. Nonetheless, further support for this hypothesis has yet to be marshaled. In particular, a demonstration of PTTH cycling *in vivo*, preferably in the hemolymph titer, would be supportive because factors other than PTTH might be driving prothoracic gland cycles, e.g., input from nerves that synapse with the prothoracic gland in *Periplaneta* (Richter, 1985).

The existence of light-dependent cyclic or rhythmic behavior by an organism or a tissue or single cell indicates the presence of a light-sensing system as well as an actual molecular-based clock. In cases where isolated brains or prothoracic glands cyclically release hormones under *in vitro* conditions, the sensing apparatus and clock(s) must reside within some or all of the cells under study. A light-sensing system implies the presence of a photoreceptive protein or a photopigment coupled to a transduction system. In the instance of PTTH and ecdysteroid cycling observed *in vitro* in *Rhodnius* tissues, such a sensing system must be extraretinal. In *Drosophila*, one such protein is known, cryptochrome, which absorbs in the blue, and functions as an extraocular photoreceptor in the lateral neurons of the brain (see Stanewsky, 2002, 2003). In a series of clever experiments involving gland transplantation, selective illumination of larval regions and phase-shifting of gut purge, Mizoguchi and Ishizaki (1982) showed that the prothoracic gland of *Samia cynthia* possesses both a photoreceptor and an endogenous clock; it seems likely that a cryptochrome is the photoreceptor in this system.

The actual cellular molecular clock has been studied extensively in *Drosophila* where the central oscillator is formed by a complex feedback loop involving the transcription, translation, and phosphorylation of at least seven genes: *period*, *timeless*, *clock*, *cycle*, *doubletime, vrille*, and *shaggy* (Stanewsky, 2002, 2003) (see **Chapter 4.11**), and possibly *cryptochrome*, which appears to function as part of the "central oscillator" in some peripheral tissues (Krishnan *et al*., 2001). The presence of cryptochrome in the prothoracic glands has not been determined but the *period* and *timeless* proteins have been immunologically detected in the *Rhodnius* gland (Terry and Steel, cited in Steel and Vafapoulou, 2002) and in the prothoracic gland portion of the *Drosophila* ring gland (Myers *et al*., 2003). At least some of the proteins involved in the *Drosophila* central oscillator shuttle between the nucleus and the cytoplasm, and cycles of abundance of both protein and mRNA are characteristic. For a more thorough understanding of these processes, (see **Chapters 3.12**, **4.11**, and Saunders (2002)).

The expression of the *period* protein in insect brains has been the subject of several immunohistochemical studies in insects other than *Drosophila*. In the *Rhodnius* brain, a small group of neurons adjacent to the prothoracicotropes (one per hemisphere) were immunopositive for the *period* and *timeless* proteins (Terry and Steel, cited in Steel and Vafapoulou, 2002). Sauman and Reppert (1996a) found that two pairs of dorsolateral neurons, per hemisphere, were immunopositive for *period* in the brain of the moth *A. pernyi*, and that the cell bodies were in contact with the two pairs of dorsolateral neurosecretory cells that were immunopositive for PTTH. Interestingly, the *period* positive cells sent their axons to the corpus cardiacum whereas PTTH-producing neurons course contralaterally to terminate in the corpus allatum. Thus, if the PTTH- and period-producing cells communicate with one another it is likely to be at the level of the cell bodies, perhaps via tight junctions, or in the corpus cardiacum, where the "*period* cells" could transmit information to boutons on the axons of the "PTTH neurons." Interestingly, only cytoplasmic and axonal *period* staining was observed in the *Antheraea* brain (Sauman and Reppert, 1996b); the expected nuclear presence was not seen, although temporal cycling of protein and mRNA was observed. Závodská *et al*. (2003) surveyed the cephalic nervous systems of 14 species of insects, representing six orders, using immunohistochemical methods and antibodies against *period*, PTTH, and pigment-dispersing hormone (PDH, also known as pigment-dispersing factor), as well as eclosion hormone. PDH-expressing neurons are also important in circadian processes, although their role appears to be information transfer between cells rather than direct participation in the intracellular molecular

clock oscillator (Stengl and Homberg, 1994; Helfrich-Förster *et al*., 2000). A detailed recapitulation of the data obtained in this study is beyond the purposes of this review but a few results are worth noting. First, *period* was widely distributed, with most species showing antibody reactivity in cells of the optic lobe, pars intercerebralis, and the dorsolateral protocerebrum. Second, *period* staining was limited to the cytoplasm. Third, PDH exhibited the most consistent immunoreactivity, being found in the optic lobes of all 14 species. Finally, none of the cells immunopositive for any one of the four antigens was also immunopositive for any of the other three antigens. Thus, to the extent that these four proteins play a role in circadian cycles, the data suggest that such cycles are controlled, at least within the brain, by a nexus of cells rather than a discrete nucleus.

Obviously, we are currently still in the early stages of understanding the molecular and physiological mechanisms that control cyclical PTTH release. The studies reviewed here, as well as others, suggest that there are several ways in which the PTTH-prothoracic gland axis may entrain rhythms in steroid levels. It is not yet possible to say whether these varied circadian systems sort out along taxonomic, or ecological, or other grounds. Furthermore, we know essentially nothing about the removal or degradation of PTTH from the insect circulation. Changes in the PTTH hemolymph titer must be regulated not only by synthesis and release, but also by processes of sequestration and/or proteolysis. Whether or not there are PTTH-specific uptake, elimination, or proteolytic pathways is totally unknown, but it seems unlikely that the prothoracic gland will be the only tissue found to be involved in these processes. More work on these problems is clearly needed, involving not only “modern” molecular genetics, but “old-fashioned” biochemistry and physiology as well.

3.2.3.6. PTTH and Diapause

All organisms face variations in their environment, and even in the most equable habitats, confront periods during which they must alter their behavior and/or their physiology to cope with stressful conditions. When such nonfavorable circumstances are neither too severe nor prolonged, behavioral changes or simple homeostatic mechanisms are generally evoked. When these changes are harsher, such as nonseasonal drought or the protracted cold of temperate zone winters, then more sweeping actions are taken. At the physiological level, insects and many other arthropods deal with unpredictable environmental challenges by becoming quiescent. Simple quiescence involves rapid and temporary cessation of activity and the animal quickly resumes its activity when the hostile conditions cease. When the adverse conditions are relatively predictable, e.g., winter or seasonal drought, then the solution of most insects is to enter diapause (see **Chapter 3.12**). Diapause is a type of dormancy that involves perception of one or more predictable environmental cues that anticipate and predict regularly occurring, unfavorable conditions. Animals in diapause express physiological mechanisms that greatly increase their ability to resist environmental challenges that range from desiccation to freeze-damage resistance. The cues that trigger diapause are not necessarily the conditions that the behavior has evolved to escape or moderate, e.g., organisms that enter a winter diapause state may be stimulated to do so by shorter day length rather than by falling temperatures. Diapause involves a genetically determined response that is controlled by the neuroendocrine system. Diapause is obligate or facultative, and undergone by embryos, larvae, pupae, or adults, depending on the species. For broader views of diapause than presented here, see Tauber *et al.* (1986); Danks (1987); and **Chapter 3.12**.

The PTTH-ecdysteroid axis has been implicated in the control of both larval and pupal diapause. *Manduca* larvae molt into diapausing pupae after being raised under a short-day (e.g., 12 h) photoperiod. Bowen *et al.* (1984a) found that such animals exhibit a much reduced hemolymph ecdysteroid titer relative to developing pupae. Low ecdysteroid titers have been documented in other Lepidoptera and other taxa that undergo a pupal diapause, e.g., the moths *H. cecropia* (McDaniel, 1979) and *Heliothis virescens* (Loeb, 1982), and the flesh flies *Sarcophaga crassipalpis* (Walker and Denlinger, 1980) and *S. argyrostoma* (Richard *et al*., 1987). Bowen *et al.* (1984a) showed that two factors resulted in the low levels of circulating ecdysteroids during diapause. First, diapause prothoracic glands were found to be refractory to PTTH stimulation when tested *in vitro*. Smith *et al*. (1986a) found that the refractory state in diapausing pupae could be simulated in nondiapausing pupae by removal of the brain, indicating that the presence of a brain-derived factor is necessary to retain prothoracic gland responsiveness. PTTH is, of course, a prime candidate to function in such a trophic context (see Section 3.2.3.6) but a role for other hormones cannot be ruled out. Smith *et al.* (1986a) also determined that the refractory “blockade” in the *Manduca* prothoracic gland is downstream from the PTTH receptor, Ca^{2+}-influx, and cAMP generation (see Section 3.2.3.4 for a discussion of the PTTH transduction cascade). The use of a calcium

ionophore and a cell-permeable cAMP analog both failed to stimulate ecdysteroid synthesis in the diapause glands, although these agents are as effective as PTTH when applied to nondiapause glands. However, PTTH stimulated cAMP accumulation within diapause glands, in fact to levels higher than seen under nondiapause conditions. This observation indicates that there is not also a block upstream from cAMP generation, such as a downregulation in the numbers of PTTH receptors or adenylate cyclase molecules. Similar data, indicating a signal transduction lesion downstream from cAMP generation, were obtained by Richard and Saunders (1987), studying diapause in the flesh fly *Calliphora vicina*. Basal protein kinase A activity in *Manduca* day 10 diapause glands was ≈20% lower than that seen in nondiapause day 0 pupal glands, but both pupal and diapause protein kinase activity approximately doubled in response to dibutyryl cAMP (Smith *et al*., 1987a). Thus, lowered protein kinase A activity is likely contributory to the refractory nature of diapause prothoracic glands but other factors are undoubtedly involved.

Bowen *et al*. (1984a) suggest that the second reason for low ecdysteroid levels in diapause pupae is the absence of PTTH. These workers found that, in *Manduca*, during the first 20 days of diapause, the content of PTTH in the brain or in the brain–retrocerebral complex did not differ significantly from that in nondiapausing, developing pupae. Bowen and colleagues construed these data to indicate that PTTH release was greatly curtailed during diapause but the interpretation of this result is, unfortunately, not straightforward since it is possible that PTTH catabolism in the hemolymph was increased; note that the hemolymph PTTH titer was not measured in this case. In fact, Bowen *et al*. (1986) studied the release of PTTH *in vitro* from *Manduca* brain-retrocerebral complexes and found no difference between complexes from nondiapause and diapause pupae. PTTH release *in vitro* from diapause complexes could have been an artifact stemming from dissection or incubation conditions, or might indicate a release from an *in vivo* inhibitory factor that is lost or inhibited itself under the *in vitro* regimen. If PTTH release is indeed inhibited *in vivo* in diapausing pupae, then data from nematodes may provide a clue to one step in its control, i.e., Tissenbaum *et al*. (2000) found that the diapause-like dauer state of *C. elegans* and *Ancylostoma caninum* was broken rapidly by muscarinic agents. Furthermore, dauer recovery was inhibited by atropine, a muscarinic antagonist. This finding is of note because both Agui (1989) and Shirai *et al*. (1994) showed that cholinergic agents caused the release *in vitro* of PTTH from lepidopteran larval complexes. A dopaminergic pathway may also play a role in diapause, but in contrast to the muscarinic system, dopamine may be involved in inducing or maintaining diapause (see **Chapter 3.12**). Some insects that undergo pupal diapause may exhibit a more complex control of the PTTH-prothoracic gland axis. Williams (1967) was not able to detect PTTH activity in brains from diapausing *H. cecropia* or *Samia cynthia*, in contrast to *A. pernyi*. These data suggest that the stronger diapause in *Hyalophora* and *Samia* is characterized by a period of very low PTTH levels in the brain; whether or not the prothoracic glands are refractory to stimulation at these times was not investigated.

Recently, Xu and Denlinger (2003) studied the levels of PTTH mRNA in brains of *H. virescens* nondiapause and prediapause larvae, and nondiapause and diapause pupae. They found that mRNA levels in nondiapause animals remained relatively constant through the fifth instar until about day 10 of pupal–adult development. However, in prediapause larvae, PTTH mRNA levels dropped at the onset of wandering and, in diapause pupae, the mRNA levels remained low through at least the first 10 days of diapause. These results may reveal the mechanism behind the low PTTH levels seen in diapausing *H. cecropia* or *S. cynthia* pupae, as reported by Williams (1967).

Data from *Manduca* indicate that the PTTH-producing neurosecretory cells undergo morphological and electrophysiological changes during diapause. Hartfelder *et al*. (1994) found no significant differences in cellular ultrastructure between developing pupae and early diapause pupae. However, as diapause progressed, a number of cellular structures exhibited changes, e.g., neurosecretory granules were concentrated into large clusters that were separated by well-organized rough endoplasmic reticulum, notably fewer Golgi complexes were seen, and interdigitations with glial cells were decreased. When diapause was terminated by exposure to warm conditions (26 °C versus 4 °C), the PTTH cells reverted to prediapause morphology within 3 days. Progressive differences in electrophysiological properties between diapausing and developing pupae were found by Tomioka *et al*. (1995), who noted that the diapausing PTTH cells gradually became less excitable. This relative refractoriness was caused by a rise in the threshold value for action potential generation and a decrease in the input resistance.

The endocrine control of larval diapause appears to be more varied than pupal diapause. In

the European corn borer, *O. nubilalis*, larval diapause is similar to *Manduca*. Hemolymph ecdysteroid titers are significantly lower in prediapause larvae than in developing animals (Bean and Beck, 1983) and brain PTTH levels were the same in diapause and nondiapause animals, for at least the first 4 weeks of diapause (Gelman *et al*., 1992). Gelman and colleagues also found that diapause prothoracic glands were highly refractory to PTTH stimulation, as seen in *Manduca*. It must be pointed out that although these results are very reminiscent of the *Manduca* findings, the only PTTH activity detected in diapause brains of *Ostrinia* had an apparent MW of ≤ 5 kDa (Gelman *et al*., 1992). In contrast, the south-western corn borer, *Diatraea grandiosella*, which undergoes up to three stationary molts during diapause, appears to maintain an active PTTH-prothoracic gland axis during larval diapause; its diapause is controlled by high JH levels (Chippendale and Yin, 1973; Yin and Chippendale, 1973; Chippendale, 1984) (see **Chapter 3.12**). High JH levels are also responsible for provoking and sustaining larval diapause in the rice-stem borer, *Chilo suppressalis* (Yagi and Fukaya, 1974); the exact state of the PTTH-prothoracic gland axis during larval life in this species appears to be unknown.

In larval and pupal diapause that are characterized by low ecdysteroid levels, the available evidence suggests that the relative quiescence of the prothoracic glands reflects decreased PTTH release and stimulation. This in turn suggests that the photoperiodic clock involved in the control of PTTH in short-day versus long-day developmental programs might reside in the brain, where PTTH is synthesized. This hypothesis was elegantly addressed with experiments that involved *in vitro* reprogramming of larval brains with altered light cycles (Bowen *et al*., 1984b). In brief, brains from short-day larvae were exposed to a long-day light treatment for 3 days *in vitro* and then implanted into short-day larvae destined to enter diapause. Approximately half of these host larvae did not enter diapause, i.e., the implanted brains reversed the diapause program. If the transplanted brains received a short-day light treatment *in vitro* prior to implantation, then very few host larvae failed to enter diapause. The composite data showed that the three main regulators of pupal diapause in *Manduca* reside in the brain: photoreceptor, clock, and hormonal effector. The main hormonal actor in this developmental program is clearly PTTH, but other factors might well be involved (see **Chapters 3.11** and **3.12**). For instance, brains in animals destined to diapause might secrete an ecdysiostatic factor to downregulate prothoracic gland activity in addition to curtailing PTTH release after pupal ecdysis (see Section 3.2.5.2).

The literature on diapause in insects is very large (see **Chapter 3.12**) and only a very small fraction of it has been reviewed here. Given the long evolutionary history of insects, it is likely that there are additional variations in the ways in which environmental cues, receptors, PTTH, and the prothoracic glands interact to induce or modulate diapause.

3.2.4. PTTH: Signal Transduction in the Prothoracic Gland

Hormones are intercellular messengers that alter the behavior of the target cells. PTTH has only one known target tissue, the prothoracic gland (the possibility that PTTH affects other tissues is discussed in Section 3.2.3.6), and the ultimate response of the gland to PTTH is to increase ecdysteroid production. This section considers the transduction changes in the prothoracic gland elicited by PTTH, and how these changes connect to increased ecdysteroidogenesis. The reader is referred to **Chapter 3.3** for a discussion of the actual synthetic pathway leading from sterols to ecdysteroids. The present discussion has been organized to present the biochemical events of PTTH signal transduction in a sequential or temporal order, so far as we currently understand them. Of course, as events proceed from the initial contact of PTTH with the prothoracic gland and the PTTH receptor, it becomes increasingly likely that multiple PTTH-dependent phenomena occur simultaneously; indeed, it would be a mistake to conceive of any signal transduction cascade as a linear, vectorial process, rather than a complex nexus of interconnected pathways. By using an "outside-in" cellular approach to this topic, a broad chronological view will not be presented, but within each of the subtopics, some history of the field will be afforded. An overview of the PTTH signal transduction pathway, as discussed below, is provided in **Figure 7**.

3.2.4.1. The PTTH Receptor and Associated Proteins

A PTTH receptor has not yet been purified or cloned, so the present section is speculation based on data primarily from *Manduca* and *Bombyx*, and using information gleaned from other characterized peptide receptor systems. PTTH is a circulating, hydrophilic protein and can be expected to bind to a receptor that exposes an extracellular binding site and traverses the cell membrane. PTTH binding to

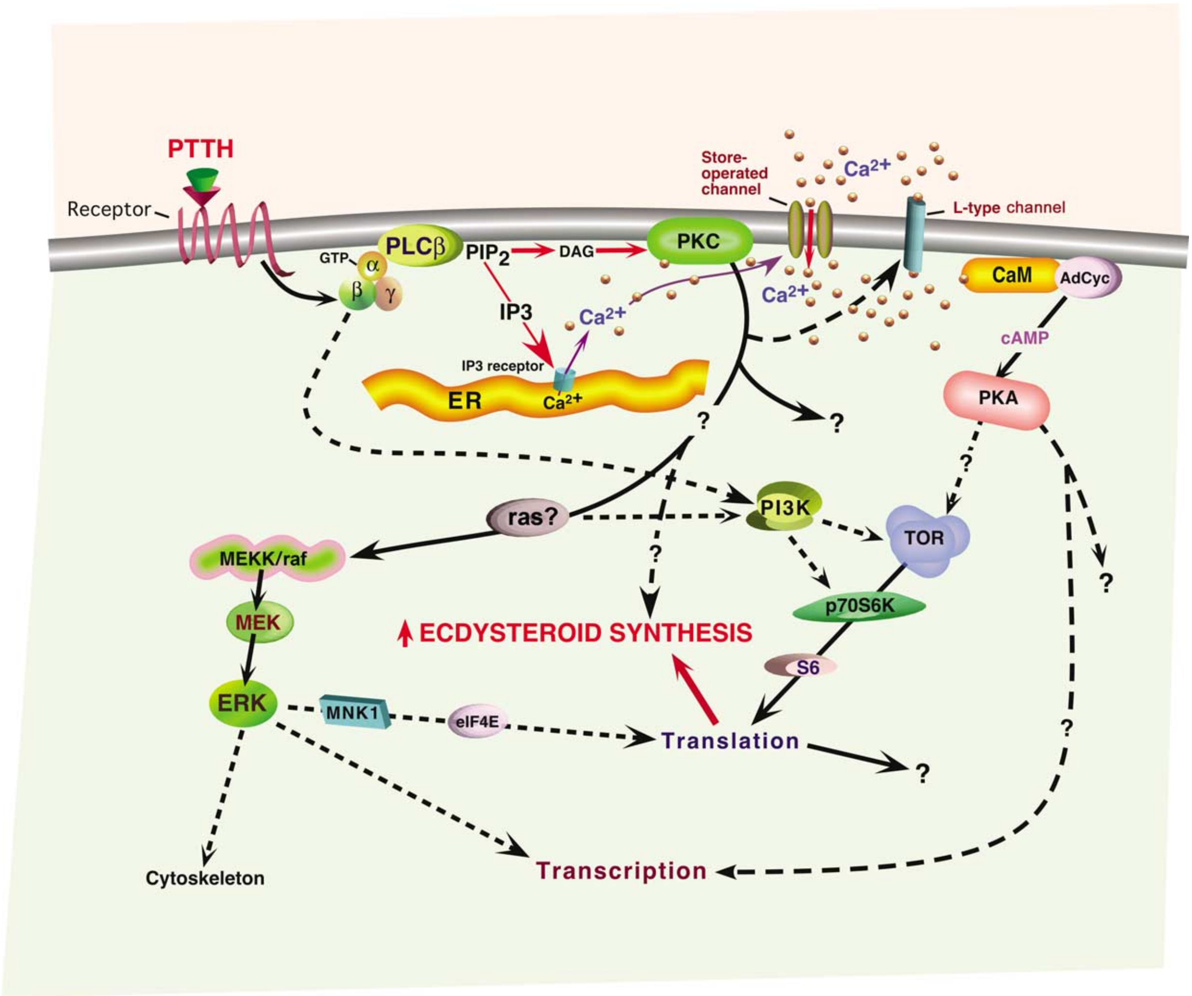


Figure 7 The PTTH signal transduction cascade in prothoracic gland cells. Solid lines indicate demonstrated or highly likely interactions while dashed lines indicate hypothetical relationships. Abbreviations: PTTH, prothoracicotropic hormone; PLCβ, phospholipase C β; PIP_2, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; CaM, calmodulin; AdCyc, adenylate cyclase; ER, endoplasmic reticulum; PKA, protein kinase A; PI3K, phosphoinositide 3OH-dependent kinase; TOR, target of rapamycin; MEKK, MEK kinase; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; S6, ribosomal protein S6; p70S6K, 70-kDa S6 kinase; MNK 1, MAP kinase-interacting kinase 1; ras, a small GTP binding protein; raf, raf serine-threonine kinase; and eIF-4E, eukaryotic translation initiation factor 4E.

the receptor is likely a high affinity interaction, with a K_D in the very low nanomolar or high picomolar range. Such an affinity is typical for peptide receptors and is also suggested by the findings that *Manduca* recombinant PTTH at ≈0.300 nM was sufficient to maximally stimulate ecdysteroidogenesis *in vitro* (Gilbert *et al*., 2000). More limited data from *Bombyx* also indicated that a similarly low concentration (≈1.7 nM) of *Bombyx* recombinant PTTH was sufficient to elicit maximal ecdysteroidogenesis *in vitro* (Dedos *et al*., 1999). Eukaryotic membrane receptors fall into several broad classes and it seem most likely that the PTTH receptor belongs to the G-protein coupled receptor (GPCR) superfamily (for an insect-oriented review, see **Chapter 5.5**). This is a very large group of proteins that bind a diverse array of ligands. GPCRs span the membrane seven times, with connecting intracellular and extracellular loops. The ligand-binding site is located in an extracellular region. Intracellular areas of the GPCRs interact with the heterotrimeric G-proteins, which are the first-level effectors of an activated ligand-bound GPCR. The direct and indirect targets of activated G-proteins, released from a GPCR by ligand-occupancy, are many and include adenylyl cyclase (see Pieroni *et al*., 1993), which generates the second messenger cAMP from ATP. PTTH evokes a rapid generation of cAMP in

prothoracic gland cells (see Section 3.2.4.2) and, currently, it appears that the generation of cAMP in response to an extracellular ligand almost always involves a GPCR. GPCRs range from <400 to ≈1000 amino acids in size, are glycosylated, and occur as hetero- or homooligomers (see Gazi *et al*., 2002).

The heterotrimeric G-proteins (α, β, and γ) that characterize GPCRs are a genetically diverse group. In both mammals and insects, a considerable number of Gα, Gβ, and Gγ proteins are expressed and the specific subunits that associate with a given GPCR determine to a large degree the particular signaling pathways activated by that GPCR. For example, $G\alpha_s$ stimulates adenylate cyclase activity and $G\alpha_q$ activates phospholipase C (see Offermanns, 2003) and some Gβγ subunits can also activate phospholipase C (see Boyer *et al*., 1994; Morris and Scarlata, 1997). The presence of G proteins in *Manduca* prothoracic glands was investigated by Meller and Gilbert (1990) using Western blotting and cross-linking techniques with antibodies against mammalian Gα and Gβ proteins. Their results suggested that several Gα and Gβ proteins are expressed in the glands and that they likely occur, as expected, in multimeric complexes. Meller and Gilbert also used an antibody against $G\alpha_S$ in an immunoprecipitation experiment and found that $G\alpha_S$ in the prothoracic gland was associated with adenylate cyclase. This observation is congruent with the proposal that the PTTH receptor is a GPCR but, obviously, the association of the G protein–cyclase complex with another receptor cannot be ruled out. Girgenrath and Smith (1996) studied the effects of pertussis and cholera toxins on PTTH-stimulated ecdysteroid synthesis in *Manduca* prothoracic glands; these toxins inhibit and stimulate, respectively, the activity of some G proteins. Pertussis toxin had no effect on PTTH-stimulated ecdysteroid synthesis, but cholera toxin stimulated ecdysteroid synthesis. Cholera toxin also stimulated ecdysteroid synthesis by *Bombyx* prothoracic glands (Dedos and Fugo, 1999c). These observations suggested that PTTH acts through a $G\alpha_s$ protein. Pertussis toxin also had no effect on mastoparan- or PTTH-stimulated Ca^{2+} influx in *Manduca* prothoracic glands (Birkenbeil, 2000).

Several additional pharmacological agents that are known to have effects on GPCR–G protein interactions have been used to investigate further the role of G proteins in PTTH signaling. Mastoparan, a wasp venom that activates G proteins among its other effects caused a dose-dependent increase in intracellular Ca^{2+} (Birkenbeil, 1999), but a dose-dependent decrease in ecdysteroid production in *Manduca* prothoracic gland cells (Birkenbeil, 2000). Injection of GDP-β-S, an inhibitor of G protein activation, failed to block PTTH-stimulated Ca^{2+} influx; PTTH-stimulated ecdysteroid synthesis was not measured in this context (Birkenbeil, 2000). In contrast, the compound suramin, which uncouples G proteins from GPCRs among other effects, blocked PTTH-stimulated Ca^{2+}influx in *Bombyx* (Birkenbeil, 2000) while in *Manduca*, suramin inhibited PTTH-stimulated ecdysteroid synthesis (Rybczynski and Gilbert, unpublished data). The collective data from these laboratories suggest that the PTTH is coupled to a G protein and that the prothoracic gland expresses a number of Gα proteins, at least one of which is not coupled to the PTTH receptor. The supposition that prothoracic glands express multiple Gα proteins is in accord with the immunoblotting results of Meller and Gilbert (1990) discussed above and it is possible that PTTH receptors couple to more than one type of G protein.

If the PTTH receptor is a GPCR, then a number of proteins, besides the G proteins, likely associate with the receptor, at least at some times. These include proteins that are involved in receptor desensitization, such as a PTTH receptor-specific kinase, second messenger kinases (like cAMP-dependent protein kinase A), regulators of G protein signaling (RGS proteins) and arrestins (which bind phosphorylated receptors). If the PTTH receptor undergoes agonist-induced internalization, then proteins comprising the relevant endocytic pathway, e.g., clathrin, and subsequent degradative or recycling pathways would be periodically associated with the receptor. Desensitization and receptor cycling are complex topics. Another fascinating group of GPCR-interacting proteins is the RAMP (receptor activity modifying proteins), a family of proteins identified in vertebrates (see Morfis *et al*., 2003). RAMPs contain a single transmembrane domain, heterodimerize with GPCRs, and the specific RAMP in the heterodimer can determine the ligand recognized by the receptor complex. Developmentally specific RAMP associations with a PTTH receptor could explain the puzzling decrease in the ability of small PTTH to stimulate *Manduca* pupal glands (see Section 3.2.2.5). Naturally, such an interaction is purely hypothetical at this point, given that a search of the *Drosophila* genome database did not reveal any obvious RAMP homologs (unpublished data).

Further speculation on the PTTH receptor and associated proteins is premature at this time and the field will only progress once an authentic receptor has been isolated and characterized. The

field of membrane receptors is a vast one and for detailed discussions on GPCRs, the interested reader should see reviews by Böhm *et al.* (1997); Vázquez-Prado *et al.* (2003), and **Chapter 5.5**, among many others.

3.2.4.2. Second Messengers: Ca^{2+}, cAMP, and Inositols

The activation of GPCRs results in a transient increase of second messenger molecules within the cell. These small molecules carry and expand the intracellular "reach" of the receptor ligand, the first messenger, by binding to numerous target molecules. Ca^{2+} is perhaps the most common of second messengers. The concentration of free Ca^{2+} in the cytoplasm is well buffered and transient increases alter the behavior of Ca^{2+}-binding proteins. Increases in Ca^{2+} can originate from the extracellular environment as well as from internal stores, such as those in the endoplasmic reticulum (see Berridge *et al.*, 2000). Smith *et al.* (1985) found that extracellular Ca^{2+} was crucial for PTTH-stimulated ecdysteroid synthesis in *Manduca* prothoracic glands and that Ca^{2+} influx was a very early event in the PTTH transduction pathway, i.e., cAMP analogs that stimulate ecdysteroid synthesis are still effective in the absence of extracellular Ca^{2+}, indicating that Ca^{2+} influx precedes cAMP synthesis (see below). Smith *et al.* (1985) reported also that basal ecdysteroidogenesis was insensitive to the presence or absence of external Ca^{2+}, as also later noted by Gu *et al.* (1998) for *Bombyx* prothoracic glands, and Henrich (1995) for *Drosophila* ring glands. In contrast, Meller *et al.* (1990) found that basal ecdysteroidogenesis in *Manduca* glands was decreased, although not absent, in Ca^{2+}-free medium, and varied with stage of development; Dedos and Fugo (1999b) uncovered a similar, developmentally specific Ca^{2+}-dependency for basal ecdysteroid synthesis in *Bombyx* glands. Thus, whether or not basal ecdysteroidogenesis is responsive to short-term changes in external Ca^{2+} is not certain; differences in experimental technique might well determine a gland's sensitivity in this regard.

External Ca^{2+} has been shown to be important for PTTH-stimulated ecdysteroidogenesis in at least two other insect species. PTTH-stimulated ecdysteroid synthesis by *Bombyx* prothoracic glands also required the presence of Ca^{2+} in the external medium (Gu *et al.*, 1998) and PTTH-containing neural extracts failed to stimulate ecdysteroid synthesis by *Drosophila* ring glands in the absence of external Ca^{2+} (Henrich, 1995). The importance of Ca^{2+} influx to PTTH-stimulated ecdysteroid synthesis was further demonstrated by the ability of the Ca^{2+} ionophore A23187 to stimulate ecdysteroidogenesis in *Manduca* (Smith and Gilbert, 1986) and *Bombyx* (Gu *et al.*, 1998) prothoracic glands.

Several studies using microfluorometric Ca^{2+} measurements explicitly verified the assumption that PTTH causes increases in prothoracic gland cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) and that the external environment was the primary source of this Ca^{2+}. Birkenbeil (1996) showed that a PTTH preparation caused a rise in $[Ca^{2+}]_i$ in prothoracic glands of the wax moth, *G. mellonella*, while Birkenbeil and Dedos (2002) confirmed the interpretation that PTTH caused Ca^{2+} influx in *Bombyx* glands. In *Manduca*, Birkenbeil (1999, 2000) showed that PTTH preparations stimulated Ca^{2+} influx, and this work has been extended with pure *Manduca* recombinant PTTH (Fellner, Rybczynski, and Gilbert, unpublished data).

The modes by which free Ca^{2+} enters the cytoplasm of prothoracic glands in response to PTTH stimulation has been addressed chiefly in *Manduca*, *Galleria*, and *Bombyx*. In *Manduca* and *Bombyx*, cationic lanthanum inhibited PTTH-stimulated ecdysteroid synthesis (Smith *et al.*, 1985; Birkenbeil and Dedos, 2002); lanthanum is a nonspecific blocker of many plasma membrane ion channels. Identification of the precise types of Ca^{2+} channels that function in PTTH signaling has proven difficult. The Ca^{2+} channel agonist Bay K8644, which activates voltage-gated Ca^{2+} channels, stimulated ecdysteroid synthesis in both *Manduca* and *Bombyx* prothoracic glands (Smith *et al.*, 1987b; Dedos and Fugo, 1999b) but it is not known how this compound interacts with PTTH-stimulated Ca^{2+} influx, i.e., is it additive, synergistic, or antagonistic? In *Manduca*, the L-type channel blocker nitrendipine partially inhibited PTTH-stimulated ecdysteroid synthesis by prothoracic glands (Smith *et al.*, 1987; Girgenrath and Smith, 1996). However, nitrendipine was not found to inhibit PTTH-stimulated Ca^{2+} influx by Birkenbeil (1996, 1999). To confuse the issue more, an inhibitory effect of nifedipine, another L-type channel blocker, was demonstrated by Fellner *et al.* (unpublished data) while Birkenbeil (1999) did not observe any effect of nifedipine. Birkenbeil did note an inhibitory effect of amiloride, a T-type Ca^{2+} channel blocker, on PTTH-stimulated Ca^{2+} influx, but, at the concentration used, amiloride affects other proteins in addition to the T-type Ca^{2+} channels. In *Bombyx*, similarly conflicting data have been obtained. The L-type channel inhibitors verapamil (Gu *et al.*, 1998) and nitrendipine (Dedos and Fugo, 2001) both inhibited PTTH-stimulated ecdysteroidogenesis in *Bombyx* prothoracic glands but nitrendipine had no apparent effect

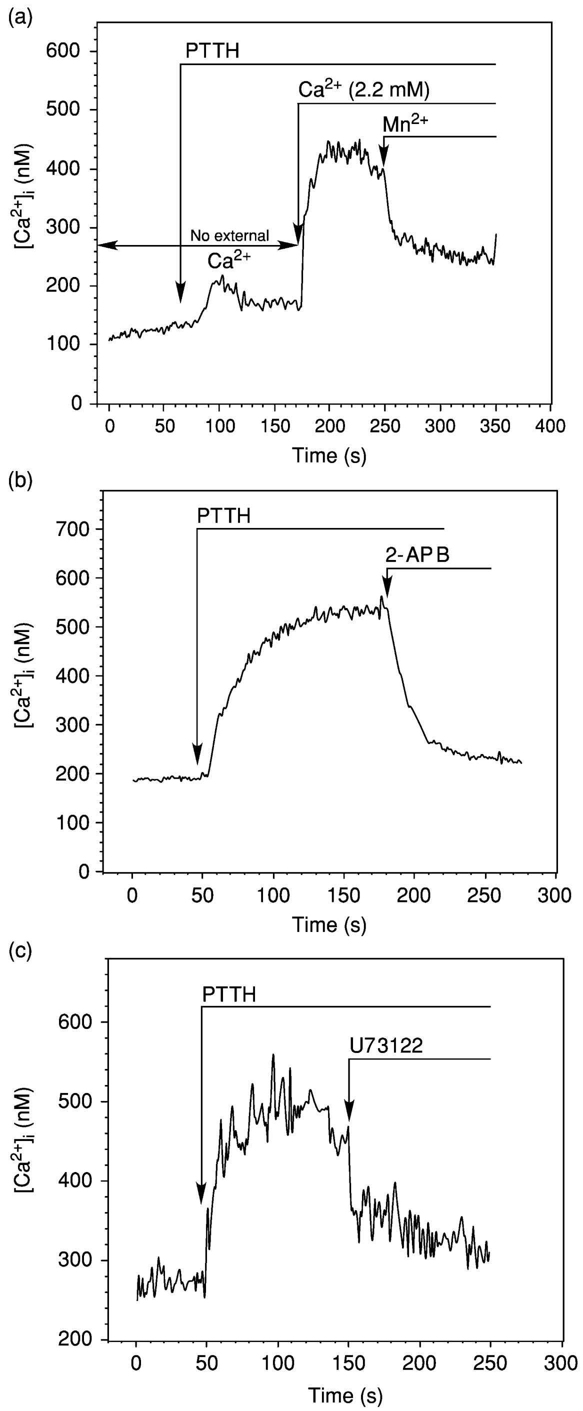


Figure 8 Free Ca^{2+} levels in prothoracic gland cells of *Manduca sexta* larvae, measured by fura-2 fluorescence. Data shown are representative plots from single cells. (a) The effect of PTTH on free cellular Ca^{2+} levels in the absence of external Ca^{2+}, and following the addition of Ca^{2+} to the incubation medium. The drop in free cellular Ca^{2+} levels following the addition of Mn^{2+} to the external medium is due to the entry of Mn^{2+} through store-operated channels. Mn^{2+}-dependent quenching of the Ca^{2+} fura-2 fluorescence indicates that the observed fluorescence results from Ca^{2+} binding to the fura, and not the simple accumulation of the dye within the cell. (b) 2-APB, an inhibitor of Ca^{2+} release from IP_3 gated stores on free cellular Ca^{2+} levels, truncates the PTTH-stimulated rise in free cellular Ca^{2+}. (c) U73122, an inhibitor of PLCβ, ablates the PTTH-stimulated rise in the concentration of free cellular Ca^{2+}. (Data from Fellner, Rybczynski, and Gilbert, unpublished data.)

on PTTH-stimulated cytoplasmic free Ca^{2+} (Birkenbeil and Dedos, 2002). The contribution of T-type Ca^{2+} channels to *Bombyx* PTTH signaling is not clear; two inhibitors of this channel class, bepridil and mibefradil, had no effect on PTTH-stimulated $[Ca^{2+}]_i$ changes, while the less specific amiloride inhibited $[Ca^{2+}]_i$ increases. These perplexing results may reflect methodological differences among the studies. It also seems likely that the identification of an insect channel type based purely on pharmacological reagents characterized in vertebrates may be risky (Girgenrath and Smith, 1996; Rybczynski and Gilbert, 2003).

Fellner *et al.* (unpublished data) investigated the kinetics of PTTH-stimulated increases in $[Ca^{2+}]_i$ in *Manduca* prothoracic glands and found that PTTH caused a rapid increase in $[Ca^{2+}]_i$ with levels plateauing within ≈30 s to 1 min. Furthermore, when prothoracic glands were incubated in the absence of external Ca^{2+} a small, transient $[Ca^{2+}]_i$ peak was seen followed by a large peak if Ca^{2+} is added to the external medium (see **Figure 8a**). These results suggested that PTTH first causes a release of Ca^{2+} from internal stores followed by a larger influx of external Ca^{2+}, with the large influx of Ca^{2+} being due substantially to the opening of store-operated channels. Store-operated channels are Ca^{2+} channels that respond to increases in internal Ca^{2+} (see Clementi and Meldolesi, 1996). The compound 2-aminoethoxydiphenyl borate (APB), an inhibitor of Ca^{2+} release from IP_3-sensitive stores in the endoplasmic reticulum, inhibited the PTTH-stimulated increase of $[Ca^{2+}]_i$, indicating that PTTH acts through the IP_3 receptor (**Figure 8b**) TMB-8, a second inhibitor of the release of Ca^{2+} from internal stores, also greatly diminished the PTTH-stimulated increase of $[Ca^{2+}]_i$, thus confirming the early participation of internal Ca^{2+} stores in the PTTH signal transduction pathway. It must be noted that Birkenbeil (1996, 1999) did not observe TMB-8 inhibition of PTTH-stimulated Ca^{2+} influx in *Galleria* or *Manduca* prothoracic glands. The reason for this is unknown. The composite data indicate that PTTH-stimulated increases in $[Ca^{2+}]_i$ originated from two Ca^{2+} pools: (1) an internal, IP_3-controlled endoplasmic reticulum store and (2) the extracellular milieu. The participation of an IP_3-controlled endoplasmic reticulum store in the early events of PTTH-stimulated Ca^{2+} mobilization was further

confirmed by the ability of a phospholipase C inhibitor (U73122) to block or interrupt $[Ca^{2+}]_i$ increases (**Figure 8c**); phospholipase C activity is required to generate IP_3 from its PIP_2 precursor.

Entry from the extracellular pool proceeds through at least two types of channels. One is a store-operated channel and the second is a voltage-gated channel, probably of the L-type. The opening of the voltage-gated channel may result from changes in transmembrane potential caused by operation of the store-operated channels, but other factors cannot be ruled out. For example, *Drosophila* dihydropyridine-sensitive Ca^{2+}channel activity can be modulated by either PLC or protein kinase A signaling pathways (Gu and Singh, 1997).

These results with *Manduca*, including sensitivity to TMB-8, were largely congruent with those reached in a similar study utilizing *Bombyx* prothoracic glands (Birkenbeil and Dedos, 2002), although at least two differences existed. First, no small increase in $[Ca^{2+}]_i$ was actually noted when *Bombyx* glands were stimulated with PTTH under Ca^{2+}-free conditions; however, the time resolution of the *Bombyx* study was too coarse to detect a transient peak such as found in *Manduca*. Second, the rapidity with which PTTH-stimulated $[Ca^{2+}]_i$ reached maximal levels was considerably slower and more variable in *Bombyx* (from ≈ 2 to >10 min) than in *Manduca* (≈ 20 s to 1 min). The drawn-out nature of the *Bombyx* $[Ca^{2+}]_i$ response is surprising; when $[Ca^{2+}]_i$ increases are a primary response to receptor activation, the kinetics of $[Ca^{2+}]_i$ increases are generally rapid and temporally stereotyped. At present it is unclear whether the differences in $[Ca^{2+}]_i$ kinetics are consequences of variant research protocols or represent biological differences between the species, e.g., the ability of a reagent to pass through the basal lamina surrounding the prothoracic gland might vary between *Manduca* and *Bombyx*. Regardless of the differences, the *Bombyx* data are consistent with the interpretation that PTTH causes release of Ca^{2+} from internal pools, followed by the opening of store-operated channels and voltage-gated channels.

The existence of a PTTH-stimulated, small $[Ca^{2+}]_i$ peak in *Manduca,* and presumably in *Bombyx*, sensitive to APB and TMB-8 inhibition, suggested that inositol metabolism played a role in PTTH-stimulated signal transduction, i.e., the generation of IP_3 by phospholipase C (PLC) and the subsequent release of IP_3-sensitive endoplasmic reticulum Ca^{2+} stores. Support for this hypothesis came from both *Bombyx* and *Manduca*. In the silkworm, the phospholipase C inhibitor, 2-nitro-4-carboxyphenyl-*N*, *N*-diphenylcarbamate, blocked PTTH-stimulated ecdysteroidogenesis but had no effect on basal synthesis. In *Manduca*, the phospholipase C inhibitor U73122, likewise blocked PTTH-stimulated ecdysteroidogenesis and was without effect on basal production (Rybczynski *et al*., unpublished data). Smith and Gilbert (1989) postulated the participation of phospholipase C in PTTH-stimulated ecdysteroidogenesis but attempts to detect PTTH-stimulated increases in PIP_2 metabolites like IP_3 have not been successful (Girgenrath and Smith, 1996; Dedos and Fugo, 2001). The failure to detect IP_3 might reflect a difficult-to-detect, small and transient IP_3 peak, if the APB-sensitive change in $[Ca^{2+}]_i$ is any guide to IP_3 behavior in the prothoracic gland. Clearly, in light of the results obtained with phospholipase C inhibitors, the question of PTTH-stimulated IP_3 generation should be readdressed. Indirect evidence supporting the participation of the IP_3 pathway in ecdysteroidogenesis have also resulted from the study of *Drosophila* carrying mutations in the IP_3 receptor gene (Venkatesh and Hasan, 1997; Venkatesh *et al*., 2001). Mutations in this gene caused molting delays and larval lethality that could be partially reversed by feeding larvae food that contained 20-hydroxyecdysone.

PTTH may stimulate changes in $[Ca^{2+}]_i$ and in Ca^{2+}-dependent events indirectly, as well as directly through receptor interactions. Electrophysiological studies using *Manduca* prothoracic glands *in vitro* revealed that Ca^{2+}-dependent action potentials could spread from cell to cell (Eusebio and Moody, 1986). PTTH and signals from nerves (see Section 3.2.5.2) are candidate triggers for such action potentials, but whether or not this occurs *in vivo* is unknown. Gap junctions and intercellular bridges have been found in *Manduca* prothoracic glands and these structures may allow the spread of signaling molecules, like cAMP, among cells (Dai *et al*., 1994). Thus, Ca^{2+}-dependent action potentials, gap junctions, and intercellular bridges all could serve to synchronize the behavior of the prothoracic gland cells in response to PTTH or other hormones. Measurements of resting $[Ca^{2+}]_i$ in *Manduca* cells indicated that cells of the prothoracic gland are indeed heterogeneous (Fellner, Rybczynski, and Gilbert, unpublished data), but, as yet, the significance of cell-to-cell differences is unknown. If cells are heterogeneous *in vivo* in their sensitivity to PTTH or other signals, signal-spreading among cells could serve to make the whole prothoracic gland respond at signal levels set by the most responsive cells. Note that a synchronization of cellular responses could downregulate the prothoracic gland's response to PTTH just as readily as facilitate it.

Both Ca^{2+} ions and cAMP are second messengers, although there are many cases in which the generation of cAMP is dependent upon prior intracellular changes in $[Ca^{2+}]_i$. This is the case in the prothoracic gland, where a role for cAMP in PTTH-stimulated ecdysteroid synthesis was first suggested by measurements of adenylate cyclase activity and intracellular cAMP in glands synthesizing large versus small amounts of ecdysteroids (Vedeckis and Gilbert, 1973; Vedeckis *et al.*, 1974, 1976). An explicit demonstration of the role of cAMP in ecdysteroid synthesis was obtained a decade later when *Manduca* PTTH preparations were shown to stimulate cAMP synthesis in a Ca^{2+}-dependent manner, i.e., extracellular Ca^{2+} was required for PTTH-stimulated cAMP synthesis (Smith *et al.*, 1984, 1985, 1986b). Further data supporting the upstream position of $[Ca^{2+}]_i$ relative to cAMP in the PTTH transduction cascade derive from observations that calcium ionophore mimics PTTH in eliciting both steroid and cAMP synthesis (Smith *et al.*, 1985, 1986b). In addition, the cell-permeable cAMP analog dibutyryl cAMP, as well as agents that activate adenylyl cyclase and inhibit cAMP phosphodiesterase activity, all stimulated ecdysteroid synthesis in prothoracic glands incubated in Ca^{2+}-free medium (Smith *et al.*, 1985, 1986b). Note that these *Manduca* studies utilized a partially purified PTTH, but identical results were later obtained with a recombinant PTTH (Gilbert *et al.*, 2000). Similar results have been obtained from *Bombyx* studies (Gu *et al.*, 1996, 1998; Dedos and Fugo, 1999b). Furthermore, *Bombyx* prothoracic glands stimulated with PTTH under Ca^{2+}-replete conditions continued to secrete ecdysteroids at elevated levels even after transfer to a Ca^{2+}-free medium (Birkenbeil and Dedos, 2002). Thus, if sufficient cAMP has been synthesized, extracellular Ca^{2+} is apparently no longer needed, at least under short-term *in vitro* conditions; any Ca^{2+}-dependent events that occur subsequent to cAMP generation must depend on $[Ca^{2+}]_i$ for time intervals of at least 2 h, a common *in vitro* incubation period.

The importance of cAMP in PTTH-stimulated ecdysteroid synthesis has been demonstrated in several studies. In *Manduca*, multiple analyses demonstrated that cell-permeable, excitatory cAMP analogs stimulated ecdysteroid synthesis (Smith *et al.*, 1984, 1985; Smith and Gilbert, 1986; Rybczynski and Gilbert, 1994). Furthermore, an antagonist form of cAMP (Rp-cAMP) substantially inhibited PTTH-stimulated ecdysteroidogenesis (Smith *et al.*, 1996). Excitatory cAMP analogs stimulated ecdysteroid synthesis in *Bombyx* prothoracic glands as well (Gu *et al.*, 1996, 1998; Dedos and Fugo, 1999c).

Adenylyl cyclase, which synthesizes cAMP from ATP, is found in multiple forms in vertebrates. The activity of these forms may be stimulated or inhibited by, or be insensitive to, G proteins or Ca^{2+}-calmodulin. In *Drosophila*, there appear to be nine isoforms of adenylyl cyclase, although five of these may be restricted to the male germline (Cann *et al.*, 2000). Meller *et al.* (1988, 1990) found that the *Manduca* prothoracic gland expresses Ca^{2+}/calmodulin-dependent adenylate cyclase activity that exhibits a developmental shift in some characteristics. Either of two calmodulin antagonists inhibited nearly all cyclase activity in membrane preparations (Meller *et al.*, 1988), suggesting that the vast majority, if not entirety, of gland adenylate cyclases are calmodulin-dependent. GTP-γ-S, a poorly hydrolyzable GTP analog, stimulated cyclase activity in both larval and pupal membrane preparations; however, in larval glands, GTP-γ-S was not effective without the simultaneous presence of calmodulin. This developmental switch in the characteristics of gland cyclase activity occurred about day 5 of the fifth larval instar, just prior to the beginning of the large premolt ecdysteroid peak. The switch was also characterized by changes in cyclase sensitivity to cholera toxin and NaF, two factors that interact with G proteins (Meller *et al.*, 1990). It is not known if the change in cyclase characteristics represents a change in cyclase isoform, a posttranslational modification, or a shift in the expression of proteins that interact with cyclase. In *Bombyx* prothoracic gland membrane preparations, calmodulin, Gpp(NH)p (a GTP analog) and NaF stimulated adenylate cyclase activity, while GDB-β-S, a GDP analog, inhibited activity (Chen *et al.*, 2001). NaF and calmodulin effects on cyclase activity were additive rather than synergistic, suggesting that both G protein- and calmodulin-dependent adenylate cyclases exist in the *Bombyx* prothoracic gland. It remains to be determined which of these putatively different adenylate cyclases is coupled to the PTTH signal transduction cascade. However, both *Bombyx* and *Manduca* prothoracic glands respond to cholera toxin with increased ecdysteroidogenesis (Girgenrath and Smith, 1996; Dedos and Fugo, 1999c); an increase in cellular cAMP levels was also measured in *Bombyx* glands. These data suggested that a $G\alpha_s$ protein might be coupled to the PTTH receptor.

Many, if not all, intracellular signaling systems exhibit downregulation after a time, with signaling intermediates returning to basal levels. During continuous presence of PTTH, $[Ca^{2+}]_i$ levels persisted above basal for at least 25 min in *Bombyx* prothoracic glands, with a peak at about 15 min after initiating PTTH stimulation; after transfer of

stimulated glands to PTTH-free conditions, $[Ca^{2+}]_i$ levels remained elevated for at least 20 min (Birkenbeil and Dedos, 2002). Similar $[Ca^{2+}]_i$ data are not available for *Manduca* prothoracic glands. Thus, how long $[Ca^{2+}]_i$ remains elevated during or after PTTH stimulation is not known. In the prothoracic glands of *Galleria* sixth and seventh (final) larval instars, Birkenbeil (1996) found developmental changes in $[Ca^{2+}]_i$, with peaks co-occurring coincident with peaks of *in vitro* ecdysteroid production. This observation suggested a relatively slow return to basal conditions and/or nonadaptation to continuous PTTH stimulation. In contrast, intracellular Ca^{2+} was essentially constant in glands of fifth instar *Manduca* larvae (Birkenbeil, 1999). The mechanisms by which prothoracic gland $[Ca^{2+}]_i$ is restored to the basal level have not been explicitly explored but presumably involve buffering by Ca^{2+}-binding proteins, reloading of internal stores and transport to the extracellular milieu.

The temporal profile of cAMP levels during PTTH stimulation of the prothoracic gland is also incomplete. PTTH elicited a rapid generation of cAMP in *Manduca* prothoracic glands, readily detectable within 2 min of applying PTTH and peaking after 5 min (Smith *et al.*, 1984). Elevated cAMP levels persisted for at least 20 min, with continuous PTTH exposure. Data from *Bombyx* prothoracic glands were similar, with a rapid rise in cAMP that reached a maximum at 10 min and continued at about twice basal for at least an hour (Gu *et al.*, 1996). Analysis of cAMP levels in *Manduca* prothoracic glands during the fifth (final) larval instar revealed a large maximum occurring at about the time of the commitment peak in ecdysteroid production (Vedeckis *et al.*, 1976), suggesting, as is the case for $[Ca^{2+}]_i$, a slow return to basal levels following stimulation and/or nonadaptation to continuous PTTH stimulation. Initially surprising were the relatively low levels of gland cAMP observed during the premolt period of high ecdysteroid production several days later (but see discussion immediately following).

Intracellular cAMP levels are a product of synthesis and breakdown. The puzzlingly low cAMP *in vivo* levels found during the premolt ecdysteroid peak (Vedeckis *et al.*, 1976), as well as in *Manduca* glands stimulated *in vitro* with PTTH at this stage and later (Smith and Gilbert, 1986; Smith and Pasquarello, 1989), appear to result chiefly from developmental differences in phosphodiesterase activity. Analysis of cAMP phosphodiesterase activity revealed a nearly six-fold increase from a minimum on days 3 and 4 to a peak just prior to pupal ecdysis; at the time of the large, premolt ecdysteroid peak, when PTTH signaling is presumably high also, phosphodiesterase activity was ≈5 times the minimum (Smith and Pasquarello, 1989). This observation suggested that the importance of the cAMP pathway in PTTH signal transduction was greatly diminished in late larval and pupal glands (Vedeckis *et al.*, 1976). This proved clearly not to be the case, as demonstrated by the inhibitory action of a cAMP antagonist on PTTH-stimulated pupal ecdysteroid synthesis (Smith *et al.*, 1996).

3.2.4.3. Protein Kinases and Phosphatases

A very common, if not universal, response to increases in second messengers is the modulation of protein kinases and phosphatase activities. In this section, known and hypothesized kinase and phosphatase contributors to PTTH signaling are discussed. These enzymes are discussed separately, perhaps giving a linear view of their participation in the PTTH signal transduction cascade. This is clearly not the case and the enzyme-by-enzyme approach is followed merely to provide a structure to the discussion, not to imply simplicity of the pathway.

3.2.4.3.1. Protein kinase A Two isoforms of cAMP-dependent protein kinase A (PKA; types I and II) have been found in the *Manduca* prothoracic gland, with the majority of the activity (≥95%) attributable to the type II isoform (Smith *et al.*, 1986b). PTTH-dependent stimulation of PKA activity was found in both larval and pupal glands, although the addition of a phosphodiesterase inhibitor was necessary to measure PTTH-dependent PKA activity in the latter. The requirement of a phosphodiesterase inhibitor to measure PTTH-dependent PKA activity in pupal glands was not surprising, given the rapid metabolism of cAMP at this stage, as discussed above (see Section 3.2.4.2). PTTH stimulated PKA activity rapidly, with a patent increase above controls following 3 min of exposure, and with elevated levels still evident after 90 min (Smith *et al.*, 1986b). PKA is composed of two subunits: one catalytic and the other regulatory. In prothoracic glands of early fifth larval instars of *Manduca*, the catalytic subunit remains relatively constant per mg protein for the first 4 days while the regulatory subunit increases approximately three-fold (Smith *et al.*, 1993). This may insure that PKA is only activated when a fairly high threshold of cAMP has accumulated in response to PTTH (Smith *et al.*, 1993), but this hypothesis is assuredly tentative, pending information not currently available, on the relative abundances of the catalytic and regulatory subunits at other stages of development.

The rapid activation of PKA in response to PTTH stimulation suggests that PKA plays a proximate role in the control of PTTH-stimulated steroidogenesis. The effects of agonist and antagonist cAMP homologs is supportive but not conclusive evidence for this interpretation, since there are other cellular targets for cAMP besides PKA. For instance, in mammals, cAMP-dependent guanine nucleotide exchange factors, which activate small G proteins, have been described (Kawasaki *et al*., 1998). These proteins can stimulate phosphorylation events, including the activation of the 70-kDa S6 kinase, a known element in PTTH action (see below), and a search of the *Drosophila* genome data base suggests that insects may possess one or more homologs to these exchange factors (data not shown). However, the PKA inhibitor H89 is effective at blocking PTTH-stimulated ecdysteroid synthesis in *Manduca* glands, albeit at fairly high doses, again implicating PKA as a regulatory factor in this pathway (Smith *et al*., 1996). The *in vivo* substrates for PTTH-activated PKA are poorly known. PTTH, dibutyryl cAMP, and Ca^{2+} ionophore all stimulate phosphorylation of a 34-kDa protein in *Manduca* prothoracic glands (Rountree *et al*., 1987, 1992; Combest and Gilbert, 1992; Song and Gilbert, 1994) that has been identified as the S6 ribosomal protein (Song and Gilbert, 1995). However, it is unclear that S6 is directly phosphorylated by PKA. When the (mammalian) catalytic subunit of PKA, or cAMP, was added to prothoracic gland homogenates, the phosphorylation of S6 was enhanced (Smith *et al*., 1987a; Rountree *et al*., 1992; Combest and Gilbert, 1992), indicating that S6 was a potential PKA substrate. However, in intact cells, the situation may be different since the immunosuppressive agent rapamycin was able to block PTTH-stimulated S6 phosphorylation (Song and Gilbert, 1994, 1995). Rapamycin acts via formation of a protein complex that inhibits p70 S6 kinase activity and PKA is neither a known substrate for rapamycin nor a component of the rapamycin–protein complex (see Section 3.2.4.4).

3.2.4.3.2. Protein kinase C The possibility that protein kinase C (PKC) was involved in the PTTH-stimulated ecdysteroid synthesis was first addressed, with negative results, by Smith *et al*. (1987a), using the potent PKC activator phorbol myristate acetate (PMA). A 90-min incubation with PMA had no effect on *Manduca* steroidogenesis, either alone or in conjunction with PTTH (see also Smith, 1993). However, in *Bombyx*, PMA boosted prothoracic gland ecdysteroid synthesis after 5 h of treatment, but no effect was apparent after 2 h (Dedos and Fugo, 2001), suggesting that PKC did not play an acute role in steroidogenesis. Furthermore, studies on inositol metabolism in both *Manduca* and *Bombyx* failed to find PTTH-related generation of PIP_2 metabolites (see Section 3.2.4.2). Diacyl glycerol (DAG), one of these metabolites, is an activator of PKCs and is formed in a 1 : 1 ratio with IP_3. The apparent absence of IP_3 suggested that DAG was also absent and hence that PKCs were not involved in PTTH action. Nevertheless, three lines of evidence do indicate that a PKC is part of the signal transduction network of PTTH, and that it occupies an early and important position in regulating prothoracic gland ecdysteroidogenesis. First, the PKC inhibitor chelerythrine Cl inhibited PTTH-stimulated ecdysteroid synthesis by both *Bombyx* (Dedos and Fugo, 2001) and *Manduca* prothoracic glands (**Figure 9a**) (Rybczynski and Gilbert, unpublished data); PTTH stimulated ERK phosphorylation was also inhibited (**Figure 9a**). Second, PTTH-stimulated the phosphorylation of several (unidentified) prothoracic gland proteins, as recognized by an antibody against phosphorylated PKC substrate sequences (**Figure 9b**), and chelerythrine Cl inhibited these phosphorylations (data not shown) (Rybczynski and Gilbert, unpublished data). Third, although measurable changes in IP_3 or other PIP_2 metabolites have not been detected following PTTH stimulation, phospholipase C inhibitors blocked PTTH-stimulated ecdysteroid synthesis in *Bombyx* (2-nitro-4-carboxyphenyl-N, *N*-diphenylcarbamate: Dedos and Fugo, 2001) and *Manduca* (U73122: Fellner *et al*., unpublished data) prothoracic glands (**Figure 9c**). U73122 also inhibited PTTH-stimulated ERK phosphorylation (**Figure 9c**) and the release of Ca^{2+} from IP_3-sensitive stores (see Section 3.2.4.2). These data suggest that PTTH stimulates inositol metabolism and activates PKCs, but the inositol increases are too small or transient to have been detected. Intracellular compartmentalization of early acting components of the signal transduction system might make small changes biologically meaningful and eliminate the need for large-scale intracellular increases in IP_3 or DAG.

The identity and function of proteins phosphorylated by PKC in response to PTTH stimulation of the prothoracic gland are currently unknown. PKCs can modulate the function of a wide variety of proteins including ion channels (see Shearman *et al*., 1989), MAP kinases (Suarez *et al*., 2003), plasma membrane receptors (Medler and Bruch, 1999), cytoskeletal elements (Gujdar *et al*., 2003), and nuclear proteins (Martelli *et al*., 2003). The observation that some putative PKC phosphorylations took place only after several hours of PTTH stimulation

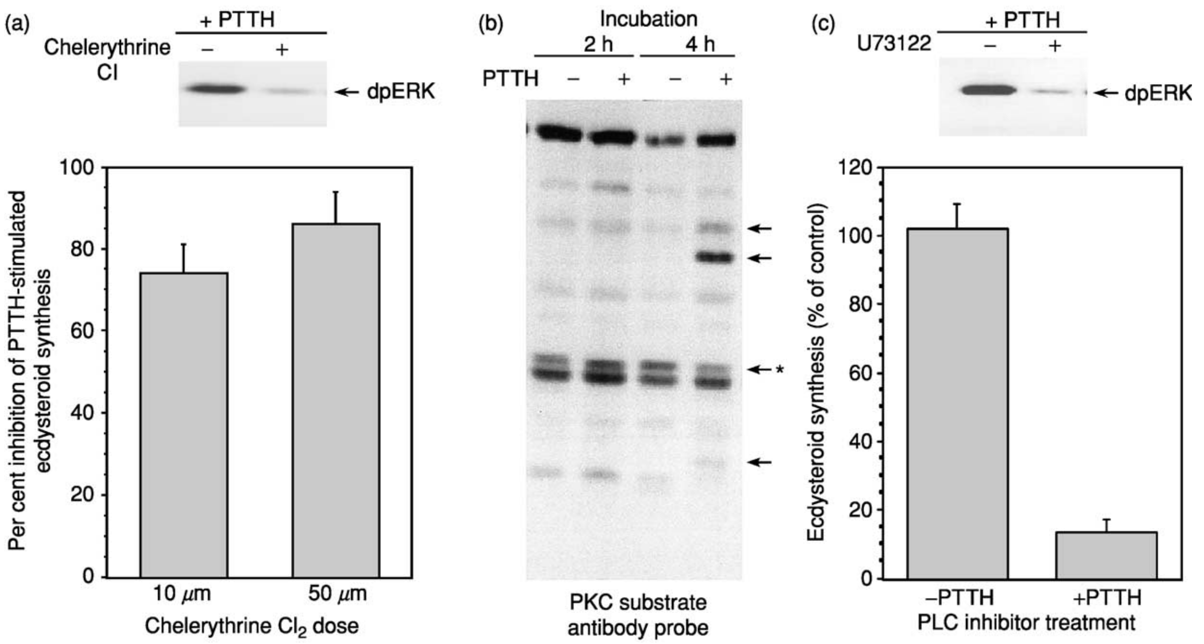


Figure 9 Evidence for the involvement of PKC and PLC in PTTH-stimulated events in the larval prothoracic gland of *Manduca sexta*. Ecdysteroids were measured with a radioimmunoassay and protein phosphorylation detected following SDS-PAGE of gland proteins, using phospho-specific antibodies and chemiluminescence reagents. (a) Chelerythrine Cl, an inhibitor of PKC, blocks PTTH-stimulated ecdysteroid synthesis (lower panel) and ERK phosphorylation (upper panel). Chelerythrine Cl had no effect on basal ecdysteroid synthesis and ERK phosphorylation (data not shown). (b) PTTH stimulates the phosphorylation of proteins recognized by an antibody against putative PKC phosphorylation substrate sites. Glands were incubated for up to 4 h ± PTTH. (c) U73122, an inhibitor of PLCβ, blocks PTTH-stimulated ecdysteroid synthesis, without affecting basal synthesis (lower panel). U73122 also inhibits PTTH-stimulated ERK phosphorylation (upper panel) but basal ERK phosphorylation is unaffected (data not shown). (Data from Rybczynski, Fellner, and Gilbert, unpublished data.)

(Figure 9b) suggested that PKC might be involved in the long-term effects of PTTH, such as transcription, as well as in the acute regulation of steroid synthesis. The position of PKC in the PTTH signaling hierarchy has not been determined but, given a probable requirement for DAG, it seems likely that PKC is among the first intracellular components to be called into action. This hypothesis is supported by the observations that (1) inhibition of PKC activity prevented PTTH-dependent ecdysteroid synthesis and (2) inhibition of PKC activity prevented PTTH-dependent MAP kinase phosphorylation (see below), i.e., PKC is upstream of MAP kinases (Rybczynski and Gilbert, unpublished data). The time course of PKC activity in the PTTH signal transduction network may be extended given the observed late appearance of some putative PKC phosphorylation events (Figure 9b).

3.2.4.3.3. Extracellular signal-regulated kinase The mitogen-activated protein kinases (MAP kinases) are a family of related proteins that are activated by a wide variety of extracellular signals, ranging from growth factors and peptide hormones to heat and osmotic stresses. MAP kinases, in turn, regulate a wide variety of intracellular processes from microtubule stability and protein synthesis to transcription. There are several families of MAP kinases, which are organized into three-level signaling modules, i.e., a MAP kinase, a MAP kinase kinase (MEK), and a MAP kinase kinase kinase (MEKK or MAP3K) (see Lewis *et al.*, 1998; Kyriakis, 2000). Perhaps the most studied of the MAP kinase modules is the extracellular signal-regulated kinase (ERK) cascade. This module typically starts with a protein called RAF, and proceeds through a MEK to the ERK protein(s); in mammals there are two ERKs while *Drosophila* and *Manduca* appear to express a single ERK (see Lewis *et al.*, 1998; Biggs and Zipursky, 1992; Rybczynski *et al.*, 2001). A number of proteins lie upstream from RAF, the small G protein RAS is a frequent but not ubiquitous activator of RAF, which itself may lie downstream of G protein-coupled receptors, tyrosine kinase receptors, or cytokine receptors (see Lewis *et al.*, 1998). MEKKs and MEKs are activated by single phosphorylations while ERKs require a dual phosphorylation on threonine and tyrosine residues for activity.

A PTTH stimulation of the ERK pathway in larval *Manduca* prothoracic glands was shown

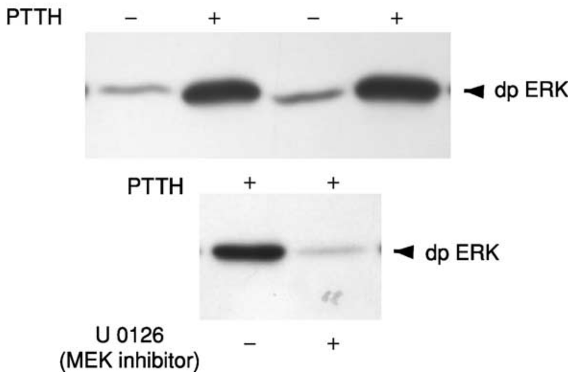


Figure 10 PTTH stimulates ERK phosphorylation in the larval prothoracic gland of *Manduca sexta* (upper panel) that is blocked by the MAPK/ERK kinase inhibitor U0126 (lower panel). ERK phosphorylation was detected following SDS-PAGE of gland proteins, using an antiphosphorylated ERK antibody and chemiluminescence reagents. (Adapted from Rybczynski, R., Bell, S.C., Gilbert, L.I. **2001**. Activation of an extracellular signal-regulated kinase (ERK) by the insect prothoracicotropic hormone. *Mol. Cell. Endocrinol. 184*, 1–11.)

recently (Rybczynski *et al.*, 2001) using activity assays, inhibitors of ERK phosphorylation, and antibodies that recognize the dually phosphorylated state (see **Figure 10**). This study revealed that PTTH caused a rapid but transient increase in ERK phosphorylation and that inhibitors of ERK activation blocked both basal and PTTH-stimulated ecdysteroid syntheses. The data suggested that ERKs might be involved in regulating some aspects of the acute PTTH response in prothoracic glands. Further examination showed that PTTH-stimulated ERK phosphorylation was primarily a larval gland response. As *Manduca* larvae progressed through the fifth instar, PTTH-stimulated phosphorylation in the prothoracic gland declined until it was undetectable in pupal glands (Rybczynski and Gilbert, 2003). Concurrently, ERK levels declined in the glands but the basal levels of phosphorylation increased in response to unknown factors. These two phenomena combined to cause the aforementioned decline in PTTH-stimulated ERK phosphorylation. Rybczynski and Gilbert (2003) also found that PTTH-stimulated ERK phosphorylation occurred via a Ca^{2+}-dependent pathway but not one that was cAMP-dependent. Some PKC isotypes require Ca^{2+} for activation and thus are candidates for the regulation of the ERK cascade. As discussed above, PKC inhibition resulted in a block of PTTH-stimulated ERK phosphorylation (**Figure 8c**). In fact, PKC may directly activate RAF, the first component of the ERK phosphorylation module, without the participation of RAS (see Liebman, 2001). However, PKC might control ERK phosphorylation in a less specific manner if PKC regulates PTTH-stimulated Ca^{2+} channel opening. Of note in this regard is the observation that PKC modulation of dihydropyridine-sensitive Ca^{2+} channels occurs in *Drosophila* tissues (Gu and Singh, 1997). The protein kinase Pyk2, which can be activated by increases in $[Ca^{2+}]_i$ and by PKC (Lev *et al.*, 1995), is also a reasonable candidate to be an upstream regulator of the ERK cascade.

The list of potential targets of ERK phosphorylation is long and encompasses transcription factors, steroid and growth factor receptors, various membrane-associated proteins, cytoskeletal proteins, kinases, and proteins involved in regulating translation (see Grewal *et al.*, 1999). Studies to discover PTTH-related ERK phosphorylation targets in the prothoracic gland are currently underway but no conclusive identifications can be made at this time. In most cell types, some fraction of dually phosphorylated ERKs migrate into the nucleus where they modulate transcription; no conclusive information about the prothoracic gland on this topic is presently available. Preliminary cell fractionation experiments suggested that dually phosphorylated ERKs do indeed move into the prothoracic gland cells' nuclei following PTTH stimulation (Rybczynski and Gilbert, unpublished data); however, the basal lamina surrounding the gland and the very large nuclei make clean subcellular fractions difficult to obtain and the conclusion must be considered tentative.

3.2.4.3.4. p70 S6 kinase The S6 protein is a prominent ribosomal phosphoprotein that can be phosphorylated by PKA and a 70-kDa S6 kinase (p70S6K) (Palen and Traugh, 1987). S6 phosphorylation is a frequent and presumably important event in various intercellular signaling pathways (see Erickson, 1991; Volarevic and Thomas, 2001). Phosphorylation of S6 by p70S6K, but apparently not by PKA, can increase the rate of translation of some mRNAs (Palen and Traugh, 1987). PTTH has long been known to induce the phosphorylation of a ≈34-kDa protein in prothoracic glands of *Manduca*, which was hypothesized to be S6 (see Gilbert *et al.*, 1988); this identification proved to be correct (see Section 3.2.4.4).

Song and Gilbert (1994) showed that p70S6K was responsible for PTTH-stimulated S6 phosphorylation in *Manduca* prothoracic glands. Using the macrolide immunosuppressant rapamycin, which blocks p70S6K phosphorylation-dependent kinase activity (see Dufner and Thomas, 1999), they found that PTTH-stimulated phosphorylation of an S6 peptide substrate was inhibited by rapamycin,

when tested with an S6K1-enriched protein fraction prepared from glands treated with both PTTH and rapamycin. Furthermore, rapamycin inhibited PTTH-stimulated ecdysteroid synthesis at low (nM) concentrations. These observations also implicate the participation of at least two additional proteins in the PTTH transduction cascade, although direct proof of their activity remains to be obtained. One is the phosphoinositide 3OH-dependent kinase (PI3K) and the second is a protein termed, among others, TOR (target of rapamycin; the *Drosophila* gene is called *dtor*) and *frap* (FKBP12-rapamycin-associated protein). TORs are proteins that contain a kinase domain similar to that of PI3K; their activity is inhibited when rapamycin binds to the small protein FKBP, forming an inhibitory complex that interacts with TORs (see Fumagalli and Thomas, 2000). Both PI3K and TOR positively regulate p70S6K activity. It is likely that another level of proteins is interposed between p70S6K and PI3K, such as the atypical PKCs (see Fumagalli and Thomas, 2000). TORs probably interact directly with p70S6K and also inhibit phosphatases that downregulate p70S6K activity (see Fumagalli and Thomas, 2000). Activation of PI3K by PTTH might take place via several intermediary proteins, such as RAS, or the βγ subunit of G proteins (see Coffer, 2000). The regulation of TOR function, in the context of PTTH, is more problematic. It may be activated by PI3K, perhaps via an intermediate kinase, but the regulation of TORs is incompletely understood (see Raught *et al.*, 2001; Abraham, 2002).

3.2.4.3.5. Tyrosine kinases Many intercellular signaling molecules evoke a series of protein phosphorylations on tyrosine residues within the target tissues. Tyrosine kinases include both intracellular proteins as well as membrane-spanning receptors. The possibility that PTTH signal transduction involves tyrosine kinase has been addressed in part by Smith *et al.* (2003). Their work revealed that PP1, an inhibitor of the Src tyrosine kinase, repressed PTTH-stimulated cAMP and ecdysteroid synthesis in *Manduca* prothoracic glands. PP1 did not appear to interact with adenylate cyclase, since forskolin was still able to stimulate cAMP production in the presence of PP1. This result suggested that a Src kinase might act very early in PTTH signaling, perhaps at the level of Ca^{2+} mobilization. Indeed, tyrosine kinases can activate phospholipases, generating IP_3 and raising $[Ca^{2+}]_i$ via the IP_3 receptor/Ca^{2+} channel (see Rhee, 2001); tyrosine kinases can also directly activate the IP_3 receptor/Ca^{2+} channel through phosphorylation (Jayaraman *et al.*, 1996). Another intriguing possibility is that stimulation of the PTTH receptor, if it is indeed a GPCR, might transactivate a tyrosine receptor kinase via G proteins and other intermediates, and consequently recruit some or all of the signaling pathway of a completely different receptor class (see Luttrell *et al.*, 1999; Wetzker and Bohmer, 2003). Perhaps relevant to this scenario is the observation that an antibody against the vertebrate insulin receptor, a tyrosine kinase receptor, cross-reacted with a protein of appropriate molecular weight in the prothoracic gland (Smith *et al.*, 1997) and a second antibody against tyrosine-phosphorylated proteins indicated that PTTH stimulated the phosphorylation of a protein of the same molecular weight (Smith *et al.*, 2003). However, care must be taken in interpreting the effects of this single kinase inhibitor, since PP1, at the micromolar concentrations employed, might inhibit other kinases, including those in the ERK pathway (see Bain *et al.*, 2003).

3.2.4.4. Ribosomal Protein S6

Ribosomal protein S6 is a small (≈34 kDa) basic protein, present at one copy per 40S ribosome, that is located near the tRNA/mRNA binding site (see Fumagalli and Thomas, 2000). Phosphorylation of S6 is associated with mitogenic stimulation of non-dividing cells and occurs in vertebrate steroidogenic cells in response to factors such as FSH and TSH (Das *et al.*, 1996; Suh *et al.*, 2003); of course, FSH and TSH have mitogenic effects as well as effects on hormone synthesis. S6 phosphorylation at six serine residues near the C-terminal of the protein has been correlated with the selective increase of certain mRNAs, especially those containing polypyrimidine tracts near the transcription initiation site (see Erickson, 1991; Fumagalli and Thomas, 2000; Meyuhaus and Hornstein, 2000; Volarevic and Thomas, 2001).

Rountree *et al.* (1987) and others (see Section 3.2.4.3.1) discovered that PTTH stimulates the phosphorylation of a ≈34 kDa-protein in *Manduca* prothoracic glands that was hypothesized to be the S6 ribosomal protein. Song and Gilbert (1994, 1995, 1997) confirmed this identification by using the p70S6K inhibitor rapamycin and, more rigorously, by two-dimensional analysis of ribosomal proteins (see **Figure 11**). As discussed earlier, they found that rapamycin blocked S6 phosphorylation and ecdysteroid synthesis; furthermore, rapamycin inhibited the accumulation of specific, newly synthesized proteins, including a heatshock 70-kDa cognate protein (Song and Gilbert, 1995), which had been hypothesized to be necessary for PTTH-stimulated ecdysteroid synthesis (Rybczynski and Gilbert, 1994, 1995a; see below).

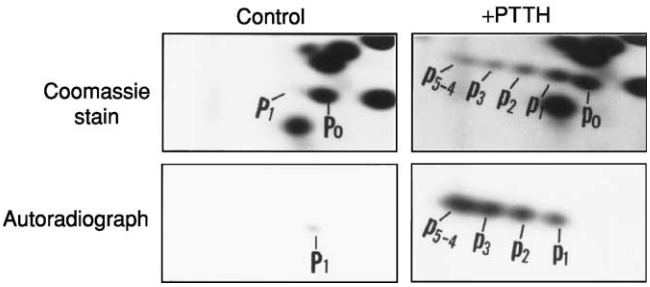


Figure 11 PTTH stimulates the phosphorylation of ribosomal protein S6 in the prothoracic gland of *Manduca sexta*. The upper two panels show the effect of PTTH on the migration pattern of the S6 protein (labeled P_0 to $P_{5–4}$) in two-dimensional gel analyses of ribosomal proteins, as visualized with Coomassie Blue staining. The lower two panels show the effect of PTTH on the migration pattern of the S6 protein, as visualized with autoradiography of ^{32}P-labeled proteins, following two-dimensional gel analyses of ribosomal proteins. The data reveal that up to five sites on S6 are phosphorylated following the stimulation of prothoracic glands with PTTH. (Adapted from Gilbert, L.I., Song, Q., Rybczynski, R. **1997**. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. *Invert. Neurobiol. 3*, 205–216.)

cDNA cloning of the *Manduca* S6 mRNA revealed that the sequence coded for a polypeptide of ≈ 29 000 MW, with an estimated isoelectric point of 11.5 (Song and Gilbert, 1997). Like the mammalian S6 proteins, the *Manduca* sequence contained a cluster of six serines in the final (C-terminal) 20 amino acids. Phosphoamino acid analysis indicated that, following PTTH stimulation, the *Manduca* S6 protein was phosphorylated solely on serines. The two-dimensional analysis of ribosomal proteins showed that PTTH-stimulation resulted in phosphorylation of up to five sites; nonstimulated glands yielded an S6 with an apparent single phosphorylation (Song and Gilbert, 1995, 1997). The location of the PTTH-stimulated serine phosphorylations is not known since, in addition to the six terminal serines, the *Manduca* protein was deduced to contain an additional 10 residues. The combination of Northern blotting, using the S6 cDNA as a probe, and immunoblotting, using polyclonal antibodies against the S6 protein terminus, showed that S6 RNA and protein levels in the prothoracic gland were not correlated (Song and Gilbert, 1997). S6 RNA per unit total RNA was highest in the first 3 days of the fifth instar and decreased linearly to a much lower level by day 7; this low level persisted through day 4 of pupal life, interrupted only by a minor increase on the first day following pupal ecdysis. In contrast, S6 proteins showed two peaks of abundance. The first occurred on days 6 and 7 of the fifth instar, when the prothoracic gland is beginning the large surge of ecdysteroid synthesis that precedes the larval–pupal molt. The second peak fell on days 3 and 4 of pupal–adult development, the beginning of the large pupal–adult period of ecdysteroid synthesis. As a control, S6 protein levels in the fat body were assayed. These showed no relationship to S6 in the prothoracic gland or to hemolymph ecdysteroid titers. Rather, S6 protein in fat body showed a steady decline from the beginning to the end of the fifth instar and then fairly constant low levels through day 4 of pupal–adult development. These data suggested that S6 levels might be a controlling factor in supporting high levels of ecdysteroid synthesis.

Although these composite data strongly support the notion that the PI3K/TOR–p70S6K–S6 axis is essential for PTTH-stimulated ecdysteroid synthesis to occur, there are some puzzling data that do not readily fit this scheme. Both Rountree *et al.* (1987) and Smith *et al.* (1987a) discovered that, like PTTH, a cAMP analog (dibutyryl cAMP (dbcAMP)) stimulated S6 phosphorylation and Rountree *et al.* (1987) found that JH and a JH analog inhibited S6 phosphorylation in glands studied *in vitro* (Rountree *et al.*, 1987). However, under the same *in vitro* conditions, JH had no effect on PTTH-stimulated cAMP synthesis and ecdysteroid synthesis (Rountree and Bollenbacher, 1986). A second puzzling observation, revolving again around cAMP, stems from the study of the effects of rapamycin on steroidogenesis. As described above, rapamycin inhibits both PTTH-stimulated S6 phosphorylation, and ecdysteroidogenesis. However, dbcAMP was able to override the rapamycin block and stimulate ecdysteroid synthesis to the usual levels above basal, even though rapamycin blocked the dbcAMP-dependent S6 phosphorylation (Song and Gilbert, 1994). These two sets of data suggest that a vigorous, six-site S6 phosphorylation response may not be required to support PTTH-stimulated ecdysteroid synthesis. In this respect, it would be interesting to use the more sensitive two-dimensional gel analysis of ribosomal proteins to determine if JH and rapamycin blocked all phosphorylations above the basal level; it might be that the single-dimension gel

analysis used was not sensitive enough to detect partial S6 phosphorylation in these experiments. It is also possible that there are alternate pathways by which PTTH stimulates ecdysteroid synthesis and that these pathways can substitute for the presumably default S6-dependent one. For instance, activation of the ERK pathway can also upregulate protein translation in some cells (see Grewal *et al.*, 1999).

3.2.4.5. Translation and Transcription

In vertebrate steroidogenic tissues, the final signal transduction step in peptide-regulated steroid hormone production is the rapid translation of one or more proteins. In these taxa, two proteins, StAR (steroidogenic acute regulatory protein) and DBI (diazepam-binding inhibitor), facilitate the delivery of cholesterol across the mitochondrial membrane to the side-chain cleavage P450 enzyme, which converts cholesterol to pregnenelone (see Stocco and Clark, 1997; Papadopoulos *et al.*, 1997; **Chapter 3.3**). This step is rate limiting and is alleviated by rapid translation of StAR and/or DBI that is stimulated by a peptide hormone such as adrenocorticotropic hormone (ACTH). A *Manduca* DBI homolog has been cloned and evidence suggests a role for DBI in basal ecdysteroid synthesis (Snyder and Feyereisen, 1993; Snyder and Van Antwerpen, 1998), but experiments to explicitly test its role in acute PTTH-stimulated ecdysteroidogenesis have yet to be performed. Analysis of the *Drosophila* and *Anopheles* genome databases indicates that an insect protein with close homology to the StAR-like vertebrate protein MLN64 exists but the similarity of the insect protein to StAR itself is lower. MLN64 contains a region homologous to the cholesterol-binding region of StAR (see **Chapter 3.3**) but the insect protein sequence contains a large interior region that has no vertebrate counterpart and *in situ* hybridizations in *Drosophila* indicate that expression is not limited to the ring gland (see **Chapter 3.3**).

In insects, it is clear that PTTH-stimulated ecdysteroid synthesis also requires translation, as revealed by the ability of translation inhibitors like cycloheximide and puromycin to block such ecdysteroid synthesis (Keightley *et al.*, 1990; Kulesza *et al.*, 1994; Rybczynski and Gilbert, 1995a); translation inhibitors have no effect on basal ecdysteroid synthesis, under standard *in vitro* protocols. In *Drosophila*, the movement of an ecdysteroid precursor(s) between intracellular compartments (endoplasmic reticulum and mitochondrion) probably involves a carrier protein, although the necessity of translation in this species has not been demonstrated (Warren and Gilbert, 1996; Warren *et al.*, 1996).

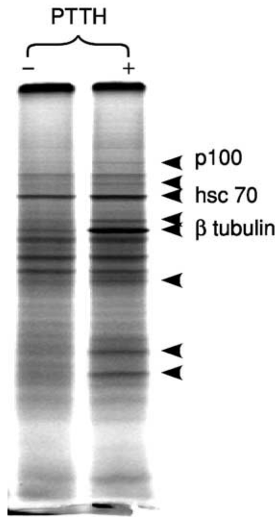


Figure 12 PTTH stimulates the synthesis of specific proteins in the prothoracic gland of *Manduca sexta*. Glands were incubated with ^{35}S-methionine ± PTTH for 1 h and then subjected to SDS-PAGE and autoradiography. The arrowheads indicate newly synthesized proteins whose accumulation in the prothoracic gland is upregulated by PTTH. Hsc 70, β tubulin and p100 were identified in the first studies on this system (Rybczynski and Gilbert 1994, 1995a; 1995b); the additional PTTH-regulated translation products were only detected after further refinement of the SDS-PAGE conditions (Rybczynski and Gilbert, unpublished data).

Analysis of protein synthesis in *Manduca* prothoracic glands, using ^{35}S-methionine to label newly synthesized proteins, revealed that PTTH stimulated overall translation at most developmental stages and that some proteins, perhaps as many as 10, were differentially translated above this general increase (see **Figure 12**) (Keightley *et al.*, 1990; Rybczynski and Gilbert, 1994; Gilbert *et al.*, 1997). Two of these proteins have been identified and cloned: a β tubulin (Rybczynski and Gilbert, 1995a, 1998) and a 70-kDa heatshock cognate protein (hsc 70) (Rybczynski and Gilbert, 1995b, 2000). PTTH-stimulated synthesis of hsc 70 rose slowly and was prolonged, suggesting that hsc 70 might function as a chaperone in supporting translation, and in other long-term effects of PTTH not directly connected to acute changes in ecdysteroidogenesis, e.g., an hsc 70 protein appears to be necessary for proper function of the ecdysone receptor (Arbeitman and Hogness, 2000). PTTH-stimulated hsc 70 synthesis above basal at all stages examined during the fifth instar and pupal–adult development; peaks occurred on the third day of the fifth instar (V_3) and the first day of pupal–adult development (P_1) (Rybczynski and Gilbert, 1995b). In contrast, PTTH-stimulated β tubulin synthesis occurred

rapidly and was transitory, suggesting a more direct role in ecdysteroidogenesis for this cytoskeletal protein (Rybczynski and Gilbert, 1995a). Peaks in PTTH-stimulated β tubulin synthesis occurred on V_3 and P_1 (Rybczynski and Gilbert, 1995a), as observed for hsc 70. Indeed, treatment of pupal prothoracic glands with microtubule-disrupting drugs inhibited PTTH-stimulated ecdysteroid synthesis (Watson *et al.*, 1996) but this effect was not seen in larval glands (Rybczynski and Gilbert, unpublished data). Furthermore, the pupal inhibition effect was associated with translation inhibition that was overridden by treatment of glands with a cAMP analog, indicating that physical coupling between the PTTH receptor and a protein acting early in the transductory cascade was disrupted by such drugs (Rybczynski and Gilbert, unpublished data). The reason for the larval–pupal difference in microtubule effects on ecdysteroid synthesis is unknown. Note that neither study (Watson *et al.*, 1996; Rybczynski and Gilbert, unpublished data) explicitly monitored the effects of the microtubule disrupting drugs on cytoskeletal structures or cell shape. Note also that the role of the microtubule cytoskeleton in vertebrate steroidogenic cells is not clear. Basal steroidogenesis seems to be generally immune to microtubule-acting drugs while peptide hormone-stimulated steroidogenesis is variably inhibited or not, depending on the cell type and drug concentration (see Feuilloley and Vaudry, 1996). In systems where peptide hormone-stimulated steroidogenesis is inhibited, the data suggest that the pharmacological disruption of microtubules disturbs early events in the signal transduction cascade, e.g., receptor–G protein–cyclase interactions (see Feuilloloey and Vaudry, 1996), a hypothesis consistent with the above-mentioned ability of a cAMP analog to override such a disruption.

The identities of other PTTH-stimulated proteins remain unknown. Attempts to use antivertebrate StAR antibodies against *Manduca* prothoracic gland proteins were not interpretable, due to multiple protein cross-reactions, at a range of MWs, in immunoblot analyses (Rybczynski and Gilbert, unpublished data). Note that the predicted molecular weight of the *Drosophila* MLN64/StAR homolog is considerably larger (≈61 kDa) than the vertebrate molecule (≈30 kDa). PTTH-stimulated protein synthesis is not limited to specific proteins but can also be detected in overall rates of translation (Keightley *et al.*, 1990; Rybczynski and Gilbert, 1994; Kulesza *et al.*, 1994). The amount by which PTTH stimulates overall translation above basal appears to vary with the stage, with V_3 glands responding at about six times the rate of V_1 glands (Rybczynski and Gilbert, 1994). The incubation of nonstimulated prothoracic glands with ^{35}S-methionine revealed that the pattern of translated proteins varied among several stages in the fifth larval instar (Lee *et al.*, 1995); how these changes relate to changes in the ecdysteroidogenic capability of the prothoracic gland, or to PTTH signaling, is unknown. Unfortunately, a more detailed developmental analysis of protein synthesis, both basal and PTTH stimulated, by larval and pupal prothoracic glands remains to be done. As mentioned above, phosphorylation of ribosomal protein S6 preferentially increases the translation of mRNAs containing a 5′-polypyrimidine tract. The identification of these RNAs is undoubtedly incomplete but recent evidence reveals that in vertebrate cells many such RNAs code for ribosomal proteins (see Pearson and Thomas, 1995), suggesting that some of the unidentified PTTH-dependent translation products are also components of the ribosome.

PTTH stimulates transcription in prothoracic glands, based on the incorporation of radiolabeled uridine into acid-precipitable RNA in response to dibutyryl cAMP (Keightley *et al.*, 1990). Inhibition of transcription using actinomycin D resulted in a partial inhibition of both PTTH- and dibutyryl cAMP-stimulated ecdysteroid synthesis (Keightley *et al.*, 1990; Rybczynski and Gilbert, 1995b). These data suggested that transcription may be necessary for a maximal ecdysteroidogenic response to PTTH. However, actinomycin D had a larger effect on translation (≈50% inhibition in a 45-min incubation) than might be expected unless an unprecedented fraction of prothoracic gland proteins was translated from mRNAs with very short half-lives (Rybczynski and Gilbert, 1995b). In other words, the possibility that actinomycin D interfered with translation, as well as with transcription, cannot be ruled out, thus making it difficult to determine the role of transcription in acute increases of ecdysteroidogenesis.

cDNA clones for *Manduca* β1 tubulin and hsc 70 were obtained, with the hope that the expression pattern of these two genes might shed some light on their functions in PTTH signaling. In the prothoracic gland, β1 tubulin mRNA exhibited a peak in the fifth instar just before wandering (Rybczynski and Gilbert, 1998). β tubulin protein levels paralleled the mRNA profile during the fifth instar but in contrast to the mRNA profile, β tubulin protein abundance exhibited a second peak on day 2 (P_2) of pupal–adult development. These data might indicate that β tubulin protein levels are important to glands that are preparing to synthesize large amounts of ecdysteroids but do little to explain

the very rapid synthesis that occurs upon PTTH stimulation (Rybczynski and Gilbert, 1995b). Hsc 70 mRNA levels also showed a peak in the fifth instar, just before wandering (V_4), with lesser peaks on V_7 and P_2 (Rybczynski and Gilbert, 2000). Hsc 70 protein levels showed peaks on V_3, V_7, and P_0 levels, yielding a pattern of expression that does not clearly relate to either prothoracic gland growth or ecdysteroid synthesis. Although the profiles of β tubulin and hsc 70 mRNAs and proteins did not lend themselves to understanding the roles these gene products play in PTTH-stimulated ecdysteroid synthesis, analyses of brain and fat body indicated that the expression patterns of these products were tissue specific (Rybczynski and Gilbert, 1998, 2000). Complicating the question of determining what special roles β tubulin and hsc 70 might play in PTTH signaling in the prothoracic gland is the fact that both these proteins are very abundant and participate in various housekeeping duties in the cell. Discerning a PTTH-specific function against this background is difficult. Clearly, the topic of transcription in PTTH-stimulated ecdysteroid synthesis has not been exhausted and is worthy of further scrutiny.

3.2.4.6. Protein Phosphatases

The stimulation of ecdysteroidogenesis *in vivo* by PTTH is transient, and with varying biosynthetic rates, depending on the developmental stage. Given that PTTH stimulates the phosphorylation of a number of kinases and an unknown number of target proteins, it is reasonable to assume that downregulation of PTTH-stimulated ecdysteroid synthesis involves the activity of protein phosphatases. This topic has received scant attention in regard to the action of PTTH. Song and Gilbert (1996) used okadaic acid and calyculin, highly effective inhibitors of the serine/threonine-specific protein phosphatases 1 and 2A, to address this topic. They found that treatment of *Manduca* prothoracic glands with either of these inhibitors enhanced the phosphorylation of S6, and of several unidentified proteins, but that PTTH-stimulated phosphorylation of S6 was not augmented by a cotreatment with the PP inhibitors. However, although okadaic acid and calyculin increased S6 phosphorylation, these drugs inhibited both basal and PTTH-stimulated ecdysteroid and protein synthesis; included in the latter effect was the apparent inhibition of the syntheses of both β tubulin and hsc 70. Finally, Song and Gilbert (1996) found that PTTH stimulated phosphatase activity capable of dephosphorylating S6, and perhaps other proteins, and that this activity was inhibited by okadaic acid, implicating PP1 and/or PP2A in the downregulation of PTTH effects. The combined data indicated that the multiple phosphorylation of S6, by itself, was not sufficient to drive increased ecdysteroidogenesis. They also suggested that the nonphosphorylated state of one or more proteins was necessary for normal ecdysteroid synthesis, assuming that neither okadaic acid nor calyculin had effects on proteins other than phosphatases. That PTTH both stimulated S6 phosphorylation and dephosphorylation processes were expected given the likelihood that negative feedback loops exist in the prothoracic gland to restore its ecdysteroidogenic activity to basal levels.

The MAP kinases, like the ERK activated by PTTH stimulation (see Section 3.2.4.3), can be dephosphorylated by a number of phosphatases (see Camps *et al*., 2000). Dephosphorylation of ERKs at either the threonine or tyrosine sites results in loss of ERK kinase activity; PP2A and several tyrosine phosphatases, e.g., PTP-SL, STEP, and HePTP, have all been shown to inactivate vertebrate ERKs (see Camps *et al*., 2000). In addition, ERKs and other MAP kinases are dephosphorylated by unique dual-specificity phosphatases (DSPs) that remove phosphates from both threonine and tyrosine residues. These DSPs are probably the major agents of vertebrate ERK inactivation (see Camps *et al*., 2000). Mammals express at least 9 DSP genes and at least one homolog is found in *Drosophila* (D-mkp) (Cornelius and Engel, 1995). Currently, the contributions of various protein phosphatases to the regulation of prothoracic gland ERK phosphorylation are unknown. Serine/threonine phosphatases are likely regulators of proteins, like MEK, that are upstream from ERK and their role can be assessed, in part, by using well-characterized inhibitors like okadaic acid. Understanding the role of DSPs is more difficult and will require, *inter alia*, the further characterization of DSP-specific inhibitors that are currently under development (Vogt *et al*., 2003).

3.2.5. The Prothoracic Gland

The summary view of the PTTH signaling system presented in **Figure 7** is no doubt incomplete and will be subject to future additions and amendments. Among the factors deliberately left out of this scheme are those that cause changes in the prothoracic gland that are either independent of PTTH or that are PTTH dependent but not linked to the rapid regulation of ecdysteroidogenesis. In this section, an overview of these factors, both demonstrated and hypothesized, is presented.

3.2.5.1. Developmental Changes in the Prothoracic Gland

Current evidence indicates that the cells of the prothoracic gland either do not divide after embryonic life or do so very infrequently (see discussion in Rybczynski and Gilbert, 2000). Nevertheless, the gland is far from being a static tissue. A number of components in the PTTH transduction cascade change as the fifth larval instar progresses, as discussed in the previous section. These include an increase in cAMP phosphodiesterase activity, decrease in the ERK phosphorylation response, an increase in the amount of the regulatory subunit of PKA, and a change in calmodulin sensitivity, rendering pupal adenylate cyclase less responsive than the larval enzyme. In addition to these events, a number of additional developmental differences have been described. First, while cell number is constant, cell size changes dynamically and rapidly, accompanied by a number of structural changes. In *Manduca* and *Spodoptera*, cell size, i.e., diameter, is roughly correlated with ecdysteroidogenic capacity during the last larval instar, with large-celled glands being the most productive (Sedlak *et al*., 1983; Zimowska *et al*., 1985; Hanton *et al*., 1993). As expected, as cell size increases, the protein content of the gland also increases. In *Manduca*, total extractable and particulate protein increases from a minimum at the beginning of the fifth instar of ≈3 μg/gland to ±20 μg/gland on V_4; protein levels stay high through V_7 before reaching a low of 10–12 μg/gland on P_0 (Smith and Pasquarello, 1989; Meller *et al*., 1990; Rybczynski and Gilbert, 1995b). Protein content rises again through at least P_4 (Rybczynski and Gilbert, 1995b). Though size matters, the ecdysteroidogenic potential of prothoracic glands is not a simple function of cell size or protein content. An analysis of protein content and ecdysteroidogenesis in V_1 to V_3 *Manduca* fifth instar prothoracic glands revealed that basal and PTTH-stimulated synthesis per milligram of protein increased during this period; that is, ecdysteroid synthesis per gland increased faster than did the gland's total protein content (Rybczynski and Gilbert, 1994). A reanalysis of data gathered throughout the fifth instar of *Manduca* (provided by Smith and Pasquarello, 1989) confirms this observation, and revealed that a peak of basal ecdysteroid synthesis per unit protein occurred on V_4. Levels after V_4 declined but never reached the low seen on V_1. PTTH-stimulated synthesis per milligram of protein exhibited a different pattern. A peak was again seen on V_4 but levels were high and relatively similar from V_2 through V_7, followed by a dip on V_8 and V_9, and then a sharp rise to near V_4 levels on P_0. Note that PTTH-stimulated synthesis per milligram of protein was essentially the same on V_2 and V_3 using the data from Smith and Pasquarello (1989), while Rybczynski and Gilbert (1994) found V_2 levels to be considerably lower than V_3 levels.

An additional developmental change in prothoracic glands has been described for *Bombyx*. *Bombyx* prothoracic glands from the early fifth instar do not respond to PTTH stimulation with either increased ecdysteroid or cAMP synthesis, and are also distinguished by an exceedingly low basal level of steroid synthesis *in vitro* (Sakurai, 1983; Gu *et al*., 1996). However, these glands apparently respond with increased ecdysteroid synthesis to dibutyryl cAMP, suggesting that there is an upstream developmental "lesion" in one or more components of the PTTH signaling pathway (Gu *et al*., 1996, 1997). In contrast, Takaki and Sakurai (2003) found that dibutyryl cAMP did not elicit ecdysteroid synthesis in early fifth instar glands. The reason for the discrepancy in results is not known but might lie in methodological or strain-specific differences among the studies. Further work indicated that early fifth instar glands from *Bombyx* possess an adenylate cyclase that is much less responsive to Ca^{2+}/calmodulin stimulation than that characterized later in the instar when the gland is responsive to PTTH (Chen *et al*., 2001). It is not known if this cyclase difference is sufficient to explain fully the refractory nature of early fifth instar glands, nor is it known what underlies the cyclase behavior, i.e., are different adenylate cyclases present at different developmental stages? The larger question – why are *Bombyx* glands refractory while *Manduca* glands are not – is conjectural. The possibility that the *Bombyx* condition is a consequence of its domestication cannot be excluded.

These observations showing developmental changes in the signaling and biosynthetic components of the prothoracic gland raise an obvious question. What factor(s) controls the growth and differentiation state of the gland? At present, we can only speculate. PTTH is, of course, a candidate. Functionally homologous vertebrate peptide hormones, acting alone or in concert with other factors, often have a differentiation and mitogenic effect on their target tissues in addition to their steroidogenic activity (see Adashi, 1994; Richards, 1994; Richards *et al*., 2002). Presently, two lines of evidence suggest that PTTH may have nonecdysteroidogenic effects on the prothoracic gland. First, PTTH hemolymph titers in *Bombyx* showed a pattern not completely consonant with ecdysteroidogenesis, indicating other roles for this peptide

hormone (see Section 3.2.3.4). Second, PTTH stimulates two categories of protein synthesis in the *Manduca* prothoracic gland (Rybczynski and Gilbert, 1994). One class of proteins comprised a small group of molecules whose translation appears to be linked to PTTH-stimulated ecdysteroidogenesis and presumably includes one or more proteins that are necessary for PTTH-stimulated increases in steroid synthesis (see also Song and Gilbert, 1995). The second class of translation products was less well defined and might include most, if not all, house-keeping mRNAs in the prothoracic gland. Translation of this group of proteins was detected by increases in the acid-precipitable, radiolabeled proteins and not by particular pattern changes discernible in protein bands following SDS-PAGE. This second class of translation products has been considered to reflect a trophic, nonsteroidogenic effect of PTTH (Rybczynski and Gilbert, 1994) and does not appear to be an obligate result of PTTH stimulation of prothoracic glands. For instance, PTTH stimulation of *Manduca* V_1 glands did not measurably increase overall protein synthesis while the increases in translation of specific proteins and in ecdysteroid synthesis were readily seen (Rybczynski and Gilbert, 1994). Furthermore, the Ca^{2+} ionophore A23187 stimulated ecdysteroid and specific protein synthesis in V_2 and V_3 glands but had no effect (V_2) or an inhibitory effect (−50%: V_3) on general, trophic protein synthesis. Simultaneous stimulation with both A23187 and dibutyryl cAMP, admittedly a pharmacologically complex situation, likewise stimulated both ecdysteroid and specific protein synthesis while decreasing general protein synthesis below basal on all 3 days studied (V_1–V_3) (Rybczynski and Gilbert, 1994). Whether the trophic effect of PTTH occurs beyond stages V_2 and V_3 is currently unknown; it would not be surprising if the effect was minimal during periods when the prothoracic gland is not growing appreciably, e.g., V_5 to P_0.

It seems likely that PTTH also stimulates transcription in the prothoracic gland that is not linked to acute regulation of ecdysteroid synthesis. Again, if parallels between vertebrate and insect steroidogenic tissues hold true, it is expected that PTTH stimulation of prothoracic glands would upregulate transcription of mRNAs that code for the enzymes that synthesize ecdysteroids from cholesterol, as well as ancillary, supportive proteins like adrenodoxin reductase (see Simpson and Waterman, 1988; Orme-Johnson, 1990; **Chapter 3.3**). The recent cloning of several of the cytochrome P450s that are involved in ecdysteroid synthesis in *Drosophila* should allow this issue to be addressed in the near future (see **Chapter 3.3**). This transcriptional trophic effect, like that of translation, may be limited to certain developmental periods. A lack of effect would not be surprising in the period immediately preceding prothoracic gland apoptosis in those species where such programmed cell death occurs (see Section 3.2.5.4).

3.2.5.2. Regulators of the Prothoracic Gland other than PTTH

The prothoracic gland, like other internal organs, is bathed in hemolymph and is potentially regulated by any number of the hormones found in the hemolymph, in addition to PTTH. The influence of the juvenile hormones has been discussed (see Section 3.2.3.3; and **Chapters 3.7**, **3.9**, **3.11**, and **3.12**) but it bears repeating that the evidence is weak for direct regulation of prothoracic gland physiology by JHs. Aside from JHs and the ecdysteroids that are covered in Section 3.2.5.3, most, if not all, of these hormones are peptides or small proteins (see **Chapter 3.10**).

Several prothoracicotropic factors, which were partially purified from lepidopteran proctodaea, are discussed briefly in Section 3.2.2.5. Rather more far afield was the observation that an ovarian-derived dipteran oostatic factor (trypsin modulating oostatic factor or TMOF) appears to exert dose-dependent stimulatory and inhibitory effects on ring gland and prothoracic gland basal and PTTH-stimulated ecdysteroid production (Hua and Koolman, 1995; Hua *et al*, 1999; Gelman and Borovsky, 2000). The ecdysiostatic effect of TMOF, a hexapeptide, on *C. vicina* ring glands was attributed to a TMOF-dependent rise in cAMP, with the latter being found to be inhibitory of basal ecdysteroidogenesis (Hua and Koolman, 1995). However, an earlier study showed that dibutyryl cAMP stimulated *Calliphora* ring gland ecdysteroidogenesis (Richard and Saunders, 1987) and it is not currently possible to reconcile these conflicting observations about cAMP. How TMOF might exert a prothoracicotropic effect rather than an ecdysiostatic effect under some conditions is also not known. Thus, these observations are intriguing but the *in vivo* significance of TMOF in regard to prothoracic gland function remains to be rigorously determined.

Another small molecule, a nonapeptide termed prothoracicostatic peptide (PTSP), has been purified from *Bombyx* brain (Hua *et al*., 1999). This peptide, identical in sequence to a myoinhibitory peptide first isolated from *Manduca* ventral nerve cord (Mas-MIP I: Blackburn *et al*., 1995), was able to inhibit basal (Hua *et al*., 1999; Dedos *et al*., 2001) and

PTTH-stimulated (Hua *et al.*, 1999) ecdysteroidogenesis *in vitro* in a dose-dependent manner. PTSP appears to block "basal" Ca^{2+} influx via dihydropyridine-sensitive channels (Dedos *et al.*, 2001; Dedos and Birkenbeil, 2003) but PTSP did not block PTTH-stimulated Ca^{2+} influx (Dedos and Birkenbeil, 2003). It is difficult to reconcile the latter observation with the finding that PTSP blocked PTTH-stimulated ecdysteroid synthesis (Hua *et al.*, 1999), given that PTTH-stimulated ecdysteroid synthesis requires Ca^{2+} influx. Furthermore, rather large doses were required ($\geq$100 nM) to achieve an ecdysteroidogenic inhibition relative to the dose needed for myoinhibition (1 nM; Blackburn *et al.*, 1995). Davis *et al.* (2003), using immunohistochemical techniques, have found that an apparent release of MIP I (PTSP) from *Manduca* epitracheal glands appears to coincide with the rapid drop in ecdysteroid hemolymph titer that occurs just prior to larval ecdysis. This intriguing observation deserves confirmation, e.g., via studies under long-day conditions, where this ecdysteroid decline occurs approximately 24 h before ecdysis, or in the fifth instar, where ecdysteroid levels begin a fall several days before pupal ecdysis (Bollenbacher *et al.*, 1981; Grieneisen *et al.*, 1993). Given these data calling into question the *in vivo* function of this peptide, it seems safest to consider PTSP a candidate prothoracicostatic factor, pending further studies. If PTSP does occur *in vivo* at high nanomolar concentrations, it is likely to have wide effects beyond the prothoracic gland, i.e., many cell types undoubtedly express dihydropyridine-sensitive Ca^{2+} channels the inhibition of which would affect cellular function throughout the organism.

The changing electrical properties of prothoracic glands during diapause were discussed above in regard to *Manduca* PTTH (see Section 3.2.3.6). However, the electrical properties of prothoracic glands may be important in another context, i.e., as a factor in species wherein the prothoracic gland is innervated. Innervation of prothoracic glands is widely distributed among taxa, but it is not clear that it is a universal feature within any given insect group, e.g., the Lepidoptera contain species with and without innervation (see Sedlak, 1985; references in Okajima and Watanabe, 1989). The role of innervation in chronic or acute regulation of ecdysteroid synthesis has not been well studied. In *P. americana*, the activity of a nerve originating from the prothoracic ganglion was correlated positively with the secretion of ecdysteroids from the prothoracic gland (Richter and Gersch, 1983) but experimental discharge of this nerve did not have a significant effect on ecdysteroidogenesis (Richter, 1985). Sectioning the prothoracic gland nerve of last instar larvae prevented the normal ecdysteroid peak seen between days 20 and 24 but the subsequent larger peak seen after day 26 was unaffected. These observations suggest a bimodal control of *Periplaneta* ecdysteroid synthesis in this instar, with the second peak presumed to be controlled solely by PTTH. However, a role for PTTH in the first peak cannot be ruled out, i.e., neuronal input could control the reactive state of the gland, in regard to PTTH, without directly regulating ecdysteroidogenesis.

In the moth *Mamestra*, the neurons innervating the prothoracic gland are inhibitory in regard to basal ecdysteroid synthesis but their effect on PTTH-stimulated synthesis is not known (Okajima and Watanabe, 1989; Okajima and Kumagai, 1989; Okajima *et al.*, 1989). Conflicting data on the role of innervation complicates any attempt to generalize at this time. For instance, Alexander (1970) proposed an inhibitory role in *Galleria* for the prothoracic gland nerve originating in the subesophageal ganglion while Mala and Sehnal (1978) argued for an excitatory (steroidogenic) influence. Neuronal input to the prothoracic gland may also originate in the brain. In both *S. crassipalpis* and *Drosophila*, species possessing the compound endocrine structure termed the ring gland, histochemical studies using back-filling techniques and reporter genes revealed that cells of the lateral protocerebrum directly innervate the ring gland (Giebultowicz and Denlinger, 1985; Siegmund and Korge, 2001). In the case of *Drosophila*, the identified cells appear to be neurosecretory, based on size and distinctive morphology. In *Sarcophaga*, several of these cells, presumably also neurosecretory, terminated in the corpus allatum region of the ring gland, as found for the PTTH-producing cells of lepidopterans. *Drosophila*, like *Sarcophaga*, contained several cells in the lateral protocerebrum that innervated the corpus allatum region. However, *Drosophila* also contained a pair of cells that innervated the prothoracic gland region of the ring gland. This observation suggests that if the neuronal cells in question regulate ecdysteroid synthesis, such regulation may not proceed as in the Lepidoptera, i.e., a soluble PTTH, released into the general circulation, might not be needed. In fact, a *Drosophila* PTTH with homology to the lepidopteran molecules might be completely lacking and its function taken over by another signaling molecule, perhaps even one that is considered a conventional neurotransmitter.

Clearly, the part that prothoracic gland innervation plays in affecting steroid synthesis deserves further attention. At present, based on structural studies, it seems likely that direct nervous input

from ganglia is primarily via peptides rather than neurotransmitters (see Sedlak, 1985). Peptidergic input may reflect a chronic or modulatory role for neuronal input in the control of prothoracic gland function; the possibility that such peptides may serve as trophic factors important for gland survival as seen for vertebrate nerve growth factor (see Levi-Montalcini *et al.*, 1996) is an intriguing and unexplored topic.

The changes in prothoracic gland cell size, protein content, and ecdysteroidogenic potential that have been documented during larval and pupal–adult development may be influenced heavily by the trophic influences of PTTH and, in some species, neurotrophic factors, as discussed above. Additional strong candidates for the regulation of prothoracic gland biology during development are the insect insulin-like proteins (ILPs). As discussed earlier (see Section 3.2.2.5), insects have a number of such genes (seven in *Drosophila*; Brogiolo *et al.*, 2001) and at least four lines of evidence suggest that one or more ILPs might affect prothoracic gland biology. First, it has been known for decades that insulin is helpful or even necessary for the growth (division) of some insect cells in culture (Mosna and Barigozzi, 1975; Davis and Shearn, 1977). Second, the cells of the prothoracic gland undergo large changes in size during development and the insect insulin pathway is involved in regulating cell size, not only cell cycling. For instance, a mutation in the *Drosophila* insulin receptor substrate gene *Chico* yields flies that are small because their cells are small (see Goberdhan and Wilson, 2003). Third, antibody studies suggest that the prothoracic gland expresses an insulin receptor (Smith *et al.*, 1997; see also Section 3.2.4.3). Fourth, there is precedence for insulin control of ecdysteroid synthesis, although in the mosquito ovary and not the prothoracic gland (Graf *et al.*, 1997; Riehle and Brown, 1999). The idea that insulin affects prothoracic glands in some way must be tested rigorously. Especially useful will be the presumed future availability of authentic insect insulin-like molecules, their use in well-characterized *in vitro* experimental regimes, and the determination of the titers of insulin-like proteins in the hemolymph during larval and pupal–adult development.

3.2.5.3. Ecdysteroid Feedback on Ecdysteroidogenesis in the Prothoracic Gland

The rise and fall of circulating hormone levels is characteristic of many endocrine signaling events, including the coordination of larval and pupal–adult development of insects by ecdysteroids (see **Chapter 3.8**). Several processes contribute to the regulation of circulating levels of active hormone, e.g., secretion rates, metabolism to inactive forms, sequestration, and excretion. The secretion rate is itself the product of multiple variables, namely: substrate availability, maximum biochemical synthesis rates, stimulation of the producing tissue by a trophic factor, product storage, presence of inhibitory factors, product feedback, etc.

Ecdysteroid feedback on the prothoracic gland, both positive and negative, can proceed through at least two paths. First, ecdysteroids might directly interact with the prothoracic gland, potentially resulting in rapid or slow changes in the PTTH signaling cascade or the ecdysteroid biosynthetic pathway. Second, ecdysteroids might act indirectly, by modifying the behavior of another tissue, which then, through a nonecdysteroid pathway, modulates the biology of the prothoracic gland.

Only a limited amount of data has been gathered to test the hypothesis that the prothoracic gland is subject to both rapid and delayed feedback from ecdysteroids. Sakurai and Williams (1989) showed that incubating larval *Manduca* prothoracic glands for 24 h with low concentrations of 20E (e.g., 0.20 μM) resulted in an inhibition of 20% to 60% of basal ecdysteroid synthesis. In contrast, pupal glands generally experienced a stimulation of synthesis. This was true of prothoracic glands from both nondiapausing and diapausing pupae but the effect of PTTH on these glands was not evaluated. A study of the effect of shorter 20E incubations on basal and PTTH-stimulated *Manduca* glands revealed that when a higher dose (10 μM) of 20E was employed a very significant inhibition of basal ecdysteroidogenesis (≈75%) was seen after only 1 h of incubation (Song and Gilbert, 1998); note that in these experiments, prothoracic glands were preincubated with exogenous 20E but their ecdysteroid production was measured subsequently in medium lacking the supplemental 20E. Somewhat surprisingly, this regime of pretreatment with 20E had no effect on PTTH-stimulated ecdysteroid synthesis, relative to controls not incubated with 10 μM 20E, unless the preincubation was for more than 3 h. These observations suggest that the repression of basal ecdysteroid synthesis after short incubations was due to a reversible block in the steroidogenic pathway that could be overcome by PTTH stimulation. What constitutes this block is open to speculation. The data currently in hand suggest that the PTTH signal transduction pathway is not measurably active in regulating steroidogenesis in the absence of PTTH. Perhaps the rapid 20E effect on basal steroidogenesis was due to direct, competitive inhibition by 20E of some step in ecdysteroid

synthesis. This possibility could be tested by using lower concentrations of 20E and non-steroidal compounds that bind to the ecdysone receptor but that would not be expected to interact with the biosynthetic enzymes involved in ecdysteroidogenesis (see **Chapters 3.3** and **3.4**). In fact, Jiang and Koolman (1999) used the non-steroidal ecdysone agonist RH-5849 in such a test, using *C. vicina* ring glands. Their results revealed that RH-5849 inhibited basal ecdysone secretion rapidly, with more than 50% inhibition seen within 30 min of incubation. These data suggest that ecdysteroid feedback on the prothoracic gland involved regulation of transcription or nongenomic effects, perhaps mediated through second messenger signaling initiated via a plasma membrane ecdysteroid receptor (see **Chapter 3.7**). How such signaling might interact with the basal synthesis pathway is unknown; it is possible that RH-5849 interferes with the synthesis pathway in a manner unrelated to its ecdysone agonist effect.

Longer incubations (>3 h) with 20E, resulted in the downregulation of steroidogenesis which was not fully reversible with PTTH (brain extract) treatment (Song and Gilbert, 1998). This effect seems likely to be due to changes in transcription and translation, and this hypothesis should be testable using newly, or soon-to-be, available molecular probes against the transcripts coding for the ecdysteroidogenic enzymes (see **Chapter 3.3**). Incubation of *Manduca* prothoracic glands with 10 μM 20E altered the expression pattern of USP proteins within 6 h (Song and Gilbert, 1998). USP is an obligate partner to the EcR protein in forming the functional ecdysteroid receptor (see **Chapter 3.5**), and this change might be a sign of an altered transcription pattern in the gland. The possibility that components of the PTTH signaling pathway were also downregulated cannot be ruled out and this supposition could also be tested using molecular probes, or by using commercially available antibodies to assess protein levels and the phosphorylation states thereof.

The above data suggested that 20E levels directly regulated basal and PTTH-stimulated ecdysteroid synthesis by the prothoracic gland. An indirect type of 20E feedback on prothoracic gland activity, involving one or more intermediate tissues, is also a possibility. In *Bombyx*, early fifth instar glands are refractory to PTTH stimulation *in vitro* (see Sections 3.2.3.3 and 3.2.5.1; and see **Chapter 3.8**). Injection of 0.5–4.0 μg 20E into intact *Bombyx* larvae also resulted in partial inhibition of basal ecdysteroid synthesis at the higher doses, when glands from treated animals were later assayed *in vitro* (Takaki and Sakurai, 2003), but a consistent effect of 20E injection on PTTH-stimulated ecdysteroid synthesis was not seen. However, when the brain and corpora allata were removed from larvae prior to control (water) or 20E injections, somewhat different results were obtained. First, at stages where basal ecdysteroid synthesis was expected to have declined between the treatment and sampling periods in response to exogenous 20E, such declines were absent or blunted. Second, when such glands were challenged with PTTH, the gland activation was larger than expected. These results suggested that 20E might downregulate prothoracic gland activity via stimulating the release of an inhibitory brain or corpus allatum factor (see Section 3.2.5.2). Whether or not both direct and indirect feedback of 20E on prothoracic glands occurs in nonmanipulated animals is an open question. The effects seen upon removal of the brain and corpora allata are suggestive, but this operation removes a Pandora's box of cell types, neuronal connections, and hormones, and the results stemming from such an operation must be interpreted with care.

3.2.5.4. Apoptosis of the Prothoracic Gland

Programmed cell death or apoptosis is a normal component of development in multicellular organisms, including insects, and involves a complex, multistep cascade of intracellular events (see Vernooy *et al*., 2000). The only demonstrated function of the prothoracic glands is to produce ecdysteroids and thus to control and coordinate the molting process. It is not surprising, therefore, that prothoracic glands undergo apoptosis during pupal–adult development or early in adult life, once sufficient ecdysteroids have been produced to accomplish this last molt. Programmed cell death of the prothoracic glands occurs in insects that possess a structurally distinct gland, like Lepidoptera, as well as in species possessing a ring gland, where the prothoracic gland is part of a composite, multi-tissue organ.

In *Drosophila*, the degeneration of the prothoracic gland portion of the ring gland during pupal–adult development takes place at a time when whole animal ecdysteroid titers are high and production of ecdysteroids *in vitro* is low (Dai and Gilbert, 1991). This observation led to the suggestion that the source of ecdysteroids at this time was either another tissue, e.g., oenocytes, or the conversion of previously produced hormone from a inactive, conjugate to an active form, with the conjugate not being recognized by the radioimmunoassay employed to assess steroid hormone levels (Dai and Gilbert, 1991). Given the uncertainties about

the control of ecdysteroid synthesis in *Drosophila* discussed earlier in this chapter, it is also possible that the production *in vitro* is not an accurate index of steroidogenesis *in vivo*; however, this hypothesis presupposes that a prothoracic gland that is undergoing apoptosis is still capable of considerable ecdysteroid synthesis.

The structural and hormonal correlates of the programmed cell death of the *Manduca* prothoracic gland, a "free-standing" tissue, have been addressed by Dai and Gilbert (1997, 1998, 1999). In this species, apoptosis of the gland was initiated on day 6 (P_6) of pupal–adult development (Dai and Gilbert, 1997). P_6 is approximately 2 days after the pupal–adult peak of ecdysteroidogenesis and is a time when fat body and hemocytes begin to envelop the gland (Dai and Gilbert, 1997). Ecdysteroid synthesis fell rapidly after P_6, as the glands continued to degenerate, and by P_{14}, only cellular debris was present.

The external factors controlling apoptosis of the prothoracic gland have been partially determined. The absence of juvenile hormones at the beginning of pupal–adult development was originally believed to be an enabling condition (Wigglesworth, 1955; Gilbert, 1962) and subsequent data supported this proposal, i.e., injection of JH into pupae at this stage prevented gland death (Gilbert, 1962; Dai and Gilbert, 1997). The absence of JH was not sufficient to initiate apoptosis because prothoracic glands extirpated from early pupal stages and placed in JH-free culture conditions do not undergo apoptosis (Dai and Gilbert, 1999). Nevertheless, a hormonal basis for gland apoptosis was indicated because the addition of 20E to these cultures efficiently promoted programmed cell death, if the steroid exposure was $\geq$24 h. The 20E doses found to be effective *in vitro* were well within the physiological range seen in the days preceding normal, *in vivo* cell death. These cultured-gland experiments also revealed an extra level of complexity in the control of prothoracic gland apoptosis, namely, that JH did not protect glands *in vitro* against 20E-induced cell death (Dai and Gilbert, 1999). Thus, once again, the role of JHs in modulating prothoracic gland physiology was indirect, involving another tissue and probably another hormone. One might also conclude that 20E-induced apoptosis of the prothoracic gland represents the ultimate negative feedback loop for this steroid hormone-producing gland.

3.2.5.5. Parasitoids and Ecdysteroidogenesis

Insect parasitoids lay their eggs on or in host insects. Following hatching, the parasitoid develops until finally emerging, with the host generally dying as a result of the infection. Parasitoids manipulate the physiology of the host organism to their advantage and this often includes disrupting the normal function of the host's endocrine system (see Beckage, 1985, 1997, 2002). It must also be pointed out that parasitoid infections are biologically complex, involving not only the parasitoid, but also ovarian proteins, symbiotic viruses, and venoms. These additional factors can have major effects on the host's physiology, even in the absence of the parasitoids.

Parasitoids utilize a wide variety of tactics to increase the suitability of their hosts as growth chambers and even a modest review of the subject is beyond the constraints of this chapter (see Beckage, 1985, 1997, 2002). However, several endocrine effects are frequent enough to deserve mention in the context of considering PTTH. One of the most common effects of parasitoid infestation appears to be elevated JH levels. These high titers result in developmental arrest of the host and stem from multiple factors, including increased secretion by the host, decreased degradation, and JH secretion by the parasitoid and perhaps by parasitoid-derived teratocytes (Cole *et al.*, 2002). Elevated levels of JH may act partly through blocking PTTH release since increased levels of PTTH in brains of parasitized *Manduca* have been observed (Žitňan *et al.*, 1995). In some Lepidoptera–parasitoid interactions, the prothoracic gland disintegrates, e.g., *H. virescens* parasitized by *Campoletis sonorensis*, an ichneumonid wasp (Dover and Vinson, 1990; Dover *et al.*, 1995), while in other species, the gland persists. In prothoracic glands from *Manduca* parasitized by *Cotesia congregata*, a braconid wasp, both basal and brain extract-stimulated ecdysteroid synthesis *in vitro* were lower than synthesis from glands of nonparasitized larvae (Kelly *et al.*, 1998). This is strongly reminiscent of the effect of experimentally augmented JH levels on prothoracic glands discussed in Section 3.2.3.3, and is consistent with a view that this parasitism interaction, which blocks development in the fifth instar, is essentially a juvenalizing event. Evidence from *in vitro* studies suggested that a portion of the elevated JH in this example was secreted by the parasitoid and did not simply result from decreased JH catabolism (Cole *et al.*, 2002). *In vivo* ecdysteroid levels at this time were not strongly suppressed relative to control early fifth instar larvae, but the high premolt peak seen normally in the second half of the fifth instar was absent. Ligation experiments revealed that it was likely that some ecdysteroids were secreted by the parasitoid *Cotesia* (Gelman *et al.*, 1998). A stronger suppression of ecdysteroid hemolymph titer has been described for precocious prepupae of

Trichoplusia ni parasitized by the wasp *Chelonus* near *curvimaculatus* (Jones *et al*., 1992); in this interaction, an ecdysteroid peak observed shortly before the emergence of the parasitoid larvae was also believed to originate with the parasitoid.

Evidence that parasitism has a direct effect on the functioning of the prothoracic gland comes primarily from work on *H. virescens* parasitized by the braconid wasp *Cardiochiles nigriceps*. In this interaction, basal ecdysteroid synthesis by the glands was considerably depressed when measured *in vitro* (Pennacchio *et al*., 1997, 1998a); JH levels were elevated and JH metabolism depressed in parasitized larvae during the first 4 days of the final larval instar (Li *et al*., 2003). Such glands showed no activation when challenged with a crude PTTH extract or forskolin (an adenylate cyclase activator) or dibutyryl cAMP; but all three reagents stimulated ecdysteroid synthesis in control glands. Further investigation revealed several biochemical lesions downstream from cAMP generation (Pennacchio *et al*., 1997, 1998a), including depressed basal protein synthesis and phosphorylation and a failure to increase protein phosphorylation and synthesis upon PTTH stimulation. No difference in RNA synthesis was found between control and parasite-derived glands (Pennacchio *et al*., 1998a). Surprisingly, the effects of *Cardiochiles* parasitism on the prothoracic gland proved to be largely the result of coinfection with the *C. nigriceps* polydnavirus that is injected into the host along with the *Cardiochiles* eggs and venom (Pennacchio *et al*., 1998b). Venoms also play a role in parasitoid manipulation of host prothoracic glands. The venom of the ectoparasitic wasp *Eulophus pennicornis*, when injected into larvae of the tomato moth (*Lacanobia oleraceae*) results in depressed basal ecdysteroid synthesis by prothoracic glands when assayed 48 h later and, furthermore, such glands failed to respond to forskolin with increased steroidogenesis (Marris *et al*., 2001). However, incubation of prothoracic glands with venom extract for 3 h had no effect. This observation suggests that either venom requires a longer time to disrupt the PTTH transduction cascade, or that an intermediate tissue is involved in the venom effect.

These and similar observations suggest that the roles of viruses and venoms in manipulating host endocrine systems can be great. How parasitoids and their coevolved, symbiotic viruses influence the PTTH-prothoracic gland axes of their hosts is far from resolved and might well involve a number of tactics that include altering hormone synthesis and prohormone conversion by their hosts (Beckage, 1985), hormone release, and feedback systems.

3.2.6. PTTH: The Future

At the end of a review article, it is both a cliché and a truism to write phrases like "While we have learned a great deal about this topic in the last few years, there is clearly much left to be understood," and "Although we have obtained the answers to many questions about this topic, the answers have raised even more questions." These comments are certainly true about PTTH, and many unanswered and new questions about PTTH have been raised in this review. Finally, a few of these questions will be iterated and a few new ones added. The answers to these questions will require not only new methodologies and model systems, but also new ways of looking at familiar topics.

What we know about PTTHs derives chiefly from studies of Lepidoptera. Are there closely related molecules in all or most other taxa, or do other pathways function, e.g., total neuronal control of ecdysteroid synthesis? Similarly, our knowledge of PTTH signaling within the prothoracic gland comes almost entirely from lepidopteran studies. Are other, significantly different control mechanisms used? Certainly, the work on putative PTTH-containing extracts from *Drosophila* suggests that the control of ecdysteroid synthesis by hormones originating outside the prothoracic gland might proceed by novel means.

Even within the scope of lepidopteran studies, a great deal of the control pathway activated by PTTH remains to be determined. We have little knowledge of negative feedback mechanisms, and the function of the developmental changes seen in prepupal prothoracic glands is currently mainly a source of speculation. The necessity for new protein synthesis in PTTH-stimulated ecdysteroidogenesis is well established but the role of such protein(s) has not been determined. If one of these proteins facilitates steroid precursor movement, as in vertebrate steroidogenesis, what do the other proteins do? Another question concerns the complexity of PTTH signal transduction (see **Figure 7**). If the prothoracic gland's only function is to make ecdysteroids, why is the PTTH signaling cascade, which we know imperfectly, so complex? Are the multiple kinase networks simply cellular fossils that reflect a lack of selection to simplify, with layer upon layer of control mechanisms, as evolution sharpened the control of steroid synthesis?

If there might be proposed one final "big" question about PTTH, perhaps the best candidate would be "What else does PTTH do?" *Bombyx* PTTH was first purified from the heads of adult animals, long

after the prothoracic glands have disintegrated via programmed cell death. Is this PTTH reservoir an artifact of the domestication of *Bombyx*, or does PTTH modulate the physiology of other cell types, steroidogenic or not?

Insects comprise a very large fraction of the animal world with which mankind must coexist and compete with for the world's resources. We can only hope that the human resources to study these questions keep pace with our need to understand and adapt to this amazing class of animals.

References

Abraham, R.T., **2002**. Identification of TOR signaling complexes: more TORC for the cell growth engine. *Cell 111*, 9–12.

Adachi-Yamada, T., Iwami, M., Kataoka, H., Suzuki, A., Ishizaki, H., **1994**. Structure and expression of the gene for the prothoracicotropic hormone of the silkmoth *Bombyx mori*. *Eur. J. Biochem. 220*, 633–643.

Adashi, E.Y., **1994**. Endocrinology of the ovary. *Hum. Reprod. 9*, 815–827.

Agui, N., **1989**. *In vitro* release of prothoracicotropic hormone (PTTH) from the cultured brain of *Mamestra brassicae* L.; effects of neurotransmitters on PTTH release. In: Mitsuhashi, J. (Ed.), Invertebrate Cell System Applications, vol. 1. CRC, Press Boca Raton, pp. 111–119.

Agui, N., Bollenbacher, W.E., Gilbert, L.I., **1983**. *In vitro* analysis of prothoracicotropic hormone specificity and prothoracic gland sensitivity in Lepidoptera. *Experientia 39*, 984–988.

Agui, N., Bollenbacher, W.E., Granger, N.A., Gilbert, L.I., **1980**. Corpus allatum is release site for insect prothoracicotropic hormone. *Nature 285*, 669–670.

Agui, N., Granger, N.A., Bollenbacher, W.E., Gilbert, L.I., **1979**. Cellular localization of the insect prothoracicotropic hormone: *In vitro* assay of a single neurosecretory cell. *Proc. Natl Acad. Sci. USA 76*, 5694–5698.

Aizono, Y., Endo, Y., Sattelle, D.B., Shirai, Y., **1997**. Prothoracicotropic hormone-producing neurosecretory cells in the silkworm, *Bombyx mori*, express a muscarinic acetylcholine receptor. *Brain Res. 763*, 131–136.

Alexander, N.J., **1970**. A regulatory mechanism of ecdysone release in *Galleria mellonella*. *J. Insect Physiol. 16*, 271–276.

Allegret, P., **1964**. Interrelationship of larval development, metamorphosis and age in a pyralid lepidopteran, *Galleria mellonella* (L.), under the influence of dietetic factors. *Exp. Gerontol. 1*, 49–66.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., **1990**. Basic local alignment search tool. *J. Mol. Evol. 215*, 403–410.

Arbeitman, M.N., Hogness, D.S., **2003**. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell 101*, 67–77.

Bain, J., McLauchlan, H., Elliott, M., Cohen, P., **2003**. The specificities of protein kinase inhibitors: an update. *Biochem. J. 371*, 199–204.

Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity during development of fifth stadium *Manduca sexta*. *Insect Biochem. 17*, 989–996.

Bean, D.W., Beck, S.D., **1983**. Haemolymph ecdysteroid titres in diapause and nondiapause larvae of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 29*, 687–693.

Beckage, N.E., **1985**. Endocrine interactions between endoparasitic insects and their hosts. *Annu. Rev. Entomol. 30*, 371–413.

Beckage, N.E., **1997**. New insights: how parasites and pathogens alter the endocrine physiology and development of insect hosts. In: Beckage, N.E. (Ed.), Parasites and Pathogens: Effects on Host Hormones and Behavior. Chapman and Hall, New York, pp. 3–36.

Beckage, N.E., **2002**. Parasite- and pathogen-mediated manipulation of host hormones and behavior. In: Pfaff, D., Arnold, A., Etgen, A., Fahrbach, S., Rubin, R. (Eds.), Hormones, Brain, and Behavior, vol. 3. Academic Press, New York, pp. 281–315.

Beckel, W.E., Friend, W., **1964**. The relation of abdominal distension and nutrition to molting in *Rhodnius prolixus* (Stahl) (Hemiptera). *Can. J. Zool. 42*, 71–78.

Berridge, M.J., Lipp, P., Bootman, M.D., **2000**. The versatility and universality of calcium signalling. *Nat. Rev.: Mol. Cell Biol. 1*, 11–21.

Biggs, III, W.H., Zipursky, S.L., **1992**. Primary structure, expression, and signal-dependent tyrosine phosphorylation of a *Drosophila* homolog of extracellular signal-regulated kinase. *Proc. Natl Acad. Sci. USA 89*, 6295–6299.

Birkenbeil, H., **1996**. Involvement of calcium in prothoracicotropic stimulation of ecdysone synthesis in *Galleria mellonella*. *Arch. Insect Biochem. Physiol. 33*, 39–52.

Birkenbeil, H., **1999**. Intracellular calcium in PTTH-stimulated prothoracic glands of *Manduca sexta* (Lepidoptera: Sphingidae). *Eur. J. Entomol. 96*, 295–298.

Birkenbeil, H., **2000**. Pharmacological study of signal transduction during stimulation of prothoracic glands from *Manduca sexta*. *J. Insect Physiol. 46*, 1409–1414.

Birkenbeil, H., Dedos, S.G., **2002**. Ca^{2+} as second messenger in PTTH-stimulated prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 32*, 1625–1634.

Blackburn, M.B., Wagner, R.M., Kochansky, J.P., Harrison, D.J., Thomas-Laemont, P., *et al.*, **1995**. The identification of two myoinhibitory peptides, with sequence similarities to the galanins, isolated from the ventral nerve cord of *Manduca sexta*. *Regulat. Pept. 57*, 213–219.

Böhm, S.K., Grady, E.F., Bunnett, N.W., **1997**. Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem. J. 322*, 1–18.

Bollenbacher, W.E., Agui, N., Granger, N.A., Gilbert, L.I., **1979**. *In vitro* activation of insect prothoracic glands by the prothoracicotropic hormone. *Proc. Natl Acad. Sci. USA 76*, 5148–5152.

Bollenbacher, W.E., Gilbert, L.I., **1981**. Neuroendocrine control of postembryonic development in insects. In: Farner, D.S., Lederi, K. (Eds.), Neurosecretion. Plenum, New York, pp. 361–370.

Bollenbacher, W.E., Granger, N.A., **1985**. Endocrinology of the prothoracicotropic hormone. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, New York, pp. 109–151.

Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., **1987**. Developmental endocrinology of larval moulting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 128*, 175–192.

Bollenbacher, W.E., Katahira, E.J., O'Brien, M.A., Gilbert, L.I., Thomas, M.K., *et al.*, **1984**. Insect prothoracicotropic hormone: evidence for two molecular forms. *Science 224*, 1243–1245.

Bollenbacher, W.E., O'Brien, M.A., Katahira, E.J., Gilbert, L.I., **1983**. A kinetic analysis of the action of the insect prothoracicotropic hormone. *Mol. Cell. Endocrinol. 32*, 27–46.

Bollenbacher, W.E., Smith, S.L., Goodman, W., Gilbert, L.I., **1981**. Ecdysteroid titer during larval-pupal development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 44*, 302–306.

Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., **1984a**. *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *Manduca sexta*. *J. Exp. Biol. 108*, 9–24.

Bowen, M.F., Gilbert, L.I., Bollenbacher, W.E., **1986**. Endocrine control of insect diapause: an *in vitro* analysis. *In Vitro Invert. Horm. Genes C210*, 1–14.

Bowen, M.F., Saunders, D.S., Bollenbacher, W.E., Gilbert, L.I., **1984b**. *In vitro* reprogramming of the photoperiodic clock in an insect brain-retrocerebral complex. *Proc. Natl Acad. Sci. USA 81*, 5881–5884.

Boyer, J.L., Graber, S.G., Waldo, G.L., Harden, T.K., Garrison, J.C., **1994**. Selective activation of phospholipase C by recombinant G-protein α- and βγ-subunits. *J. Biol. Chem. 269*, 2814–2819.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., *et al.*, **2001**. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr. Biol. 11*, 213–221.

Burtt, E.T., **1938**. On the corpora allata of dipterous insects II. *Proc. Royal Soc. Lond. B126*, 210–223.

Camps, M., Nichols, A., Arkinstall, S., **2000**. Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J. 14*, 6–16.

Cann, M.J., Chung, E., Levin, L.R., **2000**. A new family of adenylyl cyclase genes in the male germline of *Drosophila melanogaster. Devel. Genes Evol. 210*, 200–206.

Carrow, G., Calabrese, R.L., Williams, C.M., **1981**. Spontaneous and evoked release of prothoracicotropin from multiple neurohemal organs of the tobacco hornworm. *Proc. Natl Acad. Sci. USA 78*, 5866–5870.

Chen, C.H., Gu, S.H., Chow, Y.S., **2001**. Adenylate cyclase in prothoracic glands during the last larval instar of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 31*, 659–664.

Chippendale, G.M., **1984**. Environmental signals, the neuroendocrine system, and the regulation of larval diapause in the southwestern corn borer, *Diatraea grandiosella*. *Ciba Found. Symp. 104*, 259–276.

Chippendale, G.M., Yin, C.M., **1973**. Endocrine activity retained in diapause insect larvae. *Nature 246*, 511–513.

Chippendale, G.M., Yin, C.M., **1976**. Diapause of the southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Pyralidae): effects of a juvenile hormone mimic. *Bull. Entomol. Res. 66*, 75–79.

Clementi, E., Meldolesi, J., **1996**. Pharmacological and functional properties of voltage-independent Ca^{2+} channels. *Cell Calcium 19*, 269–279.

Coffer, P.J., **2000**. Phosphatidylinositol 3-kinase signaling; a tale of two kinase activities. In: Woodgett, J. (Ed.), Protein Kinase Functions. Oxford University Press, Oxford, pp. 1–39.

Cole, T.J., Beckage, N.E., Tan, F.F., Srinivasan, A., Ramaswamy, S.B., **2002**. Parasitoid–host endocrine relations: self-reliance or co-optation? *Insect Biochem. Mol. Biol. 32*, 1673–1679.

Combest, W.L., Gilbert, L.I., **1992**. Polyamines modulate multiple protein phosphorylation pathways in the insect prothoracic gland. *Mol. Cell. Endocrinol. 83*, 11–19.

Combettes-Souverain, M., Issad, T., **1998**. Molecular basis of insulin action. *Diab. Metab. 24*, 477–489.

Cornelius, G., Engel, M., **1995**. Stress causes induction of MAP kinase-specific phosphatase and rapid repression of MAP kinase activity in *Drosophila*. *Cellular Sig. 7*, 611–615.

Dai, J.-D., Gilbert, L.I., **1991**. Metamorphosis of the corpus allatum and degeneration of the prothoracic gland during larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Devel. Biol. 144*, 309–326.

Dai, J.-D., Gilbert, L.I., **1997**. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. *Insect Biochem. Mol. Biol. 27*, 69–78.

Dai, J.-D., Gilbert, L.I., **1998**. Juvenile hormone prevents the onset of programmed cell death in the prothoracic glands of *Manduca sexta*. *Gen. Comp. Endocrinol. 109*, 155–165.

Dai, J.-D., Gilbert, L.I., **1999**. An *in vitro* analysis of ecdysteroid-elicited cell death in the prothoracic gland of *Manduca sexta*. *Cell Tissue Res. 297*, 319–327.

Dai, J.-D., Mizoguchi, A., Gilbert, L.I., **1994**. Immunoreactivity of neurosecretory granules in the brain-retrocerebral complex of *Manduca sexta* to heterologous antibodies against *Bombyx* prothoracicotropic hormone and bombyxin. *Invert. Reprod. Devel. 26*, 187–196.

Dai, J.-D., Mizoguchi, A., Satake, S., Ishizaki, H., Gilbert, L.I., **1995**. Developmental changes in the prothoracicotropic hormone content of the *Bombyx mori* brain-retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Devel. Biol. 171*, 212–223.

Danks, H.V., **1987**. Insect dormancy: an ecological perspective. Biological Survey of Canada (Terrestrial Arthropods), Monograph Series I. Ottawa, pp. 439.

Das, S., Maizels, E.T., DeManno, D., St. Clair, E., Adam, S.A., *et al.*, **1996**. A stimulatory role of cyclic adenosine 3′,5′-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. *Endocrinology 137*, 967–974.

Davis, N.T., Blackburn, M.B., Golubeva, E.G., Hildebrand, J.G., **2003**. Localization of myoinhibitory peptide immunoreactivity in *Manduca sexta* and *Bombyx mori*, with indications that the peptide has a role in molting and ecdysis. *J. Exp. Biol. 206*, 1449–1460.

Davis, K.T., Shearn, A., **1977**. *In vitro* growth of imaginal disks from *Drosophila melanogaster. Science 196*, 438–440.

Dedos, S.G., Birkenbeil, H., **2003**. Inhibition of cAMP signalling cascade-mediated Ca^{2+} influx by a prothoracicostatic peptide (Mas-MIP I) via dihydropyridine-sensitive Ca^{2+} channels in the prothoracic glands of the silkworm, *Bombyx mori. Insect Biochem. Mol. Biol. 33*, 219–228.

Dedos, S.G., Fugo, H., **1996**. Effects of fenoxycarb on the secretory activity of the prothoracic glands in the fifth instar of the silkworm, *Bombyx mori. Gen. Comp. Endocrinol. 104*, 213–224.

Dedos, S.G., Fugo, H., **1999a**. Disturbance of adult eclosion by fenoxycarb in the silkworm, *Bombyx mori. J. Insect Physiol. 45*, 257–264.

Dedos, S.G., Fugo, H., **1999b**. Interactions between Ca^{2+} and cAMP in ecdysteroid secretion from the prothoracic glands of *Bombyx mori*. *Mol. Cell. Endocrinol. 154*, 63–70.

Dedos, S.G., Fugo, H., **1999c**. Downregulation of the cAMP signal transduction cascade in the prothoracic glands is responsible for the fenoxycarb-mediated induction of permanent 5th instar larvae in *Bombyx mori*. *Insect Biochem. Mol. Biol. 29*, 723–729.

Dedos, S.G., Fugo, H., **2001**. Involvement of calcium, inositol-1,45 trisphosphate and diacylglycerol in the prothoracicotropic hormone-stimulated ecdysteroid synthesis and secretion in the prothoracic glands of *Bombyx mori*. *Zool. Sci. 18*, 1245–1251.

Dedos, S.G., Fugo, H., Nagata, S., Takamiya, M., Kataoka, H., **1999**. Differences between recombinant PTTH and crude brain extracts in cAMP-mediated ecdysteroid secretion from the prothoracic glands of the silkworm, *Bombyx mori. J. Insect Physiol. 45*, 415–422.

Dedos, S.G., Nagata, S., Ito, J., Takamiya, M., **2001**. Action kinetics of a prothoracicostatic peptide from *Bombyx mori* and its possible signaling pathway. *Gen. Comp. Endocrinol. 122*, 98–108.

Dover, B.A., Tanaka, T., Vinson, S.B., **1995**. Stadium-specific degeneration of host prothoracic glands by *Campoletis sonorensis* calyx fluid and its association with host ecdysteroid titers. *J. Insect Physiol. 41*, 947–955.

Dover, B.A., Vinson, S.B., **1990**. Stage-specific effects of *Campoletis sonorensis* parasitism on *Heliothis virescens* development and prothoracic glands. *Physiol. Entomol. 15*, 405–414.

Dufner, A., Thomas, G., **1999**. Ribosomal S6 kinase signaling and the control of translation. *Exp. Cell Res. 1253*, 100–109.

Endo, K., Fujimoto, Y., Kondo, M., Yamanaka, A., Watanabe, M., *et al.*, **1997**. Stage-dependent changes of the prothoracicotropic hormone (PTTH) activity of brain extracts and of the PTTH sensitivity of the prothoracic glands in the cabbage armyworm, *Mamestra brassicae*, before and during winter and aestival pupal diapause. *Zool. Sci. 14*, 127–133.

Endo, K., Fujimoto, Y., Masaki, T., Kumagai, K., **1990**. Stage-dependent changes in the activity of the prothoracicotropic hormone (PTTH) in the brain of the Asian comma butterfly, *Polygonia c-aureum* L. *Zool. Sci. 7*, 695–702.

Erickson, R.L., **1991**. Structure, expression, and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. *J. Biol. Chem. 266*, 6007–6010.

Eusebio, E.J., Moody, W.J., **1986**. Calcium-dependent action potentials in the prothoracic gland of *Manduca sexta*. *J. Exp. Biol. 126*, 531–536.

Fain, M.J., Riddiford, L.M., **1976**. Reassessment of the critical periods for prothoracicotropic hormone and juvenile hormone secretion in the larval molt of the tobacco hornworm *Manduca sexta*. *Gen. Comp. Endocrinol. 30*, 131–141.

Fescemyer, H.W., Masler, E.P., Kelly, T.J., Lusby, W.R., **1995**. Influence of development and prothoracicotropic hormone on the ecdysteroids produced *in vitro* by the prothoracic glands of female gypsy moth (*Lymantria dispar*) pupae and pharate adults. *J. Insect Physiol. 41*, 489–500.

Feuilloley, M., Vaudry, H., **1996**. Role of the cytoskeleton in adrenocortical cells. *Endocrine Rev. 17*, 269–288.

Fujimoto, Y., Endo, K., Watanabe, M., Kumagai, K., **1991**. Species-specificity in the action of big and small prothoracicotropic hormones (PTTHs) of four species of lepidopteran insects, *Mamestra brassicae*, *Bombyx mori*, *Papilio xuthus* and *Polygonia c-aureum*. *Zool. Sci. 8*, 351–358.

Fujishita, M., Ishizaki, H., **1982**. Temporal organization of endocrine events in relation to the circadian clock during larval-pupal development in *Samia cynthia ricini. J. Insect Physiol. 28, 77–84.*

Fukuda, S., **1941**. Role of the prothoracic gland in differentiation of the imaginal characters in the silkworm pupa. *Annotationes Zool. Japon. 20*, 9–13.

Fullbright, G., Lacy, E.R., Bullesbach, E.E., **1997**. The prothoracicotropic hormone bombyxin has specific receptors on insect ovarian cells. *Eur. J. Biochem. 245*, 774–780.

Fumagalli, S., Thomas, G., **2000**. S6 phosphorylation and signal transduction. In: Sonenberg, N., Hershey, J.W.B., Mathews, M.B. (Eds.), Translational Control of Gene Expression. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 695–717.

Gazi, L., Lopez-Gimenez, J.F., Strange, P.G., **2002**. Formation of oligomers by G protein-coupled receptors. *Curr. Opin. Drug Discov. Devel. 5*, 756–763.

Gelman, D.B., Borovsky, D., **2000**. *Aedes aegypti* TMOF modulates ecdysteroid production by prothoracic glands of the gypsy moth, *Lymantria dispar. Arch. Insect Biochem. Physiol. 45*, 60–68.

Gelman, D.B., Beckage, N.E., **1995**. Low molecular weight ecdysiotropins in proctodaea of fifth instars of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), and hosts parasitized by the braconid wasp *Cotesia congregata* (Hymenoptera: Braconidae). *Eur. J. Entomol. 92*, 123–129.

Gelman, D.B., Reed, D.A., Beckage, N.E., **1998**. Manipulation of fifth-instar host (*Manduca sexta*) ecdysteroid levels by the parasitoid wasp *Cotesia congregata*. *J. Insect Physiol. 44*, 833–843.

Gelman, D.B., Thyagaraja, B.S., Kelly, T.J., Masler, E.P., Bell, R.A., *et al.*, **1991**. The insect gut: a new source of ecdysiotropic peptides. *Experentia 47*, 77–80.

Gelman, D.B., Thyagaraja, B.S., Kelly, T.J., Masler, E.P., Bell, R.A., *et al.*, **1992**. Prothoracicotropic hormone levels in brains of the European corn borer, *Ostrinia nubilalis*: diapause vs. the non-diapause state. *J. Insect Physiol. 38*, 383–395.

Gibbs, D., Riddiford, L.M., **1977**. Prothoracicotropic hormone in *Manduca sexta*. Localization by a larval assay. *J. Exp. Biol. 66*, 255–266.

Giebultowicz, J.M., **2000**. Molecular mechanism and cellular distribution of insect circadian clocks. *Annu Rev. Entomol. 45*, 769–793.

Giebultowicz, J.M., Denlinger, D.L., **1985**. Identification of neurons innervating the ring gland of the flesh fly larva, *Sarcophaga crassipalis* Macquartt (Diptera: Sarcophagidae). *Int. J. Insect Morphol. Embryol. 14*, 155–161.

Gilbert, L.I., **1962**. Maintenance of the prothoracic gland by the juvenile hormone in insects. *Nature 193*, 1205–1207.

Gilbert, L.I., Bollenbacher, W.E., Agui, N., Granger, N.A., Sedlak, B.J., *et al.*, **1981**. The prothoracicotropes: source of the prothoracicotropic hormone. *Am. Zool. 21*, 641–653.

Gilbert, L.I., Combest, W.L., Smith, W.A., Meller, V.H., Rountree, D.B., **1988**. Neuropeptides, second messengers and insect molting. *BioEssays 8*, 153–157.

Gilbert, L.I., Rybczynski, R., Song, Q., Mizoguchi, A., Morreale, R., *et al.*, **2000**. Dynamic regulation of prothoracic gland ecdysteroidogenesis: *Manduca sexta* recombinant prothoracicotropic hormone and brain extracts have identical effects. *Insect Biochem. Mol. Biol. 30*, 1079–1089.

Gilbert, L.I., Rybczynski, R., Tobe, S., **1996**. Regulation of endocrine function leading to insect metamorphosis. In: Gilbert, L.I., Tata, J., Atkinson, B.R. (Eds.), Metamorphosis: Post-Embryonic Reprogramming of Gene Expression in Insect and Amphibian Cells. Academic Press, NY, pp. 59–107.

Gilbert, L.I., Rybczynski, R., Warren, J.T., **2002**. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu Rev. Entomol. 47*, 883–916.

Gilbert, L.I., Song, Q., Rybczynski, R., **1997**. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. *Invert. Neurobiol. 3*, 205–216.

Girgenrath, S., Smith, W.A., **1996**. Investigation of presumptive mobilization pathways for calcium in the steroidogenic action of big prothoracicotropic hormone. *Insect Biochem. Mol. Biol. 26*, 455–463.

Goberdhan, D.C., Wilson, C., **2003**. The functions of insulin signaling: size isn't everything, even in *Drosophila*. *Differentiation 71*, 375–397.

Goltzene, F., Holder, F., Charlet, M., Meister, M., Oka, T., **1992**. Immunocytochemical localization of *Bombyx*-PTTH-like molecules in neurosecretory cells of the brain of the migratory locust, *Locusta migratoria*. A comparison with neuroparsin and insulin-related peptide. *Cell Tissue Res. 269*, 133–140.

Graf, R., Neuenschwander, S., Brown, M.R., Ackermann, U., **1997**. Insulin-mediated secretion of ecdysteroids from mosquito ovaries and molecular cloning of the insulin receptor homologue from ovaries of bloodfed *Aedes aegypti*. *Insect Mol. Biol. 6*, 151–163.

Granger, N.A., Bollenbacher, W.E., **1981**. Hormonal control of insect metamorphosis. In: Gilbert, L.I., Frieden, E. (Eds.), Metamorphosis. 2nd edn. Plenum, New York, pp. 105–137.

Gray, R.S., Muehleisen, D.P., Katahira, E.J., Bollenbacher, W.E., **1994**. The prothoracicotropic hormone (PTTH) of the commercial silkmoth, *Bombyx mori*, in the CNS of the tobacco hornworm, *Manduca sexta*. *Peptides 15*, 777–782.

Grewal, S.S., York, R.D., Stork, P.J., **1999**. Extracellular-signal-regulated kinase signalling in neurons. *Curr. Opin. Neurobiol. 9*, 544–553.

Grieneisen, M.L., Warren, J.T., Gilbert, L.I., **1993**. Early steps in ecdysteroid biosynthesis: evidence for the involvement of cytochrome P-450 enzymes. *Insect Biochem. Mol. Biol. 23*, 13–23.

Gruetzmacher, M.C., Gilbert, L.I., Granger, N.A., Goodman, W., Bollenbacher, W.E., **1984a**. The effect of juvenile hormone on prothoracic gland function during larval-pupal development of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 30*, 331–340.

Gu, S.-H., Chow, Y.-S., **1996**. Regulation of juvenile hormone biosynthesis by ecdysteroid levels during the early stages of the last two larval instars of *Bombyx mori*. *J. Insect Physiol. 42*, 625–632.

Gu, S.-H., Chow, Y.-S., Lin, F.-J., Wu, J.-L., Ho, R.-J., **1996**. A deficiency in prothoracicotropic hormone transduction pathway during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol. 120*, 99–105.

Gu, S.-H., Chow, Y.-S., O'Reilly, D.R., **1998**. Role of calcium in the stimulation of ecdysteroidogenesis by recombinant prothoracicotropic hormone in the prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 28*, 861–867.

Gu, S.-H., Chow, Y.-S., Yin, C.-M., **1997**. Involvement of juvenile hormone in regulation of prothoracicotropic hormone transduction during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol. 127*, 109–116.

Gu, G.-G., Singh, S., **1997**. Modulation of the dihydropyridine-sensitive calcium channels in *Drosophila* by a phospholipase C-mediated pathway. *J. Neurobiol. 33*, 265–275.

Gujdar, A., Sipeki, S., Bander, E., Buday, L., Farago, A., **2003**. Phorbol ester-induced migration of HepG2 cells is accompanied by intensive stress fibre formation, enhanced integrin expression and transient down-regulation of p21-activated kinase 1. *Cell. Signall. 15*, 307–318.

Haluska, P., Adjei, A.A., **2001**. Receptor tyrosine kinase inhibitors. *Curr. Opin. Invest. Drugs 2*, 280–286.

Hamilton, S.E., McKinnon, L.A., Jackson, D.A., Goldman, P.S., Migeon, J.C., *et al.*, **1995**. Molecular analysis of the regulation of muscarinic receptor expression and function. *Life Sci. 56*, 939–943.

Hanton, W.K., Watson, R.D., Bollenbacher, W.E., **1993**. Ultrastructure of prothoracic glands during larval-pupal development of the tobacco hornworm, *Manduca sexta*: a reappraisal. *J. Morphol. 216*, 95–112.

Hartfelder, K., Hanton, W.K., Bollenbacher, W.E., **1994**. Diapause-dependent changes in prothoracicotropic hormone-producing neurons of the tobacco hornworm, *Manduca sexta*. *Cell Tissue Res. 277*, 69–78.

Hayes, G.C., Muehleisen, D.P., Bollenbacher, W.E., Watson, R.D., **1995**. Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of calcium. *Mol. Cell. Endocrinol. 115*, 105–112.

Helfrich-Förster, C., Tauber, M., Park, J.H., Muhlig-Versen, M., Schneuwly, S., *et al.*, **2000**. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci. 20*, 3339–3353.

Henrich, V.C., **1995**. Comparison of ecdysteroid production in *Drosophila* and *Manduca*: pharmacology and cross-species neural reactivity. *Arch. Insect Biochem. Physiol. 30*, 239–254.

Henrich, V.C., Pak, M.D., Gilbert, L.I., **1987a**. Neural factors that stimulate ecdysteroid synthesis by the larval ring gland of *Drosophila melanogaster*. *J. Comp. Physiol. B 157*, 543–549.

Henrich, V.C., Rybczynski, R., Gilbert, L.I., **1999**. Peptide hormones, steroid hormones and puffs: mechanisms and models in insect development. *Vit. Horm. 55*, 73–125.

Henrich, V.C., Tucker, R.L., Maroni, G., Gilbert, L.I., **1987b**. The ecdysoneless (*ecd1ts*) mutation disrupts ecdysteroid synthesis autonomously in the ring gland of *Drosophila melanogaster*. *Devel. Biol. 120*, 50–55.

Hua, Y.-J., Koolman, J., **1995**. An ecdysiostatin from flies. *Regul. Pept. 57*, 263–271.

Hua, Y.-J., Tanaka, Y., Nakamura, K., Sakakibara, M., Nagata, S., *et al.*, **1999**. Identification of a prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. *J. Biol. Chem. 274*, 31169–31173.

Huang, E.J., Reichardt, L.F., **2003**. Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem. 72*, 609–642.

Hynes, R.O., Zhao, Q., **2000**. The evolution of cell adhesion. *J. Cell Biol. 150*, F89–F95.

Ichikawa, M., Ishizaki, H., **1961**. Brain hormone of the silkworm, *Bombyx mori*. *Nature 191*, 933–934.

Ichikawa, M., Ishizaki, H., **1963**. Protein nature of the brain hormone of insects. *Nature 198*, 308–309.

Inoue, T., Thomas, J.H., **2001**. Targets of TGF-β signaling in *Caenorhabditis elegans* dauer formation. *Devel. Biol. 217*, 192–204.

Ishibashi, J., Kataoka, H., Isogai, A., Kawakami, A., Saegusa, H., *et al.*, **1994**. Assignment of disulfide bond location in prothoracicotropic hormone of the silkworm, *Bombyx mori:* a homodimeric protein. *Biochemistry 33*, 5912–5919.

Ishizaki, H., Mizoguchi, A., Fujishita, M., Suzuki, A., Moriya, I., *et al.*, **1983b**. Species specificity of the insect prothoracicotropic hormone (PTTH): the presence of *Bombyx*- and *Samia*-specific PTTHs in the brain of *Bombyx mori*. *Devel. Growth Different. 25*, 593–600.

Ishizaki, H., Suzuki, A., **1980**. Prothoracicotropic hormone. In: Miller, T.A. (Ed.), Neurohormonal Techniques in Insects. Springer, New York, pp. 244–276.

Ishizaki, H., Suzuki, A., **1984**. Prothoracicotropic hormone of *Bombyx mori*. In: Hoffman, J., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer, New York, pp. 63–77.

Ishizaki, H., Suzuki, A., **1992**. Brain secretory peptides of the silkmoth *Bombyx mori:* prothoracicotropic hormone and bombyxin. In: Joose, J., Buijs, R.M., Tilders, F.J.H. (Eds.), Progress in Brain research, vol. 92. Elsevier, Amsterdam, pp. 1–14.

Ishizaki, H., Suzuki, A., **1994**. The brain secretory peptides that control moulting and metamorphosis of the silkmoth, *Bombyx mori*. *Int. J. Devel. Biol. 38*, 301–310.

Ishizaki, H., Suzuki, A., Moriya, I., Mizoguchi, A., Fujishita, M., *et al.*, **1983a**. Prothoracicotropic hormone bioassay: pupal-adult *Bombyx* assay. *Devel. Growth Different. 25*, 585–592.

Jayaraman, T., Ondrias, K., Ondriasova, E., Marks, A.R., **1996**. Regulation of the inositol 1,4,5-trisphosphate

receptor by tyrosine phosphorylation. *Science 272*, 1492–1494.

Jenkins, S.P., Brown, M.R., Lea, A.O., **1992**. Inactive prothoracic glands in larvae and pupae of *Aedes aegypti*: ecdysteroid release by tissues in the thorax and abdomen. *Insect Biochem. Mol. Biol. 22*, 553–559.

Jhoti, H., McLeod, A.N., Blundell, T.L., Ishizaki, H., Nagasawa, H., *et al.*, **1987**. Prothoracicotrophic hormone has an insulin-like tertiary structure. *FEBS Lett. 219*, 419–425.

Jiang, R.-J., Koolman, J., **1999**. Feedback inhibition of ecdysteroids: evidence for a short feedback loop repressing steroidogenesis. *Arch. Insect Biochem. Physiol. 41*, 54–59.

Jones, D., Gelman, D., Loeb, M., **1992**. Hemolymph concentrations of host ecdysteroids are strongly suppressed in precocious prepupae of *Trichoplusia ni* parasitized and pseudoparasitized by *Chelonus* near *curvimaculatus*. *Arch. Insect Biochem. Physiol. 21*, 155–165.

Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H., Suzuki, A., **1991**. Prothoracicotropic hormone of the silkworm, *Bombyx mori:* amino acid sequence and dimeric structure. *Agric. Biol. Chem. 55*, 73–86.

Kataoka, H., Nagasawa, H., Isogai, A., Tamura, S., Mizoguchi, A., *et al.*, **1987**. Isolation and partial characterization of prothoracicotropic hormone of the silkworm, *Bombyx mori*. *Agric. Biol. Chem. 51*, 1067–1076.

Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M., *et al.*, **1990**. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science 247*, 1333–1335.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., *et al.*, **1998**. A family of cAMP-binding proteins that directly activate Rap1. *Science 282*, 2275–2279.

Keightley, D.A., Lou, K.J., Smith, W.A., **1990**. Involvement of translation and transcription in insect steroidogenesis. *Mol. Cell. Endocrinol. 74*, 229–237.

Kelly, T.J., Gelman, D.B., Reed, D.A., Beckage, N.E., **1998**. Effects of parasitization by *Cotesia congregata* on the brain-prothoracic gland axis of its host, *Manduca sexta*. *J. Insect Physiol. 44*, 323–332.

Kelly, T.J., Kingan, T.G., Masler, C.A., Robinson, C.H., **1996**. Analysis of the ecdysiotropic activity in larval brains of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 42*, 873–880.

Kelly, T.J., Masler, E.P., Thyagaraja, B.S., Bell, R.A., Imberski, R.B., **1992**. Development of an *in vitro* assay for prothoracicotropic hormone of the gypsy moth, *Lymantria dispar* (L.) following studies on identification, titers and synthesis of ecdysteroids in last-instar females. *J. Comp. Physiol. B 162*, 81–87.

Kiguchi, K., Agui, N., **1981**. Ecdysteroid levels and developmental events during larval moulting in the silkworm, *Bombyx mori*. *J. Insect Physiol. 27*, 805–812.

Kim, A.-J., Cha, G.-H., Kim, K., Gilbert, L.I., Lee, C.C., **1997**. Purification and characterization of the prothoracicotropic hormone of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 94*, 1130–1135.

Kingan, T.G., **1981**. Purification of the prothoracicotropic hormone from the tobacco hornworm *Manduca sexta*. *Life Sci. 28*, 2585–2594.

Kiriishi, S., Nagasawa, H., Kataoka, H., Suzuki, A., Sakurai, S., **1992**. Comparison of the *in vivo* and *in vitro* effects of bombyxin and prothoracicotropic hormone on prothoracic glands of the silkworm, *Bombyx mori*. *Zool. Sci. 9*, 149–155.

Kiriishi, S., Rountree, D.B., Sakurai, S., Gilbert, L.I., **1990**. Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3b-reductase mediated conversion to ecdysone in representative insects. *Experientia 46*, 716–721.

Kobayashi, M., Kimura, J., **1958**. The "brain" hormone in the silkworm, *Bombyx mori* L. *Nature 181*, 1217.

Kobayashi, M., Yamazaki, M., **1966**. The proteinic brain hormone in an insect, *Bombyx mori* L. (Lepidoptera: Bombycidae). *Appl. Entomol. Zool. 12*, 53–60.

Kondo, H., Ino, M., Suzuki, A., Ishizaki, H., Iwami, M., **1996**. Multiple gene copies for bombyxin, an insulin-related peptide of the silkmoth *Bombyx mori*: structural signs for gene rearrangement and duplication responsible for generation of multiple molecular forms of bombyxin. *J. Mol. Evol. 259*, 926–937.

Kopeć, S., **1922**. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull. 42*, 323–342.

Krishnan, B., Levine, J.D., Lynch, M.K., Dowse, H.B., Funes, P., *et al.*, **2001**. A new role for cryptochrome in a *Drosophila* circadian oscillator. *Nature 411*, 313–317.

Kulesza, P., Lee, C.Y., Watson, R.D., **1994**. Protein synthesis and ecdysteroidogenesis in prothoracic glands of the tobacco hornworm (*Manduca sexta*): stimulation by big prothoracicotropic hormone. *Gen. Comp. Endocrinol. 93*, 448–458.

Kyriakis, J.M., **2000**. Mammalian MAP kinase pathways. In: Woodgett, J. (Ed.), Protein Kinase Functions. Oxford University Press, Oxford, pp. 40–156.

LaRochelle, W.J., Pierce, J.H., May-Siroff, M., Giese, N., Aaronson, S.A., **1992**. Five PDGF B amino acid substitutions convert PDGF A to a PDGF B-like transforming molecule. *J. Biol. Chem. 267*, 17074–17077.

Lee, C.Y., Lee, K.J., Chumley, P.H., Watson, C.J., Abdur-Rahman, A., *et al.*, **1995**. Capacity of insect (*Manduca sexta*) prothoracic glands to secrete ecdysteroids: relation to glandular growth. *Gen. Comp. Endocrinol. 100*, 404–412.

Leonardi, M.G., Cappellozza, S., Ianne, P., Cappellozza, L., Parenti, P., *et al.*, **1996**. Effects of topical application of an insect growth regulator (fenoxycarb) on some physiological parameters on the fifth instar larvae of the silkworm *Bombyx mori*. *Comp. Biochem. Physiol. 113B*, 361–365.

Lester, D.S., Gilbert, L.I., **1986**. Developmental changes in choline uptake and acetylcholine metabolism in the

larval brain of the tobacco hornworm, *Manduca sexta*. *Brain Res. 391*, 201–209.

Lester, D.S., Gilbert, L.I., **1987**. Characterization of acetylcholinesterase activity in the larval brain of *Manduca sexta*. *Insect Biochem. 17*, 99–109.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., *et al.*, **1995**. Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature 376*, 737–745.

Levi-Montalcini, R., Skaper, S.D., Dal Toso, R., Petrelli, L., Leon, A., **1996**. Nerve growth factor: from neurotrophin to neurokine. *Trends Neurosci. 19*, 514–520.

Lewis, T.S., Shapiro, P.S., Ahn, N.G., **1998**. Signal transduction through MAP kinase cascades. *Adv. Cancer Res. 74*, 49–139.

Li, S., Falabella, P., Kuriachan, I., Vinson, S.B., Borst, D.W., *et al.*, **2003**. Juvenile hormone synthesis, metabolism, and resulting haemolymph titre in *Heliothis virescens* larvae parasitized by *Toxoneuron nigriceps*. *J. Insect Physiol. 49*, 1021–1030.

Liebmann, C., **2001**. Regulation of MAP kinase activity by peptide signalling pathway: paradigms of multiplicity. *Cell. Signall. 13*, 777–785.

Loeb, M.J., **1982**. Diapause and development in the tobacco budworm, *Heliothis virescens*: a comparison of haemolymph ecdysteroid titres. *J. Insect Physiol. 28*, 667–673.

Loh, Y.P., Brownstein, M.J., Gainer, H., **1984**. Proteolysis in neuropeptide processing and other neural functions. *Annu. Rev. Neurosci. 7*, 189–222.

Lonard, D.M., Bhaskaran, G., Dahm, K.H., **1996**. Control of prothoracic gland activity by juvenile hormone in fourth instar *Manduca sexta* larvae. *J. Insect Physiol. 42*, 205–213.

Luttrell, L.M., Daaka, Y., Lefkowitz, R.J., **1999**. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr. Opin. Cell Biol. 11*, 177–183.

Mains, R.E., Dickerson, I.M., May, V., Stoffers, D.A., Perkins, S.N., *et al.*, **1990**. Cellular and molecular aspects of peptide hormone biosynthesis. *Front. Neuroendocrinol. 11*, 52–89.

Mala, J., Sehnal, F., **1978**. Role of the nerve cord in the control of prothoracic glands in *Galleria mellonella*. *Experientia 34*, 1233–1235.

Mansfield, S.G., Cammer, S., Alexander, S.C., Muehleisen, D.P., Gray, R.S., *et al.*, **1998**. Molecular cloning and characterization of an invertebrate cellular retinoic acid binding protein. *Proc. Natl Acad. Sci. USA 95*, 6825–6830.

Marris, G.C., Weaver, R.J., Bell, J., Edwards, J.P., **2001**. Venom from the ectoparasitoid wasp *Eulophus pennicornis* disrupts host ecdysteroid production by regulating host prothoracic gland activity. *Physiol. Entomol. 26*, 229–238.

Martelli, A.M., Faenza, I., Billi, A.M., Fala, F., Cocco, L., *et al.*, **2003**. Nuclear protein kinase C isoforms: key players in multiple cell functions? *Histol. and Histopathol. 18*, 1301–1312.

Massagué, J., **1998**. TGF-β signal transduction. *Annu. Rev. Biochem. 67*, 753–791.

Masumura, M., Satake, S., Saegusa, H., Mizoguchi, A., **2000**. Glucose stimulates the release of bombyxin, an insulin-related peptide of the silkworm *Bombyx mori*. *Gen. Comp. Endocrinol. 118*, 393–399.

McDaniel, C.N., **1979**. Hemolymph ecdysone concentrations in *Hyalophora cecropia* pupae, dauer pupae, and adults. *J. Insect Physiol. 25*, 143–145.

Medler, K.F., Bruch, R.C., **1999**. Protein kinase Cβ and δ selectively phosphorylate odorant and metabotropic glutamate receptors. *Chem. Senses 24*, 295–299.

Meller, V.H., Combest, W.L., Smith, W.A., Gilbert, L.I., **1988**. A calmodulin-sensitive adenylate cyclase in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol. 59*, 67–76.

Meller, V.H., Gilbert, L.I., **1990**. Occurrence, quaternary structure and function of G protein subunits in an insect endocrine gland. *Mol. Cell. Endocrinol. 74*, 133–141.

Meller, V.H., Sakurai, S., Gilbert, L.I., **1990**. Developmental regulation of calmodulin-dependent adenylate cyclase in an insect endocrine gland. *Cell Regul. 1*, 771–780.

Mesce, K.A., Fahrbach, S.E., **2002**. Integration of endocrine signals that regulate insect ecdysis. *Front. Neuroendocrinol. 23*, 179–199.

Meyuhaus, O., Hornstein, E., **2000**. Translational control of TOP mRNAs. In: Sonenberg, N., Hershey, J.W.B., Mathews, M.B. (Eds.), Translational Control of Gene Expression, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 671–693.

Mizoguchi, A., **2001**. Effects of juvenile hormone on the secretion of prothoracicotropic hormone in the last- and penultimate-instar larvae of the silkworm *Bombyx mori*. *J. Insect Physiol. 47*, 767–775.

Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., **2002**. Basic pattern of fluctuation in hemolymph PTTH titers during larval-pupal and pupal-adult development of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol. 127*, 181–189.

Mizoguchi, A., Ishizaki, H., **1982**. Prothoracic glands of the saturniid moth *Samia cynthia* ricini possess a circadian clock controlling gut purge timing. *Proc. Natl Acad. Sci. USA 79*, 2726–2730.

Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., **2001**. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol. 31*, 349–358.

Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., *et al.*, **1990**. Immunohistochemical localization of prothoracicotropic hormone-producing cells in the brain of *Bombyx mori*. *Devel. Growth Different. 32*, 591–598.

Monconduit, H., Mauchamp, B., **1998**. Effects of ultralow doses of fenoxycarb on juvenile hormone-regulated physiological parameters in the silkworm, *Bombyx mori* L. *Arch. Insect Biochem. Physiol. 37*, 178–189.

Morfis, M., Christopoulos, A., Sexton, P.M., **2003**. RAMPs: 5 years on, where to now? *Trends Pharmacol. Sci. 24*, 596–601.

Morris, A.J., Scarlata, S., **1997**. Regulation of effectors by G-protein α- and βγ-subunits. Recent insights from studies of the phospholipase c-β isoenzymes. *Biochem. Pharmacol. 54*, 429–435.

Mosna, G., Barigozzi, C., **1975**. Stimulation of growth by insulin in *Drosophila* embryonic cells *in vitro*. *Experientia 32*, 855–856.

Muehleisen, D.P., Gray, R.S., Katahira, E.J., Thomas, M.K., Bollenbacher, W.E., **1993**. Immunoaffinity purification of the neuropeptide prothoracicotropic hormone from *Manduca sexta*. *Peptides 14*, 531–541.

Mulye, H., Gordon, R., **1993**. Effects of fenoxycarb, a juvenile hormone analogue, on lipid metabolism of the eastern spruce budworm, *Choristoneura fumiferana*. *J. Insect Physiol. 39*, 721–727.

Myers, E.M., Yu, J., Sehgal, A., **2003**. Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster*. *Curr. Biol. 13*, 526–533.

Nagata, K., Maruyama, K., Kojima, K., Yamamoto, M., Tanaka, M., *et al.*, **1999**. Prothoracicotropic activity of SBRPs, the insulin-like peptides of the saturniid silkworm *Samia cynthia ricini*. *Biochem. Biophy. Res. Comm. 266*, 575–578.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., **1997**. A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int. J. Neural Syst. 8*, 581–599.

Nijhout, H.F., **1979**. Stretch-induced molting in *Oncopeltus fasciatus*. *J. Insect Physiol. 25*, 277–281.

Nijhout, H.F., **1981**. Physiological control of molting in insects. *Am. Zool. 21*, 631–640.

Nijhout, H.F., **1994**. Insect Hormones. Princeton University Press, Princeton, NJ, pp. 267.

Nijhout, H.F., Gunnert, L.W., **2002**. Bombyxin is a growth factor for wing imaginal disks in Lepidoptera. *Proc. Natl Acad. Sci. USA 99*, 15446–15450.

Nimi, S., Sakurai, S., **1997**. Development changes in juvenile hormone and juvenile hormone acid titers in the hemolymph and *in-vitro* juvenile hormone synthesis by corpora allata of the silkworm, *Bombyx mori*. *J. Insect Physiol. 43*, 875–884.

Noguti, T., Adachi-Yamada, T., Katagiri, T., Kawakami, A., Iwami, M., *et al.*, **1995**. Insect prothoracicotropic hormone: a new member of the vertebrate growth factor superfamily. *FEBS Lett. 376*, 251–256.

O'Brien, M.A., Granger, N.A., Agui, N., Gilbert, L.I., Bollenbacher, W.E., **1986**. Prothoracicotropic hormone in the developing brain of the tobacco hornworm *Manduca sexta*: Relative amounts of two molecular forms. *J. Insect Physiol. 32*, 719–725.

O'Brien, M.A., Katahira, E.J., Flanagan, T.R., Arnold, L.W., Haughton, G., *et al.*, **1988**. A monoclonal antibody to the insect prothoracicotropic hormone. *J. Neurosci. 8*, 3247–3257.

Offermanns, S., **2003**. G-proteins as transducers in transmembrane signalling. *Prog. Biophy. Mol. Biol. 83*, 101–130.

Okajima, A., Kumagai, K., **1989**. The inhibitory control of prothoracic gland activity by the neurosecretory neurones in a moth, *Mamestra brassicae*. *Zool. Sci. 6*, 851–858.

Okajima, A., Kumagai, K., Watanabe, M., **1989**. The involvement of afferent chemoreceptive activity in the nervous regulation of the prothoracic gland in a moth, *Mamestra brassicae*. *Zool. Sci. 6*, 859–866.

Okajima, A., Watanabe, M., **1989**. Electrophysiological identification of neuronal pathway to the prothoracic gland and the change in electrical activities of the prothoracic gland innervating neurones during larval development of a moth, *Mamestra brassicae*. *Zool. Sci. 6*, 459–468.

Okuda, M., Sakurai, S., Ohtaki, T., **1985**. Activity of the prothoracic gland and its sensitivity to prothoracicotropic hormone in the penultimate and last-larval instar of *Bombyx mori*. *J. Insect Physiol. 31*, 455–461.

O'Leary, P.D., Hughes, R.A., **2003**. Design of potent peptide mimetics of brain-derived neurotrophic factor. *J. Biol. Chem. 278*, 25738–25744.

Orikasa, C., Yamauchi, H., Nagasawa, H., Suzuki, A., Nagata, M., **1993**. Induction of oocyte-nurse cell differentiation in the ovary by the brain during the initial stage of oogenesis in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool. 28*, 303–311.

Orme-Johnson, N.R., **1990**. Distinctive properties of adrenal cortex mitochondria. *Biochim. Biophys. Acta 1020*, 213–231.

Pak, J.-W., Chung, K.W., Lee, C.C., Kim, K., Namkoong, Y., *et al.*, **1992**. Evidence for multiple forms of the prothoracicotropic hormone in *Drosophila melanogaster* and indication of a new function. *J. Insect Physiol. 38*, 167–176.

Pak, M.D., Gilbert, L.I., **1987**. A developmental analysis of ecdysteroids during the metamorphosis of *Drosophila melanogaster*. *J. Liq. Chromatog. 10*, 2591–2611.

Palen, E., Traugh, J.A., **1987**. Phosphorylation of ribosomal protein S6 by cAMP-dependent protein kinase and mitogen-stimulated S6 kinase differentially alters translation of globin mRNA. *J. Biol. Chem. 262*, 3518–3523.

Papadopoulos, V., Amri, H., Boujrad, N., Cascio, C., Culty, M., *et al.*, **1997**. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids 62*, 21–28.

Pearson, R.B., Thomas, G., **1995**. Regulation of p70s6k/p85s6k and its role in the cell cycle. *Progr. Cell Cycle Res. 1*, 21–32.

Pelc, D., Steel, C.G.H., **1997**. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen. Comp. Endocrinol. 108*, 358–365.

Pennacchio, F., Falabella, P., Sordetti, R., Varricchio, P., Malva, C., *et al.*, **1998a**. Prothoracic gland inactivation in *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *J. Insect Physiol. 44*, 845–857.

Pennacchio, F., Falabella, P., Vinson, S.B., **1998b**. Regulation of *Heliothis virescens* prothoracic glands by *Cardiochiles nigriceps* polydnavirus. *Arch. Insect Biochem. Physiol. 38*, 1–10.

Pennacchio, F., Sordetti, R., Falabella, P., Vinson, S.B., **1997**. Biochemical and ultrastructural alterations in prothoracic glands of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) last instar larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *Insect Biochem. Mol. Biol. 27*, 439–450.

Pieroni, J.P., Jacobowitz, O., Chen, J., Iyengar, R., **1993**. Signal recognition and integration by Gs-stimulated adenylyl cyclases. *Curr. Opin. Neurobiol. 3*, 345–351.

Raught, B., Gingras, A.C., Sonenberg, N., **2001**. The target of rapamycin (TOR) proteins. *Proc. Natl Acad. Sci. USA 98*, 7037–7044.

Redfern, C.P.F., **1983**. Ecdysteroid synthesis by the ring gland of *Drosophila melanogaster* during late-larval, pre-pupal and pupal development. *J. Insect Physiol. 29*, 65–71.

Rhee, S.G., **2001**. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem. 70*, 281–312.

Richard, D.S., Gilbert, L.I., **1991**. Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster. Experientia* 47, 1063–1066.

Richard, D.S., Saunders, D.S., **1987**. Prothoracic gland function in diapause and non-diapause destined *Sarcophaga argyrostoma* and *Calliphora vicina*. *J. Insect Physiol. 33*, 385–392.

Richard, D.S., Warren, J.T., Saunders, D.S., Gilbert, L.I., **1987**. Haemolymph ecdysteroid titres in diapause and non-diapause destined larvae and pupae of *Sarcophaga argyrostoma*. *J. Insect Physiol. 33*, 115–122.

Richards, J.S., **1994**. Hormonal control of gene expression in the ovary. *Endocr. Rev. 15*, 725–751.

Richards, J.S., Russell, D.L., Ochsner, S., Hsieh, M., Doyle, K.H., *et al.*, **2002**. Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. *Rec. Progr. Horm. Res. 57*, 195–220.

Richter, K., **1985**. Physiological investigations on the role of the innervation in the regulation of the prothoracic gland in *Periplaneta americana*. *Arch. Insect Biochem. Physiol. 2*, 319–329.

Richter, K., **2001**. Daily changes in neuroendocrine control of moulting hormone secretion in the prothoracic gland of the cockroach *Periplaneta americana* (L.). *J. Insect Physiol. 47*, 333–338.

Richter, K., Gersch, M., **1983**. Electrophysiological evidence of nervous involvement in the control of the prothoracic gland in *Periplaneta americana*. *Experientia 39*, 917–918.

Richter, K., Peschke, E., Peschke, D., **2000**. A neuroendocrine releasing effect of melatonin in the brain of an insect, *Periplaneta americana* (L.). *J. Pineal Res. 28*, 129–135.

Riddiford, L.M., **1976**. Hormonal control of insect epidermal cell commitment *in vitro*. *Nature 259*, 115–117.

Riddiford, L.M., **1994**. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Adv. Insect Physiol. 24*, 213–274.

Riddiford, L.M., **1996**. Juvenile hormone: the status of its "status quo" action. *Arch. Insect Biochem. Physiol. 32*, 271–286.

Riehle, M.A., Brown, M.R., **1999**. Insulin stimulates ecdysteroid production through a conserved signaling cascade in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 29*, 855–860.

Roberts, B., **1984**. Photoperiodic regulation of prothoracicotropic hormone release in late larval, prepupal and pupa stages of *Sarcophaga bullata*. *Ciba Found. Symp. 104*, 164–187.

Roberts, B., Gilbert, L.I., **1986**. Ring gland and prothoracic gland sensitivity to interspecific prothoracicotropic hormone extracts. *J. Comp. Physiol. B 156*, 767–771.

Roberts, B., Henrich, V., Gilbert, L.I., **1987**. Effects of photoperiod on the timing of larval wandering in *Drosophila melanogaster. Physiol. Entomol. 12*, 175–180.

Rountree, D.B., Bollenbacher, W.E., **1986**. The release of the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta,* is controlled intrinsically by juvenile hormone. *J. Exp. Biol. 120*, 41–58.

Rountree, D.B., Combest, W.L., Gilbert, L.I., **1987**. Protein phosphorylation in the prothoracic glands as a cellular model for juvenile hormone-prothoracicotropic hormone interactions. *Insect Biochem. 17*, 943–948.

Rountree, D.B., Combest, W.L., Gilbert, L.I., **1992**. Prothoracicotropic hormone regulates the phosphorylation of a specific protein in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 22*, 353–362.

Rybczynski, R., Bell, S.C., Gilbert, L.I., **2001**. Activation of an extracellular signal-regulated kinase (ERK) by the insect prothoracicotropic hormone. *Mol. Cell. Endocrinol. 184*, 1–11.

Rybczynski, R., Gilbert, L.I., **1994**. Changes in general and specific protein synthesis that accompany ecdysteroid synthesis in stimulated prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 175–189.

Rybczynski, R., Gilbert, L.I., **1995a**. Prothoracicotropic hormone elicits a rapid, developmentally specific synthesis of β tubulin in an insect endocrine gland. *Devel. Biol. 169*, 15–28.

Rybczynski, R., Gilbert, L.I., **1995b**. Prothoracicotropic hormone-regulated expression of a hsp 70 cognate protein in the insect prothoracic gland. *Mol. Cell. Endocrinol. 115*, 73–85.

Rybczynski, R., Gilbert, L.I., **1998**. Cloning of a β1 tubulin cDNA from an insect endocrine gland: Developmental

and hormone-induced changes in mRNA expression. *Mol. Cell. Endocrinol. 141*, 141–151.

Rybczynski, R., Gilbert, L.I., **2000**. cDNA cloning and expression of a hormone-regulated heat shock protein (hsc 70) from the prothoracic gland of *Manduca sexta*. *Insect Biochem. Mol. Biol. 30*, 579–589.

Rybczynski, R., Gilbert, L.I., **2003**. Prothoracicotropic hormone stimulated extracellular signal-regulated kinase (ERK) activity: the changing roles of Ca^{2+}- and cAMP-dependent mechanisms in the insect prothoracic glands during metamorphosis. *Mol. Cell. Endocrinol. 205*, 159–168.

Rybczynski, R., Mizoguchi, A., Gilbert, I.I., **1996**. *Bombyx* and *Manduca* prothoracicotropic hormones: an immunologic test for relatedness. *Gen. Comp. Endocrin. 102*, 247–254.

Safranek, L., Cymborowski, B., Williams, C.M., **1980**. Effects of juvenile hormone on ecdysone-dependent development in the tobacco hornworm, *Manduca sexta*. *Biol. Bull. 158*, 248–256.

Saitou, N., Nei, M., **1987**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol. 4*, 406–425.

Sakurai, S., **1983**. Temporal organization of endocrine events underlying larval–larval ecdysis in the silkworm, *Bombyx mori*. *J. Insect Physiol. 29*, 919–932.

Sakurai, S., **1984**. Temporal organization of endocrine events underlying larval–pupal metamorphosis in the silkworm, *Bombyx mori*. *J. Insect Physiol. 30*, 657–664.

Sakurai, S., Gilbert, L.I., **1990**. Biosynthesis and secretion of ecdysteroids by the prothoracic glands. In: Ohnishi, E., Ishizaki, H. (Eds.), Molting and Metamorphosis. Springer, Berlin, pp. 83–106.

Sakurai, S., Kaya, M., Satake, S., **1998**. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori:* role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol. 44*, 867–881.

Sakurai, S., Okuda, M., Ohtaki, T., **1989**. Juvenile hormone inhibits ecdysone secretion and responsiveness to prothoracicotropic hormone in prothoracic glands of *Bombyx mori*. *Gen. Comp. Endocrinol. 75*, 222–230.

Sakurai, S., Williams, C.M., **1989**. Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 75*, 204–216.

Sampath, T.K., Rashka, K.E., Doctor, J.S., Tucker, R.F., Hoffmann, F.M., **1993**. *Drosophila* transforming growth factor β superfamily proteins induce endochondral bone formation in mammals. *Proc. Natl Acad. Sci. USA 90*, 6004–6008.

Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., *et al.*, **1997**. Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comp. Biochem. Physiol. B 118*, 349–357.

Sauman, I., Reppert, S.M., **1996a**. Molecular characterization of prothoracicotropic hormone (PTTH) from the giant silkmoth *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relationship to circadian clock cells in central brain. *Devel. Biol. 178*, 418–429.

Sauman, I., Reppert, S.M., **1996b**. Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of *Period* protein regulation. *Neuron 17*, 889–900.

Saunders, D.S., **2002**. Insect Clocks. Elsevier, Amsterdam, p. 560.

Schneiderman, H.A., Gilbert, L.I., **1964**. Control of growth and development in insects. *Science 143*, 325–333.

Sedlak, B.J., **1985**. Structure of endocrine glands. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, New York, pp. 109–151.

Sedlak, B.J., Marchione, L., Devorkin, B., Davino, R., **1983**. Correlations between endocrine gland ultrastructure and hormone titers in the fifth larval instar of *Manduca sexta*. *Gen. Comp. Endocrinol. 52*, 291–310.

Sehnal, F., **1983**. Juvenile hormone analogues. In: Downer, R., Laufer, H. (Eds.), Endocrinology of Insects. Allan R Liss, pp. 657–672.

Sehnal, F., Hansen, I., Scheller, K., **2002**. The cDNA-structure of the prothoracicotropic hormone (PTTH) of the silkmoth *Hyalophora cecropia*. *Insect Biochem. Mol. Biol. 32*, 233–237.

Sehnal, F., Svacha, P., Zrzavy, J., **1996**. Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J., Atkinson, B.R. (Eds.), Metamorphosis: Post-Embryonic Reprogramming of Gene Expression in Insect and Amphibian Cells. Academic Press, NY, pp. 3–58.

Seidah, N.G., Prat, A., **2002**. Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem. 38*, 79–94.

Shearman, M.S., Sekiguchi, K., Nishizuka, Y., **1989**. Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacol. Rev. 41*, 211–237.

Shionoya, M., Matsubayashi, H., Asahina, M., Kuniyoshi, H., Nagata, S., *et al.*, **2003**. Molecular cloning of the prothoracicotropic hormone from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 795–801.

Shirai, Y., Aizono, Y., Iwasaki, T., Yanagida, A., Mori, H., *et al.*, **1993**. Prothoracicotropic hormone is released five times in the 5th-larval instar of the silkworm *Bombyx mori*. *J. Insect Physiol. 39*, 83–88.

Shirai, Y., Iwasaki, T., Matsubara, F., Aizono, Y., **1994**. The carbachol-induced release of prothoracicotropic hormone from brain-corpus cardiacum-corpus allatum complex of the silkworm, *Bombyx mori*. *J. Insect Physiol. 40*, 469–473.

Shirai, Y., Shimazaki, K., Iwasaki, T., Matsubara, F., Aizono, Y., **1995**. The in vitro release of prothoracicotropic hormone (PTTH) from the brain-corpus cardiacum-corpus allatum complex of silkworm,

Bombyx mori. *Comp. Biochem. Physiol. C 110*, 143–148.

Shirai, Y., Uno, T., Aizono, Y., **1998**. Small GTP-binding proteins in the brain-corpus cardiacum-corpus allatum complex of the silkworm, *Bombyx mori*: involvement in the secretion of prothoracicotropic hormone. *Arch. Insect Biochem. Physiol. 38*, 177–184.

Siegmund, T., Korge, G., **2001**. Innervation of the ring gland of *Drosophila melanogaster*. *J. Comp. Neurol. 431*, 481–491.

Simpson, E.R., Waterman, M.R., **1988**. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annu. Rev. Physiol. 50*, 427–440.

Smith, W.A., **1993**. Second messengers and the action of prothoracicotropic hormone in *Manduca sexta*. *Am. Zool. 33*, 330–339.

Smith, W.A., **1995**. Regulation and consequences of cellular changes in the prothoracic glands of *Manduca sexta* during the last larval instar: a review. *Arch. Insect Biochem. Physiol. 30*, 271–293.

Smith, W.A., Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., **1986a**. Cellular changes in the prothoracic glands of *Manduca sexta* during pupal diapause. *J. Exp. Bio. 120*, 131–142.

Smith, W.A., Combest, W.L., Gilbert, L.I., **1986b**. Involvement of cyclic AMP-dependent protein kinase in prothoracicotropic hormone-stimulated ecdysone synthesis. *Mol. Cell. Endocrinol. 47*, 25–33.

Smith, W.A., Combest, W.L., Rountree, D.B., Gilbert, L.I., **1987a**. Neuropeptide control of ecdysone biosynthesis. In: Law, J.H. (Ed.), Molecular Entomology. Alan R. Liss, New York, pp. 129–139.

Smith, W.A., Gilbert, L.I., **1986**. Cellular regulation of ecdysone synthesis by the prothoracic glands of *Manduca sexta*. *Insect Biochem. 16*, 143–147.

Smith, W.A., Gilbert, L.I., **1989**. Early events in peptide-stimulated ecdysteroid secretion by the prothoracic glands of *Manduca sexta*. *J. Exp. Zool. 252*, 262–270.

Smith, W.A., Gilbert, L.I., Bollenbacher, W.E., **1984**. The role of cyclic AMP in the regulation of ecdysone synthesis. *Mol. Cell. Endocrinol. 37*, 285–294.

Smith, W.A., Gilbert, L.I., Bollenbacher, W.E., **1985**. Calcium-cyclic AMP interactions in prothoracicotropic hormone stimulation of ecdysone synthesis. *Mol. Cell. Endocrinol. 39*, 71–78.

Smith, W.A., Koundinya, M., McAllister, T., Brown, A., **1997**. Insulin receptor-like tyrosine kinase in the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 35*, 99–110.

Smith, W.A., Pasquarello, T.J., **1989**. Developmental changes in phosphodiesterase activity and hormonal response in prothoracic glands of *Manduca sexta*. *Mol. Cell. Endocrinol. 63*, 239–246.

Smith, W.A., Priester, J., Morais, J., **2003**. PTTH-stimulated ecdysone secretion is dependent upon tyrosine phosphorylation in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 1317–1325.

Smith, W.A., Rountree, D.B., Bollenbacher, W.E., Gilbert, L.I., **1987b**. Dissociation of the prothoracic glands of *Manduca sexta* into hormone-responsive cells. In: Borkovec, A., Gelman, D. (Eds.), Progress in Insect Neurochemistry and Neurophysiology. Humana Press, Clifton, NJ, pp. 319–322.

Smith, W.A., Varghese, A.H., Lou, K.J., **1993**. Developmental changes in cyclic AMP-dependent protein kinase associated with increased secretory capacity of *Manduca sexta* prothoracic glands. *Mol. Cell. Endocrinol. 90*, 187–195.

Smith, W.A., Varghese, A.H., Healy, M.S., Lou, K.J., **1996**. Cyclic AMP is a requisite messenger in the action of big PTTH in the prothoracic glands of pupal *Manduca sexta*. *Insect Biochem. Mol. Biol. 26*, 161–170.

Snyder, M.J., Feyereisen, R., **1993**. A diazepam binding inhibitor (DBI) homolog from the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol. 94*, R1–R4.

Snyder, M.J., Van Antwerpen, R., **1998**. Evidence for a diazepam-binding inhibitor (DBI) benzodiazepine receptor-like mechanism in ecdysteroidogenesis by the insect prothoracic gland. *Cell Tissue Res. 294*, 161–168.

Song, Q., Gilbert, L.I., **1994**. S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase. *Devel. Genet. 15*, 332–338.

Song, Q., Gilbert, L.I., **1995**. Multiple phosphorylation of ribosomal protein S6 and specific protein synthesis are required for prothoracicotropic hormone-stimulated ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 591–602.

Song, Q., Gilbert, L.I., **1996**. Protein phosphatase activity is required for prothoracicotropic hormone-stimulated ecdysteroidogenesis in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Arch. Insect. Biochem. Physiol. 31*, 465–480.

Song, Q., Gilbert, L.I., **1997**. Molecular cloning, developmental expression, and phosphorylation of ribosomal protein S6 in the endocrine gland responsible for insect molting. *J. Biol. Chem. 272*, 4429–4435.

Song, Q., Gilbert, L.I., **1998**. Alterations in ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol. 28*, 849–860.

Stanewsky, R., **2002**. Clock mechanisms in *Drosophila*. *Cell Tissue Res. 309*, 11–26.

Stanewsky, R., **2003**. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J. Neurobiol. 54*, 111–147.

Steel, C.G.H., Vafopoulou, X., **2002**. Physiology of circadian systems. In: Saunders, D.S., with Steel, C.G.H., Vafopoulou, X., Lewis, R.D. (Eds.), Insect Clocks. 3rd edn. Elsevier, Amsterdam, pp. 115–188.

Stengl, M., Homberg, U., **1994**. Pigment-dispersing hormone-immunoreactive neurons in the cockroach

Leucophaea maderae share properties with circadian pacemaker neurons. *J. Comp. Physiol. A 175*, 203–213.

Stocco, D.M., Clark, B.J., **1997**. The role of the steroidogenic acute regulatory protein in steroidogenesis. *Steroids 62*, 29–36.

Suarez, C., Diaz-Torga, G., Gonzalez-Iglesias, A., Vela, J., Mladovan, A., *et al.*, **2003**. Angiotensin II phosphorylation of extracellular signal-regulated kinases in rat anterior pituitary cells. *Am. J. Physiol.: Endocrinol. Metab. 85*, E645–E653.

Suh, J.M., Song, J.H., Kim, D.W., Kim, H., Chung, H.K., *et al.*, **2003**. Regulation of the phosphatidylinositol 3-kinase, Akt/protein kinase B, FRAP/mammalian target of rapamycin, and ribosomal S6 kinase 1 signaling pathways by thyroid-stimulating hormone (TSH) and stimulating type TSH receptor antibodies in the thyroid gland. *J. Biol. Chem. 278*, 21960–21971.

Takaki, K., Sakurai, S., **2003**. Regulation of prothoracic gland ecdysteroidogenic activity leading to pupal metamorphosis. *Insect Biochem. Mol. Biol. 33*, 1189–1199.

Tanaka, M., Kataoka, H., Nagata, K., Nagasawa, H., Suzuki, A., **1995**. Morphological changes of BM-N4 cells induced by bombyxin, an insulin-related peptide of *Bombyx mori*. *Regul. Pept. 57*, 311–318.

Tauber, M.J., Tauber, C.A., Masaki, S., **1986**. Seasonal Adaptations of Insects. Oxford University Press, Oxford, pp. 411.

Tissenbaum, H.A., Hawdon, J., Perregaux, M., Hotez, P., Guarente, L., *et al.*, **2000**. A common muscarinic pathway for diapause recovery in the distantly related nematode species *Caenorhabditis elegans* and *Ancylostoma caninum*. *Proc. Natl Acad. Sci. USA 97*, 460–465.

Tomioka, K., Agui, N., Bollenbacher, W.E., **1995**. Electrical properties of the prothoracicotropic hormone cells in diapausing and non-diapausing pupae of the tobacco hornworm, *Manduca sexta*. *Zool. Sci. 12*, 165–173.

Truman, J.W., **1972**. Physiology of insect rhythms. II. The silkmoth brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol. 81*, 99–114.

Truman, J.W., Riddiford, L.M., **1974**. Physiology of insect rhythms. 3. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol. 60*, 371–382.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Vafopoulou, X., Sim, C.-H., Steel, C.G.H., **1996**. Prothoracicotropic hormone in *Rhodnius prolixus*: *in vitro* analysis and changes in amounts in the brain and retrocerebral complex during larval-adult development. *J. Insect Physiol. 42*, 407–415.

Vafopoulou, X., Steel, C.G.H., **1996**. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA 93*, 3368–3372.

Vafopoulou, X., Steel, C.G., **1997**. Ecdysteroidogenic action of *Bombyx* prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus in vitro*. *J. Insect Physiol. 43*, 651–656.

Vafopoulou, X., Steel, C.G., **1998**. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. Comp. Physiol. A 182*, 343–349.

Vafopoulou, X., Steel, C.G.H., **1999**. Daily rhythm of responsiveness to prothoracicotropic hormone in prothoracic glands of *Rhodnius prolixus*. *Arch. Insect. Biochem. Physiol. 41*, 117–123.

Vafopoulou, X., Steel, C.G.H., **2001**. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *J. Insect Physiol. 47*, 935–941.

Vafopoulou, X., Steel, C.G.H., **2002**. Prothoracicotropic hormone of *Rhodnius prolixus*: partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *Invert. Reprod. Devel. 42*, 111–120.

Vázquez-Prado, J., Casas-Gonzalez, P., Gracia-Sáinz, J.A., **2003**. G protein-coupled receptor cross-talk: pivotal roles of protein phosphorylation and protein-protein interactions. *Cell. Signall. 15*, 549–557.

Vedeckis, W.V., Gilbert, L.I., **1973**. Production of cyclic AMP and adenosine by the brain and prothoracic glands of *Manduca sexta*. *J. Insect Physiol. 19*, 2445–2457.

Vedeckis, W.V., Bollenbacher, W.E., Gilbert, L.I., **1974**. Cyclic AMP as a possible mediator of prothoracic gland activation. *Zool. Jahrb. Physiol. 78*, 440–448.

Vedeckis, W.V., Bollenbacher, W.E., Gilbert, L.I., **1976**. Insect prothoracic glands: a role for cyclic AMP in the stimulation of α ecdysone secretion. *Mol. Cell. Endocrinol. 5*, 81–88.

Venkatesh, K., Hasan, G., **1997**. Disruption of the IP3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. *Curr. Biol. 7*, 500–509.

Venkatesh, K., Siddhartha, G., Joshi, R., Patel, S., Hasan, G., **2001**. Interactions between the inositol 1,4,5-trisphosphate and cyclic AMP signaling pathways regulate larval molting in *Drosophila*. *Genetics 158*, 309–318.

Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J., *et al.*, **2000**. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol. 150*, F69–F76.

Vogt, A., Cooley, K.A., Brisson, M., Tarpley, M.G., Wipf, P., *et al.*, **2003**. Cell-active dual specificity phosphatase inhibitors identified by high-content screening. *Chem. Biol. 10*, 733–742.

Volarevic, S., Thomas, G., **2001**. Role of S6 phosphorylation and S6 kinase in cell growth. *Prog. Nucl. Acid Res. Mol. Biol. 65*, 101–127.

Walker, G.P., Denlinger, D.L., **1980**. Juvenile hormone and moulting hormone titres in diapause- and non-diapause destined flesh flies. *J. Insect Physiol. 26*, 661–664.

Warren, J.T., Bachman, J.S., Dai, J.-D., Gilbert, L.I., **1996**. Differential incorporation of cholesterol and cholesterol derivatives by the larval ring glands and adult ovaries of *Drosophila melanogaster*: a putative explanation for the *(l(3)ecd^1* mutation. *Insect Biochem. Mol. Biol. 26*, 931–943.

Warren, J.T., Gilbert, L.I., **1996**. Metabolism i*n vitro* of cholesterol and 25-hydroxycholesterol by the larval prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 26*, 917–929.

Watson, R.D., Ackerman-Morris, S., Smith, W.A., Watson, C.J., Bollenbacher, W.E., **1996**. Involvement of microtubules in prothoracicotropic hormone-stimulated ecdysteroidogenesis by insect (*Manduca sexta*) prothoracic glands. *J. Exp. Biol. 276*, 63–69.

Watson, R.D., Bollenbacher, W.E., **1988**. Juvenile hormone regulates the steroidogenic competence of *Manduca sexta* prothoracic glands. *Mol. Cell. Endocrinol. 57*, 251–259.

Watson, R.D., Yeh, W.E., Muehleisen, D.P., Watson, C.J., Bollenbacher, W.E., **1993**. Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of cyclic AMP. *Mol. Cell. Endocrinol. 92*, 221–228.

Westbrook, A.L., Regan, S.A., Bollenbacher, W.E., **1993**. Developmental expression of the prothoracicotropic hormone in the CNS of the tobacco hornworm, *Manduca sexta*. *J. Comp. Neurol. 327*, 1–16.

Wetzker, R., Bohmer, F.D., **2003**. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat. Rev. Mol. Cell Biol. 4*, 651–657.

Wigglesworth, V.B., **1933**. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera); with special reference to the function of oenocytes and of the dermal glands. *Q. J. Microsc. Sci. 76*, 296–318.

Wigglesworth, V.B., **1934**. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II Factors controlling moulting and “metamorphosis.” *Q. J. Microsc. Sci. 77*, 191–222.

Wigglesworth, V.B., **1940**. The determination of characters at metamorphosis in *Rhodnius prolixus*. *J. Exp. Biol. 17*, 201–222.

Wigglesworth, V.B., **1955**. The breakdown of the thoracic gland in the adult insect, *Rhodnius prolixus*. *J. Exp. Biol. 32*, 485–491.

Williams, C.M., **1947**. Physiology of insect diapause II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull. 93*, 89–98.

Williams, C.M., **1967**. The present status of the brain hormone. In: Beament, J.W.L., Treherne, J.E. (Eds.), Insects and Physiology. Oliver and Boyd, Edinburgh, pp. 133–139.

Xu, W.H., Denlinger, D.L., **2003**. Molecular characterization of prothoracicotropic hormone and diapause hormone in *Heliothis virescens* during diapause, and a new role for diapause hormone. *Insect Mol. Biol. 12*, 509–516.

Xu, W.H., Rinehart, J.P., Denlinger, D.L., **2003**. Structural characterization and expression analysis of prothoracicotropic hormone in the corn earworm, *Helicoverpa zea*. *Peptides 24*, 1319–1325.

Yagi, S., Fukaya, M., **1974**. Juvenile hormone as a key factor regulating larval diapause of the rice stem borer, *Chilo suppressalis*. *Appl. Ent. Zool. 9*, 247–255.

Yagi, Y., Ishibashi, J., Nagata, K., Kataoka, H., Suzuki, A., *et al.*, **1995**. The brain neurosecretory cells of the moth *Samia cynthia ricini*: immunohistochemical localization and developmental changes of the *Samia* homologues of the *Bombyx* prothoracicotropic hormone and bombyxin. *Devel. Growth Different. 37*, 505–516.

Yin, C.M., Chippendale, G.M., **1973**. Juvenile hormone regulation of the larval diapause of the southwestern corn borer, *Diatraea grandiosella*. *J. Insect Physiol. 19*, 2403–2420.

Yokoyama, I., Endo, K., Yamanaka, A., Kumagai, K., **1996**. Species-specificity in the action of big and small prothoracicotropic hormones (PTTHs) of the swallowtail butterflies, *Papilio xuthus*, *P. machaon*, *P. bianor* and *P. helenus*. *Zool. Sci. 13*, 449–454.

Závodská, R., Sauman, I., Sehnal, F., **2003**. Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *J. Biol. Rhythms 18*, 106–122.

Zimowska, G., Handler, A.M., Cymborowski, B., **1985**. Cellular events in the prothoracic glands and ecdysteroid titres during the last larval instar of *Spodoptera littoralis*. *J. Insect Physiol. 31*, 331–340.

Žitňan, D., Kingan, T.G., Kramer, S.J., Beckage, N.E., **1995**. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J. Comp. Neurol. 356*, 83–100.

Žitňan, D., Sehnal, F., Bryant, P.J., **1993**. Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed *Drosophila*. *Devel. Biol. 156*, 117–135.

Relevant Website

http://www.cbs.dtu.dk – This site provides a number of online sequence analysis tools, including programs to predict glycosylation sites in proteins.

3.3 Ecdysteroid Chemistry and Biochemistry

R Lafont and C Dauphin-Villemant, Universite Pierre et Marie Curie, Paris, France
J T Warren, University of North Carolina, Chapel Hill, NC, USA
H Rees, University of Liverpool, UK

3.3.1. Introduction

It is not the aim here, owing to the limitations of space, to review all the literature concerning the chemistry and biochemistry of ecdysteroids, and the reader should consult older articles in several excellent reviews by Denis Horn (Horn, 1971; Horn and Bergamasco, 1985) and the book edited by Jan Koolman (Koolman, 1989). After a short historical introduction, we will focus on recent data concerning (1) the techniques used for ecdysteroid analysis and (2) the biosynthesis and metabolism of these hormones. However, we will not give details about chemical synthesis procedures, and we will limit this topic to a few chemical reactions that can be performed by (trained) biologists and do not require an experienced chemist.

The endocrine control of insect molting was established in part by the pioneering work of Stefan Kopec (1922), who demonstrated the need for a diffusible factor originating from the brain (see **Chapter 3.2**) in order for metamorphosis to take place in the gypsy moth, *Lymantria dispar*. This role of the brain was further substantiated by Wigglesworth (1934) with experiments using the blood-sucking bug *Rhodnius prolixus* to define "critical periods" for the necessity of the brain to produce a molting factor (see **Chapter 3.2**). The presence in the hemolymph of a molting/metamorphosis hormone was also established by other approaches: Frew (1928) cultivated imaginal discs *in vitro* in hemolymph from either larvae or pupae and he observed their evagination in the second medium only; Koller (1929) and Von Buddenbrock (1931) observed that the injection of hemolymph of molting animals into younger ones was able to accelerate molting of the latter. Fraenkel (1935) ligated larvae of *Calliphora erythrocephala* and observed pupation in the anterior part only; this provided a basis for the establishment of a bioassay for molting hormone activity, later used for the isolation of ecdysone (E) by Butenandt and Karlson (1954). The use of double ligation experiments with *Bombyx mori* larvae allowed Fukuda (1940) to demonstrate that a factor originating in the brain (i.e., the "brain hormone") (see **Chapter 3.2**) was relayed to an endocrine gland situated in the prothorax that produced the true "molting hormone." This

prothoracic gland was first described by Ke (1930); it is termed "ventral gland" in locusts, and in Diptera it is part of a complex endocrine structure, the "ring gland." In fact, the direct demonstration that prothoracic glands indeed produce E came much later (Chino *et al.*, 1974; King *et al.*, 1974; Borst and Engelmann, 1974), by the analysis of the secretory products of prothoracic glands cultivated *in vitro*. One year later, Hagedorn *et al.* (1975) demonstrated that the ovary of adult mosquitoes was another source of E, and this was the basis for establishing the role of ecdysteroids in the control of reproduction.

The first attempts to purify the molting hormone were performed by Becker and Plagge (1939) using the *Calliphora* bioassay derived from Fraenkel's work. α-Ecdysone was isolated in 1954 by Butenandt and Karlson after 10 years of attempts to do so. These authors used *B. mori* pupae because this material was available in large amounts. Ten more years of work was necessary to elucidate E's structure, which was finally achieved using X-ray crystallography analysis (Huber and Hoppe, 1965). A detailed description of this story can be found in Karlson and Sekeris (1966), and it is of great interest to follow how E was progressively characterized and to compare this work with what can be accomplished with presently available tools.

A second molecule, 20-hydroxyecdysone (20E) (initially termed β-ecdysone) was described soon thereafter, and this molecule was also isolated from a crayfish (Hampshire and Horn, 1966). It was rapidly established that 20E was the major molting hormone of all arthropods. Amazingly, E, which was the first of these compounds to be isolated, is at best a minor compound in most insect species when detectable, with the exception of young lepidopteran pupae, i.e., the material used by Butenandt and Karlson. Other closely related molecules were then isolated and the generic term of "ecdysteroids" was proposed for this new steroid family (Goodwin *et al.*, 1978). Meanwhile, ecdysteroids had also been isolated from plants, hence the terms "zooecdysteroids" and "phytoecdysteroids."

3.3.2. Definition, Occurrence, and Diversity

Ecdysteroids could be defined either by their biological activity (molting hormones) or their chemical structure. They represent a specific family of sterol derivatives comprising more than 300 members that bear common structural features: a *cis* (5β-H) junction of rings A and B, a 7-ene-6-one chromophore, and a *trans* (14α-OH or –H) junction of rings C and D (Figure 1). Most of them also bear the 3β-OH already present in their sterol precursor, and a set of other hydroxyl groups located both on the steroid nucleus and the side chain (Figure 2) that renders them rather water soluble. The major insect ecdysteroid is 20E, a 27-carbon (27C) molecule derived from cholesterol (C), but some insect species contain its 28C or 29C homologs, makisterone A and makisterone C, respectively (Figure 3).

The huge chemical diversity of ecdysteroids results from variations in the number and position of –OH groups, which can be either free or conjugated to various polar or apolar moieties, giving rise to the 70 different molecules presently described in insects listed in Table 1 (more information about these molecules can be found on the Ecdybase website). The greatest diversity has been found in the eggs/embryos of Orthoptera and Lepidoptera, probably because they contain very large amounts of these molecules (up to 40 μg g^{-1} fresh weight, i.e., 10 to 100 times the concentrations found in larvae or pupae). The nomenclature of insect ecdysteroids is generally derived from the ecdysone name, but

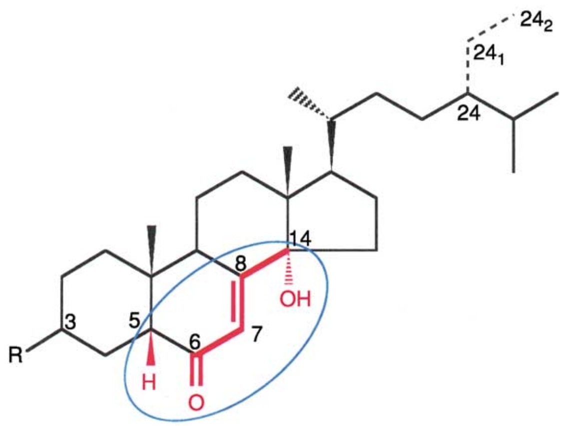


Figure 1 Common structural features of ecdysteroids (major characteristics are indicated in red/blue; R = 3β-OH in most cases).



Figure 2 Space-filling model of the 20-hydroxyecdysone molecule. Blue: carbon atoms; red: oxygen atoms; white: hydrogen atoms. (Courtesy of Pr J.-P. Girault.)

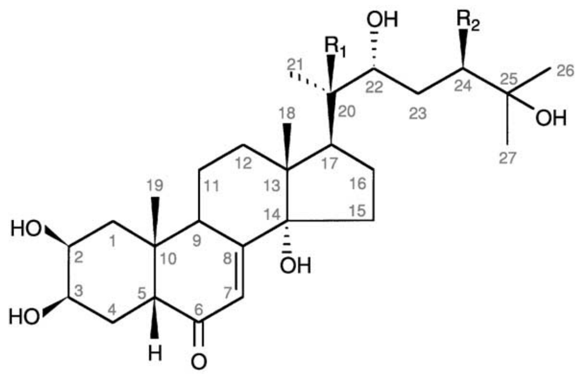


Figure 3 Structures of the major insect ecdysteroids. $R_1 = R_2 = H$: ecdysone (E); $R_1 = OH$, $R_2 = H$: 20-hydroxyecdysone (20E); $R_1 = OH$, $R_2 =$ methyl: makisterone A; $R_1 = OH$, $R_2 =$ ethyl: ethyl: makisterone C.

this is always the case, and the trivial name of many ecdysteroids (especially phytoecdysteroids) is related to the name of the species from which they have been isolated; standardized abbreviations have been proposed for insect ecdysteroids (Lafont *et al.*, 1993a) and are used throughout this chapter.

3.3.3. Methods of Analysis

3.3.3.1. Methods of Identification

3.3.3.1.1. Physicochemical properties
3.3.3.1.1.1. UV spectroscopy The 7-en-6-one group of ecdysteroids has a relatively strong absorption at $\lambda_{max} \sim 243$ nm with an ε of ~ 10–16 000: for instance E, has a λ_{max} at 242 nm and an ε of 12 400 in EtOH. This maximum of absorbance is shifted to 247 nm in water.

3.3.3.1.1.2. IR spectroscopy The presence of a number of hydroxyl groups on most ecdysteroids ensures a strong absorption in the infrared (IR) spectrum in the region of 3340–3500 cm^{-1}. The αβ-unsaturated ketone results in a characteristic cyclohexenone absorption at 1640–1670 cm^{-1}, with a weaker alkene stretch at approximately 1612 cm^{-1}. An additional carbonyl absorption is seen with 3-dehydroecdysteroids: thus 3-dehydroecdysone (3DE) and 3-dehydro-20-hydroxyecdysone (3D20E) show a carbonyl absorption at 1712 cm^{-1} and 1700 cm^{-1}, respectively, in addition to the usual 7-ene-6-one signal. Among conjugates, the absorption of phosphates ranges from 1100 to 1000 cm^{-1}. Acetates and acyl esters can be characterized by additional bands corresponding to the carbonyl and to the ester group. Ecdysteroids are not soluble in the usual infrared solvents (CS_2, CCl_4, $CHCl_3$) so IR spectra are done on KBr discs or mineral oil (Nujol) mulls. The development of Fourier transform spectrometers (FT-IR) allows the solubility problem to be overcome, as dilute solutions can be analyzed. Thus, IR spectra can even be recorded in-line during HPLC analysis (Louden *et al.*, 2001, 2002).

3.3.3.1.1.3. Fluorescence In the presence of sulfuric acid or aqueous ammonia, ecdysteroids are induced to fluoresce (Horn, 1971). Typical values for excitation and emission wavelengths are in the region of 380 and 430 nm, respectively. Some variations in excitation and emission wavelength and fluorescence intensity are observed with ecdysteroids (Koolman, 1980).

3.3.3.1.2. Mass spectrometry Mass spectrometry (MS) has been invaluable in the elucidation of ecdysteroid structures and has been particularly useful for insect samples containing low levels of ecdysteroids. Most of the early work on ecdysteroids has been performed using relatively unsophisticated direct introduction techniques (electron impact, EI), but new ionization techniques are now increasingly employed.

3.3.3.1.2.1. Modes of ionization
3.3.3.1.2.1.1. Electron impact (EI-MS) In EI-MS, an electron beam is used for the ionization and the collision will break the molecule into several characteristic fragments. With nonvolatile substances like ecdysteroids, the use of a 70 eV electron beam results in extensive fragmentation. The abundant fragments correspond to the steroid nucleus or to the side chain and are highly informative for structural elucidation.

3.3.3.1.2.1.2. Chemical ionization (CI-MS) This method uses a reagent gas that is ionized by the electron beam and gives rise to a set of ions that in turn can react with ecdysteroids (Lafont *et al.*, 1981). The ionization reaction proceeds as follows:

$$M + HR^+ \rightarrow MH^+ \text{ (pseudo-molecular ion)} + R$$

$$M + HR^+ \rightarrow MHR^+ \text{(adduct ion)}$$

where R = the reagent gas, e.g., ammonia. The relative intensity of these ions is rather high. Among the various reagent gases, ammonia is most widely used, giving both MH^+ and $(M+NH_4)^+$ ions of high intensity. This method is of interest for the determination of the molecular mass (M) of ecdysteroids. Both positive and negative ionization can be used. The CI/D techniques were analyzed in depth by Lusby *et al.* (1987) who considered the role of

Table 1 Ecdysteroids identified (and isolated in most cases) from insects (see also Rees, 1989; for a complete list of ecdysteroids and for structures, see Ecdybase)

Ecdysteroid	*Species*	*Reference*
Low polarity ecdysteroids (≤5-OH groups)		
2,14,22,25-Tetradeoxyecdysone (5β-ketol)	*Locusta migratoria* ovaries	Hetru *et al.* (1978)
2,22,25-Trideoxyecdysone (5β-ketodiol)	*Locusta migratoria* ovaries	Hetru *et al.* (1978)
2,22-Dideoxyecdysone	*Locusta migratoria* ovaries	Hetru *et al.* (1978)
2,22-Dideoxy-20-hydroxyecdysone	*Bombyx mori* ovaries	Ikekawa *et al.* (1980)
2,22-Dideoxy-23-hydroxyecdysone	*Bombyx mori*	Kamba *et al.* (2000a)
22-Deoxy-20-hydroxyecdysone	*Bombyx mori*	Kamba *et al.* (1994)
Ecdysone	*Bombyx mori* pupae	Butenandt and Karlson (1954)
2-Deoxyecdysone	*Bombyx mori* ovaries/eggs	Ohnishi *et al.* (1977)
2-Deoxy-20-hydroxyecdysone	*Schistocerca gregaria*	Isaac *et al.* (1981a)
20-Deoxy-makisterone A (=24-methyl-ecdysone)	*Drosophila* ring glands	Redfern (1984)
14-Deoxy-20-hydroxyecdysone	*Gryllus bimaculatus*	Hoffmann *et al.* (1990)
Phase I metabolites		
+ 20-OH		
20-Hydroxyecdysone	*Bombyx mori* pupae	Hocks and Wiechert (1966)
Makisterone A	*Oncopeltus fasciatus*	Kaplanis *et al.* (1975)
24-Epi-makisterone A	*Acromyrmex octospinosus*	Maurer *et al.* (1993)
Makisterone C	*Dysdercus fasciatus*	Feldlaufer *et al.* (1991)
+ 26-OH (and the subsequent oxidation products)		
20,26-Dihydroxyecdysone	*Manduca sexta* pupae	Thompson *et al.* (1967)
26-Hydroxyecdysone	*Manduca sexta* eggs	Kaplanis *et al.* (1973)
Ecdysonoic acid	*Schistocerca gregaria* eggs	Isaac *et al.* (1983)
20-Hydroxyecdysonoic acid	*S. gregaria* eggs; *Pieris brassicae* pupae	Isaac *et al.* (1983); Lafont *et al.* (1983)
20-Hydroxyecdysone 26-aldehyde (2 hemiacetal forms)	*Chironomus tentans cells*	Kayser *et al.* (2002)
+ 3-oxo/3α-		
3-Dehydroecdysone	*Calliphora erythrocephala*	Karlson *et al.* (1972)
3-Dehydro-20-hydroxyecdysone	*Calliphora vicina*	Koolman and Spindler (1977)
3-Dehydro-2-deoxyecdysone	*Locusta migratoria*	Tsoupras *et al.* (1983a)
3-Epi-20-hydroxyecdysone	*Manduca sexta*	Thompson *et al.* (1974)
3-Epiecdysone	*Manduca sexta*	Kaplanis *et al.* (1979)
3-Epi-2-deoxyecdysone	*Schistocerca gregaria*	Isaac *et al.* (1981a)
3-Epi-26-hydroxyecdysone	*Manduca sexta*	Kaplanis *et al.* (1980)
3-Epi-20,26-dihydroxyecdysone	*Manduca sexta*	Kaplanis *et al.* (1979)
3-Epi-22-deoxy-20-hydroxyecdysone	*Bombyx mori*	Mamiya *et al.* (1995)
3-Epi-22-deoxy-20,26-dihydroxyecdysone	*Bombyx mori*	Kamba *et al.* (2000b)
3-Epi-22-deoxy-16β,20-dihydroxyecdysone	*Bombyx mori*	Kamba *et al.* (2000b)
Phase II metabolites: apolar conjugates		
Acetates: 3-acetylation		
Ecdysone 3-acetate	*Schistocerca gregaria*	Isaac *et al.* (1981b)
20-Hydroxyecdysone 3-acetate	*Locusta migratoria*	Modde *et al.* (1984)
20-Hydroxyecdysone 22-acetate	*Drosophila melanogaster*	Maroy *et al.* (1988)
Acyl esters: 22-acylation		
Ecdysone 22-palmitate	*Gryllus bimaculatus*	Hoffmann *et al.* (1985)
Ecdysone 22-palmitoleate	*Gryllus bimaculatus*	Hoffmann *et al.* (1985)
Ecdysone 22-oleate	*Gryllus bimaculatus*	Hoffmann *et al.* (1985)
Ecdysone 22-linoleate	*Gryllus bimaculatus*	Hoffmann *et al.* (1985)
Ecdysone 22-stearate	*Gryllus bimaculatus*	Hoffmann *et al.* (1985)
20-Hydroxyecdysone 22-palmitate	*Heliothis virescens*	Kubo *et al.* (1987)
20-Hydroxyecdysone 22-oleate	*Heliothis virescens*	Kubo *et al.* (1987)
20-Hydroxyecdysone 22-linoleate	*Heliothis virescens*	Kubo *et al.* (1987)
20-Hydroxyecdysone 22-stearate	*Heliothis virescens*	Kubo *et al.* (1987)
Phase II metabolites: polar conjugates		
Glucosides: 22-glucosylation		
26-Hydroxyecdysone 22-glucoside	*Manduca sexta*	Thompson *et al.* (1987a)
Phosphates		
2-Phosphates		
Ecdysone 2-phosphate	*Schistocerca gregaria*	Isaac *et al.* (1984)

Table 1 Continued

Ecdysteroid	Species	Reference
26-Hydroxyecdysone 2-phosphate	*Manduca sexta*	Thompson *et al.* (1987b)
3-Epi-22-deoxy-20-hydroxyecdysone 2-phosphate	*Bombyx mori*	Mamiya *et al.* (1995)
3-Epi-22-deoxy-20,26-dihydroxyecdysone 2-phosphate	*Bombyx mori*	Kamba *et al.* (2000b)
3-Epi-22-deoxy-16β,20-dihydroxyecdysone 2-phosphate	*Bombyx mori*	Kamba *et al.* (2000b)
3α-Phosphates		
3-Epi-2-deoxyecdysone 3-phosphate	*Locusta migratoria*	Tsoupras *et al.* (1982b)
3-Epi-20-hydroxyecdysone 3-phosphate	*Pieris brassicae*	Beydon *et al.* (1987)
3β-Phosphates		
Ecdysone 3-phosphate	*Locusta migratoria*	Lagueux *et al.* (1984)
22-Deoxy-20-hydroxyecdysone 3-phosphate	*Bombyx mori*	Kamba *et al.* (1994)
2,22-Dideoxy-20-hydroxyecdysone 3-phosphate	*Bombyx mori*	Hiramoto *et al.* (1988)
2,22-Dideoxy-23-hydroxyecdysone 3-phosphate	*Bombyx mori*	Kamba *et al.* (2000a)
22-Phosphates		
2-Deoxyecdysone 22-phosphate	*Schistocerca gregaria*	Isaac *et al.* (1982)
2-Deoxy-20-hydroxyecdysone 22-phosphate	*Locusta migratoria*	Tsoupras *et al.* (1982a)
Ecdysone 22-phosphate	*Schistocerca gregaria*	Isaac *et al.* (1982)
20-Hydroxyecdysone 22-phosphate	*Locusta migratoria*	Tsoupras *et al.* (1982a)
3-Epi-2-deoxyecdysone 22-phosphate	*Bombyx mori*	Kamba *et al.* (1995)
3-Epi-ecdysone 22-phosphate	*Bombyx mori*	Kamba *et al.* (1995)
26-Phosphates		
26-Hydroxyecdysone 26-phosphate	*Manduca sexta*	Thompson *et al.* (1985b)
Phase II metabolites/complex conjugates		
Acetyl-phosphates		
3-Acetylecdysone 2-phosphate	*Schistocerca gregaria*	Isaac *et al.* (1984)
3/2-Acetylecdysone 22-phosphate	*Schistocerca gregaria*	Isaac and Rees (1984)
3-Acetyl-20-hydroxyecdysone 2-phosphate	*Locusta migratoria; Schistocerca gregaria*	Modde *et al.* (1984), Isaac and Rees (1984)
3/2-Acetyl-20-hydroxyecdysone 22-phosphate	*Locusta migratoria*	Tsoupras *et al.* (1983b)
Ecdysone 2,3-diacetate 22-phosphate	*Locusta migratoria*	Lagueux *et al.* (1984)
Nucleotides		
2-Deoxyecdysone 22-AMP	*Locusta migratoria*	Tsoupras *et al.* (1983a)
Ecdysone 22-AMP	*Locusta migratoria*	Hétru *et al.* (1984)
Ecdysone 22-isopentenyl-AMP	*Locusta migratoria*	Tsoupras *et al.* (1983b)
Ecdysteroid-related steroids		
Bombycosterol	*Bombyx mori*	Fujimoto *et al.* (1985a)
Bombycosterol 3-phosphate	*Bombyx mori*	Hiramoto *et al.* (1988)

parameters such as source pressure and source temperature on fragmentation.

3.3.3.1.2.1.3. Field desorption (FD-MS) With FD-MS, ionization proceeds in a high electrostatic field; such conditions allow ecdysteroid molecules to lose one electron (→ M^+) without excessive heating. In such cases, the organic molecules have not received any large amount of energy and, therefore, almost no fragmentation is observed (Koolman and Spindler, 1977).

3.3.3.1.2.1.4. Fast-atom bombardment (FAB-MS) FAB-MS uses a beam of neutral atoms (argon or xenon) bearing a high kinetic energy for the ionization of involatile ionic or nonionic compounds present either in the solid state or as a solution in a glycerol matrix. FAB-MS is particularly suitable for polar ecdysteroids – phosphate conjugates (see e.g., Isaac *et al.*, 1982b; Modde *et al.*, 1984), ecdysonoic acids (Isaac *et al.*, 1984) – but of course it works also with nonionic ecdysteroids (Rees and Isaac, 1985). Sodium or potassium salts of conjugates and ecdysonoic acids give, respectively, $[M+Na]^+$ and $[M+K]^+$ ions. FAB^+ has been used to characterize fatty acyl derivatives of ecdysteroids (Dinan, 1988), but FAB^- can also be used (Wilson *et al.*, 1988a). When using a deuterated matrix, extensive deuterium exchange takes place and it allows the number of hydroxyl groups present in the molecule to be determined (Pís and Vaisar, 1997).

3.3.3.1.2.1.5. Electrospray (ES-MS) ES-MS has been introduced more recently (Hellou *et al*., 1994). It allows a high intensity of molecular ions to be obtained and is recommended for fragile molecules. Moreover, it can be conveniently coupled with HPLC for in-line analysis.

3.3.3.1.2.2. Low/high-resolution mass spectrometry (HR-MS) All the above methods provide low-resolution mass spectra. Such data result, in fact, from an approximation, as the exact molecular mass of atoms (with the exception of the ^{12}C carbon atom, which by definition is equal to 12) is not a whole number (for example, the mass of ^{1}H is 1.00783 and that of ^{16}O is 15.99491). By using high-resolution instruments, the mass resolution is much increased and the masses of fragments can be obtained with a great accuracy, allowing the spectroscopist to unambiguously determine the elementary composition of ions and of their parent molecules. This technique has fully replaced the former elementary analysis.

3.3.3.1.2.3. Tandem mass spectrometry (MS-MS) The determination of fragment structure and filiation is by no means easy. The MS-MS techniques correspond to the sequential operation of several chambers (quadrupoles) where fragment separation takes place. Thus, the first quadrupole is used to select one ion, and following the passage of that ion through a "fragmentation cell," the second one is used to analyze the further fragmentation of this selected ion. An extensive analysis of this type allows the establishment of genetic relationships between all the observed fragments and is of considerable help in the understanding of fragmentation mechanisms. The same method can also be used to search for the presence of a specific compound in a crude mixture (Mauchamp *et al*., 1993).

3.3.3.1.2.4. General rules for mass spectrometry The fragmentation patterns of ecdysteroids are dominated by ions resulting from the ecdysteroid nucleus and those due to the side chain (Nakanishi, 1971; **Figure 4**). Because of the ready elimination of water from these polyhydroxy compounds, molecular ions, if present, are usually weak (<1%) when using EI-MS, although [M-18]$^{+}$ ions are often readily apparent. Indeed, the high mass region of the spectra of ecdysteroids tends to be characterized by ions 18 mass units apart resulting from the sequential losses of water (Horn, 1971). The presence of a 20,22-vicinal diol allows the cleavage of the side chain (2), giving rise to a prominent fragment at m/z 99 for 20E (otherwise dependent on the structure of the side chain), frequently forming the base peak of the EI spectrum. An ion at m/z 81 is also frequently observed, which probably results from the elimination of water from the side chain fragment (Horn, 1971; Nakanishi, 1971). In the absence of a 20,22-diol, fragmentation (1) between C_{17} and C_{20} is observed. This cleavage is less dominant than the 20,22-scission, and the result is a more complex pattern. Where the side chain has been modified to include methyl or ethyl substituents at C_{24} (e.g., the makisterones A and C), the fragments at m/z 99 and 81 are replaced by ions 14 and 28 mass units higher (Nakanishi, 1971).

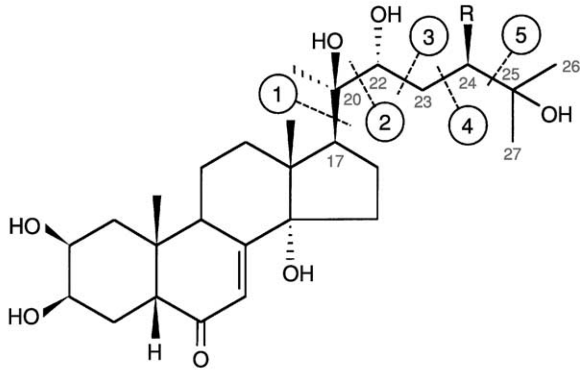


Figure 4 Major and minor fragmentations of ecdysteroid side chain. 1: C_{17}–C_{20} cleavage; 2: C_{20}–C_{22} cleavage (major if 20,22-diol); 3: C_{22}–C_{23} cleavage (minor); 4: C_{23}–C_{24} cleavage (if branching at C_{24}); 5: C_{24}–C_{25} cleavage (for TMS ethers).

3.3.3.1.3. Nuclear magnetic resonance (NMR) NMR spectroscopy is the method of choice for determining the structure of ecdysteroids. Proton ^{1}H-NMR and carbon ^{13}C-NMR are of general use, whereas the use of ^{31}P-NMR is restricted to phosphate conjugates (Hétru *et al*., 1985; Rees and Isaac, 1985) and will not be discussed here.

Obtaining a high-quality ^{1}H-NMR spectrum using modern equipment requires about 50 μg of a pure compound and this is generally sufficient to determine elementary changes like epimerization, appearance of an extra-OH group, or the position involved in a conjugation process. More important changes require ^{13}C-NMR studies, which until recently required amounts in the milligrams. Complete NMR spectra of E and 20E have been published (Kubo *et al*., 1985b; Girault and Lafont, 1988; Lee and Nakanishi, 1989), and general tables are given in several reviews (e.g., Hoffmann and Hétru, 1983; Horn and Bergamasco, 1985; Rees and Isaac, 1985; see also Ecdybase website). Most NMR data have been obtained using C_5D_5N as solvent, except for very apolar compounds (apolar conjugates or acetate derivatives analyzed in $CDCl_3$) and polar conjugates (analyzed in D_2O). These

solvents may be of interest for use with specific compounds in connection with either solubility problems or the presence of signals, which would be masked by signals due to the classical solvents. For ^{1}H-NMR studies using modern machines, the water solubility of many common ecdysteroids is high enough to obtain excellent spectra (Girault and Lafont, 1988).

3.3.3.1.3.1. Proton NMR There have been spectacular improvements in proton NMR with the introduction of high-field machines and sophisticated software, and this has resulted not only in the possibility of using smaller amounts of ecdysteroids but also of obtaining more information from the spectra. Classical proton NMR spectroscopy (in C_5D_5N) allows the easy determination of methyl groups (usually C_{18}, C_{19}, C_{21}, C_{26}, and C_{27}, δ 0.8–1.4 ppm) and of a few additional signals corresponding to 7-H (vinylic proton, δ *ca.* 6.2 ppm) and to primary or secondary alcoholic functions (2-H, 3-H and 22-H δ 4.0–4.2 ppm). Other signals (CH or CH_2 groups) overlap in the same area of the spectrum (δ 1.5–2.3 ppm), with the noticeable exception of 9-H (δ *ca.* 3.5 ppm) and 5-H (δ *ca.* 3.0 ppm) (Horn, 1971). Furthermore, two-dimensional techniques such as 2D-COSY and relayed correlated spectroscopy (COSY) have allowed the complete assignment of all protons of the ecdysone molecule (Kubo *et al.*, 1985a; Girault and Lafont, 1988). The proton NMR spectrum strongly depends upon the solvent and, to a lesser extent, on the temperature used. In most cases the solvent used was C_5D_5N or $CDCl_3$ (for acetate derivatives essentially), but for some compounds such data are lacking and the available spectra were obtained in D_2O, CD_3OD, …, so they are more difficult to compare. Coupling constants also provide useful information concerning the stereochemistry of the ecdysteroid molecule, and, for example, they allow 3α- and 3β-OH compounds to be distinguished.

3.3.3.1.3.2. Carbon-NMR The introduction of ^{13}C NMR (Lukacs and Bennett, 1972) represented a major advance in the determination of ecdysteroid structures. The signals of the carbon atoms are spread over 200 ppm. Thus, ^{13}C NMR spectra are better resolved than ^{1}H spectra. Due to the low natural abundance of ^{13}C, relatively large samples (≥10 mg) are required. Off-resonance decoupled spectra show the coupling of carbon atoms to protons, allowing the carbons to be distinguished from the number (0–3) of protons they bear and allowing an easier interpretation of spectra.

New methods have recently become available for collecting ^{13}C information thanks to inverse correlations with proton signals: the heteronuclear multiple-quantum correlation (HMQC) and the heteronuclear multiple-bond correlation (HMBC), which make use of the direct ($^{1}J^{13}$C-H) and long range ($^{2}J^{13}$C-H and $^{3}J^{13}$C-H) couplings, respectively (**Figure 5**; Girault, 1998); they allow ^{13}C data to be obtained with less than 500 μg of ecdysteroid. The production of higher field instruments (up to 800 MHz) will continue to reduce sample requirements (to 100 μg or even less, if completely pure).

3.3.3.2. Methods of Purification

Ecdysteroids form a group of rather polar compounds and, as a consequence, their extraction is usually performed using a polar solvent such as MeOH. The next step usually involves one or more solvent partitions with the aim of removing the bulk of polar and nonpolar contaminants prior to chromatography (**Figure 6**).

3.3.3.2.1. Extraction procedures Usually, a polar solvent (MeOH, at least 10× sample weight v/w) is used. Two or three repeats are necessary to extract ecdysteroids quantitatively. Alternative solvents include the less toxic EtOH, or Me_2CO, MeCN, $MeOH$-H_2O or H_2O. Extraction can be performed at room temperature, but slight heating (<60 °C) or even refluxing conditions (Soxhlet apparatus) may significantly improve its efficiency.

3.3.3.2.2. Partition techniques The crude extract is concentrated under reduced pressure to give an oily residue. The rather peculiar polarity of ecdysteroids allows their purification by solvent partitioning. This of course applies to compounds having a polarity in the same range as E and 20E. In the cases where polar or apolar ecdysteroids are present (e.g., phosphate or acyl esters), it is clear that those derivatives will partition in a very different way according to their respective polarity, and they may be lost.

3.3.3.2.2.1. Solvent partitioning In early studies (see Horn, 1971), the initial partition was made between an aqueous concentrate and Bu-1-OH. The Bu-1-OH residue, into which the ecdysteroids were extracted, was then partitioned between aqueous MeOH and hexane to remove nonpolar material such as lipids. However, reversing this order of operations was found to be beneficial and led to reduced emulsion formation during the partition step and less problems with frothing during evaporation. Other suitable solvents for removing lipids include hexane-MeOH (7:3, v/v), light petroleum (b.p. 40–60 °C), or Pr-1-OH-hexane (3:1, v/v), the

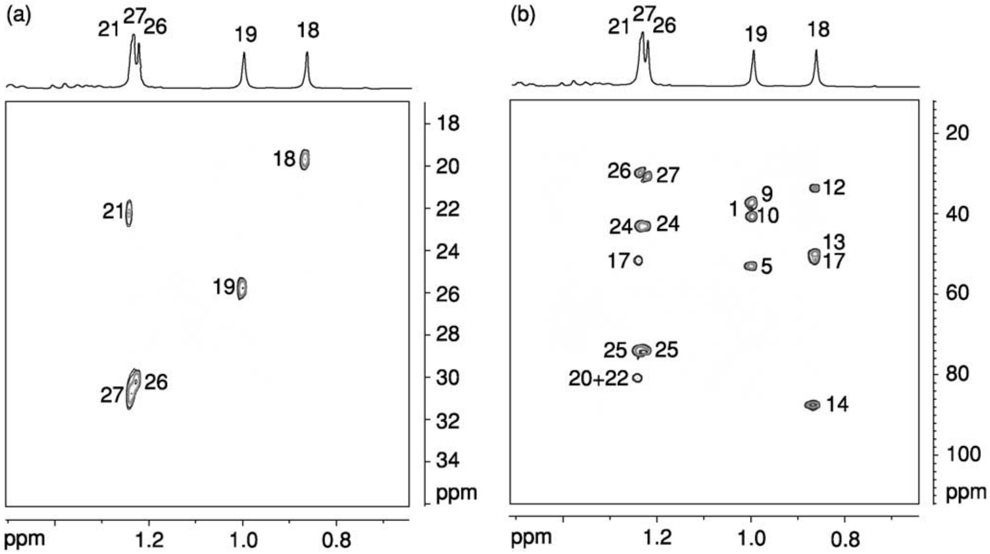


Figure 5 Inverse correlation NMR spectra of 20-hydroxyecdysone in D_2O. (a) Heteronuclear multiple-quantum correlation allows the observation of the direct $^1J^{13}$C-H couplings (e.g., between the 18-Me protons and C-18); (b) Heteronuclear multiple-bond correlation allows the observation of $^2J^{13}$C-H (e.g., between 18-Me protons and C-13) and $^3J^{13}$C-H (e.g., between 18-Me protons and C-12, C-14, and C-17) long-range couplings. (Courtesy of Dr J.-P. Girault.)

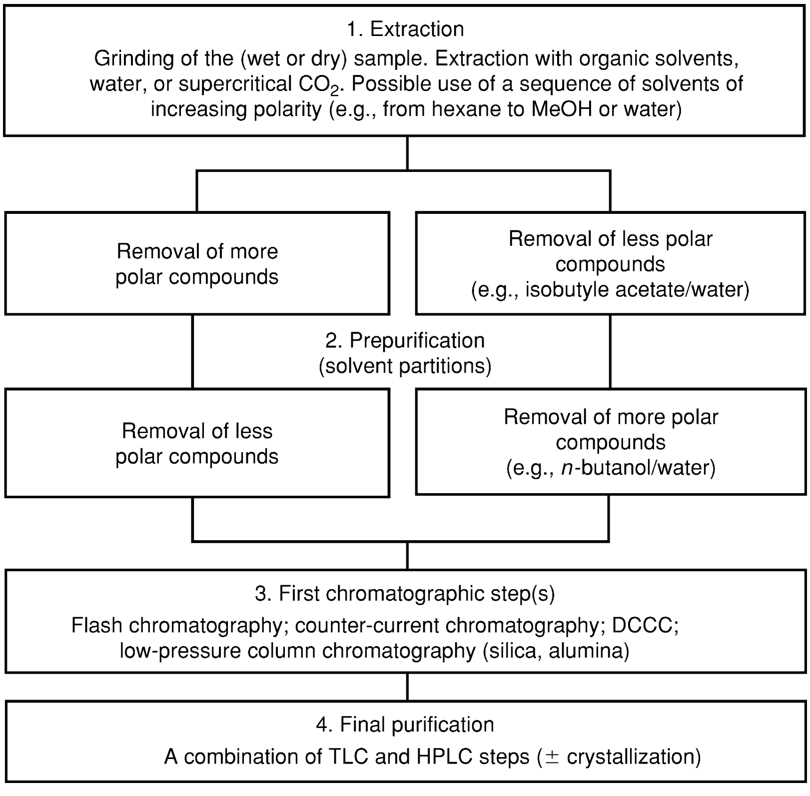


Figure 6 Example of an extraction and purification chart for the large-scale isolation of ecdysteroids. (Reprinted with permission from Lafont, R., Morgan, E.D., Wilson, I.D., **1994b**. Chromatographic procedures for phytoecdysteroids. *J. Chromatogr. 658*, 31–53; © Elsevier.)

ecdysteroids remaining in the aqueous phase. The addition of $(NH_4)_2SO_4$ improves phase separation. The separation of polar impurities from the ecdysteroids can be achieved by partition between H_2O and Bu-1-OH (ecdysteroids partition into the organic phase) and H_2O-EtOAc (ecdysteroids remain in the aqueous phase). The major factors governing the choice of solvent partition system

are the type of contaminants to be removed (i.e., mainly lipids or mainly polar compounds, etc.) and the nature of the ecdysteroids to be isolated. Thus, the addition or removal of one-OH group, or conjugation to polar (e.g., sulfate) or nonpolar (e.g., acetate or fatty acyl) groups can significantly affect partition ratios. A combination of two successive partitions allows elimination of both polar and apolar contaminants. The use of two systems having one solvent in common avoids the need for an evaporation step between the two partitions. It is thus possible to combine first $CHCl_3/H_2O$ followed by H_2O/Bu-1-OH. $CHCl_3$ can be replaced by a more polar organic solvent, such as isobutyl acetate that, allows ecdysteroids to remain in the water phase (Matsumoto and Kubo, 1989).

3.3.3.2.2.2. Counter-current distribution (CCD) Counter-current distribution between Bu-1-OH and H_2O is effective for removing polar contaminants and several other systems have been successfully used in the past (Horn and Bergamasco, 1985). For instance, Butenandt and Karlson (1954) used this method to purify ecdysone from *Bombyx* pupae by means of Craig countercurrent distribution with butanol-cyclohexane-water (6:4:10, v/v/v). This method allowed an efficient separation of two different fractions containing E and 20E, respectively. The same two ecdysteroids were also isolated from an extract of 15 kg *Calliphora* pupae (Karlson, 1956).

3.3.3.2.2.3. Droplet counter-current chromatography (DCCC) DCCC provides an efficient means for purifying samples up to the gram range. It belongs to the family of liquid–liquid partition chromatographic methods. In favorable cases, DCCC can enable the preparation of pure compounds (Kubo *et al*., 1985a). Generally, a subsequent HPLC step is required to get pure ecdysteroids. In a related procedure, high-speed countercurrent chromatography (HSCCC), the column is a multilayer coil and efficient mixing is achieved through planetary rotation (Ito, 1986). The centrifugal forces allow separations to be achieved within hours instead of days with DCCC.

3.3.3.2.3. Column chromatography (low pressure)
3.3.3.2.3.1. Preparative columns This technique can be easily scaled-up according to sample size; it just suffices to keep a low sample to sorbent ratio. It can be used with various stationary phases (NP: silica, alumina; RP: hydrophobic resins, polyamide, or C_{18}-bonded silica; ion-exchange phases, etc.). Elution usually proceeds with a step-gradient of the mobile phase with increasing eluting power; fractions are collected and assayed, for example, by TLC or HPLC.

3.3.3.2.3.2. Small columns and/or disposable cartridges
3.3.3.2.3.2.1. Normal phase Low-pressure chromatography on a small column was used in very early methods for the fractionation of crude extracts: silica or alumina columns (normal phase systems) were generally eluted with binary mixtures, e.g., a step-gradient of alcohol in chloroform or benzene.

3.3.3.2.3.2.2. Reversed phase The availability of hydrophobic phases (resins like Amberlite® or hydrocarbon-bonded silica) has led to a complete renewal of the early procedures. Small cartridges or syringes containing 0.2–1 g of HPLC phase are ideally suited for a rapid clean up of small samples (Lafont *et al*., 1982; Watson and Spaziani, 1982; Pimprikar *et al*., 1984; Rees and Isaac, 1985; Lozano *et al*., 1988b; Wilson *et al*., 1990b). They can also be used for desalting purposes, e.g., direct adsorption of ecdysteroids from culture media, from buffers used for enzymatic hydrolysis of conjugates, or from reversed phase HPLC fractions when an involatile buffer is used.

3.3.3.3. Methods of Separation

The general characteristics of separation are summarized in **Table 2**.

3.3.3.3.1. Thin-layer chromatography (TLC, HPTLC, OPLC)
3.3.3.3.1.1. Chromatographic procedures Normal-phase (absorption) chromatography on silica gel has been used extensively for the isolation of ecdysteroids and for metabolic studies (Horn, 1971; Morgan and Poole, 1976; Morgan and Wilson, 1989).

3.3.3.3.1.1.1. Normal-phase and reversed-phase systems A general normal phase system consists of 95% EtOH-$CHCl_3$ (1:4, v/v). For reproducible results, plates should be heated for 1 h at 120 °C, and then deactivated to constant activity over saturated saline, and TLC tanks should be saturated with the vapor of the solvent used for the chromatography before the plates are developed. Reversed-phase TLC on either bonded or paraffin coated plates has been widely used (Wilson *et al*., 1981, 1982b; Wilson, 1985). Bonded silicas with various alkyl chain lengths of C_2 to C_{18} (or alternatively, various degrees of coating) are available and all seem

Table 2 Methods of analysis and of characterization

Method	*Subtypes*	*Detection methods*	*Comments*	*Selected references*
TLC	1D/2D, AMD NP/RP HPTLC OPLC	UV/fluorescence quenching Color reactions Radioactivity/ autoradiography Off-line MS Immuno- or bioassay		Mayer and Svoboda (1978), Wilson (1985), Wilson and Lafont (1986), Wilson and Lewis (1987), Wilson (1988), Wilson *et al.* (1988b), Báthori *et al.* (2000), Read *et al.* (1990), Wilson *et al.* (1990c); Lafont *et al.* (1993b)
LC	Low-pressure/high-pressure (HPLC)	UV Fluorescent derivatives MS (+ IR, NMR)	Analytical or preparative	Lafont and Wilson (1990), Kubo and Komatsu (1986, 1987), Marco *et al.* (1993), Evershed *et al.* (1993), Wainwright *et al.* (1997), Louden *et al.* (2002)
	NP, RP, ion-exchange, affinity	Radioactivity Immuno- or bioassay		Lozano *et al.* (1988a, 1988b) Warren *et al.* (1986)
SFC		UV MS	Very fast and efficient	Raynor *et al.* (1988, 1989), Morgan *et al.* (1988), Morgan and Huang (1990), Shin *et al.* (1993)
DCCC	Ascending, descending	UV	Preparative	Kubo *et al.* (1985a), Bathori (1998)
CE, MCE		UV	Analytical only	Large *et al.* (1992), Davis *et al.* (1993), Yasuda *et al.* (1993)
GLC	Filled or capillary columns	FID ECD MS	Requires derivatization	Poole *et al.* (1975), Bielby *et al.* (1986), Evershed *et al.* (1987)

TLC, thin-layer chromatography; AMD, automated multiple development; NP, normal-phase; RP, reversed-phase; HPTLC, high-performance thin-layer chromatography; OPLC, over-pressure thin-layer chromatography; SFC, supercritical fluid chromatography; DCCC, droplet counter-current chromatography; CE, capillary electrophoresis; MCE, micellar CE; GLC, gas-liquid chromatography; MS, mass spectrometry; IR, infrared; NMR, nuclear magnetic resonance; FIR, flame ionization detector; ECD, electron capture detector.

suitable for ecdysteroids. MeOH-H_2O, Pr-2-OH-H_2O, EtOH-H_2O, MeCN-H_2O, and Me_2CO-H_2O solvent systems have been used for chromatography; MeOH-H_2O mixtures are most commonly used (Wilson *et al.*, 1981).

3.3.3.3.1.1.2. Two-dimensional TLC A given biological sample can contain a very complex mixture of ecdysteroids. In such cases, two-dimensional techniques have proved to be very efficient: e.g., silica plates developed with (1) toluene-Me_2CO-EtOH-25% aqueous ammonia (100:140:32:9 v/v), then (2) $CHCl_3$-MeOH-benzene (25:5:3 v/v) (Báthori *et al.*, 2000).

3.3.3.3.1.1.3. Visualization techniques Detection of ecdysteroids on the TLC plate can be accomplished using a variety of techniques of varying specificities. Non-specific techniques include iodine vapors or heating in the presence of ammonium carbonate, which produces fluorescent spots or fluorescence quenching when a fluor (ZnS) is incorporated into the silica. More specific reagents, such as the vanillin-sulfuric acid spray, can be used to give spots of characteristic color (Horn, 1971). A slightly more specific fluorescence reaction than that obtained using ammonia detection can be achieved by spraying the plate with sulfuric acid.

3.3.3.3.1.1.4. Scanners Plate analysis can benefit from the wide array of available detectors (scanners) such as UV, UV-Vis, fluorescence, FT-IR, radioactivity, or mass spectrometry (Wilson *et al.*, 1988a, 1990a, 1990c).

3.3.3.3.1.2. General rules for TLC Ecdysteroid behavior on TLC is directly related to the number and position of free hydroxyl groups and the presence of various substituents. The migration of a given compound is given by its R_f (= reference front), defined as [migration of the substance/migration of the solvent front]. From the R_f value, another parameter can be calculated, the R_m, defined as [$R_m = \log(1/R_f - 1)$]. A single modification of an ecdysteroid results in a change in its R_m, expressed as ΔR_m which is to some extent characteristic of the modification (Koolman *et al.*, 1979).

3.3.3.3.1.3. New TLC techniques Several methods providing improved resolution when compared with conventional TLC are available.

3.3.3.3.1.3.1. Automated or Programmed multiple development (AMD and PMD) In this system, the plate is developed repeatedly with the same solvent, which is allowed to migrate further and further with each development. This method allows a reconcentration of ecdysteroids at each run, in particular by suppressing tailing, and this finally results in sharper bands and improved resolution (Wilson and Lewis, 1987).

3.3.3.3.1.3.2. Overpressure thin-layer chromatography (OPLC) In OPLC, the plate is held under an inert membrane under hydrostatic pressure (*ca.* 25 bar) and the solvent is forced through the layer by an HPLC pump. Therefore, the flow of the solvent is controlled and is no longer due to capillarity, and the plate can be developed within minutes. To be efficient, this technique requires the use of HPTLC plates. OPLC has been used with ecdysteroids in only a few instances (Read *et al.*, 1990).

3.3.3.3.2. High-performance liquid chromatography (HPLC) HPLC is the most popular technique for ecdysteroid separations, both for analytical and preparative purposes. It offers a wide choice of techniques that can be optimized for polar or apolar metabolites. The identification of any ecdysteroid by comigration with a reference compound must rely on the simultaneous use of several (at least two) different HPLC systems, generally one normal-phase (NP) and one reversed-phase (RP) system (reviews: Lafont *et al.*, 1980, 1981; Touchstone, 1986; Lafont, 1988; Thompson *et al.*, 1989; Lafont and Wilson, 1990).

3.3.3.3.2.1. Chromatographic procedures

3.3.3.3.2.1.1. Normal phase (NP) systems Silica columns are usually run using mixtures of a chlorinated solvent (e.g., CH_2Cl_2, dichloroethane) and an alcohol (e.g., MeOH, EtOH, Pr-2-OH). Water added to just below saturation partially deactivates silica and this results in more symmetrical peaks; a classical mixture is CH_2Cl_2–Pr-2-OH–H_2O (Lafont *et al.*, 1979). The respective proportions of the three components can be adapted to suit the sample polarity. Thus, specific mixtures can be prepared for nonpolar compounds, e.g., acetates or E precursors (125:15:1 v/v/v), for medium-polarity compounds (125:25:2 or 125:30:2 for E and 20E), or for more polar ecdysteroids (125:40:3 for 26-hydroxyecdysteroids) including glycoside conjugates (100:40:3).

Polar bonded columns (-diol or -polyol,-APS = aminopropylsilane,) can also be used instead of silica (Dinan *et al.*, 1981). Diol-bonded columns have been used with the phasmid *Carausius morosus* for the separation of a wide array of metabolites (Fournier and Radallah, 1988), whereas the APS phase proved particularly efficient for the separation of mixtures of 3α-OH, 3β-OH, and 3-oxo ecdysteroids (Dinan *et al.*, 1981). In addition, these columns allow the use of gradients without the problems linked with the long re-equilibration times encountered with silica. CH_2Cl_2-based solvents, although very efficient for chromatographic separations, suffer from a high UV-cutoff and the quenching properties of this compound, which preclude diode-array detection or efficient in-line radioactivity monitoring (see below). This problem may be overcome with cyclohexane based mixtures (Lafont *et al.*, 1994a), which also display a different selectivity.

3.3.3.3.2.1.2. Reversed-phase systems C_{18}-bonded silicas are the most widely used phases, with elution performed with MeOH-H_2O. MeCN-H_2O or MeCN-buffer mixtures, and are more efficient, especially when polar conjugates and/or ecdysonoic acids are present. The use of ion suppression, e.g., using an acidic buffer, is absolutely essential in the case of polar conjugates or 26-acids. Otherwise, the ionized molecules do not partition well, resulting in variable retention times. Systems have been designed for polar or apolar metabolites, both of which may exist within the same animal. The use of various pHs (Pís *et al.*, 1995a) can modify selectively the retention time of polar (ionizable) metabolites and give access to the pK value of ionizable groups, which can help to characterize conjugates.

3.3.3.3.2.1.3. Detection procedures Several types of detectors can be used for the monitoring of ecdysteroids in the eluates.

UV detector. Ecdysteroids possess a chromophore that is strongly absorbing at ~245 nm, which allows the easy detection of 10 pmol amounts.

Fluorescence detector. There have been several attempts to prepare fluorescent derivatives of ecdysteroids. The potential interest is great, as this would enhance the sensitivity of detection by at least 100 times and possibly also the selectivity as compared to UV. Precolumn or postcolumn derivatization can be used. There are several prerequisites for precolumn reactions: (1) they must be simple and give a single derivative for each ecdysteroid; (2) they must be quantitative, even at very low concentrations of

ecdysteroids present in a crude sample; (3) they must be specific; and (4) the excess reagent (if fluorescent itself) must be removed prior to HPLC analysis. Two approaches have been described: the first used phenanthrene boronic acid (Poole *et al.*, 1978), which is specific for diols and would therefore react also with sugars; the second used 1-anthroyl nitrile (Kubo and Komatsu, 1986), which reacts with alcohols, in this case the 2-OH of ecdysteroids. Interest in the second case would essentially be increased sensitivity, but it is not a specific reaction in that the authors use the same reagent for the determination of prostaglandins! These methods have not received further applications, and we must emphasize that the use of precolumn derivatization would negate one major advantage of HPLC over GLC. Postcolumn derivatization could make use of the sulfuric acid-induced fluorescence (see above), but this has not been investigated.

Diode-array detectors. Such detectors represent valuable tools since they provide the absorbance spectrum of all eluted peaks. Thus, in the case of ecdysteroids, whether or not a compound that comigrates with a reference ecdysteroid has a UV absorbance with a maximum at 242 nm can be directly checked. This is an additional criterion for assessing the identity of peaks and it works equally well with very small amounts of ecdysteroids (less than 100 ng).

Radioactivity monitors. On-line radioactivity measurement in ecdysteroid metabolic or biosynthesis studies is of considerable interest because: (1) it provides an immediate result; (2) it saves time and avoids the need for collecting many fractions, filling scintillation vials, and waiting for their measurement; (3) it provides direct comparison with the signal of another detector, e.g., UV monitor, and allows an easy check for the coelution of radioactive peaks with reference compounds; and (4) it does not significantly decrease the resolution when compared with the UV signal: this means that at a flow rate of 1 ml min^{-1} for the column effluent and 3 ml min^{-1} for the scintillation cocktail, with a detector cell size of 0.5 ml, the result is equivalent or even better than collecting 0.2 ml fractions (i.e., 200 tubes for a 40 min analysis).

Mass spectrometry (MS). On-line HPLC-MS provides useful information on the structure of ecdysteroids eluting from the column, and has become a routine technique thanks to the thermospray and electrospray techniques. Two groups (Evershed *et al.*, 1993; Marco *et al.*, 1993) reported the successful use of HPLC-MS with ecdysteroids. These techniques work in the nanogram range with single ion monitoring (SIM) detection (Evershed *et al.*, 1993). Their use with acetonide derivatives results in a stabilization of the molecules and the production of abundant molecular ions (Marco *et al.*, 1993). A further improvement was obtained with atmospheric pressure chemical ionization (APCI-MS) resulting in a much lower limit of detection – 10 pg with E and 20E (Wainwright *et al.*, 1996, 1997).

Nuclear magnetic resonance (NMR). Using superheated D_2O as eluent, it has been possible to get on-line proton NMR spectra, but this requires high (100–400 μg) amounts of ecdysteroids to be injected on the column (Louden *et al.*, 2002).

3.3.3.3.2.2. Different aims of HPLC

3.3.3.3.2.2.1. Analytical and preparative HPLC Columns of different sizes exist with the same packings, and it is therefore very easy to scale up any chromatographic method. Analytical (i.d. 4.6 mm), semi-preparative (i.d. 9.4 mm), and preparative (i.d. *ca.* 20 mm or more) columns are available. By increasing the flow rate in proportion to the cross-section, one obtains similar separations with increased maximal load.

3.3.3.3.2.2.2. Microbore HPLC Microbore HPLC has been designed essentially for coupling with mass spectrometry. It uses columns of small internal diameter (e.g., 1 mm) run with a reduced solvent flow rate (*ca.* 50 μl min^{-1}), which is compatible with a direct coupling with a mass spectrometer.

3.3.3.3.2.2.3. Quantitative analyses HPLC has been used for the direct quantification of individual ecdysteroids in biological samples. This requires of course high sensitivity because of the low concentrations encountered and adequate sample clean up. Quantification is best obtained if an internal standard is added to the sample either before HPLC analysis, or better, before sample purification (Lafont *et al.*, 1982). Many phytoecdysteroids can be used as internal standards (Wilson *et al.*, 1982a).

The fundamental place of HPLC in ecdysteroid analysis will be exemplified by a detailed analytical protocol applied to locust eggs (**Figure 7**). A combination of HPLC at various pHs and of enzymatic hydrolyses may indeed allow a rational diagnosis of ecdysteroid types present in a given biological extract (**Table 3**).

3.3.3.3.3. Gas-liquid chromatography (GLC) This method was first introduced for ecdysteroids by Katz and Lensky (1970), and developed during the following years (Morgan and Woodbridge,

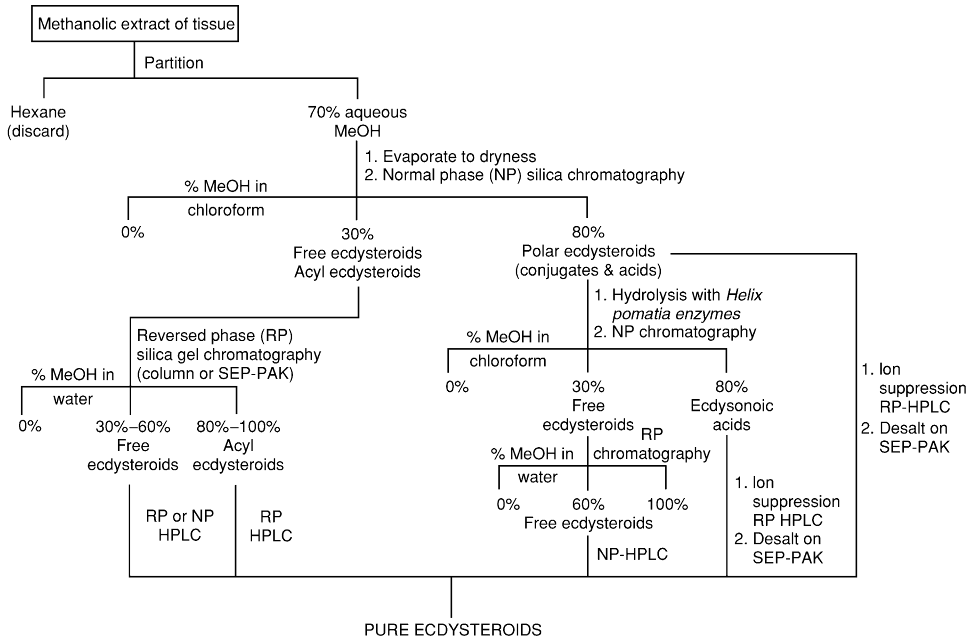


Figure 7 A general procedure for the purification of ecdysteroids combining several chromatographic steps. (From Russel, G.B., Greenwood, D.R., **1989**. Methods of isolation of ecdysteroids. In: Koolman, J. (Ed.), ecdysone from chemistry to mode of action. Georg Thieme Verlag, Stuttgart, p. 102. Reprinted by permission.)

Table 3 Strategy for a rationale diagnosis of ecdysteroids

Polarity	*Electrically charged (Yes/No)*	*pK_a (if applicable)*	*Susceptibility to hydrolysis (Yes/No)*	*Conclusion*
High		6.5–7.0	Y	Phosphate esters
	Y	4.5	N	26-Oic acids
		2.0	Y	Sulfate esters[a]
			Y	Glucosides
Medium			N	26-OH derivatives
	N	–	N	E, 20E and related
Low			N	Precursors
			Y	Acyl esters

[a]Sulfate esters have not yet been found in insects.

1971; Ikekawa *et al.*, 1972; Miyazaki *et al.*, 1973). Despite the advantages possessed by gas-liquid chromatography (GLC) in terms of sensitivity and specificity as compared to other techniques, its use for ecdysteroids has been confined to only a few groups (Borst and O'Connor, 1974; Morgan and Poole, 1976; Koreeda and Teicher, 1977; Lafont *et al.*, 1980; Webster, 1985; Bielby *et al.*, 1986; Evershed *et al.*, 1987, 1990). As all but a few biosynthetic intermediates of ecdysteroids are nonvolatile, it is necessary to convert them into volatile trimethylsilyl (TMS) ether derivatives. Detection can be carried out using either a flame ionization detector (FID, poor sensitivity), or better still, an electron capture detector (ECD) or a mass spectrometer (MS). In the 1970–1980 period, GC-MS represented a reference method for analyzing ecdysteroid mixtures (Lafont *et al.*, 1980).

With filled columns (1–3 m long), chromatography is performed on nonpolar stationary phases such as OV-1 and OV-101 coated on Gas Chrom Q and Gas Chrom P. Typical operation temperatures for the columns are in the range of 280 °C, with carrier gas flow rates of 50 ml min^{-1} (Bielby *et al.*, 1986). Retention behavior on GLC is the result of both molecular mass and polarity, depending on the degree of silylation. Capillary GC using flexible fused silica columns provides improved resolution and sensitivity. Thus, 10 pg (20E) or 100 pg (E) could be detected by GC coupled with mass spectrometric (SIM) detection (Evershed *et al.*, 1987).

3.3.3.3.4. Supercritical fluid chromatography Supercritical fluid chromatography (SFC) uses a supercritical fluid as mobile phase, which behaves like a compressible fluid of very low viscosity, where mass transfer operates very rapidly, allowing the use of high linear velocities and, therefore, very short analysis times without any loss of efficiency. SFC may use either conventional packed HPLC columns or capillary tubes, and the mobile phase is generally supercritical CO2 containing a small amount of an organic modifier, e.g., MeOH (Morgan *et al.*, 1988; Raynor *et al.*, 1988, 1989). Chromatographic parameters that can be monitored are the temperature, the pressure, and the percentage of the organic modifier. Supercritical CO_2 is a nonpolar fluid and, therefore, SFC is more or less equivalent to normal-phase HPLC; the eluting power is increased by adding MeOH. Various types of packed columns can be used, including silica or bonded silica; alternatively, fused silica capillary columns can be used (Raynor *et al.*, 1988). A major advantage of SFC over HPLC is the shorter retention times; compounds elute as sharp peaks and the sensitivity of detection is accordingly increased. A second advantage concerns the high transparency of CO_2 in the infrared (IR), which allows the use of FT-IR detectors. Furthermore, SFC is compatible with both flame ionization detection (the universal detection used with GLC) and mass spectrometry. No derivatization is required, and this represents an important advantage over GLC.

3.3.3.3.5. Capillary electrophoresis (CE) Capillary electrophoresis is a recently introduced technique that allows extremely efficient separation of peptides, oligonucleotides, and also steroid hormones. The migration of substances is due to a combination of electroendosmotic flow of buffer towards the cathode and the (slower) migration of negatively charged solutes towards the anode. CE gives very efficient separations for a wide range of ionized and unionized compounds. As most of the ecdysteroids are not ionizable, CE has to be performed using some form of micellar CE (MCE, also known as MECK or micellar electrokinetic chromatography). In this mode, a surfactant such as sodium dodecyl sulfate (SDS) is added to the buffer at a concentration above its critical micellar concentration. The compounds of interest then partition between the aqueous phase and the micelles as these are drawn through the capillary by the electroosmotic flow. Such an approach has been shown to be well suited to the CE of ecdysteroids. Two types of methodology have been used in CE: with SDS and an organic modifier such as MeOH (Large *et al.*, 1992; Davis *et al.*, 1993); or with SDS and γ-cyclodextrin as modifiers (Yasuda *et al.*, 1993). CE has been used for extracts from the eggs of *Schistocerca gregaria* (Davis *et al.*, 1993) and crayfish hemolymph (Yasuda *et al.*, 1993). The sensitivity of MCE for ecdysteroids was very good and the on-column detection of *ca.* 175 pg of ecdysone was readily achieved. In fact, due to the very small volume that can be injected on the column (5 nl), this corresponds in practice to a much larger sample containing 35 μg ml^{-1} (i.e., a 10 μl sample would have to contain 350 ng ecdysteroid).

3.3.3.4. Methods of Quantification

One can use the physicochemical techniques as discussed above (see **Table 4**) or, alternatively, bioassays and immunoassays (**Table 5**) performed either on crude extracts or on fractions collected after a chromatographic separation (TLC, HPLC).

3.3.3.4.1. Bioassays Various kinds of bioassays are used for testing ecdysteroids from animal or plant extracts, either *in vivo* or *in vitro*. Their purpose may be either the measurement of ecdysteroid concentrations in biological samples (they allowed the isolation of E by Butenandt and Karlson, 1954), to analyze the relative biological activity of various ecdysteroids (structure–activity relationships), or to screen plant extracts for ecdysteroid content in a semiquantitative way.

We may distinguish between assays where disturbances or toxic effects are assessed (e.g., abnormal molting), and bioassays designed to measure a biological activity linked to a normal physiological process or even to quantify hormones. The naturally occurring ecdysteroids exhibit a wide range of molting hormone activities when measured in bioassay systems. Bioassay systems for molting hormones have tended to be based on the larval–pupal molt of a number of dipteran species (e.g., *Calliphora*, *Sarcophaga*, and *Musca*). In these assays, last instar larvae are ligated and those showing no further development of the abdomen are selected for use in the bioassay. Extracts of (pure) compounds are then injected into the abdomen and signs of development (tanning of the cuticle) are sought. The sensitivity of the assay varies depending on the dipteran species used and the assay conditions: in *Calliphora* it is of the order of 5–50 ng per abdomen for 20E versus 5–6 ng for *Musca*. A variation on the rather specialized larval–pupal molt used in the dipteran bioassays is the locust abdomens of fourth instar *S. gregaria* and is based on a larval-to-larval molt. Another useful bioassay, which does not require the injection of the test material, is based on the use of

Table 4 Physicochemical methods of quantification: characteristics and limits of detection

Method	*Limits of detection*	*Specificity*	*References*
Direct or after a chromatographic separation			
UV absorbance (*ca.* 242 nm)	1 μg	Poor	Sardini and Krepinsky (1974)
Chugaev's color reagent (380 nm)	<10 μg	Limited	Kholodova (1977, 1981), Bondar *et al.* (1993)
Sulfuric acid induced fluorescence	10 pg	Limited	Gilgan and Zinck (1972), Koolman (1980)
Bioassay	*ca.* 5 ng	Good	Cymborowski (1989)
Immunoassay	5–10 pg	(Very) good	Porcheron *et al.* (1989)
TLC			
UV absorbance (scanner)	500 ng	Low	Wilson *et al.* (1990c), Báthori and Kalász (2001)
Fluorescence quenching (fluorescent TLC plates)	500 ng	Low	Mayer and Svoboda (1978)
Mass spectrometer			
HPLC			
UV absorbance	<10 ng	Fair	Lafont *et al.* (1982), Wright and Thomas (1983)
Fluorescence (requires derivatization)	<10 pg	?	Poole *et al.* (1978), Kubo and Komatsu (1986, 1987)
Mass spectrometer	50–100 pg	Very good	Evershed *et al.* (1993), Le Bizec *et al.* (2002)
GLC			
Flame ionization detector	50 ng	Poor	Katz and Lensky (1970)
Electron capture detector	1–10 pg	Good	Poole *et al.* (1975, 1980), Bielby *et al.* (1986)
Mass spectrometer	10–100 pg	Very good	Evershed *et al.* (1987)

ligated larvae of *Chilo suppressalis* (Sato *et al.*, 1968). These are simply dipped into methanolic solutions of the test compound of extract. An *in vitro* variation of this assay uses pieces of integument from ligated larvae of *Chilo* and has a reported sensitivity of *ca.* 15 ng per test (Agui, 1973). In all *in vivo* test systems, it is difficult to assess whether the observed biological activity derives from the injected compound or an active metabolite. An obvious example of this is the conversion of 2dE and E into the more active metabolite, 20E (as shown by comparison with their activity in *in vitro* bioassays). The same considerations may apply to 3-oxo (3-dehydro) ecdysteroids.

In vitro systems, where it is possible to determine the biological activity of a compound in the quasi-absence of its metabolism, are therefore very useful for the determination of structure–activity relationships. Among them, a microplate bioassay using a *Drosophila* cell line (B II) was designed by Clément and Dinan, 1991 and Clément *et al.*, 1993 with good sensitivity and accuracy. This assay relies on a turbidimetric measurement of cell density in a system where ecdysteroids inhibit cell proliferation. More recently, new methods have been proposed, which use either *Drosophila* Kc cells transfected with a reporter plasmid containing the 5′ region of the *hsp27* promoter and the firefly luciferase gene (Miki-Mikitani, 1995) or SL2 cells with the addition of an EcR expression plasmid (Baker *et al.*, 2000). Cells were cultured for 24 h in the presence of ecdysteroids, and then cell lysates were used to measure luciferase activity.

A number of *in vitro* bioassay systems exist, including puffing of polytene chromosomes (reviews Bergamasco and Horn, 1980; Cymborowski, 1989) and morphogenesis in imaginal discs. As Bergamasco and Horn (1980) pointed out, in bioassays where quantitative data are sought, the purity of the sample, especially the absence of other active ecdysteroids, is of paramount importance.

3.3.3.4.2. Immunoassays Immunoassay (IA) use antibodies obtained by immunization with ecdysteroids. As ecdysteroids are not directly immunogenic, they must be coupled to a suitable protein before immunization. Generally, they are derivatized to create a carboxylic function which can react with the amino groups of protein carriers (BSA, HSA or thyroglobulin). The protein–ecdysone complex is strongly immunogenic and high titers of antibodies are generally elicited after the first injection.

The specificity of antibodies is rarely absolute, as they generally will recognize both E and 20E, as well as a series of closely related ecdysteroids. The degree of hapten binding by antibodies is primarily determined by the structure of the immunogen, especially as regards the position of the linkage between the ecdysteroid and the protein. For instance, protein esterification via the C_2 hydroxyl on the A-ring or the C_{22} hydroxyl on the side chain will generally result in antisera specificity against ecdysteroid ligands exhibiting molecular changes in either the side chain or the steroid

Table 5 Bioassays and immunoassays

Method	*Subtypes*	*Detection modes*	*Comments*	*References*
Bioassays			Coupling with a separation method enhances the informative power	Review: Cymborowski (1989)
	In vivo	Induction of molting or development in hormone deprived systems	Fundamental role for ecdysone isolation; still used for some SAR[a] studies	*Calliphora* test: Adelung and Karlson (1969), Karlson and Shaaya (1964), Thomson *et al.* (1970), Fraenkel and Zdarek (1970) *Musca* test: Kaplanis *et al.* (1966) *Sarcophaga* test: Ohtaki *et al.* (1967) Diapausing *Samia cynthia* test: Williams (1968) *Chilo* dipping test: Sato *et al.* (1968), Koreeda *et al.* (1970) *Limulus* bioassay: Jegla and Costlow (1979) Blowfly abdominal bags: Agrawal and Scheller (1985) *Galleria* test: Sláma *et al.* (1993) *Dermestes* test: Sláma *et al.* (1993)
	In vitro	Organ development		Integument pieces: Agui (1973) Imaginal discs: Kuroda (1968); Oberlander (1974), Terentrou *et al.* (1993)
		Cell proliferation/ differentiation (s.l.)	SAR studies	Kc cells: Cherbas *et al.* (1980) BII cells: Clément and Dinan (1991), Clément *et al.* (1993)
		Chromosome puffing	SAR studies	Richards (1978)
		Reporter gene (e.g., luciferase or GFP) activation	SAR studies	Mikitani (1995); Baker *et al.* (2000), Swevers *et al.* (2004)
		Radio receptor assay	Uses *Drosophila* Kc cells cytosol as receptor preparation	Mikitani (1996)
Immunoassays			Coupling with a separation method enhances the informative power	Reviews: Warren and Gilbert (1988), Reum and Koolman (1989)
	RIA	Radioactivity	Radioactive tracer required	Borst and O'Connor (1972)
	CLA	Chemiluminescence	Chemiluminescent tracer required	Reum *et al.* (1984)
	EIA	Colorimetry	Enzymatic tracer required	Porcheron *et al.* (1989); Royer *et al.* (1995)
	ELISA	Colorimetry	No tracer required	Kingan (1989)

[a]SAR, structure-activity relationship.

nucleus, respectively (**Figure 8**) (Warren and Gilbert, 1988).

Immunoassays need tracer molecules. In Radioimmune Assay (RIA), tracers are radiolabeled with a high specific activity. Two kinds of analogs are used: commercially available [^{3}H]-ecdysone (50–80 Ci/mmol), and laboratory-made iodinated analogs (up to 2000 Ci/mmol), which provide a higher sensitivity (Hirn and Delaage, 1980). The RIA has several drawbacks due to the utilization of a radioisotope, which requires special precautions, and for that reason other immunoassays have been developed: a chemilumino-immunoassay (CIA), which uses ecdysone-6-carboxy-methoxime aminobutylethyl-isoluminol (ABEI) to form a chemroluminescent tracer (Reum *et al.*, 1984), and enzyme immunoassays (EIA) with an ecdysone derivative coupled to an enzyme, acetylcholinesterase (Porcheron *et al.*, 1989; Royer *et al.*, 1993) or peroxidase (Delbecque *et al.*, 1995; Von Gliscynski *et al.*, 1995).

After incubation of antibodies, tracer, and biological extract (or calibration references), the separation of free and bound fractions is performed in various ways (Reum and Koolman, 1989). The lower limit of sensitivity was estimated, in the best cases, as 4.10^{-10} mol (1.6 pg) (Hirn and Delaage,

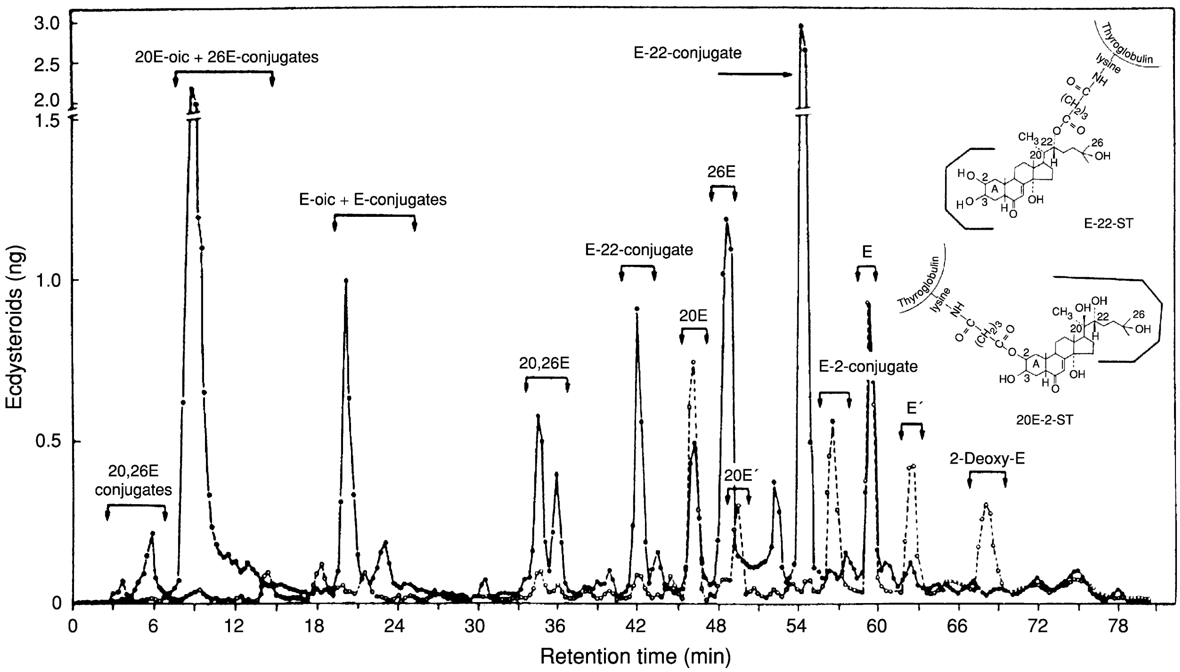


Figure 8 RP-HPLC/RIA analysis of ecdysteroids from the gut of females during day 14 of pupal–adult development in *M. sexta*. The chromatograms show the elution from a C_{18}-silica column by a neutral buffered water/acetonitrile system of a female gut sample. Analysis is by differential RIA employing two complimentary antibodies. One antibody was elicited by an ecdysone-22-succinylthyroglobulin immunogen conjugate (E-22-ST) where the ecdysteroid hapten was conjugated via its C_{22}-hydroxyl group to the immunogenic carrier protein (solid line). The other antibody was elicited by a 20-hydroxyecdysone-2-succinylthyroglobulin immunogen conjugate (20E-2-ST) where the ecdysteroid was conjugated via the C_2 hydroxyl group (dashed line). In general, substrate discrimination by antibodies is greatest for molecules that differ in their architecture at a place far from the point of hapten attachment to the immunogenic protein carrier molecule. E′: 3α-epiE, 20E′, 3α-epi20E; 26E, 26-hydroxyE; 20,26E, 20,26-dihydroxyE; E-oic, ecdysonoic acid; 20E-oic, 20-hydroxyecdysonoic acid; E-22- (or E-2)-conjugate, E conjugated at the 22 (or 2) position. (Modified from Warren *et al.*, **1986**. Metabolism of ecdysteroids during the embryogenesis of *Manduca sexta*. *J. Liquid Chromatography 918*, 1759–1782).

1980) but, in fact, the sensitivity currently achieved by most ecdysteroid immunoassays is in the range of 20–200 fmol (10–100 pg). An example of RIA performed after an HPLC separation is given in Figure 8.

3.3.3.5. Sources of Ecdysteroids

Only a few ecdysteroids are commercially available. All of them have been isolated from plants and they include only four insect ecdysteroids: 2d20E, E, 20E, and makisterone A. Because of its use as an anabolic substance by many bodybuilders (Lafont and Dinan, 2003), 20E can now be purchased in large quantities at a low price. When an ecdysteroid other than the four noted above is required, there are three ways in which it can be obtained: (1) it can be requested from someone who has already isolated (or synthesized) this compound; (2) the molecule can be isolated from an adequate plant source; or (3) it can be synthesized from an available precursor. This does not always require complicated chemistry; Table 6 lists some syntheses that are not highly complex and can be performed in the absence of an experienced chemist.

3.3.4. Ecdysteroid Biosynthesis

3.3.4.1. Sterol Precursors and Dealkylation Processes

Owing to the lack of a squalene synthase (and/or other enzymes), insects are unable to synthesize sterols *de novo* (Clayton, 1964; Svoboda and Thompson, 1985). Consequently, they rely on dietary sterols for their supply. Zoosterols are essentially cholesterol; thus, insects feeding on animal derived food get cholesterol directly. On the other hand, those feeding on plants obtain mainly phytosterols, which differ from cholesterol by the presence of various alkyl substituents (ethyl, methyl, methylene) on carbon 24. However, it should be noted that most plants also contain small but detectable amounts of cholesterol. Nevertheless, many, but not all, phytophagous insect species possess the ability to first dealkylate phytosterols to cholesterol, which is subsequently

Table 6 Just a little bit of chemistry: total or partial synthesis (side-chain modifications) and simple modifications (e.g., oxidation at C-3, removal of -OH groups (e.g., 25-OH), conjugation to acetic acid, fatty acids, glucose, sulfate)

Starting material	*Derivative*	*References*
20E	3D20E	Koolman and Spindler (1977), Spindler *et al.* (1977), Dinan and Rees (1978), Girault *et al.* (1989)
	20E′	Dinan and Rees (1978)
	Ponasterone A	Dinan (1985)
	Inokosterone	Yingyongnarongkul and Suksamrarn (2000)
	Acetates	Horn (1971)
	Glucosides	Pís *et al.* (1994)
	Poststerone	Hikino *et al.* (1969), Petersen *et al.* (1993)
	Rubrosterone	Hikino *et al.* (1969)
	2d20E	Suksamrarn and Yingyongnarongkul (1996)
	14d20E	Harmatha *et al.* (2002)
E	3DE	Spindler *et al.* (1977), Dinan and Rees (1978), Girault *et al.* (1989)
	E′	Dinan and Rees (1978)
	25dE	Pís *et al.* (1995b)
	22-Acyl esters	Dinan (1988)
	Sulfates	Pís *et al.* (1995a)
2dE	2,25dE	Pís *et al.* (1995b)
	2,22dE	Kumpun *et al.* (in preparation)

converted in endocrine tissues to the active C_{27} molting hormone 20E. By contrast, some species, mainly Hemiptera and Hymenoptera, are unable to dealkylate phytosterols and consequently produce 24-alkylated ecdysteroids (i.e., C_{28} makisterone A and/or C_{29} makisterone C; Feldlaufer *et al.*, 1986; Feldlaufer, 1989; Mauchamp *et al.*, 1993).

In some cases, due to a combination of specific mechanisms including (1) selective concentration of the dietary cholesterol (Thompson *et al.*, 1963; Svoboda *et al.*, 1980) and/or (2) its selective delivery to steroidogenic organs, the insect can produce mainly C_{27} ecdysteroids even if cholesterol is a very minor sterol component of its diet. The ecdysteroid pool usually represents only 0.1–0.2% of total sterols, as illustrated by the low conversion rate of radioactive cholesterol when injected *in vivo* for long-term labeling studies (e.g., Beydon *et al.*, 1981, 1987).

Finally, some insects have become independent of a dietary sterol supply thanks to the production of sterols by yeast-like or fungal symbionts; this situation has been described in several insect species, particularly in aphids and beetles (Svoboda and Thompson, 1985). Yeast-like symbionts provide mainly 24-alkylated sterols (e.g., ergosta-5,7,24(28)-trienol). Thus, those insects still require a

Table 7 Relationship between dietary sterols and ecdysteroid structures

Dietary sterols	*Dealkylation*	*Major ecdysteroid*
27C	Indifferent	20-Hydroxyecdysone (or Ponasterone A)
28C/29C	+	20-Hydroxyecdysone
28C	−	Makisterone A (or 24-epi-Makisterone A)
29C	−	Makisterone C

dealkylation process in order to produce cholesterol (Weltzel *et al.*, 1992; Nasir and Noda, 2003). According to the nature of their dietary sterols and their ability to dealkylate phytosterols, insects produce different ecdysteroids (**Table 7**).

3.3.4.1.1. General scheme of cholesterol formation from phytosterols A given species usually contains a complex sterol cocktail, with a few major compounds and many minor ones. Sterol diversity concerns (1) the number of carbon atoms (27 to 29) as discussed above and (2) the number and position of unsaturations, classically termed Δ: mainly Δ^5 and Δ^7 on the steroid nucleus, and Δ^{22} and Δ^{24} on the side-chain (**Figure 9**). In addition, sterols can be in free or conjugated form, i.e., sterol acyl esters with various fatty acids or sterol acylglucosides, where a sugar moiety is linked both to the 3β-OH of the sterol and to a fatty acid (Eichenberger, 1977; Grunwald, 1980).

Ingested sterols are subjected to the action of several types of enzymes: (1) hydrolytic enzymes (esterases or glycosidases) from the insect gut (see **Chapter 4.5**), which will release free sterols from their conjugates; (2) reductases and/or oxidases; and (3) enzymes involved in the so-called "dealkylation process." Reactions (2) and (3) have been investigated extensively from the late 1960s by a combination of several strategies: feeding experiments with diets containing one particular sterol; *in vivo* metabolic studies with [^{3}H] or [^{14}C] labeled sterols allowing the isolation of labeled intermediates; in vitro enzymatic studies using isolated tissues or cell-free preparations; and observing the effects of various pharmacological inhibitors of reactions known in mammals/humans (i.e., hypocholesterolemic agents). All the biochemical pathways have been fully elucidated, at least with regards to the metabolism of the most common Δ^5 plant sterols (reviews: Fujimoto *et al.*, 1985b; Svoboda and Thompson, 1985; Svoboda and Feldlaufer, 1991; Svoboda and Weirich, 1995; Svoboda, 1999), and are summarized in **Figure 10**.

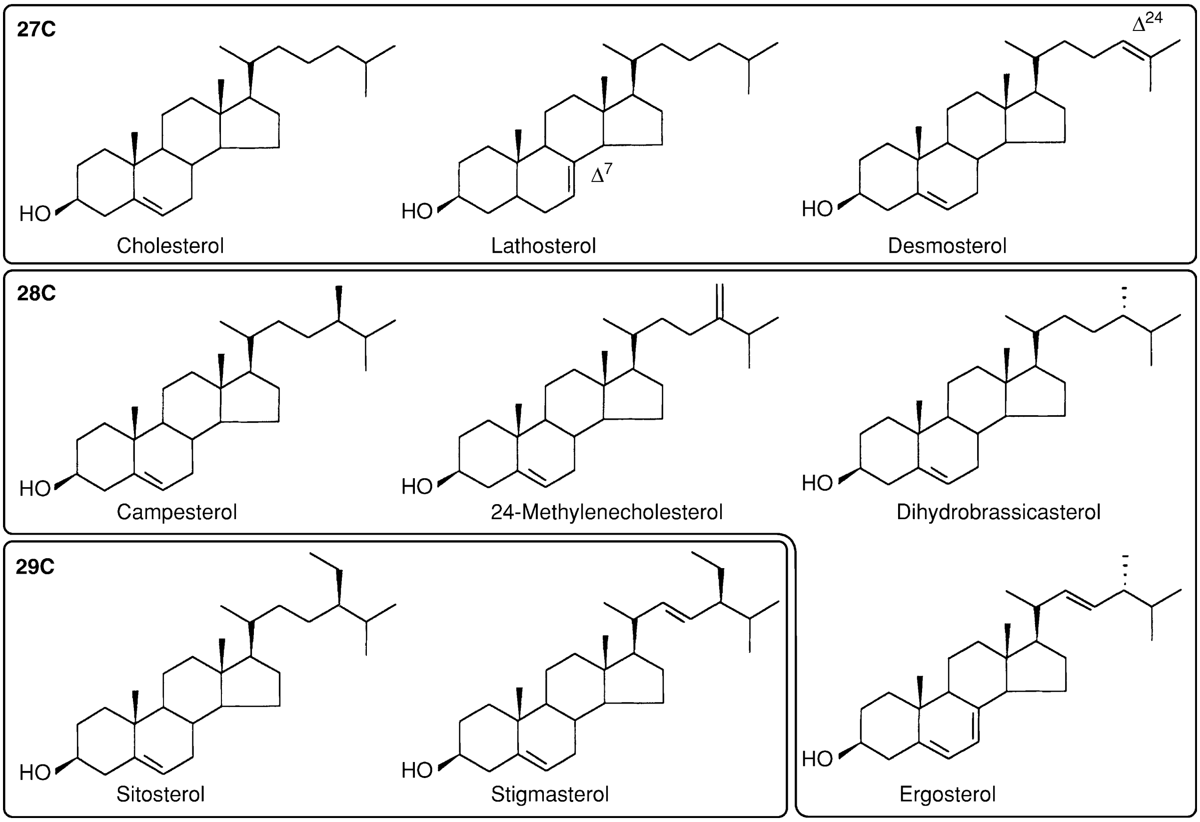


Figure 9 Structures of cholesterol and of representative phytosterols.

3.3.4.1.2. Dealkylating enzymes The first evidence for dealkylation of ergosterol into Δ^{22}-cholesterol was obtained with the cockroach *Blatella germanica* (Clark and Bloch, 1959). Further work, performed in particular with *Manduca sexta* and *Bombyx mori* larvae, allowed the identification of all intermediates. Four to five enzymes are necessary to dealkylate phytosterols. The first three reactions involve the formation of an epoxide intermediate, and the removal of the side chain is accompanied by the formation of a Δ^{24} bond, thus forming desmosterol or its $\Delta^{22,24}$ analog (Figure 10). The second part (one or two steps) concerns the reduction of the side chain. When starting for instance from sitosterol or campesterol, the dealkylation product (desmosterol) bears a single (Δ^{24}) double bond, and can be reduced to cholesterol by most insect species analyzed so far. In the case of insects fed on Δ^{22} sterol (e.g., stigmasterol or ergosterol), the dealkylation product bears a 22,24-diene and two reductases are required. It has been shown that the reduction at C_{22} must precede that at C_{24} and cannot be performed by all insect species (Svoboda and Thompson, 1985).

It was expected that dealkylation takes place in the insect gut. Indeed, cell-free extracts prepared from larval silkworm guts when incubated with adequate intermediates resulted in the formation of desmosterol and cholesterol (Fujimoto *et al.*, 1985b). Midgut microsomes are the most active source of enzymes (e.g., *Spodoptera littoralis*, Clarke *et al.*, 1985), but midgut is not the only active tissue in this respect (Awata *et al.*, 1975). Unfortunately, most of these early experiments were discontinued, and as yet none of these enzymes has been fully characterized.

The desmosterol 24-reductase is present both in insects and vertebrates, catalyzing the last step in cholesterol biosynthesis. There seems to be a significant structural conservation, as the insect enzyme can be inhibited by many hypocholesterolemic agents used in humans to reduce endogenous cholesterol biosynthesis. Indeed, azasteroids and various amines are also active on the insect enzyme and will consequently inhibit insect development (Svoboda and Weirich, 1995), but they cannot be used as insecticides due to their phytotoxicity. Moreover, there is significant sequence conservation between the human and nematode proteins and even human and plant sequences share 30% identity (Choe *et al.*, 1999; Waterham *et al.*, 2001). Unfortunately, available data on insect genomes concern

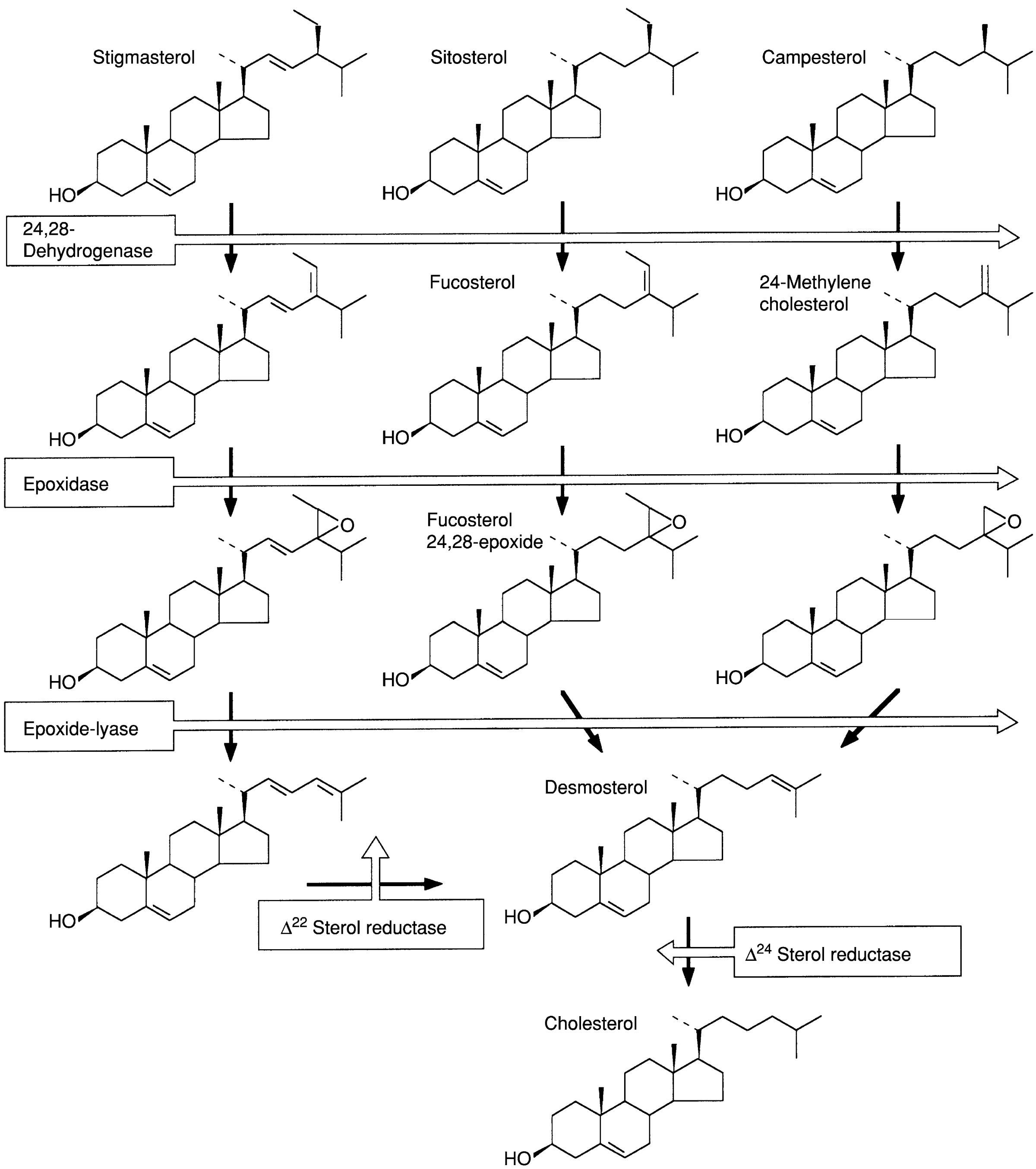


Figure 10 From phytosterols to cholesterol: the dealkylation processes.

species that are believed to be unable to dealkylate phytosterols and thus would lack the corresponding enzyme.

3.3.4.1.3. Variations among insect species Not all phytophagous species that can dealkylate phytosterols use the pathway described in Figure 10. Thus, the Mexican bean beetle, *Epilachna varivestis*, first totally reduces sitosterol (or stigmasterol) to stigmastanol, which is then dealkylated to cholestanol and the latter is then dehydrogenated to lathosterol (Δ^7), the major sterol found in this species (Svoboda *et al.*, 1975). This example highlights the importance of unsaturations present in the steroid nucleus. In some insect species, Δ^7 or $\Delta^{5,7}$ compounds can be the major sterols (Svoboda and Lusby, 1994; Svoboda, 1999), while *Drosophila pachea* has evolved specific requirements for ingesting Δ^7

sterols in order to produce E (Heed and Kircher, 1965; Warren *et al.*, 2001; see Section 3.3.4.2.1). In summary, insects' ability to modify sterol nucleus unsaturations differs greatly between species (Rees, 1985).

In some instances, species unable to dealkylate phytosterols may adapt their physiology to the nature of their sterol diet, e.g., *Drosophila melanogaster*. Apparently unable to dealkylate phytosterols (Svoboda *et al.*, 1989), *D. melanogaster* produces a mixture of 20E and makisterone A, the proportions of which reflect the composition of its sterol diet (Redfern, 1986; Feldlaufer *et al.*, 1995; Pak and Gilbert, 1987). This strategy can operate because in this species E receptors have a very similar affinity for both ecdysteroids (Dinan *et al.*, 1999; Baker *et al.*, 2000) (37). Bees (Hymenoptera) seem to follow a similar scheme (Svoboda *et al.*, 1983; Feldlaufer *et al.*, 1986). Leafcutter ants appear to be unique in that they feed mainly on fungal sterols, which differ from plant sterols in their stereochemistry at C_{24}. As a result, they produce 24-epimakisterone A instead of makisterone A (Maurer *et al.*, 1993). More generally, different enzymatic machinery and/or ecdysteroid receptor specificity among insects will result in different sterol requirements. These are revealed by feeding experiments on defined diets and so represent an aspect of the adaptation of insects to specific foods.

3.3.4.2. The Biosynthetic Pathway of Ecdysteroids from Cholesterol

Although it has received considerable attention (see reviews by Rees, 1985, 1995; Warren and Hétru, 1990; Sakurai and Gilbert, 1990; Grieneisen, 1994; Dauphin-Villemant *et al.*, 1998; Gilbert *et al.*, 2002), the biosynthetic pathway of ecdysteroids is still not understood completely. Biochemical approaches focusing on the nature of ecdysteroid synthetic intermediates have long formed the bulk of the results. However, unlike vertebrate steroidogenesis, where all the metabolites between cholesterol and the various active steroid hormones have been isolated and identified, no intermediate between the initial dehydrogenation product of cholesterol (7-dehydrocholesterol, 7dC) and the first recognizable ecdysteroid-like product, the 5β-ketodiol (2,22,25-trideoxyecdysone, 2,22,25dE) has ever been observed, much less identified. These are the reactions that comprise the mysterious "black box" of Dennis Horn (**Figure 11**). During invertebrate steroidogenesis, biosynthetic intermediates do not accumulate, perhaps because the proposed multistep oxidations of 7dC are either very efficient or the resulting compounds are very unstable, or both. A great step towards elucidating the synthetic mechanism in the insect was made when it was finally determined that the actual secreted product of lepidopteran prothoracic glands (Warren *et al.*, 1988a, 1988b; Sakurai *et al.*, 1989; Kiriishi *et al.*, 1990) and of most other insect prothoracic glands and crustacean Y-organs (Spaziani *et al.*, 1989; Böcking *et al.*, 1994) (see **Chapter 3.16**) was in fact not E but 3-dehydroecdysone (3DE). This finding greatly strengthened existing proposals of the early intermediacy of a 3-dehydro-Δ^4-sterol in arthropod ecdysteroidogenesis (Davies *et al.*, 1981; Rees, 1985; Grieneisen *et al.*, 1991), as it is a rather common moiety in mammalian steroidogenesis (Brown, 1998). However, the precise step at which the 3β-hydroxyl groups of early sterol ecdysteroid precursors become oxidized (dehydrogenated) to the ketone remains mystery. The recent molecular identification in *Drosophila* of the cytochrome P450 enzymes (CYP) catalyzing the terminal hydroxylation reactions at C_{25}, C_{22}, C_2, and C_{20} has provided new tools for studying these unresolved questions in ecdysteroid biosynthesis (see Section 3.3.4.3.1).

3.3.4.2.1. Cholesterol 7,8-dehydrogenation Early feeding studies showed that 7dC could support insect growth in the absence of C or phytosterols, suggesting that this compound was an early intermediate in ecdysteroid biosynthesis (Horn and Bergamasco, 1985). Subsequent kinetic studies revealed that 7dC (**Figure 11**) was the first intermediate in the formation of either E or 3DE from C, both in insects and crustaceans (Milner *et al.*, 1986; Warren *et al.*, 1988a; Grieneisen *et al.*, 1991, 1993; Rudolph *et al.*, 1992; Böcking *et al.*, 1993, 1994; Warren and Gilbert, 1996). Based on labeling experiments with radioactive precursors, the mechanism of cholesterol 7,8-dehydrogenation is known to involve the stereospecific removal of the 7β- and 8β-hydrogens from the more sterically hindered "top" (β-surface) of the planar C molecule (Rees, 1985). In several arthropod species, it was shown that the reaction is basically irreversible (Grieneisen, 1994) and is catalyzed by a microsomal CYP enzyme. This assumption was based on its biochemical characteristics, such as enzyme kinetics, subcellular localization, requirement for NADPH, or inhibition by CO and fenarimol (Grieneisen *et al.*, 1991, 1993; Warren and Gilbert, 1996).

Although its activity may become substrate limited, the cholesterol 7,8-dehydrogenase does not appear to be the rate-limiting enzyme in the biosynthesis of ecdysteroids. In many insect species, including *Manduca* (Warren *et al.*, 1988a; Grieneisen *et al.*, 1991) and *Bombyx* (Sakurai *et al.*, 1986), the

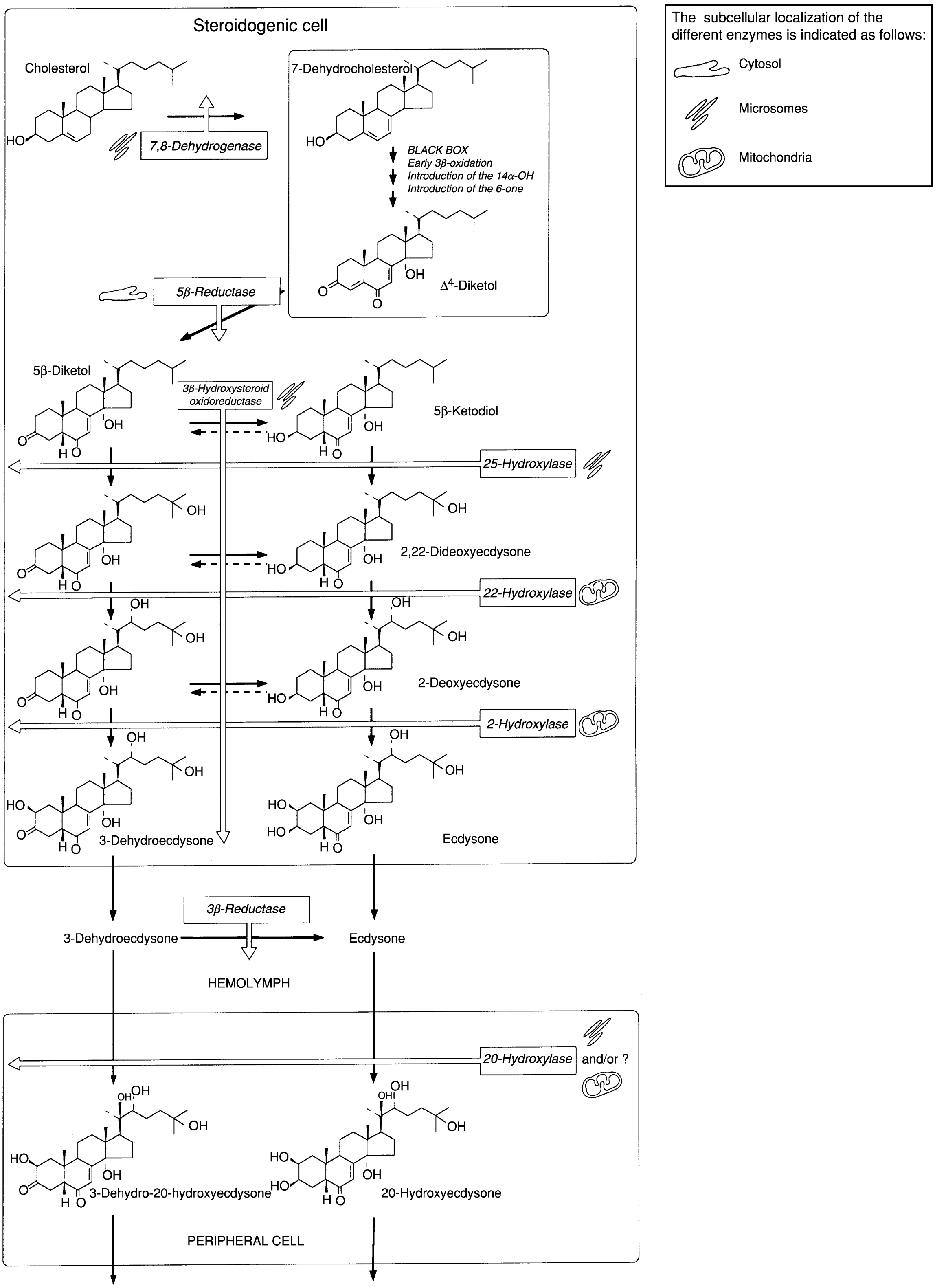


Figure 11 Putative biosynthetic pathway of ecdysteroids.

prothoracic glands always contain considerable amounts of both C and 7dC, even though both sterols undergo wild contralateral fluctuations during E biosynthesis, while in a few insect species, i.e., *Tribolium confusum, Atta cephalotes,* and *Acromyrmex octospinosus*, there is a clear preponderance of $\Delta^{5,7}$ sterols in the whole body (Grieneisen, 1994). In crabs, a ratio of 7dC:C ranging from 1:20 to 1:100 in the hemolymph was observed, while in the molting glands, the ratio was closer to 1:1 (Lachaise *et al*., 1989; Rudolph *et al*., 1992).

In the desert fly, *D. pachea*, the 7,8-dehydrogenase reaction is completely absent (Warren *et al*., 2001) and so it depends on an alternative source of 7dC. It lives on a single species of cactus that supplies it with the Δ^7-sterol lathosterol, which it alternatively dehydrogenates to 7dC (Goodnight and Kircher, 1971), i.e., similar to the synthesis of 7dC in vertebrates (Brown, 1998). Like *D. pachea*, the "low E" *Drosophila* mutant, *woc,* also shows an impairment of 7,8-dehydrogenase activity (Warren *et al*., 2001). However, in *woc*, while late third instar larval ecdysteroid production and subsequent larval–pupal metamorphosis can be rescued by the simple feeding of 7dC, continued normal development, as in *D. pachea*, is not observed in *woc* as the animals die as early pupae (see Section 3.3.5.4.3).

The 7,8-dehydrogenation of sterols appears not to have strict substrate requirements in arthropods. Cholesterol, along with the primary phytosterols campesterol, sitosterol, and stigmasterol (Sakurai *et al*., 1986), as well as the more polar synthetic substrate, 25-hydroxycholesterol (25C) (Böcking *et al*., 1994; Warren and Gilbert, 1996; Warren *et al*., 1996, 1999, 2001), are all efficiently converted into their respective 7-dehydro derivatives, both *in vivo* and *in vitro*. This is also the case for the unusual oxysterols α-5,6-epoxycholesterol (αepoC) and α-5,6-iminocholesterol, but not for their respective β-isomers (which, nevertheless, do function as competitive inhibitors of this reaction; (Warren *et al*., 1995). Only the former substrates were efficiently converted into either α-5,6-epoxy7dC, an oft-mentioned prospective "black box" intermediate (Rees, 1985) and a potentially potent alkylating agent (Nashed *et al*., 1986), or α-5,6-imino7dC, respectively. Indeed, a 46 kDa protein became increasingly radiolabeled following the incubation of ^{3}H-αepoC with *Manduca* prothoracic gland microsomes. Unfortunately, the resultant tagged product–enzyme adduct has so far proved too unstable for a classical purification and sequencing of the cytochrome. Nevertheless, the substrate specificity of the CYP enzyme for the α-isomer over the β-isomer again suggests a mechanism involving an attack by the cytochrome on the β-face of the molecule.

Even if the precise mechanism of action of the enzyme, as well as its structural characteristics, are not yet known, many studies have shown that CYPs can catalyze a great diversity of reactions, including dehydrogenation reactions. In vertebrates, for instance, dehydrogenation of the alkane CH-CH bond from testosterone is catalyzed by a CYP. The P450-dependent oxidation of this substrate not only leads to a dehydrogenated metabolite, but also to a hydroxylated metabolite, the latter not being a metabolite of the former (Mansuy, 1998). A similar mechanism might be envisioned for cholesterol 7,8-dehydrogenation, although the formation of such a hydroxylated C derivative has not been observed in insects. Nevertheless, one such possible intermediate (7β-hydroxycholesterol) did significantly inhibit the conversion of radiolabeled C to either 7dC or E (Grieneisen *et al*., 1991), but precise chemical studies are needed with a recombinant enzyme when it becomes available.

3.3.4.2.2. Early steps after the formation of 7dC: the "black box" Conversion of 7dC to the 5β-ketodiol or 5β-diketol, via the long-hypothesized 3-dehydro-Δ^4-diketol (cholesta-4,7-diene-3,6-dione-14α-ol) intermediate, constitutes the so-called "black box" (**Figure 11**). The name is most apt, as both the mechanism of this apparent multistep oxidation, as well as the subcellular location of all these reactions, is still a matter of great debate. Several modifications of 7dC appear to take place early and seemingly simultaneously, i.e., oxidation of the 3β-alcohol to the ketone (with stereospecific loss of the 3α-hydrogen), C_6 oxidation with additional loss of the 4β- and 6-hydrogens and formation of the 6-keto group, and finally 14α-hydroxylation. These observations are consistent with the intermediacy of 3-dehydro-Δ^4-diketol (Davies *et al*., 1981; Rees, 1985; Grieneisen *et al*., 1991; Dauphin-Villemant *et al*., 1998). Analogous to the rate-limiting step in mammalian steroidogenesis, it has long been thought that most or all of these reactions take place in the mitochondria and may be catalyzed by one or more P450 enzymes. That is, 7dC (or 7-dehydro25C) could not be converted into ecdysteroids by the crude microsomal fraction, only by the crude mitochondrial fraction of *Manduca* prothoracic glands. However, as no intermediates could be detected, no reaction mechanism was apparent (Grieneisen *et al*., 1993; Warren and Gilbert, 1996).

Nevertheless, some "negative" evidence has been obtained from metabolic studies concerning these

early biochemical modifications. Concerning 14α-hydroxylation, the results of incubations of *Manduca* prothoracic glands (Bollenbacher *et al.*, 1977a) or *Locusta migratoria* prothoracic glands or ovaries (Haag *et al.*, 1987) have suggested that this reaction must take place before, or in concert with, the introduction of the 6-keto group. That is, 3β-hydroxy-5β-cholest-7-en-6-one can be efficiently converted to 14-deoxy-ecdysone, but not to E.

Several hypotheses have also been tested concerning the introduction of the 6-keto group. Even though 6-hydroxyl-sterol derivatives have been isolated in some biological models, ecdysteroid analogs with a 6-hydroxyl group could not be oxidized to form true ecdysteroids with a 6-ketone group (Schwab and Hétru, 1991; Grieneisen, 1994; Gilbert *et al.*, 2002). The possibility that either a 3-dehydro-α-5,6-epoxy-7dC molecule (Grieneisen *et al.*, 1991; Warren *et al.*, 1995) or a 3-dehydro-5α,8α-epidioxide (J. Warren, C. Dauphin-Villemant, and R. Lafont, unpublished data) could be converted to a 3-dehydro-Δ^4-sterol intermediate has also been investigated. However, when incubated with prothoracic glands from insects or Y-organs from crustaceans, these compounds (either nonradioactive or as radioactive tracers) were never shown to be converted into known ecdysteroids (Gilbert *et al.*, 2002).

Finally, if the 3-dehydro-Δ^4-diketol (**Figure 11**) is an intermediate in ecdysteroid biosynthesis, its formation would also require an early oxidation step at C_3. However, as yet, every attempt to understand when and how this step takes place has failed. In studies with either prothoracic glands or Y-organs, the early oxidation of C (or 25C) to 3-dehydro-C (or 3-dehydro-25C) has never been directly observed. The facile conversion of these oxidized sterols, by either acid or base catalysis, to their distinctive isomerization products, 3-dehydro-Δ^4-C or 3-dehydro-Δ^4-25C, has also never been detected in vivo or in vitro (J. Warren and R. Lafont, unpublished data). Due to greater electron delocalization, 3-dehydro-7dC is very much more unstable, instantly converting into its more stable 3-dehydro-$\Delta^{4,7}$ sterol isomer when placed in an aqueous environment (Antonucci *et al.*, 1951; Lakeman *et al.*, 1967). However, the formation of either of these compounds (or their 25C analogues) has never been observed during ecdysteroid biosynthesis from radiolabeled precursors. Apparently, C_3 oxidation most likely occurs in concert with subsequent oxidations of these very unstable intermediates.

A hypothesis where all three modifications might possibly be performed simultaneously by a single enzyme (perhaps a CYP enzyme localized in the mitochondria) has been proposed (Gilbert *et al.*, 2002). However, at present, mitochondrial CYP involvement in these hypothetical black box oxidations is not supported for *Drosophila*. All six families of putative mitochondrial CYPs have been tentatively identified on the basis of their conserved motifs associated with the specific cofactor (adrenodoxin) binding to the cytochrome (Tijet *et al.*, 2001) (see **Chapter 4.1**). According to tissue expression studies in *Drosophila* (C. Dauphin-Villemant and M. O'Connor, unpublished data), no mitochondrial CYP has been localized to the ring glands, other than those already identified as catalyzing the final 2- and 22-hydroxylation steps (see Section 3.3.4.3.1). Nevertheless, even if the black box reactions do not involve mitochondrial P450 enzymes, these oxidation reactions could still occur in the mitochondria. Alternatively, it remains a possibility that these reactions are instead localized within an alternative membrane-bound subcellular organelle, that is, either another domain of the endoplasmic reticulum (ER) or an organelle known to be involved in the oxidation of numerous substrates (including some mammalian sterols), i.e., the peroxisome (see Section 3.3.6.2). In any case, regardless of the location and mechanisms of the black box reactions, they appear to be quite novel and so may be specific to many invertebrates.

3.3.4.2.3. Reduction steps of a proposed 3-dehydro-Δ^4-ecdysteroid intermediate Even though there is as yet no direct evidence for the intermediacy of the 3-dehydro-Δ^4-diketol in the biosynthesis of ecdysteroids, it still remains a good candidate. This is substantiated by several lines of evidence. It has been shown to be efficiently converted first to the 5β-diketol (and subsequently to 3DE and E) by a 5β-reductase present primarily in the Y-organs of crustaceans, i.e., in the crab *Carcinus maenas* (Blais *et al.*, 1996) and the crayfish *Orconectes limosus* (C. Blais, C. Dauphin-Villemant, and R. Lafont, unpublished data). The reaction is quite specific for the correct oxidation state of the substrate at C_3, as no reduction of the unoxidized 3β-OH-Δ^4-sterol intermediate was observed either in crab Y-organs (Blais *et al.*, 1996) or in insect prothoracic glands (Bollenbacher *et al.*, 1979). However, the complete characterization of an arthropod 3-dehydro-Δ^4-steroid 5β-reductase is still lacking as all attempts to demonstrate a similar reaction in insects have so far been unsuccessful. Nevertheless, in all other vertebrates or plants investigated so far, the formation of 5β[H]-cholesterol derivatives always proceeds through such 3-dehydro-Δ^4-sterol intermediates (Brown, 1998).

In the case of species producing only 3β-hydroxylated ecdysteroids, i.e., E or 20-deoxymakisterone A or C, the postulated intermediacy of the 3-dehydro-Δ^4-diketol and diketol in the biosynthetic pathway implies the presence of a 3β-reduction step involving subterminal compounds in the molting glands (**Figure 11**). Until now, the interconversion between 3-dehydro and 3-OH compounds has essentially been documented only in the hemolymph and peripheral tissues of several arthropods (see Section 3.3.5.3.2). A cytosolic NADPH requiring enzyme has been identified. It is able to rapidly and irreversibly catalyze the conversion of 3DE into E, which, in turn, is rapidly hydroxylated to 20E. This enzyme has been cloned in the lepidopterans *Spodoptera littoralis* (Chen *et al*., 1999b) and *Trichoplusia ni* (Lundström *et al*., 2002). However, this enzyme cannot be responsible for 3β-reduction in the prothoracic glands, since it retains a rather narrow substrate specificity, is unable to reduce subterminal intermediates of ecdysteroidogenesis, and, furthermore, is not expressed in the steroidogenic tissues.

However, in the crab, *Carcinus maenas*, a microsomal enzyme similar to the 3β-hydroxysteroid dehydrogenase (3βHSD) from vertebrates has been detected specifically in the Y-organs (Dauphin-Villemant *et al*., 1997). In the presence of NADH, it can catalyze the 3β-reduction not only of the diketol, but also 3-dehydro-2,22-dideoxyecdysone and 3-dehydro-2-deoxyecdysone, but not 3DE. Interestingly, in the presence of NAD^+, the same subcellular fraction can re-oxidize 2-deoxyecdysone and 2,22-deoxyecdysone back to the original 3-dehydro substrates, suggesting a reversible reaction consistent with a true 3βHSD activity.

3.3.4.2.4. Late hydroxylation steps Starting from either the 5β-diketol or 5β-ketodiol (the terminal 3-dehydro or 3-OH products of the black box), conversion initially into 3DE or E and ultimately into 3D20E or 20E (**Figure 11**) is well documented. The 5β-ketodiol appears to be a true intermediate in ecdysteroid biosynthesis since at least in one insect species, *Locusta migratoria,* it has been identified as an endogenous compound of ovaries, in addition to 7dC (Hétru *et al*., 1978, 1982). It was also identified as a conversion product from [^{3}H]-cholesterol during *in vitro* incubations of ovaries (Hétru *et al*., 1982) and following [^{3}H]-cholesterol injections into nondiapause eggs of *B. mori* (Sonobe *et al*., 1999). Furthermore, it has been demonstrated in several insect and crustacean species (Grieneisen, 1994) that both prothoracic glands and Y-organs are able to convert the 5β-ketodiol very efficiently into E via sequential hydroxylations at C_{25}, C_{22}, and C_2 (**Figure 11**). Subsequent hydroxylation of circulating E at C_{20}, catalyzed by an ecdysone 20-monooxygenase present in peripheral tissues, yields the active molting hormone 20E (see Section 3.3.5.2).

It has long been accepted that the enzymes catalyzing these reactions are four different monooxygenases belonging to the CYP superfamily (see **Chapter 4.1**), based on their biochemical characteristics, i.e., membrane subcellular localization, NADPH cofactor, and O_2 requirement and inhibition of the reaction by CO or by classical CYP inhibitors like fenarimol, metyrapone, or piperonyl butoxide observed in various arthropods (Grieneisen, 1994). In this respect, the 2-hydroxylase presents some peculiar features, since it is insensitive to CO and apparently can function with some Krebs cycle intermediates (succinate or isocitrate) in addition to NADPH (Kappler *et al*., 1986, 1988).

The subcellular distribution of these terminal hydroxylases has been studied in several arthropod models. While the tissue locations of the enzymatic activities of the first three enzymes in insects (Kappler *et al*., 1988; Grieneisen *et al*., 1993) and crustaceans (Blais *et al*., 1996; Dauphin-Villemant *et al*., 1997) are not clear-cut, it is nevertheless widely accepted that the 25-hydroxylase is a microsomal enzyme whereas the 2- and 22-hydroxylases are strictly mitochondrial. In contrast, the 20-hydroxylase has been shown to be microsomal, mitochondrial, or, in some instances, both (Bollenbacher *et al*., 1977b; Feyereisen and Durst, 1978; Smith *et al*., 1979; Smith, 1985; Mitchell and Smith, 1986; Smith and Mitchell, 1986; see Section 3.3.5.2). The subcellular localization of all these CYP enzymes has now been directly demonstrated in *Drosophila* S2 cells (see Section 3.3.4.3.1).

By analogy with steroid hormone biosynthesis in vertebrates, the identified hydroxylases might be expected to be expressed specifically in the endocrine tissues (or peripheral tissues for the 20-monooxygenase) and perhaps act through a preferential sequence of hydroxylations. Yet, early metabolic studies (Rees, 1985) have reported 2-, 22-, and 25-hydroxylase activities in a number of tissues, not only larval prothoracic glands and adult ovaries, but also in fat body, Malpighian tubules, gut, and epidermis. However, the specific activity of the observed enzymatic activities was seldom mentioned and a closer examination always revealed a substrate conversion that was much more efficient in true steroidogenic organs (prothoracic glands, Y-organs, ovaries) than in other tissues, at least in terms of the C_{22}- and C_{25}-hydroxylations (Meister *et al*., 1985; Rees, 1985, 1995; Grieneisen, 1994). While the

evidence for C_2-hydroxylation activity outside the ring gland remains strong, the C_{25} and C_{22} activities may have been due to the use of crude preparations with the possibility that other CYPs where present in the subcellular fractions. Cytochromes with broader substrate specificities (detoxification enzymes, for instance) may, to some extent, have been able to convert intermediates into ecdysteroids.

Another characteristic of the enzymes involved in vertebrate steroidogenesis is that the reactions generally occur in a preferred sequence, both due to their precise substrate specificities and their subcellular compartmentalization (Brown, 1998). This appears to be at least partly true in the case of ecdysteroid biosynthesis, even though additional enzymological studies are necessary. In fact, accumulation of many intermediates has been repeatedly reported in insect and crustacean systems when using labeled 5β-ketodiol as an exogenous precursor (Rees, 1985; Grieneisen, 1994). However, these compounds have not been identified as endogenous intermediates and might therefore be artifacts. More precisely, if hydroxylation at C_2 occurs first in either the 5β-ketodiol (2,22,25dE) or ketotriol (2,22dE), it leads to a dead end. This has been shown both in insects (Dollé *et al*., 1991; Rees, 1995) and crustaceans (Lachaise *et al*., 1989; Pís *et al*., 1995b) since both 22,25-dideoxyecdysone and 22-deoxyecdysone (Warren *et al*., 2002) are only poorly converted into E via hydroxylation at C_{22}. On the other hand, C_{25}-hydroxylation does not appear to be the last biosynthetic step, since 25-deoxyecdysone is a major secretory product in several crab species (Lachaise *et al*., 1989; Rudolph and Spaziani, 1992), but has not been found in insects. Taken together, these results suggest that the last steps in E biosynthesis within the endocrine gland also follow a preferential sequence of hydroxylations.

The present hypothesis suggests that the first terminal oxidation reaction of the newly reduced black box product (i.e., the 3-dehydro-Δ^4-diketol), whether localized in the mitochondria, ER, the peroxisome, or in the cytosol around these organelles, nevertheless occurs at C_{25} within the ER. Then, after translocation of the resulting 5β-ketotriol product to the mitochondria, sequential hydroxylations take place, the first necessarily at C_{22} and the last at C_2, followed by the secretion of the product from the cell as either 3DE and/or E. Circulating E is then converted to 20E in the peripheral tissues. Thus, in arthropods, the apparent preferential sequence of biosynthetic steps may be due more to their particular subcellular compartmentalization than to any strict substrate specificity. That is, the more convenient access of this cytosolic substrate to the 25-hydroxylase in the ubiquitous ER may determine the first reaction. Curiously, of the last two mitochondrial enzymes, the 22-hydroxylase apparently must function first, as the formation of the alternative 2-hydroxylated product (22dE), while not prohibited by any strict substrate specificity, nevertheless is not normally observed *in vivo*. Specific compartmentalization of these two enzymes within the mitochondria may somehow restrict initial substrate (2,22dE) access to the 2-hydroxylase.

3.3.4.2.5. Various secretion products: single or multiple metabolic pathways? Ecdysteroid biosynthesis has been studied in various model systems, i.e., steroidogenic tissues originating from animals producing different and sometimes exclusive ecdysteroids. There is a general implicit postulate that production of the same ecdysteroid proceeds through a common pathway in all investigated arthropods. This means that orthologous enzymes are expected to be identified in the various species. Until now, however, comparative structural data has been lacking. On the other hand, the occurrence of different secretory products in different species raises the question of whether they are the result of profound changes in ecdysteroid biosynthesis or just the effect of minor differences in pathways. The reason for the different oxidation state at C_3 in the two major ecdysteroid products identified in many arthropod species, i.e., E or 3DE, might involve just variability in the activity or expression of a terminal reductase in or near the steroidogenic tissue. Such a hypothesis is substantiated by the fact that, generally, in 3DE-producing species, the conversion of the 5β-diketol leads preferentially to 3DE, while the conversion of the 5β-ketodiol is also efficient but leads to E (Böcking *et al*., 1993; Grieneisen, 1994). Similarly, the preferential production of 25-deoxy ecdysteroids by crab Y-organs may be due simply to the absence or the very low expression of the 25-hydroxylase in crustaceans. However, the mechanisms for these differences may be more complex and could involve different substrate specificities of the corresponding enzymes.

3.3.4.3. Molecular Analysis of Ecdysteroid Biosynthesis

Different approaches have been engaged in recent years in order to gain structural information about the genes and proteins involved in ecdysteroid biosynthesis. With regard to the biosynthetic enzymes, a first approach has been to take advantage of expected sequence homologies and look for CYP enzymes expressed specifically in prothoracic glands (Snyder *et al*., 1996). Using this strategy, CYP

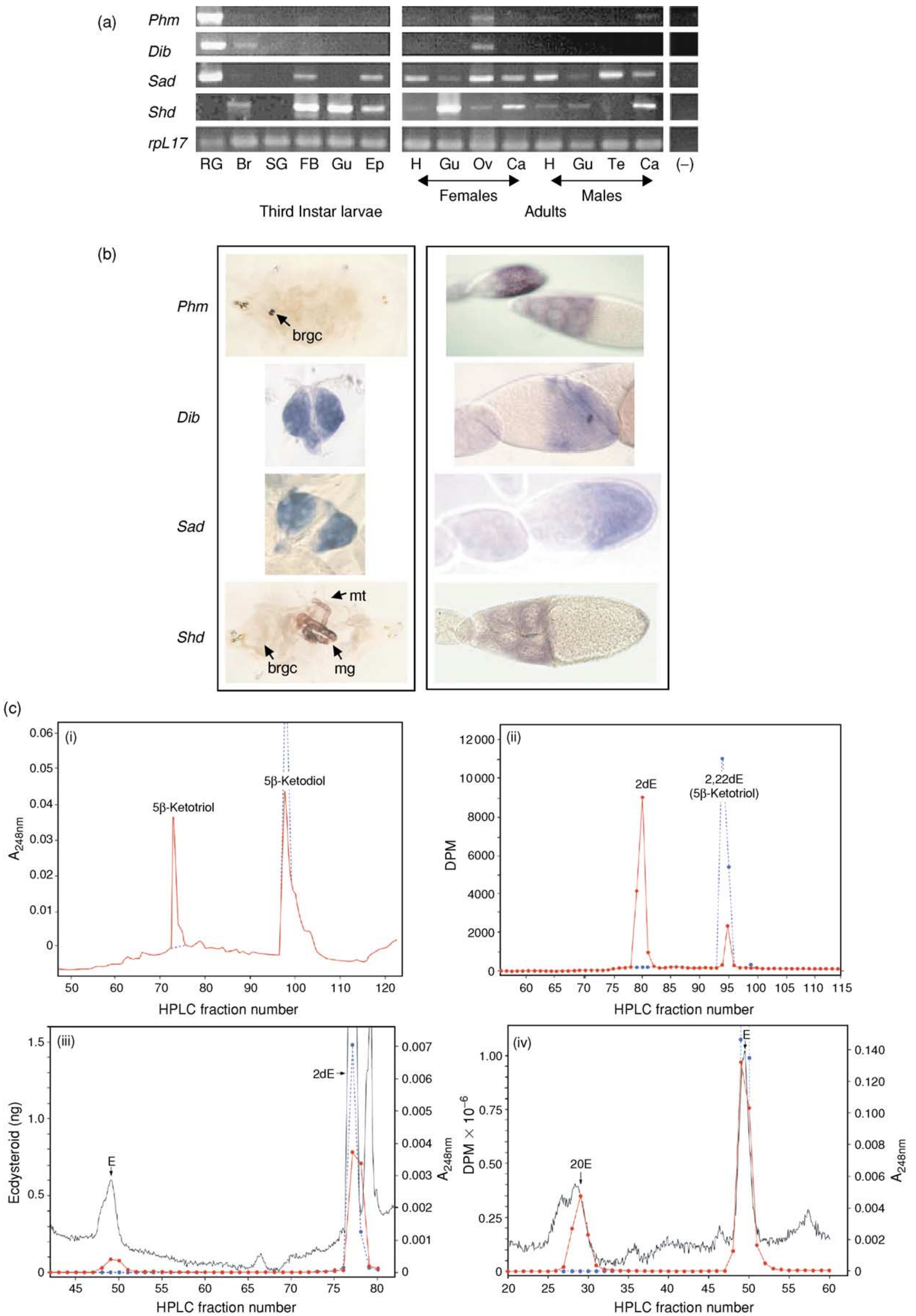
enzymes specific to *Manduca* prothoracic glands (see **Chapter 4.1**) and crayfish (*Orconectes limosus*) Y-organs (Dauphin-Villemant *et al.*, 1997, 1999) were identified. Curiously, both were of the CYP4 family, but their precise function has not yet been elucidated.

In *Drosophila*, genetic approaches as well as the recent sequencing of the genome represent powerful tools to identify genes involved in ecdysteroid biosynthesis. Several mutations, including *ecdysoneless* (*ecd*[1]), *DTS3, dre4, giant*, and *woc*, have been shown to affect E titers (Garen *et al.*, 1977; Walker *et al.*, 1987; Schwartz *et al.*, 1984; Sliter and Gilbert, 1992; Warren *et al.*, 1996; Wismar *et al.*, 2000) and consequently development and metamorphosis. However, the corresponding genes appear to be more linked either to the transcriptional regulation of ecdysteroid production or to the efficient delivery of ecdysteroid intermediates to the enzymatic machinery rather than to the biosynthetic enzymes themselves. For instance, *giant* encodes a bZIP (basic-leucine zipper) transcription factor (Capovilla *et al.*, 1992), *ecd*[1] may impair sterol availability (Warren *et al.*, 1996) while *woc* encodes a transcription factor with eight zinc fingers (Wismar *et al.*, 2000) that appears (in the least) to regulate expression of the cholesterol 7,8-dehydrogenase (Warren *et al.*, 2001). An enhancer-trap approach has also been used in recent years (Harvie *et al.*, 1998) in order to begin characterizing the function of *Drosophila* ring glands during larval development. Several genes have been identified as specifically expressed in the lateral cells of the ring gland (i.e., the prothoracic gland cells), but there is presently no evidence that they are involved in ecdysteroid biosynthesis.

3.3.4.3.1. Identification of steroidogenic cytochrome P450 enzymes While many of the early oxidation steps in ecdysteroid biosynthesis are still incompletely understood, it nevertheless appears to involve a cascade of sequential hydroxylations catalyzed by several cytochrome P450 enzymes. A combination of molecular genetics and biochemistry was used recently to further elucidate these reactions in the biosynthetic pathway in *Drosophila*. One phenotype predicted for mutations that disrupt the production of ecdysteroids would be the inability to produce a cuticle at any stage, the earliest being the synthesis of embryonic cuticle during the second half of embryogenesis. Among the mutants screened by Nüsslein-Volhard and colleagues in their landmark characterization of embryonic patterning (Jürgens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984), several were later grouped under the name of the *Halloween* mutants (Chavez *et al.*, 2000). They were selected for their similar abnormal cuticular patterning and low ecdysteroid titers, suggestive of a defect in the ecdysteroid pathway. In all of them, several developmental and physiological defects (i.e., absence of head involution, dorsal closure, and gut development) were associated with a very poor differentiation of embryonic cuticle and which led to lethality before the end of embryonic development.

While the first Halloween gene, *disembodied* (*dib*) (CYP302A1) was located by classical molecular techniques (Chavez *et al.*, 2000), in order to select the remaining genes, the *Drosophila* genome was searched for the presence of cytochrome P450 enzyme-type sequences near the known map location of these genes. At least four other genes, *phantom (phm), spook (spo), shadow (sad)*, and *shade (shd)* were shown to also code for CYP enzymes, CYP 306A1, CYP307A1, CYP315A1, and CYP314A1, respectively (Warren *et al.*, 2002; Petryk *et al.*, 2003; Warren *et al.*, 2004), as they retained the conserved domains characteristic of this superfamily (particularly the high conservation of the heme-binding domain at the C-terminal end). Owing to a high divergence in their overall sequence as compared to other already known sequences, these enzymes are members of a new CYP family (see drnelson for nomenclature) (see **Chapter 4.1**). Four of them, *phm*, *sad*, *dib*, and *shd*, have now been fully characterized, as their corresponding enzymes, when expressed in *Drosophila* S2 cells, have been shown to catalyze the last four reactions in ecdysteroid biosynthesis, i.e., the terminal 25-, 22-, 2-, and 20-hydroxylations (see below).

Dib and *sad* mRNAs are initially expressed in the epidermis during early embryogenesis and then later in classical steroidogenic tissues, i.e., in ring glands from late embryogenesis to metamorphosis and in the nurse and/or follicle cells of adult female ovaries. Their expression patterns were studied using *in situ* hybridization on different tissues in addition to RT-PCR analysis (**Figure 12 a** and **b**). More recently, it was shown that *phm* retains an almost identical expression pattern to *dib* (Warren *et al.*, 2004), while *sad* actually exhibits a much broader pattern, consistent with prior tissue activity studies, including not only expression in the steroidogenic tissues (Warren *et al.*, 2002), but also significant expression in the gut and epidermis in larvae and adults (**Figure 12 a** and **b**). In contrast, while *shd* is also expressed in the early embryonic epidermis, it is not expressed in late embryonic or larval ring glands, but instead is prominent in peripheral larval tissues and adult ovaries (Petryk *et al.*, 2003).


(a)
Phm
Dib
Sad
Shd
rpL17
RG Br SG FB Gu Ep
H Gu Ov Ca H Gu Te Ca (–)
Females
Males
Third Instar larvae
Adults
(b)
Phm
Dib
Sad
Shd
brgc
mt
mg
(c)
(i)
5β-Ketotriol
5β-Ketodiol
A248nm
HPLC fraction number
(ii)
2dE
2,22dE
(5β-Ketotriol)
DPM
HPLC fraction number
(iii)
E
2dE
Ecdysteroid (ng)
A248nm
HPLC fraction number
(iv)
E
20E
DPM × 10−6
A248nm
HPLC fraction number

The initial hypothesis that *dib* and the other Halloween gene CYPs might catalyze steps in the ecdysteroid biosynthetic pathway has now been confirmed by the biochemical characterization of the Phm, Dib, Sad, and Shd CYP enzymes. The cDNA coding sequences of these genes have been transiently transfected into *Drosophila* S2 cells as expression plasmids under the control of the actin 5C promoter. When the transfected cells are incubated with specific ecdysteroid precursor substrates, they efficiently and selectively convert them to product (**Figure 12c**), indicating the 25-, 22-, 2-, and 20-hydroxylase functions for Phm, Dib, Sad, and Shd respectively. Additional information on the substrate specificities of these enzymes was obtained in a similar manner. For instance, *dib*-transfected cells converted 2,22-dideoxyecdysone (the 5β-ketotriol) to 2-deoxyecdysone (**Figure 12c**), but could not convert 22-deoxyecdysone to E, confirming the strict substrate requirement (i.e., the absence of a 2-hydroxy function) for the 22-hydroxylase enzyme, at least in this expression system (Warren *et al.*, 2002). On the other hand, cells transfected with *sad* hydroxylated not only 2-deoxyecdysone and 2,22-dideoxyecdysone at C_2 (Warren *et al.*, 2002), but also 2,22-dideoxy-20-hydroxyecdysone (C. Blais, C. Dauphin-Villemant, and R. Lafont, unpublished data), suggesting that modifications on the side chain do not necessarily interfere with the active site of this enzyme. Similarly, cells transfected with *phm* converted not only 2,22,25-trideoxyecdysone (the 3β,5β-ketodiol) to 2,22-dideoxyecdysone, but also 25-hydroxylated both the 3β,5α-ketodiol and the 3α,5α-ketodiol to their respective isomeric ketotriol products (Warren *et al.*, 2004)). Apparently, in this case, A/B-ring and C_3 stereochemistry are relatively unimportant determinants for Phm activity. However, Phm could not metabolize 25dE to E or ponasterone A to 20E, indicating an inability (as was observed with Dib) to recognize substrates prematurely hydroxylated at C_2. Detailed enzymological and mechanistic studies are now possible following the stable, heterologous expression in nonsteroidogenic cell lines of both single and multiple (i.e., linked) steroidogenic P450 enzymes targeted to either the mitochondria or the endoplasmic reticulum, or both.

Cytochrome P450 enzymes, as membrane-bound enzymes, have so far been found to be either localized in the endoplasmic reticulum or in the mitochondria of eukaryotes (see **Chapter 4.1**). Their amino acid sequences at the N-terminus present conserved structural features that are hypothesized to direct their subcellular localization (Omura and Ito, 1991; Von Wachenfeldt and Johnson, 1995; Van den Broek *et al.*, 1996) (see **Chapter 4.1**). The N-terminal sequences deduced from *dib* and *sad* cDNAs (**Figure 13**) are consistent with their mitochondrial localization previously determined for the C_2- and C_{22}-hydroxylases from subcellular fractionation studies in *Locusta* (Kappler *et al.*, 1986, 1988) and *Manduca* (Grieneisen *et al.*, 1993). Both contain numerous positively charged residues, in addition to many polar groups within the first 20–30 amino acids. In contrast, the N-terminus of Shd (the 20-monooxygenase) and Spo (which remains

Figure 12 Identification of *Halloween* genes of *Drosophila*. Abbreviations: *phantom, phm; disembodied, dib; shadow, sad; and shade, shd*. (a) RT-PCR analysis showing larval and adult tissue expression of *phm*, *dib*, *sad*, and *shd*. RpL17 expression was used as a control, since this gene is expressed similarly in all tissues. RG, ring gland; Br, brain; SG, salivary gland; FB, fat body; Gu, gut; Ep, epidermis; H, head; Ov, ovary; Te, testis, Ca, carcass; (−) negative control. (b) *In situ* hybridization antisense digoxygenin-labeled RNA probes were used. Left part: total larvae or ring gland labeling of late L3 larvae; brcg, brain ring gland complex; mg, midgut; mt, Malpighian tubules. Right part: staining of adult egg chambers (stages 8–10). (c) CYP Enzyme characterization: Halloween genes or GFP were transfected into *Drosophila* S2 cells using DDAB. Three days later, the cells were incubated with either radiolabeled or nonradiolabeled substrates for 4–6 h prior to analysis by RP-HPLC (C_{18}-silica based column, 30–100% methanol gradient over 2 h, 1 ml min^{-1}, 1 ml/HPLC fraction). Specific ecdysteroid detection by UV absorbance (A_{248nm}) and/or radioimmunoassay (RIA, SHO3 antibodies, ng ecdysteroids) for non-labeled substrates and DPM for radiolabeled substrates. (i) S2 cells + *Phm* (red line – A_{248nm}). Substrate: non-radiolabeled 5β-ketodiol (2,22,25-trideoxyecdysone = 2,22,25dE). Control: S2 cells + GFP (blue line – A_{248nm}). Product: 5β-ketotriol (2, 22-dideoxyecdysone = 2,22dE). (ii) S2 cells + *Dib* (red line – DPM). Substrate: [^{3}H]-5β-ketotriol (2,22dE). Control: S2 cells + GFP (blue line – DPM). Product: [^{3}H]-2-deoxyecdysone (2dE). (iii) S2 cells + *Sad* (red line – ng ecdysteroids RIA; black line – A_{248nm}). Substrate: nonradiolabeled 2-deoxyecdysone (2dE). Control: S2 cells + GFP (blue line – ng ecdysteroid RIA). Product: ecdysone (E). (iv) S2 cells + *Shd* (red line – DPM; black line – A_{248nm}). Substrate: [^{3}H]-ecdysone (E). Control: S2 cells + GFP (blue line – A_{248nm}). Product: [^{3}H]-20-hydroxyecdysone (20E). (Modified from Chávez, V.M., Marqués, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., *et al.*, **2000**. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126; Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048; Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., *et al.*, **2003**. Shade: the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778; Warren J.T., Petryk, A., Marqués, G., Parvy, J-P., Shinoda, T., *et al.*, **2004**. *Phantom* encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem. Mol. Biol. 29*, 679–695.)

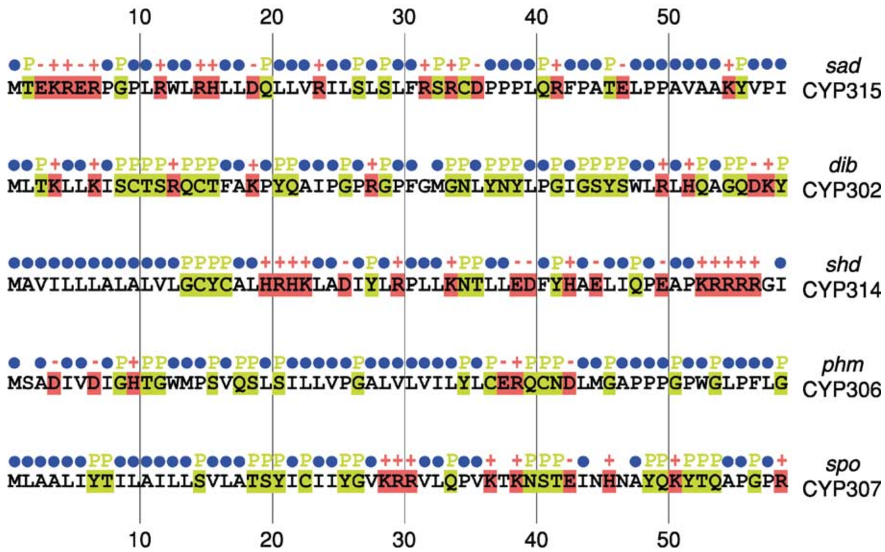


Figure 13 Predicted N-terminal amino acid sequence of the Halloween P450 genes. Red represents charged (+/−) residues, yellow shows polar (P) residues, and blue circles represent nonpolar amino acids. Prominent charged residues in the N-terminal 20–30 amino acids of P450 enzymes are thought to be involved in the targeting of the protein to the inner mitochondrial matrix. Alternatively, predominantly nonpolar N-terminal residues are thought to help anchor the protein in the membrane of the endoplasmic reticulum. (Modified from Chávez, V.M., Marqués, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Nätzle, J.E., O'Connor, M.B. **2000**. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126; Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., Dauphin-Villemant, C., O'Connor, M.B., Gilbert, L.I. **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048; Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Parvy, J.-P., Dauphin-Villemant, C., O'Connor, M.B. **2003**. Shade: the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778; Warren J.T., Petryk, A., Marqués, G., Parvy, J-P., Shinoda, T., *et al.*, **2004**. *Phantom* encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem. Mol. Biol. 29*, 679–695.).

unidentified) consists of a string of about 20 or more hydrophobic amino acids devoid of charged residues, suggesting that both these CYP enzymes are targeted to the endoplasmic reticulum.

The mitochondrial localization of the Dib and Sad cytochromes has been confirmed by *in situ* localization of the corresponding C-terminal, epitope-tagged (His or HA) proteins expressed in S2 cells (Warren *et al.*, 2002; Petryk *et al.*, 2003). However, in apparent contradiction of the above considerations, when a similarly tagged *shd* construct was transfected into S2 cells, it was determined that it, like *dib* and *sad*, also localized in the mitochondria and not in the ER (Petryk *et al.*, 2003). When phylogenetic trees with various CYPs are constructed, *shd* always clusters (along with *dib* and *sad*) with the mitochondrial CYPs, primarily because of shared sequences associated with binding to its redox partner adrenodoxin (Tijet *et al.*, 2001) (see **Chapter 4.1**). However, upon closer examination of the Shd N-terminus (**Figure 13**), a possible mitochondrial import sequence containing numerous charged residues is present immediately downstream from the apparent microsomal targeting sequence. Protease-mediated cleavage of the N-terminus of Shd, thereby eliminating the initial string of hydrophobic residues, could result in the retargeting of this enzyme to the mitochondria. Such differential posttranslational modification of Shd could explain the observed distribution of ecdysone 20-monoxygenase activity into either the mitochondria or ER or both, depending on the insect, tissue, or stage (see Section 3.3.5.2). A similar situation has been reported for mammalian CYP1A1, which has a chimeric N-terminal signal that facilitates the targeting of the protein to the ER and, following protease action, to the mitochondria (Addya *et al.*, 1997). Not clear, however, is how the P450 enzyme manages to interact with apparently separate reductase cofactors when it is present in these two different membrane environments (see Section 3.3.4.3.2).

The predicted N-terminal sequence of Phm (**Figure 13**) could present a similar anomaly, as there is no typical hydrophobic stretch in the first 20 amino acids. However, if one considers the second methionine at position 14 as being the real start of the protein, then the following 20 amino acids are mainly hydrophobic and so would suggest an ER import sequence. Indeed, a similarly tagged Phm

enzyme was found to localize in the ER of transfected S2 cells (Warren *et al.*, 2004), consistent with prior biochemical characterizations of the 25-hydroxylase in arthropods (Kappler *et al.*, 1988).

Several points should be noted regarding the molecular evolution of CYP genes and their relation to steroidogenesis. It is postulated that all cytochrome P450 enzymes are derived from a few ancient CYPs (Nelson, 1998) (see **Chapter 4.1**). As additional genomes are deciphered, genes putatively orthologous to *phm*, *dib*, *sad*, and *shd* will soon be identified in *Anopheles gambiae, Drosophila pseudoobscura*, and *Bombyx*. Comparative studies and rapid progress in insect genomics will allow confirmation that these orthologous genes actually catalyze the same reactions in mosquitoes and other insects as they do in *Drosophila*. Indeed, following similar heterologous expression in *Drosophila* S2 cells, it was recently determined that a *Bombyx* gene analogous to *phm* also codes for a P450 enzyme that catalyzes specific ecdysteroid 25-hydroxylation (Warren *et al.*, 2004). Furthermore, as was shown for the *Drosophila* ring gland-specific genes *dib*, *sad* and *phm* (Warren *et al.*, 2002; Petryk *et al.*, 2003; Warren *et al.*, 2004), *Bombyx phm* expression in the prothoracic gland cells during the last larval–larval molt undergoes a dramatic down-regulation by the beginning of the last larval stadium (Warren *et al.*, 2004), perhaps the result of feedback inhibition by the product, 20E, operating via its nuclear receptor (see **Chapter 3.8**).

Finally, recent advances in the knowledge of insect genomes will likely enable the identification of all the P450 enzymes involved in ecdysteroid biosynthesis (including the cholesterol 7,8-dehydrogenase) in the near future. For instance, all putative CYP genes have been identified in *Drosophila* (Tijet *et al.*, 2001) (see **Chapter 4.1**). There is no doubt that a systematic analysis of the tissue expression of all CYP genes will enable us to determine the best candidates coding for the remaining enzymes involved in ecdysteroid biosynthesis. More integrated approaches using microarray technology approaches are presently available and should constitute new tools to identify the large set of genes specifically expressed in steroidogenic cells and so potentially linked to ecdysteroidogenesis.

3.3.4.3.2. Electron transport involved in cytochrome P450 reactions (see **Chapter 4.1**) Monooxygenations catalyzed by cytochrome P450 enzymes require molecular oxygen and two electrons during their catalytic cycle. In vertebrate steroidogenesis, the electron transfer systems associated with the mitochondrial and microsomal CYP enzymes are different. In the mitochondrial system, reduction occurs with FAD as the flavoprotein (adrenodoxin reductase), which transfers electrons to an iron-sulfur protein (adrenodoxin) and finally to the CYP. In contrast, in the microsomal system, cytochrome P450 reductase, a flavoprotein containing both FMN and FAD, passes electrons directly from NADPH to the CYP. The second electron may alternatively arise from NADPH via cytochrome b_5, and a number of forms of cytochrome P450 show an obligatory requirement for cytochrome *b5* (Miller, 1988; Schenkman and Jansson, 2003).

There is some evidence in arthropods that ecdysteroid hydroxylations catalyzed by CYPs involve electron transfer systems analogous to those used in vertebrates (see **Chapter 4.1**). The obligatory function of cytochrome P450 reductase and the adrenodoxin/adrenodoxin reductase system in the function of microsomal and mitochondrial CYPs, respectively, has been indicated by immunoinhibition studies. Anti-adrenodoxin and anti-adrenodoxin reductase antibodies effectively inhibited ecdysone 20-monooxygenase activity in *Spodoptera* fat body mitochondria (Chen *et al.*, 1994a). Similarly, antibodies raised against cytochrome P450 reductase inhibited microsomal E 20-monooxygenase activity in *B. mori* embryos (Horike and Sonobe, 1999). EPR spectroscopic studies have corroborated the presence of ferredoxin (adrenodoxin) and cytochrome P450 enzyme in the foregoing mitochondria (Shergil *et al.*, 1995) and in *Manduca* fat body mitochondria (Smith *et al.*, 1980).

The electron transfer systems appear to be structurally and functionally conserved. The cytochrome P450 reductase has been cloned in several insect species (Koener *et al.*, 1993; Hovemann *et al.*, 1997; Horike *et al.*, 2000). Similarly, the adrenodoxin reductase gene (*dare*) has been identified in *Drosophila* (Freeman *et al.*, 1999). Taken together, these data underline the similarity of cytochrome P450 enzyme function in both vertebrates and invertebrates. As there are numerous cytochrome P450 enzymes involved in various metabolic processes, the expression patterns of the electron transfer proteins are not specific to steroidogenic tissues. However, specific transcriptional or posttranscriptional regulation may occur in steroidogenic tissues, as has been shown in vertebrates. Molecular studies are needed to substantiate such hypotheses.

3.3.4.3.3. Intracellular steroid movements During ecdysteroid biosynthesis, intracellular movements of all sterol intermediates (and not just C) must necessarily occur, as the various enzymes involved in their interconversion have been localized to different subcellular compartments. In vertebrates, several proteins (in addition to the enzymatic

machinery) are expressed more or less specifically in the steroidogenic tissues, where they appear to act to promote cholesterol movements inside the cells. These proteins play complementary roles during the acute regulation of steroidogenesis. Three major steps are thought to be involved, although the mechanisms are still not fully understood. First, a sterol carrier protein (SCP-2) mediates the transfer of C, whether dissolved in the cell membrane, the ER, or within intracellular lipid droplets, to the outer mitochondrial membrane (Gallegos *et al*., 2001). Then the binding of a ligand, diazepam binding inhibitor (DBI), to a peripheral benzodiazepine receptor (PBR) is involved in the formation of a pore complex, thereby creating contact sites between the outer and inner membranes of the mitochondria (Papadopoulos, 1993). Finally (at least in the adrenal) the steroidogenic acute regulatory protein (StAR), by means of its StAR-related lipid transfer domain (START), enables a rapid increase in the transport of cholesterol from the outer to the inner mitochondrial membrane (Stocco, 2001) where the first enzyme of steroidogenesis, the cholesterol side chain cleavage cytochrome P450 (CYP11A), resides (Miller, 1988; Stocco and Clark, 1996; Black *et al*., 1994). A similar function has been attributed to the vertebrate cholesterol transporter MLN64 (metastatic lymph node 64) within the placenta (Moog-Lutz *et al*., 1997). However, while it shares the START domain with the StAR protein, MLN64 also has an additional transmembrane region.

In arthropods, the situation is somewhat different since the first step of ecdysteroid biosynthesis is microsomal instead of mitochondrial, but intracellular sterol (and ecdysterol) trafficking may nevertheless require facilitation by mechanisms similar to those observed in vertebrates (**Figure 14a**). It would first seem necessary to increase the access of C, whether localized in the cell membrane or present in cytosolic vesicles, to the 7,8-dehydrogenase in the ER. Thus, an SCP2-like protein could be involved in these sterol movements into and maybe out of the ER. Subsequently, the newly synthesized and very nonpolar 7dC must then translocate from the ER through the cytosol to the unknown organelle where it undergoes the black box oxidations, i.e., to the mitochondria, to another domain of the ER, to the peroxisome, or to some other place. If there is mitochondrial involvement at this stage of the synthesis (or later), then a StAR-like protein, i.e., Start1 (see below), may well be involved, perhaps along with DBI/PBR. Alternatively, if peroxisomal, then an SCP2-like carrier might be indicated. This movement of 7dC out of the ER and into the location of its subsequent oxidation, in analogy with the cholesterol side chain cleavage reaction of mammalian steroidogenesis, has long been hypothesized to be the rate-limiting step of ecdysteroid biosynthesis (Rees, 1985, 1995; Warren and Hétru, 1990; Warren and Gilbert, 1996) and ultimately under the control of the brain molting factor prothoracicotropic hormone (PTTH) (see **Chapter 3.2**). The final envisioned Δ^4-diketol product could then undergo hypothesized reduction by a cytosolic or membrane-bound 5β-reductase. In any case, the resulting ecdysteroid-like product, 3-dehydro-2,22,25dE (or its 3β-OH reduction product, see Section 3.3.4.2.3), must necessarily travel back to the ER to undergo 25-hydroxylation, prior to its final sequential 22- and 2-hydroxylations within the mitochondria. The now very polar, almost sugar-like steroid product 3DE (or E) is then somehow finally secreted through the cell membrane and into the hemolymph for subsequent 3β-OH reduction (if needed, see Section 3.3.5.3.2) and/or conversion to 20E in the peripheral tissues.

Homologs of the above-mentioned proteins are found across the animal kingdom, including insects. Recent studies dealing with their role in insects are consistent with their putative implication in the regulation of ecdysteroid biosynthesis (**Figure 14a**). A SCP-2-like protein has recently been identified in *Aedes aegypti* (Krebs and Lan, 2003). It has a sequence (about 40% amino acid identity with mammalian sequences) and three-dimensional structure similar to the mammalian SCP-2/SCP-X proteins (Stolowich *et al*., 2002; Dyer *et al*., 2003), suggesting a conserved role as a cholesterol transporter. In *Drosophila*, a SCP-X gene has been reported (Kitamura *et al*., 1996), but only a 1.6 kb transcript was mentioned with high expression levels in the midgut of embryos. On the other hand, an independent gene (AeSCP-2) has been identified in *Aedes* and is similar to the vertebrate SCP-2 transcript. The *Aedes* SCP-2-like protein also has a high affinity towards cholesterol, suggesting its role as a cholesterol transporter (Krebs and Lan, 2003). It presents a broad tissue distribution, but further detailed studies are needed of the expression of a SCP-2-like protein in arthropod steroidogenic tissues.

In various vertebrate steroidogenic cell models, the activation of the mitochondrial 18 kDa peripheral-type benzodiazepine receptor (PBR) by its ligand diazepam binding inhibitor (DBI) or endozepine, confers the ability to take up and release cholesterol (Papadopoulos *et al*., 1998). The diazepam-binding inhibitor (DBI) is a 10-kDa protein, which has been identified in *M. sexta* (Snyder and Feyereisen, 1993) and *D. melanogaster* (Kolmer *et al*., 1993, 1994). Homologous sequences are also

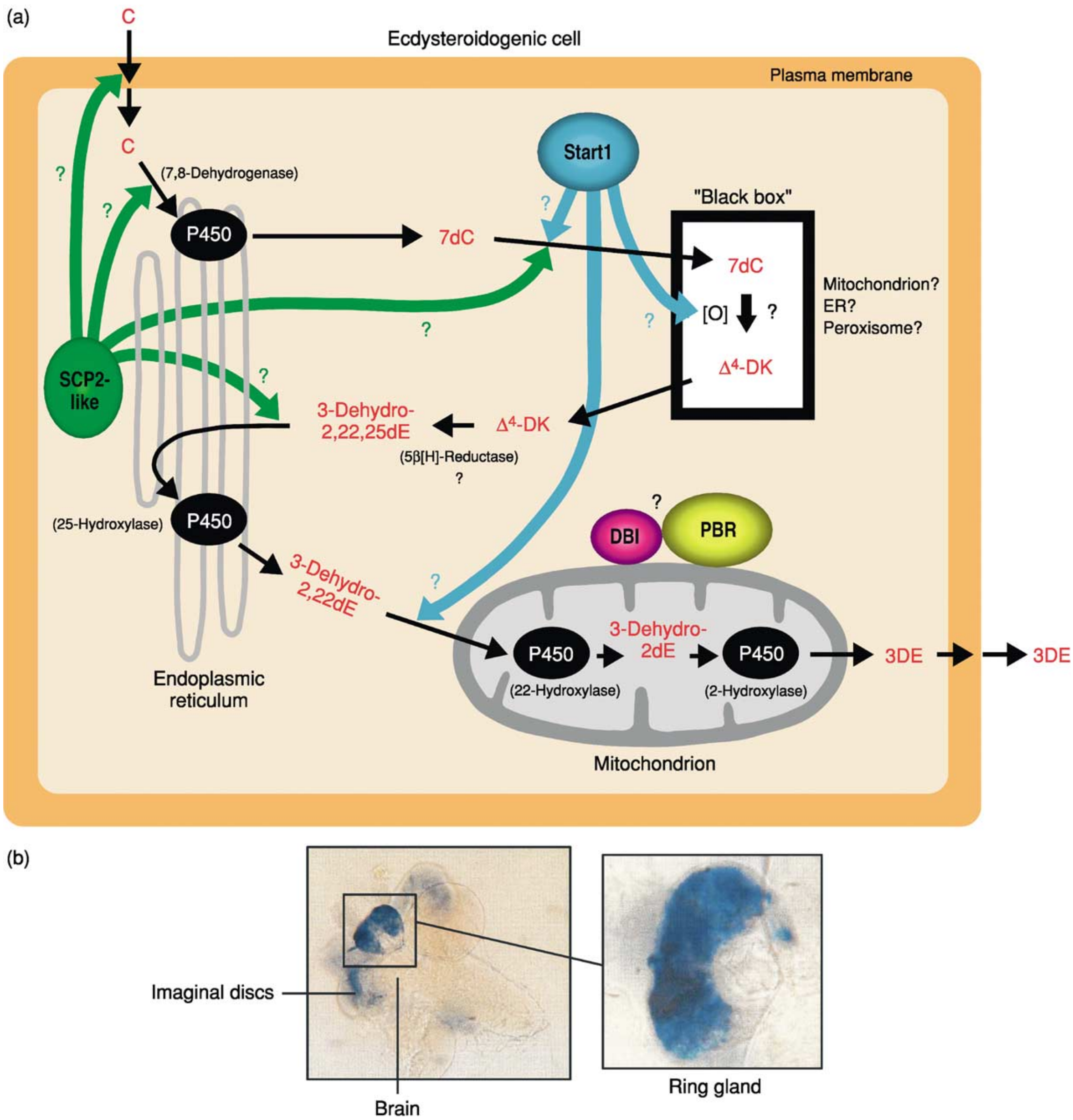


Figure 14 Proposed involvement of the hypothetical sterol carrier proteins SCP2, DBI, and StAR in insect ecdysteroidogenesis. (a) Ecdysone biosynthesis: localizations of subcellular enzymes and hypothetical intracellular movements of intermediates within the ecdysteroidogenic cell. C, cholesterol; 7dC, 7-dehydrocholesterol; D4-DK, D4-diketol; 3-dehydro-2,22,25dE, 3-dehydro-2,22,25-trideoxyecdysone (diketol); 2,22dE, 2,22-dideoxyecdysone (ketotriol); 3-dehydro-2dE, 3-dehydro-2-deoxyecdysone; 3DE, 3-dehydroecdysone; SCP2-like, sterol carrier protein 2-like; Start1, steroidogenic acute regulatory protein related lipid transfer domain protein 1; DBI, diazepam binding inhibitor; PBR, peripheral benzodiazepine receptor. (b) *In situ* hybridization of *Drosophila* L3 larvae brain ring gland complex employing an antisense *Drosophila* StAR (Start1) digoxigenin-labeled RNA probe (C. Dauphin-Villemant, A. Mesneau, and J.-P. Parvy, unpublished data).

found in other dipteran and lepidopteran species. In *M. sexta* prothoracic glands, DBI expression varies concomitantly with ecdysteroid production, and *in vitro* ecdysteroid production is stimulated by a diazepam analog (Snyder and Antwerpen, 1998). Changes in prothoracic glands of both mRNA and DBI protein levels suggest a tissue-specific regulation of this gene, consistent with a role in the regulation of ecdysteroid biosynthesis.

The capacity of steroidogenic cells during development is highly variable in arthropods, as in vertebrates, and peptide hormones induce very rapid increases in steroid production (within minutes) (see **Chapter 3.2**). The acute regulation of vertebrate

steroidogenesis seems to be the predominant role of the labile proteins StAR and its close analog MLN64 (Stocco, 2000, 2001). Similarly, stimulation of insect steroidogenesis is rapidly inhibited by cycloheximide, an inhibitor of protein synthesis, indicating the requirement of a labile protein (Keightley *et al*., 1990; Dauphin-Villemant *et al*., 1995) (see **Chapter 3.2**). Analysis of genome databases revealed related sequences in invertebrates, including *C. elegans*, *D. melanogaster*, and *A. gambiae*. One, *Start1*, is homologous not with *StAR*, but rather with *MLN64*. However, in addition to the four putative transmembrane domains (helices) of MLN64, it also contains a 122 amino acid insert of unknown function. The spatial and temporal pattern of both *Start1* message and Start1 protein expression in *Drosophila* is consistent with it being the rate-limiting factor in ecdysteroidogenesis (Roth *et al*., 2004). For instance, *in situ* hybridization using an antisense Digoxygenin-labeled probe corresponding to the sequence for *Start1* isolated from third instar total RNAs shows a strong labeling of ring glands (**Figure 14b**; C. Dauphin-Villemant, unpublished data). In a similar manner, a carotenoid binding protein (CBP) presenting similarities with the StAR family was recently identified in the *Bombyx mori* silk gland (Tabunoki *et al*., 2002). However, as it binds carotenoids rather than cholesterol, it is possible that several StAR related proteins may exist in arthropods. Clearly, further studies are needed to demonstrate the involvement of the *Drosophila* Start1 protein or any of these other proteins, in the regulation of steroidogenesis.

3.3.4.4. The Ecdysteroidogenic Tissues

3.3.4.4.1. Prothoracic glands and gonads Classically, prothoracic glands are considered as the source of ecdysteroids during postembryonic development, but they usually degenerate prior to the early adult stage. The development of new analytical techniques such as radioimmunoassay (Borst and O'Connor, 1972) allowed direct evidence to be obtained for ecdysone secretion by prothoracic glands. Using *in vitro* incubations of these organs, several authors could demonstrate that they actually secrete measurable amounts of ecdysone, and that the variations of this secretory activity are consistent with the fluctuations of hemolymph ecdysteroid levels (Chino *et al*., 1974; King *et al*., 1974; Borst and Engelmann, 1974). However, this was not a full demonstration for *de novo* synthesis. Only the *in vitro* conversion of radiolabeled cholesterol or 25-hydroxycholesterol by prothoracic glands and Y-organs provided the final argument for the *de novo* synthesis of ecdysteroids by these endocrine organs (Hoffmann *et al*., 1977; Warren *et al*., 1988a, 1988b; Böcking *et al*., 1994).

Adult insects also contain ecdysteroids, as was found in *Bombyx* (Karlson and Stamm-Menendez, 1956). The same methods used for prothoracic glands established that ovaries are a temporary ecdysteroid source, usually in adults and/or pharate adults, depending on the stage when the oocytes develop (Hagedorn *et al*., 1975; Hagedorn, 1985). Careful dissections allowed demonstration that the follicle cells surrounding terminal oocytes are able to produce these compounds *de novo* from cholesterol (Goltzené *et al*., 1978). Labeling was only observed in vitellogenic follicles (stage 10), suggesting local effects of ecdysteroids (paracrine), as expected from the inhibitory effect of ecdysteroids on selective stages of oocyte maturation (Soller *et al*., 1999). These maternal ecdysteroids can also be stored as conjugates by developing oocytes and/or are released into the hemolymph for distribution within the adult. There, they may retain specific endocrine functions such as the control of vitellogenesis, of sexual pheromone biosynthesis, or of ovarian cyclic activity.

Insect testes also contain ecdysteroids and it has been proposed that the testis interstitial tissue plays a role similar to the Leydig cells of vertebrates. When cultured *in vitro*, testes indeed release small amounts of a complex mixture of immunoreactive compounds (Loeb *et al*., 1982), and this release can be stimulated by an ecdysiotropic peptide originating in the brain (Loeb *et al*., 1988). They efficiently convert 2,22,25-trideoxyecdysone into E and 20E (Jarvis *et al*., 1994b). However, the direct evidence for a complete *de novo* biosynthetic pathway in testes is still lacking, and thus a partial synthesis starting from some downstream biosynthetic intermediate or ecdysteroid conjugate cannot be excluded at present. The amounts of ecdysteroids involved are always low as compared to those produced by ovaries, and the absence of a prominent component within the ecdysteroids released *in vitro* does not strongly argue for an endocrine function. Consistent with this hypothesis is the lack of *dib* and *phm* expression in testes, as noticed in the RT-PCR study of various *Drosophila* tissues (**Figure 12a**), although much further study is needed to affirm this result.

3.3.4.4.2. Other tissues? The idea of alternative sites of ecdysteroid production has arisen from much experimental data that do not fit with the classical scheme (Redfern, 1989; Delbecque *et al*., 1990). Prothoracic glands of *Tenebrio molitor* (Coleoptera) degenerate during the pharate pupal stage, so the pharate pupal and pupal ecdysteroid

peaks must arise from another source (Glitho *et al*., 1979). *In vitro* cultivated epidermal fragments of *Tenebrio* pupae release significant amounts of ecdysteroids, and they convert 2,22,25-trideoxyecdysone (but not C) into E. This release is connected with the epidermis cell cycle, as it can be abolished by treatment with mitomycin (Delbecque *et al*., 1990 and references therein). The prothoracic glands of larvae and pupae of the mosquito, *Ae. aegypti*, do not secrete any ecdysteroid when cultured *in vitro*, whereas fragments of thorax and abdomen do secrete ecdysteroids (Jenkins *et al*., 1992). In the same way, epidermal fragments from locust last instar nymphs secrete ecdysteroids *in vitro* (Cassier *et al*., 1980), and cell lines derived from imaginal discs display a rhythmic ecdysteroid production (Mesnier *et al*., 2000). It should be emphasized that prothoracic glands have an ectodermal origin. Therefore, the general epidermis is expected to be the source of molting hormones (see, e.g., Bückmann, 1984; Lachaise *et al*., 1993). Interestingly, it was recently demonstrated that skin is also a primary steroidogenic tissue in humans (Thiboutot *et al*., 2003).

Other approaches have raised similar questions. Insects deprived of molting glands can still molt (e.g., *Periplaneta americana*, Gersch, 1979; *M. sexta*, Sakurai *et al*., 1991); ovariectomized adult insects still contain/produce ecdysteroids (Delbecque *et al*., 1990). Thus, there are data that cannot be explained, unless an alternative ecdysteroid source is postulated. At this level, we should clearly distinguish between primary and secondary sources (Delbecque *et al*., 1990). Primary sources can be defined as tissues that are capable of the *de novo* synthesis of ecdysteroids starting from C or related phytosterols. Secondary sources release ecdysteroids following the hydroxylation of late intermediates or the hydrolysis of conjugates, and therefore do not contain the whole set of enzymes required for de novo synthesis. It is expected, with the complete characterization of all the various *Drosophila* steroidogenic enzymes, that these molecular tools will become available for other insect species as well (e.g., *Aedes*, *Tenebrio*) and they will allow the identification of the tissues that express steroidogenic enzymes in these species.

Finally, the importance and mechanism of ecdysteroid biosynthesis during early embryonic development is still not well understood. Early metabolic studies performed in *Locusta* (Koolman 1989) suggested that ecdysteroids were provided by maternal stores at this stage. However, the very existence of the lethal embryonic Halloween mutations and the results of *in situ* hybridizations using *dib*, *sad*, and *phm* probes argue for *de novo* ecdysteroid biosynthesis during early embryogenesis in *Drosophila*, as has now been described in *Bombyx* embryos (Sonobe *et al*., 1999), and not simply from the hydrolysis of maternally derived ecdysteroid stores within the developing embryo (Bownes *et al*., 1988; Grau and Gutzeit, 1990; Kozlova and Thummel, 2003).

3.3.5. Ecdysteroid Metabolism

3.3.5.1. Introduction

Early reviews are available on ecdysteroid metabolism (Rees and Isaac, 1984, 1985; Lagueux *et al*., 1984; Lafont and Koolman, 1984), including two on synthetic (Smith, 1985) and degradative (Koolman and Karlson, 1985) aspects of regulation of ecdysteroid titer in the first edition of this series, together with several articles in the book edited by Koolman (1989) and a more recent one (Rees, 1995).

In view of the diverse nature of the insect class, there is always a danger of attempting to unify their metabolic processes into common schemes. Certainly, there is considerable species variation in ecdysteroid metabolic pathways. The pathways have been primarily deduced from examination of the fate of [^{3}H]-ecdysteroids in various species of whole insects at various stages of development, or in various tissues incubated *in vitro*. Although in many cases, the metabolic products have been incompletely characterized, such studies have been complemented by the isolation and physical characterization of metabolites.

Defined changes in the ecdysteroid titer are mandatory for correct functioning of the hormone system during development. Regulation of the ecdysteroid titer involves the rates of ecdysone biosynthesis, conversion into the generally more active 20E, inactivation, and excretion. The presence of ecdysteroid carrier proteins, which may protect the hormone to a certain extent from inactivating enzymes, may influence the fate of circulating ecdysteroids. From the limited information available, there is an apparent species-dependent heterogeneity in ecdysteroid transport, with some species lacking binding proteins, whilst others differ in relation to the specificity of protein binding (Karlson, 1983; Whitehead, 1989; Koolman, 1990).

One difficulty is ensuring that some ecdysteroids that have been generally regarded as hormonally inactive metabolites do not possess biological activity in particular systems. For example, although 20E is regarded as the principal active ecdysteroid in most species, there is evidence that E, 3-dehydro-, and 26-hydroxy-ecdysteroids may have hormonal

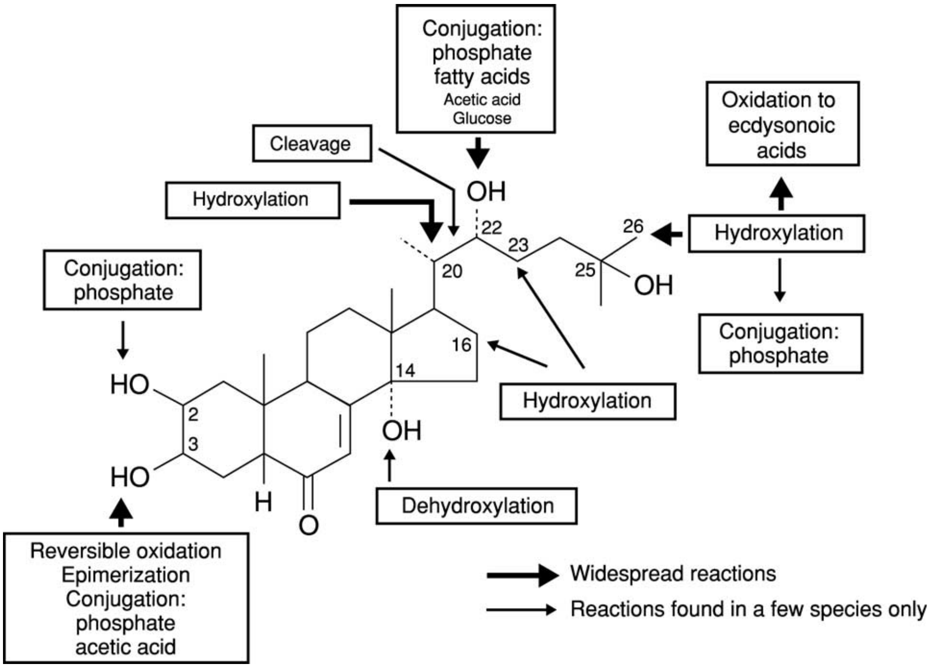


Figure 15 Major reactions of ecdysone metabolism. (Modified from Lafont, R., Connat, J.-L. **1989**. Pathways of ecdysone metabolism. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, p. 167.)

activity in certain systems (see Lafont and Connat, 1989; Dinan, 1989). The tissues that are most active in metabolizing ecdysteroids are the fat body, Malpighian tubules and the midgut. In larvae, ecdysteroids are excreted via the gut or Malpighian tubules and occur in feces as unchanged hormones and various metabolites including conjugates, ecdysteroid acids, and 3-epiecdysteroids (Lafont and Koolman, 1984; Koolman and Karlson, 1985).

Over the years, there have been a plethora of studies on the metabolism of E in various species and the major modifications that have been detected are summarized in **Figure 15**. This concentrates on insects and does not include some more specialized transformations such as found in vertebrates. Since most of the metabolic studies have utilized $[^3H]$-ecdysone labeled in the side chain, the importance of side chain cleavage may well have been underestimated, since the labeled side chain fragment may not have been detected under the conditions used. This section will concentrate on work published since 1985. Studies *in vitro* on E metabolism in different tissues of various species (Koolman and Karlson, 1985), together with the enzymes catalyzing the reactions (Weirich, 1989), have been tabulated and reviewed.

3.3.5.2. Ecdysone 20-Monooxygenase

E, either produced directly by the prothoracic glands or in many Lepidoptera via reduction of 3dE by hemolymph 3-dehydroecdysone 3β-reductase, undergoes ecdysone 20-monooxygenase-catalyzed hydroxylation to yield 20E (**Figure 11**). The 20-hydroxylation reaction is important in the production of 20E, which is generally far more active than E. Earlier work on ecdysone 20-monooxygenase (ecdysone 20-hydroxylase; EC 1.14.99.22) is covered in several reviews (Weirich *et al.*, 1984; Lafont and Koolman, 1984; Smith, 1985; Weirich, 1989; Grieneisen, 1994; Rees, 1995).

3.3.5.2.1. Properties

3.3.5.2.1.1. Species and tissues Early studies on ecdysone 20-monooxygenase in certain tissues of various insect species, together with the biochemical properties of some of these enzymes have been reviewed (Weirich *et al.*, 1984; Smith, 1985). Recently, the gene coding for ecdysone 20-monooxygenase activity in *Drosophila* (*shd*, CYP314A1) has been identified and characterized (Petryk *et al.*, 2003; see Section 3.3.4.3.1). The enzyme is expressed in several peripheral tissues, primarily in the fat body, Malpighian tubules, and midgut. Although generally lower activity may occur in certain other tissues including the ovaries and integument, it is absent from prothoracic glands.

3.3.5.2.1.2. Subcellular distribution Depending on species and tissue, ecdysone 20-monooxygenase may be either mitochondrial or microsomal or occur

in both subcellular fractions. For example, a thorough study has established the occurrence of both high mitochondrial and microsomal activities in midgut from *M. sexta* (Weirich *et al.*, 1985). Although the mitochondrial enzyme has the higher V_{max}, since it also has the higher apparent K_M (E) (1.63×10^{-5} M versus 3.67×10^{-7} M for the microsomal one), at physiological E concentrations (10^{-7}–10^{-8} M), it is only one-eighth to one-tenth as active as the microsomal enzyme. Thus, the microsomal ecdysone 20-monooxygenase is the primary enzyme activity in *Manduca* midgut (Weirich *et al.*, 1996). Similarly, in larvae of *Drosophila* and fed adult females of *Aedes aegypti*, dual localization of enzymatic activity has been shown (Smith and Mitchell, 1986; Mitchell and Smith, 1986; see Section 3.3.4.3.1). Furthermore, in larvae of the housefly, *Musca domestica*, although appreciable ecdysone 20-monooxygenase activity occurs in both mitochondrial and microsomal fractions, pretreatment of larvae with E results in an increase in V_{max} and a decrease in K_M values in mitochondria, but not in microsomes. This suggested that the mitochondrial E 20-monooxygenase is under regulatory control by E in the larval stage and that only such activity has a physiological role during development in *M. domestica* (Agosin *et al.*, 1988). Since one of the mitochondrial cytochrome P450 species fractionated from E-induced mitochondria catalyzed the reaction at significantly higher rates than the other five species in a reconstituted system, it was postulated that the 20-monooxygenase is a mitochondrial process that requires the induction of a low K_M P450 species by ecdysone (Agosin and Srivatsan, 1991). In adults of both sexes of the cricket, *Gryllus bimaculatus*, E 20-monooxygenase activity is located primarily in the microsomal fraction of the midgut, with peak activity at day 4 following ecdysis; no detectable activity was observed in fat body, ovaries, Malpighian tubules, and carcass tissues (Liebrich *et al.*, 1991; Liebrich and Hoffmann, 1991).

3.3.5.2.1.3. Cofactor requirements and cytochrome P450 properties Ecdysone 20-monooxygenase requires NADPH as a source of reducing equivalents. As in the case of vertebrates, the mitochondrial system can use other secondary sources of reducing equivalents such as NADH or Krebs cycle intermediates, presumably by intramitochondrial generation of NADPH via transhydrogenation of $NADP^+$ (Greenwood and Rees, 1984; Smith, 1985). Similarly, a requirement of the 20-monooxygenase for oxygen has been firmly established. Evidence for involvement of cytochrome P450 has been obtained by inhibition of activity with carbon monoxide, its maximal reversal with light at 450 nm as revealed by a photochemical action spectrum, use of a range of specific inhibitors of cytochrome P-450, together with demonstration of the presence of the protein by CO difference spectroscopy (see Smith, 1985).

3.3.5.2.1.4. Substrate specificity Although reconstituted pure vertebrate steroid hydroxylating cytochrome P450s exhibit substrate specificity, it is not absolute and may hydroxylate more than one position on the steroid substrate albeit at different rates (Sato *et al.*, 1978). Since pure cytochrome $P450_{ecdysone}$ has not been available for such reconstitution experiments (see Section 3.3.4.3.1), only preliminary data are available using mitochondrial or microsomal preparations where multiple P450s may occur. *Locusta migratoria* microsomes do not react with 2β-acetoxy- or 3-dehydroecdysone, nor with the 5α-epimer of E; neither will they hydroxylate 20E nor 3d20E at any other position (Feyereisen and Durst, 1978). In the case of *Manduca* fat body mitochondria, the results of a competition assay, which is presumed to reflect enzyme–substrate binding, surprisingly showed that the most effective competitor was not E, but 2,25-dideoxyecdysone, followed in order of decreasing competition by E, 22-deoxyecdysone, 2-deoxyecdysone, 22,25-dideoxyecdysone, and 2,22,25-trideoxy-ecdysone (Smith *et al.*, 1980). Interestingly, 20-hydroxylation of 26-hydroxyecdysone, ecdysonoic acid, and 3-epiecdysone has been observed in *P. brassicae* (Lafont *et al.*, 1980).

3.3.5.2.1.5. Kinetics and product inhibition The K_M and V_{max} values determined for the various mitochondrial and microsomal E 20-monooxygenase systems show substantial differences (Weirich *et al.*, 1984; Smith, 1985). However, the extent to which these differences are real or reflect different detailed methodologies is uncertain (see Smith, 1985). However, several studies have demonstrated competitive inhibition of the enzyme system by its product, 20E.

3.3.5.2.2. Control during development Early studies on regulation of E 20-monooxygenase activity have been comprehensively reviewed (Smith, 1985).

3.3.5.2.2.1. Developmental changes in ecdysone 20-monooxygenase activity Although many early studies on changes in the metabolism of E *in vivo* suggested that E 20-monooxygenase activities vary during development, definitive demonstration of this required *in vitro* studies, since several factors

could conceivably complicate the former (see Smith, 1985).

In the migratory locust, *Locusta migratoria*, peaks in E 20-monooxygenase activity, microsomal cytochrome P450 levels, and NADPH cytochrome *c* reductase activity in both fat body and Malpighian tubules coincided with the peak of 20E in the hemolymph (Feyereisen and Durst, 1980). This suggested that the increases in E 20-monooxygenase activities were due to increased amounts of the monooxygenase components. However, in several species, the peak of E 20-monooxygenase activity preceded that of the hormone titer during the last larval instar (*Schistocerca gregaria* Malpighian tubules: Johnson and Rees, 1977; Gande *et al*., 1979; *Calliphora vicina*: Young, 1976). Similarly, in the fifth larval instar of *Manduca*, fat body and midgut monooxygenase activities exhibited 10-fold and 60-fold fluctuations, respectively, that were not temporally coincident with one another, or with the major hemolymph ecdysteroid titer peak (Smith *et al*., 1983). These peak activities for fat body and midgut monooxygenases were temporally coincident and succedent, respectively, with the small hemolymph peak of ecdysteroid titer on day 4 that is responsible for the change in commitment during larval–pupal reprogramming; both activities were at basal levels during the major hemolymph ecdysteroid titer peak on day 7–8.

The exact significance of these E 20-monooxygenase developmental profiles remains obscure. Since the apparent K_M values for the fat body and midgut systems were fairly constant during the instar, whereas the apparent V_{max} values in each tissue fluctuated in a manner that was temporally and quantitatively coincident with the fluctuations in monooxygenase activity, it suggested that the changes in these activities were due to changes in amounts of the enzymes. More recently, the peak in *Manduca* midgut E 20-monooxygenase activity at the onset of wandering on day 5 has been further examined (Weirich, 1997). During the day preceding the peak, the microsomal activity increased 60-fold (total activity) or 115-fold (specific activity) and decreased gradually within 2 days after the peak. In contrast, the mitochondrial activity increased only 1.3- to 2.4-fold (total and specific activities, respectively) before the peak, but declined more rapidly than the microsomal activity after the peak. This indicates that mitochondrial and microsomal E 20-monooxygenase activities are controlled independently and that changes in the physiological rate of E 20-hydroxylation in the midgut are affected primarily by changes in the microsomal E 20-monooxygenase activities. Further studies on *M. sexta* showed that basal levels of E 20-monooxygenase activity occurred during the later stages of embryogenesis and that a peak of monooxygenase activity occurred late in the fourth instar in both fat body and midgut at the time of spiracle apolysis (Mitchell *et al*., 1999). Both the fat body and midgut hydroxylase activities were basal during the pupal stage and only rose late in pharate–adult development, just prior to adult eclosion.

In another lepidopteran, the gypsy moth *Lymantria dispar*, preliminary studies with homogenates of various tissues taken at different times in the fifth instar surprisingly showed that E 20-monooxygenase activity in fat body, midgut, and Malpighian tubules exhibited peak activity coinciding with the peak in the hemolymph ecdysteroid titer on the penultimate day of the instar (Weirich and Bell, 1997). In *P. brassicae*, E 20-hydroxylase activity occurs in microsomes of pupal wing discs, with an apparent K_M of 58 nM for E. The activity varied during pupal–adult development with a maximum on day 4, a time when ecdysteroid titers are high (Blais and Lafont, 1986). Although the activity is low, the peak activity is sufficient to hydroxylate 25% of endogenous E in pupae. In eggs of the silkworm, *B. mori*, during embryonic development, E 20-monooxygenase activity was primarily microsomal and remained low in diapause eggs, whereas that in nondiapause eggs increased from the gastrula stage (Horike and Sonobe, 1999). This increase in 20-monooxygenase activity was prevented by actinomycin D and α-amanitin, suggesting the requirement for gene transcription. In the last larval stage of *Diploptera punctata*, maximal 20-monooxygenase activity of midgut homogenates, which was primarily microsomal, occurred on days 5–9, whereas the hemolymph ecdysteroid titer exhibited a small peak on day 10 with a major one on day 17 (Halliday *et al*., 1986).

In *Drosophila*, E 20-monooxygenase activity has been assayed in homogenates throughout the life cycle (Mitchell and Smith, 1988; Petryk *et al*., 2003; see Section 3.3.4.3.1). There was a small peak of enzymatic activity in the egg that was temporally coincident with the major peak in the egg ecdysteroid titer. This was followed by several larger fluctuations in activity during the first, second, and early third larval stadia, with a large peak during the wandering stage that remained high during early pupariation, before dropping to basal levels by pupation and remaining at these low levels through to adults. The major peak in E 20-monooxygenase activity during the wandering stage and early pupariation is temporally coincident with the major ecdysteroid titer peak that occurs at puparium

formation. Similarly, in the Diptera *Neobellieria bullata* and *Parasarcophaga argyrostoma* fat body microsomal E 20-monooxygenase activity exhibits a peak at the beginning of wandering behavior in the third instar (Darvas *et al*., 1993). Clearly, the exact significance of the developmental profiles in E 20-monooxygenase activities during development and, particularly, in relation to the ecdysteroid titer in various species is far from clear.

3.3.5.2.2.2. Factors affecting ecdysone 20-monooxygenase activity Not only is little known about the physiological significance of the developmental fluctuations in ecdysone 20-monooxygenase during development, but the factors and mechanisms responsible for such changes are ill understood. As alluded to earlier, the product of the reaction, 20E, is a competitive inhibitor, although on the basis of K_i values and hormone titers, there is doubt as to whether 20E could affect the monooxygenase activity under physiological conditions in all species examined (see Smith, 1985).

In certain species, there is evidence that E or 20E may induce E 20-monooxygenase systems. For example, in last instar larvae of *L. migratoria*, where the peak in ecdysteroid titer and E 20-monooxygenase activity are coincident, injection of E or 20E at an earlier time led to selective induction of cytochrome P450 systems, including E 20-monooxygenase, lauric acid ω-hydroxylase, NADPH-cytochrome *c* reductase, and cytochrome P450 (Feyereisen and Durst, 1980). This induction is apparently a specific effect of active molting hormones since 22-isoecdysone and 22,25-dideoxyecdysone were inactive. Such induction apparently requires protein synthesis, since it is abolished after simultaneous injection of E and actinomycin D, puromycin, and cycloheximide. Furthermore, involvement of the brain in this induction by E is ruled out, since it was observed in isolated abdomens.

Studies on housefly larvae corroborate regulation of E 20-monooxygenase activity by E (Srivatsan *et al*., 1987). Interestingly, although both mitochondria and microsomes catalyze the reaction, only the former E 20-monooxygenase appears to be regulated by E.

In the midgut of final larval instar tobacco hornworm, *Manduca*, the 50-fold increase in E 20-monooxygenase activity at the onset of the wandering stage, is prevented by actinomycin D and cycloheximide, indicating a requirement for transcription and protein synthesis (Keogh *et al*., 1989). This increase in 20-monooxygenase activity could also be elicited in head (but not thoracic) ligated animals by a brain-retrocerebral complex factor(s) released at the time of prothoracicotropic hormone (PTTH) release (see **Chapter 3.2**). It was reported that E or 20E could elicit the increase in monooxygenase activity in both head and thoracic ligated animals, suggesting the operation of a neuroendocrine–endocrine axis (PTTH → prothoracic glands → E → midgut) in the induction. Furthermore, it was reported that the ecdysteroid receptor agonist, RH 5849, could also elicit the increase in midgut E 20-monooxygenase activity in head or thoracic ligated larvae (Keogh and Smith, 1991). However, the finding that neither 20E nor RH5849 induced E 20-monooxygenase activity, but rather ecdysteroid 26-hydroxylase activity, in the cotton leafworm, *Spodoptera littoralis* (Chen *et al*., 1994b; see Section 3.3.5.3.2) prompted the re-investigation of the situation in *Manduca* midgut. In this study, it was shown that although 20E and RH5849 could induce E 20-monooxygenase in subcellular fractions of midgut of *Manduca*, there was a much stronger induction of ecdysteroid 26-hydroxylase activity (Williams *et al*., 1997). In the original studies (Keogh *et al*., 1989; Keogh and Smith, 1991), 26-hydroxylation was not detected, presumably because the thin-layer chromatography system used for the assay may not have resolved 20E and 26-hydroxyecdysone.

In fat body of sixth larval instar *S. littoralis*, E 20-monooxygenase activity is predominantly mitochondrial, with much less activity in the microsomes (Hoggard and Rees, 1988). The former activity exhibits a peak of activity during the instar at 72 h, when the larvae stop feeding (Chen *et al*., 1994a). To ask the question as to what is the molecular mechanism underlying this increased activity, various antibodies raised against components of vertebrate mitochondrial steroidogenic enzyme systems have been used as potential probes for the corresponding insect proteins. Since correlation was observed between developmental changes in mitochondrial E 20-monooxygenase activity and the abundance of polypeptides recognized by cytochrome $P450_{11\beta}$ antibody and a polypeptide recognized by the adrenodoxin reductase antibody, it suggests that developmental changes in the abundance of components of the monooxygenase system may be important in developmental regulation of the enzyme expression (Chen *et al*., 1994a).

There is evidence that E 20-monooxygenase activity may also be influenced by dietary factors, including plant flavonoids and other allelochemicals. For example, assay of activities *in vitro* of E 20-monooxygenase from certain species has demonstrated that a range of plant flavonoids and other allelochemicals, including azadirachtin, significantly inhibit the activity in a dose-dependent manner,

whereas certain flavonols and other allelochemicals stimulate activity (Smith and Mitchell, 1988; Mitchell *et al*., 1993). However, none of the compounds tested elicited effects at very low concentrations. Furthermore, studies on the influence of dietary allelochemicals on midgut microsomal E 20-monooxygenase activity in the fall armyworm, *Spodoptera frugiperda*, have shown that various indoles, flavonoids, monoterpenes, sesquiterpenes, coumarins, methylenedioxyphenyl compounds, and ketohydrocarbons all caused significant stimulation of activity (Yu, 1995, 2000). Significantly, enzyme inducibility was different between E 20-monooxygenase and xenobiotic-metabolizing cytochrome P450 monooxygenases.

In addition, there have been numerous studies on the effects of a plethora of synthetic inhibitors on E 20-monooxygenase activity in various species (e.g., Kulcsar *et al*., 1991; Darvas *et al*., 1992; Jarvis *et al*., 1994a).

3.3.5.2.3. Potential modulation by covalent modification The initial indication that hormonal factors might regulate ecdysteroid metabolism was furnished by the demonstration that a forskolin-induced increase in intracellular cyclic AMP in *Calliphora* fat body *in vitro* led to decreased rates of conversion of E into 20E and of the latter to other metabolites (Lehmann and Koolman, 1986, 1989). Thus, it was suggested that since forskolin mimics the action of peptide hormones by activating the adenylate cyclase system (see **Chapter 3.2**), a peptide hormone, possibly a neuropeptide, might be involved in the regulation of E metabolism. Such activation of adenylate cyclase leads to an increase in intracellular cAMP, which enhances protein (enzyme) phosphorylation via cAMP-dependent protein kinases (see **Chapter 3.2**). Such a process as part of a reversible phosphorylation-dephosphorylation of enzymes provides a mechanism for rapid modulation of enzymatic activities. In the case of vertebrate mitochondria, such a control mechanism has been reported only for a few enzyme complexes. In such systems, relevant endogenous mitochondrial protein kinase and phosphoprotein phosphatase activities have been demonstrated (see Hoggard and Rees, 1988). In fact, experimental support for such modulation of E 20-monooxygenase activity by phosphorylation-dephosphorylation has been obtained. In the case of both the microsomal and broken mitochondrial fractions from fat body of *S. littoralis*, experiments involving treatments with phosphatase inhibitors, exogenous phosphatase, protein kinase, or the adenylate cyclase activator, forskolin, provide indirect evidence that the enzyme system may exist in an active phosphorylated state and inactive dephosphorylated state (Hoggard and Rees, 1988; Hoggard *et al*., 1989). However, confirmation of this notion requires more definitive direct evidence, including direct demonstration of the incorporation of labeled phosphate into components of the E 20-monooxygenase, together with demonstration of the occurrence of an appropriate mitochondrial protein kinase and protein phosphatase.

There are many reports of the phosphorylation of cytochrome P450s (Koch and Waxman, 1991), including steroidogenic $P450_{11\beta}$ from bovine adrenal mitochondria (Defaye *et al*., 1982), with limited evidence for the physiological significance of the phenomenon. The activity of the microsomal cytochrome P450-dependent cholesterol 7α-hydroxylase may also be modulated by phosphorylation-dephosphorylation (Goodwin *et al*., 1982).

Since the pharmacologically inactive forskolin derivative, 1,9-dideoxy-forskolin, as well as forskolin, induced a dose-dependent inhibition of E 20-monooxygenase, the effect must be direct rather than via activation of adenylate cyclase (Keogh *et al*., 1992). Thus, care must be exercised in the design of such experiments to avoid possible misinterpretation of data.

3.3.5.3. Ecdysteroid Inactivation and Storage

Insect development is absolutely dependent on defined changes in ecdysteroid titer. We have already considered the major hormone activation step, the E 20-monooxygenase-catalyzed reaction, and will now consider the major pathways contributing to hormone inactivation. As alluded to in Section 3.3.1, there are many reviews covering ecdysteroid metabolism and inactivation. It is difficult to establish unequivocally that a particular compound is an inactivation product, since an ecdysteroid that is essentially hormonally inactive in one assay may have activity in other systems (Dinan, 1989; Lafont and Connat, 1989).

Early studies on $[^3H]$-ecdysteroid metabolism in insects and other arthropods frequently reported the formation of polar products that were hydrolyzable with various hydrolytic enzymes, especially the so-called "*Helix pomatia* sulfatase," releasing free ecdysteroids. However, unless the enzyme is absolutely pure, no conclusion can be drawn regarding the nature of the conjugate.

It is convenient to consider metabolism and inactivation of ecdysteroids in relation to (1) potential re-utilization of the products from adult females during embryogenesis, or (2) removal of hormonal activity and excretion.

3.3.5.3.1. Ecdysteroid storage, utilization, and inactivation

3.3.5.3.1.1. Phosphate conjugates Ecdysteroid "storage" has been reviewed (Isaac and Slinger, 1989). Initially, a relatively high concentration of polar conjugated ecdysteroids was reported in *Bombyx* eggs (Mizuno and Ohnishi, 1975). However, the demonstration that ecdysteroids in locust ovaries/eggs occurred almost exclusively as polar conjugates (Dinan and Rees, 1981a) and their identification as the 22-phosphate derivatives in *S. gregaria* (Rees and Isaac, 1984) and *L. migratoria* (Lagueux *et al.*, 1984) opened the way for more definitive investigations in various species. Significantly, these maternal conjugates are phosphorylated at C_{22} and are presumably inactive, since a free 22*R* hydroxyl group is important for high hormonal activity. The ovarian ecdysiosynthetic tissue has been shown to be the follicle cells (see Lagueux *et al.*, 1984) and the cytosolic phosphotransferase from *S. gregaria* follicle cells has been characterized (Kabbouh and Rees, 1991a). The enzyme is dependent on ATP/Mg^{2+} for high activity, and activity varies during ovarian development, reaching a peak at the end of oogenesis in agreement with the titer of ecdysteroid 22-phosphates in the oocytes. Furthermore, enzymatic activity can be induced in terminal oocytes by E or 20E treatment, which suggests a physiological mechanism of increasing conjugation as biosynthesis of ecdysteroids in follicle cells proceeds (Kabbouh and Rees, unpublished data).

The majority of the ovarian ecdysteroid conjugates, together with some free hormone, was passed into the oocytes (Dinan and Rees, 1981a; see Rees and Isaac, 1984; Hoffmann and Lagueux, 1985). During embryogenesis, locusts undergo several molts and cycles of cuticulogenesis coincident with peak titers of free ecdysteroids (Sall *et al.*, 1983). Support for the hypothesis that the ecdysteroid 22-phosphates, following enzymatic hydrolysis, could at least provide one source of hormone in embryogenesis, particularly before differentiation of the prothoracic glands, was provided by demonstration of enzymatic hydrolysis of conjugates in a cell-free system from embryos (Isaac *et al.*, 1983). Significantly, activity of the enzyme increases around the time of greater utilization of the conjugates, which also corresponds to an increase in free ecdysteroid titer (Scalia *et al.*, 1987). Moreover, an ecdysteroid-phosphate phosphatase was recently purified and cloned from *Bombyx* embryos and shown to be active only in nondiapausing eggs (Yamada and Sonobe, 2003). In several species, there is no doubt that embryonic synthesis of ecdysteroids occurs in the later stages. Reviews on the role of ecdysteroids in reproduction and embryonic development are available (Hagedorn, 1985; Hoffmann and Lagueux, 1985).

The egg is a closed system and, therefore, decreases in the embryonic hormone titer occur largely by metabolic inactivation, with accumulation of products prior to emergence (Dinan and Rees, 1981b). This accumulation facilitated the identification of a number of new ecdysteroid derivatives, presumed to be inactivation products, including ecdysteroid 2-phosphate 3-acetate derivatives, 3-epi-2-deoxyecdysone 3-phosphate, and ecdysteroid 26-oic acids (see Rees and Isaac, 1984; Hoffmann and Lagueux, 1985; Rees and Isaac, 1985). **Figure 16** shows probable metabolic pathways of the major ecdysteroid 22-phosphates in developing eggs of *S. gregaria* (Rees and Isaac, 1984).

In *S. gregaria*, at the beginning of embryonic development, the ecdysteroids (conjugates and free) occur only in the yolk, whereas after blastokinesis, they occur in the embryo (Scalia *et al.*, 1987). *Locusta migratoria* produce diapause eggs under short-day (SD) conditions and nondiapause eggs under long-day (LD) conditions. The demonstration that the total detected ecdysteroids (primarily phosphate conjugates) in newly laid eggs was more than three times higher in nondiapause eggs than in diapause eggs suggests that ecdysteroids may be involved in the control of embryonic diapause in this species (Tawfik *et al.*, 2002).

Surprisingly, in *Manduca*, the major conjugate in newly laid eggs is 26-hydroxyecdysone 26-phosphate (Thompson *et al.*, 1985b). This compound undergoes hydrolysis during embryogenesis, with 20-hydroxylation of the released hormone occurring, yielding 20,26-dihydroxyecdysone, followed by reconjugation of 26-hydroxyecdysone to produce the 22-glucoside derivative; formation of 3α-epimers and 26-oic acids also occurs during embryogenesis (Warren *et al.*, 1986; Thompson *et al.*, 1987b, 1988). The proposed pathways of metabolism of 26-hydroxyecdysone 26-phosphate in the developing embryos of *Manduca* are given in **Figure 17**.

Early in embryogenesis, much of the 26-hydroxyecdysone 26-phosphate is hydrolysed, resulting in a peak of 26-hydroxyecdysone just prior to the appearance of the first serosal cuticle (Dorn *et al.*, 1987). The 20,26-dihydroxyecdysone titer reaches a peak at about the time of deposition of the first larval cuticle late in embryogenesis, raising the possibility that this ecdysteroid may have a hormonal role at this stage of development (Warren *et al.*, 1986). Significantly, the concentrations of E and 20E are minimal throughout embryogenesis. That the foregoing metabolic changes are related to

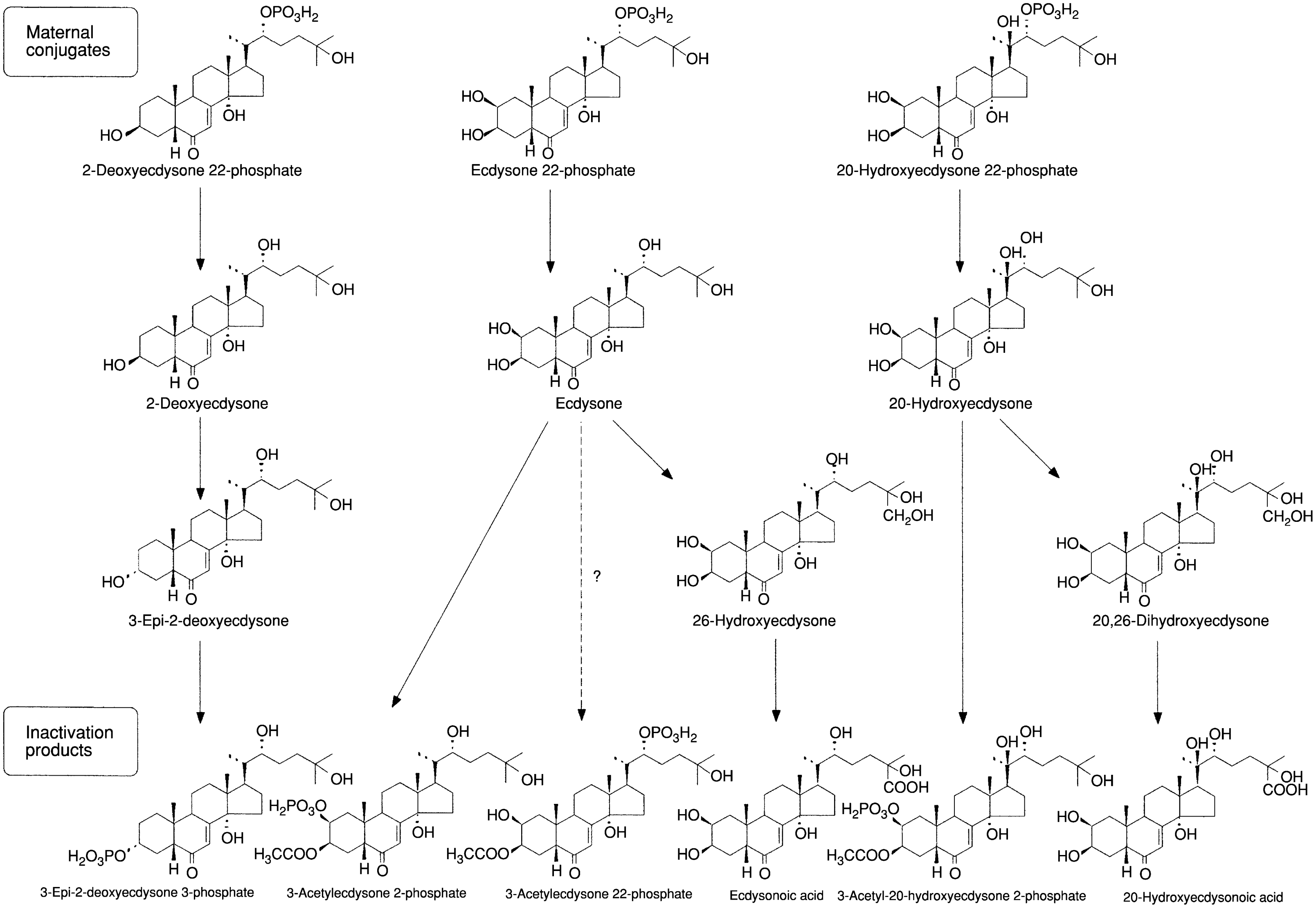


Figure 16 Probable metabolic pathways of the major ecdysteroid 22-phosphates in developing eggs of *Schistocerca gregaria*. (Modified from Rees, H.H., Isaac, R.E. **1984**. Biosynthesis of ovarian ecdysteroid phosphates and their metabolic fate during embryogenesis in *Schistocerca gregaria*. In: Hoffmann, J.A., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, pp. 181–195.)

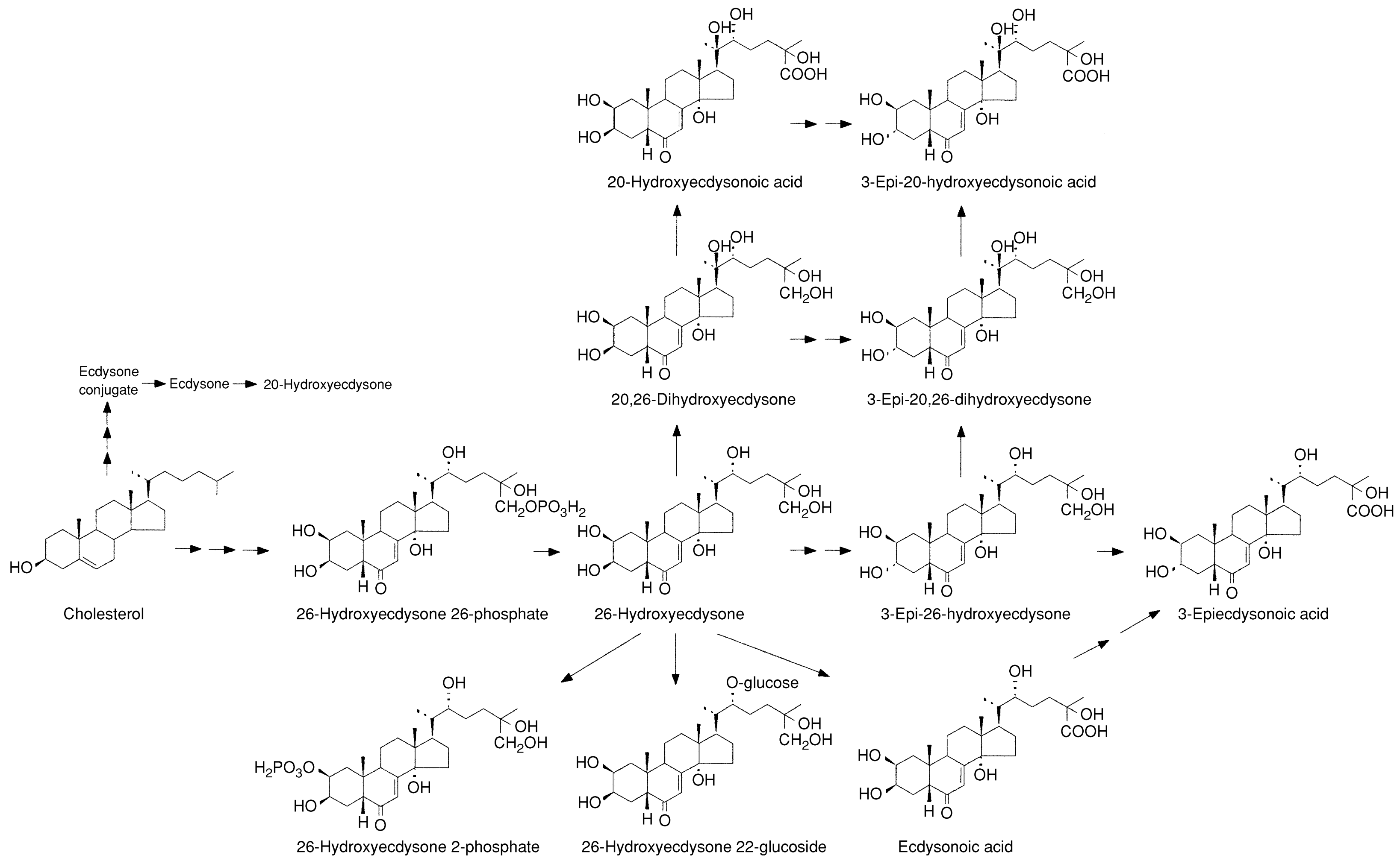


Figure 17 Proposed pathways of metabolism of 26-hydroxyecdysone 26-phosphate during embryogenesis of *M. sexta* (modified from Thompson, M.J., Svoboda, J.A., Lozano, R., Wilzer, K.R., **1988**. Profile of free and conjugated ecdysteroids and ecdysteroid acids during embryonic development of *Manduca sexta* (L.) following maternal incorporation of [^{14}C] cholesterol. *Arch. Insect Biochem. Physiol. 7*, 157–172. by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

embryogenesis is indicated by their virtual absence in incubated unfertilized eggs of *Manduca* (Feldlaufer *et al*., 1988). A plethora of free ecdysteroids and phosphate conjugates have been isolated from ovaries of the silkworm, *B. mori*, and include the following: E, 20E, 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, 2,22-dideoxy-20-hydroxyecdysone and their 22-phosphates, bombycosterol and bombycosterol 3-phosphate (Ohnishi, 1986; Ohnishi *et al*., 1989), 22-deoxy-20-hydroxyecdysone and its 3-phosphate (Kamba *et al*., 1994), 3-epi-22-deoxy-20-hydroxyecdysone and its 2-phosphate (Mamiya *et al*., 1995), 3-epi-22-deoxy-20,26-dihydroxyecdysone, plus 3-epi-22-deoxy-16β, 20-dihydroxyecdysone and their 2-phosphates (Kamba *et al*., 2000b), as well as 2,22-dideoxy-23-hydroxyecdysone and its 3-phosphate (Kamba *et al*., 2000a). Although the metabolic relationships of many of the foregoing ecdysteroids can be surmised, they await experimental verification. The occurrence of 23- and 16β-hydroxy ecdysteroids is unusual. An ATP: ecdysteroid phosphotransferase that is active on a number of ecdysteroid substrates has been demonstrated in *B. mori* ovaries (Takahashi *et al*., 1992).

Evidence has been furnished suggesting that 20E, acting via the receptor (see **Chapter 3.5**), is responsible for the developmental difference between diapause and nondiapause in *B. mori* embryos and that a continuous supply of 20E may be required to induce embryonic development (Makka *et al*., 2002). In agreement with this is evidence suggesting that 20-hydroxylation of E may be a rate-limiting step in diapause silkworm eggs (Sonobe *et al*., 1999). Furthermore, dephosphorylation of ecdysone 22-phosphate and its subsequent hydroxylation at C_{20} and C_{26} are prominent in nondiapause eggs, whereas phosphorylation of E is a major process in diapause eggs (Makka and Sonobe, 2000). Surprisingly, in *S. littoralis*, the newly laid eggs contain primarily 2-deoxyecdysone 22-phosphate (Rees, 1995).

3.3.5.3.1.2. Fatty acyl conjugates Quite different types of conjugates, viz. apolar ecdysteroid 22-fatty acyl esters, originally found in tick ovaries and eggs (Wigglesworth *et al*., 1985; Crosby *et al*., 1986a) (see **Chapter 3.16**), also occur in such tissues in certain Orthoptera. In the tick, *Boophilus microplus*, such conjugates appear to function as inactive, storage forms of maternal hormone, being utilized in early embryogenesis and undergoing subsequent inactivation by 20E-oic acid formation in conjunction with re-conjugation (Crosby *et al*., 1986b). However, the situation is complicated, since eggs of all tick species do not contain significant ecdysteroid 22-fatty acyl esters, some containing appreciable free hormones.

Analogous apolar ecdysone 22-fatty acyl esters, produced by the ovaries, have been identified in newly laid eggs of the house cricket, *Acheta domesticus* (Whiting and Dinan, 1989). The rate of fatty acylation by ovaries is developmentally regulated, increasing as the ovaries increase in size (Whiting *et al*., 1997). Evidence suggests that the ecdysone 22-fatty acyl esters undergo hydrolysis during embryogenesis, with the resulting E being converted to 20E, which is then inactivated by double conjugation, first with an uncharacterized polar moiety, followed by 22-fatty acylation (Whiting *et al*., 1993; Dinan, 1997).

The apolar esters in eggs of another orthopteran, the cockroach, *Periplaneta americana*, differ slightly in chromatographic properties from ecdysteroid 22-fatty acyl esters, although free ecdysteroids are released by hydrolysis with *Helix pomatia* enzymes or esterases (Slinger *et al*., 1986; Slinger and Isaac, 1988a). A storage role is suggested for these apolar conjugates, since they undergo hydrolysis during the first third of embryogenesis when there is also an increase in free and polar conjugated ecdysteroids (Slinger and Isaac, 1988b). Synthesis of such apolar esters by an ovary microsomal fraction from *P. americana* requires fatty acyl-CoA as cosubstrate, indicating that the enzyme is an acyl-CoA: ecdysone acyltransferase and not a hydrolase (Slinger and Isaac, 1988c).

Interestingly, significant levels of apolar or polar ecdysteroid conjugates do not occur in newly laid eggs of another cricket species, *Gryllus bimaculatus* (Espig *et al*., 1989). Similarly, it is noteworthy that significant amounts of detectable maternal ecdysteroid conjugates have not been reported in newlylaid eggs of many insect species (see e.g., Hoffmann and Lagueux 1985; Isaac and Slinger, 1989).

Comparison of the sequence similarity of *Drosophila* vitellins and part of the porcine triacylglycerol lipase, led to the intriguing hypothesis that ecdysteroid fatty acyl ester may bind to vitellin in the eggs as storage forms, the conjugates being made available for enzymic hydrolysis as the vitellin is degraded during embryogenesis (Bownes *et al*., 1988; Bownes, 1992). Such candidate ecdysteroid 22-fatty acyl esters are produced in adult female *Drosophila*, together with ecdysone 22-phosphate (Grau and Lafont, 1994b; Grau *et al*., 1995). However, the significance of binding of ecdysone 22-phosphate to an approx. 50 kDa protein in D*rosophila* ovaries is unclear at present (Grau *et al*., 1995).

3.3.5.3.2. Inactivation and excretion in immature stages Ecdysteroids such as E and 20E may be excreted *per se* or as metabolites via the gut or Malpighian tubules. Ecdysteroid metabolizing enzymes, particularly in the fat body, midgut, and Malpighian tubules, may facilitate excretion, in addition to inactivating the hormone. In fact, variation in the rate of excretion during development appears to be an important factor in determining the hormone titer. Furthermore, hormone inactivation by the gut may be important in protection of the insect from any ingested phytoecdysteroids (see below). In closed stages of insects, where excretion is impossible (egg and pupae), irreversible inactivation of ecdysteroids is important, with metabolites accumulating in the gut (Koolman and Karlson, 1985; Isaac and Slinger, 1989; Lafont and Connat, 1989).

A comparative study of the metabolism of E and 20E in representatives of arthropods, mollusks, annelids, and mammals has led to the conclusion that the metabolic pathways differ strongly between species and that each class has evolved specific reactions (Lafont *et al.*, 1986).

3.3.5.3.2.1. Conjugates and overall metabolism Aspects of conjugate formation will be considered initially, since such compounds feature prominently in the metabolism and inactivation of ecdysteroids in immature stages of most insect species. Early studies on enzymatic aspects of ecdysteroid conjugation/hydrolysis have been reviewed (Weirich, 1989). Originally, the identification of various conjugated ecdysteroid inactivation products (e.g., 3-acetylecdysone 2-phosphate), together with the ecdysteroid 26-oic acids from developed eggs of locusts before hatching facilitated elucidation of inactivation pathways in other systems (Lagueux *et al.*, 1984; Rees and Isaac, 1984). In fact, in larval stages of locusts, the inactivation pathways (Gibson *et al.*, 1984; Modde *et al.*, 1984) proved to be very similar to the embryonic routes (see **Figure 16**), with pathways in other species having much in common (Lafont and Connat, 1989). Some features of ecdysteroid metabolic pathways in various stages of a few species that have been reported since the first edition in 1985 will be considered initially. In *Manduca*, when labeled C was injected into 5th instar larvae and the metabolites isolated from day 8 pupae, the predominant ecdysteroid metabolites were 20E, 20,26-dihydroxyecdysone, 20-hydroxyecdysonoic acid, and 3-epi-20-hydroxyecdysonoic acid. Smaller amounts occurred as conjugates (phosphates) of 26-hydroxyecdysone, 3-epi-20-hydroxyecdysone, 20, 26-dihydroxyecdysone, and its 3α-epimer (Lozano *et al.*, 1988a, 1988b). At various stages throughout pupal–adult development, the predominant metabolites were 3-epi-20-hydroxyecdysonoic acid and the phosphate conjugates of 3-epi-20-dihydroxyecdysone (Lozano *et al.*, 1989).

In feeding last larval stage of another lepidopteran, *Pieris brassicae*, injected 20E was converted primarily into 20-hydroxyecdysonoic acid, 3-dehydro-20-hydroxyecdysone, and 3-epi-20-hydroxyecdysone 3-phosphate. Use of E as substrate gave the same metabolites, together with ones lacking the 20-hydroxyl group (Beydon *et al.*, 1987). Similarly, in *P. brassicae* eggs, [^{3}H]-ecdysone metabolism is similar to that in larvae and pupae viz. hydroxylation at C_{20} and C_{26}, with further oxidation to ecdysteroid 26-oic acids. Injection of [^{3}H]-cholesterol into female pharate adults indicated that the egg ecdysteroids/metabolites consisted of 20-hydroxyecdysonoic acid, ecdysonoic acid, 20E, and E (Beydon *et al.*, 1989). In another lepidopteran, the tobacco budworm, *Heliothis virescens*, incubation of testes with labeled E yielded a range of products including 20, 26-dihydroxyecdysone, 3-epi-20-hydroxyecdysone, 20E, and highly polar metabolites (Loeb and Woods, 1989).

In female adults of the cricket, *Gryllus bimaculatus*, injected [^{3}H]-ecdysone was converted into at least 24 metabolites, the highest number being in the gut and feces, and included 26-hydroxyecdysone and 14-deoxyecdysone amongst the free ecdysteroids. In addition, polar (phosphate) and apolar (fatty acyl) conjugates were also observed. Following feeding of [^{3}H] ecdysone, most of the label remained within the gut and was excreted within 48 h, with 14-deoxyecdysone being a major metabolite (Thiry and Hoffmann, 1992). Both apolar and polar ecdysteroid conjugates are widely distributed in tissues of males of this species (Hoffmann and Wagemann, 1994). In vitellogenic females of *Labidura riparia* (Dermaptera), [^{3}H] 20-E was metabolized primarily through conjugation, in particular acetylation at C_3 and phosphate ester formation at C_2 (Sayah and Blais, 1995).

Incubation of midgut cytosol of *Manduca*, where prominent 3-epimerization of ecdysteroid occurs, with [^{3}H]-ecdysone and Mg^{2+}/ATP, results in detection of four E phosphoconjugates and two 3-epiecdysone phosphoconjugates (Weirich *et al.*, 1986). ATP- and Mg^{2+}-dependent ecdysteroid phosphotransferase activities have been demonstrated in cytosolic extracts of ovaries and fat body from mature female pupae of *B. mori* (Takahashi *et al.*, 1992). The midgut cytosol of the cotton leafworm, *S. littoralis*, contains Mg^{2+}/ATP-dependent ecdysteroid 2- and 22-phosphotransferase activities, with a predominance of the former (Webb *et al.*, 1995). The

phosphotransferase activities were high early in the final larval instar and then declined. The relatively high K_M value (21 μM) of the E 2-phosphotransferase (Webb *et al.*, 1996), together with its expression during the feeding period when the hemolymph ecdysteroid titer is low, may suggest that these phosphotransferase enzymes are involved in inactivation of any dietary ecdysteroids.

The enzymes catalyzing formation of E 3-acetate and its phosphorylation in production of 3-acetylecdysone 2-phosphate have been characterized in larval tissues of *Schistocerca*. 3-Acetylation of E and 20E was characterized in gastric caeca from final larval instar *S. gregaria*, since high activity occurred in that tissue. The microsomal enzyme had a somewhat stronger affinity for 20E (K_M 53.5 μM) than E (71 μM) and utilized acetyl coenzyme A, but not acetic acid, as cosubstrate, indicating that the enzyme is an acetyl coenzyme A (CoA): ecdysteroid acetyltransferase and not a hydrolase. Furthermore, esterification of E was not observed when long-chain fatty acylCoA derivatives were substituted as cosubstrates (Kabbouh and Rees, 1991b).

In final instar *S. gregaria* larvae, ATP: E 3-acetate 2-phosphotransferase activity was low in gastric caeca and gut, but was somewhat higher in fat body than in Malpighian tubules. Thus, the soluble fat body enzyme (*ca.* 45 kDa) was characterized. The Mg^{2+}/ATP dependent phosphotransferase utilized E 3-acetate as the preferred substrate (K_M 10.5 μM), with no significant phosphorylation of E and E 2-acetate. The physiological significance of the increase in phosphotransferase activity during the second half of the instar, well before the rise in ecdysteroid titer, is unclear (Kabbouh and Rees, 1993).

In some lepidopteran species, e.g., *Heliothis armigera* (cotton bollworm, Robinson *et al.*, 1987) and *Heliothis virescens* (cotton budworm, Kubo *et al.*, 1987), a major fate of ingested ecdysteroids is formation of 22-fatty acyl esters in the gut for fecal excretion. Characterization of the ecdysteroid-22-*O*-acyltransferase activity in crude homogenates of midgut from *H. virescens* has shown that the enzyme can use fatty acyl CoA as cosubstrate, but not phosphatidylcholine or free fatty acid, indicating that the enzyme differs appreciably from the cholesterol acyltransferase, where all such cosubstrates can be utilized (Zhang and Kubo, *et al.*, 1992a). Furthermore, unlike the situation for the latter enzyme, divalent cations inhibit the ecdysteroid acyltransferase. Subsequently, it has been demonstrated that the ecdysteroid 22-*O*-acyltransferase is located in the plasma membrane of the gut epithelial cells (gut brush border membrane; Kubo *et al.*, 1994). The acyltransferase was active only during feeding stages, its activity decreased as the larvae became committed to pupation and was not enhanced by feeding ecdysteroids to the fifth instar larvae (Zhang and Kubo *et al.*, 1992b). It has been suggested that dietary phytoecdysteroid detoxification is the major function of the acyltransferase (Zhang and Kubo, 1992b, 1994). In both *H. virescens* and *B. mori*, injected E was converted into 20E, 26-hydroxyecdysone, and 20, 26-dihydroxyecdysone, which was oxidized to 20E 26-oic-acid for excretion. These transformations also occurred in the case of ingested E in these species, but as already seen in *H. virescens*, an additional prominent route involved E–22-O-acyl ester formation, which was largely excreted. In contrast, in *B. mori*, the latter pathway was replaced by formation of 3-epi-E and a polar conjugate tentatively identified as the sulfate, which was excreted, in the case of ingested E (Zhang and Kubo, 1993). In the tomato moth, *Lacanobia overacea*, and the cotton leafworm, *Spodoptera littoralis*, the situation is similar to that in *H. virescens*, where injected/endogenous ecdysteroids undergo conversion into 20-hydroxyecdysonoic acid, with ingested ecdysteroids undergoing detoxification yielding apolar 22-fatty acyl esters (Blackford and Dinan, 1997a; Blackford *et al.*, 1997). Surprisingly, the Death's head hawkmoth, *Aclerontia atropos*, is unaffected by dietary 20E, which it excretes in an unmetabolized form (Blackford and Dinan, 1997b). Interestingly, in four other Lepidoptera (*Aglais urticae, Inachis io*, *Cynthia cardini*, and *Tyria jacobaeae*) ingested and injected 20E followed the same fate, with polar compounds being prominent and no detectable formation of 22-fatty acyl esters (Blackford and Dinan, 1997c).

In adult *Drosophila melanogaster*, following injection of [^{3}H]-E, E-22-fatty acyl esters were the major metabolites, followed by 3dE, 26-hydroxyecdysone, ecdysonoic acid, 20-hydroxyecdysone, and E 22-phosphate (Grau and Lafont, 1994a; Grau *et al.*, 1995). 20E was metabolized in a similar manner (Grau and Lafont, 1994a). The E-22-phosphate was produced in the ovaries, whereas E was efficiently converted into 3DE and ecdysone 22-fatty acyl esters by gut/Malpighian tubule complexes (Grau and Lafont, 1994b). In third instar *D. melanogaster* larvae, although E metabolism proceeds through different pathways including C_{20} hydroxylation, C_{26} hydroxylation, and oxidation to 26-oic acids, C_3 oxidation and epimerization followed by conjugation, 3-dehydroecdysteroids are the major metabolites, with C_3 oxidation occurring in various tissues (Sommé-Martin *et al.*, 1988).

3.3.5.3.2.2. 26-Hydroxylation and ecdysonoic acid formation Ecdysonoic acid and 20-hydroxyecdysonoic acid were originally identified from developing eggs of *Schistocerca*, pupae of *Spodoptera* (Isaac *et al*., 1983), and *Pieris*, where their formation has been shown to occur in several tissues (Lafont *et al*., 1983). Since that time, it has become apparent that 26-oic acid formation is a widespread and prominent pathway of ecdysteroid inactivation, as alluded to in several studies considered above.

It has been shown that administration of E, 20E, or an ecdysteroid agonist, RH-5849, to last-instar larvae of the cotton leafworm, *S. littoralis*, leads to induction of ecdysteroid 26-hydroxylase activity in fat body mitochondria (Chen *et al*., 1994b). This induction occurred in both early last instar larvae and in older larvae that had been head-ligated to prevent the normal developmental increase in E 20-monooxygenase activity. The induction of hydroxylase activity requires both RNA and protein synthesis. E-26-oic acid and a compound tentatively identified as the 26-aldehyde derivative of E were also formed from E in the RH 5949-induced systems. Direct demonstration in a cell-free system of the conversion of 26-hydroxyecdysone into the aldehyde and the corresponding 26-oic acid (ecdysonoic acid), established the following inactivation pathway: ecdysteroid → 26-hydroxyecdysteroid → ecdysteroid 26-aldehyde → ecdysteroid 26-oic acid. Whether these three reactions are catalyzed by a single enzyme (as is the case of rabbit liver cytochrome P450 CYP27, which catalyzes the complete conversion of 3α,7α,12α-trihydroxy-5β-cholestane into 3α,7α,12α-trihydroxy-5β-cholestanoic acid (see Betsholtz and Wikvall, 1995) remains an open question.

Induction of the 26-hydroxylase activity might be expected to result from enhanced cytochrome P450 gene transcription and protein synthesis (Chen *et al*., 1994b). Similarly, both 20E and RH-5849 caused RNA- and protein-synthesis-dependent induction of ecdysteroid 26-hydroxylase activity in midgut mitochondria and microsomes in *Manduca* (Williams *et al*., 1997). This induction of an ecdysteroid inactivation pathway (and others, see following section) by 20E and RH-5849 is reminiscent of the regulation of vitamin D inactivation in vertebrates where 1α,25-dihydroxyvitamin D_3 stimulates its own degradation by induction of a vitamin D response element to initiate degradation to excretory metabolites (see Williams *et al*., 1997 for references). However, the possibility cannot be discounted that at least some of the observed induction events may be indirect. Evidence has been furnished that the 26-hydroxylase activity is a typical NADPH-requiring cytochrome P450-dependent monooxygenase that is sensitive to inhibition by CO and imidazole/triazole-based fungicides (Kayser *et al*., 1997; Williams *et al*., 2000). Furthermore, indirect evidence suggested that the *Manduca* mitochondrial and microsomal 26-hydroxylases could exist in a less active dephosphorylated state or more active phosphorylated state (Williams *et al*., 2000). Interestingly, long-term culture of a *Chironomus tentans* (Diptera) cell line in the presence of 20E resulted in selection of subclones that are resistant to the steroid, but respond normally to the agonist, tebufenozide (RH-5992) (see **Chapter 3.4**). Evidence was obtained that ecdysteroid resistance of the cell clones is due to high metabolic inactivation of the hormone by 26-hydroxylation (Kayser *et al*., 1997). In this cell system, 20,26-dihydroxyecdysone is further metabolized to two compounds that are slowly interconvertible geometrical isomers, which presumably arise from hemiacetal formation between the 26-aldehyde and the 22*R*-hydroxyl groups to yield a tetrahydropyrane ring in the side chain (Kayser *et al*., 2002). Induction of ecdysteroid 26-hydroxylase by the agonists RH-5849, RH-5992, and RH-0345 appears universal in lepidopteran species that show susceptibility to the agonists (Williams *et al*., 2002). It appears that the more potent ecdysteroid agonists in Lepidoptera, RH-5992, and RH-0345 show in general a greater induction of 26-hydroxylase than does RH-5849. Feeding RH-5849 to the dipteran *Musca domestica* results in induction of an ecdysteroid phosphotransferase. The low toxicity of the ecdysteroid agonists in orthopteran and coleopteran orders also correlates with a lack of induction of ecdysteroid 26-hydroxylase activity. In fact, it has been proposed that in species where ecdysteroid agonists are effective in stimulating an untimely premature molt, a response to a state of apparent hyperecdysonism elicited by the agonists is induction of enzymes of ecdysteroid inactivation (Williams *et al*., 2002) (see **Chapter 3.4**).

3.3.5.3.2.3. 3-Epimerization An oxygen and NAD(P)H-dependent "E epimerase" activity was originally reported in *Manduca* midgut cytosol which irreversibly converted E into its 3α-isomer (3-epiecdysone; Nigg *et al*., 1974; Mayer *et al*., 1979). Subsequently, this transformation has been shown to occur via the oxygen-dependent E oxidase catalyzed formation of 3DE, which is then irreversibly reduced to 3-epiecdysone, catalyzed by NAD(P)H-dependent 3-dehydroecdysteroid 3α-reductase (**Figure 18**) (*Pieris*, Blais and Lafont, 1984; *Spodoptera*, Milner and Rees, 1985; *Manduca*, Weirich, 1989).

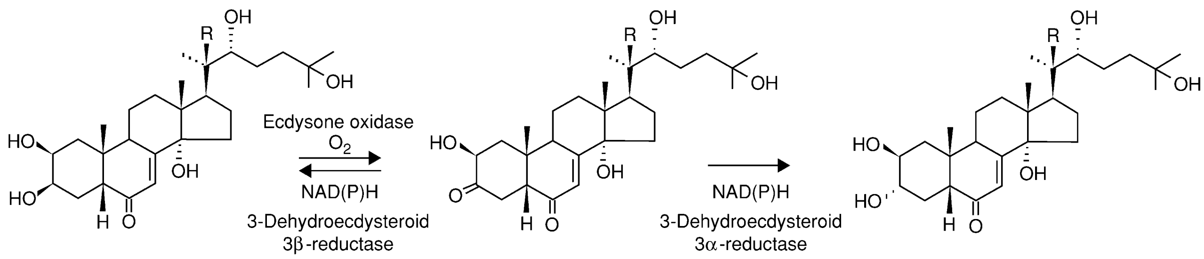


Figure 18 Enzymatic interconversion of 3β-ecdysteroids, 3-dehydroecdysteroids, and 3α-ecdysteroids. R = H: Ecdysone (metabolites); R = OH: 20-hydroxyecdysone (metabolites). (Modified from Rees, H.H. **1995**. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol. 92*, 9–39.)

The 3-epiecdysteroid product may also undergo phosphoconjugation. However, many tissues also contain an NAD(P)H-dependent 3-dehydroecdysteroid 3β-reductase, which reduces the 3DE back to E (see Lafont and Koolman, 1984); the exact significance of this apparent futile cycle is unclear. The foregoing pathways occur with E and 20E and have been reviewed (Lafont and Koolman, 1984; Koolman and Karlson, 1985; Thompson *et al.*, 1985a; Weirich, 1989; Rees, 1995). It is apparent from the foregoing sections of this chapter that 3-epiecdysteroid formation is a widespread ecdysteroid metabolic pathway amongst species, with the 3-dehydroecdysteroid 3α-reductase being apparently restricted to gut tissues. 3-Epiecdysteroid formation is certainly emphasized in Lepidoptera.

The kinetic properties and developmental expression of E oxidase, 3-dehydroecdysteroid 3α-reductase and 3β-reductase enzymes from midgut cytosol of *Manduca* (Weirich *et al.*, 1989, 1991, 1993) and *Spodoptera* have been investigated (Webb *et al.*, 1995, 1996). In the former species, the larval midgut was the only organ exhibiting substantial specific activities of E oxidase and 3α-reductase, with both activities increasing up to the seventh day after ecdysis (Weirich *et al.*, 1989). Hemolymph and fat body had only moderate to high 3β-reductase and low E oxidase activities, whereas the epidermis did not contain significant activities of any of the enzymes. Thus, apparently, only the larval midgut has a role in the inactivation of ecdysteroids by 3-epimerization in *Manduca*.

It has been shown that administration of the hormone agonist, RH5849, but not of 20E, to *Manduca* results in RNA- and protein-dependent induction of the midgut cytosol E oxidase and 3-epiecdysone phosphotransferase activities (Williams *et al.*, 1997). Clearly, elucidation of the significance of these results requires further work.

Ecdysone oxidase from *Calliphora* was amongst the first enzymes catalyzing ecdysteroid transformations to be investigated extensively (Koolman, 1978; Koolman and Karlson, 1978; Koolman, 1985) and has been purified considerably (Koolman and Karlson, 1978). In *Calliphora vicina* also, injected 3DE is rapidly reduced *in vivo*, but relative formation of E and 3-epiecdysone cannot be ascertained since the TLC fractionation system employed lacks resolution (Karlson and Koolman, 1973). The reaction catalyzed by E oxidase (EC 1.1.3.16) may be written as:

$$E + O_2 = \text{3-dehydroecdysone} + H_2O_2$$

The enzyme would be expected to possess a prosthetic group, but none has been identified so far. E oxidase efficiently oxidizes a number of ecdysteroids, but not 22,25-dideoxyecdysone nor cholesterol (Koolman and Karlson, 1978; Koolman, 1985). E oxidase represents the only oxidase in eukaryotic animals known to catalyze oxygen-dependent oxidation of steroids; in contrast, oxidation of steroids in vertebrates occurs via NAD(P) $^+$-linked dehydrogenases.

The native enzyme from *Calliphora* has an apparent molecular mass of approximately 240 kDa (Koolman, 1985). However, native E oxidase purified from midgut cytosol of *Spodoptera* might be a trimer (approximately 190 kDa), since the apparent Mr of the oxidase subunit was 64 kDa by SDS-PAGE (Chen *et al.*, 1999a). The latter was corroborated by cloning the cDNA (2.8 kb) encoding a 65 kDa protein (Takeuchi *et al.*, 2001). Northern blotting demonstrated that the mRNA transcript was expressed in midgut during the prepupal stage of the last larval instar at a time corresponding to an ecdysteroid titer peak. Conceptual translation of the cDNA followed by data base searching indicated that the enzyme is an FAD flavoprotein that belongs to the glucose-methanol-choline oxidoreductase superfamily. Injection of the agonist, RH-5992, induced the transcription of E oxidase, suggesting that it is an ecdysteroid responsive gene. The gene encoding the enzyme consists of five exons and

sequences similar to the binding motifs for Broad-Complex and FTZ-F1 occur in the 5′ flanking region, consistent with ecdysteroid regulation. Southern blotting indicated that E oxidase is encoded by a single-copy gene.

Interestingly, the entomogenous fungus, *Nomuraea rileyi*, inhibits molting of its host, *B. mori*, by production of an enzyme that oxidizes the C_{22} hydroxyl group of hemolymph ecdysteroids (Kiuchi *et al.*, 2003). Whether this enzyme is an oxidase or an $NAD(P)^+$-dependent dehydrogenase has not been established.

One major 3-dehydroecdysteroid 3α-reductase (active with both NADPH and NADH) together with three major 3-dehydroecdysteroid 3β-reductases (active only with NADPH as cosubstrate) have been fractionated from *Manduca* midgut cytosol (Weirich and Svoboda, 1992). In the case of *Spodoptera*, two forms of 3-dehydroecdysone 3α-reductase have been observed during purification, a 26 kDa form that may be a trimer with an apparent Mr of approximately 76 kDa and a second 51 kDa form that appears to be a monomer by gel filtration (Chen *et al.*, 1999a). The cDNA (1.2 kb) encoding the 26 kDa protein has been cloned and Northern blotting showed that the mRNA transcript was expressed in Malpighian tubules during the early stage of the last larval instar (Takeuchi *et al.*, 2000). Conceptual translation of the cDNA followed by database searching revealed that the enzyme belongs to the Short-chain-Dehydrogenases/Reductases (SDR) superfamily. Furthermore, the enzyme is a novel eukaryotic 3-dehydrosteroid 3α-reductase member of that family, whereas vertebrate 3-dehydrosteroid 3α-reductases belong to the Aldo-keto Reductase (AKR) superfamily. Surprisingly, no similarity was observed between the 3-dehydroecdysone 3α-reductase and a reported 3-dehydroecdysone 3β-reductase from the same species (Chen *et al.*, 1999b), which acts on the same substrate and belongs to the AKR family.

As alluded to earlier, 3-dehydroecdysteroid 3β-reductase activity has been demonstrated in the cytosol of various tissues of many species. In addition, hemolymph 3-dehydroecdysteroid 3β-reductase is involved in reduction of the 3DE produced by prothoracic glands of many lepidopterans (see Section 3.3.4.2.; Warren *et al.*, 1988b; Sakurai *et al.*, 1989; Kiriishi *et al.*, 1990).

Hemolymph NAD(P)H-dependent 3-dehydroecdysone 3β-reductase activity has been shown to undergo developmental fluctuation in several species, exhibiting high activity at the time of the peak in ecdysteroid titer near the end of the last larval instar (*Manduca*, Sakurai *et al.*, 1989; *Spodoptera*, Chen *et al.*, 1996; *Periplenta americana*, Richter *et al.*, 1999). In *P. americana*, some enzyme is located on the outer surface of the prothoracic gland cells as well. The exact source of the hemolymph 3-dehydroecdysone 3β-reductase and the mechanism regulating its expression during development have not been elucidated. The 3β-reductase has a wide tissue expression (*Pieris*, Blais and Lafont, 1984; European corn borer, *Ostrinia nubilalis*, Gelman *et al.*, 1991; *Bombyx*, Nomura *et al.*, 1996), including embryonated eggs of the gypsy moth, *Lymantria* (Kelly *et al.*, 1990). Of significance to ecdysteroid biosynthesis is the demonstration that various 3-dehydroecdysteroid precursors may be reduced to the corresponding 3β-hydroxy compounds in prothoracic glands and follicle cells of *Locusta* (Dollé *et al.*, 1991; Rees, 1995).

The 3-dehydroecdysteroid 3β-reductases have been purified from hemolymph of *Bombyx* (Nomura *et al.*, 1996) and *Spodoptera* (Chen *et al.*, 1996), demonstrating that the native enzyme is a monomer of Mr of approximately 42 kDa and 36 kDa, respectively. In the case of both enzymes, a lower K_m was observed with NADPH than with NADH as cosubstrate (*B. mori*, 0.90 μM versus 5.4 μM; *S. littoralis*, 0.94 μM versus 22.8 μM). Kinetic analysis of the *S. littoralis* hemolymph 3β-reductase, using either NADPH or NADH-as cofactor, revealed that the enzyme exhibits maximal activity at low 3DE substrate concentrations, with a drastic inhibition of activity at higher concentrations (>5 μM). Similar substrate inhibition was observed for the cytosolic NADPH-dependent 3-dehydroecdysone 3α-reductase of *Manduca* midgut which showed apparent 3DE substrate inhibition above 10 μM (Weirich *et al.*, 1989). Although the K_M for the *S. littoralis* hemolymph 3-dehydroecdysone 3β-reductase has not been determined due to the substrate inhibition, it is conceivable that the K_M value could be less than 5 μM. The results suggest that the 3β-reductase has a high-affinity (low K_M) binding site for the 3-dehydroecdysteroid substrate, together with a lower affinity inhibition site (Chen *et al.*, 1996). Cloning of the full-length 3-dehydroecdysone 3β-reductase cDNA from *S. littoralis* and conceptual translation yield a protein with a predicted Mr of 39 591 of which the first 17 amino acids appear to constitute a signal peptide to yield a predicted Mr for the mature protein of 37 689 (Chen *et al.*, 1999b), which agrees with the approx. Mr (36 kDa) of the purified protein on SDS-PAGE. Similarly, database searching suggested that the enzyme is a new member of the third (AKR) superfamily of oxidoreductases.

Northern blot analysis revealed that highest expression is detected in Malpighian tubules, followed

by midgut and fat body, with low-level expression in hemocytes and none detectable in central nervous system. Surprisingly, preliminary results of Western blotting experiments revealed 3β-reductase detection in hemolymph and hemocytes, with little in midgut and none in Malpighian tubules, in agreement with the results for *B. mori* (Nomura *et al*., 1996). This suggests that selective translation of mRNA may contribute to regulation of expression of 3-dehydroecdysteroid 3β-reductase (Chen *et al*., 1999b). The developmental profile of the mRNA revealed that the gene is only transcribed in the second half of the sixth instar essentially at the time of increasing enzymatic activity and ecdysteroid titer. Thus, it appears that the change in 3β-reductase activity is, at least in part, regulated by gene expression at the transcriptional level, although posttranscriptional regulation may also be important in certain tissues. Southern analysis indicates that the 3-dehydroecdysone 3β-reductase is encoded by a single gene, which probably contains at least one intron (Chen *et al*., 1999b). Recently, a gene encoding a protein that shows 42.5% identity to *S. littoralis* 3-dehydroecdysteroid 3β-reductase has been cloned from the cabbage loofer, *Trichoplusia ni* and the protein has been shown to possess low 3β-reductase activity (Lundström *et al*., 2002). Northern blotting has indicated that highest expression of the gene is detected in fat body, the expression being highly induced after bacterial challenge. However, by immunohistochemistry, the protein was localized exclusively in the epidermis and the cuticle, but its significance is unclear.

3.3.5.3.2.4. 22-Glycosylation The formation of 26-hydroxyecdysone 22-glucoside in late embryos of *Manduca* has already been alluded to (Thompson *et al*., 1987a).

In the baculovirus, *Autographa californica* nuclear polyhedrosis virus, expression of the *egt* gene produces UDP-glycosyl transferase, which apparently inactivates the host ecdysteroids by catalyzing production of ecdysteroid 22-glycoside. Thus, *egt* gene expression allows the virus to interfere with insect development by blocking molting in infected larvae of the fall armyworm, *Spodoptera frugiperda* (O'Reilly and Miller, 1989; O'Reilly *et al*., 1991). Although kinetic analysis indicates that the ecdysteroid UDP-glucosyltransferase (EGT) has broadly similar specificities for UDP-galactose and UDP-glucose (Evans and O'Reilly, 1998), there is evidence that *in vivo*, ecdysteroids are conjugated with galactose (O'Reilly *et al*., 1992; review: O'Reilly, 1995). EGT appears to be an oligomer of 3–5 subunits (Mr approx 56 kDa) and, surprisingly, E seems to be the optimal substrate, whereas 3DE is seven times less favorable, with 20E being conjugated very poorly, more than 34 times less readily than 3DE (Evans and O'Reilly, 1998). The physiological significance of this finding is uncertain. Analogous EGT enzymes and mechanisms have been established for the gypsy moth *Lymantria* nuclear polyhedrosis virus (Park *et al*., 1993; Burand *et al*., 1996), *Epiphyas postvittana* nucleopolyhedrovirus (Caradoc-Davies *et al*., 2001), and *Mamestra brassicae* multinucleocapsid nucleopolyhedrovirus (Clarke *et al*., 1996).

3.3.5.3.2.5. Neglected pathways? Given the huge diversity of insects, there is no reason to consider that metabolic pathways are restricted to those-described above. A few examples will illustrate that the story is not finished:

- Side chain cleavage between C_{20} and C_{22} resulting in the formation of poststerone was described long ago in *Bombyx* by Hikino *et al.* (1975); moreover, other experiments have demonstrated the ability of certain insects to produce C_{21} steroids from cholesterol in exocrine glands of aquatic beetles (Schildknecht, 1970) and also ovaries of *Manduca* (Thompson *et al*., 1985c), providing the evidence for a desmolase activity in some insect species at least. The fact that available [^{3}H]-ecdysteroids are labeled on the side chain has precluded further analysis of this problem, as the resulting poststerone would not be labeled and the radioactivity would become "volatile". This problem awaits reexamination when nuclear labeled ecdysteroids are available.
- Minor ecdysteroids have been isolated from *Bombyx* that bear an –OH group in positions 16β (Kamba *et al*., 2000b) or 23 (Kamba *et al*., 2000a), which indicates that during some developmental stages at least (here in adults) additional CYPs must be active.
- 14-Deoxy metabolites of E and 20E have been identified in *Gryllus bimaculatus* feces, and their formation is probably due to bacterial metabolism in the insect gut (Hoffmann *et al*., 1990).
- Labeling studies with *Drosophila* adults have shown very complex patterns that include some as yet unidentified metabolites (Grau and Lafont, 1994a, 1994b). Their chromatographic behavior is consistent with the presence of one additional –OH group in position 11β (?), but positions 16β and 23 as observed in *Bombyx* (see above) are also plausible.
- Finally, it should be noted that sulfate conjugates, although never isolated as endogenous metabolites, can be formed *in vitro* by insect/tissue homogenates incubated with ecdysteroids and

a sulfate donor (e.g., *Prodenia eridania* gut: Yang and Wilkinson, 1972; *Aedes togoi* whole larvae/pupae/adult: Shampengton and Wong, 1989; *Sarcophaga peregrina* fat body: Matsumoto *et al.*, 2003). In *Sarcophaga*, a 43 kDa protein responsible for this reaction was purified by affinity chromatography on a 3′-phosphoadenosine 5′-phosphate (PAP)-agarose column (Matsumoto *et al.*, 2003). However, the physiological significance of such activities *in vivo* in ecdysteroid metabolism remains to be firmly established, since it is conceivable that the activity observed might be ascribed to another primary activity, with low specificity for ecdysteroids.

3.3.6. Some Prospects for the Future

3.3.6.1. Evolution and Appearance of Ecdysteroids

Zooecdysteroids are not restricted to arthropods. Trace amounts of E, 20E, and other ecdysteroids have been detected by HPLC-immunoassays or GC-MS in annelids, nematodes, helminths, and mollusks (Spindler, 1988; Franke and Käuser, 1989; Barker *et al.*, 1990). More recently, ecdysteroids were also found in nemerteans (Okazaki *et al.*, 1997). It must be emphasized that at the moment there is no evidence that these animals are able to produce ecdysteroids themselves, as they seem to lack some key biosynthetic enzymes, a conclusion based on metabolic studies performed with biosynthetic intermediates efficiently converted in arthropods (**Table 8**, Lafont *et al.*, 1995; Lafont, 1997). An evolutionary scenario (see **Chapter 3.6**) has been proposed for the progressive appearance of enzymes, which resulted in an increasing number of hydroxyl groups on the sterol molecule (the last ones being the three mitochondrial enzymes, i.e., the 22-, 2-, and the 20-hydroxylases). A similar phylogenetic emergence seems to have operated in the case of vertebrate-type steroid hormones (Bolander, 1994).

Several findings, however, do not fit with this scheme. In particular, large amounts of ecdysteroids have been isolated from Zoanthids (e.g., Sturaro *et al.*, 1982; Suksamrarn *et al.*, 2002) and even from various sponges (Diop *et al.*, 1996; Cafieri *et al.*, 1998; Costantino *et al.*, 2000). The large amounts seem to preclude a hormonal role and rather fit with an allelochemical (protective) role, perhaps against predation, as in the case of pycnogonids (Tomaschko, 1995). In the case of Zoanthids, it is conceivable that ecdysteroids arise from their food (e.g., copepods), but this does not seem so evident in the case of sponges, which are expected to feed on bacteria, protozoa, or dissolved matter, none of

Table 8 Presence/absence of some key hydroxylases involved in ecdysteroid biosynthesis and/or metabolism in invertebrates

	Hydroxylations at positions				
Animals	*C2*	*C20*	*C22*	*C25*	*Other*
Arthropods					
Insects	+	+	+	+	16β, 23, 26
Crustaceans	+	+	+	+	26
Ticks	+	+	+	+	26
Myriapods	+	+	+	+	?
Nonarthropods					
Mollusks (Gastropods)	−	+	−	+	16β
Annelids					
Achaetes	−	+	−	+	16β
Polychaetes	−	+	−	+	26
Oligochaetes	−	−	−	+	18
Nematodes	−	?	?	?	?

?, does not mean that the reaction is absent, but only that there is at present no direct demonstration for it.
Lafont, R., Connat, J.-L., Delbecque, J.-P., Porcheron, P., Dauphin-Villemant, C., *et al.*, **1995**. Comparative studies on ecdysteroids. In: Ohnishi, E., Takahashi, S.Y., Sonobe, H. (Eds.), Recent Advances in Insect Biochemistry and Molecular Biology. University of Nagoya Press, Nagoya, pp. 45–91.

which are known to contain ecdysteroids. This awaits additional experiments to establish whether or not sponges are able to produce ecdysteroids themselves. It should be noted that sponges also contain an incredible number of steroids (Aiello *et al.*, 1999), including steroidal $\Delta^{4,7}$-3,6-diketones (Malorni *et al.*, 1978) or 4,6,8-triene-3-ones (Kobayashi *et al.*, 1992), which resemble putative ecdysteroid precursors (Grieneisen *et al.*, 1991; Grieneisen, 1994; Gilbert *et al.*, 2002). We expect that molecular approaches will allow a search for homologs of insect biosynthetic enzymes that will provide an adequate answer to this fundamental problem in comparative endocrinology.

3.3.6.2. Biosynthetic Pathway(s)

The last 2 years have resulted in a spectacular improvement of our knowledge of ecdysteroidogenic enzymes, thanks to the *Drosophila* Halloween mutants. Up to now, however, this has only led to the characterization of enzymes involved in already identified reactions (i.e., hydroxylations at positions 25, 2, 22, and 20), due to the expression of recombinant enzymes and the determination of their catalytic activity. Whether this approach will suffice in the identification of the remaining unknown reactions (the so-called "black box") is debatable. Subsequent to the conversion of C to 7dC by the 7,8-dehydrogenase present in the ER, oxidized early intermediates do not accumulate under normal conditions. In theory we may expect to increase their

concentrations by using one (or a combination) of the following strategies: (1) the use of mutants defective in one or several terminal enzymes; (2) the use of specific inhibitors of these enzymes (Burger *et al.*, 1988, 1989; Mauvais *et al.*, 1994); or (3) the use of subcellular fractions instead of whole cells/tissues in order to have only a few biosynthetic enzymes present in the preparation. An additional technical problem to overcome will be the instability of such intermediates. This problem has already been encountered when using a tritiated $\Delta^{4,7}$-3,6-diketone (Blais *et al.*, 1996) or α-5,6-epoxy7dC (Warren *et al.*, 1995). Furthermore, recent experiments (see Section 3.3.4.2.2) suggest that the long-hypothesized, rate-limiting, mitochondrial P_{450}-mediated "black box" oxidations of 7dC, leading to the Δ^4-diketol, cannot be catalyzed by a mitochondrial P450 enzyme and may not even occur in the mitochondria. Studies implicating the mitochondria in these novel transformations employed crude membrane fractions from *Manduca* prothoracic glands (Warren and Gilbert, 1996) and there was clear evidence for the co-sedimentation of a large proportion of the microsomes with the crude mitochondrial pellet, as has been observed with other insect prothoracic gland tissues (Kappler *et al.*, 1988). However, insect peroxisomes also normally sediment with the mitochondrial pellet (Kurisu *et al.*, 2003) and mammalian peroxisomes are known to participate in the oxidation of both steroids and fatty acids (Breitling *et al.*, 2001). Thus, it is possible that the oxidation of 7dC (or 3-oxo7dC) may instead occur in this membrane-bound subcellular organelle. In addition, the peroxisome has been shown to be a target of sterol carrier protein 2 (Schroeder *et al.*, 2000). Also, at least in *Spodoptera frugiperda* cells, the organelle has been shown to be devoid of catalase activity, a normal marker enzyme in mammalian peroxisomes that acts to destroy hydrogen peroxide (Kurisu *et al.*, 2003). If a normal synthesis of hydrogen peroxide is also observed in insect peroxisomes, then the result could be an ideal source of oxygen for the hypothesized black box oxidations (Gilbert *et al.*, 2002).

Up to now, the black box has been investigated through trial-and-error experiments analyzing the metabolic fate of radiolabeled putative intermediates, but this has mainly led to the elimination of several hypotheses (Grieneisen, 1994). In an alternative strategy, the so-called "isotope trap," steroidogenic organs would be incubated with a labeled early precursor in the presence of an excess of an unlabeled putative downstream intermediate. If the latter is indeed an intermediate (and is stable enough to survive the incubation), it is expected to trap radioactivity due to isotopic dilution and saturation of the subsequent steps. It could also be possible to use systems capable of producing larger amounts of ecdysteroids (other than what can be achieved through the tedious dissection of thousands of prothoracic glands or ring glands), e.g., perhaps embryos or suitable plant cell or organ cell cultures, which would allow the use of precursors labeled with stable isotopes (^{2}H, ^{13}C) and subsequent NMR analysis of terminal ecdysteroid products (Fujimoto *et al.*, 1997, 2000).

Thus, the problem of the early steps remains a very open question. In addition, it may well be that different biosynthetic pathways have been developed between insects and plants (Fujimoto *et al.*, 1989, 1997; Reixach *et al.*, 1999), and perhaps also within the diverse array of insects and other arthropods. Nevertheless, the black box reactions appear to be quite specific to arthropods (and maybe a few plants). As such, they make a very appealing target for the development of novel biochemical strategies for the control of insect pests.

3.3.6.3. Additional Functions for Ecdysteroids?

We have discussed that tissues other than prothoracic glands and gonads may produce ecdysteroids, possibly as autocrine factors (see Section 3.3.4.4.2). Whatever the biosynthetic site, this corresponds to an involvement of ecdysteroids in the control of development and reproduction processes. The recent discovery of large ecdysteroid concentrations in the defensive secretions of chrysomelid beetles (Laurent *et al.*, in press) suggests a possible allelochemical role of ecdysteroids in certain cases. The situation described in chrysomelid beetles is similar to that of the pycnogonids, where ecdysteroids have been demonstrated conclusively to represent an efficient protection against predation by crabs (Tomaschko, 1995). Another example is the phytoecdysteroid accumulation by many plant species, seen as a protection against phytophagous insects (Dinan, 2001). However, it should be emphasized that previous analyses of defensive secretions of Chrysomelid beetles allowed the isolation of 2,14,22-trideoxyecdysone 3-sophorose and other ecdysteroid related compounds lacking the Δ^7 bond (Pasteels *et al.*, 1994). Thus, the story of ecdysteroid biosynthesis could turn out to be considerably more complex than it already appears to be.

Acknowledgments

René Lafont wishes to thank Dr Jean-Pierre Girault for providing the materials of Figure 2 and 5. Huw Rees is grateful to the Biotechnology and Biological

Sciences Research Council and The Leverhulme Trust for financial support of work in his laboratory, Dr Hajime Takeuchi for preparing Figures 15–18 for this review, and Dr Lyndsay Davies for help with the manuscript. James Warren would like to thank Susan Whitfield for help in preparing Figures 12 and 14a. This work was supported in part by National Science Foundation Grant IBN0130825.

References

Addya, S., Anandatheerthavarada, H., Biswas, G., Bhagwat, S., Mullick, J., *et al.*, **1997**. Targeting of NH_2-terminal-processed microsomal protein to mitochondria: a novel pathway for the biogenesis of hepatic mitochondrial P450MT2. *J. Cell Biol. 139*, 589–599.

Adelung, D., Karlson, P., **1969**. Eine verbesserte, sehr empfindliche Methode zur biologischen Auswertung des Insektenhormones Ecdyson. *J. Insect Physiol. 15*, 1301–1307.

Agosin, M., Srivatsan, J., **1991**. Role of microsomal cytochrome P-450 in the formation of ecdysterone in larval house fly. *Comp. Biochem. Physiol. 99B*, 271–274.

Agosin, M., Srivatsan, J., Weirich, M., **1988**. On the intracellular localization of the ecdysone 20-monooxygenase in *Musca domestica*. *Arch. Insect Biochem. Physiol. 9*, 107–117.

Agrawal, O.P., Scheller, K., **1985**. An *in vivo* system has the advantages of an *in vitro* system as well: blowfly abdominal bags. *Naturwiss. 72*, 436–437.

Agui, N., **1973**. Quantitative bioassay for moulting hormone *in vitro*. *Appl. Entomol. Zool. 8*, 236–239.

Aiello, A., Fattorusso, E., Menna, M., **1999**. Steroids from sponges: recent reports. *Steroids 64*, 687–714.

Antonucci, R., Bernstein, S., Giancola, D., Sax, K., **1951**. $\Delta^{5,7}$ steroids. IX. The preparation of $\Delta^{4,7}$ and $\Delta^{4,7,9}$ 3-ketosteroid hormones. *J. Org. Chem. 16*, 1453–1457.

Awata, N., Morisaki, M., Ikekawa, N., **1975**. Carbon–carbon cleavage of fucosterol-24,28-oxide by cell-free extracts of silkworm *Bombyx mori*. *Biochem. Biophys. Res. Commun. 64*, 157–161.

Baker, K.D., Warren, J.T., Thummel, C.S., Gilbert, L.I., Mangelsdorf, D.J., **2000**. Transcriptional activation of the *Drosophila* ecdysone receptor by insect and plant ecdysteroids. *Insect Biochem. Mol. Biol. 30*, 1037–1043.

Barker, G.C., Chitwood, D.J., Rees, H.H., **1990**. Ecdysteroids in helminths and annelids. *Invert. Reprod. Devel. 18*, 1–11.

Báthori, M., **1998**. Purification and characterization of plant ecdysteroids of *Silene* species. *Trends Anal. Chem. 17*, 372–383.

Báthori, M., Blunden, G., Kalász, H., **2000**. Two-dimensional thin-layer chromatography of plant ecdysteroids. *Chromatographia 52*, 815–817.

Báthori, M., Kalász, H., **2001**. Separation methods for phytoecdysteroids. *LC-GC Europe 14*, 626–633.

Becker, E., Plagge, E., **1939**. Über das Pupariumbildung auslösende Hormon der Fliegen. *Biol. Zbl. 59*, 326.

Beckers, C., Emmerich, H., **1973**. Preparation of an ecdysone 6-carboxy-methoxyloxime complex with bovine serum albumin. *Naturwissenschaften 6*, 50.

Bergamasco, R., Horn, D.H.S., **1980**. The biological activities of ecdysteroids and ecdysteroid analogues. In: Hoffmann, J.A. (Ed.), Progress in Ecdysone Research. Elsevier/North-Holland, Amsterdam, p. 299.

Betsholtz, I.H., Wikvall, K., **1995**. Cytochrome P450 CYP27-catalyzed oxidation of C27-steroid into C27-acid. *J. Steroid Biochem. Mol. Biol. 55*, 115–119.

Beydon, P., Claret, J., Porcheron, P., Lafont, R., **1981**. Biosynthesis and inactivation of ecdysone during the pupal-adult development of the cabbage butterfly, *Pieris brassicae*. *Steroids 38*, 633–650.

Beydon, P., Girault, J.P., Lafont, R., **1987**. Ecdysone metabolism in *Pieris brassicae* during the feeding last larval instar. *Arch. Insect Biochem. Physiol. 4*, 139–149.

Beydon, P., Permana, A.D., Colardeau, J., Morinière, M., Lafont, R., **1989**. Ecdysteroids from developing eggs of *Pieris brassicae*. *Arch. Insect Biochem. Physiol. 11*, 1–11.

Bielby, C.R., Morgan, E.D., Wilson, I.D., **1986**. Gas chromatography of ecdysteroids as their trimethylsilyl ethers. *J. Chromatogr. 351*, 57–64.

Black, S.M., Harikrishna, J.A., Szklarz, G.D., Miller, W.L., **1994**. The mitochondrial environment is required for activity of the cholesterol side-chain cleavage enzyme, cytochrome P450scc. *Proc. Natl Acad. Sci. USA 91*, 7247–7251.

Blackford, M.J.P., Clarke, B.S., Dinan, L., **1997**. Distribution and metabolism of exogenous ecdysteroids in the Egyptian Cotton leafworm *Spodoptera littoralis* (Lepidoptera: Nocturidae). *Arch. Insect Biochem. Physiol. 34*, 329–346.

Blackford, M., Dinan, L., **1997a**. The tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) detoxifies ingested 20-hydroxyecdysone, but is susceptible to the ecdysteroid agonists RH-5849 and RH-5992. *Insect Biochem. Mol. Biol. 27*, 167–177.

Blackford, M., Dinan, L., **1997b**. The effects of ingested ecdysteroid agonists (20-hydroxyecdysone, RH5849 and RH5992) and an ecdysteroid antagonist (cucurbitacin B) on larval development of two polyphagous lepidopterans *Acherontia atropus* and *Lacanobia oleracea*. *Ent. Exper. Applic. 83*, 263–276.

Blackford, M.J.P., Dinan, L., **1997c**. The effects of ingested 20-hydroxyecdysone on the larvae of *Aglais urticae*, *Inachis io*, *Cynthia cardui* (Lepidoptera: Nymphalidae) and *Tyria jacobeae* (Lepidoptera: Arctiidae). *J. Insect Physiol. 43*, 315–327.

Blais, C., Dauphin-Villemant, C., Kovganko, N., Girault, J.-P., Descoins, C., Jr., *et al.*, **1996**. Evidence for the involvement of 3-oxo-Δ^4 intermediates in ecdysteroid biosynthesis. *Biochem. J. 320*, 413–419.

Blais, C., Lafont, R., **1984**. Ecdysteroid metabolism by soluble enzymes from an insect: metabolic relationship between 3β-hydroxy-, 3α-hydroxy-, and 3-oxo-ecdysteroids. *Hoppe-Seyler's Z. Physiol. Chem. 365*, 809–817.

Blais, C., Lafont, R., **1986**. Ecdysone 20-hydroxylation in imaginal wing discs of *Pieris brassicae* (Lepidoptera): correlations with ecdysone and 20-hydroxyecdysone titers in pupae. *Arch. Insect Biochem. Physiol. 3*, 501–512.

Böcking, D., Dauphin-Villemant, C., Sedlmeier, D., Blais, C., Lafont, R., **1993**. Ecdysteroid biosynthesis in molting glands of the crayfish *Orconectes limosus*: evidence for the synthesis of 3-dehydroecdysone by *in vitro* synthesis and conversion studies. *Insect Biochem. Mol. Biol. 23*, 57–63.

Böcking, D., Dauphin-Villemant, C., Toullec, J.-Y., Blais, C., Lafont, R., **1994**. Ecdysteroid formation from 25-hydroxycholesterol by arthropod molting glands *in vitro*. *C. R. Acad. Sci. Paris 317*, 891–898.

Bolander, F.F., **1994**. In: Molecular Endocrinology, 2nd edn. Academic Press, New York, p. 501.

Bollenbacher, W., Faux, A.F., Galbraith, N., Gilbert, L.I., Horn, D.H.S., *et al.*, **1979**. *In vitro* metabolism of possible ecdysone precursors by prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Steroids 34*, 509–526.

Bollenbacher, W., Galbraith, N., Horn, D.H.S., Gilbert, L.I., **1977a**. *In vitro* metabolism of 3β-hydroxy-, and 3β-,14α-hydroxy-[3α-^{3}H]-5β-cholest-7-en-6-one by the prothoracic glands of *Manduca sexta*. *Steroids 29*, 47–63.

Bollenbacher, W., Smith, S., Wielgus, J., Gilbert, L.I., **1977b**. Evidence for an α-ecdysone cytochrome P-450 mixed function oxidase in insect fat body mitochondria. *Nature 268*, 660–663.

Bondar, O.P., Saad, L.M., Shatursky, Ya.P., Kholodova, Yu.D., **1993**. Comparative characteristics of color reactions of ecdysteroids. *Ukr. Biokhim. Zh. 65(4)*, 83–87.

Borst, D.W., Engelmann, F., **1974**. *In vitro* secretion of α-ecdysone by prothoracic glands of a hemimetabolous insect, *Leucophaea maderae* (Blattaria). *J. Exp. Zool. 189*, 413–419.

Borst, D.W., O'Connor, J.D., **1972**. Arthropod molting hormone: Radioimmune assay. *Science 178*, 418–419.

Borst, D.W., O'Connor, J.D., **1974**. Trace analysis of ecdysones by gas-liquid chromatography, radioimmunoassay and bioassay. *Steroids 24*, 637–656.

Bownes, M., **1992**. Why is there sequence similarity between insect yolk proteins and vertebrate lipases? *J. Lipid Res. 33*, 677–690.

Bownes, M., Shirras, A., Blair, M., Collins, J., Coulson, A., **1988**. Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *Proc. Natl Acad. Sci. USA 85*, 1554–1557.

Breitling, R., Marijanovic, Z., Perovic, D., Adamski, J., **2001**. Evolution of 17 beta-HSD type 4, a multifunctional protein of beta-oxidation. *Mol. Cell. Endocrinol. 171*, 205–210.

Brown, G.D., **1998**. The biosynthesis of steroids and triterpenoids. *Nat. Product Rep. 15*, 653–696.

Bückmann, D., **1984**. The phylogeny of hormones and hormonal systems. *Nova Acta Leopoldina NF 56*, 437–452.

Burand, J.P., Park, E.J., Kelly, T.J., **1996**. Dependence of ecdysteroid metabolism and development in host larvae on the time of baculovirus infection and the activity of the UDP-glucosyltransferase gene. *Insect Biochem. Mol. Biol. 26*, 845–852.

Burger, A., Colobert, F., Hétne, C., Luu, B., **1988**. Acetylenic cholesteryl derivatives as irreversible inhibitors of ecdysone biosynthesis. *Tetrahedron 44*, 1141–1152.

Burger, A., Roussel, J.-P., Hétne, C., Hoffmann, J.A., Luu, B., **1989**. Allenic cholesteryl derivatives as inhibitors of ecdysone biosynthesis. *Tetrahedron 45*, 155–164.

Butenandt, A., Karlson, P., **1954**. Über die Isolierung eines Metamorphosehormons der Insekten in kristallisierter Form. *Z. Naturforsch. 9b*, 389–391.

Cafieri, F., Fattorusso, E., Taglialatela-Scafati, O., **1998**. Novel bromopyrrole alkaloids from the sponge Agelas dispar. *J. Nat. Prod. 61*, 122–125.

Capovilla, M., Eldon, E.D., Pirrotta, V., **1992**. The *giant* gene of *Drosophila* encodes a b-ZIP DNA-binding protein that regulates the expression of other segmentation gap genes. *Development 114*, 99–112.

Caradoc-Davies, K., Graves, S., O'Reilly, D.R., Evans, O.P., Ward, V.K., **2001**. Identification and *in vivo* characterization of the *Epiphyas postvittana* nucleopolyhedrovirus ecdysteroid UDP-glucosyltransferase. *Virus Genes 22*, 255–264.

Cassier, P., Baehr, J.-C., Caruelle, J.-P., Porcheron, P., Claret, J., **1980**. The integument and ecdysteroids: *in vivo* and *in vitro* studies. In: Hoffmann, J.A. (Ed.), Progress in Ecdysone Research. Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 235–246.

Chávez, V.M., Marqués, G., Delbecque, J.-P., Kobayashi, K., Hollingsworth, M., *et al.*, **2000**. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126.

Chen, J.-H., Hara, T., Fisher, M.J., Rees, H.H., **1994a**. Immunological analysis of developmental changes in ecdysone 20-monooxygenase expression in the cotton leafworm, *Spodoptera littoralis*. *Biochem. J. 299*, 711–717.

Chen, J.-H., Kabbouh, M., Fisher, M.J., Rees, H.H., **1994b**. Induction of an inactivation pathway for ecdysteroids in larvae of the cotton leafworm, *Spodoptera littoralis*. *Biochem. J. 301*, 89–95.

Chen, J.-H., Powls, R., Rees, H.H., Wilkinson, M.C., **1999a**. Purification of ecdysone oxidase and 3-dehydroecdysone 3β-reductase from the cotton leafworm, *Spodoptera littoralis*. *Insect Biochem. Mol. Biol. 29*, 899–908.

Chen, J.H., Turner, P.C., Rees, H.H., **1999b**. Molecular cloning and characterization of hemolymph 3-dehydroecdysone 3β-reductase from the cotton leafworm,

Spodoptera littoralis – a new member of the third superfamily of oxidoreductases. *J. Biol. Chem. 274*, 10551–10556.

Chen, J.-H., Webb, T.J., Powls, R., Rees, H.H., **1996**. Purification and characterization of hemolymph 3-dehydroecdysone 3β-reductase in relation to ecdysteroid biosynthesis in the cotton leafworm *Spodoptera littoralis*. *Eur. J. Biochem. 242*, 394–401.

Cherbas, L., Yonge, C.D., Cherbas, P., Williams, C.M., **1980**. The morphological response of K_c-H cells to ecdysteroids: hormonal specificity. *Wilhelm Roux' Arch. Devel. Biol. 189*, 1–15.

Chino, H., Sakurai, S., Ohtaki, T., Ikekawa, N., Miyazaki, H., *et al.*, **1974**. Biosynthesis of α-ecdysone by prothoracic glands *in vitro*. *Science 183*, 529–530.

Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Yuan, H., *et al.*, **1999**. The *Arabidopsis dwarf1* mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. *Plant Physiol. 119*, 897–907.

Clark, A.J., Bloch, K., **1959**. Conversion of ergosterol to 22-dehydrocholesterol in *Blatella germanica*. *J. Biol. Chem. 234*, 2589–2593.

Clarke, E.E., Tristem, M., Cory, J.S., O'Reilly, D.R., **1996**. Characterization of the ecdysteroid UDP-glucosyltransferase gene from *Mamestra brassicae* nucleopolyhedrovirus. *J. Gen. Virol. 77*, 2865–2871.

Clarke, G.S., Baldwin, B.C., Rees, H.H., **1985**. Inhibition of the fucosterol-24(28)-epoxide cleavage enzyme of sitosterol dealkylation in *Spodoptera littoralis* larvae. *Pestic. Biochem. Physiol. 24*, 220–230.

Clayton, R.B., **1964**. The utilization of sterols by insects. *J. Lipid Res. 5*, 3–19.

Clément, C., Bradbrook, D.A., Lafont, R., Dinan, L.N., **1993**. Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem. Mol. Biol. 23*, 187–193.

Clément, C., Dinan, L.N., **1991**. Development of an assay for ecdysteroid-like and anti-ecdysteroid activities in plants. In: Hrdy, I. (Ed.), Insect Chemical Ecology. Academia Prague and SBP Academic Publishers, The Hague, pp. 221–226.

Costantino, V., Dell'Aversano, C., Fattorusso, E., Mangoni, A., **2000**. Ecdysteroids from the Caribbean sponge *Iotrochota birotulata*. *Steroids 65*, 138–142.

Crosby, T., Evershed, R.P., Lewis, D., Wigglesworth, K.P., Rees, H.H., **1986a**. Identification of ecdysone 22-long-chain fatty acyl esters in newly laid eggs of the cattle tick *Boophilus microplus*. *Biochem. J. 240*, 131–138.

Crosby, T., Wigglesworth, K.P., Lewis, D., Rees, H.H., **1986b**. Moulting hormones in the development of the cattle tick, *Boophilus microplus*. In: Borovsky, D., Spielman, A. (Eds.), Proceedings of the Vero Beach Symposium; Host Regulated Developmental Mechanisms in Vector Arthropods. University of Florida, Florida, pp. 37–45.

Cymborowski, B., **1989**. Bioassay of ecdysteroids. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 144–149.

Darvas, B., Rees, H.H., Hoggard, N., **1993**. Ecdysone 20-monooxygenase systems in flesh-flies (Diptera: Sarcophagidae), *Neobellieria bullata* and *Parasarcophaga argyrostoma*. *Comp. Biochem. Physiol. 105B*, 765–773.

Darvas, B., Rees, H.H., Hoggard, N., El-Din, M.H.T., Kuwano, E., *et al.*, **1992**. Cytochrome P-450 inducers and inhibitors interfering with ecdysone 20-monooxygenases and their activities during postembryonic development of *Neobellieria bullata* Parker. *Pest. Sci. 36*, 135–142.

Dauphin-Villemant, C., Blais, C., Lafont, R., **1998**. Towards the elucidation of the ecdysteroid biosynthetic pathway. *Ann. NY Acad. Sci. 839*, 306–310.

Dauphin-Villemant, C., Böcking, D., Blais, C., Toullec, J.-Y., Lafont, R., **1997**. Involvement of a 3β-hydroxysteroid dehydrogenase in ecdysteroid biosynthesis. *Mol. Cell. Endocrinol. 128*, 139–149.

Dauphin-Villemant, C., Böcking, D., Sedlmeier, D., **1995**. Regulation of ecdysteroidogenesis in crayfish molting glands: involvement of protein synthesis. *Mol. Cell. Endocrinol. 109*, 97–103.

Dauphin-Villemant, C., Böcking, D., Tom, M., Maïbèche, M., Lafont, R., **1999**. Cloning of a novel cytochrome P-450 (CYP4C15) differentially expressed in the steroidogenic glands of an arthropod. *Biochem. Biophy. Res. Commun. 264*, 413–418.

Davies, T.G., Dinan, L.N., Lockley, W.J.S., Rees, H.H., Goodwin, T.W., **1981**. Formation of the A/B *cis* ring junction of ecdysteroids in the locust, *Schistocerca gregaria*. *Biochem. J. 194*, 53–62.

Davis, P., Lafont, R., Large, T., Morgan, E.D., Wilson, I.D., **1993**. Micellar capillary electrophoresis of the ecdysteroids. *Chromatographia 37*, 37–42.

Defaye, G., Mornier, N., Guidicelli, C., Chambaz, E.M., **1982**. Phosphorylation of purified mitochondrial cytochromes P-450 (cholesterol desmolase and 11β-hydroxylase) from bovine adrenal cortex. *Mol. Cell. Endocrinol. 27*, 157–168.

Delbecque, J.P., Weidner, K., Hoffmann, K.-H., **1990**. Alternative sites for ecdysteroid production in insects. *Invert. Reprod. Devel. 18*, 29–42.

Delbecque, J.-P., Beydon, P., Chapuis, L., Corio-Costet, M.-F., **1995**. *In vitro* incorporation of radiolabeled cholesterol and mevalonic acid into ecdysteroid by hairy root cultures of a Plant, Serratula tinctonia. *Eur. J. Entomol. 92*, 301–307.

Dinan, L., **1985**. Ecdysteroid receptors in a tumorous blood cell line of *Drosophila melanogaster. Arch. Insect Biochem. Physiol.* 2, 295–317.

Dinan, L., **1988**. The chemical synthesis of ecdysone 22-long-chain fatty acyl esters in high yield. *J. Steroid Biochem. 31*, 237–245.

Dinan, L., **1989**. Ecdysteroid structure and hormonal activity. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 345–354.

Dinan, L., **1997**. Ecdysteroids in adults and eggs of the house cricket, *Acheta domesticus* (Orthoptera: Gryllidae). *Comp. Biochem. Physiol. 116B*, 129–135.

Dinan, L., **2001**. Phytoecdysteroids: biological aspects. *Phytochemistry 57*, 325–339.

Dinan, L.N., Donnahey, P.L., Rees, H.H., Goodwin, T.W., **1981**. High-performance liquid chromatography of ecdysteroids and their 3-epi, 3-dehydro and 26-hydroxy derivatives. *J. Chromatogr. 205*, 139–145.

Dinan, L., Hormann, R.E., Fujimoto, T., **1999**. An extensive ecdysteroid CoMFA. *J. Comp. Aid. Mol. Design 13*, 185–207.

Dinan, L.N., Rees, H.H., **1978**. Preparation of 3-epi-ecdysone and 3-epi-20-hydroxyecdysone. *Steroids 32*, 629–638.

Dinan, L.N., Rees, H.H., **1981a**. The identification and titers of conjugated and free ecdysteroids in developing ovaries and newly-laid eggs of S*chistocerca gregaria*. *J. Insect Physiol. 27*, 51–58.

Dinan, L.N., Rees, H.H., **1981b**. Incorporation *in vivo* of [4-^{14}C]-cholesterol into the conjugated ecdysteroids in ovaries and eggs of *Schistocerca gregaria*. *Insect Biochem. 11*, 255–265.

Diop, M., Samb, A., Costantino, V., Fattorusso, E., Mangoni, A., **1996**. A new iodinated metabolite and a new alkyl sulfate from the Senegalese sponge *Ptilocaulis spiculifer*. *J. Nat. Prod. 59*, 271–272.

Dollé, F., Hétru, C., Roussel, J.P., Rousseau, B., Sobrio, F., *et al.*, **1991**. Synthesis of a tritiated 3-dehydroecdysteroid putative precursor of ecdysteroid biosynthesis in *Locusta migratoria*. *Tetrahedron 47*, 7067–7080.

Dorn, A., Bishoff, S.T., Gilbert, L.I., **1987**. An incremental analysis of the embryonic development of the tobacco hornworm, *Manduca sexta*. *Int. J. Invert. Reprod. Devel. 11*, 137–158.

Dyer, D.H., Lovell, S., Thoden, J.B., Holden, H.M., Rayment, I., *et al.*, **2003**. The structural determination of an insect sterol carrier protein-2 with a ligand bound C16 fatty acid at 1.35 Å resolution. *J. Biol. Chem. 278*, 39085–39091.

Eichenberger, W., **1977**. Steryl glycosides and acylated steryl glycosides. In: Tevini, M., Lichtenthaler, H.K. (Eds.), Lipids and Lipid Polymers in Higher Plants. Springer-Verlag, Berlin, pp. 169–182.

Espig, W., Thiry, E., Hoffman, K., **1989**. Ecdysteroids during ovarian development and embryogenesis in the cricket, *Gryllus bimaculatus* de Greer. *Invert. Reprod. Devel. 15*, 143–154.

Evans, O.P., O'Reilly, D.R., **1998**. Purification and kinetic analysis of a baculovirus ecdysteroid UDP-glucosyltransferase. *Biochem. J. 330*, 1265–1270.

Evershed, R.P., Kabbouh, M., Prescott, M.C., Maggs, J.L., Rees, H.H., **1990**. Current status and recent advances in the chromatography and mass spectrometry of ecdysteroids. In: McCaffery, A.R., Wilson, I.D. (Eds.), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, London, p. 103.

Evershed, R.P., Mercer, J.G., Rees, H.H., **1987**. Capillary gas chromatography – mass spectrometry of ecdysteroids. *J. Chromatogr. 390*, 357–369.

Evershed, R.P., Prescott, M.C., Kabbouh, M., Rees, H.H., **1993**. High-performance liquid chromatography/mass spectrometry with thermospray ionization of free ecdysteroids. *Rapid Commun. Mass Spect. 7*, 477–481.

Feldlaufer, M.F., **1989**. Diversity of molting hormones in insects. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 308–312.

Feldlaufer, M.F., Herbert, E.W., Jr., Svoboda, J.A., Thompson, M.J., **1986**. Biosynthesis of makisterone A and 20-hydroxyecdysone from labeled sterols in the honey bee, *Apis mellifera*. *Arch. Insect Biochem. Physiol. 3*, 415–421.

Feldlaufer, M.F., Svoboda, J.A., Thompson, M.J., Wilzer, K.R., **1988**. Fate of maternally-acquired ecdysteroids in unfertilized eggs of *Manduca sexta*. *Insect Biochem. 18*, 219–221.

Feldlaufer, M.F., Weirich, G.F., Imberski, R.B., Svoboda, J.A., **1995**. Ecdysteroid production in *Drosophila* reared on defined diets. *Insect Biochem. Mol. Biol. 25*, 709–712.

Feldlaufer, M.F., Weirich, G.F., Lusby, W.R., Svoboda, J.A., **1991**. Makisterone C: a 29-carbon ecdysteroid from developing embryos of the cotton stainer bug, *Dysdercus fasciatus*. *Arch. Insect Biochem. Physiol. 18*, 71–79.

Feyereisen, R., Durst, F., **1978**. Ecdysterone biosynthesis: a microsomal cytochrome P-450 linked ecdysone 20-monooxygenase from tissues of the African migratory locust. *Eur. J. Biochem. 88*, 37–47.

Feyereisen, R., Durst, F., **1980**. Development of microsomal cytochrome P-450 monooxygenases during the last larval instar of the locust, *Locusta migratoria*: correlation with the hemolymph 20-hydroxyecdysone titer. *Mol. Cell. Endocrinol. 20*, 157–169.

Fournier, B., Radallah, D., **1988**. Ecdysteroids in *Carausius morosus* eggs during embryonic development. *Arch. Insect Biochem. Physiol. 7*, 211–224.

Fraenkel, G., **1935**. A hormone causing pupation in the blowfly, *Calliphora erythrocephala*. *Proc. R. Soc. Lond. B 118*, 1–12.

Fraenkel, G., Zdarek, J., **1970**. The evaluation of the "*Calliphora* test" as an assay for ecdysone. *Biol. Bull. 139*, 138–150.

Franke, S., Käuser, G., **1989**. Occurrence and hormonal role of ecdysteroids in non-Arthropods. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 296–307.

Freeman, M.R., Dobritsa, A., Gaines, P., Segraves, W.A., Carlson, J.R., **1999**. The *dare* gene: steroid hormone production, olfactory behaviour, and neural degeneration in *Drosophila*. *Development 126*, 4591–4602.

Frew, J.G., **1928**. A technique for the cultivation of insect tissues. *Br. J. Exp. Biol. 6*, 1–11.

Fujimoto, Y., Hiramoto, M., Kakinuma, K., Ikekawa, N., **1989**. Elimination of C6-hydrogen during the formation of ecdysteroids from cholesterol in *Locusta migratoria* ovaries. *Steroids 53*, 477–485.

Fujimoto, Y., Miyasaka, S., Ikeda, T., Ikekawa, N., Ohnishi, E., *et al.*, **1985a**. An unusual ecdysteroid, (20S)-cholesta-7,14-diene-3β,5α,6α,20,25-pentaol

(bombycosterol) from the ovaries of the silkworm, *Bombyx mori*. *J. Chem. Soc., Chem. Commun.* 10–12.

Fujimoto, Y., Morisaki, M., Ikekawa, M., **1985b**. Enzymatic dealkylation of phytosterols in insects. *Meth. Enzymol. 111*, 346–352.

Fujimoto, Y., Kushiro, T., Nakamura, K., **1997**. Biosynthesis of 20-hydroxyecdysone in *Ajuga* hairy roots: hydrogen migration from c-6 to c-5 during *cis*-A/B ring formation. *Tetrahedron Letters 38*, 2697–2700.

Fujimoto, Y., Ohyama, K., Nomura, K., Hyodo, R., Takahashi, K., *et al.*, **2000**. Biosynthesis of sterols and ecdysteroids in Ajuga hairy roots. *Lipids 35*, 279–288.

Fukuda, S., **1940**. Induction of pupation in silkworm by transplanting the prothoracic gland. *Proc. Imperial Acad. (Tokyo) 16*, 417–420.

Gallegos, A.M., Atshaves, B.P., Storey, S.M., Starodub, O., Petrescu, A.D., *et al.*, **2001**. Gene structure, intracellular localization, and functional roles of the sterol carrier protein-2. *Progr. Lipid Res. 40*, 498–563.

Gande, A.R., Morgan, E.D., Wilson, I.D., **1979**. Ecdysteroid levels throughout the life cycle of the desert locust, *Schistocerca gregaria*. *J. Insect Physiol. 25*, 669–675.

Garen, A., Kaurar, L., Lepesant, J.-A., **1977**. Roles of ecdysone in Drosophila development. *Proc. Natl. Acad. Sci. USA 74*, 5099–5103.

Gelman, D.B., De Milo, A.B., Thyagaraja, B.S., Kelly, T.J., Masler, E.P., *et al.*, **1991**. 3-Oxoecdysteroid 3β-reductase in various organs of the European corn borer, *Ostrinia nubilalis* (Hubner). *Arch. Insect Biochem. Physiol. 17*, 93–106.

Gersch, M., **1979**. Molting of insects without molting gland: results with larvae of *Periplaneta americana*. *Experientia 33*, 228–230.

Gibson, J.M., Isaac, R.E., Dinan, L.N., Rees, H.H., **1984**. Metabolism of [^{3}H] ecdysone in *Schistocerca gregaria*; formation of ecdysteroid acids together with free and phosphorylated ecdysteroid acetates. *Arch. Insect Biochem. Physiol. 1*, 385–407.

Giesen, K., Lammel, U., Langehans, D., Krukkert, K., Bunse, I., *et al.*, **2003**. Regulation of glial cell number and differentiation by ecdysone and Fos signaling. *Mech. Devel. 120*, 401–413.

Gilbert, L.I., Rybczynski, R., Warren, J.T., **2002**. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol. 47*, 883–916.

Gilgan, M.W., Zinck, M.E., **1972**. Estimation of ecdysterone from sulphuric acid-induced fluorescence. *Steroids 20*, 95–104.

Girault, J.-P., **1998**. Determination of ecdysteroid structure by 1D and 2D NMR. *Russian J. Plant Physiol. 45*, 306–309.

Girault, J.-P., Lafont, R., **1988**. The complete ^{1}H-NMR assignment of ecdysone and 20-hydroxyecdysone. *J. Insect Physiol. 34*, 701–706.

Girault, J.P., Blais, C., Beydon, P., Rolando, C., Lafont, R., **1989**. Synthesis and N.M.R. study of 3-dehydroecdysteroids. *Arch. Insect Biochem. Physiol. 10*, 199–213.

Glitho, I., Delbecque, J.-P., Delachambre, J., **1979**. Prothoracic gland involution related to moulting hormone levels during the metamorphosis of *Tenebrio molitor* L. *J. Insect Physiol. 25*, 187–191.

Goltzené, F., Lagueux, M., Charlet, M., Hoffmann, J.A., **1978**. The follicle cell epithelium of maturing ovaries of *Locusta migratoria*: a new biosynthetic tissue for ecdysone. *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1427–1434.

Goodnight, K.C., Kircher, H.W., **1971**. Metabolism of lathosterol by *Drosophila melanogaster*. *Lipids 6*, 166–169.

Goodwin, C.D., Cooper, B.W., Margolis, S., **1982**. Rat liver cholesterol 7α-hydroxylase. Modulation of enzyme activity by changes in phosphorylation state. *J. Biol. Chem. 257*, 4469–4472.

Goodwin, T.W., Horn, D.H.S., Karlson, P., Koolman, J., Nakanishi, K., *et al.*, **1978**. Ecdysteroids: a new generic term. *Nature 272*, 111.

Grau, V., Gutzeit, H., **1990**. Asymmetrically distributed ecdysteroid- related antigens in follicles and young embryos of *Drosophila melanogaster*. Roux's. *Arch. Dev. Biol. 198*, 295–302.

Grau, V., Lafont, R., **1994a**. Metabolism of ecdysone and 20-hydroxyecdysone in adult *Drosophila melanogaster*. *Insect Biochem. Mol. Biol. 24*, 49–58.

Grau, V., Lafont, R., **1994b**. The distribution of ecdysone metabolites within the body of adult *Drosophila melanogaster* females and their sites of production. *J. Insect Physiol. 40*, 87–96.

Grau, V., Pis, J., Lafont, R., **1995**. Ovary-specific interaction of ecdysone 22-phosphate with proteins in adult *Drosophila melanogaster* (Diptera: Drosophilidae). *Eur. J. Entomol. 92*, 189–196.

Greenwood, D.R., Rees, H.H., **1984**. Ecdysone 20-monooxygenase in the desert locust, *Schistocerca gregaria*. *Biochem. J. 223*, 837–847.

Grieneisen, M.L., **1994**. Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. *Insect Biochem. Mol. Biol. 24*, 115–132.

Grieneisen, M., Warren, J.T., Gilbert, L.I., **1993**. Early steps in ecdysteroid biosynthesis: evidence for the involvement of cytochrome P-450 enzymes. *Insect Biochem. 23*, 13–23.

Grieneisen, M., Warren, J.T., Sakurai, S., Gilbert, L.I., **1991**. A putative route to ecdysteroids: metabolism of cholesterol in vitro by mildly disrupted prothoracic glands of *Manduca sexta*. *Insect Biochem. 21*, 41–51.

Grunwald, C., **1980**. Steroids. In: Bell, E.A., Charlwood, B.V. (Eds.), Encyclopedia of Plant Physiology, New Series, vol. 8. Secondary Plant Products. Springer Verlag, Berlin, p. 221.

Haag, T., Meister, M.-F., Hétru, C., Kappler, C., Nakatani, Y., *et al.*, **1987**. Synthesis of a labelled putative precursor of ecdysone. II. [$^3H_4$]3β-hydroxy-5β-cholest-7-ene-6-one: critical re-evaluation of its role in *Locusta migratoria*. *Insect Biochem. 17*, 291–301.

Hagedorn, H.H., **1985**. The role of ecdysteroids in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 205–262.

Hagedorn, H.H., O'Connor, J.D., Fuchs, M.S., Sage, B., Schlaeger, D.A., *et al.*, **1975**. The ovary as a source of α-ecdysone in an adult mosquito. *Proc. Natl Acad. Sci. USA 72*, 3255–3259.

Halliday, W.R., Farnsworth, D.E., Feyereisen, R., **1986**. Hemolymph ecdysteroid titer and midgut ecdysone 20-monooxygenase activity during the last larval stage of *Diploptera punctata*. *Insect Biochem. 16*, 627–634.

Hampshire, F., Horn, D.H.S., **1966**. Structure of crustecdysone, a crustacean moulting hormone. *J. Chem. Soc. Chem. Commun.* 37–38.

Harvie, P.D., Filippova, M., Bryant, P. J., **1998**. Genes expressed in the Ring Gland, the major Endocrine Organ of *Drosophila melanogastere*. *Genetics 149*, 217–231.

Heed, W.B., Kircher, H.W., **1965**. Unique sterol in the ecology and nutrition of *Drosophila pachea*. *Science 149*, 758–761.

Hellou, J., Banoub, J., Gentil, E., Taylor, D.M., O'Keefe, P.G., **1994**. Electrospray tandem mass spectrometry of ecdysteroid moulting hormones. *Spectroscopy 12*, 43–53.

Hétru, C., Fraisse, D., Tabet, J.-C., Luu, B., **1984**. Confirmation, par spectrométrie de masse en mode FAB, de l'identification des esters d'AMP d'ecdystéroïdes dans les oeufs du Criquet migrateur. *C. R. Acad. Sci. Paris (Série II) 299*, 429–432.

Hétru, C., Lagueux, M., Luu, B., Hoffmann, J.A., **1978**. Adult ovaries of *Locusta migratoria* contain the sequence of biosynthetic intermediates for ecdysone. *Life Sci. 22*, 2141–2154.

Hétru, C., Luu, B., Hoffmann, J.A., **1985**. Ecdysone conjugates: isolation and identification. *Methods Enzymol. 111*, 411–419.

Hétru, C., Kappler, C., Hoffmann, J.A., Nearn, R., Luu, B., *et al.*, **1982**. The biosynthetic pathway of ecdysone: studies with vitellogenic ovaries of *Locusta migratoria* (Orthoptera). *Mol. Cell. Endocrinol. 26*, 51–81.

Hikino, H., Hikino, Y., Takemoto, T., **1969**. Rubrosterone, a metabolite of insect metamorphosing substance from *Achyranthes rubrofusca*: synthesis. *Tetrahedron 25*, 3389–3394.

Hikino, H., Oizumi, Y., Takemoto, T., **1975**. Steroid metabolism in *Bombyx mori*, I. Catabolism of ponasterone A and ecdysterone in *Bombyx mori*. *Hoppe-Seyler's Z. Physiol. Chem. 356*, 309–314.

Hiramoto, M., Fujimoto, Y., Kakinuma, K., Ikekawa, N., Ohnishi, E., **1988**. Ecdysteroid conjugates in the ovaries of the silkworm, *Bombyx mori*: 3-phosphates of 2,22-dideoxy-20-hydroxyecdysone and bombycosterol. *Experientia 44*, 823–825.

Hirn, M.L., Delaage, M.A., **1980**. Radioimmunological approaches to the quantification of ecdysteroids. In: Hoffmann, J.A. (Ed.), Progress in Ecdysone Research. Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 69–82.

Hocks, P., Wiechert, R., **1966**. 20-Hydroxy-ecdyson, isoliert aus Insekten. *Tetrahedron Lett. 26*, 2989–2993.

Hoffmann, J.A., Hétru, C., **1983**. Ecdysone. In: Downer, R.G.H., Laufer, H. (Eds.), Invertebrate Endocrinology, vol 1: Endocrinology of Insects. Alan R. Liss, New York, pp. 65–88.

Hoffmann, J.A., Lagueux, M., **1985**. Endocrine aspects of embryonic development in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 1. Pergamon Press, Oxford, pp. 435–460.

Hoffmann, J. A., Koolman, J., Beyler, C., **1977**. Rôle des glandes prothoraciques dans la production d'ecdysone au cours du dernier stade larraire de *Locusta migratoria L.C.R. Acad. Sci. Paris, Sér. D. 280*, 733–737.

Hoffmann, K.H., Bulenda, D., Thiry, E., Schmid, E., **1985**. Apolar ecdysteroid esters in adult female crickets, *Gryllus bimaculatus*. *Life Sci. 37*, 185–192.

Hoffmann, K.H., Thiry, E., Lafont, R., **1990**. 14-Deoxyecdysteroids in an insect (*Gryllus bimaculatus*). *Z. Naturforsch. 45C*, 703–708.

Hoffmann, K.H., Wagemann, M., **1994**. Age dependency and tissue distribution of ecdysteroids in adult male crickets, *Gryllus bimaculatus* De Geer (Ensifera, Gryllidae). *Comp. Biochem. Physiol. A 109*, 293–302.

Hoggard, N., Rees, H.H., **1988**. Reversible activation–inactivation of mitochondrial ecdysone 20-monooxygenase: a possible role for phosphorylation–dephosphorylation. *J. Insect Physiol. 34*, 647–653.

Hoggard, N., Fisher, M.J., Rees, H.H., **1989**. Possible role for covalent modification in the reversible activation of ecdysone 20-monooxygenase activity. *Arch. Insect Biochem. Physiol. 10*, 241–253.

Horike, N., Sonobe, H., **1999**. Ecdysone 20-monooxygenase in eggs of the silkworm, *Bombyx mori*; enzymatic properties and developmental changes. *Arch. Insect Biochem. Physiol. 41*, 9–17.

Horike, N., Takemori, H., Nonaka, Y., Sonobe, H., Okamoto, M., **2000**. Molecular cloning of NADPH-cytochrome P450 oxidoreductase from silkworm eggs. Its involvement in 20-hydroxyecdysone biosynthesis during embryonic development. *Eur. J. Biochem. 267*, 6914–6920.

Horn, D.H.S., **1971**. The ecdysones. In: Jacobson, M., Crosby, D.G. (Eds.), Naturally Occurring Insecticides. Marcel Dekker, New York, pp. 333–459.

Horn, D.H.S., Bergamasco, R., **1985**. Chemistry of ecdysteroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 7. Pergamon Press, Oxford, pp. 185–248.

Hovemann, B.T., Sehlmeyer, F., Malz, J., **1997**. *Drosophila melanogaster* NADPH-cytochrome P450 oxidoreductase: pronounced expression in antennae may be related to odorant clearance. *Gene 189*, 213–219.

Huber, R., Hoppe, W., **1965**. Zur Chemie des Ecdysons. VII. Die Kristall-und Molekülstruktur-analyse des

Insektenverpuppungshormons Ecdyson mit des automatisierten Faltmolekülmethode. *Chem. Ber. 98*, 2403–2424.

Ikekawa, N., Ikeda, T., Mizuno, E., Ohnishi, E., Sakurai, S., **1980**. Isolation of a new ecdysteroid, 2,22-dideoxy-20-hydroxyecdysone from the ovaries of the silkworm *Bombyx mori*. *J. Chem. Soc. Chem. Commun.* 448–449.

Ikekawa, N., Hattori, F., Rubio-Lightbourn, J., Miyazaki, H., Ishibashi, M., *et al.*, **1972**. Gas chromatographic separation of phytoecdysteroids. *J. Chromatogr. Sci. 10*, 233–242.

Isaac, R.E., Desmond, H.P., Rees, H.H., **1984**. Isolation and identification of 3-acetyl-ecdysone 2-phosphate, a metabolite of ecdysone, from developing eggs of *Schistocerca gregaria*. *Biochem. J. 217*, 239–243.

Isaac, R.E., Milner, N.P., Rees, H.H., **1983**. Identification of ecdysonoic acid and 20-hydroxyecdysonoic acid isolated from developing eggs of *Schistocerca gregaria* and pupae of *Spodoptera littoralis*. *Biochem. J. 213*, 261–265.

Isaac, R.E., Rees, H.S., **1984**. Isolation and identification of ecdysteroid phosphates and acetylecdysteroid phosphates from developing eggs of the locust, *Schistocerca gregaria*. *Biochem. J. 231*, 459–464.

Isaac, R.E., Rees, H.H., Goodwin, T.W., **1981a**. Isolation of 2-deoxy-20-hydroxyecdysone and 3-epi-2-deoxyecdysone from eggs of the desert locust, *Schistocerca gregaria* during embryogenesis. *J. Chem. Soc. Chem. Commun.* 418–420.

Isaac, R.E., Rees, H.H., Goodwin, T.W., **1981b**. Isolation of ecdysone 3-acetate as a major ecdysteroid from the developing eggs of the desert locust, *Schistocerca gregaria*. *J. Chem. Soc. Chem. Commun.* 594–595.

Isaac, R.E., Rose, M.E., Rees, H.H., Goodwin, T.W., **1982**. Identification of ecdysone-22-phosphate and 2-deoxyecdysone-22-phosphate in eggs of the desert locust, *Schistocerca gregaria*, by fast atom bombardment mass spectrometry and N.M.R. spectroscopy. *J. Chem. Soc. Chem. Commun.* 249–251.

Isaac, R.E., Slinger, A.J., **1989**. Storage and excretion of ecdysteroids. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. George Thieme Verlag, Stuttgart, pp. 250–253.

Isaac, R.E., Sweeney, F.P., Rees, H.H., **1983**. Enzymic hydrolysis of ecdysteroid phosphate during embryogenesis in the desert locust (*Schistocerca gregaria*). *Biochem. Soc. Trans. 11*, 379–380.

Ito, Y., **1986**. Trends in Countercurrent chromatography. *Trends Analyt. Chem. 5*, 142–147.

Jarvis, T.D., Earley, F.G.P., Rees, H.H., **1994a**. Inhibition of the ecdysteroid biosynthetic pathway in ovarian follicle cells of *Locusta migratoria*. *Pestic. Biochem. Physiol. 48*, 153–162.

Jarvis, T.D., Earley, F.G.P., Rees, H.H., **1994b**. Ecdysteroid biosynthesis in larval testes of *Spodoptera littoralis*. *Insect Biochem. Mol. Biol. 24*, 531–537.

Jegla, T.C., Costlow, J.D., **1979**. The *Limulus* bioassay for ecdysteroids. *Biol. Bull. 156*, 103–114.

Jenkins, S.P., Brown, M.R., Lea, A.O., **1992**. Inactive prothoracic glands in larvae and pupae of *Aedes aegypti*: ecdysteroid release by tissues in the thorax and abdomen. *Insect Biochem. Mol. Biol. 22*, 553–559.

Johnson, P., Rees, H.H., **1977**. The mechanism of C-20 hydroxylation of α-ecdysone in the desert locust, *Schistocerca gregaria*. *Biochem. J. 168*, 513–520.

Jürgens, G., Wieschaus, E., Nüsslein-Volhard, C., Kluding, H, **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* II Zygotic loci on the third chromosome. *Roux's Arch. Devel. Biol. 193*, 283–295.

Kabbouh, M., Rees, H.H., **1991a**. Characterization of the ATP: 2-Deoxyecdysone 22-phosphotransferase (2-deoxyecdysone 22-kinase) in the follicle cells of *Schistocerca gregaria*. *Insect Biochem. 21*, 57–64.

Kabbouh, M., Rees, H.H., **1991b**. Characterization of acetyl-CoA: ecdysone 3-acetyltransferase in *Schistocerca gregaria* larvae. *Insect Biochem. 21*, 607–613.

Kabbouh, M., Rees, H.H., **1993**. Characterization of ATP: ecdysone 3-acetate 2-phosphotransferase (ecdysone 3-acetate 2-kinase) in *Schistocerca gregaria* larvae. *Insect Biochem. Mol. Biol. 23*, 73–79.

Kamba, M., Mamiya, Y., Sonobe, H., Fujimoto, Y., **1994**. 22-Deoxy-20-hydroxy-ecdysone and its phosphoric acid ester from ovaries of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 24*, 395–402.

Kamba, M., Sonobe, H., Mamiya, Y., Hara, N., Fujimoto, Y., **1995**. Isolation and identification of 3-epiecdysteroids from diapause eggs of the silkworm, *Bombyx mori*. *J. Sericult. Sci. Japan 64*, 333–343.

Kamba, M., Sonobe, H., Mamiya, Y., Hara, N., Fujimoto, Y., **2000a**. 2,22-Dideoxy-23-hydroxyecdysone and its 3-phosphate from ovaries of the silkworm, *Bombyx mori*. *Nat. Product Lett. 14*, 349–356.

Kamba, M., Sonobe, H., Mamiya, Y., Hara, N., Fujimoto, Y., **2000b**. 3-Epi-22-deoxy-20,26-dihydroxyecdysone and 3-epi-22-deoxy-16β,20-dihydroxyecdysone and their 2-phosphate from eggs of the silkworm, *Bombyx mori*. *Nat. Product Lett. 14*, 469–476.

Kaplanis, J.N., Dutky, S.R., Robbins, W.E., Thompson, M.J., Lindquist, E.L., **1975**. Makisterone A: A new 28-carbon hexahydroxy moulting hormone from the embryo of the milkweed bug. *Science 190*, 681–682.

Kaplanis, J.N., Robbins, W.E., Thompson, M.J., Dutky, S.R., **1973**. 26-Hydroxy-ecdysone: new insect molting hormone from the egg of the tobacco hornworm. *Science 180*, 307–308.

Kaplanis, J.N., Tabor, L.A., Thompson, M.J., Robbins, W.E., Shortino, T.J., **1966**. Assay for ecdysone (moulting hormone) activity using the housefly, *Musca domestica. L. Steroids 8*, 625–631.

Kaplanis, J.N., Thompson, M.J., Dutky, S.R., Robbins, W.E., **1979**. The ecdysteroids from the tobacco hornworm during pupal-adult development five days after peak titer of molting hormone activity. *Steroids 34*, 333–345.

Kaplanis, J.N., Thompson, M.J., Dutky, S.R., Robbins, W.E., **1980**. The ecdysteroids from young embryonated eggs of the tobacco hornworm. *Steroids 36*, 321–336.

Kappler, C., Kabbouh, M., Durst, F., Hoffmann, J.A., **1986**. Studies on the C-2 hydroxylation of 2-deoxyecdysone in *Locusta migratoria*. *Insect Biochem. 16*, 25–32.

Kappler, C., Kabbouh, M., Hétru, C., Durst, F., Hoffmann, J.A., **1988**. Characterization of the three hydroxylases involved in the final steps of biosynthesis of the steroid hormone ecdysone in *Locusta migratoria* (Insecta, Orthoptera). *J. Steroid Biochem. 31*, 891–898.

Karlson, P., **1956**. Biochemical studies on insect hormones. *Vit. Horm. 14*, 227–266.

Karlson, P., **1983**. Eighth Adolf Butenandt Lecture. Why are so many hormones steroids? *Hoppe-Seyler's Z. Physiol. Chem. (S) 364*, 1067–1087.

Karlson, P., Bugany, H., Döpp, H., Hoyer, G.A., **1972**. 3-Dehydroecdyson, ein Stoff-wechselprodukt des Ecdysons bei der Schmeissfliege *Calliphora erythrocephala* Meigen. *Hoppe-Seyler's Z. Physiol. Chem. 358*, 1610–1614.

Karlson, P., Koolman, J., **1973**. On the metabolic fate of ecdysone and 3-dehydroecdysone in *Calliphora vicina*. *Insect Biochem. 3*, 409–417.

Karlson, P., Sekeris, C.E., **1966**. Ecdysone, an insect steroid hormone, and its mode of action. *Recent Progr. Horm. Res. 22*, 473–502.

Karlson, P., Shaaya, E., **1964**. Der Ecdysontiter während der Insektenentwicklung. I. Eine Methode zur Bestimmung des Ecdysongehalts. *J. Insect Physiol. 10*, 797–804.

Karlson, P., Stamm-Menéndez, M.D., **1956**. Notiz über den Nachweis von Metamorphose-Hormon in den Imagines von *Bombyx mori*. *Hoppe-Seyler's Z. Physiol. Chem. 306*, 109–111.

Katz, M., Lensky, Y., **1970**. Gas chromatographic analysis of ecdysone. *Experientia 26*, 1043.

Kayser, H., Ertl, P., Eilinger, P., Spindler-Barth, M., Winkler, T., **2002**. Diastereomeric ecdysteroids with a cyclic hemiacetal in the side chain produced by cytochrome P450 in hormonally resistant cells. *Arch. Biochem. Biophys. 400*, 180–187.

Kayser, H., Winkler, T., Spindler-Barth, M., **1997**. 26-Hydroxylation of ecdysteroids is catalyzed by a typical cytochrome P-450-dependent oxidase and related to ecdysteroid resistance in an insect cell line. *Eur. J. Biochem. 248*, 707–716.

Ke, O., **1930**. Morphological variation of the prothoracic gland in the domestic and the wild silkworm. *Bull. Sci. Fac. Terkult, Ksuju Imp. Uni. 4*, 12–21(cited in Koolman, 1989).

Keightley, D.A., Lou, K.J., Smith, W.A., **1990**. Involvement of translation and transcription in insect steroidogenesis. *Mol. Cell. Endocrinol. 74*, 229–237.

Kelly, T.J., Thyagaraja, B.S., Bell, R.A., Masler, E.P., Gelman, D.B., *et al.*, **1990**. Conversion of 3-dehydroecdysone by a ketoreductase in post-diapause, pre-hatch eggs of the Gypsy Moth *Lymantria dispar*. *Arch. Insect Biochem. Physiol. 14*, 37–46.

Keogh, D.P., Johnson, R.F., Smith, S.L., **1989**. Regulation of cytochrome P-450 dependent steroid hydroxylase activity in *Manduca sexta*: evidence for the involvement of a neuroendocrine-endocrine axis during larval-pupal development. *Biochem. Biophys. Res. Commun 165*, 442–448.

Keogh, D.P., Mitchell, M.J., Crooks, J.R., Smith, S.L., **1992**. Effects of the adenylate cyclase activator forskolin and its inactive derivative 1, 9-dideoxy-forskolin on insect cytochrome P-450 dependent steroid hydroxylase activity. *Experientia 48*, 39–41.

Keogh, D.P., Smith, S.L., **1991**. Regulation of cytochrome P-450 dependent steroid hydroxylase activity in *Manduca sexta*: effects of the ecdysone agonist RH 5849 on ecdysone 20-monooxygenase activity. *Biochem. Biophys. Res. Commun. 176*, 522–527.

Kholodova, Yu. D., **1977**. Use of the Chugaev reaction for the quantitative determination of ecdysones. *Khim. Prir. Soedin.* 227–230.

Kholodova, Yu. D., **1981**. Chugaev's reaction for the analysis of steroids. Proc. Symp. Analysis Steroids, Eger, Hungary, p. 519.

King, D.S., Bollenbacher, W.E., Borst, D.W., Vedeckis, W.V., O'Connor, J.D., *et al.*, **1974**. The secretion of α-ecdysone by the prothoracic glands of *Manduca sexta in vitro*. *Proc. Natl Acad. Sci. USA 71*, 793–796.

Kingan, T.G., **1989**. A competitive enzyme-linked immunosorbent assay: application in the assay of peptides, steroids and cyclic nucleotides. *Anal. Biochem. 183*, 283–289.

Kinnear, J.F., Martin, M.D., Faux, A.F., Horn, D.H.S., Wilkie, J.R., **1979**. Insect moulting hormones: a study of 4-ene-3-one steroids as possible ecdysteroid precursors. *Austral. J. Chem. 32*, 2017–2024.

Kiriishi, S., Rountree, D.B., Sakurai, S., Gilbert, L.I., **1990**. Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3β-reductase mediated conversion to ecdysone in representative insects. *Experientia 46*, 716–721.

Kitamura, T., Kobayashi, S., Okada, M., **1996**. Regional expression of the transcript encoding sterol carrier protein x-related thiolase and its regulation by homeotic genes in the midgut of *Drosophila* embryos. *Develop. Growth Differ. 38*, 373–381.

Kiuchi, M., Yasui, H, Hayasaka, S., Kamimura, M., **2003**. Entomogenous fungus *Nomuraea rileyi* inhibits host insect molting by C22-oxidizing inactivation of hemolymph ecdysteroids. *Arch. Insect Biochem. Physiol. 52*, 35–44.

Kobayashi, M., Krishna, M.M., Ishida, K., Anjaneyulu, V., **1992**. Marine sterols. XXII. Occurrence of 3-oxo-4,6,8(14)-triunsaturated steroids in the sponge *Dysidea herbacea*. *Chem. Pharm. Bull. 40*, 72–74.

Koch, J.A., Waxman, D.J., **1991**. P450 phosphorylation in isolated hepatocytes and *in vivo*. *Meth. Enzymol. 206*, 305–315.

Koener, J.F., Carino, F.A., Feyereisen, R., **1993**. The cDNA and deduced protein sequence of house fly

NADPH-cytochrome P450 reductase. *Insect Biochem. Mol. Biol. 23*, 439–447.

Koller, G., **1929**. Die innere Sekretion bei wirbellosen Tiere. *Biol. Rev. 4*, 269–306.

Kolmer, M., Alho, H., Costa, E., Pani, L., **1993**. Cloning and tissue-specific functional characterization of the promoter of the rat diazepam binding inhibitor, a peptide with multiple biological actions. *Proc. Natl Acad. Sci. USA 90*, 8439–8443.

Kolmer, M., Roos, C., Tirronen, M., Myohanen, S., Alho, H., **1994**. Tissue-specific expression of the diazepam-binding inhibitor in *Drosophila melanogaster*: cloning, structure, and localization of the gene. *Mol. Cell. Biol. 14*, 6983–6995.

Koolman, J., **1978**. Ecdysone oxidase in insects. *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1315–1321.

Koolman, J., **1980**. Analysis of ecdysteroids by fluorometry. *Insect Biochem. 10*, 381–386.

Koolman, J., **1985**. Ecdysone oxidase. *Methods Enzymol. 111*, 419–429.

Koolman, J., **1989**. Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart.

Koolman, J., **1990**. Ecdysteroids. *Zool. Sci. 7*, 563–580.

Koolman, J., Karlson, P., **1978**. Ecdysone oxidase: reaction and specificity. *Eur. J. Biochem. 89*, 453–460.

Koolman, J., Karlson, P., **1985**. Regulation of ecdysteroid titer: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 7. Pergamon Press, Oxford, pp. 343–361.

Koolman, J., Reum, L., Karlson, P., **1979**. 26-Hydroxyecdysone, 20,26-dihydroxyecdysone and inokosterone detected as metabolites of ecdysone in the blowfly, *Calliphora vicina* by radiotracer experiments. *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1351–1355.

Koolman, J., Spindler, K.-D., **1977**. Enzymatic and chemical synthesis of 3-dehydro-ecdysterone, a metabolite of the moulting hormone of insects. *Hoppe-Seyler's Z. Physiol. Chem. 358*, 1339–1344.

Kopec, S., **1922**. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull. 42*, 323–342.

Koreeda, M., Nakanishi, K., Goto, M., **1970**. Ajugalactone, an insect moulting hormone inhibitor as tested by the *Chilo* dipping method. *J. Am. Chem. Soc. 92*, 7512–7513.

Koreeda, M., Teicher, B.A., **1977**. Chemical analysis of insect molting hormones. In: Turner, R.B. (Ed.), Analytical Biochemistry of Insects. Elsevier Scientific Publishing Company, Amsterdam, pp. 207–240.

Kozlova, T., Thummel, C.S., **2003**. Essential roles for ecdysone signalling during *Drosophila* mid-embryonic development. *Science 301*, 1911–1914.

Krebs, K.C., Lan, Q., **2003**. Isolation and expression of a sterol carrier protein-2 gene from the yellow fever mosquito, *Aedes aegypti*. *Insect Mol. Biol. 12*, 51–60.

Kubo, I., Komatsu, S., **1986**. Micro analysis of prostaglandins and ecdysteroids in insects by high-performance liquid chromatography and fluorescence detection. *J. Chromatogr. 362*, 61–70.

Kubo, I., Komatsu, S., **1987**. Simultaneous measurement of prostaglandins and ecdysteroids in insects by high-performance liquid chromatography and fluorescence labeling. *Agri. Biol. Chem. 51*, 1305–1309.

Kubo, I., Komatsu, S., Asaka, Y., De Boer, G., **1987**. Isolation and identification of apolar metabolites of ingested 20-hydroxyecdysone in frass of *Heliothis virescens* larvae. *J. Chem. Ecol. 13*, 785–794.

Kubo, I., Matsumoto, A., Asano, S., **1985a**. Efficient isolation of ecdysteroids from the silkworm, *Bombyx mori* by droplet counter-current chromatography. *Insect Biochem. 15*, 45–47.

Kubo, I., Matsumoto, A., Hanke, F.J., **1985b**. The ^{1}H-NMR assignment of 20-hydroxyecdysone. *Agri. Biol. Chem. 49*, 243–244.

Kubo, I., Zhang, M., de Boer, G., Uchima, K., **1994**. Location of ecdysteroid 22-O-acyltransferase in the larvae of *Heliothis virescens*. *Entomol. Exp. Appl. 70*, 263–272.

Kulcsar, P., Darvas, B., Brandtner, H., Koolman, J., Rees, H.H., **1991**. Effects of the pyridine-containing P-450 inhibitor, fenarimol, on the formation of 20-OH ecdysone in flies. *Experientia 47*, 261–263.

Kurisu, M., Morita, M., Kashiwayama, Y., Yokata, S., Hayashi, H., *et al.*, **2003**. Existence of catalase-less peroxisomes in Sf21 insect cells. *Biochem. Biophys. Res. Commun. 306*, 169–176.

Kuroda, Y., **1968**. Effects of ecdysone analogues on differentiation of eye-antennal discs of *Drosophila melanogaster* in culture. *Annu. Rep. Natl Inst. Genet. Japan 19*, 22–23.

Lachaise, F., Carpentier, G., Sommé, G., Colardeau, J., Beydon, P., **1989**. Ecdysteroid synthesis by crab Y-organs. *J. Exp. Zool. 252*, 283–292.

Lachaise, F., Le Roux, A., Hubert, M., Lafont, R., **1993**. The molting glands of crustaceans: localization, activity and endocrine control (a review). *J. Crust. Biol. 13*, 198–234.

Lafont, R., **1997**. Ecdysteroids and related molecules in animals and plants. *Arch. Insect Biochem. Physiol. 35*, 3–20.

Lafont, R., **1988**. HPLC analysis of ecdysteroids in plants and animals. In: Kalász, H., Ettre, L.S. (Eds.), Chromatography '87. Akadémiai Kiadó, Budapest, pp. 1–15.

Lafont, R., Beydon, P., Blais, C., Garcia, M., Lachaise, F., *et al.*, **1986**. Ecdysteroid metabolism – a comparative study. *Insect Biochem. 16*, 11–16.

Lafont, R., Beydon, P., Mauchamp, B., Sommé-Martin, G., Andrianjafintrimo, M., *et al.*, **1981**. Recent progress in ecdysteroid analytical methods. In: Sehnal, F., Zabza, A., Cymborowski, B. (Eds.), Regulation of Insect Development and Behaviour. Technical University of Wroclaw Press, pp. 125–144.

Lafont, R., Beydon, P., Sommé-Martin, G., Blais, C., **1980**. High-performance liquid chromatography of ecdysone metabolites applied to the cabbage butterfly, *Pieris brassicae*. *Steroids 36*, 185–207.

Lafont, R., Blais, C., Beydon, P., Modde, J.-F., Enderle, U., *et al.*, **1983**. Conversion of ecdysone and 20-hydroxyecdysone into 26-oic derivatives is a major pathway in larvae and pupae of species from three insect orders. *Arch. Insect Biochem. Physiol. 1*, 41–58.

Lafont, R., Blais, C., Harmatha, J., Wilson, I.D., **2000**. Ecdysteroids: Chromatography. In: Wilson, I.D., Adlard, E.R., Cooke, M., Poole, C.F. (Eds.), Encyclopedia of Separation Science. Academic Press, London, pp. 2631–2640.

Lafont, R., Connat, J.-L., **1989**. Pathways of ecdysone metabolism. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, p. 167.

Lafont, R., Connat, J.-L., Delbecque, J.-P., Porcheron, P., Dauphin-Villemant, C., *et al.*, **1995**. Comparative studies on ecdysteroids. In: Ohnishi, E., Takahashi, S.Y., Sonobe, H. (Eds.), Recent Advances in Insect Biochemistry and Molecular Biology. University of Nagoya Press, Nagoya, pp. 45–91.

Lafont, R., Dinan, L.N., **2003**. Practical uses for ecdysteroids in mammals and humans: an update. *J. Insect Sci. 3.7* (http://www.insectscience.org/3.7/).

Lafont, R., Kaouadji, N., Morgan, E.D., Wilson, I.D., **1994a**. Selectivity in the HPLC analysis of ecdysteroids. *J. Chromatogr. 658*, 55–67.

Lafont, R., Koolman, J., **1984**. Ecdysone metabolism. In: Hoffmann, J.A., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, pp. 196–226.

Lafont, R., Koolman, J., Rees, H.H., **1993a**. Standardized abbreviations for common ecdysteroids. *Insect Biochem. 23*, 207–209.

Lafont, R., Morgan, E.D., Wilson, I.D., **1994b**. Chromatographic procedures for phytoecdysteroids. *J. Chromatogr. 658*, 31–53.

Lafont, R., Pennetier, J.-L., Andrianjafintrimo, M., Claret, J., Modde, J.-F., *et al.*, **1982**. Sample processing for high-performance liquid chromatography of ecdysteroids. *J. Chromatogr. 236*, 137–149.

Lafont, R., Porter, C.J., Williams, E., Read, H., Morgan, E.D., *et al.*, **1993b**. The application of off-line HPTLC-MS-MS to the identification of ecdysteroids in plant and arthropod samples. *J. Planar Chromatogr. 6*, 421–424.

Lafont, R., Sommé-Martin, C., Chambet, J.-C., **1979**. Separation of ecdysteroids by using high pressure liquid chromatography on microparticulate supports. *J. Chromatogr. 170*, 185–194.

Lafont, R., Wilson, I.D., **1990**. Advances in ecdysteroid HPLC. In: McCaffery, A.R., Wilson, I.D. (Eds.), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, London, pp. 79–94.

Lagueux, M., Hoffmann, J.A., Goltzené, F., Kappler, C., Tsoupras, G., *et al.*, **1984**. Ecdysteroids in ovaries and embryos of *Locusta migratoria*. In: Hoffmann, J.A., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, pp. 168–180.

Lakeman, J., Speckamp, W.W., Huisman, H.O., **1967**. Addition to stenoid polyenes IV. *Tetrahedron Lett. 38*, 3699–3703.

Large, T., Lafont, R., Morgan, E.D., Wilson, I.D., **1992**. Micellar capillary electrophoresis of ecdysteroids. *Anal. Proc. 29*, 386–388.

Laurent, P., Braekman, J.-C., Daloze, D., Pasteels, J.M., **2003**. An ecdysteroid (22-acetyl-20-hydroxyecdysone) from the defense gland secretions of an insect: *Chrysolina carnifex* (Coleoptera: Chrysomelidae). *Chemoecology 13*, 109–111.

Le Bizec, B., Antignac, J.-P., Monteau, F., André, F., **2002**. Ecdysteroids: one potential new anabolic family in breeding animals. *Anal. Chim. Acta 473*, 89–97.

Lee, S.S., Nakanishi, K., **1989**. ^{13}C-NMR assignments of some insect molting hormones. *Kaohsiung J. Med. Sci. 5*, 564–568.

Lehmann, M., Koolman, J., **1986**. The influence of forskolin on the metabolism of ecdysone and 20-hydroxyecdysone in isolated fat body of the blowfly, *Calliphora vicina*. *Hoppe-Seyler's Z. Physiol. Chem. 367*, 387–393.

Lehmann, M., Koolman, J., **1989**. Regulation of ecdysone metabolism. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 217–220.

Liebrich, W., Dumberger, B.B., Hoffmann, K.H., **1991**. Ecdysone 20-monooxygenase in a cricket (*Gryllus bimaculatus* Ensifera, Gryllidae): activity throughout adult life-cycle. *Comp. Biochem. Physiol. A-Physiol. 99*, 597–602.

Liebrich, W., Hoffmann, K.H., **1991**. Ecdysone 20-monooxygenase in a cricket, *Gryllus bimaculatus* (Ensifera, Gryllidae): characterization of the microsomal midgut steroid hydroxylase in adult females. *J. Comp. Physiol. B 161*, 93–99.

Loeb, M.J., Brandt, E.P., Woods, C.W., Bell, R.A., **1988**. Secretion of ecdysteroid by sheets of testes of the gipsy moth, *Lymantria dispar*, and its regulation by testis ecdysiotropin. *J. Exp. Zool. 248*, 94–100.

Loeb, M.J., Woods, C.W., **1989**. Metabolism of ecdysteroid by testes of the tobacco budworm, *Heliothis virescens*. *Arch. Insect Biochem. Physiol. 10*, 83–92.

Loeb, M.J., Woods, C.W., Brandt, E.P., Borkovec, A.B., **1982**. Larval testes of the tobacco budworm. A new source of insect ecdysteroids. *Science 218*, 896–898.

Louden, D., Handley, A., Taylor, S., Lenz, E., Miller, S., *et al.*, **2001**. Spectroscopic characterization and identification of ecdysteroids using high performance liquid chromatography combined with on-line diode-array, FT-infrared, ^{1}H-Nuclear Magnetic Resonance and time-of-flight mass spectrometry. *J. Chromatogr. A 910*, 237–246.

Louden, D., Handley, A., Lafont, R., Taylor, S., Sinclair, I., *et al.*, **2002**. HPLC analysis of ecdysteroids in plant extracts using superheated deuterium oxide with multiple on-line spectroscopic analysis (UV, IR, ^{1}H NMR, and MS). *Anal. Chem. 74*, 288–294.

Lozano, R., Thompson, M.J., Lusby, W.R., Svoboda, J.A., Rees, H.H., **1988a**. Metabolism of [^{14}C]cholesterol in *Manduca sexta* pupae: isolation and identification of sterol sulfates, free ecdysteroids and ecdysteroid acids. *Arch. Insect Biochem. Physiol. 7*, 249–266.

Lozano, R., Thompson, M.J., Svoboda, J.A., Lusby, W.R., **1988b**. Isolation of acidic and conjugated ecdysteroid fractions from *Manduca sexta* pupae. *Insect Biochem. 18*, 163–168.

Lozano, R., Thompson, M.J., Svoboda, J.A., Lusby, W.R., **1989**. Profiles of free and conjugated ecdysteroids and ecdysteroid acids during pupal-adult development of *Manduca sexta*. *Arch. Insect Biochem. Physiol. 12*, 63–74.

Lukacs, G., Bennett, C.R., **1972**. Résonance magnétique du ^{13}C de produits naturels et apparentés. VII (18. L'α-ecdysone). *Bull. Soc. Chim. Fr. 10*, 3996–4000.

Lundström, A., Kang, D., Liu, G., Fernandez, C., Warren, J.T., *et al.*, **2002**. A protein from the cabbage looper, *Trichoplusia ni*, regulated by a bacterial infection is homologous to 3-dehydroecdysone 3β-reductase. *Insect Biochem. Mol. Biol. 32*, 829–837.

Lusby, W.R., Oliver, J.E., Thompson, M.J., **1987**. Application of desorption chemical ionization techniques for analysis of biologically active compounds isolated from insects. In: Rosen, J.D. (Ed.), Applications of New Mass Spectrometry Techniques in Pesticide Chemistry. John Wiley, pp. 99–115.

Makka, T., Seino, A., Tomita, S., Fujiwara, H., Sonobe, H., **2002**. A possible role of 20-hydroxyecdysone in embryonic development of the silkworm *Bombyx mori*. *Arch. Insect Biochem. Physiol. 51*, 111–120.

Makka, T., Sonobe, H., **2000**. Ecdysone metabolism in diapause eggs and non-diapause eggs of the silkworm, *Bombyx mori*. *Zool. Sci. 17*, 89–95.

Malorni, A., Minale, L., Riccio, K., **1978**. Steroids from sponges: occurence of steroidal Δ4,7-3,6-diketones in the marine sponge *Raphidostila incisa*. *Nouv. I. Chimie. 2*, 351–354.

Mamiya, Y., Sonobe, H., Yoshida, K., Hara, N., Fujimoto, Y., **1995**. Occurrence of 3-epi-22-deoxy-20-hydroxyecdysone and its phosphoric ester in diapause eggs of the silkworm, *Bombyx mori*. *Experientia 51*, 363–367.

Mansuy, D., **1998**. The great diversity of reactions catalyzed by cytochromes P450. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 121*, 5–14.

Marco, M.-P., Sánchez-Baeza, F.J., Camps, F., Coll, J., **1993**. Phytoecdysteroid analysis by high-performance liquid chromatography-thermospray mass spectrometry. *J. Chromatogr. 641*, 81–87.

Maroy, P., Kauffmann, G., Dübendorfer, A., **1988**. Embryonic ecdysteroids of *Drosophila melanogaster*. *J. Insect Physiol. 34*, 633–637.

Matsumoto, T., Kubo, I., 1989. Ecdysone from *Vitex strickeri* as insect growth inhibitor. Application JP 87-295802 (*cited in Chemical Abstracts 111:* 169367).

Matsumoto, E., Matsui, M., Tamura, H., **2003**. Identification and purification of sulfotransferase for 20-hydroxyecdysteroid from the larval fat body of a fleshfly, *Sarcophaga Peregrina*. *Biosci. Biotechnol. Biochem. 67*, 1780–1785.

Mauchamp, B., Royer, C., Kerhoas, L., Einhorn, J., **1993**. MS/MS analyses of ecdysteroids in developing *Dysdercus fasciatus*. *Insect Biochem. Mol. Biol. 23*, 199–205.

Maurer, P., Girault, J.-P., Larchevêque, M., Lafont, R., **1993**. 24-Epimakisterone A (not makisterone A) is the major ecdysteroid in the leaf-cutting ant *Acromyrmex octospinosus* (Reich) (Hymenoptera, Formicidae: Attini). *Arch. Insect Biochem. Physiol. 23*, 29–35.

Mauvais, A., Burger, A., Rousset, J.- P., Hétru, C., Luu, B., **1989**. Acetylenic inhibitors of c-22 hydrocylase of ecdysone biosynthesis. *Bioorganic Chemistry 22*, 36–50.

Mayer, R.T., Durrant, J.L., Holman, G.M., Weirich, G.F., Svoboda, J.A., **1979**. Ecdysone 3-epimerase from the midgut of *Manduca sexta* (L.). *Steroids 34*, 555–562.

Mayer, R.T., Svoboda, J.A., **1978**. Thin-layer chromatographic *in situ* analysis of insect ecdysones via fluorescence-quenching. *Steroids 31*, 139–150.

Meister, M.F., Dimarcq, J.-L., Kappler, C., Hétru, C., Lagueux, M., *et al.*, **1985**. Conversion of a labelled ecdysone precursor, 2,22,25-trideoxyecdysone, by embryonic and larval tissues of *Locusta migratoria*. *Mol. Cell. Endocrinol. 41*, 27–44.

Mesnier, M., Partiaoglou, N., Oberlander, H., Porcheron, P., **2000**. Rhythmic autocrine activity in cultured insect epidermal cells. *Arch. Insect Biochem. Physiol. 44*, 7–16.

Mikitani, K., **1995**. Sensitive, rapid and simple method for evaluation of ecdysteroid agonist activity based on the mode of action of the hormone. *J. Sericult. Sci. Japan 64*, 534–539.

Mikitani, K., **1996**. An automated ecdysteroid receptor binding assay using a 96-well microplate. *J. Sericult. Sci. Japan 65*, 141–144.

Miller, W.L., **1988**. Molecular biology of steroid hormone synthesis. *Endocr. Rev. 9*, 295–318.

Milner, N.P., Nali, M., Gibson, J.M., Rees, H.H., **1986**. Early stages of ecdysteroid biosynthesis: the role of 7-dehydrocholesterol. *Insect Biochem. 16*, 17–23.

Milner, N.P., Rees, H.H., **1985**. Involvement of 3-dehydroecdysone in the 3-epimerization of ecdysone. *Biochem. J. 231*, 369–374.

Mitchell, M.J., Crooks, J.R., Keogh, D.P., Smith, S.L., **1999**. Ecdysone 20-monooxygenase activity during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 41*, 24–32.

Mitchell, M.J., Keogh, D.P., Crooks, J.R., Smith, S.L., **1993**. Effects of plant flavonoids and other allelochemicals on insect cytochrome P-450 dependent steroid hydroxylase activity. *Insect Biochem. Mol. Biol. 23*, 65–71.

Mitchell, M.J., Smith, S.L., **1986**. Characterization of ecdysone 20-monooxygenase activity in wandering stage larvae of *Drosophila melanogaster* : evidence for mitochondrial and microsomal cytochrome P-450 dependent systems. *Insect Biochem. 16*, 525–537.

Mitchell, M.J., Smith, S.L., **1988**. Ecdysone 20-monooxygenase activity throughout the life cycle of *Drosophila melanogaster*. *Gen. Comp. Endocrinol. 72*, 467–470.

Mizuno, T., Ohnishi, E., **1975**. Conjugated ecdysone in the eggs of the silkworm, *Bombyx mori*. *Devel. Growth Differ. 17*, 219–225.

Modde, J.-F., Lafont, R., Hoffmann, J.A., **1984**. Ecdysone metabolism in *Locusta migratoria* larvae and adults. *Int. J. Invert. Reprod. Devel. 7*, 161–183.

Moog-Lutz, C., Tomasetto, C., Regnier, C. H., Wendling, C., Lutz, Y., *et al.*, **1997**. MLN64 exhibits homology with the steroidogenic acute regulatory protein (STAR) and is over-expressed in human breast carcinomas. *Int. J. Cancer 71*, 183–191.

Morgan, E.D., Huang, H-P., **1990**. Analysis of ecdysteroids by supercritical fluid chromatography. In: McCaffery, A.R., Wilson, I.D. (Eds.), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, London, pp. 95–102.

Morgan, E.D., Murphy, S.J., Games, D.E., Mylchreest, I., **1988**. Analysis of ecdysteroids by supercritical-fluid chromatography. *J. Chromatogr. 441*, 165–169.

Morgan, E.D., Poole, C.F., **1976**. The extraction and determination of ecdysones in arthropods. *Adv. Insect Physiol. 12*, 17–62.

Morgan, E.D., Wilson, I.D., **1989**. Methods for separation and physico-chemical quantification of ecdysteroids. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 114–130.

Morgan, E.D., Woodbridge, A.P., **1971**. Insect moulting hormones (ecdysones). Identification as derivatives by gas chromatography. *J. Chem. Soc. Chem. Commun.* 475–476.

Nakanishi, K., **1971**. The ecdysones. *Pure Appl. Chem. 25*, 167–195.

Nashed, N.T., Michaud, D.P., Levin, W., Jerina, D.M., **1986**. 7-Dehydrocholesterol 5,6β-oxide as a mechanism-based inhibitor of microsomal cholesterol oxide hydrolase. *J. Biol. Chem. 261*, 2510–2513.

Nasir, H., Noda, H., **2003**. Yeast-like symbiotes as a sterol source in Anobiid beetles (Coleoptera, Anobiidae): possible metabolic pathways from fungal sterols to 7-dehydrocholesterol. *Arch. Insect Biochem. Physiol. 52*, 175–182.

Nelson, D.R., **1998**. Metazoan cytochrome P450 evolution. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 121*, 175–182.

Nigg, H.A., Svoboda, J.A., Thompson, M.J., Kaplanis, J.N., Dutky, S.R., *et al.*, **1974**. Ecdysone metabolism: ecdysone dehydrogenase-isomerase. *Lipids 9*, 971–974.

Nomura, Y., Komatsuzaki, M., Iwami, M., Sakurai, S., **1996**. Purification and characterization of hemolymph 3-dehydroecdysteroid 3β-reductase of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 26*, 249–257.

Nüsslein-Volhard, C., Wieschaus, E., Kluding, H., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Roux's Arch. Devel. Biol. 183*, 267–282.

Oberlander, H., **1974**. Biological activity of insect ecdysones and analogues *in vitro*. *Experientia 30*, 1409–1410.

Ohnishi, E., **1986**. Ovarian ecdysteroids of *Bombyx mori*: retrospect and prospect. *Zool. Sci. 3*, 401–407.

Ohnishi, E., Hiramoto, M., Fujimoto, Y., Kakinuma, K., Ikekawa, N., **1989**. Isolation and identification of major ecdysteroid conjugates from the ovaries of *Bombyx mori*. *Insect Biochem. 19*, 95–101.

Ohnishi, E., Mizuno, T., Chatani, F., Ikekawa, N., Sakurai, S., **1977**. 2-Deoxy-α-ecdysone from ovaries and eggs of the silkworm, *Bombyx mori*. *Science 197*, 66–67.

Ohtaki, T., Milkman, R.D., Williams, C.M., **1967**. Ecdysone and ecdysone analogues: their assay on the flesh fly *Sarcophaga peregrina*. *Proc. Natl Acad. Sci. USA 85*, 981–984.

Okazaki, R.K., Snyder, M.J., Grimm, C.C., Chang, E.S., **1997**. Ecdysteroids in nemerteans: further characterization and identification. *Hydrobiologia 365*, 281–285.

Omura, T., Ito, A., **1991**. Biosynthesis and intracellular sorting of mitochondrial forms of cytochrome P450. *Methods Enzymol. 206*, 75–81.

O'Reilly, D.R., **1995**. Baculovirus encoded ecdysteroid UDP-glucosyl-transferases. *Insect Biochem. Mol. Biol. 25*, 541–550.

O'Reilly, D.R., Brown, M.R., Miller, L.K., **1992**. Alteration of ecdysteroid metabolism due to baculovirus infection of the fall armyworm *Spodoptera frugiperda*: host ecdysteroids are conjugated with galactose. *Insect Biochem. Mol. Biol. 22*, 313–320.

O'Reilly, D.R., Howarth, O.W., Rees, H.H., Miller, L.K., **1991**. Structure of the ecdysone glucoside formed by a baculovirus ecdysteroid UDP-glucosyltransferase. *Insect Biochem. 21*, 795–801.

O'Reilly, D.R, Miller, L.K., **1989**. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyltransferase. *Science 245*, 1110–1112.

Pak, M.D., Gilbert, L.I., **1987**. A developmental analysis of ecdysteroids during the metamorphosis of *Drosophila melanogaster*. *J. Liq. Chromatgr. 10*, 2591–2611.

Papadopoulos, V., **1993**. Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. *Endocr. Rev. 14*, 222–240.

Papadopoulos, V., Widmaier, E.P., Amri, H., Zilz, A., Li, H., *et al.*, **1998**. *In vivo* studies on the role of the peripheral benzodiazepine receptor (PBR) in steroidogenesis. *Endocr. Res. 24*, 479–487.

Park, E.J., Burand, J.P., Yin, C.-M., **1993**. The effect of baculovirus infection on ecdysteroid titer in Gypsy Moth larvae (*Lymantria dispar*). *J. Insect Physiol. 39*, 791–796.

Pasteels, J.M., Rowell-Rahier, M., Braekman, J.C., Daloze, D., **1994**. Chemical defense of adult leaf beetles updated. In: Jolivet, P.H., Cox, M.L., Petitpierre, E. (Eds.), Novel Aspects of the Biology of Chrysomelidae. Kluwer, Dordrecht, pp. 289–301.

Petersen, Q.R., Cambie, R.C., Russel, G.B., **1993**. Jones oxidation of 20-hydroxyecdysone (= crustecdysone). *Aust. J. Chem. 46*, 1961–1964.

Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., *et al.*, **2003**. Shade: the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778.

Pimprikar, G.D., Coign, M.J., Sakurai, H., Heits, J.R., **1984**. High-performance liquid chromatographic determination of ecdysteroid titers in the house fly. *J. Chromatogr. 317*, 413–419.

Pís, J., Girault, J.-P., Grau, V., Lafont, R., **1995a**. Analysis of ecdysteroid conjugates: chromatographic characterization of glucosides, phosphates and sulfates. *Eur. J. Entomol. 92*, 41–50.

Pís, J., Girault, J.-P., Larchevêque, M., Dauphin-Villemant, C., Lafont, R., **1995b**. A convenient synthesis of 25-deoxyecdysone, a major secretory product of Crustacean Y-organs and of 2,25-dideoxyecdysone, its putative immediate precursor. *Steroids 60*, 188–194.

Pís, J., Hykl, J., Budesinski, M., Harmatha, J., **1994**. Regioselective synthesis of 20-hydroxyecdysone glycosides. *Tetrahedron 50*, 9679–9690.

Pís, J., Vaisar, T., **1997**. H/D isotopic exchange in the Fast Atom Bombardment of ecdysteroids. *J. Mass Spectrom. 32*, 1050–1056.

Poole, C.F., Morgan, E.D., Bebbington, P.M., **1975**. Analysis of ecdysone by gas chromatography using electron capture detection. *J. Chromatogr. 104*, 172–175.

Poole, C.F., Singhawangcha, S., Zlatkis, A., Morgan, E.D., **1978**. Determination of bifunctional compounds. Part III. Polynuclear aromatic boronic acids as selective fluorescent reagents for HPTLC and HPLC. *J. High Resol. Chromatogr. Chromatogr. Commun. 2*, 96–97.

Poole, C.F., Zlatkis, A., Sye, W.-F., Singhawangcha, S., Morgan, E.D., **1980**. The determination of steroids with and without natural electrophores by gas chromatography and electron-capture detection. *Lipids 15*, 734–744.

Porcheron, P., Morinière, M., Grassi, J., Pradelles, P., **1989**. Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. *Insect Biochem. 19*, 117–122.

Raynor, W.M., Kithinji, J.P., Barker, I.K., Bartle, K.D., Wilson, I.D., **1988**. Supercritical fluid chromatography of ecdysteroids. *J. Chromatogr. 436*, 497–502.

Raynor, W.M., Kithinji, J.P., Bartle, K.D., Games, D.E., MylchReest, I.C., *et al.*, **1989**. Packed column supercritical fluid chromatography and linked supercritical fluid chromatography – mass spectrometry for the analysis of phytoecdysteroids from *Silene nutans* and *Silene otites*. *J. Chromatogr. 467*, 292–298.

Read, H., Wilson, I.D., Lafont, R., **1990**. Overpressure thin-layer chromatography of ecdysteroids. In: McCaffery, A.R., Wilson, I.D. (Eds.), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, London, p. 127.

Redfern, C.P.F., **1984**. Evidence for the presence of makisterone A in *Drosophila* larvae and the secretion of 20-deoxymakisterone A by the ring gland. *Proc. Natl Acad. Sci. USA 81*, 5643–5647.

Redfern, C.P.F., **1986**. Changes in patterns of ecdysteroid secretion by the ring gland of *Drosophila* in relation to the sterol composition of the diet. *Experientia 42*, 307–309.

Redfern, C.P.F., **1989**. Ecdysiosynthetic tissues. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 182–187.

Rees, H.H., **1985**. Biosynthesis of ecdysone. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 7. Pergamon Press, Oxford, pp. 249–293.

Rees, H.H., **1989**. Zooecdysteroids; structures and occurrence. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 28–38.

Rees, H.H., **1995**. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol. 92*, 9–39.

Rees, H.H., Isaac, R.E., **1984**. Biosynthesis of ovarian ecdysteroid phosphates and their metabolic fate during embryogenesis in *Schistocerca gregaria*. In: Hoffmann, J.A., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, pp. 181–195.

Rees, H.H., Isaac, R.E., **1985**. Biosynthesis and metabolism of ecdysteroids and methods of isolation and identification of the free and conjugated compounds. *Methods Enzymol. 111*, 377–410.

Reixach, N., Lafont, R., Camps, F., Casas, J., **1999**. Biotransformation of putative phytoecdysteroid biosynthetic precursors in tissue cultures of *polypodium vulgares*. *Eur. J. Biochem. 266*, 608–615.

Reum, L., Klinger, W., Koolman, J., **1984**. A new immunoassay for ecdysteroids based on chemiluminescence. In: Kricka, L.J., Stanley, P.E., Thorpe, G.H.G., Whitehead, T.C. (Eds.), Analytical Applications of Bioluminescence and Chemiluminescence. Academic Press, New York, pp. 249–252.

Reum, L., Koolman, J., **1989**. Radioimmunoassay of ecdysteroids. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, p. 131.

Richards, G., **1978**. The relative biological activity of α- and β-ecdysone and their 3-dehydro derivatives in the chromosome puffing assay. *J. Insect Physiol. 24*, 329–335.

Richter, K., Böhm, G.-A., Leubert, F., **1999**. 3-Dehydroecdysone secretion by the molting gland of the cockroach, *Periplaneta americana*. *Arch. Insect Biochem. Physiol. 41*, 107–116.

Robinson, P.D., Morgan, E.D., Wilson, I.D., Lafont, R., **1987**. The metabolism of ingested and injected [^{3}H]ecdysone by final instar larvae of *Heliothis armigera*. *Physiol. Entomol. 12*, 321–330.

Roth, G.E., Gierl, M. S., Vollborn, L., Meise, M., Lintermann, R., *et al.*, **2004**. The *Drosophila* gene start 1: a putative cholesterol transporter and key regulator of ecdysteroid synthesis. *Proc. Natl Acad. Sci. USA 101*, 1601–1606.

Royer, C., Porcheron, P., Pradelles, P., Kerhoas, L., Einhorn, J., *et al.*, **1995**. Rapid and specific enzyme immunoassay for Makisterone A. *Insect Biochem. Mol. Biol. 25*, 235–240.

Royer, C., Porcheron, P., Pradelles, P., Mauchamp, B., **1993**. Development and use of an enzymatic tracer for an enzyme immunoassay of makisterone A. *Insect Biochem. Mol. Biol. 23*, 193–197.

Rudolph, P.H., Spaziani, E., **1992**. Formation of ecdysteroids by Y-organs of the crab, *Menippe mercenaria*. II. Incorporation of cholesterol into 7-dehydrocholesterol and secretion products in vitro. *Gen. Comp. Endocrinol. 88*, 235–242.

Rudolph, P.H., Spaziani, E., Wang, W.L., **1992**. Formation of ecdysteroids by Y-organs of the crab, *Menippe mercenaria*. I. Biosynthesis of 7-dehydrocholesterol *in vivo*. *Gen. Comp. Endocrinol. 88*, 224–234.

Russell, G.B., Greenwood, D.R., **1989**. Methods of isolation of ecdysteroids. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, p. 97.

Sakurai, S., Gilbert, L.I., **1990**. Biosynthesis and secretion of ecdysteroids by the prothoracic glands. In: Ohnishi, E., Ishizaki, H. (Eds.), Molting and Metamorphosis. Japan Sci. Soc. Press, Tokyo, pp. 83–106.

Sakurai, S., Warren, J.T., Gilbert, L.I., **1989**. Mediation of ecdysone synthesis in *Manduca sexta* by a hemolymph enzyme. *Arch. Insect Biochem. Physiol. 10*, 179–197.

Sakurai, S., Warren, J.T., Gilbert, L.I., **1991**. Ecdysteroid synthesis and molting by the tobacco hornworm, *Manduca sexta,* in the absence of prothoracic glands. *Arch. Insect Biochem. Physiol. 18*, 13–36.

Sakurai, S., Yonemura, N., Fujimoto, Y., Hata, F., Ikekawa, N., **1986**. 7-Dehydrosterols in prothoracic glands of the silkworm, *Bombyx mori*. *Experientia 42*, 1034–1036.

Sall, C., Tsoupras, G., Kappler, C., Lagueux, M., Zachary, D., *et al.*, **1983**. Fate of maternal conjugated ecdysteroids during embryonic development in *Locusta migratoria*. *J. Insect Physiol. 29*, 491–507.

Sardini, D., Krepinsky, J., **1974**. Densitometric determination of ecdysteroids. *Farmaco Edizione Pratica 29*, 723–731.

Sato, H., Ashida, N., Suhara, K., Itagaki, E., Takemori, S., *et al.*, **1978**. Properties of an adrenal cytochrome P-450 (P-$450_{11\beta}$) for the hydroxylations of corticosteroids. *Arch. Biochem. Biophys. 190*, 307–314.

Sato, Y., Sakai, M., Imai, S., Fujioka, S., **1968**. Ecdysone activity of plant-originated molting hormones applied on the body surface of lepidopterous larvae. *Appl. Entomol. Zool. 3*, 49–51.

Sayah, F., Blais, C., **1995**. Ecdysteroid metabolism in *Labidura riparia* females (Insecta, Dermaptera). *Netherlands J. Zool. 45*, 89–92.

Scalia, S., Sbrenna-Miacciarelli, A., Sbrenna, G., Morgan, E.D., **1987**. Ecdysteroid titers and location in developing eggs of *Schistocerca gregaria*. *Insect Biochem. 17*, 227–236.

Schenkman, J.B., Jansson, I., **2003**. The many roles of cytochrome b5. *Pharmacol. Ther. 97*, 139–152.

Schildknecht, H., **1970**. The defensive chemistry of land and water beetles. *Angewandte Chemie, Int. Edn 9,* 1–9.

Schroeder, F., Frolov, A., Starodub, O., Atshaves, B.B., Russell, W., *et al.*, **2000**. Pro-sterol carrier protein-2: Role of the N-terminal presequence in structure, function and peroxisomal targeting. *J. Biol. Chem. 275*, 25547–25555.

Schwab, C., Hétru, C., **1991**. Synthesis and conversion study of a radiolabeled putative ecdysone precursor, 5β-cholest-7-ene-3β,6α; 14α-triol in *Locusta migratoria* prothoracic glands. *Steroids 56*, 316–319.

Schwartz, M.B., Imberski, R.B., Kelly, T.J., **1984**. Analysis of metamorphosis in *Drosophila melanogaster*: characterization of *giant*, an ecdysteroid-deficient mutant. *Devel. Biol. 103*, 85–95.

Shampengtong, L., Wong, K.P., **1989**. An *in vitro* assay of 20- hydroxyecdysone solfotransferase in the mosquito, *Aedes togoi*. *Insect Biochem. 19*, 191–196.

Shergill, J.K., Cammack, R., Chen, J.-H., Fisher, M.J., Madden, S., *et al.*, **1995**. EPR spectroscopic characterization of the iron-sulphur proteins and cytochrome P-450 in the mitochondria from the insect *Spodoptera littoralis* (cotton leafworm). *Biochemical J. 307*, 719–728.

Sláma, K., Abubakirov, N.K., Gorovits, M.B., Baltaev, U.A., Saatov, Z., **1993**. Hormonal activity of ecdysteroids from certain Asiatic plants. *Insect Biochem. Mol. Biol. 23*, 181–185.

Slinger, A.J., Dinan, L.N., Isaac, R.E., **1986**. Isolation of apolar ecdysteroid conjugates from newly-laid oothecae of *Periplaneta americana*. *Insect Biochem. 16*, 115–119.

Slinger, A.J., Isaac, R.E., **1988a**. Synthesis of apolar ecdysteroid conjugates by ovarian tissue from *Periplaneta americana*. *Gen. Comp. Endocrinol. 70*, 74–82.

Slinger, A.J., Isaac, R.E., **1988b**. Ecdysteroid titers during embryogenesis of the cockroach, *Periplaneta americana*. *J. Insect Physiol. 34*, 1119–1125.

Slinger, A.J., Isaac, R.E., **1988c**. Acyl-CoA: ecdysone acyltransferase activity from the ovary of *P. americana*. *Insect Biochem. 18*, 779–784.

Sliter, T., Gilbert, L.I., **1992**. Developmental arrest and ecdysteroid deficiency resulting from mutations at the *dre4* locus of *Drosophila* encodes a product with DNA binding activity. *Genetics 130*, 555–568.

Smith, S.L., **1985**. Regulation of ecdysteroid titer: synthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 7. Pergamon Press, Oxford, p. 295.

Smith, S.L., Bollenbacher, W., Cooper, D., Schleyer, H., Wielgus, J., *et al.*, **1979**. Ecdysone 20-monooxygenase: characterization of an insect cytochrome P-450 dependent steroid hydroxylase. *Mol. Cell. Endocrinol. 15*, 111–133.

Smith, S.L., Bollenbacher, W.E., Gilbert, L.I., **1980**. Studies on the biosynthesis of ecdysone and 20-hydroxyecdysone in the tobacco hornworm, *Manduca sexta*. In: Hoffmann, J.A. (Ed.), Progress in Ecdysone Research. Elsevier/North-Holland Biomedical Press, Amsterdam, p. 139.

Smith, S.L., Bollenbacher, W.E., Gilbert, L.I., **1983**. Ecdysone 20-monooxygenase activity during larval-pupal development of *Manduca sexta*. *Mol. Cell. Endocrinol. 31*, 227–251.

Smith, S.L., Mitchell, M.J., **1986**. Ecdysone 20-monooxygenase systems in a larval and an adult dipteran. *Insect Biochem. 16*, 49–55.

Smith, S.L., Mitchell, M.J., **1988**. Effects of azadirachtin on insect cytochrome P-450 dependent ecdysone 20-monooxygenase activity. *Biochem. Biophys. Res. Commun. 154*, 559–563.

Snyder, M.J., Antwerpen, R., **1998**. Evidence for a diazepam-binding inhibitor (DBI) benzodiazepine receptor-like mechanism in ecdysteroidogenesis by the insect prothoracic gland. *Cell Tissue Res. 294*, 161–168.

Snyder, M.J., Feyereisen, R., **1993**. A diazepam binding inhibitor (DBI) homolog from the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol. 94*, R1–4.

Snyder, M.J., Scott, J.A., Andersen, J.F., Feyereisen, R., **1996**. Sampling P450 diversity by cloning polymerase chain reaction products obtained with degenerate primers. *Methods Enzymol. 272*, 304–312.

Soller, M., Bownes, M., Kubli, E., **1999**. Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol. 208*, 337–351.

Sommé-Martin, G., Colardeau, J., Lafont, R., **1988**. Conversion of ecdysone and 20-hydroxyecdysone into 3-dehydroecdysteroids is a major pathway in third instar *Drosophila melanogaster* larvae. *Insect Biochem. 18*, 729–734.

Sonobe, H., Tokushige, H., Makka, T., Tsutsumi, H., Hara, N., *et al.*, **1999**. Comparative studies of ecdysteroid metabolism between diapause eggs and non-diapause eggs of the silkworm, *Bombyx mori*. *Zool. Sci. 16*, 935–943.

Spaziani, E., Rees, H.H., Wang, W.L., Watson, R.D., **1989**. Evidence that Y-organs of the crab *Cancer antennarius* secrete 3-dehydroecdysone. *Mol. Cell. Endocrinol. 66*, 17–25.

Spindler, K.-D., **1988**. Parasites and hormones. In: Mehlhorn, H. (Ed.), Parasites. Springer, New York, pp. 465–476.

Spindler, K.-D., Koolman, J., Mosora, F., Emmerich, H., **1977**. Catalytical oxidation of ecdysteroids to 3-dehydro products and their biological activities. *J. Insect Physiol. 23*, 441–444.

Srivatsan, J., Kuwahara, T., Agosin, M., **1987**. The effect of alpha-ecdysone and phenobarbital on the alpha-ecdysone 20-monooxygenase of house fly larva. *Biochem. Biophys. Res. Commun. 148*, 1075–1080.

Stocco, D.M., **2000**. Intramitochondrial cholesterol transfer. *Biochim. Biophys. Acta 1486*, 184–197.

Stocco, D.M., **2001**. StAR protein and the regulation of steroid hormone biosynthesis. *Annu. Rev. Physiol. 63*, 193–213.

Stocco, D.M., Clark, B.J., **1996**. Regulation of the acute production of steroids in steroidogenic cells. *Endocr. Rev. 17*, 221–244.

Stolowich, N., Petrescu, A.D., Huang, H., Martin, G.G., Scott, A.I., *et al.*, **2002**. Sterol carrier protein-2: structure reveals function. *Cell. Mol. Life Sci. 59*, 193–212.

Sturaro, A., Guerriero, A., De Clauser, R., Pietra, F., **1982**. A new unexpected marine source of a molting hormone. Isolation of ecdysterone in large amounts from the zoanthid *Gerardia savaglia*. *Experientia 38*, 1184–1185.

Suksamrarn, A., Jankam, A., Tarnchompoo, B., Putchakarn, S., **2002**. Ecdysteroids from a *Zoanthus* sp. *J. Nat. Prod. 65*, 1194–1197.

Suksamrarn, A., Yingyongnarongkul, B., **1996**. Synthesis and biological activity of 2-deoxy-20-hydroxyecdysone and derivatives. *Tetrahedron 52*, 12623–12630.

Svoboda, J.A., **1999**. Variability of metabolism and function of sterols in insects. *Crit. Rev. Biochem. Mol. Biol. 34*, 49–57.

Svoboda, J.A., Agarwal, H., Robbins, W.E., Nair, A.M.G., **1980**. Lack of conversion of C_{29} phytosterols to cholesterol in the Khapra beetle, *Trogoderma granarium*. *Experientia 36*, 1029–1030.

Svoboda, J.A., Feldlaufer, M.F., **1991**. Neutral sterol metabolism in insects. *Lipids 26*, 614–618.

Svoboda, J.A., Herbert, E.W., Jr., Thompson, M.J., **1983**. Definitive evidence for lack of phytosterol dealkylation in honey bees. *Experientia 39*, 1120–1121.

Svoboda, J.A., Imberski, R.B., Lusby, W.R., **1989**. *Drosophila melanogaster* does not dealkylate [^{14}C]sitosterol. *Experientia 45*, 983–985.

Svoboda, J.A., Lusby, W.R., **1994**. Variability of sterol utilization in stored-product insects. *Experientia 50*, 72–74.

Svoboda, J.A., Thompson, M.J., **1985**. Steroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 137–175.

Svoboda, J.A., Thompson, M.J., Robbins, W.E., Elden, T.C., **1975**. Unique pathways of sterol metabolism in the Mexican bean beetle, a plant-feeding insect. *Lipids 10*, 524–527.

Svoboda, J.A., Weirich, G.F., **1995**. Sterol metabolism in the tobacco hornworm, *Manduca sexta* – a review. *Lipids 30*, 263–267.

Swevers, L., Kravariti, L., Ciolfi, S., Xenou-Kokoletsi, M., Ragousis, N., *et al.*, **2004**. A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. *FASEB J. 18*, 134–136.

Tabunoki, H., Sugiyama, H., Tanaka, Y., Fujii, H., Banno, Y., *et al.*, **2002**. Isolation, characterization and cDNA sequence of a carobenoid binding protein from the silk gland of *Bombyx mori* Larrae. *J. Biol. Chem. 277*, 32133–32140.

Takahashi, S.Y., Okamoto, K., Sonobe, H., Kamba, M., Ohnishi, E., **1992**. *In vitro* synthesis of ecdysteroid conjugates by tissue extracts of the silkworm, *Bombyx mori*. *Zool. Sci. 9*, 169–174.

Takeuchi, H., Chen, J.-H., O'Reilly, D.R., Rees, H.H., Turner, P.C., **2000**. Regulation of ecdysteroid signaling: molecular cloning, characterization and expression of 3-dehydroecdysone 3α-reductase, a novel eukaryotic member of the short-chain dehydrogenases/reductases superfamily from the cotton leafworm, *Spodoptera littoralis*. *Biochem. J. 349*, 239–245.

Takeuchi, H., Chen, J.-H., O'Reilly, D.R., Turner, P.C., Rees, H.H., **2001**. Regulation of ecdysteroid signaling: cloning and characterization of ecdysone oxidase – a novel steroid oxidase from the cotton leafworm, *Spodoptera littoralis*. *J. Biol. Chem. 276*, 26819–26828.

Tawfik, A.I., Tanaka, Y., Tanaka, S., **2002**. Possible involvement of ecdysteroids in embryonic diapause of *Locusta migratoria*. *J. Insect Physiol. 48*, 743–749.

Terentrou, P., Blattmann, M., Bradbrook, D., Kauser, G., Koolman, J., **1993**. Biological activity and receptor-binding of ecdysteroids in imaginal discs of *Calliphora vicina*: a comparison. *Insect Biochem. Mol. 23*, 131–136.

Thiboutot, D., Jabara, S., McAllister, J.M., Sivarajah, A., Gilligand, K., *et al.*, **2003**. Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-A). *J. Invest. Dermatol. 120*, 905–914.

Thiry, Y., Hoffmann, K.H., **1992**. Dynamics of ecdysone and 20-hydroxyecdysone metabolism after injection and ingestion in *Gryllus bimaculatus*. *Zool. Jahr. – Abteil. Allgemeine Zool. Jb.-Abt. Allg. Z. Physiol. Tiere 96*, 17–38.

Thompson, M.J., Feldlaufer, M.F., Lozano, R., Rees, H.H., Lusby, W.R., *et al.*, **1987a**. Metabolism of 26-[^{14}C]hydroxyecdysone 26-phosphate in the tobacco hornworm, *Manduca sexta* (L.), to a new ecdysteroid conjugate: 26-[^{14}C]-hydroxyecdysone 22-glucoside. *Arch. Insect Biochem. Physiol. 4*, 1–15.

Thompson, M.J., Kaplanis, J.N., Robbins, W.E., Dutky, S.R., Nigg, H.N., **1974**. 3-Epi-20-hydroxyecdysone from meconium of the tobbacco hornworm. *Steroids 24*, 359–366.

Thompson, M.J., Kaplanis, J.N., Robbins, W.E., Yamamoto, R.T., **1967**. 20,26-Dihydroxyecdysone, a new steroid with moulting hormone activity from the tobacco hornworm, *Manduca sexta* (Johannson). *J. Chem. Soc. Chem. Commun.* 650–653.

Thompson, M.J., Louloudes, S.J., Robbins, W.E., Waters, J.A., Steele, J.A., *et al.*, **1963**. The identity of the major sterol from house flies reared by the CSMA procedure. *J. Insect Physiol. 9*, 615–622.

Thompson, M.J., Svoboda, J.A., Feldlaufer, M.F., **1989**. Analysis of free and conjugated ecdysteroids and polar metabolites of Insects. In: Nes, W.D., Parish, E.J. (Eds.), Analysis of Sterols and Other Biologically Significant Steroids. Academic Press, San Diego, pp. 81–105.

Thompson, M.J., Svoboda, J.A., Lozano, R., Wilzer, K.R., **1988**. Profile of free and conjugated ecdysteroids and ecdysteroid acids during embryonic development of *Manduca sexta* (L.) following maternal incorporation of [^{14}C]cholesterol. *Arch. Insect Biochem. Physiol. 7*, 157–172.

Thompson, M.J., Svoboda, J.A., Lusby, W.R., Rees, H.H., Oliver, J.E., *et al.*, **1985c**. Biosynthesis of a C21 steroid conjugate in an insect. The conversion of [^{14}C]-cholesterol to 5-[^{14}C]pregnen-3β,20β-diol glucoside in the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem. 260*, 15410–15412.

Thompson, M.J., Svoboda, J.A., Rees, H.H., Wilzer, K.R., **1987b**. Isolation and identification of 26-hydroxyecdysone 2-phosphate: an ecdysteroid conjugate of eggs and ovaries of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 4*, 183–190.

Thompson, M.J., Weirich, G.F., Rees, H.H., Svoboda, J.A., Feldlaufer, M.F., *et al.*, **1985b**. New ecdysteroid conjugate: isolation and identification of 26-hydroxyecdysone 26-phosphate from eggs of the tobacco hornworm, *Manduca sexta* (L.). *Arch. Insect Biochem. Physiol. 2*, 227–236.

Thompson, M.J., Weirich, G.F., Svoboda, J.A., **1985a**. Ecdysone 3-epimerase. *Methods Enzymol. 111*, 437–442.

Thomson, J.A., Imray, F.P., Horn, D.H.S., **1970**. An improved *Calliphora* bioassay for insect moulting hormones. *Austral. J. Exp. Biol. 48*, 321–328.

Thomson, J.A., Siddall, J.B., Galbraith, M.N., Horn, D.H.S., Middleton, E.J., **1969**. The biosynthesis of ecdysone in the blowfly *Calliphora stygia*. *J. Chem. Soc. Chem. Commun.* 669–670.

Tijet, N., Helvig, C., Feyereisen, R., **2001**. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene 262*, 189–198.

Tomaschko, K.-H., **1995**. Autoradiographic and morphological investigations of the defensive ecdysteroid glands in adult *Pycnogonum litorale* (Arthropoda: Pantopoda). *Eur. J. Entomol. 92*, 105–112.

Touchstone, J.C., **1986**. Ecdysteroids. In: CRC Handbook. Chromatography. Steroids. CRC Press, Boca Raton, FL, pp. 119–135.

Tsoupras, G., Hétru, C., Luu, B., Constantin, E., Lagueux, M., *et al.*, **1983a**. Identification and metabolic fate of ovarian 22-adenosine monophosphoric ester of 2-deoxyecdysone in ovaries and eggs of an insect, *Locusta migratoria*. *Tetrahedron 39*, 1789–1796.

Tsoupras, G., Hétru, C., Luu, B., Lagueux, M., Constantin, E., *et al.*, **1982b**. The major conjugates of ecdysteroids in young eggs and in embryos of *Locusta migratoria*. *Tetrahedron Lett. 23*, 2045–2048.

Tsoupras, G., Luu, B., Hétru, C., Müller, J.F., Hoffmann, J.A., **1983b**. Conversion *in vitro* de 20-hydroxyecdysone en métabolites phosphorylés et acétylés par des complexes tube digestif-tubes de Malpighi de larves de

Locusta migratoria. *C. R. Acad. Sci. Paris, Série III 296*, 77–80.

Tsoupras, G., Luu, B., Hoffmann, J.A., **1982a**. Isolation and identification of three ecdysteroid conjugates with a C-20 hydroxyl group in eggs of *Locusta migratoria*. *Steroids 40*, 551–560.

Tsoupras, G., Luu, B., Hoffmann, J.A., **1983c**. A cytokinin (Isopentenyl-Adenosyl-Mononucleotide) linked to ecdysone in newly laid eggs of *Locusta migratoria*. *Science 220*, 507–509.

Van den Broek, P.J.A., Barroso, M., Lechner, M.C., **1996**. Critical amino-terminal segments in insertion of rat liver cytochrome P450 3A1 into the endoplasmic reticulum membrane. *Experientia 52*, 851–855.

Von Buddenbrock, W., **1931**. Untersuchungen über die Häutungshormone des Schmetterlinge. *Z. Vergleich. Physiol. 14*, 415–428.

Von Gliscynski, U., Delbecque, J.-P., Böcking, D., Sedlmeier, D., Dircksen, H., *et al.*, **1995**. Three new antisera with high sensitivity to ecdysone, 3-dehydroecdysone and other A-ring derivatives: production and characterization. *Eur. J. Entomol. 92*, 75–79.

Von Wachenfeldt, C., Johnson, E.F., **1995**. Structures of eukaryotic cytochrome P450 enzymes. In: Ortiz de Montellano, P.R. (Ed.), Cytochromes P450, Structure, Mechanism, and Biochemistry. Plenum Press, New York, pp. 183–223.

Wainwright, G., Prescott, M.C., Lomas, L.O., Webster, S.G., Rees, H.H., **1996**. Trace analysis of arthropod hormones by liquid chromatography-atmospheric pressure chemical ionization/mass spectrometry. *Biochem. Soc. Trans. 24*, 476S.

Wainwright, G., Prescott, M.C., Lomas, L.O., Webster, S.G., Rees, H.H., **1997**. Development of a new high-performance liquid chromatography-mass-spectrometric method for the analysis of ecdysteroids in biological extracts. *Arch. Insect Biochem. Physiol. 35*, 21–31.

Walker, V.K., Watson, K.L., Holden, J.A., Steel, C.H., **1987**. Vitellogenesis and fertility in *Drosophila* females with low ecdysteroid titres: The l(3)3DTS mutation. *J. Insect Physiol. 33*, 137–142.

Warren, J.T., Bachman, J.S., Dai, J.D., Gilbert, L.I., **1996**. Differential incorporation of cholesterol and cholesterol derivatives into ecdysteroids by the larval ring glands and adult ovaries of *Drosophila melanogaster:* a putative explanation for the *l(3)ecd*[1] mutation. *Insect Biochem. Mol. Biol. 26*, 931–943.

Warren, J.T., Dai, J., Gilbert, L.I., **1999**. Can the insect nervous system synthesize ecdysteroids? *Insect Biochem. Mol. Biol. 29*, 571–579.

Warren, J.T., Gilbert, L.I., **1986**. Ecdysone metabolism and distribution during the pupal-adult development of *Manduca sexta*. *Insect Biochem. 16*, 65–82.

Warren, J.T., Gilbert, L.I., **1988**. Radioimmunoassay of ecdysteroids. In: Gilbert, L.I., Miller, T.A. (Eds.), Immunological Techniques in Insect Biology. Springer-Verlag, Berlin, p. 181.

Warren, J.T., Gilbert, L.I., **1996**. Metabolism in vitro of cholesterol and 25-hydroxycholesterol by the larval prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 26*, 917–929.

Warren, J.T., Hétru, C., **1990**. Ecdysone biosynthesis: pathways, enzymes and the early steps problem. *Invert. Reprod. Devel. 18*, 91–99.

Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster. Proc. Natl Acad. Sci., USA 99*, 11043–11048.

Warren, J.T., Petryk, A., Marqués, G., Parvy, J.-P., Shinoda, T., *et al.*, **2004**. *Phantom* encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem. Mol. Biol. 34*, 991–1010.

Warren, J.T., Rybczynski, R., Gilbert, L.I., **1995**. Stereospecific, mechanism-based, suicide inhibition of a cytochrome P450 involved in ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 29*, 679–695.

Warren, J.T., Sakurai, S., Rountree, D.R., Gilbert, L.I., **1988a**. Synthesis and secretion of ecdysteroids by the prothoracic glands of *Manduca sexta*. *J. Insect Physiol. 34*, 571–576.

Warren, J.T., Sakurai, S., Rountree, D.R., Gilbert, L.I., Lee, S.-S., *et al.*, **1988b**. Regulation of the ecdysteroid titer of *Manduca sexta*: reappraisal of the role of the prothoracic glands. *Proc. Natl Acad. Sci. USA 85*, 958–962.

Warren, J.T., Steiner, B., Dorn, A., Pak, M., Gilbert, L.I., **1986**. Metabolism of ecdysteroids during the embryogenesis of *Manduca sexta*. *J. Liquid Chromatogr. 9*, 1759–1782.

Warren, J.T., Wismar, J., Subrahmanyam, B., Gilbert, L.I., **2001**. *Woc* (*without children*) gene control of ecdysone biosynthesis in *Drosophila melanogaster. Mol. Cell. Endocrinol. 181*, 1–14.

Waterham, H.R., Koster, J., Romeijn, G.J., Hennekam, R.C.M., Vreken, P., *et al.*, **2001**. Mutations in the 3β-hydroxysterol Δ^{24}-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *Am. J. Hum. Genet. 69*, 685–694.

Watson, R.D., Spaziani, E., **1982**. Rapid isolation of ecdysteroids from crustacean tissues and culture media using Sep-Pak C18 cartridges. *J. Liq. Chromatogr. 5*, 525–535.

Webb, T.J., Powls, R., Rees, H.H., **1995**. Enzymes of ecdysteroid transformation and inactivation in the midgut of the cotton leafworm, *Spodoptera littoralis*: properties and developmental profiles. *Biochem. J. 312*, 561–568.

Webb, T.J., Powls, R., Rees, H.H., **1996**. Characterization, fractionation and kinetic properties of the enzymes of ecdysteroid 3-epimerization and phosphorylation isolated from the midgut cytosol of the cotton leafworm, *Spodoptera littoralis*. *Insect Biochem. Mol. Biol. 26*, 809–816.

Webster, S.G., **1985**. Catalyzed derivatization of trimethylsilyl ethers of ecdysterone. A preliminary study. *J. Chromatogr. 333*, 186–190.

Weirich, G.F., **1989**. Enzymes involved in ecdysone metabolism. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 174–180.

Weirich, G., **1997**. Ecdysone 20-hydroxylation in *Manduca sexta*. (Lepidoptera: Sphingidae) midgut: development-related changes of mitochondrial and microsomal ecdysone 20-monooxygenase activities in the fifth larval instar. *Eur. J. Entomol. 94*, *57–65*.

Weirich, G.F., Bell, R.A., **1997**. Ecdysone 20-hydroxylation and 3-epimerization in larvae of the Gypsy Moth, *Lymantria dispar* L: tissue distribution and developmental changes. *J. Insect Physiol. 43*, 643–649.

Weirich, G.F., Feldlaufer, M.F., Svoboda, J.A., **1993**. Ecdysone oxidase and 3-oxoecdysteroid reductases in *Manduca sexta*: developmental changes and tissue distribution. *Arch. Insect Biochem. Physiol. 23*, 199–211.

Weirich, G.F., Svoboda, J.A., **1992**. 3-Oxoecdysteroid reductases in *Manduca sexta* midgut. *Arch. Insect Biochem. Physiol. 21*, 91–102.

Weirich, G.F., Svoboda, J.A., Thompson, M.J., **1984**. Ecdysone 20-monooxygenases. In: Hoffmann, J.A., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, p. 227.

Weirich, G.F., Svoboda, J.A., Thompson, M.J., **1985**. Ecdysone 20-monooxygenase in mitochondria and microsomes of *Manduca sexta* L. midgut: is the dual localization real? *Arch. Insect Biochem. Physiol. 2*, 385–396.

Weirich, G.F., Thompson, M.J., Svoboda, J.A., **1986**. *In vitro* ecdysteroid conjugation by enzymes of *Manduca sexta* midgut cytosol. *Arch. Insect Biochem. Physiol. 3*, 109–126.

Weirich, G.F., Thompson, M.J., Svoboda, J.A., **1989**. Ecdysone oxidase and 3-oxoecdysteroid reductases in *Manduca sexta* midgut: kinetic parameters. *Arch. Insect Biochem. Physiol. 12*, 201–218.

Weirich, G.F., Thompson, M.J., Svoboda, J.A., **1991**. Enzymes of ecdysteroid 3-epimerization in midgut cytosol of *Manduca sexta:* pH optima, consubstrate kinetics, and sodium chloride effect. *Insect Biochem. 21*, 65–71.

Weirich, G.F., Williams, V.P., Feldlaufer, M.F., **1996**. Ecdysone 20-hydroxylation in *Manduca sexta* midgut: kinetic parameters of mitochondrial and microsomal ecdysone 20-monooxygenases. *Arch. Insect Biochem. Physiol. 31*, 305–312.

Weltzel, J.M., Ohnishi, M., Fujita, T., Nakanishi, K., Naya, Y., *et al.*, **1992**. Diversity in steroidogenesis of symbiotic microorganisms from planthoppers. *J. Chem. Ecol. 18*, 2083–2094.

Whitehead, D.L., **1989**. Ecdysteroid carrier proteins. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. George Thieme Verlag, Stuttgart, p. 232.

Whiting, P., Dinan, L., **1989**. Identification of the endogenous apolar ecdysteroid conjugates present in newly-laid eggs of the house cricket (*Acheta domesticus*) as 22-long-chain fatty acyl esters of ecdysone. *Insect Biochem. 19*, 759–765.

Whiting, P., Sparks, S., Dinan, L., **1993**. Ecdysteroids during embryogenesis of the house cricket, *Acheta domesticus*: occurrence of novel ecdysteroid conjugates in developing eggs. *Insect Biochem. Mol. Biol. 23*, 319–329.

Whiting, P., Sparks, S., Dinan, L., **1997**. Endogenous ecdysteroid levels and rates of ecdysone acylation by intact ovaries *in vitro* in relation to ovarian development in adult female house crickets, *Acheta domesticus*. *Arch. Insect Biochem. Physiol. 35*, 279–299.

Wieschaus, E., Nüsslein-Volhard, C., Jürgens, G., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* III Zygotic loci on the X-chromosome and fourth chromosome. *Roux's Arch. Devel. Biol. 193*, 296–307.

Wigglesworth, K.P.W., Lewis, D., Rees, H.H., **1985**. Ecdysteroid titer and metabolism to novel apolar derivatives in adult female *Boophilus microplus*. *Arch. Insect Biochem. Physiol. 2*, 39–54.

Wigglesworth, V.B., **1934**. Factors controlling moulting and "metamorphosis" in an insect. *Nature 133*, 725–726.

Williams, C.M., **1968**. Ecdysone and ecdysone-analogues: their assay and action on diapausing pupae of the *Cynthia* silkworm. *Biol. Bull. 134*, 344–355.

Williams, D.R., Chen, J.-H., Fisher, M.J., Rees, H.H., **1997**. Induction of enzymes involved in molting hormone (ecdysteroid) inactivation by ecdysteroids and an agonist, 1,2-dibenzoyl-1-*tert*-butylhydrazine (RH – 5849). *J. Biol. Chem. 272*, 8427–8432.

Williams, D.R., Fisher, M.J., Rees, H.H., **2000**. Characterization of ecdysteroid 26-hydroxylase: an enzyme involved in molting hormone inactivation. *Arch. Biochem. Biophys. 376*, 389–398.

Williams, D.R., Fisher, M.J., Smagghe, G., Rees, H.H., **2002**. Species specificity of changes in ecdysteroid metabolism in response to ecdysteroid agonists. *Pest. Biochem. Physiol. 72*, 91–99.

Wilson, I.D., **1985**. Thin-layer chromatography of ecdysteroids. *J. Chromatogr. 318*, 373–377.

Wilson, I.D., **1988**. Reversed phase thin-layer chromatography of the ecdysteroids. *J. Planar Chromatogr. 1*, 116–122.

Wilson, I.D., Bielby, C.R., Morgan, E.D., **1982a**. Evaluation of some phytoecdysteroids as internal standards for the chromatographic analysis of ecdysone and 20-hydroxyecdysone in arthropods. *J. Chromatogr. 236*, 224–229.

Wilson, I.D., Bielby, C.R., Morgan, E.D., **1982b**. Studies on the reversed-phase thin-layer chromatography of ecdysteroids on C-12 bonded and paraffin-coated silica. *J. Chromatogr. 242*, 202–206.

Wilson, I.D., Lafont, R., **1986**. Thin-layer chromatography and high-performance thin-layer chromatography of [^{3}H] metabolites of 20-hydroxyecdysone. *Insect Biochem. 16*, 33–40.

Wilson, I.D., Lafont, R., Kingston, R.G., Porter, C.J., **1990a**. Thin-layer chromatography-tandem mass

spectrometry directly from the adsorbent: application to phytoecdysteroids from *Silene otites*. *J. Planar Chromatogr. 3*, 359–361.
Wilson, I.D., Lafont, R., Porter, C.J., Kingston, R.G., Longden, K., *et al.*, **1990c**. Thin-layer chromatography of ecdysteroids: detection and identification. In: McCaffery, A.R., Wilson, I.D. (Eds.), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, London, pp. 117–126.
Wilson, I.D., Lafont, R., Wall, P., **1988a**. TLC of ecdysteroids with off-line identification by fast atom bombardment mass spectrometry directly from the adsorbent. *J. Planar Chromatogr. 1*, 357–359.
Wilson, I.D., Lewis, S., **1987**. Separation of ecdysteroids by high-performance thin-layer chromatography using automated multiple development. *J. Chromatogr. 408*, 445–448.
Wilson, I.D., Morgan, E.D., Lafont, R., Wright, B., **1998**. High-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy. Application to the ecdysteroids of *Silene otites*. *J. Chromatogr. A 799*, 333–336.
Wilson, I.D., Morgan, E.D., Murphy, S.J., **1990b**. Sample preparation for the chromatographic analysis of ecdysteroids using solid phase extraction methods. *Anal. Chim. Acta 236*, 145–155.
Wilson, I.D., Morgan, E.D., Robinson, P., Lafont, R., Blais, C., **1988b**. A comparison of radio-thin-layer and radio-high-performance liquid chromatography for ecdysteroid metabolism studies. *J. Insect Physiol. 34*, 707–711.
Wilson, I.D., Scalia, S., Morgan, E.D., **1981**. Reversed-phase thin-layer chromatography for the separation and analysis of ecdysteroids. *J. Chromatogr. 212*, 211–219.
Wismar, J., Habtemichael, N., Warren, J.T., Dai, J.D., Gilbert, L.I., *et al.*, **2000**. The mutation without children (rgl) causes ecdysteroid deficiency in third-instar larvae of *Drosophila melanogaster*. *Devel. Biol. 226*, 1–17.
Wright, J.E., Thomas, B.R., **1983**. Boll weevil: determination of ecdysteroids and juvenile hormones with high pressure liquid chromatography. *J. Liq. Chromatogr. 6*, 2055–2066.
Yamada, R., Sonobe, H. **2003**. Purification, kinetic characterization, and molecular cloning of a novel enzyme ecdysteroid-phosphate phosphatase. *J. Biol. Chem. 278*, 26365–26373.
Yang, R.S., Wilkinson, C.F., **1972**. Enzymatic sulphation of *p*-nitrophenol and steroids by larval gut tissues of the southern armyworm (*Prodenia eridania* Cramer). *Biochem. J. 130*, 487–493.
Yasuda, A., Ikeda, M., Naya, Y., **1993**. Analysis of hemolymph ecdysteroids in the crayfish *Procambarus clarkii* by high-performance capillary electrophoresis. *Nippon Suisan Gakkaishi 59*, 1793–1799.
Yingyongnarongkul, B., Suksamrarn, A., **2000**. 25-Deoxyecdysteroids: synthesis and moulting hormone activity of two C-25 epimers of inokosterone. *Science Asia 26*, 15–20.
Young, N.L., **1976**. The metabolism of ^{3}H-molting hormone in *Calliphora erythrocephala* during larval development. *J. Insect Physiol. 22*, 153–155.
Yu, S.J., **1995**. Allelochemical stimulation of ecdysone 20-monooxygenase in fall armyworm larvae. *Arch. Insect Biochem. Physiol. 28*, 365–375.
Yu, S.J., **2000**. Allelochemical induction of hormone-metabolizing microsomal monooxygenases in the fall armyworm. *Zool. Studies 39*, 243–249.
Zhang, M., Kubo, I., **1992a**. Characterization of ecdysteroid 22-O-acyltransferase from tobacco budworm, *Heliothis virescens*. *Insect Biochem. Mol. Biol. 22*, 599–603.
Zhang, M., Kubo, I., **1992b**. Possible function of ecdysteroid 22-*O*-acyltransferase in the larval gut of tobacco budworm, *Heliothis virescens*. *J. Chem. Ecol. 18*, 1139–1149.
Zhang, M., Kubo, I., **1993**. Metabolic fate of ecdysteroids in larval *Bombyx mori* and *Heliothis virescens*. *Insect Biochem. Mol. Biol. 23*, 831–843.
Zhang, M., Kubo, I., **1994**. Mechanism of *Heliothis virescens* resistance to exogenous ecdysteroids. *Bioreg. Crop Protect. Pest Contr. 557*, 182–201.

Relevant Websites

http://ecdybase.org – This website contains datasheets on more than 300 different ecdysteroids and many bibliographic data.
http://drnelson.utmem.edu – A free database for cytochrome P450 homepage by Dr David Nelson, Assistant professor, Department of Biochemistry, University of Tennessee.

3.4 Ecdysteroid Agonists and Antagonists

L Dinan, University of Exeter, Exeter, UK
R E Hormann, RheoGene Inc., Norristown, PA, USA

3.4.1. Introduction

Ecdysteroids are often regarded as the poor relatives in relation to the vertebrate steroid hormones and are in fact ignored in many reviews or textbooks on steroid hormones. Ecdysone and 20-hydroxyecdysone were identified 30 years after the vertebrate steroid hormones (Butenandt and Karlson, 1954; Huber and Hoppe, 1965) and, after a brief phase where studies on ecdysteroid regulation of puffing led the way in elucidating steroid hormone control of gene expression (Clever and Karlson, 1960; Ashburner, 1972), ecdysteroid research has returned to semiobscurity amongst the majority of biochemists and endocrinologists. This is a shame in many ways. Not only are arthropods the most numerous animals on the planet, but elucidation of the multitude of forms and developments which are ecdysteroid-dependent is an intellectual and scientific challenge (see **Chapter 3.5**). In recent years, one insect species has regained some of the lost ground, i.e., *Drosophila melanogaster*, owing to its well understood genetics, the powerful molecular biological techniques which can be applied to it and, most recently, by the complete sequencing of its genome (Adams *et al*., 2000). However, we know very little about the identities of the ecdysteroids or their metabolism during development in this species (Richards, 1981; Grau and Lafont, 1994a, 1994b) (see **Chapter 3.3**). Throughout the world there is only a handful of chemists who concern themselves with ecdysteroid chemistry. And yet, Nature has been kind to us in providing a wonderful array of ecdysteroid analogs in the form of phytoecdysteroids (Dinan, 2001). These natural analogs are an excellent starting point for structure–activity investigations of the many physiological, biochemical, or pharmacological processes affected by ecdysteroids. Ecdysteroids have considerable applied potential, ranging from the development of new, environmentally more acceptable insecticides, to gene switch elicitors and possible invertebrate endocrine disruptors. In addition to compounds with ecdysteroid-like activity, compounds which block the hormonal action of ecdysteroids (antagonists) may also have a natural role in insect–plant relationships or find application as probes for ecdysteroid-regulated gene control or even in insect pest control. The pharmacology of ecdysteroids is an almost uncharted area, but the existing data indicate intriguing prospects. Ecdysteroids provide stimulating challenges in steroid chemistry and study of their modes of action will generate new perspectives in steroid endocrinology, which will also have implications for mammalian systems. It is the purpose of this review to summarize research, especially that appearing since 1985, to reveal the diversity of analogs, strategies, and potential applications involving ecdysteroid agonists and antagonists.

3.4.2. Definitions and Conventions

The term ecdysteroid was originally defined as "all compounds structurally related to ecdysone" (Goodwin *et al*., 1978). However, Lafont and Horn (1989) differentiated between true ecdysteroids and ecdysteroid-related compounds. True ecdysteroids possess a steroid nucleus with an A/B-*cis*-ring junction and a 14α-hydroxy-7-en-6-one chromophore, irrespective of their biological activity, while ecdysteroid-related compounds do not fulfil all these criteria. Zooecdysteroids are ecdysteroids present in animals, while phytoecdysteroids are those in plants. There is overlap between these categories, since the same analog can be found in both plants and animals. The conventional numbering system for ecdysteroids (and all other steroids) is shown on the structure of the C_{29} phytoecdysteroid makisterone C in **Figure 1**. A system of standardized abbreviations for ecdysteroids has been proposed (Lafont

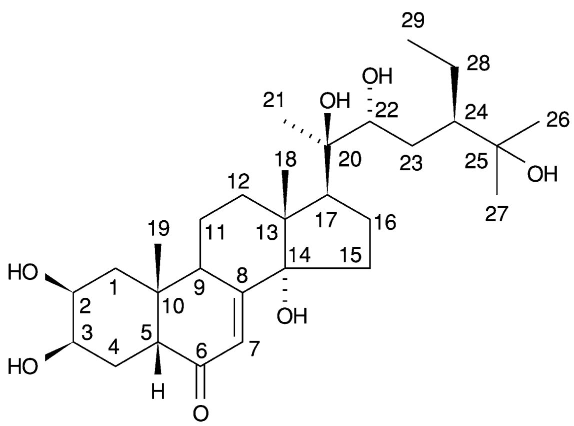


Figure 1 Structure of makisterone C showing the numbering of the carbon atoms.

et al., 1993) and is used here. We are concerned here with compounds which mimic the actions of natural ecdysteroids in specified arthropod systems (ecdysteroid agonists) or which block the actions of natural ecdysteroids in these systems (ecdysteroid antagonists). In general, we are referring to agonists and antagonists which interact with intracellular ecdysteroid receptor complexes, but it should be appreciated that there are other possible modes of action (especially for antagonists) and it is necessary to demonstrate that a particular (ant)agonist does interact with the receptor and that its affinity for the receptor should be similar to the concentration required to bring about a 50% response in the biological assay before it can be claimed as a receptor (ant)agonist. In view of the situation which prevails with certain compounds interacting with vertebrate steroid nuclear receptors, where the compound may be an agonist in one tissue, but an antagonist in another, or may be agonistic over a certain concentration range, but antagonistic over another (McDonnell *et al.*, 2002), we should be aware that a similar situation could also exist with certain ecdysteroid (ant)agonists. Such compounds would probably be called specific ecdysteroid receptor modulators (SEcRMs).

For the purposes of this review, the activity of a compound will refer to systems in which the affinity of the receptor for the compound is probably the major or only factor in determining the response. Potency will be the term used for more complicated systems, where a number of factors (i.e., penetration, sequestration, receptor affinity, metabolism, and excretion) contribute, each to a greater or lesser (and probably unknown) extent, to the magnitude of the response.

3.4.3. Studies Before 1985

3.4.3.1. Zooecdysteroids

Prior to 1985, 124 natural ecdysteroids were known, of which 27% were zooecdysteroids, 60% were phytoecdysteroids, and 13% were common to both groups (Lafont *et al.*, 2002). The zooecdysteroids fell into several categories: free ecdysteroids, polar conjugates, apolar conjugates, and ionic metabolites (ecdysonoic and 20-hydroxyecdysonoic acids). The richest sources of zooecdysteroids then known were locust (*Schistocerca gregaria* and *Locusta migratoria*) eggs, where levels reach ca. 75 $\mu g\, g^{-1}$ fresh weight (Dinan and Rees, 1981). It was generally accepted that 20-hydroxyecdysone was the hormonal molecule in invertebrate systems, although the C_{28} ecdysteroid makisterone A might replace it in systems where dealkylation of plant sterols was not fully operational (Kaplanis *et al.*, 1975; Redfern, 1984). However, the predominance of ecdysone and 2-deoxyecdysone in locust eggs and the biological activity of 3-dehydro-20-hydroxyecdysone in the induction of P1 gene transcription in fat body of *D. melanogaster* (Sommé-Martin *et al.*, 1990) indicated that other ecdysteroids might be responsible for endogenous biological activity in certain systems. The known zooecdysteroids in 1985 had mainly been isolated from insect or crustacean systems, with some compounds also being isolated and identified from coral (Guerriero and Pietra, 1985) and ecdysteroids being detected in snails (Romer, 1979), leeches (Sauber *et al.*, 1983), nematodes (Mendis *et al.*, 1983), cestodes (Mendis *et al.*, 1984), and schistosomes (Nirde *et al.*, 1983). Knowledge of the biosynthetic pathway for ecdysteroids in insects was limited to the demonstration of cholesterol as an ultimate precursor, studies on the sequence of hydroxylation reactions and studies with some stereospecifically labeled precursors (Rees, 1995). Thanks largely to the chemical synthesis of a variety of ecdysteroid analogs, the empirical understanding of ecdysteroid structure–activity studies was quite advanced in 1985 (Horn and Bergamasco, 1985). However, data had been obtained with a wide variety of *in vivo* systems, leading to a generalized understanding, which did not take account of the contributions of metabolism, sequestration, etc. to biological potency.

3.4.3.2. Phytoecdysteroids

After the almost simultaneous discovery of phytoecdysteroids by five research groups in 1966–67 (Galbraith and Horn, 1966; Nakanishi *et al.*, 1966; Heinrich and Hoffmeister, 1967; Kaplanis *et al.*, 1967; Takemoto *et al.*, 1967), a large number of analogs were identified over the next decade. Since the crystal structure of ecdysone (E) had only been finally solved in 1965 (Huber and Hoppe, 1965), the discovery of large amounts of ecdysteroids in plants was fortuitous and provided adequate amounts of certain analogs, especially 20-hydroxyecdysone (20E), for physiological and biochemical experiments on insects. Early screening studies (Imai *et al.*, 1969) indicated that ca. 6% of higher plant species contain ecdysteroids, but the concentrations vary enormously from species to species and also within the plant and during development. Ecdysteroid-containing species generally contain one or two major ecdysteroids (often 20E and polypodine B (polB)) and a plethora of minor ecdysteroids. This is reflected in the commercial price of purified ecdysteroids, since

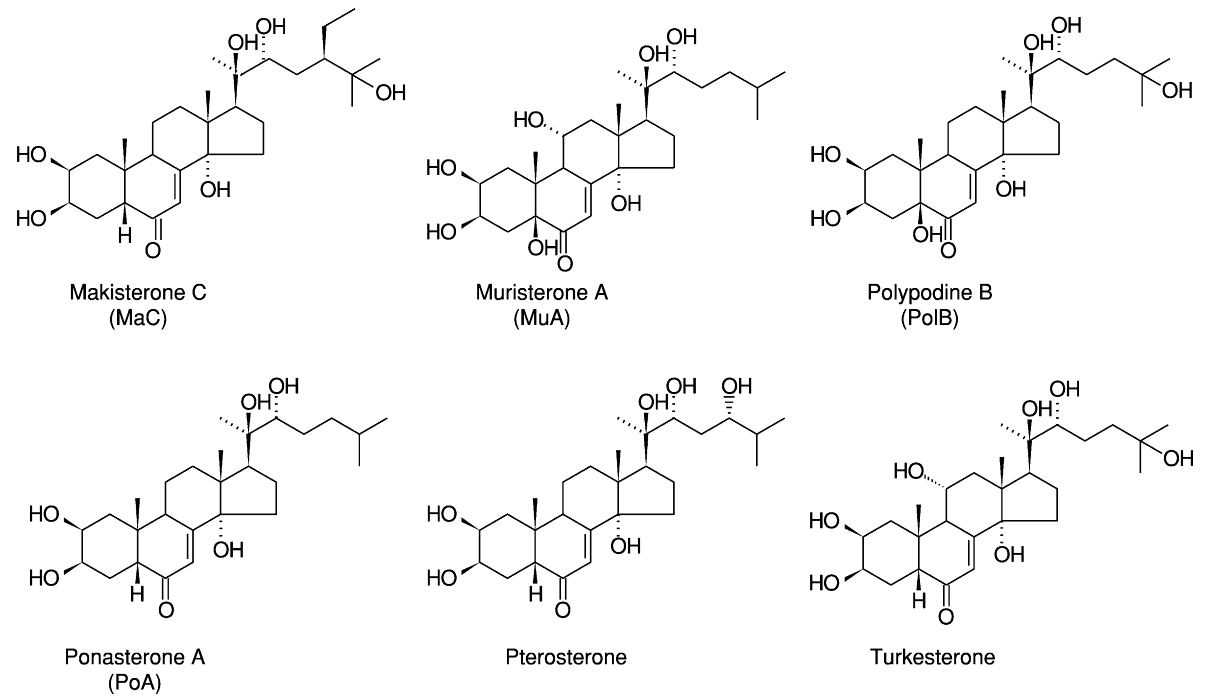


Figure 2 Commonly occurring phytoecdysteroids.

20E is the only one that is available in large amounts and at a moderate price. The structures of the most readily encountered phytoecdysteroids are given in Figure 2. It has always been appreciated that phytoecdysteroids form an excellent resource for ecdysteroid structure–activity relationship (SAR) studies, but prior to 1985 this could only be exploited in a limited way. Many of the minor ecdysteroids isolated prior to 1985 have not been isolated again since, with the result that we possess no, or very limited, information about their biological activities.

3.4.3.3. Ajugalactone

Nakanishi's group also undertook a search for "antiecdysones" in plant by using the rice stem borer *Chilo suppressalis* dipping assay to identify extracts which inhibited or delayed molting of the larvae. Ajugalactone (Figure 3) was identified as the active component from a methanol extract of *Ajuga decumbens* (Labiatae). While ajugalactone (250–500 ppm) was able to antagonize the activity of ponasterone A (PoA) (50 ppm), it had no effect against 20E (Koreeda *et al.*, 1970). The existence of a second antiecdysteroid is mentioned, which is structurally quite different and which antagonizes 20E, but not PoA. Unfortunately, no further information has been published concerning the identity or mode of action of this compound in *C. suppressalis*. In the *D. melanogaster* B_{II} bioassay, ajugalactone shows no antagonist activity against 20E or PoA (Clément *et al.*, 1993), but does possess agonist activity ($EC_{50} = 1.6 \times 10^{-7}$ M), where EC_{50} is concentration required to bring about a 50% response (Dinan *et al.*, 1999c).

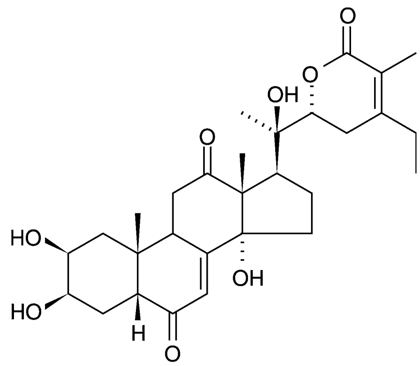
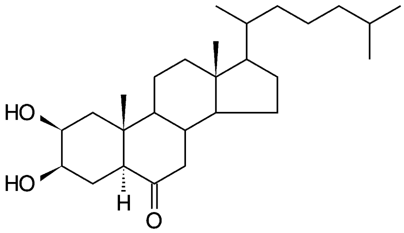
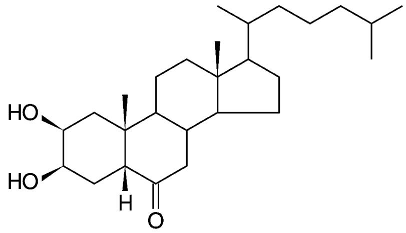


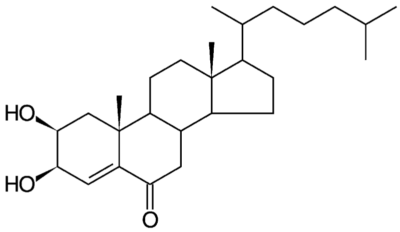
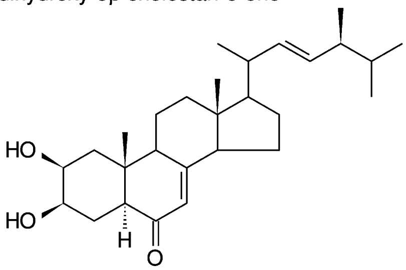
Figure 3 The structure of ajugalactone.

3.4.3.4. 6-Keto Steroids

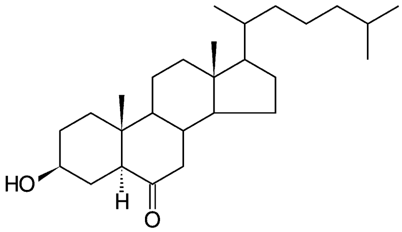
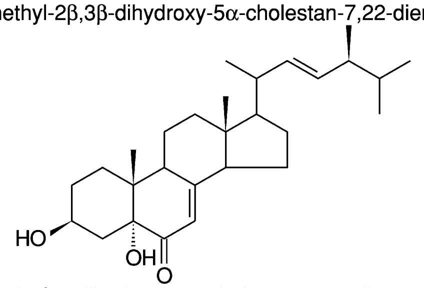
Several sterol derivatives were found to inhibit postecdysial cuticle hardening and sclerotization after injection (10–50 µg per animal) into *Pyrrhocoris apterus* (Hemiptera). The active compounds (Figure 4) all possessed a keto group at C6 and a 3β-hydroxyl. The same compounds were not active on topical application or feeding. The active compounds were believed to be ecdysteroid antagonists by the authors (Hora *et al.*, 1966; Velgová *et al.*, 1968), but no definitive evidence could be

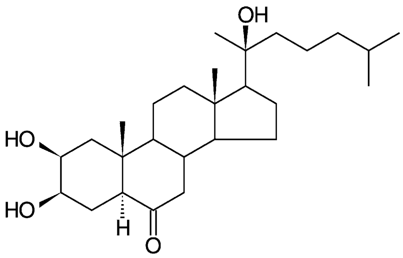
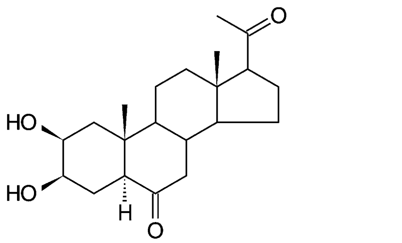
2β,3β-Dihydroxy-5α-cholestan-6-one 2β,3β-dihydroxy-5β-cholestan-6-one

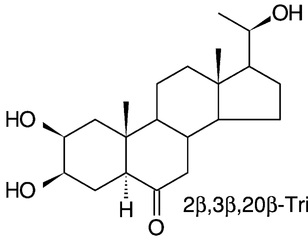
2β,3β-Dihydroxycholest-4-en-6-one 24β-methyl-2β,3β-dihydroxy-5α-cholestan-7,22-dien-6-one

3β-Hydroxy-5α-cholestan-6-one 24β-methyl-3β,5-dihydroxy-5α-cholestan-7,22-dien-6-one

2β,3β,20α-Trihydroxy-5α-cholestan-6-one 2β,3β-dihydroxy-5α-pregan-6,20-dione



2β,3β,20β-Trihydroxy-5α-pregnan-6-one

Figure 4 6-Keto steroids possessing anti-ecdysteroid activity.

provided at that time. These compounds should be reinvestigated with the currently available methods.

3.4.4. Mode of Action of Ecdysteroids

3.4.4.1. Intracellular Receptors

This topic is covered much more extensively elsewhere (see **Chapter 3.5**). Briefly, ecdysteroid intracellular receptors are members of the nuclear receptor superfamily, which have a characteristic domain structure (Kumar and Thompson, 1999). The N-terminal A/B-domain is highly variable and is associated with transcriptional activation. The C-domain is highly conserved and is involved in binding the receptor complex to specific response elements in the DNA. The D-domain is variable and represents a hinge region between the DNA-binding domain and the ligand-binding domain (E-domain).

The E-domain is not only responsible for ligand binding, but also has been implicated in receptor dimerization and interactions with other transcriptional activators. There may also be a C-terminal F-domain, which, if present, is highly variable between even closely related nuclear receptors (Kumar and Thompson, 1999). Nuclear receptors regulate gene expression as monomers or, more frequently, as dimers, either as homodimers or as heterodimers with another member of the nuclear receptor superfamily. The ecdysteroid receptor (EcR) protein forms heterodimers with Ultraspiracle (USP) (Oro *et al.*, 1990), a homolog of the vertebrate nuclear receptor RXR. Only the EcR : USP (or EcR : RXR) (Yao *et al.*, 1993) complexes are able to bind ecdysteroid with very high affinity and the presence of ecdysteroid promotes complex formation with the ecdysteroid binding to the EcR protein. For example, the affinity (K_d) for PoA of *Chilo suppressalis* EcR alone is 55 nM, which is enhanced to 1.2 nM in the presence of USP (Minakuchi *et al.*, 2003). No definitive ligand for USP has been identified, but it has been suggested that juvenile hormones (or methyl farnesoate in Crustacea) (see **Chapter 3.16**) may bind to this receptor component and modify the transactivational capacity of the complex (Jones and Sharp, 1997; Jones and Jones, 2000; Xu *et al.*, 2002).

The most extensively studied ecdysteroid receptor system in arthropods is that of *D. melanogaster*, where three isoforms (A, B1, and B2) of EcR occur (Koelle *et al.*, 1991; Talbot *et al.*, 1993). These isoforms arise through alternative promoter usage and differential splicing, resulting in different A/B-domains, but they all possess common DNA and ligand binding domains. The EcR isoforms show tissue and stage specificity. Although there is only one form of USP in *D. melanogaster*, two or more isoforms have been found in other arthropods. USP isoforms also show tissue and stage specificity (Kapitskaya *et al.*, 1996). EcR and USP gene homologs have now been characterized from a variety of arthropod species.

The biochemical characterization of intracellular ecdysteroid receptor complexes lags well behind that of vertebrate steroid hormone receptors and has been in a period of quiescence for the past decade (Luo *et al.*, 1991), as emphasis has been placed on the characterization and expression of the *EcR* and *USP* genes. The generally accepted ligand for ecdysteroid receptors in arthropods is 20E, but this does not preclude other ecdysteroids being significant at particular stages of development or in certain tissues (Wang *et al.*, 2000). *In vitro*, at least, EcR complexes recognize a wide range of ecdysteroidal and nonsteroidal analogs, as agonists or antagonists (see below).

3.4.4.2. Nongenomic Actions

In addition to the actions mediated by intracellular receptors, steroid hormones also demonstrate certain rapid (occurring within seconds) actions, which appear to be mediated by membrane effects/receptors. This is the case for vertebrate (Falkenstein *et al.*, 2000) and invertebrate (Tomaschko, 1999) steroids. One presumes that the SAR studies for ecdysteroids which have been performed to date are predominantly determined by interaction of ecdysteroids with nuclear receptors, but one should bear in mind that in certain assay systems the measured activity may be modulated by interaction with nongenomic sites.

3.4.4.3. Taste Receptors

Certain insect species have the ability to perceive ecdysteroids in their diets (Ma, 1969, 1972; Schoonhoven and Derksen-Koppers, 1973). This is probably a general phenomenon for many phytophagous insect species, since phytoecdysteroids are widespread in the plant world (Dinan, 1998). Only recently have more extensive electrophysiological studies on ecdysteroid taste receptor cells been performed (Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002), but very little is currently known about their specificity (Darazy-Choubaya *et al.*, unpublished data). This will clearly be an important area for future research, if ecdysteroids (or their analogs) are to be developed further as crop protection agents.

3.4.5. Strategy for the Identification of Ecdysteroid Agonists and Antagonists at Exeter University

3.4.5.1. Strategy

In 1994, as we started our search for ecdysteroid agonists and antagonists, approximately 200 zooecdysteroids and phytoecdysteroids were known as natural products (Lafont *et al.*, 2002), the diacylhydrazines had been identified as synthetic nonsteroidal agonists (Wing, 1988; Wing *et al.*, 1988), but no unambiguous antagonists were known. We reasoned that plants would be a good potential source of ecdysteroid antagonists because of their need to protect themselves against invertebrate predators. Almost as a byproduct of the research, further agonists (phytoecdysteroids and nonsteroidal compounds) could be identified. The plant natural products should come from two sources, plant extracts

and purified compounds. Several researchers generously provided samples of phytochemicals which they had isolated and identified for assessment of their activities. This approach led to the identification of withanolides (Dinan *et al*., 1996) and phenylalkanoids (Dinan *et al*., 2001a) as ecdysteroid antagonists and is facilitating SAR studies on several classes of molecules. Since there are estimated to be over 250 000 species of higher plants, it was clear from the start that a representative survey of the plant world would require the analysis of several thousand species. In the event, screening was done for just over 5000 species (ca. 2% of the total). To obtain this number of plant samples we took advantage of the British love for gardening and obtained seed samples from two commercial seed suppliers (Chiltern Seeds, Ulverston, Cumbria, UK and B&T World Seeds, Olonzac, France) and the seed-exchange scheme operated by botanical gardens throughout the world. The focus was put on extracts of seeds simply because there was no space or staff resources to grow and harvest plants of thousands of species, although, subsequently, the distribution of ecdysteroid agonists and antagonists in several hundred of the species was examined (Dinan *et al*., 2001b, and unpublished data). It was also imperative to miniaturize the extraction and initial analysis procedure in order to maximize the throughput. Initial analysis was to be by bioassay (see below) and ecdysteroid-specific radioimmunoassay (RIA), both of which are very sensitive techniques. Therefore crushed seed samples, each weighing ca. 25 mg were extracted with methanol (3 × 1 ml, each for 3 h at 55 °C) and the pooled methanol extracts were diluted with water (1.3 ml) and partitioned against hexane (2 × 2 ml) to remove nonpolar lipids and pigments. The extraction procedure could thus be conveniently performed in Eppendorf vials and one could prepare extracts of 120 samples in 1.5 to 2 days. Initial analyses in two or three ecdysteroid-specific RIAs (DBL-1, Black and White antisera; all generous gifts from Prof. Jan Koolman, University of Marburg, Germany) and B_{II} bioassay (agonist and antagonist versions) of the neat, 10-fold, and 100-fold diluted extracts could reveal five classes of extract (Dinan *et al*., 1999c):

- negative in RIA and bioassay (ca. 90% of seed extracts); not examined further
- positive in agonist bioassay and/or RIA (ca. 5% of seed extracts); phytoecdysteroid-containing
- positive in the agonist bioassay, but negative in the RIA (two detected); nonsteroidal agonist-containing
- antagonist bioassay positive (ca. 3% of extracts); ecdysteroid antagonist-containing.
- A proportion of the neat extracts (5-10%) were cytotoxic and these were tested at greater dilution to determine if the cytoxicity was masking agonistic or antagonistic activities.

Aliquots of significantly positive antagonistic extracts were separated on reversed-phase C_{18} solid-phase extraction (SPE) cartridges to provide preliminary information about polarity of the active compounds. For several extracts, activity disappeared after SPE separation, so activity was probably a consequence of the synergistic activity of compounds of different polarities. Such extracts have not yet been examined further. Agonist/phytoecdysteroid-positive and strong antagonist extracts were separated on a standardized wide-polarity reversed-phase gradient system (25 cm × 4.6 mm i.d. Spherisorb ODS-2 column, 5 µm particle size, eluted at 1 ml min^{-1} with a linear gradient from 30% methanol/water to 100% methanol over 30 min, followed by 10 min at 100% methanol; monitored at 242 nm), which had been characterized by the determination of the retention times of a large number of phytoecdysteroid reference compounds (Dinan, 1995a). Fractions of 1 min duration were collected and analyzed by RIA and/or bioassay as appropriate. This served three major purposes: (1) it revealed how complex the biological activity profile in the sample was, (2) it provided initial information on the polarity of the active principles, and (3) it served as a dereplication procedure, so that the same compounds were not repeatedly extracted from different sources. All this information could be obtained from the initial extract of 25 mg seeds. If the initial analyses indicated the presence of novel or potent compounds, a larger batch of seeds (10–20 g) was extracted in order to isolate the compounds of interest.

A second strand to the strategy has been the assessment of isolated natural products to determine if they possess ecdysteroid agonist or antagonist activities. Many thousands of plant natural products have been isolated and identified, but most have not been assessed for any form of biological activity. Through the generosity of other researchers in providing samples, purchase and our own isolations, we have been able to assess several hundred pure compounds for agonist and antagonist activities in the B_{II} bioassay. These compounds represent members of the following classes of compounds: alkaloids, brassinosteroids, bufadienolides, cardenolides, cucurbitacins, diterpenes, ecdysteroids, flavonoids, lignans, limonoids, phenylalkanoids,

saponins, sesquiterpenes, vertebrate steroids, with asteroids (Dinan *et al.*, 2001a).

3.4.5.2. B_{II} Bioassay

Central to the success of the above strategy was the development of the B_{II} bioassay. Previous bioassays (review: Dinan, 1989), while effective, were too cumbersome and time-consuming for the rapid screening of many hundreds of samples. The *D. melanogaster* B_{II} cell line derived from hemocytes of a tumorous blood cell mutant (*l[2]mbn*) is ecdysteroid-responsive (Gateff *et al.*, 1980). Hemocytes contribute to the remodeling of larval tissues during metamorphosis by degrading those tissues which do not survive to adulthood. In the *l(2)mbn* mutant, the hemocytes are no longer able to recognize which tissues should remain undegraded. In the cell line derived from this mutant, the addition of ecdysteroids acts as a signal to induce phagocytosis and the cells develop from a uniform layer of small cells to form clumps of larger cells, surrounded by clear areas. This response lends itself to turbidometric quantification. It could be rapidly established that the B_{II} cells would grow readily in 96-well microtiter plates and that the bioassay, in principle, could work. However, validating the bioassay in terms of the crucial aspects of specificity, sensitivity, and robustness took much longer (Clément and Dinan, 1991; Clément *et al.*, 1993). It has been already shown that the B_{II} cells do not metabolize E, 20E, or PoA (Dinan, 1985). Extracts or purified compounds are assessed for their agonist activity by determining, if they can induce the characteristic ecdysteroid-specific response (cell clumping). Antagonistic activity is revealed by the ability to inhibit the clumping induced by 20E (5×10^{-8} M). Extracts/compounds/chromatography fractions are added to the wells of the microtiter plate and the solvent allowed to evaporate under sterile conditions. B_{II} cells (200 μl of 5×10^5 cells ml^{-1} in Schneider's medium containing 10% heat-inactivated fetal calf serum) are added to the wells. The microtiter plates are covered and stored in a humid environment at 25 °C for 7 days. The response is then quantified by reading the plate in a microplate reader (at 405 nm) and the response verified by examining the cells *in situ* with an inverted microscope. The latter differentiates between antagonistic and cytotoxic responses. This bioassay has the potential to be automated. The sensitivity of the assay is 0.5 ng 20E equivalents per well with a maximal response at 5×10^{-7} M 20E equivalents (50 ng per well). The inter- and intra-assay variation is <5% (Dinan *et al.*, 2001d). Consequently, it is possible to use the bioassay to quantify the relative potencies of active compounds, leading to the application of the bioassay to quantitative SAR (QSAR) (Dinan *et al.*, 1999c; Ravi *et al.*, 2001; Hormann *et al.*, 2003; Dinan, 2003). The EC_{50} for 20E is 7.5×10^{-9} M.

3.4.6. Agonists

3.4.6.1. Zooecdysteroids

The structures of the zooecdysteroids identified since 1985 can be accessed from http://www.ecdybase.org, together with information on sources, physicochemical properties, and citations for the first descriptions. Most novel ecdysteroids in recent years have been identified from noninsect invertebrates. The number of new zooecdysteroids identified in the last two decades (42) is rather few, reflecting the lower emphasis placed on steroid biochemistry in the last decades and the greater focus on molecular biology. The structural diversity of known zooecdysteroids (present in arthropods, other invertebrates or as metabolites of exogenous ecdysteroids in vertebrates) can be summarized as follows (based on a cholestane skeleton):

- hydroxyl groups at 2β, 3α, 3β, 5α, 5β, 6α, 11α, 14α, 16β, 20*R*, 22*R*, 23α, 25, 26/27
- keto groups at C3, C6
- unsaturation: $\Delta^{7(8)}$, $\Delta^{4(5)}$, $\Delta^{14(15)}$, $\Delta^{22(23)}$
- methyl: 24α, 24β
- ethyl: 24β
- side chain cleavage: C20/C22, C17/C20
- sequential oxidation of hydroxyl to aldehyde and carboxyl: C26
- acetoxy: 2β, 3β, 22
- long-chain fatty acid ester: C22
- glycolic acid ester: C22
- 4-hydroxybutanoic acid ester: C25
- phosphoryl: 2β, 3β, 3α, 22, 26
- sulphate ester: 26
- glucosyl: 22, 25
- glycolyl: C22
- A/B junction: *cis*, *trans*.

3.4.6.2. Phytoecdysteroids

Significantly more new phytoecdysteroids have been identified since 1985 than zooecdysteroids (http://www.ecdybase.org). The website provides chemical, spectroscopic, chromatographic, and biological information about every known ecdysteroid analog. Approximately 250 phytoecdysteroids are currently known. Researchers identifying new analogs are encouraged to submit their data to the website for inclusion. Recent advances in biological aspects of phytoecdysteroids have been reviewed

(Dinan, 2001) and are also covered elsewhere in this volume (see **Chapter 3.3**). The structural diversity of phytoecdysteroids can be summarized as follows:

- hydroxyl groups: 1α, 1β, 2α, 2β, 2α, 3β, 3α, 5β, 6α, 9α, 11α, 11β, 12β, 14α, 16β, 17α, 17β, C19, 20*R*, C21, 22*R*, 23α, 23ξ, 24α, 24β, 25, 26/27, C28, C29
- keto groups: C2, C3, C6, C7, C12, C17, C20, C22
- epoxy groups: 22α/23β
- unsaturation: $\Delta^{7(8)}$, $\Delta^{4(5)}$, $\Delta^{5(6)}$, $\Delta^{8(9)}$, $\Delta^{9(11)}$, $\Delta^{14(15)}$, $\Delta^{20(21)}$, $\Delta^{22(23)}$, $\Delta^{24(25)}$, $\Delta^{24(28)}$, $\Delta^{25(26/27)}$
- methyl: 4α, 23α, 24α, 24β
- ethyl: 23ξ, 24β
- side chain cleavage: C20/C22, C17/C20
- lactone rings: C24/C20 five-membered, C26/C22 six-membered, C26/C28 five-membered, C26/C29 six-membered, C29/C25 five-membered
- carboxylic acid methyl ester: C26
- side chain ether: C25/C22, C26/C22
- acetoxy: 2β, 3α, 3β, 20, 22, 25
- benzoyl: 2β, 3β, 20, 22, 25
- benzylidene acetal: C20/C22
- cinnamoyl: 2β
- coumaroyl: 3β
- crotonic acid ester: 3β
- 3-hydroxybutanoic acid ester: 22
- methoxy: 25
- phosphoryl: 3β
- pyrrole 2-carboxylic acid ester: 24ξ
- sulfuryl: 22
- glycosyl (glucosyl, galactosyl, xylosyl): 2β, 3β, 22, 24, 25
- acetonide: C2/C3, C20/C22
- A/B-junction: *cis*, *trans*.

3.4.6.3. Biological Activities of Ecdysteroids

The published data for the biological activities of ecdysteroids in a variety of arthropod bioassay systems have been reviewed recently (Dinan, 2003) and are included in Ecdybase. The most extensive set of biological activity data for ecdysteroids has been determined with the B_{II} bioassay. This data set (**Table 1**) is unique in its scope, the verification of the purity of the compounds and that it derives from a homogeneous assay. Less extensive data sets are available for the *in vitro* Kc cell bioassay (Cherbas *et al.*, 1980) and the *in vivo Calliphora*, *Musca*, and *Chilo* bioassays (review: Dinan, 2003).

In addition to naturally occurring ecdysteroids, the range of analogs for biological testing can be extended by chemical synthesis. This approach can be used to generate natural compounds that are not readily available (e.g., Suksamrarn *et al.*, 1994; Roussel *et al.*, 1995; Suksamrarn and Yingyongnarongkul, 1997) or for the preparation of analogs that are not present in nature, but which would be informative in generating SAR models (e.g., Harmatha and Dinan, 1997; Harmatha *et al.*, 2002) or in testing their predictions (e.g., Suksamrarn *et al.*, 2002; Dinan *et al.*, 2003). Conclusions deriving from SAR and molecular modeling studies are discussed below (see Section 3.4.9). The most active ecdysteroids discovered to date are PoA ($EC_{50} = 3.1 \times 10^{-10}$ M in the B_{II} bioassay), canescensterone ($EC_{50} = 5.3 \times 10^{-10}$ M in the B_{II} bioassay) (Dinan, 2003), 26-iodoponasterone A (9.0×10^{-10} M in the Kc cell assay) (Lee *et al.*, 1991) and 14-deoxymuristerone A (1.8×10^{-10} M in the Kc cell assay) (Cherbas *et al.*, 1980). Essentially all ecdysteroids show some biological activity in the B_{II} bioassay, with activities ranging over six orders of magnitude.

3.4.6.4. Brassinosteroids

Owing to certain structural similarities between the brassinosteroids and ecdysteroids, it was suggested that each group may demonstrate the biological activities of the other group. Both groups of steroids are polyhydroxylated and retain the full sterol side chain. However, the location and orientation of the hydroxyl groups (2α,3α- and 22,23-diols in brassinosteroids and 2β,3β- and 20*R*,22*R*-diols in ecdysteroids) and the configuration at C5 (5α-H in brassinosteroids and 5β-H in ecdysteroids) differ between the two steroid groups. Both groups possess a keto group at C6, but in ecdysteroids this is part of the characteristic 14α-hydroxy-7-en-6-one chromophore, while in brassinosteroids it may be part of a lactone.

The ability of seven brassinosteroids to induce the evagination of imaginal discs of *Phormia terranovae* has been assessed. Two of them (homodolicholide and castasterone) were able weakly to induce evagination at 10^{-4} M, while all the seven were able to inhibit the evagination induced by 20E. Castasterone and 22*S*,23*S*-homobrassinolide (**Figure 5**) were the most effective, but even here 5×10^{-5} M brassinosteroid was required to antagonize 5×10^{-7} M 20E fully (Hetru *et al.*, 1986). Lehmann *et al.* (1988) demonstrated that 22*S*,23*S*-homocastasterone and 22*S*,23*S*-homobrassinolide could compete with specifically bound [^{3}H]PoA associated with an ecdysteroid receptor preparation from *Calliphora vicina*. The K_d value for homocastasterone was calculated to be 5×10^{-6} M, with that of homobrassinolide being about 10-fold higher. These two brassinosteroids also retard molting in last instar nymphs of *Periplaneta americana* (Richter *et al.*, 1987), but demonstrate an ecdysteroid-like neurotropic activity

Table 1 Agonistic potencies of ecdysteroids in the *Drosophila melanogaster* B_{II} cell assay. Where compounds show no activity at 10^{-4} M, they are described as ''inactive''. Where the solubility is lower than 10^{-4} M, the activity is given as >[maximal solubility]

Ecdysteroid [a]	EC_{50} *(M)*	*Reference*
Abutasterone	1.4×10^{-7}	Clément *et al.* (1993), Dinan *et al.* (2003)
Ajugalactone	1.6×10^{-7}	Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
Ajugasterone B	3.3×10^{-7}	Dinan *et al.* (unpublished data)
Ajugasterone C	3.0×10^{-8}	Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001), Harmatha *et al.* (2002)
	8.0×10^{-8}	Harmatha and Dinan (1997), Dinan *et al.* (1999a), Ravi *et al.* (2001), Bourne *et al.* (2002)
	6.2×10^{-8}	Odnikov *et al.* (2002)
Ajugasterone C dimer	8.2×10^{-7}	Dinan *et al.* (unpublished data)
Amarasterone A	5.6×10^{-7}	Dinan *et al.* (unpublished data)
Atrotosterone A	2.5×10^{-8}	Harmatha and Dinan (1997)
Atrotosterone B	6.5×10^{-6}	Harmatha and Dinan (1997)
Atrotosterone C	3.0×10^{-6}	Harmatha and Dinan (1997), Dinan *et al.* (1999a), Ravi *et al.* (2001)
Bombycosterol	$>2.7 \times 10^{-5}$	Dinan *et al.* (1999a)
Calonysterone	Inactive	Dinan *et al.* (1999a)
Canescensterone	5.3×10^{-10}	Dinan *et al.* (unpublished data)
Carthamosterone	4.4×10^{-7}	Dinan *et al.* (unpublished data)
Castasterone	$>10^{-3}$	Dinan *et al.* (1999a)
Cheilanthone B	1.3×10^{-7}	Dinan *et al.* (unpublished data)
Cyasterone	5.3×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	1.2×10^{-8}	Dinan *et al.* (2003)
Cyasterone 3-acetate	4.3×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Dacryhainansterone	5.2×10^{-9}	Bourne *et al.* (2002)
2-Dansyl-20-hydroxyecdysone	2.2×10^{-6}	Dinan *et al.* (unpublished data)
24(28)-Dehydroamarasterone B	5.2×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
3-Dehydro-2-deoxyecdysone (silenosterone)	4.6×10^{-5}	Dinan *et al.* (unpublished data)
3-Dehydro-2-deoxyecdysone 22-acetate	4.0×10^{-5}	Dinan *et al.* (unpublished data)
3-Dehydroecdysone	6.0×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22-Dehydroecdysone	4.5×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2-Dehydro-3-*epi*-20-hydroxyecdysone	4.0×10^{-7}	Ravi *et al.* (2001), Hormann *et al.* (2003), Sarker *et al.* (1998)
4-Dehydro-20-hydroxyecdysone	2.8×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22-Dehydro-12-hydroxycyasterone	1.3×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22-Dehydro-12-hydroxynorsengosterone	1.3×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22-Dehydro-12-hydroxysengosterone	2.7×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22-Dehydro-20-hydroxyecdysone	1.7×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
24(28)[*Z*]-Dehydro-29-hydroxymakisterone C	2.2×10^{-8}	Dinan *et al.* (unpublished data)
22-Dehydro-20-*iso*-ecdysone	3.0×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
24(28)-Dehydromakisterone A	4.0×10^{-9}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
24(25)-Dehydroprecyasterone	7.3×10^{-8}	Dinan *et al.* (unpublished data)
14-Dehydroshidasterone	1.5×10^{-5}	Dinan *et al.* (unpublished data)
14-Deoxy-14,18-cyclo-20-hydroxyecdysone	6.3×10^{-7}	Dinan *et al.* (unpublished data)
2-Deoxy-20,26-dihydroxyecdysone	5.3×10^{-6}	Dinan *et al.* (unpublished data)
3-Deoxy-1β,20-dihydroxyecdysone	1.0×10^{-7}	Dinan *et al.* (unpublished data)
2-Deoxyecdysone	5.0×10^{-5}	Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
	2.0×10^{-5}	Dinan *et al.* (unpublished data)
2-Deoxyecdysone 22-glucoside	2.1×10^{-5}	Dinan *et al.* (unpublished data)
22-Deoxyecdysone	ca. 10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
25-Deoxyecdysone	1.0×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2-Deoxyecdysone 22-acetate	1.4×10^{-6}	Clément *et al.* (1993), Dinan *et al.* (1999a), Ravi *et al.* (2001)
2-Deoxyecdysone 3.22-diacetate	$>5 \times 10^{-5}$	Dinan *et al.* (unpublished data)
2-Deoxyecdysone 25-glucoside	7.3×10^{-6}	Dinan *et al.* (unpublished data)
(20*R*)22-Deoxy-20,21-dihydroxyecdysone	2.0×10^{-7}	Ravi *et al.* (2001), Dinan *et al.* (1999b), Hormann *et al.* (2003)
(14α-H) 14-Deoxy-25-hydroxydacryhainansterone	2.2×10^{-7}	Bourne *et al.* (2002)
(14β-H)14-Deoxy-25-hydroxydacryhainansterone	1.3×10^{-6}	Bourne *et al.* (2002)
2-Deoxy-20-hydroxyecdysone	6.6×10^{-7}	Clément *et al.* (1993), Dinan *et al.* (1999a), Ravi *et al.* (2001)
	1.3×10^{-6}	Dinan *et al.* (unpublished data)
2-Deoxy-21-hydroxyecdysone	4.3×10^{-6}	Ravi *et al.* (2001)
(5α-H)2-Deoxy-21-hydroxyecdysone	9.5×10^{-5}	Ravi *et al.* (2001)

Table 1 Continued

Ecdysteroid[a]	*EC$_{50}$ (M)*	*Reference*
2-Deoxy-20-hydroxyecdysone 3-acetate	4.3×10^{-6}	Ravi *et al.* (2001)
2-Deoxy-20-hydroxyecdysone 22-acetate	2.4×10^{-5}	Ravi *et al.* (2001)
2-Deoxy-20-hydroxyecdysone 22-benzoate	6.3×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2-Deoxy-20-hydroxyecdysone 3,22-diacetate	Inactive	Ravi *et al.* (2001)
14-Deoxy(14α-H)-20-hydroxyecdysone	3.0×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	2.2×10^{-8}	Harmatha *et al.* (2002)
14-Deoxy(14β-H)-20-hydroxyecdysone	8.3×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001), Harmatha *et al.* (2002)
22-Deoxy-20-hydroxyecdysone (taxisterone)	1.4×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	9.5×10^{-8}	Odnikov *et al.* (2002)
2-Deoxyintegristerone A	7.0×10^{-6}	Dinan *et al.* (unpublished data)
(5α-H)2-Deoxyintegristerone A	5.0×10^{-6}	Dinan *et al.* (unpublished data)
(5α-H)22-Deoxyintegristerone A	8.0×10^{-8}	Dinan *et al.* (unpublished data)
5-Deoxykaladasterone	5.2×10^{-10}	Clément *et al.* (1993), Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
25-Deoxypolypodine B	1.0×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2-Deoxypolypodine B 3-glucoside	1.6×10^{-5}	Dinan *et al.* (unpublished data)
24,25-Didehydrodacryhaninansterone	2.2×10^{-8}	Dinan *et al.* (unpublished data)
25,26-Didehydrodacryhainansterone	2.7×10^{-8}	Dinan *et al.* (unpublished data)
25,26-Didehydroponasterone A	1.5×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22,25-Dideoxyecdysone	ca. 10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2,22-Dideoxy-20-hydroxyecdysone	ca. 5×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
22,23-Di-*epi*-geradiasterone	4.0×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Dihydropoststerone (two isomers)	7.4×10^{-4}, 1.2×10^{-4}	Dinan *et al.* (unpublished data)
Dihydropoststerone 2,3-acetonide	Inactive	Dinan *et al.* (unpublished data)
(20*S*)Dihydropoststerone 2,3,20-tribenzoate	Inactive	Dinan *et al.* (unpublished data)
(20*R*)Dihydropoststerone 2,3,20-tribenzoate	2.5×10^{-4}	Dinan *et al.* (unpublished data)
(5α-H)Dihydrorubrosterone	5.6×10^{-6}	Ravi *et al.* (2001)
(5β-H)Dihydrorubrosterone	$>10^{-5}$	Ravi *et al.* (2001)
Dihydrorubrosterone 17β-acetate	$>10^{-4}$	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
9,20-Dihydroxyecdysone	1.6×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
20,26-Dihydroxyecdysone (podecdysone C)	7.3×10^{-6}	Dinan *et al.* (unpublished data)
(22*S*)20-(2,2′-dimethylfuranyl)Ecdysone	7.3×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
(22*R*)20-(2,2′-dimethylfuranyl)Ecdysone	1.0×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Ecdysone	2.0×10^{-7}	Clément *et al.* (1993), Harmatha and Dinan (1997)
	1.1×10^{-6}	Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
	1.0×10^{-6}	Dinan *et al.* (unpublished data)
Ecdysone 2,3-acetonide	$>3.0 \times 10^{-5}$	Dinan *et al.* (1999a)
Ecdysone 6-carboxymethyloxime	5.0×10^{-7}	Clément *et al.* (1993)
Ecdysone 22-hemisuccinate	7.5×10^{-6}	Clément *et al.* (1993)
Ecdysone 22-myristate	1.4×10^{-5}	Dinan *et al.* (unpublished data)
24-*epi*-Abutasterone	1.2×10^{-6}	Ravi *et al.* (2001), Hormann *et al.* (2003)
24-*epi*-Castasterone	$>10^{-4}$	Dinan *et al.* (1999a)
22-*epi*-Ecdysone	3.5×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	5.3×10^{-6}	Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
3-*epi*-20-Hydroxyecdysone (coronatasterone)	1.3×10^{-7}	Ravi *et al.* (2001)
	1.6×10^{-7}	Odnikov *et al.* (2002)
14-*epi*-20-Hydroxyecdysone 2,3-acetonide	7.0×10^{-7}	Dinan *et al.* (unpublished data)
14-*epi*-20-Hydroxyecdysone 2,3;20,22-diacetonide	1.6×10^{-6}	Dinan *et al.* (unpublished data)
1-*epi*-Integristerone A	2.5×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
3-*epi*-22-*iso*-Ecdysone	4.4×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
14-*epi*-20-Hydroxyecdysone	1.7×10^{-4}	Harmatha *et al.* (2002)
24-*epi*-Makisterone A	2.2×10^{-7}	Harmatha and Dinan (1997), Dinan *et al.* (1999a), Ravi *et al.* (2001)
9α,14α-Epoxy-20-hydroxyecdysone	1.4×10^{-6}	Dinan *et al.* (unpublished data)
9β,14β-Epoxy-20-hydroxyecdysone	2.2×10^{-7}	Dinan *et al.* (unpublished data)
9α,14α-Epoxy-20-hydroxyecdysone 2,3-acetonide	$>10^{-4}$	Dinan *et al.* (unpublished data)
25-Fluoropodecdysone B	7.2×10^{-9}	Dinan *et al.* (unpublished data)
25-Fluoropolypodine B	4.7×10^{-8}	Dinan *et al.* (unpublished data)
25-Fluoroponasterone A	5.1×10^{-9}	Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)

Continued

Table 1 Continued

Ecdysteroid [a]	*EC₅₀ (M)*	*Reference*
Geradiasterone	4.0×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2β,3β,9α,20*R*,22*R*,25-Hexahydroxy-5β-cholest-7,14-dien-6-one	1.8×10^{-7}	Dinan *et al.* (unpublished data)
28-Homobrassinolide	$>10^{-4}$	Dinan *et al.* (1999a)
14α-Hydroperoxy-20-hydroxyecdysone	1.6×10^{-8}	Harmatha *et al.* (2002)
5β-Hydroxyabutasterone	2.3×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
25-Hydroxyatrotosterone A	1.0×10^{-5}	Harmatha and Dinan, 1997
25-Hydroxyatrotosterone B	5.0×10^{-5}	Harmatha and Dinan, 1997
24-Hydroxycyasterone	2.0×10^{-6}	Dinan *et al.* (unpublished data)
25-Hydroxydacryhainansterone	2.6×10^{-7}	Bourne *et al.* (2002)
5β-Hydroxy-25,26-didehydroponasterone A	6.3×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone	7.6×10^{-9}	Clément *et al.* (1993)
	7.5×10^{-9}	Harmatha and Dinan 1997; Dinan *et al.* (1999a), 2003; Ravi *et al.* (2001); Harmatha *et al.* (2002), Bourne *et al.* (2002)
(5α-H)20-Hydroxyecdysone	3.3×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 2-acetate	4.0×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 3-acetate	4.7×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 22-acetate	4.0×10^{-6}, 2.0×10^{-7}	Ravi *et al.* (2001)
20-Hydroxyecdysone 2,3-acetonide	1.5×10^{-7}	Dinan *et al.* (unpublished data)
20-Hydroxyecdysone 20,22-acetonide	3.0×10^{-7}	Dinan *et al.* (unpublished data)
20-Hydroxyecdysone 22-benzoate	4.3×10^{-9}	Ravi *et al.* (2001)
20-Hydroxyecdysone 6-carboxymethyloxime	Inactive	Clément *et al.* (1993)
20-Hydroxyecdysone dimer	2.1×10^{-7}	Harmatha *et al.* (2002)
20-Hydroxyecdysone 2-β-D-glucopyranoside	2.0×10^{-5}	Harmatha and Dinan, 1997, Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 3-β-D-glucopyranoside	1.3×10^{-5}	Harmatha and Dinan, 1997, Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 22-β-D-glucopyranoside	4.7×10^{-5}	Harmatha and Dinan, 1997, Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 25-β-D-glucopyranoside	8.5×10^{-6}	Harmatha and Dinan, 1997, Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-Hydroxyecdysone 2-hemisuccinate	8.3×10^{-7}	Clément *et al.* (1993)
20-Hydroxyecdysone 22-hemisuccinate	Inactive	Clément *et al.* (1993)
20-Hydroxyecdysone 2,3-monoacetonide	1.5×10^{-7}	Dinan *et al.* (unpublished data)
20-Hydroxyecdysone 20,22-monoacetonide	3.0×10^{-7}	Dinan *et al.* (unpublished data)
20-Hydroxyecdysone 6-oxime	2.2×10^{-6}	Dinan *et al.* (unpublished data)
20-Hydroxyecdysone 2,3,22-triacetate	$>1.6 \times 10^{-5}$	Dinan *et al.* (1999a)
20-Hydroxyecdysone 3β-D-xylopyranoside (limnantheoside A)	1.6×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
6β-Hydroxy-20-hydroxyecdysone	1.7×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
6α-Hydroxy-20-hydroxyecdysone	2.0×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
26-Hydroxypolypodine B	4.8×10^{-7}	Clément *et al.* (1993)
5β-Hydroxystachysterone C	3.5×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
(25*R*/*S*)-Inokosterone	1.1×10^{-7}	Clément *et al.* (1993), Dinan *et al.* (2003)
(25*R*)-Inokosterone	1.5×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
(25*S*)-Inokosterone	2.7×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Inokosterone 26-hemisuccinate	3.8×10^{-6}	Clément *et al.* (1993)
Integristerone A	1.8×10^{-7}, 8.3×10^{-7}, 2.0×10^{-7}	Dinan *et al.* (unpublished data)
20-*iso*-Ecdysone	1.0×10^{-4}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
20-*iso*-22-*epi*-Ecdysone	1.0×10^{-4}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
iso-Homobrassinolide	$>10^{-5}$	Dinan *et al.* (1999a)
Isostachysterone C ($\Delta^{25(26)}$)	9.2×10^{-9}, 4.2×10^{-9}	Dinan *et al.* (unpublished data)
Kaladasterone	3.4×10^{-7}	Bourne *et al.* (2002)
Ketodiol	Inactive	Dinan *et al.* (1999a)
5α-Ketodiol	$>2.3 \times 10^{-5}$	Dinan *et al.* (1999a)
Leuzeasterone	1.7×10^{-8}	Dinan *et al.* (unpublished data)
Limnantheoside C	1.3×10^{-6}	Meng *et al.* (2001b)

Table 1 Continued

Ecdysteroid [a]	*EC₅₀ (M)*	*Reference*
Makisterone A	1.3×10^{-8}	Clément *et al.* (1993), Harmatha and Dinan (1997), Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
Makisterone C	2.0×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
Malacosterone	9.0×10^{-6}	Harmatha and Dinan (1997), Dinan *et al.* (1999a), Ravi *et al.* (2001)
24-Methylecdysone (20-deoxymakisterone A)	2.1×10^{-6}	Dinan *et al.* (unpublished data)
14-Methyl-12-en-15,20-dihydroxyecdysone	2.3×10^{-6}	Dinan *et al.* (unpublished data)
14-Methyl-12-en-shidasterone	4.0×10^{-6}	Dinan *et al.* (unpublished data)
Muristerone A	2.2×10^{-8}	Clément *et al.* (1993), Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001); Harmatha *et al.* (2002), Bourne *et al.* (2002)
29-Norcyasterone	1.2×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
29-Norsengosterone	1.3×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Paxillosterone	4.2×10^{-7}	Harmatha and Dinan (1997)
Paxillosterone 20,22-*para*-hydroxybenzylidene acetal	3.0×10^{-7}	Harmatha and Dinan (1997)
14-Perhydroxy-20-hydroxyecdysone	1.6×10^{-8}	Harmatha *et al.* (2002)
Pinnatasterone	4.0×10^{-7}	Dinan *et al.* (unpublished data)
Podecdysone B	1.2×10^{-5}	Harmatha *et al.* (2002), Bourne *et al.* (2002)
Polypodine B	1.0×10^{-9}	Harmatha and Dinan (1997), Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
Polyporusterone B	2.1×10^{-9}	Dinan *et al.* (unpublished data)
Ponasterone A	3.1×10^{-10}	Clément *et al.* (1993), Harmatha and Dinan (1997), Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001)
	2.0×10^{-10}	Dinan *et al.* (unpublished data)
Ponasterone A 6-carboxymethyloxime	7.5×10^{-6}	Clément *et al.* (1993)
Ponasterone A dimer	4.6×10^{-8}	Dinan *et al.* (unpublished data)
Ponasterone A 2-hemisuccinate	3.1×10^{-7}	Clément *et al.* (1993)
Ponasterone A 22-hemisuccinate	5.0×10^{-7}	Clément *et al.* (1993)
Ponasterone A 3β-D-xylopyranoside (limnantheoside B)	1.5×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
Poststerone	ca. 2×10^{-5}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	2.0×10^{-5}	Dinan *et al.* (2003)
Poststerone 20-dansylhydrazine	6.0×10^{-6}	Dinan *et al.* (unpublished data)
Pterosterone (rhapontisterone)	2.0×10^{-9}	Dinan *et al.* (unpublished data)
Punisterone	8.3×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
Rapisterone B	2.3×10^{-7}	Dinan *et al.* (unpublished data)
Rapisterone C	3.9×10^{-7}	Dinan *et al.* (unpublished data)
Rapisterone D	1.0×10^{-9}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Rubrosterone	$>10^{-4}$	Dinan *et al.* (1999a), Ravi *et al.* (2001), Hormann *et al.* (2003)
Sengosterone	9.0×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Shidasterone (stachysterone D)	1.5×10^{-6}, 4.0×10^{-6}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
Sidisterone	4.3×10^{-6}	Dinan *et al.* (unpublished data)
Sileneoside A (20-hydroxyecdysone 22-galactoside)	4.1×10^{-5}	Dinan *et al.* (unpublished data)
Sileneoside C (integristerone A 22-glactoside)	1.0×10^{-4}	Dinan *et al.* (unpublished data)
Sileneoside D (20-hydroxyecdysone 3-galactoside)	3.0×10^{-5}	Dinan *et al.* (unpublished data)
Sileneoside E (2-deoxyecdysone 3-galactoside; blechnoside A)	Inactive	Dinan *et al.* (unpublished data)
Stachysterone B	8.2×10^{-8}	Bourne *et al.* (2002)
Stachysterone C	1.4×10^{-8}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	7.5×10^{-9}	Dinan *et al.* (unpublished data)
2,14,22,25-Tetradeoxy-5α-ecdysone	$>2.5 \times 10^{-5}$	Dinan *et al.* (1999a)
2α,3α,22*S*,25-Tetrahydroxy-5α-cholestan-6-one	$>10^{-4}$	Dinan *et al.* (1999a)
2β,3β,20*R*,22*R*-Tetrahydroxy-25-fluoro-5β-cholest-8,14-dien-6-one	7.2×10^{-9}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
2β,3β,6α-Trihydroxy-5β-cholestane	$>2.4 \times 10^{-5}$	Dinan *et al.* (1999a)
2β,3β,6β-Trihydroxy-5β-cholestane	$>2.4 \times 10^{-5}$	Dinan *et al.* (1999a)
Turkesterone	1.3×10^{-6}	Clément *et al.* (1993), Dinan *et al.* (1999a, 2003), Ravi *et al.* (2001), Bourne *et al.* (2002)
	3.0×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)
	8.0×10^{-7}	Dinan *et al.* (unpublished data)

Continued

Table 1 Continued

Ecdysteroid[a]	*EC*$_{50}$ *(M)*	*Reference*
Turkesterone 2-acetate	5.2×10^{-6}	Dinan *et al.* (2003)
Turkesterone 11α-acetate	4.0×10^{-6}	Dinan *et al.* (2003)
Turkesterone 22-acetate	6.0×10^{-7}	Dinan *et al.* (unpublished data)
Turkesterone 11α-arachidate	2.2×10^{-5}	Dinan *et al.* (2003)
Turkesterone 11α-butanoate	4.0×10^{-5}	Dinan *et al.* (2003)
Turkesterone 11α-decanoate	1.1×10^{-6}	Dinan *et al.* (2003)
Turkesterone 2,11α-diacetate	2.0×10^{-4}	Dinan *et al.* (2003)
Turkesterone 2,22-diacetate	1.0×10^{-3}	Dinan *et al.* (2003)
Turkesterone 11α-hexanoate	2.2×10^{-5}	Dinan *et al.* (2003)
Turkesterone 11α-laurate	3.4×10^{-6}	Dinan *et al.* (2003)
Turkesterone 11α-myristate	1.3×10^{-6}	Dinan *et al.* (2003)
Turkesterone 11α-propionate	8.3×10^{-6}	Dinan *et al.* (2003)
Viticosterone E	1.0×10^{-7}	Dinan *et al.* (1999a), Ravi *et al.* (2001)

[a]For structures of individual ecdysteroids, refer to http://www.ecdybase.org.

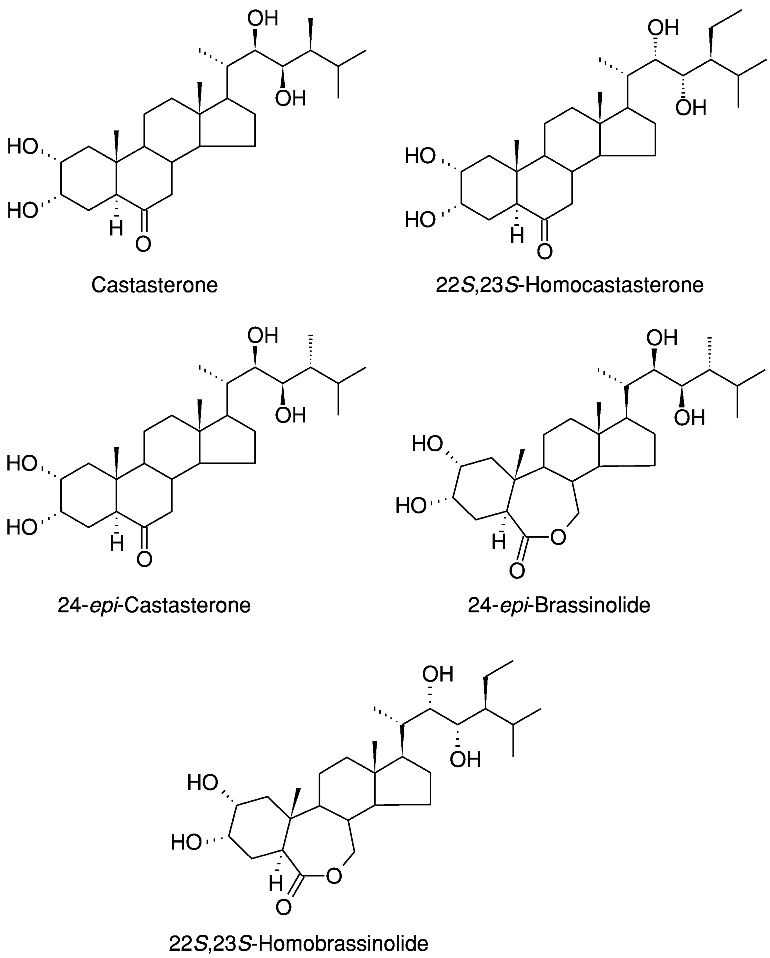


Figure 5 The structures of brassinosteroids showing ecdysteroid (ant)agonist activity.

in the same species (Richter and Adam, 1991). Spindler *et al.* (1992) examined the effects of 22*S*,23*S*-homobrassinolide and 22*S*,23*S*-homocastasterone on an ecdysteroid-responsive *Chironomus tentans* cell line and found that both were weak agonists in terms of their ability to induce ecdysteroid-specific responses and that both competed weakly for specifically-bound [^{3}H]PoA in cell-free

extracts of the *C. tentans* cells. Assessment of 10 brassinosteroids in the *D. melanogaster* B_{II} bioassay revealed no agonist or antagonist activity even at their limit of solubility (10^{-4} or 10^{-3} M) (Dinan *et al*., 2001d). In *Spodoptera littoralis*, 24-epibrassinolide and 24-epicastasterone can compete specifically-bound [^{3}H]PoA with K_d values of 3.7 and 2.0 μM, respectively (Smagghe *et al*., 2002). However, these compounds did not induce evagination of *S. littoralis* imaginal discs even at 100 μM or when fed to last instar larvae. In view of the low concentrations of brassinosteroids in plants, any ecdysteroid (ant)agonist activity they may possess will not be biologically relevant. However, given the chemical similarities between brassinosteroids and ecdysteroids, it is of interest to determine which structural modifications are required to convert molecules of one class to others with the biological activity of the other class. Hybrid brassinosteroid/ecdysteroid molecules have been generated and tested in the B_{II} bioassay (for ecdysteroid agonist/antagonist activity) and in the rice lamina inclination test (for brassinosteroid-like activity) (Voigt *et al*., 2001).

3.4.6.5. Diacylhydrazines

Owing to the chemical and physical properties of ecdysteroids, it is not possible to regard them as potential insecticidal compounds in their own right (Dinan, 1989). Prospects lie in the identification of nonsteroidal analogs which retain biological activity, but are chemically simpler and more resistant to metabolic and/or environmental degradation. The first nonsteroidal ecdysteroid agonists were identified at Rohm & Haas Co. in the late 1980s (Wing, 1988; Wing *et al*., 1988). The prototypical molecule was RH-5849 (**Figure 6**), which was shown to bind to ecdysteroid receptors with relatively low affinity, but, because it is metabolized only slowly, it is able to induce premature molting and death in treated insects. As a result of the synthesis and assessment of a very large number of diacylhydrazines (DAHs), Rohm & Haas could identify and develop analogs as commercially viable insecticides, starting with RH-5992 (tebufenozide) and followed by RH-0345 (halofenozide) and RH-2485 (methoxyfenozide). These compounds possess very low mammalian toxicities and even show some selectivity for certain orders of insects. The DAHs are treated more extensively elsewhere in this series (see **Chapter 6.3**), and the focus here will be on SARs for DAHs (see Section 3.4.9). There is considerable interest in modeling possible overlaps between DAH and ecdysteroid binding to the ligand-binding domain of the EcR (see below). Although many (165) DAHs have been assessed in the B_{II} bioassay (**Table 2**), none has yet been identified which possesses antagonistic activity.

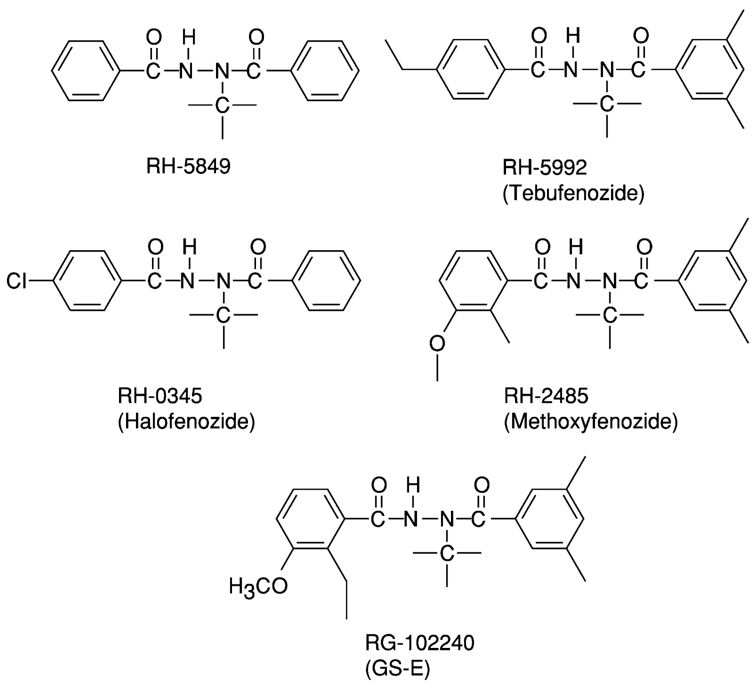
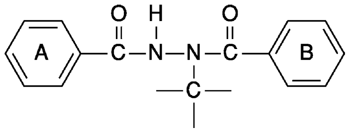


Figure 6 Structures of prototypical and commercially relevant diacylhydrazines.

Table 2 Agonistic potencies of diacylhydrazines in the *Drosophila melanogaster* B_{II} cell assay



RH number	*Substituent(s) ring A*	*Substituent(s) ring B*	*B_{II} bioassay EC_{50} (M)*
65848	3-Cl	3-Cl	1.8×10^{-6}
65849	H	H	1.8×10^{-6}
68581	4-Me	4-Me	7.4×10^{-6}
69115	3-OMe	3-OMe	1.2×10^{-5}
69116	2-NO_2	2-NO_2	5.3×10^{-6}
69117	2-Cl	2-Cl	2.6×10^{-6}
69122	4-CH_3	H	8.3×10^{-7}
69252	H	4-Cl	1.8×10^{-5}
69253	H	3-Cl	1.1×10^{-6}
69254	H	2-Cl	1.3×10^{-6}
69255	3-Me	3-Me	2.2×10^{-6}
69256	2-Me	2-Me	9.0×10^{-6}
69258	H	4-CH_3	8.0×10^{-5}
69259	H	3-CH_3	2.4×10^{-6}
69260	H	2-CH_3	3.8×10^{-6}
69261	H	4-OCH_3	8.2×10^{-5}
69262	H	3-OCH_3	1.2×10^{-5}
69263	H	2-OCH_3	3.8×10^{-6}
69266	H	4-CN	1.2×10^{-4}
69269	H	2-NO_2	1.1×10^{-6}
69600	H	4-F	3.8×10^{-6}
69601	H	3-F	3.3×10^{-6}
69602	H	2-F	6.2×10^{-6}
69662	H	4-CF_3	1.1×10^{-4}
69663	H	3-CF_3	1.1×10^{-5}
69664	H	2-CF_3	3.6×10^{-6}
69669	H	3-CN	6.2×10^{-5}
70345	4-Cl	H	9.0×10^{-7}
70389	2-Cl	H	8.0×10^{-6}
70942	3-Cl	H	5.2×10^{-6}
70947	2-CH_3	H	1.1×10^{-5}
71266	H	2-Br	8.0×10^{-7}
71267	H	4-OH	1.2×10^{-4}
71275	4-Me	2-Me	8.0×10^{-7}
71276	4-Me	3-Me	6.2×10^{-7}
71279	4-Me	2-Cl	2.2×10^{-7}
71353	4-Cl	2-Me	9.3×10^{-7}
71541	4-F	4-F	4.4×10^{-6}
71680	H	4-Phenyl	1.1×10^{-4}
71787	H	3-Br	2.1×10^{-6}
71947	4-Ethyl	H	8.2×10^{-7}
72179	4-CF_3	H	2.2×10^{-6}
72617	4-Cl	3-Cl	4.2×10^{-7}
72618	4-Cl	4-Me	1.2×10^{-5}
72773	4-Me	3-Cl	4.2×10^{-7}
73831	H	2-I	1.2×10^{-6}
73979	H	4-Acetyl	1.6×10^{-5}
74427	4-Et	2-NO_2	2.3×10^{-7}
74442	2-CF_3	H	3.5×10^{-5}
74443	2-Br	H	3.4×10^{-5}
75717	2-NO_2	H	3.2×10^{-5}
75718	3-CF_3	H	4.3×10^{-5}
75894	4-*n*-Pr	4-Cl	9.0×10^{-6}

Table 2 Continued

RH number	*Substituent(s) ring A*	*Substituent(s) ring B*	*B_{II} bioassay EC_{50} (M)*
75916	4-OCH_3	H	3.3×10^{-6}
75992	4-Et	3,5-di-Me	5.3×10^{-7}
75995	H	3-OH	2.2×10^{-4}
78295	2-Cl	2-Me	8.2×10^{-6}
78374	3-CH_3	H	1.3×10^{-5}
78404	2-Cl	2-F	1.9×10^{-5}
78434	4-NO_2	H	1.3×10^{-5}
78435	4-CN	H	3.6×10^{-5}
78447	3-OMe	3-Me	2.7×10^{-6}
78448	3-OCH_3	H	9.0×10^{-6}
78449	3-OMe	4-Cl	1.6×10^{-5}
78487	2-OMe	2-NO_2	3.1×10^{-5}
79186	4-OH	H	1.5×10^{-5}
80664	H	3-NH_2	9.3×10^{-5}
80665	H	2-NH_2	2.5×10^{-5}
80678	4-F	H	2.1×10^{-6}
81204	3-CN	3-Me	1.8×10^{-5}
81215	S-CH(OH)CH_3	3-Me	5.4×10^{-6}
81252	3-CO_2H	3-Me	8.5×10^{-5}
81398	2-F	3-Me	6.1×10^{-6}
81539	2-F	H	5.3×10^{-6}
87590	2-Et	3-Me	3.5×10^{-6}
92895	3-OMe	2-NO_2	4.0×10^{-6}
92897	3-Me	2-NO_2	1.9×10^{-6}
94496	4-I	H	9.5×10^{-7}
95028	4-Br	3-Me	8.5×10^{-7}
95075	3-Me	3-Cl	2.5×10^{-6}
96586	3-Cl	4-Me	2.1×10^{-5}
96596	4-Acetyl	H	2.3×10^{-5}
99547	2-OH	3-Me	1.2×10^{-5}
99613	3-OH	3-Me	1.3×10^{-5}
99633	3-Cl	4-F	2.3×10^{-6}
100153	2-NH_2	H	4.4×10^{-6}
101292	2-Me	2-Cl	2.2×10^{-6}
101570	3-Cl	2-Cl	1.2×10^{-6}
111016	4-Cl	2-SMe	2.4×10^{-6}
112608	4-OMe	3-F	1.6×10^{-6}
116715	4-F	3-Br	8.7×10^{-7}
120004	3-F	2-OMe	2.3×10^{-6}
120064	3-F	2-Me	3.5×10^{-6}
120230	3-F	2-F	8.0×10^{-6}
120436	3-F	H	4.4×10^{-6}
122632	4-F	3-OMe	8.3×10^{-6}
122659	3-F	3-OMe	1.1×10^{-5}
122972	4-F	3-F	1.9×10^{-6}
123238	2-F	4-Me	4.2×10^{-5}
123757	4-Et	2-OMe	6.0×10^{-7}
124863	3-OMe	2-OMe	1.1×10^{-5}
124948	3-OMe	4-F	5.4×10^{-6}
167174	2-NO_2	3-Cl	8.3×10^{-6}
167179	2-NH_2	3-Cl	1.6×10^{-6}
Test set			
75744	4-Et	3-Et	1.5×10^{-6}
75749	4-Me	4-Et	9.0×10^{-5}
75915	4-OMe	3-Me	2.8×10^{-6}
78331	2-Cl	3-Cl	5.4×10^{-6}
78677	4-CF_3	4-Cl	7.2×10^{-6}
78678	4-CF_3	3-Me	1.4×10^{-6}
79185	4-*i*-Pr	3-Me	1.7×10^{-6}

Table 2 Continued

RH number	Substituent(s) ring A	Substituent(s) ring B	B_{II} bioassay EC_{50} (M)
79268	4-CN	3-Me	4.2×10^{-5}
79337	4-OH	3-Me	4.0×10^{-5}
80771	4-Et	2-F	1.9×10^{-6}
82982	3-OCF_3	3-Me	1.4×10^{-5}
83292	4-*t*-Bu	2-NO_2	3.2×10^{-6}
83299	4-*t*-Bu	4-F	8.5×10^{-6}
111729	4-Et	2-Cl	2.2×10^{-7}
112483	4-OMe	3-Br	8.7×10^{-7}
112520	4-OMe	2-Br	1.8×10^{-7}
112621	4-Et	3-F	6.0×10^{-7}
118878	4-F	2-OMe	1.7×10^{-6}
118890	4-F	2-NO_2	6.6×10^{-7}
Unquantified weak activity			
68583	4-OMe	4-OMe	Inactive at 10^{-4}
69118	2-OMe	2-OMe	Inactive at 10^{-4}
69123	4-CN	4-CN	Inactive at 10^{-4}
69267	H	4-NO_2	Inactive at 2.5×10^{-4}
69268	H	3-NO_2	$>2.5 \times 10^{-4}$
71351	4-Cl	4-Ph	Inactive at 10^{-4}
71979	H	2-OH	Inactive at 10^{-3}
75748	H	4-I	$>2.5 \times 10^{-5}$
75750	4-Et	4-Et	$>1 \times 10^{-4}$
75977	2-Cl	4-Et	$>10^{-4}$
78327	2-F	4-Cl	Inactive at 10^{-4}
78375	3-NO_2	H	$>1 \times 10^{-4}$
78386	2-Cl	4-CN	Inactive at 10^{-4}
78483	2-OCH_3	H	Inactive at 10^{-4}
78484	2-OMe	3-Me	$>10^{-4}$
78486	2-OMe	4-Cl	Inactive at 10^{-4}
79126	4-Et	4-OCF_3	Inactive at 10^{-4}
79183	4-*n*-Bu	4-Cl	Inactive at 2.5×10^{-5}
80662	4-Et	4-CO_2Me	Inactive at 10^{-4}
80729	3-OH	H	$>1 \times 10^{-3}$
80778	4-CO_2Me	3-Me	Inactive at 10^{-4}
80779	4-CO_2H	3-Me	Inactive at 10^{-4}
81203	4-Ph	3-Me	$>10^{-4}$
83413	2-Me	4-Cl	Inactive at 10^{-4}
89842	4-Et	4-OPh	Inactive at 10^{-4}
96597	4-Ac	4-Me	$>1 \times 10^{-4}$
112350	4-$CH_3O(CO)$-	H	Inactive at 10^{-4}
119005	4-F	2-OEt	Inactive at 10^{-4}
126814	4-Ac	4-Ac	Inactive at 10^{-4}
141591	2-Pyrrole	3,5-di-Me	Inactive at 10^{-3}
141592	H	2-Pyrrole	$>1 \times 10^{-3}$
141650	4-Ethyl	2-Pyrrole	Inactive at 10^{-3}
141713	2-Pyrrole	H	Inactive at 10^{-3}

3.4.6.6. 8-*O*-Acetylharpagide

The iridoid glucoside 8-*O*-acetylharpagide (Figure 7), isolated from *Ajuga reptans* (Labiatae), was reported to possess weak ecdysteroid agonist activity (Elbrecht *et al*., 1996). Unfortunately, *A. reptans* is also a rich source of phytoecdysteroids and it is now clear that 8-O-acetylharpagide, *per se*, possesses no agonistic activity and that the activity is attributable to contaminating ecdysteroids (Dinan *et al*., 2001c).

3.4.6.7. DTBHIB

The synthetic compound 3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutylbenzamide (DTBHIB) (Figure 7) is reported to be a weak ecdysteroid agonist, as it induces ecdysteroid-specific changes in the *D. melanogaster* Kc cell line ($EC_{50} = 3 \times 10^{-6}$ M) and is able to displace [^{3}H]PoA from receptors *in vitro* ($EC_{50} = 6 \times 10^{-6}$ M). However, the isopropyl analog showed no activity in either test (Mikitani, 1996).

3.4.6.8. Maocrystal E

As part of an extensive survey of natural products to detect those showing ecdysteroid agonist or antagonist activities in the B_{II} bioassay (Dinan *et al*., 2001a), it was found that the diterpene maocrystal E (isolated from *Isodon* spp. (Labiatae)) (Li *et al*., 1985) showed agonist activity ($EC_{50} = 5 \times 10^{-6}$ M). Further, maocrystal E (Figure 7) competed with [^{3}H]PoA for the ligand binding site of *D. melanogaster* and *Choristoneura fumiferana* EcR complexes (EcR/USP) with K_i values of 8.6×10^{-6} M and 5.8×10^{-5} M, respectively. These data support the interaction of maocrystal E with the ligand-binding domain.

3.4.6.9. Tetrahydroquinolines

Researchers at FMC Corp. (Princeton, NJ, USA) have indicated that 4-phenylamino-1,2,3,4-tetrahydroquinolines also act as ecdysteroid agonists (Dixson *et al*., 2000). More recently, RheoGene investigators have pursued this chemistry in the context of EcR gene switch actuation (Smith *et al*., 2003) (see Section 3.4.9.4).

3.4.7. Antagonists

3.4.7.1. Ecdysteroids

Although many ecdysteroids have been identified (Lafont *et al*., 2002) (see **Chapter 3.3**), relatively few had until recently been assessed for their biological activity, let alone to determine whether they possess agonistic or antagonistic activity. Apart from ajugalactone (see Section 3.4.3.3), there are no literature reports concerning antagonistic ecdysteroids. Approximately 150 ecdysteroid analogs have now been assessed in the B_{II} bioassay and very recently one of these was shown to possess antagonistic activity. 24ξ-Hydroxydihydrocarthamosterone (Figure 8) had been isolated as a minor

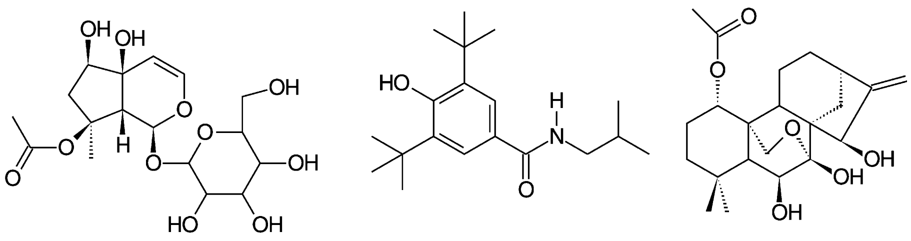


Figure 7 The structures of 8-*O*-acetylharpagide, 3,5,-di-*tert*-butyl-4-hydroxy-*N*-isobutylbenzamide (DTBHIB), and maocrystal E.

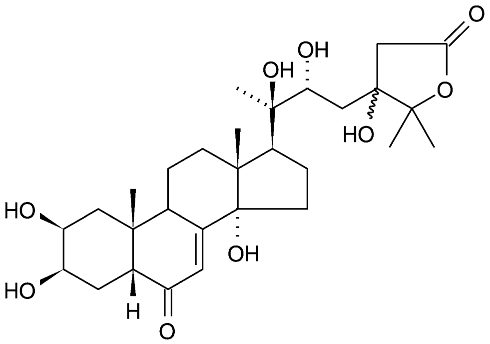


Figure 8 The structure of 24ξ-hydroxydihydrocarthamosterone.

ecdysteroid from *Leuzea carthamoides* (Compositae) and has an EC_{50} value of 2.0×10^{-5} M versus 5×10^{-8} M 20E. To verify that the compound is antagonistic, it has been shown that the biological activity co-chromatographs with the ultraviolet (UV)-absorbing peak on high-performance liquid chromatography (HPLC) (Harmatha and Dinan, unpublished data). The stereochemistry of the antagonistic ecdysteroid has not yet been fully elucidated, but it will be interesting to determine what makes this compound antagonistic, while other closely related compounds (e.g., carthamosterone) are agonistic.

3.4.7.2. Brassinosteroids

See Section 3.4.6.4 above.

3.4.7.3. Withanolides

The first evidence that certain withanolides can act as ecdysteroid antagonists was obtained in 1996, when a series of 16 withanolides isolated from *Iochroma gesnerioides* (syn. *I. coccineum*: Solanaceae; Alfonso *et al.*, 1991, 1993; Alfonso and Kapetanidis, 1994) were assessed in the B_{II} bioassay (Dinan *et al.*, 1996). None of the compounds showed agonist activity, but several exhibited distinct antagonist activity. The activities of four of these were adequate for quantification to determine EC_{50} values (versus 5.0×10^{-8} M 20E): 2,3-dihydro-3β-methoxywithaferin A ($EC_{50} = 3.5 \times 10^{-5}$ M), 2,3-dihydro-3β-methoxywithacnistine (1.0×10^{-5} M), 2,3-dihydro-3β-methoxyiochromolide (5.0×10^{-6} M), and 2,3-dihydro-3β-hydroxywithacnistine (2.5×10^{-6} M) (see **Figure 9** for corresponding structures) (note that the configuration of the C3 oxygen-containing substituent was determined as 3β after the publication of the paper; Sarker, Šik and Dinan, unpublished data). The active compounds possessed an α,β-unsaturated lactone in the side chain, a 3β-oxygen-containing function and a 5β,6β-epoxide.

A further 23 purified withasteroids have been assessed for their ecdysteroid agonist/antagonist activities (Dinan *et al.*, 1997a, 2001a). None of these demonstrated antagonistic activity, but one of them, withaperuvin D, was active as an agonist ($EC_{50} = 2.5 \times 10^{-5}$ M). This compound also possesses an α,β-unsaturated lactone in the side chain, but the side chain is linked 17α to the ring system. It also possesses a 3α,6α-epoxide with 5β-H, which is unusual amongst withasteroids. Further SAR studies are required to elucidate the structural features required for antagonism and antagonism.

3.4.7.4. Cucurbitacins

Amongst the first 200 plant extracts to be assessed with the B_{II} bioassay for the presence of ecdysteroid (ant)agonists, the methanolic extract of *Iberis umbellata* (Cruciferae) demonstrated distinct antagonist activity. A literature search revealed that cucurbitacins are characteristic of certain members of this genus (Gmelin, 1963). Bioassay-guided HPLC fractionation of the seed extract gave two UV-absorbing peaks which corresponded to the biological activity. Subsequent identification by nuclear magnetic resonance (NMR) and mass spectrometry revealed them to be cucurbitacin B and cucurbitacin D (**Figure 10**) (Dinan *et al.*, 1997b).

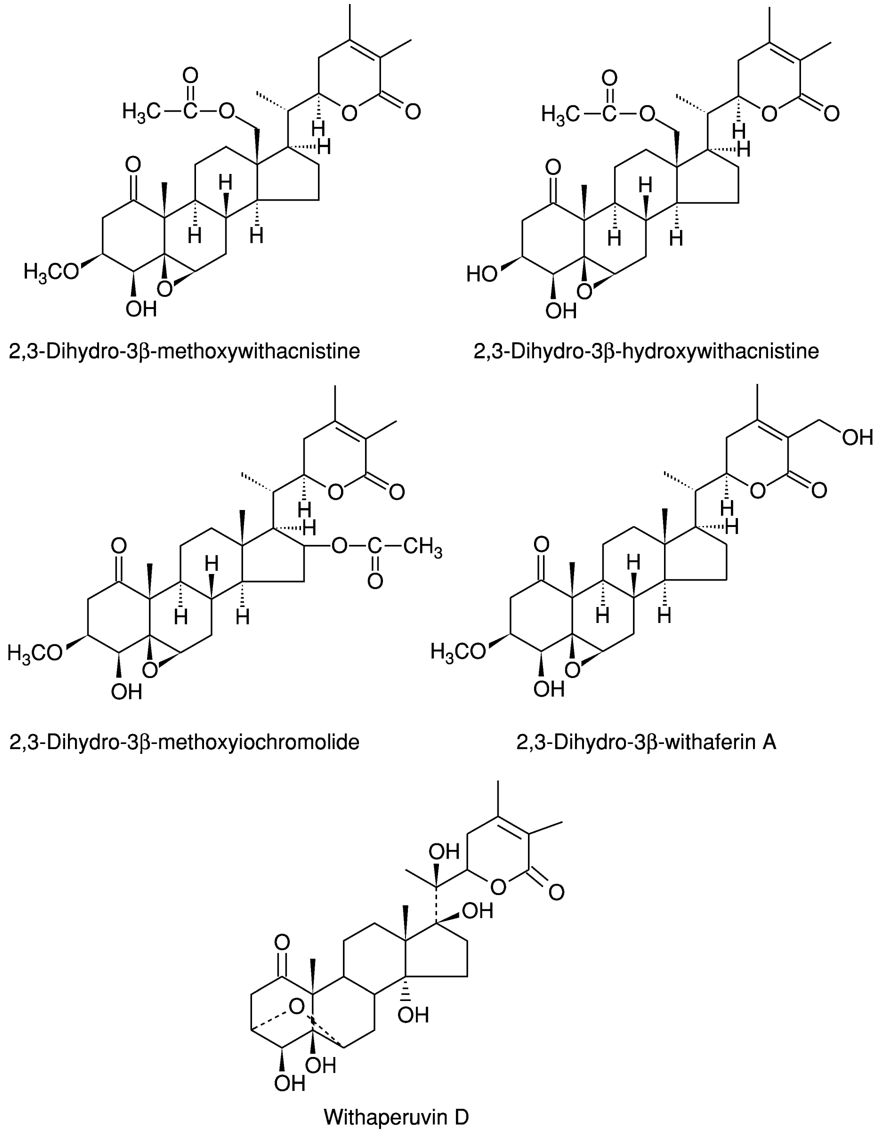


Figure 9 Structures of withasteroids active as ecdysteroid (ant)agonists.

The purified compounds antagonize the action of 20E (5×10^{-8} M) in the B_{II} cells; cucB ($EC_{50} = 1.5 \times 10^{-6}$ M) and cucD ($EC_{50} = 1.0 \times 10^{-5}$ M). Both compounds also compete with [^{3}H]PoA for the ligand binding site of EcR complexes extracted from B_{II} cells, the K_d for cucB being 5.0×10^{-6} M. CucB also antagonizes the 20E-induced stimulation of a transfected ecdysteroid-regulated reporter gene in *D. melanogaster* S2 cells and prevents the formation of 20E-induced complex formation of EcR/USP/EcRE complexes as examined by gel-shift assays. Preliminary SAR studies suggest that the Δ^{23}-22-oxo functional grouping is responsible for the antagonistic activity. In fact, hexanorcucurbitacin D, which lacks this functional grouping, is a weak agonist and 5-methylhex-3-en-2-one, which mimics C22 to C27 of the side chain of cucurbitacin D, is also a weak antagonist. Thus, cucurbitacins B and D are unequivocal EcR antagonists.

Subsequent studies with cucurbitane-type compounds isolated from *Hemsleya carnosiflora* (Cucurbitaceae) revealed that several of these antagonize 20E action in the B_{II} bioassay (Dinan *et al.*, 1997c). The active principles of further antagonistic plant extracts have been shown to result from the presence of cucurbitacins, e.g., cucurbitacin D from *Cercidiphyllum japonicum* (Cercidiphyllaceae) (Sarker *et al.*, 1997b) and cucurbitacin D, cucurbitacin F, and 3-*epi*-cucurbitacin D from *Physocarpus opulifolius* (Rosaceae) (Sarker *et al.*, 1999b).

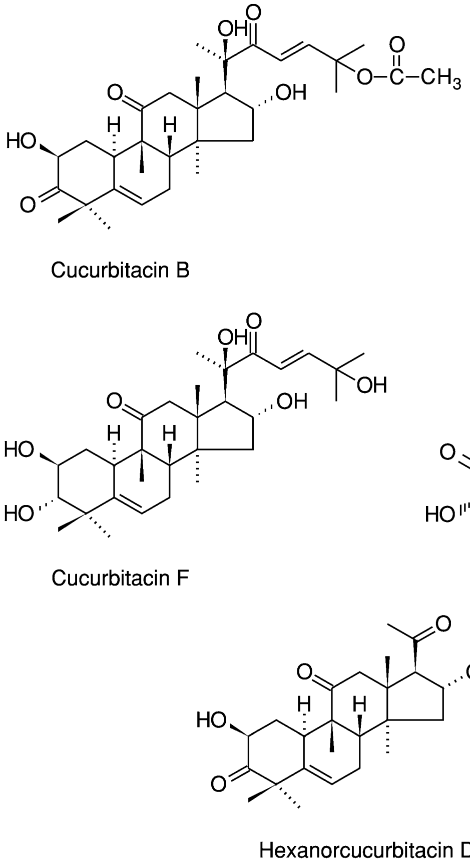


Figure 10 Structures of cucurbitacins active as ecdysteroid (ant)agonists.

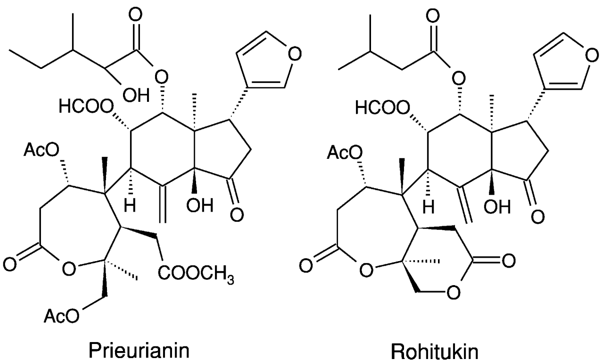


Figure 11 Structures of limonoids active as ecdysteroid antagonists.

3.4.7.5. Limonoids

The methanolic extract of seeds of *Turraea obtusifolia* (Meliaceae) showed antagonistic activity. Bioassay-guided purification resulted in the identification of two limonoids, prieurianin and rohitukin (**Figure 11**) (Sarker *et al.*, 1997a). Neither compound was particularly active (EC_{50} values $= 10^{-5}$ M and 1.3×10^{-4} M, respectively, versus 5×10^{-8} M 20E). Several other purified limonoids have been assessed for activity (Dinan *et al.*, 2001a), but only nomilin and obacunanone were found to have weak antagonistic activity. Azadirachtin, which had been suggested to act as an ecdysteroid antagonist, was also inactive (Lehmann *et al.*, 1988; Clément *et al.*, 1993). It has not yet been determined whether the active limonoids interact with the ligand binding domain of the EcR, somewhere else on the receptor complex or at some other point in the transduction pathway. In view of the wide diversity of natural limonoid structures and their general effects on

insect feeding, growth, and development (Isman, 1995), a wider range of limonoids should be assessed in the B_{II} bioassay and in a range of ecdysteroid-responsive systems from other insect species to determine the real significance of this class of ecdysteroid antagonist.

3.4.7.6. Stilbenoids

Methanol extracts of seeds of several species of *Paeonia* (Paeoniaceae) proved to be antagonistic. The antagonistic activity of one of these (*P. suffruticosa*) was examined more closely and the activity was found to be associated with *cis*-resveratrol and three oligostilbenes, named as suffruticosols A, B, and C (Sarker *et al.*, 1999a). Further, antagonistic stilbenoids have been isolated from *Iris clarkei* (Iridaceae) (Keckeis *et al.*, 2000) and *Carex pendula* (Cyperaceae) (Meng *et al.*, 2001a). The structures of the isolated stilbenoids are presented in **Figure 12**, while the biological activities are summarized in **Table 3**. The remarkable observation arising from consideration of the relationship between activity and structure is that the various stilbenoids possess similar activities, even though they differ enormously in molecular size from monostilbenes like *cis*-resveratrol through di-, tri-, and tetrastilbenes. The stilbenoids from *C. pendula* (kobophenol B, *cis*-miyabenol A, and *cis*-miyabenol C) compete with [^{3}H]PoA for the ligand binding site on the EcR complex (Meng *et al.*, 2001a). It is difficult to envisage how molecules of such differing sizes can interact with the ligand binding domain, although the planar nature of the molecules may facilitate this.

3.4.7.7. Phenylalkanoids

Purified phenylalkanoids (**Figure 13**) were assessed for ecdysteroid agonist and antagonist activity because they have a wide range of bioactivities, including insecticidal activity (Perrett and Whitfield, 1995). Six phenylalkanoids have been assessed (Dinan *et al.*, 2001a) and all were found to possess antagonistic activity, of which marginatine ($EC_{50} = 5.0 \times 10^{-5}$ M versus 5×10^{-8} M 20E) was found to be the most active.

3.4.7.8. Industrial Compounds

Endocrine disruption is currently an area of considerable, and increasing, concern. Most research to date has been conducted on aquatic vertebrates (fish and mammals) and the emphasis has been placed on disruption involving members of the nuclear receptor superfamily. As EcRs are also members of this superfamily and aquatic arthropods fill important ecological niches and are of great significance in food webs, endocrine disruption in invertebrates could have even more impact, affecting several trophic levels. Research in this area is hampered by restricted knowledge of invertebrate endocrinology (especially with regard to aquatic species) and the vast number of man-made and natural compounds released into the environment, which might potentially act as endocrine-disrupting chemicals (EDCs) (DeFur *et al.*, 1999). The simplicity and robustness of the B_{II} bioassay lend themselves to its use as a preliminary screen to identify potential EDCs, since many compounds can be rapidly tested to determine if they act as ecdysteroid agonists or antagonists. The few active compounds can then be taken forward for more extensive (and very time-consuming) *in vivo* testing using appropriate invertebrate species. *In vivo* studies alone very rarely give conclusive information on the mode of action of toxic chemicals, complicating the identification of EDCs by this approach, while a cell-based receptor assay does not reflect the full complications (penetration, metabolism, sequestration, excretion, receptor cross-talk, etc.) of a biological organism. Consequently, the two approaches are complementary and an approach using the B_{II} bioassay as a pre-screen to identify the potentially developmentally disruptive chemicals allows one to proceed to *in vivo* testing on a rational basis. Several hundred environmental chemicals have now been tested for ecdysteroid (ant)agonist activity in the B_{II} bioassay (Dinan *et al.*, 2001d; Cary *et al.*, unpublished data). Several weak antagonists have been identified, of which diethylphthalate (DEP), bisphenol A (BPA), and lindane (**Figure 14**) are the most signficant, both in terms of their potencies and the amounts released into the environment (Dinan *et al.*, 2001d). For each of these three compounds we could demonstrate that the antagonistic activity co-chromatographs with the named compound (i.e., is not associated with an impurity) and that the compound competes with [^{3}H]PoA for the ligand binding domain of the EcR complex. However, the EC_{50} values (2.0×10^{-3} M, 1.0×10^{-4} M, and 3.0×10^{-5} M for DEP, BPA, and lindane, respectively; versus 5×10^{-8} M 20E) are high and, given the concentrations of these compounds in the environment, it is unlikely that they are true invertebrate EDCs, although the caveats about the differences between *in vitro* and *in vivo* assessments mentioned above and the possibility that other species could be more susceptible than *D. melanogaster* should be borne in mind. Others are also beginning to use *in vitro* assays for the identification of potential invertebrate EDCs (Oberdörster *et al.*, 1999, 2001).

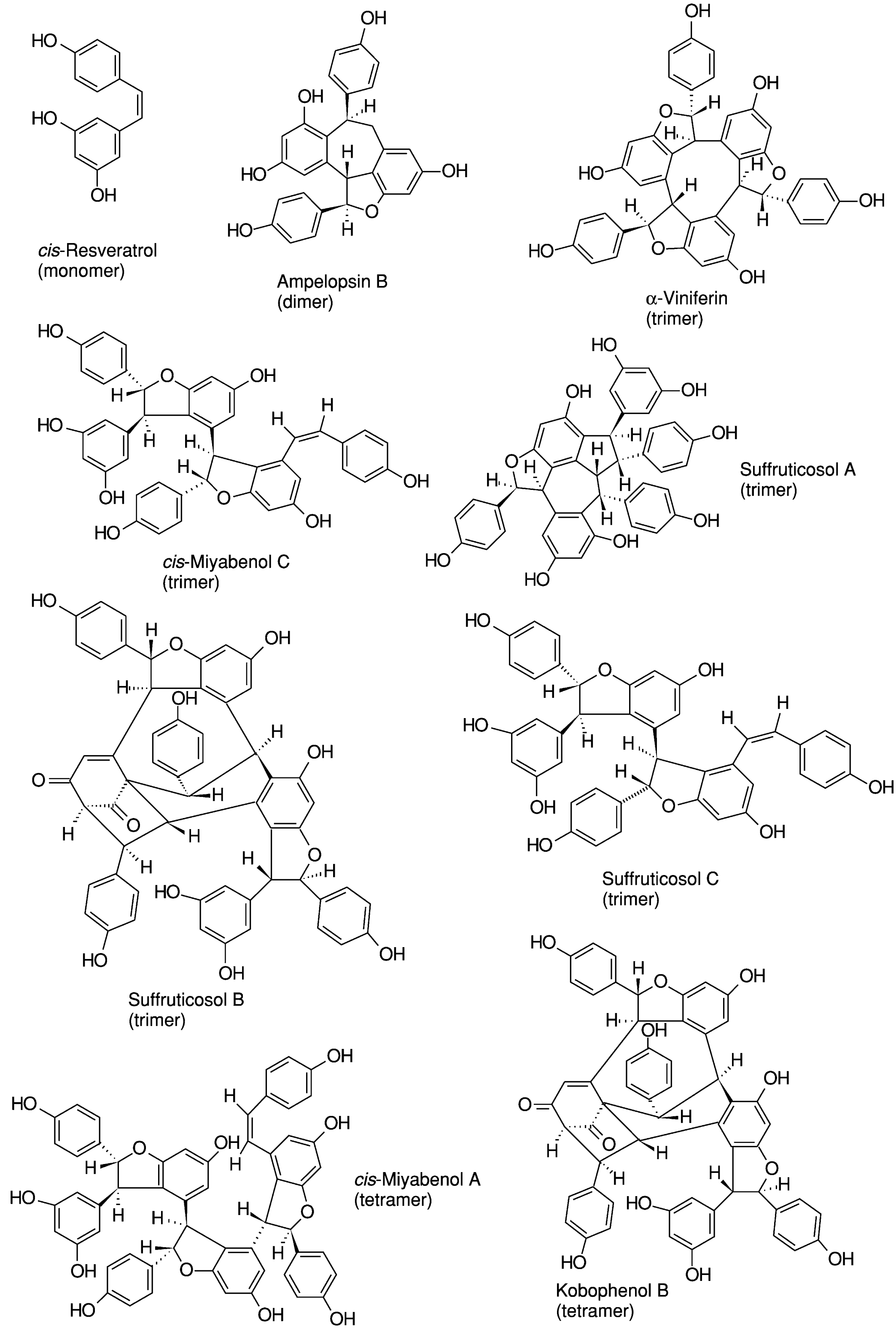


Figure 12 Structures of stilbenoids active as ecdysteroid antagonists.

Table 3 Antagonistic activities of stilbenoids in the *Drosophila melanogaster* B_{II} cell bioassay (vs. 5×10^{-8} M 20-hydroxyecdysone)

Number	*Compound*	*Source*	*EC_{50} (M)*
Monostilbenes	*cis*-Resveratrol	*Paeonia suffruticosa*	1.2×10^{-5}
	trans-Resveratrol	Sigma	Inactive
Distilbenes	Ampelopsin B	*Iris clarkei*	3.3×10^{-5}
Tristilbenes	*cis*-Miyabenol C	*Carex pendula*	1.9×10^{-5}
	Suffruticosol A	*Paeonia suffruticosa*	5.3×10^{-5}
	Suffruticosol B	*Paeonia suffruticosa*	1.4×10^{-5}
	Suffruticosol C	*Paeonia suffruticosa*	2.2×10^{-5}
	α-Viniferin	*Iris clarkei*	1.0×10^{-5}
Tetrastilbenes	Kobophenol B	*Carex pendula*	3.7×10^{-5}
	cis-Miyabenol A	*Carex pendula*	3.1×10^{-5}

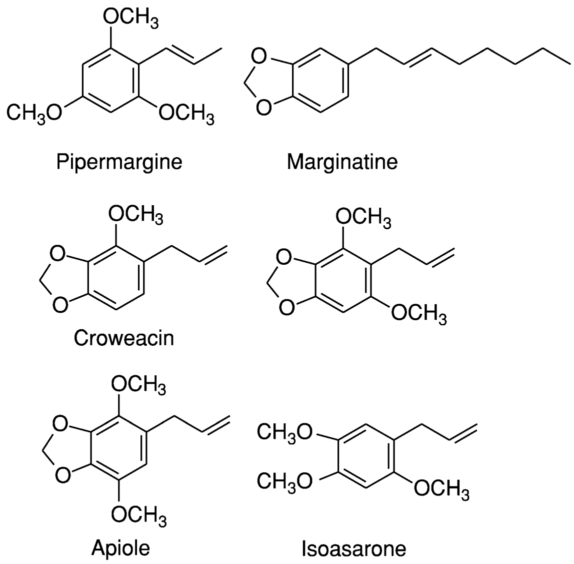


Figure 13 Structures of phenylalkanoids active as ecdysteroid antagonists.

3.4.8. Functions of Natural Ecdysteroid (Ant)Agonists in Insect–Plant Relationships

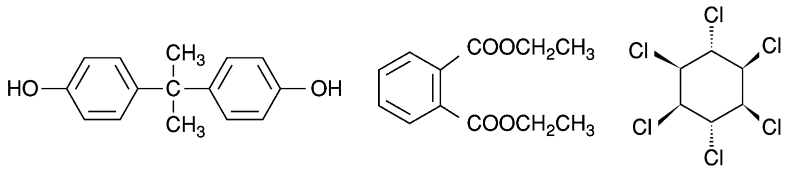
3.4.8.1. Agonists

When phytoecdysteroids were first discovered, it was suggested that they were a defense against insect predators (Galbraith and Horn, 1966). However, it soon became clear that certain insect species are highly resistant to ingested ecdysteroids (review: Dinan, 1998). It is now generally accepted that phytoecdysteroids contribute to deterrence of nonadapted phytophagous invertebrate predators, either by acting as antifeedants (mediated by taste receptors) or as hormonal toxicants on ingestion. Some 5–6% of terrestrial plant species accumulate phytoecdysteroids (Imai *et al.*, 1969), of which approximately half contain the compounds at levels which in themselves would deter nonadapted insect predators (Dinan, 1995a). Ecdysteroids may still contribute to the protection of plant species containing lower levels, since the ecdysteroids may interact synergistically with other secondary compounds. There appears to be a relationship between the occurrence and levels of phytoecdysteroids in the food plants of a particular insect species and its ability to cope with ecdysteroid in its diet (Blackford and Dinan, 1997). Thus, monophagous species such as larvae of the peacock butterfly (*Inachis io*) which feeds on ecdysteroid-negative stinging nettle (*Urtica dioica*; Urticaceae) would rather starve to death than consume nettle leaves coated with even low levels of 20E, while larvae of the oligophagous cinnabar moth (*Tyria jacobeae*) which encounter some ecdysteroid-containing plants in its host-range are moderately resistant to ingested ecdysteroids, but suffer developmental defects on the ingestion of larger amounts.

No studies have been yet performed on the role of maocrystal E in insect–plant interactions.

3.4.8.2. Antagonists

It has been realized for some time that compounds such as cucurbitacins, withanolides, stilbenoids, phenylalkanoids, and limonoids contribute to plants' protection strategies against invertebrate predators (Ascher *et al.*, 1980; Govindachari *et al.*, 1995; Miró, 1995; Perrett and Whitfield, 1995), but little was known about the mechanism(s) of action. The characterization of members of these classes of compounds as EcR antagonists has provided a testable hypothesis for the investigation of their roles in insect–plant relationships. All the active compounds identified so far have relatively weak affinities for the *D. melanogaster* EcR complex (K_d values in the μM range). This may reflect the low cost to the plant in producing these compounds and the high energy cost to phytophagous insects of detoxifying large amounts of such compounds (Dinan *et al.*, 2001b).



Bisphenol A Diethylphthalate Lindane

Figure 14 Structures of potential environmental disruptors interacting with ecdysteroid receptors.

3.4.9. QSAR and Molecular Modeling

3.4.9.1. Ecdysteroid SAR and QSAR

The early literature on ecdysteroid SAR studies has been reviewed (Bergamasco and Horn, 1980; Horn and Bergamasco, 1985; Dinan, 1989). The various bioassays employed vary in their complexity of performance and interpretation. A number of factors can affect the potency of a test compound to a greater or lesser extent depending on the assay system: penetration, metabolism, excretion rate, sequestration, and target site activity. *In vivo* assays will give a measure of all of these acting in concert, but not allow the individual contributions to be identified. On the other hand, cell-free receptor assays probably give a much more direct measure of target activity, but may bear little resemblance to the *in vivo* situation.

Horn and Bergamasco (1985) summarized their main conclusions regarding ecdysteroid SAR in insect systems as follows:

- an A/B *cis*-ring junction is essential
- a 6-oxo-7-ene grouping is essential
- a full sterol side chain is essential
- a free 14α-hydroxyl group is essential
- 2β-, 3β-, and 20*R*-hydroxyl groups enhance biological activity
- a C25 hydroxyl diminishes activity.

These conclusions were empirically derived from data combined from a number of different bioassay systems (mainly the *Calliphora*, *Musca*, and *Sarcophaga* assays, the *Chilo* dipping test and the *D. melanogaster* Kc cell assay). On the basis of the above summary, Bergamasco and Horn (1980) visualized the ecdysteroid interacting with the ligand binding pocket of the receptor at three major sites: the β-face of the A- and B-rings, the α-face in the region of the 14α-hydroxyl group, and the side chain from C22 to C27. Later, uniform data from the B_{II} bioassay enabled quantification of the potency contributions to the B_{II} EC_{50} by individual structural features, by making direct pairwise comparisons between structures which differ only in a single feature (**Table 4**) (Dinan *et al.*, 1999c).

Table 4 Ecdysteroid structural modifications and contributions to B_{II} $p(EC_{50})$

Potency-enhancing modifications	*Δp(EC50)*	*Potency-diminishing modifications*	*Δp(EC50)*
+2β-OH	2.28	3β-OH → 3-=O	−0.74
+20-OH	1.35	β-C-3 → α-C-3	−0.0
+14α-OH	0.60	+5β-OH	−0.25
+22*R*-OH	0.47	*trans* → *cis* C/D-junction	−1.44
22-OH → 22-=O	0.04	6-=O → 6-OH (α or β)	−1.89
		+11β-OH	−1.87
		20*R*-C- → 20*S*-C	−1.72
		22*R*-OH → 22*S*-OH	−0.60
		+22-OH, +25-OH	−0.34
		+24-CH_3 (*R* or *S*)	−0.85
		+25-OH	−0.89
		25-OH → 24/25 C=C	−0.91

In this way the previous SAR could be qualitatively substantiated and enhanced. Significantly, the previous SAR formulation described primarily as a brief collection of essential molecular features could now be cast in terms of the cumulative contribution, both enhancing or diminishing, of an expanded feature set (**Table 4**). In the language of pharmacophore analysis, the most highly contributing features are hydrogen bond donors or acceptors attached to C2, C3, C6, C20, and C22 together with a lipophilic group extending from C22. These groups are arranged approximately according to the geometry shown in **Figure 15**, wherein the ring sizes and ring junction stereochemistry determine the exact distances and angles. No single functional group is essential for ecdysteroidal potency; noteworthy is that the 14α-OH group can be replaced with unsaturation or can be altogether removed without abolishing B_{II} bioassay activity. Available data conflict as to a uniformly positive contribution of the 14α-OH to the pharmacophore.

More recent work involving examination of selected ecdysteroid probe sets in engineered EcR mammalian gene switch assays or in Sf-9 cells

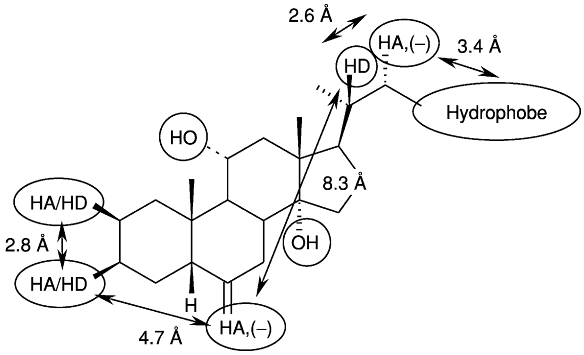


Figure 15 Ecdysteroid pharmacophore. Features are generalized where possible; HA, hydrogen bond acceptor; HD, hydrogen bond donor; (−), polar negative atom.

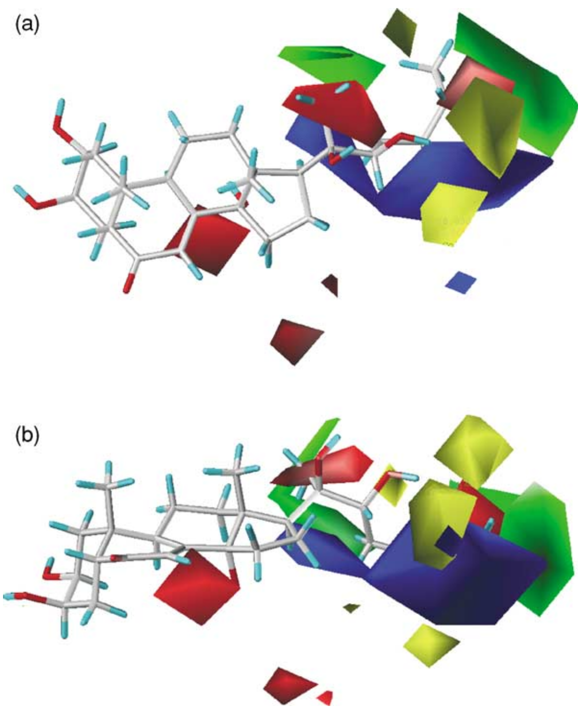


Figure 16 Comparative molecular field analysis (CoMFA) electrostatic field contour plot (standard deviation × coefficient) for favored ecdysteroid CoMFA model. Blue/red polyhedra represent regions where positive charge is favored/disfavored; contribution level = 85/8%, respectively. Green/yellow polyhedra represent sterically favored/disfavored regions; contribution level = 80/25%, respectively. Ponasterone A (PoA) is depicted. (a) View from the β-face; (b) view towards the C6/C14 edge of the steroid. (Reproduced with permission from Ravi M., Hopfinger A.J., Hormann, R.E., Dinan, L., **2001**. 4D-QSAR analysis of a set of ecdysteroids and a comparison to CoMFA modeling. *J. Chem. Inform. Comput. Sci. 41*, 1587–1604; © American Chemical Society.)

is roughly consistent with the previous SAR (Nakagawa *et al.*, 2000a; Saez *et al.*, 2000). The available ecdysteroid datasets are unfortunately too limited for quantitative analysis by classical QSAR. However, quantitative analysis was performed on the B_{II} data set using the multidimensional comparative molecular field analysis (CoMFA) approach (Dinan *et al.*, 1999c; Ravi *et al.*, 2001). By the CoMFA method, the intensity of steric and electrostatic fields at specified points in a three-dimensional lattice is measured and tallied as a function of each member ecdysteroid of the QSAR training set. Thereby, a descriptor table of many columns is generated. Partial least-squares supplies the appropriate statistical treatment to manipulate the data and derive a QSAR expression. This expression is best depicted as color-coded surfaces or polyhedra describing regions in space where the presence of generalized pharmacophoric features would be expected to either enhance or diminish efficacy.

For the ecdysteroids, a CoMFA model was developed with an $r^2 = 0.92$ and a cross-validated r^2 (q^2) of 0.593 for a 67-member training set and an average residual of fit of 1.47 p(EC_{50}) units for a 16-member test set (**Figure 16**). The model is characterized by a large (+)-charge-enhancing region (blue) wrapped around the steroid side chain and (+)-charge-diminishing regions (red) at O20, between C26 and C27, and below C7/C8. Steric-enhancing regions (green) appear distal to C21 and at the terminus of the side chain. Steric-diminishing regions (yellow) appear around the perimeter of the side chain. The CoMFA is consistent with, and extends, the simple functional group-oriented pharmacophore of **Figure 15**, and provides an abstraction useful for evaluating new or hypothetical structures which might not fit into the simple functional group schema.

The four-dimensional QSAR (4D-QSAR) method (Hopfinger *et al.*, 1997) provides another complementary view of ecdysteroid QSAR in the B_{II} assay. In 4D-QSAR, structures for analysis are passed through a molecular dynamics simulation, and the QSAR descriptors are derived from the frequency that particular atom types might populate individual cells in a three-dimensional lattice enveloping the training set structures. Thus, in contrast to CoMFA, 4D-QSAR takes conformation into account. The method can help discriminate among ligand alignment postulates and can provide optimal ligand conformation hypotheses. Data reduction is accomplished by partial least-squares, and models are selected and optimized by use of a genetic algorithm.

4D-QSAR was applied to essentially the same training set as used for the CoMFA modeling, resulting in four complementary models ($r^2 = 0.83$–0.88, $q^2 = 0.76$–0.80), one of which is depicted in **Figure 17** (Ravi *et al.*, 2001). Taken together, the 4D-QSAR

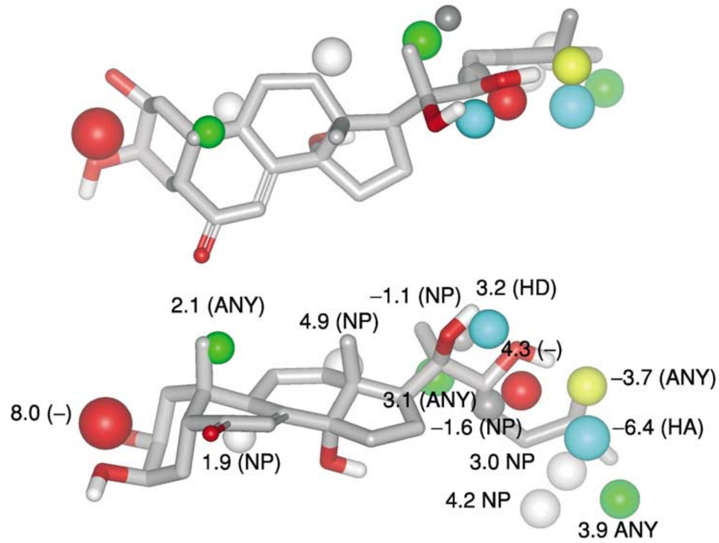


Figure 17 One of the favored ecdysteroid four-dimensional quantitative structure–activity relationship (4D-QSAR) models relative to PoA in its postulated active conformation. Alignment is based on atoms 6, 12, and 16. No external whole molecule descriptors were used in this model. The grid cell occupancy descriptors (GCODs) are shown as spheres. Interactive pharmacore element (IPE) type abbreviations: ANY, any atom (green/yellow); NP, nonpolar (white/gray); (−), polar negative (red/blue); HD, hydrogen bond donor (cyan/pink); HA, hydrogen bond acceptor (pink/cyan). Positive values of a GCOD indicate that occupancy by the appropriate IPE increases activity while a negative value indicates occupancy decreases activity. (Reproduced with modification and permission from Ravi M., Hopfinger A.J., Hormann R.E., Dinan L., **2001**. 4D-QSAR analysis of a set of ecdysteroids and a comparison to CoMFA modeling. *J. Chem. Inform. Comput. Sci. 41*, 1587–1604. © American Chemical Society.)

models corroborate the CoMFA conclusions, and also specify structural requirements more precisely. The 4D-QSAR models indicate that the hydroxyl group at C2 should be a hydrogen bond acceptor, the hydroxyl at C20 should be a hydrogen bond donor, the atom attached to C22 should be polar and bear a partial negative charge (and is a likely hydrogen-bond acceptor, although HA/HD role swapping with O20 is possible), and that there should be no hydrogen bond acceptor at C25. The general picture generated by the ecdysteroid CoMFA and 4D-QSAR models is that in the ligand binding cavity of the EcR, the steroid side chain lies in a sterically restrictive hydrophobic cylinder with hydrogen-bonding functionality near O20 and O22. Interestingly, analysis of weakly active ecdysteroids in the 4D-QSAR models indicates that the potency depression is often due not so much to an offending functional group as it is to the inability to assume a non-offending conformation.

Homology modeling of the EcR ligand-binding domain and ecdysteroid docking generated several interesting hypotheses for ecdysteroid–EcR binding, notwithstanding serious challenges owing to the absence of homologous nuclear receptors with identity any greater than ca. 30%, and, of course, the absence of a publicly available EcR crystal structure itself (see **Chapter 3.5**). Wurtz *et al.* (2000) have proposed two models for ecdysteroid interaction with the dipteran *C. tentans* EcR ligand binding domain, based on homology models built from the ligand binding domains of the human retinoic acid receptor (hsRARγ) and the human vitamin D receptor (hsVDR). Both models insert the steroid side chain in a "tail-first" orientation, such that ring-A remains closest to the enclosing Helix-12. Between these two models, the orientation of 20E differs by roughly 180°, thereby altering the interactions between the functional groups on 20E and the residues lining the ligand binding pocket. Another model built at Rohm & Haas, based on estrogen receptor (ER), thyroid harmone receptor (TR), and vitamin D receptor (VDR) and docked with 20E in the "tail-first" orientation, was successfully used to guide mutant generation for selective modulation of ecdysteroid responsiveness, while leaving DAH sensitivity unaltered (Kumar *et al.*, 2002). A more recent model has been constructed by investigators at Sankyo and Nippon Kayaku (Kasuya *et al.*, 2003).

The CoMFA and 4D-QSAR view of a hydrophobic cylinder surrounding the side chain, the hydrogen bond acceptor characteristics of 22OH, and the hydrogen bond donor characteristic of 20OH can all be easily reconciled with the CtEcR (*C. tetans*) 20E-liganded homology models based on either the retinoic acid receptor (RAR) or the VDR, as well

as with the generally hydrophobic ligand binding domain characteristics of known nuclear-receptor ligand binding domains on which these models are based (Wurtz *et al*., 2000). On the other hand, the CtEcR homology models predict that all six hydroxyls and the C6 carbonyl of 20E are implicated in H-bonding, whereas the receptor-independent 4D-QSAR and CoMFA models do not recognize 14OH in any significant way, and strongly suggest that 25OH is not involved in hydrogen bonding. *A fortiori*, simple empirical SAR of the 25 position indicates that 25OH is detrimental to EcR affinity. Despite these conflicts, the bulk of empirical SAR, multidimensional QSAR analysis, homology modeling, and docking generally provide a consistent picture of the factors involved in ecdysteroid activity.

While it is significant and reassuring that the calculational models and qualitative SAR are generally in concordance with each other and with ecdysteroid binding data and physiological data, a more stringent test is in consonance with a crystal structure of EcR bound with an ecdysteroid. The ligand-binding domains of EcR/USP from *Heliothis virescens* (HvEcR) co-crystallized with PoA at ~3 Å resolution considerably enlightens our understanding of ecdysteroid SAR (Billas *et al*., 2003). In this structure, PoA assumes a pose similar to vitamin D in VDR, in which the steroid assumes a crescent shape with its side-chain proximal to Helix-12 ("head-first"), and the A-ring is held in a chair conformation. Hydoxyl groups at carbons 2 (R383), 3 (E309), 14 (T343/T346), and 20 (Y408) as well as the C-6 carbonyl (A398) all participate in hydrogen-bonding. Moreover, the interacting residues are generally conserved across insect species, which concurs with the ubiquity of PoA recognition. The HvEcR residues near the side-chain terminus are generally hydrophobic, consistent with qualitative SAR and QSAR models, which all discount 25-OH as a pharmacophore element. The combined CoMFA and 4D-QSAR models correctly interpreted/predicted the crescent shape, A-ring chair conformation, hydrophobic quality, and steric permissiveness of the distal portion of the side-chain, and the identity of most hydrogen-bonding units (**Figure 15**). However, whereas 4D-QSAR models predicted that C-22 would be involved in hydrogen bonding as an acceptor, the HvEcR crystal structure indicates no such role. Likewise, although qualitative SAR and QSAR models lead one to the conclusion that the C-14 hydroxyl may not necessarily be advantageous for binding, the HvEcR crystal structure clearly shows not one, but two hydrogen-bonding interactions at this position.

In interpreting the immensely valuable EcR crystal structures, one should keep in mind that dynamics exert a significant role in ligand–protein interactions, and that multiple ligand-binding modes may be possible. For example, some of the homology modeling that antedates the HvEcR–PoA crystal structure hypothesizes a "tail-first" ecdysteroid orientation – a hypothesis testable through careful docking studies and possibly multiple crystal structures. Although hydrogen-bonding HvEcR residues are generally conserved across species, nonconserved binding pocket residues may provide a means for supplementary or substitute binding interactions, particularly if the specific ecdysteroid analog in question offers complementary features for such hypothetical alternative interactions.

3.4.9.2. Diacylhydrazine SAR and QSAR

3.4.9.2.1. Lepidopterans The diacylhydrazines (**Figure 18**) have enjoyed the most extensive qualitative and quantitative analysis, owing to commercial interest and the relative abundance of analogs. The chemotype was first synthesized and identified as an insecticide in the early 1980s (Hsu and Aller, 1991), and shortly thereafter reported as an ecdysteriod agonist (Wing, 1988; Wing *et al*., 1988). Using Kc cells, competitive binding studies indicated that RH-5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) is a competitive inhibitor with PoA with a K_d value of 2 μM, which implies that the two ligands share a common binding domain. Depression of the PoA kinetic on-rate, but not the off-rate, by RH-5849 are consistent with affinity for a common steroid/DAH binding site rather than an allosteric site. DAH toxicity to caterpillars is attributable to hyperecdysteroidism and premature initiation of molting. For an initial set of 28 DAHs, Kc cellular response is proportional to propensity toward head capsule apolysis in the tobacco hornworm, *Manduca sexta* (Wing *et al*., 1988). Hsu (1991) described an initial

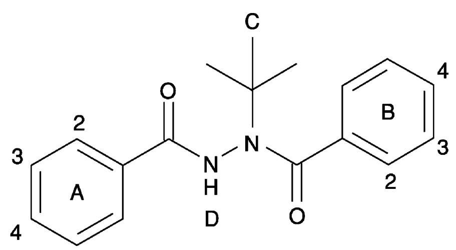


Figure 18 Diacylhydrazine (DAH) notation and numbering system.

DAH SAR for toxicity toward southern armyworm (*Spodoptera eridania*) as follows:

1. A- and B-regions: when A or B is phenyl and C is *tert*-butyl, toxicity is retained when the alternate A or B group is an aryl, aromatic heterocycle, aliphatic cycle or short alkenyl. Acyclic alkyl or benzyl are somewhat less active.
2. C-region: a bulky group is essential, but excessive size significantly truncates potency. *Tert*-butyl confers the optimum blend of foliar and plant systemic potency. Some functionality, such as cyano, is permissible.

Later, Rohm & Haas investigators updated the empirical SAR for southern armyworm toxicity (Hsu *et al.*, 1997):

1. A- and B-region: aryl > heterocyclic; asymmetric A/B-substitution > symmetric A/B-substitution.
2. A-region (monosubstituted, B = phenyl): 2-Cl, 2-CH_3, 3-Cl, 3-OCH_3, 4-Et, 4-Br, 4-I, 4-CH_3, 4-CF_3, 4-OCH_3, > H > 2-Br, 2-NO_2, 2-CF_3, 2-NH_2, 2-OCH_3, 3-CH_3, 3-Et, 3-NO_2, 4-Cl, 4-iPr, 4-*t*-Bu.
3. B-region (monosubstituted, A = phenyl): 2-ethyl, 2-I, 2-Br, 2-NO_2, 2-Cl, 3-Br, 3-ethyl, 3-methyl, 3-Cl, 4-Cl, 4-F > H > 2-OCH_3, 2-CH_3, 2-NH_2, 3-CF_3, 3-OCH_3, 3-NO_2, 3-NH_2, 4-NO_2, 4-CH_3, 4-OCH_3.
4. D-region: H or hydrolyzable group.
5. Backbone: C = O > C = S, S = O; NH–N(tbu) > CH_2–N(tbu), NH–CH(tbu).

Significantly, nonlinear, intra-ring, and interring substituent relationships were observed, thereby presenting a challenge for lead optimization which nonetheless proceeded steadily to the invention of RH-5992 (tebufenozide), and later advanced to the development of RH-2485 (methoxyfenozide), insecticides.

More recent reports from Sankyo and Nippon Kayaku Co. describe qualitative DAH SAR pertaining to structures related to chromafenozide insecticide (Sawada *et al.*, 2003a, 2003b, 2003c). Various DAHs with heterocycles fused to the 3,4-positions of the A-ring are toxic toward the common cutworm (*Spodoptera litura*). The most active compounds have oxygen and carbon-containing, five- or six-membered fused rings devoid of bulky or hydrogen bond-donating substitution. Optimized structures also bear a small substituent at the A2 position, typically methyl (a pattern shared with methoxyfenozide), while the remainder of the molecule reflects the structure of RH-5992. With respect to the C-module, considerable potency is retained when neopentyl, 1-*t*-butyl-ethyl, or 1,1-dimethylbenzyl replace the *t*-butyl group. Hence, once the A- and B-modules are optimized, the C-module becomes less refractory towards structural modulation. Many of these observations are implicit in earlier DAH patent literature; however, patent data format generally does not lend itself to SAR analysis. Additional D-module SAR from Sankyo and Nippon Kayaku (Sawada *et al.*, 2003c) and the observation that dibenzoyl cyclic hydrazides are inactive (Toya *et al.*, 2002) remain consistent with the earlier hypothesis that only hydrolyzable or readily metabolizable groups are tolerated in the D-module, and that the bioactive chemical species at the EcR is a DAH with a free amide NH. Notwithstanding, the observation that alkoxycarbonylmethyl groups are tolerated as the D-module does challenge the earlier, more restrictive hypotheses concerning the amide NH (Cao *et al.*, 2001).

The DAH chemotype is well suited for Hansch–Fujita QSAR (Hansch and Leo, 1995), and a number of QSAR relationships have been proposed by Nakagawa and colleagues to describe aspects of DAH insect toxicity under varying circumstances. The Hansch approach considers combinations of substituent physiochemical properties and other simple molecular properties as descriptors of the biological activity. The result is a multiple linear regression equation, which, if demonstrated to be robust statistically, can be quite instructive in its quantitative description of the structural factors responsible for potency.

In examination of DAH toxicity to larvae of *C. suppressalis* for a series of 46 DAHs unsubstituted on the A-ring and monosubstitued on the B-ring, it was determined that the level of toxicity is positively influenced by elevated log *P* and elevated electronic inductive effect (σ_I) of the *ortho*-substituent (Oikawa *et al.*, 1994a). Potency is suppressed by voluminous *meta*- and *para*-substituents as well as multiple (2, *n*) substitution patterns. The same training set with *Chilo* data was later examined by simply measuring the distance from the A-carbonyl oxygen to the attachment atom of the B-subsitutent for DAHs represented in a crystal structure-like conformation (Qian, 1996). A parabolic relationship was found with an optimal distance of ca. 4.6 Å. An almost identical training set was used to determine the SAR for toxicity toward larvae of the beet armyworm (*Spodoptera exigua*); this turned out to be highly correlated with toxicity toward *Chilo* larvae, and could be described by a very similar regression equation ($n = 41$; $s = 0.275$; $r^2 = 0.959$; descriptors: log *P*, σ_I, $V_{ortho, meta, para}$, indicator variables for mutliply substituted members). For a complementary training set in which the B-ring was held constant with a near-optimized 2-Cl substituent and

the A-ring pattern was varied, the following equation for *Chilo* toxicity was derived:

$$\mathrm{pLD}_{50} = (0.722)\Delta\log P - (0.74)\Sigma\Delta L_{ortho} - (0.868)\Sigma\Delta V_{\mathrm{w}}^{meta} - (0.485)\Delta L_{para} + 6.653$$
$$n = 44,\ s = 0.284,\ r = 0.896,\ F_{4,39} = 39.5 \qquad [1]$$

This equation states that for the training set with A-ring variation, toxicity to *Chilo* larvae is positively influenced by high $\log P$ and negatively influenced by the length of substituents in the *ortho*- and *para*-positions and the summed volume of the *meta*-substitutents (Oikawa *et al.*, 1994b). Thus, the most active DAHs in this series bear small lipophilic substituents which increase the $\log P$, and therefore pLD_{50}, to a greater degree than their size detracts from activity. When a very similar training set was tested against beet armyworm larvae, an exactly analogous regression equation was developed ($n=42$, $s=0.273$, $r^2=0.902$, descriptors: $\log P, \Delta L_{ortho}, V_{\mathrm{w}}^{meta}, L_{para}$) reflecting toxicity highly correlated with that of *Chilo* (Smagghe *et al.*, 1999).

Several related QSAR studies were perfomed with *Chilo* in which the ecdysteroid-agonist response was measured not by toxicity, but rather by the rates of chitin synthesis in cultured integument fragments from diapause larvae (Nakagawa *et al.*, 1995b, 2000a). The first study on 37 B-2-Cl and A-unsubstituted DAHs, members of the training sets from the two prior studies on *Chilo* larvae, also resulted in a linear regression with terms resembling the sum of terms from the two prior *Chilo* studies ($n=37$, $s=0.288$, $r^2=0.872$, $F_{9,27}= 20.43$). The second study used a training set consisting of 23 DAHs heavily represented by structures in which one or the other aryl ring was replaced by a saturated ring or chain. Here, a QSAR was developed which described integument activity in terms of a positive influence by $\log P$ and a negative influence by the distances between the carbonyl carbons and the distal carbon atoms on the same side of the hydrazine linkage. In a Hansch study using larval toxicity of the spruce budworm, a set of 10 A- or B-ring monosubstituted DAHs showed a positive correlation of toxicity to substituent π-lipophilicty values and a negative correlation to substituent length (L), more or less independent of substituent position (Mohammed-Ali *et al.*, 1995).

The classical QSAR approach is attractive for its simplicity. It can provide straightforward insights for ligand design. The method is limited, however, by application to datasets with a common core region, and classical QSAR's potential for underparameterization can easily lead to oversimplification (Hansch and Fujita, 1995). Addressing these issues in the context of DAH QSAR, the three-dimensional CoMFA approach has been applied to a balanced set of 37 A- and B-ring substituted DAHs aligned in the crystal structure conformation, and using ecdysteroidal-responsive *N*-acetylglucosamine incorporation of *Chilo* integument as the dependent variable. The steric field exerts the most dominant role in the model (52.5% contribution) with potency-diminishing contours located in A- and B-ring positions reassuringly consistent with the two *Chilo* Hansch analyses described above ($r^2=0.845$, $q^2=0.438$, $s=0.292$, four components). $\log P$ contributes to a level of 10.7%; the remainder of the descriptors arise from the electrostatic field (Nakagawa *et al.*, 1995a).

3.4.9.2.2. Nonlepidopterans The initial report of the ecdysteroidal properties of DAHs correlated the hyperecdysonistic response in larvae of a lepidopteran, *M. sexta* with the affinity for a dipteran, *D. melanogaster* EcR in Kc cells for an early set of DAHs (Wing, 1988; Wing *et al.*, 1988). This observation presaged research in defining the physiological and physiochemical reasons for the exquisite lepidopteran selectivity of DAHs. The general understanding which has emerged is that: (1) species susceptibility to DAH toxicity is primarily a function of the intrinsic affinity of the DAH to the specific EcR, (2) the general DAH chemotype has the greatest affinity for lepidopteran EcRs; DAH subsets have moderate affinity for coleopteran and dipteran EcRs, (3) ecdysteroid affinity is fairly consistent across insect species while DAH affinity modulates above or below 20E or PoA potency levels, depending upon species, (4) absorption, distribution, metabolism, excretion, and insect behavior may modulate, but do not usually significantly affect, the toxicity levels determined primarily by EcR affinity (Smagghe *et al.*, 2001). These general conclusions have been thoroughly reviewed (Hsu *et al.*, 1997; Dhadialla *et al.*, 1998; Carlson, 2000).

The isolation of the locus of biological activity and selectivity to the EcR enables the interpretation of QSARs in terms of receptor binding pocket interactions and facilitates direct quantitative comparisons across species. For the coleopteran Colorado potato beetle (*Leptinotarsa decemlineata*), fourth instar larvae were monitored for toxicity after topical application of a set of 45 A-ring unsubstitued DAHs (Nakagawa *et al.*, 2001b). Unlike a closely analogous DAH set examined in *C. suppressalis*, which showed a uniformly augmenting effect from $\log P$ and ($\sigma_{\mathrm{I}}^{ortho}$) and a negative influence by V_{w}^{meta} and multiple substitution, DAH toxicity in *L. decemlineata* exhibits a more complex convex

parabolic relationship to hydrophobicity, which can be expressed in terms of the π-value of substituents in the *meta*-position, and which is enhanced by large *ortho*-substitents ($V_w^{ortho}, \Delta B_1^{ortho}$). Only the diminishing effect of large *para*-substitents (*C. suppressalis*: V_w^{para}, *L. decemlineata*: ΔB_5^{para}) is analogous across these two species. Overall, there is no clear correlation of toxicity between these two species. Interestingly, the *ortho*-*sec*-butyl substituent was found to be quite favorable for activity against *L. decemlineata*. Examining the substituent effects on the A-ring while the B-ring is held constant as 2-Cl (another DAH set closely analogous to one described above for *C. suppressalis*) the following equations were developed to describe two distinct DAH subclasses (Nakagawa *et al.*, 1999):

$$\mathrm{pLD}_{50} = (0.887)\log P + (0.395)\Delta B_5^{ortho} + (0.435)E_s^{meta} + (0.65)E_s^{para} + (1.09)\mathrm{HB} + 2.97$$
$$n = 28, s = 0.328, r^2 = 0.842, F_{5,22} = 10.68 \quad [2]$$

$$\mathrm{pLD}_{50} = (-1.695)\log P - (0.527)\Sigma E_s^{ortho} - (0.766)\Sigma E_s^{meta} - (0.916)\Sigma E_s^{para} + 9.23$$
$$n = 16, s = 0.373, r^2 = 0.885, F_{4,11} = 9.982 \quad [3]$$

Here, the effect of hydrophobicity is expressed in two equations with a single log *P* term rather than a multiterm expression in one equation. One DAH subclass, which is more populous and tends to be monosubstituted, increases in toxicity with increasing log *P* (eqn [2]), while the second subclass, generally multisubstituted, shows the opposite effect (eqn [3]). As with *C. suppressalis* toxicity, in *L. decemlineata*, the first subclass exhibits a toxicity-intensifying effect from log *P*, but unlike *C. suppressalis*, a diminishing effect from sterically requiring substituents in all positions (cf. eqn [1]). For the second subclass, the situation is completely reversed with respect to log *P* and substituent sterics. To highlight the dichotomy of lepidopteran versus coleopteran SAR, the lepidopteran-optimized DAHs, tebufenozide and methoxyfenozide, were observed to be at least two orders of magnitude weaker than the coleopteran-optimized DAH, halofenozide, in *L. decemlineata* ($\mathrm{pLD}_{50} = 3.38$, 3.29 versus 6.09), while in the lepidopteran *S. exigua*, almost the opposite is the case ($\mathrm{pLD}_{50} = 7.5$, 8.18 versus 6.1; Smagghe *et al.*, 2003).

A direct comparison of *L. decemlineata* toxicity with that of *C. suppressalis* and *S. exigua* by the CoMFA method illuminates the disparate balance of structural factors responsible for toxicity in the larval stages of these organisms (Smagghe *et al.*, 2003). Earlier toxicity data from all three organisms for a common set of 59 DAHs exemplifying modifications on both rings was used to build CoMFA/CoMSIA (comparative molecular similarity indices analysis) models (Klebe, 1998) by a uniform procedure. All models retained a significant steric component. However, whereas the resultant *S. exigua* and *C. suppressalis* models greatly benefited from hydrophobic field and surface area contributions, as well as other factors, the favored *L. decemlineata* model was dominated by sterics with an additional contribution only from electrostatics (for all three models, $r^2 = 0.843$–0.958, $q^2 = 0.583$–0.683, 5–6 components). The models were optimized on test set r^2 (24–50 test set members) as well as training set q^2 in order to produce a QSAR tool which would be predictive as well as explanatory (adjusted test set $r^2 = 0.40$–0.465).

Another CoMFA analysis was performed on a balanced set of 100 DAHs modeled in the folded crystal structure conformation (Chan *et al.*, 1990) and tested in the *Drosophila* $\mathrm{B_{II}}$ assay (Dinan, 2003; Dinan and Hormann, unpublished data). Many of the training set members examined against *C. suppressalis* and *L. decemlineata* appear in the *Drosophila* model. Sterics, electrostatic, and hydrogen bond donor/acceptor fields all play a balanced role with log *P* in explaining ecdysonergic activity ($r^2 = 0.903$, $q^2 = 0.737$, five components). Not only do the field contributions differ from prior lepidopteran or coleopteran models, the *Drosophila* $\mathrm{B_{II}}$ field contours vary significantly as well (**Figures 19** and **20**).

In addition to the native inducible gene expression systems discussed so far, engineered heterologous systems may shed light not only on ligand–EcR recognition, but also on secondary factors which could modulate the specific EcR–ligand interaction. A set of 146 DAHs spanning considerable substituent space were analyzed in a gene switch derived from *Bombyx* EcR and utilizing endogenous RXR as partner protein and β-galactosidase as reporter in stably transfected human kidney cells (Suhr *et al.*, 1998; Hormann *et al.*, 2002). The ligands were modeled in the folded conformation. From these data, CoMFA/CoMSIA ($r^2 = 0.633$, $q^2 = 0.831$, five components) and 4D-QSAR models ($r^2 = 0.709$, $q^2 = 0.644$) were constructed and evaluated with a 30 member test set (average residual fit = 0.493–0.521 pEC_{50} units). The favored CoMFA models utilized hydrogen bonding fields and the CoMSIA hydrophobic field in addition to steric and electrostatics in rather complex contours. A large hydrophobic contour appears in the region between the two aromatic rings. The favored 4D-QSAR model is characterized by several aromatic grid cell occupancy descriptors in addition to the more common

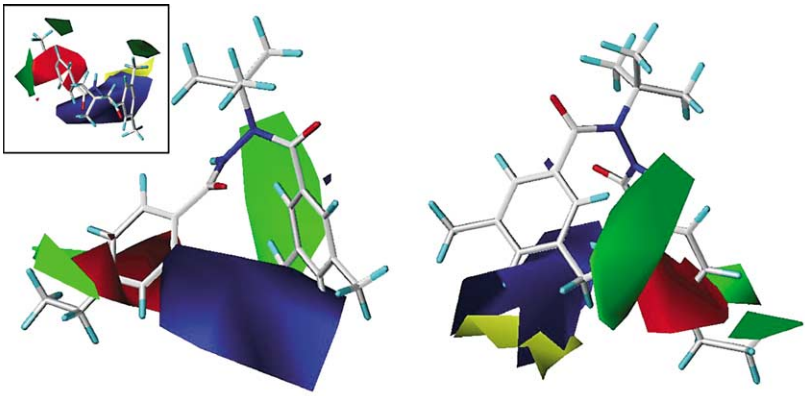


Figure 19 CoMFA electrostatic and steric field contour plot (standard deviation × coefficient) for DAH CoMFA model of B_{II} data. Blue/red polyhedra represent regions where positive charge is favored/disfavored; contribution level = 75/25%, respectively. Green/yellow polyhedra represent sterically favored/disfavored regions; contribution level = 65/35%, respectively. Tebufenozide is depicted.

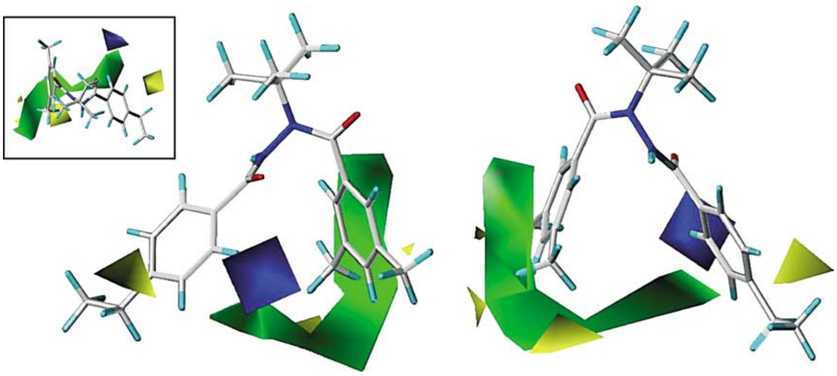


Figure 20 CoMFA hydrogen bond field contour plot (standard deviation × coefficient) for DAH CoMFA model of B_{II} data. Blue/red polyhedra represent regions where hydrogen bond donation is favored/disfavored; contribution level = 70/30%, respectively. Green/yellow polyhedra represent hydrogen bond acceptor favored/disfavored regions; contribution level = 70/30%, respectively. Tebufenozide is depicted.

any-atom and nonpolar atom type descriptors; most grid cell occupancy descriptors (GCODs) are clustered around the more distal regions of the aromatic rings. Model building with a number of trial alignment hypothesis strongly suggests that the DAHs align in the *Bombyx mori* EcR cavity principally according to one or more of the backbone atoms, in contradistinction to one or the other of the benzamide fragments.

3.4.9.2.3. Pharmacophores The pharmacophoric features common to all sensitive insect orders are:

1. Two hydrogen acceptor or polar negative atoms space approximately approximately 3.5–4.0 Å apart.
2. A bulky, conformationally-determining (see below) lipophilic group located asymmetrically between the two negative centers.
3. Moderately sized (about six carbons) groups on either side of the negative centers.
4. A hydrogen bond-donating group is highly advantageous when located near the alternate negative center.

The pharmacophore has been most aptly manifested in mono-*N*-substituted diacylhydrazides. Toxicity optimized for both lepidopterans and coleopterans favors aryl groups with substituent types which tend to be small and nonhydrogen bond donating (Figure 21). Lepidopteran activity is enhanced with A-ring substitution at the 4-position with 1–2 carbon lipophilic groups or, alternatively, with a 2,3- or a 2,[3,4]-ring substitution pattern. B-ring patterns are less specific, but substitution in the 2-, 2,5-, 3,5-, or 3,4,5-positions can be favorable. Coleopteran activity, on the other hand, is optimized only with the most parsimonious selection of one or

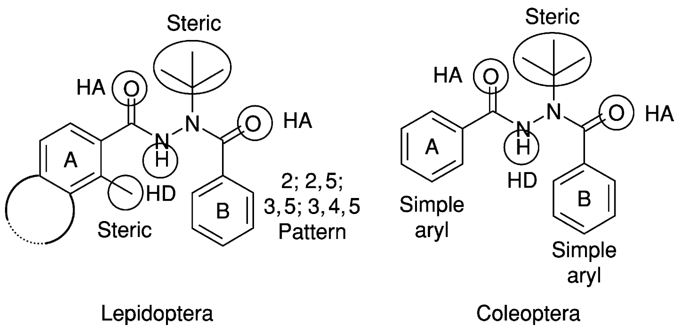


Figure 21 Diacylhydrazine pharmacophore for two insect orders.

two small groups, exemplified by halofenzozide having a single chlorine at the A4 position.

The known binding pose of the DAH chemotype in HvEcR (Billas *et al*., 2003) is a folded conformation with P-helicity (vida infra), wherein the *t*-butyl group points downward approximately toward the H6/7 loop and the A-ring is orientated towards the H7/10 interface. The A-ring *ortho*-methyl substituent of the studied ligand, BY-106830, is orientated to its exterior. Hydrogen-bonding interactions are made with the A-ring carbonyl (N504), B-ring carbonyl (T343), and NH group (Y408). The *t*-butyl group lies in a hydrophobic pocket comprising residues from H3, H11, the H6/7 loop and the H11/12 loop (Billas *et al*., 2003). Overall, this binding pose is quite unusual and considerably shifted in position relative to ligands of other nuclear receptors, which, when viewed together, maintain a fairly consistent center of mass more to be likened to PoA bound in HvEcR. EcR crystal structure-naive docking studies have generally placed DAHs in a more conventional position (Wurtz *et al*., 2000; Kumar *et al*., 2002). The DAHs are generally globular and do not exhibit completely dissymmetric and pronounced electrostatic surface markings. Thus, in homology model docking studies, DAH orientation tends to be multimodal. It remains to be seen what DAH docking to a genuine EcR structure will reveal.

The conclusions from qualitative SAR, QSAR studies, and Lepidopteran pharmacophore analysis depicted in **Figure 21** are consistent with the DAH–protein interactions implicated by the HvEcR/BY-106830 crystal structure. The authors attribute the Lepidopteran specifity of the DAH BY-106830 to the lepidopteran-conserved residue, V384, which is complementary in shape, hydrophobicity and particularly the size of the 3,5-dimethyl substitution of the BY-106830 B-ring. In other insect orders, methionine occurs at this residue position and is prohibitively sterically demanding (Billas *et al*., 2003). It will be very interesting to see in the future just how DAHs actually do bind to non-lepidopteran EcRs.

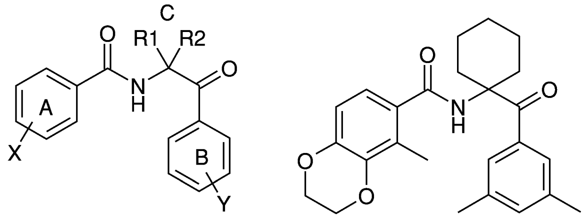


Figure 22 The amidoketone (AMK) generalized and lead structures.

3.4.9.3. Amidoketone SAR

The amidoketones (AMK) have been disclosed recently as an EcR-active chemotype (Tice *et al*., 2003a, 2003b). These compounds invoke an isosteric replacement of the B-*t*-butyl amide in the DAHs with a disubstituted ketone, thereby nullifying the long-held precept that nitrogens are required in the backbone. The AMKs are effective as gene switch inducers in systems based on BmEcR/RXR (*B. mori*) in HEK cells or on CfEcR/β-RXR-LmUSP (*C. fumiferana*, *L. migratoria*) in Chinese hamster ovary cells. AMK SARs for these two receptors indicate a pattern reflecting that found for the DAHs. Notably, the C-module, in which the C-for-N replacement is located, shows a strong preference for four or five attached carbons, either as two acyclic susbstituents or more preferably, as a five- or six-membered ring. The most active reported representative bears a 3,5-dimethyl substitution on the B-ring, a cyclohexyl group as the C-module, and a dioxan ring fused to the A-aryl group. The crystal structure of an AMK analog of methoxyfenozide shows a remarkable fit of its backbone atoms with those of a crystal structure for GS-E^{TM} (RG-102240, DAH: A = 2-ethyl, 3-methoxy; B = 3,5-dimethyl) (**Figure 22**) (Tice *et al*., 2003b). Thus, the doubly substituted backbone carbon,

isosteric to N(*t*-Bu), appears also to serve quite adequately as a conformational determinant.

3.4.9.4. Tetrahydroquinolines

The tetrahydroquinolines (THQ) are another distinctly different ecdysonergic chemotype for which an SAR is emerging. Investigators at FMC Corp., who made the initial discovery (Chaguturu *et al.*, 1999; Dixson *et al.*, 2000), have reported that representatives bind competitively with PoA to the EcR, and that they have a nanomolar affinity for the *Drosophila* receptor, exceeding their submicromolar affinity for HvEcR, the receptor from the tobacco budworm, *Heliothis virescens*. FMC Corp. has also demonstrated the compounds to be insecticidal. The optimized compounds bear small alkyl or halo groups in the *meta*- and/or *para*-positions of the D-ring and fluorine- or no substitution in the A- and C-rings. Subsequently at RheoGene, Smith *et al.* (2003) examined a designed library of THQs in an inducible gene regulation system based on AaEcR (from yellow fever mosquito, *Aedes aegypti*) in 3T3 cells and demonstrated that similarly optimized structures were nearly as potent as GS-E^{TM} as gene-switch elicitors, with induction levels reaching several hundred-fold above background (**Figure 23**). Published data do not quite yet allow definition of a THQ pharmacophore or a QSAR.

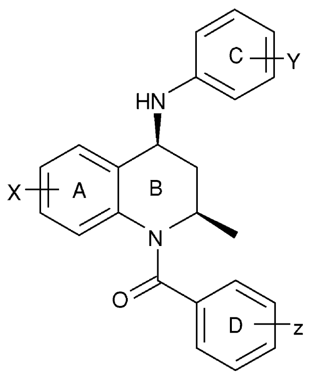


Figure 23 The tetrahydroquinoline (THQ) generalized structure.

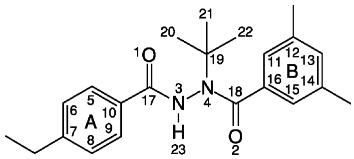
3.4.9.5. Other Chemotypes and Nonecdysonergic Effects

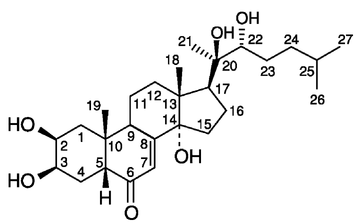
Other chemotypes reported to be E agonists or antagonists such as DTBHIB, maocrystal E, or the brassinosteroids, cucurbitacins, stilbenoids, phenylalkanoids, and harpagides are not yet sufficiently represented with an adequate span of potency for satisfactory application of QSAR. In one or two cases, true ecdysonergic properties remain to be adequately demonstrated. Only a preliminary SAR examination of the electrophysiological response of taste receptors to ecdysteroids has been conducted.

3.4.9.6. Interchemotype Comparisons

The DAH chemotype is striking not only owing to its extraordinary suitability as a crop protection agent, but also owing to the utter dissimilarity of its chemical structure to the natural ecdysteroids (**Table 5**).

Table 5 Diacylhydrazine and ecdysteroid structures, physiochemical properties, and numbering systems





	RH-75992 /tebufenozide	*Ponasterone A*
Shape	Globuar	Crescent
Volume (Å^3)	365	454
Surface area (Å^2)	465	633
Polar surface area (Å^2)	47	121
C log *P*	4.51	0.99
H bond donors	1	5
H bond acceptors	2	6
Effective rotatable bonds	2	5
Polarizability (μ)	7.79	3.72

Reproduced with permission from Hormann, R.E., Dinan, L., Whiting, P., **2003**. Superimposition evaluation of ecdysteroid agonist chemotypes through multidimensional QSAR. *J. Comp. Aided Mol. Des.* *17*(2–4), 135–153; © Kluwer Academic Publishers.

Accordingly, interchemotype comparisons have been undertaken in the area of physiological properties, structural superimposition and model building and, ultimately, might take place in the arena of comparative EcR crystal structures containing alternate chemotypes. In EcR competitive binding studies and tissue activation, lepidopteran-optimized DAHs such as tebufenozide and methoxyfenozide match or exceed PoA in potency, but even the prototype RH-5849 compares favorably with the less active 20E (Dhadialla *et al.*, 1998; Nakagawa *et al.*, 2000b). Otherwise, for other insect orders, the DAHs are generally relatively weaker. The relationship is a bit less clear in engineered inducible gene regulation systems. In the VgEcR/RXR gene-switch, which is based on the *Drosophila* receptor (DmEcR), nine out of 21 tested DAH ligands showed an ability to actuate this ecdysteroid-responsive switch transiently transfected into CV-1 cells, albeit with considerably less potency than muristerone A (Saez *et al.*, 2000). On the other hand, Chinese hamster ovary cells stably transfected with the DmEcR-derived VgEcR/RXR switch and a β-galactosidase reporter responded to GS-E^{TM} at a level comparable to PoA and muristeroneA (MuA) (Carlson *et al.*, 2001a). In gene switches derived from lepidopteran EcRs, DAHs are generally superior to the steroids as ligands (Kumar *et al.*, 2002).

The geometrical relationship of ecdysteroids and DAHs is particularly interesting, not only because of their structural dissimilarity, but also owing to the compelling evidence, solidified by holo-EcR crystal structures, that the same binding cavity recognizes both chemotypes. This is the EcR paradox: the simultaneous high affinity for both the extended, polar, hydroxyl-laden ecdysteroids and the relatively compact, lipophilic, hydrogen bond donor-deficient diacylhydrazines.

An understanding of steroid–DAH superimpositions is greatly facilitated by an appreciation for the DAH and ecdysteroid conformational space. DAHs distribute into four conformational clusters based on the *E*- or *Z*-configuration of the amide bond. Each of these is described by a trivial nomenclature (**Figure 24**). Furthermore, each conformational cluster exists in two enantiomorphs, depending upon the *M*- or *P*-helicity of the N–N bond (**Figure 25**). Since the conformation-determining amide bonds are in the backbone of the structure, each of these clusters vary quite considerably in overall shape and potential superimposition with the ecdysteroids.

DAH conformational analysis by several investigators leads to the general conclusion that the extended, but especially the folded and stacked, conformations are more favorable than the hooked conformation. However, depending upon substitution pattern and calculation method, all four clusters can appear within as little as 4 kcal of the global minimum (Hormann *et al.*, 2003). More accurate calculations will certainly result with application of improved N–N torsional parameterization (Reynolds and Hormann, 1996; Chakravarty *et al.*, 1998). Perhaps owing to the fact that the folded conformation is frequently observed in crystal structures, all DAH–steroid superimpositions so far proposed, as well as all multidimensional QSAR studies, have focused on the folded conformation with either the *M*- or the *P*-helicity. The DAH conformation observed in the HvEcR crystal structure

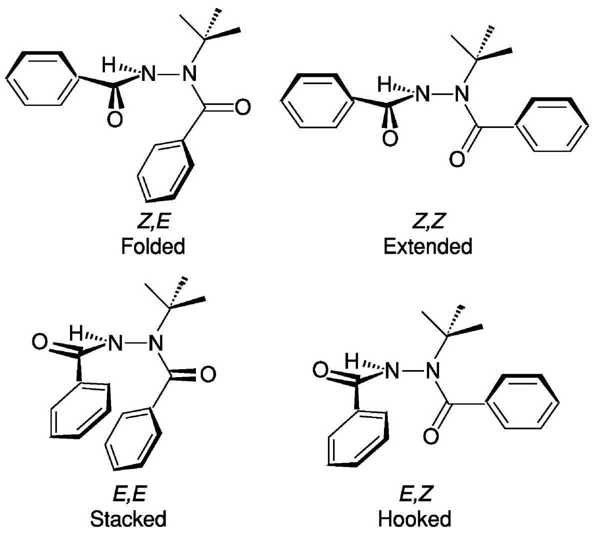


Figure 24 Diacylhydrazine conformational clusters. (Reproduced with permission from Hormann, R.E., Dinan, L., Whiting, P., **2003**. Superimposition evaluation of ecdysteroid agonist chemotypes through multidimensional QSAR. *J. Comp. Aided Mol. Des.* *17*(2–4), 135–153; © Kluwer Academic Publishers.)

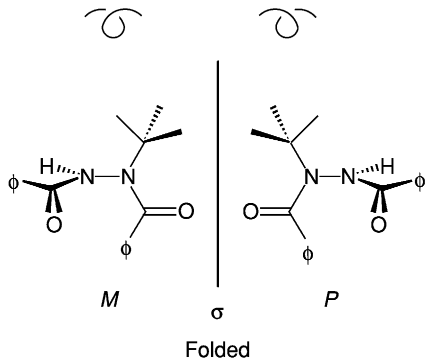


Figure 25 Diacylhydrazine enantiomorphs (folded conformation). (Reproduced with permission from Hormann, R.E., Dinan, L., Whiting, P., **2003**. Superimposition evaluation of ecdysteroid agonist chemotypes through multidimensional QSAR. *J. Comp. Aided Mol. Des.* *17*(2–4), 135–153; © Kluwer Academic Publishers.)

is indeed a folded conformation of P-helicity (Billas *et al.* 2003).

The ecdysteroid conformational space varies primarily in the A-ring conformation and the trajectory of the side chain relative to the D-ring. The A-ring may take on a chair, half-chair, or twist-boat conformation; the chair conformation is found in the E crystal structure, which, in turn, is the basis for much of the modeling that has been performed. The C16/C17/C20/C22 and C17/C20/C22/C23 torsions are most conformationally-determinant; in the crystal structure, these take on values of 62.8° and 73.9°, respectively (Huber and Hoppe, 1965), thereby conferring a crescent shape to the entire molecule. Similar conformations often appear as the favored ones in 4D-QSAR studies, in which conformation is self-optimizing. Conformations incorporating a more acute bend at the core/side chain junction appear in at least one CoMFA study (Nakagawa *et al.*, 1998) and also for some weakly active steroids in 4D-QSAR studies. Satisfyingly, the PoA conformation observed in the HvEcR crystal structure shows an A-ring in the chair conformation and an overall crescent shape.

DAH/ecdysteroid superimpositions hypotheses have been derived from or analyzed by simple pharmacophore considerations (Shimizu *et al.*, 1997), perceived substructure analogy (Mohammed-Ali *et al.*, 1995; Sawada *et al.*, 2003a), conformational studies (Qian, 1996), homology model docking (Wurtz *et al.*, 2000), and multidimensional QSAR (Nakagawa *et al.*, 1995a, 1998; Hormann *et al.*, 2003). The latter two approaches potentially offer some degree of validation. The first superimpostion tested in a QSAR model relied on a set of 37 DAHs and six ecdysteroids tested in the previously mentioned *Chilo* integument assay. Overlaps were established by aligning the DAH carbonyl oxygens with O6 and O14 of the steroid, or alternatively, O14 and O20. Models were evaluated in part on the q^2 values which ranged from 0.393 to 0.472. The favored model ($q^2 = 0.472$, $r^2 = 0.892$, $s = 0.250$, five components) involved an O14/O22 alignment with the DAH B-ring oriented toward the side chain. Shimizu *et al.* (1997) later discovered that replacement of the DAH A-ring with simple C_5–C_6 alkyl chains resulted in structures with potency approaching that of tebufenozide in the *C. suppressalis* integument assay, and, considering the sensitivity of steroid potency to O22 configuration, hypothesized that DAH carbonyl alignments with O20 and O22 might result in superior superimpositions (Nakagawa *et al.*, 1998). CoMFA models of this superimposition type (**Figure 26**) compare favorably with models based on the O14/O22 alignment

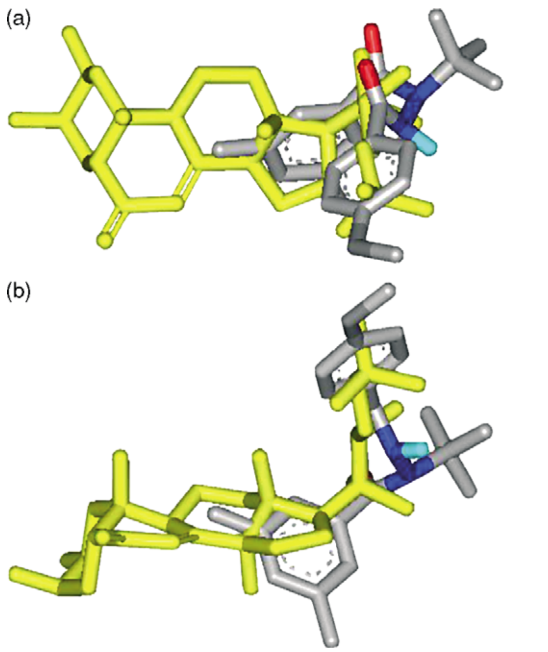


Figure 26 Favored superimposition for DAH–ecdysteroid CoMFA model of Nakagawa (1998). 20-Hydroxyecdysone and tebufenozide are depicted. (a) View from the β-face; (b) view towards the C6/C14 edge of the steroid. (Reproduced with permission from Nakagawa, Y., Hattori, K., Shimizu, B.-I., Akamatsu, M., Miyagawa, H., *et al.*, **1998**. Quantitative structure–activity studies of insect growth regulators XIV. Three-dimensional quantitative structure–activity relationship of ecdysone agonists including dibenzoylhydrazine analogs. *Pestic. Sci.* 53, 267–277.)

($q^2 = 0.359$–0.409 and 0.307–0.388, respectively), especially in view of the fact that 14OH is not necessary for receptor binding (Cherbas *et al.*, 1982). The analyses were performed with 56 DAHs and six ecdysteroids using *C. suppressalis* integument data as the target variable.

In another study, *Drosophila* B_{II} data for 97 DAHs and 66 ecdysteroids were fitted to eight different superimpositions, representing both *M* and *P* DAH enantiomorphs, using CoMFA and 4D-QSAR in a consensus strategy (Hormann *et al.*, 2003). The resultant QSAR models were evaluated on the basis of q^2 and test set r^2, using a test set comprising 19 DAHs and 33 ecdysteroids which had not been used in model construction. The best test set r^2 values, generally a more rigorous criterion of model quality than training set q^2, were obtained from a common superimposition ($q^2_{4D\text{-}QSAR} = 0.722$; $q^2_{CoMFA} = 0.607$, test set $r^2_{4D\text{-}QSAR} = 0.382$, test set $r^2_{CoMFA} = 0.428$) (**Figure 27**) which fortuitously resembled that obtained from a CtEcR homology model built from RAR-γ (Wurtz *et al.*, 2000). In this case, the DAH B-carbonyl aligns with the steroid B-ring carbonyl, but the DAH A-ring carbonyl does not

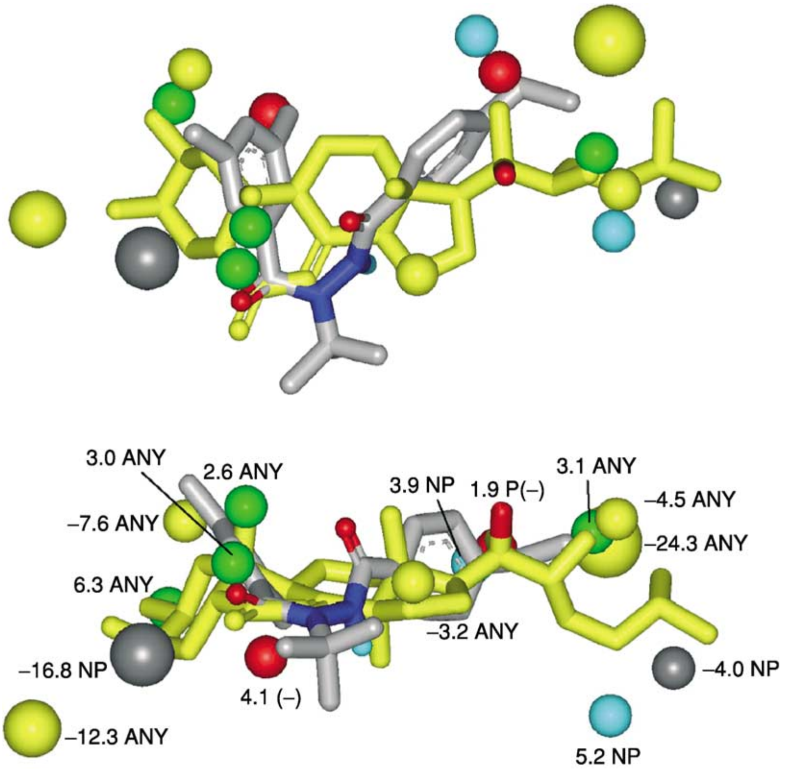


Figure 27 One of the favored DAH/ECD superimpositions depicted as its 4D-QSAR model relative to PoA and tebufenozide in their postulated active conformations with respect to each model. See explanatory text for **Figure 17**. (Reproduced with permission from Hormann, R.E., Dinan, L., Whiting, P., **2003**. Superimposition evaluation of ecdysteroid agonist chemotypes through multidimensional QSAR. *J. Comp. Aided Mol. Des. 17*(2–4), 135–153; © Kluwer Academic Publishers.)

align with any oxygen functionality. Another highly ranking superimpostion invoked a DAH carbonyl alignment with O20 of the steroid. None of these DAH/ecdysteroid superimposition hypotheses correspond to the overlap found for the HvEcR crystal structures (Billas *et al*., 2003). As previously alluded to, there is a structural reason to speculate that the excluding volume of M384 in H5 of DmEcR might possibly preclude this possibility altogether. Whatever the case may be, the HvEcR/BY-106830 structure offers a revealing answer to the EcR paradox – the recognition of widely disparate DAH and ecdysteroid chemotypes. In the HvEcR crystal structures, this is accomplished by (1) use of only partially overlapping loci (steroid side-chain and DAH B-ring/B–C=O/*t*-Bu), (2) use of only one common pharmacophore element (hydrophobe corresponding to distal portion of steroid side-chain and DAH B-ring/*t*-Bu), and (3) interaction with common hydrogen-bonding protein residues from substantially different positions (Y408 with steroid-20-OH/DAH-NH and T343 with steroid-14-OH/DAH B–C=O). It is becoming apparent that the complete set of pharmacophore elements recognized by EcR is much more expansive than those found in the ecdysteroids alone.

One must be mindful of the possibility of multiple binding modes across EcRs from different species, across analogs of the same chemotype, or even for a single ligand with respect to a single responsive EcR. Whether one adheres strictly to a crystal structure superimposition or speculates on alternate binding modes, superimposition exercises are useful because the resulting models can be used as virtual screens with quite different characteristics than scored docking.

Superimpositional studies of the alternate chemotypes are less advanced. The direct structural and SAR similarity of the AMKs strongly suggests that their steroid superimposition could be inferred from DAH considerations. The THQs, on the other hand, require an independent analysis. One illustrative possibility, generated from the genetic algorithm similarity program (GASP) algorithm, is shown in **Figure 28** (Jones *et al*., 1995). One might also speculate that the THQ A/D-rings or C/D-rings coincide with the DAH aryl rings.

3.4.9.7. Future Developments

QSAR methodology is important to ligand design because it enables conceptualization and succinct articulation of pharmacophoric design elements.

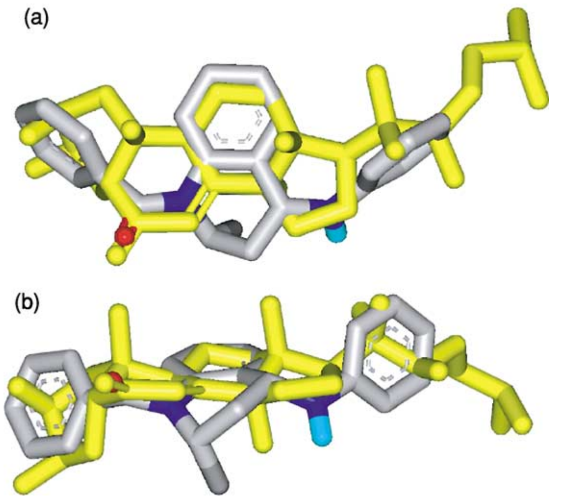


Figure 28 A possible THQ/ECD superimposition of the fully unsubstituted 2*R*,4*S*-THQ and PoA. (a) View from the β-face of PoA; (b) view towards the C6/C14 edge of PoA.

These design elements can take the form of localized physiochemical properties, hydrogen bonding potential, shape, molecular dynamics, and whole molecule properties. These features are frequently difficult or impossible to express in simple diagrams or prose, particularly in the area of multichemotype superimposition. Applied rigorously and creatively, QSAR can evoke novel perceptions of the ligands – perceptions that are useful for further design. These principles have held true for QSAR applied to ecdysteroid agonists.

Nevertheless, certain issues remain outstanding. Undersized training sets, narrow structural variation, and inadequate or imbalanced distribution across the target variable can lead to unstable models with limited explanatory power. The issue of model validation also requires more attention (Golbraikh and Tropsha, 2002). Models with low q^2 values or the absence of a test set leave the question of predictability completely unanswered and often in doubt. Moreover, even where test sets are applied, the test set selection may demonstrate only that the model is interpolative rather than extrapolative. The latter is preferred.

Examples of all of these compromising situations exist in the field of ecdysteroid agonist QSAR. To be sure, some of the aforementioned criteria have developed fairly recently as QSAR methodology has matured. Even applied imperfectly, the QSAR approach so far has exerted explanatory power and provided increased confidence during grueling synthesis programs. The indisputable mark of success will be a QSAR-based ligand design that would otherwise have been impossible. Multichemotype superimposition experiments may be headed in this direction. The demonstration that a structurally divergent active chemotype could be designed as a direct and unique consequence of a superimposition would be compelling indeed.

In the future, one may anticipate more extensive use of validating test sets and a more liberal application of consensus QSAR. The CoMFA/4D-QSAR studies open this door. Parallel models using topological descriptors or alternate variable selection and statistical methods may be especially effective in combination with multidimensional QSAR; a model using topological descriptors has been built on the ecdysteroid B_{II} data set (Golbraikh *et al.*, 2001). Additionally, judicious application of lateral QSAR using the same training sets, but different test organisms, may shed additional light on the cross-species selectivity question. One may also see improvements in multidimensional QSAR methodology which speak directly to the issue of multichemotype models.

The availability of an EcR binding domain crystal structure, in addition to answering numerous questions in structural biology, enables *de novo* ligand design. It is also possible that extracted protein information can be incorporated into ligand QSAR models (Santos-Filho *et al.*, 2001), which might be more reliably quantitative than pure docking. Both crystal structure and advanced receptor-independent QSAR models can be used as virtual screens to discover new ligands. In doing so, one must keep firmly in mind the possibility of unexpected or multiple binding modes, either within a single ligand-binding pocket or across species. It may turn out that protein dynamics simulations enhance the already illuminating perspective offered by static EcR crystal structures in explaining the level of receptor plasticity crucial for recognizing ligands of disparate structure.

3.4.10. Applications and Prospects

3.4.10.1. Insecticides

The wide diversity of chemistries that have been shown to interact with ecdysteroids receptors give hope that further commercially viable compounds can be developed as effective and selective insecticides. The lead given in this area by the DAHs is very encouraging. These compounds are chemically simple, effective, nontoxic to vertebrates, and even show some selectivity towards particular insect orders (Hsu *et al.*, 1997; Dhadialla *et al.*, 1998; Carlson, 2000; Carlson *et al.*, 2001b). The discovery of the DAHs was somewhat serendipitous, but the development of further classes of ecdysteroid signaling pathway-active compounds can be facilitated

through mechanism-based assays, QSAR, molecular modeling, and virtual screening. Prior to the identification of the DAHs, it was questioned whether ecdysteroid agonists would make effective insecticides, since it was expected that such compounds would only have an effect at the next molt. In the event, the fact that affected insects do not immediately die is not a problem because a further effect of the ecdysteroid agonist is to bring about a cessation of feeding. Ecdysteroid antagonists might be expected to be effective against pest species where the adult is the destructive stage or where reproductive disruption is sought. However, as our understanding of the pleiotropic physiological consequences of ecdysteroid (ant)agonists is still very incomplete, we should not exclude the possiblitiy that ecdysteroid antagonists may also be effective against larval insects or embryos.

3.4.10.2. Genetic Modification of Ecdysteroid Levels in Crop Plants

Very few crop species accumulate ecdysteroids. Only spinach (*Spinacia oleracea*; Chenopodiaceae) and quinoa (*Chenopodium quinoa*; Chenopodiaceae) accumulate significant amounts (Dinan, 1992, 1995b). However, available evidence indicates that most, if not all, higher plant species possess the genetic capacity to produce ecdysteroids, but that the pathway is downregulated (Dinan *et al*., 2001d). If this is true, this will considerably facilitate the elevation of ecdysteroid levels in crop species, as alteration of the regulation will result in changes in ecdysteroid level. This could require alteration of only one gene, rather than the incorporation of a series of genes for the entire biosynthetic pathway. As an absolute prerequisite to the genetic modification of ecdysteroid levels in crop species, it is necessary that the biosynthetic pathway for phytoecdysteroids should be elucidated. Significant progress is now being made in this area using hairy root cultures of *A. reptans* (Fujimoto *et al*., 2000), which produce ecdysteroids in large amounts and allow stable isotope-labeled precursors to be incorporated in high yield permitting analysis of ecdysteroidal products by ^{13}C NMR. The generality of the *Ajuga* pathway(s) will need to be assessed and the regulation of the pathways determined in order to identify the step(s) which are downregulated in nonaccumulating species. Ecdysteroid-accumulating lines of crop species could be generated by genetic engineering approaches or by traditional plant breeding techniques. Ultimately, it should be possible to modify the profile of ecdysteroids present in crop plants to maximise resistance against even polyphagous pest species which possess particular detoxification mechanisms against common major ecdysteroids (e.g., 22-acylation of 20E) and/or to elevate levels of ecdysteroids of higher biological activity (e.g., PoA). The very low vertebrate toxicity (and even benefit as nutritional supplements) and lack of taste of ecdysteroids would mean that phytoecdysteroid accumulation need not be restricted to the portions of the plant not to be consumed.

3.4.10.3. Gene Switching Elicitors

Engineered inducible gene expression systems are gaining research attention and technological significance. Gene switches permit the temporal and/or spatial expression of transfected genes, with potential application in medicine (gene therapy, tissue engineering, biotherapeutic protein production), agriculture (crop protection, yield enhancement, nutritional enhancement, pharmaceutical production) and in basic research for investigation of gene function (proteomics and drug lead discovery). Several different approaches are being developed (Fussenegger, 2001) and the use of native or hybrid ecdysteroid receptors with ecdysonergic ligands is one of the current forerunners in the field. The elicitor should be effective at low concentrations and should be target-specific (i.e., nontoxic). For medical applications, the elicitor should be nonallergenic and preferably orally available. The elicitor can also affect dynamic properties of the gene switch such as response time, cycling, dynamic range, maximum response, span of the dose-response curve, and tissue specificity. These properties should be tailored to the specific application. Ecdysteroids and DAHs, which, taken together, span a wide range of physiochemical properties, and are both highly promising chemotype candidates for many gene-switch applications.

The gene-switch potency, absorption, distribution, and known pharmacological properties of the ecdysteroids are favorable toward most uses of a gene switch. On the other hand, metabolism and elimination may be mitigating factors. When native or hybrid ecdysteroid receptors are transfected into mammalian, plant or yeast cells, the EcRs possess altered ecdysteroid specificity and potency. In some cases, 20E is hardly active at all and PoA and MuA are required at concentrations 100-fold higher than in insect systems. More extensive SAR or even QSAR studies could assist identification of the most potent ecdysteroids for these systems. A detailed discussion of ecdysteroid-regulated gene switches is beyond the scope of this review, but has been reviewed elsewhere recently (Lafont and Dinan, 2003).

The properties of DAHs that are quite favorable to gene switch applications are potency towards

lepidopteran EcRs, metabolic stability, chemical simplicity, and absence of toxicity. Available data suggest that absorption and distribution properties may be acceptable for pharmaceutical applications. However, solubility is potentially a barrier to be overcome. It remains to be seen if the DAH physical properties are an issue for agricultural gene switch applications in which plant uptake requirements may differ from those needed for the already successful insecticidal applications. An alternative DAH (GS-E^{TM}) (**Figure 6**) has been developed for use with mammalian cells. QSAR has been brought to bear on DAH potency in a mammalian cellular EcR-based gene switch system (Hormann *et al.*, 2002), and should aid future studies that pertain to pharmacokinetics.

Orthogonal gene switches, that is to say, multiple parallel switches which can be actuated simultaneously, noninteractively, and independently, would constitute a significant enhancement to the field of engineered gene regulation. A major potential benefit of EcR systems is their inherent orthogonality to the corresponding transcriptional machinery of the mammalian host. A concomitant liability, however, is the potential for immunogenicity. Likewise, ecdysteroids are not regarded as normal, interactive components of mammalian cells and may have a natural built-in orthogonality. However, the ecdysteroids are present in certain plants as phytoecdysteroids, can be present in the human diet, and may later turn out to have unacceptable pleiotropoic effects. For purposes of orthogonality, the DAHs look attractive. Certainly, the order-specificity of DAHs can be exploited to develop switches for different hybrid ecdysteroid receptors in the same system. For representative ecdysteroids, an otherwise ecdysteroid-responsive EcR has been rendered ecdysteroid-refractive by site-directed mutagenesis, auguring well for orthogonal systems which also utilize a DAH elicitor in a parallel channel, as has been addressed at RHeoGene, L.L.C. (Kumar *et al.*, 2002).

3.4.10.4. Human Health Preparations and Animal Food Supplements

The effects of ecdysteroids on mammals have recently been reviewed (Lafont and Dinan, 2003), to which the reader is referred for more extensive treatment of this topic. As a consequence of the reputed anabolic effects of ecdysteroids on animals, a large number of ecdysteroid-containing preparations are now available for use by sportsmen and body-builders. While there is evidence for the positive anabolic effects of ecdysteroids on Japanese quail (Koudela *et al.*, 1995; Sláma *et al.*, 1996) and pigs (Selepcova *et al.*, 1993; Kratky *et al.*, 1997), the effects are not dramatic (ca. 15% increase in body mass over controls). Ecdysteroids may also improve physical performance, as demonstrated on rats (Chermnykh *et al.*, 1988). Evidence has been provided for effects of ecdysteroids on cellular proliferation, protein synthesis, glucose metabolism, lipid metabolism, nerve function, hepatic function, cardiovascular function, renal function, and the immune system, indicating that ecdysteroids might contribute to the relief of conditions such as diabetes, hypercholesterolemia, wound healing, mental disorders, myocardial arrhythmia, inflammations, etc. (review: Lafont and Dinan, 2003). All these aspects need further study and verification, but the lack of toxic dose required to kill 50% of the organism ($LD_{50} > 6\,g\,kg^{-1}$), androgenic or (anti)estrogenic effects make pharmacological applications of ecdysteroids feasible and potentially attractive.

3.4.10.5. Improvement of Silk Yields

Exogenous ecdysteroids fed to larvae of the silkworm *Bombyx mori* at certain stages of development enhance synchronous development of the larvae and, when coadministered with a juvenile hormone analog, significantly elevate the yield of silk obtainable from the cocoon (Chou and Lu, 1980; Ninagi and Maruyama, 1996). Such treatments have considerable potential to improve the efficiency of silkworm-rearing by Asian farmers, especially since the hormonal treatments could be based on extracts of appropriate indigenous plant species (Chandrakala *et al.*, 1998).

3.4.10.6. Endocrine Disruption

Although it is recognized that endocrine disruption in invertebrates may be a potentially serious problem, many of the necessary experimental tools are not available for research in this area. The many gaps that still exist in our knowledge of invertebrate endocrinology limit the ability to interpret the toxic effects of exogenous chemicals. Even for the insects, where quite extensive endocrinological information is available for a number of model species, they do not correspond to aquatic species or to the sentinel species one would wish to use to monitor for EDCs. Thus, it is to be expected that there will be a revival in interest in hormone biochemistry for environmentally relevant species to provide baseline information against which exposed insects can be compared. Further, with the sequencing of the *D. melanogaster* genome and the development of DNA arrays, it is to be expected that the influence of exogenous chemicals on gene expression will be investigated more extensively.

3.4.10.7. QSAR and Molecular Modeling

If we are to understand the role of phytoecdysteroids in deterring insect predation, the QSAR/modeling approach should be extended to more analogs and to more species and to the SAR of the deterrent antifeedant/toxic effects of ecdysteroids. This would go some way to explain the diversity of phytoecdysteroid structures in plants. The pharmacological effects of ecdysteroids (almost exclusively 20E) on mammals are only now being accepted and it seems that there are many potential beneficial effects. In the future, it will be helpful to conduct QSAR and docking/scoring studies to identify the most effective analogs. In the area of gene switching using ecdysteroids as analogs, SAR will be important not only because hybrid ecdysteroid receptors in mammalian cells have altered specificities, but also because ligands will need to be tailored as specific elicitors for multiplex systems expressing several receptor types simultaneously, in order to enable selective activation of their regulated genes.

Acknowledgments

We are grateful to Y. Nakagawa for molecular structures of diacylhydrazine–ecdysteroid superimpositions. Research conducted at Exeter University was supported by the BBSRC, Rohm & Haas Co., RheoGene Inc., Astra-Zeneca plc, EU-INTAS and the Leverhulme Trust.

References

Adams, M.D., Celinker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Alfonso, D., Bernardinelli, G., Kapetanidis, I., **1993**. Withanolides from *Iochroma coccineum. Phytochemistry 34*, 517–521.

Alfonso, D., Kapetanidis, I., **1994**. Withanolides from *Iochroma gesnerioides. Phytochemistry 36*, 179–183.

Alfonso, D., Kapetanidis, I., Bernardinelli, G., **1991**. Iochromolide: a new acetylated withanolide from *Iochroma coccineum. J. Nat. Prod. 54*, 1576–1582.

Ascher, K.R.S., Nemny, N.E., Eliyahu, M., Kirson, I., Abraham, A., *et al.*, **1980**. Insect antifeedant properties of withanolides and related steroids from Solanaceae. *Experientia 36*, 998–999.

Ashburner, M., **1972**. Ecdysone induction of puffing in polytene chromosomes of *Drosophila melanogaster. Exp. Cell Res. 71*, 433–440.

Bergamasco, R., Horn, D.H.S., **1980**. The biological activities of ecdysteroids and ecdysteroid analogs. In: Hoffmann, J.A. (Ed.), Developments in Endocrinology, Vol. 7. Elsevier/North Holland, Amsterdam, pp. 299–324.

Billas, I.M.L., Iwema, T., Garnier, J.-M., Mitschler, A., Rochel, N., *et al.*, **2003**. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature 426*, 91–96.

Blackford, M.J.P., Dinan, L., **1997**. The effects of ingested 20-hydroxyecdysone on the larvae of *Aglais urticae, Inachis io, Cynthia cardui* (Lepidoptera: Nymphalidae) and *Tyria jacobaeae* (Lepidoptera: Arctiidae). *J. Insect Physiol. 43*, 315–327.

Bourne, P.C., Whiting, P., Dhadialla, T.S., Hormann, R.E., Girault, J.-P., *et al.*, **2002**. Ecdysteroid 7,9(11)-dien-6-ones as potential photoaffinity labels for ecdysteroid binding proteins. *J. Insect Sci. 2*, 11.

Butenandt, A., Karlson, P., **1954**. Über die Isolierung eines Metamorphose-hormons der Insekten in kristallisierter Form. *Z. Naturforsch. 9B*, 389–391.

Cao, S., Qian, X., Song, G., **2001**. *N′-tert*-Butyl-*N′*-aroyl-*N*-(alkoxycarbonylmethyl)-*N*-aroylhydrazines, a novel nonsteroidal ecdysone agonist: synthesis, insecticidal activity, conformational and crystal structure analysis. *Can. J. Chem. 79*, 272–278.

Carlson, G.R., **2000**. Tebufenozide: a novel caterpillar control agent with unusually high target selectivity. *ACS Symposium Series 767*, 8–17.

Carlson, G.R., Cress, D.E., Dhadialla, T.S., Hormann, R.E., Le, D.P., **2001a**. Ligands for modulating the expression of exogenous genes via an ecdysone receptor complex. US Patent 6 258 603.

Carlson, G.R., Dhadialla, T.S., Hunter, R., Jansson, R.K., Jany, C.S., *et al.*, **2001b**. The chemical and biological properties of methoxyfenozide, a new insecticidal ecdysteroid agonist. *Pest Mgt. Sci. 57*, 115–119.

Chaguturu, R., Chiu, G., Cruickshank, P., Cullen, T., Dargar, R., *et al.*, **1999**. Can you use surrogate tissue sources to optimize target site hits to whole insect leads? Presented by Cullen, T. at Changing AgroChem and AgBiotech R&D through Technology, IBC Agriculture, Biomolecular Symposium 23–24 September 1999, San Francisco, CA.

Chakravarty, S.J., Hormann, R.E., Reynolds, C.H., **1998**. Improved AMBER force field parameters for the N–N rotational barrier in diacylhydrazines. Book of Abstracts, 216th ACS National Meeting, Boston, August 23–27 (1998), COMP–114.

Chan, T.H., Ali, A., Britten, J.F., Thomas, A.W., Strunz, G.M., *et al.*, **1990**. The crystal structure of 1,2-dibenzoyl-1-*tert*-butylhydrazine, a nonsteroidal ecdysone agonist, and its effects on spruce budworm (*Choristoneura fumiferana*). *Can. J. Chem. 68*, 1178–1181.

Chandrakala, M.V., Maribashetty, V.G., Jyothi, H.K., **1998**. Application of phytoecdysteroids in sericulture. *Curr. Sci. 74*, 341–346.

Cherbas, L., Yonge, C.D., Cherbas, P., Williams, C.M., **1980**. The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. *Roux's Arch. Devel. Biol. 189*, 1–15.

Cherbas, P., Trainor, D.A., Stonard, R.J., Nakanishi, K., **1982**. 14-Deoxymuristerone, a compound exhibiting

exceptional moulting hormonal activity. *J. Chem. Soc., Chem. Commun.* 1307–1308.

Chermnykh, N.S., Shimanovsky, N.L., Shutko, G.V., Syrov, V.N., **1988**. Effects of methandrostenolone and ecdysterone on physical endurance of animals and protein metabolism in the skeletal muscles. *Farmakol. Toksikol. 6*, 57–62.

Chou, W.S., Lu, M.S., **1980**. Growth regulation and silk production in *Bombyx mori* L. from phytogenous ecdysteroids. In: Hoffmann, J.A. (Ed.), Developments in Endocrinology, vol. 7. Elsevier/North-Holland, Amsterdam, pp. 281–297.

Clément, C.Y., Dinan, L., **1991**. Development of an assay for ecdysteroid-like and anti-ecdysteroid activities in plants. In: Hrdý, I. (Ed.), Insect Chemical Ecology. Academia, Prague, pp. 221–226.

Clément, C.Y., Bradbrook, D.A., Lafont, R., Dinan, L., **1993**. Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem. Mol. Biol. 23*, 187–193.

Clever, U., Karlson, P., **1960**. Induktion von Puff-Veränderungenen in den Speicheldrüsenchromosomen von *Chironomus tentans* durch Ecdyson. *Exp. Cell Res. 20*, 623–626.

DeFur, P.L., Crane, M., Ingersoll, C., Tattersfield, L. (Eds.) **1999**. Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment. SETAC Press, Pensacola, FL.

Descoins, C., Jr., Marion-Poll, F., **1999**. Electrophysiological responses of gustatory sensilla of *Mamestra brassicae* (Lepidoptera, Noctuidae) larvae to three ecdyst- eroids: ecdysone, 20-hydroxyecdysone and ponasterone A. *J. Insect Physiol. 45*, 871–876.

Dhadialla, T.S., Carlson, G.R., Le, D.P., **1998**. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol. 43, 545–569.*

Dinan, L., **1985**. Ecdysteroid receptors in a tumorous blood cell line of *Drosophila melanogaster. Arch. Insect Biochemi. Physiol. 2*, 295–317.

Dinan, L., **1989**. Ecdysteroid structure and hormonal activity. Koolman, J. (Ed.), Ecdysone: From Chemistry to Mode of Action. Thieme Verlag, Stuttgart, pp. 345–354.

Dinan, L., **1992**. The association of phytoecdysteroids with flowering in fat hen, *Chenopodium album*, and other members of the Chenopodiaceae. *Experientia 48*, 305–308.

Dinan, L., **1995a**. A strategy for the identification of ecdysteroid receptor agonists and antagonists from plants. *Eur. J. Entomol. 92*, 271–283.

Dinan, L., **1995b**. Distribution and levels of phytoecdysteroids within individual plants of species of the Chenopodiaceae. *Eur. J. Entomol. 92*, 295–300.

Dinan, L., **1998**. A strategy towards the elucidation of the contribution made by phytoecdysteroids to the deterrence of invertebrate predators on plants. *Russ. J. Plant Physiol. 45*, 296–305.

Dinan, L., **2001**. Phytoecdysteroids: biological aspects. *Phytochemistry 57*, 325–329.

Dinan, L., **2003**. Ecdysteroid structure–activity relationships. In: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, Vol. 29, Bioactive Natural Products. Elsevier, Amsterdam, pp. 3–71.

Dinan, L., Bourne, P.C., Meng, Y., Sarker, S.D., Tolentino, R.B., *et al.*, **2001a**. Assessment of natural products in the *Drosophila melanogaster* B_{II} cell bioassay for ecdysteroid agonist and antagonist activities. *Cell. Mol. Life Sci. 58*, 321–342.

Dinan, L., Bourne, P., Whiting, P., Dhadialla, T.S., Hutchinson, T.H., **2001d**. Screening of environmental contaminants for ecdysteroid agonist and antagonist activity using the *Drosophila melanogaster* B_{II} cell *in vitro* assay. *Envir. Toxicol. Chem. 20*, 2038–2046.

Dinan, L., Bourne, P., Whiting, P., Tsitsekli, A., Saatov, Z., *et al.*, **2003**. Synthesis and biological activities of turkesterone 11α-acyl derivatives. *J. Insect Sci. 3*, 6–11.

Dinan, L., Hormann, R.E., Fujimoto, T., **1999a**. An extensive ecdysteroid CoMFA. *J. Comp. Aid. Mol. Des. 13*, 185–207.

Dinan, L., Sarker, S.D., Bourne, P., Whiting, P., Šik, V., *et al.*, **1999b**. Phytoecdysteroids in seeds and plants of *Rhagodia baccata* (Labill.) Moq. (Chenopodiaceae). *Arch. Insect Biochem. Physiol. 41*, 18–23.

Dinan, L., Rees, H.H., **1981**. The identification and titres of conjugated and free ecdysteroids in developing ovaries and newly-laid eggs of *Schistocerca gregaria. J. Insect Physiol. 27*, 51–58.

Dinan, L., Sarker, S.D., Šik, V., **1997a**. 28-Hydroxywithanolide E from *Physalis peruviana*. *Phytochemistry 44*, 509–512.

Dinan, L., Savchenko, T., Whiting, P., **2001b**. On the distribution of phytoecdysteroids in plants. *Cell. Mol. Life Sci. 58*, 1121–1132.

Dinan, L., Savchenko, T., Whiting, P., Sarker, S.D., **1999c**. Plant natural products as insect steroid receptor agonists and antagonists. *Pestic. Sci. 55*, 331–335.

Dinan, L., Whiting, P., Alfonso, D., Kapetanidis, I., **1996**. Certain withanolides from *Iochroma gesnerioides* antagonize ecdysteroid action in a *Drosophila melanogaster* cell line. *Entomol. Exp. Applic. 80*, 415–420.

Dinan, L., Whiting, P., Bourne, P., Coll, J., **2001c**. 8-O-Acetylharpagide is not an ecdysteroid agonist. *Insect Biochem. Mol. Biol. 31*, 1077–1082.

Dinan, L., Whiting, P., Girault, J.-P., Dhadialla, T.S., Cress, D.E., *et al.*, **1997b**. Cucurbitacins are insect stroid hormone antagonists acting at the ecdysteroid receptor. *Biochem. J. 327*, 643–650.

Dinan, L., Whiting, P., Sarker, S.D., Kasai, R., Yamasaki, K., **1997c**. Cucurbitane-type compounds from *Hemsleya carnosiflora* antagonize ecdysteroid action in the *Drosophila melanogaster* B_{II} cell line. *Cell. Mol. Life Sci. 53*, 271–274.

Dixson, J.A., Elshenawy, Z.M., Eldridge, J.R., Dungan, L.B., Chiu, G., *et al.*, **2000**. A New Class of Potent Ecdysone Agonists: 4-Phenylamino-1,2,3,4-Tetrahydroquinolines. Presentation at the Middle Atlantic Regional ACS Meeting, University of Delaware.

Elbrecht, A., Chen, Y., Jurgens, T., Hensens, O.D., Zink, D.L., *et al.*, **1996**. 8-*O*-Acetylharpagide is a non-steroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol. 26*, 519–523.

Falkenstein, E., Tillmann, H.-C., Christ, M., Feuring, M., Wehling, M., **2000**. Multiple actions of steroid hormones: a focus on rapid, nongenomic effects. *Pharmacol. Rev. 52*, 513–555.

Fujimoto, Y., Ohyama, K., Nomura, K., Hyodo, R., Takahashi, K., *et al.*, **2000**. Biosynthesis of sterols and ecdysteroids in *Ajuga* hairy roots. *Lipids 35*, 279–288.

Fussenegger, M., **2001**. The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering, and advanced gene therapies. *Biotechnol. Progr. 17*, 1–51.

Galbraith, M.N., Horn, D.H.S., **1966**. An insect-moulting hormone from a plant. *J. Chem. Soc., Chem. Commun.* 905–906.

Gateff, E., Gissman, L., Shrestha, R., Plus, N., Pfister, H., *et al.*, **1980**. Characterization of two tumorous blood cell lines of *Drosophila melanogaster* and the viruses they contain. In: Kurstak, E., Maramorosch, K., Dübendorfer, A. (Eds.), Invertebrate Systems *in Vitro*. Elsevier/North-Holland, Amsterdam, pp. 517–533.

Gmelin, R., **1963**. Die Verbreitung von Cucurbitacinen und Cucubitacinglykosiden in der Gattung *Iberis* (Cruciferae). *Arzneimittelforschung 13*, 771–776.

Golbraikh, A., Bonchev, D., Tropsha, A., **2001**. Novel chirality descriptors derived from molecular topology. *J. Chem. Inform. Comput. Sci. 41*, 147–158.

Golbraikh, A., Tropsha, A., **2002**. Beware of q2! *J. Mol. Graphics Model. 20*, 269–276.

Goodwin, T.W., Horn, D.H.S., Karlson, P., Koolman, J., Nakanishi, K., *et al.*, **1978**. Ecdysteroids: a new generic term. *Nature 272*, 122.

Govindachari, T.R., Narasimhan, N.S., Sureshi, G., Partho, P.D., Gopalakrishnan, G., *et al.*, **1995**. Structure-related insect antifeedant and growth regulating activities of some limonoids. *J. Chem. Ecol. 21*, 1585–1600.

Grau, V., Lafont, R., **1994a**. The distribution of ecdysone metabolites within the body of adult *Drosophila melanogaster* females and their sites of production. *J. Insect Physiol. 40*, 87–96.

Grau, V., Lafont, R., **1994b**. Metabolism of ecdysone and 20-hydroxyecdysone in adult *Drosophila melanogaster. Insect Biochem. Mol. Biol. 24*, 49–58.

Guerriero, A., Pietra, F., **1985**. Isolation, in large amounts, of the rare plant ecdysteroid ajugasterone-C from the Mediterranean zoanthid *Gerardia savaglia*. *Comp. Biochem. Physiol. B 80*, 277–278.

Hansch, C., Leo, A., **1995**. Exploring QSAR. American Chemical Society Books, Washington, DC.

Hansch, C., Fujita, T., **1995**. Status of QSAR at the end of the 20th century. In: Hansch, C., Fujita, T. (Eds.), Classical and Three-Dimensional QSAR in Agrochemistry. American Chemical Society, Washington, DC, pp. 288–301.

Harmatha, J., Dinan, L., **1997**. The biological activity of natural and synthetic ecdysteroids in the B_{II} bioassay. *Arch. Insect Biochem. Physiol. 35*, 219–225.

Harmatha, J., Dinan, L., Lafont, R., **2002**. Biological activities of a specific ecdysteroid dimer and selected monomeric structural analogues in the B_{II} bioassay. *Insect Biochem. Mol. Biol. 32*, 181–185.

Heinrich, G., Hoffmeister, H., **1967**. Ecdyson als Begleitsubstanz des Ecdysterons in *Polypodium vulgare* L. *Experientia 23*, 995.

Hetru, C., Roussel, J.-P., Mori, K., Nakatani, Y., **1986**. Activité antiecdystéroïde de brassinosteroïdes. *C. R. Acad. Sci. Paris, Série II, 302*, 417–420.

Hopfinger, A.J., Wang, S., Tokarski, J.S., Jin, B., Albuquerque, M., *et al.*, **1997**. Construction of 3D-QSAR models using the 4D-QSAR analysis formalism. *J. Am. Chem. Soc. 119*, 10509–10524.

Hora, J., Lábler, L., Kasal, A., Černý, V., Šorm, F., **1966**. On steroids CIII. Molting deficiencies produced by some sterol derivatives in an insect (*Phyrrhocorus apterus* L.). *Steroids 8*, 887–914.

Hormann, R.E., Chortyk, O., Cress, D.E., Hopfinger, A.J., Thompson, C.S., **2002**. 4D-QSAR and CoMFA of ligand elicitors for the ecdysone receptor-mediated gene transcription system. Abstracts of Papers 224th ACS National Meeting, Boston, MA, August 18–22, 2002, COMP-058.

Hormann, R.E., Dinan, L., Whiting, P., **2003**. Superimposition evaluation of ecdysteroid agonist chemotypes through multidimensional QSAR. *J. Comp. Aided Mol. Des. 17*(2–4), 135–153.

Horn, D.H.S., Bergamasco, R., **1985**. Chemistry of ecdysteroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, Vol. 7. Pergamon Press, Oxford, pp. 185–248.

Hsu, A.C.-T., **1991**. 1,2-Diacyl-1-alkylhydrazines. In: Baker, D.R., Fenyes, J.G., Moberg, W.K. (Eds.), Synthesis and Chemistry of Agrochemicals, Vol. 2. American Chemical Society, Washington, DC, pp. 478–490.

Hsu, A.C.-T., Aller, H.E., **1991**. Insecticidal N′-substituted-N,N′-diacylhydrazines. US Patent 4 985 461.

Hsu, A.C.-T., Fujimoto, T.T., Dhadialla, T.S., **1997**. Structure–activity study and conformational analysis of RH-5992, the first commercialized nonsteroidal ecdysone agonist. *ACS Sym. Ser. 658*, 206–219.

Huber, R., Hoppe, W., **1965**. Die Kristall- und Molekülstrukturanalyse des Insektenverpuppungshormons Ecdyson mit der automatisierten Faltmolekülmethode. *Chem. Berichte 98*, 2403–2404.

Imai, S., Toyosata, T., Sakai, M., Sato, Y., Fujioka, S., *et al.*, **1969**. Screening results of plants for phytoecdysones. *Chem. Pharmaceut. Bull. 17*, 335–339.

Isman, M.B., **1995**. Leads and prospects for the development of new botanical insecticides. *Rev. Pestic. Toxicol. 3*, 1–20.

Jones, G., Jones, D., **2000**. Considerations on the structural evidence of a ligand-binding function of ultraspiracle, an insect homolog of vertebrate RXR. *Insect Biochem. Mol. Biol. 30*, 671–679.

Jones, G., Sharp, P.A., **1997**. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA 94*, 13499–13503.

Jones, G., Willett, P., Glen, R.C., **1995**. A genetic algorithm for flexible molecular overlay and pharmacophore elucidation. *J. Comp. Aided Mol. Des. 9*, 532–549.

Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., Rhaikel, A.S., **1996**. The mosquito ultraspiracle homologue, a partner of ecdysteroid receptor heterodimer: Cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell. Endocrinol. 12*, 119–132.

Kaplanis, J.N., Dutky, S.R., Robbins, W.E., Thompson, M.J., Lindquist, E.L., *et al.*, **1975**. Makisterone A: a 28-carbon hexahydroxy moulting hormone from the embryo of the milkweed bug. *Science 190*, 681–682.

Kaplanis, J.N., Thompson, M.J., Robbins, W.E., Bryce, M., **1967**. Insect hormones: alpha ecdysone and 20-hydroxyecdysone in bracken fern. *Science 157*, 1436–1438.

Kasuya, A., Sawada, Y., Tsukamoto, Y., Tanaka, K., Toya, T., *et al.*, **2003.** Binding mode of ecdysone agonists to the receptor: comparative modeling and docking studies. *J. Mol. Mod. 9*, 58–65.

Keckeis, K., Sarker, S.D., Dinan, L., **2000**. Resveratrol-type oligostilbenes from *Iris clarkei* antagonize 20-hydroxyecdysone action in the *Drosophila melanogaster* B_{II} cell line. *Cell. Mol. Life Sci. 57*, 333–336.

Klebe, G., **1998**. Comparative molecular similarity indices: CoMSIA. In: Kubinyi, H., Folkers, G., Martin, Y.C. (Eds.), 3D QSAR in Drug Design: Recent Advances, Vol. 3. Kluwer Academic Publishers, Dordrecht, pp. 87–104.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.

Koudela, K., Tenora, J., Bajer, J., Mathova, A., Sláma, K., **1995**. Stimulation of growth and development in Japanese quails after oral administration of ecdysteroid-containing diet. *Eur. J. Entomol. 92*, 349–354.

Koreeda, M., Nakanishi, K., Goto, M., **1970**. Ajugalactone, an insect moulting inhibitor as tested by the *Chilo* dipping method. *J. Am. Chem. Soc. 92*, 7512–7513.

Kratky, F., Opletal, L., Hejhalek, J., Kucharova, S., **1997**. Effect of 20-hydroxyecdysone on the protein synthesis of pigs. *Zivocisna Vyroba 42*, 445–451.

Kumar, M.B., Fujimoto, T., Potter, D.W., Deng, Q., Palli, S.R., **2002**. A single point mutation in ecdysone receptor leads to increased ligand specificity: implications for gene switch applications. *Proc. Natl Acad. Sci. USA 99*, 14710–14715.

Kumar, R., Thompson, E.B., **1999**. The structure of the nuclear hormone receptors. *Steroids 64*, 310–319.

Lafont, R., Dinan, L., **2003**. Practical uses for ecdysteroids in mammals including humans: an update. *J. Insect Sci. 3*, 7–30.

Lafont, R., Harmatha, J., Marion-Poll, F., Dinan, L., Wilson, I.D., **2002**. The Ecdysone Handbook, 3rd edn. Cybersales a.s., Prague. Available online at http://ecdybase.org.

Lafont, R., Horn, D.H.S., **1989**. Phytoecdysteroids: structures and occurrence. In: Koolman, J. (Ed.), Ecdysone: From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 39–64.

Lafont, R., Koolman, J., Rees, H.H., **1993**. Standardized abbreviations for common ecdysteroids. *Insect Biochem. Mol. Biol. 23*, 207–209.

Lee, S.-S., Nakanishi, K., Cherbas, P., **1991**. Synthesis of 26-iodoponasterone, a new and very active ecdysteroid. *J. Chem. Soc., Chem. Commun.* 51–52.

Lehmann, M., Vorbrodt, H.-M., Adam, G., Koolman, J., **1988**. Antiecdysteroid activity of brassinosteroids. *Experientia 44*, 355–356.

Li, C., Zhou, J., Sun, H., **1985**. Structures of new diterpenoids, maocrystal A-E from *Rabdosia eriocalyx*. *Acta Botan. Yunnan. 7*, 115–116.

Luo, Y., Amin, J., Voellmy, R., **1991**. Ecdysterone receptor is a sequence-specific transcription factor involved in the developmental regulation of heat shock genes. *Mol. Cell. Biol. 11*, 3660–3675.

Ma, W.-C., **1969**. Some properties of gustation in the larva of *Pieris brassicae*. *Entomol. Exp. Applic. 12*, 584–590.

Ma, W.-C., **1972**. Dynamics of feeding responses in *Pieris brassicae* as a function of chemosensory input: a behavioural, ultrastructural and electrophysiological study. *Mededel. Landbouw. Wageningen 11*, 1–162.

Marion-Poll, F., Descoins, C., **2002**. Taste detection of phytoecdysteroids in larvae of *Bombyx mori*, *Spodoptera littoralis* and *Ostrinia nubilalis*. *J. Insect Physiol. 48*, 467–476.

McDonnell, D.P., Connor, C.E., Wijayarantine, A., Chang, C.-Y., Norris, J.D., **2002**. Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. *Rec. Prog. Hormone Res. 57*, 295–316.

Mendis, A.H.W., Rees, H.H., Goodwin, T.W., **1984**. The occurrence of ecdysteroids in the cestode, *Moniezia expansa*. *Mol. Biochem. Parasitol. 10*, 123–138.

Mendis, A.H.W., Rose, M.E., Rees, H.H., Goodwin, T.W., **1983**. Ecdysteroids in adults of the nematode, *Dirofilaria immitis*. *Mol. Biochem. Parasitol. 9*, 209–226.

Meng, Y., Bourne, P.C., Whiting, P., Šik, V., Dinan, L., **2001a**. Identification and ecdysteroid antagonist activity of three oligostilbenes from the seeds of *Carex pendula* (Cyperaceae). *Phytochemistry 57*, 393–400.

Meng, Y., Whiting, P., Šik, V., Rees, H.H., Dinan, L., **2001b**. Limnantheoside C (20-hydroxyecdysone 3-*O*-β-D-glucopyranosyl-[1→3]-β-D-xylopyranoside), a novel phytoecdysteroid from seeds of *Limnanthes alba* (Limnanthaceae). *Z. Naturforsch. 56C*, 988–994.

Mikitani, K., **1996**. A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide shows apparent insect molting hormone activities at molecular and cellular

levels. *Biochem. Biophys. Res. Commun. 227*, 427–432.

Minakuchi, C., Nakagawa, Y., Kamimura, M., Miyagawa, H., **2003**. Binding affinity of nonsteroidal ecdysone agonists against the ecdysone receptor complex determines the strength of their molting hormonal activity. *Eur. J. Biochem. 270*, 4095–4104.

Miró, M., **1995**. Cucurbitacins and their pharmacological effects. *Phytother. Res. 9*, 159–168.

Mohammed-Ali, A.K., Chan, T.-H., Thomas, A.W., Strunz, G.M., Jewett, B., **1995**. Structure–activity relationship study of synthetic hydrazines as ecdysone agonists in the control of spruce budworm (*Choristoneura fumiferana*). *Can. J. Chem. 73*, 550–557.

Nakagawa, Y., Hattori, K., Minukuchi, C., Kugimiya, S., Ueno, T., **2000a**. Relationship between structure and molting hormonal activity of tebufenozide, methoxyfenozide, and their analogs in cultured integument system of *Chilo suppressalis* Walker. *Steroids 65*, 117–123.

Nakagawa, Y., Minakuchi, C., Ueno, T., **2000b**. Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Sf-9 cell line. *Steroids 65*, 537–542.

Nakagawa, Y., Shimizu, B.-I., Oikawa, N., Akamatsu, M., Nishimura, K., *et al.*, **1995a**. Three-dimensional quantitative structure–activity analysis of steroidal and dibenzylhydrazine-type ecdysone agonists. In: Hansch, C., Fujita, T. (Eds.), Classical and Three-Dimensional QSAR in Agrochemistry. American Chemical Society, Washington, DC, pp. 288–301.

Nakagawa, Y., Soya, Y., Nakai, K., Oikawa, N., Nishimura, K., *et al.*, **1995b**. Quantitative structure-activity studies of insect growth regulators. XI. Stimulation and inhibition of *N*-acetylglucosamine incorporation in a cultured integument system by substituted *N*-tert-butyl-*N*,*N*′-dibenzoylhydrazines. *Pestic. Sci. 43*, 339–345.

Nakagawa, Y., Smagghe, G., Tirry, L., Fujita, T., **2001a**. Quantitative structure–activity studies of insect growth regulators XIX. Effects of substituents on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the beet armyworm *Spodoptera exigua*. *Pest Mgt Sci. 58*, 131–138.

Nakagawa, Y., Hattori, K., Shimizu, B.-I., Akamatsu, M., Miyagawa, H., *et al.*, **1998**. Quantitative structure–activity studies of insect growth regulators XIV. Three-dimensional quantitative structure–activity relationship of ecdysone agonists including dibenzoylhydrazine analogs. *Pestic. Sci. 53*, 267–277.

Nakagawa, Y., Smagghe, G., Kugimiya, S., Hattori, K., Ueno, T., *et al.*, **1999**. Quantitative structure–activity studies of insect growth regulators. XVI. Substituent effects of dibenzoylhydrazines on the insecticidal activity to Colorado potato beetle *Leptinotarsa decemlineata*. *Pestic. Sci. 55*, 909–918.

Nakagawa, Y., Smagghe, G., Van Paemel, M., Tirry, L., Fujita, T., **2001b**. Quantitative structure–activity studies of insect growth regulators. XVIII. Effects of substituents on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the Colorado potato beetle *Leptinotarsa decemlineata*. *Pest Mgt. Sci. 57*, 858–865.

Nakanishi, K., Koreeda, M., Sasaki, L., Chang, M.L., Hsu, H.Y., **1966**. Insect hormones I. The structure of ponasterone A, an insect molting hormone from the leaves of *Podocarpus nakaii* H. *J. Chem. Soc., Chem. Commun.* 915–917.

Ninagi, O., Maruyama, M., **1996**. Utilization of 20-hydroxyecdysone extracted from a plant in sericulture. *Jap. Agric. Res. Q. 30*, 123–128.

Nirde, P., Torpier, G., De Reggi, M.L., Capron, A., **1983**. Ecdysone and 20-hydroxyecdysone: new hormones for the human parasite *Schistosoma mansoni*. *FEBS Lett. 151*, 223–227.

Oberdörster, E., Cottam, D.M., Wilmot, F.A., Milner, M.J., McLachlan, J.A., **1999**. Interaction of PAHs and PCBs with ecdysone-dependent gene expression and cell proliferation. *Toxicol. Appl. Pharmacol. 160*, 101–108.

Oberdörster, E., Clay, M.A., Cottam, D.M., Wilmot, F.A., McLachlan, J.A., *et al.*, **2001**. Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. *J. Steroid Biochem. Mol. Biol. 77*, 229–238.

Odnikov, V.N., Galyautdinov, I.V., Nedopekin, D.V., Khalilov, L.M., Shashkov, A.S., *et al.*, **2002**. Phytoecdysteroids from the juice of *Serratula coronata* L. (Asteraceae). *Insect Biochem. Mol. Biol. 32*, 161–165.

Oikawa, N., Nakagawa, Y., Nishimura, K., Ueno, T., Fujita, T., **1994a**. Quantitative structure–activity analysis of larvicidal 1-(substituted benzoyl)-2-benzoyl-1-*tert*-butylhydrazines against *Chilo suppressalis*. *Pestic. Sci. 41*, 139–148.

Oikawa, N., Nakagawa, Y., Nishimura, K., Ueno, T., Fujita, T., **1994b**. Quantitative structure–activity studies of insect growth regulators. X. Substituent effects of 1-*tert*-butyl-1-(2-chlorobenzoyl)-2-(substituted benzoyl) hydrazines against *Chilo suppressalis*. *Pestic. Biochem. Physiol. 48*, 135–144.

Oro, A.E., McKeown, M., Evans, R.M., **1990**. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature 347*, 298–301.

Perrett, S., Whitfield, P.J., **1995**. Antihelmintic and pesticidal activity of *Acorus gramineus* (Araceae) is associated with phenylpropanoid asarones. *Phytother. Res. 9*, 405–409.

Qian, X., **1996**. Molecular modeling study on the structure–activity relationship of substituted dibenzoyl-1-*tert*-butylhydrazines and their strucutral similarity to 20-hydroxyecdysone. *J. Agric. Food Chem. 44*, 1538–1542.

Ravi, M., Hopfinger, A.J., Hormann, R.E., Dinan, L., **2001**. 4D-QSAR analysis of a set of ecdysteroids and a comparison to CoMFA modeling. *J. Chem. Inform. Comput. Sci. 41*, 1587–1604.

Redfern, C.P.F., **1984**. Evidence for the presence of makisterone A in *Drosophila* larvae and the secretion of 20-deoxymakisterone A by the ring gland. *Proc. Natl Acad. Sci. USA 81*, 5643–5647.

Rees, H.H., **1995**. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol. 92*, 9–39.

Reynolds, C.H., Hormann, R.E., **1996**. Theoretical study of the structure and rotational flexibility of diacylhydrazines: implications for the structure of nonsteroidal ecdysone agonists and azapeptides. *J. Am. Chem. Soc. 118*, 9395–9401.

Richards, G., **1981**. The radioimmune assay of ecdysteroid titers in *Drosophila melanogaster. Mol. Cell. Endocrinol. 21*, 181–197.

Richter, K., Adam, G., **1991**. Neurodepressing effect of brassinosteroids in the cockroach *Periplaneta americana*. *Naturwissenschaften 78*, 138–139.

Richter, K., Adam, G., Vorbrodt, H.M., **1987**. Inhibiting effect of 22S,23S-homobrassinolide on the moult of the cockroach *Periplaneta americana* (L.) (Orthopt., Blattidae). *J. Appl. Entomol. 103*, 532–534.

Romer, F., **1979**. Ecdysteroids in snails. *Naturwissenschaften 66*, 471–472.

Roussel, P.G., Turner, N.J., Dinan, L., **1995**. Synthesis of shidasterone and the unambiguous determination of its configuration at C-22. *J. Chem. Soc., Chem. Commun.* 933–934.

Saez, E., Nelson, M.C., Eshelman, B., Banayo, E., Koder, A., *et al.*, **2000**. Identification of ligands and coligands for the ecdysone-regulated gene switch. *Proc. Natl Acad. Sci. USA 97*, 14512–14517.

Santos-Filho, O.A., Mishra, R.K., Hopfinger, A.J., **2001**. Free energy force field (FEFF) 3D-QSAR analysis of a set of *Plasmodium falciparum* dihydrofolate reductase inhibitors. *J. Comp. Aided Mol. Des. 15*, 787–810.

Sarker, S.D., Savchenko, T., Whiting, P., Šik, V., Dinan, L., **1997a**. Two limonoids from *Turraea obtusifolia* (Meliaceae), prieurianin and rohitukin, antagonise 20-hydroxyecdysone action in a *Drosophila* cell line. *Arch. Insect Biochem. Physiol. 35*, 211–217.

Sarker, S.D., Šik, V., Rees, H.H., Dinan, L., **1998**. 2-Dehydro-3-*epi*-20-hydroxyecdysone from *Froelichia floridana* (Amaranthaceae). *Phytochemistry 49*, 2311–2314.

Sarker, S.D., Whiting, P., Dinan, L., Šik, S., Rees, H.H., **1999a**. Identification and ecdysteroid antagonist activity of three resveratrol trimers (suffruticosols A, B and C) from *Paeonia suffruticosa*. *Tetrahedron 55*, 513–524.

Sarker, S.D., Whiting, P., Lafont, R., Girault, J.-P., Dinan, L., **1997b**. Cucurbitacin D from *Cercidiphyllum japonicum*. *Biochem. System. Ecol. 25*, 79–80.

Sarker, S.D., Whiting, P., Šik, V., Dinan, L., **1999b**. Ecdysteroid antagonists (cucurbitacins) from *Physocarpus opulifolius* (Rosaceae). *Phytochemistry 50*, 1123–1128.

Sauber, F., Reuland, M., Berchtold, J.-P., Hetru, C., Tsoupras, G., *et al.*, **1983**. Cycle de mue et ecdystéroïdes chez une sangsue, *Hirudo medicinalis*. *C. R. Acad. Sci.* Paris, *296*, 413–418.

Sawada, Y., Yanai, T., Nakagawa, H., Tsukamoto, Y., Yokoi, S., *et al.*, **2003a**. Synthesis and insecticidal activity of benzoheterocyclic analogues of *N*′-benzoyl-*N*-(*tert*-butyl)benzohydrazide. I. Design of benzoheterocyclic analogues. *Pest Mgt. Sci. 59*, 25–35.

Sawada, Y., Yanai, T., Nakagawa, H., Tsukamoto, Y., Yokoi, S., *et al.*, **2003b**. Synthesis and insecticidal activity of benzoheterocyclic analogues of *N*′-benzoyl-*N*-(*tert*-butyl)benzohydrazide. II. Introduction of substituents on the benzene rings of the benzoheterocycle moiety. *Pest Mgt. Sci. 59*, 36–48.

Sawada, Y., Yanai, T., Nakagawa, H., Tsukamoto, Y., Tamagawa, Y., *et al.*, **2003c**. Synthesis and insecticidal activity of benzoheterocyclic analogues of *N*′-benzoyl-*N*-(*tert*-butyl)benzohydrazide. III. Modification of *N*-*tert*-butylhydrazine moiety. *Pest Mgt. Sci. 59*, 49–57.

Schoonhoven, L.M., Derksen-Koppers, I., **1973**. Effects of secondary plant substances on drinking behaviour in some Heteroptera. *Entomol. Exp. Applic. 16*, 141–145.

Selepcova, L., Magic, D., Vajda, V., **1993**. Use of *Rhaponticum carthamoides* Wild. in animals nutrition. Presented at mtg on Cultivation, Harvesting and Processing of Herbs, The High Tatras, Slovak Republic, June 15–17. Book of Abstracts, p. 76.

Shimizu, B.-I., Nakagawa, Y., Hattori, K., Nishimura, K., Kurihara, N., *et al.*, **1997**. Molting hormonal and larvicidal activities of aliphatic acyl analogs of dibenzoylhydrazine insecticides. *Steroids 62*, 638–642.

Sláma, K., Koudela, K., Tenora, J., Mathova, A., **1996**. Insect hormones in vertebrates: anabolic effects of 20-hydroxyecdysone in Japanese quails. *Experientia 52*, 702–706.

Smagghe, G., Carton, B., Decombel, L., Tirry, L., **2001**. Significance of absorption, oxidation, and binding to toxicity of four ecdysone agonists in multi-resistant cotton leafworm. *Arch. Insect Biochem. Physiol. 46*, 127–139.

Smagghe, G., Decombel, L., Carton, B., Voigt, B., Adam, G., *et al.*, **2002**. Action of brassinosteroids in the cotton leafworm *Spodoptera littoralis*. *Insect Biochem. Mol. Biol. 32*, 199–204.

Smagghe, G., Nakagawa, Y., Carton, B., Mourad, A.K., Fujita, T., *et al.*, **1999**. Comparative ecdysteroid action of ring-substituted dibenzoylhydrazines in *Spodoptera exigua*. *Arch. Insect Biochem. Physiol. 41*, 42–53.

Smagghe, G., Nakagawa, Y., Hormann, R., **2003**. Multidimensional QSAR of Diacylhydrazine Toxicity in Beet Armyworm, Rice Stem Borer and Colorado Potato Beetle. In: The 31st Symposium of Structure–Activity Relationships, Hoshi University of Pharmacy, Shinagawa-ku, Tokyo, Japan.

Smith, H.C., Cavanaugh, C.K., Friz, J.L., Thompson, C.S., Saggers, J.A., *et al.*, **2003**. Synthesis and SAR of *cis*-1-benzoyl-1,2,3,4-tetrahydroquinoline ligands for control of gene expression in ecdysone responsive systems. *Bioorg. Med. Chem. Lett. 13*(11), 1943–1946.

Sommé-Martin, G., Colardeau, J., Beydon, P., Blais, C., Lepesant, J.-A., *et al.*, **1990**. P1 gene expression in

Drosophila larval fat body: induction by various ecdysteroids. *Arch. Insect Biochem. Physiol. 15*, 43–56.

Spindler, K.-D., Spindler-Barth, M., Turberg, A., Adam, G., **1992**. Action of brassinosteroids on the epithelial cell line from *Chironomus tentans*. *Z. Naturforsch. 47C*, 280–284.

Suhr, S.T., Gil, E.B., Senut, M.-C., Gage, F., **1998**. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl Acad. Sci. USA 95*, 7999–8004.

Suksamrarn, A., Ganpinyo, P., Sommechai, C., **1994**. Base-catalyzed autoxidation of 20-hydroxyecdysone: synthesis of calonysterone and 9,20–dihydroxyecdysone. *Tetrahedron Lett. 35*, 4445–4448.

Suksamrarn, A., Pattanaprateep, P., Tanachatchairatana, T., Haritakun, W., Yingyongnarongkul, B.-E., *et al.*, **2002**. Chemical modifications at the 22-hydroxyl group of ecdysteroids: alternative structural requirements for high moulting activity. *Insect Biochem. Mol. Biol. 32*, 193–197.

Suksamrarn, A., Yingyongnarongkul, B.-E., **1997**. Synthesis and moulting hormone activity of 3-*epi*-2-deoxy-20-hydroxyecdysone and analogues. *Tetrahedron 53*, 3145–3154.

Takemoto, T., Ogawa, S., Nishimoto, N., **1967**. Studies on the constituents of *Achyranthis radix*. I. *Yakugaku Zasshi 87*, 1463–1468.

Talbot, W.S., Swyryd, E.A., Hogness, D.S., **1993**. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell 73*, 1323–1337.

Tice, C.M., Hormann, R.E., Thompson, C.S., Friz, J.L., Cavanaugh, C.K., *et al.*, **2003a**. Synthesis and SAR of α-acylaminoketone ligands for control of gene expression. *Bioorg. Med. Chem. Lett. 13*, 475–478.

Tice, C.M., Hormann, R.E., Thompson, C.S., Friz, J.L., Cavanaugh, C.K., *et al.*, **2003b**. Optimization of α-acylaminoketone ecdysone agonists for control of gene expression. *Bioorg. Med. Chem. Lett. 13*, 1883–1886.

Tomaschko, K.-H., **1999**. Nongenomic effects of ecdysteroids. *Arch. Insect Biochem. Physiol. 41*, 89–98.

Toya, T., Yamaguchi, K., Endo, Y., **2002**. Cyclic dibenzoylhydrazines reproducing the conformation of ecdysone agonists, RH-5849. *Bioorg. Med. Chem. Lett. 10*, 953–961.

Velgová, H., Lábler, L., Černý, V., Šorm, F., Sláma, K., **1968**. On steroids. CXI. Some further compounds producing molting deficiencies in an insect. *Coll. Czech. Chem. Commun. 33*, 242–256.

Voigt, B., Whiting, P., Dinan, L., **2001**. The ecdysteroid agonist/antagonist and brassinosteroid-like activities of synthetic brassinosteroid/ecdysteroid hybrid molecules. *Cell. Mol. Life Sci. 58*, 1133–1140.

Wang, S.F., Ayer, S., Segraves, W.A., Williams, D.R., Raikhel, A.S., **2000**. Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol. Cell. Biol. 20*, 3870–3879.

Wing, K.D., **1988**. RH 5849, a nonsteroidal ecdysone agonist: effects on a *Drosophila* cell line. *Science 241*, 467–469.

Wing, K.D., Slawecki, R.A., Carlson, G.R., **1988**. RH5849, a nonsteroidal ecdysone agonist: effects on larval Lepidoptera. *Science 241*, 470–472.

Wurtz, J.-M., Guillot, B., Fagart, J., Moras, D., Tietjen, K., *et al.*, **2000**. A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: a homology modeling and docking approach. *Protein Sci. 9*, 1073–1084.

Xu, Y., Fang, F., Chu, Y., Jones, D., Jones, G., **2002**. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle. *Eur. J. Biochem. 269*, 6026–6036.

Yao, T.P., Forman, B.M., Jiang, Z.Y., Cherbas, L., Chen, J.D., *et al.*, **1993**. Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature 366*, 476–479.

3.5 The Ecdysteroid Receptor

V C Henrich, University of North Carolina-Greensboro, Greensboro, NC, USA

3.5.1. Overview

3.5.1.1. Molecular Identity of the Insect Ecdysteroid Receptor

The morphogenetic events associated with insect development are largely triggered by the action of a single class of steroid hormones, the ecdysteroids (see **Chapter 3.3**).[1] Among all insect orders examined so far, it has been established that the ecdysteroid-induced orchestration of molting and metamorphosis is mediated by a heterodimer comprised of the ecdysone receptor (EcR; Koelle *et al.*, 1991) and Ultraspiracle (USP; Oro *et al.*, 1990; Shea *et al.*, 1990; Henrich *et al.*, 1990) that is stabilized by 20-hydroxyecdysone (20E) and which recognizes specific promoter elements in the insect genome to regulate transcription (Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993). Both proteins belong to a superfamily of nuclear receptors which mediate transcriptional responses to steroids and other lipophilic molecules (Blumberg and Evans, 1998) (see **Chapter 3.6**). Recently, a second ecdysteroid-signaling pathway involving USP and another nuclear receptor DHR38 (Sutherland *et al.*, 1995) has been discovered (Baker *et al.*, 2003).

The insect EcR is a distant relative of the vertebrate farnesol X receptor (FXR; Forman *et al.*, 1995) and LXR (Willy *et al.*, 1995). EcR was first identified in the fruit fly, *Drosophila melanogaster*, and has since been found in several insects, in the ixodid tick (*Amblyomma americanum*; Guo *et al.*, 1997) and in the fiddler crab (*Uca pugilator*; Durica *et al.*, 2002). No functional EcR ortholog has been reported outside the arthropod phylum (Bonneton

[1]Ecdysteroids refers to the family of ecdysteroids including natural and artificial steroids. Individual forms, including the classic active "molting hormone," 20-hydroxyecdysone (20E, formerly β-ecdysone), will be specified throughout the chapter, as will its precursor, ecdysone (formerly α-ecdysone). Ecdysteroid agonists refers to molecules which are not steroidal, but which are capable of inducing one or more insect EcR genes in a given experimental regime.

et al., 2003) (see **Chapter 3.6**). The Ultraspiracle (USP) protein is an ortholog of the retinoid X receptor (RXR), which in turn is a heterodimeric partner for several other nuclear receptors (Mangelsdorf *et al.*, 1990; Oro *et al.*, 1990). Among noninsect invertebrates, an RXR ortholog has been reported from *A. americanum* (Guo *et al.*, 1998), *U. pugilator* (Chung *et al.*, 1998), and the parasitic nematode, *Dirofilaria immitis* (Shea *et al.*, 2004). RXR is widely found in humans and other vertebrates, reflecting its early evolutionary origins (see **Chapter 3.6**). Both EcR and USP have diverged evolutionarily among the insect orders (see **Chapter 3.6**), indicating that the functional properties of EcR and USP have also diverged among them.

3.5.1.2. Historical Perspective

The pursuit of the insect ecdysteroid receptor and its activities has led to a convergence of several basic and applied research disciplines. The ability of a single steroid hormone to induce widespread and coordinated changes in gene transcription has attracted basic scientists interested in the mechanistic basis of its variable gene and cellular action. For insect biologists, the diversity of developmental responses triggered by ecdysteroids among the insect orders reveals the essential importance of this process for understanding the evolutionary diversity and adaptability seen among individual insect species. In turn, the variety of responses among the insect orders has led to attempts to disrupt ecdysteroid receptor action with species-specific agonists and antagonists such as the bisacylhydrazines (Wing, 1988) for insecticidal purposes (Dhadialla *et al.*, 1998, and references therein; see **Chapter 3.4**). Finally, because ecdysteroids exert no harmful effects in humans and other organisms (see **Chapter 3.4**), the ecdysteroid receptor is now viewed as a potential inducer of beneficial transcriptional responses in plant and animal cells. Much of the work on ecdysteroid action that was reported prior to 1985 appeared in the previous edition of this series and will not be repeated here (Riddiford, 1985; O'Connor, 1985; Yund and Osterbur, 1985), except for those early events which have had an obvious bearing on continuing investigations.

The effects of ecdysteroids upon gene transcriptional activity became apparent through studies of the effects of "molting hormone," 20E, on the polytene chromosomes of insects such as *D. melanogaster* and *Chironomus tentans* (midge). Puffing was first reported in 1933 in *D. melanogaster* by E. Heitz, and in the early 1950s Wolfgang Beermann performed seminal work on puff changes in *C. tentans*. Later, Ulrich Clever demonstrated that the insect "molting hormone" induced puffing changes and thus reported the first evidence that steroid hormones act directly upon the transcriptional activity of specific target genes, now regarded as a central feature of steroid hormone action in all animals. A variety of biochemical studies also established the presence of a protein in cellular extracts that binds to ecdysteroids, further indicating that an intracellular receptor mediates the transcriptional response (O'Connor, 1985; Yund and Osterbur, 1985, and references therein).

Conceptually, the polytene chromosomes can be viewed as an *in situ* expression microarray (**Figure 1**), since the puff sites disclose the transcriptional activity of specific genes (by chromosomal location rather than by sequence). This natural array has the added and unique benefit of showing temporal changes in puff size that roughly indicate continuous changes in transcriptional rate. Hans Becker noted in 1959 that "early" puffs appear when incubated with the ecdysteroidogenic ring gland and regress as other "late puffs" appear (Becker, 1959). Ulrich Clever and Michael Ashburner later showed that blocking the protein translation of early puff RNAs with cycloheximide treatment prevents the regression of some early puffs, and simultaneously prevents the appearance of many late puffs (Asburner *et al.*, 1974). From these findings, Ashburner postulated that an intracellular

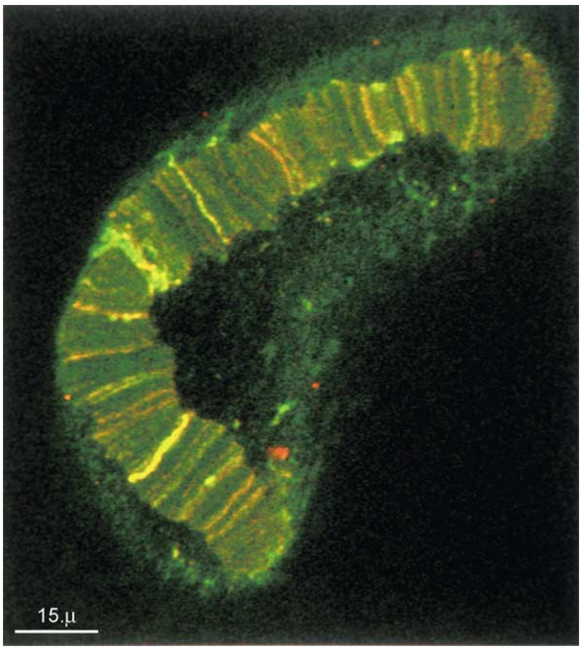


Figure 1 Prepupal chromosome III from *Chironomus tentans* labeled with antibodies against EcR (green) and RNA polymerase II (red). Yellow signals indicate colocalization of the two antibodies. Green signal is a fixation artifact. See Wegmann *et al.* (1995) for methods. (Photograph courtesy of Markus Lezzi.)

ecdysteroid receptor directs a transcriptional response at early puff sites in the presence of 20E. Further, the early puff gene products regulate the appearance of later puffs and also feedback to repress their own expression. This original model for ecdysteroid action has proven remarkably durable over the years, though it is often overlooked that several puffs described by Ashburner have not been placed within the regulatory hierarchy and that some features of the model have not yet been pursued at the molecular level.

The role of a receptor was also implicated by the identification of an ecdysone response element (EcRE) in the ecdysteroid-inducible promoter of the 27 kDa heat shock protein (*hsp27*) of *D. melanogaster* (Riddihough and Pelham, 1987). The palindromic inverted nucleotide repeat sequence of this ecdysteroid receptor target resembles the motifs that at the time of its discovery were associated with a growing class of nuclear receptors for several vertebrate hormones, including the glucocorticoids and estrogen (Hollenberg *et al.*, 1985; Green *et al.*, 1986), raising anticipation that the ecdysteroid receptor is evolutionarily conserved, just as the process of steroid action on transcriptional activity is mechanistically conserved.

Much of the progress made on the molecular genetic basis of ecdysteroid action can be traced to 1968 when, during a sabbatical at CalTech, David Hogness met Ashburner, who was a postdoctoral associate there. Hogness later visited the laboratory of Beermann at Tubingen, and decided to identify the "puff" genes targeted by ecdysteroid action. The conviction to this goal was strong enough that Hogness isolated overlapping genomic DNA segments over a span of several hundred kilobases to find them. His laboratory successfully "walked" through the chromosomal region associated with two early puff genes using this positional cloning approach (Burtis *et al.*, 1990; Segraves and Hogness, 1990). One of the early puff genes, E75,[2] encodes a member of the nuclear hormone receptor superfamily (Segraves and Hogness, 1990), as defined by two cysteine–cysteine zinc fingers that are responsible for the receptor protein's recognition of specific promoter elements.

[2]The puff sites in *Drosophila melanogaster* were originally identified by their cytological location among the 102 subintervals arbitrarily delineated across the sex chromosome (X or chromosome 1, subintervals 1–20) and three autosomes (chromosome 2, subintervals 21–60; chromosome 3, subintervals 61–100, chromosome 4, subintervals 101–102) that comprise the *D. melanogaster* genome. The puffs are further designated as E (early or early-late) or L (late) based on their temporal appearance after 20E incubation.

E75, several other ecdysteroid-responsive gene products in *Drosophila*, and numerous other superfamily members are "orphan receptors," that is, they carry the zinc finger configuration but do not mediate transcriptional activity through any known ligand. When E75 was used as a probe to screen a cDNA library, one of the recovered candidate clones proved to be the *D. melanogaster* ecdysone receptor gene (*Dm*EcR; Koelle *et al.*, 1991), and a functional receptor was also recovered by biochemical purification from a *Drosophila* embryonic cell line (Luo *et al.*, 1991). In reality, EcR dimerizes with a second nuclear receptor, Ultraspiracle (*Dm*USP; Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993), whose structural resemblance to the vertebrate RXR suggests its conservation as a heterodimeric partner for EcR. The recovery of EcR, USP, and orphans such as E75 inspired a more exhaustive search for nuclear receptors in *D. melanogaster*, and the annotated genome now lists 21 nuclear receptors. Among them, only EcR unambiguously interacts physically with an activating ligand, though there is evidence that USP binds to juvenile hormone (Jones and Sharp, 1997; Jones *et al.*, 2001).

The recovery and characterization of EcR, USP, other orphan receptors, and various ecdysteroid-inducible targets in other insects has proceeded at a quickening pace in recent years, and, along with it, a proliferation of DNA probes and antibodies have been developed to examine these players. Several insect researchers have actively identified orthologs for EcR and USP and its puff gene targets so that a rapidly expanding body of comparative information is appearing in the literature. The abundance of information accumulated in the *Drosophila* system has heavily influenced mechanistic interpretations of ecdysteroid action, though it is apparent from the comparative studies done so far that ecdysteroid action at the developmental level varies among insect species, consistent with their wide range of adaptations to the environment. Complete EcR and USP sequences have so far been reported for a few dozen species among four of the insect orders (Diptera, Lepidoptera, Orthoptera, and Coleoptera) and two other arthropod classes. If the variety of results obtained from these studies provide any indication, then only a fraction of the potential information concerning the ecdysteroid receptor has so far been retrieved and assimilated.

3.5.1.3. Chapter Organization

The straightforward thesis of the ecdysteroid hierarchy based on the chromosomal puff response seems to contrast with the complexity found in many

in vivo experiments. It is apparent that ecdysteroid responsiveness varies widely by tissue or cell type, species, incubation conditions, developmental time, activating ligand, ligand concentration, and treatment duration, presumably because the functional capacity of the receptor itself varies in these regimes. Nevertheless, several common and conserved themes have emerged from this work that are valuable not only for insect biologists, but also for other geneticists, cell biologists, and endocrinologists.

The organization of this chapter is based largely on the criteria by which the EcR of *D. melanogaster* was originally defined: (1) the deduced EcR amino acid sequence includes the zinc fingers and ligand-binding domain helices that typify nuclear receptors, (2) extracts of cells expressing EcR bind to ^{125}I-iodoponasterone and this binding disappears with the addition of anti-EcR antibodies, (3) insect cellular extracts carry a protein that binds specifically to an *hsp27* EcRE and this binding disappears in the presence of anti-EcR antibodies, (4) ecdysteroid-inducible transcription is restored to an ecdysone-insensitive *Drosophila* cell line by transfecting the cells with EcR cDNA, and (5) the pattern of spatial and temporal expression of EcR during premetamorphic development is consistent with its role as a mediator of the ecdysteroid response (Koelle *et al*., 1991).

This chapter will examine the information gathered about the ecdysteroid receptor at three levels, and provide brief descriptions and references for the tools used to undertake those experiments. First, the receptor will be viewed as a structural entity, with particular emphasis on the sequence characteristics of EcR and USP as well as their biophysical and biochemical properties. These studies build upon the first three properties noted for EcR in its original characterization. Second, and as in the original report, the transcriptional function of the ecdysteroid receptor will be examined, with particular regard to its interactions with ligand, other protein factors, and promoter elements. This section will also describe a few of the ecdysteroid-inducible systems which have been developed for various applications. Third, the ecdysteroid receptor will be viewed from a cellular and developmental perspective *in vivo*, with special emphasis on the spatial and temporal diversity of ecdysteroid-mediated action, along with the approaches used to address these questions. Finally, the chapter will offer a prognosis concerning important and unresolved problems surrounding ecdysteroid action via its receptors, along with possible experimental technologies and strategies that might clarify them.

3.5.2. Ecdysteroid Receptor Structure, Biophysics, and Biochemistry

3.5.2.1. Domain Organization and Amino Acid Alignment

Both EcR and USP belong to the superfamily of nuclear receptors first described for several steroid hormones and vitamins among the vertebrates. Like their evolutionary counterparts, EcR and USP are structurally modular, that is, they are composed of distinct domains responsible for specific molecular functions (**Figure 2**). Individual domains are at least partially autonomous in their function, since domains of different nuclear receptors can be swapped to create structural and functional chimeras. Chimeras derived from the EcR and USP of different insect species have been used to compare and differentiate their functional capabilities (e.g., Suhr *et al*., 1998; Wang *et al*., 2000; Henrich *et al*., 2000).

The basic organization of nuclear receptors has been described extensively in other reviews, and will be examined here exclusively in terms of the functional features found in insects and associated with each of the domains: (1) the N-terminal (A/B) domain through which nuclear receptors interact with other transcriptional factors, (2) the DNA binding domain (DBD) or C domain which is responsible for the receptor's recognition of specific DNA response elements in the genome and which is comprised of two cysteine–cysteine zinc fingers that define proteins as members of the nuclear receptor superfamily, (3) the hinge region (D domain), which has been implicated in ligand-dependent heterodimerization along with the E domain, nuclear localization, and DNA recognition, (4) the E- or ligand-binding domain (LBD), which is usually comprised of 12 alpha-helices that form a ligand-binding pocket, and (5) the F domain, which is found as a nonconserved sequence in all of the insect EcRs but not in any known USP or RXR sequence.

3.5.2.1.1. The A/B domain This domain is sometimes referred to as the *trans*-activation domain because this portion of the nuclear receptor interacts with the cell's transcriptional machinery and is responsible for a ligand-independent transcriptional activation function (AF1). The A/B domain tends to be variable among nuclear receptors, though there is modest similarity in this portion of EcR and USP among insect species. In several species, multiple isoforms of EcR and/or USP with different A/B domains arise through the activity of alternative promoters and/or alternative pre-mRNA splicing. The occurrence of multiple isoforms for EcR and

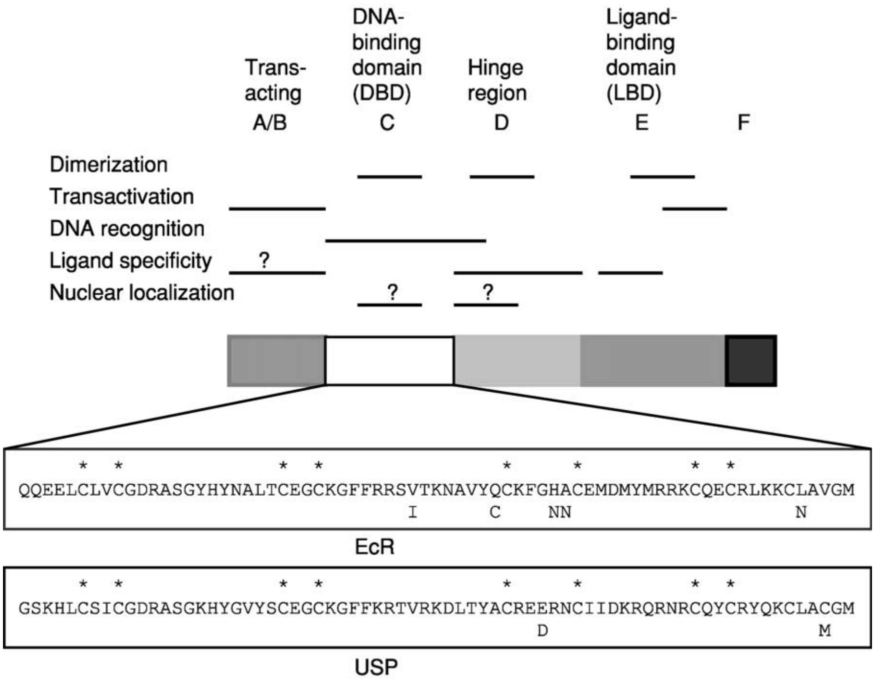


Figure 2 Domain organization of nuclear receptor superfamily members. Bars denote regions of the receptor associated with specific subfunctions or suspected subfunctions (as indicated with a ?). Boxed regions indicate consensus amino acid sequences for the DNA-binding domain of insect EcR and USP based on Bonneton *et al.* (2003). Asterisks (*) indicate positions of two sets of four cysteines which ligand to zinc ion to form each of the two zinc fingers.

USP does not follow any apparent pattern and therefore does not seem to be tied to an obvious evolutionary mechanism. For instance, so far only the *Dm*EcR gene among insects encodes three isoforms (A, B1, and B2; Talbot *et al.*, 1993). Among cyclorapphous flies the Mediterranean fruit fly (*Ceratitis capitata, Cc*) EcR gene specifies both the A and B1 isoforms (Verras *et al.*, 2002), whereas only the B1 isoform has been found so far in the sheep blowfly (*Lucilia cuprina, Lc*) genome (Hannan and Hill, 1997).

Among other Diptera, the mosquito (*Aedes aegypti, Aa*) EcR encodes the A and B1 isoforms (Wang *et al.*, 2002), but the midge (*Chironomus tentans,Ct*) encodes only the B1-like isoform (Imhof *et al.*, 1993). The same variable pattern is found among the lepidopteran species. The corn earworm (*Heliothis virescens, Hv*; Martinez *et al.*, 1999a) has only EcR-B1, whereas the spruce budworm (*Choristoneura fumiferana, Cf*; Perrera *et al.*, 1999a), the rice stem borer (*Chilo suppressalis, Cs*), the tobacco hornworm (*Manduca sexta, Ms*; Jindra *et al.*, 1996), and the silkmoth (*Bombyx mori, Bm*; Swevers *et al.*, 1995; Kamimura *et al.*, 1997) express both an A and B1 isoform. An A and B1 isoform of EcR has also been recovered from the mealworm (*Tenebrio molitor, Tm*) genome (Mouillet *et al.*, 1997) and a single EcR isoform has been recovered from the migratory locust (*Locusta migratoria, Lm*; Saleh *et al.*, 1998). The most complex EcR gene organization is found in a noninsect species, *A. americanum*, wherein at least three A/B isoforms have been identified, each of which resembles the *D. melanogaster* A isoform sequence (Guo *et al.*, 1997). The functional properties of the insect EcR isoforms and their distinct developmental roles will be discussed later in the chapter, though it is noteworthy that the B2 isoform uniquely associated with *D. melanogaster* is also the most efficient for rescuing larval development in EcR homozygous mutants that would otherwise die during embryonic development (Li and Bender, 2000). This observation simultaneously highlights the possible uniqueness of the fruit fly EcR and the potential significance of the A/B domain for comparison among the insects.

In one species, *C. tentans*, multiple USP isoforms differing in their A/B domain occur along with only a single EcR isoform (Vogtli *et al.*, 1999). The migratory locust (*Locusta migratoria; Lm*) also expresses multiple USP isoforms along with the single EcRB1 isoform found so far, though the USP variants do not arise from differential splicing in the A/B domain (Hayward *et al.*, 1999, 2003). Along with two EcR isoforms, two USP isoforms exist in *A. aegypti* (Kapitskaya *et al.*, 1996), *M. sexta* (Jindra *et al.*, 1997), and *B. mori* (Tzertzinis

et al., 1994). Two RXR/USP isoforms also exist in *A. americanum*, along with its three EcR isoforms (Guo *et al.*, 1998). Other complete USP sequences have been reported for *L. cuprina* (Hannan and Hill, 2001) and *C. fumiferana* (Perera *et al.*, 1998).

3.5.2.1.2. The C domain or DNA-binding domain (DBD) The members of the nuclear receptor superfamily are defined by a 66–68 amino acid region known as the cysteine–cysteine zinc finger (**Figures 2 and 3**). The EcR DBD closely resembles the vertebrate FXR, and both EcR/USP and FXR/RXR recognize a palindromic inverted repeat sequence separated by a single nucleotide (IR1); the *hsp27* EcRE follows this arrangement. A consensus sequence based on the investigation of several functional IR1 elements is: 5′-PuG(G/T)T(C/G)A(N)TG(C/A(C/A(C/t)Py (Antoniewski *et al.*, 1993). As will be noted later, the EcR/USP complex is also capable of recognizing direct repeat elements (Antoniewski *et al.*, 1996; Vogtli *et al.*, 1998). The DBD amino acid sequence is highly conserved among the Diptera and Lepidoptera, but specific residues are substituted in the EcR DBD of other insects (Henrich and Brown, 1995; Bonneton *et al.*, 2003) (see **Chapter 3.6**).

3.5.2.1.3. The D domain or hinge region The D domain includes the amino acids which lie between the zinc fingers and the ligand binding domain (LBD). Portions of this region are highly conserved among all the insect EcRs, including a T-box motif that plays a role in DNA recognition (Devarakonda *et al.*, 2003; **Figure 3**) and an A-box, a helical structure that is essential for high affinity recognition of a DNA response element (Niedziela-Majka *et al.*, 2000). The D region of EcR is also essential for ligand dependent heterodimerization with USP (Suhr *et al.*, 1998; Perera *et al.*, 1999b). The USP hinge region includes a highly conserved

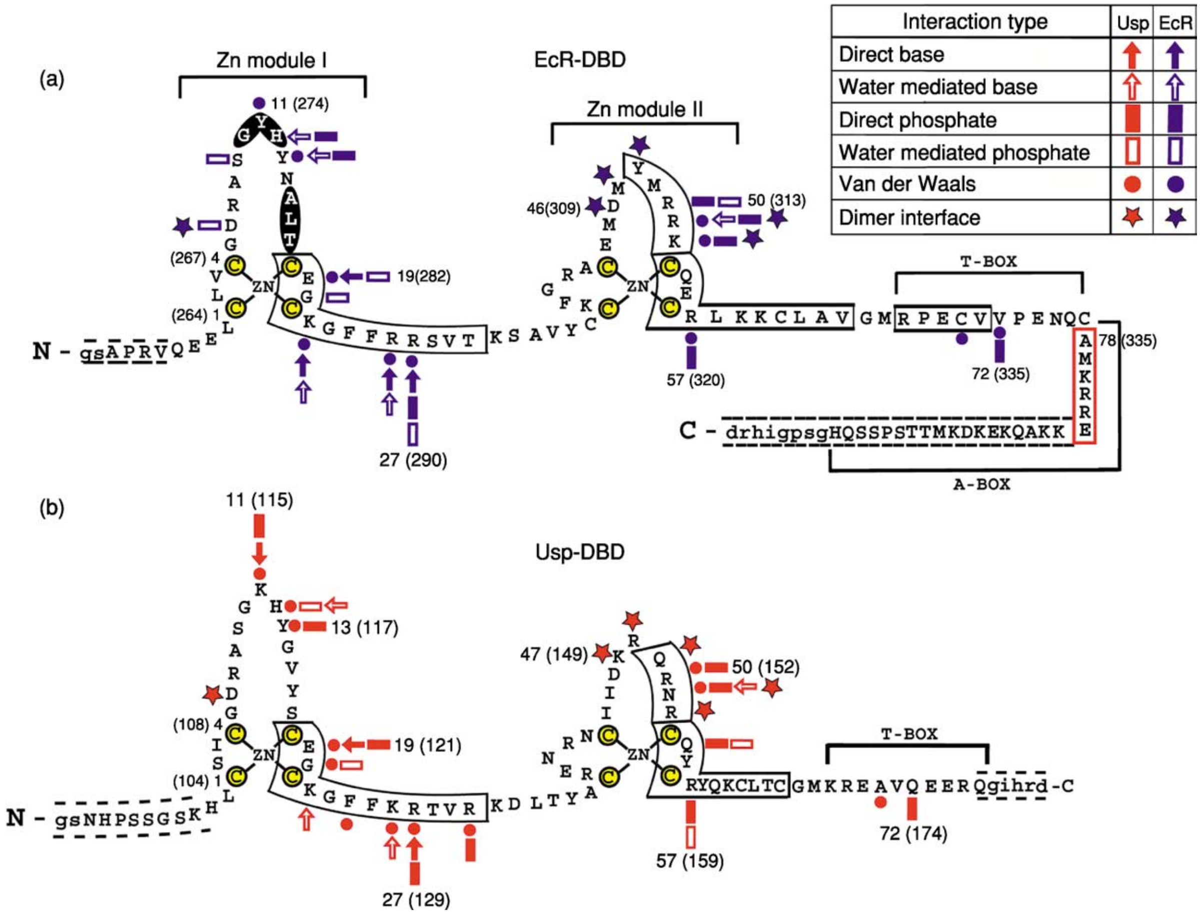


Figure 3 The protein constructs used for a crystallization study, designating amino acid contact sites with noted DNA interactions. (a) Sequence of *Drosophila melanogaster* EcR DBD with liganded zinc fingers, T-box, and A box. (b) Sequence of *D. melanogaster* USP DBD with liganded zinc fingers and T-box. Lower case letters designate artifacts of cloning in the constructs used for the study. (Reproduced with permission from Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., **2003**. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J. 22*, 5827–5840.)

T-box motif, which plays a role in site recognition of DNA response elements but is not essential for heterodimerization (**Figure 3**; Niedziela-Majka *et al*., 2000; Devarakonda *et al*., 2003).

3.5.2.1.4. The E domain or ligand binding domain (LBD) The domain of the nuclear receptor which is responsible for interacting with the receptor's cognate ligand is the E domain or LBD. The EcR and USP LBD fall within the canonical organization observed for almost all other members of the nuclear receptor superfamily. The region is comprised of 12 α-helices that form a ligand-binding pocket which holds the cognate ligand. The sequence of the EcR LBD is highly conserved among the insects, consistent with the widespread occurrence of the molting hormone, 20E, among the insect orders (see **Figure 4**). Nevertheless, the EcR LBD sequences from some insect orders have not been reported, and possibly important divergences among them remain unknown. For EcR, a ligand-dependent transcriptional activation function (AF2) is localized in the most carboxy-terminal helix 12, which folds over the pocket to hold the ligand molecule inside. This folding thereby creates an interactive surface with other proteins that ultimately modulates the receptor's transcriptional activity. For nuclear receptors generally, a dimerization interface lies along helices 9 and 10 (Perlmann *et al*., 1996) and ligand-independent transcriptional functions (AF1) have also been associated with various portions of the EcR LBD (Hu *et al*., 2003). Later portions of the chapter will explore not only the functional features of the LBD based on biochemical, cellular, and *in vivo* experiments, but will also discuss the effects of LBD modification and species-based sequence differences upon EcR and USP capabilities.

As noted elsewhere (see **Chapter 3.6**), the USP LBD has diverged considerably over evolutionary time. Whereas the USP LBD of some insect orders bears a fairly close resemblance to the vertebrate RXR, the dipteran and lepidopteran USP LBDs include a loop between helices 1 and 3 (**Figure 5**; Billas *et al*., 2001; Clayton *et al*., 2001). The diversity is further elaborated in the fly sequences, which include several additional, glycine-rich stretches that are not found in other insects. The aforementioned *Lm* USP includes short and long variants in the LBD which arise by an unidentified molecular process (Hayward *et al*., 2003).

Among the 21 nuclear receptors in *D. melanogaster*, all but two of these proteins (Knirps, Nauber *et al*., 1988; and Knirps-related, Oro *et al*., 1988) carry the 12 alpha-helices that typify the LBD. Except for EcR and possibly USP, none of these 19 superfamily members are known to interact physically with a cognate ligand. Several show patterns of expression that suggest a role in mediating ecdysteroid-inducible responses (Sullivan and Thummel, 2003) and one, DHR38, is activated by ecdysteroids via a novel mechanism (Baker *et al*., 2003).

3.5.2.1.5. F domain Only a few members of the nuclear receptor superfamily possess the F domain, but the EcR family members carry up to 226 amino acids (in *D. melanogaster*) beyond the helix 12 region, though these are not conserved among the insect EcR sequences. Apparently, the F-domain of *Dm*EcR is totally dispensible, since its cleavage produces a receptor that has the same transcriptional capabilities as the full-length EcR (Hu *et al*., 2003). Recently, a variant of the *Aedes albopictus* EcR has been discovered which lacks seven amino acids in this carboxy-terminal region. While the functional significance, if any, of this deviation is not known, only one of the forms normally predominates in a mosquito cell line (Jayachandran and Fallon, 2001).

3.5.2.2. Strategies for Identifying Insect Receptors

The identity of EcR described earlier by using a DNA probe derived from the E75 "early puff" gene quickly led to similar strategies for identifying EcR orthologs in other insects. These attempts have sometimes employed conventional probes, typically derived from the DBD sequence of *Dm*EcR, to screen cDNA libraries (e.g., Mouillet *et al*., 1997; Guo *et al*., 1997). In many instances a combination of approaches utilizing cDNA library screening along with polymerase chain reaction (PCR) using primers from conserved EcR regions have been utilized to obtain full-length EcR cDNA clones (e.g., Swevers *et al*., 1995). Similarly, the use of PCR to amplify a small portion of the EcR DBD sequence, followed by cDNA library searches has also resulted in the successful recovery of EcR cDNA clones (e.g., Hannan and Hill, 1997). Because many insects encode multiple EcR isoforms, 5′ and 3′ rapid amplification of cDNA ends (RACE) has been used with primers derived from regions of the EcR cDNA shared among all the isoforms. These extensions from a common region within the EcR sequence such as the DBD or LBD permit the rapid recovery of novel transcripts with isoform-specific coding regions (e.g., Minakuchi *et al*., 2002).

The recovery of the *D. melanogaster ultraspiracle* (*usp*) gene resulted from three independent screens using vertebrate RXR as a probe (Oro *et al*., 1990),

EcR

```
                                         Helix 1          Helix 2         Helix 3                 Helix 4
Drosophila melanogaster(B1)  416  LTYNQLA--VIYKLIWYQDGYEQPSEEDLRRIMS---QPDENESQTDVSFRHITEITILTVQLIVEFAKGLPAFTKIPQEDQITLLKA
Lucilia cuprina              439  LSYNQLA--VIYKLIWYQDGYEQPSEEDLKRIMS---SPDENESQHDASFRHITEITILTVQLIVEFAKGLPAFTKIPQEDQITLLKA
Aedes aegypti                428  LTANQMA--VIYKLIWYQDGYEQPSEEDLKRIMIG--SPNEEEDQHDVHFRHITEITILTVQLIVEFAKGLPAFTKIPQEDQITLLKA
Chironomus tentans           275  LTANQVA--VIYKLIWYQDGYEQPSEEDLKRITTE--LEEEEDQEHEANFRYITEVTILTVQLIVEFAKGLPAFIKIPQEDQITLLKA
Heliothis virescens          283  LTANQKS--LIARLVWYQEGYEQPSEEDLKRVTQ---SDEDDED-SDMPFRQITEMTILTVQLIVEFAKGLPGFAKISQSDQITLLKA
Choristoneura fumiferana     285  LTANQQF--LIARLIWYQDGYEQPSDEDLKRITQTWQQADDENEESDTPFRQITEMTILTVQLIVEFAKGLPGFAKISQPDQITLLKA
Bombyx mori                  349  LSANQKS--LIARLVWYQEGYEQPSDEDLKRVTQTWQSDEEDEE-SDLPFRQITEMTILTVQLIVEFAKGLPGFSKISQSDQITLLKA
Manduca sexta                290  LSANQKS--LIARLVWYQEGYEQPSEEDLKRVTQTWQLEEEEEEETDMPFRQITEMTILTVQLIVEFAKGLPGFSKISQSDQITLLKA
Locusta migratoria           305  VSPEQEE--LIHRLVYFQNEYESPSEEDLRRVTS---QPTEGEDQSDVRFRHITEITILTVQLIVEFAKRLPGFDKLLREDQIALLKA
Tenebrio molitor             249  ISPEQEELILIHRLVYFQNEYEHPSEEDVKRIIN---QPIDGEDQCEIRFRHTTEITILTVQLIVEFAKRLPGFDKLLQEDQIALLKA
Amblyomma americanum (A1)    324  LSSSQE-DLINKLVYYQQEFESPSEEDMKKTTPF---PLGDSEEDNQRRFQHITEITILTVQLIVEFSKRVPGFDTLAREDQITLLKA
Rat FXR                      242  LTVDQQTLLD-----YIMDSY-SKQRMPQEITNKI---LKDEEFSAEENFLILTEMATSHVQILVEFTKRLPGFQTLDHEDQIALLKG
                                                    o                                o  o

                        Helix 5                     Helix 6   Helix 7           Helix 8                     Helix 9
Drosophila melanogaster      CSSEVMMLRMARRYDHSSDSIFFANNRSYTRDSYKMAGMADNIEDLLHFCRQMFSMKVDNVEYALLTAIVIFSDRPGLEKAQLVEAIQSYYIDTLRIYILNR
Lucilia cuprina              CSSEVMMLRMARRYDHNSDSIFFANNRSYTRDSYKMAGMADNIEDLLHFCRQMYSMKVDNVEYALLTAIVIFSDRPGLEEAELVEAIQSYYIDTLRIYILNR
Aedes aegypti                CSSEVMMLRMARRYDAATDSILFANNRSYTRDSYRMAGMADTIEDLLHFCRQMFSLTVDNVEYALLTAIVIFSDRPGLEQAELVEHIQSYYIDTLRIYILNR
Chironomus tentans           CSSEVMMLRMARRYDHDSDSILFANNTAYTKQTYQLAGMEETIDDLLHFCRQMYALSIDNVEYALLTAIVIFSDRPGLEKAEMVDIIQSYYTETLKVYIVNR
Heliothis virescens          CSSEVMMLRVARRYDAATDSVLFANNQAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALLTAIVIFSDRPGLEQPLLVEEIQRYYLNTLRVYILNQ
Choristoneura fumiferana     CSSEVMMLRVARRYDAASDSVLFANNQAYTRDNYRKAGMAYVIEDLLHFCRCMYSMALDNIHYALLTAVVIFSDRPGLEQPQLVEEIQRYYLNTLRIYILNQ
Bombyx mori                  SSSEVMMLRVARRYDAASDSVLFANNKAYTRDNYRQGGMAYVIEDLLHFCRCMFAMGMDNVHFALLTAIVIFSDRPGLEQPSLVEEIQRYYLNTLRIYIINQ
Manduca sexta                SSSEVMMLRVARRYDAATDSVLFANNQAYTRDNYRKAGMSYVIEDLLHFCRCMYSMSMDNVHYALLTAIVIFSDRPGLEQPLLVEEIQRYYLKTLRVYILNQ
Locusta migratoria           CSSEVMMFRMARRYDVNSDSILFANNQPYTKDSYNLAGMGETIEDMLRFCRQMYAMKVDNAEYALLTAIVIFSERPSLVEGWKVEKIQEIYLEALKAYVDNR
Tenebrio molitor             CSSEVMMFRMARRYDVQSDSILFVNNQPYPRDSYNLAGMGETIEDLLHFCRTMYSMKVDNAEYALLTAIVIFSERPSLIEGWKVEKIQEIYLEALRAYVDNR
Amblyomma americanum         CSSEVMMLRGARKYDVKTDSIVFANNQPYTRDNYRSASVGDSADALFRFCRKMCQLRVDNAEYALLTAIVIFSERPSLVDPHKVERIQEYYIETLRMYSENH
Rat FXR                      SAVEAMFLRSAEIFNKKLP---AGHADLLEERIRKSCISDEYITPMFSFYKSVGELKMTQEEYALLTAIVILSPDRQYIKDREAVELQEPLLDVLQKLCKIY
                                       o              o         o

                                  Helix 10       Helix 11      Helix 12
Drosophila melanogaster      HCGDSMSLVFYAKLLSILTELRTLGNQNAEMCFSLKLKNRKLPKFLEEIWDVHAI      Koelle et al., 1991
Lucilia cuprina              HCGDPMSLVFFAKLLSILTELRTLGNQNAEMCFSLKLKNRKLPKFLEEIWDVHAI      Hannan and Hill., 1997
Aedes aegypti                HAGDPKCSVIFAKLLSILTELRTLGNQNSEMCFSLKLKNRKLPRFLEEIWDVQDI      Wang et al., 1998
Chironomus tentans           HGGESRCSVQFAKLLGILTELRTMGNKNSEMCFSLKLRNRKLPRFLEEVWDVGDV      Imhof et al., 1993
Heliothis virescens          NSASPRGAVIFGEILGILTEIRTLGMQNSNMCISLKLKNRKLPPFLEEIWDVADV      Martinez et al., 1999
Choristoneura fumiferana     LSGSARSSVIYGKILSILSELRTLGMQNSNMCISLKLKNRKLPPFLEEIWDVADM      Kathapalli et al., 1995
Bombyx mori                  NSASSRCAVIYGRILSVLTELRTLGTQNSNMCISLKLKNRKLPPFLEEIWDVAEV      Swevers et al., 1995
Manduca sexta                HSASPRCAVLFGKILGVLTELRTLGTQNSNMCISLKLKNRKLPPFLEEIWDVAEV      Jindra et al., 1996
Locusta migratoria           R--RPKSGTIFAKLLSVLTELRTLGNQNSEMCFSLKLKNKKLPPFLAEIWDVIP-      Saleh et al., 1998
Tenebrio molitor             R--SPSRGTIFAKLLSVLTELRTLGNQNSEMCISLKLKNKKLPPFLDEIWDVDLK      Mouillet et al., 1997
Amblyomma americanum         R---PPGKNYFARLLSILTELRTLGNMNAEMCFSLKVQNKKLPPFLAEIWDIQE       Guo et al., 1997
Rat FXR                      ---QPENPQHFACLLGRLTELRTFNHHHAEMLMSWRVNDHKFTPLLCEIWDV         Forman et al., 1995
```

Figure 4 Amino acid alignment of representative insect EcR sequences (order Diptera: *D. melanogaster, Lucilia cuprina, Aedes aegypti, C. tentans*; order Lepidoptera: *Heliothis virescens, Bombyx mori, Manduca sexta, Choristoneura fumiferana*; order Orthoptera: *Locusta migratoria*; order Coleoptera: *Tenebrio molitor*). Also included: ixodid tick (*Ambyomma americanum*), and rat FXR. Amino acids designate number of first residue in alignment. References indicate original report. Bars designate each of the 12 helices, and shaded residues indicate those which have been mutated and reported (references given in the text). Binding sites of *Hv*EcR with 20E indicated with circle (o).

USP

```
                                       Helix 1            Helix 2         Helix 3
Drosophila melanogaster     233  SVSR---DFSIERIIEAEQRAETQCGDRALTFLRVG-PYSTVQPDYKGAVS--ALCQVVNKQLFQMVEYARMMPHFAQVP
Lucilia cuprina             203  SSLR---DLTIERIIEAEQKAESLSGDNVLPFLRVG-NNSMVQHDYKGAVS--HLCQMVNKQLYQMVEYARRTPHFTHLQ
Aedes aegypti USP-A         229  SSVR---DVTIERIHEAEQLSEQKSGDNAIPYLRVG-SNSMIPPEYKGAVS--HLCQMVNKQIYQLIDFARRVPHFINLP
Chironomus tentans          295  GPGR---DITVERLMEADQMSEARCGDKSIQYLRVAASNTMIPPEYRAPVS--AICAMVNKQVFQHMDFCRRLPHFTKLP
Heliothis virescens         201  SSVQV--ELSIERLLEMESLVADPSEE--FQFLRVG-PDSNVPPKFRAPVS--SLCQIGNKQIAALVVWARDIPHFSQLE
Choristoneura fumiferana    210  SSVQVSDELSIERLTEMESLVADPSEE--FQFLRVG-PDSNVPPRYRAPVS--SLCQIGNKQIAALVVWARDIPHFGQLE
Bombyx mori                 202  SSVQ---ELSIERLLELEALVADSAEE--LQILRVG-PESGVPAKYRAPVS--SLCQIGNKQIAALIVWARDIPHFGQLE
Manduca sexta               201  SSVQ---ELSIERLLEIESLVADPPEE--FQFLRVG-PESGVPAKYRAPVS--SLCQIGNKQIAALVVWARDIPHFGQLE
Locusta migratoria          177  SSLH--TDMPVERILEAEKRVECKAEN------QVEY-----ESTMNNICQAANICQATNKQLFQLVEWAKHIPHFTSLP
Tenebrio molitor            176  SNMQ--AEMPLDRIIEAEKRIECTPAG------GSGG-----VGEQHDGVN--NICQATNKQLFQLVQWAKLIPHFTSLP
Amblyomma americanum        158  TSGGAPPEMPLERILEAELRVES-----QTGTLSESAQQQD-------PVS--SICQAADRQLHQLVQWAKHIPHFEELP
Homo sapiens RXRa           224  SSAN--EDMPVERILEAELAVEPKTET------YVEANMGLNPSSPNDPVT--NICQAADKQLFTLVEWAKRIPHFSELP

                                     Helix 4 | Helix 5                                          Helix 6
Drosophila melanogaster          LDDQVILLKAAWIELLIANVAWCSIVSLDDGGAGGGGGGLGHDGSFERRSPGLQPQQLFLNQSFSYHRNSAIKAGVSAI
Lucilia cuprina                  REDQILLLKAGWNELLIANVAWCSIESLDAEYASPG---TVHDGSFGRRSPVRQPQQLFLNQNFSYHRNSAIKANVVSI
Aedes aegypti USP                RDDQVMLLRCGWNEMLIAAVAWRSMEYIETER--------SSDGSRITV---RQPQLMCLGPNFTLHRNSAQQAGVDTL
Chironomus tentans               LNDQMYLLKQSLNELLILNIAYMSIQYVEPDRR-------NADGSLERR---QISQQMCLSRNYTLGRNMAVQAGVVQI
Heliothis virescens              MEDQILLIKGSWNELLLFAIAWRSMEFLTEERD-------GVDGTGNRT--TSPPQLMCLMPGMTLHRNSALQAGVGQI
Choristoneura fumiferana         LDDQVVLIKASWNELLLFAIAWRSMEYLEDERE-------NGD--GTRS--TTQPQLMCLMPGMTLHRNSAQQAGVGAI
Bombyx mori                      IDDQILLIKGSWNELLLFAIAWRSMEFLNDERE-------NVD---SRN--TAPPQLICLMPGMTLHRNSALQAGVGQI
Manduca sexta                    LEDQILLIKNSWNELLLFAIAWRSMEYLTDERE-------NVD---SRS--TAPPQLMCLMPGMTLHRNSALQAGVGQI
Locusta migratoria               LEDQVLLLRAGWNELLIAAFSHRSVDVKDG---------------------------IVLATGLTVHRNSAHQAGVGTI
Tenebrio molitor                 MSDQVLLLRAGWNELLIAAFSHRSIQAQDA---------------------------IVLATGLTVNKTSAHAVGVGNI
Amlyomma americanum              LEDRMVLLKAGWNELLIAAFSHRSVDVRDG---------------------------IVLATGLVVQRHSAHGAGVGAI
Homo sapiens RXRa                LDDQVILLRAGWNELLIASFSHRSIAVKDG---------------------------ILLATGLHVHRNSAHSAGVGAI

                                    Helix 7           Helix 8                Helix 9                Helix 10
Drosophila melanogaster          FDRILSELSVKMKRLNLDRRELSCLKAIILYNPDIRGIKSRAEIEMCREKVYACLDEHCRLEHPGDDGRFAQLLLRLPALR
Lucilia cuprina                  FDRILSELSIKMKRLNIDRSELSCLKAIILFNPDIRGLKCRADVEVCREKIYACLDEHCRTEHPGDDGRFAQLLLRLPALR
Aedes aegypti USP                FDRILCELGIKMKRLDVTRAELGVLKAIILFNPDIRGLKCQKEIDGMREKIYACLDEHCKQQHPSEDGRFAQLLLRLPALR
Chironomus tentans               FDRILSELSVKMKRLDLDATELCLLKSIVVFNPDVRTLDDRKSIDLLRSRIYASLDEYCRQKHPNEDGRFAQLLLRLPALR
Heliothis virescens              FDRVLSELSLKMRTLRVDQAEYVALKAIILLNPDVKGLKNRQEVEVLREKMFLCLDEYCRRSRSSEEGRFAALLLRLPALR
Choristoneura fumiferana         FDRVLSELSLKMRTLRMDQAEYVALKAIVLLNPDVKGLKNRQEVDVLREKMFSCLDDYCRRSRSNEEGRFASLLLRLPALR
Bombyx mori                      FDRVLSELSLKMRSLRMDQAECVALKAIILLNPDVKGLKNKQEVDVLREKMFLCLDEYCRRSRGGEEGRFAALLLRLPALR
Manduca sexta                    FDRVLSELSLKMRTLRMDQAEYVALKAIILLNPDVKGLKNKPEVVVLREKMFSCLDEYVRRSRCAEEGRFAALLLRLPALR
Locusta migratoria               FDRVLTELVAKMREMKMDKTELGCLRSVILFNPEVRGLKSAQEVELLREKVYAALEEYTRTTHPDEPGRFAKLLLRLPSLR
Tenebrio molitor                 YDRVLSELVNKMKEMKMDKTELGCLRAIILYNPTCRGIKSVQEVEMLREKIYGVLEEYTRTTHPNEPGRFAKLLLRLPALR
Amblyomma americanum             FDRVLTELVAKMREMKMDRTELGCLLAVVLFNPEAKGLRTCPSGGPEGESVASALEEHCRQQYPDQPGRFAKLLLRLPALR
Homo sapiens RXRa                FDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPDSKGLSNPAEVEALREKVYASLEAYCKHKYPEQPGRFAKLLLRLPALR

                                     | Helix 11       Helix 12
Drosophila melanogaster          SISLKCQDHLFLFRITSDRPLEELFLEQLEAPP            Oro et al.(1990), Shea et al.(1990), Henrich et al.(1990)
Lucilia cuprina                  SISLKCLDHLFFFRLIGERALEELIAEQLEAPI            Hannan and Hill (2001)
Aedes aegypti USP                SISLKCLDHLNFIRLLSDKHLDSFIVEMLDMPI            Kapitskaya et al. (1996)
Chironomus tentans               SISLKCLDHLFYFQLIDDKNVENSVIEEFHKLN            Vogtli et al. (1999)
Heliothis virescens              SISLKSFEHLFFFHLVADTSIAGYIRDALRNHA            unpublished
Choristoneura fumiferana         SISLKSFEHLYFFHLVAEGSISGYIREALRNHA            Perera et al. (1998)
Bombyx mori                      SISLKSFEHLYLFHLVAEGSVSSYIRDALCNHA            Tzertzinis et al. (1994)
Manduca sexta                    SISLKCFEHLYFFHLVADTSIASYIHDALRNHA            Jindra et al. (1997)
Locusta migratoria               SIGLKCLEHLFFFRLIGDVPIDTFLMEMLESPS            Hayward et al. (1999)
Tenebrio molitor                 SIGLKCSEHLFFFKLIGDVPIDTFLMEMLESPA            delaChambre (unpublished)
Amblyomma americanum             SIGLKCLEHLFFFKLIGDTPIDNFLLSMLEAPS            Guo et al. (1998)
Homo sapiens RXRa                SIGLKCLEHLFFFKLIGDTPIDTFLMEMLEAPH            Mangelsdorf et al. (1990)
```

Figure 5 Continued

employing an oligonucleotide derived from a highly conserved portion of the DBD to screen an embryonic cDNA library (Henrich *et al*., 1990), and by using a promoter element in the s15 chorion gene to screen an expression library (Shea *et al*., 1990). A genomic clone residing near the tip of the *D. melanogaster* X chromosome rescues larvae homozygous for the early larval lethal mutation, *ultraspiracle* (*usp*; Oro *et al*., 1990), so named because mutant larvae fail to shed their first instar cuticle and appear to develop an extra row of spiracles (Perrimon *et al*., 1985). The same combination of PCR-based and library screening approaches used for finding EcR have led to the recovery of complete USP sequences from other insects.

The discovery that E75 was not only a target of ecdysteroid action, but a nuclear receptor itself, proved to be the first of several serendipitous discoveries of other nuclear receptors in the *Drosophila* genome that are associated directly with the salivary gland puffing response mediated by EcR and USP. Another "early late" puff gene product, E78, is a member of the nuclear receptor superfamily (Stone and Thummel, 1993) as is DHR3, which had been recovered in the same library screen that yielded EcR (Koelle *et al*., 1992); DHR3 is also a heterodimeric partner of EcR (White *et al*., 1997). Still another nuclear receptor, βFTZ-F1, a transcriptional regulator of the embryonic segmentation gene *fushi tarazu* (Lavorgna *et al*., 1991) is the product of the stage-specific 75CD chromosomal puff that occurs during the mid-prepupal ecdysteroid peak (Woodard *et al*., 1994).

More systematic screens have yielded other *Drosophila* hormone receptors (DHRs), including DHR4 (Sullivan and Thummel, 2003), DHR39 (Horner *et al*., 1995), and DHR78 (Fisk and Thummel, 1998), which are orphans and ecdysteroid-regulated. Based on USP's possible homology to RXR, a yeast two-hybrid assay employing USP as bait led to the isolation of an interacting orphan receptor, DHR38, that is orthologous to the vertebrate nerve growth factor 1B (NGF1B; Sutherland *et al*., 1995). The *seven-up* (*svp*) gene, originally defined by a mutation that disrupts photoreceptor function, encodes the *Drosophila* ortholog of another vertebrate orphan, the chicken ovalbumin upstream promoter-transcription factor (COUP-TF; Mlodzik *et al*., 1990). COUP-TF heterodimerizes with RXR, and analogously, SVP forms a functional heterodimer with USP as shown by its ability to compete for dimerization with EcR in both *D. melanogaster* (Zelhof *et al*., 1995a) and *A. aegypti* (Zhu *et al*., 2003a).

Therefore, the orphan nuclear receptors not only resemble EcR and USP structurally, it is increasingly apparent that they also play a role in the orchestration of ecdysteroid response, both as targets of EcR and USP-mediated transcriptional activity and as heterodimeric partners for the two functional components of the ecdysteroid receptor. Each of these orphans is sufficiently unique in sequence that a combination of library screening and PCR approaches have led to the successful recovery of orphan orthologs. A listing of the orphans implicated in some aspect of ecdysteroid regulation from other insect species is given in **Table 1**. Later in the chapter, the regulation and developmental roles of these orphans in *D. melanogaster* and other insects will be explored and compared further. The transcript levels of all 21 nuclear receptors found in the *D. melanogaster* genome have been analyzed preliminarily to reveal their potential connection with ecdysteroid-regulated developmental events. Some of these have not been functionally associated with ecdysteroid action and play a role in other developmental processes (Sullivan and Thummel, 2003, and references therein); these will not be considered further in this chapter.

3.5.2.3. Homology Models and Crystal Structure for Ecdysteroid Receptor Components

The need to understand the interaction of EcR with its natural ligand and other ecdysteroid agonists has prompted efforts to elucidate the crystal structure of the EcR LBD. Homology models based on a comparison of the EcR LBD with the known crystal structures of the vertebrate retinoic acid receptor (RAR) and vitamin D receptor (VDR) have been employed to formulate predictions about the three-dimensional structure of the EcR LBD (Wurtz *et al*., 2000). The docking model resulting from this comparison with known crystal structures suggests that the ligand-binding pocket of EcR consists of a shallow tube that is just large enough to accommodate 20E and a bulky envelope. The shape and size of the

Figure 5 Amino acid alignment of representative insect USP sequences (order Diptera: *D. melanogaster, L. cuprina, A. aegypti, C. tentans*; order Lepidoptera: *H. virescens, B. mori, M. sexta, C. fumiferana*; order Orthoptera: *L. migratoria*; order Coleoptera: *T. molitor*). Also included: ixodid tick (*A. americanum*) and human RXR-α. Amino acids designate number of first residue in alignment. References indicate original report. Bars designate each of the 12 helices, and shaded residues indicate those which have been mutated and reported (references given in the text).

Table 1 Nuclear receptors experimentally associated with ecdysteroid action in *Drosophila melanogaster*. Relevant references given in text

Drosophila *receptor*	*Noninsect ortholog*	*Insect orthologs*	*Mutational effect in* Drosophila
EcR	vert. FXR	see **Figure 4**	Embryonic/larval lethal
USP	vert. RXR	see **Figure 5**	Larval lethal
DHR4		*Manduca*	Not reported
DHR38	vert. NGF1B	*Aedes*	Disrupted cuticle development
DHR39			No discernible effect
E75	*Metapanaeus* (shrimp) Vert. PPARγ	*Aedes, Bombyx, Choristoneura, Galleria, Manduca*	Larval/adult lethal
E78	*Dirofilaria* (filarial parasite)		Disrupted chromosome puffing
DHR3	*C. elegans* CHR3, ROR	*Aedes, Choristoneura Galleria, Manduca, Bombyx, Tenebrio*	Embryonic lethal
DHR78		*Bombyx, Tenebrio*	Larval lethal
FTZF1	vert. Steroidogenic factor-1 (SF1)	*Aedes, Bombyx, Manduca, Tenebrio*	Embryonic/larval lethal
SVP	vert. COUP-TF	*Aedes, Tenebrio*	Disrupted eye development

pocket predicted by this model also indicate that the more compact bisacylhydrazines that behave as nonsteroidal agonists of EcR fill up only a portion of the pocket. The crystal structure of *Hv*EcR has shown that the EcR LBD is highly flexible, and that the shape of its ligand-binding pocket adapts to the ligand that occupies it, whether it be 20E, or a nonsteroidal agonist (**Figure 6**; Billas *et al.*, 2003). Just as the crystal structure predicts, substitution of an amino acid that normally contacts 20E (A398P in *Hv*EcR; see **Figure 4** for its position in other EcR sequences) destroys *Hv*EcR's ability to mediate an inducible transcriptional response to 20E, but the same mutation does not affect the inducibility caused by a nonsteroidal agonist. The crystal structure further predicts that a valine residue residing in helix 5 of Lepidoptera EcR sequences (V384 in *Hv*EcR) is responsible for the high affinity of a lepidopteran-specific agonist, BY106830. Most insect EcRs from other orders encode a methionine at this position in helix 5 (see **Figure 4**). The flexibility of the EcR LBD which allows it to bind structurally disparate ligands, presumably is stabilized by heterodimerization with USP.

When USP is purified for crystal structure analysis, a phospholipid is copurified that is partially embedded in the relatively large ligand-binding pocket of USP. As a consequence, the USP ligand-binding domain is held in an antagonistic, apo-conformation in which the carboxy-terminal region including the last alpha-helix (helix 12) is held out (**Figure 7**; Billas *et al.*, 2001; Clayton *et al.*, 2001). Another modeling study has shown that the USP LBD possesses the theoretical capability to reconfigure itself into an agonist position and interact with protein cofactors, although the residues involved in these interactions would be different from those exposed on the RXR-α homolog when it assumes an agonist conformation (Sasorith *et al.*, 2002). Modelling has also been employed to determine the capability for the three major juvenile hormones (JH1, JH2, and JH3) to bind to the ligand-binding pocket of USP. At least two ligand-binding configurations were identified, one associated with USP forms that carry an arginine residue at a critical position in helix 5 (see **Figure 5**). This residue prevails among the non-dipteran species for which the model predicted that JH acids could form a stable binding configuration.

For the dipteran species, which typically carry a nonpolar amino acid at this position in helix 5, JH esters showed better affinity than JH acids, consistent with the results of biochemical binding studies performed on the *Drosophila* USP in which a purified USP fusion protein shows half-maximal binding in the range of 1–5 μM using a tryptophan fluorescence assay (Jones *et al.*, 2001). While the modeling demonstrates the theoretical capability for various JH forms to bind to either lepidopteran or dipteran forms of USP, the occupancy rate of the ligand-binding pocket for them is relatively low for nuclear receptors. Nevertheless, other studies indicate that biological relevance does not always depend upon a high ligand binding affinity (Forman *et al.*, 1995; Kitareewan *et al.*, 1996; Staudinger *et al.*, 2001). For instance, the affinity of the vertebrate peroxisome proliferator activating receptors (PPARs) involves ligand interactions of low specificity and affinity that approximates the levels predicted for USP and JH forms (Schmidt *et al.*, 1992). Viewed from this perspective, the low affinity of a receptor for its activating ligand actually suggests a

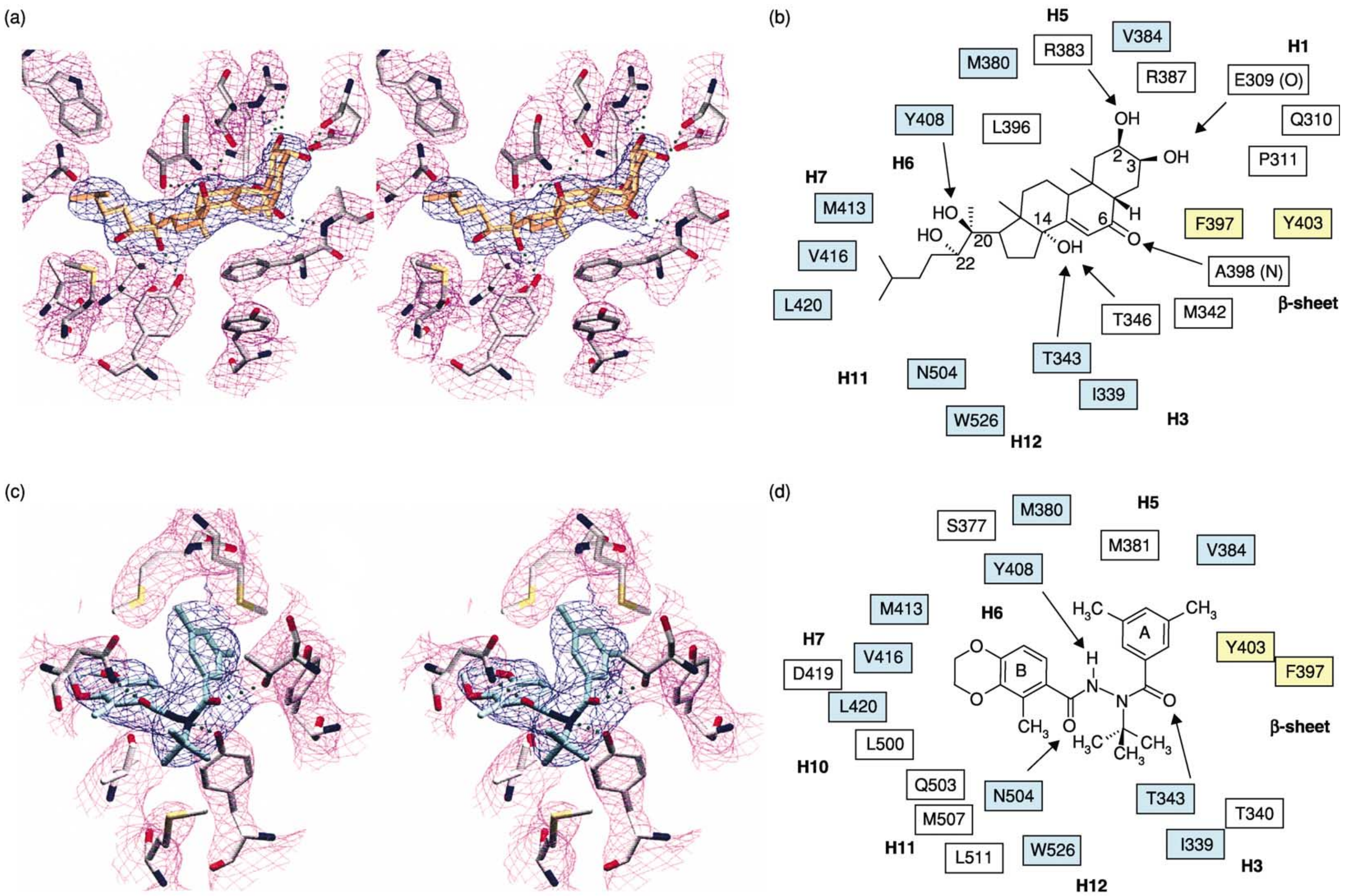


Figure 6 Two ligand binding modes for *Hv*EcR LBD as described in Billas *et al*. (2003). (a) Two weighted omit stereoview maps of the electron density for ponasterone A-bound HvEcR-LBD at 2.9 Å resolution. Ligand shown in blue and selected amino acid residues shown in magenta. Interactions between residues and ligand indicated as green dotted lines. (b) Schematic representation of the interactions between ligand-binding residues and ponasterone A. Arrows correspond to hydrogen bonds between ligand and amino acid residues. Residues in blue are common to both stereoview structures in (a), residues in white are those depicted only in the first stereoview map, and those in yellow are depicted only in the second (righthand) map. (c) two weighted stereoview maps, as above, with the nonsteroidal agonist, BYI06830. (d) schematic representation of the interactions between ligand-binding residues and BYI06830. (Adapted with permission from Billas, I.M., Iwema, T., Garnier, J-M., Mitschier, A., Rochel, N., *et al*., **2003**. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature 426*, 91–96.)

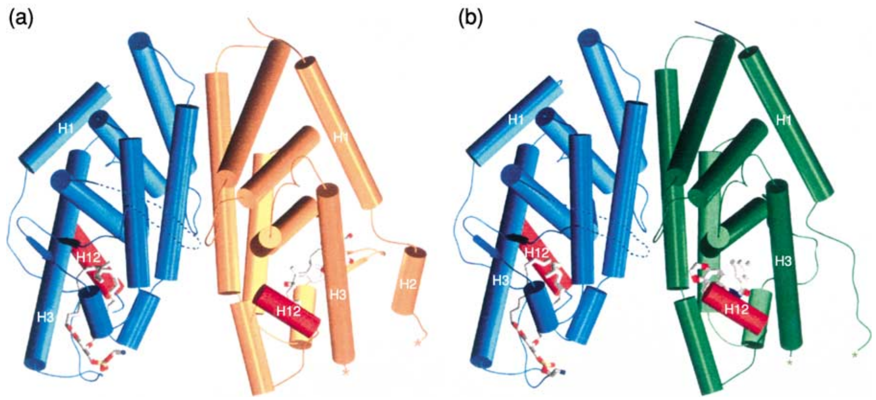


Figure 7 The dimerization structures of *Hv*EcR LBD and *Hv*USP LBD. (a) EcR/USP LBD heterodimer with bound ponasterone A. (b) EcR/USP LBD heterodimer with nonsteroidal agonist, BYI06830. Helix 12 is shown in red, USP shown in blue. The missing loop that connects USP Helix 5 to the β-sheet is shown as a dashed blue line that projects closely to EcR helix 9–10. (Adapted with permission from Billas, I.M., Iwema, T., Garnier, J-M., Mitschier, A., Rochel, N., *et al.*, **2003**. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature 426*, 91–96.)

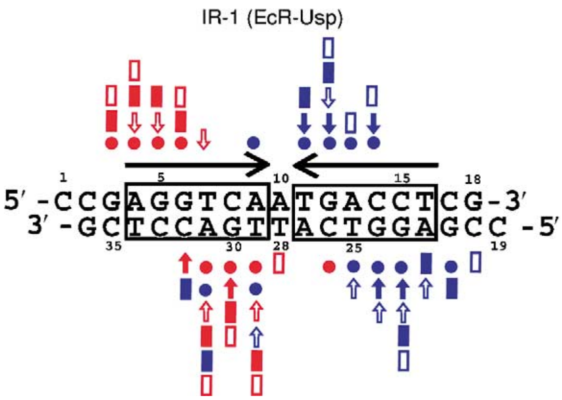


Figure 8 An idealized palindromic repeat element with a single nucleotide spacer used in the EcR-USP complex. Symbols as in Figure 3. Red indicates USP interaction sites, blue indicates EcR interaction sites. Solid arrows indicate a direct base interaction and hollow arrows indicate a water-mediated base interaction. Solid bars indicate a direct phosphate interaction, and hollow bars indicate a water-mediated phosphate interaction. Circles indicate van der Waals interaction. (Reproduced with permission from Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., **2003**. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J. 22*, 5827–5840.)

mechanism by which fairly tight regulation is achieved when ligand titers reach relatively high levels in the cell. A discussion of functional experiments concerning the effects of JH on the ecdysteroid receptor components, EcR and USP, is presented later.

The crystal structure of the EcR and USP DBD at a canonical EcRE DNA-binding site reveals the nature and location of DNA-binding residues for the two DBD regions, as well as their points of protein-protein interaction (Figure 8). While RXR forms a functional dimer with EcR (Christopherson *et al.*, 1992; Yao *et al.*, 1993), the physical interaction between USP/RXR and EcR is different than it is for RXR with several of its natural partners (**Figure 9**; Devarakonda *et al.*, 2003).

The ability of the DHR38/USP heterodimer to induce transcription in response to ecdysteroids, even though the complex does not physically interact with its ligand, has prompted a crystal structure analysis of the DHR38 LBD. DHR38 lacks a true ligand-binding pocket – the space is filled with four phenylalanine side chains. The dimerization interface apparently lies along helix 10, but DHR38 lacks helix 12, and its AF2 function may reside in an unique sequence lying between helix 9 and 10 in an activated (agonistic) conformation (Baker *et al.*, 2003).

3.5.2.4. Biochemical Analysis of EcR and USP

Numerous biochemical experiments have been performed to assess the properties associated with EcR and USP function in insects, including: (1) the affinity of EcR for ecdysteroids and nonsteroidal agonists, (2) the ability of EcR and USP to dimerize and interact with DNA target sequences, (3) the physical interaction of EcR and USP with other orphan receptors and proteins, and (4) the detection and demonstration of posttranslational and other modified forms of EcR and USP.

The necessity for heterodimerization between EcR and USP to produce a functional ecdysteroid receptor was demonstrated by showing that the *in vitro* translated EcR and USP products bind to a radiolabelled *hsp27* EcRE, but that neither product alone is capable of binding to the same element, based on the results of an electrophoretic mobility shift assay (EMSA). Moreover, this effect is enhanced by the simultaneous presence of muristerone A or

20E, indicating that the hormone stabilizes the heterodimer at an EcRE site. Further, the affinity of radiolabelled ^{125}I-iodoponasterone, an ecdysteroid with high specific activity and affinity for EcR (Cherbas *et al.*, 1988), is substantially higher for EcR/USP dimers than for EcR alone ($\sim K_d = 1$ nM; Yao *et al.*, 1993). A similar level of ligand affinity is obtained with extracts taken from cultured *Drosophila* Kc and S2 cells, which contain endogenously expressed USP (Koelle *et al.*, 1991). EcR also heterodimerizes with the vertebrate RXR and this heterologous dimer is responsive to muristerone A, but not 20E, indicating that USP plays some role in determining the ligand specificity of the complex (Christopherson *et al.*, 1992; Yao *et al.*, 1993). The ability of EcR and USP candidates to heterodimerize on an *hsp27* EcRE has become a standard for verification (e.g., Swevers *et al.*, 1996; Hannan and Hill, 2001; Durica *et al.*, 2002; Minakuchi *et al.*, 2002).

The EcR/USP heterodimers from other insects show an affinity for ^{125}I-iodoponasterone and [^{3}H]-ponasterone A that approximates the *Dm*EcR/USP heterodimer (e.g., $K_d = 1.1$ nM; Swevers *et al.*, 1996). For *Aa*EcR and *Aa*USP, the detection of EcR/USP heterodimers in a mammalian cell culture extract increases approximately 25-fold with the addition of 5 μM 20E. This accompanies a decrease in the quantity of *Aa*USP/*Aa*SVP heterodimers, thus illustrating the competitive dynamics of these two protein–protein interactions involving USP (Zhu *et al.*, 2003a).

The ability of nonsteroidal agonists, such as the diacylhydrazines, RH5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) and RH5992 (tebufenozide), to displace radiolabelled ponasterone has been used to assess EcR specificity for these compounds. When extracts from a lepidopteran (Sf9) and dipteran insect cell line (Kc) are compared for the ability of the diacylhydrazines to displace [^{3}H]-ponasterone A, the affinity of all RH compounds is considerably lower in the Kc cells (Nakagawa *et al.*, 2002). Also, none of several RH compounds fail to displace [^{3}H]-ponasterone A in extracts containing the migratory locust's EcR and USP, indicating that the orthopteran EcR, like those of other ancient insect orders, possesses substantially different binding properties than those of the Lepidoptera (Hayward *et al.*, 2003). Ligand binding requires the entire LBD, and deletion of a portion of helix 12 at the carboxy-terminal end of EcR is sufficient to eliminate ponasterone A binding (Perera *et al.*, 1999b; Hu *et al.*, 2003; Grebe *et al.*, 2003).

Fusion proteins expressing wild-type and mutated versions of *Dm*EcR and *Dm*USP LBDs in yeast cells have also been subjected to tests of their affinity for ponasterone A in cell extracts (Grebe *et al.*, 2003). The association rate of a *Dm*EcR LBD fusion protein is modestly elevated by the presence of USP, and the dissociation rate is reduced by about 20-fold, so that the dissociation constant is reduced from about 40 nM in EcR alone to about 0.5 nM in the presence of USP. At least one mutation of the *Drosophila* EcR LBD (N626K) alters the rate of dissociation without affecting association rate, suggesting that an ecdysteroid enters and exits the EcR ligand-binding pocket by different routes, though this effect has not been tested in the intact EcR or *in vivo*. Purified *Dm*USP has the potential to form homodimers and other oligomers, even in the absence of a DNA-binding site, though this capability is reduced by removal of the A/B domain (Rymarczyk *et al.*, 2003).

Along with its ligand-binding properties, the ability to recognize a DNA element, usually the consensus *hsp27* EcRE by an EcR/USP or EcR/RXR dimer, is a standard for identifying a functional ecdysteroid receptor. The *hsp27* EcRE was originally localized by DNAse I footprinting and its ability to confer ecdysteroid-inducibility on an otherwise noninducible promoter in S1 *Drosophila* cultured cells. The sequence is 23 bp long and arranged in an inverted palindrome separated by a single nucleotide (Riddihough and Pelham, 1987). The EcR and USP zinc finger interactions with an idealized palindromic EcRE and with each other are described diagrammatically (**Figure 3**; Ozyhar and Pongs, 1993; Devarakonda *et al.*, 2003) and shown graphically (**Figure 8**). A diagrammatic depiction of the nature of individual nucleotide interactions between the element and the zinc fingers is also shown in **Figure 9** (Devarakonda *et al.*, 2003). A closely related response element taken from an ecdysteroid-inducible gene in Kc cells, Eip28/29, has also been used experimentally (Cherbas *et al.*, 1991). The EcR/USP heterodimer also recognizes half sites arranged into direct repeats (DR) separated by 0–5 nucleotide spacers (DR0–DR5) with the DR4 element showing the highest affinity. Various direct repeat elements were tested with the ecdysteroid-inducible and fat body-specific *fbp1* promoter. When these direct repeat elements are connected to the minimal *fbp1* promoter, they are unable to induce higher transcription in the presence of ecdysteroids. However, when the DR0 and DR3 elements are substituted for the natural *fbp1* EcRE in its normal promoter context, both elements are ecdysteroid-inducible and fat body specific (Antoniewski *et al.*, 1996).

Context is also important for the intermolt, *sgs4* gene promoter, which requires that an EcRE be surrounded by serum element binding protein

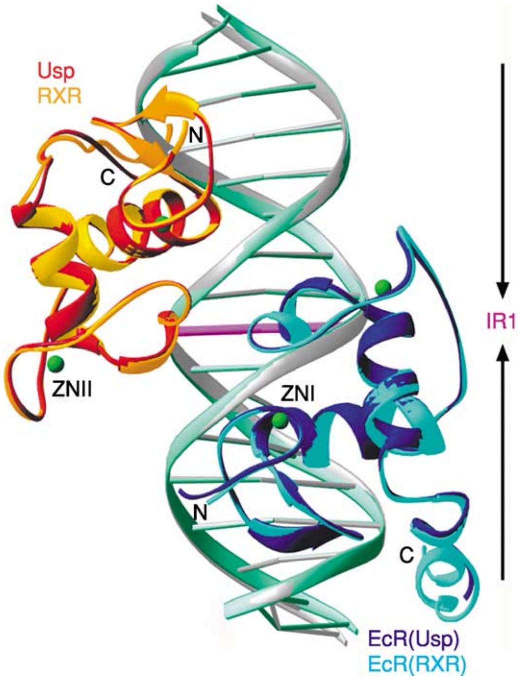


Figure 9 A superposition of the *Dm*EcR/*Dm*USP/IR1 DNA complex and the *Dm*EcR/*Hs*RXR/IR1 DNA complex. USP is shown in red, RXR is shown in gold. EcR with USP is shown in purple, and EcR with RXR is shown in light blue. Zinc ions are shown in green and the single base pair spacer nucleotide is shown as magenta. (Reproduced with permission from Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., **2003**. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J. 22*, 5827–5840.)

(SEBP) binding sites in order to mediate a receptor-inducible response (Lehman and Korge, 1995).

A perfect palindrome carrying the half-site sequence, GAGGTCA separated by a single A/T nucleotide (PAL1) shows the highest affinity for the *Dm*EcR/*Dm*USP complex in extracts taken from *Drosophila* S2 cells. By testing the ability of other elements to compete for this element, the order of affinity is been determined: PAL1 > DR4 > DR5 > PAL0 > DR2 > DR1 > hsp27,DR3 > DR0. In all cases, affinity is elevated by the presence of muristerone A. In cell culture experiments testing the inducibility of these elements when attached to a minimal promoter, inducibility correlates with affinity, except that the *hsp27* EcRE is a stronger inducing element than DR1, DR2, or PAL0. When DR4 is placed in a reverse orientation relative to the promoter, it maintains its ability to mediate a transcriptional response (Vogtli *et al.*, 1998). A similar correlation between DNA affinity and inducibility has been noted for the *Aa*EcR/*Aa*USP complex and its response elements (Wang *et al.*, 1998).

A natural direct repeat EcRE has been identified in the ecdysteroid-regulated intermolt 3C puff of *D. melanogaster* that is separated by 12 nucleotides (DR12) which is capable of conferring ecdysteroid-responsiveness to a heterologous promoter in cell cultures (D'Avino *et al.*, 1995). The affinity of the EcR/USP complex for the DR12 element is offset by the presence of other orphan receptors (DHR38, DHR39, *β*FTZF1), and a weak inductive response to 20E is also offset by the presence of these orphans (Crispi *et al.*, 1998). A similar competition scenario exists between *Dm*EcR and *Dm*SVP as heterodimeric partners with *Dm*USP. The SVP/USP dimer preferentially binds to a DR1 element, whereas the EcR/USP dimer displays a heightened affinity for an Eip28/29 EcRE (Zelhof *et al.*, 1995a).

The recognition of these canonical elements also depends upon specific features of the ecdysteroid receptor itself. In at least one instance, DNA recognition depends upon isoform-specific EcR and USP combinations. The EcR heterodimers formed with each of the two USP/RXR isoforms in the ixodid tick, *A. americanum,* display substantially different affinities for DNA response element sequences, with an RXR1/EcR dimer showing strong affinity for both palindromic and direct repeat sequences, whereas an RXR2/EcR dimer shows only weak affinity for a PAL1 element (Palmer *et al.*, 2002).

Ligand-binding specificity also plays a role in determining the affinity of EcR/USP for a response element. *Aa*EcR/*Dm*USP recognition of an *hsp27* EcRE on EMSAs is normally enhanced by the presence of ecdysone, but only 20E enhances affinity of the *Dm*EcR in this assay. Domain swapping between the *Aa*EcR and the *Dm*EcR localizes a region within the LBD that is responsible for the differential effect of ecdysone on the two EcRs. Within the swapped region, a single amino acid conversion in the *Dm*EcR (Y611F) to the corresponding *Aa*EcR sequence results in a mutated receptor that shows the ability to dimerize in the presence of both 20E and ecdysone (Wang *et al.*, 2000). Interestingly, all insect EcR sequences encode either a tyrosine or phenylalanine at this position in helix 10, which lies along a dimerization interface. The subtle yet significant difference in sequence, when considered in the framework of the numerous residue differences that exist among the insect EcR LBDs, illustrates the dimensions of potential functional diversity among them.

Generally, the interpretation of EMSA results must be handled circumspectively because purified EcR/USP proteins do not always exhibit the same DNA binding properties as cell extracts which include the receptor components (e.g., Lan *et al.*, 1999; Palmer *et al.*, 2002). This is exemplified in the extreme by the observation that affinity column

purified *Dm*EcR and *Dm*USP, when mixed together, fail to bind to the *hsp27* EcRE unless other chaperone proteins are added. While no single chaperone is essential for DNA binding, the elimination of any one of them proportionally reduces EcRE recognition. The formation of this chaperone/receptor complex also requires ATP, although none of these chaperones is necessary for ligand binding. Only two of the six chaperones described, Hsp90 and Hsc70, are *Drosophila* proteins; the others used in the study were human chaperone proteins (Arbeitman and Hogness, 2000).

By expressing GST fusion proteins in *E. coli* and using affinity chromatography to purify them, preparations of *Chironomus* EcR and USP have been recovered which retain essential DNA-binding and ligand-binding characteristics as long as detergents are absent from the purification process. Scatchard plots of bacterially expressed *Ct*EcR reveal two high-affinity ecdysteroid binding sites, as do extracts from a *Chironomus* epithelial cell line (Grebe *et al*., 2000). Bacterial GST-fusion proteins expressing the *Drosophila* EcR and USP DNA-binding domains have also been isolated for the purpose of testing their affinity to DNA elements, although the GST motif alters the DNA-binding properties of the fusion protein (Niedziela-Majka *et al*., 1998; Grebe and Spindler-Barth, 2002). Similarly, the recovery of a fusion protein encoding the *Dm*EcR LBD is enhanced by cleaving the C-terminal F-domain, which is considerably longer in *D. melanogaster* than other species (Halling *et al*., 1999); this cleavage exerts no discernible effect on transcriptional activity (Hu *et al*., 2003).

Since EcR and USP function as part of a protein complex, orphan receptors and cofactors described throughout the chapter typically require the discovery and/or demonstration of a physical interaction. These demonstrations include the use of yeast two-hybrid assays (e.g., Sutherland *et al*., 1995; Beckstead *et al*., 2001), the interaction of *in vitro* translated EcR and USP with a protein or protein domain (e.g., Bai *et al*., 2000), and the identification of interacting proteins that coprecipitate with EcR and/or USP (e.g., Sedkov *et al*., 2003). A member of another class of proteins, the immunophilins, which are known to interact with vertebrate steroid receptor complexes, coprecipitates as part of an EcR/USP complex taken from *M. sexta* prothoracic (ecdysteroidogenic) glands. Ligand affinity of the complex falls within an expected range, though it is unknown whether the interaction between the immunophilin, FKBP46, and EcR/USP is direct and also unknown whether this interaction affects transcriptional activity (Song *et al*., 1997).

The colocalization of proteins such as RNA polymerase II and EcR on *Chironomus* polytene chromosomes by immunostaining illustrates a possible relationship between the complex and the cell's transcriptional machinery (see **Figure 1**; Yao *et al*., 1993; Wegmann *et al*., 1995). A zinc finger protein that preferentially localizes at active puffs along *Drosophila* polytene chromosomes, known as peptide on ecdysone puffs (PEP), has been characterized by anti-PEP antibody decoration of polytene chromosomes, but has not yet been functionally associated with ecdysteroid action (Amero *et al*., 1991).

The effect of posttranslational modifications upon ecdysteroid activity has also not been explored extensively, though a phosphorylated form of *Dm*USP in fly larvae has been identified by showing that phosphatase activity eliminates a high molecular weight band in larval extracts. The phosphorylated form of USP in larval salivary glands is elevated by incubation with 20E (Song *et al*., 2003). Similarly, the presence of a phosphorylated form of both *Ct*EcR and *Ct*USP extracted from *C. tentans* larvae has been demonstrated on Western immunoblots (Rauch *et al*., 1998). The relative abundance of phosphorylated forms of the two USP isoforms in the *M. sexta* prothoracic gland is altered by the presence of 20E and has been associated with changes in ecdysteroidogenic activity, suggesting a feedback mechanism by which ecdysteroid synthesis is downregulated in prothoracic glands (Song and Gilbert, 1998).

While several groups have employed DNA cloning methods to recover receptor genes from which protein sequences are deduced, other efforts have demonstrated receptor purification through measurements of their activity. Partial purification of an ecdysteroid-binding protein from *Drosophila* embryos produces two peptides that are radiolabelled by a 20E derivative, one of 150 kDa and another of 90 kDa (Strangmann-Diekmann *et al*., 1990), the latter being the molecular weight predicted from the *Drosophila* EcR cDNA sequence (Koelle *et al*., 1991). Purification of the protein through its affinity to a magnetic *hsp27* EcRE failed to resolve the disparity between the measured size of the purified product and the predicted size of the protein (Ozyhar *et al*., 1992). Purification of the 20E receptor yielded a protein that binds to the appropriate response element sequence of the *hsp27* promoter, as well as an *hsp23* promoter element, and elevates transcription rates by 100-fold (Luo *et al*., 1991). The presence of ecdysteroids increases the affinity of the receptor protein as measured by EMSA.

3.5.3. Functional Characterization of the Ecdysteroid Receptor

3.5.3.1. Cell Culture Studies: Rationale

The study of EcR and USP *in vivo* is complicated because ecdysteroid responses vary both spatially and temporally. In fact, the effects of ecdysteroids can be obscured by the heterogeneity of response at the promoter of a single gene (Andres and Cherbas, 1992; Andres and Cherbas, 1994). Therefore, it is often difficult to unravel *in vivo* transcriptional and cellular responses mechanistically, even in a single cell type, since the response to ecdysteroids typically triggers ongoing changes in the target cell, that in turn, affect later ecdysteroid responses.

Cell cultures provide the benefit of working with a stable, relatively homogeneous cell type that does not require the rigorous staging and rearing conditions necessary for meaningful studies *in vivo*. Thus, cultured cells can provide a variety of important and useful insights about EcR and USP by establishing a foundation for subsequent *in vivo* hypothesis testing. Because they are easy to grow, it is also relatively easy to recover cellular extracts for biochemical testing. Nevertheless, stably cultured cells probably do not duplicate any actual cell exactly, so that observations reflect what a cell can do, but not necessarily what a cell does. Therefore, subsequent *in vivo* verification is essential for insights garnered through cell culture experiments.

Numerous experiments involving cultured cell lines have been conducted over the years to test the ability of the ecdysteroid receptor to regulate transcriptional activity induced by ecdysteroid treatment. Early experiments focused on endogenous changes in cell morphology and gene expression in insect cells challenged with ecdysteroids such as 20E, which express EcR and USP endogenously. As transfection technology has developed, increasingly sophisticated cell systems have been developed to analyze and dissect receptor functions. In recent years these systems have been developed to screen for novel agonists and devise "new and improved" ecdysteroid induction systems for agricultural, biomedical, and commercial application (see **Chapter 3.4**).

3.5.3.2. Cell Cultures: Characterization

The most prominent cell line for early studies of ecdysteroid action was the *D. melanogaster* Kc cell line, and derivatives of it are still employed today (Hu *et al.*, 2003). In response to 20E at 10^{-7} to 10^{-8} M, Kc cells start to develop extensions within hours, produce acetylcholinesterase, and undergo a proliferative arrest a few days later. These cells are even more responsive to the phytoecdysteroids, ponasterone A and muristerone A, than to 20E (Cherbas *et al.*, 1980 and references therein). With prolonged exposure to ecdysteroids, Kc cells become insensitive to 20E and EcR levels become depressed (Koelle *et al.*, 1991). The nonsteroidal agonist RH5849 not only mimics the effects of 20E when tested on sensitive Kc cells, it also fails to evoke a response from insensitive Kc cells, thus arguing that the RH5849 acts via a common mechanism with ecdysteroids (Wing, 1988).

Other cell lines, Schneider 2 and 3 (S2 and S3), also become insensitive to ecdysteroid action by reducing their titer of EcR, and have been used because they are relatively easy to transfect transiently with fusion and reporter genes. As noted earlier, the identity of the *Dm*EcR gene was partly demonstrated by introducing the *Dm*EcR cDNA into ecdysteroid-insensitive S2 cells and thus restoring their responsiveness to 20E (Koelle *et al.*, 1991). More recently, Kc cell lines have been improved by developing protocols for stable integration of transgenes by P-element transposition into the genome (Segal *et al.*, 1996) and by using parahomologous gene targeting to "knock out" an endogenous gene target (Cherbas and Cherbas, 1997). In this way, a cell line, L57-3-11 containing no endogenous EcR has been produced which allows the introduction of modified versions of EcR for subsequent experimentation, without the complications normally posed by the presence of endogenous EcR (Hu *et al.*, 2003).

The Kc cell line has been used to identify ecdysteroid-regulated transcriptional targets. By comparing hormone-treated and control Kc cells, Savakis *et al.* (1980) isolated and identified three ecdysteroid-inducible peptides (EIPs) named by their molecular mass in kiloDaltons, EIP28, EIP29, and EIP40, whose synthesis is elevated 10-fold at 10^{-8} M 20E. JH supplementation of the cell culture medium inhibits acetylcholinesterase induction but not EIP induction (Cherbas *et al.*, 1989).

The use of ecdysteroid-responsive lines for other insects is expanding continuously, largely in order to understand the causes of insecticidal resistance to ecdysteroid agonists and to identify novel insecticidal candidates. A *C. tentans* epithelial cell line was used to isolate several stable and ecdysteroid-resistant clones that metabolizes 20E relatively quickly (Spindler-Barth and Spindler, 1998). Clones from the same line were later selected for their resistance to the inductive effects of RH5992 and differential metabolism of RH5992 was ruled out as a cause for the resistance. While the profile of EcR in these cells is approximately normal, some

lines shows a modest decline in the abundance of phosphorylated USP forms that is not reversible by addition of 20E. A variety of differences in EcR ligand-binding characteristics were noted among the cell lines tested that presumably reflect the role of unknown factors underlying the resistance (Grebe *et al*., 2000).

Another widely used lepidopteran cell line is Sf9, derived from ovarian cells of the fall armyworm, *Spodoptera frugiperda*. The affinity of ponasterone A for Sf9 and Kc cells is very similar, but the nonsteroidal agonist ANS-118 (chromafenozide) displays a much higher affinity for extracts derived from Sf9 cells than from Kc cells (Toya *et al*., 2002). Similarly, cell lines derived from *C. fumeriferana* show a much greater responsiveness to RH5992 than *Drosophila* cultured cells, which do not respond to the compound and excrete it an elevated rate via an ABC transporter system (Retnakaran *et al*., 2001). *Choristoneura* cell lines, with prolonged exposure to RH5992 become irreversibly insensitive to ecdysteroids and to the agonist, though the mechanism is unknown (Hu *et al*., 2004). A more comprehensive discussion of ecdysteroid agonist properties is provided elsewhere in this series (see **Chapter 3.4**), though it is clear that the differences among species are derived primarily from structural diversifications in EcR and possibly, USP (see **Chapter 3.6**).

Other lines are particularly useful for studying ecdysteroid action in economically important insect species even when a complete EcR and USP have not been yet been isolated. These lines include the European corn borer (*Ostrinia nubilalis*; Trisyono *et al*., 2000), the tent caterpillar (*Malacosoma disstria*; Palli *et al*., 1995), the fall armyworm (*Spodoptera frugiperda*; Chen *et al*., 2002a), and the cotton boll weevil (*Anthonomus grandis*; Dhadialla and Tzertzinis, 1997). The ecdysteroid responsiveness has been established for at least some of these lines by showing that orthologs for ecdysteroid responsive *D. melanogaster* genes, notably the genes encoding E75 (early puff) and DHR3 (early-late puff), are activated transcriptionally when the cells are challenged with 20E or an agonist.

A major benefit of using insect cell lines is that they provide the cofactors and machinery that are necessary for transcription to occur. On the other hand, other factors may modify or obscure an aspect of ecdysteroid response in a given experimental regime as silent and unidentified participants. As is evident from *in vivo* studies, a given experiment will generate substantially different cellular responses depending upon the milieu of proteins that contribute to ecdysteroid responsiveness in a given cell type. For this reason, mammalian cell lines containing no endogenous ecdysteroid responsiveness have been used to reconstruct an ecdysteroid responsive transcriptional system by introducing the genes encoding EcR, USP, cofactors, and reporter genes in order to analyze their individual effects on ecdysteroid action (e.g., Christopherson *et al*., 1992; Yao *et al*., 1993; Palli *et al*., 2003; Henrich *et al*., 2003). Heterologous cells and cell lines also pose special difficulties, since it is conceivable that endogenous proteins provide novel functions or act as surrogates for similar insect proteins. Conversely, these cells might render false negative results in some experiments because they are missing one or more crucial cofactors.

3.5.3.3. Cell Cultures: Functional Receptor Studies

A variety of studies have examined the capability of EcR and USP to function in insect and other cell lines. Using the same approach, several orphan receptors from *D. melanogaster* have also been tested for their ability to respond to candidate ligands. These studies have generally focused on: (1) the responsiveness and activity of target DNA sequences in promoters, (2) the effect of various ecdysteroids, agonists, and antagonists upon receptor activity, (3) the properties of specific domains on receptor function and the effect of structural modifications upon transcriptional activity, and (4) the effect of heterodimerization and other cofactors, including orphan receptors, upon transcriptional activity.

3.5.3.3.1. Promoter studies The *hsp27* EcRE connected to a weak constitutive promoter is capable of inducing the transcription of a reporter gene in a *Drosophila* cell line by over 100-fold when challenged with 20E (Riddihough and Pelham, 1987). When *hsp27* EcREs are organized in a tandem repeat and attached to a weak promoter, the gene is not only responsive to 20E, but also is repressed in the absence of hormone, suggesting a repressive role for the ecdysteroid receptor via this element (Dobens *et al*., 1991). A plethora of cell culture transcriptional tests and accompanying EMSAs have been reported which followed from this early work, and this is an important criterion for determining the functionality of an EcR/USP heterodimer, as it was in the original report (Koelle *et al*., 1991).

The most fundamental task concerning promoter activity is to define the functional promoter elements to which the ecdysteroid receptor complex binds and exerts its effects on transcription. This was accomplished for the genes encoding the aforementioned EIP28 and 29, which are translated from

alternatively spliced mRNAs of the same gene (Schulz *et al.*, 1986). Three specific ecdysone response elements are ecdysteroid-responsive *in vivo* (Andres *et al.*, 1992; Andres and Cherbas, 1994). One of these Eip28/29 EcREs lies in the promoter region though it is not required for ecdysteroid-inducibility in Kc cells, possibly because this element is not used in the embryonic hemocytes from which the cells are derived. The other two elements lie downstream from the polyadenylation site, that is, on the 3′ side of the gene (Cherbas *et al.*, 1991). The EIP40 gene is also ecdysteroid-inducible *in vivo* (Andres and Cherbas, 1992) and the genomic region that includes the EIP40 gene has been analyzed (Rebers, 1999). Just as cell culture experiments have been used to identify EcREs *in vitro* for subsequent *in vivo* analysis, the reverse relationship has also been undertaken successfully – the ecdysteroid-responsive promoter elements in the β3 tubulin gene, which is ecdysteroid-responsive *in vivo* (Sobrier *et al.*, 1989), were identified by testing their inducibility by 20E in Kc cells (Bruhat *et al.*, 1993).

Cell culture experiments have been used not only to dissect the functional ecdysteroid response elements within a promoter region, but also to examine the possibility that different factors compete for these sites to modulate transcriptional activity. The relationship of *M. sexta* EcRB1 and two *Ms*USP isoforms has been explored in *M. sexta* GV1 cell cultures by observing the ecdysteroid-inducible MHR3 promoter (Lan *et al.*, 1997). Both EcR/USP complexes display about the same level of ligand affinity, but the EcRB1/USP1 complex induces much higher levels of expression in response to 20E from an intact promoter and also represses basal expression in the absence of 20E. Further, the EcRB1/USP1 complex binds to a canonical EcRE in the promoter and activates transcription, but the addition of USP2 prevents transcription and blocks binding to the EcRE. *In vitro* translated EcRB1/USP2 can bind to this same EcRE, indicating that other cellular cofactors are responsible for the differential action of the two *Ms*USP isoforms (Lan *et al.*, 1999).

The two isoforms of *Ct*USP show somewhat different ligand-binding capabilities, and one of the forms, when purified, recognizes a DR1 element that is not recognized by the other. However, both show about the same ability to induce transcription with DmEcR in human HeLa cells. The CtEcR does not possess the ability to induce transcription in cell cultures (Vogtli *et al.*, 1999).

3.5.3.3.2. Transactivation studies The appearance of multiple EcR isoforms in *D. melanogaster* has prompted comparisons of their transcriptional responsiveness in cell cultures and in yeast. The B1 and B2 N-terminal domains from *Dm*EcR, when combined with a GAL4 DBD and and transfected into yeast, are capable of mediating transcription via a GAL4-responsive universal activation site (UAS) in the promoter. This AF1 (ligand-independent) transcriptional function is further elevated by removing an inhibitory region within the B1 domain (Mouillet *et al.*, 2001). In human HeLa cells, *Dm*EcRB1 and *Dm*EcRB2 showed about the same level of AF1 function and both also possessed AF2 (ligand-dependent) transcriptional activity. By contrast, EcRA reduced basal transcriptional activity below the level obtained from an EcR with no A/B domain at all.

Similar results have been obtained in a mammalian Chinese hamster ovary (CHO) line, except that the B1 isoform displays a highly elevated AF1 function compared to the A and B2 isoforms (Henrich *et al.*, 2003). This activity difference measured for B1 in the two cell lines suggests that the HeLa cell line carries a repressor that interacts with the B1 domain which does not exist in CHO cells. It also illustrates the diversity of function that is possible among different cell types owing to differences in the cellular milieu. In the EcR-deficient L57-3-11 line, all three EcR isoforms are responsive to ecdysteroids, though the A domain contributes no AF1 function, nor does the *Dm*USP A/B domain. The B1 domain contains several regions that when deleted impose a modest impact on AF1 functions. By contrast, specific point mutations in the B2 domain dramatically reduce AF1 functions (Hu *et al.*, 2003), suggesting that a localized interaction with other transcriptional factors is essential for activity.

Lezzi *et al.* (2002) devised a yeast two-hybrid assay which fused the EcR and USP LBD to the yeast GAL4 activation domain and DNA-binding domain, respectively. A variety of site-directed mutations of the LBDs were used to test their effects on functional dimer formation and both AF1 and AF2 (ligand-dependent) transcriptional activity as measured by UAS-mediated lacZ activity. The wild-type LBDs dimerize and induce activity at a low rate even in the absence of muristerone A, and this rate increases more than tenfold in the presence of muristerone A (but not other ecdysteroids such as 20E). Mutations of critical residues in EcR's helix 10 all but eliminate both dimerization and transcriptional activity, as expected. Deletion or mutation of helix 12, which normally folds over the ligand-filled pocket, eliminates EcR's AF2 function, as do mutations that affect ligand binding, but AF1 function is still detected in these mutant forms of

EcR. Substitution at a consensus cofactor-interacting residue, K497E, results in a strong elevation of basal transcriptional activity, though this mutant EcR has a reduced capacity for ligand-dependent induction and low ligand affinity (Bergman *et al.*, 2004; Grebe *et al.*, 2003). Virtually all point mutations in the *Dm*USP LBD eliminate transcriptional activity, though many retain the capability to dimerize with EcR, suggesting that USP is required for normal AF1 activity. Deletion of the carboxy-terminal region of *Dm*USP, including helix 12, does not disrupt homodimerization detected by gel filtration, but eliminates ligand-binding. Substitutions of specific residues in helix 12 modestly reduce basal transcriptional activity in yeast. However, while ligand-binding for the mutant USP is only slightly reduced, the ligand-dependent transcription of the EcR/USP complex is virtually eliminated (Przibilla *et al.*, 2004). This result is counterintuitive since the crystallized *Dm*USP is locked into an antagonistic conformation which implies that AF2 activity is impossible. It also contrasts with the normal activity seen in several helix 12 mutations of USP that were tested in the *Drosophila* L57-3-11 cell line, which may express enough *Dm*USP to mask the mutational effects (Hu *et al.*, 2003).

3.5.3.3.3. Ligand effects At least three issues pertaining to ligand responsiveness of the ecdysteroid receptor have been addressed through the use of cell cultures. First, one has focused on the responsiveness of a given receptor to ecdysteroids and agonists, particularly novel compounds. Second, modified receptors have been tested for their capacity to respond to a given ligand or to assess the structural basis of ligand specificity. Finally, recent studies have focused on the possibility that other orphan receptors are responsive to ecdysteroids and/or other ligands.

By testing ecdysteroid-induced transcriptional activity in cell culture, new nonsteroidal agonists, including both artificial compounds (Mikitani, 1996) and a variety of phytochemicals which act as agonists, including 8-O-acetylharpagide (Elbrecht *et al.*, 1996) or antagonists, including several flavones (Oberdorster *et al.*, 2001). Environmental chemicals including polyaromatic hydrocarbons (PAHs) and polychorinated biphenyls (PCBs) induce ecdysteroid-responsive genes, suggesting an EcR-mediated basis for molting defects commonly found among aquatic species residing in polluted water (Oberdorster *et al.*, 1999). The use of cell culture assays has also been utilized to analyze the effects of juvenile hormone and its analogs upon transcriptional activity. The topic of juvenile hormone and its effects upon EcR and USP will be addressed later in the chapter.

Based on lines of evidence gleaned from experimental studies over the years, the possibility that ecdysteroids other than 20E display biological activity has been speculated about and even suspected for years (Somme-Martin *et al.*, 1990; Grau and Lafont, 1994; Henrich and Brown, 1995). In S2 cells, the 20-hydroxylated derivates of the major and natural ecdysteroid products of the *D. melanogaster* larval ring gland, 20E and makisterone A are the most efficacious in activating a reporter gene whose promoter carried three tandem copies of the *hsp27* EcRE. By contrast, precursor and metabolite ecdysteroids, as well as nonsteroidal agonists, evoke considerably less activity in the cell culture assay. As noted in other studies, ponasterone A displays the highest potency, and two other phytoecdysteroids, muristerone A and cyasterone, also are more inducible than 20E in these cells (Baker *et al.*, 2000).

Receptor modifications have also been utilized to delineate the basis for differences in ligand specificity, as noted earlier for the effects of the A398P mutation to test predictions based on the *Hv*EcR crystal structure (Billas *et al.*, 2003). Similarly, a substitution in the *Cf*EcR (A393P) eliminates both its ligand-binding affinity for ponasterone A and its transcriptional inducibility in cell cultures, possibly by impairing the receptor's interaction with a coactivator (GRIP1), but the mutation exerts no debilitative effect on responsiveness to a nonsteroidal agonist (Kumar *et al.*, 2002). A chimeric *Drosophila/Bombyx* EcR carrying only the *Bombyx* D-domain is responsive to RH5992 in mammalian CV-1 cells. The effect is largely attributable to the relatively high level of RH5992-dependent dimerization between the D-domain of the chimera and endogenous RXR or cotransfected USP (Suhr *et al.*, 1998). Based on a deletion analysis of EcR, both its D and E domain are required for ligand-dependent dimerization with USP (Perera *et al.*, 1999b).

As already noted, insect cells may provide endogenous components that influence ecdysteroid activity. EcR itself masks a second ecdysteroid responsive activity in *Drosophila* S2 cells. A fusion protein carrying the DHR38 LBD was tested for its ability to dimerize with *Dm*USP in response to several ecdysteroids using S2 cells from which endogenous EcR activity was eliminated by RNA interference. A response to 20E occurs that is further induced by adding an RXR activator, indicating that the DHR38/RXR heterodimer is responsible for the effect. Similarly, cotransfection with a chimeric and constitutively active form of USP also confers the ability of DHR38 to respond to 20E (Baker *et al.*, 2003). The DHR38/USP dimer also responds to at least six other ecdysteroids, including some, such as

3-dehydro-20E and 3-dehydromakisterone A, which are abundant during late larval development in *Drosophila* and display unusually high inducibility in fat body (Somme-Martin *et al.*, 1990). The relevance of this pathway for ecdysteroid-driven events represents an important avenue for investigation in *Drosophila* and other insects.

Cell culture experiments have also been used to demonstrate the effect of orphan receptors and receptor cofactors upon ecdysteroid response, including AHR38 (Zhu *et al.*, 2000), *Aa*SVP (Zhu *et al.*, 2003a), *Dm*SVP (Zelhof *et al.*, 1995a), and DHR78 (Zelhof *et al.*, 1995b). These will be discussed in connection with their biological relevance for modulating ecdysteroid receptor-mediated activities.

3.5.3.4. Ecdysteroid-Inducible Cell Systems

The use of ecdysteroid-inducible cellular systems has emerged rapidly in recent years since ecdysteroids and nonsteroidal agonists are inexpensive and harmless to humans. Yeast assays have been developed to screen for ecdysteroid agonists of potential commercial value. When EcR by itself is transformed into yeast cells, it is capable of inducing a high level of transcription that is ligand-independent and which is not appreciably increased by the presence of USP or RXR (De la Cruz and Mak, 1997). Through modifications of the A/B domain, an inducible system was successfully produced (De la Cruz *et al.*, 2000).

A second ecdysteroid-inducible yeast system was produced by making at least two modifications. First, by removing the *Aa*EcR A/B domain, the receptor's AF1 function is reduced when transformed into yeast. Second, the addition of the mammalian receptor coactivator GRIP1 confers yeast cells with the ability to be induced more strongly by a range of ecdysteroid agonists. Conversely, the addition of the receptor corepressor SMRT represses transcription (Tran *et al.*, 2001a). A yeast inducible assay utilizing the *Cf*EcR and *Cf*USP also requires the removal of the A/B domain from both EcR and USP, along with the addition of the GRIP1 coactivator (Tran *et al.*, 2001b). In fact, the USP A/B domain often displays repressive properties or is inactive in heterologous cells, thus necessitating its deletion and/or replacement with a constitutively active N-terminal domain, such as VP16 (Tran *et al.*, 2001b; Hu *et al.*, 2003; Henrich *et al.*, 2003).

The field use of the bisacylhydrazines as insecticides has also inspired the introduction of ecdysteroid-inducible systems into plants which could be used to promote the expression of beneficial genes. Nonsteroidal agonist-inducible expression has been obtained from *Zea mays* (corn) protoplasts (Martinez *et al.*, 1999b), tobacco, and *Arabidopsis* (Padidam *et al.*, 2003) via EcR fusion proteins. This induction in *Arabidopsis* has been successfully linked to the expression of a coat protein gene from the tobacco mosaic virus (TMV), which confers resistance to TMV infection (Koo *et al.*, 2004) and also induces the expression of a factor in maize that restores fertility to a male-sterile strain (Unger *et al.*, 2002).

As noted, ecdysteroids evoke no discernible responses upon mammalian cells, and therefore, attempts to implant an ecdysteroid-inducible gene expression system into cells for therapeutical purposes has been undertaken. EcR dimerizes with endogenously expressed RXR to form a functional heterodimer that responds to biologically inert ecdysteroids or nonsteroidal agonists (Christopherson *et al.*, 1992; Palli *et al.*, 2003). By producing and testing a variety of chimeras, a heterologous receptor system has been developed in mammalian cells that evokes induction rates of almost 9000-fold, with very rapid reduction when the ecdysteroid agonist is removed (Palli *et al.*, 2003). A mouse strain carrying a transgenically introduced *Drosophila* EcR gene has been produced in which tissues are capable of responding to ecdysteroids without any other discernible phenotypes other than the expression of an *hsp27* EcRE regulated reporter gene (No *et al.*, 1996). The ecdysone switch has been shown to be responsive to several ecdysteroids and ecdysteroid agonists, and the maximal response can be further elevated by the addition of RXR activators. RXR superinduction requires that EcR be previously bound by its cognate ligand since RXR activators by themselves exert no effect upon EcR/RXR activity (Saez *et al.*, 2000). Ecdysteroid-inducible expression of medically important genes has been accomplished in human cell lines (Choi *et al.*, 2000), as well as the introduction of an ecdysteroid-inducible gene into rats via an adenovirus vector used for somatic gene therapy (Hoppe *et al.*, 2000).

3.5.4. Cellular, Developmental, and Genetic Analysis

3.5.4.1. The Salivary Gland Hierarchy: A Model for Steroid Hormone Action

Many of the insights concerning steroid hormone regulation of transcriptional activity were first recognized in the larval salivary gland of several insect species. By observing the location, timing, and size of these chromosomal puffs, the activity of an ecdysteroid-inducible gene can be inferred. The progression of events, and their relation to

developmental changes in the salivary gland and the whole animal are described here for *D. melanogaster* (see Thummel, 2002 and references therein). During the period of the third instar that precedes the wandering stage, "intermolt puffs" appear at several specific chromosomal sites. In response to the late larval ecdysteroid peak, these puffs regress and another set of "early" ecdysteroid-responsive puffs appear. The timing and duration of these "early" puffs varies (some are referred to as "early-late" puffs) and these eventually regress as another set of "late puffs" appear at other chromosomal sites. The puffing pattern accompanies the production and secretion of glue protein from the salivary gland, which is extruded as the larva becomes immobile and tanning of the larval cuticle begins (puparium formation).

Several hours later, a second smaller pulse of ecdysteroid induces a second wave of "early" puff activity, which is followed by a second wave of "late" puff activity. The two peaks evoke similar, but not identical, transcriptional changes, and these subtle differences underlie important developmental consequences. The second wave culminates in the expression of βFTZ-F1, a specific isoform of an orphan receptor encoded by the stage-specific (that is, the prepupal specific) gene puff located at the chromosomal interval, 75C. βFTZ-F1, in turn, sets off a cascade that ultimately includes the expression of another stage-specific puff, E93, involved in the histolysis of the gland (Broadus *et al*., 1999; Lee *et al*., 2002).

As expected, 20E shows the greatest potency in terms of puff induction, followed by 3-dehydro-20E, which is an endogenously produced ecdysteroid in the *Drosophila* third instar (Somme-Martin *et al*., 1988). Significantly, however, while the potency of individual ecdysteroids varies, no individual ecdysteroid evokes an unique puffing site, or alternatively, fails to evoke puffing at a site induced by other ecdysteroids. In other words, there is no evidence for multiple ecdysteroid signaling pathways in the *Drosophila* salivary gland (Richards, 1976).

3.5.4.2. Molecular and Genetic Characterization of the Puff Hierarchy

Numerous genetic and molecular studies have verified the basic tenets of the Ashburner model. Ashburner proposed that the ecdysteroid receptor directly induces the early puffs while simultaneously repressing late puff genes, and that the early puff gene products not only increase late puff transcription, but also feed back to downregulate their own expression. The use of chromosomal deletions and duplications of the region that contains E74 and E75 provides a way to alter the intracellular dosage of early puff gene products and thereby test the model. Late puffs appear earlier and are larger when the early puff genes are duplicated, consistent with the accumulation of an activating factor. On the other hand, late puff appearance is delayed in chromosomes with early puff deletions (Walker and Ashburner, 1981).

Further evidence that late puffing events are dependent upon early puffing events was shown ingeniously by testing permeabilized salivary glands 20E in combination with cellular extracts (Myohara and Okada, 1987). When ecdysteroid and a cellular extract from unstimulated salivary glands are mixed together, no response is registered from the late puff at 78C, but this puff appears when 20E, along with a cellular extract from stimulated salivary glands, is tested on permeabilized salivary glands.

Another line of inquiry has focused on demonstrating that EcR and USP interact directly with puff site regions. Immunostaining has shown specific instances in which EcR and USP colocalize to early puff sites (Yao *et al*., 1993), and that EcR and RNA polymerase II antibodies colocalize on *C. tentans* polytene chromosomes (see **Figure 1**). EcR has also been associated with late puff sites (Talbot *et al*., 1993), inferring that it directly influences the regulation of late puff genes. Further, the products of the early puff gene, E75, have been associated by immunostaining with early and late puff sites, as predicted by the Ashburner model (Hill *et al*., 1993).

The puff hierarchy actually begins before the onset of pupariation in *Drosophila* with the appearance of several intermolt puffs (see Lehman, 1995 and references therein). The *sgs4* gene encodes a salivary glue protein and is regulated by the synergistic interplay of an ecdysone receptor element, and a secretion enhancer binding protein (SEBP3) site. A second SEBP binding site has been associated with products of the early puff product, Broad, indicating that the proper regulatory interplay between the ecdysone receptor and other transcriptional factors depends upon the cellular milieu (von Kalm *et al*., 1994; Lehman and Korge, 1995). As ecdysteroid titers increase, the intermolt puffs regress.

Three early puff genes have been the subject of intensive ongoing study in *D. melanogaster* and other insects. All three genes encode transcription factors, consistent with a role in regulating late puff expression: the Broad-Complex (Br-C) encodes a zinc finger protein (DiBello *et al*., 1991), E74 is an *ets* protooncogene (Burtis *et al*., 1990), and E75 encodes an orphan receptor (Segraves and Hogness, 1990). All three possess a level of organizational

complexity that is not evident from the cytological studies alone. Br-C encodes several different isoforms derived from alternative splicing (DiBello *et al.*, 1991). Broad proteins carry a common core domain and a subset of several different variable domains (see Bayer *et al.*, 1997), and the expression of each isoform varies both spatially and temporally (e.g., Huet *et al.*, 1993; Mugat *et al.*, 2000; Brennan *et al.*, 2001; Riddiford *et al.*, 2003). The switchover of expression among combinations of Br-C isoforms has been associated with the modulatory regulation of many ecdysteroid-inducible genes including the transcription factor gene *hedgehog* (*hh*; Brennan *et al.*, 1998), micro-RNA (Sempere *et al.*, 2003), and *fbp1* (Mugat *et al.*, 2000). The E74 puff encodes two isoforms, one of which is transcribed at low ecdysteroid levels (E74B; Karim and Thummel, 1991) and disappears as titers become elevated while the transcript which specifies the E74A isoform becomes more abundant. Finally, three isoforms exist for the nuclear receptor at the E75 puff (A, B, and C; Segraves and Hogness, 1990) resulting from alternative splicing. One of these, E75B, carries only the second zinc finger within its DBD. Several other ecdysteroid responsive puff genes have also been identified which play cellular roles (see Thummel 2002, and references therein).

3.5.4.3. Developmental Regulation of Ecdysteroid Response in *D. melanogaster*

Transcript levels of EcR, the intermolt genes, and the individual isoforms of the early puff genes (BR-C, E74, E75) vary temporally among individual tissues during the late larval/prepupal period in *D. melanogaster* (Huet *et al.*, 1993). It is apparent that the relative abundance of the early puff isoforms changes continuously as ecdysteroid titers surge and decline, that the profile of "early puff" expression varies among tissues at any given time, and that the pattern changes are specific for a given tissue. For instance, EcR levels are very low throughout the larval/prepupal period in imaginal discs, whereas they remain elevated in the gut throughout this period. The relative abundance of EcR and its early puff products plays some role in the timing of subsequent gene expression, as exemplified by the Ddc (dopa decarboxylase) gene. It behaves as an "early late" gene and is induced by the combined action of EcR and Br-C, which is offset, in turn, by a repressive promoter element in epidermis and imaginal discs (Chen *et al.*, 2002b). A similar mechanism has been described for the suppression of Ddc in *M. sexta* (Hiruma *et al.*, 1995).

The complexity of *in vivo* response to ecdysteroids is also revealed by comparing the puffing response seen along the polytene chromosomes of the larval fat body during the same time that the salivary gland puff response occurs (Richards, 1982). While many aspects of the fat body response resemble those seen in the salivary gland, individual fat body puff sites are responsive to both ecdysone and 20E in ranges below 10^{-6} M, whereas salivary gland puffing is much more responsive to 20E. While one obvious explanation lies with differences in metabolism (see **Chapter 3.3**), the fat body response to ecdysone does not lag the response to 20E in the fat body, as would be expected if conversion to 20E preceded receptor-mediated response. Fat body chromosomes also evoke different puffing patterns than salivary gland chromosomes, and these presumably reflect functional differences between the two compared cell types.

Diversity of response has been seen for other ecdysteroid-inducible genes. For instance, the fat body protein-1 (*fbp1*) gene is ecdysteroid-inducible and expressed only in the fat body during the onset of metamorphosis (Maschat *et al.*, 1991). The aforementioned Eip28/29 gene is ecdysteroid-responsive but displays several different patterns of expression in individual tissues at the onset of metamorphosis (Andres and Cherbas, 1992, 1994). The complexity of *in vivo* regulation is also exemplified by the E93 gene, which falls at an endpoint in the salivary gland hierarchy because the E93 gene product plays a central role in salivary gland histolysis (Lee *et al.*, 2002). Nevertheless, its expression in other tissues is not responsive to 20E (Baehrecke and Thummel, 1995).

The *D. melanogaster* imaginal discs and ring gland are notable in that they apparently fail to respond to ecdysteroids during the larval/prepupal period, as measured with a GAL4-EcR/GAL4-USP *in vivo* system in which lacZ staining reports the level of ligand-dependent heterodimerization between the two fusion proteins (Kozlova and Thummel, 2002). The lack of responsiveness may reflect the effect of cofactors (or their absence) and/or mechanisms by which 20E is either removed from the cells via a transporter mechanisms (Hock *et al.*, 2000) or metabolized.

The same GAL4 *in vivo* reporter system indicates that EcR and USP heterodimerization occurs in response to the ecdysteroid peak during *D. melanogaster* mid-embryogenesis and that the receptor plays a role in germ band retraction and head involution (Kozlova and Thummel, 2003a). Moreover, the transcriptional relationship among ecdysteroid-regulated genes is generally maintained during the course of individual ecdysteroid peaks throughout premetamorphic development (Sullivan and

Thummel, 2003). Mutations of the early-late gene, DHR3, cause embryonic lethality, further implicating not only EcR but the ecdysteroid hierarchy itself (Carney *et al.*, 1997).

Of course, the existence of functionally distinct EcR isoforms provides an important basis for differential ecdysteroid responses. During the late larval stage, the EcRA isoform of *D. melanogaster* is generally predominant in imaginal discs, whereas the B isoforms are associated primarily with larval tissues that undergo histolysis during metamorphosis (Talbot *et al.*, 1993). Issues surrounding the diversity of isoform function will be discussed further in Section 3.5.4.5.

3.5.4.4. Conservation of Ecdysteroid Response Among Insects

The isolation of EcR and USP from other insects and the assembly of their developmental profiles now allows direct analysis of ecdysteroid action in other organisms. So far, these studies show general consistency with those obtained in *D. melanogaster*, specifically, EcR expression varies over time and peak levels of expression coincide with ecdysteroid peaks, and different isoforms predominate among different tissues. For instance, the expression patterns of the two EcR isoforms in *M. sexta* vary among cell types (Jindra *et al.*, 1996) and expression levels of the two USP isoforms switch in conjunction with molts (Jindra *et al.*, 1997), providing a regulatory framework for the early-late response of MHR3 discussed earlier (Lan *et al.*, 1999).

The recovery of ecdysteroid-inducible target genes such as E74, E75, FTZF1, and HR3 orthologs also allows a comparison of the ecdysteroid-inducible cascade in other organisms, and the resulting proteins play biological roles in a variety of regulatory processes that is too extensive to discuss here. The comparative analysis over preadult development is most complete in *M. sexta*, and the essential features of the ecdysteroid-inducible hierarchy are maintained (Riddiford *et al.*, 2003, and references therein). Similarly, the players and timing of ecdysteroid-responsive genes during *B. mori* choriogenesis are conserved (Swevers and Iatrou, 2003), as they are in *A. aegypti* vitellogenesis (Zhu *et al.*, 2003a) (see **Chapter 3.9**). Interestingly, however, USP's other partners, DHR38 and SVP, have not been associated with whole body responses in any organism. In flies, SVP is involved in eye differentiation (Mlodzik *et al.*, 1990) and DHR38 is involved in cuticle formation (Fisk and Thummel, 1998) at the onset of metamorphosis; both players are involved in mosquito vitellogenesis (Zhu *et al.*, 2003a) (see **Chapter 3.9**).

At least two points emerge from these considerations. First, while the early response at the core of the ecdysteroid hierarchy appears to be highly conserved among insects at the global level, local ecdysteroid responses likely recruit specific players to mediate specific developmental events. Second, combinations of the EcR/SVP/DHR38/USP orphan receptor group might be incorporated into various ecdysteroid-regulated processes for the purpose of regulating a linear series of events such as mosquito vitellogenesis and fly eye differentiation. One intriguing aspect of this orphan group is that the broad spectrum response of DHR38/USP might be important for regulating responses via the ecdysone precursor to 20E and via 20E metabolites occurring after 20E exerts its effects, with SVP providing activation of an alternative pathway by competing with the 20E-inducible response itself. The context of EcR action will be interesting to explore in other insect processes, such as the color pattern formation of butterfly wings (Koch *et al.*, 2003) and insect diapause (see **Chapter 3.12**).

Historically, numerous studies of puffing on polytene chromosomes preceded and have continued along with the work on *D. melanogaster*. The salivary gland response of *Sciara coprophila* (fungal fly) provides an interesting comparison to *D. melanogaster*, and illustrates the importance of investigating the features of each system without pretense. The *S. coprophila* II/9A puff appearing in response to ecdysteroid treatment reflects the initiation of both bi-directional DNA replication (Liang *et al.*, 1993) and the elevated transcription of two similar mRNA species which bear no resemblance to ecdysteroid-responsive genes found in *Drosophila* (DiBartolomeis and Gerbi, 1989). When the promoter region of the *S. coprophila* II/9-1 gene is transgenically introduced into *D. melanogaster*, it is transcriptionally responsive to ecdysteroids in the salivary gland, indicating that the ecdysteroid-inducible transcriptional machinery is conserved evolutionarily between the two species (Bienz-Tadmor *et al.*, 1991). However, there is no evidence that the DNA amplification is induced by 20E in flies or mediated by the ecdysteroid receptor.

In *Trichosia pubescens*, Amabis and Amabis (1984) found that ecdysteroid treatment of salivary glands generates both DNA and RNA "early" puffs, whose subsequent regression is accompanied by the appearance of other "late" DNA amplification and RNA puffs. Cycloheximide treatments after early puff appearance block late puff expansion. In this species, large puffs appear after considerable delay, and the appearance of early puffs is not obvious, suggestive of a single broad ecdysteroid peak, rather

than the two peaks that occur in *D. melanogaster* over the same developmental timespan. In this respect, the response pattern is comparable to *C. tentans*, which in turn relies upon an interplay of ecdysone and 20E to achieve ecdysteroid-induced regulation (Stocker and Pavan, 1977).

In yet another sciarid fly, *Bradysia hygida*, 20E induces DNA amplification and transcription of a temporally "late" puff gene (BhC4-1). When this gene is transgenically introduced into *D. melanogaster*, it retains its "late" puff characteristics in terms of salivary gland timing, but transcription of the gene is not blocked by repression of protein translation with cycloheximide, suggesting that the late induction is actually a form of derepression in the *D. melanogaster* salivary gland (Basso *et al*., 2002). This interpretation is also based on the observation that 20E exerts both repressive and inductive effects upon the protein composition of the *B. hygida* salivary gland during the fourth and final larval stage (de Carvalho *et al*., 2000).

As evidenced by the cases presented here, beyond the superficial similarity, there are numerous unsolved mysteries about the integration of ecdysteroid response into biological processes, especially when one considers that the most complete hierarchical comparisons so far involve only lepidopteran and dipteran species. Several ecdysteroid-inducible genes have been identified for the coleopteran, *T. molitor* (Mouillet *et al*., 1999), at least indicating that several of the orphan receptors involved in mediating ecdysteroid responses exist in more primitive insects. Further, the existence of an E78 ortholog in the filarial parasite, *D. immitis*, implies that at least portions of the hierarchy are conserved in other invertebrates (Crossgrove *et al*., 2002).

3.5.4.5. *In Vivo* Molecular and Genetic Analysis of EcR and USP

The pattern of EcR gene transcription during premetamorphic development fluctuates dramatically, with high levels accompanying each of the embryonic and larval molts, followed by peaks of expression during the prepupal and pupal-adult stages of metamorphosis (Koelle *et al*., 1991). Moreover, the EcR gene encodes three different isoforms with the A isoform's transcription governed by a promoter and two other isoforms (B1 and B2) generated by alternative splicing via a second promoter (Talbot *et al*., 1993). All three isoforms share a common sequence that includes a small portion of the A/B domain and the other domains, but the amino-terminal side of the A/B region for each isoform is unique. The A isoform predominates in fly tissues which undergo morphogenetic changes during metamorphosis, including the imaginal discs, the ring gland, the imaginal rings of the foregut, hindgut, and salivary gland (Talbot *et al*., 1993), and in a heterogeneous subset of neurons that degenerate after adult emergence (Truman *et al*., 1994) (see **Chapter 2.4**), whereas the B1 isoform is found in larval tissues including the salivary gland, the fat body, larval muscle (Talbot *et al*., 1993), and proliferative neurons (Truman *et al*., 1994).

Functional studies of EcR based on the isolation of mutations within the *D. melanogaster* gene have led to further insights about EcR's developmental role, and essentially confirmed its ecdysteroid-mediating function. As might be expected, null mutations of a common region in EcR, and thereby disrupt all three isoforms and result in embryonic lethality, whereas mutations that retain a reduced ability to mediate ecdysteroid responses allow mutant survival through some or all of the larval stages. Some of these weaker mutations affect nonconserved residues, but at least one of these prepupal lethal mutations involves a highly conserved phenylalanine in the DBD; conversely, there are nonconserved residues that cause embryonic lethality when substituted. One of the lethal mutations (A483T) replaces the amino acid residue that interacts with the SMRTER corepressor and is a conditional third instar lethal mutation. Presumably, the lability of the mutant protein disrupts the normal interaction between EcR and SMRTER at higher temperatures (29 °C). The A483T mutation (along with other EcR mutations; **Table 2**) also disrupts adult female fecundity at its restrictive temperature, indicating that the corepressor interaction is essential for at least late larval development and oogenesis (Bender *et al*., 1997; Carney and Bender, 2000). B-specific mutations cause early larval lethality (Schubiger *et al*., 1998); an A-specific EcR mutation reduces but does not eliminate mutant survival to the adult stage and disrupts the normal expression of EcRB1 (D'Avino and Thummel, 2000; Schubiger *et al*., 2003) (see **Chapter 2.4**). Other A-specific mutations cause pupal lethality (Carney *et al*., 2004). A gain-of-function mutation of *Dm*EcR (K497A) that dimerizes in the absence of hormone, possibly because of disrupted corepressor binding, has also been identified in cell cultures, though its potential *in vivo* effect is unknown (Bergman *et al*., 2004).

Over developmental time, the B2 isoform, when expressed under the control of a heat shock promoter, rescues larval development in EcR-null mutants, though heat shocks are required in each instar to accomplish it. The A and B1 isoforms rescue development through the first instar, but fail thereafter, suggesting that a common function is required

Table 2 A selected list of EcR mutations and their functional consequences. Mutations correspond to shaded residues in **Figure 4**

Mutation	*Species*	*Effect*
A393P	*C. fumiferana*	Destroys 20E response, but not nonsteroidal agonist response (Kumar *et al.*, 2002; Billas *et al.*, 2003). Impaired coactivator (GRIP1) interaction (*Cf*EcR only; Kumar *et al.*, 2002)
A398P	*H. virescens*	
A522P	*D. melanogaster*	
A534P	*A. aegypti*	
A559P	*B. mori*	
Y403A	*H. virescens*	Reduces nonsteroidal agonist response, but not 20E response (Billas *et al.*, 2003)
A483T	*D. melanogaster*	Conditional late larval lethal mutation; disrupts oogenesis (Li and Bender, 2000); binding site for SMRTER corepressor (Tsai *et al.*, 1999)
K497A, E	*D. melanogaster*	Constitutive transcriptional activity in cell culture (Bergman *et al.*, 2004)
Y611F	*D. melanogaster*	Elevated binding to *hsp27* EcRE in presence of ecdysone (Wang *et al.*, 2000)
F645A	*D. melanogaster*	Dimerizes normally but is not 20E inducible; dominant negative lethal mutation *in vivo* (Cherbas *et al.*, 2003)
W650A	*D. melanogaster*	No dimerization (Cherbas *et al.*, 2003)

during the first instar and more differentiated EcR functions are essential later. The rescued B2 transformants become sluggish during the wandering stage of the late third instar, then immobile, and eventually die (Li and Bender, 2000).

Isoform-specific mutations in conjunction with transformation rescue have further delineated EcR-based functions in fly development. As expected, an EcRB1-specific mutation disrupts developmental activities in those cells where it is expressed. For instance, the salivary gland's ecdysteroid-induced puffing is disrupted in B1 mutants (but not eliminated), and only transformation with EcRB1 restores normal puffing of various early and early-late genes, though B2 exerts a partial rescue. Similarly, B1-expressing abdominal histoblasts and midgut cells develop abnormally (Bender *et al.*, 1997). Neuronal remodeling during metamorphosis is disrupted in genetic mosaics that do not express either of the two B isoforms in proliferating neurons (Schubiger *et al.*, 1998) (see **Chapter 2.4**), but remodeling is rescued by the expression of either B isoform within these cells (Schubiger *et al.*, 2003). Remodeling is not disrupted in mutations of the early puff genes, indicating that this aspect of the cascade is not specifically tied to the failure of B isoform mutant effects (Lee *et al.*, 2000).

A related strategy for discriminating EcR functions involves introducing an isoform or fusion protein transgenically into flies, expressing the transgene ectopically, and then observing the dominant negative phenotypic consequences of such expression. At least three variations have been reported: (1) expression of a wild-type isoform ectopically under the control of an UAS promoter regulated by the yeast GAL4 transcription factor (Schubiger *et al.*, 2003), (2) the expression of a GAL4-EcR LBD fusion protein that forms an inactive dimer with cellular USP in a nonisoform specific manner (Kozlova and Thummel, 2002, 2003a), and (3) UAS-controlled expression of a dominant negative EcRB1 isoform (F645A in *D. melanogaster*) that forms an inactive dimer with intracellular USP, and is then tested for rescuability by the concomitant expression of a specific isoform (Cherbas *et al.*, 2003). In the case of wild-type overexpression, each isoform generates a unique pattern of phenotypes. Overexpression of EcRA suppresses posterior puparial tanning, and affects ecdysteroid-inducible gene expression in posterior compartments of the wing disc but does not affect viability. By contrast, overexpression of EcRB1 and B2 during puparial formation greatly reduces viability (Schubiger *et al.*, 2003) (see **Chapter 2.4**). Ectopic expression of a GAL4-EcR during the late third instar causes a failure of puparial contraction and cuticular tanning that resembles the traits displayed by mutant EcR larvae (Kozlova and Thummel, 2002). The isoform-specific rescue of the EcR^{F645A} mutants, however, reveals a poor correlation between tissue-specific effects and intracellular titers of each isoform, though the accumulation of disrupted responses in several individual tissues leads to a stage-specific developmental arrest in the third instar (Cherbas *et al.*, 2003).

Further insights about the functional differences associated with the *D. melanogaster* EcR isoforms will likely emerge by understanding the basis for isoform-specific mRNA transcription. Two promoters, one associated with the A isoform, and the other associated with the two B isoforms, regulate the appearance of these transcripts. Promoter segments responsible for high levels of EcRA expression during metamorphosis have been identified (Sung and Robinow, 2000). While the level of EcRA detected is homogeneous among those neurons which express

EcRA during metamorphosis, the underlying promoter regulation is surprisingly heterogeneous among them, indicating that the observed pattern of expression obscures an underlying regulatory complexity. A TGF-β/activin signaling pathway has been implicated in the regulation of EcRB1 transcription. In activin mutants, EcRB1 transcription is reduced and neurons in the mushroom bodies of the brain fail to undergo remodeling during metamorphosis. The expression of EcRB1 rescues the remodeling defects, indicating that EcRB1 levels are regulated through the activin signaling pathway (Zheng *et al*., 2003).

By comparison with EcR's complexity, the regulation of the *usp* gene in *D. melanogaster* is relatively simple, with no introns and no alternative splicing forms, though the profile is more complex in species with multiple USP isoforms (Jindra *et al*., 1997; Vogtli *et al*., 1999). In flies, the expression of USP through development is relatively stable, though it is unclear whether EcR or USP is the rate-limiting partner in developing tissues (Henrich *et al*., 1994). The *usp* gene is defined by several recessive early larval lethal mutations: three missense substitutions (usp^3, usp^4, and usp^5) that mutate amino acid residues in the USP DBD and directly contact phosphate residues in the DNA backbone, along with a nonsense mutation, usp^2, that truncates the DBD (Oro *et al*., 1990; Henrich *et al*., 1994; Lee *et al*., 2000). The null-usp^2 allele evokes a different effect on gene expression than the other *usp* alleles. Whereas all the mutations disrupt the normal repression of the BrC-Z1 isoform, only USP2 is incapable of activating BrC-Z1 transcription. USP3 and USP4 are also able to mediate ecdysteroid-induced gene transcription through an *hsp27* EcRE-regulated promoter (Ghbeish *et al*., 2001).

This dual capability has been analyzed *in vivo* during ommatidial assembly and differentiation in the eye disc. Briefly, the differentiation of the eight retinula cells in each of the 700 or so ommatidia that become the compound eye occurs through the recruitment of undifferentiated cells as a wave moves from the posterior to anterior end of the eye disc. Cells along this progressing wave, the morphogenetic furrow, undergo a host of transcriptional changes and some aspects of the process are ecdysteroid-dependent *in vitro*. Furrow advancement accelerates in mutant *usp* patches on the eye disc (Zelhof *et al*., 1997), whereas an ecdysteroid deficit retards its advancement (Brennan *et al*., 1998). The apparently paradoxical action results from the failure of a repressive *usp* function as evidenced by the abnormal appearance of Br-C Z1 expression in mutant patches lying along the front of the moving furrow. The usp^2 allele causes an absence of Z1 behind the furrow, where it is normally present, further demonstrating the dual roles. USP has been similarly implicated in both wing margin bristle differentation (Ghbeish and McKeown, 2002), and the repression of premature neuronal differentiation in the wing imaginal discs (Schubiger and Truman, 2000) (see **Chapter 2.4**). The maintenance of USP3 and USP4 activation functions likely explains the normal appearance of mutant *usp* imaginal clones (Oro *et al*., 1992).

Maternal contribution of normal *usp* transcript is essential for the completion of embryogenesis (Oro *et al*., 1992). Mutant *usp* larvae are rescued through the third instar with a USP connected to a heat shock promoter. The lethal phenotype of these partially rescued larvae is reminiscent of the effects seen with larval EcR mutations and internal morphology is similar at the time of larval arrest. Only the *usp* mutants, however, develop a supernumerary larval cuticle and fail to wander off the food (Hall and Thummel, 1998; Li and Bender, 2000). DHR38 mutants also undergo abnormal cuticle apolysis (Kozlova *et al*., 1998), suggesting that the DHR38/USP dimer may be essential for this aspect of premetamorphic development, rather than the EcR/USP dimer.

It is beyond the realm of this chapter to explore the effects of ecdysteroid-inducible genes in depth and the reader is directed to other reviews (Henrich *et al*., 1999; Riddiford *et al*., 2000; Thummel, 2002). Isoform-specific mutational analysis of ecdysteroid-responsive genes has generally confirmed essential roles at metamorphosis with the two isoforms of E74 (Fletcher *et al*., 1995), two of the E75 isoforms (Bialecki *et al*., 2002), DHR3 (Lam *et al*., 1999), and the *β*FTZF1 isoform (Broadus *et al*., 1999). The complex genetic complementation of several Br-C mutations (Kiss *et al*., 1988) has been associated with each of four isoforms (Z1–Z4; Bayer *et al*., 1997). The *npr1* (nonpupariation) third instar lethal mutations of Br-C fail to complement all other alleles by impairing a common and essential premetamorphic function. Some Br-C mutations affect the gene's own expression (Gonzy *et al*., 2002), consistent with predictions of the Ashburner model. As noted elsewhere, Br-C isoforms play a variety of inductive and repressive roles, often through switchover of isoform expression, in a large spectrum of premetamorphic processes.

3.5.4.6. Ecdysteroid Receptor Cofactors

As noted earlier, the ecdysteroid receptor is part of a complex of proteins which affect both its inductive

and repressive transcriptional functions. In the case of Hsc70, a chaperone that physically interacts with *Dm*EcR, this functional importance is demonstrated by the genetic interaction between EcR and Hsc70 mutations (*hsc4* gene) in *D. melanogaster*. Trans-heterozygotes for these two mutations develop severely blistered wings and bent legs typically associated with the mutational impairment of EcR function (Bender *et al*., 1997; Arbeitman and Hogness, 2000).

Based on findings from experiments with vertebrate nuclear receptors (see **Chapter 3.6**), transcriptional cofactors and their roles have begun to be recognized and explored; the process of eye ommatidial differentiation during the third instar of *D. melanogaster* that was described earlier has become the focus of efforts to understand the role of cofactors in development. The dominant negative EcR^{F645A} mutation severely disrupts eye development (Cherbas *et al*., 2003; Sedkov *et al*., 2003), and USP function is also required for normal furrow progression (Zelhof *et al*., 1997).

One cofactor implicated in the process is the product of the trithorax-related (*trr*) gene, a histone methyltransferase that conceivably plays a role in remodeling the chromatin in promoter regions, just as vertebrate factors do to facilitate receptor-mediated transcription (Stallcup, 2001). The role of TRR as an ecdysone-dependent coactivator is evidenced by the fact that it is recovered as part of a complex that includes EcR and a trimethylated form of the histone-3 protein (presumably modified by TRR) from the ecdysteroid inducible promoter of the *Drosophila hedgehog (hh)* gene in extracts derived from 20E challenged S2 cells. The methylation is reduced in complexes taken from *trr* mutant embryos. Further, the trans-heterozygotic combination of a *trr* mutation and the EcR^{F645A} mutation causes almost complete lethality, revealing an essential *in vivo* interaction. The aforementioned Hedgehog (Hh) protein regulates the progression of eye cell differentiation, and *trr*-mutant somatic cell clones express Hh at reduced levels. It follows that TRR is normally a coactivator associated with elevated ecdysteroid-inducible activity leading to higher Hh levels.

EcR retains its repressive capabilities in the absence of its inducible activity, and another cofactor, the corepressor SMRTER, has also been implicated in the regulation of Hh expression and eye differentiation. The lethal and mutant phenotypic effects of the dominant negative EcR^{F645A} mutation are reversed by a hypomorphic mutation of the *Smr* corepressor (i.e., a partial loss of repression), just as EcR mutant effects are overcome by increasing TRR coactivator activity (Sedkov *et al*., 2003).

Both TRR and SMRTER carry the LXXLL amino acid motifs (L refers to leucine and X refers to any amino acid) associated with nuclear receptor interactions, and physical interaction sites with EcR have been mapped in both cases (Tsai *et al*., 1999; Sedkov *et al*., 2003). Moreover, the SMRTER interactive site in *Dm*EcR has been mapped to an amino acid residue in helix 5 that when mutated (A483T) results in conditional larval lethality (Bender *et al*., 1997; Tsai *et al*., 1999). Another *Drosophila* coactivator, Taiman (TAI), was first identified in a screen for mutations that disrupt oogenesis. TAI resembles a human ortholog (AIB1) belonging to the p160 steroid receptor coactivator family (SRC), whose members are typified by a basic helix-loop-helix (bHLH) domain, a PAS domain of unknown function, several LXXLL motifs associated with nuclear receptor interaction, and several glutamine-rich stretches. These proteins form a bridge between hormone receptors, chromatin modifying enzymes such as the histone acetyl transferases, and the transcriptional machinery. TAI expression colocalizes with EcR and USP in the border cells of the ovary and colocalizes with USP on the polytene chromosomes of *Drosophila* larval salivary glands. TAI also elevates ecdysteroid-inducible transcription in a cell culture and coprecipitates with EcR, but not USP (Bai *et al*., 2000). Its role in premetamorphic processes has not yet been elucidated.

Still another EcR-interacting protein containing the LXXLL motif, *rigor mortis* (*rig*), is required for ecdysteroid signaling during larval development. Mutant *rig* larvae fail to survive beyond the advent of metamorphosis, while displaying phenotypes resembling other mutations defective in ecdysteroid synthesis or response. Rig is required as a coactivator for induction of the E74A isoform which normally appears as ecdysteroid titers increase, but is not required for E75A, EcR, or USP transcription; the effect on transcription is likely to be indirect since Rig contains no DNA-binding motifs. The protein interacts physically with EcR and USP, and also with the orphan receptors DHR3, βFTZ-F1, and SVP. Even when helix 12 is deleted from βFTZ-F1, its interaction with Rig is detectable, suggesting that the relationship between Rig and nuclear receptors is ligand-independent (Gates *et al*., 2004). A *D. melanogaster* corepressor, Alien, that is highly conserved phylogenetically, also interacts with several receptors, including EcR, SVP, and βFTZ-F1 (but not DHR3, DHR38, DHR78, or DHR96; Dressel *et al*., 1999). No mutations of Alien have been reported so far.

The pattern of receptor interactions seen with TAI, Rig, and Alien suggests that a level of regulation

remains to be elucidated in connection with the ecdysteroid hierarchy. This is further highlighted by the effects of another cofactor, Bonus (Bon), which belongs to a class of proteins (TIF1) that do not bind to DNA directly, but which repress transcriptional activity. Homozygous *bon* mutants display many of the defective *β*FTZ-F1 phenotypes noted earlier (Broadus *et al.*, 1999), and Bon physically interacts with helix 12 of *β*FTZ-F1 via an LXXLL motif. In some *bon* mutants, transcript levels of EcR B1, E74A and B, and BR-C are reduced, but DHR3 transcript levels are elevated (Beckstead *et al.*, 2001). Non-DNA binding cofactors, MBF1 and MBF2, associated with *Bombyx* *β*FTZ-F1 activity have also been identified which interact with the TATA binding protein to induce transcription (Li *et al.*, 1994).

Finally, it is notable that the methoprene-resistant (Met) mutation in *D. melanogaster* defines a gene which specifies another bHLH-PAS transcription factor (Ashok *et al.*, 1998) (see **Chapters 3.7** and **3.9**). Cellular extracts from flies homozygous for Met mutations show little binding to the juvenile hormone analog methoprene (Shemshidini and Wilson, 1990). The implications of this identity and its significance for explaining the modulatory effects of JH on ecdysteroid-inducible transcriptional activity will be addressed later.

3.5.4.7. Orphan Receptor Interactions with EcR and USP

In addition to those players which have been tied directly to the transcriptional response elicited by the EcR/USP heterodimer (DHR3, E75, E78, *β*FTZF1), several other nuclear receptors also modulate receptor activity by either dimerizing directly with USP, or by competing for EcR/USP promoter sites.

The DHR38/USP interaction is particularly interesting because *in vitro* results suggest that it is this "receptor" complex that responds to a broad spectrum of ecdysteroids, including many known to exist in *D. melanogaster* larvae. Further, DHR38 mutations disrupt cuticle formation, as do *usp* mutations, but EcR mutations do not impair this aspect of development, leaving open the possibility that this aspect of ecdysteroid regulation does not involve EcR at all (Hall and Thummel, 1998; Kozlova *et al.*, 1998). DHR38 is broadly expressed but transcript levels appear to be in low abundance (Sullivan and Thummel, 2003), though the DHR38/USP heterodimer is more responsive to 20E than EcR/USP (Baker *et al.*, 2003). In any case, the mechanism of action is novel, since the transcriptional activation via DHR38/USP is not associated with a physical ligand interaction.

A similar dimer between AHR38 and *Aa*USP has been noted prior to vitellogenesis in *A. aegypti*. The nonresponsive heterodimer is displaced by the EcR/USP heterodimer in response to a 20E titer peak following a bloodmeal (Zhu *et al.*, 2000) (see **Chapter 3.9**). As noted earlier, USP also dimerizes with SevenUp (SVP), the aforementioned ortholog of COUP-TF. As the 20E peak that stimulates *A. aegypti* vitellogenin expression ensues, a similar competition between EcR and SVP for USP as a heterodimeric partner leads to a downregulation of *A. aegypti* vitellogenin gene transcription after egg-laying (Zhu *et al.*, 2003a), illustrating a mechanism by which an ecdysteroid response can be terminated.

Ectopic expression of the svp^+ gene in flies causes lethality, but this effect can be rescued by simultaneous ectopic expression of usp^+ (Zelhof *et al.*, 1995a). This interaction apparently is a relevant aspect of photoreceptor differentiation, since both SVP and USP function are essential for this process to occur normally (Mlodzik *et al.*, 1990; Zelhof *et al.*, 1997). The potential relevance of the SVP/USP interaction has also been established by demonstrating that SVP competes with EcR for USP's partnership, and thereby reduces ecdysteroid-induced transcription in cell cultures. Further, the functional SVP/USP dimer preferentially interacts with DR1 DNA elements, whereas the EcR/USP dimer interacts with the Eip28/29 element.

Still another orphan receptor that is essential for normal metamorphosis in flies is DHR78. Mutations of this receptor cause late larval lethality, disruptions of the tracheal system, and the impairment of EcR, E74B, and BR-C transcription. DHR78 binds to numerous ecdysteroid inducible sites in salivary glands (Fisk and Thummel, 1998; Astle *et al.*, 2003). DHR78 inhibits 20E-dependent induction of transcription in cell culture assays (Zelhof *et al.*, 1995b) and it has been proposed that DHR78 activity may interact with an "upstream" ligand in the period that precedes the late third instar ecdysteroid peak to prime a later ecdysteroid response (Fisk and Thummel, 1998). A *Bombyx* ortholog, BHR78, has been shown to dimerize directly with BmUSP (Hirai *et al.*, 2002) suggesting that DHR78 may block 20E-dependent transcription by competing for USP with EcR.

3.5.4.8. Ecdysteroid Action and Other Developmental Processes

The ecdysteroid response is highly heterogeneous among cell types, owing to differences in the

quantitative levels of EcR and USP isoforms, rates of 20E conversion from ecdysone, 20E metabolism and cellular exclusion, and yet it is evident that the circulation of ecdysteroids in the insect hemolymph provides the organism with a means to coordinate its individual developmental programs. Therefore, it is expected that ecdysteroids set off general responses and also specific responses that involve either EcR and USP or targets that are regulated by them.

For organismal investigations, the dramatic changes associated with the late larval ecdysteroid peak in *D. melanogaster* evoke substantial changes in transcript levels which can be detected using genomic approaches. As already noted, a combination of standard technology and a genomic outlook has motivated the assembly of a detailed profile of orphan receptor transcript levels through preadult development (Sullivan and Thummel, 2003). A limitation of the whole body approach is evident in the case of DHR38, which is widely expressed but at such low levels that the transcript is barely evident on Northern blots. An earlier study using subtractive hybridization led to the characterization of several ecdysteroid-inducible genes, most of which were unknown and not associated with ecdysteroid-inducible events directly (Hurban and Thummel, 1993). Screens of hematopoietic Kc cells (Savakis *et al.*, 1980) and imaginal discs (Apple and Fristrom, 1991) have yielded several ecdysteroid-dependent genes which do not overlap with those recovered from other screens, and these transcripts are largely associated with tissue-specific responses. However, while the timing of early ecdysteroid gene expression is delayed in the discs (Huet *et al.*, 1993), at least one ecdysteroid-responsive pupal cuticle gene, EDG84A, is regulated by FTZ-F1, which as noted, also regulates metamorphic processes in the salivary gland (Murata *et al.*, 1996).

Microarray analysis has been used more recently to investigate the changes in transcription on a genome-wide basis, using the onset of the white prepupal (puparial) stage as a reference point. The early puff genes change in a predictable, ecdysteroid-regulated manner, and genes involved in processes such as myogenesis, apoptosis, and imaginal disc differentiation are upregulated at appropriate times during the period. As ecdysteroid levels peak, transcription of most genes encoding glycolytic enzymes are substantially reduced, as are genes whose enzymatic products regulate the citric acid and fatty acid cycles, oxidative phosphorylation, and amino acid metabolism (White *et al.*, 1999). Adult flies heterozygous for a lethal EcR mutation survive longer than wild-type flies and show greater resistance to oxidative stress, heat, and dry conditions, though activity levels are normal and a possible connection to metabolic function has not been made (Simon *et al.*, 2003).

Important mechanistic activities at the cellular level, however, will continue to require careful functional analysis. In the case of eye differentiation in flies, morphogenetic furrow movement during the late third instar is dependent on 20E, but not on normal EcR gene activity (Brennan *et al.*, 2001). The repressive action of USP described earlier for this process (Ghbeish and McKeown, 2002) is offset by the positive regulation of Hedgehog (Hh) transcription that involves EcR (Sedkov *et al.*, 2003). Hh, in turn, facilitates a switchover from the Z2 to the Z1 isoform at the ecdysteroid-responsive BR-C gene locus. The temporal coordination of hormonal signaling with the regulatory activities along the furrow, therefore, provide a balance of signals that both stimulates morphogenetic furrow movement (in the form of EcR-mediated Hh expression) and regulates its rate of progression (in the form of a repressive USP function). The mechanism of furrow movement is not fully elucidated, and it is conceivable that a second hormonal signaling pathway is involved in the process.

The interaction between JH and ecdysteroid action remains elusive, despite the clear recognition that there must be cross-talk between these pathways. The JH analog methoprene induces several disruptions of the central nervous system that resemble those caused by mutations of the BR-C gene. These defects are not seen in flies homozygous for the methoprene-resistant (MET) mutation (Restifo and Wilson, 1998).

3.5.4.9. Juvenile Hormone Effects upon Ecdysteroid Action

A central tenet of insect biology is that ecdysteroids mediate larval-larval molts in the presence of the sesquiterpenoid, JH (see **Chapter 3.7**). In the absence of JH, by contrast, ecdysteroid induces a larval-pupal transition. Implicit in this view is the recognition that JH via its receptor competes with the EcR/USP dimer at an EcRE (Berger *et al.*, 1992) or modifies components of the ecdysteroid receptor itself (Jones *et al.*, 2001; Henrich *et al.*, 2003). Nevertheless, no uniform explanation for the action of JH, alone or in conjunction with ecdysteroids, has been forthcoming.

The structural similarity of 9-*cis* retinoic acid, the cognate ligand of vertebrate RXR, with JH inspired experiments to test the possibility that the insect RXR ortholog, USP, is the JH receptor, and like RXR, switches between a homodimeric and heterodimeric state to process incoming hormonal

signals. This enticing possibility is further bolstered by the observation that RXR is inducible by binding to the acid metabolite of the JH analog, methoprene (Harmon *et al*., 1995). Jones and Sharp (1997) reported that a *Drosophila* USP fusion protein binds to JH forms found in flies, as measured by the dose-dependent suppression by JH3 and JH3-bis epoxide on the fluorescence of tryptophan residues in the ligand binding domain of USP. Later studies have demonstrated that purified USP fusion proteins exist in homodimeric and homotetrameric forms which show a saturable ability to bind to JH3, with about 50% binding at 4 μM, while the closely related farnesol exerts no effect. Using nuclear extracts from the lepidopteran Sf9 cell line, it was further shown that USP binds specifically as a homodimer to a direct repeat response element separated by 12 nucleotides (DR12). Further, JH3 strongly induces a reporter gene in which the DR12 element is attached to the core promoter of the gene encoding the *Trichoplisia ni* JH esterase (Jones *et al*., 2001). A DR4 element in the *C. fumiferana* JH esterase gene promoter is induced by JH1 and suppressed by 20E, suggesting that an interaction between these hormones occurs at the DNA level (Kethidi *et al*., 2004). The biochemical examination of these promoters, along with those of JH-inducible genes found recently in cell cultures through microarray analysis, will hopefully bring a convergence of views on JH action (Dubrovsky *et al*., 2000).

Along a different line of reasoning, experiments have been undertaken based on the sequence similarity between the EcR LBD and the vertebrate FXR LBD, which surprisingly, is responsive to JH3 in the 10–50 μM range with an RXR dimer partner in mammalian cell cultures (Forman *et al*., 1995). When tested in this regime, JH3 potentiates the ability of the EcR/USP complex to induce a weak transcriptional induction via an *hsp27* EcRE in the presence of submaximal muristerone A levels. The potentiation apparently requires prior binding of EcR to ecdysteroids and there is no evidence yet that JH3 is binding with USP in this assay, and no response is noted with EcR/USP to bile acid, the most potent FXR activator. When tested with 20E, only EcRB2 among the three *Drosophila* isoforms is additionally potentiated with JH3 (Henrich *et al*., 2003), and, as noted, the B2 isoform is the only one capable of rescuing larval development in EcR mutants (Li and Bender, 2000). The potentiation is not analogous to the responsiveness of an EcR/RXR heterodimer described earlier (Saez *et al*., 2000), and the low ecdysteroid levels which accompany JH titers during larval development suggests its possible biological relevance. In both *Drosophila* and *Manduca*, one of the isoforms of Broad (Z1) is ectopically induced in cuticle by the application of JH analogs, further supporting the possibility that JH acts directly upon the ecdysteroid receptor's transcriptional capability (Zhou and Riddiford, 2002), since the BR-C gene is a direct target of the receptor in both *Drosophila* and *Manduca*.

In reality, differences such as the promoter and receptor constructs among the experimental regimes leave open the possibility that USP acts by multiple mechanisms, particularly if one considers the possibility that posttranslational modifications such as phosphorylation also can modify receptor activity. Possibly distinct roles of USP during larval and metamorphic development are evidenced *in vivo* by the fact that a chimeric USP transgene (in which the *Drosophila* USP LBD is replaced by the equivalent domain from *C. tentans*) completely rescues larval development in *usp* mutants that otherwise die in the first instar. Such genetically rescued larvae, however, suddenly die as the onset of the prepupal stage approaches (Henrich *et al*., 2000). Another potentially important factor is the protein defined by the Methoprene-tolerant (Met) mutation that belongs to the bHLH-PAS family of transcription factors, and includes several cofactors known to interact with nuclear receptors (Ashok and Wilson, 1998). The effects of Met upon the ecdysteroid receptor await further experimentation in *Drosophila* and other insects.

JH3 plays an essential role for the translation of *β*FTZ-F1 from existing transcripts in the fat body of newly emerged *A. aegypti* females. In turn, FTZF1 activity is required for the subsequent transcription of the mosquito "early puff" genes regulating vitellogenesis (Zhu *et al*., 2003b) (see **Chapter 3.9**). While this may be seen as another mode of action, the effect on *β*FTZF1 translation may be indirect, that is, JH3 may regulate the transcription of genes whose products play a role in mRNA stability and/or protein translation.

In summary, the diverse functional effects of JH upon ecdysteroid-inducible gene expression have elicited several possible mechanisms from investigators over the years. As it becomes increasingly apparent that EcR and USP are the two most recognizable components of a protein complex of diverse functional capability, it seems highly plausible that there is not a single JH receptor, but rather, a JH receptive protein complex (or complexes).

3.5.5. Prognosis

An explosion of information about the ecdysteroid receptor has occurred recently which will

undoubtedly influence the field far into the future. There are three levels of progress that will be central to the development of the field: (1) continued progress on understanding the biological action of ecdysteroids using functional genomics and genetics in *D.melanogaster*, (2) the adaptation of transgenic and genomic methods into receptor studies of other insects, and (3) continued modeling of insect receptor complexes along with tests of these models through both *in vitro* and *in vivo* experimentation.

The emergence of clever new methodologies, notably the *in vivo* lacZ reporter system reported by Kozlova and Thummel (2003b), provides *in vivo* tools that overcome the limitations of biochemical isolation and testing from flies. Microarray analysis has already been employed to investigate changes in gene transcription at the onset of metamorphosis (White *et al*., 1999) and to compare expression in Kc167 and SL2 cell lines (Neal *et al*., 2003). Microarray analysis has also been employed in *D. melanogaster* to follow expression events throughout larval and metamorphic development. The results suggest that while the larval stage is relatively quiescent, there are unique patterns of expression associated with this early developmental time which in some cases, may prove to be repressive functions (Arbeitman *et al*., 2002). In this respect, microarrays also may prove important for gaining a better grasp of JH's mode of action (Dubrovsky *et al*., 2000) during larval stages. Inevitably, the genomic approach will unravel entire gene networks that interact with and operate within the ecdysteroid-regulated hierarchy (Arbeitman *et al*., 2002; Stathopoulos and Levine, 2002). In any event, investigating the entire genome is important for identifying temporal connections at a gene expression level which potentially coincide with important functional relationships, as the connection between the glycolytic pathway and the onset of metamorphosis has already exemplified (White *et al*., 1999). Sooner rather than later, it is likely that genome-wide approaches will be applied to identify EcR and/or USP binding sites (Ren *et al*., 2000).

Another feature of ecdysteroid action which has received virtually no attention is the role that nutritional and cellular states play in determining and modifying ecdysteroid action (Zinke *et al*., 2002). As the case with glycolysis shows, it seems inevitable that a relationship exists here, given that the most important problem for premetamorphic insects stems from their need to process nutrients as they grow and undergo molting. The role of orphan receptors and their ligands seems an obvious place to explore this possibility, since most of the identified orphans among vertebrates have proven to be responsive to a variety of dietary compounds and intracellular metabolites, including EcR's vertebrate relatives, FXR and LXR.

The regulation of EcR and USP expression has begun to be undertaken and should also lead to important insights about endocrine regulation generally, and the interaction of ecdysteroid regulation with other biological processes (Sung and Robinow, 2000; Zheng *et al*., 2003). EcR undergoes fairly dramatic fluctuations in transcription, though little is known about the relative titers of EcR and USP intracellularly (Koelle *et al*., 1991). In other words, it is not clear which one of the two is rate-limiting in any given cell type, and thus, a quantitative question remains to be fully considered. In fact, there has been a tendency to ascribe regulation to the roles of individual players, without regard to the fact that cellular protein titers, ligand titers, rate of metabolism, and the relative affinity of promoter elements all play a role in determining the level of transcriptional response at any given gene at any given time. When ecdysteroid action is viewed as a challenge of cellular equilibrium, it is seen as a continuous and interactive process rather than a directed and responsive one. Interestingly, there are still unanswered questions about ecdysteroid regulation within the *Drosophila* salivary gland hierarchy which will be particularly useful for examining ecdysteroid response and processing, particularly with the judicious use of transgenic constructs.

A second important cornerstone for future progress will depend upon a more complete depiction of ecdysteroid receptor function among the insect orders. A survey of known EcR and USP sequences shows that only Diptera and Lepidoptera are extensively represented at this time. Based upon the variations in agonist responsiveness already discovered among the insect receptors in these two orders alone, the need for obtaining and testing other insect receptors is plainly obvious.

The ability to assess the effects of EcR, USP, and genes targeted by the ecdysteroid receptor in other insects will be greatly enhanced by continued development of transformation procedures. The ability to produce "null" mutations via RNA interference with transgenic constructs will be particularly important for assessing functional processes in other insects. Uhlirova *et al*. (2003) illustrated this possibility by showing that Sindbis virus-induced transformation with an RNAi eliminates Br-C expression in *Bombyx mori*. The interference exacerbates the same developmental defects in *Bombyx* as previously noted for the effects of Br-C null mutations in flies.

Related to the discovery and characterization of ecdysteroid responses in insect processes will be the

continued examination of the insect receptors themselves. The use of chimeras and mutational analysis, in conjunction with the predictive powers provided by improved modeling and crystal structures, will lead to further insights concerning the capabilities of receptor function and its response to ligands, which may include a broad spectrum based on the flexibility of its ligand-binding pocket (Billas *et al*., 2003). Many mutations affect receptor function nonspecifically (Bender *et al*., 1997; Bergman *et al*., 2004), and site-directed mutagenesis provides a particularly useful approach for finding mutations that specifically disrupt EcR and USP subfunctions essential for interpreting receptor function in cellular and *in vivo* systems.

In summary, the information reported here represents only a fraction of the progress on ecdysteroid receptor action that has taken place over the last 20 years. The convergence of research interests mentioned at the outset has created a synergy among approaches that will continue to yield important new insights about endocrine action and its effects on development, new possibilities for insecticidal discovery, and new applications for the use of ecdysteroid-regulated transcriptional cell systems.

Acknowledgments

The author wishes to thank Drs. Dino Moras, Markus Lezzi, and Fraydoon Rastinejad for contributing illustration figures for this chapter. The author also acknowledges Drs Margarithe Spindler-Barth, Klaus Spindler, Markus Lezzi and Jean-Antoine Lepesant for useful discussions related to the material presented in this manuscript, along with Dr. Larry Gilbert for his advice on the writing and his friendship. The author is responsible for any inaccuracies in the content and apologizes for the exclusion of many excellent contributions because of space constraints. VCH is currently supported by the US Department of Agriculture's Competitive Grants program (2003-35302-13474).

References

Amabis, D.C., Amabis, J.M., **1984**. Effects of ecdysterone in polytene chromosomes of *Trichosia pubescens*. *Devel. Biol. 102*, 1–9.

Amero, S.A., Elgin, S.C.R., Beyer, A.L., **1991**. A unique zinc finger protein is associated preferentially with active ecdysone-responsive loci in *Drosophila*. *Genes Devel. 5*, 188–200.

Andres, A.J., Cherbas, P., **1992**. Tissue-specific ecdysone responses: Regulation of the *Drosophila* genes Eip28/29 and Eip40 during larval development. *Development 116*, 865–876.

Andres, A.J., Fletcher, J.C., Karim, F.D., Thummel, C.S., **1993**. Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. *Devel. Biol. 160*, 388–404.

Andres, A.J., Cherbas, P., **1994**. Tissue-specific regulation by ecdysone: distinct patterns of Eip28/29 expression are controlled by different ecdysone response elements. *Devel. Genet. 15*, 320–331.

Antoniewski, C., Laval, M., Lepesant, J.A., **1993**. Structural features critical to the activity of an ecdysone receptor binding site. *Insect Biochem. Mol. Biol. 23*, 105–114.

Antoniewski, C., Mugat, B., Delbac, F., Lepesant, J-A., **1996**. Direct repeats bind the EcR/Usp receptor and mediate ecdysteroid responses in *Drosophila melanogaster. Mol. Cell. Biol. 16*, 2977–2986.

Apple, R.T., Fristrom, J.W., **1991**. 20-hydroxyecdysone is required for, and negatively regulates, transcription of *Drosophila* pupal cuticle protein genes. *Devel. Biol. 146*, 569–582.

Arbeitman, M.N., Hogness, D.S., **2000**. Molecular chaperones activate the *Drosophila* ecdysone recepetor, an RXR heterodimer. *Cell 101*, 67–77.

Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., *et al*., **2002**. Gene expression during the life cycle of *Drosophila melanogaster. Science 297*, 2270–2275.

Asahina, M., Jindra, M., Riddiford, L.M., **1997**. Developmental expression of Ultraspiracle proteins in the epidermis of the tobacco hornworm, *Manduca sexta*, during larval life and the onset of metamorphosis. *Devel. Genes Evol. 207*, 381–388.

Ashburner, M., Chihara, C., Meltzer, P., Richards, G., **1974**. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol. 38*, 655–662.

Ashok, M., Turner, C., Wilson, T.G., **1998**. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl Acad. Sci. USA 95*, 2761–2766.

Astle, J., Kozlova, T., Thummel, C.S., **2003**. Essential roles for the Dhr78 orphan nuclear receptor during molting of the *Drosophila* tracheal system. *Insect Biochem. Mol. Biol. 33*, 1201–1209.

Baehrecke, E.H., Thummel, C.S., **1995**. The *Drosophila* E93 gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. *Devel. Biol. 17*, 85–97.

Bai, J., Uehara, Y., Montell, D.J., **2000**. Regulation of invasive cell behavior by Taiman, a *Drosophila* protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. *Cell 103*, 1047–1058.

Baker, K.D., Warren, J.T., Thummel, C.S., Gilbert, L.I., Mangelsdorf, D.J., **2000**. Transcriptional activation of the *Drosophila* ecdysone receptor by insect and

plant ecdysteroids. *Insect Biochem. Mol. Biol. 30*, 1037–1043.

Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., *et al.*, **2003**. The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell 113*, 731–742.

Bashirullah, A., Pasquinelli, A.E., Kiger, A.A., Perrimon, N., Ruvkun, G., *et al.*, **2003**. Coordinate regulation of small temporal RNAs at the onset of *Drosophila* metamorphosis. *Devel. Biol. 259*, 1–8.

Basso, L.R., Vasconcelos, C., Fontes, A.M., Hartfelder, K., Silva, J.A., *et al.*, **2002**. The induction of DNA puff BhC4-1 is a late response to the increase in 20-hydroxyecdysone titers in last instar dipteran larvae. *Mech. Devel. 110*, 15–26.

Bayer, C.A., von Kalm, L., Fristrom, J.W., **1997**. Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the Broad-Complex during *Drosophila metamorphosis*. *Devel. Biol. 191*, 311–312.

Becker, H.J., **1959**. Die puffs der speicheldrüsenchromosomen von *Drosophila melanogaster. Chromosoma 10*, 654–678.

Beckstead, R., Ortiz, J.A., Sanchez, C., Prokopenko, S.N., Chambon, P., *et al.*, **2001**. Bonus, a *Drosophila* homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit βFTZ-F1-dependent transcription. *Mol. Cell. 7*, 753–765.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., Hogness, D.S., **1997**. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell 91*, 777–788.

Berger, E.M., Goudie, K., Klieger, L., Berger, M., DeCato, R., **1992**. The juvenile hormone analog, methoprene, inhibits ecdysterone induction of small heat shock protein gene expression. *Devel. Biol. 151*, 410–418.

Bergman, T., Henrich, V.C., Schlattner, U., Lezzi, M., **2004**. Ligand control of interaction *in vivo* between ecdysteroid receptor and ultraspiracle ligand-binding domain. *Biochem. J. 378*, 779–784.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., **2002**. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Devel. Cell 3*, 209–220.

Bienz-Tadmor, B., Smith, H.S., Gerbi, S.A., **1991**. The promoter of DNA puff gene II/9–1 *Sciara coprophila* is inducible by ecdysone in late prepupal salivary glands of *Drosophila melanogaster. Cell Regul. 2*, 875–888.

Billas, I.M., Moulinier, I., Rochel, N., Moras, D., **2001**. Crystal structure of the ligand-binding domain of the ultraspiracle protein Usp, the ortholog of retinoid X receptors in insects. *J. Biol. Chem. 276*, 7465–7474.

Billas, I.M., Iwema, T., Garnier, J-M., Mitschier, A., Rochel, N., *et al.*, **2003**. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature 426*, 91–96.

Blumberg, B., Evans, R.M., **1998**. Orphan nuclear receptors – new ligands and new possibilities. *Genes Devel. 12*, 3149–3155.

Bonneton, F., Zelus, D., Iwema, T., Robinson-Rechavi, M., Laudet, V., **2003**. Rapid divergence of the ecdysone receptor in Diptera and Lepidoptera suggests coevolution between EcR and Usp-RXR. *Mol. Biol. Evol. 20*, 541–555.

Brennan, C.A., Ashburner, M., Moses, K., **1998**. Ecdysone pathway is required for furrow progression in the developing *Drosophila* eye. *Development 125*, 2653–2664.

Brennan, C.A., Ashburner, M., Moses, K., **2001**. Broad-Complex, but not ecdysone receptor, is required for progression of the morphogenetic furrow in the *Drosophila* eye. *Development 128*, 1–11.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., Woodard, C.T., **1999**. The *Drosophila* βFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell 3*, 143–149.

Bruhat, A., Dreau, D., Drake, M.E., Tourmente, S., Chapel, S., *et al.*, **1993**. Intronic and 5′ flanking sequences of the *Drosophila* beta 3 tubulin gene are essential to confer ecdysone responsiveness. *Mol. Cell. Endocrinol. 94*, 61–71.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., Hogness, D.S., **1990**. The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. *Cell 61*, 85–99.

Carney, G.E., Bender, M., **2000**. The *Drosophila* ecdysone receptor (EcR) gene is required maternally for normal oogenesis. *Genetics 154*, 1203–1211.

Carney, G.E., Robertson, A., Davis, M.B., Bender, M., **2004**. Creation of EcR isoform-specific mutations in *Drosophila melanogaster* via local P element transposition, imprecise P element excision, and male recombination. *Mol. Genet. Genomics 271*, 282–290.

Carney, G.E., Wade, A.A., Sapra, R., Goldstein, E.S., Bender, M., **1997**. DHR3, an ecdysone-inducible early-late gene encoding a *Drosophila* nuclear receptor, is required for embryogenesis. *Proc. Natl Acad. Sci. USA 94*, 12024–12029.

Chan, S.M., **1998**. Cloning of a shrimp (*Metapanaeus ensis*) cDNA encoding a nuclear receptor superfamily member: an insect homologue of E75 gene. *FEBS Lett. 436*, 395–400.

Chen, J.H., Turner, P.C., Rees, H.H., **2002a**. Molecular cloning and induction of nuclear receptors from insect cell lines. *Insect Biochem. Mol. Biol. 32*, 657–667.

Chen, L., O'Keefe, S.L., Hodgetts, R.B., **2002b**. Control of Dopa decarboxylase gene expression by the Broad-Complex during metamorphosis in *Drosophila*. *Mech. Devel. 119*, 145–156.

Cherbas, L., Cherbas, P., **1997**. "Parahomologous" gene targeting in *Drosophila* cells: an efficient, homology-dependent pathway of illegitimate recombination near a target site. *Genetics 145*, 349–358.

Cherbas, P., Cherbas, L., Lee, S-S., Nakanishi, K., **1988**. 26-[^{125}I]Iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl Acad. Sci. USA 85*, 2096–2100.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., Cherbas, P., **2003**. EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development 130*, 271–284.

Cherbas, L., Koehler, M.M.D., Cherbas, P., **1989**. Effects of juvenile hormone on the ecdysone response in *Drosophila* Kc cells. *Devel. Genet. 10*, 177–188.

Cherbas, L., Lee, K., Cherbas, P., **1991**. Identification of ecdysone response elements by analysis of the *Drosophila* Eip28/29 gene. *Genes Devel. 5*, 120–131.

Cherbas, L., Yonge, C.D., Cherbas, P., Williams, C.M., **1980**. The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. *Wilhelm Roux's Archives 189*, 1–15.

Choi, D.S., Wang, D., Tolbert, L., Sadee, W., **2000**. Basal signaling activity of human dopamine D2L receptor demonstrated with an ecdysone-inducible mammalian expression system. *J. Neurosci. Methods 94*, 217–225.

Christopherson, K.S., Mark, M.R., Bajaj, V., Godowski, P.J., **1992**. Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl Acad. Sci. USA 89*, 6314–6318.

Chung, A.C-K., Durica, D.S., Clifton, S.W., Roe, B.A., Hopkins, P.M., **1998**. Cloning of crustacean EcR and RXR homologs and elevation of RXR mRNA by retinoic acid. *Mol. Cell. Endocrinol. 139*, 209–237.

Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., **2001**. The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA 98*, 1549–1554.

Crispi, S., Giordano, E., D'Avino, P.P., Furia, M., **1998**. Cross-talking among *Drosophila* nuclear receptors at the promiscuous response element of the ng-1 and ng-2 intermolt genes. *J. Mol. Biol. 275*, 561–574.

Crossgrove, K., Laudet, V., Maina, C.V., **2002**. *Dirofilaria immitis* encodes Di-nhr-7, a putative ortholog of the *Drosophila* ecdysone regulated E78 gene. *Mol. Biochem. Parasitol. 119*, 169–177.

D'Avino, P.P., Thummel, C.S., **2000**. The ecdysone regulatory pathway controls wing morphogenesis and integrin expression during *Drosophila* metamorphosis. *Devel. Biol. 220*, 211–224.

D'Avino, P.P., Drispi, S., Cherbas, L., Cherbas, P., Furia, M., **1995**. The moulting hormone ecdysone is able to recognize target elements composed of direct repeats. *Mol. Cell. Endocrinol. 113*, 1–9.

de Carvalho, D.P., Coelho, P.S.R., de Almeida, J.C., **2000**. A dual role of 20-hydroxyecdysone in the control of protein synthesis related to DNA puff activity in the anterior region of *Bradysia hygida* (Diptera, Sciaridae) salivary gland. *Insect Biochem. Mol. Biol. 30*, 541–548.

De la Cruz, F.E., Kirsch, D.R., Heinrich, J.N., **2000**. Transcriptional activity of *Drosophila melanogaster* ecdysone receptor isoforms and ultraspiracle in *Saccharomyces cerevisiae*. *J. Mol. Endocrinol. 24*, 183–191.

De la Cruz, F., Mak, P., **1997**. *Drosophila* ecdysone receptor functions as a constitutive activator in yeast. *J. Steroid Biochem. Mol. Biol. 62*, 353–369.

Devarakonda, S., Harp, J.M., Kim, Y., Ozyhar, A., Rastinejad, F., **2003**. Structure of the heterodimeric ecdysone receptor DNA-binding complex. *EMBO J. 22*, 5827–5840.

Dhadialla, T., Carlson, G.R., Le, D.P., **1998**. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol. 43*, 545–569.

Dhadialla, T.S., Tzertzinis, G., **1997**. Characterization and partial cloning of ecdysteroid receptor from a cotton boll weevil embryonic cell line. *Arch. Insect Biochem. Physiol. 35*, 45–57.

DiBartolomeis, S.M., Gerbi, S.A., **1989**. Molecular characterization of DNA puff II/9A genes in *Sciara coprophila*. *J. Mol. Biol. 210*, 531–540.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., Guild, G.M., **1991**. The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics 129*, 385–397.

Dobens, L., Rudolph, K., Berger, E.M., **1991**. Ecdysterone regulatory elements function as both transcriptional activators and repressors. *Mol. Cell. Biol. 11*, 1846–1853.

Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., *et al.*, **1999**. Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Mol. Cell. Biol. 19*, 3383–3394.

Dubrovsky, E.B., Dubrovskaya, V.A., Bilderback, A.L., Berger, E.M., **2000**. The isolation of two juvenile hormone-inducible genes in *Drosophila melanogaster*. *Devel. Biol. 224*, 486–495.

Durica, D.S., Wu, X., Anilkumar, G., Hopkins, P.M., Chung, A.C-K., **2002**. Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation. *Mol. Cell. Endocrinol. 189*, 59–76.

Elbrecht, A., Chen, Y., Jurgens, T., Hensens, O.D., Zink, D.L., *et al.*, **1996**. 8-O-acetylharpagide is a nonsteroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol. 26*, 519–523.

Elke, C., Vogtli, M., Rauch, P., Spindler-Barth, M., Lezzi, M., **1997**. Expression of EcR and Usp in *Escherichia coli*; purification and functional studies. *Arch. Insect Biochem. Physiol. 35*, 59–69.

Fisk, G.J., Thummel, C.S., **1995**. Isolation, regulation, and DNA-binding properties of three *Drosophila* nuclear hormone receptor superfamily members. *Proc. Natl Acad. Sci. USA 92*, 10604–10608.

Fisk, G.J., Thummel, C.S., **1998**. The DHR78 nuclear receptor is required for ecdysteroid signaling during the onset of *Drosophila* metamorphosis. *Cell 93*, 543–555.

Fletcher, J.C., Burtis, K.C., Hogness, D.S., Thummel, C.S., **1995**. The *Drosophila* E74 gene is required for

metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development 121*, 1455–1465.

Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., *et al.*, **1995**. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell 81*, 687–693.

Gates, J., Lam, G., Ortiz, J.A., Losson, R., Thummel, C.S., **2004**. rigor mortis encodes a novel nuclear receptor interacting protein required for ecdysone signaling during *Drosophila* larval development. *Development 131*, 25–36.

Ghbeish, N., Tsai, C-C., Schubiger, M., Zhou, J.Y., Evans, R.M., *et al.*, **2001**. The dual role of ultraspiracle, the *Drosophila* retinoid X receptor, in the ecdysone response. *Proc. Natl Acad. Sci. USA 98*, 3867–3872.

Ghbeish, N., McKeown, M., **2002**. Analyzing the repressive function of ultraspiracle, the *Drosophila* RXR, in *Drosophila* eye development. *Mech. Devel. 111*, 89–98.

Gonzy, G., Pokholkova, G.V., Perronet, F., Mugat, B., Demakova, O.V., *et al.*, **2002**. Isolation and characterization of novel mutations of the Broad-Complex, a key regulatory gene of ecdysone induction in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 32*, 121–132.

Grau, V., Lafont, R., **1994**. Metabolism of ecdysone and 20-hydroxyecdysone in adult *Drosophila melanogaster. Insect Biochem. Mol. Biol. 24*, 49–58.

Grebe, M., Przibilla, S., Henrich, V.C., Spindler-Barth, M., **2003**. Characterization of the ligand-binding domain of the ecdysteroid receptor from *Drosophila melanogaster. Biol. Chem. 384*, 93–104.

Grebe, M., Rauch, P., Spindler-Barth, M., **2000**. Characterization of subclones of the epithelial cell line from *Chironomus tentans* resistant to the insecticide RH5992, a nonsteroidal moulting hormone agonist. *Insect Biochem. Mol. Biol. 30*, 591–600.

Grebe, M., Spindler-Barth, M., **2002**. Expression of ecdysteroid receptor and ultraspiracle from *Chironomus tentans* (Insecta, Diptera) in *E. coli* and purification in a functional state. *Insect Biochem. Mol. Biol. 32*, 167–174.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.-M., *et al.*, **1986**. Human oestrogen receptor cDNA: sequence, expression, and homology to v-erb-A. *Nature 320*, 134–139.

Guo, X., Harmon, M.A., Laudet, V., Mangelsdorf, D.J., Palmer, M.J., **1997**. Isolation of a functional ecdysteroid receptor homologue form the ixodid tick *Ambloymma americanum* (L.). *Insect. Biochem. Mol. Biol. 27*, 945–962.

Guo, X., Xu, Q., Harmon, M., Jin, X., Laudet, V., *et al.*, **1998**. Isolation of two functional retinoid X receptor subtypes from the ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol. 27*, 945–962.

Hall, B.L., Thummel, C.S., **1998**. The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. *Development 125*, 4709–4717.

Halling, B.P., Yuhas, D.A., Eldridge, R.R., Gilbery, S.N., Deutsch, V.A., *et al.*, **1999**. Expression and purification of the hormone binding domain of the *Drosophila* ecdysone and ultraspiracle receptors. *Protein Expr. Purif. 17*, 373–386.

Hannan, G.N., Hill, R.J., **1997**. Cloning and characterization of LcEcR: a functional ecdysone receptor from the sheep blowfly *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 27*, 479–488.

Hannan, G.N., Hill, R.J., **2001**. Lcusp, an ultraspiracle gene from the sheep blowfly, *Lucilia cuprina*: cDNA cloning, developmental expression of RNA and confirmation of function. *Insect Biochem. Mol. Biol. 31*, 771–781.

Harmon, M.A., Boehm, M.F., Heyman, R.A., Mangelsdorf, D.J., **1995**. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl Acad. Sci. USA 92*, 6157–6160.

Hayward, D.C., Bastiani, M.J., Truman, J.W., Riddiford, L.M., Ball, E.E., **1999**. The sequence of Locusta RXR, homologous to *Drosophila* Ultraspiracle, and its evolutionary implications. *Devel. Genes Evol. 209*, 564–571.

Hayward, D.C., Dhadialla, T.S., Zhou, S., Kuiper, M.J., Ball, E.E., *et al.*, **2003**. Ligand specificity and developmental expression of RXR and ecdysone receptor in the migratory locust. *J. Insect Physiol. 49*, 1135–1144.

Henrich, V.C., Brown, N.E., **1995**. Insect nuclear receptors; a developmental and comparative perspective. *Insect Biochem. Mol. Biol. 25*, 881–897.

Henrich, V.C., Burns, E., Yelverton, D.P., Christensen, E., Weinberger, C., **2003**. Juvenile hormone potentiates ecdysone receptor-dependent transcription in a mammalian cell culture system. *Insect Biochem. Mol. Biol. 33*, 1239–1247.

Henrich, V.C., Rybczynski, R., Gilbert, L.I., **1999**. Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. In: Litwack, G. (Ed.), Vitamins and Hormones, Vol. 55. Academic Press, San Diego, pp. 73–125.

Henrich, V.C., Sliter, T.J., Lubahn, D.B., MacIntyre, A., Gilbert, L.I., **1990**. A steroid-thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. *Nucleic Acids Res. 18*, 4143–4148.

Henrich, V.C., Szekely, A.A., Kim, S.J., Brown, N., Antoniewski, C., *et al.*, **1994**. Expression and function of the ultraspiracle (*usp*) gene locus during development in *Drosophila melanogaster. Devel. Biol. 165*, 38–52.

Henrich, V.C., Vogtli, M., Grebe, M., Przibilla, S., Spindler-Barth, M., *et al.*, **2000**. Developmental effects of chimeric ultraspiracle gene derived from *Drosophila* and *Chironomus*. *Genesis 28*, 125–133.

Hill, R.J., Segraves, W.A., Choi, D., Underwood, P.A., Macavoy, E., **1993**. The reaction with polytene chromosomes of antibodies raised against *Drosophila* E75A protein. *Insect Biochem. Mol. Biol. 23*, 99–104.

Hirai, M., Shinoda, T., Kamimura, M., Tomita, S., Shiotsuki, T., **2002**. *Bombyx mori* orphan receptor,

BmHR78: cDNA cloning, testis abundant expression, and putative dimerization partner for *Bombyx* ultraspiracle. *Mol. Cell. Endocrinol. 189*, 201–211.

Hiruma, K., Carter, M.S., Riddiford, L.M., **1995**. Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Devel. Biol. 169*, 195–209.

Hiruma, K., Shinoda, T., Malone, F., Riddiford, L.M., **1999**. Juvenile hormone modulates 20-hydroxyecdysone-inducible ecdysone receptor and *ultraspiracle* gene expression in the tobacco hornworm, *Manduca sexta*. *Devel. Genes Evol. 209*, 18–30.

Hock, T., Cottrill, T., Keegan, J., Garza, D., **2000**. The E23 early gene of *Drosophila* encodes an ecdysone-inducible ATP-binding cassette transporter capable of repressing ecdysone-mediated gene activation. *Proc. Natl Acad. Sci. USA 15*, 9519–9524.

Hollenberg, S.M., Weinberger, C., Ong, E.S., Cerelli, G., Oro, A., *et al.*, **1985**. Domain structure of human glucocorticoid receptor and its relationship to the v-erb-A oncogene product. *Nature 318*, 635–641.

Hoppe, U.C., Marban, E., Johns, D.C., **2000**. Adenovirus-mediated inducible gene expression *in vivo* by a hybrid ecdysone receptor. *Mol. Therap. 1*, 159–164.

Horner, M.A., Chen, T., Thummel, C.S., **1995**. Ecdysteroid regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Devel. Biol. 168*, 490–502.

Hu, W., Cook, B.J., Ampasala, D.R., Zheng, S., Caputo, G., *et al.*, **2004**. Morphological and molecular effects of 20-hydroxyecdysone and its agonist tebufenozide on CF-203, a midgut derived cell line from the spruce budworm, *Choristoneura fumiferana*. *Arch. Insect Biochem. Physiol. 55*, 68–78.

Hu, X., Cherbas, L., Cherbas, P., **2003**. Transcription activation by the ecdysone receptor (EcR/Usp): Identification of activation functions. *Mol. Endocrinol. 17*, 716–731.

Huet, F., Ruiz, C., Richards, G., **1993**. Puffs and PCR: the *in vivo* dynamics of early gene expression during ecdysone responses in *Drosophila*. *Development 118*, 613–627.

Hurban, P., Thummel, C.S., **1993**. Isolation and characterization of fifteen ecdysone-inducible *Drosophila* genes reveal unexpected complexities in ecdysone regulation. *Mol. Cell. Biol. 13*, 7101–7111.

Imhof, M.O., Rusconi, S., Lezzi, M., **1993**. Cloning of a *Chironomus tentans* cDNA encoding a protein (cEcRH) homologous to the *Drosophila melanogaster* ecdysteroid receptor (dEcR). *Insect Biochem. Mol. Biol. 23*, 115–124.

Jayachandran, G., Fallon, A.M., **2000**. Evidence for expression of EcR and Usp components of the 20-hydroxyecdysone receptor by a mosquito cell line. *Arch. Insect Biochem. Physiol. 43*, 87–96.

Jayachandran, G., Fallon, A.M., **2001**. Antisense expression of the 20-hydroxyecdysone receptor (EcR) in transfected mosquito cells uncovers a new EcR isoform that varies at the C-terminal end. *In Vitro Cell Devel. Biol. Anim. 37*, 522–529.

Jindra, M., Sehnal, F., Riddiford, L.M., **1994a**. Isolation, characterization, and developmental expression of the ecdysteroid-induced E75 gene of the wax moth *Galleria mellonella*. *Eur. J. Biochem. 221*, 665–675.

Jindra, M., Sehnal, F., Riddiford, L.M., **1994b**. Isolation and developmental expression of the ecdysteroid-induced GHR3 gene of the wax moth *Galleria mellonella*. *Insect Biochem. Mol. Biol. 24*, 763–773.

Jindra, M., Sehnal, F., Riddiford, L.M., **1995**. Ecdysteroid-induced expression of the GmE75 and GHR3 orphan receptor genes in *Galleria mellonella* (Lepidoptera: Pyralidae) larvae and cultured silk glands. *Eur. J. Entomol. 92*, 235–236.

Jindra, M., Huang, J-Y., Malone, F., Asahina, M., Riddiford, L.M., **1997**. Identification and developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. *Insect Mol. Biol. 6*, 41–53.

Jindra, M., Malone, F., Hiruma, K., Riddiford, L.M., **1996**. Developmental profiles and ecdysteorid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 180*, 258–272.

Jones, G., Sharp, P.A., **1997**. Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA 94*, 13499–13503.

Jones, G., Wozniak, M., Chu, Y., Dhar, S., Jones, D., **2001**. Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. *Insect Biochem. Mol. Biol. 32*, 33–49.

Kamimura, M., Tomita, S., Kiuchi, M., Fujiwara, H., **1997**. Tissue-specific and stage-specific expression of two silkworm ecdysone receptor isoforms – ecdysteroid-dependent transcription in cultured anterior silk glands. *Eur. J. Biochem. 248*, 786–793.

Kapitskaya, M.Z., Li, C., Miura, K., Segraves, W., Raikhel, A.S., **2000**. Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol. Cell. Endocrinol. 160*, 25–37.

Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., Raikhel, A.S., **1996**. The mosquito ultraspiracle homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell Endocrinol. 12*, 119–132.

Karim, F.D., Thummel, C.S., **1991**. Ecdysone coordinates the timing and amounts of E74B and E74A transcription in *Drosophila*. *Genes Devel. 5*, 1067–1079.

Kethidi, D.R., Perera, S.C., Zheng, S.C., Feng, Q.L., Krell, P.J., *et al.*, **2004**. Identification and characterization of a JH response region in the juvenile hormone esterase gene from the spruce budworm, *Choristoneura fumiferana*. *J. Biol. Chem. 279*, 19634–19642.

Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., Fristrom, J.W., **1988**. Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. *Genetics 118*, 247–259.

Kitareewan, S., Burka, L.T., Tomer, K.B., Parker, C.E., Deterding, L.J., *et al*., **1996**. Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Mol. Biol. Cell 7*, 1153–1166.

Koch, P.B., Merk, R., Reinhardt, R., Weber, P., **2003**. Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly *Precis coenia* (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. *Devel. Genes Evol. 212*, 571–584.

Koelle, M.R., Segraves, W.A., Hogness, D.S., **1992**. DHR3: A *Drosophila* steroid receptor homolog. *Proc. Natl Acad. Sci. USA 89*, 6167–6171.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al*., **1991**. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 4*, 59–77.

Koo, J.C., Asurmendi, S., Bick, J., Woodford-Thomas, T., Beachy, R.N., **2004**. Ecdysone agonist-inducible expression of a coat protein gene from tobacco mosaic virus confers viral resistance in transgenic *Arapidopsis*. *Plant J. 37*, 439–448.

Kostrouchova, M., Karause, M., Kostrouch, Z., Rall, J.E., **1998**. CHR3: a *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. *Development 125*, 1617–1626.

Kothopalli, R., Palli, S.R., Ladd, T.R., Sohi, S.S., Cress, D., *et al*., **1995**. Cloning and developmental expression of the ecdysone receptor gene from the spruce budworm, *Choristoneura fumiferana*. *Devel. Genet. 17*, 319–330.

Kozlova, T., Pokholkova, G.V., Tzertzinis, G., Sutherland, J.D., *et al*., **1998**. *Drosophila* hormone receptor 38 functions in metamorphosis: a role in adult cuticle formation. *Genetics 149*, 1465–1475.

Kozlova, T., Thummel, C.S., **2002**. Spatial patterns of ecdysteroid receptor activation during the onset of *Drosophila* metamorphosis. *Development 129*, 1739–1750.

Kozlova, T., Thummel, C.S., **2003a**. Essential roles for ecdysone signaling during *Drosophila* mid-embryonic development. *Science 301*, 1911–1914.

Kozlova, T., Thummel, C.S., **2003b**. Methods to characterize *Drosophila* nuclear receptor activation and function *in vivo*. *Methods Enzymol. 364*, 475–490.

Kumar, M.B., Fujimoto, T., Potter, D.W., Deng, Q., Palli, S.R., **2002**. A single point mutation in ecdysone receptor leads to increased specificity: implications for gene switch applications. *Proc. Natl Acad. Sci. USA 99*, 14710–14715.

Lafont, R., Connat, J-L., **1989**. Pathways of ecdysone metabolism. In: Koolman, J. (Ed.), Ecdysone. Thieme, Stuttgart, pp. 167–173.

Lam, G., Hall, B.L., Bender, M., Thummel, C.S., **1999**. DHR3 is required for the prepupal–pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. *Devel. Biol. 212*, 204–216.

Lan, Q., Hiruma, K., Hu, X., Jindra, M., Riddiford, L.M., **1999**. Activation of a delayed early gene encoding MHR3 by the ecdysone receptor heterodimer EcR-B1-Usp-1 but not by EcR-B1-Usp-2. *Mol. Cell. Biol. 19*, 4897–4906.

Lan, Q., Wu, Z-N., Riddiford, L.M., **1997**. Regulation of the ecdysone receptor, Usp, E75, and MHR3 genes by 20-hydroxyecdysone in the GV1 cell line of the tobacco hornworm, *Manduca sexta*. *Insect Mol. Biol. 6*, 3–10.

Lavorgna, G., Ueda, H., Clos, J., Wu, C., **1991**. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu. *Science 252*, 848–851.

Lee, C.Y., Simon, C.R., Woodard, C.T., Baehrecke, E.H., **2002**. Genetic mechanism for the stage and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Devel. Biol. 252*, 138–148.

Lee, T., Marticke, S., Sung, C., Robinow, S., Luo, L., **2000**. Cell-autonomous requirement of the Usp/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron 28*, 807–818.

Lehman, M., **1995**. *Drosophila* Sgs genes: stage and tissue specificity of hormone responsiveness. *BioEssays 18*, 47–54.

Lehmann, M., Korge, G., **1995**. Ecydsone regulation of the *Drosophila* Sgs-4 gene is mediated by the synergistic action of ecdysone receptor and SEBP 3. *EMBO J. 14*, 716–726.

Lezzi, M., Bergman, T., Henrich, V.C., Vogtli, M., Fromel, C., *et al*., **2002**. Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle. *Eur. J. Biochem. 269*, 3237–3245.

Li, F-Q., Ueda, H., Hirose, S., **1994**. Mediators of activation of fushi tarazu gene transcription by BmFTZ-F1. *Mol. Cell. Biol. 14*, 3013–3021.

Li, T-R., Bender, M., **2000**. A conditional rescue system reveals essentials functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in *Drosophila*. *Development 127*, 2897–2905.

Liang, C., Spitzer, J.D., Smith, H.S., Gerbi, S.A., **1993**. Replication initiates at a confined region during DNA amplification in *Sciara* DNA puff II/9A. *Genes Devel. 7*, 1072–1084.

Luo, Y., Amin, J., Voellmy, R., **1991**. Ecdysterone receptor is a sequence-specific transcription factor involved in the developmental regulation of heat shock genes. *Mol. Cell. Biol. 11*, 3660–3675.

Mangelsdorf, D.J., Ong, E.S., Dyck, J.A., Evans, R.M., **1990**. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature 345*, 224–229.

Martinez, A., Scanlon, D., Gross, B., Perara, S.C., Palli, S.R., *et al*., **1999a**. Transcriptional activation of the cloned *Heliothis virescens* (Lepidoptera) ecdysone receptor (HvEcR) by muristerone A. *Insect Biochem. Mol. Biol. 29*, 915–930.

Martinez, A., Sparks, C., Drayton, P., Thompson, J., Greenland, A., *et al*., **1999b**. Creation of ecdysone receptor chimeras in plants for controlled regulation of gene expression. *Mol. Gen. Genet. 261*, 546–552.

Maschat, F., Dubertret, M.L., Lepesant, J.A., **1991**. Transformation mapping of the regulatory elements of the

ecdysone-inducible P1 gene of *Drosophila melanogaster. Mol. Cell. Biol. 11*, 2913–2917.

Matusuoka, T., Fujiwara, H., **2000**. Expression of ecdysteroid-regulated genes is reduced specifically in the wing discs of the wing-deficient mutant (fl) of *Bombyx mori. Devel. Genes Evol. 210*, 120–128.

Mikitani, K., **1996**. A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3, 5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamid shows apparent insect molting hormone activities at molecular and cellular levels. *Biochem. Biophys. Res. Comm. 227*, 427–432.

Minakuchi, C., Nakagawa, Y., Kiuchi, M., Tomita, S., Kamimura, M., **2002**. Molecular cloning, expression analysis and functional confirmation of two ecdysone receptor isoforms from the rice stem borer, *Chilo suppressalis*. *Insect Biochem. Mol. Biol. 33*, 561–562.

Miura, K, Zhu, J., Dittmer, N.T., Chen, L., Raikhel, A.S., **2002**. A COUP-TF/Svp homolog is highly expressed during vitellogenesis in the mosquito *Aedes aegypti*. *J. Mol. Endocrinol. 29*, 223–238.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., Rubin, G.M., **1990**. The *Drosophila* seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell 60*, 211–224.

Mouillet, J.F., Bousquet, F., Sedano, N., Alabouvette, J., Nicolai, M., *et al.*, **1999**. Cloning and characterization of new orphan nuclear receptors and their developmental profiles during *Tenebrio* metamorphosis. *Eur. J. Biochem. 265*, 972–981.

Mouillet, J.F., Delbecque, J.P., Quennedey, B., Delachambre, J., **1997**. Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. *Eur. J. Biochem. 248*, 856–863.

Mouillet, J-F., Henrich, V.C., Lezzi, M., Vogtli, M., **2001**. Differential control of gene activity by isoforms A, B1, and B2 of the *Drosophila* ecdysone receptor. *Eur. J. Biochem. 268*, 1811–1819.

Mugat, B., Brodu, V., Kejzloarova-Lepesant, J., Antoniewski, C., Bayer, C.A., *et al.*, **2000**. Dynamic expression of broad-complex isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Devel. Biol. 227*, 104–117.

Murata, T., Kageyama, Y., Hirome, S., Ueda, H., **1996**. Regulation of the EDG84A gene by FTZ-F1 during metamorphosis in *Drosophila melanogaster. Mol. Cell. Biol. 16*, 6509–6515.

Myohara, M., Okada, M., **1987**. Induction of ecdysterone-stimulated chromosomal puffs in permeabilized *Drosophila* salivary glands: A new method for assaying the gene-regulating activity of cytoplasm. *Devel. Biol. 122*, 396–406.

Nakagawa, Y., Minakuchi, C., Takahashi, K., Ueno, T., **2002**. Inhibition of [^{3}H] ponasterone A binding by ecdysone agonists in the intact Kc cell line. *Insect Biochem. Mol. Biol. 32*, 175–180.

Nauber, U., Pankratz, M.J., Kienlin, A., Seifert, E., Klemm, U., *et al.*, **1988**. Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene knirps. *Nature 336*, 489–492.

Neal, S.J., Gibson, M.L., Westwood, J.T., **2003**. Construction of a cDNA-based microarray for *Drosophila melanogaster*: a comparison of gene transcription profiles from SL2 and Kc167 cells. *Genome 46*, 879–892.

Niedziela-Majka, A., Kochman, M., Ozyhar, A., **2000**. Polarity of the ecdysone receptor complex interaction with the palindromic response element from the hsp27 gene promoter. *Eur. J. Biochem. 267*, 507–519.

Niedziela-Majka, A., Rymarczyk, G., Kochman, M., Ozyhar, A., **1998**. Pure, bacterially expressed DNA-binding domains of the functional ecdysteroid receptor capable of interacting synergistically with the hsp27 20-hydroxyecdysone response element. *Protein Exp. Purif. 14*, 208–220.

No, D., Yao, T-P., Evans, R.M., **1996**. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl Acad. Sci. USA 93*, 3346–3351.

Oberdorster, E., Clay, M.A., Cottam, D.M., Wilmot, F.A., McLachlan, J.A., *et al.*, **2001**. Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. *J. Steroid Biochem. Mol. Biol. 77*, 229–238.

Oberdorster, E., Cottam, D.M., Wilmot, F.A., Milner, M.J., McLachlan, J.A., **1999**. Interaction of PAHs and PCBs with ecdysone-dependent gene expression and cell proliferation. *Toxicol. Appl. Pharmacol. 160*, 101–108.

O'Connor, J.D., **1985**. Ecdysteroid action at the molecular level. In: Kerkur, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 8, Endocrinology II. Pergamon, Oxford, pp. 85–98.

Oro, A.E., McKeown, M., Evans, R.M., **1990**. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature 347*, 298–301.

Oro, A.E., McKeown, M., Evans, R.M., **1992**. The *Drosophila* retinoid X receptor homolog ultraspiracle functions in both female reproduction and eye morphogenesis. *Development 115*, 449–462.

Oro, A.E., Ong, E.S., Margolis, J.S., Posakony, J.W., McKeown, M., *et al.*, **1988**. The *Drosophila* gene knirps-related is a member of the steroid-receptor gene superfamily. *Nature 336*, 493–496.

Ozyhar, A., Gries, M., Kiltz, H-H., Pongs, O., **1992**. Magnetic DNA affinity purification of ecdysteroid receptor. *J. Steroid Biochem. Mol. Biol. 43*, 629–634.

Ozyhar, A., Pongs, O., **1993**. Mutational analysis of the interaction between ecdysteroid receptor and its response element. *J. Steroid Biochem. Mol. Biol. 46*, 135–145.

Padidam, M., Gore, M., Lu, D.L., Smirnova, O., **2003**. Chemical inducible, ecdysone receptor based gene expression system for plants. *Transgenic Res. 12*, 101–109.

Palli, S.R., Hiruma, K., Riddiford, L.M., **1992**. An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. *Devel. Biol. 150*, 305–318.

Palli, S.R., Kaptiskaya, M.Z., Kumar, M.B., Cress, D.E., **2003**. Improved ecdysone receptor based inducible gene regulation system. *Eur. J. Biochem. 270*, 1308–1315.

Palli, S.R., Ladd, T.R., Retnakaran, A., **1997**. Cloning and characterization of a new isoform of *Choristoneura* hormone receptor 3 from the spruce budworm. *Arch. Insect Biochem. Physiol. 35*, 33–44.

Palli, S.R., Sohi, S.S., Cook, B.J., Lambert, D., Ladd, T.R., *et al.*, **1995**. Analysis of ecdysteroid action in *Malacosoma disstria* cells: cloning selected regions of E75 and MHR3-like genes. *Insect Biochem. Mol. Biol. 25*, 697–707.

Palmer, M.J., Warren, J.T., Jin, X., Guo, X., Gilbert, L.I., **2002**. Developmental profiles of ecdysteroids, ecdysteroid receptor mRNAs and DNA-binding properties of ecdysteroid receptors in the Ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol. 32*, 465–476.

Perera, S.C., Ladd, T.R., Dhadialla, T.S., Krell, P.J., Sohi, S.S., *et al.*, **1999a**. Studies on two ecdysone receptor isoforms of the spruce budworm, *Choristoneura fumiferana*. *Mol. Cell. Endocrinol. 152*, 73–84.

Perera, S.C., Palli, S.R., Ladd, T.R., Krell, P.J., Retnakaran, A., **1998**. The ultraspiracle gene of the spruce budworm, *Choristoneura fumiferana*: cloning of cDNA and developmental expression of mRNA. *Devel. Genet. 22*, 169–179.

Perera, S.C., Sundaram, M., Krell, P.J., Retnakaran, A., Dhadialla, T.S., *et al.*, **1999b**. An analysis of ecdysone receptor domains required for heterodimerization with Ultraspiracle. *Arch. Insect Biochem. Physiol. 41*, 61–70.

Perlmann, T., Umesono, K., Rangarajan, P.N., Froman, B.M., Evans, R.M., **1996**. Two distinct dimerization interfaces differentially modulate target gene specificity of nuclear hormone receptors. *Mol. Endocrinol. 10*, 958–966.

Perrimon, N., Engstrom, L., Mahowald, A.P., **1985**. Developmental genetics of the 2C-D region of the *Drosophila* X chromosome. *Genetics 126*, 4581–4589.

Przibilla, S., Hitchcock, W.W., Szecsi, M., Grebe, M., Beatty, J., *et al.*, **2004**. Functional studies on the ligand-binding domain of Ultraspiracle from *Drosophila melanogaster. Biol. Chem. 385*, 21–30.

Rauch, P., Grebe, M., Elke, C., Spindler, K-D., Spindler-Barth, M., **1998**. Ecdysteroid receptor and ultraspiracle from *Chironomus tentans* (Insecta) are phosphoproteins and are regulated differently by molting hormone. *Insect Biochem. Mol. Biol. 28*, 265–275.

Rebers, J., **1999**. Overlapping antiparallel transcripts induced by ecdysone in a *Drosophila* cell line. *Insect Biochem. Mol. Biol. 29*, 293–302.

Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., *et al.*, **2000**. Genome-wide location and function of DNA binding proteins. *Science 290*, 2306–2309.

Retnakaran, A., Gelbic, I., Sundaram, M., Tomkins, W., Ladd, T., *et al.*, **2001**. Mode of action of the ecydsone agonist tebufenozide (RH-5992), and an exlusion mechanism to explain resistance to it. *Pest. Manag. Sci. 57*, 951–957.

Restifo, L.L., Wilson, T.G., **1998**. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible Broad Complex transcription factors. *Devel. Genet. 22*, 141–159.

Richards, G.P., **1976**. The control of prepupal puffing patterns *in vitro*; implications for prepupal ecdysone titres in *Drosophila melanogaster. Devel. Biol. 48*, 91–95.

Richards, G., **1982**. Sequential gene activation by ecdysteroids in polytene chromosomes of *Drosophila melanogaster* VII. Tissue specific puffing. *Wilhelm Roux's Arch. 191*, 103–111.

Riddiford, L., **1985**. Hormone action at the cellular level. In: Kerkur, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 8, Endocrinology II. Pergamon, Oxford, pp. 37–84.

Riddiford, L.M., Cherbas, P., Truman, J.W., **2000**. Ecdysone receptors and their biological actions. *Vitam. Horm. 60*, 1–73.

Riddiford, L.M., Hiruma, K., Zhou, X., Nelson, C.A., **2003**. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster. Insect Biochem. Mol. Biol.* 1327–1338.

Riddihough, G., Pelham, H.R.B., **1987**. An ecdysone response element in the *Drosophila* hsp27 promoter. *EMBO J. 6*, 3729–3734.

Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., **1993**. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development 119*, 1251–1259.

Rymarczyk, G., Grad, I., Rusek, A., Oswiecimska-Rusin, K., Niedziela-Majli, A., *et al.*, **2003**. Purification of *Drosophila melanogaster* ultraspiracle protein and analysis of its a/B region-dependent dimerization *in vitro*. *Biol. Chem. 384*, 59–69.

Saez, E., Nelson, M.C., Eshelman, B., Banayo Koder, E.A., Cho, G.J., *et al.*, **2000**. Identification of ligands and coligands for the ecdysone-regulated gene switch. *Proc. Natl Acad. Sci. USA 97*, 14512–14517.

Saleh, D.S., Zhang, J., Wyatt, G.R., Walker, V.K., **1998**. Cloning and characterization of an ecdysone receptor cDNA from *Locusta migratoria*. *Mol. Cell. Endocrinol. 143*, 91–99.

Sasorith, S., Billas, I., Iwema, T., Moras, D., Wurtz, J-M., **2002**. Structure-based analysis of the ultraspiracle protein and docking studies of putative ligands. *J. Insect Sci. 2*, 25–36.

Savakis, C., Demetri, G., Cherbas, P., **1980**. Ecdysteroid-inducible polypeptides in a *Drosophila* cell line. *Cell 22*, 665–674.

Schmidt, A., Endo, N., Rutledge, S.J., Vogel, R., Shinar, D., *et al.*, **1992**. Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol. 6*, 1634–1641.

Schubiger, M., Tomita, S., Sung, C., Robinow, S., Truman, J.W., **2003**. Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Devel. 120*, 909–918.

Schubiger, M., Truman, J.W., **2000**. The RXR ortholog Usp suppresses early metamorphic processes in *Drosophila* in the absence of ecdysteroids. *Development 127*, 1151–1159.

Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., Bender, M., **1998**. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development 125*, 2053–2062.

Schulz, R.A., Cherbas, L., Cherbas, P., **1986**. Alternative splicing generates two distinct Eip28/29 gene transcripts in *Drosophila* Kc cells. *Proc. Natl Acad. Sci. USA 83*, 9428–9432.

Sedkov, Y., Cho, E., Petruk, S., Cherbas, L., Smith, S.T., *et al.*, **2003**. Methylation at lysine 4 of histone H3 in ecdysone-dependent development of *Drosophila*. *Nature 426*, 78–83.

Segal, D., Cherbas, L., Cherbas, P., **1996**. Genetic transformation of *Drosophila* cells in culture by P-element mediated transposition. *Somat. Cell Mol. Genet. 22*, 159–165.

Segraves, W.A., Hogness, D.S., **1990**. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Devel. 4*, 204–219.

Segraves, W.A., Woldin, C., **1993**. The E75 gene of *Manduca sexta* and comparison with its *Drosophila* homolog. *Insect Biochem. Mol. Biol. 23*, 91–97.

Sempere, L.F., Sokol, N.S., Dubrovsky, E.B., Berger, E.M., Ambros, V., **2003**. Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and broad-Complex gene activity. *Devel. Biol. 259*, 9–18.

Shea, C., Hough, D., Xiao, J., Tzertzinis, G., Maina, C.V., **2004**. An rxr/usp homolog from the parasitic nematode, *Dirofilaria immitis*. *Gene 324*, 171–182.

Shea, M.J., King, D.L., Conboy, M.J., Mariani, B.D., Kafatos, F.C., **1990**. Proteins that bind to *Drosophila* chorion *cis*-regulatory elements: A new C2H2 zinc finger protein and a C2C2 steroid receptor-like component. *Genes Devel. 4*, 1128–1140.

Shemshidini, L., Wilson, T.G., **1990**. Resistance to juvenile hormone and an insect growth regulator in *Drosophila* is associated with an altered cytosolic juvenile hormone-binding protein. *Proc. Natl Acad. Sci. USA 87*, 2072–2086.

Simon, A.F., Shih, C., Mack, A., Benzer, S., **2003**. Steroid control of longevity in *Drosophila melanogaster. Science 299*, 1407–1410.

Sobrier, M.L., Chapel, S., Couderc, J.L., Micard, D., Lecher, P., *et al.*, **1989**. 20-OH-ecdysone regulates 60C beta tubulin gene expression in Kc cells and during *Drosophila* development. *Exp. Cell Res. 184*, 241–249.

Somme-Martin, G., Colardeau, J., Beydon, P., Blais, C., Lepesant, J.A., *et al.*, **1990**. P1 gene expression in *Drosophila* larval fat body: induction by various ecdysteroids. *Arch. Insect Biochem. Physiol. 15*, 43–56.

Somme-Martin, G., Colardeau, J., Lafont, R., **1988**. Conversion of ecdysone and 20-hydroxyecdysone into 3-dehydroecdysteroids is a major pathway in third instar *Drosophila melanogaster* larvae. *Insect Biochem. 395*, 398–402.

Song, Q., Alnemri, E.S., Litwack, G., Gilbert, L.I., **1997**. An immunophilin is a component of the insect ecdysone receptor (EcR) complex. *Insect Biochem. Mol. Biol. 27*, 973–982.

Song, Q., Gilbert, L.I., **1998**. Alterations in ultraspiracle (Usp) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol. 28*, 849–860.

Song, Q., Sun, X., Jin, X.Y., **2003**. 20E regulated Usp expression and phosphorylation in *Drosophila melanogaster. Insect Biochem. Mol. Biol.* 1211–1218.

Spindler-Barth, M., Spindler, K-D., **1998**. Ecdysteroid resistant subclones of the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). I. Selection and characterization of resistant clones. *In Vitro Cell Devel. Biol. Anim. 36*, 116–122.

Stallcup, M.R., **2001**. Role of protein methylation in chromatin remodeling and transcriptional regulation. *Oncogene 20*, 3014–3020.

Stathopoulos, A., Levine, M., **2002**. Whole-genome expression profiles identify gene batteries in *Drosophila*. *Devel. Cell 3*, 464–465.

Staudinger, J., Goodwin, B., Jones, S.A., Hawkins-Brown, D., MacKenzie, K.I., *et al.*, **2001**. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc. Natl Acad. Sci. USA 98*, 3369–3374.

Stocker, A.J., Pavan, C., **1977**. Developmental puffing patterns in salivary gland chromosomes of *Rhynchosciara hollanderi*. *Chromosoma 62*, 17–47.

Stone, B.L., Thummel, C.S., **1993**. The *Drosophila* 78C early late puff contains E78, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone receptor superfamily. *Cell 75*, 307–320.

Strangmann-Diekmann, M., Klone, A., Ozyhar, A., Kreklau, F., Kiltz, H-H., *et al.*, **1990**. Affinity labeling of a partially purified ecdysteroid recpetor with a bromoacetylated 20-OH ecdysone derivative. *Eur. J. Biochem. 189*, 137–145.

Sung, C., Robinow, S., **2000**. Characterization of the regulatory elements controlling neuronal expression of the

A-isoform of the ecdysone receptor gene of *Drosophila melanogaster. Mech. Devel. 91*, 237–248.

Suhr, S.T., Gil, E.B., Senut, M-C., Gage, F.H., **1998**. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl Acad. Sci. USA 95*, 7999–8004.

Sullivan, A.A., Thummel, C.S., **2003**. Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. *Mol. Endocrinol. 17*, 2125–2137.

Sutherland, J.D., Kozlova, T., Tzertzinis, G., Kafatos, F.C., **1995**. *Drosophila* hormone receptor 38: a second partner for *Drosophila* Usp suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. *Proc. Natl Acad. Sci. USA 92*, 7966–7970.

Swevers, L., Cherbas, L., Cherbas, P., Iatrou, K., **1996**. *Bombyx* EcR (BmEcR) and *Bombyx* Usp (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Mol. Biol. 26*, 217–221.

Swevers, L., Drevet, J.R., Lunke, M.D., Iatrou, K., **1995**. The silkmoth homolog of the *Drosophila* ecdysone receptor (B1 isoform): cloning and analysis of expression during follicular cell differentation. *Insect Biochem. Mol. Biol. 25*, 857–866.

Swevers, L., Iatrou, K., **2003**. The ecdysone regulatory cascade and ovarian development in lepidopteran insects: insights from the silkmoth paradigm. *Insect Biochem. Mol. Biol. 33*, 1285–1297.

Talbot, W.S., Swywryd, E.A., Hogness, D.S., **1993**. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell 73*, 1323–1337.

Thomas, H.E., Stuttenberg, H.G., Stewart, A.F., **1993**. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptors and ultraspiracle. *Nature 362*, 471–475.

Thummel, C.S., **2002**. Ecydsone-regulated puff genes 2000. *Insect Biochem. Mol. Biol. 32*, 113–120.

Toya, T., Fukasawa, H., Masui, A., Endo, Y., **2002**. Potent and selective partial ecdysone agonist activity of chromafenozide in Sf9 cells. *Biochem. Biophys. Res. Commun. 292*, 1087–1091.

Tran, H.T., Askari, H.B., Shaaban, S., Price, L., Palli, S.R., *et al.*, **2001b**. Reconstruction of ligand-dependent transactivation of *Choristoneura fumiferana* ecdysone receptor in yeast. *Mol. Endocrinol. 15*, 1140–1153.

Tran, H.T., Shaaban, S., Askari, H.B., Walfish, P.G., Raikhel, A.S., *et al.*, **2001a**. Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J. Mol. Endocrinol. 27*, 191–209.

Trisyono, A., Goodman, C.L., Grasela, J.J., McIntosh, A.H., Chippendale, G.M., **2000**. Establishment and characterization of an *Ostrinia nubilalis* cell line and its response to ecdysone agonists. *In Vitro Cell. Devel. Biol. 36*, 300–404.

Truman, J.T., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., **1994**. Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development 120*, 219–234.

Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M., Evans, R.M., **1999**. SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR mediated repression is critical for development. *Mol. Cell. 4*, 175–186.

Tzertzinis, G., Malecki, A., Kafatos, F.C., **1994**. BmCF1, a *Bombyx mori* RXR-type receptor related to the *Drosophila* ultraspiracle. *J. Mol. Biol. 238*, 479–486.

Uhlirova, M., Foy, B.D., Beaty, B.J., Olson, K.E., Riddiford, L.M., *et al.*, **2003**. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proc. Natl Acad. Sci. USA 23*, 15607–15612.

Unger, E., Cigan, A.M., Trimnell, M., Xu, R.J., Kendall, T., *et al.*, **2002**. A chimeric ecdysone receptor facilitates methoxfenozide-dependent restoration of male fertility in ms45 maize. *Transgenic Res. 11*, 455–465.

Verras, M., Gourzi, P., Zacharapoulou, A., Mintzas, A.C., **2002**. Developmental profiles and ecdysone regulation of the mRNAs for two ecdysone receptor isoforms in the Mediterranean fruit fly *Ceratitis capitata*. *Insect. Mol. Biol. 11*, 553–565.

Vogtli, M., Elke, C., Imhof, M.O., Lezzi, M., **1998**. High level transactivation by the ecdysone receptor complex at the core recognition motif. *Nucleic Acids Res. 10*, 2407–2414.

Vogtli, M., Imhof, M.O., Brown, N.E., Rauch, P., Spindler-Barth, M., *et al.*, **1999**. Functional characterization of two Ultraspiracle forms (CtUsp-1 and CtUsp-2) from *Chironomus tentans*. *Insect Biochem. Mol. Biol. 29*, 931–942.

vonKalm, L., Crossgrove, K., Von Seggern, D., Guild, G.M., Beckendorf, S.K., **1994**. The Broad-Complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J. 13*, 3505–3516.

Walker, V.K., Ashburner, M., **1981**. The control of ecdysterone-regulated puffs in *Drosophila* salivary glands. *Cell 26*, 269–277.

Wang, S-F., Ayer, S., Segraves, W.A., Williams, D.R., Raikhel, A.S., **2000**. Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol. Cell. Biol. 20*, 3870–3879.

Wang, S.F., Li, C., Sun, G., Zhu, J., Raikhel, A.S., **2002**. Differential expression and regulation by 20-hydroxyecdysone of mosquito ecdysteroid receptor isoforms A and B. *Mol. Cell. Endocrinol. 196*, 29–42.

Wang, S-F., Miura, K., Miksicek, R.J., Segraves, W.A., Raikhel, A.S., **1998**. DNA-binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex. *J. Biol. Chem. 273*, 27531–27540.

Weller, J., Sun, G.C., Zhou, B., Lan, Q., Hiruma, K., *et al.*, **2001**. Isolation and developmental expression of two nuclear receptors, MHR4 and βFTZ-F1, in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 22*, 827–837.

Wegmann, I.S., Quack, S., Spindler, K-D., Dorsch-Hasler, K., Vogtli, M., *et al.*, **1995**. Immunological studies on

the developmental and chromosomal distribution of ecdysteroid receptor proteins in *Chironomus tentans*. *Arch. Insect Biochem. Physiol. 30*, 95–114.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., **1997**. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science 276*, 114–117.

White, K.P., Rifkin, S.A., Hurban, P., Hogness, D.S., **1999**. Microarray analysis of *Drosophila* development during metamorphosis. *Science 286*, 2179–2184.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., *et al.*, **1995**. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Devel. 9*, 1033–1045.

Wing, K.D., **1988**. RH5849, a nonsteroidal ecdysone agonist: effects on a *Drosophila* cell line. *Science 241*, 467–469.

Woodard, C.T., Baehrecke, E.H., Thummel, C.S., **1994**. A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell 18*, 607–615.

Wurtz, J.-M., Guilot, B., Fagart, J., Moras, D., Tietjen, K., *et al.*, **2000**. A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: a homology modeling and docking approach. *Protein Sci. 9*, 1073–1084.

Yao, T., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., *et al.*, **1992**. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell 71*, 63–72.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., *et al.*, **1993**. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. *Nature 366*, 476–479.

Yund, M.A., Osterbur, D.L., **1985**. Ecdysteroid receptors and binding proteins. In: Kerkur, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 7, Endocrinology I. Pergamon, Oxford, pp. 473–490.

Zelhof, A.C., Ghbeish, N., Tsai, C., Evans, R.M., McKeown, M., **1997**. A role for Ultraspiracle, the *Drosophila* RXR, in morphogenetic furrow movement and photoreceptor cluster formation. *Development 124*, 2499–2505.

Zelhof, A.C., Yao, T.P., Chen, J.D., Evans, R.M., McKeown, M., **1995a**. Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. *Mol. Cell. Biol. 15*, 6736–6745.

Zelhof, A.C., Yao, T., Evans, R.M., McKeown, M., **1995b**. Identification and characterization of a *Drosophila* nuclear receptor with the ability to inhibit the ecdysone response. *Proc. Natl Acad. Sci. USA 92*, 10477–10481.

Zheng, X., Wang, J., Haerry, T.E., Wu, A.Y-H., Martin, J., *et al.*, **2003**. TGF-B signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell 112*, 303–315.

Zhou, Z., Riddiford, L., **2002**. Broad specifies pupal development and mediates the ‘status quo’ action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development 129*, 2259–2269.

Zhu, J., Miura, K., Chen, L., Raikhel, A.S., **2000**. AHR38, a homolog of NGF1-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*. *EMBO J. 19*, 253–262.

Zhu, J., Miura, K., Chen, L., Raikhel, A.S., **2003a**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue, Ultraspiracle. *Proc. Natl Acad. Sci. USA 100*, 544–549.

Zhu, J., Chen, L., Raikhel, A.S., **2003b**. Posttranscriptional control of the competence factor βFTZ-F1 by juvenile hormone in the mosquito *Aedes aegypti*. *Proc. Natl Acad. Sci. USA 100*, 13338–13343.

Zinke, I., Schutz, C.S., Katzenberger, J.D., Bauer, M., Pankratz, M.J., **2002**. Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *EMBO J. 21*, 6162–6173.

3.6 Evolution of Nuclear Hormone Receptors in Insects

V Laudet and F Bonneton, Ecole Normale Supérieure de Lyon, Lyon, France

3.6.1. Introduction

Nuclear receptors form a superfamily of ligand activated transcription factors, which regulate various physiological functions from development or reproduction to homeostasis and metabolism (Laudet and Gronemeyer, 2002). This superfamily is present in all metazoans, and only in metazoans – no nuclear receptors have been found in the complete genome sequences currently available for plants, fungi, or unicellular eukaryotes (Escriva *et al.*, in press). In insects, nuclear receptors are implicated in embryogenesis as well as in the control of the postembryonic development, namely molting and metamorphosis (Kozlova and Thummel, 2000) (see **Chapters 3.3** and **3.5**). One of the most important insect hormones, 20-hydroxyecdysone (20E) is a ligand for a nuclear receptor, the ecdysone receptor (EcR) (Koelle *et al.*, 1991) (see **Chapter 3.5**). Although EcR is commonly called the ecdysone receptor, it is really the receptor for the principal molting hormone of insects, 20E. Ecdysone is in most cases the precursor of 20E. Although it is strongly debated, it is interesting to note that several studies have suggested that juvenile hormone (JH), a hormone that counteracts the action of ecdysone, might also act through nuclear receptors (Jones and Sharp, 1997) (see **Chapter 3.7**). It is of note that the superfamily contains not only receptors for known ligands but also a large number of so-called orphan receptors for which ligands do not exist or have not been identified. It is not known whether all of these orphan receptors indeed have a ligand, if they activate transcription in a constitutive manner, or if they have alternative transcriptional regulation mechanisms (Giguere, 1999). In insects, many orphan receptors are direct target genes of the EcR and participate in the gene regulatory hierarchy that controls metamorphosis.

Nuclear receptors are modular proteins, with three major domains arranged linearly from the N-terminus to the C-terminus (**Figure 1**). At the N-terminus there is a transactivation domain, most often called AF-1, which is of variable length and sequence in the different family members and which is recognized by other proteins in the transcription complex. The DNA binding domain (DBD), the site for specific interaction with DNA, is in the mid region of the protein and harbors two zinc finger motifs common to all members of the family. This domain is absent in two divergent members of the superfamily DAX-1 and SHP that are found only in vertebrates (Laudet and Gronemeyer, 2002). The C-terminal contains the ligand binding domain (LBD), whose overall architecture is well conserved between the various family members but which is nonetheless different enough to permit the high degree of ligand specificity characteristic of each member of the family. This domain harbors a ligand dependent activation function, called AF-2, that is responsible for the recruitment of transcriptional coactivators. As with DBD, the LBD is absent in

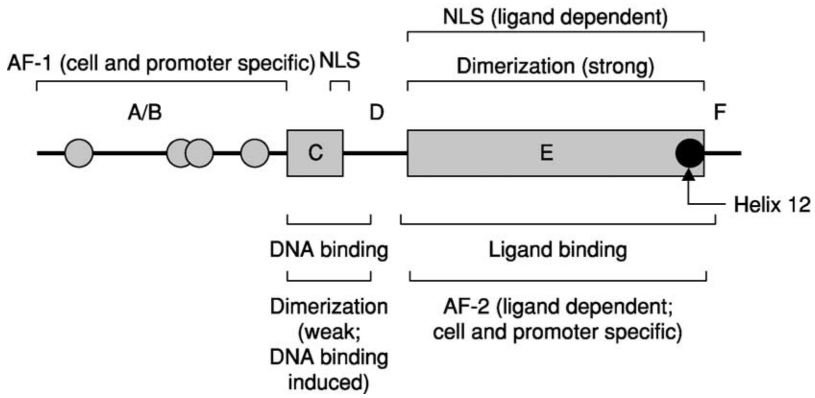


Figure 1 Schematic illustration of the structural and functional organization of nuclear receptors. The evolutionary conserved regions C and E are indicated as boxes and a black bar represents the divergent regions A/B, D, and F. Note that region F may be absent in some receptors. The functions of the domains are depicted below and above the scheme; most of these are derived from analyses of vertebrate receptors. Within the transcription activation functions, AF-1 and AF-2, autonomous transactivation domains have been defined in most receptors. These are depicted as circles in the A/B and E domain. The activation domain of the E region has been mapped within the C-terminal helix 12. Note that there are many receptor genes that encode different isoforms harboring different A/B regions, and thus different AF-1 and activation domains.

some divergent members of the superfamily such as the Knirps group in insects (Laudet and Gronemeyer, 2002).

The three-dimensional structure of the LBD has been determined for several nuclear receptors, including both unliganded (apo) or liganded (holo) forms, allowing increased understanding of the mechanisms involved in ligand binding and transactivation functions (**Figure 2**). Interestingly, the structure of the EcR-USP LBD heterodimer from the moth *Heliothis virescens* has recently been established (Billas *et al.*, 2003). These crystal structures show that the E domain undergoes a major conformational change upon ligand binding, allowing the interaction with coactivators and the transactivation of target genes. Many nuclear receptors are transcriptional silencers in the absence of ligand (apo-receptor) as a result of interaction with intermediary factors (i.e., corepressors). The determination of the crystal structure of nuclear receptors has shown that the LBD region is made up of a three-layered α-helical antiparallel sandwich of 11–12 helices forming a hydrophobic pocket (Wurtz *et al.*, 1996). Upon ligand binding (holo-receptor), the ligand makes different contacts with amino acid residues, promoting a conformational change that closes the “lid” (helix 12, H12) on the pocket and the corepressor complex dissociates. Thus, the activation domain within the H12 (AF2-AD) is able to interact with coactivators and promotes transcription of target genes. The conformational change of the LBD upon ligand binding is therefore necessary for the transactivation function of nuclear receptors (Steinmetz *et al.*, 2001; Laudet and Gronemeyer, 2002).

Nuclear receptors bind as homodimers or heterodimers to the regulatory regions of target genes (usually to the sequence PuGGTCA) called the hormone response element (HRE) (Laudet and Gronemeyer, 2002) (see **Chapter 3.5**). However, mutation, extension, and duplication, as well as distinct relative orientations of repeats of this motif, generate response elements that are selective for a given (class of) receptor(s). For example, steroid receptors bind as homodimers to HREs containing two core motifs separated by 1–3 nucleotides and organized as palindromes. Other receptors (e.g., RARs, TRs, HR38) heterodimerize with retinoid X receptors (RXRs, homologous to the insect *USP* gene) and bind to direct repeats (DRs) of the core motif. The spacing between the two halves of the DR dictates the type of heterodimer that will bind (e.g., a DR separated by five nucleotides (DRs) will be recognized by RXR:RAR and a DR4 by RXR:TR). Some receptors, such as the FTZ-F1, HR3, or E75 orphan receptors, can bind to DNA as monomers through a single core sequence. In this case, an A/T-rich region 5′ to the core element governs the binding specificity (Laudet and Adelmant, 1995).

The development of nuclear receptor research has largely been driven using data from mouse and *Drosophila* and it is interesting to note that the knowledge from these two organisms are largely complementary. In the mouse, many *in vitro* functional data are available and much effort has been deployed to analyze the mode of action of nuclear

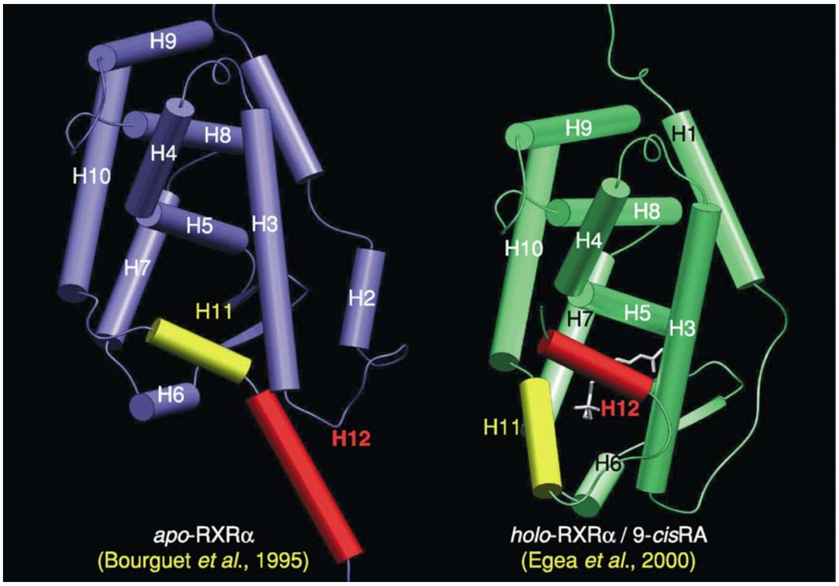


Figure 2 Three-dimensional structure of RXR in the apo or holo form. There are 11 helices that form a compact structure with a ligand binding pocket, the size of which varies between the various family members. The entrance to the pocket is guarded by a twelfth helix (H12), which forms a movable lid over the pocket and contains residues critically required for the function of AF2. The orientation of H12 is determined by the size and shape of the ligand occupying the pocket. In most cases, without ligand the LBD interacts with corepressors through a region called the CoRNR box in the region of helices H3 and H4. The ligand promotes a complex conformational change inducing the repositioning of H12, modifying the CoRNR box and thus leading to corepressor complex dissociation. This repositioning of H12 also allows the recruitment of coactivators involving the interaction with short LxxLL-like motifs called ''NR boxes'' present in most coactivators. The LxxLL motif recognizes a hydrophobic cleft formed by H3, H4, and H12 that has an important role in stabilizing this LxxLL-LBD interaction. The conformational change of the LBD upon ligand binding is therefore necessary for the transactivation function of NRs. (Data from Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., Moras, D., **1995**. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature 375*, 377–382; and Egea, P.F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., *et al.*, **2000**. Crystal structure of the human RXRα ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *EMBO J. 19*, 2592–2601.)

receptors. Indeed, much of our knowledge on how nuclear receptors modulate transcription comes from studies on mammalian cell systems. In contrast, *Drosophila* work has provided essentially an *in vivo* oriented picture of the developmental role of nuclear receptors. Nevertheless, two important perspectives are missing from the field. First, very few structure/function analyses are available for insect (including *Drosophila*) nuclear receptors, most often what we know of their mode of action being derived from data obtained on mammals. This obviously hampers our global understanding of insect nuclear receptor action *in vivo*. Second, most of the data concerning nuclear receptors in insects arise from very few model systems, mainly *Drosophila* and other Diptera or lepidopterans such as *Bombyx mori* and *Manduca sexta*.

Taking these limitations into account, this chapter aims to put our current knowledge of insect nuclear receptors in a broader evolutionary perspective. This will be done first by discussing insect nuclear receptors in the context of metazoan phylogeny and evolution of the whole nuclear receptor superfamily. In the second part, we focus on the evolution of nuclear receptors in arthropods, providing specific data on liganded and orphan receptors in this phylum. Nevertheless, we must emphasize that an enormous amount of functional, genetic, and structural data is available on nuclear receptors and it is unrealistic to attempt to present a complete view of this exponentially expanding field. The interested reader can find several excellent reviews (Truman and Riddiford, 2002; Thummel, 2001 among others, as well as many chapters in this volume (see **Chapters 3.7** and **3.9**)) and the web specific information in Nurebase, a database specializing in nuclear receptors maintained in Lyon (Nurebase; Duarte *et al.*, 2002; Ruau *et al.*, 2004).

3.6.2. Nuclear Receptors in the Animal Kingdom

3.6.2.1. The Phylogeny of Nuclear Receptors

3.6.2.1.1. Classification of the nuclear receptors Molecular phylogeny has been applied to better delineate the evolutionary history of the nuclear

receptor superfamily (Amero *et al.*, 1992; Laudet *et al.*, 1992; Laudet, 1997; Thornton and DeSalle 2000; review: Escriva Garcia *et al.*, 2003). Since two main functional domains, the DBD and the LBD, are conserved in most nuclear receptors, phylogenetic trees have been produced using each of these domains alone or in combination. It is now clear that the DBD alone is too short and too conserved to contain a significant phylogenetic signal. Thus, if DBD-based trees contain accurate information on the grouping of closely related receptors (such as TRs and RARs) they are unable to correctly decipher deep relationships. Using the LBD alone provides a better resolution of the phylogeny but this is less robust than the phylogenies arising from the use of both DBD and LBD. Thus, despite several attempts it has been very difficult to compare the evolution of the DBD and the LBD using their phylogenies. The question of some degree of independence between the two domains during evolution is still open (see conflicting views in Escriva *et al.*, 2000 and Thornton and DeSalle, 2000). All the trees discussed in this review were constructed using both domains. It is important to note that several phylogenetic methods, namely maximum parsimony, distance analysis using neighbor-joining, or probabilistic approaches using maximum likelihood, have been used to produce nuclear receptor phylogeny. Despite some differences in specific regions of the trees (respective placement of ER, ERR, and steroid receptors, for example; Laudet, 1997; Thornton *et al.*, 2003), the topology of these trees and the conclusions that can be drawn from them are very similar. Thus, the phylogeny of the nuclear receptor superfamily is now relatively consensual and widely accepted.

Based on these results, the family has been divided into six subfamilies that are supported by high bootstrap values (**Figure 3**). It is worth noting that the relationships between these subfamilies are not supported and it is impossible to say which one is the most ancient, based only on sequence phylogeny.

1. The first subfamily contains several important liganded groups of nuclear receptors such as the thyroid hormone receptors (TRs), the retinoic acid receptors (RARs), the PPARs, the xenobiotic receptors PXR and CAR with their homolog in insect DHR96 (which is still considered an orphan receptor), the bile acid receptor FXR, the ecdysone receptor (ECR), and the oxysterol receptors (LXRs). In addition, this subfamily also contains two groups of orphan receptors, Rev-erb/E75 and ROR/HR3. It is interesting to note that ligands (namely cholesterol and retinoic acid) were recently proposed for the RAR-related orphan receptor (ROR) (Stehlin-Gaon *et al.*, 2003; Kallen *et al.*, 2002). The relationship between these groups is poorly resolved: if TR and RAR appear very often to cluster together with weak bootstrap values, such as for REV-ERB and ROR, the only clear and robust grouping joins LXR, FXR, and PXR groups.
2. The second subfamily clusters RXR, the 9-cis retinoic acid receptor, and its arthropod homolog USP that apparently does not bind this compound, and many groups of orphan receptors: TR2 and TR4 and their *Drosophila* homolog HR78, HNF4, the TLL group (with four genes in *Drosophila*: *TLL*, *DSF*, *PNR*, and *FAX1*), and the COUP-TF/SVP group. Of note is the presence of unusual receptors that contain only the LBD sequence, DAX and SHP clearly being members of this subfamily, probably related to TLL receptors (Laudet, 1997).
3. The third subfamily clusters steroid receptors (SRs), the estrogen receptor (ER), and the estrogen related receptors (ERRs) that are still orphan receptors. There is one ERR homolog in *Drosophila* but not for SRs and ERs. As their names indicate ERRs appear more closely related to ERs than to steroid receptors but this view has been challenged recently by data suggesting that ERs and SRs cluster together and are then joined by ERR. The respective placement of SRs, ERs, and ERRs is an important issue regarding ligand binding evolution and is still under discussion.
4. The fourth subfamily contains only the NGFIB group of orphan receptors with their unique arthropod homolog HR38.
5. The fifth subfamily contains the orphan receptor SF1 group, which contains a *Drosophila* homolog, FTZ-F1, as well as another gene, DHR39, whose origin is still unclear.
6. The last subfamily contains the orphan receptor GCNF with its ortholog in *Drosophila*, HR4.

It is important to note that the placement of three *Drosophila* genes that contain no LBD (KNI, KNRL, and EAGLE) is still unclear. They may be related to HR96 but, given the poor phylogenetic information present in the DBD sequences, it will be difficult to resolve their origin using phylogenetic analysis alone (Laudet, 1997).

Given the plethora of names available for the same sequence, it has proved useful to construct a nomenclature based on the nuclear receptor sequences using molecular phylogeny (Nuclear Receptor Nomenclature Committee, 1999). The system is based on the nomenclature system that was developed

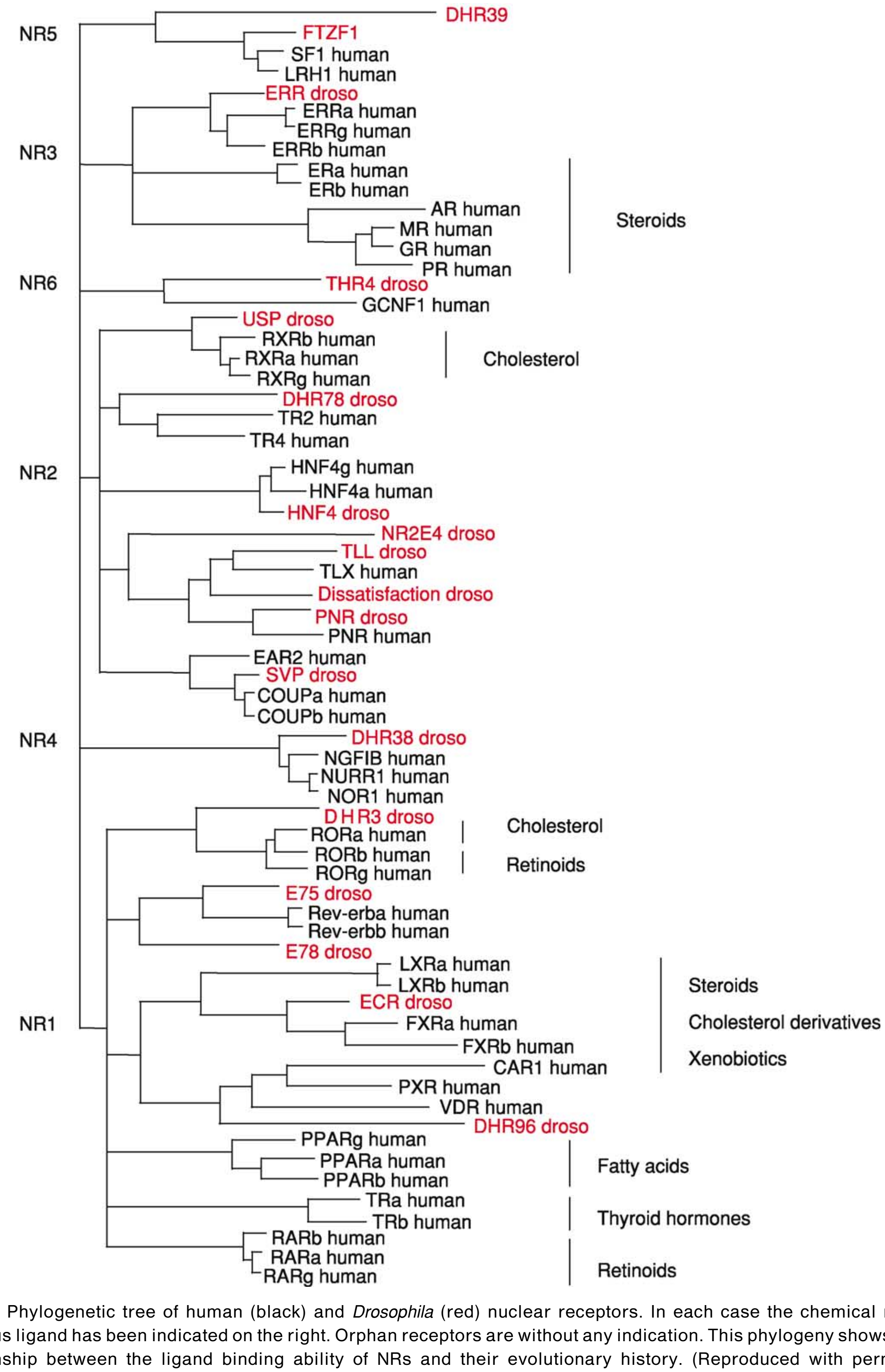


Figure 3 Phylogenetic tree of human (black) and *Drosophila* (red) nuclear receptors. In each case the chemical nature of the endogenous ligand has been indicated on the right. Orphan receptors are without any indication. This phylogeny shows that there is no relationship between the ligand binding ability of NRs and their evolutionary history. (Reproduced with permission from Robinson-Rechavi, unpublished data.)

for cytochrome P450. Each receptor is described by the letters NR (for "nuclear receptor") and a three-digit nomenclature: the subfamily to which a given receptor belongs is indicated by Arabic numbers, groups by capital letters, and individual genes by Arabic numbers. Thus, the *Drosophila* EcR that belongs to subfamily I, to group H in this subfamily, and which is the first gene described in that group

will be called NR1H1. The receptors that contain only one of the two functional domains (KNI group, DAX/SHP, and some nematode receptors such as Odr-7) are artificially clustered in subfamily 0. This system has proven to be flexible enough to integrate nuclear receptors from invertebrates as well as sequences generated from genome projects for which no pharmacological data are yet available. In order to follow the evolution of this nomenclature, the new names are available on a specific website (http://www.ens-lyon.fr/LBMC/laudet/nomenc.html) as well as on Nurebase.

3.6.2.1.2. Evolution of nuclear receptor functions It is always interesting to compare results from phylogenetic analysis with functional or morphological features of the group of sequences or organisms that are compared. For nuclear receptors, evolution of the superfamily with major functional characterizations of the receptors, namely DNA binding, dimerization abilities, and ligand binding (reviews: Escriva *et al*., 2000; Escriva *et al*., in press), is compared.

As discussed above, the vast majority of nuclear receptors recognize the core sequence RGGTCA organized either as DRs, inverted repeats (mostly palindromes), or alone with an upstream A/T-rich sequence. Nuclear receptors bind to these elements as homodimers, heterodimers, or monomers. In most cases, heterodimers are formed with RXRs (or USP in insects). When these functional characteristics are compared with the tree there is a very good correlation with the evolutionary history. For example, all members of subfamily II are able to form homodimers on direct repeat sequence, whereas all members of subfamily III can form homodimers on palindromic elements. Monomeric receptors able to bind unique RGGTCA sequences are found in many places on the tree (subfamilies I, IV, and V, for example). One of the most striking cases is the RXRs that are able to form heterodimers only with members of subfamilies I and IV. The only exception to the rule could be the case of COUP-TF and SVP, which have been described as being able to interact with RXRs (Kliewer *et al*., 1992; Cooney *et al*., 1993). Nevertheless, this finding is strongly debated and it is not clear whether this interaction represents a real heterodimer formation or could be viewed more as a classical protein–protein interaction (Butler and Parker, 1995). The only physiologically relevant data in favor of this interaction is the ECR-USP/SVP interaction found in *Aedes aegypti* (Miura *et al*., 2002; Zhu *et al*., 2003). It should be noted that the dimer interface of this complex has not been mapped and thus we cannot be sure that it represents a real heterodimer, such as RXR-TR. Despite this, it is clear that the DNA binding and dimerization abilities are reasonably well correlated with the evolutionary history, suggesting that the first nuclear receptor was able to bind the RGGTCA core sequence, most probably as a homodimer recognizing direct repeat sequences, and that subsequently there was divergence on this theme following the various gene duplications that gave rise to the present diversity of nuclear receptors.

A comparison of the phylogenetic tree with the ligand binding ability of the nuclear receptors reveals a very different picture. The following observations clearly suggest that the evolutionary relationships of nuclear receptors and the nature of their ligands are not correlated:

i. the presence of nuclear receptors for different ligands within subfamilies (e.g., TRs and RARs, which bind totally different compounds, are related inside subfamily I);
ii. closely related ligands recognized by different receptors (e.g., retinoids, which are ligands for RARs, RXRs, and RORβ); and
iii. the widespread distribution of orphan receptors in the phylogenetic tree.

This situation could be explained by an independent gain of ligand binding capacity during nuclear receptor evolution. This model implies that the first member of the nuclear receptor superfamily was an orphan receptor. In accordance with this notion, nuclear receptor sequences described from early metazoans are mostly orthologs of orphan receptors (Escriva Garcia *et al*., 1997, 2003; Grasso *et al*., 2001) with the notable exception of the RXR, whose endogenous ligand is still a matter of controversy (Kostrouch *et al*., 1998; Wiens *et al*., 2003). Although this model is not discussed in detail here, it is interesting to briefly mention some of its implications.

The C-terminal moiety of the nuclear receptor, which is called the ligand binding domain, is well conserved when compared across the whole superfamily, including orphan receptors for which structural data suggest an absence of ligand (e.g., NURR1; Wang *et al*., 2003). We know that the LBD carries a transcriptional activation domain that can be turned on by a conformational change that allows coactivator binding. It is clear that this conformational change can be induced not only by ligand binding but also by other mechanisms. For example, it is well known that phosphorylation events in the LBD can induce this conformational change (review: Weigel and Zhang, 1998). Alternatively, protein–protein interactions (such as the one occurring between SF1 and Ptx1 on two SF1

target genes), pituitary luteinizing hormone beta and Mullerian inhibiting substance may also induce coactivator binding (Tremblay *et al.*, 1999). Indeed, the Evans laboratory has suggested that the LBD exists in an equilibrium between an OFF and an ON conformation and that ligand binding directs this equilibrium in favor of the ON (holo) conformation (Schulman *et al.*, 1996). In conclusion, it is believed that ligand binding is only one of the possible switches and that this equilibrium can also be modified by phosphorylation or protein–protein interactions, among other mechanisms. Thus, it is suggested that the sequence conservation of the LBD is not the result of the conservation of ligand binding activity but is rather the hallmark of the existence of this conformational change in all nuclear receptors. The LBD should in fact be viewed as a domain responsible for an allosteric modification of the receptor allowing the exchange between corepressor and coactivator complexes. Seen in this perspective, it is not surprising that the LBD is conserved in orphan receptors.

Recent structural data have shed new light on the ligand binding ability of nuclear receptors. First, there is now substantial evidence that "real" orphan receptors (i.e., nuclear receptors that are not recognized by cognate endogenous ligands) may exist. This is the case for NURR1 and its *Drosophila* homolog HR38, which harbors a LBD folded in an active conformation but with a putative ligand binding pocket filled by large hydrophobic side chains, such that it is difficult to imagine a ligand occupying the same space (Baker *et al.*, 2003; Wang *et al.*, 2003). Other recent reports suggest that in some cases a structural "ligand" (e.g., fatty acid for *Drosophila* USP or for HNF4) may be present inside the LBD without playing any functional role (Billas *et al.*, 2001; Clayton *et al.*, 2001; Wisely *et al.*, 2002). In other cases (ERRγ and LRH-1), empty pockets are found in the nuclear receptor, which nevertheless has an active conformation that can be recognized by coactivators (Greschik *et al.*, 2002; Sablin *et al.*, 2003). Thus, the LBD of orphan nuclear receptors can contain pockets filled with side chain residues, pockets with structural ligands or empty pockets, demonstrating an unanticipated large number of possible states between receptors for well-known hormones and orphan receptors. This may render it difficult to define with precision endogenous ligands for a given receptor. Such a debate is currently raging about the RXR and its pharmacological ligand, 9-*cis* retinoic acid.

All these structural results may have evolutionary implications. It has been proposed that the first nuclear receptor had an exogenous molecule such as a fatty acid playing a structural role buried inside its LBD (Sladek, 2002). This compound cannot be called a ligand as it cannot regulate the activity of the receptor and cannot even exit the LBD. A receptor enclosing such a molecule could thus be considered as an orphan receptor such as the *Drosophila* USP. Second, some of these orphan receptors may have weakened the interaction between these structural ligands and the LBD and "invented" ligand regulated transcriptional activity (**Figure 4**). Although tempting, this model cannot be generalized and is not widely accepted at present, as recent structural data have shown that LBDs with an empty pocket or with no

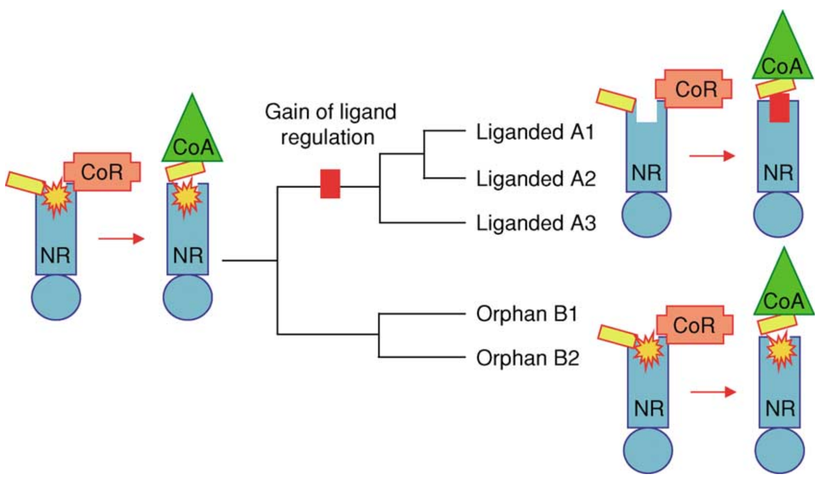


Figure 4 A possible scheme for the evolution of ligand binding abilities of nuclear receptors. The original nuclear receptor may have been able to bind a lipophilic compound that was a ''structural ligand,'' i.e., an integral component of the protein structure. In contrast, modern ligands are interchangeable elements that induce allosteric functional changes of the LBD. This model may explain why the LBD has been conserved during evolution. NR, nuclear receptor; CoR, corepressor; CoA, coactivator. Helix H12 is shown as a yellow box, the ligand as a red box, and the structural ligand as an irregular yellow form.

pocket are possible and apparently stable. The existence of "structural ligands" is thus perhaps less frequent than anticipated.

3.6.2.2. Metazoan Phylogeny and Nuclear Receptors: the Ecdysozoan Problem

3.6.2.2.1. Distribution of nuclear receptors in metazoans Recent molecular data have considerably refreshed our ideas and concepts on metazoan phylogeny (Aguinaldo *et al.*, 1997; de Rosa *et al.*, 1999). **Figure 5** depicts the present consensus on metazoan phylogeny. Although lively debates are ongoing on many aspects of this tree, several points should nevertheless be briefly discussed in relation to nuclear receptor evolution. The focus will be on the main metazoan phyla since for the vast majority of the 35 phyla no sequence information was available except some classical phylogenetic markers such as mitochondrial genes or ribosomal rRNA genes.

1. The identity of the unicellular eukaryote that is most closely related to metazoans has long been a mystery, but it is now clear that choanoflagellates, which share several cytological and molecular synapomorphies with metazoans, are the sister group of metazoans. Despite some ancient claims based on cross hybridization data or poor sequence identities, it is clear that nuclear receptors are not found outside the Metazoa. There is no known nuclear receptor homolog in the various plant or fungi genomes available and since more than a dozen such genomes are now available, the hypothesis of a secondary loss can be excluded. But this information is not sufficient to conclude that nuclear receptors are specific to metazoans. Indeed, nothing is known about the presence of nuclear receptors in choanoflagellates. Expressed sequence tag (EST) projects in choanoflagellates have not revealed nuclear receptors, but these genes are usually not frequent in these data because of a relatively low level of expression (King *et al.*, 2003). Indeed, the isolation of a nuclear receptor from the sister group of the Metazoa could illuminate our views on nuclear receptor origins, which remains obscure. Several reports have suggested low scores of sequence identity for the LBD and DBD regions of nuclear receptors with a peroxisomal membrane protein, Pex11p, and the LIM/GATA zinc finger domain, respectively. This has prompted some groups to suggest that the first nuclear receptor arose from the fusion of two genes encoding these various proteins (Clarke and Berg, 1998; Barnett *et al.*, 2000). In the absence of structural data that can confirm the significance of these low similarity scores, it is hard to draw firm conclusions on this matter. The question of the origin of the first nuclear receptor is thus still open.
2. The metazoans are monophyletic and sponges (Porifera) are clearly the first group that diverged from the metazoan trees. It is now believed that the sponges are not a monophyletic group and the precise phylogeny of the early metazoan tree is far from being resolved but the basal position of sponges when compared to cnidarians is widely accepted. Cnidarians (sea anemone, corals, *Hydra*) and other related groups such as the Ctenophora diverged later on.

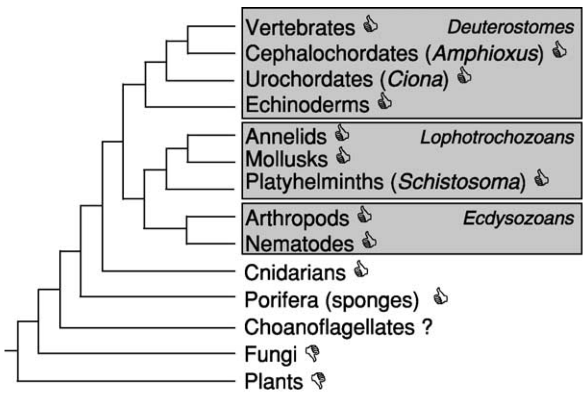


Figure 5 Simplified phylogeny of metazoans. Clades where NRs have been isolated are labeled with 👍, clades where complete genome sequences exist without any NR gene are labeled with 👎, and clades where the presence of NRs is not yet known are labeled with ? (Modified from Aguinaldo, A.M., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., *et al.*, **1997**. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature 387*, 489–493; and Adoutte, A., Balavoine, G., Lartillot, N., de Rosa, R., **1999**. Animal evolution: the end of the intermediate taxa? *Trends Genet. 15*, 104–108.)

The presence of nuclear receptors in sponges has long been debated. In a polymerase chain reaction (PCR) screen, we found nuclear receptor signatures in all animal phyla analyzed from diploblastic animals (e.g., cnidarians; Escriva Garcia *et al.*, 1997) to vertebrates, but not in sponges. Recently, however, Werner Muller's group reported the presence of an RXR ortholog in the sponge *Suberites domuncula* (Wiens *et al.*, 2003). Even if the precise identity of this receptor is unclear, it is undoubtedly a member of subfamily II that currently contains, to the exclusion of the RXR, only orphan receptors and which appears to be the most ancient one.

The ancestry of subfamily II has been clearly established in our PCR screen that yields subfamily II homologs (COUP-TF, RXR) together with a subfamily V homolog (FTZ-F1) in *Hydra* and *Anemonia*, two cnidarians (Escriva Garcia *et al.*,

1997). This has been confirmed by the isolation of several nuclear receptors (RXR (NR2B), TR2/4 (NR2C), TLL (NR2E), COUP-TF (NR2F)) in various cnidarians such as *Montipora verrucosa, Tripedalia cystophora*, and *Acropora millepora* (Kostrouch *et al*., 1998; Grasso *et al*., 2001). Of note, is the presence of subfamily V members in cnidarians, which has not been confirmed by independent evidence and should thus be regarded as tentative. Thus, only subfamily II is clearly present in all metazoans, suggesting that the first nuclear receptor and thus the origin of the superfamily probably belonged to this subfamily.

3. The bilaterians, metazoans that possess three embryonic layers and bilateral symmetry, are split in two main groups, the protostomians and deuterostomians, which differ on many morphological and molecular characters. Interestingly, all bilaterians are now included in one of these two groups. Protostomians are split into two stable groups: the ecdysozoans (animals that molt) including arthropods and nematodes; and the lophotrochozoans including mollusks, annelids, and flatworms (Aguinaldo *et al*., 1997). Several complete genomes of members of the Ecdysozoa are available (*Caenorhabditis elegans, Caenorhabditis briggsae, Drosophila melanogaster, Anopheles gambiae*), while the lophotrochozoans are a genomic desert with very few data available and no complete genome sequence available as yet. Thus, many surprises are expected to come from the molecular and comparative genomic study of this group.

Indeed, the comprehensive phylogenetic analysis of all nuclear receptors found in complete genome sequences, as well as the identification of an ER ortholog in a mollusk, strongly suggest that liganded receptors such as TRs, RARs, or SRs that were believed to be specific to the vertebrate lineage are probably more widespread among metazoan animals than previously thought (Thornton *et al*., 2003; Bertrand *et al*., in press). Importantly, analysis of the complete nuclear receptor set in a given genome allows one to establish unambiguously whether this genome has lost some nuclear receptor if these genes are present in other metazoan species. For example, the fact that *Drosophila, C. elegans*, and *Ciona* genomes do not harbor an ER may be viewed as an argument in favor of the fact that ERs are found only in recent chordates, even if the topology of the tree indicates an ancestry of ER in bilaterians. But the recent cloning of a clear ER ortholog in a mollusk (even if this receptor apparently does not bind estrogens!) permits reconciliation of the tree topology with the model and leads to the suggestion that indeed an ER ortholog was present in the common ancestor of all bilaterians and was lost independently in nematodes and the *Drosophila* genomes as well as in *Ciona*. This example illustrates very clearly the derived nature of the *Drosophila* and nematode genomes and the importance of lophotrochozoan analysis. Thus, the search for nuclear receptor genes in lophotrochozoans is clearly a priority for future research.

The analysis of all nuclear receptors present in bilaterians allows us to propose the basic set of nuclear receptors in metazoan phyla. The principle of this inference is that for each group of nuclear receptors we can locate the speciation event that led to the deuterostome and protostome lineages by the divergence between chordate, insect, and nematode orthologs. Then all genes resulting from duplications, which occurred before this speciation, were probably present in Urbilateria. Using this reasoning, which remains correct even if secondary loss occurred in some lineages, it appears that the genome of the common ancestor to nematodes, insects, and chordates had at least 25 nuclear receptor genes (Bertrand *et al*., in press). Any variation from this number should be explained by lineage specific events such as gene loss (i.e., arthropods and nematodes) or gene duplications that arose often in certain lineages (i.e., vertebrates or nematodes).

3.6.2.2.2. Nuclear receptors, nematodes, and the ecdysozoan The case of ecdysozoans is of course particularly relevant for the analysis of nuclear receptor evolution in arthropods. Nematodes, arthropods, and other phyla such as onychophorans are grouped in the Ecdysozoa, animals that share the developmental trait of molting, although this grouping is still debated (Aguinaldo *et al*., 1997; Blair *et al*., 2002). Genomic data reveal that nematodes contain a very large number of nuclear receptor genes (up to 270) whereas insects (*Drosophila* and *Anopheles*) harbor 21 nuclear receptor genes. Three questions are relevant for nuclear receptor evolution in ecdysozoans: (1) what was the extent of gene loss events that occurred during ecdysozoan evolution; (2) in this context, by what mechanisms did nematodes reach such an impressive number of nuclear receptors and did gene duplication play any role in nuclear receptor evolution in arthropods; and (3) are nuclear receptors linked to the grouping of ecdysozoans and their molting behavior?

As discussed above, the common ancestor of all bilaterians probably had 25 nuclear receptor genes in its genome. Since Diptera genomes contain 21

genes, a simple conclusion could be that arthropods lost four genes. In fact, the phylogenetic analysis of the nuclear receptors of bilaterians reveals a much more complex and interesting picture (Bertrand *et al.*, in press). Both *Drosophila* and nematode genomes lost several genes such as TR, RAR, PPAR, ER, SR, EAR2, and LXR. Whether these genes have been lost only once in the common ancestor of *Drosophila* and nematodes (i.e., the ancestor of all Ecdysozoa) or twice independently in these two lineages remains unclear. We will need information on other ecdysozoans to resolve this issue. In addition, it is clear that nematodes (or at least *C. elegans*) lost other genes such as *HR39, ERR, RXR, TLL, DSF, PNR, EcR*, and *E75*. It is possible that some of these genes still exist in nematode genomes but that their assignation is obscured by strong sequence divergence since nematode nuclear receptors genes, like many other genes, evolved rapidly (Robinson-Rechavi *et al.*, unpublished data).

It is of course very interesting to see that the analysis of dipteran genomes in the context of other bilaterians reveals the loss of seven nuclear receptor genes among which six are encoded liganded receptors. It is important to note, however, that this conclusion is reached only by the analysis of the nuclear receptor set present in the *Drosophila* and *Anopheles* complete genomes. There is a long road from the common ancestor of Ecdysozoa to the common ancestor of Diptera and it is not absolutely clear when these losses occurred and whether they occurred simultaneously. Thus, we can expect that some of these genes will be found in some ecdysozoans and even in some arthropods or primitive insects. Since many researchers working on arthropods have been influenced by the set of nuclear receptors present in *Drosophila*, it came as no surprise that for example SR, ER, or PPARs were not searched for in more primitive insects or arthropods. These data would allow us to revise the nuclear receptor set present in Ecdysozoa. Another basic question suggested by this analysis is why arthropods lost so many liganded receptors and what are the remnants of these signaling pathways still present in the genome of these organisms. This will be the subject of future analysis.

Returning to the premise that the urbilateria genome probably had 25 nuclear receptors, of which seven were lost in Ecdysozoa leading to 18 nuclear receptor genes, we then find that dipteran genomes contain 21 nuclear receptor genes. Interestingly, the three supplementary receptors are the three highly unusual members of the KNI group (knirps, knirps-related, and eagle) that clearly form a group of relatively recent duplicates. In nematodes, the situation is much more striking since the nematode genome lost more genes ($7+8=15$) but contains more than 270 receptors. The phylogenetic analysis of nematode nuclear receptors led to show that the *C. elegans* genome contains 13 ancient nuclear receptors and approximately 250 supplementary receptors that are the result of an explosive diversification of a unique gene, HNF4. In fact, it can be concluded that *C. elegans* as well as *C. briggsae* and probably others contain approximately 250 HNF4 genes! These genes are highly divergent and not functionally or structurally linked and the reason for such a massive increase in gene number is still far from being clear (Robinson-Rechavi *et al.*, unpublished data). Nevertheless, it should be emphasized that examples of lineage specific expansion in gene number are found for other types of genes and in other genomes suggesting that this is an event more widespread than expected.

In the perspective of nuclear receptor evolution, it is fundamental to recall, given the important role played by EcR and several other nuclear receptors, that the ecdysozoan group is composed of animals that molt. In this context, it is tempting to speculate that the ecdysone signaling cascade, including the EcR-USP heterodimer would be an obvious ecdysozoan synapomorphy (de Mendonca *et al.*, 1999). It is thus striking, and even irritating, to find neither an ecdysone receptor nor an RXR in the *C. elegans* genome. Did molting evolve several times independently in Ecdysozoa? The lack of resolution of this issue illustrates once again that we should be very cautious in the conclusions that we draw from classical model organisms such as *C. elegans* or *Drosophila*. Indeed, if *C. elegans* effectively lacks EcR and USP homologs, this unexpected property is not widespread in nematodes. *Dirofilaria immitis*, a parasitic nematode, does contain an ortholog of EcR and USP (Shea *et al.*, 2003). Thus, before concluding that a given receptor is absent in a large zoological group such as nematodes one should always also look at data from several species not too closely related. Unfortunately, this striking situation also suggests that *C. elegans* will be a poor model for comparing events governing molting in nematodes with the same events in arthropods.

The other question related to the ecdysozoan issue is whether the molting and EcR-USP signaling cascades are widespread outside the Ecdysozoa. If the answer is yes, then this no longer can be viewed as an ecdysozoan synapomorphy. The lack of resolution of this question underlines the profound ignorance of molecular mechanisms regulating the development in protostomians (besides *C. elegans* and *D. melanogaster*) in general and in lophotrochozoans in

particular. Ecdysteroids have been described in *Schistosoma* but the significance of this observation is far from clear (de Mendonça *et al.*, 2000a). To date, no EcR ortholog has been found in *Schistosoma*, which is an interesting observation given that a very large number of EST sequences, including many nuclear receptors, are available in this species (Verjovski-Almeida *et al.*, 2003). Thus, with our present, very limited knowledge, the proposal that EcR-USP signaling represents an ecdysozoan synapomorphy, although probably oversimplistic, is still acceptable.

3.6.3. Phylogeny of Nuclear Receptors in Arthropods

3.6.3.1. Towards an Inventory

Despite their functional importance, the evolution of arthropod nuclear receptors has been largely overlooked (Henrich and Brown, 1995). Three reasons may account for this disappointing situation. First, the sequencing effort has focused mainly on Diptera (*Drosophila*, malaria vector *An. gambiae*, yellow fever mosquito *Ae. aegypti*) and Lepidoptera (silk worm *B. mori*, tobacco hornworm *M. sexta*), which are two closely related and highly derived holometabolous orders of insects. Therefore, the sample of available sequences is small and evolutionarily incomplete (Figure 6). Second, these proteins have not been studied in insects as a homogeneous family of transcription factors, such as the products of *Hox* genes for example. Rather, they were analyzed separately through their involvement in various developmental pathways, like embryonic segmentation (*knirps*, *tailless*, *Ftz-F1*), molting and metamorphosis (*EcR*, *Usp*, *Ftz-F1*, *E75*...), or eye morphogenesis (*seven-up*). However, their common structure and mode of action requires an integrated view of their evolution. Third, to the exclusion of EcR, ligands still remain to be identified for the nuclear receptors of arthropods. Whether they are all real orphans or not is still an open question and an essential issue is to understand their function and their evolution. Nevertheless, we can draw here a summary of the present knowledge, in order to suggest the orientations of the future work that should help to understand both the evolution of nuclear receptors and their role during diversification of insects and other arthropods.

All the arthropod nuclear receptors known to date can be classified into the seven subfamilies previously described (Figure 1) (Laudet, 1997). For many of them it is easy to identify a sequence relationship with a chordate receptor. However, it appears that rapid evolutionary divergence produced nuclear

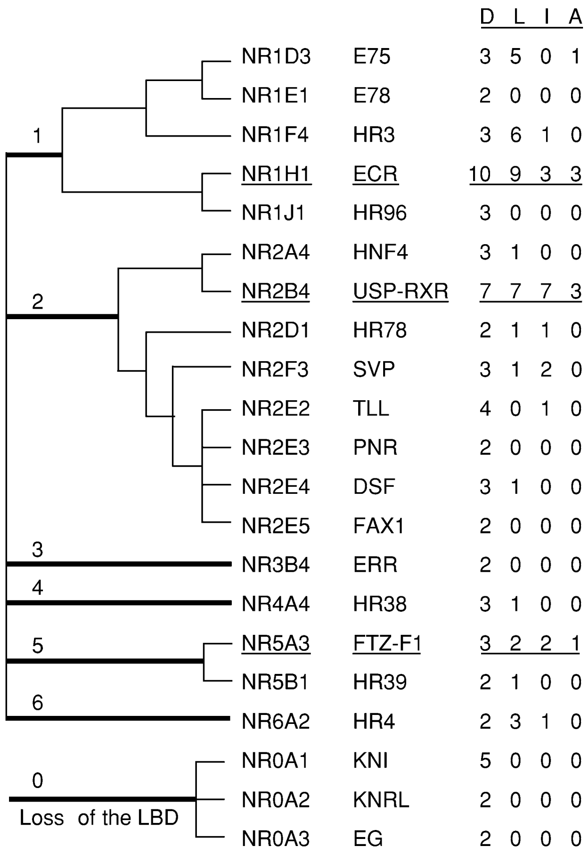


Figure 6 Phylogeny of identified nuclear receptors in arthropods. The schematic unrooted tree of the six subfamilies is a simplified version of that from the Nuclear Receptors Nomenclature Committee (1999). In addition, the NR0 subfamily is shown at the bottom of the figure. Branches in bold are those leading to a subfamily with the corresponding number written above the branch. Two names are given for each nuclear receptor: on the left is the official nomenclature and on the right is the trivial name used in this chapter. The number of species where one given receptor has been identified (update: July 2003) is also indicated. Four groups of species have been defined: D, Diptera; L, Lepidoptera; I, other insects; and A, other arthropods (Bonneton *et al.*, 2003). When a nuclear receptor is found in all of these four groups, its name is underlined.

receptors that may be specific to insects or other arthropods. Because ECR and USP-RXR show such a divergence and have been intensively studied (see **Chapter 3.5**), their evolution will be considered separately.

3.6.3.1.1. Subfamily 1: all members of the ecdysone pathway In insects, subfamily NR1 contains five receptors, which are all directly involved in the ecdysone pathway of *D. melanogaster* (Figure 7). They can be separated into two distinct groups, based on structural as well as functional data (Laudet, 1997; Thornton and Desalle, 2000). The first group includes E75 (NR1D3), E78 (NR1E1), and HR3 (NR1F4). All of their vertebrate homologs

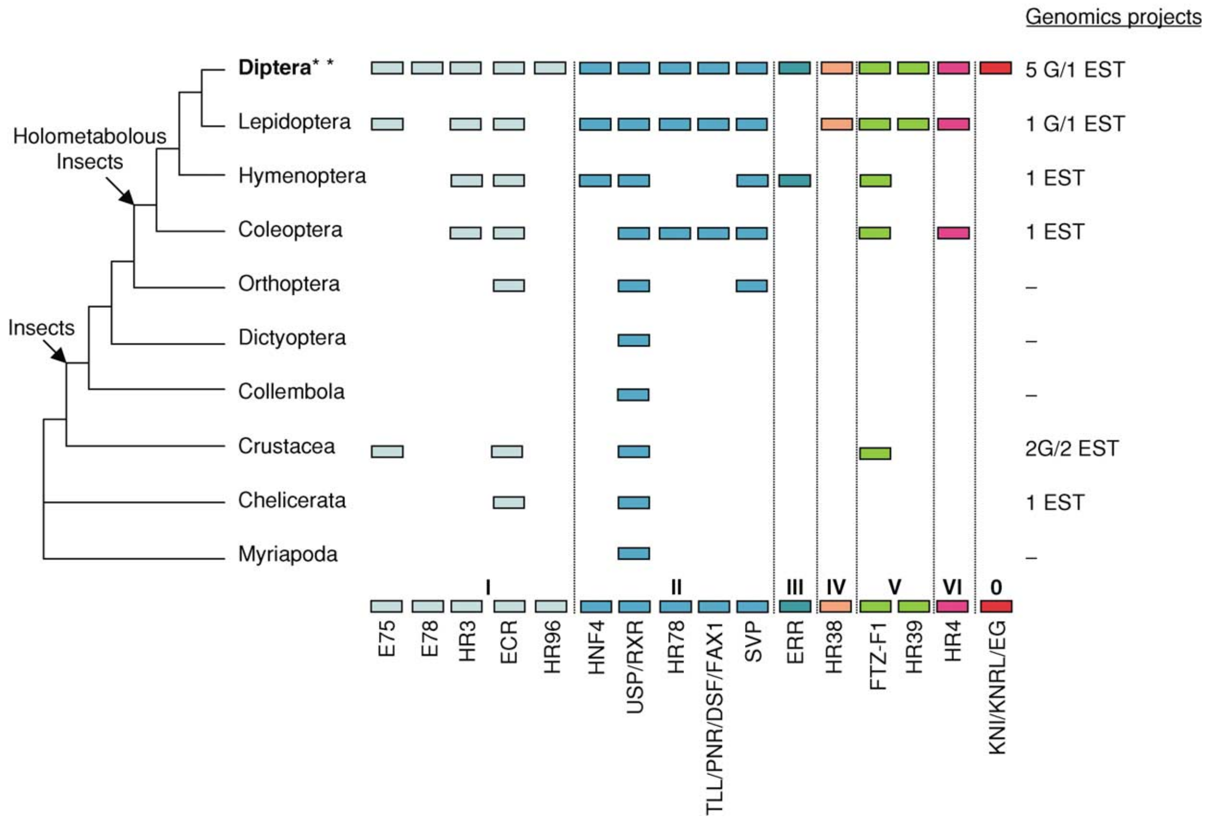


Figure 7 Phylogenetic distribution of identified nuclear receptors in arthropods. On the left, a tree indicates the evolutionary relationships between the groups where nuclear receptors are known (Kristensen, 1981; Whiting *et al.*, 1997; Hwang *et al.*, 2001; Giribet *et al.*, 2001). Diptera is written in bold with two asterisks (*) to highlight the fact that two complete genome sequences are available for this order (*D. melanogaster* and *An. gambiae*), allowing for an accurate account of nuclear receptors (Bertrand *et al.*, in press). Concerning Hymenoptera, we have found HR3, HNF4, SVP, and ERR in *Apis* ESTs. The 16 groups of arthropod nuclear receptors are indicated by rectangular boxes colored according to their subfamilies, which are shown at the bottom of the figure (subfamilies 0, I, II, III, IV, V, VI). The ongoing complete genome (G) and EST sequencing projects are listed on the right (GOLD); –, no known project.

are orphan receptors that are unable to form heterodimers with RXR. The second group contains the famous ecdysone receptor, EcR (NR1H1), and the less well-known HR96 (NR1J1). Both proteins may be specific to Ecdysozoa. Note that several nuclear receptors of subfamily 1 found in chordates have no homologs in arthropods and nematodes: thyroid hormone receptors (TRs, NR1A), retinoic acid receptors (RARs, NR1B), and peroxisome proliferator-activated receptors (PPAR, NR1C) that bind fatty acids (Laudet and Gronemeyer, 2002). In fact, a recent analysis of data provided by genomic projects suggests that this group of "classical" hormone receptors may be very ancient (present in the ancestor of bilateria) and was lost in ecdysozoans (Bertrand *et al.*, in press).

3.6.3.1.1.1. E75 The E75 gene (*Eip75B* in the FlyBase, NR1D3) has been identified in *D. melanogaster* (Feigl *et al.*, 1989; Segraves and Hogness, 1990), *An. gambiae* (Bertrand *et al.*, in press), *Ae. aegypti* (Pierceall *et al.*, 1999), and several species of Lepidoptera (Segraves and Woldin, 1993; Jindra *et al.*, 1994a; Palli *et al.*, 1995; Palli *et al.*, 1997b; Zhou *et al.*, 1998; Swevers *et al.*, 2002). It was also isolated from the shrimp *Metapeneus ensis* (Chan, 1998). These proteins are homologous to the vertebrate receptors REVERB-α and REVERB-β, which have been shown to be constitutive transcriptional repressors involved in the pacemaker controlling the circadian clock in vertebrates (Preitner *et al.*, 2002) (see **Chapter 4.11**). In insects, E75 acts as a repressor of HR3, probably through direct interaction, both in *Drosophila* (White *et al.*, 1997) and in *Bombyx* (Swevers *et al.*, 2002). The possible implication of E75 in circadian rhythmicity is still not known (see **Chapter 4.11**). In *Drosophila*, inactivation of all E75 functions causes first instar larval lethality, but isoform specific null mutations reveal different functions for each of the three isoforms (Bialecki *et al.*, 2002). The complex role of this gene is far from being fully understood but expression

and hormonal induction data suggest that its involvement in early ecdysone responses during molting and metamorphosis may be shared among arthropods (Jindra *et al.*, 1994a; Palli *et al.*, 1997b; Chan, 1998; Zhou *et al.*, 1998). It also plays a role during oogenesis and vitellogenesis in *Drosophila* (Bryant *et al.*, 1999), *Aedes* (Pierceall *et al.*, 1999; Raikhel *et al.*, 2002) (see **Chapter 3.9**), and *Bombyx* (Swevers *et al.*, 2002).

3.6.3.1.1.2. E78 The E78 gene (*Eip78C* in the FlyBase, NR1E1) is only known in Diptera (*Drosophila* and *Anopheles*) and although it is related to E75 and REVERB, it is extremely divergent from both of them. It could be a paralog of E75 that experienced a rapid evolutionary rate. Interestingly, the same situation is observed in nematodes, with the presence of an ortholog of E75 (nhr-85 in *C. elegans*) together with an ortholog of E78 (SEX-1 in *C. elegans*) (Kostrouch *et al.*, 1995; Carmi *et al.*, 1998; Unnasch *et al.*, 1999; Sluder and Maina, 2001; Crossgrove *et al.*, 2002). It is therefore possible that, in addition to one NR1D gene related to REVERB, all ecdysozoans also possess one specific fast-evolving NR1E gene that has been lost in chordates (Bertrand *et al.*, in press). However, mutant phenotypes indicate that this NR1E gene probably plays different roles in insects and in nematodes. Indeed, in *Drosophila*, E78 homozygous mutants are viable and fertile, with subtle defects in regulation of some puffs and in formation of dorsal chorionic appendages (Russel *et al.*, 1996; Bryant *et al.*, 1999). By contrast, the E78 ortholog (SEX-1) of *Caenorhabditis* is required for sex determination and viability of hermaphrodites (Carmi *et al.*, 1998).

3.6.3.1.1.3. HR3 Orthologs of HR3 (*Hr46* in FlyBase, NR1F4) have been identified in Diptera (Koelle *et al.*, 1992; Kapitskaya *et al.*, 2000; Bertrand *et al.*, in press) and Lepidoptera (Palli *et al.*, 1992; Jindra *et al.*, 1994b; Palli *et al.*, 1995, 1996; Matsuoka and Fujiwara, 2000; Debernard *et al.*, 2001), and a short sequence (partial DBD) is also available from the coleopteran *Tenebrio molitor* (Mouillet *et al.*, 1999). This receptor is well conserved outside insects, with one gene in *Caenorhabditis* (Kostrouch *et al.*, 1995) and three homologs in vertebrates: ROR α, β, γ, which are transcriptional activators (Jetten *et al.*, 2001). It has been shown recently that cholesterol and all-trans retinoic acid are ligands for RORα (Kallen *et al.*, 2002) and RORβ (Stehlin-Gaon *et al.*, 2003), respectively. RORs and REVERB bind to the same response element and RORs are thought to be competitors for REVERB. As such, they are believed to play an important role in circadian rhythm (Andre *et al.*, 1998). HR3 is an ecdysone inducible early–late gene that plays a key role during *Drosophila* metamorphosis by repressing early genes and directly inducing the prepupal regulator FTZ-F1 (Lam *et al.*, 1997; White *et al.*, 1997). This function in molting and metamorphosis is probably conserved throughout insects, as suggested by studies of expression and hormonal induction in *Manduca* (Palli *et al.*, 1992; Lan *et al.*, 1997, 1999; Langelan *et al.*, 2000), *Choristoneura fumiferana* (Palli *et al.*, 1996, 1997a), and *Tenebrio* (Mouillet *et al.*, 1999). Interestingly, inhibition of HR3 expression using RNAi showed that this gene is also required for *Caenorhabditis* molting, suggesting a conserved role in Ecdysozoa (Kostrouchova *et al.*, 1998, 2001). As for E75, HR3 is a functionally complex gene involved in many aspects of the ecdysone pathway. For example, a role in oogenesis and vitellogenesis has been described for *Aedes* (Kapitskaya *et al.*, 2000; Li *et al.*, 2000; Raikhel *et al.*, 2002) (see **Chapter 3.9**), *Bombyx* (Eystathioy *et al.*, 2001), and *Caenorhabditis* (Kostrouchova *et al.*, 1998). HR3 is also expressed in embryos of *Drosophila* (Carney *et al.*, 1997) and *Caenorhabditis* (Kostrouchova *et al.*, 1998). Furthermore, *Drosophila* HR3 mutants have defects in their nervous system and die during embryogenesis (Carney *et al.*, 1997). Given the wide variety of tissues and stages of expression for HR3, it is clear that other functions will be identified by detailed analysis of mutants. This is nicely illustrated by two studies showing a specific role for HR3 during the formation of wings in *Drosophila* and in *Bombyx*. Clonal analysis reveals requirements for *Drosophila* HR3 in the development of adult wings, bristles, and cuticle, but no apparent function in eye or leg development (Lam *et al.*, 1999). In a *Bombyx* wing deficient mutant called *flügellos*, HR3 expression is reduced only in wing discs, and not in the testis and fat body, while ECR, USP, E75, and HR38 are not affected (Matsuoka and Fujiwara, 2000). The wings are one of many organs where HR3 is expressed, and the role of this gene in their development in two different insects could only be revealed with appropriate genetic manipulations.

3.6.3.1.1.4. HR96 The HR96 (NR1J1) gene has been identified in *Drosophila* (Fisk and Thummel, 1995) and in *Anopheles* (Bertrand *et al.*, in press). In *Caenorhabditis*, three homologs are known: DAF-12, NHR-8, and NHR-48 (Sluder and Maina, 2001). All these ecdysozoan receptors are related to the vertebrate receptors NR1I: VDR, which is the receptor for vitamin D, PXR and CAR, which both bind a wide variety of xenobiotics (Laudet, 1997). Given the fact that the vertebrate homologs of

HR96 bind steroid-derived compounds, it is likely that HR96 also binds such a ligand. Since ECR and its vertebrate homologs LXR and FXR can bind steroids, it is even tempting to speculate that all the related nuclear receptors NR1H/J have the same property. It is also possible that HR96 requires USP-RXR to bind DNA in the same way as the other NR1H/I/J receptors. Little is known about the functions of HR96, which is inducible by 20E and is expressed during the onset of metamorphosis (Fisk and Thummel, 1995). The *Caenorhabditis* homologous gene *daf-12*, which functions downstream of the insulin and TGF-β signaling pathways, regulates diapause, developmental age, and adult longevity (Antebi *et al*., 1998, 2000; Gerisch *et al*., 2001; Jia *et al*., 2002). Interestingly, a growing number of studies suggest that common regulatory mechanisms control developmental timing in *Caenorhabditis* and *Drosophila*, with a central role for the ecdysone pathway in insects (Thummel, 2001) (see **Chapters 3.3** and **3.5**).

3.6.3.1.2. Subfamily 2: a large collection of old and new genes Only subfamilies NR2 and NR5 have been found in all metazoans, suggesting that they are probably at the origin of the superfamily (Escriva *et al*., in press). Most of the NR2 proteins are orphans (except HNF4 and RXR) and do not form heterodimers (except USP-RXR). In insects, subfamily NR2 contains eight genes, including the most conserved nuclear receptors: HNF4, seven-up (SVP-COUP), and tailless (TLL). The tailless group (NR2E) contains four different genes that exhibit a rather restricted pattern of expression: TLL, PNR, DSF, and FAX1. Probably they all share a primary function in the developing nervous system (see **Chapter 2.4**).

3.6.3.1.2.1. HNF4 HNF4 (NR2A) is one of the best-conserved nuclear receptors between arthropods and vertebrates. This gene was identified in *Drosophila* (Zhong *et al*., 1993), *Aedes* (Kapitskaya *et al*., 1998), *Anopheles* (Bertrand *et al*., in press), and *Bombyx* (Swevers and Iatrou, 1998). Two homologous HNF4 genes exist in mammals. In *Caenorhabditis*, a surprising explosive burst of duplications of HNF4 produced most of the (>270) supplementary nuclear receptors found in this species (Robinson-Rechavi *et al*., unpublished data). The embryonic expression pattern of this receptor is remarkably well conserved between insects and vertebrates. Indeed, HNF4 is transcribed zygotically in several analogous organs: the midgut, Malpighian tubules, and fat body of insects (Zhong *et al*., 1993; Kapitskaya *et al*., 1998; Swevers and Iatrou, 1998) and the intestine, kidney, and liver of vertebrates (Laudet and Gronemeyer, 2002). Furthermore, analyses of mutants show that the development of these organs requires HNF4 activity in *Drosophila* (Zhong *et al*., 1993) as well as in the mouse (Chen *et al*., 1994). If conflicting results were obtained concerning the role of HNF4 in the fat body of *Drosophila* embryos (Zhong *et al*., 1993; Hoshizaki *et al*., 1994), this gene is clearly transcribed in the fat body of *Aedes* adults (Kapitskaya *et al*., 1998) as well as in the *Bombyx* larvae and pupae (Swevers and Iatrou, 1998). In the mosquito, three isoforms have been identified, each having a specific temporal pattern during the vitellogenesis that takes place in the female fat body (Kapitskaya *et al*., 1998). Therefore, HNF4 probably performs similar functions during the formation of the gut and its derivatives in a large range of metazoans. Another conserved pattern is the maternal expression of HNF4, but its function has not been studied. It has been shown recently that the mammalian HNF4 receptor binds fatty acids constitutively (Dhe-Paganon *et al*., 2002; Wisely *et al*., 2002). Given the strong similarity (85%) observed for the HNF4 LBD between insects and vertebrates, it is tempting to speculate that this type of ligand may also be used in insects.

3.6.3.1.2.2. HR78 The HR78 gene (NR2D1) has been identified in *Drosophila* (Fisk and Thummel, 1995; Zelhof *et al*., 1995b), *Anopheles* (Bertrand *et al*., in press), *Bombyx* (Hirai *et al*., 2002), and *Tenebrio* (Mouillet *et al*., 1999). These genes are distantly related to the vertebrate's orphan receptors TR2 and TR4, and to the very divergent nematode genes *nhr-41* (*Caenorhabditis*; Sluder and Maina, 2001) and *nhr-2* (filarial nematode *Brugia malayi*; Moore and Devaney, 1999). As with most of the early genes of the ecdysone cascade (including ECR, E75, and E78), the developmental expression of HR78 is globally stable between the species of the *D. melanogaster* subgroup (Rifkin *et al*., 2003). HR78 is required for ecdysteroid signaling during the onset of metamorphosis of *Drosophila* (Fisk and Thummel, 1995; Zelhof *et al*., 1995b; Fisk and Thummel, 1998). This receptor is inducible by 20E and binds to over 100 sites on polytene chromosomes, many of which correspond to ecdysteroid regulated puff loci. By contrast with the important sequence divergence of the proteins, the temporal profile of expression of HR78 is very well conserved between *Drosophila* and *Tenebrio*, with an early activation at the end of larval stages, followed by a rather constant level during prepupal stages (Fisk

and Thummel, 1995; Zelhof *et al*., 1995b; Mouillet *et al*., 1999). *Drosophila* HR78 null mutants die during the second and third larval instar with tracheal defects, showing that, despite a uniform expression in the embryo, this receptor is not essential for early development (Fisk and Thummel, 1998; Astle *et al*., 2003). A surprising result was obtained by studying the *Bombyx* homolog. Indeed, yeast two hybrid and pull down assays showed that *Bombyx* HR78 could interact with USP-RXR (Hirai *et al*., 2002). If we consider that HR78 and ECR bind to the same DNA sites (Fisk and Thummel, 1995; Zelhof *et al*., 1995b), and have the same USP-RXR partner, it is therefore possible that competition occurs between these two proteins during the onset of metamorphosis.

3.6.3.1.2.3. SVP The *seven-up* gene (SVP, NR2F3) is a member of the COUP-TFs group, the most conserved nuclear receptors. It has been identified in various insects: *Drosophila* (Henrich *et al*., 1990; Mlodzik *et al*., 1990), *Aedes* (Miura *et al*., 2002), *Bombyx* (Togawa *et al*., 2001), and partial clones were also recovered from *Tenebrio* (Mouillet *et al*., 1999) and the grasshopper *Schistocerca gregaria* (Broadus and Doe, 1995). One homolog was studied in *Caenorhabditis* (Zhou and Walthall, 1998), and three COUP-TF genes are present in vertebrates (Pereira *et al*., 2000). These orphan receptors have a very broad pattern of expression and are essential for development in all the organisms studied. In the embryos of *Drosophila* and *Schistocerca*, SVP was found in the developing eyes, the central and peripheral nervous systems, the Malpighian tubules, and the fat body (Mlodzik *et al*., 1990; Hoshikazi *et al*., 1994; Broadus and Doe, 1995; Kerber *et al*., 1998; Sudarsan *et al*., 2002). Analysis of mutants demonstrated the functional role of these expressions in *Drosophila*. In addition, SVP participates in the diversification of cardioblasts in the heart tube of *Drosophila* embryos (Lo and Frasch, 2001; Ponzielli *et al*., 2002). The SVP protein is regulated by different pathways, such as Ras in eyes, EGF in Malpighian tubules, and hedgehog in heart, and thus plays multiple roles from cell fate determination to regulation of cell cycle and differentiation. Interestingly, SVP/COUP-TF inhibits USP-RXR signaling pathways, both in vertebrates and in insects. This has been tested *in vivo* with *Drosophila* in which the overexpression of SVP that leads to lethality at the onset of metamorphosis can be rescued by a concomitant overexpression of USP-RXR (Zelhof *et al*., 1995a). It is of note that SVP is expressed at the beginning of metamorphosis, when the ecdysteroid titer is low, both in *Drosophila* and in *Tenebrio* (Mouillet *et al*., 1999). A similar inhibition of 20E response occurs during vitellogenesis in the mosquito *Aedes* (see **Chapter 3.9**). Furthermore, in this species, it was shown *in vitro* that the repression of the ecdysone pathway is probably due to a direct interaction between SVP and USP-RXR. The replacement of ECR by SVP in USP-RXR heterodimers would be essential for the termination of vitellogenesis, when the ecdysteroid titer declines (Miura *et al*., 2002; Zhu *et al*., 2003). However, it must be noted that no physical interaction between SVP and USP-RXR was detected in *Drosophila* (Zelhof *et al*., 1995a).

3.6.3.1.2.4. TLL The *tailless* gene (TLL, NR2E2) is one of the most conserved nuclear receptors. It was first identified in *Drosophila* as a terminal gap gene determining embryo segmentation (Jürgens *et al*., 1984). Homologs have been studied in *Drosophila virilis* (Liaw and Lengyel, 1993), the house fly *Musca domestica* (Sommer and Tautz, 1991), and the coleopteran *Tribolium castaneum* (Schröder *et al*., 2000). Nematodes and vertebrates also have one *tailless* gene. The early terminal expression is necessary for the establishment of the nonmetameric domains at the anterior and posterior poles of the *Drosophila* embryo (Jürgens *et al*., 1984; Pignoni *et al*., 1990). This pattern is very well conserved in Diptera (Sommer and Tautz, 1991; Liaw and Lengyel, 1993). By contrast, the early posterior expression of *tailless* in *Tribolium* reveals a temporal divergence. This difference suggests that tailless may not function as a gap gene in *Tribolium*, but may be involved in an earlier specification of terminal fate (Schröder *et al*., 2000). Thus, it appears that an important shift occurred in *tailless* function during the transition from short-germ to long-germ embryogenesis. This situation contrasts with the conservation of *tailless* late expression in the developing forebrain of insects and vertebrates. Using mutants demonstrated an essential role for *tailless* in eye formation of *Drosophila* (Daniel *et al*., 1999; Hartmann *et al*., 2001) and the mouse (Monaghan *et al*., 1997; Yu *et al*., 2000). In conclusion, the primary conserved function for *tailless* would be in the development of the forebrain, while its role in segmentation was probably acquired during the evolution of long-germ holometabolous insects. Other gap genes such as *orthodenticle*, *empty spiracles*, or *hunchback* are known to be part of a conserved neural network that was recruited for insect segmentation (Reichert, 2002).

3.6.3.1.2.5. PNR One NR2E3 gene, homologous to the vertebrate photoreceptor-specific nuclear receptor (PNR; Kobayashi *et al.*, 1999), was identified in the genome of *Drosophila* (Flybase: CG16801) and *Anopheles* (Bertrand *et al.*, in press). Nothing is known about this gene in insects. It is of note that PNR expression is restricted to the retina and plays a critical role in the development of photoreceptors (Kobayashi *et al.*, 1999; Haider *et al.*, 2000). Therefore, both *tailless* and PNR play an important role during vertebrate eye development. We have seen that the role of *tailless* in the formation of the visual system is conserved between insects and vertebrates. Whether the same conservation exists for PNR remains to be established.

3.6.3.1.2.6. DSF The NR2E4 nuclear receptor (*dissatisfaction*, DSF) may be specific to insects. It has been identified in *D. melanogaster* (Finley *et al.*, 1998), *D. virilis* (Pitman *et al.*, 2002), *Anopheles* (Bertrand *et al.*, in press), and *Manduca* (Pitman *et al.*, 2002), but no homologs are found in nematodes and chordates. Recent analysis of genomic data suggest that DSF is one of the four nuclear receptors (along with HR39, E78, and FAX1) that were lost in the chordate lineage (Bertrand *et al.*, in press). DSF is necessary for appropriate sexual behavior and sex-specific neural development. It acts downstream of the genes *Sex lethal* and *transformer* in the sex determination cascade. Mutant females resist male courtship and fail to lay eggs, while mutant males are bisexual and mate poorly (Finley *et al.*, 1997; O'Kane and Asztalos, 1999). The *dsf* gene is expressed in a very limited set of neurons in the brain of larvae, pupae, and adults, with no sex specificity (Finley *et al.*, 1998). The DSF protein acts as a repressor, through its LBD and an unusually very long hinge region (Pitman *et al.*, 2002). It will be very interesting to test whether DSF can also act as a ligand-dependent activator, since no sex hormones are known in insects (De Loof and Huybrechts, 1998).

3.6.3.1.2.7. FAX1 The NR2E5 gene, called FAX-1, was first identified in *Caenorhabditis* as a regulator of axon pathfinding and neurotransmitter expression (Wightman *et al.*, 1997). FAX-1 is expressed in embryonic neurons after neurogenesis but just prior to axon extension (Much *et al.*, 2000). Homologs were identified in the genome of *Drosophila* (Flybase: CG10296) and *Anopheles* (Bertrand *et al.*, in press). The *fax-1* genes probably experienced a rapid evolution in Ecdysozoa, since they are very divergent from all the other genes of the NR2E group of nuclear receptors.

3.6.3.1.3. Subfamily 3: ERR a classical steroid receptor in insects? The subfamily NR3 comprises the receptors for sex and adrenal steroid hormones: estrogens (ER), androgens (AR), progesterone (PR), glucocorticoids (GR), and mineralocorticoids (MR), as well as ERR which is an orphan receptor related to ER. Until recently, these proteins were believed to be restricted to vertebrates. However, not only is one ERR gene present in the urochordate *Ciona intestinalis* (Yagi *et al.*, 2003) and in Diptera, but an ER ortholog was isolated from a mollusk (Thornton *et al.*, 2003). Therefore, it appears that, to the exclusion of ERR, steroid receptors from the subfamily NR3 were specifically lost in ecdysozoans (Bertrand *et al.*, in press). Unfortunately, the precise relationships between ER, ERR, and the other steroid receptors are difficult to determine with the current data.

One ERR gene (NR3B4) is present in the genome of *Drosophila* (Adams *et al.*, 2000; Östberg *et al.*, 2003) and *Anopheles* (Bertrand *et al.*, in press). It was also found independently in a screen for *Drosophila* embryonic neural precursor genes (Brody *et al.*, 2002). There is no ortholog of ERR in the *Caenorhabditis* genome (Maglisch *et al.*, 2001). In vertebrates, the three ERR genes have broad expression patterns and influence a wide range of physiological processes, including those governed by ERs (Giguere, 2002). A striking contrast between vertebrate and insect endocrinology is the lack of sex determining hormones in insects (De Loof and Huybrechts, 1998). Obtaining functional data for *Drosophila* and/or *Anopheles* ERR could help to tackle this intriguing problem.

3.6.3.1.4. Subfamily 4: HR38, an alternative partner for USP-RXR The subfamily NR4 is a small group of orphan nuclear receptors: the vertebrate's NGFIB α βγ, divergent nematode receptors (NHR-6 in *C. elegans*) and the insect HR38 (Sluder and Maina, 2001; Laudet and Gronemeyer, 2002). The HR38 gene (NR4A4) has been cloned in *Drosophila* (Fisk and Thummel, 1995; Sutherland *et al.*, 1995; Komonyi *et al.*, 1998), *Aedes* (Zhu *et al.*, 2000), and *Bombyx* (Sutherland *et al.*, 1995).

Remarkably, like their vertebrate homologs NGFIB, insect HR38 can bind DNA either as a monomer or through an interaction with USP-RXR (Sutherland *et al.*, 1995; Crispi *et al.*, 1998; Zhu *et al.*, 2000). This interaction plays a very interesting role in the mosquito *Aedes*. In this insect, vitellogenesis requires a blood meal that triggers a 20E cascade to activate yolk synthesis (see **Chapter 3.9**). Before a blood meal, this regulation is inhibited despite the presence of the hormone.

A key factor of this inhibition is the competitive binding of HR38 to USP-RXR, which prevents the formation of the ECR/USP-RXR heterodimer, the functional EcR (Zhu *et al.*, 2000; Raikhel *et al.*, 2002). Therefore, although HR38 is not directly regulated by 20E, it can participate in the 20E pathway as an alternative partner to USP-RXR. In *Drosophila*, HR38 is expressed in ovaries and during all stages of development. Different mutant alleles have different lethal phases, from larval stages to adults, showing a role in metamorphosis and adult epidermis formation (Kozlova *et al.*, 1998). Recent results have shown that the *Drosophila* heterodimer HR38/USP-RXR responds to a distinct class of ecdysteroids independently of ECR and without direct binding of the ligand to either HR38 or USP-RXR. In fact, crystal structure analysis reveals that the *Drosophila* HR38 LBD lacks both a conventional ligand binding pocket and a bona fide AF2 transactivation domain (Baker *et al.*, 2003). Interestingly, the vertebrate NGFIB receptors are ligand independent transcriptional activators that are considered to be real orphans, a view which was recently reinforced by structural analysis (Wang *et al.*, 2003). These data provide strong evidence that NR4 receptors operate through an atypical mechanism of activation.

3.6.3.1.5. Subfamily 5: competition in the ecdysone pathway The subfamily NR5 contains orphan nuclear receptors that, together with NR2, are believed to be at the origin of the superfamily (Escriva *et al.*, in press). In mammals, the subfamily NR5 contains three homologs, including the steroidogenic factor 1 (SF1), an essential regulator of endocrine function (Parker *et al.*, 2002). One ortholog (*nhr-25*) is present in the genome of *Caenorhabditis* (Gissendanner and Sluder, 2000). In insects, the two NR5 genes, FTZ-F1 and HR39, are important in the ecdysone (20E) pathway.

3.6.3.1.5.1. FTZ-F1 The FTZ-F1 gene (NR5A3) is one of the best-known nuclear receptors of insects. This gene encodes two protein isoforms, α and β, each having a specific role. The αFTZ-F1 isoform acts during segmentation of the early embryo, a function that is likely to be an innovation of holometabolous insects. The βFTZ-F1 isoform is a major regulator of metamorphosis, a role probably shared by all ecdysozoans. Interestingly, FTZ-F1 has been cloned not only in Diptera (Lavorgna *et al.*, 1991, 1993; Li *et al.*, 2000) and Lepidoptera (Sun *et al.*, 1994; Weller *et al.*, 2001), but also in the honeybee, *Apis mellifera* (Hepperle and Hartfelder, 2001), *Tenebrio* (Mouillet *et al.*, 1999), and in the shrimp *Metapeneus ensis* (Chan and Chan, 1999). Only ECR and USP-RXR have been identified in such a wide sample of arthropod species (**Figure 6**).

The *Drosophila* αFTZ-F1 is a direct regulator of the pair-rule gene *fushi-tarazu* (*ftz*), which governs the formation of embryonic metameres (Ueda and Hirose, 1990; Lavorgna *et al.*, 1991; Ohno *et al.*, 1994). Later, it was also found that αFTZ-F1 and FTZ are mutually dependent cofactors for regulation of target genes such as *engrailed*, *wingless*, or *ftz* itself (Florence *et al.*, 1997; Guichet *et al.*, 1997; Yu *et al.*, 1997). This interaction requires the DBD of both proteins as well as a contact between a LRALL domain of the FTZ protein with the AF-2 activation domain of αFTZ-F1 (Schwartz *et al.*, 2001; Suzuki *et al.*, 2001; Yussa *et al.*, 2001). The LRALL domain matches the consensus box LxxLL that is found in many cofactors of nuclear receptors (Laudet and Gronemeyer, 2002). Such a partnership between a homeodomain protein and a nuclear receptor is still a unique example in insects (Yussa *et al.*, 2001). It raises the exciting possibility that nuclear receptors may interact with *Hox* genes. Indeed, *ftz* is a member of the arthropod *Hox* gene family. However, it is a very derived *Hox* gene, which has lost its homeotic function in insects and acquired new roles in neurogenesis and segmentation (Hughes and Kaufman, 2002). Thus, it was interesting to determine whether the LxxLL box was conserved in the FTZ proteins. Elegant experiments, both *in vitro* and *in vivo*, revealed a progressive evolution of FTZ in insects. The pair-rule function is moderately conserved in *T. castaneum* and absent in the *Schistocerca*. Furthermore, this functional evolution correlates with the presence of a LxxLL domain in the FTZ protein of the holometabolous insects *Drosophila* and *Tribolium*, and its absence in the hemimetabolous grasshopper *Schistocerca* (Alonso *et al.*, 2001; Lohr *et al.*, 2001). In conclusion, it appears that the *ftz* gene gained one of the domains that facilitate the interaction between αFTZ-F1 and FTZ somewhere in the emergence of holometabolous insects.

The isoform βFTZ-F1 plays a central role during molting and metamorphosis of *Drosophila*. It is repressed by 20E and its expression, which occurs during mid-prepupa when the titer of 20E is low, activates the late prepupal genes (Lavorgna *et al.*, 1993; Woodard *et al.*, 1994). Its activation requires HR3 (Kageyama *et al.*, 1997; White *et al.*, 1997), an interaction that is also used during vitellogenesis of *Aedes* (Li *et al.*, 2000) (see **Chapter 3.9**). Analysis of mutants shows that βFTZ-F1 provides competence for stage-specific responses to 20E throughout the organism (Broadus *et al.*, 1999; Yamada

et al., 2000). These general characteristics seem to be well conserved in Lepidoptera, as shown by expression and induction experiments made with *Bombyx* (Sun *et al.*, 1994) and *Manduca* (Hiruma and Ridifford, 2001; Weller *et al.*, 2001). In fact, the patterns of expression observed in *Tenebrio* (Mouillet *et al.*, 1999) and the shrimp *M. ensis* (Chan and Chan, 1999), together with the mutant analysis of the *Caenorhabditis* homologous gene *nhr-25* (Asahina *et al.*, 2000; Gissendanner and Sluder, 2000) suggest that the role of βFTZ-F1 may be very similar in all Ecdysozoa. A promising result is that βFTZ-F1 could be one of the factors controlling honeybee caste development (see **Chapter 3.13**). In this social insect, JH and ecdysteroids induce a dramatic reduction in ovariole number during the fourth larval instar of workers but not in queens. In a search for genes controlling this phenotypic plasticity, the βFTZ-F1 of *A. mellifera* was identified by differential display of RT-PCR as a 20E down-regulated gene in the larval ovary (Hepperle and Hartfelder, 2001). This pioneering work highlights one of the most interesting putative functions for nuclear receptors: molecular regulation of polyphenism (Nijhout, 2003) (see **Chapter 3.13**).

3.6.3.1.5.2. HR39 The HR39 gene (NR5B1) exists in *Drosophila* (Ohno and Petkovich, 1992; Ayer *et al.*, 1993), *Anopheles* (Bertrand *et al.*, in press), and *Bombyx* (Niimi *et al.*, 1997) but not in vertebrates. Note that this gene was initially described with the misleading name of FTZ-F1β but should not be confused with the β isoform of FTZ-F1 (NR5A3). This second group of subfamily NR5 was previously seen as the result of a duplication that arose during insect evolution (Laudet and Gronemeyer, 2002). However, the identification of an HR39 homolog in *Schistosoma mansoni*, a platyhelminth parasite of humans, suggests that the FTZ-F1/HR39 duplication occurred very early during protostomian evolution (de Mendonça *et al.*, 2002). The *Caenorhabditis* genome has one FTZ-F1 gene (*nhr-25*) but no HR39 homolog (Sluder and Maina, 2001). The HR39 gene of *Drosophila* is induced by 20E and expressed at every stage of development, with a maximum at the end of third larval and prepupal stages (Horner *et al.*, 1995). Therefore, contrary to βFTZ-F1, it behaves like an early gene during the onset of metamorphosis. Remarkably, the pattern of expression of *Schistosoma* HR39 resembles that of *Drosophila*, with the highest amounts in the larval form (de Mendonça *et al.*, 2002). Both HR39 and FTZ-F1 bind as monomers to the same response elements (Ayer and Benyajati, 1992, 1993; Ohno and Petkovich, 1992; Crispi *et al.*, 1998). Overexpression of HR39 or FTZ-F1 have opposite effects on promoter activity, suggesting competition between these two nuclear receptors (Ayer *et al.*, 1993; Ohno *et al.*, 1994).

3.6.3.1.6. Subfamily 6: HR4, a new early–late gene? The HR4 gene (NR6A2) is homologous to germ cell nuclear factor (GCNF), an orphan nuclear receptor of vertebrates. It has been identified in the genome of *Drosophila* (Adams *et al.*, 2000) and *Anopheles* (Bertrand *et al.*, in press), in the lepidopterans *Manduca* (Weller *et al.*, 2001), *Bombyx* (Charles *et al.*, 1999), and *Trichoplusia ni* (Chen *et al.*, 2002) as well as in *Tenebrio* (Mouillet *et al.*, 1999). One homolog is also found in nematodes (Sluder and Maina, 2001), showing that there might be one single NR6A gene in all metazoans. For once, *Drosophila* is not the leading model organism in the study of an insect nuclear receptor, since most of our current information on HR4 come from *Tenebrio* and lepidopteran species. Several lines of evidence suggest that HR4 may be a new early–late gene involved in the onset of metamorphosis. First, HR4 is directly inducible by 20E in *Manduca* (Hiruma and Riddiford, 2001) and in a *Trichoplusia* cell line (Chen *et al.*, 2002). Then, its activation during molting and metamorphosis starts after the early gene HR3 and before the mid-prepupal gene FTZ-F1. *Manduca*, *Bombyx*, and *Tenebrio* share this pattern of expression (Charles *et al.*, 1999; Mouillet *et al.*, 1999; Hiruma and Riddiford, 2001; Weller *et al.*, 2001). Finally, preliminary results obtained with *Drosophila* confirm that HR4 plays an essential role during molting and metamorphosis (King-Jones and Thummel, FlyBase). However, as for many other nuclear receptors, the most conserved functions are probably in embryogenesis and gametogenesis. Indeed, in adult vertebrates, GCNF is predominantly expressed in the germ cells of gonads, and loss of GCNF function causes embryonic lethality (Chung and Cooney, 2001). Furthermore, the *Drosophila* HR4 gene was also identified in an enhancer-trap screen for novel X-linked essential genes. The P-insertion (PL78) located in this gene causes embryonic lethality with observable cuticular defects and reveals that *Drosophila* HR4 is expressed in embryonic gut and ovary (Bourbon *et al.*, 2002).

3.6.3.1.7. Subfamily 0: atypical transcription factors KNI, KNRL, and EG The subfamily 0 was artificially created to encompass all the proteins that contain only one of the two conserved domains (DBD: NR0A or LBD: NR0B) of the nuclear receptors (nuclear receptors nomenclature committee, 1999). In insects, three transcription factors have

been classified in the subfamily NR0A because they lack a LBD but contain a DBD similar to the one found in the superfamily (Laudet, 1997). This group contains three genes that have been cloned only in Diptera: *knirps* (KNI, NR0A1), *knirps-related* (KNRL, NR0A2), and *eagle* (EG, NR0A3). No homolog can be found in nematodes and vertebrates, suggesting that this group may be specific to insects or arthropods. On phylogeny based with the DBD, these genes are distantly related to HR96 and ECR, typical nuclear receptors of subfamily NR1 (Laudet, 1997).

The gap gene *knirps* has been identified in *D. melanogaster* (Nauber *et al*., 1988), *D. virilis* (Gerwin *et al*., 1994), *D. americana*, *D. novamexicana* (Wittkopp *et al*., 2003), and *Musca* (Sommer and Tautz, 1991) but, surprisingly, it is absent from the genome of *Anopheles* (Bertrand *et al*., in press). KNRL and EG are present both in *Drosophila* and *Anopheles* (Oro *et al*., 1988; Rothe *et al*., 1989; Higashijima *et al*., 1996; Bertrand *et al*., in press). The chromosomal location of these genes, at the proximal part of the left arm of the third chromosome, is conserved between *Drosophila* and *Anopheles*. These data suggest that *knirps* may be the result of a duplication of an ancestral *knrl* gene, which occurred during the evolution of brachyceran Diptera. This hypothesis is reinforced by the strong conservation of expression and function between *Drosophila knirps* and *knrl*. Indeed, both genes are functionally redundant for their role in determining the anterior head structures at the blastoderm stage (Gonzalez-Gaitan *et al*., 1994). Furthermore, they control together the development of several other organs, such as trachea (Chen *et al*., 1998), wing veins (Lunde *et al*., 1998, 2003), foregut, and hindgut (Fuss *et al*., 2001). Thus, it appears that *knirps* and *knrl*, which are very close together on the third chromosome, have conserved the same regulatory regions after duplication. An interesting difference, however, is the size of their intron sequences: one kb in *knirps* and 19 kb in *knrl*. The consequence of this difference in intron size is that *knrl* can complement *knirps* mutations only as an artificial intronless transgene. This effect is explained by the required coordination of mitotic cycle length and gene size during the rapid developmental period of gap gene expression (Rothe *et al*., 1992).

The *eagle* gene (initially named *egon*, for embryonic gonads) is required for the specification of serotonergic neurons and other neuroblasts in the embryonic and larval central nervous system of *Drosophila* (Higashijima *et al*., 1996; Dittrich *et al*., 1997; Lundell and Hirsch, 1998). This gene is present in the genome of *Anopheles* (Bertrand *et al*., in press), but nothing is known about *eagle* in other species of insects.

3.6.3.2. Conservations and Innovations

What general conclusions can be drawn from our present knowledge of the evolution of nuclear receptors in insects? First, it is likely that all insects have at least 17 different nuclear receptors, but DSF, KNI, KNRL, and EG are not included in this number, since their origin by domain recombination is poorly understood. They might well be insect-specific duplications, but this hypothesis has yet to be tested. There are 21 nuclear receptor genes in the genomes of both the brachyceran dipteran *Drosophila* (Adams *et al*., 2000) and the nematoceran dipteran *Anopheles* (Bertrand *et al*., in press). In other insects, given the absence of complete genomic sequences, the full number of these genes is not yet known. However, thanks to a wide interest in the genetics of metamorphosis, several nuclear receptors have been cloned by classical means outside Diptera. In Lepidoptera, 12 nuclear receptors have been identified (11 in *B. mori*), eight in Coleoptera (seven in *Tenebrio molitor*) seven in Hymenoptera and only three in Orthoptera (**Figure 7**). Different ongoing genome and EST sequencing projects should help to clarify this fundamental point (GOLD). Indeed, among the model organisms chosen for these projects are representatives of Hymenoptera (the honeybee *A. mellifera*), Coleoptera (the corn rootworm *D. virgifera*), crustaceans (the water flea *Daphnia*, the crab *C. pugilator*, and the shrimp *F. chinensis*), and Chelicerates (the tick *Amblyomma americanum*). Despite the current lack of heterometabolous and ametabolous insects in these projects, the sequence data will be essential to draw a comprehensive phylogenetic analysis of arthropod nuclear receptors.

With respect to functional evolution, three major trends can be seen (**Figure 8**). First, a strong conservation of structure and regulation characterize HNF4, SVP, and TLL. These proteins are the most conserved nuclear receptors in metazoans and although they may have acquired new functions in insects, their fundamental roles in patterning the embryo are likely to be very similar in all species. There is little doubt that HNF4, SVP, and TLL genes will be found in all insects and other arthropods. Second, by contrast, KNI, KNRL, and EG show an extreme divergence, with the lack of a LBD. In that respect, they cannot be considered as bona fide nuclear receptors. Third, it appears that most of the other nuclear receptors have a broad pattern of expression and participate, directly or indirectly, in

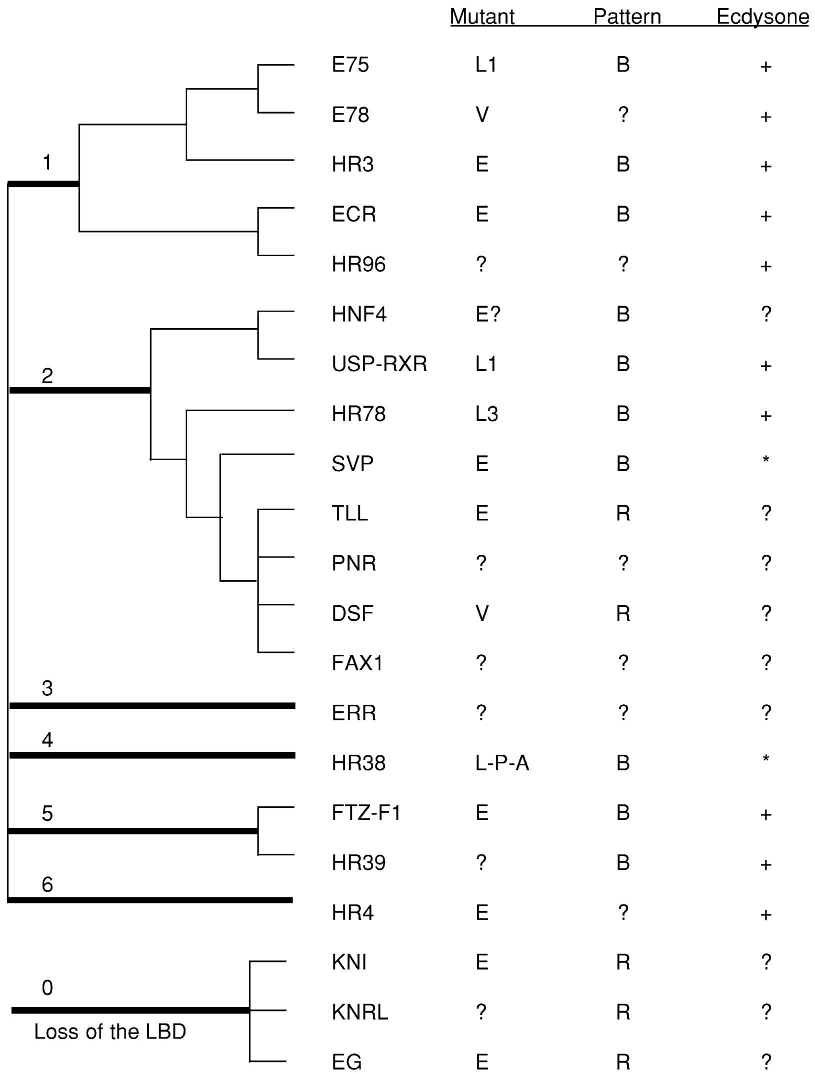


Figure 8 Developmental characteristics of insect nuclear receptors. The tree is the same as in **Figure 6**. The column on the left (mutant) indicates the stage of lethality of the *D. melanogaster* mutants: E, embryo; L, larva; L1, first instar larva; L3, third instar larva; P, pupa; A, adult. In addition, the viable phenotypes (V) are also indicated. The central column (pattern) summarizes the spatial and temporal patterns of expression in insects as broad (B) or restricted (R). The column on the right (ecdysone) indicates whether one given nuclear receptor is part of the ecdysone pathway (+) or simply interacts with it (*).

the 20E pathway (**Figure 8**) (Sullivan and Thummel, 2003). It is a general characteristic of nuclear receptors to be expressed at various stages in many different organs, therefore participating in several physiological processes. The organism uses them as powerful gene regulators whose activity can be coordinated in time and space through the availability of the appropriate ligand. The current view may be biased by the fact that the single hormone mostly related to the insect's nuclear receptor is 20E. However, this signal has undoubtedly acquired a central role in the developmental timing controlled by nuclear receptors.

3.6.4. Evolution of the Ecdysone (20E) Receptor

3.6.4.1. The Arthropod Ecdysone Receptor is a Heterodimer between ECR and USP-RXR

Within the superfamily of nuclear receptors, ECR (NR1H1) belongs to the same group as the vertebrate liver X receptors (LXRα and LXRβ__NR1H3, and NR1H2_), which are receptors for oxysterols (Laudet and Gronemeyer, 2002). USP-RXR (NR2B4) is the ortholog of vertebrate retinoid X receptors (RXRα, β, .γ NR2B1, 2, 3) (Laudet and Gronemeyer, 2002). The name USP (*ultraspiracle*)

comes from the phenotype of *Drosophila* mutants (Perrimon *et al.*, 1985), whereas RXR refers to the mammalian ligand (9-*cis* retinoic acid) (Mangelsdorf *et al.*, 1990). Both genes have not only been isolated in a large number of dipteran and lepidopteran species, but also in several other insect species and arthropods (**Figure 6**).

The functional *Drosophila* ecdysone (20E) receptor is a heterodimer of the products of the ecdysone receptor (*EcR*) and ultraspiracle (*usp*) genes (Koelle *et al.*, 1991; Oro *et al.*, 1992; Yao *et al.*, 1993). The requirement of heterodimerization between ECR and USP-RXR has been found in all the other species studied, including several dipterans (Wang *et al.*, 1998; Vögtli *et al.*, 1999), lepidopterans (Swevers *et al.*, 1996; Perera *et al.*, 1998; Lan *et al.*, 1999; Minakuchi *et al.*, 2003), the crab *C. pugilator* (Durica *et al.*, 2002), and even the tick *Am. americanum* (Guo *et al.*, 1997, 1998). Therefore, it appears that the heterodimer ECR/USP-RXR is the functional ecdysone (20E) receptor in all arthropods. It must be noted that these tests of heterodimerization were made using artificial or real *Drosophila* EcREs, and that the natural binding sites are unknown in other species. However, in *Aedes* (Wang *et al.*, 1998), as well as in *Drosophila* (Vögtli *et al.*, 1998), the heterodimer ECR/USP-RXR exhibits a broad DNA binding specificity, which is likely to be conserved among arthropods.

3.6.4.2. Divergence of the Ecdysone Receptor in Diptera and Lepidoptera

Understanding the evolution of ecdysteroid regulation in insects requires comparative analysis of both partners of the heterodimer. It has been shown that ECR and USP-RXR experienced a strong acceleration of evolutionary rate in Diptera and Lepidoptera compared with other insects (Bonneton *et al.*, 2003). In the LBD of USP-RXR there is only 49% identity between Diptera–Lepidoptera and other insects, as opposed to 68% between these other insects and other arthropods, and 70% between the other insects and chordates (**Table 1**). Thus, the USP-RXR LBD of many insects is less similar to Diptera and Lepidoptera than it is to the chordate RXRs. A similar situation holds for the DBD and LBD domains of ECR, although the divergence is less pronounced. Thus, both ECR and USP-RXR LBD sequences of Diptera and Lepidoptera have evolved at significantly different rates than other species (**Table 1**). This acceleration defines a clear separation within holometabolous insects. Diptera and Lepidoptera belong to the clade Panorpida (Kristensen, 1981), with Hymenoptera as a sister group. Panorpida also includes: Trichoptera (caddisflies), the sister group of Lepidoptera, as well as Mecoptera (scorpionflies), and Siphonaptera (fleas) which are more closely related to Diptera (**Figure 6**). A unique event of acceleration could be responsible for the accelerated evolutionary rates at the base of and within these groups.

3.6.4.3. Evolution of USP-RXR

Several protein domains for which sequence divergence is specific to Diptera and Lepidoptera have been identified (**Figure 9**). The most impressive differences affects the LBD of USP-RXR. In addition, ECR sequences show variability in other domains, namely the DNA binding and the C-terminal F domains (Bonneton *et al.*, 2003). In the *Drosophila* and *Heliothis* USP-RXR structures, the loop between helices H1 and H3 is located inside the hydrophobic furrow of the LBD, thereby preventing the repositioning of helix H12 and interactions with coactivators, and locking these USP-RXRs in an unusual antagonist conformation (Billas *et al.*, 2001; Clayton *et al.*, 2001). In the light of these results, our observation of Diptera- and Lepidoptera-specific sequence diversity in both the loop H1–H3 and the helix H12 suggests a form of concerted evolution between these two interacting regions of the USP-RXR LBD. This evolution may have changed the ligand-dependent transactivation activity of

Table 1 Identity and evolutionary rates for USP-RXR and ECR LBD

		USP-RXR LBD		*ECR LBD*	
Groups of species		*Identity (%)*	*Evolutionary rate*	*Identity (%)*	*Evolutionary rate*
Diptera-Lepidoptera/	Other insects	49	0.31***	64	0.12*
Diptera-Lepidoptera/	Other arthropods	44	0.36***	58	0.13*
Other insects/	Other arthropods	68	0.06 NS	68	0.006 NS

Values indicated for comparisons of evolutionary rates are substitution rate difference. The probability associated to the test is indicated as follows: not significant (NS) $>5\%$; * $\leq 5\%$; *** $\leq 0.5\%$. (Adapted from Bonneton, F., Zelus, D., Iwema, T., Robinson-Rechavi, M., Laudet, V., **2003**. Rapid divergence of the ecdysone receptor in Diptera and Lepidoptera suggests coevolution between ECR and USP-RXR. *Mol. Biol. Evol. 20*, 541–553.)

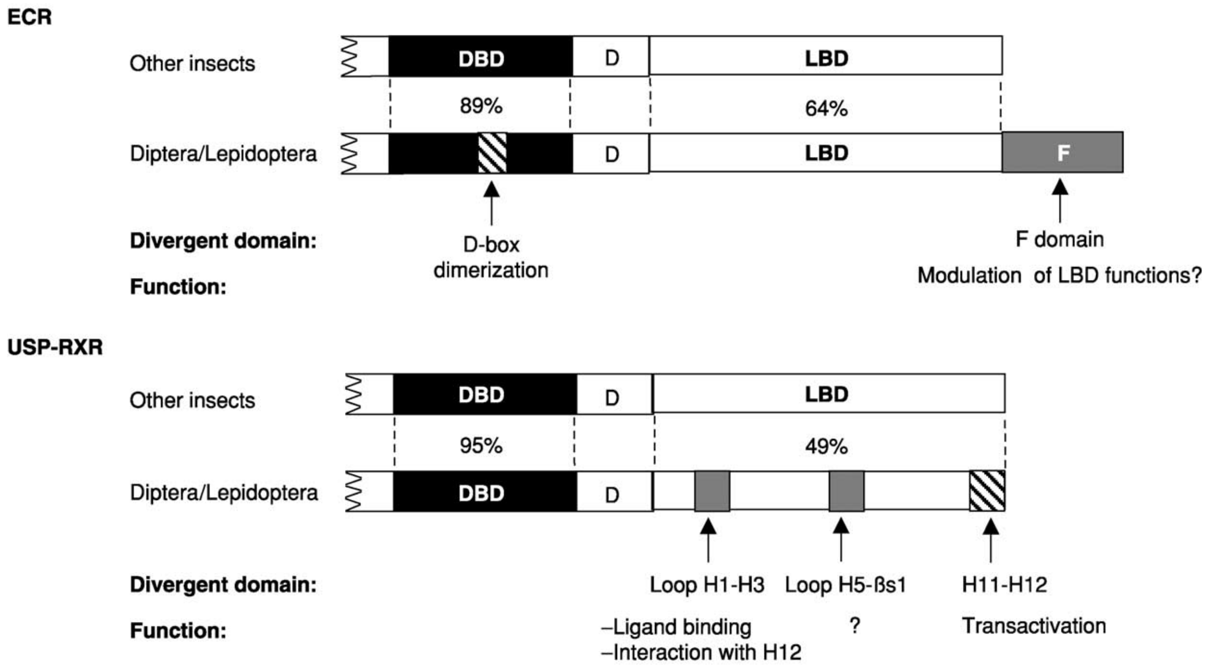


Figure 9 Protein divergence and functional domains of ECR and USP-RXR in insects. On this diagram (not to scale), the DBDs are shown in black, the LBDs in white, and the Diptera-Lepidoptera specific insertions are in grey. Dashed lines indicate regions with a strong Diptera/Lepidoptera divergence. The N-terminal A/B domain (not shown) and the linker region (D domain) are highly divergent in size and sequences and, thus, are not compared in this figure. The average identity percentages of pairwise comparisons for DBD and LBD of USP-RXR and ECR are from Bonneton *et al.* (2003). The name of each divergent domain is indicated, with its known or putative function written below.

the protein. It may also have had an effect on the ligand binding activity, since the loop H1–H3 contains residues that interact with the phospholipid cocrystallized with *Drosophila* and *Heliothis* LBD. However, given the very strong conservation of Helix H10, it is likely that the dimerization activity of USP-RXR LBD remained largely unchanged during evolution. This is important, since USP-RXR is the heterodimeric partner of numerous nuclear receptors, both in vertebrates (TR, RAR, PPAR, LXR, FXR, CAR, NGFIB, etc.) and in insects (HR38, SVP, HR78).

All these hypotheses can now be tested both *in vitro* and, more importantly, *in vivo* using transgenesis. Small changes at the molecular level can lead to important functional differences that may be revealed only by studying their consequences in whole organisms. For example, replacing the domains D and E of the *Drosophila usp* gene with the equivalent domains of another dipteran, *Chironomus*, allows the rescue of usp mutants from first instar larvae lethality, but do not restore a normal metamorphosis (Henrich *et al.*, 2000). Therefore, despite the 72% of similarity between the exchanged domains, this chimeric USP-RXR fails to function normally in *Drosophila*. Similar studies involving smaller chimeric regions and site directed mutations should help in the future to identify which domains contributed to the functional evolution of USP-RXR in insects.

3.6.4.4. Evolution of ECR

It is intriguing that the LBD of ECR underwent a significant increase of substitution rate in Diptera and Lepidoptera, while its structure remained apparently largely unchanged. In all insects, and presumably in all arthropods, ECR LBD binds 20E (Riddiford *et al.*, 2000). This fundamental interaction may represent the primary selective constraint acting on this domain. However, the ligand affinity of ECR can show subtle differences in insects. This is best illustrated within Diptera. A homology model of the *Chironomus* ECR LBD showed that the putative ligand binding pocket is strongly conserved in all arthropods (Wurtz *et al.*, 2000). Nevertheless, comparative tests of toxicity and receptor affinity of nonsteroid insecticides revealed that the *Chironomus* ecdysone (20E) receptor behaves more like a lepidopteran than like *Drosophila* (Smagghe *et al.*, 2002). Also of great interest is the discovery that the *Aedes* receptor is significantly much more sensitive to ecdysone than the *Drosophila* receptor, whereas both proteins exhibit the same sensitivity to 20E, the natural biologically active hormone. Surprisingly, the genetic origin of this difference in

ligand specificity was mapped into the helix H10 of the ECR LBD, a region known to be essential for dimerization (Wang *et al.*, 2000). These results show that the conservation of the structure of ECR LBD can hide a significant plasticity, which can only be revealed through appropriate functional tests.

Nuclear receptor LBDs are also involved in heterodimerization activity. The rapid evolution of ECR can be explained by adaptation to the extremely divergent USP-RXR, and eventually acquisition of new partners, such as HR38 (Baker *et al.*, 2003). It may be that the stability of the heterodimer required compensatory changes in ECR and USP-RXR, suggestive of coevolution. The differences seen in ECR DBD also suggest functional changes in dimerization (Bonneton *et al.*, 2003). Indeed, four of the six substitutions that are conserved among Diptera and Lepidoptera are located in the second zinc finger, at positions known to be involved in protein dimerization but not in DNA contact or nuclear localization signal (Black *et al.*, 2001; Khorasanizadeh and Rastinejad, 2001).

Another evolutionary acquisition of Diptera and Lepidoptera is the presence of a C-terminal F domain of variable length, which does not show any sequence conservation between species (**Figure 8**; Bonneton *et al.*, 2003). Most nuclear receptors do not contain this domain, including mammalian homologs LXRs. This difference is interesting, since it is known that when present (ERα, HNF-4) the F domain of nuclear receptors can modulate different functions of the LBD (Montano *et al.*, 1995; Nichols *et al.*, 1998; Peters and Khan, 1999). The F domain of *Drosophila* ECR may also contribute modestly to the activation function of the LBD (Thornmeyer *et al.*, 1999; Hu *et al.*, 2003).

3.6.4.5. Ecdysone Receptor and the Evolution of Insects

Ecdysteroids control reproduction and development of arthropods. As a result, the pattern of expression of the ecdysone (20E) receptor correlates very well in time and space with the localization and the level of these hormones. Indeed, both ECR and USP-RXR are expressed in a wide range of tissues during oogenesis, embryogenesis, molting, and metamorphosis (see **Chapter 3.5**). Therefore, any evolutionary change in the mode of action of the ecdysone receptor can have important consequences for development and/or reproduction. This major issue has only started to be addressed, and most of the questions are still unanswered.

Changing the regulation of a key developmental gene is probably one of the most effective ways to create new functions rapidly during the course of evolution. In that respect, very interesting results have shown that alterations in the timing of ovarian ECR and USP-RXR expression characterize the evolution of larval reproduction in two species of gall midges (Hodin and Riddiford, 2000). In these dipterans, pedogenesis involves the precocious growth and differentiation of the ovary and subsequent parthenogenetic reproduction in an otherwise larval form. Genetic analysis has shown that ECR and USP-RXR regulate the timing of ovarian morphogenesis during *Drosophila* metamorphosis (Hodin and Riddiford, 1998). These data suggest that heterochronic shifts in the ovarian regulation of both genes may play an important role in the evolution of dipteran pedogenesis. In a larger perspective, a growing number of evidence suggest that the genetic cascade triggered by ecdysteroids may play a central role in the control of developmental timing in Ecdysozoa (Thummel, 2001). Therefore, we can expect that future studies will identify changes in the expression of the ecdysone receptor associated with the evolution of different temporally regulated processes of insects such as, for example, molting, metamorphosis, diapause, and adult longevity.

Regarding the evolution of postembryonic development, it is observed that the divergence of EcR does not correlate with the different types of insect metamorphosis (Bonneton *et al.*, 2003). Moreover, several nuclear receptors such as E75, HR3, HR78, βFTZ-F1, and HR4 play very similar roles in the control of molting and metamorphosis amongst insects (see **Chapter 3.5**). These results suggest that the ecdysteroid-induced genetic cascade (at least the upstream part of this cascade) is a well-conserved developmental module in insects; this conclusion may even be extended to all the molting metazoans. Actually, theories that try to explain the evolution of insect metamorphosis usually consider that JH is the key player, rather than 20E (Sehnal *et al.*, 1996; Truman and Riddiford, 1999, 2002). A heterochronic shift in embryonic JH secretion, with an earlier appearance of this hormone, could well have been the major event in the transition from hemimetabolous to holometabolous insects. In the light of these interpretations, the possibility that JH is a natural ligand of USP-RXR (Jones and Sharp, 1997; Jones *et al.*, 2001; Sasorith *et al.*, unpublished data) is one of the most important point to clarify in order to understand the emergence of metamorphosis in insects.

Acknowledgments

We thank Barbara Demeinex and Hector Escriva for their very helpful comments on this text, and Gilles

Parmentier for the search of nuclear receptors in honeybee's EST. Work from our laboratory is funded by the CNRS, the MENRT, the Région Rhône-Alpes, and the ARC.

References

Adams, M.D., Celniker, S.E., Holt, R.A., *et al*., **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Adoutte, A., Balavoine, G., Lartillot, N., de Rosa, R., **1999**. Animal evolution: the end of the intermediate taxa? *Trends Genet. 15*, 104–108.

Aguinaldo, A.M., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., *et al*., **1997**. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature 387*, 489–493.

Alonso, C.R., Maxton-Kuechenmeister, J., Akam, M., **2001**. Evolution of Ftz protein function in insects. *Curr. Biol. 11*, 1473–1478.

Amero, S.A., Kretsinger, R.H., Moncrief, N.D., Yamamoto, K.R., Pearson, W.R., **1992**. The origin of nuclear receptor proteins: a single precursor distinct from other transcription factors. *Mol. Endocrinol. 6*, 3–7.

Andre, E., Conquet, F., Steinmayr, M., Stratton, S.C., Porciatti, V., *et al*., **1998**. Disruption of retinoid-related orphan receptor beta changes circadian behavior, causes retinal degeneration and leads to vacillans phenotype in mice. *EMBO J. 17*, 3867–3877.

Antebi, A., Culotti, J.G., Hedgecock, E.M., **1998**. *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. *Development 125*, 1191–1205.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., Riddle, D.L., **2000**. *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. *Genes Devel. 14*, 1512–1527.

Asahina, M., Ishihara, T., Jindra, M., Kohara, Y., Katsura, I., *et al*., **2000**. The conserved nuclear receptor Ftz-F1 is required for embryogenesis, moulting and reproduction in *Caenorhabditis elegans*. *Genes Cells 5*, 711–723.

Astle, J., Kozlova, T., Thummel, C.S., **2003**. Essential roles for the *Dhr78* orphan nuclear receptor during molting of the *Drosophila* tracheal system. *Insect Biochem. Mol. Biol. 33*, 1201–1209.

Ayer, S., Benyajati, C., **1992**. The binding site of a steroid hormone receptor-like protein within the *Drosophila Adh* adult enhancer is required for high levels of tissue-specific alcohol dehydrogenase expression. *Mol. Cell. Biol. 12*, 661–673.

Ayer, S., Walker, N., Mosammaparast, M., Nelson, J.P., Shilo, B.Z., *et al*., **1993**. Activation and repression of *Drosophila* alcohol dehydrogenase distal transcription by two steroid hormone receptor superfamily members binding to a common response element. *Nucleic Acids Res. 21*, 1619–1627.

Baker, K.D., Shewchuk, L.M., Kozlova, T., *et al*., **2003**. The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell 113*, 731–742.

Barnett, P., Tabak, H.F., Hettema, E.H., **2000**. Nuclear receptors arose from pre-existing protein modules during evolution. *Trends Biochem. Sci. 25*, 227–228.

Bertrand, S., Brunet, F., Laudet, V., Robinson-Rechavi, M. Evolutionary genomics of nuclear receptors: from 25 ancestral genes to derived endocrine systems. *Mol. Biol. Evol.* (in press).

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., **2002**. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Devel. Cell 3*, 209–220.

Billas, I.M., Iwema, T., Garnier, J.M., Mitschler, A., Rochel, N., *et al*., **2003**. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature 426*, 91–96.

Billas, I.M., Moulinier, L., Rochel, N., Moras, D., **2001**. Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. *J. Biol. Chem. 276*, 7465–7474.

Black, B.E., Holaska, J.M., Rastinejad, F., Paschal, B.M., **2001**. DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr. Biol. 11*, 1749–1758.

Blair, J.E., Ikeo, K., Gojobori, T., Hedges, S.B., **2002**. The evolutionary position of nematodes. *BMC Evol. Biol. 2*, 1–7.

Bonneton, F., Zelus, D., Iwema, T., Robinson-Rechavi, M., Laudet, V., **2003**. Rapid divergence of the ecdysone receptor in Diptera and Lepidoptera suggests coevolution between ECR and USP-RXR. *Mol. Biol. Evol. 20*, 541–553.

Bourbon, H.M., Gonzy-Treboul, G., Peronnet, F., *et al*., **2002**. A P-insertion screen identifying novel X-linked essential genes in *Drosophila*. *Mech. Devel. 110*, 71–83.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., Moras, D., **1995**. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature 375*, 377–382.

Broadus, J., Doe, C.Q., **1995**. Evolution of neuroblast identity: *seven-up* and *prospero* expression reveal homologous and divergent neuroblast fates in *Drosophila* and *Schistocerca*. *Development 121*, 3989–3996.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., Woodard, C.T., **1999**. The *Drosophila* beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell 3*, 143–149.

Brody, T., Stivers, C., Nagle, J., Odenwald, W.F., **2002**. Identification of novel *Drosophila* neural precursor genes using a differential embryonic head cDNA screen. *Mech. Devel. 113*, 41–59.

Bryant, Z., Subrahmanyan, L., Tworoger, M., LaTray, L., Liu, C.R., *et al*., **1999**. Characterization of differentially expressed genes in purified *Drosophila* follicle cells:

toward a general strategy for cell type-specific developmental analysis. *Proc. Natl Acad. Sci. USA 96*, 5559–5564.

Butler, A.J., Parker, M.G., **1995**. COUP-TF II homodimers are formed in preference to heterodimers with RXR alpha or TR beta in intact cells. *Nucleic Acids Res. 23*, 4143–4150.

Carmi, I., Kopczynski, J.B., Meyer, B.J., **1998**. The nuclear hormone receptor SEX-1 is an X-chromosome signal that determines nematode sex. *Nature 396*, 168–173.

Carney, G.E., Wade, A.A., Sapra, R., Goldstein, E.S., Bender, M., **1997**. DHR3, an ecdysone-inducible early-late gene encoding a *Drosophila* nuclear receptor, is required for embryogenesis. *Proc. Natl Acad. Sci. USA 94*, 12024–12029.

Chan, S.M., **1998**. Cloning of a shrimp (*Metapanaeus ensis*) cDNA encoding a nuclear receptor superfamily member: an insect homologue of *E75* gene. *FEBS Lett. 436*, 395–400.

Chan, S.M., Chan, K.M., **1999**. Characterization of the shrimp eyestalk cDNA encoding a novel fushi tarazu-factor 1 (FTZ-F1). *FEBS Lett. 454*, 109–114.

Charles, J.P., Shinoda, T., Chinzei, Y., **1999**. Characterization and DNA-binding properties of GRF, a novel monomeric binding orphan receptor related to GCNF and betaFTZ-F1. *Eur. J. Biochem. 266*, 181–190.

Chen, C.K., Kuhnlein, R.P., Eulenberg, K.G., Vincent, S., Affolter, M., *et al.*, **1998**. The transcription factors KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during *Drosophila* tracheal development. *Development 125*, 4959–4968.

Chen, J.H., Turner, P.C., Rees, H.H., **2002**. Molecular cloning and induction of nuclear receptors from insect cell lines. *Insect Biochem. Mol. Biol. 32*, 657–667.

Chen, W.S., Manova, K., Weinstein, D.C., Duncan, S.A., Plump, A.S., *et al.*, **1994**. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Devel. 8*, 2466–2477.

Chung, A.C., Cooney, A.J., **2001**. Germ cell nuclear factor. *Int. J. Biochem. Cell Biol. 33*, 1141–1146.

Clarke, N.D., Berg, J.M., **1998**. Zinc fingers in *Caenorhabditis elegans*: finding families and probing pathways. *Science 282*, 2018–2022.

Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., **2001**. The structure of the *ultraspiracle* ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA 98*, 1549–1554.

Cooney, A.J., Leng, X., Tsai, S.Y., O'Malley, B.W., Tsai, M.J., **1993**. Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. *J. Biol. Chem. 268*, 4152–4160.

Crispi, S., Giordano, E., D'Avino, P.P., Furia, M., **1998**. Cross-talking among *Drosophila* nuclear receptors at the promiscuous response element of the *ng-1* and *ng-2* intermolt genes. *J. Mol. Biol. 275*, 561–574.

Crossgrove, K., Laudet, V., Maina, C.V., **2002**. *Dirofilaria immitis* encodes *Di-nhr-7*, a putative orthologue of the *Drosophila* ecdysone-regulated *E78* gene. *Mol. Biochem. Parasitol. 119*, 169–177.

Daniel, A., Dumstrei, K., Lengyel, J.A., Hartenstein, V., **1999**. The control of cell fate in the embryonic visual system by *atonal*, *tailless* and EGFR signaling. *Development 126*, 2945–2954.

De Loof, A., Huybrechts, R., **1998**. "Insects do not have sex hormones": a myth? *Gen. Comp. Endocrinol. 111*, 245–260.

de Mendonça, R.L., Bouton, D., Bertin, B., Escriva, H., Noel, C., *et al.*, **2002**. A functionally conserved member of the FTZ-F1 nuclear receptor family from *Schistosoma mansoni*. *Eur. J. Biochem. 269*, 5700–5711.

de Mendonça, R.L., Escriva, H., Bouton, D., Zelus, D., Vanacker, J.M., *et al.*, **2000b**. Structural and functional divergence of a nuclear receptor of the RXR family from the trematode parasite *Schistosoma mansoni*. *Eur. J. Biochem. 267*, 3208–3219.

de Mendonça, R.L., Escriva, H., Vanacker, J.M., Bouton, D., Delannoy, S., *et al.*, **1999**. Nuclear hormone receptors and evolution. *Am. Zool. 39*, 704–713.

de Rosa, R., Grenier, J.K., Andreeva, T., Cook, C.E., Adoutte, A., *et al.*, **1999**. *Hox* genes in brachiopods and priapulids and protostome evolution. *Nature 399*, 772–776.

Debernard, S., Bozzolan, F., Duportets, L., Porcheron, P., **2001**. Periodic expression of an ecdysteroid-induced nuclear receptor in a lepidopteran cell line (IAL-PID2). *Insect Biochem. Mol. Biol. 31*, 1057–1064.

Dhe-Paganon, S., Duda, K., Iwamoto, M., Chi, Y.I., Shoelson, S.E., **2002**. Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand. *J. Biol. Chem. 277*, 37973–37976.

Dittrich, R., Bossing, T., Gould, A.P., Technau, G.M., Urban, J., **1997**. The differentiation of the serotonergic neurons in the *Drosophila* ventral nerve cord depends on the combined function of the zinc finger proteins *Eagle* and *Huckebein*. *Development 124*, 2515–2525.

Duarte, J., Perriere, G., Laudet, V., Robinson-Rechavi, M., **2002**. NUREBASE: database of nuclear hormone receptors. *Nucleic Acids Res. 30*, 364–368.

Durica, D.S., Wu, X., Anilkumar, G., Hopkins, P.M., Chung, A.C., **2002**. Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation. *Mol. Cell. Endocrinol. 189*, 59–76.

Egea, P.F, Mitschler, A., Rochel, N., Ruff, M., Chambon, P., *et al.*, **2000**. Crystal structure of the human RXRα ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *EMBO J. 19*, 2592–2601.

Escriva Garcia, H., Laudet, V., Robinson-Rechavi, M., **2003**. Nuclear receptors are markers of animal genome evolution. *J. Struct. Funct. Genomics 3*, 177–184.

Escriva, H., Bertrand, S., Laudet, V. The evolution of the nuclear receptor superfamily, vol. 40. In: Nuclear Receptor Superfamily, Essays in Biochemistry, (in press).

Escriva, H., Delaunay, F., Laudet, V., **2000**. Ligand binding and nuclear receptor evolution. *BioEssays 22*, 717–727.

Escriva Garcia, H., Safi, R., Hanni, C., Langlois, M.C., Saumitou-Laprade, P., *et al.*, **1997**. Ligand binding was acquired during evolution of nuclear receptors. *Proc. Natl Acad. Sci. USA 94*, 6803–6808.

Eystathioy, T., Swevers, L., Iatrou, K., **2001**. The orphan nuclear receptor BmHR3A of *Bombyx mori*: hormonal control, ovarian expression and functional properties. *Mech. Devel. 103*, 107–115.

Feigl, G., Gram, M., Pongs, O., **1989**. A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in *Drosophila melanogaster. Nucleic Acids Res. 17*, 7167–7178.

Finley, K.D., Edeen, P.T., Foss, M., Gross, E., Ghbeish, N., *et al.*, **1998**. *Dissatisfaction* encodes a tailless-like nuclear receptor expressed in a subset of CNS neurons controlling *Drosophila* sexual behavior. *Neuron 21*, 1363–1374.

Finley, K.D., Taylor, B.J., Milstein, M., McKeown, M., **1997**. *dissatisfaction*, a gene involved in sex-specific behavior and neural development of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 94*, 913–918.

Fisk, G.J., Thummel, C.S., **1995**. Isolation, regulation, and DNA-binding properties of three *Drosophila* nuclear hormone receptor superfamily members. *Proc. Natl Acad. Sci. USA 92*, 10604–10608.

Fisk, G.J., Thummel, C.S., **1998**. The DHR78 nuclear receptor is required for ecdysteroid signaling during the onset of *Drosophila* metamorphosis. *Cell 93*, 543–555.

Florence, B., Guichet, A., Ephrussi, A., Laughon, A., **1997**. Ftz-F1 is a cofactor in *Ftz* activation of the *Drosophila engrailed* gene. *Development 124*, 839–847.

Fuss, B., Meissner, T., Bauer, R., Lehmann, C., Eckardt, F., *et al.*, **2001**. Control of endoreduplication domains in the *Drosophila* gut by the knirps and knirps-related genes. *Mech. Devel. 100*, 15–23.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., Antebi, A., **2001**. A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life span. *Devel. Cell 1*, 841–851.

Gerwin, N., La Rosee, A., Sauer, F., Halbritter, H.P., Neumann, M., *et al.*, **1994**. Functional and conserved domains of the *Drosophila* transcription factor encoded by the segmentation gene *knirps*. *Mol. Cell. Biol. 14*, 7899–7908.

Giguere, V., **1999**. Orphan nuclear receptors: from gene to function. *Endocr. Rev. 20*, 689–725.

Giguere, V., **2002**. To ERR in the estrogen pathway. *Trends Endocrinol. Metab. 13*, 220–225.

Giribet, G., Edgecombe, G.D., Wheeler, W.C., **2001**. Arthropod phylogeny based on eight molecular loci and morphology. *Nature 413*, 157–161.

Gissendanner, C.R., Sluder, A.E., **2000**. nhr-25, the *Caenorhabditis elegans* ortholog of ftz-f1, is required for epidermal and somatic gonad development. *Devel. Biol. 221*, 259–272.

Gonzalez-Gaitan, M., Rothe, M., Wimmer, E.A., Taubert, H., Jackle, H., **1994**. Redundant functions of the genes *knirps* and *knirps-related* for the establishment of anterior *Drosophila* head structures. *Proc. Natl Acad. Sci. USA 91*, 8567–8571.

Grasso, L.C., Hayward, D.C., Trueman, J.W., Hardie, K.M., Janssens, P.A., *et al.*, **2001**. The evolution of nuclear receptors: evidence from the coral *Acropora*. *Mol. Phylogenet. Evol. 21*, 93–102.

Greschik, H., Wurtz, J.M., Sanglier, S., Bourguet, W., van Dorsselaer, A., *et al.*, **2002**. Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. *Mol. Cell 9*, 303–313.

Guichet, A., Copeland, J.W., Erdelyi, M., *et al.*, **1997**. The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature 385*, 548–552.

Guo, X., Harmon, M.A., Laudet, V., Mangelsdorf, D.J., Palmer, M.J., **1997**. Isolation of a functional ecdysteroid receptor homologue from the ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol. 27*, 945–962.

Guo, X., Xu, Q., Harmon, M.A., Jin, X., Laudet, V., *et al.*, **1998**. Isolation of two functional retinoid X receptor subtypes from the Ixodid tick, *Amblyomma americanum* (L.). *Mol. Cell. Endocrinol. 139*, 45–60.

Haider, N.B., Jacobson, S.G., Cideciyan, A.V., *et al.*, **2000**. Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. *Nat. Genet. 24*, 127–131.

Hartmann, B., Reichert, H., Walldorf, U., **2001**. Interaction of gap genes in the *Drosophila* head: *tailless* regulates expression of *empty spiracles* in early embryonic patterning and brain development. *Mech. Devel. 109*, 161–172.

Henrich, V.C., Brown, N.E., **1995**. Insect nuclear receptors: a developmental and comparative perspective. *Insect Biochem. Mol. Biol. 25*, 881–897.

Henrich, V.C., Sliter, T.J., Lubahn, D.B., MacIntyre, A., Gilbert, L.I., **1990**. A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. *Nucleic Acids Res. 18*, 4143–4148.

Henrich, V.C., Vogtli, M.E., Antoniewski, C., Spindler-Barth, M., Przibilla, S., *et al.*, **2000**. Developmental effects of a chimeric *ultraspiracle* gene derived from *Drosophila* and *Chironomus*. *Genesis 28*, 125–133.

Hepperle, C., Hartfelder, K., **2001**. Differentially expressed regulatory genes in honey bee caste development. *Naturwissenschaften 88*, 113–116.

Higashijima, S., Shishido, E., Matsuzaki, M., Saigo, K., **1996**. *eagle*, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the *Drosophila* CNS. *Development 122*, 527–536.

Hirai, M., Shinoda, T., Kamimura, M., Tomita, S., Shiotsuki, T., **2002**. *Bombyx mori* orphan receptor, *BmHR78*: cDNA cloning, testis abundant expression

and putative dimerization partner for *Bombyx ultraspiracle*. *Mol. Cell. Endocrinol. 189*, 201–211.

Hiruma, K., Riddiford, L.M., **2001**. Regulation of transcription factors MHR4 and betaFTZ-F1 by 20-hydroxyecdysone during a larval molt in the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 232*, 265–274.

Hodin, J., Riddiford, L.M., **1998**. The ecdysone receptor and *ultraspiracle* regulate the timing and progression of ovarian morphogenesis during *Drosophila* metamorphosis. *Devel. Genes Evol. 208*, 304–317.

Hodin, J., Riddiford, L.M., **2000**. Parallel alterations in the timing of ovarian ecdysone receptor and *ultraspiracle* expression characterize the independent evolution of larval reproduction in two species of gall midges (Diptera: Cecidomyiidae). *Devel. Genes Evol. 210*, 358–372.

Horner, M.A., Chen, T., Thummel, C.S., **1995**. Ecdysteroid regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Devel. Biol. 168*, 490–502.

Hoshizaki, D.K., Blackburn, T., Price, C., Ghosh, M., Miles, K., *et al.*, **1994**. Embryonic fat-cell lineage in *Drosophila melanogaster. Development 120*, 2489–2499.

Hu, X., Cherbas, L., Cherbas, P., **2003**. Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. *Mol. Endocrinol. 17*, 716–731.

Hughes, C.L., Kaufman, T.C., **2002**. Hox genes and the evolution of the arthropod body plan. *Evol. Devel. 4*, 459–499.

Hwang, U.W., Friedrich, M., Tautz, D., Park, C.J., Kim, W., **2001**. Mitochondrial protein phylogeny joins myriapods with chelicerates. *Nature 413*, 154–157.

Jetten, A.M., Kurebayashi, S., Ueda, E., **2001**. The ROR nuclear orphan receptor subfamily: critical regulators of multiple biological processes. *Prog. Nucleic Acid Res. Mol. Biol. 69*, 205–247.

Jia, K., Albert, P.S., Riddle, D.L., **2002**. DAF-9, a cytochrome P450 regulating *C. elegans* larval development and adult longevity. *Development 129*, 221–231.

Jindra, M., Sehnal, F., Riddiford, L.M., **1994a**. Isolation, characterization and developmental expression of the ecdysteroid-induced E75 gene of the wax moth *Galleria mellonella*. *Eur. J. Biochem. 221*, 665–675.

Jindra, M., Sehnal, F., Riddiford, L.M., **1994b**. Isolation and developmental expression of the ecdysteroid-induced GHR3 gene of the wax moth *Galleria mellonella*. *Insect Biochem. Mol. Biol. 24*, 763–773.

Jones, G., Sharp, P.A., **1997**. *Ultraspiracle*: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA 94*, 13499–13503.

Jones, G., Wozniak, M., Chu, Y., Dhar, S., Jones, D., **2001**. Juvenile hormone III-dependent conformational changes of the nuclear receptor *ultraspiracle*. *Insect Biochem. Mol. Biol. 32*, 33–49.

Jürgens, G., Wieschaus, E., Nüsslein-Volhard, C., Kluding, H., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster. Roux's Arch. Devel. Biol. 193*, 283–295.

Kageyama, Y., Masuda, S., Hirose, S., Ueda, H., **1997**. Temporal regulation of the mid-prepupal gene FTZ-F1: DHR3 early late gene product is one of the plural positive regulators. *Genes Cells 2, 559–569.*

Kallen, J.A., Schlaeppi, J.M., Bitsch, F., Geisse, S., Geiser, M., *et al.*, **2002**. X-ray structure of the hRORalpha LBD at 1.63 A: structural and functional data that cholesterol or a cholesterol derivative is the natural ligand of RORalpha. *Structure (Camb.) 10*, 1697–1707.

Kapitskaya, M.Z., Dittmer, N.T., Deitsch, K.W., Cho, W.L., Taylor, D.G., *et al.*, **1998**. Three isoforms of a hepatocyte nuclear factor-4 transcription factor with tissue- and stage-specific expression in the adult mosquito. *J. Biol. Chem. 273*, 29801–29810.

Kapitskaya, M.Z., Li, C., Miura, K., Segraves, W., Raikhel, A.S., **2000**. Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol. Cell. Endocrinol. 160*, 25–37.

Kerber, B., Fellert, S., Hoch, M., **1998**. Seven-up, the *Drosophila* homolog of the COUP-TF orphan receptors, controls cell proliferation in the insect kidney. *Genes Devel. 12*, 1781–1786.

Khorasanizadeh, S., Rastinejad, F., **2001**. Nuclear-receptor interactions on DNA-response elements. *Trends Biochem. Sci. 26*, 384–390.

King, N., Hittinger, C.T., Carroll, S.B., **2003**. Evolution of key cell signaling and adhesion protein families predates animal origins. *Science 301*, 361–363.

Kliewer, S.A., Umesono, K., Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., *et al.*, **1992**. Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc. Natl Acad. Sci. USA 89*, 1448–1452.

Kobayashi, M., Takezawa, S., Hara, K., Yu, R.T., Umesono, Y., *et al.*, **1999**. Identification of a photoreceptor cell-specific nuclear receptor. *Proc. Natl Acad. Sci. USA 96*, 4814–4819.

Koelle, M.R., Segraves, W.A., Hogness, D.S., **1992**. DHR3: a *Drosophila* steroid receptor homolog. *Proc. Natl Acad. Sci. USA 89*, 6167–6171.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.

Komonyi, O., Mink, M., Csiha, J., Maroy, P., **1998**. Genomic organization of DHR38 gene in *Drosophila*: presence of Alu-like repeat in a translated exon and expression during embryonic development. *Arch. Insect Biochem. Physiol. 38*, 185–192.

Kostrouch, Z., Kostrouchova, M., Love, W., Jannini, E., Piatigorsky, J., *et al.*, **1998**. Retinoic acid X receptor in the diploblast, *Tripedalia cystophora*. *Proc. Natl Acad. Sci. USA 95*, 13442–13447.

Kostrouch, Z., Kostrouchova, M., Rall, J.E., **1995**. Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA 92*, 156–159.

Kostrouchova, M., Krause, M., Kostrouch, Z., Rall, J.E., **1998**. CHR3: a *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. *Development 125*, 1617–1626.

Kostrouchova, M., Krause, M., Kostrouch, Z., Rall, J.E., **2001**. Nuclear hormone receptor CHR3 is a critical regulator of all four larval molts of the nematode *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA 98*, 7360–7365.

Kozlova, T., Pokholkova, G.V., Tzertzinis, G., Sutherland, J.D., Zhimulev, I.F., *et al.*, **1998**. *Drosophila* hormone receptor 38 functions in metamorphosis: a role in adult cuticle formation. *Genetics 149*, 1465–1475.

Kozlova, T., Thummel, C.S., **2000**. Steroid regulation of postembryonic development and reproduction in *Drosophila*. *Trends Endocrinol. Metab. 11*, 276–280.

Kristensen, N.P., **1981**. Phylogeny of insect orders. *Annu. Rev. Entomol. 26*, 135–157.

Lam, G., Hall, B.L., Bender, M., Thummel, C.S., **1999**. DHR3 is required for the prepupal–pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. *Devel. Biol. 212*, 204–216.

Lam, G.T., Jiang, C., Thummel, C.S., **1997**. Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development 124*, 1757–1769.

Lan, Q., Hiruma, K., Hu, X., Jindra, M., Riddiford, L.M., **1999**. Activation of a delayed-early gene encoding MHR3 by the ecdysone receptor heterodimer EcR-B1-USP-1 but not by EcR-B1-USP-2. *Mol. Cell. Biol. 19*, 4897–4906.

Lan, Q., Wu, Z., Riddiford, L.M., **1997**. Regulation of the ecdysone receptor, USP, E75 and MHR3 mRNAs by 20-hydroxyecdysone in the GV1 cell line of the tobacco hornworm, *Manduca sexta*. *Insect Mol. Biol. 6*, 3–10.

Langelan, R.E., Fisher, J.E., Hiruma, K., Palli, S.R., Riddiford, L.M., **2000**. Patterns of MHR3 expression in the epidermis during a larval molt of the tobacco hornworm *Manduca sexta*. *Devel. Biol. 227*, 481–494.

Laudet, V., **1997**. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J. Mol. Endocrinol. 19*, 207–226.

Laudet, V., Adelmant, G., **1995**. Nuclear receptors. Lonesome orphans. *Curr. Biol. 5*, 124–127.

Laudet, V., Gronemeyer, H., **2002**. The Nuclear Receptor FactsBook. Academic Press, London.

Laudet, V., Hanni, C., Coll, J., Catzeflis, F., Stehelin, D., **1992**. Evolution of the nuclear receptor gene superfamily. *EMBO J. 11*, 1003–1013.

Lavorgna, G., Karim, F.D., Thummel, C.S., Wu, C., **1993**. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl Acad. Sci. USA 90*, 3004–3008.

Lavorgna, G., Ueda, H., Clos, J., Wu, C., **1991**. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. *Science 252*, 848–851.

Li, C., Kapitskaya, M.Z., Zhu, J., Miura, K., Segraves, W., *et al.*, **2000**. Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. *Devel. Biol. 224*, 96–110.

Liaw, G.J., Lengyel, J.A., **1993**. Control of *tailless* expression by *bicoid*, *dorsal* and synergistically interacting terminal system regulatory elements. *Mech. Devel. 40*, 47–61.

Lo, P.C., Frasch, M., **2001**. A role for the COUP-TF-related gene *seven-up* in the diversification of cardioblast identities in the dorsal vessel of *Drosophila*. *Mech. Devel. 104*, 49–60.

Lohr, U., Yussa, M., Pick, L., **2001**. *Drosophila fushi tarazu*: a gene on the border of homeotic function. *Curr. Biol. 11*, 1403–1412.

Lunde, K., Biehs, B., Nauber, U., Bier, E., **1998**. The *knirps* and *knirps-related* genes organize development of the second wing vein in *Drosophila*. *Development 125*, 4145–4154.

Lunde, K., Trimble, J.L., Guichard, A., Guss, K.A., Nauber, U., *et al.*, **2003**. Activation of the *knirps* locus links patterning to morphogenesis of the second wing vein in *Drosophila*. *Development 130*, 235–248.

Lundell, M.J., Hirsch, J., **1998**. *eagle* is required for the specification of serotonin neurons and other neuroblast 7–3 progeny in the *Drosophila* CNS. *Development 125*, 463–472.

Maglisch, J.M., Sluder, A., Guan, X., Shi, Y., McKee, D.D., *et al.*, **2001**. Comparison of complete nuclear receptor sets from the human, *Caenorhabditis elegans* and *Drosophila* genomes. *Genome Biol. 2*, RESEARCH0029.

Mangelsdorf, D.J., Ong, E.S., Dyck, J.A., Evans, R.M., **1990**. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature 345*, 224–229.

Matsuoka, T., Fujiwara, H., **2000**. Expression of ecdysteroid-regulated genes is reduced specifically in the wing discs of the wing-deficient mutant (fl) of *Bombyx mori*. *Devel. Genes Evol. 210*, 120–128.

Minakuchi, C., Nakagawa, Y., Kiuchi, M., Seino, A., Tomita, S., *et al.*, **2003**. Molecular cloning and expression analysis of *ultraspiracle* (USP) from the rice stem borer *Chilo suppressalis*. *Insect Biochem. Mol. Biol. 33*, 41–49.

Miura, K., Zhu, J., Dittmer, N.T., Chen, L., Raikhel, A.S., **2002**. A COUP-TF/Svp homolog is highly expressed during vitellogenesis in the mosquito *Aedes aegypti*. *J. Mol. Endocrinol. 29*, 223–238.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., Rubin, G.M., **1990**. The *Drosophila seven-up* gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. *Cell 60*, 211–224.

Monaghan, A.P., Bock, D., Gass, P., Schwager, A., Wolfer, D.P., *et al.*, **1997**. Defective limbic system in mice lacking the *tailless* gene. *Nature 390*, 515–517.

Montano, M.M., Muller, V., Trobaugh, A., Katzenellenbogen, B.S., **1995**. The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol. Endocrinol. 9*, 814–825.

Moore, J., Devaney, E., **1999**. Cloning and characterization of two nuclear receptors from the filarial nematode *Brugia pahangi*. *Biochem. J. 344*, 245–252.

Mouillet, J.F., Bousquet, F., Sedano, N., Alabouvette, J., Nicolai, M., *et al.*, **1999**. Cloning and characterization of new orphan nuclear receptors and their developmental profiles during *Tenebrio* metamorphosis. *Eur. J. Biochem. 265*, 972–981.

Much, J.W., Slade, D.J., Klampert, K., Garriga, G., Wightman, B., **2000**. The *fax-1* nuclear hormone receptor regulates axon pathfinding and neurotransmitter expression. *Development 127*, 703–712.

Nauber, U., Pankratz, M.J., Kienlin, A., Seifert, E., Klemm, U., *et al.*, **1988**. Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene *knirps*. *Nature 336*, 489–492.

Nichols, M., Rientjes, J.M., Stewart, A.F., **1998**. Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *EMBO J. 17*, 765–773.

Niimi, T., Morita, S., Yamashita, O., **1997**. The profiles of mRNA levels for BHR39, a *Bombyx* homolog of *Drosophila* hormone receptor 39, and *Bombyx* FTZ-F1 in the course of embryonic development and diapause. *Devel. Genes Evol. 207*, 410–412.

Nijhout, H.F., **2003**. Development and evolution of adaptive polyphenisms. *Evol. Devel. 5*, 9–18.

Nuclear Receptor Nomenclature Committee, **1999**. A unified nomenclature system for the nuclear receptor superfamily. *Cell 97*, 161–163.

Ohno, C.K., Petkovich, M., **1992**. FTZ-F1 beta, a novel member of the *Drosophila* nuclear receptor family. *Mech. Devel. 40*, 13–24.

Ohno, C.K., Ueda, H., Petkovich, M., **1992**. The *Drosophila* nuclear receptors FTZ-F1 alpha and FTZ-F1 beta compete as monomers for binding to a site in the *fushi tarazu* gene. *Mol. Cell. Biol. 14*, 3166–3175.

O'Kane, C.J., Asztalos, Z., **1999**. Sexual behaviour: courting dissatisfaction. *Curr. Biol. 9*, R289–R292.

Oro, A.E., McKeown, M., Evans, R.M., **1992**. The *Drosophila* retinoid X receptor homolog *ultraspiracle* functions in both female reproduction and eye morphogenesis. *Development 115*, 449–462.

Oro, A.E., Ong, E.S., Margolis, J.S., Posakony, J.W., McKeown, M., *et al.*, **1988**. The *Drosophila* gene *knirps-related* is a member of the steroid-receptor gene superfamily. *Nature 336*, 493–496.

Ostberg, T., Jacobsson, M., Attersand, A., Mata de Urquiza, A., Jendeberg, L., **2003**. A triple mutant of the *Drosophila* ERR confers ligand-induced suppression of activity. *Biochemistry 42*, 6427–6435.

Palli, S.R., Hiruma, K., Riddiford, L.M., **1992**. An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. *Devel. Biol. 150*, 306–318.

Palli, S.R., Ladd, T.R., Retnakaran, A., **1997a**. Cloning and characterization of a new isoform of *Choristoneura* hormone receptor 3 from the spruce budworm. *Arch. Insect Biochem. Physiol. 35*, 33–44.

Palli, S.R., Ladd, T.R., Ricci, A.R., Sohi, S.S., Retnakaran, A., **1997b**. Cloning and development expression of *Choristoneura* hormone receptor 75: a homologue of the *Drosophila* E75A gene. *Devel. Genet. 20*, 36–46.

Palli, S.R., Ladd, T.R., Sohi, S.S., Cook, B.J., Retnakaran, A., **1996**. Cloning and developmental expression of *Choristoneura* hormone receptor 3, an ecdysone-inducible gene and a member of the steroid hormone receptor superfamily. *Insect Biochem. Mol. Biol. 26*, 485–499.

Palli, S.R., Sohi, S.S., Cook, B.J., Lambert, D., Ladd, T.R., *et al.*, **1995**. Analysis of ecdysteroid action in *Malacosoma disstria* cells: cloning selected regions of E75- and MHR3-like genes. *Insect Biochem. Mol. Biol. 25*, 697–707.

Parker, K.L., Rice, D.A., Lala, D.S., *et al.*, **2002**. Steroidogenic factor 1: an essential mediator of endocrine development. *Recent Prog. Horm. Res. 57*, 19–36.

Pereira, F.A., Tsai, M.J., Tsai, S.Y., **2000**. COUP-TF orphan nuclear receptors in development and differentiation. *Cell. Mol. Life Sci. 57*, 1388–1398.

Perera, S.C., Palli, S.R., Ladd, T.R., Krell, P.J., Retnakaran, A., **1998**. The *ultraspiracle* gene of the spruce budworm, *Choristoneura fumiferana*: cloning of cDNA and developmental expression of mRNA. *Devel. Genet. 22*, 169–179.

Perrimon, N., Engstrom, L., Mahowald, A.P., **1985**. Developmental genetics of the 2C-D region of the *Drosophila* X chromosome. *Genetics 111*, 23–41.

Peters, G.A., Khan, S.A., **1999**. Estrogen receptor domains E and F: role in dimerization and interaction with coactivator RIP-140. *Mol. Endocrinol. 13*, 286–296.

Pierceall, W.E., Li, C., Biran, A., Miura, K., Raikhel, A.S., *et al.*, **1999**. E75 expression in mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol. Cell. Endocrinol. 150*, 73–89.

Pignoni, F., Baldarelli, R.M., Steingrimsson, E., Diaz, R.J., Patapoutian, A., *et al.*, **1990**. The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell 62*, 151–163.

Pitman, J.L., Tsai, C.C., Edeen, P.T., Finley, K.D., Evans, R.M., *et al.*, **2002**. DSF nuclear receptor acts as a repressor in culture and *in vivo*. *Devel. Biol. 245*, 315–328.

Ponzielli, R., Astier, M., Chartier, A., Gallet, A., Therond, P., *et al.*, **2002**. Heart tube patterning in *Drosophila* requires integration of axial and segmental information provided by the Bithorax Complex genes and hedgehog signaling. *Development 129*, 4509–4521.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., *et al.*, **2002**. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell 110*, 251–260.

Raikhel, A.S., Kokoza, V.A., Zhu, J., *et al.*, **2002**. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochem. Mol. Biol. 32*, 1275–1286.

Reichert, H., **2002**. Conserved genetic mechanisms for embryonic brain patterning. *Int. J. Devel. Biol. 46*, 81–87.

Riddiford, L.M., Cherbas, P., Truman, J.W., **2000**. Ecdysone receptors and their biological actions. *Vitam. Horm. 60*, 1–73.

Rifkin, S.A., Kim, J., White, K.P., **2003**. Evolution of gene expression in the *Drosophila melanogaster* subgroup. *Nat. Genet. 33*, 138–144.

Rothe, M., Nauber, U., Jackle, H., **1989**. Three hormone receptor-like *Drosophila* genes encode an identical DNA-binding finger. *EMBO J. 8*, 3087–3094.

Rothe, M., Pehl, M., Taubert, H., Jackle, H., **1992**. Loss of gene function through rapid mitotic cycles in the *Drosophila* embryo. *Nature 359*, 156–159.

Ruau, D., Duarte, J., Ourjdal, T., Perriere, G., Laudet, V., *et al.*, **2004**. Update of NUREBASE: nuclear hormone receptor functional genomics. *Nucleic Acids Res. 32*, D165–167.

Russell, S.R., Heimbeck, G., Goddard, C.M., Carpenter, A.T., Ashburner, M., **1996**. The *Drosophila Eip78C* gene is not vital but has a role in regulating chromosome puffs. *Genetics 144*, 159–170.

Sablin, E.P., Krylova, I.N., Fletterick, R.J., Ingraham, H.A., **2003**. Structural basis for ligand-independent activation of the orphan nuclear receptor LRH-1. *Mol. Cell 11*, 1575–1585.

Schröder, R., Eckert, C., Wolff, C., Tautz, D., **2000**. Conserved and divergent aspects of terminal patterning in the beetle *Tribolium castaneum*. *Proc. Natl Acad. Sci. USA 97*, 6591–6596.

Schulman, I.G., Juguilon, H., Evans, R.M., **1996**. Activation and repression by nuclear hormone receptors: hormone modulates an equilibrium between active and repressive states. *Mol. Cell. Biol. 16*, 3807–3813.

Schwartz, C.J., Sampson, H.M., Hlousek, D., Percival-Smith, A., Copeland, J.W., *et al.*, **2001**. FTZ-Factor1 and Fushi tarazu interact via conserved nuclear receptor and coactivator motifs. *EMBO J. 20*, 510–519.

Segraves, W.A., Hogness, D.S., **1990**. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Devel. 4*, 204–219.

Segraves, W.A., Woldin, C., **1993**. The E75 gene of *Manduca sexta* and comparison with its *Drosophila* homolog. *Insect Biochem. Mol. Biol. 23*, 91–97.

Sehnal, F., Svàcha, P., Zrzavy, J., **1996**. Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, San Diego, pp. 3–58.

Shea, C., Hough, D., Xiao, J., Tzertzinis, G., Maina, C.V., **2003**. An *rxr/up* homolog from the parasitic nematode, *Dirofilaria immitis*. *Gene 324*, 171–182.

Sladek, F., **2002**. Desperately seeking...something. *Mol. Cell 10*, 219–221.

Sluder, A.E., Maina, C.V., **2001**. Nuclear receptors in nematodes: themes and variations. *Trends Genet. 17*, 206–213.

Smagghe, G., Dhadialla, T.S., Lezzi, M., **2002**. Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. *Insect Biochem. Mol. Biol. 32*, 187–192.

Sommer, R., Tautz, D., **1991**. Segmentation gene expression in the housefly *Musca domestica*. *Development 113*, 419–430.

Stehlin-Gaon, C., Willmann, D., Zeyer, D., Sanglier, S., Van Dorsselaer, A., *et al.*, **2003**. All-trans retinoic acid is a ligand for the orphan nuclear receptor ROR beta. *Nat. Struct. Biol. 10*, 820–825.

Steinmetz, A.C., Renaud, J.P., Moras, D., **2001**. Binding of ligands and activation of transcription by nuclear receptors. *Annu. Rev. Biophys. Biomol. Struct. 30*, 329–359.

Sudarsan, V., Pasalodos-Sanchez, S., Wan, S., Gampel, A., Skaer, H., **2002**. A genetic hierarchy establishes mitogenic signalling and mitotic competence in the renal tubules of *Drosophila*. *Development 129*, 935–944.

Sullivan, A.A., Thummel, C.S., **2003**. Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. *Mol. Endocrinol. 24*, 24.

Sun, G.C., Hirose, S., Ueda, H., **1994**. Intermittent expression of BmFTZ-F1, a member of the nuclear hormone receptor superfamily during development of the silkworm *Bombyx mori*. *Devel. Biol. 162*, 426–437.

Sutherland, J.D., Kozlova, T., Tzertzinis, G., Kafatos, F.C., **1995**. *Drosophila* hormone receptor 38: a second partner for *Drosophila* USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. *Proc. Natl Acad. Sci. USA 92*, 7966–7970.

Suzuki, T., Kawasaki, H., Yu, R.T., Ueda, H., Umesono, K., **2001**. Segmentation gene product Fushi tarazu is an LXXLL motif-dependent coactivator for orphan receptor FTZ-F1. *Proc. Natl Acad. Sci. USA 98*, 12403–12408.

Swevers, L., Cherbas, L., Cherbas, P., Iatrou, K., **1996**. *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Mol. Biol. 26*, 217–221.

Swevers, L., Eystathioy, T., Iatrou, K., **2002**. The orphan nuclear receptors BmE75A and BmE75C of the silkmoth *Bombyx mori*: hormonal control and ovarian expression. *Insect Biochem. Mol. Biol. 32*, 1643–1652.

Swevers, L., Iatrou, K., **1998**. The orphan receptor BmHNF-4 of the silkmoth *Bombyx mori*: ovarian and zygotic expression of two mRNA isoforms encoding polypeptides with different activating domains. *Mech. Devel. 72*, 3–13.

Thornmeyer, D., Tenbaum, S.P., Renkawitz, R., Baniahmad, A., **1999**. EcR interacts with corepressors and harbours an autonomous silencing domain functional in both *Drosophila* and vertebrate cells. *J. Steroid Biochem. Mol. Biol. 68*, 163–169.

Thornton, J.W., DeSalle, R., **2000**. A new method to localize and test the significance of incongruence: detecting domain shuffling in the nuclear receptor superfamily. *Syst. Biol. 49*, 183–201.

Thornton, J.W., Need, E., Crews, D., **2003**. Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. *Science 301*, 1714–1717.

Thummel, C.S., **2001**. Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. *Devel. Cell 1*, 453–465.

Togawa, T., Shofuda, K., Yaginuma, T., Tomino, S., Nakato, H., *et al.*, **2001**. Structural analysis of gene encoding cuticle protein BMCP18, and characterization of its putative transcription factor in the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 31*, 611–620.

Tremblay, J.J., Marcil, A., Gauthier, Y., Drouin, J., **1999**. Ptx1 regulates SF1 activity by an interaction that mimics the role of the ligand-binding domain. *EMBO J. 18*, 3431–3441.

Truman, J.W., Riddiford, L.M., **1999**. The origins of insect metamorphosis. *Nature 401*, 447–452.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insight into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Ueda, H., Hirose, S., **1990**. Identification and purification of a *Bombyx mori* homologue of FTZ-F1. *Nucleic Acids Res. 18*, 7229–7234.

Unnasch, T.R., Bradley, J., Beauchamp, J., Tuan, R., Kennedy, M.W., **1999**. Characterization of a putative nuclear receptor from *Onchocerca volvulus*. *Mol. Biochem. Parasitol. 104*, 259–269.

Verjovski-Almeida, S., De Marco, R., Martins, E.A., *et al.*, **2003**. Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*. *Nature Genet. 35*, 148–157.

Vögtli, M., Elke, C., Imhof, M.O., Lezzi, M., **1998**. High level transactivation by the ecdysone receptor complex at the core recognition motif. *Nucleic Acids Res. 26*, 2407–2414.

Vögtli, M., Imhof, M.O., Brown, N.E., Rauch, P., Spindler-Barth, M., *et al.*, **1999**. Functional characterization of two *Ultraspiracle* forms (CtUSP-1 and CtUSP-2) from *Chironomus tentans*. *Insect Biochem. Mol. Biol. 29*, 931–942.

Wang, S.F., Li, C., Zhu, J., Miura, K., Miksicek, R.J., *et al.*, **2000**. Differential expression and regulation by 20-hydroxyecdysone of mosquito *ultraspiracle* isoforms. *Devel. Biol. 218*, 99–113.

Wang, S.F., Miura, K., Miksicek, R.J., Segraves, W.A., Raikhel, A.S., **1998**. DNA binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex. *J. Biol. Chem. 273*, 27531–27540.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., *et al.*, **2003**. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature 423*, 555–560.

Weigel, N.L., Zhang, Y., **1998**. Ligand-independent activation of steroid hormone receptors. *J. Mol. Med. 76*, 469–479.

Weller, J., Sun, G.C., Zhou, B., Lan, Q., Hiruma, K., *et al.*, **2001**. Isolation and developmental expression of two nuclear receptors, MHR4 and betaFTZ-F1, in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 31*, 827–837.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., **1997**. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science 276*, 114–117.

Whiting, M.F., Carpenter, J.C., Wheeler, Q.D., Wheeler, W.C., **1997**. The streptisera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *Syst. Biol. 46*, 1–68.

Wiens, M., Batel, R., Korzhev, M., Muller, W.E., **2003**. Retinoid X receptor and retinoic acid response in the marine sponge *Suberites domuncula*. *J. Exp. Biol. 206*, 3261–3271.

Wightman, B., Baran, R., Garriga, G., **1997**. Genes that guide growth cones along the *C. elegans* ventral nerve cord. *Development 124*, 2571–2580.

Wisely, G.B., Miller, A.B., Davis, R.G., *et al.*, **2002**. Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. *Structure (Camb.) 10*, 1225–1234.

Wittkopp, P.J., Williams, B.L., Selegue, J.E., Carroll, S.B., **2003**. *Drosophila* pigmentation evolution: divergent genotypes underlying convergent phenotypes. *Proc. Natl Acad. Sci. USA 100*, 1808–1813.

Woodard, C.T., Baehrecke, E.H., Thummel, C.S., **1994**. A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell 79*, 607–615.

Wurtz, J.M., Bourguet, W., Renaud, J.P., Vivat, V., Chambon, P., *et al.*, **1996**. A canonical structure for the ligand-binding domain of nuclear receptors. *Nat. Struct. Biol. 3*, 87–94.

Wurtz, J.M., Guillot, B., Fagart, J., Moras, D., Tietjen, K., *et al.*, **2000**. A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: a homology modeling and docking approach. *Protein Sci. 9*, 1073–1084.

Yagi, K., Satou, Y., Mazet, F., Shimeld, S.M., Degnan, B., *et al.*, **2003**. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. III. Genes for Fox, ETS, nuclear receptors and NFkappaB. *Devel. Genes Evol. 213*, 235–244.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., *et al.*, **2000**. Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*. *Development 127*, 5083–5092.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., *et al.*, **1993**. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature 366*, 476–479.
Yu, R.T., Chiang, M.Y., Tanabe, T., Kobayashi, M., Yasuda, K., *et al.*, **2000**. The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision. *Proc. Natl Acad. Sci. USA 97*, 2621–2625.
Yu, Y., Li, W., Su, K., Yussa, M., Han, W., *et al.*, **1997**. The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz. *Nature 385*, 552–555.
Yussa, M., Lohr, U., Su, K., Pick, L., **2001**. The nuclear receptor Ftz-F1 and homeodomain protein Ftz interact through evolutionarily conserved protein domains. *Mech. Devel. 107*, 39–53.
Zelhof, A.C., Yao, T.P., Chen, J.D., Evans, R.M., McKeown, M., **1995a**. *Seven-up* inhibits *ultraspiracle*-based signaling pathways *in vitro* and *in vivo*. *Mol. Cell. Biol. 15*, 6736–6745.
Zelhof, A.C., Yao, T.P., Evans, R.M., McKeown, M., **1995b**. Identification and characterization of a *Drosophila* nuclear receptor with the ability to inhibit the ecdysone response. *Proc. Natl Acad. Sci. USA 92*, 10477–10481.
Zhong, W., Sladek, F.M., Darnell, J.E., Jr., **1993**. The expression pattern of a *Drosophila* homolog to the mouse transcription factor HNF-4 suggests a determinative role in gut formation. *EMBO J. 12*, 537–544.
Zhou, B., Hiruma, K., Jindra, M., Shinoda, T., Segraves, W.A., *et al.*, **1998**. Regulation of the transcription factor E75 by 20-hydroxyecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*, during larval molting and metamorphosis. *Devel. Biol. 193*, 127–138.
Zhou, H.M., Walthall, W.W., **1998**. UNC-55, an orphan nuclear hormone receptor, orchestrates synaptic specificity among two classes of motor neurons in *Caenorhabditis elegans*. *J. Neurosci. 18*, 10438–10444.
Zhu, J., Miura, K., Chen, L., Raikhel, A.S., **2000**. AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*. *EMBO J. 19*, 253–262.
Zhu, J., Miura, K., Chen, L., Raikhel, A.S., **2003**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue *Ultraspiracle*. *Proc. Natl Acad. Sci. USA 100*, 544–549.

Relevant Websites

http://www.genomesonline.org – GOLD.
http://www.ens-lyon.fr – Nurebase.

3.7 The Juvenile Hormones

W G Goodman, University of Wisconsin–Madison,
Madison, WI, USA
N A Granger, University of North Carolina,
Chapel Hill, NC, USA

3.7.1. Introduction

The juvenile hormones (JHs) represent a family of acyclic sesquiterpenoids that are, with a single known exception, unique to the class Insecta. They are the principal products of the corpora allata (CA), retrocerebral paired or unpaired endocrine glands of ectodermal origin (Wigglesworth, 1970). One or more JHs have been identified in approximately 100 insect species spanning at least 10 insect orders, from the most ancestral to the most highly derived. From an evolutionary standpoint, the JHs may have originally been involved in orchestration of reproductive processes such as control of gonadal development and vitellogenin synthesis. The role of JH in the more highly derived insect orders has expanded considerably to include regulation of metamorphosis, caste determination, behavior, diapause, and various polyphenisms (Nijhout, 1994). This chapter will present the current status of our knowledge about the JHs, their evolution, roles in embryological and larval development, biosynthesis, transport, catabolism, and molecular mode of action. The role of JH in reproduction is covered elsewhere (see **Chapter 3.9**).

3.7.2. Chemistry of the Juvenile Hormones

3.7.2.1. Discovery of the Major Juvenile Hormone Homologs

Elucidation of the chemical structures of the JHs began with Röller and colleagues, who identified the principal JH in lipid extracts of *Hyalophora cecropia* (Röller *et al.*, 1967). This first JH, methyl (2*E*,6*E* 10-*cis*)-10,11-epoxy-7-ethyl-3,11-dimethyl-2, 6-tridecadienoate, was termed JH I, and since that time, eight molecules with similar aliphatic sesquiterpene structures have been identified in insects (**Figure 1**). The structure was confirmed as the 2*E*,6*E*, 10 *cis* isomer (Dahm *et al.*, 1968), while the absolute configuration of JH I at its chiral centers (C10 and C11) was determined to be 10*R*,11*S* (Faulkner and Petersen, 1971; Nakanishi *et al.*, 1971; Meyer *et al.*, 1971).

Meyer *et al.* (1968) subsequently identified a minor component in the *H. cecropia* extracts that differed from JH I by a methyl, instead of an ethyl group at C7 (**Figure 1**). Termed JH II, methyl (2*E*, 6*E* 10-*cis*)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate), like JH I, displays an *E,E* configuration at C2, C3 and C6, C7. The absolute configuration at the C10, C11 positions for the naturally occurring JH II has not been rigorously determined (Baker, 1990); however, in binding studies, (10*R*,11*S*)-JH II bound with nearly the same affinity to the *Manduca sexta* hemolymph juvenile hormone binding protein as (10*R*,11*S*)-JH I (Park *et al.*, 1993). Thus, it is likely that the naturally occurring enantiomers of JH I and II display the same 10*R*,11*S* configuration.

The third JH homolog, JH III, methyl 10,11 epoxyfarnesoate, was identified from medium in which CA of the tobacco hornworm, *M. sexta*, had been maintained (Judy *et al.*, 1973) (**Figure 1**). JH III differs from the higher homologs in that the three branches of the carbon skeleton at C3, C7, and C11 are methyl groups; however, it displays the same 2*E*,6*E* geometry. The hormone has only one chiral carbon, C10, which, in the naturally occurring hormone, displays the 10*R* configuration. JH III is the only, or predominant, JH in all insects except Lepidoptera (Schooley *et al.*, 1984). Interestingly, JH III had been chemically synthesized a decade earlier, before it was recognized as a naturally occurring hormone (Bowers *et al.*, 1965). A fourth and fifth JH (**Figure 1**), the trihomosesquiterpenoids JH 0 and its isomer, 4-methyl JH I (iso-JH 0), were identified in *M. sexta* eggs (Bergot *et al.*, 1981a), but nothing is currently known of their functions.

More recently, JH III with a second epoxide substitution at C6,C7 was isolated and identified from *in vitro* cultures of larval ring glands of *Drosophila melanogaster* (Richard *et al.*, 1989) (**Figure 1**). This homolog, termed JH III bisepoxy (JHB_3), is active in a *D. melanogaster* bioassay and has been found in other higher Diptera (Borovsky *et al.*, 1994a; Moshitzky and Applebaum, 1995; Yin *et al.*, 1995).

3.7.2.2. Hydroxylated Juvenile Hormones

Hydroxylation reactions are frequently associated with the inactivation of bioreactive molecules but in certain cases, hydroxylation can actually increase the biological potency of a compound. This important biochemical reaction appears to be involved in

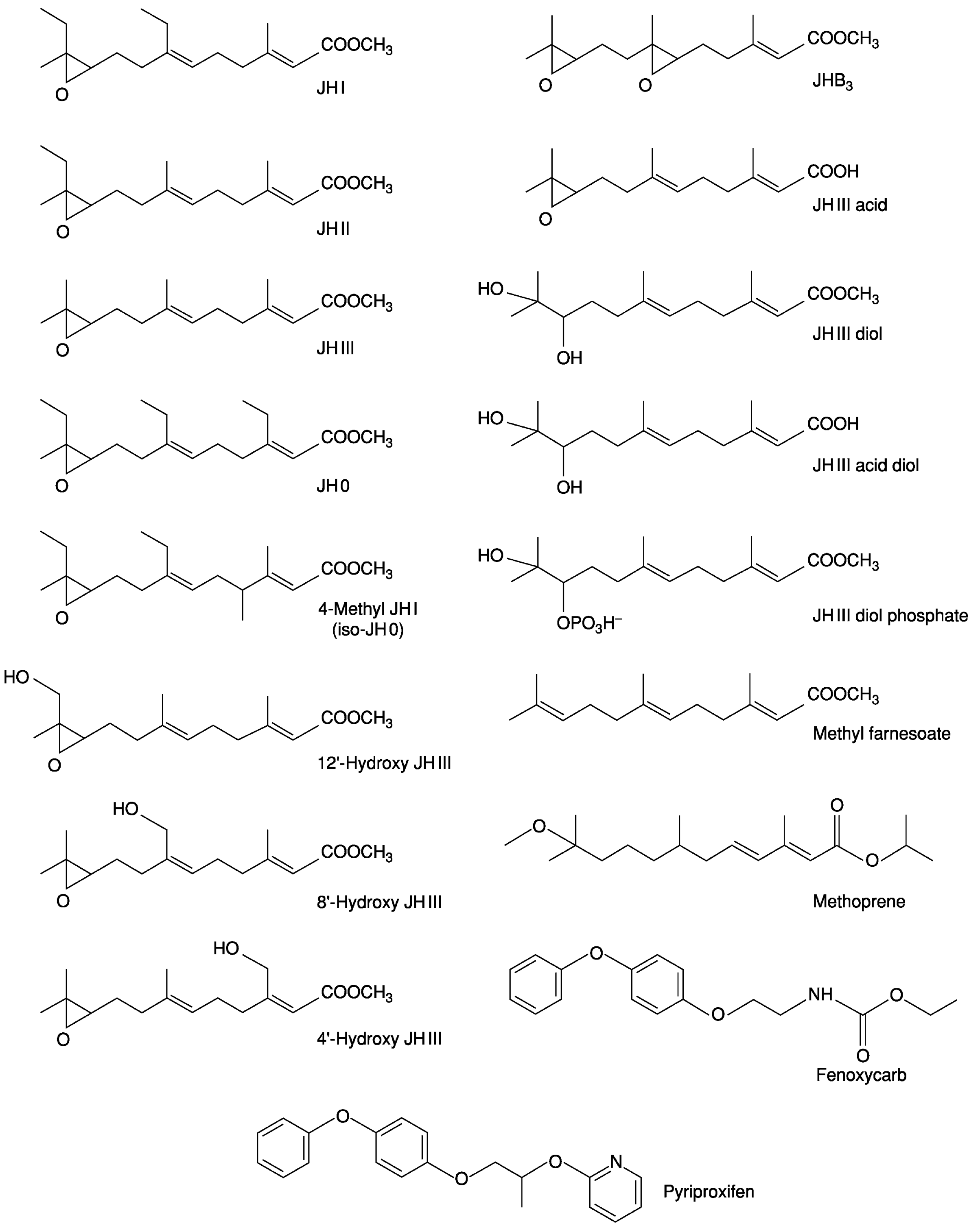


Figure 1 Chemical structures of the naturally occurring juvenile hormone (JH) homologs, hydroxylated JHs, JH metabolites, and commonly used JH analogs.

the production of a new group of JH homologs, the hydroxylated JHs (HJHs). These HJHs, 4-OH, 8-OH, and 12-OH JH III, are synthesized and released by the CA of the African locust, *Locusta migratoria* (Darrouzet *et al*., 1997, 1998; Mauchamp *et al*., 1999) (**Figure 1**). The biological activity of synthetic 12-OH JH III was bioassayed utilizing *Tenebrio molitor* and was found to be 100-fold more active than JH III (Darrouzet *et al*., 1997). This result may reflect the cross-species nature of the bioassay,

since the assay using *L. migratoria* is not sufficiently sensitive (see Section 3.7.5.1).

While the biosynthetic pathway for the synthesis of the HJHs has not been determined, two pathways have been suggested: (1) hydroxylation of the JH III structure itself or (2) incorporation of hydroxylated precursors into the JH backbone via the isoprenoid biosynthetic pathway (Darrouzet *et al.*, 1998). A cytochrome P450, *CYP4C7*, identified in the cockroach *Diploptera punctata*, can catalyze the formation of 12-OH JH III and (10*E*)-12-OH farnesol, indicating that hydroxylation of JH III appears to be a primary reaction in the CA (Sutherland *et al.*, 1998). However, there is a sharp increase in the expression of this enzyme when JH is absent or when there is an intrinsic repression of JH synthesis, suggesting that hydroxylation of JH is a mechanism for inactivating the hormones. The pathways of synthesis and the physiological roles of the HJHs remain a fertile area for investigation.

3.7.3. Other Naturally Occurring Juvenile Hormones

Davey (2000a), in a thought-provoking essay, posed an intriguing question: "How many JHs are there?" Using a chemical definition, i.e., that JH is a farnesoid molecule secreted by the CA, one can count but a handful. However, if a JH is defined by its biological activity, the JHs could include HJHs, catabolic products, and nontraditional molecules, increasing the number significantly and creating the difficult problem of sorting out which molecules are actually JHs.

3.7.3.1. Metabolites of Juvenile Hormones as Hormones?

An open mind is needed when considering whether metabolites of JH could serve as substrate for conversion to the biologically active hormone or perhaps could act as hormones themselves. A group of molecules that has long been overlooked and frequently relegated to a mere biochemical curiosity are the JH conjugates (Roe and Venkatesh, 1990). Polar metabolites of JH I that are cleaved by glucosidases and sulfatases were identified in flies nearly three decades ago (Yu and Terriere, 1978), and more recent studies hint at the existence of very polar metabolites of JH. A major metabolite resulting from the injection or application of JH II into wandering larvae and prepupae of the cabbage looper, *Trichoplusia ni*, was found to be an unidentified, water-soluble polar product of the hormone (Kallapur *et al.*, 1996). Identification by radio high-performance liquid chromatography (HPLC), as well as mass spectrometry, has confirmed that the molecule is a novel polar metabolite and that it is not a JH diol phosphate (M. Roe, personal communication). *In vitro* studies on the biosynthetic products of the CA from *M. sexta* and two other lepidopteran species indicate that the glands are synthesizing JH conjugates not recognized by antibodies highly specific for the JHs and their acids; nevertheless, when these compounds are hydrolyzed by esterases, JH III acid is formed (Granger *et al.*, 1995a). This product also appears to exist in the hemolymph, and the results of this study suggest the moiety is either a glucuronide of JH III or an acylglycerol with JH III as the side chain(s).

Schooley's group identified other polar metabolites in the hemolymph of *M. sexta* as phosphate conjugates of JH I and JH III diol and found that JH I diol phosphate (**Figure 1**) is the principal end product of JH I metabolism in *M. sexta* (Halarnkar and Schooley, 1990; Halarnkar *et al.*, 1993). More recently, Maxwell *et al.* (2002a, 2002b) identified the enzyme responsible for catalyzing the conversion of the JH diol to the JH diol phosphate, JH diol kinase (JHDK), and proposed that cellular JH epoxide hydrolase (JHEH) and JHDK are primarily responsible for the irreversible inactivation of JH early in the last stadium of *M. sexta*, when the JH titer rapidly decreases (Baker *et al.*, 1987) (see Section 3.7.9).

At the present time, there appear to be only two definitive pathways for the catabolism of JH: (1) hydrolysis of the methyl ester of JH by hemolymph esterases, yielding JH acid, and (2) hydration of the epoxide by JHEH, yielding JH diol (Roe and Venkatesh, 1990) (**Figures 1** and **2**). Can any of these catabolites act as a JH? There is certainly evidence that JH acid can be converted to JH and that it may also act as a hormone. It has been known for a number of years that JH acids are secreted by the CA of *M. sexta* beginning early in the last larval stadium (Janzen *et al.*, 1991) and that they are apparently the sole product of the CA by the wandering stage in this stadium (Sparagana *et al.*, 1984; Janzen *et al.*, 1991) (**Figure 3**). JH acid is also detected in the hemolymph of *M. sexta* (Baker *et al.*, 1987) and of the silkworm *Bombyx mori*, where, at certain critical stages, the titer of JH acid surpasses that of JH (Niimi and Sakurai, 1997). JH acid is found as a product of the CA in other lepidopterans including the adult male loreyi leafworm, *Mythimna loreyi* (Ho *et al.*, 1995a, 1995b), the adult male black cutworm, *Agrotis ipsilon* (Duportets *et al.*, 1998), and the larval tomato moth, *Lacanobia oleracea* (Audsley *et al.*, 2000).

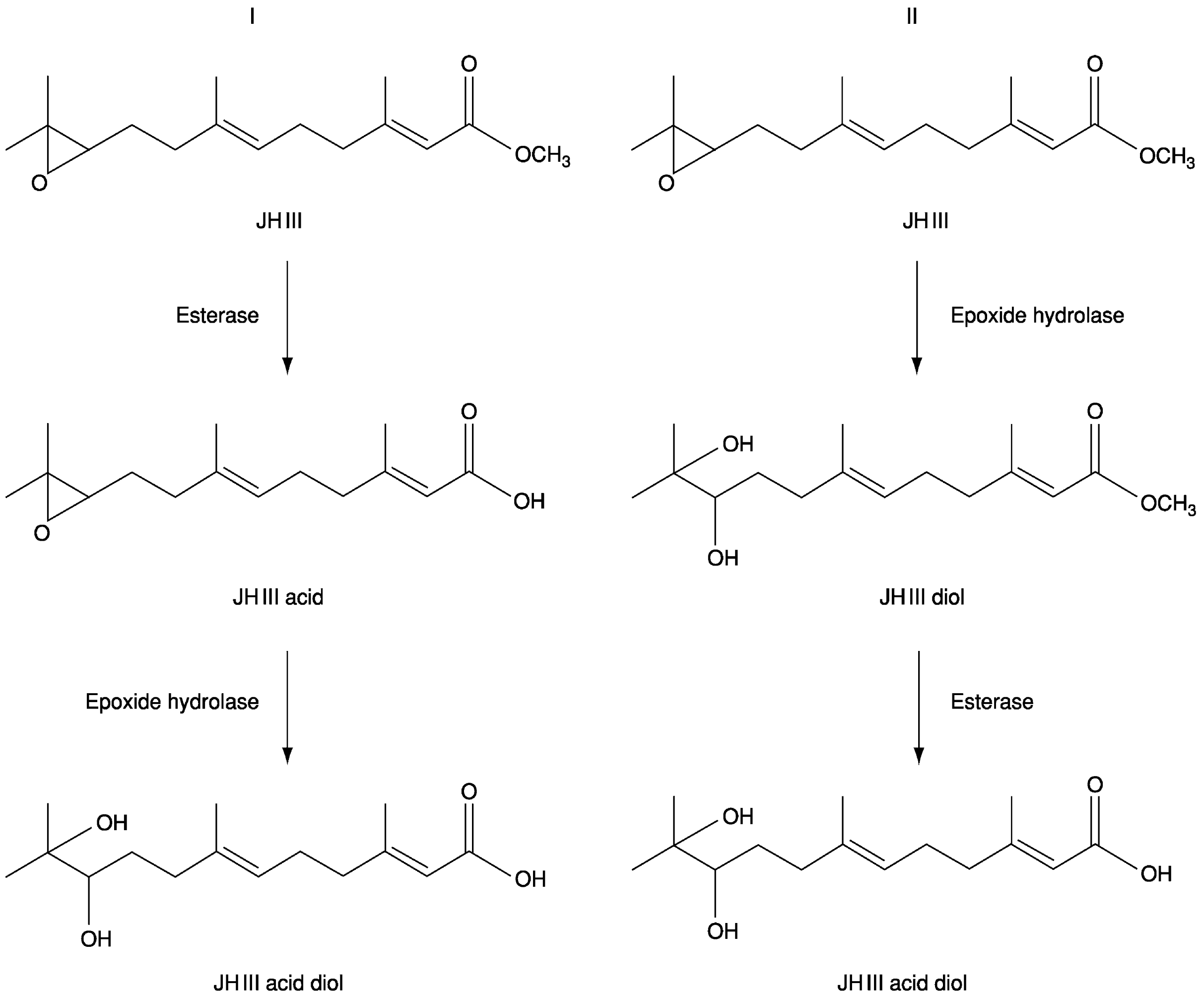


Figure 2 Primary routes of JH metabolism. Route I represents hydrolysis of the methyl ester via an esterase followed by hydrolysis of the epoxide ring via an epoxide hydrolase. Route II represents hydrolysis of the epoxide ring via an epoxide hydrolase followed by hydrolysis of the methyl ester via an esterase. The end product in both routes is JH acid diol.

Studies by Bhaskaran and his colleagues suggest that JH acid may be a hormone in its own right. The ability of *M. sexta* fat body cells to respond to the JH analog methoprene (**Figure 1**) with enhanced production of yolk protein and its mRNA is acquired after ecdysteroid-initiated commitment to pupation, but it also requires prior exposure to JH II acid or methoprene acid (Ismail *et al*., 1998). In subsequent work, Ismail *et al*. (2000) demonstrated that for two metamorphic events, the production of pupal proteins by Verson's gland, and the loss of the ability of crochet epidermis to produce larval crochets, exposure to JH acid or methoprene acid plus a low dose of RH5992 (an ecdysteroid analog) is required. Use of RH5992 or methoprene acid alone does not induce these changes. Gilbert *et al*. (2000) recently outlined unpublished experiments by Bhaskaran and colleagues to determine whether JH acids played a role in the acquisition of metamorphic competence in abdominal rings of first stadium *D. melanogaster*. Preliminary results indicate that JH acid plus ecdysteroid induce in first instar *D. melanogaster* both Broad, a transcription factor that appears in response to ecdysteroids early in the last larval stadium (see Section 3.7.12.3), and the adult promoter of alcohol dehydrogenase. However, attempts to repeat this work have been inconclusive, and the results of the previous works have been questioned on technical grounds.

3.7.3.2. Other Juvenile Hormones

It is a curious fact that the JH of *Rhodnius prolixus*, the hemipteran used by Wigglesworth in his pioneering experiments on the nature of the hormones controlling growth and reproduction (Wigglesworth, 1936), does not correspond to any other known JH (Wyatt and Davey, 1996). Early

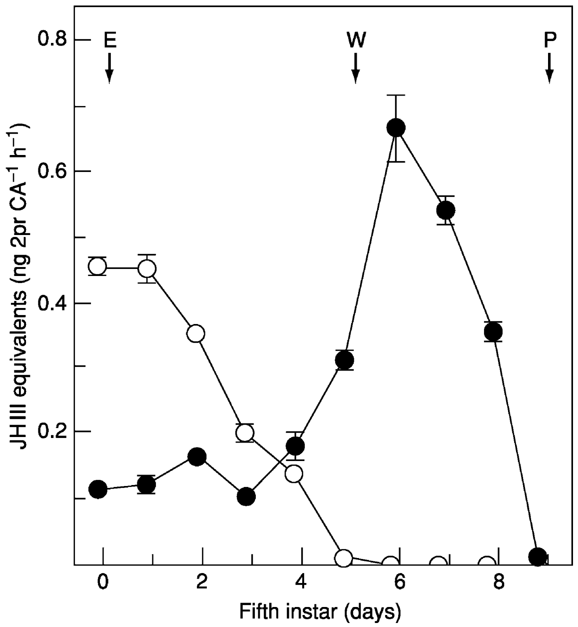


Figure 3 Biosynthesis of JH and JH acid by *Manduca sexta* corpora allata (CA) *in vitro*. Biosynthesis of JH I (open circles) and JH I acid (closed circles) by CA *in vitro* from fifth instars as measured by radioimmunoassay after separation of JH and JH acid by phase partition. Synthesis is expressed as ng JH I radioimmunoassay equivalents. E, the time of larval ecdysis; W, the onset of wandering behavior; P, the time of pupal ecdysis. Each datum point is the mean of 9–12 incubations (±SEM). (Reproduced from Janzen, W.P., Menold, M., Granger, N.A., **1991**. Effects of endogenous esterases and an allatostatin on the products of *Manduca sexta* corpora allata *in vitro*. *Physiol. Entomol. 16*, 283–293.)

attempts to identify the hemipteran JHs did not meet with success (Baker *et al*., 1988), but a more recent report suggests that JH I is the JH of Hemiptera (Numata *et al*., 1992). Unfortunately, this latter identification seems doubtful in light of the results of Kotaki (1993, 1996). These studies revealed that while the product of the CA of the stink bug, *Plautia stali*, has a sesquiterpenoid skeleton similar to JH III, the addition of the JH III precursors farnesoic acid or farnesol to incubations of CA stimulates the production of neither JH III nor JHB_3, but one that differs chromatographically from any known JH (Kotaki, 1997).

Among the possible groups of known, but nontraditional JHs, are the biosynthetic precursors of JH (**Figures 4** and **5**). Methyl farnesoate (MF) (**Figure 1**) may be the crustacean form of the insect JH (see Section 3.7.6; **Chapter 3.16**), but it is also present during embryogenesis in the cockroach *Nauphoeta cinerea* (Bürgin and Lanzrein, 1988) (see Section 3.7.10.2) and is synthesized by the embryonic CA of *D. punctata* (Cusson *et al*., 1991a) and the adult CA of the dipteran *Phormia regina* (Yin *et al*., 1995). In *P. regina*, MF alone has limited JH-like biological activity, but appears to work best in a coordinated role together with JH III and JHB_3.

Farnesoic acid (FA) (**Figure 4**), another JH precursor, has also been identified as a product of the *D. punctata* nymphal CA; moreover, it is proposed to have a hormonal or prohormonal function during the latter half of the fourth stadium, when release of JH III ceases. Alternatively, it may be secreted as a by-product of *O*-methyl transferase activity in the terminal steps of JH III biosynthesis (Yagi *et al*., 1991). Cusson *et al*. (1991a) speculate that continued synthesis of FA would allow for the rapid resumption of JH III synthesis when it is needed.

Plants possess compounds that exhibit JH-like activity in insect bioassays, and these molecules are considered to play a defensive role. For the most part, these "phytojuvenoids" are structurally distinct from the insect JHs and, with the exception of farnesol, which occurs widely in flowering plants, do not resemble the JH homologs (Bergamasco and Horn, 1983). Curiously, (10*R*)-JH III and MF have been conclusively identified in the sedges *Cyperus iria* and *C. aromaticus* (Toong *et al*., 1988). Recently, it has been possible to produce JH III and its precursors and to define its biosynthetic pathway in cell suspension cultures of *C. iria* (Bede *et al*., 1999, 2001). Given the large amount of JH III present in the plants, one might suspect that it acts as a naturally occurring insect growth regulator to deter feeding. However, it is possible that the plant uses the hormone as a plant growth inhibitor to retard the growth of other nearby species.

There exist a few compounds with no structural similarity to the JHs that display hormonal activity. The ecdysteroids top this short list. Ovarian ecdysteroids have long been known to have JH functions in adult females, stimulating the synthesis of vitellogenin in some species, and terminating previtellogenic reproductive diapause and promoting uptake of yolk proteins by oocytes in others, all roles traditionally associated with JH (Hagedorn, 1983; Girardie and Girardie, 1996; Richard *et al*., 1998) (see **Chapter 3.9**). There may also exist peptides that mimic the action of JH. In *R. prolixus*, JH stimulates protein synthesis in the male accessory gland *in vitro*, a function also supported by an uncharacterized neuropeptide from the brain (Barker and Davey, 1983; Gold and Davey, 1989). In addition, there are reports of peptides stimulating the synthesis of vitellogenin in *L. migratoria*, a role usually attributed to JH (Girardie and Girardie, 1996; Girardie *et al*., 1998). Finally, there is the proposal of Davey (2000b) that thyroid hormones may function

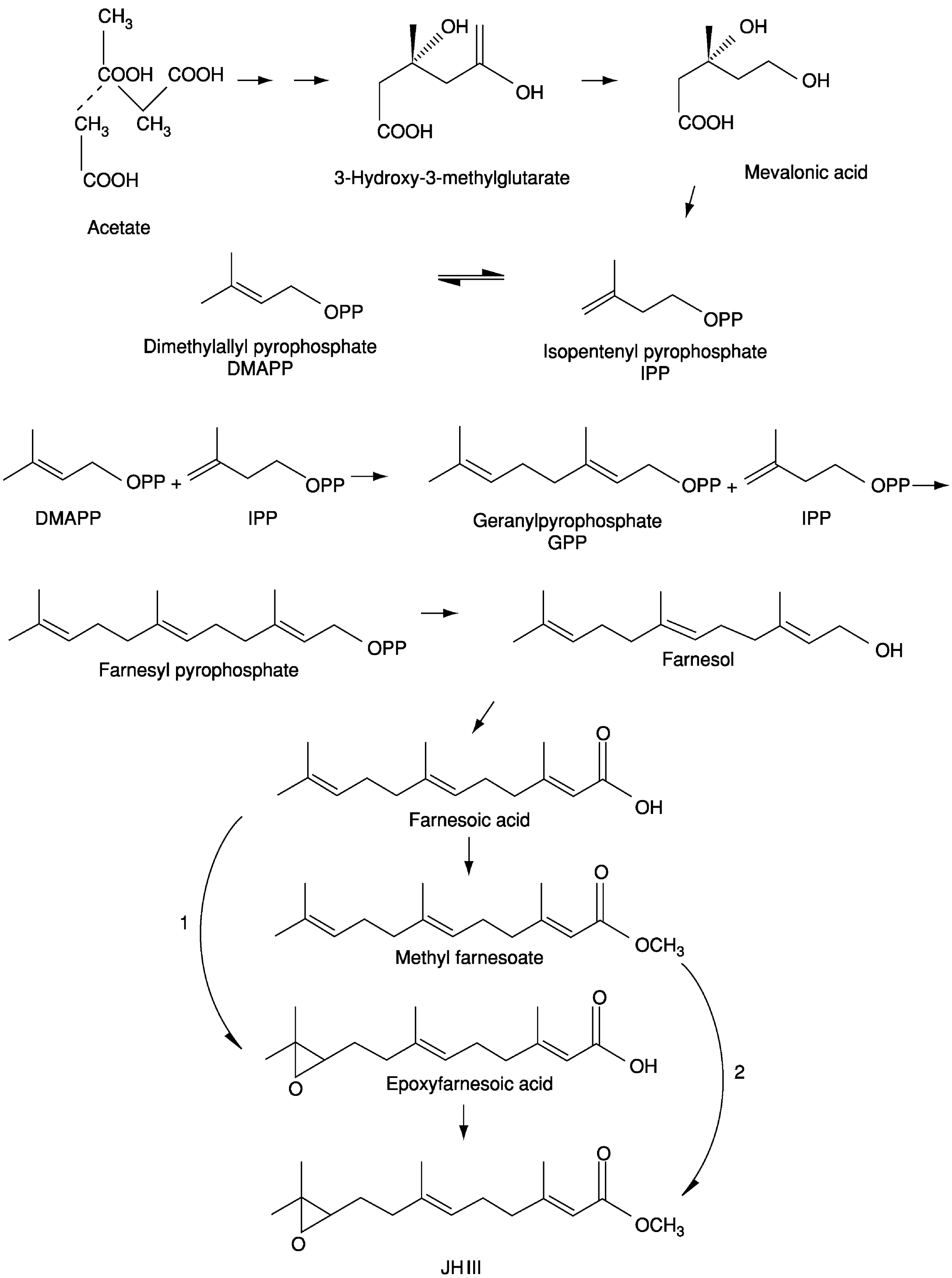


Figure 4 Biosynthetic pathway for JH III. In Lepidoptera, the last two steps in the biosynthetic pathway convert farnesoic acid to JH acid, which is then methylated to form JH III (**1**). In Orthoptera and Dictyoptera, the last two steps in the biosynthetic pathway convert farnesoic acid to methyl farnesoate, which is then epoxidized to form JH III (**2**).

in insects. Phenoxy-phenyl compounds such the JH analog fenoxycarb (Figure 1), thyroxine (T4) and triiodothyronine (T3) mimic the effects of JH III in reducing the volume of follicle cells in *L. migratoria* (Davey and Gordon, 1996; Kim *et al.*, 1999). In many species, follicle cells respond to JH by altering their cytoskeletal structure, resulting in the appearance of lateral spaces between the cells (patency) via which vitellogenin gains access to the oocyte surface (Davey *et al.*, 1993). T3 binds to the same receptor

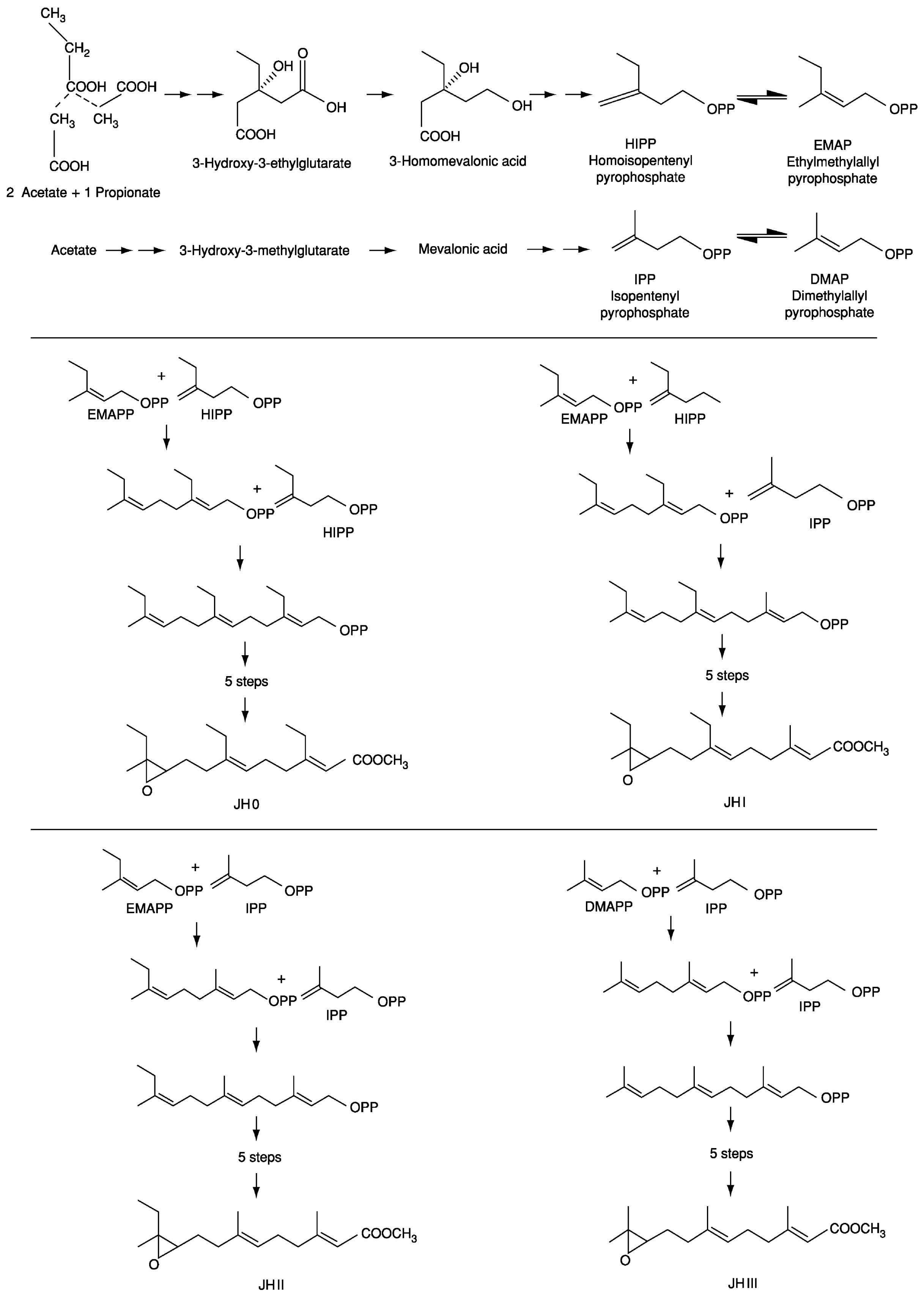


Figure 5 Biosynthetic pathways for JH 0, I, II, and III.

on locust follicle cells as JH III (Davey and Gordon, 1996; Kim *et al*., 1999), and T3 immunoreactivity can be found in locust hemolymph (Davey, 2000a, 2000b). Locust food (wheat shoots and bran) was found to contain both T3 and T4, thus suggesting that insect diets could serve as supplementary sources of compounds which act as JH (Davey, 2000b). The fact that T3 apparently binds to a receptor that also binds JH has interesting implications in the search for potential JH receptor(s) (see Section 3.7.12).

The increased sensitivity afforded by modern analytical tools should make easier the difficult task of deciphering the biological roles of these new JH-like molecules. Are they biologically relevant or are they the result of promiscuous biochemical reactions that yield end products with no real biological activity? It remains to be determined whether the results of studies using incubations *in vitro* or homogenates of CA to generate new JH-like molecules under experimental conditions bear any resemblance to the situation *in vivo*. There is good evidence from vertebrate endocrinology to believe that it is a real problem. A very early study on corticosteroid biosynthesis in the adrenal gland (Heftmann and Mosettig, 1960) lists more than 40 corticosteroids present in the human adrenal glands, even with the limited technology of that time. Of those, only a handful have ever been detected in the blood. A similar situation has been found with the insect ecdysteroids (Rees, 1985) (see **Chapter 3.3**).

3.7.4. Biosynthetic Pathways for the Juvenile Hormones

The biosynthetic pathway for the JHs, now known for many years, has been described in exquisite detail by Schooley and Baker (1985). Briefly, biosynthesis of the most ubiquitous JH, JH III, proceeds by the normal terpenoid pathway: the formation of five-carbon (5C) isoprenoid units from acetate via mevalonic acid, with the sequential head to tail condensation of three 5C units to form farnesyl pyrophosphate (**Figure 4**). Farnesyl pyrophosphate then undergoes esteratic cleavage to farnesol, which is then oxidized to FA. The last two steps in the biosynthetic pathway diverge depending upon the insect order. In Lepidoptera, a C10,C11 epoxidase converts the FA to the epoxy acid (JH acid), which is then methylated by an *O*-methyl transferase to form the methyl ester. In Orthoptera and Dictyoptera, the converse appears to be the case: epoxidation follows methylation (Schooley and Baker, 1985; **Figure 4**).

Synthesis of the higher homologs, JH 0, I, and II, appears to be a unique biochemical feature limited to certain insect species (Schooley *et al*., 1984). These insects can synthesize homoisoprenoid (6C) units from acetate and propionate via 3′-homomevalonate, and from a mixture of these isoprenoid (5C) and homoisoprenoid (6C) units form the respective homologs (**Figure 5**). Thus, three homoisoprenoid units (ethylmethylallyl pyrophosphates) form the skeleton of JH 0, two homoisoprenoid units and one isoprenoid unit (dimethylallyl pyrophosphate) form JH I, and one homoisoprenoid unit and two isoprenoid units form JH II.

With regard to the pathway for JHB_3, Moshitzky and Applebaum (1995) have presented evidence that synthesis in *D. melanogaster* proceeds from FA, which is epoxidized twice, at C6,C7 and C10, C11 to form 6,7;10,11-bisepoxyfarnesoic acid (FABE). FABE is then methylated to yield JHB_3 (**Figure 6**). It should be noted that JH III is not epoxidized to JHB_3.

3.7.4.1. Specificity of the Biosynthetic Pathways

The coexistence of multiple JH homologs in a single species at the same developmental stage, together with the discovery of new forms of JH in insects previously thought to have only one, presents an enigma not only as to their functions, but also as to the substrate specificity of the pathways by which they are synthesized and the regulatory mechanisms that effect those steps. Schooley and Baker (1985) concluded that biogenesis of the JH skeleton occurs via the condensation of isoprenoid and homoisoprenoid units and that overwhelming evidence suggests low substrate specificity at most steps in the biosynthetic pathway, even in enzyme preparations from CA, or incubations of CA, from insects known to produce JH III only. This is particularly evident in the two terminal steps, where lack of substrate specificity has been demonstrated in a number of species. For example, the CA of the locust *Schistocerca gregaria*, which normally synthesize only JH III *in vitro*, will produce JH I as their sole product if incubated with dihomofarnesoic acid (Schooley *et al*., 1978a). Schooley and Baker (1985) concluded that the proportions of the precursors (propionate, mevalonate, and homomevalonate) added to incubation medium in which CA are maintained dictate the proportions of the JH homologs produced.

The precursor propionate is essential for the formation of the ethyl side chain of JH 0, I and II (**Figure 5**), and within the CA, propionate is derived principally from the branched-chain amino acids

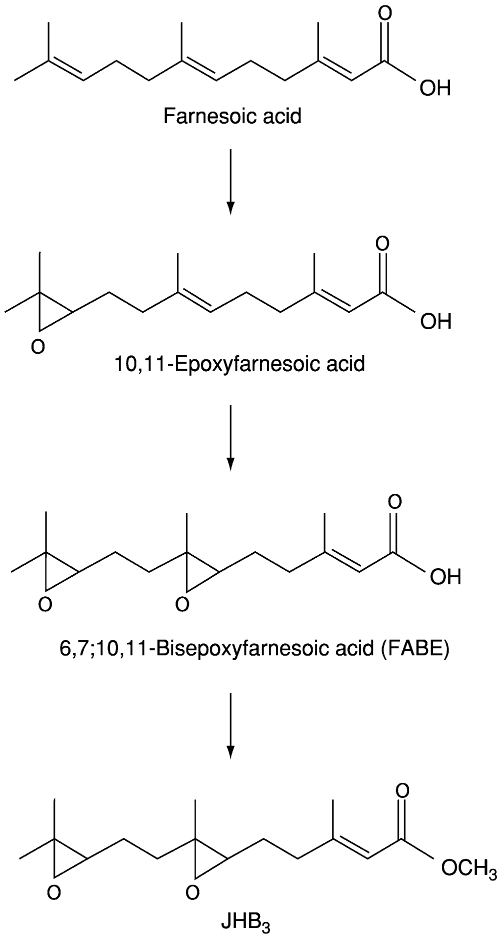


Figure 6 Biosynthetic pathway for JHB_3.

isoleucine and valine (Brindle *et al.*, 1988). The CA of *D. punctata* produce only trace amounts of JH II *in vitro* when propionate is the sole carbon donor, while continuing to produce copious amounts of JH III, the sole naturally occurring JH in this species (Feyereisen and Farnsworth, 1988). The CA of such insects are unable to convert branched-chain amino acids into propionate due to the lack of an active branched-chain amino acid transaminase (Brindle *et al.*, 1987, 1992). Cusson *et al.* (1996) tested the hypothesis that shifts in the proportions of the different JHs and JH acids released by lepidopteran CA are associated with changes in the activity of the transaminase. They found that the age-related increase in the biosynthesis of JH I acid by adult male CA of the moth *Pseudaletia unipuncta* was linked to an age-related increase in transaminase activity in CA homogenates. This increase was not observed with female CA, which synthesized more JH II than JH I during this same time period. The authors propose the existence of a factor regulating propionate production in lepidopteran CA. This factor is not the *M. sexta* allatostatin (see Section 3.7.7.2) that inhibits JH biosynthesis by female *P. unipuncta* CA, since this peptide has no effect on the rate of isoleucine metabolism by either male of female glands.

Thus, as predicted, the availability of propionate within the CA and the activity of the transaminase has a significant impact on the proportions of JH I and II and their acids, but has no effect on the production of JH III, which is synthesized from the nonbranched isoprenoid units (**Figure 5**). The specificity and activity of other enzymes may therefore be involved in creating the blend of JH homologs and their acids synthesized by lepidopteran CA. As noted by Schooley *et al.* (1976) and Cusson *et al.* (1996), these enzymes may be the isopentenyl diphosphate isomerase and the farnesyl diphosphate synthase that catalyze the condensation of the isoprenoid and homoisoprenoid units to form the backbone of the JH homologs. Due to their low abundance, the substrate specificity of these enzymes has not been studied in enzyme preparations from CA homogenates. The cloning and sequencing of farnesyl diphosphate synthase in *A. ipsilon* (GenBank Accession no. AJ009962) and *B. mori* (GenBank Accession no. AB072589) have been reported (Castillo-Gracia and Couillaud, 1999; Kikuchi *et al.*, 2001), and recently Sen and Sperry (2002) have partially purified native farnesyl diphosphate synthase from whole-body preparations of *M. sexta*. Thus, substrate specificity for this enzyme may be known in the near future.

Sen and her colleagues have also examined other enzymes in the JH biosynthetic pathway, including the specificity of prenyltranferase activity of the larval CA of *M. sexta* (Sen and Ewing, 1997). This enzyme catalyzes the head-to-tail condensation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) units to form the skeleton of JH III, and of homoisopentenyl phosphate and ethylmethylallyl diphosphate (EMAPP) to form the skeletons of JH 0, I, II and 4-methyl JH I (**Figures 5** and **6**). Using homodimethylallyl diphosphate (HDMAPP) and the natural and unnatural homologs of geranyl diphosphate (GPP) (formed from the first coupling of one DMAPP and one IPP in the synthesis of JH III) (**Figure 5**), they found that this enzyme has a high tolerance for homologous substrates. EMAPP was a better substrate than DMAPP, while the unnatural geranyl homolog was a poorer substrate for the prenyltransferase than the natural homolog, suggesting that this enzyme contributes to the specificity of the JH skeleton.

Sen and Garvin (1995) examined the specificity of the farnesol dehydrogenase, an enzyme that

catalyzes the oxidation of farnesol to farnesal. Their results suggest that this enzyme is a unique dehydrogenase with high specificity for alcohols with Δ-2,3 unsaturation and alkyl chain hydrophobicity corresponding to at least three isoprenoid units, and hence is a selective enzyme for JH biosynthesis. In a subsequent study, Sperry and Sen (2001) concluded that, in fact, the oxidation of farnesol to farnesal did not involve a unique dehydrogenase, but rather a specific oxygen-dependent enzyme, possibly a flavin- or nonheme iron-dependent oxidase. Further studies on these enzymes will identify targets for compounds with anti-JH activity, as well as foster a better understanding of the key enzymes that are targets of allatotropins and allatostatins (see Section 3.7.7).

3.7.4.2. Possible Feedback Loops in the Biosynthetic Pathways

The formation of farnesyl pyrophosphate is an intermediate step in the synthesis of JH from mevalonate. A mammalian "orphan" nuclear receptor activated by farnesyl pyrophosphate metabolites has been identified (Forman *et al.*, 1995) and termed farnesoid X receptor (FXR) (Weinberger, 1996). FXR is activated by farnesol, farnesal, FA, and methyl farnesoate (**Figure 4**), and is expressed in isoprenoidogenic tissues. It has been suggested that these farnesyl pyrophosphate metabolites (farnesoids) may be signals for transcriptional feedback control of cholesterol biosynthesis, since cholesterol is synthesized from isoprenoids (Weinberger, 1996). As noted by Gilbert *et al.* (2000), Weinberger and his colleagues used a transactivation assay in Chinese hamster ovary cells by transfecting with plasmid DNAs expressing FXR, RXR (retinoid X receptor) and a farnesol responsive chloramphenicol acetyltransferase (CAT) reporter to demonstrate that JH, methoprene, and farnesol can all affect mammalian FXR. Farnesoids are JH biosynthetic agonists; they stimulate the formation of JH when added to incubations of CA and are derived from farnesyl pyrophosphate by a metabolic pathway that may serve to excrete surplus isoprenoid precursors (Schooley and Baker, 1985). Since insects cannot synthesize cholesterol *de novo* and require a dietary source of sterols (Rees, 1985), FXR may be involved in the feedback control of JH biosynthesis via these farnesoids. This possibility certainly deserves further study and opens the door to an exciting field of investigation of the role of orphan nuclear receptors in the regulation of JH biosynthesis (Chawla *et al.*, 2001) (see Section 3.7.7.3)

3.7.4.3. What Constitutes a Juvenile Hormone Titer?

As discussed by Darrouzet *et al.* (1998), the identification of new JHs, whether metabolites, conjugates, precursors, or hydroxylated forms, illustrates the variability of compounds released as end products from the CA; these end products are what Darrouzet *et al.* (1998) term "bouquets" of compounds. The "bouquets" are different in different orders, and undoubtedly vary between species within orders, and even within species by sex and stage in the life cycle (Couillaud, 1995). This is certainly true for *M. sexta*, where JH I, II, and III and their acids have been measured in larval hemolymph (Baker *et al.*, 1987), and for *P. regina*, in which the CA secrete JH III, JHB_3, and MF (Yin *et al.*, 1995). Thus, it may be naive to think of the circulating titer of a single JH as responsible for the production of a JH effect. Rather, it may be a particular ratio or balance of homologs that is important for biological activity.

Sites outside the CA may also contribute to the circulating titers of the different hormones by metabolism, catabolism, hydroxylation, or conjugation. The JH I originally isolated and identified from the abdomens of male *H. cecropia* pupae (Röller *et al.*, 1967) was, in fact, synthesized by male accessory glands from JH acid secreted by the CA (Dahm *et al.*, 1976; Peter *et al.*, 1981). The male accessory glands in the mosquito *Aedes aegypti* synthesizes JH I, JH III, JHB_3, and MF from acetate (Borovsky *et al.*, 1994a), and the mosquito ovary synthesizes JH III from FA (Borovsky *et al.*, 1994b). Non-JHs such as the thyroid hormones, which can be ingested by the insect, may function as a JH in some JH-linked events. Are we safe in assuming that an event is truly JH independent if it occurs in an insect from which the CA have been removed (Davey, 2000a)? Is the JH titer in the hemolymph truly reflective of what elicits a JH-dependent event?

Thus, in considering JH titers and any temporally linked JH-dependent event, the event must be viewed from the perspective of a coordinated interplay between products of the CA, the products of other tissues in the body, the catabolism of these products (see Section 3.7.9), and the receptors for these products on the target tissues (see Section 3.7.12.1). The list of products possibly involved in this interplay is long and getting longer.

3.7.5. Measurement of the Juvenile Hormones

The lipophilic nature of JH, its occurrence in exceptionally low concentrations in biological samples

(parts per billion or lower), its lability, and its tendencies to bind nonspecifically to substrates, contribute to making this one of the most difficult of all hormones to measure accurately. There are three methods employed to quantitatively assess JH titers: bioassays, physicochemical assays, and radioimmunoassays. Relative JH titers can be determined using the radiochemical assay.

3.7.5.1. Bioassays

The first techniques used to measure JH titers were an assortment of bioassays that quantified a JH effect on a morphological or physiological process (Sláma *et al.*, 1974). Initially, these bioassays were used to assess the activity of transplanted CA, but are now more commonly used to quantify JH indirectly by comparing the biological response of an unknown preparation to a known amount of JH. These types of assays have been well described and critiqued in previous reviews (Feyereisen, 1985; Baker, 1990). Their use is in decline for a number of reasons: (1) they are cumbersome; (2) they are time-consuming, requiring maintenance of a test insect colony; (3) they are subjective in their scoring; (4) they lack specificity, because they measure only total JH; and (5) they show differential sensitivity to the various homologs. Nevertheless, several assays including the *Galleria* wax test (Gilbert and Schneiderman, 1961; deWilde *et al.*, 1968) and the *Manduca black* larval assay (Fain and Riddiford, 1975) are still used, since there are certain instances where bioassays play an important role. These include testing biological extracts for JH activity, especially when the active ingredient is thought to have a non-JH-like structure, or monitoring biological activity of a JH during its purification process.

3.7.5.2. Physicochemical Assays

With growing sophistication in the technology for studying organic compounds, a number of physicochemical methods have been developed for measuring JH in biological samples. Unfortunately, none of these methods is rapid, simple, or inexpensive. Moreover, the literature surrounding the physicochemical detection methods is awash in methods that can be bewildering for a biologist. The current physicochemical methods reflect several decades of experimentation that emphasized specificity and ultrasensitivity at the expense of simplicity. There are normally two phases to the physicochemical assay; the sample extraction/clean-up phase, and the quantification phase. Because JH represents only a minute fraction of the extractable lipids, especially in whole-body extracts, protocols generally require some type of clean-up procedure(s) such as column chromatography, thin layer chromatography, separatory cartridge purification, HPLC, and/or gas chromatography (GC). Preparation of a biological sample for JH analysis has been discussed in several excellent reviews that cover extraction methods, solvents, and partition techniques (Baker, 1990; Halarnkar and Schooley, 1990). Further cautions with regard to degradation, volatility, and nonspecific adsorption have also been extensively outlined (Granger and Goodman, 1983, 1988; Goodman *et al.*, 1995).

Once the clean-up procedure is completed, JH can be quantified by a number of different procedures that employ a gas chromatograph interfaced with an electron-capture detection unit or mass spectrometer (Baker, 1990). In the early analyses of the hormones, GC separation of underivatized JH was coupled with electron impact (EI) or chemical ionization (CI) detection systems to monitor levels of the hormone. While these methods required no derivatization, they often resulted in complex spectra, poor recoveries, and an accumulation of breakdown products (Baker, 1990). Moreover, JH is not particularly stable under most GC conditions, due to instability of the epoxide ring. The use of CI, with its softer ionization process and its improved sensitivity and specificity, yields fewer fragment ions than the harsher EI ionization mode. Nevertheless, problems remain with the misidentification of the JH homologs (Baker, 1990).

To enhance sensitivity and specificity, methods were developed that generated JH derivatives with unique chemical tags. The first to be developed were the organohalide derivatives that are detected by a GC interfaced with an electron capture detection (ECD) system. While this method greatly increased sensitivity of the assay, it had serious drawbacks in uniformity of derivatization and in identification of the homologs. To overcome these difficulties, Rembold *et al.* (1980) and Bergot *et al.* (1981b) developed GC-mass spectrometry (MS) methods which, while still requiring derivatization of the hormones, were far more efficient and sufficiently selective to allow each JH homolog to be identified using selected ion monitoring. GC-MS-EI and GC-MS-CI analyses are now performed with slight variations on these procedures (Neese *et al.*, 2000; Smith *et al.*, 2000; Cole *et al.*, 2002).

Teal *et al.* (2000) have recently developed a method for quantifying JH from biological samples that allows for direct analysis of hormone without derivatization. These investigators use GC interfaced with ion-trap MS and CI. This method

requires no clean-up procedure or sample derivatization and is highly sensitive, and thus may be the assay of choice for future studies. While this procedure has been used to titer JH in hemolymph (Burns *et al.*, 2002) and culture medium (Bede *et al.*, 2001), in which lipid content is relatively low, it will be interesting to see if the assay can be successfully used with whole-body extracts.

The advantage of the physicochemical assays lies in their sensitivity and their unequivocal identification of the different JHs and metabolites. Their drawback lies in accessibility to the prohibitively costly equipment and the relatively low throughput.

3.7.5.3. Radioimmunoassays

Radioimmunoassays (RIAs) are a rapid, sensitive, and inexpensive alternative to measuring JH, both in biological samples and in medium from incubations of CA. The JH RIA is a competitive protein binding assay in which JH from a biological sample competes with a fixed amount of radiolabeled JH for a limited number of binding sites on JH-directed antibodies. The radiolabeled JH bound to the antibody in the presence of the unknown is compared to a standard curve derived with known amounts of radioinert JH.

Since JH itself is not antigenic, antibodies against JH are produced by chemically linking JH to a protein such as human serum albumin or thyroglobulin. The hormone can be conjugated to the protein via the C1 (ester) terminus of JH or through C10,C11 (see Granger and Goodman, 1983, 1988; Baker, 1990; Goodman, 1990, for reviews of the technique). The resulting antiserum is carefully screened for specificity, since it may recognize a particular JH homolog exclusively (Granger *et al.*, 1979, 1982; Goodman, 1990), two or more homologs to differing extents (Granger and Goodman, unpublished data), or all homologs equivalently (Goodman *et al.*, 1993, 1995).

Like the physicochemical methods, JH from whole-body or hemolymph extracts must be partially purified prior to conducting the RIA. The partial purification process is essential for a successful assay since a number of lipids can nonspecifically interfere with the assay. Inattention to this process invariably results in excessively large and inaccurate JH titers due to micelle formation of nonspecific lipids that either trap JH or inactivate the JH-directed antibodies. Misidentification of the JH homologs and overestimation of titers can also occur when chromatographic systems contaminated with high levels of JH standards are also used for the purification of biological samples (Baker *et al.*, 1984). It is for these reasons that RIA technology has rightly been criticized (Feyereisen, 1985; Tobe and Stay, 1985). A number of excellent clean-up procedures have been suggested (Strambi, 1981; Strambi *et al.*, 1981; Goodman *et al.*, 1995; Niimi and Sakurai, 1997; Noriega *et al.*, 2001). It is important to note that despite differences in extraction methods and JH-directed antibodies, excellent agreement between assays can be achieved (Goodman *et al.*, 1993).

JH RIAs have been used most effectively to measure CA activity *in vitro*, since the products are secreted into defined incubation medium that lacks the massive amount of lipid found in whole-body or hemolymph extracts. RIAs have been used extensively in studies on CA activity and in control of JH biosynthesis in the tobacco hornworm (Bollenbacher *et al.*, 1987; Granger and Janzen, 1987; Janzen *et al.*, 1991; Granger *et al.*, 1994), locusts (Couillaud *et al.*, 1984; Baehr *et al.*, 1986), honeybees (Huang and Robinson, 1995), and bumblebees (Cnaani *et al.*, 2000). Furthermore, certain extant RIAs recognize JH and JH acid equivalently (Janzen *et al.*, 1991), which can be a benefit in light of the fact that the CA of a number of species secrete JH acid at certain times in their life cycle (Sparagana *et al.*, 1984; Janzen *et al.*, 1991; Ho *et al.*, 1995a, 1995b; Niimi and Sakurai, 1997; Duportets *et al.*, 1998; Audsley *et al.*, 2000).

One of the major problems that has plagued research on the chemistry and biology of JH is the lack of commercially available, enantiomerically pure JH; nowhere is this more evident than in RIA technology. Depending upon the antigen used for immunization and immunocompetence of the rabbit, antibodies can be highly specific and capable of recognizing a single enantiomer of each homolog. Thus, using racemic JH as a standard may pose difficulties in establishing an accurate titer. A very complex binding reaction is generated when the radiotracer is racemic, but JH from the biological sample is enantiomerically pure; the analysis becomes even more complex when the radioinert standards are racemic. This problem has been eliminated by the use of a chiral HPLC matrix that can resolve the JH enantiomers (Cusson *et al.*, 1997). Moreover, the use of the appropriate enantiomers leads to increased sensitivity of the assay. Based on the ED_{50} values (effective dose required to inhibit binding of 50% of the radiolabeled tracer), the naturally occurring enantiomers of JH I, JH II, and JH III in this study were between 30 and 87 times more immunoreactive than the unnatural isomers. In summary, the JH RIA is a relatively rapid, inexpensive, and accurate method for titering JH. If

measurement of more than one homolog is required, then confirmation by physicochemical methods is recommended.

3.7.5.4. Radiochemical Assays

The radiochemical assay (RCA), originally developed by Pratt and Tobe (Pratt and Tobe, 1974; Tobe and Pratt, 1974), has been employed for many years to measure JH biosynthesis *in vitro* by the CA of a wide variety of insects (Feyereisen and Tobe, 1981; Tobe and Feyereisen, 1983; Tobe and Stay, 1985; Yagi and Tobe, 2001). The RCA measures the rate of incorporation of the methyl group from either [^{14}C]methyl methionine or [^{3}H]methyl methionine into JH in isolated CA. The availability of assay components, the relative simplicity of the protocol, its sensitivity (0.1 pmol), and the short incubation time (1–3 h), have made this assay popular among insect endocrinologists.

There is a general assumption that isolated glands lacking neural connections function in the same fashion as glands *in vivo*. This assumption has been challenged by Horseman *et al.* (1994), who demonstrated that nerve transection in adult *L. migratoria* led to a rapid, 16-fold increase in JH production. A similar result has also been found in isolated CA of *A. aegypti* (Noriega, personal communication). Thus, the RCA may, at certain times, seriously overestimate the expected *in vivo* production of JH (Pratt *et al.*, 1990). Another potential problem is the use of [^{3}H]methyl methionine as the methyl donor (Yagi and Tobe, 2001). The lack of definitive data from the manufacturers on the purity and specific activity of this compound can make determination of the biosynthesized JH difficult. Yagi and Tobe (2001) recommend either comparing incorporation of the [^{3}H]methyl group with the [^{14}C]methyl group in a dual-label experiment or switching altogether to [^{14}C]methyl methionine. It appears that isotopic discrimination by the *O*-methyl transferase leads to preferential incorporation of the [^{14}C]-labeled methyl group vs. the [^{3}H]-labeled methyl group, but only when concentrations of [^{3}H]methyl methionine above the normal range for the RCA are used.

The measurements of JH biosynthesis using the RCA can also be affected by the incubation medium, which in most instances is TC199. TC199 has a high $Na^{+}:K^{+}$ ratio (17.8) and thus is a suitable medium for the CA from insects where the hemolymph ratio of $Na^{+}:K^{+}$ is similar, for example, *Leptinotarsa decemlineata* or *T. molitor* (Granger *et al.*, 1986). This medium has been used extensively in the RCA, irrespective of insect species. For insects such as *M. sexta*, where the hemolymph $Na^{+}:K^{+}$ ratio is low (0.1) (Nowock and Gilbert, 1976), TC199 has been found to depress JH synthesis in comparison to Grace's medium, in which the $Na^{+}:K^{+}$ ratio is 0.33 (Granger *et al.*, 1986; Watson *et al.*, 1986). More recently, L-15B, a medium widely used in the culture of arthropod tissues and found to be isoosmotic with cockroach hemolymph, was demonstrated to be superior to TC199 for both long- and short-term cultures of cockroach CA (Holbrook *et al.*, 1997).

3.7.6. Evolution of the Juvenile Hormones

3.7.6.1. Juvenile Hormone Homolog Distribution among the Insect Orders

One or more of the JH homologs have been identified in nearly all species of insects in which they have been sought (Sehnal, 1984) and have even been found in Collembola, an order once thought basal to insects but now considered to represent a more ancestral group, distinct from insects (Nardi *et al.*, 2003). A JH effect on ecomorphosis of a collembolan species, *Hypogastrura tulbergi*, has been reported (Lauga-Reyrel, 1985), and on this basis, it seems likely that JH or a JH-like molecule functioned in the ancestors of modern insects at least 400 million years ago. **Figure 7** illustrates the evolutionary time line in which the various orders of insects were thought to have appeared, the orders in which JHs or JH-like molecules have been identified, and the identity of these compounds.

3.7.6.2. Evolutionary Connections among the Arthropods

Arthropods, which appeared more than 500 million years ago during the Cambrian period, include four extant classes: Hexapoda, Chelicerata, Myriapoda, and Crustacea. Of these, the Hexapoda and Crustacea appear to be the most closely related, based on molecular data, and are now considered together as Pancrustacea (Boore *et al.*, 1995; Friedrich and Tautz, 1995; Tobe and Bendena, 1999). **Figure 8** illustrates a time line of the appearance of the extant classes of Arthropoda and those classes in which JH effects have been demonstrated.

Outside the class Insecta, JH-like molecules have been identified in Crustacea (see **Chapter 3.16**), and functions for these molecules have been proposed. Both FA and MF, the immediate precursors of JH III (**Figure 4**), are major biosynthetic products of the mandibular organs of many crustaceans. The similarities in function and embryological origin of the

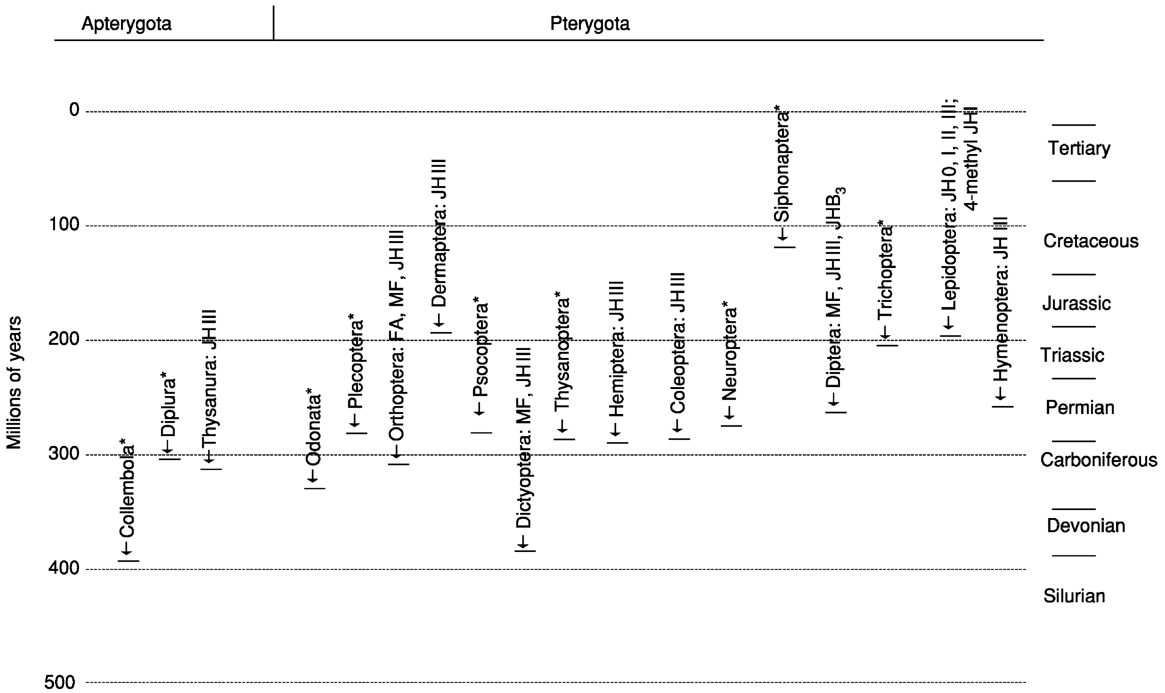


Figure 7 Evolutionary timeline for the appearance of the insect orders based on the earliest documented fossil record (Heming, 2003). Orders in which JH effects have been demonstrated are noted with an asterisk (*). Orders in which JH or JH-like molecules have been conclusively identified are noted by the homolog.

mandibular organs and CA, and the fact that the biosynthetic pathway for MF and FA are the same in both crustaceans and insects (see Section 3.7.4.1), suggests that these products may be the JHs of Crustacea. FA can act as a JH in crustacean reproduction (Laufer *et al.*, 1993), and MF has a juvenilizing role in the prawn (Abdu *et al.*, 1998) and the barnacle (Smith *et al.*, 2000) during metamorphosis from the larval to the juvenile form. MF also delays metamorphosis in larvae of the lobster, *Homerus americanus* (Borst *et al.*, 1987). Nevertheless, definitive physiological and developmental roles for these two JH precursors remain vague (Homola and Chang, 1997), and further work is needed to determine their exact functions.

Although it is not clear whether FA or MF are prohormones, hormones, or simply side products of JH biosynthesis in insects, the esterification of JH acids in the accessory glands of male *H. cecropia* (Shirk *et al.*, 1983) and the imaginal discs of *M. sexta* (Sparagana *et al.*, 1985) certainly suggests that FA can be converted to MF, and MF esterified to JH III, in tissues outside the CA. Research is needed on the existence, location, and activity of the enzymes *O*-methyl transferase and MF epoxidase to elucidate whether FA and MF are prohormones or hormones in their own right.

3.7.6.3. Evolutionary Inferences

FA is released from the nymphal CA of the cockroach, *D. punctata*, while homo/dihomo MF and FA appear to be products of the CA of adult female and male *P. unipuncta* (Cusson *et al.*, 1991a). It has recently been shown that in addition to JH III, the embryonic CA of *D. punctata* produce MF, with a shift to predominantly JH production as development proceeds (Stay *et al.*, 2002) (see Section 3.7.10.2). This evidence has led Stay *et al.* (2002) to postulate that the production of MF by the early embryonic CA is an ancient trait in Arthropoda, and that the original metamorphic and reproductive hormone in this more basal order was MF rather than JH. In light of the proposal that the ancestral role of JH was involved in embryogenesis (Truman and Riddiford, 1999), MF may have fulfilled this role in the early evolution of the Arthropoda, with a shift to JH III production as metamorphosis in the Insecta evolved.

Sehnal *et al.* (1996) have suggested that the postembryonic development in the ancestors of ametabolous insects occurred in the absence of JH and that the original function of JH in postembryonic life was the regulation of reproduction. As the occurrence of metamorphic molts evolved in ancient

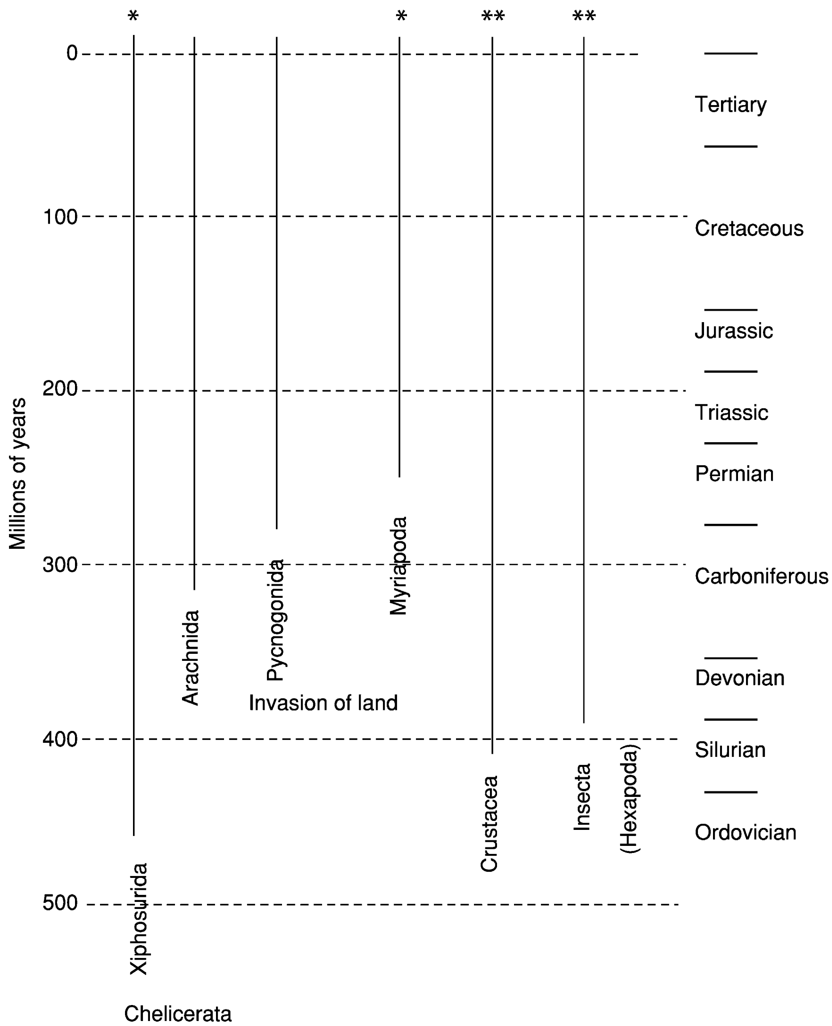


Figure 8 Evolutionary timeline of the appearance of the extant classes of Arthropoda. Classes in which JH effects have been demonstrated are noted with an asterisk (*). Classes in which JH or JH-like molecules have been identified are noted with a double asterisk (**).

arthropods, it is possible that a hormone (JH) that functioned in the embryo and the mature adult, but played no role in postembryological development, was coopted to regulate development before and after these metamorphic molts (Tobe and Bendena, 1999). In some insect species, JH appears to regulate the types of responses that would facilitate evolution of a larval stage, such as premature differentiation of the embryo and alterations in morphology (Truman and Riddiford, 1999). As outlined below (see Section 3.7.10.7), Truman and Riddiford (1999, 2002) have proposed that an advancement in the time of appearance of JH during embryogenesis may have been key to the evolutionary transformation of a pronymphal stage to the larval stage of holometabolous insects.

The production of more complex forms of JH may have occurred with the evolution of holometabolous development. At the present time, JH III is the only known form of JH in most insect species, particularly the Hemimetabola. With the exception of the HJHs (see Section 3.7.2.2), the more complex forms of JH, JH 0, iso-JH 0, JH I, methyl-JH I, JH III, and JHB_3, appear to occur only in two highly derived orders of holometabolous insect, the Lepidoptera and the Diptera. Furthermore, only Diptera appear to have evolved the ability to produce JHB_3. The production of the higher homologs and their multiple occurrence in some species appears to be related to the evolutionary development of intricate physiological events and their regulation. The exact physiological or developmental role of the different JHs or JH-like molecules in each class and order needs to be determined to further elucidate the role of JH in arthropod and insect evolution and by extension, the evolution of these molecules themselves.

3.7.7. Regulation of Juvenile Hormone Biosynthesis

A great deal of progress has been made since 1985 in understanding the regulation of JH biosynthesis by neurohormones, neuromodulators, and neurotransmitters (Feyereisen, 1985). At that time it was generally believed, based on a considerable body of *in vivo* studies, that physiological factors, mainly from the central nervous system, either stimulated (allatotropins) or inhibited (allatostatins) JH synthesis by the CA. Their chemical nature was unknown, but their existence was accepted, based on distinct and predictable changes in the hemolymph JH titer that related directly to physiological events, such as ecdysis of the larva and reproduction in the adult. By 1985, methods for maintenance of the CA *in vitro* had been established for a number of species, and two approaches to the measurement of JH biosynthesized *in vitro* had been developed: the radiochemical assay (Pratt and Tobe, 1974) (see Section 3.7.5.4) and the radioimmunoassay (Baehr *et al.*, 1976, 1979; Strambi, 1981) (see Section 3.7.5.3). Using these methods, the JH homologs synthesized by the CA *in vitro* have been found to be the same as those in hemolymph, although the homolog ratio may vary for CA that synthesize more than one JH. Moreover, developmental changes in JH biosynthetic activity by the CA *in vitro* parallel changes in the hemolymph JH titers (Feyereisen, 1985; Tobe and Stay, 1985). What factors elicit these changes in JH biosynthetic activity?

3.7.7.1. Neuropeptides

3.7.7.1.1. Allatotropins The first peptide to be isolated that affected JH synthesis *in vitro* was the allatotropin (AT) of *M. sexta* (Manse-AT) (Kataoka *et al.*, 1989). While this amidated tridecapeptide did not stimulate JH biosynthesis by larval or pupal CA *in vitro*, it did stimulate synthesis by adult CA at a concentration of 10^{-9} M. The gene for Manse-AT was isolated a decade later and was found to express three different mRNAs that differ from one another by alternative splicing (GenBank Accession no. U62100, U62101, U62102) (Taylor *et al.*, 1996). It has been suggested that the different mRNAs encode three distinct prohormones. Immunocytochemistry with polyclonal antibodies to Manse-AT and *in situ* hybridization with riboprobes for its mRNAs were used to show that Manse-AT exists in the central nervous system of *M. sexta* larvae. The former study found immunoreactivity in cerebral neurosecretory cells as well as axons in the corpora cardiaca (CC) and CA (Žitňan *et al.*, 1995), while the latter found only low levels in nonneurosecretory cells of the larval brain and subesophageal ganglion (Bhatt and Horodyski, 1999). Although Manse-AT stimulates the adult CA in this species, its mRNAs were not detected in prepupal, pharate pupal, or adult brains (Bhatt and Horodyski, 1999).

Is Manse-AT a functional AT in other lepidopteran species? There is direct evidence from several different studies that this is indeed the case. Manse-AT may be the true allatotropin in *P. unipuncta*: it has been cloned in this species (GenBank Accession no. AF212926) and Manse-AT immunoreactivity has been found in the CA (Truesdell *et al.*, 2000). Manse-AT has also been cloned from the silkworm *B. mori* (Park *et al.*, 2002) and has been purified from methanolic brain extracts of the fall armyworm, *Spodoptera frugiperda*, in which it stimulates adult CA (Oeh *et al.*, 2000, 2001). A cDNA encoding 134 amino acids, including an allatotropic peptide, has recently been cloned in this species (GenBank Accession no. AJ488181, AJ508061) (Abdel-Latief *et al.*, 2003), and the mature peptide is identical to Manse-AT. The *Spodoptera* peptide precursor has 84%, 93%, and 83% amino acid sequence identity with those of *M. sexta*, *P. unipuncta*, and *B. mori*, respectively. Manse-AT also stimulates JH synthesis by both larval and adult CA of *L. oleracea* (Audsley *et al.*, 1999a, 1999b, 2000), and the adult CA of another moth, *Heliothis virescens* (Kataoka *et al.*, 1989; Teal, 2002).

Manse-AT may be a functional AT in other orders as well. Immunological analyses indicate it is present in the abdominal nervous system of the cockroach *Periplaneta americana* (Rudwall *et al.*, 2000) and in the brain of the fly *P. regina* (Tu *et al.*, 2001). Moreover, it stimulates JH synthesis by the larval CA of the honeybee, *Apis mellifera* (Rachinsky and Feldlaufer, 2000; Rachinsky *et al.*, 2000), and the adult female CA of *P. regina* (Tu *et al.*, 2002).

3.7.7.1.2. Other roles for Manse-AT Since Manse-AT does not affect larval *M. sexta* CA, could it have another function in the larva? Indeed, Manse-AT has been shown to be a multifunctional molecule in *M. sexta*, as well as in other species. It exerts an apparently species-specific inhibition of ion transport in the midgut of *M. sexta* fifth instars (Lee *et al.*, 1998) and accelerates the heart rate in pharate adults, but not larvae (Veenstra *et al.*, 1994). It stimulates foregut contractions in the moths *Helicoverpa armigera* and *L. oleracea* (Duve *et al.*, 1999, 2000); acts as a cardioaccelerator in *Leucophaea maderae*, *P. americana* (Rudwall *et al.*, 2000),

and *P. unipuncta* (Koladich *et al.*, 2002); and is involved in the photic entrainment of the circadian clock of *L. maderae* (Petri *et al.*, 2002). Thus, a phenomenon observed with a number of other insect neuropeptides is illustrated by Manse-AT: it has multiple functions and these functions are stage-, tissue-, and species-specific.

3.7.7.1.3. Molecular biology of the allatotropins Horodyski and his colleagues have undertaken a detailed study of the molecular biology of the ATs. They have found that while levels of Manse-AT mRNAs are low or nonexistent in the brain, the Manse-AT gene is expressed in a variety of cells in other regions of the central nervous system and frontal ganglion in larval, pupal, and adult stages of *M. sexta* and that the cellular expression pattern in the nerve cord, as indicated by levels of Manse-AT mRNA, changes during metamorphosis (Bhatt and Horodyski, 1999).

Manse-AT-like peptides can be derived from each of three distinct precursor proteins that are the predicted products of alternatively spliced mRNAs (Taylor *et al.*, 1996). The basis for differences in the spliced mRNAs is the inclusion of one or two exons at the same location within the open reading frame. These exons contain a sequence that codes for a peptide with limited structural identity to Manse-AT (Horodyski *et al.*, 2001; Lee and Horodyski, 2002). The three expressed peptides have overlapping but nonidentical activities in JH biosynthesis assays with adult CA *in vitro* and in the inhibition of active ion transport across larval posterior midgut epithelium *in vitro*. The presence of the mRNA isoforms resulting from the alternative splicing is regulated in a tissue-and developmentally specific manner, suggesting changing roles for the resulting peptides at different life stages (Lee *et al.*, 2002). Finally, the expression of one of these mRNA isoforms is increased in the ventral nerve cord of *M. sexta* last instars subjected to treatments that normally reduce feeding and increase levels of JH in the larva: starvation, parasitization, or ingestion of the ecdysteroid agonist RH5992 (K.Y. Lee and Horodyski, 2002). These discoveries are the first to identify the pathway by which these treatments first have their effect. Whether this effect involves downstream regulation of the endocrine system, midgut ion transport, or another target is yet unknown.

3.7.7.1.4. Other allatotropins Until recently, Manse-AT was the only AT identified. However, an allatotropic immunoreactive peptide has been isolated from the abdominal ganglia of the mosquito *A. aegypti* (Veenstra and Costes, 1999) and found to have a unique sequence. It has now been shown that Aedae-AT has a stimulatory effect on adult female *A. aegypti* CA *in vitro*, with maximum stimulation at dosages of 10^{-8}–10^{-9} M, suggesting that this moiety is the true AT in *A. aegypti* (Li *et al.*, 2003). Furthermore, while neither the JH precursor FA nor Aedae-AT stimulates JH III synthesis by the CA of newly emerged females, when both are added to the incubation medium, high levels of JH III production occur, indicating that Aedae-AT makes the CA competent to make JH III from FA.

There are a number of studies that suggest the existence of at least seven other allatotropins (Stay, 2000; Li *et al.*, 2003). In the wax moth, *Galleria mellonella*, a protein fraction of larval brains was found to stimulate JH II synthesis by CA *in vitro*, and monoclonal antibodies raised to that fraction were used to identify a 20 kDa peptide by immunoblotting (Bogus and Scheller, 1996). Two pairs of immunoreactive neurosecretory cells in the *G. mellonella* brain and in the CC were identified with these monoclonal antibodies. One of the pairs of cerebral neurosecretory cells was immunoreactive with antibodies to Manse-AT as well, but because of the size of the putative Galme-AT, it is unlikely to be a homolog of Manse-AT. The investigators suggest that Manse-AT and Galme-AT have common epitopes due to splicing from the same preprohormone. Recent attempts to isolate the gene for this AT using an expression library and monoclonal antibodies have not been successful (Sehnal, personal communication).

A putative AT has been extracted from the subesophageal ganglion of the cricket *Gryllus bimaculatus*, and stimulates the adult CA of not only *Gryllus*, but also of another cricket, *Acheta domesticus* (Lorenz and Hoffmann, 1995). Extracts of the brain–subesophageal ganglion–CC–CA from the true bug, *Pyrrhocoris apterus*, stimulate JH biosynthesis by the CA of adults raised under long-day photoperiodic conditions (Hodkova *et al.*, 1996), and methanolic extracts of brain or CC from adult vitellogenic females of the locust *L. migratoria* stimulate JH III release from the CA of vitellogenic females 20–40-fold (Gadot *et al.*, 1987). These extracts also stimulate JH production by the CA of adult male *Locusta*, but not without some manipulation, since the CA must be chilled at 4 °C for 24 h to obtain the stimulatory response (Lehmberg *et al.*, 1992). Surgical manipulations and immunological studies suggest that the brain is the source of this factor (Couillaud *et al.*, 1984; Ulrich, 1985). Allatotropic activity was also extracted from the subesophageal ganglion and CC of adult male *M. loreyi*, and found to stimulate JH III acid and iso-JH II

synthesis by male CA *in vitro* approximately twofold (Kou and Chen, 2000).

3.7.7.1.5. Nonneural allatotropins The lack of effect of Manse-AT and other putative ATs on CA when one would assume an active stimulation of JH biosynthesis implies that there exist ATs of different structures than those isolated to date from neural tissue. Studies suggesting the existence of allatotropic molecules from nonneural sources represent the most intriguing line of research on the control of JH biosynthesis. There currently appear to be two sources for such factors: the ovary and the male accessory sex gland. Ovarian factors that effect JH production have been found in two species, the cockroach *D. punctata* and the cricket *G. bimaculatus*. In *D. punctata*, ovaries stimulate increased JH production in a stage-specific manner: ovaries just prior to ovulation are stimulatory while ovaries of pregnant females are not (Rankin and Stay, 1985). Stimulation is directly related to the size of the basal oocytes; ovaries with basal oocytes smaller than 0.8 mm or larger than 1.5 mm were not stimulatory. More recently, it has been demonstrated that *Diploptera* ovaries, fat body, and muscle can all stimulate JH synthesis by CA *in vitro* (Unnithan *et al*., 1998). Stimulation by the ovary is dose-dependent and sensitive, although the moiety responsible does not appear to be a peptide. In this species, however, the overall control of JH biosynthesis is very much dictated by allatostatins (see Section 3.7.7.2). In *G. bimaculatus* (Hoffmann *et al*., 1996), an increase in JH biosynthesis follows the implantation of ovaries into adult males. Whether this effect is mediated by a true AT is not clear, since an extract of ovaries did not stimulate JH synthesis *in vitro*.

A 36 amino acid peptide of known sequence from the male accessory sex gland of *D. melanogaster* (Chen *et al*., 1988; Schmidt *et al*., 1993) stimulates JH synthesis when transferred to the female during mating and also increases JH synthesis *in vitro* by the CA of adult female *D. melanogaster* (Moshitzky *et al*., 1996). The peptide has the same effect *in vitro* on CA from adult females of the moth *H. armigera* (Fan *et al*., 1999). By contrast, fragments of the C-terminal and a truncated N-terminal peptide from this *Drosophila* sex peptide (SP) inhibit JH synthesis *in vitro* by CA from both species. Since two N-terminal peptides stimulate JH synthesis, similar to the full-length molecule, it appears that the first five N-terminal amino acid residues are essential for CA stimulation by SP in both species (Fan *et al*., 2000). Moshitzky *et al*. (2003) recently reported that the *Drosophila* SP downregulates JH III bisepoxide synthesis *in vitro* by CA of another dipteran, the medfly *Ceratitis capitata*.

3.7.7.1.6. Future directions of research on allatotropins As proposed by Gilbert *et al*. (2000), the future directions of research on ATs should concentrate in several areas: (1) the identification of ATs in other orders and the sequences of the Manse-AT-like molecules in those lepidopteran species where Manse-AT has an effect; (2) the exploration of other functions for identified ATs; (3) the elucidation of the interaction of ATs and allatostatins in the overall control of JH biosynthesis; and (4) studies of their receptors and ligand–receptor interactions leading to downstream events that modulate synthesis. To this list should be added research on nonneural ATs.

3.7.7.2. Allatostatins

Allatostatins (ASTs), compounds that inhibit JH biosynthesis by the CA, can be grouped into three families. More than 170 different peptides belong to the largest of the families, the FGLamide ASTs, or the AST-A group. This family is characterized by the presence of a highly conserved pentapeptide in the C-terminus, Y/F-X-F-G-L-amide but displays considerable variability in length and sequence in their N-termini (Tobe and Stay, 2004). These ASTs, first identified by Stay *et al*. (1991a) in *D. punctata*, have now been found in many different insect species (Gade *et al*., 1997; Stay, 2000; Li and Noriega, personal communication). They have also been found in groups outside the insects, including Crustacea (Dircksen *et al*., 1999; Skiebe, 1999; Duve *et al*., 2002) and freshwater pulmonate snails (Rudolph and Stay, 1997). In *D. punctata* alone, there are 14 FGLamide ASTs, with one terminating in an isoleucine (Stay, 2000; Tobe and Stay, 2004). Although Lepidoptera have their own family of ASTs, an FGLamide AST was isolated and identified from *M. sexta* (Davis *et al*., 1997). FGLamide immunoreactivity is found in the brain, abdominal ganglia, neurohemal organs, and thoracic motor neurons of *M. sexta* larvae, but the processes of immunoreactive neurons in the CA are sparse. The authors concluded that in this species, the FGLamides are probably neuromodulatory, myomodulatory, and myotrophic.

The second family of ASTs, or AST-B group, has been identified in crickets, locusts, and stick insects and contains a common amino acid sequence of W_2W_9amide, that is, tryptophan residues at the 2 and 9 positions of the N-terminus (Lorenz *et al*., 1999, 2000; Tobe and Stay, 2004). Because these peptides in stick insects are variable in length, a

designation of W-X_6-Wamide has been assigned to this family (Tobe and Stay, 2004). At the present time there appear to be 17 of these peptides (Stay, personal communication): five in *G. bimaculatus*, six in *Carausius morosus*, with the remainder in orders as diverse as Lepidoptera (*M. sexta* and *B. mori*), Diptera (*D. melanogaster*), Orthoptera (*L. migratoria*), and Dictyoptera (*P. americana*) (Stay, 2000). A considerable number of these are not active in the species from which they were isolated, being identified solely on the basis of amino acid sequence.

The third family of ASTs, the AST-C group, has been identified in Lepidoptera and has been designated the PISCF family. The first to be discovered, an AST (Manse-AST) in *M. sexta*, was purified from pharate adult heads by Kramer *et al.* (1991) and shown to be a 15 residue, nonamidated peptide with a free C-terminus and with no homology to the cockroach ASTs. This neuropeptide is unique in another sense, in that it can completely inhibit JH production in *M. sexta*. Immunochemical and molecular techniques have shown that Manse-AST is present in other insects, including *P. unipuncta* (Jansons *et al.*, 1996), *L. oleracea* (Audsley *et al.*, 1998), *Spodoptera littoralis* (Audsley *et al.*, 1999a), and *D. melanogaster* (unpublished information cited in Stay, 2000). As with the other AST families, there is some question as to whether Manse-AST is the functional AST in all these species. Synthetic Manse-AST does not affect JH biosynthesis *in vitro* by *Drosophila* ring glands; in *P. unipuncta*, it does not inhibit larval CA or newly emerged adults, and inhibits adult female CA only 60% at a concentration of 10^{-6} M (Jansons *et al.*, 1996). Manse-AST inhibits JH synthesis *in vitro* by adult CA of *Heliothis* (*Helicoverpa) zea* (Kramer *et al.*, 1991) and *S. frugiperda* (Oeh *et al.*, 1999), but with the latter, only when the CA are first stimulated by Manse-AT. More recently, a synthetic Manse-AST was shown to inhibit JH II synthesis *in vitro* by CA from larval *L. oleracea* by approximately 70% (Audsley *et al.*, 2000). Manse-AST has also been demonstrated to modulate JH biosynthesis by the CA of insects of other orders, including *A. mellifera* (Rachinsky and Feldlaufer, 2000) and *P. regina* (Tu *et al.*, 2001). Thus, the PISCF ASTs do not appear to be limited to Lepidoptera, and may be the functional AST in other insect orders as well.

3.7.7.2.1. Effect of allatostatins on the juvenile hormone biosynthetic pathway Soon after the discovery of the ASTs, Pratt and his colleagues (Pratt *et al.*, 1989, 1991) showed that addition of exogenous farnesoate or mevalonate reverses the inhibition of JH biosynthesis *in vitro* by AST, suggesting that the action of this neuropeptide occurs before the synthesis of the isoprenoid and homoisoprenoid units that condense to form the JH backbone (Granger, 2003) (see Section 3.7.4.1). Tobe and his colleagues (Wang *et al.*, 1994) provided evidence that *D. punctata* allatostatin 4 (Dippu-AST 4) inhibits the activity of *O*-methyl transferase, the enzyme catalyzing the penultimate step in JH III biosynthesis in this species (see Section 3.7.4.1), but only at the moderately high concentration of 10^{-8} M. The authors concluded that this effect is probably secondary since Dippu-AST 4 inhibits JH synthesis at a much lower concentration and because a variety of precursors such as FA and (*E*,*E*)-farnesol were found in this and other studies to reverse the inhibition by Dippu-AST 4. Wang *et al.* (1994) concluded that Dippu-AST 4 is acting primarily on the earlier, rather than later, stages of JH biosynthesis. More recent work has confirmed that the most probable targets for the ASTs are the first committed steps in the synthesis of JH III, that is, either the transfer of citrate to the cytoplasm across the mitochondrial membrane or the cleavage of citrate to yield cytoplasmic acetyl CoA, a building-block for the isoprenoid and homoisoprenoid units (Sutherland and Feyereisen, 1996).

3.7.7.2.2. Locations of allatostatins in the insect AST-positive cerebral neurosecretory cells have been shown for all three families of ASTs by immunocytochemistry and *in situ* hybridization. Immunocytochemistry has demonstrated that these cells in *M. sexta*, *D. punctata*, and *G. bimaculatus* are usually located in the lateral protocerebrum, project to the CA on the contralateral side, and arborize in the CC and the CA (see Stay, 2000 for a summary). In some cases, medial neurosecretory cells are immunoreactive, as are some cells in the tritocerebrum, and these cells, together with some of the lateral cells, have been observed to arborize within the brain and thus can be considered interneurons (Stay *et al.*, 1992; Žitňan *et al.*, 1995). With the use of immunocytochemistry, ASTs have been demonstrated in other regions of the central nervous system including the peripheral nerves, the gut, the ovary, and the oviducts (Stay, 2000; Witek and Hoffmann, 2001; Garside *et al.*, 2002) (see Section 3.7.7.2.4). *In situ* hybridization has been used less frequently to locate ASTs within the insect, usually in combination with immunocytochemistry – for example, the identification of ASTs in hemocytes of *D. punctata* (Skinner *et al.*, 1997). The wide distribution of the ASTs indicated by these results reinforces their pleiotropic roles.

3.7.7.2.3. Pleiotropic roles of allatostatins It is clear from the enormous bulk of work on the ASTs that, as with the ATs, their actions are not limited to the modulation of the production of JH and that they frequently do not have an allatostatic role in the insect from which they are isolated. The functions of ASTs have been loosely grouped into six categories: (1) inhibition of JH biosynthesis; (2) modulation of muscle contraction in the heart and gut; (3) modulation of neural activity in the central nervous system; (4) inhibition of vitellogenin synthesis/release; (5) inhibition of the production of ecdysteroids by prothoracic glands and ovaries *in vitro*; and (6) modulation of digestive enzyme activity (Bellés *et al.*, 1999; Stay, 2000; Gade, 2002; Tobe and Stay, 2004). The same AST can exert several different tissue-specific effects at various developmental stages in different insect species.

The ubiquitous nature of these peptides in insects and in other invertebrates, plus the conservation of core sequences within them, speaks to their ancient origin. It has been suggested that their function as regulators of JH biosynthesis evolved before the rise of the insects (Tobe and Stay, 2004). The great profusion of ASTs, even within a single species, suggests flexibility and overlap in their functions, although it is clear that in some instances, a single AST or structurally similar group can have a narrowly defined function and only minimal activity in other roles. It has been noted that in insect species more recently evolved than cockroaches, there are fewer ASTs (13–14 in cockroaches compared to 5–9 in Diptera and Lepidoptera), and a concurrent loss of their role as inhibitors of JH biosynthesis (Tobe and Stay, 2004). Since a single AST can have different effects at different times, it is not possible to conclude that the loss of AST function is the direct result of the loss of their numbers, and studies of the molecular evolution of the ASTs are needed to resolve this question (Donly *et al.*, 1993a; Ding *et al.*, 1995; Bellés *et al.*, 1999).

3.7.7.2.4. Other types of allatostatins A partially identified factor from the brains of late last instars of *M. sexta* has been found to inhibit JH production irreversibly in a combination *in vivo/in vitro* assay (Bhaskaran *et al.*, 1990; Unni *et al.*, 1993). CA exposed *in vitro* to the brain factor are then implanted into penultimate instars to assay their activity. Because this factor appears to be responsible for stable inhibition of JH biosynthesis until after metamorphosis, it was given the name allatinhibin to distinguish it from ASTs, whose effect is fast and reversible.

There are also nonneural factors of unknown structure that can inhibit JH synthesis by the CA. The ovary appears to be a prime source for these factors, which is not surprising, given the fact that JH regulates reproduction. Early work by Stay and Rankin (Stay *et al.*, 1980; Rankin and Stay, 1985) indicated that the ovary of *D. punctata* has a role in the decrease of JH synthesis at the end of vitellogenesis. Allatostatic peptides have been extracted from prechorionogenic ovaries of *G. bimaculatus* (Hoffmann *et al.*, 1996) and *L. migratoria* (Ferenz and Aden, 1993). The partially purified *Locusta* ovarian factor is small (1–1.3 kDa) and inhibits JH synthesis *in vitro* in a dose-dependent manner. The ovarian extract from *G. bimaculatus* also inhibits JH synthesis by CA *in vitro* in a dose-dependent manner, with the potency of inhibition dependent on the stage of the animal from which the CA are taken. Immunoassays using separate polyclonal antisera to *Gryllus* ASTs A1 (FGLamide) and B1 (W-X_6-Wamide) have recently been employed to demonstrate AST-A-like and -B-like immunoreactivity in ovary extracts and partially purified HPLC fractions (Witek and Hoffmann, 2001). The immunoreactive fractions inhibit JH biosynthesis *in vitro*. The AST- and AST-B-epitopes are immunolocalized to the cortical cytoplasm of oocytes in ovaries of both larval and adult crickets; no neural structures within the oocytes are stained. In *D. punctata*, where the ovary has a clear role in the decrease of JH biosynthesis at the end of vitellogenesis, the ovarian factor responsible could be either a neuropeptide sequestered by the ovary or an ovarian peptide with an AST epitope. With the use of a competitive reverse transcriptase polymerase chain reaction assay (RT-PCR) to quantify AST expression, it has been demonstrated that both the lateral and common oviducts and ovaries express message for ASTs and that there are changes in the pattern of expression during the reproductive cycle (Garside *et al.*, 2002). Unlike the situation in *G. bimaculatus*, however, there are no immunoreactive cell bodies in the oviducts or ovary, but there is immunoreactivity in the terminal abdominal ganglion and ventral nerve 7, branches of which innervate the oviducts. Thus, the transcripts quantified by RT-PCR may be generated in the axonal compartment of the allatostatin-containing nerves innervating these structures.

3.7.7.2.5. Molecular biology of the allatostatins The first AST gene to be cloned was that from *D. punctata*, which encoded for a 41.5 kDa precursor polypeptide (GenBank Accession no. U00444) (Donly *et al.*, 1993a, 1993b). This polypeptide

contains the 13-member family of AST peptides that exist in this species, with appropriate processing sites for endoproteolytic cleavage and amidation. The ASTs are clustered in the precursor, and separating the clusters are three acidic spacers. These spacers are believed to stabilize the precursor, but they have also been proposed to code for a related peptide (Donly *et al.*, 1993a; Ding *et al.*, 1995). Southern blot analysis revealed that there is a single copy of the AST gene per haploid genome. The prepro-ASTs for all cockroach species examined are similar in size, the organization of the ASTs within them (13 or 14) is conserved, and the separation of groups of peptides by acidic domains is maintained (Ding *et al.*, 1995; Bellés *et al.*, 1999). The different AST sequences in the gene of one species were derived from duplication events. Thus, the AST-A family in cockroaches is a good example of a family of peptides with similar sequences derived from a single precursor encoded by a single gene.

The *Drosophila* Genome Project database was screened for sequences corresponding to various insect ASTs. The resulting alignments enabled the cloning of the cDNA for a prepro-*Drosophila* AST containing four putative A-type ASTs (GenBank Accession no. AF263923) (Lenz *et al.*, 2000). A similar approach was taken to identify a prepro-B-type AST that contains two B-type ASTs (GenBank Accession no. AF312379) (Williamson *et al.*, 2001a, 2001c), and a prepro-C-type AST that contains a single C-type AST with one amino acid residue difference from the *M. sexta* C-type AST. The sequence of the C-type AST is identical to that of previously identified dipteran ASTs (GenBank Accession no. AF316433) (Williamson *et al.*, 2001b).

The mRNA for the *G. bimaculatus* AST encodes a hormone precursor that contains at least 14 putative hormones (GenBank Accession no. AJ302036) (Meyering-Vos *et al.*, 2001). Five of these were previously identified in this species, while the remaining ones are known from other species. The deduced prepro-AST sequence is similar to that in cockroaches, but shorter than that of locusts. Regions of the acidic spacers that separate clusters of hormones are conserved between cockroaches and crickets, providing additional evidence that the spacers have a real function.

Identification of the prepro-AST message has been made in two other species. In the mosquito *A. aegypti* the message is about 3×10^3 bases in length and encodes five ASTs (GenBank Accession no. U66841) (Veenstra *et al.*, 1997). The prepro-AST genes are expressed in both abdominal ganglia and midgut. Three cDNAs of 1506 bases encode ASTs in the German cockroach, *Blattella germanica* (Yang *et al.*, 2000) and all have a nucleotide sequence with strong similarity (~80%) to that of other cockroach cDNAs for the prepro-AST.

3.7.7.2.6. Control of allatostatin titers The effect of ASTs on JH biosynthesis may occur in a paracrine fashion, i.e., local release within the CA from the axons of cerebral cells that produce these neuropeptides, or in a true endocrine fashion, via the hemolymph. By either means, receptors would mediate the effect of the neuropeptide (see Section 3.7.7.2.7) and it is ultimately the combination of titer and receptor that determines the effect of a neuropeptide. Recent work indicates that degradative mechanisms targeting ASTs can control the levels to which the CA are exposed. Initial work by Bendena *et al.* (1997) demonstrated that, like most neuropeptides, *Diploptera* ASTs are susceptible to rapid degradation. One of the ASTs of *B. germanica* has a half-life of 3–6 min in the intact insect, thus explaining why high doses are required for biological effects *in vivo* (Peralta *et al.*, 2000). A study of the degradation of Manse-AST by enzymes in foregut extracts of *L. oleracea* revealed a half-life of 5 min for the AST. Its degradation resulted in two products, both of which are cleaved at the C-terminal side of arginine residues (Audsley *et al.*, 2002).

Dippu-AST 7 and Dippu-AST 9 are subject to two primary catabolic cleavage steps: cleavage by a putative endopeptidase, yielding a C-terminal hexapeptide, and subsequent cleavage of this product by an aminopeptidase to yield a C-terminal pentapeptide. Neither of these hemolymph enzymes inactivates the ASTs, since the C-terminal pentapeptide contains the minimal sequence necessary for the inhibition of JH synthesis (Garside *et al.*, 1997a). However, there are membrane-bound enzymes in the brain, gut, and CA that cleave ASTs at the C-terminus, inactivating them in a two-step process (Garside *et al.*, 1997b). Pseudopeptide mimetic analogs of Dippu-ASTs have been synthesized that are resistant to degradation by hemolymph and tissue-bound peptidases, and these can significantly inhibit JH biosynthesis by CA when injected into mated *Diploptera* females, as measured by an *in vitro* assay using CA explanted from these females (Nachman *et al.*, 1999; Garside *et al.*, 2000). An outcome of this research is the creation of tools with which the actions of ASTs can be studied in detail, using degradation-resistant analogs.

3.7.7.2.7. Allatostatin receptors The type and number of receptors for ASTs in the CA control

the timing, duration, and potency these neuropeptides will have on JH biosynthesis. Work on AST receptors has also flourished, particularly with the discovery of such receptors in *D. melanogaster*, the model insect for genetic and molecular biological investigations. The first study to identify AST receptors was carried out by Cusson *et al.* (1991b), who used photoaffinity labeling to demonstrate two putative receptors in *Diploptera* CA. Subsequently, a single receptor for Dippu-AST 7 with a K_d of 7.2×10^{-10} M was found in the CA (Yu *et al.*, 1995), in addition to a single 37 kDa receptor in adult female brains that recognized both Dippu-AST 5 and Dippu-AST 7. Since a single K_d was obtained with AST 5 (9×10^{-10} M), but two with AST 7 (1.5×10^{-9} and 3.8×10^{-9} M), it was suggested that there were two receptor sites for Dippu-AST 7 in the brain. A subsequent structure–activity study using synthetic analogs of Dippu-AST 2 revealed two receptor types in *Diploptera* CA (Pratt *et al.*, 1997). The investigators proposed that the C-terminal portion of ASTs contains both the "message" determining its full effect, as well as "address information" determining its binding affinity for the receptor, and that divergent evolution of receptor types occurred with the evolution of multiple ASTs from a common ancestor. More recently, a radioligand-binding assay was used to identify a single class of binding sites for Dippu-AST 7 in midgut membranes with a K_d of 2.1×10^{-10} M (Bowser and Tobe, 2000). In competitive binding assays, Dippu-AST 7 and AST 2 exhibit a higher affinity for the midgut receptor than AST 5, 9, 10, or 11, while the other ASTs did not compete. Clearly, cloning and sequencing of these receptors is needed to sort out the ubiquitous occurrence and varied functions of the multiple ASTs.

Two laboratories have cloned G-protein coupled receptors (see **Chapter 5.5**) from *D. melanogaster* that are structurally related to mammalian somatostatin/galanin/opioid receptors (GenBank Accession no. AF163775: Birgul *et al.*, 1999; GenBank Accession no. AF253526: Lenz *et al.*, 2000), but which bind a peptide related to the AST family and thus appear to be AST receptors. Lenz *et al.* (2000) termed their receptor DAR-1. Screening the *Drosophila* genome database, these investigators found a second G-protein coupled receptor, termed DAR-2, with a 47% amino acid residue similarity with DAR-1 (Lenz *et al.*, 2001). Expression studies of DAR-2 in Chinese hamster ovary cells indicated it is the cognate receptor for four *Drosophila* A-type ASTs that bind to the receptor differentially. The DAR-2 gene is expressed in embryos, larvae, pupae, and adults, and is mainly expressed in the gut of third instars. Since the larval gut of *D. melanogaster* contains cells expressing the gene for the prepro-ASTs, the authors concluded that DAR-2 mediates AST-induced inhibition of gut motility. In a simultaneous study, both DAR-1 and DAR-2 were expressed in Chinese hamster ovary cells and tested for activation using synthetic *Drosophila* ASTs and selected *Diploptera* ASTs (Larsen *et al.*, 2001). Both types of ASTs activate DAR-1 and DAR-2, indicating ligand redundancy and cross-species activity.

An apparent A-type AST receptor has been cloned from the cockroach *P. americana*, with about 60% amino acid similarity in the transmembrane regions to the two identified *Drosophila* receptors. When functionally expressed in *Xenopus* oocytes, this receptor exhibits high affinity for cockroach ASTs (GenBank Accession no. AF336364) (Auerswald *et al.*, 2001). An A-type AST receptor has also been cloned from the silkworm *B. mori* (GenBank Accession no. AH011256) (Secher *et al.*, 2001). This receptor (BAR) shows 60% amino acid residue identity with DAR-1 and 48% with DAR-2. The genomic structure of BAR displays two introns that are coincident with, and have the same intron phasing as, two introns in DAR-1 and DAR-2, and the authors conclude that these three receptors are both structurally and genomically related. BAR mRNA is expressed mainly in the gut, and thus this receptor appears to be a gut peptide hormone receptor. Further studies should focus on identifying AST receptors in the CA to define the second messenger cascade that translates the AST signal in the gland, and to elucidate the evolutionary path by which this large class of peptides evolved to regulate JH biosynthesis.

3.7.7.2.8. Corpora allata sensitivity to allatostatins Humoral factors may play a role in regulating the sensitivity of the CA to ASTs. Unnithan and Feyereisen (1995) found that acquisition of sensitivity to AST by CA from adult mated *Diploptera* females occurs just before choriogenesis and is dependent on a humoral factor. The investigators could manipulate sensitivity by various experimental techniques, e.g., ovariectomy of vitellogenic females disrupts both JH biosynthesis and acquisition of sensitivity. This line of research deserves more investigation, since it could represent the upregulation of AST receptors by other circulating hormones.

3.7.7.3. Other Factors Regulating Juvenile Hormone Biosynthesis

3.7.7.3.1. Neurotransmitters It has long been thought that regulation of JH biosynthesis is exerted

via the axons of cerebral neurons that innervate the CA. Yet, it is clear from research on the ASTs that this control could occur via the hemolymph, with release from the CC, the neurohemal organ for cerebral neuropeptides, or other sites of AST-producing cells. Control of JH synthesis by neurotransmitters is also likely, since some of the nerve cells innervating the CA are ordinary neurons (Sedlak, 1985).

Octopamine, a primary insect neurotransmitter, was the first neurotransmitter to be identified in the CA of locusts and cockroaches (Evans, 1985). Studies of octopamine effects on JH biosynthesis *in vitro* have shown either stimulation or inhibition, depending on the species. Octopamine stimulates JH biosynthesis in adult *L. migratoria* (Lafon-Cazal and Baehr, 1988) and in larvae (Rachinsky, 1994) and adults (Kaatz *et al.*, 1994) of *A. mellifera*, but inhibits synthesis in adults of the cockroach *D. punctata* (Thompson *et al.*, 1990) and the cricket *G. bimaculatus* (Woodring and Hoffmann, 1994). In *D. punctata*, inhibition by octopamine is bimodal, with peaks occurring at 10^{-10} M and 10^{-4} M (Thompson *et al.*, 1990).

Recent work on adult females of *D. punctata* has provided the first indication that JH biosynthesis by the insect CA may be affected by the fast excitatory neurotransmitter L-glutamate, acting via ionotropic receptors to modulate the intracellular Ca^{2+} concentration (Pszczolkowski *et al.*, 1999) (see Section 3.7.7.4.3). Since high Mg^{2+} and kynurenate treatments inhibit the L-glutamate-induced stimulation of JH biosynthesis, it was proposed the NMDA (magnesium-sensitive) and non-NMDA (kynurenate-sensitive) receptors are present in the *Diploptera* CA and are involved in the regulation of JH synthesis. A subsequent study, monitoring rates of JH biosynthesis by *Diploptera* CA *in vitro* in response to L-glutamate agonists and antagonists, identified the receptors present as NMDA-, kainate-, and/or quisqualate-sensitive subtypes of ionotropic receptors. These receptors are coupled to calcium ion channels and are thus ionotropic channels rather than metabotropic channels, the latter being coupled to inositol (IP3)-stimulated Ca^{2+} release (Chiang *et al.*, 2002a).

The first suggestion that dopamine might be involved in the regulation of JH production was found in a study of dopamine in the brains of two cockroach species, where its levels fluctuated significantly in relation to events during the JH regulated ovarian cycle (Owen and Foster, 1988; Pastor *et al.*, 1991a). A similar situation has recently been described in the fire ant, *Solenopsis invicta* (Boulay *et al.*, 2001). Shortly after the study by Pastor *et al.* (1991a), it was reported that dopamine affects JH synthesis *in vitro* by the CA of adult female *B. germanica*, and that its effect was either stimulatory or inhibitory, depending on the stage of the ovarian cycle (Pastor *et al.*, 1991b). A screen of neurotransmitters in the larval *Manduca* CA using electrochemical detection-HPLC revealed that dopamine was the only biogenic amine in the gland (Granger *et al.*, 1996). Immunocytochemical analysis further corroborated these studies and demonstrated that dopamine is present in both the brain and CA (Granger, unpublished data). Dopamine affects JH synthesis by larval *Manduca* CA *in vitro* differentially, stimulating both JH synthesis and cAMP production by CA in very early fifth stadium glands but inhibiting production of both JH and cAMP after day 2 (Granger *et. al.*, 1996) (**Figure 9**).

Another line of evidence implicating dopamine in the regulation of larval development, possibly through an effect on the CA, derives from studies of the effects of parasitism of the armyworm *Pseudaletia separata* by the wasp *Cotesia kariyai*. Parasitism by this species elevates dopamine levels in the nerve cord and hemolymph, slows normal development, and delays pupation of the host (Noguchi *et al.*, 1995; Noguchi and Hayakawa, 1996). Dopamine levels are significantly elevated in the hemolymph of *M. sexta* larvae parasitized by the wasp *Cotesia congregata* (Hopkins *et al.*, 1998), although no developmental effects are related to the increase. Significantly higher levels of dopamine have also been found in the hemolymph and central nervous system of diapause-bound pupae of the armyworm *Mamestra brassicae* (Noguchi and Hayakawa, 1997) (see **Chapter 3.12**). The relationship of elevated dopamine levels and delayed pupation is unclear, although persistent levels of JH are known to delay pupation in Lepidoptera. It is tempting to think that elevated levels of dopamine are stimulating CA activity. However, the converse could equally be true: persistent JH synthesis delaying pupation could elevate dopamine levels. A similar situation occurs in the honeybee: JH has been implicated in the control of honeybee division of labor, where elevated levels of octopamine and serotonin in the antennal lobes of adult workers are associated with foraging behavior (Schulz and Robinson, 2001) (see **Chapter 3.13**). Treatment of honeybees with methoprene elevates octopamine levels, and these authors concluded that JH modulates octopamine levels in the honeybee brain, and that octopamine acts downstream of JH to influence behavior (Schulz *et al.*, 2002).

Neurotransmitter receptors exist in the CA, which is not surprising given that these glands

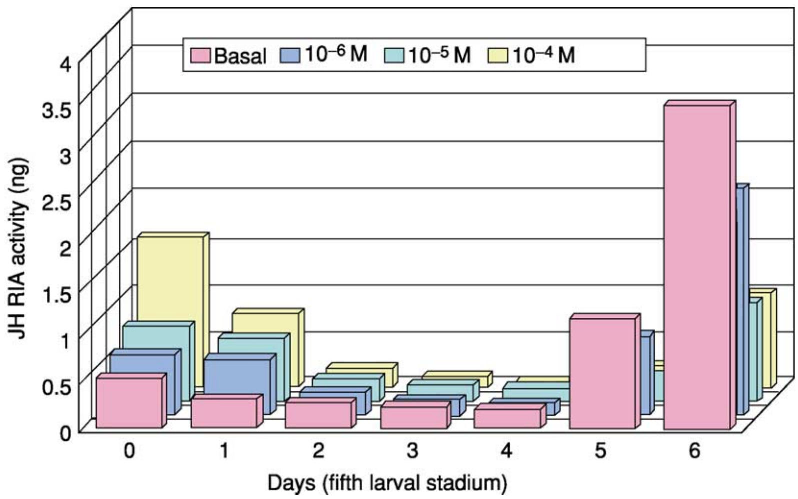


Figure 9 Effect of dopamine synthesis on JH biosynthesis in *vitro. Manduca sexta* CA from different days during the last larval stadium were incubated with increasing concentrations of dopamine. JH III/JH III acid synthesis was measured by radioimmunoassay (RIA) and expressed as ng of JH III radioimmunoassay equivalents. (Adapted from Granger, N.A., Sturgis, S.L., Ebersohl, R., Geng, C.X., Sparks, T.C., **1996**. Dopaminergic control of corpora allata activity in the larval tobacco hornworm, *Manduca sexta. Arch. Insect Biochem. Physiol. 32*, 449–466.)

are extensively innervated by the axons of cerebral neurons and neurosecretory cells. The receptors for biogenic amines belong to a large superfamily of G-protein coupled receptors, and the nucleotide sequences of the octopamine, tyramine, dopamine D1 and serotonin receptors are known for *D. melanogaster, A. mellifera, H. virescens, B. mori*, and *L. migratoria* (von Nickisch-Rosenegk *et al*., 1996; Blenau and Baumann, 2001). Binding of dopamine to D1 receptors increases adenylyl cyclase activity, while binding to D2 receptors either has no effect or inhibits adenylyl cyclase (Gingrich and Caron, 1993; Vernier *et al*., 1995; Huang *et al*., 2001). D2-like receptors have not yet been cloned in any insect, although pharmacological studies suggest they exist (Blenau and Baumann, 2001), and receptors for biogenic amines in the CA have not been pharmacologically characterized except for the dopamine receptors in the CA of *M. sexta* last instars (Granger *et al*., 2000). Because dopamine both stimulates and inhibits JH synthesis and adenylyl cylase activity, depending on developmental stage, these glands undoubtedly possess both D1-like and D2-like receptors (Granger *et al*., 1996). The CA D2-like receptor was found to have some pharmacological resemblance to vertebrate D2 receptors, but certain vertebrate D1 receptor agonists/antagonists were found to be equally effective as D2 receptor agonists/antagonists. By contrast, the CA D1-like receptor was found to be pharmacologically distinct from vertebrate D1 receptors (Granger *et al*., 2000). This fits with the overall picture of dopamine receptors that have been cloned from insects–they display pharmacological properties that set them apart from vertebrate receptors (Blenau and Baumann, 2001).

3.7.7.3.2. Interendocrine control by ecdysteroids If, according to the axiom of Carroll Williams (1976), JH is the handmaiden of ecdysone, then one might ask: Do ecdysteroids regulate JH biosynthesis? The evidence that ecdysteroids may regulate CA activity comes primarily from Granger, Bollenbacher, and their colleagues, who determined that regulation is interendocrine and occurs via the brain. The increase in the ecdysteroid titer, specifically 20-hydroxyecdysone (20E), that elicits pupal commitment in the last stadium of *M. sexta* and other Lepidoptera, also stimulates the synthesis of JH acids by *Manduca* CA. This effect, exerted only when the CA are incubated as a complex with the brain–CC, occurs in response to physiological concentrations of 20E, and results in the postcommitment increase in the hemolymph titers of JH acids that have been shown to be necessary for development to the pupa (Whisenton *et al*., 1985; Watson *et al*., 1986; Granger *et al*., 1987). A subsequent investigation of the kinetics of the 20E effect revealed that maximum stimulation was achieved in 1 h, supporting the idea of an indirect effect, probably via the brain, and suggesting the existence of a mediating factor (Whisenton *et al*., 1987). Physiological concentrations of 20E were also shown to stimulate JH synthesis in *Manduca* fourth stadium CA, taken before the normal rise in CA activity at the end of the stadium. This suggests that interendocrine control of JH biosynthesis during larval molting is also exerted by 20E (Whisenton *et al*., 1987), and that this effect

is specific for a biologically active ecdysteroid, since two biologically inactive ecdysteroids, 22-isoecdysone and 5-α-ecdysone, failed to stimulate the CA. The effective concentrations that stimulate the CA differ by about 200-fold, $\sim 1\,\mu g\,ml^{-1}$ during larval development and $0.02\,\mu g\,ml^{-1}$ during pupal commitment, in keeping with the hemolymph concentrations of 20E at those times (Bollenbacher *et al.*, 1981). Interestingly, both of these concentrations stimulate CA from last instars at the time of commitment, and there are no other maxima between these two concentrations (**Figure 10**) (Granger, unpublished data). Given the fact that Manse-AT does not affect *Manduca* larval CA, it would appear that 20E acts in lieu of an AT in larvae.

Incubation of brain–CC–CA complexes from pharate *Manduca* pupae with physiological concentrations of 20E resulted in a 40% decrease in the production of JH acids (Granger *et al.*, 1987). This decrease did not occur if the glands were incubated with, but separated from, the brain–CC complex. While the level of inhibition seen in these experiments is not sufficient to account for the inactivation of the CA by the end of the stadium, this mechanism could act in concert with Manse-AST, which does inhibit larval glands.

Granger *et al.* (1996) have demonstrated that the dopamine stimulation of JH synthesis by CA from day 0 last instars of *M. sexta* may be downregulated by 20E. Ecdysteroid receptors (see **Chapter 3.5**) have been detected in the nuclei of *Manduca* CA as early as day 3 of the last stadium (Bidmon *et al.*, 1992), suggesting the possibility that the negative effect of ecdysteroids on JH biosynthesis involves nuclear receptors and transcriptional control. In a study of the ecdysteroid receptors in *Manduca* CA, it was found that both components of the heterodimeric receptor complex – ecdysteroid receptor (ECR) and Ultraspiracle protein (USP) – are expressed (Gilbert *et al.*, 2000; Granger and Rybczcynski, unpublished data). The CA contain two or more USP isoforms that are probably the phosphorylation states of two primary translation products. Preliminary results show that the p49 USP isoform in the CA increases during the feeding period and after commitment to pupation in the last larval stadium (**Figure 11**). The larger USP isoforms show a maxima surrounding the time of the commitment peak in ecdysteroid titer and during the premolt ecdysteroid peak. This pattern directly contrasts with that of the ECRs which show few differences in relative abundance or isoform diversity in this stadium (Gilbert *et al.*, 2000). Culture *in vitro* of day 1 CA with ecdysteroids causes a decrease in the predominance of the larger USP isoforms and an increase in p49 (**Figure 11**). This response is also seen in prothoracic glands (Song and Gilbert, 1998). It may be that the changes in ecdysteroid titer are responsible for changes in USP isoform number and abundance during the last stadium and thus might modulate the number and/or kind of genes (for dopamine receptors, for example, which are affected by the ecdysteroid titer) activated by a given concentration of ecdysteroid receptor.

With regard to other possible molecular interactions of ecdysteroids and the JH biosynthetic pathway, the evidence is largely conjectural. As discussed above (see Section 3.7.4.2), a mammalian farnesoid receptor, FXR, is affected by JH, methoprene, and farnesol in a mammalian transactivation assay. Weinberger (1996) has demonstrated that bile acids, 3-α-hydroxysteroids, and oxysterols are endogenous FXR affectors. Is it possible that another class of steroids, the ecdysteroids, also affect FXR, and could this be a mechanism by which ecdysteroids affect JH biosynthesis?

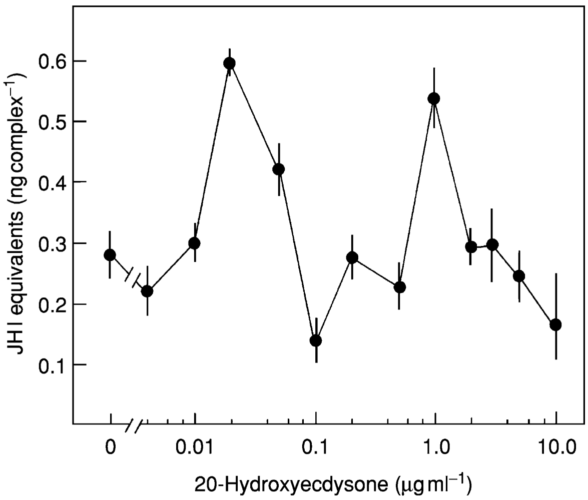


Figure 10 Effect of increasing concentrations of 20-hydroxyecdysone on JH I acid biosynthesis *in vitro* by CA incubated as a brain–CC–CA complex, from gate II, day 3 fifth instars of *M. sexta*. Each datum point represents the mean (±) SEM, where $n = 5$–6 incubations. Results are expressed as ng JH I RIA equivalents synthesized per complex in a 6 h incubation. (From Granger, unpublished data.)

3.7.7.3.3. Regulation of juvenile hormone biosynthesis by juvenile hormone

There is a paucity of direct evidence that JH regulates its own synthesis. An early study by Granger *et al.* (1986) indicated that neither exogenous JH I nor JH III can inhibit JH I acid biosynthesis by CA from last instars of *M. sexta*. A later, more detailed study of JH feedback in this system confirmed this result (Granger, unpublished data). Richard and Gilbert (1991), using *Drosophila* ring glands, demonstrated that

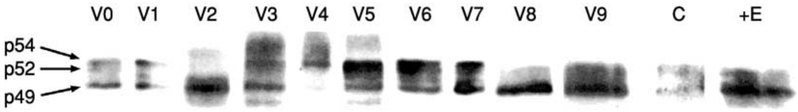


Figure 11 Ultraspiracle expression in the corpus allatum during the fifth larval stadium of *M. sexta*. The proportion of the p49 isoform increases as the stadium progresses, and this shift can be replicated *in vitro* by incubating CA with 20-hydroxyecdysone as described by Song and Gilbert (1998). CA were dissected on the indicated days and subjected to sodium dodeylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with an anti-USP antibody (Song and Gilbert, 1998). V0–V9, days of the fifth larval stadium; C, control, V1 CA; E, V1 CA incubated with 10 μM 20E for 18 h. Arrows indicate isoforms of USP. (Rybczynski, Moshitzky, and Granger, unpublished data.)

JH III and JHB_3, but not MF, reversibly inhibited biosynthesis of JH III, JHB_3, and MF. These results are in agreement with the generally accepted mechanism of negative feedback regulation of an endocrine gland by its product(s) (see **Chapter 3.8**).

As discussed above (see Section 3.7.4.2), mammalian orphan nuclear receptors now appear to be involved in the coordination of the lipid-based metabolic signaling cascade (Chawla *et al*., 2001). One of these nuclear receptors, RXR, is affected by farnesol and JH, and thus might be of significance in considering regulatory feedback loops of JH on JH biosynthesis. Molecular approaches have led to spectacular advances in our understanding of insect endocrinology, and the future holds great promise with respect to the elucidation of interendocrine control mechanisms.

3.7.7.4. Second Messengers

Receptors for neuropeptides and neurotransmitters are usually coupled to intracellular reaction cascades, regulating the level of second messengers that transduce these signals (see **Chapter 3.2**). The influence of a variety of second messenger systems on JH biosynthesis has been investigated both independently and as signal transducers, including cyclic AMP (cAMP), cyclic GMP (cGMP), phospholipid/protein kinase C, inositol phosphates, diacyl glycerol (DAG), and calcium (Ca^{2+}).

3.7.7.4.1. Cyclic AMP The role of cAMP in the regulation of JH biosynthesis has been studied most extensively in the cockroach *D. punctata*. In an initial study, compounds that increased intracellular cAMP were found to inhibit JH synthesis *in vitro* (Meller *et al*., 1985). These data suggest that the effect of the ASTs is mediated by cAMP. Of various compounds tested, 8-bromo-cAMP, which is resistant to hydrolysis by phosphodiesterases, and forskolin, a diterpene that activates adenylyl cyclases, were both potent inhibitors of JH synthesis *in vitro*. Furthermore, developmental changes in CA sensitivity to forskolin were found to parallel those to ASTs (Stay *et al*., 1991a), and exposure of CA to crude brain extracts resulted in both an increase in cAMP and a decrease in JH production (Aucoin *et al*., 1987). However, a subsequent study by Tobe (1990) showed that cAMP levels were low in virgin female CA, whose JH biosynthesis is inhibited. More cGMP than cAMP was found in these CA, and cGMP was lowest when JH biosynthesis was at a maximum in mated females. Levels of cAMP and cGMP were then measured in the CA of both virgin and mated females exposed to synthetic Dippu-ASTs 1–4 (Cusson *et al*., 1992). No significant changes in the levels of either cyclic nucleotide were found, strongly suggesting that neither of these cyclic nucleotides is involved in the signal transduction of the ASTs. Octopamine inhibition of JH biosynthesis in this species is bimodal (see Section 3.7.7.3.1), but only the inhibition in response to the higher concentration of octopamine (10^{-4} M) results in a rise in the levels of intracellular cAMP (Thompson *et al*., 1990). Thus, the caveat expressed by Meller *et al*. (1985), that the effects of pharmacological agents might only mimic the cellular response to inhibitory factors, appears to hold in *D. punctata*, where only a moderately high dose of octopamine exerts an inhibitory effect via an octopamine-sensitive cAMP.

By contrast, cAMP has a stimulatory effect on larval CA activity in *M. sexta* and is involved in transduction of the dopamine signal. Granger *et al*. (1994) found that relatively inactive CA, such as those of day 4 of the last stadium or from the *black larval* (*bl*) mutant, were sensitive to a variety of compounds *in vitro* that elicited, or mimicked the effects of elevated intracellular cAMP levels. It was also discovered that the stage-specific effects of dopamine on JH/JH acid biosynthesis by *M. sexta* larval CA *in vitro* were mediated by a calcium/calmodulin sensitive adenylyl cyclase (Granger *et al*., 1995b). Dopamine at concentrations of 10^{-6} and 10^{-7} M stimulated adenylyl cyclase activity in homogenates of glands taken from day 0 last instars, when this neurotransmitter stimulates JH biosynthesis, but inhibited enzyme activity in homogenates of day 6 glands, when dopamine inhibits CA activity (Granger *et al*., 1995b, 1996). A similar approach demonstrated that octopamine had no effect on

enzyme activity in gland homogenates over a wide range of concentrations. It was subsequently found that effects of dopamine receptor agonists and antagonists on JH biosynthesis *in vitro* were mirrored by changes in adenylyl cyclase activity (Granger *et al*., 2000). Thus, in this species, cAMP appears to be important in neurotransmitter effects on the CA.

Transduction of a neurotransmitter signal appears to be mediated by cAMP in the CA of adult male *L. migratoria* as well (Lafon-Cazal and Baehr, 1988). In this species, stimulation of JH biosynthesis by dopamine correlates with an increase in adenylyl cyclase activity. In honeybee larvae, both octopamine and serotonin elicit a dose-dependent increase in JH release from intraglandular stores of JH in the CA (Rachinsky, 1994). Incubation of these glands with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and a threshold concentration of octopamine (10^{-8} M) potentiated the octopamine effect on JH release, indicating that the action of octopamine is mediated by cAMP.

3.7.7.4.2. Inositol phosphates and diacylglycerol Other possible candidates for the transduction of neuropeptide signals in insects are IP3 and diacylglycerol (DAG), which are products of phosphatidylinositol-4,5 bisphosphate hydrolysis. The binding of the neuropeptide to a cell membrane receptor, which then interacts with membrane-bound G proteins, initiates this cascade. Increased IP3 then elicits the release of Ca^{2+} from intracellular storage sites, while DAG activates protein kinase C (PKC) (Berridge, 1993). The IP3 second messenger pathway exists in insects (Berridge, 1993). Work by Feyereisen and Farnsworth (1987) demonstrated that phorbol esters, which activate PKC, are potent inhibitors of JH biosynthesis in *D. punctata*, suggesting this pathway as central to the transduction of the AST signal. In a subsequent study, Rachinsky *et al*. (1994) found that treatment of *Diploptera* CA *in vitro* with inhibitors of DAG kinase elevates the concentration of DAG, resulting in a significant, dose-dependent inhibition of JH biosynthesis. A further enhancement of this effect was obtained when the CA were exposed *in vitro* to both DAG kinase inhibitors and ASTs, suggesting that the DAG pathway is indeed involved in the cellular response to the AST signal. Treatment of the CA with thapsigargin, a drug that mobilizes calcium stores without generation of inositol phosphates, significantly stimulates JH biosynthesis by, and also reverses the effect of ASTs on, highly active CA from mated females. It does not affect inactive CA from virgin females. The authors concluded that IP3, the other product of phosphoinositide hydrolysis, could modulate JH biosynthesis at specific development time points and thus could be responsible for the decreasing sensitivity of the CA of mated females to ASTs (Stay *et al*., 1991b). Furthermore, the stage-specific effects of IP3 on intracellular free Ca^{2+} in turn modulate the antagonistic action of DAG (Rachinsky and Tobe, 1996).

In adult male and female *M. sexta*, Manse-AT induces the production of inositol phosphates, including IP3, in CA *in vitro*, and two intracellular Ca^{2+} releasing agents were found to stimulate JH biosynthesis *in vitro*, similar to the effect of Manse-AT (Reagan *et al*., 1992). On this basis, these investigators concluded that Manse-AT activates the IP3 pathway in adult *Manduca* CA, increasing the intracellular concentration of free Ca^{2+} and thus stimulating JH biosynthesis. Staurosporine, a PKC inhibitor, had no effect on the inositol phosphates, but did stimulate the rate of JH biosynthesis *in vitro* and indicated a role for DAG as well. DAG involvement must be confirmed and the mechanism of staurosporine-induced stimulation must be established.

3.7.7.4.3. Calcium Calcium is unique as a second messenger in the CA, in that a transmembrane channel is involved, which may open either in response to ligand–receptor binding or to a change in membrane potential. The resulting increase in cytosolic free Ca^{2+} then interacts with the same cascades of biochemical events as cyclic nucleotides and inositol phosphates. While JH biosynthesis by *Diploptera* CA *in vitro* can be stimulated by the release of intracellular Ca^{2+} stores (Rachinsky *et al*., 1994; Rachinsky and Tobe, 1996), it appears that calcium stimulation of JH production occurs primarily by its influx from the extracellular environment. Calcium was first indicated as a player in the control of JH biosynthesis in *D. punctata* with the observation that elevated extracellular Ca^{2+} substantially reduces the effects of brain extract (ASTs) on JH biosynthesis (Aucoin *et al*., 1987). It was then shown that JH synthesis is nearly totally inhibited in Ca^{2+}-free medium but is stimulated in media with increasing concentrations of this ion (Kikukawa *et al*., 1987). Studies by Thompson and Tobe (1986) indicated that Ca^{2+} acts as a second messenger to stimulate JH biosynthesis by entering CA cells through voltage-gated Ca^{2+} channels. Recent work by Chiang and colleagues demonstrated that another type of Ca^{2+}-permeable membrane channel is involved in regulation of JH production by Ca^{2+}; the receptor-operated, ionotropic L-glutamate receptor (Pszczolkowski *et al*., 1999; Chiang *et al*., 2002b).

Elevated Ca^{2+} concentrations in incubation medium stimulate the synthesis of JH *in vitro* by the ring glands of *D. melanogaster* (Richard *et al.*, 1990) and the larval blowfly *Lucilia cuprina* (East *et al.*, 1997), the larval CA of *M. sexta* (Allen *et al.*, 1992a), and adult male CA of the cricket *G. bimaculatus* (Klein *et al.*, 1993). By contrast, JH biosynthesis by the CA of *L. migratoria* appears to be relatively independent of changes in extracellular Ca^{2+}, although calcium ionophores can elicit and increase synthesis, depending on the physiological status of the adult source (gregarious but not solitary) (Dale and Tobe, 1988a).

With *M. sexta* CA, Ca^{2+} levels may vary with the stage of development. Larval CA were found to require extracellular Ca^{2+} $\geq$0.1 mM for maximal JH biosynthesis *in vitro*, while JH acid synthesis by glands taken after pupal commitment continues in the absence of extracellular Ca^{2+} (Allen *et al.*, 1992a). Both calcium ionophores and caffeine, which initiate the release of Ca^{2+} from intracellular stores, stimulate JH acid synthesis by postcommitment CA, suggesting that intracellular Ca^{2+} may be the principal source of this ion after commitment. Calcium channel antagonists and calcium channel blockers decrease JH biosynthesis by both larval and postcommitment CA, indicating that Ca^{2+} channels exist in the CA cell membrane and that these channels may be both voltage-gated and receptor-operated.

Measurements of cytosolic free Ca^{2+} in isolated *Manduca* CA cells by use of the fluorescent Ca^{2+} indicator Fura-2 and digitized microscopy revealed that the highest levels of intracellular Ca^{2+} occur on days 0 and 6 of the last stadium, when *in vitro* synthesis of JH and JH acid is at the highest levels (Allen *et al.*, 1992a, 1992b) (**Figure 12**). Concentrations of free Ca^{2+} in *Manduca* hemolymph peak on days 0 and 7 of the last stadium (Allen *et al.*, 1992a); thus, there is an optimal concentration of Ca^{2+} in the hemolymph at the time when the CA require extracellular Ca^{2+} for maximal synthesis. This finding suggests that extracellular free Ca^{2+} could be responsible, at least in part, for the high activity of the gland at this time. Furthermore, free cytosolic Ca^{2+} levels decrease significantly by day 1, when the hemolymph titers in JH have dropped precipitously (Baker *et al.*, 1987). However, the concentrations of free Ca^{2+} in the hemolymph throughout the last stadium never drop below millimolar concentrations (Allen *et al.*, 1992a). Thus, it is clear that free Ca^{2+} is a significant player in the control of CA biosynthetic activity, but probably as a partner to another signal. It is interesting that steroid hormones are known to stimulate Ca^{2+} influx in certain biological systems (Blackmore *et al.*, 1990) and that free Ca^{2+} is highest in the CA cells at times when the hemolymph ecdysteroid titer in *M. sexta* is high (Bollenbacher, 1988). Thus, another possible mechanism for interendocrine control of JH synthesis by ecdysteroids (see Section 3.7.7.3.2) could involve the calcium ion.

A similar approach to measuring free Ca^{2+} in isolated cells of *Diploptera* CA was used to study the effects of thapsigargin and Dippu-AST (Rachinsky and Tobe, 1996). It was found that thapsigargin elevated intracellular Ca^{2+}, confirming the stimulatory effect of thapsigargin on JH biosynthesis via elevated Ca^{2+}; this effect could not be blocked by the later addition of Dippu-AST. The simultaneous addition of Dippu-AST with thapsigargin prevented the thapsigargin-induced increase in intracellular Ca^{2+}, although, as mentioned previously, the inhibition of JH biosynthesis by Dippu-AST is abolished by thapsigargin (Rachinsky *et al.*, 1994). These conflicting results are not yet resolved.

It has been known for many years that the calcium ion also can act indirectly as a second messenger, by binding to calmodulin (CaM) and eliciting a conformational modification of CaM (see **Chapter 3.2**). This enhances the affinity of the Ca^{2+}/CaM complex for target effectors, such as membrane-associated and cytosolic enzymes and transmembrane ion channels, including that for Ca^{2+} (Stoclet *et al.*, 1987). It is of interest to note that a Ca^{2+}/CaM-sensitive adenylyl cyclase has been identified in the CA of *Manduca* last instars (Granger *et al.*, 1995b).

3.7.7.4.4. Potassium Postassium also affects JH biosynthesis. Treatment of adult female *L. migratoria* CA *in vitro* with high levels of K^+ stimulates JH biosynthesis, either by stimulating the release of ASTs from nerve endings within the CA or by a direct effect on the CA cells (Dale and Tobe, 1988a). A K^+-elicited inhibition of JH synthesis was also reported for *D. punctata* (Rankin *et al.*, 1986), but in both cases, the effect of high concentrations of K^+ proved to be Ca^{2+}-dependent because it was abolished by Ca^{2+} channel blockers (Dale and Tobe, 1988b).

In summary, based on the evidence to date, it appears that the allatotropic and allatostatic neurotransmitters, which are in evolutionary terms the more primitive intercellular signaling molecules, operate via cyclic nucleotide second messengers. The neuropeptidergic signals that evolved later are transduced by phosphoinositides and DAG. Calcium plays a central role in the function of both types

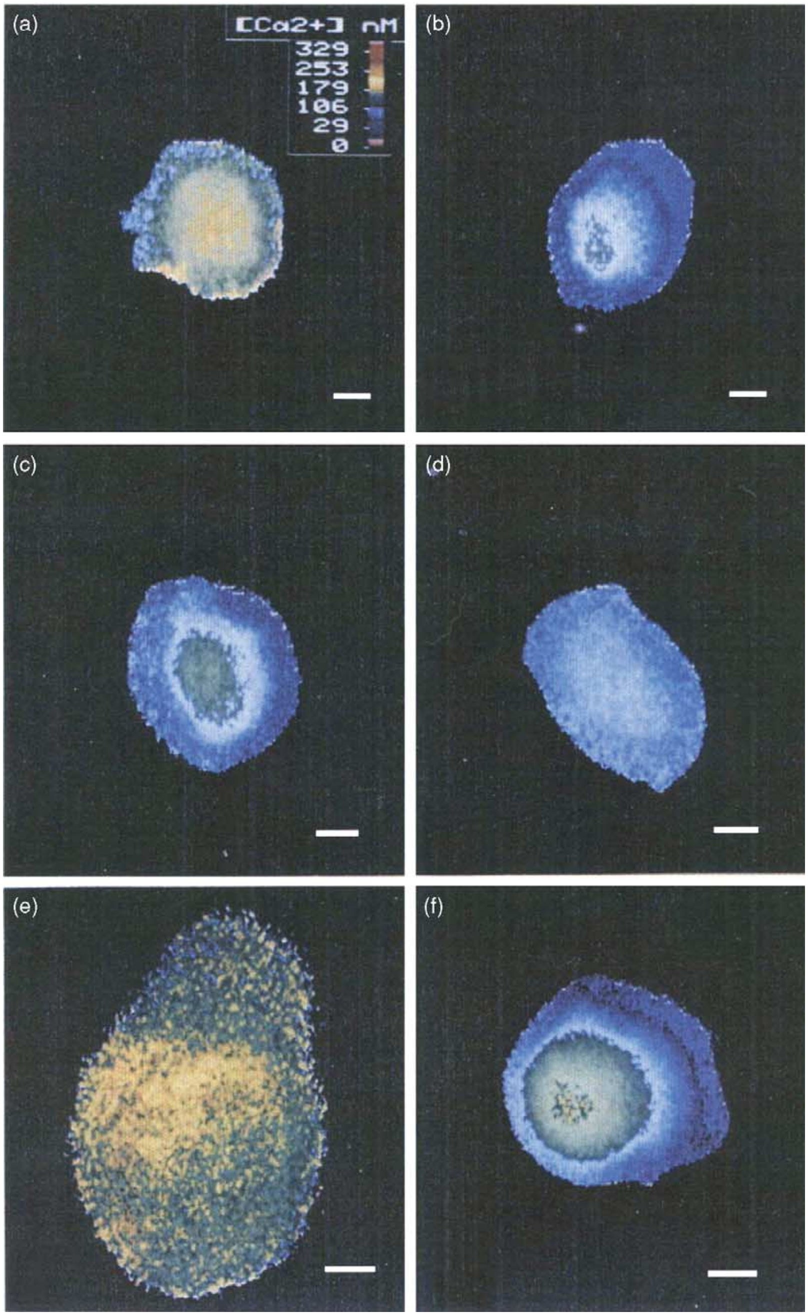


Figure 12 Measurements of cytosolic free Ca^{2+} in isolated *Manduca* CA cells. Pseudocolored ratio images of CA cells from fifth stadium larvae and early pupae. Cells loaded with Fura-2 and incubated in Grace's medium containing 0.1 mmol l^{-1} Ca^{2+}. (a) Day 0; (b) day 1; (c) day 2; (d) day 4; (e) day 6; (f) day 0 pupal period. The color spectrum in A corresponds to the concentration range (nmol l^{-1}) of Ca^{2+}. Scale bars represent 120 μm. (Reproduced with permission from Allen, C.U., Janzen, W.P., Granger, N.A., **1992a**. Manipulation of intracellular calcium affects *in vitro* juvenile hormone synthesis by larval corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol. 84*, 227–241.)

of second messengers, and may itself contribute to the upregulation of gland activity at certain critical times when JH biosynthesis levels are high.

3.7.7.5. Putting It All Together

Given the long list of factors that can affect the biosynthetic activity of the CA, and perhaps also its product(s) – something that has not yet been explored, attempts to create an overall mechanism of endocrine control is fraught with hazards. An excellent summary of the roles of calcium and phosphoinositides in second messenger signaling in the regulation of JH production was made by Rachinsky and Tobe (1996), using three different

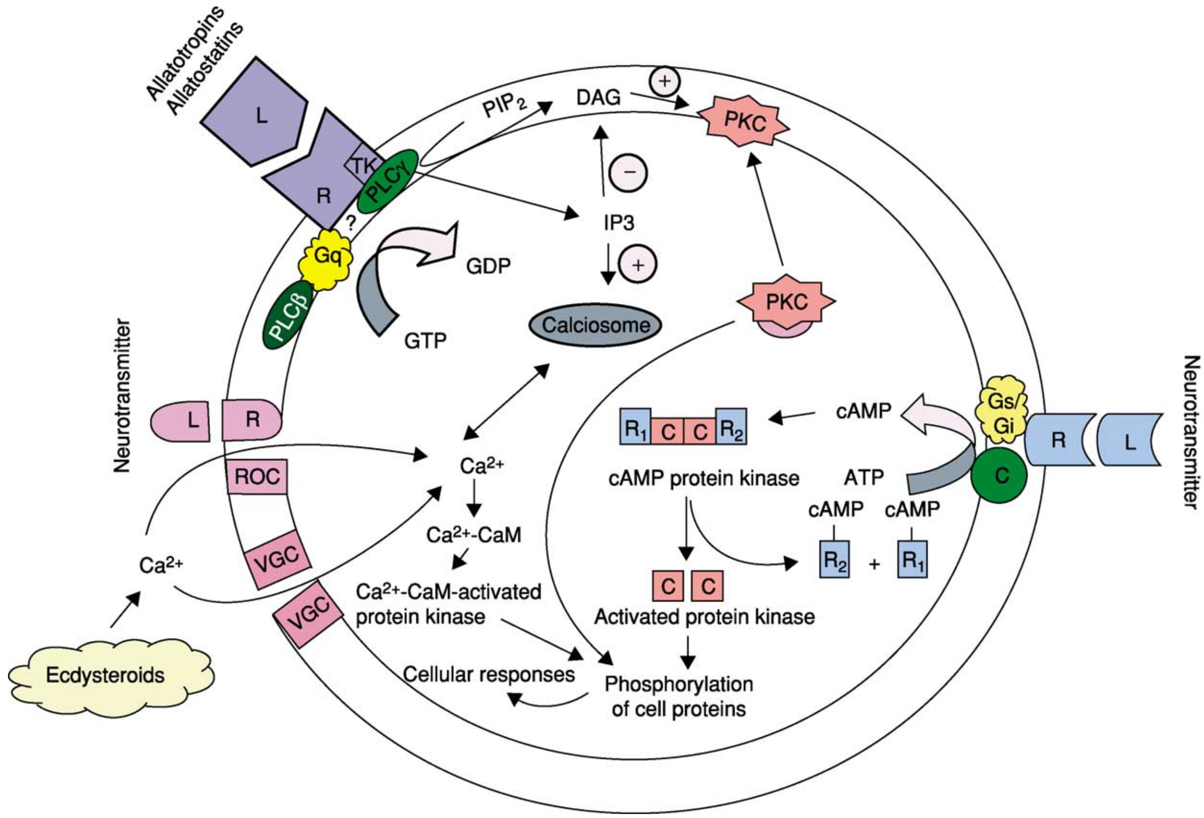


Figure 13 Hypothetical scheme for the regulation of JH biosynthesis by the CA. Factors thought to be involved include neurohormones, neurotransmitters, ecdysteroids, and second messengers. L, ligand; R, receptor; ROC, receptor-operated channel; VGC, voltage-gated channel; c, catalytic subunit; $R_{1,2}$, regulatory subunit; C, cyclase; G_s, stimulatory G protein; G_i, inhibitory G protein; G_q, G protein associated with phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; IP3, inositol triphosphate; PIP_2, phosphatidylinositol-4,5 bisphosphate; TK, tyrosine kinase; PLC, phospholipase C.

insects as model systems: *L. migratoria*, in which JH biosynthesis appears to be governed principally by allatotropic signals; *D. punctata*, where this role is filled by ASTs; and *M. sexta*, in which both types of signals occur, perhaps as the result of the complex nature of the products of the CA in this species and possibly coupled with its holometabolous development. They concluded that differences in signal transduction between species reflect, in part, differences in the signals themselves, and that these differences may be stage-specific.

With the complicated nature of this discussion, which excludes a consideration of signals other than neuropeptides, neurotransmitters, and selected hormones, we have chosen to create a scheme for a CA cell that is representative of all insects, responding to these various types of signals and containing all of the second messenger systems shown to exist thus far (Figure 13). Thus, this cell responds to ATs and ASTs, neurotransmitters, ecdysteroids, and Ca^{2+}, operating via either voltage-gated or receptor-operated channels. It contains adenylyl cyclase, phosphoinositide/DAG, and Ca^{2+} second messenger systems, involving PKC. The complexity of this scheme indicates how much work is yet to be done on the basic mechanisms regulating JH biosynthesis at the level of the gland.

3.7.8. Hemolymph Transport Proteins for the Juvenile Hormones

The physicochemical nature of JH presents the insect with a serious problem, that of dispersing a lipid hormone via an aqueous circulatory system. While the JH homologs are water-soluble at levels that far exceed the physiological titers normally encountered (Kramer *et al.*, 1976), their amphiphilic nature promotes surface binding (Law, 1980). When dispersed in an aqueous medium, JH displays a marked propensity for nonspecific binding to nearly any surface, making its distribution problematic. Early in the evolution of insects and crustaceans, hemolymph proteins arose that interacted with JH in a noncovalent fashion, yet allowed them to be dispersed in the aqueous environment of the hemolymph (Li and Borst, 1991; King *et al.*, 1995). These

transport proteins, with their hitch-hiking hormones buried in a hydrophobic pocket, provided early arthropods with a readily available source of hormone that could dissociate from the protein and interact with target tissues. These transport molecules have evolved into highly specific hormone carriers that have been extensively studied since their discovery in the 1970s (Trautmann, 1972; Whitmore and Gilbert, 1972).

The hemolymph of most insect species contains two classes of JH-binding macromolecules: (1) non-specific binding molecules characterized by a high equilibrium dissociation constant ($K_d = >10^{-6}$ M; low affinity), and (2) specific binding molecules exhibiting a low equilibrium dissociation constant ($K_d = <10^{-6}$ M; high affinity) (Goodman, 1990). JH binding proteins of both types have been reported in approximately 50 species (Goodman, 1983; Goodman *et al*., 1991; Trowell, 1992). The association of JH with low affinity proteins may be biologically irrelevant, given the surface active nature of the hormone and the high probability of its metabolism. Examples of low affinity interactions between JH and proteins can be observed when photoaffinity-labeled JH probes are incubated *in vitro* with hemolymph proteins (Prestwich, 1987; Prestwich *et al*., 1994). In contrast to the low affinity binders, the high affinity JH binding proteins have captured considerable experimental attention, and it is these hemolymph proteins that will be discussed.

3.7.8.1. High Affinity, High Molecular Weight Hemolymph Juvenile Hormone Binding Proteins

The circulatory system of a number of insect species contains high affinity, high molecular weight hormone binding proteins, here termed hemolymph juvenile hormone binding proteins (hJHBP) to distinguish them from intracellular hormone binding proteins. Their high molecular weight appellation stems from the fact that the native molecular weight of these binding proteins routinely exceeds 300 kDa. The high molecular weight hJHBPs can be divided into two subgroups, the lipophorins and the storage proteins or hexamerins. These proteins were originally considered low affinity binders but more recent evidence, using the appropriate homologs and enantiomers, indicates the affinity is much higher than first reported (Trowell, 1992; deKort and Granger, 1996).

3.7.8.1.1. Larval lipophorins as juvenile hormone transporters Lipophorins are multimeric hemolymph proteins that transport dietary lipids, pheromones, and cuticular lipids to their sites of utilization (Soulages and Wells, 1994). These multifaceted transport proteins also transport JH in a number of insect species belonging to an evolutionarily diverse array of insect orders, including Dictyoptera, Isoptera, Coleoptera, Diptera, and Hymenoptera. Trowell (1992) examined this area extensively and we here focus briefly on lipophorin–JH transport proteins studies that have been reported since that review.

Much of the recent work on JH–lipophorin interaction has centered on the adult insects, and as noted by King and Tobe (1993), the information derived from the adult stage may not be applicable to the larval stage. Indeed, the situation in adults becomes more complicated as both lipophorins and vitellogenins interact with JH during the adult stage (Engelmann and Mala, 2000). Nevertheless, if one assumes that immunological identity between lipophorins of the nymphal and adult stages indicates these proteins have a similar if not identical structure (King and Tobe, 1993), then various characteristics of the larval JH binding lipophorin (JHBL) can be extrapolated from those of the adult. The JHBL from the adult female *D. punctata* is a multimer of 680 kDa, composed of subunits of apolipophorin I (230 kDa) and apolipophorin II (80 kDa) (King and Tobe, 1988). Covalent labeling with a JH III photoaffinity probe indicates that the binding site resides on the larger subunit only. The protein appears to be specific for the naturally occurring enantiomer (10*R*)-JH III and displays a dissociation constant of 3 nM (King and Tobe, 1988). These binding characteristics, high affinity, and selectivity towards the correct enantiomer are in keeping with the JHBLs from other species (Trowell, 1992; deKort and Granger, 1996). Since lipophorin levels in the hemolymph are typically high, ranging from 6 to 16 mg ml^{-1} during the last nymphal stadium (10-40% of the total hemolymph protein), titers of the transporter are significant and yield a large excess of unoccupied binding sites (King and Tobe, 1993). These investigators also confirmed that JHBL offers protection from enzymatic degradation. As might be expected, titers of the JHBL follow total hemolymph protein levels, with a notable exception approximately one-third of the way though the stadium. At this time, levels of JHBL jump from 4 mg ml^{-1} to 12 mg ml^{-1} with no corresponding increase in total hemolymph protein. An even larger rise in JHBL is noted during a corresponding period in *L. maderae* (Engelmann and Mala, 2000). Unfortunately, the rapid increase in titer has yet to be linked to any biological event.

Trowell *et al.* (1994) characterized a JHBL in the hemolymph of larval *L. cuprina*. As is the case with other JHBLs, the blowfly protein is composed of two types of subunits, apolipohorin I (228 kDa) and apoliphorin II (70 kDa). Binding studies indicate that JH III is the preferred homolog followed by JH II > JH I > JH III acid > JH diol. Curiously, no displacement is observed with JHB_3 even though it is the predominant *in vitro* product of the blowfly CA (Trowell *et al.*, 1994). The dissociation constant for JH–JHBL is approximately 30 nM and, as with the other JHBLs studied to date, the fat body is the site of synthesis.

3.7.8.1.2. Larval hexamerins as juvenile hormone transporters A second type of high affinity, high molecular weight JH transport molecule has been discovered, having the characteristics of a hexamerin. The hexamerins, composed of six 70–80 kDa subunits, are widely distributed throughout the phylum Arthropoda and have been found in insects, crustaceans, and certain chelicerates (Burmester, 2002); however, they are not typically employed as hemolymph JH transporters. To date, only species in the order Orthoptera, including *L. migratoria* (Koopmanschap and deKort, 1988; Braun and Wyatt, 1996) and *Melanoplus sanguinipes* (Ismail and Gillott, 1995), are known to exploit hexamerins as JH transport proteins.

The most thoroughly studied of the JH-binding hexamerins (JHBHs) is that of *L. migratoria*. This protein, a 74.4 kDa protein, as deduced by cDNA analysis (Braun and Wyatt, 1996), contains 15% lipid (Koopmanschap and deKort, 1988). Binding analysis indicates the dissociation constant for (10*R*)-JH III–JHBH is 1–4 nM. According to Koopmanschap and deKort (1988), the hexamerin is present at relatively low concentrations that never exceed 2% of the total hemolymph protein, yet its hexameric structure allows a single native molecule to bind up to six molecules of hormone. As a result, this JHBH contains very large number of unoccupied hormone binding sites. The 4.3 kb hexamerin mRNA encodes a 668 amino acid protein and contains 2 kb of 3′ untranslated region (GenBank Accession no. U74469) (Braun and Wyatt, 1996). Functional assays to locate the JH binding site suggest that it resides in the N-terminus, since a truncated JHBH lacking this region does not bind the hormone. A comparison of its amino acid sequence with other members of the hexamerin superfamily indicates that the JHBH from *L. migratoria* represents a new form that is most closely aligned with the hemocyanins (Braun and Wyatt, 1996).

3.7.8.2. High Affinity, Low Molecular Weight Hemolymph Juvenile Hormone Binding Proteins

While the high affinity, low molecular weight hJHBPs are limited to only Lepidoptera and Diptera (review: Trowell, 1992), they have, nevertheless, been characterized extensively because relatively large quantities of larval hemolymph can be easily obtained from just a few insects. The low molecular weight hJHBPs are usually monomeric and range in molecular weight from 25 to 35 kDa. Although low molecular weight hJHBPs have been identified in a number of lepidopterans, only those in *B. mori*, *G. mellonella*, and *M. sexta* have been extensively studied.

3.7.8.2.1. Chemical and physical characteristics The lepidopteran hJHBPs are monomers composed of approximately 220–240 amino acid residues. In species where the primary sequence of hJHBP has been deduced, four to six cysteine residues have been reported. *Manduca sexta* hJHBP, which has six cysteine residues (Park and Goodman, 1993) and *G. mellonella* hJHBP, which has four cysteine residues (Kolodziejczyk *et al.*, 2001), contain two disulfide bridges.

The results of CNBr cleavage, together with extrapolated data from the study of *G. mellonella* hJHBP, suggest that the disulfide bridges in *M. sexta* hJHBP are probably formed from the Cys9 to Cys16 and Cys151 to Cys195 residues. That the cysteine residues at position 9 and 16 are important to JH binding was demonstrated with the *H. virescens* hJHBP. This hJHBP displays a nearly identical alignment of cysteine residues with those in the *M. sexta* hJHBP (Wojtasek and Prestwich, 1995). Using the *H. virescens* hJHBP construct, Wojtasek and Prestwich (1995) generated mutant hJHBPs in which an alanine was substituted for a cysteine residue at each of the cysteine positions. They discovered that Cys9 and Cys16 are critical for JH binding but that cysteine residues at other sites are much less important. Park and Goodman (1993) demonstrated that of the two remaining cysteine residues not involved in disulfide bond formation, one is inaccessible to alkylating agents, implying an internal location within the protein. The other cysteine residue is easily modified, suggesting a location on the surface of the protein. The role of disulfide bridges in overall structure and hormone binding has yet to be determined.

Initial studies indicated that the *M. sexta* hJHBP is not glycosylated (Goodman *et al.*, 1978a); however, more recent evidence suggests hJHBPs from both

G. mellonella and *M. sexta* are indeed glycosylated. Using matrix assisted laser desorption ionization time of flight (MALDI-TOF) measurements to determine the molecular mass of the native protein and to calculate the deduced amino acid residue mass, it has been determined that carbohydrates account for approximately 10% of the molecular mass of the *M. sexta* hJHBP (Goodman, unpublished data). Computer analysis of potential glycosylation sites indicates only one potential motif, that surrounding the Asn66 residue of the mature, secreted protein. The carbohydrate moiety can be enzymatically cleaved from the hJHBP using *N*-glycosidase F; however, hJHBP is resistant to cleavage by other glycosidases such as neuraminidase and endoglycosidase H (Goodman, unpublished data). Computer analysis of potential glycosylation motifs in the hJHBP from *G. mellonella* indicate multiple sites for glycosidation, including an *O*-GalNAc site (Thr116), *N*-glycosylation sites (Asn3, Asn93) and *O*-β *N*-acetylglucosamine (*O*-βGLcNAc) sites (Ser12, Thr116, Thr186) (Duk *et al*., 1996). Duk *et al*. (1996) confirmed this observation using lectin binding assays, gel staining, and mass spectrometry and suggested there are five major types of oligosaccharide chains, each containing two GlcNAc residues and two, three, or five mannose residues. The function of these carbohydrate moieties is unknown; however, glycosylation of hormone transport proteins in vertebrates is thought to limit proteolytic cleavage of the protein and thereby increase its half-life in the circulatory system (Westphal, 1986). While other potential posttranslational modifications can be predicted from computational programs, no experimental evidence for the modifications has been reported.

The lepidopteran hJHBPs interact with their ligands at concentrations in the low nanomolar range (Goodman, 1990; Trowell, 1992; deKort and Granger, 1996); however, the homologs are not all bound with the same affinity. It was originally thought that the least polar of the homologs was bound with the highest affinity (Goodman *et al*., 1978b), following the polarity rule which states that within a series of small nonpolar homologous hormones, the least polar of the group will be bound with the highest affinity (Westphal, 1986). Park *et al*. (1993), in an extensive study of *M. sexta* hJHBP binding kinetics, demonstrated that the polarity of the JH homologs was not as important to the binding equilibrium as first proposed. This study revealed that the dissociation constants for the binding protein hormone complex are approximately the same for JH I or II, but as previously observed, the interaction with JH III is considerably weaker. Although the polarity rule may not apply to the equilibrium constants, it is quite clear that the more polar the homolog, the shorter the half-life of the hormone–protein complex (Park *et al*., 1993). Thus, JH I, the least polar of the homologs tested, has the longest half-life (29 s), while JH III, the most polar homolog, has the shortest (13 s). These half-times of dissociation are consistent with vertebrate hormone transport proteins (Mendel, 1989).

The hJHBP of *M. sexta* preferentially binds the naturally occurring enantiomers of (10*R*)-JH III (Schooley *et al*., 1978b), (10*R*,11*S*)-JH I, and (10*R*, 11*S*)-JH II (Park *et al*., 1993). This enantiomeric specificity appears universal among the high affinity JH binding proteins, whether they are lipophorins or hexamerins (Trowell, 1992; deKort and Granger, 1996), indicating the binding site for the hormone is reasonably selective.

In addition to binding studies using the JH homologs and their respective enantiomers, studies have been conducted using geometrical isomers of the hormones, hormone metabolites, and biologically active analogs (Goodman *et al*., 1976; Peterson *et al*., 1977, 1982). The results indicate that highly active JH analogs and JH metabolites do not interact with hJHBP. Armed with this information, several investigators have speculated on the nature of the ligand binding domain and its constituent residues. Goodman *et al*. (1978b) suggested the binding site may be a hydrophobic cleft on the surface of the protein. The primary interactions between the hormone and the binding site would occur along the alkyl side chains of the hormone and the methyl ester moiety at C1, which together form a distinct hydrophobic surface. Perturbation of that surface, especially by the introduction of highly polar groups at C1, significantly reduces binding. However, the site is not sterically hindered since nonpolar additions such as ethyl and propyl groups at the C1 position reduce binding only moderately (Peterson *et al*., 1977). Hydrogen bonding, which involves electrophilic residues in the binding site, potentially anchors the hormone at the epoxide and C1 positions and may be more important for interaction with JH III than JH I. Support for this idea comes from the studies of Prestwich and Wawrzeńczyk (1985), who found that the interaction between the naturally occurring JH I enantiomer and hJHBP was only several fold greater than its antipode, suggesting that the binding site could not only accommodate the 10*R*,11*S* enantiomer but the 10*S*,11*R* enantiomer as well. In contrast, the significant binding energy derived from the (10*R*)-JH III epoxide–hJHBP interaction is probably disrupted in the (10*S*) configuration. Thus, the reduction in

the hydrophobic "face" of JH III places considerably more importance on the hydrogen bonding between the epoxide and methyl ester of JH III and the hJHBP.

Using photoaffinity labeling, Touhara and Prestwich (1992) attempted to map the binding site of the *M. sexta* hJHBP. On the basis of labeling patterns, they proposed that the region from Ala184 to Asn226, a region predicted to be a hydrophobic domain, is involved in the recognition of the lipophilic backbone of JH. Another region, Asp1 to Glu34, also appears to be involved, leading these investigators to propose a model in which the two regions are connected by disulfide bridges to form a two-sided hormone binding pocket. More recently, Tesch and Goodman (unpublished data) discovered that trypsin treatment of hormone-saturated (holo-hJHBP) and hormone-free hJHBP (apo-hJHBP) from *M. sexta* yields strikingly different results, raising questions about the Touhara and Prestwich model. When holo-hJHBP is treated with trypsin, the C-terminus of hJHBP from Lys180 onward is rapidly removed. The N-terminus (first 180 residues) of the molecule remains intact for approximately 1 h, despite the fact that it contains at least 10 well-documented tryptic cleavage sites. The trypsin-truncated hJHBP is still capable of binding JH, indicating that the C-terminus (last 46 residues) of the protein, despite its elevated level of hydrophobicity, is not directly involved in binding. Conversely, apo-hJHBP, when treated with trypsin, is completely destroyed within a few minutes. Similar findings were observed in *G. mellonella* (Wieczorek and Kochman, 1991). These results suggest that the binding site is not located in the C-terminus as proposed by the Touhara and Prestwich model but rather lies upstream, closer to the N-terminus. Rodriguez-Parkitna *et al.* (2002) hypothesize that the C-terminus may serve as a "barrel cover" that changes its position upon hormone binding. Thus, while the C-terminus may not be directly involved in hormone binding, it may have other important roles, such as target cell docking functions or hormone transfer to the cell.

Hormone-induced conformational changes have been observed in certain of the vertebrate serum steroid hormone binding proteins (Grishkovskaya *et al.*, 2002), and there is good evidence indicating that hJHBP undergoes a conformational change upon interaction with JH, but the degree of change appears to vary with the species. While the hormone appears to induce conformational changes that mask certain regions, no differences were detected between apo- and holo-hJHBP in sedimentation rate or electrophoretic migration studies in *M. sexta* (Tesch and Goodman, unpublished data). The studies of Touhara *et al.* (1993) on recombinant *M. sexta* hJHBP did not reveal differences in circular dichroism spectra of apo- and holo-hJHBP, indicating that the hormone does not induce changes in secondary structure. In contrast to these studies, Wieczorek and Kochman (1991) demonstrated a shift in the sedimentation coefficient between the apo-hJHBP (2.30S) and holo-hJHBP (2.71S) forms of the hJHBP from *G. mellonella*, as well as slight changes in electrophoretic mobility between these two forms. More recently, the Kochman group used circular dichroism analyses to confirm their earlier discovery that hormone binding leads to changes in the secondary structure of hJHBP in *G. mellonella* (Krzyzanowska *et al.*, 1998). It is unclear why these changes in secondary structure were not detected in the *M. sexta* hJHBP. It may well be that small but significant differences between the primary and secondary structures of the *M. sexta* and *G. mellonella* hJHBPs are responsible. These initial studies on ligand-induced conformational change are highly intriguing but unfortunately offer only a glimpse into the structure and dynamic conformational changes. Crystallographic studies, such as those currently under way with the *G. mellonella* hJHBP (Kolodziejczyk *et al.*, 2003) will provide the desired information on the nature of the JH binding site and ligand-induced conformational change in the protein.

3.7.8.2.2. Protein structures The high affinity, low molecular weigh hJHBPs that have been sequenced to date display a reasonable degree of identity in alignment (~25%), but as noted by Rodriguez-Parkitna *et al.* (2002), no one region shows a higher than average homology, thus precluding the identification of a putative JH-binding domain (**Figure 14**). These investigators examined other JH-associating proteins for potential sequence similarities and concluded that there was little similarity among the hJHBPs and ligand binding domains of potential nuclear receptors, insect transferrins, or the high affinity, high molecular weight JHBPs. From this analysis, it may be inferred that the composition and sequence(s) of amino acids residues making up the hydrophobic JH binding domain are not under extreme selective pressure, as long as the site is lined with hydrophobic residues that permit interaction with the aliphatic side chains of the hormone. However, the site must contain the hydrophilic residues to permit interaction with the epoxide moiety.

As more protein sequences enter the data bases, a picture is emerging that suggests the lepidopteran

```
M. sexta         MNGFKVFLFL LYAKCVLSDQ ---GALFEPC STQDIACLSR ATQQFLEKAC RGVPEYDIRP
B. mori          MASLKVFLVF VFARYVASDG ---DALLKPC KLGDMQCLSS ATEQFLEKTS KGIPQYDIWP
H. virescens     MAAYTSFLLL AFASCVLSEG ---GVFFNPC YKSDIKCLSN ATETFLEKTC NGYPNTEIKA
G. mellonella    MITLNIFLVL VIYQCALSDG SKLNLSTEPC DVSDIECISK ATQVFLDNTY QGIPEYNIKK
                 *   . **.:      . *:     .   :**    *: *:*  **: **:::  .* *: :*

M. sexta         IDPLIIPSLD VAAYDDIGLI FHFKNLNVTG LKNQKISDFR MDTTRKSVLL KTQADLNVVA
B. mori          IDPLVVTSLD VIAPSDAGIV IRFKNLNITG LKNQQISDFQ MDTKAKTVLL KTKADLHIVG
H. virescens     IDPLVIPELK VVVDESMGLV FDFKNINIVG LKNQQISDFK MDTDKKSVVL KTKAILNIVA
G. mellonella    LDPITIPSLE KSIEKIN-LN VRYNNLKVTG FKNQKISHFT LVRDTKAVNF KTKVNFTAEG
                 :**: :..*.     .   :  . ::*:::.* :***:**.*  :    *:* : **:. :   .

M. sexta         DVVIELSKQS KSFAGVMNIQ ASIIGGAKYS YDLQDDSKGV KHFEVGQETI SCESIGEPAV
B. mori          DIVIELTEQS KSFTGLYTAD TNVIGAVRYG YNLKNDDDGV QHFEVQPETF TCESIGEPKV
H. virescens     DLKIEFTKQN KVFNGPYIAK ATALGSSQYG YSFTKKDD-K EYFVVGSEEN ACEIIGEPDV
G. mellonella    KLVIELPKSS KTYTGEVTIE ASAEGGAAYS YSVKTDDKGV EHYEAGPETV SCEIFGEPTL
                 .: **:.:.. * : *    . :.  *.  *. *..  ...   ::: .  *   :** :*** :

M. sexta         NLNPELADAL LKDPDTTHYR KDYEAHRVSI RQRSLCKIVE LCYVDVVHNI RAVAKILPST
B. mori          TLSSDLSSAL EKDSGNNSLE PDMEPLKT-L RQAAICKIAE ACYISVVHNI RASAKILPAS
H. virescens     EIGEELQKAL LNDADAKAMK PDYEANKVAL RKKTLCHIVE AAYVTVIHNI RAVAKLFPKE
G. mellonella    SVSSTLEDAL KLDSDFKKIF TGYGKQLTEG RKQTACRIVE TVYAVSVHNI RAAARILPKS
                  :.  * .**   *.. .     .     .   *: : *:*.*   *   :*** ** *:::*

M. sexa          AFFTDVN
B. mori          SFFENLN
H. virescens     AFFLDI-
G. mellonella    AYFKNV-
                 ::* ::
```

Figure 14 Multiple alignment of the sequenced hemolymph JH binding proteins (hJHBPs) from Lepidoptera. Amino acid sequences of *M. sexta* (Madison wild-type) hJHBP (GenBank Accession no. AAF45309), *B. mori* hJHBP 2 (AAF19268), *H. virescens* hJHBP (AAA68242), and *G. mellonella* hJHBP (AAN06604). Alignment was performed using the CLUSTAL W algorithm. Letters in bold represent residues in the signal peptide. Symbols beneath the columns of amino acid residues represent the degree of conservation in each column: (*) indicates residues completely conserved; (:) indicates residues with only very conservative substitutions; (.) indicates residues with mostly conservative substitutions.

hJHBPs belong to a superfamily of proteins, the calycins (Rodriguez-Parkitna *et al.*, 2002), which include fatty acid binding proteins, avidins, lipocalins (see **Chapter 4.8**), and metalloprotease inhibitors (Flower, 1996, 2000). Proteins in this family are typically low molecular weight, secreted proteins that bind small, hydrophobic molecules. Members of the calycin protein family share a β-barrel structure composed of 8 or 10 β-sheets that are flanked by α-helices in the N- and C-termini. While sequence similarity between the hJHBPs and other members of the superfamily is low, modeling of the *G. mellonella* and *M. sexta* hJHBP secondary structure indicates considerable similarity to insecticyanin and human plasma retinol binding protein, both well-studied lipocalins (Rodriguez-Parkitna *et al.*, 2002). Given the structural information gleaned to date, it will not be surprising if in the near future, the lepidopteran hJHBPs are firmly placed in the lipocalin subfamily of calycins.

3.7.8.2.3. Genomic structures At the present time, the genomic structure of only one *hJHBP*, that from *M. sexta* (Madison wild-type strain), has been examined (Orth *et al.*, 2003a). The *hJHBP* locus (GenBank Accession no. AF226857) consists of five exons spanning 6.7 kb and is flanked by typical eukaryotic and insect-specific transcriptional control elements. The first exon encodes the 5′ untranslated region, a signal peptide plus the first two amino acids of the open reading frame, while the other exons encode the mature, secreted protein and the 3′ untranslated region. The proximal promoter region contains a A/T-rich region similar in position, but slightly longer than that of the JH esterase gene from *T. ni* (Jones *et al.*, 1998); however, both of these genes lack the canonical TATAA motif normally associated with the proximal promoter region. Computational analysis indicates numerous potential transcription factor binding sites in the 5′-flanking region of the gene. Most intriguing are

two closely spaced ECR response elements (see **Chapter 3.5**) approximately 5 kb upstream from the start site. The JHs are presumed to act on the ecdysteroid signaling pathway (Riddiford, 1994), regulating gene expression by modulating the transcriptional activity of the ecdysteroid receptor and its heterodimeric partner, Ultraspiracle (Jones and Sharp, 1997) (see Section 3.7.12.2). Considering that both the protein concentration (Hidayat and Goodman, 1994) and the *hJHBP* gene expression (Orth *et al*., 1999) undergo a significant decline during a larval-to-larval molting period, it is reasonable to speculate that ecdysteroids, acting via these ecdysteroid response elements, may be involved in the regulation of *hJHBP* expression and may indirectly modulate JH titers.

Recent studies reveal that strain-related polymorphisms in the *hJHBP* nucleotide sequence, outside the open reading frame, lead to significant differences in the titers of this protein. A comparison of hJHBP levels in two different strains of *M. sexta* revealed that one strain, the Seattle wild-type (*Swt*), has levels that are nearly 60% lower than those of the Madison wild-type strain (*Mwt*) (Young *et al*., 2003). The *Swt* strain exhibits an *hJHBP* allele, *Snb*, that differs significantly from the previously described *Md* allele found in the *Mwt* strain (Orth *et al*., 2003a) by the presence of a novel insertion in intron 2. The relatively small size of the insert (<500 bp), its position between direct repeats, its integration into an A/T rich region, and its lack of an open reading frame all suggest that the element is similar to a small, integrated nuclear element (Adams *et al*., 1986; Robertson and Lampe, 1995; Jurka and Klonowski, 1996). This element, termed Ms1, is abundant in the genome of *Mwt* (Young *et al*., 2003); however, the *Md* gene locus of the strain does not contain Ms1. While lepidopteran insertion elements are not particularly novel, the effect of Ms1 on *Swt* expression of *hJHBP* is significant. A potential mechanism by which *Snb* may depress hJHBP titers can be deduced from the sequence of Ms1. Zhou and Liu (2001) report that sequences within the *B. mori* repetitive insertion element Bm1, termed matrix-associating regions (MARs), interact differently with the nonchromatin scaffolding than do insertion elements lacking MARs. The insertion of MARs into a gene or locus may have significant effects on gene expression by hindering DNA unwinding during transcription (Walter *et al*., 1998; Carey and Smale, 2000); thus, expression of hJHBP by the *Swt* strain may be downregulated by this unique insertion element. From the physiological standpoint, the reduced levels of hJHBP in individuals expressing the *Snb* gene locus appear to have negligible effects on JH titers at hour 50 of the fourth stadium, the only time point examined in this study. Due to the high levels of hJHBP in wild-type animals (Hidayat and Goodman, 1994) and the high affinity of hJHBP for JH (Park *et al*., 1993), it may well be that the insect can withstand a loss of as much as 60% of the circulating hJHBP and still maintain levels of the hormone sufficient to prevent metamorphosis. The discovery of Ms1 serves as a warning that laboratory strains of the same species, held in reproductive isolation for long periods of time, are rapidly diverging from their common ancestor. The belief that all wild-type strains of the same species will respond to physiological challenges in an identical fashion may be a naive assumption.

3.7.8.2.4. Regulation The discovery of the high affinity JHBPs naturally leads to the question of whether these proteins have a direct influence on JH titers. In most insects, the exceedingly high ratio of hJHBP to JH makes it unlikely that even significant shifts in hJHBP levels would play a direct role in hormone regulation (Hidayat and Goodman, 1994). As noted above, a 60% reduction in hJHBP titers in a strain of *M. sexta* does not markedly affect JH titer. Nevertheless, the hJHBP titers fluctuate significantly during development (Goodman, 1990; Hidayat and Goodman, 1994; Rodriguez-Parkitna *et al*., 2002), and these changes do not mirror the changes in total hemolymph protein levels. Assessment of bound and unbound hormone levels indicates that virtually all of the hormone is bound at any given time and that the level of holo-hJHBP is a tiny fraction of apo-hJHBP. Why should the fat body, the site of synthesis of hJHBP (Nowock *et al*., 1975), be engaged in excessive synthesis of this protein, and why should its levels be so precisely controlled? The presence of excess hJHBP remains a mystery, but one intriguing hypothesis suggests that the protein acts as a high affinity scavenger to sequester the hormone in the hemolymph where it can be metabolized by hemolymph enzymes (Touhara *et al*., 1996). This scavenger hypothesis has also been proposed for the role of hJHBP in the embryo, where maternal JH may need to be compartmentalized to prevent interference with embryonic development (Orth *et al*., 2003b). While this hypothesis is provocative, it assumes that cellular degradation of the hormone is minimal, and as noted below (see Section 3.7.9.1), this may not be the case.

Although it is unclear why the levels of hJHBP need to be controlled so precisely during development, a picture of the elements controlling the

expression of its gene is beginning to emerge. As previously noted, the presence of ecdysteroid response elements in the 5′ flanking region of *hJHBP* suggests ecdysteroids may be involved in the regulation of *hJHBP* expression. Conversely, there is reasonably good evidence that JH itself has a role in regulating the expression of this gene. It has been observed that an inverse relationship exists between JH and hJHBP titers in *M. sexta* (Hidayat and Goodman, 1994). Hormone titers at the beginning of the penultimate stadium are high, but hJHBP titers are low. When JH titers drop during the late intermolt period, levels of hJHBP rise (Hidayat and Goodman, 1994). Topical application of JH during the late intermolt period leads to a striking fivefold increase in hJHBP mRNA, beginning approximately 2 h after hormone application and persisting for approximately 18 h (Orth *et al*., 1999). The response is both dose-dependent and saturable, with higher levels of JH (>1 ng) inducing a downregulation of hJHBP mRNA abundance. Curiously, hormone application at other times during the stadium does not elicit this response, which has lead to the suggestion that JH-induced transcription of *hJHBP* is already maximally stimulated at these times and further elevation of hormone levels by topical application has a dampening response. Thus, the control of hJHBP levels appears to be tied to fluctuating JH titers during the intermolt period; elevated titers of the hormone downregulate hJHBP mRNA and protein, while reduced JH titers elevate both message and protein. During the molting period, the elevated levels of ecdysteroids required for molting reduce expression of the *hJHBP* gene. If the sole function of hJHBP is hormone delivery, then the developmental pattern and excess titer remain a mystery, but as with the vertebrate serum hormone binding proteins, the hJHBPs may be more than transport macromolecules.

3.7.8.3. Functions

Facilitating the transport and dispersal of JH to distant target sites is the most obvious function of the hJHBPs, yet other equally important roles for the binding protein have been hypothesized or experimentally determined (Westphal, 1986; Goodman, 1990). These functions include: (1) reducing the levels of promiscuous binding to nontarget tissues; (2) reducing enzymatic degradation of the hormone (Hammock *et al*., 1975); (3) acting as a scavenger to enhance JH metabolism in larval hemolymph (Touhara *et al*., 1996) or the developing embryo (Orth *et al*., 2003b); (4) providing a peripheral reservoir of hormone that is readily available at the target tissue; (5) enhancing JH synthesis in the CA by acting as a sink; and (6) aiding hormone movement from the hemolymph into the target cell. The first four functions are either well established or have at least gained a level of scientific credibility.

Preliminary evidence now suggests that hJHBP may promote the synthesis of JH by dispersing the hormone from the immediate vicinity of the CA. Using the RCA to assess the effect of several JH-interacting proteins (bovine serum albumin, JH-directed antiserum, and *M. sexta* hJHBP) on JH biosynthesis *in vitro*, it was observed that only hJHBP significantly increased the incorporation of radiolabeled tracer into JH (Granger and Goodman, unpublished data). *Manduca sexta* CA synthesized ~60% more JH when incubated in medium containing physiological levels of hJHBP. While the results are preliminary, they do suggest that the binding proteins aid the transit of JH from the CA and thus could prevent hormone from accumulating nonspecifically in the vicinity of the glands. The binding proteins, in essence, create a longer feedback loop and thus block short-circuiting of JH biosynthesis.

The most intriguing unexplored aspect of the hJHBP functions is that of its action at the surface of a target cell. A considerable body of evidence has convinced most vertebrate endocrinologists that hormone binding proteins release their ligands into the circulatory system, thus allowing them to enter the cell in the unbound state (Westphal, 1986; Mendel, 1989; Grasberger *et al*., 2002). For some hormones, this is a reasonable conclusion; however, several protein-bound hormones and vitamins have specific cell surface receptors for their respective transport proteins (review: Kahn *et al*., 2002). Retinol binding protein (RBP), for example, binds to a plasma membrane receptor that participates in transfer of the ligand from the serum transport protein to the cytoplasmic retinol binding protein (Sundaram *et al*., 1998, 2002). As levels of apo-RBP far exceed those of the holo-RBP, a conformational change in the protein must occur so that cell surface receptors are not interacting with ligand-free RBP (Chau *et al*., 1999).

Even more striking are the roles of sex hormone binding globulin (SHBG), a vertebrate serum protein that binds and transports the sex steroids. As is the case for RBP, target tissues requiring the sex steroids display a plasma membrane receptor for SHBG. Unlike the RBP receptor, the SHBG receptor binds only the unloaded form of the transport protein; the hormone-loaded SHBG complex does not bind (Kahn *et al*., 2002). Sex steroids then bind to the receptor-bound SHBG on the cell surface which, in turn, activates adenylyl cyclase. The role of the

activated second messenger cascade remains unknown. The extracellular transport protein is more than just a vehicle for hormone dispersal; indeed, it may need to be anchored at the cell surface to facilitate uptake. Moreover, the receptor–protein complex may have its own intrinsic signaling ability. Whether this complex cascade of hormone transfer, cell surface receptors, and activation of second messengers occurs in insects remains to be investigated.

3.7.9. Catabolism of the Juvenile Hormones

The maintenance of effective hormone titers at the target site is a delicate balancing act among various processes: synthesis, delivery, metabolism, and cellular uptake. Endocrine signals, by their very nature, must be transitory to effect their exquisite control of the target response. Soon after the chemical structures of JHs were elucidated in the late 1960s, a search for hormone-inactivating enzymes began that has continued unabated to the present, with better than 350 articles describing some aspect of JH metabolism. This prodigious number of publications reflects the agricultural interest in targeting catabolism as a means of insect pest control, since it is assumed that dramatically increasing or decreasing catabolism of JH may lead to developmental derailment (Bonning and Hammock, 1996). While this novel means of pest control has yet to be commercially exploited, the biological information gleaned from studies on JH metabolism is of considerable importance in understanding hormone titer regulation. For earlier reviews of JH catabolism, the reader is referred to Hammock (1985), Roe and Venkatesh (1990), deKort and Granger (1996), and Gilbert *et al*. (2000).

The initial step in JH catabolism may occur in the hemolymph or in target cells, and results in different products; however, the end point is the same: biological inactivation of the hormone. Hemolymph inactivation of JH is the result of enzymatic activity of several different esterases capable of hydrolyzing the hormone at the C1 position to form the metabolite, JH acid (Hammock, 1985; Roe and Venkatesh, 1990) (**Figure 2**). Although cellular inactivation of JH is primarily the result of epoxide hydrolases that hydrate the epoxide at the C10, C11 position to form the JH diol (**Figure 2**), cells also possess esterase activity that yields JH acid (Lassiter *et al*., 1995; Roe and Venkatesh, 1990). Our examination of JH catabolism begins with the hemolymph where esterases represent the primary mechanism of hormone inactivation.

3.7.9.1. Juvenile Hormone Esterases

The hemolymph of a number of species contains esterases that are capable of hydrolyzing the ester at the C1 position to form JH acid. While the early literature suggested that there are two classes of esterases responsible for JH hydrolysis, JH-specific esterase (JHE) and nonspecific or general esterases (Sanburg *et al*., 1975), recent work has been less clear about the role of the nonspecific enzyme(s) (Gilbert *et al*., 2000). General esterases were originally defined as those enzymes that could metabolize both a general substrate, α-naphthyl acetate, and JH (Sanburg *et al*., 1975) and were inhibited by the inhibitor *O*,*O*-diisopropyl phosphorofluoridate (DFP). More recent evidence suggests that the general esterases are less important in JH metabolism than first thought (Roe and Venkatesh, 1990; Gilbert *et al*., 2000).

Because conversion to JH acid can be carried out by a number of enzymes, Hammock (1985) proposed a working definition for a JHE. From a biochemical standpoint, such an enzyme should have a low apparent K_m for JH and it should therefore hydrolyze JH with a high k_{cat}:K_m ratio (see Section 3.7.9.1.2). Furthermore, the enzyme must be able to hydrolyze JH in the presence or absence of a JH binding protein. From a biological standpoint, the JHE must have activity that correlates with a decline in JH titer. The premise that these enzymes are vital to hormone titer regulation has thus received considerable attention.

3.7.9.1.1. Physical properties JH-specific esterases (EC 3.1.1.1) are members of the carboxylesterase family and have been studied in at least six orders of insects, including Thysanura, Orthoptera, Hemiptera, Coleoptera, Diptera, and Hymenoptera (Hammock, 1985); as molecular cloning becomes more widely used, more insect orders will undoubtedly be added to this list. To date, the best characterized of the JHEs are those from the hemolymph of Lepidoptera.

The JHEs of Lepidoptera appear to be composed of a single polypeptide chain with a relative molecular mass of approximately 63–67 kDa (Roe and Venkatesh, 1990; Hinton and Hammock, 2003). The hemolymph of the coleopteran *L. decemlineata*, contains a JHE with a native Mr of 120 kDa; it appears to be composed of two polypeptide chains of approximately 57 kDa each (Vermunt *et al*., 1997a). A similar situation exists in the orthopteran *Gryllus assimilis*, where the native JHEs displays a Mr of 98 kDa and is composed of two identical subunits of 52 kDa (Zera *et al*., 2002). The

isoelectric points of all the JHEs studied are acidic, falling into the range of 5.2 to 6.1 (Abdel-Aal and Hammock, 1986; Hinton and Hammock, 2003). Isoelectric focusing of apparently pure JHE from several species yields two to four distinct proteins, suggesting heterogeneity in posttranslational modifications such as glycosylation. The JHE of the lepidopteran *T. ni* is glycosylated (Hanzlik and Hammock, 1987); however, the JHE of *M. sexta* lacks glycosylation, even though computational analysis of its sequence indicates three potential glycosylation sites (Kamita *et al.*, 2003). It should be noted that even a baculovirus expression system containing a single JHE construct still yielded several isoforms that could not be explained by limited proteolysis or differential deglycosylation (Hinton and Hammock, 2003).

3.7.9.1.2. Kinetic parameters and specificity JHE kinetics and substrate specificity have long been a central focus of JHE studies, owing to the obvious agricultural potential of this information. Elucidation of JHE kinetic constants also permits predictions of the rate limits of JH metabolism, as well as provide valuable insight into to how peripheral JH levels are regulated. A number of reports from the 1980s indicate that among lepidopterans, the apparent Michaelis constant (K_m) for the naturally occurring JHs ranged from 10^{-8} to 10^{-6} M (Roe and Venkatesh, 1990). JH titers in the species tested are at least 10–100 times lower than the estimated K_m concentration, indicating that the enzyme is very sensitive to changes in JH concentration, but suggesting that its catalytic potential is wasted. Since the K_m values seem unduly high with regard to substrate concentration, investigators have examined the individual rate components of the enzymatic reaction to better explain the observed data (Sparks and Rose, 1983; Hammock, 1985; Abdel-Aal and Hammock, 1986).

In the simplest of terms, the velocity of JHE or any enzymatic reaction can be viewed as the sum of various rate components and the concentration of the reactants, and can thus be defined by the familiar equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad [1]$$

where E represents enzyme, S represents substrate, ES represent the Michaelis complex, and P the product. Under physiological conditions, where the concentration of JH is several orders of magnitude lower than the K_m, the rate at which JH acid is formed is a function of JH interaction with JHE (k_1 and k_{-1}) and the rate of product formation (k_2). Several important lessons can be drawn from the kinetic data for JHE. First, JHE has a very high affinity for JH. Second, it has a relatively low turnover number or k_{cat}, where k_{cat} is the maximum number of substrate molecules (JH) converted to product (JH acid) per active site per unit time. These factors make the enzyme a very effective scavenger that can "find" JH at physiological concentrations and convert it to JH acid (Abdel-Aal and Hammock, 1986).

Substrate specificity of JHE represents another characteristic that seems counterintuitive. The K_m and V_{max} that the JHEs display towards the JH homologs is surprisingly low when compared to other substrates, such as α-naphthyl acetate. For example, recombinant *M. sexta* JHE displays a K_m (410 μM) and V_{max} (21 μmol min^{-1} mg protein^{-1}) for α-naphthyl acetate that is considerably higher than for its natural substrate, JH III (K_m = 0.052 μM, V_{max} = 1.4 μmol min^{-1} mg protein^{-1}) (Hinton and Hammock, 2003). As noted by Fersht (1985), when specificity is used to discriminate between two competing compounds, it should be determined by the k_{cat}/K_m ratios, and not K_m alone. The k_{cat}/K_m ratio for JH III and α-naphthyl acetate are 27 and 0.04, respectively, underscoring the high degree of specificity of JHE for JH III (Hinton and Hammock, 2003). While these kinetic parameters suggest that the enzyme has a high degree of specificity, it appears that JHEs from several species also hydrolyze methyl and ethyl esters of JH I and III at very similar rates (Grieneisen *et al.*, 1997). Moreover, even *n*-propyl and *n*-butyl esters of these homologs can serve effectively as substrates, albeit at a lower rate of hydrolysis. As might be expected, the unnatural (2*Z*,6*E*)-JH I ethyl ester isomer is not metabolized by JHEs from *M. sexta* or *H. virescens*.

Since hJHBPs have a significant effect on substrate availability (Peter *et al.*, 1979; Peter, 1990; King and Tobe, 1993), studies on JHE specificity must be performed with relatively pure preparations of the enzyme. In those few studies using highly purified or recombinant *T. ni* JHE, it was determined that the JHE hydrolyzed JH I more rapidly than JH II or III and that it displayed a faster catalytic rate for the naturally occurring enantiomer, (10*R*,11*S*)-JH II, than for the (10*S*,11*R*)-JH II form (Hanzlik and Hammock, 1987). However, a comparison of the k_{cat}/K_m ratios for the enantiomers indicated that hydrolysis of the two forms was equivalent (Hanzlik and Hammock, 1987). The same holds true for the gypsy moth, *Lymantria dispar*, in which the JHE shows no enantiomeric selectivity and appears unable to discriminate between

homologs (Valaitis, 1991). One of the most unexpected groups of JHE substrates is found in the naphthyl and *p*-nitrophenyl series. While it was originally concluded that hemolymph JHE from some species, such as *M. sexta*, could not use α-naphthyl acetate as a substrate (Coudron, 1981), the JHEs of other species can recognize members of the naphthyl series (Rudnicka and Kochman, 1984; Hanzlik and Hammock, 1987). More recently it was demonstrated that recombinant JHE from *M. sexta*, *H. virescens*, and *T. molitor* can hydrolyze naphthyl compounds with chain derivatives eight carbons long, thus dispelling the long-held belief that JHE from *M. sexta* is unable to use naphthyl derivatives as substrates (Kamita *et al.*, 2003).

Another counterintuitive observation is that while the major role of JHE is the conversion of JH to JH acid, both native and recombinant JHEs from several species can, under the appropriate conditions, transesterify JH to form the higher ester homologs, i.e., JH ethyl, JH *n*-propyl, and JH *n*-butyl esters (Grieneisen *et al.*, 1997). Although JHE-mediated JH transesterification may be a curiosity limited to the test tube, Debernard *et al.* (1995) demonstrated that when JH III, dissolved in ethanol (10 μl), was injected into *L. migratoria*, it was both converted to JH III acid and transesterified to the JH III ethyl ester. With regard to this particular study, it should be noted that care must be taken to avoid artifacts when alcohols are used as carrier solvents for the hormone in JHE assays. Nevertheless, while JHE in a biological milieu clearly serves as an esterase, these new findings imply that the enzyme may have other physiological roles (see Anspaugh *et al.* (1995) for other possible roles for JHE).

3.7.9.1.3. Protein structure and catalytic site While the catalytic action of JHE is probably similar to that of other esterases, there is some uncertainty about the molecular mechanism by which it operates and some question as to how it can effectively hydrolyze a chemically stable conjugated ester (Hinton and Hammock, 2003). A putative three-dimensional structure of the JHE of *H. zea* has been generated using homology modeling, a structure-building process that uses computer algorithms, based on the assumption that proteins with homologous sequences have similar three-dimensional structures (Thomas *et al.*, 1999). Using the structures of two well-defined carboxylesterases (sequence identity ~28%) as models, these investigators demonstrated that JHE belongs to the α/β hydrolase fold family. The *H. zea* JHE (GenBank Accession no AF037196) possesses a putative catalytic triad of amino acids, including the nucleophilic Ser224, as part of the characteristic motif, G-X-S-X-G (X represents any amino acid residue). The second and third components to the triad are a base, His465, which is part of the G-X-X-H-X-X-D/E motif , and an acid, Glu353, that lie in a deep cleft of approximately 23 Å. The importance of these residues to JH catalysis was confirmed through site-directed mutagenesis (Ward *et al.*, 1992). In addition to the catalytic site, the cleft is lined with a number of hydrophobic amino acids, a situation that might be expected, considering the hydrophobic nature of JH.

One might reasonably expect the catalytic site of JHEs of other species to show some sequence similarity and, not surprisingly, they do. A comparison of sequences among the known lepidopteran JHEs, those of *B. mori* (Hirai *et al.*, 2002), *Choristoneura fumiferana* (Feng *et al.*, 1999), *H. virescens* (Hanzlik *et al.*, 1989), and *M. sexta*, (Hinton and Hammock, 2001), reveals better than 30% identity. The residues associated with the catalytic site are in complete agreement and display the appropriate alignment. Even in species outside the order, such as *D. melanogaster* (Campbell *et al.*, 1992) and *T. molitor* (Hinton and Hammock, 2003), the catalytic sites align with those in Lepidoptera (Feng *et al.*, 1999; Kamita *et al.*, 2003). The only species that does not show alignment of the putative catalytic site is *L. decemlineata* (Vermunt *et al.*, 1997b), which is surprising given the consensus displayed by another beetle, *T. molitor.* With the exception the putative catalytic site residues and a sequence surrounding Ser224, the JHEs as a family are not highly conserved. This might be expected, however, since only the cleft into which the substrate fits needs to be lined with hydrophobic residues (Kamita *et al.*, 2003).

3.7.9.1.4. Juvenile hormone esterase inhibitors The development of effective JHE inhibitors has been an ongoing process since the first studies more than 25 years ago. While JHE inhibitors have yet not been commercialized for agricultural use, they have, nevertheless, been important tools in the discovery of the role of JH catabolism *in vivo*. Two important series of JHE inhibitors have emerged from these studies, the trifluoromethyl ketones (TFKs) and the phosphoramidothiolates (Roe and Venkatesh, 1990). The TFKs, such as 1,1,1-trifluorotetradecan-2-one (TFT), were initially suspected to act as transitional state analogs, mimicking the α,β saturation of JH (Hammock *et al.*, 1982) (**Figure 15**). The transitional state, where the enzyme and substrate form a complex, represents a transitory period in which the chemical bonds of the substrate

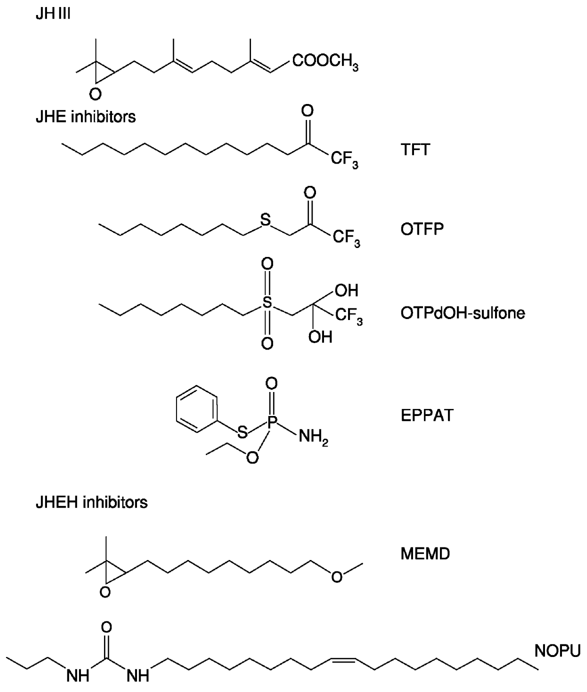


Figure 15 Chemical structures of JH III, JH esterase and JH epoxide hydrolase inhibitors. TFT, 1,1,1-trifluorotetradecan-2-one; OTFP, 3-octylthio-1,1,1-trifluoro-2-propanone; OTPdOH-sulfone, 1-octyl[1-(3,3,3-trifluoropropan-2,2-hydroxy)] sulfone; EPPAT, *O*-ethyl *S*-phenyl phosphoramidothiolate; MEMD, methyl 10,11-epoxy-11-methyldodecanoate; NOPU, *N*-[(*Z*)-9-octadecenyl]-*N*′-propyl urea.

are in the process of being broken and made. Thus, a transitional state analog has the properties of the unstable intermediate. In the catalytic site, the electron-withdrawing inhibitor increases the electrophilicity of the carbonyl group, enhancing its susceptibility to nucleophilic attack by the reactive serine (Hammock *et al.*, 1982). While it was thought that mimicking the JH backbone was important to recognition, further modifications of TFT to resemble JH structure did not improve JHE inhibition. However, introducing a thioether beta to the carbonyl 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) (**Figure 15**) increased the potency of inhibition at least 100-fold (Abdel-Aal and Hammock, 1985). More recently, it has been demonstrated that 1-octyl[1-(3,3,3-trifluoropropan-2,2-dihydroxy)] sulfone (OTPdOH-sulfone) is a better inhibitor of JHE than OTFP (Roe *et al.*, 1997). The increased inhibition displayed by OTFP-sulfone stems from the increased hydration (Roe *et al.*, 1997; Wheelock *et al.*, 2001). The attractiveness of the TFK-containing inhibitors lies in their reversible binding of the enzyme, which permits their use in affinity-matrix purification of JHEs. Moreover, the TFKs are useful for *in vivo* studies since they are highly specific and appear to have little effect on nonspecific esterases (Roe *et al.*, 1997).

In contrast to the TFKs, the organophosphate inhibitors of JHE, most importantly *O*-ethyl *S*-phenyl phosphoramidothiolate (EPPAT) (**Figure 15**), bind irreversibly to JHE (Hammock, 1985). Like the TFKs, EPPAT appears to be highly selective for JHE and can be used *in vivo*. While EPPAT is only a moderately effective inhibitor of JHE, it has a much longer life *in vitro* than the TFKs.

3.7.9.1.5. Genomic structures At the present time, the genomic structures of only two *JHE*s are completely known, those of *H. virescens* (GenBank Accession no. J04955) (Hanzlik *et al.*, 1989; Harshman *et al.*, 1994) and *D. melanogaster* (GenBank Accession no. AF304352) (Campbell *et al.*, 2001). The *JHE* locus of *H. virescens* is 8 kb in length and is composed of five exons that give rise to an open reading frame of 1.8 kb (Harshman *et al.*, 1994). Unfortunately, nothing is known about the 5′ upstream region in this gene, although the core promoter region for *JHE* has been elucidated in another noctuid, *T. ni* (Jones *et al.*, 1998). In *D. melanogaster,* the *JHE* is localized on the second chromosome and covers approximately 2.5 kb in region 52F1-2 (FlyBase). The gene is composed of five exons (note: Flybase incorrectly indicates six exons) that contain an open reading frame of 1.7 kb. In the 5′ position immediately adjacent to the *JHE* gene is another carboxylesterase gene (Flybase CG 8424) that displays 42% identity with *JHE* and contains the putative catalytic sites (Kamita *et al.*, 2003). This gene is nearly identical in length and is suggested to be a gene duplication (Campbell *et al.*, 2001). Just downstream from *JHE* is the gene *spin,* which is reported to be implicated in female behavior (FlyBase CG8428). Since gene expression may be coordinately regulated by domain control elements (Spitz *et al.*, 2003), a knowledge of neighboring genes may provide new insights into the regulation of *JHE* and potential molecular cascades in which the gene is involved.

3.7.9.1.6. Regulation The importance of JHE in regulating JH titers has prompted a number of investigators to examine its physiological and genetic regulation (review: Gilbert *et al.*, 2000). The primary site of synthesis for the hemolymph JHEs, the fat body (Whitmore *et al.*, 1974; Hammock *et al.*, 1975; Wing *et al.*, 1981), has been the primary focus of many studies that have been reviewed previously in some detail (Roe and Venkatesh, 1990; Roe *et al.*, 1993; deKort and Granger, 1996; Gilbert *et al.*, 2000). Tissues other than the fat body can also

express the enzyme (see Roe and Venkatesh, 1990 for a review of the different tissues; Feng *et al.*, 1999). Even the CA have been found to have JHE activity in a number of species (Sparagana *et al.*, 1984; Wisniewski *et al.*, 1986; Meyer and Lanzrein, 1989a, 1989b), and in one case, the glands secrete measurable, and developmentally fluctuating, amounts of the enzyme into incubation medium (Sparks *et al.*, 1989; Janzen *et al.*, 1991). Most studies conclude that the JHEs of different tissues and at different developmental time points are similar, if not identical, to the hemolymph form of JHE; however, their regulation may be different (Jesudason *et al.*, 1992). Sparks *et al.* (1989) made the novel suggestion that the multiple isoforms of hemolymph JHE may be the result of its production and release into the hemolymph from a variety of tissues.

To best understand JHE regulation, a brief description of hemolymph JHE levels is in order. In *M. sexta*, a peak in hemolymph JHE is observed prior to ecdysis in larval stadia two through five (Roe and Venkatesh, 1990), with activity in each stadium increasing in direct proportion to larval weight (Roe *et al.*, 1993). Although levels of JHE activity during the earlier stadia are lower, the precise timing and appearance of JHE and its developmental profile in relation to total hemolymph protein suggests specific regulation. However, the role of the JHE in the early instars is unclear, since hemolymph JH titers remain relatively high even in the presence of enzyme (Hidayat and Goodman, 1994).

In contrast to the earlier stadia, the developmental profiles of JHE activity and of JH titers during the last stadium of *M. sexta* reveal two peaks (Vince and Gilbert, 1977; Baker *et al.*, 1987; Roe and Venkatesh, 1990). Titers of JH are highest in the hours immediately following the molt from the fourth to the fifth stadium (**Figure 16**). They then drop dramatically, becoming undetectable by 48 h after ecdysis. JH titers rise a second time to peak at 156 h and then decline prior to metamorphosis. JHE activity begins to climb midway through the feeding period and peaks shortly before wandering. A smaller rise in JHE activity appears after the second peak of JH but drops at pupation (Baker *et al.*, 1987). Similar developmental profiles are seen in the last stadium of *S. littoralis* (Zimowska *et al.*, 1989) and *L. decemlineata* (deKort, 1990). Most studies have pinpointed the fat body as the primary source of JHE in the hemolymph (Wing *et al.*, 1981; Wroblewski *et al.*, 1990). However, Jesudason *et al.* (1992) suggest that the first peak of JHE, which occurs during the feeding stage, is of fat body origin, while the second peak, during the prepupal phase, is not. Alternatively, it could be that in this study, the preparation of prepupal fat body samples in the absence of protease inhibitors led to the destruction of JHE at this stage. While the JHE profile in hemolymph appears to be similar for most lepidopterans, it appears to differ outside of this order: some species have a single burst of JHE activity that declines either midway through the last stadium or at the very end of larval life (review: Roe and Venkatesh, 1990).

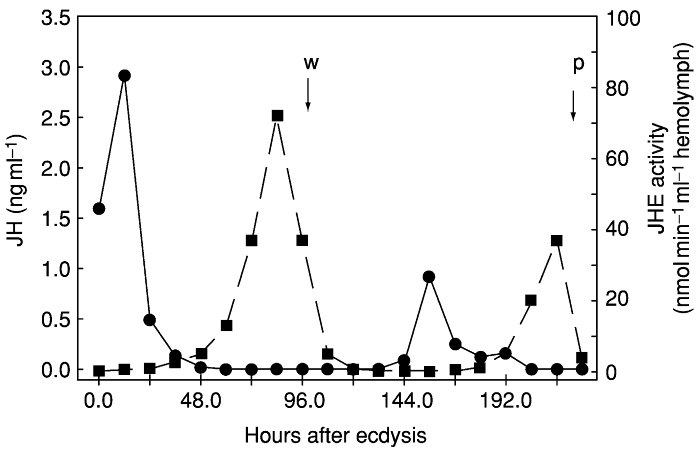


Figure 16 Hemolymph JH titer and JHE activity during the fifth stadium of *M. sexta* larval development. Closed circles, total titer for all homologs detected (JH 0, I, II, III). JH acids are not included. JH titers were combined for both females and males and expressed as the average. Closed squares, JHE activity. JHE activities were combined for both females and males and expressed as the average. W, onset of wandering behavior; P, time of pupal ecdysis. (Adapted from Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem.* *17*, 989–996.)

As can be seen in **Figure 16**, the rise in JHE activity after the JH titer drops does not support the concept that the enzyme is directly responsible for the decline in JH titers. Nevertheless, the enzyme displays exceptional scavenging properties, and it may be that the increase in enzyme activity occurs to ensure that no detectable JH is present during commitment to pupation. More problematic is the observation that JH is not totally eliminated when JHE is present. For example, a comparison of JH levels in the penultimate stadium of *M. sexta* (Hidayat and Goodman, 1994) with JHE levels during this same period (Roe and Venkatesh, 1990) reveals measurable JH in the presence of JHE. The results of similar studies of the fifth stadium in this species strongly suggest that the pre-wandering peak of JHE has no functional relationship to metamorphosis (Browder *et al*., 2001). While not stated explicitly, the same conclusion can be drawn from studies in *T. ni* (Sparks *et al*., 1979; Hanzlik and Hammock, 1988; Jones *et al*., 1990b), and in still another case, JH levels appear refractory to JHE in diapausing larvae of the European cornborer, *Ostrinia nubilalis* (Bean *et al*., 1982). Although hemolymph JHE levels follow a developmental pattern that suggests the enzyme may be directly involved in JH catabolism, there remains a degree of uncertainty about the assumed role of JHE in eliminating the hormone from the circulatory system.

That JHE levels do not follow a developmental pattern typical of other hemolymph proteins prompted a search for possible regulatory mechanisms modulating its activity. A logical candidate for JHE regulation is JH itself, but regulation of JHE by JH in lepidopteran larvae, especially during the last larval stadium, depends on whether the insect is in the pre-wandering or post-wandering phase. In the pre-wandering phase of the last larval stadium, JH regulation of hemolymph JHE levels is ambiguous. The brain appears to play an inhibitory role in control of JHE activity, and there are other factors, such as tissue competence, that seem to be involved as well (Venkatesh and Roe, 1988; Jesudason *et al*., 1992; Roe *et al*., 1993). In contrast, manipulation of JH titers by surgical and chemical means during the post-wandering period of the last stadium has a direct impact on JHE activity (Roe *et al*., 1993; review: Gilbert *et al*., 2000). Treatment of larvae with JH or JH analogs leads to an increase in JHE activity in the hemolymph, while allatectomy leads to a decline in JHE that can be reversed by supplying exogenous hormone. At the molecular level, nuclear run-on experiments using the fat body of *T. ni* have confirmed that exogenous JH or JH analogs stimulate *JHE* expression and that the response can be detected within 3 h of treatment (Venkataraman *et al*., 1994). Using a cell line derived from the midgut of *C. fumiferana,* Feng *et al*. (1999) demonstrated that JH can increase the abundance of JHE mRNA within 1 h, suggesting a potential upregulation of *JHE* expression by JH. However, these investigators rightfully caution that the apparent rise in message could also be due to stabilization of JHE mRNA. Curiously, the potent protein synthesis inhibitor, cyclohexamide, mimicked the action of JH, prompting the suggestion that an inhibition of protein synthesis leads to an increase in the accumulation of JHE message or, alternatively, stabilization of the message. In contrast to the apparent stimulation of expression levels by JH, the presence of 20E in the medium leads to a decrease in JHE mRNA, in a dose-dependent manner (Feng *et al*., 1999). This discovery indicates that regulation of *JHE* expression is more complex than first thought and that ecdysteroids must be considered in the overall regulatory system.

The endocrine system is an important bridge between the target cell and the environment, and it is axiomatic that environmental factors are major players in regulation of the endocrine system. Factors such as stress (Gruntenko *et al*., 2000), circadian cues that induce diapause (Bean *et al*., 1982, 1983), nutrition (Cymborowski *et al*., 1982; Sparks *et al*., 1983; Venkatesh and Roe, 1988; Roe and Venkatesh, 1990), and parasitism (Hayakawa, 1990; see Edwards *et al*., 2001 and references therein) are all thought to play a role in regulating JHE levels and are undoubtedly transduced by the nervous system.

3.7.9.1.7. Catabolism of juvenile hormone esterase

After a rapid increase midway through the feeding period of the last stadium in Lepidoptera, JHE levels drop dramatically immediately prior to the onset of wandering. Experimental evidence corroborates this observation: the half-life of recombinant JHE when injected into second instars of *M. sexta* is approximately 20 min, while other exogenously supplied proteins of similar molecular mass display half-lives measured in days (Ichinose *et al*., 1992). Since no cleavage products of JHE can be detected in the hemolymph, Ichinose *et al*. (1992) suggested that the enzyme is removed from the circulatory system by cellular uptake. It was discovered that the pericardial cells were responsible for uptake and destruction of the hemolymph JHE (Ichinose *et al*., 1992). Pericardial cells are a collection of cells that surround the insect heart in various configurations, depending upon the order (Wigglesworth, 1972; Crossley, 1985). In *M. sexta,*

the pericardial mass is punctuated with numerous labyrinthine channels that increase exposure of the cells to the hemolymph (Brockhouse *et al.*, 1999), and it is thought that this tissue mass is responsible for the removal of hemolymph proteins and small colloidal particles from the hemolymph (Crossley, 1985).

It is speculated that the rapid removal of JHE from the hemolymph by the pericadial cells occurs via receptor-mediated endocytosis, followed by transport to lysosomes for destruction (Bonning *et al.*, 1997). During the passage from the endosome to the lysosome, JHE interacts with putative heat shock cognate proteins with relative molecular masses of 100 and 140 kDa. These pericardial cell-specific proteins cross-react with a universal antiserum directed towards heat shock protein 70, hence the designation as cognate (Shanmugavelu *et al.*, 2000). In addition to the heat shock proteins, a novel 29 kDa protein, termed P29, appears to bind and target JHE for lysosomal destruction. This protein has been detected in fat body and pericardial cells of insects from all five larval stadia in *M. sexta*. The P29 gene has been identified using a phage display library and has been sequenced (GenBank Accession no. AF233526).

3.7.9.2. Juvenile Hormone Epoxide Hydrolases

The preponderance of literature on JH catabolism has focused on the role of JHE in metamorphosis, yet the less well-studied pathway, i.e., epoxide hydration via JH epoxide hydrolase (JHEH), may actually be more biologically relevant in some species (Gilbert *et al.*, 2000). For example, when JH I is injected into early fifth instars of *M. sexta*, the major metabolite is not JH acid or JH acid-diol, but a JH diol phosphate conjugate (Halarnkar *et al.*, 1993). Further evidence that JHEH plays a significant role in the reduction of JH titers at critical times can be found in the mosquito *Culex quinquefasciatus*, in which JHEH activity is two to four times higher than JHE activity throughout most of the life cycle (Lassiter *et al.*, 1994). Moreover, two peaks of JHEH are seen during the last stadium, suggesting that the enzyme is involved in JH metabolism during critical developmental periods (Lassiter *et al.*, 1995). Even in *T. ni*, with which so many of the JHE studies have been done, JH diol is a major metabolite or intermediate (Kallapur *et al.*, 1996). It appears that the contribution of JHEH to the overall catabolism of JH varies extensively, even within the same order (Hammock, 1985).

3.7.9.2.1. Physical properties The JHEHs (EC.3.3.2.3) belong to a large family of proteins that display the αβ hydroxylase fold (Debernard *et al.*, 1998) and they are responsible for hydration of the JH epoxide to a diol. Since hydrolases are involved in a wide range of enzymatic reactions and utilize multiple substrates, it has been justifiably cautioned that JHEHs may not be as specific as they are portrayed (Harris *et al.*, 1999). Nevertheless, the term JHEH will be employed as it is commonly used in the literature.

A number of species have been catalogued as having JHEH activity (Hammock, 1985), but in only a few species have the JHEHs been well characterized. Because a single species may have more than one form of JHEH (Harshman *et al.*, 1991; Keiser *et al.*, 2002), it has been suggested that the different JHEHs represent tissue-specific enzymes (Harshman *et al.*, 1991). Epoxide hydrolases are present in both soluble (cytoplasmic) and insoluble (microsomal) forms, but the EHs responsible for hydration of the epoxide are found associated mainly with the microsomal fraction (Wisniewski *et al.*, 1986; Touhara and Prestwich, 1993; Wojtasek and Prestwich, 1996; Harris *et al.*, 1999; Keiser *et al.*, 2002). JHEH appears to be a monomer, with a relative molecular mass ranging from 46 to 53 kDa (Harshman *et al.*, 1991; Touhara and Prestwich, 1993; Harris *et al.*, 1999; Keiser *et al.*, 2002). In general, the pH range for JHEH activity is broad, extending from approximately pH 5 to 9, probably reflecting the involvement of the reactive histidine (pK_a 6.5 for the imidazole group) in the catalytic site (Debernard *et al.*, 1998). The JHEHs of *D. melanogaster* are heat- and organic solvent-tolerant, withstanding incubation at 55 °C and concentrations of ethanol exceeding 40% (Harshman *et al.*, 1991). Moreover, the recombinant enzyme from *M. sexta* retains full activity in the presence of low levels of the reducing agent dithiothreitol and a sulphydryl modifying reagent, iodoacetamide (Debernard *et al.*, 1998).

3.7.9.2.2. Kinetic parameters, specificity, and the catalytic site Determinations of the kinetic parameters of the JHEH from the microsomal fraction of *M. sexta* eggs revealed K_m values for JH I, II, and III of 0.61, 0.55, and 0.28 μM, respectively (Touhara and Prestwich, 1993). Interestingly, the V_{max}:K_m ratio for JH III, the least abundant egg homolog (Bergot *et al.*, 1981a), was 25 times greater than that for JH I, suggesting that the enzyme displays considerably more specificity for JH III than the higher homologs (Touhara and Prestwich, 1993). This observation was confirmed by Debernard *et al.* (1998), using recombinant JHEH from *M. sexta*; while the enzyme could hydrolyze

JH I, II, and several synthetic substrates, JH III was the most favored substrate. JH III is also a better substrate for JHEH than JH acid (Touhara and Prestwich, 1993; Kallapur *et al.*, 1996), and in *D. melanogaster*, JH III is a more suitable substrate than JHB_3 (Casas *et al.*, 1991). If JH III is the optimal substrate for JHEH, then JHEH may indeed be the enzyme responsible for intracellular JH catabolism, especially since with the exception of JHB_3, JH III is the only or predominant JH in all insect orders but Lepidoptera (see Section 3.7.2.1). However, as noted by Harris *et al.* (1999), more direct evidence, such as a correlation between inhibition of the enzyme *in vivo* and a decrease in JH metabolism, is required before assuming the enzyme is JH III-specific.

As noted by several groups, the high degree of conservation of the catalytic site suggests that the mechanism of epoxide hydration is similar to that found in mammals (Roe *et al.*, 1996; Wojtasek and Prestwich, 1996; Debernard *et al.*, 1998). Using the JHEH of *M. sexta* as a model, Debernard *et al.* (1998) proposed a two-step catalytic process. The first step involves a nucleophilic attack of the epoxide at the least hindered position, C10, by Asp227. This, in turn, opens the ring, leading to the formation of an hydroxyl-alkyl enzyme. The neighboring Trp228 is thought to activate the epoxide for a nucleophilic attack (Harris *et al.*, 1999). The second step involves the hydrolysis of this covalent intermediate by a water molecule that is activated by His428 and Asp350. The histidine residue activates water with an Asp or Glu residue acting as the proton scavenger from the histidine to reactivate the enzyme. The resulting product is (10*S*,11*S*)-JH diol. A variation on this putative reactive site was found by Linderman *et al.* (2000), in which the site uses Glu403 instead of Asp350 as the charge relay partner with the histidine residue. There is remarkable sequence conservation of the catalytic triad and its surrounding residues among both insects and mammals. All JHEHs, like their mammalian counterparts, contain a tryptophan residue at positions 150–155, forming part of the oxyanion hole that may stabilize the hydroxyl-alkyl intermediate (Lacourciere and Armstrong, 1993; Wojtasek and Prestwich, 1996; Keiser *et al.*, 2002).

3.7.9.2.3. Juvenile hormone epoxide hydrolase inhibitors In contrast to the early and successful discoveries of highly effective JHE inhibitors, the search for JHEH inhibitors has been less productive until recently (Hammock, 1985; Casas *et al.*, 1991; Harshman *et al.*, 1991; Roe *et al.*, 1996). Investigations have taken one of two directions, examining either compounds that mimic the JH backbone (Roe *et al.*, 1996; Linderman *et al.*, 2000) or compounds based on urea and amide pharmacophores that are not subject to metabolism through epoxide degradation (Severson *et al.*, 2002).

Roe *et al.* (1996) demonstrated that methyl 10,11-epoxy-11-methyldodecanoate (MEMD), a long-chain aliphatic epoxide (**Figure 15**) displays an I_{50} (molar concentration needed to inhibit 50% of enzyme activity) in the low nanomolar range. A promising group of MEMD analogs was investigated by Linderman *et al.* (2000), who re-examined the structure–activity relationship of MEMD epoxide substitution and enantioselectivity. Two classes of MEMD analogs were synthesized, a glycidol-ester series and an epoxy-ester series. As a group, the glycidol-esters were more potent inhibitors than the corresponding epoxy-esters, by an order of magnitude. The inhibitory activity in both classes was found to be dependent upon the absolute configuration of the epoxide at C10, with the *R* configuration displaying the higher degree of inhibition. The inhibitory activity of the most potent compound of the series ($I_{50} = 1.2 \times 10^{-8}$ M) is thought to be due to the hydroxyl group in the active site, which forms an additional hydrogen bond. This bond may stabilize the enzyme–inhibitor complex by inducing a conformational change, or it could reduce the rate at which the diol product dissociates from the enzyme's active site.

The other class of JHEH inhibitors is the analogs of the ureas and amide pharmacophores that have been demonstrated to be potent inhibitors of mammalian soluble and microsomal epoxide hydrolases in mammals (Severson *et al.*, 2002). To date, none of the nearly 60 compounds tested are as active as the glycidol-esters in the inhibition of recombinant *M. sexta* JHEH (**Figure 15**), a surprising result, given their action on mammalian enzymes. The most potent of the series, *N*-[(*Z*)-9-octadecenyl]-*N*′-propyl urea (NOPU), has an $I_{50} = 8.0 \times 10^{-8}$ M. Severson *et al.* (2002) suggest that when the inhibitor enters the catalytic site, the carbonyl group of the urea interacts with two tyrosine residues in the oxyanion hole. Since the inhibitor lacks an epoxide, it does not covalently bind to the reactive Asp227 residue, but it does block the catalytic site from further activity.

3.7.9.2.4. Genomic structures At the present time, the genomic structure of only one insect *JHEH* gene is known, that of *D. melanogaster*. The gene cluster, approximately 8.6 kb, is located on chromosome 2 in region 55F7-8, near the *JHE* locus at 52F1. As suggested by Harshman *et al.* (1991) and confirmed

through genomic studies (FlyBase), there are multiple forms of *JHEH* in *D. melanogaster.* Annotation of the *JHEH* locus identifies three putative genes that display approximately 37% identity in their amino acid sequence. The genomic region containing *JHEH1* (GenBank Accession no. NM_137541; CG15101) is approximately 1.8 kb and contains four exons. Approximately 0.7 kb downstream from *JHEH1* lies *JHEH2* (GenBank Accession no. NM_137542; CG15102), which is 2.2 kb in length and has two potential transcripts. One transcript has four exons (GenBank Accession no. NM_137524; CG15102) while the other has three (GenBank Accession no. NM_176233; CG15102). At 0.3 kb downstream from *JHEH2* lies the gene *Bari* which encodes a transposable element. *JHEH3* (GenBank Accession no. NM_137543; CG15106) lies about 0.3 kb downstream from the transposable element and is composed of three exons. No genes of obvious relation to JH action or catabolism lie close to the *JHEH* locus. *JHEH1* and *2* are more related to each other than to *JHEH3*; all three putative genes display the residues of the catalytic triad in the correct sequence, and all are of about the same length. Computational analysis using CLUSTALW indicates that *JHEH1* and *3* are most divergent in their first 70 residues, which might be expected if the *N*-terminal is needed for attachment to cell membranes. Since JHEH activity has been demonstrated in other cell fractions besides microsomes (Casas *et al.*, 1991; Harshman *et al.*, 1991), it may well be that the first exons of *JHEH1* and *3* code for sequences that target these enzymes for different locations within the cell.

Recent evidence suggests that the N-terminal of EHs may play another role. The soluble human EH, EPXH2, also possesses phosphatase activity, so the enzyme can not only transform epoxy fatty acids to their corresponding diols but also dephosphorylate dihydroxy lipid phosphates (Newman *et al.*, 2003). The phosphatase activity localized to the *N*-terminal domain is unaffected by a number of classic phosphatase inhibitors. Alignment of EPXH2 with several of the insect JHEHs shows less than 25% amino acid sequence similarity under nonstringent conditions, and regions of similarity are limited to the C-terminal domain. Nevertheless, the possibility of an insect JHEH with phosphatase activity presents an interesting twist in the metabolic pathway for JH catabolism (see Section 3.7.9.3).

3.7.9.3. Secondary Metabolism of Juvenile Hormone: Juvenile Hormone Diol Kinase

Secondary metabolism of JH has been examined in a number of investigations (Roe and Venkatesh, 1990; Halarnkar *et al.*, 1993; Grieneisen *et al.*, 1995), but as noted by Halarnkar *et al.* (1993), the results of these studies may be misleading, since the enzymes used may have contained multiple hydrolytic activities. In only one instance has an actual JH conjugate been unequivocally identified. The conjugate, (10*S*,11*S*)-JH diol phosphate (**Figure 1**), is the product of a two-step enzymatic process: conversion of JH to JH diol and then addition of a phosphate group to C10 (Halarnkar *et al.*, 1993). The enzyme responsible for the phosphorylation of JH diol is JH diol kinase (JHDK), which has been characterized from the Malpighian tubules of early fifth instars of *M. sexta* (Grieneisen *et al.*, 1995; Maxwell *et al.*, 2002a, 2000b). The discovery of JHDK (EC 2.1.7.3) was made when an analysis of JH I metabolites *in vivo* yielded, in addition to JH diol and JH acid, a very polar JH I conjugate that was subsequently identified as JH I diol phosphate (Halarnkar and Schooley, 1990).

3.7.9.3.1. Physical properties JHDK from *M. sexta* Malpighian tubules is a cytosolic protein composed of two identical subunits of 20 kDa, as determined by mass spectrometry (Maxwell *et al.*, 2002a). Gel filtration studies indicate it has a molecular mass of approximately 43 kDa, and it has been suggested that the native form of the enzyme is homodimeric. JHDK displays a K_m in the nanomolar range for JH I diol, which is appropriate for an enzyme responsible for clearance of a hormone whose titers rarely exceeds 10 nM. Most significantly, the developmental profile of catalytic activity for JHDK parallels that for JHEH, a requisite if JH diol phosphate is a legitimate terminal metabolite. Analysis of the k_{cat}/K_m parameter for the diols of JH I, II, and III indicates that JH I diol is the preferred substrate, suggesting a preference for an ethyl group at the C7 position. JHDK requires both Mg^{2+} and ATP for activity (Grieneisen *et al.*, 1995; Maxwell *et al.*, 2002a), although excess Mg^{2+} or Ca^{2+} inhibits its activity (Maxwell *et al.*, 2002a). When stored in the appropriate buffers, the enzyme is reasonably stable but it is very sensitive to various metal ions.

The specificity of JHDK for JH I diol is relatively high, considering the multitude of potential phosphate acceptor groups present in a cell. The enzyme does not recognize methyl geranoate diol (one isoprenyl unit shorter than JH) nor methyl geranylgeranoate diol (one isoprenyl group longer than JH), yet it does recognize JH I ethyl ester diol. It also recognizes both JH diol enantiomers, indicating that absolute stereospecificity of the hydroxyl groups is of minor importance.

Most surprising is the enzyme's inability to recognize JH acid diols. If JH acid diol cannot be phosphorylated by JHDK, the fact that JH diol phosphate is a significant metabolite (Halarnkar *et al.*, 1993) must be explained otherwise. JH acid diol could undergo further catabolism by cytochrome P-450s (Sutherland *et al.*, 1998). However, the role of cellular JHE becomes problematic if the pathway catalyzed by JHEH and JHDK is the major pathway for JH catabolism in the cell. There may yet be undiscovered enzymes in cellular JH catabolism.

3.7.9.3.2. Genomic structures The sequence and hypothetical structure of the *M. sexta* and *D. melanogaster JHDK* genes have recently been examined by Maxwell *et al.* (2002b). A partial characterization of JHDK from whole body homogenates of *D. melanogaster* indicates that it is similar to the enzyme in *M. sexta*, with the exception of its subunit structure. The active *D. melanogaster* JHDK is composed of a single monomer of ~20 kDa, while the active *M. sexta* JHDK is composed of two identical 20 kDa subunits. This latter gene (GenBank Accession no. AJ430670) has been sequenced and found to code for an enzyme that has 59% sequence identity and >80% similarity to sarcoplasmic calcium-binding protein 2 (dSCP2) of *D. melanogaster* (GenBank Accession no. AF093240; CG14904). Similarities in chromatographic properties, isoelectric point, and enzyme activity led Maxwell *et al.* (2002b) to conclude that dSCP2 is the probable *D. melanogaster* homolog of *M. sexta* JHDK.

Maxwell *et al.* (2002b) used computer modeling and docking programs to generate a three-dimensional model of JHDK. They capitalized on two facts: (1) the catalytic site of JHDK must contain both a purine binding site (MgATP or MgGTP) and a hydrophobic pocket for JH diol; (2) the scaffolding for another sarcoplasmic calcium-binding protein is known. Both the *M. sexta* and *D. melanogaster* JHDKs contain the three conserved nucleotide-binding elements common to nucleotide binding proteins, surrounding the putative substrate-binding site. The model further demonstrates that the protein contains four domains that form two pairs of a helix–loop–helix motif (EF-hand) (Branden and Tooze, 1999). The model's charge interactions in the hydrophobic binding pocket, as well as its depth (19 Å), are complementary to the extended conformation of the diol. Moreover, the hydrophobic nature of the binding pocket complements the C1 ester of the substrate and supports the observation that JH diol is the only substrate for this enzyme (Maxwell *et al.*, 2002a).

3.7.9.4. Juvenile Hormone Catabolism and New Directions

The field of JH catabolism is changing with the application of sophisticated analytical tools and the use of metabolism studies *in vivo* to uncover potential catabolic pathways. The combination of the two approaches has led to the surprising discovery that JH diol phosphate conjugates are major JH catabolites, thus emphasizing the role of JHEHs in clearing JH from the body (Halarnkar *et al.*, 1993; Gilbert *et al.*, 2000). That discovery has revealed a significant problem in the JHE phase-separation assay. While this assay is a powerful tool, it also detects polar metabolites resulting from enzymatic activity other than that of JHE. In addition, it is critical that the correct controls be employed. This point is driven home by the fact that JHEs from several species can, under the appropriate conditions, transesterify JH (Debernard *et al.*, 1995; Grieneisen *et al.*, 1997). Thus, the JHE phase separation assay should be employed only as the first step in the search for new JH catabolites. The assay should be followed by the use of the advanced chromatographic tools and detection systems now available for identifying trace polar metabolites. The need to demonstrate biological relevance applies not only to the newly discovered JH-like molecules (see Section 3.7.3.1), but also to the enzymes involved in JH catabolism. It may well be that some of the enzymes are really not JH-directed, but will, under experimental conditions, generate metabolites not seen under *in vivo* conditions.

Analysis of JHE activity is further complicated by the existence of both hemolymph and tissue JH binding proteins that preferentially bind certain homologs (Goodman *et al.*, 1976; Park *et al.*, 1993) and enantiomers (Schooley *et al.*, 1978b). These proteins have a significant influence on JHE activity (Hammock *et al.*, 1975) due to preferential binding of one enantiomer (Peter, 1990) or homolog (Halarnkar *et al.*, 1993) over another (see Section 3.7.8.2.1). The recent development of chromatographic methods to separate racemic preparations of JH should alleviate some of these problems (Cusson *et al.*, 1997).

Finally, in the drive to discover a unifying theory to explain JH catabolism, the diversity of the class Insecta is often overlooked. While synthesis of the results of different studies is unavoidable, to assume that all insect species use a single common pathway for JH catabolism would be a gross

oversimplification of the real situation. One need only compare JH metabolism in the flea *Ctenocephalides felis*, which utilizes JHE (Keiser *et al*., 2002), *D. melanogaster*, which utilizes JHEH (Casas *et al*., 1991), and the moth *T. ni*, which utilizes both pathways (Kallapur *et al*., 1996), to understand that multiple pathways for JH catabolism have evolved in this diverse class.

3.7.10. Juvenile Hormones in Embryological Development

The role of JHs in embryonic development remains an enigma despite the discovery of the hormones in the eggs of *H. cecropia* more than 40 years ago (Gilbert and Schneiderman, 1961). However, the paradigm that so elegantly describes the developmental action of JH during larval to pupal metamorphosis has not, until recently, seemed applicable to the embryonic development of insects. The observation that JH titers rise at different times during embryogenesis of hemimetabolous and holometabolous insects has led Truman and Riddiford (1999) to speculate that JH may have played a significant role in the evolutionary divergence of these two groups. As the evidence presented below indicates, the role of JH during embryonic development warrants further consideration.

3.7.10.1. Juvenile Hormone Homologs and Precursors Present during Embryogenesis

The JH homologs from embryos of only a few species have been identified thus far (**Table 1**), giving us a very incomplete picture. What does emerge from these limited data is confirmation that JH III in nonlepidopteran insects is the predominant embryonic hormone, and following the pattern observed in other stages (Schooley *et al*., 1984). Furthermore, JH homologs and precursors not detected in larvae or adults exist in eggs, including JH 0, 4-methyl JH I, and the JH precursor, MF. Even more remarkable are the measurable titers of these homologs and precursors in whole-body extracts of embryos. While it is difficult to compare titers in whole-body extracts with those in hemolymph, the levels of JH in the embryo do appear significantly higher than those in hemolymph at any time during larval development (Edwards *et al*., 2001).

3.7.10.2. Role of Methyl Farnesoate during Embryogenesis

One of the more interesting JH-like molecules found in embryos is MF, the final precursor in the pathway to JH III biosynthesis. While MF possesses the farnesyl backbone of the JHs, it lacks the terminal epoxide at the C10 position (**Figure 1**) and exhibits relatively low activity in bioassays when compared to JH III (Sláma *et al*., 1974). Several investigators have reported high levels of JH III and MF in *N. cinerea* midway through embryonic development (Baker *et al*., 1984; Lanzrein *et al*., 1984; Brüning *et al*., 1985). Using the RCA, Bürgin and Lanzrein (1988) demonstrated that the embryonic CA are indeed synthesizing JH III and MF. The CA of *N. cinerea* become differentiated just before dorsal closure of the embryo, and synthesis of MF and JH III begins shortly thereafter (Bürgin and Lanzrein, 1988). MF levels rise first, followed by JH III about a day later. Both MF and JH III levels remain elevated for several days, exceeding 800 ng g^{-1}, and then decline during the later stages of embryonic development with MF decreasing to undetectable levels (Brüning *et al*., 1985).

Table 1 Juvenile hormone homologs present during insect embryogenesis

Species	*Hormones*	*Reference*
Thermobia domestica	JH III	Baker *et al.* (1984)
Blatella orientalis	JH III	Short and Edwards (1992)
Nauphoeta cinerea	JH III, methyl farnesoate	Baker *et al.* (1984), Brüning *et al.* (1985)
Locusta migratoria	JH III	Temin *et al.* (1986), Pener *et al.* (1986)
Telogryllus commodus	JH III	Loher *et al.* (1983)
Oncopeltus fasciatus	JH III	Bergot *et al.* (1981a)
Leptinotarsa decemlineata	JH III	deKort *et al.* (1982)
Melolontha melolontha	JH III	Trautmann *et al.* (1974)
Heliothis virescens	JH 0, I, II	Bergot *et al.* (1981a)
Spodoptera littoralis	JH I, II, III	Steiner *et al.* (1999)
Manduca sexta	JH 0, I, II, 4-Me JH I	Bergot *et al.* (1981a)
Hyalophora cecropia	JH 0, I, II	Bergot *et al.* (1981a)
Bombyx mori	JH I, II, III	Gharib *et al.* (1983)
Apis mellifera	JH III	Rembold *et al.* (1992)

Recent studies on another cockroach, *D. punctata*, support the hypothesis that MF is a major product of the embryonic CA (Stay *et al.*, 2002). Using the RCA to monitor rates of MF and JH III synthesis, these investigators demonstrated that production of MF increases approximately a day before that of JH III and, as in the case of *N. cinerea*, synthesis of both compounds begin shortly after dorsal closure. Ultrastructural studies of the CA confirm the synthetic activity of the glands after dorsal closure (Lee and Chiang, 1997). In contrast to *N. cinerea*, where MF and JH III are found in equivalent amounts, the biosynthetic rate of JH in *D. punctata* exceeds that of MF by 30-fold.

Stay *et al.* (2002), examining the role of *D. punctata* allatostatin 7 (see Section 3.7.7.2) in regulating MF and JH biosynthesis in *D. punctata* embryos, found the process complex. This potent inhibitor of JH III biosynthesis in adults also inhibits synthesis of MF and JH after dorsal closure. However, prior to dorsal closure, the allatostatin has the reverse effect, stimulating MF biosynthesis. Prior to innervation by neurons transporting the allatostatin, the CA produce MF and can be stimulated by Dippu-AST to produce MF in a dose-dependent manner. Following innervation by Dippu-AST-containing neurons, the glands begin to synthesize both MF and JH, and exogenous Dippu-AST downregulates synthesis of both molecules.

A study with *N. cinerea* embryos is a reminder that MF and JH III cannot be viewed as equivalent or interchangeable (Brüning and Lanzrein, 1987). Treatment of *N. cinerea* embryos with ethoxy-precocene eliminates JH III titers but has little effect on MF levels; yet, the investigators were able to rescue ethoxy-precocene treated embryos with JH III. Interestingly, although ethoxy-precocene is known to induce destruction of the CA in certain insect species, it does not chemically allatectomize this species. Histological studies revealed that as late as 10 days after ethoxy-precocene treatment, the CA were similar to control CA in size and cell number but the nuclei appeared pycnotic. It remains to be seen whether MF is important to embryonic development in *N. cinerea* and *D. punctata*, or in any other species.

3.7.10.3. Juvenile Hormone Titers during Embryogenesis: Correlation with Developmental Events

As noted previously (see Section 3.7.10.1), the action of JH during embryogenesis is unclear and may be different from its role of maintaining the status quo role during the larval stage. Associating changes in JH titers to events in organogenesis could provide clues to the role(s) of JH during embryogenesis. Short and Edwards (1992) measured JH titers in embryos of the cockroach *Blattella orientalis*, and demonstrated that JH III is present at very low levels at oviposition and in the period preceding dorsal closure. Midway through embryogenesis, JH III titers rise dramatically, reaching better than 600 ng g^{-1} of tissue before decreasing. Unfortunately, the investigators did not associate the JH titers with specific embryological events, thus making it difficult to link the peak with any specific developmental process. By contrast, Brüning *et al.* (1985) were able to demonstrate that JH III appears in the embryo of *N. cinerea* only after dorsal closure; no hormone was detected prior to dorsal closure. As in the case of *B. orientalis*, JH III in embryos of *N. cinerea* rises to a very high level (800 ng g^{-1} of tissue). The titer remains high for about 8 days and then falls to undetectable levels prior to emergence.

Temin *et al.* (1986) conducted a closely timed study of JH titers during embryonic development of *L. migratoria*. During the first 60 h after oviposition, JH III levels remain reasonably constant at about 15 pg per egg. Blastokinesis, a movement of the embryo in relation to the yolk mass that consists of two phases (anatrepsis and katatrepsis) (Johannsen and Butt, 1941), occurs from 60 to 108 h after oviposition. During this time, JH is undetectable; however, at dorsal closure, which occurs at approximately 120 h, JH titers begin to rise and reach their highest level at 180 h. Unlike the massive peak in the JH titer observed in *B. orientalis* and *N. cinerea*, the peak titer in *L. migratoria* is approximately 45–50 pg per egg. Levels then fall slowly and are undetectable at the time of nymphal emergence. It is interesting that no JH is detected during the period between the initiation of blastokinesis and dorsal closure, a time that corresponds to the most active period of organogenesis (Temin *et al.*, 1986). However, as noted below, exposure to JH during this period can have profound effects on the development of the embryo and larva.

Dorn (1975), using a bioassay to titer embryonic JH in *Oncopeltus fasciatus*, found that newly oviposited eggs contain low, but detectable, levels of JH. He suggests that the hormone found at oviposition is maternal in origin. The titers remain stable during the first 2 days of embryonic development, but then rise rapidly during the third day. This period in *O. fasciatus* is marked by the completion of CA development and katatrepsis. Levels of JH continue to rise during the fourth day of embryonic development, a period in which final dorsal closure occurs, and peak during the fifth day at levels nearly

15 times that found at oviposition. JH levels drop at the time of hatching.

Titers of JH in the embryos of Lepidoptera present a more confusing picture. Using a bioassay, Gilbert and Schneiderman (1961) were the first to demonstrate that unfertilized eggs of *H. cecropia* contained JH. Bergot *et al.* (1981a) subsequently used GC-MS to detect low levels of JH I at 108 h after fertilization, a period following dorsal closure in this species (Riddiford, 1970). After dorsal closure, JH titers rise to a peak at 156 h (520 pg tissue g^{-1}) and then slowly decline to 50 pg tissue g^{-1} at the time of emergence, approximately 250 h after oviposition.

In *M. sexta* embryos, organogenesis has been extensively studied and correlated to JH titers. JH titers are undetectable by GC-MS prior to provisional dorsal closure (~54 h after oviposition) (Bergot *et al.*, 1981a; Dorn *et al.*, 1987). The titers rise to their highest level between 60 and 72 h after oviposition, when a true larval cuticle appears. Interestingly, this peak in JH is composed of several homologs, including JH 0 and 4-methyl JH I, that are unique to the embryonic stage of Lepidoptera. The titers drop to less than 50 pg g^{-1} total JH at the time of hatching, approximately 120 h after oviposition.

Using an HPLC/bioassay method to measure JH levels in the embryo of the noctuid *T. ni*, Grossniklaus-Bürgin and Lanzrein (1990) showed that JH titers rise during the period of early segmentation, prior to dorsal closure, and remain detectable throughout the period of early organogenesis. As has been observed in other species, the JH titer increases to its highest level shortly after dorsal closure and then declines at hatching. The extensive embryological landmarks available for *T. ni* permit a direct correlation of the peak in the JH titer with the formation of the first larval cuticle.

A similar JH profile is observed in another noctuid, *S. littoralis* (Steiner *et al.*, 1999). GC-MS quantitation was used to demonstrate that low levels of JH II are detectable prior to dorsal closure, during a period in which segmentation and limb growth is extensive. JH titers are at their highest after dorsal closure and more precisely, highest at the time the true larval cuticle is being formed. Titers drop as the embryo develops, but in contrast to other species where the hormone declines to undetectable levels at hatching, *S. littoralis* maintains elevated titers of JH through to emergence.

Despite the extensive body of work concerning JH and caste differentiation in *A. mellifera* (Huang *et al.*, 1991; Schulz *et al.*, 2002) (see **Chapter 3.13**), there is little information linking embryological markers with JH titers in this species. Rembold *et al.* (1992) found that JH III levels are undetectable by GC-MS until very late in embryogenic development (66 h after oviposition). The late peak in the JH titer (72–76 h) may reflect the fact that the CA are not fully developed until 62–64 h after oviposition (Nelson, 1915). Moreover, dorsal closure occurs late in this species, (62–66 h) which is in keeping with the established developmental correlation between dorsal closure and the peak in the embryonic JH titer. It should be noted that the *A. mellifera* embryo does not undergo blastokinesis, thus making it difficult to relate changes in the JH titer with events surrounding and within this process.

As previously noted, the very early presence of JH in the egg of some species has led several investigators to suggest the origin of the hormone at this stage is maternal. In other words, hemolymph JH from the adult female is sequestered by the embryo during the process of vitellogenesis. Gilbert and Schneiderman (1961), in their pioneering work on JH, demonstrated that newly oviposited eggs contain low levels of JH; however, allatectomy of adult gravid females leads to eggs devoid of JH. They, as well as later investigators (Temin *et al.*, 1986; Steiner *et al.*, 1999), proposed that JH is taken up passively by the embryo. Indeed, presupposing that all JH is derived from the CA, this premise would be true, but the possibility remains that extra-allatal sites are involved in JH biosynthesis. Hartmann *et al.* (1987) demonstrated that the serosa of *L. migratoria* is capable of methylation of JH III acid to form JH III. Whether this process can yield sufficient JH to account for the amount of JH detected before dorsal closure is uncertain, nor is it clear whether other insects utilize this unique tissue for biosynthesis of JH. A summary of JH titers during embryogenesis is seen in **Table 2**.

Do these profiles indicate that JH is requisite at key times during embryonic development? Unfortunately, the markers that denote embryological development vary widely among species and make comparisons difficult. For example, blastokinesisis varies radically among species (Johannsen and Butt, 1941) and does not provide an adequate morphological marker to make generalizations. Moreover, while the movements of blastokinesis may appear similar in many species, this process may not necessarily signify a developmentally defining homologous event (Heming, 2003). By contrast, dorsal closure is an anatomical reference point that signifies a specific developmental event. However, an embryonic cell layer, more commonly referred to as a membrane, is known to create a provisional dorsal closure that occurs earlier than the final

Table 2 JH levels during insect embryogenesis[a]

Order and species	*Oviposition*	*Blastokinesis*	*Dorsal closure or larval cuticulogenesis*	*Reference*
Hemimetabolous				
Orthoptera				
Locusta migratoria	Low[b]	None	High[d]	Temin *et al.* (1986)
Dictyoptera				
Nauphoeta cinerea	None	None	High	Brüning *et al.* (1985)
Blatta orientalis	Low	Low	High	Short and Edwards (1992)
Hemiptera				
Oncopeltus fasciatus	Low	Medium[c]	High	Dorn (1975)
Holometabolous				
Lepidoptera				
Manduca sexta	None	None	High	Bergot *et al.* (1981a)
Spodoptera littoralis	Low	Not determined	High	Steiner *et al.* (1999)
Trichoplusia ni	None	Low	High	Grossniklaus-Bürgin and Lanzrein (1990)
Hyalophora cecropia	Low	Low	High	Gilbert and Schneiderman (1961), Bergot *et al.* (1981)
Hymenoptera				
Apis mellifera	None	None[e]	High	Rembold *et al.* (1992)
Diptera				
Drosophila melanogaster	None	None[e]	None	Bownes and Rembold (1987)

[a]JH titers were determined by GC/MS or HPLC/bioassay.
[b]Low JH titers are those that are less than one-third of the highest level reported.
[c]Medium JH levels are those that fall between one-third and two-thirds of the highest level reported.
[d]High JH levels are those that are greater than two-thirds of the highest level reported.
[e]Blastokinesis is not observed in these species; in these species, JH titers are reported for the period corresponding to approximately half way between oviposition and dorsal closure.

dorsal closure. An example of the confusion this can cause is readily seen in studies of the embryo of *M. sexta*, where a provisional dorsal closure is evident at about 50 h after oviposition (Broadie *et al.*, 1991), while the final dorsal closure becomes apparent only at 102 h after oviposition (Dorn *et al.*, 1987). While knowledge of JH titers offers correlative information when linked to a key point in embryogenesis, it can provide only indirect evidence for the role(s) the hormone plays during this period.

3.7.10.4. Roles of Juvenile Hormone during Embryogenesis

To better understand the embryological roles of JH, two experimental approaches have been taken: chemical allatectomy using precocene and the application of exogenous JH or a JH agonist. Application of JH I or II to eggs of *Thermobia domestica*, an ametabolous insect, any time from oviposition to 2 days before hatching, leads to highly deformed embryos and poor emergence rates (Rohdendorf and Sehnal, 1973). Injeyan *et al.* (1979) applied JH III to *S. gregaria* embryos and found a different pattern of JH-induced disruption. JH applied shortly after oviposition has little effect on embryogenesis, but when applied from days 3 to 9 postoviposition has a profound effect on development, including disruption of blastokinesis, inhibition of postblastokinesis development, and failure of the vermiform larva either to initiate or to complete ecdysis. JH has no effect on embryonic development when applied between day 10 and day 16 (hatching). Disruption of development is accompanied by increased pigmentation of the embryos, and it was discovered that the pigmented regions of treated insects have a much thinner procuticle. In contrast to these results, Sbrenna-Micciarelli (1977) demonstrated that high doses of the analog farnesyl methyl ether applied to the embryo just before blastokinesis induces premature development of the cuticle. Although the results are conflicting, both authors conclude that JH may have a role in cuticulogenesis.

Application of precocene to *N. cinerea* embryos following dorsal closure results in abnormal midgut development, which appears to stem from the failure of ectodermal cells to migrate from the developing stomodaeum and proctodaeum into the midgut rudiments to form the gut epithelium (Brüning *et al.*, 1985). In addition, the absence of JH after dorsal closure leads to disintegration of the fat body and abnormalities in the cuticle. *Oncopeltus fasciatus*

embryos treated with precocene at blastokinesis fail to undergo final dorsal closure, suggesting that JH is involved in development of ectodermal tissue (Dorn, 1982). This developmental aberration can be rescued by topical application of JH. Enslee and Riddiford (1977) demonstrated that blastokinesis in *P. apterus* was vulnerable to exogenous JH, which interfered with dorsal closure and induced truncated appendage development and early pigmentation of developing eye and abdominal structures. These authors suggested that the extraembryonic "membranes" might be the target of JH. Day-old embryos of *R. prolixus*, when treated with high levels of fenoxycarb, do not develop past katatrepsis, and patterns of protein synthesis, as determined by two-dimensional gel electrophoresis, are significantly altered (Kelly and Huebner, 1987). In yet another hemipteran, *Aphis fabae*, precocene inhibits embryogenesis, but the process can be rescued by JH (Hardie, 1987). Interestingly, JH appears to accelerate embryonic development via a mechanism independent of the parthenogenic reproductive strategy employed by aphids.

The effect of JH or analogs on the embryogenesis of holometabolous insects is similar to that seen in hemimetabolous insects. Unfortunately fewer studies have been carried out and all of them utilize the addition of exogenous JH to determine an effect. In Lepidoptera, JH injected into the female prior to oviposition halts embryogenesis at formation of the blastoderm, while JH applied directly to the egg shortly after oviposition has no effect on blastoderm formation, but blocks blastokinesis (Riddiford, 1970). Even without blastokinesis, embryos continue larval differentiation and form recognizable but incomplete first instars, as in *P. apterus* (Enslee and Riddiford, 1977). In another lepidopteran embryo, that of *Plodia interpunctella*, JH agonists induce aberrant dorsal closure and a loss of trachea (Dyby and Silhacek, 1997). A very different situation exists in *D. melanogaster*, a species in which no JH is detected during embryogenesis (Bownes and Rembold, 1987). While JH analogs can disrupt development if applied very early, large doses of JH I do not have an effect on embryogenesis (Smith and Arking, 1975).

The underlying molecular role of JH during embryogenesis is even more obscure. In an early study, Rao and Krishnakumaran (1974) attempted to elucidate the role of JH at the molecular level. When these investigators examined morphological alterations and changes in DNA synthesis in embryos of *A. domesticus* challenged with JH I, they found a qualitative drop in incorporation of thymidine into DNA, as monitored by autoradiography. Embryonic epidermal tissue is the first to show reduced incorporation of thymidine in the presence of exogenous JH; however, some tissues appear refractory to the hormone. Interestingly, JH-treated *A. domesticus* embryos develop legs and antennae that are short and stumpy, which led Rao and Krishnakumaran (1974) to suggest that JH may suppress DNA synthesis and cell division in the embryonic appendages.

Despite the paucity of information, it is tempting to speculate that embryonic ectodermal tissue is a major target of JH. The effects of JH on cuticular morphology, dorsal closure, and pigmentation of embryonic epidermal structures support this idea, and are in keeping with the function of JH during larval development. The fact that JH acts on midgut and fat body development in at least some species indicates there also may be a wider role for the hormone.

3.7.10.5. Juvenile Hormone Binding Proteins of the Embryo

The JHBPs have long been known to modulate the catabolism of hemolymph JH during the larval period (Hammock *et al*., 1975; Roe and Venkatesh, 1990; deKort and Granger, 1996) (see Section 3.7.9). It is generally assumed that JHBP has a unique hormone-binding domain whose steric arrangement hinders the access of catabolic enzymes to JH (Goodman, 1990). While the roles of the hJHBP in larval development are well defined (see Section 3.7.8.3), the role of embryonic JHBP is less clear. It is now clear that significant levels of JHBP are present throughout embryogenesis even though the circulatory system is not yet fully functional until late in development, and JH levels, as noted above, do not rise until the insect is midway through embryonic development.

In *L. migratoria*, a JHBP similar to that found in nymphal hemolymph is present in both the embryo and serosa (Hartmann *et al*., 1987), prior to the development of the CA and a functional circulatory system. These investigators demonstrated that the serosa has a methyl transferase capable of converting JH III acid to JH III, and surmised that the embryonic JHBP serves either to distribute the hormone or to act as a buffer, protecting the embryo from excess maternal JH. The hypothesis of a protective function for embryonic JHBP is supported by the fact that JH levels in the gravid female are at least an order of magnitude higher than in the eggs (Temin *et al*., 1986). Thus, the JHBP could act as a "sponge", keeping the JH accessible to catabolic enzymes that can readily inactivate the hormone.

In *M. sexta*, a hJHBP-like molecule is present at oviposition and remains at detectable levels throughout embryogenesis (Touhara and Prestwich, 1994; Touhara *et al.*, 1994). A re-examination of the characteristics and titers of the embryonic JHBP demonstrated that the embryonic JHBP is identical in binding characteristics (Touhara and Prestwich, 1994; Touhara *et al.*, 1994), molecular mass, and immunochemical properties to the larval hemolymph form (Orth *et al.*, 2003a). Furthermore, the nucleotide sequence of the embryonic *JHBP* gene is identical to that of the larval gene (Orth *et al.*, 2003b); thus, it can be concluded that the embryonic JHBP is identical to hJHBP. Expression of the *JHBP* gene begins in both the embryo and the serosa approximately 15 h after oviposition, peaks between 24 and 36 h, and then declines to undetectable levels at 72 h. The burst of *JHBP* expression occurs before emergence of functional CA, fat body, or circulatory system. In contrast to its gene expression, titers of JHBP are at their highest during the first half of embryogenesis (**Figure 17**). The titer declines rapidly between 36 and 60 h after oviposition and then slowly drops to its lowest level at the time of emergence. The peak of gene expression does not appear to enhance the already high levels of JHBP and the reason for this temporal discordance between gene expression and the JHBP titer is unclear. As in the case of *L. migratoria*, it appears that both the serosa and the embryo of *M. sexta* are capable of expressing *JHBP*. Curiously, the expression of *JHBP* in *M. sexta* begins about 15 h after oviposition, corresponding to the time at which the serosa becomes active in secretion of the first of several embryonic "membranes" (Lamer and Dorn, 2001).

Most of the embryonic JHBP in *M. sexta*, especially in the early hours following oviposition, is maternally derived and is assumed to be sequestered from hemolymph during the process of vitellogenesis. The presence of relatively high levels of the protein prior to development of the CA or circulatory system suggests that, as in *L. migratoria*, high levels of JHBP in the early embryo of *M. sexta* ensure low levels of unbound JH, thus protecting the embryo from excess JH (Orth *et al.*, 2003b). JH bioassay data indicate that hemolymph JH titers in adult female *M. sexta* are as high as those found in the early fourth stadium larvae (Judy, personal communication). Thus, during the early part of embryogenesis, the maternally derived JHBP acts as an effective buffer, constantly binding and releasing the hormone in accordance with equilibrium conditions. Embryonic JHE displays a developmental profile similar to that of JHBP (Share *et al.*, 1988) and together, these proteins regulate bioavailability of the hormone to the developing embryo.

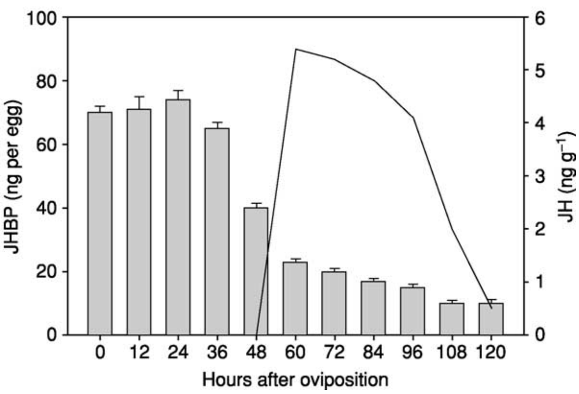


Figure 17 Titers of JH and juvenile hormone binding protein during embryological development of *M. sexta*. Bars, JHBP mean titers (± SD) (JHBP adapted from Orth, A.P., Tauchman, S.J., Doll, S.C., Goodman, W.G., **2003b**. Embryonic expression of juvenile hormone binding protein and its relationship to the toxic effects of juvenile hormone in *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 1275–1284. Line (—), total JH titer data adapted from Bergot, B.J., Baker, F.C., Cerf, D.C., Jamieson, G., Schooley, D.A., **1981a**. Qualitative and quantitative aspects of juvenile hormone titers in developing embryos of several insect species: discovery of a new JH-like substance extracted from eggs of *Manduca sexta*. In: Pratt, G.E., Brooks, G.T. (Eds.), Juvenile Hormone Biochemistry. Elsevier/North-Holland, Amsterdam, pp. 33–45.

3.7.10.5.1. Juvenile hormone catabolism during embryogenesis: methyl ester hydrolysis Embryonic titers of JH are the result of both biosynthesis and catabolism. While this area has received considerable attention in the larval stage (see Section 3.7.9), scant attention has been focused on these catabolic enzymes in the egg. Roe *et al.* (1987a, 1987b) examined the metabolism of JH in the eggs of the cricket *A. domesticus*. Their studies found that the major route of metabolism is the conversion of JH to JH acid. They reported that general esterase activity, as determined using α-naphthyl acetate as a substrate, remains relatively constant throughout embryonic development. In contrast, JHE activity is high in unfertilized eggs and remains high until the time of dorsal closure, when it drops to its lowest level and remains relatively low until nymphal emergence.

In a very thorough analysis of JH titers (Brüning *et al.*, 1985), JH biosynthetic rates, and JHE activity in *N. cinerea* embryos, Lanzrein and her colleagues demonstrated that JHE activity is very low following dorsal closure (20 days after oviposition) (Bürgin and Lanzrein, 1988). **Figure 18** shows that the massive peak representing both JH III and MF begins to decline very rapidly when JHE activity

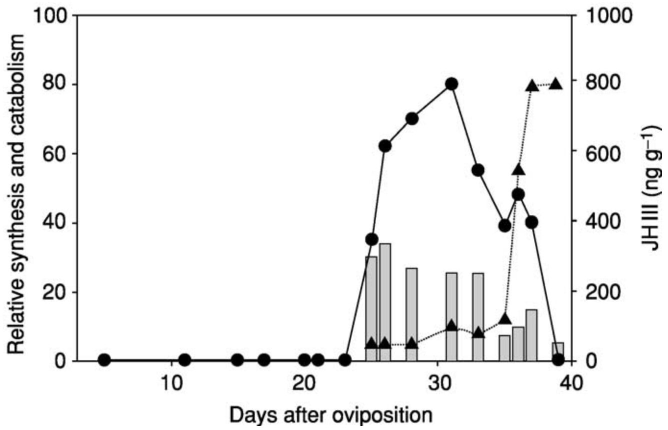


Figure 18 JH titers, JH biosynthetic rates, and JHE activity from *Nauphoeta cinerea* embryos. Closed circles, JH III titers (Brüning *et al.*, 1985); bars, relative JH III biosynthesis as determined by the radiochemical assay (Bürgin and Lanzrein, 1988); closed triangles, JHE activity (Bürgin and Lanzrein, 1988). Dorsal closure occurs at day 20.

rises at day 35. According to these investigators, the rise in JHE corresponds to the period of chorion breakdown; as a result, the increase in available oxygen significantly changes the physiology of the rapidly developing embryo. Interestingly, JHE activity does not appear to be localized to the hemolymph, but rather is distributed generally throughout the entire embryo (Bürgin and Lanzrein, 1988). Since JH III titers in the first instar nymphs are low (1ng g^{-1}), the dramatic rise in JHE activity may be necessary to clear both JH and MF prior to nymphal emergence (Brüning *et al.*, 1985).

The rapid rise in JHE activity observed at the end of embryonic development in *N. cinerea* contrasts with that observed in *M. sexta*. In this insect, JHE activity is high in preovipositional eggs, but declines well before provisional dorsal closure (Share *et al.*, 1988). JHE activity remains relatively constant throughout the rest of embryonic development and does not show the dramatic increase in activity that is observed in *N. cinerea* (Bürgin and Lanzrein, 1988). Despite the presence of JHE activity, JH titers increase dramatically in the period surrounding larval cuticulogenesis (Bergot *et al.*, 1981a). The rise in the JH titer, in the presence of significant JHE activity, contradicts the generally held view that JHE is involved in inactivation of the hormone (see Section 3.7.9.1). It may well be that JH and JHE are sufficiently compartmentalized at the onset of cuticulogenesis that catabolism of the hormone is limited. Once the larval circulatory system is fully functional, approximately 24 h later as determined by vigorous dorsal vessel contraction (Broadie *et al.*, 1991), contact between the hormone and the catabolic enzymes result in a reduced JH titer as the enzyme carries out its anticipated function.

3.7.10.5.2. Juvenile hormone catabolism during embryogenesis: epoxide hydrolysis While most studies on JH catabolism have been focused on the JHEs, a handful of investigators have examined the action of the JHEH (see Section 3.7.9.2). Share *et al.* (1988), in their studies on JH metabolism during embryogenesis in *M. sexta*, demonstrated that JHEH activity is responsible for only a small amount of the total catabolic activity. They found that this enzyme hydrolyzes JH III about six times better than JH I, but they did not take into account the fact that JH I binds to the JHBP with a significantly higher affinity and thus protects the hormone from degradation (Park *et al.*, 1993).

The first characterization of a specific JHEH early in development was carried out by Touhara and Prestwich (1993) in a study of JH metabolism in eggs of *M. sexta*. Using the JH photoaffinity label [^{3}H]epoxyfarnesyl diazoacetate, these investigators demonstrated that the radiolabeled analog covalently attaches to two proteins in the egg, the juvenile hormone binding protein and a 50 kDa protein that was identified as a JHEH. The majority of the JHEH activity is found in the microsomal fraction of the egg homogenate. The purified JHEH displays an apparent K_m of 0.61 μM for JH I and 0.28 μM for JH III, confirming the earlier observation by Share *et al.* (1988) that JH III appears to be the preferred substrate. A subsequent characterization of recombinant JHEH from *M. sexta* reconfirmed this substrate specificity (Debernard *et al.*, 1998). This study also found that the hJHBP protects the hormone from epoxide hydration, but whether this occurs *in vivo* is uncertain since the binding protein is present in the hemolymph (with the exception of the fat body), while the JHEH is localized to the cellular compartment.

3.7.10.6. Juvenile Hormones and the Evolution of Insect Metamorphosis

Truman and Riddiford (1999, 2002) have proposed that the temporal appearance of JH during embryonic development may have played an important role in the evolution of holometabolic insects. Their hypothesis is based, in part, on the idea that ancestral insects displayed three stages after embryogenesis: the pronymph, the nymph, and the adult. The pronymphal stage represents a specialized hatching stage in ametabolous and hemimetabolous insects that is (1) nonfeeding, (2) often mobile, (3) often surrounded by an embryonic cuticle, and (4) different in body proportions from that of the first instar. Depending upon the species, the pronymphal stage is initiated after completion of blastokinesis and may end prior to hatching or persist for several days after hatching. The Truman and Riddiford hypothesis states that the pronymphal and nymphal stages of this insect are developmentally equivalent to the larval and pupal stages, respectively, of the holometabolus insect. Moreover, there is a requirement for a JH-free period in ametabolous and hemimetabolous species that allows nymphal development to proceed towards adult stage.

Can experimental manipulation mimic the evolutionary processes that led to the transition to the holometabolous state? As noted earlier, JH can induce certain tissues in hemimetabolous species to undergo apparent premature differentiation before those tissues are completely patterned. This curious phenomenon has led Truman and Riddiford (1999, 2002) to suggest that the early appearance of JH during embryogenesis, either experimentally or naturally, modifies the developmental trajectory of the holometabolous insect to the adult stage, giving rise to a protolarval stage. From an evolutionary standpoint, advancing the time at which JH appears during embryogenesis may have been crucial to the transition from the pronymphal stage of the hemimetabolous insect to the protolarval stage of holometabolous species. While this hypothesis deserves much merit for simplicity and elegance, it is not without criticism (Heming, 2003). As demonstrated in **Table 2**, JH titers have been rigorously determined in only a handful of species, thus leaving any generalizations about the endocrine-driven rise of holometabolous development open to question. Moreover, working definitions of key embryogenic events, such as blastokinesis and dorsal closure, may be interpreted differently, depending on the investigator. The problem is further compounded by contradictory data on the time at which JH titers rise in relation to the key embryonic events. Truman and Riddiford (2002) suggest that JH titers in the embryo of the lepidopteran *M. sexta* are highest at the time of katatrepsis, yet Ziese and Dorn (2003) place katatrepsis at a time when JH titers (Bergot *et al*., 1981a) are undetectable. Assigning an embryological role to JH when the levels are low raises yet another issue: low JH titers at oviposition and at blastokinesis in some species contradict the hypothesis that a JH-free period is required for further normal development (**Table 2**). While the Truman and Riddiford hypothesis about the rise of holometabolous insects presents an intriguing model that deserves further critical study, the role that JH may have had in this process is still open.

3.7.11. Juvenile Hormones in Premetamorphic Development

3.7.11.1. Juvenile Hormone Titers

The premetamorphic titers of JH appear to vary greatly among the insect orders, with some orders, such as Dictyoptera, displaying levels 100-fold greater than Lepidoptera (Gilbert *et al*., 2000). Yet one pattern has remained constant since the discovery of JH in the 1930s: JH titers, on average, are high while the larva is growing and feeding but drop at a well-defined point to permit metamorphosis. This pattern can be further refined to distinguish between hemimetabolous and holometabolous insects (Riddiford, 1994). In hemimetabolous insects, JH titers are low to undetectable during the final stadium. In holometabolous insects, JH titers are relatively high at the beginning of the final larval stadium but decline to undetectable levels prior to the cessation of feeding. The absence of JH permits the release of the prothoracicotropic hormone (PTTH) from specific cerebral neurosecretory cells (Rountree and Bollenbacher, 1986), and PTTH then induces ecdysteroid synthesis by the prothoracic glands, initiating a small rise in hemolymph ecdysteroid levels (Bollenbacher *et al*., 1981) (see **Chapter 3.2**). This rise in the ecdysteroid titer in the absence of JH initiates metamorphosis. In contrast to the hemimetabolous insects, a second increase in the hemolymph JH titer is typically observed after the insect has found a suitable pupation site. This peak in the titer is thought to prevent precocious adult differentiation of imaginal discs and other imaginal precursors (Riddiford, 1994).

3.7.11.2. Potential Problems with Titer Determinations

One of the major impediments to better understanding the role(s) of JH is the lack of precise titers

during biologically relevant periods. Virtually all hormones studied in vertebrates display daily oscillations in their titer and release and are, in many cases, linked to critical homeostatic events (Czeisler and Klerman, 1999). Early studies demonstrated a daily, bimodal, rhythmic fluctuation in the size of the nuclei in CA of adult *D. melanogaster* (Rensing, 1964). While size of the CA cells does not necessarily indicate a gland active in JH biosynthesis (Tobe and Stay, 1985), there is ample evidence that indicates enlarged glands are biosynthetically active (Chiang *et al*., 1989, 1991). Thus, fluctuations in CA cell size suggests circadian fluctuations in JH synthesis.

The importance of examining titer fluctuations in detail can be seen in the elegant work from Steel's laboratory, which demonstrates a circadian rhythmicity of ecdysteroid titers in nymphal *R. prolixus* (Steel and Ampleford, 1984; Vafopoulou and Steel, 2001) (see **Chapter 3.11**). A single-point JH determination every 24 h may not be a serious problem for the student of metamorphosis, where JH-regulated events stretch over a period of several days. However, it does make a difference when relatively rapid changes in JH titer regulate critical cellular events that occur during a short time period. Recent studies demonstrating that JH titers fluctuate rapidly in adult insects supports the premise that detailed studies of JH titers during the premetamorphic stages are needed. Elekonich *et al.* (2001) found that honeybee foragers show a diurnal increase in hemolymph JH titers, from about 100 ng ml^{-1} in the late morning to over 350 ng ml^{-1} by late evening, a period spanning less than 12 h. A similar change is seen in the fourth stadium of *M. sexta*, where titers initially rise and then drop significantly over an 8 h period and may have some influence on levels of the transport protein, hJHBP (Fain and Riddiford, 1975; Orth *et al*., 1999). This is not to infer that JH titers must be absolutely accurate; the important information is knowledge of changes in the relative titers at well-defined times.

However, even if precise staging is possible, the measurement of JH in samples from species or stages displaying low levels of hormone, or from smaller sized species, requires that tissue collections be pooled from a number of individuals. Valuable information about population variability is thus lost. Ideally, a single individual should be sampled sequentially over time, but this process leads to wound-induced changes (Caveney, 1970) that may significantly alter JH titers. In our hands, wounding has rapid and significant effects on hJHBP mRNA expression, which potentially modulates JH titers (Orth and Goodman, unpublished data). Even moderate handling prior to sample collection may have an effect on JH titers (Varjas *et al*., 1992).

3.7.11.3. Premetamorphic Roles of the Juvenile Hormones

The roles of JH in the stages preceding metamorphosis to the adult are ubiquitous, affecting behavior, organs and tissues, cellular organelles, and biochemical pathways. Several excellent reviews have covered these areas (Nijhout, 1994; Riddiford, 1994, 1996), and the focus here is on research since that time.

3.7.11.3.1. Behavioral and neuronal responses There are a number of behavioral phenomena ascribed to JH during the adult stage, including pheromone production and calling (review: Cusson *et al*., 1994), migration (review: Dingle and Winchell, 1997), phonotaxis (Stout *et al*., 1992, 1998; Bronsert *et al*., 2003), and caste determination and anatomical changes in the brain of honeybees (reviews: Robinson and Vargo, 1997; Elekonich and Robinson, 2000) (see **Chapter 3.13**). However, little is known about the effect of JH on behavior during the premetamorphic period, and most of these studies have been done in social or migratory insects.

Late in the third stadium, honeybee larvae destined to become queens have JH titers five times higher than larvae destined to become workers (Rachinsky and Hartfelder, 1991; Rembold *et al*., 1992) (see **Chapter 3.13**). In another hymenopteran, the ant *Phiedole bicarinata*, large doses of methoprene, if applied during a critical period in the last stadium, induce worker-destined larvae to become soldiers (Wheeler and Nijhout, 1981). Higher JH titers during the last stadium of certain migratory species appear to elicit a stationary adult stage, rather than migration (Yagi and Kuramochi, 1976; Nijhout and Wheeler, 1982). Yin *et al*. (1987) demonstrated that methoprene can affect the circadian system in the lepidopteran *Diatraea grandiosella*, inducing a dose-dependent phase shift in adult eclosion.

At the neuronal level, most endocrine studies have focused on the effect of ecdysteroids on the neural circuitry during metamorphsis (see **Chapter 2.4**), although JH appears to play a role as well. Truman and Reiss (1988) demonstrated that reorganization of neurons during metamorphosis of *M. sexta* is, in part, under the control of JH, but the regulatory elements involved are still undefined. Recent work suggests that JH affects larval neurons innervating the prothoracic gland of the cockroach *P. americana* (Richter and Gronert, 1999). Exposure of the insect to exogenous JH III and methoprene both *in vivo* and *in vitro* induces a short-term depression of spike

activity in neurons innervating the prothoracic gland, but has no effect on the nervous connectives of the stomatogastric nervous system. This response to JH III is very rapid, occurring within 3 min of treatment and reaching a low (75% reduction) within 15 min. Curiously, fenoxycarb, a potent JH analog, was only half as active as JH III and methoprene; methyl laurate, a control lipid, had no effect. The authors speculate that JH is acting directly on membrane receptors to elicit the reduced neurotropic activity; this observation relates to an earlier hypothesis that JH can influence inhibitory neurotransmitter receptors such as γ-aminobutyric acid (GABA) (Stout *et al.*, 1992).

3.7.11.3.2. Epidermal responses It has long been known that JH has a marked influence on epidermal and cuticular structure. The early work of Williams (1952) demonstrated that JH-containing extracts, when applied at a developmentally sensitive time during the pupal period, can induce a second pupal cuticle. This discovery prompted Williams to suggest that JH is a "status quo" hormone, a label that is still in frequent use (Willis, 1996). The integument of the insect is composed of an outer layer of secreted proteins, lipids, pigments, and complex carbohydrates termed the cuticle. The single layer of cells immediately beneath the cuticle, the epidermis, is responsible for synthesis and secretion of most, but not all, of the proteins found in the cuticle (Willis, 1996). The epidermis itself contains another subset of proteins, pigment-associating proteins, the expression and position of which in the cells appear to be regulated by JH. The radical changes in morphology at metamorphosis led Wigglesworth (1959) to predict that each stage of development had its own set of unique genes. While there does appear to be a limited group of stage-specific cuticular proteins, especially in the case of the higher flies, current evidence demonstrates that many cuticular proteins can be found in more than one stage of the life cycle (review: Willis, 1996).

Hormonal regulation of epidermal gene expression has been examined in a number of insect species, but the most extensive studies, by Riddiford and her colleagues, have been carried out on the epidermal genes of *M. sexta* (Riddiford, 1994, 1996; Riddiford *et al.*, 2001, 2003). These include the larval cuticular protein 14 (LCP14) (GenBank Accession no. 813279) (Rebers and Riddiford, 1988), LCP14.6 (GenBank Acc. No. U65902) (Rebers *et al.*, 1997), LCP16/17 (GenBank Accession no. M25486) (Horodyski and Riddiford, 1989), the biliverdin-associating proteins, insecticyanin a and b (GenBank Accession nos. 864714 and 864715) (Li and Riddiford, 1992), and dopa decarboxylase (DDC) (GenBank Accession no. U03909) (Hiruma and Riddiford, 1988; Hiruma *et al.*, 1995). Studies using the epidermis are particularly compelling, since the results of *in vitro* manipulations mirror those observed *in vivo*, and importantly, the genomic structure and flanking regions of their genes have been determined.

LCP14 is a larval-specific gene that encodes for a 14 kDa protein expressed during the feeding period of each stadium (Riddiford, 1994). At the onset of a larval molt, mRNA for LCP14 rapidly becomes undetectable and remains so until the insect molts to the next stadium, at which time the expression levels rise again and the process repeats itself. In the last stadium, levels of LCP14 message rise during the first several days, but fall sharply prior to wandering and are no longer detectable. *In vitro* manipulation indicates that 20E suppresses the expression of this gene. If JH is present when 20E titers rise, suppression by 20E is only transient; if JH is absent, the gene is permanently silenced. While it is uncertain how JH acts at the molecular level to maintain the expression of *LCP14*, its function may involve the transcription factor βFTZ-F1. It has been shown that there are three potential binding sites for βFTZ-F1 in the *LCP14* gene, one approximately 2 kb upstream from the start site and two in the first intron (Lan, personal communication). The expression of *βFTZ-F1* begins about 16 h before molting, peaks at about 9 h before molting, and then ceases approximately 3 h before molting (Weller *et al.*, 2001). Functional analysis of the putative *LCP14* promoter indicates that the βFTZ-F1 response elements in the first intron are involved in downregulating *LCP14* expression (Lan, personal communication). The decline in βFTZ-F1 expression coincides with the rise in JH at the very beginning of the fifth stadium and with it, the increase in *LCP14* expression. Whether JH acts directly on the gene, or is involved in modulating the ecdysteroid effect, remains unclear.

LCP14.6 is another hormonally controlled cuticular gene, which, like *LCP14*, is downregulated by 20E, but in contrast to *LCP14*, is suppressed *in vitro* by large doses of methoprene (Riddiford, 1986). Its expression is temporally and spatially complex and is not stage-specific, since it occurs in the larval, pupal, and adult stages. A comparison of its expression pattern with the JH titer profile suggests that, *in vivo*, *LCP14.6* expression may be suppressed by the hormone. *LCP16/17* encodes a multigene family of three proteins that appear midway through the feeding period of the fifth stadium (Horodyski and Riddiford, 1989). The developmental appearance of

these proteins correlates with a thinning of cuticular lamellae and a corresponding increase in stiffness. Like *LCP14.6*, expression of the *LCP16/17* is suppressed by large doses of methoprene.

One of the more striking effects elicited by JH is its action on larval pigmentation (Nijhout, 1994; Applebaum *et al.*, 1997). Nowhere is this more evident than in larval *M. sexta*, where two mutants have been discovered with anomalous pigmentation and aberrant JH titers. In the *bl* mutant (Safranek and Riddiford, 1975), JH titers are low at certain critical developmental points and the larvae are highly melanized; in the white mutant, the opposite is the case (Panchapakesan *et al.*, 1994). To maintain the normal wild-type phenotype following a molt, i.e., a transparent cuticle and an epidermis with intracellular vesicles containing the biliverdin-associating protein, insecticyanin (Riley *et al.*, 1984; Goodman *et al.*, 1985), JH titers must be sufficiently high at the time of head capsule slippage (Hiruma and Riddiford, 1988; Riddiford, 1994). If JH titers are too high at the time of head capsule slippage, the resulting cuticle will be transparent, and the underlying epidermal cells will lack insecticyanin vesicles, causing the insect to appear white (Panchapakesan *et al.*, 1994). Conversely, if JH titers are too low at the time of head capsule slippage, the resulting cuticle will be highly melanized and the epidermis devoid of insecticyanin vesicles, causing the insect to appear black (Goodman *et al.*, 1987).

Insecticyanin (Ins) is a 21 kDa protein that associates with biliverdin IX to yield an intensely blue protein important to larval camouflage (Goodman *et al.*, 1985). Ins is found in epidermal cells, where it is sequestered in 1 μm vesicles (**Figure 19**). These vesicles, together with other epidermal and cuticular pigments, give the wild-type insect its distinctive blue–green hue during the feeding period (Riddiford *et al.*, 1990). Analysis of epidermal ultrastructure indicates that the densely packed vesicles appear to be held in position by a web of microtubules (Goodman, unpublished data). A typical wild-type epidermal cell contains approximately 100 of the membrane-coated vesicles, localized in the apical region. The epidermal cell also secretes significant quantities of Ins into the hemolymph, making it one of the more prominent hemolymph proteins (Riddiford *et al.*, 1990). At commitment to pupation, when JH is no longer present and ecdysteroid levels rise, the entire population of Ins-containing vesicles is secreted from the epidermal cell (Sedlak *et al.*, 1983) and the insect ceases production of the protein (Goodman *et al.*, 1987; Riddiford *et al.*, 1990). Interestingly, the *bl* mutant, which has a low JH titer at head capsule slippage, lacks the epidermal Ins-containing vesicles, thus mimicking the ultrastructure of the wandering fifth stadium epidermis. Topical application of JH to *bl* mutants, just prior to head capsule slippage, will induce the appearance of the Ins vesicles in the next stadium, similar in number and position to those in the wild-type (Goodman *et al.*, 1987) (**Figure 19**). Moreover, JH, when applied at head capsule slippage, prevents cuticular melanization in the next stadium (Safranek and Riddiford, 1975). The role of JH in this process is still unclear but ultrastructural data suggests that the cytoskeleton may be involved in retaining the Ins vesicles in the apical portion of the epidermal cell.

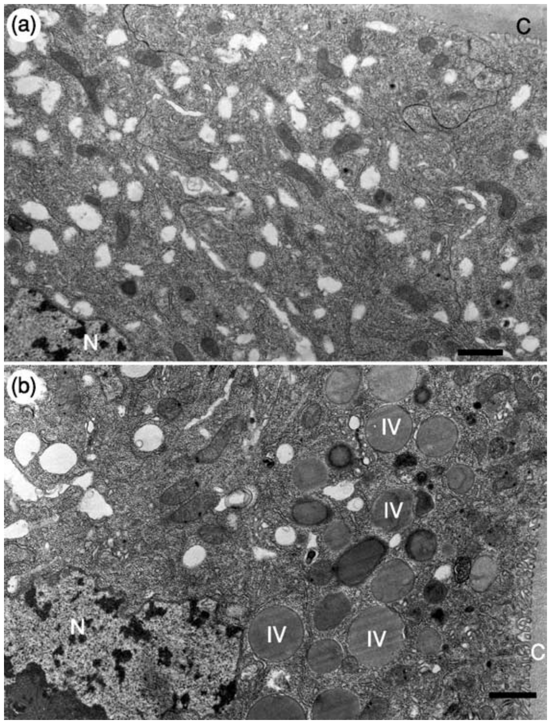


Figure 19 Electron micrographs of the epidermis of the *bl* strain of *M. sexta*. Fourth instars, just prior to head capsule slippage, were treated with either acetone (a) or 50 ng JH I dissolved in acetone (b). Tissue was collected approximately 30 h later, at the time of the fifth larval ecdysis, and prepared for microscopy. C, cuticle; IV, insecticyanin vesicle; N, nucleus. Scale bars represent 1 μm. (Goodman, unpublished data.)

In addition to the presence of Ins mRNA in the epidermis of wild-type larvae, Ins mRNA has also been found in the fat body (Li and Riddiford, 1994; Li, 1996); however, the protein could not be detected immunologically unless pericardial cells were included in the preparation (Goodman *et al.*, 1987). Curiously, JH appears to upregulate epidermal Ins mRNA abundance, while it downregulates this mRNA in the fat body (Li, 1996). As in the case of *LCP14*, 20E downregulates the epidermal Ins mRNA (Riddiford *et al.*, 1990) during the molting period.

Cloning of the *Ins* gene revealed a pair of duplicated genes termed *Ins a* and *Ins b* (Li and Riddiford, 1992) that are expressed in both the epidermis and fat body. Only the product encoded by the *Ins b* gene is found in the hemolymph (Riddiford, 1994). The difference in expression patterns between *Ins a* and *Ins b* led Li (1996) to investigate the structure of the genes, to determine whether different promoters were involved. Each of the genes displays a unique sequence in the 3′ noncoding region, suggesting differential expression. Preliminary computational analysis of the 5′ upstream region of *Ins a* indicated no known response elements (Lan, personal communication).

DDC is the enzyme responsible for catalyzing the conversion of dopa to dopamine (Hiruma and Riddiford, 1984) while granular phenoloxidase (PO) catalyzes the oxidative dehydrogenation of diphenols to quinones (Hopkins and Kramer, 1992) (see **Chapter 4.4**). For cuticular melanization to occur, vesicles containing dopamine, the granular proenzyme form of PO, and other enzymes necessary for melanin synthesis are secreted into the new endocuticle following head capsule slippage. If JH titers are low or absent at the time of head capsule slippage, the proenzyme form of PO will be synthesized by the epidermis and deposited in the endocuticle for a period of approximately 12–14 h. Approximately 3 h before ecdysis, PO is activated, possibly by proteolytic activation (Jiang *et al*., 2003), and melanin begins to appear in the new cuticle. Topical application of JH to *bl* larvae, or to wild-type larvae that have been neck-ligated at head capsule slippage, will block phenoloxidase activity, and a new transparent cuticle will be formed (Riddiford *et al*., 2003). While JH suppresses cuticular melanization in a striking fashion, it remains unclear whether the hormone is acting on transcription or translational events that regulate PO activity.

Hormonal regulation of epidermal DDC (EC 4.1.1.28) in *D. melanogaster* and *M. sexta* has been extensively studied by the Hodgett (Chen *et al*., 2002a, 2002b) and Riddiford laboratories (Riddiford, 1994; Riddiford *et al*., 2003). In *M. sexta*, 20E works directly on epidermis to block DDC synthesis; however, as the ecdysteroid titers decline during the head capsule slippage period, levels of DDC begin to rise and they peak near the time of the larval molt. A study of the 5′ upstream region of the *DDC* gene indicates that there are several transcription factor binding sites, including a βFTZ-F1 response element between the TATA box and the transcriptional start site (Hiruma *et al*., 1995). Recently, Beckstead *et al*. (2001) demonstrated that the *D. melanogaster* gene *bonus* (*bon*) encodes a homolog of the vertebrate TIF1 transcriptional cofactors. Among the many phenotypes associated with this gene is pigmentation (Beckstead *et al*., 2000). *Bon* binds to the AF-2 activation domain present in the ligand-binding domain of βFTZ-F1 and behaves as a transcriptional inhibitor *in vivo*. A transcription factor implicated in regulation of melanization as well as metamorphosis is the Broad-complex (Br-C). In the metamorphosis of *D. melanogaster*, Br-C is a required mediator in the *DDC* response to ecdysteroids. At pupariation and at eclosion, Br-C uncouples *DDC* from an active silencing mechanism that functions through two distinct *cis*-acting regions of the *DDC* locus (Chen *et al*., 2002a, 2002b). While much is known about *DDC* and its regulation via ecdysteroids, the role of JH in its control remains unclear (Hiruma *et al*., 1995; Chen *et al*., 2002a, 2002b; Riddiford *et al*., 2003). It has been demonstrated that the epidermis of allatectomized *M. sexta* larvae displays approximately 50% more DDC activity than epidermis from sham-operated insects, and that activity of the enzyme can be suppressed by moderate levels of JH I. While this observation is certainly provocative, it remains to be seen whether the JH effect on *DDC* expression is direct, or as some speculate, indirect (Hiruma, personal communication).

3.7.11.3.3. Fat body responses The premetamorphic fat body is responsible for metabolism of nutrients, synthesis of most hemolymph proteins, and detoxification of xenobiotics (Locke, 1984; Sondergaard, 1993; Haunerland and Shirk, 1995). Given the central roles this tissue plays, it is not surprising that an extensive body of literature has focused on the role of JH in regulating cellular and molecular events occurring in the fat body.

Numerous studies involving hormonal regulation of ultrastructural changes in adult fat body, especially those regarding vitellogenesis (see **Chapter 3.9**), have been conducted, but few have focused on the ultrastructural changes induced by JH in the larval fat body. Such experiments are problematic since JH titers are already relatively high during most of larval life, and challenging the fat body with additional JH may result in pharmacological responses that mask the actual underlying events. Thus, the most comprehensive studies are developmental, correlating JH titers with ultrastructural changes occurring during the premetamorphic and metamorphic periods (Locke, 1984; Dean *et al*., 1985). In one series of studies, it was found that during the last stadium of both hemimetabolous

and holometabolous insects, when JH titers are dropping, the fat body synthesizes and secretes several hexameric proteins collectively termed storage proteins (Levenbook, 1985). These proteins are retrieved from the hemolymph by the fat body and stored in relatively large vesicles for later use during the pupal–adult transformation (Locke, 1984; Dean *et al.*, 1985). It has also been demonstrated that a JH analog affects fat body nuclei. Treatment of nymphal *L. migratoria* with high levels of methoprene leads to an increase in the size of fat body nuclei, which could be linked to either increased transcription rates or to increased ploidy levels (Cotton and Anstee, 1991). The latter option seems likely, since Jensen and Brasch (1985) demonstrated that allatectomy of adult *L. migratoria* prevents polyploidization, while methoprene restores the process.

At the molecular level, the role of JH on fat body gene expression has been studied by a number of investigators in several different insect species. Certain of these gene targets are discussed below (see Section 3.7.12), and a comprehensive discussion of the various fat body genes thought to be under the control of JH has been presented by Riddiford (1994). One of the most well-studied groups of premetamorphic fat body genes and proteins is the hexamerins (Burmester *et al.*, 1998; Burmester, 2002). The insect hexamerins encompass at least five different subgroups based on amino acid sequence, including: the coleopteran and dictyopteran JH-suppressible arylphorins, the lepidopteran JH-supressible hexamerins, lepidopteran aromatic amino acid-rich arylphorins, the lepidopteran methionine-rich hexamerins, and the dipteran arylphorins (Beintema *et al.*, 1994). The products encoded by the hexamerin genes are expressed predominantly during late larval life, and thus appear to be negatively regulated by JH. Another group of proteins in the hexamerin superfamily are the hemolymph cyanoproteins (Miura *et al.*, 1998) and the orthopteran hemolymph JH binding proteins (Koopmanschap and deKort, 1988; Braun and Wyatt, 1996). As first demonstrated by Jones *et al.* (1988, 1990a), the expression patterns of certain fat body genes are downregulated by JH and JH analogs; however, exceedingly high doses of JH analogs were used to elicit the responses. More recently, Hwang *et al.* (2001) and Cheon *et al.* (2002) have isolated two genes encoding hexamerins that are JH-suppressible at low doses of JH analog. Expression of these genes is not dose-dependent, but can be downregulated within a 6 h period, suggesting that JH is acting directly on transcription. Thus, a general pattern is emerging that indicates fat body hexamerin expression is suppressed in the presence of JH.

While few insect endocrinologists would argue with the idea that the fat body is a target for JH, there remain technical difficulties in working with the fat body. Its cellular heterogeneity, massive tracheal penetration, and apparent sensitivity to wounding or to culture *in vitro* make experimental manipulation of the premetamorphic fat body difficult. More troubling is the fact that many of the studies lack a dose–response curve, and data are based on a single large dose of hormone or analog. As demonstrated by Orth *et al.* (1999) in their study on JH regulation of *hJHBP* expression, the effect of increasing doses of JH on the fat body is stimulatory only within a limited range; after peak stimulation is reached, further increases in JH result in a negative response. In this case, a single large dose of hormone would have obscured very important data. Another issue with many of the studies is the lack of authentic controls, especially for experiments *in vitro*. Large doses of JH can form a monolayer at the surface of the medium, hindering gaseous exchange and compromising the physiology of the fat body in culture. Unless a comparable lipid is used as a control, the results may be meaningless. It is anticipated that technological advances, particularly in molecular analyses, coupled with a more physiological approach to hormone treatment, should ultimately reveal how JH functions with respect to the fat body.

3.7.11.3.4. Muscle responses Myogenesis occurs during two developmental periods: the embryonic period in which larval muscles are developed and the period of larval–pupal metamorphosis, in which adult muscles are formed. In addition to myogenesis, existing larval muscles can undergo cell death, or become restructured and/or reoriented for a new role in the adult (Riddiford, 1994; Roy and Vijay Raghavan, 1999). At the present time there is little evidence to suggest that JH has a direct role on embryonic myogenesis; however, JH involvement in the regulation of muscle development and muscle fate during metamorphosis is well documented (Schwartz, 1992; Riddiford, 1994; Hegstrom *et al.*, 1998; Roy and VijayRaghavan, 1999; Buszczak and Segraves, 2000; Cascone and Schwartz, 2001; Lee *et al.*, 2002). The fate of larval muscles is determined by a finely tuned interplay between JH and ecdysteroids, an example of which is found in the process of proleg retraction in *M. sexta* (Weeks and Truman, 1986a, 1986b). After the larva wanders in the last larval stadium, the prolegs are no longer required, and muscles that move these structures begin to degenerate. The signal for this process is the commitment peak in the ecdysteroid titer, which occurs in the absence of JH. Under these conditions,

the retractor muscles become committed to undergo apoptosis (programmed cell death) (see **Chapter 2.5**), with the actual process of degradation elicited by the large prepupal peak of ecdysteroids that occurs several days later. Despite its concurrent presence at the time of the prepupal peak in the ecdysteroid titer, JH can no longer inhibit apoptosis of the retractor muscles.

Adult-specific muscles are formed from specific myoblasts that proliferate and differentiate during the late larval or early pupal stages (Roy and VijayRaghavan, 1999) (see **Chapters 2.1** and **2.3**). In those muscles that make up the larval ventral diaphragm of *M. sexta*, proliferation and differentiation are controlled by ecdysteroids, but these two events have different hormonal requirements (Champlin *et al*., 1999). Moderate levels of ecdysteroids induce proliferation of the ventral diaphragm myoblasts, while high or low doses arrest cellular proliferation in the G_2 phase of the cell cycle in these cells. High doses of ecdysteroids, such as those observed at the prepupal peak, induce the myoblasts to exit the cell cycle and express muscle myosin. The ability of critical titers of ecdysteroids to initiate either proliferation or differentiation is modified in the presence of methoprene. For example, high levels of ecdysteroids induce myoblast proliferation, but are unable to induce myoblast differentiation, in the presence of methoprene. These experimental observations correlate well with myoblast development *in vivo*. The prepupal peak of ecdysteroids, which is sufficient to induce differentiation, is accompanied by a concomitant rise in JH that prevents the myoblasts from differentiating. The myoblasts continue to proliferate until day 7 of the pupal stage when, in the presence of the pupal ecdysteroid peak but in the absence of JH, they then begin to differentiate. While both the prepupal and pupal surges of ecdysteroids are sufficient to cause differentiation, the pupal surge is not accompanied by a rise in JH levels, thus allowing the myoblasts to differentiate.

A similar interplay between JH and ecdysteroids is observed in the development of flight muscle in the desert locust, *S. gregaria*. Flight muscle development is initiated at the beginning of the last nymphal stadium and completed shortly after adult metamorphosis (Wang *et al*., 1993). In addition to its fully differentiated myofibrils, functional flight muscle contains a high level of a fatty acid binding protein that is involved in sequestering lipids for energy. Flight muscle development begins during the fifth nymphal stadium, when JH is absent and ecdysteroid titers are high. When a large dose of methoprene is applied to the last-stadium nymph, the insect undergoes another nymphal molt, and flight muscles that should have formed working myofibrils remain undifferentiated and lack fatty acid binding protein. Thus, elevated JH titers in the last nymphal stadium can lead to either retention of nymphal muscle or delay in the development of adult muscles.

In the adult stage, JH can have the opposite effect on muscles, inducing them to undergo degradation to yield precursors for vitellogenin production (Rose *et al*., 2001). Treating the alate or winged form of the aphid *Acyrthosiphon pisum* with a JH analog initiates histolysis of indirect flight muscles via the ubiquitin-dependent pathway for apoptosis (Kobayashi and Ishikawa, 1994). Conversely, treatment with precocene II, a compound that reduces JH biosynthesis in this species, prevents flight muscle breakdown. A similar situation is found in another aphid species, *A. fabae* (Hardie *et al*., 1990). The development of flight muscle in Orthoptera appears to follow this same pattern, i.e., JH either induces and/or is involved in the histolysis of these muscles. Application of JH III or methoprene to the cricket *Modicogryllus confirmatus* induces the degeneration of flight muscles within 3 days (Tanaka, 1994). In addition to JH, there may be neural factors that are required for the degeneration of specific flight muscles (Shiga *et al*., 2002).

3.7.11.3.5. Prothoracic gland responses It has long been known that the prothoracic glands, the site of synthesis of ecdysteroids, are regulated by ecdysone itself, via short positive and negative feedback loops (Williams, 1952; Siew and Gilbert, 1971; Sakurai and Williams, 1989) (see **Chapter 3.8**). Given the close relationship between JH and ecdysteroids in regulation of metamorphosis, the effect of JH on the prothoracic glands has also been examined (Gilbert, 1962; Siew and Gilbert, 1971). JH has two separate and distinct effects: prevention of precocious degeneration of the glands and regulation of ecdysteroid synthesis/secretion during the larval stage. In *M. sexta*, the prothoracic glands undergo apoptosis between 5 and 6 days after pupation, at the time of the pupal peak in the ecdysteroid titer. Dai and Gilbert (1999) demonstrated that early pupal glands, in the presence of ecdysteroids *in vitro*, will also undergo apoptosis. Glandular degeneration can be delayed for up to a week, if a newly molted pupa is injected with a large dose of JH II. Moreover, the glands remain capable of synthesizing ecdysone (Dai and Gilbert, 1998). The underlying mechanisms by which JH prevents this important developmental event is still unclear.

Nijhout (1994), in his elegant review of JH effects on ecdysteroid synthesis, came to the conclusion

that JH does not act directly on the prothoracic glands to modulate ecdysone biosynthesis. His model suggests that elevated levels of JH during the early part of the last larval stadium act on the brain (or other intermediary tissue) to suppress synthesis or release of PTTH, the hormone responsible for stimulating ecdysteroidogenesis in the prothoracic glands. It is only when the JH titers drop that PTTH can be released to initiate commitment to pupation by stimulating synthesis of the commitment peak in the ecdysteroid titer (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). The idea that the brain is the major target is supported by the work of Gruetzmacher *et al.* (1984), who demonstrated that although PTTH could stimulate *M. sexta* prothoracic gland activity *in vitro*, none of the JH homologs or methoprene, even at a number of different doses, were able to do so. Evidence presented by Sakurai *et al.* (1989) suggests that JH not only acts on the brain but also on the glands themselves, to directly suppress the acquisition of competence to respond to PTTH. Thus, under normal physiological conditions in the last larval stadium, the JH-regulated inhibition of PTTH secretion and suppression of gland competence probably evolved as a safety mechanism to prevent the premature onset of the metamorphic molt (Nijhout, 1994).

While it is accepted that high levels of JH inhibit PTTH production or secretion during the feeding period of the last larval stadium, this does not appear to be the case either in the earlier stadia or after commitment to pupation. In the earlier stadia, JH titers are high to maintain the larval state. Presumably, these high levels of JH should inhibit PTTH secretion, yet the insects undergo normal molts. In early studies, the implantation of extra CA into penultimate and last instars of *G. mellonella* invariably led to perfect supernumerary molts, often more than one (Granger and Sehnal, 1974; Sehnal and Granger, 1975). In more recent studies, supplementing normal JH titers with an analog, fenoxycarb, did not block third and fourth instars of *B. mori* from undergoing normal molts to fourth and fifth instars; however, an extra molt to a perfect sixth instar was noted (Kamimura and Kiuchi, 2002). An identical result was seen in *M. sexta* using methoprene (Lonard *et al.*, 1996). An *in vitro* study provides a basis for the results of these *in vivo* experiments. Addition of fenoxycarb to incubations *in vitro* of *B. mori* prothoracic glands taken throughout the last stadium results in an extreme suppression of ecdysteroidogenesis (Dedos and Fugo, 1996). Although a recent report has shown that JH I, applied in large doses early in the last larval stadium, can transiently elevate PTTH levels in the hemolymph of *B. mori* (Mizoguchi, 2001), the majority of the studies done so far indicate that in contrast to the model for JH–ecdysteroid interaction during larval–pupal metamorphosis, JH is capable of acting directly on the prothoracic glands and does so by suppressing ecdysteroid synthesis.

Biochemical evidence supports this contention: hydroprene, a JH analog, and JH I significantly delay the ability of the prothoracic glands to respond to PTTH or MIX, an inhibitor of cyclic nucleotide phosphodiesterase (Gu *et al.*, 1997). Since PTTH activates an intracellular second messenger system involving cyclic nucleotides (Gilbert *et al.*, 2000) (see **Chapter 3.2**), MIX can serve as a surrogate for PTTH by inhibiting their catabolism. The fact that neither PTTH nor MIX can activate ecdysteroidogenesis in the presence of JH strongly reinforces the idea that JH can act directly on the glands.

Several groups have demonstrated that the effect of JH on the prothoracic glands changes after commitment to pupation. Insects that have been debrained or neck-ligated to remove the source of PTTH respond to JH or analog treatment with increased ecdysteroid synthesis (Hiruma *et al.*, 1978; Safranek *et al.*, 1980; Gruetzmacher *et al.*, 1984; Dedos and Fugo, 1996; Dai and Gilbert, 1998). Thus, a reversal in hormone action appears to have taken place at, and possibly as a result of, commitment. As we have seen with other tissues, the response to JH can vary depending upon the developmental period. In the case of the prothoracic glands, the regulatory processes that control ecdysteroidogenesis and involve JH are very complex.

3.7.12. Molecular Mode of Action of the Juvenile Hormones

No sooner had the chemical structure of JH I been reported by Röller *et al.* (1967), one of the first of many reviews on JH action at the molecular level appeared in print (Kroeger, 1968). Since that time, there have been a number of major reviews focusing on the topic, with at least nine appearing during the last dozen years (Kumaran, 1990; Riddiford, 1994, 1996; Jones, 1995; Wyatt and Davey, 1996; Gade *et al.*, 1997; Gilbert *et al.*, 2000; Lafont, 2000; Wheeler and Nijhout, 2003).

Despite the volumes written about the subject, the molecular action of JH remains very much a mystery. This has led some to suggest that JH may have multiple roles in cellular and molecular mechanisms (Jones, 1995; Wheeler and Nijhout, 2003). Our assessment of the literature suggests that some of the problems in deciphering the molecular action

of JH lie not in unfathomable molecular mechanisms, but rather in the quality of the hormone, choice of experimental model, and development of experimental design.

A major experimental pitfall is the quality of the hormone, especially commercially available JH III from a US supplier. The supplier acknowledges that the hormone is racemic and the level of contamination is significant. Yet there appears to be a tacit assumption among investigators that the unnatural racemate and contaminants have no effect on the experimental procedure, an assumption that is unwarranted until proven. Long-term storage of the hormone and its metabolites may also be problematic and a means of repurifying the compounds must be available. A similar criticism can be leveled at the use of commercial-grade JH agonists that may contain significant amounts of incipients with unknown biological functions.

Metamorphosis is undoubtedly one of the most striking phenomena in the biological world, yet it has not yielded its molecular secrets easily, particularly with regard to JH action. While major strides have been made in the last few years, using a paradigm that depends on the absence of JH makes dissecting the molecular action of the hormone challenging. This problem is further compounded by the fact that the metamorphic actions of JH on cellular and molecular events often require lengthy incubation periods. These long periods make separating key regulatory events from secondary events difficult. It may well be that the extreme complexity of metamorphosis does not lend itself well to isolating the actions of JH, and, as Jones (1995) and Wheeler and Nijhout (2003) note, the hormone may act on a number of molecular pathways in the same cell. As our knowledge of larval physiology and molecular biology expands, nonmetamorphic events regulated by JH, such as pigmentation and metabolic processes, may provide more tractable experimental models with which to study the molecular actions of JH at the genomic level.

3.7.12.1. Juvenile Hormone Interaction with Ultraspiracle

One of the more intriguing discoveries since the last major reviews is that presented by Jones and Sharp (1997), who demonstrated that the nuclear receptor Ultraspiracle (USP) can bind JH III (see **Chapter 3.5**). USP, an orphan receptor for which no endogenous ligand has been unambigiously established (Billas *et al.*, 2001), is a heterodimeric partner that interacts with ECR to form the functional ECR complex (Thomas *et al.*, 1993; Yao *et al.*, 1993) or with DHR38, the *D. melanogaster* ortholog of the mammalian NGFI-B subfamily of orphan nuclear receptors (Baker *et al.*, 2003) (see **Chapter 3.5**). USP is an ortholog of the vertebrate retinoid X receptor (RXR) (Oro *et al.*, 1990), which is responsive to very high levels of methoprene (Harmon *et al.*, 1995).

Using a fluorescence assay that detects changes in protein conformation induced by ligand binding, Jones and Sharp (1997) demonstrated that JH III and JH III acid change the conformation of recombinant *D. melanogaster* USP, while farnesol and 20E do not. The USP–JH III acid interaction generates anomalous spectra, suggesting that it may not trigger a conformational change that is identical to that obtained with JH III. These investigators estimate the dissociation constant of the USP: JH III complex to be approximately 1 μM, a value considerably higher than might be expected for a nuclear receptor that must sequester hormone directly from the circulatory system. Further refinement of the original studies indicated that methoprene competes with JH III for a binding site on USP (Jones *et al.*, 2001). It should be cautioned that these studies used only partially purified (75%) racemic JH III, yet the investigators chose to express ligand concentrations as "concentrations of the natural active isomer in the respective binding reaction" (Jones *et al.*, 2001). Arbitrarily overlooking the impurities in the hormone preparation and using an estimated 10*R* JH III concentration may present problems in the interpretation of the binding constants.

Nevertheless, evidence from functional transcription assays supports this model. Using modified USP response elements coupled to a JHE core promoter (−61 to +28 relative to the start site) and a reporter gene, Jones *et al.* (2001) demonstrated that construct expression could be induced by JH III in a dose-dependent fashion. The promoter construct was only weakly responsive to all *trans*-retinoic acid and did not respond to triiodo-L-thyronine (Jones *et al.*, 2001). Functional transcriptional assays also demonstrate that point mutations in the putative JH III binding site of USP, which abolish JH binding, cause the mutant receptor to act as a dominant negative and suppress JH III activation (Xu *et al.*, 2002).

Jones *et al.* (2001) suggested that high levels of JH III induce a conformational change in structure to stabilize dimeric/oligomeric forms of USP. This interaction may be important in dimerization of USP with its ecdysteroid receptor partner, but how it occurs and the role of USP–DNA binding in stabilization of the complex is not yet understood. It is well known that ecdysteroids induce enhanced

heterodimer formation even when the binding of the heterodimers to cognate DNA response elements does not occur (Lezzi *et al*., 2002).

The initial crystallographic analyses of the USP ligand binding domains from *H. virescens* (Billas *et al*., 2001) and *D. melanogaster* (Clayton *et al*., 2001) indicated that the putative ligand binding site is locked in an antagonist conformation. More recently, Sasorith *et al*. (2002) reinvestigated the site and found that recombinant USP could bind ligands and attempted, using computer algorithms, to dock putative ligands in the binding site. Their studies indicate that the ligand binding site is relatively large and predominantly lined with hydrophobic residues. Interestingly, the binding site of the recombinant USP contains a phospholipid inserted by the expression host, and it would be of considerable interest to know how this lipid can affect JH binding.

Computation docking of JH and analogs in the binding site of recombinant *H. virescens* USP suggests that JH may fit the putative site; however, the percentage of occupancy of the ligand binding sites by these ligands lies in the bottom range of values for classical nuclear receptors, thus raising concern about the validity of USP as the juvenile hormone receptor (Sasorith *et al*., 2002).

USP, with its large ligand binding site and a low level of occupancy, behaves more like a sensor than a classical high affinity receptor. Indeed, using the sensor model (Chawla *et al*., 2001), one might envision an entirely different role for USP, based on nutritional levels in the premetamorphic larva. When sufficient levels of a key dietary lipid(s) have been attained, the USP binding site becomes saturated with that nutritional ligand, which, in turn, alters the conformation of the protein. Ligand-activated USP might then enhance ecdysteroid binding by its partner, ECR. Ligand activation of the appropriate ecdysteroid receptor isoforms leads to changes in neuronal activity (Hewes and Truman, 1994; Truman, 1996) that may trigger activity in the prothoracicotropes or dendritic fields enervating them. While this hypothesis is simplistic, it could account for the very early signaling events that initiate ecdysis. It now seems clear that USP binds JH, albeit with low affinity, as well as other lipids. It may be that refinement of experiments presented by the Jones laboratory will shed new light on how the hormone acts at the molecular level.

3.7.12.2. Juvenile Hormone Interaction with the *Methoprene-Tolerant* Gene

Using mutagenesis studies to dissect the molecular action of JH, Wilson and his group have examined a locus in *D. melanogaster*, the *Methoprene-tolerant* (*Met*) locus, that encodes for a protein similar in sequence to proteins in the basic-helix–loop–helix–PAS (bHLH-PAS) family of transcriptional regulators (Wilson and Ashok, 1998). Treating wild-type flies with high levels of methoprene at certain times during larval development leads to a distinct array of developmental abnormalities, including aberrations in the central nervous system, salivary glands, and muscles (Restifo and Wilson, 1998). In contrast to wild-types, flies carrying the *Met* mutation are resistant to high levels of methoprene and appear to develop and reproduce normally (Wilson and Fabian, 1986).

The *Met* gene (GenBank Accession no. AF034859) encodes for a 716-residue protein which in wild-type flies displays a dissociation constant of 6 nM for JH III (Shemshedini *et al*., 1990). Competitive displacement studies comparing the homologs indicate that JH I, JH II, JH III acid, and methoprene are weak competitors when compared with JH III; methoprene binding is 100-fold weaker than that of JH III. Flies carrying the *Met* mutation exhibited a significantly reduced (sixfold) binding affinity for JH III (38 nM). The reduced affinity of the 85 kDa Met protein for methoprene translates into a 50–100-fold increase in resistance to both its toxic and morphogenetic effects. Flies carrying the *Met* mutation are also resistant to toxic levels of the naturally occurring hormones JH III and JHB_3, but not to various other classes of insecticides. This important distinction demonstrates that *Met* is not a general insecticide-resistance gene, but is specific for JH and JH analogs (Wilson *et al*., 2003).

Analysis of the *Met* sequence indicates it has three regions displaying similarity to the bHLH–PAS family of transcriptional regulators. It is instructive to note that the bHLH–PAS gene family was named for three important members of the group: the *Period* gene, the *Aryl hydrocarbon receptor* gene, and the *Single-minded* gene. Of particular importance is the similarity of *Met* to the *Aryl hydrocarbon receptor* (*Ahr*) gene that encodes for a xenobiotic binding protein (Ashok *et al*., 1998). The fact that methoprene interacts with a member of the AHR family of receptors may explain why so many compounds structurally distant from JH have JH-like activity (Stahl, 1975). With its similarity to AHRs, Met could bind hydrophobic ligands that marginally resemble JH and regulate certain JH-responsive genes (Ashok *et al*., 1998). Equally interesting is the fact that the vertebrate AHR partners with another protein, the aryl hydrocarbon nuclear translocator, to form an active transcriptional regulator that activates genes responsible for mediating the

response to xenobiotics. While no partner has yet been found for Met, the potential for such an interaction has been noted (Restifo and Wilson, 1998).

Immunolocalization studies suggest that Met is limited to nuclei (Pursley *et al.*, 2000), thus ruling out the earlier suggestion that Met acts as a cytosolic "sink" (Shemshedini *et al.*, 1990). Developmentally, it is present in all cells of the *Drosophila* embryo from the 256 cell stage until early gastrulation, when the signal begins to decline. In second and third stadium larvae Met is found in salivary glands, fat body, imaginal discs, and gut primordium. *Met* is also present in pupal histoblasts and in adult reproductive tissues, including the female ovarian follicle cells and spermatheca and the male accessory glands (Pursley *et al.*, 2000).

To date, over a dozen alleles of *Met* have been recovered in genetic screens (Wilson *et al.*, 2003), but one in particular, Met^{27}, poses a perplexing question about the role of Met in JH action. When examined by Northern analysis and RT-PCR, flies homozygous for Met^{27} lack *Met* transcripts, yet these insects survive and develop into adults. The lack of *Met* expression becomes evident only in the adult female, in which there is an 80% reduction in oogenesis (Wilson and Ashok, 1998). If flies carrying a null mutation in *Met* can undergo seemingly normal development and limited oogenesis, both events being under JH control, the role of Met in JH action is open to question. Pursley *et al.* (2000) suggest that "genetic redundancy" (Krakauer and Plotkin, 2002), the equivalent of back-up systems for biological phenomena, may be involved in this situation. Given the apparent lack of Met functions during the larval stages, but its significant role in both male and female reproduction (Wilson and Ashok, 1998; Wilson *et al.*, 2003), it may be that JH utilizes several different and distinct molecular pathways to control development and reproduction.

3.7.12.3. Juvenile Hormone and Metamorphosis: The Role of the *Broad*-Complex

A growing body of evidence has implicated the transcription factor Broad (FlyBase identification no. FBgn0010011) as a key component in the initiation of metamorphosis (Riddiford *et al.*, 2003). Mutations in *Broad*, a member of the Broad-Tramtrack-Bric-a-Brac family, allow normal larval development to occur but prevent pupation (Kiss *et al.*, 1988; Bayer *et al.*, 1996; Riddiford *et al.*, 2003). Less severe Broad phenotypes have wings that are shorter and wider than wild-types. The *Broad* gene is large (100 kb) and in *Drosophila* encodes for at least six isoforms of the protein, which all share a common N-terminal of approximately 425 amino acids (Bayer *et al.*, 1996). Through alternative splicing, the C-terminal can be represented by any one of four pairs of C_2H_2-type zinc finger domains, Z1, Z2, Z3, and Z4. The N- and C-terminals are connected by linkage domains of varying lengths. The Z4 transcript encodes a protein of 877 amino acids.

After the onset of metamorphosis, the nuclei of all larval and imaginal cells display Broad; however, each tissue appears to have its own specific constellation of isoforms that appear in a temporally unique sequence (Mugat *et al.*, 2000). For example, Mugat *et al.* (2000), using an isoform-specific antibody in their studies on *D. melanogaster* fat body, followed a shift from Z2 to the other isoforms as metamorphic events proceed. Bayer *et al.* (1996) demonstrated that Z1 is the predominant isoform during pupal cuticle formation in the abdominal epidermis and imaginal discs.

Ingestion of the JH analog pyriproxifen by first instar *D. melanogaster* prolongs the third stadium by several days (Riddiford *et al.*, 2003) and results in a 12 h delay in the appearance of Broad in abdominal epidermis. By the time of pupation, however, Broad protein levels were similar to controls, indicating that this potent JH analog cannot prevent the appearance of this protein. The adult abdominal epidermis, formed from nests of tissue called histoblasts, begins to proliferate and spread over the abdomen after puparium formation. These new cells displace the existing larval cells. Broad is found in larval cells that are destined to die, but cannot be found in imaginal cells once they have spread across the abdomen. Interestingly, JH treatment delays the loss of Broad in the imaginal cells and causes these cells to produce proteins indicative of a pupal-like cuticle. Increasing Z1 levels through misexpression at the onset of adult cuticle formation mimics the effects of JH, causing the reappearance of mRNAs encoding pupal cuticular proteins and the suppression of mRNAs encoding adult cuticular proteins (Zhou and Riddiford, 2002). These investigators suggest that JH prolongs the expression of the Z1 isoform, and suppresses the onset of adult cuticular formation.

In *M. sexta*, only three Broad isoforms have been discovered, Z2, Z3, and Z4, with Z4 being most prominent in epidermal tissue involved in pupal cuticle formation (Zhou *et al.*, 1998; Zhou and Liu 2001). As observed in *D. melanogaster*, the protein's appearance is well-defined spatially and temporally, following the pattern of pupal commitment, as defined by the loss of sensitivity to JH. Most significantly, within 6 h of 20E treatment, epidermal

preparations *in vitro* display a marked rise in Broad mRNA (Zhou *et al*., 1998; Zhou and Riddiford, 2002). This rise signals the onset of commitment to pupation. In cells that are not yet committed, JH I prevents the induction of Broad mRNA synthesis by 20E (Zhou *et al*., 1998). Once Broad appears in the cells, JH can no longer suppress the accumulation of Broad mRNA, and its levels remain elevated, even during the rise in JH titers later in the stadium. *Broad* expression eventually disappears during the early pupal stage coinciding with the onset of adult cuticular synthesis. The role of Broad was further defined by applying sufficient JH to pupae to elicit the formation of a second pupal cuticle; in this case, Broad mRNA and protein rose after exposure to 20E, then dropped after the second pupal cuticle was formed (Zhou and Riddiford, 2002). Thus, Broad's presence in the cell appears to inhibit larval cuticular synthesis, while permitting pupal cuticular synthesis to proceed. Moreover, Broad appears to suppress the synthesis of the adult cuticle. Exogenous JH can delay but not prevent the expression of Broad, but the molecular actions of the hormone remain unclear.

3.7.12.4. Juvenile Hormone Regulation of Cytosolic Malate Dehydrogenase

JH has long been implicated in the regulation of cellular energy production and of biochemical and second messenger pathways. This long history includes research on such diverse mechanisms as modification of oxidative phosphorylation (Clarke and Baldwin, 1960; Chefurka, 1978); shifts in cellular potassium levels (Kroeger, 1968); membrane perturbation (Baumann, 1969; Barber *et al*., 1981); second messenger regulation (Everson and Feir, 1976; Kensler *et al*., 1978; Yamamoto *et al*., 1988); changes in cytoskeletal structure (Capella and Hartfelder, 2002); and the development of asymmetric organs (Adam *et al*., 2003). Interest in certain of these areas has been rekindled by a series of studies carried out by Farkas and his associates on the regulation of cytosolic malate dehydrogenase in *D. melanogaster* (Farkas and Knopp, 1997, 1998; Farkas and Sutakova, 2001; Farkas *et al*., 2002).

Cytosolic malate dehydrogenase (EC 1.1.1.40), also known as NADP-malic enzyme (ME), is involved in production of cytoplasmic NADPH. The enzyme is particularly prevalent in tissues that are involved in fatty acid synthesis and it is a target for various hormones in vertebrates (Farkas *et al*., 2002). In a series of papers, Farkas and his group have demonstrated that ME, a product of the *Men* gene (CG 10120; FBgn 0002719), is under the regulation of JH during the mid-third stadium of *D. melanogaster*. A dose of 5 pg of JH III will elicit half-maximal ME activity and while this level is higher than physiological levels (0.02 pg per larva) (Sliter *et al*., 1987), it is still lower than the dose typically used to induce morphological abberations (Riddiford and Ashburner, 1991).

The response to JH III occurs in two phases, an early phase occurring 3 h after treatment, in which ME activity doubles, and a late phase occurring 12 h after treatment, in which a threefold increase over the control is observed. The transcriptional inhibitor actinomycin D does not block the early ME rise, but does block the late rise, suggesting that JH can increase ME activity without new protein synthesis. That JH added directly to the cytosol can not induce the ME rise indicates that the hormone may be acting through a pre-existing pathway that is immediately affected, such as that of a second messenger cascade (Farkas *et al*., 2002). In contrast to the early rise, the late rise in ME activity is sensitive to actinomycin D, indicating that this rise is dependent on *de novo* transcription of ME. The authors have yet to examine the levels of ME mRNA that would confirm the inhibitor studies.

Using developmentally staged, wild-type larvae and the temperature-sensitive mutants ecd^{1} and $su(f)^{ts67g}$, Farkas *et al*. (2002) demonstrated that ecdysteroids are also involved in the JH-regulated ME rise. Wild-type flies respond to JH only near the time of pupariation, when ecdysteroid titers begin to rise. By contrast, in temperature-sensitive, ecdysone-deficient mutants, JH is unable to initiate the expected ME rise at elevated temperatures. These observations suggest that a complex synergistic regulatory mechanism is acting to control ME levels. A low level of ecdysteroid must be present before JH can induce activation of ME, yet high levels of ecdysteroid, such as those seen at pupariation, downregulate the activity of ME. The physiological role of JH in upregulating this event cannot be well understood unless placed in the context of lipogenesis. Farkas *et al*. (2002) suggest that the ME–JH interaction may play a role in increasing the levels of unsaturated fatty acids. JH and certain JH analogs have been shown to induce a rise in unsaturated fatty acids (Schneider *et al*., 1995) and phospholipid levels (Della-Cioppa and Engelmann, 1984) in adult *S. gregaria* and *L. maderae*. In *D. melanogaster*, the ecdysteroid-induced onset of metamorphosis leads to the cessation of feeding; it may be that high levels of JH during the earlier stadia and the early part of the last stadia ensure availability of lipid resources during metamorphosis. While the pathways are still not clear, this paradigm of JH

action at the molecular level holds much promise for better understanding how the hormone may regulate homeostatic mechanisms that are necessary for insect growth and development.

References

Abdel-Aal, Y.A.I., Hammock, B.D., **1985**. 3-Octylthio-1,1,1-trifluoro-2-propanone, a high affinity and slow binding inhibitor of juvenile hormone esterase from *Trichoplusia ni*. *Insect Biochem. 15*, 111–122.

Abdel-Aal, Y.A.I., Hammock, B.D., **1986**. Transition state analogs as ligands for affinity purification of juvenile hormone esterase. *Science 233*, 1073–1076.

Abdel-Latief, M., Meyering-Vos, M., Hoffmann, K.H., **2003**. Molecular characterization of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin. *Insect Biochem. Mol. Biol. 33*, 467–476.

Abdu, U., Takac, P., Laufer, H., Sagi, A., **1998**. Effect of methyl farnesoate on late larval development and metamorphosis in the prawn *Macrobrachium rosenbergii*: a juvenoid-like effect? *Biol. Bull. 195*, 112–119.

Adam, G., Perrimon, N., Noselli, S., **2003**. The retinoic-like juvenile hormone controls the looping of left-right asymmetric organs in *Drosophila*. *Development 130*, 2397–2405.

Adams, D.S., Eickbush, T.H., Herrera, R.J., Lizardi, P.M., **1986**. A highly reiterated family of transcribed oligo(A)-terminated, interspersed DNA elements in the genome of *Bombyx mori*. *J. Mol. Biol. 187*, 465–478.

Allen, C.U., Janzen, W.P., Granger, N.A., **1992a**. Manipulation of intracellular calcium affects *in vitro* juvenile hormone synthesis by larval corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol. 84*, 227–241.

Allen, C.U., Herman, B., Granger, N.A., **1992b**. Fura-2 measurement of cytosolic free Ca^{2+} concentration in corpus allatum cells of larval *Manduca sexta*. *J. Exp. Biol. 166*, 253–266.

Anspaugh, D.D., Kennedy, G.G., Roe, R.M., **1995**. Purification and characterization of a resistance-associated esterase from the Colorado potato beetle, *Leptinotarsa decemlineata*. *Pestic. Biochem. Physiol. 53*, 84–96.

Applebaum, S.W., Avisar, E., Heifetz, Y., **1997**. Juvenile hormone and locust phase. *Arch. Insect Biochem. Physiol. 35*, 375–391.

Ashok, M., Turner, C., Wilson, T.G., **1998**. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl Acad. Sci. USA 95*, 2761–2766.

Aucoin, R.R., Rankin, S.M., Stay, B., Tobe, S.S., **1987**. Calcium and cyclic AMP involvement in the regulation of juvenile hormone biosynthesis in *Diploptera punctata*. *Insect Biochem. 17*, 965–969.

Audsley, N., Weaver, R.J., Edwards, J.P., **1998**. Enzyme linked immunosorbent assay for *Manduca sexta* allatostatin (Mas-AS), isolation and measurement of Mas-AS immunoreactive peptide in *Lacanobia oleracea*. *Insect Biochem. Mol. Biol. 28*, 775–784.

Audsley, N., Weaver, R.J., Edwards, J.P., **1999a**. Juvenile hormone synthesis by corpora allata of tomato moth, *Lacanobia oleracea*, and the effects of allatostatins and allatotropin *in vitro*. *Eur. J. Entomol. 96*, 287–297.

Audsley, N., Weaver, R.J., Edwards, J.P., **1999b**. Significance of *Manduca sexta* allatostatin in the tomato moth *Lacanobia oleracea*. In: Sandman, C.A., Strand, F.L., Beckman, B., Cronwall, B.M., Flynbn, F.W., Nachman, F.W. (Eds.), Neuropeptides, Structure and Function in Biology and Behavior, vol. 897. New York Academy of Science, New York, pp. 330–341.

Audsley, N., Weaver, R.J., Edwards, J.P., **2000**. Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. *Insect Biochem. Mol. Biol. 30*, 681–689.

Audsley, N., Weaver, R.J., Edwards, J.P., **2002**. Degradation of *Manduca sexta* allatostatin and allatotropin by proteases associated with the foregut of *Lacanobia oleracea* larvae. *Peptides 23*, 2015–2023.

Auerswald, L., Birgul, N., Gade, G., Kreienkamp, H.J., Richter, D., **2001**. Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. *Biochem. Biophys. Res. Commun. 282*, 904–909.

Baehr, J.-C., Caruelle, J.P., Poras, M., **1986**. The activity of denervated corpora allata in a diapausing strain of *Locusta migratoria*: *in vivo* and *in vitro* studies. *Int. J. Invertebr. Reprod. Devel. 10*, 143–150.

Baehr, J.-C., Porcheron, P., Papillon, M., Dray, F., **1979**. Haemolymph levels of juvenile hormone, ecdysteroids and protein during the last two larval instars of *Locust migratoria*. *J. Insect Physiol. 25*, 371–460.

Baehr, J.-C., Pradelles, P., Lebreux, C., Cassier, P., Dray, F., **1976**. A simple and sensitive radioimmunoassay of insect juvenile hormone using an iodinated tracer. *FEBS Lett. 69*, 123–128.

Baker, F.C., **1990**. Techniques for identification and quantification of juvenile hormones and related compounds in arthropods. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, NJ, pp. 389–444.

Baker, F.C., Lanzrein, B., Miller, C.A., Tsai, L.W., Jamieson, G.C., *et al*., **1984**. Detection of only JH III in several life-stages of *Nauphoeta cinerea* and *Thermobia domestica*. *Life Sci. 35*, 1553–1560.

Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., *et al*., **2003**. The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell 113*, 731–742.

Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem. 17*, 989–996.

Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1988**. The absence of significant levels of the known

juvenile hormones and related compounds in the milkweed bug, *Oncopeltus fasciatus*. *Insect Biochem. 18*, 453–462.

Barber, R.F., Downer, R.G.H., Thompson, R.G.H., **1981**. Perturbation of phospholipid membranes by juvenile hormone. *Biochim. Biophys. Acta 643*, 593–600.

Barker, J.F., Davey, K.G., **1983**. A polypeptide from the brain and corpus cardiacum of male *Rhodnius prolixus* which stimulates *in vitro* protein synthesis in the transparent accessory reproductive gland. *Insect Biochem. 13*, 7–10.

Baumann, G., **1969**. Juvenile hormone: effects on bimolecular lipid membranes. *Nature 223*, 316–317.

Bayer, C., von, Kalm L., Fristrom, J.W., **1996**. Gene regulation in imaginal discs and salivary gland development during metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Academic Press, London, pp. 321–361.

Bean, D.W., Beck, S.D., Goodman, W.G., **1982**. Juvenile hormone esterases in diapause and nondiapause larvae of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 6*, 485–492.

Bean, D.W., Goodman, W.G., Beck, S.D., **1983**. Regulation of juvenile hormone esterase activity in the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 29*, 877–883.

Beckstead, R., Ortiz, J.A., Sanchez, C., Prokopenko, S.N., Chambon, P., *et al.*, **2001**. *Bonus*, a *Drosophila* homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit beta *FTZ-F1*-dependent transcription. *Mol. Cell 7*, 753–765.

Beckstead, R., Prokopenko, S.N., Losson, R., Chambon, P., Bellen, H., **2000**. *Bonus*, a *Drosophila* RIF1 homolog, plays a role in hormone response. *Drosophila Res. Conf. 41*, 54.

Bede, J.C., Goodman, W.G., Tobe, S.S., **1999**. Developmental distribution of insect juvenile hormone III in the sedge, *Cyperus iria* L. *Phytochemistry 52*, 1269–1274.

Bede, J.C., Teal, P.E.A., Goodman, W.G., Tobe, S.S., **2001**. Biosynthetic pathway of insect juvenile hormone III in cell suspension cultures of the sedge *Cyperus iria*. *Plant Physiol. 127*, 584–593.

Beintema, J.J., Stam, W.T., Hazes, B., Smidt, M.P., **1994**. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). *Mol. Biol. Evol. 11*, 493–503.

Bellés, X., Graham, L.A., Bendena, W.G., Ding, Q., Edwards, J.P., *et al.*, **1999**. The molecular evolution of the allatostatin precursor in cockroaches. *Peptides 20*, 11–22.

Bendena, W.G., Garside, C.S., Yu, C.G., Tobe, S.S., **1997**. Allatostatins: diversity in structure and function of an insect neuropeptide family. *Ann. New York Acad. Sci. 814*, 53–66.

Bergamasco, R., Horn, D.H.S., **1983**. Distribution and role of insect hormones in plants. In: Downer, R.G.H., Laufer, H. (Eds.), Invertebrate Endocrinology. Alan R. Liss, New York, pp. 627–656.

Bergot, B.J., Baker, F.C., Cerf, D.C., Jamieson, G., Schooley, D.A., **1981a**. Qualitative and quantitative aspects of juvenile hormone titers in developing embryos of several insect species: discovery of a new JH-like substance extracted from eggs of *Manduca sexta*. In: Pratt, G.E., Brooks, G.T. (Eds.), Juvenile Hormone Biochemistry. Elsevier/North-Holland, Amsterdam, pp. 33–45.

Bergot, B.J., Ratcliff, M., Schooley, D.A., **1981b**. A method for quantitative determination of juvenile hormones by mass spectroscopy. *J. Chromatogr. 204*, 231–244.

Berridge, M.J., **1993**. Inositol trisphosphate and calcium signaling. *Nature 361*, 315–325.

Bhaskaran, G., Dahm, K.H., Barrera, P., Pacheco, J.L., Peck, K.E., *et al.*, **1990**. Allatinhibin, a neurohormonal inhibitor of juvenile hormone biosynthesis in *Manduca sexta*. *Gen. Comp. Endocrinol. 78*, 123–136.

Bhatt, T.R., Horodyski, F., **1999**. Expression of the *Manduca sexta* allatotropin gene in cells of the central and enteric nervous system. *J. Comp. Neurol. 403*, 407–420.

Bidmon, H.J., Stumpf, W.E., Granger, N.A., **1992**. Ecdysteroid receptors in the neuroendocrine–endocrine axis of a moth. *Experientia 48*, 42–47.

Billas, I.M.L., Moulinier, L., Rochel, N., Moras, D., **2001**. Crystal structure of the ligand-binding domain of the ultraspiracle protein, usp, the ortholog of retinoid x receptors in insects. *J. Biol. Chem. 276*, 7465–7474.

Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., **1999**. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J. 18*, 5892–5900.

Blackmore, P.F., Beebe, S.J., Danforth, D.R., Alexander, N., **1990**. Progesterone and 17 alpha-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. *J. Biol. Chem. 265*, 1376–1380.

Blenau, W., Baumann, A., **2001**. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol. 48*, 13–38.

Bogus, M., Scheller, K., **1996**. Allatotropin released by the brain controls larval molting *Galleria mellonella* by affecting juvenile hormone synthesis. *Int. J. Devel. Biol. 40*, 205–210.

Bollenbacher, W.E., **1988**. The interendocrine regulation of larval–pupal development in the tobacco hornworm, *Manduca sexta*: a model. *J. Insect Physiol. 34*, 941–948.

Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., **1987**. Developmental endocrinology of larval moulting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 128*, 175–192.

Bollenbacher, W.E., Smith, S.L., Goodman, W.G., Gilbert, L.I., **1981**. Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 44*, 302–306.

Bonning, B.C., Hammock, B.D., **1996**. Development of recombinant baculoviruses for insect control. *Annu. Rev. Entomol. 41*, 191–210.

Bonning, B.C., Ward, V.K., Vanmeer, M.M.M., Booth, T.F., Hammock, B.D., **1997**. Disruption of lysosomal targeting is associated with insecticidal potency of juvenile hormone esterase. *Proc. Natl Acad. Sci. USA 94*, 6007–6012.

Boore, J.L., Collins, T.M., Stanton, D., Daehler, L.L., Brown, W.M., **1995**. Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangements. *Nature 376*, 163–165.

Borovsky, D., Carlson, D.A., Hancock, R.G., Rembold, H., Vanhandel, E., **1994a**. *De novo* biosynthesis of juvenile hormone III and I by the accessory glands of the male mosquito. *Insect Biochem. Mol. Biol. 24*, 437–444.

Borovsky, D., Carlson, D.A., Ujvary, I., Prestwich, G.D., **1994b**. Biosynthesis of (10*R*)-juvenile hormone III from farnesoic acid by *Aedes aegypti* ovary. *Arch. Insect Biochem. Physiol. 27*, 11–25.

Borst, D.W., Laufer, H., Landau, M., Chang, E.S., Hertz, W.A., *et al.*, **1987**. Methyl farnesoate and its role in crustacean reproduction and development. *Insect Biochem. 17*, 1123–1127.

Boulay, R., Hooper-Bui, L.M., Woodring, J., **2001**. Oviposition and oogenesis in virgin fire ant females, *Solenopsis invicta*, are associated with a high level of dopamine in the brain. *Physiol. Entomol. 26*, 294–299.

Bowers, W.S., Thompson, M.J., Vebel, E., **1965**. Juvenile and gonadotropic hormone activity of 10,11-epoxyfarnesoic acid methyl ester. *Life Sci. 4*, 2323–2331.

Bownes, M., Rembold, H., **1987**. The titer of juvenile hormone during the pupal and adult stages of the life cycle of *Drosophila melanogaster. Eur. J. Biochem. 164*, 709–712.

Bowser, P.R.F., Tobe, S.S., **2000**. Partial characterization of a putative allatostatin receptor in the midgut of the cockroach *Diploptera punctata*. *Gen. Comp. Endocrinol. 119*, 1–10.

Branden, C., Tooze, J., **1999**. Introduction to Protein Structure, 2nd edn. Garland Press, New York.

Braun, R.P., Wyatt, G.R., **1996**. Sequence of the hexameric juvenile hormone-binding protein from the hemolymph of *Locusta migratoria*. *J. Biol. Chem. 271*, 31756–31762.

Brindle, P.A., Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. Sources of propionate for the biogenesis of ethyl-branched insect juvenile hormone: role of isoleucine and valine. *Proc. Natl Acad. Sci. USA 84*, 7906–7910.

Brindle, P.A., Baker, F.C., Tsai, L.W., Schooley, D.A., **1992**. Comparative metabolism of isoleucine by corpora allata of nonlepidopteran insects versus lepidopteran insects in relation to juvenile hormone biosynthesis. *Arch. Insect Biochem. Physiol. 19*, 1–15.

Brindle, P.A., Schooley, D.A., Tsai, L.W., Baker, F.C., **1988**. Comparative metabolism of branched-chain amino acids to precursors of juvenile hormone biogenesis in corpora allata of lepidopterous versus nonlepidopterous insects. *J. Biol. Chem. 263*, 10653–10657.

Broadie, K.S., Bate, M., Tublitz, N.J., **1991**. Quantitative staging of embryonic development of the tobacco hawkmoth, *Manduca sexta*. *Roux's Arch. Devel. Biol. 199*, 327–334.

Brockhouse, A.C., Horner, H.T., Booth, T.F., Bonning, B.C., **1999**. Pericardial cell ultrastructure in the tobacco hornworm, *Manduca sexta*. *Int. J. Insect Morphol. Embryol. 28*, 261–271.

Bronsert, M., Bingol, H., Atkins, G., Stout, J., **2003**. Prolonged response to calling songs by the L3 auditory interneuron in female crickets (*Acheta domesticus*): possible roles in regulating phontactic threshold and selectiveness for call carrier frequency. *J. Exp. Zool. 296*, 72–85.

Browder, M.H., D'Amico, L.J., Nijhout, H.F., **2001**. The role of low levels of juvenile hormone esterase in the metamorphosis of *Manduca sexta*. *J. Insect Sci. 1*, 1–4.

Brüning, E., Lanzrein, B., **1987**. Function of juvenile hormone III in embryonic development of the cockroach, *Nauphoeta cinera*. *Int. J. Invertebr. Reprod. Devel. 12*, 29–44.

Brüning, E., Saxer, A., Lanzrein, B., **1985**. Methyl farnesoate and juvenile hormone III in normal and precocene treated embryos of the ovoviviparous cockroach *Nauphoeta cinerea*. *Int. J. Invertebr. Reprod. Devel. 8*, 269–278.

Bürgin, C., Lanzrein, B., **1988**. Stage dependent biosynthesis of methyl farnesoate and juvenile hormone III and metabolism of juvenile hormone III in embryos of the cockroach, *Nauphoeta cinera*. *Insect Biochem. 18*, 3–9.

Burmester, T., **2002**. Origin and evolution of arthropod hemocyanins and related proteins. *J. Comp. Physiol. B 172*, 95–107.

Burmester, T., Massey, H.C., Zakharkin, S.O., Benes, H., **1998**. The evolution of hexamerins and the phylogeny of insects. *J. Mol. Evol. 47*, 93–108.

Burns, S.N., Teal, P.E.A., Meer, R.K.V., Nation, J.L., Vogt, J.T., **2002**. Identification and action of juvenile hormone III from sexually mature alate females of the red imported fire ant, *Solenopsis invicta*. *J. Insect Physiol. 48*, 357–365.

Buszczak, M., Segraves, W.A., **2000**. Insect metamorphosis: out with the old, in with the new. *Curr. Biol. 10*, R830–R833.

Campbell, P.M., Harcourt, R.L., Crone, E.J., Claudianos, C., Hammock, B.D., *et al.*, **2001**. Identification of a juvenile hormone esterase gene by matching its peptide mass fingerprint with a sequence from the *Drosophila* genome project. *Insect Biochem. Mol. Biol. 31*, 513–520.

Campbell, P.M., Healy, M.J., Oakeshott, J.G., **1992**. Characterisation of juvenile hormone esterase in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 22*, 665–677.

Capella, I.C.S., Hartfelder, K., **2002**. Juvenile-hormone-dependent interaction of actin and spectrin is crucial for polymorphic differentiation of the larval honey bee ovary. *Cell Tissue Res. 307*, 265–272.

Carey, M., Smale, S.T., **2000**. Transcriptional Regulation in Eukaryotes: Concepts, Strategies, Techniques. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Casas, J., Harshman, L.G., Hammock, B.D., **1991**. Epoxide hydrolase activity on juvenile hormone in *Manduca sexta*. *Insect Biochem. 21*, 17–26.

Cascone, P.J., Schwartz, L.M., **2001**. Post-transcriptional regulation of gene expression during the programmed death of insect skeletal muscle. *Devel. Genes Evol. 211*, 397–405.

Castillo-Gracia, M., Couillaud, F., **1999**. Molecular cloning and tissue expression of an insect farnesyl fdiphosphate synthase. *Eur. J. Biochem. 262*, 365–370.

Caveney, S., **1970**. Juvenile hormone and wound modeling of *Tenebrio* cuticle architecture. *J. Insect Physiol. 16*, 1087–1107.

Champlin, D.T., Reiss, S.E., Truman, J.W., **1999**. Hormonal control of ventral diaphragm myogenesis during metamorphosis of the moth, *Manduca sexta*. *Devel. Genes Evol. 209*, 265–274.

Chau, P.L., Vanaalten, D.M.F., Bywater, R.P., Findlay, J.B.C., **1999**. Functional concerted motions in the bovine serum retinol-binding protein. *J. Comp. Aided Mol. Des. 13*, 11–20.

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., **2001**. Nuclear receptors and lipid physiology: opening the X-files. *Science 294*, 1866–1870.

Chefurka, W., **1978**. Sesquiterpene juvenile hormones: novel uncouplers of oxidative phosphorylation. *Biochem. Biophys. Res. Commun. 83*, 571–578.

Chen, L., OKeefe, S.L., Hodgetts, R.B., **2002a**. Control of dopa decarboxylase gene expression by the Broad-Complex during metamorphosis in *Drosophila*. *Mech. Devel. 119*, 145–156.

Chen, L., Reece, C., O'Keefe, S.L., Hawryluk, G.W.L., Engstrom, M.M., *et al.*, **2002b**. Induction of the early-late *Ddc* gene during *Drosophila* metamorphosis by the ecdysone receptor. *Mech. Devel. 114*, 95–107.

Chen, P.S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M., *et al.*, **1988**. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. *Cell 54*, 291–298.

Cheon, H.M., Hwang, S.J., Kim, H.J., Jin, B.R., Chae, K.S., *et al.*, **2002**. Two juvenile hormone suppressible storage proteins may play different roles in *Hyphantria cunea*. *Arch. Insect Biochem. Physiol. 50*, 157–172.

Chiang, A.S., Lin, W.Y., Liu, H.P., Pszczolkowski, M.A., Fu, T.F., *et al.*, **2002a**. Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA 99*, 37–42.

Chiang, A.S., Gadot, M., Burns, E.L., Schal, C., **1991**. Developmental regulation of juvenile hormone synthesis: ovarian synchronization of volumetric changes of corpus allatum cells in cockroaches. *Mol. Cell. Endocrinol. 75*, 141–147.

Chiang, A.S., Pszczolkowski, M.A., Liu, H.P., Lin, S.C., **2002b**. Ionotropic glutamate receptors mediate juvenile hormone synthesis in the cockroach, *Diploptera punctata*. *Insect Biochem. Mol. Biol. 32*, 669–678.

Chiang, A.S., Gadot, M., Schal, C., **1989**. Morphometric analysis of corpus allatum cells in adult females of three cockroach species. *Mol. Cell. Endocrinol. 67*, 179–184.

Clarke, K.U., Baldwin, R.W., **1960**. The effect of insect hormones and of 2:2-dinitrophenol on the mitochondrion of *Locusta migratoria*. *J. Insect Physiol. 5*, 37–46.

Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., **2001**. The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA 98*, 1549–1554.

Cnaani, J., Robinson, G.E., Hefetz, A., **2000**. The critical period for caste determination in *Bombus terrestris* and its juvenile hormone correlates. *J. Comp. Physiol. A 186*, 1089–1094.

Cole, T.J., Beckage, N.E., Tan, F.F., Srinivasan, A., Ramaswamy, S.B., **2002**. Parasitoid–host endocrine relations: self-reliance or co-option? *Insect Biochem. Mol. Biol. 32*, 1673–1679.

Cotton, G., Anstee, J.H., **1991**. A biochemical and structural study on the effects of methoprene on fat body development in *Locusta migratoria* L. *J. Insect Physiol. 37*, 525–539.

Coudron, T.A., **1981**. Preparation of homogeneous juvenile hormone specific esterase from the haemolymph of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. 11*, 453–461.

Couillaud, F., **1995**. Juvenile hormone biosynthesis by insect corpora allata: variations on a central theme. *Netherlands J. Zool. 45*, 79–82.

Couillaud, F., Girardie, J., Tobe, S.S., Girardie, A., **1984**. Activity of disconnected corpora allata in *Locusta migratoria*: juvenile hormone biosynthesis *in vitro* and physiological effects *in vitro*. *J. Insect Physiol. 30*, 551–556.

Crossley, A.C., **1985**. Nephrocytes and pericardial cells. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3. Pergamon, Oxford, pp. 487–515.

Cusson, M., Lepage, A., McNeil, J.N., Tobe, S.S., **1996**. Rate of isoleucine metabolism in lepidopteran corpora allata: Regulation of the proportion of juvenile hormone homologues released. *Insect Biochem. Mol. Biol. 26*, 195–201.

Cusson, M., Miller, D., Goodman, W.G., **1997**. Characterization of antibody 444 using chromatographically purified enantiomers of juvenile hormones I, II, and III: Implications for radioimmunoassays. *Analyt. Biochem. 249*, 83–87.

Cusson, M., Prestwich, G.D., Stay, B., Tobe, S.S., **1991b**. Photoaffinity labeling of allatostatin receptor proteins in the corpora allata of the cockroach, *Diploptera*

punctata. *Biochem. Biophys. Res. Commun. 181*, 736–742.

Cusson, M., Tobe, S.S., McNeil, J.N., **1994**. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm moth, *Pseudaletia unipuncta*. *Arch. Insect Biochem. Physiol. 25*, 329–345.

Cusson, M., Yagi, K.J., Guan, X.C., Tobe, S.S., **1992**. Assessment of the role of cyclic nucleotides in allatostatin-induced inhibition of juvenile hormone biosynthesis in *Diploptera punctata*. *Mol. Cell. Endocrinol. 89*, 121–125.

Cusson, M., Yagi, K.J., Ding, Q., Duve, H., Thorpe, A., *et al.*, **1991a**. Biosynthesis and release of juvenile hormone and its precursors in insects and crustaceans: The search for a unifying arthropod endocrinology. *Insect Biochem. Mol. Biol. 21*, 1–6.

Cymborowski, B., Bogus, M., Beckage, N.E., Williams, C.M., Riddiford, L.M., **1982**. Juvenile hormone titers and metabolism during starvation-induced supernumerary larval moulting of the tobacco hormworm, *Manduca sexta*. *J. Insect Physiol. 28*, 129–135.

Czeisler, C.A., Klerman, E.B., **1999**. Circadian and sleep-dependent regulation of hormone research in humans. *Rec. Progr. Hormone Res. 57*, 97–130.

Dahm, K.H., Bhaskaran, G., Peter, M.G., Shirk, P.D., Seshan, K.R., *et al.*, **1976**. On the identity of the juvenile hormones in insects. In: Gilbert, L.I. (Ed.), The Juvenile Hormones. Plenum, New York, pp. 19–47.

Dahm, K.H., Röller, H., Trost, B.M., **1968**. The JH: IV. Stereochemistry of JH and biological activity of some of its isomers and related compounds. *Life Sci. 7*, 129–137.

Dai, J.D., Gilbert, L.I., **1998**. Juvenile hormone prevents the onset of programmed cell death in the prothoracic glands of *Manduca sexta*. *Gen. Comp. Endocrinol. 109*, 155–165.

Dai, J.D., Gilbert, L.I., **1999**. An *in vitro* analysis of ecdysteroid-elicited cell death in the prothoracic gland of *Manduca sexta*. *Cell Tissue Res. 297*, 319–327.

Dale, J.F., Tobe, S.S., **1988a**. Differences in the stimulation by calcium ionophore of juvenile hormone-III release from corpora allata of solitarious and gregarious *Locusta migratoria*. *Experientia 44*, 240–241.

Dale, J.F., Tobe, S.S., **1988b**. The effect of a calcium ionophore, a calcium channel blocker and calcium-free medium on juvenile hormone release *in vitro* from corpora allata of *Locusta migratoria*. *J. Insect Physiol. 34*, 451–456.

Darrouzet, E., Rossignol, F., Couillaud, F., **1998**. The release of isoprenoids by locust corpora allata *in vitro*. *J. Insect Physiol. 44*, 103–111.

Darrouzet, E., Mauchamp, B., Prestwich, G.D., Kerhoas, L., Ujvary, I., *et al.*, **1997**. Hydroxy juvenile hormones: new putative juvenile hormones biosynthesized by locust corpora allata *in vitro*. *Biochem. Biophys. Res. Commun. 240*, 752–758.

Davey, K.G., **2000a**. The modes of action of juvenile hormones: some questions we ought to ask. *Insect Biochem. Mol. Biol. 30*, 663–669.

Davey, K.G., **2000b**. Do thyroid hormones function in insects? *Insect Biochem. Mol. Biol. 30*, 877–884.

Davey, K.G., Gordon, D.R.B., **1996**. Fenoxycarb and thyroid hormones have JH-like effects on the follicle cells of *Locusta migratoria in vitro*. *Arch. Insect Biochem. Physiol. 32*, 613–622.

Davey, K.G., Sevala, V.L., Gordon, D.R.B., **1993**. The action of juvenile hormone and antigonadotropin on the follicle cells of *Locusta migratoria*. *Invertebr. Reprod. Devel. 24*, 39–46.

Davis, N.T., Veenstra, J.A., Feyereisen, R., Hildebrand, J.G., **1997**. Allatostatin-like-immunoreactive neurons of the tobacco hornworm, *Manduca sexta*, and isolation and identification of a new neuropeptide related to cockroach allatostatins. *J. Comp. Neurol. 385*, 265–284.

Dean, R.L., Locke, M., Collins, J.V., **1985**. Structure of the fat body. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3. Pergamon, Oxford, pp. 155–210.

Debernard, S., Morisseau, C., Severson, T.F., Feng, L., Wojtasek, H., *et al.*, **1998**. Expression and characterization of the recombinant juvenile hormone epoxide hydrolase (JHEH) from *Manduca sexta*. *Insect Biochem. Mol. Biol. 28*, 409–419.

Debernard, S., Rossignol, F., Malosse, C., Mauchamp, B., Couillaud, F., **1995**. Transesterification of juvenile hormone occurs *in vivo* in locust when injected in alcoholic solvents. *Experientia 51*, 1220–1224.

Dedos, S.G., Fugo, H., **1996**. Effects of fenoxycarb on the secretory activity of the prothoracic glands in the fifth instar of the silkworm *Bombyx mori*. *Gen. Comp. Endocrinol. 104*, 213–225.

deKort, C.A.D., **1990**. Thirty-five years of diapause research with the Colorado potato beetle. *Entomol. Exp. Applic. 56*, 1–13.

deKort, C.A.D., Bergot, B.J., Schooley, D.A., **1982**. The nature and titre of juvenile hormone in the Colorado potato beetle *Leptinotarsa decemlineata*. *J. Insect Physiol. 28*, 471–474.

deKort, C.A.D., Granger, N.A., **1996**. Regulation of JH titers: the relevance of degradative enzymes and binding proteins. *Arch. Insect Biochem. Physiol. 33*, 1–26.

Della-Cioppa, G., Engelmann, F., **1984**. Juvenile hormone regulation of phospholipid synthesis in the endoplasmic reticulum of vitellogenic fat body cells from *Leucophaea maderae*. *Insect Biochem. 14*, 27–36.

deWilde, J., Staal, G.B., deKort, C.A.D., deLoof, A., Baard, G., **1968**. JH titer in the hemolymph as a function of photoperiodic treatment in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). *Proc. Kon. Ned. Wetensch. Ser. C Biol. Med. Sci. 71C*, 321–326.

Ding, Q., Donly, B.C., Tobe, S.S., Bendena, W.G., **1995**. Comparison of the allatostatin neuropeptide precursors

in the distantly related cockroaches *Periplaneta americana* and *Diploptera punctata*. *Eur. J. Biochem. 234*, 737–746.

Dingle, H., Winchell, R., **1997**. Juvenile hormone as a mediator of plasticity in insect life histories. *Arch. Insect Biochem. Physiol. 35*, 359–373.

Dircksen, H., Skiebe, P., Abel, B., Agricola, H., Buchner, K., *et al.*, **1999**. Structure, distribution, and biological activity of novel members of the allatostatin family in the crayfish *Orconectes limosus*. *Peptides 20*, 695–712.

Donly, B.C., Ding, Q., Tobe, S.S., Bendena, W.G., **1993a**. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. *Proc. Natl Acad. Sci. USA 90*, 8807–8811.

Donly, B.C., Ding, Q., Tobe, S.S., Bendena, W.G., **1993b**. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. *Proc. Natl Acad. Sci. USA 90*, 12055(correction of 1993a).

Dorn, A., **1975**. Struktur und funktion des embryonalen corpus allatum von *Oncopeltus fasciatus*. *Verhandl. Deutsche Zool. Gesell. 67*, 85–89.

Dorn, A., **1982**. Precocene-induced effects and possible role of juvenile hormone during embryogenesis of the milkweed bug *Oncopeltus fasciatus*. *Gen. Comp. Endocrinol. 42*, 42–52.

Dorn, A., Bishoff, S.T., Gilbert, L.I., **1987**. An incremental analysis of the embryonic development of the tobacco hornworm, *Manduca sexta*. *Int. J. Invertebr. Reprod. Devel. 11*, 137–158.

Duk, M., Krotkiewski, H., Forest, E., Parkitna, J.M.R., Kochman, M., *et al.*, **1996**. Evidence for glycosylation of the juvenile-hormone-binding protein from *Galleria mellonella* hemolymph. *Eur. J. Biochem. 242*, 741–746.

Duportets, L., Dufour, M.C., Couillaud, F., Gadenne, C., **1998**. Biosynthetic activity of corpora allata, growth of sex accessory glands and mating in the male moth *Agrotis ipsilon* (Hufnagel). *J. Exp. Biol. 201*, 2425–2432.

Duve, H., Johnsen, A.H., Scott, A.G., Thorpe, A., **2002**. Allatostatins of the tiger prawn, *Penaeus monodon*. *Peptides 23*, 1039–1051.

Duve, H., Audsley, N., Weaver, R.J., Thorpe, A., **2000**. Triple co-localisation of two types of allatostatin and an allatotropin in the frontal ganglion of the lepidopteran *Lacanobia oleracea*: innervation and action on the foregut. *Cell Tissue Res. 300*, 153–163.

Duve, H., East, P.D., Thorpe, A., **1999**. Regulation of lepidopteran foregut movement by allatostatins and allatotropin from the frontal ganglion. *J. Comp. Neurol. 413*, 405–416.

Dyby, S., Silhacek, D.L., **1997**. Juvenile hormone agonists cause abnormal midgut closure and other defects in the moth *Plodia interpunctella*. *Invertebr. Reprod. Devel. 32*, 231–244.

East, P.D., Sutherland, T.D., Trowell, S.C., Herlt, A.J., Rickards, R.W., **1997**. Juvenile hormone synthesis by ring glands of the blowfly *Lucilia cuprina*. *Arch. Insect Biochem. Physiol. 34*, 239–253.

Edwards, J.P., Weaver, R.J., Marris, G.C., **2001**. Endocrine changes in lepidopteran larvae: potential challenges to parasitoid development and survival. In: Edwards, J.P., Weaver, R.J. (Eds.), Endocrine Interactions of Insect Parasites and Pathogens. Bios Scientific Publishers, Oxford, pp. 1–32.

Elekonich, M.M., Robinson, G.E., **2000**. Organizational and activational effects of hormones on insect behavior. *J. Insect Physiol. 46*, 1509–1515.

Elekonich, M.M., Schulz, D.J., Bloch, G., Robinson, G.E., **2001**. Juvenile hormone levels in honey bee (*Apis mellifera* L.) foragers: foraging experience and diurnal variation. *J. Insect Physiol. 47*, 1119–1125.

Engelmann, F., Mala, J., **2000**. The interactions between juvenile hormone (JH), lipophorin, vitellogenin, and JH esterases in two cockroach species. *Insect Biochem. Mol. Biol. 30*, 793–803.

Enslee, E.C., Riddiford, L.M., **1977**. Morphological effects of JH mimics on embryonic development in the bug *Pyrrochoris apterus*. *Roux's Arch. Devel. Biol. 181*, 163–181.

Evans, P.D., **1985**. Octopamine. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemstry and Pharmacology, vol. 11. Pergamon, Oxford, pp. 500–530.

Everson, R.D., Feir, D., **1976**. Juvenile hormone regulation of cyclic AMP and cAMP phosphodiesterase activity in *Oncopeltus fasciatus*. *J. Insect Physiol. 22*, 781–784.

Fain, M., Riddiford, L.M., **1975**. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta*. *Biol. Bull. 149*, 506–521.

Fan, Y.L., Rafaeli, A., Moshitzky, P., Kubli, E., Choffat, Y., *et al.*, **2000**. Common functional elements of *Drosophila melanogaster* seminal peptides involved in reproduction of *Drosophila melanogaster* and *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol. 30*, 805–812.

Fan, Y.L., Rafaeli, A., Gileadi, C., Kubli, E., Applebaum, S.W., **1999**. *Drosophila melanogaster* sex peptide stimulates juvenile hormone synthesis and depresses sex pheromone production in *Helicoverpa armigera*. *J. Insect Physiol. 45*, 127–133.

Farkas, R., Sutakova, G., **2001**. Swelling of mitochondria induced by juvenile hormone in larval salivary glands of *Drosophila melanogaster*. *Biochem. Cell Biol. 79*, 755–764.

Farkas, R., Knopp, J., **1998**. Genetic and hormonal control of cytosolic malate dehydrogenase activity in *Drosophila melanogaster*. *Gen. Physiol. and Biophys. 17*, 37–50.

Farkas, R., Knopp, J., **1997**. Ecdysone-modulated response of *Drosophila* cytosolic malate dehydrogenase to juvenile hormone. *Arch. Insect Biochem. Physiol. 35*, 71–83.

Farkas, R., Danis, P., Medvedova, L., Mechler, B.M., Knopp, J., **2002**. Regulation of cytosolic malate dehydrogenase by juvenile hormone in *Drosophila melanogaster*. *Cell Biochem. Biophys. 37*, 37–52.

Faulkner, D.J., Petersen, H.R., **1971**. Synthesis of C18 cecropia juvenile hormone to obtain optically active forms of known absolute configuration. *J. Am. Chem. Soc. 93*, 3766–3767.

Feng, Q.L., Ladd, T.R., Tomkins, B.L., Sundaram, M., Sohi, S.S., *et al.*, **1999**. Spruce budworm (*Choristoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning. *Mol. Cell. Endocrinol. 148*, 95–108.

Ferenz, H.J., Aden, E., **1993**. Ovarian control of juvenile hormone biosynthesis in *Locusta migratoria*. *Entomol. General. 18*, 9–17.

Fersht, A., **1985**. Enzyme Structure and Mechansim. W.H. Freeman, San Francisco, CA.

Feyereisen, R., **1985**. Regulation of juvenile hormone titer: Synthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemstry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 391–430.

Feyereisen, R., Farnsworth, D.E., **1988**. Forced synthesis of trace amounts of juvenile hormone II from propionate by corpora allata of a juvenile hormone III producing insect. *Experientia 44*, 47–49.

Feyereisen, R., Farnsworth, D.E., **1987**. Inhibition of insect juvenile hormone synthesis by phorbol 12-myristate 13-acetate. *FEBS Lett. 222*, 345–348.

Feyereisen, R., Tobe, S.S., **1981**. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. *Analyt. Biochem. 111*, 372–375.

Flower, D.R., **1996**. The lipocalin protein family: structure and function. *Biochem. J. 318*, 1–14.

Flower, D.R., **2000**. Experimentally determined lipocalin structures. *Biochim. Biophys. Acta 1482*, 46–56.

Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., *et al.*, **1995**. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell 81*, 687–693.

Friedrich, M., Tautz, D., **1995**. Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. *Nature 376*, 165–167.

Gade, G., **2002**. Allatoregulatory peptides: molecules with multiple functions. *Invertebr. Reprod. Devel. 41*, 127–135.

Gade, G., Hoffmann, K.H., Spring, J.H., **1997**. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev. 77*, 963–1032.

Gadot, M., Rafaeli, A., Applebaum, S.W., **1987**. Partial purification and characterization of locust allatotropin I. *Arch. Insect Biochem. Physiol. 4*, 213–224.

Garside, C.S., Koladich, P.M., Bendena, W.G., Tobe, S.S., **2002**. Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata*. *Insect Biochem. Mol. Biol. 32*, 1089–1099.

Garside, C.S., Hayes, T.K., Tobe, S.S., **1997a**. Degradation of dip-allatostatins by hemolymph from the cockroach, *Diploptera punctata*. *Peptides 18*, 17–25.

Garside, C.S., Hayes, T.K., Tobe, S.S., **1997b**. Inactivation of Dip-allatostatin 5 by membrane preparations from the cockroach *Diploptera punctata*. *Gen. Comp. Endocrinol. 108*, 258–270.

Garside, C.S., Nachman, R.J., Tobe, S.S., **2000**. Injection of dip-allatostatin or dip-allatostatin pseudopeptides into mated female *Diploptera punctata* inhibits endogenous rates of JH biosynthesis and basal oocyte growth. *Insect Biochem. Mol. Biol. 30*, 703–710.

Gharib, B., deReggi, M., Connat, J.L., Chaix, J.C., **1983**. Ecdysteroid and juvenile hormone changes in *Bombyx mori* eggs, related to the initiation of diapause. *FEBS Lett. 160*, 119–123.

Gilbert, L.I., **1962**. Maintenance of the prothoracic gland by the juvenile hormone in insects. *Nature 193*, 1205–1207.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Gilbert, L.I., Schneiderman, H.A., **1961**. The content of juvenile hormone and lipid in Lepidoptera: sexual differences and developmental changes. *Gen. Comp. Endocrinol. 1*, 453–472.

Gingrich, J.A., Caron, M.G., **1993**. Recent advances in the molecular biology of dopamine receptors. *Annu. Rev. Neurosci. 16*, 299–321.

Girardie, J., Girardie, A., **1996**. Lom OMP, a putative ecdysiotropic factor for the ovary in *Locusta migratoria*. *J. Insect Physiol. 42*, 215–222.

Girardie, J., Huet, J.C., Atay-Kadiri, Z., Ettaouil, S., Delbecque, J.P., *et al.*, **1998**. Isolation, sequence determination, physical and physiological characterization of the neuroparsins and ovary maturating parsins of *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 28*, 641–650.

Gold, S.M.W., Davey, K.G., **1989**. The effect of juvenile hormone on protein synthesis in the transparent accessory gland of male *Rhodnius prolixus*. *Insect Biochem. 19*, 139–143.

Goodman, W.G., **1983**. Hemolymph transport of ecdysteroids and juvenile hormones. In: Downer, R.G.H., Laufer, H. (Eds.), Endocrinology of Insects. Alan R. Liss, New York, pp. 147–160.

Goodman, W.G., **1990**. Biosynthesis, titer regulation and transport of juvenile hormones. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, NJ, pp. 83–124.

Goodman, W.G., Adams, B., Trost, J.T., **1985**. Purification and characterization of a biliverdin-associated protein from the hemolymph of *Manduca sexta*. *Biochem. 24*, 1168–1175.

Goodman, W.G., Bollenbacher, W.E., Zvenko, H.L., Gilbert, L.I., **1976**. A competitive binding protein assay for juvenile hormone. In: Gilbert, L.I. (Ed.), The Juvenile Hormones. Plenum, New York, pp. 75–95.

Goodman, W.G., Huang, Z.H., Robinson, G.E., Strambi, C., Strambi, A., **1993**. Comparison of two juvenile hormone radioimmunoassays. *Arch. Insect Biochem. Physiol. 23*, 147–152.

Goodman, W.G., Maxfield, B., Park, Y.C., **1991**. Immunochemical similarities among the hemolymph juvenile hormone binding proteins of moths. *Experientia 47*, 945–948.

Goodman, W.G., O'Hern, P.A., Zaug, R.H., Gilbert, L.I., **1978a**. Purification and characterization of a juvenile hormone binding protein from the hemolymph of the fourth instar tobacco hornworm, M*anduca sexta*. *Mol. Cell. Endocrinol. 11*, 225–242.

Goodman, W.G., Orth, A.P., Toong, Y.C., Ebersohl, R., Hiruma, K., *et al.*, **1995**. Recent advances in radioimmunoassay technology for the juvenile hormones. *Arch. Insect Biochem. Physiol. 30*, 295–305.

Goodman, W.G., Schooley, D.A., Gilbert, L.I., **1978b**. Specificity of the juvenile hormone binding protein: the geometrical isomers of juvenile hormone I. *Proc. Natl Acad. Sci. USA 75*, 185–189.

Goodman, W.G., Tatham, G., Nesbit, D.J., Bultmann, H., Sutton, R.D., **1987**. The role of juvenile hormone in endocrine control of pigmentation in *Manduca sexta*. *Insect Biochem. 17*, 1065–1070.

Granger, N.A., **2003**. Chemistry of juvenile hormones. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones. Elsevier Science, New York, pp. 547–554.

Granger, N.A., Allen, L.G., Sturgis, S.L., Combest, W., Ebersohl, R., **1995b**. Corpora allata of the larval tobacco hornworm contain a calcium/calmodulin-sensitive adenyl cyclase. *Arch. Insect Biochem. Physiol. 30*, 149–164.

Granger, N.A., Bollenbacher, W.E., Vince, R., Gilbert, L.I., Baehr, J.-C., *et al.*, **1979**. *In vitro* biosynthesis of juvenile hormone by the larval corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol. 16*, 1–17.

Granger, N.A., Ebersohl, R., Sparks, T.C., **2000**. Pharmacological characterization of dopamine receptors in the corpus allatum of *Manduca sexta* larvae. *Insect Biochem. Mol. Biol. 30*, 755–766.

Granger, N.A., Goodman, W.G., **1983**. Juvenile hormone radioimmunoassays: theory and practice. *Insect Biochem. 13*, 333–340.

Granger, N.A., Goodman, W.G., **1988**. Radioimmunoassays: Juvenile Hormones. In: Gilbert, L.I., Miller, T.A. (Eds.), Immunological Techniques in Insect Biology. Springer, New York, pp. 215–252.

Granger, N.A., Janzen, W.P., **1987**. Inhibition of *Manduca sexta* corpora allata *in vitro* by a cerebral allatostatic neuropeptide. *Mol. Cell. Endocrinol. 49*, 237–248.

Granger, N.A., Janzen, W.P., Ebersohl, R., **1995a**. Biosynthetic products of the corpus allatum of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 427–439.

Granger, N.A., Macdonald, J.D., Menold, M., Ebersohl, R., Hiruma, K., *et al.*, **1994**. Evidence of a stimulatory effect of cyclic AMP on corpus allatum activity in *Manduca sexta*. *Mol. Cell. Endocrinol. 103*, 73–80.

Granger, N.A., Mitchell, L.J., Niemiec, S.M., **1986**. Biosynthesis of juvenile hormones I and III by the corpora allata of *Manduca sexta*: effects of *in vitro* conditions on gland activity. In: Kurstak, E. (Ed.), *In Vitro* Invertebrate Hormones and Genes. Elsevier, London, pp. 1–15.

Granger, N.A., Niemiec, S.M., Gilbert, L.I., Bollenbacher, W.E., **1982**. Juvenile hormone III biosynthesis *in vitro* by larval and pupal corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol. 28*, 587–604.

Granger, N.A., Sehnal, F., **1974**. Regulation of larval corpora allata in *Galleria mellonella*. *Nature 21*, 415–417.

Granger, N.A., Sturgis, S.L., Ebersohl, R., Geng, C.X., Sparks, T.C., **1996**. Dopaminergic control of corpora allata activity in the larval tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 32*, 449–466.

Granger, N.A., Whisenton, L.R., Janzen, W.P., Bollenbacher, W.E., **1987**. Interendocrine control by 20-hydroxyecdysone of the corpora allata of *Manduca sexta*. *Insect Biochem. 17*, 949–954.

Grasberger, H., Golcher, H.M.B., Fingerhut, A., Janssen, O.E., **2002**. Loop variants of the serpin thyroxine-binding globulin: implications for hormone release upon limited proteolysis. *Biochem. J. 365*, 311–316.

Grieneisen, M.L., Mok, A., Kieckbusch, T.D., Schooley, D.A., **1997**. The specificity of juvenile hormone esterase revisited. *Insect Biochem. Mol. Biol. 27*, 365–376.

Grieneisen, M.L., Kieckbusch, T.D., Mok, A., Dorman, G., Latli, B., *et al.*, **1995**. Characterization of the juvenile hormone epoxide hydrolase (JHEH) and juvenile hormone diol phosphotransferase (JHDPT) from *Manduca sexta* Malpighian tubules. *Arch. Insect Biochem. Physiol. 30*, 255–270.

Grishkovskaya, I., Avvakumov, G.V., Hammond, G.L., Catalano, M.G., Muller, Y.A., **2002**. Steroid ligands bind human sex hormone-binding globulin in specific orientations and produce distinct changes in protein conformation. *J. Biol. Chem. 277*, 32086–32093.

Grossniklaus-Bürgin, C., Lanzrein, B., **1990**. Qualitative and quantitative analyses of juvenile hormone and ecdysteroids from the egg to the pupal molt in *Trichoplusia ni*. *Arch. Insect Biochem. Physiol. 14*, 14–30.

Gruetzmacher, M.C., Gilbert, L.I., Granger, N.A., Goodman, W., Bollenbacher, W.E., **1984**. The effect of juvenile hormone on prothoracic gland function during the larval–pupal development of *Manduca sexta*: an *in situ* and *in vitro* analysis. *J. Insect Physiol. 30*, 331–340.

Gruntenko, N.E., Wilson, T.G., Monastirioti, M., Rauschenbach, I.Y., **2000**. Stress-reactivity and juvenile hormone degradation in *Drosophila melanogaster* strains having stress-related mutations. *Insect Biochem. Mol. Biol. 30*, 775–783.

Gu, S.H., Chow, Y.S., Yin, C.-M., **1997**. Involvement of juvenile hormone in regulation of prothoracicotropic hormone transduction during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol. 127*, 109–116.

Hagedorn, H.H., **1983**. The role of ecdysteroids in adult insects. In: Downer, R.G.H., Laufer, H. (Eds.), Endocrinology of Insects. Alan R. Liss, New York, pp. 271–304.

Halarnkar, P.P., Jackson, G.P., Straub, K.M., Schooley, D.A., **1993**. Juvenile hormone catabolism in *Manduca sexta*: homologue selectivity of catabolism and identification of a diol-phosphate conjugate as a major end product. *Experientia 49*, 988–994.

Halarnkar, P.P., Schooley, D.A., **1990**. Reversed-phase liquid chromatographic separation of juvenile hormone and its metabolites and its application for an *in vivo* juvenile hormone catabolism study in *Manduca sexta*. *Analyt. Biochem. 188*, 394–397.

Hammock, B.D., **1985**. Regulation of juvenile hormone titer: Degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 431–472.

Hammock, B.D., Nowock, J., Goodman, W.G., Stamoudis, V., Gilbert, L.I., **1975**. The influence of hemolymph-binding protein on juvenile hormone stability and distribution in *Manduca sexta* fat body and imaginal discs *in vitro*. *Mol. Cell. Endocrinol. 3*, 167–184.

Hammock, B.D., Wing, K.D., McLaughlin, J., Lovell, V.M., Sparks, T.C., **1982**. Trifluoromethylketones as possible transition state analog inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol. 17*, 76–88.

Hanzlik, T.N., Abdel-aal, Y.A.I., Harshman, L.G., Hammock, B.D., **1989**. Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from *Heliothis virescens*: evidence for a catalytic mechanism for the serine carboxylesterases different from that of the serine proteases. *J. Biol. Chem. 264*, 12419–12425.

Hanzlik, T.N., Hammock, B.D., **1987**. Characterization of affinity purified JHE from *Trichoplusia ni*. *J. Biol. Chem. 262*, 13584–13589.

Hanzlik, T.N., Hammock, B.D., **1988**. Characterization of juvenile hormone hydrolysis in early larval development of *Trichoplusia ni*. *Arch. Insect Biochem. Physiol. 9*, 135–156.

Hardie, J., **1987**. Juvenile hormone stimulation of oocyte development and embryogenesis in the parthenogenetic ovaries of an aphid, *Aphis fabae*. *Int. J. Invertebr. Reprod. Devel. 11*, 189–202.

Hardie, J., Mallory, A.C.L., Quashie-Williams, C.A., **1990**. Juvenile hormone and host-plant colonization by the black bean aphid, *Aphis fabae*. *Physiol. Entomol. 15*, 331–336.

Harmon, M.A., Boehm, M.F., Heyman, R.A., Mangelsdorf, D.J., **1995**. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl Acad. Sci. USA 92*, 6157–6160.

Harris, S.V., Thompson, D.M., Linderman, R.J., Tomalski M.D., Roe, R.M., **1999**. Cloning and expression of a novel juvenile hormone-metabolizing epoxide hydrolase during larval-pupal metamorphosis of the cabbage looper, *Trichoplusia ni*. *Insect Mol. Biol. 8*, 85–96.

Harshman, L.G., Ward, V.K., Beetham, J.K., Grant, D.F., Grahan, L.J., *et al.*, **1994**. Cloning, characterization, and genetics of the juvenile hormone esterase gene from *Heliothis virescens*. *Insect Biochem. Mol. Biol. 24*, 671–676.

Harshman, L.G., Casas, J., Dietze, E.C., Hammock, B.D., **1991**. Epoxide hydrolase activities in *Drosophila melanogaster*. *Insect Biochem. 21*, 887–894.

Hartmann, R., Jendrsczok, C., Peter, M.G., **1987**. The occurrence of a juvenile hormone binding protein and *in vitro* synthesis of juvenile hormone by the serosa of *Locusta migratoria* embryos. *Roux's Arch. Devel. Biol. 196*, 347–355.

Haunerland, N.H., Shirk, P.D., **1995**. Regional and functional differentiation in the insect fat body. *Annu. Rev. Entomol. 40*, 121–145.

Hayakawa, Y., **1990**. Juvenile hormone esterase activity repressive factor in the plasma of parasitized insect larvae. *J. Biol. Chem. 265*, 10813–10816.

Heftmann, E., Mosettig, E., **1960**. Biochemistry of Steroids. Reinhold Publishing, New York, NY.

Hegstrom, C.D., Riddiford, L.M., Truman, J.W., **1998**. Steroid and neuronal regulation of ecdysone receptor expression during metamorphosis of muscle in the moth, *Manduca sexta*. *J. Neurosci. 18*, 1786–1794.

Heming, B.S., **2003**. Insect Development and Evolution. Cornell University Press, Ithaca, NY.

Hewes, R.S., Truman, J.W., **1994**. Steroid regulation of excitability in identified insect neurosecretory cells. *J. Neurosci. 14*, 1812–1819.

Hidayat, P., Goodman, W.G., **1994**. Juvenile hormone and hemolymph juvenile hormone binding protein titers and their interaction in the hemolymph of fourth stadium *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 709–715.

Hinton, A.C., Hammock, B.D., **2001**. Purification of juvenile hormone esterase and molecular cloning of the cDNA from *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 57–66.

Hinton, A.C., Hammock, B.D., **2003**. Juvenile hormone esterase (JHE) from *Tenebrio molito*r: full-length cDNA sequence, *in vitro* expression, and characterization of the recombinant protein. *Insect Biochem. Mol. Biol. 33*, 477–487.

Hirai, M., Kamimura, M., Kikuchi, K., Yasukochi, Y., Kiuchi, M., *et al.*, **2002**. cDNA cloning and characterization of *Bombyx mori* juvenile hormone esterase: an inducible gene by the imidazole insect growth regulator KK-42. *Insect Biochem. Mol. Biol. 32*, 627–635.

Hiruma, K., Carter, M.S., Riddiford, L.M., **1995**. Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Devel. Biol. 169*, 195–209.

Hiruma, K., Riddiford, L.M., **1984**. Regulation of melanization of tobacco hornworm larval cuticle *in vitro*. *J. Exp. Zool. 230*, 393–403.

Hiruma, K., Riddiford, L.M., **1988**. Granular phenoloxidase involved in cuticular melanization in the tobacco hornworm: regulation of its synthesis in the epidermis by juvenile hormone. *Devel. Biol. 130*, 87–97.

Hiruma, K., Shimada, H., Yagi, S., **1978**. Activation of the prothoracic gland by juvenile hormone and prothoracicotropic hormone in *Mamestra brassicae*. *J. Insect Physiol. 24*, 215–220.

Ho, H.Y., Tu, M.P., Chang, C.Y., Yin, C.-M., Kou, R., **1995a**. Identification of *in vitro* release products of corpora allata in female and male loreyi leafworms, *Leucania loreyi*. *Experientia 51*, 601–605.

Ho, H.Y., Tu, M.P., Chang, C.Y., Yin, C.-M., Kou, R., **1995b**. Identification of *in vitro* release products of corpora allata in female and male loreyi leafworms, *Leucania loreyi*. *Experientia 51*, 1225(correction of 1995a).

Hodkova, M., Okuda, T., Wagner, R., **1996**. Stimulation of corpora allata by extract from neuroendocrine complex; Comparison of reproducing and diapausing *Pyrrhocoris apterus*. *Eur. J. Entomol. 93*, 535–543.

Hoffmann, K.H., Lorenz, M.W., Klein, P.M., **1996**. Ovarian control of juvenile hormone-III biosynthesis in the cricket species *Gryllus bimaculatus*. *Entomol. General. 20*, 157–167.

Holbrook, G.L., Chiang, A.S., Schal, C., **1997**. Improved conditions for culture of biosynthetically active cockroach corpora allata. *In Vitro Cell Devel. Biol. – Animal 33*, 452–458.

Homola, E., Chang, E.S., **1997**. Crustacean juvenile hormone in search of functions. *Comp. Biochem. Physiol. B 117*, 347–356.

Hopkins, T.E., Kramer, K.J., **1992**. Insect cuticle sclerotization. *Annu. Rev. Entomol. 37*, 273–302.

Hopkins, T.L., Starkey, S.R., Beckage, N.E., **1998**. Tyrosine and catecholamine levels in the hemolymph of tobacco hornworm larvae, *Manduca sexta*, parasitized by the braconid wasp, *Cotesia congregata*. *Arch. Insect Biochem. Physiol. 38*, 193–201.

Horodyski, F.M., Bhatt, S.R., Lee, K.Y., **2001**. Alternative splicing of transcripts expressed by the *Manduca sexta* allatotropin (Mas-AT) gene is regulated in a tissue-specific manner. *Peptides 22*, 263–269.

Horodyski, F.M., Riddiford, L.M., **1989**. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. *Devel. Biol. 132*, 292–303.

Horseman, G., Hartmann, R., Virantdoberlet, M., Loher, W., Huber, F., **1994**. Nervous control of juvenile hormone biosynthesis in *Locusta migratoria*. *Proc. Natl Acad. Sci. USA 91*, 2960–2964.

Huang, X.M., Lawler, C.P., Lewis, M.M., Nichols, D.E., Mailman, R.B., **2001**. D-1 dopamine receptors. *Int. Rev. Neurobiol. 48*, 65–139.

Huang, Z.Y., Robinson, G.E., **1995**. Seasonal changes in juvenile hormone titers and rates of biosynthesis in honey bees. *Comp. Biochem. Physiol. B 165*, 18–28.

Huang, Z.Y., Robinson, G.E., Tobe, S.S., Yagi, K.J., Strambi, C., *et al.*, **1991**. Hormonal regulation of behavioural development in the honeybee is based on changes in the rate of juvenile hormone biosynthesis. *J. Insect Physiol. 37*, 733–741.

Hwang, S.J., Cheon, H.M., Kim, H.J., Chae, K.S., Chung, D.H., *et al.*, **2001**. cDNA sequence and gene expression of storage protein 2. A juvenile hormone-suppressible hexamerin from the fall webworm, *Hyphantria cunea*. *Comp. Biochem. Physiol. B 129*, 97–107.

Ichinose, R., Nakamura, A., Yamoto, T., Booth, T.F., Maeda, S., *et al.*, **1992**. Uptake of juvenile hormone esterase by pericardial cells of *Manduca sexta*. *Insect Biochem. Mol. Biol. 22*, 893–904.

Injeyan, H.S., Tobe, S.S., Rapport, E., **1979**. The effects of exogenous juvenile hormone treatment on embryogenesis in *Schistocerca gregaria*. *Can. J. Zool. 57*, 838–845.

Ismail, S.M., Gillott, C., **1995**. Identification, characterization, and developmental profile of a high molecular weight, juvenile hormone binding protein in the hemolymph of the migratory grasshopper, *Melanoplus sanguinipes*. *Arch. Insect Biochem. Physiol. 29*, 415–430.

Ismail, S.M., Goin, C., Muthumani, K., Kim, M., Dahm, K.H., *et al.*, **2000**. Juvenile hormone acid and ecdysteroid together induce competence for metamorphosis of the Verson's gland in *Manduca sexta*. *J. Insect Physiol. 46*, 59–68.

Ismail, S.M., Satyanarayana, K., Bradfield, J.Y., Dahm, K.H., Bhaskaran, G., **1998**. Juvenile hormone acid: evidence for a hormonal function in induction of vitellogenin in larvae of *Manduca sexta*. *Arch. Insect Biochem. Physiol. 37*, 305–310.

Jansons, I.S., Cusson, M., McNeil, J.N., Tobe, S.S., Bendena, W.G., **1996**. Molecular characterization of a cDNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). *Insect Biochem. Mol. Biol. 26*, 767–773.

Janzen, W.P., Menold, M., Granger, N.A., **1991**. Effects of endogenous esterases and an allatostatin on the products of *Manduca sexta* corpora allata *in vitro*. *Physiol. Entomol. 16*, 283–293.

Jensen, A.L., Brasch, K., **1985**. Nuclear development in locust fat body: the influence of juvenile hormone on inclusion bodies and the nuclear matrix. *Tissue Cell 17*, 117–130.

Jesudason, P., Anspaugh, D.D., Roe, R.M., **1992**. Juvenile hormone metabolism in the plasma, integument, midgut, fat body, and brain during the last instar of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 20*, 87–105.

Jiang, H., Wang, Y., Yu, X.-Q., Zhu, Y., **2003**. Prophenoloxidase-activating proteinase-3 from *Manduca sexta* hemolymph clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem. Mol. Biol. 33*, 1049–1060.

Johannsen, O.A., Butt, F.M., **1941**. Embryology of Insects and Myriapods. McGraw-Hill, New York.

Jones, G., **1995**. Molecular mechanisms of action of juvenile hormone. *Annu. Rev. Entomol. 40*, 147–169.

Jones, G., Brown, N., Manczak, M., Hiremath, S., Kafatos, F.C., **1990a**. Molecular cloning, regulation, and complete sequence of a hemocyanin-related, juvenile hormone-suppressible protein from insect hemolymph. *J. Biol. Chem. 265*, 8596–8602.

Jones, G., Hanzlik, T., Hammock, B.D., Schooley, D.A., Miller, C.A., *et al.*, **1990b**. The juvenile hormone titre during the penultimate and ultimate larval stadia of *Trichoplusia ni*. *J. Insect Physiol. 36*, 77–83.

Jones, G., Hiremath, S.T., Hellmann, G.M., Rhoads, R.E., **1988**. Juvenile hormone regulation of mRNA levels for a highly abundant hemolymph protein in larval *Trichoplusia ni*. *J. Biol. Chem. 263*, 1089–1092.

Jones, G., Manczak, M., Schelling, D., Turner, H., Jones, D., **1998**. Transcription of the juvenile hormone esterase gene is under the control of both an initiator and AT-rich motif. *Biochem. J. 335*, 79–84.

Jones, G., Sharp, P.A., **1997**. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA 94*, 13499–13503.

Jones, G., Wozniak, M., Chu, Y.X., Dhar, S., Jones, D., **2001**. Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. *Insect Biochem. Mol. Biol. 32*, 33–49.

Judy, K.J., Schooley, D.A., Dunham, L.L., Hall, M.S., Bergot, J., *et al.*, **1973**. Isolation, structure, and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proc. Natl Acad. Sci. USA 70*, 1509–1513.

Jurka, J., Klonowski, P., **1996**. Integration of retroposable elements in mammals: selection of target sites. *J. Mol. Evol. 43*, 685–689.

Kaatz, H., Eichmuller, S., Kreissl, S., **1994**. Stimulatory effect of octopamine on juvenile hormone biosynthesis in honey bees (*Apis mellifera*): physiological and immunocytochemical evidence. *J. Insect Physiol. 40*, 865–872.

Kahn, S.M., Hryb, D.J., Nakhla, A.M., Romas, N.A., Rosner, W., **2002**. Sex hormone-binding globulin is synthesized in target cells. *J. Endocrinol. 175*, 113–120.

Kallapur, V.L., Majumder, C., Roe, R.M., **1996**. *In vivo* and *in vitro* tissue specific metabolism of juvenile hormone during the last stadium of the cabbage looper, *Trichoplusia ni*. *J. Insect Physiol. 42*, 181–190.

Kamimura, M., Kiuchi, M., **2002**. Applying fenoxycarb at the penultimate instar triggers an additional ecdysteroid surge and induces perfect extra larval molting in the silkworm. *Gen. Comp. Endocrinol. 128*, 231–237.

Kamita, S.G., Hinton, A.C., Wheelock, C.E., Wogulis, M.D., Wilson, D.K., *et al.*, **2003**. Juvenile hormone esterase: Why are you so JH specific? *Insect Biochem. Mol. Biol. 33*, 1261–1273.

Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., *et al.*, **1989**. Identification of an allatotropin from adult *Manduca sexta*. *Science 243*, 1481–1483.

Keiser, K.C.L., Brandt, K.S., Silver, G.M., Wisnewski, N., **2002**. Cloning, partial purification and in vivo developmental profile of expression of the juvenile hormone epoxide hydrolase of *Ctenocephalides felis*. *Arch. Insect Biochem. Physiol. 50*, 191–206.

Kelly, G.M., Huebner, E., **1987**. Juvenoid effects on *Rhodnius prolixus* embryogenesis. *Insect Biochem. 17*, 1079–1083.

Kensler, T.W., Verma, A.K., Boutwell, R.K., Mueller, G.C., **1978**. Effects of retinoic acid and juvenile hormone on the induction of ornithine decarboxylase activity by 12–*O*-tetradecanoylphorbol-13-acetate. *Cancer Res. 38*, 2896–2899.

Kikuchi, K., Hirai, M., Shiotsuki, T., **2001**. Molecular cloning and tissue distribution of farnesyl pyrophosphate synthase from the silkworm, *Bombyx mori*. *J. Insect Biotechnol. Sericol. 70*, 167–172.

Kikukawa, S., Tobe, S.S., Solowiej, S., Rankin, S.M., Stay, B., **1987**. Calcium as a regulator of juvenile hormone biosynthesis and release in the cockroach *Diploptera punctata*. *Insect Biochem. 17*, 179–187.

Kim, Y., Davari, E.D., Sevala, V., Davey, K.G., **1999**. Functional binding of a vertebrate hormone, L-3,5,3′-triiodothyronine (T-3), on insect follicle cell membranes. *Insect Biochem. Mol. Biol. 29*, 943–950.

King, L.E., Ding, Q., Prestwich, G.D., Tobe, S.S., **1995**. The characterization of a haemolymph methyl farnesoate binding protein and the assessment of methyl farnesoate metabolism by the haemolymph and other tissues from *Procambrus clarkii*. *Insect Biochem. Mol. Biol. 25*, 495–501.

King, L.E., Tobe, S.S., **1988**. The identification of an enantioselective JH-III binding protein from the haemolymph of the cockroach, *Diploptera punctata*. *Insect Biochem. 18*, 793–805.

King, L.E., Tobe, S.S., **1993**. Changes in the titre of a juvenile hormone III binding lipophorin in the hemolymph of *Diploptera punctata* during development and reproduction. *J. Insect Physiol. 39*, 241–252.

Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., Fristrom, J.W., **1988**. Interactions and developmental effects of mutations in the Broad-Complex of *Drosophila melanogaster*. *Genetics 118*, 247–259.

Klein, P.M., Lorenz, M.W., Huang, K.H., Hoffmann, K.H., **1993**. Age dependency and regulatory properties of juvenile hormone III biosynthesis in adult male crickets, *Gryllus bimaculatus*. *J. Insect Physiol. 39*, 315–324.

Kobayashi, M., Ishikawa, H., **1994**. Involvement of juvenile hormone and ubiquitin-dependent proteolysis in flight muscle breakdown of an alate aphid (*Acyrthosiphon pisum*). *J. Insect Physiol. 40*, 107–111.

Koladich, P.M., Cusson, M., Bendena, W.G., Tobe, S.S., McNeil, J.N., **2002**. Cardioacceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth, *Pseudaletia unipuncta*. *Peptides 23*, 645–651.

Kolodziejczyk, R., Dobryszycki, P., Ozyhar, A., Kochman, M., **2001**. Two disulphide bridges are present in juvenile hormone binding protein from *Galleria mellonella*. *Acta Biochim. Polon. 48*, 917–920.

Kolodziejczyk, R., Kochman, M., Bujacz, G., Dobryszycki, P., Ozyhar, A., *et al.*, **2003**. Crystallization

and preliminary crystallographic studies of juvenile hormone-juvenile hormone binding protein from *Galleria mellonella*. *Acta Crystallogr. D 59*, 519–521.

Koopmanschap, A.B., deKort, C.A.D., **1988**. Isolation and characterization of a high molecular weight JH-III transport protein in the hemolymph of *Locusta migratoria*. *Arch. Insect Biochem. Physiol. 7*, 105–118.

Kotaki, T., **1993**. Biosynthetic products by heteropteran corpora allata *in vitro*. *Appl. Entomol. Zool. 28*, 242–245.

Kotaki, T., **1996**. Evidence for a new juvenile hormone in a stink bug, *Plautia stali*. *J. Insect Physiol. 42*, 279–286.

Kotaki, T., **1997**. A putative juvenile hormone in a stink bug, *Plautia stali:* the corpus allatum releases a JH-active product different from any known JHs *in vitro*. *J. Invertebr. Reprod. Devel. 31*, 225–230.

Kou, R., Chen, S.J., **2000**. Allatotropic activity in the suboesophageal ganglia and corpora cardiaca of the adult male loreyi leafworm, *Mythimna loreyi*. *Arch. Insect Biochem. Physiol. 43*, 78–86.

Krakauer, D.C., Plotkin, J.B., **2002**. Redundancy, antiredundancy, and the robustness of genomes. *Proc. Natl Acad. Sci. USA 99*, 1405–1409.

Kramer, K.J., Dunn, P.E., Peterson, R., Law, J.H., **1976**. Interaction of juvenile hormone with binding proteins in insect hemolymph. In: Gilbert, L.I. (Ed.), The Juvenile Hormones. Plenum, New York, pp. 327–341.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., *et al.*, **1991**. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. *Proc. Natl Acad. Sci. USA 88*, 9458–9462.

Kroeger, H., **1968**. Gene activities during insect metamorphosis and their control by hormones. In: Etkin, W., Gilbert, L.I. (Eds.), Metamorphosis: A Problem in Developmental Biology. Appleton-Century-Crofts, New York, NY, pp. 185–220.

Krzyzanowska, D., Lisowski, M., Kochman, M., **1998**. UV-difference and CD spectroscopy studies on juvenile hormone binding to its carrier protein. *J. Peptide Res. 51*, 96–102.

Kumaran, A.K., **1990**. Action of JH at cellular and molecular levels. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, NJ, pp. 183–227.

Lacourciere, G.M., Armstrong, R.N., **1993**. The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate. *J. Am. Chem. Soc. 115*, 10466–10467.

Lafon-Cazal, M., Baehr, J.-C., **1988**. Octopaminergic control of corpora allata activity in an insect. *Experientia 44*, 895–896.

Lafont, R., **2000**. Understanding insect endocrine systems: molecular approaches. *Entomol. Exp. Applic. 97*, 123–136.

Lamer, A., Dorn, A., **2001**. The serosa of *Manduca sexta*: ontogeny, secretory activity, structural changes, and functional consideration. *Tissue Cell 33*, 580–595.

Lanzrein, B., Imboden, H., Bürgin, C., Brüning, E., Gfeller, G., **1984**. On titers, origin, and functions of juvenile hormone III, methyl farnesoate and ecdysteroids in embryonic development of the ovoviviparous cockroach, *Nauphoeta cinerea*. In: Hoffmann, J., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer, Berlin, pp. 454–465.

Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., *et al.*, **2001**. Type A allatostatins from *Drosophila melanogaster* and *Diplotera puncata* activate two *Drosophila* allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. *Biochem. Biophys. Res. Commun. 286*, 895–901.

Lassiter, M.T., Apperson, C.S., Crawford, C.L., Roe, R.M., **1994**. Juvenile hormone metabolism during adult development of *Culex quinquefasciatus*. *J. Med. Entomol. 32*, 586–93.

Lassiter, M.T., Apperson, C.S., Roe, R.M., **1995**. Juvenile hormone metabolism during the fourth stadium and pupal stage of the southern house mosquito, *Culex quinquefasciatus*. *J. Insect Physiol. 41*, 869–876.

Laufer, H., Ahl, J.S.B., Sagi, A., **1993**. The role of juvenile hormones in crustacean reproduction. *Am. Zool. 33*, 365–374.

Lauga-Reyrel, F., **1985**. Experimental establishment and rupture of the ecomorphosis of *Hypogastrura tullbergi* by exogenous contribution of precocene or juvenile hormone. *Bull. Soc. Hist. Nat. Toulouse 120*, 61–74.

Law, J.H., **1980**. Lipid–protein interactions in insects. In: Locke, M., Smith, D.S. (Eds.), Insect Biology in the Future: VBW80. Academic Press, New York, pp. 295–310.

Lee, C.Y., Simon, C.R., Woodard, C.T., Baehrecke, E.H., **2002**. Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Devel. Biol. 252*, 138–148.

Lee, K.Y., Chamberlin, M.E., Horodyski, F.M., **2002**. Biological activity of *Manduca sexta* allatotropin-like peptides, predicted products of tissue-specific and developmentally regulated alternatively spliced mRNAs. *Peptides 23*, 1933–1941.

Lee, K.Y., Horodyski, F.M., **2002**. Restriction of nutrient intake results in the increase of a specific *Manduca sexta* allatotropin (Manse-AT) mRNA in the larval nerve cord. *Peptides 23*, 653–661.

Lee, K.Y., Horodyski, F.M., Chamberlin, M.E., **1998**. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* FLRFamides in the tobacco hornworm *Manduca sexta*. *J. Exp. Biol. 201*, 3067–3074.

Lee, Y.J., Chiang, A.S., **1997**. Ultrastructural changes in corpora allata during a cycle of juvenile hormone biosynthesis in embryos of the viviparous cockroach *Diploptera punctata*. *Tissue Cell 29*, 715–726.

Lefevre, K.S., Lacey, M.J., Smith, P.H., Roberts, B., **1993**. Identification and quantification of juvenile hormone biosynthesized by larval and adult Australian sheep blowfly *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 23*, 713–720.

Lehmberg, E., Ferenz, H.J., Applebaum, S.W., **1992**. Maturation and responsiveness to extracts of corpora allata from male *Locusta migratoria* containing allatotropic factors. *Z. Naturforsch. 47C*, 449–452.

Lenz, C., Williamson, M., Hansen, G.N., Grimmelikhuijzen, C.J.P., **2001**. Identification of four *Drosophila* allatostatins as the cognate ligands for the *Drosophila* orphan receptor DAR-2. *Biochem. Biophys. Res. Commun. 286*, 1117–1122.

Lenz, C., Williamson, M., Grimmelikhuijzen, C.J.P., **2000**. Molecular cloning and genomic organization of an allatostatin preprohormone from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 273*, 1126–1131.

Levenbook, L., **1985**. Insect storage proteins. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 10. Pergamon, Oxford, pp. 307–346.

Lezzi, M., Bergman, T., Henrich, V.C., Vogtli, M., Fromel, C., *et al.*, **2002**. Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle. *Eur. J. Biochem. 269*, 3237–3245.

Li, H., Borst, D.W., **1991**. Characterization of a methyl farnesoate binding protein in hemolymph from *Libinia emarginata. Gen. Comp. Endocrinol. 81*, 335–342.

Li, W.C., **1996**. Differential expression of the two duplicated insecticyanin genes, ins-a and ins-b, in the black mutant of *Manduca sexta. Arch. Biochem. Biophys. 330*, 65–70.

Li, W.C., Riddiford, L.M., **1992**. Two distinct genes encode two major isoelectric forms of insecticyanin in the tobacco hornworm, *Manduca sexta. Eur. J. Biochem. 205*, 491–499.

Li, W.C., Riddiford, L.M., **1994**. The two duplicated insecticyanin genes, ins-a and ins-b are differentially expressed in the tobacco hornworm, *Manduca sexta. Nucl. Acids Res. 22*, 2945–2950.

Li, Y., Unnithan, G.C., Veenstra, J.A., Feyereisen, R., Noriega, F.G., **2003**. Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti in vitro*: effect of farnesoic acid and *Aedes* allatotropin. *J. Exp. Biol. 206*, 1825–1832.

Linderman, R.J., Roe, R.M., Harris, S. V., Thompson, D.M., **2000**. Inhibition of insect juvenile hormone epoxide hydrolase: asymmetric synthesis and assay of glycidol-ester and epoxy-ester inhibitors of *Trichoplusia ni* epoxide hydrolase. *Insect Biochem. Mol. Biol. 30*, 767–774.

Locke, M., **1984**. The structure and development of the vacuolar system in the fat body of insects. In: King, R.C., Akai, H. (Eds.), Insect Ultrastructure. Plenum, New York, pp. 151–197.

Loher, W., Ruzo, F., Baker, F.C., Miller, C.A., Schooley, D.A., **1983**. Identification of the juvenile hormone from the cricket, *Teleogryllus commodus*, and juvenile hormone titre changes. *J. Insect Physiol. 29*, 585–589.

Lonard, D.M., Bhaskaran, G., Dahm, K. H., **1996**. Control of prothoracic gland activity by juvenile hormone in fourth instar *Manduca sexta* larvae. *J. Insect Physiol. 42*, 205–214.

Lorenz, M.W., Hoffmann, K.H., **1995**. Allatotropic activity in the subesophageal ganglia of crickets, *Gryllus bimaculatus* and *Acheta domesticus*. *J. Insect Physiol. 41*, 191–196.

Lorenz, M.W., Hoffmann, K.H., Gade, G., **1999**. Juvenile hormone biosynthesis in larval and adult stick insects, *Carausius morosus*. *J. Insect Physiol. 45*, 443–452.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., Gade, G., **2000**. Identification of multiple peptides homologous to cockroach and cricket allatostatins in the stick insect *Carausius morosus*. *Insect Biochem. Mol. Biol. 30*, 711–718.

Mauchamp, B., Darrouzet, E., Malosse, C., Couillaud, F., **1999**. 4′-OH-JH-III: an additional hydroxylated juvenile hormone produced by locust corpora allata *in vitro*. *Insect Biochem. Mol. Biol. 29*, 475–480.

Maxwell, R.A., Welch, W.H., Horodyski, F.M., Schegg, K.M., Schooley, D.A., **2002b**. Juvenile hormone diol kinase. II. Sequencing, cloning, and molecular modeling of juvenile hormone-selective diol kinase from *Manduca sexta*. *J. Biol. Chem. 277*, 21882–21890.

Maxwell, R.A., Welch, W.H., Schooley, D.A., **2002a**. Juvenile hormone diol kinase. I. Purification, characterization, and substrate specificity of juvenile hormone-selective diol kinase from *Manduca sexta*. *J. Biol. Chem. 277*, 21874–21881.

Meller, V., Aucoin, R.R., Tobe, S. S., Feyereisen, R., **1985**. Evidence for an inhibitory role of cyclic AMP in the control of juvenile hormone biosynthesis by cockroach corpora allata. *Mol. Cell. Endocrinol. 43*, 155–163.

Mendel, C.M., **1989**. The free hormone hypothesis: a physiologically based mathematical model. *Endocrine Rev. 10*, 232–74.

Meyer, A.S., Hanzmann, E., Murphy, R. C., **1971**. Absolute configuration of cecropia juvenile hormone. *Proc. Natl Acad. Sci. USA 68*, 2312–2315.

Meyer, A.S., Schneiderman, H.A., Hanzmann, E., Ko, J., **1968**. The two juvenile hormones from the cecropia silk moth. *Proc. Natl Acad. Sci. USA 60*, 853–860.

Meyer, W.R., Lanzrein, B., **1989a**. Degradation of juvenile hormone and methylation of juvenile hormone acid by corpora cardiaca–corpora allata of the cockroach, *Nauphoeta cinerea*. 1. Biochemical aspects. *Arch. Insect Biochem. Physiol. 10*, 303–316.

Meyer, W.R., Lanzrein, B., **1989b**. Degradation of juvenile hormone and methylation of juvenile hormone acid by corpora cardiaca–corpora allata of the cockroach, *Nauphoeta cinerea*. 2. Physiological aspects. *Arch. Insect Biochem. Physiol. 10*, 317–331.

Meyering-Vos, M., Wu, X., Huang, J., Jindra, M., Hoffmann, K.H., *et al.*, **2001**. The allatostatin gene of the cricket *Gryllus bimaculatus*. *Mol. Cell. Endocrinol. 184*, 103–114.

Miura, K., Shinoda, T., Yura, M., Nomura, S., Kamiya, K., *et al.*, **1998**. Two hexameric cyanoprotein subunits from an insect, *Riptortus clavatus*, sequence, phylogeny

and developmental and juvenile hormone regulation. *Eur. J. Biochem. 258*, 929–940.

Mizoguchi, A., **2001**. Effects of juvenile hormone on the secretion of prothoracicotropic hormone in the last- and penultimate-instar larvae of the silkworm *Bombyx mori*. *J. Insect Physiol. 47*, 767–775.

Moshitzky, P., Applebaum, S.W., **1995**. Pathway and regulation of JH III-bisepoxide biosynthesis in adult *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol. 30*, 225–238.

Moshitzky, P., Gilbert, L.I., Applebaum, S.W., **2003**. Biosynthetic maturation of the corpus allatum of the female adult medfly, *Ceratitis capitata*, and its putative control. *J. Insect Physiol. 49*, 603–609.

Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klauser, S., *et al.*, **1996**. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol. 32*, 363–374.

Mugat, B., Brodu, V., Kejzlarova-Lepesant, J., Antoniewski, C., Bayer, C.A., *et al.*, **2000**. Dynamic expression of Broad-complex isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Devel. Biol. 227*, 104–117.

Nachman, R.J., Garside, C.S., Tobe, S.S., **1999**. Hemolymph and tissue-bound peptidase-resistant analogs of the insect allatostatins. *Peptides 20*, 23–29.

Nakanishi, K., Schooley, D.A., Koreeda, M., Dillon, J., **1971**. Absolute configuration of the C18-juvenile hormone: application of a new circular dichroism method using tris(dipivaloylmethanato) praseodymium. *J. Chem. Soc., Chem. Commun.* 1235–1236.

Nardi, F., Spinsanti, G., Boore, J.L., Carapelli, A., Dallai, R., *et al.*, **2003**. Hexapod origins: monophyletic or paraphyletic. *Science 299*, 1887–1889.

Neese, P.A., Sonenshine, D.E., Kallapur, V.L., Apperson, C.S., Roe, R.M., **2000**. Absence of insect juvenile hormones in the American dog tick, *Dermacentor variabilis* (Say) (Acari: Ixodidae), and in *Ornithodoros parkeri* Cooley (Acari: Argasidae). *J. Insect Physiol. 46*, 477–490.

Nelson, J.A., **1915**. The Embryology of the Honeybee. Princeton University Press, Princeton, NJ.

Newman, J.W., Morisseau, C., Harris, T.R., Hammock, B.D., **2003**. The soluble epoxide hydrolase encoded by EPXH2 is a bifunctional enzyme with novel lipid phosphate phosphatase activity. *Proc. Natl Acad. Sci. USA 100*, 1558–1563.

Niimi, S., Sakurai, S., **1997**. Developmental changes in juvenile hormone and juvenile hormone acid titers in the hemolymph and *in vitro* juvenile hormone synthesis by corpora allata of the silkworm, *Bombyx mori*. *J. Insect Physiol. 43*, 875–884.

Nijhout, H.F., **1994**. Insect Hormones. Princeton, University Press, Princeton, NJ.

Nijhout, H.F., Wheeler, D.E., **1982**. Juvenile hormone and the physiological basis of insect polymorphisms. *Q. Rev. Biol. 57*, 109–133.

Nijhout, H.F., Williams, C.M., **1974**. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta*: cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol. 61*, 493–501.

Noguchi, H., Hayakawa, Y., **1997**. Role of dopamine at the onset of pupal diapause in the cabbage armyworm *Mamestra brassicae*. *FEBS Lett. 413*, 157–161.

Noguchi, H., Hayakawa, Y., **1996**. Mechanism of parasitism-induced elevation of dopamine levels in host insect larvae. *Insect Biochem. Mol. Biol. 26*, 659–665.

Noguchi, H., Hayakawa, Y., Downer, R.G.H., **1995**. Elevation of dopamine levels in parasitized insects. *Insect Biochem. Mol. Biol. 25*, 197–201.

Noriega, F.G., Edgar, K.A., Goodman, W.G., Shah, D.K., Wells, M.A., **2001**. Neuroendocrine factors affecting the steady-state levels of early trypsin mRNA in *Aedes aegypti*. *J. Insect Physiol. 47*, 515–522.

Nowock, J., Gilbert, L.I., **1976**. *In vitro* analysis of factors regulating the juvenile hormone titer of insects. In: Kurstak, E., Maramorosch, K. (Eds.), Invertebrate Tissue Culture: Applications in Medicine, Biology, and Agriculture. Academic Press, New York, pp. 203–212.

Nowock, J., Goodman, W.G., Bollenbacher, W.E., Gilbert, L.I., **1975**. Synthesis of juvenile hormone binding proteins by the fat body of *Manduca sexta*. *Gen. Comp. Endocrinol. 27*, 230–239.

Numata, H., Numata, A., Takahashi, C., Nakagawa, Y., Iwatani, K., *et al.*, **1992**. Juvenile hormone I is the principal juvenile hormone in a hemipteran insect, *Riptortus clavatus*. *Experientia 48*, 606–610.

Oeh, U., Dyker, H., Losel, P., Hoffmann, K.H., **2001**. *In vivo* effects of *Manduca sexta* allatotropin and allatostatin on development and reproduction in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). *Invertebr. Reprod. Devel. 39*, 239–247.

Oeh, U., Lorenz, M.W., Dyker, H., Losel, P., Hoffmann, K.H., **2000**. Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol. 30*, 719–727.

Oeh, U., Lorenz, M.W., Dyker, H., Loesel, P.H.K.H., **1999**. Interaction between *Manduca* allatotropin and *Manduca* allatostatins in the fall armyworm *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol. 30*, 719–727.

Oro, A.E., McKeown, M., Evans, R.M., **1990**. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature 347*, 298–301.

Orth, A.P., Doll, S.C., Goodman, W.G., **2003a**. Sequence, structure and expression of the hemolymph juvenile hormone binding protein gene in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 93–102.

Orth, A.P., Tauchman, S.J., Doll, S.C., Goodman, W.G., **2003b**. Embryonic expression of juvenile hormone binding protein and its relationship to the toxic effects of juvenile hormone in *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 1275–1284.

Orth, A.P., Lan, Q., Goodman, W.G., **1999**. Ligand regulation of juvenile hormone binding protein mRNA in mutant *Manduca sexta*. *Mol. Cell. Endocrinol. 149*, 61–69.

Owen, M.D., Foster, J., **1988**. The association of changes in cerebral ganglion dopamine metabolism with ootheca development in *Periplaneta americana*. *Comp. Biochem. Physiol. C 91*, 403–408.

Panchapakesan, K., Lampert, E.P., Granger, N.A., Goodman, W.G., Roe, R.M., **1994**. Biology and physiology of the white mutant of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 40*, 423–429.

Park, C., Hwang, J.S., Kang, S.W., Lee, B.H., **2002**. Molecular characterization of a cDNA from the silk moth *Bombyx mori* encoding *Manduca sexta* allatotropin peptide. *Zool. Sci. 19*, 287–292.

Park, Y.C., Goodman, W.G., **1993**. Analysis and modification of thiols in the hemolymph juvenile hormone binding protein of *Manduca sexta*. *Arch. Biochem. Biophys. 302*, 12–18.

Park, Y.C., Tesch, M.J., Toong, Y.C., Goodman, W.G., **1993**. Affinity purification and binding analysis of the hemolymph juvenile hormone binding protein from *Manduca sexta*. *Biochemistry 32*, 7909–7915.

Pastor, D., Artigas, F., Martinez, E., Bellés, X., **1991a**. Brain levels of 5-hydroxytryptamine and dopamine in *Blattella germanica* adult females during the first gonadotropic cycle. *Biogen. Amines 8*, 101–114.

Pastor, D., Piulachs, M.-D., Cassier, P., Andres, M., Bellés, X., **1991b**. Etude *in vivo* et *in vitro* de l'action de la dopamine sur la croissance des oocytes et la production d'hormone juvenile chez *Blattella germanica*. *C. R. Acad. Sci. Paris 313*, 207–212.

Pener, M.P., Dessberg, D., Lazarovici, P., Reuter, C.C., Tsai, L.W., *et al.*, **1986**. The effect of a synthetic precocene on juvenile hormone III titer in late *Locusta* eggs. *J. Insect Physiol. 32*, 853–857.

Peralta, E., Vilaplana, L., Pascual, N., Carreno, C., Piulachs, M.D., *et al.*, **2000**. A microdialysis study of allatostatin degradation in *Blattella germanica*. *Physiol. Entomol. 25*, 254–259.

Peter, M.G., **1990**. Chiral recognition in insect juvenile hormone metabolism. In: Testa, B. (Ed.), Chirality and Biological Activity. Alan, R. Liss, New York, pp. 111–117.

Peter, M.G., Shirk, P.D., Dahm, K.H., Röller, H., **1981**. On the specificity of juvenile hormone biosynthesis in the male cecropia. *Z. Naturforsch. 36C*, 579–585.

Peter, M.G., Sunawan, S., Gellissen, G., Emmerich, H., **1979**. Difference in hydrolysis and binding of homologous juvenile hormones in *Locusta migratoria* hemolymph. *Z. Naturforsch. 34C*, 588–598.

Peterson, R.C., Dunn, P.E., Seballos, H.L., Barbeau, B.K., Keim, P.S., *et al.*, **1982**. Juvenile hormone carrier protein of *Manduca sexta* haemolymph. Improved purification procedure: protein modification studies and sequence of the amino terminus of the protein. *Insect Biochem. 12*, 643–650.

Peterson, R.C., Reich, M.F., Dunn, P.E., Law, J.H., Katzenellenbogen, J.A., **1977**. Binding specificity of the juvenile hormone carrier protein from the hemolymph of the tobacco hornworm *Manduca sexta*. *Biochemistry 16*, 2305–2311.

Petri, B., Homberg, U., Loesel, R., Stengl, M., **2002**. Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol. 205*, 1459–1469.

Pratt, G.E., Tobe, S.S., **1974**. Juvenile hormone radiobiosynthesized by corpora allata of adult female locusts *in vitro*. *Life Sci. 14*, 575–586.

Pratt, G.E., Unnithan, G.C., Fok, K.F., Siegel, N.R., Feyereisen, R., **1997**. Structure–activity studies reveal two allatostatin receptor types in corpora allata of *Diploptera punctata*. *J. Insect Physiol. 43*, 627–634.

Pratt, G.E., Farnsworth, D.E., Fok, K.F., Siegel, N.R., McCormack, A.L., *et al.*, **1991**. Identity of a second type of allatostatin from cockroach brains: An octadecapeptide amide with a tyrosine-rich address sequence. *Proc. Natl Acad. Sci. USA 88*, 2412–2416.

Pratt, G.E., Farnsworth, D.E., Feyereisen, R., **1990**. Changes in the sensitivity of adult cockroach corpora allata to a brain allatostatin. *Mol. Cell. Endocrinol. 70*, 185–195.

Pratt, G.E., Farnsworth, D.E., Siegel, N.R., Fok, K.F., Feyereisen, R., **1989**. Identification of an allatostatin from adult *Diploptera punctata*. *Biochem. Biophys. Res. Commun. 163*, 1243–1247.

Prestwich, G.D., **1987**. Chemistry of pheromone and hormone metabolism in insects. *Science 237*, 999–1006.

Prestwich, G.D., Touhara, K., Riddiford, L.M., Hammock, B.D., **1994**. Larva lights: a decade of photoaffinity labeling with juvenile hormone analogues. *Insect Biochem. Mol. Biol. 24*, 747–761.

Prestwich, G.D., Wawrzeńczyk, C., **1985**. High specific activity enantiomerically enriched juvenile hormones: synthesis and binding assay. *Proc. Natl Acad. Sci. USA 82*, 5290–5294.

Pszczolkowski, M.A., Lee, W.S., Liu, H.P., Chiang, A.S., **1999**. Glutamate-induced rise in cytosolic calcium concentration stimulates *in vitro* rates of juvenile hormone biosynthesis in corpus allatum of *Diploptera punctata*. *Mol. Cell. Endocrinol. 158*, 163–171.

Pursley, S., Ashok, M., Wilson, T.G., **2000**. Intracellular localization and tissue specificity of the methoprene-tolerant (*met*) gene product in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol. 30*, 839–845.

Rachinsky, A., **1994**. Octopamine and serotonin influence on corpora allata activity in honey bee (*Apis mellifera*) larvae. *J. Insect Physiol. 40*, 549–554.

Rachinsky, A., Feldlaufer, M.F., **2000**. Responsiveness of honey bee (*Apis mellifera*) corpora allata to allatoregulatory peptides from four insect species. *J. Insect Physiol. 46*, 41–46.

Rachinsky, A., Hartfelder, K., **1991**. Differential production of juvenile hormone and its deoxy precursor by corpora allata of honeybees during a critical

period of caste development. *Naturwissenschaften 78*, 270–272.

Rachinsky, A., Tobe, S.S., **1996**. Role of second messengers in the regulation of juvenile hormone production in insects, with particular emphasis on calcium and phosphoinositide signaling. *Arch. Insect Biochem. Physiol. 33*, 259–282.

Rachinsky, A., Tobe, S.S., Feldlaufer, M.F., **2000**. Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera*): developmental changes in sensitivity to JH precursors and allatotropin. *Insect Biochem. Mol. Biol. 30*, 729–737.

Rachinsky, A., Zhang, J., Tobe, S.S., **1994**. Signal transduction in the inhibition of juvenile hormone biosynthesis by allatostatins: roles of diacylglycerol and calcium. *Mol. Cell. Endocrinol. 105*, 89–96.

Rankin, S.M., Stay, B., **1985**. Ovarian inhibition of juvenile hormone synthesis in the viviparous cockroach *Diploptera punctata*. *Gen. Comp. Endocrinol. 59*, 230–238.

Rankin, S.M., Stay, B., Aucoin, R.R., Tobe, S.S., **1986**. *In vitro* inhibition of juvenile hormone synthesis by corpora allata of the viviparous cockroach *Diploptera punctata*. *J. Insect Physiol. 32*, 151–156.

Rao, K.D.P., Krishnakumaran, A., **1974**. Effect of juvenile hormone on DNA synthesis during embryogenesis in *Acheta domesticus*. *Roux's Arch. Devel. Biol. 174*, 276–284.

Reagan, J.D., Miller, W.H., Kramer, S.J., **1992**. Allatotropin-induced formation of inositol phosphates in the corpora allata of the moth, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 20*, 145–155.

Rebers, J.E., Niu, J., Riddiford, L.M., **1997**. Structure and spatial expression of the *Manduca sexta* MSCP14.6 cuticle gene. *Insect Biochem. Mol. Biol. 27*, 229–240.

Rebers, J.E., Riddiford, L.M., **1988**. Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. *J. Mol. Biol. 203*, 411–423.

Rees, H.H., **1985**. Biosynthesis of ecdysones. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 249–293.

Rembold, H., Czoppelt, C., Grune, M., Lackner, B., Pfeffer, J., *et al.*, **1992**. Juvenile hormone titers during honeybee embryogenesis and metamorphosis. In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), Insect Juvenile Hormone Research: Chemistry, Biochemistry and Mode of Action. INRA, Paris, pp. 37–43.

Rembold, H., Hagenguth, H., Rascher, J., **1980**. A sensitive method for detection and estimation of juvenile hormones from biological samples by glass capillary combined gas chromatography-selected ion monitoring mass spectrometry. *Analyt. Biochem. 101*, 356–363.

Rensing, L., **1964**. Daily rhythmicity of corpus allatum and neurosecretory cells in *Drosophila melanogaster. Science 144*, 1586–1587.

Restifo, L.L., Wilson, T.G., **1998**. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible Broad complex transcription factors. *Devel. Genet. 22*, 141–159.

Richard, D.S., Gilbert, L.I., **1991**. Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster. Experientia 47*, 1063–1066.

Richard, D.S., Applebaum, S.W., Gilbert, L.I., **1990**. Allatostatic regulation of juvenile hormone production *in vitro* by the ring gland of *Drosophila melanogaster. Mol. Cell. Endocrinol. 68*, 153–161.

Richard, D.S., Applebaum, S.W., Sliter, T.J., Baker, F.C., Schooley, D.A., *et al.*, **1989**. Juvenile hormone bisepoxide biosynthesis *in vitro* by the ring gland of *Drosophila melanogaster*: a putative juvenile hormone in the higher Diptera. *Proc. Natl Acad. Sci. USA 86*, 1421–1425.

Richard, D.S., Watkins, N.L., Serafin, R.B., Gilbert, L.I., **1998**. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol. 44*, 637–644.

Richter, K., Gronert, M., **1999**. Neurotropic effect of juvenile hormone III in larvae of the cockroach, *Periplaneta americana*. *J. Insect Physiol. 45*, 1065–1071.

Riddiford, L.M., **1970**. Effects of juvenile hormone on the programming of postembryonic development in eggs of the silkworm, *Hyalophora cecropia*. *Devel. Biol. 22*, 249–263.

Riddiford, L.M., **1986**. Hormonal regulation of sequential larval cuticular gene expression. *Arch. Insect Biochem. Physiol. 3*(Suppl. 1), 75–86.

Riddiford, L.M., **1994**. Cellular and molecular actions of the juvenile hormones. 1. General considerations and premetamorphic actions. *Adv. Insect Physiol. 24*, 213–274.

Riddiford, L.M., **1996**. Molecular aspects of juvenile hormone action in insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Academic Press, New York, pp. 223–251.

Riddiford, L.M., Ashburner, M., **1991**. Effects of juvenile hormone mimics on larval development and metamorphosis of *Drosophila melanogaster. Gen. Comp. Endocrinol. 82*, 172–183.

Riddiford, L.M., Cherbas, P., Truman, J.W., **2001**. Ecdysone receptors and their biological actions. *Vitamins and Hormones – Adv. Res. Applic. 60*, 1–73.

Riddiford, L.M., Hiruma, K., Zhou, X., **2003**. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster. Insect Biochem. Mol. Biol. 33*, 1327–1338.

Riddiford, L.M., Palli, S.R., Hiruma, K., Li, W., Green, J., *et al.*, **1990**. Developmental expression, synthesis, and secretion of insecticyanin by the epidermis of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 14*, 171–190.

Riley, C.T., Barbeau, B.K., Keim, P.S., Kezdy, F.J., Heinrikson, R.L., *et al.*, **1984**. The covalent protein

structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem. 259*, 13159–13165.

Robertson, H.M., Lampe, D.J., **1995**. Distribution of transposable elements in arthropods. *Annu. Rev. Entomol. 40*, 333–357.

Robinson, G.E., Vargo, E.L., **1997**. Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker. *Arch. Insect Biochem. Physiol. 35*, 559–583.

Rodriguez Parkitna, J.M., Ozyhar, A., Wisniewski, J.R., Kochman, M., **2002**. Cloning and sequence analysis of *Galleria mellonella* juvenile hormone binding protein: a search for ancestors and relatives. *Biol. Chem. 383*, 1343–1355.

Roe, R.M., Venkatesh, K., **1990**. Metabolism of juvenile hormones: degradation and titer regulation. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, NJ, pp. 126–179.

Roe, R.M., Anspaugh, D.D., Venkatesh, K., Linderman, R.J., Graves, D.M., **1997**. A novel geminal diol as a highly specific and stable *in vivo* inhibitor of insect juvenile hormone esterase. *Arch. Insect Biochem. Physiol. 36*, 165–179.

Roe, R.M., Crawford, C.L., Clifford, C.W., Woodring, J.P., Sparks, T.C., *et al.*, **1987a**. Characterization of the juvenile hormone esterases during embryogenesis of the house cricket, *Acheta domesticus*. *Int. J. Invertebr. Reprod. Devel. 12*, 57–72.

Roe, R.M., Crawford, C.L., Clifford, C.W., Woodring, J.P., Sparks, T.C., *et al.*, **1987b**. Role of juvenile hormone metabolism during embryogenesis of the house cricket, *Acheta domesticus*. *Insect Biochem. 17*, 1023–1026.

Roe, R.M., Jesudason, P., Venkatesh, K., Kallapur, V.L., Anspaugh, D.D., *et al.*, **1993**. Developmental role of juvenile hormone metabolism in Lepidoptera. *Am. Zool. 33*, 375–383.

Roe, R.M., Kallapur, V., Linderman, R.J., Viviani, F., Harris, S.V., *et al.*, **1996**. Mechanism of action and cloning of epoxide hydrolase from the cabbage looper, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol. 32*, 527–535.

Rohdendorf, E.B., Sehnal, F., **1973**. Inhibition of reproduction and embryogenesis in the firebrat, *Thermobia domestica*, by juvenile hormone analogues. *J. Insect Physiol. 19*, 37–56.

Röller, H., Dahm, K.H., Sweely, C.C., Trost, B.M., **1967**. The structure of the juvenile hormone (giant silkworm moth *Hyalophora cecropia*). *Angew. Chem. Int. Edn* 6, 179–180.

Rose, U., Ferber, M., Hustert, R., **2001**. Maturation of muscle properties and its hormonal control in an adult insect. *J. Exp. Biol. 204*, 3531–3545.

Rountree, D., Bollenbacher, W.E., **1986**. The release of the PTTH in the tobacco hornworm, *Manduca sexta*, is controlled intrinsically by juvenile hormone. *J. Exp. Biol. 120*, 41–59.

Roy, S., VijayRaghavan, K., **1999**. Muscle pattern diversification in *Drosophila*: the story of imaginal myogenesis. *BioEssays 21*, 486–498.

Rudnicka, M., Kochman, M., **1984**. Purification of the juvenile hormone esterase from the haemolymph of the wax moth *Galleria mellonella*. *Insect Biochem. 14*, 189–198.

Rudolph, P.H., Stay, B., **1997**. Cockroach allatostatin-like immunoreactivity in the central nervous system of the freshwater snails *Bulinus globosus* (Planorbidae) and *Stagnicola elodes* (Lymnaeidae). *Gen. Comp. Endocrinol. 106*, 241–250.

Rudwall, A.J., Sliwowska, J., Nassel, D.R., **2000**. Allatotropin-like neuropeptide in the cockroach abdominal nervous system: myotropic actions, sexually dimorphic distribution and co-localization with serotonin. *J. Comp. Neurol. 428*, 159–173.

Safranek, L., Cymborowski, B., Williams, C.M., **1980**. Effects of juvenile hormone on ecdysone-dependent development in the tobacco hornworm, *Manduca sexta*. *Biol. Bull. 158*, 248–256.

Safranek, L., Riddiford, L.M., **1975**. The biology of the black larval mutant of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 21*, 1931–1938.

Sakurai, S., Williams, C.M., **1989**. Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 75*, 204–216.

Sakurai, S., Okuda, M., Ohtaki, T., **1989**. Juvenile hormone inhibits ecdysone secretion and responsiveness to prothoracicotropic hormone in prothoracic glands of *Bombyx mori*. *Gen. Comp. Endocrinol. 75*, 222–230.

Sanburg, L.L., Kramer, K.J., Kezdy, F.J., Law, J.H., Oberlander, H., **1975**. Role of juvenile hormone esterases and carrier proteins in insect development. *Nature 253*, 266–267.

Sasorith, S., Billas, I.M.L., Iwema, T., Moras, D., Wurtz, J.M., **2002**. Structure-based analysis of the Ultraspiracle protein and docking studies of putative ligands. *J. Insect Sci. 2*, 25–00.

Sbrenna-Micciarelli, A., **1977**. Effects of farnesyl methyl ether on embryos of *Schistocerca gregaria*. *Acta Embryol. Exp. 3*, 295–303.

Schmidt, T., Choffat, Y., Klauser, S., Kubli, E., **1993**. The *Drosophila melanogaster* sex peptide: a molecular analysis of structure–function relationships. *J. Insect Physiol. 39*, 361–368.

Schneider, M., Wiesel, G., Dorn, A., **1995**. Effects of JH III and JH analogues on phase-related growth, egg maturation and lipid metabolism in *Schistocerca gregaria* females. *J. Insect Physiol. 41*, 23–31.

Schooley, D.A., Baker, F.C., **1985**. Juvenile hormone biosynthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 363–390.

Schooley, D.A., Baker, F.C., Tsai, L.W., Miller, C.A., Jamieson, G.C., **1984**. Juvenile hormones 0, I, II exist only in Lepidoptera. In: Hoffmann, J., Porchet, M.

(Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer, New York, pp. 373–383.

Schooley, D.A., Judy, K.J., Baker, F.C., Lee, E., Bergot, B.J., *et al.*, **1978a**. Biosynthesis of the juvenile hormones: the role of the homoisoprenoid intermediates. In: Gaillard, P.J., Boer, H.H. (Eds.), Comparative Endocrinology. Elsevier, London, pp. 499–502.

Schooley, D.A., Bergot, B.J., Goodman, W.G., Gilbert, L.I., **1978b**. Synthesis of both optical isomers of insect juvenile hormone JH III and their affinity for the JH-specific binding protein of *Manduca sexta*. *Biochem. Biophys. Res. Commun. 81*, 743–749.

Schooley, D.A., Judy, K., Bergot, B.J., Hall, M.S., Jennings, R.C., **1976**. Determination of the physiological levels of juvenile hormone in several insects and biosynthesis of the carbon skeleton of the juvenile hormones. In: Gilbert, L.I. (Ed.), The Juvenile Hormones. Plenum, New York, pp. 101–117.

Schulz, D.J., Robinson, G.E., **2001**. Octopamine influences division of labor in honey bee colonies. *J. Comp. Physiol. A 187*, 53–61.

Schulz, D.J., Sullivan, J.P., Robinson, G.E., **2002**. Juvenile hormone and octopamine in the regulation of division of labor in honeybee colonies. *Hormones Behav. 42*, 222–231.

Schwartz, L.M., **1992**. Insect muscle as a model for programmed cell death. *J. Neurobiol. 23*, 1312–1326.

Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., *et al.*, **2001**. Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. *J. Biol. Chem. 276*, 47052–47060.

Sedlak, B.J., **1985**. Structure of endocrine glands. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 25–60.

Sedlak, B.J., Marchione, L., Devorkin, B., Davino, R., **1983**. Correlations between endocrine gland ultrastructure and hormone titers in the fifth larval instar of *Manduca sexta*. *Gen. Comp. Endocrinol. 52*, 291–310.

Sehnal, F., **1984**. The juvenile hormones of insects. *Nov. Acta Leopold. 56*, 251–266.

Sehnal, F., Granger, N.A., **1975**. Control of corpora allata functions in the last instar larvae of *Galleria mellonella*. *Biol. Bull. 148*, 106–116.

Sehnal, F., Svacha, P., Zrzavy, J., **1996**. Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Academic Press, London, pp. 3–58.

Sen, S.E., Sperry, A.E., **2002**. Partial purification of a farnesyl diphosphate synthase from whole-body *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 889–899.

Sen, S.E., Ewing, G.J., **1997**. Natural and unnatural terpenoid precursors of insect juvenile hormone. *J. Organ. Chem. 62*, 3529–3536.

Sen, S.E., Garvin, G.M., **1995**. Substrate requirements for lepidopteran farnesol dehydrogenase. *J. Agric. Food Chem. 43*, 820–826.

Severson, T.F., Goodrow, M.H., Morisseau, C., Dowdy, D.L., Hammock, B.D., **2002**. Urea and amide-based inhibitors of the juvenile hormone epoxide hydrolase of the tobacco hornworm *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 1741–1756.

Shanmugavelu, M., Baytan, A.R., Chesnut, J.D., Bonning, B.C., **2000**. A novel protein that binds juvenile hormone esterase in fat body tissue and pericardial cells of the tobacco hornworm *Manduca sexta*. *J. Biol. Chem. 275*, 1802–1806.

Share, M.R., Venkatesh, K., Jesudason, P., Roe, R.M., **1988**. Juvenile hormone metabolism during embryogenesis in the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 8*, 173–186.

Shemshedini, L., Lanoue, M., Wilson, T.G., **1990**. Evidence for a juvenile hormone receptor involved in protein synthesis in *Drosophila melanogaster*. *J. Biol. Chem. 265*, 1913–1918.

Shiga, S., Yasuyama, K., Okamura, N., Yamaguchi, T., **2002**. Neural- and endocrine control of flight muscle degeneration in the adult cricket, *Gryllus bimaculatus*. *J. Insect Physiol. 48*, 15–24.

Shirk, P., Bhaskaran, G., Röller, H., **1983**. Developmental physiology of corpora allata and accessory sex glands in the cecropia silkmoth. *J. Exp. Zool. 227*, 69–79.

Short, J.E., Edwards, J.P., **1992**. Levels of juvenile hormone III during embryonic development in the oriental cockroach, *Blattella orientalis* (L.). In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), Insect Juvenile Hormone Research: Chemistry, Biochemistry and Mode of Action. INRA, Paris, pp. 19–25.

Siew, Y.C., Gilbert, L.I., **1971**. Effects of molting hormone and juvenile hormone on insect endocrine gland activity. *J. Insect Physiol. 17*, 2095–2104.

Skiebe, P., **1999**. Allatostatin-like immunoreactivity in the stomatogastric nervous system and the pericardial organs of the crab *Cancer pagurus*, the lobster *Homarus americanus*, and the crayfish *Cherax destructor* and *Procambarus clarkii*. *J. Comp. Neurol. 403*, 85–105.

Skinner, J.R., Fairbairn, S.E., Woodhead, A.P., Bendena, W.G., Stay, B., **1997**. Allatostatin in hemocytes of the cockroach *Diploptera punctata*. *Cell Tissue Res. 290*, 119–128.

Sláma, K., Romaňuk, M., Šorm, F., **1974**. Insect Hormones and Bioanalogues. Springer, Berlin.

Sliter, T.J., Sedlak, B.J., Baker, F.C., Schooley, D.A., **1987**. Juvenile hormone in *Drosophila melanogaster*: identification and titer determination during development. *Insect Biochem. 17*, 161–166.

Smith, P.A., Clare, A.S., Rees, H.H., Prescott, M.C., Wainwright, G., *et al.*, **2000**. Identification of methyl farnesoate in the cypris larva of the barnacle, *Balanus amphitrite*, and its role as a juvenile hormone. *Insect Biochem. Mol. Biol. 30*, 885–890.

Smith, R.F., Arking, R., **1975**. The effects of juvenile hormone analogues on embryogenesis in *Drosophila melanogaster*. *J. Insect Physiol. 21*, 723–732.

Sondergaard, L., **1993**. Homology between the mammalian liver and the *Drosophila* fat body. *Trends Genet. 9*, 193–000.

Song, Q.S., Gilbert, L.I., **1998**. Alterations in Ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol. 28*, 849–860.

Soulages, J.L., Wells, M.A., **1994**. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Protein Chem. 45*, 371–415.

Sparagana, S.P., Bhaskaran, G., Barrera, P., **1985**. Juvenile hormone acid methyltransferase activity in imaginal discs of *Manduca sexta* prepupae. *Arch. Insect Biochem. Physiol.* 2, 191–202.

Sparagana, S.P., Bhaskaran, G., Dahm, K.H., Riddle, V., **1984**. Juvenile hormone production, juvenile hormone esterase, and juvenile hormone acid methyltransferase in corpora allata of *Manduca sexta*. *J. Exp. Zool. 230*, 309–314.

Sparks, T.C., Rose, R.L., **1983**. Inhibition and substrate specificity of the haemolymph juvenile hormone esterase of the cabbage looper, *Trichoplusia ni*. *Insect Biochem. 13*, 633–640.

Sparks, T.C., Allen, L.G., Schneider, F., Granger, N.A., **1989**. Juvenile hormone esterase activity from *Manduca sexta* corpora allata *in vitro*. *Arch. Insect Biochem. Physiol. 11*, 93–108.

Sparks, T.C., Hammock, B.D., Riddiford, L.M., **1983**. The hemolymph juvenile hormone esterase of *Manduca sexta* inhibition and regulation. *Insect Biochem. 13*, 529–541.

Sparks, T.C., Willis, W.S., Shorey, H.H., Hammock, B.D., **1979**. Haemolymph juvenile hormone esterase activity in synchronous last instar larvae of the cabbage looper, *Trichoplusia ni*. *J. Insect Physiol. 25*, 125–132.

Sperry, A.E., Sen, S.E., **2001**. Farnesol oxidation in insects: evidence that the biosynthesis of insect juvenile hormone is mediated by a specific alcohol oxidase. *Insect Biochem. Mol. Biol. 31*, 171–178.

Spitz, F., Gonzalez, F.D., Duboule, D., **2003**. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell 113*, 405–417.

Stahl, G.B., **1975**. Insect growth regulators with juvenile hormone activity. *Annu. Rev. Entomol. 20*, 417–460.

Stay, B., **2000**. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. *Insect Biochem. Mol. Biol. 30*, 653–662.

Stay, B., Zhang, J.R., Tobe, S.S., **2002**. Methyl farnesoate and juvenile hormone production in embryos of *Diploptera punctata* in relation to innervation of corpora allata and their sensitivity to allatostatin. *Peptides 23*, 1981–1990.

Stay, B., Chan, K.K., Woodhead, A.P., **1992**. Allatostatin-immunoreactive neurons projecting to the corpora allata of adult *Diploptera punctata*. *Cell Tissue Res. 270*, 15–23.

Stay, B., Woodhead, A.P., Joshi, S., Tobe, S.S., **1991a**. Allatostatins, neuropeptide inhibitors of juvenile hormone biosynthesis in brain and corpora allata of cockroaches, *Diploptera punctata*. In: Menn, J.J., Kelly, T.J., Masler, E.P. (Eds.), Insect Neuropeptides. American Chemical Society, Washington, DC, pp. 164–176.

Stay, B., Joshi, S., Woodhead, A.P., **1991b**. Sensitivity to allatostatins of corpora allata from larval and adult female *Diploptera punctata*. *J. Insect Physiol. 37*, 63–70.

Stay, B., Friedel, T., Tobe, S.S., Mundall, E.C., **1980**. Feedback control of juvenile hormone synthesis in cockroaches: possible role for ecdysterone. *Science 207*, 898–900.

Steel, C.G.H., Ampleford, E.J., **1984**. Circadian control of haemolymph ecdysteroid titers and ecdysis rhythms in *Rhodnius prolixus*. In: Porter, R., Collins, G.M. (Eds.), Photoperiodic Regulation of Insect and Molluscan Hormones. Pitman, London, pp. 150–169.

Steiner, B., Pfisterwilhelm, R., Grossniklaus-Bürgin, C., Rembold, H., Treiblmayr, K., *et al.*, **1999**. Titres of juvenile hormone I, II and III in *Spodoptera littoralis* (Noctuidae) from the egg to the pupal moult and their modification by the egg-larval parasitoid *Chelonus inanitus* (Braconidae). *J. Insect Physiol. 45*, 401–413.

Stoclet, J.C., Gerard, D., Kilhoffer, M.C., Lugnier, C., Miller, R., *et al.*, **1987**. Calmodulin and its role in intracellular calcium regulation. *Progr. Neurobiol. 29*, 321–364.

Stout, J., Hao, J., Kim, P., Mbungu, D., Bronsert, M., *et al.*, **1998**. Regulation of the phonotactic threshold of the female cricket, *Acheta domesticus*: juvenile hormone III, allatectomy, auditory neuron thresholds and environmental factors. *J. Comp. Physiol. A 182*, 635–645.

Stout, J., Hayes, V., Zacharias, D., Henley, J., Stumper, A., *et al.*, **1992**. Juvenile hormone controls phonotactic responsivness of female crickets by genetic regulation of the response properties of identified auditory interneurons. In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), Insect Juvenile Hormone Research: Fundamental and Applied Approaches. INRA, Paris, pp. 265–283.

Strambi, C., **1981**. Some data obtained by radioimmunoassay of juvenile hormones. In: Pratt, G.E., Brooks, G.E. (Eds.), Juvenile Hormone Biochemistry. Elsevier/North Holland, Amsterdam, pp. 59–66.

Strambi, C., Strambi, A., De Reggi, M.L., Hirn, M.H., DeLagge, M.A., **1981**. Radioimmunoassay of insect juvenile hormones and of their diol derivatives. *Eur. J. Biochem. 118*, 401–406.

Sundaram, M., van Aalten, D.M.F., Findlay, J.B.C., Sivaprasadarao, A., **2002**. The transfer of transthyretin and receptor-binding properties from the plasma retinol-binding protein to the epididymal retinoic acid-binding protein. *Biochem. J. 362*, 265–271.

Sundaram, M., Sivaprasadarao, A., Desousa, M.M., Findlay, J.B.C., **1998**. The transfer of retinol from serum retinol-binding protein to cellular retinol-binding protein is mediated by a membrane receptor. *J. Biol. Chem. 273*, 3336–3342.

Sutherland, T.D., Feyereisen, R., **1996**. Target of cockroach allatostatin in the pathway of juvenile hormone biosynthesis. *Mol. Cell. Endocrinol. 120*, 115–123.

Sutherland, T.D., Unnithan, G.C., Andersen, J.F., Evans, P.H., Murataliev, M.B., *et al.*, **1998**. A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA 95*, 12884–12889.

Tanaka, S., **1994**. Endocrine control of ovarian development and flight muscle histolysis in a wing dimorphic cricket, *Modicogryllus confirmatus*. *J. Insect Physiol. 40*, 483–490.

Taylor, P.A., Bhatt, T.R., Horodyski, F.M., **1996**. Molecular characterization and expression analysis of *Manduca sexta* allatotropin. *Eur. J. Biochem. 239*, 588–596.

Teal, P.E.A., **2002**. Effects of allatotropin and allatostatin on *in vitro* production of juvenile hormones by the corpora allata of virgin females of the moths of *Heliothis virescens* and *Manduca sexta*. *Peptides 23*, 663–669.

Teal, P.E.A., Proveaux, A.T., Heath, R.R., **2000**. Analysis and quantitation of insect juvenile hormones using chemical ionization ion-trap mass spectrometry. *Analyt. Biochem. 277*, 206–213.

Temin, G., Zander, M., Roussel, J.P., **1986**. Physico-chemical (GC-MS) measurements of juvenile hormone III titers during embryogenesis of *Locusta migratoria*. *Int. J. Invertebr. Reprod. Devel. 9*, 105–112.

Thomas, B.A., Church, W.B., Lane, T.R., Hammock, B.D., **1999**. Homology model of juvenile hormone esterase from the crop pest, *Heliothis virescens*. *Proteins – Struct. Funct. Genet. 34*, 184–196.

Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., **1993**. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid-x receptor and Ultraspiracle. *Nature 362*, 471–475.

Thompson, C.S., Tobe, S.S., **1986**. Electrical properties of membranes and cells of the corpora allata of the cockroach *Diploptera punctata*: evidence for the presence of voltage-sensitive calcium channels. In: Borkovec, A.B., Gelman, D.B. (Eds.), Insect Neurochemistry and Neurophysiology. Humana Press, Totowa, NJ, pp. 375–378.

Thompson, C.S., Yagi, K.J., Chen, Z.F., Tobe, S.S., **1990**. The effects of octopamine on juvenile hormone biosynthesis, electrophysiology, and cAMP content of the corpora allata of the cockroach *Diploptera punctata*. *J. Comp. Physiol. B 160*, 241–249.

Tobe, S.S., **1990**. Role of intracellular messengers in the regulation of juvenile hormone biosynthesis in the cockroach, *Diploptera punctata*. In: Epple, A., Scanes, C.G., Stetson, M.H. (Eds.), Progress in Comparative Endocrinology. Wiley-Liss, New York, pp. 174–179.

Tobe, S.S., Stay, B., **2004**. Neuropeptides: roles in the regulation of juvenile hormone production. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones. Elsevier Science, Oxford. (in press).

Tobe, S.S., Bendena, W.G., **1999**. The regulation of juvenile hormone production in arthropods. *Ann. New York Acad. Sci. 897*, 300–310.

Tobe, S.S., Stay, B., **1985**. Structure and regulation of the corpus allatum. *Adv. Insect Physiol. 18*, 305–432.

Tobe, S.S., Feyereisen, R., **1983**. Juvenile hormone biosynthesis: Regulation and assay. In: Downer, R.G.H., Laufer, H. (Eds.), Endocrinology of Insects. Alan R. Liss, New York, pp. 161–178.

Tobe, S.S., Pratt, G.E., **1974**. The influence of substrate concentrations on the rate of insect juvenile hormone biosynthesis by corpora allata of the desert locust *in vitro*. *Biochem. J. 144*, 107–113.

Toong, Y.C., Schooley, D.A., Baker, F.C., **1988**. Isolation of insect juvenile hormone III from a plant. *Nature 333*, 170–171.

Touhara, K., Lerro, K.A., Bonning, B.C., Hammock, B.D., Prestwich, G.D., **1993**. Ligand binding by a recombinant insect juvenile hormone binding protein. *Biochemistry 32*, 2068–2075.

Touhara, K., Prestwich, G.D., **1992**. Binding site mapping of a photoaffinity-labeled juvenile hormone binding protein. *Biochem. Biophys. Res. Commun. 182*, 466–473.

Touhara, K., Prestwich, G.D., **1993**. Juvenile hormone epoxide hydrolase: photoaffinity labeling, purification, and characterization from tobacco hornworm eggs. *J. Biol. Chem. 268*, 19604–19609.

Touhara, K., Prestwich, G.D., **1994**. Role of juvenile hormone binding protein in modulating function of JH epoxide hydrolase in eggs of *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 641–646.

Touhara, K., Soroker, V., Prestwich, G.D., **1994**. Photoaffinity labeling of juvenile hormone epoxide hydrolase and JH-binding proteins during ovarian and egg development in *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 633–640.

Touhara, K., Wojtasek, H., Prestwich, G.D., **1996**. *In vitro* modeling of the ternary interaction in juvenile hormone metabolism. *Arch. Insect Biochem. Physiol. 32*, 399–406.

Trautmann, K.H., **1972**. *In vitro* Studium der Tragerproteine von 3H-markierten Juvenilhormonwirksamen verbindungen in der Hämolymphe von *Tenebrio molitor* L. *Larvae. Z. Naturforsch. 27c*, 263–273.

Trautmann, K.H., Masner, P., Schuler, A., Suchy, M., Wipf, H.K., **1974**. Evidence of the juvenile hormone III in insects of four orders. *Z. Naturforsch. 29c*, 757–759.

Trowell, S.C., **1992**. High affinity juvenile hormone carrier proteins in the haemolymph of insects. *Comp. Biochem. Physiol. B 103*, 795–808.

Trowell, S.C., Hines, E.R., Herlt, A.J., Rickards, R.W., **1994**. Characterization of a juvenile hormone binding lipophorin from the blowfly, *Lucilia cuprina*. *Comp. Biochem. Physiol. B 109*, 339–359.

Truesdell, P.F., Koladich, P.M., Kataoka, H., Kojima, K., Suzuki, A., *et al.*, **2000**. Molecular characterization of a cDNA from the true armyworm *Pseudaletia unipuncta*

encoding *Manduca sexta* allatotropin peptide. *Insect Biochem. Mol. Biol. 30*, 691–702.

Truman, J.W., **1996**. Metamorphosis of the insect nervous system. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Academic Press, New York, pp. 283–320.

Truman, J.W., Reiss, S.E., **1988**. Hormonal regulation of the shape of identified motorneurons in the moth *Manduca sexta*. *J. Neurosci. 8*, 765–775.

Truman, J.W., Riddiford, L.M., **1999**. The origins of insect metamorphosis. *Nature 401*, 447–452.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Tu, M.P., Kou, R., O'Remus, G., Yin, C.-M., Stoffolano, J.G., **2002**. Allatotropic activity in the brain of female *Phormia regina*. *J. Insect Physiol. 48*, 733–741.

Tu, M.P., Kou, R., Wang, Z.S., Stoffolano, J.G., Yin, C.-M., **2001**. Immunolocalization and possible effect of a moth allatotropin-like substance in a fly, *Phormia regina*. *J. Insect Physiol. 47*, 233–244.

Ulrich, G.M., **1985**. Elimination of the allatotropic activity in locusts by microsurgical and immunological methods: evidence for humoral control of the corpora allata, hemolymph proteins and ovary development. *Gen. Comp. Endocrinol. 59*, 120–130.

Unni, B., Barrera, P., Muszynskapytel, M., Bhaskaran, G., Dahm, K.H., **1993**. Partial characterization of allatinhibin, a neurohormone of *Manduca sexta*. *Arch. Insect Biochem. Physiol. 24*, 173–185.

Unnithan, G.C., Feyereisen, R., **1995**. Experimental acquisition and loss of allatostatin sensitivity by corpora allata of *Diploptera punctata*. *J. Insect Physiol. 41*, 975–980.

Unnithan, G.C., Sutherland, T.D., Cromey, D.W., Feyereisen, R., **1998**. A factor causing stable stimulation of juvenile hormone synthesis by *Diploptera punctata* corpora allata *in vitro*. *J. Insect Physiol. 44*, 1027–1037.

Vafopoulou, X., Steel, C.G.H., **2001**. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine zeitgebers. *J. Insect Physiol. 47*, 935–941.

Valaitis, A.P., **1991**. Characterization of hemolymph juvenile hormone esterase from *Lymantria dispar*. *Insect Biochem. 21*, 583–595.

Varjas, L., Kulcsar, P., Fekete, J., Bihatsi-Karsai, E., Lelik, L., **1992**. JH titres measured by GC-MS in the hemolymph of *Mamestra oleracea* larvae reared under different photoperiodic conditions. In: Mauchamp, B., Couillaud, F., Baehr, J.-C. (Eds.), Insect Juvenile Hormone Research: Fundamental and Applied Approaches. INRA, Paris, pp. 45–50.

Veenstra, J.A., Costes, L., **1999**. Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. *Peptides 20*, 1145–1151.

Veenstra, J.A., Lehman, H.K., Davis, N.T., **1994**. Allatotropin is a cardioacceleratory peptide in *Manduca sexta*. *J. Exp. Biol. 188*, 347–354.

Veenstra, J.A., Noriega, F.G., Graf, R., Feyereisen, R., **1997**. Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti*. *Peptides 18*, 937–942.

Venkataraman, V., Omahony, P.J., Manzcak, M., Jones, G., **1994**. Regulation of juvenile hormone esterase gene transcription by juvenile hormone. *Devel. Genet. 15*, 391–400.

Venkatesh, K., Roe, R.M., **1988**. The role of juvenile hormone and brain factor(s) in the regulation of plasma juvenile hormone esterase activity during the last larval stadium of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 34*, 415–426.

Vermunt, A.M.W., Vermeesch, A.M.G., deKort, C.A.D., **1997a**. Purification and characterization of juvenile hormone esterase from hemolymph of the Colorado potato beetle. *Arch. Insect Biochem. Physiol. 35*, 261–277.

Vermunt, A.M.W., Koopmanschap, A.B., Vlak, J.M., deKort, C.A.D., **1997b**. Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase from the Colorado potato beetle. *Insect Biochem. Mol. Biol. 27*, 919–928.

Vernier, P., Cardinaud, B., Valdenaire, O., Philippe, H., Vincent, J.D., **1995**. An evolutionary view of drug–receptor interaction: the bioamine receptor family. *Trends Pharmacol. Sci. 16*, 375–381.

Vince, R., Gilbert, L.I., **1977**. Juvenile hormone esterase activity in precisely timed instar larvae and pharate pupae of *Manduca sexta*. *Insect Biochem. 7*, 115–120.

von Nickisch-Rosenegk, E., Krieger, J., Kubick, S., Laage, R., Strobel, J., *et al.*, **1996**. Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol. 26*, 817–827.

Walter, W.R., Sing, G.B., Krawetz, S.A., **1998**. MARs mission update. *Biochem. Biophys. Res. Commun. 242*, 419–422.

Wang, X., Chen, X., Haunerland, N.H., **1993**. Flight muscle development in juvenile and adult forms of the desert locust, *Schistocerca gregaria*. *J. Insect Physiol. 39*, 325–333.

Wang, Z.W., Ding, Q., Yagi, K.J., Tobe, S.S., **1994**. Terminal stages in juvenile hormone biosynthesis in corpora allata of *Diploptera punctata*: developmental changes in enzyme activity and regulation by allatostatins. *J. Insect Physiol. 40*, 217–223.

Ward, V.K., Bonning, B.C., Huang, T., Shiotsuki, T., Griffeth, V.N., *et al.*, **1992**. Analysis of the catalytic mechanism of juvenile hormone esterase by site-directed mutagenesis. *Int. J. Biochem. 24*, 1933–1942.

Watson, R.D., Whinsenton, L.R., Bollenbacher, W.E., Granger, N.A., **1986**. Interendocrine regulation of the corpora allata and prothoracic glands of *Manduca sexta*. *Insect Biochem. 16*, 149–155.

Weeks, J.C., Truman, J.W., **1986a**. Hormonally mediated reprogramming of muscles and motorneurones during the larval–pupal transformation of the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 125*, 1–14.

Weeks, J.C., Truman, J.W., **1986b**. Steroid control of neuron and muscle development during the metamorphosis of an insect. *J. Neurobiol. 17*, 249–267.

Weinberger, C., **1996**. A model for farnesoid feedback control in the mevalonate pathway. *Trends Endocrinol. Metab. 7*, 1–6.

Weller, J., Sun, G.C., Zhou, B.H., Lan, Q., Hiruma, K., *et al.*, **2001**. Isolation and developmental expression of two nuclear receptors, MHR4 and beta FTZ-F1, in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 31*, 827–837.

Westphal, U., **1986**. Steroid–Protein Interactions II. Springer, Berlin.

Wheeler, D.E., Nijhout, H.F., **1981**. Soldier determination in ants: new role for juvenile hormone. *Science 213*, 361–363.

Wheeler, D.E., Nijhout, H.F., **2003**. A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. *BioEssays 25*, 994–1001.

Wheelock, C.E., Severson, T.F., Hammock, B.D., **2001**. Synthesis of new carboxylesterase inhibitors and evaluation of potency and water solubility. *Chem. Res. Toxicol. 14*, 1536–1572.

Whisenton, L.R., Bowen, M.F., Granger, N.A., Gilbert, L.I., Bollenbacher, W.E., **1985**. Brain-mediated 20-hydroxyecdysone regulation of juvenile hormone synthesis by the corpora allata of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 58*, 311–318.

Whisenton, L.R., Granger, N.A., Bollenbacher, W.E., **1987**. A kinetic analysis of brain-mediated 20-hydroxyecdysone stimulation of the corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol. 54*, 171–178.

Whitmore, E., Gilbert, L.I., **1972**. Haemolymph lipoprotein transport of juvenile hormone. *J. Insect Physiol. 18*, 1153–1167.

Whitmore, D., Gilbert, L.I., Ittycheriah, P.I., **1974**. The origin of hemolymph carboxylesterases "induced" by the insect juvenile hormone. *Mol. Cell. Endocrinol. 1*, 37–54.

Wieczorek, E., Kochman, M., **1991**. Conformational change of the haemolymph juvenile hormone binding protein from *Galleria mellonella*. *Eur. J. Biochem. 201*, 347–353.

Wigglesworth, V.B., **1936**. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus*. *Q. J. Microsc. Sci. 79*, 91–121.

Williams, C.M., **1952**. Physiology of insect diapause IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull. 103*, 120–138.

Wigglesworth, V.B., **1959**. Metamorphosis, polymorphism, differentiation. *Sci. Am. 200*, 100–106.

Wigglesworth, V.B., **1970**. Insect Hormones. W.H. Freeman, San Francisco.

Wigglesworth, V.B., **1972**. The Principles of Insect Physiol. Chapman and Hall, London.

Williams, C.M., **1976**. Juvenile hormone ... in retrospect and prospect. In: Gilbert, L.I. (Ed.), The Juvenile Hormones. Plenum, New York, pp. 1–14.

Williamson, M., Lenz, C., Winther, A.M.E., Nassel, D.R., Grimmelikhuijzen, C.J.P., **2001a**. Molecular cloning, genomic organization and expression of a B-Type (Cricket-Type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun. 281*, 544–550.

Williamson, M., Lenz, C., Winther, A.M.E., Nassel, D.R., Grimmelikhuijzen, C.J.P., **2001b**. Molecular cloning, genomic organization, and expression of a C-type (*Manduca sexta*-type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun. 282*, 124–130.

Williamson, M., Lenz, C., Winther, A.M.E., Nassel, D.R., Grimmelikhuijzen, C.J.P., **2001c**. Molecular cloning, genomic organization and expression of a B-Type (Cricket-Type) allatostatin preprohormone from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun. 282*, 368 (correction of 2001b).

Willis, J.H., **1996**. Metamorphosis of the cuticle, its proteins, and their genes. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis. Academic Press, New York, pp. 253–282.

Wilson, T.G., Ashok, M., **1998**. Insecticide resistance resulting from an absence of target-site gene product. *Proc. Natl Acad. Sci. USA 95*, 14040–14044.

Wilson, T.G., Fabian, J., **1986**. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. *Devel. Biol. 118*, 190–201.

Wilson, T.G., DeMoor, S., Lei, J., **2003**. Juvenile hormone involvement in *Drosophila melanogaster* male reproduction. *Insect Biochem. Mol. Biol. 33*, 1167–1175.

Wing, K.D., Sparks, T.C., Lovell, V.M., Levinson, S.O., Hammock, B.D., **1981**. The distribution of juvenile hormone esterase and its interrelationship with other proteins influencing juvenile hormone metabolism in the cabbage looper, *Trichoplusia ni*. *Insect Biochem. 11*, 473–485.

Wisniewski, J.R., Muszynska-Pytel, M., Kochman, M., **1986**. Juvenile hormone degradation in brain and corpora cardiaca–corpora allata complex during the last larval instar of *Galleria mellonella*. *Experientia 42*, 167–168.

Witek, G., Hoffmann, K.H., **2001**. Immunological evidence for FGLamide- and W_2W_9-allatostatins in the ovary of *Gryllus bimaculatus*. *Physiol. Entomol. 26*, 49–57.

Wojtasek, H., Prestwich, G.D., **1995**. Key disulfide bonds in an insect hormone binding protein: cDNA cloning of a juvenile hormone binding protein of *Heliothis virescens* and ligand binding by native and mutant forms. *Biochemistry 34*, 5234–5241.

Wojtasek, H., Prestwich, G.D., **1996**. An insect juvenile hormone-specific epoxide hydrolase is related to vertebrate microsomal epoxide hydrolases. *Biochemi. Biophys. Res. Communi. 220*, 323–329.

Woodring, J., Hoffmann, K.H., **1994**. The effects of octopamine, dopamine and serotonin on juvenile hormone synthesis, *in vitro*, in the cricket, *Gryllus bimaculatus*. *J. Insect Physiol. 40*, 797–802.

Wroblewski, V.J., Harshman, L.G., Hanzlik, T.N., Hammock, B.D., **1990**. Regulation of juvenile hormone esterase gene expression in the tobacco budworm. *Arch. Biochem. Biophys. 278*, 461–466.

Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of juvenile hormone. 2. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.

Xu, Y., Fang, F., Chu, Y.X., Jones, D., Jones, G., **2002**. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle. *Eur. J. Biochem. 269*, 6026–6036.

Yagi, K.J., Tobe, S.S., **2001**. The radiochemical assay for juvenile hormone biosynthesis in insects: problems and solutions. *J. Insect Physiol. 47*, 1227–1234.

Yagi, K.J., Konz, K.G., Stay, B., Tobe, S.S., **1991**. Production and utilization of farnesoic acid in the juvenile hormone biosynthetic pathway by corpora allata of larval *Diploptera punctata*. *Gen. Comp. Endocrinol. 81*, 284–294.

Yagi, S., Kuramochi, K., **1976**. The role of juvenile hormone in larval duration and spermiogenesis in relation to phase variation in the tobacco cutworm, *Spodoptera litura*. *Applic. Entomol. Zool. 11*, 133–138.

Yamamoto, C.K., Chadarevian, A., Pellegrini, M., **1988**. Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. *Science 239*, 916–919.

Yang, J.H., Park, J.-C., Lee, B.H., **2000**. Molecular characterization of allatostatins cDNA in central nervous system and midgut from the German cockroach *Blattella germanica*. *Korean J. Entomol. 30*, 131–137.

Yao, T.P., Forman, B.M., Jiang, Z.Y., Cherbas, L., Chen, J.D., *et al.*, **1993**. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. *Nature 366*, 476–479.

Yin, C.-M., Zou, B.X., Jiang, M.G., Li, M.F., Qin, W.H., *et al.*, **1995**. Identification of juvenile hormone III bisepoxide (JHB_3), JH III and methyl farnesoate secreted by the corpus allatum of *Phormia regina, in vitro* and function of JHB_3 either applied alone or as a part of a juvenoid blend. *J. Insect Physiol. 41*, 473–488.

Yin, C.-M., Takeda, M., Wang, Z.-S., **1987**. Juvenile hormone analogue, methoprene as a circadian and development modulator in *Diatraea grandiosella*. *J. Insect Physiol. 33*, 95–102.

Young, J.K., Orth, A.P., Goodman, W.G., **2003**. Allelic variation in the hemolymph juvenile hormone binding protein gene of *Manduca sexta*. *Mol. Cell. Endocrinol. 208*, 41–50.

Yu, C.G., Hayes, T.K., Strey, A., Bendena, W.G., Tobe, S.S., **1995**. Identification and partial characterization of receptors for allatostatins in brain and corpora allata of the cockroach *Diploptera punctata* using a binding assay and photoaffinity labeling. *Regul. Peptides 57*, 347–358.

Yu, S.J., Terriere, L.C., **1978**. Metabolism of juvenile hormone I by microsomal oxidase, esterase and epoxide hydratase of *Musca domestica* and some comparisons with *Phormia regina* and *Sarcophaga bullata*. *Pestic. Biochem. Physiol. 9*, 237–246.

Zera, A.J., Sanger, T., Hanes, J., Harshman, L., **2002**. Purification and characterization of hemolymph juvenile hormone esterase from the cricket, *Gryllus assimilis*. *Arch. Insect Biochem. Physiol. 49*, 41–55.

Zhou, B.H., Hiruma, K., Shinoda, T., Riddiford, L.M., **1998**. Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 203*, 233–244.

Zhou, C., Liu, B., **2001**. Identification and characterization of a silk gland-related matrix association region in *Bombyx mori*. *Gene 277*, 139–144.

Zhou, X.F., Riddiford, L.M., **2002**. Broad specifies pupal development and mediates the “status quo” action of juvenile hormone on the pupal–adult transformation in *Drosophila* and *Manduca*. *Development 129*, 2259–2269.

Ziese, S., Dorn, A., **2003**. Embryonic integument and “molts” in *Manduca sexta*. *J. Morphol. 255*, 146–161.

Zimowska, G., Rembold, H., Bayer, G., **1989**. Juvenile hormone identification, titer, and degradation during the last larval stadium of *Spodoptera littoralis*. *Arch. Insect Biochem. Physiol. 12*, 1–14.

Žitňan, D., Kingan, T.G., Kramer, S.J., Beckage, N.E., **1995**. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J. Comp. Neurol. 356*, 83–100.

3.8 Feedback Regulation of Prothoracic Gland Activity

S Sakurai, Kanazawa University, Kanazawa, Japan

3.8.1. Introduction

The physiologic effects of insect hormones depend to a large extent on their concentration in the hemolymph. The hormone concentration exposed to target cells is determined by three factors: synthesis and secretion, the downregulation of hormone secretion, and the rates of degradation and elimination. The synthesis, secretion, and downregulation are to a great extent mediated by positive and negative feedback circuits. In the vertical sequences like the hypothalamic–pituitary–thyroid axis of adult vertebrates for example, thyroid hormone suppresses the synthesis and release of thyrotropin releasing hormone (TRH) from neurosecretory cells (NSCs) in the paraventricular nucleus of the hypothalamus as well as thyroid stimulating hormone (TSH) from the thyrotrophs in the adenohypophysis. The control of hypothalamic TRH release by thyroid hormone is referred to as long-loop feedback, and that of TSH release from the pituitary gland as short-loop feedback, while the control of the thyroid gland itself by thyroid hormone is called ultra-short-loop feedback. The definition of feedback is, however, rather vague among insect endocrinologists and the term "feedback" has been used occasionally to denote a "horizontal" connection between two or more secretory organs, i.e., the reciprocal interaction between the prothoracic gland and corpus allatum, although these organs are not organized vertically. In the present chapter, the term feedback indicates both feedback in the vertical sequences and horizontal connections, thus covering an intricate network. In addition, the terms, long-loop, short-loop, and ultra-short-loop will not be used, those being simply denoted as feedback unless otherwise indicated.

The endocrine control in insects governs diverse biological events in gamete formation, embryonic development, postembryonic development, homeostasis, and behavior, many of which are believed to be under the control, directly or indirectly, of neuropeptides, and therefore neurosecretion. The insect endocrine system is significantly neuroendocrine, as nearly 17% of the cells of the central nervous system (CNS) produce neuropeptides (Gäde *et al.*, 1997; Predel and Eckert, 2000) (see **Chapter 3.10**). Although each ganglion in the CNS contains a number of NSCs with different characteristics with regard to

both size and developmental changes in their shapes and content, only a few NSCs have been characterized on the basis of their product. Even in the secretory cells whose products have been identified and their roles established, very little is known about the feedback regulation of cell activity.

In insects, a few endocrine glands are part of the peripheral secretory organ system. These are the prothoracic gland that secretes ecdysone (see **Chapter 3.3**), the corpus allatum that secretes juvenile hormone (JH) and functions as a neurohemal organ for various neuropeptides produced by the brain NSCs (see **Chapter 3.7**), the corpus cardiacum that secretes peptide hormones involved in the control of energy metabolism (Gäde *et al.*, 1997), the epitracheal gland that includes the Inka cell that releases ecdysis triggering hormone (Zitnan *et al.*, 1996) (see **Chapter 3.1**), the ovary and testes, both of which are known to produce ecdysone in a distinct period of the insect life cycle (Hagedorn *et al.*, 1975; Lagueux *et al.*, 1977; Hetru *et al.*, 1978; Delbecque *et al.*, 1990) (see **Chapter 3.9**), the gut that contains a number of myotropic factors (Gäde *et al.*, 1997) (see **Chapter 4.5**), epidermal cells, a source of dopamine (Noguchi and Hayakawa, 1996) and ecdysone in the argasid tick (Zhu *et al.*, 1991) (see **Chapter 3.16**), and the fat body secreting an insect cytokine, growth-blocking peptide (Hayakawa *et al.*, 1998) and growth factors, one of which is imaginal disc growth factor (IDGF) known to stimulate cell proliferation of wing imaginal discs in *Drosophila* (Kawamura *et al.*, 1999). Among those peripheral glands, feedback regulation is known only in the axis of the brain–Inka cell at ecdysis (see **Chapter 3.1**) and the brain–prothoracic gland axis (see **Chapter 3.2**). No information is available on the feedback regulation of the production and/or secretion of metabolic hormones such as hypertrehalosemic hormone and adipokinetic hormone from the corpora cardiaca (see **Chapter 3.10**), although they are crucially involved in the homeostasis of blood sugars and lipids, and even of allatotropin and allatostatin that control JH secretion from the corpora allata (see **Chapter 3.7**).

In addition to feedback in the vertical axis, horizontal interactions are known between the prothoracic glands and corpora allata. Ecdysteroids and JHs interact, and we cannot study one without considering the other (Gilbert *et al.*, 2000). Thus, the feedback regulation of the brain–prothoracic gland axis and the horizontal controls involved in ecdysteroidogenesis will be the main focus of this chapter (**Figure 1**).

3.8.2. Feedback Regulation of Brain–Prothoracic Gland Axis

3.8.2.1. Feedback Regulation of Brain Neurosecretory Cells by 20-Hydroxyecdysone

20-Hydroxyecdysone (20E) has been considered to be involved in the regulation of production and/or secretion of neurosecretory materials from brain NSCs based on the changes in NSC shape and stain intensity (e.g., Steel and Harmsen, 1971; Steel, 1975; Agui and Hiruma, 1977a, 1977b; Khalil *et al.*, 1988) (see **Chapter 3.11**), but the evidence is only circumstantial. The weakest point of those earlier studies is the lack of knowledge of the products of the NSCs in question. Most of these studies were concerned with the four pairs of large NSCs in the pars intercerebralis of the brain, which were once believed to be prothoracicotropic hormone (PTTH) cells (Steel and Harmsen, 1971; Steel, 1975; Agui and Hiruma, 1977a) but are not now believed to be so (Agui *et al.*, 1979; Mizoguchi *et al.*, 1990; Gilbert *et al.*, 2000). This indicates that ecdysteroids affect other NSCs besides the PTTH cells, although it is not truly feedback regulation. It should be noted that the nomenclature of negative and positive feedback in the earlier literatures is somewhat confusing. When 20E injections increase the stain intensity of NSCs, this was described as a positive feedback effect, although it is not certain whether the increase in stain intensity is a result of feedback inhibition of neurosecretion or activation of the gene encoding the neuropeptide or both. Both *in vivo* and *in vitro* experiments show that 20E elicits an increase in the stain intensity of most brain NSCs in *Rhodnius prolixus* (Steel, 1975) and *Mamestra brassicae* (Agui and Hiruma, 1977a). Removal of the prothoracic glands from *M. brassicae* larvae decreases the stain intensity and 20E injection recovers it (Agui and Hiruma, 1977b). Since the increased stain intensity may be caused by an increased production of neurosecretory materials or suppression of their release, or both, the results indicate that 20E stimulates the production of neurosecretory material (positive feedback) but suppresses its release (negative feedback) (Steel, 1975). The observations in those reports have been focused on the median NSCs, of which four pairs of the large cells are now believed to be bombyxin cells (Mizoguchi *et al.*, 1987; Iwami *et al.*, 1990; Moto *et al.*, 1999) (see **Chapter 3.2**), suggesting that 20E affects bombyxin secretion and/or its production. In Inka cells, that produce a peptide hormone, ecdysis triggering hormone (ETH), rising ecdysteroid levels upregulate ETH gene

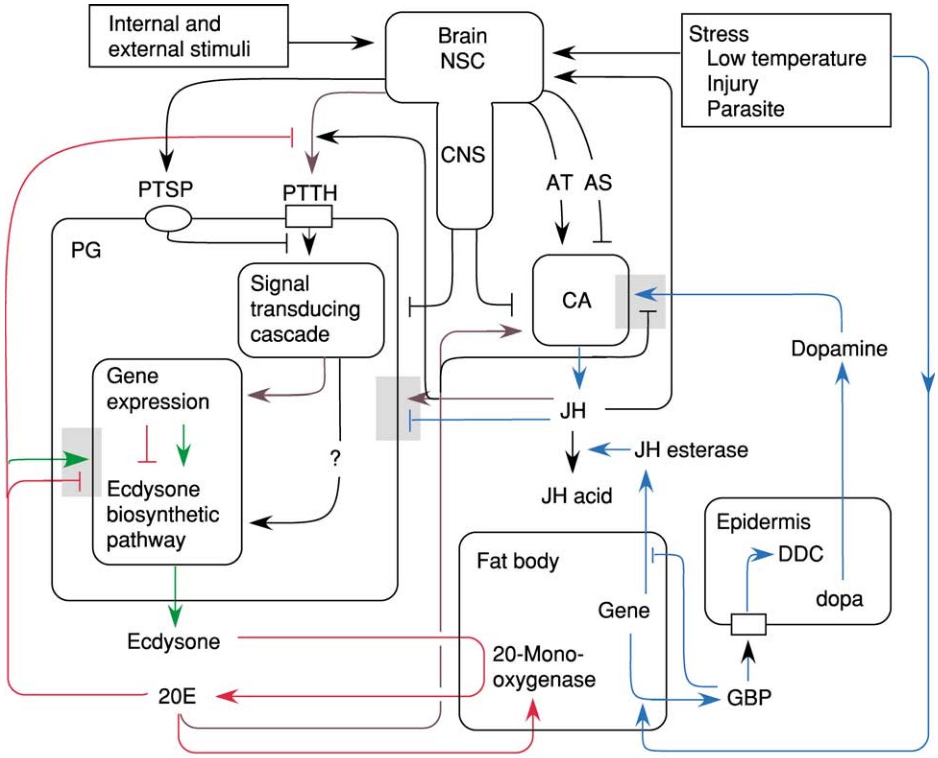


Figure 1 Vertical sequence and horizontal network of regulation of prothoracic gland, based on the information obtained mainly from studies on *Manduca sexta* and *Bombyx mori* except for the growth-blocking peptide (GBP) network. Red lines indicate the vertical sequence that provides the feedback inhibition of the brain–prothoracic gland axis. Green lines indicate partly the feedback stimulation loop. Blue lines show the horizontal regulation of the prothoracic glands through the stress–GBP network (partly hypothetical). Arrowheads and ⊥ symbols indicate stimulatory and inhibitory regulation, respectively. Shadowed area indicates that the feedback effects change along with postembryonic development: 20E inhibits the larval glands with moderate to high secretory activity, while stimulating the glands with low secretory activity as seen in early fifth instar larvae, the first day of nondiapausing pupae, and diapausing pupae of *M. sexta*. JH suppresses prothoracic glands before the onset of wandering, but stimulates the glands in the early pupal period. In the stress–GBP pathway, dopamine suppresses the corpus allatum until the middle of the fifth instar but not thereafter, probably due to the change in the type of dopamine receptor (Granger *et al.*, 1996) (see **Chapter 3.7**). AS, allatostatin; AT, allatotropin; CA, corpus allatum; CNS, central nervous system; DDC, DOPA decarboxylase; GBP, growth-blocking peptide; NSC, neurosecretory cell; PG, prothoracic gland cell; PTSP, prothoracicostatic peptide; PTTH, prothoracicotropic hormone. See text for details.

expression, while the subsequent ecdysteroid decline triggers ETH release (Zitnan *et al.*, 1999) (see **Chapter 3.1**). Although Inka cells are not NSCs, neuropeptide gene expression could be similarly upregulated in the brain NSCs, which is in accordance with the earlier observations described above.

3.8.2.2. Feedback Regulation of PTTH Cells by 20-Hydroxyecdysone

There is no firm evidence to show the feedback regulation of PTTH production and its release, but 20E appears to elicit feedback control on PTTH cells, as seen in the diapausing pupal brain of *M. brassicae* (Agui and Hiruma, 1977a) (see **Chapter 3.12**). Diapausing pupal brains were incubated with 20E for 2 days followed by incubation in fresh medium for another 2 days, and then inactive diapausing pupal prothoracic glands were placed in the latter conditioned medium. When ecdysteroid activity in the final medium was assessed biologically by adding a piece of integument, apolysis (secretion of new cuticles from the underlying epidermal cells) was induced, indicating that the conditioned medium in which prothoracic glands from diapausing pupae had been added did contain ecdysteroids. This suggested that the 20E in the first medium might have caused the brain to release PTTH. They also showed increases in the stain intensity of all median and posterior brain NSCs, but not in the lateral NSCs. The intensity of the latter actually decreased after incubation of the brain with 20E for 2 days, but it is not obvious whether these lateral NSCs are the PTTH cells that have been described as

being in the lateral region of other Lepidoptera (Agui *et al*., 1979; Mizoguchi *et al*., 1990; Gilbert *et al*., 2000) (see **Chapter 3.2**) since there are at least three types of NSCs of similar size in the lateral region. Nevertheless, the data suggest that 20E stimulates PTTH release at a moderate concentration while suppressing its release at a high concentration (Agui and Hiruma, 1977a).

Circumstantial evidence for the above hypothesis also comes from comparing the changes in hemolymph ecdysteroid and PTTH concentrations in the fourth and fifth larval instars and the pupal period of *Bombyx mori* (Mizoguchi *et al*., 2001, 2002). In the fourth instar, the PTTH titer begins to decline as the ecdysteroid titer peaks. Brain extirpation or neck ligation has been utilized to estimate the time after which the brain is not required for inducing the following larval ecdysis, referred to as the critical period, and thereby the time when sufficient PTTH is secreted (Truman, 1972). This time is approximately 14 h prior to the peak of the ecdysteroid titer in *Manduca* (Bollenbacher *et al*., 1987) and 12 h in *Bombyx* fourth instar larvae (Sakurai, 1983). This time is well correlated with the change in PTTH titer. Nevertheless, the question remains concerning the extremely low PTTH titer as low as 18 pg ml^{-1} hemolymph in *Bombyx* (Mizoguchi *et al*., 2001), which is an order of magnitude lower than the critical concentration (0.2–0.3 ng ml^{-1}) to stimulate the prothoracic glands *in vitro* (Kataoka *et al*., 1987; Kiriishi *et al*., 1992; Hua *et al*., 1999). Accordingly, it is not conclusive at present if the measured PTTH titer in the fourth instar larvae reflects the actual PTTH concentration or if there are other factors involved in the upregulation of prothoracic gland activity, although brain extracts effectively induce a larval–larval molt in neck ligated larvae (Suzuki and Ishizaki, 1986). In *Manduca*, PTTH titers in the fourth instar larval hemolymph were measured by an indirect bioassay method using paired prothoracic glands (Bollenbacher *et al*., 1987). The peak titer of PTTH was found to be 0.2 units of PTTH, just enough to activate the glands, and occurred 9 h prior to the ecdysteroid peak (Bollenbacher *et al*., 1987). When the ecdysteroid titer peaks, the PTTH titer has decreased, indicating the feedback inhibition of PTTH synthesis and/or release by 20E. At that stage any ecdysone formed would be promptly converted to 20E (Kamimura and Kiuchi, 2002). In the fifth instar, the PTTH titer declines sharply in concurrence with the beginning of the increase in ecdysteroid titer approximately 12 h later (Mizoguchi *et al*., 2002). This supports the idea of feedback inhibition of PTTH release by high ecdysteroid titers. A similar observation was made for the pupal period (Mizoguchi *et al*., 2002). In *Bombyx* pupae, the PTTH titer peaks on day 1.25 and declines after considerable fluctuations. The peak ecdysteroid titer occurs 1 day later, although both peaks are broad and overlap. The ecdysteroid titer was measured by radioimmunoassay (RIA) of crude extracts of hemolymph and the changes in individual ecdysteroids were determined by a combination of high-performance liquid chromatography (HPLC) separation and RIA measurement, a paradigm that showed that the 20E peak follows the ecdysone peak by 3 days in *Manduca* (Warren and Gilbert, 1986). Taking into consideration that pupal–adult development takes 9 days in *Bombyx,* which was the object of the studies by Mizoguchi *et al.* (2001), while the comparable period in *Manduca* is about 20 days, the 20E peak in *Bombyx* pupae should appear at least 1 day after the measured ecdysteroid peak. In that case, the PTTH titer would decline when the 20E titer has peaked. Accordingly, 20E may downregulate PTTH secretion and/or synthesis throughout the postembryonic development, except in the case of diapausing animals.

It has not been shown unequivocally that 20E exerts feedback regulation on PTTH production. At each of the developmental stages of *Bombyx*, a high ecdysteroid titer does not elicit an accumulation of PTTH in the brain (Mizoguchi *et al*., 2001) nor in the PTTH cell axon ending of the corpus allatum of *Manduca* (Dai *et al*., 1995), a neurohemal organ for PTTH, in contrast to brain NSCs indicated by the above-mentioned earlier observations (Steel, 1975; Agui and Hiruma, 1977a). It is conceivable that once 20E elicits its inhibitory effects on the PTTH cells at its high concentration at the end of the fourth and fifth instars, 20E may not only suppress PTTH secretion but also downregulate the PTTH gene. In the hypothalamic–pituitary–adrenal or –thyroid axes in vertebrates, corticosteroid or thyroid hormone downregulates the genes of tropic components in the hypothalamic NSCs and pituitary cells. The hypothalamic TRH and pituitary TSH subunit genes are downregulated by the active form of thyroid hormone, triiodothyronine (T3) (Shibusawa *et al*., 2003). There is no indication if such is also the case in PTTH gene regulation, since we know nothing about this gene, but 20E could exert its effect by downregulating the PTTH gene. Another interpretation is suggested by the study of deiodinase activity in the thyrotrophs of the anterior pituitary gland (Huang *et al*., 2001; Schneider *et al*., 2001). When TSH genes are suppressed strongly by thyroid hormone, type II iodothyronine deiodinase (D2) activity

increases in the thyrotrophs to promote the conversion of T4 to T3, the latter being stronger in its ability to bind to the thyroid hormone receptor and thereby promote binding to *trans*-regulatory elements of the TRH and TSH genes (Shibusawa *et al*., 2003). Such locally synthesized T3 acts as a negative feedback factor in suppressing the TSH gene, and therefore the localized upregulation of D2 activity may be the first step in the negative feedback loop (Huang *et al*., 2001). Although it is not known if circulating 20E in insect hemolymph is positively released from the midgut and fat body, major tissues expressing 20-monooxygenase activity (Smith *et al*., 1983), i.e., the enzyme that mediates the conversion of ecdysone to 20E, or only leaks from peripheral tissues. It might be of interest to measure this enzyme activity in PTTH cells when they are presumably negatively controlled by 20E. If the 20-monooxygenase is upregulated locally in the PTTH cells, feedback inhibition of the PTTH gene may occur earlier than the time when a 20E increase occurs in the hemolymph. Since the gene encoding the 20-monooxygenase is now known (see **Chapter 3.3**), one could use *in situ* hybridization studies to quantify that gene's transcripts in the PTTH cells.

In addition to PTTH, prothoracicostatic peptide (PTSP) appears to be present in *Bombyx* fifth instar larvae, although it is identical to the *Manduca* myoinhibitory peptide (Mas-MIP I) (Hua *et al*., 1999). PTSP does not alter the basal secretory activity of prothoracic glands *in vitro* but suppresses the PTTH-stimulated glands, presumably by inhibiting PTTH action (Hua *et al*., 1999) by blocking the calcium channel that is tightly associated with the earliest step of the PTTH signaling pathway (Dedos and Birkenbeil, 2003). PTSP has been isolated from the *Bombyx* brain, and therefore the site of synthesis is probably in the brain, but nothing is known about the location of the PTSP cells or the effects of 20E on its production and/or secretion.

3.8.3. Feedback Regulation of the Prothoracic Gland by Ecdysteroid

A decrease in the hemolymph ecdysteroid titer may be as important as its increase in various aspects of insect development. At the larval–larval molt, the DOPA decarboxylase (DDC) gene in the epidermal cells is activated in response to a decreasing ecdysteroid titer (Hiruma *et al*., 1995). DDC mediates the conversion of DOPA to dopamine, a precursor of DOPA quinone that mediates sclerotization by chemically bridging cuticular proteins (see **Chapters 4.2** and **4.4**) and which also serves as an intermediate in melanin production for melanization. Therefore, an artificial increase in ecdysteroid concentration delays the melanization process. Similarly, adult eclosion is delayed by 20E injection (see **Chapter 3.1**). In spite of the recognition of the importance of feedback regulation of prothoracic gland secretory activity, only six papers on this subject have been published in the last 50 years (Williams, 1952; Siew and Gilbert, 1971; Beydon and Lafont, 1983; Bodnaryk, 1986; Sakurai and Williams, 1989; Song and Gilbert, 1998; review: Gilbert *et al*., 1997). The next section is based on those publications.

3.8.3.1. Feedback Activation of Ecdysteroid Production

The first paper that indicated the feedback activation of prothoracic glands appeared in 1952, although the term "feedback" was not used. Rather, it was stated that "prothoracic glands can be triggered also by the prothoracic gland hormone itself" in the famous experiments involving the parabiosis of eight brainless diapausing pupae and chained pupal abdomens of *Hyalophora cecropia* (Williams, 1952). This study doubtlessly describes the positive feedback regulation of prothoracic gland activity. There are three reports to support feedback activation in *Manduca* (Sakurai and Williams, 1989), the bertha armyworm *Mamestra configurata* (Bodnaryk, 1986), and *Bombyx* (Sakurai and Imokawa, 1988) although Bodnaryk does not mention positive feedback. The prothoracic glands of day 2 fifth instar *Manduca* larvae were inserted into diapausing pupae that had had their brains removed, and gland activity was then measured *in vitro* every day after implantation. In such pupal preparations, the implants exhibited periodic changes in secretory activity, as the peaks of 50–60 ng per gland appeared 4, 7, and 12 days after the implantation. Each peak was separated by a nadir of activity of approximately 20 ng per gland. The same was also the case in pupal preparations lacking both brain and prothoracic glands. The implanted day 2 glands may be stimulated by ecdysteroid they synthesize. When the activity attains a considerably higher level than a threshold in the host pupae, the glands are inhibited. Once the gland activity decreases to a low level, they are again capable of being activated by their ecdysteroids or those of the host prothoracic glands that may have been activated by the ecdysteroid produced by the implanted glands. These *in vivo* observations indicate the presence of feedback activation. However, the glands of feeding larvae are barely activated by either ecdysone or 20E *in vitro*. By contrast, *in vitro* administration of 20E stimulates diapausing pupal

glands as well as day 1 nondiapausing pupal glands (Sakurai and Williams, 1989). A similar autonomous change in the gland activity is observed in *Bombyx* fifth instar larvae (Sakurai and Imokawa, 1988). Larvae lacking the retrocerebral complex (brain–corpora cardiaca–corpora allata) are capable of gut purge followed by pupation if the surgical operation was done on day 4 of the instar, 2 to 3 days before gut purge. After the operation, the gland activity gradually increases and gut purge is induced. Subsequently, the activity sharply decreases to its lowest level before pupation. Although *in vitro* challenge by ecdysteroids failed to activate glands of feeding *Manduca* larvae, the composite data indicate that 20E causes stimulation of ecdysteroid production of glands when their secretory activity is as low as those of diapausing pupae, but the feedback activation of young last instar larval glands of relatively low secretory activity is conjectural.

In *M. configurata* pupae, injection of 20E (500 ng g^{-1} fresh weight) into postdiapause, pupae 1 day after transfer to 20 °C from 6 to 8 months of storage at 0 °C accelerates the increase in ecdysone concentration in the hemolymph in about 1 day and in the 20E concentration in about 2 days. In contrast, a higher 20E dose, as much as 2500 ng g^{-1}, suppressed the occurrence of both ecdysteroid peaks (Bodnaryk, 1986), as will be discussed below (see Section 3.8.3.2). In postdiapause pupae, ecdysone and 20E titers begin to increase 4 and 7 days after being transferred to 20 °C, showing that the glands 1 day after the transfer are apparently not yet activated, and therefore, indicates that the exogenous 20E stimulated the glands having very low or no secretory activity, as in the *H. cecropia* and *Manduca* diapausing pupal glands.

In diapausing pupae, either photoperiod induced or artificially induced by brain removal from nondiapausing pupae at pupation, adult development initiates spontaneously if it is not a deep diapause, as for the univoltine *H. cecropia*. In brainless pupae, there is no PTTH source, but in such *Manduca* pupae, ecdysone is the major ecdysteroid and [^{3}H]-cholesterol is incorporated into ecdysone at the beginning of spontaneous adult development, indicating that the prothoracic glands were activated in those pseudodiapausing pupae (Sakurai *et al.*, 1991). Since they lack a brain, the prothoracic glands may be activated by signals other than PTTH, possibly by the positive feedback of ecdysteroid that the prothoracic glands secreted. Although feedback activation has been recorded for pupal prothoracic glands, there is no evidence that such activation is responsible for the increase in ecdysteroid titer that triggers pupal–adult development.

3.8.3.2. Feedback Inhibition

Siew and Gilbert (1971) first showed the negative feedback regulation of prothoracic gland activity. This is more easily explainable than that discussed above, i.e., to downregulate the glands once they have secreted enough ecdysteroid to initiate the molting process. Later, feedback inhibition of the prothoracic glands was described in the large cabbage butterfly *Pieris brassicae* (Beydon and Lafont, 1983), *M. configurata* (Bodnaryk, 1986), and *M. sexta* (Sakurai and Williams, 1989; Song and Gilbert, 1998). In *Pieris* pupae, the inhibition of ecdysteroid biosynthesis by exogenous 20E is dose dependent (Beydon and Lafont, 1983). In *Manduca*, when prothoracic glands at peak activity 2 days after the onset of wandering were implanted into pupae from which the brain–corpora cardiaca–corpora allata complex and prothoracic glands had been removed, the 3-dehydroecdysone (see **Chapter 3.3**) secretory activity of the implanted glands decreased at the same rate as those in intact larvae. Similar inhibition is elicited by 20E *in vitro*, indicating that feedback inhibition solely accounts for the decrease of secretory activity after the peak of activity that occurs prior to pupation (Sakurai and Williams, 1989).

Feedback inhibition by 20E was demonstrated clearly using the prothoracic glands of day 5 *Manduca* larvae (Song and Gilbert, 1998). The glands were incubated in the presence or absence of 20E for various time periods and then incubated in medium alone for a further 1 h to assess the basal secretory activity (**Figure 2**). The basal level of ecdysteroid production was markedly inhibited by the 1 h incubation with 20E to approximately 25% of the control. When these glands were challenged with PTTH after exposure to 20E for 24 h, the enhanced level of ecdysteroidogenesis was far less than that of the control glands. Comparison of basal ecdysteroidogenesis with PTTH-stimulated activity after gland incubation for 6 h with 20E, showed that the glands were still capable of responding to PTTH, although the basal level was very low due to the inhibition by 20E. By contrast, after 24 h incubation in medium alone, the activation ratio (amount of ecdysteroids produced by one of a paired set of glands incubated with PTTH/amount of ecdysteroids produced by the contralateral gland incubated in medium alone) (Agui *et al.*, 1979) was 11 while it was 2.5 after the incubation with 20E. In addition, the basal secretory activity after the incubation with 20E for 6 h was similar to that after incubation in medium alone for 24 h, but the activation ratio for the former glands was 4.5 whereas that for the latter glands

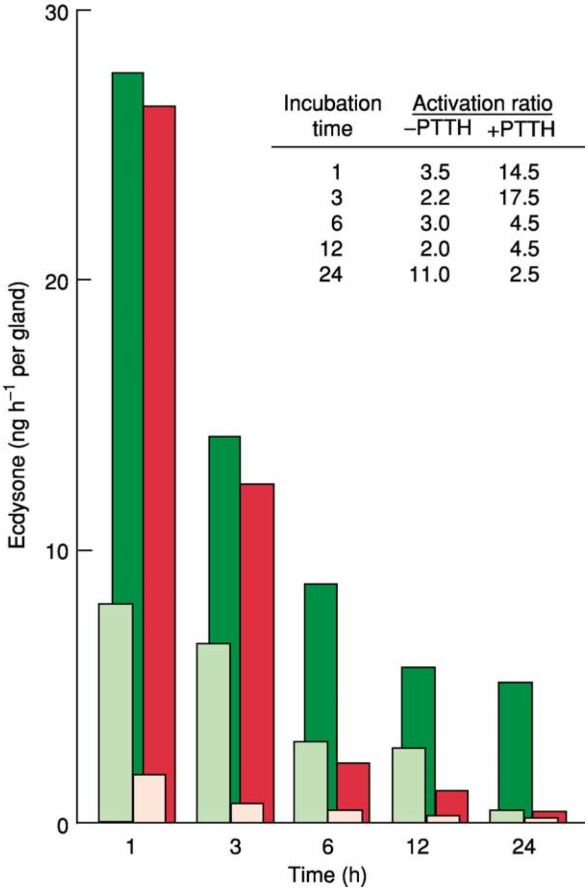


Figure 2 Inhibition by 20E of basal secretory activity and the responsiveness to PTTH of the prothoracic glands. One of a paired prothoracic glands of day 5 fifth instar *Manduca* larvae were incubated with 10 μM 20E (red and pink bars) or in medium alone (green and light green bars) for an indicated period and then incubated for another 1 h in a medium containing PTTH (red and green) or in medium alone (light green and pink) for assessing the basal secretory activity and the gland responsiveness to PTTH. Inset table: activation ratio (amount of ecdysteroids produced in the presence of PTTH/amount of ecdysteroids produced in medium alone) calculated from the paired values in the figure. Note that 20E suppresses the basal secretory activity at 1 h of incubation with 20E (light green vs. pink), while hardly affecting the PTTH-stimulated secretory activity (green vs. red). The latter is suppressed by a 6 h incubation with 20E. (Modified from Song, Q., Gilbert, L.I., **1998**. Alterations in ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol. 28*, 849–860.)

was 11, indicating that the 6 h incubation with 20E partially suppressed the gland responsiveness to PTTH. Thus, the prothoracic glands retained their competence to PTTH after a short incubation with 20E, suggesting that 20E simply suppressed the ecdysteroidogenic machinery, and that an exposure to 20E for a considerably longer period inhibited PTTH-stimulated ecdysteroidogenesis as well.

Feedback inhibition is known in crustaceans (see **Chapter 3.16**). The secretory activity of the Y-organs of the crayfish *Orconectes limosus* was greatly inhibited within 1 h after a single injection of 20E and slightly, but significantly, by a 1 h incubation with an ecdysteroid antagonist, RH-5849 (Dell *et al.*, 1999). This result is very similar to the *in vitro* feedback inhibition of *Manduca* prothoracic glands. They were markedly inhibited within 1 h by incubation with 20E (Song and Gilbert, 1998) or RH-5849 (Sakurai and Gilbert, unpublished data). Therefore, the feedback inhibition of the ecdysteroidogenic organs could be common to arthropods.

Ecdysone exerts feedback inhibition of *Manduca* day 3 fifth instar prothoracic glands *in vitro*, although the effect is faint (Sakurai and Williams, 1989). When the *Manduca* glands were inhibited by ecdysteroids (see Section 3.8.5.1), USP-1 and USP-2 proteins appeared in complementary patterns with USP-2 being predominant (see **Chapter 3.5**). Both suppression of USP-1 protein synthesis and simultaneous up-regulation of USP-2 synthesis are brought about by ecdysone and 20E. The effective concentration of ecdysone is, however, 100-fold as much as 20E (Song and Gilbert, 1998). This weak effect of ecdysone is consistent with the K_d of ecdysone for the nuclear receptor complex, which is lower than that of 20E by a factor of more than 100 (Cherbas *et al.*, 1980) (see **Chapters 3.5** and **3.6**). Since the prothoracic glands express no 20-monooxygenase activity mediating the conversion of ecdysone to 20E (Warren *et al.*, 1988), the result still indicates a weak action of ecdysone. Although ecdysone metabolites have been proposed to have morphogenetic roles of their own (Gilbert *et al.*, 2000; Gilbert, personal communication), no information is available on the possibility that 20,26-dihydroxyecdysone, the immediate metabolite of 20E, exerts feedback inhibition on prothoracic glands.

3.8.3.3. Complete and Incomplete Inactivation

There are two types of suppression of prothoracic gland secretory activity. One is incomplete shutdown, as seen at the ecdysis from the third (second penultimate) to the fourth (penultimate) instar and also at the end of the fifth (last) instar associated with the climax of pupal metamorphosis. In those periods, the gland activity declines greatly but still remains at a low level as the glands are still capable of secreting considerable amounts of ecdysone when examined *in vitro* (Okuda *et al.*, 1985; Bollenbacher *et al.*, 1987). In addition, they retain competence to respond to PTTH (Okuda *et al.*, 1985; Gu *et al.*, 2000; Takaki and Sakurai, 2003). By contrast, at the time of ecdysis from the penultimate to the last larval instar in *Bombyx*, gland activity is completely shut down for a period of 2–3 days (Okuda *et al.*,

1985; Gu *et al.*, 1997). In *Manduca*, the *in vitro* secretory activity of the glands on the second day of the fifth instar is 0.01–0.03 ng h^{-1} per gland (Smith and Pasquarello, 1989; Rybczynski and Gilbert, 1994) (see **Chapter 3.2**), indicating that activity is at an almost negligible level. In this stage in *Bombyx*, the glands are insensitive to PTTH. It is not clear if this insensitivity is due to the disappearance of the PTTH receptor from the gland cell membranes or a deficiency of one or more factors involved in the PTTH signaling. Whatever the cause, JH is involved in maintaining this inactivity. When the corpora allata are removed from newly molted *Bombyx* fifth instar larvae, the basal secretory activity of their glands reappears 9 h after the allatectomy. Three hours before this time, the glands exhibit a normal response to PTTH (Sakurai *et al.*, 1989a). This indicates that all the apparatus for ecdysteroidogenesis has been recovered before initiating spontaneous ecdysone secretion, and JH acts to maintain the suppressed condition (Takaki and Sakurai, 2003). Thus, feedback inhibition late in the penultimate instar may affect some steps of ecdysteroidogenesis and such a deficiency is maintained by JH. However, there is no information about whether the PTTH receptor itself has disappeared, either due to repression of its gene expression or it is simply sequestered away by the involution of the plasma membrane into the inside of the cells. Because dibutyryl cAMP and IBMX, a phosphodiesterase inhibitor, have lost their ability to enhance ecdysteroidogenesis at this stage (Gu *et al.*, 2000; Takaki and Sakurai, 2003), a simple deficiency of PTTH receptors can hardly account for the gland's inactivity.

3.8.4. Mechanisms of Decrease in Hemolymph Ecdysteroid Titer: Biochemical Aspects

3.8.4.1. Inactivation of 20-Hydroxyecdysone

There are at least two possible reasons for the decrease in the hormone titer, i.e., an increase in inactivation and excretion, and/or a decrease in hormone production. When ecdysone is injected into the body cavity of either a fifth (last) instar larvae or adult of *Locusta migratoria*, the ecdysteroid concentration at first increases, but promptly declines (Hoffmann *et al.*, 1974). The fate of the injected ecdysone is stage specific. On day 7 of the 11 day long instar when the hemolymph ecdysteroid titer is at its highest, ecdysone is promptly converted to 20E within 2 h and then is slowly excreted with the feces. In contrast, in either early or late fifth instar larvae or adults, the injected ecdysone is excreted promptly into the feces as is, or after conversion to inactivation products. This indicates that at the time of peak ecdysteroid titer, feedback inhibition by 20E may act to increase the excretion process, since at this time, 20-monooxygenase activity is maximal while the excretion rate is at its lowest. This results in the accumulation of 20E in the hemolymph.

In the metabolism of ecdysteroids, there are two immediate inactivation steps, epimerization and hydroxylation (see **Chapter 3.3**). Epimerization occurs at C3 to form 3-epiecdysteroid via an intermediate, 3-dehydroecdysteroid (Webb *et al.*, 1995). Since the hemolymph contains a high level 3-dehydroecdysone 3β-reductase activity (Sakurai *et al.*, 1989b; Kiriishi *et al.*, 1990), it is not possible to reduce 3-dehydroecdysteroid to 3-epiecdysteroid in hemolymph by the 3α-ketoreductase. Accordingly, inactivation of ecdysteroid through 3-epimerization is restricted only to tissues. Another immediate inactivation reaction of 20E, and probably the most important for 20E metabolism, is brought about by hydroxylation at the terminal carbon of the side chain to form 20,26-dihydroxyecdysone. This is further oxidized to the 26-oic acid and exhibits no biological activity. In the midgut cytosolic fraction prepared from feeding larvae of *Manduca*, the 26-hydroxylase is induced by 20E through the upregulation of its gene (Williams *et al.*, 1997). Although we have no information on 20,26-dihydroxyecdysone concentrations in larval hemolymph, it is highly possible that it achieves a maximum following that of 20E, similar to what occurs in *Manduca* pharate adults. Since the 26-hydroxylase is induced by 20E during the larval period, it would be of interest to examine the feedback effects of 20,26-dihydroxyecdysone on ecdysteroidogenesis. In *Manduca* pharate adults, the 20,26-dihydroxyecdysone concentration increases after the peak in the 20E titer, and becomes the sole free ecdysteroid metabolite among the various radioimmunoreactive steroids in the hemolymph at this stage (Warren and Gilbert, 1986). Accordingly, the upregulation of the 26-hydroxylase gene by 20E may be the ultimate cause of the decrease in the hemolymph 20E titer during the pupal–adult development. However, this may not be the sole reason for the decrease in 20E titer, since at this period, ecdysone secretion has almost ceased due to the degeneration of prothoracic glands, the result of programmed cell death induced by 20E (Dai and Gilbert, 1997, 1998).

3.8.4.2. Suppression of Ecdysteroidogenesis

There may be at least three different targets for the negative feedback induced by 20E in prothoracic gland cells: the enzymes of the ecdysone biosynthetic

pathway, the hypothetical system for the subcellular movement of intermediary ecdysteroid metabolites, and the signaling pathway for PTTH (**Figure 3**). Ecdysone synthesis is a complicated pathway and our knowledge is still rudimentary. We have extensive knowledge of only some of the steps occurring in the prothoracic glands (Gilbert *et al.*, 2002) (see **Chapter 3.3**). The first reaction is the dehydrogenation of cholesterol to 7-dehydrocholesterol by a P450 (CYP) enzyme in the endoplasmic reticulum (ER) (Grieneisen *et al.*, 1993; Warren and Gilbert, 1996). It is followed by a series of complex and uncharacterized oxidation reactions occurring in a location that is presently unknown, although it has long been thought to be the mitochondria in analogy with mammalian steroidogenesis (see below). The product of these reactions, an ecdysteroid precursor, is then hydroxylated at C25 by another CYP enzyme present in the ER (Kappler *et al.*, 1988), followed by additional hydroxylations at C22 and finally C2, both catalyzed by different CYP enzymes present in the mitochondria (Warren *et al.*, 2002) (see **Chapter 3.3**). The product, either 3DE and/or E (Warren *et al.*, 1988), is then secreted from the prothoracic gland cell by another unknown process and subsequently is taken up into peripheral tissues and converted to 20E by ecdysone-20-monooxygenase, another CYP enzyme variously localized in both the ER and mitochondria (Petryk *et al.*, 2003). 20E possibly affects one or more of these enzymes involved in the biosynthesis of ecdysone, but the effects of 20E on P450 activities have not yet been investigated.

Involvement of both the ER and mitochondria in ecdysone biosynthesis indicates that both cholesterol and ecdysone precursors must necessarily be conveyed between critical subcellular domains, including but not limited to the ER and the mitochondria, during ecdysone biosynthesis. Some of

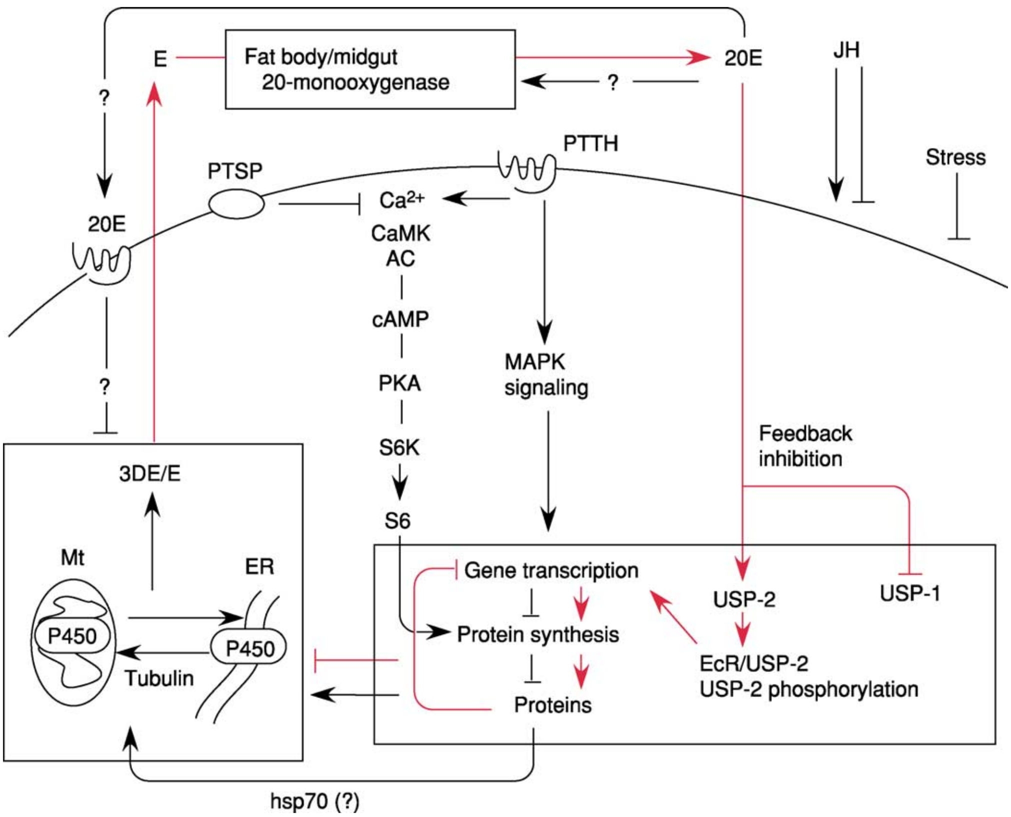


Figure 3 Feedback regulation of ecdysteroidogenesis in the prothoracic gland cell. Red lines indicate the ultra-short-loop feedback inhibition, which has been so far revealed. Arrowheads and ⊥ symbols indicate activation and inhibition, respectively. Note that 20E binds to USP-2 and activates EcR/USP-2 though the responsible isoform of EcR has not yet been identified. Then the nuclear receptor heterodimer acts as a transcription activator and the resulting proteins may suppress the genes whose products are involved in ecdysteroidogenesis (Song and Gilbert, 1998). See **Chapter 3.2**; for details of the PTTH signaling pathway and see **Chapter 3.3** for the ecdysone synthesis pathway. The membrane receptor for 20E is hypothetical. AC, adenylyl cyclase; cAMP, cyclic AMP; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; 3DE, 3-dehydroecdysone; E, ecdysone; ER, endoplasmic reticulum; MAPK, mitogen activated protein kinase; MT, mitochondrion; PKA, cAMP-dependent protein kinase; PTSP, prothoracicostatic peptide; PTTH, prothoracicotropic hormone; S6, ribosomal protein S6; S6K, S6 kinase.

these sterol translocations may be rate limiting in *Manduca* and *Drosophila* ecdysone synthesis (Warren and Gilbert, 1996; Watson *et al*., 1996), and therefore, a possible target of negative feedback regulation. In mammalian steroidogenic cells, the primary substrate, cholesterol, is transported from its intracellular reservoirs, the cell membrane and/or the ER, first to the outer mitochondrial membrane and then to the CYP enzyme present in the inner mitochondrial membrane (P450ssc), perhaps with the aid of the cytoskeleton and/or various "translocation" factors, and these processes are accelerated upon stimulation by a tropic hormone (Hall, 1995, 1997). In *Manduca* prothoracic glands, PTTH stimulates the synthesis of β-tubulin, which is rapid and transitory, suggesting a direct role of microtubules in the upregulation of ecdysteroidogenesis by PTTH (Rybczynski and Gilbert, 1995a) (see **Chapter 3.2**). Indeed, microtubule-disrupting drugs inhibit PTTH-stimulated ecdysteroidogenesis in the pupal prothoracic glands (Watson *et al*., 1996). Although the drugs do not inhibit the basal secretory activity of pupal glands, nor PTTH-stimulated larval glands (Gilbert *et al*., 2002), the turnover of cytoskeletal proteins is a possible target of acute feedback inhibition, since vertebrate and insect steroidogenic cells are surprisingly homologous in the mechanisms that regulate the changes in steroidogenesis (Gilbert *et al*., 2002).

A third possibility includes factors that modulate or control, directly or indirectly, the PTTH signaling pathway (see **Chapter 3.2**). PTTH stimulation is known to require *de novo* gene expression and protein synthesis (Keightley *et al*., 1990; Rybczynski and Gilbert, 1994), and the latter may be mediated by S6 kinase (S6K) which mediates ribosomal S6 phosphorylation (Song and Gilbert, 1994, 1995, 1997). Circumstantial evidence deduced from time-course studies indeed shows that PTTH-stimulated S6 phosphorylation is involved in the upregulation of ecdysteroidogenesis. Since S6K activates several transcription factors, including c-Jun and Atf-2, PTTH signaling could involve activation of the transcription factors. Interestingly, the dephosphorylation of S6 protein occurs in temporal synchrony with the decline in ecdysone biosynthesis (Song and Gilbert, 1995), indicating that the feedback inhibition of ecdysteroidogenesis does not necessarily involve the direct inhibition of the enzymes in the ecdysone biosynthetic pathway. Rather, the regulation of the synthesis of proteins that control such enzyme activities could be the primary target of negative feedback regulation by 20E. In PTTH-stimulated prothoracic gland cells, hsp70 synthesis is also upregulated (Rybczynski and Gilbert, 1995b, 2000), implying its participation in feedback control of ecdysteroidogenesis by the modulation of the assembly of the ecdysteroid receptor complex. In this regard, USP proteins are suggested to exert important roles in the negative feedback (Gilbert *et al*., 1997) (see Section 3.8.5). Although not yet identified, molecular chaperones besides hsp70 could also be involved in modulating the rate of ecdysteroidogenesis by assisting the movement into the mitochondria of catalytic enzymes synthesized in the cytosol.

3.8.5. Molecular Mechanism of Feedback Inhibition

3.8.5.1. Feedback Mediated by the EcR/USP Heterodimer

In the feedback inhibition of prothoracic glands, the primary action of 20E is most likely mediated by a heterodimeric nuclear receptor composed of the ecdysone receptor (EcR) and Ultraspiracle (USP) (see **Chapters 3.5** and **3.6**). In this interaction, the USP isoform is of primary importance (Gilbert *et al*., 1997; Song and Gilbert, 1998) (**Figure 3**). Developmental profiles of two USP isoforms in the prothoracic glands of *Manduca* last instar larvae revealed that USP-1 and USP-2 are expressed in complementary patterns. At first, USP-1 is predominant while USP-2 is barely detectable until day 6 at which time the hemolymph ecdysteroid level only begins to rise. After day 6, USP-2 is upregulated and expressed maximally on days 7–9, i.e., during the peak in the ecdysteroid titer, while USP-1 is simultaneously downregulated to almost undetectable levels. The net result is that USP-2 comprises about 85% of the total USP proteins (Gilbert *et al*., 1997). The downregulation of USP-1 and simultaneous upregulation of USP-2, both being elicited by 20E, may alter a set of genes that ultimately leads to downregulation of ecdysteroidogenesis. *In vitro* incubation with 20E inhibits gland activity (**Figure 2**). Incubation of day 5 prothoracic glands with 20E reduced the basal secretory activity in 6 h to one-sixth that of the control glands incubated in medium alone. During the 6 h incubation with 20E, USP-1 is downregulated while USP-2 is upregulated. In day 7 glands, only the EcR/USP-2 heterodimer is present, but not EcR/USP-1, indicating that USP-2 becomes the predominant component of the receptor complex in response to a high ecdysteroid titer, forming EcR/USP-2, presumed to be functional in feedback inhibition (Song and Gilbert, 1998). After the binding of 20E to EcR, the functional heterodimer probably leads to the downregulation of the expression of one or more critical

components of the ecdysteroidogenic pathway (Gilbert *et al.*, 1997). 20E is more effective than ecdysone by a factor of 100 in eliciting the expression of USP-2 in day 5 prothoracic glands. This difference in activity coincides well with the difference in binding affinities of these two ecdysteroids for the EcR/USP complex (Cherbas *et al.*, 1980; Minakuchi *et al.*, 2003) (see **Chapter 3.5**). Such a difference is reflected in the activities of ecdysone and 20E in the *in vitro* feedback inhibition of the prothoracic glands (Sakurai and Williams, 1989). Two EcR isoforms, EcR-A and EcR-B1, have been identified in Lepidoptera. However, it remains to be seen which isoform participates in forming the functional EcR/USP-2 heterodimer and whether the EcR/USP-2 heterodimer is the primary transcriptional factor providing for the downregulation of USP-1.

Although incubation of day 5 *Manduca* prothoracic glands with 20E for 6 h markedly reduces the basal secretory activity, they still retain their responsiveness to PTTH, e.g., as PTTH elicits an activation ratio of 5 (**Figure 2**). This activation ratio, however, decreases from approximately 15 after 1 h incubation with 20E, to 5 after 6 h, indicating that 20E does affect the PTTH signaling pathway. The complementary patterns with USP-2 being predominant during the incubation with 20E could cause the loss of sensitivity to PTTH (Song and Gilbert, 1998), and thereby act to sustain the inhibited state of ecdysteroidogenesis.

It is of interest that the developmental profiles of individual USP isoform expression in epidermis are quite similar to those in the prothoracic glands (Hiruma *et al.*, 1999). USP-1 mRNA levels are high during the feeding period, but decline to low levels shortly after the onset of the wandering stage when the hemolymph ecdysteroid titer begins to increase. In contrast, the USP-2 mRNA level remains low until the increase in ecdysteroid titer, after which its expression is enhanced. A similar control over the expression of USP isoforms by 20E is reported in the adult mosquito *Aedes aegypti* (Wang *et al.*, 2000). After a blood meal, the fat body initiates the production of yolk protein (vitellogenin), needed for egg maturation, in response to 20E originating from the ecdysone secreted by the ovaries that are stimulated by the blood meal (see **Chapter 3.9**). USP-A (*A. aegypti* homolog of USP-1) is downregulated at the time of the increase in 20E titer, while USP-B (homolog of USP-2) is upregulated. Incubation of the fat body with 20E results in the upregulation of USP-B while USP-A transcription is downregulated. EcR/USP-B elicits a stronger transactivating activity on the vitellogenin gene than does EcR/USP-A. The function of the receptor complex, of course, varies among the tissues that exhibit tissue-specific responses to 20E, e.g., the feedback inhibition of ecdysteroidogenesis in the prothoracic glands, the triggering of pupal differentiation in the epidermis, and yolk protein expression in the ovaries. However, the fundamental molecular mechanisms underlying the control of gene expression by EcR/USP could be similar in individual tissues.

We presently have no information to speculate about whether feedback inhibition affects one or more of the enzyme activities involved in the ecdysone biosynthetic pathway. A decrease in ecdysone production may be as important as an increase with respect to the regulation of insect growth and development, but little is known at present about feedback regulation at either the biochemical or molecular levels.

3.8.5.2. Acute and Delayed Inhibition

Incubation of day 5 fifth instar *Manduca* prothoracic glands with 20E for 1 h strongly suppresses the basal secretory activity to about 20% of the control, but the glands retain their competence to respond to PTTH (Song and Gilbert, 1998) (**Figure 2**). Similarly, RH-5849 significantly suppresses the secretory activity within 10 min while retaining sensitivity to PTTH (Sakurai and Gilbert, unpublished data). Acute inhibition is also reported in the crayfish *Orconectes limosus*, in which the Y-organ is the ecdysteroidogenic gland, and 20E injection to the crayfish larvae inhibits the gland activity. Ecdysteroid release from intermolt Y-organs is reduced sixfold within 1 h after 20E injection (Dell *et al.*, 1999). In *Manduca* prothoracic glands, the complementary pattern of USP-1 and USP-2 expression appears only after a 6 h of incubation with 20E, but nothing is observed after only 1 h (Song and Gilbert, 1998). In an injection study in *Orconectes*, the degree of inhibition caused by RH-5849 at its minimum effective dose of 5 μg is less than that resulting from 0.25 μg 20E. Thus, RH-5849 is at least 20 times less effective than 20E. The binding affinity of 20E for the *Chironomus tentans* EcR/USP complex is in the range of 10^{-7} M ($K_d = 230$ nM), and is similar to that of RH-5849 ($K_d = 278$ nM) (Smagghe *et al.*, 2002) (see **Chapter 3.5**). The binding affinities of 20E and RH-5849 to the *in vitro* translated EcR/USP complex of *Chilo suppressalis* shows that 20E affinity is only threefold higher than RH-5849 (Minakuchi *et al.*, 2003). However, it is not obvious whether the direct application of this K_d data to *Orconectes* is appropriate, since the binding affinity of some ecdysteroid agonists differs greatly in different orders of insects, e.g., the binding affinity of RH-2485, another potent nonsteroidal ecdysone

agonist, for crude nuclear extracts is 70-fold higher than that of 20E in the moth *Plodia interpunctella*, while in the beetle *Anthonomus grandis*, the former value is less than the latter by a factor of 50 (Dhadialla and Tzertzinis, 1997). Nevertheless, in *Chironomus*, *Chilo*, and *Orconectes*, in spite of the similarity in the affinities of 20E and RH-5849 to the EcR/USP complex, 20E is 20-fold more effective than RH-5849 in inhibiting ecdysone secretion, indicating that the acute inhibition may not be due to a genomic action of 20E. Hence, the acute inhibition of basal secretory activity indicates that the rapid feedback inhibition of ecdysteroid secretion might be mediated by a nongenomic pathway, rather than a conventional genomic pathway. The acute inhibition does not, however, suppress the prothoracic gland responsiveness to PTTH (Song and Gilbert, 1998).

The above discussion gives rise to a more complex model for the role of feedback inhibition. There may be two different signaling pathways underlying the feedback inhibition; one is acute and the other is delayed. Acute inhibition may result in the rapid decline of ecdysone secretion after the physiological peak concentration of 20E is attained in the hemolymph and is probably mediated by a nongenomic action of 20E. After this acute inhibition, a second genomic action via the EcR/USP-2 heterodimer ensures that the glands will not produce ecdysone again once the 20E titer declines below the threshold level for the acute feedback inhibition. Thereby, the glands are maintained inactive until the next PTTH stimulation. During this period with low secretory activity, developmental events are allowed to occur in response to a declining ecdysteroid concentration.

3.8.6. Horizontal Control of the Prothoracic Gland

20E also affects CA activity while JH alters prothoracic gland ecdysteroidogenic activity (see **Chapter 3.7**). JH is believed to elicit the control of prothoracic gland activity in two ways: one through the control of PTTH release and the other by a direct effect on the glands, although the evidence for this is not yet firm. In addition, JH effects, at least in the last larval instar, change from a primarily static response to a tropic response on the brain–prothoracic gland axis at a time shortly before the onset of wandering (Hiruma *et al*., 1978; Cymborowski and Stolarz, 1979; Safranek *et al*., 1980). In this section, JH effects on PTTH secretion from the brain and ecdysone secretion from the prothoracic glands will be discussed. The effect of ecdysteroids on CA secretory activity is detailed elsewhere (see **Chapter 3.7**).

3.8.6.1. Effects of JH on PTTH Secretion

Injection or topical application of JH or its analogs methoprene (effective in *Bombyx*) or hydroprene (effective in *Manduca*), at an appropriate period during the feeding stage of the last larval instar of Lepidoptera, delays the onset of the prepupal period in *Manduca* (Nijhout and Williams, 1974b; Safranek *et al*., 1980; Rountree and Bollenbacher, 1986; Watson and Bollenbacher, 1988), *Spodoptera littoralis* (Cymborowski and Stolarz, 1979), and *Bombyx* (Akai *et al*., 1973; Sakurai and Imokawa, 1988; Gu *et al*., 1997). In *Manduca*, the prothoracic glands from hydroprene- or JH I-treated larvae are capable of responding to PTTH *in vitro*. Therefore, the delay is supposed to be due to the inhibition of PTTH release from the brains (Rountree and Bollenbacher, 1986). In *Bombyx*, JH analog (JHA) application before day 3 of the fifth instar results in a delay of the onset of wandering, while its application on day 3 produces dauer (permanent) larvae that never undergo metamorphosis, but die after surviving for more than 2 weeks (Akai *et al*., 1973; Sakurai and Imokawa, 1988). In such larvae, the prothoracic glands retain their sensitivity to PTTH, when assessed by an *in vitro* PTTH challenge. In addition, PTTH injection 10 days after the JHA treatment restores spinning behavior and is followed by normal pupation, just as if the days between the JHA treatment and PTTH injection did not exist (Sakurai and Imokawa, 1988). These data indicate that JHA can disturb the program of PTTH secretion in the brain if JHA is applied at a critical time point in the fifth instar.

If JH suppresses PTTH release, then what is the effect of JH on the brain–prothoracic gland axis in the penultimate instar when the JH titer is high? Removal of the corpora allata from early penultimate instar larvae results in the induction of precocious pupation. In *Mamestra* and *Bombyx*, the hemolymph ecdysteroid titer peaks on day 3 of the fourth instar and induces larval ecdysis. Allatectomy abolishes the appearance of the ecdysteroid peak and the ecdysteroid titer remains at low levels for several days until the precocious onset of wandering (Hiruma, 1986; Ohtaki *et al*., 1986). The prothoracic glands, after allatectomy, retain their responsiveness to PTTH, indicating that the decline of the JH titer after allatectomy may act on the brain to alter the PTTH secretion program for larval ecdysis. JH application to the allatectomized larvae increases the hemolymph ecdysteroid concentration (Ohtaki *et al*., 1986), as well as the basal secretory activity of the prothoracic glands within 24 h of the JHA treatment (Sakurai, unpublished data). In

addition, the brains of early penultimate instar larvae of *M. brassicae* contain higher PTTH activity than do those of allatectomized larvae (Hiruma, 1986). This may be due to an accumulation of PTTH in the brain, the result of a declining JH titer suppressing PTTH release. These observations suggest that in the penultimate instar, JH may serve to maintain the brain to produce and/or release PTTH, and the brain ceases PTTH release in the absence of JH in the hemolymph. A similar situation is found in the late feeding stage of the last larval instar of *Manduca* and *Mamestra*. Allatectomy delays their pupation by delaying the appearance of the ecdysteroid peak in the prepupal period (Hiruma, 1986; Sakurai, 1990). This could be interpreted as a result of a delay in PTTH release due to low JH titer, although there are no data documenting either the release of PTTH from the brain, nor the PTTH titer in the hemolymph. Only one instance of the direct measurement of PTTH in the hemolymph is available to assess these effects of JH (Mizoguchi, 2001). On days 0–2 of the last larval instar in *Bombyx*, topically applied JH I increases the PTTH concentration in hemolymph significantly. When day 0 larvae were treated with 3 μg JH I, the PTTH titer increased from 28 to 50 pg ml^{-1} in 12 h. Whether or not this increase is of significant biological meaning, it is at least clear that JH does not inhibit PTTH secretion, even in early fifth instar. This is contrary to the data of Rountree and Bollenbacher (1986) who showed that JH inhibits PTTH release in feeding last instar larvae in *Manduca*. Rather, JH seems to act as a positive regulator of brain PTTH secretion, as has been proposed in *Galleria mellonella* (Sehnal *et al*., 1981). Then, how can we interpret the earlier observations that JH induces dauer larvae or causes a delay in the onset of wandering if applied early in the fifth instar? Induction of dauer larvae require an injection of 10 μg JH I (Akai and Kobayashi, 1971) or 1 μg methoprene (Sakurai and Imokawa, 1988). However, the highest titer of JH in the last instar is less than 10 ng JH I equivalent ml^{-1} (Fain and Riddiford, 1975; Baker *et al*., 1987; Niimi and Sakurai, 1997), which indicates that although the dose of JH is pharmacological, JH nevertheless disturbs the PTTH release program in the brain, directly or indirectly, through an unknown nervous network participating in the control of PTTH cells. Apart from the effects of JH on PTTH release, it is obvious that the brain also responds to an increase and decrease in both ecdysteroid and JH. Quoting Gilbert *et al*. (2000), "ecdysteroids and JHs interact and we cannot study one without considering the other." Ecdysteroids and JH must affect the production and release of PTTH, although the role of JH on the brain is still obscure due to the lack of *in vitro* information on PTTH secretion from the brain.

3.8.6.2. Effects of JH on Ecdysteroidogenesis

Effects of JH on ecdysteroidogenesis are somewhat complicated and not yet understood. JH stimulation of prothoracic gland activity was first demonstrated in diapausing pupae of *H. cecropia*; JH injection broke the diapause (Gilbert and Schneiderman, 1959; Williams, 1959). However, the brain may not be involved in the diapause-terminating effects of JH, since JH effects were observed in pupae whose brains had been removed prior to JH treatment (Gilbert and Schneiderman, 1959). This indicates that the brain of a diapausing pupa is not the primary target of the applied JH. Rather, JH appears to directly stimulate the pupal prothoracic glands.

In *H. cecropia* diapausing pupae (Gilbert and Schneiderman, 1959), JH probably stimulated prothoracic glands to secrete ecdysone so that a positive feedback loop was initiated, operating in a manner similar to that demonstrated for the *Manduca* pupal prothoracic glands (Sakurai and Williams, 1989). In *Manduca* pupae, ecdysteroids stimulate prothoracic glands of diapausing pupae, but also those in a very early nondiapausing pupal stage. Although JH stimulation of pupal prothoracic glands has been demonstrated *in vivo*, JH has not been detected in the pupal stage, at least before degeneration of the prothoracic glands. Thus, JH stimulation of the pupal glands cannot occur in the normal insect life cycle.

In the last larval instar of Lepidoptera, JH delays the increase in the hemolymph ecdysteroid titer when topically applied to prewandering larvae. However, its effect on the steroid titer changes from inhibitory to stimulatory shortly before or at the wandering stage (Hiruma *et al*., 1978; Safranek *et al*., 1980; Sakurai, 1990). In the prepupal stage, JH has been considered to have a stimulatory effect on prothoracic glands. The hemolymph JH titer begins to increase concomitantly with the onset of wandering and reaches a broad peak at the midpoint of the prepupal period, after which it decreases towards pupation (Baker *et al*., 1987; Niimi and Sakurai, 1997). Removal of the corpora allata from the last instar larvae during the prewandering stage results in a delay of pupation in *Mamestra* and *Manduca* (Hiruma, 1980; Hiruma *et al*., 1999), indicating that the JH peak may affect ecdysteroidogenesis. Neck ligation of *Manduca* feeding larvae on the day of wandering suppresses pupation, and JHA (hydroprene) application restores the delay (Safranek *et al*., 1980). The ligation suppressed the

increase in hemolymph ecdysteroid titer after wandering, while the administration of JHA to those larvae increased these levels to that found in intact larvae. However, *in vitro* treatment of wandering larval glands with JHA fails to stimulate gland activity (Sakurai, 1990).

In the tropical moth *Omphisa fuscidentalis*, larvae enter diapause at a time after the cessation of feeding, but before gut purge, i.e., probably at the wandering stage. Larval diapause lasts for 9 months, during which the secretory activity of prothoracic glands is detectable (Singtripop *et al*., 1999, 2000). Nevertheless, the hemolymph ecdysteroid titer remains at low levels (less than 10 ng ml^{-1}) in the first half of the diapause period, indicating that there is no feedback activation in spite of the satisfaction of a circumstantial requirement, i.e., low secretory activity of the glands and low ecdysteroid titer driving positive feedback. Topical application of JHA (methoprene) to those larvae breaks the diapause with associated increases in ecdysteroid titer and prothoracic gland secretory activity. This activation by JHA is also seen in larvae whose brains have been removed prior to JHA treatment, indicating that JHA does not stimulate brain PTTH release. Transplantation of the prothoracic glands of JHA-treated larvae to nontreated larvae breaks the diapause of the recipients. Successive transplantation after JHA treatment showed that JHA stimulation for only 1 day was enough to stimulate the prothoracic glands (Singtripop and Sakurai, unpublished data), but gland activity began to increase only 7 days after JHA treatment (Singtripop *et al*., 2000). What occurred in the glands during the 6 days immediately after JHA stimulation? During the diapause period, the prothoracic glands do not respond to either circulating ecdysteroids or the application of exogenous 20E (Singtripop *et al*., 2002). Second, JHA may not stimulate the glands directly. Thus, an assumption can be made that the glands are suppressed to respond to feedback activation by ecdysteroids, and JH may release the glands from such suppression. Such glands are capable of responding to ecdysteroids very gradually because the initial ecdysteroid titer is fairly low. This seems to account for the above-mentioned disagreement of *in vivo* and *in vitro* results for the effects of JHA on prothoracic glands in the prepupal period in *Manduca* and *Mamestra*. JH may act on prothoracic glands as a modulator of feedback activation in the wandering stage and the following prepupal period, and therefore JH application restores the increase of hemolymph ecdysteroid titer in the neck ligated larvae but cannot stimulate the glands *in vitro*.

In the last larval instar before the onset of wandering, prothoracic glands are suppressed by JH, but there have been no *in vitro* data, except for experiment using *Drosophila* ring glands (Richard and Gilbert, 1991). Both juvenile hormone bisepoxide (JHB_3), a *Drosophila* JH, and JH III well inhibit the ring glands, but the effective concentration is in the mM range, a rather pharmacological concentration. Application of hydroprene, an effective JH analog in *Manduca*, to larvae before the onset of wandering inhibits the increase in the hemolymph ecdysteroids that normally occurs with wandering, and the prothoracic gland activity as well (Rountree and Bollenbacher, 1986; Sakurai, 1990). In *Bombyx* feeding larvae, JHA application on day 3 of the fifth instar induces dauer larvae although the prothoracic glands after JHA treatment exhibit a low, but significant secretory activity that remains at low levels for as long as the larvae survive. By contrast, the gland activity in larvae whose brains have been removed increases autonomously after the operation to a degree sufficient to induce pupation (Sakurai and Imokawa, 1988). This indicates that JHA applied on day 3 might suppress the glands ability to respond to feedback activation, in addition to disturbing the PTTH secretion program of the brain discussed below (see Section 3.8.6.3). Thus, the switchover of the JH effects on prothoracic glands, occurring around the time of wandering, does not mean the switching from direct suppression to activation by JH. Rather, it may be caused by a change in the gland's responsiveness to feedback regulation. After wandering, JH supports feedback activation and/or interferes with feedback inhibition, although JH may directly suppress the glands before wandering, if the ecdysteroid titer is low.

There is no information at the molecular level on the effects of JH on prothoracic glands although JH modulates the 20E-inducible EcR and USP gene expressions in the *Manduca* epidermis (Hiruma *et al*., 1999). In day 2 fourth instar larval epidermis, USP-1 is downregulated by 20E, while USP-2 is upregulated. Coexistence of JH I with 20E suppresses the downregulation of USP-1 expression, indicating that JH may act antagonistically against 20E. USP is suggested to bind JH and act as a JH nuclear receptor (Jones and Sharp, 1997; Jones *et al*., 2001) (see **Chapter 3.7**), although the binding affinity of JH to USP is very low, and therefore USP is not yet accepted as a true JH nuclear receptor. JH is reported to activate EcR-dependent transcription in Chinese hamster ovary cells (Henrich *et al*., 2003). 20E activated the reporter gene expression in Chinese hamster ovary cells transfected with plasmids carrying a *Drosophila* EcR gene, a USP

gene, and an ecdysteroid-inducible reporter gene with five ecdysone response elements. In this system, the additional presence of JH III enhanced the response. The effective concentration of JH III was 40 μM (10 600 ng ml^{-1}), which is much higher than the JH concentration in larval hemolymph so far reported as less than 10 ng ml^{-1}, and thus the effect of JH III is only pharmacological. Accordingly, it is not known for sure if JH interacts directly with the EcR/USP heterodimer *in situ* to modulate the function of 20E-EcR/USP, but those reports indicate that JH is at least capable of binding to EcR/USP. If this is the case in the prothoracic glands, then JH may modulate the action of 20E by binding to USP-2 and reducing the feedback inhibition by 20E-EcR/USP-2, at least, in the prepupal period when the 20E titer is high enough to induce the complementary expressions of USP-1 and USP-2. If so, the delay in pupation caused by removal of corpora allata at the wandering stage could be interpreted as implying that JH in the prepupal period suppresses the feedback inhibition by suppressing the USP-2 expression by 20E, so that the glands secrete ecdysteroids until the peak titer. In the absence of JH, ecdysteroidogenesis is suppressed by 20E, resulting in an ecdysteroid titer that does not increase. The result is a delay in pupation. This interpretation of JH interaction with USP appears to account well for the fact that the USP-2 upregulation in *Manduca* prothoracic glands occurs only 1 day before the peak in ecdysteroid titer. In the prepupal period, the threshold concentration of 20E needed to induce USP-2 in the presence of JH may be higher than that in the absence of JH, and it is therefore of interest to examine the effects of JH on USP gene expression in the prothoracic glands.

3.8.6.3. Growth-Blocking Peptide–Corpora Allata–Prothoracic Gland Pathway

Endocrine control of larval growth could be more complicated than previously considered. There is another factor, besides PTTH, 20E and JH, that is indirectly involved in the control of ecdysteroidogenesis. The factor is growth-blocking peptide (GBP), an insect cytokine, which was first identified from the hemolymph of lepidopteran larvae that had been parasitized by a wasp (Hayakawa, 1991). When last (sixth) instar larvae of the armyworm *Pseudaletia separata* were parasitized by the parasitoid wasp *Cotesia kariyai*, larval growth was delayed so that the parasitoid wasp larvae had sufficient time to complete larval growth inside the host larvae and escape from them in order to pupate. The GBP concentration in the hemolymph is high in the penultimate instar and gradually declines early in the last larval instar. During normal growth, JH esterase in the hemolymph increases in early last instar larvae (Sparks *et al.*, 1983; Baker *et al.*, 1987), and the GBP concentration decreases concomitantly with the increase in esterase activity (Ohnishi *et al.*, 1995). GBP is produced by the larval fat body and acts to suppress JH esterase gene expression. Accordingly, suppression of GBP expression results in increased esterase activity, leading to a decline in JH concentration in the hemolymph. In the parasitized larvae, the GBP gene is activated by parasitization, and the elevated GBP suppresses the decline of JH titer by repressing the JH esterase gene (Hayakawa, 1990), which causes the hemolymph JH titer to remain high and thereby suppress the reactivation of the prothoracic glands. Thus, the parasite delays the growth of the host larvae. GBP also acts on epidermal cells by upregulating the DDC gene, resulting in an enhanced production of dopamine (Noguchi and Hayakawa, 1996; Noguchi *et al.*, 2003). Dopamine is released into the hemolymph and acts to enhance JH secretion from the corpora allata in early last instar larvae (Granger *et al.*, 1996) (see **Chapter 3.7**). These all figure in the sequential control of prothoracic gland activity (**Figure 1**). In the early last larval instar, the GBP gene is downregulated, although the mechanism is not known, resulting in a decrease in hemolymph GBP concentration. This decrease causes a downregulation of the DDC gene and results in a low dopamine concentration in the hemolymph. Although dopamine may not be the sole factor to maintain activity in the corpora allata, nevertheless, this activity may decline due to a low dopamine concentration. Simultaneously, JH esterase activity increases and as a result, the JH titer declines sharply, although it remains to be seen whether dopamine acts directly to suppress esterase gene expression or if the decreasing GBP titer directly elicits the upregulation of the JH esterase gene in the fat body. In addition to the GBP pathway, 20E is known to maintain or even stimulate the JH synthetic activity of the corpora allata (Whisenton *et al.*, 1987a, 1987b; Gu and Chow, 1996). Accordingly, the extremely low ecdysteroid titer in the early fifth instar (Bollenbacher *et al.*, 1981; Sakurai *et al.*, 1998) also contributes to the decrease in activity in the corpora allata. Finally, the declining JH titer releases the prothoracic glands from their suppression by JH and allows them to begin ecdysteroidogenesis, as well as to respond to PTTH (Sakurai *et al.*, 1989a). Because GBP is tightly involved in the changes in JH titer, the GBP–JH–prothoracic gland axis could be of importance in the control of prothoracic gland activity and therefore in

the hormonal control of insect growth and development. Since the parasitization of *P. separata* by the parasitoid *A. kariyai* appears to affect the host brain in a manner to suppress the PTTH release (Tanaka *et al.*, 1987), GBP could be affecting the brain PTTH cells.

There are conditions other than parasitization that delay larval growth, including low temperature (Malá *et al.*, 1987; Ohnishi *et al.*, 1995), insufficient food supply (Bounhiol, 1938; Nijhout and Williams, 1974a; Morita and Tojo, 1985; Miner *et al.*, 2000), and injury (O'Farrell *et al.*, 1960; Kunkel, 1977; Malá *et al.*, 1987). When last instar *P. separata* larvae are transferred from 25 to 10 °C, pupation is delayed by 11 days, and after the transfer, the GBP concentration is elevated and maintained at high levels for a certain period (Ohnishi *et al.*, 1995), indicating that the cold-stress suppressed, or even upregulated, the GBP gene in the fat body, which probably led to the delayed activation of prothoracic glands through a suppression of the decline of JH titer. Chilling of last instar *G. mellonella* larvae elicits an extra larval molt or a delay of pupation (Malá *et al.*, 1987). It is of interest that GBP is involved in the delay of larval growth under certain stressed circumstances. In the starved larvae, the feedback regulation of prothoracic gland activity seems to be more complicated. Starvation in last instar larvae of *Manduca* results in the maintainance of a high level of hemolymph JH, rather than the decline observed in normal feeding larvae (Cymborowski *et al.*, 1982), apparently due to the suppression of the decline in hemolymph JH esterase activity. Thus, the resulting high JH level may modulate the brain–prothoracic gland axis as mentioned above. This could be accounted for by the suppression of GBP expression that normally ceases after the last larval molt. However, suppression of larval growth by starvation is not simply caused by the suppression of prothoracic gland activity. Starvation suppresses the release of growth factors, like bombyxin and IDGFs, which are needed for normal growth. Nevertheless, 20E is required simultaneously to sustain larval growth (Nijhout and Grunert, 2002). Accordingly, the horizontal regulation of prothoracic glands appears to participate in helping the animal survive unexpectedly severe circumstances.

For more than four decades, injury has been recognized to delay larval growth, known as the injury effect. Following an operation such as the sham operation for allatectomy or autotomy of larval legs, the larval or even pupal period is usually prolonged by one or more days over that of intact animals. Similar sham operations on the last instar larvae of *G. mellonella* either prolongs the intermolt periods or produces supernumerary larvae. The injury is supposed to stimulate JH production (Malá *et al.*, 1987). In the sixth instar larvae of the cockroach *Blatta orientalis*, autotomy results in a delay of ecdysis by 4 days and also delays the appearance of the hemolymph ecdysteroid peak by 4 days (Kunkel, 1977), indicating that injury alters the ecdysone secretion program by affecting the brain–prothoracic gland axis. These two results are well explained by the operation of the GBP pathway (**Figure 1**). Injury elicits an increase in GBP titer, which stimulates the corpora allata to produce JH and also suppresses JH esterase expression, resulting in a high JH titer. The high titer suppresses the brain–prothoracic gland axis, delaying the appearance of the peak ecdysteroid titer. As a result, injury either delays ecdysis or induces supernumerary larvae. Thus, it seems highly possible that the humoral factor induced by injury is GBP, and that the GBP system is the cause for the delay in development.

3.8.7. Role of Feedback Regulation in Larval Growth and Metamorphosis: Meaning of the Shapes of the Ecdysteroid Peak

In contrast to the pupal (pharate adult) stage, during which the prothoracic glands degenerate and are unable to produce additional ecdysone, at the end of every larval stage including the last larval instar, gland activity decreases to a very low level. However, the degree of inactivation achieved is different at the end of each of at least the third, fourth, and fifth (last) instars of *Manduca* (Bollenbacher *et al.*, 1987; Smith and Pasquarello, 1989; Rybczynski and Gilbert, 1994; Smith, 1995) and *Bombyx* (Okuda *et al.*, 1985; Sakurai *et al.*, 1989b; Gu *et al.*, 1996, 2000; Takaki and Sakurai, 2003). Therefore, the mode of feedback inhibition of prothoracic glands may change developmentally.

When the ecdysteroid titer peaks occurring at the end of the third, fourth, and fifth larval instars and pupal stage are compared to each other, it is easily recognized that the peak titer, as well as its width, are developmental stage specific in *Manduca* (Bollenbacher *et al.*, 1981) and *Bombyx* (Kiguchi and Agui, 1981; Mizoguchi *et al.*, 2001; Takaki and Sakurai, 2003). In *Bombyx*, the peak width at the point of 50% peak height in the third, fourth, and fifth instars and pupal stage are 7 h (peak value of 1.2 μM), 9 h (1 μM), 18 h (3.2 μM), and 24 h (15.4 μM), respectively. The duration of the period with the titer above 1×10^{-7} M, a value similar to the binding affinity of 20E for the EcR/USP heterodimer, is 18 h, 32 h, 3 days, and 6

days, respectively (estimated from Takaki and Sakurai (2003) for the third and fourth instars, and from Mizoguchi *et al*. (2001) for the fifth instar and pupal periods). Thus, the shape of the ecdysteroid titer peak prior to the stationary molt in the third and fourth instars is sharper and narrower than those in the prepupal and pupal period. At the pupal molt the peak value is threefold higher than that for the stationary molt. This value in the pupal period is fivefold higher than that in the prepupal period. This is also the case in *Manduca* (Bollenbacher *et al*., 1981, 1987; Warren and Gilbert, 1986). Although little attention has been focused on such differences so far, these changes must possess an important meaning in the control of insect growth and metamorphosis.

In *Bombyx*, the composition of hemolymph ecdysteroids is strikingly different at the peak titers occurring between the fourth instar and prepupal period (Kamimura and Kiuchi, 2002). At day 2.5, 0.5 days prior to the peak in hemolymph ecdysteroids occurring in the fourth instar that lasts 4.5 days, the 20E concentration is fivefold higher than that of ecdysone, while at the peak, the 20E titer is 17 times as great as that of ecdysone. In contrast, 0.5 days prior to the peak titer in the prepupal period, ecdysone is the predominant ecdysteroid, as the 20E concentration is approximately 70% that of ecdysone. 20E becomes predominant by the time of the peak titer, although 20E concentrations are only 30% higher than ecdysone. In addition, the absolute concentrations of 20E at peak titer are 620 $ng\,ml^{-1}$ (1.3 μM) in the fourth instar and 480 $ng\,ml^{-1}$ (1 μM) in the fifth instar. In *Manduca* prothoracic glands of day 5 fifth instar larvae, 1 μM 20E significantly suppresses USP-1 while at the same time it stimulates USP-2 expression (Song and Gilbert, 1998). The fourth instar glands are also inhibited by 20E *in vitro*, similar to those of the fifth instar (Sakurai and Gilbert, unpublished data). These composite data from *Manduca* and *Bombyx* indicate that in the fourth instar, 20-monooxygenase activity sharply increases when prothoracic gland activity increases, and therefore secreted ecdysone is promptly converted to 20E, which provides for an acute induction of USP-2 and thereby elicits a strong negative feedback along with the increased sensitivity of the glands to 20E (Takaki and Sakurai, 2003). By contrast, in the prepupal period, 20-monooxygenase activity may only gradually increase, while the degree of feedback inhibition is not so strong as compared with that in the fourth instar. While receiving a weak feedback inhibition by 20E in the early period of feedback inhibition, the prothoracic glands are capable of responding to PTTH in the presence of 20E (Sakurai and Williams, 1989; Song and Gilbert, 1998), and therefore the glands may be able to respond to PTTH and secrete ecdysone even in the face of the feedback inhibition that occurs before the peak titer in the prepupal period.

The degree of sharpness of both the increase and decrease in the titer cannot be accounted for only by feedforward (PTTH) and feedback factors. As discussed elsewhere in this volume (see **Chapters 3.2, 3.3**, and **3.7**) and above, various factors are involved in the control of prothoracic gland secretory activity resulting in both upregulation and downregulation (**Figure 1**). JH acts as a modulator in a stage-specific manner. Nerves from the prothoracic ganglion suppress gland activity in at least some insects (Okajima and Kumagai, 1989; Richter and Böhm, 1997). Decreases in the titer also depend on ecdysteroid metabolism, starting with the conversion of ecdysone to 20E by the 20-monooxygenase followed by 26-hydroxylation mediated by the 26-hydroxylase that is induced by 20E (Hoffman *et al*., 1974; Williams *et al*., 1997). Thus, the stage-specific expression of these enzymes is also tightly involved in the decrease in the ecdysteroid titer.

The peak value, composition of ecdysteroids, and duration of ecdysteroid titer above a certain concentration are controlled precisely in a stage-specific manner, but the overall output of the gland is dominant and governs every aspect, directly or indirectly, of insect postembryonic development. Ecdysone has been regarded as an inactive prohormone because its affinity to EcR/USP is far less than that of 20E (Minakuchi *et al*., 2003). This low affinity well accounts for its very low biological activity measured using an *in vitro* system (Cherbas *et al*., 1980) where exogenous 20-monooxygenase activity is not a factor. A hormonal role for ecdysone is still conjectural, but one has been suggested over the last three decades (Mandaron, 1973; Tanaka and Takeda, 1993; Tanaka and Naya, 1995; Gilbert *et al*., 1996; Kamimura and Kiuchi, 2002). Nevertheless, 20E must be the main feedback factor in the brain–prothoracic gland axis.

3.8.8. Future Directions

In the study of feedback regulation of the endocrine system, one of the advantages of insects over mammals is that the regulation of most endocrine systems is tightly associated with embryonic and postembryonic development, including growth, pupal–adult differentiation, and cell death. In vertebrates, although hormonal regulation is seen in embryonic development, feedback and feedforward regulation

in the endocrine system is focused to a great extent on the adult stage and therefore the regulation is rather stereotypic. In insects, all the cells of the body are targets of two hormones, 20E and JH, regardless of their developmental stages. The regulation of prothoracic gland ecdysteroidogenesis is believed to be under the control of a complicated regulatory network. The regulatory mechanism of prothoracic gland activity includes almost every aspect found in a mammalian system, i.e., involving tropic and static factors, as well as a horizontal network of the control, neural control, and feedback regulation. At the cellular level, those factors may act through signal transducing pathways mediated by second messengers, a variety of kinases, and different combinations of transcription factors, including the nuclear receptors, EcRs and USPs, and their coactivators. All these factors could be involved in the regulation of gene expression, protein synthesis, and the transport of steroid intermediates in the prothoracic gland cells. As a result of the integration of all of those regulatory pathways, ecdysone secretion is either stimulated or suppressed. With the recent advances of analytical equipment and techniques, macrolepidopteran prothoracic glands (*Manduca*) are sufficiently large in mass to perform biochemical and pharmacological analysis of the aforementioned complicated regulation. However, *Drosophila* is different from other insects; for example, the involvement of JH in the stationary molt is not understood. Nevertheless, precocious metamorphosis can be induced by genetic manipulation (Bialecki *et al*., 2002), indicating that there should be a control mechanism to ensure the stationary molt. The most sophisticated genetic manipulation is available in *Drosophila*, which will be of great help in understanding the complicated feedback regulation systems in the endocrine systems of insects.

References

Agui, N., Granger, N.A., Gilbert, L.I., Bollenbacher, W.E., **1979**. Cellular localization of the insect prothoracicotropic hormone: *in vitro* assay of a single neurosecretory cell. *Proc. Natl Acad. Sci. USA 76*, 5694–5698.

Agui, N., Hiruma, K., **1977a**. *In vitro* activation of neurosecretory brain cells in *Mamestra brassicae* by β-ecdysone. *Gen. Comp. Endocrinol. 33*, 467–472.

Agui, N., Hiruma, K., **1977b**. Ecdysone as a feedback regulator for the neurosecretory brain cells in the cabbage armyworm, *Mamestra brassicae* L. *J. Insect Physiol. 23*, 1393–1396.

Akai, H., Kiguchi, K., Mori, K., **1973**. The influence of juvenile hormone on the growth and metamorphosis of *Bombyx mori*. *Bull. Sericult. Exp. Station, Japan 25*, 287–305.

Akai, H., Kobayashi, M., **1971**. Induction of prolonged larval instar by the juvenile hormone in *Bombyx mori* L. *Appl. Entomol. Zool. 6*, 138–139.

Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity, during development of fifth stadium *Manduca sexta*. *Insect Biochem. 17*, 989–996.

Beydon, P., Lafont, R., **1983**. Feedback inhibition of ecdysone production by 20-hydroxyecdysone in *Pieris brassicae* pupae. *J. Insect Physiol. 29*, 529–533.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., **2002**. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Devel. Cell 3*, 209–220.

Bodnaryk, R.P., **1986**. Feedback inhibition of ecdysone production by 20-hydroxyecdysone during pupal–adult metamorphosis of *Mamestra configurata* Wlk. *Arch. Insect Biochem. Physiol. 3*, 53–60.

Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., **1987**. Developmental endocrinology of larval moulting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 128*, 175–192.

Bollenbacher, W.E., Smith, S.L., Goodman, W., Gilbert, L.I., **1981**. Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm *Manduca sexta*. *Gen. Comp. Endocrinol. 44*, 302–306.

Bounhiol, J.J., **1938**. Recherches expérimentales sur le déterminisme de la métamorphose chez les Lépidoptères. *Bull. Biol. France Belgique* (Suppl. 24), 1–199.

Cherbas, L., Yonge, C.D., Cherbas, P., Williams, C.M., **1980**. The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. *Roux's Arch. Devel. Biol. 189*, 1–15.

Cymborowski, B., Bogus, M., Beckage, N.E., Williams, C.M., Riddiford, L.M., **1982**. Juvenile hormone titers and metabolism during starvation-induced supernumerary larval moulting of the tobacco hornworm, *Manduca sexta* L. *J. Insect Physiol. 28*, 129–135.

Cymborowski, B., Stolarz, G., **1979**. The role of juvenile hormone during larval–pupal transformation of *Spodoptera littoralis*: switchover in the sensitivity of the prothoracic gland to juvenile hormone. *J. Insect Physiol. 25*, 939–942.

Dai, J.-D., Gilbert, L.I., **1997**. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal–adult metamorphosis. *Insect Biochem. Mol. Biol. 27*, 69–78.

Dai, J.-D., Gilbert, L.I., **1998**. Juvenile hormone prevents the onset of programmed cell death in the prothoracic glands of *Manduca sexta*. *Gen. Comp. Endocrinol. 109*, 155–165.

Dai, J.-D., Mizoguchi, A., Satake, S., Ishizaki, H., Gilbert, L.I., **1995**. Developmental changes in the prothoracicotropic hormone content of the *Bombyx mori* brain–retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Devel. Biol. 171*, 212–223.

Dedos, S.G., Birkenbeil, H., **2003**. Inhibition of cAMP signaling cascade-mediated Ca^{2+} influx by a prothoracicostatic peptide (Mas-MIP I) via dihydropyridine-sensitive Ca^{2+} channels in the prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 33*, 219–228.

Delbecque, J.-P., Weidner, K., Hoffmann, K.H., **1990**. Alternative sites for ecdysteroid production in insects. *Inverteb. Reprod. Devel. 18*, 29–42.

Dell, S., Sedimeier, D., Böcking, D., Dauphin-Villemant, C., **1999**. Ecdysteroid biosynthesis in crayfish Y-organs: feedback regulation by circulating ecdysteroids. *Arch. Insect Biochem. Physiol. 41*, 148–155.

Dhadialla, T.S., Tzertzinis, G., **1997**. Characterization and partial purification of ecdysteroid nuclear receptor for a cotton boll weevil embryonic cell line. *Arch. Insect Biochem. Physiol. 35*, 45–57.

Fain, M.J., Riddiford, L.M., **1975**. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull. 149*, 506–521.

Gäde, G., Hoffmann, K.H., Spring, J.H., **1997**. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev. 77*, 963–1032.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Gilbert, L.I., Rybczynski, R., Song, Q., Mizoguchi, A., Morreale, R., *et al.*, **2000**. Dynamics regulation of prothoracic gland ecdysteroidogenesis: *Manduca sexta* recombinant prothoracicotropic hormone and brain extracts have identical effects. *Insect Biochem. Mol. Biol. 30*, 1079–1089.

Gilbert, L.I., Rybczynski, R., Tobe, S.S., **1996**. Regulation of endocrine function leading to insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, New York, pp. 59–107.

Gilbert, L.I., Rybczynski, R., Warren, J.T., **2002**. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol. 47*, 883–916.

Gilbert, L.I., Schneiderman, H.A., **1959**. Prothoracic gland stimulation by juvenile hormone extracts of insects. *Nature 184*, 171–173.

Gilbert, L.I., Song, Q., Rybczynski, R., **1997**. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. *Invertebr. Neurosci. 3*, 205–216.

Granger, N.A., Sturgis, S.L., Ebersohl, R., Geng, C., Sparks, T.C., **1996**. Dopamine control of corpora allata activity in the larval tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 32*, 449–466.

Grieneisen, M.L., Warren, J.T., Gilbert, L.I., **1993**. Early steps in ecdysteroid biosynthesis: evidence for the involvement of P-450 enzymes. *Insect Biochem. Mol. Biol. 23*, 13–23.

Gu, S.H., Chow, Y.S., **1996**. Regulation of juvenile hormone biosynthesis by ecdysteroid levels during the early stages of the last two larval instars of *Bombyx mori*. *J. Insect Physiol. 42*, 625–632.

Gu, S.H., Chow, Y.S., Lin, F.J., Wu, J.L., Ho, R.J., **1996**. A deficiency in prothoracicotropic hormone transduction pathway during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol. 120*, 99–105.

Gu, S.H., Chow, Y.S., Yin, C.M., **1997**. Involvement of juvenile hormone in regulation of prothoracicotropic hormone transduction during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol. 127*, 109–116.

Gu, S.H., Tsia, W.H., Chow, Y.S., **2000**. Temporal analysis of ecdysteroidogenic activity of the prothoracic glands during the fourth larval instar of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 30*, 499–505.

Hagedorn, H.H., O'Connor, J.D., Fuchs, M.S., Sage, B., Schlaeger, D.A., *et al.*, **1975**. The ovary as a source of alpha-ecdysone in an adult mosquito. *Proc. Natl Acad. Sci. USA 72*, 3255–3259.

Hall, P.F., **1995**. The roles of microfilaments and intermediate filaments in the regulation of steroid synthesis. *J. Steroid Biochem. Mol. Biol. 55*, 601–605.

Hall, P.F., **1997**. The roles of calmodulin, actin, and vimentin in steroid synthesis by adrenal cells. *Steroids 62*, 185–189.

Hayakawa, Y., **1990**. Juvenile hormone esterase activity repressive factor in the plasma of parasitized insect larvae. *J. Biol. Chem. 265*, 10813–10816.

Hayakawa, Y., **1991**. Structure of a growth-blocking peptide present in parasitized insect hemolymph. *J. Biol. Chem. 266*, 7982–7984.

Hayakawa, Y., Ohnishi, A., Endo, Y., **1998**. Mechanism of parasitism-induced elevation of hemolymph growth-blocking peptide levels in host insect larvae (*Pseudaletia separata*). *J. Insect Physiol. 44*, 859–866.

Henrich, V.C., Burns, E., Yelverton, D.P., Christensen, E., Weinberger, C., **2003**. Juvenile hormone potentiates ecdysone receptor-dependent transcription in a mammalian cell culture system. *Insect Biochem. Mol. Biol. 33*, 1239–1247.

Hetru, C., Lagueux, M., Bang, L., Hoffmann, J.A., **1978**. Adult ovaries of *Locusta migratoria* contain the sequence of biosynthetic intermediates for ecdysone. *Life Sci. 22*, 2141–2154.

Hiruma, K., **1980**. Possible roles of juvenile hormone in the prepupal stage of *Mamestra brassicae*. *Gen. Comp. Endocrinol. 41*, 392–399.

Hiruma, K., **1986**. Regulation of prothoracicotropic hormone release by juvenile hormone in the penultimate and last instar larvae of *Mamestra brassicae*. *Gen. Comp. Endocrinol. 63*, 201–211.

Hiruma, K., Carter, M.S., Riddiford, L.M., **1995**. Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Devel. Biol. 169*, 195–209.

Hiruma, K., Shimada, H., Yagi, S., **1978**. Activation of the prothoracic gland by juvenile hormone and prothoracicotropic hormone in *Mamestra brassicae*. *J. Insect Physiol. 24*, 215–220.

Hiruma, K., Shinoda, T., Malone, F., Riddiford, L.M., **1999**. Juvenile hormone modulates 20-hydroxyecdysone-inducible ecdysone receptor and ultraspiracle gene expression in the tobacco hornworm, *Manduca sexta*. *Devel. Genes Evol. 209*, 18–30.

Hoffmann, J.A., Koolman, J., Karlson, P., Joly, P., **1974**. Molting hormone titer and metabolic fate of injected ecdysone during the fifth larval instar and in adults of *Locusta migratoria* (Orthoptera). *Gen. Comp. Endocrinol. 22*, 90–97.

Hua, Y.-J., Tanaka, Y., Nakamura, K., Sakakibara, M., Kataoka, H., **1999**. Identification of a prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. *J. Biol. Chem. 274*, 31169–31173.

Huang, H., Cai, L., Remo, B.F., Brown, D.D., **2001**. Timing of metamorphosis and the onset of the negative feedback loop between the thyroid gland and the pituitary is controlled by type II iodothyronine deiodinase in *Xenopus laevis*. *Proc. Natl Acad. Sci. USA 98*, 7348–7353.

Iwami, M., **1990**. The genes encoding bombyxin, a brain secretory peptide of *Bombyx mori*: structure and expression. In: Ohnishi, E., Ishizaki, H. (Eds.), Molting and Metamorphosis. Japan Science Society Press, Tokyo, pp. 49–56.

Jones, G., Sharp, P.A., **1997**. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA 94*, 13499–13503.

Jones, G., Wozniak, M., Chu, Y., Dhar, S., Jones, D., **2001**. Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. *Insect Biochem. Mol. Biol. 32*, 33–49.

Kamimura, M., Kiuchi, M., **2002**. Appling fenoxycarbo at the penultimate instar triggers an additional ecdysteroid surge and induces perfect extra larval molting in the silkworm. *Gen. Comp. Endocrinol. 128*, 231–237.

Kappler, C., Kabbouh, M., Hetru, C., Durst, F., Hoffmann, J.A., **1988**. Characterization of three hydroxylases involved in the final steps of biosynthesis of the steroid hormone ecdysone in *Locusta migratoria* (Insecta, Orthoptera). *J. Steroid Biochem. 31*, 891–898.

Kataoka, H., Nagasawa, H., Isogai, A., Tamura, S., Mizoguchi, A., *et al.*, **1987**. Isolation and partial characterization of a prothoracicotropic hormone of the silkworm, *Bombyx mori*. *Agric. Biol. Chem. 51*, 1067–1076.

Kawamura, K., Shibata, T., Saget, O., Peel, D., Bryant, P.J., **1999**. A new family of growth factors produced by the fat body and active on *Drosophila* imaginal disc cells. *Development 126*, 211–219.

Keightley, D.A., Lou, K.J., Smith, W.A., **1990**. Involvement of translation and transcription in insect steroidogenesis. *Mol. Cell. Endocrinol. 74*, 229–237.

Khalil, G.M., Marzouk, A.S., Darwish, Z.E., **1988**. Effect of 20-hydroxyecdysone on neurosecretory cell activity in female *Hyalomma dromedarii* synganglion (Acari: Ixodidae). *Exp. Appl. Acarol. 5*, 101–119.

Kiguchi, K., Agui, N., **1981**. Ecdysteroid level and developmental events during larval moulting in the silkworm, *Bombyx mori*. *J. Insect Physiol. 27*, 805–812.

Kiriishi, S., Nagasawa, H., Kataoka, H., Suzuki, A., Sakurai, S., **1992**. Comparison of the *in vivo* and *in vitro* effects of bombyxin and prothoracicotropic hormone on prothoracic glands of the silkworm, *Bombyx mori*. *Zool. Sci. 9*, 149–155.

Kiriishi, S., Rountree, D.B., Sakurai, S., Gilbert, L.I., **1990**. Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3β-reductase mediated conversion to ecdysone in representative insects. *Experientia 46*, 716–721.

Kunkel, J.G., **1977**. Cockroach molting. 2. The nature of regeneration-induced delay of molting hormone secretion. *Biol. Bull. 153*, 145–162.

Lagueux, M., Hirn, M., Hoffmann, J.A., **1977**. Ecdysone during ovarian development in *Locusta migratoria*. *J. Insect Physiol. 23*, 109–119.

Malá, J., Sehnal, F., Kumaran, A.K., Granger, N.A., **1987**. Effects of starvation chilling and injury on endocrine gland function in *Galleria mellonella*. *Arch. Insect Biochem. Physiol. 4*, 113–128.

Mandaron, P., **1973**. Effects of α-ecdysone, β-ecdysone and inokosterone on the *in vitro* evagination of *Drosophila* leg discs and the subsequent differentiation of imaginal integumentary structures. *Devel. Biol. 31*, 101–113.

Minakuchi, C., Nakagawa, Y., Kamimura, M., Miyagawa, H., **2003**. Binding affinity of nonsteroidal ecdysone agonists against the ecdysone receptor complex determines the strength of their molting hormone activity. *Eur. J. Biochem. 270*, 4095–4104.

Miner, A.L., Rosenberg, A.J., Nijhout, H.F., **2000**. Control of growth and differentiation of the wing imaginal disk of *Precis coenia* (Lepidoptera: Nymphalidae). *J. Insect Physiol. 46*, 251–258.

Mizoguchi, A., **2001**. Effects of juvenile hormone on the secretion of prothoracicotropic hormone in the last- and penultimate-instar larvae of the silkworm *Bombyx mori*. *J. Insect Physiol. 47*, 767–775.

Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., **2002**. Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol. 15*, 181–189.

Mizoguchi, A., Ishizaki, H., Nagasawa, H., Kataoka, H., Isogai, A., *et al.*, **1987**. A monoclonal antibody against a synthetic fragment of bombyxin (4K-prothoracicotropic hormone) from the silkmoth, *Bombyx mori*: characterization and immunohistochemistry. *Mol. Cell. Endocrinol. 51*, 227–235.

Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., **2001**. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol. 15*, 349–358.

Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., *et al.*, **1990**. Immunohistochemical localization of prothoracicotropic hormone-producing neurosecretory cells in the brain of *Bombyx mori*. *Devel. Growth Differ. 32*, 591–598.

Morita, M., Tojo, S., **1985**. Relationship between starvation and supernumerary ecdysis and recognition of the penultimate larval instar in the common cutworm *Spodoptera litura*. *J. Insect Physiol. 31*, 307–314.

Moto, K., Abdel Salam, S.E., Sakurai, S., Iwami, M., **1999**. Gene transfer into insect brain and cell-specific expression of bombyxin gene. *Devel. Genes Evol. 209*, 447–450.

Niimi, S., Sakurai, S., **1997**. Developmental changes in juvenile hormone and juvenile hormone acid titers in the hemolymph and *in vitro* juvenile hormone synthesis by corpora allata of the silkworm, *Bombyx mori*. *J. Insect Physiol. 43*, 875–884.

Nijhout, H.F., Grunert, L.W., **2002**. Bombyxin is a growth factor for wing imaginal discs in Lepidoptera. *Proc. Natl Acad. Sci. USA 99*, 15446–15450.

Nijhout, H.F., Williams, C.M., **1974a**. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *J. Exp. Biol. 61*, 481–491.

Nijhout, H.F., Williams, C.M., **1974b**. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol. 61*, 493–501.

Noguchi, H., Hayakawa, Y., **1996**. Mechanism of parasitism-induced elevation of dopamine levels in host insect larvae. *Insect Biochem. Mol. Biol. 26*, 659–665.

Noguchi, H., Tsuzuki, S., Tanaka, K., Matsumoto, H., Hiruma, K., *et al.*, **2003**. Isolation and characterization of a dopa decarboxylase cDNA and the induction of its expression by an insect cytokine, growth-blocking peptide in *Pseudaletia separata*. *Insect Biochem. Mol. Biol. 33*, 209–217.

O'Farrell, A.F., Stock, A., Rae, C.A., Morgan, J.A., **1960**. Degeneration and development in the cockroach *B. germanica*. *Acta Entomol. Bohemoslovaca 57*, 317–324.

Ohnishi, A., Hayakawa, Y., Matsuda, Y., Kwon, K.W., Takahashi, T.A., *et al.*, **1995**. Growth-blocking peptide titer during larval development of parasitized and cold-stressed armyworm. *Insect Biochem. Mol. Biol. 25*, 1121–1127.

Ohtaki, T., Yamanaka, F., Sakurai, S., **1986**. Differential timing of pupal commitment in various tissues of the silkworm, *Bombyx mori*. *J. Insect Physiol. 32*, 635–642.

Okajima, A., Kumagai, K., **1989**. The inhibitory control of prothoracic gland activity by neurosecretory neurons in a moth, *Mamestra brassicae*. *Zool. Sci. 6*, 851–858.

Okuda, M., Sakurai, S., Ohtaki, T., **1985**. Activity of the prothoracic gland and its sensitivity to prothoracicotropic hormone in the penultimate and last-larval instar of *Bombyx mori*. *J. Insect Physiol. 31*, 455–461.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., *et al.*, **2003**. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778.

Predel, R., Eckert, M., **2000**. Neurosecretion: peptidergic systems in insects. *Naturwissenschaften 87*, 343–350.

Richard, D.S., Gilbert, L.I., **1991**. Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster*. *Experientia 47*, 1063–1066.

Richter, K., Böhm, G.-A., **1997**. The molting gland of the cockroach *Periplaneta americana*: secretory activity and its regulation. *Gen. Pharmacol. 29*, 17–21.

Rountree, D.B., Bollenbacher, W.E., **1986**. The release of the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta*, is controlled intrinsically by juvenile hormone. *J. Exp. Biol. 120*, 41–58.

Rybczynski, R., Gilbert, L.I., **1994**. Changes in general and specific protein synthesis that accompany ecdysteroid synthesis in stimulated prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 175–189.

Rybczynski, R., Gilbert, L.I., **1995a**. Prothoracicotropic hormone elicits a rapid, developmentally specific synthesis of β-tubulin in an insect endocrine gland. *Devel. Biol. 169*, 15–28.

Rybczynski, R., Gilbert, L.I., **1995b**. Prothoracicotropic hormone-regulated expression of hsp70 cognate protein in the insect prothoracic gland. *Mol. Cell. Endocrinol. 115*, 73–85.

Rybczynski, R., Gilbert, L.I., **2000**. cDNA cloning and expression of a hormone-regulated heat shock protein (hsc 70) from the prothoracic gland of *Manduca sexta*. *Insect Biochem. Mol. Biol. 30*, 579–589.

Safranek, L., Cymborowski, B., Williams, C.M., **1980**. Effects of juvenile hormone on ecdysone-dependent development in the tobacco hornworm, *Manduca sexta*. *Biol. Bull. 158*, 248–256.

Sakurai, S., **1983**. Temporal organization of endocrine events underlying larval–larval ecdysis in the silkworm *Bombyx mori*. *J. Insect Physiol. 29*, 919–932.

Sakurai, S., **1990**. Hormonal mechanism underlying the switch in the response of the prothoracic glands to juvenile hormone. *Adv. Invertebr. Reprod. 5*, 435–440.

Sakurai, S., Imokawa, H., **1988**. Developmental arrest induced by juvenile hormone in larvae of *Bombyx mori*. *Arch. Insect Biochem. Physiol. 8*, 219–228.

Sakurai, S., Kaya, M., Satake, S.-I., **1998**. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval–pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol. 44*, 867–881.

Sakurai, S., Okuda, M., Ohtaki, T., **1989a**. Juvenile hormone inhibits ecdysone secretion and responsiveness to prothoracicotropic hormone in prothoracic glands of *Bombyx mori*. *Gen. Comp. Endocrinol. 75*, 222–230.

Sakurai, S., Warren, J.T., Gilbert, L.I., **1989b**. Mediation of ecdysone synthesis in *Manduca sexta* by a hemolymph enzyme. *Arch. Insect Biochem. Physiol. 10*, 179–197.

Sakurai, S., Warren, J.T., Gilbert, L.I., **1991**. Ecdysteroid synthesis and molting by the tobacco hornworm, *Manduca sexta*, in the absence of prothoracic glands. *Arch. Insect Biochem. Physiol. 18*, 13–36.

Sakurai, S., Williams, C.M., **1989**. Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm *Manduca sexta*. *Gen. Comp. Endocrinol. 75*, 204–216.

Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St. Germain, D.L., *et al.*, **2001**. Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. *Mol. Endocrinol. 15*, 2137–2148.

Sehnal, F., Maróy, P., Malá, J., **1981**. Regulation and significance of ecdysteroid titre fluctuations in lepidopterous larvae and pupae. *J. Insect Physiol. 27*, 535–544.

Shibusawa, N., Hollenberg, A.N., Wondisford, F.E., **2003**. Thyroid hormone receptor DNA binding is required for both positive and negative gene regulation. *J. Biol. Chem. 278*, 732–738.

Siew, Y.C., and Gilbert, L.I., **1971**. Effects of moulting hormone and juvenile hormone on insect endocrine gland activity. *J. Insect Physiol. 17*, 2095–2104.

Singtripop, T., Tungjitwitayakul, J., Sakurai, S., **2002**. Intensity of larval diapause in the bamboo borer, *Omphisa fuscidentalis*. *Zool. Sci. 19*, 577–582.

Singtripop, T., Wanichacheewa, S., Sakurai, S., **2000**. Juvenile hormone-mediated termination of larval diapause in the bamboo borer, *Omphisa fuscidentalis*. *Insect Biochem. Mol. Biol. 30*, 847–854.

Singtripop, T., Wanichacheewa, S., Tsuzuki, S., Sakurai, S., **1999**. Larval growth and diapause in a tropical moth *Omphisa fuscidentalis* Hampton. *Zool. Sci. 16*, 725–733.

Smagghe, G., Dhadialla, T.S., Lezzi, M., **2002**. Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. *Insect Biochem. Mol. Biol. 32*, 187–192.

Smith, S.L., Bollenbacher, W.E., Gilbert, L.I., **1983**. Ecdysone 20-monooxygenase activity during larval–pupal development of *Manduca sexta*. *Mol. Cell. Endocrinol. 31*, 227–251.

Smith, W.A., **1995**. Regulation and consequences of cellular changes in the prothoracic glands of *Manduca sexta* during the last larval instar. *Arch. Insect Biochem. Physiol. 30*, 271–293.

Smith, W.A., Pasquarello, T.J., **1989**. Developmental changes in phosphodiesterase activity and hormonal response in the prothoracic glands of *Manduca sexta*. *Mol. Cell. Endocrinol. 63*, 239–246.

Song, Q., Gilbert, L.I., **1994**. S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase. *Devel. Genet. 15*, 332–338.

Song, Q., Gilbert, L.I., **1995**. Multiple phosphorylation of ribosomal protein S6 and specific protein synthesis are required for prothoracicotropic hormone-stimulated ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 591–602.

Song, Q., Gilbert, L.I., **1997**. Molecular cloning, developmental expression, and phosphorylation of ribosomal protein S6 in the endocrine gland responsible for insect molting. *J. Biol. Chem. 272*, 4429–4435.

Song, Q., Gilbert, L.I., **1998**. Alterations in ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol. 28*, 849–860.

Sparks, T.C., Hammock, B.D., Riddiford, L.M., **1983**. The haemolymph juvenile hormone esterase of *Manduca sexta* (L.): inhibition and regulation. *Insect Biochem. 13*, 529–541.

Steel, C.G.H., **1975**. A neuroendocrine feedback mechanism in the insect moulting cycle. *Nature 253*, 267–269.

Steel, C.G.H., Harmsen, R., **1971**. Dynamics of the neurosecretory system in the brain of an insect, *Rhodnius prolixus*, during growth and molting. *Gen. Comp. Endocrinol. 17*, 125–141.

Suzuki, C., Ishizaki, H., **1986**. Prothoracicotropic hormone bioassay: *Bombyx* larval assay. *Int. J. Invertebr. Reprod. Devel. 10*, 259–274.

Takaki, K., Sakurai, S., **2003**. Regulation of prothoracic gland steroidogenic activity leading to pupal metamorphosis. *Insect Biochem. Mol. Biol. 33*, 1189–1199.

Tanaka, T., Agui, N., Hiruma, K., **1987**. The parasitoid *Apanteles kariyai* inhibits pupation of its host, *Pseudaletia separata*, via disruption of prothoracicotropic hormone release. *Gen. Comp. Endocrinol. 67*, 364–374.

Tanaka, Y., Naya, S., **1995**. Dietary effect of ecdysone and 20-hydroxyecdysone on larval development of two lepidopteran species. *Appl. Entomol. Zool. 30*, 285–294.

Tanaka, Y., Takeda, S., **1993**. Ultranumerary larval ecdysis of the silkworm, *Bombyx mori* induced by ecdysone. *Naturwissenschaften 80*, 131–132.

Truman, J.W., **1972**. Physiology of insect rhythms. 1. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol. 57*, 805–820.

Wang, S.F., Li, C., Zhu, J., Miura, K., Miksicek, R.J., *et al.*, **2000**. Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. *Devel. Biol. 218*, 99–113.

Warren, J.T., Gilbert, L.I., **1986**. Ecdysone metabolism and distribution during the pupal–adult development of *Manduca sexta*. *Insect Biochem. 6*, 65–82.

Warren, J.T., Gilbert, L.I., **1996**. Metabolism *in vitro* of cholesterol and 25-hydroxycholesterol by the larval prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol. 26*, 917–929.

Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic

pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048.

Warren, J.T., Sakurai, S., Rountree, D.B., Lee, S.-S., Nakanishi, K., *et al.*, **1988**. Regulation of the ecdysteroid titer of *Manduca sexta*: reappraisal of the role of the prothoracic glands. *Proc. Natl Acad. Sci. USA 85*, 958–962.

Watson, R.D., Bollenbacher, W.E., **1988**. Juvenile hormone regulates the steroidogenic competence of *Manduca sexta* prothoracic glands. *Mol. Cell. Endocrinol. 57*, 251–259.

Watson, R.D., Ackerman-Morris, S., Smith, W.A., Watson, C.J., Bollenbacher, W.E., **1996**. Involvement of microtubules in prothoracicotropic hormone-stimulated ecdysteroidogenesis by insect (*Manduca sexta*) prothoracic glands. *J. Exp. Zool. 276*, 63–69.

Webb, T.J., Powls, R., Rees, H.H., **1995**. Enzymes of ecdysteroid transformation and inactivation in the midgut of the cotton leafworm, *Spodoptera littoralis*: properties and developmental profiles. *Biochem. J. 312*, 561–568.

Whisenton, L.R., Granger, N.A., Bollenbacher, W.E., **1987a**. A kinetics analysis of brain-mediated 20-hydroxyecdysone stimulation of the corpora allata of *Manduca sexta*. *Mol. Cell. Endocrinol.* 171–178.

Whisenton, L.R., Watson, R.D., Granger, N.A., Bollenbacher, W.E., **1987b**. Regulation of juvenile hormone biosynthesis by 20-hydroxyecdysone during the fourth larval instar of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 66*, 62–70.

Williams, C.M., **1952**. Physiology of insect diapause. 4. The brain and prothoracic glands as an endocrine system in the *Cecropia* silkworm. *Biol. Bull. 103*, 120–138.

Williams, C.M., **1959**. The juvenile hormone. 1. Endocrine activity of the corpora allata of the adult *Cecropia* silkworm. *Biol. Bull. 116*, 323–338.

Williams, D.R., Chen, J.-H., Fisher, M.J., Rees, H.H., **1997**. Induction of enzymes involved in molting hormone (ecdysteroid) inactivation by ecdysteroids and an agonist, 1,2-dibenzoyl-*t*-butylhydrazine (RH-5849). *J. Biol. Chem. 272*, 8427–8432.

Zhu, X.X., Oliver, J.H., Jr., Dotson, E.M., **1991**. Epidermis as the source of ecdysone in an argasid tick. *Proc. Natl Acad. Sci. USA 88*, 3744–3747.

Zitnan, D., Kingan, T.G., Hermesman, J.L., Adams, M.E., **1996**. Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science 271*, 88–91.

Zitnan, D., Ross, C.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23*, 523–535.

3.9 Hormonal Control of Reproductive Processes

A S Raikhel, University of California, Riverside, CA, USA
M R Brown, University of Georgia, Athens, GA, USA
X Belles, Institut de Biologia Molecular de Barcelona (CSIC), Spain

3.9.1. Introduction

The years following the 1985 publication of *Comprehensive Insect Physiology, Biochemistry and Pharmacology* have been marked by stunning developments in insect science. A technological revolution in biochemistry, molecular biology, and genetics has swept all areas of biological science, and has profoundly influenced insect science as well. With this technological revolution, the small size of insects is no longer a barrier to discovering their biochemical make-up, cloning or characterizing genes, or uncovering genetic and hormonal signals governing their functions. Sequencing of insect genomes, particularly that of *Drosophila melanogaster*, has led to the identification of previously unknown genes and to the development of functional genomic approaches that lead to further elucidation of genetic regulatory networks.

During this period, insect endocrinology has shifted its focus from physiology and biochemistry to molecular biology and genetics. The latter are the subjects of the present volume. This chapter specifically reviews the progress that has been achieved since 1985 in our understanding of the hormonal control of insect reproduction. Particular attention is paid to those areas where significant progress has been made at the molecular and genetic levels.

There are a number of recent reviews on hormonal regulation of yolk protein (*yp*) genes (Raikhel *et al*., 2003; Belles, 2004; Bownes, 2004; Wang *et al*., 2004); vitellogenins and their processing (Sappington *et al*., 2002; Telfer, 2002; Tufail *et al*., 2004); nonvitellogenin yolk proteins (Bownes and Pathirana, 2002; Telfer, 2002; Masuda *et al*., 2004; Yamahama *et al*., 2004), and the cell biology of the insect fat body (Giorgi *et al*., 2004).

3.9.2. Endocrine Control of Female Reproduction

3.9.2.1. Evolution of Endocrine Control of Vitellogenesis and Egg Development

The insect female reproductive system consists of the ovaries, which contain ovarioles, oviducts, spermatheca, accessory glands, vagina, and ovipositor (Davey, 1985; Chapman, 1998). Ovarioles are the egg producing units in the ovary. Typically, the insect ovary has four to ten ovarioles; however, some species contain many more ovarioles. Ovarioles are tubular and consist of both somatic and germline cells. At the apex of each ovariole, a germarium houses the primary germline cells. The follicles or egg chambers form within the germarium

and continue to mature along the ovariole tube. There are three types of ovaries in insects (Büning, 1994). Ovaries of primitive groups of insects contain panoistic ovarioles with egg chambers consisting of the oocytes surrounded by the follicular epithelium (panoistic ovarioles). In more advanced insects, where the structure of ovarioles is more complex (meroistic), some germ cells are set aside to form nurse cells that produce massive amounts of numerous products for the developing oocyte. In polytrophic meroistic ovarioles, each egg chamber contains its own group of nurse cells connected to the oocyte. Follicle cells surround the oocyte and nurse cells. In telotrophic meroistic ovaries, each ovariole contains the trophic chamber with large groups of nurse cells. The trophic chamber is connected to the egg chambers by nutritive cords. Egg chambers of telotrophic ovarioles contain only the oocyte surrounded by the follicle cells.

Endocrine control of female reproduction is governed by different types of hormones: neuropeptides (see **Chapter 3.10**), juvenile hormones (JHs; see **Chapter 3.7**), and ecdysteroids (see **Chapters 3.3** and **3.5**). In keeping with common practice among researchers in the field, ecdysteroid is used as the generic term for steroidal insect molting hormones, reserving the term ecdysone for the specific chemical compound 2β, 3β, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one, originally known as α-ecdysone. The abbreviation 20E will be used to refer to 20-hydroxyecdysone (2β, 3β, 14α, 20R, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one), the ecdysone metabolite believed to serve as the active hormone in most well-characterized responses. Individually, or in concert, the regulation of particular events during female reproduction by these hormones has evolved along with their effects on molting and metamorphosis. Control of female reproduction in Apterygota remains the most enigmatic. Studies of the firebrat, *Thermobia domestica* (Zygentoma), have shed some light on the hormonal regulation of vitellogenesis and ovarian development in primitive apterous insects (Bitsch and Bitsch, 1984; Bitsch *et al.*, 1986). In this insect, molting occurs continually into the adult stage, and oogenesis is coordinated with the ecdysteroid regulated adult molting cycle (Bitsch *et al.*, 1986). Treatment with the anti-allatal drug precocene blocks ovarian maturation, which indicates its dependence upon juvenile hormone secreted by the corpora allata (CA) (Bitsch and Bitsch, 1984; Bitsch *et al.*, 1986). Further studies are required in order to understand the precise roles of JH and ecdysteroids in regulating vitellogenesis and oogenesis in apterous insects.

It is well established that major events of reproduction in all insect orders with incomplete metamorphosis (Hemimetabola) are governed by JH. In the orders with complete metamorphosis (Holometabola), control strategies have evolved differently. In beetles (Coleoptera), JH remains the major regulatory hormone of reproductive events. In Hymenoptera, the role of JH is elaborated in eusocial species having a single or a few reproductive females in a colony (see **Chapter 3.13**). In Lepidoptera, female reproduction is regulated either by JH or ecdysteroids. In many such species, egg maturation occurs during the pharate adult stage and requires coordination with hormonal signals controlling metamorphosis. In dipteran insects, mosquitos, and flies, ecdysteroids have the leading role as hormonal regulators, but JH has an important role as a regulator in dipteran females in preparing reproductive tissues for ecdysteroid mediated events, such as vitellogenesis. For all insects, neuropeptides play a key role regulating the production of JH and ecdysteroids. A summary of the primary hormones involved in reproduction according to the phylogeny of insect orders is presented in **Figure 1**; for some orders, this is not known.

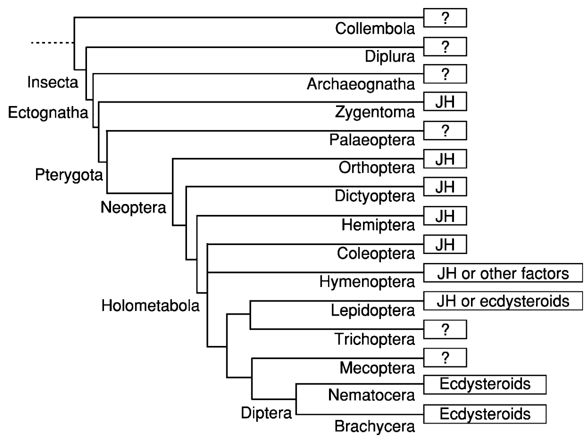


Figure 1 Utilization of juvenile hormone or ecdysteroids as major regulators of vitellogenesis and reproduction among insect orders. Some hemipterans show incomplete dependence on JH. In hymenopterans, JH plays a vitellogenic role in nonsocial and primitive social groups, but not in advanced groups (see **Chapter 3.13**). In lepidopterans, those species that begin vitellogenesis after adult emergence are JH dependent, and those in which vitellogenesis proceeds between pupal and adult stages are partially dependent on JH, whereas species in which vitellogenesis proceeds within or before the pupal stage are independent of JH. Dipterans, in general, are ecdysteroid dependent, although JH may play an accessory role in vitellogenesis (Modified from Belles, X., **2004**. Vitellogenesis directed by juvenile hormone. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 157–198).

3.9.2.1.1. Juvenile hormone directed female reproduction In the adult females of Hemimetabola (Dictyoptera to Hemiptera) and Coleoptera, JH is the main regulator and pleiotropically controls most aspects of female reproduction (**Figure 2**). The major role of JH in reproduction is to regulate vitellogenin (Vg) gene expression in the fat body, generally, and in ovarian follicular epithelium (Wyatt and Davey, 1996; Engelmann, 1983, 2003; Belles, 2004). Some gryllid and hemipteran species show an incomplete dependence upon JH (Wyatt and Davey, 1996; Strambi *et al.*, 1997).

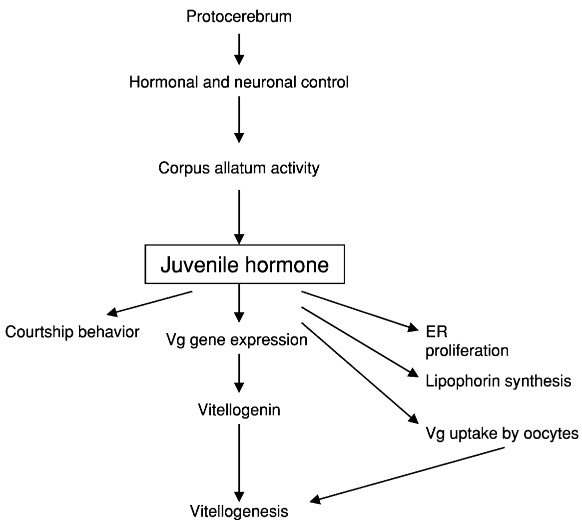


Figure 2 The pleiotropic role of juvenile hormone in insect reproduction. ER, endoplasmic reticulum; Vg, vitellogenin. (Reproduced with permission from Engelmann, F., **2003**. Juvenile hormone action in insect reproduction. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, Vol. 2. Academic Press, San Diego, pp. 536–539.)

Cockroaches (Dictyoptera) are the classical model for studies of JH dependent vitellogenesis. In the oviparous *Periplaneta americana* (Blattidae), Weaver and Edwards (1990) have shown that allatectomy or treatment with inhibitors of JH synthesis blocks Vg production, oocyte growth, and ootheca formation, whereas JH treatment restores these processes. The regulation of vitellogenesis by JH has been most thoroughly studied in *Blattella germanica* (Blattellidae). In this typical JH dependent species, in which the females carry the ootheca externally until the first inside larvae emerge, JH production (Belles *et al.*, 1987), Vg titers (Martín *et al.*, 1995), and Vg mRNA levels (Comas *et al.*, 1999) show cyclical and approximately parallel patterns during the reproductive cycle (**Figure 3**). The pseudoviviparous cockroach, *Leucophaea maderae*, has been one of the favorite models for biochemical studies of JH regulation of reproduction. Effective doses to induce vitellogenesis in females *in vivo* range from 1 μg of JH I to 25 μg of JH III *in vivo*. Methoprene, a potent JH analog (JHA), induces vitellogenesis in adult males, as well (Don-Wheeler and Engelmann, 1997). In another pseudoviviparous cockroach, *Blaberus discoidalis*, allatectomy prevents ovarian maturation that can be restored by JH III treatment (Keeley and McKercher, 1985). In decapitated adult females, methoprene or JH III induces Vg protein synthesis (Keeley *et al.*, 1988) and a 6.5 kb mRNA, presumably corresponding to the Vg transcript (Bradfield *et al.*, 1990). The neuropeptide, hypertrehalosemic hormone, enhances the vitellogenic

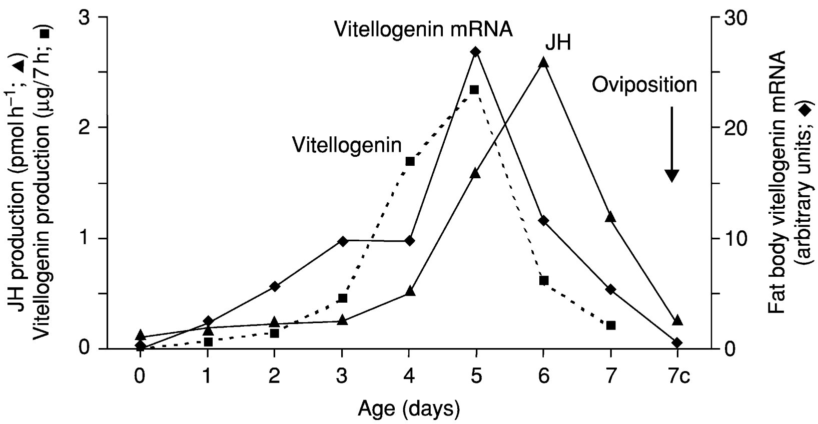


Figure 3 Production rates of juvenile hormone (JH) from the corpora allata, vitellogenin (Vg) from the periovarial fat body, and fat body Vg mRNA during the first reproductive cycle in the cockroach *Blattella germanica*. (Based on data from Belles, X., Casas, J., Messenguer, A., Piulachs, M.D., **1987**. *In vitro* biosynthesis of JH III by the corpora allata of *Blattella germanica* (L.) adult females. *Insect Biochem. 17*, 1007–1010; Martin, D., Piulachs, M.D., Belles, X., **1995**. Patterns of hemolymph vitellogenin and ovarian vitellin in the German cockroach, and the role of juvenile hormone. *Physiol. Entomol. 20*, 59–65; and Martin, D., Comas, D., Piulachs, M.D., Belles, X., **1998**. Isolation and sequence of a partial vitellogenin cDNA from the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae), and characterization of the vitellogenin gene expression. *Arch. Insect Biochem. Physiol. 38*, 137–146.)

effects of JH (Lee and Keeley, 1994a). In *Nauphoeta cinerea*, in which hemolymph Vg levels show a cyclic pattern during the gonadotropic cycle, JH I, II, and III can induce Vg production (Buschor and Lanzrein, 1983).

In Dermaptera, the CA are required for reproduction in female earwigs, *Anisolabis maritima*, as first shown by Ozeki (1951; see Rankin *et al.*, 1997), and JH is the main gonadotropic hormone in female *Euborellia annulipes*, which have vitellogenic cycles (Rankin *et al.*, 1997). Rouland *et al.* (1981) reported that allatectomized females *Labidura riparia* do not produce vitellogenic proteins, and 0.1 µg of farnesol, a precursor of JH, restores vitellogenesis.

In Orthoptera, the regulation of vitellogenesis by JH has been demonstrated in a number of locusts and grasshoppers (Acrididae) through classical experiments involving allatectomy and JH treatment (Wyatt, 1991; Wyatt and Davey, 1996). The most thoroughly studied orthopteran species has been *Locusta migratoria*, in which Vg synthesis is cyclic (Chinzei and Wyatt, 1985). Induction of Vg synthesis in female *L. migratoria* by JH homologs is only achieved with repeated doses or with high doses coinjected with a JH esterase inhibitor (Wyatt *et al.*, 1987), whereas synthetic JHAs at doses between 2 and 30 µg have a much more potent vitellogenic action (Edwards *et al.*, 1993; Zhang *et al.*, 1993). Neuropeptides may also be involved, since a brain factor enhances vitellogenesis induced by JH in fat body incubated *in vitro* (Glinka *et al.*, 1995). JH titer, and Vg protein and mRNA levels have been described during the reproductive cycle of the locust *Schistocerca gregaria* (Mahamat *et al.*, 1997) and the grasshopper *Romalea microptera* (= *Romalea guttata*) (Borst *et al.*, 2000).

In contrast to other orthopteroids, the phasmid *Carausius morosus* is independent of JH for vitellogenesis (Bradley *et al.*, 1995). Allatectomy does not prevent production of viable eggs in this insect, as first reported by Pflugfelder in 1930, and later confirmed with antibodies against *C. morosus* Vg that detected its production in allatectomized and intact females (Bradley *et al.*, 1995). However, it is not clear whether the independence from JH for vitellogenesis applies to all Phasmida.

In crickets (Gryllidae), both JH and ecdysteroids are involved in the control of vitellogenesis (review: Strambi *et al.*, 1997). In *Acheta domesticus*, the CA are necessary for oocyte growth, and JH restores vitellogenesis in allatectomized or decapitated females (Renucci *et al.*, 1987; Loher *et al.*, 1992). Conversely, female *Teleogryllus commodus* emerging from allatectomized larvae can still produce eggs (Loher and Giannakakis, 1990). Although allatectomy of *Gryllus bimaculatus* reduced the rate of egg production, oocytes in allatectomized females contained a similar amount of yolk protein as oocytes in controls; JH or JHA restored egg production to a variable extent (Hoffmann and Sorge, 1996). Notably, low doses of ecdysteroids stimulated oocyte growth in females of this species (Chudakova *et al.*, 1982; Behrens and Hoffmann, 1983). These results suggest that in crickets in which allatectomy does not completely suppress oocyte growth, ecdysteroids may play a vitellogenic role (Hoffmann and Sorge, 1996).

For Hemiptera, the CA were shown to be necessary for vitellogenesis in *Rhodnius prolixus* by V.B. Wigglesworth in the 1930s, and this observation has since been confirmed for several other hemipteran species (Wyatt and Davey, 1996; Davey, 1997; Belles, 2004). Later studies demonstrated that allatectomy of *R. prolixus* does not totally abolish Vg synthesis, but JH treatment does restore normal production (Wang and Davey, 1993). Female *Oncopeltus fasciatus* chemically allatectomized with precocenes produced the Vg precursor, but its conversion to mature Vg, which is incorporated into the oocytes, did not take place, as shown with electrophoresis of native proteins and immunodiffusion (Kelly and Hunt, 1982). A later study of this species found that the synthesis of two female specific Vg components, 200 and 170 kDa, was inhibited in precocene treated specimens and restored by administration of JH (Martinez and Garcerá, 1987). This discrepancy may be due to differences in the precocene treatment. Only one study has demonstrated a role for JH in a homopteran species. In the black bean aphid, *Aphis fabae*, ovarian development begins prenatally in this parthenogenic species, in such a manner that oocytes differentiate in the embryonic germaria, and at emergence each ovariole of a first instar aphid already contains one or two developing embryos. Precocene treatment of aphid nymphs inhibited oocyte development in embryos inside the parental ovaries, whereas JH reversed this inhibition (Hardie, 1987).

For Coleoptera, JH is the main gonadotropic hormone, but surprisingly little is known about the reproductive endocrinology of this holometabolous order with the greatest number of insect species. A long day regimen for the Colorado potato beetle, *Leptinotarsa decemlineata*, leads to reproductive activity and a short day induces diapause. JH or JHA (pyriproxyfen) treatment of short-day females induces Vg synthesis, and the JHA also induces Vg production in last instar larvae (de Kort *et al.*, 1997). Similarly, females of *Coccinella septempunctata*

reared on suboptimal artificial diets fail to reproduce, but Vg synthesis can be induced by treatment with JHA (Zhai *et al.*, 1984, 1987; Guan, 1989). In the spruce weevil, *Pissodes strobi*, treatment of previtellogenic females with JH III induces the precocious appearance of Vg transcripts, as determined by the identification of Vg mRNA (Leal *et al.*, 1997). This study also showed that Vg mRNA accumulates slower in beetles feeding on Sitka spruce trees (*Picea sitchensis*), which are resistant to *P. strobi* attack, thus suggesting that this plant contains anti-juvenoid compounds.

3.9.2.1.2. From nonsocial sawflies to eusocial ants and bees Within the Hymenoptera, JH appears to play a role in female reproduction among nonsocial and eusocial species and through this action may affect caste determination in the eusocial groups (see **Chapter 3.13**). In the primitive suborder Symphyta, only *Athalia* sawflies have received attention. JH applied to male *Athalia rosae* induced an increase in Vg production, and ovaries implanted into these males incorporated the Vg (Hatakeyama and Oishi, 1990). Later, it was shown that ovaries of *Athalia rosae* implanted into males of the closely related species, *Athalia infumata*, incorporated the heterospecific Vg that was induced in the host by JH III treatment (Hatakeyama *et al.*, 1995). Although these studies did not examine vitellogenesis in females, the results suggest a role for JH in females.

The regulation of reproduction by JH varies among the suborder Apocrita, which encompasses a range of species from solitary types to highly social groups like ants and honeybees (for reviews of JH action in social Hymenoptera see Robinson and Vargo, 1997; Hartfelder, 2000; **Chapter 3.13**). In the primitive eusocial paper wasps (Polistidae), *Polistes dominulus* (= *Polistes gallicus*) and *P. metricus*, JH acts as a gonadotropin. Queens or dominant females of *Polistes* show high JH titers associated with growing ovaries, whereas subordinate females have low JH titers. In general, allatectomy of dominant females leads to a reduction in the dominance status. The bumble bee, *Bombus terrestris* (Apidae), shows a relatively primitive social structure, and JH titers are high in egg laying queens. A regulatory role for JH is substantiated by the positive correlation between ovary development and rate of JH production in queenless workers (Block *et al.*, 2000). The role of JH remains unresolved among the studies of ants and bees with a complex social structure. JH treatment of isolated virgin queens of the fire ant, *Solenopsis invicta* (Myrmicinae) leads to wing shedding and oviposition, which is prevented by allatectomy. Subsequent JH treatment of allatectomized queens induces these phenomena (see Robinson and Vargo, 1997). Conversely, JH titers are low in reproductive female ants in the genus *Diacamma* (Ponerine; Sommer *et al.*, 1993). In the highly eusocial honeybee, *Apis mellifera* (Apidae), Vg synthesis in laying queens was only slightly reduced by allatectomy, and JH treatment increased ovary development only weakly (see Robinson and Vargo, 1997). Exogenous JH administered to queen and workers of *A. mellifera* advances the timing of vitellogenin appearance (Barchuk *et al.*, 2002); together, these results indicate that JH does not play a primary role in honeybee reproduction.

3.9.2.1.3. Butterflies and moths: endocrine compatibility of metamorphosis and vitellogenesis In Lepidoptera, some species begin vitellogenesis after adult emergence, and studies of Papilionoidea (e.g., *Pieris brassicae*, *Nymphalis antiopa*, *Polygonia caureum*, *Vanessa cardui*, and *Danaus plexippus*) and Noctuoidea (e.g., *Heliothis virescens*, *Helicoverpa zea*, and *Pseudaletia unipuncta*) show that JH stimulates vitellogenesis (see Cusson *et al.*, 1994a; Ramaswamy *et al.*, 1997). In *P. unipuncta*, the release rate of JH from CA *in vitro* is positively correlated with Vg synthesis (Cusson *et al.*, 1994b). In this species and *H. virescens*, vitellogenesis is abolished in decapitated females, and JH treatment restores it (Cusson *et al.*, 1994a; Ramaswamy *et al.*, 1997).

For other groups of Lepidoptera, egg development proceeds between the pupal and pharate adult stages. Studies of species in the Tortricoidea have shown that decapitation of female *Choristoneura fumiferana* and *C. rosaceana* reduces egg production, but Vg remains in the hemolymph (Delisle and Cusson, 1999). Treatment of the decapitated females with methoprene restores egg production. Similarly, treatment of female codling moths (*Cydia pomonella*) with fenoxycarb, a JHA, did not affect protein yolk content, but it did stimulate chorionation (Webb *et al.*, 1999). In this species, ecdysteroids may have a priming effect on vitellogenesis, since treatment with ecdysteroid agonists (tebufenozide and methoxyfenozide) increased levels of circulating Vg but did not affect its incorporation into the oocytes (Sun *et al.*, 2003). These results suggest that vitellogenesis is not completely dependent on JH and that it plays a primary role in Vg uptake by the oocyte and in chorionation in this family.

In the Sphingid (Bombycoidea) *Manduca sexta*, vitellogenesis starts 3–4 days before adult emergence and proceeds in the absence of the pupal CA; thereafter, JH is necessary to complete oocyte growth and chorionation (Satyanarayana *et al.*,

1994). Vg was present in prepupae of both sexes at low levels, had disappeared by pupal ecdysis, and reappeared in the late pharate adult females. Methoprene treatment induced Vg synthesis and Vg mRNA in prepupae and freshly molted pupae, and simultaneous administration of ecdysteroids abolished the vitellogenic action of JH (Satyanarayana *et al.*, 1994). The pyraloid moths studied to date have a similar reproductive physiology. In *Plodia interpunctella*, the declining ecdysteroid titer triggers vitellogenesis in early pupae, and ecdysteroid treatment inhibits vitellogenin uptake by the oocytes (Shirk *et al.*, 1992). Similarly, vitellogenesis in *Diatrea grandiosella* proceeds in the absence of ecdysteroids, whereas choriogenesis is completed with JH in the pharate adult (Shu *et al.*, 1997).

In non-Sphingid bombycoids, such as *Bombyx mori* (Bombycidae), *Hyalophora cecropia* (Saturniidae), and *Malacosoma pluviale* (Lasiocampidae), and in the lymantrid (Noctuoidea) *Lymantria dispar*, vitellogenesis proceeds before adult emergence in the absence of JH. In these groups, vitellogenesis seems totally independent of JH, as shown by allatectomy and JH treatment, which did not influence oocyte growth (see Wyatt and Davey, 1996 for references). Other studies of *L. dispar*, in which vitellogenesis starts as early as day 3 of the last larval instar, found that treatment with JHAs on day 2 of this instar selectively prevents the production of Vg (Fescemyer *et al.*, 1992) and Vg mRNA (Hiremath and Jones, 1992).

3.9.2.1.4. Mosquitos and flies: the shift to ecdysteroid mediated vitellogenesis For the Diptera, edysteroids have the primary role of regulating reproduction, as demonstrated in detail almost exclusively in mosquitos and flies. JH plays an important priming role in these females by preparing reproductive tissues for ecdysteroid mediated processes. Regulation of Vg production by ecdysteroids was first demonstrated in the yellow fever mosquito, *Aedes aegypti*. Vg synthesis in the mosquito fat body is stimulated by a blood meal and inhibited by removal of ovaries prior to blood feeding (Hagedorn and Fallon, 1973). The ovaries secrete the factor required for Vg production by the fat body, and this was found to be ecdysone (E), which is converted into the active form of the hormone 20-hydroxyecdysone (20E) (Hagedorn *et al.*, 1975). Remarkably, mosquitos and flies are the only insects known to use ecdysteroids as the key regulator of reproduction. How or why this shift away from JH- to ecdysteroid-mediated reproduction occurred in Diptera remains an enigma.

The endocrinology of vitellogenesis in mosquitos (suborder Nematocera) has been reviewed in detail for *A. aegypti* (Hagedorn, 1985; Raikhel, 1992a; Dhadialla and Raikhel, 1994; Sappington and Raikhel, 1998a; Raikhel *et al.*, 2003; Wang *et al.*, 2004) and investigated in *Culex pipiens*, *Aedes atropalpus*, and *Anopheles stephensi* (Hagedorn, 1985; Klowden, 1997). In *A. aegypti*, vitellogenesis is separated into four phases: previtellogenic (PV) preparation, arrest, yolk protein (YP) synthesis (vitellogenesis), and termination of vitellogenesis (Raikhel, 1992b; Dhadialla and Raikhel, 1994). A newly emerged female needs about 3 days to become competent for the physiological demands of intense vitellogenesis. During this phase, the fat body and ovary acquire responsiveness to 20E and become competent for blood meal-activated vitellogenesis and oogenesis, respectively (Flanagan *et al.*, 1977; Li *et al.*, 2000; Zhu *et al.*, 2003a). After 3 days of PV preparation, the fat body and ovary enter a state of arrest that persists until a blood meal is taken. During the YP synthesis stage, proteins are produced by the fat body and accumulated by developing oocytes (Raikhel, 1992a; Raikhel *et al.*, 2002). This massive synthesis peaks at around 24 h post-blood meal (PBM), then drops sharply, and terminates by 36–42 h PBM. The fat body undergoes remodeling, and the first batch of eggs completes chorion formation and is oviposited.

Titers of JH III and ecdysteroids in female *A. aegypti* are presented in **Figure 4**. During PV preparation, JH III titer increases and stabilizes during the arrest phase, and the CA remains active (Shapiro *et al.*, 1986). JH is required for the fat body to attain competence for YP synthesis in response to 20E. Following blood feeding, the JH III titer drops as a result of a rapid decrease in CA activity and elevation of JH esterase titer in the hemolymph (Shapiro *et al.*, 1986). Blood feeding triggers the release of the ovarian ecdysteroidogenic hormone (OEH) from the medial neurosecretory cells of the brain for up to 12 h postfeeding (**Figure 5**; Lea, 1972; Brown *et al.*, 1998). In response to OEH, the ovary produces ecdysteroids, and the hemolymph titer of ecdysteroids in female mosquitos is correlated with the rate of YP synthesis in the fat body (Hagedorn *et al.*, 1975). The ecdysteroid titers are only slightly elevated at 4 h PBM, rise sharply at 6–8 h PBM to a maximum level at 18–20 h PBM, and then decline to previtellogenic levels by 30 h PBM (Hagedorn *et al.*, 1975).

Numerous studies have clearly established that ecdysteroid control of YP synthesis is a central event in the blood meal-activated regulatory cascade

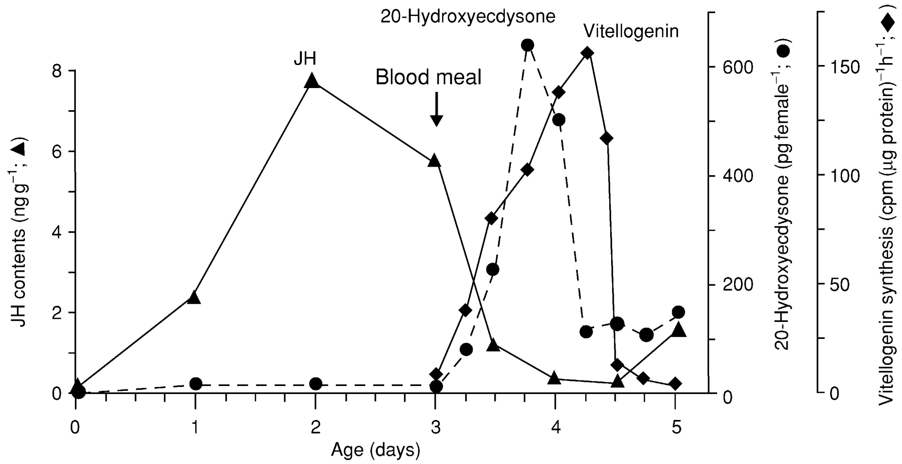


Figure 4 Juvenile hormone (JH) and 20-hydroxyecdysone titers and the level of Vg during the first reproductive cycle in the mosquito *Aedes aegypti*. (Based on data from Hagedorn, H., **1985**. The role of ecdysteroids in reproduction. Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 8. Pergamon, Oxford, pp. 205–261; and Dhadialla, T., Raikhel, A.S., **1994**. Endocrinology of mosquito vitellogenesis. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 275–281.)

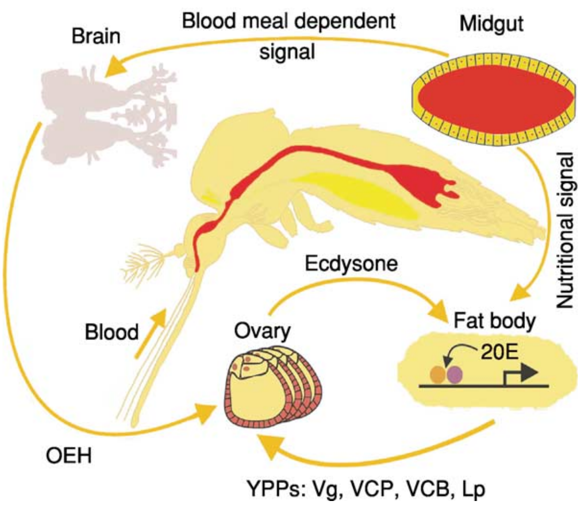


Figure 5 Schematic representation of the nutritional and hormonal activation of vitellogenesis in the mosquito *Aedes aegypti*. A blood meal results in a direct signal to the fat body, which is required to initiate yolk protein precursor (*YPP*) gene expression. The brain also receives a signal from the midgut, which activates medial neurosecretory cells to release a peptide hormone, ovarian ecdysteroidogenic hormone (OEH). OEH activates follicular cells of the primary follicles to produce ecdysone. Ecdysone is converted in the fat body to the active steroid hormone, 20-hydroxyecdysone (20E), which activates *YPP* gene expression via an ecdysone hierarchy. Yolk protein precursors, vitellogenin (Vg), vitellogenic carboxypeptidase (VCP), vitellogenic cathepsin B (VCB), and lipophorin (Lp) are secreted by the fat body into the hemolymph and selectively accumulated by developing oocytes.

leading to successful egg maturation (reviews: Hagedorn, 1985; Raikhel, 1992b; Dhadialla and Raikhel, 1994; Wang *et al*., 2004). Consistent with the proposed role of 20E in activating mosquito YP synthesis, experiments using fat body *in vitro* have shown that physiological doses of 20E (10^{-6} M) activate yolk protein precursor (*YPP*) genes, which are described below (Deitsch *et al*., 1995; Cho *et al*., 1999).

Molecular studies have demonstrated that although the ecdysteroid triggered regulatory hierarchies, such as those implicated in the initiation of metamorphosis, are reiteratively utilized in the control of mosquito vitellogenesis, the unique interplay of hierarchical factors is determined by the mosquito's biology (Raikhel *et al*., 2002, 2003; Wang *et al*., 2004). Of particular importance are the cyclicity of vitellogenic response and its dependence upon blood feeding. The demands for a very high level of expression of *YPP* genes, especially *Vg*, put additional restraints on the hormonal regulation of these genes.

The endocrine control of vitellogenesis in higher flies (suborder Cyclorrhapha) has been studied extensively in several species (reviews: Bownes, 1986, 1994, 2004; Raikhel *et al*., 2003) and is altered according to whether the species lays eggs in batches or continuously. In species laying eggs in batches, the maturation of a synchronous group of oocytes is controlled by changes in hormone levels, as described best for the housefly, *Musca domestica* (Adams and Filipi, 1983; Adams *et al*., 1985).

In this species, the fat body starts to synthesize YPs after the female fly feeds on a protein meal, and YP uptake also commences in the ovary. This phase is controlled by the circulating ecdysteroids, and the fat body later shuts down YP synthesis as egg development is completed. There is a strong correlation

between ecdysteroid titers in the hemolymph and the progress of vitellogenesis, as well as between ecdysteroid titers and the amount of YP produced in the fat body (Adams *et al*., 1985). Ovaries produce ecdysteroids *in vitro*, and ovariectomized flies have reduced ecdysteroid levels early in the cycle (Adams *et al*., 1985). In the autogenic strain of *M. domestica*, decapitation of females blocks Vg gene expression in fat body, but 20E restores accumulation of Vg mRNA. Moreover, males were induced to produce YPs in response to the hormone (Agui *et al*., 1991). JH acts at an early stage to prime the fat body for YP synthesis and ensures that the oocytes are arrested in a previtellogenic stage (Adams, 1974, 1981). Interestingly, Adams *et al*. (1989) also have shown that the response to 20E in male houseflies was enhanced by application of JH. Many aspects of the regulation of vitellogenesis in the housefly are quite similar to those described for *A. aegypti*.

In the blowfly, *Calliphora vicina*, YP-containing secretory granules become visible in the fat body when vitellogenesis is initiated by ingestion of a protein meal, but the granules are not detected in the fat body of ovariectomized females (Thomsen and Thomsen, 1974, 1978). Their appearance can be restored in ovariectomized females by injection of 20E (Thomsen *et al*., 1980). These observations have suggested the involvement of the ovary and ecdysteroids in the initiation of YP synthesis in the fat body of *C. vicina* and is reminiscent of the response of *A. aegypti* to a blood meal, which triggers the ovary to produce ecdysteroids and initiate a cycle of Vg production in the fat body.

In the blowflies, *Sarcophaga* and *Phormia*, non-protein-fed females respond to 20E by inducing YP synthesis in fat body (Huybrechts and De Loof, 1982). Unlike *C. vicina*, ovariectomized houseflies and blowflies still produce YPs indicating additional complexity of hormonal regulation in these insects (Engelmann and Wilkens, 1969; Jensen *et al*., 1981; Huybrechts and De Loof, 1982). Interestingly, male *Sarcophaga*, *Phormia*, and *Lucilia* also produce YPs in fat body in response to 20E injection (Huybrechts and De Loof, 1982). JH alone cannot induce YP synthesis in males in *Musca* or *Calliphora* (Adams, 1974; Huybrechts and De Loof, 1982; Adams *et al*., 1989).

In those species continuously laying eggs, each individual egg chamber differentiates, and its progress through vitellogenesis is modulated by hormonal signals connecting its development to environmental factors such as mating and food intake. This type of vitellogenesis is characteristic of *D. melanogaster*, and many related species. The regulation of vitellogenesis in *D. melanogaster* has been the subject of numerous studies using molecular, genetic, developmental, and physiological approaches (Bownes, 2004). In this species, adult sex determination (see **Chapter 1.5**) is the primary factor for correct expression of yolk protein genes. Once the genes are active in the female, the level of expression is modulated by ecdysteroids and JH in a complex way (Bownes *et al*., 1993; Bownes, 2004). JH seems crucial for vitellogenesis, especially in the ovary (Bownes *et al*., 1996; Soller *et al*., 1999), but it appears that JH does not directly affect the transcription of *yp* genes in the fat body. More recent data reported by Richard *et al*. (1998, 2001) points to a more prominent role for ecdysteroids in regulating vitellogenesis. It is interesting that in the Caribbean fruit fly, *Anastrepha suspensa*, in which the YPs are only produced in the ovary, there is no evidence of hormonal regulation by JH or 20E in females, and 20E injection is unable to stimulate YP synthesis in males (Handler, 1997).

3.9.2.2. Yolk Protein Precursors

3.9.2.2.1. Sites of yolk protein production In most insects, the fat body is the exclusive site for production of yolk protein precursors (YPPs) and, in others, the ovary is a complementary vitellogenic organ. **Table 1** provides a list of species in which fat body and ovarian YPP production have been reported, and in cyclorrhaphan Diptera, YPP synthesis occurs in both the fat body and the ovary. In some dipteran species, such as the stable fly *Stomoxys calcitrans*, *Anastrepha suspensa* and perhaps the tsetse fly *Glossina austeni*, the ovary is the exclusive site of YPP synthesis (review: Wyatt and Davey, 1996). Insect YPs are the subject of several recent reviews (Bownes and Pathirana, 2002; Sappington *et al*., 2002; Telfer, 2002; Tufail *et al*., 2004; Masuda *et al*., 2004; Yamahama *et al*., 2004).

3.9.2.2.2. The fat body In female insects, the main function of the fat body is to produce massive amounts of YPPs. Multiple lobes of this organ are distributed mainly in the abdomen and to a lesser extent in the thorax and head. In many insects, fat body consists of a single cell type, the trophocyte (= adipocyte), and in cockroaches and some other insects, mycetocytes and urate cells are also present (Kelly, 1985; Locke, 1998). The multilobed structure of this tissue enhances its interaction with hemolymph. Also, the fat body is responsible for intermediary metabolism; storage of carbohydrates,

Table 1 Vitellogenic organs in insects

Order–suborder	Species	Vitellogenic organ: Fat body	Ovary	Type of ovarioles
Zygentoma	*Thermobia domestica*[a]	X	X	Panoistic
Dictyoptera	*Blattella germanica*[b]	X		Panoistic
Dictyoptera	*Leucophaea maderae*[b]	X		Panoistic
Orthoptera–Caelifera	*Locusta migratoria*[b]	X		Panoistic
Orthoptera–Ensifera	*Acheta domesticus*[b]	X		Panoistic
Phasmida	*Bacillus rossius*[b]	X		Panoistic
Hemiptera	*Rhodnius prolixus*[c]	X	X	Telotrophic meroistic
Coleoptera	*Leptinotarsa decemlineata*[b]	X	X	Telotrophic meroistic
Coleoptera	*Coccinella septempunctata*[b]	X	X	Telotrophic meroistic
Hymenoptera	*Apis mellifera*[d]	X	X	Polytrophic meroistic
Lepidoptera	*Plodia interpunctella*[b]	X		Polytrophic meroistic
Lepidoptera	*Hyalophora cecropia*[b]	X		Polytrophic meroistic
Lepidoptera	*Manduca sexta*[b]	X		Polytrophic meroistic
Diptera–Nematocera	*Rhyncosciara americana*[b]	X		Polytrophic meroistic
Diptera–Nematocera	*Aedes aegypti*[b]	X		Polytrophic meroistic
Diptera–Brachycera	*Dacus oleae*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Drosophila melanogaster*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Musca domestica*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Sarcophaga bullata*[b]	X		Polytrophic meroistic
Diptera–Brachycera	*Calliphora vicina*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Stomoxys calcitrans*[b]		X	Polytrophic meroistic
Diptera–Brachycera	*Anastrepha suspensa*[e]		X	Polytrophic meroistic
Diptera–Brachycera	*Glossina austeni*[b]	?	X	Polytrophic meroistic

[a]Rousset and Bitsch (1993).
[b]See Valle (1993) for references.
[c]Melo *et al.* (2000).
[d]K.R. Guidugli, M.D. Piulachs, X. Belles, and Z.L.P. Simões, unpublished data.
[e]Handler (1997).

lipids, and proteins; and synthesis of hemolymph proteins. These functions are hormonally controlled and successively change in accordance with the demands of different life stages.

Trophocytes are equipped with abundant cytoplasmic organelles that accomplish a great variety of synthetic and secretory functions. The basal lamina enveloping the fat body allows diffusion of large oligomeric proteins with molecular sizes of over 300 000 (even up to 500 000 Da) and keeps the apical plasma membrane of trophocytes structurally differentiated. This apical membrane is highly infolded and resembles a plasma membrane reticular system in which secretory granules are being released by exocytosis (Locke and Huie, 1983; Raikhel and Lea, 1983; Dean *et al.*, 1985; Raikhel and Snigirevskaya, 1998; Mazzini *et al.*, 1989; Giorgi *et al.*, 2004).

3.9.2.2.3. Fat body derived yolk protein precursors

3.9.2.2.3.1. Vitellogenins In most female insects, the major constituent of protein yolk is Vg, a large, conjugated protein that is taken into oocytes and stored as vitellin (Vn). The amino acid sequence, structure, and composition of Vg are sufficiently conserved between insects and other oviparous animals to indicate origin from a common ancestral protein (Chen *et al.*, 1994; Sappington and Raikhel, 1998a; Sappington *et al.*, 2002) and may share homology with other, more distantly related lipoproteins (Blumenthal and Zucker-Aprison, 1987; Spieth *et al.*, 1991; Sappington *et al.*, 2002). Insect Vgs are encoded by mRNAs of 6–7 kb that are translated as primary products of ~200 kDa. Vg primary products have been characterized at the molecular level for Hemimetabola and Holometabola species (Sappington *et al.*, 2002; Tufail *et al.*, 2004).

The primary pre-proVg is cleaved into subunits (apoproteins), ranging from 50 to 180 kDa, by subtilisin-like endoproteases and proprotein convertases (Barr, 1991; Rouille *et al.*, 1995). These enzymes recognize a consensus motif (R/K)XX(R/K) preceding the cleavage site, and the motif is found in all known insect Vgs (Chen *et al.*, 1994; Sappington and Raikhel, 1998a; Sappington *et al.*, 2002; Tufail *et al.*, 2004). Vg convertase (VC) has been characterized from the vitellogenic fat body of

A. aegypti (Chen and Raikhel, 1996) and is a homolog of human and *D. melanogaster* furins and a *D. melanogaster* convertase (Barr *et al*., 1991; Roebroek *et al*., 1991, 1992a, 1992b; Hayflick *et al*., 1992).

In hemimetabolous insects like *L. maderae*, *P. americana*, and *Riptortus clavatus*, the Vg primary product is cleaved into large and small polypeptides, including polypeptides of ~80–110 kDa (**Figure 6**) (Della-Cioppa and Engelmann, 1987; Hirai *et al*., 1998; Tufail *et al*., 2001; Tufail and Takeda, 2002). It has also been reported that Vgs from some hemimetabolous insects, such as *L. maderae* and *R. clavatus*, are processed further in the oocyte (Hirai *et al*., 1998; Tufail and Takeda, 2002).

In holometabolous insects, the Vg primary product is cleaved into a single large and small polypeptide (**Figure 6**) (Dhadialla and Raikhel, 1990; Chen *et al*., 1994, 1996). As with other apocritan Hymenoptera, Vg is not cleaved in the parasitic wasp, *Pimpla nipponica* (**Figure 6**; Nose *et al*., 1997). In the fall armyworm moth, *Spodoptera frugiperda*, only a single Vg apoprotein was detected in hemolymph and ovarian extracts, although other lepidopteran Vgs normally are processed into two subunits (Hiremath and Lehtoma, 1997; Sorge *et al*., 2000).

Following extensive co- and posttranslational modifications, Vg subunits form high molecular weight oligomeric phospholipoglycoproteins (400–600 kDa) that are secreted into the hemolymph of females (Osir *et al*., 1986a; Wojchowski *et al*., 1986; Dhadialla and Raikhel, 1990; Sappington and Raikhel, 1998a; Giorgi *et al*., 1998; Tufail *et al*., 2004). Mature vitellogenins generally exist as oligomers, but monomeric molecules of about 300 kDa may exist in the cockroach *N. cinerea* (Imboden *et al*., 1987).

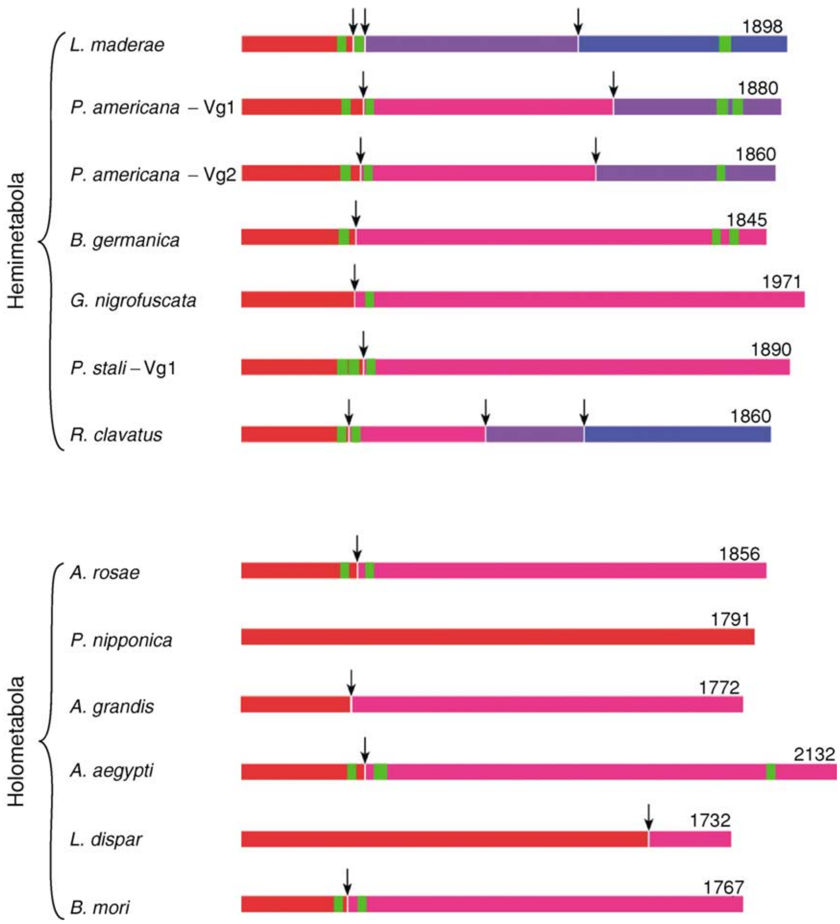


Figure 6 Schematic representation of the cleavage sites and polyserine domains in vitellogenins from 12 insect species. The arrows and white lines indicate the putative or determined cleavage sites following the consensus RXXR cleavage site sequence. The green segments show the polyserine stretches. Numbers indicate the amino acid residues deduced from the N-termini (excluding the signal peptides). Color code is used to indicate the number of vitellogenin subunits resulting from proteolytic cleavage. (Modified from Tufail, M., Raikhel, A.S., Takeda, M., **2004**. Biosynthesis and processing of insect vitellogenins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK.)

One to several *Vg* genes have been identified in different insect species (reviews: Telfer, 2002; Tufail *et al.*, 2004). In the silkworm *B. mori*, *Vg* is encoded by a single gene (Tufail *et al.*, 2004), and there are five *Vg* genes in the mosquito *A. aegypti* (Romans *et al.*, 1995). Regulatory regions of *Vg* genes that govern hormone dependent expression have been characterized in great detail for *A. aegypti* (Kokoza *et al.*, 2001) and only partially for *L. migratoria* and *B. germanica* (Wyatt *et al.*, 1984; Locke *et al.*, 1987; Belles, 2004).

3.9.2.2.3.2. Yolk polypeptides of the cyclorrhaphous Diptera In higher Diptera, the number of YPs ranges from one to five, and they are different from the Vg of other insects (review: Bownes and Pathirana, 2002). There are three major YPs of 46, 45, and 44 kDa (Barnett *et al.*, 1980; Bownes *et al.*, 1993) in *D. melanogasti* and up to five YPs ranging from 40 to 51 kDa in *C. erythrocephala* (Fourney *et al.*, 1982), *Neobellieria* (= *Sarcophaga*) *bullata* (Huybrechts and De Loof, 1982), *M. domestica* (Adams and Filipi, 1983), *A. suspensa* (Handler, 1997), *Phormia regina* (Zou *et al.*, 1988), *Ceratitis capitata* (Rina and Savakis, 1991), and eight other species of *Drosophila* (Bownes, 1980). The primary translation products of cyclorrhaphan YPs are close to the size of each mature polypeptide. Their post-translational modification includes glycosylation and phosphorylation and tyrosine sulfation (Brennan *et al.*, 1980; Minoo and Postlethwait, 1985; Baeuerle and Huttner, 1985).

Yolk protein genes have been characterized for *D. melanogaster* (Hung and Wensink, 1983; Garabedian *et al.*, 1987; Yan *et al.*, 1987), *C. capitata* (Rina and Savakis, 1991), and *C. erythrocephala* (Martinez and Bownes, 1994). Deduced amino acid sequences for the three YPs of *D. melanogaster* show that they are related to each other and to other cyclorrhaphan YPs. These YPs are not lipoproteins (see **Chapter 4.6**), likely constitute subunits of a larger native protein (Fourney *et al.*, 1982; Adams and Filipi, 1983; Zou *et al.*, 1988), and more closely resemble a family of vertebrate lipases and not the insect Vgs (Bownes *et al.*, 1988; Terpstra and Ab, 1988).

3.9.2.2.3.3. Additional yolk proteins secreted by the fat body A number of supplemental proteins are secreted by the fat body of vitellogenic females and selectively accumulated by developing oocytes. Typically, these YPs are minor yolk components but in some species can be as abundant as Vg (Telfer, 2002; Masuda *et al.*, 2004). Most are female-specific products, but some are found in the hemolymph of both sexes. They are likely to serve specialized functions necessary for embryonic development and, as yet, it is difficult to draw any unifying conclusions, since very few of these YPs have been characterized for insects.

3.9.2.2.3.3.1. Microvitellogenin Microvitellogenin (mVg) is a small yolk protein of 30 kDa (Pan, 1971; Kawooya *et al.*, 1986; Telfer and Pan, 1988; Pan *et al.*, 1994). In lepidopteran species, mVg is synthesized and secreted by the fat body (Cole *et al.*, 1987) and incorporated by ovarian follicles (Telfer and Kulakosky, 1984; Kulakosky and Telfer, 1989). *Manduca sexta* mVg is a monomeric protein with no detectable carbohydrate, lipid, or phosphate (Kawooya *et al.*, 1986). *Bombyx mori* produces multiple 30 kDa proteins that are the principal components of both male and female hemolymph during late larval and pupal stages (Izumi *et al.*, 1981) and constitute 35% of the egg total soluble protein (Zhu *et al.*, 1986). Deduced amino acid sequences from five *B. mori* mVg cDNA clones revealed their high similarity (Sakai *et al.*, 1988), and *M. sexta* mVg shares 70% sequence similarity with a *B. mori* mVg (Wang *et al.*, 1989). Furthermore, antigenic similarity between mVgs in *M. sexta* and *H. cecropia* (Kawooya *et al.*, 1986) supports the homology of lepidopteran mVgs.

3.9.2.2.3.3.2. Lipophorin as a yolk protein Lipids are a critical source of energy during insect embryogenesis (Beenakkers *et al.*, 1985) and can represent as much as 30–40% of the egg's dry weight (Troy *et al.*, 1975; Kawooya and Law, 1988; Briegel, 1990). Lipophorins (Lp) are lipid transport proteins in insects (see **Chapter 4.6**) and play a prominent role in the accumulation of lipids in insect oocytes (review: Antwerpen van *et al.*, 2004). Kinetic analyses have indicated that lipid transfer is affected by a saturable mechanism in both *M. sexta* (Kawooya and Law, 1988) and *H. cecropia* (Kulakosky and Telfer, 1990), thus indicating that lipid uptake occurs via receptor-mediated endocytosis. Specific ovarian receptors for Lp have been cloned from *L. migratoria* (Dantuma *et al.*, 1997), *A. aegypti* (Cheon *et al.*, 2001), and *B. germanica* (Ciudad, Piulachs, and Belles, unpublished data). In saturniid and sphingid moths, Lp is the second most abundant YP (Chino *et al.*, 1977b; Telfer *et al.*, 1991; Telfer and Pan, 1988), and it has been found in the yolk of the fall webworm *H. cunea* (Arctiidae) (Yun *et al.*, 1994). In mosquito eggs, Lp makes up only 3% of total egg proteins (Sun *et al.*, 2001). In *B. germanica*, Lp facilitates hydrocarbon uptake by maturing oocytes (Fan *et al.*, 2002).

3.9.2.2.3.3.3. Pro-proteases Yolk proteins are essential reserves of amino acid and other nutrients for the developing insect embryos, and sequential cleavage of YPs into smaller molecules occurs throughout embryogenesis (Zhu *et al.*, 1986; Yamashita and Indrasith, 1988; Masetti and Giorgi, 1989; Yamamoto and Takahashi, 1993; Izumi *et al.*, 1994; Takahashi *et al.*, 1996; Cho *et al.*, 1999; Yamahama *et al.*, 2004). This proteolytic degradation of YPs is probably regulated through a battery of proteases (see **Chapter 4.7**).

At present, two female-specific proenzymes are known to be deposited in the protein yolk of *A. aegypti*. One is a serine carboxypeptidase, vitellogenic carboxypeptidase (VCP), and it is a glycosylated 53 kDa protein secreted by the female fat body in synchrony with vitellogenin (Hays and Raikhel, 1990). An antigenically similar protein of the same size occurs in the yolk, and the enzyme is activated during embryogenesis by cleavage to a 48 kDa polypeptide (Cho *et al.*, 1991). A 44 kDa fat body protein, vitellogenic cathepsin B (VCB), with sequence similarity to vertebrate cathepsin B is also incorporated into the yolk of *A. aegypti* (Cho *et al.*, 1999). It is converted to 42 kDa after internalization in developing oocytes, and then to 33 kDa in developing embryos, coinciding with the onset of YP degradation. The 33 kDa protein degrades mosquito YPs *in vitro*. Secretory pathways for VCP and VCB in the fat body and their endocytosis in the oocyte were shown by immunocytochemistry to be the same as those of vitellogenin (Snigirevskaya *et al.*, 1997a). Notably, they are deposited in the amorphous, peripheral layer of the yolk bodies surrounding the central core of the vitellin crystal (Snigirevskaya *et al.*, 1997b).

An acid cysteine proteinase (see **Chapter 4.7**) was first purified from *Bombyx* eggs (Kageyama and Takahashi, 1990) and named *Bombyx* cysteine proteinase (BCP). It has broad substrate specificity and hydrolyzes various protein substrates, including *B. mori* yolk proteins. Since BCP accumulates in hemolymph, it is secreted by the fat body as shown by Northern blots. Ovarian follicle cells also synthesize the enzyme, as established by Northern blot and its synthesis from ovarian RNA (Yamamoto *et al.*, 2000).

3.9.2.2.3.3.4. Other fat body products used as yolk proteins For several insects, there are reports of other proteins that are incorporated into yolk. In *L. migratoria* females, 21 kDa and 25 kDa proteins are produced by the fat body in synchrony with the synthesis of Vg (Zhang *et al.*, 1993; Zhang and Wyatt, 1996) and accumulate in oocytes along with Vg. These additional yolk proteins are significant constituents of the yolk in *L. migratoria* eggs. In the lepidopteran *M. sexta*, a blue biliprotein (insecticyanin) composed of four 21.4 kDa subunits is a component of larval hemolymph and is present in eggs (Chino *et al.*, 1969; Kang *et al.*, 1995). An arylphorin-like cyanoprotein that is a hemolymph storage hexamer is also deposited in eggs of the bean bug *R. clavatus* (Chinzei *et al.*, 1990; Miura *et al.*, 1994). The incorporation of these pigmented proteins may help to conceal eggs from predators (Chino *et al.*, 1969). The iron transport protein, transferrin (see **Chapter 4.10**) has been shown to be selectively deposited in yolk of the flesh fly *Sarcophaga peregrina* (Kurama *et al.*, 1995) and the bean bug *R. clavatus* (Hirai *et al.*, 2000). Hemolymph and oocytes of *Rhodnius prolixus* also contain a 15 kDa heme binding protein (Oliveira *et al.*, 1995). Eggs of the stick insect *C. morosus* contain a minor yolk protein that is secreted by the fat body and sulfated by the follicle cells (Giorgi *et al.*, 1995).

3.9.2.2.4. Yolk proteins synthesized by ovarian follicle cells Irrespective of insect ovary type, only the follicle cells in egg chambers engage in the production of proteins that are utilized as YPs. As demonstrated for several insects, follicle cells secrete a protein that has a similar antigenic reactivity and subunit composition as the Vg produced by fat body. In the firebrat *T. domestica*, there is the dual origin of YP from fat body and ovaries (Rousset and Bitsch, 1993) (**Table 1**). The synthesis of YPs by both tissues in adult females of this order, where molting and reproduction alternate, indicates that it could be an ancestral condition. YP production has also been observed in ovarian follicles of the heteropteran *R. prolixus* (Melo *et al.*, 2000) and two coleopterans, *C. septempunctata* (Zhai *et al.*, 1984) and *L. decemlineata* (Peferoen and De Loof, 1986). In the honeybee, *A. mellifera*, Vg expression has been reported in the fat body (Piulachs *et al.*, 2003), but a more recent study has revealed that it is also expressed in the ovaries (K.R. Guidugli, M.D. Piulachs, X. Belles, and Z.L.P. Simões, unpublished data). These findings in *R. prolixus* and *A. mellifera* suggest the importance of reassessing the vitellogenic role of the ovary in more species.

In higher flies (suborder Cyclorrhapha), the ovarian origin of YPs was first detected in *D. melanogaster* (Bownes and Hames, 1978), and in this and other species, these YPs have been investigated extensively (see Section 3.9.2.2.3.2). Follicle cells were identified as the site of synthesis in *D. melanogaster*

by their ability to secrete YPs when manually separated from follicles labeled with [^{35}S]methionine, and by *in situ* hybridization to contain YP mRNAs (Brennan *et al.*, 1982). Follicle cells are implicated in production of YPs in *C. erythrocephala* (Rubacha *et al.*, 1988) and in *S. bullata* (Geysen *et al.*, 1986). In the stable fly, *S. calcitrans* (Houseman and Morrison, 1986; Chen *et al.*, 1987), the Caribbean fruit fly *A. suspensa* (Handler and Shirk, 1988; Handler, 1997), and the tsetse fly, *glossina austeni* (Huebner *et al.*, 1975), female specific proteins do not occur in the hemolymph of reproductive adults, thus indicating that YPs are synthesized only in the ovaries.

In some cyclorrphan flies, YP genes are expressed differently in the fat body and follicular epithelium. In *C. erythrocephala*, ovaries produced 51 and 49 kDa YPs, while fat body secreted a 46 kDa YP and possibly a different 49 kDa YP (Fourney *et al.*, 1982). In *D. melanogaster*, the two tissues secrete the same three YPs, but the smallest one, YP3, is underrepresented in ovarian synthesis (Brennan *et al.*, 1982; Isaac and Bownes, 1982). Ovarian and fat body YPs are mixed in the eggs of these flies.

In several species of Lepidoptera, follicle cells secrete proteins that are incorporated into yolk and may account for up to 25% of the total soluble protein, as in mature eggs of *B. mori* (Zhu *et al.*, 1986). In the moth *H. cecropia*, a 55 kDa protein is present in the intercellular spaces and yolk bodies (Bast and Telfer, 1976). Follicles of *B. mori* produce an ovary specific protein of 225 kDa with three 72 kDa subunits (Ono *et al.*, 1975; Indrasith *et al.*, 1988; Sato and Yamashita, 1991a); one of which is converted to a 64 kDa polypeptide by egg maturation. Sequencing of a cDNA clone revealed its similarities to human gastric and rat lingual lipases, especially to a noncatalytic lipid binding domain (Inagaki and Yamashita, 1989; Sato and Yamashita, 1991b). Two follicle specific YPs have also been isolated from *M. sexta* (Tsuchida *et al.*, 1992). One is a 130 kDa protein consisting of two glycosylated and phosphorylated 65 kDa subunits, similar to those of the follicle cell derived yolk protein of *B. mori*. The second is a slightly larger 140 kDa protein that is glycosylated but not phosphorylated. In the pyralid moth *P. interpunctella*, two yolk polypeptides originate in the follicle cells, and the 235–264 kDa protein with subunits of 69 and 33 kDa is an ortholog of the egg specific protein in *B. mori* (Shirk *et al.*, 1984; Bean *et al.*, 1988; Shirk and Perera, 1998). Sequences for a cDNA clone encoding YP4 of *P. interpunctella* (Perera and Shirk, 1999) showed it to be an ortholog of a follicular product in *G. mellonella* (Rajaratnam, 1996).

3.9.2.3. Mechanisms of Juvenile Hormone Action in Vitellogenesis

3.9.2.3.1. Induction of vitellogenin gene expression Vitellogenic action of JH is one of the hallmarks of insect reproduction. Although JH is known to initiate vitellogenesis in many insects (see **Chapter 3.7**), its precise mechanism of action is poorly understood. Studies of the induction of *Vg* gene transcription by JH have focused on two species: the migratory locust, *L. migratoria*, and the German cockroach, *B. germanica* (Wyatt, 1991, 1997; Belles, 2004).

Two *Vg* genes have been partially characterized in *L. migratoria*, and studies at the molecular level have demonstrated the absence of detectable Vg mRNA in the fat body of JH deprived specimens (Dhadialla *et al.*, 1987). Transcription of both *Vg* genes is coordinately induced *in vivo* by JH or JHA (Dhadialla *et al.*, 1987; Glinka and Wyatt, 1996), but allatectomized locust females have a low sensitivity to JH, since doses up to 100 μg of JH III do not induce Vg production. JHAs, such as methoprene or pyriproxyfen, are more potent and stable (Wyatt *et al.*, 1996). When JHA is administered to females chemically allatectomized with the allatocide precocene within 1 day of adult emergence, there is a lag of 12–24 h before Vg mRNA can be detected (Edwards *et al.*, 1993). This lag period can be shortened by prior administration of a subeffective dose of JH or JHA that is insufficient by itself to induce *Vg* gene transcription. This dose likely primes the fat body cells for an accelerated response to a subsequent effective dose (**Figure 7**) (Edwards *et al.*, 1993; Wyatt *et al.*, 1996). The lag period between JHA treatment and vitellogenesis can be extended by inhibition of protein synthesis with cycloheximide (**Figure 7**) (Edwards *et al.*, 1993). These results suggest that the action of JH on *Vg* genes in locust females is indirect and requires the synthesis of protein factors involved in transcription. In this respect, the priming action of JH on fat body for a vitellogenic response is similar to that in mosquitos in which the molecular nature of this JH action has been recently elucidated (Zhu *et al.*, 2003a).

Studies on the regulation of vitellogenesis in *B. germanica* also were facilitated by the cloning of a Vg cDNA (Martin *et al.*, 1998; Comas *et al.*, 2000). Experiments *in vivo* with allatectomized females have shown that Vg mRNA can be detected as early as 2 h after treatment with 1 μg of JH III, whereas Vg protein can be detected 2 h later (**Figure 8**). In addition, dose–response studies show that doses of 0.1, 1, and 10 μg of JH III induced Vg

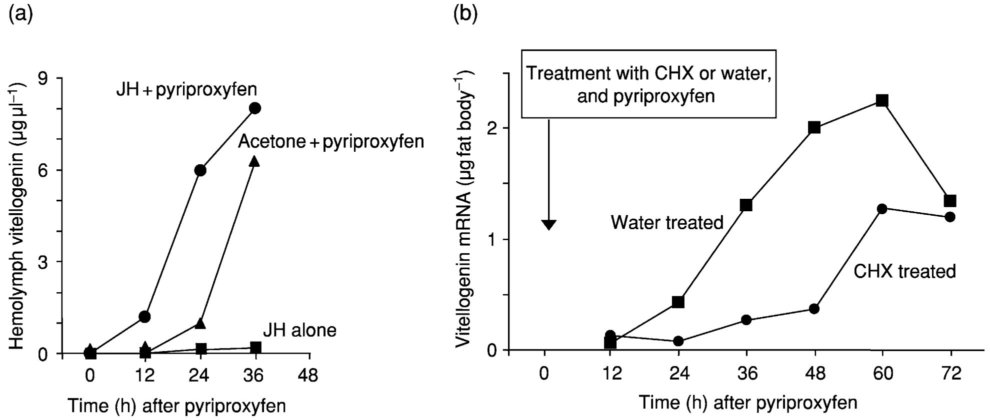


Figure 7 Induction of vitellogenin transcription in chemically allatectomized females of the migratory locust, *Locusta migratoria*. (a) Shortening of the response lag time by administration of a subeffective dose of JH. Specimens pretreated with low doses of JH III in acetone (four applications of 10 μg each, over 48 h) and then treated with 10 μg of pyriproxyfen, synthesize vitellogenin earlier (circles) than those receiving an equivalent treatment of acetone alone and pyriproxyfen (triangles). Equivalent pretreatment with JH III, but no pyriproxyfen, did not induce detectable vitellogenin synthesis (squares). (Data from Wyatt, G.R., Braun, R.P., Zhang, J., **1996**. Priming effect in gene activation by juvenile hormone in locust fat body. *Arch. Insect Biochem. Physiol. 32*, 633–640.) (b) Inhibitory effects of cycloheximide (CHX) upon vitellogenesis induced by pyriproxyfen. Insects were treated with cycloheximide (62 μg) in water or with water alone, and 1 h later treated with 10 μg pyriproxyfen. Cycloheximide delayed vitellogenin transcription by about 1 day, which is approximately equal to the duration of inhibition of protein synthesis. (Reproduced with permission from Edwards, G.C., Braun, R.P., Wyatt, G.R., **1993**. Induction of vitellogenin synthesis in *Locusta migratoria* by the juvenile hormone analog, Pyriproxyfen. *J. Insect Physiol. 39*, 609–614.)

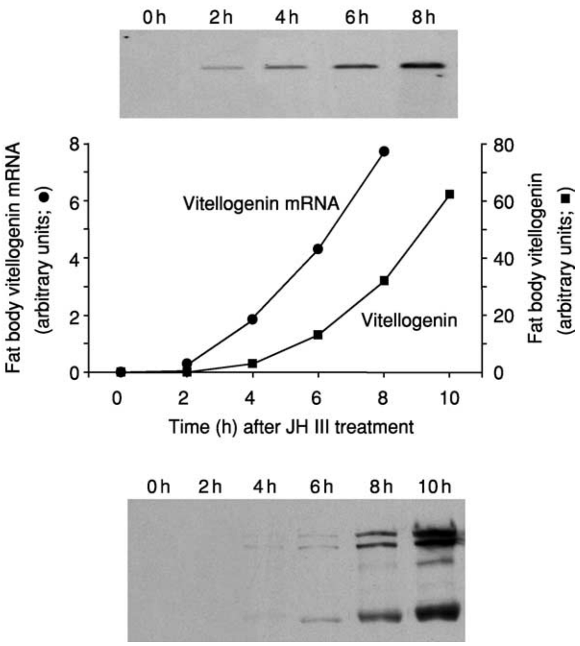


Figure 8 Production of vitellogenin mRNA and vitellogenin protein *in vivo* after JH treatment of allatectomized females of *Blattella germanica*. A dose of 1 μg of JH III was topically applied to 48 h old allatectomized females; the fat body was dissected 2, 4, 6, 8, or 10 h later, and analyzed for vitellogenin mRNA (Northern blot, above), or vitellogenin protein (Western blot, below). (Reprinted with permission from Comas, D., Piulachs, M.D., Belles, X., **1999**. Fast induction of vitellogenin gene expression by juvenile hormone III in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Insect Biochem. Mol. Biol. 29*, 821–827, with permission from Elsevier.)

synthesis, but not 0.01 μg (Comas *et al.*, 1999, 2001). Cycloheximide applied to fat body *in vitro* abolishes the vitellogenic effects of JH (Comas *et al.*, 2001), again suggesting that the effect of JH on the *Vg* gene involves the synthesis of protein factors involved in transcription.

3.9.2.3.2. Potential response elements in JH-dependent genes related to vitellogenesis Results from the above studies of JH action on vitellogenesis in the locust and cockroach led to the hypothesis that JH may affect proteins belonging to the nuclear hormone receptor superfamily (see **Chapters 3.5** and **3.6**), which in turn would mediate transcription of Vg genes (Wyatt *et al.*,1996; Belles, 2004; see **Chapter 3.5**). Signature response elements for the binding of the putative JH transcription factors could be found within the regulatory regions of these genes, in the same way as those for the ecdysteroid receptor (EcR) and related proteins.

Analysis of the *jhp21* gene of *L. migratoria* revealed the partially palindromic, 13-nucleotide motif AGGTTCGAG^{A}/$_{T}$CCT that is found in three copies from the transcription start point (Zhang and Wyatt, 1996). This motif is suggestive of a hormone response element, given that it is similar to the consensus ecdysteroid response element (see **Chapter 3.5**), as defined by Jiang *et al.* (2000). Furthermore, this nucleotide motif is very similar to the canonical sequence IR-1 (AGGTCAATGACCT), a

consensus-inverted repeat with a single nucleotide spacer that is recognized by the ecdysteroid receptor and that confers JH responsiveness upon genes in mammalian cells that contain farnesoid x receptor (FXR), a member of the nuclear receptor superfamily (Forman *et al*., 1995).

3.9.2.3.3. Molecular mechanisms in juvenile hormone action Hypothesized JH specific DNA motifs require functional characterization, either by demonstrated activity in gene regulation or by binding to a known JH receptor or a transcription factor involved in JH response. Significant progress in this regard has been made on the 13-nucleotide motif AGGTTCGAG$^{A}/_{T}$CCT from the upstream region of the *jhp21* gene in *L. migratoria*. A cell-free transcription system was developed that uses nuclear proteins extracted from locust fat body, and transcription is measured with reporter constructs containing a short DNA sequence, lacking G in the transcribed strand, fused to the promoter sequence of interest (Zhang *et al*., 1996). When transcription is carried out with the transcription terminator *O*-methyl-GTP and not GTP, only the DNA sequence lacking G is transcribed, and the transcript can be resolved by gel electrophoresis. With nuclear extracts from untreated adult female *L. migratoria*, constructs that include the promoter region of the vitellogenin or *jhp21* genes are transcribed, as is also the nonspecific promoter of the adenovirus major late antigen (*AdML*), which is used as a positive control. However, extracts from precocene treated females, while still transcribing *AdML*, fail to transcribe from the *jhp21* promoter (Zhang *et al*., 1996). After transcription was found to be specific for the reproductive female fat body, truncated constructs of the promoter region of the *jhp21* gene were used to show that the DNA between nucleotides −1056 and −1200 from the transcription start site strongly enhanced transcription. In synthetic constructs, the incorporation of two tandem copies of the 15 nucleotide element GAGGTTCGAGACCTC (found at −1152) stimulated transcription as strongly as the native 145 nucleotide sequence, whereas the 15 nucleotide element, mutated at four positions and inserted into two copies, was inactive (Zhang *et al*., 1996). These results suggested that the sequence GAGGTTCGAGACCTC might be a JH response element. Tests of nuclear extracts for specific protein binding to the putative JH response element with the electrophoretic mobility shift assay demonstrated the occurrence of a specific DNA binding protein in extracts from JH exposed fat body, whereas it was found to be lacking in JH deprived tissue (Zhang *et al*., 1996). Furthermore, Zhou *et al*. (2002) have reported that this binding shows a preference for the inverted repeat GAGGTTC in the left half-site and that it is abolished by phosphorylation catalyzed by a protein kinase C present in the nuclear extracts. These results further support the identification of a putative JH response element that is bound by a transcription factor brought to an active state by JH. It is still uncertain whether the binding protein may be the nuclear JH receptor, a dimerization partner of the receptor, or another protein factor involved in the transcriptional process (Wyatt, 1997).

A putative JH response element has been identified in the JH esterase gene (*Cfjhe*) from the spruce budworm, *C. fumiferana* (Kethidi *et al*., 2004). This 30 bp region contains two conserved hormone response element half-sites separated by a 4 nucleotide spacer similar to the direct repeat 4 (DR-4). The response element designated as JHRE is located between −604 and −574 of the *Cfjhe* promoter and is sufficient to support JH I induction. At the same time, the same region is responsive for 20E mediated repression of this gene. When a luciferase reporter was placed under the control of JHRE, a minimal promoter was induced by JH I in a dose and time dependent manner in a cell transfection assay. Moreover, the gel-retardation assay revealed the presence of a JHRE binding protein in the nuclear extract isolated from JH I treated CF-203 cells. The results described in the following sections suggest the hypothesis that JH acts via a nuclear receptor mode of action at the transcriptional level.

3.9.2.3.4. Is there a juvenile hormone nuclear receptor? Early experiments studying farnesol related molecules as possible ligands for the retinoid x receptor (RXR)–FXR mammalian receptor complex showed that JH III activated this complex (although it did not activate FXR or RXR alone), whereas methoprene did not (Forman *et al*., 1995). Curiously enough, an independent study reported that RXR alone could be activated by methoprene acid but not by JH III (Harmon *et al*., 1995). Although the physiological significance of these experiments remained unclear, attention has been drawn to *Ultraspiracle* (USP) (insect ortholog of RXR and obligatory dimerization partner for the EcR) as a possible candidate for a JH receptor.

Work along this line was published by Jones and Sharp (1997), who reported that JH at micromolar concentrations binds to *D. melanogaster* USP, modifying its conformation and inducing USP dependent transcription, whereas yeast two-hybrid assays indicated that JH could promote USP homodimerization (see **Chapters 3.5** and **3.7**). In response to ligand binding, *D. melanogaster* USP undergoes

conformational change to form a multihelix hydrophobic groove for recruitment of transcriptional coactivators (Jones and Jones, 2000). In addition, JH III binds to the ligand binding pocket of USP, and application of JH III to cells activates a transfected reporter construct containing a JH esterase core promoter and a DR12 hormone response element. The DR12 element confers enhanced transcriptional JH responsiveness, and it binds with specificity to recombinant USP (Jones *et al*., 2001; Xu *et al*., 2002).

In more primitive insects, USP has a ligand binding domain closer to vertebrate RXR than to dipteran or lepidopteran USP (Bonneton *et al*., 2003). This has raised the question of whether an RXR ortholog of primitive insects might bind JH with higher affinity than USPs of higher insects. This possibility has been explored in *L. migratoria*, in which two isoforms of RXR are known: LmRXR-L is more closely related to vertebrate RXR and LmRXR-S has a deletion of 66 nucleotides in the ligand binding domain (Hayward *et al*., 2003). Both LmRXR-S and LmRXR-L formed heterodimers with locust LmEcR *in vitro*, which bound the active ecdysteroid ponasterone A, as expected. However, neither LmRXR isoform alone nor LmRXR heterodimerizing with LmEcR bound JH III at nanomolar concentrations (Hayward *et al*., 2003). Parallel work in *B. germanica* has led to cloning of the orthologs BgRXR-L and BgRXR-S. Interestingly, despite the highly fluctuating levels of circulating JH III, mRNA levels of both BgRXR-L and BgRXR-S isoforms in the fat body remain constant throughout the vitellogenic cycle (Maestro, Martin and Belles, unpublished data).

If either insect USP or RXR is a JH receptor, it behaves in a very different way to other members of the steroid/thyroid hormone nuclear receptor family, especially since its binding of JH is not saturable and is of relatively low affinity. One possibility is that USP or RXR form a heterodimer with a nuclear receptor other than EcR and that this complex might play the role of a JH receptor. Another possibility is inspired by promiscuous nuclear receptors like RXR, PPAR, or PXR of vertebrates (Harmon *et al*., 1995; Zomer *et al*., 2000; Watkins *et al*., 2001), which are activated by a variety of effectors and have K_d values in the micromolar range. These nuclear receptors have been considered as chemical sensors rather than canonical specific receptors (Watkins *et al*., 2001), a concept that could be extended to the interaction of JHs with the RXR/USP of insects.

The *Methoprene resistant* (*Met*) gene of *Drosophila* may also be a useful model for gaining insight into the molecular action of JH. Mutant *D. melanogaster* resistant to methoprene were generated in the 1980s, and these *Met* mutants were also highly resistant to JH III, JH bisexpoxide (JHB_3), and other JHAs, but not to conventional insecticides. Biochemical work later showed the presence of an 85 kDa protein in various tissues of wild-type *D. melanogaster* that bound specifically to JH III with high affinity ($K_d = 6.7$ nM), whereas in *Met* mutants the affinity of this protein for the hormone was lower by six-fold. In addition, JH III stimulation of protein synthesis in male accessory glands was clearly less pronounced in *Met* mutants than in wild-type flies, which suggested that *Met* is involved in the mechanism of action of JH (Shemshedini and Wilson, 1990; Shemshedini *et al*., 1990; see **Chapter 3.7**). Sequencing of the corresponding gene showed that *Met* is a member of the bHLH-PAS family of proteins that behave as transcriptional regulators (Ashok *et al*., 1998). These proteins show a basic helix–loop–helix domain (bHLH), which is characteristic of a number of transcription factors, and a PAS domain, named after the first members of the family: the products of the genes *period* (*Per*) (see **Chapter 4.11**) and *single-minded* (*Sim*) from *D. melanogaster*, and the aryl hydrocarbon receptor (AhR) and the Ah receptor nuclear translocator (ARNT) from vertebrates. Interestingly, *Per* and *Sim* have dimerization partners but no known ligands. AhR and ARNT form a functional dimer in the presence of a ligand, which upregulates a series of genes, the products of which metabolize foreign chemicals. Although *Met* mutants show no serious problems during embryonic and larval development or during metamorphosis (Wilson and Ashok, 1998), it is tempting to consider that the *Met* product could be involved in the transcription of JH-target genes, either by being the JH receptor or a transcription factor of the cascade triggered by JH.

These results also support the hypothesis that *Met* could be a receptor coactivator for the JH receptor, playing a role similar to that reported for the steroid hormone coactivator-1, the disruption of which results in hormone resistance in mice (Xu *et al*., 1998). In this context, it is worth noting that the steroid hormone coactivator-1 also belongs to the bHLH-PAS family and that it and *Met* have the LXXLL motif, which in vertebrate transcriptional activators is needed for binding to nuclear receptors.

The identification of JH response elements in JH responsive genes might be the most promising approach to the characterization of the JH receptor. This strategy is being followed for the *jhp21* gene in *L. migratoria* and for the *cfjhe* gene in

C. fumiferana, with the encouraging results described above. Also promising has been the discovery of JH responsive genes in *D. melanogaster*, such as *JhI-1*, *JhI-21*, *JhI-26*, and *minidiscs* (*mnd*) (Dubrovsky, 2002), whose promoter regions may provide the needed JH response elements more easily, given the genetic, conceptual, and technical background available in studies of this fly. Along this line, promoter regions of the *JhI-26* and *mnd* genes, which are directly inducible by JH, have already been tested for their ability to confer JH inducibility on heterologous reporter genes transfected to S2 cells. Constructs prepared with a 3 kb fragment from a region upstream of either the *JhI-26* or the *mnd* transcription initiation site cloned in front of the *hsp70-lacZ* reporter gene have served for the first experiments. After transfection and methoprene induction, the *JhI-26* promoter insertion conferred between a 6- and 15-fold increase in the 8-galactosidase activity, whereas that of *mnd* conferred between a three- and fourfold increase (Dubrovsky, 2002). The results suggest that these 3 kb promoter fragments contain JH response elements, and thus further study of shorter fragments should lead to the determination of their role. In the meantime, *in silico* studies on the promoter fragments used in the transfections have revealed several motifs showing a significant similarity with the estrogen response element (ERE), suggesting that they can be considered putative response elements (Dubrovsky, 2002).

3.9.2.4. Molecular Mechanisms of Ecdysteroid Action in Vitellogenesis

The early 20E-inducible gene *E75* has been implicated in the JH signaling pathway (Dubrovsky *et al.*, 2004). JH induces the E75A orphan nuclear receptor in *D. melanogaster.* The induction by JH is rapid and does not require protein synthesis, indicating direct action of JH on the *E75* gene. *E75A* mRNA is reduced in ovaries of *apterous*[4] *Drosophila* mutant adults defective in JH secretion. However, *E75A* mRNA expression can be rescued by topical JH analog application. Furthermore, ectopic expression of E75A is sufficient to perform several functions of the JH signaling pathway. Ectopic E75 can downregulate its own transcription and potentiate the JH inducibility in the JH gene, *JhI-21*. In the presence of JH, E75A can also repress 20E activation of early genes including *Broad*. The occurrence of a putative response element for E75 (the motif TGACCAAATT) (Belles, 2004) in the promoter region of the Vg gene of *B. germanica*, led to clone the three isoforms of this transcription factor, BgE75A, BgE75B, and BgE75C, in this cockroach. The pattern of expression of the three isoforms and results of RNAi experiments suggest that JH enhances E75 induction by 20E and that E75 is involved in the modulation of the cycles of oocyte maturation in *B. germanica* (D. Mañé, D. Martin and X. Belles, unpublished).

In contrast to JH, the molecular mechanisms governing 20E regulation of reproductive events have been elucidated in detail for the yellow fever mosquito, *A. aegypti*, and the fruit fly, *D. melanogaster* (see **Chapters 3.5** and **3.6**). Owing to variations in their reproductive biology, studies of these insects often cover different events in female reproductive endocrinology, thus providing complementary information.

3.9.2.4.1. Genetic regulation of ecdysteroid response Ashburner *et al.* (1974) proposed a hierarchical model for the genetic regulation of polytene chromosome puffing by 20E (see **Chapter 3.5**). According to this model, the binding of 20E to its cognate receptor triggers the expression of a small set of early genes, which in turn activate expression of a large set of late genes and which, meanwhile, repress their own transcription. Subsequent genetic and molecular biological studies have revealed events underlying this ecdysteroid regulatory hierarchy in *D. melanogaster* (Thummel, 1996, 2002; Bender, 2003). Responses to ecdysteroids are mediated by the EcR complex, which consists of two members of the nuclear receptor gene family, EcR and USP, the latter being the retinoid X receptor homolog (Koelle *et al.*, 1991; Yao *et al.*, 1992, 1993). Upon binding 20E, this heterodimer recognizes a sequence specific DNA motif, ecdysteroid response element (EcRE), and directly induces the transcription of a small set of so-called early genes. These early genes encode mainly transcription factors, including E74, E75, and Broad-Complex (BR-C), of the Ets, nuclear receptor, and zinc-finger type, respectively, that transfer and amplify the hormonal signal through the regulation of numerous late ecdysone-responsive genes that specify stage and tissue specific effects of 20E (Thummel, 1996, 2002). The *E74*, *E75*, and *BR-C* early genes each encode a family of transcription factors by use of multiple promoters and alternative splicing. Biochemical and mutational analysis has shown that each of these genes acts as a 20E induced transcription factor and is required for the hormonal response in target tissues (Thummel, 2002; Bender, 2003).

3.9.2.4.2. Ecdysteroid receptor (EcR) The diversity of cellular responses to 20E may require particular combinations of EcR isoforms. Indeed,

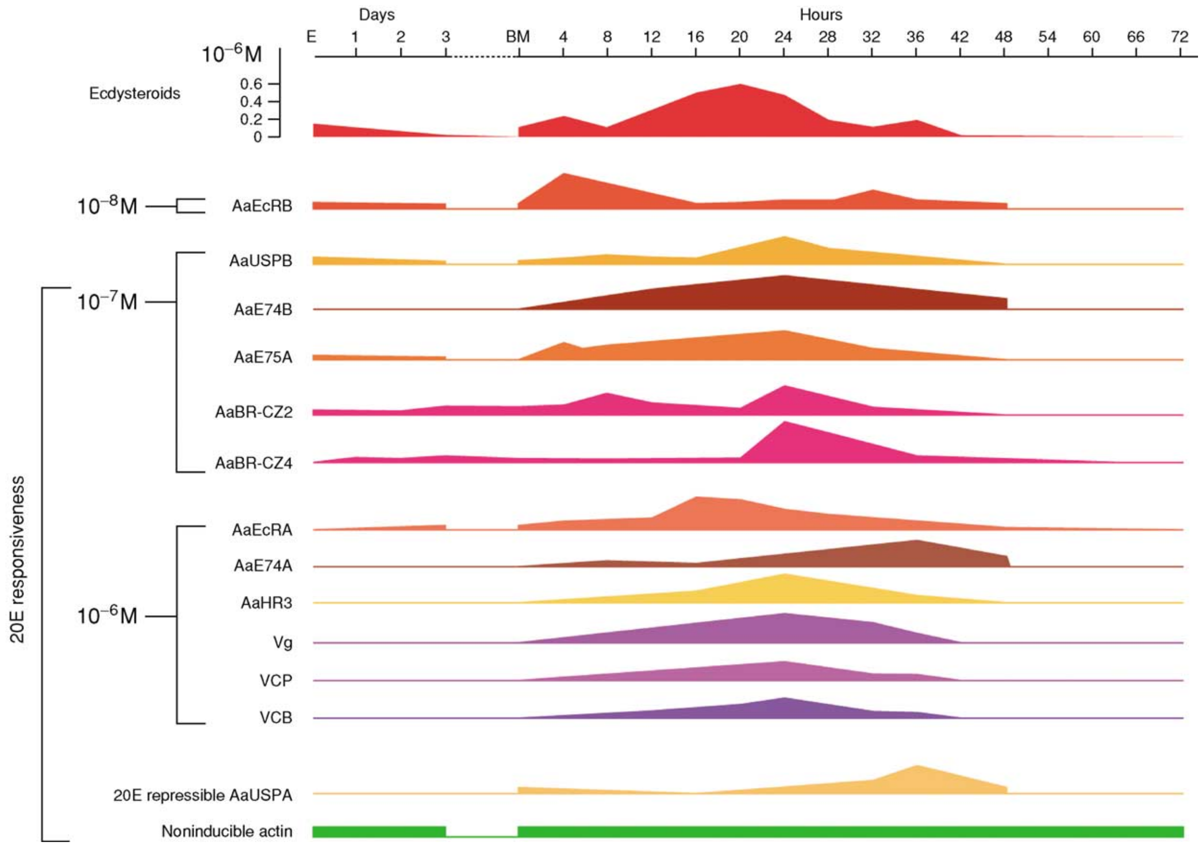


Figure 9 Expression profiles and hormone responsiveness of the key factors of the 20-hydroxyecdysone (20E) regulatory hierarchy and their target genes, yolk protein precursors (*YPP*), during vitellogenesis in *Aedes aegypti*. Hormone titers of 20E in *A. aegypti* females (Hagedorn *et al.*, 1975; Shapiro *et al.*, 1986). Transcript profiles and sensitivities of the transcription factors to 20E (AaEcRA and AaEcRB, AaUSPA and AaUSPB, AaE74A and AaE74B, and AaHR3) and the late genes (*Vg*, *VCP,* and *VCB*) were determined by Northern or RT-PCR analyses. Adapted from Cho and Raikhel (1992; *Vg*), Cho *et al.* (1991; *VCP*), Cho *et al.* (1999; *VCB*), Wang *et al.* (2002; AaEcRA and AaEcRB), Wang *et al.* (2000b; AaUSPB and the 20E repressible AaUSPA), Sun *et al.* (2002; AaE74A and AaE74B), Pierceall *et al.* (1999; AaE75A), Kapitskaya *et al.* (2000; AaHR3), and L. Chen, J. Zhu, and A.S. Raikhel (unpublished data; BR-C isoforms Z2 and Z4). E, eclosion; BM, blood meal. (Modified with permission from Sun, G.Q., Zhu, J.S., Raikhel, A.S., **2004**. The early gene E74B isoform is a transcriptional activator of the ecdysteriod regulatory hierarchy in mosquito vitellogenesis. *Mol. Cell. Endocrinol. 218*, 95–105.)

expression of specific *D. melanogaster* EcR isoforms could be correlated with patterns of responses of particular cell or tissue types to 20E (Talbot *et al.*, 1993). Genetic studies have confirmed that different EcR proteins are functionally distinct, with the greatest difference being between the EcR-A and EcR-B isoforms and the greatest overlap being between the EcR-B1 and EcR-B2 functions (Bender *et al.*, 1997; Schubiger *et al.*, 1998; Lee *et al.*, 2000; see **Chapter 3.5**).

Cloning of the *A. aegypti* EcR isoforms, AaEcR-A and AaEcR-B, has facilitated evaluation of their expression during mosquito vitellogenesis. Significantly, transcripts of both isoforms exhibit different patterns of expression after a blood meal triggers fat body vitellogenesis in female mosquitos (**Figure 9**). The AaEcR-B transcript level rose sharply by 4 h PBM, which coincides with the small ecdysteroid peak, and then declined and reached its lowest level at 16–24 h PBM. In contrast, the AaEcR-A transcript peaked at 16–20 h PBM, coinciding with the large ecdysteroid peak (Cho *et al.*, 1995; Wang *et al.*, 2002). Both isoform mRNAs were transcribed in a cycloheximide independent manner, suggesting that they are direct targets of 20E. However, AaEcR-A transcription requires the continuous presence of 20E, while the AaEcR-B mRNA level rose for 4 h and then declined under the same conditions. These results indicate that the mosquito EcR isoforms play distinct physiological functions during vitellogenesis in the mosquito fat body.

3.9.2.4.3. USP isoforms Like the EcR isoforms, two USP cDNA isoforms (AaUSP-A and AaUSP-B) were cloned from *A. aegpyti* (Kapitskaya *et al.*, 1996). They differ only at the N-terminus, thus

indicating their origin from the same gene, which recently has been confirmed by cloning the *USP* gene from *A. aegypti* (Wang, Zhu, and Raikhel, unpublished data). The isoform specific expression patterns in the *Aedes* fat body during vitellogenesis suggest that the two mosquito USP isoforms may carry out distinct functions. The level of AaUSP-B mRNA correlates with the high titer of ecdysteroid at 16–20 h PBM (Wang *et al*., 2000b). Intriguingly, USP-B is activated with 20E, whereas USP-A is inhibited by the hormone (**Figure 9**) (Wang *et al*., 2000b). AaEcR heterodimerizes with either mosquito USP isoform; the AaEcR-USP-B heterodimer displays stronger DNA binding and transactivation activities than the AaEcR-USP-A heterodimer (Wang *et al*., 2000b).

3.9.2.4.4. Ecdysteroid response elements (EcREs) The canonical steroid receptor binding site is a DNA element with an AGGTCA consensus sequence that binds one or two receptor molecules as monomers or as homodimers and/or heterodimers (Tsai and O'Malley, 1994). The binding sites can be arranged as single half sites or as inverted, direct, or everted repeats. Although DNA binding by EcR-USP is highly sequence specific, a surprising variety of sequences have been identified as ecdysteroid response elements in *D. melanogaster* (see **Chapter 3.5**), suggesting that variability of EcREs provides yet another level of specificity in 20E gene regulation (Riddihough and Pelham, 1987; Cherbas *et al*., 1991; Antoniewski *et al*., 1994, 1995, 1996; D'Avino *et al*., 1995; Horner *et al*., 1995; Lehmann and Korge, 1995).

The DNA binding properties of the AaEcR-AaUSP heterodimer have been systematically analyzed with respect to the effects of nucleotide sequence, orientation, and spacing between half sites in natural *D. melanogaster* and synthetic EcREs (Wang *et al*., 1998). AaEcR-AaUSP exhibits a broad binding specificity, forming complexes with inverted repeats (IRs) and direct repeats (DRs) of the nuclear receptor response element half-site consensus sequence AGGTCA separated by spacers of varying length. A single nucleotide spacer was optimal for both imperfect (IR^{hsp}-1) and perfect (IR^{per}-1) inverted repeats. Spacer length was less important in DRs of AGGTCA (DR-0 to DR-5). Although 4 bp was optimal, DR-3 and DR-5 bound AaEcR-AaUSP almost as efficiently as DR-4. Furthermore, AaEcR-AaUSP also bound DRs separated by 11–13 nucleotide spacers. Cotransfection assays utilizing CV-1 cells have demonstrated that the mosquito EcR-USP heterodimer is capable of transactivating reporter constructs containing either IR-1 or DR-4. The levels of transactivation are correlated with the respective binding affinities of the response elements (IR^{per}-1 > DR-4 > IR^{hsp}-1). Taken together, these analyses predict broad variability in the EcREs of mosquito ecdysteroid responsive genes (Wang *et al*., 1998). These analyses enabled the identification of a functionally active EcRE that binds the heterodimer EcR-USP in the *A. aegypti Vg* gene. A direct repeat with a 1 bp spacer (DR-1) with the sequence AGGCCAaTGGTCG is the major part of the EcRE in the *Vg* gene (Martin *et al*., 2001a).

3.9.2.4.5. Ecdysteroid early response genes The *A. aegypti E75* homolog consists of three overlapping transcription units encoding E75A, E75B, and E75C isoforms (Pierceall *et al*., 1999), which are members of the nuclear receptor family. As in *D. melanogaster*, these E75 transcripts arise by alternative promoter usage and splicing of transcription unit-specific 5′ exons onto a set of shared downstream exons. All three transcripts are induced at the onset of vitellogenesis by a blood meal and are highly expressed in the mosquito ovary and fat body, which suggests that they are involved in the regulation of oogenesis and vitellogenesis, respectively. Furthermore, *in vitro* fat body experiments have demonstrated that AaE75 isoforms are induced by 20E. In contrast to the yolk protein transcripts, which show only a small gradual increase during the first 4 h PBM, E75 transcripts exhibit a sharp rise immediately after the onset of vitellogenesis, reaching a peak at 3–4 h PBM, when the hormone titer shows a first peak. After falling from their initial peak, the level of mosquito E75 transcripts gradually increase again, reaching the second peak at 18–24 h PBM, which coincides with the accumulation of yolk protein RNA in the fat body (**Figure 9**). E75 isoform transcription is appropriately 10 times more sensitive to 20E than is the *Vg* gene (Pierceall *et al*., 1999).

Two isoforms of the homolog to the *D. melanogaster* transcription factor E74, which share a common C-terminal Ets DNA binding domain, yet have unique N-terminal sequences, are present in the mosquito. They exhibit a high level of similarity to DmE74 isoforms A and B and show structural features typical for members of the Ets transcription factor superfamily (Sun *et al*., 2002). Furthermore, both mosquito E74 isoforms bind to a *D. melanogaster* E74 binding site with the consensus motif C/AGGAA. E74B mRNA reaches its peak at 24 h PBM and drops sharply thereafter, correlating with peak expression of the *Vg* gene. The AaE74B transcript is induced by a blood meal activated hormonal cascade in fat body and peaks at 24 h PBM, the peak of

vitellogenesis (**Figure 9**). However, unlike E75 transcripts, E74B does not have an early peak at 3–4 h. AaE74A is activated at the termination of vitellogenesis and exhibits a peak at 36 h PBM in the fat body and 48 h PBM in the ovary. The AaE74A and AaE74B isoforms most likely play different roles in regulation of vitellogenesis in mosquitos, as an activator and a repressor of YPP gene expression, respectively. Both AaE74 isoform mRNAs are induced by 20E in fat body *in vitro* and display superinduction when cycloheximide is applied together with 20E (Sun *et al*., 2004). While both E74 isoforms are capable of binding to the E74 consensus sequence, only E74B induces a reporter gene expression through the consensus E74 binding site in cell transfection assays. Furthermore, a transient transfection assay using the *Vg* promoter region containing putative E74 binding sites has shown that E74B functions as an activator, whereas E74A serves as a repressor (Sun *et al*., 2004).

The BR-C plays a key role in the genetic control of 20E responses. The BR-C encodes a family of DNA binding proteins that share a common (core) N-terminus fused by alternative splicing to one of four pairs of C2H2-type zinc finger domains, Z1, Z2, Z3, and Z4. All four BR-C isoforms are present in *A. aegypti* (Chen, Zhu, and Raikhel, unpublished data). In female fat body, expression of Z1, Z2, and Z4 is induced immediately after a blood meal. Both Z1 and Z4 transcripts exhibit a peak at 24 h PBM. The Z2 transcript exhibits a sharp increase after onset of vitellogenesis, reaching initial peak at 8 h PBM. After an initial decline, the level of Z2 rises again at 24 h PBM (**Figure 9**). The expression of BR-C Z1, Z2, and Z4 in the fat body is attenuated between 24 h and 36 h PBM. Z3 transcript expression in the fat body is very low throughout the vitellogenic cycle. The overall level of Z4 has been found to be much higher than those of the other isoforms. In the fat body *in vitro* experiments, the mRNA levels of all four isoforms were rapidly induced by 20E and increased in a dose dependent manner. Functional analysis using RNAi experiments has suggested that Z1 and Z4 serve as repressors and that Z2 serves as an activator of *Vg* gene expression. Remarkably, RNAi knockdown of Z1 or Z4 extends *Vg* expression to 36 h PBM, which suggests that these factors regulate hormone dependent timing of this gene transcription (Chen, Zhu, and Raikhel, unpublished data).

Analyses of 20E sensitivity by transcripts of several key mediators and their expression profiles in vitellogenic fat body of the mosquito have revealed potential roles in the 20E gene regulatory hierarchy cascade, thus providing a road map for future studies (Pierceall *et al*., 1999; Kapitskaya *et al*., 1998; Li *et al*., 2000; Wang *et al*., 2000b, 2002; Sun *et al*., 2002, 2004) (**Figure 9**). At the top of the 20E cascade, AaEcRB, the dominant isoform for initiating mosquito vitellogenesis *in vivo*, exhibits high sensitivity to 20E at 10^{-8} M, and it is expressed predominantly during the first hours after blood meal activation of vitellogenesis (Wang *et al*., 2002) (**Figure 9**). Its obligatory partner AaUSP-B, early gene products AaE74B and AaE75A, and BR-C isoforms constitute the group with the second highest sensitivity to 20E at 10^{-7} M (Pierceall *et al*., 1999; Wang *et al*., 2000; Sun *et al*., 2004; Chen, Zhu, and Raikhel, unpublished data). AaEcR-A, AaE74A, and the early late gene *AaHR3* form the lowest 20E sensitivity group, being maximally activated at 10^{-6} M, which is similar to the target genes *Vg*, *VCP*, and *VCB* (**Figure 9**).

3.9.2.4.6. Hormonal enhancers in the regulatory region of mosquito yolk protein genes Transcriptional activation of hormonally controlled genes in specific tissues depends on interactions between sequence specific transcription factors and enhancer/promoter elements of these genes. Analysis of the 5′-upstream regulatory region of the mosquito *Vg* gene has revealed putative binding sites for EcR-USP and the early genes, E74, E75, and BR-C, which indicate this gene is regulated through a combination of direct and indirect hierarchies (**Figure 10**). Analyses of *D. melanogaster* and *A. aegypti* transformations, as well as DNA binding assays, have identified *cis*-regulatory sites in the *Vg* gene for stage and fat body specific activation via a blood meal triggered cascade (Kokoza *et al*., 2001). Three regulatory regions in the 2.1 kb, 5′ region of the *Vg* gene are required for its blood meal activation and high-level expression (**Figure 11**). The proximal region, adjacent to the basal transcription start site, contains binding sites for several transcription factors that direct tissue and stage specific expression: EcR/USP, GATA transcription factor (GATA), CAAT binding protein (C/EBP), and hepatocyte nuclear factor 3/forkhead transcription factor (HNF3/fkh). It appears that a combinatorial action of these transcription factors brings about fat body specific expression. EcR/USP acts as a timer, allowing the gene to be turned on, but the level of expression driven through this response element is low. The median region contains the sites for early gene factors E74 and E75, and transgenic studies have shown that this region is required for a stage specific hormonal enhancement of the *Vg* gene expression

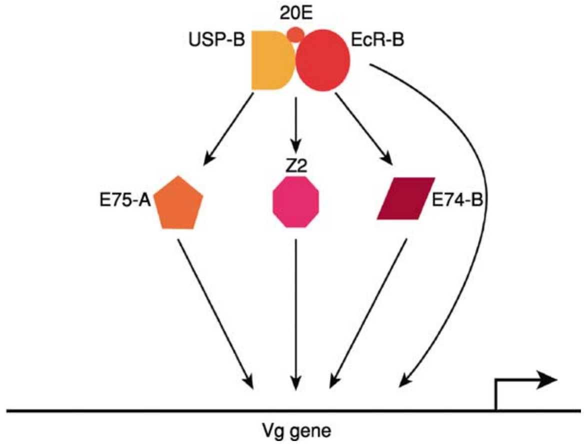


Figure 10 Direct and indirect regulation of vitellogenin (*Vg*) gene by 20-hydroxyecdysone in the mosquito fat body. The activated ecdysteroid receptor, consisting of EcR-B/USP-B binds to the EcRE response element in the regulatory region of the *Vg* gene. This binding is required to permit the *Vg* gene expression. After binding 20E, the EcR-USP heterodimer also activates early genes, *E74, E75*, and *BR-C*. The products of these genes E74-B, E75-A, and BR-C Z2 act as powerful activators of the *Vg* gene. (Based on Pierceall, W.E., Li, C., Biran, A., Miura, K., Raikhel, A.S., *et al.*, **1999**. E75 expression in mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol. Cell. Endocrinol. 150*, 73–89; Kokoza, V.A., Martin, D., Mienaltowski, M.J., Ahmed, A., Morton, C.M., *et al.*, **2001**. Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene 274*, 47–65; Martin, D., Wang, S.F., Raikhel, A.S., **2001b**. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol. Cell. Endocrinol. 173*, 75–86; Sun, G.Q., Zhu, J.S., Li, C., Tu, Z.J., Raikhel, A.S., **2002**. Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, *Aedes aegypti*. *Mol. Cell. Endocrinol. 190*, 147–157; L. Chen, J. Zhu, and A.S. Raikhel, unpublished data.)

(Kokoza *et al*., 2001). Furthermore, *in vitro* cell transfection experiments have demonstrated that the ecdysteroid receptor and E74B act synergistically to bring about a high-level, 20E activated *Vg* gene expression (Sun and Raikhel, unpublished data). Finally, the distal portion is characterized by multiple response elements for a GATA transcription factor (Martin *et al*., 2001b). In transgenic experiments using both *A. aegypti* and *D. melanoagaster*, this GATA- rich region is required for high expression levels characteristic to the *Vg* gene (**Figure 11**).

Recent analyses of the *A. aegypti Vg* promoter region have identified many binding sites for the BR-C transcription factors: one for Z1, two for Z2, three for Z3, and seven for Z4. When BR-C isoform expression vectors were introduced into Kc cells together with EcR and USP expression vectors in the presence of 20E, Z1 and Z4 repressed the activation mediated by EcR-USP, while Z2 enhanced this activation. The role of Z3 was negligible. When the Z1 binding site was mutated, luciferase activity driven by the *Vg* promoter was no longer inhibited by the overexpression of Z1. After removal of Z2 binding sites in the *Vg* promoter, Z2 lost the capability to increase luciferase activity. However, because there are seven binding sites of BR-C Z4 on the *Vg* promoter, the repression of *Vg* promoter by Z4 could not be completely released after mutation of all the Z4 binding sites. These experiments support the RNAi test performed *in vivo* and suggest that BR-C Z1 and Z4 serve as repressors while Z2 is an activator of the *Vg* gene expression. Z4 may not directly bind to the *Vg* promoter (Chen, Zhu, and Raikhel, unpublished data).

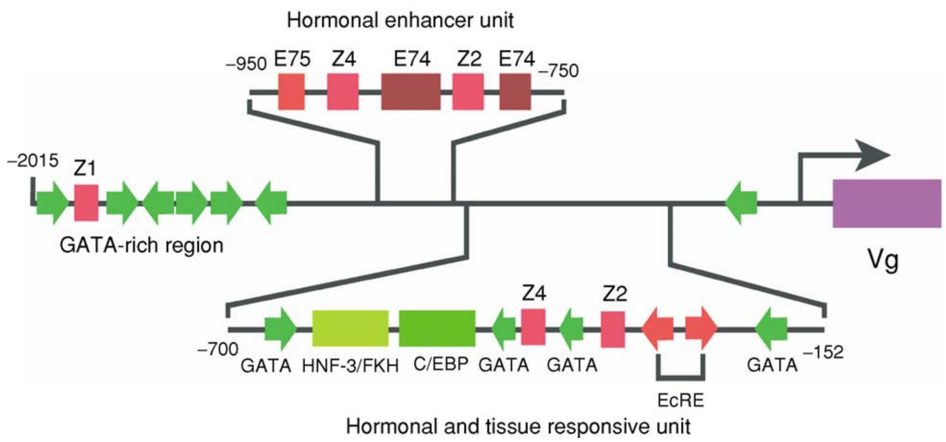


Figure 11 Schematic illustration of the regulatory regions of the *Aedes aegypti Vg* gene. Numbers refer to nucleotide positions relative to the transcription start site. Binding sites for hormonal transcription factors are depicted in red and those for tissue specific factors in green. C/EBP, response element of C/EBP transcription factor; EcRE, ecdysteroid response element; E74 and E75, response elements for respective early gene product of the ecdysone hierarchy; GATA, response element for GATA transcription factor; HNF3/fkh, response element for HNF3/forkhead factor; Vg (purple), coding region of the *Vg* gene; Z1, Z2, and Z4 are binding sites for corresponding Broad-Complex isoforms. (Modified with permission from Kokoza, V.A., Martin, D., Mienaltowski, M.J., Ahmed, A., Morton, C.M., *et al.*, **2001**. Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene 274*, 47–65.)

3.9.2.4.7. Competence to 20E response Female mosquitos, such as *A. aegypti*, require a previtellogenic preparatory period to attain the capability for host seeking behavior and blood feeding. During this time, the fat body becomes competent for massive yolk protein synthesis and secretion, and the ovary for accumulation of YPs. In the fat body, this process is manifested in the development of the endoplasmic reticulum and Golgi complexes, proliferation of ribosomes, and increase in cell ploidy (Dittmann *et al*., 1989; Raikhel and Lea, 1990). JH III titers rise tenfold over the first 2 days after emergence and then slowly decline over the next 5 days. Blood ingestion causes an immediate decline of JH, which falls to its lowest level at 24 h PBM (Shapiro *et al*., 1986). Activation of fat body nucleoli for ribosomal RNA production and ribosomal production is blocked by removal of the CA in newly eclosed adult females, but it can be restored by either implantation of CA or topical application of JH III to allatectomized females. These developmental events most likely are controlled by JH from the CA (Raikhel and Lea, 1990, 1991). The exposure of a newly emerged female mosquito to JH III is essential for the fat body to become responsive to 20E (Flanagan *et al*., 1977; Li *et al*., 2000; Zhu *et al*., 2003a).

To dissect the molecular mechanism governing the acquisition of competence for vitellogenesis in the *A. aegypti* fat body, the mosquito homolog of the ecdysteroid response competence factor, βFTZ-F1, in *D. melanogaster* has been cloned (Li *et al*., 2000). During metamorphosis in *D. melanogaster*, the stage specificity of the genetic response to 20E is set up by βFTZ-F1, an orphan nuclear receptor. Ectopic *βFTZ-F1* expression leads to elevated transcription levels for 20E-inducible *BR-C* and the early genes *E74* and *E75*. *βFTZ-F1* mutants pupate normally in response to the late larval 20E pulse but display defects in stage specific responses to the subsequent 20E pulse in prepupae (Lam *et al*., 1997; White *et al*., 1997). Mosquito *βFTZ-F1* is transcribed highly in the late pupa and in the adult female fat body during pre- and postvitellogenic periods, when ecdysteroid titers are low, and the transcripts nearly disappear in midvitellogenesis, when ecdysteroid titers are high. Each rise in the level of *βFTZ-F1* transcripts is preceded by a high expression of another nuclear receptor (HR3) that coincides with the 20E peaks (Kapitskaya *et al*., 2000; Li *et al*., 2000). This observation is consistent with the role of *HR3* in *D. melanogaster*, which facilitates induction of *βFTZ-F1* in mid-prepupa.

Although the genetic tools are limited for the *A. aegypti*, experiments with the fat body *in vitro* lend support at the functional level to the hypothesis that FTZ-F1 serves as a competence factor for the ecdysteroid response initiating vitellogenesis. In these experiments, FTZ-F1 transcription is inhibited by 20E and is superactivated by its withdrawal (Li *et al*., 2000). Electrophoretic mobility-shift assay analysis of nuclear extracts from *A. aegypti* fat body demonstrated that the onset of ecdysone response competence in this tissue is correlated with the appearance of the functional FTZ-F1 protein at the end of previtellogenic development (Li *et al*., 2000). Western blot analysis using antibodies to *A. aegypti* βFTZ-F1 show that the βFTZ-F1 protein is not detectable in the fat body nuclear extracts of newly emerged and 1-day-old mosquitos but is abundant at 3–5 days posteclosion (PE), and then not dectected shortly after blood feeding (Zhu *et al*., 2003a). Furthermore, when fat body from females was isolated at 6 h PE and incubated for 18 h in medium with JH III, the nuclei contained βFTZ-F1 protein, in contrast to controls incubated with acetone (Zhu *et al*., 2003a). Taken together, these findings indicate that JH III is quite likely the crucial factor modulating production of βFTZ-F1 protein in the fat body through posttranscriptional regulation of its gene in the previtellogenic stage (**Figure 12**).

To confirm that *βFTZ-F1* may encode a competence factor in the fat body of female *A. aegypti*, it was silenced by RNAi, which involves the introduction of homologous double-stranded RNA (dsRNA) in order to target specific mRNAs for degradation (Zhu *et al*., 2003a). Compared with naïve females, the mRNA and protein levels of *βFTZ-F1* declined substantially in females treated with *βFTZ-F1* dsRNA but not in those treated with control dsRNA, which indicates that *βFTZ-F1* was selectively inhibited by RNAi. Expression of the *Vg* gene was dramatically diminished after blood feeding in the *βFTZ-F1* dsRNA treated mosquitos. These results suggest that *A. aegypti* βFTZ-F1 is essential for the stage specific 20E response in fat body during vitellogenesis, which is reminiscent of the role βFTZ-F1 plays in *D. melanogaster* during the prepupa to pupa transition. Functional analysis of *βFTZ-F1* by the RNAi technique suggests that βFTZ-F1 is in fact a competence factor that defines the stage specific 20E response during vitellogenesis in the mosquito (Zhu *et al*., 2003a).

Despite extensive studies, the mode of JH action is still not well understood. During previtellogenic development in female mosquitos, JH controls the synthesis of βFTZ-F1 protein, but this posttranscriptional control of gene expression can occur at one or more levels: pre-mRNA splicing pattern, mRNA

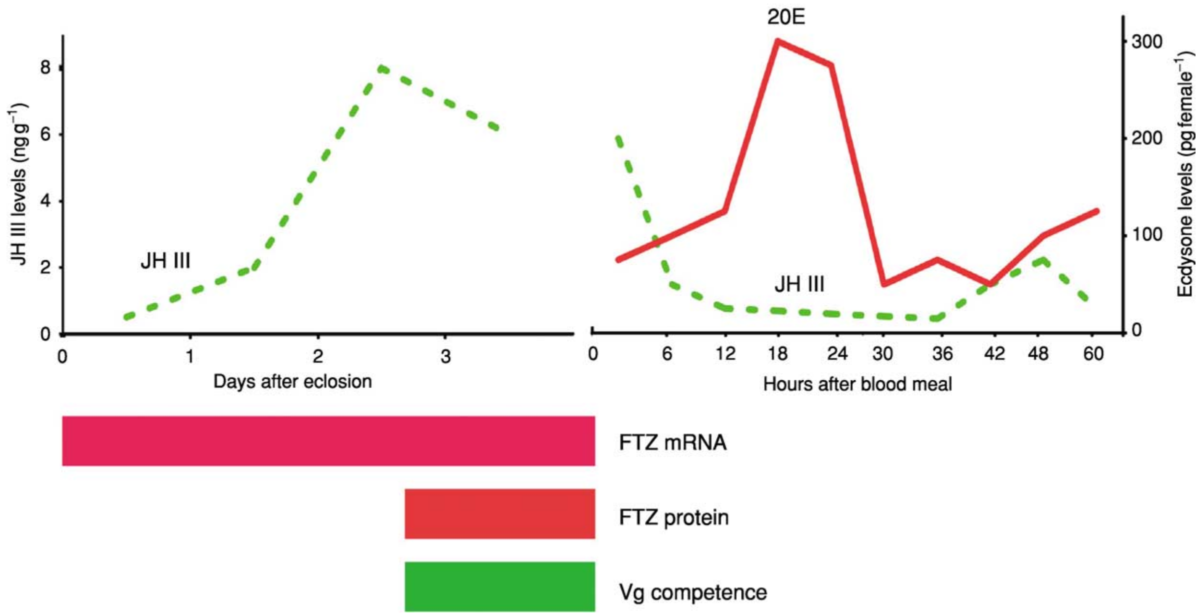


Figure 12 βFTZ-F1, the orphan nuclear factor (see **Chapter 3.5**), is implicated as a competence factor for stage specific response to ecdysteroid during vitellogenesis in the *Aedes aegypti* fat body. The *AaFTZ-F1* mRNA is present at late pupal and previtellogenic stages in newly eclosed females; however, the appearance of active AaFTZ-F1 factor coincides with the onset of competence for 20E response (Li *et al.*, 2000; Zhu *et al.*, 2003a). (Reprinted with permission from Raikhel, A.S., Kokoza, V.A., Zhu, J.S., Martin, D., Wang, S.F., *et al.*, **2002**. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochem. Mol. Biol. 32*, 1275–1286; © Elsevier.)

stability, mRNA transport, or translation rate. Although the mechanism underlying this regulation remains obscure, these findings represent another step towards understanding the mode of JH action.

3.9.2.4.8. Regulation of vitellogenesis cyclicity by ecdysteroid mediated signaling through heterodimerization with the RXR homolog Ultraspiracle In many insects, vitellogenesis and egg maturation arecyclic and, as a consequence, these insects produce batches of eggs, unlike others that lay eggs continuously. Generally, the mode of egg production – continuous or cyclic – depends upon feeding and lifestyle adaptations. Cyclic egg production occurs in all insect genera irrespective of the type of hormonal control. In insects, in which reproduction is governed by JH, these mechanisms are poorly understood at the molecular level. However, for insects with cyclic egg production that depends primarily on ecdysteroids, recent studies of the mosquito, *A. aegypti*, have shed some light on these mechanisms.

Zhu *et al*. (2000, 2003b) and Miura *et al*. (2002) have demonstrated that during mosquito vitellogenesis, two nuclear receptors, HR38 and Svp, regulate the cyclicity of ecdysteroid mediated signaling via heterodimerization with USP. Both AaEcR and AaUSP proteins are abundant in nuclei of the previtellogenic female fat body at the state of arrest; however, the EcR-USP heterodimer capable of binding to the specific ecdysone response elements is barely detectable in these nuclei. Studies have shown that EcR is a primary target of 20E signaling modulation in target tissues at the state of arrest. A possible mechanism through which the formation of ecdysteroid receptor activity can be regulated is a competitive binding of other factors to either EcR or USP. Indeed, at this stage, AaUSP exists as a heterodimer with the orphan nuclear receptor, AHR38. This protein is a homolog of the *D. melanogaster* DHR38 and vertebrate NGFI-B/Nurr1 orphan receptors, and it acts as a repressor by disrupting EcR binding to DNA response elements and by interacting strongly with AaUSP. However, in the presence of 10^{-6} M of 20E, EcR efficiently displaces AHR38 and forms an active heterodimer with USP, as happens after a blood meal (**Figures 13 and 14**).

To regulate cyclicity in egg production, termination of Vg gene expression is needed in female mosquitos, so that egg maturation and deposition can be completed and the arrest stage restored until another blood meal can be obtained. To identify other negative regulators of the AaEcR/AaUSP mediated 20E response during vitellogenesis, a

mosquito homolog (AaSvp) of the vertebrate orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF), and *D. melanogaster* Seven-up (Svp) has been cloned (Miura *et al.*, 2002). Coimmunoprecipitation experiments with nuclear extracts (Zhu *et al.*, 2003b) have clearly shown that in the female fat body AaSvp associates only with AaUSP. Furthermore, formation of AaSvp-AaUSP heterodimers occurs in a precise, timely manner at 30–33 h PBM, after the 20E titer declines to the previtellogenic level and when the expression of *YPP* genes, including *Vg*, is terminated (**Figure 13**). *In vitro* experiments have suggested that the declining titer of 20E could be a critical factor facilitating the formation of Svp-USP heterodimers (Zhu *et al.*, 2003a). Mosquito Svp, thus, represses the 20E mediated transactivation of vitellogenesis through its heterodimerization with USP (Zhu *et al.*, 2003b).

The modulation of vitellogenesis cyclicity appears to be regulated through alternative heterodimerization of the RXR homolog USP that blocks ecdysteroid mediated signaling (**Figure 14**). AaUSP exerts its functions by associating with distinct partners at different stages of vitellogenesis. During the arrest stage, AHR38 prevents the formation of the functional EcR complex by sequestering AaUSP, which blocks 20E dependent transactivation. After a blood meal, the AaEcR-AaUSP heterodimerization becomes dominant, and AaEcR-AaUSP binding to EcREs on the Vg gene permits its expression. When vitellogenesis proceeds to the termination stage, falling 20E titers shift AaUSP heterodimerization towards AaSvp, repressing USP-based hormone responses (**Figure 14**).

In summary, these studies clearly show that the cyclicity of vitellogenesis in the mosquito fat body is regulated through USP, which sequentially forms inactive or active heterodimers with either repressors (HR38 and Svp) or the activator (EcR) and directly affects ecdysteroid mediated signaling.

3.9.2.5. Molecular Endocrinology of Vitellogenesis in the Cyclorrhaphan Diptera

3.9.2.5.1. Vitellogenesis and hormones in *D. melanogaster* Like the mosquito, the fat body in fruit flies matures and differentiates at eclosion under the control of JH, but it immediately begins to synthesize YPs and secrete them into the hemolymph. Simultaneously, the uptake of YPs is initiated by developing oocytes that mature into eggs, which are fertilized and laid continuously. If the fly does not mate or has enough food, mature eggs are retained, and oocytes

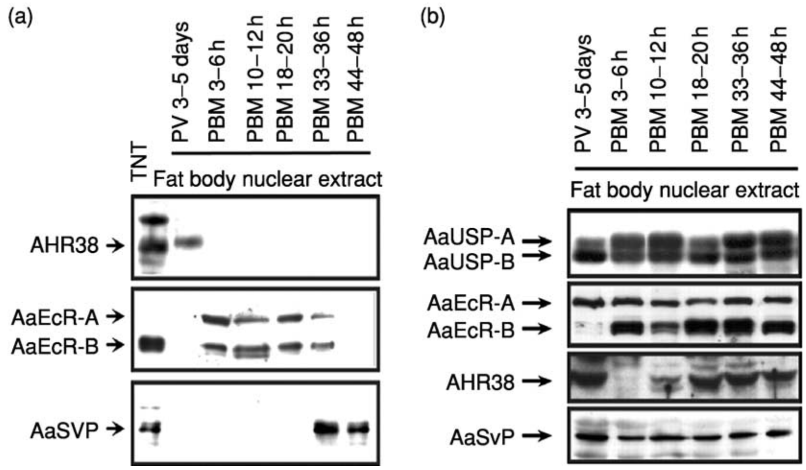


Figure 13 *Aedes aegypti Ultraspiracle* (AaUSP) interacts sequentially with a repressor AHR38, an activator AaEcR, and then with repressor AaSvp in the fat body during the vitellogenic cycle. Nuclear extracts were prepared from the fat bodies of 500 adult females for each time point. (a) Coimmunoprecipitation analysis. A protein aliquot equivalent to 100 mosquitos was incubated with anti-*Drosophila* USP monoclonal antibody. The resulting immune complexes were then precipitated by the addition of protein A-agarose beads. After extensive washing, immune complexes were dissociated and separated by means of SDS-PAGE followed by immunoblotting using rabbit anti-AHR38, rabbit anti-AaEcR, chicken anti-AaSvp antibodies, or the respective preimmune sera. *In vitro* translated proteins (AHR38, AaEcR-B, and AaSvp) were used as controls in Western blot analysis. (b) Western blot analysis of USP proteins during the vitellogenic cycle shows that two isoforms of USP are present throughout the vitellogenic cycle in fat body nuclei. A protein aliquot equivalent to 25 fat bodies was loaded in each lane for SDS-PAGE. Western blot analysis was performed using the anti-DmUSP monoclonal antibody. *In vitro* TNT expressed USP-A and USP-B proteins were used as positive controls. (Reproduced with permission from Zhu, J.S., Miura, K., Chen, L., Raikhel, A.S., **2003b**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue *Ultraspiracle*. *Proc. Natl Acad. Sci. USA 100*, 544–549.)

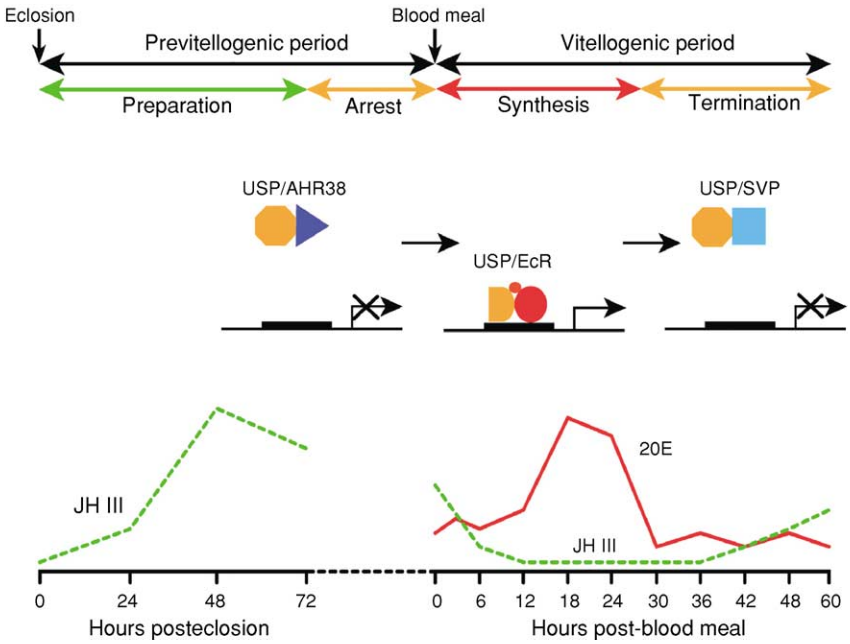


Figure 14 Heterodimerization of *Ultraspiracle* (USP), the obligatory partner of EcR, with repressor nuclear receptors during the first vitellogenic cycle in the mosquito *Aedes aegypti*. At the state of arrest, AaUSP was associated with AHR38, preventing activation of YPP production prior to blood feeding. After a blood meal, AaUSP heterodimerizes with AaEcR, and induces expression of YPP genes in the presence of an elevated 20E titer. Around 30–36 h PBM, AaSvp attaches to AaUSP, decelerating the massive protein synthesis. Top panel: stages of mosquito vitellogenic cycle; bottom panel: titers of juvenile hormone and ecdysteroids. (Modified with permission from Zhu, J.S., Miura, K., Chen, L., Raikhel, A.S., **2003b**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue *Ultraspiracle*. *Proc. Natl Acad. Sci. USA 100*, 544–549.)

arrest at a previtellogenic stage and do not enter vitellogenesis (Bownes, personal communication).

Despite the fact that in most cyclorrhaphan Diptera, including *D. melanogaster*, YPs are made in fat body and follicular epithelial cells surrounding the oocyte, the regulation of *yp* gene expression is different in these tissues (Bownes, personal communication). Ecdysteroids stimulate YP synthesis in the fat body of males and upregulate it in the fat body of females (Postlethwait *et al*., 1980; Jowett and Postlethwait, 1980; Bownes, 1982; Bownes *et al*., 1983). This is consistent with the finding that EcREs have been mapped to regions flanking the *yp* genes (Bownes *et al*., 1996). JH also increases YP synthesis in the fat body of females (Bownes and Blair, 1986; Bownes *et al*., 1987; Soller *et al*., 1997), but it does not induce YP synthesis in males. No potential JH elements have been found in these genes. Walker *et al*. (1991) have shown that ecdysteroids increased CAT expression of *YP* promoter CAT fusion in the S3 cell line. JH had no effect on the same reporter construct. Thus, it is unlikely that JH plays any role in directly affecting transcription of *yp* genes in the fat body. JH may play a role in the regulation of yolk protein synthesis by follicle cells, as shown in studies of the *D. melanogaster* mutant ap^{56f} (Richard *et al*., 1998, 2001). Female ap^{56f} produces low levels of JH and normal to elevated levels of ovarian ecdysteroids and are fertile but have delayed vitellogenesis. JH application to female ap^{56f} reversed the delay in vitellogenesis.

Many of the response genes in the ecdysteroid regulatory hierarchy, including *EcR/USP E74, E75*, and *BR-C*, that have been shown to be crucial for 20E regulation of *Vg* gene expression in the fat body of *A. aegypti* have not been studied in *D. melanogaster* with respect to YP synthesis in the fat body. This is mostly because in normal, mated, well-fed females the genes are constitutively active rather than regulated in response to a blood meal (Raikhel *et al*., 2003; Bownes, personal communication).

3.9.2.5.2. Regulatory regions of yolk protein genes in *D. melanogaster* Relatively little is known about the *cis*-acting regions of the *yp* genes in any species of cyclorrhaphan Diptera. Several EcREs have been identified in the 5′ and 3′ regions and within the coding sequences of the *yp* genes of *D. melanogaster* (see **Figures 15 and 16**), and they appear to confer ecdysone inducible expression in males, when injected with 20E. These putative EcRE sequences are similar to those shown to bind the EcRE/USP heterodimer and lead to transcription of other genes in insect metamorphosis and the *Vg*

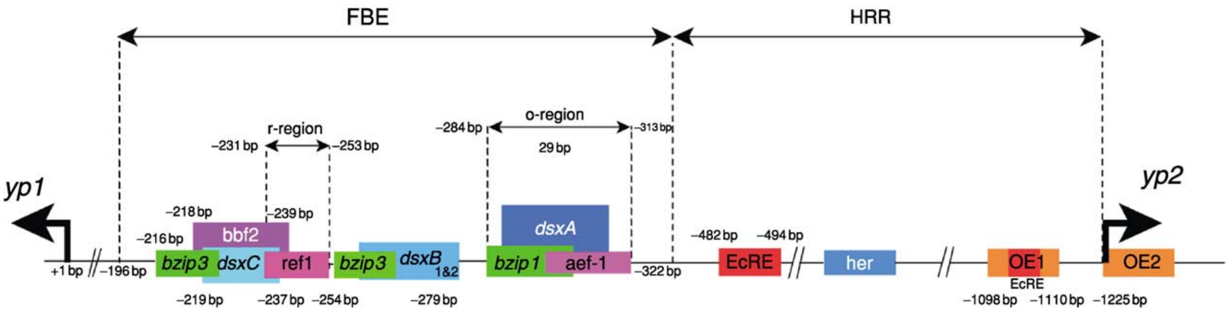


Figure 15 *Drosophila melanogaster yp* genes 1 and 2 are located in close proximity in opposite orientation and share a regulatory region called the intergenic enhancer region. The 1225 bp intergenic enhancer region of the *yp1* and *yp2* genes contains four enhancer regions, the fat body enhancer (FBE), the hermaphrodite response region (HRR), and two ovary enhancers (OE1 and OE2); aef-1, the adult enhancer factor-1; *bzip3*; C/EBP, CAAT enhancer binding protein; *dsx*, doublesex binding site; EcRE, ecdysone response element; her, binding site for the hermaphrodite protein. (Data kindly provided by M. Bownes.)

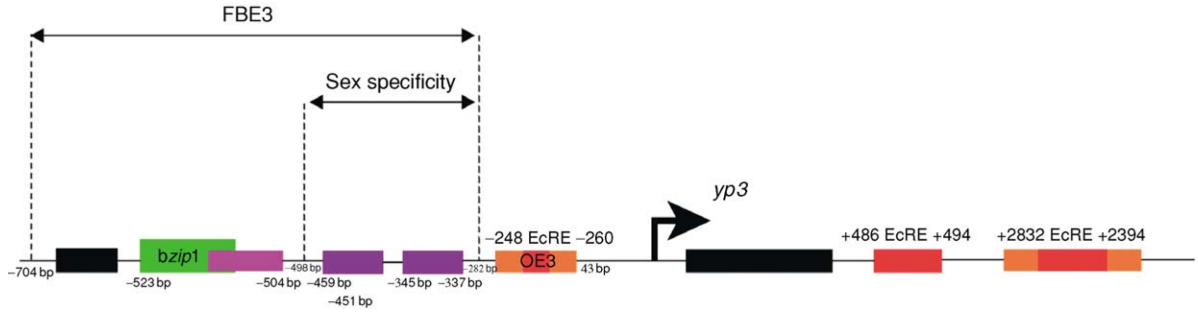


Figure 16 The *Drosophila yp3* gene is separated from two others on the X-chromosome and has a separate regulatory region, the upstream enhancer 3. aef-1, adult enhancer factor 1; bbf, binding site for the box-B binding factor; EcRE, ecdysone response element. (Data kindly provided by M. Bownes.)

genes in the mosquito (Bownes *et al.*, 1996). Although these EcREs have been mapped on the *yp* genes, it is not clear how much of the 20E inducibility of *yp* gene expression is a direct action through the binding of EcR and how much is due to downstream genes such as *BR-C, E74*, and *E75* (Bownes, personal communication).

Despite the fact that the hormonal and molecular control of *yp* gene transcription in *D. melanogaster* is poorly understood, the sex specific expression of these genes has been elucidated in detail (see **Chapters 1.5** and **1.7**). Doublesex (*dsx*) exerts the most important control of *yp* gene expression (Bownes and Nothiger, 1981; Coschigano and Wensink, 1993; Belote *et al.*, 1985). Alternate transcript splicing of *dsx* produces different proteins in male and female adults, Dsx^{M} and Dsx^{F}, respectively, that have similar DNA binding domains but different C-terminal extensions (Baker and Wolfner, 1988; Burtis and Baker, 1989; MacDougall *et al.*, 1995). The male and female proteins are essential for maintaining *yp* transcription in the female fat body and repressing it in males (Bownes and Nothiger, 1981; Belote *et al.*, 1985; Burtis *et al.*, 1991; Bownes, personal communication). In males, 20E injection overrides sex determination inhibition as shown by the transient expression of *yp* genes in the fat body of males (Bownes *et al.*, 1996; Bownes, personal communication). In females, *yp* gene expression in the ovarian follicle cells does not depend upon the sex determination pathway (Logan *et al.*, 1989; Logan and Wensink, 1990; Lossky and Wensink, 1995), and 20E is probably a key hormone regulating this expression in such cells (Bownes, 2004).

In *D. melanogaster*, *yp* genes 1 and 2 are located in close proximity in opposite orientation and share a regulatory region called the intergenic enhancer region (**Figure 15**). In females, expression of *yp1* and *yp2* occurs in fat body and follicle cells of developing egg chambers at stages 8–10 (Bownes, 2004). The 1225 bp intergenic enhancer region of these *yp* genes contains four enhancer regions: fat body enhancer (FBE), hermaphrodite response region (HRR), and two ovary enhancers (OE1 and OE2) (Logan *et al.*, 1989; Logan and Wensink,

1990; Garabedian *et al*., 1985, 1986; Abrahamsen *et al*., 1993; Lossky and Wensink, 1995). FBE drives expression of these genes in fat body, and HRR responds to the *hermaphrodite* gene that acts along with Dsx to control their sex specific expression (Li and Baker, 1998). OE1 and OE2 direct expression of these genes in follicle cells (Logan *et al*., 1989; Logan and Wensink, 1990; Lossky and Wensink, 1995). With base pairing in respect to the *yp1* start site, several binding sites for activator or repressor molecules have been mapped, and binding has been demonstrated for some of these molecules (**Figure 16**). A putative binding site for *bzip3* overlaps a weak binding site for a Dsx, *dsxC*. Two overlapping binding sites for the mammalian CAAT enhancer binding protein (C/EBP) and the adult enhancer factor-1 (AEF-1) have been found in other fat body enhancers and the *Adh* gene of the mammalian liver to activate or repress transcription, respectively (Abel *et al*., 1992). These two sites, in turn, overlap the strongest binding site for Dsx, *dsxA* (Bownes *et al*., 1996). Further upstream, between −322 and −1225 bp, up to the *yp2* transcription start site, lies the HRR, which contains the binding site for the hermaphrodite protein, and OE1. A putative EcRE lies within OE1. A second EcRE is found between −482 and −494 bp of the HRR region. OE2 is located at the start site for *yp2* transcription (Logan *et al*., 1989; Logan and Wensink, 1990; Lossky and Wensink, 1995; Bownes, ; Bownes, personal communication).

The *D. melanogaster yp3* gene is separated from two others on the X-chromosome and has a separate regulatory region, the upstream Enhancer 3 (Hutson and Bownes, 2003). The organization of the regulatory regions of this gene differs from those of *yp1* and *yp2*, resembling instead those in *Musca* and *Calliphora yp* genes (Hutson and Bownes, 2003). The 703 bp upstream enhancer sequence of the *yp3* transcription start site contains three regions that confer tissue specificity, sex specificity, and ovary specificity (**Figure 16**). The 419 bp fat body enhancer 3 (FBE3) is the furthest 5′ regulatory region spanning from −704 to −285 bp and controls expression of *yp3* in the female fat body (Hutson and Bownes, 2003). Most 5′ in the FBE3 is the binding site for *dsxA*. Downstream of the *dsxA* site is the overlapping binding site, also found in FBE1/2, for the activator molecule CAAT enhancer binding protein (C/EBP), and the repressor, adult enhancer factor 1 – AEF-1 (Falb and Maniatis, 1992a, 1992b). The region between −498 and −252 bp is essential for sex specificity. There are two binding sites for the box-B binding factor (BBF), a transcriptional activator (Abel *et al*., 1992). A possible mechanism also involves a putative homolog of the unknown activator R that has also been found to be the final determinant needed for female specific fat body expression of *yp1* and 2 (Ronaldson and Bownes, 1995; Hutson and Bownes, 2003).

Hutson and Bownes (2003) have analyzed the regulation of *yp3* gene expression. JH increased stability of *yp3* transcripts, and 20E plays an important role in regulating transcription. Three putative EcREs have been mapped in FBE3 and *yp3* (Hutson and Bownes, 2003). Immediately 3′ to the FBE3 is the ovarian enhancer 3 (OE3) that is necessary for regulation of *yp3* transcription in the follicle cells of the developing egg chamber between stages 8 and 10. Within this region is an EcRE, and a second EcRE is located 3′ of the intronic sequence of *yp3*. The final EcRE is found within the second exon of *yp3*. 20E can override the negative effects of Dsx^{M} while maintaining the tissue specificity of *yp3* gene expression (Hutson and Bownes, 2003). Experiments using upstream, downstream, and coding sequences of *yp3* fused to reporter genes agree well with the locations of the putative EcREs, because there are ecdysteroid inducible sites upstream, downstream, and in the coding region (Hutson and Bownes, 2003).

3.9.2.6. Hormones and Ovarian Maturation

3.9.2.6.1. Previtellogenic development of the follicle Several aspects of ovarian previtellogenic development in insects are controlled by JH. Stimulation of previtellogenic growth of follicles by JH is best supported by past studies (Strong, 1965; Gwardz and Spielman, 1973; Tobe and Pratt, 1975; Tobe and Stay, 1977; Lanzrein *et al*., 1978; Moobola and Cupp, 1978; Tobe and Langley, 1978; McCaffery and McCaffery, 1983). Differentiation of the follicular epithelium is another aspect of oocyte development that is regulated by JH (Davey and Hubner, 1974; Abu-Hakima and Davey, 1975; Elliot and Gillot, 1976; Koeppe and Wellman, 1980; Koeppe *et al*., 1980). In *M. domestica*, JH is released soon after adult emergence, and it is essential for ovarian maturation, since without it ovaries remain immature (Adams, 1974, 1980). JH stimulates endopolyploidy in the nurse cells, primes the oocyte for growth, and potentially plays a role in follicle morphogenesis. Although, JH affects the previtellogenic stages of *M. domestica* oogenesis, it does not induce vitellogenesis. JH also is required for oocyte development in the mosquito *A. aegypti*, where it controls growth and differentiation of the follicular epithelium. These events are blocked by the removal of CA and restored by either implantation of CA or application of JH (Raikhel and Lea, 1991).

3.9.2.6.2. The role of 20E in ovarian development and egg maturation in Diptera Ecdysteroids are produced by the ovaries of many insects, including mosquitos and flies (Hagedorn *et al*., 1975; Bownes *et al*., 1984; Hagedorn, 1985). However, their role in ovarian development is not completely understood. In *D. melanogaster*, ecdysteroids can have an antagonistic effect on oocyte progression and lead to apoptosis of egg chambers. Ecdysteroid mutants, such as *ecd*[1] (Redfern and Bownes, 1982) and *l(3)3*^{DTS} (Walker *et al*., 1987), have defects in the progress of oogenesis. One of the early genes in the ecdysteroid regulatory hierarchy, *BR-C*, is crucial at several stages of oogenesis. It is first expressed in all follicle cells surrounding the oocyte prior to the control point (Deng and Bownes, 1997), and this early *BR-C* induction may be controlled by ecdysteroids. An exciting development is the isolation of the *D. melanogaster dare* gene that encodes adrenodoxin reductase, which is expressed in the ovary and plays a key role in ecdysteroid biosynthesis (Freeman *et al*., 1999) (see **Chapter 3.3**). These authors postulated that ovarian expression of *dare* provides a maternal source of this enzyme to the developing embryo. Since it is first expressed just prior to activation of the *BR-C*, it is also possible that it could also support the synthesis of ecdysteroids, which would activate *BR-C* in nearby follicle cells at this stage of oogenesis. This scenario suggests the existence of an egg chamber autonomous timing event that regulates when and where ecdysteroids are available to control oocyte progression. *BR-C* expression in oogenesis is crucial for the regulation of endoreplication of polyploid follicle cells, specific amplification of the chorion genes, and later for positioning of the chorionic appendages (Deng and Bownes, 1997; Tzolovsky *et al*., 1999).

Although *BR-C* is regulated by ecdysteroids in early oogenesis, the epidermal growth factor signaling pathway in the egg chamber is crucial for positioning the late *BR-C* expression pattern, which in turn positions the chorionic appendages (Deng *et al*., 1999). Since the effects of 20E are mediated by EcR and USP, mutations in these genes in *D. melanogaster* affect oogenesis. EcR-A and Ec-R-B1 are expressed in the nurse cells and follicle cells throughout oogenesis (Deng, Mauchline, and Bownes, personal communication). A temperature sensitive mutant affecting these isoforms, EcR^{A483T}, reduced egg laying, induced abnormal egg clusters, and led to a loss of vitellogenic stages (Carney and Bender, 2000). Loss of EcR in germline clones leads to egg chamber arrest at around stage 6–7 of follicle development (previtellogenic stage). Carney and Bender (2000) proposed the existence of an ecdysone dependent checkpoint at this stage. Buszczak *et al*. (1999) have shown that the early ecdysone response genes, *E75* and *E74*, along with the *BR-C*, are crucial for egg chamber morphogenesis in mid-oogenesis. Germline clones of cells lacking *E75, dare*, or EcR functions results in the degeneration of these egg chambers at stage 8 (Buszczak *et al*., 1999).

3.9.2.6.3. Regulation of patency by follicle cells The appearance of large intercellular spaces in the follicular epithelium during vitellogenesis, a phenomenon termed patency, has been reported in many insects, from the less modified species having panoistic ovaries, like *B. germanica* (**Figure 17**)

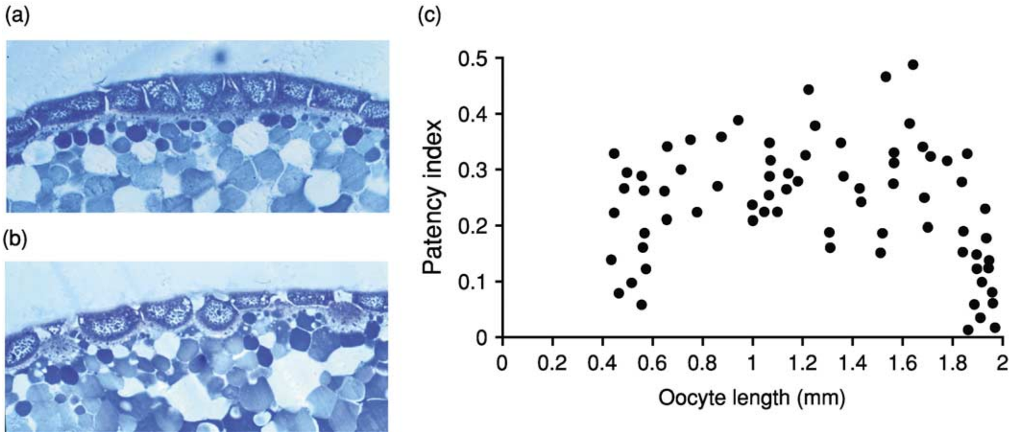


Figure 17 Patency of follicle cells in *Blattella germanica* during yolk protein uptake. (a, b) Sections at the equatorial zone in basal oocytes of (a) 0.68 mm length (3 days old) and (b) 1.52 mm length (5 days old). (c) Relation between patency index (PI) and basal oocyte length. PI = SE/SC, where SE is the surface of the intercellular spaces stained with Evans' blue, and SC the surface of the follicle cells. (Reprinted with permission from Pascual, N., Cerdà, X., Benito, B., Tomás, J., Piulachs, M.D., *et al*., **1992**. Ovarian ecdysteroid levels and basal oocyte development during maturation in the cockroach *Blattella germanica* (L.). *J. Insect Physiol. 38*, 339–348, with permission from Elsevier.)

(Pascual *et al.*, 1992) to the most modified ones having polytrophic ovaries, like flies or mosquitos (Wyatt and Davey, 1996). Studies on *Rhodnius prolixus* by Davey and co-workers have suggested that patency is under JH control (Wyatt and Davey, 1996). Further studies in this bug have shown that JH increases the activity of Na^+/K^+ ATPase in membrane preparations from vitellogenic follicle cells incubated *in vitro*, and experiments with inhibitors and activators of protein kinase C indicated that this enzyme is involved in the activation of the ATPase (Sevala and Davey, 1989). Incubation of membrane preparations with JH I resulted in the phosphorylation of a 100 kDa protein, a process that was dependent on protein kinase C and was inhibited by ouabain. Based on these experiments, it was suggested that the 100 kDa protein could be the α-subunit of the ATPase (Sevala and Davey, 1993). According to this model, JH would act on the follicle cells via a protein kinase C dependent cascade to stimulate a membrane bound Na^+/K^+ ATPase (**Figure 18**). Then, changes in the ionic balance would cause water loss and shrinkage of the cells with the associated apparition of large intercellular spaces. An equivalent system seems to operate in the locust *L. migratoria* (Davey *et al.*, 1993) and the mealworm beetle, *Tenebrio molitor* (Webb *et al.*, 1997). The model proposed to explain these mechanisms acting in follicle cell patency (**Figure 18**) suggests the existence of a membrane receptor that binds JH. The first efforts to characterize such a receptor were carried out in *R. prolixus*. In this species, Ilenchuk and Davey (1985) have shown that JH I binds to membrane preparations of follicle cells with a K_d of 6.54 nM in a specific and saturable fashion. Neither JH II nor JH III exhibited biological activity, and they did not compete for the binding site (Ilenchuk and Davey, 1987). A 35 kDa protein in solubilized follicle cell membranes from *L. migratoria* was found to bind a photoaffinity analog of JH III, [^{3}H]EFDA (Sevala *et al.*, 1995). The membrane preparations bound [^{3}H]JH III with a K_d of 3.68 nM in a specific and saturable fashion, and the binding of EFDA was blocked when membranes were previously treated with JH III. Interestingly, EBDA, a photoaffinity analog of JH I, did not show any specific binding in equivalent assays; EHDA, a photoaffinity analog of JH II, bound specifically to a 35 kDa protein, and MDK, a photoaffinity analog of methoprene, bound specifically to a different 17 kDa protein (Sevala *et al.*, 1995). Although relatively small, these JH binding proteins may serve as membrane receptors; certainly the 35 kDa protein is within the size range of G protein-coupled receptors (see **Chapter 5.5**). Further investigations are required to elucidate the nature of these proteins.

Webb and Hurd (1995) have reported specific and saturable binding of JH III to microsomal preparations of vitellogenic follicle cells from the beetle, *T. molitor.* Results of Scatchard analysis indicated the occurrence of two sites of different affinity, one with a K_d of 10 nM and the other one of 400 nM. Both sites showed modest affinity for JH I. The specificity of JH binding by follicle cell membranes from the above species is intriguing, given the structural similarity of JH homologs: *R. prolixus* to JH I only, *L. migratoria* to JH III and II, and *T. molitor* to JH III and also JH I. Coincidently, JH III is found in *L. migratoria* and *T. molitor*, while that of *R. prolixus* is unknown.

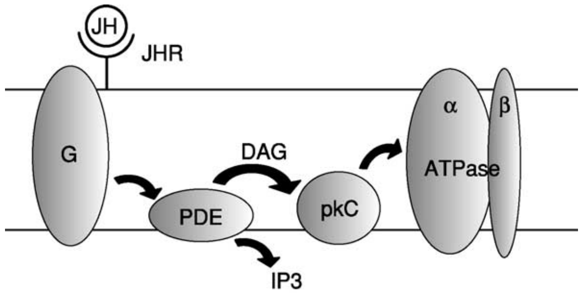


Figure 18 Model for the action of JH on the follicle cell membrane. The binding of JH to the JH receptor (JHR) on the outer surface of the membrane triggers a cascade involving a G protein, with which the receptor site is closely associated. Protein kinase C (pkC) is activated through phosphodiesterase (PDE) and diacylglycerol (DAG) and phosphorylates the α-subunit of the Na^+K^+ ATPase, thus activating the enzyme. (Modified from Wyatt, G.R., Davey K.G., **1996**. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.)

3.9.2.6.4. Development of oocyte endocytic complex and endocytosis When YPs reach the plasma membrane of oocytes (oolemma), they are internalized into the ooplasm through receptor mediated endocytosis, via coated pits and vesicles, and transferred to early endosomes in the cortical ooplasm. From early endosomes they are conveyed to yolk spheres, while receptors are recycled back to the oocyte surface (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998a; Snigirevskaya and Raikhel, 2004; Raikhel *et al.*, 2002). In *D. melanogaster*, the endocytic pathway leading to the production of yolk spheres was visualized by Giorgi *et al.* (1993) with exposure to peroxidase *in vivo* or *in vitro*. The Golgi apparatus and the yolk spheres were then labeled by fixation with osmium zinc iodide (OZI). Starvation resulted in the OZI labeling being restricted to the Golgi apparatus and to an

extended tubular network, whereas feeding or treatment with JH caused the yolk spheres to become labeled with OZI and to incorporate peroxidase. Similar results were obtained in equivalent experiments with the mutant *ap⁴*, which is defective in JH biosynthesis, or with vitellogenic follicular tissue incubated *in vitro* and treated or not with JH. The results of this study indicated that JH facilitates endocytic uptake by inducing the fusion of coated vesicles and tubules with the yolk spheres (Giorgi *et al.*, 1993).

D. melanogaster ap⁴ mutant flies produce low levels of JH and ovarian ecdysteroids and are infertile (Richard *et al.*, 1998, 2001) This situation suggests that JH has a prominent role in regulating YP uptake by the ovary, given that the endocytic organelles are absent in *ap⁴* oocytes and YPs are present at normal levels in the hemolymph. The development of endocytic organelles can be restored in *ap⁴* flies by application of JH in a fashion similar to that of mosquito. In insects with continuous egg production such as *D. melanogaster*, it is not clear whether JH regulates the formation of endocytic organelles, stimulates the endocytosis itself or acts in both events, because these events are difficult to separate.

In insects with cyclic egg production, early developmental and vitellogenic events in egg chambers are separated at the arrest stage, which is broken by either food intake or other stimuli. Normally, these insects mature only a single egg per ovariole in each cycle. Raikhel and Lea (1985) have shown that during the JH dependent previtellogenic period of follicle development in the mosquito *A. aegypti*, a highly specialized endocytic complex consisting of microvilli, numerous coated vesicles, and endosomes appears in the oocyte cortex. The formation of this endocytic complex is controlled by JH III. It was blocked by CA ablation at eclosion, but restored by either CA implantation or the application of JH III. Analysis of several genes involved in receptor mediated endocytosis of YPs, e.g., clathrin heavy chain (Kokoza and Raikhel, 1997), Vg receptor (Sappington *et al.*, 1996), and ovarian lipophorin receptor (Cheon *et al.*, 2001), has revealed that their expression occurs very early in the previtellogenic development of mosquito ovaries, even before the formation of the primary follicle. Immunocytochemistry, however, shows that the Vg receptor is present as the development of the endocytic complex occurs in the oocyte cortex (Sappington *et al.*, 1995). These findings suggest that JH likely acts at the posttranscriptional level on these genes in a manner similar to that in the fat body (Zhu *et al.*, 2003a).

3.9.2.6.5. The ovary as an endocrine organ Many different cell types and tissues make up the insect ovary, and any of these cells or tissues may produce factors that affect itself (autocrine), neighboring cells (paracrine), or more distant cells (endocrine). The ovary is a primary source of ecdysteroids in female insects, as demonstrated in many different insect groups (e.g., Whiting *et al.*, 1997; Marti *et al.*, 2003), and autocrine, paracrine, and endocrine effects have been reported or ascribed to ovarian ecdysteroids in female insects (Hagedorn, 1985; Lanot *et al.*, 1989). The follicle cells surrounding the oocyte are presumed to be the specific cell source, as first demonstrated for locusts (Kappler *et al.*, 1986; 1988), although this has not been confirmed in higher orders of insects (e.g., Diptera). Ecdysteroids are sequestered by developing oocytes in all insects and are present in hemolymph to a lesser or greater degree depending on whether they are required for vitellogenesis. In general, ovarian ecdysteroidogenesis proceeds in dictyopteran females in parallel to vitellogenesis (Pascual *et al.*, 1992; Romañá *et al.*, 1995), but in female mosquitos and higher flies, this process is initiated by neurohormones (see Section 3.9.3.8.3) released in response to the ingestion of a blood or protein meal, respectively (Hagedorn *et al.*, 1975; Trabalon *et al.*, 1990).

For insects, the detailed and complete biosynthetic pathway for the conversion of cholesterol to ecdysteroids is not known, but it is believed to mimic vertebrate steroidogenesis in that precursor steroid molecules shuttle between the endoplasmic reticulum and the inner mitochondrial membrane during processing (Gilbert *et al.*, 2002; see **Chapter 3.3**). Key proteins and enzymes involved in the ovarian ecdysteroid biosynthetic process have been identified by genetic analysis of *D. melanogaster*. Females with the *ecdysoneless* conditional mutation appear to lack the ability to transport an intermediate to the inner mitochondrial membrane for further processing at the restrictive temperature (Warren *et al.*, 1996), but the protein responsible for the mutant phenotype has not yet been identified. Expression of the adrenodoxin reductase (*dare*) gene in ovaries of *D. melanogaster* females is required in the vitellogenic stage of oogenesis (Buszczak *et al.*, 1999; Freeman *et al.*, 1999). This mitochondrial enzyme mediates the transport of electrons from NADPH to adrenodoxin, which in turn donates them to the mitochondrial cytochrome P450 enzymes responsible for steroidogenesis. The *disembodied*, *shadow*, *phantom*, and *shade* genes encode cytochrome P450 enzymes involved in the final

hydroxylation steps of ecdysteroid biosynthesis (Gilbert *et al*., 2002; Warren *et al*., 2002; Petryk *et al*., 2003) (see **Chapter 3.3** for details). These hydroxylases were localized within the adult ovary, and *disembodied* was expressed at the beginning of the vitellogenic stage of oocyte maturation (Chavez *et al*., 2000). Ecdysone is the major ecdysteroid secreted by *A. aegypti* ovaries and is converted to the more active form of 20E by the ecdysone 20-monoxygenase in peripheral tissues, such as the fat body and ovaries (Hagedorn *et al*., 1975; Borovsky *et al*., 1986; Smith and Mitchell, 1986).

The ovary may be a source of JH and peptide or protein messengers. The ovaries of *A. aegypti* synthesize *in vitro* physiologically significant amounts of JH III and other JH-like compounds from radiolabeled farnesoic acid, methionine, and acetate (Borovsky *et al*., 1994). Only one other study has investigated this phenomenon in a female insect, and under similar conditions, ovaries of *D. melanogaster* failed to produce JH (Richard *et al*., 2001). For female Diptera, ovaries with mature eggs may be the source of peptide or protein factors shown to inhibit vitellogenesis directly or indirectly (see Sections 3.9.3.8.7 and 3.9.3.8.8). Unfortunately, hemolymph titers for such factors have not been profiled in these female insects during oogenesis; thus, their physiological function remains conjectural. Several different neuropeptides have been identified in neurons and neurosecretory cells associated with oviducts (see Section 3.9.2.8), and these peptides may have paracrine and endocrine effects when released at specific times during reproduction.

3.9.2.7. Hormone Action in Female Accessory Glands

3.9.2.7.1. The role of juvenile hormone in the colleterial glands Among the insect accessory sex glands and other organs associated with fertilization and oviposition, the colleterial glands of female cockroaches have been studied preferentially with regard to the endocrine basis of development and biosynthetic activity. The asymmetrical left and right colleterial glands form the ootheca, the hard egg case deposited by female cockroaches. The right gland produces an 8-glucosidase, whereas the left gland produces calcium oxalate, protocatechuic acid-8-glucoside, other 8-glucosides, a diphenol oxidase, and a group of structural proteins called oothecins. When the secretions of both glands mix, the glucosidase reacts with the 8-glucosides, and the resulting phenols are oxidized to quinones, which cross-link the oothecins through phenolic bridges (see Koeppe *et al*., 1985). This gives the typical hard consistency of the ootheca.

The first studies demonstrating the key role of CA for accessory gland development in the cockroach *L. maderae* were reported by B. Scharrer in the 1940s. Subsequent research in other cockroach species, *P. americana* and *B. germanica*, involving allatectomy and hormonal treatment, have demonstrated that JH is essential for the production of oothecins and the other molecules in the left colleterial gland (**Figure 19**). JH does not influence production of 8-glucosidase in the right colleterial gland (see Koeppe *et al*., 1985; Wyatt and Davey, 1996).

3.9.2.7.2. Oothecins – structural proteins regulated by juvenile hormone Oothecin synthesis in *P. americana* has been used as a model to investigate the effects of JH. Oothecins are composed of a 39 kDa protein, which is rich in valine and proline, and five smaller proteins (types A–E), which are rich in glycine and tyrosine. The sequence of a C type oothecin displays a number of similarities with chorion proteins of silk moths (Pau *et al*., 1987). Expression studies in *P. americana* females have shown that the first appearance of oothecin mRNAs coincides

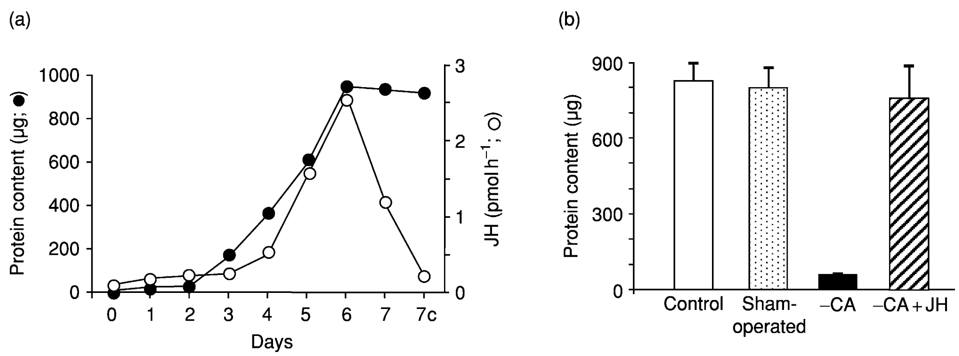


Figure 19 The increase of protein content in the female left colleterial gland of the *Blattella germanica* is paralleled by the increase in production of juvenile hormone (JH) by the corpora allata (a). Protein accumulation is regulated by JH, as shown by experiments of allatectomy (–CA) and subsequent treatment with 10 μg of JH III (–CA + JH) (b). (Based on data from Belles and Piulachs, 1983 and Danès and Piulachs, unpublished data.)

with rising rates of JH biosynthesis, whereas allatectomy abolishes their expression. No correlation was observed with the ecdysteroid concentration in the hemolymph, whereas ovariectomy, which depresses ecdysteroid titer, did not affect oothecin production (Pau *et al.*, 1987). Thus, ecdysteroids probably do not play a significant role in regulating oothecin expression. In a related study, 20E was shown to inhibit RNA synthesis *in vitro* in the left colleterial gland (Iris and Sin, 1988). This may be a pharmacological rather than physiological effect, given that the 20E dose ($1.2\,\mu g\,ml^{-1}$) was more than two orders of magnitude higher than maximal concentrations of ecdysteroids in female *P. americana* (Weaver *et al.*, 1984).

3.9.2.8. Oviposition

Oviposition by female insects is regulated by the central nervous system (CNS) in response to male accessory gland factors passed during fertilization (see **Chapter 1.5**), oocyte maturity, and physical site characteristics. Neurotransmitters and neuropeptides, acting as such, would have a direct action in activating or inhibiting this process, but JH and ecdysteroids may modulate nervous system regulation. Diverse neurotransmitters and neuropeptides have been localized in cells of the terminal ganglion that have axons extending to different oviduct regions in insects, and their effect on oviduct muscle contractions *in vitro* has been documented. Tachykinins (Kwok *et al.*, 1999), SchistoFLRFa (Kwok and Orchard, 2002), crustacean cardioactive peptide (Donini and Lange, 2002), proctolin and serotonin (Lange, 2002) stimulated such contractions, whereas octopamine and myosuppressin were inhibitory (HVFLRFa; Starratt *et al.*, 2000). The spermatheca also are innervated from the terminal ganglion, and octopamine and Arg-Phe amide peptides alter muscle contractions in these organs (Clark and Lange, 2003). Although allatostatin-A has no demonstrated effects on insect oviducts, it greatly increases in concentration in oocytes and oviduct epithelium and nerves of the female cockroach, *Diploptera punctata*, during vitellogenesis, possibly functioning as an anti-JH factor in ovaries (Woodhead *et al.*, 2003). In the cricket *A. domesticus*, ovipositor movements in a typical oviposition sequence are completely abolished in allatectomized females, whereas JH treatment restores that behavior (Strambi *et al.*, 1997). Oviposition by coleopteran females is inhibited by the application of 20E antagonists, largely due to their induction of abnormal egg maturation (hyperecdysonism) (Taïbi *et al.*, 2003).

Parturition hormone (PH) activity is present not only in the uterus of the tsetse fly *Glossina morsitans* but also in the oviducts of *Bombyx* and *Schistocerca*, as well as the ejaculatory duct of *S. gregaria* males (Zdarek *et al.*, 2000). Activity thus appears to be present in the reproductive ducts of diverse insect taxa. To determine whether any of the common insect neuropeptides are capable of mimicking the effect of PH, 35 identified neuropeptides and analogs were evaluated for PH activity. Modest PH activity was observed for only high doses of proctolin and a pyrokinin analog, thus suggesting that PH is unlikely to be closely related to any of the identified neuropeptides tested. While proctolin was highly effective in stimulating contractions of the *S. gregaria* oviduct, the extract from the tsetse fly uterus elicited only a weak response in this bioassay. PH activity, however, was effectively mimicked with an injection of 8 bromo-cyclic GMP, suggesting a potential role for this cyclic nucleotide in mediating the PH response. Neck-ligated, pregnant females were responsive to PH, other neuropeptides and cyclic nucleotides, whereas in intact females, the brain presumably negates the effects of the exogenous compounds.

3.9.2.9. Peptide Hormones Involved in Female Reproduction

As described above, JH and ecdysteroids are considered to be the primary hormones affecting female reproduction by acting separately or coordinately to stimulate oogenesis and vitellogenesis, depending on the insect group or species. Much is known about specific neuropeptides that either stimulate or inhibit JH or ecdysteroid synthesis in insects, but little evidence points to the direct effects of peptide hormones on physiological processes associated with egg maturation (reviews: Gade *et al.*, 1997; Klowden, 1997; De Loof *et al.*, 2001; Nässel, 2002; Taghert and Veenstra, 2003). In effect, neuropeptides regulating the secretion of JH and ecdysteroids could be considered the “master” hormones, because their secretion is directed by the CNS as an integrated response to external and internal cues, such as day length and nutrient stores (see **Chapter 3.2**).

Peptides acting as hormones or neurotransmitters have innumerable effects on behavior, ion and metabolite homeostasis, and locomotion, which are key to successful reproduction by insects (see **Chapter 3.10**). Only recently have comprehensive catalogs for peptide messenger genes been made available for *D. melanogaster* (Taghert and Veenstra, 2003) and *Anopheles gambiae* (Riehle *et al.*, 2002), thanks to their respective genome projects.

Approximately 40 different genes encoding peptide messengers are present in these dipterans, and many of these genes contain multiple copies of variant peptides. New peptide messenger genes await discovery in these insects, and future studies should elucidate the conservation of peptide messenger genes among insect orders. In addition, receptors for these peptides and elements of diverse signal transduction pathways have been revealed by bioinformatics in concert with gene expression and mutation for *D. melanogaster* (Taghert and Veenstra, 2003). Most importantly, elucidation of these pathways will lead to a greater understanding of how peptide messengers modulate biochemical processes and gene expression in insect cells.

3.9.2.9.1. Allatotropins The allatotropin (AT) family of structurally related peptides is so-named because the first peptide was isolated based on its stimulation of the CA *in vitro* to produce JH (see **Chapter 3.7**). In insects where vitellogenesis is JH dependent, this peptide is probably an important initiator of reproduction. The first peptide shown to have this activity was purified from head extracts of pharate adult *M. sexta* (Manse-AT; GFKNVEMMTARGFa; Kataoka *et al.*, 1989). Identical peptides and genes encoding related ones have been identified in other species of Lepidoptera (Taylor *et al.*, 1996; Oeh *et al.*, 2000; Truesdell *et al.*, 2000; Park *et al.*, 2002; Abdel-latief *et al.*, 2003). In *M. sexta*, three alternatively spliced mRNAs result from AT gene expression, which has been localized by *in situ* hybridization and immunocytochemistry to cells in the brain, frontal ganglion ventral ganglia, and midgut of different life stages (review: Elekonich and Horodyski, 2003). In *P. unipuncta*, expression of the AT gene was observed first in late pupae and continued during the adult stage (Truesdell *et al.*, 2000).

Related peptides and genes encoding such peptides have been identified in *L. migratoria* (Paemen *et al.*, 1991), a beetle (*L. decemlineata*; Spittaels *et al.*, 1996), two species of Diptera, *A. aegypti* (Veenstra and Costes, 1999) and *A. gambiae*, and in a few other invertebrates (Elekonich and Horodyski, 2003). Immunocytochemical studies with Manse-AT antisera have revealed the presence of AT-like peptides in cockroaches and two other dipterans, including *D. melanogaster* (Elekonich and Horodyski, 2003), but no ortholog AT gene has been identified in the *D. melanogaster* genome as yet (Taghert and Veenstra, 2003).

In vitro assays with adult CA are routinely used to investigate the pathway of JH biosynthesis. Manse-AT stimulates the CA to produce JH I, II, and III, but is limited by availability of farnesoic acid, a precursor (Elekonich and Horodyski, 2003). This AT also stimulates JH secretion *in vitro* by larval and adult CA of other lepidopteran species and the adult CA of the honeybee, *A. mellifera* (Rachinsky *et al.*, 2000) and blowfly, *Phormia regina* (Tu *et al.*, 2001). Similarly, JH secretion by the CA of older sugar fed female *A. aegypti* is increased *in vitro* by this species' AT or farnesoic acid alone, whereas both are required to activate JH synthesis in CA from newly eclosed females (Li *et al.*, 2003).

In adult insects, ATs have demonstrated effects on other processes associated with reproduction. *L. migratoria* AT may play a role in oviposition, as suggested by its myotropic activity on oviducts from locust and cockroach females, *Leucophaea maderae*, the basis for its isolation from male accessory glands (Paemen *et al.*, 1991). Immunocytochemistry showed that AT-immunoreactivity had a widespread and sexually dimorphic distribution in the nervous system of adult locusts and cockroaches (Nässel, 2002).

3.9.2.9.2. Allatostatins Three groups of neuropeptides in insects have been isolated based on their inhibition of JH secretion by CA *in vitro* and are known as allatostatins (ASTs). The first peptides with this bioactivity were isolated from the cockroach, *D. punctata* (Woodhead *et al.*, 1989) and are now termed the A- or cockroach-type of AST (AST-A). Typically, they are short peptides with the signature terminal sequence of F/YXFGLa, and identified AST-A cDNAs and genes encode a propeptide containing 13 or 14 peptides with slight sequence variations that are posttranscriptionally cleaved and processed (Belles *et al.*, 1999; Meyering-Vos *et al.*, 2001). The second group, known as the B- or cricket-type of AST (AST-B), is a family of peptides with a common sequence of W(X)$_6$Wa, e.g., GWQDLNGGWa and AWERFHGSWa, first identified in the cricket, *G. bimaculatus* and shown to inhibit JH biosynthesis *in vitro* in CA from virgin females (Lorenz *et al.*, 1995). Related peptides were isolated first from a locust, *L. migratoria*, based on their inhibition of oviduct contractions (Schoofs *et al.*, 1991). The third group or C-type of AST (AST-C) is represented by the 15 amino acid, non-amidated peptide (PEVRFRQCYFNPISCF), first discovered in *M. sexta* (Kramer *et al.*, 1991), based on its inhibition of JH synthesis by CA from adult moths.

All three AST types probably exist in insects, as indicated by the annotation of peptide messenger genes for *D. melanogaster* (Taghert and Veenstra, 2003) and *A. gambiae* (Riehle *et al.*, 2002), and other reports detail the isolation of related peptides

or characterization of encoding cDNAs or genes from a variety of insects (reviews: Bendena *et al.*, 1999; Stay, 2000). Numerous immunocytochemical studies have examined the distribution of AST types in a great variety of insects and even different life stages and organs (Nassel, 2002). Each AST type appears to have a unique distribution in the CNS, and the presence of an AST type in the CA is dependent on whether it affects JH secretion by the CA. Interestingly, AST-A is also present in midgut endocrine cells and hemocytes (Stay, 2000), thus suggesting an even greater endocrine repertory.

In general, it appears that one AST type inhibits JH secretion by CA in one or more life stages of a particular species, and the other types do not. The dipteran ASTs are exceptional in that none affect JH secretion by dipteran larval CA, but blowfly AST-A (callatostatin) inhibits JH biosynthesis by CA from cockroaches (Duve *et al.*, 1993). Notably, peptides from all three AST types have been shown to inhibit muscle contractions in a variety of organs from different insect groups. Receptors for ASTs have been identified in a cockroach (AST-A; Auerswald *et al.*, 2001), a silk moth (AST-A; Secher *et al.*, 2001) and *D. melanogaster*, after candidate G protein-coupled receptors (GPCR) were expressed in cell systems and shown to bind AST-A (two different GPCRs; Larsen *et al.*, 2001), AST-B (Johnson *et al.*, 2003), and AST-C (Kreienkamp *et al.*, 2002).

In addition, ASTs have demonstrated effects on female reproduction. An AST-A inhibited vitellogenin release *in vitro* by fat body of cockroach females, *B. germanica* (Marin *et al.*, 1996). The inhibitory effect was counteracted by the addition of mevalonolactone, thus suggesting that the AST-A inhibited synthesis of mevalonate, and hence dolichol, and therefore impairing the glycosylation and export of vitellogenin from the fat body. *In vitro* ecdysteroid biosynthesis by ovaries from a cricket, *G. bimaculatus*, was inhibited by an AST-B (Lorenz *et al.*, 1997), the same action as prothoracicostatic peptides in the AST-B family have on larval prothoracic glands from the silk moth, *B. mori* (Hua *et al.*, 1999; **Chapter 3.2**). Surprisingly, there are only a few reports on the *in vivo* effects of ASTs in female insects. After showing that bioactive AST-As were circulating in *D. punctata*, three synthetic AST-As were injected every 12 h for 3 days into mated females and were found to significantly decrease oocyte length and *in vitro* JH synthesis by the CA in treated females (Woodhead *et al.*, 1993). Similar injections of cricket AST-A and AST-B into female crickets, *G. bimaculatus*, resulted in decreased body and ovary weight, eggs/ovary, and ovary ecdysteroid biosynthesis and increased vitellogenin titer in hemolymph. The peptides had little or no effect on JH biosynthesis; however, these trends were not statistically significant when compared to controls (Lorenz *et al.*, 1998).

3.9.2.9.3. Ovary ecdysteroidogenic hormone and neuroparsin Ovaries in female insects produce ecdysteroids, but only in Diptera do these hormones direct the increased gene expression required for vitellogenesis. In a series of classic endocrine studies on female mosquitos, the first "egg development neurosecretory hormone" or gonadotropin was described for insects (Lea, 1972). Almost 30 years later, this hormone was isolated from the heads of female *A. aegypti*, structurally characterized as an 86 residue peptide, and renamed "ovary ecdysteroidogenic hormone" (OEH), based on its direct stimulation of ecdysteroid synthesis by ovaries *in vitro* (Brown *et al.*, 1998). Immunocytochemistry with OEH antiserum stained clusters of medial neurosecretory cells in brains, as well as other cells in the ventral ganglia and midguts of larvae and both sexes of *A. gambiae* and *A. aegypti* (Brown and Cao, 2001). Cloning of the OEH cDNA revealed the OEH prohormone and led to the bacterial expression of a recombinant OEH that was shown to have the same bioactivity *in vitro* and stimulated vitellogenesis and subsequent yolk deposition; this is considered to be an indirect effect of increased ovarian ecdysteroidogenesis (Brown *et al.*, 1998). An ortholog OEH gene has been identified in another mosquito, *A. gambiae*, but not in *D. melanogaster* (Riehle *et al.*, 2002).

In Diptera, activation of ovarian ecdysteroidogenesis by OEH or other brain factors may occur through either an insulin signaling pathway (see below) or a G protein-coupled receptor (GPCR)/cAMP pathway (Shapiro, 1983). Factors with OEH activity have been extracted from brains of higher flies (Adams and Li, 1998), and in the blowfly, *Phormia regina*, ovarian steroidogenesis is stimulated *in vitro* by cAMP analogs and preceded by a peak in ovarian cAMP levels after a protein meal (Manière *et al.*, 2000). This study also showed that crude brain extracts, as well as separate extracts of the medial neurosecretory region and the rest of the brain, stimulated ovarian ecdysteroidogenesis *in vitro* but, surprisingly, the medial neurosecretory extracts did not elicit an increase in ovarian cAMP levels, whereas the crude brain and nonneurosecretory region extracts did. These results offer additional support for the existence of ecdysteroidogenic brain factors in female flies that act through different signaling pathways.

Mosquito OEHs are closely related to the neuroparsins first isolated from the corpora cardiaca of

L. migratoria (Girardie *et al.*, 1987). Experiments showed that the purified native A and B forms (83 and 78 residues, respectively) administered to adult females inhibited oocyte growth, whereas injection of neuroparsin antibody alone had the opposite effect (Girardie *et al.*, 1987). These are considered to be anti-JH effects, since JH is required for vitellogenesis in locusts, and ecdysteroids have no known role, but the mechanism for these effects remains unknown. Neuroparsins also elevated trehalose and lipid levels in hemolymph of male locusts (Moreau *et al.*, 1988) and act as antidiuretics on rectal pads (Girardie and Fournier, 1993).

As revealed by the cloning of *L. migratoria* neuroparsin cDNA, both neuroparsin forms are processed from a prohormone (Langueux *et al.*, 1992). Other neuroparsins have been isolated and identified in the locust, *Schistocerca gregaria*, and have a similar bioactivity (Girardie *et al.*, 1998a). More recently, four different neuroparsin transcripts/cDNAs have been identified in *S. gregaria* (Janssen *et al.*, 2001; Claeys *et al.*, 2003); two of the transcripts were present only in brains of all life stages, whereas the other two transcripts were in the CNS, fat body, and male accessory glands and testis. Northern blot analyses showed that levels of all four transcripts changed throughout the life stages, with a significant increase in the two widely distributed ones preceding the sexual activity of males and females. Peptides for three of the transcripts have yet to be isolated, thus their role in reproduction is unknown.

Locust neuroparsins and mosquito OEHs constitute a peptide family, as substantiated by an analysis of nucleotide and protein sequence databases that revealed neuroparsin related peptides in the honeybee and diverse arthropod and mollusk species (Claeys *et al.*, 2003). This analysis further suggested that conserved features of these peptides are shared by insulin-like growth factor binding proteins, which modulate growth factor activity, in vertebrates.

3.9.2.9.4. Ovary maturing parsin A neurohormone was isolated from female locusts, *L. migratoria*, based on its stimulation of vitellogenesis and oocyte growth, and was thus designated "ovary maturating parsin" (OMP; Girardie *et al.*, 1991). The peptide of 65 residues occurs in two isoforms, differing only at one residue position. With immunocytochemistry, OMP was localized in brain neurosecretory cells and the corpora cardiaca of locusts in all stages (Richard *et al.*, 1994). In a later study, sets of female *L. migratoria* were injected daily with different amounts of OMP over 10 days after eclosion and found to have significantly increased hemolymph titer of ecdysteroids and oocyte length over controls (Girardie *et al.*, 1998b). OMP has been identified in another locust, *S. gregaria*, and exists as isoforms: two long forms corresponding to those of *L. migratoria* OMP and two shorter forms found only in mature adults (Girardie *et al.*, 1998b). Injection of mixed OMPs in female *S. gregaria* over 12 days similarly affected ecdysteroid and vitellogenin levels in hemolymph and stimulated oocyte growth, relative to controls (Girardie *et al.*, 1998b). Although locust OMPs are considered ecdysteroidogenic and gonadotrophic peptides *in vivo*, no direct effects have been reported for OMP on isolated locust ovaries or fat body, thus, the mode of action needs further study. OMPs are known to exist in acridian Orthoptera, but not in other insects (Richard *et al.*, 1994), as indicated by the failure to identify related peptides in the *D. melanogaster* and *A. gambiae* genome databases.

3.9.2.9.5. Short neuropeptide F, neuropeptide F, and head peptides The sequences of a great diversity of insect neuropeptides end in Arg-Phe-NH_2, and three such peptides of relevance were isolated from the Colorado potato beetle, *L. decemlineata*, (ARGPQLRLRFa and APSLRLRFa; Cerstiaens *et al.*, 1999) and the locust, *S. gregaria* (YSQVARPRFa; Schoofs *et al.*, 2001). In a subsequent experiment, multiple injections of the beetle peptides into virgin female locusts, *L. migratoria*, significantly increased oocyte growth relative to controls (Cerstiaens *et al.*, 1999). Injection of the *S. gregaria* peptide stimulated ovarian growth in female *S. gregaria*, presumably due to the increased vitellogenin levels in the peptide treated females (Schoofs *et al.*, 2001). It has not been determined whether these effects are specific to vitellogenesis or protein synthesis in general.

The two beetle peptides, along with peptides identified in other insect and arthropod species, belong to a "short neuropeptide F" (sNPF) family, as clearly indicated by ortholog genes in *D. melanogaster* (Taghert and Veenstra, 2003) and *A. gambiae* (Riehle *et al.*, 2002) that encode related short peptides ending in RLRFa or RLRWa. A functional GPCR for the *D. melanogaster* sNPFs has been characterized and its tissue expression characterized (Mertens *et al.*, 2002; Feng *et al.*, 2003), but no other bioactivities are known for insect sNPFs. The "head peptides" first identified in *A. aegypti* and later shown to inhibit host seeking behavior in nonoogenic females (Brown *et al.*, 1994) are not members of the sNPF family, as indicated by their terminus, KTRFa, and the structure of the *Aedes* head peptide cDNA (Stracker *et al.*, 2002).

Surprisingly, analysis of the *D. melanogaster* and *A. gambiae* genome databases revealed no ortholog "head peptide" genes (Riehle *et al*., 2002).

The sequence of the *S. gregaria* peptide (probably a fragment) is more similar to the C-terminus of longer peptides (36 residues almost invariably) in the "neuropeptide F/Y" family of invertebrate and vertebrate peptides (Stanek *et al*., 2002). A functional GPCR for the *D. melanogaster* NPF has been characterized (Garczynski *et al*., 2002), and in *D. melanogaster*, NPF controls foraging and social behavior by larvae (Wu *et al*., 2003), but no bioactivities are known for adults.

3.9.2.9.6. Insulin-like peptides and the insulin signaling pathway Insulin-like peptides (ILPs) are known for only a few insect species: *L. migratoria*, three species of Lepidoptera, and *D. melanogaster* (review: Claeys *et al*., 2002). In all but the locust, multiple ILP genes have been identified, with *B. mori* having an astonishing 32 ILP genes (also known as "bombyxins") and *D. melanogaster* and *A. gambiae*, each with 7 ILP genes (Krieger *et al*., 2004). In insects, clusters of neurosecretory cells in the medial, dorsal region of brains are immunostained by insulin and ILP antisera in different life stages and species (Cao and Brown, 2001; Rulifson *et al*., 2002). The gut and reproductive tract may be another source of ILPs as reported for a few insect species (Montuenga *et al*., 1989; Iwami *et al*., 1996); these organs are the primary sources of insulin and related peptides in vertebrates. The presence of ILPs in hemolymph of larvae and adult silk moths has been established, and the ILP titer is much higher in males after eclosion (Satake *et al*., 1999).

As characterized for vertebrates and invertebrates, insulin and related peptides act primarily through a receptor tyrosine kinase and an insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K)/protein kinase B/Akt signaling pathway (Oldham and Hafen, 2003). For insects, the expression and function of proteins comprising this pathway have been characterized in detail only for *D. melanogaster* (Garofalo, 2002), and ortholog genes of the proteins were identified in *A. gambiae* (Riehle *et al*., 2002). An ever-increasing number of studies show that this pathway is a nexus for the transcriptional and translational regulation of growth and longevity in *D. melanogaster* and other animals (Oldham and Hafen, 2003; Tatar *et al*., 2003).

Perturbations in any gene encoding ILPs or proteins in this pathway result in multiple and dramatic effects on not only embryonic and postembryonic development, but also oogenesis and vitellogenesis in female *D. melanogaster*. Females with an ILP gene mutation had at most a single vitellogenic oocyte in each ovariole and thus a much reduced fecundity, in comparison to wild-type females (Ikeya *et al*., 2002). Mutations in the gene encoding the insulin receptor (IR) result in sterile females due to reduced ovariole development and yolk deposition (Chen *et al*., 1996). Females with an IR mutation are longer lived but deficient in JH synthesis by the CA and ovarian ecdysteroid production (Tatar *et al*., 2001; Tu *et al*., 2002). Treatment of these mutant flies with a JH analog initiates vitellogenesis and restores normal life expectancy. Vitellogenesis is blocked in females with a homozygous mutation in the IRS gene (Drummond-Barbosa and Spradling, 2001), yet another step in this key pathway.

There are only a few studies of the insulin signaling pathway in other insects. In the mosquito *A. aegypti*, the expression pattern and phosphorylation states of the IR and PKB/Akt in ovaries were characterized through previtellogenic arrest and a gonotrophic cycle after a blood meal (Riehle and Brown, 2002, 2003). Bovine insulin stimulates ecdysteroidogenesis by *A. aegypti* ovaries directly in a dose-dependent manner *in vitro*, whereas specific inhibitors of tyrosine kinase activity and PI3K inhibit ecdysteroid production (Riehle and Brown, 1999). In Lepidoptera, a putative IR was identified in tissues from *M. sexta* larvae with an IR antiserum (Smith *et al*., 1997), and ovarian cells from three species showed high-affinity binding to a silk moth ILP, presumably to ovarian IRs recognized by an IR antiserum (Fullbright *et al*., 1997).

Recently, the panoply of insulin-like peptide regulation in *D. melanogaster*, in part, has been shown to act through forkhead transcription factors and is nutrition dependent (Junger *et al*., 2003). The possibility also exists that insect ILPs may activate MAP kinases and G protein-coupled receptor/cAMP pathways (see **Chapter 3.2**), as shown for insulin related peptides in vertebrates (Hsu *et al*., 2002; Oldham and Hafen, 2003), and even elicit cross-talk between signaling pathways. Conceptually, this activation of multiple pathways by ILPs has the potential to exhibit as profound an effect on the expression of genes required for reproduction in insects, as do JH and ecdysteroids.

3.9.2.9.7. Ovary peptides that block vitellogenesis Unrelated peptides have been isolated from mature ovaries of dipteran species based on their ability to block egg maturation. The first such peptide (YDPAPPPPPP) was extracted from the ovaries of the mosquito, *A. aegypti*, and shown to inhibit yolk deposition in blood-fed females (Borovsky,

2003). Later, the direct action of this peptide was determined to be inhibition of proteolytic enzyme biosynthesis (see **Chapter 4.7**) in the midgut (see **Chapter 4.5**), and thus is known as a "trypsin modulating oostatic factor" (TMOF). With a TMOF antiserum, this peptide was localized to the follicular epithelium surrounding oocytes only after 24 h PBM and was not present in other tissues (Borovsky *et al*., 1994). This localization is supported by the characterization of a gene encoding a vitelline membrane protein, which contains TMOF sequences (Lin *et al*., 1993), and this protein is secreted by follicular epithelium cells.

A different peptide (NPTNLH) with this same activity was purified from ovarian extracts of the flesh fly *Neobellieria bullata* (Bylemans *et al*., 1994; Borovsky *et al*., 1996). Injection of the hexapeptide inhibited trypsin synthesis by the midgut of liver-fed flesh fly females, resulting in a reduction of circulating vitellogenin and oocyte growth. Immunoassays determined that *N. bullata* TMOF-staining was localized exclusively over yolk granules in oocytes and that a 75 kDa precursor protein was present in ovary extracts (Bylemans *et al*., 1996). A more recent study has shown that this peptide is probably a substrate for an angiotensin converting enzyme circulating in the hemolymph of female flies, and feeding of an inhibitor of this enzyme enhanced hemolymph vitellogenin levels (Vandingenen *et al*., 2001). A second peptide (SIVPLGLPVPIGPIVVGPR) with a similar effect was also purified from ovarian extracts of this fly (Bylemans *et al*., 1995) and named "colloostatin," given its sequence similarity to collagen. When administered *in vivo*, the peptide inhibits yolk uptake by previtellogenic oocytes and reduces the levels of circulating vitellogenin, but it does not inhibit trypsin biosynthesis in the gut.

3.9.2.9.8. Termination of vitellogenesis A few studies have shown that peptide hormones are involved in the transition from vitellogenesis to chorionogenesis so that egg maturation and ultimately oviposition can occur. Oostatic factors originating from ovaries are known for insects, and these factors may play a role in this transition through distinctly different actions, including inhibition of ovary ecdysteroidogenesis (Kelly *et al*., 1986; Adams and Li, 1998), than that reported for TMOF and other such "oostatic" factors. In dipteran females, termination of ovary ecdysteroidogenesis indirectly may end vitellogenesis, and recent studies indicate that ovarian steroidogenesis is inhibited through signaling pathways that use Ca^{2+} or cGMP as intermediates. *In vitro* ecdysteroidogenesis by vitellogenic ovaries from the blowfly, *P. regina*, was inhibited by Ca^{2+} ionophores or thapsigargin, whereas inhibitors of Ca^{2+}-calmodulin phosphodiesterases increased ecdysteroidogenesis (Manière *et al*., 2002). Analogs of cGMP inhibited *in vitro* steroid biosynthesis in vitellogenic ovaries from *P. regina*, thus correlating with the peak levels of cGMP detected in ovaries at the termination of vitellogenesis (Manière *et al*., 2003). Notably, paracrine or autocrine factors, such as nitric oxide, were implicated in this inhibition, and not brain factors, thus pointing to an even greater degree of complexity in the regulation of insect reproduction.

Th adipokinetic hormone (AKH) family has key roles in regulating lipid and carbohydrate metabolism in all life stages of diverse insects (see **Chapter 3.10**). These peptides also are known to inhibit protein and vitellogenin synthesis and RNA synthesis in female fat body and circulate in the hemolymph of ovipositing locust females (Moshitzky and Appelbaum, 1990; Glinka *et al*., 1995; Kodrik and Goldsworthy, 1995). AKH regulation occurs through a GPCR, now identified in *D. melanogaster* and *B. mori* (Staubli *et al*., 2002), and a signaling pathway with cAMP and Ca^{2+} intermediates (Gäde *et al*., 1997).

A GPCR/cAMP signaling pathway is involved in the termination of vitellogenesis in mosquitos (Dittmer *et al*., 2003). Characterization of the gene encoding a cAMP response-element binding protein in the mosquito, *A. aegypti* (AaCREB) revealed signature domains for this family of transcription factors, and this gene was constitutively expressed in female fat body, where YPs are synthesized. Elicitors of the cAMP signal transduction pathway attenuated ecdysteroid stimulated YP gene expression by fat body *in vitro*. In cell transfection assays, AaCREB served as a potent repressor of transcription, and analysis of electrophoretic mobility shift assays detected CREB specific band-shift complexes in nuclear extracts from vitellogenic fat bodies at 24 h and 36 h post-blood meal, when YP gene expression reaches its peak then terminates. Examination of the regulatory regions of Vg and vitellogenic carboxypeptidase revealed putative CREB response elements, which bound *in vitro* expressed AaCREB and offers further support for its termination of YP gene expression in the fat body of this mosquito.

3.9.3. Hormones and Male Reproduction

3.9.3.1. Spermatogenesis

Insect spermatogenesis can be divided into three main steps: (1) mitotic proliferation of spermatogonia, leading to spermatocytes; (2) meiosis of

spermatocytes, giving the spermiocytes; and (3) spermiogenesis of spermiocytes, leading to spermatozoa. As for female reproduction, ecdysteroids, JHs, and peptides have demonstrated effects on male reproductive processes.

3.9.3.1.1. Ecdysteroids and early spermatogenesis In the early steps of spermatogenesis, involving mitoses and meioses, the stimulatory role of ecdysteroids has been demonstrated in many species of Orthoptera, Hemiptera, Lepidoptera, and Diptera (Hagedorn, 1985). In *R. prolixus*, for example, mitotic division of spermatogonia takes place in the last larval instar in the absence of JH and in the presence of high levels of ecdysteroids. In addition, it has been shown experimentally that 20E increases the rate of mitosis in the spermatogonial cells (Dumser, 1980). Further corroboration of the importance of ecdysteroids in early spermatogenesis has been provided by Jacob (1992) who reported that fragments of testes from the rhinoceros beetle, *Oryctes rhinoceros*, incubated *in vitro* require ecdysteroids and the testis sheath to complete the mitotic and meiotic processes.

The stimulatory function of ecdysteroids in early stages of spermatogenesis gives physiological sense to the discovery of ecdysteroid production in the testes of the lepidopteran *Heliothis virescens* (Loeb *et al*., 1982). These authors observed that the testis sheath of tobacco budworm larvae, when incubated *in vitro*, secreted ecdysteroids into the culture medium. Testes ecdysteroid production has been demonstrated for other Lepidoptera belonging to the genera *Lymantria*, *Ostrinia*, *Mamestra*, *Leucania*, and *Spodoptera* (see Loeb *et al*., 2001 and references therein).

3.9.3.1.2. The role of juvenile hormone The role of JH in spermatogenesis is less clear. Early reports suggested that JHs antagonize the stimulatory effects of ecdysteroids, especially in early stages of spermatogenesis (see Dumser, 1980; Koeppe *et al*., 1985; Wyatt and Davey, 1996). However, other contributions have shown that JH seems to have a stimulatory effect on late spermatogenesis, especially in diapausing species when JH simultaneously accelerates spermiogenesis and interrupts the diapause (Koeppe *et al*., 1985). For example, administration of a JHA to diapausing adult leafhoppers, *Draeculacephala crassicornis*, did not influence mitosis of spermatogonia but promoted the formation of spermatozoa (Reissig and Kamm, 1974). Results obtained in the beetle, *Oryctes rhinoceros*, in addition to showing that ecdysteroids are necessary in early spermatogenesis (see above), also demonstrated that spermiogenesis took place *in vitro* only if an active CA pair was present in the incubation medium (Jacob, 1992). The stimulatory role of JH on spermiogenesis may be related to polyamine synthesis, especially putrescine, spermidine, and spermine, given that JH promotes the production of these compounds, at least in fat body and neural tissue of crickets (Cayre *et al*., 1995).

Another effect of JH related to sperm physiology has been discovered by Dean and Meola (1997) in the cat flea, *Ctenocephalides felis*. These authors have shown that sperm transfer into the epididymis is stimulated when the fleas are exposed to a filter paper treated with JH III or JHAs. As the concentration of the JHA or the exposure time increases, the percentage of fleas that transfer sperm also increases.

3.9.3.1.3. Peptides and proteins involved in spermatogenesis In relation to ecdysteroid biosynthesis in the testes, Wagner *et al*. (1997) have identified a 21 amino acid peptide (ISDFDEYEPLNDADNNEVLDF) in brain extracts of the gypsy moth, *L. dispar*, that has ecdysteroidogenic properties. Given that the peptide induces the synthesis of ecdysteroids specifically in the testes (induction experiments using prothoracic glands were unsuccessful), it has been called "testes ecdysiotropin."

Other peptide or protein factors are postulated to be directly involved in the regulation of spermatogenesis. As early as 1953, C. Williams reported the occurrence of a macromolecular factor in the hemolymph of *Hyalophora cecropia* that stimulated spermatogenesis in spermatocysts incubated *in vitro*. In 1971, Kambysellis and Williams showed that 20E allowed the entry of this factor into the testis (see Hagedorn, 1985), but since then, no such factor has been isolated. More recently, spermatid differentiation in *Drosophila* was shown to require a peptidyl peptidase ortholog of the mammalian angiotensin converting enzyme (ACE) (Hurst *et al*., 2003), and ACE mRNA is found mainly in large primary spermatocytes, whereas it is not detectable in cyst cells.

3.9.3.2. Male Accessory Gland Function

The male accessory glands of insects generally are of mesodermal origin and exhibit a wide morphological diversity, from single pairs of histologically and morphologically identical tubules to multi-paired heterogeneous tubules, with multiple forms and contents (Happ, 1992). Development of male accessory glands is regulated by ecdysteroids (review: Happ, 1992), as with many other developmental processes. This section will focus on the endocrine regulation of the biosynthetic and secretory activity of the

glands. The secretions produced by male accessory glands have a wide diversity of functions (see **Chapter 1.5**), and some of these secretions, when transferred to the female through copulation, may affect the process of vitellogenesis, as described below.

3.9.3.2.1. Products of the male accessory glands The male accessory glands synthesize and secrete a complex mixture of proteins, carbohydrates, lipids, and amino acids (Chen, 1984; see **Chapter 1.5**) that are transferred to the female during copulation. The primary function of the accessory gland products is to facilitate sperm transfer to the female. For example, these glands produce the structural proteins needed for spermatophore formation, and these spermatophore proteins may serve as nutrient resources for the female. In addition, many secretory products of the accessory glands cause physiological and behavioral changes in the mated female. These changes may be sperm related (sperm protection, storage and activation, competition with the sperm of previous males) or may alter the behavior and reproductive physiology of the mated female. The most prominent behavioral effects are the reduction of attractiveness to males and induction of refractoriness. The effects on reproductive physiology include enhancement of oocyte growth and induction of ovulation and oviposition (Chen, 1984; Gillott, 2003).

In recent years, research has been focused on Drosophilid dipterans, and more than 75 accessory gland proteins have been molecularly characterized in *D. melanogaster* (Swanson *et al*., 2001; **Chapter 1.5**). Of these proteins, the so-called sex peptide has been the most thoroughly studied compound (see **Chapter 1.5**). The sex peptide of *D. melanogaster* is synthesized as a 55 amino acid precursor, and the processed peptide depresses sexual receptivity and enhances oviposition. Ortholog peptides have been identified in other *Drosophila* species (Chen, 1996; Wolfner, 1997). In *D. melanogaster*, sex peptide stimulates the biosynthesis of JH *in vitro* by the CA of the adult female (Moshitzky *et al*., 1996). This activity is possibly related to the results of Soller *et al*. (1999), which showed that the same peptide stimulates vitellogenic oocyte progression in *D. melanogaster* and that a JHA mimics such an effect. Interestingly, the sex peptide of *D. melanogaster* stimulates JH synthesis in *Helicoverpa armigera* (Fan *et al*., 1999), which suggests that this Noctuid moth may have ortholog peptides with these allatotropic properties. Male accessory glands are an important source of JHs and related metabolites (see Section 3.9.3.2.2). These JHs can be transferred to females through copulation and enhance oocyte growth, as shown for certain lepidopterans in which JH regulates vitellogenesis (Shirk *et al*., 1980; Park *et al*., 1998).

3.9.3.2.2. Regulation of male accessory gland function by juvenile hormone In the 1940s, Wigglesworth carried out the first studies showing the importance of CA in male accessory gland function for *Rhodnius*. In the cockroach, *B. germanica*, JH influences the growth not only of the accessory glands (Piulachs *et al*., 1992) (**Figure 20**) but also of the conglobate gland (Vilaplana *et al*., 1996). Further research on other insect species, mainly orthopterans, dictyopterans, lepidopterans, and dipterans, has demonstrated that allatectomy depresses to a greater or lesser degree the accumulation of gland secretions, whereas JH treatment restores the usual secretion levels (Gillott, 1996; Wyatt and Davey, 1996). In a number of species, it has been reported that the pattern of secretion accumulation in the accessory glands is parallel to that of JH concentration in the hemolymph or to that of JH synthetic rates by the CA (Wyatt and Davey, 1996). Incubation of the male accessory glands from *D. melanogaster* with nanomolar concentrations of JH induced a nearly threefold stimulation of protein synthesis (Yamamoto *et al*., 1988). Since many accessory gland proteins in *D. melanogaster* have been characterized at the molecular level (see above and **Chapter 1.5**), monitoring expression of these genes in response to JH has become easier. For example, Cho *et al*. (2000) have shown that the protein Mst57Dc is highly expressed after eclosion, when the titer of JH III peaks, and that in the JH deficient mutant *ap56f*, the levels of Mst57Dc mRNA are about 60% of those of the wild-type.

Subsequent studies have monitored the effect of JH on the pattern of proteins, or even on particular proteins, present in accessory glands for species of Hemiptera, Orthoptera, and Dictyoptera (Wyatt and Davey, 1996). For example, allatectomy differentially affects the accumulation of various proteins present in the glands, whereas treatment with JH affects the protein pattern in the grasshopper, *Melanoplus sanguinipes* (Gillott, 1996), and in *B. germanica* (Belles and Piulachs, 1992). In *M. sanguinipes*, treatment with JH III induces the accumulation of most proteins but depresses the accumulation of two (Gillott, 1996). A later study of *M. sanguinipes* showed that JH acts directly on the accessory glands to promote synthesis of a specific protein, LHPI, which constitutes more than 50% of the protein content (Gillott and Gaines, 1992). Juvenile hormone could not stimulate LHPI synthesis in glands from allatectomized males, unless there was an

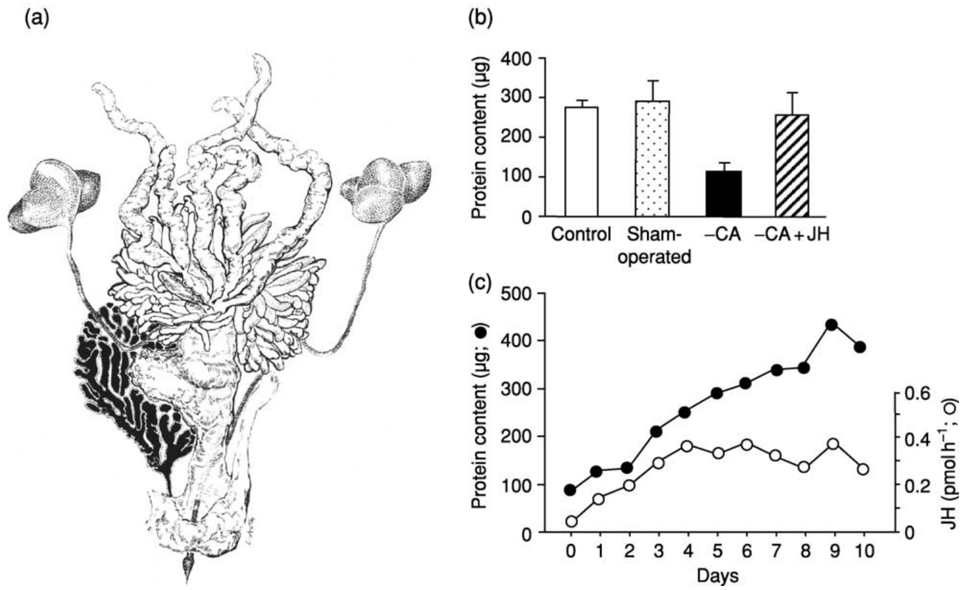


Figure 20 Protein accumulation in the male accessory glands of *Blattella germanica* (a) is regulated by juvenile hormone (JH), as shown by allatectomy experiments (−CA) and subsequent treatment with 10 μg of JH III (−CA + JH) (b). In addition, the patterns of increase in protein contents in the first days of adult life, and that of JH production by the corpora allata incubated *in vitro* are approximately parallel (c). (Based on data from Piulachs, M.D., Maestro, J.L., Belles, X., **1992**. Juvenile hormone production and accessory reproductive gland development during sexual maturation of male *Blattella germanica* (L.) Dictyoptera, Blattellidae). *Comp. Biochem. Physiol. 102A*, 477–480; and Belles and Piulachs, unpublished data.

overnight exposure to the hormone. Cycloheximide temporally abolishes the stimulatory effect of JH on LHPI synthesis in the glands of male *M. sanguinipes* (Ismail *et al.*, 1995), thus suggesting that the action of JH is mediated by protein factors involved in the transcription of LHPI. A similar priming effect of JH on protein synthesis in the accessory glands of male *L. migratoria* has been reported (Braun and Wyatt, 1995). It is worth noting that the protein Met of *D. melanogaster*, which may be involved in the action of JH action, is localized in the accessory glands and ejaculatory duct cells of adult males (Pursley *et al.*, 2000).

3.9.3.2.3. Production of juvenile hormone by the accessory glands As mentioned above, JH is a product of the accessory gland. In fact, the first active extracts of JH were obtained by Williams in the 1950s from the abdomen of the adult male of the moth, *Hyalophora cecropia*, and these glands are still known to be the most copious, natural source. It was later shown that the CA of the cecropia moth produce the acids of JH I and II and that these are converted into the corresponding JHs by the male accessory glands (Shirk *et al.*, 1983). Production of JH in the accessory glands has been reported in males of *Heliothis* as well (Park *et al.*, 1998).

JH production by male accessory glands may enable transfer of JH to females during copulation to directly enhance vitellogenesis and oocyte growth or indirectly to stimulate the female's CA. As shown for *H. virescens*, mating has an allatotropic effect, and JH measurements in males and females before and after copulation suggest the transfer of JH to females by the male (Park *et al.*, 1998). Also, production or sequestration of JH by male accessory glands may be another mechanism to regulate JH hemolymph titer or biosynthesis by CA.

3.9.3.2.4. Other hormones regulating male accessory glands Hormones other than JH also may regulate the biosynthetic activity of male accessory glands. In *R. prolixus* males, removal of certain brain neurosecretory cells impairs protein accumulation in the accessory glands, whereas treatment with JH I only partially restores protein accumulation; a peptide extracted from *R. prolixus* brains stimulates protein synthesis by isolated accessory glands (Wyatt and Davey, 1996). As these results indicate, a neuropeptide and JH are required for accessory gland function. Cerebral neurosecretion and JH are involved in male accessory gland secretory activity in another blood-sucking bug, *Panstrongylus megistus* (Regis *et al.*, 1987). In *M. sanguinipes*, cardioallatectomy inhibits the accumulation of protein LHPI to a greater degree than that provoked by allatectomy alone (Cheeseman and Gillott, 1988), which suggests again the

participation of a neurosecretory factor in the regulation of protein secretion by the male accessory glands.

Finally, 20E appears to stimulate total protein synthesis in the male accessory glands of the male lepidopterans, *S. littoralis* and *Chilo partellus* (Gillott, 1996). More detailed studies of *M. sanguinipes* have shown that 20E promotes protein accumulation in the accessory glands incubated *in vitro* (Ismail and Gillott, 1995) and that these glands incubated *in vitro* are able to produce ecdysteroids (Gillott and Ismail, 1995).

3.9.4. Future Directions

Our understanding of how JH, ecdysteroids, and peptide hormones control various aspects of insect reproduction has advanced but also broadened greatly over the last two decades. Of particular importance is the elucidation of genetic expression heirarchies regulating ecdysteroid action at the cellular level and hormonal networks controlling reproduction at the organismal level. Despite the fact that the vitellogenic action of JH is one of the hallmarks of reproduction in most insects, its precise mechanism of action remains poorly understood. Elucidating this mechanism represents the most challenging task for future research. Genetic ecdysteroid networks governing insect development and metamorphosis have been studied in detail. Our understanding of similar networks occurring in reproduction is still limited. Future studies should take advantage of techniques for gene knockout and functional genomics to gain further insight into the regulation of female and male reproduction. Likewise, these techniques offer many advantages for research on peptide hormones involved in insect reproduction.

Acknowledgments

We are grateful to Professor Mary Bownes for her kind permission to use unpublished results. We thank Dr Guoqiang Sun for his help with the manuscript, Mr. Ray E. Hardesty for editing the manuscript, Mr. Geoffrey Attardo for his help in producing figures, and Ms. Sue Gerdes for secretarial assistance.

References

Abdel-Latief, M., Meyering-Vos, M., Hoffmann, K.H., **2003**. Molecular characterisation of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides. *Insect. Biochem. Mol. Biol. 33*, 467–476.

Abel, T., Bhatt, R., Maniatis, T., **1992**. A *Drosophila* CREB/ATF transcriptional activator binds to both fat body- and liver-specific regulatory elements. *Genes Devel. 6*, 466–480.

Abrahamsen, N., Martinez, A., Kjaer, T., Sondergaard, L., Bownes, M., **1993**. *Cis*-regulatory sequences leading to female-specific expression of yolk protein genes 1 and 2 in the fat body of *Drosophila melanogaster. Mol. Gen. Genet. 237*, 41–48.

Abu-Hakima, R., Davey, K.G., **1975**. Two actions of juvenile hormone on the follicle cells of *Rhodnius prolixus* Stal. *Can. J. Zool. 53*, 1187–1188.

Adams, T.S., **1974**. The role of juvenile hormone in housefly ovarian follicle morphogenesis. *J. Insect Physiol. 20*, 263–276.

Adams, T.S., **1980**. The role of ovarian hormones in maintaining cyclical egg production in insects. In: Clark, W.H., Jr., Adams, T.S. (Eds.), Advances in Invertebrate Reproduction, vol. 10. Elsevier, New York, pp. 109–125.

Adams, T.S., **1981**. Activation of successive ovarian gonotrophic cycles by the corpus allatum in the housefly, *Musca domestica* (Diptera, Muscidae). *Int. J. Invertebr. Reprod. 3*, 41–48.

Adams, T.S., Filipi, P.A., **1983**. Vitellin and vitellogenin concentrations during oogenesis in the first gonotrophic cycle of the house fly, *Musca domestica*. *J. Insect Physiol. 29*, 723–733.

Adams, T.S., Filipi, P.A., Kelly, T.J., **1989**. Effect of 20-hydroxy-ecdysone and a juvenile hormone analogue on Vg production in male house flies, *Musca domestica*. *J. Insect Physiol. 35*, 765–773.

Adams, T.S., Hagedorn, H.H., Wheelock, G.D., **1985**. Haemolymph ecdysteroid in the house fly, *Musca domestica*, during oogenesis and its relationship with vitellogenin levels. *J. Insect Physiol. 31*, 91–97.

Adams, T.S., Li, Q.J., **1998**. Ecdysteroidostatin from the house fly, *Musca domestica*. *Arch. Insect Biochem. Physiol. 38*, 166–176.

Agui, N., Shimada, T., Izumi, S., Tomino, S., **1991**. Hormonal-control of vitellogenin messenger-RNA levels in the male and female housefly, *Musca domestica*. *J. Insect Physiol. 37*, 383–390.

Antoniewski, C., Laval, M., Dahan, A., Lepesant, J.A., **1994**. The ecdysone response enhancer of the fbp1 gene of *Drosophila melanogaster* is a direct target for the EcR/USP nuclear receptor. *Mol. Cell. Biol. 14*, 4465–4474.

Antoniewski, C., Mugat, B., Delbac, F., Lepesant, J.A., **1996**. Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster. Mol. Cell. Biol. 16*, 2977–2986.

Antoniewski, C., Ogrady, M.S., Edmondson, R.G., Lassieur, S.M., Benes, H., **1995**. Characterization of an EcR/USP heterodimer target site that mediates ecdysone responsiveness of the *Drosophila* Lsp-2 gene. *Mol. Gen. Genet. 249*, 545–556.

Antwerpen van R., Pham, D.O.-D., Ziegler, R., **2004**. Accumulation of lipids in insect oocytes. In: Raikhel, A.S., Sappington, T. (Eds.), Reproductive Biology of

Invertebrates, vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 265–287.

Ashburner, M., Chihara, C., Meltzer, P., Richards, G., **1974**. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harb. Symp. Quant. Biol. 38*, 655–662.

Ashok, M., Turner, C., Wilson, T.G., **1998**. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl Acad. Sci. USA 95*, 2761–2766.

Auerswald, L., Birgul, N., Gade, G., Kreienkamp, H.J., Richter, D., **2001**. Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. *Biochem. Biophys. Res. Commun. 282*, 904–909.

Baeuerle, P., Huttner, W., **1985**. Tyrosine sulfation of yolk proteins 1, 2 and 3 in *Drosophila melanogaster. J. Biol. Chem. 260*, 6434–6439.

Baker, B.S., Wolfner, M.F., **1988**. A molecular analysis of doublesex, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster. Genes Devel. 2*, 477–489.

Barchuk, R.A., Bitondi, M.M.G., Simões, Z.L.P., **2002**. Effects of juvenile hormone and ecdysone on the timing of vitellogenin appearance in hemolymph of queen and worker pupae of *Apis mellifera. J. Insect Sci.* 2.1. (Available online at. http://www.insectscience.org/2.1.)

Barnett, T., Pachl, C., Gergen, J., Wensink, P., **1980**. The isolation and characterization of *Drosophila* yolk protein genes. *Cell 21*, 729–738.

Barr, P.J., **1991**. Mammalian subtilisins: the long-sought dibasic processing endoproteases. *Cell 66*, 1–3.

Bast, R., Telfer, W., **1976**. Follicle cell protein synthesis and its contribution to the yolk of the cecropia moth. *Devel. Biol. 52*, 83–97.

Bean, D., Shirk, P.D., Brookes, V., **1988**. Characterization of yolk proteins from the eggs of the Indianmeal moth *Plodia interpunctella. Insect Biochem. 18*, 199–210.

Beenakkers, A.M., Van der Horst, D.J., Van Marrewijk, W.J., **1985**. Insect lipids and lipoproteins, and their role in physiological processes. *Prog. Lipid Res. 24*, 19–67.

Behrens, W., Hoffmann, K. H., **1983**. Effect of exogenous ecdysteroids on reproduction in crickets, *Gryllus bimaculatus. Int. J. Invert. Reprod. 6*, 149–159.

Belles, X., **2004**. Vitellogenesis directed by juvenile hormone. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 157–198.

Belles, X., Casas, J., Messenguer, A., Piulachs, M.D., **1987**. In vitro biosynthesis of JH III by the corpora allata of *Blattella germanica* (L.) adult females. *Insect Biochem. 17*, 1007–1010.

Belles, X., Graham, L.A., Bendena, W.G., Ding, Q.I., Edwards, J.P., *et al.*, **1999**. The molecular evolution of the allatostatin precursor in cockroaches. *Peptides 20*, 11–22.

Belles, X., Piulachs, M.D., **1992**. Juvenile hormone and maturation of male accessory reproductive glands in the cockroach *Blattella germanica* (L.). In: Mauchamp, B., Couillaud, F., Baehr, J.C. (Eds.), Recent Progress in Insect Juvenile Hormone Research: Fundamental and Applied Approach. INRA, Paris, pp. 101–109.

Belote, J.M., Handler, A.M., Wolfner, M.F., Livak, K.J., Baker, B.S., **1985**. Sex-specific regulation of yolk protein gene expression in *Drosophila. Cell 40*, 339–348.

Bendena, W.G., Donly, B.C., Tobe, S.S., **1999**. Allatostatins: a growing family of neuropeptides with structural and functional diversity. *Ann. N.Y. Acad. Sci. 897*, 311–329.

Bender, M., **2003**. Ecdysone actin in insect development. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, Vol. 1. Academic Press, San Diego, pp. 438–446.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., Hogness, D.S., **1997**. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell 91*, 777–788.

Bitsch, C., Bitsch, J., **1984**. Antigonadotropic and antiallatal effects of precocene II in the firebrat, *Thermobia domestica* (Thysanura: Lepismatidae). *J. Insect Physiol. 30*, 463–470.

Bitsch, C., Delbecque, J.P., Mathelin, J., Bitsch, J., **1986**. Effects of precocenes on intermoult length and ecdysteroid titres in relation to ovarian maturation in *Thermobia domestica* (Thysanura: Lepismatidae). *J. Insect Physiol. 32*, 535–541.

Block, G., Borts, D.W., Huang, Z.Y., Robinson, G.E., Cnaani, J., *et al.*, **2000**. Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris. J. Insect Physiol. 46*, 47–57.

Blumenthal, T., Zucker-Aprison, E., **1987**. Evolution and regulation of vitellogenin genes. In: O'Connor, J.D. (Ed.), Molecular Biology of Invertebrate Development. Alan R. Liss, New York, pp. 3–19.

Bonneton, F., Zelus, D., Iwema, T., Robinson-Rechavi, M., Laudet, V., **2003**. Rapid divergence of the ecdysone receptor in Diptera and Lepidoptera suggests coevolution between ECR and USP-RXR. *Mol. Biol. Evol. 20*, 541–553.

Borovsky, D., **2003**. Trypsin-modulating oostatic factor: a potential new larvicide for mosquito control. *J. Exp. Biol. 206* (21), 3869–3875.

Borovsky, D., Carlson, D.A., Hancock, R.G., Rembold, H., Van Handel, E., **1994**. De novo biosynthesis of juvenile hormone III and I by the accessory glands of the male mosquito. *Insect Biochem. Mol. Biol. 24*, 437–444.

Borovsky, D., Janssen, I., Vanden Broeck, J., Huybrechts, R., Verhaert, P., *et al.*, **1996**. Molecular sequencing and modeling of *Neobellieria bullata* trypsin. Evidence for translational control by *Neobellieria* trypsin-modulating oostatic factor. *Eur. J. Biochem. 237*, 279–287.

Borovsky, D., Powell, C.A., Nayor, J.K., Blalock, J.E., Hayes, T.K., **1994**. Characterization and localization

of mosquito gut receptors for trypsin-modulating oostatic factor using a complementary peptide and immunocytochemistry. *FASEB J. 8*, 350–355.

Borovsky, D., Whisenton, L.R., Thomas, B.R., Fuchs, M.S., **1986**. Biosynthesis and distribution of ecdysone and 20-OH-ecdysone in *Aedes aegypti*. *Arch. Insect Biochem. Physiol. 3*, 19–30.

Borst, D.W., Eskew, M.R., Wagner, S.J., Shores, K., Hunter, J., *et al.*, **2000**. Quantification of juvenile hormone III, vitellogenin, and vitellogenin-mRNA during the oviposition cycle of the lubber grasshopper. *Insect Biochem. Mol. Biol. 30*, 813–819.

Bownes, M., **1980**. The use of yolk protein variations in *Drosophila* species to analyze the control of vitellogenesis. *Differentiation 16*, 109–116.

Bownes, M., **1982**. Hormonal and genetic regulation of vitellogenesis in *Drosophila*. *Q. Rev. Biol. 57*, 247–274.

Bownes, M., **1986**. Expression of the genes coding for vitellogenin (yolk protein). *Annu. Rev. Entomol. 31*, 507–553.

Bownes, M., **1994**. The regulation of the yolk protein genes, a family of sex differentiation genes in *Drosophila melanogaster. Bioessays 10*, 745–752.

Bownes, M., **2004**. The regulation of yolk protein gene expression and vitellogenesis in higher Diptera. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 95–128.

Bownes, M., Blair, M., **1986**. The effects of a sugar diet and hormones on the expression of the *Drosophila* yolk protein genes. *J. Insect Physiol. 32*, 493–501.

Bownes, M., Blair, M., Kozma, R., Dempster, M., **1983**. 20-Hydroxyecdysone stimulates tissue-specific yolk-proteingene transcription in both male and female *Drosophila*. *J. Embryol. Exp. Morphol. 78*, 249–268.

Bownes, M., Dubendorfer, A., Smith, T., **1984**. Ecdysteroids in adult male and female of *Drosophila melanogaster. J. Insect Physiol. 30*, 823–830.

Bownes, M., Hames, B.D., **1978**. Genetic analysis of vitellogenesis in *Drosophila melanogaster*: the identification of a temperature-sensitive mutation affecting one of the yolk proteins. *J. Embryol. Exp. Morphol. 47*, 111–120.

Bownes, M., Nothiger, R., **1981**. Sex determining genes and vitellogenin synthesis in *Drosophila melanogaster. Mol. Gen. Genet. 182*, 222–228.

Bownes, M., Pathirana, S., **2002**. The yolk proteins in higher Diptera. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part A: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth UK, pp. 103–130.

Bownes, M., Ronaldson, E., Mauchline, D., **1996**. 20-Hydroxyecdysone, but not juvenile hormone, regulation of yolk protein gene expression can be mapped to *cis*-acting DNA sequences. *Devel. Biol. 173*, 475–489.

Bownes, M., Ronaldson, E., Mauchline, D., Martinez, A., **1993**. Regulation of vitellogenesis in *Drosophila*. *Int. J. Insect Morphol. Embryol. 22*, 349–367.

Bownes, M., Scott, A., Blair, M., **1987**. Hormonal and genetic factors regulating the expression of the genes encoding the *Drosophila* yolk proteins. *Development 101*, 931–941.

Bownes, M., Shirras, A., Blair, M., Collins, J., Coulson, A., **1988**. Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *Proc. Natl Acad. Sci. USA 85*, 1554–1557.

Bradfield, J.Y., Berlin, R.L., Keeley, L.L., **1990**. Contrasting modulations of gene expression by a juvenile hormone analogue. *Insect Biochem. 20*, 105–111.

Bradley, J.T., Masetti, M., Cecchettini, A., Giorgi, F., **1995**. Vitellogenesis in the allatectomized stick insect *Carausius morosus* (Br.) (Phasmatodea: Lonchodinae). *Comp. Biochem. Physiol. 110B*, 255–266.

Braun, R.P., Wyatt, G.R., **1995**. Growth of the male accessory gland in adult locusts: roles of juvenile hormone, esterase, and binding proteins. *Arch. Insect Biochem. Physiol. 30*, 383–400.

Brennan, M., Warren, T., Mahowald, A., **1980**. Signal polypeptides and signal peptidase in *Drosophila melanogaster. J. Cell Biol. 87*, 516–520.

Brennan, M.D., Weiner, A.J., Goralski, T.J., Mahowald, A.P., **1982**. The follicle cells are a major site of vitellogenin synthesis in *Drosophila melanogaster. Devel. Biol. 89*, 225–236.

Briegel, H., **1990**. Fecundity, metabolism, and body size in *Anopheles* (Diptera; Culicidae), vectors of malaria. *J. Med. Entomol. 27*, 839–850.

Brown, M.R., Cao, C., **2001**. Distribution of ovary ecdysteroidogenic hormone I in the nervous system and gut of mosquitoes. *J. Insect Sci.* 1.3. (Available online at http://www.insectscience.org/1.3).

Brown, M.R., Graf, R., Swiderek, K.M., Fendley, D., Stracker, T.H., *et al.*, **1998**. Identification of a steroidogenic neurohormone in female mosquitoes. *J. Biol. Chem. 273*, 3967–3971.

Brown, M.R., Klowden, M.J., Crim, J.W., Young, L., Shrouder, L., *et al.*, **1994**. Endogenous regulation of mosquito host-seeking behavior by a neuropeptide. *J. Insect Physiol. 40*, 399–406.

Büning, J., **1994**. The Insect Ovary. Chapman and Hall, London.

Burtis, K.C., Baker, B.S., **1989**. *Drosophila* doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell 56*, 997–1010.

Burtis, K.C., Coschigano, K.T., Baker, B.S., Wensink, P.C., **1991**. The doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. *EMBO J. 10*, 2577–2582.

Buschor, J., Lanzrein, B., **1983**. Isolation and quantification of vitellogenin in the hemolymph of the ovoviviparous cockroach *Nauphoeta cinerea*. *Comp. Biochem. Physiol. 76B*, 65–72.

Buszczak, M., Freeman, M.R., Carlson, J.R., Bender, M., Cooley, L., *et al.*, **1999**. Ecdysone response genes govern egg chamber development during mid-oogenesis in *Drosophila*. *Development 126*, 4581–4589.

Bylemans, D., Borovsky, D., Hunt, D.F., Shabanowitz, J., Grauwels, L., *et al.*, **1994**. Sequencing and characterization of trypsin modulating oostatic factor (TMOF) from the ovaries of the grey fleshfly, *Neobellieria (Sarcophaga) bullata*. *Regul. Pept. 50*, 61–72.

Bylemans, D., Proost, P., Samijn, B., Borovsky, D., Grauwels, L., *et al.*, **1995**. Neb-colloostatin, a second folliculostatin of the grey fleshfly, *Neobellieria bullata*. *Eur. J. Biochem. 228*, 45–49.

Bylemans, D., Verhaert, P., Janssen, I., Vanden Broeck, J., Borovsky, D., *et al.*, **1996**. Immunolocalization of the oostatic and prothoracicostatic peptide, Neb-TMOF, in adults of the fleshfly, *Neobellieria bullata*. *Gen. Comp. Endocrinol. 103*, 273–280.

Cao, C., Brown, M.R., **2001**. Localization of an insulin-like peptide in brains of two flies. *Cell Tissue Res. 304*, 317–321.

Carney, G.E., Bender, M., **2000**. The *Drosophila* ecdysone receptor (EcR) gene is required maternally for normal oogenesis. *Genetics 154*, 1203–1211.

Cayre, M., Strambi, C., Tirard, A., Renucci, M., Charpin, P., *et al.*, **1995**. Effects of juvenile hormone on polyamines of the fat body and neural tissue of the cricket *Acheta domesticus*. *Comp. Biochem. Physiol. 111A*, 241–250.

Cerstiaens, A., Benfekih, L., Zouiten, H., Verhaert, P., De Loof, A., *et al.*, **1999**. Led-NPF-1 stimulates ovarian development in locusts. *Peptides 20*, 39–44.

Chapman, R.F., **1998**. The Insects: Structure and Function. Cambridge University Press, Cambridge, 298–312.

Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., *et al.*, **2000**. The *Drosophila* disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126.

Cheeseman, M.T., Gillott, C., **1988**. Corpus allatum and corpus cardiacum regulation of long hyaline gland protein synthesis in the male grasshopper, *Melanoplus sanguinipes*. *Gen. Comp. Endocrinol. 72*, 416–423.

Chen, A., Kim, H., Mayer, R., Norman, J., **1987**. Vitellogenesis in the stable fly, *Stomoxys calcitrans*. *Comp. Biochem. Physiol 88B*, 897–903.

Chen, C., Jack, J., Garofalo, R.S., **1996**. The *Drosophila* insulin receptor is required for normal growth. *Endocrinology 137*, 846–856.

Chen, J.S., Cho, W.L., Raikhel, A.S., **1994**. Analysis of mosquito vitellogenin cDNA, similarity to vertebrate phosvitins and arthropod serum proteins. *J. Mol. Biol. 237*, 641–647.

Chen, J.-S., Raikhel, A.S., **1996**. Subunit cleavage of insect pro-vitellogenin by a subtilisin-like convertase; characterization of the mosquito convertase. *Proc. Natl Acad. USA 93*, 6186–6190.

Chen, P.S., **1984**. The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annu. Rev. Entomol. 29*, 233–255.

Chen, P.S., **1996**. The accessory gland proteins in male *Drosophila*: structural, reproductive, and evolutionary aspects. *Experientia 52*, 503–510.

Cheon, H.M., Seo, S.J., Sun, J., Sappington, T.W., Raikhel, A.S., **2001**. Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 31*, 753–760.

Cherbas, L., Lee, K., Cherbas, P., **1991**. Identification of ecdysone response elements by analysis of the *Drosophila* Eip28/29 gene. *Genes Devel. 5*, 120–131.

Chino, H., Downer, R., Takahashi, K., **1977b**. The role of diacylglycerol-carrying lipoprotein I in lipid transport during insect vitellogenesis. *Biochim. Biophys. Acta 487*, 508–516.

Chino, H., Murakami, S., Harashima, K., **1969**. Diglyceride-carrying lipoproteins in insect hemolymph. *Isolation, purification and properties. Biochim. Biophys. Acta 176*, 1–26.

Chino, H., Yamagata, M., Sato, S., **1977a**. Further characterization of lepidopteran vitellogenin from hemolymph and mature eggs. *Insect Biochem. 7*, 125–131.

Chinzei, Y., Haruna, T., Miura, K., Numata, H., Nakayama, S., **1990**. Purification and characterization of biliverdin-associated cyanoprotein from eggs and hemolymph of the bean bug, *Riptortus clavatus* (Heteroptera: Alydidae). *Insect Biochem. 20*, 545–555.

Chinzei, Y., Wyatt, G.R., **1985**. Vitellogenin titre in haemolymph of *Locusta migratoria* in normal adults, after ovariectomy, and response to methoprene. *J. Insect Physiol. 31*, 441–445.

Cho, K.S., Won, D.H., Cha, G.H., Lee, C.C., **2000**. Regulation of Mst57Dc expression in male accessory glands of *Drosophila melanogaster. Mol. Cell. 10*, 180–185.

Cho, W.L., Deitsch, K., Raikhel, A.S., **1991**. An extra-ovarian protein accumulated in mosquito oocytes is a carboxypeptidase activated in embryos. *Proc. Natl Acad. Sci. USA 88*, 10821–10824.

Cho, W.L., Kapitskaya, M.Z., Raikhel, A.S., **1995**. Mosquito ecdysteroid receptor – analysis of the cDNA and expression during vitellogenesis. *Insect Biochem. Mol. Biol. 25*, 19–27.

Cho, W.L., Raikhel, A.S., **1992**. Cloning of cDNA for mosquito lysosomal aspartic protease. Sequence analysis of an insect lysosomal enzyme similar to cathepsins D and E. *J. Biol. Chem. 267*, 21823–21829.

Cho, W.L., Tsao, S., Hays, A., Walter, R., Chen, J.S., *et al.*, **1999**. Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *J. Biol. Chem. 274*, 13311–13321.

Chudakova, I., Maslennikova, V., Luchnikova, E., **1982**. Effect of 20-hydroxyecdysone on *Acheta domesticus* L. (Orthoptera) and *Drosophila melanogaster* Meig. (Diptera) reproduction. *Zool. Jb. Physiol. 86*, 45–52.

Claeys, I., Simonet, G., Poels, J., Van Loy, T., Vercammen, L., *et al.*, **2002**. Insulin-related peptides and their

conserved signal transduction pathway. *Peptides 23*, 807–816.

Claeys, I., Simonet, G., Van Loy, T., De Loof, A., Vanden Broeck, J., **2003**. cDNA cloning and transcript distribution of two novel members of the neuroparsin family in the desert locust, *Schistocerca gregaria*. *Insect Mol. Biol. 12*, 473–481.

Clark, J., Lange, A.B., **2003**. Octopamine modulates spermathecal muscle contractions in *Locusta migratoria*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 189*, 105–114.

Cole, K., Fernando-Warnakulasuriya, G., Boduski, M., Freeman, M., Gordan, J., *et al.*, **1987**. Primary structure and comparative sequence analysis of an insect apolipoprotein. *J. Biol. Chem. 262*, 11794–11800.

Comas, D., Piulachs, M.D., Belles, X., **1999**. Fast induction of vitellogenin gene expression by juvenile hormone III in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Insect Biochem. Mol. Biol. 29*, 821–827.

Comas, D., Piulachs, M.D., Belles, X., **2000**. The vitellogenin of *Blattella germanica* (L.) (Dictyoptera, Blattellidae): nucleotide sequence of the cDNA and analysis of the protein primary structure. *Arch. Insect Biochem. Physiol. 44*, 1–11.

Comas, D., Piulachs, M.D., Belles, X., **2001**. Induction of vitellogenin gene transcription in vitro by juvenile hormone in *Blattella germanica*. *Mol. Cell. Endocrinol. 183*, 93–100.

Coschigano, K.T., Wensink, P.C., **1993**. Sex-specific transcriptional regulation by the male and female doublesex proteins of *Drosophila*. *Genes Devel. 7*, 42–54.

Cusson, M., Tobe, S.S., McNeil, J.N., **1994a**. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm moth *Pseudaletia unipuncta*. *Arch. Insect Biochem. Physiol. 25*, 329–345.

Cusson, M., Yu, C.G., Carruthers, K., Wyatt, G.R., Tobe, S.S., *et al.*, **1994b**. Regulation of vitellogenin production in armyworm moths, *Pseudaletia unipuncta*. *J. Insect Physiol. 40*, 129–136.

Dantuma, N.P., Pijnenburg, M.A., Diederen, J.H., Van der Horst, D.J., **1997**. Developmental down-regulation of receptor-mediated endocytosis of an insect lipoprotein. *J. Lipid Res. 38*, 254–265.

Davey, K.G., **1985**. The female reproductive tract. In: Kerhut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 1. Pergamon, Oxford, pp. 1–14.

Davey, K.G., **1997**. Hormonal controls on reproduction in female Heteroptera. *Arch. Insect Biochem. Physiol. 35*, 443–453.

Davey, K.G., Huebner, E., **1974**. The response of the follicle cells of *Rhodinus proxilus* to juvenile hormone and antigonadotropin *in vitro*. *Canad. J. Zool. 52*, 1407–1412.

Davey, K.G., Sevala, V.L., Gordon, D.R.B., **1993**. The action of juvenile hormone and antigonadotropin on the follicle cells of *Locusta migratoria*. *Invertebr. Reprod. Devel. 23*, 189–193.

D'avino, P.P., Crispi, S., Cherbas, L., Cherbas, P., Furia, M., **1995**. The moulting hormone ecdysone is able to recognize target elements composed of direct repeats. *Mol. Cell. Endocrinol. 113*, 1–9.

de Kort, C.A.D., Koopmanschap, A.B., Vermunt, A.M.W., **1997**. Influence of pyriproxyfen on the expression of hemolymph protein genes in the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol. 43*, 363–371.

De Loof, A., Baggerman, G., Breuer, M., Claeys, I., Cerstiaens, A., *et al.*, **2001**. Gonadotropins in insects: an overview. *Arch. Insect Biochem. Physiol. 47*, 129–138.

Dean, R.L., Locke, M., Collins, J.V., **1985**. Structure of the fat body. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 3: Integument, Respiration and Circulation. Plenum, London, pp. 155–210.

Dean, S.R., Meola, R.W., **1997**. Effect of juvenile hormone and juvenile hormone mimics on sperm transfer from the testes of the male cat flea (Siphonaptera: Pulicidae). *J. Med. Entomol. 34*, 485–488.

Deitsch, K.W., Dittmer, N., Kapitskaya, M.Z., Chen, J.S., Cho, W.L., *et al.*, **1995**. Regulation of gene-expression by 20-hydroxyecdysone in the fat body of *Aedes aegypti* (Diptera, Culicidae). *Eur. J. Entomol. 92*, 237–244.

Delisle, J., Cusson, M., **1999**. Juvenile hormone biosynthesis, oocyte growth and vitellogenin accumulation in *Choristoneura fumiferana* and *C. rosaceana*: a comparative study. *J. Insect Physiol. 45*, 515–523.

Della-Cioppa, G., Engelmann, F., **1987**. The vitellogenin of *Leucophaea maderae*: synthesis as a large phosphorylated precursor. *Insect Biochem. 17*, 401–415.

Deng, W., Leaper, K., Bownes, M., **1999**. A targeted gene silencing technique shows that *Drosophila* myosin VI is required for egg chamber and imaginal disc morphogenesis. *J. Cell Sci. 112*, 3677–3690.

Deng, W.M., Bownes, M., **1997**. Two signaling pathways specify localised expression of the Broad-Complex in *Drosophila* eggshell patterning and morphogenesis. *Development 124*, 4639–4647.

Dhadialla, T., Raikhel, A.S., **1990**. Biosynthesis of mosquito vitellogenin. *J. Biol. Chem. 265*, 9924–9933.

Dhadialla, T., Raikhel, A.S., **1994**. Endocrinology of mosquito vitellogenesis. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 275–281.

Dhadialla, T.S., Cook, K.E., Wyatt, G.R., **1987**. Vitellogenin mRNA in Locust fat body: coordinate induction of two genes by a juvenile hormone analog. *Devel. Biol. 123*, 108–114.

Dittmann, F., Kogan, P.H., Hagedorn, H.H., **1989**. Ploidy levels and DNA synthesis in fat body cells of the adult mosquito, *Aedes aegypti*. The role of juvenile hormone. *Arch. Insect Biochem. Physiol. 12*, 133–143.

Dittmer, N.T., Sun, G., Wang, S.F., Raikhel, A.S., **2003**. CREB isoform represses yolk protein gene expression in the mosquito fat body. *Mol. Cell. Endocrinol. 210*, 39–49.

Donini, A., Lange, A.B., **2002**. The effects of crustacean cardioactive peptide on locust oviducts are calcium-dependent. *Peptides 23*, 683–691.

Don-Wheeler, G., Engelmann, F., **1997**. The biosynthesis and processing of vitellogenin in the fat bodies of females and males of the cockroach *Leucophaea maderae*. *Insect Biochem. Mol. Biol. 27*, 901–918.

Drummond-Barbosa, D., Spradling, A.C., **2001**. Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. *Devel. Biol. 231*, 265–278.

Dubrovsky, E.B., **2002**. Juvenile hormone regulated gene expression in *Drosophila melanogaster. Recent Res. Devel. Mol. Cell. Biol. 3*, 527–547.

Dubrovsky, E.B., Dubrovskaya, V.A., Berger, E.M., **2004**. Hormonal regulation and functional role of *Drosophila* E75A orphan nuclear receptor in the juvenile hormone signaling pathway. *Devel. Biol. 263*, 258–270.

Dumser, J.B., **1980**. The regulation of spermatogenesis in insects. *Annu. Rev. Ent. 25*, 341–369.

Duve, H., Johnsen, A.H., Scott, A.G., Yu, C.G., Yagi, K.J., *et al.*, **1993**. Callatostatins: neuropeptides from the blowfly *Calliphora vomitoria* with sequence homology to cockroach allatostatins. *Proc. Natl Acad. Sci. USA 90*, 2456–2460.

Edwards, G.C., Braun, R.P., Wyatt, G.R., **1993**. Induction of vitellogenin synthesis in *Locusta migratoria* by the juvenile hormone analog, Pyriproxyfen. *J. Insect Physiol. 39*, 609–614.

Elekonich, M.M., Horodyski, F.M., **2003**. Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. *Peptides 24*, 1623–1632.

Elliot, R.H., Gillot, C., **1976**. Histological changes in the ovary in relation to yolk deposition, allatectomy, and destruction of the median neurosecretory cells in *Melanoplus sanguinipes*. *Can. J. Zool. 54*, 185–192.

Engelmann, F., **1983**. Vitellogenesis controlled by juvenile hormone. In: Downer, R.G.H., Laufer, H. (Eds.), Endocrinology of Insects. Alan R. Liss, New York, pp. 259–270.

Engelmann, F., **2003**. Juvenile hormone action in insect reproduction. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, Vol. 2. Academic Press, San Diego, pp. 536–539.

Engelmann, F., Wilkens, J.L., **1969**. Synthesis of digestive enzyme in the fleshfly *Sarcophaga bullata* stimulated by food. *Nature 222*, 798.

Falb, D., Maniatis, T., **1992a**. A conserved regulatory unit implicated in tissue-specific gene expression in *Drosophila* and man. *Genes Devel. 6*, 454–465.

Falb, D., Maniatis, T., **1992b**. *Drosophila* transcriptional repressor protein that binds specifically to negative control elements in fat body enhancers. *Mol. Cell. Biol. 12*, 4093–4103.

Fan, Y., Chase, J., Sevala, V.L., Schal, C., **2002**. Lipophorin-facilitated hydrocarbon uptake by oocytes in the German cockroach *Blattella germanica* (L.). *J. Exp. Biol. 205*, 781–790.

Fan, Y.L., Rafaeli, A., Gileadi, C., Kubli, E., Applebaum, S.W., **1999**. *Drosophila melanogaster* sex peptide stimulates juvenile hormone synthesis and depresses sex pheromone production in *Helicoverpa armigera*. *J. Insect Physiol. 45*, 127–133.

Feng, G., Reale, V., Chatwin, H., Kennedy, K., Venard, R., *et al.*, **2003**. Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster. Eur. J. Neurosci. 18*, 227–238.

Fescemyer, H.W., Masler, E.P., Davis, R.E., Kelly, T.J., **1992**. Vitellogenin synthesis in female larvae of the gypsy moth, *Lymantria dispar* (L.): suppression by juvenile hormone. *Comp. Biochem. Physiol. 103B*, 533–542.

Flanagan, T.R., Hagedorn, H.H., **1977**. Vitellogenin synthesis in mosquito – role of juvenile hormone in development of responsiveness to ecdysone. *Physiological Entomology 2*, 173–178.

Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., *et al.*, **1995**. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell 81*, 687–693.

Fourney, R., Pratt, G., Harnish, D., Wyatt, G., White, B., **1982**. Structure and synthesis of vitellogenin from *Calliphora erythrocephala*. *Insect Biochem. 12*, 311–321.

Freeman, M.R., Dobritsa, A., Gaines, P., Segraves, W.A., Carlson, J.R., **1999**. The dare gene: steroid hormone production, olfactory behavior, and neural degeneration in *Drosophila*. *Development 126*, 4591–4602.

Fullbright, G., Lacy, E.R., Bullesbach, E.E., **1997**. The prothoracicotropic hormone bombyxin has specific receptors on insect ovarian cells. *Eur. J. Biochem. 245*, 774–780.

Gade, G., Hoffmann, K.H., Spring, J.H., **1997**. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev. 77*, 963–1032.

Garabedian, M.J., Hung, M.C., Wensink, P.C., **1985**. Independent control elements that determine yolk protein gene expression in alternative *Drosophila* tissues. *Proc. Natl Acad. Sci. USA 82*, 1396–1400.

Garabedian, M.J., Shepherd, B.M., Wensink, P.C., **1986**. A tissue-specific transcription enhancer from the *Drosophila yolk protein 1* gene. *Cell 45*, 859–867.

Garabedian, M., Shirras, A., Bownes, M., Wensink, P., **1987**. The nucleotide sequence of the gene coding for *Drosophila melanogaster* yolk protein 3. *Gene 55*, 1–8.

Garczynski, S.F., Brown, M.R., Shen, P., Murray, T.F., Crim, J.W., **2002**. Characterization of a functional neuropeptide F receptor from *Drosophila melanogaster. Peptides 23*, 773–780.

Garofalo, R.S., **2002**. Genetic analysis of insulin signalling in *Drosophila*. *Trends Endocrinol. Metabol. 13*, 156–162.

Geysen, J., Cardeon, J., De Loof, A., **1986**. Distribution of yolk polypeptides in the follicle cells during differentiation of the follicular epithelium in *Sarcophaga bullata* egg follicles. *Development 101*, 33–44.

Gillott, C., **1996**. Male insect accessory glands: functions and control of secretory activity. *Invertebr. Reprod. Devel. 30*, 199–205.

Gillott, C., **2003**. Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annu. Rev. Entomol. 48*, 163–184.

Gillott, C., Gaines, S.B., **1992**. Endocrine regulation of male accessory gland development and activity. *Can. Entomol. 124*, 871–886.

Gillott, C., Ismail, P.M., **1995**. In vitro synthesis of ecdysteroid by the male accessory reproductive glands, testis and abdominal integument of the adult migratory grasshopper, *Melanoplus sanguinipes*. *Invertebr. Reprod. Devel. 27*, 65–71.

Giorgi, F., Cecchettini, A., Falleni, A., Masetti, M., Gremigni, V., **1998**. Vitellogenin is glycosylated in the fat body of the stick insect *Carausius morosus* and not further modified upon transfer to the ovarian follicle. *Micron 29*, 451–460.

Giorgi, F., Cecchettini, A., Locci, M., Masetti, M., Bradley, J., **1995**. A fat body-derived protein is selectively sulfated during transit to ovarian follicles in the stick insect *Carausius morosus*. *Devel. Biol. 167*, 379–387.

Giorgi, F., Lucchesi, P., Morelli, A., Bownes, M., **1993**. Ultrastructural analysis of *Drosophila* ovarian follicles differing in yolk polypeptide (yps) composition. *Development 117*, 319–328.

Giorgi, F., Snigirevskaya, E.S., Raikhel, A.S., **2004**. The cell biology of yolk protein synthesis and secretion. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 33–68.

Gilbert, L.I., Rybczynski, R., Warren, J.T., **2002**. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol. 47*, 883–916.

Girardie, J., Boureme, D., Couillaud, F., Tamarelle, M., Girardie, A., **1987**. Anti-juvenile effect of neuroparsin A, a neuroprotein isolated from locust corpora cardiaca. *Insect Biochem. 17*, 977–983.

Girardie, J., Fournier, B., **1993**. Chemistry and physiology of neuroparsin: an antidiuretic neurohormone of the African locust. In: Beyenbach, K.W. (Ed.), Structure and Function of Primary Messengers in Invertebrates: Insect Diurectic and Antidiuretic Peptides, vol. 12. Karger, Basel, pp. 67–83.

Girardie, J., Geoffre, S., Delbecque, J.-P., **1998a**. Arguments for two distinct gonadotropic activities triggered by different domains of the ovary maturating parsin of *Locusta migratoria*. *J. Insect Physiol. 44*, 1063–1071.

Girardie, J., Huet, J.C., Atay-Kadiri, Z., Ettaouil, S., Delbecque, J.P., *et al.*, **1998b**. Isolation, sequence determination, physical and physiological characterization of the neuroparsins and ovary maturing parsins of *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 28*, 641–650.

Girardie, J., Richard, O., Huet, J.C., Nespoulous, C., Van Dorsselaer, A., *et al.*, **1991**. Physical characterization and sequence identification of the ovary maturating parsin. A new neurohormone purified from the nervous corpora cardiaca of the African locust (*Locusta migratoria migratorioides*). *Eur. J. Biochem. 202*, 1121–1126.

Glinka, A.V., Kleiman, A.M., Wyatt, G.R., **1995**. Roles of juvenile hormone, a brain factor, and adipokinetic hormone in regulation of vitellogenin biosynthesis in *Locusta migratoria*. *Biochem. Mol. Biol. Int. 35*, 323–328.

Glinka, A.V., Wyatt, G.R., **1996**. Juvenile hormone activation of gene transcription in locust fat body. *Insect Biochem. Mol. Biol. 26*, 13–18.

Guan, X.-C., **1989**. Effects of exogenic juvenile hormone analogue on haemolymph vitellogenin content in *Coccinella septempunctata* L. *Acta Entomol. Sinica 32*, 6–11 (in Chinese with English summary).

Gwardz, R.W., Spielman, A., **1973**. Corpus allatum control of ovarian development in *Aedes aegypti*. *J. Insect Physiol. 19*, 1441–1448.

Hagedorn, H., **1985**. The role of ecdysteroids in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 205–261.

Hagedorn, H.H., Fallon, A.M., **1973**. Ovarian control of vitellogenin synthesis by the fat body in *Aedes aegypti*. *Nature 244*, 103–105.

Hagedorn, H.H., Oconnor, J.D., Fuchs, M.S., Sage, B., Schlaeger, D.A., *et al.*, **1975**. Ovary as a source of alpha-ecdysone in an adult mosquito. *Proc. Natl Acad. Sci. USA 72*, 3255–3259.

Handler, A., **1997**. Developmental regulation of yolk protein genes in *Anastrepha suspensa*. *Arch. Insect Biochem. Physiol. 36*, 25–35.

Handler, A., Shirk, P., **1988**. Identification and analysis of the major yolk polypeptide from the Caribbean fruit fly *Anastrepha suspensa* (loew). *Arch. Insect Biochem. Physiol. 9*, 91–106.

Happ, G.M., **1992**. Maturation of the male reproductive system and its endocrine regulation. *Annu. Rev. Entomol. 37*, 303–320.

Hardie, J., **1987**. Juvenile hormone stimulation of oocyte development and embryogenesis in the parthenogenetic ovaries of an aphid *Aphis fabae*. *Int. J. Invert. Reprod. Devel. 11*, 189–202.

Harmon, M.A., Boehm, M.F., Heyman, R.A., Mangelsdorf, D.J., **1995**. Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl Acad. Sci. USA 92*, 6157–6160.

Hartfelder, K., **2000**. Insect juvenile hormone: from "status quo" to high society. *Braz. J. Med. Biol. Res. 33*, 157–177.

Hatakeyama, M., Kageyama, Y., Kinoshita, T., Oishi, K., **1995**. Completion of development in *Athalia rosae* (Hymenoptera) eggs matured with heterospecific *Athalia infumata* yolk protein. *J. Insect Physiol. 41*, 351–355.

Hatakeyama, M., Oishi, K., **1990**. Induction of vitellogenin synthesis and maturation of transplanted

previtellogenic eggs by juvenile hormone III in males of the sawfly, *Athalia rosae*. *J. Insect Physiol. 36*, 791–797.

Hayflick, J.S., Wolfgang, W.J., Forte, M.A., Thomas, G., **1992**. A unique Kex2-like endoprotease from *Drosophila melanogaster* is expressed in the central nervous system during early embryogenesis. *J. Neurosci. 12*, 705–717.

Hays, A.R., Raikhel, A.S., **1990**. A novel female-specific protein produced by the vitellogenic fat body and accumulated in ovaries in the mosquito *Aedes aegypti*. *Roux's Arch. Devel. Biol. 199*, 114–121.

Hayward, D.C., Dhadialla, T.S., Zhou, S., Kuiper, M.J., Ball, E.E., *et al.*, **2003**. Ligand specificity and developmental expression of RXR and ecdysone receptor in the migratory locust. *J. Insect Physiol. 49*, 1135–1144.

Hirai, M., Watanabe, D., Chinzei, Y., **2000**. A juvenile hormone-repressible transferrin-like protein from the bean bug *Riptortus clavatus*: cDNA sequence analysis and protein identification during diapause and vitellogenesis. *Arch. Insect Biochem. Physiol. 44*, 17–26.

Hirai, M., Watanabe, D., Kiyota, A., Chinzei, Y., **1998**. Nucleotide sequence of vitellogenin mRNA in the bean bug, *Riptortus clavatus*: analysis of processing in the fat body and ovary. *Insect Biochem. Mol. Biol. 28*, 537–547.

Hiremath, S., Jones, D., **1992**. Juvenile hormone regulation of vitellogenin in the gypsy moth, *Lymantria dispar*: suppression of vitellogenin mRNA in the fat body. *J. Insect Physiol. 6*, 461–474.

Hiremath, S., Lehtoma, K., **1997**. Complete nucleotide sequence of the vitellogenin mRNA from the gypsy moth: novel arrangement of the subunit encoding regions. *Insect Biochem. Mol. Biol. 27*, 27–35.

Hoffmann, K.H., Sorge, D., **1996**. Vitellogenin levels in allatectomized female crickets, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae). *Arch. Insect Biochem. Physiol. 32*, 549–558.

Horner, M.A., Chen, T.H., Thummel, C.S., **1995**. Ecdysteroid regulation and DNA-binding properties of *Drosophila* nuclear hormone-receptor superfamily members. *Devel. Biol. 168*, 490–502.

Houseman, J.G., Morrison, P.E., **1986**. Absence of female-specific protein in the hemolymph of stable fly *Stomoxys calcitrans* (L.) (Diptera: Muscidae). *Arch. Insect Biochem. Physiol. 3*, 205–213.

Hsu, S.Y., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., *et al.*, **2002**. Activation of orphan receptors by the hormone relaxin. *Science 295*, 671–674.

Hua, Y.J., Tanaka, Y., Nakamura, K., Sakakibara, M., Nagata, S., *et al.*, **1999**. Identification of a prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. *J. Biol. Chem. 274*, 31169–31173.

Huebner, E., Tobe, S.S., Davey, K.G., **1975**. Structural and functional dynamics of oogenesis in *Glossina austeni*: vitellogenesis with special reference to the follicular epithelium. *Tissue Cell 7*, 535–558.

Hung, M.C., Wensink, P., **1983**. Sequence and structure conservation in yolk proteins and their genes. *J. Mol. Biol. 164*, 481–492.

Hurst, D., Rylett, C.M., Isaac, R.E., Shirras, A.D., **2003**. The *Drosophila* angiotensin-converting enzyme homologue Ance is required for spermiogenesis. *Devel. Biol. 254*, 238–247.

Hutson, S.F., Bownes, M., **2003**. The regulation of yp3 expression in the *Drosophila melanogaster* fat body. *Devel. Genes Evol. 213*, 1–8.

Huybrechts, R., De Loof, A., **1982**. Similarities in vitellogenin and control of vitellogenin synthesis within the genera *Sarcophaga*, *Phormia*, and *Lucilia* (Diptera). *Comp. Biochem. Physiol. B 72*, 339–344.

Inagaki, S., Yamashita, O., **1989**. Complete amino acid sequence of *Bombyx* egg-specific protein. *Arch. Insect Biochem. Physiol. 10*, 131–140.

Indrasith, L., Sasaki, T., Yaginuma, T., Yamashita, O., **1988**. The occurrence of a premature form of egg-specific protein in vitellogenic follicles of *Bombyx mori*. *J. Comp. Physiol. B 158*, 1–7.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E., **2002**. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Curr. Biol. 12*, 1293–1300.

Ilenchuk, T.T., Davey, K.G., **1985**. The binding of juvenile hormone to the membranes of the follicle cells in *Rhodnius prolixus*. *Can. J. Biochem. Cell. Biol. 63*, 102–106.

Ilenchuk, T.T., Davey, K.G., **1987**. Effects of various compounds on Na/K ATPase activity, JH I binding capacity and patency response in the follicles of *Rhodnius prolixus*. *Insect Biochem. 17*, 1085–1089.

Imboden, H., Konig, R., Ott, P., Lustig, A., Kampfer, U., *et al.*, **1987**. Characterization of the native vitellogenin and vitellin of the cockroach, *Nauphoeta cinerea*, and comparison with other species. *Insect Biochem. 17*, 353–365.

Iris, F.J.M., Sin, F.Y.T., **1988**. The role of JH III and ecdysterone in the regulation of the left colleterial gland activities in *Periplaneta americana* during the reproductive cycle. *Gen. Comp. Endocrinol. 71*, 105–115.

Isaac, P.G., Bownes, M., **1982**. Ovarian and fat-body vitellogenin synthesis in *Drosophila melanogaster. Eur. J. Biochem. 123*, 527–534.

Ismail, P.M., Gillott, C., **1995**. 20-Hydroxyecdysone and juvenile hormone regulation of specific protein synthesis in the male accessory reproductive gland of *Melanoplus sanguinipes*. *J. Insect Physiol. 41*, 911–920.

Iwami, M., Tanaka, A., Hano, N., Sakurai, S., **1996**. Bombyxin gene expression in tissues other than brain detected by reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization. *Experientia 52*, 882–887.

Izumi, S., Fikie, J., Yamada, S., Tomino, S., **1981**. Molecular properties and biosynthesis of major plasma proteins in *Bombyx mori*. *Biochim. Biophys. Acta 670*, 222–229.

Izumi, S., Yano, K., Yamamoto, Y., Takahashi, S.Y., **1994**. Yolk proteins from insect eggs: structure, biosynthesis

and programmed degradation during embryogenesis. *J. Insect Physiol.* *40*, 735–746.

Jacob, M., **1992**. *In vitro* spermatogenesis in *Oryctes rhinoceros* (Coleoptera: Scarabaeidae): the role of ecdysone and juvenile hormone. *Zool. Sci.* *9*, 457–461.

Janssen, T., Claeys, I., Simonet, G., De Loof, A., Girardie, J., *et al.*, **2001**. cDNA cloning and transcript distribution of two different neuroparsin precursors in the desert locust, *Schistocerca gregaria*. *Insect Mol. Biol.* *10*, 183–189.

Jensen, P.V., Hansen, B.L., Hansen, G.N., Thomsen, E., **1981**. Vitellogenin and vitellin from the blowfly *Calliphora vicina*: occurrence, purification and antigenic characterization. *Insect Biochem.* *11*, 129–135.

Jiang, C.A., Lamblin, A.F.J., Steller, H., Thummel, C.S., **2000**. A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. *Mol. Cell* *5*, 445–455.

Johnson, E.C., Bohn, L.M., Barak, L.S., Birse, R.T., Nassel, D.R., *et al.*, **2003**. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. *J. Biol. Chem.* *278* (52), 52172–52178.

Jones, G., Jones, D., **2000**. Considerations on the structural evidence of a ligand-binding function of ultraspiracle, an insect homolog of vertebrate RXR. *Insect Biochem. Mol. Biol.* *30*, 671–679.

Jones, G., Sharp, P.A., **1997**. *Ultraspiracle*: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA* *94*, 13499–13503.

Jones, G., Wozniak, M., Chu, Y., Dhar, S., Jones, D., **2001**. Juvenile hormone III-dependent conformational changes of the nuclear receptor *ultraspiracle*. *Insect Biochem. Mol. Biol.* *32*, 33–49.

Jowett, T., Postlethwait, J.H., **1980**. The regulation of yolk polypeptide synthesis in *Drosophila* ovaries and fat body by 20-hydroxyecdysone and a juvenile hormone analog. *Devel. Biol.* *80*, 225–234.

Junger, M.A., Rintelen, F., Stocker, H., Wasserman, J.D., Vegh, M., *et al.*, **2003**. The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J. Biol.* *2* (3), 20.

Kageyama, T., Takahashi, S.Y., **1990**. Purification and characterization of a cysteine protease from silkworm eggs. *Eur. J. Biochem.* *193*, 203–210.

Kang, Y., Kulakosky, P., Vanantwerpen, R., Law, J., **1995**. Sequestration of insecticyanin, a blue hemolymph protein, into eggs of the hawkmoth, *Manduca sexta*. Evidence for receptor mediated endocytosis. *Insect Biochem. Mol. Biol.* *25*, 503–520.

Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., Raikhel, AS., **1996**. The mosquito ultraspiracle homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell. Endocrinol.* *121*, 119–132.

Kapitskaya, M.Z., Dittmer, N.T., Deitsch, K.W., Cho, W.L., Taylor, D.G., *et al.*, **1998**. Three isoforms of a hepatocyte nuclear factor-4 transcription factor with tissue- and stage-specific expression in the adult mosquito. *J. Biol. Chem.* *273*, 29801–29810.

Kapitskaya, M.Z., Li, C., Miura, K., Segraves, W., Raikhel, A.S., **2000**. Expression of the early-late gene encoding the nuclear receptor HR3 suggests its involvement in regulating the vitellogenic response to ecdysone in the adult mosquito. *Mol. Cell. Endocrinol.* *160*, 25–37.

Kappler, C., Golzené, F., Lagueux, M., Hetru, C., Hoffmann, J.A., **1986**. Role of follicle cells and the oocytes in ecdysone biosynthesis and esterification in vitellogenic females of *Locusta migratoria*. *Int. J. Invert. Reprod. Devel.* *9*, 17–34.

Kappler, C., Kabbouh, M., Hetru, C., Durst, F., Hoffmann, J.A., **1988**. Characterization of three hydroxylases involved in the final steps of biosynthesis of the steroid hormone ecdysone in *Locusta migratoria* (Insecta, Orthoptera). *J. Steroid Biochem.* *31*, 891–898.

Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., *et al.*, **1989**. Identification of an allatotropin from adult *Manduca sexta*. *Science* *243*, 1481–1483.

Kawooya, J., Law, J., **1988**. Uptake of the major hemolymph lipoprotein and its transformation in the insect egg. *J. Biol. Chem.* *263*, 8740–8747.

Kawooya, J., Osir, E., Law, J., **1986**. Physical and chemical properties of microvitellogenin, a protein from the egg of the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem.* *261*, 10844–10849.

Keeley, L.L., McKercher, S.R., **1985**. Endocrine regulations of ovarian maturation in the cockroach, *Blaberus discoidalis*. *Comp. Biochem. Physiol. A* *80*, 115–121.

Keeley, L.L., Sowa, S.M., Hayes, T.K., Bradfield, J.Y., **1988**. Neuroendocrine and juvenile hormone effects on ovarian maturation and protein synthesis by the in vitro fat body from adult female *Blaberus discoidalis* cockroaches. *Gen. Comp. Endocrinol.* *72*, 364–373.

Kelly, R.B., **1985**. Pathways of protein secretion in eukaryotes. *Science* *230*, 25–31.

Kelly, T.J., Hunt, L.M., **1982**. Endocrine influence upon the development of vitellogenic competency in *Oncopeltus fasciatus*. *J. Insect Physiol.* *28*, 935–941.

Kelly, T.J., Masler, E.P., Schwartz, M.B., Haught, S.B., **1986**. Inhibitory effects of oostatic hormone on ovarian maturation and ecdysteroid production in Diptera. *Insect Biochem.* *16*, 273–279.

Kethidi, D.R., Perera, S.C., Zheng, S.C., Fena, Q.L., Krell, P.J., *et al.*, **2004**. Identification and characterization of a JH response region in the juvenile hormone esterase gene from the spruce budworm, *Choristoneura fumiferana*. *J. Biol. Chem.* *279*, 19634–19642.

Klowden, M.J., **1997**. Endocrine aspects of mosquito reproduction. *Arch. Insect Biochem. Physiol.* *35*, 491–512.

Kodrik, D., Goldsworthy, G.J., **1995**. Inhibition of RNA synthesis by adipokinetic hormone of brain factor(s) in adult fat body of *Locusta migratoria*. *J. Insect Physiol.* *41*, 127–133.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid-receptor superfamily. *Cell 67*, 59–77.

Koeppe, J.K., Fuchs, M., Chen, T.T., Hunt, L.M., Kovalick, G.E., *et al.*, **1985**. The role of juvenile hormone in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 165–203.

Koeppe, J.K., Hobson, K., Wellman, S.E., **1980**. Juvenile hormone regulation of structural changes and DNA synthesis in the follicular epithelium of *Leucophaea maderae*. *J. Insect Physiol. 26*, 229–240.

Koeppe, J.K., Wellman, S.E., **1980**. Ovarian maturation in *Leucophaea maderae:* juvenile hormone regulation of thymidine uptake into follicle cell DNA. *J. Insect Physiol. 26*, 219–228.

Kokoza, V.A., Martin, D., Mienaltowski, M.J., Ahmed, A., Morton, C.M., *et al.*, **2001**. Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene 274*, 47–65.

Kokoza, V.A., Raikhel, A.S., **1997**. Ovarian- and somatic-specific transcripts of the mosquito clathrin heavy chain gene generated by alternative 5′-exon splicing and polyadenylation. *J. Biol. Chem. 272*, 1164–1170.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., *et al.*, **1991**. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. *Proc. Natl Acad. Sci. USA 88*, 9458–9462.

Kreienkamp, H.J., Larusson, H.J., Witte, I., Roeder, T., Birgul, N., *et al.*, **2002**. Functional annotation of two orphan G-protein-coupled receptors, Drostar1 and -2, from *Drosophila melanogaster* and their ligands by reverse pharmacology. *J. Biol. Chem. 277*, 39937–39943.

Krieger, M.J.B., Jahan, N., Riehle, M.A., Cao, C., Brown, M.R., **2004**. Molecular characterization of insulin-like peptide genes and their expression in the African malaria mosquito, *Anopheles gambiae*. *Insect Mol. Biol. 13*, 305–315.

Kulakosky, P., Telfer, W., **1989**. Kinetics of yolk precursor uptake in *Hyalophora cecropia*: stimulation of microvitellogenin endocytosis by vitellogenin. *Insect Biochem. 19*, 367–373.

Kulakosky, P., Telfer, W., **1990**. Lipophorin as a yolk precursor in *Hyalophora cecropia*: uptake kinetics and competition with vitellogenin. *Arch. Insect Biochem. Physiol. 14*, 269–285.

Kurama, T., Kurata, S., Natori, S., **1995**. Molecular characterization of an insect transferrin and its selective incorporation into eggs during oogenesis. *Eur. J. Biochem. 228*, 229–235.

Kwok, R., Nassel, D.R., Lange, A.B., Orchard, I., **1999**. Locustatachykinin isoforms in the locust: distribution and quantification in the central nervous system and action on the oviduct muscle. *Peptides 20*, 687–694.

Kwok, R., Orchard, I., **2002**. Characterization of a putative SchistoFLRFamide receptor in the CNS of *Locusta migratoria*. *Peptides 23*, 765–772.

Lam, G.T., Jiang, C.A., Thummel, C.S., **1997**. Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development 124*, 1757–1769.

Lange, A.B., **2002**. A review of the involvement of proctolin as a cotransmitter and local neurohormone in the oviduct of the locust, *Locusta migratoria*. *Peptides 23*, 2063–2070.

Langueux, M., Kromer, E., Girardie, A., **1992**. Cloning of a *Locusta* cDNA encoding neuroparsin A. *Insect Biochem. Mol. Biol. 22*, 511–516.

Lanot, R., Dorn, A., Günster, B., Thiebold, J., Lagueux, M., *et al.*, **1989**. Functions of ecdysteroids in oocyte maturation and embryonic development of insects. In: Koolman, J. (Ed.), Ecdysone: From Chemistry to Mode of Action. Georg Thieme Veralag, Stuttgart, pp. 262–270.

Lanzrein, B., Gentinetta, V., Fehr, R., Lüscher, M., **1978**. Correlation between haemolymph juvenile hormone titre, corpus allatum volume, and corpus allatum *in vivo* and *in vitro* activity during oocyte maturation in a cockroach (*Nauphoeta cinerea*). *Gen. Comp. Endocrinol. 36*, 339–345.

Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., *et al.*, **2001**. Type A allatostatins from *Drosophila melanogaster* and *Diplotera puncata* activate two *Drosophila allatostatin* receptors, DAR-1 and DAR-2, expressed in CHO cells. *Biochem. Biophys. Res. Commun. 286*, 895–901.

Lea, A.O., **1972**. Regulation of egg maturation in the mosquito by the neurosecretory system: the role of the corpus cardiacum. *Gen. Comp. Endocrinol. 3* (Suppl.), 602–608.

Leal, I., White, E.E., Sahota, T.S., Manville, J.F., **1997**. Differential expression of the vitellogenin gene in the spruce terminal weevil feeding on resistant versus susceptible host trees. *Insect Biochem. Mol. Biol. 27*, 569–575.

Lee, T., Marticke, S., Sung, C., Robinow, S., Luo, L., **2000**. Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron 28*, 807–218.

Lee, Y.-H., Keeley, L.L., **1994a**. Hypertrehalosemic hormone effects on transcriptional activity in the fat body of the cockroach, *Blaberus discoidalis*. *Insect Biochem. Mol. Biol. 24*, 357–362.

Lee, Y.-H., Keeley, L.L., **1994b**. Intracellular transduction of trehalose synthesis by hypertrehalosemic hormone in the fat body of the tropical cockroach, *Blaberus discoidalis*. *Insect Biochem. Mol. Biol. 24*, 473–480.

Lehmann, M., Korge, G., **1995**. Ecdysone regulation of the *Drosophila* sgs-4 gene is mediated by the synergistic action of ecdysone receptor and sebp-3. *EMBO J. 14*, 716–726.

Li, C., Kapitskaya, M.Z., Zhu, J., Miura, K., Segraves, W., *et al.*, **2000**. Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. *Devel. Biol. 224*, 96–110.

Li, H., Baker, B.S., **1998**. *hermaphrodite* and *doublesex* function both dependently and independently to control

various aspects of sexual differentiation in *Drosophila*. *Development 125*, 2641–2651.

Li, Y., Unnithan, G.C., Veenstra, J.A., Feyereisen, R., Noriega, F.G., **2003**. Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti* in vitro: effect of farnesoic acid and *Aedes allatotropin*. *J. Exp. Biol. 206*, 1825–1832.

Lin, Y.G., Hamblin, M.T., Edwards, M.J., Barillas-Mury, C., Kanost, M.R., *et al.*, **1993**. Structure, expression, and hormonal control of genes from the mosquito, *Aedes aegypti*, which encode proteins similar to the vitelline membrane proteins of *Drosophila melanogaster*. *Devel. Biol. 155*, 558–568.

Locke, J., White, B.N., Wyatt, G.R., **1987**. Cloning and 5′-end nucleotide sequences of two juvenile hormone-inducible vitellogenin genes of the African migratory locust. *DNA 6*, 331–342.

Locke, M., **1998**. The fat body. In: Harrison, F.W., Locke, M. (Eds.), Microscopic Anatomy of Invertebrates, 11B Insecta. Wiley-Liss, New York, pp. 641–686.

Locke, M., Huie, P., **1983**. A function for plasma membrane reticular systems. *Tissue Cell 15*, 885–902.

Loeb, M., Woods, C.V., Brandt, E.P., Borkovec, A.B., **1982**. Larval testes of the tobacco budworm: a new source of insect ecdysteroids. *Science 218*, 896–898.

Loeb, M.J., De Loof, A., Gelman, D.B., Hakim, R.S., Jaffe, H., *et al.*, **2001**. Testis ecdysiotropin, an insect gonadotropin that induces synthesis of ecdysteroid. *Arch. Insect Biochem. Physiol. 47*, 181–188.

Logan, S.K., Garabedian, M.J., Wensink, P.C., **1989**. DNA regions that regulate the ovarian transcriptional specificity of *Drosophila* yolk protein genes. *Genes Devel. 3*, 1453–1461.

Logan, S.K., Wensink, P.C., **1990**. Ovarian follicle cell enhancers from the *Drosophila* yolk protein genes: different segments of one enhancer have different cell-type specificities that interact to give normal expression. *Genes Devel. 4*, 613–623.

Loher, W., Giannakakis, A.M., **1990**. Allatectomy in the penultimate larval instar of *Teleogryllus commodus*: morphological, physiological and behavioural consequences. *Zool. Jb. Physiol. 94*, 167–179.

Loher, W., Weber, T., Rembold, H., Huber, F., **1992**. Persistence of phonotaxis in females of four species of crickets following allatectomy. *J. Comp. Physiol. 171A*, 325–341.

Lorenz, J.I., Lorenz, M.W., Hoffman, K.-H., **1997**. Factors regulating juvenile hormone and ecdysteroid biosynthesis in *Gryllus bimaculatus* (Ensifera: Gryllidae). *Eur. J. Entomol. 94*, 369–379.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., **1995**. A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. *J. Biol. Chem. 270*, 21103–21108.

Lorenz, M.W., Lorenz, J.I., Treiblmayr, K., Hoffmann, K.H., **1998**. In vivo effects of allatostatins in crickets, *Gryllus bimaculatus* (Ensifera: Gryllidae). *Arch. Insect Biochem. Physiol. 38*, 32–43.

Lossky, M., Wensink, P.C., **1995**. Regulation of *Drosophila* yolk protein genes by an ovary-specific GATA factor. *Mol. Cell. Biol. 15*, 6943–6952.

MacDougall, C., Harbison, D., Bownes, M., **1995**. The developmental consequences of alternate splicing in sex determination and differentiation in *Drosophila*. *Devel. Biol. 172*, 353–376.

Mahamat, H., Hassanali, A., Ferenz, H.J., **1997**. Vitellogenin titres in normal and accelerated maturation of gregarious-phase *Schistocerca gregaria*. *Comp. Biochem. Physiol. 116B*, 447–451.

Manière, G., Vanhems, E., Delbecque, J.P., **2000**. Cyclic AMP-dependent and independent stimulations of ovarian steroidogenesis by brain factors in the blowfly, *Phormia regina*. *Mol. Cell. Endocrinol. 168*, 31–40.

Manière, G., Vanhems, E., Gautron, F., Delbecque, J.P., **2002**. Calcium inhibits ovarian steroidogenesis in the blowfly, *Phormia regina*. *J Endocrinol. 173*, 533–544.

Manière, G., Vanhems, E., Gautron, F., Delbecque, J.P., **2003**. Inhibition of ovarian steroidogenesis by cyclic GMP in a fly. *J. Endocrinol. 177*, 35–44.

Marti, D., Grossniklaus-Burgin, C., Wyder, S., Wyler, T., Lanzrein, B., **2003**. Ovary development and polydnavirus morphogenesis in the parasitic wasp *Chelonus inanitus*. I. Ovary morphogenesis, amplification of viral DNA and ecdysteroid titers. *J. Gen. Virol. 84*, 1141–1150.

Martin, D., Comas, D., Piulachs, M.D., Belles, X., **1998**. Isolation and sequence of a partial vitellogenin cDNA from the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae), and characterization of the vitellogenin gene expression. *Arch. Insect Biochem. Physiol. 38*, 137–146.

Martin, D., Piulachs, M.D., Belles, X., **1995**. Patterns of hemolymph vitellogenin and ovarian vitellin in the German cockroach, and the role of juvenile hormone. *Physiol. Entomol. 20*, 59–65.

Martin, D., Piulachs, M.D., Belles, X., **1996**. Inhibition of vitellogenin production by allatostatin in the German cockroach. *Mol. Cell. Endocrinol. 121*, 191–196.

Martin, D., Piulachs, M.D., Raikhel, A.S., **2001a**. A novel GATA factor transcriptionally represses yolk protein precursor genes in the mosquito *Aedes aegypti* via interaction with the CtBP corepressor. *Mol. Cell. Biol. 21*, 164–174.

Martin, D., Wang, S.F., Raikhel, A.S., **2001b**. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol. Cell. Endocrinol. 173*, 75–86.

Martinez, A., Bownes, M., **1994**. The sequence and expression pattern of the *Calliphora erythrocephala* yolk protein A and B genes. *J. Mol. Evol. 38*, 336–351.

Martinez, T., Garcerá, M.D., **1987**. Effects of precocene II on female-specific hemolymph polypeptides in *Oncopeltus fasciatus*. *Arch. Insect Biochem. Physiol. 6*, 49–58.

Masetti, M., Giorgi, F., **1989**. Vitellin degradation in developing embryos of the stick insect, *Carausius morosus*. *J. Insect Physiol. 35*, 689–697.

Masuda, H., Braz, G.R.C., Paiva-Silva, G.O., Silva-Neto, M.A.C., Oliveira, P.L., **2004**. Non-vitellin yolk proteins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitetlogenesis. Science Publishers, Enfield/USA-Plymouth, UK, pp. 289–314.

Mazzini, M., Burrini, A., Giorgi, F., **1989**. The secretory pathway of vitellogenin in the fat body of the stick insect *Bacillus rossius*: an ultrastructural and immunocytochemical study. *Tissue Cell 21*, 535–541.

McCaffery, A.R., McCaffery, V.A., **1983**. Corpus allatum activity during overlapping cycles off oocyte growth in adult female *Melanoplus sanguinipes*. *J. Insect Physiol. 29*, 259–266.

Melo, A.C., Valle, D., Machado, E.A., Salerno, A.P., Paiva-Silva, G.O., *et al.*, **2000**. Synthesis of vitellogenin by the follicle cells of *Rhodnius prolixus*. *Insect Biochem. Mol. Biol. 30*, 549–557.

Mertens, I., Meeusen, T., Huybrechts, R., De Loof, A., Schoofs, L., **2002**. Characterization of the short neuropeptide F receptor from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 297*, 1140–1148.

Meyering-Vos, M., Wu, X., Huang, J., Jindra, M., Hoffmann, K.H., *et al.*, **2001**. The allatostatin gene of the cricket *Gryllus bimaculatus* (Ensifera, Gryllidae). *Mol. Cell. Endocrinol. 184*, 103–114.

Minoo, P., Postlethwait, J., **1985**. Biosynthesis of *Drosophila* yolk polypeptides. *Arch. Insect Biochem. Physiol. 2*, 7–27.

Miura, K., Nakagawa, M., Chinzei, Y., Shinoda, T., Nagao, E., *et al.*, **1994**. Structural studies of biliverdin-associated cyanoprotein from the bean bug, *Riptortus clavatus*. *Zool. Sci. 11*, 537–545.

Miura, K., Zhu, J., Dittmer, N.T., Chen, L., Raikhel, A.S., **2002**. A COUP-TF/Svp homolog is highly expressed during vitellogenesis in the mosquito *Aedes aegypti. J. Mol. Endocrinol. 29*, 223–238.

Montuenga, L.M., Barrenechea, M.A., Sesma, P., Lopez, J., Vazquez, J.J., **1989**. Ultrastructure and immunocytochemistry of endocrine cells in the midgut of the desert locust, *Shistocerca gregaria* (Forskal). *Cell Tissue Res. 258*, 577–583.

Moobola, S.M., Cupp, E.W., **1978**. Ovarian development in the stable fly, *Stomoxys calcitrans*, in relation to diet and juvenile hormone control. *Physiol. Entomol. 3*, 317–321.

Moreau, R., Gourdoux, L., Girardie, J., **1988**. Neuroparsin: a new energetic neurohormone in the African locust. *Arch. Insect Biochem. Physiol. 8*, 135–145.

Moshitzky, P., Applebaum, S.W., **1990**. The role of adipokinetic hormone in the control of vitellogenesis in locusts. *Insect Biochem. 20*, 319–323.

Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klauser, S., *et al.*, **1996**. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol. 32*, 363–374.

Nassel, D.R., **2002**. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol. 68*, 1–84.

Nose, Y., Lee, J., Ueno, T., Hatekayama, M., Oishi, K., **1997**. Cloning of cDNA for vitellogenin of the parasitoid wasp *Pimpla nipponica* (Hymenoptera: Apocrita: Ichneumonidae): vitellogenin primary structure and evolutionary considerations. *Insect Biochem. Mol. Biol. 27*, 1047–1056.

Oeh, U., Lorenz, M.W., Dyker, H., Losel, P., Hoffmann, K.H., **2000**. Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol. 30*, 719–727.

Oldham, S., Hafen, E., **2003**. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol. 13*, 79–85.

Oliveira, P., Kawooya, J., Ribeiro, J., Meyer, T., Poormam, R., *et al.*, **1995**. A heme-binding protein from hemolymph and oocytes of the blood-sucking insect, *Rhodnius prolixus*. *J. Biol. Chem. 270*, 10897–10901.

Ono, S., Nagayama, H., Shimura, K., **1975**. The occurrence and synthesis of female- and egg-specific proteins in silkworm, *Bombyx mori*. *Insect Biochem. 5*, 313–329.

Osir, E., Anderson, D., Grimes, W., Law, J., **1986b**. Studies on the carbohydrate moiety of vitellogenin from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. 16*, 471–478.

Osir, E., Wells, M., Law, J., **1986a**. Studies on vitellogenin from the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 3*, 217–233.

Paemen, L., Tips, A., Schoofs, L., Proost, P., Van Damme, J., *et al.*, **1991**. Lom-AG-myotropin: a novel myotropic peptide from the male accessory glands of *Locusta migratoria*. *Peptides 12*, 7–10.

Pan, M., **1971**. The synthesis of vitellogenin in the cecropia silkworm. *J. Insect Physiol. 17*, 677–689.

Pan, M., Wiemerslage, L., Telfer, W., **1994**. Male-grown eggs in *Hyalophora*: deficient follicle cell secretion as well as protein and lipid yolk deposition. *J. Insect Physiol. 40*, 765–773.

Park, C., Hwang, J.S., Kang, S.W., Lee, B.H., **2002**. Molecular characterization of a cDNA from the silk moth *Bombyx mori* encoding *Manduca sexta* allatotropin peptide. *Zool. Sci. 19*, 287–292.

Park, Y.I., Shu, S., Ramaswamy, S.B., Srinivasan, A., **1998**. Mating in *Heliothis virescens*: transfer of juvenile hormone during copulation by male to female and stimulation of biosynthesis of endogenous juvenile hormone. *Arch. Insect Biochem. Physiol. 38*, 100–107.

Pascual, N., Cerdà, X., Benito, B., Tomás, J., Piulachs, M.D., *et al.*, **1992**. Ovarian ecdysteroid levels and basal oocyte development during maturation in the cockroach *Blattella germanica* (L.). *J. Insect Physiol. 38*, 339–348.

Pau, R.N., Birnstingl, S., Edwards-Jones, K., Gillen, C.U., Matsakis, E., **1987**. The structure and organization of genes coding for juvenile hormone-regulated 16 Kdalton oothecins in the cockroach *Periplaneta americana*. *UCLA Symp. Mol. Cell. Biol. N.S. 66*, 265–277.

Peferoen, M., De Loof, A., **1986**. Synthesis of vitellogenic and non-vitellogenic yolk proteins by fat body and the ovary of *Leptinotarsa decemlineata*. *Comp. Biochem. Physiol. B 83*, 251–254.

Perera, O., Shirk, P., **1999**. cDNA of YP4, a follicular epithelium yolk protein subunit, in the moth, *Plodia interpunctella*. *Arch. Insect Biochem. Physiol. 40*, 157–164.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., *et al.*, **2003**. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778.

Pierceall, W.E., Li, C., Biran, A., Miura, K., Raikhel, A.S., *et al.*, **1999**. E75 expression in mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol. Cell. Endocrinol. 150*, 73–89.

Piulachs, M.D., Guidugli, K.R., Barchuk, A.R., Cruz, J., Simoes, Z.L., *et al.*, **2003**. The vitellogenin of the honey bee, *Apis mellifera*: structural analysis of the cDNA and expression studies. *Insect Biochem. Mol. Biol. 33*, 459–465.

Piulachs, M.D., Maestro, J.L., Belles, X., **1992**. Juvenile hormone production and accessory reproductive gland development during sexual maturation of male *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Comp. Biochem. Physiol. 102A*, 477–480.

Postlethwait, J.H., Bownes, M., Jowett, T., **1980**. Sexual phenotype and vitellogenin synthesis in *Drosophila melanogaster. Devel. Biol. 79*, 379–387.

Pursley, S., Ashok, M., Wilson, T.G., **2000**. Intracellular localization and tissue specificity of the Methoprene-tolerant (Met) gene product in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 30*, 839–845.

Rachinsky, A., Tobe, S.S., Feldlaufer, M.F., **2000**. Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera* L.): developmental changes in sensitivity to JH precursor and allatotropin. *Insect Biochem. Mol. Biol. 30*, 729–737.

Rahman, M.M., Bosch, L.V., Baggerman, G., Clynen, E., Hens, K., *et al.*, **2002**. Search for peptidic molecular markers in hemolymph of crowd-(gregarious) and isolated-reared (solitary) desert locusts, *Schistocerca gregaria*. *Peptides 23*, 1907–1914.

Raikhel, A.S., **1992a**. Biochemistry and molecular biology of the mosquito vitellogenesis. In: Bennetova, B., Gelbil, I., Soldan, T. (Eds.), Advances in Regulation of Insect Reproduction. Institute of Entomology, Czech Academy of Science, Czeske Budovize, Czech Republic, pp. 243–246.

Raikhel, A.S., **1992b**. Vitellogenesis in mosquitoes. *Adv. Dis. Vect. Res. 9*, 1–39.

Raikhel, A.S., Dhadialla, T.S., **1992**. Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol. 37*, 217–251.

Raikhel, A.S., Kokoza, V.A., Zhu, J.S., Martin, D., Wang, S.F., *et al.*, **2002**. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochem. Mol. Biol. 32*, 1275–1286.

Raikhel, A.S., Lea, A.O., **1983**. Previtellogenic development and vitellogenin synthesis in the fat body of a mosquito: an ultrastructural and immunocytochemical study. *Tissue Cell 15*, 281–300.

Raikhel, A.S., Lea, A.O., **1985**. Hormone-mediated formation of the endocytic complex in mosquito oocytes. *Gen. Comp. Endocrinol. 57*, 422–433.

Raikhel, A.S., Lea, A.O., **1990**. Juvenile hormone controls previtellogenic proliferation of ribosomal RNA in the mosquito fat body. *Gen. Comp. Endocrinol. 77*, 423–434.

Raikhel, A.S., Lea, A.O., **1991**. Control of follicular epithelium development and vitelline envelope formation in the mosquito – role of juvenile-hormone and 20-hydroxyecdysone. *Tissue Cell 23*, 577–591.

Raikhel, A.S., McGurk, L., Bownes, M., **2003**. Ecdysteroid action in insect reproduction. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, Vol. 1. Academic Press, San Diego, pp. 451–459.

Raikhel, A.S., Snigirevskaya, E.S., **1998**. Vitellogenesis. In: Harrison, F.W., Locke, M. (Eds.), Microscopic Anatomy of Invertebrates, Vol. 11c. Insecta. Wiley-Liss & Sons, New York, pp. 933–955.

Rajaratnam, V., **1996**. Isolation, characterization and complete nucleotide sequence of *Galleria mellonela* cDNA coding for the follicle cell-specific yolk protein, YP4. *Insect Biochem. Mol. Biochem. 26*, 545–555.

Ramaswamy, S.B., Shu, S.Q., Park, Y.I., Zeng, F.R., **1997**. Dynamics of juvenile hormone-mediated gonadotropism in the Lepidoptera. *Arch. Insect Biochem. Physiol. 35*, 539–558.

Rankin, S.M., Chambers, J., Edwards, J.P., **1997**. Juvenile hormone in earwigs: roles in oogenesis, mating, and maternal behaviors. *Arch. Insect Biochem. Physiol. 35*, 427–442.

Redfern, C.P.F., Bownes, M., **1982**. Yolk polypeptide synthesis and uptake in ovaries of the *apterous* mutant of *Drosophila melanogaster. Mol. Gen. Genet. 185*, 181–183.

Regis, L., Furtado, A.F., Gomes, Y.M., Coutinho, V.B., Schuetz, M.J., *et al.*, **1987**. Secretory activity and endocrine regulation of male accessory glands in the blood-sucking bug *Panstrongylus megistus* (Hemiptera: Reduviidae). *Mem. Inst. Oswaldo Cruz 82* (Suppl. 3), 75–78.

Reissig, W.H., Kamm, J.A., **1974**. Effects of foliage sprays of juvenile hormone analogues on development of *Draeculacephala crassicornis*. *J. Econ. Entomol. 67*, 181–183.

Renucci, M., Strambi, A., Strambi, C., **1987**. Ovarian development and endocrine control of the vitellogenesis in the house cricket, *Acheta domesticus*. *Gen. Endocrinol. (Life Sci. Adv.) 6*, 83–92.

Richard, D.S., Gilbert, M., Crum, B., Hollinshead, D.M., Schelble, S., *et al.*, **2001**. Yolk protein endocytosis by oocytes in *Drosophila melanogaster*: immunofluorescent

localization of clathrin, adaptin and the yolk protein receptor. *J. Insect Physiol. 47*, 715–723.

Richard, D.S., Jones, J.M., Barbarito, M.R., Cerula, S., Detweiler, J.P., *et al.*, **2001**. Vitellogenesis in diapausing and mutant *Drosophila melanogaster*: further evidence for the relative roles of ecdysteroids and juvenile hormones. *J. Insect Physiol. 47*, 905–913.

Richard, D.S., Watkins, N.L., Serafin, R.B., Gilbert, L.I., **1998**. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol. 44*, 637–644.

Richard, O., Tamarelle, M., Geoffre, S., Girardie, J., **1994**. Restricted occurrence of *Locusta migratoria* ovary maturing parsin in the brain-corpora cardiaca complex of various insect species. *Histochemistry 102*, 233–239.

Riddihough, G., Pelham, H.R.B., **1987**. An ecdysone response element in the *Drosophila* hsp27 promoter. *EMBO J. 6*, 3729–3734.

Riehle, M.A., Brown, M.R., **1999**. Insulin stimulates ecdysteroid production through a conserved signaling cascade in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 29*, 855–860.

Riehle, M.A., Brown, M.R., **2002**. Insulin receptor expression during development and a reproductive cycle in the ovary of the mosquito *Aedes aegypti*. *Cell Tissue Res. 308*, 409–420.

Riehle, M.A., Brown, M.R., **2003**. Molecular analysis of the serine/threonine kinase Akt and its expression in the mosquito, *Aedes aegypti*. *Insect Mol. Biol. 12*, 225–232.

Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., **2002**. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science 298*, 172–175.

Rina, M., Savakis, C., **1991**. A cluster of vitellogenin genes in the Mediterranean fruit fly *Ceratitis capitata*: sequence and structural conservation in dipteran yolk proteins. *Genetics 127*, 769–780.

Robinson, G.E., Vargo, E.L., **1997**. Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker. *Arch. Insect Biochem. Physiol. 35*, 559–583.

Roebroek, A.J., Pauli, I.G., Zhang, Y., van de Ven, W.J., **1991**. cDNA sequence of a *Drosophila melanogaster* gene, Dfur1, encoding a protein structurally related to the subtilisin-like proprotein processing enzyme furin. *FEBS Lett. 289*, 133–137.

Roebroek, A.J., Creemers, J.W., Pauli, I.G., Kurzik-Dumke, U., Rentrop, M., *et al.*, **1992a**. Cloning and functional expression of Dfurin2, a subtilisin-like proprotein processing enzyme of *Drosophila melanogaster* with multiple repeats of a cysteine motif. *J. Biol. Chem. 267*, 17208–17215.

Roebroek, A.J., Pauli, I.G., Zhang, Y., van de Ven, W.J., **1992b**. cDNA sequence of a *Drosophila melanogaster* gene, Dfur1, encoding a protein structurally related to the subtilisin-like proprotein processing enzyme furin. FEBS cloning and functional expression of Dfurin2, a subtilisin-like proprotein processing enzyme of *Drosophila melanogaster* with multiple repeats of a cysteine motif. *J. Biol. Chem. 267*, 17208–17215.

Romañá, I., Pascual, N., Belles, X., **1995**. The ovary is a source of circulating ecdysteroids in *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Eur. J. Entomol. 92*, 93–103.

Romans, P., Tu, Z., Ke, Z., Hagedorn, H.H., **1995**. Analysis of a vitellogenin gene of the mosquito, *Aedes aegypti* and comparisons to vitellogenins from other organisms. *Insect Biochem. Mol. Biol. 25*, 939–958.

Ronaldson, E., Bownes, M., **1995**. Two independent *cis*-acting elements regulate the sex- and tissue-specific expression of yp3 in *Drosophila melanogaster. Genet. Res. 66*, 9–17.

Rouille, Y., Duguay, S.J., Lund, K., Furuta, M., Gong, Q., *et al.*, **1995**. Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convartases. *Frontiers Neuroendocrinol. 16*, 322–361.

Rouland, C., Brousse-Gaury, P., Goudey-Perrière, F., **1981**. Influence d'un farnesol de synthèse sur la vitellogenèse de femelles de *Labiduria riparia* Pallas (Insecte, Dermaptère), allatectomisées ou parsectomisées. *C.R. Acad. Sci. Paris 292*, 105–108.

Rousset, A., Bitsch, C., **1993**. Comparison between endogenous and exogenous yolk proteins along an ovarian cycle in the firebrat *Thermobia domestica* (Insecta, Thysanura). *Comp. Biochem. Physiol. 104B*, 33–44.

Rubacha, A., Tucker, M., DeValoir, T., Berlikoff, E., Beckingham, K., **1988**. Genes with specific functions in ovarian follicles of *Calliphora erythrocephala*. *Devel. Biol. 129*, 449–463.

Rulifson, E.J., Kim, S.K., Nusse, R., **2002**. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science 296*, 1118–1120.

Sakai, N., Mori, S., Izumi, S., Haino-Fukushima, K., Ogura, T., *et al.*, **1988**. Structures and expression of mRNAs coding for major plasma proteins of *Bombyx mori*. *Biochim. Biophys. Acta 949*, 224–232.

Sappington, T., Oishi, K., Raikhel, A.S., **2002**. Structural characteristics of insect vitellogenins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, vol. 12. Part A: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 69–101.

Sappington, T.W., Hays, A.R., Raikhel, A.S., **1995**. Mosquito vitellogenin receptor: purification, developmental and biochemical characterization. *Insect Biochem. Mol. Biol. 25*, 807–817.

Sappington, T.W., Kokoza, V.A., Cho, W.L., Raikhel, A.S., **1996**. Molecular characterization of the mosquito vitellogenin receptor reveals unexpected high homology to the *Drosophila* yolk protein receptor. *Proc. Natl Acad. Sci. USA 93*, 8934–8939.

Sappington, T.W., Raikhel, A.S., **1998a**. *Aedes aegypti*. In: Encyclopaedia of Reproduction, vol. 1. Academic Press, New York, pp. 61–77.

Sappington, T.W., Raikhel, A.S., **1998b**. Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem. Mol. Biol. 28*, 277–300.
Sappington, T.W., Raikhel, A.S., **2004**. Insect vitellogenin/yolk protein receptors. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 229–264.
Satake, S., Nagata, K., Kataoka, H., Mizoguchi, A., **1999**. Bombyxin secretion in the adult silkmoth *Bombyx mori*: sex-specificity and its correlation with metabolism. *J. Insect Physiol. 45*, 939–945.
Sato, Y., Yamashita, O., **1991a**. Structure and expression of a gene coding for egg-specific protein of the silkworm, *Bombyx mori. Insect Biochem 21*, 233–238.
Sato, Y., Yamashita, O., **1991b**. Structure and expression of a gene coding for egg-specific protein of the silkworm, *Bombyx mori. Insect Biochem. 21*, 495–505.
Satyanarayana, K., Bradfield, J.Y., Bhaskaran, G., Dahm, K.H., **1994**. Stimulation of vitellogenin production by methoprene in prepupae and pupae of *Manduca sexta. Arch. Insect Biochem. Physiol. 25*, 21–37.
Schoofs, L., Clynen, E., Cerstiaens, A., Baggerman, G., Wei, Z., *et al.*, **2001**. Newly discovered functions for some myotropic neuropeptides in locusts. *Peptides 22*, 219–227.
Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., De Loof, A., **1991**. Isolation, identification and synthesis of locustamyoinhibiting peptide (LOM-MIP), a novel biologically active neuropeptide from *Locusta migratoria. Regul. Pept. 36*, 111–119.
Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., Bender, M., **1998**. *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development 125*, 2053–2062.
Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., *et al.*, **2001**. Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori. J. Biol. Chem. 276*, 47052–47060.
Sevala, V.L., Davey, K.G., **1989**. Action of juvenile hormone on the follicle cells of *Rhodnius prolixus*: evidence for a novel regulatory mechanism involving protein kinase C. *Experientia 45*, 355–356.
Sevala, V.L., Davey, K.G., **1993**. Juvenile hormone dependent phosphorylation of 100 kDa polypeptide is mediated by protein kinase C in the follicle cells of *Rhodnius prolixus. Invertebr. Reprod. Devel. 24*, 39–46.
Sevala, V.L., Davey, K.G., Prestwich, G.D., **1995**. Photoaffinity labelling and characterization of a juvenile hormone binding protein in the membranes of follicle cells of *Locusta migratoria. Insect Biochem. Mol. Biol. 25*, 267–273.
Shapiro, A.B., Wheelock, G.D., Hagedorn, H.H., Baker, F.C., Tsai, L.W., *et al.*, **1986**. Juvenile-hormone and juvenile-hormone esterase in adult females of the mosquito *Aedes aegypti. J. Insect Physiol. 32*, 867–877.
Shapiro, J.P., **1983**. Ovarian cyclic AMP and response to a brain hormone from the mosquito *Aedes aegypti. Insect Biochem. 13*, 273–279.
Shemshedini, L., Lanoue, M., Wilson, T.G., **1990**. Evidence for a juvenile hormone receptor involved in protein synthesis in *Drosophila melanogaster. J. Biol. Chem. 265*, 1913–1918.
Shemshedini, L., Wilson, T.G., **1990**. Resistance to juvenile hormone and an insect growth regulator in *Drosophila* is associated with an altered cytosolic juvenile hormone-binding protein. *Proc. Natl Acad. Sci. USA 87*, 2027–2076.
Shirk, P.D., Bean, D., Millemann, A., Brookes, V., **1984**. Identification, synthesis and characterization of yolk polypeptide of *Plodia interpunctella. J. Exp. Zool. 232*, 87–98.
Shirk, P.D., Bhaskaran, G., Roller, H., **1980**. The transfer of juvenile hormone from male to female during mating in the cecropia silkmoth. *Experientia 36*, 682–683.
Shirk, P.D., Bhaskaran, G., Roller, H., **1983**. Developmental physiology of corpora allata and accessory sex glands in the cecropia silkmoth. *J. Exp. Zool. 227*, 69–79.
Shirk, P.D., Perera, O., **1998**. 5′ coding region of the follicle epithelium yolk polypeptide 2 cDNA in the moth *Plodia interpunctella* contains an extended coding region. *Arch. Insect Biochem. Physiol. 39*, 98–108.
Shirk, P.D., Bean, D., Millemann, A.M., Brooks, V.J., **1984**. Identification, synthesis and characterization of yolk polypeptide of *Plodia interpunctella. J. Exp. Zool. 232*, 87–98.
Shirk, P.D., Zimowska, G., Silhacek, D.L., Shaaya, E., **1992**. Initiation of vitellogenesis in pharate adult females of the Indian-meal moth, *Plodia interpunctella. Arch. Insect Biochem. Physiol. 21*, 53–63.
Shu, S., Park, Y.I., Ramaswamy, S.B., Srinivasan, A., **1997**. Hemolymph juvenile hormone titers in pupal and adult stages of southwestern corn borer [*Diatraea grandiosella* (Pyralidae)] and relationship with egg development. *J. Insect Physiol. 43*, 719–726.
Smith, S.L., Mitchell, M.J., **1986**. Ecdysone 20-monoxygenase systems in a larval and adult dipteran: an overview of their biochemistry, physiology and pharmacology. *Insect Biochem. 16*, 49–55.
Smith, W.A., Koundinya, M., McAllister, T., Brown, A., **1997**. Insulin receptor-like tyrosine kinase in the tobacco hornworm, *Manduca sexta. Arch. Insect. Biochem. Physiol. 35*, 99–110.
Snigirevskaya, E., Sappington, T., Raikhel, A.S., **1997b**. Internalization and recycling of vitellogenin receptor in the mosquito oocyte. *Cell Tissue Res. 290*, 175–183.
Snigirevskaya, E.S., Hays, A.R., Raikhel, A.S., **1997a**. Secretory and internalization pathways of mosquito yolk protein precursors. *Cell Tissue Res. 290*, 129–142.
Snigirevskaya, E.S., Raikhel, A.S., **2004**. Receptor-mediated endocytosis of yolk proteins in insect oocytes. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress

in Vitellogenesis. Science Publishers, Enfield, USA/ Plymouth, UK, pp. 199–228.

Soller, M., Bownes, M., Kubli, E., **1997**. Mating and sex peptide stimulate the accumulation of yolk in oocytes of *Drosophila melanogaster. Eur. J. Biochem. 243*, 732–738.

Soller, M., Bownes, M., Kubli, E., **1999**. Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol. 208*, 337–351.

Sommer, K., Hölldobler, B., Rembold, H., **1993**. Behavioral and physiological aspects of reproductive control in *Diacamma* species from Malaysia (Formicidae, Ponerinae). *Ethology 94*, 162–170.

Sorge, D., Nauen, R., Range, S., Hoffmann, K., **2000**. Regulation of vitellogenesis in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *J. Insect Physiol. 46*, 969–976.

Spieth, J., Nettleton, M., Zucker-Aprison, E., Lea, K., Blumenthal, T., **1991**. Vitellogenin motifs conserved in nematodes and vertebrates. *J. Mol. Evol. 32*, 429–438.

Spittaels, K., Vankeerberghen, A., Schoofs, L., Proost, P., Van Damme, J., *et al.*, **1996**. Isolation and characterization of *Locusta migratoria* accessory gland myotropin I (Lom-Ag-MT-I) from the brain of the Colorado potato beetle, *Leptinotarsa decemlineata*. *Arch. Insect Biochem. Physiol. 31*, 149–155.

Stanek, D.M., Pohl, J., Crim, J.W., Brown, M.R., **2002**. Neuropeptide F and its expression in the yellow fever mosquito, *Aedes aegypti*. *Peptides 23*, 1367–1378.

Starratt, A.N., Lange, A.B., Orchard, I., **2000**. N-terminal modified analogs of HVFLRF amide with inhibitory activity on the locust oviduct. *Peptides 21*, 197–203.

Staubli, F., Jorgensen, T.J., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99*, 3446–3451.

Stay, B., **2000**. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. *Insect Biochem. Mol. Biol. 30*, 653–662.

Stracker, T.H., Thompson, S., Grossman, G.L., Riehle, M.A., Brown, M.R., **2002**. Characterization of the AeaHP gene and its expression in the mosquito *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol. 39*, 331–342.

Strambi, A., Strambi, C., Cayre, M., **1997**. Hormonal control of reproduction and reproductive behavior in crickets. *Arch. Insect Biochem. Physiol. 35*, 393–404.

Strambi, C., Cayre, M., Sattelle, D.B., Augier, R., Charpin, P., *et al.*, **1998**. Immunocytochemical mapping of an RDL-like GABA receptor subunit and of GABA in brain structures related to learning and memory in the cricket *Acheta domesticus*. *Learn Mem. 5*, 78–89.

Strong, L., **1965**. The relationship between the brain, corpora allata, and oocyte growth in the Central American locust, *Schistocerca* sp. I. The cerebral neurosecretory system, the corpora allata, and the oocyte growth. *J. Insect Physiol. 11*, 135–146.

Sun, G.Q., Zhu, J.S., Li, C., Tu, Z.J., Raikhel, A.S., **2002**. Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, *Aedes aegypti*. *Mol. Cell. Endocrinol. 190*, 147–157.

Sun, G.Q., Zhu, J.S., Raikhel, A.S., **2004**. The early gene E74B isoform is a transcriptional activator of the ecdysteroid regulatory hierarchy in mosquito vitellogenesis. *Mol. Cell. Endocrinol. 218*, 95–105.

Sun, J., Hiraoka, T., Dittmer, N., Cho, K., Raikhel, A.S., **2001**. Lipophorin as a yolk protein precursor in the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 30*, 1161–1171.

Sun, X., Song, Q., Barrett, B., **2003**. Effect of ecdysone agonists on vitellogenesis and the expression of EcR and USP in codling moth (*Cydia pomonella*). *Arch. Insect Biochem. Physiol. 52*, 115–129.

Swanson, W.J., Clark, A.G., Waldrip-Dail, H.M., Wolfner, M.F., Aquadro, C.F., **2001**. Evolutionary EST analysis identifies rapidly evolving male reproductive proteins in *Drosophila*. *Proc. Natl Acad. Sci. USA 98*, 7375–7379.

Taghert, P.H., Veenstra, J.A., **2003**. *Drosophila* neuropeptide signaling. *Adv. Genet. 49*, 1–65.

Taibi, F., Smagghe, G., Amrani, L., Soltani-Mazouni, N., **2003**. Effect of ecdysone agonist RH-0345 on reproduction of mealworm, *Tenebrio molitor. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 135C*, 257–267.

Takahashi, S.Y., Yamamoto, Y., Zhao, X., Watabe, S., **1996**. Bombyx acid cysteine proteinase. *Int. J. Invertebr. Reprod. Devel. 30*, 265–281.

Talbot, W.S., Swyryd, E.A., Hogness, D.S., **1993**. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell 73*, 1323–1337.

Tatar, M., Bartke, A., Antebi, A., **2003**. The endocrine regulation of aging by insulin-like signal. *Science 299*, 1346–1351.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M., *et al.*, **2001**. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science 292*, 107–110.

Taylor, P.A., III, Bhatt, T.R., Horodyski, F.M., **1996**. Molecular characterization and expression analysis of *Manduca sexta* allatotropin. *Eur. J. Biochem. 239*, 588–596.

Telfer, W., Kulakosky, P., **1984**. Isolated hemolymph proteins as probes of endocytotic yolk formation in *Hyalophora cecopia*. *Adv. Invert. Reprod. 3*, 81–86.

Telfer, W., Pan, M., **1988**. Adsorptive endocytosis of vitellogenin, lipophorin, and microvitellogenin during yolk formation in *Hyalophora*. *Arch. Insect Biochem. Physiol. 9*, 339–355.

Telfer, W., Pan, M., Law, J., **1991**. Lipophorin in developing adults of *Hyalophora cecropia*: support for yolk formation and preparation for flight. *Insect Biochem. 21*, 653–663.

Telfer, W.H., **2002**. Insect yolk proteins: a progress report. In: Raikhel, A.S., Sappington, T.W. (Eds.),

Reproductive Biology of Invertebrates, Vol. 12, Part A: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 29–67.

Terpstra, P., Ab, G., **1988**. Homology of *Drosophila* yolk proteins and the triacylglycerol lipase family. *J. Mol. Biol. 202*, 663–665.

Thomsen, E., Hansen, B.L., Hansen, G.N., Jensen, P.V., **1980**. Ultrastructural immunocytochemical localization of vitellogenin in the fat body of the blowfly, *Calliphora vicina* Rob.-Desv. (*erythrocephala* Meig.) by use of the unlabeled antibody-enzyme method. *Cell Tissue Res. 208*, 445–455.

Thomsen, E., Thomsen, M., **1974**. Fine structure of the fat body of the female of *Calliphora erythrocephala* during the first egg-maturation cycle. *Cell Tissue Res. 152*, 193–217.

Thomsen, E., Thomsen, M., **1978**. Production of specific-protein secretion granules by the fat body cells of the blowfly *Calliphora erythrocephala*. Substitution of an ovarian key factor by β-ecdysone. *Cell Tissue Res. 193*, 25–33.

Thummel, C.S., **1996**. Flies on steroids – *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet. 12*, 306–310.

Thummel, C.S., **2002**. Ecdysone-regulated puff genes 2000. *Insect Biochem. Mol. Biol. 32*, 113–120.

Tobe, S.S., Langley, P.A., **1978**. Reproductive physiology of *Glossina*. *Annu. Rev. Entomol. 23*, 283–307.

Tobe, S.S., Pratt, G.E., **1975**. Corpus allatum activity in vitro during ovarian maturation in the desert locust. *Schistocerca gregaria*. *J. Exp. Biol. 62*, 611–627.

Tobe, S.S., Stay, B., **1977**. Corpus allatum activity in vitro during the reproductive cycle of the viviparous cockroach, *Diploptera punctata* (Eschoscholtz). *Gen. Comp. Endocrinol. 31*, 138–147.

Trabalon, M., Campan, M., Porcheron, P., Clement, J.L., Baehr, J.C., *et al.*, **1990**. Relationships among hormonal changes, cuticular hydrocarbons, and attractiveness during the first gonadotropic cycle of the female *Calliphora vomitoria* (Diptera). *Gen. Comp. Endocrinol. 80*, 216–222.

Troy, S., Anderson, W.A., Spielman, A., **1975**. Lipid content of maturing ovaries of *Aedes aegypti* mosquitoes. *Comp. Biochem. Physiol. B 50*, 457–461.

Truesdell, P.F., Koladich, P.M., Kataoka, H., Kojima, K., Suzuki, A., *et al.*, **2000**. Molecular characterization of a cDNA from the true armyworm *Pseudaletia unipuncta* encoding *Manduca sexta* allatotropin peptide. *Insect Biochem. Mol. Biol. 30*, 691–702.

Tsai, M.J., O'Malley, B.W., **1994**. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem. 63*, 451–486.

Tsuchida, K., Kawooya, J., Law, J., Wells, M., **1992**. Isolation and characterization of two follicle-specific proteins from eggs of *Manduca sexta*. *Insect Biochem. Mol. Biol. 22*, 89–98.

Tu, M.-P., Kou, R., Wang, Z., Stoffolano, J.G., Jr., Yin, C., **2001**. Immunolocalization and possible effect of a moth allatotropin-like substance in a fly, *Phormia regina* (Diptera: Calliphoridae). *J. Insect Physiol. 47*, 233–244.

Tu, M.-P., Yin, C.-M., Tatar, M., **2002**. Impaired ovarian ecdysone synthesis of *Drosophila melanogaster* insulin receptor mutants. *Aging Cell 1*, 158–160.

Tufail, M., Hatakeyama, M., Takeda, M., **2001**. Molecular evidence for two vitellogenin genes and processing of vitellogenins in the American cockroach, *Periplaneta americana*. *Arch. Insect Biochem. Physiol. 48*, 72–80.

Tufail, M., Raikhel, A.S., Takeda, M., **2004**. Biosynthesis and processing of insect vitellogenins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK.

Tufail, M., Takeda, M., **2002**. Vitellogenin of the cockroach, *Leucophaea maderae*: nucleotide sequence, structure and analysis of processing in the fat body and oocytes. *Insect Biochem. Mol. Biol. 32*, 117–134.

Tzolovsky, G., Deng, W.M., Schlitt, T., Bownes, M., **1999**. The function of the broad-complex during *Drosophila melanogaster* oogenesis. *Genetics 153*, 1371–1383.

Valle, D., **1993**. Vitellogenesis in insects and other groups. A review. *Mem. Inst. Oswaldo Cruz 88*, 1–26.

Vandingenen, A., Hens, K., Macours, N., Zhu, W., Janssen, I., *et al.*, **2001**. Captopril, a specific inhibitor of angiotensin converting enzyme, enhances both trypsin and vitellogenin titers in the grey fleshfly *Neobellieria bullata*. *Arch. Insect. Biochem. Physiol. 47*, 161–167.

Veenstra, J.A., Costes, L., **1999**. Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. *Peptides 20*, 1145–1151.

Vilaplana, L.L., Piulachs, M.D., Belles, X., **1996**. The conglobate gland of *Blattella germanica* (L.) (Dictyoptera, Blattellidae). Maturation, juvenile hormone dependency and changes during spermatophore formation. *Invert. Reprod. Devel. 29*, 167–172.

Wagner, R.M., Loeb, M.J., Kochansky, J.P., Gelman, D.B., Lusby, W.R., *et al.*, **1997**. Identification and characterization of an ecdysiotropic peptide from brain extracts of the gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol. 34*, 175–189.

Walker, V.K., Schreiber, M., Purvis, C., George, J., Wyatt, G.R., *et al.*, **1991**. Yolk polypeptide gene expression in cultured *Drosophila* cells. *In Vitro Cell. Devel. Biol. A 27*, 121–127.

Walker, V.K., Watson, K.L., Holden, J.J.A., Steel, C.G.H., **1987**. Vitellogenesis and fertility in *Drosophila* females with low ecdysteroid titres; the $l(3)3^{DTS}$ mutation. *J. Insect Physiol. 33*, 137–142.

Wang, S.F., Ayer, S., Segraves, W.A., Williams, D.R., Raikhel, A.S., **2000a**. Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol. Cell. Biol. 20*, 3870–3879.

Wang, S.F., Li, C., Sun, G.Q., Zhu, J.S., Raikhel, A.S., **2002**. Differential expression and regulation by

20-hydroxyecdysone of mosquito ecdysteroid receptor isoforms A and B. *Mol. Cell. Endocrinol. 196*, 29–42.

Wang, S.F., Li, C., Zhu, J.S., Miura, K., Miksicek, R.J., *et al.*, **2000b**. Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. *Devel. Biol. 218*, 99–113.

Wang, S.F., Miura, K., Miksicek, R.J., Segraves, W.A., Raikhel, A.S., **1998**. DNA binding and transactivation characteristics of the mosquito ecdysone receptor-*Ultraspiracle* complex. *J. Biol. Chem. 273*, 27531–27540.

Wang, S.F., Zhu, J., Martin, D., Raikhel, A.S., **2004**. Regulation of yolk protein genes by ecdysone. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress of Vitellogenesis. Science Publishers, Enfield, USA/ Plymouth, UK, pp. 69–94.

Wang, X.Y., Cole, K., Law, J., **1989**. The nucleotide sequence of a microvitellogenin encoding gene from the tobacco hornworm, *Manduca sexta*. *Gene 80*, 259–268.

Wang, Z., Davey, K.G., **1993**. The role of juvenile hormone in vitellogenin production in *Rhodnius prolixus*. *J. Insect Physiol. 39*, 471–476.

Warren, J.T., Bachmann, J.S., Dai, J.D., Gilbert, L.I., **1996**. Differential incorporation of cholesterol and cholesterol derivatives into ecdysteroids by the larval ring glands and adult ovaries of *Drosophila melanogaster*: a putative explanation for the l(3)ecd1 mutation. *Insect Biochem. Mol. Biol. 26*, 931–943.

Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 99*, 11043–11048.

Watkins, R.E., Wisely, G.B., Moore, L.B., Collins, J.L., Lambert, M.H., *et al.*, **2001**. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science 292*, 2329–2333.

Weaver, R.J., Edwards, J.P., **1990**. The role of the corpora allata and associated nerves in the regulation of ovarian cycles in the oviparous cockroach *Periplaneta americana*. *J. Insect Physiol. 36*, 51–59.

Weaver, R.J., Strambi, A., Strambi, C., **1984**. The significance of free ecdysteroids in the haemolymph of adult cockroaches. *J. Insect Physiol. 30*, 705–711.

Webb, T.J., Ellams, K.M., Major, M., Hurd, H., **1997**. The interplay between patency, microsomal Na(+)/ K(+) ATPase activity and juvenile hormone, in *Tenebrio molitor* parasitized by *Hymenolepsis diminuta*. *J. Insect Physiol. 43*, 337–343.

Webb, T.J., Hurd, H., **1995**. Microsomal juvenile hormone binding proteins in the follicle cells of *Tenebrio molitor. Insect Biochem. Mol. Biol. 25*, 631–637.

Webb, T.J., Shu, S.Q., Ramaswamy, S.B., Dorn, S., **1999**. Influence of juvenile hormone and mating on oogenesis and oviposition in the codling moth, *Cydia pomonella*. *Arch. Insect Biochem. Physiol. 41*, 186–200.

White, K.P., Hurban, P., Watanabe, T., Hogness, D.S., **1997**. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science 276*, 114–117.

Whiting, P., Sparks, S., Dinan, L., **1997**. Endogenous ecdysteroid levels and rates of ecdysone acylation by intact ovaries in vitro in relation to ovarian development in adult female crickets, *Acheta domesticus*. *Arch. Insect Biochem. Physiol. 35*, 279–299.

Wilson, T.G., Ashok, M., **1998**. Insecticide resistance resulting from an absence of target-site gene product. *Proc. Natl Acad. Sci. USA 95*, 14040–14044.

Wojchowski, D.M., Parsons, P., Nordin, J.H., Kunkel, J.G., **1986**. Processing of pro-vitellogenin in insect fat body: a role for high mannose oligosaccharides. *Devel. Biol. 116*, 422–430.

Wolfner, M.F., **1997**. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem. Mol. Biol. 27*, 179–192.

Woodhead, A.P., Asano, W.Y., Stay, B., **1993**. Allatostatins in the hemolymph of *Diploptera punctata* and their effect *in vivo*. *J. Insect Physiol. 39*, 1001–1005.

Woodhead, A.P., Stay, B., Seidel, S.L., Khan, M.A., Tobe, S.S., **1989**. Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA 86*, 5977–6001.

Woodhead, A.P., Thompson, M.E., Chan, K.K., Stay, B., **2003**. Allatostatin in ovaries, oviducts, and young embryos in the cockroach *Diploptera punctata*. *J. Insect Physiol. 49*, 1103–1114.

Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N., *et al.*, **2003**. Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron 39*, 147–161.

Wyatt, G.R., **1991**. Gene regulation in insect reproduction. *Invertebr. Reprod. Devel. 20*, 1–35.

Wyatt, G.R., **1997**. Juvenile hormone in insect reproduction – a paradox? *Eur. J. Entomol. 94*, 323–333.

Wyatt, G.R., Braun, R.P., Zhang, J., **1996**. Priming effect in gene activation by juvenile hormone in locust fat body. *Arch. Insect Biochem. Physiol. 32*, 633–640.

Wyatt, G.R., Cook, K.E., Firko, H., Dhadialla, T.S., **1987**. Juvenile hormone action on locust fat body. *Insect Biochem. 17*, 1071–1073.

Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.

Wyatt, G.R., Locke, J., Bradfield, J.Y., **1984**. The vitellogenin genes for *Locusta migratoria* and other insects. In: Engles, W. (Ed.), Advances in Invertebrate Reproduction, vol. 3. Elsevier, Amsterdam, pp. 73–80.

Xu, J., Qiu, Y., DeMayo, F.J., Tsai, S.Y., Tsai, M.J., *et al.*, **1998**. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science 279*, 1922–1925.

Xu, Y., Fang, F., Chu, Y., Jones, D., Jones, G., **2002**. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle.

Eur. J. Biochem. 269, 6026–6036. (Erratum in: *Eur. J. Biochem*. 2003; *270*, *565*.)

Yamahama, Y., Yamamoto, Y., Watabe, S., Takahashi, S.Y., **2004**. Yolk protein degradation during insect embryogenesis. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 315–354.

Yamamoto, K., Chadarevian, A., Pellegrini, M., **1988**. Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. *Science 239*, 916–919.

Yamamoto, Y., Takahashi, S.Y., **1993**. Cysteine proteinase from *Bombyx* eggs: role in programmed degradation of yolk proteins during embryogenesis. *Comp. Biochem. Physiol. B 106*, 35–45.

Yamamoto, Y., Yamahama, Y., Katou, K., Watabe, S., Takahashi, S.Y., **2000**. *Bombyx* acid cysteine protease (BCP): hormonal regulation of biosynthesis and accumulation in the ovary. *J. Insect Physiol. 46*, 783–791.

Yamashita, O., Indrasith, L.S., **1988**. Metabolic fates of yolk proteins during embryogenesis in arthropods. *Devel. Growth Differ. 30*, 337–346.

Yan, Y., Kunert, C., Postlethwait, J., **1987**. Sequence homologies among the three yolk polypeptides (YP) genes in *Drosophila melanogaster. Nucl. Acids Res. 15*, 67–85.

Yao, T.P., Forman, B.M., Jiang, Z.Y., Cherbas, L., Chen, J.D., *et al*., **1993**. Functional ecdysone receptor is the product of ecr and ultraspiracle genes. *Nature 366*, 476–479.

Yao, T.P., Segraves, W.A., Oro, A.E., Mckeown, M., Evans, R.M., **1992**. *Drosophila ultraspiracle* modulates ecdysone receptor function via heterodimer formation. *Cell 71*, 63–72.

Yun, H., Kim, W., Kim, H., **1994**. Immunological analysis of lipophorin in the haemolymph, ovaries and testes of the fall webworm, *Hyphantria cunea* (Drury). *Arch. Insect Biochem. Physiol.* 27, 153–167.

Zdarek, J., Nachman, R.J., Denlinger, D.L., **2000**. Parturition hormone in the tsetse *Glossina morsitans*: activity in reproductive tissues from other species and response of tsetse to identified neuropeptides and other neuroactive compounds. *J. Insect Physiol. 46*, 213–219.

Zhai, Q.H., Postlethwait, J., Bodley, J., **1984**. Vitellogenin synthesis in the lady beetle *Coccinella septempunctata*. *Insect Biochem. 14*, 299–305.

Zhai, Q.-H., Zhang, J.-Z., Gong, H., **1987**. Regulation of vitellogenin synthesis by juvenile hormone analogue in *Coccinella septempunctata*. *Insect Biochem. 17*, 1059–1064.

Zhang, J., McCracken, A., Wyatt, G.R., **1993**. Properties and sequence of a female-specific, juvenile hormone-induced protein from locust hemolymph. *J. Biol. Chem. 268*, 3282–3288.

Zhang, J., Saleh, D.S., Wyatt, G.R., **1996**. Juvenile hormone regulation of an insect gene: a specific transcription factor and a DNA response element. *Mol. Cell. Endocrinol. 122*, 15–20.

Zhang, J., Wyatt, G.R., **1996**. Cloning and upstream sequence of a juvenile hormone regulated gene from the migratory locust. *Gene 175*, 193–197.

Zhou, S., Zhang, J., Hirai, M., Chinzei, Y., Kayser, H., *et al*., **2002**. A locust DNA-binding protein involved in gene regulation by juvenile hormone. *Mol. Cell. Endocrinol. 190*, 177–185.

Zhu, J.S., Chen, L., Raikhel, A.S., **2003a**. Posttranscriptional control of the competence factor betaFTZ-F1 by juvenile hormone in the mosquito *Aedes aegypti*. *Proc. Natl Acad. Sci. USA 100*, 13338–13343.

Zhu, J., Indrasith, L., Yamashita, O., **1986**. Characterization of vitellin, egg-specific protein and 30-kDa protein from *Bombyx* eggs, and their fates during oogenesis and embryogenesis. *Biochim. Biophys. Acta 882*, 427–436.

Zhu, J.S., Miura, K., Chen, L., Raikhel, A.S., **2000**. AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*. *EMBO J. 19*, 253–262.

Zhu, J.S., Miura, K., Chen, L., Raikhel, A.S., **2003b**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue *Ultraspiracle*. *Proc. Natl Acad. Sci. USA 100*, 544–549.

Zomer, A.W., Van der Burg, B., Jansen, G.A., Wanders, R.J., Poll-The, B.T., *et al*., **2000**. Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. *J. Lipid. Res. 41*, 1801–1807.

Zou, B., Stoffolono, J., Nordin, J., **1988**. Subunit composition of vitellin, and concentration profiles of vitellogenin, and vitellin in *Phormia regina* (Meig.) following a protein meal. *Comp. Biochem. Physiol. B 90*, 861–867.

3.10 Hormones Controlling Homeostasis in Insects

D A Schooley, University of Nevada, Reno, NV, USA
F M Horodyski, Ohio University, Athens, OH, USA
G M Coast, Birkbeck College, London, UK

3.10.1. Introduction

Insects, like other animals, face a variety of challenges to their survival in the varying conditions of their environment and food availability. Many changes in their physiological status must be controlled to achieve homeostasis. In this chapter the hormonal controllers of the supply of endogenous metabolic energy, as well as the hormonal control of fluid and electrolyte balance are discussed. A review on the same subject has appeared recently, but is significantly less detailed (Gäde, 2004).

Due to the wide variety of environmental niches inhabited by insects, the mechanisms for control of homeostasis may show considerable variation between genera, although basic underlying themes are conserved. Insects are an extremely ancient grouping of organisms, with short lifespans and frequently multiple generations per year. On completion of sequencing of the *Anopheles gambiae* genome, the genome and proteome of this dipteran insect were compared with that of the related dipteran *Drosophila melanogaster* (Zdobnov *et al*., 2002). One of the salient conclusions is that these two "closely related" dipterans show greater genetic divergence than do the genomes of the human and the pufferfish, although the estimated time of divergence between these two dipterans (~250 million years ago) is considerably more recent than that between the higher organisms (Zdobnov *et al*., 2002). This rapid rate of evolution is reflected in differences in the nature of main substrates used as metabolic fuels for flight, in considerable interspecific diversity in structure between hormones in certain families that regulate homeostasis, especially diuretic hormones, and also in differences in which family of diuretic hormones appears to dominate as the major controllers of fluid excretion between different genera. It has also become apparent that signal transduction pathways for a given family of homeostatic hormones may differ between genera.

It is interesting to compare and contrast the depth of our current knowledge in this area to that when the first edition of this series was published. At that time, the sequences of only two insect neuropeptides were known: proctolin and adipokinetic hormone (AKH) from *Locusta migratoria* (herein called Locmi-AKH-I). Technical advances in peptide isolation and analysis made in the early 1980s were responsible for an explosive growth in the isolation and identification of new insect neuropeptides. The

availability of synthetic peptide hormones then allowed detailed studies of their mechanism of physiological action. It has also become clear, as will be seen in this chapter, that insect peptide hormones, like their vertebrate homologs, frequently have more than one function. Well over 100 insect neuropeptides have been identified, belonging to a number of families. The majority of these were isolated on the basis of their myotropic activity, because of the ease and rapidity of bioassays for such effects. Owing to a huge literature, only those myotropic peptides are covered (certain forms of AKH and the myokinins) that are known to be important homeostatic regulators.

3.10.2. Hormonal Control of Energy Stores

It is difficult to discuss this section without considering comparative endocrinology. In vertebrates the storage of fats in adipose tissue and storage of glycogen in muscles and liver are increased by high levels of insulin. Mobilization of fats from adipocytes and glycogen from liver and muscles is triggered by high levels of the counter-regulatory hormone glucagon. Glucagon acts through the cyclic AMP signal transduction pathway to activate hormone sensitive lipase in adipocytes, to activate glycogen phosphorylase and inactivate glycogen synthase in muscle and liver, and to activate gluconeogenesis and inactivate glycolysis in liver and kidney. Insulin, by activating cAMP phosphodiesterase and phosphoprotein phosphatase I, has the opposite effects on these pathways.

In insects the adipokinetic-hypertrehalosemic hormones have the same role as glucagon in animals. However, a great deal of research has failed to reveal a hormone with a counter-regulatory action, although a very recent discovery by Clynen *et al.* (2003a) may shed light on this area.

3.10.2.1. Adipokinetic Hormone and Hypertrehalosemic Hormone

3.10.2.1.1. Biological role of AKH Peptides in the AKH family stimulate metabolism by mobilizing energy stores in the fat body. The actions mediated by the AKH peptides are critical to supply energy to tissues, such as the flight muscles, with substrates necessary to maintain long distance flight. Depending on the insect species, either lipids, carbohydrates, proline, or a combination thereof, are released from the fat body during times of metabolic need. For example, the energy substrate released into the hemolymph of cockroaches is trehalose, a disaccharide of glucose and the major hemolymph carbohydrate of insects (Friedman, 1985). When energy demands are high, locusts release predominantly diacylglycerols from the fat body, which are transported by lipophorins in the hemolymph (Chino and Gilbert, 1964, 1965; Soulages and Wells, 1994) (see **Chapter 4.6**), but also release carbohydrate under some conditions (Goldsworthy, 1969). Proline is released into the hemolymph to provide fuel for the contraction of flight muscles in the tsetse fly (*Glossina morsitans*; Bursell, 1963) and in coleopteran insects (De Kort *et al.*, 1973; Gäde and Auerswald, 2002). Peptides that primarily mobilize lipids in the species in which they were isolated are referred to as AKH (Mayer and Candy, 1969a), while peptides whose predominant role is to mobilize carbohydrates are known as hypertrehalosemic hormones (HrTH), since the increase in total carbohydrate is accounted for by an increase in trehalose. The adipokinetic and hypertrehalosemic functions of AKH peptides are similar to the metabolic responses induced by the vertebrate hormone, glucagon.

AKH peptides are synthesized by, and released from, the cells of the glandular lobe of the corpus cardiacum (CC) (Goldsworthy *et al.*, 1972a), an endocrine organ attached to the brain. The effect of the CC on metabolism was first described in *Periplaneta americana*, where injection of a CC extract into adult cockroaches caused an increased level of trehalose in the hemolymph and a decrease in fat body glycogen content (Steele, 1961). In the locusts *Schistocerca gregaria* and *L. migratoria*, injection of a CC extract into the hemolymph increases levels of diacylglycerol (Beenakkers, 1969; Mayer and Candy, 1969a), resembling the changes in lipid composition that occur during flight (Beenakkers, 1965; Mayer and Candy, 1967). This elevation of diacylglycerol reflects the oxidation of lipids as the primary fuel during prolonged flight in locusts (Weis-Fogh, 1952). The onset of flight is rapidly followed by the release of AKH into the hemolymph to mobilize energy substrates from the fat body (Cheeseman and Goldsworthy, 1979; Orchard and Lange, 1983a; Candy, 2002). The AKH concentration in the hemolymph during flight (Cheeseman and Goldsworthy, 1979; Candy, 2002) is similar to the concentration necessary to induce lipid release from the fat body *in vivo* (Goldsworthy *et al.*, 1986a). In addition to the adipokinetic effect in *L. migratoria*, injection of a CC extract also causes an increase in hemolymph carbohydrate levels in young male locusts whose fat body contains sufficient glycogen stores (Goldsworthy, 1969). In the moth *Manduca sexta*, AKH stimulates glycogen breakdown in larval insects and lipid mobilization

in adults (Siegert and Ziegler, 1983; Ziegler and Schulz, 1986; Ziegler *et al*., 1990).

The similarity of AKH and HrTH was demonstrated before their structures were known; a *P. americana* CC extract induced an adipokinetic response in *L. migratoria* and a *L. migratoria* CC extract induced a hyperglycemic response in *P. americana* (Goldsworthy *et al*., 1972a). The differences in the metabolic responses reflect the strategy of the insect in the nature of the fuel stored in the fat body. An adipokinetic hormone was first identified in *L. migratoria* (Stone *et al*., 1976). The structural similarity of the *L. migratoria* and *P. americana* peptides was confirmed when three groups isolated two octapeptides in the same year from *P. americana* based on myotropic assays (Baumann and Penzlin, 1984; Scarborough *et al*., 1984; Witten *et al*., 1984). However, Scarborough *et al*. (1984) also showed that the less abundant peptide from *P. americana*, Peram-CAH-II (**Figure 1**) has a very significant sequence identity to glucagon (4 of 8 residues are identical; 2 others are highly conserved), and that both peptides would elevate hemolymph carbohydrate in an assay where trehalose is hydrolyzed to glucose. Later Siegert and Mordue (1986a) isolated these factors as specific hypertrehalosemic agents from cockroach. Because of the functional relatedness of AKH peptides, heterologous bioassays, such as the lipid mobilization assay in *L. migratoria* and carbohydrate mobilization assay in *P. americana*, are often used for the isolation of metabolic neuropeptides in a large number of insect species (Gäde, 1980).

The biochemical effect of a CC extract in the *P. americana* fat body is the activation of glycogen phosphorylase (Steele, 1963). Similarly in *L. migratoria*, glycogen phosphorylase is activated by a CC extract (Van Marrewijk *et al*., 1980) or by synthetic AKH (Gäde, 1981). The effect of AKH on lipid mobilization in *M. sexta* is mediated by the activation of triacylglycerol lipase (TAG lipase) which converts TAG into diacylglycerol, which is subsequently released into the hemolymph (Arrese *et al*., 1996). Injection of Locmi-AKH I into *S. gregaria* resulted in a twofold increase of TAG lipase in the fat body (Ogoyi *et al*., 1998).

As is common among neuropeptides, AKH possesses numerous biological activities in addition to its well-known metabolic functions. Some of these actions include the acceleration of heart rate in *P. americana* (Baumann and Gersch, 1982; Scarborough *et al*., 1984) and *Blaberus discoidalis* (Keeley *et al*., 1991), the stimulation of myotropic contractions in *P. americana* (O'Shea *et al*., 1984; Witten *et al*., 1984), the inhibition of protein synthesis

Locmi-AKH-I	-	p	Q	L	N	F	T	P	N	W	G	T	a	-	-
Rommi-CC	-	p	Q	V	N	F	T	P	N	W	G	T	a	-	-
Phymo-AKH	-	p	Q	L	N	F	T	P	N	W	G	S	a	-	-
Psein-AKH	-	p	Q	V	N	F	T	P	G	W	a	-	-	-	-
Libau-AKH	-	p	Q	V	N	F	T	P	S	W	a	-	-	-	-
Emppe-AKH	-	p	Q	V	N	F	T	P	N	W	a	-	-	-	-
Micvi-CC	-	p	Q	I	N	F	T	P	N	W	a	-	-	-	-
Polae-HrTH	-	p	Q	I	T	F	T	P	N	W	a	-	-	-	-
Phymo-AKH-III	-	p	Q	I	N	F	T	P	W	W	a	-	-	-	-
Locmi-AKH-III	-	p	Q	L	N	F	T	P	W	W	a	-	-	-	-
Erisi-AKH	-	p	Q	L	N	F	T	P	S	W	a	-	-	-	-
Pyrap-AKH	-	p	Q	L	N	F	T	P	N	W	a	-	-	-	-
Grybi-AKH	-	p	Q	V	N	F	S	T	G	W	a	-	-	-	-
Onyay-CC	-	p	Q	Y	N	F	S	T	G	W	a	-	-	-	-
Schgr-AKH-II	-	p	Q	L	N	F	S	T	G	W	a	-	-	-	-
Tenar-HrTH	-	p	Q	L	N	F	S	T	G	W	G	G	a	-	-
Locmi-AKH-II	-	p	Q	L	N	F	S	A	G	W	a	-	-	-	-
Panbo-RPCH	-	p	Q	L	N	F	S	P	G	W	a	-	-	-	-
Scade-CC-I	-	p	Q	F	N	Y	S	P	D	W	a	-	-	-	-
Scade-CC-II	-	p	Q	F	N	Y	S	P	V	W	a	-	-	-	-
Melme-CC	-	p	Q	L	N	Y	S	P	D	W	a	-	-	-	-
Placa-HrTH-I†,II	-	p	Q	V	N	F	S	P	S	W	G	N	a	-	-
Anaim-AKH	-	p	Q	V	N	F	S	P	S	W	a	-	-	-	-
Bladi-HrTH	-	p	Q	V	N	F	S	P	G	W	G	T	a	-	-
Peram-CAH-I	-	p	Q	V	N	F	S	P	N	W	a	-	-	-	-
Declu-CC	-	p	Q	L	N	F	S	P	N	W	G	N	a	-	-
Tenmo-HrTH	-	p	Q	L	N	F	S	P	N	W	a	-	-	-	-
Carmo-HrTH-I*	-	p	Q	L	T	F	T	P	N	W	G	T	a	-	-
Carmo-HrTH-II	-	p	Q	L	T	F	T	P	N	W	G	T	a	-	-
Phyle-CC	-	p	Q	L	T	F	T	P	N	W	G	S	a	-	-
Peram-CAH-II	-	p	Q	L	T	F	T	P	N	W	a	-	-	-	-
Tabat-HoTH	-	p	Q	L	T	F	T	P	G	W	G	Y	a	-	-
Tabat-AKH	-	p	Q	L	T	F	T	P	G	W	a	-	-	-	-
Phote-HrTH	-	p	Q	L	T	F	S	P	D	W	a	-	-	-	-
Vanca-AKH	-	p	Q	L	T	F	T	S	S	W	G	G	K	-	-
Manse-AKH	-	p	Q	L	T	F	T	S	S	W	G	a	-	-	-
Helze-HrTH	-	p	Q	L	T	F	S	S	G	W	G	N	a	-	-
Locmi-HrTH	-	p	Q	V	T	F	S	R	D	W	S	P	a	-	-
Human-Glucagon	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D

Figure 1 Alignment of sequences of the known AKH/HrTH from the Insecta, from a sample of 39 peptides with a unique primary structure. Peptide sequences were compiled largely from Gäde *et al*. (1997) with the inclusion of additional unique sequences found since 1997 (Gäde and Kellner, 1999; Siegert, 1999; Kodrík *et al*., 2000; Köllisch *et al*., 2000; Siegert *et al*., 2000; Lorenz *et al*., 2001; Gäde *et al*., 2003). The sequence of one peptide from *Carausius morosus* (Carmo-HrTHI*) contains a hexose modification on the Trp8 residue (Gäde *et al*., 1992), and an additional peptide from *Platypleura capensis* (Placa-HrTHI†) contains a modification that has not been characterized (Gäde and Janssens, 1994). Species names are as given in the Swiss-Prot database as recommended previously (Coast *et al*., 2002). Those not defined in the text are: Anaim, *Anax imperator*; Bladi, *Blaberus discoidalis*; Declu, *Decapotoma lunata*; Emppe, *Empusa pennata*; Erysi, *Erythemis simplicicollis*; Grybi, *Gryllus bimaculatus*; Libau, *Libellula auripennis*; Micvi, *Microhodotermes viator*; Onyay, *Onymacris aygulus*; Phote, *Phormia terranova*; Phymo, *Phymateus morbillosus*; Phyle, *Phymateus leprosus*; Polae, *Polyphaga aegyptiaca*; Psein, *Pseudagrion inconspicuum*; Pyrap, *Pyrrhocoris apterus*; Rommi, *Romalea microptera*; Scade, *Scarabaeus deludens*; Tenar, *Tenthredo arcuata*; Vanca, *Vanessa cardui*. The 15 N-terminal residues of glucagon are shown (16–29 deleted); Gln3 is conserved in all AKH and the motif Thr-Phe-Thr from glucagon is 100% conserved in nine sequences, and Ser8 and Asp9 of glucagon are conserved in certain AKH sequences and are conservative substitutions in others. Tyr10 is a conservative substitution for the completely conserved Trp.

(Carlisle and Loughton, 1979; Cusinato *et al.*, 1991) and RNA synthesis (Kodrik and Goldsworthy, 1995), the inhibition of lipid synthesis (Gokuldas *et al.*, 1988), the stimulation of heme synthesis in *B. discoidalis* (Keeley *et al.*, 1991), a neuromodulatory effect in *P. americana* (Wicher *et al.*, 1994), the stimulation of locomotory activity in *M. sexta* (Milde *et al.*, 1995) and *Pyrrhoccoris apterus* (Socha *et al.*, 1999; Kodrík *et al.*, 2000), and a lipogenic effect in which lipids are translocated from the hemolymph to the fat body in *P. americana* (Oguri and Steele, 2003).

Although AKH is widely viewed as an energy mobilization hormone synthesized by and released from the CC, effects on the nervous system have been documented. This was first shown by demonstrating that neurohormone D, an HrTH neuropeptide in *P. americana*, elicits an increase in the spontaneous spike frequency in DUM neurons of the cockroach terminal ganglion (Wicher *et al.*, 1994). This action of neurohormone D on Ca^{2+} currents is through the upregulation of the mid/low voltage activated (M-LVA) Ca^{2+} current by the actions of cAMP and protein kinase A (PKA), suggesting a role for phosphorylation of the channel protein by PKA (Wicher, 2001). The contribution of protein kinase C in the regulation of Ca^{2+} influx through voltage activated Ca^{2+} channels was also documented.

Injection of Manse-AKH into the mesothoracic neuropil increases motor activity in *M. sexta*, similar to the effects of octopamine (Milde *et al.*, 1995). A stimulation of locomotory activity in the firebug, *P. apterus* was observed after injection of Locmi-AKH I or the endogenous peptide, Pyrap-AKH (Socha *et al.*, 1999; Kodrík *et al.*, 2000). The mechanism for the peptide's action on the nervous system was not determined, but dispersal activity is associated with starvation and the need to mobilize energy reserves from the fat body (Socha *et al.*, 1998). Thus, in *P. apterus*, the neuromodulatory function of AKH integrates behavior with the metabolic needs of the insect (Socha *et al.*, 1999). Some investigators reported the presence of Locmi-AKH I in the brain (Moshitzky *et al.*, 1978; Bray *et al.*, 1993). Using mass spectrometric (MS) analysis, none of the Locmi-AKHs were detected in the pars intercerebralis (PI), but a material with the M_r of Locmi-HrTH (Siegert, 1999) was present in the PI and in the CC (Clynen *et al.*, 2001).

The interaction of AKH with both the humoral and cellular immune system was demonstrated in *L. migratoria* (Goldsworthy *et al.*, 2002, 2003a). One component of the insect defense response to wounding and infection is the activation of phenoloxidase via a serine proteinase cascade triggered by the recognition of bacterial and fungal cell wall components resulting in the production of toxic quinones (Gillespie *et al.*, 1997). Injection of Locmi-AKH I or II enhanced the activation of phenoloxidase in response to a challenge with laminarin (a β-1,3-glucan) and activated phenoloxidase in the presence of bacterial lipopolysaccharide (LPS) (Goldsworthy *et al.*, 2002). The different potencies of Locmi-AKH I and II in phenoloxidase activation are consistent with their relative potencies in the locust lipid mobilization assay (Goldsworthy *et al.*, 1986b), suggesting a direct link between the immune and lipid mobilization responses (Goldsworthy *et al.*, 2002). The enhancement of phenoloxidase activation by AKH is age dependent, and restricted to the mature adult stage (Mullen and Goldsworthy, 2003). This time dependence correlates with the lipid mobilization response to AKH (Mwangi and Goldsworthy, 1977a) and to the concentration of apolipophorin III (ApoLp-III) in the hemolymph (Mwangi and Goldsworthy, 1977b; Mullen and Goldsworthy, 2003). In addition to the role of ApoLp-III in lipid metabolism, the lipid associated form of ApoLp-III stimulates antimicrobial activity in the hemolymph of *Galleria mellonella* (Wiesner *et al.*, 1997; Detloff *et al.*, 2001). These data suggest that AKH may be exerting its effect on phenoloxidase activation in part through its effects on ApoLp-III metabolism (Mullen and Goldsworthy, 2003). An effect on phenoloxidase activation by LPS in the absence of AKH was observed in starved locusts, presumably because of the effects of starvation on lipid mobilization (Goldsworthy *et al.*, 2003a).

An important component of the cellular immune response is the formation of nodules by hemocyte aggregation to engulf or entrap foreign bodies (Lavine and Strand, 2002). AKH increases nodule formation when coinjected with LPS (Goldsworthy *et al.*, 2003a). Unlike the effects of AKH on phenoloxidase activation, which is specific to mature locusts (Goldsworthy *et al.*, 2002), its effects on nodule formation are apparent from the fifth instar nymphal stage through the mature adult stage. AKH may be exerting its effect on the humoral immune system (activation of prophenoloxidase) and the cellular immune system (nodule formation) via separate mechanisms, since they exhibit differing sensitivities to pharmacological agents and show different correlations to the nutritional state and lipid composition (Goldsworthy *et al.*, 2003b).

3.10.2.1.2. Structural features of AKH Evidence for multiple AKHs within a species was first found in locusts (Carlsen *et al.*, 1979), and has been extended to include many insect species (Gäde

et al., 1997), with three biologically active peptides present in some insects (Oudejans *et al.*, 1991; Siegert *et al.*, 2000; Köllisch *et al.*, 2003). Each AKH is derived from a unique mRNA (Schulz-Aellen *et al.*, 1989; Noyes and Schaffer, 1990; Fischer-Lougheed *et al.*, 1993; Bogerd *et al.*, 1995). In *L. migratoria*, a fourth member of the AKH family (Locmi-HrTH) was characterized based on its hypertrehalosemic activity in *P. americana*, but no function has yet been identified in *L. migratoria* (Siegert, 1999). The three Locmi-AKHs differ in amount, with Locmi-AKH I being the most abundant and Locmi-AKH III the least abundant (Oudejans *et al.*, 1993; Clynen *et al.*, 2001). One rationale for multiplicity of peptides is their varying potencies in different biological assays, which may enable the fine tuning of an energy-demanding response such as long-distance flight (Vroemen *et al.*, 1998a). Locmi-AKH I and II differ in potency. Locmi-AKH I is more effective than Locmi-AKH II in the lipid mobilization assay, the ability to alter circulating lipoproteins, and the ability to activate glycogen phosphorylase, while Locmi-AKH II is more effective in increasing cAMP levels in the fat body (Orchard and Lange, 1983a; Goldsworthy *et al.*, 1986b). These data suggest that Locmi-AKH I is the predominant hyperlipemic hormone, and Locmi-AKH II is largely responsible for carbohydrate mobilization. Locusts employ carbohydrate as an energy source for the initial stages of flight and lipid for more prolonged flight (Weis-Fogh, 1952; Mayer and Candy, 1969b; Jutsum and Goldsworthy, 1976), suggesting that Locmi-AKH II is most important at the onset of flight and Locmi-AKH I assumes a major role in prolonged flight activity (Vroemen *et al.*, 1997). Locmi-AKH III is present at low levels and seems to have a high turnover, suggesting it may be a modulatory entity that provides the locust with energy when not flying.

The peptides from the AKH family are among the most thoroughly characterized insect peptides with respect to their structural diversity (Gäde *et al.*, 1997). At least 38 distinct peptides have been structurally characterized from at least 90 species that include representatives from most insect orders (**Figure 1**). A distinct sequence of an additional family member, the red pigment-concentrating hormone (Panbo-RPCH), long thought to be unique to crustaceans (Fernlund and Josefsson, 1972), has recently been found in insects (Gäde *et al.*, 2003). RPCH has been shown to induce hyperlipemia in locusts (Herman *et al.*, 1977; Mordue and Stone, 1977). In contrast to the insect peptides whose roles include the mobilization of metabolic stores, the role of RPCH in crustaceans is to induce aggregation of pigment granules in chromatophores (Rao, 2001). Thus, these orthologous peptides found in insects and crustacea do not retain a common function. Although AKH-like immunoreactivity exists in invertebrate phyla other than the Arthropoda (Schooneveld *et al.*, 1987), confirmation of the presence of related peptides is lacking. The most compelling evidence thus far for an AKH related peptide in a nonarthropod is the detection of Locmi-AKH I-like immunoreactivity in the nematode *Panagrellus redivivus* and the induction by a peptide fraction of an adipokinetic response in *S. gregaria* and a hypertrehalosemic response in *P. americana* (Davenport *et al.*, 1991).

All AKH precursor proteins share a common organization; an N-terminal signal peptide followed by AKH and the AKH precursor-related peptide (APRP) (Martínez-Pérez *et al.*, 2002). The organization of the RPCH precursor protein (Linck *et al.*, 1993) is similar to that of AKH/HrTH peptides, supporting the hypothesis that they are derived from a common ancestor (Martínez-Pérez *et al.*, 2002). Close examination of the precursor structures and gene organization of AKH/RPCH peptides with that of the APGWamide peptide family of mollusks revealed similarities including a common overall architecture of the precursor protein, a common predicted secondary structure, and similar position of introns, suggesting that they could have arisen from a common ancestral gene or from recombination of an ancestral AKH/RPCH-like gene with an APGWamide-like gene (Martínez-Pérez *et al.*, 2002).

While significant structural variability was observed when comparing AKH peptides sequenced in different insect species, several common characteristics are defining features of this family (Gäde *et al.*, 1997) (**Figure 1**). The sizes of AKHs range from 8 to 11 amino acids. All AKHs are blocked at their N-terminus with a pyroglutamate residue, and all are amidated at their C-terminus with one exception (Köllisch *et al.*, 2000). An aromatic amino acid is present in each AKH at position 4, and Trp is conserved at position 8. The most critical residues for bioactivity are probably the most prevalent (pGlu1, Phe4, Trp8) and might be involved in binding to the receptor, stability of the peptide, or formation of an intrapeptide interaction stabilizing the conformation needed to achieve optimal activity (Hayes and Keeley, 1990; Gäde, 1992). For example, the conserved Trp8 was proposed to form a hydrogen bond with Ser5 or Phe4 (Hayes and Keeley, 1990), and the hydrophobic nature and relative spacing of residues 2, 4, and 8 may be important in determining receptor binding.

The biological activity of neuropeptides is mediated by their interaction with a membrane bound receptor on the target cell; most then alter the concentration of second messengers that result in biochemical changes and ultimately lead to a physiological response. Small peptides, such as AKH, are flexible structures that adopt many conformations in solution, and their potency is related to their structural conformation upon interaction with the receptor (Nachman *et al.*, 1993). The identification of AKH receptors and their expression allows the direct assay of peptide (or analog) binding to the receptor and subsequent response of the target cell to identify the critical structural features of the peptides that are required for receptor binding and activation. However, considerable information on the importance of individual amino acids or parts of the peptide for binding to the receptor has been acquired using bioassays in structure–activity studies in several insect species, but these results are also influenced by such factors as the relative stability of the peptides *in vivo*. The overall conclusion that can be drawn from structure–activity studies is that the endogenous peptides are usually the most potent in the respective bioassays, and coevolution of the peptide and the receptor took place to achieve optimal binding (Hayes and Keeley, 1990; Fox and Reynolds, 1991a).

Stone *et al.* (1978) predicted that many members of the AKH/RPCH family have the potential to adopt a β-turn conformation. While AKH peptides do not form an ordered structure in aqueous solution, the presence of sodium dodecylsulfate (SDS) causes a change in the circular dichroism (CD) spectra that is characteristic of a β-turn (Goldsworthy and Wheeler, 1989; Cusinato *et al.*, 1998). However, the CD spectra of Locmi-AKH II and Manse-AKH, peptides that lack a proline residue in position 6, are not affected by the presence of SDS and lack the capacity to form a β-turn. The capacity of some AKH peptides to form a β-turn between residues 4 and 8 was also confirmed using nuclear magnetic resonance (NMR) spectroscopy (Zubrzycki and Gäde, 1994; Zubrzycki and Gäde, 1999; Nair *et al.*, 2001). It was suggested that the activity of AKH in the *L. migratoria* lipid mobilization assay is correlated with the ability to form a β-structure in SDS micelles, which in turn quantitatively affects its interactions with the receptor (Goldsworthy *et al.*, 1997; Cusinato *et al.*, 1998). Locmi-AKH II and Manse-AKH, which lack the ability to form a β-structure, exhibit low potency in the *L. migratoria* lipid mobilization assay (Cusinato *et al.*, 1998). Analogs with substitutions in residues 6–8 of AKHs exhibit reduced potency since the substitutions apparently hinder the formation of a β-turn (Lee *et al.*, 1996). However, the capacity to form a β-turn does not correlate with potency when assayed for inhibition of acetate uptake into fat body *in vitro*, since *Acheta domesticus* AKH and Locmi-AKH II are equally active using this assay (Cusinato *et al.*, 1998). This pleiotropic response may reflect the heterogeneity of receptors, which exhibit differing structural preferences for their ligand. Structural analysis of AKH peptides from *Melolontha melolontha* (Melme-CC), *Tenebrio molitor* (Tenmo-HrTH), and *Decapotoma lunata* (Declu-CC) using NMR supports the hypothesis that the β-turn is important for receptor binding in the *L. migratoria* lipid mobilization assay (Nair *et al.*, 2001). The Asn residue at position 7 (Asn7), and the Phe residue at position 4 (Phe4) are essential for a potent response, and it was shown that the Asn7 projects outward from the β-turn, and the orientation of Asn7 and the tightness of the β-turn are influenced by the aromatic residue at position 4. Data on the biological activity of a large number of natural AKHs and synthetic analogs in the *L. migratoria* lipid mobilization assay and acetate uptake assay were compiled to attempt to construct quantitative structure–activity relationships (QSAR) (Lee *et al.*, 2000). Little new understanding was gained in this study since the peptides used were not designed with QSAR in mind, but this approach might be used to understand the requirements of peptide–receptor interactions and to predict sequences that possess improved biological potency as used successfully with substance P (Norinder *et al.*, 1997).

Structure–activity studies were also carried out using the activation of glycogen phosphorylase in *M. sexta* fat body to assay several naturally occurring AKH peptides and synthetic analogs (Ziegler *et al.*, 1991, 1998). Manse-AKH lacks the ability to form a β-turn (Goldsworthy and Wheeler, 1989), and among the peptides tested, the most inactive were the ones with the highest probability of forming a β-turn (Ziegler *et al.*, 1991, 1998). The substitution of Ser6 of Manse-AKH with Pro, which favors the formation of a β-turn, drastically reduced its abilities to activate glycogen phosphorylase and to bind to the receptor in a fat body membrane preparation. This indicates that the *M. sexta* AKH receptor does not bind peptides that contain a β-turn (Ziegler *et al.*, 1998).

Removal of pGlu1 from Locmi-AKH I abolishes activity (Gäde, 1990), but replacement of pGlu1 by an unblocked amino acid partially restored lipid mobilization activity *in vivo* to varying degrees depending on the identity of the substituted amino acid, and activity was further enhanced by N-terminal blockage (Lee *et al.*, 1997). Replacement of pGlu1 in

Manse-AKH, however, did not restore potency in a bioassay measuring activity of glycogen phosphorylase unless the N-terminus was blocked (Herman *et al.*, 1977; Ziegler *et al.*, 1998). These data imply that pGlu1 is not absolutely essential for activity, and that one of the roles of the blocked N-terminus might be to impart increased stability to the peptide by preventing aminopeptidase attack (Lee *et al.*, 1997; Ziegler *et al.*, 1998).

3.10.2.1.3. AKH synthesis and release Like most neuropeptides, AKH is derived by processing of a larger precursor protein (Hekimi and O'Shea, 1987). The steps involved in AKH synthesis have been most thoroughly characterized in *S. gregaria* (O'Shea and Rayne, 1992), and subsequent studies in other insects have shown that the basic features of AKH biosynthesis are conserved. AKH precursor proteins share a similar organization, an N-terminal signal peptide, followed by AKH and the AKH precursor related peptide (APRP) (O'Shea and Rayne, 1992), as first shown in *S. gregaria* (Schulz-Aellen *et al.*, 1989) and *M. sexta* (Bradfield and Keeley, 1989). The locust AKH precursor exists as a dimer formed by oxidation of the Cys residues present in the APRP. This occurs prior to proteolytic processing in the trans-Golgi at basic amino acid residues and C-terminal amidation (Rayne and O'Shea, 1994). The dimeric structure of the precursor might confer a conformation that facilitates or allows the correct processing of the precursor. Since multiple AKHs (Diederen *et al.*, 1987; Hekimi *et al.*, 1991) and their mRNAs (Bogerd *et al.*, 1995) are present in the same glandular cells, random formation of dimeric precursors gives rise to both homodimers and heterodimers (Hekimi *et al.*, 1991; Huybrechts *et al.*, 2002). Further processing of *L. migratoria* APRPs from the AKH I and AKH II precursors takes place to yield additional peptides designated the adipokinetic hormone joining peptides (AKH-JP I and AKH-JP II), but their release was not demonstrated (Baggerman *et al.*, 2002). Biological roles for the APRPs or the AKH-JPs have not been found (Oudejans *et al.*, 1991; Hatle and Spring, 1999; Baggerman *et al.*, 2002). Relative peptide levels in the glandular cells are controlled by multiple mechanisms. The 4.5:1 ratio of AKH I to AKH II present in the glandular cells (Hekimi *et al.*, 1991) is regulated by a 1.7:1 ratio of AKH I to AKH II mRNA and the more efficient translation of AKH I mRNA (Fischer-Lougheed *et al.*, 1993).

Antisera specific to AKH I or AKH II have demonstrated their colocalization in the same secretory granules in the glandular cells of the CC (Diederen *et al.*, 1987). Since an AKH III-specific antiserum is not available, the colocalization of each APRP implies that AKH III is also colocalized with AKH I and II (Harthoorn *et al.*, 1999). The amount of AKHs increase dramatically in the CC during development (Siegert and Mordue, 1986b), due to their continued synthesis (Fischer-Lougheed *et al.*, 1993; Oudejans *et al.*, 1993) and to the increase in the number of cells in the glandular lobe of the CC (Kirschenbaum and O'Shea, 1993). The continued synthesis of AKH generates a large pool of secretory granules in the glandular cells (Diederen *et al.*, 1992), and newly synthesized AKH is preferentially released over AKHs stored in older secretory granules (Sharp-Baker *et al.*, 1995), which constitutes a pool of peptides that cannot be released (Sharp-Baker *et al.*, 1996; Harthoorn *et al.*, 2002). Therefore, only a small percentage of the total AKH present in the glandular cells is released during flight (Cheeseman *et al.*, 1976) or upon stimulation by CCAP (Harthoorn *et al.*, 2002). The AKHs are released in the same proportion as their levels in the CC (Harthoorn *et al.*, 1999), and the continuous synthesis of AKH is required for the secretion of peptides from the glandular cells in response to metabolic need (Harthoorn *et al.*, 2002).

In *S. gregaria* and *L. migratoria*, the hemolymph trehalose levels during flight are about 50% that of preflight values (Mayer and Candy, 1969b; Jutsum and Goldsworthy, 1976), and injection of a high concentration of trehalose prevents AKH release assayed by quantifying lipid mobilizing activity in the hemolymph (Cheeseman *et al.*, 1976). Trehalose and glucose exert a direct action on the glandular cells of the CC, since high trehalose concentrations decreased both spontaneous AKH release and AKH release induced by the neuropeptide Locmi-TK I, 3-isobutyl-l-methylxanthine (IBMX), or high potassium concentrations *in vitro* (Passier *et al.*, 1997). It was suggested that trehalose exerts this effect, in part, after its conversion to glucose. The decrease in trehalose concentration in response to the energy demands of flight relieves this inhibition, and is one of the factors that contributes to AKH release that is observed during flight (Cheeseman and Goldsworthy, 1979; Orchard and Lange, 1983b). It was also suggested that high levels of diacylglycerol resulting from mobilization of lipid energy stores may exert negative feedback on AKH release (Cheeseman and Goldsworthy, 1979).

The neural and hormonal factors that modulate AKH release have been most thoroughly characterized in *L. migratoria* (Vullings *et al.*, 1999). Implanted CC do not show the ultrastructural signs of enhanced AKH release during flight, suggesting that AKH secretion is under neural control by cells

that make direct contact with the glandular lobe cells (Rademakers, 1977a). Neuroanatomical studies defined the secretomotor neurons in the lateral part of the protocerebrum that project through the nervi corporis cardiaci II (NCC II) to innervate the glandular lobe of the CC (GCC) and make synaptic contact with the AKH cells, and the axon terminals of the GCC are derived solely from the secretomotor cells (Rademakers, 1977b; Konings *et al.*, 1989). Electrical stimulation of the NCC II resulted in the release of AKH I and II from the GCC and was accompanied by an increase in cAMP levels in cells of the GCC (Orchard and Loughton, 1981; Orchard and Lange, 1983a). While stimulation of the NCC I had no direct effect on AKH release, an enhancement of the NCC II-stimulated release of AKH from the CC was observed (Orchard and Loughton, 1981). Hormonally mediated lipid mobilization during flight is abolished in locusts in which the NCC I and NCC II were severed (Goldsworthy *et al.*, 1972b). Together, these data indicate that the secretomotor neurons are involved in the control of AKH release, and compounds present in the NCC I modulate AKH secretion.

An antiserum raised against locustatachykinin I (Locmi-TK I), a member of a family of structurally related neuropeptides (Schoofs *et al.*, 1993), stained a subset of secretomotor neurons projecting to the GCC and made synaptoid contacts with AKH-immunoreactive glandular cells, suggesting a role for a Locmi-TK I-like peptide in AKH release (Nässel *et al.*, 1995). Indeed, Locmi-TK I and II induced AKH release from the CC *in vitro* and increased cAMP levels in the GCC, but an effect on AKH release required concentrations in the 50–200 μM range (Nässel *et al.*, 1995, 1999). Furthermore, four Locmi-TK isoforms were identified in *L. migratoria* CC extracts (Nässel *et al.*, 1999). The anatomical features of the Locmi-TK-containing secretomotor neurons suggest that these peptides might act on synaptic receptors, and the high concentration of peptide required for AKH release is consistent with this hypothesis, particularly since synaptic receptors might be less accessible for an *in vitro* effect and thus would require higher peptide concentrations for an effect to be observed.

SchistoFLRFamide is a member of a large family of neuropeptides known as the FMRFamide-related peptides (FaRPs) that are widely found in insects (Orchard *et al.*, 2001). Antisera to FMRFamide and SchistoFLRFamide label a subset of secretomotor neurons that are distinct from the Locmi-TK-containing cells which make synaptoid contacts with glandular cells of the CC (Vullings *et al.*, 1998). FMRFamide and SchistoFLRFamide have no effect on the spontaneous release of AKH *in vitro*, but reduced IBMX-induced AKH release at a 10 μM peptide concentration. Like the case with Locmi-TK neuropeptides, the high concentration of FaRPs required for an effect is consistent with a direct supply of peptides on the glandular cells. Thus, FaRPs are inhibitory neuromodulators that act to fine-tune the release of AKH to meet the energetic demands of the flying animal (Vullings *et al.*, 1998).

A direct approach was used to isolate a compound, the neuropeptide CCAP, from a *S. gregaria* brain extract that potently stimulates release of AKH from *L. migratoria* and *S. gregaria* CC *in vitro* (Veelaert *et al.*, 1997). CCAP is a multifunctional neuropeptide and is best known for its stimulatory activity on heartbeat (Tublitz and Truman, 1985) and its effect as trigger for ecdysis behavior (Gammie and Truman, 1997; see **Chapter 3.1**). Unlike the Locmi-TK and SchistoFLRFamide-like peptides, there are no CCAP containing fibers in the GCC (Dircksen and Homberg, 1995; Veelaert *et al.*, 1997); CCAP is active in the nM range indicating that it may act as a neurohormonal releasing factor for AKH (Veelaert *et al.*, 1997).

Flight activity increases the octopamine levels in the hemolymph of *S. gregaria* (Goosey and Candy, 1980), and in *L. migratoria*, octopamine was shown to stimulate AKH release from the CC in the presence of IBMX (Pannabecker and Orchard, 1986). It was later found that in this experimental design, octopamine potentiates the stimulatory effect of IBMX mediated cAMP elevation on AKH release, and has no effect on its own (Passier *et al.*, 1995). This suggests that octopamine has a neurohormonal role in modulating AKH release (Veelaert *et al.*, 1997).

3.10.2.1.4. AKH degradation After secretion of AKHs into the hemolymph, their degradation will have a significant effect on peptide levels, and the differential rates of degradation will affect the ratios of peptides and, ultimately, the physiological response. AKHs are blocked at both termini, and the initial step in their degradation is by an endopeptidase that yields inactive peptide fragments (Siegert and Mordue, 1987; Fox and Reynolds, 1991b; Rayne and O'Shea, 1992). The endopeptidase has been localized to the external surface of several tissues (Rayne and O'Shea, 1992), including the Malpighian tubules (Baumann and Penzlin, 1987; Siegert and Mordue, 1987), but in *M. sexta*, AKH is cleaved by an enzyme circulating in the hemolymph (Fox and Reynolds, 1991b).

The half-life of AKH I in *L. migratoria* was initially determined to be 24 min by quantifying the disappearance of AKH from the hemolymph subsequent to its release using an *in vivo* bioassay (Cheeseman

et al., 1976). Subsequently, a half-life of 30 min was determined for AKH I and II in *S. gregaria* by measuring the disappearance of AKH after injecting a large, nonphysiological dose (Rayne and O'Shea, 1992). The synthesis of high-specific activity tritiated AKHs allowed the quantification of the half-lives of AKH I, II, and III in *L. migratoria* after injecting a physiological dose of 1 pmol (Oudejans *et al.*, 1996). This study clearly demonstrated that each AKH has a different rate of breakdown, with AKH III being significantly less stable in the hemolymph. The half-lives of AKH I, II, and III at rest are 51, 40, and 5 min, respectively, and AKH I and III are degraded more rapidly during flight, suggesting that more than one protease is involved in AKH degradation. In contrast to the relatively long half-lives of Locmi-AKH I and II, injected *Gryllus bimaculatus* AKH has a half-life of about 3 min and is degraded by enzymes released from hemocytes (Woodring *et al.*, 2002).

3.10.2.1.5. Signal transduction for AKH There is substantial evidence from a number of systems that inositol-1,4,5-trisphosphate (IP_3) serves as the second messenger to transduce the hormonal signal of AKH/HrTH neuropeptides in the fat body and, in some cases, cAMP is also involved (Van der Horst *et al.*, 1997; Gäde and Auerswald, 2003). In *L. migratoria*, the involvement of cAMP in AKH mediated lipid mobilization was shown by demonstrating the accumulation of cAMP in the fat body following an injection of a CC extract, and the increased hemolymph lipid concentration after treatment with dibutyryl-cAMP (db-cAMP) (Gäde and Holwerda, 1976). A similar effect of a CC extract on cAMP levels was also shown *in vitro* in *S. gregaria* (Spencer and Candy, 1976). The effect of the CC extract on cAMP levels was shown to be due to AKH (Gäde, 1979; Goldsworthy *et al.*, 1986b).

Although the mobilization of lipids is a crucial role of AKH in *L. migratoria*, the mechanism of AKH action in the target cell has been studied most extensively using glycogen phosphorylase activation as the assay for AKH activity (Van Marrewijk *et al.*, 1980). The potencies of each AKH in cAMP elevation and glycogen phosphorylase activation differed when assayed at physiological levels, decreasing in the order AKH III > AKH II > AKH I (Vroemen *et al.*, 1995a). These differences in potency support the hypothesis that the action of AKH II is more directed to carbohydrate metabolism than that of AKH I (Orchard and Lange, 1983a). The increase in cAMP levels is mediated by the G protein G_s, since cholera toxin, an irreversible activator of G_s, enhanced cAMP levels and glycogen phosphorylase activity (Vroemen *et al.*, 1995a). Pertussis toxin, an irreversible inhibitor of G_i, had no effect on cAMP levels or glycogen phosphorylase activation.

In *L. migratoria*, the uptake of Ca^{2+} into the fat body was stimulated by the presence of AKH, and the effects of AKH on diacylglycerol levels and glycogen phosphorylase activation are dependent on extracellular Ca^{2+} and can be mimicked by the calcium ionophore A23187 (Van Marrewijk *et al.*, 1991). A similar Ca^{2+} dependence for AKH mediated lipid mobilization was demonstrated *in vitro* (Lum and Chino, 1990; Wang *et al.*, 1990). In addition, the release of Ca^{2+} from intracellular stores leads to the subsequent influx of Ca^{2+} into the cell (Van Marrewijk *et al.*, 1993) by a process known as capacitative Ca^{2+} entry (Berridge, 1995), confirming a requirement for both intracellular and extracellular Ca^{2+} for the full effect of AKH on the fat body. Although each of the three AKHs increase Ca^{2+} uptake with similar potency, the three AKHs also enhance the efflux of Ca^{2+} into the fat body cytosol, but the increase in efflux increased in the order AKH I < AKH II < AKH III (Vroemen *et al.*, 1995b), so the effect of AKH I on intracellular Ca^{2+} levels that result from flux across the cell membrane is the greatest of the three AKHs. The mobilization of intracellular Ca^{2+} by each of the AKHs in locusts is mediated by the stimulation of inositol phosphate (InsPn) formation, most notably IP_3 (Stagg and Candy, 1996; Van Marrewijk *et al.*, 1996), but differences in potency were documented for each AKH in *L. migratoria*, decreasing in the order AKH III > AKH I > AKH II (Vroemen *et al.*, 1997). IP_3, generated by activation of phospholipase C (PLC), plays a critical role in Ca^{2+} mobilization that leads to activation of protein kinase C (PKC) for a wide variety of neuropeptides (Berridge, 1993). The stronger effect of AKH I on IP_3 combined with its weak effect on Ca^{2+} efflux relative to AKH II suggest that AKH I is the major lipid mobilizing hormone, particularly since Ca^{2+} is required for translocation of hormone sensitive lipases from the cytosol to lipid droplets to elicit lipolysis (Clark *et al.*, 1991; Egan *et al.*, 1992). The activation of glycogen phosphorylase by each of the AKHs is mediated by PLC activation, since a PLC inhibitor, U73122, dampened the response to the peptides, and the residual activity might be due to a cAMP pathway not influenced by IP_3 (Vroemen *et al.*, 1997). Elevation of IP_3 levels by AKH is mediated by the G protein G_q, since a G_q antagonist dampened AKH I induced glycogen phosphorylase activity (Vroemen *et al.*, 1998b). Stimulation of glycogen phosphorylase by cAMP is independent of extracellular Ca^{2+} (Van Marrewijk *et al.*, 1993), and the elevation of cAMP by forskolin or db-cAMP did not affect the enhancement of InsPn levels by AKH I, so a direct linkage between

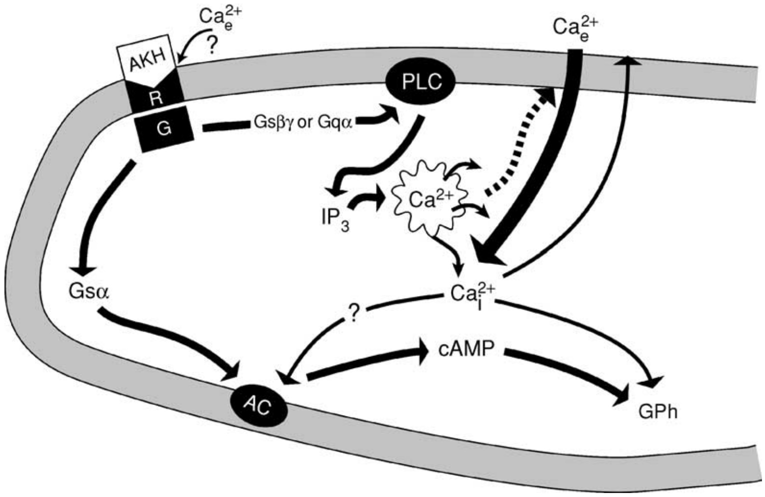


Figure 2 Proposed model for the coupling of AKH signaling pathways in *Locusta migratoria* fat body cells for carbohydrate mobilization. R, receptor; G, G protein; PLC, phospholipase C; IP_3, inositol-1,4,5-trisphosphate; AC, adenylate cyclase; GPh, glycogen phosphorylase. (Reprinted from Van der Horst,D.J., Vroemen, S.F., Van Marrewijk, W.J.A., **1997**. Metabolism of stored reserves in insect fat body: hormonal signal transduction implicated in glycogen mobilization and biosynthesis of the lipophorin system. *Comp. Biochem. Physiol. 117B*, 463–474, with permission from Elsevier.)

the AKH receptor and PLC activation must exist that is not dependent on the elevation of cAMP. These data have led to a model describing AKH signaling in *L. migratoria* (Van der Horst *et al*., 1997) (**Figure 2**). The AKH receptor is coupled to at least two distinct G proteins, G_s and G_q, and the activation of both pathways results in a complete enhancement of glycogen phosphorylase activation by AKH. AKH induced Ca^{2+} influx is mediated by voltage independent channels, possibly calcium release activated channels, since La^{3+}, a universal Ca^{2+} channel blocker, prevents glycogen phosphorylase activation (Vroemen *et al*., 1998b).

In *P. americana*, the effects of HrTH on phosphorylase activation in the fat body and trehalose release are dependent on the presence of extracellular Ca^{2+} (McClure and Steele, 1981; Orr *et al*., 1985; Steele and Paul, 1985), and the entry of Ca^{2+} into intact fat body (Steele and Paul, 1985), or fat body trophocytes (Steele and Ireland, 1999), is stimulated by HrTH. A key role for Ca^{2+} in glycogen breakdown is its requirement, together with calmodulin, for the activity of phosphorylase kinase, the activator of glycogen phosphorylase (Pallen and Steele, 1988). The mode of HrTH action in *P. americana* was examined in disaggregated trophocytes, the fat body cells responsible for trehalose synthesis (Steele and Ireland, 1994). This system was used to provide the ability to test the effect of peptides or varying conditions on a large number of samples to better correlate changes in a discrete population of responsive cells. It was demonstrated that in *P. americana* HrTH I and II stimulated IP_3 formation with comparable potencies (Steele *et al*., 2001). The HrTH stimulated formation of IP_3 and the increase in intracellular Ca^{2+} concentration are probably involved in phosphorylase activation and trehalose efflux from trophocytes, since a PLC inhibitor blocked these effects. HrTH I and II increase Ca^{2+} levels in fat body trophocytes by the release of intracellular Ca^{2+} followed by the subsequent capacitative influx of extracellular Ca^{2+} (Sun and Steele, 2001). Capacitative Ca^{2+} entry is inhibited by activation of protein kinase C or inhibition of calmodulin (Sun and Steele, 2001). HrTH stimulates the activity of phospholipase A_2 in trophocytes (Sun and Steele, 2002), which leads to the increased production of free fatty acids to provide an essential source of energy for trehalose synthesis (Ali and Steele, 1997). Phospholipase A_2 activation and the stimulation of trehalose efflux are dependent on G proteins, protein kinase C and calmodulin, and occur following the increase in cytosolic Ca^{2+} mediated by HrTH (Sun *et al*., 2002; Sun and Steele, 2002). In *P. americana*, a CC extract elevated cAMP levels in the fat body, but this effect was not due to the action of HrTH (Orr *et al*., 1985).

Transduction of the HrTH signal in *B. discoidalis* does not involve cyclic AMP, since HrTH does not elevate fat body cAMP levels (Park and Keeley, 1995), and elevating cAMP levels by IBMX or db-cAMP treatment does not lead to elevated trehalose synthesis (Lee and Keeley, 1994) or glycogen phosphorylase activity (Park and Keeley, 1995). Likewise, no effect of cAMP on trehalose production was observed in the cockroaches *P. americana* (Orr *et al*., 1985) or *Blaptica dubia* (Becker *et al*., 1998).

The release of Ca^{2+} from intracellular stores by thimerosal or thapsigargin increased trehalose synthesis (Keeley and Hesson, 1995) and glycogen phosphorylase activation (Park and Keeley, 1996), even in the absence of extracellular Ca^{2+}. In contrast, stimulation of the influx of extracellular Ca^{2+} did not affect trehalose synthesis (Keeley and Hesson, 1995) and had only a modest effect on phosphorylase activation (Park and Keeley, 1996). However, a maximal response to hormone requires the presence of extracellular Ca^{2+} (Keeley and Hesson, 1995; Park and Keeley, 1996). Since HrTH was shown to increase IP_3 levels in the fat body, it was suggested that HrTH leads to the release of intracellular Ca^{2+} followed by an influx of extracellular Ca^{2+} to achieve a maximal effect on the fat body (Park and Keeley, 1996). A similar dependence on extracellular and intracellular Ca^{2+} was demonstrated for HrTH mediated trehalose production in *B. dubia* (Becker *et al.*, 1998). However, the HrTH mediated decrease in concentration of the metabolic signaling molecule, fructose 2,6-bisphosphate (Becker and Wegener, 1998), is largely dependent on Ca^{2+} entry, since this effect could be fully mimicked by the calcium ionophore A23187 (Becker *et al.*, 1998). This decrease in fructose 2,6-bisphosphate (Becker and Wegener, 1998) is a key regulatory step towards the inhibition of glycolysis and directing the products of glycogen breakdown towards the production of trehalose that results from HrTH action (Wiens and Gilbert, 1967).

The signal transduction pathway for the action of AKH on lipid mobilization in adult *M. sexta* involves both cAMP and Ca^{2+} (Arrese *et al.*, 1999). The magnitude of diacylglycerol mobilization that was induced by Ca^{2+} mobilizing agents was similar to the response to the peptide, while treatments that increase cAMP levels did not induce a maximal response. For this reason, it was proposed that Ca^{2+} potentiates the action of cAMP. AKH induces an increase in PKA activity that precedes the activation of TAG lipase and release of diacylglycerol, further supporting the role for cAMP as a second messenger for AKH action in *M. sexta*.

In the fruit beetle, *Pachnoda sinuata*, which mobilizes carbohydrate reserves and increases proline synthesis in response to AKH (Auerswald *et al.*, 1998), the signal transduction pathway involves both cAMP and Ca^{2+} (Gäde and Auerswald, 2003). Injection of the endogenous AKH, Melme-CC (first isolated from *M. melolontha*), elevates cAMP levels in the fat body, and injection of a membrane-permeable cAMP analog or IBMX elevates proline levels in the hemolymph supporting the involvement of cAMP (Auerswald and Gäde, 2000). The induction of cAMP by AKH requires the presence of extracellular Ca^{2+} (Auerswald and Gäde, 2001a), and results in the rapid influx of Ca^{2+} into the fat body cell (Auerswald and Gäde, 2001b). The release of intracellular Ca^{2+} is required for the full stimulation of proline production by stimulating the influx of extracellular Ca^{2+} by capacitative Ca^{2+} entry (Auerswald and Gäde, 2001a). In contrast to the effect of cAMP on proline production, it is not involved in carbohydrate mobilization or glycogen phosphorylase activation (Auerswald and Gäde, 2000), which requires the presence of extracellular Ca^{2+} and the release of Ca^{2+} from intracellular stores (Auerswald and Gäde, 2001b). Injection of Melme-CC causes an increase in IP_3 levels in the fat body, but coinjection with a PLC inhibitor, U73122, affects only the carbohydrate mobilizing activity, and not the hyperprolinemic effect of the peptide (Auerswald and Gäde, 2002). These data suggest that two separate pathways exist for AKH action in *P. sinuata*, and that the release of the IP_3 dependent Ca^{2+} stores from the endoplasmic reticulum is not involved in proline production.

3.10.2.1.6. AKH receptor The *M. sexta* AKH receptor was characterized using tritium labeled Manse-AKH with a binding assay to larval fat body membrane preparations (Ziegler *et al.*, 1995). Specific and saturable binding, which required the presence of Ca^{2+}, was detected with a K_d of 7×10^{-10} M and was consistent with binding to one type of receptor. Smaller amounts of peptide are needed for a full response in a bioassay indicating that only a fraction of receptors must bind peptide for a maximal response. Specific binding to membrane preparations of *M. sexta* heart and muscle was not detected, but a low level of specific binding to the pterothoracic ganglion membrane preparations of adult *M. sexta* was observed (Ziegler *et al.*, 1995), consistent with the action of AKH that was observed on central nervous system neurons (Milde *et al.*, 1995).

Two independent methods were used to identify the AKH receptor cDNA from *D. melanogaster* (Park *et al.*, 2002; Staubli *et al.*, 2002). In one study, the *D. melanogaster* receptor cDNA related to the gonadotropin releasing hormone (GnRH) receptor (AF077299) (Hauser *et al.*, 1998) was expressed in a CHO cell line expressing the promiscuous G protein G16 (CHO/G16) and aequorin, and Drome-AKH was identified as the ligand present in a 3rd instar larval extract that induced a bioluminescent response (Staubli *et al.*, 2002). A dose–response curve showed that the response induced by synthetic Drome-AKH had an EC_{50} of 8×10^{-10} M, and all other *D. melanogaster* peptides tested were inactive.

The *D. melanogaster* AKH receptor was independently identified by injecting *in vitro* synthesized

RNA encoding the GPCR from the vasopressin receptor group (AF522194) into *Xenopus* oocytes and measuring the inward current in response to application of AKH (Park *et al.*, 2002). Oocytes responded specifically to Drome-AKH with an EC_{50} of 3×10^{-10} M. *Xenopus* oocytes expressing a second GPCR (AF522188) reacted with AKH, but with a much lower potency ($EC_{50} = 2.4 \times 10^{-8}$ M). AF522188 also reacted with CCAP with an EC_{50} of 1.2×10^{-8} M suggesting that this GPCR might interact with multiple ligands. This suggestion is supported by the overlap in the functions of AKH and CCAP, since both peptides possess cardioacceleratory activity (Scarborough *et al.*, 1984; Witten *et al.*, 1984; Tublitz and Truman, 1985). The *D. melanogaster* AKH receptors independently identified are derived from the same gene, and differ only by a small deletion of 8 bp in AF522194 causing a frameshift in its predicted sequence at the C-terminus, and several point mutations, only one of which results in an amino acid substitution (Park *et al.*, 2002). These differences did not affect the potency and specificity of their interactions with Drome-AKH.

The characterization of an AKH receptor cDNA in one species leads towards the development of strategies to identify the AKH receptor in a wide variety of insect species (Staubli *et al.*, 2002). Primers were designed to conserved regions of the *D. melanogaster* AKH receptor and a second receptor, that for *D. melanogaster* corazonin (Park *et al.*, 2002), to clone the AKH receptor from *Bombyx mori* (Staubli *et al.*, 2002). Aequorin expressing CHO/G16 cells transfected with the *B. mori* AKH receptor cDNA were activated by Helze-HrTH (Jaffe *et al.*, 1988), a peptide not yet identified in *B. mori*, with an EC_{50} of 3×10^{-10} M. The known AKH from *B. mori* (Ishibashi *et al.*, 1992), identical to Manse-AKH and Helze-AKH, activated the *B. mori* AKH receptor at a lower affinity, suggesting that *B. mori* possesses a second AKH that is more related to Helze-HrTH.

The presence of multiple AKH peptides in many insect species (Gäde *et al.*, 1997), the differing rank order of potencies of these AKHs depending on the assay used (Goldsworthy *et al.*, 1986b), and the age related changes in sensitivity to AKHs (Mwangi and Goldsworthy, 1977a; Ziegler, 1984; Woodring *et al.*, 2002) raise the question of whether AKH(s) interacts with multiple receptors. The presence of multiple AKH receptors in some insect species has been suggested based on biphasic responses to peptide analogs (Gäde and Hayes, 1995). In *P. sinuata*, AKH probably acts through two receptors, since peptide analogs possess differential effects on carbohydrate mobilization and proline synthesis (Auerswald and Gäde, 1999), and these biochemical effects are independently mediated by different signal transduction pathways (Auerswald and Gäde, 2000, 2002). However, evidence in *M. sexta* is consistent with the presence of one type of receptor responsible for the adipokinetic response, since analogs that are partial agonists are capable of reducing the response to the endogenous peptide (Fox and Reynolds, 1991a), and none of the AKH analogs tested exhibited a biphasic response, which would provide evidence for multiple receptors (Ziegler *et al.*, 1998).

Receptor desensitization, the increase in refractoriness in response to sustained exposure to the ligand, is mediated by phosphorylation of the agonist-bound receptor by G protein-coupled receptor kinases (Perry and Lefkowitz, 2002). Preincubation of *L. migratoria* fat body with any of the three Locmi-AKHs renders the tissue insensitive to each of the AKHs (Vroemen *et al.*, 1998a). While this result might imply that all three AKHs share the same receptor, an alternative interpretation is that each receptor recognizes all three AKHs with differing affinities, and that prolonged preincubation with a high dose of one peptide could desensitize all receptors.

3.10.2.1.7. Effects on gene expression Flight activity is the only natural stimulus known for AKH release, but neither AKH mRNA levels nor prohormone synthesis were altered by flight activity (Harthoorn *et al.*, 2001). Therefore, the availability of releasable AKH is dependent on its continuous biosynthesis.

The first example of the regulation of HrTH gene expression by nutritional status was obtained in *B. discoidalis* (Lewis *et al.*, 1998). It was demonstrated that starvation induced a twofold increase in HrTH mRNA levels in the CC, which returned to essentially normal levels after 2 days of refeeding. This starvation induced increase in HrTH mRNA level is accompanied by a similar increase in HrTH synthesis (Sowa *et al.*, 1996). The downregulation of HrTH mRNA levels by feeding is not simply a response to elevated hemolymph carbohydrate levels or a neural response to feeding, but is more likely due to the consumption of a complex of nutrients (Lewis *et al.*, 1998).

The first example of an effect on downstream gene expression in response to an insect neuropeptide (HrTH) was demonstrated in the fat body of *B. discoidalis* (Bradfield *et al.*, 1991). The mRNA level of a cytochrome P450 (P4504C1) was increased directly in response to physiological levels of HrTH, and was also upregulated by starvation in a CC-dependent manner (Bradfield *et al.*, 1991; Lu

et al., 1995, 1996). It was suggested that P4504C1 is involved in mobilization of fat body resources in response to starvation either by the control of fatty acid oxidation to provide energy for the conversion of glycogen to trehalose in response to the release of HrTH (Bradfield *et al.*, 1991), or by providing an alternative route for carbohydrate synthesis during exposure to HrTH or starvation (Lu *et al.*, 1996).

3.10.2.2. A Counter-Regulatory Hormone for AKH?

Despite extensive research, until recently there was no evidence for a counter-regulatory hormone for the AKH family. Bombyxin is an insect homolog of insulin, both in structural properties (Kawakami *et al.*, 1989) and receptor architecture and properties (Fullbright *et al.*, 1997). However, treatment of the natural host with bombyxin results in an activation of glycogen phosphorylase and a decrease in glycogen stores (Satake *et al.*, 1997), the opposite of the effects of insulin in vertebrates. An important role of insulin in vertebrates is lowering blood glucose, to not only maximize the utility of this expensive fuel for anaerobic metabolism, but to prevent damage to body proteins via formation of aldimines from the less abundant, but reactive, aldose form of glucose. Aldimines can rearrange to ketimines, which cannot hydrolyze like aldimines and thus permanently alkylate proteins with adverse effects. Because insect hemolymph contains the nonreducing sugar trehalose, this function of insulin would be of no importance to insects. Clynen *et al.* (2003a) used proteomic techniques to identify a small peptide, QSDLFLLSPK, from the GCC of *L. migratoria*; the mass spectral sequence was ambiguous, because this technique is unable to distinguish Leu from Ile, or Gln from Lys. However, a basic local alignment search tool (BLAST) search revealed a 100% match to a fragment of the locust insulin related peptide (Lagueux *et al.*, 1990). This fragment is termed a "co-peptide" which is immediately downstream of the signal peptide in the prepro-insulin related peptide of *L. migratoria*. The co-peptide is just upstream of the B-chain, which is followed by the C-peptide, and then the A-chain. Each peptide is separated by dibasic amino acid cleavage sites. In the mature insulin related peptide, the A- and B-chains are joined by two disulfide bonds, with an additional, internal disulfide bond in the A-chain. The C-peptide is not known to have a biological role in vertebrates, and the co-peptide might be presumed to have no activity. Synthesis of the *L. migratoria* co-peptide and bioassay revealed that it inhibits glycogen phosphorylase in the fat body, an effect expected of an insulin-like hormone. However, it has no effect on hemolymph lipid levels. Bombyxin, the first insulin related peptide discovered in insects, actually lowers storage carbohydrates in *Bombyx mori*, a paradoxical effect for a homolog of insulin (Satake *et al.*, 1997). The co-peptide certainly merits more detailed physiological study. Of some concern is the generality of these results: examination of the precursors of insulin related peptides in *B. mori*, *A. gambiae*, and *D. melanogaster* revealed that none of them contain the corresponding co-peptide; the B-chain in each begins immediately after the signal peptide.

In the horsefly *Tabanus atratus*, two peptides with AKH activity have been identified: Tabat-AKH and Tabat-HoTH (Jaffe *et al.*, 1989) (**Figure 1**). While Tabat-HoTH, a decapeptide, was claimed to have hypotrehalosemic activity (that expected for an insulin-like peptide rather than a member of the AKH/HrTH family), examination of **Figure 5b** in Jaffe *et al.* (1989) reveals that the curves showing dose-dependent differences between hemolymph sugars in control versus Tabat-HoTH treated larvae are but a trend and have overlapping standard deviations; no statistical tests were applied. Thus, the claims for hypotrehalosemic activity are unsupported by the published data.

3.10.3. Hormonal Control of Water and Electrolyte Homeostasis

Insects are small and hence have a high surface area to volume ratio, making them vulnerable to desiccation. Nevertheless, insects have evolved to be the most populous and diverse of all terrestrial animals. Insects have cuticles covered with water-impervious hydrocarbons to minimize water loss, and employ a variety of other behavioral, morphological, and physiological adaptations to keep water loss to a minimum, particularly in species that occupy very dry habitats. To maintain fluid homeostasis, water gained from the diet, metabolism and, in some species, from the atmosphere, must equal water lost by evaporation, respiration, and excretion. Evaporative and respiratory losses vary with environmental factors and the state of hydration, but the major site of regulation is the excretory system, and water loss via this route can change dramatically depending on the physiological status of the insect. Excretory water loss is determined by the rate at which fluid enters the hindgut from the midgut and Malpighian tubules (MT), and the rate of reabsorption therein. Fluid and ion homeostasis are under endocrine and possibly neural control, which allows the insect to regulate hemolymph volume and composition while permitting nitrogenous waste, toxic substances, and excess ions and/or water to be voided. The

endocrine factors affecting the regulation are referred to as diuretic hormones (DHs) and antidiuretic hormones (ADHs) although this may not precisely describe their actions. Generally, DHs stimulate primary urine secretion by MTs, whereas ADHs increase fluid reabsorption from the hindgut, but there are exceptions to this.

3.10.3.1. Molecular Basis for Urine Excretion

Urine excretion in vertebrates is primarily driven by blood pressure, but there is a complex interplay between a number of hormones in the control of fluid and ion homeostasis. Aldosterone and vasopressin (antidiuretic hormone) are largely responsible for maintaining electrolyte and water balance via reabsorption, while atrial natriuretic factor and angiotensin II primarily control blood volume (Beyenbach, 1993). In contrast, insects have low pressure circulatory systems and primary urine isosmotic to the hemolymph is secreted by the MT. Diuresis must therefore be driven by active ion transport, not by blood pressure. Diuretic hormones increase MT secretion by stimulating ion transport, whereas antidiuretic hormones either reduce MT secretion or stimulate fluid and vital solute reabsorption in the hindgut (Phillips, 1983) (**Figure 3**). A large literature exists on diuresis and its regulation in insects; early physiological studies have been reviewed comprehensively (Phillips, 1983; Spring, 1990). However, most early physiological studies on hormonal control of fluid homeostasis in insects utilized crude extracts of insect neuroendocrine tissues to affect target organs. It is now known that such studies dealt with a mixture of different active factors, so that unraveling mode of action of any single factor was impossible. A fundamental understanding of the control of fluid homeostasis requires that the controlling factors be identified, synthesized, and tested for their effects. Only availability of synthetic factors can allow a detailed

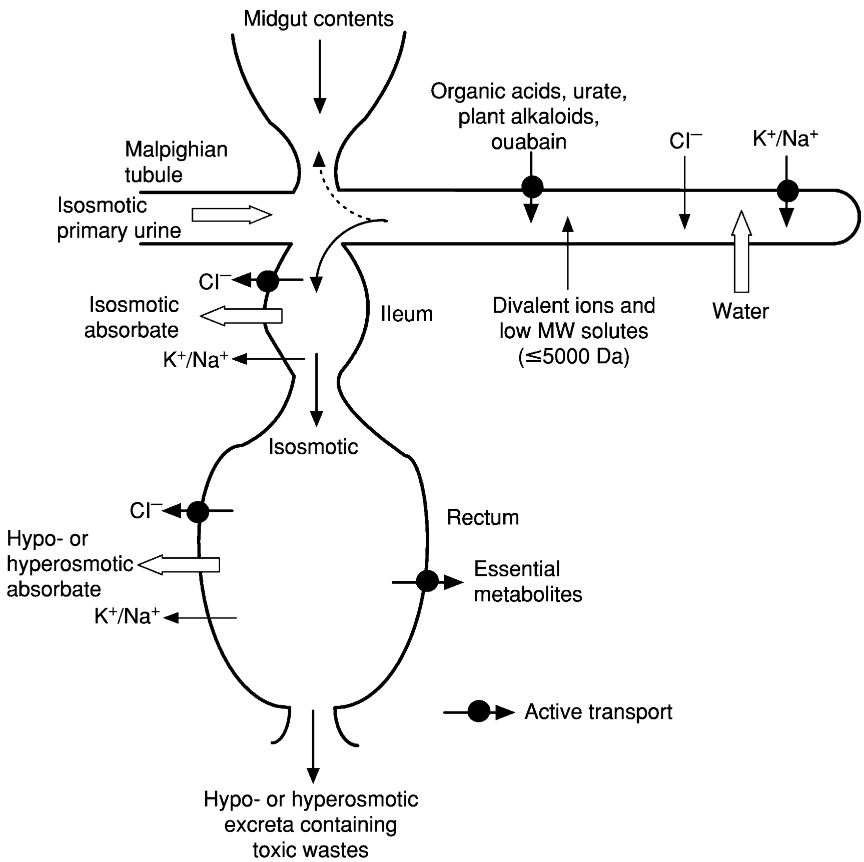


Figure 3 A generalized scheme of the classic view of the excretory process in insects. Malpighian tubule secretion is driven by the active transport of KCl and/or NaCl into the lumen, which draws water by osmosis. Other ions and metabolites enter the lumen by passive or active transporters. The primary urine enters the gut in most species at the midgut–hindgut junction and generally is directed posteriorly into the ileum and rectum where it is modified by isosmotic fluid reabsorption. The fluid entering the ileum and rectum is extensively modified by reabsorption of essential metabolites and fluid, which may be hypo- or hyperosmotic to the luminal contents. The driving force for ion transport is an apical electrogenic Cl^- pump, with cations entering passively, in contrast to the Malpighian tubules. Toxic wastes are retained in the hindgut lumen and are voided in the excreta, which can be strongly hypo- or hyperosmotic to hemolymph. (Modified with permission from Coast, G.M., Orchard, I., Phillips, J.E., Schooley, D.A., **2002a**. Insect diuretic and antidiuretic hormones. *Adv. Insect Physiol. 29*, 279–409.)

molecular understanding of the actions of factors controlling excretion and their physiological interplay, and whether prime control is due to diuresis or antidiuresis. There have been a number of reviews of the hormonal control of MT, and of hindgut function, focusing on identified hormones (Audsley *et al.*, 1994; Phillips and Audsley, 1995; Coast, 1996, 1998; Phillips *et al.*, 1998a; O'Donnell and Spring, 2000), and an extremely comprehensive review of both MT and hindgut function was published recently (Coast *et al.*, 2002b). The thrust of this review is to focus on advances in excretory physiology that concentrate on the effects of pure, defined factors.

An understanding of the molecular basis of basal urine production in the Insecta has emerged only during the last 15 years, and has been reviewed by Nicolson (1993) and Beyenbach (1995, 2003). Ion transport leading to basal secretion of urine by the MT is driven by an apical vacuolar-type H^+-ATPase (V-ATPase), in parallel with an apical cation/H^+ antiporter (Grinstein and Wieczorek, 1994). In a yellow fever mosquito, *Aedes aegypti*, MT, this transporter is believed to be a 1 cation:1 H^+ antiporter (Beyenbach, 2001), while in *M. sexta* midgut it is believed to be a 1 cation:2 H^+ antiporter (Azuma *et al.*, 1995). The activity of the V-ATPase is stimulated by treatment of tubules with cAMP or peptides elevating cAMP, and cGMP also stimulates its activity in *D. melanogaster* (O'Donnell *et al.*, 1996). The precise mechanism of stimulation is unknown; there is no evidence for phosphorylation of the V-ATPase, which lacks consensus sites for protein kinase A phosphorylation (Wieczorek *et al.*, 2000). A body of evidence suggests that activation of the *M. sexta* protein is related to association of the V_1 and V_0 subunits via a redox process (Grüber *et al.*, 2001); formation of a disulfide bond between Cys254 and Cys532 of the catalytic A protein of the yeast V_1 unit leads to reversible inactivation of the V-ATPase (Forgac, 2000), apparently via dissociation of the knob-like V_1 unit from the stalk-like V_0 unit.

The V-ATPase generates an electrochemical gradient favoring the entry of protons from the lumen in exchange for K^+ and/or Na^+ (Bertram *et al.*, 1991; Maddrell and O'Donnell, 1992; Weltens *et al.*, 1992) (**Figure 4**). The alkali cation-H^+ antiports of the hematophagous insect *R. prolixus* have a

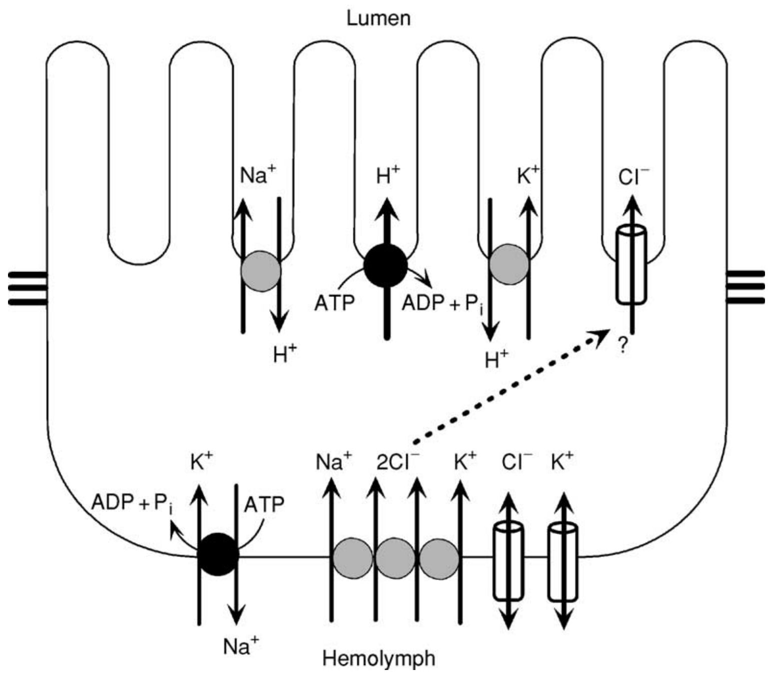


Figure 4 A generalized model for ion transport by Malpighian tubule principal cells. Active cation transport is energized by an apical (luminal) membrane H^+ pump (V-ATPase), which generates a gradient for protons to return to the cell via parallel K^+/H^+ and Na^+/H^+ antiports. Where there is a favorable electrochemical gradient, cations may enter principal cells by passive diffusion through ion channels in the basolateral membrane (notably K^+, but also Na^+ in *Aedes aegypti*). Other routes for cation uptake include primary (Na^+–K^+–ATPase) and secondary (Na^+–K^+–2Cl^- and K^+–Cl^- cotransport) active transport. Na^+ entry may also be coupled to the transport of organic solutes (not shown). Cl^- that enters the cells via cation-Cl^- cotransporters probably exits through channels in the apical membrane. Arrows with circles indicate primary (black circles) or secondary (gray circles) active transport, while arrows through cylinders represent membrane ion channels. (Modified with permission from Coast, G.M., Orchard, I., Phillips, J.E., Schooley, D.A., **2002a**. Insect diuretic and antidiuretic hormones. *Adv. Insect Physiol. 29*, 279–409.)

preference for Na^+ over K^+ (Maddrell, 1978; Maddrell *et al*., 1993b), whereas there appears to be no such preference in the house cricket, *A. domesticus* (Coast *et al*., 1991). Alkali cations enter principal cells through ion channels in the basolateral membrane, if the electrochemical gradient permits, or by secondary (e.g., cation/Cl^- cotransport) transport. The basolateral primary (Na^+–K^+–ATPase) active transporter has long been proposed to be important in MT transport processes, yet its known stoichiometry in vertebrate systems (3 Na^+ exported per 2 K^+ imported) is one opposing the observed direction of cation transport in MT. In fact, serotonin has been reported to inhibit the activity of the Na^+–K^+–ATPase in MT of *Rhodnius prolixus*, with this inhibition claimed to be responsible for some of the diuretic activity of this amine (Grieco and Lopes, 1997). However, indirect evidence suggests it may not play a significant role in *R. prolixus*, because ouabain, a potent inhibitor of this transporter, has no effect on fluid secretion (Ianowski *et al*., 2002). The Na^+–K^+–ATPase does not appear to be of functional importance in MT of *A. aegypti* (Weng *et al*., 2003) or in *M. sexta* (E. Chidembo and D.A. Schooley, unpublished data), as judged by a lack of vanadate or ouabain sensitivity of ATP hydrolase activity. In contrast, in *T. molitor* the Na^+–K^+–ATPase seems to play an important role in maintenance of cell K^+ concentration, and ouabain is an irreversible inhibitor of MT fluid secretion (Wiehart *et al*., 2003). These species differences may be explained by the nature of cations secreted in primary urine; in the cryptonephric tubules of *T. molitor*, activities of K^+ exceeding $3\,mol\,l^{-1}$ have been reported (O'Donnell and Machin, 1991), whereas *A. aegypti* secrete large amounts of Na^+ (Hegarty *et al*., 1991). Species or order differences in the relative activity of the Na^+–K^+–ATPase may also underlie the high levels of hemolymph K^+ versus Na^+ in many Lepidoptera, Coleoptera, and Hymenoptera (see Sutcliffe, 1963 and Section 3.10.3.12.1): a low activity of Na^+–K^+–ATPase in epithelial tissues would remove the driving force for keeping blood K^+ levels low versus Na^+ in hemolymph of most insect orders.

In general, the electrochemical gradient over the epithelium favors Cl^- diffusion into the lumen through a shunt pathway. The Cl^--selective shunt described by Pannabecker *et al*. (1993) in MT of *A. aegypti* does not appear to be in the principal cells, and is either paracellular or through a second cell type, the stellate cells, which form thin (3–5 μm deep) windows between the hemolymph and tubule lumen. Not all species have stellate cells; recently, they have been shown to have a different embryonic origin than principal cells (Denholm *et al*., 2003). Agents elevating intracellular Ca^{2+} increase Cl^- conductance, but do not stimulate the V-ATPase.

3.10.3.2. A Multiplicity of Peptides Regulate Diuresis and Antidiuresis in Insects

A number of "model species" have been adopted by different investigators for studying diuresis and antidiuresis. The classic work of Ramsay (1954) was performed with a stick insect, *Carausius morosus*. A great deal of pioneering work on diuresis and its control has been done with the hematophagous hemipteran *R. prolixus* (Maddrell, 1963) and *D. melanogaster* has been proposed as a useful model for studying diuresis in insects (Dow *et al*., 1998; MacPherson *et al*., 2001). The Beyenbach group has studied ion transport mechanisms in *A. aegypti* for decades (Beyenbach, 2003). Locusts have been the preferred model for studying the role of the hindgut in fluid reabsorption, largely through the work of the Phillips group (Phillips and Audsley, 1995).

The first hint that more than two different classes of peptides acted on MT was supplied by the Beyenbach group's studies of regulation of *A. aegypti* tubules by nervous system extracts separated on HPLC (Petzel *et al*., 1985). The first undisputed identification of an insect DH was the identification from *M. sexta* of a 41 amino acid peptide with high sequence similarity to the sauvagine/urotensin/urocortin/corticotropin releasing factor (CRF) family (Kataoka *et al*., 1989b). To date, 13 "CRF-like" DH structures have been identified by isolation and Edman degradation; four were identified by BLAST searches of the *D. melanogaster*, *A. gambiae*, and *B. mori* genomes (**Figure 5**). Somewhat later, the leucokinins (myotropic peptides originally isolated based on their ability to cause contractions of the cockroach hindgut) were also shown to have potent diuretic effects (Hayes *et al*., 1989). Currently, 33 different myokinin sequences are known from nine species of insects. In the last few years, it has become evident that a "cocktail" of neuropeptides are implicated in the regulation of MT secretion (Coast *et al*., 2002a; Skaer *et al*., 2002). The majority of these peptides have diuretic activity, stimulating fluid secretion. Others have antidiuretic activity and reduce the rate of secretion. From the mid-90s onwards, peptides belonging to four families in addition to the CRF-like DHs and myokinins have been shown to influence tubule secretion: the calcitonin-like (CT-like) DHs, CAP_{2b}-like, tachykinin

related peptides, and two antidiuretic factors, ADFa and ADFb. The greatest amount of research has been carried out on the first two families discovered, i.e., the CRF-like and myokinin families; these appear to be ubiquitous among insects, although there is considerable interspecific variation in activity. For example, the CRF-like DH of *A. domesticus* stimulates maximal secretion, and is about threefold more active than the native myokinins (Coast *et al.*, 1992). Alternatively, in *D. melanogaster* and in *M. domestica* (Holman *et al.*, 1999), the CRF-like DHs have poor activity compared with the native kinin. The situation is even more extreme in *R. prolixus*, where CRF-like peptides can elicit maximal secretion and myokinins appear to be inactive (Te Brugge *et al.*, 2002).

CT-like, CAP_{2b}-like, and tachykinin related peptides may also be of widespread occurrence in insects, but have been investigated less extensively than the CRF-like DHs and myokinins. Indeed, to date, CT-like peptides have only been identified in three species, *Diploptera punctata*, *D. melanogaster*, and *Formica polyctena* (a partial sequence), and three in the genomes of *A. gambiae*, *B. mori*, and *Apis mellifera*, but these represent very diverse orders (Dictyoptera, Diptera, Lepidoptera and Hymenoptera, respectively). There is immunological evidence for the existence of these factors in six other insect species representing the Orthoptera, Coleoptera, Hemiptera, and Lepidoptera (see Section 3.10.3.5). Even less is known of the antidiuretic factors controlling MT secretion since these have only been described in *T. molitor*. Intriguingly, peptides that have diuretic activity in one species have been shown to have antidiuretic activity in another. Manse-CAP_{2b} is a diuretic in Diptera (*D. melanogaster* and *M. domestica*), but an antidiuretic in *R. prolixus* and *T. molitor*. CAP_{2b} has no effect on secretion by MT of *M. sexta* at 1 μmol l^{-1} (Skaer *et al.*, 2002), the latter organism being the host from which it was identified. Tenmo-ADFb is antidiuretic in *T. molitor* and *A. aegypti*, but a diuretic in *A. domesticus* (G.M. Coast, unpublished data). Even when comparing quite similar species, peptide activity can vary considerably, as for CAP_{2b} in *D. melanogaster* and *M. domestica*. In *D. melanogaster*, CAP_{2b} acts on principal cells to activate a calcium-calmodulin dependent NO synthase, and the rise in NO levels increases cGMP production by a soluble guanylate cyclase. Diuresis results from the cGMP dependent stimulation of an apical membrane V-type ATPase that powers fluid secretion (Davies *et al.*, 1995). Conversely, CAP_{2b} has no effect on cGMP levels in *M. domestica* tubules or on the activity of the V-type ATPase. Instead, diuresis results from the opening of a passive chloride shunt pathway, which has previously been attributed to myokinin stimulation (C.S. Garside and G.M. Coast, unpublished data).

3.10.3.3. CRF-Like DH

The first insect DH identified was Manse-DH, a 41 residue peptide isolated from 10 000 heads of pharate adult *M. sexta* using an *in vivo* assay (Kataoka *et al.*, 1989b). This assay for monitoring the isolation utilized beheaded, newly eclosed adults of *Pieris rapae*, similar to a technique described by Nicolson for *Pieris brassicae* (Nicolson, 1976). Manse-DH has 41% sequence identity with sauvagine, a peptide isolated from skin of the frog *Phyllomedusa sauvagei* (Montecucchi and Henschen, 1981) and believed for some time to be the amphibian form of CRF. The direct sequence identity with CRF is somewhat lower. Subsequently a smaller peptide, Manse-DPII, was isolated from dissected neuroendocrine cells of brains of *M. sexta*. Again, an *in vivo* bioassay was used to monitor the isolation using adults of *M. sexta*. Due to the extremely small amount of tissue extracted in this "surgical purification" method, the peptide was obtained in a pure state after a single step of reversed-phase liquid chromatography (RPLC) (Blackburn *et al.*, 1991). However, the amount of peptide isolated was so small that only a partial sequence was determined by Edman degradation; complete structure proof required mass spectral sequencing. As aligned in **Figure 5**, Manse-DPII has only nine residues identical with those in Manse-DH. Also in 1991, CRF-like DHs were isolated from *A. domesticus* and *L. migratoria*; the former was isolated based on its ability to increase levels of cAMP in MT of *A. domesticus* maintained *in vitro* (Kay *et al.*, 1991a). This 46 amino acid peptide, Achdo-DP, is clearly a third member of the "CRF-like" DH. Two publications reported the sequence of the same DH from *L. migratoria* within a few weeks of each other; both utilized purifications by multiple steps of RPLC. Lehmberg *et al.* required only three RPLC purification steps to isolate this peptide to homogeneity from 4000 dissected locust brains using a direct enzyme-linked immunosorbent assay (ELISA) with antibodies raised against Manse-DH (Lehmberg *et al.*, 1991). Kay and colleagues utilized whole heads of *L. migratoria* to isolate Locmi-DH, monitoring the four-step RPLC isolation procedure with the *in vitro* assay for cAMP production with MTs of the locust (Kay *et al.*, 1991b).

In 1992 Kay *et al.* identified from *P. americana* a 46 residue DH, which differs from Locmi-DH at 13 residues, and from Achdo-DP at 18 residues. MT of

L. migratoria were used to monitor the isolation of this peptide, because of extreme variability between cockroach tubules (G.M. Coast, unpublished data). Much later, Furuya *et al.* (2000b) isolated a 46 residue DH from the Pacific beetle roach, *Diploptera punctata*, which still differs from Peram-DH at nine residues. Another 46 residue DH from *Zootermopsis nevadensis* (Baldwin *et al.*, 2001), a dampwood termite, differs from Peram-DH at only three residues, and thus is ranked phylogenetically between the two cockroach DHs by the sequence alignment program utilized, Clustal W version 1.8 (**Figure 5**). It is curious that these 46 residue DHs, while being relatively more related to one another than the other CRF-like DH, still vary highly in sequence. An interesting observation with *Z. nevadensis* is that this species contains another DH that elevates cAMP in tubules of *M. sexta*, the species used to assay the isolation of Zoone-DH. This second factor is more basic, but less hydrophobic, than Zoone-DH; two attempts to isolate it were unsuccessful, because of its lability in the acidic solvents used for extraction (Baldwin *et al.*, 2001).

The first, and to date only, case of two species having an identical CRF-like DH sequence was the identification of Musdo-DP from *M. domestica* and *Stomoxys calcitrans* (Clottens *et al.*, 1994). The assay to monitor the purification scheme was based on elevation of cAMP in MT of *M. sexta* maintained *in vitro*. This 44 amino acid peptide was accompanied by another, more hydrophobic active factor in *S. calcitrans*. The existence of this unknown factor has been reported (see Schooley, 1994), but, unfortunately, no details of its properties were ever published. Audsley *et al.* (1995) advocated the use of *M. sexta* MT for a general heterologous bioassay for isolation of DH from other species, because these MT proved to cross-react more promiscuously with all CRF-like DH tested than MT of *L. migratoria* or *A. domesticus*. A DH was isolated from the salt water mosquito, *Culex salinarius*, the isolation of which has never been reported, although its sequence and biological properties have been published (Clark *et al.*, 1998a). This 42 residue peptide differs from the Musdo-DH sequence at nine positions. Two more dipteran CRF-like DHs have been identified from the genome of *D. melanogaster* (Cabrero *et al.*, 2002) and *A. gambiae*. Drome-DH_{44} differs from Musdo-DH at only one residue: the substitution of a Ser at position 31 for a Thr in Musdo-DH. In contrast, Anoga-DH_{44} differs from Musdo-DH by seven residues. All dipteran DHs isolated to date are two residues (in one case four residues) shorter than the 46-mers isolated from the Orthoptera, Dictyoptera, and a single isopteran.

```
                1               15              30              45
Manse-DH      ------RMPSLSIDL  PMSVLRQKLSLEKER  KVHALRA----AANR  NFLNDI-NH2
Hylli-DH41    ------RMPSLSIDL  PMSVLRQKLSLEKER  KVQALRA----AANR  NFLNDI-NH2
Bommo-DH41    ------RMPSLSIDM  PMSVLRQKLSLENER  KLQSLRA----MANR  NFLNDI-NH2
Musdo-DP      ------NKPSLSIVN  PLDVLRQRLLLEIAR  RQMKENT-RQVELNR  AILKNV-NH2
Drome-DH44    ------NKPSLSIVN  PLDVLRQRLLLEIAR  RQMKENS-RQVELNR  AILKNV-NH2
Anoga-DH44    ------TKPSLSIVN  PLDVLRQRIILEIAR  RQMRENT-RQVELNK  ALLREI-NH2
Culsa-DH      --------PSLSIVN  PLDVLRQRIILEMAR  RQMRENT-RQVERNK  AILREI-NH2
Peram-DP      ----TGSGPSLSIVN  PLDVLRQRLLLEIAR  RRMRQSQ-DQIQANR  EILQTI-NH2
Zoone-DH      ----TGAVPSLSIVN  PLDVLRQRLLLEIAR  RRMRQSQ-DQIQANR  EMLQTI-NH2
Dippu-DH46    ----TGTGPSLSIVN  PLDVLRQRLLLEIAR  RRMRQTQ-NMIQANR  DFLESI-NH2
Locmi-DH      ----MGMGPSLSIVN  PMDVLRQRLLLEIAR  RRLRDAE-EQIKANK  DFLQQI-NH2
Achdo-DP      -----TGAQSLSIVA  PLDVLRQRLMNELNR  RRMRELQGSRIQQNR  QLLTSI-NH2
Tenmo-DH47    AGALGESGASLSIVN  SLDVLRNRLLLEIAR  KKAKEG----ANRNR  QILLSL-OH
Manse-DP2     ---------SFSVNP  AVDILQH-------R  YMEKVAQ-----NNR  NNLNRV-NH2
Hylli-DH30    ---------SFSVNP  AVEILQH-------R  YMEKVAQ-----NNR  NFLNRV-NH2
Bommo-DH34    ---------SFSVNP  AVDTLQR---GAYNH  FLEKVVQ-----SNR  DYLNRI-NH2
Tenmo-DH37    -------SPTISITA  PIDVLRK--TWEQER  ARKQMV------KNR  EFLNSLN-OH

sauvagine     ------QGPPISIDL  SLELLRK--MIEIEK  QEKEKQQAA---NNR  LLLDTI-NH2
wsUrot I      -----NDDPPISIDL  TFHLLRN--MIEMAR  NENQREQAG---LNR  KYLDEV-NH2
urocortin     ------DDPPLSIDL  TFHLLRT--LLELAR  TQSQRERAE---QNR  IIFDSV-NH2
xenlaCRF      -----AEEPPISLDL  TFHLLRE--VLEMAR  AEQIAQQAH---SNR  KLMDII-NH2
h,r,c,eCRF    -----SEEPPISLDL  TFHLLRE--VLEMAR  AEQLAQQAH---SNR  KLMEII-NH2
```

Figure 5 The CRF-like DHs and the CRF superfamily of peptides – the blue colored residues emphasize the similarities among the CRF-like DHs. Sauvagine, urotensin I, and urocortin 1 are paralogs of CRF. The CRF-like DHs are shown using the Swiss Prot species abbreviations (defined in the text except for Bommo (*Bombyx mori*, whose genome sequence appeared while the article was in press)); wsUrot I, *Catostomus commersoni* urotensin I; Xenla, *Xenopus laevis*. The four identical CRFs are from human (h), rat (r), goat (c), and horse (e). Two residues (shown in red) are conserved in the lepidopteran DHs and the CRF superfamily. The unique C-termini of the *Tenebrio molitor* DH are shown in green.

An unusual CRF-like DH was identified in *T. molitor* (Furuya *et al*., 1995); its isolation was monitored by the elevation of cAMP from MT of this species maintained *in vitro*. This 37 residue DH, Tenmo-DH_{37}, was isolated from 8400 pupal heads of *T. molitor*, and was accompanied by a less abundant, more hydrophobic factor isolated in insufficient quantity to sequence. Only later was the structure of this second factor reported, after extraction of an additional 20 000 heads, combined with the active fractions from identification of Tenmo-DH_{37}. The peptide isolated, Tenmo-DH_{47}, is the largest CRF-like DH identified to date (Furuya *et al*., 1998) at 47 residues, and is much less abundant than its small congener. Structurally, both peptides differ from all other CRF-like DH in having their C-terminal residue existing in the free carboxylate form rather than being amidated. In addition, Tenmo-DH_{37} has a one-residue "extension" of its sequence: all sequences from organisms other than *T. molitor* have a C-terminal Ile-amide or Val-amide, whereas Tenmo-DH_{37} has a Leu-Asn-OH C-terminus, and Tenmo-DH_{47} has a Leu-OH, lacking the Asn extension of its smaller congener. A physiological consequence of this structural difference is that Tenmo-DH_{37} is without any detectable biological activity on MT of *M. sexta*: its activity is $\leq 10^4$ lower than Manse-DH in this assay. This is consistent with data on the free acid form of Manse-DH which is about 1000-fold less active than the natural, amidated form in the *P. rapae* assay (Kataoka *et al*., 1989b). The more abundant, smaller Tenmo-DH_{37} was found to be about 600-fold more potent than the larger peptide in an *in vitro* assay measuring cAMP production with adult MT (Furuya *et al*., 1998), and about 200-fold more potent in a Ramsay fluid secretion assay with larval MT (Wiehart *et al*., 2002). However, Manse-DH is only 17-fold less active than Tenmo-DH_{37} in the cAMP assay with adult MT (Furuya *et al*., 1995). The two latter DHs have only 15 residues in common in the alignment in **Figure 5**, with Tenmo-DH_{37} being more similar to Manse-DPII, and Tenmo-DH_{47} being more similar to the "longer" CRF-like DH.

Two DHs were isolated from the white-lined Sphinx, *Hyles lineata*, a close relative of *M. sexta* (Furuya *et al*., 2000a). The larger of these, Hylli-DH_{41}, differs from Manse-DH only at residue 27, which is Gln rather than His (Manse-DH). Similarly, the shorter DH, Hylli-DH_{30}, differs from Manse-DPII only at residue 9, by a conservative substitution (Glu in the *H. lineata* peptide for Asp in Manse-DPII). Thus, the sequences of two DHs from each of three species are currently known, and it is apparent that these form a group of paralogous sequences. It is fascinating to contrast the CRF-like DH with the CRF superfamily of peptides in vertebrates. In 1991, two CRF sequences were identified by cDNA cloning in the white sucker *Catostomus commersoni*. The sucker CRFs differ from the sequences of human, horse, rat, and dog CRF (all identical) at only one and two residues (Morley *et al*., 1991), yet are only 54% identical with urotensin, the fish equivalent of sauvagine. The following year, a similar situation was discovered with *Xenopus laevis*, which has two forms of CRF differing from the human sequence at only 2 and 3 residues (Stenzel-Poore *et al*., 1992). Consequently a sauvagine (or urotensin)-like peptide was sought and found in rat brain: urocortin 1, a vertebrate ortholog of urotensin and sauvagine (Vaughan *et al*., 1995). Thus, the CRF superfamily in vertebrates consists of two paralogous subfamilies of sequences, a situation mirrored in the CRF-like DH, although the sequences are very highly conserved in vertebrates in contrast with those known from insects.

Examination of the sequences of the 15 known CRF-like DHs (**Figure 5**) reveals only four amino acids that are completely conserved in this family. Two of these are a Ser and a Leu, but they are separated by seven amino acids. The others are an Asn and a Leu in the C-terminal domain, separated by three amino acids. The Leu is separated from the C-terminal residue by two amino acids (three in Tenmo-DH_{37}). Consequently, the prospects for using degenerate probes to attempt to isolate additional new members of this family from cDNA or mRNA are very poor. Curiously, in the CRF superfamily of peptides, again only four residues are completely conserved; one is the Ser also conserved in the CRF-like DH, and another the Asn near the C-terminus. The other two totally conserved residues in the CRF superfamily differ from those in the CRF-like DH. Despite the exceptional sequence conservation of the various forms of CRF, the paralogs sauvagine, urotensin, and urocortins diverge considerably (Hauger *et al*., 2003).

Aside from the genome sequences for Drome-DH_{44}, Boomo-DH_{41}, Bommo-DH_{34}, and Anoga-DH_{44}, to date only one gene sequence encoding a CRF-like DH has been determined by cloning; that is for Manse-DH (Digan *et al*., 1992). The prepropeptide has a 19 residue signal peptide for cellular secretion, followed by a 61 residue propeptide terminating in Lys-Arg (the cleavage site for a Kex-2 like processing enzyme), then the 41 residues of Manse-DH, followed by a Gly-Lys-Arg plus 15 additional amino acids of the propeptide. The

Lys-Arg processing site results in a C-terminal Gly, which provides the amidation on the C-terminal Ile following action of peptidylglycine amidating monooxygenase (Prigge *et al*., 2000) on the Gly residue.

Another aspect of the sequence alignment is worth noting. A Pro residue is situated either exactly between the totally conserved Ser and Leu residues in the N-terminal domain, or one residue closer to the Ser in two of the three "short" CRF-like DHs. The residue just upstream of this Pro is Asn in 11 of the 15 CRF-like DH, Ala in two, and Leu in two. The Asp-Pro bond, not observed in any DH isolated to date, is exceptionally labile to acid on standing in solution, and the Asn codon requires but a single base change to give Asp. The inability to isolate the second CRF-like DH from *Z. nevadensis* was probably due to the presence of an Asp-Pro bond (Baldwin *et al*., 2001); multiple attempts to isolate a CRF-like factor from heads of *A. mellifera*, assayed by its strong stimulation of MT of *M. sexta*, were totally unsuccessful because the activity would disappear after only a few steps (E. Lehmberg and D.A. Schooley, unpublished data).

There are very few studies on structure–activity relationships with the CRF-related DHs because of their size and difficulty of solid-phase chemical synthesis. Studies with Manse-DH and Achdo-DP show that a region near the N-terminus is important for receptor activation while the remaining portion of the peptide is required for receptor binding. An analog Manse-DH(13–41), truncated by 12 residues at the N-terminus, binds Manse-DHR expressed in Sf9 cells with high affinity (IC_{50} ~2.8 nmol l^{-1} versus 0.16 nmol l^{-1} for the intact peptide) but does not stimulate cAMP production, whereas Manse-DH (3–41) has high receptor binding affinity and is a potent stimulant of adenylate cyclase (Reagan, 1995a). Manse-DH(21–41) and Manse-DH(26–41) have binding affinities reduced 100-fold and 1000-fold, respectively, while Manse-DH(31–41) has no binding activity at 1 μmol l^{-1} (Reagan *et al*., 1993). Deletion of the first four residues from Manse-DH decreases activity, while deletion of the next residue essentially abolishes it (P. Dey and D.A. Schooley, unpublished data). Taken together with the results of Reagan (1995a), this suggests that the receptor activating domain of Manse-DH lies very close to the N-terminus. Studies with N-terminal truncated analogs of Achdo-DP in a cricket tubule fluid secretion assay confirm the importance of the N-terminus for receptor activation (Coast *et al*., 1994). The activities of Achdo-DP(6–46) and Achdo-DP(7–46) are essentially indistinguishable from intact Achdo-DP, but the activity of Achdo-DP(11–46) is reduced by 60% and Achdo-DP(23–46) is inactive. These data could suggest that the domain necessary for receptor activation is farther from the N-terminus in Achdo-DP than in Manse-DH. However, results for peptides with low activity in a series of deletion peptides must be interpreted with caution; such peptides are made by removing peptide synthesis resin at various stages of the synthesis, which proceeds from the C- to the N-terminus. Usually all synthetic deletion peptides are purified using the same preparative RPLC column; traces of biological activity from a full-length, or nearly so, peptide will bleed out of a column essentially permanently (D.A. Schooley, unpublished data). Thus, the shortest peptides must be purified first on a new column. Intact Met residues may be important for activity; one of the Met residues (at position 1, 3, or 13) in Locmi-DH can become oxidized, with a resultant loss of activity (I. Kay and G.M. Coast, unpublished data). This may explain why $[Nle^{2,11}]$-Manse-DH (with Nle replacing Met^2 and Met^{11}) maximally stimulates cricket tubule secretion, whereas Manse-DH gives only a 60% response (Coast *et al*., 1992). In addition, Locmi-DH(1–23) and Locmi-DH(24–46) are inactive whether tested separately or together (Nittoli *et al*., 1999) (G.M. Coast, unpublished data); thus, the binding and activation domains must be joined together to have a biologically active peptide.

Manse-DH with a free C-terminus (acid) stimulates cAMP production by adult *M. sexta* MT (Audsley *et al*., 1995) and posteclosion diuresis in decapitated newly emerged *P. rapae* (Kataoka *et al*., 1989b), but in both assays it was 1000-fold less potent than Manse-DH. Reagan *et al*. (1993) were unable to detect any binding of this ligand to the Manse-DHR, which they attributed to rapid degradation of the nonblocked peptide (presumably by carboxylpeptidases) in the receptor binding assay.

3.10.3.4. Diuretic and Myotropic Peptides: the Kinins

Insect kinins (originally called myokinins) were isolated from whole head extracts of the Madeira cockroach, *Leucophaea maderae* (leucokinins; Leuma-Ks) (Holman *et al*., 1986a, 1986b, 1987a, 1987b) based on their potent myotropic activity in a cockroach (*L. maderae*) hindgut assay (Holman *et al*., 1991). The eight leucokinins differ in myotropic potency. They were later found to be potent stimulants of fluid secretion in *A. aegypti*, also causing a depolarization of the transepithelial potential (Hayes *et al*., 1989). Soon thereafter, five kinins (achetakinins; Achdo-Ks) were isolated from *A. domesticus* using the *L. maderae* hindgut assay; they were shown to also possess high diuretic activity on MT

of *A. domesticus* (Coast *et al.*, 1990). Similarly, locustakinin was isolated from 9000 brain–corpora cardiaca–corpora allata–subesophageal complexes of *L. migratoria* using the *L. maderae* hindgut assay (Schoofs *et al.*, 1992). It is curious that only a single kinin, locustakinin (Locmi-K), was isolated from this species, whereas all other orthopterans and dictyopterans studied to date have 5–8 kinins. Using hindgut of *P. americana*, eight kinins were isolated from 800 corpora cardiaca–corpora allata of this species (Predel *et al.*, 1997), five of which are unique sequences, while three occur in other species: Locmi-K, Leuma-K-7, and Leuma-K-8. Availability of endogenous kinins is crucial to understanding their role in any species. For example, muscakinin is about 10^6 more potent in *M. domestica* (Holman *et al.*, 1999) than leucokinin I.

The first kinins to be identified from a holometabolous species were those from *A. aegypti*, which were isolated using an ELISA rather than a functional assay (Veenstra, 1994). While these were termed "*Aedes* leucokinins 1–3," this nomenclature suggests they may be the same sequence as those from *L. maderae*, but they are in fact unique. Here the Swiss-Prot-NCBI five-letter standards for species abbreviation, Aedae-K-1, Aedae-K-2, and Aedae-K-3, as previously recommended is used (Coast *et al.*, 2002a). Later, the sequence of the single cDNA encoding these kinins was determined, and their biological properties determined (Veenstra *et al.*, 1997). While all three Aedae-K peptides are capable of depolarizing the mosquito MT at doses between 0.1 and 1 $nmol\,l^{-1}$, only Aedae-K-3 gave good stimulation of fluid secretion, with an EC_{50} near 10 $nmol\,l^{-1}$; Aedae-K-3 was appreciably less active and Aedae-K-2 was without effect on fluid secretion at 1 $\mu mol\,l^{-1}$. Three kinins were isolated from wild-collected salt marsh mosquitos, 94% of them *C. salinarius*, and the first kinin was christened "culekinin depolarizing peptide" (Hayes *et al.*, 1994). They were isolated using the *L. maderae* hindgut assay, as well as an electrophysiological assay with tubules of *A. aegypti*. The sequence of only the heptamer Culsa-K-1 was published (Hayes *et al.*, 1994). Some years later, the biological properties of Culsa-K-2 and Culsa-K-3, along with the sequences, were published by other workers (Cady and Hagedorn, 1999a, 1999b), but details of their isolation and identification remain unpublished. These peptides stimulate inositol trisphosphate (IP_3) concentrations in MT of *A. aegypti*, with no effect on cAMP levels (Cady and Hagedorn, 1999b). Further, they stimulate *in vivo* urine production in adult female mosquitos (Cady and Hagedorn, 1999a). Using the sequences of the Aedae-K as a BLAST query of the *A. gambiae* genome (Holt *et al.*, 2002), a single gene encoding three different kinins of 15, 10, and 21 residues was located: Anoga-K-1, -K-2, and -K-3. These peptides occur in the gene in the size order shown; the first and second are separated only by the amino acid residues Gly-Lys-Arg, as are the second and third (residues necessary for cleavage from the propeptide and formation of the amidated C-terminus). There are no reports of their synthesis and biological properties as yet.

Holman *et al.* (1999) isolated a 15 residue kinin from another fly species, *M. domestica*, using an ELISA with an antibody raised against Leuma-K-1. Musdo-K strongly stimulates fluid secretion in *M. domestica* MT, whereas the hexapeptide Achdo-K-5, identical with the six C-terminal residues of Musdo-K, is 1000-fold less potent in this assay. Thus, the longer sequence is crucial for full activity in *M. domestica*. Musdo-K also stimulates the housefly hindgut, possibly aiding in excretion. Later that year, Terhzaz *et al.* (1999) reported the isolation of a single 15 residue kinin from *D. melanogaster*, using a purification strategy based on that for the Aedae-K peptides. The sequence differs from Musdo-K at the second residue, a Thr in Musdo-K versus a Ser in Drome-K. A portion of the gene encoding a partial propeptide was determined from a BLAST search of sequenced bacterial artificial chromosomes of the then-incomplete *D. melanogaster* genome. Studies of the effects of Aedae-K on MT showed that it elevates intracellular Ca^{2+}, but not cAMP. A BLAST search of the now-complete genome of this species verifies that the *pp* gene encodes but a single kinin.

The isolation of kinins from a lepidopteran insect, the corn earworm *Helicoverpa zea*, is of interest because only the first step utilized a functional assay. Blackburn *et al.* (1995) used ~2000 abdominal ventral nerve cords (VNC) from adult moths as tissue source. Homogenized tissues were prepurified on a Sep-Pak C_{18}, and then fractionated on RPLC. Fractions were assayed using a Ramsay assay with MT of adult *M. sexta*. Three fractions with diuretic activity coincided with peaks having strong absorbance at 280 nm, which gave second derivative UV spectra characteristic of Trp, one of three amino acids totally conserved in the kinins. Subsequent purification steps were monitored based on the diagnostic UV spectral behavior of Trp, rather than by using a functional assay. The peak eluting first was purified to homogeneity in one more RPLC step, and sequenced by Edman degradation (Helze-K-III; see **Figure 6**). The other two peptides, present in

```
Leuma-K-1   -------------DPAFNSWGa
Peram-K-5   -------------SPAFNSWGa
Peram-K-1   -------------RPSFNSWGa
Peram-K-3   -------------DPSFNSWGa
Leuma-K-3   -------------DQGFNSWGa
Leuma-K-7   -------------DPAFSSWGa
Locmi-K     ---------------AFSSWGa
Leuma-K-2   -------------DPGFSSWGa
Leuma-K-4   -------------DASFHSWGa
Peram-K-2   -------------DASFSSWGa
Musdo-K     ------NTVVLGKKQRFHSWGa
Drome-K     ------NSVVLGKKQRFHSWGa
Achdo-K-5   ---------------AFHSWGa
Leuma-K-6   -------------QSSFHSWGa
Culsa-K-1   --------------NPFHSWGa
Anoga-K-3   NMPRTHKQPKVVIRNPFHSWGa
Aedae-K-2   --------------NPFHAWGa
Culsa-K-3   --------WKYVSKQKFFSWGa
Aedae-K-1   -------NSKYVSKQKFYSWGa
Anoga-K-1   ------DTPRYVSKQKFHSWGa
Leuma-K-8   -------------GADFYSWGa
Achdo-K-1   ------------SGADFYPWGa
Peram-K-4   -------------GAQFSSWGa
Leuma-K-5   -------------GSGFSSWGa
Achdo-K-3   -------------ALPFSSWGa
Achdo-K-2   ---------------AYFSPWGa
Helze-K-1   ----------------YFSPWGa
Culsa-K-2   -----------NNANVFYPWGa
Aedae-K-3   -----------NNPNVFYPWGa
Anoga-K-2   -----------NTAQVFYPWGa
Achdo-K-4   -------------NFKFNPWGa
Helze-K-2   ---------------VRFSPWGa
Helze-K-3   -------------KVKFSAWGa
```

Figure 6 A multiple sequence alignment (Clustal W, V. 1.8) of the 33 known insect kinins from nine species. Boxed residues are identical. At the C-terminus, a five amino acid motif is highly conserved: FX_1X_2WGamide. X_1 is N, S, Y, or H in all known sequences. X_2 is usually S, but may be P or A. The first kinins were isolated from *Leucophaea maderae* (Leuma). For the definition of other species' abbreviations, see text. Note most of the dipteran kinins cluster towards the center of the alignment. There is immunocytochemical evidence for the presence of kinins in over 15 species of insects. Interested parties are referred to a recent, massively comprehensive review (Coast *et al.*, 2002a).

smaller quantity, required two additional steps for purification to homogeneity. The Trp residue could not be detected by Edman sequencing in these two kinins, which were additionally sequenced by tandem MS sequencing methods. The deduced sequences of all Helze-Ks were synthesized, and bioassayed on MT of *M. sexta*. No EC_{50} values were determined, only "threshold values": the lowest concentration at which a significant effect on fluid secretion can be measured. These were $\sim$7 pmol l^{-1} for Helze-K-I, 0.6 pmol l^{-1} for Helze-K-II, and 6 pmol l^{-1} for Helze-K-III. None of these peptides had activity *in vivo* in *H. zea*, but they do not appear to have been tested *in vitro* in *H. zea*, or *in vivo* in *M. sexta*, so no valid comparison can be drawn. While kinins have not yet been identified in *R. prolixus*, partially purified extracts from neuroendocrine tissue of this species, which contain kinin-like immunoreactivity, do not stimulate tubule secretion, but are active on the hindgut (Te Brugge *et al.*, 2002).

3.10.3.5. Calcitonin-Like DH

The first member of this family was identified together with the CRF-like Dippu-DH_{46} isolated from 4000 brains of *D. punctata* (Furuya *et al.*, 2000b). During the first step of RPLC purification of extracts, only one fraction (corresponding to Dippu-DH_{46}) stimulated cAMP production by MT of *M. sexta*, yet this fraction and a faster eluting fraction both elevated cAMP production by MT of *S. americana*. Because the *M. sexta* assay is easier, it was utilized to isolate Dippu-DH_{46}. Isolation to homogeneity of the second factor was accomplished using MT of *S. americana*. Upon sequence analysis, it was immediately apparent that this 31 residue peptide was not a member of the CRF-like DH. A BLAST search revealed similarities to certain proteins, but no highly significant resemblance to any bioactive peptides. Nevertheless, a manual search through the catalog of a commercial supplier of peptides (Peninsula Laboratories) for the unusual GP-NH_2 C-terminal sequence revealed an interesting similarity to

calcitonin; in fact, only the calcitonin family has a Pro-NH_2 at the C-terminus of all bioactive peptides listed in this catalog. In the alignment shown in **Figure 7**, only 6 of 31 residues of Dippu-DH_{31} are well conserved in the calcitonin family. However, chicken calcitonin is only 30-fold less potent in a fluid secretion assay than Dippu-DH_{31} using MT of *D. punctata* (Furuya *et al*., 2000b), suggesting this to be a genuine homology: one of the main actions of calcitonin is lowering blood Ca^{2+} by increasing Ca^{2+} excretion by the kidneys, in addition to increasing Ca^{2+} deposition in bone, which require active transport mechanisms. Interestingly, Dippu-DH_{31} and Dippu-DH_{46} have potent synergistic interaction in *D. punctata* (Furuya *et al*., 2000b). The actions of Dippu-DH_{31} in *L. migratoria* are more consistent with an elevation of intracellular Ca^{2+} in this species (Furuya *et al*., 2000b). These apparent differences in signaling are not surprising in light of the fact that a single, cloned calcitonin receptor has been shown to be capable of activating both the adenylate cyclase and phospholipase C signal transduction systems (Chabre *et al*., 1992; Force *et al*., 1992).

To date, the only other characterized CT-like DH is that cloned "*in silico*" from the genome of *D. melanogaster* (gene Dh31 CG13094); this peptide, Drome-DH_{31}, was synthesized and shown to be a potent diuretic in fruit fly tubules, and to act via adenylate cyclase (Coast *et al*., 2001). Another homolog of Dippu-DH_{31} exists in the genome of *A. gambiae* (BX038390) (Riehle *et al*., 2002), Anoga-DH_{31}, but its biological function has not yet been studied. Moreover, with the recent availability of preliminary genome sequence data for *B. mori* and *A. mellifera*, genes encoding CT-like homologs exist in these two species (Bommo-DH_{31} and Apime-DH_{31}). Using a direct ELISA with an affinity-purified Dippu-DH_{31} antibody, immunoreactivity was observed in head extracts of *M. sexta*, *T. molitor*, *R. prolixus*, *A. domesticus*, *Blaptica dubia*, and the Mormon cricket, *Anabrus simplex* (V.C. Lombardi, J.J. Hull, V. Te Brugge, and D.A. Schooley, unpublished data). At the time of writing, isolation of the *M. sexta* ortholog of Dippu-DH_{31} is imminent. Thus, it is likely that the CT-like DHs constitute another family of DHs of widespread occurrence within the Insecta. The partial sequence of a similar peptide isolated from the Belgian forest ant, *Formica polyctena*, was published in a Ph.D. thesis (Laenen, 1999). The isolation of the factor was monitored by observing its stimulation of MT writhing in *L. migratoria*: sequence analysis of this peptide was only possible out to 29 residues, but all are identical with the first 29 residues of Dippu-DH_{31} (see **Figure 7**). MS analysis revealed that the sequence was incomplete; the last two amino acids not detected must be different from those in Dippu-DH_{31} because the M_r is lower than that of the latter peptide (Laenen, 1999). Because the peptide lacked any diuretic activity on tubules of *F. polyctena*, from which it was isolated, it was not investigated further. Unfortunately, this discovery predated the publication of the sequence of Dippu-DH_{31}; the sequence identity might have spurred completion of the sequence of the ant peptide despite its lack of diuretic activity in the host species.

Chick CT	CASLSTCVLGKLSQE	LHKLQTYPRTDVGAG	TP-NH_2
Eel CT	CSALSTCVLGKLSQE	LHKLQTYPRTDVGAG	TP-NH_2
Human CT	CGNLSTCMLGTYTQD	FNKFHTFPQTAIGVG	AP-NH_2
Porcine CT	CSNLSTCVLSAYWRN	LNNFHRFSGMGFGPE	TP-NH_2
Rat CT	CGNLSTCMLGTYTQD	LNKFHTFPQTSIGVG	AP-NH_2
Salmon CT	CSNLSTCVLGKLSQE	LHKLQTYPRTNTGSG	TP-NH_2
Apime-DH_{31}	GLDLGLSRGFSGSQA	AKHLMGLAAANY-AG	GP-NH_2
Dippu-DH_{31}	GLDLGLSRGFSGSQA	AKHLMGLAAANY-AG	GP-NH_2
Forpo-DH	GLDLGLSRGFSGSQA	AKHLMGLAAANY-AG	XX
Drome-DH_{31}	TVDFGLARGYSGTQE	AKHRMGLAAANF-AG	GP-NH_2
Anoga-DH_{31}	TVDFGLSRGYSGAQE	AKHRMAMAVANF-AG	GP-NH_2
Bommo-DH_{31}	AFDLGLGRGYSGALQ	AKHLMGLAAANF-AG	GP-NH_2

Figure 7 The known calcitonin-like DHs are shown using the Swiss Prot species abbreviation convention: Forpo, *Formica polyctena*; other species abbreviations are given in text. In this figure, red is conserved in the calcitonin family; blue is conserved in Dippu-DH_{31} and homologs. Very recently (while this article was in press), the *Bombyx mori* (Bommo) genome became available. Limited genome sequence data also became available for *Apis mellifera* (Apime).

3.10.3.6. CAP_{2b}/Periviscerokinins

Manse-CAP_{2b} was isolated from *M. sexta* as a cardioactive factor (Huesmann *et al*., 1995). Davies *et al*. (1995) found that it stimulates fluid secretion from MT of *D. melanogaster* by the unusual action (for a peptide hormone) of elevating production of nitric oxide, which in turn activates a soluble guanylate cyclase. This peptide was reported, based exclusively on the retention time of biological activity from *D. melanogaster* tissue extracts on RPLC, to be an endogenous peptide in *D. melanogaster*. However, a BLAST search of the *D. melanogaster* genome (Vanden Broeck, 2001) revealed the existence of three homologs of CAP_{2b} with 8, 10, and 12 amino acid residues, having 6, 7, and 5 amino acid identities, respectively, to Manse-CAP_{2b} (**Figure 8**). Garside and Coast (unpublished data) have studied the activity of Manse-CAP_{2b} and Drome-capa-2 on MT of houseflies; in this species they are diuretic but signal transduction is not via NO or cGMP, but appears to result from an increase in intracellular Ca^{2+}. Predel *et al*. (1995) isolated a myotropin from perisympathetic organs of the cockroach *P. americana* christened periviscerokinin, which

Drome-capa-1	-	-	-	G	A	N	M	G	L	Y	A	F	P	R	V	a
Manse-CAP_{2b}	-	-	-	-	-	-	p	Q	L	Y	A	F	P	R	V	a
Anoga-CAP_{2b12}	-	-	-	G	P	T	V	G	L	F	A	F	P	R	V	a
Locmi-PVK-1	-	-	-	-	-	A	A	G	L	F	Q	F	P	R	V	a
Drome-capa-2	-	-	-	-	-	A	S	G	L	V	A	F	P	R	V	a
Anoga-CAP_{2b9}	-	-	-	-	-	p	Q	G	L	V	P	F	P	R	V	a
Peram-PVK-1	-	-	-	G	A	S	-	G	L	I	P	V	M	R	N	a
Leuma-PVK-1	-	-	-	G	S	S	-	G	L	I	P	F	G	R	T	a
Leuma-PVK-3	-	-	-	G	S	S	-	G	M	I	P	F	P	R	V	a
Peram-PVK-2	-	-	-	G	S	S	S	G	L	I	S	M	P	R	V	a
Leuma-PVK-2	-	-	-	-	G	S	S	G	L	I	S	M	P	R	V	a
Drome-capa-3	T	G	P	S	A	S	S	G	L	W	G	-	P	R	L	a

Figure 8 The *Manduca sexta* CAP_{2b} family of peptides. All are amidated at the C-terminus (indicated with lower case a), and two (Manse-CAP_{2b} and Anoga-CAP_{2b9} have pyroglutamic acid as the N-terminal residue (denoted by pQ, because Gln (Q) is the precursor of pGlu). At least one of the peptides with low sequence similarity in the C-terminal domain (FPRVa), Peram-PVK-1 (from *Periplaneta americana*), has no diuretic activity, suggesting that the conserved C-terminal motif is crucial for activity.

seemed to be a peptide unrelated to other families of peptides. However, subsequently, this group (Predel *et al.*, 1998) isolated a second peptide from this source, Peram-PVK-2, whose sequence was obviously related to not only Peram-PVK-1 but also to CAP_{2b}. The CAP_{2b} family now consists of 14 members, with the addition of three peptides from the cockroach *L. maderae* (Predel *et al.*, 2000), one from the locust (Predel and Gäde, 2002), and three from the genome of *A. gambiae* (Riehle *et al.*, 2002) (**Figure 8**). Recently, partial sequences for two new members of this family were identified using highly sensitive mass spectrometric assays (Clynen *et al.*, 2003b) on single perisympathetic organs from two fly species, *Neobelleria bullata* and *M. domestica*. These sequences are incomplete because of the inability of MS analysis to distinguish between Ile and Leu in peptides, and because of lack of certain crucial diagnostic ions and are therefore not given in **Figure 8**. It is novel that this approach to isolation and identification does not require a bioassay.

3.10.3.7. Arginine Vasopressin-Like Insect Diuretic Hormone

Proux *et al.* (1982) found that a factor in the subesophageal ganglia of *L. migratoria* stimulated *in vivo* clearance of injected amaranth from the hemolymph of locusts, an established assay for diuresis in this species (Mordue, 1969), and obtained evidence that this material was identical to a factor that cross-reacts with arginine vasopressin (AVP). This factor was isolated from 51 000 dissected subesophageal and thoracic ganglia using RPLC techniques with monitoring of fractions with a radioimmunoassay for AVP. In the first step of isolation two factors were separated, both of which were purified to homogeneity (Schooley *et al.*, 1987). Injection of the purified factors into *S. gregaria* revealed that only the less abundant, slower eluting factor (F2) caused amaranth excretion. The faster eluting F1 and slower eluting F2 were found to have identical amino acid compositions and identical primary sequences: Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2. This peptide was synthesized in both the amidated and free acid forms, and after reduction and carboxymethylation was compared with the similarly modified forms of F1 and F2 prepared for sequence analysis. This comparison established that F1 was identical to the nonapeptide whose sequence is shown above. Analysis of both materials by size exclusion chromatography suggested that F2 was a dimer of F1. Two separate, difficult specific syntheses were performed to prepare pure F2 as parallel and antiparallel dimers (Proux *et al.*, 1987). The synthetic antiparallel dimer proved to have identical retention properties on RPLC with F2 (Proux *et al.*, 1987). Natural and synthetic F2 were shown to promote fluid secretion in an unusual fluid secretion assay in which a group of *L. migratoria* MT attached to the ampulla were removed, maintained in oxygenated saline, and the high combined flow of all tubules monitored (Proux *et al.*, 1988).

Later, this work was called into question by Coast *et al.* (1993), who reported that synthetic F2 had no stimulatory activity on a single *L. migratoria* MT in a conventional Ramsay assay, although Locmi-DH (a CRF-like peptide) had potent activity. Later, Montuenga *et al.* (1996) showed that there are endocrine cells in the ampulla of *L. migratoria* that contain not only Locmi-DH, but a second, as yet uncharacterized peptide, which is recognized by a Locmi-DH antiserum but is faster eluting on RPLC than the latter peptide. It is conceivable that the "AVP-like DH" may stimulate the release of Locmi-DH from these endocrine cells. The localization of AVP-like immunoreactivity has been determined in the locust (Thompson *et al.*, 1991); the titer of AVP-like immunoreactivity changes in the hemolymph in response to unknown stimuli and neurons containing it innervate nonocular photoreceptors (Thompson and Bacon, 1991). Analysis by RPLC of AVP-like containing neurons showed them to contain not only the antiparallel dimer F2, but also the parallel dimer and the monomer F1 (Baines *et al.*, 1995). Thus, there is strong evidence that the AVP-like factor is a *bona fide* neuropeptide, although its role remains controversial.

3.10.3.8. Other Peptides with Diuretic Activity

Skaer *et al.* (2002) tested three different tachykinin-related peptides (TRP) on MT of pharate adult

M. sexta: locustatachykinin-1 from *L. migratoria* (Locmi-TK-1; GPSGFYGVRamide; Schoofs *et al.*, 1993) and two TRPs from *L. maderae* (Leuma TRP-1, APSGFLGVRamide; and Leuma-TRP-4, APSGFMGMRamide; Muren and Nässel, 1996). Each TRP was tested at four concentrations from 1 nmol l^{-1} to 1 μmol l^{-1}. Each TRP exhibited a dose dependent increase in the rate of fluid secretion, but a maximal response was reached only 30–40 min after application. While no EC_{50} values were measured, they appeared to be in the range of 10–100 nmol l^{-1}. Leuma-TRP-1 gave the highest maximal secretion of the three; at 1 μmol l^{-1}, it increased the rate of fluid secretion 2.83-fold compared to that of the control. In contrast to the CAPs and leucokinin, TRP effects on tubule secretion activity were not long lasting. Two cardioactive peptides of as yet unknown sequence, CAP_{1a} and CAP_{1b} (Tublitz *et al.*, 1991), strongly stimulate *M. sexta* MT; there is a distinct probability they could be kinins. The very slow response (20–30 min delay) observed as a result of treatment of tubules with all agonists may be attributable to their use of 8–12 cm long pieces of a single MT (Skaer *et al.*, 2002); *M. sexta* tubules tend to collapse on dissection and the delay in fluid secretion may result from the filling time of the tubule (G.M. Coast, unpublished data).

Recently, Locmi-TK-1 has been reported to have an EC_{50} of 1.2 nmol l^{-1} in *L. migratoria*, with an identical value reported for *S. gregaria* (Johard *et al.*, 2003). The response observed was about 75% of that observed in response to treatment with Locmi-DH; the latter was found to have synergistic activity with both Locmi-TK-1 and serotonin. In light of the response observed to this peptide in these two distantly related species (*M. sexta* versus locusts), further studies of the effects of these peptides will be of very great interest.

Three fractions were isolated from a head extract of *A. aegypti* by RPLC based on their effect on the transepithelial potential (TEP) of isolated perfused MT (Petzel *et al.*, 1985). Each fraction contained a pronase-sensitive peptide with M_r values estimated by gel filtration chromatography to be 2400 (fraction I), 2700 (fraction II), and 1860 Da (fraction III) (Petzel *et al.*, 1986). Fraction I depolarized the TEP. While it has no effect on fluid secretion, it increases tritiated water (THO) loss and urine output from intact flies, possibly by inhibiting fluid uptake from the hindgut (Wheelock *et al.*, 1988). Fraction II also depolarized the TEP, with a biphasic response; the TEP first depolarized, and then hyperpolarized. Fractions II and III both have diuretic activity and selectively stimulate secretion of NaCl-rich urine. Fraction III was the more potent of these two; its diuretic and natriuretic activity were indistinguishable from that of exogenous cyclic AMP, although the latter only hyperpolarized the TEP (Petzel *et al.*, 1985). Fraction III was named mosquito natriuretic peptide (MNP) and was shown subsequently to stimulate cyclic AMP production in isolated tubules (Petzel *et al.*, 1987). Hegarty *et al.* (1991) found that dibutyryl cAMP stimulated Na^+ secretion from *A. aegypti* MT, with a concomitant decrease in K^+ secretion. Unfortunately, this factor is still unidentified.

3.10.3.9. Serotonin and Other Biogenic Amines

The biogenic amine serotonin (5-hydroxytryptamine; 5HT) is known to stimulate diuresis in a variety of insects, including *R. prolixus* (Maddrell *et al.*, 1969), *L. migratoria* (Morgan and Mordue, 1984; cAMP independent), *P. brassicae* (Nicolson and Millar, 1983), and *M. sexta* (Skaer *et al.*, 2002). In most cases, levels required for stimulation are high: in *R. prolixus*, it stimulates distal tubules up to 1000-fold via a cAMP dependent mechanism with EC_{50} ~30–40 nmol l^{-1} (Maddrell *et al.*, 1993a), whereas the response of *L. migratoria* tubules is only 25% of the maximum obtained with a CC extract and is mediated by a different second messenger, possibly Ca^{2+} (EC_{50} 10–100 nM; Morgan and Mordue, 1984). The response of *A. aegypti* tubules to serotonin varies with the stage of development. In larvae, it acts via cAMP to stimulate maximal secretion (EC_{50} ~100 nM; Clark and Bradley, 1996; Clark *et al.*, 1998a). In contrast, the response of adult tubules is ~25% of maximal (EC_{50} ~1 μM) and both cAMP and inositol trisphosphate (IP_3) production are increased (Veenstra, 1988; Cady and Hagedorn, 1999b). In *M. sexta* four concentrations were studied; while no EC_{50} value was reported, it appears to be in the range of 100–300 μmol l^{-1} (Skaer *et al.*, 2002). Octopamine also stimulates *M. sexta* tubules, but is much less potent. Fournier *et al.* (1994) reported that serotonin has antidiuretic effects in the rectum of *L. migratoria*.

Recently, Blumenthal (2003) has shown tyramine to be a potent stimulant of *D. melanogaster* MT, acting at levels as low as 1 nmol l^{-1}. Tyramine is several orders of magnitude more active than octopamine or dopamine. Interestingly, tyramine is produced in the MT from tyrosine taken up from the hemolymph, or culture medium, via tyrosine decarboxylase, and a tyramine receptor is also expressed in MT of this species. Tyramine is believed to play an autocrine or paracrine role; it causes fluctuations in the basolateral membrane transepithelial potential.

Clearly this discovery merits studies in other species; this is the first demonstration of a physiological role for a tyramine receptor in *D. melanogaster* (Blumenthal, 2003).

3.10.3.10. Antidiuretic Factors Inhibiting Malpighian Tubule Secretion

Spring *et al.* (1988) characterized an antidiuretic hormone from hemolymph of *A. domesticus* kept under very dry conditions; an extract of this hemolymph inhibited secretion by MT of control insects. Hemolymph extract from insects reared under normal humidity did not inhibit MT secretion. Until recently, no factor had been isolated with such activity. Lavigne *et al.* (2001) published a partial purification of a factor from *Leptinotarsa decemlineata* through five RPLC steps using only two different columns. The protocol they used involved minimal changes of parameters between successive purifications, and utilized evaporation steps between RPLC purifications likely to result in poor recovery. While no peak was visible in the final separation step, they reported that the apparent molecular size of the factor (estimated by dialysis) was in the range of 25–50 amino acids. The first factor to be identified that inhibits MT secretion, Tenmo-ADFa, was isolated from pupal heads of *T. molitor*, based on its ability to elevate cyclic GMP (cGMP) levels in MT of this species. Tenmo-ADFa was found to be a 14-mer with the sequence VVNTPGHAVSYHVY-OH, lacking the amidation usually found in regulatory peptides. It is exquisitely potent with an EC_{50} of 10 fmol l^{-1} (0.01 pmol l^{-1}) (Eigenheer *et al.*, 2002), but receptor downregulation occurs at high concentrations (such as 1 nmol l^{-1}!). A second factor isolated from the same source, Tenmo-ADFb, is a 13-mer with the sequence YDDGSYKPHIYGF-OH, again nonamidated. It is 24 000 times less potent than Tenmo-ADFa, but still has $EC_{50} = 0.24$ nmol l^{-1} (Eigenheer *et al.*, 2003). These factors have poor solubility in the acidic solvents usually used for extracting neuropeptides from tissues. Following typical isolation protocols resulted in low yields. However, upon discovering that the bulk of the activity remained in the pellet from acid extraction, the pellet was extracted with a neutral solvent, which by then had so many impurities removed that only three RPLC purification steps were required to purify the factors from the neutral extract to homogeneity. Curiously, these factors have extremely high sequence similarity to two cuticular proteins of this beetle: Tenmo-ADFa is identical to the C-terminus of the protein CAA03880 (Mathelin *et al.*, 1998) at all residues, except that Thr4 in the peptide is an Ala residue in the protein (**Figure 9**). Tenmo-ADFb is 100% identical with the 13 C-terminal residues of *T. molitor* putative cuticle protein 9.2 (TmPCP9.2) (Baernholdt and Andersen, 1998). Consequently, the identification of ADFb was not published until immunohistochemical evidence showed specific staining for Tenmo-ADFb-like material in two pairs of bilaterally symmetrical neurosecretory cells of the protocerebrum of *T. molitor* (Eigenheer *et al.*, 2003). While the cuticle protein TmPCP9.2 lacks obvious enzymatic cleavage sites upstream of the portion identical with ADFb, this identity suggested the possibility that it could be a proteolysis fragment of the cuticle protein, and that this fragment might have coincidental biological activity. Tenmo-ADFa does have an interesting sequence identity to big endothelin 1 (**Figure 9**). The sequence identity to rabbit endothelin 1 (8 residues) and human endothelin 1 (7 residues) begins at exactly that bond where endothelin converting enzyme cleaves big endothelin into the potent vasoconstrictor endothelin 1. This cleavage site is marked with an arrow in **Figure 9**.

Recently Coast (unpublished data) has found that *M. sexta* allatotropin (Kataoka *et al.*, 1989a; Manse-AT) inhibits the Manse-DH stimulated secretion of fluid by larval MT of this species at 50–200 nmol l^{-1} levels. This is the same concentration found earlier to inhibit fluid uptake from the anterior midgut (Lee *et al.*, 1998) of *M. sexta*.

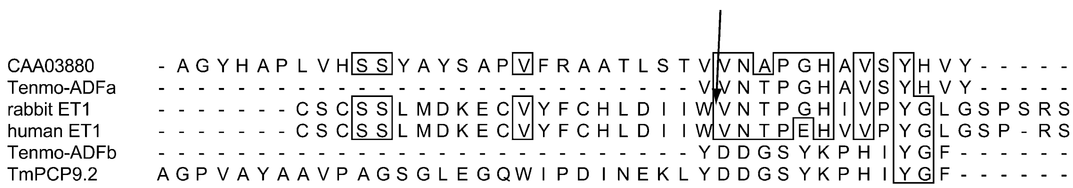


Figure 9 Two antidiuretic factors from *Tenebrio molitor*: Tenmo-ADFa and Tenmo-ADFb. Tenmo-ADFa is identical at all but one residue with the C-terminus of the cuticle protein CAA03880 from this species (Mathelin *et al.*, 1998), and Tenmo-ADFb is 100% identical with the 13 C-terminal residues of another cuticle protein from this species, TmPCP9.2 (Baernholdt and Andersen, 1998). In addition, Tenmo-ADFa has an interesting sequence identity with big endothelin 1, a precursor of the potent vasoconstrictor endothelin 1. Endothelin converting enzyme cleaves big endothelin at the bond indicated by an arrow in the figure; this is exactly where the sequence identity with ADFa commences. Thus, the identity is to the apparently inactive endothelin fragment removed in processing.

Incubation of 1 nmol l^{-1} Manse-DH with and without 50 nmol l^{-1} Manse-AT (E. Chidembo and D.A. Schooley, unpublished data) shows that Manse-AT seems to act via lowering cAMP levels.

3.10.3.11. Antidiuretic Factors that Promote Fluid Reabsorption in the Hindgut

Hormonal control of fluid reabsorption in the hindgut has been heavily studied in locusts (*S. gregaria* and *L. migratoria*). Three neuropeptides have been either isolated or characterized from the CC of locusts (reviews: Phillips *et al.*, 1988, 1986): neuroparsins, ion transport peptide (ITP), and chloride transport stimulating hormone (CTSH). Only the first two have been fully sequenced; CTSH is labile to the usual conditions used for RPLC isolation of peptides, due to its instability in acidic conditions.

Herault *et al.* (1985) reported that the nervous and glandular lobes of the CC of *L. migratoria* each contain a factor acting as an ADH on the rectum; these factors differ in size and extraction properties. The glandular lobe (GCC) factor was not purified, but Herault and Proux (1987) reported that GCC extracts cause a peak in rectal tissue cAMP levels coinciding with elevated short circuit current (I_{sc}). This stimulation is mimicked by forskolin, a stimulant of adenylate cyclase. The factor from the CC was reported to be a neuroparsin (Np), because all antidiuretic activity in crude CC of *L. migratoria* was abolished by an antibody to this neuropeptide (Herault *et al.*, 1988). Neuroparsins are proteins (NpA, NpB) isolated and sequenced from CC of *L. migratoria* (Girardie *et al.*, 1989, 1990). They have also been reported to have hypertrehalosemic (Moreau *et al.*, 1988) and antijuvenile hormone activity (Girardie *et al.*, 1987). NpB was reported to be a homodimer of a 78-residue polypeptide. NpA is identical to NpB except for having a heterogeneous N-terminus, the longest form of which has 83 residues. NpB is thought to be formed from NpA by enzymatic cleavage of the N-terminal sequence. Subsequently Hietter *et al.* (1991) confirmed the sequences of these small proteins, including the N-terminal heterogeneity, but revised the structural assignment for neuroparsins involving three internal disulfide bridges, in which the chains are monomeric. Fournier (1991) presented a body of data consistent with NpB acting on rectal fluid reabsorption by stimulating the inositol phosphate (IP) cascade with subsequent elevation of cytosolic Ca^{2+}. They concluded that neuroparsins are the only ADH in *L. migratoria* GCC and that these peptides act via the IP-Ca^{2+} second messenger system, whereas serotonin from the NCC acts on the rectum via a cAMP mediated Ca^{2+} increase (Fournier *et al.*, 1994). They did not study actions of purified or synthetic Nps on rectal solute transport processes. Jeffs and Phillips (1996) found no effect of Locmi-Nps on either rectal ion or fluid transport in the locust *S. gregaria*. They attributed this either to fundamental differences in transport properties in these closely related insects or, more likely, to the use of Cl^--free saline in the assay used (Fournier *et al.*, 1987) for preincubation of everted rectal sacs for 1 h.

Chloride transport stimulating hormone, a peptide occurring in the CC of *S. gregaria*, was characterized by its ability to increase short circuit current in *S. gregaria* rectal sheets maintained in Ussing chambers (Phillips *et al.*, 1980). In this assay, either cAMP or CC extracts give a ten-fold increase in I_{sc} for >8 h after stimulation. A single zone is seen on size exclusion chromatography with M_r of about 8000 Da. The peptide nature is proved by its sensitivity to trypsin, and it is active at what appear to be nmol l^{-1} concentrations (Phillips *et al.*, 1986). The peptide can be extracted with water, saline, and aqueous ethanol, but biological activity is lost rapidly below pH 6. This may be consistent with the presence of an Asp-Pro bond in the primary sequence, which, as mentioned in Section 3.10.3.3, is suspected of causing an inability to isolate two CRF-like DH by RPLC techniques as well. Proux *et al.* (1985) provided some evidence that CTSH is produced in the pars intercerebralis and is transferred down nerves to the glandular lobe of the CC, after passing through the nervous lobe of the CC. CTSH is similar in size to Locmi-Nps, but is distinguished from the latter by the stability of Locmi-Nps to acidic conditions. Also, Locmi-Nps are reported to act via stimulation of phospholipase C.

Audsley and Phillips (1990) used an assay similar to that for CTSH, substituting locust ileum for rectum, and found stimulants of ion transport in both the CC and the ventral ganglia. The effects elicited by CC and ganglia extracts differ in properties. The factor in the CC, unlike CTSH, is stable to acid. This stability led to its successful isolation by RPLC, and a partial sequence was obtained to 31 amino acids (Audsley *et al.*, 1992b). This peptide was christened ion transport peptide (Schgr-ITP), to distinguish it from CTSH. It was found to be 44–59% identical to the crustacean hyperglycemic (CHH), molt inhibiting (MIH), and vitellogenesis inhibiting (VIH) hormones, a group of highly related 72 amino acid peptides (Audsley *et al.*, 1992b). This was the first direct evidence for the existence of a peptide related to this family outside crustaceans. Audsley *et al.* (1992a) tested purified Schgr-ITP on the rectum and found that it had weak effects on short circuit

current compared with CTSH, and no effect on the rate of fluid reabsorption. They concluded that separate neuropeptides act on the ileum and rectum of the locust.

Meredith *et al.* (1996) used degenerate primers designed from the partial protein sequence to clone by PCR a partial sequence of Schgr-ITP from a brain cDNA library. Use of 5′ and 3′ rapid amplification of cDNA ends (RACE) strategies led to isolation of a cDNA of 517 bp encoding a complete open reading frame for Schgr-ITP prepropeptide of 130 amino acid residues (Meredith *et al.*, 1996). The prepropeptide has a 55-residue signal peptide and a dibasic cleavage site preceding the start of the partial ITP sequence determined by Audsley *et al.* (1992b). The C-terminal Leu is followed by Gly-Lys-Lys-stop, which is consistent with C-terminal amidation and a second dibasic cleavage site to give the complete ITP sequence of 72 amino acid residues. Meredith *et al.* (1996) also used ITP cDNA sequence primers to probe a locust ileal mRNA library, and isolated an ITP-like clone, which was sequenced. It was identical to the brain cDNA for ITP except for an additional 121 bp insert at amino acid position 40 of ITP, suggesting alternative C-termini splicing of genomic DNA. The open reading frame for ITP-L (134 residues) is four residues longer than that of ITP (**Figure 10**). All six cysteines are conserved. The unique C-terminus of ITP-L has only 14 of the last 36 amino acid residues in common with ITP, most of the difference being over the last 20 residues. Using reverse transcriptase PCR (RT-PCR) techniques, ITP-L mRNA was detected in tissues (flight muscle, hindgut, and MTs) whose extracts have no stimulatory effect in the locust ileal I_{sc} bioassay, while ITP mRNA was found only in the brain and CC, which do stimulate ileal I_{sc}. The ITP-L peptide does not stimulate the locust ileal I_{sc} bioassay, but acts as a weak antagonist (Wang *et al.*, 2000). The Schgr-ITP synthesized by solid-phase peptide synthesis was oxidized to the appropriately folded form with three disulfide bonds (King *et al.*, 1999); this was found to exhibit biological actions identical to that of the native peptide isolated from tissue.

While in the 1990s there was concern over conflicts in results between the proponents of neuroparsins versus Schgr-ITP, three publications in the area of crustacean neuroendocrinology strongly support the role of ITP as an authentic osmoregulator (see **Chapter 3.16**). Chung *et al.* (1999) reported a large, precisely timed release of CHH from gut endocrine cells in the crab *Carcinus maenas* at ecdysis. This release appears to trigger the water and ion uptake required during molting, which allows the swelling necessary for successful ecdysis and the subsequent increase in size during postmolt. The endogenous ortholog of CHH of the freshwater crayfish *Pachygrapsus marmoratus* (Spanings-Pierrot *et al.*, 2000) has been shown to play a crucial role in control of gill ion transport. In the crayfish *Astacus leptodactylus*, injection of CHH was found to increase the hemolymph osmolality and Na^+ concentration 24 h after injection (Serrano *et al.*, 2003). Two other CHH related peptides caused a smaller increase in Na^+ concentration.

Liao *et al.* (2000) characterized one of two factors that separate on RPLC of brain–CC–corpora allata extracts of larval *M. sexta*. These factors trigger fluid reabsorption in an everted rectal sac bioassay with *M. sexta*. Owing to the cryptonephric anatomy of larvae of this species, the CRF-like peptide Manse-DH also causes rectal fluid reabsorption. However, the effect of Manse-DH is blocked by bumetanide (an inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter), bafilomycin A_1, and amiloride, while that of the more potent of the two factors, Manse-ADFB, is not blocked by any of these inhibitors. The Cl^- channel blockers 4,4′-diisothiocyanatostilbene-2, 2′-disulforic acid disodium salt (DIDS) and diphenylamine-2-carboxylic acid (DPC) both block

```
Schgr-ITPL   - S F F D I Q C K G V Y D K S I F A R L D R I C E D C Y N L F R E P Q L H S L C R K D
Schgr-ITP    - S F F D I Q C K G V Y D K S I F A R L D R I C E D C Y N L F R E P Q L H S L C R S D
Bommo-ITP    - S F F T L E C K G V F D A A I F A R L D R I C D D C F N L F R E P Q L Y T L C R A E
Drome-ITP    S N F F D L E C K G I F N K T M F F R L D R I C E D C Y Q L F R E T S I H R L C K Q E
Carma-CHH    pQ I Y D T S C K G V Y D R A L F N D L E H V C D D C Y N L Y R T S Y V A S A C R S N
Homam-MIH    pQ V F D Q A C K G V Y D R N L F K K L D R V C E D C Y N L Y R K P F V A T T C R E N

Schgr-ITPL   C F T S D Y F K G C I D V L L L Q D D M D K I Q S W I K Q I H G A E P G V a
Schgr-ITP    C F K S P Y F K G C L Q A L L L I D E E E K F N Q M V E I L a - - - - - - -
Bommo-ITP    C F T T P Y F K G C M E S L Y L Y D E K E Q I D Q M I D F V a - - - - - - -
Drome-ITP    C F G S P F F N A C I E A L Q L H E E M D K Y N E W R D T L a - - - - - - -
Carma-CHH    C Y S N L V F R Q C M D D L L M M D E F D Q Y A R K V Q M V a - - - - - - -
Homam-MIH    C Y S N W V F R Q C L D D L L L S D V I D E Y V S N V Q M V a - - - - - - -
```

Figure 10 Alignment of Schgr-ITP with its *Bombyx mori* (Bommo-) and *Drosophila melanogaster* paralogs (Clustal W, V. 1.8), as well as the variant form Schgr-ITPL. Boxed residues are identical. Also shown are the highly similar *Carcinus maenas* (Carma-) crustacean hyperglycemic hormone, and *Homarus americanus* (Homam-) molt inhibiting hormone. All peptides are amidated at the C-terminus (denoted by lower case a); the two crustacean peptides are blocked at the N-terminus with pQ (pyroglutamate) residues.

the action of Manse-ADFB, as does H-89, a potent and specific inhibitor of protein kinase A. These data suggest that this factor of still unknown structure has a mechanism of action similar to Schgr-ITP. Homologs of ITP have been identified in *B. mori* (Endo *et al*., 2000) and in the genome of *D. melanogaster* (**Figure 10**): While neither has been synthesized or subjected to an activity assay, combined with the data on osmoregulatory effects of CHH, it seems likely that Manse-ADFB is similar to this family of peptides.

3.10.3.12. Cellular Mechanisms of Action

3.10.3.12.1. Introduction Does the diverse control of tubule secretion between different orders, and even between similar species belonging to the same order, have any rationale? Do differences in hemolymph composition, diet (and hence ion uptake), water availability, and the structure of the excretory system explain the diverse control strategies employed by different species? Primitive insect orders have a hemolymph characterized by a high concentration of Na^+ relative to K^+, and the sum of total anions and cations accounts for a considerable part of the total osmolarity (Sutcliffe, 1963). In contrast, more highly evolved insect orders have a greater proportion of hemolymph osmolarity attributable to high levels of amino acids and organic acids and, in some (Lepidoptera, Hymenoptera, and certain Coleoptera; Sutcliffe, 1963), K^+ is the dominant cation, with elevated concentrations of Mg^{2+} and Ca^{2+}. Since tubule secretion is driven by secondary active cation transport, differences in hemolymph composition might place some constraint on how fluid secretion is regulated. The same could apply to the dietary intake of ions, most notably K^+ and Na^+. If the diet is K^+-rich, as in herbivorous insects, then it is appropriate for K^+ to be the dominant cation secreted by the MT and to control secretion by manipulating the rate of K^+ transport. Conversely, hematophagous insects take infrequent blood meals, which imposes a considerable challenge on the excretory system for the removal of imbibed salt (NaCl) and water.

Not surprisingly, the MTs of mosquitos (*A. aegypti*) respond to released diuretics with a dramatic increase in secretion driven by a switch from K^+ transport to Na^+ transport (Beyenbach, 2003). Hence, the diuretic hormone (so far unidentified, but thought to be a CRF-like or CT-like DH) exerts both diuretic and natriuretic activity. Interestingly, in the laboratory, the same mechanism has been shown to operate in the tubules of male mosquitos (Plawner *et al*., 1991), which do not feed on blood but on nectar (Clements, 1992), resulting in a diet that is not rich in NaCl. Periods of diuresis that do not require a concomitant natriuresis must be initiated by the release of a different DH, possibly a myokinin, which, because it stimulates anion movement, has a neutral effect on cation transport. Can a similar story be developed for other species? *D. melanogaster* feeds on fermentation products (the alcoholic of the Insecta!), whereas the closely related *M. domestica* imbibes a mixture of saliva and the products of extracorporal digestion. A major drawback in this analysis is that most researchers have studied effects on fluid secretion rather than on ion transport. Peptides with apparently similar diuretic activity could well differ in their effect on urine composition and hence on ion transport (the product of concentration and the rate of secretion). An early demonstration of the possibility of such an occurrence is in MT of *A. aegypti* treated with bumetanide, a drug that inhibits the Na^+–K^+–$2Cl^-$ cotransporter stimulated by cAMP. Bumetanide treatment has little effect on basal fluid secretion, but decreases K^+ secretion with a concomitant increase in Na^+ secretion (Hegarty *et al*., 1991). Coast (1995) has shown that Locmi-DH (CRF-like) and locustakinin have fairly similar diuretic potency, but have quite different effects on ion transport. The majority of physiological studies to date on the action of synthetic peptides affecting MT function have focused on cellular effects, such as elevation of second messengers, or on fluid transport. Relatively few have focused on the effects of synthetic factors on ion transport, which, given the complex interplay of factors controlling fluid and ion homeostasis in vertebrates, is a serious omission. Moreover, in the cricket *Teleogryllus oceanicus*, the distal portion of the MT have been reported to secrete a hyperosmotic urine containing 125 mM Mg^{2+}, together with a lesser concentration of Na^+ (Xu and Marshall, 1999). There are few studies on Mg^{2+} transporters in insect systems, yet in a number of insect species with high hemolymph K^+, the concentration of Mg^{2+} is actually higher (Sutcliffe, 1963; Cohen and Patana, 1982; Dow *et al*., 1984). Thus, data on specific ion transport is an underinvestigated area likely to shed some light on why so many peptides are involved in control of water and salt balance in insects.

Water availability may also play a role in determining the cocktail of diuretics and antidiuretics used to control tubule secretion. The simplistic view that diuretics stimulate tubule secretion whereas antidiuretics increase fluid reabsorption in the hindgut is no longer tenable (at least in some species). To date, antidiuretic activity inhibiting the MT has been identified in three species. In *R. prolixus*,

the antidiuretic activity of CAP_{2b} has been linked to the need to rapidly turn off tubule secretion (Quinlan *et al*., 1997). Normally, the rapid degradation of a DH (probable half-life in the circulation measured in minutes) (Li *et al*., 1997) would suffice to end a period of diuresis. The situation in *R. prolixus* is very different, however, because the DH has such a dramatic effect on secretion, which at maximal rates would be sufficient to deplete the total store of water in the hemolymph within minutes. Under these conditions, it is essential that diuresis be switched off coincident with reduced fluid uptake from the midgut (Quinlan *et al*., 1997), and the insect now conserves water until the next blood meal.

In this respect, *R. prolixus* resembles a xeric insect in the period between its intermittent blood meals. It is of considerable interest that another insect shown to use antidiuretic factors to control tubule secretion is *T. molitor*, an extreme example of a xeric species able to survive very dry conditions (O'Donnell and Machin, 1991). In both *T. molitor* and *R. prolixus* (for which a native antidiuretic factor has still to be identified), antidiuresis appears to result from the activation of a cGMP dependent cAMP phosphodiesterase, which lowers intracellular levels of cAMP, the second messenger used by both CRF-like and CT-like DH. Not surprisingly, the antidiuretic factors of *T. molitor* are extremely potent (Eigenheer *et al*., 2002, 2003) and effectively counter the diuretic activity of native CRF-like DH (Wiehart *et al*., 2002). To grasp the importance of this, it is important to note that coleopteran larvae and adults have a cryptonephric system in which the distal ends of the MTs are closely associated with the rectum (Ramsay, 1964; Section 3.10.3.12.7). Fluid is reabsorbed from the rectum into the cryptonephric region of the tubules and flows into the gut at the midgut–hindgut junction, where it is postulated to move anteriorly directed and to moisten the dry food for digestion prior to reabsorption by the midgut (Nicolson, 1991). To gain water (the cryptonephric system is used to take up water from subsaturated atmospheres), fluid absorption from the free portion of the tubules must exceed fluid secretion, although these two processes could be taking place in functionally different segments. Unfortunately, nothing is known of fluid reabsorption from *T. molitor* tubules or whether antidiuretic factors have any effect on urine composition. Recent work (G.M. Coast, unpublished data) with MT from *M. sexta* larvae, which also have a cryptonephric system, shows that one segment (the yellow segment) can display net secretion or net reabsorption depending on the composition of the bathing fluid and the complement of peptides present. The two processes appear to be proceeding in parallel, with the overall balance tilted towards secretion or reabsorption by stimulants of each activity. The likelihood must be that similar mechanisms operate in *T. molitor* tubules.

3.10.3.12.2. CRF-like DH:cellular actions The CRF-like DHs studied to date all elevate cAMP in response to treatment of MT *in vitro*. As noted by Rafaeli *et al.* (1984), the cAMP is usually released from the tubule, which allows assay of the medium without having to homogenize and extract the tubules. This fact facilitates *in vitro* assays for isolating new members of this family. When using conspecific assays, CRF-like DH stimulate secretion by MTs of *A. domesticus* (Coast and Kay, 1994), *L. migratoria* (Patel *et al*., 1995), *D. punctata* (Furuya *et al*., 2000b), *M. domestica* (Iaboni *et al*., 1998), *T. molitor* (both Tenmo-DH_{37} and Tenmo-DH_{47}) (Wiehart *et al*., 2002), and both CRF-like DH of *M. sexta*, Manse-DH (Audsley *et al*., 1993, 1995), and Manse-DPII (Blackburn and Ma, 1994) at low nanomolar concentrations. Diuretic activity varies among species, from a maximal response in *A. domesticus* (Coast and Kay, 1994), equivalent to that obtained with a CC extract, to <25% of a maximal response in *M. domestica* (Iaboni *et al*., 1998), and probably depends upon whether anion or cation transport is the rate-limiting step in tubule secretion.

The potency of CRF-like DH varies much more in heterologous assays. Tubules of *M. sexta* are extremely flexible in cross-reacting with all members of the CRF-like DHs tested (Audsley *et al*., 1995), with the exceptions noted of the free acid form of Manse-DH and the two DHs of *T. molitor*, which have free acids at the carboxyl terminus. In contrast, tubules of *L. migratoria* seem to be very selective for their endogenous ligand; Peram-DP has an EC_{50} over 300-fold higher than Locmi-DH, and other ligands were less effective (Audsley *et al*., 1995). The CRF related peptide of *Culex salinarius* (Culsa-DH) has weak diuretic and natriuretic activity in *A. aegypti* (Clark *et al*., 1998b), compared with the pronounced effects of an unidentified head factor, mosquito natriuretic peptide (MNP). Culsa-DH appeared to elevate intracellular Ca^{2+} at $nmol\,l^{-1}$ concentrations, but to activate adenylate cyclase at ~100 $nmol\,l^{-1}$ levels (Clark *et al*., 1998b). These data are based upon the electrophysiological signatures of the peptide being mimicked by thapsigargin and cAMP: the response to Culsa-DP is biphasic with thapsigargin mimicking the initial depolarization at $nmol\,l^{-1}$ concentrations of peptide, and cAMP reproducing the subsequent

hyperpolarization at ~100 nmol l^{-1} levels of Culsa-DP. However, this work involved a cross-species assay, and preliminary data for synthetic Anoga-DH_{44} (G.M. Coast, K.M. Schegg, and D.A. Schooley, unpublished data) using *A. gambiae* MT suggest that this CRF related DH may have a major role in the postblood meal diuresis of this species.

The increase in cAMP is believed to activate a bumetanide sensitive Na^+–K^+–$2Cl^-$ cotransporter (see **Figure 11**) in *M. sexta* (Audsley *et al.*, 1993), *R. prolixus* (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Ianowski *et al.*, 2002), *A. domesticus* (G.M. Coast, unpublished data), and adults of *A. aegypti* (Beyenbach, 2003), but not in larval tubules (Clark and Bradley, 1996). The Na^+–K^+–$2Cl^-$ cotransporter has been cloned from *M. sexta* (Reagan, 1995b); it is a large protein with 12 putative transmembrane helices resembling orthologous proteins from other species, and it contains a consensus site for phosphorylation by protein kinase A (Reagan, 1995b). Similar transporters encoded in the *D. melanogaster* genome (CG2509 and CG31547) lack the protein kinase A site (Ser1025) reported by Reagan (1995b), which might explain the relatively low sensitivity of this species to the CRF-like DH compared with kinins. Thr963 in the *A. gambiae* transporter (XM307782) is a likely protein kinase A site (determined with the program NetPhos 2.0 at the Center for Biological Sequence Analysis, Technical University of Denmark; NetPhos, 2004). This program uses neural network predictions for serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins.

3.10.3.12.2.1. CRF-like DH receptors Reagan (1994) utilized expression cloning to deduce the sequence of a receptor from *M. sexta* (Manse-DHR), and subsequently a similar one from *A. domesticus* (Reagan, 1996) (Achdo-DHR). Two orthologs of these receptors are encoded in the *D. melanogaster* genome (CG8422 and CG12370); recently one of these (CG8422) was shown to be the receptor (Drome-DHR) for Drome-DH_{44} (Johnson *et al.*, 2004). A BLAST search of the *A. gambiae* genome using the Manse-DHR as a query returned a highly similar receptor (XM 315466) tentatively denoted in **Figure 12** as Anoga-DHR. All are members of Family B of seven transmembrane domain

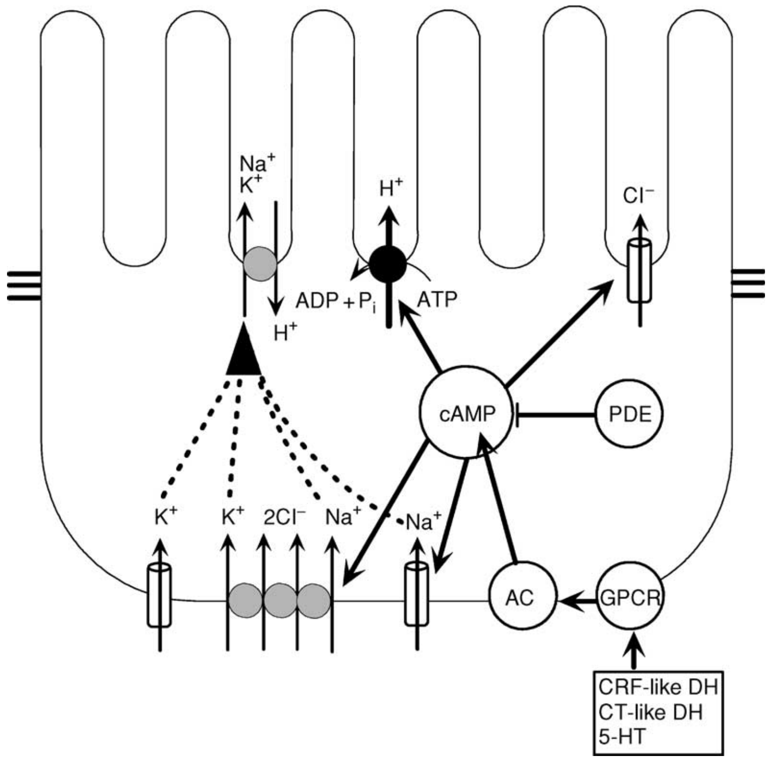


Figure 11 Proposed actions of cAMP in principal cells. CRF related DH, CT-like DH, and serotonin stimulate adenylate cyclase (AC) activity and increase intracellular levels of cAMP. Diuresis results from the stimulation of ion transport by cAMP which, depending on species, activates the apical membrane V-ATPase (*Drosophila melanogaster*, *Tenebrio molitor*, and *Rhodnius prolixus*), increases Na^+–K^+–$2Cl^-$ cotransport (*R. prolixus*, *Manduca sexta*, *Aedes aegypti*, and *Acheta domesticus*) and opens a Na^+ selective conductance in the basolateral membrane (*A. aegypti*). In addition, cAMP opens an apical membrane Cl^- conductance in *R. prolixus* tubules. Cyclic AMP levels are reduced by phosphodiesterase activity, which may account for the antidiuretic effects of Tenmo-ADFa,b and CAP_{2b} (see text for details).

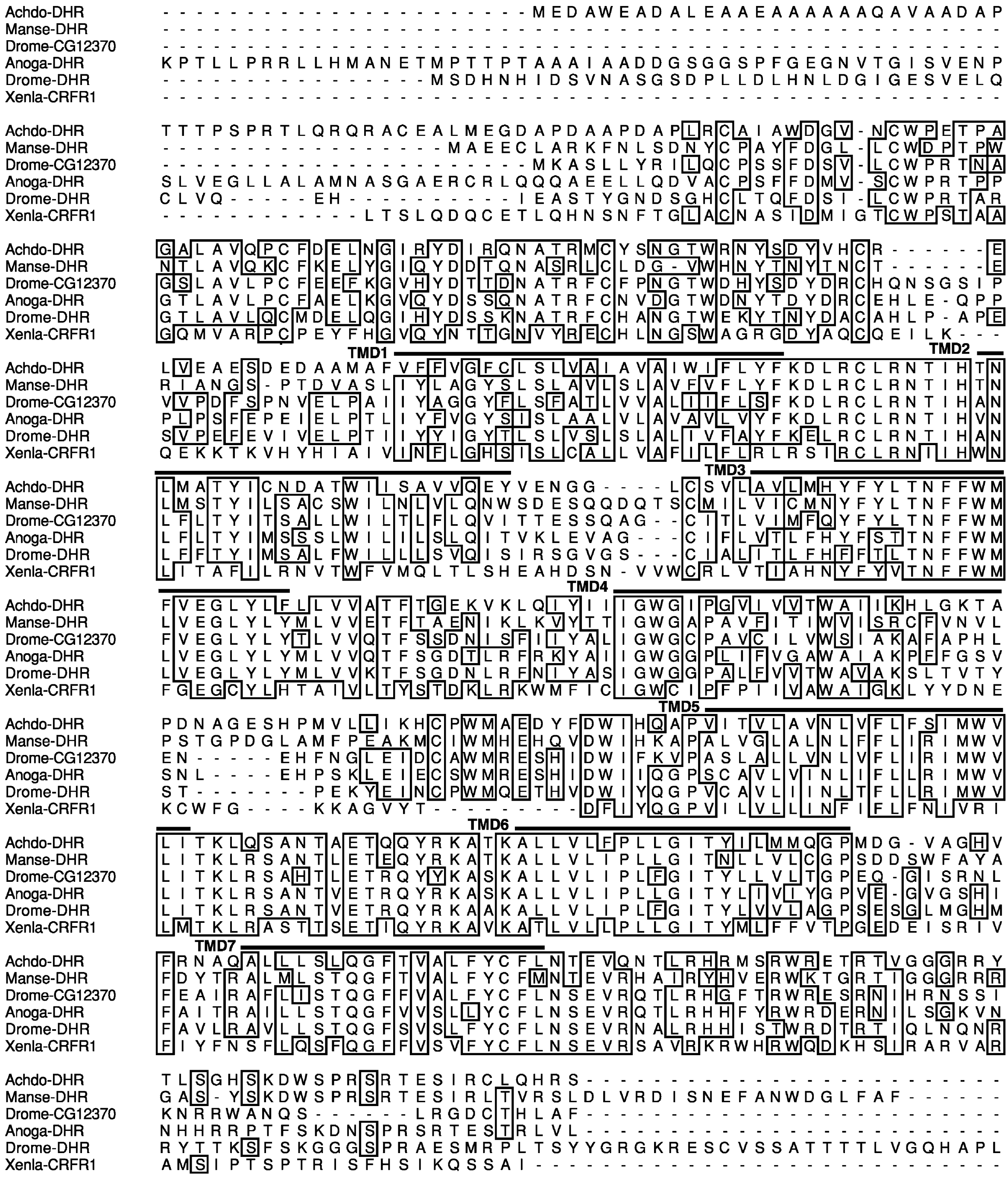


Figure 12 The CRF-like DH receptors are shown with identical residues boxed. Sequences were aligned with Clustal W, V. 1.8. The seven transmembrane domains (TMD) are indicated with a bold line and the domain number. Their approximate location was determined with the program TMHMM Server v. 2.0 at the Center for Biological Sequence Analysis, Technical University of Denmark (TMHMM, 2004). This program determines the location of transmembrane (TM) domains using hidden Markov models (HMM). The bold lines were determined for the *Manduca sexta* CRF-like DH receptor (Manse-DHR); for the *Drosophila melanogaster* CRF-like DH receptor (Drome-DHR) the location of the first TMD agreed exactly, whereas for the second it started and continued one residue downstream of that in Manse-DHR, for the third it started and continued one residue upstream, for the fourth it started and ended seven residues upstream of that in Manse-DHR, for the fifth it started seven residues upstream and ended one residue upstream of that in Manse-DHR, for the sixth it started three residues upstream and ended in the same position of that in Manse-DHR, and for the seventh it started five residues upstream and ended at the same position as that of Manse-DHR. The final 47 C-terminal residues of Drome-DHR are not shown for clarity. Drome-CG12730 is an orphan receptor with high similarity to this family of receptors. A homologous receptor is found in the genome of *Anopheles gambiae* (Anoga-DHR), and the *Acheta domesticus* DH receptor (Achdo-DHR) has been cloned (Reagan, 1996). The *Xenopus laevis* CRF1 receptor is shown for comparison.

G protein-coupled receptors (a family that includes the CRF receptors of vertebrates; Dautzenberg and Hauger, 2002). As in most receptor families, the transmembrane domains (TMD) are highly conserved (**Figure 12**). Other domains differ in their degree of similarity; intracellular domain 1 (between TMD 1 and 2) and domain 3 (between TMD 5 and 6) are quite highly conserved. In the CRF receptors, intracellular domain 3 is believed to be the site for G protein coupling (Hauger *et al.*, 2003). All of the CRF-like DH receptors have six Cys residues in the N-terminal extracellular domain, a structural feature that has been shown to be critical for ligand interaction in the CRF receptor (Qi *et al.*, 1997); the orphan receptor Drome-CG12730 has but five Cys in this domain. The location of five of these is completely conserved, while the Cys closest to the N-terminus is variable in position (**Figure 12**). The disulfide pairings in the CRF receptor have been inferred (Qi *et al.*, 1997); they will add a great deal of conformation rigidity to the N-terminal domain, believed to be key in CRF binding (Dautzenberg *et al.*, 1998), together with the fourth extracellular loop (Sydow *et al.*, 1997, 1999). Certain amino acid residues in the N-terminal domain of *X. laevis* (Dautzenberg *et al.*, 1998) and human (Wille *et al.*, 1999) CRF_1 receptors have been identified as crucial for ligand binding via site-directed mutagenesis. The intense research interest in these receptors has been driven in part by the development of small molecule antagonists of the receptors as drugs targeted at depression and anxiety (Kehne and De Lombaert, 2002); it is conceivable that a similar approach with agonists or antagonists of the insect receptors could lead to new insecticides.

It seems highly likely that separate receptors exist for the paralogous, shorter CRF-like DHs, Manse-DPII, Hylli-DH_{30}, and Tenmo-DH_{37}, but there is currently no evidence for this. The CRF_1 receptor binds both CRF and urocortin 1 (Hauger *et al.*, 2003), but discriminates against urocortin 2 and 3; whereas the $CRF_{2(a)}$, $CRF_{2(b)}$, and $CRF_{2(c)}$ receptors show a preference for urocortin forms over CRF (Hauger *et al.*, 2003). In *M. sexta*, both Manse-DH and Manse-DPII are active in adults *in vivo* (Kataoka *et al.*, 1989b; Blackburn *et al.*, 1991) as well as *in vitro* (Blackburn and Ma, 1994; Audsley *et al.*, 1995). However, in larvae only Manse-DH has been shown to be active; Manse-DPII is without apparent effect on the cryptonephric MT (Audsley *et al.*, 1995) and the ascending portion of the larval MT (G.M. Coast, unpublished data). Curiously, upon treatment of leaf slices with Manse-DPII, neonatal larvae of *M. sexta* behaved as though the peptide had antifeedant effects, and some mortality was observed at the ensuing molt (Ma *et al.*, 2000). Similarly, Keeley *et al.* (1992) showed that injection of last instar larvae of *Heliothis virescens* with synthetic Manse-DH had an antifeedant effect in addition to effects on fluid secretion; MT of larvae treated *in vitro* with this peptide showed increased fluid excretion. These actions are suggestive of effects of both peptides on receptors in sites likely to control feeding, such as the CNS.

3.10.3.12.3. Kinins: cellular actions Different models have been advanced for the mode of action of kinins in mosquitos versus fruit flies (see **Figure 13**). In both models, kinins bind to a cell membrane receptor, resulting in activation of phospholipase Cβ (PLCβ) and a rapid rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$). The stellate cells of fruit fly tubules express a gene (CG10626) encoding a G protein-coupled kinin receptor (Drome-K-R). The binding of Drome-K to this receptor activates PLCβ and increases inositol (1,4,5)-trisphosphate (IP_3) production and $[Ca^{2+}]_i$ in S2 cells transfected with Drome-K-R (Radford *et al.*, 2002). Two *Drosophila* genes encode PLCβ (*norpA* and *Plc21C*) and Drome-K-stimulated diuresis is greatly reduced in tubules from *norpA* mutants (Pollock *et al.*, 2003). An increase in $[Ca^{2+}]_i$ precedes the fastest physiological response of tubules to kinin stimulation. It results from the release of Ca^{2+} from endoplasmic reticulum stores triggered by IP_3 binding a receptor (IP_3R) that is a ligand gated Ca^{2+} channel, and mutations of the IP_3R gene (*itpr*) attenuate kinin stimulated diuresis and the rise in $[Ca^{2+}]_i$ (Pollock *et al.*, 2003). CAP_{2b} peptides also use IP_3 as a second messenger in fruit fly tubules, but act on principal cells rather than stellate cells (see Section 3.10.3.12.4).

In marked contrast to the fruit fly, kinins act through a Ca^{2+} signaling pathway in principal cells of *A. aegypti* tubules (Yu and Beyenbach, 2002). The molecular genetic tools available for *D. melanogaster* are not yet available for mosquitos, but Beyenbach's group has shown that the distinctive electrophysiological signature accompanying kinin stimulation, namely depolarization of the transepithelial potential (V_{tep}) and a reduction in transepithelial resistance (R_t), are identical in tubule segments with and without stellate cells (Yu and Beyenbach, 2004). Indeed, the fact that it is possible to measure increased levels of IP_3 in intact tubules of *A. aegypti* challenged with Aedae-Ks (Cady and Hagedorn, 1999b) suggests the response is not restricted to stellate cells, which are fewer in number and smaller in size than principal cells.

Irrespective of the cell type towards which kinin activity is directed, V_{tep} depolarizes due to the

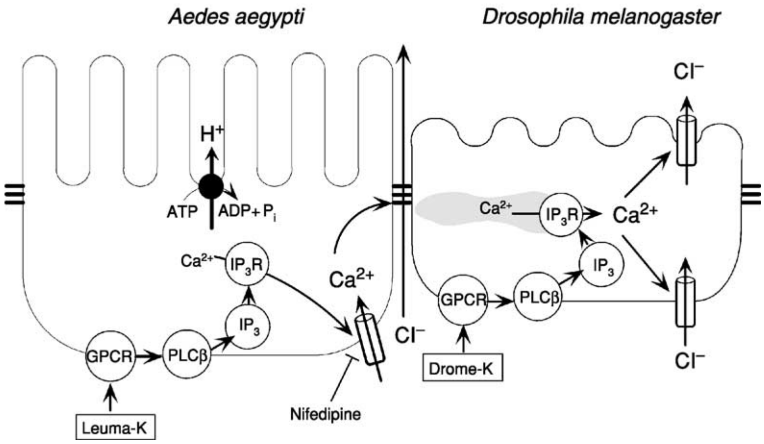


Figure 13 Kinin activity in Malpighian tubules of *Aedes aegypti* and *Drosophila melanogaster*. Kinins bind a PLCβ-coupled receptor and stimulate production of IP_3, which initiates Ca^{2+} release from endoplasmic reticulum IP_3 sensitive stores. The resultant increase in $[Ca^{2+}]_i$ opens a Cl^- selective shunt pathway that lies outside of the principal cells. Kinins (Leuma-K-8) act on *A. aegypti* principal cells, which have small IP_3 sensitive stores. Store emptying opens nifedipine sensitive store-operated Ca^{2+} channels (SOCC) in the basolateral membrane. Ca^{2+} entry through SOCC raises $[Ca^{2+}]_i$, which in some unknown way opens a paracellular conductance allowing Cl^- to diffuse into the lumen through intercellular septate junctions. In marked contrast, Drome-K binds a receptor (Drome-K-R) on the stellate cells of *D. melanogaster* tubules to stimulate production of IP_3. Release of Ca^{2+} from IP_3 sensitive stores elevates stellate cell $[Ca^{2+}]_i$, which opens a transcellular conductance pathway through Cl^- channels in the apical and basolateral membranes. GPCR, $G_{s\beta}$-coupled receptor.

opening of a Cl^- conductance pathway that lies outside of the principal cells. This pathway is believed to be through the stellate cells in fruit fly tubules because: (1) a high density of "maxi-Cl^-" channels are found in a small number of apical membrane patches, befitting the small number of stellate cells; and (2) vibrating probe analysis reveals Cl^- dependent current-density "hot spots" over stellate cells (O'Donnell *et al.*, 1998). Alternatively, Beyenbach's group argue that the Cl^- conductance pathway in *A. aegypti* tubules is paracellular through septate junctions (Wang *et al.*, 1996; Yu and Beyenbach, 2001); although Cl^- channels are present in the apical membrane of stellate cells, they are not maxi channels and are not activated by kinins (O'Connor and Beyenbach, 2001). Indeed, the work of Yu and Beyenbach, 2004 (see above) shows that stellate cells neither signal nor mediate the kinin response. The differences between kinin activity in *D. melanogaster* and *A. aegypti* have been viewed as somewhat controversial; nevertheless, these species have very different diets (blood versus fruit), which mandate different excretory demands. It seems probable that each is correct for that species. As noted earlier, Zdobnov *et al.* (2002) concluded that *D. melanogaster* and another mosquito, *A. gambiae*, are somewhat more divergent in evolution than the human and the pufferfish!

Malpighian tubules of the house cricket, *A. domesticus*, lack stellate cells, but respond to kinin stimulation with an increase in fluid secretion that is Cl^- dependent and probably mediated by a rise in $[Ca^{2+}]_i$ (Coast, 2001). As in dipteran tubules, kinins open a Cl^- conductance pathway causing V_{tep} to depolarize. Without stellate cells, this conductance can only be paracellular or through principal cells. The effects of ion substitutions in the bathing fluid on membrane potentials indicate that principal cells have a negligible basolateral Cl^- conductance both before and after kinin stimulation, which appears to rule out a transcellular pathway for Cl^- movement into the lumen (G.M. Coast, unpublished data). It is also worth noting that kinins have no effect on fluid secretion in *R. prolixus* tubules (Te Brugge *et al.*, 2002), which might be explained by the unusual lumen negative V_{tep} (Ianowski and O'Donnell, 2001) opposing passive diffusion of Cl^- through a paracellular conductance pathway.

3.10.3.12.3.1. Kinin receptors Recently, one of a group of putative neuropeptide receptors from *D. melanogaster* (Hewes and Taghert, 2001) has been identified as the probable receptor for Drome-K (Radford *et al.*, 2002). When heterologously expressed, the receptor shows an EC_{50} of 40 pmol l^{-1} and a t(1/2) <1 s for Ca^{2+} elevation. Earlier, Cox *et al.* (1997) cloned a receptor from a *Lymnaea stagnalis* (pond snail) cDNA library (see **Figure 14**), and also identified the endogenous kinin, lymnokinin (PSFHSWS-amide), in this same paper. When heterologously expressed in CHO cells, this receptor

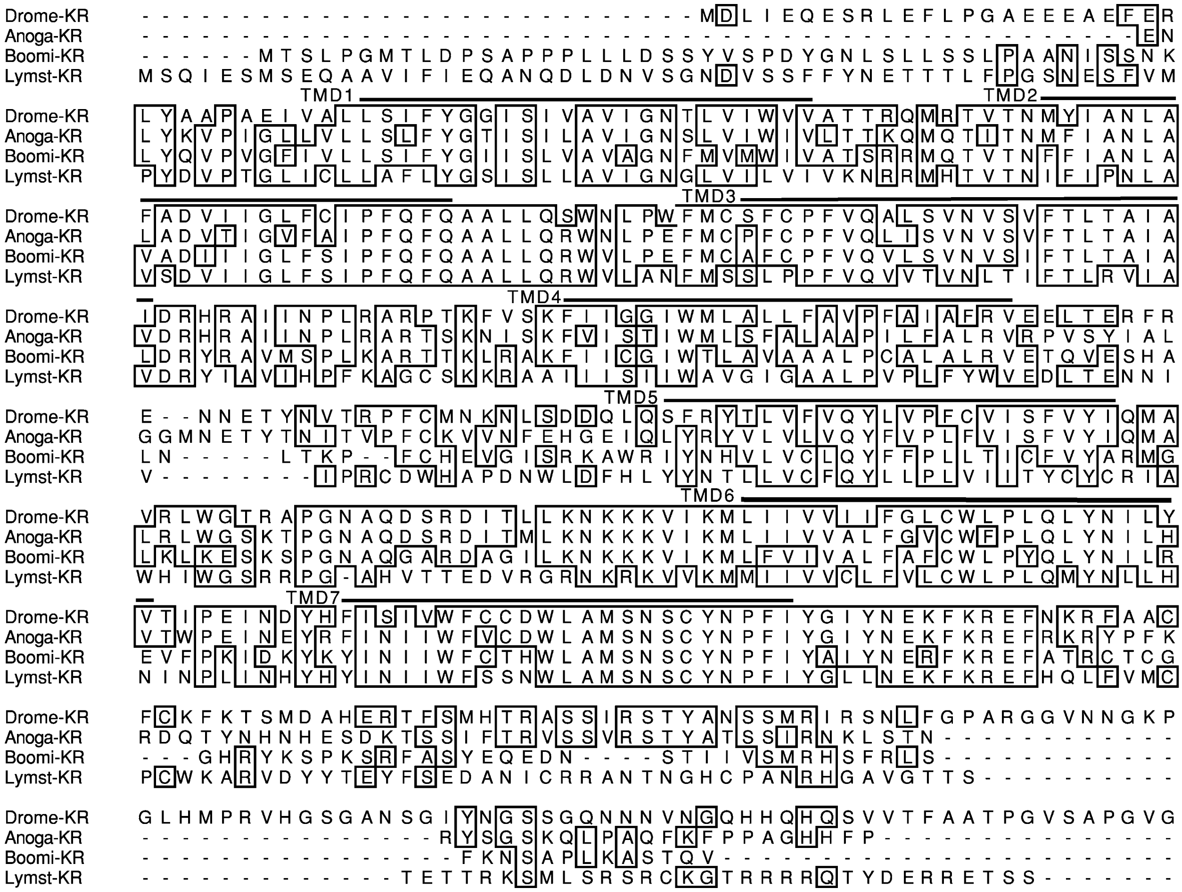


Figure 14 The kinin receptors (KR) are shown with identical residues boxed. Sequences were aligned with Clustal W, V. 1.8. The seven transmembrane domains (TMD) are indicated with a bold line and the domain number. Their approximate location was determined with the program TMHMM Server v. 2.0 at the Center for Biological Sequence Analysis, Technical University of Denmark (TMHMM, 2004). The bold lines were determined for the *Drosophila melanogaster* kinin receptor (Drome-KR); for the *Boophilus microplus* receptor (Boomi-KR) the location of TMD1, -2, -3, -4, -5, and -7 were identical with those of the Drome-KR, for TMD6 the domain started in the same place as the Drome-KR but ended 4 residues upstream. The final 70 C-terminal residues of Drome-KR are not shown for clarity. This receptor has an unusually long C-terminal intracellular domain.

was stimulated by lymnokinin with an EC_{50} for a Ca^{2+} response of 1.1 nmol l^{-1}. A "leucokinin-like" receptor has also been cloned from the cattle tick *Boophilus microplus* (Holmes *et al.*, 2000); it is clearly an ortholog to the above two kinin receptors. However, no ligand is known for this receptor in the tick, so no affinity data are available. In addition, an ortholog of these receptors occurs in the genome of *A. gambiae*.

3.10.3.12.4. CAP_{2b} family: cellular actions The diuretic activity of CAP_{2b} peptides has been most thoroughly investigated in fruit fly tubules in which they act through a Ca^{2+}-NO-cGMP signaling pathway to stimulate cation transport by increasing V-ATPase activity in principal cells, as evidenced by hyperpolarization of the transepithelial potential (V_{tep}) and acidification of the urine (O'Donnell *et al.*, 1996; Kean *et al.*, 2002). The binding of CAP_{2b} to a phospholipase C (PLCβ)-coupled receptor on principal cells in the main secretory segment stimulates the production of inositol (1,4,5)-trisphosphate (IP_3) which initiates the release of Ca^{2+} from endoplasmic reticulum IP_3-sensitive stores (see Figure 15 for model). In support of this, the tubules express genes encoding PLCβ (*norpA* and *plc21*) (Pollock *et al.*, 2003) and the IP_3 receptor (*itpr*) (Blumenthal, 2001), and the CAP_{2b}-stimulated diuresis is significantly reduced in tubules from *norpA* and *itpr* mutant flies (Pollock *et al.*, 2003). The amount of Ca^{2+} released from principal cell IP_3-sensitive stores is too small to be detected, but store emptying probably activates store-operated Ca^{2+} channels (SOCCs) and the influx of extracellular Ca^{2+}. This is essential for the CAP_{2b} stimulated diuresis, accounting for the relatively slow rise in $[Ca^{2+}]_i$. Fruit fly tubules express genes that encode a variety of Ca^{2+} channels involved in

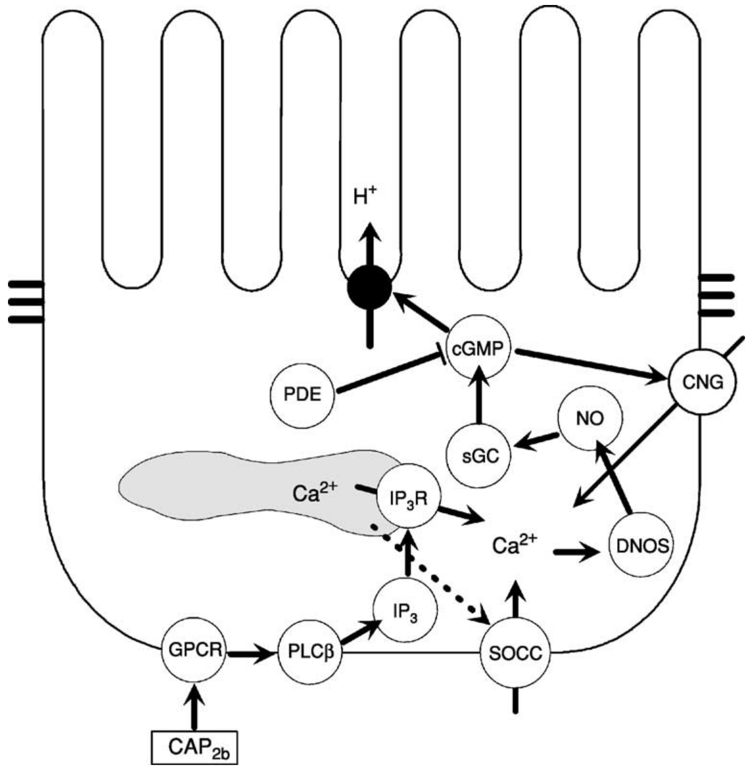


Figure 15 A model for the action of CAP_{2b} and orthologs via a nitridergic signaling pathway in *Drosophila melanogaster* principal cells. The binding of CAP_{2b} to a $G_{s\beta}$-coupled receptor (GPCR) stimulates production of IP_3, which initiates Ca^{2+} release from endoplasmic reticulum stores by opening ligand gated Ca^{2+} channels (IP_3R). Emptying of these IP_3 sensitive stores activates store-operated Ca^{2+} channels (SOCC) and the resultant rise in $[Ca^{2+}]_i$ stimulates a Ca^{2+}-calmodulin nitric oxide synthase (*Drosophila* NOS, or DNOS) and elevates nitric oxide (NO) production. NO activates a soluble guanylate cyclase (sGC), increasing production of cGMP, which activates the apical membrane V-ATPase. Cyclic GMP also opens cyclic nucleotide gated Ca^{2+} channels (CNG) in the basolateral membrane, which augments the Ca^{2+}signal. The hydrolysis of cGMP by phosphodiesterase (PDE) appears to be important in modulating the stimulation of fluid secretion by CAP_{2b}.

Ca^{2+} transport and signaling. These include the genes *trp* and *trpl* that encode Trp and Trp-like (Trpl) channel proteins. Analysis of *trp* and *trpl* mutants implicates TRPL in the diuretic response to CAP_{2b} and this could therefore be the elusive SOCC. The CAP_{2b} stimulated rise in $[Ca^{2+}]_i$ is sufficient to activate nitric oxide synthase, which is a Ca^{2+}/calmodulin sensitive *D. melanogaster* nitric oxide synthase (NOS) that is expressed only in principal cells (Davies *et al*., 1997; Rosay *et al*., 1997; Davies, 2000). The NO generated by NOS activates a soluble guanylate cyclase (sGC) resulting in elevated levels of cGMP, which are confined to the principal cells (Kean *et al*., 2002). Interestingly, fruit fly tubules express a gene encoding a cyclic nucleotide gated channel (CNG), and activation of this channel by cGMP may contribute to the influx of extracellular Ca^{2+} and help sustain the rise in $[Ca^{2+}]_i$ (MacPherson *et al*., 2001). Other possible targets for the cGMP produced in response to CAP_{2b} stimulation include cGMP dependent protein kinases (cGKs), and two genes encoding cGK (*dg1* and *dg2*) are expressed in tubules. Significantly, tubules from *dg2* mutant flies are hypersensitive to cGMP, and the cGK could therefore be involved in activating the apical V-ATPase, the ultimate target of CAP_{2b} (Dow and Davies, 2003).

The detailed dissection of the elaborate signal transduction pathway for CAP_{2b} in fruit fly tubules is testament to the advantages afforded by the powerful combination of molecular biology and genetic analysis in unravelling complex physiological processes. Nevertheless, it is important to remember that however elegant a model, it provides only a description of what occurs in the fruit fly and may be quite different in other insects. Indeed, when CAP_{2b} peptides have been tested in other species their actions differ from those described in the fruit fly. For example, CAP_{2b} stimulates fluid secretion by housefly (*M. domestica*) tubules by a NO-cGMP independent mechanism that results in V_{tep} depolarizing rather than hyperpolarizing and is not accompanied by urine acidification (C.S. Garside and G.M. Coast, unpublished data). The cellular actions

of CAP_{2b} in the housefly are very similar to those of the kinins in that the depolarization of V_{tep} is due to the opening of a Cl^- conductance pathway that lies outside of the principal cells and may be paracellular or transcellular through the stellate cells (see above in this section). Moreover, CAP_{2b} activity is not dependent on Ca^{2+} influx from the bathing medium, but most likely involves Ca^{2+} release from intracellular stores, because prior treatment of tubules with thapsigargin blocks CAP_{2b} stimulated diuresis. If housefly principal cells have only small stores of releasable Ca^{2+}, as in fruit fly tubules, then the assumption is that CAP_{2b} acts on stellate cells. In this context, it is noteworthy that high concentrations (100 nmol l^{-1}) of CAP_{2b} peptides increase $[Ca^{2+}]_i$ in the stellate cells of fruit fly tubules (Kean *et al.*, 2002). The response to Drome-capa-1 is large enough to open the stellate cell Cl^- conductance and, in combination with the stimulation of active cation transport by the principal cells, this might explain the more potent diuretic activity of this peptide compared with Drome-capa-2 and Manse-CAP_{2b}.

In contrast to the diuretic activity of CAP_{2b} peptides in the housefly and fruit fly, CAP_{2b} acts via cGMP to reduce secretion by MT from *R. prolixus* and *T. molitor* larvae, and *A. aegypti* adult females. CAP_{2b} (and cGMP) antagonizes the actions of cAMP and the diuretic hormones serotonin (*R. prolixus*; Quinlan *et al.*, 1997) and Tenmo-DH_{37} (*T. molitor*; Wiehart *et al.*, 2002), both of which act via cAMP dependent mechanisms to stimulate tubule fluid secretion. The antagonist effects of CAP_{2b} and cGMP can be explained by the activation of a cGMP dependent cAMP phosphodiesterase (Quinlan *et al.*, 1997), which would lower intracellular levels of cAMP and thus reduce tubule secretion.

3.10.3.12.5. Cellular actions of antidiuretic factors inhibiting Malpighian tubule secretion The first insect neuropeptide shown to inhibit MT secretion was Manse-CAP_{2b}, first isolated as a myotropin from *M. sexta*. This peptide has no effect on MT of the species from which it was isolated (Skaer *et al.*, 2002). However, Quinlan *et al.* (1997) reported CAP_{2b} to be antidiuretic in *R. prolixus*, and that low levels of cGMP or Manse-CAP_{2b} would decrease the serotonin stimulated secretion of MT. These effects could be reversed by addition of 1 mmol l^{-1} cAMP. Subsequently, they studied further the antagonistic effects of cGMP and cAMP on *R. prolixus* MT; an inhibitory level of cGMP was without effect on the concentrations of K^+ and Na^+ of secreted fluid (Quinlan and O'Donnell, 1998). Transport of organic anions into upper tubules involves at least two different transporters: one for acylamides (e.g., *p*-aminohippuric acid) and another for sulfonates (e.g., amaranth and phenol red). Amaranth and phenol red blocked the actions of both cGMP and cAMP, whereas *p*-aminohippuric acid was without effect, suggesting that exogenous cyclic nucleotides are carried into the MT cell by the sulfonate transporter (Quinlan and O'Donnell, 1998). These workers hypothesized that cGMP activated a cAMP phosphodiesterase, but have apparently not provided any proof for this.

In *T. molitor*, both CAP_{2b} and the two *T. molitor* antidiuretics, ADFa and ADFb, act by stimulating a guanylate cyclase, probably membrane bound. As found in *R. prolixus*, Wiehart *et al.* (2002) showed that ADFa and Tenmo-DH_{37} have antagonistic effects, the former elevating cGMP levels and the latter elevating cAMP levels. It also seems likely in this species that cGMP activates a cAMP specific phosphodiesterase. Attempts to prove this by using *T. molitor* MT incubated with 1 μmol l^{-1} erythro-9-(2-Hydroxy-3-nonyl)adenine. HCl (EHNA), a selective inhibitor of the cGMP activated cAMP phosphodiesterase II (Podzuweit *et al.*, 1995), and 100 fmol l^{-1} Tenmo-ADFa were unfortunately inconclusive (S.W. Nicolson, R.J. Eigenheer, and D.A. Schooley, unpublished data).

Tenmo-ADFa has been recently found to have antidiuretic action in *A. aegypti*; at 1 nmol l^{-1} concentrations in a Ramsay assay it significantly inhibited the rate of fluid secretion without significant effects on the concentrations of Na^+, K^+, and Cl^- in secreted fluid and also significantly increased intracellular cGMP concentration, consistent with a second messenger role (Massaro *et al.*, 2004). Low doses of cGMP (20 μmol l^{-1}) inhibit isosmotic fluid secretion without inducing electrophysiological effects, whereas cGMP at 500 μmol l^{-1} significantly depolarized the basolateral membrane, but hyperpolarized the transepithelial potential. Low concentrations of Tenmo-ADFa and cGMP inhibit isosmotic fluid secretion by reducing electroneutral transport, whereas high levels of cGMP increase an unknown conductance of the basolateral membrane and depolarize the basolateral membrane voltage supplemental to and independent of the electroneutral mechanism of action of low concentrations of cGMP (Massaro *et al.*, 2004).

While CAP_{2b} has been termed an "antidiuretic hormone" in *R. prolixus* (O'Donnell and Spring, 2000), this may be premature: in *T. molitor*, only two zones of material that elevated cGMP levels in MT were isolated and sequenced, even though ADFb (Eigenheer *et al.*, 2003) is 24 000 times less potent than ADFa (Eigenheer *et al.*, 2002). CAP_{2b}

($EC_{50} = 85\,nmol\,l^{-1}$) is less potent still than ADFb; the presence of CAP_{2b} or a paralog in *T. molitor* would probably have been detected. It is quite interesting that cGMP has so far been found to be antidiuretic in two phylogenetically disparate blood feeders, *R. prolixus* and *A. aegypti*, and a starkly xeric species, *T. molitor.* Quinlan and O'Donnell (1997) have argued that an antidiuretic factor could be of great importance in a blood feeder to cause a rapid decrease in postblood meal diuresis, in order to avoid depleting the hemolymph volume. In fact, they observed that day 2 postblood meal MT were far more sensitive to the effects of CAP_{2b} than day 12 MT, with day 22 postblood meal MT still less sensitive. *Tenebrio molitor* is so highly evolved for water absorption that activities of K^+ in its cryptonephric MT exceeding 3 M have been measured (O'Donnell and Machin, 1991), allowing absorption of water vapor from the rectum above 88% relative humidity. (Despite this species having high hemolymph Na^+, the active transport mechanism in MT is quite selective for K^+.) Thus, antidiuretic factors could be quite important in conserving water in this species.

It seems surprising that cGMP has been found to have the opposite effect, diuresis, in *D. melanogaster* (Davies *et al*., 1995) and *M. sexta* (Skaer *et al*., 2002). However, cGMP is only weakly diuretic in the dipteran *M. domestica*, and Drome-capa-2 (one of three endogenous Manse-CAP_{2b}-like peptides encoded by the *D. melanogaster* genome) is diuretic with actions that resemble those of Musdo-K (G.M. Coast, unpublished data). It is possible that the diuretic effect of cGMP in certain species may be due to stimulation of a cGMP stimulates cAMP phosphodiesterase (PDE type III).

3.10.3.12.6. Cellular actions of ITP: an antidiuretic factor that promotes fluid reabsorption in the hindgut Schgr-ITP acts via cAMP to stimulate salt (NaCl/KCl) and water uptake from the locust ileum (Phillips *et al*., 1998b). Its actions have been studied extensively in flat sheet preparations of the locust ileum mounted between two Ussing chambers (this method is suitable only for large insects, even the hindgut of adult *M. sexta* is too small (S. Liao, J. Kenyon, and D.A. Schooley, unpublished data)). Addition of Schgr-ITP or cAMP to the hemolymph side results in a ten-fold stimulation of the current (short circuit current; I_{scc}) needed to clamp the tissue at a transepithelial potential (V_{tep}) of 0 mV. The ITP stimulated I_{scc} is Cl^- dependent and reflects a ten-fold increase in net active Cl^- transport from the luminal to the basal surface of the epithelium against an overall electrochemical potential gradient (see Figure 16 for model). Extensive

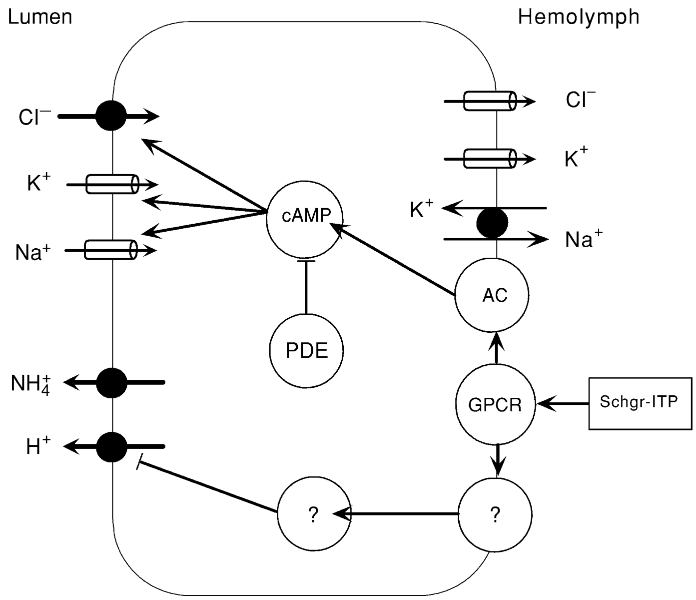


Figure 16 Proposed actions of Schgr-ITP on ion transport by locust ileum. Schgr-ITP stimulates ion transport and isosmotic fluid reabsorption from the lumen of the ileum. Acting via cAMP, it stimulates the apical electrogenic Cl^- pump, which is the prime driving force for ion and fluid transport. Cyclic AMP, also opens conductance pathways for Na^+ and K^+ in the apical membrane. K^+ and Cl^- exit to the hemolymph side of the epithelium through conductance pathways in the basolateral membrane, while Na^+ is removed via Na^+-K^+-ATPase activity. Schgr-ITP also inhibits ileal acid (H^+) secretion via a cAMP independent pathway. AC, adenylate cyclase; PDE, phosphodiesterase; GPCR, $G_{s\alpha}$-coupled receptor.

electrophysiological studies reveal that the active transport step takes place at the apical (luminal) membrane and involves an unusual (for vertebrates) electrogenic Cl^- pump that is stimulated by cAMP (Phillips *et al.*, 1998b). Chloride exits the cells to the hemolymph side by passive diffusion through a large basolateral membrane Cl^- conductance. The major cations (Na^+ and K^+) follow Cl^- passively across the apical membrane through separate conductance pathways both of which are increased by cAMP. At the hemolymph side, K^+ exits the cells passively via a large K^+ conductance in the basolateral membrane, whereas Na^+ is actively removed from the cells by a Na^+–K^+–ATPase pump. Fluid uptake from the locust ileum is driven by active salt (NaCl/KCl) transport and isosmotic fluid reabsorption is stimulated fourfold by Schgr-ITP and cAMP (Phillips *et al.*, 1998b). Interestingly, while both Schgr-ITP and crude extracts of corpora cardiaca inhibit active acid (H^+) secretion into the lumen of the ileum, cAMP is without effect indicating the involvement of another second messenger in the actions of this peptide (Phillips *et al.*, 1998a).

Schgr-ITP has been shown to have no effect on secretion of MT from *S. gregaria* (Coast *et al.*, 1999); similarly, Locmi-K and Locmi-DH have no effect on the ilea or recta of *S. gregaria in vitro*. Locmi-K and Locmi-DH have a synergistic effect on MT of *S. gregaria*, and this effect is much faster than the effect of Schgr-ITP on the ileum. These data would suggest that in early phases of feeding, urine secretion would commence before reabsorption, allowing voiding of excess water (Coast *et al.*, 1999).

3.10.3.12.7. The cryptonephric complex and cellular actions of factors acting on it The cryptonephric complex of Coleoptera and larval Lepidoptera is a composite tissue consisting of both excretory and reabsorptive tissue. In this anatomy, which differs between the two orders, the rectum is closely associated with the distal part of the MTs, which lie within a perinephric space that is separated from the hemolymph by a water impermeable perinephric membrane. The cryptonephric tubules of *T. molitor* contact the perinephric membrane at regions called "boursouflures" (or "bulges"). Here the perinephric membrane is greatly reduced and forms a "blister" over an underlying leptophragma cell (Ramsay, 1964). Ramsay postulated that KCl is transported from the hemolymph into the cryptonephric tubules across the leptophragma cells without an accompanying movement of water, thereby establishing a high osmotic concentration (as much as 6.8 Osm in *T. molitor* larvae, close to water-saturation for KCl) in the tubule lumen (O'Donnell and Machin, 1991). The perinephric space also has a high osmolarity consisting largely of nonionic solutes (melting point depression up to $-10\,°C$) with a Na^+–K^+ ratio similar to that of hemolymph (Ramsay, 1964), unlike the distal parts of the cryptonephric tubules, which absorb K^+ to the near exclusion of Na^+ (O'Donnell and Machin, 1991). Using double-barrel microelectrodes and dye injections, O'Donnell and Machin (1991) also found that the perinephric space is an important functional part of the cryptonephric complex. They reported large pH (as much as 2 units, averaging 1.1 units) and electrochemical gradients (70 mV) across the apical membrane of the tubule, with the average K^+ activity across the membrane exceeding the Nernst equilibrium by 75-fold. They favor a model where cations enter the perinephric space through the anterior, more permeable part of the perinephric sheath. Anions may enter the perinephric space separately, possibly through the leptophragma cells, present in the *T. molitor* cryptonephric complex but absent from *M. sexta*. This could be consistent with the leptophragma cells being equivalent to stellate cells in other species. The highest activity of K^+ measured in their studies was apparently near the distal end of the cryptonephric MT. The high osmotic concentration in the tubule accounts for the osmotic withdrawal of water from first the perinephric space and ultimately the rectal lumen. This exceptional osmotic gradient may be necessary to drive water transport across at least two membranes and can account for the uptake of water vapor from air of >89% relative humidity (RH) (Coutchié and Machin, 1984). A very recent paper describes a microcalorimetric study on larvae of *T. molitor* exposed to a dry atmosphere; there was a doubling of metabolic activity prior to commencement of water vapor absorption from the air (Hansen *et al.*, 2004), which could indicate hormonal involvement in this metabolic activity. The consequence of this anatomy is that DH (at least CRF-like DH) promote salt uptake from the hemolymph, but water uptake from the rectum. In fact, Ramsay(1964) hypothesized that the cryptonephric complex in beetles evolved to conserve water. Nicolson (1991) speculated that a DH in similar Coleoptera might serve as a clearance hormone rather than a diuretic hormone, with the fluid flow directed into the midgut to moisten the dry food for digestion, and the fluid reabsorbed in the midgut. This model raises the question of where reabsorption of electrolytes, at least other than KCl, from the hindgut occurs in these species. It is clear that the model developed for fluid reabsorption in locusts is not

applicable for Coleoptera given the cryptonephric anatomy.

The cryptonephric complex of lepidopteran larvae differs significantly from that of *T. molitor* in that it is restricted to three longitudinal bands running the length of the rectum, corresponding to the three pairs of MTs (Ramsay, 1976). Between each band, a patch of "normal" rectal epithelium separates the luminal contents from the hemocoel. Additionally, boursouflures appear to be absent in the lepidopteran cryptonephric complex, which nevertheless is assumed to function in a manner similar to that of *T. molitor*, although high osmotic concentrations are not established. Ramsay (1976) argued that the cryptonephric complex in Lepidoptera evolved to handle the osmotic challenge of ingesting massive quantities of salt, because he found that *M. sexta* larvae were able to feed on a diet containing sufficient NaCl to represent 1 kg of salt per day for an adult human! However, most lepidopterans feed on plants with high K^+ and low Na^+. Ramsay did not test KCl; perhaps this ability to excrete high Na^+ is more linked to the high hemolymph K^+ content of this species (Sutcliffe, 1963). About 90% of the fluid entering the rectum of day 1 fifth instar larvae is reabsorbed (Reynolds and Bellward, 1989), a quarter of which enters the cryptonephric tubules and is recycled to the midgut via the straight ascending and descending tubule segments (Moffett, 1994). At the same time, K^+ in the tubule lumen is returned to the midgut in exchange for Na^+ (Moffett, 1994). The remaining fluid is reabsorbed across the "normal" epithelium, which is composed of cells with extensive infoldings of the apical and basolateral membranes and associated mitochondria (Reynolds and Bellward, 1989). The latter group argued that the ileum of *M. sexta* has no ultrastructural characteristics of a reabsorptive tissue, suggesting that fluid reabsorption in this species is dependent on the "normal" rectal epithelium.

Audsley *et al.* (1993) presented evidence that Manse-DH has a net antidiuretic effect on the cryptonephric complex of *M. sexta*. They showed that Manse-DH stimulates a bumetanide sensitive Na^+–K^+–$2Cl^-$ cotransporter, causing an influx of salt from the hemolymph, with fluid following from the rectal lumen. Transport processes that are consistent with their findings are summarized in **Figure 17**. Crucial to this work was development of an assay using everted rectal sacs from fifth instar larvae. In this preparation, the rectal lumen is on the outside, while the hemolymph side of the tissue is sealed around a polypropylene tube so that reabsorption into the hemolymph is manifest by a weight gain over time. Further details of the preparation are given by Liao *et al.* (2000), who used it to demonstrate the existence of two factors in CC–CA of larval *M. sexta* that are distinct from Manse-DH and trigger fluid reabsorption by the cryptonephric complex of this species (see above in this section). The more abundant of these two factors, Manse-ADF-B = Manse-ADF-2, is believed to stimulate a Cl^--ATPase via cAMP as second messenger, a mechanism of action apparently identical to that of Schgr-ITP. Elucidating the mechanism of action of Manse-ADF-2 could only be accomplished via use of selective inhibitors, because Manse-DH also exerts its antidiuretic effect on the tubules via cAMP. However, incubation of everted rectal sacs with bafilomycin A_1 (an inhibitor of the V-ATPase) on the hemolymph side of the tissue was found to block the basal reabsorption by this tissue, masking the effect of Manse-DH, as does incubation with the far cheaper amiloride on the lumen side, an inhibitor of the H^+-alkali metal antiporter. Addition of Manse-ADF-2 to these inhibited preparations caused a highly significant increase in fluid reabsorption; the reabsorption was easier to measure in the presence of the inhibitors due to the strong decrease in basal reabsorption. Incubation of rectal sacs with H-89, a potent and selective inhibitor of protein kinase A, blocks the effect of Manse-ADF-2. In addition, incubation of rectal sacs with the Cl^- channel blockers DIDS or DPC caused a significant decrease in reabsorption, and the reabsorption could not be relieved by treatment with Manse-ADF-2. The ionic transport believed to occur in the "normal" rectal epithelium as a result of this antidiuretic factor is summarized in **Figure 17**.

In examining the alignment (**Figure 5**) of the 15 known CRF-like DHs, the sequences from two lepidopteran species and one beetle species differ significantly from those of other species, as reflected by the fact that this Clustal W alignment ranks them differently from the other sequences (phylogenetically, Tenmo-DH_{47} is aligned between Achdo-DP and Manse-DPII, but for this figure it was moved up next to the lepidopteran sequences). It is tempting to hypothesize that the divergence of these sequences from the other CRF-like DHs may have to do with the existence of the cryptonephric complex in larvae of *M. sexta* and larvae and adults of *T. molitor.*

3.10.3.13. Synergism

In general, the transport of cations (K^+ and Na^+) and anions (Cl^-) by Malpighian tubules appears to be regulated separately (O'Donnell *et al.*, 1996). Thus, depending on species, CRF-related, CT-like,

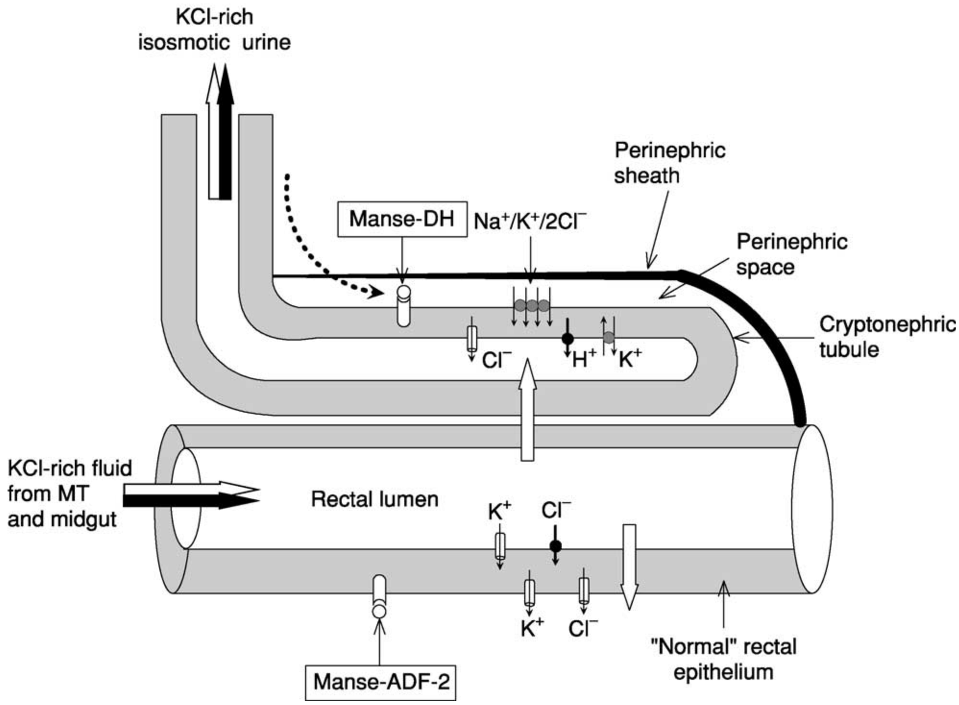


Figure 17 A model for the hindgut of *Manduca sexta* and similar lepidopteran larvae. At the top is shown one of three longitudinal segments of cryptonephric complex; on the opposing side of the hindgut lies a longitudinal segment of ''normal'' rectal epithelium. There are three radially symmetrical bands of cryptonephric tubules and ''normal'' rectal epithelium. Fluid entering the rectal lumen from the midgut and Malpighian tubules is reabsorbed by the cryptonephric tubules (CNT) and by the ''normal'' epithelium. The CNT lie in a perinephric space (PNS) isolated from the hemolymph by a water impermeable perinephric membrane (PNM). Ion transport by CNT is similar to that described in principal cells, with cation uptake from the PNS occurring via Na^{+}-K^{+}-$2Cl^{-}$ cotransport. Manse-DH acts via cAMP to stimulate the cotransporter and hence increase secretion of KCl-rich fluid into the CNT lumen. The withdrawal of water from the PNS creates an osmotic gradient favoring the passive withdrawal of water from the rectal lumen. Ions may enter the PNS from the rectal lumen (not shown) or across a thinner and more permeable (to ions) anterior region of the PNM (dotted arrow). In contrast to the actions of Manse-DH, Manse-ADF-2 acts via cAMP to stimulate ion transport and hence fluid reabsorption across the ''normal'' rectal epithelium. Ion transport by the rectal epithelium appears to be driven by an apical electrogenic Cl^{-} pump (c.f. the locust ileum) that is stimulated by cAMP. Cl^{-} exits the cells by passive diffusion through Cl^{-} channels in the basolateral membrane. The active transport of Cl^{-} from lumen to hemocoel establishes a favorable gradient for passive reabsorption of K^{+} through conductance pathways in the apical and basolateral membranes.

and CAP_{2b} peptides (*D. melanogaster*) stimulate active cation transport, whereas kinins and CAP_{2b} peptides (*M. domestica*) open a Cl^{-} selective shunt pathway to increase anion movement into the tubule lumen. By stimulating different transport processes, either through different second messengers or by acting on different cell types, combinations of these diuretics can have an effect on tubule fluid secretion that is greater than the sum of their separate effects. An electrophysiological basis for this synergism can be derived from an electrical equivalent circuit used to model transepithelial ion transport (Clark *et al.*, 1998a) (**Figure 18**). The circuit comprises elements representing the active transport pathway for K^{+} and Na^{+} through the principal cells, in parallel with a passive transport pathway for Cl^{-} that lies outside of the principal cells. Within the active pathway, E_{cell} and R_{cell} represent the electromotive force and the resistance to cation transport, respectively, whereas R_{shunt} defines the passive transport pathway. The two pathways are coupled on the apical and basal sides of the epithelium by the conductances of the urine and hemolymph, respectively. It follows that any increase in ion transport through one pathway must be matched by equivalent movement through the other pathway to maintain electrical neutrality. The current flow through the circuit (I_{loop}) is described by Ohm's law, where $I_{loop} = E_{cell}/R_t$, with R_t being the transepithelial resistance ($R_t = R_{cell}R_{shunt}/(R_{cell} + R_{shunt})$). Reducing either R_{cell} or R_{shunt} will increase transepithelial ion secretion as evidenced by the increase in I_{loop}. However, a reduction in both R_{cell} and R_{shunt} will increase ion secretion (I_{loop}) by far more than the sum of their separate effects (Clark *et al.*, 1998a). Such synergistic control of tubule secretion has been demonstrated in tubules of *L. migratoria* (Coast, 1995) and *M. domestica*

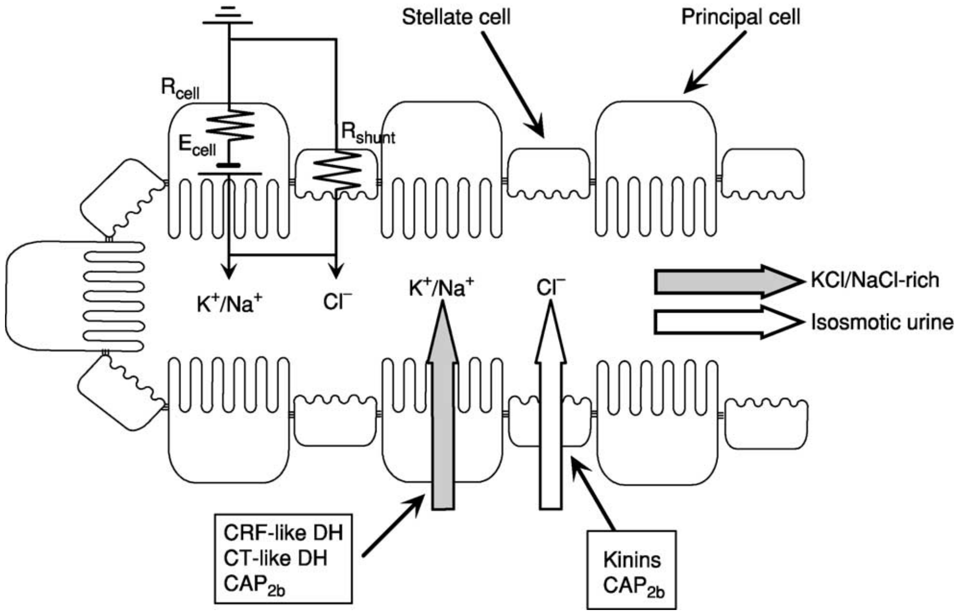


Figure 18 An electrical equivalent circuit model for the secretion of KCl and/or NaCl by Malpighian tubules. The circuit comprises parallel active cation (K^+ and Na^+) and passive anion (Cl^-) transport pathways, the former through principal cells and the latter through either stellate cells or intercellular junctions (not shown). E_{cell} is the electromotive force of the active pathway and is primarily generated by the apical membrane V-ATPase. R_{cell} is the transcellular resistance to active transport, while R_{shunt} is the resistance of the anion pathway to the diffusion of Cl^- into the lumen. Current flow (the equivalent of ion transport) through the separate pathways must be equal in order to preserve electrical neutrality. Synergism between factors that separately stimulate cation and anion transport can in part be explained by the combined effect of reducing R_{cell} and R_{shunt} on intraepithelial current flow, which is greater than the sum of their single effects. (Adapted with permission from Clark, T.M., Hayes, T.K., Beyenbach, K.W., **1998a**. Dose-dependent effects of CRF-like diuretic peptide on transcellular and paracellular transport pathways. *Am. J. Physiol. Renal Physiol. 274*, F834–F840.)

(Iaboni *et al*., 1998) challenged with a combination of their CRF related peptides and kinins to stimulate cation and anion transport, respectively. Interestingly, the CRF related peptide (Dippu-DH_{46}) and CT-like peptide (Dippu-DH_{31}) act synergistically to stimulate secretion by *D. punctata* tubules, although in this instance the peptides appear to use the same second messenger, namely cAMP (Furuya *et al*., 2000b). A possible explanation is that the peptides act on different cell types as seen in fruit fly tubules with the segregation of Ca^{2+} signaling pathways stimulated by CAP_{2b} (principal cells) and Drome-K (stellate cells) (Rosay *et al*., 1997). Synergism has also been demonstrated between serotonin and a peptide diuretic hormone from the mesothoracic ganglion mass of *R. prolixus* (Maddrell *et al*., 1993a). The peptide is unlikely to be a CRF related DH, but may be a CT-like peptide (V. Te Brugge and I. Orchard, unpublished data). Its mode of action will be of some interest, because serotonin itself stimulates both cation and anion transport in *R. prolixus* tubules (Ianowski and O'Donnell, 2001).

Synergism between two (or more?) diuretics offers a number of advantages arising from the fact that less hormone needs to be released into the circulation to stimulate diuresis. For example, diuresis can be turned on and off more rapidly, because there is less hormone to inactivate, and there will be energy savings in terms of the cost of hormone synthesis. The steeper dose–response curve that characterizes synergism (Maddrell *et al*., 1993a; Coast, 1995) means that a small change in hormone concentration can have a pronounced impact on fluid secretion, resulting in more precise control of diuresis and it is noteworthy that Locmi-DH and Locmi-K, which are co-localized in abdominal ganglion neurosecretory cells (Patel *et al*., 1994), are not co-localized in the brain and corpora cardiaca. This requires that hormone release be coordinated and it is significant that the receptor for Drome-K (Drome-K-R) is present on neurosecretory cells in the pars intercerebralis that express Drome-DH, and in their axons in the retrocerebral complex (Radford *et al*., 2002).

3.10.3.14. Possible Utility of Research on Hormonal Control of Fluid Homeostasis

There is a long-term practical significance to this research. Over 30 years ago a *Nature* paper reported that insects poisoned with conventional insecticides were found to have undergone a profound diuresis, which probably contributed to their death (Maddrell

and Casida, 1971). Control of fluid homeostasis has long been recognized as a point of great vulnerability of the Insecta, because for most species all water ingested comes from the diet. Discovery of low molecular weight agonists or antagonists of diuretic or antidiuretic hormones could lead to a new class of safe, insect specific pesticides. Screening for such molecules will be possible with the implementation of rapid, simple receptor binding assays for important classes of neuropeptides crucial in controlling water balance. Such an assay has been described for the tachykinin receptor by Torfs *et al.* (2002), who engineered *D. melanogaster* S2 cells to express recombinant aequorin together with the tachykinin receptor, and also for the Drome-K receptor using the identical approach by Radford *et al.* (2002). Agents binding to this receptor, in particular agonists, trigger an easily measured bioluminescent response suitable for high-throughput screening. Such research is one of the major thrusts of pharmaceutical companies seeking to develop new drugs. Two classes of insecticides with hormonal activity are known: (1) juvenoids, agonists of the sesquiterpenoid juvenile hormones, are useful for controlling many insects where the adult is the pest; (2) ecdysone agonists, such as tefubenozide, or MIMIC®, are useful for control of even larval stages of insects. One of the chief liabilities of these chemicals is that they work by altering gene transcription, and so are slow acting. Peptide hormone mimics should work within seconds to minutes, surmounting one of the principal shortcomings of prior hormonal pesticides.

Acknowledgments

F. Horodyski thanks the National Science Foundation (IBN-9807907) for financial support. D. Schooley and G. Coast thank the National Institutes of Health (GM-48172-11) for financial support. We also thank numerous colleagues (Drs C. Garside, E. Lehmberg, K. Schegg, R. Eigenheer, S. Nicolson, S. Liao, J. Kenyon, V. Te Brugge, and I. Orchard, as well as Ms. E. Chidembo and P. Dey) for allowing us to cite unpublished data. D. Schooley would like to thank his wife Eleanor and his editor Larry for their patience.

References

Ali, I., Steele, J.E., **1997**. Evidence that free fatty acids in trophocytes of *Periplaneta americana* fat body may be regulated by the activity of phospholipase A_2 and cyclooxygenase. *Insect Biochem. Mol. Biol. 27*, 681–692.

Arrese, E.L., Flowers, M.T., Gazard, J.L., Wells, M.A., **1999**. Calcium and cAMP are second messengers in the adipokinetic hormone-induced lipolysis of triacylglycerols in *Manduca sexta* fat body. *J. Lipid Res. 40*, *556–564*.

Arrese, E.L., Rojas-Rivas, B.I., Wells, M.A., **1996**. The use of decapitated insects to study lipid mobilization in adult *Manduca sexta*: effects of adipokinetic hormone and trehalose on fat body lipase activity. *Insect Biochem. Mol. Biol. 26*, 775–782.

Audsley, N., Coast, G.M., Schooley, D.A., **1993**. The effects of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex of *Manduca sexta*. *J. Exp. Biol. 178*, 231–243.

Audsley, N., Kay, I., Hayes, T.K., Coast, G.M., **1995**. Cross reactivity studies of CRF-related peptides on insect Malpighian tubules. *Comp. Biochem. Physiol. A 110A*, 87–93.

Audsley, N., McIntosh, C., Phillips, J.E., **1992a**. Actions of ion-transport peptide from locust corpus cardiacum on several hindgut transport processes. *J. Exp. Biol. 173*, 275–288.

Audsley, N., McIntosh, C., Phillips, J.E., **1992b**. Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *J. Exp. Biol. 173*, 261–274.

Audsley, N., McIntosh, C., Phillips, J.E., Schooley, D.A., Coast, G.M., **1994**. Neuropeptide regulation of ion and fluid reabsorption in the insect excretory system. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 74–80.

Audsley, N., Phillips, J.E., **1990**. Stimulants of ileal salt transport in neuroendocrine system of the Desert Locust. *Gen. Comp. Endocrinol. 80*, 127–137.

Auerswald, L., Gäde, G., **1999**. Effects of metabolic neuropeptides from insect corpora cardiaca on proline metabolism on the African fruit beetle, *Pachnoda sinuata*. *J. Insect Physiol. 45*, 535–543.

Auerswald, L., Gäde, G., **2000**. Cyclic AMP mediates the elevation of proline by AKH peptides in the cetoniid beetle, *Pachnoda sinuata*. *Biochim. Biophys. Acta 1495*, 78–89.

Auerswald, L., Gäde, G., **2001a**. Hormonal stimulation of proline synthesis in the fat body of the fruit beetle, *Pachnoda sinuata*, is calcium dependent. *Insect Biochem. Mol. Biol. 32*, 23–32.

Auerswald, L., Gäde, G., **2001b**. The role of calcium in the activation of glycogen phosphorylase in the fat body of the fruit beetle, *Pachnoda sinuata*, by hypertrehalosemic hormone. *Biochim. Biophys. Acta 1499*, 199–208.

Auerswald, L., Gäde, G., **2002**. The role of Ins(1,4,5)P3 in signal transduction of the metabolic neuropeptide Mem-CC in the cetoniid beetle, *Pachnoda sinuata*. *Insect Biochem. Mol. Biol. 32*, 1793–1803.

Auerswald, L., Schneider, P., Gäde, G., **1998**. Utilisation of substrates during tethered flight with and without lift generation in the African fruit beetle, *Pachnoda sinuata* (Cetoniinae). *J. Exp. Biol. 201*, 2333–2342.

Azuma, M., Harvey, W.R., Wieczorek, H., **1995**. Stoichiometry of K^+/H^+ antiport helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett. 361*, 153–156.

Baernholdt, D., Andersen, S.O., **1998**. Sequence studies on post-ecdysial cuticular proteins from pupae of the yellow mealworm, *Tenebrio molitor. Insect Biochem. Mol. Biol. 28*, 517–526.

Baggerman, G., Huybrechts, J., Clynen, E., Hens, K., Harthoorn, L., *et al.*, **2002**. New insights in adipokinetic hormone (AKH) precursor processing in *Locusta migratoria* obtained by capillary liquid chromatography-tandem mass spectrometry. *Peptides 23*, 635–644.

Baines, R.A., Thompson, K.S.J., Rayne, R.C., Bacon, J.P., **1995**. Analysis of the peptide content of the locust vasopressin-like immunoreactive (VPLI) neurons. *Peptides 16*, 799–807.

Baldwin, D., Schegg, K.M., Furuya, K., Lehmberg, E., Schooley, D.A., **2001**. Isolation and identification of a diuretic hormone from *Zootermopsis nevadensis*. *Peptides 22*, 147–152.

Baumann, E., Gersch, M., **1982**. Purification and identification of neurohormone D, a heart accelerating peptide from the corpora cardiaca of the cockroach *Periplaneta americana*. *Insect Biochem. 12*, 7–14.

Baumann, E., Penzlin, H., **1984**. Sequence analysis of neurohormone D, a neuropeptide of an insect *Periplaneta americana*. *Biomed. Biochim. Acta 43*, K13–K16.

Baumann, E., Penzlin, H., **1987**. Inactivation of neurohormone D by Malpighian tubules in an insect, *Periplaneta americana*. *J. Comp. Physiol. B 157*, 511–517.

Becker, A., Liewald, J.F., Wegener, G., **1998**. Signal transduction in isolated fat body from the cockroach *Blaptica dubia* exposed to hypertrehalosemic neuropeptide. *J. Comp. Physiol. B 168*, 159–167.

Becker, A., Wegener, G., **1998**. Hypertrehalosemic neuropeptides decrease levels of the glycolytic signal fructose 2,6-bisphosphate in cockroach fat body. *J. Exp. Biol. 201*, 1939–1946.

Beenakkers, A.M.T., **1965**. Transport of fatty acids in *Locusta migratoria* during sustained flight. *J. Insect Physiol. 11*, 879–888.

Beenakkers, A.M.T., **1969**. The influence of corpus cardiacum on lipid metabolism in *Locusta migratoria*. *Gen. Comp. Endocrinol. 13*, 492.

Berridge, M.J., **1993**. Inositol trisphosphate and calcium signalling. *Nature 361*, 315–325.

Berridge, M.J., **1995**. Capacitative calcium entry. *Biochem. J. 312*, 1–11.

Bertram, G., Schleithoff, L., Zimmermann, P., Wessing, A., **1991**. Bafilomycin A_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*: is a vacuolar ATPase involved in ion and fluid secretion? *J. Insect Physiol. 37*, 201–209.

Beyenbach, K.W., **1993**. Extracellular fluid homeostasis in insects? In: Beyenbach, K.W. (Ed.), Structure and Function of Primary Messengers in Invertebrates: Insect Diuretic and Antidiuretic Peptides, vol. 12. Karger, Basel, pp. 146–173.

Beyenbach, K.W., **1995**. Mechanism and regulation of electrolyte transport in Malpighian tubules. *J. Insect Physiol. 41*, 197–207.

Beyenbach, K.W., **2001**. Energizing epithelial transport with the vacuolar $H(^+)$-ATPase. *News Physiol. Sci. 16*, 145–151.

Beyenbach, K.W., **2003**. Transport mechanisms of diuresis in Malpighian tubules of insects. *J. Exp. Biol. 206*, 3845–3856.

Blackburn, M.B., Kingan, T.G., Bodnar, W., Shabanowitz, J., Hunt, D.F., *et al.*, **1991**. Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*. *Biochem. Biophys. Res. Commun. 181*, 927–932.

Blackburn, M.B., Ma, M.C., **1994**. Diuretic activity of Mas-DP II, an identified neuropeptide from *Manduca sexta*: an *in vivo* and *in vitro* examination in the adult moth. *Arch. Insect Biochem. Physiol. 27*, 3–10.

Blackburn, M.B., Wagner, R.M., Shabanowitz, J., Kochansky, J.P., Hunt, D.F., *et al.*, **1995**. The isolation and identification of three diuretic kinins from the abdominal ventral nerve cord of adult *Helicoverpa zea*. *J. Insect Physiol. 41*, 723–730.

Blumenthal, E.M., **2001**. Characterization of transepithelial potential oscillations in the *Drosophila* Malpighian tubule. *J. Exp. Biol. 204*, 3075–3084.

Blumenthal, E.M., **2003**. Regulation of chloride permeability by endogenously produced tyramine in the *Drosophila* Malpighian tubule. *Am. J. Physiol. Cell Physiol. 284*, C718–728.

Bogerd, J., Kooiman, F.P., Pijenburg, M.A.P., Hekking, L.H.P., Oudejans, R.C.H.M., *et al.*, **1995**. Molecular cloning of three distinct cDNAs, each encoding a different adipokinetic hormone precursor, of the migratory locust, *Locusta migratoria*. *J. Biol. Chem. 270*, 23038–23043.

Bradfield, J.Y., Keeley, L.L., **1989**. Adipokinetic hormone gene sequence from *Manduca sexta*. *J. Biol. Chem. 264*, 12791–12793.

Bradfield, J.Y., Lee, Y.-H., Keeley, L.L., **1991**. Cytochrome P450 family 4 in a cockroach: molecular cloning and regulation by hypertrehalosemic hormone. *Proc. Natl Acad. Sci. USA 88*, 4558–4562.

Bray, M.M., Shafi, S., Wheeler, C.H., Goldsworthy, G.J., **1993**. Quantification by radioimmunoassay of adipokinetic hormone-I in neural tissues in the head of *Locusta migratoria*. *Comp. Biochem. Physiol. 106A*, 257–262.

Bursell, A., **1963**. Aspects of the metabolism of amino acids in the tsetse fly, *Glossina* (Diptera). *J. Insect Physiol. 9*, 439–452.

Cabrero, P., Radford, J.C., Broderick, K.E., Costes, L., Veenstra, J.A., *et al.*, **2002**. The DH gene of *Drosophila melanogaster* encodes a diuretic peptide that acts through cyclic AMP. *J. Exp. Biol. 205*, 3799–3807.

Cady, C., Hagedorn, H.H., **1999a**. The effect of putative diuretic factors on in vivo urine production in the mosquito, *Aedes aegypti*. *J. Insect Physiol. 45*, 317–325.

Cady, C., Hagedorn, H.H., **1999b**. Effects of putative diuretic factors on intracellular second messenger levels in the Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol. 45*, 327–337.

Candy, D.J., **2002**. Adipokinetic hormone concentrations in the haemolymph of *Schistocerca gregaria* measured by radioimmunoassay. *Insect Biochem. Mol. Biol. 32*, 1361–1367.

Carlisle, J.A., Loughton, B.G., **1979**. Adipokinetic hormone inhibits protein synthesis in *Locusta*. *Nature 282*, 420–421.

Carlsen, J., Herman, W.S., Christensen, M., Josefsson, L., **1979**. Characterisation of a second peptide with adipokinetic and red pigment-concentrating activity from the locust corpora cardiaca. *Insect Biochem. 9*, 497–501.

Chabre, O., Conklin, B.R., Lin, H.Y., Lodish, H.F., Wilson, E., *et al.*, **1992**. A recombinant calcitonin receptor independently stimulates 3′,5′-cyclic adenosine monophosphate and $Ca2^{+}$/inositol phosphate signaling pathways. *Mol. Endocrinol. 6*, 551–556.

Cheeseman, P., Goldsworthy, G.J., **1979**. The release of adipokinetic hormone during flight and starvation in *Locusta*. *Gen. Comp. Endocrinol. 37*, 35–43.

Cheeseman, P., Jutsum, A.R., Goldsworthy, G.J., **1976**. Quantitative studies on the release of locust adipokinetic hormone. *Physiol. Ent. 1*, 115–121.

Chino, H., Gilbert, L.I., **1964**. Diglyceride release from insect fat body. *Science 143*, 359–361.

Chino, H., Gilbert, L.I., **1965**. Lipid release and transport in insects. *Biochim. Biophys. Acta 98*, 94–110.

Chung, J.S., Dircksen, H., Webster, S.G., **1999**. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc. Natl Acad. Sci. USA 96*, 13103–13107.

Clark, J.D., Lin, L.-L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., *et al.*, **1991**. A novel arachidonic acid-selective PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell 65*, 1043–1051.

Clark, T.M., Bradley, T.J., **1996**. Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. *J. Insect Physiol. 42*, 593–602.

Clark, T.M., Hayes, T.K., Beyenbach, K.W., **1998a**. Dose-dependent effects of CRF-like diuretic peptide on transcellular and paracellular transport pathways. *Am. J. Physiol. Renal Physiol. 274*, F834–F840.

Clark, T.M., Hayes, T.K., Holman, G.M., Beyenbach, K.W., **1998b**. The concentration-dependence of CRF-like diuretic peptide: mechanisms of action. *J. Exp. Biol. 201*, 1753–1762.

Clements, A.N., **1992**. The Biology of Mosquitoes. Chapman & Hall, London.

Clottens, F.L., Holman, G.M., Coast, G.M., Totty, N.F., Hayes, T.K., *et al.*, **1994**. Isolation and characterization of a diuretic peptide common to the house fly and stable fly. *Peptides 15*, 971–979.

Clynen, E., Baggerman, G., Veelaert, D., Cerstiaens, A., Van der Horst, D., *et al.*, **2001**. Peptidomics of the pars intercerebralis-corpus cardiacum complex of the migratory locust, *Locusta migratoria*. *Eur. J. Biochem. 268*, 1929–1939.

Clynen, E., Huybrechts, J., Baggerman, G., Van Doorn, J., Van Der Horst, D., *et al.*, **2003a**. Identification of a glycogenolysis-inhibiting peptide from the corpora cardiaca of locusts. *Endocrinology 144*, 3441–3448.

Clynen, E., Huybrechts, J., De Loof, A., Schoofs, L., **2003b**. Mass spectrometric analysis of the perisympathetic organs in locusts: identification of novel periviscerokinins. *Biochem. Biophys. Res. Commun. 300*, 422–428.

Coast, G.M., **1995**. Synergism between diuretic peptides controlling ion and fluid transport in insect Malpighian tubules. *Regul. Pept. 57*, 283–296.

Coast, G.M., **1996**. Neuropeptides implicated in the control of diuresis in insects. *Peptides 17*, 327–336.

Coast, G.M., **1998**. The regulation of primary urine production in insects. In: Webster, S.G. (Ed.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge, pp. 189–209.

Coast, G.M., **2001**. The neuroendocrine regulation of salt and water balance in insects. *Zoology (Jena) 103*, 179–188.

Coast, G.M., Chung, J.-S., Goldsworthy, G.J., Patel, M., Hayes, T.K., *et al.*, **1994**. Corticotropin releasing factor related diuretic peptides in insects. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 67–73.

Coast, G.M., Cusinato, O., Kay, I., Goldsworthy, G.J., **1991**. An evaluation of the role of cyclic AMP as an intracellular 2nd messenger in Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol. 37*, 563–573.

Coast, G.M., Hayes, T.K., Kay, I., Chung, J.S., **1992**. Effect of *Manduca sexta* diuretic hormone and related peptides on isolated Malpighian tubules of the house cricket *Acheta domesticus* (L.). *J. Exp. Biol. 162*, 331–338.

Coast, G.M., Holman, G.M., Nachman, R.J., **1990**. The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol. 36*, 481–488.

Coast, G.M., Kay, I., **1994**. The effects of acheta diuretic peptide on isolated Malpighian tubules from the house cricket *Acheta domesticus*. *J. Exp. Biol. 187*, 225–243.

Coast, G.M., Meredith, J., Phillips, J.E., **1999**. Target organ specificity of major neuropeptide stimulants in locust excretory systems. *J. Exp. Biol. 202*, 3195–3203.

Coast, G.M., Orchard, I., Phillips, J.E., Schooley, D.A., **2002a**. Insect diuretic and antidiuretic hormones. *Adv. Insect Physiol. 29*, 279–409.

Coast, G.M., Rayne, R.C., Hayes, T.K., Mallet, A.I., Thompson, K.S.J., *et al.*, **1993**. A comparison of the

effects of 2 putative diuretic hormones from *Locusta migratoria* on isolated locust malpighian tubules. *J. Exp. Biol. 175*, 1–14.

Coast, G.M., Webster, S.G., Schegg, K.M., Tobe, S.S., Schooley, D.A., **2001**. The *Drosophila melanogaster* homologue of an insect calcitonin-like diuretic peptide stimulates V-ATPase activity in fruit fly Malpighian tubules. *J. Exp. Biol. 204*, 1795–1804.

Coast, G.M., Zabrocki, J., Nachman, R.J., **2002b**. Diuretic and myotropic activities of N-terminal truncated analogs of *Musca domestica* kinin neuropeptide. *Peptides 23*, 701–708.

Cohen, A.C., Patana, R., **1982**. Ontogenetic and stress-related changes in hemolymph chemistry of Beet Armyworms. *Comp. Biochem. Physiol. 71A*, 193–198.

Coutchié, P.A., Machin, J., **1984**. Allometry of water-vapor absorption in 2 species of tenebrionid beetle larvae. *Am. J. Physiol. 247*, R230–R236.

Cox, K.J.A., Tensen, C.P., Van der Schors, R.C., Li, K.W., Van Heerikhuizen, H., *et al.*, **1997**. Cloning, characterization, and expression of a G-protein-coupled receptor from *Lymnaea stagnalis* and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. *J. Neurosci. 17*, 1197–1205.

Cusinato, O., Drake, A.F., Gäde, G., Goldsworthy, G.J., **1998**. The molecular conformations of representative arthropod adipokinetic peptides determined by circular dichroism spectroscopy. *Insect Biochem. Mol. Biol. 28*, 43–50.

Cusinato, O., Wheeler, C.H., Goldsworthy, G.J., **1991**. The identity and physiological actions of an adipokinetic hormone in *Acheta domesticus*. *J. Insect Physiol. 37*, 461–469.

Dautzenberg, F.M., Hauger, R.L., **2002**. The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharmacol. Sci. 23*, 71–77.

Dautzenberg, F.M., Wille, S., Lohmann, R., Spiess, J., **1998**. Mapping of the ligand-selective domain of the *Xenopus laevis* corticotropin-releasing factor receptor 1: Implications for the ligand-binding site. *Proc. Natl Acad. Sci. USA 95*, 4941–4946.

Davenport, T.R.B., Isaac, R.E., Lee, D.L., **1991**. The presence of peptides related to the adipokinetic hormone/red pigment-concentrating hormone family in the nematode, *Panagrellus redivivus*. *Gen. Comp. Endocrinol. 81*, 419–425.

Davies, S.A., **2000**. Nitric oxide signalling in insects. *Insect Biochem. Mol. Biol. 30*, 1123–1138.

Davies, S.A., Huesmann, G.R., Maddrell, S.H.P., O'Donnell, M.J., Skaer, N.J.V., *et al.*, **1995**. CAP_{2b}, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol. Regul. Integr. Comp. Physiol. 269*, R1321–R1326.

Davies, S.A., Stewart, E.J., Huesmann, G.R., Skaer, N.J.V., Maddrell, S.H.P., *et al.*, **1997**. Neuropeptide stimulation of the nitric oxide signaling pathway in *Drosophila melanogaster* Malpighian tubules. *Am. J. Physiol. Regul. Integr. Comp. Physiol. 273*, R823–R827.

De Kort, C.A.D., Bartlink, A.K.M., Schuurmans, R.R., **1973**. The significance of L-proline for oxidative metabolism in the flight muscles of the Colorado beetle, *Leptinotarsa decemlineata*. *Insect Biochem. 3*, 11–17.

Denholm, B., Sudarsan, V., Pasalodos-Sanchez, S., Artero, R., Lawrence, P., *et al.*, **2003**. Dual origin of the renal tubules in *Drosophila*: mesodermal cells integrate and polarize to establish secretory function. *Curr. Biol. 13*, 1052–1057.

Detloff, M., Wittwer, D., Weise, C., Wiesner, A., **2001**. Lipophorin of lower density is formed during immune responses in the lepidopteran insect *Galleria mellonella*. *Cell Tissue Res. 306*, 449–458.

Diederen, J.H.B., Maas, H.A., Pel, H.J., Schooneveld, H., Jansen, W.F., *et al.*, **1987**. Co-localization of the adipokinetic hormones I and II in the same glandular cells and in the same secretory granules of corpus cardiacum of *Locusta migratoria* and *Schistocerca gregaria*. *Cell Tissue Res. 249*, 379–389.

Diederen, J.H.B., Peppelenbosch, M.P., Vullings, H.C.B., **1992**. Ageing adipokinetic cells in *Locusta migratoria*: an ultrastructural morphometric study. *Cell Tissue Res. 268*, 117–121.

Digan, M.E., Roberts, D.N., Enderlin, F.E., Woodworth, A.R., Kramer, S.J., **1992**. Characterization of the precursor for *Manduca sexta* diuretic hormone Mas-DH. *Proc. Natl Acad. Sci. USA 89*, 11074–11078.

Dircksen, H., Homberg, U., **1995**. Crustacean cardioactive peptide-immunoreactive neurons innervating brain neuropils, retrocerebral complex and stomatogastric nervous system of the locust, *Locusta migratoria*. *Cell Tissue Res. 279*, 495–515.

Dow, J.A., Gupta, B.L., Hall, T.A., Harvey, W.R., **1984**. X-ray microanalysis of elements in frozen-hydrated sections of an electrogenic K^+ transport system: the posterior midgut of tobacco hornworm (*Manduca sexta*) in vivo and in vitro. *J. Membr. Biol. 77*, 223–241.

Dow, J.A.T., Davies, S.A., Sözen, M.A., **1998**. Fluid secretion by the *Drosophila* Malpighian tubule. *Am. Zool. 38*, 450–460.

Dow, J.T., Davies, S.A., **2003**. Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev. 83*, 687–729.

Egan, J.J., Greenberg, A.S., Chang, M.-K., Wek, S.A., Moos, M.C.Jr., *et al.*, **1992**. Mechanisms of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. *Proc. Natl Acad. Sci. USA 89*, 8537–8541.

Eigenheer, R.A., Nicolson, S.W., Schegg, K.M., Hull, J.J., Schooley, D.A., **2002**. Identification of a potent antidiuretic factor acting on beetle Malpighian tubules. *Proc. Natl Acad. Sci. USA 99*, 84–89.

Eigenheer, R.A., Wiehart, U.M., Nicolson, S.W., Schoofs, L., Schegg, K.M., *et al.*, **2003**. Isolation, identification and localization of a second beetle antidiuretic peptide. *Peptides 24*, 27–34.

Endo, H., Nagasawa, H., Watanabe, T., **2000**. Isolation of a cDNA encoding a CHH-family peptide from the silkworm *Bombyx mori*. *Insect Biochem. Mol. Biol. 30*, 355–361.

Fernlund, P., Josefsson, L., **1972**. Crustacean color-change hormone: amino acid sequence and chemical synthesis. *Science 177*, 173–175.

Fischer-Lougheed, J., O'Shea, M., Cornish, I., Losberger, C., Roulet, E., *et al.*, **1993**. AKH biosynthesis: transcriptional and translational control of two co-localized prohormones. *J. Exp. Biol. 177*, 223–241.

Force, T., Bonventre, J.V., Flannery, M.R., Gorn, A.H., Yamin, M., *et al.*, **1992**. A cloned porcine renal calcitonin receptor couples to adenylyl cyclase and phospholipase C. *Am. J. Physiol. 262*, F1110–1115.

Forgac, M., **2000**. Structure, mechanism and regulation of the clathrin-coated vesicle and yeast vacuolar H(+)-ATPases. *J. Exp. Biol. 203*, 71–80.

Fournier, B., **1991**. Neuroparsins stimulate inositol phosphate formation in locust rectal cells. *Comp. Biochem. Physiol. B 99*, 57–64.

Fournier, B., Guerineau, N., Mollard, P., Girardie, J., **1994**. Effects of two neuronal antidiuretic molecules, neuroparsin and 5-hydroxytryptamine, on cytosolic free calcium monitored with indo-1 in epithelial and muscular cells of the African locust rectum. *BBA-Mol. Cell. Res. 1220*, 181–187.

Fournier, B., Herault, J.P., Proux, J., **1987**. Study of an antidiuretic factor from the nervous lobes of the migratory locust corpora cardiaca. Improvement of an existing bioassay. *Gen. Comp. Endocrinol. 68*, 49–56.

Fox, A.M., Reynolds, S.E., **1991b**. Degradation of adipokinetic hormone family peptides by a circulating endopeptidase in the insect *Manduca sexta*. *Peptides 12*, 937–944.

Fox, A.M., Reynolds, S.E., **1991a**. The pharmacology of the lipid-mobilising response to adipokinetic hormone family peptides in the moth, *Manduca sexta*. *J. Insect Physiol. 37*, 373–381.

Friedman, S., **1985**. Carbohydrate metabolism. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 43–70.

Fullbright, G., Lacy, E.R., Büllesbach, E.E., **1997**. The prothoracicotropic hormone bombyxin has specific receptors on insect ovarian cells. *Eur. J. Biochem. 245*, 774–780.

Furuya, K., Harper, M., Schegg, K.M., Schooley, D.A., **2000a**. Isolation and characterization of CRF-related diuretic hormones from the whitelined sphinx moth *Hyles lineata*. *Insect Biochem. Mol. Biol. 30*, 127–133.

Furuya, K., Milchak, R.J., Schegg, K.M., Zhang, J., Tobe, S.S., *et al.*, **2000b**. Cockroach diuretic hormones: characterization of a calcitonin-like peptide in insects. *Proc. Natl Acad. Sci. USA 97*, 6469–6474.

Furuya, K., Schegg, K.M., Schooley, D.A., **1998**. Isolation and identification of a second diuretic hormone from *Tenebrio molitor*. *Peptides 19*, 619–626.

Furuya, K., Schegg, K.M., Wang, H., King, D.S., Schooley, D.A., **1995**. Isolation and identification of a diuretic hormone from the mealworm *Tenebrio molitor*. *Proc. Natl Acad. Sci. USA 92*, 12323–12327.

Gammie, S.C., Truman, J.W., **1997**. Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. *J. Neurosci. 17*, 4389–4397.

Gäde, G., **1979**. Studies on the influence of synthetic adipokinetic hormone and some analogs on cyclic AMP levels in different arthropod systems. *Gen. Comp. Endocrinol. 37*, 122–130.

Gäde, G., **1980**. Further characteristics of adipokinetic and hyperglycaemic factor(s) of stick insects. *J. Insect Physiol. 26*, 351–360.

Gäde, G., **1981**. Activation of fat body glycogen phosphorylase in *Locusta migratoria* by corpus cardiacum extract and synthetic adipokinetic hormone. *J. Insect Physiol. 27*, 155–162.

Gäde, G., **1990**. Structure-function studies on hypertrehalosemic and adipokinetic hormones: activity of naturally occurring analogues and some N- and C-terminal modified analogues. *Physiol. Ent. 15*, 299–316.

Gäde, G., **1992**. Structure-activity relationships for the carbohydrate-mobilizing action of further bioanalogues of the adipokinetic hormone/red pigment-concentrating hormone family of peptides. *J. Insect Physiol. 38*, 259–266.

Gäde, G., **2004**. Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Annu. Rev. Entomol. 49*, 93–113.

Gäde, G., Auerswald, L., **2002**. Beetles' choice – proline for energy output: control by AKHs. *Comp. Biochem. Physiol. 132B*, 117–129.

Gäde, G., Auerswald, L., **2003**. Mode of action of neuropeptides from the adipokinetic hormone family. *Gen. Comp. Endocrinol. 132*, 10–20.

Gäde, G., Auerswald, L., Simek, P., Marco, H.G., Kodrik, D., **2003**. Red pigment-concentrating hormone is not limited to crustaceans. *Biochem. Biophys. Res. Commun. 309*, 967–973.

Gäde, G., Hayes, T.K., **1995**. Structure-activity relationships for *Periplaneta americana* hypertrehalosemic hormone I: the importance of side chains and termini. *Peptides 16*, 1173–1180.

Gäde, G., Hoffmann, K.-H., Spring, J.H., **1997**. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev. 77*, 963–1032.

Gäde, G., Holwerda, D.A., **1976**. Involvement of adenosine 3′:5′-cyclic monophosphate in lipid mobilization in *Locusta migratoria*. *Insect Biochem. 6*, 535–540.

Gäde, G., Janssens, M.P., **1994**. Cicadas contain novel members of the AKH/RPCH family peptides with hypertrehalosemic activity. *Biol. Chem. Hoppe-Seyler 375*, 803–809.

Gäde, G., Kellner, R., **1999**. Dragonfly *Erythemis simplicicollis* contains a novel adipokinetic neuropeptide. *Arch. Insect. Biochem. Physiol. 40*, 99–106.

Gäde, G., Kellner, R., Rinehart, K.L., Proefke, M.L., **1992**. A tryptophan-substituted member of the AKH/RPCH family isolated from a stick insect corpus cardiacum. *Biochem. Biophys. Res. Commun. 189*, 1303–1309.

Gillespie, J.P., Kanost, M.R., Trenczek, T., **1997**. Biological mediators of insect immunity. *Annu. Rev. Entomol. 42*, 611–643.

Girardie, J., Boureme, D., Couilland, F., Tamarelle, M., Girardie, A., **1987**. Anti-juvenile effect of neuroparsin A, a new protein isolated from the locust corpora cardiaca. *Insect Biochem. 17*, 977–983.

Girardie, J., Girardie, A., Huet, J.-C., Pernollet, J.-C., **1989**. Amino acid sequence of locust neuroparsins. *FEBS Lett. 248*, 4–8.

Girardie, J., Huet, J.C., Pernollet, J.C., **1990**. The locust neuroparsin-A – sequence and similarities with vertebrate and insect polypeptide hormones. *Insect Biochem. 20*, 659–666.

Gokuldas, M., Hunt, P.A., Candy, D.J., **1988**. The inhibition of lipid synthesis *in vitro* in the locust *Schistocerca gregaria* by factors from the corpora cardiaca. *Physiol. Ent. 13*, 43–48.

Goldsworthy, G., Chandrakant, S., Opoku-Ware, K., **2003a**. Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide. *J. Insect Physiol. 49*, 795–803.

Goldsworthy, G., Mullen, L., Opoku-Ware, K., Chandrakant, S., **2003b**. Interactions between the endocrine and immune systems in locusts. *Physiol. Ent. 28*, 54–61.

Goldsworthy, G., Opoku-Ware, K., Mullen, L., **2002**. Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. *J. Insect Physiol. 48*, 601–608.

Goldsworthy, G.J., **1969**. Hyperglycaemic factors from the corpus cardiacum of *Locusta migratoria*. *J. Insect Physiol. 15*, 2131–2140.

Goldsworthy, G.J., Johnson, R.A., Mordue, W., **1972b**. *In vivo* studies on the release of hormones from the corpora cardiaca of locusts. *J. Comp. Physiol. 79*, 85–96.

Goldsworthy, G.J., Lee, M.J., Luswata, R., Drake, A.F., Hyde, D., **1997**. Structures, assays and receptors for locust adipokinetic hormones. *Comp. Biochem. Physiol. 117B*, 483–496.

Goldsworthy, G.J., Mallison, K., Wheeler, C.H., **1986b**. The relative potencies of two known locust adipokinetic hormones. *J. Insect Physiol. 32*, 95–101.

Goldsworthy, G.J., Mallison, K., Wheeler, C.H., Gäde, G., **1986a**. Relative adipokinetic activities of members of the adipokinetic hormone/red pigment concentrating hormone family. *J. Insect Physiol. 32*, 433–438.

Goldsworthy, G.J., Mordue, W., Guthkelch, J., **1972a**. Studies on insect adipokinetic hormones. *Gen. Comp. Endocrinol. 18*, 545–551.

Goldsworthy, G.J., Wheeler, C.H., **1989**. Physiological and structural aspects of adipokinetic hormone function in locusts. *Pest. Sci. 25*, 85–95.

Goosey, M.W., Candy, D.J., **1980**. The D-octopamine content of the haemolymph of the locust *Schistocerca americana gregaria* and its elevation during flight. *Insect Biochem. 10*, 393–397.

Grieco, M.A.B., Lopes, A.G., **1997**. 5-Hydroxytryptamine regulates the (Na^+-K^+)ATPase activity in Malpighian tubules of *Rhodnius prolixus*: evidence for involvement of G-protein and cAMP-dependent protein kinase. *Arch. Insect Biochem. Physiol. 36*, 203–214.

Grinstein, S., Wieczorek, H., **1994**. Cation antiports of animal plasma membranes. *J. Exp. Biol. 196*, 307–318.

Grüber, G., Wieczorek, H., Harvey, W.R., Muller, V., **2001**. Structure-function relationships of A-, F- and V-ATPases. *J. Exp. Biol. 204*, 2597–2605.

Hansen, L.L., Ramlov, H., Westh, P., **2004**. Metabolic activity and water vapour absorption in the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae): real-time measurements by twochannel microcalorimetry. *J. Exp. Biol. 207*, 545–552.

Harthoorn, L.F., Diederen, J.H.B., Oudejans, R.C.H.M., Van der Horst, D.J., **1999**. Differential location of peptide hormones in the secretory pathway of insect adipokinetic cells. *Cell Tissue Res. 298*, 361–369.

Harthoorn, L.F., Oudejans, R.C.H.M., Diederen, J.H.B., Van de Wijngaart, D.J., Van der Horst, D.J., **2001**. Absence of coupling between release and synthesis of peptide hormones in insect neurosecretory cells. *Eur. J. Cell Biol. 80*, 451–457.

Harthoorn, L.F., Oudejans, R.C.H. M., Diederen, J.H.B., Van der Horst, D.J., **2002**. Coherence between biosynthesis and secretion of insect adipokinetic hormones. *Peptides 23*, 629–634.

Hatle, J.D., Spring, J.H., **1999**. Tests of potential adipokinetic hormone precursor related peptide (APRP) functions: Lack of responses. *Arch. Insect Biochem. Physiol. 42*, 163–166.

Hauger, R.L., Grigoriadis, D.E., Dallman, M.F., Plotsky, P.M., Vale, W.W., *et al.*, **2003**. International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. *Pharmacol. Rev. 55*, 21–26.

Hauser, F., Søndergaard, L., Grimmelikhuijzen, C.J.P., **1998**. Molecular cloning, genomic organization and developmental regulation of a novel receptor from *Drosophila melanogaster* structurally related to gonadotropin-releasing hormone receptors from vertebrates. *Biochem. Biophys. Res. Commun. 249*, 822–828.

Hayes, T.K., Holman, G.M., Pannabecker, T.L., Wright, M.S., Strey, A.A., *et al.*, **1994**. Culekinin depolarizing peptide: A mosquito leucokinin-like peptide that influences insect Malpighian tubule ion transport. *Regul. Pept. 52*, 235–248.

Hayes, T.K., Keeley, L.L., **1990**. Structure-activity relationships on hyperglycemia by representatives of the adipokinetic/hyperglycemic hormone family in

Blaberus cockroaches. *J. Comp. Physiol. 160B*, 187–194.

Hayes, T.K., Pannabecker, T.L., Hinckley, D.J., Holman, G.M., Nachman, R.J., *et al.*, **1989**. Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci. 44*, 1259–1266.

Hegarty, J.L., Zhang, B., Pannabecker, T.L., Petzel, D.H., Baustian, M.D., *et al.*, **1991**. Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol. 261*, C521–C529.

Hekimi, S., Fischer-Lougheed, J., O'Shea, M., **1991**. Regulation of neuropeptide stoichiometry in neurosecretory cells. *J. Neurosci. 11*, 3246–3256.

Hekimi, S., O'Shea, M., **1987**. Identification and purification of two precursors of the insect neuropeptide adipokinetic hormone. *J. Neurosci. 7*, 2773–2784.

Herault, J.P.E., Girardie, J., Proux, J.P., **1985**. Separation and characteristics of antidiuretic factors from the glandular lobes of the migratory locust corpora cardiaca. *Int. J. Invert. Reprod. Devel. 8*, 325–335.

Herault, J.P.E., Girardie, J., Proux, J.P., **1988**. Further characterization of the antidiuretic factor from the glandular part of the corpora cardiaca of the migratory locust. *Int. J. Invert. Reprod. Devel. 13*, 183–192.

Herault, J.P.E., Proux, J.P., **1987**. Cyclic AMP, the second messenger of an antidiuretic hormone from glandular lobes of migratory locust CC. *J. Insect Physiol. 33*, 487–492.

Herman, W.S., Carlsen, J.B., Christensen, M., Josefsson, L., **1977**. Evidence for an adipokinetic function of the RPCH activity present in the desert locust neuroendocrine system. *Biol. Bull. 153*, 527–539.

Hewes, R.S., Taghert, P.H., **2001**. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res. 11*, 1126–1142.

Hietter, H., Van Dorsselaer, A., Luu, B., **1991**. Characterization of 3 structurally-related 8–9 kDa monomeric peptides present in the corpora cardiaca of *Locusta* – a revised structure for the neuroparsins. *Insect Biochem. 21*, 259–264.

Holman, G.M., Cook, B.J., Nachman, R.J., **1986a**. Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. *Comp. Biochem. Physiol. 84C*, 205–211.

Holman, G.M., Cook, B.J., Nachman, R.J., **1986b**. Primary structure and synthesis of two additional neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. *Comp. Biochem. Physiol. 84C*, 271–276.

Holman, G.M., Cook, B.J., Nachman, R.J., **1987a**. Isolation, primary structure and synthesis of leucokinins V and VI: myotropic peptides of *Leucophaea maderae*. *Comp. Biochem. Physiol. 88C*, 27–30.

Holman, G.M., Cook, B.J., Nachman, R.J., **1987b**. Isolation, primary structure and synthesis of Leucokinins VII and VIII: the final members of this new family of cephalomyotropic peptides isolated from head extracts of *Leucophaea maderae*. *Comp. Biochem. Physiol. 88C*, 31–34.

Holman, G.M., Nachman, R.J., Coast, G.M., **1999**. Isolation, characterization and biological activity of a diuretic myokinin neuropeptide from the housefly, *Musca domestica*. *Peptides 20*, 1–10.

Holman, G.M., Nachman, R.J., Schoofs, L., Hayes, T.K., Wright, M.S., *et al.*, **1991**. The *Leucophaea maderae* hindgut preparation – a rapid and sensitive bioassay tool for the isolation of insect myotropins of other insect species. *Insect Biochem. 21*, 107–112.

Holmes, S.P., He, H., Chen, A.C., lvie, G.W., Pietrantonio, P.V., **2000**. Cloning and transcriptional expression of a leucokinin-like peptide receptor from the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). *Insect Mol. Biol. 9*, 457–465.

Holt, R.A., Subramanian, G.M., Hacpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Huesmann, G.R., Cheung, C.C., Loi, P.K., Lee, T.D., Swiderek, K.M., *et al.*, **1995**. Amino acid sequence of CAP_{2b}, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*. *FEBS Lett. 371*, 311–314.

Huybrechts, J., Clynen, E., Baggerman, G., De Loof, A., Schoofs, L., **2002**. Isolation and identification of the AKH III precursor-related peptide from *Locusta migratoria*. *Biochem. Biophys. Res. Commun. 296*, 1112–1117.

Iaboni, A., Holman, G.M., Nachman, R.J., Orchard, I., Coast, G.M., **1998**. Immunocytochemical localisation and biological activity of diuretic peptides in the housefly, *Musca domestica*. *Cell Tissue Res. 294*, 549–560.

Ianowski, J.P., Christensen, R.J., O'Donnell, M.J., **2002**. Intracellular ion activities in Malpighian tubule cells of *Rhodnius prolixus*: evaluation of Na^+–K^+–$2Cl^-$ cotransport across the basolateral membrane. *J. Exp. Biol. 205*, 1645–1655.

Ianowski, J.P., O'Donnell, M.J., **2001**. Transepithelial potential in Malpighian tubules of *Rhodnius prolixus*: lumen-negative voltages and the triphasic response to serotonin. *J. Insect Physiol. 47*, 411–421.

Ishibashi, J., Kataoka, H., Nagasawa, H., Isogai, A., Suzuki, A., **1992**. Isolation and identification of adipokinetic hormone of the silkworm, *Bombyx mori*. *Biosci. Biotech. Biochem. 56*, 66–70.

Jaffe, H., Raina, A.K., Riley, C.T., Fraser, B.A., Bird, T.G., *et al.*, **1988**. Isolation and primary structure of a neuropeptide hormone from *Heliothis zea* with hypertrehalosemic and adipokinetic activities. *Biochem. Biophys. Res. Commun. 155*, 344–350.

Jaffe, H., Raina, A.K., Riley, C.T., Fraser, B.A., Nachman, R.J., *et al.*, **1989**. Primary structure of two neuropeptide hormones with adipokinetic and hypotrehalosemic activity isolated from the corpora cardiaca of horse flies (Diptera). *Proc. Natl Acad. Sci. USA 86*, 8161–8164.

Jeffs, L.B., Phillips, J.E., **1996**. Pharmacological study of the second messengers that control rectal ion and fluid transport in the desert locust (*Schistocerca gregaria*). *Arch. Insect Biochem. Physiol. 31*, 169–184.

Johard, H.A., Coast, G.M., Mordue, W., Nassel, D.R., **2003**. Diuretic action of the peptide locustatachykinin I: cellular localisation and effects on fluid secretion in Malpighian tubules of locusts. *Peptides 24*, 1571–1579.

Johnson, E.C., Bohn, L.M., Taghert, P.H., **2004**. *Drosophila* CG8422 encodes a functional diuretic hormone receptor. *J. Exp. Biol. 207*, 743–748.

Jutsum, A.R., Goldsworthy, G.J., **1976**. Fuels for flight in *Locusta*. *J. Insect Physiol. 22*, 243–249.

Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., *et al.*, **1989a**. Identification of an allatotropin from adult *Manduca sexta*. *Science 243*, 1481–1483.

Kataoka, H., Troetschler, R.G., Li, J.P., Kramer, S.J., Carney, R.L., *et al.*, **1989b**. Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. Natl Acad. Sci. USA 86*, 2976–2980.

Kawakami, A., Iwami, M., Nagasawa, H., Suzuki, A., Ishizaki, H., **1989**. Structure and organization of 4 clustered genes that encode bombyxin, an insulin–related brain secretory peptide of the silkmoth *Bombyx mori*. *Proc. Natl Acad. Sci. USA 86*, 6843–6847.

Kay, I., Coast, G.M., Cusinato, O., Wheeler, C.H., Totty, N.F., *et al.*, **1991a**. Isolation and characterization of a diuretic peptide from *Acheta domesticus* – evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe-Seyler 372*, 505–512.

Kay, I., Wheeler, C.H., Coast, G.M., Totty, N.F., Cusinato, O., *et al.*, **1991b**. Characterization of a diuretic peptide from *Locusta migratoria*. *Biol. Chem. Hoppe-Seyler 372*, 929–934.

Kean, L., Cazenave, W., Costes, L., Broderick, K.E., Graham, S., *et al.*, **2002**. Two nitridergic peptides are encoded by the gene capability in *Drosophila melanogaster. Am. J. Physiol. Regul. Integr. Comp. Physiol. 282*, R1297–R1307.

Keeley, L.L., Chung, J.S., Hayes, T.K., **1992**. Diuretic and antifeedant actions by *Manduca sexta* diuretic hormone in lepidopteran larvae. *Experientia 48*, 1145–1148.

Keeley, L.L., Hayes, T.K., Bradfield, J.Y., Sowa, S.M., **1991**. Physiological actions by hypertrehalosemic hormone and adipokinetic peptides in adult *Blaberus discoidalis* cockroaches. *Insect Biochem. 21*, 121–129.

Keeley, L.L., Hesson, A.S., **1995**. Calcium-dependent signal transduction by the hypertrehalosemic hormone in the cockroach fat body. *Gen. Comp. Endocrinol. 99*, 373–381.

Kehne, J., De Lombaert, S., **2002**. Non-peptidic CRF1 receptor antagonists for the treatment of anxiety, depression and stress disorders. *Curr. Drug Target CNS Neurol. Disord. 1*, 467–493.

King, D.S., Meredith, J., Wang, Y.J., Phillips, J.E., **1999**. Biological actions of synthetic locust ion transport peptide (ITP). *Insect Biochem. Mol. Biol. 29*, 11–18.

Kirschenbaum, S.R., O'Shea, M., **1993**. Postembryonic proliferation of neuroendocrine cells expressing adipokinetic hormone peptides in the corpora cardiaca of the locust. *Development 118*, 1181–1190.

Kodrik, D., Goldsworthy, G.J., **1995**. Inhibition of RNA synthesis by adipokinetic hormones and brain factor(s) in adult fat body of *Locusta migratoria*. *J. Insect Physiol. 41*, 127–133.

Kodrík, D., Socha, R., Simek, P., Zemek, R., Goldsworthy, G.J., **2000**. A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera). *Insect Biochem. Mol. Biol. 30*, 489–498.

Köllisch, G.V., Lorenz, M.W., Kellner, R., Verhaert, P.D., Hoffmann, K.H., **2000**. Structure elucidation and biological activity of an unusual adipokinetic hormone from corpora cardiaca of the butterfly, *Vanessa cardui*. *Eur. J. Biochem. 267*, 5502–5508.

Köllisch, G.V., Verhaert, P., Hoffmann, K.H., **2003**. *Vanessa cardui* adipokinetic hormone (Vanca-AKH) in butterflies and a moth. *Comp. Biochem. Physiol. 135A*, 303–308.

Konings, P.N.M., Vullings, H.G.B., Kok, O.J.M., Diederen, J.H.B., Jansen, W.F., **1989**. The innervation of the corpus cardiacum of *Locusta migratoria*: A neuroanatomical study with the use of Lucifer yellow. *Cell Tissue Res. 258*, 301–308.

Laenen, B., **1999**. Purification, Characterization and Mode of Action of Endogenous Neuroendocrine Factors in the Forest Ant, *Formica polyctena*. Ph.D. thesis, Limburgs Universitair Centrum.

Lagueux, M., Lwoff, L., Meister, M., Goltzene, F., Hoffmann, J.A., **1990**. cDNAs from neurosecretory cells of brains of *Locusta migratoria* (Insecta, Orthoptera) encoding a novel member of the superfamily of insulins. *Eur. J. Biochem. 187*, 249–254.

Lavigne, C., Embleton, J., Audy, P., King, R.R., Pelletier, Y., **2001**. Partial purification of a novel insect antidiuretic factor from the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), which acts on Malpighian tubules. *Insect Biochem. Mol. Biol. 31*, 339–347.

Lavine, M.D., Strand, M.R., **2002**. Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol. 32*, 1295–1309.

Lee, K.Y., Horodyski, F.M., Chamberlin, M.E., **1998**. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* FLRFamides in the tobacco hornworm *Manduca sexta*. *J. Exp. Biol. 201*, 3067–3074.

Lee, M.J., Cusinato, O., Luswata, R., Wheeler, C.H., Goldsworthy, G.J., **1997**. N-terminal modifications to AKH-I from *Locusta migratoria*: assessment of biological potencies *in vivo* and *in vitro*. *Reg. Peptides 69*, 69–76.

Lee, M.J., de Jong, S., Gäde, G., Poulos, C., Goldsworthy, G.J., **2000**. Mathematical modeling of insect neuropeptide potencies. Are quantitatively predictive models possible? *Insect Biochem. Mol. Biol. 30*, 899–907.

Lee, M.J., Drake, A.F., Goldsworthy, G.J., **1996**. Locusta-AKH-III and related peptides containing two tryptophan residues have unusual CD spectra. *Biochem. Biophys. Res. Commun. 226*, 407–412.

Lee, Y.-H., Keeley, L.L., **1994**. Intracellular transduction of trehalose synthesis by hypertrehalosemic hormone in the fat body of the tropical cockroach, *Blaberus discoidalis*. *Insect Biochem. Mol. Biol. 24*, 473–480.

Lehmberg, E., Ota, R.B., Furuya, K., King, D.S., Applebaum, S.W., *et al.*, **1991**. Identification of a diuretic hormone of *Locusta migratoria*. *Biochem. Biophys. Res. Commun. 179*, 1036–1041.

Lewis, D.K., Bradfield, J.Y., Keeley, L.L., **1998**. Feeding effects on gene expression of the hypertrehalosemic hormone in the cockroach, *Blaberus discoidalis*. *J. Insect Physiol. 44*, 967–972.

Li, H., Wang, H., Schegg, K.M., Schooley, D.A., **1997**. Metabolism of an insect diuretic hormone by Malpighian tubules studied by liquid chromatography coupled with electrospray ionization mass spectrometry. *Proc. Natl Acad. Sci. USA 94*, 13463–13468.

Liao, S., Audsley, N., Schooley, D.A., **2000**. Antidiuretic effects of a factor in brain/corpora cardiaca/corpora allata extract on fluid reabsorption across the cryptonephric complex of *Manduca sexta*. *J. Exp. Biol. 203*, 605–615.

Linck, B., Klein, J.M., Mangerich, S., Keller, R., Weidemann, W.M., **1993**. Molecular cloning of crustacean red pigment concentrating hormone precursor. *Biochem. Biophys. Res. Commun. 195*, 807–813.

Lorenz, M.W., Kellner, R., Völkl, W., Hoffmann, K.H., Woodring, J., **2001**. A comparative study on hypertrehalosemic hormones in the Hymenoptera: sequence determination, physiological actions and biological significance. *J. Insect Physiol. 47*, 563–571.

Lu, K.-H., Bradfield, J.Y., Keeley, L.L., **1995**. Hypertrehalosemic hormone-regulated gene expression of cytochrome P4504C1 in the fat body of the cockroach, *Blaberus discoidalis*. *Arch. Insect Biochem. Physiol. 28*, 79–90.

Lu, K.-H., Bradfield, J.Y., Keeley, L.L., **1996**. Age and starvation effects on hypertrehalosemic hormone-dependent gene expression of cytochrome P4504C1 in the cockroach, *Blaberus discoidalis*. *J. Insect Physiol. 42*, 925–930.

Lum, P.Y., Chino, H., **1990**. Primary role of adipokinetic hormone in the formation of low density lipophorin in insects. *J. Lipid. Res. 31*, 2039–2044.

Ma, M., Emery, S.B., Wong, W.K.R., De Loof, A., **2000**. Effects of *Manduca* diuresin on neonates of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol. 118*, 1–7.

MacPherson, M.R., Pollock, V.P., Broderick, K.E., Kean, L., O'Connell, F.C., *et al.*, **2001**. Model organisms: new insights into ion channel and transporter function. L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster*. *Am. J. Physiol. Cell. Physiol. 280*, C394–C407.

Maddrell, S.H., Casida, J.E., **1971**. Mechanism of insecticide-induced diuresis in *Rhodnius*. *Nature 231*, 55–56.

Maddrell, S.H., Pilcher, D.E., Gardiner, B.O., **1969**. Stimulatory effect of 5-hydroxytryptamine (serotonin) on secretion by malpighian tubules of insects. *Nature 222*, 784–785.

Maddrell, S.H.P., **1963**. Excretion in the blood-sucking bug, *Rhodnius prolixus* Stål. I. The control of diuresis. *J. Exp. Biol. 40*, 247–256.

Maddrell, S.H.P., **1978**. Transport in insect excretory epithelia. In: Ussing, H.H. (Ed.), Membrane Transport in Biology. Springer-Verlag, Heidelberg, pp. 239–271.

Maddrell, S.H.P., Herman, W.S., Farndale, R.W., Riegel, J.A., **1993a**. Synergism of hormones controlling epithelial fluid transport in an insect. *J. Exp. Biol. 174*, 65–80.

Maddrell, S.H.P., O'Donnell, M.J., **1992**. Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol. 172*, 417–429.

Maddrell, S.H.P., O'Donnell, M.J., Caffrey, R., **1993b**. The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed *Rhodnius prolixus*. *J. Exp. Biol. 177*, 273–285.

Martínez-Pérez, F., Becerra, A., Valdés, J., Zinker, S., Aréchiga, H., **2002**. A possible molecular ancestor for mollusk APGWamide, insect adipokinetic hormone, and crustacean red pigment concentrating hormone. *J. Mol. Evol. 54*, 703–714.

Massaro, R.C., Lee, L.W., Patel, A.B., Wu, D.S., Yu, M.-J., *et al.*, **2004**. The mechanism of action of the antidiuretic pepide Tenmo ADFa in Malpighian tubules of *Aedes aegypti*. *J. Exp. Biol. 207*, 2877–2888.

Mathelin, J., Quennedey, B., Bouhin, H., Delachambre, J., **1998**. Characterization of two new cuticular genes specifically expressed during the post-ecdysial molting period in *Tenebrio molitor. Gene 211*, 351–359.

Mayer, R.J., Candy, D.J., **1967**. Changes in haemolymph lipoproteins during locust flight. *Nature 215*, 987.

Mayer, R.J., Candy, D.J., **1969b**. Changes in energy reserve during flight of the desert locust, *Schistocerca gregaria*. *Comp. Biochem. Physiol. 31*, 409–418.

Mayer, R.J., Candy, D.J., **1969a**. Control of haemolymph lipid concentration during locust flight: an adipokinetic hormone from the corpora cardiaca. *J. Insect Physiol. 15*, 611–620.

McClure, J.B., Steele, J.E., **1981**. The role of extracellular calcium in hormonal activation of glycogen phosphorylase in cockroach fat body. *Insect Biochem. 11*, 605–613.

Meredith, J., Ring, M., Macins, A., Marschall, J., Cheng, N.N., *et al.*, **1996**. Locust ion transport peptide (ITP): primary structure, cDNA and expression in a baculovirus system. *J. Exp. Biol. 199*, 1053–1061.

Milde, J.J., Ziegler, R., Wallstein, M., **1995**. Adipokinetic hormone stimulates neurones in the insect central nervous system. *J. Exp. Biol. 198*, 1307–1311.

Moffett, D.F., **1994**. Recycling of K^+, acid-base equivalents, and fluid between gut and hemolymph in lepidopteran larvae. *Physiol. Zool. 67*, 68–81.

Montecucchi, P.C., Henschen, A., **1981**. Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvagei*. *Int. J. Pept. Protein Res. 18*, 113–120.

Montuenga, L.M., Zudaire, E., Prado, M.A., Audsley, N., Burrell, M.A., *et al.*, **1996**. Presence of *Locusta* diuretic hormone in endocrine cells of the ampullae of locust Malpighian tubules. *Cell Tissue Res. 285*, 331–339.

Mordue, W., **1969**. Hormonal control of Malpighian tubule and rectal function in the desert locust, *Schistocerca gregaria*. *J. Insect Physiol. 15*, 273–285.

Mordue, W., Stone, J.V., **1977**. Relative potencies of locust adipokinetic hormone and prawn red-pigment concentrating hormone in insect and crustacean systems. *Gen. Comp. Endocrinol. 33*, 103–108.

Moreau, R., Gourdeux, L., Girardie, J., **1988**. Neuroparsin: a new energetic neurohormone in the African locust. *Arch. Insect Biochem. Physiol. 8*, 135–145.

Morgan, P.J., Mordue, W., **1984**. 5-Hydroxytryptamine stimulates fluid secretion in locust Malpighian tubules independently of cAMP. *Comp. Biochem. Physiol. 79C*, 305–310.

Morley, S.D., Schonrock, C., Richter, D., Okawara, Y., Lederis, K., **1991**. Corticotropin-releasing factor (CRF) gene family in the brain of the teleost fish *Catostomus commersoni* (white sucker): molecular analysis predicts distinct precursors for two CRFs and one urotensin I peptide. *Mol. Mar. Biol. Biotechnol. 1*, 48–57.

Moshitzky, P., Henzel, W.J., Rafaeli, A., Ramachandran, J., Applebaum, S.W., **1978**. Synthesis of adipokinetic hormone (AKH-I) in the locust brain. *Insect Biochem. 17*, 1133–1137.

Mullen, L., Goldsworthy, G., **2003**. Changes in lipophorins are related to the activation of phenoloxidase in the hemolymph of *Locusta migratoria* in response to injection of immunogens. *Insect Biochem. Mol. Biol. 33*, 661–670.

Muren, J.E., Nässel, D.R., **1996**. Isolation of five tachykinin-related peptides from the midgut of the cockroach *Leucophaea maderae*: existence of N-terminally extended isoforms. *Regul. Pept. 65*, 185–196.

Mwangi, R.W., Goldsworthy, G.J., **1977a**. Age-related changes in the response to adipokinetic hormone in *Locusta migratoria*. *Physiol. Ent. 2*, 37–42.

Mwangi, R.W., Goldsworthy, G.J., **1977b**. Diglyceride-transporting lipoproteins in *Locusta*. *J. Comp. Physiol. B 114*, 177–190.

Nachman, R.J., Holman, G.M., Hadden, W.F., **1993**. Leads for insect neuropeptide mimetic development. *Arch. Ins. Biochem. Physiol. 22*, 181–197.

Nair, M.M., Jackson, G.E., Gäde, G., **2001**. Conformation study of insect adipokinetic hormones using NMR constrained molecular dynamics. *J. Computer-Aided Mol. Design 15*, 259–270.

Nässel, D.R., Passier, P.C.C.M., Elekes, K., Dircksen, H., Vullings, H.G.B., *et al.*, **1995**. Evidence that locustatachykinin I is involved in release of adipokinetic hormone from locust corpora cardiaca. *Reg. Peptides 57*, 297–310.

Nässel, D.R., Vullings, H.G.B., Passier, P.C.C.M., Lundquist, C.T., Schoofs, L., *et al.*, **1999**. Several isoforms of locustatachykinins may be involved in cyclic AMP-mediated release of adipokinetic hormones from the locust corpora cardiaca. *Gen. Comp. Endocrinol. 113*, 401–412.

Nicolson, S.W., **1976**. Diuresis in the cabbage white butterfly, *Pieris brassicae*: fluid secretion by the Malpighian tubules. *J. Insect Physiol. 22*, 1347–1356.

Nicolson, S.W., **1991**. Diuresis or clearance – is there a physiological role for the diuretic hormone of the desert beetle *Onymacris*? *J. Insect Physiol. 37*, 447–452.

Nicolson, S.W., **1993**. The ionic basis of fluid secretion in insect Malpighian tubules – Advances in the last 10 years – Review. *J. Insect Physiol. 39*, 451–458.

Nicolson, S.W., Millar, R.P., **1983**. Effects of biogenic amines and hormones on butterfly Malpighian tubules: dopamine stimulates fluid secretion. *J. Insect Physiol. 29*, 611–615.

Nittoli, T., Coast, G.M., Sieburth, S.M., **1999**. Evidence for helicity in insect diuretic peptide hormones: computational analysis, spectroscopic studies, and biological assays. *J. Pept. Res. 53*, 99–108.

Norinder, U., Rivera, C., Unden, A., **1997**. A quantitative structure-activity study of some substance P-related peptides. *J. Pept. Res. 49*, 155–162.

Noyes, B.E., Schaffer, M.H., **1990**. The structurally similar neuropeptides adipokinetic hormone I and II are derived from similar, very small mRNAs. *J. Biol. Chem. 265*, 483–489.

O'Connor, K.R., Beyenbach, K.W., **2001**. Chloride channels in apical membrane patches of stellate cells of Malpighian tubules of *Aedes aegypti*. *J. Exp. Biol. 204*, 367–378.

O'Donnell, M.J., Dow, J.A.T., Huesmann, G.R., Tublitz, N.J., Maddrell, S.H.P., **1996**. Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol. 199*, 1163–1175.

O'Donnell, M.J., Machin, J., **1991**. Ion activities and electrochemical gradients in the mealworm rectal complex. *J. Exp. Biol. 155*, 375–402.

O'Donnell, M.J., Maddrell, S.H., **1984**. Secretion by the Malpighian tubules of *Rhodnius prolixus* stal: electrical events. *J. Exp. Biol. 110*, 275–290.

O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., *et al.*, **1998**. Hormonally controlled chloride movement across *Drosophila* tubules is via ion channels in stellate cells. *Am. J. Physiol. 274*, R1039–R1049.

O'Donnell, M.J., Spring, J.H., **2000**. Modes of control of insect Malpighian tubules: synergism, antagonism, cooperation and autonomous regulation. *J. Insect Physiol. 46*, 107–117.

Ogoyi, D.O., Osir, E.O., Olembo, N.K., **1998**. Fat body triacylglycerol lipase in solitary and gregarious phases of *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae). *Comp. Biochem. Physiol. 119B*, 163–169.

Oguri, E., Steele, J.E., **2003**. A novel function of cockroach (*Periplaneta americana*) hypertrehalosemic hormone: translocation of lipid from hemolymph to fat body. *Gen. Comp. Endocrinol. 132*, 46–54.

Orchard, I., Lange, A.B., **1983a**. Release of identified adipokinetic hormones during flight and following neural stimulation in *Locusta migratoria*. *J. Insect Physiol. 29*, 425–429.

Orchard, I., Lange, A.B., **1983b**. The hormonal control of haemolymph lipid during flight in *Locusta migratoria*. *J. Insect Physiol. 29*, 639–642.

Orchard, I., Lange, A.B., Bendena, W.G., Evans, P.D., **2001**. FMRFamide-related peptides: a multifunctional family of structurally-related neuropeptides in insects. *Adv. Insect Physiol. 28*, 267–329.

Orchard, I., Loughton, B.G., **1981**. The neural control of release of hyperlipaemic hormone from the corpus cardiacum of *Locusta migratoria*. *Comp. Biochem. Physiol. 68A*, 25–30.

Orr, G.L., Gole, J.W.D., Jahagirdar, A.P., Downer, R.G.H., Steele, J.E., **1985**. Cyclic AMP does not mediate the action of synthetic hypertrehalosemic peptides from the corpus cardiacum of *Periplaneta americana*. *Insect Biochem. 15*, 703–709.

O'Shea, M., Rayne, R.C., **1992**. Adipokinetic hormones: Cell and molecular biology. *Experientia 48*, 430–438.

O'Shea, M., Witten, J., Schaffer, M., **1984**. Isolation and characterization of two myoactive neuropeptides: Further evidence for an invertebrate peptide family. *J. Neurosci. 4*, 521–529.

Oudejans, R.C.H.M., Kooiman, F.P., Heerma, W., Versluis, C., Slotboom, A.J., *et al.*, **1991**. Isolation and structure elucidation of a novel adipokinetic hormone (Lom-AKH-III) from the glandular lobes of the corpus cardiacum of the migratory locust, *Locusta migratoria*. *Eur. J. Biochem. 195*, 351–359.

Oudejans, R.C.H.M., Mes, T.H.M., Kooiman, F.P., Van der Horst, D., **1993**. Adipokinetic peptide hormone content and biosynthesis during locust development. *Peptides 14*, 877–881.

Oudejans, R.C.H.M., Vroemen, S.F., Jansen, R.F.R., Van der Horst, D.J., **1996**. Locust adipokinetic hormones: carrier-independent transport and differential inactivation at physiological concentrations during flight and rest. *Proc. Natl Acad. Sci. USA 93*, 8654–8659.

Pallen, C.J., Steele, J.E., **1988**. A putative role for calmodulin in corpus cardiacum stimulated trehalose synthesis in fat body of the American cockroach (*Periplaneta americana*). *Insect Biochem. 18*, 577–584.

Pannabecker, T., Orchard, I., **1986**. Octopamine and cyclic AMP mediate release of adipokinetic hormone I and II from isolated neuroendocrine tissue. *Mol. Cell. Endocrinol. 48*, 153–159.

Pannabecker, T.L., Hayes, T.K., Beyenbach, K.W., **1993**. Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol. 132*, 63–76.

Park, J.H., Keeley, L.L., **1995**. *In vitro* hormonal regulation of glycogen phosphorylase activity in fat body of the tropical cockroach, *Blaberus discoidalis*. *Gen. Comp. Endocrinol. 98*, 234–243.

Park, J.H., Keeley, L.L., **1996**. Calcium-dependent action of hypertrehalosemic hormone on activation of glycogen phosphorylase in cockroach fat body. *Mol. Cell. Endocrinol. 116*, 199–205.

Park, Y., Kim, Y.-J., Adams, M.E., **2002**. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc. Natl Acad. Sci. USA 99*, 11423–11428.

Passier, P.C.C.M., Vullings, H.G.B., Diederen, J.H.B., Van der Horst, D.J., **1995**. Modulatory effects of biogenic amines on adipokinetic hormone secretion from locust corpora cardiaca. *Gen. Comp. Endocrinol. 97*, 231–238.

Passier, P.C.C.M., Vullings, H.G.B., Diederen, J.H.B., Van der Horst, D.J., **1997**. Trehalose inhibits the release of adipokinetic hormones from the corpus cardiacum in the African migratory locust, *Locusta migratoria*, at the level of the adipokinetic cells. *J. Endocrinol. 153*, 299–305.

Patel, M., Chung, J.S., Kay, I., Mallet, A.I., Gibbon, C.R., *et al.*, **1994**. Localization of Locusta-DP in locust CNS and hemolymph satisfies initial hormonal criteria. *Peptides 15*, 591–602.

Patel, M., Hayes, T.K., Coast, G.M., **1995**. Evidence for the hormonal function of a CRF-related diuretic peptide (*Locusta*-DP) in *Locusta migratoria*. *J. Exp. Biol. 198*, 793–804.

Perry, S.J., Lefkowitz, R.J., **2002**. Arresting developments in heptahelical receptor signaling and regulation. *Trends Cell Biol. 12*, 130–138.

Petzel, D.H., Berg, M.M., Beyenbach, K.W., **1987**. Hormone-controlled cAMP-mediated fluid secretion in yellow-fever mosquito. *Am. J. Physiol. 253*, R701–R711.

Petzel, D.H., Hagedorn, H.H., Beyenbach, K.W., **1985**. Preliminary isolation of mosquito natriuretic factor. *Am. J. Physiol. 249*, R379–R386.

Petzel, D.H., Hagedorn, H.H., Beyenbach, K.W., **1986**. Peptide nature of two mosquito natriuretic factors. *Am. J. Physiol. 250*, R328–R332.

Phillips, J.E., **1983**. Endocrine control of salt and water balance: excretion. In: Downer, R.G.H., Laufer, H. (Eds.), Endocrinology of Insects. A.R. Liss, New York, pp. 411–425.

Phillips, J.E., Audsley, N., **1995**. Neuropeptide control of ion and fluid transport across locust hindgut. *Am. Zool. 35*, 503–514.

Phillips, J.E., Audsley, N., Lechleitner, R., Thomson, B., Meredith, J., *et al.*, **1988**. Some major transport mechanisms of insect absorptive epithelia. *Comp. Biochem. Physiol. A 90*, 643–650.

Phillips, J.E., Hanrahan, J., Chamberlin, M., Thomson, B., **1986**. Mechanisms and control of reabsorption in insect hindgut. *Adv. Insect Physiol. 19*, 329–422.

Phillips, J.E., Meredith, J., Audsley, N., Richardson, N., Macins, A., *et al.*, **1998a**. Locust ion transport peptide

(ITP): a putative hormone controlling water and ionic balance in terrestrial insects. *Am. Zool. 38*, 461–470.

Phillips, J.E., Meredith, J., Audsley, N., Ring, M., Macins, A., *et al.*, **1998b**. Locust ion transport peptide (ITP): function, structure, cDNA and expression. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge, pp. 210–226.

Phillips, J.E., Mordue, W., Meredith, J., Spring, J., **1980**. Purification and characteristics of the chloride transport stimulating factor from locust corpora cardiaca: a new peptide. *Can. J. Zool. 58*, 1851–1860.

Plawner, L., Pannabecker, T.L., Laufer, S., Baustian, M.D., Beyenbach, K.W., **1991**. Control of diuresis in the yellow fever mosquito *Aedes aegypti*: evidence for similar mechanisms in the male and female. *J. Insect Physiol. 37*, 119–128.

Podzuweit, T., Nennstiel, P., Mueller, A., **1995**. Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hydroxy-3-nonyl) adenine. *Cell. Signal. 7*, 733–738.

Pollock, V.P., Radford, J.C., Pyne, S., Hasan, G., Dow, J.A., *et al.*, **2003**. NorpA and itpr mutants reveal roles for phospholipase C and inositol (1,4,5)-trisphosphate receptor in *Drosophila melanogaster* renal function. *J. Exp. Biol. 206*, 901–911.

Predel, R., Gäde, G., **2002**. Identification of the abundant neuropeptide from abdominal perisympathetic organs of locusts. *Peptides 23*, 621–627.

Predel, R., Kellner, R., Baggerman, G., Steinmetzer, T., Schoofs, L., **2000**. Identification of novel periviscerokinins from single neurohaemal release sites in insects-MS/MS fragmentation complemented by Edman degradation. *Eur. J. Biochem. 267*, 3869–3873.

Predel, R., Kellner, R., Rapus, J., Penzlin, H., Gáde, G., **1997**. Isolation and structural elucidation of eight kinins from the retrocerebral complex of the American cockroach, *Periplaneta americana*. *Regul. Pept. 71*, 199–205.

Predel, R., Linde, D., Rapus, J., Vettermann, S., Penzlin, H., **1995**. Periviscerokinin (Pea-PVK): a novel myotropic neuropeptide from the perisympathetic organs of the American cockroach. *Peptides 16*, 61–66.

Predel, R., Rapus, J., Eckert, M., Holman, G.M., Nachman, R.J., *et al.*, **1998**. Isolation of periviscerokinin-2 from the abdominal perisympathetic organs of the American cockroach, *Periplaneta americana*. *Peptides 19*, 801–809.

Prigge, S.T., Mains, R.E., Eipper, B.A., Amzel, L.M., **2000**. New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *Cell. Mol. Life Sci. 57*, 1236–1259.

Proux, J., Proux, B., Phillips, J., **1985**. Source and distribution of factors in locust nervous system which stimulate rectal Cl^- transport. *Can. J. Zool. 63*, 37–41.

Proux, J., Rougon, G., Cupo, A., **1982**. Enhancement of excretion across locust Malpighian tubules by a diuretic vasopressin-like hormone. *Gen. Comp. Endocrinol. 47*, 449–457.

Proux, J.P., Miller, C.A., Li, J.P., Carney, R.L., Girardie, A., *et al.*, **1987**. Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. *Biochem. Biophys. Res. Commun. 149*, 180–186.

Proux, J.P., Picquot, M., Herault, J.P., Fournier, B., **1988**. Diuretic activity of a newly identified neuropeptide – the arginine-vasopressin-like insect diuretic hormone: use of an improved bioassay. *J. Insect Physiol. 34*, 919–927.

Qi, L.J., Leung, A.T., Xiong, Y.T., Marx, K.A., Abou-Samra, A.B., **1997**. Extracellular cysteines of the corticotropin-releasing factor receptor are critical for ligand interaction. *Biochemistry 36*, 12442–12448.

Quinlan, M.C., O'Donnell, M.J., **1998**. Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stal: antagonistic actions of cAMP and cGMP and the role of organic acid transport. *J. Insect Physiol. 44*, 561–568.

Quinlan, M.C., Tublitz, N.J., O'Donnell, M.J., **1997**. Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stal: The peptide CAP_{2b} and cyclic GMP inhibit Malpighian tubule fluid secretion. *J. Exp. Biol. 200*, 2363–2367.

Rademakers, L.H.P.M., **1977a**. Effects of isolation and transplantation of the corpus cardiacum on hormone release from its glandular cells after flight in *Locusta migratoria*. *Cell Tissue Res. 184*, 213–224.

Rademakers, L.H.P.M., **1977b**. Identification of a secretomotor centre in the brain of *Locusta migratoria* controlling the secretory activity of the adipokinetic hormone producing cells of the corpus cardiacum. *Cell Tissue Res. 184*, 381–395.

Radford, J.C., Davies, S.A., Dow, J.A., **2002**. Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem. 277*, 38810–38817.

Rafaeli, A., Pines, M., Stern, P.S., Applebaum, S.W., **1984**. Locust diuretic hormone-stimulated synthesis and excretion of cyclic-AMP: a novel Malpighian tubule bioassay. *Gen. Comp. Endocrinol. 54*, 35–42.

Ramsay, J.A., **1954**. Active transport of water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. Exp. Biol. 31*, 104–113.

Ramsay, J.A., **1964**. The rectal complex of the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). *Philos. Trans. R. Soc. Lond. B Biol. Sci. 248*, 279–314.

Ramsay, J.A., **1976**. The rectal complex in the larvae of Lepidoptera. *Philos. Trans. R. Soc. Lond. B Biol. 274*, 203–226.

Rao, K.R., **2001**. Crustacean pigmentary-effector hormones: Chemistry and functions of RPCH, PDH, and related peptides. *Am. Zool. 41*, 364–379.

Rayne, R.C., O'Shea, M., **1992**. Inactivation of neuropeptide hormones (AKH I and AKH II) studied *in vivo* and *in vitro*. *Insect Biochem. Mol. Biol. 22*, 25–34.

Rayne, R.C., O'Shea, M., **1994**. Reconstitution of adipokinetic hormone biosynthesis *in vitro* indicates steps in prohormone processing. *Eur. J. Biochem. 219*, 781–789.

Reagan, J.D., **1994**. Expression cloning of an insect diuretic hormone receptor – a member of the calcitonin/secretin receptor family. *J. Biol. Chem. 269*, 9–12.

Reagan, J.D., **1995a**. Functional expression of a diuretic hormone receptor in baculovirus-infected insect cells: evidence suggesting that the N-terminal region of diuretic hormone is associated with receptor activation. *Insect Biochem. Mol. Biol. 25*, 535–539.

Reagan, J.D., **1995b**. Molecular cloning of a putative Na^+–K^+–$2Cl^-$ cotransporter from the Malpighian tubules of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 875–880.

Reagan, J.D., **1996**. Molecular cloning and function expression of a diuretic hormone receptor from the house cricket, *Acheta domesticus*. *Insect Biochem. Mol. Biol. 26*, 1–6.

Reagan, J.D., Li, J.P., Carney, R.L., Kramer, S.J., **1993**. Characterization of a diuretic hormone receptor from the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 23*, 135–145.

Reynolds, S.E., Bellward, K., **1989**. Water balance in *Manduca sexta* caterpillars: water recycling from the rectum. *J. Exp. Biol. 141*, 33–45.

Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., **2002**. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science 298*, 172–175.

Rosay, P., Davies, S.A., Yu, Y., Sozen, A., Kaiser, K., *et al.*, **1997**. Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell. Sci. 110*, 1683–1692.

Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., *et al.*, **1997**. Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comp. Biochem. Physiol. [B] 118B*, 349–357.

Scarborough, R.M., Jamieson, G.C., Kalish, F., Kramer, S.J., McEnroe, G.A., *et al.*, **1984**. Isolation and primary structure of two peptides with cardioacceleratory and hyperglycemic activity from the corpora cardiaca of *Periplaneta americana*. *Proc. Natl Acad. Sci. USA 81*, 5575–5579.

Schoofs, L., Holman, G.M., Proost, P., Van Damme, J., Hayes, T.K., *et al.*, **1992**. Locustakinin, a novel myotropic peptide from *Locusta migratoria*, isolation, primary structure and synthesis. *Regul. Pept. 37*, 49–57.

Schoofs, L., Vanden Broeck, J., De Loof, A., **1993**. The myotropic peptides of *Locusta migratoria*: structures, distribution, functions and receptors. *Insect Biochem. Mol. Biol. 23*, 859–881.

Schooley, D.A., **1994**. Peptide hormones regulating water balance: workshop report. *Arch. Insect Biochem. Physiol. 27*, 323–324.

Schooley, D.A., Miller, C.A., Proux, J.P., **1987**. Isolation of two arginine vasopressin-like factors from ganglia of *Locusta migratoria*. *Arch. Insect Biochem. Physiol. 5*, 157–166.

Schooneveld, H., van Herp, F., van Minnen, J., **1987**. Demonstration of substances immunologically related to the identified arthropod neuropeptides AKH/RPCH in the CNS of several invertebrate species. *Brain Res. 406*, 224–232.

Schulz-Aellen, M.-F., Roulet, E., Fischer-Lougheed, J., O'Shea, M., **1989**. Synthesis of homodimer neurohormone precursor of locust adipokinetic hormone studied by *in vitro* translation and cDNA cloning. *Neuron 2*, 1369–1373.

Serrano, L., Blanvillain, G., Soyez, D., Charmantier, G., Grousset, E., *et al.*, **2003**. Putative involvement of crustacean hyperglycemic hormone isoforms in the neuroendocrine mediation of osmoregulation in the crayfish *Astacus leptodactylus*. *J. Exp. Biol. 206*, 979–988.

Sharp-Baker, H.E., Diederen, J.H.B., Mäkel, K.M., Peute, J., Van der Horst, D.J., **1995**. The adipokinetic cell in the corpus cardiacum of *Locusta migratoria* preferentially release young secretory granules. *Eur. J. Cell Biol. 68*, 268–274.

Sharp-Baker, H.E., Oudejans, R.C.H.M., Kooiman, F.P., Diederen, J.H.B., Peute, J., *et al.*, **1996**. Preferential release of newly synthesized, exportable neuropeptides by insect neuroendocrine cells and the effect of ageing of secretory granules. *Eur. J. Cell Biol. 71*, 72–78.

Siegert, K.J., **1999**. Locust corpora cardiaca contain an inactive adipokinetic hormone. *FEBS Letters 447*, 237–240.

Siegert, K.J., Kellner, R., Gäde, G., **2000**. A third active AKH is present in the phygomorphid grasshoppers *Phymateus morbillosus* and *Dictyophorus spumans*. *Insect Biochem. Mol. Biol. 30*, 1061–1067.

Siegert, K.J., Mordue, W., **1986a**. Elucidation of the primary structures of the cockroach hyperglycaemic hormones I and II using enzymatic techniques and gas phase sequencing. *Physiol. Ent. 11*, 205–211.

Siegert, K.J., Mordue, W., **1986b**. Quantification of adipokinetic hormones I and II in corpora cardiaca of *Schistocerca gregaria* and *Locusta migratoria*. *Comp. Biochem. Physiol. 84A*, 279–284.

Siegert, K.J., Mordue, W., **1987**. Breakdown of locust adipokinetic hormone I by Malpighian tubules of *Schistocerca gregaria*. *Insect Biochem. 17*, 705–710.

Siegert, K.J., Ziegler, R., **1983**. A hormone from the corpora cardiaca controls fat body glycogen phosphorylase during starvation in tobacco hornworm larvae. *Nature 301*, 526–527.

Skaer, N.J., Nassel, D.R., Maddrell, S.H., Tublitz, N.J., **2002**. Neurochemical fine tuning of a peripheral tissue: peptidergic and aminergic regulation of fluid secretion by Malpighian tubules in the tobacco hawkmoth *M. sexta*. *J. Exp. Biol. 205*, 1869–1880.

Socha, R., Kodrik, D., Zemek, R., **1999**. Adipokinetic hormone stimulates insect locomotory activity. *Naturwissenschaften 86*, 85–86.

Socha, R., Sula, J., Zemek, R., **1998**. Feeding behavior, digestive physiology and lipid content in macropterous females of *Pyrrhocoris apterus* (L.) (Heteroptera: Pyrrhocoridae). *Physiol. Ent. 23*, 91–96.

Soulages, J.L., Wells, M.A., **1994**. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Prot. Chem. 45*, 371–415.

Sowa, S.M., Lu, K.-H., Park, J.H., Keeley, L.L., **1996**. Physiological effectors of hyperglycemic neurohormone biosynthesis in an insect. *Mol. Cell. Endocrinol. 123*, 97–105.

Spanings-Pierrot, C., Soyez, D., Van Herp, F., Gompel, M., Skaret, G., *et al.*, **2000**. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol. 119*, 340–350.

Spencer, I.M., Candy, D.J., **1976**. Hormonal control of diacyl glycerol mobilization from fat body of the desert locust, *Schistocerca gregaria*. *Insect Biochem. 6*, 289–296.

Spring, J.H., **1990**. Endocrine regulation of diuresis in insects. *J. Insect Physiol. 36*, 13–22.

Spring, J.H., Morgan, A.M., Hazelton, S.R., **1988**. A novel target for antidiuretic hormone in insects. *Science 241*, 1096–1098.

Stagg, L.E., Candy, D.J., **1996**. The effect of adipokinetic hormones on the levels of inositol phosphates and cyclic AMP in the fat body of the desert locust *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 26*, 537–544.

Staubli, F., Jørgensen, T.J.D., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99*, 3446–3451.

Steele, J.E., **1961**. Occurrence of a hyperglycaemic factor in the corpus cardiacum of an insect. *Nature 192*, 680–681.

Steele, J.E., **1963**. The site of action of insect hyperglycemic hormone. *Gen. Comp. Endocrinol. 3*, 46–52.

Steele, J.E., Garcha, K., Sun, D., **2001**. Inositol trisphosphate mediates the action of hypertrehalosemic hormone on fat body of the American cockroach, *Periplaneta americana*. *Comp. Biochem. Physiol. 130B*, 537–545.

Steele, J.E., Ireland, R., **1994**. The preparation of trophocytes from the disaggregated fat body of the cockroach (*Periplaneta americana*). *Comp. Biochem. Physiol. 107A*, 517–522.

Steele, J.E., Ireland, R., **1999**. Hormonal activation of phosphorylase in cockroach fat body trophocytes: a correlation with trans-membrane calcium flux. *Arch. Insect Biochem. Physiol. 42*, 233–244.

Steele, J.E., Paul, T., **1985**. Corpus cardiacum stimulated trehalose efflux from cockroach (*Periplaneta americana*) fat body: Control by calcium. *Can. J. Zool. 63*, 63–66.

Stenzel-Poore, M.P., Heldwein, K.A., Stenzel, P., Lee, S., Vale, W.W., **1992**. Characterization of the genomic corticotropin-releasing factor (CRF) gene from *Xenopus laevis*: two members of the CRF family exist in amphibians. *Mol. Endocrinol. 6*, 1716–1724.

Stone, J.V., Mordue, W., Batley, K.E., Morris, H.R., **1976**. Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. *Nature 263*, 207–211.

Stone, J.V., Mordue, W., Broomfield, C.E., Hardy, P.M., **1978**. Structure-activity relationships for the lipid-mobilizing action of locust adipokinetic hormone. Synthesis and activity of a series of hormone analogs. *Eur. J. Biochem. 89*, 195–202.

Sun, D., Garcha, K., Steele, J.E., **2002**. Stimulation of trehalose efflux from cockroach (*Periplaneta americana*) fat body by hypertrehalosemic hormone is dependent on protein kinase C and calmodulin. *Arch. Insect Biochem. Physiol. 50*, 41–51.

Sun, D., Steele, J.E., **2001**. Regulation of intracellular calcium in dispersed fat body trophocytes of the cockroach, *Periplaneta americana*, by hypertrehalosemic hormone. *J. Insect Physiol. 47*, 1399–1408.

Sun, D., Steele, J.E., **2002**. Regulation of phospholipase A_2 activity in cockroach (*Periplaneta americana*) fat body by hypertrehalosemic hormone: evidence for the participation of protein kinase C. *J. Insect Physiol. 48*, 537–546.

Sutcliffe, D.W., **1963**. Chemical composition of haemolymph in insects and some other arthropods, in relation to their phylogeny. *Comp. Biochem. Physiol. 9*, 121–135.

Sydow, S., Flaccus, A., Fischer, A., Spiess, J., **1999**. The role of the fourth extracellular domain of the rat corticotropin-releasing factor receptor type 1 in ligand binding. *Eur. J. Biochem. 259*, 55–62.

Sydow, S., Radulovic, J., Dautzenberg, F.M., Spiess, J., **1997**. Structure-function relationship of different domains of the rat corticotropin-releasing factor receptor. *Mol. Brain Res. 52*, 182–193.

Te Brugge, V.A., Schooley, D.A., Orchard, I., **2002**. The biological activity of diuretic factors in *Rhodnius prolixus*. *Peptides 23*, 671–681.

Terhzaz, S., O'Connell, F.C., Pollock, V.P., Kean, L., Davies, S.A., *et al.*, **1999**. Isolation and characterization of a leucokinin-like peptide of *Drosophila melanogaster*. *J. Exp. Biol. 202*, 3667–3676.

Thompson, K.S.J., Bacon, J.P., **1991**. The vasopressin-like immunoreactive (VPLI) neurons of the locust, *Locusta migratoria*. 2. Physiology. *J. Comp. Physiol. A 168*, 619–630.

Thompson, K.S.J., Tyrer, N.M., May, S.T., Bacon, J.P., **1991**. The vasopressin-like immunoreactive (VPLI) neurons of the Locust, *Locusta migratoria*. 1. Anatomy. *J. Comp. Physiol. A 168*, 605–617.

Torfs, H., Poels, J., Detheux, M., Dupriez, V., Van Loy, T., *et al.*, **2002**. Recombinant aequorin as a reporter for receptor-mediated changes of intracellular Ca^{2+}-levels in *Drosophila* S2 cells. *Invert. Neurosci. 4*, 119–124.

Tublitz, N.J., Brink, D., Broadie, K.S., Loi, P.K., Sylwester, A.W., **1991**. From behaviour to molecules: an integrated approach to the study of neuropeptides. *Trends Neurosci. 14*, 254–259.

Tublitz, N.J., Truman, J.W., **1985**. Insect cardioactive peptides II. Neurohormonal control of heart activity by two cardioacceleratory peptides in the tobacco hawkmoth, *Manduca sexta*. *J. Exp. Biol. 114*, 381–395.

Van der Horst, D.J., Vroemen, S.F., Van Marrewijk, W.J.A., **1997**. Metabolism of stored reserves in insect fat body: hormonal signal transduction implicated in glycogen mobilization and biosynthesis of the

lipophorin system. *Comp. Biochem. Physiol. 117B*, 463–474.

Van Marrewijk, W.J.A., Van den Broek, A.T.M., Beenakkers, A.M.T., **1980**. Regulation of glycogenolysis in the locust fat body during flight. *Insect Biochem. 10*, 675–679.

Van Marrewijk, W.J.A., Van den Broek, A.T.M., Beenakkers, A.M.T., **1991**. Adipokinetic hormone is dependent on extracellular Ca^{2+} for its stimulatory action on the glycogenolytic pathway in locust fat body *in vitro*. *Insect Biochem. 21*, 375–380.

Van Marrewijk, W.J.A., Van den Broek, A.T.M., Gielbert, M.-L., Van der Horst, D.J., **1996**. Insect adipokinetic hormone stimulates inositol phosphate metabolism: roles for both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in signal transduction? *Mol. Cell. Endocrinol. 122*, 141–150.

Van Marrewijk, W.J.A., Van den Broek, A.T.M., Van der Horst, D.J., **1993**. Adipokinetic hormone-induced influx of extracellular calcium into insect fat body cells is mediated through depletion of intracellular calcium stores. *Cell. Signal. 5*, 753–761.

Vanden Broeck, J., **2001**. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster. Peptides 22*, 241–254.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., *et al.*, **1995**. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature 378*, 287–292.

Veelaert, D., Passier, P., Devreese, B., Vanden Broeck, J., Van Beeumen, J., *et al.*, **1997**. Isolation and characterization of an adipokinetic hormone release-inducing factor in locusts: the crustacean cardioactive peptide. *Endocrinology 138*, 138–142.

Veenstra, J.A., **1988**. Effects of 5-hydroxytryptamine on the Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol. 34*, 299–304.

Veenstra, J.A., **1994**. Isolation and identification of three leucokinins from the mosquito *Aedes aegypti*. *Biochem. Biophys. Res. Commun. 202*, 715–719.

Veenstra, J.A., Pattillo, J.M., Petzel, D.H., **1997**. A single cDNA encodes all three *Aedes* leucokinins, which stimulate both fluid secretion by the Malpighian tubules and hindgut contractions. *J. Biol. Chem. 272*, 10402–10407.

Vroemen, S.F., De Jonge, H., Van Marrewijk, W.J.A., Van der Horst, D.J., **1998b**. The phospholipase C signaling pathway in locust fat body is activated via G_q and not affected by cAMP. *Insect Biochem. Mol. Biol. 28*, 483–490.

Vroemen, S.F., Van der Horst, D.J., Van Marrewijk, W.J.A., **1998a**. New insights into adipokinetic hormone signaling. *Mol. Cell. Endocrinol. 141*, 7–12.

Vroemen, S.F., Van Marrewijk, W.J.A., De Meijer, J., Van den Broek, A.T.M., Van der Horst, D.J., **1997**. Differential induction of inositol phosphate metabolism by three adipokinetic hormones. *Mol. Cell. Endocrinol. 130*, 131–139.

Vroemen, S.F., Van Marrewijk, W.J.A., Schepers, C.C.J., Van der Horst, D.J., **1995b**. Signal transduction of adipokinetic hormones involves Ca^{2+} fluxes and depends on extracellular Ca^{2+} to potentiate cAMP-induced activation of glycogen phosphorylase. *Cell Calcium 17*, 459–467.

Vroemen, S.F., Van Marrewijk, W.J.A., Van der Horst, D.J., **1995a**. Stimulation of glycogenolysis by three locust adipokinetic hormones involves G_s and cAMP. *Mol. Cell. Endocrinol. 107*, 165–171.

Vullings, H.G.B., Diederen, J.H.B., Veelaert, D., Van der Horst, D.J., **1999**. The multifactorial control of the release of hormones from the locust retrocerebral glandular complex. *Micro. Res. Tech. 45*, 142–153.

Vullings, H.G.B., Ten Voorde, S.E.C.G., Passier, P.C.C.M., Diederen, J.H.B., Van der Horst, D.J., *et al.*, **1998**. A possible role of SchistoFLRFamide in inhibition of adipokinetic hormone release from locust corpora cardiaca. *J. Neurocytol. 27*, 901–913.

Wang, S., Rubenfeld, A.B., Hayes, T.K., Beyenbach, K.W., **1996**. Leucokinin increases paracellular permeability in insect Malpighian tubules. *J. Exp. Biol. 199*, 2537–2542.

Wang, Y.J., Zhao, Y., Meredith, J., Phillips, J.E., Theilmann, D.A., *et al.*, **2000**. Mutational analysis of the C-terminus in ion transport peptide (ITP) expressed in *Drosophila* Kc1 cells. *Arch. Insect Biochem. Physiol. 45*, 129–138.

Wang, Z., Hayakawa, Y., Downer, R.G.H., **1990**. Factors influencing cyclic AMP and diacylglycerol levels in fat body of *Locusta migratoria*. *Insect Biochem. 20*, 325–330.

Weis-Fogh, T., **1952**. Fat combustion and metabolic rate of flying locusts (*Schistocerca gregaria* Forsk.). *Phil. Trans. R. Soc. Ser. B 237*, 1–36.

Weltens, R., Leyssens, A., Zhang, S.L., Lohrmann, E., Steels, P., *et al.*, **1992**. Unmasking an apical electrogenic H pump in isolated Malpighian tubules (*Formica polyctena*) by the use of barium. *Cell Physiol. Biochem. 2*, 101–116.

Weng, X.H., Huss, M., Wieczorek, H., Beyenbach, K.W., **2003**. The V-type H(+)-ATPase in Malpighian tubules of *Aedes aegypti*: localization and activity. *J. Exp. Biol. 206*, 2211–2219.

Wheelock, G.D., Petzel, D.H., Gillett, J.D., Beyenbach, K.W., Hagedorn, H.H., **1988**. Evidence for hormonal control of diuresis afer a blood meal in the mosquito *Aedes aegypti*. *Arch. Insect Biochem. Physiol. 7*, 75–89.

Wicher, D., **2001**. Peptidergic modulation of insect voltage-gated Ca^{2+} currents: role of resting Ca^{2+} current and protein kinases A and C. *J. Neurophysiol. 86*, 2353–2362.

Wicher, D., Walther, C., Penzlin, H., **1994**. Neurohormone D induces ionic current changes in cockroach central neurones. *J. Comp. Physiol. A 174*, 507–515.

Wieczorek, H., Gruber, G., Harvey, W.R., Huss, M., Merzendorfer, H., *et al.*, **2000**. Structure and regulation of insect plasma membrane H^+ V-ATPase. *J. Exp. Biol. 203*, 127–135.

Wiehart, U.I., Nicolson, S.W., Van Kerkhove, E., **2003**. K^+ transport in Malpighian tubules of *Tenebrio*

molitor L.: a study of electrochemical gradients and basal K^+ uptake mechanisms. *J. Exp. Biol. 206*, 949–957.

Wiehart, U.I.M., Nicolson, S.W., Eigenheer, R.A., Schooley, D.A., **2002**. Antagonistic control of fluid secretion by the Malpighian tubules of *Tenebrio molitor*: effects of diuretic and antidiuretic peptides and their second messengers. *J. Exp. Biol. 205*, 493–501.

Wiens, A.W., Gilbert, L.I., **1967**. Regulation of carbohydrate mobilization and utilization in *Leucophaea maderae*. *J. Insect Physiol. 13*, 779–794.

Wiesner, A., Losen, S., Kopacek, P., Weise, C., Gotz, P., **1997**. Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect. *J. Insect Physiol. 43*, 383–391.

Wille, S., Sydow, S., Palchaudhuri, M.R., Spiess, J., Dautzenberg, F.M., **1999**. Identification of amino acids in the N-terminal domain of corticotropin-releasing factor receptor 1 that are important determinants of high-affinity ligand binding. *J. Neurochem. 72*, 388–395.

Witten, J.L., Schaffer, M.H., O'Shea, M., Cook, J.C., Hemling, M.E., *et al.*, **1984**. Structures of two cockroach neuropeptides assigned by fast atom bombardment mass spectrometry. *Biochem. Biophys. Res. Commun. 124*, 350–358.

Woodring, J., Lorenz, M.W., Hoffmann, K.H., **2002**. Sensitivity of larval and adult crickets (*Gryllus bimaculatus*) to adipokinetic hormone. *Comp. Biochem. Physiol. 133A*, 637–644.

Xu, W., Marshall, A.T., **1999**. Magnesium secretion by the distal segment of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. *J. Insect Physiol. 45*, 777–784.

Yu, M.J., Beyenbach, K.W., **2001**. Leucokinin and the modulation of the shunt pathway in Malpighian tubules. *J. Insect Physiol. 47*, 263–276.

Yu, M.J., Beyenbach, K.W., **2002**. Leucokinin activates Ca(2+)-dependent signal pathway in principal cells of *Aedes aegypti* Malpighian tubules. *Am. J. Physiol. Renal Physiol. 283*, F499–F508.

Yu, M.J., Beyenbach, K.W., **2004**. Effects of leucokinin-VIII on *Aedes* Malpighian tubule segments lacking stellate cells. *J. Exp. Biol. 207*, 519–526.

Zdobnov, E.M., von Mering, C., Letunic, I., Torrents, D., Suyama, M., *et al.*, **2002**. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. *Science 298*, 149–159.

Ziegler, R., **1984**. Developmental changes in the response of the fat body of *Manduca sexta* to injections of corpora cardiaca extracts. *Gen. Comp. Endocrinol. 54*, 51–58.

Ziegler, R., Cushing, A.S., Walpole, P., Jasensky, R.D., Morimoto, H., **1998**. Analogs of *Manduca* adipokinetic hormone tested in a bioassay and in a receptor-binding assay. *Peptides 19*, 481–486.

Ziegler, R., Eckart, K., Jasensky, R.D., Law, J.H., **1991**. Structure-activity studies on adipokinetic hormones in *Manduca sexta*. *Arch. Insect Biochem. Physiol. 18*, 229–237.

Ziegler, R., Eckart, K., Law, J.H., **1990**. Adipokinetic hormone controls lipid metabolism in adults and carbohydrate metabolism in larvae of *Manduca sexta*. *Peptides 11*, 1037–1040.

Ziegler, R., Jasensky, R.D., Morimoto, H., **1995**. Characterization of the adipokinetic hormone receptor from the fat body of *Manduca sexta*. *Reg. Pept. 57*, 329–338.

Ziegler, R., Schulz, M., **1986**. Regulation of lipid metabolism during flight in *Manduca sexta*. *J. Insect Physiol. 32*, 903–908.

Zubrzycki, I.Z., Gäde, G., **1994**. Conformational study on an insect neuropeptide of the AKH/RPCH-family by combined ^{1}H-NMR spectroscopy and molecular mechanics. *Biochem. Biophys. Res. Commun. 198*, 228–235.

Zubrzycki, I.Z., Gäde, G., **1999**. Conformational study on a representative member of the AKH/RPCH neuropeptide family, Emp-AKH, in the presence of SDS micelle. *Eur. J. Entomol. 96*, 337–340.

Relevant Websites

http://www.cbs.dtu.dk – NetPhos, **2004**.
http://www.cbs.dtu.dk – TMHMM, **2004**.

3.11 Circadian Organization of the Endocrine System

X Vafopoulou and C G H Steel, York University, Toronto, ON, Canada

3.11.1. Introduction

The subject of this chapter has not been reviewed previously. In the 1985 edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, circadian biology (Page, 1985) was not included in the two volumes on endocrinology. This fact illustrates the enormous extent of the changes in both fields that have taken place since that time.

Since 1985, endocrinologists have encountered increasingly complex patterns of regulatory interactions among insect hormones, the dissection of which has revealed circadian rhythms in the synthesis and hemolymph titer of numerous hormones. The quest to decipher the functional significance of these phenomena has drawn endocrinologists into the relatively unfamiliar territory of circadian biology (discussed by Steel and Vafopoulou, 2002). From this quest, a new level of understanding of the roles of hormones is emerging. Once regarded as agents that pulled switches that would activate or inhibit certain processes, hormones are increasingly recognized as agents that convey information about time (of day) to diverse cells and tissues that lack other access to such temporal information. Target cells use this information to organize their cellular activities into temporal sequences. Such internal temporal organization is a prerequisite for life; organisms maintained in continuous light (LL) often fail to develop or reproduce, or else die prematurely (Pittendrigh, 1993). In addition, rhythms in hormones expose target cells throughout the insect to the same temporal information simultaneously, creating synchronization of the activities of cells in otherwise unconnected anatomical locations. These circadian rhythms are propelled by specialized regions of the insect (often, but not always, in the nervous system) known as circadian clocks, which endogenously generate rhythms of roughly 24 h periodicity and transmit this rhythmicity to other cells, primarily via nerves or hormones. Until 10 years ago, these clocks could be localized only by crude methods such as lesioning areas of tissue, which precluded analysis of both the cellular mechanism of timekeeping and its relationships with the endocrine system.

Even more profound revolutions have occurred since 1985 in the field of circadian biology. A comprehensive survey is given by Saunders (2002). A number of genes has been cloned, initially from *Drosophila melanogaster,* which seem to have no role other than to participate in molecular oscillations that generate circadian rhythms (see **Chapter 4.11**). These genes are known as clock genes, of which *period* (*per*) and *timeless* (*tim*) are the best known. These molecular oscillators appear to be functional only in a limited number of groups of

cells in the insect. The ease with which clock gene transcripts and their proteins can be detected and quantified has resulted in the localization of circadian clocks to identified cells and in the discovery of many novel loci of circadian clock gene expression. Thus, endocrinologists (among others) have been given a powerful set of new tools with which to unravel the relations between circadian clocks and the endocrine system.

The confluence of endocrinology and circadian biology is especially clear in the mechanisms that regulate development and reproduction. Both of these processes involve intricate hormonal controls and both are intimately linked with environmental signals that govern their timing. The nature, significance, and implications of clock control over these sets of hormones comprise the bulk of this chapter. This is relatively a new subject and leaves greater room for speculation and suggestions for future research than the reader may encounter in other chapters in this volume. The confluence of the two fields is most visible at the cellular level, where recent evidence reveals the close physical association between clock cells and endocrine cells in several anatomical locations. But hormones are not only the controlled output of clocks; several hormones are now known to act on clocks, where they regulate critical features of rhythmicity such as phase. Such findings are leading to the recognition that hormones also function to couple circadian clocks together, so that they function as a coordinated timing system which orchestrates the myriad events that occur in an insect every day.

3.11.2. Clocks and Oscillators: An Introduction for Endocrinologists

The later sections of this chapter describe evidence of the presence of circadian clocks and oscillators in numerous organs and tissues in insects. A variety of different methods has been employed to obtain this evidence. Therefore, it is important to keep in mind the virtues and limitations of the various methods. These are discussed briefly here. Definitive proof that particular cells comprise a circadian clock cannot be derived from any one of these alone, a combination of approaches being needed. The most convincingly documented clocks, such as the optic lobe clock that controls the locomotor rhythm (Page, 1985; Helfrich-Förster *et al.*, 1998; Helfrich-Förster, 2003), have been dissected by all these methods. As with all clocks, the locomotor clock may control various overt rhythms, not merely the rhythm that was used to identify it (see Section 3.11.3.1).

All efforts to localize clocks must be preceded by a careful analysis of the characteristics of the overt rhythm being studied (e.g., locomotion) under a variety of lighting conditions. Such studies are sometimes referred to as revealing the "formal properties" of the rhythm. Essential information includes the free-running characteristics of the rhythm (in both continuous dark (DD) and continuous light (LL) and measurement of the free-running period lengths. This information is necessary in order to detect perturbations of these characteristics in later experiments. A uniform sequence of experimental steps has then been followed for many rhythms. The traditional first steps to clock localization involve ablation and transplantation of candidate structures. Elimination of photoreceptors (e.g., eyes, ocelli, brain) will lead to a free-running rhythm similar to that seen in DD. The removal of certain structures may lead to arrhythmicity (i.e., loss of rhythmicity in the phenomenon, not loss of the phenomenon). Arrhythmicity can be produced by removal of the clock itself, but will also be produced if the clock is disconnected from the outputs that it controls. For example, removal of the subesophageal ganglion (SEG) leads to arrhythmic locomotion not because the clock is located in the SEG, but because the optic lobe clock controls the locomotor centers in the thoracic ganglia by nerve pathways that run through the SEG. Consequently, several structures will be found whose removal causes arrhythmicity in a phenomenon. The clock is usually assigned tentatively to the site that is anatomically closest to the photoreceptors. This tissue is then examined *in vitro* for its ability to generate circadian rhythmicity of nervous activity, hormone release, etc. When an endocrine output is suspected, the structure is implanted into arrhythmic hosts and the restoration of rhythmicity is examined. The fact that such experiments often work, illustrates that circadian clocks and endocrine cells are frequently located close to each other in the same piece of tissue (see Section 3.11.3.2). Since this sequence of experimental steps relies on perturbations of an overt rhythm, it is clear that the clock identified has an output that communicates temporal information to other parts of the organism. These clocks are therefore true clocks in the sense that they not only keep time but they communicate timing to other cells. A nervous output is traditionally associated with the control of local, behavioral, rhythms and an endocrine output with more widespread effects in diverse cells and tissues. Consequently, clocks that control hormones are able to exert the most profound and widespread effects, making such clocks the most important ("master clocks") in the

organism. Thus, hormones represent a key controlled output of master clocks (see Section 3.11.3.2).

The elucidation of clock genes and molecular oscillators in *Drosophila* (see **Chapter 4.11**) has both revolutionized and complicated the methods for localization of clocks. Studies of the expression of the clock genes (mainly *per* and *tim*) and of their coded proteins have resulted in the description of numerous cellular loci that are potential cellular clocks. In situations where traditional techniques have shown that a circadian clock is contained in a particular tissue, the presence of cells within it that exhibit cycling of clock gene products provides compelling evidence that these are the cells within the tissue that are responsible for the generation of rhythmicity, i.e., the "clock cells" in the tissue. Indeed, the molecular oscillator (see **Chapter 4.11**) was unraveled in *Drosophila* primarily in the context of the cells in the optic lobe that express clock genes. In addition, molecular oscillators have been found in a number of sites, both within the central nervous system (CNS) and in other tissues, the functional significance of which has not been studied. Consequently, it is not yet known whether or not such cells do in fact convey information about time to other cells. Since communication of temporal information to other cells is an essential feature of true circadian clocks, it is often unclear whether these cells are functional clocks. The functions of these novel loci of clock cells and the mechanisms by which they are coordinated with one another represent important new areas of circadian biology.

It is important to note that the models of the molecular oscillator require that the clock genes are transcribed with circadian periodicity, resulting in circadian changes in the levels of clock gene mRNA and the resulting clock proteins (PER, TIM, etc.) in the cell. These proteins act (indirectly) as transcription regulators that move into the nucleus with circadian periodicity. Consequently, cells that express clock genes are candidate clock cells only if the mRNA and/or protein levels show circadian cycling. Specifically, it should be demonstrated that these levels free-run in DD, and that clock protein migration into the nucleus also occurs with a rhythmicity that free-runs in DD. These criteria must all be fulfilled by true clock cells. These clock cells are normally found in groups of ten or more cells, and the cells are coupled together by gap junctions and/or common synaptic or hormonal inputs (examples in sections below). These features suggest that a single clock cell may not have all the properties of a complete circadian clock. The only known case of a single cell that has all the properties of a clock is the chick pinealocyte, which is photosensitive and generates a rhythmic output of melatonin *in vitro* (Pickard and Tang, 1994).

In some cells that show daily cycling of clock gene expression and clock protein abundance, these rhythms cease in DD. The failure of rhythmicity to free-run in such cases is evidence that it is not generated endogenously within the cells that express the clock genes. Rhythmicity in such cells must be driven, usually by rhythmic inputs from hormones or nerves. Thus, hormones play an important role as inputs that drive rhythmicity in oscillators of this type (see Section 3.11.3.3). These cellular oscillators are not clock cells, because their rhythmicity is driven by other cells. In other words, true clocks are responsible for generating the driving inputs to these cellular oscillators. This arrangement leads to the notion that oscillators are organized into a hierarchy, with master clocks driving rhythmicity in other oscillators via hormones or nerves. Driven oscillators are found in the CNS as well as in peripheral tissues and true clocks are found in peripheral tissues as well as the CNS. Examples are found throughout the sections below. In the context of hormones, any feedback loop (see **Chapter 3.8**) can become an oscillator (i.e., become rhythmic) if it receives rhythmic driving input from a true clock. Removal of the clock input will cause such oscillations to cease. If oscillations continue after removal of the known clock inputs, the presence of unidentified clocks within the feedback loop is implied.

In yet other cells, the clock genes and their products may show no cycling, even in L : D. Such cells presumably have no molecular oscillators and therefore have no role in timekeeping. That clock genes may be expressed in cells with no role in timekeeping has raised the possibility that clock proteins may be multifunctional and have roles in cells other than as components of the molecular oscillator. This notion gains support from the fact that PER and TIM are normally found in cells in great abundance, at levels comparable to those of secretory proteins. Such high levels would be quite unnecessary for their roles as transcriptional regulators. Further, PER and TIM proteins may be found at great distances from the nucleus, such as along the length of axons (Sauman and Reppert, 1996a). Functions of clock genes in noncircadian phenomena include cocaine sensitization (Andretic *et al.*, 1999) and the ultradian male courtship rhythm of *Drosophila* (Hall, 2002, 2003).

3.11.3. Brain Clocks and the Endocrine System

There are two distinct areas in the insect brain that are well documented as centers of circadian timekeeping. One is located in the optic lobe and has been studied in depth; the second is located in the protocerebrum (**Figure 1**). Both of theses areas have been elucidated using the adult locomotor rhythm as the monitored output rhythm and are frequently referred to as locomotor clocks. This terminology does not imply that no other rhythmic phenomena are controlled by these areas, but rather that other outputs have received little experimental attention. Recent studies of the neuroarchitecture of these clocks, summarized below, reveal a complex association between the clock cells and cerebral neuropeptides and reveal pathways by which these clock areas, either separately or in cooperation with each other, control the rhythmic release of various hormones.

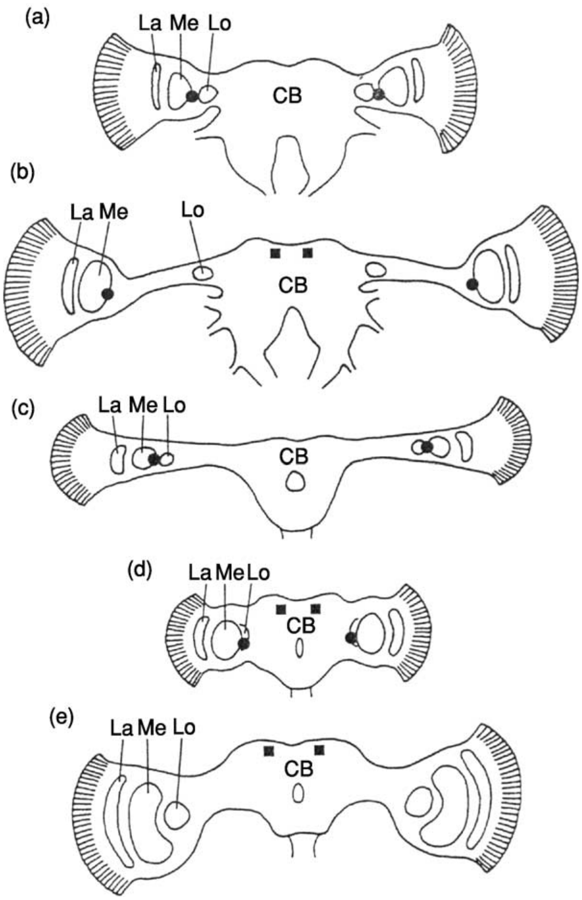


Figure 1 Diagrams of the brains of several insects, indicating the presence and location of the optic lobe clock (filled circles) and protocerebral clock (filled squares). (a) Cockroach; (b) cricket; (c) beetle; (d) fly; and (e) moth. The optic lobe neuropiles are lamina (La), medulla (Me), and lobula (Lo). Optic lobe clock neurons near the accessory medulla project to the protocerebrum in the central brain (CB) and distally to the medulla (see text). (Reproduced from Helfrich-Förster, C., Stengl, M., Homberg, U., **1998**. Organization of the circadian system in insects. *Chronobiol. Internat. 15*, 567–597.)

The structure, physiology, and molecular biology of these clocks has been reviewed extensively (Helfrich-Förster *et al*., 1998; Hall, 2003) but always in the context of behavioral rhythms. The treatment below differs significantly from previous reviews because it is focused on the relevance of this information to endocrinologists and the control of endocrine rhythms. Two central conclusions emerge. First, these clocks function to control rhythmicity in both behavioral and endocrine rhythms and constitute a key component of the circadian system in insects, with similarities to the suprachiasmatic nucleus in mammals. Second, hormones perform at least two functions in the circadian system; they are key controlled outputs of clocks which convey temporal signals to distant tissues through the hemolymph, but they also participate in the regulation of clocks (e.g., control of phase of the output) and consequently are components of the circadian timekeeping system itself.

3.11.3.1. Optic Lobe Clock and Hormones

The optic lobe has been the subject of a comprehensive series of experiments employing the classical techniques of ablation, transplantation, and lesions in several species of cockroach (reviews: Page, 1985, 1988). The optic lobe of *Leucophaea maderae* isolated *in vitro* generates a circadian rhythm of compound action potentials that free-runs in DD (Colwell and Page, 1990). When the optic lobe is transplanted into a cockroach previously rendered behaviorally arrhythmic by bilateral lobectomy, locomotion gradually becomes rhythmic as the transplanted optic lobe develops neural connections with the host brain (Page, 1983). The location of this circadian locomotor clock within the optic lobe started to become apparent when an antiserum to crustacean pigment dispersing hormone (PDH) was employed in immunohistochemical investigations of the brain (Homberg *et al*., 1991a, 1991b; Nässel *et al*., 1991). The PDH-immunoreactive perikarya were located between the neuropiles of the medulla and the lobula, adjacent to the accessory medulla (a proximal appendix of the medulla). Immunoreactivity was present throughout the axons, enabling the projections to be traced. The cells possess one projection to the protocerebrum, a second that ramifies in the medulla, and commissures connecting to the contralateral cells (**Figure 2**). On anatomical

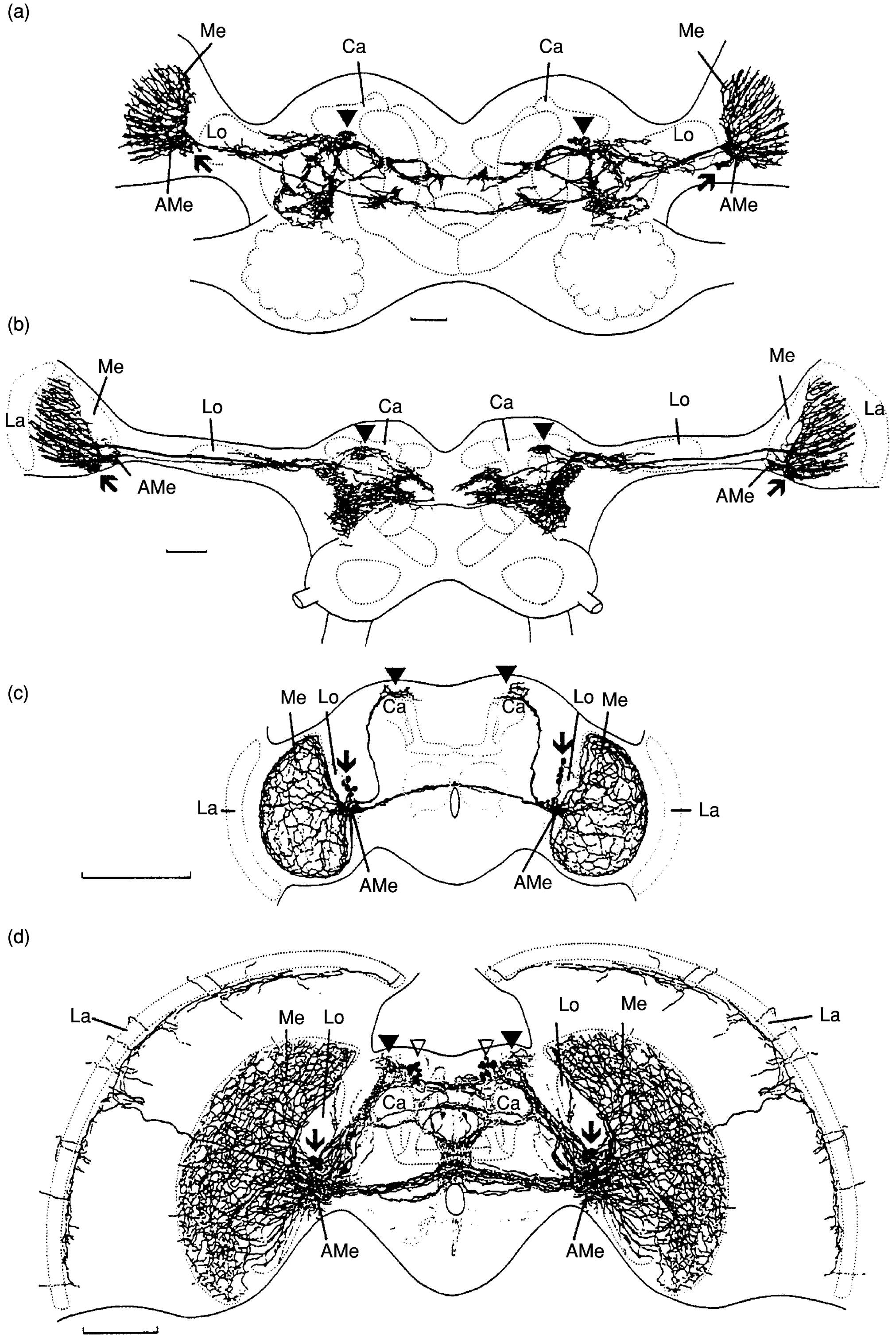


Figure 2 Axonal pathways of the presumed clock neurons of the optic lobe as revealed by pigment dispersing factor (PDF) immunoreactivity. (a) The cockroach *Leucophaea maderae*; (b) the cricket *Teleogryllus commodus*; (c) *Drosophila melanogaster*, (d) the blowfly *Phormia terraenovae*. Arrows point to the perikarya located adjacent to the accessory medulla (AMe). In all species, extensive ramifications of axons occur in the medulla (Me) and central projections occur to an area of the protocerebrum (filled arrowheads) just anterior to the calyces (Ca) of the corpora pedunculata (outlined with dots). Commisures connecting contralateral sets of neurons are also visible. In *P. terraenovae* (d), additional PDF-reactive neurons are found in the protocerebrum (open arrowheads), which project to the corpus cardiacum (not visible in the figure). Scale bar = 200 μm. (Reproduced from Helfrich-Förster, C., Stengl, M., Homberg, U., **1998**. Organization of the circadian system in insects. *Chronobiol. Internat. 15*, 567–597.)

grounds, these neurons are well suited to receive photic input from the eye and to relay it to the central brain (Stengl and Homberg, 1994). The position of these cells corresponded closely to the region of the optic lobe to which the clock had been previously assigned using microlesions (Sokolove, 1975). Following knife cuts between the lobula and protocerebrum, cockroaches regained behavioral rhythmicity in synchrony with regeneration of the PDH-reactive fibers (Stengl and Homberg, 1994). Ablation and transplantation of the accessory medulla confirmed that this small region of the optic lobe contained a clock (Reischig and Stengl, 1997). A similar pattern of branching of PDH-reactive neurons is seen in diverse insect species (Sehadová *et al.*, 2003; **Figure 2**). Orthologs of PDH are found in insects and are termed pigment dispersing factors (PDFs) (Rao and Riehm, 1993). An intimate association between neuropeptide(s) and circadian clocks was thus established.

Concomitant studies of *per* expression in *Drosophila* had identified two major sites of *per* cycling in the adult brain (Ewer *et al.*, 1992; Frisch *et al.*, 1994). These sites were dubbed the "lateral neurons," located at the junction of protocerebrum and optic lobes, and the "dorsal neurons," located above the corpora pedunculata in the protocerebrum. These are the only sites of cycling *per* expression in the brain that are present from the first instar onwards (Kaneko *et al.*, 1997). Critically, the PDF-reactive neurons expressed *per* (Helfrisch-Förster, 1995, 1997), showing that these neuropeptide-containing cells were indeed clock cells. But PDF-reactivity was not seen in *all* of the lateral neurons (only in a "subset") and was absent from all other cells that expressed *per*, including the dorsal neurons. Therefore, no universal role of PDF in clock cells can be expected. Together, these studies show that the optic lobe clock consists of a small group of neurons in which *per* expression cycles. Most, but not all, of these cells coexpress PDF, which enables the axons to be traced both distally towards the eyes and centrally into the protocerebrum. These cells are commonly associated with the accessory medulla.

The accessory medulla contains a variety of neuropeptides other than PDF, including allatostatins, FMRFamide, gastrin/cholecystokin, leucokinin I, and corazonin (Petri *et al.*, 1995). Local injection close to the accessory medulla of PDF, allatotropin, serotonin and γ-aminobutyric acid (GABA) all induce phase shifts (Petri and Stengl, 1997; Petri *et al.*, 2002; Saifullah and Tomioka, 2002, 2003) (see **Figure 20**). These recent findings imply that the optic lobe clock is subject to a sophisticated array of neurochemical manipulations. These seem to act primarily to adjust the phase of the rhythm generated by the clock cells and may either reinforce or contradict the photic information received from the environment. The complexity of organization of the optic lobe clock invites the expectation that it is not solely involved with the locomotor rhythm.

Several points of contact between the PDF-expressing lateral neurons and the neuroendocrine system in the head of adult *Drosophila* were revealed by Siegmund and Korge (2001) using a GAL4 enhancer trap system. The clock cells (sPDFMe cells in **Figure 3**) form synaptic connections in the protocerebrum with two large neurosecretory cells that terminate on the corpus allatum (CA) cells of the ring gland (**Figure 3**). At least one of these cells was thought to contain a *Manduca*-allatostatin-like peptide. These connections provide a structural pathway for circadian control of the CA (see Section 3.11.5.1). The clock cells follow the axons of two other neurosecretory cells for about 70 μm and seemed to directly contact their collaterals. These cells were very similar in size, position, and morphology to the prothoracicotropic hormone (PTTH) cells of *Bombyx mori* (Mizoguchi *et al.*, 1990) and *Manduca sexta* (Westbrook and Bollenbacher, 1990) and were presumed to contain the PTTH of *Drosophila* (see **Chapter 3.2**). Thus, a structural pathway for circadian regulation of PTTH was postulated. A close association between clock cells and PTTH cells has been described in many species (see Section 3.11.4.1.2) but this association is more commonly with clock cells in the protocerebrum than in the optic lobe. An interesting feature of these cells in *Drosophila* was that the neurosecretory axons formed terminal arborizations within the prothoracic gland (PG) region of the ring gland. This arrangement suggested that *Drosophila* PTTH was delivered directly to the PG cells and might not enter the hemolymph as a classical hormone.

3.11.3.2. Protocerebral Clock and Hormones

The picture that is emerging from recent studies is that the optic lobe and protocerebral clocks are not the distinct entities assumed in the earlier literature. The presence of a circadian clock in the protocerebrum of various insects has been inferred from traditional lesion experiments and is only now receiving analysis at the level of neuroarchitecture and molecular biology. Some species appear to have both optic lobe and protocerebral clocks, while other species seem to have one but not the other (see **Figure 1**). But the absence of evidence is not evidence of absence; for example, *per/tim* expressing

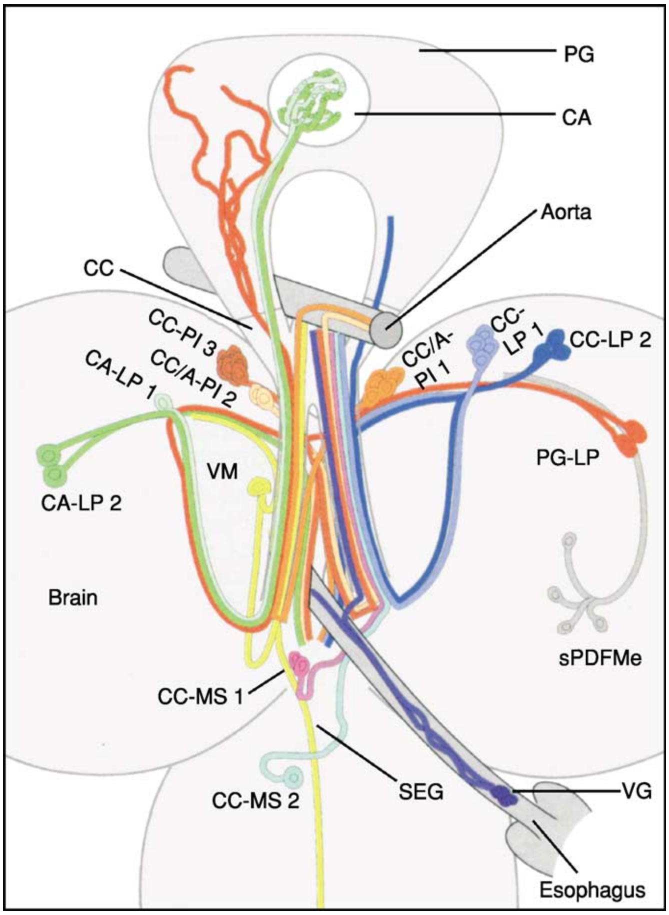


Figure 3 Axon pathways in the brain of *Drosophila melanogaster* that connect the lateral neurons of the optic lobe (sPDFMe) with the cerebral neuroendocrine system. In this diagram, the subesophageal ganglion is depicted in the lower part (SEG) and the ring gland in the upper part. CA is the corpus allatum, CC the corpus cardiacum, and PG the prothoracic gland. Arborizations of the sPDFMe neurons overlap with those of the two PG-LP neurons that directly innervate the PG and probably represent the PTTH neurons (see text). The CA is innervated by three neurons arranged in two groups (CA-LP1 and CA-LP2) and thought to contain allatostatin-like peptide, one of which passes in close proximity to the terminals of the sPDFMe neurons. The CC is innervated from the CC-LP1 and CC/A-PI 1, 2, and 3 neurons, which may receive input from the dorsal group of clock neurons (not shown here; see **Figure 4**). VM neurons are immunoreactive for eclosion hormone. (Siegmund T., Korge G., **2001**. Innervation of the ring gland of *Drosophila*. *J. Comp. Neurol.* *431*, 481–491; © Wiley. Reprinted by permission of Wiley-Liss Inc., a subsidiary of John Wiley & Sons, Inc.)

neurons close to the accessory medulla of *Manduca* (not shown in **Figure 1e**) have been recently described (Wise *et al.*, 2002). In crickets and flies (**Figure 1b** and **d**) the failure of optic lobe lesions to completely abolish the locomotor rhythm has been ascribed to the presence of a protocerebral clock. In saturniid moths, the optic lobes were entirely unnecessary for rhythmic locomotion (Truman, 1974), which was found to be regulated by a protocerebral clock entrained by extraocular photoreception. Similarly, the eclosion of silkmoths (see Section 3.11.4.3) was also found to be controlled in this way (Truman and Riddiford, 1970; Truman, 1972b). The release of PTTH that terminates pupal diapause is regulated by a photoperiodic clock in the protocerebrum (Bowen *et al.*, 1984). A close association between the PTTH cells and "clock cells" in the protocerebrum has been described by Sauman and Reppert (1996b) in *Antheraea pernyi* and by Terry and Steel (2004) in *Rhodnius prolixus* (see Section 3.11.4.1.2).

Studies of *per* expression in the brain of adult *Drosophila* revealed two groups (of two and 15 cells) of dorsal neurons in the protocerebrum (Ewer *et al.*, 1992). These cells do not express PDF and consequently their connections could not be traced by immunohistochemical methods and the cells were not accessible to targeted molecular lesioning in order to study their functions. Consequently, much less is known of these cells than the lateral neurons. Important information regarding the neuroarchitecture of clock cells in the *Drosophila* brain

was obtained using a GAL4 expression system (Kaneko and Hall, 2000). A third group of dorsal neurons (about 40 cells) was revealed (**Figure 4**), together with interconnections between the various dorsal and lateral neurons. The lateral neurons of the optic lobe clock (see above) were found to project to terminals in the dorsal protocerebrum, as previously found using PDF-immunoreactivity for tracing. These endings appeared to be connections with the dorsal neurons, some of which sent reciprocal processes back to the lateral neurons. Therefore, the protocerebral and optic lobe clocks are not distinct entities, but rather components of an integrated mosaic of clock cells in the brain.

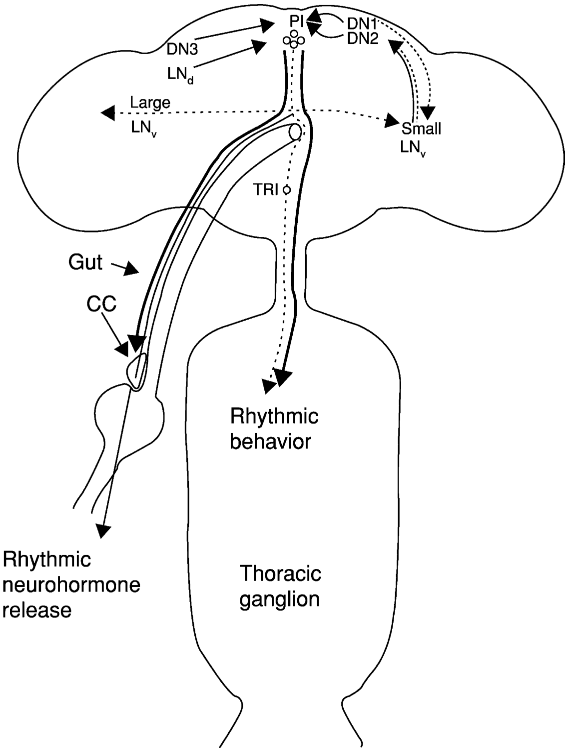


Figure 4 Deduced patterns of interactions between clock neurons and neurosecretory cells in adult *Drosophila melanogaster* and their probable connections with neuroendocrine cells of the pars intercerebralis. These connections may drive the rhythms in neurohormone release reported for other insects (see text). The LN_v (small and large) comprise the putative optic lobe clock of **Figure 1**. The large cells give rise to the arborizations in the medulla and the commissures between the hemispheres (see **Figure 2**). The small cells connect with the dorsal neurons (DN1, DN2) and receive reciprocal innervation from the DN1. All three groups of dorsal neurons (DN1, DN2, DN3) innervate the pars intercerebralis where we suggest they drive rhythmic activity in the neurosecretory cells and therefore, rhythmic neurohormone release from the corpus cardiacum (CC). The dorsal lateral neurons (LN_d) may also contribute to this input. Thus, clock cells throughout the brain converge on neurosecretory cells. Rhythmic locomotor behavior is also driven through (unspecified) neurons in the protocerebrum that project to the thoracic ganglion. (Modified with permission from a diagram of the clock neurons that control behavior in *Drosophila* by Kaneko, M., Hall, J.C., **2000**. Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol. 422*, 66–94.)

All three groups of dorsal neurons appeared to terminate close to the neurosecretory cells in the pars intercerebralis. Kaneko and Hall (2000) did not consider the relevance of their data to the neuroendocrine system. We interpret their evidence as indicating that the medial neurosecretory cells receive direct circadian input from the "dorsal neurons." The dorsal neurons are neurally coupled with the lateral neurons of the optic lobe. Therefore, the lateral neurons provide an indirect input to the neurosecretory cells, via the dorsal neurons (**Figure 4**). In some of the dorsal neurons, *per* expression cycles in antiphase to other dorsal neurons (and to the lateral neurons) (Kaneko *et al*., 1997), raising the intriguing prospect that the rhythms (in hormones?) driven by this system of clock cells may not all have the same phase. The data of Siegmund and Korge (2001) (see Section 3.11.3.1) indicate that some of the lateral neurons may bypass the dorsal neurons and make direct synaptic contact with neurosecretory cells. Thus, the neurosecretory cells appear to receive circadian input from all the major groups of "clock cells" in the brain. Therefore, we infer that most, if not all, of the neurosecretory cells would express rhythmicity.

In conclusion, the anatomically separate groups of clock cells in the brain appear to be interconnected. The nature of these interconnections may vary between species and thereby account for apparent species variations in the location and properties of the clock controlling a particular rhythm. The network of clock cells regulates rhythmicity in both behaviors and the endocrine system. As such, it is able to influence, if not drive, rhythmicity throughout the organism. It probably represents a master clock that is analogous to the suprachiasmatic nucleus (SCN) of mammals. The SCN is known to be influenced by an array of inputs, including inputs from other oscillators such as the ocular retinae, pineal gland, and parietal eye. The sections below illustrate that there is accumulating evidence that oscillators outside the brain provide input to the timing system in the brain of insects, which raises the prospect that the insect circadian system will also be comprised of multiple coupled oscillators in several anatomical locations, which communicate with each other primarily by hormones.

3.11.3.3. Peripheral Oscillators and Hormones

A variety of cells and tissues has been identified that display circadian cycling of a physiological process and/or clock gene expression. Some of these loci are able to generate rhythmicity *in vitro*, free-run in aperiodic conditions, and transmit a rhythmic output to other cells; such cells are therefore true clocks. Three such peripheral clocks have been described. The most fully characterized of these is the PG clock of *Rhodnius* (see Section 3.11.4.1.3) that generates rhythmic synthesis of ecdysteroids; PGs can be entrained by light *in vitro*, exhibit circadian cycling of PER, and communicate rhythmicity to ecdysteroid-sensitive cells via the rhythm of ecdysteroids in the hemolymph. In *Drosophila*, chemosensory hairs of the antennae exhibit cyclic expression of *per* (Krishnan *et al.*, 1999) (**Figure 5**) and this cycling is necessary for the circadian rhythm of olfactory responsiveness. In several moths, rhythmic movement of sperm along the duct system is regulated by a circadian clock, but its location is unclear (see Section 3.11.5.2.2); *per* expression occurs in part of the duct epithelium but this may not be the site of the clock that controls sperm movement.

A number of other loci have been described for which the criteria of a true clock have either not been met or have not been examined. These loci are called peripheral oscillators, not clocks. In *Drosophila*, Plautz *et al.* (1997) used a luciferase reporter driven from the promoter of *per* to identify oscillatory structures. Numerous epidermal structures exhibited a rhythmic glow. Some of these rhythms free-ran in DD and could be reset by light in pieces of the insect (e.g., a leg). The significance of these oscillators remains obscure since no output from them is known. Hege *et al.* (1997) obtained similar data for Malpighian tubules. Giebultowicz *et al.* (2000) found that tubules that were transplanted into another insect appeared to lose light sensitivity and were unable to respond to humoral signals from the host. The data are consistent with a requirement for entrainment via intact connections between the tubules and the rest of the animal. It is not known whether the tubule rhythm has a functional significance nor whether rhythmicity can be transmitted to other cell types.

In order to determine whether such peripheral oscillators possess clock properties, it will be necessary to employ *in vitro* techniques and eventually to study cell lines for timekeeping properties. These approaches are extensively used in mammalian studies. For example, SCN cell lines produce a wide array of signaling molecules (Hurst *et al.*, 2002) and can communicate rhythmicity to other cells (Allen *et al.*, 2001), confirming the central importance of the SCN cells in circadian timekeeping. Fibroblasts (Yagita *et al.*, 2001) and smooth muscle cells (Nonaka *et al.*, 2001) are able to express the same clock genes as SCN cells and do so with circadian periodicity, but expression of these genes is both induced (Nonaka *et al.*, 2001) and phase-set (McNamara *et al.*, 2001) by hormones. The induced rhythmicity in these cells is not transmitted to other cells. In mammals, peripheral oscillators exhibit circadian expression of clock genes, but this rhythmicity is driven by external factors and is not transmitted to other cells. Such oscillators are not clocks (Allen *et al.*, 2001). The

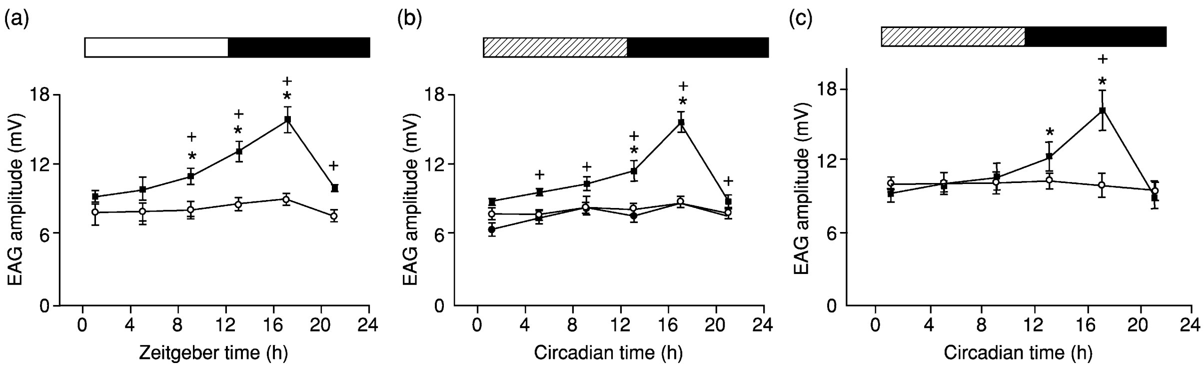


Figure 5 The circadian rhythm of olfactory responsiveness in *Drosophila melanogaster* as seen in electroantennogram records (EAG). Dark portions of horizontal bars indicate scotophase in (a) and subjective scotophase during continuous darkness in (b) and (c); light portions of the bar are photophase in (a), whereas subjective photophase in (b) and (c) is shown as a gray bar. Filled squares in (a), (b), and (c) are wild-type flies that show a rhythm in EAG amplitude that free-runs in DD ((b) and (c)). Flies with null mutations of clock genes (*per*^{0}, open circles; *tim*^{0}, filled circles) show no rhythmicity. Open squares in panel (c) depict a transgenic *per* strain (7.2.2) in which the peripheral *per* oscillator fails to function. (Reprinted with permission from Nature (Krishnan, B., Dryer, S.E., Hardin, P.E., **1999**. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature 400*, 375–378); © Macmillan Publishers Ltd.)

most common entraining signals to such oscillators are hormones (Herzog and Tosini, 2001; McNamara *et al*., 2001; Balsalobre, 2002). In insects, the roles of hormones as entraining agents to peripheral oscillators have not yet been studied. This will be a fruitful new area for insect endocrinologists.

In conclusion, insects posses numerous peripheral oscillators, but their properties and significance in all but a few cases have yet to be studied. Related research with mammals creates the expectation that hormones will emerge as the major entraining signals to such oscillators. In insects, a number of peripheral oscillators are photosensitive (Giebultowicz, 2000), but this does not reduce the potential importance of hormones as time signals. A striking example is provided by the PG clock of *Rhodnius*, which is entrainable both by light and by PTTH (see Section 3.11.4.1.3). Indeed, it has been argued that the known diversity of rhythms in any organism requires complex communication pathways between central timing systems in the brain and tissue-specific peripheral oscillators (Ikonomov *et al*., 1998; Gillette and Mitchell, 2002; Kalsbeek and Buijs, 2002). Such communication is most readily achieved by hormones.

3.11.4. Developmental Processes and Hormones

3.11.4.1. Circadian Regulation of Developmental Hormones

There is evidence that all of the hormones involved in the regulation of development are under circadian control. The literature relating to PTTH, ecdysteroids, the juvenile hormones (JHs), and melatonin is presented below. The circadian regulation of the hormones that control ecdysis behavior is also reviewed as this is a behavior that is under circadian control which is also closely integrated with development. We suspect that it will be found that all of these hormones are integrated together under the control of a circadian system that governs the temporal organization of developmental events. This notion is implied by the pervasive nature of circadian control mechanisms at all levels of development and is supported by various lines of evidence presented below. The literature has yet to integrate these lines of evidence into a comprehensive regulatory scheme.

3.11.4.1.1. Gated head critical periods One of the oldest observations in the entire field of insect endocrinology was that decapitation resulted in an arrest of the molting process if performed early in the molt, whereas decapitation later had no effect (Kopec, 1922). The period of time over which this independence of molting from the head was achieved in a population of insects became known as the head critical period (HCP). The acquisition of head independence was believed to be quite abrupt in individuals, i.e., the HCP did not occur in individual insects, only in populations. To this day, the events that give rise to the HCP are not understood. Initially, it was surmised that release of PTTH gradually activated the PGs and then its release ceased at the HCP once activation was completed (Wigglesworth, 1934). This view prevailed for decades, despite contradictory findings. For example, it was found in *Rhodnius* that brains of insects prior to the HCP (presumably releasing PTTH) were unable to promote molting when implanted into headless hosts (Wigglesworth, 1934). It was later found (Wigglesworth, 1940) that this brain implantation experiment would only promote molting if the brains were taken from animals that had *passed* the HCP. This experiment clearly showed that PTTH release did not cease at the HCP (as later studies confirmed) (see Section 3.11.4.1.2), but failed to revise the understanding of the significance of the HCP.

Much later, neck ligation experiments showed that the HCP in a population of *Manduca* larvae was gated (Truman, 1972a). This finding was the first experimental evidence that a developmental event in insects was subject to circadian timing. In these experiments, the HCP was assumed to represent the length of time during which release of PTTH occurred (in a population?). In 1972, there was no reliable assay for PTTH with which to test this assumption. It was concluded that the time of PTTH release was controlled by a circadian clock. Several other visible events (prodroma) that occur during the molt to the pupal stage were also found to be gated, including evacuation of the gut when feeding ceases ("gut purge") and the onset of "wandering" behavior (Truman and Riddiford, 1974). Together, these findings demonstrate that a number of events during molting in *Manduca* are subject to circadian timing and laid a foundation for later studies of the circadian organization of development (see sections below). However, numerous subsequent studies, outlined below, clearly refute the notion that the HCP corresponds to the time of PTTH release.

During the 1980s, doubts were raised about the adequacy of neck ligation experiments for indentifying times of release of PTTH (see **Chapter 3.2**). Using *Samia cynthia*, Fujishita and Ishizaki (1981) found that the HCP for larval molting could be

phase-shifted and that gating of the HCP persisted in DD and was lost in LL. The HCP for gut purge at the pupal molt was also responsive to phase shifts (Fujishita and Ishizaki, 1982). These findings demonstrated clearly that the gating reported earlier in *Manduca* was indeed subject to formal circadian control. However, Fujishita and Ishizaki (1982) were not convinced that the HCP represented the time of PTTH release and observed that release could begin before the HCP and/or continue after it. Sakurai (1984) made similar observations using *Bombyx* and noted that if release of PTTH took place outside the HCP, traditional neck ligation experiments would be unable to detect it. Overall, these studies showed that the HCP was a manifestation of a circadian control mechanism governing many aspects of development, but its underlying nature remained elusive. Clearly, techniques that quantified the hormones involved were required.

A bioassay for PTTH was employed by Bollenbacher *et al.* (1987), who reported PTTH in the hemolymph of fourth instar *Manduca* during the scotophase of the HCP for gate one larvae. The absence of PTTH from the hemolymph during both the preceding and the subsequent photophases supported the notion of gated PTTH release during the HCP. But no data were obtained for the adjacent scotophases, so it was not known if this release was repeated. The release of PTTH from brains of *Bombyx* was examined every 12 h for all 12 days of the fifth instar by Shirai *et al.* (1993). PTTH release was reported on all but three of the 24 time points studied, clearly showing that PTTH release was not confined to the HCP. Their published data show that PTTH release from the brain was higher during the scotophase than during the following photophase, suggesting that PTTH release occurs with a daily rhythm. However, Shirai *et al.* (1993) preferred to interpret their data as five separate periods of PTTH release over 12 days, not as a rhythm. In *Rhodnius*, PTTH is released throughout larval–adult development with a clear daily rhythm (Vafopoulou and Steel, 1996a). The work with *Rhodnius* is discussed in greater detail in the sections below. In the context of the HCP, it should be noted that the amplitude of the rhythm increased greatly *after* the HCP, showing that most of the PTTH released during development was unrelated to the HCP. This finding incidentally explains the originally paradoxical finding of Wigglesworth (1940) that brains of animals that had passed the HCP were more effective at inducing the molting of decapitated hosts than were brains of animals before the HCP. *Rhodnius* is one of the many insects in which the HCP is not gated (Knobloch and Steel, 1987). A fluoroimmunoassay (FIA) for PTTH was employed by Dai *et al.* (1995) to examine PTTH release from brains of both fourth and fifth instar larvae of *Bombyx*. PTTH release was found on every day of both instars and again differences are visible in the data between photophase and scotophase. FIA revealed PTTH in the hemolymph on all these days and also throughout the pupal stage (Mizoguchi *et al.*, 2001, 2002). PTTH release for at least three consecutive days after the HCP was found in *Periplaneta americana* (Richter, 2001).

All these reports demonstrate clearly that release of PTTH is not confined to the HCP. Rather, PTTH is released throughout large portions, if not all, of an instar. Therefore, the notion that the HCP represents the time of PTTH release is not correct. This conclusion has been reached by Vafopoulou and Steel (1996a), Sakurai *et al.* (1998), and Mizoguchi *et al.* (2002), with each set of authors employing different pathways of reasoning. Sakurai *et al.* (1998) suggested that the HCP represents a time during which the PGs become more responsive to PTTH and the targets of ecdysteroids become more responsive. Mizoguchi *et al.* (2002) suggested that the HCP might be mediated by the innervation of the PGs found in moths. None of these possibilities requires any change in PTTH release at the HCP. It should be recalled that the HCP represents the time when PGs become able to maintain steroidogenesis without input from the head. As such, the HCP signals a change in the behavior of the PGs, rather than of the head. Despite the continuing enigmatic nature of the HCP, it must be emphasized that its definition and its gated nature (in certain species) have not been questioned. The HCP remains the original well-documented event in insect development that is under circadian control.

3.11.4.1.2. Prothoracicotropic hormone The timing and pattern of release of PTTH during development has been studied extensively in the blood-feeding bug *Rhodnius*. *Rhodnius* represents a particularly favorable experimental animal for the study of circadian phenomena in the endocrine system because of the remarkable precision it exhibits in the timing of developmental events following feeding. Many animals can be fed a large blood meal simultaneously and thus the growth and development of a whole population is synchronized with remarkable precision. This synchrony is maintained throughout development, probably because the insects do not feed after the initiation of development and will not feed until after ecdysis to the next instar. Feeding is known to affect developmental rates in other insects (Steel and Davey, 1985).

Further, the long length of the last larval instar (21 days) makes it possible to distinguish events that occur daily from those which occur on the longer timescale of development. The first clear evidence of a circadian rhythm in PTTH release was thus obtained in *Rhodnius*. *In vitro* incubation of *Rhodnius* brains and quantification of the amount of PTTH released by an *in vitro* bioassay (Vafopoulou *et al*., 1996) revealed that release of PTTH occurred as a daily rhythm throughout most of larval–adult development (Vafopoulou and Steel, 1996a). Indeed, PTTH immunoreactive peptide was released with a daily rhythm as shown on dot blots (Vafopoulou and Steel, 2002). PTTH release was not confined to a short period of time at the initiation of development. The rhythm of release consisted of prominent maxima during each scotophase and minima during each photophase (**Figure 6**). The rhythm free-ran in DD for at least five cycles with a period length close to 24 h and was temperature compensated (Vafopoulou and Steel, 1996b); it is therefore controlled by an endogenous circadian clock. The rhythm also free-ran in LL for three to four cycles, but with a period length somewhat shorter than that seen in DD. No rhythm was apparent after the fourth cycle in LL (Vafopoulou and Steel, 1996b). When animals were kept in LL for 30 days prior to sampling, rhythmicity in PTTH release was entirely lost (Vafopoulou and Steel, 2001); such "damping" of a circadian rhythm following chronic exposure to LL is a common phenomenon. Most interestingly, PTTH release in such animals was not just arrhythmic; it had ceased completely (**Figure 7**, left panels). When animals were transferred from LL to DD, the rhythmic release of PTTH was promptly reinitiated and continued to free-run in DD (**Figure 7**, right panels) (Vafopoulou and Steel, 2001). This clearly demonstrated that the circadian clock that regulates the rhythm of PTTH release is photosensitive. The rhythm of PTTH in the hemolymph of *Rhodnius* is synchronous with the daily rhythm of PTTH release from the brain (**Figure 8a**); the PTTH content of the brain also cycles during a day; it undergoes a sharp drop in the middle of each scotophase in synchrony with release of PTTH into the hemolymph (**Figure 8b**). About one-third of the PTTH content of the brain is released every scotophase; the level is

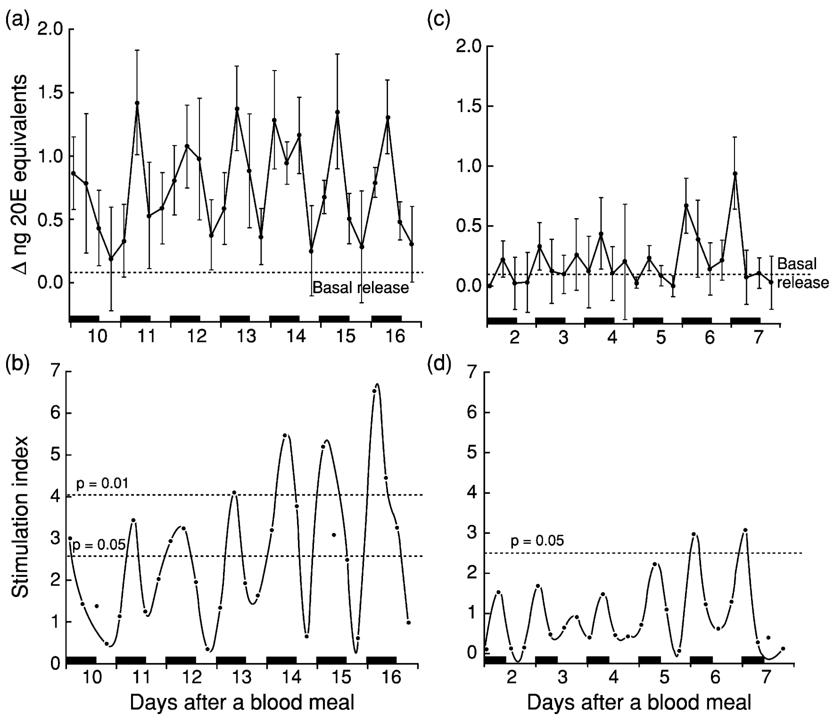


Figure 6 Circadian rhythm of prothoracicotropic hormone (PTTH) release from the brain–retrocerebral complex during larval–adult development of *Rhodnius prolixus*. Clear peaks of PTTH release occur every scotophase throughout days 10–16 of development (a), whereas deep troughs, close to background (basal release), occur every photophase. PTTH release is close to basal level in the beginning of the instar (days 2–5) (c). At the head critical period (HCP) on day 6, PTTH release increases sharply during the scotophase and marks the onset of rhythmicity. Panels (b) and (d) show the statistical significance of the levels of PTTH release shown in panels a and b, respectively. (Reproduced with permission from Vafopoulou, X., Steel, C.G.H., **1996a**. The insect neuropeptide prothoracicotropic hormone is released with a dialy rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA 93*, 3368–3372; © National Academy of Sciences, USA.)

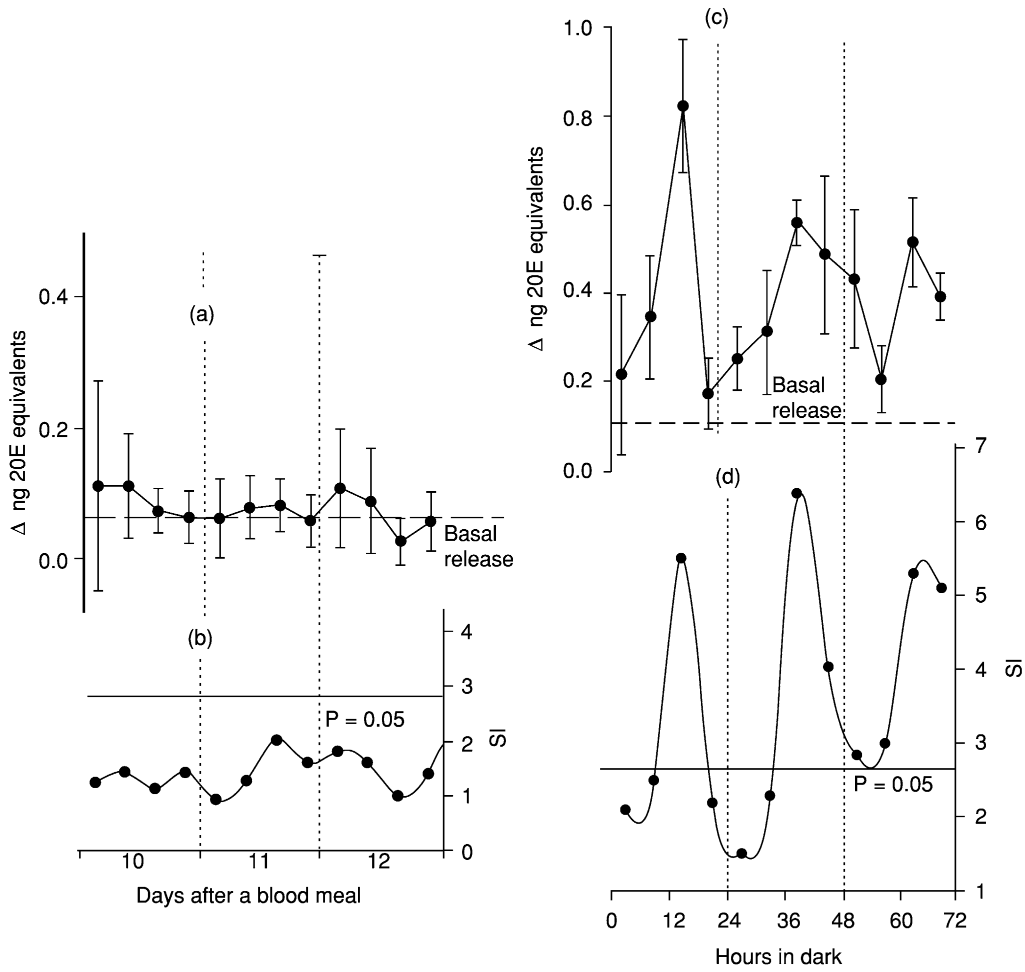


Figure 7 Induction of a daily rhythm of PTTH release from brain–retrocerebral complexes of *Rhodnius prolixus* by transfer from continuous light (LL) to continuous dark (DD). Larvae are arrhythmic following chronic exposure to LL, and there is near absence of PTTH release (a, b). Transfer to DD initiates a clear rhythm in PTTH release (c) with statistically significant peaks of roughly 24 h intervals in DD (d). (Reprinted with permission from Vafopoulou, X., Steel, C.G.H., **2001**. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *J. Insect Physiol. 47*, 935–941; © Elsevier.)

restored during the photophase each day, presumably by synthesis of the hormone. Thus, in *Rhodnius*, synthesis, release, and hemolymph levels of PTTH are all rhythmic events and are coupled to one another.

Synthesis of PTTH in *Rhodnius* occurs in a pair of large neurosecretory cells in the lateral region on each side of the protocerebrum (Vafopoulou *et al.*, 2004b). These PTTH cells lie adjacent to a small group of neurons immunopositive for the clock proteins PER and TIM (**Figure 16**) (Terry and Steel, 2004). These neurons are true clock cells since both PER and TIM exhibit circadian cycling in abundance and in migration in and out of the nucleus. The location of these clock cells in the brain is equivalent to the lateral group of clock neurons in *Drosophila*. PTTH has also been localized to the lateral neurosecretory cell cluster in other species as in *Bombyx* (Mizoguchi *et al.*, 1990), *Manduca* (Agui *et al.*, 1979; O'Brien *et al.*, 1988), and *Samia* (Yagi *et al.*, 1995) but not in all species examined (Závodská *et al.*, 2003). A similar proximity between PER-containing neurons and PTTH cells is seen in the moth *A. pernyi*, where this arrangement is believed to regulate the release of PTTH that terminates diapause in response to long days (Sauman and Reppert, 1996b). This information raises the interesting possibility that a similar, if not the same, neuronal clock mechanism may be involved in both circadian and photoperiodic regulation of PTTH release (see **Chapter 3.12**). In another moth, *Bombyx*, the PTTH levels in the hemolymph show a daily rhythmicity during larval (**Figure 9**) (Dedos and Fugo, 1999; Mizoguchi

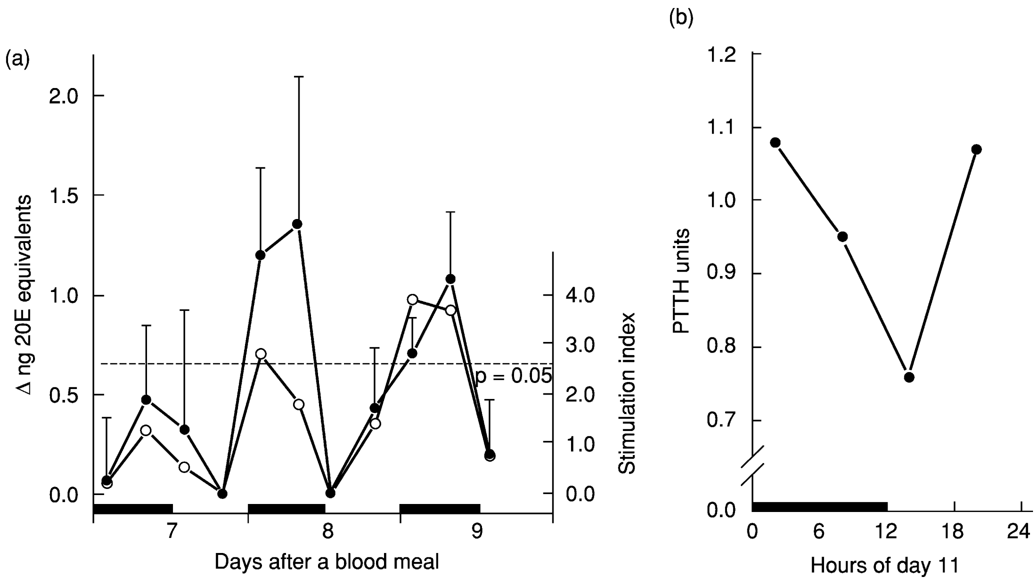


Figure 8 The daily PTTH rhythm in the hemolymph of fifth (last) larval instar of *Rhodnius prolixus* showing peaks during each scotophase (a). The pattern of the hemolymph rhythm parallels that of the PTTH release from the brain (compare with **Figure 6a**). Amounts of PTTH release (filled circles) are shown on the left axis and statistical significance (open circles) on right axis. Panel (b) shows rhythmicity in PTTH content of the *Rhodnius* brain; the brain PTTH content drops during the scotophase when the PTTH titer in the hemolymph is rising (compare a with b), suggesting release at night, whereas the brain content is replenished during the photophase, presumably by PTTH synthesis. (Reproduced with permission from Vafopoulou, X., Steel, C.G.H., **1996a**. The insect neuropeptide prothoracicotropic hormone is released with a dialy rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA 93*, 3368–3372; © National Academy of Sciences, USA.)

et al., 2002) and pupal development (**Figure 9a**) (Mizoguchi *et al*., 2001) suggesting clock control of rhythmicity. Rhythmic release of PTTH from the brain is also reported for *Periplaneta* (Richter, 2001). The first of the daily peaks in the fifth instar of *Bombyx* was found to be sensitive to phase shifts of the light : dark (LD) cycle (Mizoguchi *et al*., 2002).

Rhythmicity in release of PTTH may be widespread in insects. An anatomically comparable system of clock neurons is seen in *Drosophila*. The lateral neurons are the principle circadian locus in *Drosophila* (see Section 3.11.3.1); their terminal arborizations in the protocerebrum overlap extensively with a pair of neurons that innervate the part of the ring gland that corresponds to the PGs (**Figure 3**) (Siegmund and Korge, 2001). This pair of neurons (PG-LP neurons in **Figure 3**) resembles the PTTH neurons seen in moths in terms of position, number of cells, the dense collateral fibers along the axon, and the contralateral projection pattern (for details, see Siegmund and Korge (2001) and Section 3.11.3.1), suggesting that the neuronal system for clock control of PTTH is probably also present in *Drosophila*. These presumed PTTH neurons of *Drosophila* make direct contact with the PG cells in the ring gland (**Figure 3**) (see Section 3.11.3.1). Therefore, it is possible that PTTH in *Drosophila* may function as a neurotransmitter or neuromodulator rather than a hormone. Notwithstanding this difference, an essential common feature in all insects examined is the close proximity of clock neurons to the PTTH cells. Control of PTTH release by clock cells in the brains of both hemimetabolous and holometabolous insects suggests that circadian control of PTTH release appears to be conserved among insects and represents a unifying feature in the regulation of PTTH release. It should be noted that this arrangement of neuronal circadian oscillators in the brain and their control of rhythmic release of neuropeptide hormones is found in the SCN of vertebrates (see Section 3.11.3.2).

The continuous rhythmic release of PTTH throughout development implies that the function of PTTH is not merely to activate the PGs at the HCP. Classical studies interpreted the HCP as the only time when PTTH was released, but this view is questioned by many authors (see Section 3.11.4.1.1) The studies above show that PTTH is indeed released at the HCP but this release is only the first of many daily releases. In fact, the greatest quantities of PTTH are released after the HCP. As shown below (see Section 3.11.4.1.3), the circadian rhythm of PTTH appears to play a central role

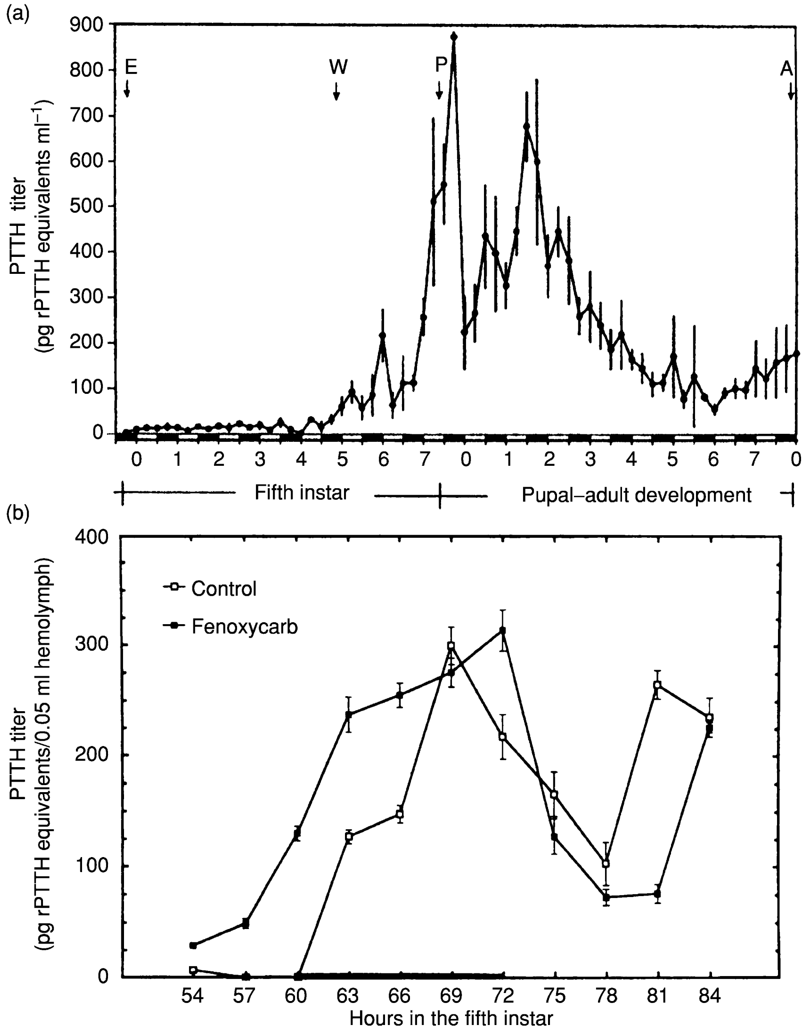


Figure 9 Daily rhythms in PTTH level in the hemolymph of *Bombyx mori*: (a) during the fifth larval instar and pupal–adult development, and (b) during a day of the fifth instar larva. Both patterns of rhythmicity show highs during the scotophase and lows during the photophase. (Reprinted with permission from (a) Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., **2002**. Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori. Gen. Comp. Endocrinol. 127*, 181–189 and (b) Dedos, S.G., Fugo, H. **1999**. Induction of dauer larvae by application of fenoxycarb early in the 5th instar of the silkworm, *Bombyx mori. J. Insect Physiol. 45*, 769–775; © Elsevier.)

in the circadian organization of larval development and possibly also of reproductive processes in adults.

3.11.4.1.3. Molting hormones Ecdysteroids are major integrators of development affecting numerous physiological processes (see **Chapters 3.3** and **3.5**). Virtually all cells in the body are exposed to and respond to changes in titer of hemolymph ecdysteroids during development. Expression of genes during specific times of development is firmly understood to be a consequence of these changes in the titer of ecdysteroids. Equivalent actions of ecdysteroids appear to operate on the circadian timescale. Circadian modulation of the ecdysteroid titer appears to provide a temporal framework upon which all the ecdysteroid-responsive cells receive information concerning the time of the day. Ecdysteroids could thus aid in the organization of gene expression within a circadian cycle. Further, all tissues are exposed to the same temporal changes in ecdysteroid levels, providing a mechanism that synchronizes and coordinates gene expression in anatomically distant tissues. In this sense, ecdysteroids can act as internal Zeitgebers conveying time information to diverse developing cells and tissues. This recently formulated capacity of ecdysteroids to act as messengers of time has led to numerous studies concerning clock regulation of ecdysteroid production.

In the early 1980s, the presence of a circadian clock in the PGs of *S. cynthia* was proposed on the basis of a series of ingenious but indirect *in vivo* experiments (Mizoguchi and Ishizaki, 1982, 1984a, 1984b). The phenomenon studied was gut purge, a behavioral change at the end of the feeding, when the larva expels the contents from its gut in preparation for pupation. Gut purge is a gated phenomenon i.e., a population rhythm (Fujishita *et al.*, 1982). The gut purge rhythm free-ran in DD, was abolished in LL, and responded to light pulses (Mizoguchi and Ishizaki, 1984b). Light pulses of 15 min yielded a Type 0 phase response curve (PRC), which showed that gut purge was controlled by a circadian clock. Fujishita *et al.* (1982) associated gut purge with a small, transient increase in the hemolymph ecdysteroid titer. This ecdysteroid peak was phase shifted by light pulses in parallel with phase shifts in gut purge (Mizoguchi and Ishizaki, 1984a). Similar phase shifts in ecdysteroids were obtained in decapitated larvae, emphasizing that the brain was not required for phase-shifting. Gut purge was not monitored in these experiments because it was severely disrupted by decapitation. In the following experiments, the timing of gut purge was employed as an indicator of the timing of ecdysteroid secretion, even though it is not certain that the ecdysteroid increase is the cause of gut purge. Larvae from DD were inserted into holes in light-tight partitions, with the partition located at various different points along the larva in different experiments (Mizoguchi and Ishizaki, 1982). Thus, selected parts of the body could be exposed to 15 min light pulses, while the rest of the body remained in darkness (**Figure 10**). When the whole larva was illuminated, the timing of gut purge was phase-delayed. A phase delay was not observed when only the head and prothorax were illuminated, but a phase delay became evident when the mesothorax and metathorax were also illuminated. Full phase delay resulted when both the head and the whole thorax were illuminated. These experiments implicated a thoracic center (presumably the PGs) in the control of the timing of gut purge. In another experiment, three pairs of PGs were transplanted into the abdomens of intact larvae which were then transferred to DD and given 10 s light pulses to various parts of the body, i.e., anterior (brain and intrinsic PGs), posterior (implanted PGs), or whole body (**Figure 10**). Invariably, the time of gut purge was dictated by the implanted PGs. The authors assumed that the phase shifts in the time of gut purge were due to hase shifts in the time of the ecdysteroid rise. It was concluded that the PGs possessed a photosensitive circadian clock. However, these ingenious

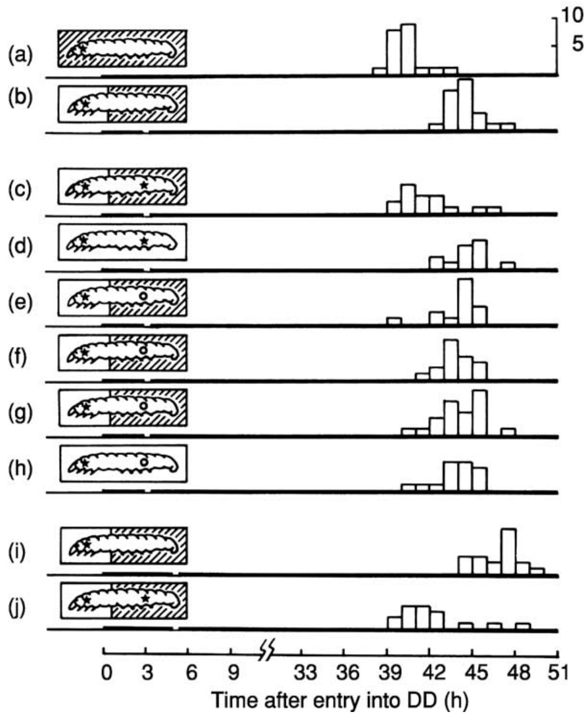


Figure 10 Effects of light pulses on the phase of gut purge of *Bombyx* larvae with and without implants of various organs in the abdomen. Larvae were transferred to DD after implantation and were given 10 s light pulses to various body parts. Pulses of light were administered 3 h (a–h) or 5 h (i and j) after transfer to DD. Clear areas on the diagrams of larvae on the left show the body areas that were illuminated. Histograms on the right show the time of gut purge. Asterisk denotes implanted prothoracic glands (PGs) and open circles other transplanted organs; (e) and (h) three brains; (f) three mesothoracic ganglia; (g) fat body. (a) Control; larvae in DD without implanted PGs and not exposed to light pulse. (b) Illumination of the head and thorax (intrinsic PGs) causes phase delay in gut purge. (c) Light pulse to anterior part of the body of a larvae with three pairs of PGs implanted (implanted PGs in dark, intrinsic PGs in light) does not phase-shift gut purge (compare c with b). (d) Whole body illumination of larvae with both sets of PGs causes phase delay. (e–h) Transplantation of various other tissues and illumination of either the anterior or the whole body of host delays gut purge as in (b). Light pulses given 5 h after transfer to DD produce similar results as those given 3 h after transfer (compare i, j with b, c). (Reproduced with permission from Mizoguchi, A., Ishizaki, H., **1982**. Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. Natl Acad. Sci. USA 79*, 2726–2730; © National Academy of Sciences, USA.)

experiments are difficult to interpret. For example, both the implanted and intrinsic PGs were presumably receiving daily stimulation from PTTH (see Section 3.11.4.1.2) and the four pairs of PGs would doubtlessly interact both with each other and with PTTH from the brain in ways that cannot be predicted (Steel and Davey, 1985).

Clear evidence that a circadian clock controls ecdysteroid levels in the hemolymph was reported

for *Rhodnius* (Steel and Ampleford, 1984; Ampleford and Steel, 1985). In *Rhodnius*, the hemolymph ecdysteroid titer during the week preceding ecdysis to the adult displays a strong daily rhythm with a massive daily peak during each scotophase and a deep trough during each photophase (**Figure 11**). The rhythm is controlled by a circadian clock since it free-runs in aperiodic conditions (e.g., DD) with a period length that is temperature compensated. The rhythm is seen throughout larval–adult development (Vafopoulou and Steel, 1989, 1991). The daily scotophase peaks in titer are three to five times higher than the values seen in adjacent photophases. It was suggested that the daily rhythm of ecdysteroid titer probably involved both synthetic (see below) and catabolic rhythmicities. Indeed, the daily decrease in titer from the top of the scotophase peak to the bottom of the photophase trough was equal to the daily amount of ecdysteroids excreted in the feces. The removal of ecdysteroids from the hemolymph was assumed to be accomplished by a combination of conjugation, sequestration in the tissues, and excretion via the Malpighian tubules and possibly also the midgut (Steel and Ampleford, 1984).

Subsequently, rhythmicities in hemolymph ecdysteroid titers were also unraveled in various other insects. In the wax moth, *Galleria mellonella*, a circadian rhythm in the titer was seen in the last larval instar (Cymborowski *et al.*, 1989). The rhythm was also present in decapitated animals emphasizing that expression of the rhythm did not require the presence of the head (Cymborowski *et al.*, 1991). Daily rhythms in ecdysteroid titer were found in both larvae and pupae of *Bombyx* (Sakurai *et al.*, 1998; Mizoguchi *et al.*, 2001, 2002). In larvae of *Manduca*, the titer displays a series of daily "steps" (Schwartz and Truman, 1983). A daily rhythm was also seen in larvae of *Periplaneta* (Richter, 2001).

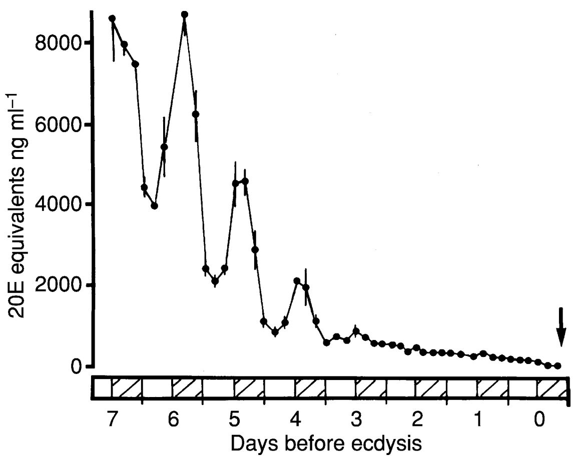


Figure 11 Rhythm of hemolymph ecdysteroid titer during larval–adult development of *Rhodnius*. Cross-hatched areas indicate scotophases and clear areas indicate photophases. Arrow marks time of ecdysis to the adult. Massive daily peaks in ecdysteroid titer occur during each scotophase. (Reprinted with permission from Ampleford, E.J., Steel, C.G.H., **1985**. Circadian control of a daily rhythm in hemolymph ecdysteroid titer in the insect *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrinol. 59*, 453–459; © Elsevier.)

The pronounced rhythmicity in the hemolymph ecdysteroid titer of *Rhodnius* implied the existence of an underlying rhythmicity in synthesis. Subsequent experiments focused on the timed ecdysteroid synthesis by the PGs. Synthesis of ecdysteroids was studied *in vitro* following explantation of PGs every 4–5 h for many days during larval–adult development (Vafopoulou and Steel, 1991). Ecdysteroids are not stored within the PG cells, thus the rate of ecdysteroid release equals the rate of synthesis (Vafopoulou and Steel, 1989). The titer of hemolymph ecdysteroids was measured for all donors of PGs, enabling the synthesis by PGs *in vitro* to be correlated with the hemolymph titer of the same animals *in vivo*. A daily rhythm of ecdysteroid synthesis was seen that was synchronous with the hemolymph titer *in vivo*. Synthesis in the scotophase was three to five times higher than synthesis in the photophase. The synthesis rhythm free-ran in both DD and LL and was temperature compensated. Therefore, the PGs contain a circadian clock. Ecdysteroid synthesis by PGs *in vitro* showed acceleration when the PGs were transferred from LL to DD *in vitro* (Vafopoulou and Steel, 1992). This finding showed that ecdysteroid synthesis by PGs was directly photosensitive and suggested that the clock controlling synthesis might also be photosensitive. Daily rhythms in ecdysteroid synthesis have also been observed in *Galleria* (Cymborowski *et al.*, 1991), *Bombyx* (Sakurai *et al.*, 1998), and *Periplaneta* (Richter, 2001).

In *Rhodnius*, the underlying complexity of this clock began to emerge when it was found that the phase of the oscillations in synthesis differs depending on whether the animals were transferred to DD or LL (Vafopoulou and Steel, 1991). In animals that were transferred from 12 : 12 to DD, the rhythm of ecdysteroid synthesis by PGs free-ran with a phase similar to the entrained state. But transfer to LL resulted in abolition of the rhythm for one cycle; then the rhythm reemerged abruptly and free-ran with peaks in the subjective photophase, i.e., the rhythm in LL free-runs in antiphase to that in DD or the entrained state. The period length in LL was slightly shorter than that in the entrained state or DD. The hemolymph titer of animals transferred to LL also undergoes the same abrupt phase inversion

after a lag of one cycle. Period length and phase of a free-running rhythm are expressions of the underlying oscillator that regulates them. The differential behavior of the rhythm in DD and LL was the first indication that more than one oscillator is responsible for driving the overt rhythm of ecdysteroid synthesis. One oscillator is phase-set by lights-off, free-runs in DD, and has a period length of about 24 h. The other oscillator is phase-set by lights-on, free-runs in LL, and has a period length of less than 24 h. Apparently, the clock mechanism underlying the *Rhodnius* rhythm is complex and consists of several components. In order to dissect the components of this clock it was necessary to adopt entirely *in vitro* approaches that would eliminate hormonal influences on PGs. Rhythmic ecdysteroid synthesis persists for 42 h *in vitro* by explanted PGs in the absence of light cues (Vafopoulou and Steel, 1998). While these observations demonstrate that the PGs synthesize ecdysteroids rhythmically entirely *in vitro*, they do not exclude the possibility that this rhythm had been established by inputs received by the PGs *in vivo* and whose effects persisted for several cycles *in vitro*. Definitive demonstration that the PGs possess a photosensitive circadian oscillator came from the induction of rhythmicity in synthesis *in vitro* by light signals in PGs that were arrhythmic (Vafopoulou and Steel, 1998). Explanted PGs from arrhythmic LL animals were transferred *in vitro* to DD and ecdysteroid synthesis monitored every 3–4 h for 42 h (**Figure 12**). These glands responded to the transfer to DD signal with the sharp acceleration of synthesis reported in 1992 but also initiated a free-running circadian rhythm of ecdysteroid synthesis entirely *in vitro*. Therefore, the PGs possess a photosensitive circadian clock that regulates rhythmic ecdysteroid synthesis. This rhythmic synthesis drives the upward side of the hemolymph titer rhythm every day. Thus, the PGs meet all the criteria that classify them as endocrine clocks. These findings confirm and extend the PG clock model first invoked by Mizoguchi and Ishizaki (1982) in *Samia*. A PG clock was also invoked in *Galleria* where decapitations of larvae did not affect the rhythm of hemolymph ecdysteroids (Cymborowski *et al.*, 1991). In contrast, neck ligation in *Periplaneta* larvae severely depressed ecdysteroid synthesis to such an extent that no rhythm was detectable (Richter, 2001). In *Bombyx*, it is still unclear whether the rhythm of hemolymph ecdysteroids (Sakurai *et al.*, 1998; Mizoguchi *et al.*, 2001) is driven by the PTTH rhythm (Mizoguchi *et al.*, 2001) or by the PG clock proposed earlier in *Samia* by Mizoguchi and Ishizaki (1982). In *Drosophila* there is good evidence that the PG cells of the ring gland possess an endogenous circadian clock (Emery *et al.*, 1997) but its function is unknown. Expression of the *per* gene exhibits a daily rhythm in the region of the ring gland that corresponds to the PG. The rhythm of *per* expression is entrained by light *in vitro* in incubations of the ring gland attached to the CNS. Addition of tetrodotoxin (TTX) to the incubation medium at concentrations sufficient to inhibit nerve conduction did not affect the *per* rhythm, demonstrating that the rhythm was not driven by the nervous system but was controlled locally by the PG cells. However, there is no evidence of rhythmic ecdysteroid synthesis by these cells, nor is rhythmicity evident in any of the numerous publications on *Drosophila* ecdysteroid titers (Richards, 1981). Therefore, in *Drosophila*, the function of this PG clock is unclear.

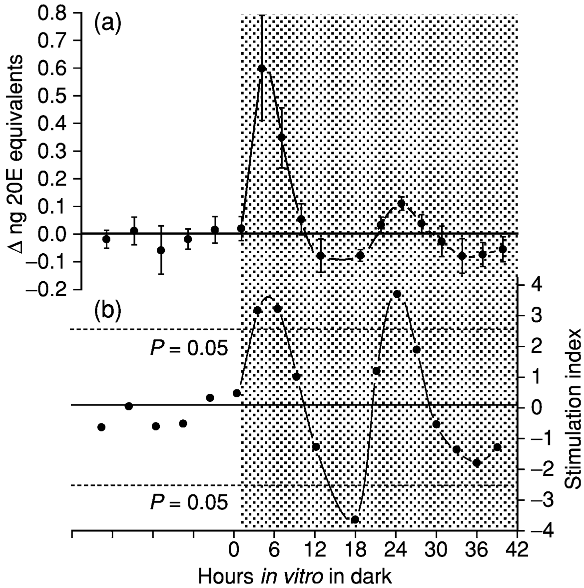


Figure 12 *In vitro* induction of rhythmic ecdysteroid synthesis by *Rhodnius* PGs following transfer from LL (clear area) to DD (stippled area). (a) Glands in LL are clearly arrhythmic (points in clear area), but become rhythmic on transfer to DD. The period length of the induced oscillations is close to 24 h. Lower panel (b) shows the statistical significance of the actual values in the upper panel (a). (Reproduced with permission from Vafopoulou, X., Steel, C.G.H. **1998**. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. Comp. Physiol. A* 182, 343–349; © Springer-Verlag.)

PGs comprise an ultrastructurally uniform population of about 200 cells (Beaulaton *et al.*, 1984; Dai *et al.*, 1994), which exhibit action potentials and are electrically coupled (Eusebio and Moody, 1986) by gap junctions (Dai *et al.*, 1994). Synchrony of the whole gland would therefore result from mutual coupling between its component cells. Each PG cell, when dissociated from the whole, appears

capable of synthesizing ecdysteroids (Smith *et al*., 1986; Asahina *et al*., 1994). The PG cells in *Rhodnius* do not receive any nerve supply (Wigglesworth, 1952) by which the cells might be synchronized, whereas those in at least some Lepidoptera are richly innervated (Lee, 1948). There is evidence that all the cells of the paired PGs of *Rhodnius* are circadian oscillators. All the cells show immunoreactivity to antibodies against the clock proteins PER and TIM (**Figure 13**) (Terry and Steel, 2004) and these proteins exhibit robust circadian cycling in abundance and nuclear migration which free-runs in DD; strong immunolocalization to the nucleus occurs during the scotophase whereas the cells are almost devoid of stain during the photophase. Therefore, each PG is a group of cellular oscillators that function in unison as a result of cellular coupling. The construction of a clock from individual cellular oscillators coupled by gap junctions and local potentials is also seen in neuronal clocks in molluscs (Block *et al*., 1993) and the mammalian SCN. Thus the PG clock, though an endocrine gland, is remarkably similar to neuronal clocks. It is of interest that PGs (at least in *Bombyx*) are, like the nervous system, of ectodermal origin (Lee, 1948).

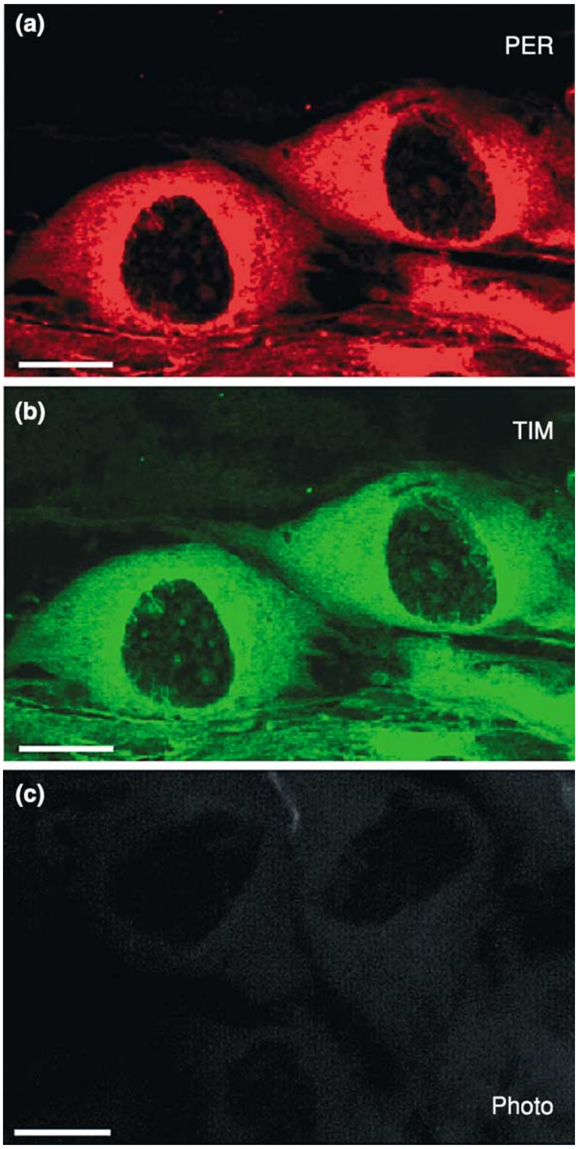


Figure 13 PER (a, red) and TIM (b, green) are colocalized in PG cells of *Rhodnius* and also show cycling. During the scotophase (a, b) immunoreactivity is high in the cytoplasm but some is also seen in the nucleus. During the photophase (c), immunoreactivity is minimal. *Rhodnius* PGs synthesize ecdysteroids during the scotophase but not during the photophase. Scale bar = 30 μm.

The rhythms of both ecdysteroid synthesis and hemolymph titer *in vivo* occur in the presence of a PTTH rhythm (see Section 3.11.4.1.2). Since the PGs are the only known targets of PTTH in larvae, it is expected that PTTH would affect the function of the PGs. However, as shown above, the PGs possess their own photosensitive clock and this does not support the possibility that the rhythmicity in synthesis *in vivo* is driven by rhythmic PTTH. The functional relationship between the PTTH clock in the brain and the PG clock was investigated by decapitation or paralysis with TTX of whole *Rhodnius* larvae in order to reveal the properties of the rhythm of ecdysteroid synthesis in the absence of rhythmic neuropeptide input (Pelc and Steel, 1997). TTX was used at a sublethal dose that caused flaccid paralysis of the whole animal and prevented release of PTTH for 4 days. Rhythmicity was maintained in both ecdysteroid synthesis and the hemolymph ecdysteroid titer; the rhythms retained entrainment to LD cycles and free-ran in both DD and LL. The rhythms showed no evidence of damping or loss of entrainment, confirming that the PG clock described *in vitro* is operative *in vivo*. This finding probably explains why decapitation (after the HCP) does not arrest development; the rhythmicity in hemolymph ecdysteroids is maintained by the PG clock, which becomes directly entrained to light in headless animals. Therefore, rhythmic neuropeptide input is not required to drive rhythmic steroidogenesis by the PGs *in vivo*. This conclusion contrasts with comparable systems in vertebrates, where rhythms in steroidogenesis are regarded as passive slaves driven by rhythmic neuropeptide input (see Turek, 1994). An important finding was that in both decapitated and TTX-paralyzed animals the rhythms of synthesis and titer always adopted a phase 12 h different from those seen when PTTH was present, regardless of the conditions of illumination. Therefore, the rhythm of steroidogenesis adopted a daytime peak

when entrained to light *in vivo* in paralyzed animals (no PTTH), but adopted a night-time peak when PTTH was present (normal animals). It was concluded that in intact animals, PTTH is the dominant entraining agent to the PG clock; photic entrainment of steroidogenesis is revealed only when rhythmic PTTH input is removed. In the presence of both light and PTTH the PGs entrain preferentially to PTTH. Thus, both light and PTTH act as Zeitgebers to the PG clock, but they contradict rather than reinforce each other. There are several examples in the literature in which circadian systems can be entrained by both light and neurochemical agents. Examples include neuropeptide Y (Yannielli and Harrington, 2000; Maywood *et al*., 2002) and melatonin (Lewy *et al*., 1995) actions on the mammalian circadian system and the action of serotonin on the circadian system in the eye of the mollusc *Aplysia californica* (Corrent *et al*., 1982; Block *et al*., 1993) and in the optic lobe of *Gryllus bimaculatus* (Saifullah and Tomioka, 2003).

The function of PTTH as an entraining agent to the PG clock shows that PTTH can act on the clock, not just on the pathway of steroidogenesis. But PTTH action on the clock could be mediated by the same intracellular pathway by which PTTH mediates stimulation of steroidogenesis. In *Manduca*, PTTH regulates calcium influx into the PG cells, which leads to stimulation of calmodulin-sensitive adenylate cyclase in the PGs (Gilbert *et al*., 2002) (see **Chapter 3.2**). Calcium and cAMP are known to induce phase shifts in many circadian systems; they act directly on the clock in the SCN (Tischkau *et al*., 2003) and frequently produce PRCs which are 12 h displaced from the PRCs for light pulses (Edmunds, 1988). Therefore, this signal transduction pathway is an appropriate pathway by which PTTH could regulate the PG clock.

The information above indicates that both PTTH and ecdysteroids are regulated by photosensitive circadian oscillators and their circadian rhythms interact every day. It is now evident that these two hormones do not act consecutively in a "cascade" but are continuously integrated together into a daily "tango" throughout the course of development (Steel and Vafopoulou, 1999) (see Section 3.11.4.1.4).

3.11.4.1.4. Hormones as agents that couple clocks The preceding sections have documented that the circadian system of *Rhodnius*, and probably that of other insects, consists of multiple photosensitive clocks coupled to the neuroendocrine axis. Two of these clocks are found in the brain where they are located in two symmetrical groups of neurons in the dorsal protocerebrum that control rhythmic release of PTTH. Coupling of the left and right clocks in the brain to each other is probably achieved by neural connections across the midline as is the case with the paired optic lobe clocks (**Figure 2**). Each PG is also a photosensitive clock that generates rhythmicity in ecdysteroid synthesis. All cells of each PG appear to be cellular oscillators since they all exhibit circadian cycling of PER/TIM clock proteins and are coupled into a functional unit by cell junctions. Thus, the circadian system that drives the downstream rhythm of the hemolymph ecdysteroid titer consists of (at least) four anatomically discrete, photosensitive clocks. Four similar clock loci, but less fully documented, are seen in moths where the information is distributed between the three genera, *Bombyx*, *Antheraea*, and *Samia*. In *Drosophila*, the number of loci is reduced to three because the normally paired PGs are fused into one ring gland. Close association between clock cells in the brain and PTTH cells is seen in the three divergent genera, *Drosophila*, *Antheraea*, and *Rhodnius*. The situation in insects therefore, is similar to the one seen in vertebrates where overt hormone rhythms express the output of multiple coupled clocks. The intricacy of functional coordination of the four (or three) circadian clocks in whole animals was studied in *Rhodnius in vivo* (Vafopoulou and Steel, 2001). PTTH release ceased completely after a few cycles in animals maintained in LL; free-running rhythmic release was promptly initiated by transfer to DD, demonstrating that the circadian oscillators that control PTTH rhythmicity are photosensitive. Transfer to DD also initiated a free-running rhythm of ecdysteroid synthesis in spite of the thick, brown overlying cuticle. Interestingly, the first initiated peak of steroidogenesis occurred several hours *prior* to the first peak of PTTH release and was not impaired when PTTH release was prevented by prior injections of TTX. These findings suggested that the initial response to a light cue involves direct response of the PG clocks to light. However, in intact animals, the second peak of steroidogenesis becomes phase-delayed until it synchronizes with the rhythm of PTTH release. Thus, the PTTH rhythm free-ran with a stable period and phase following its initiation, whereas the ecdysteroid rhythm became shifted by PTTH after its initiation. This experiment clearly showed that both clocks in brain and PGs operate *in vivo* and can initiate rhythmicity in their respective hormones following the same light cue. However, when the PTTH rhythm is present, it overrides light as a Zeitgeber to PGs and phase-sets the PG clocks. The neuropeptide therefore acts as an entraining agent for the PG clocks in whole animals

and conveys temporal information to them. Therefore, in *Rhodnius*, and probably in other insects as well, PTTH acts as a hormonal Zeitgeber to the PGs. This action synchronizes the PTTH clocks and PG clocks together into a functional unit. Therefore, the function of PTTH is integral to the circadian organization of the endocrine system.

It has been shown that the PGs are able to recognize the daily time-cues from the PTTH rhythm (Vafopoulou and Steel, 1999). Treatment of entrained PGs of *Rhodnius* with PTTH *in vitro* leads to an augmentation of ecdysteroid synthesis, but only in PGs taken from animals at certain times of the day; PGs are maximally responsive to PTTH around lights-off and insensitive to PTTH around lights-on. Therefore, PGs exhibit a daily rhythm of responsiveness to PTTH, which implies the existence of a daily up- and downregulation of PTTH receptor availability. It is interesting that the rhythm of responsiveness phase-leads that of PTTH release. Whether the responsiveness rhythm is driven by the PTTH rhythm or is endogenously controlled by the PGs themselves is not known at present. The ability of PTTH to set the phase of the PG clock (discussed above) shows that it is able to act on the clock, not just on steroidogenesis. Thus, the brain clocks communicate daily with the PG clocks.

It is probable that this communication is reciprocal, with ecdysteroids providing daily information to the brain. Feedback actions of ecdysteroids on PTTH release by the brain is cited frequently in the literature (see **Chapter 3.8**), but has not been studied from a circadian perspective. Nevertheless, it is well known that the nervous system is a major target for the action of ecdysteroids (see **Chapter 2.4**). It is possible that the rhythm of ecdysteroids acts on the clock cells in the brain or on brain neurons that provide input to the clock cells, leading to affects on PTTH release. Indeed, studies of ecdysteroid receptors (EcR) in *Rhodnius* (Vafopoulou *et al.*, 2001, 2004a) using immunohistochemistry revealed the presence of EcR in all of the 17 medial neurosecretory cells of the protocerebrum as well as the dorsal and lateral clock cells. Moreover, nuclear EcR exhibits cycling in all three locations. In the dorsal (**Figure 14**) as well as the lateral regions of the brain, EcR immunobinding exhibits cycling between subcellular locations within a day; immunobinding is exclusively nuclear during scotophase (**Figure 14a**) and exclusively cytoplasmic during photophase (**Figure 14b**). Therefore, there are several pathways by which ecdysteroids could provide inputs to the brain clocks. Unlike EcR in epidermal cells, which cycles in synchrony with the hemolymph ecdysteroid rhythm, the rhythm of nuclear EcR in the brain is many hours out of synchrony with both the hemolymph ecdysteroid rhythm and the EcR rhythm in epidermal cells. These findings are consistent with the reported induction of EcR in epidermal cells by ecdysteroids, but argue against direct induction of EcR in brain neurons by ecdysteroids, raising the prospect of more complex action of ecdysteroids on the brain (Vafopoulou *et al.*, 2004a).

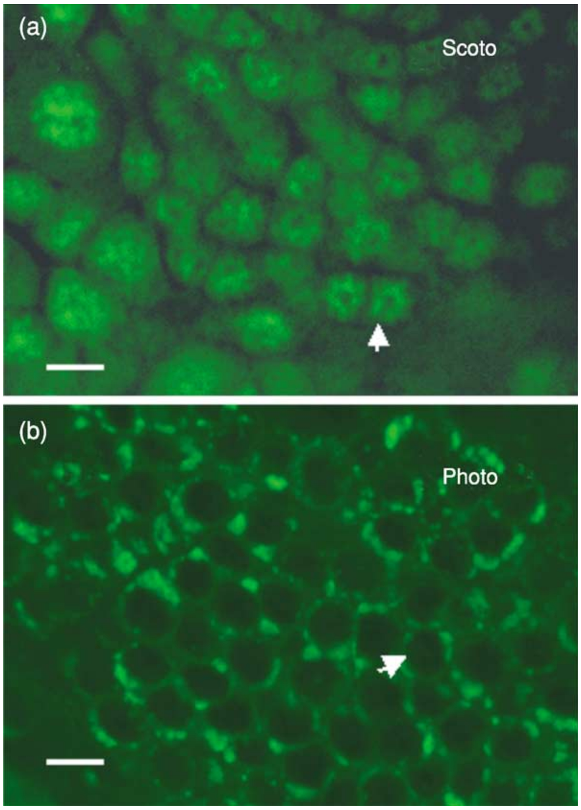


Figure 14 Ecdysteroid receptor (EcR) cycles in the dorsal clock cells (adjacent to the mushroom body) in the protocerebrum of *Rhodnius*. (a) EcR immunoreactivity is abundant and confined to nuclei during the scotophase. (b) During the photophase, EcR is confined to the cytoplasm. Arrows indicate nuclei. Scale bar = 12 μm.

Yet another endocrine component of the multi-oscillator circadian system may be melatonin. Melatonin has been detected in tissue extracts of several species (Vivien-Roels and Pevet, 1993; Hardeland and Poeggeler, 2003) including hemolymph (Itoh *et al.*, 1995a, 1995b; Linn *et al.*, 1995) where changes in levels within a day have been reported. To date, true circadian studies have been conducted only in *Rhodnius* (Gorbet and Steel, 2003, 2004). A robust daily rhythm in hemolymph levels has been found, with peaks in the scotophase and minimal values in the photophase (as commonly seen in vertebrates) (**Figure 15**). The melatonin rhythm is

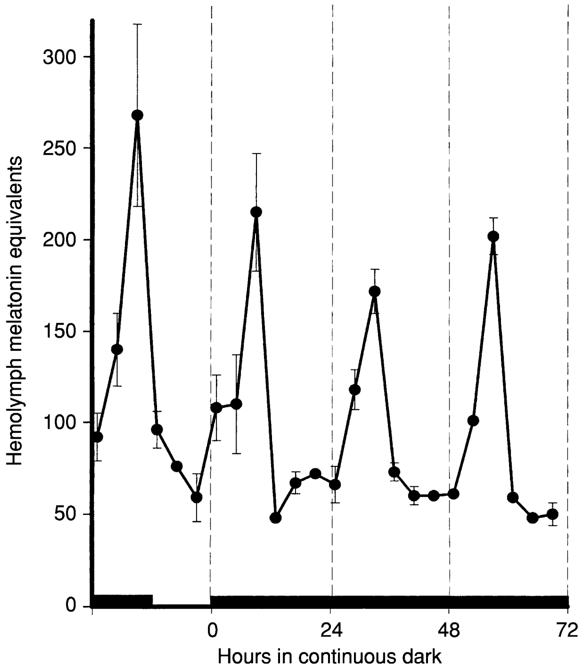


Figure 15 Rhythm of melatonin in the hemolymph of *Rhodnius*. Entrained rhythm in 12 : 12 (first peak) peaks in the late scotophase and free-runs in DD for at least three cycles (dark bar), illustrating circadian control of the rhythm.

controlled by a circadian clock since it free-runs in DD and damps out after three cycles in LL. The rhythmic pattern and the daily peak levels of melatonin in *Rhodnius* are very similar to those seen in circulating melatonin in vertebrate blood (Gorbet and Steel, 2003). Melatonin is known to act on steroidogenic tissues in vertebrates where it influences the phase of steroid release (Pang *et al.*, 1998). Melatonin may therefore participate in the circadian regulation of the PTTH–ecdysteroid neuroendocrine axis. In *Periplaneta*, no effect of melatonin on ecdysteroid synthesis by the PGs was detected, but it appears to facilitate the release of PTTH from brains *in vitro* (Richter *et al.*, 2000). It is possible that rhythmic levels of melatonin in the *Rhodnius* hemolymph modulate rhythmic release of PTTH by the brain by acting on some component of the PTTH clock in the brain. Such an arrangement would be strikingly analogous to mammalian systems, where melatonin modulates the phase of clock neurons in the SCN (Gillette and McArthur, 1995). Another possible function of melatonin may be to modulate EcR levels in the brain. In mammals, melatonin modulates steroid hormone receptor levels (Roy and Wilson, 1981; Danforth *et al.*, 1983; Molis *et al.*, 1994). Further, many brain regions of mammals exhibit rhythmic electrical activity. Such rhythms are driven by neuronal circadian oscillators in discrete brain loci such as the SCN; these regions can regulate the phase of rhythmic expression of steroid nuclear receptors (van Esseveldt *et al.*, 2000). The neuronal brain oscillators are characterized by the expression of PER/TIM clock proteins. Since two comparable loci of PER/TIM oscillators have been found in the lateral and dorsal regions of the *Rhodnius* brain, this raises the possibility that neuronal rhythms in the brain, and by association rhythms in EcR, could be driven from within the brain. Thus, there exist several possible mechanisms whereby the phase of EcR rhythmicity in the brain of *Rhodnius* described above may be regulated.

It is concluded that the circadian organization of developmental hormones, at least in *Rhodnius*, is achieved by four circadian clocks located in anatomically distinct sites, two in the brain and one in each PG. They are coupled into a functional unit *in vivo* by nerves within the brain and by hormonal pathways involving PTTH, ecdysteroids, and possibly melatonin (Steel and Vafopoulou, 2001). It should be noted that we are portraying these hormones as agents that communicate temporal information between circadian oscillators. This portrayal is still novel for insect hormones although it is well established in mammalian systems (Reppert and Weaver, 2001; Balsalobre, 2002; Kalsbeek and Buijs, 2002).

The output of the above complex circadian system is the circadian rhythm of hemolymph ecdysteroids. Diverse cells and tissues of the developing organism are thereby exposed to a tightly regulated sequence of increases and decreases in ecdysteroid titer on a circadian timescale. Do cells respond to these daily changes in hormone levels? Apparently they do. EcR is found in many target tissues; among them, the epidermis is a primary target of ecdysteroid action and EcR in epidermal cells is well studied (see **Chapter 3.5**). In *Rhodnius*, the relative abundance of nuclear EcR immunoreactivity in epidermal cells shows a striking daily rhythm that is synchronous with the rhythm of ecdysteroids in the hemolymph (Vafopoulou *et al.*, 2001, 2004a). These close temporal relations between hormone and EcR cycling are indicative of the regulation of EcR expression by the levels of circulating ecdysteroids (see **Chapter 3.5**). Indeed when hormonally naive epidermal cells of a *Chironomus* cell line are exposed to 20E, EcR immunoreactivity disappears from the cytoplasm and increases in the nucleus (Lammerding-Köppel *et al.*, 1998). These findings further indicate that the circadian time signals embedded in the rhythmic

ecdysteroid titer are detected by the primary target cells of the hormone, i.e., the epidermis.

This complexity of the multioscillator system in *Rhodnius* and its control over the rhythmicity of hormones is shown in **Figure 16**. Since most insect cells possess ecdysteroid-responsive genes which are known to be expressed in temporally orchestrated sequences during the course of development (Riddiford *et al*., 2000; Thummel, 2002) (see **Chapter 3.5**), it is possible that the rhythm in hemolymph ecdysteroids also orchestrates sequential gene expression in target cells within the circadian cycle. A case in point is the transcriptional activity of region I-18C of the salivary gland polytene chromosomes of the midge *Chironomus tentans* (Lezzi *et al*., 1991). This chromosomal region exhibits a daily rhythm of condensation and decondensation (associated with transcriptional activity). Decondensation occurs during the scotophase and parallels increased responsiveness to exogenous 20-hydroxyecdysone (20E).

Since most cells in an organism lack mechanisms which enable them to measure time or to synchronize themselves with the environment, the circadian system above appears to be the primary mechanism that maintains internal temporal organization during development both in the insect as a whole and within cells. Since all the hormones in the circadian system persist into the adult stage (see Section 3.11.5.1), it is possible that they constitute a central timing system throughout life.

3.11.4.2. Rhythmicity in Cuticle Deposition

In many insects, new cuticle is deposited in morphologically visible "daily growth layers." Such daily layers are found in all instars of many hemimetabolous insects and in the adults of various holometabolous species (Neville, 1983). In many species, this daily rhythm is under circadian control (Neville, 1965). These early reports demonstrate that epidermal cells exhibit circadian rhythmicity. Since the activities of epidermal cells are regulated primarily by ecdysteroids, the possibility is raised that daily growth layers in cuticle are driven by rhythms in ecdysteroids, or perhaps other hormones. The literature on daily growth layers does not address this possibility since it largely predates the discovery of rhythmicity in the endocrine system. This literature is briefly revisited below. It is suggested that daily growth layers may reflect rhythmicity in epidermal cells that is driven by ecdysteroids or other hormones (see **Chapters 4.2–4.4**).

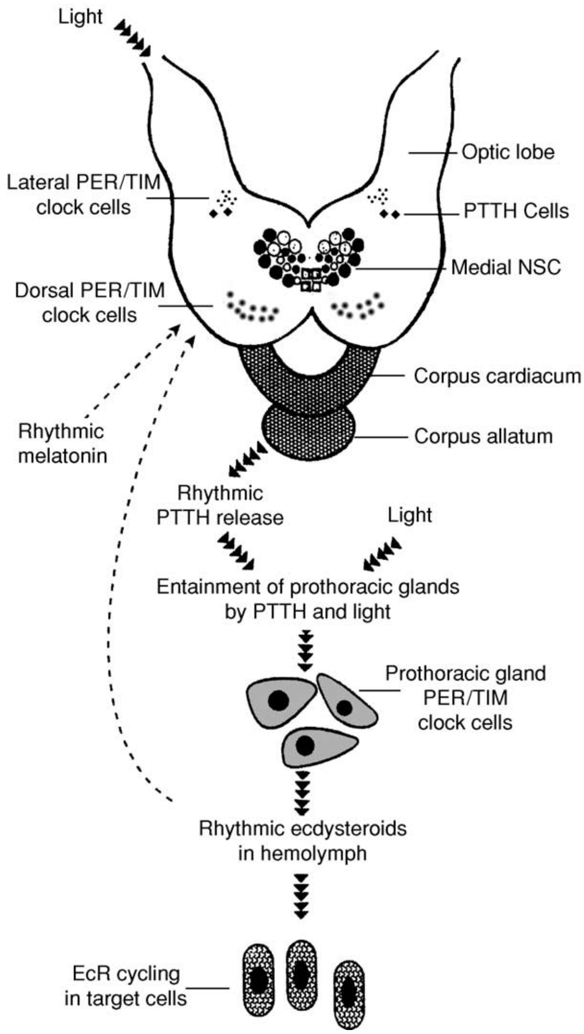


Figure 16 Schematic diagram of circadian regulation of developmental hormones in *Rhodnius*. PTTH is released rhythmically from the retrocerebral complex. The PTTH cells (diamonds) in the brain are adjacent to the lateral group of clock neurons. At least in *Drosophila*, these clock neurons project to the neurosecretory cells of the pars intercerebralis and also interact with the dorsal clock neurons (see **Figure 4**). PTTH acts on the PGs to entrain a PER/TIM-based clock in the PG cells (**Figure 13**) that is directly photosensitive (**Figure 12**). Rhythmicity in ecdysteroid synthesis is generated by the PG clock but is entrained by both PTTH and light. The resulting hemolymph titer of ecdysteroids is rhythmic, and distributes temporal information to target cells. Circadian cycling of EcR occurs in target cells, indicating rhythmic responses to ecdysteroids. The hemolymph ecdysteroid rhythm may act back on the brain (dashed arrow) via cycling EcR in the brain (**Figure 14**). The circadian rhythm of melatonin (**Figure 15**) provides a further possible input into the system. This system contains at least four circadian clocks (two in the brain, one in each PG) that are coupled by nerves and hormones (see text). Ecdysteroids are the primary controlled output of this system, distributing temporal information to all cells that possess EcR. They are potentially able to orchestrate target cell responses within a circadian cycle and to synchronize cells in distant locations with each other.

The first part of the cuticle to be deposited during a molt is a thin epicuticle composed of several layers (Hepburn, 1985). Deposition of epicuticle occurs around the middle of the molt cycle in the presence of peak titers of ecdysteroids. The bulk of the cuticle (procuticle) is deposited during decreasing titers of ecdysteroids. Frequently, by the time of ecdysis only the outer part of procuticle (the future exocuticle) has been secreted. The deposition of procuticle continues after ecdysis and may continue until the next apolysis; this region is usually termed endocuticle. Cuticle consists of chitin microfibrils which are embedded in a matrix of proteins and are laid down as plaques on the surface of the epidermal cells (Neville, 1975). At any particular depth in the cuticle, the microfibrils lie parallel to one another in the plane of the cuticle. However, when viewed from above, the orientation of the microfibrils rotates anticlockwise through successive layers within the cuticle; this arrangement of microfibrils forms a helicoidal pattern between successive levels within the procuticle. A vertical section of this type of procuticle gives the appearance of lamellae because each orientation of microfibrils is seen to repeat every 180° of the helix. Thus, each lamella consists of several layers of microfibrils whose orientation changes progressively with the depth of the cuticle. In many insects, procuticle may be lamellate throughout resulting in endocuticle and exocuticle regions that are morphologically indistinguishable. However, there are many species in which the endocuticle consists of microfibrils arranged in alternating regions of lamellate layers (with helicoidal orientation) and nonlamellate layers (with uniform orientation). In these species, the cuticle normally grows by one pair of these bands each day; for part of the day lamellate cuticle is laid down, and for the rest of the day nonlamellate cuticle is laid down. The physiological mechanism regulating the deposition of endocuticle is largely unknown (Riddiford, 1985). Nevertheless, the presence of these daily growth layers in diverse insect species (Neville, 1983) has spawned interest in the circadian control of cuticle secretion and its implications for rhythmicity in the epidermis and the hormones that regulate cuticle secretion.

There is considerable evidence that daily deposition of alternate layers of lamellate and nonlamellate cuticle is under circadian regulation in a number of insects. In adults of the locusts *Schistocerca gregaria* and *Locusta migratoria*, the daily rhythm of bands in the hind tibia exhibited all the characteristics of an endogenously controlled rhythm; the rhythm free-ran in DD, was abolished in LL, was temperature compensated, and was entrained by light (Neville, 1965). Removal of the eyes and ocelli or painting of the head capsule black or transplantation of cylinders of tibia into the hemocoel of imaginal hosts did not affect the cuticle rhythm in locusts (Neville, 1967). These experiments demonstrated that rhythmic cuticle deposition was independent of organized photoreceptors and did not require nervous regulation. The results are consistent with the possibility that rhythmic cuticle secretion is sustained by rhythmic humoral factors in the hemolymph. However, the cuticle rhythm in cockroaches such as *Blaberus cranifer* and *L. maderae*, exhibits many of the characteristics of circadian regulation (temperature compensation, for example) (Wiedenmann *et al.*, 1986; Weber, 1995), but is not entrained by LD cycles (Wiedenmann *et al.*, 1986). When animals were transferred to aperiodic conditions such as LL for a prolonged period prior to ecdysis, rhythmicity in cuticle deposition still commenced at ecdysis throughout the animal and was maintained daily thereafter (Wiedenmann *et al.*, 1986). It was inferred that the initiation of banding in the cuticle occurs at ecdysis by some unidentified signal. This signal could be a hemolymph factor, perhaps a hormone, released around the time of ecdysis (Lukat *et al.*, 1989). This unknown signal was apparently unnecessary for the maintenance of the rhythm after its initiation, since the banding rhythm continued in leg pieces that were implanted into older animals in which cuticle rhythmicity had ceased (Lukat *et al.*, 1989). In some species, this signal may occur prior to ecdysis because several daily bands may already be present in the cuticle immediately after ecdysis (Kristensen, 1966). Together, these studies of cuticular banding suggest that rhythmicity is initiated at or before ecdysis by a humoral agent, perhaps a hormone. The rhythm appears not to require additional inputs after ecdysis. These features sustain the possibility that rhythmicity is initiated by (rhythmic) hemolymph ecdysteroids at or prior to ecdysis. Once the rhythm is initiated, continuous input seems to be unnecessary; indeed the hemolymph ecdysteroid levels seen after ecdysis are very low (Steel and Vafopoulou, 1989).

A further consideration in understanding the regulation of rhythmic cuticle deposition involves *per*-based oscillators that may reside in, or close to, the epidermis. Rhythmic *per* expression was found in several peripheral tissues in *Drosophila*, including the thorax, legs, and wings (Plautz *et al.*, 1997). In this insect, the thoracic apodemes exhibit a daily rhythm in cuticle bands (Johnston and Ellison, 1982). It is therefore possible that *per*-based peripheral oscillators may drive the cuticular rhythm in

apodemes. However, the general body cuticle of *Drosophila* does not show daily growth bands. It seems important to determine whether *per* expression is seen specifically in epidermal cells and whether the peripheral oscillators noted in appendages are entrainable by humoral signals.

3.11.4.3. Circadian Regulation of Ecdysis

The endocrine regulation of ecdysis is treated elsewhere (see **Chapter 3.1**). Previous reviews include Reynolds (1980, 1987), Truman and Morton (1990), Truman *et al.* (1991), Hesterlee and Morton (1996), Horodyski (1996), Ewer *et al.* (1997), Nässel (2000), Predel and Eckert (2000), Jackson *et al.* (2001), Ewer and Reynolds (2002), and Mesce and Fahrbach (2002). This section addresses only those aspects of the subject that bear on the control of the timing of ecdysis.

The term ecdysis refers to the complex of behaviors by which an insect extracts itself from an old exoskeleton. Eclosion refers specifically to the adult ecdysis of holometabolous insects. Since every particular ecdysis occurs only once in the lifetime of an insect, circadian rhythms in ecdysis can be detected only in a population of insects of mixed ages. Such circadian "gating" of ecdysis has been described in numerous species of insects. The subject has long been of particular importance to circadian biology, the conceptual foundations of which were elucidated using the eclosion rhythm of *Drosophila* (see Pittendrigh, 1993). *Drosophila* continues to be the organism of choice for genetic manipulations of both eclosion and the molecular machinery of circadian clocks in general (Stanewsky, 2002, 2003; Hall, 2003) (see **Chapter 4.11**). By contrast, knowledge of the physiology and endocrinology of ecdysis derives almost exclusively from the large moths *A. pernyii*, *Hyalophora cecropia*, *M. sexta*, and *B. mori*.

Ecdysis occurs as soon as sufficient new cuticle is deposited below the old cuticle to provide sufficient structural support for both the insect as a whole and for its internal organs. A preecdysis sequence of behaviors then occurs, which serves to loosen remaining attachments between the new and old cuticles, followed by an ecdysis sequence characterized by rhythmic peristaltic contractions of the abdomen that propel the old cuticle away, revealing the new cuticle. The new cuticle is then inflated by swallowing air or water and then hardened by quinone tanning of cuticular proteins (see **Chapter 4.4**). At the time of ecdysis, the new cuticle is usually no more than half its final thickness. Cuticle secretion continues for days or weeks after ecdysis (as do many other components of the molting process, including muscle development and remodeling of neural circuits). Ecdysis is often, but incorrectly, described as the final event in molting or as occurring at the end of the molting process.

Insects seem to have experienced strong selective pressure to undergo ecdysis as early as possible during the molting process. The presence of two cuticles around the body, one of which is loose, encumbers locomotion and makes chewing difficult. Further, the presence of two cuticles within the tracheal system impairs gas exchange. Insects seek secluded locations away from predators once new cuticle formation is under way. These considerations illustrate that the time of ecdysis must be intimately coordinated with the progress of cuticle secretion. The latter is governed primarily by systematic changes in the titer of hemolymph ecdysteroids (Steel and Vafopoulou, 1989). Thus, the time of ecdysis is coordinated with molting via ecdysteroids. There is considerable experimental support for this view, summarized below. Coordination of ecdysis with molting does not necessarily involve circadian timing. The fact that circadian timing usually finetunes the relationship with the timing of molting is often ascribed to the need for ecdysis to take place at a time of day when predators are inactive.

There is an additional set of reasons why circadian control is needed in the case of the adult ecdysis. Closely related species may have eclosion gates that are separated by many hours. Many species mate soon after adult eclosion. Gating of eclosion may therefore serve to ensure that mating occurs between members of a single species and therefore may represent a mechanism for reproductive isolation. A secondary consequence of the timing of mating by the timing of eclosion is that the reproductive states of males and females become synchronized with each other; this in turns creates synchrony in the processes of reproduction within the population as a whole. The gating of eclosion has classically been ascribed to the action of a brain-centered "eclosion clock" (Truman and Riddiford, 1970; Truman, 1972b). Only two inputs have been documented that influence the timing of ecdysis: ecdysteroids and an eclosion clock. Whether or not the mechanisms that control ecdysis differ between larval and adult ecdyses has been debated extensively in the literature (Truman *et al.*, 1981; Ewer and Reynolds, 2002).

The importance of ecdysteroids in timing ecdysis was first shown by Slama (1980). He found that injection of 20E into pharate adult *Tenebrio molitor* delayed ecdysis. Using *Manduca*, Truman *et al.* (1983) reported that 20E injection appeared to affect both the pupal ecdysis and eclosion, but

in different ways (Figure 17a and b). When physiological doses of 20E (1 µg or less) were injected within 8 h of ecdysis, the delay in the time of ecdysis was progressive and dose dependent in both larvae and pupae. But at supraphysiological doses (5 µg or more) the pupal ecdysis was prevented whereas eclosion was not. The high doses of 20E induced discontinuous delays in eclosion, with the animals "jumping" to progressively later eclosion gates as the dose of 20E was increased (Figure 17b). This effect was attributed to a direct circadian control over eclosion (by the brain) that was presumed to be absent from larvae. This conclusion was consistent with earlier studies (Truman, 1972a) reporting that the ecdysis gate for larvae of *Manduca* became fixed at the HCP for decapitation (see Section 3.11.4.1.1) and that phase shifts of the light cycle applied after the HCP failed to generate phase shifts in the time of ecdysis. Since the HCP is a gated event, it was inferred that the gating of larval ecdyses was set by prior endocrine events that control molting. These events were presumed to involve the circadian control of PTTH release and, by association, ecdysteroid levels (Ewer and Reynolds, 2002) (see Section 3.11.4.1). Collectively, these experiments showed that ecdysis of all instars requires a decline in the

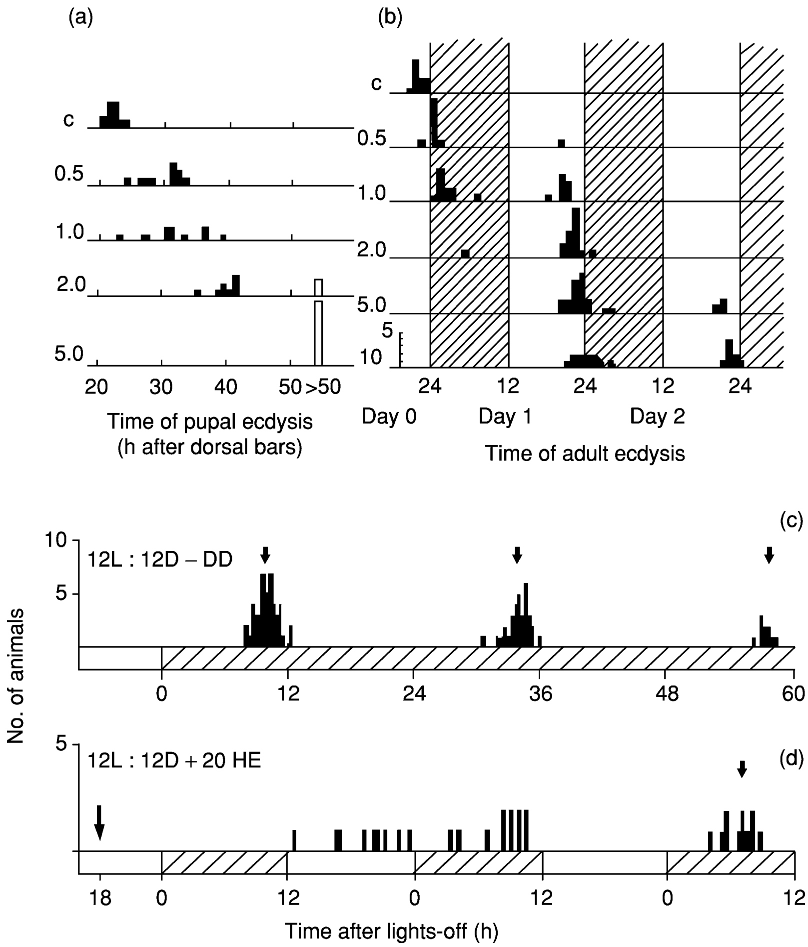


Figure 17 Upper panel: injection of increasing amounts of 20-hydroxyecdysone (20E) produces increasing delays of the time of (a) pupal ecdysis and (b) adult eclosion in *Manduca sexta*. The time of pupal ecdysis is delayed progressively as the dose of 20E increases. The same is true for eclosion for small doses of 20E; however, at higher doses of 20E the time of eclosion is displaced into the following gate. Open bars indicate animals that did not undergo ecdysis and (c) indicates the ecdysis time of control animals. Amounts of 20E injected range from 0.5 to 5.0 µg. (Reprinted with permission from Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., **1983**. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 29*, 895–900; © Elsevier.) Lower panel: in *Rhodnius prolixus*, the imaginal ecdysis rhythm free-runs in DD (c). Injection of 40 ng 20E (8% of the lowest dose used in *Manduca*) at the long arrow in (d) abolishes the first gate after injection, but timing recovers in subsequent gates. The response resembles (a) rather than (b) in *Manduca*. (Reproduced with permission from Steel, C.G.H., Ampleford, E.J., **1984**. Circadian control of hemolymph ecdysteroid titres and the ecdysis rhythm in *Rhodnius prolixus*. In: Porter, R., Collins, G.M. (Eds.), Photoperiodic Regulation of Insect and Molluscan Hormones. Pitman Press, London, pp. 150–169; © John Wiley & sons Ltd.)

titer of ecdysteroids. Such a decline is characteristic of the period of exocuticle deposition during molting (Steel and Vafopoulou, 1989), and illustrates the role of ecdysteroids in coordinating the time of ecdysis with the progress of molting. Thus, a steadily declining ecdysteroid titer would provide the ecdysis control system with information about which of the daily ecdysis gates should be employed by an individual insect. This has been the preferred model for the eclosion of moths for many years; the silkmoths *Antheraea* and *H. cecropia* appear to have a circadian clock in the brain that is specific for the control of eclosion (Truman and Riddiford, 1970; Truman, 1972b) and the hemolymph ecdysteroid titer of *Manduca* contains no information about circadian time as it exhibits no clear circadian rhythmicity (Schwartz and Truman, 1983).

In a growing number of species, the hemolymph ecdysteroid titer shows circadian oscillations (see Section 3.11.4.1.3). In such species, the circadian time signals conveyed by the ecdysteroid titer could regulate the circadian gating of ecdysis without the involvement of a specific ecdysis clock. For example, ecdysis in the silkmoth *Bombyx* is tightly gated (Sakurai, 1983) and the ecdysteroid titer exhibits a daily rhythm (Sakurai *et al*., 1998; Mizoguchi *et al*., 2001). Interestingly, the time of ecdysis is set early in molting in both *Manduca* (Truman, 1972a) and *Bombyx* (Sakurai, 1983); in *Bombyx* the time is set on the day in which rhythmicity in ecdysteroids commences (Sakurai *et al*., 1998). It is possible that the precision with which ecdysteroid titers are timed in various species influences the precision with which ecdysis is gated.

All ecdyses in *Rhodnius* are tightly gated (**Figure 17c**) (Ampleford and Steel, 1982) and the circadian regulation of ecdysteroids has been extensively studied in this species (see Section 3.11.4.1.3). In *Rhodnius*, unlike *Bombyx*, the gate for ecdysis can be phase-shifted for many days after the onset of rhythmicity in the ecdysteroid titer (Ampleford and Steel, 1982, 1985), presumably because the rhythm in ecdysteroids is itself entrainable by light (see Section 3.11.4.1.3); it is not known if the same occurs in *Bombyx*. In *Rhodnius*, injection of a much smaller dose of 20E than was used with *Manduca* much longer in advance of ecdysis has a dramatic influence on the gating of ecdysis (**Figure 17d**). The first ecdysis gate is abolished and animals emerge outside the gate until the time of the second gate, when gating is restored. It seems that *Rhodnius* ecdysis is extremely sensitive to small perturbations of ecdysteroid levels (see discussion by Ewer and Reynolds, 2002). The intimate relation between the gating of ecdysis and the ecdysteroid titer rhythm was revealed in *Rhodnius* by experiments showing that manipulations of the ecdysteroid titer led to predictable changes in the ecdysis rhythm (Ampleford and Steel, 1986). Thus, circadian time signals present in the circulating ecdysteroid titer provide important signals that regulate the gating of ecdysis. Nevertheless, factors other than the ecdysteroid rhythm appeared to be involved in generating the rhythm of ecdysis (Ampleford and Steel, 1986).

From the above it is apparent that the ecdysis control system reads the circulating levels of ecdysteroids to determine the developmental readiness for ecdysis, i.e., which of the daily gates will be used by an individual insect. This information is contained in the ecdysteroid titer whether or not it is under circadian control. In those species where the ecdysteroid titer also contains circadian information, this information contributes to the timing of ecdysis within a day, i.e., to the gated nature of ecdysis. Indeed, it would be strange if such circadian input to the ecdysis control system was ignored.

How and where do ecdysteroids act on the system that controls ecdysis? No clear evidence exists in the literature, but some possibilities have been raised. The ecdysis control system consists of eclosion hormone (EH) synthesized in ventromedial (VM) neurons in the protocerebrum of the brain, ecdysis triggering hormone (ETH) and preecdysis triggering hormone (PETH) synthesized in Inka cells of segmentally repeated epitracheal glands and crustacean cardioactive peptide (CCAP) synthesized in segmentally repeated neurons in the abdominal ganglia (see **Chapter 3.1**). The nature and mechanism of interactions between these peptides and their roles in coordination of ecdysis are the subjects of continuing research with many regulatory schemes in the literature. Some recent schemes are given by Žitňan and Adams (2000), Jackson *et al*. (2001), and Ewer and Reynolds (2002) (see **Chapter 3.1**). EH was the first of these neuropeptides to be discovered and has the most extensive literature (see Truman, 1992). Early evidence indicated two links between ecdysteroids and EH. The decline in the ecdysteroid titer to some critical value is necessary for both the initiation of release of EH and for the acquisition of the ability of the CNS to respond to EH (Truman *et al*., 1983; Morton and Truman, 1988). EH synthesis is apparently not regulated by ecdysteroids, since EH mRNA is continuously present in the cells from the embryo to the adult (Riddiford *et al*., 1994). The VM neurons of adult *Drosophila* possess a branch that terminates in the vicinity of the lateral clock neurons (Siegmund and Korge, 2001), suggesting a site for clock input at eclosion. This branch is not seen in the larval stages

of *Manduca* (Truman and Copenhaver, 1989), consistent with earlier evidence of a brain clock controlling eclosion in *Manduca*. However, the VM neurons do not seem to be necessary for either larval ecdysis or for eclosion. An expression system was used to drive transcription of cell death genes in the VM neurons of *Drosophila* (McNabb *et al*., 1997; Baker *et al*., 1999). These “EH knockout” flies exhibited important defects in ecdysis behavior in most cases, but about one-third of the flies completed all ecdyses and emerged as adults. Moreover, these flies exhibited a normal circadian rhythm of eclosion (McNabb *et al*., 1997). These findings suggest that although EH may be a normal component of the ecdysis control system, in the absence of EH both ecdysis behavior and the mechanism that times the behavior can be activated by alternate pathways. Jackson *et al*. (2001) concluded that multiple pathways must be present by which timed ecdysis can be initiated.

Ecdysteroids affect the Inka cells directly and in several ways. The rising ecdysteroid titers early in the molt induces EcR and then expression of the *eth* gene, leading to synthesis of both ETH and PETH in the Inka cells. The *eth* gene contains ecdysone response elements (Žitňan *et al*., 1999). By contrast with EH (above), deletion of the *eth* gene prevented ecdysis in 98% of animals and death occurred at the transition from first to second instar (Park *et al*., 2002). Ecdysteroids also suppress the development of responsiveness of the Inka cells to EH (Kingan and Adams, 2000) and are necessary for the abdominal nervous system to develop sensitivity to ETH. EH and ETH each promote the release of the other (Ewer *et al*., 1997; Kingan *et al*., 1997); thus, timed ecdysteroid signals to either the VM cells or the Inka cells would result in timed release of both EH and ETH. Both peptides activate the CCAP neurons in the abdominal ganglia; local release of CCAP acts as a proximate trigger of the ecdysis motor program (Gammie and Truman, 1997). The relative importance of EH (Ewer *et al*., 1997; Gammie and Truman, 1997) and ETH (Žitňan and Adams, 2000) in activating the CCAP pathway is currently debated. The CCAP neurons themselves may be additional targets of ecdysteroid action (Žitňan and Adams, 2000; Jackson *et al*., 2001) (see **Chapter 2.4**).

In summary, ecdysteroids appear to influence all the neuropeptides comprising the ecdysis control system, creating numerous potential control points for the timing of ecdysis by ecdysteroids. Operating in concert, these controls would effectively confine ecdysis behavior to a very narrow time window, even without input from a brain clock. Such may be the case in those larval ecdyses that are rather loosely gated. If the ecdysteroid titer is itself under circadian control, this circadian timing could readily drive the ecdysis control system and result in circadian gating of ecdysis. The additional “direct” circadian timing of eclosion by the brain in some species may result from the differentiation during development of contacts between the VM neurons and the clock neurons in the brain. This additional source of timing information might increase the precision of timing with which EH is released, with consequent tighter synchrony between the release of EH and PETH/ETH and also enhanced synchrony among the segmentally repeated Inka cells. The advent of a brain-centered timing mechanism at eclosion could be associated with a deterioration in ecdysteroid-based timing associated with degeneration of the prothoracic glands at the end of larval life. As noted earlier in this section, precision of timing is especially critical at the adult ecdysis; therefore, direct circadian timing of EH release might represent a compensation for deterioration of ecdysteroid-based timing at this critical time.

3.11.4.4. Circadian Regulation of Egg Hatching

Egg hatching refers to the behaviors by which the first larval instar liberates itself from the confines of the egg shell, employing muscular contractions of the body and/or gnawing or chewing movements using cuticular structures known as egg bursters. Egg hatching is therefore the first manifestation in the life cycle of an insect of its ability to express complex behavioral patterns. Hatching occurs only once in the life of an insect; it is inextricably coordinated with the timed events of embryonic development. In an insect population entrained to stable LD cycles, egg hatching occurs with regularity and consistency at restricted times of the day; it is therefore a gated phenomenon. The time of hatching is not determined by the time of oviposition. Numerous studies have shown that the hatching rhythm is circadian in nature; it free-runs under continuous light conditions and is entrainable by light and/or temperature cycles (see below). Early experiments by Minis and Pittendrigh (1968), for example, showed that eggs of *Pectinophora gossypiella* raised in LD exhibited a distinct daily rhythm of hatching, whereas eggs raised in aperiodic conditions (LL) showed arrhythmic hatching. The rhythm could be initiated by transfer of eggs from LL to DD. Similarly, daily rhythms of egg hatching have been described in various Lepidoptera and Orthoptera; for example, the hatching rhythms of the southwestern corn borer *Diatraea grandiosella* (Takeda, 1983), the silkmoth *Antheraea* (Sauman *et al*.,

1996), and the house cricket *Gryllus* (Tomioka *et al*., 1991) all free-run in DD. Collectively, these studies show that egg hatching is controlled by an endogenous circadian oscillator, which differentiates and becomes functional prior to hatching. Thus investigations concerning egg hatching have focused on how hatching is coordinated with the progression of embryogenesis and when during embryonic development the clock that controls hatching becomes detectable.

Minis and Pittendrigh (1968) transferred arrhythmic eggs from LL to DD at various times throughout embryonic development. They obtained rhythmic hatching only from eggs transferred to DD at 50–60% of development or later; transfers at earlier times failed to induce rhythmicity in hatching. In a converse experimental design, when eggs of *Gryllus* were transferred to LL at 60% of embryogenesis or later, hatching remained rhythmic. But if transfer to LL occurred prior to 60% of embryonic development, then the larvae hatched arrhythmically (Tomioka *et al*., 1991). Similar findings were reported by Sauman *et al*. (1996) for *Antheraea*. Interestingly, Itoh and Sumi (2000) suggested that the egg hatching rhythm of *Gryllus* was not strictly dependent on entrainment of the embryos to a light regime but appeared to be influenced by the mother as well; this implies the involvement of a maternal factor in the development of the circadian time keeping system in the embryo (possibly melatonin) (**Figure 19**). Together, these findings show that an endogenous photosensitive circadian clock that controls egg hatching becomes functional at about 50–60% of development. This suggests that some component of the timekeeping system, such as the light entrainment pathway or the clock itself, is either not differentiated or not fully functional prior to this point in embryonic development. Minis and Pittendrigh (1968) found that entrainment of hatching to temperature cycles became possible at about the same time as light entrainment became possible, indicating that both clock and entrainment pathways become functional at about the same time of embryogenesis. Further, exposure of eggs of *Pectinophora* to various wavelengths of monochromatic light during the second half of embryonic development demonstrated that the embryos were most sensitive to monochromatic light of 390–480 nm. This range of light sensitivity suggested that carotenoid-based photoreceptors could be responsible for light entrainment in these embryos. By contrast, carotenoid-depleted eggs of *Bombyx* (raised on a carotenoid-deprived diet) showed normal patterns of egg hatching rhythmicity, even though the resulting first instar larvae exhibited massively reduced ocellar photosensitivity and suppressed phototaxis (Sakamoto and Shimizu, 1994). It is therefore possible that the light input pathway for the hatching rhythm may be different from that of other major rhythms such as the locomotion rhythm. In conclusion, the above observations show that both clock and entrainment pathways become established and functional at 50–60% of embryogenesis.

What developmental events occur in the embryo at this time and are they implicated in the physiological control of hatching? Neurogenesis begins at about 20–25% of embryonic development in *Drosophila* (Doe and Goodman, 1985), *Manduca* (Dorn *et al*., 1987a), and grasshoppers (Jan and Jan, 1982). By 30% of development differentiated neurons, glial cells and sheath cells appear (Thompson and Siegler, 1993). In the CNS of *L. migratoria*, synapses and action potentials become detectable at about 70% of development (Leitch *et al*., 1992). Nerve cells that are essential to both development and circadian time keeping become differentiated and functional during embryogenesis. Neurosecretory cells of *Manduca* become immunopositive for PTTH around 24–30% of development (Westbrook and Bollenbacher, 1990). At this young age, PTTH biological activity can also be extracted from embryos (Dorn *et al*., 1987b). By 80% of development, the basic neuroarchitecture of these cells has been established, including axons that terminate in the CA (Westbrook and Bollenbacher, 1990). PTTH material active in bioassays was also extracted from *Bombyx* embryos in the second half of embryogenesis (Chen *et al*., 1987). In *Locusta*, immunoreactivity for PTTH was detected in late embryos (Goltzene *et al*., 1992). Differentiation of PGs begins around 40% of development in *Manduca* (Dorn *et al*., 1987a) and *Locusta* (Lagueux *et al*., 1979). Whether or not these young PGs are engaged in the synthesis of ecdysteroids is unclear (see below). Eggs are deposited with a rich supply of maternal ecdysteroids, mainly in the form of inactive conjugates. It is generally accepted that ecdysteroids are essential for the embryonic molts (Hoffmann and Lagueux, 1985; Kadono-Okuda *et al*., 1994). Embryos undergo one or more embryonic molts, the number varying with the insect species. For example, embryos of *L. migratoria* deposit three embryonic cuticles prior to hatching; the first, the serosal cuticle, appears at 20% of development. It is followed by the first embryonic molt at about 35% and then by the second embryonic molt at about 45% of development. At 75–80% of development the cuticle of the first larval instar is deposited. Peaks of ecdysteroids precede each embryonic molt

(Hoffmann and Lagueux, 1985). *Manduca* undergoes two embryonic molts (Dow *et al*., 1988). However, Diptera (including *Drosophila)* do not form embryonic cuticles prior to that of the first instar larva at about 75–80% of development. Embryonic molts closely correlate with increases in biologically active ecdysteroid (Dorn, 1983; Lanzrein *et al*., 1985; Slinger and Isaac, 1988). Whether active ecdysteroids are synthesized *de novo* in the embryos (Beydon *et al*., 1989; Dorn, 1983; Slinger and Isaac, 1988; Espig *et al*., 1989; Horike and Sonobe, 1999; Warren *et al*., 2002) or are produced by conversion of the inactive stored deposits (Sall *et al*., 1983; Bownes *et al*., 1988; Thompson *et al*., 1988; Kadono-Okuda *et al*., 1994) seems to vary among species. In *Bombyx,* Kadono-Okuda *et al.* (1994) applied an inhibitor of ecdysone synthesis to females at various stages of vitellogenesis, resulting in eggs with various amounts of stored ecdysteroids. The authors found that these eggs containing different amounts of ecdysteroids completed different degrees of embryonic development, and they concluded that ecdysteroids provided by the mother were essential for the normal progress of embryogenesis and egg hatching. Regardless of the origin of active ecdysteroids, the embryo apparently possesses the ability to count time and to determine when active ecdysteroids are needed for molting.

Several other neurohormones potentially important for the insect timekeeping system are also present in embryos. Truman *et al.* (1981) found that *Hyalophora* embryos contained material that was active in eclosion hormone bioassays. The amount of this material dropped at the times of embryonic ecdyses, but not at hatching. However, Fugo *et al.* (1985), obtained converse results in *Bombyx*: eclosion hormone activity did not fluctuate during embryonic molts but dropped abruptly following hatching. JH was also detected in embryos of several insects such as the cockroach *Nauphoeta cinerea* (Lanzrein *et al*., 1985), the locust *Locusta* (Temin *et al*., 1986), and *Bombyx* (Gharib and DeReggi, 1983) (review: Truman and Riddiford, 2002).

Where is the clock that controls egg hatching located in the embryo? The first detection of photic entrainment of the egg hatching rhythm in *A. pernyi* (50–60% of development) coincides with the first appearance of PER and TIM clock proteins in four pairs of cells of the embryonic brain (Sauman *et al*., 1996). Staining in the brain was exclusively cytoplasmic and did not exhibit daily oscillations in and out of the nuclei, as is required of cells possessing a molecular oscillator (see **Chapter 4.11**). At 60–70% of development, PER and TIM immunoreactivity was detected in the nuclei of midgut epithelial cells. In this location, staining exhibited robust daily oscillations that free-ran in DD. Treatment of embryos the day before hatching with *per* antisense oligodeoxynucleotide not only decreased PER immunoreactivity dramatically, but also abolished the egg hatching rhythm. Control embryos injected with a reverse orientation antisense oligonucleotide of *per* retained rhythmicity. Therefore, the *per* gene products are essential for the expression of egg hatching rhythm. Sauman and Reppert (1998) then examined the roles of brain and midgut in the control of the egg hatching rhythm (**Figure 18**). Brains from embryos 1 day before hatching were transplanted into recipient embryos, which were entrained to a light cycle that was phase delayed by 8 h relative to the donor brains. Hatching was then monitored in DD. It was observed that the implanted brain dictated the phase of hatching (**Figure 18**). By contrast, transplants of midgut did not affect the time of hatching. It became clear from these experiments that the brain is responsible for the regulation of the timing of hatching within a day (i.e., the hatching gate). This regulation is apparently achieved by the release of a "chronoactive" humoral factor from the brain, possibly a neurohormone. It was inferred that the PER-positive cells in the brain constitute the clock responsible for the egg hatching rhythm despite the failure of PER to oscillate in these cells. These cells appear to correspond to the PER-positive cells found in the brain of adult *Antheraea* (Sauman and Reppert, 1996a), which appear to regulate the release of PTTH (see Section 3.11.4.1.2). The brain also regulates the circadian cycling of PER into the nuclei of midgut epithelial cells, but this regulation requires intact nervous connection of the midgut with the brain and does not depend on a diffusible chronoactive substance from the brain. The role of this midgut oscillator is unknown. It is possible that it regulates the timing of deconjugation of maternal ecdysteroids during embryonic molts. These considerations suggest another possible pathway by which the brain might regulate ecdysteroids during embryonic molts.

Melatonin is a well-established mediator of photoperiodic information in vertebrates and a crucial element of their timekeeping system. Embryos of *Gryllus* contain both melatonin (**Figure 19b**) and *N*-acetyltransferase (NAT) (**Figure 19a**), a key enzyme in the synthetic pathway of melatonin (Itoh and Sumi, 1998a, 1998b). Freshly laid eggs contain a rich supply of melatonin of maternal origin (**Figure 19b**) (Itoh *et al*., 1994, 1995a, 1995b). At about 20% of embryonic development, the level of melatonin in the eggs begins to show a clear daily

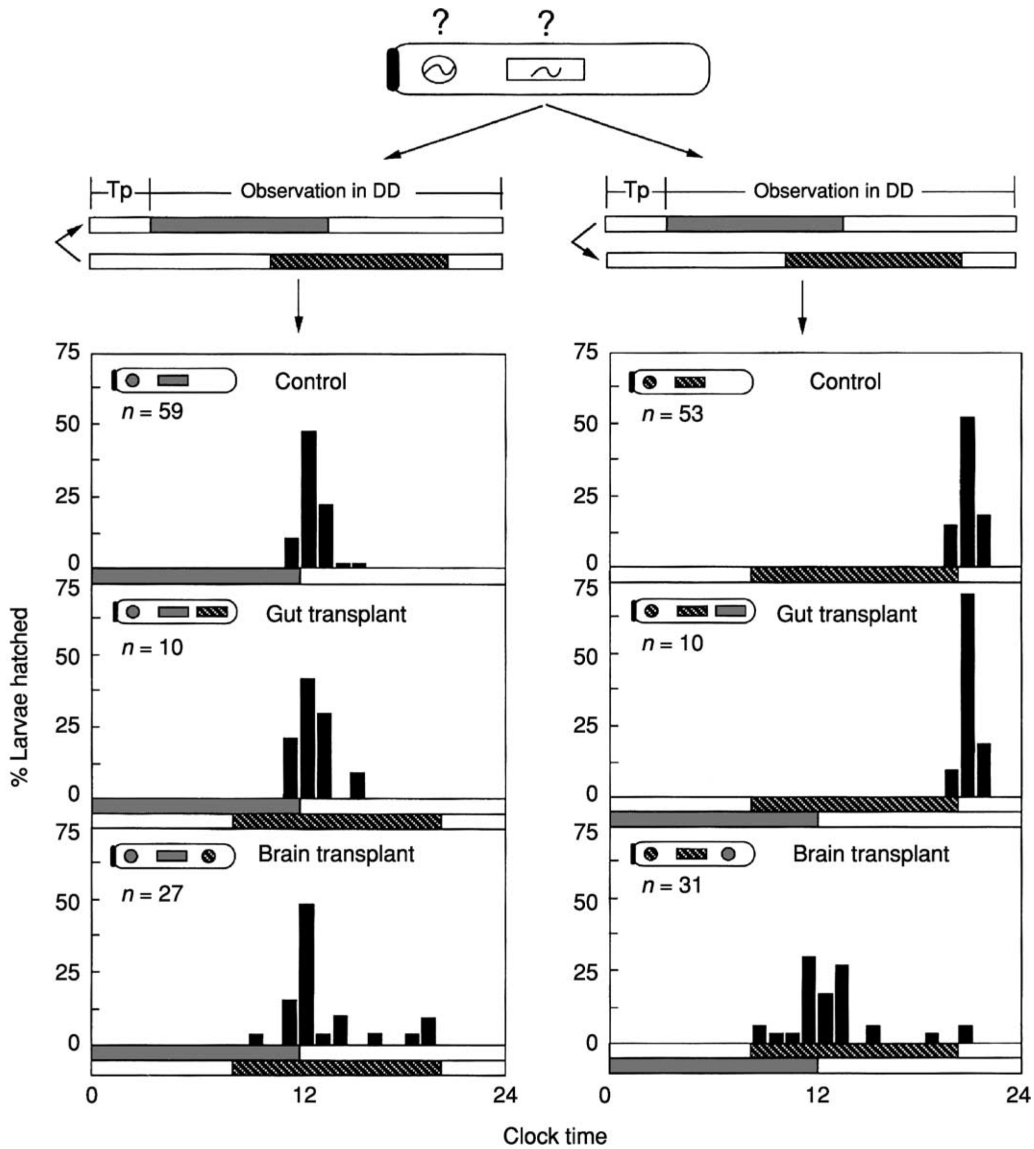


Figure 18 Experiments demonstrating a diffusible factor from the brain of developing first instar larvae of the silkmoth *Antheraea pernyi* that controls the circadian gating of egg hatching behavior. Brains or midguts of embryos were transplanted (Tp) into host embryos 1 day before egg hatching. Host embryos were entrained to a light cycle that was 8 h delayed (panels on left) or 8 h advanced (panels on right) relative to the light cycle of donor embryos. The embryos were transferred to DD following surgery and allowed to hatch. The experiments on the left show that when donor guts (middle panel) or donor brains (bottom panel) were obtained from embryos that were 8 h phase delayed relative to the host, the time of hatching of host embryos occurred at the same time as that of controls (top panel). This shows that the circadian gate of hatching is determined by the host and the subsequent release of any factors from implanted tissues is redundant. Right panels show equivalent experiments using implants that were 8 h phase advanced relative to the host. Implantation of midgut did not affect the hatching time (middle panel; compare with control panel above). However, implantation of brain advanced the time of hatching to 8 h earlier than it would normally occur in controls (bottom panel). This shows that the implanted brain releases a factor that times hatching. This factor can be detected when it is released earlier than that of the intrinsic brain but not when it is released later. (Reprinted with permission from Sauman, I., Reppert, S.M., **1998**. Brain control of embryonic circadian rhythms in the silkmoth *Antheraea pernyi*. *Neuron 20*, 741–748; © Elsevier.)

rhythm; this time coincides with the first appearance of NAT in the eggs (Itoh and Sumi, 1998a, 1998b). By 40% of development, NAT was shown to free-run in DD, suggesting that clock control of NAT was operative at this time. The rhythm was also light entrainable. These experiments show that circadian control of NAT and possibly of melatonin synthesis are initiated around mid-embryogenesis in crickets. The significance of melatonin in timekeeping in vertebrates suggests that work with melatonin in insects merits further investigation.

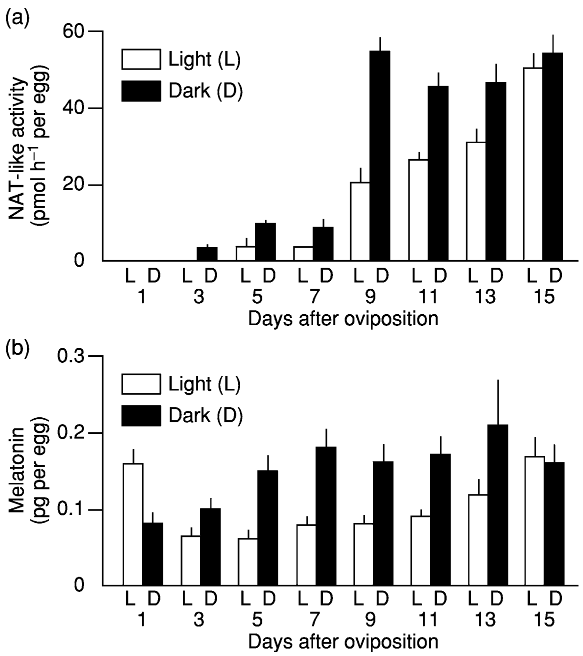


Figure 19 Changes in *N*-acetyltransferase (NAT)-like activity (a) and melatonin (b) between photophase (L) and scotophase (D) during embryogenesis of the cricket *Gryllus bimaculatus*. First instar larvae hatch around days 16–18. Newly laid eggs are devoid of NAT activity; activity is first detected at day 3 and from then on it shows daily periodic changes until hatching with highs in scotophase and lows in photophase. In contrast, melatonin is present in newly oviposited eggs. After day 3, the level of melatonin changes rhythmically with highs during scotophase and lows during photophase, suggesting intrinsic synthesis by the embryo. Photosensitivity and ability for timekeeping in the embryos of *Gryllus* is apparently established around the middle of embryogenesis. (Reprinted with permission from Itoh, M.T., Sumi, Y. **1998a**. Melatonin and serotonin *N*-acetyltransferase activity in developing eggs of the cricket *Gryllus bimaculatus*. *Brain Res. 781*, 91–99; © Elsevier.)

3.11.5. The Adult Endocrine System

3.11.5.1. Circadian Regulation of the Adult Endocrine System

All the major developmental hormones (PTTH, ecdysteroids, and JH) persist in the adult. Their roles in circadian timekeeping may also persist into the adult. Rhythms in hemolymph JH levels have been reported and pathways of clock control over the CA are known. By constrast, PTTH and ecdysteroid titers have not yet been shown to be rhythmic in adults, even though there are extensive data on these rhythms in larvae. Ecdysteroids and JH have long been known to play key roles in the regulation of reproduction. There are numerous circadian rhythms in endocrine and behavioral events that are organized around and within reproductive cycles, which raises the prospect that these hormones may also be involved in the circadian organization of reproduction.

PTTH, whose role in the circadian organization of larvae was discussed above (see Section 3.11.4.1.2), is also found in various adult insects. The first purification of PTTH employed heads of adult *Bombyx* (Ishizaki and Suzuki, 1994); PTTH was later identified at high levels in adult hemolymph (Mizoguchi *et al.*, 2001). Likewise, the brain of adult *Rhodnius* both contains and releases high quantities of biologically active PTTH (Vafopoulou *et al.*, 1996); further, the PTTH cells in the larval brain and their associated clock neurons persist in the adult brain (Terry and Steel, 2004). Similarly, the PTTH cells and the clock neurons that regulate PTTH release are also present in the brain of adult *Antheraea* (Sauman and Reppert, 1996b). There is therefore considerable evidence that the machinery for circadian regulation of PTTH persists in the adult. Even though the function of PTTH in the adult is currently unknown (see **Chapter 3.2**), it is conceivable that PTTH is as important to the circadian organization of the adult as it is in larvae. It is tempting to speculate that PTTH of adults continues to function in the regulation of ecdysteroids.

Ecdysteroids are present in the adult, where they are involved in various aspects of egg and sperm production and, along with JH, could be considered as the gonadotropins of insects (De Loof *et al.*, 2001). The primary source of ecdysteroids in larvae, the PGs, degenerate soon after ecdysis to the adult, but production of ecdysteroids continues in the adult by other tissues, such as the ovaries and testes. The hemolymph levels of ecdysteroids are much lower in adults than in larvae and consequently rhythmicity in ecdysteriod levels in adult insects has not yet been detected.

Synthesis and release of JH by the CA continues in the adult stage (see **Chapters 3.7** and **3.9**) and there is recent evidence suggesting that it may play an important role in the circadian organization of the adult. In some instances, JH synthesis is under the control of clock cells in the brain, the hemolymph level of JH is rhythmic, and JH is intimately involved in the expression of many circadian rhythms in the adult. Some of this evidence is discussed below.

JH synthesis by the CA is regulated by nerves from the brain and involves stimulatory (allatotropin) and inhibitory (allatostatin) neuropeptide inputs (see **Chapter 3.7**). The pattern of distribution of allatotropin/allatostatin immunopositive neurons in the lateral region of the protocerebrum that project to the retrocerebral complex is quite similar among different groups of insects. In *Manduca*, for

example, lateral allatostatin neurons are organized into two groups; one neuron group, the Ib cells, project immunopositive ipsilateral axons directly to the CA (Žitňan *et al.*, 1995b). Similar situations of lateral allatostatin-positive neurons that arborize terminally in the CA were found in cockroaches (Stay *et al.*, 1994), crickets (Stay *et al.*, 1994), locusts (Veelaert *et al.*, 1995), several other lepidopterans (Jeon and Lee, 1999; Audsley *et al.*, 2000), and in the ring gland of *Drosophila* (Žitňan *et al.*, 1992). Allatotropin-immunopositive lateral neurons in the brains of both *Manduca* (Žitňan *et al.*, 1995a) and *Bombyx* (Park *et al.*, 2001) also possess axons that terminate in the CA. Thus, the regulation of JH synthesis by these compounds could occur locally in the CA. In addition, these peptides may be released into the hemolymph where they may exert other regulatory functions. There is neuroanatomical evidence of connections of the circadian system to the CA by direct connection with clock cells in the brain or by indirect connection of these clock cells with the allatotropin/allatostatin cells in the brain. In adults of *Antheraea*, four PER-immunopositive cells were localized in each brain hemisphere in the protocerebrum that form a neural network; the axons of these cells coalesce to form one neural tract that projects to the CA (Sauman and Reppert, 1996a). Therefore, there is a neural pathway in *Antheraea* between clock cells and the CA that could provide circadian input directly into the CA. The situation in *Drosophila* is slightly different. In *Drosophila*, two groups of neurosecretory neurons in the protocerebrum terminate at synaptic endings within the CA (**Figure 3**); both these groups of neurons have dendritic fields that synapse with, and presumably receive input from, the lateral clock neurons that are PDF-positive and show cycling of *per* and *tim* (Siegmund and Korge, 2001). Thus, clock neurons are connected to the CA in both *Antheraea* and *Drosophila*, potentially providing circadian input to the gland.

In contrast to the above evidence of allatotropins as targets of clock control, there is evidence that allatotropins may provide an input to the clock. In *L. maderae*, allatotropin-immunoreactive neurons are present in the accessory medulla (Petri *et al.*, 1995), adjacent to the optic lobe clock (see Section 3.11.3.1). Injections of allatotropin in the vicinity of the accessory medulla resulted in stable phase shifts in the rhythm of locomotor activity (Petri *et al.*, 2002). The phase-response curves produced by injection of allatotropin were closely similar to those produced by light pulses (**Figure 20**). In other words, pulses of allatotropin have a similar action on the clock as do pulses of light. The authors suggested that allatotropin-like compounds are either a functional unit of the clock itself or modulators of photic input to the optic lobe clock in cockroaches.

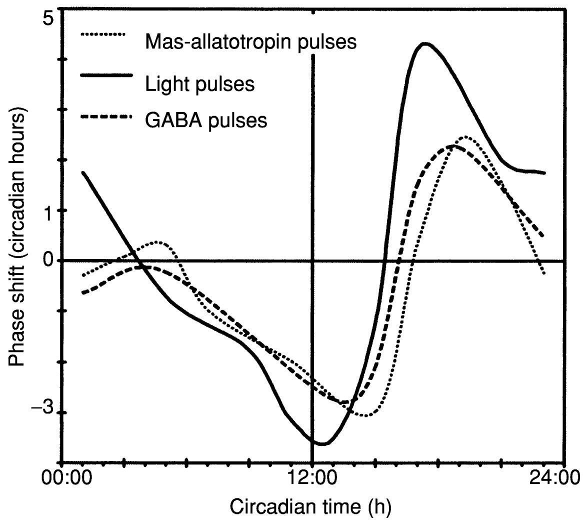


Figure 20 Phase-response curves of locomotor activity of the cockroach *Leucophaea maderae* obtained in response to injections of *Manduca* allatotropin (Mas-allatotropin), γ-aminobutyric acid (GABA), and 6 h light pulses. The close similarities of all phase-response curves suggests that both compounds may be released in response to light. (Reproduced with permission from Petri, B., Homberg, U., Loesel, R., Stengl, M., **2002**. Evidence for the role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol. 205*, 1459–1469; © The company of Biologists Ltd.)

Another mechanism that may be involved in the regulation of rhythmicity in the circulating levels of JH is clock-controlled availability of JH-binding proteins (JHBP) in the hemolymph (see **Chapter 3.7**). In *Drosophila*, the transcripts of the clock-controlled gene *take-out* (*to*) undergo daily cycling and free-run in DD; the circadian rhythm of *to* transcription is regulated by the *clock* and *cycle* clock genes (So *et al.*, 2000). Phylogenetic analysis of TO classifies it as a member of an insect protein superfamily that includes JHBPs from the hemolymph of *Manduca* and *Heliothis virescens*, a nuclear JHBP from epidermal cells of *Manduca* (Touhara and Prestwich, 1992; Touhara *et al.*, 1993; Wojtasek and Prestwich, 1995) and a putative protein from the head epidermis of *Manduca* that may be involved in JH binding (Du *et al.*, 2003). TO is more closely related to the hemolymph JHBPs than to the nuclear JHBPs. These observations suggest that the genes for JHBPs might also be clock-controlled. Though it is not known at present if TO binds the same ligands (if any) as JHBPs, these findings raise the exciting possibility that JH transport

and/or cell action may be regulated by the circadian availability of JHBPs.

Further indication of circadian regulation of JH comes from its involvement in rhythms of sensory responsiveness. JH plays an important role in the central nervous processing of sex pheromone olfactory input in males of *Agrotis ipsilon* (see **Chapter 3.15**). Males of this moth exhibit a daily rhythmicity of responsiveness to female sex pheromone that is synchronous with the daily rhythms of calling and pheromone release by females (Gemeno and Haynes, 2000) (see Section 3.11.5.4.1). Olfactory information is detected by antennal receptor neurons and then integrated centrally by interneurons in the antennal lobe (AL) of the brain (Anton and Gadenne, 1999; Gadenne *et al.*, 2001; Greiner *et al.*, 2002). Allatectomy of young males abolished behavioral responsiveness to sex pheromone (Gadenne *et al.*, 1993; Duportets *et al.*, 1996), even though the antennal olfactory neurons remained sensitive to pheromone signal (Gadenne *et al.*, 1993). Intracellular recording from the AL interneurons revealed that allatectomy significantly reduced their sensitivity to pheromone; moreover, injection of JH restored their sensitivity (Anton and Gadenne, 1999; Gadenne and Anton, 2000). Further, the proportion of AL interneurons that show sensitivity to pheromone stimulation was significantly lower in allatectomized males, but could be restored to normal levels with JH injections (Gadenne and Anton, 2000). The sensitivity to pheromones of antennal lobe interneurons increased with age and JH, whereas their sensitivity to plant volatiles was unaffected (Greiner *et al.*, 2002). Therefore, it appears that JH acts specifically on the central neural processing of sex pheromone input in males of *Agrotis*. Gadenne *et al.* (2001) showed that *Agrotis* males mate once every scotophase. Newly mated males exhibited a marked reduction in sensitivity in the antennal lobe neurons when exposed to female sex pheromones, whereas the peripheral olfactory neurons in the antennae remained highly sensitive. Sensitivity of the antennal lobe neurons recovered by the next scotophase, when the males were able to mate again. These findings show that the central neural processing system for sex pheromones undergoes daily fluctuations in sensitivity. Since this sensitivity is JH dependent, the possibility is raised that a daily rhythm of JH levels might underlie these rhythms of sensitivity.

Another example demonstrating that JH mediates rhythmicity in sensory interneurons is reported in the house cricket, *Acheta domesticus*. The males of *Acheta* exhibit a circadian rhythm of stridulation (Rence *et al.*, 1988) (see Section 3.11.5.4.2). Females recognize and respond to this acoustic sexual signal by exhibiting positive phonotactic behavior. Phonotactic behavior in females is regulated primarily by JH (Koudele *et al.*, 1987; Atkins and Stout, 1994; Stout *et al.*, 1998) (see **Chapter 3.15**). Allatectomy of females exhibiting phonotaxis resulted in decline in the directionality of behavior and sexual responsiveness, whereas topical JH application (Koudele *et al.*, 1987; Atkins and Stout, 1994) or CA transplantations (Stout *et al.*, 1976) restored them. It was found that phonotactic thresholds were regulated by changes in the level of JH, apparently by JH influencing the thresholds for firing action potentials in the L1 auditory interneuron; the L1 interneuron is a prothoracic ascending neuron that is essential for phonotactic responses to low-intensity calling songs. Intracellular and extracellular recordings of the firing of action potentials of the L1 interneuron revealed that allatectomy increased the phonotactic thresholds (Stout *et al.*, 1998). Topical application of JH onto the prothoracic ganglion or JH injection directly into the ganglion reduced both the firing thresholds of the L1 interneurons to nearly normal levels and the loudness of a mock calling song required to elicit phonotaxis (Stout *et al.*, 1998). The effects of applied JH were rapid and seen within 2 h. JH applications to other ganglia were ineffective (Stout *et al.*, 1991). Moreover, a daily rhythm in the threshold levels for phonotaxis was detected in day 3 females (Stout *et al.*, 1998), suggesting that a daily rhythm in JH levels may be the underlying cause for the observed changes in thresholds.

Evidence of daily rhythms in JH titers in hemolymph is accumulating. A clear daily rhythm is seen in *Gryllus firmus* (Zhao and Zera, 2004) (**Figure 21**), and day–night differences in JH titers are seen in *Apis mellifera* (Elekonich *et al.*, 2001) and *H. virescens* (Shu *et al.*, 1998). In the case of *Gryllus*, a clear JH rhythm is seen in the flight-capable (long-winged) morph, but only a weak rhythm is seen in the flightless (short-winged) morph; this finding again indicates a relationship between JH rhythmicity and behavior. In the case of honeybees, the transition from behavioral arrhythmicity of nursing bees to the rhythmic behavior of foraging bees was tentatively linked to the expression of the clock gene *per* in the brain (Toma *et al.*, 2000) (see **Chapter 3.13**). *per* expression in foragers cycles in a circadian fashion and free-runs in DD. Cycling of *per* is evident in both nurses and foragers. However, the relative levels of *per* mRNA and the amplitude of cycling increased significantly with the transition from nursing to foraging, consistent with the

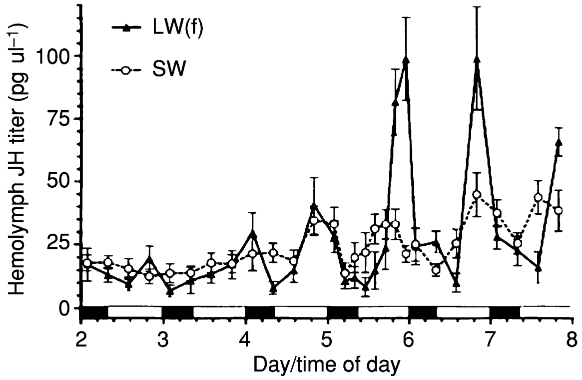


Figure 21 Daily rhythm in the hemolymph JH titer of the flight-capable (LW, long-winged) female morphs of *Gryllus firmus* (solid line). Note the daily peaks during day to night transitions. Flightless, short-winged (SW) females (dashed line) show only a weak daily rhythm in JH titer. (Reproduced with permission from Zhao, Z., Zera, A.J., **2004**. The hemolymph JH titer exhibits a large-amplitude, morph-dependent, diurnal cycle in the wing-polymorphic cricket, *Gryllus firmus*. *J. Insect Physiol. 50*, 93–102.)

pathways discussed above for control of JH by clock cells in the brain. In the honeybee *Apis cerana japonica*, *per* transcripts also oscillate daily in the brains of foragers; no comparisons were made with *per* transcrips in the brains of nurses (Shimizu *et al.*, 2001). These findings show that rhythmicity in JH titer does occur and that it may be driven by the brain.

A relationship between JH and behavioral activity rhythms is also seen in *Blattella germanica*. Males of this cockroach exhibit a bimodal circadian rhythm of locomotor activity that persists throughout their life cycle (Leppla *et al.*, 1989). Alternatively, females show a more complex pattern of locomotor activity that is clearly synchronized with the timing of each gonadotropic cycle. Immediately after emergence, the females exhibit a daily rhythm of locomotor activity. However, when they become sexually receptive during the first gonadotropic cycle, rhythmicity in locomotion is lost and the females become active throughout the day (Lee and Wu, 1994). Relative inactivity follows the expulsion of the first ootheca and then the females again become rhythmic for several days until the second gonadotropic cycle begins. Thus, rhythmic locomotion is confined to periods of expected high JH titer synchronized with gonadotropic cycles. Ovariectomy of sexually receptive females resulted in a stable circadian rhythm of locomotor activity, which was disrupted and reduced in amplitude by allatectomy (Lin and Lee, 1998). Therefore, factors associated with reproduction (perhaps ovarian factors) seem to mask the underlying circadian rhythm of locomotor activity. Topical application of JH increased the overall level of locomotor activity but it did not restore the behavioral circadian rhythm. However, JH applied topically enters the hemolymph continuously and over many days; this method of application does not provide a rhythmic supply of JH. Therefore, it is necessary to determine if JH synthesis is rhythmic in the cockroach in order to define its role in behavioral rhythms.

JH also appears to play a role in the regulation of rhythmic pheromone production in moths (see Section 3.11.5.3.1). In the females of the moth *A. ipsilon*, the pheromone level in the gland (Gemeno and Haynes, 2000), pheromone production by the gland (Picimbon *et al.*, 1997), and calling behavior (Gemeno and Haynes, 2000) all exhibit synchronous daily rhythmicities. Allatectomy of newly emerged females inhibited calling behavior; operated females neither contained nor released pheromone, even though their brain–SEG complexes contained high levels of activity of pheromone biosynthesis activating neuropeptide (PBAN) (Picimbon *et al.*, 1995). Apparently, the inhibitory effect of allatectomy on pheromone production was not solely due to disruptive effects on the gonadotropic cycle. Injections of PBAN or JH restored pheromone production but not rhythmicity in allatectomized females, suggesting that allatectomy prevented PBAN release. In contrast, JH III injections into decapitated females lacking a brain–SEG complex were ineffective in inducing pheromone production, suggesting that JH did not act directly upon the pheromone gland. Rather, JH appears to be necessary for the release of PBAN and it may do so by promoting release at “precisely gated times” (Picimbon *et al.*, 1995). Similarly, in *Helicoverpa armigera*, both pheromone production and PBAN levels are rhythmic (Rafaeli *et al.*, 1991). In this moth, JH treatment induced precocious pheromone production in newly emerged females (Fan *et al.*, 1999). However, if the animals were decapitated immediately after emergence, injections of neither JH nor PBAN were effective, whereas simultaneous injection with both compounds was stimulatory. Likewise, *in vitro* treatment with both JH and PBAN, but not either alone, induced pheromone production in inactive pheromone glands from pharate adults. These findings were interpreted as evidence that the role of JH is to prime the PBAN system. The possibility should be acknowledged that JH may play a permissive role in the above systems, i.e., its presence is required to allow these rhythmic phenomena to occur as opposed to actually controlling these rhythmicities. Elucidation of the role of JH in the regulation of daily pheromone release requires experiments on animals that

have commenced pheromone release and calling behavior.

Some general conclusions can be drawn from all the above. First, it appears that JH rhythmicity may be a widespread phenomenon among adult insects. Second, rhythmicity in JH could be responsible for the coordination of numerous circadian rhythms related to reproduction. Third, PTTH and ecdysteroids, which are known to be controlled by a circadian system in larvae, as well as the neuronal pathways that mediate their circadian control, all persist in the adult. Therefore, it is conceivable that circadian regulation of all three hormones, PTTH, ecdysteroids, and JH, participate in the regulation of rhythmic reproductive processes. Therefore, the putative central timing system in larvae that was discussed in the previous sections, may well continue functioning in the adult through circadian control of the same hormones. Unfortunately, evidence of a central circadian mechanism coordinating reproductive processes is currently fragmentary.

3.11.5.2. Circadian Regulation of Gamete Production

The existence of circadian rhythms in gametogenesis has received scant direct experimental attention. However, accumulating evidence of rhythmicity in the hormones that regulate gametogenesis, combined with substantial evidence that the output of mature gametes (at oviposition or mating) is also under circadian control, indicate that rhythmicity pervades the entire process of gamete production. Evidence of this view will be summarized prior to an examination of detailed rhythmic mechanisms in males and females below. There are three lines of evidence that are collectively compelling.

First, the evidence of circadian control of reproductive hormones is growing rapidly (see Section 3.11.5.1). The primary function of these hormones is regulation of gametogenesis. It would be surprising if rhythmicity in the secretion of regulatory hormones was not accompanied by rhythmicity in gametogenesis itself. The photoperiodic regulation of reproductive diapause (see **Chapter 3.12**) is a clear illustration that clock control of reproductive hormones is employed to regulate gametogenesis. It is firmly established that both JH and ecdysteroids exert a huge variety of actions on reproductive processes in both sexes (Hagedorn, 1985, 1989; Koeppe *et al.*, 1985; Wyatt and Davey, 1996; De Loof *et al.*, 2001) (see **Chapter 3.9**); therefore, there are numerous potential pathways by which rhythms in these hormones could evoke rhythmicity in egg or sperm production. For example, JH regulates vitellogenin production by the fat body and its uptake by the ovary, and in males it is involved in the regulation of accessory gland functions and affects reproductive behavior and spermatogenesis. Ecdysteroids are produced by both testes and ovaries (Gillott, 1995) and are regarded as possible insect sex steroid hormones (De Loof *et al.*, 2001). In females, ecdysteroids promote cell growth in egg follicles and in Diptera they regulate vitellogenin synthesis by both fat body and ovary. In males, ecdysteroids promote cell divisions in spermatocytes. Rhythmicity in any of these hormonal control pathways would likely lead to rhythmicity in gamete production.

Second, the discharge of mature eggs or sperm from the animal is frequently under circadian control. For example, the oviposition of mature eggs is often regulated by a circadian clock as is the mating activity of males (see Section 3.11.5.4.3). These behavioral rhythms require the presence of mature gametes at appropriate times of the day. The necessary coordination between these behavioral rhythms and gametogenesis could be achieved most directly by circadian control of gametogenesis.

Third, cyclic expression of the clock gene *per* has been reported in both ovaries and testes. In both ovarian follicle cells (Hardin, 1994; Hall, 1998) (see Section 3.11.5.2.1) and vas deferens epithelial cells (Gvakharia *et al.*, 2000) (see Section 3.11.5.2.2), *per* is expressed in a cycling fashion in LD but fails to free-run in DD or LL. These experiments are described in detail later. In other words, *per* cycles but not with the circadian properties that are required of a true clock. Such behavior of *per* is characteristic of oscillators that require a driving input (see Section 3.11.3.3 and **Chapter 4.11**). This requirement for a driving input to sustain *per* cycling in the reproductive system is further evidence of daily, rhythmic inputs to the system, most probably from rhythms in reproductive hormones. Hormones are well known as agents that entrain oscillators in vertebrates (review: Balsalobre, 2002) (see Section 3.11.3.3), and also in insect development (Steel and Vafopoulou, 1999) (see Section 3.11.4.1.4).

The above considerations lead to a testable model for the circadian regulation of gametogenesis. We suggest that a central timing system (in the brain?) controls the rhythmic release of neuropeptides that regulate rhythms in JH and/or ecdysteroids. JH and/or ecdysteroids provide the driving input to a *per*-based oscillator in the reproductive system, leading to rhythmic formation of gametes. Such a rhythm of gamete formation would be synchronized with rhythmic transport of eggs or sperm through the ducts of the reproductive system. These rhythms serve to make mature eggs and sperm available rhythmically for oviposition and mating behaviors,

respectively. These behaviors are themselves frequently rhythmic and under circadian control. This synchrony with behaviors implies action of JH and/or ecdysteroids on the nervous system, where they could act as modulators of the neural pattern generators for reproductive behaviors. Synchrony with behavioral rhythms could also be achieved centrally, by coupling of the putative neuroendocrine clock with the locomotor clock in the brain. Circadian rhythmicity potentially pervades reproductive mechanisms at all levels and in both sexes. Some of the known mechanisms are described below.

3.11.5.2.1. Oogenesis and egg transport Early studies with *Drosophila* by Allemand (1976a, 1976b) raised the possibility that oogenesis was rhythmic. Daily variations were reported in the frequency of egg chambers in various stages of development. These observations were interpreted as a daily rhythm in vitellogenesis, specifically in the time of onset of vitellogenic stages 8–10. The rhythm free-ran in DD for at least 5 days, implying that vitellogenesis (or at least some aspect of it), was under circadian control. Supporting evidence of such an "ovarian clock" derives from the localization of *per* transcripts and PER protein in the follicle cells surrounding small previtellogenic oocytes of *Drosophila*. However, this ovarian *per* transcript did not cycle (Hardin, 1994) and the PER protein was exclusively cytoplasmic, showing no cyclic migration to the nucleus (Liu *et al*., 1988; Saez and Young, 1988). This unclocklike behavior of *per* argues against the presence of a true clock involving *per* in the follicle cells (Hall, 1998). However, the notion of an oscillator residing within the follicle cells merits further exploration partly because it is these cells that synthesize ecdysteroids in many adult insects (Hagedorn, 1985, 1989). In addition, follicle cells are important targets of both JH (Wyatt and Davey, 1996) and ecdysteroids (Carney and Bender, 2000). Possibly, follicle cell oscillators are entrained by (rhythmic) hormonal signals and regulate rhythmicity in vitellogenesis by the oocyte that they surround. In other words, a follicle cell oscillator could be part of a pathway by which rhythmic endocrine signals lead to a rhythm in vitellogenesis.

Rhythmic mating can also lead to rhythmic egg development, again through endocrine pathways. As in many insects (see Section 3.11.5.4.2), mating in *Drosophila* is rhythmic and under circadian control (Sakai and Ishida, 2001). Mating accelerates egg maturation in *Drosophila*. After mating, there is an acceleration of yolk protein accumulation in the developing eggs, so that a large number of mature eggs is available for insemination. The sex peptide that is transferred to females by males at mating (see **Chapter 1.5**) is responsible for enhanced transcription of yolk proteins in the ovary, for enhanced yolk protein uptake by the ovary (Soller *et al*., 1997), and also for ovulation (Ottiger *et al*., 2000). These effects of sex peptide can be simulated by methoprene application (Soller *et al*., 1999). Sex peptide has also been shown to stimulate JH biosynthesis (Moshitzky *et al*., 1996). Mating also affects ecdysteroid levels, which affect vitellogenin synthesis by both ovary and fat body (Harshman *et al*., 1999; Soller *et al*., 1999). Therefore, rhythmic mating activity could readily lead to rhythmic changes in both JH and ecdysteroid levels and consequential rhythmic stimulation of vitellogenesis.

In many species, oviposition occurs with a circadian rhythm (see Section 3.11.5.4.3). The time interval between ovulation and oviposition is often both constant and quite brief, implying that rhythmic oviposition may be synchronized with, or may even be a consequence of, rhythmic ovulation. The rhythmic removal of eggs from the reproductive system requires that mature eggs are available for oviposition at circadian intervals. The simplest and most efficient mechanism by which this could be achieved would be by circadian regulation of egg maturation.

The control of ovulation and oviposition involves a multiplicity of hormones acting in species-variable patterns (Nijhout, 1994). The factors that regulate contractions of the oviducts have been extensively studied in *L. migratoria* (Donini *et al*., 2001). In this insect, transverse nerves, the caudal sympathetic system, and local neurons innervate the oviducts. Numerous hormones and neurotrasmitters such as CCAP, FMRF-related peptides, proctolin, glutamate, and octopamine are all involved in regulation of the oviduct contractions. The orchestration of these various factors into the observed circadian rhythm of oviposition has not yet been examined in any insect.

3.11.5.2.2. Spermatogenesis and sperm transport In the male reproductive system, circadian involvement has been demonstrated clearly in the duct system that transports sperm, but not in spermatogenesis itself. The absence of data does not imply that rhythmicity is absent; it could readily result from rhythmicity in the endocrine factors that regulate spermatogenisis. Both JHs and ecdysteroids regulate spermatogenesis in a complex and apparently species-variable manner (Dumser, 1980; Hagedorn, 1985; Koeppe *et al*., 1985; Hardie, 1995; Wyatt and Davey, 1996). For example, it has been shown that both hormones act directly on the testis where they regulate rates of cell division at specific stages

of spermatogenesis. In *B. mori*, JH affects the rate of division in spermatocytes (Kajiura *et al*., 1993). Ecdysteroids stimulate mitotic divisions in spermatogonia of *Rhodnius* (Dumser, 1980) and meiotic divisions in both *Manduca* (Friedländer and Reynolds, 1988) and the European corn-borer, *Ostrinia nubilalis* (Gelman *et al*., 1988). Thus, rhythms in ecdysteroids and/or JH (see Section 3.11.5.1) could result in rhythms of cell division in the testes. Also, other rhythmic inputs, such as mating, could impose rhythmicity on these endocrine control pathways and result in rhythmicity in spermatogenesis.

In several Lepidoptera, the movement of the sperm through the reproductive system is under circadian control. In stark contrast to the female system, it is alleged that neither nerves nor hormones are involved in the regulation of this movement (Giebultowicz, 2000). In Lepidoptera, sperm that are released from the testis are transported along the vas deferens by muscular contractions as aggregates known as bundles. These bundles may remain intact until after mating. In whole animals or isolated abdomens of *P. gossypiella* and *Ephestia* (*Anagasta*) *kuehniella* and *Spodoptera litura*, the sperm bundles move along the ducts in a series of distinct steps that is repeated as a daily rhythm (Riemann and Thorson, 1971; Riemann *et al*., 1974; LaChance *et al*., 1977; Thorson and Riemann, 1977; Seth *et al*., 2002). This rhythmic movement of sperm bundles was also found in *Spodoptera littoralis* and *Lymantria dispar*, using *in vitro* incubations of organ complexes which contained the testis and upper vas deferens (UVD) and in some instances the seminal vesicle (SV) as well (Giebultowicz *et al*., 1988; Bebas *et al*., 2001). This rhythm of bundle transport free-ran in DD and was phase-shifted by light cues *in vitro* showing that a clock that controls the rhythm of transport resides within the reproductive system. A rhythm of sperm transport is also seen in *E. kuehniella* and *Cydia pomonella in vivo*, but fails to persist *in vitro* (Bebas *et al*., 2001); in these species it appears that rhythmicity in sperm transport is driven by factors outside the reproductive system.

Thus, in some moths, the reproductive system contains a photosensitive circadian clock that regulates rhythmic transport of sperm. The literature refers to this as a rhythm in "sperm release," in which the liberation of sperm from the testis and their subsequent movement by the ducts are considered as two component steps of a single rhythmic event. If correct, this view requires that liberation of sperm from the testis is also rhythmic, a phenomenon that suggests rhythmicity in spermatogenesis itself. However, studies of the "sperm release rhythm" generally involve only studies of transport and not of release itself. Consequently, the contribution of rhythmic sperm release from the testis to the observed rhythmic movement of sperm along the ducts is unknown. However, these two processes are influenced differentially by ecdysteroids (see below), suggesting that they should be regarded as distinct events.

As mentioned above, translocation of sperm from the testis along the ducts results from muscular contractions of the wall of the UVD. The UVD of *Ephestia* consists of layers of longitudinal and circular muscle and a single cell layer of inner epithelium (Riemann and Thorson, 1976). The nerve supply to the vas deferens is abundant and evident in the lower part, and sparse (but not absent) in the UVD (Riemann and Thorson, 1976; Thorson and Riemann, 1977). In *Lymantria*, structures resembling axons were seen between the circular muscle and the basement membrane of the UVD epithelium (Giebultowicz *et al*., 1996). Evidence of a local nerve supply is derived from analysis of the complex patterns of muscular contractions exhibited *in vitro* by the testis–UVD–SV complex (Giebultowicz *et al*., 1996). Several distinct patterns of contractions occur in the UVD, each of which requires coordination of contraction of the circular and longitudinal muscle layers. Further, abrupt switches between these patterns also occur. One of these contraction patterns was responsible for the mass transfer of sperm bundles down the UVD to the SV (Giebultowicz *et al*., 1996). The onset of this pattern could occur *in vitro*, showing that it was not driven directly from the CNS. This pattern commenced at the normal time of the day for sperm transfer *in vivo*, implying that the clock may control the daily time of switching between motor patterns in the UVD. The overall complexity of the contraction patterns as well as the abrupt switching between them both strongly imply the presence of local neural regulation. By contrast, the literature states that the musculature of the UVD appears to receive no innervation and that the changes in contractile pattern occur autonomously (Giebultowicz *et al*., 1996). The functional requirements for the transport of sperm bundles along the male ducts are basically the same as those for transport of eggs along the female ducts. The importance of the local nerve supply employing a multitude of neurotransmitters has been well documented for control of the movement of eggs along oviducts (Orchard and Lange, 1988; Donini *et al*., 2001) (see Section 3.11.5.2.1). Therefore, we suggest that transport of sperm bundles is regulated by a

currently unidentified local nerve supply. Viewed in this light, the mechanisms of gamete transport in the male system become more comparable to those of the female system than the current literature suggests.

The location of the clock that controls the rhythmicity of sperm transport is unclear. In *C. pomonella* both *per* transcripts and PER were localized to epithelial cells lining the UVD wall. Both transcript and protein cycled in LD, but the *per* transcript showed "statistically insignificant" fluctuations in DD and the rhythm was "disrupted" in LL (Gvakharia *et al.*, 2000). It should be recalled that this behavior of *per* is very similar to that seen in the ovaries, where it was dismissed as unclocklike (Hall, 1998) (see Section 3.11.5.2.1). Such *per* oscillations that are not self-sustaining are characteristic of peripheral oscillators, that are driven by rhythmic inputs such as hormones (see Section 3.11.3.3), as has been argued for the ovarian *per* oscillator (see Section 3.11.5.2.1). The epithelial UVD oscillator seems unlikely to represent the clock that controls sperm transport since (1) the oscillator is not self-sustaining (i.e., does not free-run), whereas the rhythm in sperm transport does free-run, and (2) there is no known pathway or mechanism by which epithelial cells could regulate patterns of contraction by the overlying layers of muscles. We conclude that the epithelial oscillator likely regulates activities of the epithelial cells themselves but is not the clock that regulates rhythmic sperm movement. Indeed, these epithelial cells exhibit rhythmic secretion into the duct lumen (Riemann and Giebultowicz, 1991; Giebultowicz *et al.*, 1994) and regulate the rhythmic acidification of the lumen (Bebas *et al.*, 2002), which contributes to the maturation of sperm in the UVD (Riemann and Thorson, 1976).

The organ system used above *in vitro* to study rhythmicity in sperm movement consisted of most of the reproductive system including the testis, which is the largest endocrine organ in adult males. The testis is the only confirmed site of ecdysteroid synthesis in adult male moths (Loeb *et al.*, 1984, 1988). Sperm movement from the testis into the vas deferens was temporarily inhibited by the injection of 20E into adults of *Anagasta* (Thorson and Riemann, 1982) or infusion of 20E into pharate adults of *Lymantria* (Giebultowicz *et al.*, 1990), both in a dose-dependent manner; higher doses of 20E produced greater inhibition. The effectiveness of injections of 20E into pharate adults of *Lymantria* decreased as development proceeded, suggesting that a decline in the ecdysteroid titer was necessary for the initiation of sperm transport. However, Thorson and Reimann (1982) had shown earlier that 20E injection into isolated abdomens of adults of *Anagasta* also inhibited sperm transport, even though the normal decline in ecdysteroids titer was complete in these animals. These authors reported that the inhibition of sperm release by injected 20E did not appear to be due to inhibition of spermatogenesis because there was an accumulation of unreleased eupyrene sperm cysts at the junction of the testis and UVD in animals injected with 20E. Interestingly, the release of apyrene sperm was unaffected. Further, the rhythm of transport of sperm down the UVD was also unaffected by 20E (**Figure 22**). These differential actions of 20E on sperm release and sperm transport suggest that these two processes may represent separate distinct rhythms. It is possible that at least some of the reported events occuring in the testis–UVD–SV complex *in vitro* could be affected, or even caused, by ecdysteroids released from the testis *in vitro*. It seems important to examine the moth testis for rhythmicity in ecdysteroid synthesis, since this could drive rhythmicity in various events associated with sperm release and transport. This organ system is also potentially the source of a plethora of neurotransmitters, neuromodulators, and hormones from unidentified tissue adhering to the reproductive system, as is the case with the female system. It is therefore likely that a local nerve supply, various neuropeptides, neuromodulators,

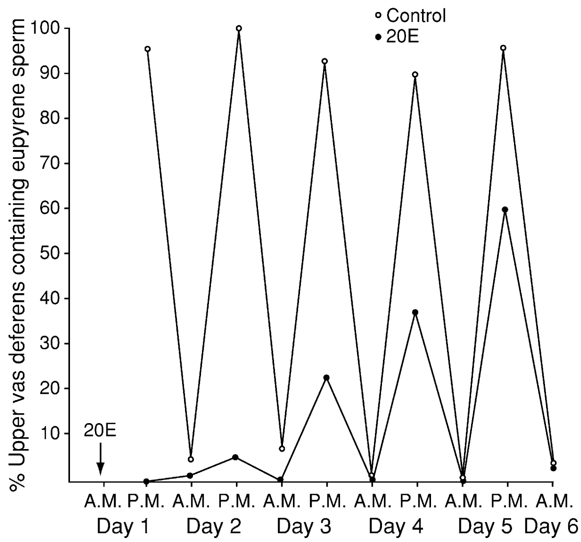


Figure 22 Injection of 20E into isolated abdomens of *Anagasta kuehniella* temporarily reduced the number of eupyrene sperm released into the upper vas deferens, but did not appear to affect the daily rhythm of sperm release. (Reprinted with permission from Thorson, B.J., Riemann, J.G., **1982**. Effects of 20-hydroxyecdysone on sperm release from the testes of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller). *J. Insect Physiol. 28*, 1013–1019; © Elsevier.)

and ecdysteroids all interact to regulate the timing of sperm movement.

The time during adult development at which the clock that controls rhythmic sperm transport becomes operational was examined in *Lymantria* (Giebultowicz and Joy, 1992). At various times during adult development, pupae were transferred from LD to DD, or given a light pulse in DD. Pupal day 6 was found to be the earliest day on which a light cue could initiate rhythmicity in sperm release, i.e., 2 days before sperm transport normally commences. However, this is also the time of peak ecdysteroids in the hemolymph (Giebultowicz *et al.*, 1990) as well as the testis (Loeb *et al.*, 1984, 1988), suggesting that the clock becomes functional in the presence of ecdysteroids. In an attempt to determine if 20E interacted with the clock in the reproductive system 10–20 μg 20E were injected into animals that were maintained in LD (Giebultowicz *et al.*, 1996). Appearance of sperm in the UVD was abolished for 1 day but then resumed, as previously found in isolated abdomens of adult *Anagasta* by Thorson and Reimann (1982). It was inferred from this experiment that 20E does not affect the phase of sperm release. However, it is unlikely that any phase shift could be produced by 20E in this experiment due to the presence of simultaneous but conflicting light entrainment of the rhythm.

Some general conclusions can be drawn from the above studies with the male system. Sperm bundles are transported through the male duct with a circadian rhythm under the control of a photosensitive clock that probably acts on the muscles via a local nerve supply. Sperm release from the testis may also be rhythmic and possibly regulated by ecdysteroids, even *in vitro*. The *per*-based oscillator in the UVD epithelium regulates rhythmic secretion by these cells and seems to be a driven oscillator, possibly entrained by ecdysteroids. This system offers valuable opportunities for the study of interactions between nerves and hormones in the regulation of peripheral oscillators. The shortage of information regarding nervous and hormonal regulation is not an evidence that the clock is autonomous.

3.11.5.3. Rhythmic Neuroendocrine Control of the Sex Pheromone Signaling System

Communication between the sexes prior to mating may be performed by visual, auditory, or chemical (pheromonal) signals. The use of sex pheromones is widespread among insects (see **Chapter 3.14**). Sex pheromones are a heterogeneous collection of small, volatile chemical compounds, which may be released by either sex (usually by the female) depending on the species, to advertise sexual receptivity and availability. Sex pheromones vary greatly among species and there are extensive studies of their chemical composition, pathways and sites of biosynthesis in various insect groups (see **Chapters 3.14** and **3.15**). A common site of pheromone synthesis is the pheromone gland, but there are notable exceptions (e.g., in Diptera). Release of sex pheromones is achieved when the advertiser displays a characteristic pattern of sexual behavior called "calling," which involves a repertoire of body movements that expose the gland to the air and facilitate dispersal of pheromone in the environment.

In almost all insect groups with a long adult life, including cockroaches, beetles, Diptera, and moths, both the behavior of calling and the accompanied release of sex pheromones occur with a daily rhythm. The females of the brown-banded cockroach, *Supella longipalpa*, for example, engage in nightly rhythmic calling behavior during which they release volatile sex pheromone; the calling rhythm is truly circadian since it free-runs in both DD and LL and is phase-set by light (Smith and Schal, 1991). Release of pheromone with a daily periodicity has also been shown in several female beetles including the scarab beetle *Anomala albopilosa* (Leal *et al.*, 1996) and the bruchid beetle *Callosobruchus maculatus* (Shu *et al.*, 1996). In some flies, the males release sex pheromones and the females respond to them. Sex pheromones in Diptera are synthesized by abdominal oenocytes and then are deposited onto the surface of the cuticle following transportation via the hemolymph to epidermal cells (Tillman *et al.*, 1999). In males of the Caribbean fruit fly, *Anastrepha suspensa*, a daily rhythm in the amount of sex pheromone circulating in the hemolymph was observed, which suggested possible daily changes in synthesis (Teal *et al.*, 1999b). These daily variations in the level of sex pheromone in the hemolymph correspond closely with the daily rhythms of calling and pheromone release (Nation, 1990; Epsky and Heath, 1993). The response of females to the release of sex pheromones by males is also rhythmic in other Diptera, such as *Rhagoletis ceraci* (Katsoyannos, 1982), *Culex quinquefasciatus* (Jones and Gubbins, 1979), and *Musca domestica* (Sybchev *et al.*, 1986) and this is usually taken to signify the occurrence of rhythmic pheromone release by the males.

Moths are the most widely studied group for rhythmicities in both calling behavior and pheromone production/release. In moths, the pheromone glands are usually located beneath the intersegmental membrane between the 8th and 9th posterior abdominal segments and they often form extrusible sacs (Bjostad *et al.*, 1987). These sacs do not usually have reservoirs for pheromone storage, so that release follows synthesis quickly. Release usually

occurs as a result of calling behavior, which facilitates the exposure of the pheromone gland. However, in some Lepidoptera such as *Bombyx*, reversal of the heartbeat causes changes in the hemolymph pressure, which results in eversion of the gland and pheromone dispersal (Ichikawa and Ito, 1999). In moths calling occurs rhythmically every day until mating. Mating then inhibits calling behavior either temporarily or permanently. Numerous studies have documented daily rhythms in calling and regulation of the rhythm by an endogenous circadian clock. In *Lymantria*, calling occurs with a daily rhythm which is abolished in LL but reinstated by transfer back to DD (Webster and Yin, 1997). In the tiger moth, *Holomelina lamae*, the calling rhythm free-runs in both DD and LL, but damps out quickly in LL. Reintroduction of LD reentrains the rhythm (Schal and Cardé, 1986) (**Figure 23**). Rhythmicity in calling was abolished when these moths were reared in DD from the second instar onwards. When these DD moths were grouped according to the time of eclosion, the authors were able to detect a clear rhythm. This suggests that the clock that controls the calling rhythm may be phase-set at ecdysis. Temperature compensation of the calling rhythm was seen in *Congether punctiferalis* (Kaneko, 1986). Circadian rhythms in calling behavior were also seen in *Heliothis armigera* (Kou and Chow, 1987), *Pseudaletia unipuncta* (Delisle and McNeil, 1987), *Helicoverpa assulta* (Kamimura and Tatsuki, 1993, 1994), and *Manduca* (Itagaki and Conner, 1988). It therefore appears that in moths rhythmicity in the behavior of calling is a widespread, if not universal, phenomenon which is governed by an endogenous circadian clock mechanism.

The striking periodicity of calling behavior in moths implies that pheromone production/release is also rhythmic. Indeed, many studies have shown

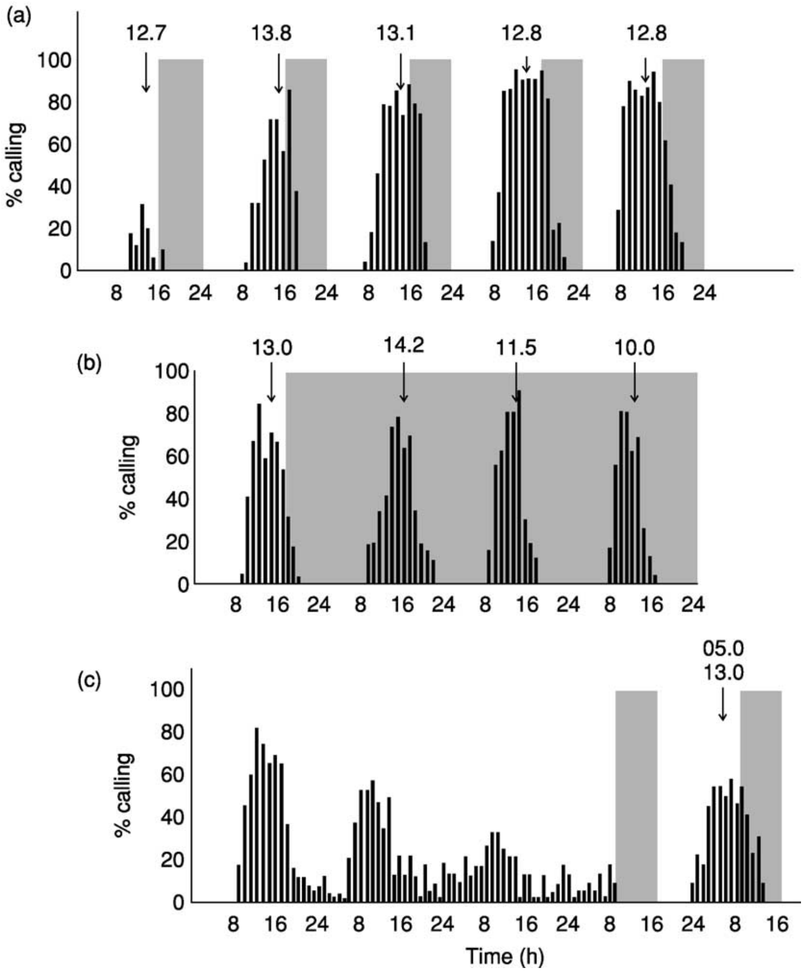


Figure 23 Entrained rhythm of calling behavior of females of the tiger moth, *Holomelina lamae* (Freeman) (a). The rhythm free-runs in DD (b) and damps out after a few cycles in LL (c). Reintroduction of the light cycle reestablishes the rhythm (right end of c). (Reproduced with permission from Schal, C., Cardé, R.T., **1986**. Effects of temperature and light on calling in the tiger moth *Holomelina lamae* (Freeman) (Lepidoptera: Arctiidae). *Physiol. Entomol. 11*, 75–87; © Blackwell Publishing Ltd.)

clear, circadian rhythmicities in pheromone content of the gland and/or pheromone release. Frequently, a close temporal relationship is found between daily changes in content of the gland, release of pheromone, and calling. For example, in the diurnal moth *L. dispar*, the pheromone content of the gland (Tang *et al*., 1992), the rate of pheromone emission from the gland (Charlton and Cardé, 1982), and the calling behavior (Webster and Yin, 1997) all exhibit synchronous daily rhythms with maxima in photophase and minima in scotophase. In the nocturnal moth *H. virescens*, the daily rhythmic changes in content of the gland (Rafaeli and Soroker, 1989; Mbata and Ramaswamy, 1990) and emission from the gland (Pope *et al*., 1982) are also synchronous, but occur with peaks in the scotophase and troughs in the photophase. In *H. assulta*, the quantities of all five individual components of the moth's pheromone cocktail in the gland fluctuated rhythmically during the day (Choi *et al*., 1998a) (**Figure 24a**) in synchrony with the calling rhythm (Kamimura and Tatsuki, 1993). Circadian control of rhythmicity was also shown in *Pseudaletia* (Delisle and McNeil, 1987), *Adoxophyes* spp. (Kou, 1992), *Agrotis segetum* (Löfstedt *et al*., 1982), *Bombyx* (Kuwahara *et al*., 1983; Sasaki *et al*., 1984), *Choristoneura fumiferana* (Ramaswamy and Cardé, 1984), *Mamestra brassicae* (Noldus and Potting, 1990; Iglesias *et al*., 1999), *Phthorimaea poerculella* (Ono *et al*., 1990), *Platynota sultana* (Webster and Cardé, 1982), *Sesamia nonagrioides* (Babilis and Mazomenos, 1992), and *S. littoralis* (Dunkelblum *et al*., 1987). In *Epiphyas postvittana* (Foster, 2000), rhythmicity in the pheromone content of a gland with no reservoir appears to result solely from periodic changes in pheromone synthesis, which is tightly coupled to release; there is no evidence of rhythmic degradation.

Thus, rhythmicity in the release of sex pheromone seems to be regulated directly at the level of synthesis. This fact implies that the factors that regulate synthesis may also be rhythmic (see Sections 3.11.5.3.1 and 3.11.5.3.2). However, there are some notable exceptions; in *Trichoplusia ni* release of pheromone occurs rhythmically (Sower *et al*., 1970), but pheromone production and release are not synchronous (Hunt and Haynes, 1990). Rather, synthesis of pheromone appears to be continuous and the daily rhythmicity in content of the gland results from rhythmic release caused by rhythmicity in abdominal movements during calling. Thus, circadian regulation of pheromone release in *Trichoplusia* may be driven by a clock governing behavior rather than a clock that regulates pheromone synthesis.

The synchrony of the pheromone release rhythm with the behavioral rhythm and their common responses to light manipulations suggest that they share common components in the underlying circadian clock mechanism. In other words, the pheromone clock and the behavioral clock may be closely linked. The clock for the calling rhythm and possibly that for the pheromone rhythm appear to be entrained by a brain photoreception center in *Andevidia peponis* (Sasaki *et al*., 1987). Surgical removal of both compound eyes and ocelli did not interfere with the ability of the calling rhythm to respond to phase shifts of the LD cycle. Localized illumination of various regions in the brain with 100 μm fiber optic light guides revealed that such phase shifts were induced by illumination of the mediodorsal protocerebrum near the medial neurosecretory cells. Illumination of other brain areas, including the optic lobes, was ineffective.

3.11.5.3.1. Rhythmicity in hormonal regulation of pheromone production The head is required for both pheromone production and calling behavior. Decapitation of females of the nocturnal moth *S. littoralis* either at the onset of a scotophase prior to the nocturnal peak of pheromone production or at the preceding photophase abolished both the production of pheromone and calling behavior (Martinez and Camps, 1988). Injection of an extract from the brain–SEG–retrocerebral complex restored both pheromone production and calling behavior equally well when administered either in the scotophase or photophase, but did not restore rhythmicity to either process. These findings imply that the expression of the pheromone release rhythm by the gland is dependent on the continuous presence of the head. Apparently the pheromone rhythm depends on circadian input from the head. The obvious implication was that the head contained a factor(s) that was essential for driving the oscillations of the overt pheromone rhythm.

A head factor with pheromonotropic activity (PBAN) was eventually identified in many moths (Raina and Klun, 1984; Martinez and Camps, 1988; Rafaeli and Soroker, 1989; Raina *et al*., 1989; Ma and Roelofs, 1995; Zhu *et al*., 1995; Zhao *et al*., 2002) and was subsequently isolated and characterized as a small, 33–34 amino acid neuropeptide with an amidated C-terminus (see also **Chapters 3.14** and **3.15**). PBAN has been sequenced in *Bombyx* (Kitamura *et al*., 1989), *Helicoverpa zea* (Raina *et al*., 1989), and *Lymantria* (Masler *et al*., 1994) and deduced from cDNA sequences in *A. ipsilon* (Duportets *et al*., 1998), *H. assulta* (Choi *et al*., 1998b), *Mamestra* (Jacquin-Joly *et al*., 1998),

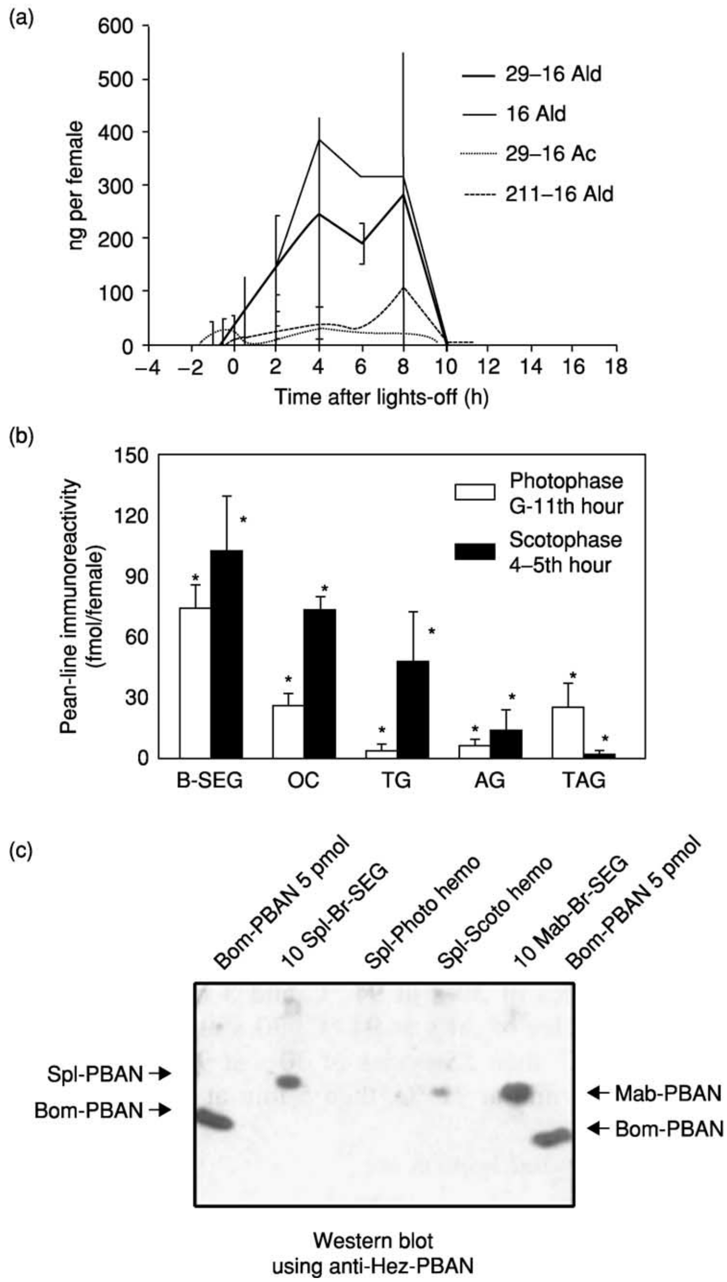


Figure 24 Daily rhythm of the five pheromone components in the pheromone gland of day 1 females of the tobacco budworm, *Helicoverpa assulta* in a 15 h light : 9 h dark cycle. The dark bar on the abscissa indicates scotophase (a). (Reprinted with permission from Choi, M.Y., Tatsuki, S., Boo, K.S., **1998a**. Regulation of sex pheromone biosynthesis in the Oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol. 44*, 653–658; © Elsevier.) (b) Day and night differences in the level of PBAN-like immunoreactivity in several neural tissues of females of *Helicoverpa armigera*. B-SEG indicates brain–subesophageal ganglia complex; CC, corpus cardiacum; TG, prothoracic ganglia; AG, abdominal ganglia, excluding the terminal abdominal ganglion (TAG). (Rafaeli, A., Hirsch, J., Soroker, V., Kamensky, B., Raina, A.K., **1991**. Spatial and temporal distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa* (*Heliothis*) *armigera* using RIA and *in vitro* bioassay. *Arch. Insect Biochem. Physiol. 18*, 119–129; © Wiley. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.) (c) Western blot showing the presence of a single immunoreacitve peptide to antibody against PBAN of *Helicoverpa zea* in the hemolymph of scotophase (Spl-Scoto hemo) but not photophase (Spl-Photo hemo) females of *Spodoptera littoralis*. The peptide is also present in brain–subesophageal ganglia complexes (Spl-Br-SEG) of *Spodoptera*. Controls, synthetic PBAN-I from *Bombyx mori* (Bom-PBAN) and Br-SEG extracts from *Mamestra brassicae* (Mab-Br-SOG). (Reprinted with permission from Iglesias, F., Marco, P., François, M.-C., Camps, F., Fabriàs, F., *et al.*, **2002**. A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualization in scotophase hemolymph. *Insect Biochem. Mol. Biol. 32*, 901–908; © Elsevier.)

and *S. littoralis* (Iglesias *et al.*, 2002). All PBANs share a high degree of sequence homology, the C-terminal pentapeptide being the most conserved. In *Bombyx*, PBAN is synthesized as a preprohormone that is trimmed to several peptides posttranslationally; one of these peptides bears striking similarity to the *Bombyx* embryonic diapause hormone (Sato *et al.*, 1993), possibly suggesting common regulation for both diapause peptide and PBAN.

In two species of *Helicoverpa* and in *Ostrinia*, immunohistochemistry and assays for both biological activity and expression of the PBAN gene have identified small groups of nerve cells in the SEG as the primary (but not necessarily the only) (see Section 3.11.5.3.2) source of PBAN (Ma and Roelofs, 1995; Choi *et al.*, 1998b; Ma *et al.*, 1998, 2000). Mapping of the axonal pathways from the PBAN-immunopositive cells in SEG in *Bombyx* revealed connections to the corpus cardiacum (CC), which suggested that the material produced in these PBAN cells in SEG may be transported to the CC for release (Ichikawa *et al.*, 1995). PBAN activity in the CC was detected in several moths. In *Ostrinia*, removal of the retrocerebral complex (CC and CA) significantly reduced the production of pheromone by the gland (Ma and Roelofs, 1995). The CC also immunostained intensely for PBAN and CC extracts exhibited strong pheromonotropic activity in both *Helicoverpa* spp. (Rafaeli *et al.*, 1991) and *Ostrinia* (Ma and Roelofs, 1995). These findings implied that the CC is probably the site of release of PBAN into the hemolymph.

Clear daily fluctuations in the PBAN content of the CC were found using a radioimmunoassay (RIA) for PBAN and assays for biological activity. In *Helicoverpa armigera*, the level of PBAN in the CC was significantly higher in scotophase than in photophase suggesting that transport to CC and/or release of PBAN from the CC were rhythmic (Rafaeli *et al.*, 1991; Rafaeli, 1994) (**Figure 24b**). In contrast, the level of PBAN in brain–SEG extracts did not change during the course of a day, suggesting that PBAN synthesis may be continuous (Rafaeli *et al.*, 1991; Rafaeli, 1994). Similar observations were also made in *A. segetum* in which PBAN biological activity in the brain–SEG did not show daily changes (Rosén, 2002). The view that PBAN release from CC is rhythmic was strengthened by extracellular recordings of the action potentials in the NCC-V nerve of *Bombyx;* this nerve contains the axonal projections from the PBAN-producing cells in SEG to CC (Ichikawa, 1998). A clear daily rhythm of bursting firing activity was found. This pattern of firing is associated with the release of neuropeptide in many other systems. The rhythm of bursting firing was synchronous with both the daily rhythm of calling behavior and with the pheromone content of the gland. The bursting activity rhythm free-ran in both DD (**Figure 25a**) and LL (**Figure 25b**), revealing endogenous circadian control (Tawata and Ichikawa, 2001). Collectively, these findings indicate that release of PBAN into the hemolymph occurs with a daily rhythm that is under circadian control.

Daily cycling in PBAN-immunoreactivity and/or biological activity in the hemolymph has also been detected in several moths. In the hemolymph of calling females of *Heliothis zea*, a peptide fraction with PBAN activity was detected in scotophase

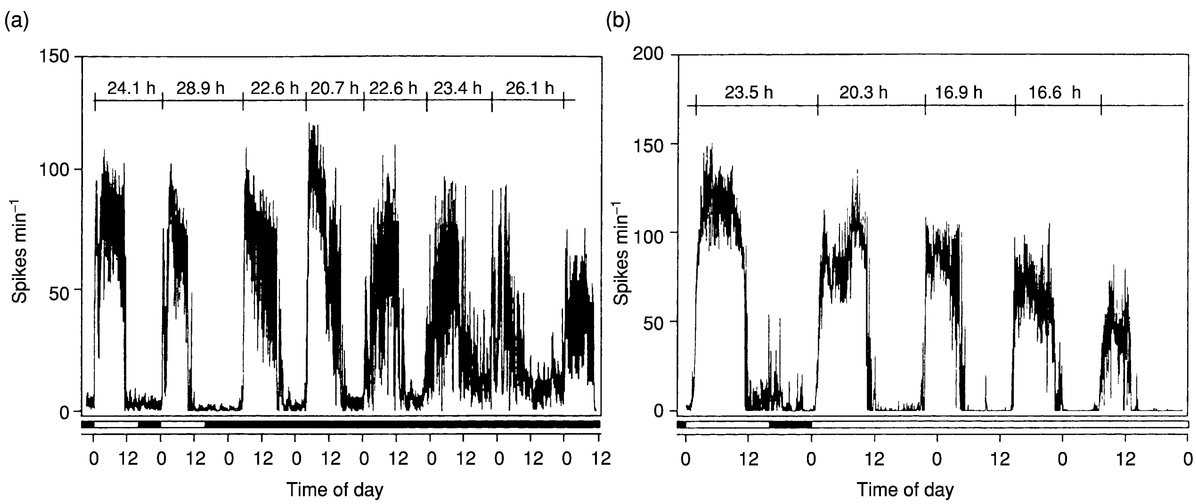


Figure 25 Entrained rhythm of the firing activity of the PBAN-producing cells, recorded from the nervus corporis cardiacum (NCC-V) of virgin females of the silkmoth, *Bombyx mori* (a). The rhythm free-runs in both DD (dark bar in a) and LL (light bar in b). (Reproduced with permission from Tawata, M., Ichikawa, T., **2001**. Circadian firing of neurosecretory cells releasing pheromonotropic neuropeptides in the silkmoth, *Bombyx mori*. *Zool. Sci. 18*, 645–649; © The Zoological Society of Japan.)

(Ramaswamy *et al.*, 1995) but not in photophase. Daily cycling of PBAN-like immunoreactivity was also detected in the hemolymph of females of *Mamestra*. This rhythm was also synchronous with the daily rhythms of pheromone production in the pheromone gland and calling behavior (Iglesias *et al.*, 1999). Similarly, in *S. littoralis*, Western blot analysis of hemolymph peptides of virgin females revealed the presence of PBAN-like compounds during the scotophase but not during the photophase (Iglesias *et al.*, 2002) (**Figure 24c**). In another moth, *A. segetum*, a bioassay for PBAN revealed that the hemolymph of females contained high PBAN activity in the scotophase but low in the photophase (Rosén, 2002). All these findings taken together support the view that at least in some moths, PBAN is released in the hemolymph where it exhibits a strong daily cycling. In the hemolymph, PBAN appears to function as a classical hormone, its principal target being the pheromone gland. Indeed, isolated pheromone glands were successfully stimulated by PBAN *in vitro* (Rafaeli and Soroker, 1989; Jurenka *et al.*, 1991; Matsumoto *et al.*, 1995; Zhao *et al.*, 2002). *In vitro* assays showed that PBAN acts on pheromone glands via a Ca^{2+}–calmodulin-dependent cyclase (review: Ramaswamy *et al.*, 1994), implying the presence of PBAN receptors on the surface of the pheromone gland cells. A putative cell membrane receptor for PBAN was identified recently in pheromone glands of *H. armigera* (Rafaeli and Gileadi, 1997; Altstein *et al.*, 1999, 2001). These findings imply that rhythmicity in the level of PBAN in the hemolymph drives rhythmicity in the production of pheromone by the pheromone gland.

However, there is a wide distribution of PBAN in the nervous system, and in some species the pheromone gland is directly innervated by the terminal abdominal ganglion (TAG). For example, the pheromone glands of two *Heliothis* species (Teal *et al.*, 1989, 1999a; Christensen *et al.*, 1991) and *Manduca* (Christensen *et al.*, 1994) are innervated from the TAG, whereas those in *Ostrinia* are not (Ma and Roelofs, 1995). PBAN immunoreactivity, gene expression (Rafaeli *et al.*, 1991; Ma and Roelofs, 1995; Ma *et al.*, 1998), and biological activity (Rosén, 2002) have been localized not only to the brain–SEG complexes and CC, but also to the thoracic and abdominal ganglia. In *H. armigera*, both PBAN immunoreactivity and biological activity cycled during a day in the thoracic and abdominal ganglia (Rafaeli *et al.*, 1991) (**Figure 24b**), suggesting possibly novel functional significance and circadian control. PBAN was also found in other neural sites. In *H. zea*, processes from the PBAN-positive cells in the SEG project into the ventral nerve cord (Kingan *et al.*, 1990). In *A. segetum*, PBAN activity was localized in the ventral nerve cord (VNC) where it fluctuated daily (Rosén, 2002). All the above raise the interesting possibility that there may be novel sites of synthesis and/or release of PBAN in the nervous system. Furthermore, the findings that PBAN and/or PBAN gene expression is found in males (Rafaeli *et al.*, 1993; Zhu *et al.*, 1995; Choi *et al.*, 1998b) and that putative PBAN receptors were located in thoracic muscles and the CNS (Elliot *et al.*, 1997) imply novel sites of action and functions of PBAN in addition to regulation of pheromone production in the pheromone gland. The significance of this information is at the moment unclear.

JH is also involved in the regulation of pheromone production as shown in the armyworm moth, *Pseudaletia* (Cusson and McNeil, 1989; Cusson *et al.*, 1994) and the black cutworm, *A. ipsilon* (Picimbon *et al.*, 1995) (see Section 3.11.5.1). Allatectomy at emergence inhibited pheromone production; injections of JH or CA extracts restored it. When females of the above species or of *H. armigera* (Fan *et al.*, 1999) were decapitated at emergence, injections of JH alone were ineffective in restoring pheromone production; however, injection of JH together with PBAN (or brain extract) restored it. This strongly suggested that the presence of JH was necessary for rhythmic, PBAN-dependent, pheromone production. The role of JH could be to regulate the production/release of PBAN by the brain–SEG (Cusson *et al.*, 1994; Picimbon *et al.*, 1995), or JH could be needed to prime the pheromone glands for the action of PBAN (Fan *et al.*, 1999). The latter may be achieved by upregulation of putative PBAN receptors in pheromone glands by JH (Rafaeli *et al.*, 2003). Either of these possibilities raises yet again the prospect that JH synthesis may be under circadian control. A fruitful line of enquiry would be the examination of JH levels in relation to rhythms of PBAN release and/or pheromone production.

3.11.5.3.2. Regulation of pheromone glands by rhythms in octopamine In moths where the pheromone gland is innervated, there is considerable evidence that neural control from the VNC is essential for the regulation of pheromone production. Octopamine release by efferent neurons from the TAG to the pheromone gland was implicated as exerting stimulatory and/or inhibitory control over the gland and, by implication, regulating the daily rhythm of pheromone production. Transection of the nerves from the TAG to the gland inhibited

pheromone production in *Heliothis zea*, *H. virescens* (Teal *et al*., 1989, 1999a; Christensen *et al*., 1991, 1992, 1994), and *A. segetum* (Rosén, 2002). Electrical stimulation of the nerve connectives anterior to the TAG induced pheromone synthesis in females in photophase, a time when they are not normally engaged in pheromone production (Christensen *et al*., 1991, 1992). In these moths, intact nerve connections to the pheromone gland are essential for pheromone production. Octopamine induced pheromone production by the gland when it was injected into intact females in photophase (no pheromone synthesis) or isolated abdomens of females in scotophase with transected nerve connectives (inhibition of pheromone synthesis) (Christensen *et al*., 1991, 1992). In *H. zea*, the level of octopamine in the TAG exhibited daily cycling, which occurred in antiphase to the daily cycle of the octopamine level in the pheromone gland (**Figure 26**). In mid-photophase, the TAG contained more octopamine than the pheromone gland. However, a few hours prior to lights-off, the octopamine levels in these two locations changed swiftly; the level of octopamine in the TAG decreased sharply, whereas the level in the gland increased significantly. About 1–2 h after lights-off the octopamine levels in both the TAG and the pheromone gland returned to the mid-photophase levels. The increase in octopamine level in

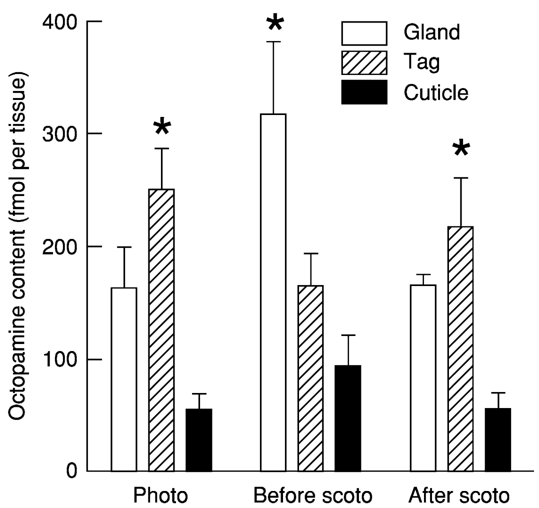


Figure 26 Day and night differences in octopamine levels in the pheromone gland, terminal abdominal ganglion (TAG), and abdominal cuticle (control) in females of *Heliothis zea*. ''Photo'' indicates mid-photophase; ''Before scoto'' indicates 0–2 h prior to lights-off; ''After scoto'' indicates 0–2 h after lights-off. Asterisks indicate statistically significant differences from controls. (Reprinted with permission from Christensen, T.A., Lehman, H.K., Teal, P.E.A., Itagaki, H., Tumlinson, J.H., *et al.*, **1992**. Diel changes in the presence and physiological actions of octopamine in the female sex-pheromone glands of *Heliothine* moths. *Insect Biochem. Mol. Physiol. 22*, 841–849; © Elsevier.)

the pheromone gland occurred at the time of pheromone emission and calling behavior. These findings provide strong circumstantial evidence that a rhythm in octopamine is involved in rhythmic pheromone synthesis. Experiments with administration of both octopamine and PBAN led the authors to suggest that the role of PBAN in the regulation of pheromone synthesis may be indirect. It was proposed that PBAN is transported to the TAG via the VNC, where it stimulates the octopaminergic innervation of the pheromone gland. However, Jurenka *et al.* (1991) and Ramaswamy *et al.* (1995) were unable to reproduce the above findings using the same moth and with the same experimental approach. It has also been proposed that octopamine may inhibit pheromone production. In *H. armigera*, octopamine or its agonist clonidine inhibited the pheromonotropic activity of PBAN using *in vitro* preparations of the pheromone gland (Rafaeli and Gileadi, 1997; Rafaeli *et al*., 1997), or *in vivo* in photophase animals that were injected with PBAN (Rafaeli *et al*., 1997). *In vivo*, the inhibition by octopamine or its agonist was reversed by injection of adrenergic antagonists only if the females were decapitated. Clonidine also inhibited normal pheromone increase in scotophase animals (Rafaeli *et al*., 1997). These findings led to the hypothesis that the daily declines during photophase in pheromone content of the gland may be attributable to daily inhibition of PBAN action mediated by octopaminergic receptors of the α_2-type.

3.11.5.4. Integration of Rhythmic Reproductive Behaviors with Hormones

The sequence of physiological processes that lead to the development of mature eggs and sperm is under complex endocrine control (see **Chapter 3.9**). Evidence of circadian regulation of these hormones is discussed above (see Section 3.11.5.1). Successful reproduction involves detecting and attracting a mate, copulation, and subsequent oviposition of fertilized eggs. In the context of hormones and circadian systems, these behaviors are significant in two ways, that may not be as distinct as the literature describes them. First, each of these behaviors occurs only at a temporally very precise part of the reproductive cycle, different behaviors being confined to different parts of the cycle. This point illustrates that the onset and termination of each behavior is intimately coordinated with the progress of egg and sperm development and therefore implies that the behaviors are modulated by signals from reproductive hormones. It further implies that different behaviors are executed in the presence of (and potentially influenced by) different hormonal

milieux. Second, all of these many distinct behaviors are performed rhythmically and are under circadian control. This point indicates integration between the reproductive hormones and the circadian system. The nature of this integration has received little experimental attention. The evidence summarized below indicates that it is unlikely that hormones directly drive reproductive behaviors. Rather, it appears that the clocks that drive behaviors are subject to sophisticated modulation by the endocrine milieu. For example, stridulation, courtship, mating, and oviposition all appear to be influenced by the locomotor clock (in the optic lobe) but each behavior takes place at a different time in the reproductive process. A model is envisaged in which the locomotor clock drives rhythmicity in the pattern generators for these behaviors, but which pattern generator becomes activated at any particular moment is influenced by the endocrine milieu. JH, octopamine, and various neuropeptides have been invoked as modulators of these behaviors, but much remains to be learned about how and where they act.

3.11.5.4.1. Rhythms in olfactory responses In many species, olfactory responses to odors (see **Chapter 3.15**) occur with a daily rhythm which is regulated by a circadian clock. The presence of a daily periodicity in olfactory responses is most apparent in the detection and response to sex pheromones. Successful mating depends on the daily coordination of the activities of males and females and it relies on temporal synchrony between the daily rhythm of release of sex pheromone by one sex (see Section 3.11.5.3) and the daily rhythm of responsiveness by the other. Daily rhythmicity in the behavioral rhythm of responsiveness to sex pheromones has been seen in a variety of insect groups including cockroaches (Liang and Schal, 1990), beetles (Shu *et al*., 1996), flies (Katsoyannos, 1982; Pivnic, 1993), and moths (Shorey and Gaston, 1965; Castrovillo and Cardé, 1979; Linn and Roelofs, 1992). The responsiveness rhythm is entrained by light cues in *Trichoplusia* (Shorey, 1966; Linn and Roelofs, 1992) and *Lymantria* (Linn *et al*., 1992), and free-runs in DD in *L. dispar* (Linn *et al*., 1992), *Trichoplusia* (Shorey, 1966; Lin and Roelofs, 1992), *Laspeyresia pomonella* (Castrovillo and Cardé, 1979), and the cockroach *Supella* (Liang and Schal, 1990), thus showing that it is controlled by an underlying circadian clock.

Circadian changes in responsiveness to sex pheromones could theoretically derive from circadian control over changes in olfactory sensitivity in the antennal sense organs or equally in the central processing of the olfactory input. Evidence suggests that both control mechanisms may in fact be operational in different insects. Explanted antennae of *Drosophila* incubated *in vitro* exhibited a daily rhythmicity in the expression of the *per* gene, which free-ran in DD for many cycles (Plautz *et al*., 1997); the rhythm was entrained by light *in vitro*. Electroantennogram responses of *Drosophila* to odorants such as ethyl acetate or benzaldehyde revealed a daily rhythm of responsiveness, which free-ran in DD and damped out quickly in LL (Krishnan *et al*., 1999). A *per* transgenic line (Krishnan *et al*., 1999) and *cry* mutants (Krishnan *et al*., 2001) confirmed that olfactory responses in *Drosophila* are controlled by a circadian clock residing in the antennae (**Figure 5**) (see Section 3.11.3.3). This antennal clock may be involved in the regulation of the rhythm of responsiveness to sex pheromones via regulation of the sensory neurons or binding proteins to pheromones that have been localized in the antennae of several insects (see **Chapter 3.15**).

A central mechanism in the antennal lobes appears to control the daily rhythmicity in pheromone sensitivity in the moth *A. ipsilon*, which is modulated by JH (Gadenne *et al*., 2001) (see Section 3.11.5.1). In addition, injection of octopamine affects many aspects of the responsiveness of male moths to sex pheromones, including the circadian rhythm of responsiveness (Linn *et al*., 1996; Linn, 1997). The complexity of octopamine effects on behavior suggests that octopamine may exert its influence on the male responsiveness by acting on the CNS. Experiments with phase advances and phase delays of the responsiveness rhythms showed that octopamine injections were effective only when administered within a narrow time window in the daily cycle prior to the daily peaks of responsiveness, suggesting a circadian component to the regulation of responsiveness by octopamine. In support of this notion is the finding that the levels of octopamine in the brain of *Trichoplusia* males exhibited a daily periodicity that was synchronous with the daily rhythm of responsiveness (Linn *et al*., 1996); the octopamine rhythm in the brain free-ran in DD and damped out rapidly in LL. All this implies that the mechanism of control of the responsiveness rhythm is centrally located and possibly related to the locomotor clock (Linn *et al*., 1996), whose output to the tritocerebrum may be modulated by circadian changes in octopamine.

There is also evidence that octopamine acts peripherally by modulating the sensitivity of the sensory neurons that detect sex pheromone in the antennae of males of *Antheraea polyphemus* (Pophof, 2000), *Mamestra* (Grosmaitre *et al*., 2001), and *Bombyx*

(Pophof, 2002). Octopamine receptors were cloned from the antennae of *Bombyx* and *H. virescens* and localized near the base of the olfactory hairs in *Heliothis* (Nickisch-Rosenegk *et al.*, 1996). The effects of octopamine on pheromone perception may thus be mediated by these antennal receptors; the modulatory action of octopamine may occur either at the level of generation of action potentials in the antennal receptor neurons (Pophof, 2002) or by acting on the accessory cells of the sensillum (Dolzer *et al.*, 2001). The olfactory receptor neurons in the antennae project axons directly into the antennal lobes, from whence projection neurons connect the antennal lobes with the lateral protocerebrum either directly or indirectly via the mushroom bodies (Heimbeck *et al.*, 2001; Hansson, 2002). *Drosophila* transgenic flies in which the function of these projection neurons was disrupted by transgenic expression of tetanus toxin show defects in both odor detection and courtship behavior of males (Heimbeck *et al.*, 2001). There is therefore a neural pathway that connects olfactory inputs with rhythmic behavioral outputs. In general, it appears that octopamine modulates the antennal olfactory clock whose output is integrated with the central locomotor clock. In turn, the outputs of the locomotor clock are responsible for driving various rhythmic behaviors such as upwind flight of the males, courtship, and mating. Further, JH appears to be involved in the modulation of the central neural pathways of the above rhythmic behaviors (see Section 3.11.5.1).

3.11.5.4.2. Bioluminescence, courtship song, and stridulation Several species, primarily Coleoptera, use bioluminescence for sexual attraction. The best-studied examples are the nightly displays of flashes of light by fireflies as a means of photic dialogue between the sexes. During flashing the animals are capable of emitting, receiving, and interpreting visual signals from the other sex. One sex, usually the male, emits a characteristic rhythmic pattern of light flashes during flight that is recognized by conspecific females; the females respond with an equally stereotyped pattern of flashes. In some firefly species, flashing by males may become synchronized in a local population of fireflies. In these synchronously flashing populations of males, the rhythmic pattern of flashing is self-sustained, runs with a stable free-running period, is entrained and phase-shifted to periodic pulses of light; therefore, it exhibits all the characteristics of an ultradian rhythm that is endogenously controlled (Buck, 1988). Flashing occurs with a circadian rhythm, within which there is a strong ultradian rhythm (Buck, 1988). Both rhythms are interrelated in terms of formal clock properties and physiological control (see below).

The efficacy of flashing as a mating signal depends also on the ability of females to receive and interpret rhythmic photic signals from the males. A daily rhythm in visual responsiveness to light flashes was observed in *Photuris versicolor* that free-runs in DD and is synchronized with the rhythm of flying and flashing (Lall, 1993). Therefore, the synchronous occurrence of all three rhythmic phenomena – bioluminescence, visual responsiveness, and flight – suggests that they may all be regulated by a common circadian clock. Indeed, it has been suggested that the ultradian flashing oscillator is associated with the locomotor clock in the brain (Bagnoli *et al.*, 1976; Case, 1984).

The regulation of courtship behavior in *Drosophila* provides another example of a behavior that has both circadian and ultradian properties. In wild-type flies of *Drosophila*, mating is preceded by courtship during which the male orients himself and directs auditory signals (the courtship song) towards the female by extension and vibration of the wings. One of the components of this song is a train of pulses with an interpulse interval that oscillates sinusoidally with a mean value of 55 s. This ultradian rhythm free-runs in LL and is temperature compensated; it is therefore regulated endogenously (Kyriakou and Hall, 1980). Mutations in *per* altered both this ultradian rhythm and the circadian locomotor rhythm. Construction of genetic mosaic flies for *per* showed that song phenotype was determined by expression of the *per* allele in the thoracic ganglia but not in the brain (Konopka *et al.*, 1996). The only cells that express *per* in the thoracic ganglia were glial cells (Ewer *et al.*, 1992). A general conclusion from both fireflies and *Drosophila* is that the oscillators for ultradian and circadian rhythms appear to share common components at both the cellular and anatomical levels.

Many insects also communicate using rhythmic acoustic signals produced by specialized structures. The best-known example is stridulation in crickets. In numerous species, stridulation is under circadian control. For example, in *Teleogryllus commodus*, stridulation occurs with a strong daily rhythm that free-runs in both DD and LL (Sokolove and Loher, 1975); it is, therefore, controlled by an endogenous circadian clock. In these species, removal of the eyes or severance of the eyes from the optic lobes results in a free-running rhythm of stridulation in either LD or LL, but with a period length characteristic of DD (Sokolove and Loher, 1975). These experiments are consistent with the view that the

stridulation rhythm is controlled by the same clock that controls the locomotor rhythm. Subsequent experiments showed that the stridulation rhythm could be uncoupled from the locomotor rhythm, but the clocks controlling both rhythms were both located in the optic lobes (Wiedenmann, 1983; Wiedenmann and Loher, 1984; Wiedenmann *et al*., 1988). It was suggested that each optic lobe contains two circadian clocks (one for each rhythm) that are normally coupled together.

3.11.5.4.3. Mating and oviposition Daily periodicities in mating behavior have been identified in all major insect groups. It is generally accepted that an endogenous circadian oscillator controls rhythmicity in mating, although there have been only a few true circadian studies. Endogenous circadian control of mating has been observed in beetles (Walker, 1979; Boucher and Pierre, 1988), fruit flies (Loher and Zervas, 1979; Sakai and Ishida, 2001), and moths (Han *et al*., 2000). In *Drosophila*, mating occurs with a circadian rhythm that free-runs in DD and is abolished in *per* and *tim* null mutants (Sakai and Ishida, 2001). Further, in *disconnected* mutants which lack some of the lateral clock neurons that control locomotion and have severe defects in the optic lobes, rhythmicity in mating was also abolished. Taken together, these observations showed that rhythmicity in mating behavior may be controlled by the locomotor clock. Thus, the *Drosophila* mating repertoire becomes an illustration of the fine coordination of clock-controlled mating activities between the sexes. Successful mating contact depends on olfactory responses of males to the release of female sex pheromones; these responses are controlled by a *per*-based olfactory clock in the antennae (Krishnan *et al*., 1999) (see Section 3.11.5.4.1). Males respond to the female chemical signal by performing the courtship song, which is also *per* controlled (Kyriakou and Hall, 1982) (see Section 3.11.5.4.2). Courtship song, in turn, affects female receptivity (Kyriakou and Hall, 1982; Hall, 2002). Thus it appears probable, as Sakai and Ishida (2001) suggested, that the mating rhythm in females may be a consequence of circadian control over pheromone release and/or responsiveness to male courtship song.

Oviposition behavior is also under circadian control in numerous insects, such as bugs (Ampleford and Davey, 1989), crickets (Sefiani, 1987), moths (Riedl and Loher, 1980; Bell, 1981; Yamaoka and Hirao, 1981; Shearer *et al*., 1995), flies and mosquitoes (Allemand, 1983; Joshi, 1996). The physiological links between mating and oviposition are both complex and species-variable (see **Chapter 1.5**). Peptides transferred to the female at mating have been shown to enhance fertility, promote oviposition, or inhibit further mating by the female (Kubli, 1992; Stanley-Samuelson, 1994). Further, proteinaceous material transferred to the female at mating can be utilized as nutrient to accelarate oogenesis; amino acids and proteins from the spermatophore have been detected in the eggs of many species (Huignard, 1983; Mullins *et al*., 1992). In some insects such as the tettigonids and crickets, the female feasts on the spermatophore whose peptide components can be found later in eggs (Simmons and Gwynne, 1993). Thus, the male contributes to both completion of egg development by the female and to the behavioral changes that follow mating. These points emphasize the close integration between the mechanisms that control egg and sperm development with those that control rhythmic behavior.

The circadian control of oviposition behavior must be synchronized with the presence and movement of eggs along the oviducts. Complex neuroendocrine mechanisms control the process of egg movement, extensively studied in orthopterans. There are pattern generators in the TAG for both digging (Thompson *et al*., 1999) and regulation of oviducal contractions (Kalogianni and Theophilidis, 1993), and contractions are influenced by proctolin, octopamine, and FMRFamide-related peptides (Noronha and Lange, 1997; Orchard *et al*., 1997). Despite extensive physiological analysis, the origin of circadian control of this system is unexplored.

References

Agui, N., Granger, N.A., Gilbert, L.I., Bollenbacher, W.E., **1979**. Cellular localization of the insect prothoracicotropic hormone: *in vitro* assay of a single neurosecretory cell. *Proc. Natl Acad. Sci. USA* 76, 5694–5698.

Allemand, R., **1976a**. Les rythmes de vitellogenèse et d'ovulation en photopériode LD 12:12 de *Drosophila melanogaster. J. Insect Physiol.* 22, 1031–1035.

Allemand, R., **1976b**. Influence de modifications des conditions lumineuses sur les rythmes circadiens de vitellogenèse et d'ovulation chez *Drosophila melanogaster. J. Insect Physiol.* 22, 1075–1080.

Allemand, R., **1983**. The circadian oviposition rhythm of *Drosophila melanogaster*. 2. Influence of biotic factors. *Biol. Behav.* 8, 273–288.

Allen, G., Rappe, J., Earnest, D.J., Cassone, V.M., **2001**. Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. *J. Neurosci.* 21, 7937–7943.

Altstein, M., Ben-Aziz, O., Daniel, S., Zeltser, I., Gilon, C., **2001**. Pyrokinin/PBAN radioreceptor assay: development and application for the characterization of a

putative receptor from the pheromone gland of *Heliothis peltigera*. *Peptides 22*, 1379–1389.

Altstein, M., Gabay, T., Ben-Aziz, O., Daniel, S., Zeltser, I., *et al*., **1999**. Characterization of a putative pheromone biosynthesis activating neuropeptide (PBAN) receptor from the pheromone gland of *Heliothis peltigera*. *Invert. Neurosci. 4*, 33–40.

Ampleford, E.J., Davey, K.G., **1989**. Egg laying in the insect *Rhodnius prolixus* is timed in a circadian fashion. *J. Insect Physiol. 35*, 183–188.

Ampleford, E.J., Steel, C.G.H., **1982**. Circadian control of ecdysis in *Rhodnius prolixus* (Hemiptera). *J. Comp. Physiol. A 147*, 281–286.

Ampleford, E.J., Steel, C.G.H., **1985**. Circadian control of a daily rhythm in hemolymph ecdysteroid titer in the insect *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrinol. 59*, 453–459.

Ampleford, E.J., Steel, C.G.H., **1986**. Induction of rhythmic modulation of hemolymph ecdysteroids in the insect *Rhodnius prolixus* by treatments which elicit rhythmic ecdysis. *Gen. Comp. Endocrinol. 63*, 353–361.

Andretic, R., Chaney, S., Hirsh, J., **1999**. Requirement of circadian genes for cocaine sensitization in *Drosophila*. *Science 285*, 1066–1068.

Anton, S., Gadenne, C., **1999**. Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. *Proc. Natl Acad. Sci. USA 96*, 5764–5767.

Asahina, M., Fugo, H., Takeda, S., **1994**. Ecdysteroid synthesis in dissociated cells of the prothoracic gland of the silkworm, *Bombyx mori*. *Zool. Sci. 11*, 107–111.

Atkins, G., Stout, J., **1994**. Processing of song signals in the cricket and its hormonal control. *Am. Zool. 34*, 655–669.

Audsley, N., Duve, H., Thorpe, A., Weaver, R.J., **2000**. Morphological and physiological comparisons of two types of allatostatin in the brain and retrocerebral complex of the tomato moth, *Lacanobia oleracea* (Lepidoptera: Noctuidae). *J. Comp. Neurol. 424*, 27–46.

Babilis, N.A., Mazomenos, B.E., **1992**. Pheromone production in *Sesamia nonagrioides*: diel periodicity and effect of age and mating. *J. Insect Physiol. 38*, 561–564.

Bagnoli, P., Brunelli, M., Magni, F., Musumeci, D., **1976**. Neural mechanisms underlying spontaneous flashing and its modulation in the firefly *Luciola lusitanica*. *J. Comp. Physiol. 108*, 133–156.

Baker, J.D., McNabb, S.L., Truman, J.W., **1999**. The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. *J. Exp. Biol. 202*, 3037–3048.

Balsalobre, A., **2002**. Clock genes in mammalian peripheral tissues. *Cell Tissue Res. 309*, 193–199.

Beaulaton, J., Porcheron, P., Gras, R., Cassier, P., **1984**. Cytophysiological correlations between prothoracic gland activity and hemolymph ecdysteroid concentration in *Rhodnius prolixus* during the fifth larval instar: further studies in normal and decapitated larvae. *Gen. Comp. Endocrinol. 53*, 1–16.

Bebas, P., Cymborowski, B., Giebultowicz, J.M., **2001**. Circadian rhythm of sperm release in males of the cotton leafworm, *Spodoptera littoralis*: *in vivo* and *in vitro* studies. *J. Insect Physiol. 47*, 859–866.

Bebas, P., Cymborowski, B., Giebultowicz, J.M., **2002**. Circadian rhythm of acidification in insect vas deferens regulated by rhythmic expression of vacuolar H^+-ATPase. *J. Exp. Biol. 205*, 37–44.

Bell, C.H., **1981**. The influence of light cycle and circadian rhythm on oviposition in 5 pyralid moth pests of stored products. *Physiol. Entomol. 6*, 231–240.

Beydon, P., Permana, A.D., Colardeau, J., Moriniere, M., Lafont, R., **1989**. Ecdysteroids from developing eggs of *Pieris brassicae*. *Arch. Insect Biochem. Physiol. 11*, 1–12.

Bjostad, L.B., Wolf, W.A., Roelofs, W.L., **1987**. Pheromone biosynthesis in lepidopterans: desaturation and chain shortening. In: Blomquist, G.J., Prestwich, G.D. (Eds.), Pheromone Biochemistry. Academic Press, San Diego, CA, pp. 77–120.

Block, G.D., Khalsa, S.B.S., McMahon, D.G., Michel, S., Guesz, M., **1993**. Biological clocks in the retina cellular mechanisms of biological timekeeping. *Int. Rev. Cytol. 146*, 83–144.

Bollenbacher, W.E., Granger, N.A., Katahira, E.J., O'Brien, M.A., **1987**. Developmental endocrinology of larval molting in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 128*, 175–192.

Boucher, L., Pierre, D., **1988**. Mating rhythm of *Caryedon serratus* (Coleoptera: Bruchidae) in laboratory and natural conditions. *Ann. Soc. Entomol. France 24*, 151–160.

Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., **1984**. *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *Manduca sexta*. *J. Exp. Biol. 108*, 9–24.

Bownes, M., Shirras, A., Blair, M., Collins, J., Coulson, A., **1988**. Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *Proc. Natl Acad. Sci. USA 85*, 1554–1557.

Buck, J., **1988**. Synchronous rhythmic flashing of fireflies. 2. *Q. Rev. Biol. 63*, 265–289.

Carney, G.E., Bender, M., **2000**. The *Drosophila ecdysone receptor* (*EcR*) gene is required maternally for normal oogenesis. *Genetics 154*, 1203–1211.

Case, J.F., **1984**. Vision in mating behavior of fireflies. *Symp. Roy. Entomol. Soc. London 12*, 195–222.

Castrovillo, P.J., Cardé, R.T., **1979**. Environmental regulation of female calling and male pheromone response periodicities in the codling moth (*Laspeyresia pomonella*). *J. Insect Physiol. 25*, 659–668.

Charlton, R.E., Cardé, R.T., **1982**. Rate and diel periodicity of pheromone emission from female gypsy moths (*Lymantria dispar*), determined with a glass-adsorption collection system. *J. Insect Physiol. 28*, 423–430.

Chen, J.H., Fugo, H., Nakajima, M., Nagasawa, H., Suzuki, A., **1987**. Neurohormones in developing embryos of the silkworm, *Bombyx mori*: the presence and characteristics of prothoracicotropic hormone, B. *J. Insect Physiol. 33*, 407–411.

Choi, M.Y., Tanaka, M., Kataoka, H., Boo, K.S., Tatsuki, S., **1998b**. Isolation and identification of the cDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the Oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol. 28*, 759–766.

Choi, M.Y., Tatsuki, S., Boo, K.S., **1998a**. Regulation of sex pheromone biosynthesis in the Oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol. 44*, 653–658.

Christensen, T.A., Itagaki, H., Teal, P.E.A., Jasensky, R.D., Tumlinson, J.H., *et al.*, **1991**. Innervation and neural regulation of the sex pheromone gland in female *Heliothis* moths. *Proc. Natl Acad. Sci. USA 88*, 4971–4975.

Christensen, T.A., Lashbrook, J.M., Hildebrand, J.G., **1994**. Neural activation of the sex-pheromone gland in the moth *Manduca sexta*: real-time measurement of pheromone release. *Physiol. Entomol. 19*, 265–270.

Christensen, T.A., Lehman, H.K., Teal, P.E.A., Itagaki, H., Tumlinson, J.H., *et al.*, **1992**. Diel changes in the presence and physiological actions of octopamine in the female sex-pheromone glands of *Heliothine* moths. *Insect Biochem. Mol. Physiol. 22*, 841–849.

Colwell, C.S., Page, T.L., **1990**. A circadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. *J. Comp. Physiol. A 166*, 643–649.

Corrent, G., Eskin, A., Kay, I., **1982**. Entrainment of the circadian rhythm from the eye of *Aplysia*: role of serotonin. *Am. J. Physiol. 242*, R326–R332.

Cusson, M., McNeil, J.N., **1989**. Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. *Science 243*, 210–212.

Cusson, M., Tobe, S.S., McNeil, J.N., **1994**. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm, *Pseudaletia unipuncta*. *Arch. Insect Biochem. Physiol. 25*, 329–345.

Cymborowski, B., Muszynska-Pytel, M., Porcheron, P., Cassier, P., **1991**. Hemolymph ecdysteroid titers controlled by a circadian clock mechanism in larvae of the wax moth, *Galleria mellonella*. *J. Insect Physiol. 37*, 35–40.

Cymborowski, B., Smietanko, A., Delbecque, J.P., **1989**. Circadian modulation of ecdysteroid titer in *Galleria mellonella* larvae. *Comp. Biochem. Physiol. A 94*, 431–438.

Dai, J.-D., Costello, M.J., Gilbert, L.I., **1994**. The prothoracic glands of *Manduca sexta*: a microscopic analysis of gap junctions and intercellular bridges. *Invert. Reprod. Devel. 25*, 93–110.

Dai, J.-D., Mizoguchi, A., Satake, S., Ishizaki, H., Gilbert, L.I., **1995**. Developmental changes in the prothoracicotropic hormone content of the *Bombyx mori* brain–retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Devel. Biol. 171*, 212–223.

Danforth, D.N., Jr., Tamarkin, L., Lippman, M.E., **1983**. Melatonin-induced increase in cytoplasmic estrogen receptor activity in hamster uteri. *Endocrinology 113*, 81–85.

Dedos, S.G., Fugo, H., **1999**. Induction of dauer larvae by application of fenoxycarb early in the 5th instar of the silkworm, *Bombyx mori*. *J. Insect Physiol. 45*, 769–775.

Delisle, J., McNeil, J.N., **1987**. Calling behavior and pheromone titer of the true armyworm *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae) under different temperature and photoperiodic conditions. *J. Insect Physiol. 33*, 315–324.

De Loof, A., Baggerman, G., Breuer, M., Claeys, I., Cerstiaens, A., *et al.*, **2001**. Gonadotropins in insects: an overview. *Arch. Insect Biochem. Physiol. 47*, 129–138.

Doe, C.Q., Goodman, C.S., **1985**. Early events in insect neurogenesis. 1. Development and segmental differences in the pattern of neuronal precursor cells. *Devel. Biol. 111*, 193–205.

Dolzer, J., Krannich, S., Fischer, K., Stengl, M., **2001**. Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol. 204*, 2781–2794.

Donini, A., Agricola, H.-J., Lange, A.B., **2001**. Crustacean cardioactive peptide is a modulator of oviduct contractions in *Locusta migratoria*. *J. Insect Physiol. 47*, 277–285.

Dorn, A., **1983**. Hormones during embryogenesis of the milkweed bug *Oncopeltus fasciatus* (Heteroptera: Lygaeida). *Entomol. Gen. 8*, 193–214.

Dorn, A., Bishoff, S.T., Gilbert, L.I., **1987a**. An incremental analysis of the embryonic development of the tobacco hornworm, *Manduca sexta*. *Int. J. Invert. Reprod. Devel. 11*, 137–158.

Dorn, A., Gilbert, L.I., Bollenbacher, W.E., **1987b**. Prothoracicotropic hormone activity in the embryonic brain of the tobacco hornworm, *Manduca sexta*. *J. Comp. Physiol. B 157*, 279–283.

Dow, R.C., Carlson, S.D., Goodman, W.D., **1988**. A scanning electron microscope study of the developing embryo of *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Int. J. Insect Morphol. Embryol. 17*, 231–242.

Du, J., Hiruma, K., Riddiford, L.M., **2003**. A novel gene in the *takeout* gene family is regulated by hormones and nutrients in *Manduca* larval epidermis. *Insect Biochem. Mol. Biol. 33*, 803–814.

Dumser, J.B., **1980**. The regulation of spermatogenesis in insects. *Annu. Rev. Entomol. 25*, 341–369.

Dunkelblum, E., Kehat, M., Harel, M., Gordon, D., **1987**. Sexual behavior and pheromone titer of the *Spodoptera littoralis* female moth. *Entomol. Exp. Applic. 44*, 241–248.

Duportets, L., Dufour, M.C., Bécard, J.M., Gadenne, C., Couillaud, F., **1996**. Inhibition of male corpora allata activity and sexual pheromone responsiveness in the black cutworm, *Agrotis ipsilon*, by the hypocholesterolemic agent, fluvastatin. *Arch. Insect Biochem. Physiol. 32*, 601–611.

Duportets, L., Gadenne, C., Dufour, M.C., Couillaud, F., **1998**. The pheromone biosynthesis activating neuropeptide (PBAN) of the black cutworm moth, *Agrotis ipsilon*: immunohistochemistry, molecular characterization and bioassay of its peptide sequence. *Insect Biochem. Mol. Biol. 28*, 591–599.

Edmunds, L.N., Jr., **1988**. Cellular and Molecular Bases of Biological Clocks: Models and Mechanisms for Circadian Timekeeping. Springer, Berlin.

Elekonich, M.M., Schultz, D.J., Bloch, G., Robinson, G.E., **2001**. Juvenile hormone levels in honey bee (*Apis mellifera* L.) foragers: foraging experience and diurnal variation. *J. Insect Physiol. 47*, 1119–1125.

Elliot, J.T., Jurenka, R.A., Prestwich, G.D., Roelofs, W.L., **1997**. Identification of soluble proteins for an insect neuropeptide. *Biochem. Biophys. Res. Commun. 238*, 925–930.

Emery, I.F., Noveral, J.M., Jamison, C.F., Siwicki, K.K., **1997**. Rhythms of *Drosophila period* gene expression in culture. *Proc. Natl Acad. Sci. USA 94*, 4092–4096.

Epsky, N.D., Heath, R.R., **1993**. Pheromone production by male *Anastrepha suspensa* (Diptera: Tephritidae) under natural light cycles in greenhouse studies. *Environ. Entomol. 22*, 464–469.

Espig, W., Thiry, E., Hoffmann, K.H., **1989**. Ecdysteroids during ovarian development and embryogenesis in the cricket *Gryllus bimaculatus* de Geer. *Invert. Reprod. Devel. 15*, 143–154.

Eusebio, E.J., Moody, W.J., **1986**. Calcium-dependent action potentials in the prothoracic gland of *Manduca sexta*. *J. Exp. Biol. 126*, 531–636.

Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbach, M., Hall, J.C., **1992**. Expression of the *period* gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci. 12*, 3321–3349.

Ewer, J., Gammie, S.C., Truman, J.W., **1997**. Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. *J. Exp. Biol. 200*, 869–881.

Ewer, J., Reynolds, S., **2002**. Neuropeptide control of molting in insects. In: Pfaff, D., Arnold, A., Etgen, A., Fahrbach, S., Rubin, A.R. (Eds.), Hormones, Brain and Behavior, vol. 3. Academic Press, New York, pp. 1–92.

Fan, Y., Rafaeli, A., Gileadi, C., Applebaum, S.W., **1999**. Juvenile hormone induction of pheromone gland PBAN-responsiveness in *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol. 29*, 635–641.

Foster, S.P., **2000**. Periodicity of sex pheromone biosynthesis, release and degradation in the lightbrown apple moth *Epiphyas postvittana* (Walker). *Arch. Insect Biochem. Physiol. 43*, 125–136.

Friedländer, M., Reynolds, S.E., **1988**. Meiotic metaphases are induced by 20-hydroxyecdysone during spermatogenesis of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 34*, 1013–1019.

Frisch, B., Hardin, P.E., Hamblen-Coyle, M.J., Rosbach, M., Hall, J.C., **1994**. A promotorless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system. *Neuron 12*, 555–570.

Fugo, H., Saito, H., Nagasawa, H., Suzuki, A., **1985**. Eclosion hormone activity in developing embryos of the silkworm, *Bombyx mori*. *J. Insect Physiol. 31*, 293–298.

Fujishita, M., Ishizaki, H., **1981**. Circadian clock and prothoracicotropic hormone secretion in relation to the larval–larval ecdysis rhythm of the saturniid *Samia cynthia ricini*. *J. Insect Physiol. 27*, 121–128.

Fujishita, M., Ishizaki, H., **1982**. Temporal organization of circadian events in relation to the circadian clock during larval–pupal development in *Samia cynthia ricini*. *J. Insect Physiol. 28*, 77–84.

Fujishita, M., Ohnishi, E., Ishizaki, H., **1982**. The role of ecdysteroids in the determination of gut-purge timing in the saturniid, *Samia cynthia ricini*. *J. Insect Physiol. 28*, 961–968.

Gadenne, C., Anton, S., **2000**. Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. *J. Insect Physiol. 46*, 1195–1206.

Gadenne, C., Dufour, M.C., Anton, S., **2001**. Transient post-mating inhibition of behavioral and central responses to sex pheromones in an insect. *Proc. R. Soc. London B 268*, 1631–1635.

Gadenne, C., Renou, M., Sreng, L., **1993**. Hormonal control of pheromone responsiveness in the male black cutworm, *Agrotis ipsilon*. *Experientia 49*, 721–724.

Gammie, S.C., Truman, J.W., **1997**. Neuropeptide hierachies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. *J. Neurosci. 17*, 4389–4397.

Gelman, D.B., Woods, C.W., Borkovec, A.B., **1988**. Effects of ecdysone and 20-hydroxyecdysone on apyrene spermiogenesis in European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 34*, 733–738.

Gemeno, C., Haynes, K.F., **2000**. Periodical and age-related variation in chemical communication system of black cutworm moth, *Agrotis ipsilon*. *J. Chem. Ecol. 26*, 329–342.

Gharib, B., DeReggi, M., **1983**. Changes in ecdysteroid and juvenile hormone levels in developing eggs of *Bombyx mori*. *J. Insect Physiol. 29*, 871–876.

Giebultowicz, J.M., **2000**. Molecular mechanism and cellular distribution of insect circadian clocks. *Annu. Rev. Entomol. 45*, 769–793.

Giebultowicz, J.M., Blackburn, M.B., Thomas-Laemont, P.A., Weyda, F., Raina, A.K., **1996**. Daily rhythm in myogenic contractions of vas deferens associated with sperm release cycle in a moth. *J. Comp. Physiol. A 178*, 629–636.

Giebultowicz, J.M., Feldlaufer, M., Gelman, D.B., **1990**. Role of ecdysteroids in the regulation of sperm release from the testis of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol. 36*, 567–571.

Giebultowicz, J.M., Joy, J.E., **1992**. Ontogeny of the circadian system controlling release of sperm from the insect testis. *J. Biol. Rhythms 7*, 203–212.

Giebultowicz, J.M., Joy, J.E., Riemann, J.G., Raina, A.K., **1994**. Changes in protein patterns in sperm and vas deferens during the daily rhythm of sperm release in the gypsy moth. *Arch. Insect Biochem. Physiol. 27*, 65–75.

Giebultowicz, J.M., Riemann, J.G., Raina, A.K., Ridgway, R.L., **1988**. Circadian system controlling release of sperm in the insect testes. *Science 245*, 1098–1099.

Giebultowicz, J.M., Stanewsky, R., Hall, J.C., Hege, D.M., **2000**. Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. *Curr. Biol. 10*, 107–110.

Gilbert, L.I., Rybczynski, R., Warren, J.T., **2002**. Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol. 47*, 883–916.

Gillette, M.U., McArthur, A.J., **1995**. Circadian actions of melatonin at the suprachiasmatic nucleus. *Behav. Brain Res. 73*, 135–139.

Gillette, M.U., Mitchell, J.W., **2002**. Signalling in the suprachiasmatic nucleus: selectively responsive and integrative. *Cell Tissue Res. 309*, 99–107.

Gillott, C., **1995**. Insect male mating systems. In: Leather, S.R., Hardie, J. (Eds.), Insect Reproduction. CRC Press, Boca Raton, FL, pp. 33–55.

Goltzene, F., Holder, F., Charlet, M., Meister, M., Oka, T., **1992**. Immunocytochemical localization of *Bombyx*-PTTH-like molecules in neurosecretory cells of the brain of the migratory locust *Locusta migratoria*: a comparison with neuroparsin and insulin-related peptide. *Cell Tissue Res. 269*, 133–140.

Gorbet, D.J., Steel, C.G.H., **2003**. A miniature radioimmunoassay for melatonin for use with small samples from invertebrates. *Gen. Comp. Endocrinol. 134*, 193–197.

Gorbet, D.J., Steel, C.G.H., 2004. Circadian regulation of a daily rhythm in the level of melatonin in the hemolymph of the insect *Rhodnius prolixus*. *J. Insect Physiol.* (in press).

Greiner, B., Gadenne, C., Anton, S., **2002**. Central processing of plant volatiles in *Agrotis ipsilon* males is age-independent in contrast to sex pheromone processing. *Chem. Senses 27*, 45–48.

Grosmaitre, X., Marion-Poll, F., Renou, M., **2001**. Biogenic amines modulate olfactory receptor neurons firing activity in *Mamestra brassicae*. *Chem. Senses 26*, 653–661.

Gvakharia, B.O., Kilgore, J.A., Bebas, P., Giebultowicz, J.M., **2000**. Temporal and spatial expression of the *period* gene in the reproductive system of the codling moth. *J. Biol. Rhythms 15*, 4–12.

Hagedorn, H.H., **1985**. The role of ecdysteroids in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 205–262.

Hagedorn, H.H., **1989**. Physiological roles of hemolymph ecdysteroids in the adult insect. In: Koolman, J. (Ed.), Ecdysone. Georg Thieme Verlag, Stuttgart, pp. 279–289.

Hall, J.C., **1998**. Genetics of biological rhythms in *Drosophila*. *Adv. Genet. 38*, 135–184.

Hall, J.C., **2002**. Courtship lite: a personal history of reproductive behavioral neurogenetics in *Drosophila*. *J. Neurogenet. 16*, 135–163.

Hall, J.C., **2003**. Genetics and Molecular Biology of Rhythms in *Drosophila* and Other Insects. Academic Press, New York.

Han, G., Du, J., Li, J., **2000**. Mating behavioral ecology of *Ancylis sativa* adult. *Yingyong Shengtai Xuebao 11*, 99–102.

Hansson, B.S., **2002**. A bug's smell-research into insect olfaction. *Trends Neurosci. 25*, 270–274.

Hardeland, R., Poeggeler, B., **2003**. Non-vertebrate melatonin. *J. Pineal Res. 34*, 233–241.

Hardie, J., **1995**. Hormones and reproduction. In: Leather, S.R., Hardie, J. (Eds.), Insect Reproduction. CRC Press, Boca Raton, FL, pp. 95–108.

Hardin, P.E., **1994**. Analysis of *period* mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. *Mol. Cell Biol. 14*, 7211–7218.

Harshman, L.G., Loeb, A.M., Johnson, B.A., **1999**. Ecdysteroid titers in mated and unmated *Drosophila melanogaster* females. *J. Insect Physiol. 45*, 571–577.

Hege, D.M., Stanewsky, R., Hall, J.C., Giebultowicz, J.M., **1997**. Rhythmic expression of a *per*-reporter in the Malpighian tubules of decapitated *Drosophila*: evidence for a brain independent circadian clock. *J. Biol. Rhythms 12*, 300–308.

Heimbeck, G., Bugnon, V., Gendre, N., Keller, A., Stocker, R.F., **2001**. A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 98*, 15336–15341.

Helfrich-Förster, C., **1995**. The *period* clock gene is expressed in central nervous system, which also produces a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 92*, 612–616.

Helfrich-Förster, C., **1997**. Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster. J. Comp. Neurol. 380*, 335–354.

Helfrich-Förster, C., **2003**. The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster. Microsc. Res. Tech. 62*, 94–102.

Helfrich-Förster, C., Stengl, M., Homberg, U., **1998**. Organization of the circadian system in insects. *Chronobiol. Internat. 15*, 567–597.

Hepburn, H.R., **1985**. Structure of the integument. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3. Pergamon, Oxford, pp. 1–58.

Herzog, E.D., Tosini, G., **2001**. The mammalian circadian clock shop. *Seminars Cell Devel. Biol. 12*, 295–303.

Hesterlee, S., Morton, D.B., **1996**. Insect physiology: the emerging story of ecdysis. *Curr. Biol. 6*, 648–650.

Hoffmann, J.A., Lagueux, M., **1985**. Endocrine aspects of embryonic development in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 1. Pergamon, Oxford, pp. 435–460.

Homberg, U., Davis, N.T., Hildebrand, J.G., **1991b**. Peptide immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta*. *J. Comp. Neurol. 303*, 35–52.

Homberg, U., Würden, S., Dircksen, H., Rao, K.R., **1991a**. Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. *Cell Tissue Res. 266*, 343–357.

Horike, N., Sonobe, H., **1999**. Ecdysone 20-monooxygenase in eggs of the silkworm, *Bombyx mori*: enzymatic properties and developmental changes. *Arch. Insect Biochem. Physiol. 41*, 9–17.

Horodyski, F.M., **1996**. Neuroendocrine control of insect ecdysis by eclosion hormone. *J. Insect Physiol. 42*, 917–924.

Huignard, J., **1983**. Transfer and fate of male secretions deposited in the spermatophore of females of *Acanthoscelides obtectus* Say. *J. Insect Physiol. 29*, 55–63.

Hunt, R.E., Haynes, K.F., **1990**. Periodicity in the quantity and blend ratios of pheromone components in glands and volatile emissions of mutant and normal cabbage looper moths, *Trichoplusia ni*. *J. Insect Physiol. 36*, 769–774.

Hurst, W.J., Earnest, D., Gillette, M.U., **2002**. Immortalized suprachiasmatic nucleus cells express components of multiple circadian regulatory pathways. *Biochem. Biophys. Res. Commun. 292*, 20–30.

Ichikawa, T., **1998**. Activity patterns of neurosecretory cells releasing pheromonotropic neuropeptides in the moth *Bombyx mori*. *Proc. Natl Acad. Sci. USA 95*, 4055–4060.

Ichikawa, T., Hasegawa, K., Shimizu, I., Katsuno, K., Kataoka, H., *et al.*, **1995**. Structure of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide in the *Bombyx mori*. *Zool. Sci. 12*, 703–712.

Ichikawa, T., Ito, K., **1999**. Calling behavior modulates heartbeat reversal rhythm in the silkmoth, *Bombyx mori*. *Zool. Sci. 16*, 203–209.

Iglesias, F., Jacquin-Joly, E., Marco, M.-P., Camps, F., Fabriàs, G., **1999**. Temporal distribution of PBAN-like immunoreactivity in the hemolymph of *Mamestra brassicae* females in relation to sex pheromone production and calling behavior. *Arch. Insect Biochem. Physiol. 40*, 80–87.

Iglesias, F., Marco, P., François, M.-C., Camps, F., Fabriàs, F., *et al.*, **2002**. A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualization in scotophase hemolymph. *Insect Biochem. Mol. Biol. 32*, 901–908.

Ikonomov, O.C., Stoynev, A.G., Shisheva, A.C., **1998**. Integrative coordination of circadian mammalian diversity: neuronal networks and peripheral clocks. *Prog. Neurobiol. 54*, 87–97.

Ishizaki, H., Suzuki, A., **1994**. The brain secretory peptides that control molting and metamorphosis of the silkmoth, *Bombyx mori*. *Int. J. Devel. Biol. 38*, 301–310.

Itagaki, H., Conner, W.E., **1988**. Calling behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. *Ann. Entomol. Soc. America 81*, 795–807.

Itoh, M.T., Hatori, A., Sumi, Y., Suzuki, T., **1994**. Identification of melatonin in different organs of the cricket, *Gryllus bimaculatus*. *Zool. Sci. 11*, 577–581.

Itoh, M.T., Hatori, A., Sumi, Y., Suzuki, T., **1995a**. Day–night changes in melatonin levels in different organs of the cricket (*Gryllus bimaculatus*). *J. Pineal Res. 18*, 165–169.

Itoh, M.T., Nomura, T., Sumi, Y., Suzuki, T., **1995b**. Melatonin and arylkylamine *N*-acetyltransferase activity in the silkworm, *Bombyx mori*. *Mol. Cell. Endocrinol. 115*, 59–64.

Itoh, M.T., Sumi, Y., **1998a**. Melatonin and serotonin *N*-acetyltransferase activity in developing eggs of the cricket *Gryllus bimaculatus*. *Brain Res. 781*, 91–99.

Itoh, M.T., Sumi, Y., **1998b**. Circadian clock controlling arylalkylamine *N*-acetyltransferase-like activity in the cricket (*Gryllus bimaculatus*) eggs. *Brain Res. 799*, 172–175.

Itoh, M.T., Sumi, Y., **2000**. Circadian clock controlling egg hatching in the cricket (*Gryllus bimaculatus*). *J. Biol. Rhythms 121*, 241–245.

Jackson, R.R., Schroeder, A.J., Roberts, M.A., McNeil, G.P., Kume, K., *et al.*, **2001**. Cellular and molecular mechanisms of circadian control in insects. *J. Insect Physiol. 47*, 833–842.

Jacquin-Joly, E., Burnet, M., François, M.C., Amma, D., Nagnan-LeMeillour, P., *et al.*, **1998**. cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of *Mamestra brassicae*: a new member of the PBAN family. *Insect Biochem. Mol. Biol. 28*, 251–258.

Jan, L.Y., Jan, Y.N., **1982**. Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *Proc. Natl Acad. Sci. USA 79*, 2700–2704.

Jeon, S.K., Lee, B.H., **1999**. Immunocytochemical identification of allatostatin producing neurons in developing central nervous system of the silk moth, *Bombyx mori*. *Korean J. Entomol. 29*, 233–238.

Johnston, J.S., Ellison, J.R., **1982**. Exact age determination in laboratory and field-caught *Drosophila*. *J. Insect Physiol. 28*, 773–779.

Jones, M.D.R., Gubbins, S.J., **1979**. Modification of female circadian flight-activity by a male accessory gland pheromone in the mosquito, *Culex pipiens quinquefasciatus*. *Physiol. Entomol. 4*, 345–352.

Joshi, D.S., **1996**. Psi-mutation affects phase angle difference, free-running period and phase shifts in *Aedes krombeini* (Stegomyia). *Biol. Rhythm Res. 27*, 421–430.

Jurenka, R.A., Jacquin, E., Roelofs, W.L., **1991**. Stimulation of pheromone biosynthesis in the moth

Helicoverpa zea: action of a brain hormone on pheromone glands involves Ca^{2+} and cAMP as second messengers. *Proc. Natl Acad. Sci. USA 88*, 8621–8625.

Kadono-Okuda, K., Amornsak, W., Yamashita, O., **1994**. Controlled ecdysteroid accumulation in eggs of the silkworm, *Bombyx mori*, by an imidazole compound (KK-42) and embryogenesis in these eggs. *Arch. Insect Biochem. Physiol. 25*, 121–135.

Kajiura, Z., Nakagaki, M., Takei, R., **1993**. Spermiogenesis of the testes of juvenile hormone-treated silkworm larvae, *Bombyx mori*. *Comp. Biochem. Physiol. A 106*, 495–499.

Kalogianni, E., Theophilidis, G., **1993**. Centrally generated rhythmic activity and modulatory function of the oviductal dorsal unpaired median (DUM) neurons in two orthopteran species (*Calliptamus* sp. and *Decticus albifrons*). *J. Exp. Biol. 174*, 123–138.

Kalsbeek, A., Buijs, R.M., **2002**. Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell Tissue Res. 309*, 109–118.

Kamimura, M., Tatsuki, S., **1993**. Diel rhythms of calling behavior and pheromone, production of Oriental tobacco budworm moth, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Chem. Ecol. 19*, 2953–2963.

Kamimura, M., Tatsuki, S., **1994**. Effects of photoperiodic changes on calling behavior and pheromone production in the Oriental tobacco budworm moth, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol. 40*, 731–734.

Kaneko, J., **1986**. Effect of temperature on the timing of calling of the yellow peach moth, *Congethes punctiferalis* (Guenee) (Lepidoptera: Pyralidae). *Jap. J. Appl. Entomol. Zool. 30*, 239–246.

Kaneko, M., Hall, J.C., **2000**. Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol. 422*, 66–94.

Kaneko, M., Helfrich-Förster, C., Hall, J.C., **1997**. Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. *J. Neurosci. 17*, 6745–6760.

Katsoyannos, B.I., **1982**. Male sex pheromone of *Rhagoletis ceraci* (Diptera: Tephritidae): factors affecting release and response and its role in the mating behavior. *Z. Angew. Entomol. 94*, 187–198.

Kingan, T.G., Adams, M.E., **2000**. Ecdysteroids regulate secretory competence in Inka cells. *J. Exp. Biol. 203*, 3011–3018.

Kingan, T.G., Gray, W., Žitnan, D., Adams, M.E., **1997**. Regulation of ecdysis-triggering hormone secretion by eclosion hormone. *J. Exp. Biol. 200*, 3245–3256.

Kingan, T.G., Raina, A.K., Blackburn, M., Ma, M., **1990**. Distribution of PBAN-like immunoreactivity in the CNS of the corn earworm, *Heliothis zea*. *Neurosci. Abstr. 16*, 856.

Kitamura, A., Nagasawa, H., Kataoka, H., Inoue, T., Matsumoto, S., *et al.*, **1989**. Amino acid sequence of pheromone biosynthesis-activating-neuropeptide (PBAN) of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun. 163*, 520–526.

Knobloch, C.A., Steel, C.G.H., **1987**. Effects of decapitation at the head critical period for molting on hemolymph ecdysteroid titers in final-instar male and female *Rhodnius prolixus* (Hemiptera). *J. Insect Physiol. 33*, 967–972.

Koeppe, J.K., Fuchs, M., Chen, T.T., Hunt, L.-M., Kovalick, G.E., *et al.*, **1985**. The role of juvenile hormone in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 165–203.

Konopka, R.J., Kyriakou, C.P., Hall, J.C., **1996**. Mosaic analysis in the *Drosophila* CNS of circadian and courtship-song rhythms affected by a *period* clock mutation. *J. Neurogen. 11*, 117–139.

Kopec, S., **1922**. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull. 42*, 323–342.

Kou, R., **1992**. Calling behavior and pheromone titer in the smaller tea tortrix moth, *Adoxophyes* spp. (Lepidoptera: Tortricidae). *J. Chem. Ecol. 18*, 855–861.

Kou, R., Chow, Y.S., **1987**. Calling behavior of the cotton bollworm, *Heliothis armigera* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. America 80*, 490–493.

Koudele, K., Stout, J.F., Reichert, D., **1987**. Factors which influence female crickets' (*Acheta domesticus*) phonotactic and sexual responsiveness to males. *Physiol. Entomol. 12*, 67–80.

Krishnan, B., Dryer, S.E., Hardin, P.E., **1999**. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature 400*, 375–378.

Krishnan, B., Levine, J.D., Lynch, K.S., Dowse, H.B., Funes, P., *et al.*, **2001**. A novel role for cryptochrome in a *Drosophila* circadian oscillator. *Nature 411*, 313–317.

Kristensen, B.I., **1966**. Incorporation of tyrosine into the rubber-like cuticle of locusts studied by autoradiography. *J. Insect Physiol. 12*, 173–177.

Kubli, E., **1992**. The sex peptide. *BioEssays 14*, 779–784.

Kuwahara, Y., Adachi, S., Tsuchida, N., **1983**. Bombykol content in female silkworm moth, *Bombyx mori* (Lepidoptera: Bombycidae): effect of age, mating and body weight. *Appl. Entomol. Zool. 18*, 182–190.

Kyriakou, C.P., Hall, J.C., **1980**. Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc. Natl Acad. Sci. USA 77*, 6729–6733.

Kyriakou, C.P., Hall, J.C., **1982**. The function of courtship song rhythms in *Drosophila*. *Anim. Behav. 30*, 794–801.

LaChance, L.E., Richard, R.D., Ruud, R.L., **1977**. Movement of eupyrene sperm bundles from the testis and storage in the ductus ejaculatoris duplex of the male

pink bollworm: effects of age, strain, irradiation, and light. *Ann. Entomol. Soc. America 70*, 647–651.

Lagueux, M., Hetru, C., Goltzene, E., Kappler, C., Hoffmann, J.A., **1979**. Ecdysone titer and metabolism in relation to cuticulogenesis in embryos of *Locusta migratoria*. *J. Insect Physiol. 25*, 709–723.

Lall, A.B., **1993**. Nightly increase in visual sensitivity correlated with bioluminescent flashing activity in the firefly *Photuris versicolor* (Coleoptera: Lampyridae). *J. Exp. Zool. 265*, 609–612.

Lammerding-Köppel, M., Spindler-Barth, M., Steiner, E., Lezzi, M., Drews, U., *et al.*, **1998**. Immunohistochemical localization of ecdysteroid receptor and ultraspiracle in the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). *Tissue Cell 30*, 187–194.

Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F.C., Miller, C.A., *et al.*, **1985**. Titers of ecdysone, 20-hydroxyecdysone and juvenile hormone III throughout the life cycle of a hemimetabolous insect, the ovoviviparous cockroach *Nauphoeta cinerea*. *Experientia 41*, 913–917.

Leal, W.S., Hasegawa, M., Sawada, M., Ono, M., Tada, S., **1996**. Scarab beetle *Anomala albopilosa* utilizes a more complex sex pheromone system than a similar species *A. cuprea*. *J. Chem. Ecol. 22*, 2001–2010.

Lee, H.J., Wu, Y.L., **1994**. Mating effects on the feeding and locomotion of the German cockroach, *Blattella germanica*. *Physiol. Entomol. 19*, 39–45.

Lee, H.T.Y., **1948**. A comparative morphological study of prothoracic glandular bands of some lepidopteran larvae with special reference to their innervation. *Ann. Entomol. Soc. America 41*, 200–205.

Leitch, B., Laurent, G., Shepherd, D., **1992**. Embryonic development of synapses on spiking local interneurons in locust. *J. Comp. Neurol. 324*, 213–236.

Leppla, N.C., Koehler, P.G., Agee, H.R., **1989**. Circadian rhythms of the German cockroach (Dictyoptera: Blattellidae): locomotion in response to different photoperiods and wavelengths of light. *J. Insect Physiol. 35*, 63–66.

Lewy, A.J., Sack, R.L., Blood, M.L., Bauer, V.K., Cutler, N.L., *et al.*, **1995**. Melatonin marks circadian phase position and resets the endogenous circadian pacemaker in humans. In: Chadwick, D.J., Ackrill, K. (Eds.), Circadian Clocks and their Adjustment. Wiley, New York, pp. 303–321.

Lezzi, M., Gatzka, F., Ineichen, H., Gruzdev, A.D., **1991**. Transcriptional activation of puff site I-18C of *Chironomus tentans*: hormonal responsiveness changes in parallel with diurnal decondensation cycle. *Chromosoma 100*, 235–241.

Liang, D., Schal, C., **1990**. Circadian rhythmicity and development of the behavioral response to sex pheromone in male brown-banded cockroaches, *Supella longipalpa*. *Physiol. Entomol. 15*, 355–361.

Lin, T.M., Lee, H.J., **1998**. Parallel control mechanisms underlying locomotor activity and sexual receptivity of the female German cockroach, *Blattella germanica* (L.). *J. Insect Physiol. 44*, 1039–1051.

Linn, C.E., **1997**. Neuroendocrine factors in the photoperiodic control of male moth responsiveness to sex pheromone. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, London, pp. 194–209.

Linn, C.E., Cambell, M.G., Poole, K.R., Wu, W.-Q., Roelofs, W.L., **1996**. Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: relationship to the modulatory action of octopamine. *J. Insect Physiol. 42*, 881–891.

Linn, C.E., Cambell, M.G., Roelofs, W.L., **1992**. Photoperiodic cues and the modulatory action of octopamine and 5–hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar. Arch. Insect Biochem. Physiol. 20*, 265–284.

Linn, C.E., Poole, K.R., Wu, W.Q., Roelofs, W.L., **1995**. Circadian changes in melatonin in the nervous system and hemolymph of the cabbage looper moth, *Trichoplusia ni*. *J. Comp. Physiol. A 176*, 761–771.

Linn, C.E., Roelofs, W.L., **1992**. Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. *Arch. Insect Biochem. Physiol. 20*, 285–302.

Liu, X., Lorenz, L., Yu, Q., Hall, J.C., Rosbash, M., **1988**. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster. Genes Devel. 2*, 228–238.

Loeb, M.J., Brandt, E.P., Birnbaum, M.J., **1984**. Ecdysteroid production by testes of the tobacco budworm, *Heliothis virescens*, from last larval instar to adult. *J. Insect Physiol. 30*, 375–381.

Loeb, M.J., Brandt, E.P., Woods, C.W., Bell, R.A., **1988**. Secretion of ecdysteroid by sheaths of testes of the gypsy moth, *Lymantria dispar*, and its regulation by testis ecdysiotropin. *J. Exp. Zool. 248*, 94–100.

Löfstedt, L., Van Der Pers, J.N.C., Lofqvist, J., Lanne, B.S., Appelgren, M., *et al.*, **1982**. Sex pheromone components of the turnip moth, *Agrotis segetum*: chemical identification, electrophysiological evaluation and behavioral activity. *J. Chem. Ecol. 8*, 1305–1321.

Loher, W., Zervas, G., **1979**. The mating rhythm of the olive fruit fly, *Dacus oleae*. *Z. Angew. Entomol. 88*, 425–435.

Lukat, R., Weber, F., Wiedenmann, G., **1989**. Cyclic layer deposition in the cockroach (*Blaberus craniifer*) endocuticle: a decentral circadian clock? *J. Insect Physiol. 35*, 321–329.

Ma, P.W.K., Garden, R.W., Niermann, J.T., O'Connor, M., Sweedler, J.V., *et al.*, **2000**. Characterizing the Hez-PBAN gene products in neuronal clusters with immunocytochemistry and MALDI MS. *J. Insect Physiol. 46*, 221–230.

Ma, P.W.K., Knipple, D.C., Roelofs, W.L., **1998**. Expression of a gene that encodes pheronome biosynthesis activating neuropeptide in the central nervous system of corn earworm, *Helicoverpa zea*. *Insect Biochem. Mol. Biol. 28*, 373–385.

Ma, P.W.K., Roelofs, W.L., **1995**. Sites of synthesis and release of PBAN-like factor in the female European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 41*, 339–350.

Martinez, T., Camps, F., **1988**. Stimulation of sex pheromone production by head extract in *Spodoptera littoralis* at different times of the photoperiod. *Arch. Insect Biochem. Physiol. 9*, 211–220.

Masler, E.P., Raina, A.K., Wagner, R.M., Kochansky, J.P., **1994**. Isolation and identification of a pheromonotropic neuropeptide from the brain–suboesophageal ganglion complex of *Lymantria dispar*: a new member of the PBAN family. *Insect Biochem. Mol. Biol. 24*, 829–836.

Matsumoto, S., Ozawa, R., Nagamin, T., Gil-Hah, K., Uchiumi, K., *et al.*, **1995**. Intracellular transduction in the regulation of pheromone biosynthesis of the silkworm, *Bombyx mori*: suggested involvement of calmodulin and phosphoprotein phosphatase. *Biosci. Biotechnol. Biochem. 59*, 560–562.

Maywood, E.S., Okamura, H., Hastings, M.H., **2002**. Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. *Eur. J. Neurosci. 15*, 216–220.

Mbata, G.N., Ramaswamy, S.B., **1990**. Rhythmicity of sex pheromone content in female *Heliothis virescens*: impact of mating. *Physiol. Entomol. 15*, 423–432.

McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., *et al.*, **1997**. Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron 19*, 813–823.

McNamara, P., Seo, S.B., Rubic, R.D., Seghali, A., Chakravarti, D., *et al.*, **2001**. Regulation of CLOCK and MOP4 by nuclear hormone receptors in vasculature: a humoral mechanism to reset a peripheral clock. *Cell 105*, 877–889.

Mesce, K.A., Fahrbach, S.E., **2002**. Integration of endocrine signals that regulate insect ecdysis. *Frontiers Neuroendocrin. 23*, 179–199.

Minis, D.H., Pittendrigh, C.S., **1968**. Circadian oscillation controlling hatching: its ontogeny during embryogenesis of a moth. *Science 159*, 534–536.

Mizoguchi, A., Dedos, S.G., Fugo, H., Kataoka, H., **2002**. Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol. 127*, 181–189.

Mizoguchi, A., Ishizaki, H., **1982**. Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. Natl Acad. Sci. USA 79*, 2726–2730.

Mizoguchi, A., Ishizaki, H., **1984a**. Circadian clock controlling gut-purge rhythm of the saturniid *Samia cynthia ricini*: its characterization and entrainment mechanism. *J. Comp. Physiol. A 155*, 639–647.

Mizoguchi, A., Ishizaki, H., **1984b**. Further evidence for the presence of a circadian clock in the prothoracic glands of the suturniid moth *Samia cynthia ricini*: decapitated larvae can respond to light-dark changes. *Devel. Growth Differ. 26*, 607–611.

Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., **2001**. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol. 31*, 349–358.

Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., *et al.*, **1990**. Immunohistochemical localization of prothoracicotropic-hormone producing neurosecretory cells in the brain of *Bombyx mori*. *Devel. Growth Differ. 32*, 501–598.

Molis, T.M., Spriggs, L.L., Hill, S.M., **1994**. Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. *Mol. Endocrinol. 8*, 1681–1690.

Morton, D.B., Truman, J.W., **1988**. The EGPs: the eclosion hormone and cyclic GMP-regulated phosphoproteins. 2. Regulation of appearance by the steroid hormone 20-hydroxyecdysone in *Manduca sexta*. *J. Neurosci. 8*, 1338–1345.

Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klauser, S., *et al.*, **1996**. Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol. 32*, 363–374.

Mullins, D.E., Keil, C.B., White, R.H., **1992**. Maternal and paternal nitrogen investment in *Blattella germanica* (L.) (Dictyoptera: Blattellidae). *J. Exp. Biol. 162*, 55–72.

Nässel, D.R., **2000**. Functional roles of neuropeptides in the insect central nervous system. *Naturwissenschaften 87*, 439–449.

Nässel, D.R., Shiga, S., Wikstrand, E.M., Rao, K.R., **1991**. Pigment-dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual system. *Cell Tissue Res. 265*, 511–523.

Nation, J.L., **1990**. Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *J. Chem. Ecol. 16*, 553–572.

Neville, A.C., **1965**. Circadian organization of chitin in some insect skeletons. *Q. J. Microsc. Sci. 106*, 315–325.

Neville, A.C., **1967**. A dermal light sense influencing skeletal structures in locusts. *J. Insect Physiol. 13*, 933–939.

Neville, A.C., **1975**. Biology of Arthropod Cuticle. Springer, Berlin.

Neville, A.C., **1983**. Daily cuticular growth layers and the teneral stage in adult insects: a review. *J. Insect Physiol. 29*, 211–219.

Nickisch-Rosenegk, E. von, Krieger, J., Kubick, S., Laage, R., Strobel, J., *et al.*, **1996**. Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol. 26*, 817–827.

Nijhout, H.F., **1994**. Insect Hormones. Princeton University Press, Princeton, NJ.

Noldus, L.P.J.J., Potting, R.P.J., **1990**. Calling behavior of *Mamestra brassicae* (Lepidopter: Noctuidae): effects of age and photoperiod. *Entomol. Exp. Applic. 56*, 23–30.

Nonaka, H., Emoto, N., Ikeda, K., Fukuya, H., Rohman, M.S., *et al.*, **2001**. Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells. *Circulation 104*, 1746–1748.

Noronha, K.F., Lange, A.B., **1997**. Proctolin's role in neurally evoked contractions of the locust oviducts. *J. Neurobiol. 33*, 139–150.

O'Brien, M.A., Katahira, E.J., Flanagan, T.R., Arnold, L.W., Haughton, G., *et al.*, **1988**. A monoclonal antibody to the insect prothoracicotropic hormone. *J. Neurosci. 8*, 3247–3257.

Ono, T., Charlton, R.E., Cardé, R.T., **1990**. Variability in pheromone composition and periodicity of pheromone titer in potato tuberworm moth, *Phthorimaea oerculella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol. 16*, 531–542.

Orchard, I., Donly, B.C., Fuse, M., Lange, A.B., Tobe, S.S., *et al.*, **1997**. FMRFamide-related peptides in insects, with emphasis on the myosuppressins. *Neuropept. Devel. Aging 814*, 307–309.

Orchard, I., Lange, A.B., **1988**. The regulation of insect visceral muscle by octopamine. In: Boulton, A.A., Juorio, A.V., Downe, R.G. (Eds.), Trace Amines. Humana Press, Totowa, NJ, pp. 41–51.

Ottiger, M., Soller, M., Stocker, R.F., Kubli, E., **2000**. Binding sites of *Drosophila melanogaster* sex peptide pheromones. *J. Neurobiol. 44*, 57–71.

Page, T.L., **1983**. Regeneration of the optic lobe tracts and circadian pacemaker activity in the cockroach *Leucophaea maderae*. *J. Comp. Physiol. A 152*, 231–240.

Page, T.L., **1985**. Clocks and circadian rhythms. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 6. Pergamon, Oxford, pp. 577–652.

Page, T.L., **1988**. Circadian organization and the representation of circadian information in the nervous system of invertebrates. *Adv. Biosci. 73*, 67–79.

Pang, S.F., Li, L., Ayre, E.A., Pang, C.S., Lee, P.P.N., *et al.*, **1998**. Neuroendocrinology of melatonin in reproduction: recent developments. *J. Chem. Neuroanat. 14*, 157–166.

Park, C., Jeon, S.K., Kim, M.Y., Han, S.S., Yu, C.H., *et al.*, **2001**. Postembryonic localization of allatotropin- and allatostatin-producing cells in central nervous system of the silk moth, *Bombyx mori*. *Zool. Sci. 18*, 367–379.

Park, Y., Filippov, V., Gill, S.S., Adams, M.E., **2002**. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development 129*, 493–503.

Pelc, D., Steel, C.G.H., **1997**. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen. Comp. Endocrinol. 108*, 358–365.

Petri, B., Homberg, U., Loesel, R., Stengl, M., **2002**. Evidence for the role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol. 205*, 1459–1469.

Petri, B., Stengl, M., **1997**. Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci. 17*, 4087–4093.

Petri, B., Stengl, M., Wuerden, S., Homberg, U., **1995**. Immunocytochemical characterization of the accessory medulla in the cockroach *Leucophaea maderae*. *Cell Tissue Res. 282*, 3–19.

Picimbon, J.-F., Bécard, J.-M., Sreng, L., Clément, J.-L., Gadenne, C., **1995**. Juvenile hormone stimulates pheromonotropic brain factor release in the female black cutworm, *Agrotis ipsilon*. *J. Insect Physiol. 41*, 377–382.

Picimbon, J.-F., Gadenne, C., Bécard, J.M., Clément, J.L., Sreng, L., **1997**. Sex pheromone of the French black cutworm moth, *Agrotis ipsilon* (Lepidoptera: Noctuidae): identification and regulation of a multicomponent blend. *J. Chem. Ecol. 23*, 211–229.

Pickard, G.E., Tang, W.-X., **1994**. Pineal photoreceptors rhythmically secrete melatonin. *Neurosci. Lett. 171*, 109–112.

Pittendrigh, C.S., **1993**. Temporal organization: reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol. 55*, 17–54.

Pivnic, K.A., **1993**. Response of males to female sex pheromone in the orange blossom midge, *Sitodiplosis mosellana* (Gehin) (Diptera: Cecidomyiidae). *J. Chem. Ecol. 19*, 1677–1689.

Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A., **1997**. Independent photoreceptive circadian clocks throughout *Drosophila*. *Science 278*, 1632–1635.

Pope, M.M., Gaston, L.K., Baker, T.C., **1982**. Composition, quantification and periodicity of sex pheromone gland volatiles from individual *Heliothis virescens* females. *J. Chem. Ecol. 8*, 1043–1056.

Pophof, B., **2000**. Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. *J. Comp. Physiol. A 186*, 307–313.

Pophof, B., **2002**. Octopamine enhances moth olfactory responses to pheromone, but not those to general odorants. *J. Comp. Physiol. A 188*, 659–662.

Predel, R., Eckert, M., **2000**. Neurosecretion: peptidergic systems in insects. *Naturwissenschaften 87*, 343–350.

Rafaeli, A., **1994**. Pheromonotropic stimulation of moth pheromone gland cultures *in vitro*. *Arch. Insect Biochem. Physiol. 25*, 287–299.

Rafaeli, A., Gileadi, C., **1997**. Neuroendocrine control of pheromone production in moths. *Invert. Neurosci. 3*, 223–229.

Rafaeli, A., Gileadi, C., Fan, Y., Cao, M., **1997**. Physiological mechanisms of pheromonostatic responses: effects of adrenergic agonists and antagonists on moth (*Helicoverpa armigera*) pheromone biosynthesis. *J. Insect Physiol. 43*, 261–269.

Rafaeli, A., Hirsch, J., Soroker, V., Kamensky, B., Raina, A.K., **1991**. Spatial and temporal distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa* (*Heliothis*) *armigera* using RIA and *in vitro* bioassay. *Arch. Insect Biochem. Physiol. 18*, 119–129.

Rafaeli, A., Soroker, V., **1989**. Influence of diel rhythm and brain hormone on pheromone production in two lepidopteran species. *J. Chem. Ecol. 15*, 447–456.

Rafaeli, A., Soroker, V., Hirch, J., Kamensky, B., Raina, A.K., **1993**. The influence of photoperiod and age on the competence of pheromone glands and the distribution of immunoreactive PBAN in *Helicoverpa* spp. *Arch. Insect Biochem. Physiol. 22*, 169–180.

Rafaeli, A., Zakharova, T., Lapsker, Z., Jurenka, R.A., **2003**. The identification of an age- and female-specific putative PBAN membrane-receptor protein in pheromone glands of *Helicoverpa armigera*: possible up-regulation by juvenile hormone. *Insect Biochem. Mol. Biol. 33*, 371–380.

Raina, A.K., Jaffe, H., Kempe, T.G., Keim, P., Blacher, R.W., *et al.*, **1989**. Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science 244*, 796–798.

Raina, A.K., Klun, J.A., **1984**. Brain factor control of sex pheromone production in the female corn earworm moth. *Science 225*, 531–533.

Ramaswamy, S.B., Cardé, R.T., **1984**. Rate of release of spruce budworm (*Choristoneura fumiferana*) pheromone from virgin females and synthetic lures. *J. Chem. Ecol. 10*, 1–8.

Ramaswamy, S.B., Jurenka, R.A., Linn, C.E., Roelofs, W.L., **1995**. Evidence for the presence of a pheromonotropic factor in hemolymph and regulation of sex pheromone production in *Helicoverpa zea*. *J. Insect Physiol. 41*, 501–508.

Ramaswamy, S.B., Mbata, G.N., Cohen, N.E., Moore, A., Cox, N.M., **1994**. Pheromonotropic and pheromonostatic activity in moths. *Arch. Insect Biochem. Physiol. 25*, 301–315.

Rao, K.R., Riehm, J.P., **1993**. Pigment-dispersing hormones. *Ann. New York Acad. Sci. 680*, 78–88.

Reischig, T., Stengl, M., **1997**. The accessory medulla is the presumptive circadian pacemaker in the cockroach *Leucophaea maderae*. *Verhandl. Deut. Zool. Gesells. 89–3*, 45.

Rence, B.G., Lisy, M.T., Garves, B.R., Quinlan, B.J., **1988**. The role of ocelli in circadian singing rhythms of crickets. *Physiol. Entomol. 13*, 201–212.

Reppert, S.M., Weaver, D.R., **2001**. Molecular analysis of mammalian circadian rhythms. *Ann. Rev. Physiol. 63*, 647–676.

Reynolds, S.E., **1980**. Integration of behavior and physiology in ecdysis. *Adv. Insect Physiol. 15*, 475–696.

Reynolds, S.E., **1987**. Endocrine timing signals that direct ecdysial physiology and behavior. In: Borkovec, A.B., Gelman, D. (Eds.), Insect Neurochemistry and Neurophysiology. Humana Press, Totowa, NJ, pp. 53–77.

Richards, G., **1981**. Insect hormones in development. *Biol. Rev. 56*, 501–549.

Richter, K., **2001**. Daily changes in neuroendocrine control of molting hormone secretion in the prothoracic gland of the cockroach, *Periplaneta americana* (L.). *J. Insect Physiol. 47*, 333–338.

Richter, K., Pescjke, E., Peschke, D., **2000**. A neuroendocrine releasing effect of melatonin in the brain of an insect, *Periplaneta americana* (L). *J. Pineal Res. 28*, 129–135.

Riddiford, L.M., **1985**. Hormone action at the cellular level. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 37–84.

Riddiford, L.M., Cherbas, P., Truman, J.W., **2000**. Ecdysone receptors and their biological actions. *Vitamins Hormones 60*, 1–73.

Riddiford, L.M., Hewes, R.S., Truman, J.W., **1994**. Dynamics and metamorphosis of an identifiable peptidergic neuron in an insect. *J. Neurobiol. 25*, 819–830.

Riedl, H., Loher, W., **1980**. Circadian control of oviposition in the codling moth, *Laspeyresia pomonella*, Lepidoptera: Olethreutidae. *Entomol. Exp. Applic. 27*, 38–49.

Riemann, J.G., Giebultowicz, J.M., **1991**. Secretion in the upper vas deferens of the gypsy moth correlated with the circadian rhythm of sperm release from the testes. *J. Insect Physiol. 37*, 53–62.

Riemann, J.G., Thorson, B.J., **1971**. Sperm maturation in the male and female genital tract of *Anagasta kühniella* (Lepidoptera: Pyralididae). *Int. J. Insect Morphol. Embryol. 1*, 11–19.

Riemann, J.G., Thorson, B.J., **1976**. Ultrastructure of the vasa deferentia of the Mediterranean flour moth. *J. Morphol. 149*, 483–506.

Riemann, J.G., Thorson, B.J., Ruud, R.L., **1974**. Daily cycle of release of sperm from the testes of the Mediterranean flour moth. *J. Insect Physiol. 20*, 195–207.

Rosén, W.-Q., **2002**. Endogenous control of circadian rhythms of pheromone production in the turnip moth, *Agrotis segetum*. *Arch. Insect Biochem. Physiol. 50*, 21–30.

Roy, E.J., Wilson, M.A., **1981**. Diurnal rhythm of cytoplasmic estrogen receptors in the rat brain in the absence of circulating estrogens. *Science 213*, 1525–1527.

Saez, L., Young, M.W., **1988**. In *situ* localization of the PER clock protein during development of *Drosophila melanogaster*. *Mol. Cell Biol. 8*, 5378–5385.

Saifullah, A.S.M., Tomioka, K., **2002**. Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket, *Gryllus bimaculatus*. *J. Exp. Biol. 205*, 1305–1314.

Saifullah, A.S.M., Tomioka, K., **2003**. Pigment-dispersing factor sets the night state of the medulla bilateral neurons in the optic lobe of the cricket, *Gryllus bimaculatus*. *J. Insect Physiol. 49*, 231–239.

Sakai, T., Ishida, N., **2001**. Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natl Acad. Sci. 98*, 9221–9225.

Sakamoto, K., Shimizu, I., **1994**. Photosensitivity in the circadian hatching rhythm of the carotenoid-depleted silkworm, *Bombyx mori*. *J. Biol. Rhythms 9*, 61–70.

Sakurai, S., **1983**. Temporal organization of endocrine events underlying larval–larval ecdysis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* *29*, 919–932.

Sakurai, S., **1984**. Temporal organization of endocrine events underlying larval–pupal metamorphosis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* *30*, 657–664.

Sakurai, S., Kaya, M., Satake, S., **1998**. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the larval–pupal stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval–pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol.* *44*, 867–881.

Sall, C., Tsoupras, G., Kappler, C., Lagueux, M., Zachary, D., *et al.*, **1983**. Maternal conjugated ecdysteroids during embryonic development in *Locusta migratoria*. *J. Insect Physiol.* *29*, 491–507.

Sasaki, M., Jibiki, F., Hirobe, T., **1984**. Comparison of the daily rhythmic behavior between wild and domestic silkmoths, *Bombyx mandarina* and *Bombyx mori*. *Bull. Fac. Agric. Tamagawa Univ.* *24*, 26–42.

Sasaki, M., Yamazaki, S., Chiba, K., **1987**. Brain photoreception in the calling rhythm of a noctuid moth, *Anadevidia peponis*: method of making eyeless moth and micro-irradiation of brain with fiber optics. *Bull. Fac. Agric. Tamagawa Univ.* *27*, 81–92.

Sato, Y., Oguchi, M., Menjo, N., Imai, K., Saito, H., *et al.*, **1993**. Precursor polyprotein for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. *Proc. Natl Acad. Sci. USA* *90*, 3251–3255.

Sauman, I., Reppert, S.M., **1996a**. Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. *Neuron* *17*, 889–900.

Sauman, I., Reppert, S.M., **1996b**. Molecular characterization of prothoracicotropic hormone (PTTH) from the giant silkmoth *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relationship to circadian clock cells in central brain. *Devel. Biol.* *178*, 418–429.

Sauman, I., Reppert, S.M., **1998**. Brain control of embryonic circadian rhythms in the silkmoth *Antheraea pernyi*. *Neuron* *20*, 741–748.

Sauman, I., Tsai, T., Roca, A.L., Reppert, S.M., **1996**. Period protein is necessary for circadian control of egg hatching behavior in the silkmoth *Antheraea pernyi*. *Neuron* *17*, 901–909.

Saunders, D.S., **2002**. (with Steel, C.G.H., Vafopoulou, X., Lewis, R.D.). Insect Clocks. Elsevier, Amsterdam, p. 560.

Schal, C., Cardé, R.T., **1986**. Effects of temperature and light on calling in the tiger moth *Holomelina lamae* (Freeman) (Lepidoptera: Arctiidae). *Physiol. Entomol.* *11*, 75–87.

Schwartz, L.M., Truman, J.W., **1983**. Hormonal control of rates of metamorphic development in the tobacco hornworm *Manduca sexta*. *Devel. Biol.* *99*, 103–114.

Sefiani, M., **1987**. Regulation of egg laying and *in vitro* contractions in *Gryllus bimaculatus*. *J. Insect Physiol.* *33*, 215–222.

Sehadová, H., Sauman, I., Sehnal, F., **2003**. Immunocytochemical distribution of pigment-dispersing hormone in the cephalic ganglia of polyneopteran insects. *Cell Tissue Res.* *312*, 113–125.

Seth, R.K., Rao, D.K., Reynolds, S.E., **2002**. Movement of spermatozoa in the reproductive tract of adult male *Spodoptera litura*: daily rhythm of sperm descent and the effect of light regime on male reproduction. *J. Insect Physiol.* *48*, 119–131.

Shearer, P.W., Jones, V.P., Riedl, H., **1995**. Diel periodicity and circadian control of oviposition by *Cryptophlebia illepida* (Lepidoptera: Torticidae). *Environ. Entomol.* *24*, 1229–1233.

Shimizu, I., Kawai, Y., Taniguchi, M., Aoki, S., **2001**. Circadian rhythm and cDNA cloning of the clock gene *period* in the honeybee, *Apis cerana japonica*. *Zool. Sci.* *18*, 779–789.

Shirai, Y., Aizono, Y., Iwasaki, T., Yanagida, A., Mori, H., *et al.*, **1993**. Prothoracicotropic hormone is released five times in the 5th-larval instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* *39*, 83–88.

Shorey, H.H., **1966**. The biology of *Trichoplusia ni*. 4. Environmental control of mating. *Ann. Entomol. Soc. America* *59*, 502–506.

Shorey, H.H., Gaston, L.K., **1965**. Sex pheromones of noctuid moths. 5. Circadian rhythm of pheromone-responsiveness in males of *Autographa californica, Heliothis virescens, Spodoptera exigua* and *Trichoplusia ni*. *Ann. Entomol. Soc. America* *58*, 597–611.

Shu, S., Koepnick, W.L., Mbata, G.N., Cork, A., Ramaswamy, S.B., **1996**. Sex pheromone production in *Callosobruchus maculatus* (Coleoptera: Bruchidae): electroantennographic and behavioral responses. *J. Stored Prod. Res.* *32*, 21–30.

Shu, S., Park, Y.I., Ramaswamy, S.B., Srinivasan, A., **1998**. Temporal profiles of juvenile hormone titers and egg production in virgin and mated females of *Heliothis virescens* (Noctuidae). *J. Insect Physiol.* *44*, 1111–1117.

Siegmund, T., Korge, G., **2001**. Innervation of the ring gland of *Drosophila*. *J. Comp. Neurol.* *431*, 481–491.

Simmons, L.W., Gwynne, D.T., **1993**. Reproductive investment in bushcrickets: the allocation of male and female nutrients to offspring. *Proc. R. Soc. London B* *252*, 1–5.

Slama, K., **1980**. Homeostatic function of ecdysteroids in ecdysis and oviposition. *Acta Entomol. Bohemoslavaca* *77*, 145–168.

Slinger, A.J., Isaac, R.E., **1988**. Ecdysteroid titers during embryogenesis of the cockroach *Periplaneta americana*. *J. Insect Physiol.* *34*, 1119–1126.

Smith, A.F., Schal, C., **1991**. Circadian calling behavior of the adult female brown-banded cockroach, *Supella longipalpa* (F.) (Dictyoptera: Blattellidae). *J. Insect Behav.* *4*, 1–14.

Smith, W.A., Rountree, D.B., Bollenbacher, W.E., Gilbert, L.I., **1986**. Dissociation of the prothoracic glands of *Manduca sexta* into hormone-responsive cells. In: Borkovec, A.B., Gelman, D.B. (Eds.), Insect Neurochemistry and Neurophysiology. Humana Press, Totowa, NJ, pp. 319–322.

So, W.V., Sarov-Blat, L., Kotarski, C.K., McDonald, M.J., Allada, R., *et al.*, **2000**. *Takeout*, a novel *Drosophila* gene under circadian clock transcriptional regulation. *Mol. Cell Biol. 20*, 6935–6944.

Sokolove, P.G., **1975**. Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res. 87*, 13–21.

Sokolove, P.G., Loher, W., **1975**. Role of eyes, optic lobes and pars intercerebralis in locomotor and stridulatory circadian rhythms of *Teleogryllus commodus*. *J. Insect Physiol. 21*, 785–799.

Soller, M., Bownes, M., Kubli, E., **1997**. Mating and sex-peptide stimulate the accumulation of yolk in oocytes of *Drosophila melanogaster. Eur. J. Biochem. 243*, 732–738.

Soller, M., Bownes, M., Kubli, E., **1999**. Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol. 208*, 337–351.

Sower, L.L., Shorey, H.H., Gaston, L.K., **1970**. Sex pheromones of noctuid moths. 21. Light–dark cycle regulation and light inhibition of the sex pheromone release by females of *Trichoplusia ni*. *Ann. Entomol. Soc. America 63*, 1090–1092.

Stanewsky, R., **2002**. Clock mechanisms in *Drosophila*. *Cell Tissue Res. 309*, 11–26.

Stanewsky, R., **2003**. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J. Neurobiol. 54*, 111–147.

Stanley-Samuelson, D.W., **1994**. Prostaglandins and related eicosanoids in insects. *Adv. Insect Physiol. 24*, 115–212.

Stay, B., Tobe, S.S., Bendena, W.G., **1994**. Allatostatins: identification, primary structures, functions and distribution. *Adv. Insect Physiol. 25*, 267–337.

Steel, C.G.H., Ampleford, E.J., **1984**. Circadian control of hemolymph ecdysteroid titres and the ecdysis rhythm in *Rhodnius prolixus*. In: Porter, R., Collins, G.M. (Eds.), Photoperiodic Regulation of Insect and Molluscan Hormones. Pitman Press, London, pp. 150–169.

Steel, C.G.H., Davey, K.G., **1985**. Integration in the insect endocrine system. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 1–35.

Steel, C.G.H., Vafopoulou, X., **1989**. Ecdysteroid titer profiles during growth and development of arthropods. In: Koolman, J. (Ed.), Ecdysone. Georg Thieme Verlag, Stuttgart, pp. 221–231.

Steel, C.G.H., Vafopoulou, X., **1999**. A tango of hormone rhythms directs insect development: circadian regulation of prothoracicotropic hormone and ecdysteroids. In: Roubos, E.W., Wenderlaar-Bonga, S.E., Vaudry, H., DeLoof, A. (Eds.), Recent Developments in Comparative Endocrinology and Neurobiology. Shaker, Maastricht, pp. 94–97.

Steel, C.G.H., Vafopoulou, X., **2001**. Regulation of rhythmic steroidogenesis by light and neuropeptide inputs during development in the insect *Rhodnius prolixus*. In: Goos, H.J.T., Rastogi, R.K., Vaudry, H., Peirantoni, R. (Eds.), Perspectives in Comparative Endocrinology: Unity and Diversity. Monduzzi Editore, Bologna, pp. 301–307.

Steel, C.G.H., Vafopoulou, X., **2002**. Physiology of circadian systems. In: Saunders, D.S., with Steel, C.G.H., Vafopoulou, X., Lewis, R., Insect Clocks. Elsevier, Amsterdam, pp. 115–188.

Stengl, M., Homberg, U., **1994**. Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J. Comp. Physiol. A 175*, 203–213.

Stout, J.F., Atkins, G., Zacharias, D., **1991**. Regulation of cricket phonotaxis through hormonal control of the threshold of an identified auditory neuron. *J. Comp. Physiol. A 169*, 765–772.

Stout, J.F., Gerard, G., Hasso, S., **1976**. Sexual responsiveness mediated by the corpora allata and its relationship to phonotaxis in the female cricket, *Acheta domesticus*. *J. Comp. Physiol. A 108*, 1–10.

Stout, J.F., Hao, J., Kim, P., Mbungu, D., Bronsert, M., *et al.*, **1998**. Regulation of the phonotactic threshold of the female cricket, *Acheta domesticus*: juvenile hormone III, allatectomy, L1 auditory neuron thresholds and environmental factors. *J. Comp. Physiol. A 182*, 635–645.

Sybchev, M.A., Stamenova, M.B., Borisova, Y.A., **1986**. Effect of the time of the day, age of insects and pheromone dose on the sexual reaction of *Musca domestica* (Diptera; Muscidae). *Ekologiya (Sofia) 1*, 62–67.

Takeda, M., **1983**. Ontogeny of the circadian system governing ecdysial rhythms in a holometabolous insect, *Diatraea grandiosella* (Pyralidae). *Physiol. Entomol. 8*, 321–331.

Tang, J.D., Charlton, R.E., Cardé, R.T., Yin, C.M., **1992**. Diel periodicity and influence of age and mating on sex pheromone titer in gypsy moth *Limantria dispar* (L.). *J. Chem. Ecol. 18*, 749–760.

Tawata, M., Ichikawa, T., **2001**. Circadian firing of neurosecretory cells releasing pheromonotropic neuropeptides in the silkmoth, *Bombyx mori*. *Zool. Sci. 18*, 645–649.

Teal, P.E.A., Davis, N.T., Meredith, J.A., Christensen, T.A., Hildebrand, J.G., **1999a**. Role of the ventral nerve cord and terminal abdominal ganglion in the regulation of sex pheromone production in the tobacco budworm (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. America 92*, 891–901.

Teal, P.E.A., Meredith, J.A., Gomez, S.Y., **1999b**. Isolation and identification of terpenoid sex pheromone components from extracts of hemolymph of males of the Caribbean fruit fly. *Arch. Insect Biochem. Physiol. 42*, 225–232.

Teal, P.E.A., Tumlinson, J.H., Oberlander, H., **1989**. Neural regulation of sex pheromone biosynthesis in *Heliothis* moths. *Proc. Natl Acad. Sci. USA 86*, 2488–2492.

Temin, G., Zander, M., Roussel, J.P., **1986**. Physico-chemical (GC-MS) measurements of juvenile hormone III titers during embryogenesis of *Locusta migratoria*. *Int. J. Invert. Reprod. 9*, 105–112.

Terry, K.L., Steel, C.G.H., **2004**. Confocal analysis of PERIOD- and TIMELESS-immunoreactivity in the brain and prothoracic glands of *Rhodnius prolixus*: localization of potential circadian oscillators in the neuroendocrine system. *J. Comp. Neurol.* (in press).

Thompson, K.G., Siegler, M.V.S., **1993**. Development of segment specificity in identified lineages of the grasshopper CNS. *J. Neurosci. 13*, 3309–3318.

Thompson, K.J., Sivanesan, S.P., Campell, H.R., Sanders, K.J., **1999**. Efferent neurons and specialization of abdominal segments in grasshoppers. *J. Comp. Neurol. 415*, 65–79.

Thompson, M.J., Svodoba, J.A., Lozano, R., Wilzer, K.R., Jr., **1988**. Profile of free and conjugated ecdysteroids and ecdysteroid acids during embryonic development of *Manduca sexta* (L.) following maternal incorporation of carbon-14 cholesterol. *Arch. Insect Biochem. Physiol. 7*, 157–172.

Thorson, B.J., Riemann, J.G., **1977**. Abdominally entrained periodicities of testis and vas deferens activity in the Mediterranean flour moth. *J. Insect Physiol. 23*, 1189–1197.

Thorson, B.J., Riemann, J.G., **1982**. Effects of 20-hydroxyecdysone on sperm release from the testes of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller). *J. Insect Physiol. 28*, 1013–1019.

Thummel, C.S., **2002**. Ecdysone-regulated puff genes 2000. *Insect Biochem. Mol. Biol. 32*, 113–120.

Tillman, J.A., Seybold, S.J., Jurenka, R.A., Blomquist, G.J., **1999**. Insect pheromones: an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol. 29*, 481–514.

Tischkau, S.A., Mitchell, J.W., Tyan, S.H., Buchanan, G.F., Gilette, M.U., **2003**. Ca^{2+}/cAMP response element protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus clock. *J. Biol. Chem. 278*, 718–723.

Toma, D.P., Bloch, G., Moore, D., Robinson, G.E., **2000**. Changes in *period* mRNA levels in the brain and division of labour in honey bee colonies. *Proc. Natl Acad. Sci. USA 97*, 6914–6919.

Tomioka, K., Wakatsuki, T., Shimono, K., Chiba, Y., **1991**. Circadian control of hatching in the cricket, *Gryllus bimaculatus*. *J. Insect Physiol. 37*, 365–371.

Touhara, K., Lerro, K.A., Bonning, B.C., Hammock, B.D., Prestwich, G.D., **1993**. Ligand binding by a recombinant insect juvenile hormone binding protein. *Biochemistry 32*, 2068–2075.

Touhara, K., Prestwich, G.D., **1992**. Binding site mapping of a photoaffinity-labelled juvenile hormone binding protein. *Biochem. Biophys. Res. Commun. 182*, 466–473.

Truman, J.W., **1972a**. Physiology of insect rhythms. 1. Circadian organization of the endocrine events underlying the molting cycle of larval tobacco hornworm. *J. Exp. Biol. 57*, 805–820.

Truman, J.W., **1972b**. Physiology of insect rhythms. 2. The silkworm brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol. 81*, 99–114.

Truman, J.W., **1974**. Physiology of insect rhythms. 4. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. *J. Comp. Physiol. A 95*, 281–296.

Truman, J.W., **1992**. The eclosion hormone system of insects. *Progr. Brain Res. 92*, 361–374.

Truman, J.W., Copenhaver, P.F., **1989**. The larval eclosion hormone neurones in *Manduca sexta*: identification of the brain–proctodeal neurosecretory system. *J. Exp. Biol. 147*, 457–470.

Truman, J.W., Horodyski, F.M., Hewes, R.S., Riddiford, L.M., **1991**. Eclosion hormone: from genes to behavior. In: Menn, J.J., Kelly, T.J., Masler, E.P. (Eds.), Insect Neuropeptides: Chemistry, Biology and Action. American Chemical Society, Washington, pp. 95–99.

Truman, J.W., Morton, D.B., **1990**. The eclosion hormone system: an example of coordination of endocrine activity during the molting cycle of insects. In: Epple, A., Scane, C.G., Stetsonn, M.H. (Eds.), Progress in Comparative Endocrinology. Wiley-Liss, New York, pp. 300–308.

Truman, J.W., Riddiford, L.M., **1970**. Neuroendocrine control of ecdysis in silkmoths. *Science 167*, 1624–1626.

Truman, J.W., Riddiford, L.M., **1974**. Physiology of insect rhythms. 3. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol. 60*, 371–382.

Truman, W.J., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., **1983**. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 29*, 895–900.

Truman, J.W., Taghert, P.H., Copenhaver, P.F., Tublitz, N.J., **1981**. Hormonal control of ecdysis through embryonic and post-embryonic development. In: Sehnal, F., Zabza, A., Menn, J.J., Cymborowski, B. (Eds.), Regulation of Insect Development and Behavior. Wrocław Technical University Press, Wrocław, pp. 1011–1020.

Turek, F.W., **1994**. Circadian rhythms. *Recent Progr. Hormone Res. 49*, 43–90.

Vafopoulou, X., Sim, C.-H., Steel, C.G.H., **1996**. Prothoracicotropic hormone in *Rhodnius prolixus*: *in vitro* analysis and changes in amounts in the brain and retrocerebral complex during larval–adult development. *J. Insect Physiol. 42*, 407–415.

Vafopoulou, X., Steel, C.G.H., **1989**. Developmental and diurnal changes in ecdysteroid biosynthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera) *in vitro* during the last larval instar. *Gen. Comp. Endocrinol. 74*, 484–493.

Vafopoulou, X., Steel, C.G.H., **1991**. Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: evidence of a dual oscillator system. *Gen. Comp. Endocrinol. 83*, 27–34.

Vafopoulou, X., Steel, C.G.H., **1992**. *In vitro* photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrinol. 86*, 1–9.

Vafopoulou, X., Steel, C.G.H., **1996a**. The insect neuropeptide prothoracicotropic hormone is released with a dialy rhythm: re-evaluation of its role in development. *Proc. Natl Acad. Sci. USA 93*, 3368–3372.

Vafopoulou, X., Steel, C.G.H., **1996b**. Circadian regulation of a daily rhythm of release of prothoracicotropic hormone from the brain–retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval–adult development. *Gen. Comp. Endocrinol. 102*, 123–129.

Vafopoulou, X., Steel, C.G.H., **1998**. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. Comp. Physiol. A 182*, 343–349.

Vafopoulou, X., Steel, C.G.H., **1999**. Daily rhythm of responsiveness to prothoracicotropic hormone in prothoracic glands of *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol. 41*, 117–123.

Vafopoulou, X., Steel, C.G.H., **2001**. Induction of rhythmicity in prothoracicotropic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuroendocrine Zeitgebers. *J. Insect Physiol. 47*, 935–941.

Vafopoulou, X., Steel, C.G.H., **2002**. Prothoracicotropic hormone of *Rhodnius prolixus:* Partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *Invert. Reprod. Devel. 42*, 111–120.

Vafopoulou, X., Steel, C.G.H., Terry, K.L., **2001**. Ecdysteroid receptor (EcR) cycles with tissue-specific phases in the insect *Rhodnius prolixus*: relationships with circadian regulation of blood ecdysteroid levels. In: Goos, H.J.T., Rastogi, R.K., Vaudry, H., Pierantoni, R. (Eds.), Perspective in Comparative Endocrinogy: Unity and Diversity. Monduzzi Editore, Bologna, pp. 837–844.

Vafopoulou, X., Steel, C.G.H., Terry, K.L., **2004a**. Circadian cycling of the insect ecdysteroid hormone nuclear receptor (EcR) occurs in some tissues, but not others, during development in *Rhodnius prolixus*. *Cell Tissue Res.* (in press).

Vafopoulou, X., Steel, C.G.H., Terry, K.L., **2004b**. Immunohistochemical localization and developmental studies of neurons expressing prothoracicotropic hormone- and bombyxin-like peptides in the brain of the insect *Rhodnius prolixus*. *J. Comp. Neurol.* (in press).

van Esseveldt, L.K.E., Lehman, M.N., Boer, G.J., **2000**. The suprachiasmatic nucleus and the circadian timekeeping system revisited. *Brain Res. Revs. 33*, 34–77.

Veelaert, D., Schoofs, L., Tobe, S.S., Yu, C.G., Vullings, H.G.B., *et al.*, **1995**. Immunological evidence for an allatostatin-like neuropeptide in the central nervous system of *Schistocerca gregaria, Locusta migratoria* and *Neobelliera bullata*. *Cell Tissue Res. 279*, 601–611.

Vivien-Roels, B., Pevet, P., **1993**. Melatonin: presence and formation in invertebrates. *Experientia 49*, 642–647.

Walker, W.F., **1979**. Mating behavior in *Oncopeltus fasciatus*: circadian rhythms of coupling, copulation duration and "rocking" behavior. *Physiol. Entomol. 4*, 275–283.

Warren, J.T., Petryk, A., Marques, G., Jarcho, M.P., Purvy, J.P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila Melanogaster. Proc. Natl Acad. Sci. USA 99*, 11043–11048.

Weber, F., **1995**. Cyclic layer deposition in the cockroach (*Blaberus cranifer*) endocuticle: a circadian rhythm in leg pieces cultured *in vitro*. *J. Insect Physiol. 41*, 153–161.

Webster, R.P., Cardé, R.T., **1982**. Relationships among pheromone titer, calling and age in the omnivorous leaf-roller moth (*Platynota stultana*). *J. Insect Physiol. 28*, 925–934.

Webster, R.P., Yin, C.M., **1997**. Effects of photoperiod and temperature on calling behavior of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). *Can. Entomol. 129*, 843–854.

Westbrook, A.L., Bollenbacher, W.E., **1990**. The development of identified neurosecretory cells in the tobacco hornworm, *Manduca sexta*. *Devel. Biol. 140*, 291–299.

Wiedenmann, G., **1983**. Splitting in a circadian activity rhythm: the expression of bilaterally paired oscillators. *J. Comp. Physiol. 150*, 51–60.

Wiedenmann, G., Krüger-Alef, K., Martin, W., **1988**. The circadian control of calling song and walking activity patterns in male crickets (*Teleogryllus commodus*). *J. Exp. Biol. 47*, 127–137.

Wiedenmann, G., Loher, W., **1984**. Circadian control of singing in crickets: two different pacemakers for early-evening and before-dawn activity. *J. Insect Physiol. 30*, 145–151.

Wiedenmann, G., Lukat, R., Weber, F., **1986**. Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? *J. Insect Physiol. 32*, 1019–1027.

Wigglesworth, V.B., **1934**. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). 2. Factors controlling molting and "metamorphosis" *Q. J. Microsc. Sci. 77*, 191–222.

Wigglesworth, V.B., **1940**. The determination of characters at metamorphosis *in Rhodnius prolixus* (Hemiptera). *J. Exp. Biol. 17*, 201–222.

Wigglesworth, V.B., **1952**. The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in molting. *J. Exp. Biol. 29*, 561–570.

Wise, S., Davis, N.T., Tyndale, E., Noveral, J., Folwell, M.G., *et al.*, **2002**. Neuroanatomical studies of *period* gene expression in the hawkmoth, *Manduca sexta*. *J. Comp. Neurol. 447*, 366–380.

Wojtasek, H., Prestwich, G.D., **1995**. Key disulfide bonds in an insect hormone binding protein: cDNA cloning of a juvenile hormone binding protein of *Heliothis virescens* and ligand binding by native and mutant forms. *Biochemistry 34*, 5234–5241.

Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of juvenile hormone. 2. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.

Yagi, Y., Ishibashi, J., Nagata, K., Kataoka, H., Suzuki, A., *et al.*, **1995**. The brain neurosecretory cells of the moth *Samia cynthia ricini*: immunohistochemical localization and developmental changes of the *Samia* homologues of the *Bombyx* prothoracicotropic hormone and bombyxin. *Devel. Growth Differ. 37*, 505–516.

Yagita, K., Tamanini, F., van der Horst, G.T.J., Okamura, H., **2001**. Molecular mechanisms of the biological clock in cultured fibroblasts. *Science 292*, 278–281.

Yamaoka, K., Hirao, T., **1981**. Mechanisms of ovipositional behavior in *Bombyx mori:* time-gating and accumulation of the internal factor. *Int. J. Invert. Reprod. 4*, 169–180.

Yannielli, P.C., Harrington, M.E., **2000**. Neuropeptide Y applied *in vitro* can block the phase shifts induced by light *in vivo*. *Neuroreport 11*, 1587–1591.

Závodská, R., Šauman, I., Sehnal, F., **2003**. Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *J. Biol. Rhythms 18*, 106–122.

Zhao, C.-H., Li, Q., Gao, W., **2002**. Stimulation of sex pheromone production by PBAN-like substance in the pine caterpillar moth, *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae). *Arch. Insect Biochem. Physiol. 49*, 137–148.

Zhao, Z., Zera, A.J., **2004**. The hemolymph JH titer exhibits a large-amplitude, morph-dependent, diurnal cycle in the wing-polymorphic cricket, *Gryllus firmus*. *J. Insect Physiol 50*, 93–102.

Zhu, J.W., Millar, J., Löfstedt, C., **1995**. Hormonal regulation of sex pheromone biosynthesis in the turnip moth, *Agrotis segetum*. *Arch. Insect Biochem. Physiol. 30*, 41–59.

Žitňan, D., Adams, M.E., **2000**. Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioral sequence. *J. Exp. Biol. 203*, 1329–1340.

Žitňan, D., Kingan, T.G., Beckage, N.E., **1995a**. Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 669–678.

Žitňan, D., Kingan, T.G., Kramer, S.J., Beckage, N.E., **1995b**. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manducas sexta* larvae parasitized by the braconid wasp, *Cotesia congregata*. *J. Comp. Neurol. 356*, 83–100.

Žitňan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S.S., *et al.*, **1999**. Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron 23*, 523–535.

Žitňan, D., Sehnal, F., Bryant, P.J., **1992**. Neurons producing specific neuropeptides in the central nervous system and pupariation-delayed *Drosophila*. *Devel. Biol. 156*, 117–135.

3.12 Hormonal Control of Diapause

D L Denlinger, Ohio State University, Columbus, OH, USA
G D Yocum, Red River Agricultural Research Center, Fargo, ND, USA
J P Rinehart, Ohio State University, Columbus, OH, USA

3.12.1. Introduction

The evolution of diapause is arguably one of the most critical events bolstering the success of insects. Having the capacity to periodically shut down development has enabled insects and their arthropod relatives to invade environments that are seasonally hostile. Indeed, very few environments permit continuous insect development. Even in the tropics, where temperatures throughout the year may be compatible with poikilotherm development, season patterns of rainfall drive cycles of plant growth that favor insects with the ability to periodically become dormant.

Diapause is an arrest in development accompanied by a major shutdown in metabolic activity. Unlike a simple quiescence that is an immediate response to an unfavorable environmental condition, diapause is a genetically programmed response that occurs at a specific stage for each species. Sometimes the insect will enter diapause at this particular stage in each generation regardless of the environmental conditions it receives (e.g., pharate first instar of the gypsy moth *Lymantria dispar*), a developmental program referred to as an obligatory diapause. Much more frequently, the decision to enter diapause is determined by environmental factors, usually daylength, received by that individual or its mother at an earlier developmental stage. This is referred to as a facultative diapause.

Development can effectively be interrupted at many points, as evidenced by the rich diversity of developmental stages used by different species for diapause. Embryonic diapauses have been documented for nearly all possible stages of embryonic development, ranging from early blastoderm formation to pharate larvae. Larval diapauses are most common in late instars, but early instar diapauses are not at all uncommon. Pupal diapause is well documented, and a few species diapause as pharate adults, but, not surprisingly, there appear to be no instances where development is halted once pharate adult development has been initiated. The most prevalent cases of adult diapause involve newly emerged adults that have not yet reproduced, but there are also examples of diapause interrupting periods of reproduction. Most commonly, insects enter diapause only once during their life cycle, but some species, especially those living at higher latitudes, may extend their development over several years, and thus spend successive winters in diapauses of different stages. Some long-lived adults may also go through multiple diapauses to coordinate egg laying with favorable conditions.

The fact that diapause is programmed well in advance of its onset implies that there is a prediapause period that can be used to prepare the insect for the special conditions that lie ahead. Hence it is common for insects that are destined for diapause to have a somewhat prolonged period of prediapause development, attain a larger size, accumulate additional lipid reserves, and add additional waterproofing to their cuticle. Distinct coloration, often to

match the insect's overwintering environment, may accompany the entry into diapause. Insects entering a winter diapause frequently undergo metabolic adjustments to enhance cold hardiness. In some cases the cold hardiness is a component of the diapause program, but in other cases cold hardiness is invoked by a different set of environmental cues.

The term diapause perhaps falsely suggests a period of arrest in which development comes to a complete halt. The period of diapause should not be regarded as a simple stop and restart of development. Andrewartha (1952) originally coined the term "diapause development" to refer to the ongoing progression of events that occurs during diapause and eventually results in the termination of diapause. It is evident that diapause is a dynamic process, as evidenced by characteristic changes in the utilization of energy reserves, systematic changes in patterns of oxygen consumption, changes in responsiveness to environmental stress and hormones, and distinct patterns and changes in gene expression. A progression of events must transpire before diapause can be terminated. In some cases, development is not completely halted. There are several examples in which developmental processes continue during diapause, albeit at much slower rates, e.g., ovarian maturation during the adult diapause of the mosquito *Culex pipiens* (Readio *et al.*, 1999) and embryonic development during diapause in the pea aphid, *Acyrthosiphon pisum* (Shingleton *et al.*, 2003). In some cases there can be profound differences even within the body of a single individual. For example, during pupal diapause in the tobacco hornworm, *Manduca sexta*, most tissues of the body are arrested, but the testicular stem cells continue to undergo mitotic division (Friedlander and Reynolds, 1992). These unique events of diapause, coupled with prediapause distinctions in diapause-programmed individuals, suggest that it is perhaps most appropriate to view diapause as an alternate developmental pathway.

Comprehensive coverage of ecological aspects of insect diapause can be found in excellent reviews by Tauber *et al.* (1986) and Danks (1987), clock mechanisms are discussed in detail by Saunders (2002), and molecular aspects of diapause regulation are discussed by Denlinger (2002). The goal in this review is to highlight the major discoveries relevant to the hormonal control of diapause that have occurred subsequent to 1985. The earlier literature was covered previously (Denlinger, 1985), and in this review the earlier findings are briefly summarized and new developments in this field are emphasized.

3.12.2. The Preparative Phase

During the preparative phase, the insect must make the decision to enter diapause (and sometimes how long to remain in diapause) and make the accompanying alterations that will result in the sequestration of additional energy reserves and waterproofing agents, and initiate specific behavioral programs needed to find protected sites appropriate for overwintering. Some species, such as the Monarch butterfly, *Danaus plexippus*, engage in long-distance flights to reach their overwintering site, but more commonly, insects programmed for diapause migrate to buffered sites that are closer at hand. When diapause is viewed as an alternate developmental program, these phases unique to diapause, even if they occur prior to diapause, must also be viewed as part of the diapause syndrome.

These early distinctions underscore the fact that we can not simply focus on the endocrine events that dictate the onset and termination of diapause. A comprehensive view of diapause also calls for an understanding of the control mechanisms regulating these early events, but regrettably we know very little of the events that regulate early phases of this developmental pathway. For example, what endocrine signals direct a diapause-programmed flesh fly (Sarcophagidae) larva to sequester twice as much lipid as a larva not destined for diapause? This observation suggests that the set points for lipid accumulation differ in diapause- and nondiapause-destined individuals, a distinction most likely regulated by the endocrine system. What signals lead to formation of the extra layer of hydrocarbons that line the puparium of a flesh fly destined for diapause? A brain factor whose action can be mimicked with cAMP appears to be involved (Yoder and Denlinger, 1992), but its identity remains unknown.

3.12.2.1. Making the Decision to Enter Diapause

For an obligate diapause, the decision to enter diapause is hard-wired. Diapause will be entered at a specific stage regardless of the environmental conditions. This is an arrangement common to species that have single generation each year, e.g., the gypsy moth and the Cecropia silkmoth, although in both of these examples a few individuals fail to enter diapause. By contrast, a facultative diapause enables the insect to complete several generations per year, and the individual "decides" its own developmental fate based on the environmental cues it perceives. The plasticity of this arrangement offers flexibility and enables the insect to more closely coordinate development with the appropriate seasons. An

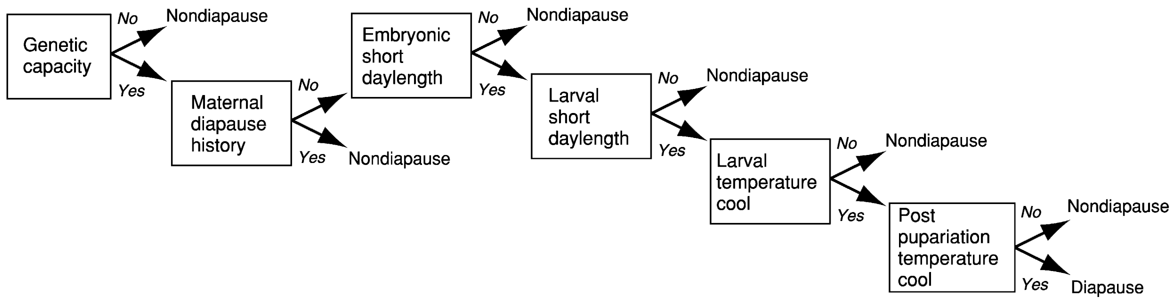


Figure 1 Sequence of signals required for the programming of pupal diapause in flesh flies (*Sarcophaga*). (Reproduced with permission from Denlinger, D.L., **1985**. Hormonal control of diapause. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 353–412.)

additional variation of this theme is when the individual does not directly decide but the decision is made by the mother.

The decision to diapause should not be viewed as a simple "yes/no" decision made at a single moment in time, but rather it is the culmination of a series of events, as exemplified for the flesh fly example shown in Figure 1. This example shows that the programming of diapause can be averted at many points along the way, right up to the time that diapause is normally manifested. In order for the program to be enacted, all of the previous conditions must have been fulfilled. Thus, the diapause program can be aborted at the last minute, but attempts to induce diapause by offering the correct signals only during the later phases of development will not be sufficient to induce diapause.

3.12.2.1.1. Reading the environmental cues Short daylength, a reliable harbinger of winter, is the most common cue used for the programming of an overwintering diapause (Figure 2). The precision of this environmental token makes it a seasonal indicator of unparalleled reliability. The critical photoperiod, the transition point between photoperiods that elicit diapause and nondiapause development, is usually quite sharp, thus implying that insects can readily distinguish daylength differences as small as 15 min. Insects that enter a summer diapause may use long daylengths instead of short daylengths for the programming of diapause (Masaki, 1980), and there are numerous examples of photoresponse curves showing restricted portions of the curve to be diapause inductive. Daylength appears to be measured as a threshold character, and only a few examples suggest that insects are capable of actually measuring changes in daylength (Tauber *et al*., 1986). The widespread importance of photoperiodism in regulating diapause induction is reflected by the fact that it is used almost universally by insects in all geographic areas excluding areas within approximately

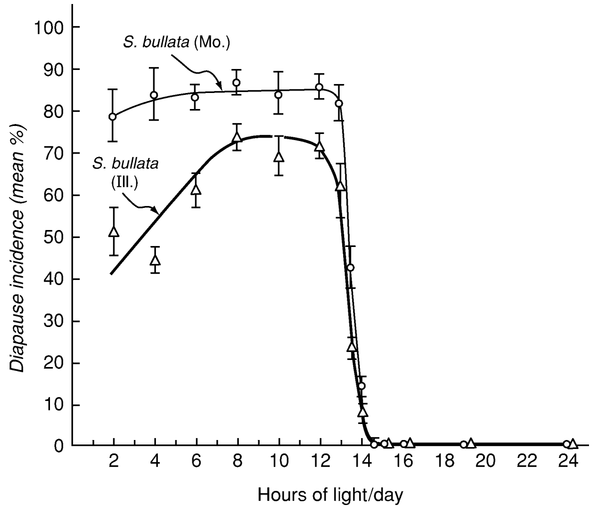


Figure 2 Critical photoperiod for diapause induction in populations of *Sarcophaga bullata* from Illinois (40° 15′ N) and Missouri (38° 30′ N) at 25 °C. (Reproduced with permission from Denlinger, D.L., **1972**. Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae). *Biol. Bull. Wood's Hole 142*, 11–24.)

5° of the equator. Diapause is still prevalent in the equatorial region, but other cues such as temperature, rainfall, or plant volatiles replace photoperiod as the cue for diapause induction in that region (Denlinger, 1986).

With a few exceptions, temperature also influences the decision to enter diapause. While it may act alone as the environmental cue used for diapause induction in some tropical species, more commonly it acts in concert with photoperiod. As shown in Figure 3a, low temperature commonly increases the diapause incidence observed at short daylength without influencing the critical photoperiod, but in some cases the critical photoperiod will shift toward longer daylengths at low temperature (Figure 3b). Under natural conditions, insects are simultaneously subjected to thermoperiods as well as photoperiods. In a number of cases, thermoperiod can substitute for a

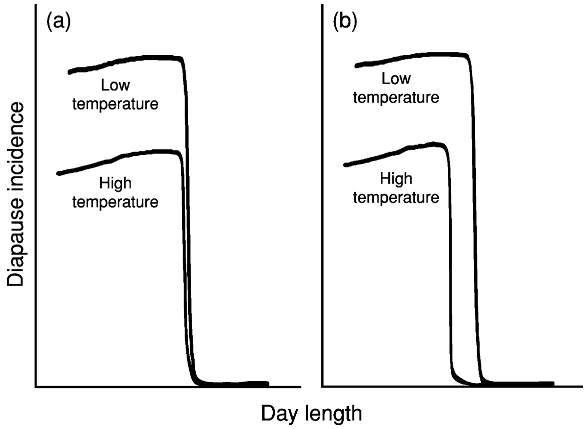


Figure 3 Low temperature can impact the photoperiodic response curve in two ways. (a) In some cases, the critical photoperiod remains the same but the incidence of diapause at short daylengths is higher. (b) In other instances, the critical photoperiod is also shifted toward longer daylengths at low temperature. (Reproduced with permission from Denlinger, D.L., **2001**. Interrupted development: the impact of temperature on insect diapause. In: Atkinson, D., Thorndyke, M. (Eds.), Environment and Animal Development: Genes, Life Histories and Plasticity. BIOS Scientific Publishers, Oxford, pp. 235–250.)

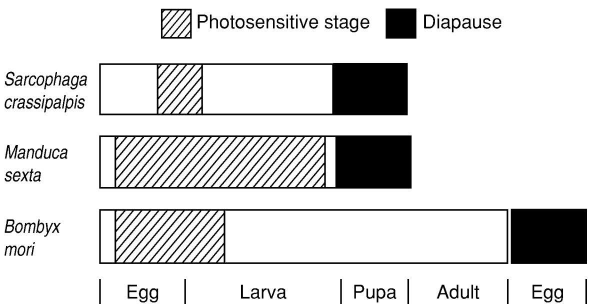


Figure 4 The relationship between the photosensitive stage and the stage of diapause in three insect examples. (Reproduced with permission from Denlinger, D.L., **1985**. Hormonal control of diapause. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 353–412.)

photoperiodic signal, and it presumably does so by linkage to a common mechanism (van Houten and Veerman, 1990). Other factors that may contribute to the diapause decision include food presence (Fielding, 1990), food quality (Hare, 1983; Overmeer *et al.*, 1989), plant volatiles (Ctvrtecka and Zdarek, 1992), pheromones (Kipyatkov, 2001), and crowding (Desroches and Huignard, 1991; Saunders *et al.*, 1999).

The photosensitive stage, the stage that receives the photoperiodic cues used to program diapause, usually occurs fairly far in advance of the actual diapause stage, as noted in **Figure 4**. The photosensitive stage may be as brief as 4 days as observed in *Sarcophaga crassipalpis*, or it may encompass much of the prediapause period as noted in *Manduca sexta*. In the extreme example noted in the silkmoth *Bombyx mori*, the photosensitive period actually occurs in the previous generation. If diapause is to be programmed by daylength, it is imperative that the correct photoperiodic signals be received during this photosensitive period. At other times during development the length of the day is of no consequence for the programming of diapause.

Thus, insects that rely on short days to program their diapause need to extract two types of information: (1) was the day short? and (2) how many short days were received? Answering the first question requires a sophisticated clock that can measure daylength with precision, and answering the second question requires a counter capable of summing the number of short days that comprise the photosensitive period (Saunders, 2002).

3.12.2.1.2. Letting mother decide In a number of parasitic Hymenoptera, higher Diptera, and some Lepidoptera it is the mother that decides the diapause fate of her progeny. This arrangement may be particularly useful in cases where the mother has access to environmental cues not readily accessible to her progeny. Most of these cases involve the mother determining the diapause status of her recently deposited eggs. In these cases the embryos are often in an early stage of development and may not yet be equipped with the sophisticated neural systems required to receive and respond to photoperiodic cues. *Bombyx mori* is in this category, and since quite a bit is known about diapause in this species it will be discussed separately (see Section 3.12.3.1.1). In other cases, however, the mother influences the diapause fate of her progeny in their larval (e.g., *Nasonia vitripennis*) or pupal (e.g., *S. bullata*) stages. This clearly implies that the mother is not simply exerting some direct effect on her progeny as might be expected in an embryonic diapause, but she is initiating an effect that is expressed at a much later stage of development.

In spite of the intriguing nature of these maternal effects very little is known about the mechanisms that regulate these transgenerational effects. In the flesh fly *S. bullata* a critical message that influences the maternal effect is transferred from the brain of the female to her ovary sometime between pupariation and shortly after adult emergence (Rockey *et al.*, 1989). Gamma aminobutyric acid (GABA) and octopamine possibly are involved in the transfer of information from mother to progeny (Webb and Denlinger, 1998). Differential display of mRNA revealed a transcript unique to the ovaries of females

that express the maternal effect, but the function of the protein it encodes is still unknown (Denlinger, 1998). How the mother can influence the diapause fate of her offspring offers an intriguing, and still unanswered, problem in development.

3.12.2.2. The Brain as Photoreceptor and Programmable Center

The conspicuous visual centers, the compound eyes and ocelli, are not the photoreceptors commonly used for perception of photoperiodic information. Most species that have been examined rely on extraretinal reception of the light signal (Shimizu and Hasegawa, 1988; Saunders and Cymborowski, 1996; Numata *et al*., 1997). Blackening or ablating the compound eyes frequently has no impact on the insect's ability to respond to photoperiod. In some cases translucent cuticular windows or lightened portions of cuticle overlay portions of the brain, thus facilitating the transfer of light to the photoreceptors in the brain. In classic experiments with the giant silkmoth, *Antheraea pernyi*, Williams and Adkisson (1964) elegantly demonstrated that the brain is the direct recipient of the light signal. This species terminates pupal diapause in response to long daylength, and by using a divided photoperiod chamber that offered diapause-terminating conditions to one half of the body and diapause-maintaining conditions to the other half, they were able to demonstrate that diapause can be broken only when the portion of the body containing the brain is exposed to diapause-terminating conditions. This is true even when the brain is transplanted to the abdomen of the pupa. In yet another elegant demonstration of the brain's role in photoreception, Bowen *et al*. (1984) demonstrated that the brain of *M. sexta,* when exposed *in vitro* to short days, is capable of eliciting diapause when implanted into a debrained pupa. Likewise, larval brain–subesophageal ganglion complexes of *B. mori* can be photoperiodically programmed for diapause *in vitro* and elicit a diapause response when reimplanted into larvae (Hasegawa and Shimizu, 1987).

More recently, however, several cases have been reported in which the compound eyes do play a role. Females of the fly *Protophormia terraenovae* (Shiga and Numata, 1997), and the bugs *Plautia crossota stali* (Morita and Numata, 1999), *Graphosoma lineatum* (Nakamura and Hodkova, 1998), and *Poecilocoris lewisi* (Miyawaki *et al*., 2003) cannot distinguish between short days and long days after surgical removal of the compound eyes. Painting the compound eyes of the bug *Riptortus clavatus* with phosphorescent paint effectively extends the photoperiod, resulting in the termination of diapause (Numata and Hidaka, 1983). By contrast, painting the head region above the pars intercerebralis fails to terminate diapause, thus suggesting that the compound eyes are the dominant photoreceptors for this species. Not all of the ommatidia of the compound eye are involved in the photoperiodic response of *R. clavatus*. By selectively removing the ommatidia from various regions of the compound eyes, it is evident that only the ommatidia in the central region of the compound eye are involved in this response (Morita and Numata, 1997b). Evidence from circadian rhythms suggests that in some cases, e.g., *Protophormia terraenovae*, both retinal and extraretinal pathways contribute to rhythm entrainment (Hamasaka *et al*., 2001). Such may also prove to be the case for photoperiodism in some species.

The diapause program resides within the brain, and can be transferred from one individual to another by transplantation of the programmed brain, as demonstrated in the embryonic diapause of *B. mori* (Hasegawa and Shimizu, 1987), pupal diapause of *M. sexta* (Safranek and Williams, 1980; Bowen *et al*., 1984) and *S. crassipalpis* (Giebultowicz and Denlinger, 1986), and adult diapause of the linden bug, *Pyrrhocoris apterus* (Hodkova, 1992). In *M. sexta* the duration of diapause is also programmed within the brain prior to the onset of diapause. In this species, the number of short days the larva receives determines the length of the pupal diapause. Short days throughout produce a high incidence of diapause but such a diapause is of short duration. By contrast, exposure to only a few short days yields a low incidence of diapause, but a diapause of long duration. Brain transplantation experiments show that information about the duration of the diapause can be transplanted along with the brain (Denlinger and Bradfield, 1981).

Precisely which cells within the brain are involved in photoreception and storage of diapause information remains unknown in most cases, but future studies pinpointing photoreceptor pigments and specific clock genes involved in photoperiodism should reveal the correct target sites for this critical information. In the vetch aphid, *Megoura viciae*, antibodies directed against invertebrate and vertebrate opsins and phototransduction proteins consistently label an anterior ventral neuropile region of the protocerebrum (Gao *et al*., 1999). Identification of this region is consistent with previous experiments using localized illumination and microlesions to identify the photoreceptor region.

3.12.2.3. Transduction of the Environmental Signals

When photoperiod is the primary cue leading to the induction of diapause, several key molecular components are essential. The first key component is a photoreceptor capable of distinguishing between the photophase and scotophase. Secondly, the insect must have the capacity to measure the length of each photophase (or scotophase), and thirdly, the insect must be able to sum the number of short (or long) days it has seen. Although our understanding of the molecular events involved in the transduction of the environmental signals remains in its infancy, several recent studies have proven insightful, and in addition the early literature offers valuable clues about the processes involved. As discussed above, the photoreceptive organ, and in some cases candidate cells, have been identified for several diapause models. Action spectra that elicit diapause have been described for several species, and in most cases the blue–green range of the visual spectrum is most sensitive to diapause induction (Truman, 1976), thus indicating that any putative photoreceptor involved in the diapause response must have features consistent with these early observations.

How temperature exerts its effect on the diapause response is poorly understood. One attractive model proposed by Saunders (1971) for flesh flies suggests that low temperature simply increases the number of short days the larvae receive: in this system, 14 short days are required for diapause induction, and lowering the temperature retards development and hence a larger portion of the population experiences the required number of short days. While this model is attractive for species with a long photosensitive period, such a system seem less likely to operate in other insects, including some closely related flesh fly species, that have a very short photosensitive period. In some cases, thermoperiod can completely substitute for photoperiod as an environmental cue. Adult diapause is induced in the predatory mite *Amblyseius potentillae* by a short day photoperiod or by rearing under constant darkness with a thermoperiod of 16 h at 15 °C and 8 h at 27 °C (van Houten *et al.*, 1987). Exposing *A. potentillae* to short day photoperiods or to short day thermoperiods either concurrently or in succession is successful for inducing diapause, thus suggesting that a single physiological mechanism is integrating photoperiodic and thermoperiodic information to regulate diapause (van Houten and Veerman, 1990). The argument that a single mechanism is involved is further strengthened by the observation that mites reared on a diet deficient in carotenoids/vitamin A (see Section 3.12.3.2.1) lose their ability to respond to either photoperiod or thermoperiod (van Houten *et al.*, 1987). By contrast, carotenoids/vitamin A are essential for the photoperiod response but not for the thermoperiodic response in the cabbage butterfly, *Pieris brassicae* (Claret, 1989). Clearly, species differences must again be evident in the integration of photoperiodic and thermoperiodic information.

3.12.2.3.1. Cryptochromes and opsins One of the most important quests in diapause research is to identify the photoreceptor pigment involved in this response. While a number of candidate proteins have been identified, most appear to be involved in visual perception. Only a few are candidates for involvement in photoperiodism, especially given the blue light sensitivity of the diapause response and the common involvement of extraocular light reception. One such candidate is the flavin-based blue light photoreceptor cryptochrome (CRY). This protein has been directly linked to the circadian pacemaker of the fruit fly *Drosophila*, acting to daily reset the clock upon initiation of the photophase as detailed below. Null mutants of CRY exhibit poor synchronization during light cycling, while rhythmicity is present in constant darkness (Stanewsky *et al.*, 1998), features that support a role for CRY in entrainment of the circadian clock by photoperiod. The possible role of CRY in diapause induction has yet to be established, although *cry* expression is upregulated in adult *S. crassipalpis* when the flies are reared at short as opposed to long daylengths (Goto and Denlinger, 2002b).

Another group of proteins that have been linked to photoperiodism are the carotenoid/vitamin A-associated opsin photoreceptors. A causal link with diapause has been established by rearing insects on carotenoid-free diets. Although several species, including *D. melanogaster* (Zimmerman and Goldsmith, 1971) and *B. mori* (Shimizu *et al.*, 1981), retain circadian behavioral patterns when reared on a carotenoid-free diet, the induction of diapause can be affected in a number of species, including adults of the predatory mite *A. potentilla* (van Zon *et al.*, 1981; Veerman *et al.*, 1985) and spider mite *Tetranychus urticae* (Bosse and Veerman, 1996), embryonic diapause of *B. mori* (Shimizu and Kato, 1984), and pupal diapause of *Pieris brassicae* (Claret, 1989; Claret and Volkoff, 1992). The opsin proteins have been linked to photoperiodism as well. Melanopsin has been implicated in circadian mechanisms in vertebrates. In mice, null mutants of melanopsin retain circadian rhythmicity in constant darkness, but lack photoperiodic entrainment

(Panda *et al*., 2002; Ruby *et al*., 2002). Similar work has yet to be conducted in insects, although boceropsin, which shows central nervous system (CNS)-specific expression, has been cloned from *B. mori* (Shimizu *et al*., 2001). A comparison of the phenotypes of null mutants of opsin-deficient and carotenoid-deficient silkworms will be be insightful.

3.12.2.3.2. Clock genes Regardless of the type of photoreceptor involved in diapause, the photoreceptor must be functionally connected to a timekeeping mechanism to exert its effect. The molecular basis of timekeeping is particularly well described in *D. melanogaster* (Schotland and Sehgal, 2001), although other species are beginning to be characterized as well (see **Chapter 4.11**). In *Drosophila*, the clock is composed of a multiple component autoregulatory feedback loop (reviews: Reppert and Weaver, 2000; Williams and Sehgal, 2001; Stanewsky, 2002). The best-described components involve the interactive oscillations of the proteins period (PER), timeless (TIM), clock (CLK), and cycle (CYC). In this system, the proteins PER and TIM heterodimerize to enter the nucleus and transcriptionally repress the expression of CLK and CYC. Conversely, CLK and CYC act to transcriptionally upregulate both PER and TIM. Thus, when levels of the proteins PER and TIM are high, they repress the transcription of CLK and CYC. As levels of CLK and CYC diminish, transcription of PER and TIM are decreased, until they are low enough that they no longer repress CLK and CYC. Once the levels of CLK and CYC are restored, PER and TIM will again be transcribed. The genes in this autoregulatory loop, in addition to several others including *vrille, doubletime*, and *CREB2*, create two interacting phases of protein oscillations (PER with TIM and CLK with CYC) thus creating a circadian rhythm of gene expression. The connection of this circadian pacemaker to photoperiod is attained through the interaction of the proteins TIM and CRY. TIM is degraded in the photophase through the action of CRY, thereby "resetting the clock" according to the daily light cycle. Although most of these genes appear to be conserved, it is evident that details of the clock mechanism may differ among species. For example, in *Antheraea pernyi,* PER apparently does not enter the nucleus (Sauman and Reppert, 1996).

These major clock genes have thus far been studied intensely in association with circadian rhythms, yet it is not absolutely clear whether diapause has a circadian basis. Although much evidence points to a role of circadian rhythms in the programming of diapause (Saunders, 2002), several arguments defend the position that diapause does not have a circadian basis (Veerman, 2001; Veerman and Veenendaal, 2003). Even if diapause does have a circadian basis it is not clear whether the same clock genes and response mechanisms are involved in both circadian rhythmicity and photoperiodism (diapause). In fact, several experiments suggest two distinct mechanisms. In both spider mites (Veerman and Veenendaal, 2003) and the blowfly *Calliphora vicina* (Saunders and Cymborowski, 2003), it is possible to segregate the photoperiodic response mechanism from the mechanism governing daily behavioral rhythms. Experiments utilizing mutants suggest that distinct mechanisms may operate in these two systems. *per* null mutants of *D. melanogaster* are arrhythmic, yet they retain the ability to enter adult diapause (Saunders *et al*., 1989; Saunders, 1990). Similarly, in the drosophilid *Chymomyza costata,* mutants with reduced rhythmicity continue to enter larval diapause (Lankinen and Riihimaa, 1992), and *per* null mutants retain the capacity for diapause as well (Shimada, 1999; Kostal and Shimada, 2001). It is, of course, possible that there is enough redundancy in the system so that knocking out a single gene does not render the timekeeping mechanism involved in photoperiodism inoperative. In *S. crassipalpis, period, cycle, clock,* and *cryptochrome* show little differences in expression patterns when the flies are reared under short day or long day. However, the expression of *timeless* is greatly suppressed in long days as compared to short days (Goto and Denlinger, 2002b). *tim* thus emerges as a potential player in the mechanism distinguishing short and long days.

Though we can not yet be certain which clock-related genes are involved in programming diapause, there are already sufficient experiments in the literature suggesting that the gene interplay described so elegantly for the control of circadian rhythms of behavior in *D. melanogaster* may not be fully relevant to diapause induction. Resolution of these disparities is pivotal for understanding diapause induction at the molecular level.

3.12.2.4. Storage Proteins

The approach to diapause is frequently accompanied by the accumulation of high concentrations of hemolymph storage proteins that persist throughout diapause. This feature of diapause, first noted in larvae of the southwestern corn borer, *Diatraea grandiosella* (Brown and Chippendale, 1978), has been frequently observed in larvae, pupae, and adults destined for diapause. Although such proteins are sometimes referred to as diapause-associated proteins, they are not truly unique to diapause and

instead can be included in the broad category of storage proteins. These same proteins are also present in nondiapausing individuals. What is unique to diapause is the long time interval over which they persist, and in some cases the concentrations in the hemolymph during diapause are much higher than ever observed in nondiapausing individuals.

In nondiapausing individuals, such proteins are abundant during metamorphosis. At this time insects endure periods of nonfeeding as they transit from one developmental stage to the next. This period of nonfeeding and high energy demand is particularly acute in holometabolous insects as they go through the larval to pupal to adult transformation. To help meet this need for metabolic reserves, feeding larvae synthesize storage proteins in the fat body and secrete them into the hemolymph where their titers reach extraordinary levels just prior to pupation. Most of the storage proteins are reabsorbed into the fat body and stored in granules at the time of the pupal molt. The granules are broken down and the amino acids are used for synthesizing the proteins needed to construct adult tissues. Insect storage proteins form a diverse group of proteins, differing significantly in amino acid composition and ranging in size from the high molecular weight hexameric proteins (200–500 kDa) to 35 kDa. Several excellent reviews discuss insect storage proteins (Telfer and Kunkel, 1991; Haunerland, 1996; Burmester *et al*., 1998). Here the focus is only on those aspects of storage proteins that directly relate to diapause.

For insects that diapause in the larval stage, the high weight hemolymph proteins can be divided into two classes based on their relationship to diapause. Proteins in the first class are present in both diapausing and nondiapausing individuals; e.g., *D. grandiosella* (Lenz *et al*., 1987), the pink bollworm, *Pectinophora gossypiella* (Salama and Miller, 1992), the spruce budworm, *Choristoneura fumiferana* (Palli *et al*., 1998), the stem-borer *Busseola fusca* (Osir *et al*., 1989), and the wax moth *Galleria mellonella* (Godlewski *et al*., 2001). The second class includes hemolymph proteins whose expression is restricted to, or greatly enhanced in, diapause-programmed individuals, e.g., the codling moth, *Cydia pomonella* (Brown, 1980), *D. grandiosella* (Dillwith and Chippendale, 1984; Lenz *et al*., 1987), *B. fusca* (Osir *et al*., 1989), *P. gossypiella* (Salama and Miller, 1992). One species may synthesize one or both of these classes of proteins. In insects that diapause as adults, two peaks of storage protein synthesis commonly occur during development, one in later instars and the other in diapausing adults. The cycle of storage protein synthesis is best exemplified by the Colorado potato beetle, *Leptinotarsa decemlineata* and *R. clavatus*. In *L. decemlineata* diapause protein 1 (DP-1) is first detected on day 2 of the fourth larval instar (de Kort and Koopmanschap, 1994). The DP-1 titer continues to increase throughout the fourth instar then decreases once the larvae starts digging, as they seek a pupation site. Trace levels of DP-1 transcript are detectable in newly emerged diapause-programmed adults. DP-1 expression increases again 2 days after emergence and is still detectable 2 months into diapause. A somewhat more complicated storage protein expression pattern occurs in *R. clavatus* (Chinzei *et al*., 1991), which synthesizes four cyanoproteins, CP-1 to CP-4. CP-4 is expressed cyclically in the first to fourth nymphal instars, with maximum expression occurring in the middle of each instar. During the final instar all four CPs are expressed. All four CPs are present in newly emerged nondiapausing adults but quickly disappear by day 2. CP-1 is again expressed on day 4 and deposited into the developing ovaries. In diapause-programmed bugs all four CPs are expressed beginning around day 4 post-emergence and continuing through to at least 70 days (Chinzei *et al*., 1992). One of the most interesting aspects of storage protein physiology is their continuous expression in some species during diapause, e.g., *P. gossypiella* (Salama and Miller, 1992), *L. decemlineata* (de Kort and Koopmanschap, 1994), *Choristoneura fumiferana* (Palli *et al*., 1998), and *G. mellonella* (Godlewski *et al*., 2001). It is not at all clear why a diapausing insect would invest scarce metabolic reserves in the synthesis of a storage protein. This pattern of continuous synthesis may suggest some alternative use for such proteins during diapause.

Although the fat body is the primary site of synthesis of storage proteins, other tissues have also been shown to synthesize these proteins, e.g., the midgut, epidermis, and pericardial cells of the lepidopteran *Calpodes ethlius* (Palli and Locke, 1987) and testis of the tobacco budworm, *Heliothis virescens* (Miller *et al*., 1990). Within the testis of *H. virescens* two storage proteins (p82 and 76) are synthesized by the sheath and exported only into the testis lumen. The testis cyst cells actively import p82 which is then deposited into the sperm mitochondria (Miller *et al*., 1990). Exposing *H. virescens* larvae to elevated temperature induces a summer diapause characterized by arrested sperm development (Henneberry and Butler, 1986). Larvae and pupae exposed to high temperature synthesize significantly more p82 than control males. Within 7 days after returning heat-treated pupae to the normal rearing temperature, p82 synthesis rate decreases to levels comparable to that of the control males. In response to heat treatment p82 is transported into

the spermatocytes' nuclei instead of the mitochondria as noted in control males. Seo and Kim (1992) speculate that p82 may function in arresting sperm development.

Juvenile hormones and ecdysteroids are both known to regulate storage proteins. Treating diapausing females of *R. clavatus* with the juvenile hormone (JH) analog methoprene induces a shift from the diapause expression pattern to the nondiapause pattern. Methoprene treatment inhibits synthesis of CP-4 (the principal diapause CP), and increases synthesis of CP-1 (the principal nondiapause CP) and vitellogenin (Miura *et al.*, 1991). Treating day 1 short-day *L. decemlineata* with the JH analog pyriproxyfen shifts the initiation of DP-1 synthesis from day 2 to day 15 and induces synthesis of the vitellogenin subunits (de Kort *et al.*, 1997). Treating the last larval instar of *G. mellonella* with 20-hydroxyecdysone (20E) inhibits synthesis of the storage proteins Lhp76 and 82 (Ray *et al.*, 1987). Abdomens deprived of ecdysteroids by ligating the larvae behind the prothoracic segment synthesize both Lhp76 and 82 for at least 15 days postligation. Lhp76 and 82 exhibit differential sensitivity to 20E regulation, with Lph 72 being significantly more responsive to 20E than Lhp82 (Ray *et al.*, 1987). *Manduca sexta* has two distinct members of the methionine-rich storage protein family (SP1A and SP1B) that are differentially regulated by 20E and JH (Corpuz *et al.*, 1991). Ligating fifth instar larvae of *M. sexta* between the first and second abdominal segments inhibits the expression of both SP1A and SP1B. Expression of SP1A and SP1B can recover in these ligated larvae in response to injection of 20E. 20E induces twice the level of expression of SP1B than of SP1A, demonstrating a clear difference in sensitivity to 20E induction. Topically applying the JH mimic fenoxycarb to day 2 and 3 fifth instar larvae inhibits expression of SP1A and SP1B. In summary, the regulation of storage proteins appears to be dictated by the overall regulatory contol of development, with JH and ecdysteroids as the major players. Thus, the unique patterns of storage protein synthesis and utilization observed during diapause are presumably directed by these key endocrine regulators as they preside over the diapause program.

3.12.3. Endocrine Regulators

3.12.3.1. Hormonal Basis for Embryonic Diapause

Embryonic diapauses are common among the Orthoptera, Hemiptera, Lepidoptera, and Diptera (especially species of *Aedes* mosquitoes), but there are reports from other orders as well. The diverse stages in which embryonic diapauses occur suggest a potentially rich diversity of regulatory schemes. Thus far, hormonal regulation has been examined only in a few species of Lepidoptera, and it is evident from the Lepidoptera that no single regulatory scheme prevails.

The most intensely studied embryonic diapause is that of *Bombyx mori*. Classic experiments, begun in the 1950s by Fukuda (1951, 1952) and Hasegawa (1952), firmly set the stage for a wealth of papers documenting the regulatory mechanism controlling the early embryonic diapause in this commercially important species. More recently, work from the Yamashita laboratory provided major contributions to the hormonal, biochemical, and molecular characterization of diapause in *B. mori*.

Diapause in *B. mori* differs from that of most model species. While both temperature and daylength are key components of the diapause decision, the specifics are reversed as compared to other diapause models. High temperature and long daylength prompt diapause induction, while low temperature and short daylength lead to nondiapause development. Diapause induction in this species is under strict maternal control. The photosensitive stage occurs far in advance of the actual diapause stage: exposure of developing female embryos and larvae to diapause-inducing conditions will cause the female to produce eggs that are destined to enter diapause. This effect is mediated by diapause hormone (DH), a neuropeptide released by the female adult during her period of egg maturation. DH acts upon the ovarioles, leading to the production of diapause-destined eggs, as described below. After being oviposited, the silkworm embryos enter a diapause characterized by a G_2 cell cycle arrest (Nakagaki *et al.*, 1991). Once diapause has been established, this cell cycle arrest can be broken only after the embryos have been chilled for 1–3 months at 5 °C. Following this mandatory chilling period, development can be initiated when the embryos are transferred to a higher temperature.

As early as 1926, ovary transplantation experiments indicated that the factor controlling diapause induction is bloodborne (Umeya, 1926). Later studies by Fukuda and Hasegawa identified the brain and subesophageal ganglion as the tissues controlling the release of the diapause-inducing factor now known as DH, and bioassays performed by injecting nondiapause-type individuals with tissue extracts showed DH activity in subesophageal ganglion and brain extracts.

3.12.3.1.1. Diapause hormone and its action in *Bombyx mori*

Although the activity of DH was established quite early, purification and sequencing of

the peptide progressed more slowly. An initial chemical characterization was made from extracts of 15 000 isolated brain–subesophageal ganglion complexes (Hasegawa, 1957). Further analysis, including an assessment of amino acid composition, was made from the extracts of 1 million isolated male adult heads (Isobe *et al.*, 1973). A final push for determining the amino acid sequence began in 1987, when extracts from 100 000 subesophageal ganglia yielded enough material for isolation and subsequent analysis. Amino acid sequencing finally yielded a 24 amino acid peptide (Imai *et al.*, 1991). Subsequent cloning of the cDNA for DH confirmed the amino acid sequence (**Figure 5**), and led to further characterization of the hormone (Sato *et al.*, 1992, 1993).

The amino acid sequence of DH indicates that it is a member of the FXPRL amide peptide family and shares the same five amino acid sequence at

TDMKDESDRGAHSERGALWFGPEL-NH_2

Figure 5 Amino acid sequence of diapause hormone from *Bombyx mori*.

the C-terminus with a variety of hormones including pheromone biosynthesis activating neuropeptide (PBAN) and pyrokinin. Although the sequence of PRL-NH_2 is the minimum structure required for DH activity (Imai *et al.*, 1998), substantially greater activity is noted from the C-terminal pentapeptide. By synthesizing a series of DH analogs of different lengths, it was determined that two other regions of the peptide in the center and near the N-terminus also contribute to biological activity (Saito *et al.*, 1994). The C-terminal amide group is essential for biological activity (Suwan *et al.*, 1994).

Cloning of the cDNA for DH resulted in a 800 bp nucleic acid sequence with a 192 amino acid open reading frame, a size much larger than the 24 amino acid DH peptide (Sato *et al.*, 1992, 1993). Computer analysis of the deduced amino acid sequence reveals that not only DH, but several additional neuropeptides, including PBAN and three other members (subesophageal ganglion neuropeptides) of the FXPRL family of neuropeptides are encoded in the single open reading frame (**Figure 6**).

```
+1 CAACAACAAAA ATG TAT AAA ACC AAC ATT GTT TTC AAC GTT TTA GCT TTG GCA TTG   56
+1              M   Y   K   T   N   I   V   F   N   V   L   A   L   A   L   15

   TTC AGT ATT TTC TTC GCG AGT TGC ACG GAT ATG AAG GAT GAA AGC GAC AGA GGA  110
    F   S   I   F   F   A   S   C   T   D   M   K   D   E   S   D   R   G   33
                                   DH

   GCT CAC AGT GAG CGG GGC GCT CTC TGG TTC GGC CCC AGA CTC GGG AAG CGA TCA  164
    A   H   S   E   R   G   A   L   W   F   G   P   R   L   G   K   R   S   51

   ATG AAG CCA TCC ACT GAA GAT AAC AGG CAA ACC TTC CTG AGG CTG CTC GAG GCG  218
    M   K   P   S   T   E   D   N   R   Q   T   F   L   R   L   L   E   A   69

   GCT GAT GCC CTC AAA TTT TAC TAC GAC CAG CTA CCT TAC GAG AGG CAA GCG GAT  272
    A   D   A   L   K   F   Y   Y   D   Q   L   P   Y   E   R   Q   A   D   87

   GAA CCG GAA ACC AAA GTA ACA AAG AAG ATC ATC TTC ACC CCC AAA CTC GGG AGG  326
    E   P   E   T   K   V   T   K   K   I   I   F   T   P   K   L   G   R  105
                                           α

   AGC GTC GCC AAA CCC CAG ACG CAT GAA AGC CTC GAA TTC ATC CCC CGG CTC GCA  380
    S   V   A   K   P   Q   T   E   E   S   L   E   F   I   P   R   L   G  123
   β

   AGG CGG CTC TCT GAC GAC ATG CCT GCT ACG CCA GCT GAC CAG GAA ATG TAC CAA  434
    R   R   L   S   E   D   M   P   A   T   P   A   D   Q   E   M   Y   O  141
           PBAN

   CCT GAC CCC GAA GAA ATG GAG TCA AGA ACA AGA TAC TTC TCG CCC AGG CTG GGG  488
    P   D   P   E   E   M   E   S   R   T   R   Y   F   S   P   R   L   G  159

   CGC ACC ATG AGC TTT TCG CCC AGA CTG GGA AGG GAG CTT TCG TAC GAT TAC CCT  542
    R   T   M   S   F   S   P   R   L   G   R   E   L   S   Y   D   Y   P  177
       γ

   ACA AAA TAT AGG GTT GCC AGA AGC GTT AAC AAG ACA ATG GAC AAC TAA ACG AAT  596
    T   K   Y   R   V   A   R   S   V   N   K   T   M   D   N              192

   TAT GGT CCG CTT GAG GTA CCT CAT TTG AGG TCT CGA TCF ACT CCG ACG AAC GGT  650
   TAC GGG TAA ACG GCG ACA ATG TTA ATG TTT TGG ACG AAA CAA TTG TTA ATT AAT  704
   AAA TTC ACG TGA TTT TGG AAT TGT AAT TTA TAA GTG AAT AAA AAA ATA AAC TAT  758
   TTA AAA TAA ATG AGT CAT TAT TAT TAT TAA AAA AAA AAA AAA                  800
```

Figure 6 The cDNA sequence and deduced amino acid sequences of diapause hormone–pheromone biosynthesis activating neuropeptide (DH-PBAN) from *Bombyx mori*, showing the sequences and locations of DH, PBAN, and other putative hormones within the preprotein. (Adapted from Sato, Y., Oguchi, M., Menjo, N., Imai, K., Saito, H., *et al.*, **1993**. Precursor polyprotein for multiple neuropeptides secreted from the subesophageal ganglion of the silkworm *Bombyx mori*: characterization of the DNA-encoding diapause hormone precursor and identification of additional peptides. *Proc. Natl Acad. Sci. USA 90*, 3251–3255.)

The resulting polyprotein is a prohormone that is post-translationally processed to excise the active peptides. The presence of PBAN and other neuropeptides in the DH prohormone stresses the importance of posttranslational control of hormone release. This also helps to explain the formerly perplexing data indicating the presence of DH activity from a wide variety of developmental stages, from both sexes, and from both diapausing and nondiapausing individuals. It is now assumed that the large peak of activity seen in developing pharate adults (Sonobe *et al.*, 1977) can be attributed not to a function of DH, but to the synthesis of PBAN as the moth completes its maturation and prepares for the synthesis of pheromones.

Genomic characterization of DH has also been achieved: Southern blotting reveals a single copy of DH-PBAN in the *Bombyx* genome, and linkage analysis suggests that it is located on chromosome 11 (Pinyarat *et al.*, 1995). The gene contains six exons separating five introns; the sequence encoding DH is located in the first two exons. The region upstream of the open reading frame includes an ecdysone response element and five homeodomain TAAT binding core domains (Xu *et al.*, 1995a).

Even in the absence of purified hormone or the DH clone, early researchers were able to characterize the DH control mechanism, as well as the site of DH synthesis and release. Early transplantation experiments indicated high DH activity in the subesophageal ganglion, and histological studies from Fukuda's laboratory identified a single pair of cells in the subesophageal ganglion as the likely site of DH release. More sensitive molecular methods have largely confirmed these results. Using real-time polymerase chain reaction (RT-PCR), it has been determined that DH is exclusively expressed in the subesophageal ganglion; no DH expression is observed in the brains, or in thoracic or abdominal ganglia, or in nonneural tissues (Sato *et al.*, 1994). *In situ* hybridization has been used to further detail the expression of DH within the subesophageal ganglion. Expression is concentrated in 12 cells grouped into three clusters along the midline of the subesophageal ganglion (Sato *et al.*, 1994). Surgical manipulation indicates that the different clusters are responsible for different activities of the prohormone. Females retaining only the two cells in the posterior cluster continue to be capable of inducing diapause in their progeny, while females with only the six cells in the medial cluster retain PBAN function. Surgical removal of the anterior cluster of four cells does not substantially affect either function, indicating that this cluster may be involved in the function of other portions of the prohormone

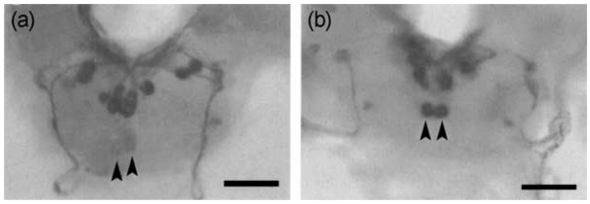


Figure 7 Localization of neurosecretory cells containing diapause hormone (DH) within the subesophageal ganglion of (a) diapause and (b) nondiapause type adults of *Bombyx mori*. Release of DH leads to a decrease of staining in diapause type individuals. Arrows indicate the somata containing DH. (Adapted from Sato, Y., Shiomi, K., Saito, H., Imai, K., Yamashita, O., **1998**. Phe-X-Pro-Arg-Leu-NH2 peptide producing cells in the central nervous system of the silkworm, *Bombyx mori*. *J. Insect Physiol.* *44*, 333–342.)

(Ichikawa *et al.*, 1996). Further supporting evidence for the identity of the DH cells comes from an immunohistochemical study using antibodies recognizing the FXPRL amide. This antibody recognizes several cells in the brain–subesophageal ganglion complex that contain peptides in this family. Of the 12 cells in the subesophageal ganglion that exhibit antigenicity, two show decreased staining in diapause-type as compared to nondiapause-type individuals (**Figure 7**), indicating release of the peptide in diapause-type females (Sato *et al.*, 1998). Immunocytochemistry combined with intracellular Lucifer Yellow injections reveals that the axons of cells in all three clusters pass through the brain and into the corpus cardiacum (Ichikawa *et al.*, 1995), the known site of DH release.

While previous studies showed DH activity in larval, pupal, and adult stages (Hasegawa, 1964), further resolution of DH expression patterns was not possible until more sensitive molecular techniques were implemented. Using RT-PCR, it was determined that the transcription of DH-PBAN is under the control of both development and temperature (Xu *et al.*, 1995b; Morita *et al.*, 2004). When *B. mori* is raised under diapause-inducing conditions of 25 °C, five peaks of synthesis are noted throughout development: the first peak occurs during embryogenesis, one peak during both the fourth and fifth instars, and two peaks during pupal and adult development. By contrast, when the insects are rearing under a nondiapausing-temperature of 15 °C, only the final peak of gene expression during pupal–adult development is observed (**Figure 8**). This final peak of transcription is most likely associated with PBAN, rather than DH, activity.

Several studies offer insights into the neural control of DH expression and release. Both *in vivo* and *in vitro* studies have shown that translation of the gene encoding DH is under control of the

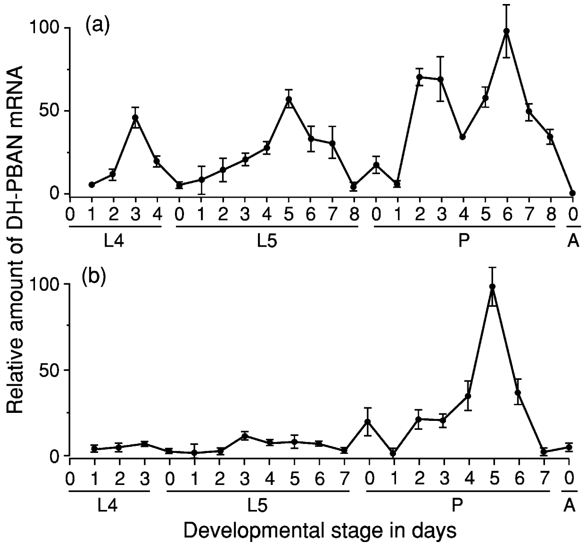


Figure 8 Levels of DH-PBAN mRNA in (a) diapause and (b) nondiapause type *Bombyx mori* during the course of development. L4, L5, fourth and fifth instars; P, pupa; A, adult. (Adapted from Xu, W.-H., Sato, Y., Ikeda, M., Yamashita, O., **1995a**. Stage-dependent and temperature controlled expression of the gene encoding the precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide in the silkworm, *Bombyx mori*. *J. Biol. Chem. 270*, 3804–3808.)

neurotransmitter dopamine. Dopamine levels in both the hemolymph and brain–subesophageal ganglion complexes of diapause-type individuals is significantly higher than in nondiapause-type individuals during the late larval and early pupal stages (Noguchi and Hayakawa, 2001). The high dopamine levels correlate with an increase in dopamine decarboxylase expression. Interestingly, a specific subset of cells appears to be responsible for the elevated dopamine titer. A critical role for dopamine associated with DH activity is further suggested by experiments in which DOPA is fed to nondiapause-type larvae during the final larval instar or injected into nondiapause-type pupae. Both of these treatments can elicit the production of diapausing eggs by nondiapause-type females. Additionally, pupal injections elevate DH mRNA in the brain–subesophageal ganglion complexes. *In vitro* studies further confirm the effects of dopamine. DH mRNA levels in isolated brain–subesophageal ganglion complexes from nondiapause-type pupae are elevated within 2 h after addition of either DOPA or dopamine to the medium.

Neuronal effects on DH are not restricted to transcriptional control. The release of DH from the subesophageal ganglion is under control of GABAergic neurons. Culturing isolated brain–subesophageal complexes from diapause-egg producing females in the presence of the inhibitory neurotransmitter GABA causes a substantial reduction in the release of DH , while incubation with picrotoxin, the GABA inhibitor, promotes DH release from the cultured brain–subesophageal complexes (Hasegawa and Shimizu, 1990; Shimizu *et al*., 1997). Further insight into the control of DH release may be gleaned from data for PBAN, which shares a common proprotein with DH. PBAN release is under circadian control, and under a light–dark cycle, PBAN-releasing neurons fire during the scotophase. This firing pattern persists with a periodicity of ~24 h (free runs) even in constant darkness, thus indicating the circadian nature of this firing pattern (Tawata and Ichikawa, 2001). At this point, however, there is no direct evidence indicating a circadian pattern of DH release.

The ovary appears to be the sole target for DH. Its action is most certainly mediated through a second messenger system, although specifics of the signal transduction system remain unclear. *In vitro* experiments suggest that early events, such as the upregulation of trehalase described below, are regulated by Ca^{2+} ion influx. Addition of the chelating agent egtazic acid (EGTA) to the incubation medium suppresses DH activity on trehalase, while the addition of Ca^{2+} restores this activity (Ikeda *et al*., 1993). Later events may also involve cyclic nucleotide cascades. DH acts to reduce the concentration of cyclic GMP in diapause-destined eggs through the regulation of guanylate cyclase activity (Chen *et al*., 1988; Chen and Yamashita, 1989).

A key indicator of DH activity reported in the older literature is a metabolic switch, resulting in the accumulation of glycogen in diapause-destined eggs. This accumulation is a result of the activity of the enzyme trehalase, which exhibits transcriptional upregulation after DH injection (Su *et al*., 1994). Accumulated glycogen is then converted to sorbitol, which acts as a classic antifreeze to lower the supercooling point of the diapausing eggs (Chino, 1957, 1958). Upon diapause termination, sorbitol is reverted to glycogen, which is subsequently used as an energy reserve. Recent data revealed the important observation that sorbitol accumulation plays a regulatory role as well (Horie *et al*., 2000). When diapause-destined eggs are dechorionated and cultured in Grace's medium, diapause is averted. When sorbitol is added to the medium the ability to diapause is restored in a dose-dependent fashion. Substantial inhibition of development is noted at biologically relevant concentrations of sorbitol. Trehalose elicits similar inhibitory effects, while glycerol is less effective. A regulatory role for sorbitol is further supported by data showing that subsequent removal of sorbitol induces resumption

of development, and that nondiapause-destined eggs arrest development when incubated in sorbitol.

3.12.3.1.2. Other schemes for regulating embryonic diapause There is no evidence to suggest that the regulatory scheme carefully documented in *B. mori* is relevant to other forms of embryonic diapause. Unfortunately other species have not received nearly the attention devoted to the silkworm, but the fragments of information that are available indicate a potentially rich diversity of regulatory mechanisms operating to halt development in embryos. Embryonic diapauses are enormously diverse, as indicated by the wide range of stages at which development is arrested, and the challenge of working on embryos has limited the number of researchers willing to address this difficult stage. Thus far, three distinct regulatory schemes have been suggested for embryonic diapause as alternatives to the scheme noted in *B. mori*.

The gypsy moth, *Lymantria dispar,* diapauses as a pharate first instar larva. Several lines of evidence indicate that this diapause is induced and maintained by an elevated ecdysteroid titer. Using synthesis of a 55 kDa gut protein, now known to be hemolin (Lee *et al.*, 2002), as an indicator of diapause, Lee and Denlinger (1997) demonstrated that placing a ligature behind the prothoracic gland blocks hemolin synthesis, and coculturing the gut with an active prothoracic gland promotes hemolin synthesis by the gut. Synthesis of this diapause indicator protein, hemolin, can also be induced if an isolated abdomen is injected with 20E or an ecdysteroid agonist RH-5992. KK-42, an imidazole derivative thought to block ecdysteroid synthesis, averts diapause in this species (Suzuki *et al.*, 1993; Bell, 1996; Lee and Denlinger, 1996), but this effect can be reversed with the application of ecdysteroids (Lee and Denlinger, 1997). And, at the end of the mandatory chilling period, diapause can be readily terminated if pharate larvae are transferred to high temperatures, but such individuals exposed to ecdysteroids fail to break diapause. Collectively, these results suggest that ecdysteroids are essential for diapause induction and maintenance. Experiments with a mutant, nondiapausing strain of *L. dispar* are also consistent with this idea (Lee *et al.*, 1997): all of the characteristics of the nondiapause strain could be switched to characteristics of the diapause strain by artificially subjecting pharate larvae from the mutant strain to elevated concentrations of ecdysteroid. Although ecdysteroids of maternal origin are well known to be incorporated into eggs, at this late stage of embryonic development the ecdysteroids are likely to be synthesized by the pharate larva's prothoracic gland. In the wild-type strain, the prothoracic gland appears to synthesize ecdysteroids continuously throughout diapause, and then shut down synthesis to terminate diapause. A diapause-promoting effect for ecdysteroids at first may seem paradoxical, especially considering the fact that a lack of ecdysteroids is the dominant feature of most larval and pupal diapauses (see Section 3.12.3.3.2). Yet, it is well known that a drop in the ecdysteroid titer is an essential signal permitting the ongoing progression of development in other systems (Slama, 1980), and the gypsy moth appears to have exploited this feature to bring about a halt to development.

The opposite effect can also be elicited by ecdysteroids, as reported for the embryonic diapauses of two locust species: the Australian plague locust *Chortoicetes terminifera* (Gregg *et al.*, 1987) and the migratory locust, *Locusta migratoria* (Tawfik *et al.*, 2002b). In both of these cases, nondiapausing eggs contain approximately three times the amount of ecdysteroids as found in diapausing eggs. In these species the diapauses occur early in embryonic development, and the ecdysteroids present are likely to be exclusively of maternal origin. In nondiapause eggs and those in which diapause was averted by high temperature, ecdysteroid titers increase after the stage at which diapause normally occurs (Gregg *et al.*, 1987), and likewise after diapause is terminated, the ecdysteroid titers increase (Tawfik *et al.*, 2000b). Whether this elevation at diapause termination is the result of *de novo* synthesis or hydrolysis of conjugated ecdysteroids of maternal origin remains unkown.

The pharate first instar larval diapause of the silkmoth *Antheraea yamamai* represents yet another intriguing regulatory scenario (Suzuki *et al.*, 1990). These experimental conclusions rely on differences in the efficacy of the imidazole KK-42 to terminate diapause following exposure to different portions of the body. KK-42 is highly efficacious in terminating diapause when the pharate first instar larva is intact, and it also works well to terminate diapause in a headless body. But diapause persists in the head–thorax compartment, thus suggesting the presence of a repressive factor from the thorax. Isolated abdomens break diapause whether or not KK-42 is added. From these results, Suzuki *et al.* (1990) conclude the presence of a diapause-promoting factor produced in the mesothorax, and a development-promoting factor produced in the region of the second to fifth abdominal segments. During diapause the diapause-promoting factor prevails and development is halted. Although the identities of these factors remain unknown, the effects can not be mimicked by

any of the obvious candidates: prothoracicotropic hormone, ecdysteroids, or JH.

3.12.3.1.3. Associated changes in gene expression In *B. mori,* expression patterns of several genes associated with the G_2 cell cycle arrest have been monitored. Flow cytometery indicates that 98% of the embryonic cells are in the G_2 phase 3.5 days after oviposition. Conversely, within 24 h after the artificial termination of diapause, up to 50% of the cells are in S phase (Nakagaki *et al*., 1991). Considerable interest has focused on the molecular basis of this cell cycle arrest, especially the genes that encode cyclin B and cyclin dependent kinase 2 (cdc2), the two genes most likely involved in mediating this arrest. Northern blotting and RT-PCR have demonstrated that levels of cyclin B drop substantially in both diapausing and nondiapausing eggs within two days after oviposition. Interestingly, incubating diapausing eggs at 5 °C to initiate diapause termination leads to a partial restoration of cyclin B levels, yet artificial termination of diapause has little effect on cyclin B mRNA levels. Together, these data suggest that there is not necessarily a connection between cyclin B transcription and mitotic activity (Takahashi *et al*., 1996). Analysis of *Bombyx cdc2* and *Bombyx cdc-2 related kinase* (*Bcdrk*) expression proved more promising: mRNA levels for both genes is substantially lower in diapausing individuals than in nondiapausing ones. Exposure to 5 °C boosts expression, as does artificial diapause termination (Takahashi *et al*., 1998). Together, these results suggest that *cdc2*, and perhaps *Bcdrk* are involved in the regulation of this G_2 cell cycle arrest during diapause.

Several other genes of interest to silkmoth diapause have also been characterized. Differential display was used to isolate a diapause upregulated gene, *BmEts* (Suzuki *et al*., 1999). This gene is highly upregulated in early diapause, but mRNA levels diminish to trace amounts after 50 days. Interestingly, in the nondiapause egg mutant *pnd*, *BmEts* expression is suppressed, indicating that *BmEts* expression is downstream of the *pnd* mutation. This information is especially intriguing because, as the name suggests, *BmEts* belongs to the *ETS* gene family of transcription factors which includes several genes such as *Drosophila E74*, a gene known to be important in the cell's response to ecdysone.

Differential display has also been used to investigate the molecular events occurring during the period of chilling required for diapause termination. These experiments resulted in the cloning of *Samui*, a novel gene containing a BAG domain (Moribe *et al*., 2001). This gene is inducible by low temperatures in nondiapausing individuals, but the response is much more pronounced in diapausing individuals. Upregulation persists for 30 days at low temperatures, and the gene is quickly downregulated when the eggs are returned to a higher temperature. Although the function of *Samui* during chilling remains unclear, it may be involved in protection against chilling injury. Similar to other genes of the BAG family, *Samui* can bind Hsp70, which suggests that it may affect low temperature survival. *Samui* may also be involved in the signal cascade of diapause termination. Downregulation of *Samui* occurs concurrently with the upregulation of sorbitol dehydrogenase, the enzyme involved in converting sorbitol to glycogen. As such it could be involved in removing the important sorbitol blockade to embryonic development, as noted earlier.

Beyond *Bombyx*, few species with an embryonic diapause have been examined for diapause-specific gene expression. Several mRNAs have been sequenced from the early embryonic diapause of *Aedes triseriatus,* but most encode unknown proteins and those that have been cloned do not appear to be specific for diapause (Blitvich *et al*., 2001). In the pharate first instar diapause of *L. dispar,* two defense-related proteins are upregulated during diapause: a 70 kDa heat shock protein begins to be synthesized at the onset of chilling (Denlinger *et al*., 1992), and the gene encoding hemolin is highly upregulated for the first 100 days of diapause, the period encompassing the mandatory chilling period (Lee *et al*., 2002). One of the cytoplasmic actins in the brain is conspicuously downregulated during diapause (Lee *et al*., 1998), and its absence thus serves as a good biomarker for diapause.

3.12.3.2. Hormonal Basis for Larval and Pupal Diapauses

Larval diapauses are best known from the Lepidoptera, but there are good examples of larval (or nymphal) diapauses in other orders as well. Diapause in the last larval instar is reported most frequently, but some larvae diapause in earlier instars or as prepupae. Pupal diapause has been examined extensively in Lepidoptera and the higher Diptera, but appears to be rare in other taxa. It is indeed the true pupal stage that is usually involved in diapause, but occasional reports indicate a diapause late in pharate adult development. Larval and pupal diapauses share much in common. Central to both is a failure to progress to the next metamorphic stage. This suggests that one of the key endocrine events regulating such a diapause is likely to be a failure of the prothoracic gland to produce the ecdysteroids

needed to initiate the next molt. Invariably, this has proven to be a key element of larval and pupal diapauses. One might also predict a shutdown until the end of diapause in the production of prothoracicotropic hormone (PTTH), the hormone from the brain that stimulates the prothoracic gland to produce ecdysone (see **Chapter 3.2**). This may often be the case but not always. The other major metamorphic hormone, JH, may or may not play a role, depending on the species.

3.12.3.2.1. Role of the brain–prothoracic gland axis The older literature is replete with experiments showing that activation of the brain is essential for terminating diapause. Of special significance are the classic experiments by Carroll Williams demonstrating that the brain of the giant silkworm *Hyalophora cecropia*, when activated by chilling, is capable of terminating diapause when implanted into a brainless pupa (Williams, 1946, 1947, 1952). These experiments were critical not only for understanding the mechanism of diapause but also for deciphering the endocrine basis for insect metamorphosis. One can now be more specific and attribute these brain effects not to the whole brain, but to the production of PTTH by a pair of neurosecretory cells in the pars intercerebralis of each brain hemisphere (Agui *et al.*, 1979).

In the pupal diapause of *M. sexta* intracellular electrical recordings of the cells that produce PTTH show all the characteristics of silent cells: high voltage thresholds, low input resistance, and few spontaneous action potentials (Tomioka *et al.*, 1995). Electrical differences between the PTTH cells of diapausing and nondiapausing pupae are already evident within 1 day after pupation. As diapause progresses in chilled pupae, the cells gradually display more spontaneous action potentials, and more excitatory postsynaptic potentials are observed. In spite of the early curtailment of neurophysiological activity in the PTTH cells, there are no corresponding ultrastructural changes that would appear to reflect the electrical silencing of the cells in early diapause (Hartfelder *et al.*, 1994).

One common method for monitoring PTTH activity is to culture prothoracic glands *in vitro* and monitor ecdysone production when brains or brain extracts are added to the culture medium. When this has been done, low PTTH activity associated with larval and pupal diapause has been observed in some cases. In *Diatraea grandiosella,* a species that diapauses late in the final larval instar, PTTH activity is far lower in brains and brain extracts from diapause-destined larvae and diapausing larvae than in nondiapausing larvae (Yin *et al.*, 1985). Likewise, in the European corn borer, *Ostrinia nubilalis*, PTTH activity is lower in brains of diapausing larvae than in nondiapausing brains (Gelman *et al.*, 1992). Similar low levels of PTTH activity are noted in the pupal diapause of the cabbage armyworm, *Mamestra brassicae* (Endo *et al.*, 1997). Yet, the brains from all of these species clearly show some PTTH activity, and pupal brain extracts from *M. sexta* (Bowen *et al.*, 1984) and *S. argyrostoma* (Richard and Saunders, 1987) actually have as much or more PTTH activity than brain extracts from nondiapausing pupae. Likewise, PTTH activity is higher in larvae of *S. peregrina* that are destined for pupal diapause than in larvae not destined for diapause (Moribayashi *et al.*, 1992). These observations suggest that the hormone may be present during diapause but is simply not released.

In *Heliothis virescens,* expression of the transcript that encodes PTTH persists at relatively high levels throughout larval development, but at the onset of wandering behavior, expression in larvae programmed for pupal diapause drops and remains low during diapause, while the transcript continues to be highly expressed in nondiapause-destined larvae and nondiapausing pupae (Xu and Denlinger, 2003). These observations suggest that, in this species, PTTH is regulated at the level of transcription. This conclusion is not easily reconciled with the above observations suggesting that PTTH is made but not released during diapause, but it is quite likely that considerable species variation may occur. Both a halt in PTTH synthesis or a failure of PTTH release could result in a developmental shutdown.

Several recent experiments suggest that high dopamine levels in the brain may be critical for diapause induction, possibly by preventing the release of PTTH. High levels of dopamine in the brains of diapause-destined larvae of *Chymomyza costata* (Kostal *et al.*, 1998), diapause-destined pupae of *M. brassicae* (Noguchi and Hayakawa, 1997), and pupae of *Pieris brassicae* (Puiroux *et al.*, 1990) suggest a causal relationship. In diapausing pupae of *Antheraea pernyi*, a drop in brain dopamine is noted shortly before the surge of ecdysteroids that elicits diapause termination (Matsumoto and Takeda, 2002). But the most compelling evidence suggesting a role for dopamine is the fascinating observation that feeding L-DOPA to last instar larvae of *M. brassicae* reared under long daylengths (not diapause-inducing) elicits a diapause-like state in the pupae (Noguchi and Hayakawa, 1997). Other than DH in *Bombyx*, there are a few examples of chemical agents capable of inducing diapause or a diapause-like state, thus this result with DOPA feeding is quite striking. A potential role is also discussed for dopamine in the induction of embryonic diapause of

B. mori (Noguchi and Hayakawa, 2001) (see Section 3.12.3.1.1). Among genes that are upregulated by both short day conditions and the feeding of DOPA in *M. brassicae*, the most striking is an upregulated gene with high identity to a *H. virescens* gene that encodes a receptor for activated protein kinase C (RACK) (Uryu *et al*., 2003). *In situ* hybridization with a RACK probe reveals expression of this gene within a few cells in the medial protocerebral neuropile. As a member of the protein kinase C (PKC) signaling cascade, RACK has the potential to be an important gene contributing to transduction of the signals involved in diapause induction. Noguchi and Hayakawa (1997) propose that the elevation of dopamine may be key to the prevention of PTTH release.

Elevation of brain serotonin (5-hydroxytryptamine) has also been linked to diapause in several cases (Kostal *et al*., 1999; L'Helias *et al*., 2001), but not in others (Puiroux *et al*., 1990). In *C. costata*, depletion of serotonin does not alter the fly's capacity to enter larval diapause (Kostal *et al*., 1999), thus suggesting that the observed elevation of this monoamine is not essential for diapause induction.

The shutdown in the brain–prothoracic gland axis is not exclusively the result of a failure of the PTTH cells to produce or release PTTH. The prothoracic gland also becomes refractory to PTTH at this time, thus providing a double assurance that the neuroendocrine system will not provide the signal needed to promote development. Assays for PTTH activity indicate that prothoracic glands dissected from diapausing individuals are refractory to stimulation, as shown in larvae of *Calliphora vicina* (Richard and Saunders, 1987) and *O. nubilalis* (Gelman *et al*., 1992), and in pupae of *Manduca sexta* (Bowen *et al*., 1984) and *S. argyrostoma* (Richard and Saunders, 1987). This loss of competency can be rather rapid (**Figure 9a**): in *S. argyrostoma* the prothoracic glands lose their competency to produce ecdysone within a 1–2 day period at the onset of diapause (Richard and Saunders, 1987). In the larval diapause of *C. vicinia* the loss of competency is more gradual, occurring over a 6 day period, and even during diapause the gland maintains limited competency to produce ecdysone (**Figure 9b**). At termination of diapause, prothoracic gland competency is again restored quickly. When diapausing larvae of *C. vicina* are transferred from 11 to 25 °C, the prothoracic gland regains competency to produce ecdysone within 24 h (Richard and Saunders, 1987).

PTTH stimulation of the prothoracic gland in nondiapausing insects is mediated by cyclic AMP as a second messenger, but the diapausing glands of *M. sexta* (Smith *et al*., 1986) and *S. argyrostoma* (Richard and Saunders, 1987) fail to respond to cyclic AMP or agents known to elevate cyclic AMP, thus implying that the block in gland function occurs beyond the stage of cyclic nucleotide action.

Reports from several species of Lepidoptera indicate that cells of the prothoracic gland are greatly reduced in size during diapause, both the cytoplasm and nuclei are shrunken, and the mitochondria take on a swollen configuration (Denlinger, 1985). In *S. crassipalpis,* there is a slight reduction in the size of the cells in the prothoracic gland component of the ring gland, but this difference is not as conspicuous as noted in the Lepidoptera (Joplin *et al*., 1993). Results of an ultrastructural examination of the fly prothoracic gland during diapause are rather surprising. Instead of being inactive, the presence of extensive arrays of rough endoplasmic reticulum suggests that the glands remain active in protein synthesis. Pulse labeling of the ring gland further supports the contention that the gland continues to synthesize proteins during diapause. The function of this curious diapause activity remains unknown, but it would appear to be unrelated to ecdysone production since all evidence indicates that this function is shut down at this time.

Though there appears to be species variation as to whether the synthesis or release of PTTH is regulated, the net effect, a shutdown in the synthesis of ecdysteroids, is consistent. The earlier literature provides numerous examples documenting a low or undetectable ecdysteroid titer in larvae or pupae in diapause and an elevation in the ecdysteroid titer at the termination of diapause. More recent studies continue to provide evidence that this is the case, e.g., larval diapauses of *O. nubilalis* (Gelman and Woods, 1983; Gelman and Brents, 1984; Peypelut *et al*., 1990) and *Omphisa fuscidentalis* (Singtripop *et al*., 2002) and pupal diapauses of *Mamestra configurata* (Bodnaryk, 1985), *Manduca sexta* (Friedlander and Reynolds, 1992), *S. argyrostoma* (Richard *et al*., 1987), and *S. peregrina* (Moribayashi *et al*., 1988). As will be discussed below (see Section 3.12.3.2.4), some larvae periodically undergo a stationary molt during diapause, and in these cases a burst of ecdysteroid synthesis may occur during diapause and thus prompt the larva to molt. The surge in ecdysteroids that normally occurs at the end of diapause may come as two distinct waves. In *M. configurata* the release of ecdysone into the hemolymph is followed several days later by the appearance of 20E (Bodnaryk, 1985). Since these two peaks are nearly identical in size, they may simply represent the conversion of ecdysone to 20E. In *Ostrinia nubilalis* a small transient peak of 20E is followed several days later by a major peak of

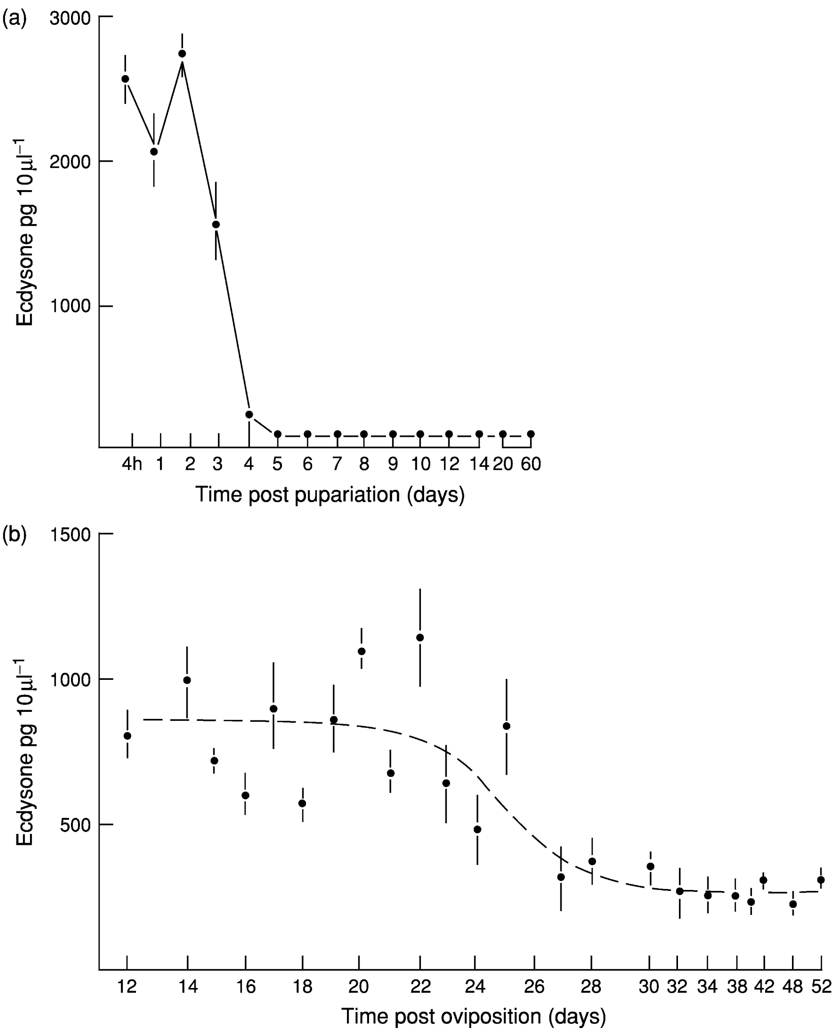


Figure 9 Decline in the competency of ring glands from diapause-destined (a) *Sarcophaga argyrostoma* and (b) *Calliphora vicina* to synthesize ecdysone in response to incubation with prothoracicotropic hormone (PTTH)-producing brains. As *S. argyrostoma* enters pupal diapause and as *C. vicina* enters larval diapause their ring glands become refractory to PTTH stimulation. (Adapted from Richard, D.S., Saunders, D.S., **1987**. Prothoracic gland function in diapause and non-diapause *Sarcophaga argyrostoma* and *Calliphora vicina*. *J. Insect Physiol.* *33*, 385–392.)

20E (Peypelut *et al.*, 1990). In this case, imaginal wing disc development is thought to be initiated by the small initial peak, but the later phases of postdiapause development require the sustained presence of 20E.

Consistent with the concept that the absence of ecdysteroids is central to maintaining the diapause state is the observation that an application of exogenous ecdysteroids or ecdysteroid mimics can terminate diapause. Again, the older literature includes numerous examples supporting this response, and more recent work continues to document this effect, e.g., larval diapauses of *O. nubilalis* (Gadenne *et al.*, 1990) and *Diprion pini* (Hamel *et al.*, 1998), and pupal diapauses of *Mamestra configurata* (Bodnaryk, 1985), *Pieris brassicae* (Pullin and Bale, 1989), and *Manduca sexta* (Friedlander and Reynolds, 1992; Sielezniew and Cymborowski, 1997; Champlin and Truman, 1998).

3.12.3.2.2. The missing pieces The role of the brain–prothoracic gland axis in diapause, as presented above, is reasonably cohesive. In a simplified version, the prothoracic gland fails to produce ecdysone because it has not been stimulated by PTTH from the brain. Yet, some pieces of information are not consistent with this simple story and remain unexplained. For *Hyalophora cecropia* the above story is just fine. The brain, when chilled, becomes activated to release PTTH and this in turn prompts the prothoracic glands to secrete the ecdysteroids needed to initiate development. Without a brain,

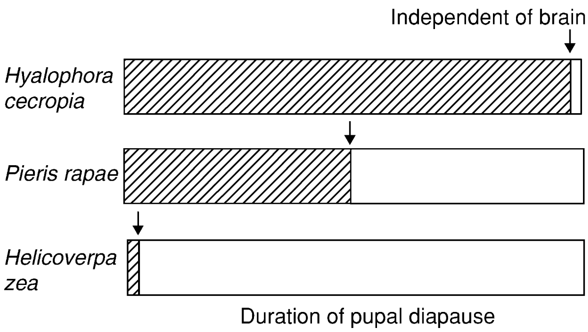


Figure 10 Time during pupal diapause at which several species are no longer dependent on the brain for initiation of adult development. (Adapted from Denlinger, D.L., **1985**. Hormonal control of diapause. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 353–412.)

the pupa is locked into a permanent diapause. But, in several cases, this is not true (Denlinger, 1985). In *M. sexta, P. rapae*, and *Antheraea polyphemus* removal of the brain during the first month or so of diapause will produce a permanent diapause, but in later stages removal of the brain has no impact (**Figure 10**). The extreme case is noted in *Helicoverpa zea*: brain removal within the first 4 h after pupation will cause a permanent diapause, but within 24 h the diapausing pupa is already independent of the brain. Clearly the *Hyalophora cecropia* model can not explain these observations. Meola and Adkisson (1977) argue that PTTH exerts it effect very early in *H. zea*, and after receiving the PTTH signal, the prothoracic glands take on the role of the regulatory organ: high temperature (27 °C) promotes ecdysteroid synthesis, while low temperature (21 °C) prevents ecdysteroid synthesis. In these cases it is clear that one can not simply explain the end of diapause as an event triggered by the brain's release of PTTH. What is lacking is a good explanation of this peculiar interaction between the brain and prothoracic gland. What does it mean for the brain, presumably PTTH, to stimulate the prothoracic gland so early and for the gland to delay the onset of ecdysteroid synthesis by many months?

Several lines of evidence suggest that there may be differences in the way the prothoracic gland is activated in nondiapausing individuals and at the termination of diapause. In *S. crassipalpis*, cyclic AMP levels in the brain and ring gland are high in nondiapausing pupae and low in diapausing pupae at the onset of diapause, and diapause can easily be averted in the diapause-programmed pupae if cyclic AMP levels are artificially boosted around the time of expected diapause entry (Denlinger, 1985). This observation is thus compatible with other evidence suggesting that cyclic AMP is used as a second messenger for PTTH (see **Chapter 3.2**). One might then assume it would also be possible to employ this same tactic of cyclic AMP elevation to initiate development in a pupa that has been in diapause for some time. Surprisingly, this does not work. In fact, cyclic AMP injected along with ecdysteroids actually retards the termination of diapause. By contrast, cyclic GMP, which is ineffective in averting diapause, can readily break diapause by itself and will enhance the diapause-breaking effect of ecdysteroids (Denlinger and Wingard, 1978). The fact that Richard and Saunders (1987) were unable to stimulate ecdysteroid production with PTTH or cyclic nucleotides in prothoracic glands cultured from diapausing pupae of *S. argyrostoma* or diapausing larvae of *C. vicina* also suggests that the glands are somehow activated in a different way at the termination of diapause.

Although prothoracic glands dissected from *C. vicina* are competent to synthesis ecdysone within 24 h after a transfer of the diapausing larvae to a high temperature, brain–ring gland complexes cultured *in vitro* fail to gain competency when subjected to the same temperature switch (Richard and Saunders, 1987). This suggests that some additional component from another part of the body may contribute to reactivation of the prothoracic gland. The pieces of the fly puzzle are these: cyclic GMP readily breaks diapause when injected *in vivo*, but fails to activate prothoracic glands cultured *in vitro*, and the brain–prothoracic gland can regain competency in response to high temperature *in vivo*, but not *in vitro*. A minimal interpretation of these observations is that some additional prothoracicotropic factor, perhaps acting through cyclic GMP, contributes to activation of the prothoracic gland at the termination of diapause.

3.12.3.2.3. Ecdysone receptor and Ultraspiracle

Two proteins, Ecdysone receptor (EcR) and Ultraspiracle (USP), dimerize to form the functional receptor for ecdysone (see **Chapter 3.5**). Ecdysone binds to this complex, which in turn binds directly to the ecdysone response elements to elicit specific gene action. The central role for ecdysteroids in the regulation of larval and pupal diapauses suggests that these proteins could contribute to the diapause control mechanism, but thus far few experiments have addressed this question. During pupal diapause of *S. crassipalpis* expression of EcR mRNA persists throughout diapause, but the expression of EcR's dimerization partner, USP, gradually declines at the onset of diapause and is undetectable after 20 days in diapause (Rinehart *et al.*, 2001). USP transcripts

again appear in late diapause (beginning on day 50) and are further upregulated 9 h after pupae are stimulated to break diapause. This 9 h time-frame coincides precisely with the rise in the ecdysteroid titer following hexane application. It is interesting to note that upregulation of the USP transcripts at day 50 coincides with the pupa's increased sensitivity to injected ecdysteroids. This suggests the possibility that the reappearance of the USP transcript is a preparatory step, perhaps a critical one, leading to the eventual termination of diapause. In the second instar larval diapause of *Choristoneura fumiferana*, genes encoding both isoforms of EcR are expressed throughout diapause, as is the gene encoding USP (Palli *et al*., 2001). However, the ecdysone-inducible transcription factors (CHR75A and CHR75B) and hormone receptor 3 (CHR3) are not present during diapause but reappear at diapause termination.

In *M. sexta* the transcript encoding EcR disappears at the onset of diapause and reappears when diapause is terminated (Fujiwara *et al*., 1995). In the larval diapause of the midge *Chironomus tentans* EcR transcripts are detectable throughout diapause, and an increase is noted at diapause termination (Imhof *et al*., 1993). The patterns of USP mRNA expression are unknown for these two species. Although the data base remains small at this point, the results do not suggest the emergence of a universal pattern in relation to diapause. What is clear, though, is that the presence or absence of these receptor proteins could very well be an important component of the regulatory scheme for diapause. Turning off the expression of one or both of these genes could promote diapause, while the presence of these gene products is likely to be essential for the resumption of development.

3.12.3.2.4. Role of the corpora allata The early views of larval and pupal diapause focused strictly on the brain–prothoracic gland axis, leaving little reason to suspect involvement by JH. This view was challenged by a fine set of experiments on *Diatraea grandiosella* by Chippendale, Yin and their colleagues beginning in the early 1970s (review: Chippendale, 1977). What launched their work on JH was the surprising discovery that injection of ecdysteroids into a diapausing larva of *D. grandiosella* prompted a stationary larval molt rather than pupation. The stationary molt, of course, suggested the presence of JH because the larva would have been expected to pupate in the absence of JH. This suspicion of JH involvement was followed by verification that the hemolymph titer of JH remains high and the corpora allata are active during diapause. At the end of diapause, the JH titer drops, and the larva goes ahead and pupates. A similar regulatory scenario is evident in some of the *Chilo* stemborers. What these two taxa share is the fact that they both are species that undergo stationary molts during diapause, and the only way to undergo a stationary molt rather than a progressive molt is to maintain a high JH titer. The high JH titer recently reported for larval diapause in the Mediterranean corn borer, *Sesamia nonagrioides*, suggests that it too has a diapause maintained by JH (Eizaguirre *et al*., 1998).

Species that do not undergo stationary molts presumably do not need to maintain a high JH titer during diapause, and in a number of lepidopteran larvae JH appears to play no role, e.g., *O. nubilalis* and *Laspeyresia pomonella* (Denlinger, 1985). In these species JH is actually high at the onset of diapause, but the titer drops to low levels shortly after the diapause is initiated. In these species an injection of ecdysteroids elicits pupation, not a stationary molt. Why the JH titer is high at the onset of diapause is unclear, but it does not appear to be involved in the induction process. Application of JH to nondiapausing larvae at this stage does not cause the induction of larval diapause.

An application of JH can terminate larval diapause in *Omphisa fuscidentalis* (Singtripop *et al*., 2000), as reported previously for a number of other larval and pupal diapauses (Denlinger, 1985), and JH presumably exerts this effect by stimulating the prothoracic gland to produce ecdysone. There is no evidence to suggest that JH, by itself, is the natural trigger for diapause termination. A combined role for JH with ecdysteroids is, however, likely is some cases. In flesh flies, JH by itself has no effect in terminating pupal diapause, but dramatic synergism is noted when JH is applied in concert with a small dose of ecdysteroid (Denlinger, 1979). When diapause is terminated naturally in flesh flies, a small peak of JH activity precedes the sustained elevation of the ecdysteroid titer, thus the combined application of JH and ecdysteroid likely is an effective mimic of the natural reactivation process.

The JH may contribute to the metabolic events occurring during pupal diapause, but it does not appear to play a regulatory role in the induction or termination of diapause. In flesh flies, JH is involved in regulating metabolic cycles that persist during diapause (Denlinger *et al*., 1984). In these flies the metabolic rate is not constant but instead occurs in cycles having periodicities of 4–6 days. These cycles are driven by JH. The hemolymph JH titer progressively increases during the trough of the cycle, reaches a critical point, triggers a bout of high oxygen consumption, and then drops precipitously. The

rapid decline in the JH titer is aided by a rise in JH esterase (Denlinger and Tanaka, 1989). When the ring gland containing the corpora allata is removed, the diapausing pupae become acyclic. Application of a high dose of JH analog to the pupae just before diapause will not alter the diapause fate of the pupa, but such pupae fail to exhibit oxygen consumption cycles and instead consume oxygen at a high rate throughout diapause. Pupae treated this way have a much shorter diapause, presumably because their high metabolic rate has prematurely exhausted their energy reserves. This conclusion assumes a mechanism used by the pupae to monitor and respond to depleted energy reserves (Denlinger *et al*., 1988).

3.12.3.2.5. A role for diapause hormone? Diapause hormone (DH) is well known for its role in diapause induction in *B. mori* (see Section 3.12.3.1.1), but attempts to induce diapause in other species with DH have failed. Thus, DH appeared to be a neuropeptide with some other primary function that was simply captured by *B. mori* to serve as a diapause regulator (Denlinger, 1985). We now know, however, that a gene encoding a DH-like sequence is present and indeed expressed in all Lepidoptera that have been examined, even those that lack an embryonic diapause. DH and pheromone biosynthesis activating neuropeptide (PBAN), along with several additional subesophageal ganglion neuropeptides (SGNPs), are encoded by a common precursor (Sato *et al*., 1993; Ma *et al*., 1994). However, the function of this DH-like peptide in species other than *B. mori* has remained a puzzle.

Might DH contribute to diapause in other species? DH-like peptides from *Helicoverpa armigera* (Zhang *et al*., 2004) and *Heliothis virescens* (Xu and Denlinger, 2003) appear to have no stimulatory effect on diapause induction, but quite surprisingly, diapausing pupae of both species readily respond to the DH-like peptides by terminating diapause. This, of course, could represent a nonspecific effect, but nonamidated forms of DH and other injected proteins fail to terminate diapause, thus suggesting that the effect is specific for DH. The possibility that a drop in DH contributes to diapause induction is further supported by the observation that expression of the mRNA encoding DH-PBAN declines at the onset of larval wandering behavior and remains low throughout pupal diapause, whereas expression remains high in larvae and pupae not destined for diapause. When diapausing pupae of *Helicoverpa armigera* are transferred to high temperatures to terminate diapause, the mRNA encoding DH-PBAN is quickly elevated (Zhang *et al*., unpublished data). In *Heliothis virescens*, Northern blots indicate that the DH-PBAN message is most highly expressed in the subesophageal ganglion complex (Xu and Denlinger, 2003), and this is supported by immunocytochemical evidence in *Helicoverpa armigera* (Zhang *et al*., unpublished data). The hemolymph titer of DH-like peptide is also much higher in nondiapausing pupae than in diapausing ones (Zhang *et al*., unpublished data). Injection of DH into diapausing pupae prompts a major increase in hemolymph ecdysteroids 3–7 days after DH injection. Although this is a rather radical concept of DH action that will need further validation, the current evidence from these noctuid moths is consistent with a drop in DH being essential for pupal diapause induction and DH elevation serving as a possible trigger for the ecdysteroid surge needed for diapause termination.

3.12.3.2.6. Associated changes in gene expression The endocrine mechanism that dictates the diapause fate of the insect can be viewed as a developmental switch that brings into play the upregulation and downregulation of huge suites of genes. Some diapause-associated patterns of gene expression may actually be upstream of the endocrine switch, while many others are likely to be downstream events set in motion by the endocrine program. We remain in an early stage of deciphering this vast gene regulatory hierarchy, and currently we have a limited view of the genes involved. This section serves to summarize our current understanding of a small sampling of the genes likely to be involved in larval and pupal diapause.

Diapause represents both a shutdown in the expression of many genes as well as the upregulation of a smaller subset of genes (Joplin *et al*., 1990; Flannagan *et al*., 1998). Estimates based on two-dimensional gel electrophoresis of proteins, subtractive hybridization, and differential display of mRNA suggest that ~4% of the genes in flesh fly pupae are diapause upregulated. Representative patterns of gene expression noted during pupal diapause in the flesh fly *Sarcophaga crassipalpis* are shown in **Figure 11**. The expression patterns fall into several discrete categories (Denlinger, 2002): genes unaffected by diapause, genes downregulated throughout diapause, genes upregulated throughout diapause, early diapause genes, late diapause genes, and those expressed intermittently throughout diapause.

Genes unaffected by diapause in the flesh fly include those encoding ecdysone receptor (EcR), heat shock 70 cognate protein (HSC 70), and 28S ribosomal protein. In the larval diapause of *Choristoneura fumiferana*, the genes encoding S-transferase and ubiquitin are in this category (Feng *et al*., 2001; Palli *et al*., 2001). Presumably many genes essential

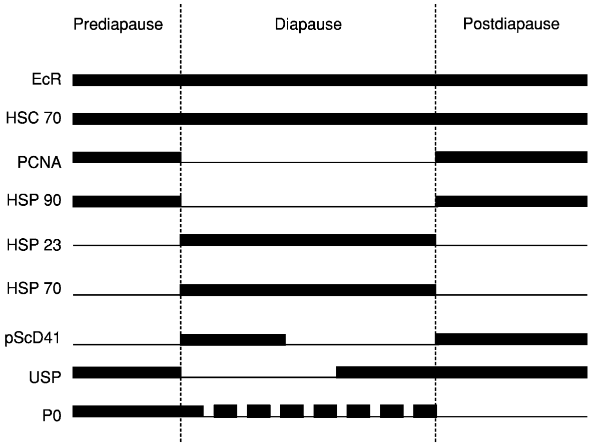


Figure 11 Representative patterns of gene expression noted in the flesh fly *Sarcophaga crassipalpis* in relation to pupal diapause. Proteins encoded by the genes are indicated on the left of the diagram. Categories include genes that are not influenced by diapause (*ecr* and *hsc70*), genes that are downregulated (*pcna* and *hsp90*), genes that are upregulated throughout diapause (*hsp23* and *hsp70*), early diapause genes (*pScD41*), late diapause genes (*usp*), and genes expressed intermittently during diapause (*po*). (Reproduced with permission from Denlinger, D.L., **2002**. Regulation of diapause. *Annu. Rev. Entomol. 47*, 93–122.)

for both diapause and nondiapause activities will be in this category.

Diapause downregulated genes represent another large category. In the flesh fly, such genes include those that encode proliferating cell nuclear antigen (PCNA) and a 90 kDa heat shock protein (Hsp90). In *C. fumiferana*, a mitochondrial phosphate transport protein is downregulated (Feng *et al.*, 1998). Although many genes in this category may simply reflect the major shutdown in metabolism that characterizes diapause, some of the downregulated genes may be of particular importance from a regulatory perspective. One such gene is *pcna*, a gene involved in cell cycle regulation. The brains of flesh fly pupae are in a G_0/G_1 cell cycle arrest, and the downregulation of *pcna* may contribute to this arrest (Tammariello and Denlinger, 1998).

Among the most highly diapause-upregulated genes in the flesh fly are those that encode the 70 kDa heat shock protein (Hsp70) (Rinehart *et al.*, 2000) and Hsp23 (Yocum *et al.*, 1998). Both of these genes are quickly upregulated at the very onset of diapause and remain upregulated throughout diapause, even at moderate temperatures. When diapause is terminated with hexane, both genes are downregulated within 6 h. This response is unusual in several ways: these are the heat-inducible forms of these genes, yet they are in this case developmentally regulated; instead of being expressed briefly as they are under stress-induced conditions, here expression persists for months. There is also a dissynchrony in this heat shock protein response. In response to most environmental stresses all of the heat shock proteins are synchronously turned on, but during diapause, Hsp23 and Hsp70 are upregulated, while Hsp90 is actually downregulated (Rinehart and Denlinger, 2000). The upregulation of Hsp70 appears to be common during diapause in other insects as well (Denlinger *et al.*, unpublished data), and it is speculated that this upregulation may have two functions (Denlinger *et al.*, 2001): (1) upregulation of heat shock proteins can be incompatible with further development, thus this upregulation may serve to bring about the halt in development that characterizes diapause, and/or (2) the presence of heat shock proteins during diapause may serve a cryoprotective role during the low temperatures the fly is likely to encounter during its overwintering diapause. In support of this second function is the observation that knocking down Hsp70 expression during diapause by use of RNA interference does indeed reduce the fly's low temperature tolerance (Rinehart, unpublished data).

Unlike the heat shock proteins that are upregulated throughout diapause, expression of a few genes is restricted to early diapause. Early-diapause genes are of interest because they could be important in the processes that shut down development. A gene in this category in flesh flies is one that encodes cystatin, a cysteine proteinase inhibitor (Goto and Denlinger, 2002a). Its mRNA is abundant for the first 20 days of diapause but then remains low for the remainder of diapause. Another gene in this category encodes a retrotransposon (Denlinger, 2002).

Genes restricted in their expression to late diapause, the late-diapause genes, are also of potential interest because such genes could be involved in the events leading to diapause termination. The gene encoding USP (see Section 3.12.3.2.3) is in this category, and we speculate that its upregulation late during pupal diapause in flesh flies may help to set the stage for diapause termination (Rinehart *et al.*, 2001). In larvae of *C. fumiferana* the gene that encodes defensin begins to rise 5 weeks after the larvae are placed at 2 °C for diapause maintenance (Palli *et al.*, 2001).

One final category of genes evident in the diapause of flesh flies includes genes that are intermittently expressed during diapause. One gene noted so far is a gene that encodes 60S ribosomal protein PO, an apurinic/apyrimidinic endonuclease (Craig and Denlinger, 2000). Expression of this gene appears to be linked to the infradian cycles of oxygen consumption characteristic of *Sarcophaga* diapause. During periods of high oxygen consumption

expression levels of this gene are low, but during the intervening days when oxygen consumption is low, the gene is highly expressed. Interestingly, this is a gene known to be involved in hypoxic stress responses.

3.12.3.3. Hormonal Basis for Adult Diapause

The central feature of adult diapause is a cessation in reproduction. For females this implies an arrest in oocyte development, and in males the most conspicuous feature is their failure to mate with receptive females. The status of the testes is not a consistently reliable indicator of diapause in males (Pener, 1992): in some cases the testes are underdeveloped, while in other cases the testes may be full of mature sperm cysts. In both males and females the accessory glands are usually reduced in size during diapause. Although a period of long-distance flight may precede the entry into diapause, once the adults arrive at their diapause site, flight muscles typically degenerate for the duration of diapause and then regenerate when diapause is terminated. Adults remain fairly inactive in diapause, but some local movements may be noted. Mating may occur either before the entry into diapause or after diapause has been completed. When mating occurs prior to diapause, the males usually die without entering diapause and only the females bridge the diapause period. In cases where both sexes diapause, mating usually occurs only after diapause has been terminated.

Most of the early work on the hormonal control of adult diapause focused on JH. The pioneering work on adult diapause, initiated in the late 1950s by Jan de Wilde and his colleagues in the Netherlands, utilized the Colorado potato beetle as their model system (de Kort, 1990). They observed that the JH titers in diapause-programmed *Leptinotarsa decemlineata* decrease after adult emergence, remain low throughout diapause, and then rise after termination of diapause. Further evidence supporting the role of JH in adult diapause was provided by the responsiveness of diapausing adults to JH, histological studies of the corpora allata (CA), and experimental manipulations of the CA. The application of synthetic JH and JH analogs to diapausing females prompts egg-laying (although such periods are not sustained). The CA from diapausing *L. decemlineata* are small and inactive. Surgical removal of the CA induces a diapause-like state in long-day beetles (those not programmed for diapause): they stop feeding, leave the food, and burrow into the soil. Chemical destruction of the CA in long-day beetles with precocene II elicits the same diapause-like response. In nondiapausing adults, cauterizing the pars intercerebralis, the region of the brain that controls CA activity, also induces a diapause-like state. This same surgery performed with diapausing adults blocks the beetle's ability to become reproductively active when placed under long daylengths (the environmental signal used to promote reproduction). Severing the axons between the pars intercerebralis and the CA does not appear to alter the diapause decision, thus suggesting that the CA in this species is under hormonal, rather than neural, control.

In the early experimental years there would have been no reason to suspect a role for ecdysteroids as a regulator of adult diapause because the well-known larval source of ecdysteroid synthesis, the prothoracic gland, degenerates in adults. But the discovery in the late 1970s that adults can produce ecdysteroids from other tissues such as ovaries raised the possibility that ecdysteroids could also be involved in the regulation of reproductive events. In the Colorado potato beetle, the hemolymph ecdysteroid titer is nearly twice as high in diapause-programmed beetles as it is in nondiapause-programmed beetles, but the titer drops sharply a few days after adult eclosion. The functional significance of this elevated ecdysteroid titer associated with diapause has not been determined.

While the scheme for the hormonal control of adult diapause that has been developed from experiments in *L. decemlineata* is applicable to many species that diapause as adults, there are also clear species differences. These differences may include whether both sexes enter diapause, sexual distinctions in the environmental cues used for regulating diapause, and the formation of stable intermediate phenotypes that lie somewhere between being fully sexually active and being complete inactive. The regulatory mechanisms controlling the CA during adult diapause vary among species. The brain's regulation of the CA can be either hormonal as in *L. decemlineata* or neural as in *Pyrrhocoris apterus*, *Tetrix undulata*, and *Locusta migratoria*. The limited number of species examined through 1985 indicated clear unifying themes within adult diapause, yet distinct differences between species were also apparent.

3.12.3.3.1. Role of the corpora allata Recent research on the endocrine basis of adult diapause continues to focus primarily on the role of JH. In substantially more species we now have new or additional evidence that JH plays a major role in regulating diapause, e.g., the green bug *Plautia stali* (Kotaki and Yagi, 1989), the pear psylla *Cacopsylla pyricola* (Krysan, 1990), the Colorado potato beetle *Leptinotarsa decemlineata* (Koopmanschap *et al.*,

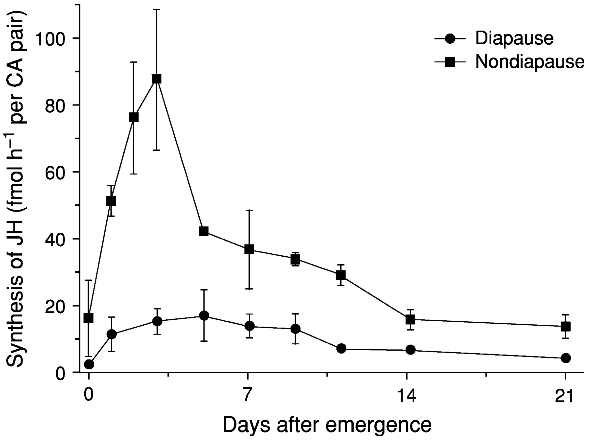


Figure 12 *In vitro* activity of the corpora allata (CA) from diapausing and nondiapausing females of the mosquito *Culex pipiens* on different days following adult emergence. (Reproduced with permission from Readio, J., Chen, M.-H., Meola, R., **1999**. Juvenile hormone biosynthesis in diapausing and nondiapausing *Culex pipiens* (Diptera: Culicidae). *J. Med. Entomol. 36*, 355–360.)

1989), the fungus beetle *Stenotarsus rotundus* (Tanaka *et al*., 1987), the beetle *Aulacophora nigripennis* (Watanabe and Tanaka, 1998), the butterfly *Speyeria idalia* (Kopper *et al*., 2001), the bumblebee *Bombus terrestris* (Larrere *et al*., 1993), the mosquito *Culex pipiens* (Readio *et al*., 1999), and the blowfly *Protophormia terraenovae* (Matsuo *et al*., 1997).

Earlier results arguing a role for JH in regulating the adult diapause of *C. pipiens* were based on the ability of JH applications to terminate diapause; more recently this evidence was supplemented by demonstrating that cultured CA dissected from diapausing females secrete very little JH (**Figure 12**), while those dissected from nondiapausing females are quite active, especially during the first week after adult eclosion (Readio *et al*., 1999). Females of *C. pipiens* that are allatectomized and then transferred to diapause-terminating conditions remain in a diapause-like state and fail to initiate the blood feeding noted in control groups. In *P. terraenovae* JH applications or implantations of active CA into diapausing adults are fully capable of initiating ovarian development (Matsuo *et al*., 1997).

The CA, of course, does not function autonomously. Activity of the CA is coordinated by the brain, and the brain exerts its control through both hormonal and neuronal conduits. Active suppression of the CA by the brain during diapause has been demonstrated in the following insect orders: Coleoptera: *L. decemlineata* (Khan *et al*., 1983, 1988); Diptera: *P. terraenovae* (Matsuo *et al*., 1997); Heteroptera: *Plautia stali* (Kotaki and Yagi, 1989), *Riptotus clavatus* (Morita and Numata, 1997a), *Pyrrhocoris apterus* (Hodkova *et al*., 2001); Orthoptera: *Locusta migratoria* (Okuda and Tanaka, 1997). In the diapause of *Protophormia terraenovae* the inhibition is maintained by neurons that originate in the pars lateralis and terminate within the CA (Shiga and Numata, 2000), but in several other species, including *Pyrrhocoris apterus* (Hodkova, 1994), the inhibition appears to originate in the pars intercerebralis. The diapause program may actually influence the organization of the synapses: rearing *Leptinotarsa decemlineata* throughout its life under short-day conditions boosts both the number and activity of neurosecretory synapses within the CA as compared to beetles reared under long-day conditions (Khan and Buma, 1985). Hormonal control of the CA is also evident in a number of species. CA activity in diapausing *Locusta migratoria* is suppressed by neuropeptide(s) stored in the corpora cardiaca (CC) (Okuda and Tanaka, 1997). Neural regulation of the CA during diapause may not be as simple as the presence or absence of an allatostatin. Brain extracts from both diapausing and nondiapausing *P. apterus* stimulate increased JH production in CA from nondiapausing donors but has no effect on JH synthesis in CA from diapausing donors (Hodkova *et al*., 1996). Hodkova *et al*. (1996) speculated that CA from diapausing adults may lack receptors for allatotropins, or there may be a high level of an allato-inhibiting factor in the CA or CC that blocks the action of the allatotropins. Extracts of the brain–suboesophageal ganglion–CC–CA complex from diapausing *P. apterus* inhibit the CA activity of *Plautia stali,* indicating that this neurosecretory complex from diapausing bugs may contain both allato-inhibiting and allato-stimulating factors (Hodkova *et al*., 1996).

The JH hemolymph titer is a function of both JH synthesis and its degradation (de Kort and Granger, 1996). JH esterases (JHE) play a central role in regulating the JH titer in diapause-destined adults of *Leptinotarsa decemlineata*. There is an inverse relationship between the JH hemolymph titer and JHE activity in prediapausing adults, with peak esterase activity occurring just prior to diapause initiation. This peak in JHE activity contributes to the low JH titer noted during the first 2 days of adult life (Kramer, 1978). The effect of JH on JHE activity is indirect and appears to require a factor from the brain. JHE isolated from the hemolymph of fourth instar larvae of *L. decemlineata* is a dimer consisting of two 57 kDa subunits (Vermunt *et al*., 1997a). cDNA clones of two different JHEs have been isolated from fourth instar larvae (Vermunt

et al., 1997b, 1998). JHE-A appears to encode the protein first isolated from the hemolymph, whereas the JHE-B encodes a protein that does not appear to be secreted into the hemolymph. Significant levels of expression of JHE-A are detected in the brain of fourth instar larvae and fat body of both fourth instar larvae and short-day adults; low expression is noted in long-day adults (Vermunt *et al.*, 1999). Treating diapause-destined day 1 adults with the JH analog pyriproxyfen strongly suppresses JHE-A expression. JHE-A sensitivity to pyriproxyfen decreases as beetles near diapause initiation. Vermunt *et al.* (1999) conclude that JHE activity in the hemolymph is critical for the initiation of diapause in the Colorado potato beetle. However, a role for JHEs in regulation of the JH titer appears not to be essential for some other cases of adult diapause. In the locusts *Nomadacris japonica* and *Locusta migratoria* there appear to be no differences in JHE hemolymph activity between long-day and short-day adults (Okuda *et al.*, 1996).

Though the evidence supporting involvement of JH in reproductive diapause is compelling, there are also results indicating that the absence of JH is not the exclusive cause of diapause induction and maintenance. Results, even in the Colorado potato beetle, suggest that lack of JH cannot account for all the observations. The fact that JH application, at best, can stimulate only a short burst of oviposition in diapausing females suggests that the actual termination of diapause requires more than a single boost in the JH titer. This observation has usually been interpreted as the need for sustained stimulation of the CA by the brain (Denlinger, 1985). Thus, a minimal interpretation is that, at the end of diapause, the brain must become reactivated in order to promote JH synthesis by the CA. Brain activation clearly needs to precede activation of the CA, and quite possibly the brain independently directs specific events that are associated with diapause termination but occur prior to activation of the CA. That such may be the case is evident from the observation that adults of *Leptinotarsa decemlineata* actually dig their way out of the soil prior to activation of the CA (Lefevere *et al.*, 1989). This suggests that activation of the CA may be the consequence of diapause termination, rather than the cause.

The regulatory scheme for males is not always the same as that in females. In *Pyrrhocoris apterus* (Hodkova, 1994) and *Protophormia terraenovae* (Tanigawa *et al.*, 1999), regulation of diapause in females clearly involves the CA, but the CA does not appear to be involved in males. Allatectomy has little effect on the manifestation of diapause in the males; they can respond to photoperiod and display mating behavior even in the absence of a CA. The inhibitory center regulating mating behavior resides in the brain's pars intercerebralis, but this important inhibitory pathway does not involve the CA.

When the CA are involved in regulating diapause, they presumably preside over the whole diapause syndrome, not just the regulation of reproduction. This appears to be the case in *Pyrrhocoris apterus,* a species in which photoperiod dictates not only the reproductive status of the female, but also the profile of phospholipid molecules in cell membranes (Hodkova *et al.*, 2002). The unique winter pattern of phosphatidylethanolamines, associated with cold hardening, is programmed by short daylength, not low temperature. Nondiapausing females deprived of their CA assume the lipid profile normally associated with diapausing females. Similarly, earlier studies with the Colorado potato beetle demonstrated involvement of the CA in determining the status of flight muscles during diapause.

3.12.3.3.2. Role of ecdysteroids The role of ecdysteroids in the regulation of adult diapause has been investigated most thoroughly in *Drosophila melanogaster.* A reproductive arrest occurs at the previtellogenic stage of ovarian follicular development in females of *D. melanogaster* that eclose under short day length and low temperatures (Saunders *et al.*, 1989). Yolk proteins (YPs) are present in the hemolymph of diapausing females, but the oocytes fail to incorporate the YPs (Saunders *et al.*, 1990). The ecdysteroids are proposed to activate endocytosis at the termination of diapause and thus allow the initiation of vitellogenesis (Richard *et al.*, 1998). The evidence for this intriguing scenario is compelling.

During diapause the ovaries produce low level of ecdysteroids; the rate of synthesis begins to increase 4 h after transferring the flies to 25 °C to terminate diapause, and reaches maximum levels by 12 h (Richard *et al.*, 1998). Injection of 20E terminates diapause in a dose-dependent manner as measured by ovarian maturation (Richard *et al.*, 2001b). YP uptake by the oocytes is by a receptor-mediated endocytotic mechanism (Bownes *et al.*, 1993), a mechanism that involves at least three key proteins. Clathrin coats the pit, and adaptin binds to both clathrin and the transmembrane YP receptor (Gao *et al.*, 1991; Smythe and Warren, 1991). Once the vesicle is internalized the vesicle breaks down, and the clathrin and adaptin are recycled (Gao *et al.*, 1991). Clathrin, adaptin, and YP receptor are undetectable by immunostaining in diapausing females. Terminating diapause by transferring the flies to 25 °C or injecting them with 20E induces the expression of clathrin, adaptin, and the YP receptor

in nurse cells adjacent to the oocytes (Richard *et al.*, 2001a). Incubating ovaries from diapausing females with 20E fails to induce the expression of clathrin, adaptin, or the YP receptor, indicating that diapause termination is not regulated by the ovaries and that some external factor is needed (Richard *et al.*, 2001a).

How can these ecdysteroid results be reconciled with the huge body of data suggesting that vitellogenesis in higher Diptera is mediated by JH? Application of JH III or JHB_3 to diapausing adults of *D. melanogaster* does indeed break diapause (Saunders *et al.*, 1990), but this response is perhaps an indirect effect caused by JH triggering the ecdysteroid-mediated initiation of vitellogenesis (Richard *et al.*, 1998, 2001b). When flies are transferred to diapause-terminating conditions, it is the edysteroid titer that goes up quickly (within hours), while the JH titer remains low.

Roles for ecdysteroids are suggested in a number of other species as well, but the results are sometimes conflicting. Significantly lower ecdysteroid titers are reported for diapausing females of *Locusta migratoria* than in nondiapausing females (Tawfik *et al.*, 2002a), a result that is consistent with the diapause of *D. melanogaster.* The ecdysteroid titer in *L. migratoria* fluctuates between 22 and 40 ng ml^{-1} in diapausing females, whereas the titer reaches a peak of 354 ng ml^{-1} in 18–22 day old nondiapausing females. Treating diapausing females with three consecutive application of JH III induces ovarian development and increases ovarian and hemolymph titers of ecdysteroids to levels similar to those noted in sexually active females (Tawfik *et al.*, 2002a). This relationship between the ecdysteroid titer and diapause induction is, however, opposite of that noted for *Leptinotarsa decemlineata* (Briers and de Loof, 1981): in the beetles, high titers of ecdysteroids are noted in diapause-destined beetles shortly after adult eclosion but then drop within the first week, whereas the titer is initially low in nondiapausing adults and then increases in females at the onset of reproduction. During diapause, the ecdysteroid titer gradually increases (Briers *et al.*, 1982). This may be essential for diapause termination. Interestingly, an injection of a combination of 20E and JH is much more effective in breaking diapause than either hormone by itself (Lefevere, 1989), a response also noted for pupal diapause termination in flesh flies (Denlinger, 1979). Lefevere *et al.* (1989) suggest that the ecdysteroid injection enhances diapause termination by blocking the activation of JHE, thus facilitating elevation of the JH titer.

A role for ecdysteroids has also been proposed for diapause in *P. apterus* (Sauman and Sehnal, 1997). Under long-day conditions, maturation of the male accessory glands and testes of *P. apterus* occurs during the fourth nymphal instar. Injecting an ecdysteroid (makisterone A) or implanting brain–suboesophageal ganglion complexes from long-day nymphs into early fourth instar nymphs reared at short days induces sexual development in the normally immature reproductive tract. Sauman and Sehnal (1997) conclude that the prediapause physiology in the fourth instar nymph of *P. apterus* is regulated by ecdysteroids and neurohormones, an observation that is consistent with other reports showing a low ecdysteroid titer contributing to the diapause fate of the adult.

It seems unlikely that adult diapause will prove to be based simply on either a JH or ecdysteroid deficiency. Most likely, both hormones are involved in orchestrating this response. In all of the above examples showing evidence for ecdysteroid involvement, JH also has been implicated in the regulation of reproduction and diapause.

3.12.3.3.3. Associated changes in gene expression Diapause-associated genes isolated thus far fall into four broad categories. The first category consists of genes that encode proteins having a putative regulatory function, e.g., the genes that encode the JHEs isolated from *L. decemlineata*. JHEs are involved in regulating the JH titer and their expression appears to be critical for the initiation of diapause in *L. decemlineata* (see Section 3.12.3.3.1). cDNA clones for *JHE-A* and *JHE-B* have been isolated and characterized: each contains an open reading frame encoding a deduced protein of 515 amino acids with 77% identity to each other (Vermunt *et al.*, 1997b, 1998). Both of the deduced proteins have a similar signaling peptide consisting of 24 amino acids. Southern blot analysis indicates that the *L. decemlineata* genome contains only single copies of *JHE-A* and *JHE-B* (Vermunt *et al.*, 1998).

The second category of genes includes those that encode proteins having protective functions. Overwintering insects face a number of environmental and biological challenges. Besides the well-documented threats of low temperature exposure and desiccation, insects are subjected to various pathogens during diapause. Using subtractive hybridization Daibo *et al.* (2001) isolated two diapause-upregulated transcripts from *Drosophila triauraria* with high identities to drosomycin, the antifungal protein family of *D. melanogaster.* Both clones share an identical 3′ untranslated region (UTR) and open reading frame but have unique 5′ UTRs, indicating that the two clones are separate members of the same family. Analysis of the two deduced proteins

from *D. triauraria* and members of the *D. melanogaster* drosomycin family reveals that they share a specific arrangement of eight cysteines, a signaling peptide, and a Knot 1 domain (Daibo *et al*., 2001).

Bacterial infection in *Aedes aegypti*, *A. albopictus*, and *D. melanogaster* induces expression of the iron binding protein transferrin (Yoshiga *et al*., 1999), and this transcript is also upregulated during adult diapause in *R. clavatus* (Hirai *et al*., 1998). When diapause is terminated with the JH analog methoprene, expression of the transferrin transcript is suppressed. Yet the protein persists in the fat body and hemolymph for some time after diapause is terminated (Hirai *et al*., 2000).

Another group of protective proteins noted during adult diapause is the heat shock proteins, highly conserved proteins that are induced in response to protein denaturation and developmental cues. In nonstressed cells, heat shock proteins serve as molecular chaperones, assisting in protein folding and the assembly of protein complexes. During stress heat shock proteins inhibit formation of and help dissolve protein aggregates, refold proteins, and target certain proteins for degradation (Parsell and Lindquist, 1993; Denlinger *et al*., 2001). *Leptinotarsa decemlineata* has at least two members (*Hsp70A* and *B*) of the inducible 70 kDa Hsp family (Yocum, 2001). *Hsp70A,* but not *Hsp70B,* is upregulated during diapause at normal rearing temperatures. Differential expression of these two *Hsp70*s is also seen in their response to low temperature exposure. Following a cold shock at −10 °C for 2 h, diapausing beetles express high levels of *Hsp70B* during recovery at 15 °C, but *Hsp70A* expression is only modestly increased (Yocum, 2001).

The genes that encode storage proteins represent a third category of genes associated with adult diapause. Genes encoding various classes of these proteins are expressed as a normal part of the diapause program in insects that diapause as adults (see Section 3.12.2.4).

The final class of genes associated with adult diapause are genes whose functions remain unknown. In *L. decemlineata* trace levels of *diapause-associated transcript* (*DAT*)*-1* and *DAT-2* are first detected 3 days postemergence in diapause-destined adults. Expression levels of these transcripts increase on day 6 postemergence and remain high for at least 60 days into diapause. On day 12 postemergence a low level of *DAT-3* is detected. Its level of expression increases on day 15 and remains high for at least 60 days into diapause (Yocum, 2003). The expression patterns for *DAT-1*, *DAT-2*, and *DAT-3* clearly demark two preparatory physiological events as the beetles enter diapause. In the leaf beetle *Gastrophysa atrocyanea* a 220 kDa glycoprotein of unknown function appears in the hemolymph at the onset of diapause and disappears at the end of diapause (Ichimori *et al*., 1990). Although the functions of these proteins remain unknown, the genes and/or proteins currently prove useful as biomarkers for diapause and for the timing of physiological events during diapause development.

3.12.4. Parallels to Other Dormancies

Diverse endocrine mechanisms are capable of halting development at different developmental stages and in different species that enter diapause at the same stage. And, sometimes the same net effect, diapause, is elicited by contrasting responses to the very same hormone. For example, a shutdown in ecdysteroid production is key to most pupal diapauses, but an elevation in ecdysteroids elicits diapause in pharate first instar larvae of the gypsy moth. Similarly, the absence of JH characterizes many adult diapauses, while some larval diapauses are maintained by a high JH titer. Progression through the different stages of embryonic and postembryonic development and the initiation of reproduction by the adult is orchestrated by a well-defined series of endocrine events requiring periods of hormone release and other equally important phases of hormone absence. This requirement for precisely timed periods of hormone presence or absence to elicit uninterrupted development suggests that nearly any interruption in this normal sequence could lock the insect into a developmental arrest at the point of interruption. This is presumably why we observe diverse endocrine mechanisms causing the same developmental shutdown we recognize as diapause. Presumably, this means that the endocrine control mechanism for halting development has evolved independently numerous times. Certainly there are taxa in which the diapause stage and the control mechanism are highly conserved. For example, all the flesh fly species (Sarcophagidae) from around the temperate and tropical world that have a diapause rely on a pupal diapause that represents a shutdown in the brain–prothoracic gland axis, thus suggesting a highly conserved trait within this family. By contrast, different members of the Drosophilidae are known to enter diapause as larvae, pupae, and adults, a feature suggesting that the endocrine basis for diapause has evolved several times within this taxon.

The challenge to identify a unifying endocrine basis for diapause becomes even more difficult when one seeks parallels in the endocrine control mechanisms for dormancies in nonarthropods. A connection between the insulin-like receptor *daf-2* in the production of dauerlarvae of the nematode *Caenorhabditis*

elegans (Kimura *et al*., 1997; Lee *et al*., 2003) and the involvement of a homologous receptor that influences JH synthesis in *D. melanogaster* (Tatar *et al*., 2001; Tatar and Yin, 2001) offers a tantalizing connection with some adult diapauses, but the fact that JH is not an active player in many forms of insect diapause suggests that this connection may not be applicable to all insect diapauses. It is quite likely that many different nonarthropod species, like the insects, will have tapped into diverse endocrine mechanisms to regulate their development and arrests of development.

This argument, however, does not preclude the possibility that, at the molecular level, diapause and other forms of dormancy may target the same or similar cellular processes to bring about a developmental arrest. Several results already hint of some commonality to the molecular events associated with diapause. For example, the diapause-associated upregulation of genes encoding heat shock proteins was first noted in flesh fly pupae, but heat shock protein upregulation during insect diapause has now been documented, not only in several pupal diapauses, but also in embryonic, larval, and adult diapauses of other insects, as well as in the dormancies of brine shrimp, nematodes, and some plants (Denlinger *et al*., 2001). This common feature possibly represents a widespread contribution of heat shock proteins to cellular defense and/or a contribution to the mechanism for shutting down development. Another feature that would appear to be an essential cellular event common to most diapauses is a shutdown of the cell cycle (Tammariello, 2001). Again, there may be diversity in the phase of the cell cycle that is arrested (e.g., G_0/G_1 in pupae of *Sarcophaga crassipalpis,* G_2 in pupae of *Manduca sexta* and in embryos of *Bombyx mori*) and in the cell cycle regulator that is targeted, but the overall targeting of the cell cycle is a likely ultimate target of whatever endocrine mechanism is utilized.

In numerous plant and animal taxa cellular changes in pH can induce or terminate the dormant state (Footitt and Cohn, 2001). Most commonly an increase in cellular pH is associated with induction of the dormant state, while cellular acidification is associated with termination of dormancy. Diapause in quite a few insects can also be terminated by pH change, but no convincing evidence has been provided to show that pH change is part of the normal sequence of events regulating insect diapause. Yet the widespread reliance upon pH changes to regulate dormancies in other systems suggests that this is a mechanism worthy of further consideration.

Evidence from *D. melanogaster* suggesting that elevation in nitric oxide (NO) can reversibly arrest embryonic development and create a diapause-like state (Teodoro and O'Farrell, 2003) raises the intriguing question whether this important molecule could be part of a general signaling pathway involved in diapause. NO's ability to rapidly shut down gene expression and the turnover of RNA and protein products is a feature that could be exploited by insects to initiate a developmental arrest. However, there is yet no evidence indicating such a link with a naturally occuring diapause. Although NO or pH changes may not ultimately prove to be the target molecule or mechanism common to diverse diapauses, these observations raise the specter that the wealth of hormonal regulators may eventually be reduced to a simple, conserved response at the cellular level.

References

Agui, N., Granger, N.A., Gilbert, L.I., Bollenbacher, W.E., **1979**. Cellular localization of insect prothoracicotropic hormone. *Proc. Natl Acad. Sci. USA 76*, 5684–5690.

Andrewartha, H.G., **1952**. Diapause in relation to the ecology of insects. *Biol. Rev. 27*, 50–107.

Bell, R.A., **1996**. Manipulation of diapause in the gypsy moth, *Lymantria dispar* L., by application of KK-42 and precocious chilling of eggs. *J. Insect Physiol. 42*, 557–563.

Blitvich, B.J., Rayms-Keller, A., Blair, C.D., Beaty, B.J., **2001**. Identification and sequence determination of mRNAs detected in dormant (diapausing) *Aedes triseriatus* mosquito embryos. *DNA Sequ. 12*, 197–202.

Bodnaryk, R.P., **1985**. Ecdysteroid levels during post-diapause development and 20-hydroxyecdysone-induced development in male pupae of *Mamestra configurata* Wlk. *J. Insect Physiol. 31*, 53–58.

Bosse, T.C., Veerman, A., **1996**. Involvement of vitamin A in the photoperiodic induction of diapause in the spider mite *Tetranychus urticae* is demonstrated by rearing an albino mutant on a semi-synthetic diet and without β-carotene or vitamin A. *Physiol. Entomol. 21*, 188–192.

Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., **1984**. *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *Manduca sexta*. *J. Exp. Biol. 108*, 9–24.

Bownes, M., Ronaldson, E., Mauchline, D., Martinez, A., **1993**. Regulation of vitellogenesis in *Drosophila*. *J. Insect Morphol. Embryol. 22*, 349–367.

Briers, T., de Loof, A., **1981**. Moulting hormone activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* Say in relation to reproduction and diapause. *Int. J. Invert. Reprod. 3*, 145–155.

Briers, T., Peferoen, M., de Loof, A., **1982**. Ecdysteroids and adult diapause in the Colorado potato beetle, *Leptinotarsa decemlineata*. *Physiol. Entomol. 7*, 379–386.

Brown, J.J., **1980**. Haemolymph protein reserves of diapausing and non-diapausing codling moth, *Cydia pomonella*. *J. Insect Physiol. 26*, 487–492.

Brown, J.J., Chippendale, G.M., **1978**. Juvenile hormone and a protein associated with the larval diapause of the southwestern corn borer, *Diatraea grandiosella*. *Insect Biochem. 8*, 359–367.

Burmester, T., Massey, H., Jr., Zakharkin, S.O., Benes, H., **1998**. The evolution of hexamerins and the phylogeny of insects. *J. Mol. Evol. 47*, 93–108.

Champlin, D.T., Truman, J.W., **1998**. Ecdysteroid control of cell proliferation during optic lobe neurogenesis in the moth *Manduca sexta*. *Development 125*, 269–277.

Chen, J.H., Yaginuma, T., Yamashita, O., **1988**. Effect of diapause hormone on cyclic nucleotide metabolism in developing ovaries of the silkmoth, *Bombyx mori*. *Comp. Biochem. Physiol. B 91*, 631–637.

Chen, J.H., Yamashita, O., **1989**. Activity changes of guanylate cyclase and cyclic GMP phosphodiesterase related to the accumulation of cyclic GMP in developing ovaries of the silkmoth, *Bombyx mori*. *Comp. Biochem. Physiol. B 93*, 385–390.

Chino, H., **1957**. Carbohydrate metabolism in diapause eggs of the silkworm, *Bombyx mori*. I. Diapause and the change of glycogen content. *Embryologia 3*, 295–316.

Chino, H., **1958**. Carbohydrate metabolism in the diapause egg of the silkworm, *Bombyx mori*. II. Conversion of glycogen into sorbitol and glycerol during diapause. *J. Insect Physiol. 2*, 1–12.

Chinzei, Y., Miura, K., Kobayashi, L., Shinoda, T., Numata, H., **1992**. Cyanoprotein: development stage, sex and diapause-dependent expression, and synthesis regulation by juvenile hormone in the bean bug, *Riptortus clavatus*. *Arch. Insect Biochem. Physiol. 20*, 61–73.

Chinzei, Y., Shinoda, T., Miura, K., Numata, H., **1991**. Quantitative changes and synthesis of cyanoprotein in whole body and tissues during development of the bean bug, *Riptortus clavatus*. *Insect Biochem. 21*, 313–320.

Chippendale, G.M., **1977**. Hormonal regulation of larval diapause. *Annu. Rev. Entomol. 22*, 121–138.

Claret, J., **1989**. Vitamine A et induction photopériodique ou thermopériodique de la diapause chez *Pieris brassicae* (Lepidoptera). *C. R. Acad. Sci. Paris, Série III 308*, 347–352.

Claret, J., Volkoff, N., **1992**. Vitamin A is essential for two processes involved in the photoperiodic reaction in *Pieris brassicae*. *J. Insect Physiol. 38*, 569–574.

Corpuz, L.M., Choi, H., Muthukrishnan, S., Kramer, K.J., **1991**. Sequences of two cDNAs and expression of the genes encoding methionine-rich storage proteins of *Manduca sexta*. *Insect Biochem. 21*, 265–276.

Craig, T.L., Denlinger, D.L., **2000**. Sequence and transcription patterns of 60S ribosomal protein PO, a diapause-regulated AP endonuclease in the flesh fly, *Sarcophaga crassipalpis*. *Gene 255*, 381–388.

Ctvrtecka, R., Zdarek, J., **1992**. Reproductive diapause and its termination in the apple blossom weevil (*Anthonomus pomorum*) (Coleoptera, Curculionidae). *Acta Entomol. Bohem. 89*, 281–286.

Daibo, S., Kimura, M.T., Goto, S.G., **2001**. Upregulation of genes belonging to the drosomycin family in diapausing adults of *Drosophila triauraria*. *Gene 278*, 177–184.

Danks, H.V., **1987**. Insect Dormancy: An Ecological Perspective. Biological Survey of Canada, Ottawa.

de Kort, C.A.D., **1990**. Thirty-five years of diapause research with the Colorado potato beetle. *Entomol. Exp. Applic. 56*, 1–13.

de Kort, C.A.D., Granger, N.A., **1996**. Regulation of JH titers: the relevance of degradative enzymes and binding proteins. *Arch. Insect Biochem. Physiol. 33*, 1–26.

de Kort, C.A.D., Koopmanschap, A.B., **1994**. Nucleotide and deduced amino acid sequence of a cDNA clone encoding diapause protein 1, an arylphorin-type storage hexamer of the Colorado potato beetle. *J. Insect Physiol. 40*, 527–535.

de Kort, C.A.D., Koopmanschap, A.B., Vermunt, A.M.W., **1997**. Influence of pyriproxyfen on the expression of haemolymph protein genes in the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol. 43*, 363–371.

Denlinger, D.L., **1972**. Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae). *Biol. Bull. Wood's Hole 142*, 11–24.

Denlinger, D.L., **1979**. Pupal diapause in tropical flesh flies: environmental and endocrine regulation, metabolic rate and genetic selection. *Biol. Bull. Woods Hole 156*, 31–46.

Denlinger, D.L., **1985**. Hormonal control of diapause. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 353–412.

Denlinger, D.L., **1986**. Dormancy in tropical insects. *Annu. Rev. Entomol. 31*, 239–264.

Denlinger, D.L., **1998**. Maternal control of fly diapause. In: Mousseau, T.A., Fox, C.A. (Eds.), Maternal Effects as Adaptations. Oxford University Press, Oxford, pp. 275–287.

Denlinger, D.L., **2001**. Interrupted development: the impact of temperature on insect diapause. In: Atkinson, D., Thorndyke, M. (Eds.), Environment and Animal Development: Genes, Life Histories and Plasticity. BIOS Scientific Publishers, Oxford, pp. 235–250.

Denlinger, D.L., **2002**. Regulation of diapause. *Annu. Rev. Entomol. 47*, 93–122.

Denlinger, D.L., Bradfield, J.Y., IV, **1981**. Duration of pupal diapause in the tobacco hornworm is determined by number of short days received by the larva. *J. Exp. Biol. 91*, 331–337.

Denlinger, D.L., Giebultowicz, J.M., Adedokun, T.A., **1988**. Insect diapause: dynamics of hormone sensitivity and vulnerablility to environmental stress. In: Sehnal, F., Zabza, A., Denlinger, D.L. (Eds.), Endocrinological Frontiers in Physiological Insect Ecology. Wrocław Technical University Press, Wrocław, pp. 885–898.

Denlinger, D.L., Lee, R.E., Yocum, G.D., Kukal, O., **1992**. Role of chilling in the acquisition of cold tolerance and the capacitation to express stress proteins in diapausing pharate larvae of the gypsy moth,

Lymantria dispar. *Arch. Insect Biochem. Physiol. 21*, 271–280.

Denlinger, D.L., Rinehart, J.P., Yocum, G.D., **2001**. Stress proteins: a role in insect diapause? In: Denlinger, D.L., Giebultowicz, J.M., Saunders, D.S. (Eds.), Insect Timing: Circadian Rhythmicity to Seasonality. Elsevier, Amsterdam, pp. 155–171.

Denlinger, D.L., Shukla, M., Faustini, D.L., **1984**. Juvenile hormone involvement in pupal diapause of the flesh fly *Sarcophaga crassipalpis*: regulation of infradian cycles of O_2 consumption. *J. Exp. Biol. 109*, 191–199.

Denlinger, D.L., Tanaka, S., **1989**. Cycles of juvenile hormone esterase activity during the juvenile hormone-driven cycles of oxygen consumption in pupal diapause of flesh flies. *Experientia 45*, 474–476.

Denlinger, D.L., Wingard, P., **1978**. Cyclic GMP breaks pupal diapause in the flesh fly *Sarcophaga crassipalpis*. *J. Insect Physiol. 24*, 715–719.

Desroches, P., Huignard, J., **1991**. Effect of larval density on development and induction of reproductive diapause in *Bruchidius atrolineatus*. *Entomol. Exp. Applic. 61*, 255–263.

de Wilde, J., Duintjer, C.S., Mook, L., **1959**. Physiology of diapause in the adult Colorado potato beetle (*Leptinotarsa decemlineata* Say). I. The photoperiod as a controlling factor. *J. Insect Physiol. 3*, 75–85.

Dillwith, J.W., Chippendale, G.M., **1984**. Purification and properties of a protein that accumulates in the fat body of pre-diapause larvae of the southwestern corn borer *Diatraea grandiosella*. *Insect Biochem. 14*, 369–382.

Eizaguirre, M., Prats, J., Abellana, M., Lopez, C., Llovera, M., *et al.*, **1998**. Juvenile hormone and diapause in the Mediterranean corn borer, *Sesamia nonagrioides*. *J. Insect Physiol. 44*, 419–425.

Endo, K., Fujimoto, Y., Kondo, M., Yamanaka, A., Watanabe, M., *et al.*, **1997**. Stage-dependent changes of the prothoracicotropic hormone (PTTH) activity of brain extracts and of the PTTH sensitivity of the prothoracic glands in the cabbage armyworm, *Mamestra brassicae,* before and during winter and aestival pupal diapause. *Zool. Sci. 14*, 127–133.

Feng, Q.L., Davey, K.G., Pang, A.S.D., Ladd, T.R., Retnakaran, A., *et al.*, **2001**. Developmental expression and stress induction of glutathione S-transferase in the spruce budworm, *Choristoneura fumiferana*: developmental expression and the induction by various stresses. *J. Insect Physiol. 47*, 1–10.

Feng, Q.L., Palli, S.R., Ladd, T.R., Retnakaran, A., Davey, K.G., **1998**. Identification and developmental expression of the mitochondrial phosphate transport protein gene from the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol. 28*, 791–799.

Fielding, D.J., **1990**. Photoperiod and food regulate termination of diapause in the squash bug, *Anasa tristis*. *Entomol. Exp. Applic. 55*, 119–124.

Flannagan, R.D., Tammariello, S.P., Joplin, K.H., Cikra-Ireland, R.A., Yocum, G.D., *et al.*, **1998**. Diapause-specific gene expression in pupae of the flesh fly *Sarcophaga crassipalpis*. *Proc. Natl Acad. Sci. USA 95*, 5616–5620.

Footitt, S., Cohn, M.A., **2001**. Developmental arrest: from sea urchins to seeds. *Seed Sci. Res. 11*, 3–16.

Friedlander, M., Reynolds, S., **1992**. Intratesticular ecdysteroid titres and the arrest of sperm production during pupal diapause in the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 38*, 693–703.

Fujiwara, H., Jindra, M., Newirtt, R., Palli, S.R., Hiruma, K., *et al.*, **1995**. Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. *Insect Biochem. Mol. Biol. 25*, 845–856.

Fukuda, S., **1951**. Factors determining the production of non-diapause eggs in the silkworm. *Proc. Jap. Acad. 27*, 582–586.

Fukuda, S., **1952**. Function of the pupal brain and subesophageal ganglion in the production of non-diapause and diapause eggs in the silkworm. *Annot. Zool. Japan 25*, 149–155.

Gadenne, C., Varjas, L., Mauchamp, B., **1990**. Effects of the non-steroidal ecdysone mimic, RH-5849, on diapause and non-diapause larvae of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 36*, 555–559.

Gao, B., Biosca, J., Craig, E.A., Greene, L.E., Eisenberg, E., **1991**. Uncoating of coated vesicles by yeast hsp70 proteins. *J. Biol. Chem. 266*, 19565–19571.

Gao, N., von Schantz, M., Foster, R.G., Hardie, J., **1999**. The putative brain photoperiodic photoreceptors in the vetch aphid, *Megoura viciae*. *J. Insect Physiol. 45*, 1011–1019.

Gelman, D.B., Brents, L.A., **1984**. Haemolymph ecdysteroid levels in diapause- and nondiapause-bound fourth and fifth instars and in pupae of the European corn borer, *Ostrinia nubilalis* (Hubner). *Comp. Biochem. Physiol. A 78*, 319–325.

Gelman, D.B., Thyagaraja, B.S., Kelly, T.J., Masler, E.P., Bell, R.A., *et al.*, **1992**. Prothoracicotropic hormone levels in brains of the European corn borer, *Ostrinia nubilalis*: diapause vs. the non-diapause state. *J. Insect Physiol. 38*, 383–395.

Gelman, D.B., Woods, C.W., **1983**. Haemolymph ecdysteroid titers of diapause- and nondiapause-bound fifth instars and pupae of the European corn borer, *Ostrinia nubilalis* (Hubner). *Comp. Biochem. Physiol. A 76*, 367–375.

Giebultowicz, J.M., Denlinger, D.L., **1986**. Role of the brain and ring gland in relation to pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *J. Insect Physiol. 32*, 161–166.

Godlewski, J., Kludkiewicz, B., Grzelak, K., Cymborowski, B., **2001**. Expression of larval hemolymph proteins (*Lhp*) genes and protein synthesis in the fat body of greater wax moth (*Galleria mellonella*) larvae during diapause. *J. Insect Physiol. 47*, 759–766.

Goto, S.G., Denlinger, D.L., **2002a**. Genes encoding two cystatins in the flesh fly *Sarcophaga crassipalpis* and their distinct expression patterns in relation to pupal diapause. *Gene 292*, 121–127.

Goto, S.G., Denlinger, D.L., **2002b**. Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. *J. Insect Physiol. 48*, 803–816.

Gregg, P.C., Roberts, B., Wentworth, S.L., **1987**. Levels of ecdysteroids in diapause and non-diapause eggs of the Australian plague locust, *Chortoicetes terminifera* (Walker). *J. Insect Physiol. 33*, 237–242.

Hamasaka, Y., Watari, Y., Arai, T., Numata, H., Shiga, S., **2001**. Retinal and extraretinal pathways for entrainment of the circadian activity rhythm in the blow fly, *Protophormia terraenovae*. *J. Insect Physiol. 47*, 967–975.

Hamel, M., Geri, C., Auger-Rozenberg, A., **1998**. The effects of 20-hydroxyecdysone on breaking diapause of *Diprion pini* L. (Hym., Diprionidae). *Physiol. Entomol. 23*, 337–346.

Hare, J.D., **1983**. Seasonal variation in plant–insect associations: utilization of *Solanum dulcamara* by *Leptinotarsa decemlineata*. *Ecology 64*, 345–361.

Hartfelder, K., Hanton, W.K., Bollenbacher, W.E., **1994**. Diapause-dependent changes in prothoracicotropic hormone-producing neurons of the tobacco hornworm, *Manduca sexta*. *Cell Tissue Res. 277*, 69–78.

Hasegawa, K., **1952**. Studies on the voltinism of the silkworm, *Bombyx mori* L., with special reference to the organs concerning determination of voltinism. *J. Fac. Agric. Tottori Univ. 1*, 83–124.

Hasegawa, K., **1957**. The diapause hormone of the silkworm, *Bombyx mori*. *Nature 179*, 1300–1301.

Hasegawa, K., **1964**. Studies on the mode of action of the diapause hormone in the silkworm, *Bombyx mori* L. II. Content of diapause hormone in the subesophageal ganglion. *J. Exp. Biol. 41*, 855–863.

Hasegawa, K., Shimizu, I., **1987**. *In vivo* and *in vitro* photoperiodic induction of diapause using isolated brain–suboesophageael ganglion complexes of the silkworm, *Bombyx mori*. *J. Insect Physiol. 33*, 959–966.

Hasegawa, K., Shimizu, I., **1990**. Gabaergic control of the release of diapause hormone from the esophageal ganglion of the silkworm, *Bombyx mori*. *J. Insect Physiol. 36*, 909–915.

Haunerland, N.H., **1996**. Insect storage proteins: gene families and receptors. *Insect Biochem. Mol. Biol. 26*, 755–765.

Henneberry, T.J., Butler, G.D., Jr., **1986**. Effects of high temperature on tobacco budworm (Lepidoptera: Noctuidae) reproduction, diapause, and spermatocyst development. *J. Econ. Entomol. 79*, 410–413.

Hirai, M., Watanabe, D., Chinzei, Y., **2000**. A juvenile hormone-repressible transferrin-like protein from the bean bug, *Riptortus clavatus*: cDNA sequence analysis and protein identification during diapause and vitellogenesis. *Arch. Insect Biochem. Physiol. 44*, 17–26.

Hirai, M., Yuda, M., Shinoda, T., Chinzei, Y., **1998**. Identification and cDNA cloning of novel juvenile hormone responsive genes from the fat body of the bean bug, *Riptortus clavatus* by mRNA differential display. *Insect Biochem. Mol. Biol. 28*, 181–189.

Hodkova, M., **1992**. Storage of the photoperiodic information within the implanted neuroendocrine complexes in females of the linden bug *Pyrrhocoris apterus* (L.) (Heteroptera). *J. Insect Physiol. 38*, 357–363.

Hodkova, M., **1994**. Photoperiodic regulation of mating behaviour in the linden bug, *Pyrrhocoris apterus,* is mediated by a brain inhibitory factor. *Experientia 50*, 742–744.

Hodkova, M., Berkova, P., Zahradnickova, H., **2002**. Photoperiodic regulation of the phospholipid molecular species composition in thoracic muscles and fat body of *Pyrrhocoris apterus* (Heteroptera) via an endocrine gland, corpus allatum. *J. Insect Physiol. 48*, 1009–1019.

Hodkova, M., Okuda, T., Wagner, R., **1996**. Stimulation of corpora allata by extract from neuroendocrine complex: comparison of reproducing and diapausing *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). *Eur. J. Entomol. 93*, 535–543.

Hodkova, M., Okuda, T., Wagner, R., **2001**. Regulation of corpora allata in females of *Pyrrhocoris apterus* (Heteroptera) (a mini-review). *In Vitro Cell. Devel. Biol. 37*, 560–563.

Horie, Y., Kanda, T., Mochida, Y., **2000**. Sorbitol as an arrester of embryonic development in diapausing eggs of the silkworm, *Bombyx mori*. *J. Insect Physiol. 46*, 1009–1016.

Ichikawa, T., Hasegawa, K., Shimizu, I., Katsuno, K., Kataoka, H., *et al.*, **1995**. Structure or neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide in the silkworm, *Bombyx mori*. *Zool. Sci. 12*, 703–712.

Ichikawa, T., Shiota, T., Shimizu, I., Kataoka, H., **1996**. Functional differentiation of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide of the moth, *Bombyx mori*. *Zool. Sci. 13*, 21–25.

Ichimori, T., Ohtomo, R., Suzuki, K., Kurihara, M., **1990**. Specific protein related to adult diapause in the leaf beetle, *Gastrophysa atrocyanea*. *J. Insect Physiol. 36*, 85–91.

Ikeda, M., Su, Z.H., Saito, H., Imai, K., Sato, Y., *et al.*, **1993**. Induction of embryonic diapause and stimulation of ovary trehalase activity in the silkworm, *Bombyx mori*, by synthetic diapause hormone. *J. Insect Physiol. 39*, 889–895.

Imai, K., Konno, T., Nakazawa, Y., Komiya, T., Isobe, M., *et al.*, **1991**. Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. *Proc. Jap. Acad. B 67*, 98–101.

Imai, K., Nomura, T., Katsuzaki, H., Komiya, T., Yamashita, O., **1998**. Minimum structure of diapause hormone required for biological activity. *Biosci. Biotechnol. Biochem. 62*, 1875–1879.

Imhof, M.O., Rusconi, S., Lezzi, M., **1993**. Cloning of the *Chironomus tentans* cDNA encoding a protein (cEcRH) homologous to the *Drosophila melanogaster* ecdysteroid receptor (dEcR). *Insect Biochem. Mol. Biol. 23*, 115–124.

Isobe, M., Hasegawa, K., Goto, T., **1973**. Isolation of the diapause hormone from the silkworm, *Bombyx mori*. *J. Insect Physiol. 19*, 1221–1240.

Joplin, K.H., Stetson, D.L., Diaz, J.G., Denlinger, D.L., **1993**. Cellular differences in ring glands of flesh fly pupae as a consequence of diapause programming. *Tissue and Cell 25*, 245–257.

Joplin, K.H., Yocum, G.D., Denlinger, D.L., **1990**. Diapause specific proteins expressed by the brain during pupal diapause of the flesh fly, *Sarcophaga crassipalpis*. *J. Insect Physiol. 36*, 775–783.

Khan, M.A., **1988**. Brain-controlled synthesis of juvenile hormone in adult insects. *Entomol. Exp. Applic. 46*, 3–17.

Khan, M.A., Buma, P., **1985**. Neural control of the corpus allatum in the Colorado potato beetle, *Leptinotarsa decemlineata*: an electron microscope study utilizing the *in vitro* tannic acid Ringer incubation method. *J. Insect Physiol. 31*, 639–645.

Khan, M.A., Koopmanschap, A.B., de Kort, C.A.D., **1983**. The relative importance of nervous and hormonal control of corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Gen. Comp. Endocrinol. 52*, 214–221.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., Ruvkun, G., **1997**. *daf-2,* an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science 277*, 942–946.

Kipyatkov, V.E., **2001**. A distantly perceived primer pheromone controls diapause termination in the ant *Myrmica rubra* L. (Hymenoptera, Formicidae). *J. Evol. Biochem. Physiol. 37*, 405–416.

Koopmanschap, A.B., Oouchi, H., de Kort, C.A.D., **1989**. Effects of juvenile hormone analogue on the eggs, post-embryonic development, metamorphosis and diapause induction of the Colorado potato beetle, *Leptinotarsa decemlineata*. *Entomol. Exp. Applic. 50*, 255–263.

Kopper, B.J., Shu, S., Charlton, R.E., Ramaswamy, S.B., **2001**. Evidence for reproductive diapause in the fritillary *Speyeria idalia* (Lepidoptera: Nymphalidae). *Ann. Entomol. Soc. America 94*, 427–432.

Kostal, V., Noguchi, H., Shimada, K., Hayakawa, Y., **1998**. Developmental changes in dopamine levels in larvae of the fly *Chymomyza costata*: comparison between wild-type and mutant-nondiapause strains. *J. Insect Physiol. 44*, 605–614.

Kostal, V., Noguchi, H., Shimada, K., Hayakawa, Y., **1999**. Dopamine and serotonin in the larval CNS of a drosophilid fly, *Chymomyza costata*: are they involved in the regulation of diapause? *Arch. Insect Biochem. Physiol. 42*, 147–162.

Kostal, V., Shimada, K., **2001**. Malfunction of circadian clock in the non-photoperiodic-diapause mutants of the drosophilid fly, *Chymomyza costata*. *J. Insect Physiol. 47*, 1269–1274.

Kotaki, T., Yagi, S., **1989**. Hormonal control of adult diapause in the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Appl. Entomol. Zool. 24*, 42–51.

Kramer, S.J., **1978**. Regulation of the activity of JH-specific esterases in the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol. 24*, 743–747.

Krysan, J.L., **1990**. Fenoxycarb and diapause: a possible method of control for pear psylla (Homoptera: Psyllidae). *J. Econ. Entomol. 83*, 293–299.

Lankinen, P., Riihimaa, A., **1992**. Weak circadian eclosion rhythmicity in *Chymomyza costata* (Diptera: Drosophilidae), and its independence of diapause type. *J. Insect Physiol. 38*, 801–811.

Larrere, M., Lavenseau, L., Tasei, J.N., Couillaud, F., **1993**. Juvenile hormone biosynthesis and diapause termination in *Bombus terrestris*. *Invert. Reprod. Devel. 23*, 7–14.

Lee, K.-Y., Denlinger, D.L., **1996**. Diapause-regulated proteins in the gut of pharate first instar larvae of the gypsy moth, *Lymantria dispar*, and the effects of KK-42 and neck ligation on expression. *J. Insect Physiol. 42*, 423–431.

Lee, K.-Y., Denlinger, D.L., **1997**. A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol. 43*, 289–296.

Lee, K.-Y., Hiremath, S., Denlinger, D.L., **1998**. Expression of actin in the central nervous system is switched off during diapause in the gypsy moth, *Lymantria dispar*. *J. Insect Physiol. 44*, 221–226.

Lee, K.-Y., Horodyski, F.M., Valaitis, A.P., Denlinger, D.L., **2002**. Molecular characterization of the insect immune protein hemolin and its high induction during embryonic diapause in the gypsy moth, *Lymantria dispar*. *Insect Biochem. Mol. Biol. 32*, 1457–1467.

Lee, K.-Y., Valaitis, A.P., Denlinger, D.L., **1997**. Further evidence that diapause in the gypsy moth, *Lymantria dispar*, is regulated by ecdysteroids: a comparison of diapause and nondiapause strains. *J. Insect Physiol. 43*, 897–903.

Lee, S.S., Kennedy, S., Tolonen, A.C., Ruvkun, G., **2003**. DAF-16 target genes that control *C. elegans* life-span and metabolism. *Science 300*, 644–647.

Lefevere, K.S., **1989**. Endocrine control of diapause termination in the adult female Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol. 35*, 197–203.

Lefevere, K.S., Koopmanschap, A.B., de Kort, C.A.D., **1989**. Juvenile hormone metabolism during and after diapause in the female Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol. 35*, 129–135.

Lenz, C.J., Venkatesh, K., Chippendale, G.M., **1987**. Major plasma proteins of larvae of the southwestern corn borer, *Diatraea grandiosella*. *Arch. Insect Biochem. Physiol. 5*, 271–284.

L'Helias, C., Beaudry, P., Callebert, J., Launay, J.M., **2001**. Head tryptophan hydroxylase activity and serotonin content during premetamorphosis of *Pieris brassicae*. *Biogen. Amines 16*, 497–522.

Ma, P.W.K., Knipple, D.C., Roelofs, W.L., **1994**. Structural organization of the *Helicoverpa zea* gene encoding the precursor protein for pheromone biosynthesis-activating neuropeptide and other neuropeptides. *Proc. Natl Acad. Sci. USA 91*, 6506–6510.

Masaki, S., **1980**. Summer diapause. *Annu. Rev. Entomol. 25*, 1–25.

Matsumoto, M., Takeda, M., **2002**. Changes in brain monoamine contents in diapausing pupae of *Antheraea pernyi* when activated under long-day conditions. *J. Insect Physiol. 48*, 765–771.

Matsuo, J., Nakayama, S., Numata, H., **1997**. Role of the corpus allatum in the control of adult diapause in the blow fly, *Protophormia terraenovae*. *J. Insect Physiol. 43*, 211–216.

Meola, R.W., Adkisson, P.L., **1977**. Release of prothoracicotropic hormone and potentiation of developmental ability during diapause in the bollworm, *Heliothis zea*. *J. Insect Physiol. 23*, 683–688.

Miller, S.G., Leclerc, R.F., Seo, S.-J., Malone, C., **1990**. Synthesis and transport of storage proteins by testes in *Heliothis virescens*. *Arch. Insect Biochem. Physiol. 14*, 151–170.

Miura, K., Chinzei, Y., Shinoda, T., Numata, H., **1991**. Cyanoprotein: quantitative changes and synthesis in diapause and juvenile hormone analog treated bean bug, *Riptortus clavatus*. *Insect Biochem. 21*, 553–562.

Miyawaki, R., Tanaka, S.I., Numata, H., **2003**. Photoperiodic receptor in the nymph of *Poecilocoris lewisi* (Heteroptera: Scutelleridae). *Eur. J. Entomol. 100*, 301–303.

Moribayashi, A., Kurahashi, H., Ohtaki, T., **1988**. Different profiles of ecdysone secretion and its metabolism between diapause- and nondiapause-destined cultures of the fleshfly, *Boettcherisca peregrina*. *Comp. Biochem. Physiol. A 91*, 157–164.

Moribayashi, A., Kurahashi, H., Ohtaki, T., **1992**. Physiological differentiation of the ring glands in mature larvae of the flesh fly, *Boettcherisca peregrina,* programmed for diapause or non-diapause. *J. Insect Physiol. 38*, 177–183.

Moribe, Y., Niimi, T., Yamashita, O., Yaginuma, T., **2001**. *Samui*, a novel cold-inducible gene, encoding a protein with a BAG-domain similar to silencer of death domains (SODD/BAG-4), isolated from *Bombyx* diapause eggs. *Eur. J. Biochem. 268*, 3432–3442.

Morita, A., Niimi, T., Yamashita, O., **2004**. Physiological differentiation of DH-PBAN-producing neurosecretory cells in the silkworm embryo. *J. Insect Physiol. 49*, 1093–1102.

Morita, A., Numata, H., **1997a**. Role of the neuroendocrine complex in the control of adult diapause in the bean bug, *Riptotus clavatus*. *Arch. Insect Biochem. Physiol. 35*, 347–355.

Morita, A., Numata, H., **1997b**. Distribution of the photoperiodic receptor in the compound eyes of the bean bug, *Riptortus clavatus*. *J. Comp. Physiol. A 180*, 181–185.

Morita, A., Numata, H., **1999**. Localization of the photoreceptor for photoperiodism in the stink bug, *Platutia crossota stali*. *Physiol. Entomol. 24*, 189–195.

Nakagaki, M., Takei, R., Nagashima, E., Yaginuma, T., **1991**. Cell-cycles in embryos of the silkworm, *Bombyx mori*: G2 arrest at diapause stage. *Roux's Arch. Devel. Biol. 200*, 223–229.

Nakamura, K., Hodkova, M., **1998**. Photoreception in entrainment of rhythms and photoperiodic regulation of diapause in a hemipteran, *Graphosoma lineatum*. *J. Biol. Rhythms 13*, 159–166.

Noguchi, H., Hayakawa, Y., **1997**. Role of dopamine at the onset of pupal diapause in the cabbage armyworm, *Mamestra brassicae*. *FEBS Lett. 413*, 157–161.

Noguchi, H., Hayakawa, Y., **2001**. Dopamine is a key factor for the induction of egg diapause of the silkworm, *Bombyx mori*. *Eur. J. Biochem. 268*, 774–780.

Numata, H., Hidaka, T., **1983**. Compound eyes as the photoperiodic receptors in the bean bug. *Experientia 39*, 868–869.

Numata, H., Shiga, S., Morita, A., **1997**. Photoperiodic receptors in arthropods. *Zool. Sci. 14*, 187–197.

Okuda, T., Tanaka, S., **1997**. An allatostatic factor and juvenile hormone synthesis by corpora allata in *Locusta migratoria*. *J. Insect Physiol. 43*, 635–641.

Okuda, T., Tanaka, S., Kotaki, T., Ferenz, H.-J., **1996**. Role of the corpora allata and juvenile hormone in the control of imaginal diapause and reproduction in three species of locusts. *J. Insect Physiol. 42*, 943–951.

Osir, E.O., Labongo, L.V., Unnithan, G.C., **1989**. A high molecular weight diapause-associated protein from the stem-borer *Busseola fusca*: purification and properties. *Arch. Insect Biochem. Physiol. 11*, 173–187.

Overmeer, W.P.J., Nelis, H.J.C.F., de Leenheer, A.P., Calis, J.N.M., Veerman, A., **1989**. Effect of diet on the photoperiodic induction of diapause in three species of predatory mite, *Amblyseius potentillae, Amblyseius cucumeris* and *Typhlodromus pyri*. *Exp. Appl. Acarol. 7*, 281–287.

Palli, S.R., Kothapalli, R., Feng, Q., Ladd, T., Perera, S.C., *et al.*, **2001**. Molecular analysis of overwintering diapause. In: Denlinger, D.L., Giebultowicz, J.M., Saunders, D.S. (Eds.), Insect Timing: Circadian Rhythmicity to Seasonality. Elsevier, Amsterdem, pp. 133–144.

Palli, S.R., Ladd, T.R., Ricci, A.R., Primavera, M., Mungrue, I.N., *et al.*, **1998**. Synthesis of the same two proteins prior to larval diapause and pupation in the spruce budworm, *Choristoneura funiferana*. *J. Insect Physiol. 44*, 509–524.

Palli, S.R., Locke, M., **1987**. The synthesis of hemolymph proteins by the larval epidermis of an insect, *Calpodes ethlius* (Lepidoptera: Hesperiidae). *Insect Biochem. 17*, 711–722.

Panda, S., Sato, T.K., Castrucci, A.M., Rollag, M.D., De Grip, W.J., *et al.*, **2002**. Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science 298*, 2213–2216.

Parsell, D.A., Lindquist, S., **1993**. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet. 27*, 437–496.

Pener, M.P., **1992**. Environmental cues, endocrine factors, and reproductive diapause in male insects. *Chronobiol. Int. 9*, 102–113.

Peypelut, L., Beydon, P., Lavenseau, L., **1990**. 20-Hydroxyecdysone triggers the resumption of imaginal wing disc development after diapause in the European corn borer, *Ostrinia nubilalis*. *Arch. Insect Biochem. Physiol. 15*, 1–19.

Pinyarat, W., Shimada, T., Xu, W.-H., Sato, Y., Yamashita, O., *et al.*, **1995**. Linkage analysis of the gene encoding precursor protein of diapause hormone and pheromone biosynthesis-activating neuropeptide in the silkworm, *Bombyx mori*. *Genet. Res. 65*, 105–111.

Puiroux, J., Moreau, R., Gourdoux, L., **1990**. Variations of biogenic amine levels in the brain of *Pieris brassicae* pupae during nondiapausing and diapausing development. *Arch. Insect Biochem. Physiol. 14*, 57–69.

Pullin, A.S., Bale, J.S., **1989**. Effects of ecdysone, juvenile hormone and haemolymph transfer on cryoprotectant metabolism in diapausing and non-diapausing pupae of *Pieris brassicae*. *J. Insect Physiol. 35*, 911–918.

Ray, A., Memmel, N.A., Krishna Kumaran, A., **1987**. Developmental regulation of the larval hemolymph protein gene in *Galleria mellonella*. *Roux's Arch. Devel. Biol. 196*, 414–420.

Readio, J., Chen, M.-H., Meola, R., **1999**. Juvenile hormone biosynthesis in diapausing and nondiapausing *Culex pipiens* (Diptera: Culicidae). *J. Med. Entomol. 36*, 355–360.

Reppert, S.M., Weaver, D.R., **2000**. Comparing clockworks: mouse vs. fly. *J. Biol. Rhythms 15*, 357–364.

Richard, D.S., Gilbert, M., Crum, B., Hollinshead, D.M., Schelble, S., *et al.*, **2001a**. Yolk protein endocytosis by oocytes in *Drosophila melanogaster*: immunofluorescent localization of clathrin, adaptin and the yolk protein receptor. *J. Insect Physiol. 47*, 715–723.

Richard, D.S., Jones, J.M., Barbarito, M.R., Cerula, S., Detweiler, J.P., *et al.*, **2001b**. Vitellogenesis in diapausing and mutant *Drosophila melanogaster:* further evidence for the relative roles of ecdysteroids and juvenile hormones. *J. Insect Physiol. 47*, 905–913.

Richard, D.S., Saunders, D.S., **1987**. Prothoracic gland function in diapause and non-diapause *Sarcophaga argyrostoma* and *Calliphora vicina*. *J. Insect Physiol. 33*, 385–392.

Richard, D.S., Warren, J.T., Saunders, D.S., Gilbert, L.I., **1987**. Haemolymph ecdysteroid titres in diapause- and non-diapause-destined larvae and pupae of *Sarcophaga argyrostoma*. *J. Insect Physiol. 33*, 115–122.

Richard, D.S., Watkins, N.L., Serafin, R.B., Gilbert, L.I., **1998**. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol. 44*, 637–644.

Rinehart, J.P., Cikra-Ireland, R.A., Flannagan, R.D., Denlinger, D.L., **2001**. Expression of ecdysone receptor is unaffected by pupal diapause in the flesh fly, *Sarcophaga crassipalpis,* while its dimerization partner, USP, is downregulated. *J. Insect Physiol. 47*, 915–921.

Rinehart, J.P., Denlinger, D.L., **2000**. Heat shock protein 90 is downregulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Mol. Biol. 9*, 641–645.

Rinehart, J.P., Yocum, G.D., Denlinger, D.L., **2000**. Developmental upregulation of inducible Hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochem. Mol. Biol. 30*, 515–521.

Rockey, S.J., Miller, B.B., Denlinger, D.L., **1989**. A diapause maternal effect in the flesh fly, *Sarcophaga bullata*: transfer of information from mother to progeny. *J. Insect Physiol. 35*, 553–558.

Ruby, N.F., Brennan, T.J., Xie, X.M., Cao, V., Franken, P., *et al.*, **2002**. Role of melanopsin in circadian responses to light. *Science 298*, 2211–2213.

Safranek, L., Williams, C.M., **1980**. Studies on the prothoraciotropic hormone in the tobacco hornworm, *Manduca sexta*. *Biol. Bull. 158*, 141–153.

Saito, H., Takeuchi, Y., Takeda, R., Hayashi, Y., Watanabe, K., *et al.*, **1994**. The core and complementary sequence responsible for biological activity of the diapause hormone of the silkworm, *Bombyx mori*. *Peptides 15*, 1173–1178.

Salama, M., Miller, T., **1992**. A diapause associated protein of the pink bollworm *Pectinophora gossypiella*. *Arch. Insect Biochem. Physiol. 21*, 1–11.

Sato, Y., Ikeda, M., Yamashita, O., **1994**. Neurosecretory cells expressing the gene for common precursor for diapause hormone and phermone biosynthesis-activating neuropeptide in the subesophageal ganglion of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol. 96*, 27–36.

Sato, Y., Nakazawa, Y., Menjo, N., Imai, K., Komiya, T., *et al.*, **1992**. A new diapause hormone molecule of the silkworm, *Bombyx mori*. *Proc. Jap. Acad. B 68*, 75–79.

Sato, Y., Oguchi, M., Menjo, N., Imai, K., Saito, H., *et al.*, **1993**. Precursor polyprotein for multiple neuropeptides secreted from the subesophageal ganglion of the silkworm *Bombyx mori*: characterization of the DNA-encoding diapause hormone precursor and identification of additional peptides. *Proc. Natl Acad. Sci. USA 90*, 3251–3255.

Sato, Y., Shiomi, K., Saito, H., Imai, K., Yamashita, O., **1998**. Phe-X-Pro-Arg-Leu-NH2 peptide producing cells in the central nervous system of the silkworm, *Bombyx mori*. *J. Insect Physiol. 44*, 333–342.

Sauman, I., Reppert, S.M., **1996**. Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. *Neuron 17*, 889–900.

Sauman, I., Sehnal, F., **1997**. Immunohistochemistry of the products of male accessory glands in several hemimetabolous insects and the control of their secretion in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). *Eur. J. Entomol. 94*, 349–360.

Saunders, D.S., **1971**. The temperature-compensated photoperiodic clock "programming" development and pupal diapause in the flesh fly, *Sarcophaga argyrostoma*. *J. Insect Physiol. 17*, 801–812.

Saunders, D.S., **1987**. Insect photoperiodism: the linden bug, *Pyrrhocoris apterus*, a species that measures daylength rather than nightlength. *Experientia 43*, 935–937.

Saunders, D.S., **1990**. The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: is the *period* gene causally involved in photoperiodic time measurement? *J. Biol. Rhythms 5*, 315–331.

Saunders, D.S., **2002**. Insect Clocks, 3rd edn. Elsevier, Amsterdam.

Saunders, D.S., Cymborowski, B., **1996**. Removal of the optic lobes of adult blow flies (*Calliphora vicina*) leaves photoperiodic induction of larval diapause intact. *J. Insect Physiol. 42*, 807–811.

Saunders, D.S., Cymborowski, B., **2003**. Selection for high diapause incidence in blow flies (*Calliphora vicina*) maintained under long days increases the maternal critical daylength: some consequences for the photoperiodic clock. *J. Insect Physiol. 49*, 777–784.

Saunders, D.S., Henrich, V.C., Gilbert, L.I., **1989**. Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutants on time measurement. *Proc. Natl Acad. Sci. USA 86*, 3748–3752.

Saunders, D.S., Richard, D.S., Applebaum, S.W., Ma, M., Gilbert, L.I., **1990**. Photoperiodic diapause in *Drosophila melanogaster* involves a block to juvenile hormone regulation of ovarian maturation. *Gen. Comp. Endocrinol. 79*, 174–184.

Saunders, D.S., Wheeler, I., Kerr, A., **1999**. Survival and reproduction of small blow flies (*Calliphora vicina*; Diptera: Calliphoridae) produced in severely overcrowded short-day larval cultures. *Eur. J. Entomol. 96*, 19–22.

Schotland, P., Sehgal, A., **2001**. Molecular control of *Drosophila* circadian rhythms. In: Denlinger, D.L., Giebultowicz, J.M., Saunders, D.S. (Eds.), Insect Timing: Circadian Rhythmicity to Seasonality. Elsevier, Amsterdam, pp. 15–30.

Seo, S.J., Kim, C.H., **1992**. Localization and function of a storage protein in testes of summer diapause males in tobacco budworm *Heliothis virescens*. *Kor. J. Entomol. 22*, 59–66.

Shiga, S., Numata, H., **1997**. Induction of reproductive diapause via perception of photoperiod through the compound eyes in the adult blow fly, *Protophormia terraenovae*. *J. Comp. Physiol. A 181*, 35–40.

Shiga, S., Numata, H., **2000**. The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae*. *Naturwissenschaften 87*, 125–128.

Shimada, K., **1999**. Genetic linkage analysis of photoperiodic clock genes in *Chymomyza costata* (Diptera: Drosophilidae). *Entomol. Sci. 2*, 575–578.

Shimizu, I., Aoki, S., Ichikawa, T., **1997**. Neuroendocrine control of diapause hormone secretion in the silkworm, *Bombyx mori*. *J. Insect Physiol. 43*, 1101–1109.

Shimizu, I., Hasegawa, K., **1988**. Photoperiodic induction of diapause in the silkworm, *Bombyx mori*: location of the photoreceptor using a chemiluminescent paint. *Physiol. Entomol. 13*, 81–88.

Shimizu, I., Kato, M., **1984**. Carotenoid functions in photoperiodic induction in the silkworm, *Bombyx mori*. *Photochem. Photobiophys. 7*, 47–52.

Shimizu, I., Kitabatake, S., Kato, M., **1981**. Effect of carotenoid deficiency on photosensitvities in the silkworm, *Bombyx mori*. *J. Insect Physiol. 27*, 593–599.

Shimizu, I., Yamakawa, Y., Shimazaki, Y., Iwasa, T., **2001**. Molecular cloning of *Bombyx* cerebral opsin (Boceropsin) and cellular localization of its expression in the silkworm brain. *Biochem. Biophys. Res. Commun. 287*, 27–34.

Shingleton, A.W., Sisk, G.C., Stern, D.L., **2003**. Diapause in the pea aphid (*Acyrthosiphon pisum*) is a slowing but not a cessation of development. *Devel. Biol. 3*, 7.

Sielezniew, M., Cymborowski, B., **1997**. Effects of ecdysteroid agonist RH-5849 on pupal diapause of the tobacco hornworm (*Manduca sexta*). *Arch. Insect Biochem. Physiol. 35*, 191–197.

Singtripop, T., Oda, Y., Wanichacheewa, S., Sakurai, S., **2002**. Sensitivities to juvenile hormone and ecdysteroid in diapause larvae of *Omphisa fuscidentalis* based on the hemolymph trehalose dynamics index. *J. Insect Physiol. 48*, 817–824.

Singtripop, T., Wanichacheewa, S., Sakurai, S., **2000**. Juvenile hormone-mediated termination of larval diapause in the bamboo borer, *Omphisa fuscidentalis*. *Insect Biochem. Mol. Biol. 30*, 847–854.

Slama, K., **1980**. Homeostatic functions of ecdysteroids in ecdysis and oviposition. *Acta Entomol. Bohem. 77*, 145–168.

Smith, W.A., Bowen, M.F., Bollenbacher, W.E., Gilbert, L.I., **1986**. Cellular changes in the prothoracic glands of diapausing pupae of *Manduca sexta*. *J. Exp. Biol. 120*, 131–142.

Smythe, E., Warren, G., **1991**. The mechanism of receptor mediated endocytosis. *Eur. J. Biochem. 202*, 689–699.

Sonobe, H., Hiyama, Y., Keino, H., **1977**. Changes in the amount of the diapause factor in the subesophageal ganglion during development of the silkworm, *Bombyx mori*. *J. Insect Physiol. 23*, 633–637.

Stanewsky, R., **2002**. Clock mechanisms in *Drosophila*. *Cell Tissue Res. 309*, 11–26.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., *et al.*, **1998**. The *cry(b)* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell 95*, 681–692.

Su, Z.H., Ikeda, M., Sato, Y., Saito, H., Imai, K., *et al.*, **1994**. Molecular characterization of ovary trehalase of the silkworm, *Bombyx mori* and its transcriptional

activation by diapause hormone. *Biochim. Biophys. Acta – Gene Struct. Expr. 1218*, 366–374.

Suwan, S., Isobe, M., Yamashita, O., Minakata, H., Imai, K., **1994**. Silkworm diapause hormone, structure-activity-relationships, indispensable role of C-terminus amide. *Insect Biochem. Mol. Biol. 24*, 1001–1007.

Suzuki, K., Minagawa, T., Kumagai, T., Naya, S.-I., Endo, Y., *et al.*, **1990**. Control mechanism of diapause of the pharate first-instar larvae of the silkmoth *Antheraea yamamai*. *J. Insect Physiol. 36*, 855–860.

Suzuki, K., Nakamura, T., Yanbe, T., Kurihara, M., Kuwano, E., **1993**. Termination of diapause in pharate first-instar larvae of the gypsy moth *Lymantria dispar japonica* by an imidazole derivative KK-42. *J. Insect Physiol. 39*, 107–110.

Suzuki, M.G., Terada, T., Kobayashi, M., Shimada, T., **1999**. Diapause-associated transcription of *BmEts*, a gene encoding an ETS transcription factor homolog in *Bombyx mori*. *Insect Biochem. Mol. Biol. 29*, 339–347.

Takahashi, M., Iwasaki, H., Niimi, T., Yamashita, O., Yaginuma, T., **1998**. Changing profiles of mRNA levels of cdc2 and a novel cdc2-related kinase (Bcdrk) in relation to ovarian development and embryonic diapause of *Bombyx mori*. *Appl. Entomol. Zool. 33*, 551–559.

Takahashi, M., Niimi, T., Ichimura, H., Sasaki, T., Yamashita, O., *et al.*, **1996**. Cloning of a B-type cyclin homolog from *Bombyx mori* and the profiles of its mRNA level in non-diapause and diapause eggs. *Devel. Genes Evol. 206*, 288–291.

Tammariello, S.P., **2001**. Regulation of the cell cycle during diapause. In: Denlinger, D.L., Giebultowicz, J.M., Saunders, D.S. (Eds.), Insect Timing: Circadian Rhythmicity to Seasonality. Elsevier, Amsterdam, pp. 173–183.

Tammariello, S.P., Denlinger, D.L., **1998**. G_0/G_1 cell cycle arrest in the brains of *Sarcophaga crassipalpis* during pupal diapause and the expression pattern of the cell cycle regulator, proliferating cell nuclear antigen. *Insect Biochem. Mol. Biol. 28*, 83–89.

Tanaka, S., Denlinger, D.L., Wolda, H., **1987**. Daylength and humidity as environmental cues for diapause termination in a tropical beetle. *Physiol. Entomol. 12*, 213–224.

Tanigawa, N.A., Shiga, S., Numata, H., **1999**. Role of the corpus allatum in the control of reproductive diapause in the male blow fly, *Protophormia terraenovae*. *Zool. Sci. 16*, 639–644.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.-P., Yin, C.-M., *et al.*, **2001**. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science 292*, 107–110.

Tatar, M., Yin, C.-M., **2001**. Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. *Exp. Gerontol. 36*, 723–738.

Tauber, M.J., Tauber, C.A., Masaki, S., **1986**. Seasonal Adaptations of Insects. Oxford University Press, Oxford.

Tawata, M., Ichikawa, T., **2001**. Circadian firing activities of neurosecretory cells releasing pheromonotropic neuropeptides in the silkmoth, *Bombyx mori*. *Zool. Sci. 18*, 645–649.

Tawfik, A.I., Tanaka, Y., Tanaka, S., **2002a**. Possible involvement of ecdysteroids in photoperiodically induced suppression of ovarian development in a Japanese strain of the migratory locust, *Locusta migratoria*. *J. Insect Physiol. 48*, 411–418.

Tawfik, A.I., Tanaka, Y., Tanaka, S., **2002b**. Possible involvement of ecdysteroids in embryonic diapause of *Locusta migratoria*. *J. Insect Physiol. 48*, 743–749.

Telfer, W., Kunkel, J.G., **1991**. The function and evolution of insect storage hexamers. *Annu. Rev. Entomol. 36*, 205–228.

Teodoro, R.O., O'Farrell, P.H., **2003**. Nitric oxide-induced suspended animation promotes survival during hypoxia. *EMBO J. 22*, 580–587.

Tomioka, K., Agui, N., Bollenbacher, W.E., **1995**. Electrical properties of the cerebral prothoracicotropic hormone cells in diapausing and nondiapausing pupae of the tobacco hornworm, *Manduca sexta*. *Zool. Sci. 12*, 165–173.

Truman, J.W., **1976**. Extraretinal photoreception in insects. *Photochem. Photobiol. 23*, 215–225.

Umeya, Y., **1926**. Experiments of ovarian transplantation and blood transfusions in silkworms, with special reference to the alternation of voltinism. (*Bombyx mori* L.) *Bull. Sericult. Exp. Station, Chosen 1*, 1–26.

Uryu, M., Ninomiya, Y., Yokoi, T., Tsuzuki, S., Hayakawa, Y., **2003**. Enhanced expression of genes in the brains of larvae of *Mamestea brassicae* (Lepidoptera; Noctuidae) exposed to short daylengths or fed Dopa. *Eur. J. Entomol. 100*, 245–250.

van Houten, Y.M., Overmeer, W.P.J., Veerman, A., **1987**. Thermoperiodically induced diapause in a mite in constant darkness is vitamin A dependent. *Experientia 43*, 933–935.

van Houten, Y.M., Veerman, A., **1990**. Photoperiodism and thermoperiodism in the predatory mite, *Amblyseius potentillae* are probably based on the same mechanism. *J. Comp. Physiol. A 167*, 201–209.

van Zon, A.Q., Overmeer, W.P.J., Veerman, A., **1981**. Carotenoids function in photoperiodic induction of diapause in a predacious mite. *Science 213*, 1131–1133.

Veerman, A., **2001**. Photoperiodic time measurement in insects and mites: a critical evaluation of the oscillator-clock hypothesis. *J. Insect Physiol. 47*, 1097–1109.

Veerman, A., Slagt, M.E., Alderlieste, M.F.J., Veenendaal, R.L., **1985**. Photoperiodic induction of diapause in an insect is vitamin A dependent. *Experientia 41*, 1194–1195.

Veerman, A., Veenendaal, R.L., **2003**. Experimental evidence for a non-clock role of the circadian system in spider mite photoperiodism. *J. Insect Physiol. 49*, 727–732.

Vermunt, A.M.W., Koopmanschap, A.B., Vlak, J.M., de Kort, C.A.D., **1997b**. Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase

from the Colorado potato beetle. *Insect Biochem. Mol. Biol. 27*, 919–928.

Vermunt, A.M.W., Koopmanschap, A.B., Vlak, J.M., de Kort, C.A.D., **1998**. Evidence for two juvenile hormone esterase-related genes in the Colorado potato beetle. *Insect Mol. Biol. 7*, 327–336.

Vermunt, A.M.W., Koopmanschap, A.B., Vlak, J.M., de Kort, C.A.D., **1999**. Expression of the juvenile hormone esterase gene in the Colorado potato beetle, *Leptinotarsa decemlineata*: photoperiodic and juvenile hormone analog response. *J. Insect Physiol. 45*, 135–142.

Vermunt, A.M.W., Vermeesch, A.M.G., de Kort, C.A.D., **1997a**. Purification and characterization of juvenile hormone esterase from hemolymph of the Colorado potato beetle. *Arch. Insect Biochem. Physiol. 35*, 261–277.

Watanabe, M., Tanaka, K., **1998**. Effect of juvenile hormone analogs on diapause termination and myo-inositol content in *Aulacophora nigripennis* adults (Coleoptera: Chrysomelidae). *Appl. Entomol. Zool. 33*, 259–292.

Webb, M.-L.Z., Denlinger, D.L., **1998**. GABA and picrotoxin alter expression of a maternal effect that influences pupal diapause in the flesh fly, *Sarcophaga bullata*. *Physiol. Entomol. 23*, 184–191.

Williams, C.M., **1946**. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm *Platysamia cecropia*. *Biol. Bull. 90*, 234–243.

Williams, C.M., **1947**. Physiology of insect diapause. II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull. 93*, 89–98.

Williams, C.M., **1952**. Physiology of insect diapause. VI. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull. 103*, 120–138.

Williams, C.M., Adkisson, P.L., **1964**. Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm *Antheraea pernyi*. *Biol. Bull. 127*, 511–525.

Williams, J.A., Sehgal, A., **2001**. Molecular components of the circadian system in *Drosophila*. *Annu. Rev. Physiol. 63*, 729–755.

Xu, W.-H., Denlinger, D.L., **2003**. Molecular characterization of prothoracicotropic hormone and diapause hormone in *Heliothis virescens* during diapause, and a new role for diapause hormone. *Insect Mol. Biol. 12*, 509–516.

Xu, W.-H., Sato, Y., Ikeda, M., Yamashita, O., **1995a**. Stage-dependent and temperature controlled expression of the gene encoding the precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide in the silkworm, *Bombyx mori*. *J. Biol. Chem. 270*, 3804–3808.

Xu, W.-H., Sato, Y., Ikeda, M., Yamashita, O., **1995b**. Molecular characterization of the gene encoding the precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) of the silkworm, *Bombyx-mori* and its distribution in some insects. *Biochim. Biophys. Acta 1261*, 83–89.

Yin, C.-M., Wang, Z.S., Chaw, W.D., **1985**. Brain neurosecretory cell and ecdysiotropin activity of the non-diapausing, pre-diapausing and diapausing southwestern corn borer, *Diatraea grandiosella* Dyar. *J. Insect Physiol. 31*, 659–667.

Yocum, G.D., **2001**. Differential expression of two *HSP70* transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. *J. Insect Physiol. 47*, 1139–1145.

Yocum, G.D., **2003**. Isolation and characterization of three diapause-associated transcripts from the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol. 49*, 161–169.

Yocum, G.D., Joplin, K.H., Denlinger, D.L., **1998**. Upregulation of a 23 kDa small heat shock protein transcript during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochem. Mol. Biol. 28*, 677–682.

Yoder, J.A., Denlinger, D.L., **1992**. Evidence for a brain factor that stimulates deposition of puparial hydrocarbons in diapausing flesh flies. *J. Exp. Biol. 162*, 339–344.

Yoshiga, T., Georgieva, T., Dunkov, B.C., Harizanova, N., Ralchev, K., *et al.*, **1999**. *Drosophila melanogaster* transferrin: cloning, deduced protein sequence, expression during the life cycle, gene localization and up-regulation on bacterial infection. *Eur. J. Biochem. 260*, 414–420.

Zhang, T.-Y., Sun, J.-S., Zhang, L.-B., Shen, J.-L., Xu, W.-H., **2004**. Cloning and expression of the cDNA encoding FXPRL family of peptides and a functional analysis of their effect on breaking pupal diapause in *Helicoverpa armigera*. *J. Insect Physiol. 50*, 25–33.

Zimmerman, W.F., Goldsmith, T.H., **1971**. Photosensitivity of the circadian rhythm and of visual receptors in carotenoid-depleted *Drosophila*. *Science 171*, 1167–1169.

3.13 Endocrine Control of Insect Polyphenism

K Hartfelder, Universidade de São Paulo,
Ribeirão Preto, Brazil
D J Emlen, University of Montana, Missoula,
MT, USA

3.13.1. Polyphenism and Polymorphism: Discontinuity in the Variation of Insects

Historically, the terms polymorphism and polyphenism have the same meaning, namely intraspecific variation in sets of characters. This phenomenon is seen as an adaptive response that allows genotypes to track short-term cycles of environmental variation and, at the same time, maintains cohesiveness of adapted gene complexes. With recent advances in population genetics, enzymology, genomics, and proteomics the term polymorphism has now gained a much wider meaning. It has come to denote variation in nucleotide sequences in general. This genetic variation may or may not have consequences for phenotypic character states, depending on whether it occurs in coding regions, promotor and regulatory regions, or in selectively neutral DNA such as microsatellites.

For the purpose of this review the term polyphenism is used to describe, in a more narrow sense, the occurrence of intraspecific variation in phenotypes. The focus is specifically on relatively discrete, or discontinuous variation in phenotype expression. Polyphenic mechanisms can be described at two levels: cues that serve as proximate triggers of developmental alternatives, and endogenous response cascades that drive the developmental responses themselves. Triggering stimuli may consist of external environmental factors (e.g., photoperiod, crowding) or internal genetic factors (e.g., alleles at polymorphic loci), or a mixture of the two; endogenous response cascades involve a series of systems that relay, synchonize, and coordinate differentiative processes in target systems.

The most common form of polyphenism in animals is sexual dimorphism. This, in most cases, is triggered by genetic factors that generate distinct phenotypes through a series of mainly endocrine response cascades. Even though sexual polymorphism occurs to some degree in all insects, it is considered only peripherally in this review. Rather, and following the tradition already set in the review on this subject by Hardie and Lees (1985) in the first edition of this series, distinct phenotypes (in either or both sexes) that are triggered by environmental or other exogenous factors are focused. Recent studies on the endogenous response cascades underlying polyphenic trait expression in insects are reviewed, and discuss general properties shared by these mechanisms.

3.13.2. Polyphenism in the Hemimetabola

The most predominant form of polyphenism in hemimetabolous insects is that of wing length. Wing length polyphenism involves differential investment in wings and wing muscles, and appears to reflect an ecophysiological tradeoff in resource allocation between dispersal and reproduction. It is observed in species that colonize and exploit ephemeral resources, in locust and aphid phase polyphenism, and in termite caste polyphenism. In the following sections, the current status of endocrine regulation of wing, phase, and caste polyphenism encountered in the Hemimetabola are reviewed.

3.13.2.1. Hemiptera/Homoptera Wing Polyphenism

3.13.2.1.1. Soapberry bugs A number of hemipteran species have polyphenic mechanisms of wing expression (Tanaka and Wolda, 1987; Dingle and Winchell, 1997). The hemipteran *Jadera haematoloma* (the soapberry bug) exhibits a wing polymorphism with four morphs: two winged forms, one that always has viable flight muscles, and one that histolyzes flight muscles part way through the adult stage, a winged form that never produces viable flight musculature, and a completely wingless form that also produces inviable flight musculature (Dingle and Winchell, 1997). *Jadera haemotoloma* has undergone rapid recent evolution following a host-shift that occurred within the past 50 years (Carroll and Boyd, 1992; Carroll *et al*., 1997, 2001), and one characteristic that has changed dramatically is the proportion of the population expressing wings: derived populations (on the new host) have significantly fewer winged animals than ancestral populations.

Dingle and Winchell (1997) demonstrated that this polyphenism was regulated by a threshold mechanism, and that this threshold behaved like a polygenic character with high levels of additive genetic variance. Manipulations of the amount of nymphal food predictably affected the percent of winged offspring, as did topical applications of the juvenile hormone (JH) mimic methoprene (Dingle and Winchell, 1997). In laboratory animals sampled from an ancestral population, increases in the amount of food decreased the percent developing with wings. Similarly, experimental augmentation of JH levels at the beginning of the final nymphal instar also decreased the proportion of winged animals, suggesting that high levels of JH result in a reduction of the relative amount of wing growth (Dingle and Winchell, 1997).

There is at present no information regarding the sensitivity of animals to JH during the penultimate or earlier instars, nor of the relative levels of ecdysteroids. However, results available at this time show striking similarities with the polyphenic mechanism described below for crickets (see Section 3.13.2.2.1). The timing of the sensitive period, the implicated role for JH, and the direction of the effect of JH, all agree with the basic model for cricket wing polyphenism.

3.13.2.1.2. Planthoppers Planthoppers often occur in both winged and wingless forms (Kisimoto, 1965; Morooka *et al*., 1988; Denno and Perfect, 1994), and dimorphism in these species results from a threshold mechanism with heritable variation for the threshold (Denno *et al*., 1986, 1996; Morooka and Tojo, 1992; Matsumara, 1996; Peterson and Denno, 1997). Where studied, the environmental factor most relevant to wing expression appears to be population density, or crowding, and as with the wing polyphenism already described, the physiological response to nymphal crowding appears to be mediated by levels of JH.

To date, the endocrine regulation of wing polyphenism has been best studied in the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). The default developmental pathway appears to involve wing production, but Iwanaga and Tojo (1986) showed that both exposure to solitary (low-density) conditions, and topical applications of JH, could suppress wing development and result in a greater proportion of wingless individuals. They identified two sensitive periods, one each in the prepenultimate and penultimate instars (Iwanaga and Tojo, 1986). The timing of these sensitive periods, and the effect of JH, were corroborated by Ayoade *et al*. (1999), who were able to induce wingless morphs in a strain of planthoppers that had been selected to be entirely winged. Bertuso *et al*. (2002) then induced expression of wings in a line selected to be entirely wingless by applying precocene to animals at these same sensitive periods. Precocene disrupts JH synthesis (see **Chapter 3.7**).

Thus, wing polyphenism in planthoppers, as with wing polyphenisms in soapberry bugs and crickets, appears to be regulated by JH during brief sensitive periods late in nymphal life, though in planthoppers the sensitive periods end in the penultimate, rather than the final, nymphal instar. This conclusion has now also received support from JH titer determinations by gas chromatography-mass spectrometry (GC-MS) and a JH binding protein assay (Bertuso *et al*., 2002) showing similar titers between brachypterous and macropterous final instar nymphs. The two morphs then showed divergent titers in the adult stage, where a higher JH titer in the short-winged morph was associated with an earlier development of oocytes (Bertuso *et al*., 2002), thus providing evidence for the dual (developmental and reproductive) role of JH in the divergent life history strategies of planthoppers.

3.13.2.1.3. Aphid phase and caste polyphenism In terms of life cycle complexity, aphids are outstanding champions. Aphid life cycles can include at least two different forms of polyphenism: cyclical switching between asexual reproduction (viviparous parthenogenesis) and sexual reproduction (associated with the production of haploid eggs capable of overwintering after fertilization), and switching between

a wingless sedentary morphology, and a winged morph capable of dispersal. This switching capacity enables aphids to successfully colonize and thrive on highly ephemeral habitats. In some cases these polyphenic switches occur simultaneously (e.g., the switch from asexual to sexual reproduction may coincide with a switch from wingless to winged morphologies), or they may occur separately (the switch from wingless to winged morphologies can occur without coincident changes in mode of reproduction or host-plant preference). The large variation in species-specific aphid life cycles stems from the fact that these elements of polyphenism can be shuffled and integrated as seemingly independent modules in the evolutionary history of each species.

In this review the ontogenetic mechanisms that generate aphid polyphenism is focused (for reviews of aphid life cycles and their ecological significance, see Dixon (1977, 1998) and Moran (1992)). In an effort to facilitate the discussion of aphid polyphenism, and to place these mechanisms in the context of insect polyphenism in general, a simplified description of aphid life cycles is presented and adapt a simplified terminology (Blackman, 1994).

3.13.2.1.3.1. Asexual versus sexual reproduction

All present-day aphids reproduce parthenogenetically, a capacity that appears to have been acquired from a common ancestor some 250 million years ago. Yet only a small number of derived species (i.e., at the tips of the different branches in the phylogenetic tree) are strictly parthenogenetic (Simon *et al*., 2002). Instead, the majority of aphid species facultatively switch between parthenogenetic and sexual modes of reproduction.

During the asexual phase, females give birth to parthenogenetic progeny which develop completely within the ovaries. While these progeny develop, their own ovaries become active, and these embryos begin ovulation before being born. In this extreme telescoping of generations, both daughters and granddaughters develop simultaneously within a single adult female. This parthenogenetic/paedogenetic reproductive strategy of wingless female aphids is unusually efficient for colonizing ephemeral habitats because it can generate large numbers of progeny extremely rapidly. However, rapid production of parthenogenetic daughters can also lead to overcrowding, and with this, the need to disperse to new host plants (see Section 3.13.2.1.3.2).

At the end of a parthenogenetic phase of reproduction, and generally, at the end of the favorable season, female aphids begin producing sexually reproductive offspring. Often, this transition from asexual to sexual reproduction is coupled with a shift from the primary to a secondary host plant species. Aphids that live on a primary host plant with a short seasonal duration may switch to a secondary host plant, and this can occur in two ways, depending on the species. Asexual females may produce a generation of winged daughters that first disperses to the new host plant, and then produces sexually reproductive (male and female) offspring or asexual females may produce winged males and females directly, and these then disperse to the secondary host and reproduce sexually. In either case, the cycle is complete when daughters disperse back to the primary host the following season, and begin parthenogenetic reproduction anew.

As may be expected given the strict seasonality in both host plant alternation and cyclical parthenogenesis, photoperiod appears to be an important environmental cue for this polyphenism. The effects of photoperiod on these key elements in aphid life cycles have long been recognized (review: Hardie and Lees, 1985), and have been studied extensively, especially in the black bean aphid, *Aphis fabae*, and the vetch aphid, *Megoura vicinae* (Hardie, 1981a, 1981b; Hardie and Lees, 1983). The photoperiodic response in the switch from parthenogenetic to sexual forms depends primarily on the length of the scotophase (Hardie *et al*., 1990), and immunocytochemical analyses of opsins and phototransduction proteins suggest that photoperiodic receptors are located in the anterior dorsal region of the aphid protocerebrum (Hardie and Nunes, 2001). In this respect, recent molecular approaches have also been succesful showing that a cDNA encoding a putative amino acid transporter in γ-aminobutyric acid (GABA)-ergic neurons is overexpressed under short-day conditions (Ramos *et al*., 2003).

Once the external trigger (changes in photoperiod, and to a lesser extent, temperature) has been detected, the polyphenic switch between modes of reproduction is coordinated by circulating levels of hormones, in particular JH. Specifically, in *A. fabae,* topical applications of JH inhibit the production of haploid eggs (and, hence, the switch from asexual to sexual reproduction), and promote parthenogenetic development (Hardie, 1981a, 1981b). JH also affected host plant preference behaviors at this same stage (Hardie, 1980, 1981c; Hardie *et al*., 1990), which makes sense, since these events occur simultaneously in *A. fabae*, and since both appear to involve a response to photoperiod. Based on these results, Hardie and Lees (1985) proposed that the endogenous JH titer should be high during long-day conditions, when animals feed on the primary host plant and reproduce parthenogenetically, and low

later in the season, when aphids switch from asexual to sexual reproduction, and from primary to secondary host plants.

3.13.2.1.3.2. Wingless versus winged morphology The switch from wingless to winged morphologies can occur in two different situations. In the first case, parthenogenetically reproducing aphids overcrowd their primary host plant, and must disperse to other (also primary) host individuals. In this case, as conditions become crowded, females begin producing asexual daughters with wings. These winged females disperse to new plants and begin parthenogenetic production of wingless daughters all over again.

At the end of the favorable growing season, however, conditions begin to deteriorate everywhere (e.g., as the primary host plants begin to die). When this occurs, aphids again produce a winged generation of offspring, except that this time the switch between wingless and winged morphologies may coincide with both the switch from primary to secondary host plants, and with the switch from asexual to sexual reproduction. Thus, the first manifestation of the wingless versus winged switch is not associated with a simultaneous switch in the mode of reproduction, but the second one is. This suggests that at least partially independent mechanisms may be involved. Indeed, in some species the winged forms produced at these two times are morphologically distinct (Hille Ris Lambers, 1966), consistent with the idea that separate environmental cues, and/or endocrine mechanisms, may regulate wing expression at these two stages.

Early research on the mechanism of aphid wing polyphenism produced equivocal results, probably due to difficulties distinguishing between hormonally induced "juvenilization" of animals, and polyphenism-specific effects on wing expression *per se* (review: Hardie and Lees, 1985). The complex life cycles of aphids, and the simultaneous involvement of several different forms of polyphenism, made this problem still more difficult. However, several clever experiments with *A. fabae* finally resolved this dilemma, and these clearly establish a role for JH in one form of aphid wing polyphenism (Lees, 1977, 1980; Hardie, 1980, 1981a, 1981b). JH appears to regulate the switch from wingless to winged morphologies that occurs at the end of the growing season (and coincident with the switch from asexual to sexual reproduction). Animals harvested at a stage that would typically begin production of winged offspring (i.e, at the end of the season) could, if exposed to either long days or high temperatures (i.e., summer conditions), be induced to forego wing production and produce wingless daughters instead. This effect of environmental stimuli could be mimicked by topical applications of JH I at this same time, suggesting that high levels of JH are associated with continued production of wingless offspring, and that a seasonal decline in levels of JH may underlie the polyphenic switch from wingless to winged morphologies (as it did with the end-of-season switch between asexual and sexual reproduction).

Interestingly, the most important environmental trigger of wing production is crowding. Animals reared under crowded conditions produce winged progeny irrespective of photoperiod or temperature, and even when exposed to topical applications of JH (Hardie, 1980). These results suggest that the two forms of wing polyphenism are, in fact, regulated by independent endocrine mechanisms. In particular, they suggest that polyphenic production of winged morphologies during the period of parthenogenetic reproduction occurs by a mechanism other than circulating levels of JH, and one that can operate independent of levels of JH (i.e., by a mechanism capable of inducing wing expression even when environmental conditions stimulate high levels of JH).

Major obstacles to direct assessment of JH titers and corpus allatum (CA) activity in aphids are their small size and telescoping of generations. Chemical allatectomy by precocenes turned out to be a valuable research tool in this context, even though not all aphid species are equally susceptible (Hardie, 1986), and not all precocene compounds acted in the same direction (Hardie *et al.*, 1995, 1996). JH rescue experiments on precocene-treated larvae of a pink pea aphid, *Acyrthosiphon pisum* clone (Gao and Hardie, 1996) showed that the destruction of the CA by precocene-II or precocene-III resulted in precocious adult development. The alate-promoting property of these compounds appeared to be unrelated to the decreased JH titer, and instead depended heavily on population density (Hardie *et al.*, 1995). In contrast, flight muscle breakdown in alate adult *A. pisum*, that had undertaken a migratory flight to a new host plant, has been shown to be under JH control (Kobayashi and Ishikawa, 1993, 1994).

Consistent with the notion that genetic variation must underly differing response thresholds to environmental cues, a single locus on the X chromosome has recently been demonstrated to affect the propensity for winged male production in different, coexisting clones of *A. pisum* (Cauillaud *et al.*, 2002). And precocene treatment, in contrast, was much more effective in apterizing presumptive alate

females than males in this species (Christiansen-Weniger and Hardie, 2000).

Interestingly, apterization of presumptive alates can also occur as a consequence of parasitization during the early larval instars (Johnson, 1959). Parasitization effects on wing development appear to be regulated relatively independent of metamorphosis, and in particular, seem to occur independent of JH (Hardie and Lees, 1985). In pea aphids, parasitization by *Aphidius pisum* caused apterization and other correlated changes in body shape (Christiansen-Weniger and Hardie, 2000), yet parasitization of the same species by *A. ervi* had the opposite effect, resulting in a higher proportion of winged offspring (Sloggett and Weisser, 2002). The developmental mechanisms underlying the divergent responses to parasitization are not yet understood.

3.13.2.1.3.3. Soldier aphids It is worth noting that aphids, the masters of polyphenism, exhibit yet another form of polyphenism. Some aphid species facultatively produce a behaviorally and morphologically distinct soldier caste. Since its original description (Aoki, 1977), this phenomenon has been detected in over 50 aphid species in the families Pemphigidae and Hormaphididae (Stern *et al.*, 1997). To date, most studies of aphid soldiers have focused on clonal relatedness and kin selection (Carlin *et al.*, 1994; Abbot *et al.*, 2001), as well as colony division of labor and defense against predators (Schutze and Maschwitz, 1991; Foster and Rhoden, 1998; Rhoden and Foster, 2002). Little is known about proximate mechanisms that induce soldier formation, except for a positive correlation between soldier proportion and colony size (Ito *et al.*, 1995), and the fact that, ontogenetically, the development of a soldier morphology appears to be linked to the replacement of the reproductive system by fat body cells and the lack of endosymbionts (Fukatsu and Ishikawa, 1992).

In conclusion, the mechanisms of endocrine control in aphid phase polyphenism are still far from resolved. In fact, there seem to be more questions than answers. It now appears that the existing grouping of aphid polyphenic mechanisms may be biologically misleading. Wing polyphenism occurs at two different stages in the annual life cycle of aphids. On the surface, these two switch mechanisms appear to be similar – even one and the same. Yet, they are regulated in response to different environmental cues, and they involve completely different endocrine mechanisms. Thus, wing polyphenism represents not one, but two developmental mechanisms. Again, on the surface, reproductive polyphenism would appear to be very different from wing polyphenism. Yet reproductive polyphenism occurs at the same time as one of the wing polyphenisms, occurs in response to the same environmental stimuli, and is regulated in the same direction by the same hormone (JH) (**Figure 1**). Thus, a more useful description of aphid polyphenism may involve a new classification scheme: an "autumn" polyphenism that includes both wing morphology and mode of reproduction, and which is regulated by a seasonal change in photoperiod and a corresponding decline in levels of JH, and a "summer" polyphenism that only involves wing expression, that occurs in response to changes in crowding, and that incorporates an as-yet unidentified endocrine mechanism that operates independently from circulating levels of JH.

3.13.2.2. Orthoptera

3.13.2.2.1. Wing polyphenism in crickets Facultative dispersal strategies in crickets involve the relative amounts of growth of the wings and associated wing musculature. Animals can have full-size, functional wings (macropters), miniature wings (micropters), or be entirely wingless (brachypters). Differences in wing expression (and associated flight capability) may occur among species (e.g., Harrison, 1980), among populations of a single species (e.g., Harrison, 1979), or even among time periods within a single individual's lifetime (e.g., some crickets shed their wings and histolyze flight muscles after mating (Srihari *et al.*, 1975)).

In a number of cricket species, wing expression is facultative, and depends on environmental conditions encountered during nymphal development, such as temperature (Ghouri and McFarlane, 1958; McFarlane, 1962), photoperiod (Masaki and Oyama, 1963; Saeki, 1966a; Alexander, 1968; Mathad and McFarlane, 1968; Tanaka *et al.*, 1976), diet (McFarlane, 1962), and population density (Fuzeau-Braesch, 1961; Saeki, 1966b; Zera and Tiebel, 1988; Zera and Denno, 1997). In these taxa, wing expression appears to be regulated by a threshold mechanism, and population comparison, controlled-breeding, and artificial selection studies all suggest high levels of additive genetic variation for the threshold of this polyphenism (Harrison, 1979; Roff, 1986, 1990; Fairbairn and Yadlowski, 1997).

The physiology of wing polyphenism has been most thoroughly studied in *Gryllus rubens* (Orthoptera: Gryllidae). Wing expression in this species is sensitive to population density; in particular, the level of crowding experienced by nymphs as they develop (Zera and Tiebel, 1988). Experiments that transferred animals between experimentally staged high and low

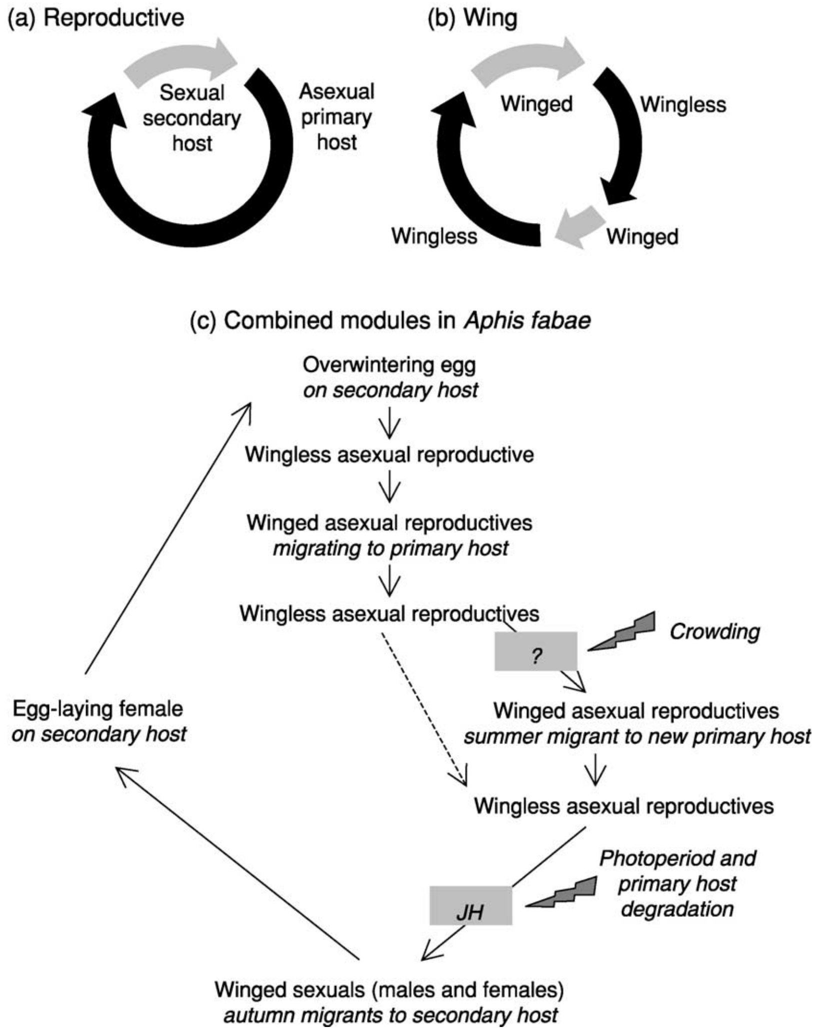


Figure 1 Polyphenism in aphid life cycles. Traditional classifications of aphid polyphenism group these mechanisms into two modules. (a) Module 1 is a switch in reproductive mode, sometimes associated with a switch from a primary to a secondary host plant. (b) Module 2 represents the switch from a wingless to a winged form. However, wing polyphenism includes two distinct events that occur at different periods of the aphid life cycle, and that incorporate different endocrine mechanisms, only one of which involves JH. (c) The modular combination is exemplified in the beam aphid, *Aphis fabae*, showing three switches from wingless to winged in the annual cycle. The first one occurs when wingless (parthenogenetic) females that arose from overwintered eggs produce winged (parthenogenetic) daughters that migrate to the primary host. The mechanisms underlying this switch are little understood. As a large population of wingless (parthenogenetic) females builds up on this host and crowding reaches critical levels, some winged (parthenogenetic) females are produced that colonize new primary hosts to initiate a new cycle of wingless generations. This switch in wing morphs does not involve a switch in reproductive mode and occurs independent of JH levels. It represents a JH-independent ''summer'' polyphenism (bottom gray arrow in b). The third switch from a wingless to a winged morph occurs at the end of the favorable season when primary host plants degrade. This is now also a switch in reproductive mode and a move of mated females to a secondary host plant where they either produce overwintering eggs or another generation of egg-laying females. This third switch in wing expression is controlled by photoperiod, resulting in decreased JH titers that allow growth of the wing anlagen. It represents the JH-dependent ''autumn'' polyphenism (top gray arrow in b). Graph compiled from data by Hardie, J., Lees, A.D., **1985**. Endocrine control of polymorphism and polyphenism. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon, Oxford, pp. 441–489; Hardie, J., Mallory, A.C.L., Quashiewilliams, C.A., **1990**. Juvenile hormone and host plant colonization by the black bean aphid, *Aphis fabae*. *Physiol. Entomol. 15*, 331–336; and Hardie, J., Gao, N., Timar, T., Sebok, P., Honda, K., **1996**. Precocene derivatives and aphid morphogenesis. *Arch. Insect Biochem. Physiol. 32*, 493–501.

densities revealed two sensitive periods relevant to expression of wings: during the middle of the penultimate and final nymphal instars, respectively (Zera and Tiebel, 1988). The default developmental pattern appeared to be winged, but animals could be switched to a wingless fate if they were exposed to crowded conditions during either or both of these sensitive periods (Zera and Tiebel, 1988).

This developmental response to nymphal crowding could be mimicked by the topical application of either JH III, or methoprene, during these same two critical periods: augmented levels of JH reduced the relative amount of wing growth (Zera and Tiebel, 1988). This suggested that titer differences of JH might underlie wing polyphenism. These presumed titer differences were corroborated by direct measures of JH titers in presumptive winged and wingless animals (Zera *et al*., 1989). In addition, a second titer difference was observed. Animals destined to produce wings had higher levels of ecdysteroids than animals destined not to produce wings (Zera *et al*., 1989).

Subsequent experiments have refined this basic model considerably. Zera and Tobe (1990) compared rates of JH biosynthesis in presumptive winged and wingless animals, and found no differences, suggesting that morph-specific variation in levels of JH result from differential clearance of JH from the blood, rather than from differential rates of hormone biosynthesis (see **Chapter 3.7**). Zera and Tiebel (1989) demonstrated that degradation of JH in *G. rubens* is primarily due to the enzyme juvenile hormone esterase (JHE), and they found higher levels of JHE in winged animals than in wingless animals. Titer differences in JHE were accompanied by corresponding differences in rates of JH clearance from hemolymph *in vivo* (Zera and Holtmeier, 1992). Finally, the timing of these morph-specific differences in levels of JHE coincided with the two sensitive periods already described, and with the observed morph-specific differences in JH titer.

Based on these results, Zera has proposed the following model (after Zera and Holtmeier (1992) and Zera and Denno (1997)). Crickets have two sensitive periods, occurring during the middle of the penultimate and final nymphal instars. During these periods, wing development is sensitive to external environmental factors (crowding), and these external stimuli appear to result in morph-specific differences in levels of two hormones: JH and 20-hydroxyecdysone (20E). Animals destined to produce wings have lower levels of JH, and higher levels of ecdysteroids, than animals destined to mature without wings (**Figure 2**). Morph-specific differences in levels of JH appear to result from subtle differences in the timing of the decline in JH titers during these sensitive periods, and these differences in JH titers result from winged animals having higher levels of the degradative enzyme JHE than wingless animals. Thus the default pattern of development entails production of wings, but a subset of individuals (animals with levels of JH above a genetically mediated threshold level) experience a delay in the rise in levels of ecdysteroids, and low levels of ecysteroids during the sensitive period probably “reprograms” these individuals towards production of a wingless body form.

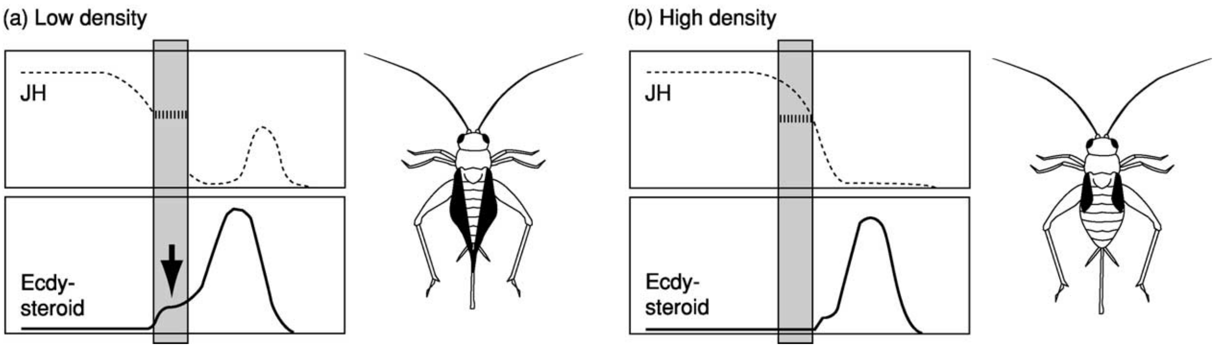


Figure 2 Facultative wing-length polyphenism in the cricket *Gryllus rubens* is a response to environmental conditions, especially nymphal crowding, and is controlled by a hormonal threshold mechanism acting during critical periods (shaded bars) in the two final instars of postembryonic development. At low population densities, the JH titer drops below a critical threshold level due to enhanced JH esterase activity, whereas the premolt ecdysteroid titer exceeds a threshold level. This endocrine situation permits wing development to a macropterous phenotype. In contrast, high population densities lead to an above-threshold JH titer and a low ecdysteroid titer during this critical period. Consequently, wing development is inhibited and brachypterous adults are formed. Graph compiled from data and models by Zera, A.J., Tiebel, K.C., **1988**. Brachypterizing effect of group rearing, juvenile hormone III and methoprene in the wing-dimorphic cricket, *Gryllus rubens. J. Insect Physiol. 34*, 489–498; Zera, A.J., Holtmeier, C.L., **1992**. *In vivo* and *in vitro* degradation of juvenile hormone-III in presumtive long-winged and short-winged *Gryllus rubens. J. Insect Physiol. 38*, 61–74; and Zera, A.J., Denno, R.F., **1997**. Physiology and ecology of dispersal polymorphism in insects. *Annu. Rev. Entomol. 42*, 207–230.

A genetic background for threshold mechanisms in wing dimorphic gryllids has been established for *Gryllus firmus* and now serves as a model system to investigate functional causes of adult life history evolution. In a fusion of resource allocation studies and metabolic biochemistry, Zhao and Zera (2002) and Zera and Zhao (2003) demonstrated a genetic bias for flight-fuel synthesis (mainly triglycerides) in the flight-capable genotype versus a bias for ovarian lipids (phospholipids) in the flightless one. When JH was applied topically the adult flight-capable morph shifted its lipid metabolism towards that of the flightless morph.

3.13.2.2.2. Phase polyphenism in locusts Locust phase polyphenism (a switch between solitary and gregarious forms) has dramatically impacted human history. The gregarization phenomenon can lead to staggering densities of animals, and these migratory swarms of locusts are one of the world's most devastating plagues. On the other hand, their capacity to switch between solitary and gregarious forms constitutes a remarkable developmental feat that has facilitated study of the hormonal control of complex animal life cycles. This polyphenism is common in several species of locusts, but is most clearly expressed in the migratory locust (*Locusta migratoria*), the desert locust (*Schistocerca gregaria*), and the red (*Nomadacris septemfasciata*) and brown (*Locustana pardalina*) locusts of Africa.

Phase polyphenism is a complex phenomenon that involves changes in body coloration, wing morphology, reproductive physiology, energy metabolism, and behavior (Uvarov, 1921). "Solitary" phase animals generally avoid conspecifics, and have cryptic, green color patterns, and reduced wing morphologies and musculature, whereas "gregarious" phase animals aggregate – actively seek conspecifics – and have a dark background pigmentation with yellow or orange color pattern, and more fully developed wings and wing musculature. The complexity of this syndrome sets locust phase polyphenism apart from the apparently simpler wing polyphenisms already discussed for other hemimetabolans (including other orthopterans). Studies comparing patterns of protein and gene expression between solitary and gregarious animals reveal numerous and complicated differences between the forms (Wedekind-Hirschberger *et al*., 1999; Clynen *et al*., 2002; Rahman *et al*., 2003), again reflecting the diversity of characters involved in this polyphenism. Perhaps the most important difference between locust phase polyphenism and the other polyphenisms discussed so far is that the switch between solitary and gregarious forms takes longer: the full transition requires multiple generations.

Gradual phase transition from solitary to gregarious locust forms was one of the earliest of the studied polyphenisms (e.g., Uvarov, 1921), and it has been clear for decades that the endocrine system, and in particular, JH, was involved (Joly, 1954; Staal and de Wilde, 1962; Couillaud *et al*., 1987). However, results from many of these early hormone studies were equivocal, and for some time the role of JH has been controversial (Pener, 1991; Applebaum *et al*., 1997; Pener and Yerushalmi, 1998; Dorn *et al*., 2000). The gradual, multigeneration nature of the response has compounded this problem in two ways. First, it has made interpretation of results and comparison across studies difficult, because not all of the involved traits respond at the same time. Some, like color, change rapidly, while others, like full migratory behavior, require much longer – often several generations. Second, the gradual nature of this phase transition meant that wild-caught, fully gregarious animals were not the same morphologically, physiologically, or behaviorally, as "gregarious" animals raised from one generation of rearing under crowded conditions in the laboratory. Yet, as pointed out by Dorn *et al*. (2000), these terms were used interchangeably, leading to confusion in the search for general patterns. Fortunately, an impressive series of recent studies has brought resolution to some of these apparent discrepancies.

3.13.2.2.2.1. Changes in color and wing morphology Changes in locust color may be triggered by a variety of environmental cues (e.g., humidity; Dorn *et al*. (2000)), but one important cue is the level of crowding experienced by nymphs as they develop. Exposure to crowded conditions induces a shift from the solitary (green) to the gregarious (black with yellow (*S. gregaria*) or orange (*L. migratoria*)) form, and much of this shift appears to be mediated by hormones. Several recent studies suggest that the neurohormone *Locusta migratoria* dark color-inducing neurohormone (Lom-DCIN) (now chemically characterized as [His^7]-corazonin) induces the expression of the black pigmentation and "foreground" color patterns typical of the gregarious form (Pener *et al*., 1992; Tanaka and Pener, 1994; Tanaka, 2000a, 2000b, 2000c, 2001). Controlled application experiments showed that increases in [His^7]-corazonin levels could switch animals from a solitary to a gregarious color pattern (Tanaka, 2000c, 2001). Juvenile hormone also affects locust color, but these effects occur independent from, and in the opposite direction to, the effects of [His^7]-corazonin.

Average levels of JH regulate the darkness of the "background" pattern (Tanaka, 2000a, 2001): higher levels of JH are associated with greener backgrounds, and hence a more solitary morphology, whereas the darker gregarious background pigmentation is associated with low levels of JH.

It is now recognized that the solitary–gregarious phase transition is associated with changes in a suite of additional morphological traits, including aspects of wing morphology and relative wing size. Recent multivariate morphometric studies found clear morphological separation of solitary and gregarious forms (Dorn *et al.*, 2000). Topical applications of a JH analog (JHA) to gregarious locust nymphs (*S. gregaria* and *L. migratoria*) caused many – but not all – of these morphological characters to shift towards "solitary" values by the time animals reached the adult molt (Dorn *et al.*, 2000). Interestingly, these "solitary" animals (i.e., animals reared under crowded conditions, but exposed to topical applications of JHA) also resembled juvenile animals. Specifically, they were morphological intermediates between fifth instar nymphs and adults reared under crowded conditions (Dorn *et al.*, 2000). However, unlike the situation with aphids, this "juvenilization" effect of JHA does not appear to be an artifact. Solitary animals normally resemble "juvenile" versions of the gregarious form, and "juvenilization" and "solitarization" may be overlapping concepts with respect to some traits (Dorn *et al.*, 2000).

Application of the neurohormone [His[7]]-corazonin also affected these morphological characteristics (in addition to the effect on color already discussed), and again, these effects occurred in the opposite direction to those induced by JH. Injections of [His[7]]-corazonin shifted animals from a solitary towards a gregarious morphology (Tanaka *et al.*, 2002).

3.13.2.2.2.2. Hormone titers of solitary and gregarious nymphs In a pioneering study of CA activity, Injeyan and Tobe (1981) showed that during the fourth nymphal instar, CA were more active in animals reared under isolated conditions than they were in animals reared under crowded conditions. More recently, Botens *et al.* (1997) measured JH III titers in laboratory animals reared under these same (isolated and crowded) conditions, and they identified a critical period during the middle of the fourth (penultimate) nymphal instar when solitary and gregarious animals differed in their level of JH. Juvenile hormone levels dropped sooner in gregarious animals (Botens *et al.*, 1997), so that during this critical window, gregarious animals had lower levels of JH than solitary animals (**Figure 3**).

Furthermore, when animals reared for three instars under isolated conditions were transferred to crowded conditions (at the beginning of the fourth instar), their JH titers decreased within 3 days to levels detected in nymphs reared entirely under crowded conditions (Botens *et al.*, 1997). Precisely the opposite effect (i.e., an increase in JH) occurred when animals were moved from crowded to isolated conditions. Finally, Botens *et al.* (1997) measured JH III titers for wild-caught solitary and

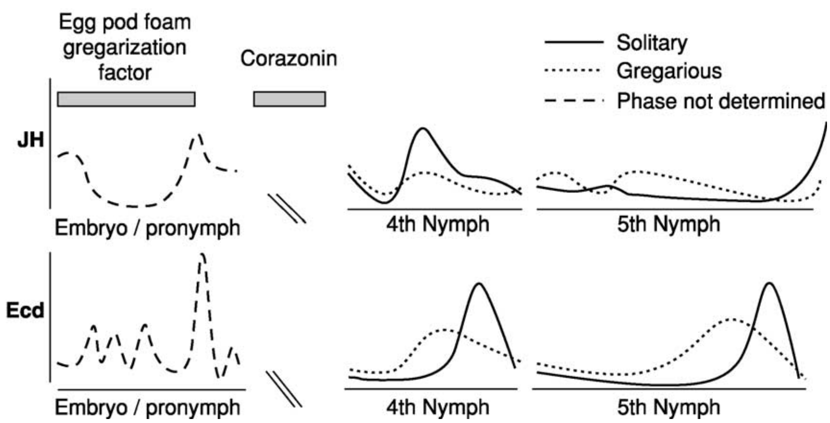


Figure 3 Juvenile hormone and ecdysteroid titers during embryonic and postembryonic development of solitary and gregarious morphs of migratory locusts (*Locusta migratoria* and *Schistocerca gregaria*). Hormone titers during embryonic development were determined by Lageux *et al.* (1977) and were not specified with respect to phase. The ecdysteroid titer peaks during embryonic development are associated with embryonic molts and the JH-free period in the middle of embryonic development is required for correct blastokinesis (Truman and Riddiford, 1999). Larval hormone titers were determined by Botens *et al.* (1997), Tawfik *et al.* (1997a), and Tawfik and Sehnal (2003). Juvenile hormone application to gregarious/crowded fourth instar larvae was shown to shift several, but not the entire complex of solitary characters (see text). Corazonin application to isolated/solitary second and third instar larvae induces the gregarious-phase foreground coloration (Tanaka, 2000a, 2000b, 2000c). Exposure of eggs of isolated/solitary females to extracts egg pod foam from gregarious/crowded females shifts offspring to the gregarious phase (Hägele *et al.*, 2000).

gregarious animals, and found the same result: JH titers during the middle of the fourth nymphal instar were higher in solitary animals than in gregarious animals. Thus, it appears that circulating levels of JH may underlie at least part of the physiological response to nymphal crowding.

Solitary and gregarious locusts also differ in their 20E levels (**Figure 3**). Animals reared under crowded conditions have a lower but more prolonged premolting ecdysteroid peak than animals reared under solitary conditions (Tawfik *et al.*, 1996; Tawfik and Sehnal, 2003) in the penultimate and last nymphal instar. These differences in ecdysteroids coincide with the phase-specifically modulated JH titer, suggesting a synergistic interaction of these major morphogenetic hormones in establishing the expression of a set of divergent phase characters, particularly during the critical penultimate instar.

Clearly, expression of multiple characters is affected by crowding, and multiple hormones appear to be involved. Although JH and ecdysone likely coordinate much of at least the more rapid shifts in locust form, other factors like the neurohormone [His^7]-corazonin are involved as well. There may even be multiple forms of JH in locusts, each with effects on specific subsets of the suite of phase-related traits. Darrouzet *et al.* (1997) recently found that the CA of *L. migratoria* synthesize two hydroxy-juvenile hormones (12′-OH JH III and 8′-OH JH III) in addition to JH III. These products have not been detected by the standard radiochemical assays (e.g., Tobe and Pratt (1974)), and whether these are secreted into the hemolymph, or whether they affect phase polyphenism, remains to be explored.

3.13.2.2.2.3. Changes in adult behavior and physiology Solitary and gregarious adult locusts differ in behavior and physiology, as well as morphology. For example, gregarious animals show an affinity for other locusts (aggregative behavior), and a strong propensity for migratory flight, in contrast with solitary animals. These behavioral differences are accompanied by corresponding differences in adult physiology related to the two different life-history strategies. Phase differences are noticable in the strength of the adipokinetic reaction which mobilizes fat body lipids during long distance flight (Ayali and Pener, 1992; Chino *et al.*, 1992; Schneider and Dorn, 1994; Ogoyi *et al.*, 1996), and in the onset of egg production (solitary females generally begin egg production earlier than gregarious females).

As with the morphological differences already discussed, behavioral differences between solitary and gregarious forms also appear to be driven – at least in part – by hormones, except that in this case, the relevant endocrine differences between morphs are observed after animals are adults (rather than during nymphal instars). In adult females, both solitary and gregarious animals show a large increase in JH levels within the first 2 weeks post eclosion (Dale and Tobe, 1986; Tawfik *et al.*, 2000). However, this rise in JH occurs earlier in solitary females than it does in gregarious females (Dale and Tobe, 1986; Tawfik *et al.*, 2000). Similar differences in the timing of hormone secretion were observed for ecdysteroids: ecdysone levels rose earlier in solitary females than in gregarious ones (Tawfik *et al.*, 1997a, 1997b; Tawfik and Sehnal, 2003).

These differences in the timing of adult female JH and ecdysteroid release correspond precisely with phase-specific differences in the timing of the first oogenesis cycle (**Figure 4**); females raised under isolated conditions begin producing eggs sooner than females reared under crowded conditions (Tawfik *et al.*, 1997a, 1997b, 2000). These same hormone titer differences also correspond with migratory flight fuel metabolism, but in the opposite direction (Wiesel *et al.*, 1996). Low levels of JH (as in gregarious females) stimulate utilization of stored lipids for flight, whereas high levels of JH (as in solitary females) reduce this use, and instead result in vitellogenin synthesis and lipid allocation to oocytes (Wiesel *et al.*, 1996; see **Chapter 3.9**). Thus, hormones appear to underlie a fundamental difference in patterns of stored-resource allocation between solitary and gregarious adult females, with these resources being directly allocated to eggs in solitary animals, but delayed and first used for migratory flight in gregarious animals.

In adult males, the principal behavioral difference between solitary and gregarious phase individuals involves production and secretion of phenylacetonitrile, the main component of the aggregation pheromone (Njagi *et al.*, 1996) (see **Chapter 3.14**) and this also appears to be regulated by phase-specific titer differences in hormones. As with females, solitary and gregarious male locusts differ in their JH and ecdysteroid titers (**Figure 4**). In particular, during a critical sensitive period approximately 15 days post eclosion, animals reared under isolated conditions have lower levels of JH, and higher levels of 20E, than animals reared under crowded conditions (Tawfik *et al.*, 1997a, 1997b, 2000). Synthesis of phenylacetonitrile is stimulated by high levels of JH, and inhibited by high levels of 20E, and this results in high levels of pheromone production in gregarious (but not solitary) animals (Tawfik *et al.*, 1997a, 1997b, 2000).

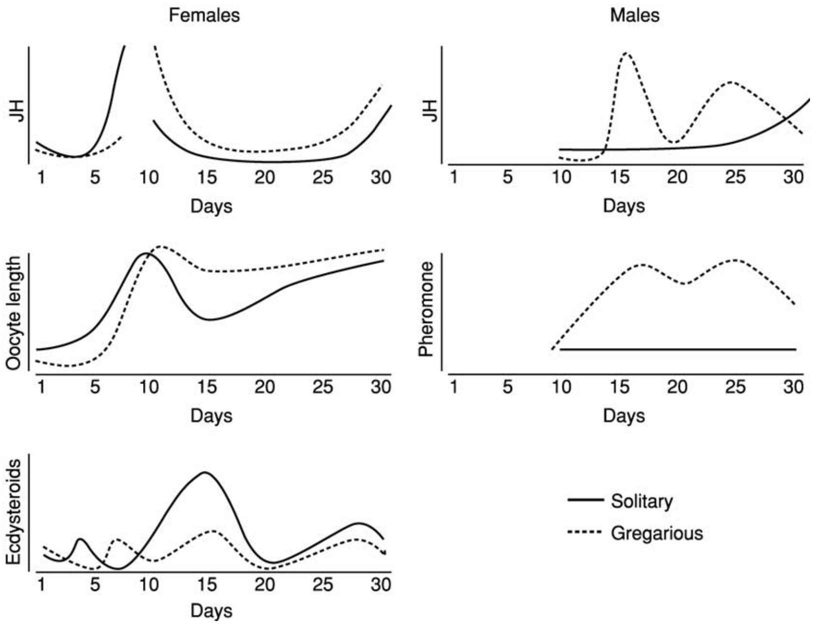


Figure 4 Hormone titers in relation to terminal oocyte length in solitary and gregarious-phase locust females (*Locusta migratoria* and *Schistocerca gregaria*), and the phase relationship between JH titer and pheromone production (phenylacetonitrile) in adult males. The first oogenic cycle is slightly advanced in solitary females, and is probably elicited by the earlier increase in JH titer and the advanced ecdysteroid titer peak. In gregarious-phase males, the sharp increase in JH titer between days 10 and 15 correlates with a strongly enhanced production of a gregarization pheromone. Graph compiled from data by Tawfik, A.I., Osir, E.O., Hassanali, A., Ismail, S.H., **1997a**. Effects of juvenile hormone treatment on phase changes and pheromone production in the desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae). *J. Insect Physiol. 43*, 1177–1182; Tawfik, A.I., Vedrova, A., Li, W.W., Sehnal, F., ObengOfori, D., **1997b**. Haemolymph ecdysteroids and the prothoracic glands in the solitary and gregarious adults of *Schistocerca gregaria*. *J. Insect Physiol. 43*, 485–493; Tawfik, A.I., Vedrova, A., Sehnal, F., **1999**. Ecdysteroids during ovarian development and embryogenesis in solitary and gregarious *Schistocerca gregaria*. *Arch. Insect Biochemi. Physiol. 41*, 134–143; Tawfik, A.I., Treiblmayr, K., Hassanali, A. Osir, E.O., **2000**. Time-course haemolymph juvenile hormone titres in solitarious and gregarious adults of *Schistocerca gregaria*, and their relation to pheromone emission, CA volumetric changes and oocyte growth. *J. Insect Physiol. 46*,1143–1150; Tawfik, A.I., Sehnal, F., **2003**. A role for ecdysteroids in the phase polymorphism of the desert locust. *Physiol. Entomol. 28*, 19–24; and Dorn, A., Ress, C., Sickold, S., Wedekind-Hirschberger, S., **2000**. Arthropoda – Insecta: endocrine control of phase polymorphism. In: Dorn, A. (Ed.), Progress in Developmental Endocrinology, vol. X, part B. John Wiley, Chichester, pp. 205–253.

3.13.2.2.2.4. Maternal effects on offspring development Interestingly, female locusts transfer ecdysteroids to their eggs. High levels of ecdysteroids have been found in vitellogenic ovaries (Lageux *et al.*, 1977), and may, in fact, be synthesized there. These ecdysteroids are transferred to the developing eggs and together with JH appear to affect molting events during embryonic development (Lageux *et al.*, 1979; Truman and Riddiford, 1999) (**Figure 3**).

Tawfik *et al.* (1999) and Tawfik and Sehnal (2003) found that the ecdysteroid content of *S. gregaria* ovaries in females reared under crowded conditions were up to four times higher than those in the ovaries of females reared in isolation (8.9 ng mg^{-1} versus 2.3 ng mg^{-1} tissue before egg-laying), and these phase-specific differences in egg ecdysteroid levels persisted after the eggs were laid (89 ng versus 14 ng per egg), and even into newly hatched larvae (Tawfik *et al.*, 1999). These studies raise the exciting possibility that endocrine differences in the mothers (resulting from their exposure to crowding as they developed) are carried over to their offspring – and this may help explain some aspects of the multigenerational nature of this phase transition.

Pheromones and semiochemicals transferred to eggs also may direct embryonic development. These compounds, when coated on the eggs, attract other gravid females to the area and result in clustered oviposition (Saini *et al.*, 1995), but they also lead to increases in the expression of "gregarious" characteristics in offspring. Washing freshly laid eggs from gregarious females shifts phase characteristics from gregarious towards solitary forms, and application of female accessory gland products to these washed eggs restores expression of the gregarious characters (McCaffery *et al.*, 1998; Hägele *et al.*, 2000).

Thus, there are several ways in which maternal compounds can direct the development of embryos and early instar animals. Expression of offspring traits may be influenced by differences in the levels

of maternally deposited egg steroids, and/or levels of maternally deposited semiochemicals and pheromones, and all of these may contribute to the multigenerational aspects of this remarkable insect polyphenism.

3.13.2.3. Isoptera (Termites): Caste Polyphenism in the Hemimetabola

The characterization of termites as "social cockroaches" suggests a very special postion of termites within insects expressing caste polyphenism. Caste polyphenism in termites differs from that of the holometabolous Hymenoptera in three very important ways. First, all isopteran species are social, whereas sociality has evolved in only a few branches of the Hymenoptera. Second, caste polyphenism in termites is a larval polyphenism – i.e., it affects primarily the morphologies and behavior of larvae, rather than adults. Finally, in termite societies both males and females form castes and, thus, contribute equally to the social organization (hymenopteran societies are female).

Hemimetabolous development permits postembryonic stages to participate actively in termite colony life, and these postembryonic larval and nymphal stages, in fact, constitute the major workforce of termite colonies (in termites, early postembryonic instars are usually called "larvae," whereas later instars are called "nymphs"; for simplicity, all postembryonic termite instars are referred to as larvae). In general, the only imagos encountered in a termite colony are the sexuals (primary reproductives (king and queen) and, at certain times of year, the predispersal sexual alates). In the adults, there is little visible dimorphism between the sexes, and the differences that exist result from the high degree of physogastry of the egg-laying queen (Bordereau, 1971). In contrast, many termites exhibit marked sexual dimorphism in the larval stages, especially in taxa that show a sex bias with respect to caste phenotypes. For termite sociality, the limelight is thus on the polyphenism exhibited by the larval stages.

Larval polyphenism means that in subsequent developmental stages, an individual can progressively specialize for different colony tasks ("temporal polyphenism" according to Noirot and Bordereau (1991)). As such, caste polyphenism is tightly linked to the molting process, and to the endocrine factors regulating molting. Interestingly, molting in termites is no longer necessarily connected with larval growth (Noirot, 1989), and larval molting has instead been co-opted as a mechanism for polyphenic changes in animal shape.

Recent reviews of termite societies and on trajectories of caste development within these (Noirot, 1985a, 1985b, 1985c, 1989; Noirot and Pasteels, 1987; Roisin, 2000) emphasize a distinction between caste systems (and polyphenic mechanisms) of "lower" (Mastotermitidae, Kalotermitidae, Hodotermitidae, and Rhinotermitidae) and "higher" termites (Termitidae). These groups differ primarily in the development of the worker caste. In the higher termites, workers represent a clear developmental trajectory, and a terminal molt. In contrast, in the lower termites, a true worker caste does not exist. Instead, most of the larval stages perform worker functions (i.e., colony maintenance). As such, workers in the lower termites do not undergo a terminal molt, and these individuals retain the capacity to develop subsequently into either soldiers or reproductives. In the lower termites, these late-instar larvae that comprise the major workforce of a colony are generally described as pseudergates.

Caste polyphenism in the termites involves several possible developmental switches (e.g., larva to pre-reproductive (with wingpads) to reproductive, larva to presoldier to soldier, and, in the higher termites, larva to worker). These developmental transformations may require several subsequent molts, and several different critical periods (**Figure 5**). Several of these developmental transformations can be reversed midway through the process, permitting extraordinarily flexible adjustment of the production of castes in the regulation of colony structure (this is particularly common in the lower termites, which are famous for undergoing stationary, and even regressive molts). Here, studies examining larva to reproductive, and larva to soldier polyphenisms are reviewed. Larva to worker development has yet barely been explored.

3.13.2.3.1. Larva to reproductive polyphenism Termite larvae occasionally molt into reproductives. The most commonly encountered reproductives are the alates – the winged males and females that disperse from termite colonies to breed and find new colonies. After mating, each new royal pair sheds its wings and finds a colony. A second type of reproductive consists of a replacement king or queen within an existing colony. When colonies bud, or when one of the original pair dies, replacement reproductives can be produced. In either case (dispersing alates or replacement reproductives), early commitment towards a sexual fate is first evidenced by the appearance of rudimentary wingpads, which may appear several molts before the terminal, adult molt. However, in the developmental trajectory of "replacement" reproductives, these wingpads fail to fully develop (replacement kings or queens do not have functional wings). Termites that do not develop

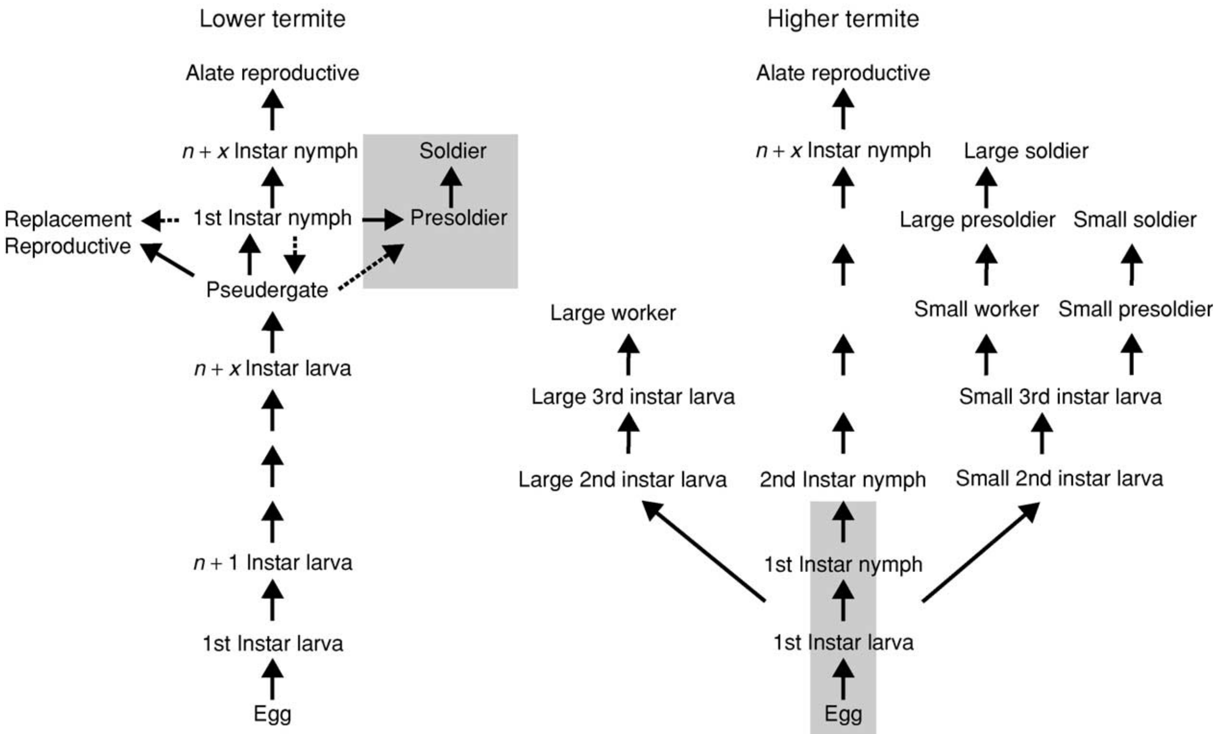


Figure 5 Generalized developmental pathways in lower and higher termites. In termite studies it is customary to denote all immatures stages without wingpads as larvae. Nymphs are stages exhibiting wingpads, and thus are potentially developing into sexual alates. Lower termites pass through a variable number of larval stages before the pseudergate stage which is the main branching point for nymphal to alate sexual (imaginal) development, to neotenic replacement reproductives, or to soldiers. There is no definitive worker caste since tasks of colony maintenance are performed by larvae. In higher termites, the branch point for developmental pathways is set early in larval development, leading alternatively to the nymphal/adult line, the soldier line, and to a definitive worker caste. Shading emphasizes points where hormone titers (JH and/or ecdysteroids) differ between castes, or where JH applications bias development, principally into presoldier/soldier differentiation. (Modified from Noirot, C., **1990**. Sexual castes and reproductive strategies in termites. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 5–35; Hartfelder, K., **2000**. Arthropoda – Insecta: caste differentiation. In: Dorn, A. (Ed.), Progress in Developmental Endocrinology, vol. X, part B. John Wiley, Chichester, pp. 185–204; and Miura, T., **2001**. Morphogenesis and gene expression in the soldier-caste differentiation of termites. *Insectes Sociaux 48*, 216–223.)

wingpads during any of these decisive molts are committed to becoming either workers or soldiers.

Interestingly, the number of larval instars that an individual completes prior to committing to a reproductive or a neuter (worker or soldier) differs considerably among species. In the higher termites, this decision usually occurs during the first or second instar, while lower termites may pass through six or more precommitment molts (**Figure 5**). The general evolutionary trend appears to involve a gradual diminution of the uncommitted larval instars.

Environmental triggers of termite reproductive caste development primarily involve demographic aspects of the colony itself, as communicated through chemical signals transmitted from workers to larvae (i.e., pheromones (Noirot, 1990)). In fact, the term "pheromone" was derived from early studies of chemical communication in termites (Karlson and Lüscher, 1959). Most early work on this polyphenism focused on the differentiation of *Kalotermes flavicollis* larvae after removal of the primary reproductive pair (e.g., Lüscher, 1964; review: Wilson, 1971). Once the king or queen had been removed, larvae began developing into replacement reproductives, suggesting that levels of chemical signals from the primary pair triggered the switch between nonreproductive and reproductive development. These studies led to the now classic model of negative feedback, whereby the king and queen each secrete compounds that repress the development of replacement reproductives of their respective sex (Lüscher, 1964), possibly via the neuroendocrine system (Lüscher, 1976).

However, recent critical revision has called this classic model into question (Pasteels and Roisin, 2001), and it is now clear that if it does apply, it does so only in the lower termites. There is little evidence for pheromonal inhibition of reproductive development in the higher termites, where this developmental option is extremely rare, and

restricted to only one or a few early larval instars (Noirot, 1990).

In addition, termite colonies annually produce large numbers of winged reproductives in the presence of the king and queen (and presumably in the presence of their pheromonal secretions). Large numbers of these "alates" gather inside colonies and disperse en masse in response to external environmental triggers such as torrential rains at the onset of the wet season. Because this switch from nonreproductive to reproductive development occurs in the presence of king and queen pheromones (i.e., it is not inhibited by these compounds), and appears instead to be triggered by external seasonal cues, the switch to reproductive development must involve not one, but at least two different environmental triggers, and very likely two different polyphenic mechanisms.

Unfortunately, the hormonal control of termite reproductive development is not well understood. In part this is due to the complexity of the polyphenism (many different "switches" are involved, and several of these have multiple critical periods spread over several subsequent molts), and in part due to the lack of sensitive bioassays for the activity of termite endocrine glands. The best assay to date was developed by Greenberg and Tobe (1985) to monitor *in vitro* CA activity for *Zootermopsis angusticollis*. In this species, the pheromonal secretions of the royal pair inhibited JH biosynthesis rates in larvae, suggesting a possible link between pheromonal cues and polyphenic regulation of larval development – at least for the switch between larvae and replacement kings and queens (Greenberg and Tobe, 1985). Specifically, this suggested that high levels of JH might be required for development into the reproductive form.

The endocrine regulation of seasonal alate production is more difficult to study, because production of alates depends on seasonal factors, and not just the presence or absence of the social pair. However, studies of Lanzrein *et al.* (1985) suggest that termite queens may transfer hormones to their eggs, and in this fashion affect the production of alate reproductives. Specifically, they found that in two *Macrotermes* species, queens transferred both JH and ecdysteroids to their eggs, and elevated levels of these hormones during embryonic development biased larvae towards becoming alate reproductives. Although there was considerable individual and seasonal variation in the hormone levels detected in this study, leading the authors to be very cautious in their conclusions, both these results, and those of Greenberg and Tobe (1985), suggest that high levels of JH may be associated with a switch to reproductive forms.

3.13.2.3.2. Larva to soldier polyphenism Much more effort has been put into studies of the endocrine regulation of soldier development. Soldier castes are the hallmark of termite societies. Soldiers have heavily sclerotized exoskeletons and specialized structures for colony defense, including either enlarged mandibles, or enlarged heads that squirt sticky glandular secretions capable of entangling attacking insects.

In the higher termites, soldiers develop from larvae through two successive molts. Larvae develop first into a presoldier, and then undergo a terminal molt into a soldier. As with reproductive development, termite soldier development is triggered by exposure to pheromones within the colony. For example, in *Nasutitermes lujae*, existing soldiers secrete a frontal gland substance that inhibits larvae from differentiating into new soldiers, whereas the royal pair appears to stimulate soldier development via another pheromone (Lefeuve and Bordereau, 1984; Bordereau and Han, 1986). Exposure to colony pheromones is thought to affect larval development through changes in levels of JH, as it did with reproductive development. Specifically, high levels of JH coinciding with high levels of ecdysteroid promotes differentiation of larvae first into presoldiers, and then into soldiers (review: Hartfelder, 2000). In *Macrotermes michaelseni*, increased JH titers during or after the high ecdysteroid peaks of both the presoldier or the soldier molt appear to enhance cuticle deposition in soldier head capsules and mandibles (Okot-Kotber, 1983). Such increases in JH titers have also been shown to be associated with the presence of JH binding proteins in different species of termites (Okot-Kotber and Prestwich, 1991a, 1991b).

In summary, levels of JH during at least two critical periods underlie polyphenic switching between termite castes. Elevated levels appear to set the stage for development into reproductives, and into soldiers, whereas low JH titers appear to favor development into a terminal worker caste (higher termites) or into a pseudergate (lower termites) that still remains flexible in its developmental potential. A critical and yet unresolved question in this context is how a larva can distinguish between soldier and reproductive development, given that both are triggered by high levels of JH. One possibility is that the critical periods of these polyphenisms are separated in time. High levels of JH that occur early during critical larval instar(s) (i.e., before the molting pulse

of ecdysteroids) could be biasing development into prereproductives and reproductives, whereas levels of JH present during the molting pulse of ecdysteroids may open the path to soldier development. This would suggest a synergistic interaction between JH and ecdysteroids in soldier development, as indicated by the findings of Okot-Kotber (1983).

The problem of caste differentiation in termites has gained new impetus from pioneering studies of differential gene expression in the soldier developmental pathway (Miura *et al*., 1999; Miura, 2001). These studies led to the identification of a gene with soldier-specific expression (*SOL1*) in the mandibular gland of the damp-wood termite *Hodotermopsis japonica*. This gene encodes a novel protein with a putative signal peptide, indicating that it may be a soldier-specific secretory product of this gland. The gland develops from a disc-like structure once a presoldier-differentiating molt has been induced by a high JH titer (Ogino *et al*., 1993; Miura and Matsumoto, 2000). Such studies are now capable of providing direct links from hormone titer modulation through gene expression to morphogenesis.

3.13.3. Polyphenism in the Holometabola

In contrast with the Hemimetabola, wing length polyphenism does not play a prominent role in the Holometabola. In this group, the prevalent forms of polyphenism are related either to camouflage, such as in color and wing pattern polyphenism in butterflies, to reproductive strategies, such as the development of weaponry in male stag and rhinoceros beetles, or to reproductive division of labor as in the female castes of many Hymenoptera.

3.13.3.1. Lepidoptera

3.13.3.1.1. Pupal color polyphenism Lepidopteran pupae display a remarkable crypsis; this immobile life stage is especially vulnerable to predators, and pupae rely on hiding for survival (Baker, 1970; Wiklund, 1975; Hazel, 1977; West and Hazel, 1982, 1985; Sims, 1983; Hazel *et al*., 1998). Most butterflies pupate in the soil or leaf-litter, and pupae are generally constitutively brown (West and Hazel, 1979, 1996). However, a number of species crawl out of the leaf-litter to pupate on the undersides of leaves or branches. Wandering larvae in these species encounter a variety of substrate "backgrounds," and effective pupal crypsis requires a facultative mechanism of pigment production (West and Hazel, 1979, 1982, 1996; Hazel and West, 1996; Hazel *et al*., 1998).

Over a century ago it was observed that some butterfly species switch between light and dark pupal forms (e.g., green versus brown), depending on the background substrate (Wood, 1867; Poulton, 1887; Merrifield and Pouldton, 1899). Today, numerous representatives of four lepidopteran families (Danaidae, Nymphalidae, Papilionidae, and Pieridae) are known to exhibit facultative pupal color polyphenism. Depending on the specific habitats and pupation substrates of each species, larvae couple pupal color production with exposure to a variety of external stimuli, e.g., photoperiod (Ishizaki and Kato, 1956; Sheppard, 1958, West *et al*., 1972), relative humidity (Ishizaki and Kato, 1956; Smith, 1978), background color (Wiklund, 1972; Gardiner, 1974; Smith, 1978), and background texture and substrate shape/size/geometry (Sevastopulo, 1975; Hazel, 1977; Hazel and West, 1979).

Pupal color polyphenism appears to be regulated by a threshold (Hazel, 1977; Sims, 1983). Although a continuous range of pupal phenotypes is possible (e.g., West *et al*., 1972), natural populations tend to be dimorphic for pupal color, and genetic studies indicate heritable differences among populations for the sensitivity of animals to background substrate characteristics (Hazel, 1977; Hazel and West, 1979; Sims, 1983).

Prepupal larvae pass through a "sensitive period" (West and Hazel, 1985) when environmental cues associated with pupation substrate influence the release of a neuroendocrine factor (e.g., Hidaka, 1961a, 1961b; Smith, 1978, 1980; Awiti and Hidaka, 1982; Bückmann and Maisch, 1987; Starnecker and Bückmann, 1997). Interestingly, the effect of this factor differs among the lepidopteran families studied.

In Papilionidae, release of this factor (called browning hormone) results in production of a dark (brown) pupa; inhibition of release of this factor results in a light (green) pupa, e.g., *Papilio xuthus*, *P. polytes*, *P. demoleus*, *P. polyxenes*, *P. glaucus*, *P. troilus*, *Eurytides marcellus*, and *Battus philenor* (Hidaka, 1961a, 1961b; Smith, 1978; Awiti and Hidaka, 1982; Hallffter and Edmonds, 1982; Starnecker and Haze, 1999). In contrast, in Nymphalidae (*Inachis io*), Pieridae (*Pieris brassicae*), and Danaidae (*Danaus chrysippus*), the default pupal color appears to be relatively dark (green), and release of the neuroendocrine factor (called pupal melanization-reducing factor (PMRF)) stimulates production of a light (yellow) pupa (Ohtaki, 1960, 1963; Maisch and Bückmann, 1987; Smith *et al*., 1988; Starnecker, 1997). Thus secretion of the neuroendocrine factor has opposite effects on the relative darkness of pupae.

Starnecker and Hazel (1999) extracted the respective neuroendocrine factor from ventral nerve chains

of both a nymphalid (*I. io*) and a papilionid (*Papilio polyxenes*), and injected these neurohormones into sensitive-stage larvae of both the appropriate and the opposite species. In all cases, animals responded to neuroendocrine injections in the species-appropriate way. For example, nymphalid larvae responded to injection of either PMRF (appropriate) or browning factor (inappropriate) by production of light pupae, while papilionid larvae responded to injection of these same substances by production of dark pupae. Cross-reactivity of these neuroendocrine factors suggests that they are in fact the same factor, and indicates that nymphalid and papilionid butterflies may have independently evolved the capacity to facultatively regulate pupal color (Starnecker and Hazel, 1999). Interestingly, although these butterfly lineages appear to have co-opted the same neuroendocrine factor, they coupled it with downstream processes of pigment synthesis that are very different (Starnecker and Hazel, 1999).

3.13.3.1.2. Seasonal wing pattern polyphenism in butterflies A multitude of butterfly species display seasonal polyphenisms for wing pattern and color. Here two of the more common, and better characterized, of these forms of wing pattern polyphenism (light versus dark hindwings and presence or absence of ventral forewing eyespots) are reviewed.

3.13.3.1.2.1. Light versus dark hindwings The most common form of butterfly wing polyphenism involves the overall lightness or darkness of wings. Numerous species in at least four lepidopteran families are characterized by distinct seasonal light and dark forms, and where it has been studied, this seasonal variation in wing pigmentation results from larval sensitivity to both photoperiod and temperature (Weismann, 1875; Süffert, 1924; Müller, 1955, 1956; Aé, 1957; Watt, 1968, 1969; Reinhardt, 1969; Hoffman, 1973, 1978; Shapiro, 1976; Endo and Funatsu, 1985; Endo and Kamata, 1985; Endo *et al*., 1985, 1988; Kingsolver, 1987; Koch and Bückmann, 1987; Kingsolver and Wiesnarz, 1991; Smith, 1991; Nylin, 1992; Jacobs and Watt, 1994).

Wing darkness is known to affect the solar absorption and thermal characteristics of butterflies (Watt, 1968, 1969; Kingsolver and Watt, 1983; Kingsolver and Wiesnarz, 1991; Jacobs and Watt, 1994), and natural selection for physiological performance under seasonally variable temperature regimes likely underlies many of these wing-darkness polyphenisms (Kingsolver, 1987, 1995a, 1995b; Kingsolver and Wiesnarz, 1991). Animals with dark wings are effective at absorbing solar radiation (Watt, 1968, 1969; Kingsolver and Watt, 1983; Kingsolver, 1987). These animals warm quickly, and perform well under cool conditions (e.g., spring), but these same animals are prone to lethal overheating under warmer (e.g., summer) conditions. Most butterfly species with light–dark polyphenisms produce dark forms when animals are reared under short days and cool temperatures (i.e., animals that will emerge in the spring), and lighter forms when they develop under longer days and warmer conditions.

To date, five species have been examined for endocrine regulation of wing melanism polyphenism: the lycaenid *Lycaena phlaeas* (Endo and Kamata, 1985), the papilionid *Papilio xuthus* (Endo and Funatsu, 1985; Endo *et al*., 1985), and the nymphalids *Polygonia c-aureum* (Fukuda and Endo, 1966; Endo, 1984; Endo *et al*., 1988), *Araschnia levana* (Koch and Bückmann, 1985, 1987), and *Junonia (Precis) coenia* (Rountree and Nijhout, 1995).

Although these species represent three families, and they differ in the direction of the polyphenism (i.e., which morph is induced under each set of conditions), and in whether or not the polyphenism is tied with facultative induction of diapause, the underlying physiological mechanisms show many similarities. All of these polyphenisms respond to both photoperiod and temperature, and although each factor can influence wing pattern expression independently, the effects of these environmental variables are additive in all cases. All of these polyphenisms involve critical periods of hormone sensitivity that occur early in the pupal period, and all but one involve the relative timing of the rise in ecdysteroids: when ecdysteroid levels are low during the critical period (i.e., when the pupal peak in ecdysteroid levels starts after the polyphenism sensitive period), then the default wing morph develops (e.g., *L. phlaeas* (Endo and Kamata, 1985); *A. levana* (Koch and Bückmann, 1987); *P. coenia* (Rountree and Nijhout, 1995); and probably *P. c-aureum*, (Endo *et al*., 1988) (see also Rountree and Nijhout (1995)). When the ecdysteroid pulse is induced to start earlier, then high levels of 20E are present during the critical period, and animals switch to production of the alternate morph (**Figure 6**).

The link between external stimulation (e.g., exposure to temperature) and the timing of ecdysteroid secretion involves the brain, and secretion of neurohormones – either prothoracicotropic hormone (PTTH) (see **Chapter 3.2**) (presumed for *A. levana* (Koch and Bückmann, 1987) and *P. coenia* (Rountree and Nijhout, 1995)), or another neurohormone called summer morph producing hormone (SMPH) (Fukuda and Endo, 1966; Endo and Funatsu, 1985; Endo and Kamata, 1985), that has

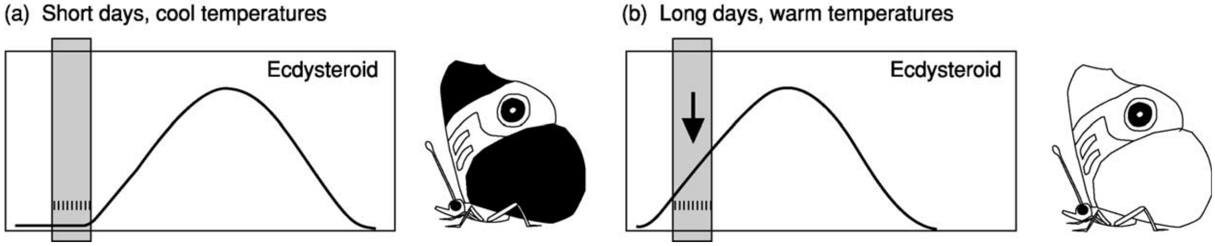


Figure 6 Seasonal wing color polyphenism in butterflies, as exemplified in *Junonia (Precis) coenia,* represents the outcome of an interaction between allelic differences (genetic polymorphism for wing color) and environmental conditions. (a) Larvae exposed to a short-day photoperiod and low temperature regime secrete low levels of a small prothoracicotropic hormone (PTTH)-like neurohormone at the onset of the pupal phase. Consequently, the pupal ecdysteroid peak builds up late and reaches threshold levels only after the critical period (grey bar). This permits the expression of dark pigments in the developing wings which are, thus, adapted for higher solar absorption. (b) In contrast, exposure of larvae to a long-day photoperiod and high temperatures leads to an early and enhanced PTTH release, and consequently above-threshold ecdysteroid levels during the critical period. These conditions favor the expression of the light-colored, alternative wing phenotype. Based on data by Masaki, T., Endo, K., Kumagai, K., **1988**. Neuroendocrine regulation of development of seasonal morphs in the Asian comma butterfly, *Polygonia c-aureum* L.: is the factor producing summer morphs (SMPH) identical to the small prothoracicotropic hormone (4K-PTTH)? *Zool. Sci. 5*, 1051–1057; Rountree, D.B., Nijhout, H.F., **1995**. Genetic control of a seasonal morph in *Precis coenia* (Lepidoptera, Nymphalidae). *J. Insect Physiol. 41*, 1141–1145, and other references cited in the text.

structural similarities to both bombyxin and small PTTH (Masaki *et al*., 1988; Rountree and Nijhout, 1995). Extirpation of brains in animals prevents release of these neurohormones, and results in complete production of the default morph (e.g., Fukuda and Endo, 1966; Endo and Funatsu, 1985; Endo and Kamata, 1985; Endo *et al*., 1988; Rountree and Nijhout, 1995). However, injection of ecdysteroids as these brainless animals pupate (i.e., prior to the critical period) can restore the polyphenism, and switch animals to the alternate form (Endo and Kamata, 1985; Koch and Bückmann, 1987; Rountree and Nijhout, 1995).

These results suggest the following model for light–dark polyphenism in butterflies (summarized for *P. coenia* in **Figure 6**): environmental stimuli affect the timing of ecdysteroid secretion indirectly, via neurohormone secretion by the brain. Larvae exposed to warm temperatures and long days produce high levels of neurohormone. High neurohormone levels stimulate early secretion of ecdysteroids, and early secretion of ecdysteroids switches animals to production of the alternate (generally the lighter) morph.

3.13.3.1.2.2. Presence/absence of eyespots Some butterfly species exhibit seasonal polyphenism for pattern elements, in addition to, or instead of, seasonal variation in overall levels of melanization (Condamin, 1973; Shapiro, 1976; Brakefield and Larsen, 1984; Brakefield and Reitsma, 1991; Roskam and Brakefield, 1999). A striking example occurs in the satyrid *Bicyclus anynana*, which exhibits a wet versus dry season polyphenism for presence (and size) of eyespots (Brakefield and Reitsma, 1991; Windig *et al*., 1994; Kooi *et al*., 1996; Brakefield *et al*., 1998). Wet-season animals express pronounced ventral wing eyespots that are reduced or absent in dry-season animals.

Ventral eyespots are thought to aid animals in surviving attacks by avian predators because they misdirect the aim of the predator (Brakefield and Larsen, 1984; Wourms and Wassermann, 1985; Windig *et al*., 1994). Wet-season animals are active (feeding, mating, and ovipositing), and are regularly exposed to avian predators, and it is presumed that these animals survive better with pronounced eyespots. Dry-season animals are almost completely dormant, and escape predators through crypsis. These animals achieve better crypsis if they do not produce conspicuous markings such as eyespots (Brakefield and Larsen, 1984; Brakefield and Reitsma, 1991; Windig *et al*., 1994).

Eyespot polyphenism in *B. anynana* results from larval sensitivity to temperature (Brakefield and Reitsma, 1991; Kooi *et al*., 1994; Windig *et al*., 1994; Brakefield and Mazotta, 1995; Kooi and Brakefield, 1999). Late-stage larvae exposed to temperatures above 23 °C produce the “wet-season” pattern with large eyespots. Larvae exposed to temperatures below 19 °C produce the “dry-season” form with tiny or no eyespots (Brakefield and Mazotta, 1995; Brakefield *et al*., 1998). Interestingly, although natural populations of these butterflies are dimorphic, this developmental mechanism (like the light–dark polyphenism described in the previous section, incidentally) does not seem to involve a threshold: intermediate temperatures lead to intermediate eyespot sizes, and animals reared in the laboratory can be induced to produce a continuous

range of wing patterns (Windig *et al.*, 1994). Instead, dimorphism appears to arise from distinct seasonal temperature regimes encountered by sequential generations of larvae in the wild (Windig *et al.*, 1994).

Temperature-induced variation in eyespot size appears to be mediated by the timing of the pupal pulse of ecdysteroids (Koch *et al.*, 1996; Brakefield *et al.*, 1998). Just as with the light–dark polyphenism in *A. levana* and *P. coenia*, animals have a hormone-sensitive period during the first few days of the pupal period. The default developmental pathway appears to be a pupal pulse of ecdysteroids that starts after this critical period. When ecdysteroid levels are low during the sensitive period, no eyespots form (the dry-season form). Wet-season (warm-temperature) animals have an earlier pulse of ecdysteroids that precedes the sensitive period, and the presence of ecdysteroids during this period results in the production of large eyespots (Koch *et al.*, 1996; Brakefield *et al.*, 1998).

Genetic strains of *B. anynana* that have been selected for continuous expression of the no-eyespot morph have late pulses of ecdysteroids identical to those of the cool-temperature-induced dry-season animals (Koch *et al.*, 1996; Brakefield *et al.*, 1998). However, these genetically eyespotless animals can be induced to produce eyespots by injections of 20E prior to the sensitive period (Koch *et al.*, 1996). These results all point to a mechanism where polyphenic expression of butterfly eyespots results from a coupling of larval exposure to temperature with variation in the timing of secretion of ecdysone during the first 2 days of the pupal period (**Figure 7**).

Butterfly eyespots have served as a model system for characterizing the developmental control of pattern formation (Kühn and von Engelhardt, 1933; Nijhout, 1985, 1986, 1991; French and Brakefield, 1995; French, 1997; Brunetti *et al.*, 2001; Beldade and Brakefield, 2002; McMillan *et al.*, 2002), and recent genetic experiments provide exciting glimpses into the patterns of gene expression that underlie eyespot formation in general, and temperature-sensitive modulation of eyespot size in particular.

Butterfly eyespots result from a patterning mechanism that takes place in the wings of late-larval and early stage pupae (Nijhout, 1985, 1986, 1991; French and Brakefield, 1995; French, 1997; Brunetti *et al.*, 2001; Beldade and Brakefield, 2002). Briefly, the eyespot consists of a series of concentric pigmented rings around an organizing center, or focus. Cells in the focus of an eyespot are critical for normal formation of the eyespot: if these cells are ablated at the end of the larval period, eyespots fail to form (Nijhout, 1980, 1991; Nijhout and Grunert, 1988). Likewise, if foci cells are transplanted, they can induce ectopic eyespots in other parts of the developing wing (Nijhout, 1991; French and Brakefield, 1995; Brakefield *et al.*, 1996). It appears that foci establish the eyespot by secreting a diffusable chemical morphogen into the surrounding epidermis, and that this signal is interpreted by the surrounding cells in a way that affects the color of the pigment synthesized; gradient contours in the diffusable substance interact with the relative sensitivities of the surrounding cells to generate the concentric rings of the eyespot.

Although the identity of the morphogen released from eyespot foci is not yet known, recent genetic studies using antibodies for *Drosophila* patterning genes have identified several signaling molecules and transcription factors expressed in the appropriate areas, and at the appropriate times (Carlin *et al.*, 1994; Brakefield *et al.*, 1996; Keys *et al.*, 1999; Weatherbee *et al.*, 1999; Brunetti *et al.*, 2001; Beldade and Brakefield, 2002). For example, *distal-less*, *engrailed*, and *spalt* all have circular regions of expression that correspond with the position of eyespots (Brakefield *et al.*, 1996; Keys *et al.*, 1999; Brunetti *et al.*, 2001; Beldade *et al.*, 2002) and their expression occurs at the time of eyespot pattern formation (late-larval and early pupal period). All three genes are expressed together in the organizing focus of the eyespot, but they are expressed separately in the surrounding color rings, lending credence to the idea that they may in some way contribute to the patterning of the rings themselves (Brunetti *et al.*, 2001; Beldade and Brakefield, 2002).

Brakefield and colleagues have taken the next step by linking expression patterns of these genes – *distal-less* in particular – with quantitative variation in eyespot size. Genetic lines selected for large and small eyespots differ in the relative size of the eyespot focus, as measured by the region of *distal-less* expression (Monteiro *et al.*, 1994, 1997; Beldade and Brakefield, 2002; Beldade *et al.*, 2002). Beldade *et al.* (2002) then showed that genetic variants of *distal-less* expression cosegregate with interindividual variation in eyespot size.

Combined, these studies suggest an explicit model for the developmental basis of variation in eyespot size (after Beldade and Brakefield (2002)): differences in the relative amount of growth of the organizing focus lead to differences in the size of the focus at the time of pattern formation. This translates into the strength of the signal emitted from this organizing center, and results in relatively larger or smaller eyespot diameters. Plasticity in the expression of eyespot size could then result from a coupling of the amount of growth of the organizing

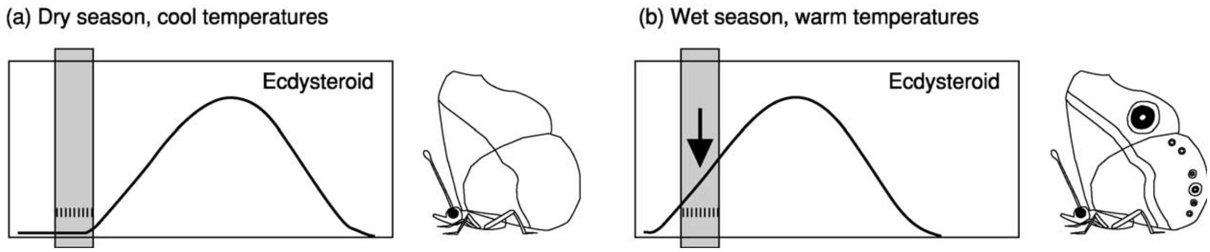


Figure 7 Eyespot size polyphenism as exemplified in the tropical butterfly *Bicyclus anynana*. (a) The less active dry-season (low temperature) morph has no or tiny eyespots on the ventral side of the forewings, as a result of a low ecdysteroid titer during the critical period (shaded bar) at the onset of pupal development. (b) In contrast, the wings of the wet-season (high temperature) morph develop large eyespots in response to an above-threshold ecdysteroid titer during this critical period. In the wing discs of last instar larvae, eyespot focal cells express the Distal-less protein. The earlier pupal ecdysteroid peak causes enhanced synthesis of dark pigment from these focal centers and consequently generates large eyespots in the wet-season morph. Based on data and models by Brakefield, P.M., Reitsma, N., **1991**. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecol. Entomol. 16*, 291–304; Kooi, R.E., Brakefield, P.M., Schlatmann, E.G.M., **1994**. Description of the larval sensitive period for polyphenic wing pattern induction in the tropical butterfly *Bicyclus anynana* (Satyrinae). *Proc. Exp. Appl. Entomol. 5*, 47–52; Brakefield, P.M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P.J., *et al.*, **1996**. Development, plasticity and evolution of butterfly eyespot patterns. *Nature 384*, 236–242; Brakefield, P.M., Kesbeke, F., Koch, P.B., **1998**. The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *Am. Nat. 152*, 853–860; Koch, P.B., Brakefield, P.M., Kesbeke, F., **1996**. Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly *Bicyclus anynana* (Lepidoptera: Satyridae). *J. Insect Physiol. 42*, 223–230; Koch, P.B., Merk, R., Reinhardt, R., Weber, P., **2003**. Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly *Precis coenia* (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. *Devel. Genes Evol. 212*, 571–584; Brunetti, C., Selegue, J.E., Monterio, A., French, V., Brakefield, P.M., *et al.*, **2001**. The generation and diversification of butterfly eyespot patterns. *Curr. Biol. 11*, 1578–1585; and Beldade, P., Brakefield, P.M., **2002**. The genetics and evo-devo of butterfly wing patterns. *Nature Rev. Genetics 3*, 442–452. For further references see text.

focus with the amount of ecdysteroid present during the patterning period (Koch *et al.*, 2003). Wet-season (higher temperature) animals would have early rises in pupal ecdysteroid levels, stimulating growth of large eyespot foci, and resulting in correspondingly large adult eyespots; whereas dry-season (lower temperature) animals would have later rises in pupal ecdysteroid levels, less hormone present during the sensitive period, less growth of the eyespot foci, and relatively smaller final eyespots (**Figure 7**).

Interestingly, not all eyespots are plastic – even within the same individual. In *B. anynana* ventral forewing eyespots are exquisitely sensitive to the larval thermal environment, but dorsal eyespots are not. Dorsal eyespots are expressed in all individuals, and display no detectable plasticity. Brakefield *et al.* (1998) propose that this difference in the environmental sensitivity of expression of dorsal and ventral eyespots results from the presence of ecdysteroid receptors (see **Chapter 3.5**) in the foci of ventral eyespots, and the absence of these same receptors in the focal cells of the dorsal eyespots. Thus patterns of ecdysone receptor expression may underlie the coupling of an adult wing pattern element expression (eyespots) with seasonally variable components of the larval environment (temperature).

3.13.3.2. Coleoptera: Male Dimorphism for Weaponry

Males of many species face intense competition from rival males over access to reproduction (Darwin, 1871; Thornhill and Alcock, 1983; Andersson, 1994). In these species, it is not uncommon for dominant and subordinate individuals to adopt different behavioral tactics: dominant (generally large) males fight to guard territories frequented by females, or display or sing to attract females, while subordinate (generally smaller) males of these same species adopt less aggressive alternative tactics, such as sneaking up to, or mimicking, females (Darwin, 1871; Austad, 1984; Dominey, 1984; Gross, 1996; Iguchi, 1998; Shuster, 2002).

In the most extreme cases, large and small males differ in morphology as well as reproductive behavior. Rarely, these alternative male “morphs” result from allelic differences among the male types (e.g., Shuster, 1989; Zimmerer and Kallmann, 1989; Ryan *et al.*, 1990; Lank *et al.*, 1995). However, the majority of male dimorphisms appear to result from polyphenic developmental processes that switch among alternative phenotypic possibilities depending on larval growth, and/or the resulting body size attained by each individual (e.g., Kukuk, 1996; Eberhard, 1982; Goldsmith, 1985, 1987; Rasmussen, 1994; Clark, 1997; Tomkins, 1999). Males encountering

favorable conditions grow large, and produce one morphology, while genetically similar (e.g., sibling) individuals encountering poor conditions remain small, and produce an alternative morphology. These male-dimorphic polyphenisms are characterized by a relatively abrupt switch between morphs that corresponds with a critical, or threshold, body size (e.g., Diakonov, 1925; Kukuk, 1966; Cook, 1987; Danforth, 1991; Eberhard and Gutierrez, 1991; Rasmussen, 1994; Kawano, 1995; Tomkins and Simmons, 1996; Iguchi, 1998).

To date, only one male dimorphism that we are aware of has been characterized physiologically: the alternative horn morphologies of the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). This is a European species that has been introduced into both Australia and the USA, where it is now an abundant inhabitant of horse and cow manure (Fincher and Woodruff, 1975; Tyndale-Biscoe, 1996).

Beetles fly into fresh manure pads and excavate tunnels into the soil below. Females dig the primary tunnels, and spend a period of days pulling fragments of dung to the ends of these tunnels, where they pack them into oval masses called "brood balls" (e.g., Fabre, 1899; Hallffter and Edmonds, 1982; Emlen, 1997; Moczek and Emlen, 2000). A single egg is laid inside each brood ball, and larvae complete their development in isolation within these buried balls of dung (Fabre, 1899; Main, 1922; Emlen and Nijhout, 1999, 2001).

Male behavior revolves around methods of gaining entry to tunnels containing females. Large males fight to guard tunnel entrances, while smaller males sneak into these tunnels on the sly (Emlen, 1997; Moczek and Emlen, 2000). Large males produce a pair of long, curved horns that aid them in contests over tunnel occupancy (Moczek and Emlen, 1999). Smaller males dispense with horn production altogether.

Both overall body size and male horn length are sensitive to the larval nutritional environment (Emlen, 1994; Hunt and Simmons, 1997; Moczek and Emlen, 1999). Specifically, the amount and quality of larval food predictably influence the final body size and horn lengths of males (Emlen, 1994; Hunt and Simmons, 1997; Moczek and Emlen, 1999). Controlled breeding experiments, artificial selection experiments, population comparisons, and "common garden" experiments all suggest that horn expression is regulated by a threshold mechanism, and that natural populations contain measurable levels of additive genetic variation for the body size threshold, i.e., the size associated with the switch between horned and hornless morphologies (Emlen, 1996; Moczek *et al*., 2002). Somehow, then, the relative amount of growth of the horns must be influenced by the overall growth – or body size – attained by each animal. Specifically, this polyphenism appears to involve a reprogramming of animals that fall beneath a genetically mediated threshold body size, so that in these animals, growth of the horns is reduced.

Beetles pass through three larval instars before molting into a pupa (Main, 1922; Emlen and Nijhout, 1999). Larvae feed on dung supplies that are provided in the brood ball, and gain weight steadily. When these food supplies are depleted, a stereotyped series of events is commenced which ultimately results in the metamorphic molt from larva to pupa (Emlen and Nijhout, 1999). The cessation of feeding appears to trigger a rapid drop in JH titers analogous to the drop that occurs with attainment of a critical size for metamorphosis in *Manduca sexta* (Nijhout, 1994). Animals stop feeding, they begin to purge their gut, they begin forming a mud/fecal shell that will protect them as pupae, and the imaginal structures (legs, wings, genitalia, horns) begin growth.

A combination of hormone-application perturbation experiments (JH) and radioimmune assays of hormone titer profiles (ecdysteroids) revealed two critical periods relevant to this developmental polyphenism. Perturbation of hormone levels during either of these sensitive periods influences the expression of male horns, though the effects of hormone application at these two times are distinct (Emlen and Nijhout, 1999, 2001). The first of these critical periods occurs at the end of the feeding period, before initiation of the metamorphic molt. Animals at this time have attained their largest sizes, and larval weight during this period exactly predicts patterns of male horn expression: male larvae with sustained weights equal to or heavier than 0.12 g end up producing horns, whereas male larvae not sustaining this critical weight end up not producing horns (Emlen and Nijhout, 2001). Furthermore, male larvae with weights beneath this critical size have a small pulse of ecdysteroids that is not present in larger males (**Figure 8**). These results suggest that body size is assessed at this time, and that animals beneath a critical larval weight are "reprogrammed" by a morph-specific pulse of ecdysteroids. Levels of JH appear to be involved in this size-assessment process. Topical application of the JH analog methoprene during this critical period raised the threshold body size associated with horn expression, so that methoprene-treated animals needed to attain a larger body size (heavier larval weight) for horn production than acetone-treated control animals (Emlen and Nijhout, 2001).

A second critical period occurs after the metamorphic molt has been initiated, as animals purge their guts and enter the prepupa period. This is the period of horn growth. All of the imaginal structures, including the horns, are growing rapidly at this time, and all animals have very high levels of ecdysteroids irrespective of sex or morph (Emlen and Nijhout, 2001). Here again, levels of JH appear correlated with body size, and perturbation of levels of JH influences the relative amount of growth of imaginal structures. In this case, augmentation of levels of JH (by topical application of methoprene) caused animals to produce disproportionately large horns. Specifically, it caused small, typically hornless males, to produce horns (Emlen and Nijhout, 1999).

In summary, experiments to date suggest that there are two periods during larval development when horn expression is sensitive to endocrine events. A first critical period occurs as animals attain their largest body sizes, and animals not attaining (or sustaining) a threshold size get reprogrammed by a small, morph-specific pulse of ecdysteroids at this time (**Figure 8**). Ecdysteroids affect patterns of gene transcription and are known to reprogram the developmental fates of specific structures (e.g., epidermis, imaginal discs) (review: Nijhout, 1994) (see **Chapter 3.5**)). In this case, a pulse of ecdysteroids appears to reprogram the relative sensitivity of cells destined to become the horns, so that their growth is inhibited during the second critical period, the period when all of the imaginal structures grow to their final sizes (not shown).

3.13.3.3. Hymenoptera: Caste Polyphenism and Division of Labor

Except for termite caste polyphenism, most of the examples discussed so far can easily be accomodated within the framework of classic Darwinian fitness concepts, wherein alternative phenotypes prove to be intimately related to alternative reproductive strategies. In principle, each of the selective environments encountered by insects (e.g., spring versus summer) favors a different optimal phenotype, and individuals maximize their reproductive success by expressing the appropriate morphology in the appropriate circumstance.

Caste polyphenisms, in contrast, are associated with a severe reduction in fertility of the subdominant (worker) caste. In fact, workers in most social insect colonies never reproduce. Social insect societies with their complex caste systems thus do not obey the rules of classical Darwinian theory, a fact that has clearly and succinctly been stated by Darwin (1859). The dilemma of inserting social insect castes into a fully acceptable Darwinian framework was only resolved by the introduction of the theory of "inclusive fitness" (Hamilton, 1964). Since then, a great deal of work has centered on the evolutionary significance of sterile worker individuals within social insect colonies, and in

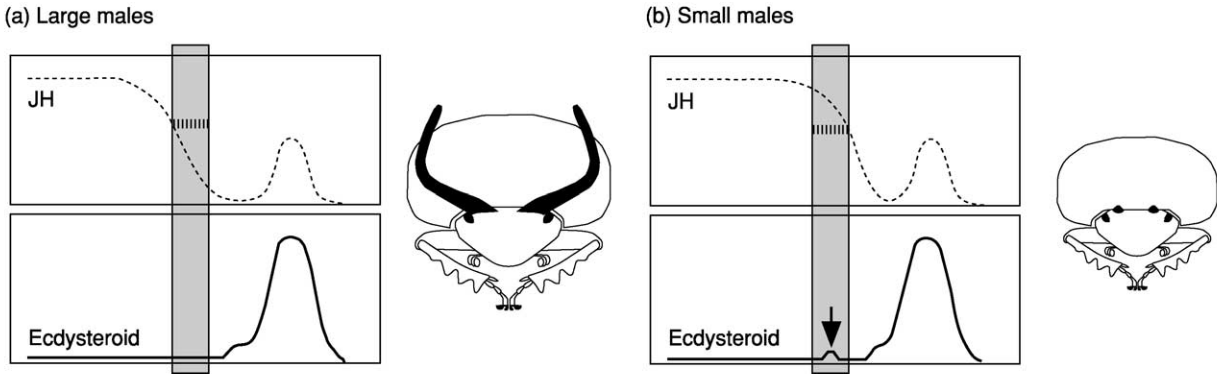


Figure 8 Hormonal control of horn length in males of the dung beetle *Onthophagus taurus*. Horn length is associated with a male's capacity to defend the entry tunnel to a burrow of an egg-laying female. It follows a nonisometric (sigmoid) relationship with respect to variation in male size. Growth of the horn morphogenetic field is determined during two hormone-sensitive periods in the last larval instar. The first one (shaded bars) occurs at the end of the feeding period when the larvae have reached their maximal weight. (a) In male larvae that passed the critical weight limit, the JH titer drops below a critical threshold during this first critical period. (b) In contrast, smaller larvae conserve an elevated JH titer and exhibit a small ecdysteroid peak which is supposed to reprogram the horn morphogenetic field so as not to permit horn growth during metamorphosis. The second critical period occurs during the prepupal phase (not shown) when the elevated JH titer promotes horn growth in the large males only. Graph based on studies by Emlen, D.J., **1994**. Environmental control of horn length dimorphism in the beetle *Onthophagus acuminathus* (Coleoptera: Scarabaeidae). *Proc. Roy. Soc. B 256*, 131–136; Emlen, D.J., Nijhout, H.F., **1999**. Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J. Insect Physiol. 45*, 45–53; and Emlen, D.J., Nijhout, H.F., **2001**. Hormonal control of male horn length dimorphism in *Onthophagus taurus* (Coleoptera: Scarabaeidae): a second critical period of sensitivity to juvenile hormone. *J. Insect Physiol. 47*, 1045–1054.

terms of ultimate explanations, the principal framework of caste evolution in social insects has now become firmly established.

What remain to be elucidated are (1) the proximate mechanisms that drive an individual insect to become a sterile worker or a full reproductive, and especially (2) the evolution of these proximate mechanisms, i.e., the evolution of developmental pathways that generate the distinct queen/worker and also the soldier phenotypes. Even though consistent in argumentation, most attempts to describe evolutionary tendencies in caste development are notorious for their lack of connection to physiological mechanisms, especially hormonal regulation, underlying the divergent differentiation pathways for the caste phenotypes. Only if it becomes possible to map these physiological mechanisms onto a phylogenetic framework may a unifying picture of caste evolution emerge which satisfies both proximate and ultimate explanations.

The diversity in caste syndromes and the manifold stimuli that trigger caste differentiation remain a challenge to any unifying view of developmental regulation. In part, the root of this problem lies in the conceptual framework of caste, that is, which aspects of caste are emphasized by different authors. In the most general sense, the term "caste" is used to describe functional roles in reproduction and division of labor, such as the performance of different tasks by different members of an insect society. In a more restricted sense, caste is seen as a manifestation of preimaginal developmental diversification resulting in distinct phenotypes which are then ever more prone to perform distinct functions in division of labor. Distinct caste phenotypes are a hallmark of the highly eusocial insect societies, and in a discussion of insect polyphenism, one might tend to concentrate on this latter aspect. Yet, in terms of evolutionary explanations, that is, how such distinct phenotypes may have emerged from modifications in developmental physiologies and gene expression, it is informative to start with incipient social systems that arise from individual differences in reproductive potential. Subsequently, we will try to discern these two aspects of caste and the underlying endocrine mechanisms that govern behavioral and morphological diversification.

A first and major distinction in social insect organization and caste systems sets apart the hemimetabolous termites from the holometabolous Hymenoptera (wasps, bees, and ants). As already outlined above, termites are diplo-diploid hemimetabolans that descended from presocial cockroaches, and caste development in termites is essentially a problem of how endocrine regulation of postembryonic development can be "manipulated" to maintain immatures as a worker caste, while permitting terminal differentiation of a soldier and a reproductive caste. Sociality in the Hymenoptera, in contrast, arose from asymmetries of genetic relationship generated by the haplo-diploid system of sex determination (Hamilton, 1964), and arose several times independently. Multiple evolutionary origins of sociality makes Hymenoptera, and in particular wasps and bees, useful objects to study the environmental, genetic, and endocrine background that sets the stage for the development of the exclusively female caste phenotypes, the primary ones being queens and workers. Ants, in contrast, are all highly social, and thus are less ideally suited for comparative studies with such a focus. They are most valuable, however, when it comes to testing hypotheses on the evolution of multiple sterile female caste phenotypes, in particular, worker versus soldier development. It is important to emphasize at this point that the soldier caste in ants is a highly derived phenotype which makes its appearance only in a few genera, in contrast to the soldier caste in termites, which is one of the essential features of termite sociality.

In general terms, holometabolous caste phenotypes can be seen as a progressive fixation of roles, first in reproduction, and second in the specialization to specific tasks related to colony maintenance (Wilson, 1971). Evolutionarily, the monopolization of colony reproduction by a queen caste starts out either with asymmetries in the reproductive potential of females that are cofounders of a nest, or from the repression of the reproductive potential of offsping daughters by the mother who persists in the nest. Obviously, in the first case, reproductive dominance should become established through a series of dominance interactions between adult and potentially reproductive females, whereas in the latter case, which requires an overlap of generations, the dominant egg-laying mother may repress reproductive activity in her daughters by manipulating a preimaginal caste bias.

3.13.3.3.1. Wasps As illustratingly stated by West-Eberhard (1996), "wasps are a microcosm for the study of development and evolution" of insect sociality, and they serve as a logical baseline for endocrine studies of caste polyphenism. All social Hymenoptera evolved from wasps (Wilson, 1971), and most levels of sociality can be found within extant wasp taxa. Three of the subfamilies in the Vespidae, the Stenogastrinae, Polistinae, and Vespinae, contain social species (Carpenter, 1991), with the Polistinae being of special interest since they

represent practically the entire range of social evolution. Many of the Old World Polistinae are open-nesting wasps, where colonies can be founded by one or more mated females. In such incipient colonies, a dominance hierarchy is gradually established by aggressive interactions between the females (Sledge *et al.*, 2001). Thereafter, the dominant female signals her status to the subordinate females (Sledge *et al.*, 2001) and whenever she encounters an egg laid by another female she removes it. In such a system, foregoing reproduction with the perspective to eventually substitute the dominant female and take over the nest, has a relatively high value in the payoff matrix, since survival chances of individual nest-founding females generally are quite low.

The endocrine basis of this reproductive dominance has, so far, only been investigated in *Polistes dominulus* (formerly *gallicus*). These studies revealed a synergistic interaction between JH synthesis, ecdysteroid titer, and ovarian activity. Dominant females have a higher CA activity, and consequently a higher JH titer, than subordinate females. In addition, the higher ovarian activity in dominant females also correlates with an elevated ecdysteroid titer. JH and/or ecdysteroid treatment of subordinate females resulted in a rise in their social rank (Barth *et al.*, 1975; Röseler *et al.*, 1984, 1985; Strambi, 1990). Such an interaction in hormonal control of physiology and behavior is a general trait in insect reproduction (see **Chapter 3.9**) and should be expected also in solitary or incipiently social Hymenoptera. These studies, however, also pointed out an important gap in our comprehension of reproductive dominance, that is the distinction between the presence of an activated ovary and high social rank (Sledge *et al.*, 2001). Even when ovariectomized, some *Polistes* foundress females continued to maintain their dominance status (Röseler *et al.*, 1985). Generally these were females with large CA. A large CA volume (and thus presumed high CA activity), however, was not linked consistently to dominance, since in other cases formerly subordinate females that became dominant over ovariectomized foundresses did not have larger CA.

With increasing social complexity the choice between alternative reproductive strategies evidently becomes restricted and, in parallel, morphological caste differences become implemented and increase in degree. This is nicely illustrated in the Ropalidiini where a gradual shift towards a preimaginal caste bias has been demonstrated convincingly (Gadagkar *et al.*, 1988), together with an age-dependent pattern of task performance (temporal or age polyethism) in adult workers (Naug and Gadagkar, 1998). In the Neotropical, swarm-founding Epiponini, morphological caste phenotypes are clearly expressed, ranging from subtle differences between the extremes in a unimodal body size range, to clearly dimorphic castes, resulting from nonisometric growth in the preimaginal phase. In the latter, queens are not necessarily always the largest individuals. Careful allometric analyses suggest size-independent allometries in many of these cases, evidencing preimaginal caste determination marked by a resetting of growth parameters in critical phases of development (Jeanne *et al.*, 1995; Noll *et al.*, 1997; O'Donnell, 1998; Keeping, 2002). Such preimaginal bias of the worker caste towards subfertility and decreased mating tendency has been attributed to nutritional castration by the brood-rearing females (Hunt, 1991). Neither the Ropalidiini nor the Epiponini have been investigated with regard to endocrine system functions in caste development, and only in two species has hormonal control in division of labor among the adult wasps been addressed. The results are of interest as they indicate a split in reaction patterns. In the primitively social *Ropalidia marginata*, an elevated JH titer stimulated egg development (Agrahari and Gadagkar, 2003), whereas in the socially more advanced *Polybia occidentalis*, methoprene accelerated behavioral development in workers (O'Donnell and Jeanne, 1993), similar to what is observed in honeybees (Robinson and Vargo, 1997).

3.13.3.3.2. Ants Ants (and termites) are an apex in caste complexity, and thus also a challenge to any unifying hypothesis on caste development and function. In this review, only a glimpse into the ant empire of behavioral and developmental diversity is provided. Luckily, we can direct any reader interested in a more detailed presentation on ant biology to a masterpiece in scientific literature written by Hölldobler and Wilson (1990). Prior to any further discussion it is important to emphasize that there are no solitary or primitively social ants. Possibly related to a major switch in foraging biology from flying to walking in search of prey, a Mesozoic sphecoid wasplike ancestor ant appears to have been exposed to relaxed selective constraints on the aerodynamics of body shape, making possible a wide variation in worker ant morphologies. This resulted in an eminent wing polyphenism between queens and workers which is present already in the earliest ant fossils (Wilson, 1987). Many early studies on ant sociobiology capitalized on such variation in form, analyzing size allometries in relation to functions performed by colony members and in relation to phylogenetic patterns (reviews: Wilson, 1971; Hölldobler and Wilson, 1990; Wheeler,

1991). Yet, even though there is a wealth of data on complex trait allometries in ants, there still appears to be a lack of information linking these data to rules of how changes in shape are actually generated in development, and how these specific developmental pathways have evolved in the colony context (Tschinkel, 1991; Tschinkel *et al*., 2003).

3.13.3.3.2.1. The queen/worker decision As in all highly social insects, ant queens differ markedly from workers. They are generally bigger, have a fully developed reproductive system, and have a well-developed flight apparatus for dispersal. Even though this queen/worker distinction is an ancestral character present in all ant species, we still have very limited knowledge as to how these caste differences arise ontogenetically. Many of the early studies on mechanisms governing queen production in ants were carried out on temperate climate species, and these indicated strong seasonal triggers of queen production (review: Wheeler, 1986). In many species, queens only are produced from overwintered eggs or larvae, illustrating a requirement for a state of diapause in the development of queens (see **Chapter 3.12**).

For some ants, the critical period for the queen/worker decison has been studied by JH application experiments. For example, topical application of JH to larvae developing from queen-biased overwintered brood in *Myrmica rubra* increased the number of queens (Brian, 1974). In *Aphaenogaster senilis*, application of JH caused the appearance of queen-like workers (Ledoux, 1976), and this same pattern occurred in the Argentinian fire ant, *Solenopsis invicta* (Vinson and Robeau, 1974). In the latter species, metamorphosis was delayed significantly by the application of JH, and the “queenlike” appearance of workers was initially attributed to a simple increase in worker size (associated with the delay in metamorphosis, and extended period of larval feeding), rather than to a direct effect of JH on queen production (Wheeler, 1990).

Ecdysteroids also have been implicated in divergent queen/worker development. In *Plagiolepis pygmea*, worker-biased larvae have higher ecdysteroid titers during the last larval instar than queen-biased larvae (Suzzoni *et al*., 1983).

In all examples cited so far, caste development is overwhelmingly dependent on environmental factors. Yet there is increasing evidence for a genetic basis to the determination of queen/worker polyphenism in at least some species of ants. Such genetic factors (allelic differences between queens and workers) were originally proposed for the slave-making ant *Harpagoxenus sublaevis* (Winter and Buschinger, 1986), but have recently also been demonstrated for a *Camponotus* (Fraser *et al*., 2000) and two *Pogonomyrmex* species (Julian *et al*., 2002; Volny and Gordon, 2002). How these alleles interact with the endocrine system to direct caste development remains to be explored.

Environmental triggers of ant caste development primarily involve pheromonal signals within the colony itself. Pheromonal regulation of ant reproduction has been studied primarily in *S. invicta*, which has completely sterile workers due to the lack of functional ovaries (Hölldobler and Wilson, 1990). Virgin *Solenopsis* queens normally leave on a mating flight, and thereafter shed their wings, activate their ovaries, and soon initate egg-laying. If prevented from flying, these queens keep their wings and maintain their ovaries in an inactive state, and animals are prevented from maturing their ovaries as long as an egg-laying queen is present in the nest (Fletcher and Blum, 1981). This integrated behavioral and physiological response in *Solenopsis* virgin queens has led to the identification of a pheromone produced by the poison gland of the dominant queen which inhibits precocious dealation and ovary activation in virgin queens as long as they are in the nest (Fletcher and Blum, 1983). The pheromone supposedly maintains high brain dopamine levels (Boulay *et al*., 2001) which are thought to suppress CA activity (Vargo and Laurel, 1994; Burns *et al*., 2002). Low CA activity in turn prevents vitellogenin uptake by the ovary. Vitellogenin synthesis appears to occur independently of JH (Vargo and Laurel, 1994), and the decision to initiate oogenesis or not seems to be taken by the activation of receptor-mediated vitellogenin uptake. In *Camponotus festinatus*, such a regulation has also been shown to prevent ovary activation in queenless workers (Martinez and Wheeler, 1991).

The species considered so far all belong to a large group of ants which do not exhibit a marked polyphenism within the worker caste. Instead, they all have a worker caste with unimodal or only slightly bimodal size frequency distributions. In these species, the principal polyphenism is between queens (reproductives) and workers (nonreproductives). In contrast, the genus *Pheidole* is characterized by multiple polyphenisms: between queen and worker, and also between different types of workers (including true soliders). In fact, the genus *Pheidole* exhibits the most spectacular polyphenism known for social insect worker castes (see below). In these taxa, the queen/worker switch occurs much earlier in development than the worker/soldier switch. Queen development in *Pheidole* is determined very early in development, while animals are still embryos,

and appears to be determined largely by hormone levels deposited by the egg-laying mothers.

Despite this change in timing, however, the roles for the two basic hormones appear the same. Queens that laid worker-biased eggs had higher ecdysteroid levels than those laying queen-biased eggs, and, correspondingly, worker-biased eggs also exhibited a higher ecdysteroid content (Suzzoni *et al.*, 1980). Furthermore, JH application to eggs increased the proportion of females developing into queens (Passera and Suzzoni, 1979). These results suggest that even during this early critical period, high levels of ecdysteroids are associated with worker development, and high levels of JH stimulate queen development.

3.13.3.3.2.2. The worker/soldier decision In contrast to the other highly eusocial Hymenoptera, division of labor within ant worker castes does not seem to be governed by age-related shifts in task performance. Rather, it is the relatively large size range of workers that underlies task preference and morphological specialization. Developmental mechanisms underlying worker caste polyphenism have been extensively reviewed (Wheeler, 1991) and mathematical models have been proposed that explicitely address the problem of growth rule reprogramming (Nijhout and Wheeler, 1994). Worker/soldier reprogramming is thought to take place during late larval instars, in response to different feeding conditions or other forms of social influence on larval growth. These, in turn, appear to impact the larval endocrine system, as a regulator of growth and the onset of metamorphosis. Studies exploring this aspect were carried out on *S. invicta*, which has a unimodal distribution of worker size (Wheeler, 1990), and on the strongly polyphenic *Pheidole bicarinata* (Wheeler, 1983, 1984).

Methoprene application to late larval instars led to an increase in size in *S. invicta* workers due to a retarded onset of metamorphosis. In *P. bicarinata*, such treatment resulted in the expression of soldier characters in the emerging brood, indicating a discrete JH-dependent developmental switch in the last larval instar. In species with a bimodal or multimodal size distribution, larvae that reach a critical size and consequently experience a different endocrine milieu can thus be shunted into alternative developmental pathways leading to overt polyphenism, generally between a soldier and a worker caste (Wheeler, 1994). Obviously, by introducing the worker/soldier decision into the late larval stages, the primary decision (queen versus worker) had to be shifted to earlier stages, as clearly occurred in the genus *Pheidole*.

While the role of JH seems to be pretty well established in *Pheidole* soldier/worker polyphenism, the primary triggering factors are still largely hypothetical. Two aspects could be relevant in this context: first, the larva/worker ratio which determines the amount of nursing activity devoted to each larva and, thus, affects body size (Porter and Tschinkel, 1985), and second, social pheromones which inhibit the development of further soldiers once a soldier level appropriate to colony size has been reached (Wheeler, 1991). In terms of colony fitness, such regulatory mechanisms represent the generalized developmental basis for the concept of adaptive colony demographies postulated by Oster and Wilson (1978).

Although our knowledge of the regulatory cascade from feeding and/or pheromonal factors to endocrine activity and consequent programming of growth parameters is still fragmentary, a landmark study on gene networks underlying wing development in ants (Abouheif and Wray, 2002) has provided remarkable insight into downstream consequences of developmental reprogramming. In the wing buds of three ant species exhibiting different degrees of polyphenism, these authors investigated expression patterns of six regulatory genes (*Ultrabithorax, extradenticle, engrailed, wingless, scalloped*, and *spalt*), all of which are functionally conserved in wing development of holometabolous insects. Cessation of wing bud development in worker-determined larvae is a characteristic element in all ants, and in species of the genus *Pheidole* this cessation can occur in two steps. *Pheidole morrisi* soldier larva develop large vestigial forewing discs but no visible hindwing discs, while worker-destined larvae develop neither of these wing discs. Of these six genes, all except for the most downstream one (*spalt*) showed correlated expression patterns for the large forewing disc in soldier-biased larvae. None of these genes, however, was expressed in the forewing disc of worker larvae, or in the hindwing discs of both soldier and worker larvae. Tracing the gene expression patterns through earlier development suggested (1) a gradual shutdown of the gene regulatory network between mid-embryogenesis and the last larval instar and (2) that this patterning network was not interrupted at a single step, as might be expected under the concept of a classical switch mechanism. Instead, this study suggested that many different points in the gene expression cascade were involved. Extending the study to other ant species with lesser degrees of polyphenism (*Neoformica nitidiventris* and *Crematogaster lineolata*) corroborated the findings from *P. morrisi*. This led Abouheif and Wray (2002) to the conclusion that

natural selection may be playing an active role in determining the most efficient route to halting wing development, and may operate directly on different genes in different species. It is a view that opens up exciting perspectives for hormonal effects on the expression of individual genes in the regulatory networks and should stimulate the search for putative hormone response elements in the upstream control region of these genes in a multispecies comparison.

3.13.3.3.2.3. Queen polyphenism, queen loss, and queenless ants, and wing dimorphism in males Notwithstanding its fascination, phenotypic diversity in the worker caste, which includes both continuous size variation and polyphenism, is restricted to only 15% of the ant genera and is mainly found in the highly derived groups. Yet ants in the subfamilies Myrmeciinae and Ponerinae, which are characterized by a number of ancestral traits and are commonly believed to be socially less complex than the “higher” ants, are by no means boring creatures. These subfamilies are of interest because they exhibit graded transitional series between queens and workers (Heinze, 1998), and workers in these ants are often not sterile: they are equipped with large ovaries and spermathecae (Crossland *et al.*, 1988; Ohkawara *et al.*, 1993; Dietemann *et al.*, 2002). In some species workers attain a queenlike morphology but contribute relatively little to colony reproduction, while in others workers contribute substantially (e.g., the colony-founding queen may be substituted by mated intercastes (Heinze and Buschinger, 1987; Peeters and Hölldobler, 1995)). In the most extreme taxa, the queen caste has been completely lost, and all reproduction is carried out by workers which are denominated gamergates (Peeters, 1991).

Since in such queenless ants all females can potentially mate and lay eggs, a reproductive conflict of interest among colony females (as is typical in primitively social insects) is a secondarily evolved consequence. These species resolve this conflict by employing a mixed strategy of aggression by a dominant female and chemical signaling (Peeters *et al.*, 1999; Liebig *et al.*, 2000; Cuvillier-Hot *et al.*, 2001; Monnin and Ratnieks, 2001).

Juvenile hormone plays an interesting role in social dominance and fertility in these queenless ants. In contrast with primitively social wasps, where the JH titer correlates positively with dominance, and especially fertility (see Section 3.13.3.3.1), application of the JH analog pyriproxyfen to queenless ants resulted in a decrease in fertility in the alpha female, and in a loss in dominance rank (Sommer *et al.*, 1993). This decline in social rank was accompanied by corresponding changes in the cuticular hydrocarbon profile (Cuvillier-Hot *et al.*, 2003). Consequently, the idea that queenless ants represent a reversion to a primitive social system appears to be a superficial oversimplification, and does not withstand a critical analysis once underlying developmental mechanisms and hormonal regulation of reproduction are taken into account. An interesting aspect in this context are the wing-disc-derived gemmae, which deserve to be studied in the context of gene regulatory networks underlying wing development in ants.

Shutdown in wing development may not be exclusive to the female sex in ants, but has recently also been shown to occur in males of *Cardiocondyla obscurior*, where a winged and a wingless male morph were found (Cremer *et al.*, 2002). Just as in workers, the loss of wings is not an isolated character state, but is part of a correlated modification in other characters, including reduced eye size. These “workerlike” males have a much prolonged spermatogenesis compared to winged conspecifics and mate exclusively in the nest. This finding is a complete novelty and should stimulate comparative analyses on physiological and genomic events underlying concurrent polyphenism in the two sexes.

3.13.3.3.3. Caste in social bees Social systems with incipient caste differences between dominant egg-laying and subordinate females have originated, multiply and apparently independently, within the Apidea (Michener, 2000). This happened most notably in the Halictinae, the Xylocopinae, and of course, the Apinae, which contain the closely related social tribes Euglossini, Bombini, Apini, and Meliponini.

The halictine bees are an enormous and abundant worldwide group of bees, yet only a few species exhibit some degree of social interaction between females, and even fewer have been studied with respect to their social biology (review: Michener, 1990b). Even in the socially most advanced halictines, caste differences between females in a nest are minimal and are mainly related to body size. Furthermore, with the loss of the dominant female, a new social rank order is established and formerly subordinate females can become dominant, mate, and lay eggs. Since caste is mainly related to adult size, it is not surprising that the size of the pollen ball deposited in the individual brood chambers is a good indicator of sex and caste (Plateaux-Quénu, 1983). Yet slight differences in size do not predict dominance, suggesting that a female’s capacity

to become a foundress is probably determined multifactorially (Yanega, 1989).

In the xylocopine bees, two tribes (Ceratini and Allodapini) present incipient social organization (review: Michener, 1990a). *Ceratina* nests are very small, containing rarely more than two females. These females may be of different size, and both are capable of laying eggs. Yet it is not necessarily the larger bee that produces the majority of offspring (Sakagami and Maeta, 1985).

In the allodapine bees, colony size may be considerably larger, with at least one Australian species reaching a colony size range approximating that of highly eusocial species (Schwarz *et al*., 2002). In the best-studied allodapine species, *Exoneura bicolor*, it is the temporal order in which females emerge that is critical to reproductive dominance (Schwarz and O'Keefe, 1991). The first female(s) to emerge are the first ones to activate their ovaries, and apparently they repress ovarian activity in their nestmates.

3.13.3.3.3.1. The primitively social bumblebees In comparison to the aforementioned bees, where females still can facultatively adopt a solitary or social strategy, sociality in the bumblebees is obligatory and colonies can become quite large. The Bombini are large bees adapted to nesting in colder climates. Virgin queens emerge in autumn, mate, and hibernate before founding a new nest in spring. These queens are long-lived and survive for the entire seasonal nest cycle. The first brood emerging in early spring consists of few rather small females (workers) that aid their mother in rearing the subsequent batches of exclusively female brood. Workers can vary considerably in size, and there is even slight overlap with queens in size–frequency distributions. During midsummer, the queen starts to lay haploid (male-producing) eggs, in addition to further female eggs. Soon after this "switch point," a few workers of high social rank also begin to lay (haploid) eggs, contributing to male production. The onset of worker reproduction has been termed the "competition point" (Duchateau and Velthuis, 1988), and marks an important incision in the colony life cycle, since from this point onwards social integration deteriorates. By the end of the season, the colony produces large queens, which disperse and subsequently mate and hibernate. These components of the annual nest cycle have best been studied in *Bombus terrestris* (Röseler, 1985), where concentrated efforts in breeding and colony-rearing programs have been made to introduce this species successfully as a pollinator in greenhouses.

Although females produced before the competition point can attain a relatively large size, they never become queens (Cnaani *et al*., 1997; Cnaani and Hefetz, 2001). This inhibition of queen production during the early stages of the colony cycle can theoretically be attributed either to "nutritional castration," that is feeding of less or lower-quality food to these larvae, or to a direct inhibitory effect from the egg-laying queen (Röseler, 1970). Long-term videorecording of feeding acts, larval food analysis, and manipulation of feeding frequency (Ribeiro, 1999; Ribeiro *et al*., 1999; Pereboom, 2000; Pereboom *et al*., 2003) have now established clearly that nurse bees do not manipulate the larvae in their feeding program, but rather respond to hunger signals given off by the larvae. So, it is obviously the intrinsic feeding program of the larvae themselves that is already defined during the early larval instars.

The decision as to which feeding program the larvae will adopt has been attributed to a queen signal, which inhibits larvae from developing into queens before the competition point in the colony cycle (Röseler, 1974). Although supposed to be a primer pheromone (Röseler *et al*., 1981), its source and nature have not yet been determined unambiguously (Bloch and Hefetz, 1999b). Meticulous studies on the endocrine response elicited in the larvae pinpointed the first and early second larval instar as the critical window for this queen signal (**Figure 9**). From the fifth day of the second instar, pre-competition-point larvae show markedly reduced levels of JH synthesis (Cnaani *et al*., 1997), as well as lower JH titers (Cnaani *et al*., 2000b), when compared to queens. This response in the CA correlates with caste-specific differences in the ecdysteroid titer (Hartfelder *et al*., 2000), indicating a synergistic interaction of the CA and prothoracic gland during the second and third larval instars. Interestingly, these hormone titer differences vanish during the feeding phase of the last larval instar, when both castes exhibit low JH and ecdysteroid titers (Cnaani *et al*., 2000b; Hartfelder *et al*., 2000). Hormonal caste differences make their reappearance during the spinning and prepupal stages (Strambi *et al*., 1984). Yet at that point titers do not exhibit quantitative modulation but only a temporal one in the position of the prepupal JH and ecdysteroid titer peaks (**Figure 9**).

Social conditions, especially the phase in the colony cycle, have a strong impact on the endocrine system, and thus on the switch from worker to queen development in the individual larvae. This was demonstrated when comparing JH release rates in larvae that were reared shortly before and shortly after the competition point (Cnaani *et al*., 2000a).

This cascade from pheromonal effects on the feeding program to associated endocrine events in

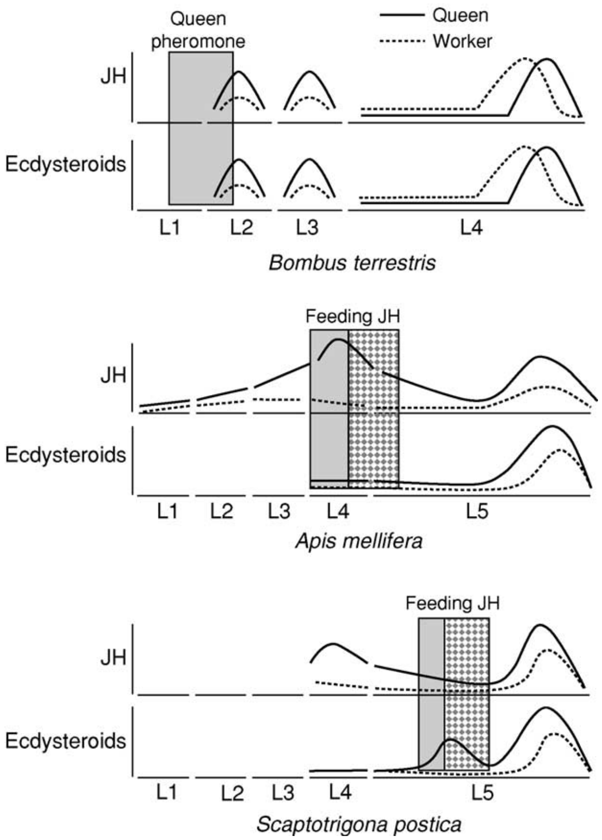


Figure 9 JH and ecdysteroid titer profiles in critical stages of caste development of the primitively eusocial bumblebee, *Bombus terrestris*, and the highly eusocial bees *Apis mellifera* and *Scaptotrigona postica*. In the primitively eusocial bumblebee, prospective queen and worker larvae differ in their JH and ecdysteroid titers during the early larval stages. Exposure during a critical period (shaded bar) to a repressor pheromone emitted by the egg-laying queen affects the hormone titers in the subsequent larval instars (L2 and L3). In the last larval instar (L4), the differences in hormone titers between queens and workers vanish and only a temporal shift in titer peaks is observed in the prepupal stage. In contrast, in the highly eusocial bees, *Apis mellifera* and *Scaptotrigona postica*, the JH and ecdysteroid titers exhibit marked differences during the late larval stages. In *Apis mellifera*, the major JH titer differences overlap with the nutritional switch from royal jelly to worker jelly in the fourth and early fifth instar (shaded bar). In *Scaptotrigona postica*, queen development is dependent on a prolonged feeding period (shaded bar) in the fifth instar. Both in *Apis* and in *Scaptotrigona*, these critical stages overlap with a JH-sensitive period (dotted bar). Morphological differences between queens and workers make their appearance in the highly eusocial bees but not in bumblebees. In the latter, queens and workers differ mainly by size and in some physiological parameters. The expression of morphological caste differences thus appears to depend on sustained hormone titer differences in the last larval instar. Hormone titer profiles for *B. terrestris* were compiled from data by Strambi, A., Strambi, C., Röseler, P.-F., Röseler, I., **1984**. Simultaneous determination of juvenile hormone and ecdysteroid titers in the hemolymph of bumblebee prepupae (*Bombus hypnorum* and *B. terrestris*). *Gen. Comp. Endocrinol. 55*, 83–88; Cnaani, J., Robinson, G.E., Bloch, G., Borst, D., Hefetz, A., **2000a**. The effect of queen–worker conflict on caste determination in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol. 47*, 346–352; and Hartfelder, K., Cnaani, J., Hefetz, A., **2000**. Caste-specific differences in ecdysteroid titers in early larval stages of the bumblebee *Bombus terrestris*. *J. Insect Physiol. 46*, 1433–1439. Data on *A. mellifera* were published by Rembold, H., **1987**. Caste-specific modulation of juvenile hormone titers in *Apis mellifera*. *Insect Biochem. 17*, 1003–1006; and Rembold, H., Czoppelt, C., Grüne, M., Lackner, B., Pfeffer, J., *et al.*, **1992**. Juvenile hormone titers during honey bee embryogenesis and metamorphosis. In: Mauchamp, B., Couillaud, F., Baehr, J.C. (Eds.), Insect Juvenile Hormone Research. INRA, Paris, pp. 37–43, as well as by Rachinsky, A., Strambi, C., Strambi, A., Hartfelder, K., **1990**. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen. Comp. Endocrinol. 79*, 31–38. Hormone titer curves for *S. postica* were adapted from Hartfelder, K., Rembold, H., **1991**. Caste-specific modulation of juvenile hormone-III content and ecdysteroid titer in postembryonic development of the stingless bee, *Scaptotrigona postica depilis*. *J. Comp. Physiol. B. 160*, 617–620. For data on critical periods and JH application experiments see text.

queen/worker differentiation does not necessarily represent a generalized chain of events in bumblebee caste polyphenism. Of the few comparative studies that have been carried out on other bumblebee species, the most noteworthy ones were on *B. hypnorum*, which was chosen for its different strategy in provisioning the larvae (Röseler, 1970). In this species, the queen/worker polyphenism involves a much later critical period. Caste fate is not determined during the early instars as it is in *B. terrestris*; instead, it is the feeding conditions in the last larval instar that appear to be the decisive factor. Yet again, the feeding differences converge in a caste-specific differential response in the endocrine system, and as in *B. terrestris*, the prepupal JH and ecdysteroid titer peaks of *B. hypnorum* workers precede the corresponding peaks in queens (Strambi *et al*., 1984).

As mentioned above, adult bumblebee workers and queens differ in size – despite some overlap in the bimodal size frequency distribution – and to some extent in their physiology, mainly in their energy metabolism (Röseler and Röseler, 1986), yet not in their capacity to lay eggs. Even in the presence of the queen, a considerable percentage of *B. terrestris* workers can exhibit signs of ovary activation. However, fewer than 40% of the workers complete oogenesis and also these latter ones do not lay eggs before the switch point in the colony cycle (Duchateau and Velthuis, 1989). Until this time point, egg-laying in workers is presumably inhibited by a queen-produced pheromone. The first studies pointing out a correlation between a queen inhibitory signal, JH production, and egg-laying by workers were carried out by Röseler (1977) and Röseler

and Röseler (1978). Reproductive workers had elevated CA activity and JH titer, relative to non-"activated" workers, and the queen inhibition of worker egg-laying could be overcome by topical application of JH. A closer look revealed the existence of a dominance hierarchy within the worker caste in both queenright and queenless colonies. Dominant workers exhibit agonistic behavior towards lower-ranking workers, resulting in decreased CA activity and inhibition of egg-laying in the latter (Röseler *et al*., 1981; Doorn, 1987).

More recent studies further explored the apparent dichotomy in worker reproduction, that is, reproduction in the absence of the queen, and worker reproduction after the competition point, yet in the presence of the queen. In small groups of queenless workers, a dominance hierarchy soon becomes established, which is reflected in elevated JH titers in the high-ranking egg-laying workers. The sequence in the response to queen removal was documented when following JH synthesis, JH titer, ecdysteroid titer, and egg development during the first 6 days in groups of queenless as compared to queenright workers (Bloch *et al*., 1996; Bloch *et al*., 2000a, 2000b). Levels of JH synthesis and JH titer were significantly elevated in workers by 3 days after removal of the queen, followed by a similar increase in ecdysteroid titer and terminal oocyte length at day 6 (**Figure 10**). This enhancement in JH release rates occurred independently of the colony cycle, that is, JH release was equally elevated in workers made queenless before and after the competition point (Bloch *et al*., 1996), giving little support to the hypothesis that (queenright) worker reproduction might be related to a decrease in queen inhibition after the competition point. Yet there was a strong correlation with worker age, since it was mainly the older workers that had elevated levels of JH release, and thus became dominant egg layers, both in queenless groups and in post-commitment-point colonies. Thus, the absence of the queen is not the *sine qua non* for worker reproduction, since individually kept workers did not initiate egg-laying and, correspondingly, maintained a low JH titer (Larrere and Couillaud, 1993). In accordance with previous studies on worker dominance hierarchies, summarized by Röseler and van Honk (1990), it appears to be the high-ranking (older) workers that inhibit JH synthesis and ovary activation in lower-ranking (younger) workers (Bloch and Hefetz, 1999b). If so, worker reproduction should be inhibited differentially during the two main phases of colony development, that is, prior to the competition point it is a queen inhibitory signal that suppresses egg development in high-ranking workers, and this acts synergistically with an inhibitory effect of dominant workers on ovary activation in the lower-ranking workers. After the competition point, it is primarily the high-ranking workers that control egg production in their

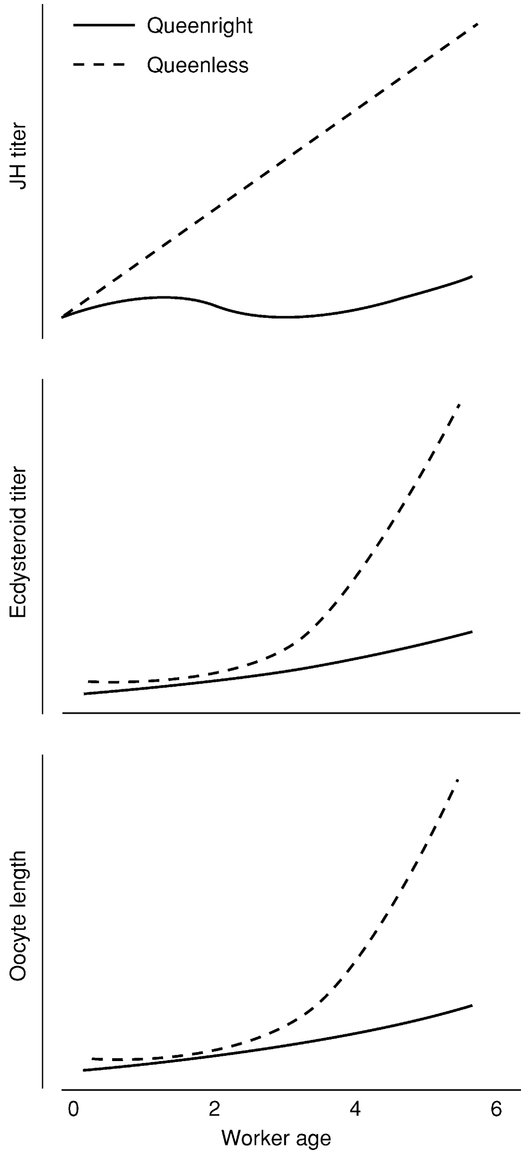


Figure 10 Queen effect on worker reproduction in *Bombus terrestris*. Removal of the queen elicits a rise in the JH titer of adult bumblebee workers, and subsequently results in a correlated response in oocyte growth and hemolymph ecdysteroid titer. This association strongly indicates that JH is involved in worker fertility, and that the presence of an egg-laying queen represses CA activity. (Modified from Bloch, G., Borst, D.W., Huang, Z.Y., Robinson, G.E., Cnaani, J., *et al*., **2000a**. Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris*. *J. Insect Physiol. 46*, 47–57; and Bloch, G., Hefetz, A., Hartfelder, K., **2000b**. Ecdysteroid titer, ovary status, and dominance in adult worker and queen bumble bees (*Bombus terrestris*). *J. Insect Physiol. 46*, 1033–1040.)

nestmates. Both of these inhibitory signals appear to be mediated through the neuroendocrine axis, particularly via JH release. The elevated ecdysteroid titer observed concomitantly with progressive oogenesis (Bloch *et al.*, 2000b) should be interpreted primarily as a consequence of JH-stimulated ovary activation. However, one cannot exclude an ecdysteroid-mediated synergistic feedback effect on dominance (just as in the *Polistes* wasps) (see Section 3.13.3.3.1).

Surprisingly, the strong correlation observed between JH titer and ovary activation in workers (Bloch *et al.*, 2000b) was not as striking in queens, which have lower JH titers than egg-laying, queenless workers (Larrere *et al.*, 1993; Bloch *et al.*, 2000a). A high JH titer is therefore not an absolute requirement for egg production, at least in queens, and may be more related to a combination of dominance status and egg production in the workers than with queen reproduction. In fact, ecdysteroids may play a more important role in queen reproduction than in worker reproduction, as evidenced by the elevated ecdysteroid titers in overwintered queens, as compared to virgin queens and queenless workers (Bloch *et al.*, 2000b). Thus it seems that in bumblebees we are confronted with the dual role of JH and ecdysteroids in female reproduction.

3.13.3.3.3.2. The highly social honeybees and stingless bees Honeybees (Apini) and their sister group Meliponini, colloquially refered to as stingless bees, have been companions of mankind for almost as long as locusts. Their origin and social organization have been narrated in picturesque myths, and their biology is a well-represented element in ethnobiology. The common themes to these myths are the nature of social integration and the origin of the differences in form and function between queens and workers. One of the main difficulties in explanations for queen/worker dimorphism, both for myths and the modern scientific approach, resides in the identification of the mode of action of the initial triggers in caste development.

As in most social insects, this is a problem of larval nutrition. In the honeybees, larvae are fed continuously during larval development, and during the early larval instars, both queen and worker larvae receive mainly a glandular proteinaceous secretion (royal jelly). In the fourth and fifth larval instar this type of food is provided in large quantities only to queen larvae, whereas worker-destined larvae are fed a mixture of glandular secretions, honey, and pollen. Apart from the switch in diet, worker larvae are also visited much less frequently than queen larvae (Beetsma, 1985). Attempts to identify specific queen-determining factors in royal jelly resulted in a partial purification of labile factors (Rembold *et al.*, 1974b), but did not provide unequivocal evidence for a "queen determinator." Rather, they suggest the necessity for a balanced diet.

This view is also consistent with results of larval food analyses in stingless bees (Hartfelder and Engels, 1989), which do not feed their brood progressively but rather mass provision the brood cells shortly after they are built. Due to mass provisioning of the brood cells and lack of further interaction of worker bees with the developing brood, other than regulation of the colony microclimate, there is no evidence for a nutritional switch in the stingless bees that could serve as a signal for queen/worker development. In the majority of the stingless bee species, that is those belonging to the highly diverse trigonine genera, queen development depends on the quantity of larval food provided to the larvae, and this is reflected in the size of the cells. Large queen cells containing two- to threefold more larval food than the worker cells are often built at the margins of the horizontal brood combs (Engels and Imperatriz-Fonseca, 1990), or can result from the fusion of two brood cells sitting on top of one another, thus providing a larva with a second portion of larval food. This strategy of queen production is also observed in the context of emergency queen rearing in some species, that is when a colony has lost its queen (Faustino *et al.*, 2002).

An exception to the queen/worker-determining mechanism by modulation of larval food quantity is the genus *Melipona*. Their brood cells are of equal size, irrespective of whether queens, workers, or males are reared within them. The fact that up to 25% of the female brood can be queens led Kerr (1950) to propose the hypothesis of a genetic predisposition with a two-locus, two-allele system, in which only double heterozygotes can become queens. Yet even in this system, larval food quality has a modulating effect on caste differentiation (Kerr *et al.*, 1966), since maximal proportions in queen production are only observed in strong colonies that have sufficiently large stocks of honey and pollen. Under suboptimal conditions the proportion of queens in the female brood is well below 25%. Even though frequently contested and still awaiting final support from genetic analyses and identification of the proposed loci, this hypothesis has a higher heuristic value than an alternative hypothesis that attributes "self-determining" capacity to the larvae (Ratnieks, 2001). Irrespective of the distinct modalities in initial triggers – nutritional switch in honeybees, large food quantities in trigonine bees, or a genetic predisposition in *Melipona*

species – all these inputs eventually converge in caste-specific activity patterns in the endocrine system which governs subsequent differentiation events in the target tissues.

3.13.3.3.3.2.1. The endocrine regulation of caste development in honeybees This endocrine cascade has been best explored in the honeybee. JH application experiments carried out in the 1970s indicated a prominent role for JH in caste development, and established a critical window for its action in the fourth and early fifth larval instar (Rembold *et al.*, 1974a; Dietz *et al.*, 1979; and further references in Hartfelder, 1990). Subsequent analyses of endogenous JH titers by specific radioimmunoassays (RIAs) and highly sensitive GC/MS corroborated these experimental JH effects, revealing a marked difference in JH titers between queen- and worker-biased larvae (**Figure 9**) during larval, prepupal, and the late pupal stages (Rembold, 1987; Rachinsky *et al.*, 1990; Rembold *et al.*, 1992). From these meticulous JH titer analyses it was possible to ask how the JH titer differences may be generated, and how these may affect caste-specific differentiation processes. Analyses of JH biosynthesis and release rates by a radiochemical assay revealed a markedly elevated CA activity in prospective queen larvae between the fourth and the early fifth larval instar (Rachinsky and Hartfelder, 1990), proving that the JH titer differences are primarily a result of differential CA activity (rates of JH degradation were generally low in honeybee development) (Mane and Rembold, 1977).

An interesting facet was the finding that the terminal steps in JH biosynthesis are the critical ones for the differential JH release rates in early fifth instar queen and worker larvae (Rachinsky and Hartfelder, 1991; Rachinsky *et al.*, 2000). Blocking the terminal steps from farnesoate to JH III is an efficient means to generate and guarantee low JH titers in worker larvae during the fourth and early fifth instar (see **Chapter 3.7**). Furthermore, since this block is reversible, as required to generate the elevated JH titers in prepupae (Rachinsky and Hartfelder, 1990, 1991), the *ortho*-methylfarnesoate transferase and the JH-epoxidase are candidate targets for allatoregulatory factors. Radiochemical assays on the CA of worker larvae showed that the *Manduca sexta* allatotropin can stimulate JH synthesis in honeybees in a dose-dependent manner, yet does not overcome the block of the terminal steps (Rachinsky and Feldlaufer, 2000; Rachinsky *et al.*, 2000). Corpus allatum regulation becomes more complex considering that a set of peptides from the brain and suboesophageal ganglion (Rachinsky, 1996) and also biogenic amines, especially serotonin (5HT) and octopamine (Rachinsky, 1994), may modulate JH biosynthesis to generate the observed caste-specific profile.

This search for allatoregulatory factors represents an upstream walk to close the gap between the initial nutritional signal that induces caste development and its translation into an endocrine response. In addition to the identification of the respective players, this also requires mapping of the structural pathways involved in such signal processing and transmission. The only pathways mapped in this context are the serotonergic system (Boleli *et al.*, 1995; Seidel and Bicker, 1996) and the stomatogastric nervous system (Boleli *et al.*, 1998). Immunocytochemical mapping of serotonergic neurons in honeybee larvae and pupae revealed an apparent heterochrony in their development. Whereas serotonergic neurons in the ventral ganglia exhibited a seemingly mature architecture already in the larval stages, 5HT-neurons in the brain matured only during late pupal–adult development (Boleli *et al.*, 1995). These results preclude an allatoregulatory function for protocerebral 5HT-neurons during preimaginal development. Instead, the detection of immunoreactive cell bodies in the vicinity of the CA, close to their connection to the nervi corporis cardiaci III, implicates the stomatogastric nervous system in the modulation of CA activity. Since the stomatogastric nervous system provides a direct link between sensory cells for food quality in the labral region of honeybee larvae (Goewie, 1978), food-intake-mediating neurons of the stomatogastric nervous system, and the retrocerebral endocrine complex (Boleli *et al.*, 1998), the nutritional switch information could certainly take this (alternative) pathway. Despite the considerable progress on this subject, the information on perception and processing of the nutritional switch signal in bees is still rudimentary and remains a major “black box” in our understanding of events upstream of the caste-specific endocrine response.

One of the downstream effects resulting from differences in JH titers between honeybee queen and worker larvae involves the endocrine system itself, as an internal circuit between the CA and the prothoracic glands. The latter were identified as loose agglomerates of cells projecting from the larval foregut to the retrocerebral complex (Hartfelder, 1993), and their activity pattern closely reflects the ecdysteroid titer in the last larval instar (Rachinsky *et al.*, 1990). The ecdysteroid titer, with makisterone A as the main compound, is low at the beginning of the last instar (**Figure 9**), and exhibits a marked peak during the prepupal stage in both

castes. Caste-related differences in the larval ecdysteroid titer are evident in the cocoon-spinning phase, with an earlier increase in queens than in workers. Mimicking a queenlike JH titer by JH application during the fourth to early fifth instar resulted in an earlier increase in prothoracic gland activity (Rachinsky and Engels, 1995). This positive interaction between JH titer and prothoracic gland activity may involve a prothoracicotropic hormone (PTTH)-like factor, which was detected immunohistochemically in honeybee brain sections (Simões *et al.*, 1997).

Alternatively, JH could stimulate ecdysteroid synthesis and release directly in the prothoracic glands, as demonstrated by *in vitro* assays exposing these glands to different doses of methoprene (Hartfelder and Engels, 1998). The positive action of JH on the ecdysteroid titer in worker larvae seems to be restricted to the larval–pupal transition, since JH application to pupae inhibits and retards the formation of the large pupal ecdysteroid peak (Zufelato *et al.*, 2000), which also consists mainly of makisterone A (Feldlaufer *et al.*, 1985).

In terms of target tissues, it is the ovary that has received most attention, as it best reflects the functional caste differences. A typical queen ovary consists of 180–200 ovarioles, whereas a worker ovary most often contains between two and four ovarioles, rarely exceeding 12 of these serial units. These differences arise primarily during the final larval instars, even though some of the differentiation steps are already visible by the second instar (Dedej *et al.*, 1998; Reginato and Cruz-Landim, 2001). Caste-specific differentiation of the ovary consists primarily of a reduction in ovariole number in the worker caste, from an initially equal number of ovariole anlage in the two castes – generally over 150 anlagen per ovary in the fourth instar – to the final set of ovarioles at pupation. Histologically, this process is marked by a reduced number of rosette-like cystocyte clusters in early fifth instar worker ovarioles, and a large number of autophagic vacuoles (Hartfelder and Steinbrück, 1997).

As shown by BrdU (bromo deoxy uridine) labeling, the reduced number of cystocyte clusters is not due to a diminished mitotic activity in the ovaries of worker larvae (Schmidt Capella and Hartfelder, 1998), but rather results from a disintegrating actin cytoskeleton in the germ cells (Schmidt Capella and Hartfelder, 2002). During the transition from the fourth to the fifth larval instar (which exactly coincides with the critical window for JH), actin loses its affinity to spectrin and forms large unstructured aggregates in the germ cell region of worker ovarioles. It is exactly at this location that shortly thereafter signs of apoptotic cell death appear. These degradative processes initiated by actin–spectrin dissociation could be prevented by a single topical JH application to late fourth instar workers (Schmidt Capella and Hartfelder, 1998, 2002). Even though these analyses permit us to pinpoint a cell biological target for JH in an important caste differentiation process, we cannot yet establish whether JH acts directly on the affinity of actin to spectrin, or whether this results from a transcriptional effect on factors that mediate the interaction between these cytoskeletal components.

3.13.3.3.3.2.2. Honeybee caste development: genomic studies As in functional genomics in general, complex transcriptional regulation can also be expected to be a key factor in our comprehension of caste development, and in this respect, the honeybee stands out as a model organism amongst the social insects. The early studies on mRNA differences between queen and worker larvae (Severson *et al.*, 1989) have gained considerable depth and impetus from powerful molecular methods, such as differential display (Corona *et al.*, 1999; Hepperle and Hartfelder, 2001) and suppression–subtractive hybridization protocols (Evans and Wheeler, 1999). These efforts, and particularly the coupling of high-throughput sequencing of expressed sequence tags (ESTs) to powerful clustering algorithms (Evans and Wheeler, 2000) represent a major breakthrough and turning point in our understanding of the complexity of caste differentiation. Two dichotomies emerge from cluster analyses of these gene expression screens (**Figure 11**). The first one is a dichotomy in gene expression patterns for early versus late larval stages, independent of caste. The second one is a queen–worker dichotomy in gene expression that is initiated in the late larval stages (Evans and Wheeler, 2001). Many of the genes that exhibited caste-specific expression encode metabolic enzymes, and thus represent an early response to the nutritional switch occurring during the fourth larval instar.

Whereas these results give us a view of the formidable complexity in genomic regulatory changes that take place during caste development, they still leave open the fundamental question as to the role of JH and ecdysteroids in this process. In fact, they only permit *post hoc* inference on hormone effects by correlations of hormone titers with the observed gene expression patterns. Direct evidence for hormone effects in caste differentiation and a glimpse into their mode of action, so far, has only been obtained for ecdysteroids in the context of ovary development. Two-dimensional analysis of protein

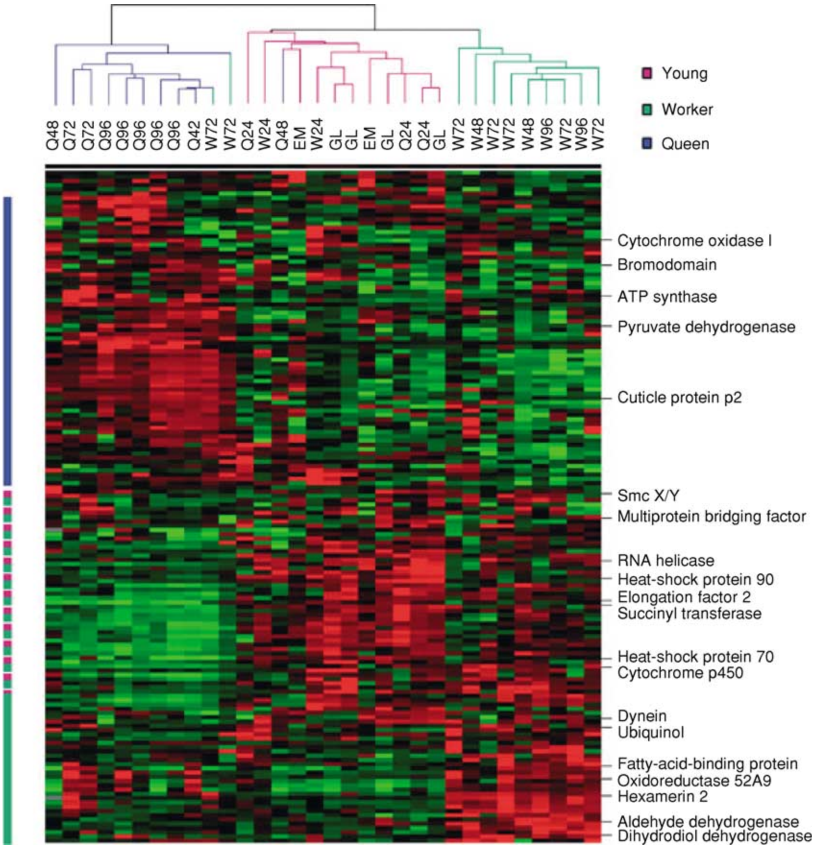


Figure 11 Microarray analysis of complex gene expression patterns in caste development of the honeybee. Clusterization revealed three sets of developmentally coregulated genes. The first cluster is characterized by genes exhibiting higher expression levels in young (still bipotential) larvae vs. older larvae. The second cluster joins genes which are higly expressed in worker larvae, particularly in the last larval instar, and the third cluster represents genes with elevated expression levels in fifth instar queen larvae. (Reproduced with permission from Evans, J.D., Wheeler, D.E., **2000**. Expression profiles during honeybee caste determination. *Genome Biol. 2,1*, 6 pages, available online http://genomebiology.com; USDA; Genome Biology is an Open Access Journal.)

synthesis patterns in larval ovaries exposed to physiological doses of makisterone A *in vitro* revealed a suppressive effect of this hormone on two low molecular weight proteins (Hartfelder *et al*., 1995) and reflected *in vivo* changes in protein synthesis that can be attributed to the caste-specific ecdysteroid titers. A subsequent differential display polymerase chain reaction (PCR) analysis of gene expression in ovaries of honeybee worker larvae showed that makisterone A has a repressive effect on the transcription of numerous genes, including transcription factor homologs, such as *FTZ-F1* and a *cut* homolog (Hepperle and Hartfelder, 2001), as well as on factors involved in cell signaling and in metabolic enzymes. As in the above-mentioned gene expression screens, metabolic enzymes constitute an important group in the ecdysone-responsive genes in the honeybee. One of these, a short-chain reductase/dehydrogenase, has been fully sequenced permitting a detailed analysis of its ecdysone regulated expression *in vivo* and *in vitro* (Guidugli *et al*., 2004).

3.13.3.3.3.2.3. Honeybee reproduction and division of labor The high rate of egg production in the honeybee queen requires an enormous production of vitellogenin by the fat body. In contrast to most insects (see **Chapter 3.9**) hormonal regulation of vitellogenin expression in honeybees is rather puzzling. Experiments with JH application and allatectomy, and also JH titer analysis, seemed to rule out any role for this hormone in the regulation of the vitellogenin titer, which exhibits a tremendous increase during the first days after emergence of the young queen from its brood cell (Engels, 1974; and other references cited in Hartfelder and Engels, 1998). Recent investigations, however, showed that the rising JH titer in late pupal development of queens and the slight increase occurring during the first 24 h in adult workers coincide with the appearance of vitellogenin in hemolymph. Further, application of the JH analog pyriproxifen caused a precocious increase in vitellogenin levels in late pupae of both castes (Barchuk *et al*., 2002).

In contrast to JH, no apparent role in reproduction and division of labor could so far be attributed to ecdysteroids in female honeybees. Except for a transient small peak early in the adult life cycle, ecdysteroid titers fluctuated at basal levels, independent of caste, reproductive status, and colony social conditions (Hartfelder *et al.*, 2002). This is all the more striking when considering that considerable levels of ecdysteroids were detected in the ovaries of adult queens (Feldlaufer *et al.*, 1986). This apparent lack of function for ecdysteroids in the reproductive physiology of highly eusocial bees, when compared to the more primitively social bees or wasps, however, may need to be reviewed in new light. Real-time PCR analysis utilizing primers directed against the recently described honeybee vitellogenin cDNA (Piulachs *et al.*, 2003) detected vitellogenin expression already during larval stages (Guidugli and Simões, personal communication), that is, during a period when ecdysteroids are primarily responsible for synchronizing and pacing development. Another novel molecular tool, namely gene silencing by injection of double-stranded RNA, should also soon shed new light on the long-discussed connection between vitellogenin, lifespan regulation, and hormonal regulation. Promising results utilizing such a strategy have recently been reported from RNA interference experiments with vitellogenin (Amdam *et al.*, 2003b).

Caste and reproduction in the highly eusocial insects are intimately related to lifespan, and recent studies have now provided evidence that vitellogenin production is directly implicated in honeybee lifespan. Besides being of interest to gerontology (Page and Peng, 2001), these findings also stimulated novel models that reconsider division of labor in honeybee colonies as an aspect integral to reproduction (Amdam and Omholt, 2002, 2003). In the center of these models stands the regulation of the vitellogenin titer during the adult lifespan of honeybee workers, since vitellogenin is not only an essential protein for oogenesis but also is a major zinc transporting protein, and as such, is involved in the delay of aging. Since JH represses vitellogenin synthesis in fat body preparations (Engels *et al.*, 1990), and thus explains the decrease in vitellogenin observed in older bees (Engels, 1974), zinc levels and hemocyte quality were also shown to decrease as honeybee workers age and start to perform high-risk tasks (Amdam and Omholt, 2002; Amdam, personal communication). These studies attribute a new functional role to the high vitellogenin titers in nurse bees, which for a long time have been considered an enigmatic by-product of supposedly leaky hormonal regulation. The detection of putative vitellogenin receptors on the larval food-producing hypopharyngeal glands of nurse bees suggests that vitellogenin, in fact, retains a reproductive function also in honeybee workers, yet instead of being incorporated into eggs, it is converted into larval food (Amdam *et al.*, 2003a).

The hormonal regulation of vitellogenin synthesis thus also consitutes an integral element in the division of labor among honeybee workers, that is, in the sequence of tasks that they perform during their short adult lifespan. Since hormonal regulation of division of labor in honeybees has been the subject of excellent recent reviews (Fahrbach, 1997; Robinson and Vargo, 1997; Page and Peng, 2001; Bloch *et al.*, 2002; Page and Erber, 2002), this topic is being considered only briefly. Early experiments by Jaycox (1976) indicated a straightforward role of JH in the division of labor, as JH application to young bees promoted a precocious switch from activities within the nest, especially brood care, to foraging activities (typical of older workers). Subsequent analyses of JH titers and CA activity confirmed this hypothesis, showing that rates of JH synthesis and hemolymph JH titers in worker bees strongly increase as the bees age and become foragers (Huang *et al.*, 1991; Robinson *et al.*, 1991, 1992). Recent studies revealed that seasonal factors (Pearce *et al.*, 2001) and the genetic constitution of a worker bee also have a strong effect on behavioral development and task preference (Giray *et al.*, 1999, 2000). The most surprising finding was, however, that allatectomy performed on newly emerged workers did not abolish but only retarded by a few days the behavioral switch from nursing to foraging activities (Sullivan *et al.*, 2000). Interestingly, this retardation effect could be prevented by a topical application of the JH analog methoprene.

Age-related polyethism, the performance of different tasks by bees of different age, can now also be addressed at the molecular level. Expression analyses in the adult honeybee brain spearheaded by Whitfield *et al.* (2002) and Kucharski and Maleszka (2000) resulted in the establishment of microarray slides for genomic analyses of age polyethism and division of labor, which may help to elucidate the still enigmatic role of JH in aging and division of labor.

3.13.3.3.3.2.4. Hormonal control of caste development and reproduction in stingless bees The stingless bees are not only the sister group to the monogeneric tribe Apini, but are also represented by an enormous number of species (Camargo and Pedro, 1992). Thus, they supply us with ample material for evolutionary insights into caste polyphenism and

reproduction in highly social bees. As aforementioned, it is the nutritional conditions that, in this group also, serve as initial trigger in the divergence of the queen/worker developmental pathways, even though in the genus *Melipona* there is strong evidence for a genetic predisposition to caste fate (Kerr, 1950).

As in the honeybee, the initial investigations on the role of hormones in stingless bee caste differentiation all relied on JH application experiments. These comparative investigations on different species of stingless bees (Campos *et al*., 1975; Campos, 1978, 1979; Buschini and Campos, 1994; Bonetti *et al*., 1995) established the spinning stage of the last larval instar as the critical phase for JH-dependent induction of queen development. These findings subsequently received support from investigations on CA activity and JH titer measurements in *Scaptotrigona postica* (Hartfelder, 1987; Hartfelder and Rembold, 1991). Except for clear JH titer differences between queen- and worker-destined larvae during the spinning stage, further caste-related JH titer differences also became apparent during the penultimate and early last larval instar, as well as at the end of pupal development (**Figure 9**). These characteristics demonstrate the close similarity between stingless bees and the honeybee with respect to developmental profiles of JH titers, despite the fact that the critical window for JH-induced queen development and, thus, the divergence of queen/worker pathways, occur much later in stingless bees than they do in the honeybee (Hartfelder, 1990). This may reflect a strong input from the differences in larval nutritional programs in the two groups noted previously. Consequently, queen development in the trigonine species takes longer than worker development, with the opposite being true for honeybees and the genus *Melipona*. These differences in nutritional programs between the three groups (*Apis, Melipona*, Trigonini) are reflected in larval and especially pupal ecdysteroid titers (Hartfelder and Rembold, 1991; Pinto *et al*., 2002), suggesting a correlated regulation of caste development by JH and ecdysteroid in all groups of highly social bees.

The high species diversity in the stingless bees, their variation in colony size, nesting sites, and the large number of further aspects in social lifestyles obviously provide material for variation on the basic theme of caste development, reproduction, and division of labor, and possible hormonal regulation therein. An important difference between honeybees and stingless bees lies in the degree of morphological differences between the sexes and castes. The males of stingless bees are morphologically much more similar to workers than they are to queens (Kerr, 1987, 1990), even though growth rules for the different morphogenetic fields along the body axes appear to be adjusted rather independently (Hartfelder and Engels, 1992).

A striking phenomenon calling for functional explanations is the strong variation in queen size in some species. The occurrence and reproductive performance of miniature queens were studied closely and compared to normal-sized queens in *Schwarziana quadripunctata* and *Plebeia remota* (Ribeiro and Alves, 2001; Ribeiro *et al*., 2002), indicating functional differences in reproductive performance related to colony conditions.

Since monopolization of reproduction by the queen is a key element in the social evolution of bees, the large variation in reproductive activities by the workers among stingless bee species can provide insight into different evolutionary solutions to the queen–worker conflict over reproduction. Worker oviposition can take two forms: trophic eggs that are laid shortly before the queen oviposits, and reproductive eggs that are laid after a queen's oviposition. Trophic eggs are unfertilized eggs that are specially produced as nutrition for the queen to maintain her high reproductive rates. Worker vitellogenin is thus directly shunted into egg production by the queen. Since the production of (trophic) worker eggs is clearly in the interest of the queen, she does not discourage worker production of these unfertilized eggs. It is therefore not surprising that worker-produced eggs can make a significant contribution to male production in a colony (Engels and Imperatriz-Fonseca, 1990, and references cited therein). Some species, such as *Frieseomelitta varia*, have opted for an entirely different solution to this conflict, as their workers are sterile, due to the complete degeneration of their ovariole anlagen during pupal development (Boleli *et al*., 1999, 2000).

The involvement of hormones in the regulation of reproduction and division of labor in adult stingless bee queens and workers has only been investigated marginally; and so far, there is only negative evidence to this end in *Melipona quadrifasciata*. As in the honeybee, ecdysteroids do not seem to play any role in queen or worker reproduction in *M. quadrifasciata* (Hartfelder *et al*., 2002), and JH titer analyses by RIA also did not provide any lead that could imply the role of this hormone in female reproduction or division of labor (Santana and Hartfelder, unpublished data).

3.13.3.3.4. Caste in polyembryonic wasps Polyembryony represents a dramatic divergence from developmental regulation in insects. It requires a reorganization of axis and positional information

in the myriads of embryos forming after syncytial cleavage in the egg of parasitic wasps, where it has been studied extensively in *Copidosoma floridanum* (review: Strand and Grbic, 1997). While it represents a highly adaptive mode of reproduction for the tiny parasitoid female to colonize a valuable yet perishable resource by laying a single egg into an insect host larva, the more fascinating is the observation that different morphs can arise from these embryos. Most embryos develop into reproductive larvae that undergo pupal and adult molts, yet a small number of embryos develop into precocious larvae that form a sterile soldier caste whose function is to manipulate the sex ratio of reproductive larvae and to protect the brood from other parasites that attack the same host (Cruz, 1981; Strand *et al*., 1990; Grbic *et al*., 1992).

As in the equally clonal aphids, regulatory mechanisms governing divergence of developmental pathways into precocious soldier larvae and normal reproductive ones are not well understood. The host endocrine milieu is clearly a prime factor that affects and synchronizes larval development and metamorphosis of the parasite with that of the host, and a combination of *in vivo* and *in vitro* experiments suggests that host ecysteroids, but not JH, are important factors in caste development of these wasps (Strand and Grbic, 1997). To exert their effects, however, prospective soldier and normal larvae must have acquired different degrees of competency to respond to the circulating host hormones since, a priori, the two phenotypes should be exposed to the same endocrine milieu.

What regulates the differential acquisition of competency in *C. floridanum* embryos in response to host ecdysteroids is still unclear. Evidence from paedogenetic gall midges, however, may provide a lead. Comparative analyses on *ecdysone receptor* (*EcR*) and *ultraspiracle* (*USP*) expression in *Heteropeza pygmea* and *Mycophila speyeri* suggest that the timing or the expression of these genes in the larval ovaries is a critical factor in the life-cycle switch from paedogenesis to metamorphosis (Hodin and Riddiford, 2000; see also **Chapter 3.5**).

3.13.4. Synthesis, Patterns, and Future Directions

In the above sections, we detailed insect polyphenisms and their hormonal regulation in a phylogenetic context to facilitate drawing parallels to general modes of development and reproduction in hemi- and holometabolous insects. Yet a functional perspective would be an equally valid approach. The most common form of polyphenism in hemimetabolous insects is a polyphenism for wing length. The development of wings has played a major role in the extraordinary evolutionary and ecological radiation of insects, and it appears that aspects of this mechanism have proven especially amenable to investigations of the evolution of facultative or polyphenic patterns of wing expression. The switch between winged and wingless developmental pathways most often occurs during a late larval instar, and involves modulation of the JH titer during a critical physiological “sensitive period.”

Aphid phase polyphenism continues to be a great enigma in terms of developmental regulation, and despite considerable progress in this field since the last review in this series by Hardie and Lees (1985), there seem to be more questions than answers. This is especially so with respect to the role of JH, which, in view of present evidence cannot be upheld as the sole – or even the principal – endocrine factor.

In locusts, much of the JH controversy has now been resolved. Phase polyphenism in locusts is undoubtedly a complex phenomenon, and phase transition results from cumulative pheromonal and hormonal signals that act during all life-cycle stages, and across several consecutive generations. Hoppers strongly respond to pheromones and can quickly change their endogenous hormone titers in response to changing population density (Botens *et al*., 1997). In turn, the JH titer during critical periods of larval development, in concert with other hormones, such as the neuropeptide [His^7]-corazonin, is capable of altering phase-specific body coloration (Tanaka, 2000a, 2000b, 2000c). In the adult stages of the life cycle, flight physiology, particularly the adipokinetic reaction, is strongly associated with phase (Dorn *et al*., 2000). Pheromones stimulate aggregation and egg-laying behavior and may thus act as a bridge between generations, in concert with phase-specific differences in hormone titers, especially the ecdysteroid content of ovaries and eggs. Such cumulative effects on phase characteristics over the entire life cycle are the hallmark of locust polyphenism, but these can also be found in other groups that exhibit complex life-cycle syndromes (Moran, 1994). Elucidating the architecture of the multiple and synergistic developmental switches remains a challenging task for future work.

In termites, hormonal regulation of caste development has been exploited for pest control, since application of JH analogs can shift the caste-ratio balance towards soldiers (and away from reproductives). Mechanistically, JH has been identified as a competence factor which interacts with ecdysteroids to enhance cuticle deposition after the presoldier and soldier molts (Okot-Kotber, 1983). Furthermore, it

may also be involved in regulating the expression of soldier specific genes, such as *SOL1* (Miura *et al.*, 1999). Establishing such networks of developmental regulation in termite caste morphogenesis will, certainly, require a focus on carefully selected model species in both lower and higher termites. Furthermore, it would be desirable to link such studies to those on hormonal regulation of development in other hemimetabolous species, particularly in cockroaches of the genus *Cryptocerus* which exhibit many similarities to life histories of primitive termites (Nalepa, 1984). Caste polyphenism studies in termites could also profit from developmental programming studies performed on embryonic stages of hemimetabolans, such as the ones performed by Truman and Riddiford (1999) on locusts, which shed new light on the evolution of holometabolous development from hemimetabolan ancestors. In this context, it would be interesting to reanalyze two sets of questions: (1) the relative immaturity of termite larvae, which, especially in the higher termites, need to be cared for by workers and (2) the stationary and regressive molts in lower termites, an astonishing phenomenon in insect metamorphosis which emphasizes the importance of molting events in termite societies.

Wing polyphenism in holometabolans takes two very different forms, that of seasonal wing pattern differences in lepidopterans, and that of wing reduction in ant workers. Wing pattern polyphenism in butterflies is an allelic polymorphism in camouflage in response to predator pressure, which, in its expression, depends on the timing of the pupal ecdysteroid peak (Koch *et al.*, 1996; Brakefield *et al.*, 1998). Winglessness in ant workers, in contrast, is an integral element of caste polyphenism in the Formicidae. It is a result of the shutdown of a gene expression cascade regulating wing disc development (Abouheif and Wray, 2002) in response to embryonic/larval hormone titers.

The focus on *Apis mellifera* as a model organism in research on caste development in social Hymenoptera has certainly been promoted by the honeybee's economic importance, a factor which more recently has also stimulated much of the research on the bumblebee, *Bombus terrestris*. Synthesis and titers of morphogenetic hormones have been monitored throughout the entire life cycle of these insects, and these studies have shown that a correlated modulation of JH and ecdysteroid titers drives caste development during the larval stages (Hartfelder and Engels, 1998). Furthermore, the full cascade from initial environmental triggers through the endocrine response to these triggers, and finally to downstream differentiative processes in target organs, has now been mapped – at least roughly – in honeybees, a process which is now rapidly advancing due to the concentrated efforts in honeybee genomics (Corona *et al.*, 1999; Evans and Wheeler, 1999, 2000, 2001; Hepperle and Hartfelder, 2001).

A general conclusion to be drawn from developmental studies on insect polyphenism is its generally strong dependency on JH; except, of course, for butterfly wing pattern polyphenism where patterning occurs during the pupal period and, consequently, is under ecdysteroid control. Occasionally this may have lead to the oversimplified view that JH may act as a master regulator in generating polyphenism. Rather, the multifaceted appearance that JH makes in this context should be attributed to its pleiotropic general function in insect metamorphosis and reproduction, which facilitated its co-option. Especially in complex life cycles, such as observed in aphids, locusts, and social insects, JH never is the sole factor, but appears integrated in its action with that of other hormones and/or neuropeptides.

Functional genomics has just begun to transform studies of insect polyphenism. Initial efforts have focused on whole-genome, or transcriptome, sequencing, and all efforts thus far have been concentrated on a single species, the honeybee (Beye *et al.*, 1998; Evans and Wheeler, 1999, 2000; Robinson *et al.*, 2002; Tomkins *et al.*, 2002; Whitfield *et al.*, 2002). Even though still at a primitive stage, these genomic studies have already injected new impetus into the field, and led to the application of differential gene expression screens with narrower foci in aphids, locusts, termites, and other social Hymenoptera. Undoubtedly, the near future will see similar screens being performed in a large number of polyphenic species.

We expect two questions to dominate and direct such screens. One is the search for differentially expressed genes in the JH response. This response cascade is still a major question mark in insect physiology, and its elucidation is largely hampered by the lack of unequivocal data on JH receptor(s) (Gilbert *et al.*, 2000; Jones and Jones, 2000). Despite the small amount of genomic information currently available for polyphenic insects, their naturally selected alternative phenotypes may make these insects especially useful tools for identifying and studying the function of JH response cascade elements. Many of these studies may profit from newly established RNA interference protocols (Amdam *et al.*, 2003b).

An alternative approach lies in the specific search for expression patterns of conserved regulatory

genes in relation to alternative pathways in tissue development. The power of this approach has recently been demonstrated in the analyses of interruption points for wing disc development in ants (Abouheif and Wray, 2002). Even more extensively, it has been put to use in the studies of butterfly wing patterns where the link between the expression of patterning genes and EcR, and thus, between hormone titer modulation and eyespot size, has become apparent (Brunetti *et al*., 2001; Koch *et al*., 2003). These studies provide a glimpse into the complexity of regulatory networks underlying polyphenism. What lies ahead in this direction will be a broadened insight into the hormonal modes of action on such genomic regulatory networks. Critical steps towards this end would involve the sequencing of upstream regulatory regions for candidate genes and functional analyses of hormone response elements.

Perhaps the greatest challenge will be to link conceptually the results of such genomic studies with organismic-level studies of physiology, behavior, and ecology. Once again, we expect two questions to remain and/or become predominant. The first one is in the realm of developmental biology and will have to address molecular and cellular mechanisms underlying reaction norms and phenotypic plasticity. Many aspects of phenotypic plasticity in insects can be ascribed to general size-related allometries. These have been mathematically formulated in growth models that assume competition for available nutrients between growing morphogenetic fields (Nijhout and Wheeler, 1994). Such intuitive tradeoffs have, however, not been detected in the only genetically accessible system for this question, that is in *Drosophila* mutants for the insulin receptor antagonist *DPTEN* (Goberdhan and Wilson, 2002). These authors suggest, instead, that tissues may be kept to proportional size by their similarity in response to the evolutionarily conserved insulin-receptor signaling pathway. To our knowledge, the components of this pathway, which could be a major link between reaction norms and developmental mechanisms, have never been studied in relation to insect polyphenisms.

The second question is much broader, and will involve linking evolutionary aspects in the life histories of polyphenic insects to the evolution of regulatory mechanisms underlying the development of alternative phenotypes (West-Eberhard, 2003). This, obviously, is a long-term program with few definable guidelines. The convergence on pleiotropic hormones in life history considerations on the one hand, and developmental regulation on the other, however, should continue to drive investigations into endocrine system functions in this context.

Note added in proof

In December 2003 a first draft version of the honeybee genome was released by the Human Genome Sequencing Center at Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/projects/honeybee/). This genomic sequence information provides an unprecedented resource for functional genomic studies and it is predicted that it will be useful not only for research on the honey bee but also work on other social Hymenoptera. A similarly rich resource, not for sequence information but for concepts, is the recently published book by M.J. West-Eberhard (2003) which leaves no doubt about the importance of alternative phenotypes and their development regulation in evolutionary processes.

References

Abbot, P., Withgott, J.H., Moran, N.A., **2001**. Genetic conflict and conditional altruism in social aphid colonies. *Proc. Natl Acad. Sci. USA 98*, 12068–12071.

Abouheif, E., Wray, G.A., **2002**. Evolution of the genetic network underlying wing polyphenism in ants. *Science 297*, 249–252.

Aé, S.A., **1957**. Effects of photoperiod on *Colias eurytheme*. *Lepidopt. News 11*, 207–214.

Agrahari, M., Gadagkar, R., **2003**. Juvenile hormone accelerates ovarian development and does not affect age polyethism in the primitively eusocial wasp, *Ropalidia marginata*. *J. Insect Physiol. 49*, 217–222.

Alexander, R.D., **1968**. Life cycle origins, speciation, and related phenomena in crickets. *Q. Rev. Biol. 43*, 1–41.

Amdam, G.V., Omholt, S.W., **2002**. The regulatory anatomy of honeybee lifespan. *J. Theoret. Biol. 21*, 209–228.

Amdam, G.V., Omholt, S.W., **2003**. The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *J. Theoret. Biol. 223*, 451–464.

Amdam, G.V., Norberg, K., Hagen, A., Omholt, S.W., **2003a**. Social exploitation of vitellogenin. *Proc. Natl Acad. Sci. USA 100*, 1799–1802.

Amdam, G.V., Simões, Z.L.P., Guidugli, K.R., Norberg, K., Omholt, S.W., **2003b**. Disruption of vitellogenin gene function in adult honeybees by intraabdominal injection of double-stranded RNA. *BioMed. Centr. Biotech. 3, 1*, 8 pages, available online: http://www.biomedcentral.com/1472-6750/3/1.

Andersson, M., **1994**. Sexual Selection. Princeton University Press, Princeton, NJ.

Aoki, S., **1977**. *Colophina clematis*, an aphid species with "soldiers." *Kontyu 45*, 276–282.

Applebaum, S.W., Avisar, E., Heifetz, Y., **1997**. Juvenile hormone and locust phase. *Arch. Insect Biochem. Physiol. 35*, 375–391.

Austad, S.N., **1984**. A classification of alternative reproductive behaviors and methods for field-testing ESS models. *Am. Zool. 24*, 309–319.

Awiti, L.R., Hidaka, T., **1982**. Neuroendocrine mechanisms involved in pupal color dimoprhism in swallowtail *Papilio xuthus*. *Insect Sci. Appl. 3*, 181–192.

Ayali, A., Pener, M.P., **1992**. Density-dependent phase polymoprhism affects response to adipokinetic hormones in *Locusta*. *Comp. Biochem. Physiol. A 101*, 549–552.

Ayoade, O., Morooka, S., Tojo, S., **1999**. Enhancement of short wing formation and ovarian growth in the genetically defined macropterous strain of the brown planthopper, *Nilaparvata lugens*. *J. Insect Physiol. 45*, 93–100.

Baker, R., **1970**. Bird predation as a selective pressure of the cabbage butterflies, *Pieris rapae* and *P. brassicae*. *J. Zool. 152*, 43–59.

Barchuk, A.R., Bitondi, M.M.G., Simões, Z.L.P., **2002**. Effects of juvenile hormone and ecdysone on the timing of vitellogenin appearance in hemolymph of queen and worker pupae of *Apis mellifera*. *J. Insect Sci. 2, 1*, 8 pages, available online: http://www.insectscience.org/2.1.

Barth, R.H., Lester, L.J., Sroka, P., Kessler, T., Hearn, R., **1975**. Juvenile hormone promotes dominance behavior and ovarian development in social wasps (*Polistes annularis*). *Experientia 31*, 691–692.

Beetsma, J., **1985**. Feeding behaviour of nurse bees, larval food composition and caste differentiation in the honey bee (*Apis mellifera* L.). In: Hölldobler, B., Lindauer, M. (Eds.), Experimental Behavioral Ecology and Sociobiology: *In Memoriam* Karl von Frisch, 1886–1982. Sinauer, Sunderland, MA, pp. 407–410.

Beldade, P., Brakefield, P.M., **2002**. The genetics and evo-devo of butterfly wing patterns. *Nature Rev. Genetics 3*, 442–452.

Beldade, P., Brakefield, P.M., Long, A.D., **2002**. Contribution of *Distal-less* to quantitative variation in butterfly eyespots. *Nature 415*, 315–318.

Bertuso, A.G., Morooka, S., Tojo, S., **2002**. Sensitive periods for wing development and precocious metamorphosis after Precocene treatment of the brown planthopper, *Nilaparvata lugens*. *J. Insect Physiol. 48*, 221–229.

Beye, M., Poch, A., Burgtorf, C., Moritz, R.F.A., **1998**. A gridded genomic library of the honeybee (*Apis mellifera*): a reference library for basic and comparative genetic studies of a hymenopteran genome. *Genomics 49*, 317–320.

Blackman, R.L., **1994**. The simplification of aphid terminology. *Eur. J. Entomol. 91*, 139–141.

Bloch, G., Borst, D.W., Huang, Z.Y., Robinson, G.E., Cnaani, J., *et al.*, **2000a**. Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris*. *J. Insect Physiol. 46*, 47–57.

Bloch, G., Borst, D.W., Huang, Z.Y., Robinson, G.E., Hefetz, A., **1996**. Effects of social conditions on juvenile hormone mediated reproductive development in *Bombus terrestris* workers. *Physiol. Entomol. 21*, 257–267.

Bloch, G., Hefetz, A., **1999a**. Reevaluation of the role of mandibular glands in regulation of reproduction in bumblebee colonies. *J. Chem. Ecol. 25*, 881–896.

Bloch, G., Hefetz, A., **1999b**. Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. *Behav. Ecol. Sociobiol. 45*, 125–135.

Bloch, G., Hefetz, A., Hartfelder, K., **2000b**. Ecdysteroid titer, ovary status, and dominance in adult worker and queen bumble bees (*Bombus terrestris*). *J. Insect Physiol. 46*, 1033–1040.

Bloch, G., Wheeler, D.E., Robinson, G.E., **2002**. Endocrine influences on the organization of insect societies. In: Pfaff, D.W., Arnold, A.P., Ettgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain, and Behavior, vol. 3. Academic Press, San Diego, CA, pp. 195–237.

Boleli, I.C., Hartfelder, K., Simões, Z.L.P., **1995**. Serotonin-like immunoreactivity in the central nervous and neuroendocrine system of honey bee (*Apis mellifera*) larvae. *Zool.-Anal. Complex Syst. 99*, 58–67.

Boleli, I.C., Simões, Z.L.P., Bitondi, M.M.G., **1999**. Cell death in ovarioles causes permanent sterility in *Frieseomelitta varia* worker bees. *J. Morphol. 242*, 271–282.

Boleli, I.C., Simões, Z.L.P., Bitondi, M.M.G., **2000**. Regression of the lateral oviducts during the larval–adult transformation of the reproductive system of *Melipona quadrifasciata* and *Frieseomelitta varia*. *J. Morphol. 243*, 141–151.

Boleli, I.C., Simões, Z.L.P., Hartfelder, K., **1998**. The stomatogastric nervous system of the honey bee (*Apis mellifera*) in a critical phase of caste development. *J. Morphol. 236*, 139–148.

Bonetti, A.M., Kerr, W.E., Matusita, S.H., **1995**. Effects of juvenile hormones I, II and III, in single and fractionated doses in *Melipona* bees. *Rev. Brasil. Biol. 55* (Suppl. 1), 113–120.

Bordereau, C., **1971**. Dimorphisme sexuel du système trachéen chez les imagos ailés de *Bellicositermes natalensis* Haviland (Isoptera, Termitidae): rapports avec la physogastrie de la reine. *Arch. Zool. Exp. Gén. 112*, 33–54.

Bordereau, C., Han, S.H., **1986**. Stimulatory influence of the queen and king on soldier differentiation in the higher termites *Nasutitermes lujae* and *Cubitermes fungifaber. Insectes Sociaux 33*, 296–305.

Botens, F.F.W., Rembold, H., Dorn, A., **1997**. Phase-related juvenile hormone determinations in field catches and laboratory strains of different *Locusta migratoria* subspecies. In: Kawashima, S., Kikuyama, S. (Eds.), Advances in Comparative Endocrinology. Monduzzi, Bologna, pp. 197–203.

Boulay, R., Hooper-Bui, L.M., Woodring, J., **2001**. Oviposition and oogenesis in virgin fire ant females *Solenopsis invicta* are associated with a high level of dopamine in the brain. *Physiol. Entomol. 26*, 294–299.

Brakefield, P.M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P.J., *et al.*, **1996**. Development, plasticity and evolution of butterfly eyespot patterns. *Nature 384*, 236–242.

Brakefield, P.M., Kesbeke, F., Koch, P.B., **1998**. The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *Am. Nat. 152*, 853–860.

Brakefield, P.M., Larsen, T.B., **1984**. The evolutionary significance of dry and wet season forms in tropical butterflies. *Biol. J. Linn. Soc. 22*, 1–22.

Brakefield, P.M., Mazotta, V., **1995**. Matching field and laboratory experiments: effects of neglecting daily temperature variation in insect reaction norms. *J. Evol. Biol. 8*, 559–573.

Brakefield, P.M., Reitsma, N., **1991**. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecol. Entomol. 16*, 291–304.

Brian, M.V., **1974**. Caste determination in *Myrmica rubra*: the role of hormones. *J. Insect Physiol. 20*, 1351–1365.

Brunetti, C., Selegue, J.E., Monterio, A., French, V., Brakefield, P.M., *et al.*, **2001**. The generation and diversification of butterfly eyespot patterns. *Curr. Biol. 11*, 1578–1585.

Bückmann, D., Maisch, A., **1987**. Extraction and partial purification of the pupal melanization reducing factor (PMRF) from *Inachis io* (Lepidoptera). *Insect Biochem. 17*, 841–844.

Burns, S.N., Teal, P.E.A., Vander Meer, R.K., Vogt, J.T., **2002**. Identification and action of juvenile hormone III from sexually mature alate females of the red imported fire ant, *Solenopsis invicta*. *J. Insect Physiol. 48*, 357–365.

Buschini, M.L.T., Campos, L.A.O., **1994**. Caste determination in *Trigona spinipes* (Hymenoptera: Apidae): influence of the available food and the juvenile hormone. *Revi. Brasil. Biol. 55* (Suppl. 1), 121–129.

Camargo, J.M.F., Pedro, S.R.M., **1992**. Systematics, phylogeny and biogeography of the Meliponinae (Hymenoptera, Apidae): a mini-review. *Apidologie 23*, 509–522.

Campos, L.A.O., **1978**. Sex determination in bees. 6. Effect of a juvenile hormone analog in males and females of *Melipona quadrifasciata* (Apidae). *J. Kansas Entomol. Soc. 51*, 228–234.

Campos, L.A.O., **1979**. Determinação do sexo em abelhas. 14. Papel do hormônio juvenil na diferenciação das castas na subfamilia *Meliponinae* (Hymenoptera: Apidae). *Rev. Brasil. Biol. 39*, 965–971.

Campos, L.A.O., Velthuis, H.H.W., Velthuis-Kluppell, F.M., **1975**. Juvenile hormone and caste determination in a stingless bee. *Naturwissenschaften 62*, 98–99.

Carlin, N.F., Gladstein, D.S., Berry, A.J., Pierce, N.E., **1994**. Absence of kin discrimination behavior in a soldier-producing aphid, *Ceratovacuna japonica* (Hemiptera, Pemphigidae, Cerataphidini). *J. New York Entomol. Soc. 102*, 287–298.

Carpenter, J.M., **1991**. Phylogenetic relationships and the origin of social behaviour in the Vespidae. In: Ross, K.G., Matthews, R.W. (Eds.), The Social Biology of Wasps. Cornell University Press, Ithaca, NY, pp. 7–32.

Carroll, S.P., Boyd, C., **1992**. Host radiation in the sopaberry bug: natural history, with the history. *Evolution 51*, 1052–1069.

Carroll, S.P., Dingle, H., Famula, T.R., Fox, C.W., **2001**. Genetic architecture of adaptive differentiation in evolving host races of the soapberry bug, *Jadera hematoloma*. *Genetica 112*, 257–272.

Carroll, S.P., Dingle, H., Klassen, S.P., **1997**. Genetic differentiation of fitnes-associated traits among rapidly evolving populations of the soapberry bug. *Evolution 51*, 1182–1188.

Cauillaud, M.C., Boutin, M., Braendle, C., Simon, J.C., **2002**. A sex-linked locus controls wing polymorphism in males of the pea aphid, *Acyrthosiphon pisum* (Harris). *Heredity 89*, 346–352.

Chino, H., Lum, P.Y., Nagao, E., Hiraoka, T., **1992**. The molecular and metabolic essentials for long distance flights in insects. *J. Comp. Physiol. B 162*, 101–106.

Christiansen-Weniger, P., Hardie, J., **2000**. The influence of parasitism on wing development in male and female pea aphids. *J. Insect Physiol. 46*, 861–867.

Clark, R., **1997**. Dimorphic males display alternative reproductive strategies in the marine amphipod *Jassa marmorata*. *Ethology 7*, 531–553.

Clynen, E., Stubbe, D., de Loof, A., Schoofs, L., **2002**. Peptide differential display: a novel approach for phase transition in locusts. *Comp. Biochem. Physiol. B 132*, 107–115.

Cnaani, J., Borst, D.W., Huang, Z.Y., Robinson, G.E., Hefetz, A., **1997**. Caste determination in *Bombus terrestris*: differences in development and rates of JH biosynthesis between queen and worker larvae. *J. Insect Physiol. 43*, 373–381.

Cnaani, J., Hefetz, A., **2001**. Are queen *Bombus terrestris* giant workers or are workers dwarf queens? Solving the "chicken and egg" problem in a bumblebee species. *Naturwissenschaften 88*, 85–87.

Cnaani, J., Robinson, G.E., Bloch, G., Borst, D., Hefetz, A., **2000a**. The effect of queen–worker conflict on caste determination in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol. 47*, 346–352.

Cnaani, J., Robinson, G.E., Hefetz, A., **2000b**. The critical period for caste determination in *Bombus terrestris* and its juvenile hormone correlates. *J. Comp. Physiol. A 186*, 1089–1094.

Condamin, M., **1973**. Monographe du genre *Bicyclus* (Lepidoptera, Satyridae). *Mém. d'Inst. e Fond. Afrique Noire 88*, 1–324.

Cook, D., **1987**. Sexual selection in dung beetles. 1. A multivariate study of the morphological variation in two species of *Ontophagus* (Scarabeidae: Ontophagini). *Austral. J. Zool. 35*, 123–132.

Corona, M., Estrada, E., Zurita, M., **1999**. Differential expression of mitochondrial genes between queens and workers during caste determination in the honeybee *Apis mellifera*. *J. Exp. Biol. 202*, 929–938.

Couillaud, F., Mauchamp, B., Girardie, A., **1987**. Biological, radiochemical and physicochemical evidence

for the low activity of disconnected corpora allata in locust. *J. Insect Physiol. 33*, 223–228.

Cremer, S., Lautenschläger, B., Heinze, J., **2002**. A transitional stage between the ergatoid and winged male morph in the ant *Cardiocondyla obscurior. Insectes Sociaux 49*, 221–228.

Crossland, M.W.J., Crozier, R.H., Jefferson, E., **1988**. Aspects of the biology of the primitive ant genus *Myrmecia* F. (Hymenoptera: Formicidae). *J. Austral. Entomol. Soc. 27*, 305–309.

Cruz, Y.P., **1981**. A sterile defender morph in a polyembryonic hymenopterous parasite. *Nature 294*, 446–447.

Cuvillier-Hot, V., Cobb, M., Malosse, C., Peeters, C., **2001**. Sex, age and ovarian activity affect cuticular hydrocarbons in *Diacamma ceylonense*, a queenless ant. *J. Insect Physiol. 47*, 485–493.

Cuvillier-Hot, V., Lenoir, A., Peeters, C., **2003**. Reproductive monopoly enforced by sterile police workers in a queenless ant. *Behav. Ecol. Sociobiol.* in press.

Dale, J.F., Tobe, S.S., **1986**. Biosynthesis and titre of juvenile hormone during the first gonotrophic cycle in isolated and crowded *Locusta migratoria* females. *J. Insect Physiol. 32*, 763–769.

Danforth, B.N., **1991**. The morphology and behavior of dimorphic males in *Perdita portalis* (Hymenoptera: Andrenidae). *Behav. Ecol. Sociobiol. 29*, 235–247.

Darrouzet, E., Mauchamp, B., Prestwich, G.D., Kerhoas, L., Ujvary, I., *et al.*, **1997**. Hydroxy juvenile hormones: new putative juvenile hormones biosynthesized by locust corpora allata *in vitro*. *Biochem. Biophys. Res. Commun. 240*, 752–758.

Darwin, C.R., **1859**. On the Origin of Species by Means of Natural Selection. John Murray, London.

Darwin, C.R., **1871**. The Descent of Man and Selection in Relation to Sex. John Murray, London.

Dedej, S., Hartfelder, K., Rosenkranz, P., Engels, W., **1998**. Caste determination is a sequential process: effect of larval age on ovariole number, hind leg size and cephalic volatiles in the honey bee (*Apis mellifera carnica*). *J. Apicult. Res. 37*, 183–190.

Denno, F., Perfect, T.J., **1994**. Planthoppers: Their Ecology and Management. Chapman and Hall, London.

Denno, R.F., Douglass, L.W., Jacobs, D., **1986**. Effects of crowding and host plant nutrition on a wing dimorphic planthopper. *Ecology 67*, 116–123.

Denno, R.F., Roderick, G.K., Peterson, M.A., Huberty, A.F., Döbel, H., *et al.*, **1996**. Habitat persistence underlies intraspecific variation and dispersal strategies of planthoppers. *Ecol. Monogr. 66*, 389–408.

Diakonov, D.M., **1925**. Experimental and biometrical investigations on dimoprhic variability of *Forficula. J. Genet. 15*, 201–232.

Dietemann, V., Hölldobler, B., Peeters, C., **2002**. Caste specialization and differentiation in reproductive potential in the phylogenetically primitiva ant *Myrmecia gulosa. Insectes Sociaux 49*, 289–298.

Dietz, A., Hermann, H.R., Blum, M.S., **1979**. The role of exogenous JH I, JH III and anti-JH (Precocene II) on queen induction in 4.5-day-old worker honeybee larvae. *J. Insect Physiol. 25*, 503–512.

Dingle, H., Winchell, R., **1997**. Juvenile hormone as a mediator of plasticity in insect life histories. *Arch. Insect Biochem. Physiol. 35*, 359–373.

Dixon, A.F.G., **1977**. Aphid ecology: life cycles, polymorphism, and population regulation. *Annu. Rev. Ecol. Syst. 8*, 329–353.

Dixon, A.F.G., **1998**. Aphid Ecology. Chapman and Hall, London.

Dominey, W.J., **1984**. Alternative tactics and evolutionarily stable strategies. *Am. Zool. 24*, 385–396.

Doorn, A. van., **1987**. Investigations into the regulation of dominace behaviour and the division of labour in bumblebee colonies (*Bombus terrestris*). *Netherlands J. Zool. 37*, 255–276.

Dorn, A., Ress, C., Sickold, S., Wedekind-Hirschberger, S., **2000**. Arthropoda – Insecta: endocrine control of phase polymorphism. In: Dorn, A. (Ed.), Progress in Developmental Endocrinology, vol. X, part B. John Wiley, Chichester, pp. 205–253.

Duchateau, M.J., Velthuis, H.H.W., **1988**. Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour 107*, 186–207.

Duchateau, M.J., Velthuis, H.H.W., **1989**. Ovarian development and egg laying in workers of *Bombus terrestris. Entomol. Exp. Applic. 51*, 199–213.

Eberhard, W.G., **1982**. Beetle horn dimorphism: making the best of a bad lot. *Am. Nat. 119*, 420–426.

Eberhard, W.G., Gutierrez, E.E., **1991**. Male dimorphism in beetles and earwigs and the question of developmental constraints. *Evolution 45*, 18–28.

Emlen, D.J., **1994**. Environmental control of horn length dimorphism in the beetle *Onthophagus acuminathus* (Coleoptera: Scarabaeidae). *Proc. Roy. Soc. B 256*, 131–136.

Emlen, D.J., **1996**. Artificial selection on horn length-body size allometry in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Evolution 50*, 1219–1230.

Emlen, D.J., **1997**. Alternative reproductive tactics and male dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Behav. Ecol. Sociobiol. 41*, 335–341.

Emlen, D.J., Nijhout, H.F., **1999**. Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J. Insect Physiol. 45*, 45–53.

Emlen, D.J., Nijhout, H.F., **2001**. Hormonal control of male horn length dimorphism in *Onthophagus taurus* (Coleoptera: Scarabaeidae): a second critical period of sensitivity to juvenile hormone. *J. Insect Physiol. 47*, 1045–1054.

Endo, K., **1984**. Neuroendocrine regulation of the development of seasonal forms of the Asian comma butterfly *Polygonia c-aureum. Devel. Growth Different. 26*, 217–222.

Endo, K., Funatsu, S., **1985**. Hormonal control of seasonal morph determination in the swallowtail butterfly,

Papilio xuthus L. (Lepidoptera: Papilionidae). *J. Insect Physiol. 31*, 669–674.

Endo, K., Kamata, Y., **1985**. Hormonal control of seasonal morph determination in the small copper butterfly, *Lycaena phlaeas daimio* Seitz. *J. Insect Physiol. 31*, 701–706.

Endo, K., Masaki, T., Kumagai, K., **1988**. Neuroendocrine regulation of the development of seasonal morphs in the Asian comma butterfly, *Polygonia c-aureum* L.: difference in activity of summer-morph-producing hormone from brain extracts of the long-day and short-day pupae. *Zool. Sci. 5*, 145–152.

Endo, K., Yamashita, I., Chiba, Y., **1985**. Effect of photoperiodic transfer and brain surgery on the photoperiodic control of pupal diapause and seasonal morphs in the swallowtail *Papilio xuthus*. *Appl. Entomol. Zool. 20*, 470–478.

Engels, W., **1974**. Occurrence and significance of vitellogenins in female castes of social Hymenoptera. *Am. Zool. 14*, 1229–1237.

Engels, W., Imperatriz-Fonseca, V.L., **1990**. Caste development, reproductive strategies, and control of fertility in honey bees and stingless bees. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 168–230.

Engels, W., Kaatz, H., Zillikens, A., Simões, Z.L.P., Trube, A., *et al*., **1990**. Honey bee reproduction: vitellogenin and caste-specific regulation of fertility. *Adv. Invert. Reprod. 5*, 495–502.

Evans, J.D., Wheeler, D.E., **1999**. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. *Proc. Natl Acad. Sci. USA 96*, 5575–5580.

Evans, J.D., Wheeler, D.E., **2000**. Expression profiles during honeybee caste determination. *Genome Biol. 2,1*, 6 pages, available online http://genomebiology.com/2000/2/1/research/0001.1.

Evans, J.D., Wheeler, D.E., **2001**. Gene expression and the evolution of insect polyphenisms. *BioEssays 23*, 62–68.

Fabre, J.H., **1899**. Souvenirs Entomologiques, excerpts transl. A.T. de Mattos. In: More Beetles (1922). Hodder and Stoughton, London.

Fahrbach, S.E., **1997**. Regulation of age polyethism in bees and wasps by juvenile hormone. *Adv. Study Behav. 26*, 285–315.

Fairbairn, D.J., Yadlowski, D.E., **1997**. Coevolution of traits determining migratory tendency: correlated response of a critical enzyme, juvenile hormone esterase, to selection on wing morphology. *J. Evol. Biol. 10*, 495–513.

Faustino, C.D., Silva-Matos, E.V., Mateus, S., Zucchi, R., **2002**. First record of emergency queen rearing in stingless bees (Hymenoptera, Apinae, Meliponini). *Insectes Sociaux 49*, 111–113.

Feldlaufer, M.F., Herbert, E.W.J., Svoboda, J.A., **1985**. Makisterone A: the major ecdysteroid from the pupae of the honey bee, *Apis mellifera*. *Insect Biochem. 15*, 597–600.

Feldlaufer, M.F., Svoboda, J.A., Herbert, E.W.J., **1986**. Makisterone A and 24-methylenecholesterol from the ovaries of the honey bee, *Apis mellifera* L. *Experientia 42*, 200–201.

Fincher, G.T., Woodruff, R.E., **1975**. A European dung beetle, *Onthophagus taurus* Schreber, new to the US (Coleoptera: Scarabeidae). *Coleopt. Bull. 29*, 349–350.

Fletcher, D.J.C., Blum, M.S., **1981**. Pheromonal control of dealation and oogenesis in virgin queen fire ants. *Science 212*, 73–75.

Fletcher, D.J.C., Blum, M.S., **1983**. The inhibitory pheromone of queen fire ants: effects of disinhibition on dealation and oviposition by virgin queens. *J. Comp. Physiol. A 153*, 467–475.

Foster, W.A., Rhoden, P.K., **1998**. Soldiers effectively defend aphid colonies against predators in the field. *Anim. Behav. 55*, 761–765.

Fraser, V.S., Kaufmann, B., Oldroyd, B.P., Crozier, R.H., **2000**. Genetic influence on caste in the ant *Camponotus consobrinus*. *Behav. Ecol. Sociobiol. 47*, 188–194.

French, V., **1997**. Pattern formation in colour on butterfly wings. *Curr. Opin. Genet. Devel. 7*, 524–529.

French, V., Brakefield, P.M., **1995**. Eyespot development in butterfly wings: the focal signal. *Devel. Biol. 168*, 112–123.

Fukatsu, T., Ishikawa, H., **1992**. Soldier and male of an eusocial aphid *Colophina arma* lack endosymbiont: implications for physiological and evolutionary interaction between host and symbiont. *J. Insect Physiol. 38*, 1033–1042.

Fukuda, S., Endo, K., **1966**. Hormonal control of the development of seasonal forms in the butterfly *Polygonia c-aureum* L. *Proc. Japan. Acad. 42*, 1082–1987.

Fuzeau-Braesch, S., **1961**. Variations dans la longeur des ailes en fonction de l'effect de groupes chez quelques espèces de Gryllides. *Bull. Soc. Zool. France 86*, 785–788.

Gadagkar, R., Vinutha, C., Shanubhogue, A., Gore, A.P., **1988**. Pre-imaginal biasing of caste in a primitively eusocial insect. *Proc. Roy. Soc. B 233*, 175–189.

Gao, N., Hardie, J., **1996**. Pre- and post-natal effects of precocenes on aphid morphogenesis and differential rescue. *Arch. Insect Biochemi. Physiol. 32*, 503–510.

Gardiner, B.O.C., **1974**. Observations on green pupae in *Papilio machaon* L. and *Pieris brassicae* L. *Wilhelm Roux Archiv Entwicklungsmech. 176*, 13–22.

Ghouri, A.S.K., McFarlane, J.E., **1958**. Occurrence of a macropterous form of *Gryllodes sigillatus* (Walker) (Othoptera: Gryllidae) in laboratory culture. *Can. J. Zool. 36*, 837–838.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Giray, T., Guzmán-Novoa, E., Aron, C.W., Zelinsky, B., Fahrbach, S.E., *et al*., **2000**. Genetic variation in worker temporal polyethism and colony defensiveness in the honey bee, *Apis mellifera*. *Behav. Ecol. 11*, 44–55.

Giray, T., Huang, Z.Y., Guzman-Novoa, E., Robinson, G.E., **1999**. Physiological correlates of genetic variation

for rate of behavioral development in the honeybee, *Apis mellifera*. *Behav. Ecol. Sociobiol. 47*, 17–28.

Goberdhan, D.C.I., Wilson, C., **2002**. Insulin receptor-mediated organ overgrowth in *Drosophila* is not restricted by body size. *Devel. Genes Evol. 212*, 196–202.

Goewie, E.A., **1978**. Regulation of caste differentiation in the honey bee (*Apis mellifera*). *Medelingen Landbouwhoogeschool Wageningen 78-15*, 1–75.

Goldsmith, S.K., **1985**. Male dimorphism in *Dendrobias mandibularis* Audinet-Serville (Coleoptera: Cerambycidae). *J. Kansas Entomol. Soc. 58*, 534–538.

Goldsmith, S.K., **1987**. The mating system and alternative reproductive behaviors of *Dendrobias mandibularis* (Coleoptera: Cerambycidae). *Behav. Ecol. Sociobiol. 20*, 111–115.

Grbic, M., Ode, P.J., Strand, M.R., **1992**. Sibling rivalry and brood sex ratios in polyembryonic wasps. *Nature 360*, 254–256.

Greenberg, S., Tobe, S.S., **1985**. Adaptation of a radiochemical assay for juvenile hormone biosynthesis to study caste differentiation in a primitive termite. *J. Insect Physiol. 31*, 347–352.

Gross, M.R., **1996**. Alternative reproductie strategies and tactics: diversity within sexes. *Trends Ecol. Evol. 11*, 92–98.

Guidugli, K.R., Hepperle, C., Hartfelder, K., **2003**. A member of the short-chain dehydrogenase/reductase (SDR) superfamily is a target of the ecdysone response in honey bee (*Apis mellifera* L.) caste development. *Apidologie 35*, 37–47.

Hägele, B., Oag, V., Bouaichi, A., McCaffery, A.R., Simpson, S.J., **2000**. The role of female accessory glands in maternal inheritance of phase in the desert locust *Schistocerca gregaria*. *J. Insect Physiol. 46*, 275–280.

Hallffter, G., Edmonds, W.G., **1982**. The Nesting Behavior of Dung Beetles (Scarabaeidae): An Ecological and Evolutive Approach. Instituto de Ecologia, Mexico, Federal District.

Hamilton, W.D., **1964**. The genetical theory of social behaviour. 1 and 2. *J. Theoret. Biol. 7*, 1–16, 17–52.

Hardie, J., **1980**. Juvenile hormone mimics the photoperiodic apterization of the alate gynopara of aphid, *Aphis fabae*. *Nature 286*, 602–604.

Hardie, J., **1981a**. The effect of juvenile hormone on host plant preference in the black bean aphid, *Aphis fabae*. *Physiol. Entomol. 6*, 369–374.

Hardie, J., **1981b**. Juvenile hormone and photoperiodically controlled polymorphism in *Aphis fabae*: postnatal effects on presumptive gynoparae. *J. Insect Physiol. 27*, 347–355.

Hardie, J., **1981c**. Juvenile hormone and photoperiodically controlled polymorphism in *Aphis fabae*: prenatal effects on presumptive oviparae. *J. Insect Physiol. 27*, 257–265.

Hardie, J., **1986**. Morphogenetic effects of precocenes on three aphid species. *J. Insect Physiol. 32*, 813–818.

Hardie, J., Gao, N., Timar, T., Sebok, P., Honda, K., **1996**. Precocene derivatives and aphid morphogenesis. *Arch. Insect Biochem. Physiol. 32*, 493–501.

Hardie, J., Honda, K., Timar, T., Varjas, L., **1995**. Effects of 2,2-dimethylchromene derivatives on wing determination and metamorphosis in the pea aphid, *Acyrthosiphon pisum*. *Arch. Insect Biochem. Physiol. 30*, 25–40.

Hardie, J., Lees, A.D., **1983**. Photoperiodic regulation of the development of winged gynoparae in the aphid, *Aphis fabae*. *Physiol. Entomol. 8*, 385–391.

Hardie, J., Lees, A.D., **1985**. Endocrine control of polymorphism and polyphenism. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 441–489.

Hardie, J., Mallory, A.C.L., Quashiewilliams, C.A., **1990**. Juvenile hormone and host plant colonization by the black bean aphid, *Aphis fabae*. *Physiol. Entomol. 15*, 331–336.

Hardie, J., Nunes, M.V., **2001**. Aphid photoperiodic clocks. *J. Insect Physiol. 47*, 821–832.

Harrison, R.G., **1979**. Flight polymorphism in the field cricket *Gryllus pennsylvannicus*. *Oecologia 40*, 125–132.

Harrison, R.G., **1980**. Dispersal polymorphism in insects. *Annu. Rev. Ecol. Syst. 11*, 95–118.

Hartfelder, K., **1987**. Rates of juvenile hormone synthesis control caste differentiation in the stingless bee *Scaptotrigona postica depilis*. *Roux's Arch. Devel. Biol. 196*, 522–526.

Hartfelder, K., **1990**. Regulatory steps in caste development of eusocial bees. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 245–264.

Hartfelder, K., **1993**. Structure and function of the prothoracic gland in honey bee (*Apis mellifera* L.) development. *Invert. Reprod. Devel. 23*, 59–74.

Hartfelder, K., **2000**. Arthropoda – Insecta: caste differentiation. In: Dorn, A. (Ed.), Progress in Developmental Endocrinology, vol. X, part B. John Wiley, Chichester, pp. 185–204.

Hartfelder, K., Bitondi, M.M.G., Santana, W.C., Simões, Z.L.P., **2002**. Ecdysteroid titers and reproduction in queens and workers of the honey bee and of a stingless bee: loss of ecdysteroid function at increasing levels of sociality? *Insect Biochem. Mol. Biol. 32*, 211–216.

Hartfelder, K., Cnaani, J., Hefetz, A., **2000**. Caste-specific differences in ecdysteroid titers in early larval stages of the bumblebee *Bombus terrestris*. *J. Insect Physiol. 46*, 1433–1439.

Hartfelder, K., Engels, W., **1989**. The composition of larval food in stingless bees: evaluating nutritional balance by chemosystematic methods. *Insectes Sociaux 36*, 1–14.

Hartfelder, K., Engels, W., **1992**. Allometric and multivariate analysis of sex and caste polymorphism in the neotropical stingless bee, *Scaptotrigona postica*. *Insectes Sociaux 39*, 251–266.

Hartfelder, K., Engels, W., **1998**. Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. *Curr. Topics Devel. Biol. 40*, 45–77.

Hartfelder, K., Kostlin, K., Hepperle, C., **1995**. Ecdysteroid-dependent protein synthesis in caste-specific development of the larval honey bee ovary. *Roux's Arch. Devel. Biol. 205*, 73–80.

Hartfelder, K., Rembold, H., **1991**. Caste-specific modulation of juvenile hormone-III content and ecdysteroid titer in postembryonic development of the stingless bee, *Scaptotrigona postica depilis*. *J. Comp. Physiol. B. 160*, 617–620.

Hartfelder, K., Steinbrück, G., **1997**. Germ cell cluster formation and cell death are alternatives in caste-specific differentiation of the larval honey bee ovary. *Invert. Reprod. Devel. 31*, 237–250.

Hazel, W.N., **1977**. The genetic basis of pupal colour dimorphism and its maintenance by natural selection in *Papilio polyxenes* (Papilionidae: Lepidoptera). *Heredity 38*, 227–236.

Hazel, W.N., Ante, S., Strongfellow, B., **1998**. The evolution of environmentally-cued upal colour in swallowtail butterflies: natural selection for pupation site and pupation colour. *Ecol. Entomol. 23*, 41–44.

Hazel, W.N., West, D.A., **1979**. Environmental control of pupal colour in swallowtail butterflies (Lepidoptera: Papilionidae: *Battus philenor* (L.) and *Papilio polyxenes* Fabr. *Ecol. Entomol. 4*, 393–408.

Hazel, W.N., West, D.A., **1996**. Pupation site preference and environmentally-cued pupal colour dimorphism in the swallowtail butterflies *Papilio polyxenes* Fabr. (Lepidoptera: Papilionidae). *Biol. J. Linn. Soc. 57*, 81–87.

Heinze, J., **1998**. Intercastes, intermorphs and ergatoids: who is who in ant reproduction? *Insectes Sociaux 45*, 113–124.

Heinze, J., Buschinger, A., **1987**. Queen polymorphism in a non-parasitic *Leptothorax* species (Hymenoptera, Formicidae). *Insectes Sociaux 34*, 28–43.

Hepperle, C., Hartfelder, K., **2001**. Differentially expressed regulatory genes in honey bee caste development. *Naturwissenschaften 88*, 113–116.

Hidaka, T., **1961a**. Mise en évidence de l'activité sécrétoire du ganglion prothoracique dans l'adaptation chromatique de la nymphe du *Papilio xuthus* L. *C. R. Soc. Biol. Paris 154*, 1682–1685.

Hidaka, T., **1961b**. Recherches sur le méchanisme endocrine de l'adaptation chromatique morphologique chez les nymphes du *Papilio xuthus* L. *J. Fac. Sci. Univ. Tokyo 9 (Sec. IV)*, 223–261.

Hille Ris Lambers, D., **1966**. Polymorphism in Aphididae. *Annu. Rev. Entomol. 11*, 47–78.

Hodin, J., Riddiford, L.M., **2000**. Parallel alterations in the timing of ovarian *Ecdysone receptor* and *Ultraspiracle* expression characterize the independent evolution of larval reproduction in two species of gall midges (Diptera: Cecidomyidae). *Devel. Genes and Evol. 210*, 358–372.

Hoffman, R.J., **1973**. Environmental control of seasonal variation in the butterfly *Colias eurytheme*. 1. Adaptive aspects of a photoperiodic response. *Evolution 27*, 387–397.

Hoffman, R.J., **1978**. Environmental uncertainty and evolution of physioplogical adaptation in *Colias* butterflies. *Am. Nat. 112*, 999–1015.

Hölldobler, B., Wilson, E.O., **1990**. The Ants. Belknapp Press of Harvard University Press, Cambridge, MA.

Huang, Z.Y., Robinson, G.E., Tobe, S.S., Yagi, K.J., Strambi, C., *et al.*, **1991**. Hormonal regulation of behavioral development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. *J. Insect Physiol. 37*, 733–741.

Hunt, J., Simmons, L.W., **1997**. Patterns of fluctuating assymetry in beetle horns: an experimental examination of the honest signalling hypothesis. *Behav. Ecol. Sociobiol. 41*, 109–114.

Hunt, J.H., **1991**. Nourishment and the evolution of social Vespidae. In: Ross, K.G., Matthews, R.W. (Eds.), The Social Biology of Wasps. Cornell University Press, Ithaca, NY, pp. 426–450.

Iguchi, Y., **1998**. Horn dimorphism in *Allomyrina dichotomia septentrionalis* (Coleoptera: Scarabaeidae) affected by larval nutrition. *Ann. Entomol. Soc. America 91*, 845–847.

Injeyan, H.S., Tobe, S.S., **1981**. Phase polymorphism in *Schistocerca gregaria*: assessment of juvenile hormone synthesis in relation to vitellogenesis. *J. Insect Physiol. 27*, 203–210.

Ishizaki, H., Kato, M., **1956**. Environmental factors affecting the formation of orange pupae in *Papilio xuthus*. *Mem. Coll. Sci. Kyoto Univ. B 3*, 11–18.

Ito, Y., Tanaka, S., Yukawa, J., Tsuji, K., **1995**. Factors affecting the proportion of soldiers in eusocial bamboo aphid, *Pseudoregma bambucicola*, colonies. *Ethol. Ecol. Evol. 7*, 335–345.

Iwanaga, K., Tojo, S., **1986**. Effects of juvenile hormone and rearing density on wing dimoprhism and oocyte development in the brown planthopper *Niloparvata lugens*. *J. Insect Physiol. 32*, 585–590.

Jacobs, M.D., Watt, W.B., **1994**. Seasonal adaptation vs. physiological constraint: photoperiod, thermoregulation and flight in *Colias* butterflies. *Funct. Ecol. 8*, 366–376.

Jaycox, E.R., **1976**. Behavioral changes in worker honey bees (*Apis mellifera*) after injection with synthetic juvenile hormone (Hymenoptera: Apidae). *J. Kansas Entomol. Soc. 49*, 165–170.

Jeanne, R.L., Graf, C.A., Yandell, B.S., **1995**. Non-size-based morphological castes in a social insect. *Naturwissenschaften 82*, 296–298.

Johnson, B., **1959**. Effect of parasitization by *Aphidius platensis* Brèthes on the developmental physiology of its host, *Aphis craccivora* Koch. *Entomol. Exp. Applic. 2*, 82–99.

Joly, L., **1954**. Résultats d'implantations systématiques de corpora allata à des jeunes larves de *Locusta migratoria*. *C. R. Soc. Biol. Paris 148*, 579–583.

Jones, G., Jones, D., **2000**. Considerations on the structural evidence of a ligand-binding function of *ultraspiracle*, an insect homolog of vertebrate RXR. *Insect Biochem. Mol. Biol. 30*, 671–679.

Julian, G.E., Fewell, J.H., Gadau, J., Johnson, R.A., Lorrabee, D., **2002**. Genetic determination of the queen caste in an ant hybrid zone. *Proc. Natl Acad. Sci. USA 99*, 8157–8160.

Karlson, P., Lüscher, M., **1959**. "Pheromones": a new term for a class of biologically active substances. *Nature 183*, 55–56.

Kawano, K., **1995**. Horn and wing allometry and male dimorphism in giant rhinoceros beetles (Coleoptera: Scarabaeidae) of tropical Asia and America. *Ann. Entomol. Soc. America 88*, 92–98.

Keeping, M.G., **2002**. Reproductive and worker caste in the primitively eusocial wasp *Belonogaster petiolata* (DeGeer) (Hymenoptera: Vespidae): evidence for pre-imaginal differentiation. *J. Insect Physiol. 48*, 867–879.

Kerr, W.E., **1950**. Evolution of the mechanism of caste determination in the genus *Melipona*. *Evolution 4*, 7–13.

Kerr, W.E., **1987**. Sex determination in bees. 17. Systems of caste determination in the Apinae, Meliponinae and Bombinae and their phylogenetic implications. *Rev. Brasil. Genét. 10*, 685–694.

Kerr, W.E., **1990**. Sex determination in bees. 26. Masculinism of workers in the Apidae. *Rev. Brasil. Genét. 13*, 479–489.

Kerr, W.E., Stort, A.C., Montenegro, M.S., **1966**. Importância de alguns fatores ambientais na determinação das castas do gênero *Melipona*. *An. Acad. Brasil. Ciências 38*, 149–168.

Keys, D.N., Lewis, D.L., Selegue, J.E., Pearson, B.J., Goodrich, L.V., *et al.*, **1999**. Recruitment of a *hedgehog* regulatory circuit in butterfly eyespot evolution. *Science 283*, 532–534.

Kingsolver, J.G., **1987**. Evolution and coadaptation of thermoregulatory behavior and wing pigmentation in pierid butterflies. *Evolution 41*, 472–490.

Kingsolver, J.G., **1995a**. Fitness consequences of seasonal polyphenism in western white butterflies. *Evolution 49*, 942–954.

Kingsolver, J.G., **1995b**. Viability selection on seasonally polyphenic traits: wing melanin pattern in western white butterflies. *Evolution 49*, 932–941.

Kingsolver, J.G., Watt, W.B., **1983**. Thermoregulatory strategies in *Colias* butterflies: thermal stress and the limits of adaptation in temporally varying environments. *Am. Nat. 121*, 32–55.

Kingsolver, J.G., Wiesnarz, D.C., **1991**. Seasonal polyphenism in wing-melanin pattern and thermoregulatory behavior pigmentation in *Pieris* butterflies. *Am. Nat. 137*, 816–829.

Kisimoto, R., **1965**. Studies on the polymorphism and its role playing in the population growth of the brown planthopper, *Nilaparvata lugens* Stal. *Bull. Shikoku Agric. Exp. Station 13*, 1–106.

Kobayashi, M., Ishikawa, H., **1993**. Breakdown of indirect flight muscles of alate aphids (*Acyrthosiphon pisum*) in relation to their flight, feeding and reproductive behavior. *J. Insect Physiol. 39*, 549–554.

Kobayashi, M., Ishikawa, H., **1994**. Involvement of juvenile hormone and ubiquitin-dependent proteolysis in flight muscle breakdown of alate aphid (*Acyrthosiphon pisum*). *J. Insect Physiol. 40*, 107–111.

Koch, P.B., Brakefield, P.M., Kesbeke, F., **1996**. Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly *Bicyclus anynana* (Lepidoptera: Satyridae). *J. Insect Physiol. 42*, 223–230.

Koch, P.B., Bückmann, D., **1985**. The seasonal dimorphism of *Araschnia levana* L. (Nymphalidae) in relation to hormonal controlled development. *Verhandl. Deutsch. Zool. Gesell. 78*, 260.

Koch, P.B., Bückmann, D., **1987**. Hormonal control of seasonal morphs by the timing of ecdysteroid release in *Araschnia levana* L. (Nymphalidae: Lepidoptera). *J. Insect Physiol. 33*, 823–829.

Koch, P.B., Merk, R., Reinhardt, R., Weber, P., **2003**. Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly *Precis coenia* (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. *Devel. Genes Evol. 212*, 571–584.

Kooi, R.E., Brakefield, P.M., **1999**. The critical period for wing pattern induction in the polyphenic tropical butterfly *Bicyclus anynana* (Satyrinae). *J. Insect Physiol. 45*, 201–212.

Kooi, R.E., Brakefield, P.M., Rossie, W.E.M., **1996**. Effects of food plant on phenotypic plasticity in the tropical butterfly *Bicyclus anynana*. *Entomol. Exp. Applic. 80*, 149–151.

Kooi, R.E., Brakefield, P.M., Schlatmann, E.G.M., **1994**. Description of the larval sensitive period for polyphenic wing pattern induction in the tropical butterfly *Bicyclus anynana* (Satyrinae). *Proc. Exp. Appl. Entomol. 5*, 47–52.

Kucharski, R., Maleszka, R., **2000**. Evaluation of differential gene expression during behavioral development in the honeybee using microarray and northern blots. *Genome Biol. 3,2*, 9 pages, available online http://genomebiology.com/2000/3/2/research/0007.

Kühn, A., von Engelhardt, M., **1933**. Über die Determination des Symmetriesystems auf dem Vorderfügel von *Ephestia kühniella*. *Wilhelm Roux' Archiv Entwicklungsmech. Organismen 130*, 660.

Kukuk, P.F., **1996**. Male dimorphism in *Lasioglossum (Chilalictus) hemiochalceum*: the role of larval nutrition. *J. Kansas Entomol. Soc. 69* (Suppl.), 147–157.

Lageux, M., Hetru, H., Goltzene, F., Kappler, C., Hoffmann, J.A., **1979**. Ecdysone titer and metabolism in relation to cuticulogenesis in embryos of *Locusta migratoria*. *J. Insect Physiol. 25*, 709–725.

Lageux, M., Hirn, M., Hoffmann, J.A., **1977**. Ecdysone during ovarian development in *Locusta migratoria*. *J. Insect Physiol. 23*, 109–119.

Lank, D.B., Smith, C.M., Hanotte, O., Burke, T., Cooke, F., **1995**. Genetic polymorphism for alternative mating behavior in lekking male ruff *Philomachus pugnax*. *Nature 378*, 59–62.

Lanzrein, B., Gentinetta, V., Fehr, R., **1985**. Titres of juvenile hormone and ecdysteroids in reproducion and eggs of *Macrotermes michaelseni*: relation to caste determination. In: Watson, J.A.L., Okot-Kotber, B.M.,

Noirot, C. (Eds.), Caste Differentiation in Social Insects. Pergamon Press, Oxford, pp. 307–327.

Larrere, M., Couillaud, F., **1993**. Role of juvenile hormone biosynthesis in dominance status and reproduction of the bumblebee, *Bombus terrestris*. *Behav. Ecol. Sociobiol. 33*, 335–338.

Larrere, M., Lavenseau, L., Tasei, J.N., Couillaud, F., **1993**. Juvenile hormone biosynthesis and diapause termination in *Bombus terrestris*. *Invert. Reprod. Devel. 23*, 7–14.

Ledoux, A., **1976**. Action d'un dérivé du farnesol sur l'apparition des femelles ailées chez *Aphaenogaster senilis* (Hym. Formicoidea). *C. R. Acad. Sci. Paris D 228*, 569–570.

Lees, A.D., **1977**. Action of juvenile hormone mimics on the regulation of larval–adult anf alary polymorphism in aphids. *Nature 267*, 46–48.

Lees, A.D., **1980**. Development of juvenile hormone sensitivity in alatae of the aphid *Megoura viciae*. *J. Insect Physiol. 26*, 143–151.

Lefeuve, P., Bordereau, C., **1984**. Soldier formation regulated by a primer pheromone from the soldier frontal gland in a higher termite. *Proc. Natl Acad. Sci. USA 81*, 7665–7668.

Liebig, J., Peeters, C., Oldham, N.J., Markstädter, C., Hölldobler, B., **2000**. Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator? Proc. Natl Acad. Sci. USA 97*, 4124–4131.

Lüscher, M., **1964**. Die spezifische Wirkung männlicher und weiblicher Ersatzgeschlechtstiere auf die Entstehung von Geschlechtstieren bei der Termite *Kalotermes flavicollis* (Fab.). *Insectes Sociaux 11*, 79–90.

Lüscher, M., **1976**. Evidence for endocrine control of caste determination in higher termites. In: Lüscher, M. (Ed.), Phase and Caste Determination in Insects. Pergamon Press, Oxford, pp. 91–103.

Main, H., **1922**. Notes on the metamorphosis of *Onthophagus taurus* L. *Proc. Entomol. Soc. London 1922*, 14–16.

Maisch, A., Bückmann, D., **1987**. The control of cuticular melanin and lutein incorporation in the morphological colour adaptation of a nymphalid pupa, *Inachis io* L. *J. Insect Physiol. 33*, 393–402.

Mane, S.D., Rembold, H., **1977**. Developmental kinetics of juvenile hormone inactivation in queen and worker castes of the honey bee, *Apis mellifera*. *Insect Biochem. 7*, 463–467.

Martinez, T., Wheeler, D.E., **1991**. Effect of the queen, brood annd worker caste on haemolymph vitellogenin titre in *Camponotus festinatus* workers. *J. Insect Physiol. 37*, 347–352.

Masaki, S., Oyama, N., **1963**. Photoperiodic control of growth and wing form in *Nemobius yezoensis* Shiraki. *Kontyu 31*, 16–26.

Masaki, T., Endo, K., Kumagai, K., **1988**. Neuroendocrine regulation of development of seasonal morphs in the Asian comma butterly, *Polygonia c-aureum* L.: is the factor producing summer morphs (SMPH) identical to the small prothoracicotropic hormone (4K-PTTH)? *Zool. Sci. 5*, 1051–1057.

Mathad, S.B., McFarlane, J.E., **1968**. Two effects of photoperiod on wing development in *Grylloides sigillatus* (Walk.). *Can. J. Zool. 46*, 57–60.

Matsumara, M., **1996**. Genetic analysis of a threshold trait: density-dependent wing dimorphism in *Sogatella furcifera* (Horvath) (Hemiptera: Delphacidae), the whitebacked planthopper. *Heredity 76*, 229–237.

McCaffery, A.R., Simpson, S.J., Islam, M.S., Roessingh, P., **1998**. A gregarizing factor in the egg pod foam of the desert locust *Schistocerca gregaria*. *J. Exp. Biol. 201*, 347–363.

McFarlane, J.E., **1962**. Effect of diet and temperature on wing development of *Grylloides sigillatus* (Walk.) (Orthoptera: Gryllidae). *Ann. Soc. Entomol. Québec 7*, 28–33.

McMillan, W.O., Monterio, A., Kapan, D.D., **2002**. Development and evolution on the wing. *Trends Ecol. Evol. 17*, 125–133.

Merrifield, F., Pouldton, E.B., **1899**. The color relation between the pupae of *Papilio machaon, Pieris napai* and many other species, and the surroundings of the larvae preparing to pupate, etc.. *Trans. Entomol. Soc. London 1899*, 369–433.

Michener, C.D., **1990a**. Castes in xylopine bees. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 123–146.

Michener, C.D., **1990b**. Reproduction and caste in social halictine bees. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 77–121.

Michener, C.D., **2000**. The Bees of the World. John Hopkins University Press, Baltimore, MD.

Miura, T., **2001**. Morphogenesis and gene expression in the soldier-caste differentiation of termites. *Insectes Sociaux 48*, 216–223.

Miura, T., Kamikouchi, A., Sawata, M., Takeuchi, H., Natori, S., *et al.*, **1999**. Soldier caste-specific gene expression in the mandibular glands of *Hodotermopsis japonica* (Isoptera: Termopsidae). *Proc. Natl Acad. Sci. USA 96*, 13874–13879.

Miura, T., Matsumoto, T., **2000**. Soldier morphogenesis in a nasute termite: discovery of a disk-like structure forming a soldier nasus. *Proc. Roy. Soc. B 267*, 1185–1189.

Moczek, A.P., Emlen, D.J., **1999**. Proximate determination of male horn dimorphism in the beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J. Evol. Biol. 12*, 27–37.

Moczek, A.P., Emlen, D.J., **2000**. Male horn dimorphism in the scarab beetle *Ontophagus taurus:* do alternative reproductive tactics favor alternative phenotypes? *Anim. Behav. 59*, 459–466.

Moczek, A.P., Hunt, J., Emlen, D.J., Simmons, L.W., **2002**. Threshold evolution in exotic populations of a polyphenic beetle. *Evol. Ecol. Res. 4*, 587–601.

Monnin, T., Ratnieks, F.L., **2001**. Policing in queenless ants. *Behav. Ecol. Sociobiol. 50*, 97–108.

Monteiro, A.F., Brakefield, P.M., French, V., **1994**. The evolutionary genetics and developmental basis of wing pattern variation in the butterfly *Bicyclus anynana*. *Evolution 48*, 1147–1157.

Monteiro, A.F., Brakefield, P.M., French, V., **1997**. Butterfly eyespots: the genetics and development of the color rings. *Evolution 51*, 1207–1216.

Moran, N.A., **1992**. The evolutionary maintenance of alternative phenotypes. *Am. Nat. 139*, 971–989.

Moran, N.A., **1994**. Adaptation and constraint in complex life cycles of animals. *Annu. Rev. Ecol. Syst. 25*, 573–600.

Morooka, S., Ishibashi, N., Tojo, S., **1988**. Relationships between wing form response to nymphal density and black colouration in the brown planthopper *Nilaparvata lugens* (Homoptera: Delphacidae). *Appl. Entomol. Zool. 23*, 449–458.

Morooka, S., Tojo, S., **1992**. Maintenance and selection of strains exhibiting specific wing form and body colour under high density conditions in the brown planthopper *Nilaparvata lugens* (Homoptera: Delphacidae). *Appl. Entomol. Zool. 27*, 445–454.

Müller, H.J., **1955**. Die Saisonformenbildung von *Araschnia levana*: ein photoperiodisch gesteuerter Diapauseeffekt. *Naturwissenschaften 42*, 134–135.

Müller, H.J., **1956**. Die Wirkung verschiedener diurnaler Licht-Dunkel-Relationen auf die Saisonformenbildung von *Araschnia levana*. *Naturwissenschaften. 43*, 503–504.

Nalepa, C.A., **1984**. Colony composition, protozoan transfer and some life history characteristics of the woodroach *Cryptocerus punctulatus* Scudder (Dictyoptera: Cryptocercidae). *Behav. Ecol. Sociobiol. 14*, 273–279.

Naug, D., Gadagkar, R., **1998**. The role of age in temporal polyethism in a primitively eusocial wasp. *Behav. Ecol. Sociobiol. 42*, 37–47.

Nijhout, H.F., **1980**. Pattern formation of lepidopteran wings: determination of an eyespot. *Devel. Biol. 80*, 267–274.

Nijhout, H.F., **1985**. The developmental physiology of color patterns in Lepidoptera. *Adv. Insect Physiol. 18*, 181–247.

Nijhout, H.F., **1986**. Pattern and diversity of lepidopteran wings. *BioScience 36*, 527–533.

Nijhout, H.F., **1991**. The Development and Evolution of Butterfly Wing Patterns. Smithsonian Institution Press, Washington, DC.

Nijhout, H.F., **1994**. Insect Hormones. Princeton University Press, Princeton, NJ.

Nijhout, H.F., Grunert, L.W., **1988**. Color pattern regulation after surgery on the wing discs of *Precis coenia* (Lepidoptera: Nymphalidae). *Development 102*, 377–385.

Nijhout, H.F., Wheeler, D.E., **1994**. Growth models of complex allometries in holometabolous insects. *Am. Nat. 148*, 40–56.

Njagi, P.G.N., Torto, B., Obeng-Ofori, D., Hassanali, A., **1996**. Phase-independent responses to phase-specific aggregation pheromone in adult desert locusts *Schistocerca gregaria* (Orthoptera: Acrididae). *Physiol. Entomol. 21*, 131–137.

Noirot, C., **1985a**. Differentiation of reproductives in higher termites. In: Watson, J.A.L., Okot-Kotber, B.M., Noirot, C. (Eds.), Caste Differentiation in Social Insects. Pergamon Press, Oxford, pp. 177–186.

Noirot, C., **1985b**. Pathways of caste development in higher termites. In: Watson, J.A.L., Okot-Kotber, B.M., Noirot, C. (Eds.), Caste Differentiation in Social Insects. Pergamon Press, Oxford, pp. 75–86.

Noirot, C., **1985c**. Pathways of caste development in the lower termites. In: Watson, J.A.L., Okot-Kotber, B.M., Noirot, C. (Eds.), Caste Differentiation in Social Insects. Pergamon Press, Oxford, pp. 41–57.

Noirot, C., **1989**. Social structure in termite societies. *Ethol. Ecol. Evol. 1*, 1–17.

Noirot, C., **1990**. Sexual castes and reproductive strategies in termites. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 5–35.

Noirot, C., Bordereau, C., **1988**. Termite polymorphism and morphogenetic hormones. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, NJ, pp. 293–324.

Noirot, C., Pasteels, J.M., **1987**. Ontogenetic development and evolution of the worker caste in termites. *Experientia 43*, 851–860.

Noll, F.B., Mateus, S., Zucchi, R., **1997**. Morphological caste differences in the neotropical swarm-founding and polygynous polistine wasps, *Polybia scutellaris*. *Stud. Neotrop. Fauna Environ. 21*, 76–80.

Nylin, S., **1992**. Seasonal plasticity in life history traits: growth and development in *Polygonia c-album* (Lepidoptera: Nymphalaidae). *Biol. J. Linn. Soc. 47*, 301–323.

O'Donnell, S., **1998**. Reproductive caste determination in eusocial wasps (Hymenoptera: Vespidae). *Annu. Rev. Entomol. 43*, 323–346.

O'Donnell, S., Jeanne, R.L., **1993**. Methoprene accelerates age polyethism in workers of a social wasp *(Polybia occidentalis)*. *Physiol. Entomol. 18*, 189–194.

Ogino, K.Y., Hirono, Y., Matsumoto, T., Ishikawa, H., **1993**. Juvenile hormone analogue, S-31183, causes a high level induction of presoldier differentiation in the Japanese damp-wood termite. *Zool. Sci. 10*, 361–366.

Ogoyi, D.O., Osir, E.O., Olembo, N.K., **1996**. Effect of phase status on response to AKH I in the desert locust *Schistocerca gregaria*. *Arch. Insect Biochem. Physiol. 32*, 173–185.

Ohkawara, K., Ito, F., Higashi, S., **1993**. Production and reproductive function of intercastes in *Myrmecina graminicola nipponica* colonies (Hymenoptera: Formicidae). *Insectes Sociaux 40*, 1–10.

Ohtaki, T., **1960**. Humoral control of pupal coloration in the cabbage white butterfly *Pieris rapae crucivora*. *Annot. Zool. Japon. 33*, 97–103.

Ohtaki, T., **1963**. Further studies on the development of pupal colouration in the cabbage white butterfly *Pieris rapae crucivora* Bois. *Serio-Seiti 9*, 84–89.

Okot-Kotber, B.M., **1983**. Ecdysteroid levels associated with epidermal events during worker and soldier differentiation in *Macrotermes michaelseni* (Isoptera: Macrotermitinae). *Gen. Comp. Endocrinol. 52*, 409–417.

Okot-Kotber, B.M., Prestwich, G.D., **1991a**. Identification of a juvenile hormone binding protein in the castes of the termite, *Reticulitermes flavipes*, by photoaffinity labeling. *Insect Biochem. 21*, 775–784.

Okot-Kotber, B.M., Prestwich, G.D., **1991b**. Juvenile hormone binding proteins of termites detected by photoaffinity labeling: comparison of *Zootermopsis nevadensis* with two rhinotermitids. *Arch. Insect Biochem. Physiol. 17*, 119–128.

Oster, G.F., Wilson, E.O., **1978**. Caste and Ecology in the Social Insects. Princeton University Press, Princeton, NJ.

Page, R.E.J., Erber, J., **2002**. Levels of behavioral organization and the evolution of division of labor. *Naturwissenschaften 89*, 91–106.

Page, R.E.J., Peng, C. Y.-S., **2001**. Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Exp. Gerontol. 36*, 695–711.

Passera, L., Suzzoni, J.P., **1979**. Le rôle de la reine de *Pheidole pallidula* (Nyl.) (Hymenoptera, Formicidae) dans la sexualisation du couvain après traitement per l'hormone juvénile. *Insectes Sociaux 26*, 343–353.

Pasteels, J.M., Roisin, Y., **2001**. Primer pheromones in termites: state of the art. Proc. 2001 Berlin Meeting of the Eur. Sections of IUSSI, p. 102.

Pearce, A.N., Huang, Z.Y., Breed, M.D., **2001**. Juvenile hormone and aggression in honey bees. *J. Insect Physiol. 47*, 1243–1247.

Peeters, C., **1991**. Ergatoid queens and intercastes in ants: two distinct adult forms which look morphologically intermediate between workers and winged queens. *Insectes Sociaux 38*, 1–15.

Peeters, C., Hölldobler, B., **1995**. Reproductive cooperation between queens and their mated workers: the complex life history of an ant with a valuable nest. *Proc. Natl Acad. Sci. USA 92*, 10977–10979.

Peeters, C., Monnin, T., Malosse, C., **1999**. Cuticular hydrocarbons correlated with reproductive status in a queenless ant. *Proc. Roy. Soc. B 266*, 1323–1327.

Pener, M.P., **1991**. Locust phase polymorphism and its endocrine relations. *Adv. Insect Physiol. 23*, 1–79.

Pener, M.P., Ayali, A., Benm-Ami, E., **1992**. Juvenile hormone is not a major factor in locust phase changes. In: Mauchamp, B., Couillaud, F., Baehr, J.C. (Eds.), Insect Juvenile Hormone Research, Fundamental and Applied Approaches. INRA, Paris, pp. 125–134.

Pener, M.P., Yerushalmi, Y., **1998**. The physiology of locust phase polymorphism: an update. *J. Insect Physiol. 44*, 365–377.

Pereboom, J.J.M., **2000**. The composition of larval food and the significance of exocrine secretions in the bumblebee *Bombus terrestris*. *Insectes Sociaux 47*, 11–20.

Pereboom, J.J.M., Velthuis, H.H.W., Duchateau, M.J., **2003**. The organisation of larval feeding in bumblebees (Hymenoptera, Apidae) and its significance to caste differentiation. *Insectes Sociaux 50*, 127–133.

Peterson, M.A., Denno, R.F., **1997**. The influence of intraspecific variation in dispersal strategies on the genetic structure of planthopper populations. *Evolution 51*, 1189–1206.

Pinto, L.Z., Hartfelder, K., Bitondi, M.M.G., Simões, Z.L.P., **2002**. Ecdysteroid titers in pupae of highly social bees relate to distinct modes of caste development. *J. Insect Physiol. 48*, 783–790.

Piulachs, M.D., Cruz, J., Simões, Z.L.P., Bellés, X., **2003**. The vitellogenin of the honey bee, *Apis mellifera*: structural analysis of the cDNA and expression studies. *Insect Biochem. Mol. Biol. 33*, 459–465.

Plateaux-Quénu, C., **1983**. Le volume d'un pain d'abeille influence-t-il le sexe de l'oeuf pondu sur lui? Étude expérimentale, portant sur la première couvée d'*Evylaeus calcateus* (Scop.). *Ann. Sci. Nat. Zool. 5*, 41–52.

Porter, S.D., Tschinkel, W.R., **1985**. Fire ant polymorphisms: factors affecting worker size. *Ann. Entomol. Soc. America 78*, 381–386.

Poulton, E.B., **1887**. An enquiry into the cause and extent of a special colour-relation between certain exposed lepidopterous pupae and the surfaces which immediately surround them. *Phil. Trans. Roy. Soc. London 178*, 311–441.

Rachinsky, A., **1994**. Octopamine and serotonin influence on corpora allata activity in honey bee (*Apis mellifera*) larvae. *J. Insect Physiol. 40*, 549–554.

Rachinsky, A., **1996**. Brain and suboesophageal ganglion extracts affect juvenile hormone biosynthesis in honey bee larvae (*Apis mellifera carnica*). *Zool.-Anal. Complex Syst. 99*, 277–284.

Rachinsky, A., Engels, W., **1995**. Caste development in honeybees (*Apis mellifera*): juvenile hormone turns on ecdysteroids. *Naturwissenschaften 82*, 378–379.

Rachinsky, A., Feldlaufer, M.F., **2000**. Responsiveness of honey bee (*Apis mellifera* L.) corpora allata to allatoregulatory peptides from four insect species. *J. Insect Physiol. 46*, 41–46.

Rachinsky, A., Hartfelder, K., **1990**. Corpora allata activity, a prime regulating element for caste-specific juvenile hormone titre in honey bee larvae (*Apis mellifera carnica*). *J. Insect Physiol. 36*, 189–194.

Rachinsky, A., Hartfelder, K., **1991**. Differential production of juvenile hormone and its deoxy precursor by corpora allata of honeybees during a critical period of caste development. *Naturwissenschaften 78*, 270–272.

Rachinsky, A., Strambi, C., Strambi, A., Hartfelder, K., **1990**. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen. Comp. Endocrinol. 79*, 31–38.

Rachinsky, A., Tobe, S.S., Feldlaufer, M.F., **2000**. Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera* L.): developmental changes in sensitivity to JH precursor and allatotropin. *Insect Biochem. Mol. Biol. 30*, 729–737.

Rahman, M.M., Vandigenen, A., Begum, M., Breuer, M., de Loof, A., *et al.*, **2003**. Search for phase specific genes

in the brain of desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) by differential display polymerase chain reaction. *Comp. Biochem. Physiol. A 135*, 221–228.

Ramos, S., Moya, A., Martínez-Torres, D., **2003**. Identification of a gene overexpressed in aphids reared under short photoperiod. *Insect Biochem. Mol. Biol. 33*, 289–298.

Rasmussen, J., **1994**. The influence of horn and body size on the reproduvctive behavior of the horned rainbow scarab beetle *Phanaeus difformis* (Coleoptera: Scarabaeidae). *J. Insect Behav. 7*, 67–82.

Ratnieks, F.L.W., **2001**. Heirs and spares: caste conflict and excess queen production in *Melipona* bees. *Behav. Ecol. Sociobiol. 50*, 467–473.

Reginato, R.D., Cruz-Landim, C., **2001**. Differentiation of the worker's ovary in *Apis mellifera* L. (Hymenoptera, Apidae) during life of the larvae. *Invert. Reprod. Devel. 39*, 127–134.

Reinhardt, R., **1969**. Über den Einfluss der Temperatur auf den Sasiondimorphismus von *Araschnia levana* L. (Lepidopt. Nymphalidae) nach photoperiodischer Diapauseinduktion. *Zool. Jb. Physiol. 75*, 41–75.

Rembold, H., **1987**. Caste-specific modulation of juvenile hormone titers in *Apis mellifera*. *Insect Biochem. 17*, 1003–1006.

Rembold, H., Czoppelt, C., Grüne, M., Lackner, B., Pfeffer, J., *et al.*, **1992**. Juvenile hormone titers during honey bee embryogenesis and metamorphosis. In: Mauchamp, B., Couillaud, F., Baehr, J.C. (Eds.), Insect Juvenile Hormone Research. INRA, Paris, pp. 37–43.

Rembold, H., Czoppelt, C., Rao, P.J., **1974a**. Effect of juvenile hormone on caste differentiation in the honey bee, *Apis mellifera*. *J. Insect Physiol. 20*, 1193–1202.

Rembold, H., Lackner, B., Geistbeck, J., **1974b**. The chemical basis of queen bee determinator from royal jelly. *J. Insect Physiol. 20*, 307–314.

Rhoden, P.K., Foster, W.A., **2002**. Soldier behaviour and division of labor in the aphid genus *Pemphigius* (Hemiptera: Aphididae). *Insectes Sociaux 49*, 257–263.

Ribeiro, M.F., **1999**. Long-duration feedings and caste differentiation in *Bombus terrestris* larvae. *Insectes Sociaux 46*, 315–322.

Ribeiro, M.F., Alves, D.A., **2001**. Size variation in *Schwarziana quadripunctata* queens (Hymenoptera, Apidae, Meliponini). *Rev. Etol. 3*, 59–65.

Ribeiro, M.F., Imperatriz-Fonseca, V.L., Santos Filho, P.S., **2002**. Exceptional high queen production in the Brazilian stingless bee *Plebeia remota*. *Stud. Neotrop. Fauna Environ. 37*, 111–114.

Ribeiro, M.F., Velthuis, H.H.W., Duchateau, M.J., van der Tweet, I., **1999**. Feeding frequency and caste differentiation in *Bombus terrestris* larvae. *Insectes Sociaux 46*, 306–314.

Robinson, G.E., Aronstein, K., Evans, J.E., Fahrbach, S.E., Johnston, S.K., *et al.*, **2002**. Proposal for the sequencing of a new target genome: white paper for a honey bee genome project. The Honey Bee Genome Sequencing Consortium. http://www.nhb.org/bee-r/HoneyBeeWhitePaper.pdf.

Robinson, G.E., Strambi, C., Strambi, A., Feldlaufer, M.F., **1991**. Comparison of juvenile hormone and ecdysteroid hemolymph titers in adult worker and queen honey bees (*Apis mellifera*). *J. Insect Physiol. 37*, 929–935.

Robinson, G.E., Strambi, C., Strambi, A., Huang, Z.-Y., **1992**. Reproduction in worker honey bees is associated with low juvenile hormone titers and rates of biosynthesis. *Gen. Comp. Endocrinol. 87*, 471–480.

Robinson, G.E., Vargo, E.L., **1997**. Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker. *Arch. Insect Biochem. Physiol. 35*, 559–583.

Roff, D.A., **1986**. The genetic basis of wing dimorphism in the sand cricket, *Gryllus firmus* and its relevance to the evolution of wing dimorphism in insects. *Heredity 57*, 221–231.

Roff, D.A., **1990**. Selection for changes in the incidence of wing dimorphism in *Gryllus firmus*. *Heredity 65*, 163–168.

Roisin, Y., **2000**. Diversity and evolution of caste patterns. In: Abe, T., Bignell, D.E., Higashi, M. (Eds.), Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers, , pp. 95–119.

Röseler, P.-F., **1970**. Unterschiede in der Kastendetermination zwischen den Hummelarten *Bombus hypnorum* und *Bombus terrestris*. *Z. Naturforsch. 25*, 543–548.

Röseler, P.-F., **1974**. Grössenpolymorphismus, Geschlechtsregulation und Stabilisierung der Kasten im Hummelvolk. In: Schmidt, G.H. (Ed.), Sozialpolymorphismus bei Insekten. Wissenschaftliche Verlagsgesellschaft, Stuttgart, pp. 298–335.

Röseler, P.-F., **1977**. Juvenile hormone control of oogenesis in bumblebee workers, *Bombus terrestris*. *J. Insect Physiol. 23*, 985–992.

Röseler, P.-F., **1985**. A technique for year-round rearing of *Bombus terrestris* (Apidae, Bombini) colonies in captivity. *Apidologie 16*, 165–170.

Röseler, P.-F., Röseler, I., **1978**. Studies on the regulation of the juvenile hormone titre in bumblebee workers, *Bombus terrestris*. *J. Insect Physiol. 24*, 707–713.

Röseler, P.-F., Röseler, I., **1986**. Caste-specific differences in fat body glycogen metabolism of the bumblebee, *Bombus terrestris*. *Insect Biochem. 16*, 501–508.

Röseler, P.F., Röseler, I., Strambi, A., **1985**. Role of ovaries and ecdysteroids in dominance hierarchy establishment among foundresses of the primitively social wasp, *Polistes gallicus*. *Behav. Ecol. Sociobiol. 18*, 9–13.

Röseler, P.F., Röseler, I., Strambi, A., Augier, R., **1984**. Influence of insect hormones on the establishment of dominance hierarchies among foundresses of the paper wasp, *Polistes gallicus*. *Behav. Ecol. Sociobiol. 15*, 133–184.

Röseler, P.-F., Röseler, I., van Honk, C.G.J., **1981**. Evidence for inhibition of corpora allata activity in

workers of *Bombus terrestris* by a pheromone from the queen's mandibular glands. *Experientia 37*, 348–351.

Röseler, P.-F., van Honk, C.G.J., **1990**. Castes and reproduction in bumblebees. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 147–166.

Roskam, J.C., Brakefield, P.M., **1999**. Seasonal polyphenism in *Bicyclus* (Lepidoptera: Satyridae) butterflies: different climates need different cues. *Biol. J. Linn. Soc. 66*, 345–356.

Rountree, D.B., Nijhout, H.F., **1995**. Genetic control of a seasonal morph in *Precis coenia* (Lepidoptera, Nymphalidae). *J. Insect Physiol. 41*, 1141–1145.

Ryan, M.J., Hews, D.W., Wagner, W.E.J., **1990**. Sexual selection on alleles that determine body size in the swordtail *Xiphophorus nigrensis*. *Behav. Ecol. Sociobiol. 26*, 231–237.

Saeki, H., **1966a**. The effect of day length on the occurrence of the macropterous form in a cricket, *Scapsipedus aspersus* Walker (Orthoptera: Gryllidae). *Jap. J. Ecol. 16*, 49–52.

Saeki, H., **1966b**. The effect of population density on the occurrence of the macropteropou form in a cricket, *Scapsipedus aspersus* Walker (Orthoptera: Gryllidae). *Jap. J. Ecol. 16*, 1–4.

Saini, R.K., Rai, M.M., Hassanali, A., Wawiye, J., Odongo, H., **1995**. Semiochemicals from froth of egg pods attract ovipositing female *Schistocerca gregaria*. *J. Insect Physiol. 41*, 711–716.

Sakagami, S.F., Maeta, Y., **1985**. Multifemale nests and rudimentary castes in the normally solitary bee *Ceratina japonica*. *J. Kansas Entomol. Soc. 57*, 639–656.

Schmidt Capella, I.C., Hartfelder, K., **1998**. Juvenile hormone effect on DNA synthesis and apoptosis in caste-specific differentiation of the larval honey bee (*Apis mellifera* L.) ovary. *J. Insect Physiol. 44*, 385–391.

Schmidt Capella, I.C., Hartfelder, K., **2002**. Juvenile-hormone-dependent interaction of actin and spectrin is crucial for polymorphic differentiation of the larval honey bee ovary. *Cell Tissue Res. 307*, 265–272.

Schneider, M., Dorn, A., **1994**. Lipid storage and mobilization by flight in relation to phase and age of *Schistocerca gregaria* females. *Insect Biochem. Mol. Biol. 24*, 883–889.

Schutze, M., Maschwitz, U., **1991**. Enemy recognition and defense within trophobiotic associations with ants by the soldier caste of *Pseudoregma sundanica* (Homoptera, Aphidoidea). *Entomol. Generalis 16*, 1–12.

Schwarz, M.P., O'Keefe, K.J., **1991**. Order of eclosion and reproductive diiferentiation in a social allodapine bee. *Ethol. Ecol. Evol. 3*, 233–245.

Schwarz, M.D., Tierney, S.M., Bull, N.J., **2002**. Long-term evolution of sociality and sex allocation in allodapine bees. In: Proc. 14th Int. Congr. IUSSI, pp. 173.

Seidel, C., Bicker, G., **1996**. The developmental expression of serotonin-immunoreactivity in the brain of the pupal honeybee. *Tissue and Cell 28*, 663–672.

Sevastopulo, D.G., **1975**. Dimorphism in *Papilio* pupae. *Entomol. Record J. Variation 87*, 109–111.

Severson, D.W., Williamson, J.L., Aiken, J.M., **1989**. Caste-specific transcription in the female honey bee. *Insect Biochem. 19*, 215–220.

Shapiro, A.M., **1976**. Seasonal polymorphism. *Evol. Biol. 9*, 259–333.

Sheppard, P.M., **1958**. Natural Selection and Heredity. Hutchinson, London.

Shuster, S.M., **1989**. Male alternative reproductive strategies in a marine isopod crustacean (*Paracerceis sculpta*): the use of genetic markers to measure diferences in fertilization success among alpha, beta, and gamma males. *Evolution 43*, 1683–1698.

Shuster, S.M., **2002**. Mating strategies, alternative. In: Pagel, M. (Ed.), Encyclopedia of Evolution, vol. 2. Oxford University Press, Oxford, pp. 688–693.

Simões, Z.L.P., Boleli, I.C., Hartfelder, K., **1997**. Occurrence of a prothoracicotropic hormone-like peptide in the developing nervous system of the honey bee (*Apis mellifera*). *Apidologie 28*, 399–409.

Simon, J.-C., Rispe, C., Sunnucks, P., **2002**. Ecology and evolution of sex in aphids. *Trends Ecol. Evol. 17*, 34–39.

Sims, S.R., **1983**. The genetic and environmental basis of pupal colour dimorphism in *Papilio zelicaon* (Lepidoptera: Papilionidae). *Heredity 50*, 159–168.

Sledge, M.F., Boscaro, F., Turillazzi, S., **2001**. Cuticular hydrocarbons and reproductive status in the social wasp *Polistes dominulus*. *Behav. Ecol. Sociobiol. 49*, 401–409.

Sloggett, J.J., Weisser, W.W., **2002**. Parasitoids induce production of dispersal morph of the aphid *Acyrthosiphon pisum*. *Oikos 98*, 323–333.

Smith, A.G., **1978**. Environmental factors influencing pupal colour determination in Lepidoptera. 1. Experiments with *Papilio polytes, Papilio demoleus* and *Papilio polyxenes*. *Proc. Roy. Soc. B 200*, 295–329.

Smith, A.G., **1980**. Environmental factors influencing pupal colour determination in Lepidoptera. 2. Experiments with *Pieris rapae, Pieris napi* and *Pieris brassicae*. *Proc. Roy. Soc. B 207*, 163–186.

Smith, D.A., Shoesmith, E.A., Smith, A.G., **1988**. Pupal polyphenism in the butterfly *Danaus chrysippus* (L): environmental, seasonal and genetic influences. *Biol. J. Linn. Soc. 33*, 17–50.

Smith, K.C., **1991**. The effects of temperature and daylength on the rosa polyphenism in the buckeye butterfly, *Precis coenia* (Lepidoptera: Nymphalidae). *J. Res. Lepidoptera 30*, 225–236.

Sommer, K., Hölldobler, B., Rembold, H., **1993**. Behavioral and physiological aspects of reproductive control in a *Diacamma* species from Malaysia (Formicidae, Ponerinae). *Ethology 94*, 162–170.

Srihari, T., Gutmann, E., Novak, V.J.A., **1975**. Effect of ecdysterone and juvenoid on the developmental involution of flight muscles in *Acheta domestica*. *J. Insect Physiol. 21*, 1–8.

Staal, G.B., de Wilde, J., **1962**. Endocrine influences on the development of phase characters in *Locusta*. *Coll. Int. Centre Natl Recherches Scientifique 114*, 89–105.

Starnecker, G., **1997**. Hormonal control of lutein incorporation into pupal cuticle of the butterfly *Inachis io* and the pupal melanization reducing factor. *Physiol. Entomol. 22*, 73–78.

Starnecker, G., Bückmann, D., **1997**. Temporal occurrence of pupal melanization reducing factor during development of the butterfly *Inachis io*. *Physiol. Entomol. 22*, 79–85.

Starnecker, G., Hazel, W.N., **1999**. Convergent evolution of neuroendocrine control of phenotypic plasticity in pupal colour in butterflies. *Proc. Roy. Soc. B 266*, 2409–2412.

Stern, D.L., Whitfield, J.A., Foster, W.A., **1997**. Behavior and morphology of monomorphic soldiers from the aphid genus *Pseudoregma* (Cerataphidini, Hormaphididae): implications for the evolution of morphological castes in social aphids. *Insectes Sociaux 44*, 379–392.

Strambi, A., **1990**. Physiology and reproduction in social wasps. In: Engels, W. (Ed.), Social Insects: An Evolutionary Approach to Castes and Reproduction. Springer-Verlag, Heidelberg, pp. 59–75.

Strambi, A., Strambi, C., Röseler, P.-F., Röseler, I., **1984**. Simultaneous determination of juvenile hormone and ecdysteroid titers in the hemolymph of bumblebee prepupae (*Bombus hypnorum* and *B. terrestris*). *Gen. Comp. Endocrinol. 55*, 83–88.

Strand, M.R., Grbic, M., **1997**. The development and evolution of polyembryonic insects. *Curr. Topics Devel. Biol. 35*, 121–159.

Strand, M.R., Johnson, J.A., Culin, J.D., **1990**. Intrinsic interspecific competition between the polyembryonic parasite *Copidosoma floridanum* and solitary endoparasite *Microplitis demolitor* in *Pseudoplusia includens*. *Entomol. Exp. Applic. 55*, 275–284.

Süffert, F., **1924**. Bestimmungsfaktoren des Zeichnungsmusters beim Saisondimorphismus von *Araschnia levana-prorsa*. *Biol. Zentralblatt 44*, 173–188.

Sullivan, J.P., Jassim, O., Fahrbach, S.E., Robinson, G.E., **2000**. Juvenile hormone paces behavioral development in the adult worker honey bee. *Horm. Behav. 37*, 1–14.

Suzzoni, J.P., Passera, L., Strambi, A., **1980**. Ecdysteroid titer and caste determination in the ant *Pheidole pallidula* (Nyl) (Hymenoptera, Formicidae). *Experientia 36*, 1228–1229.

Suzzoni, J.P., Passera, L., Strambi, A., **1983**. Ecdysteroid production during caste differentiation in larvae of the ant *Plagiolepis pygmaea*. *Physiol. Entomol. 8*, 93–96.

Tanaka, S., **2000a**. Hormonal control of body-color polymorphism in *Locusta migratoria*: interaction between [His^7]-corazonin and juvenile hormone. *J. Insect Physiol. 46*, 1535–1544.

Tanaka, S., **2000b**. Induction of darkening by corazonins in several species of Orthoptera and their possible presence in ten insect orders. *Appl. Entomol. Zool. 35*, 509–517.

Tanaka, S., **2000c**. The role of [His^7]-corazonin in the control of body-color polymorphism in the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae). *J. Insect Physiol. 46*, 1169–1176.

Tanaka, S., **2001**. Endocrine mechanisms controlling body-color polymorphism in locusts. *Arch. Insect Biochem. Physiol. 47*, 139–149.

Tanaka, S., Matsuka, M., Sakai, T., **1976**. Effect of change in photoperiod on wing form in *Pteronemobius taprobanensis* (Orthoptera: Gryllidae). *Appl. Entomol. Zool. 11*, 27–32.

Tanaka, S., Pener, M.P., **1994**. A neuropeptide controlling the dark pigmentation in color polymorphism of the migratory locust, *Locusta migratoria*. *J. Insect Physiol. 40*, 997–1005.

Tanaka, S., Wolda, H., **1987**. Seasonal wing dimorphism in a tropical seed bug: ecological significance of the short-winged form. *Oecologia 73*, 559–565.

Tanaka, S., Zhu, D.H., Hoste, B., Breuer, M., **2002**. The dark-color inducing neuropeptide, [His^7]-corazonin, causes a shift in morphometric characteristics towards the gregarious phase in isolated-reared (solitarious) *Locusta migratoria*. *J. Insect Physiol. 48*, 1065–1074.

Tawfik, A.I., Mathova, A., Sehnal, F., Ismail, S.H., **1996**. Haemolymph ecdysteroids in the solitary and gregarious larvae of *Schistocerca gregaria*. *Arch. Insect Biochem. Physiol. 31*, 427–438.

Tawfik, A.I., Osir, E.O., Hassanali, A., Ismail, S.H., **1997a**. Effects of juvenile hormone treatment on phase changes and pheromone production in the desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae). *J. Insect Physiol. 43*, 1177–1182.

Tawfik, A.I., Sehnal, F., **2003**. A role for ecdysteroids in the phase polymorphism of the desert locust. *Physiol. Entomol. 28*, 19–24.

Tawfik, A.I., Treiblmayr, K., Hassanali, A., Osir, E.O., **2000**. Time-course haemolymph juvenile hormone titres in solitarious and gregarious adults of *Schistocerca gregaria*, and their relation to pheromone emission, CA volumetric changes and oocyte growth. *J. Insect Physiol. 46*, 1143–1150.

Tawfik, A.I., Vedrova, A., Li, W.W., Sehnal, F., ObengOfori, D., **1997b**. Haemolymph ecdysteroids and the prothoracic glands in the solitary and gregarious adults of *Schistocerca gregaria*. *J. Insect Physiol. 43*, 485–493.

Tawfik, A.I., Vedrova, A., Sehnal, F., **1999**. Ecdysteroids during ovarian development and embryogenesis in solitary and gregarious *Schistocerca gregaria*. *Arch. Insect Biochemi. Physiol. 41*, 134–143.

Thornhill, R., Alcock, J., **1983**. The Evolution of Insect Mating Systems. Belknapp Press of Harvard University Press, Cambridge, MA.

Tobe, S.S., Pratt, G.E., **1974**. The influence of substrate concentrations on the rate of insect juvenile hormone biosynthesis by corpora allata of the desert locust *in vitro*. *Biochem. J. 144*, 107–113.

Tomkins, J.L., **1999**. Environmental and genetic determinants of the male forceps length dimorphism in the European earwig *Forficula auricularia* L. *Behav. Ecol. Sociobiol. 47*, 1–8.

Tomkins, J.L., Simmons, L.W., **1996**. Dimorphism and fluctuating asymmetry in the forceps of male earwigs. *J. Evol. Biol. 9*, 753–770.

Tomkins, J.P., Luo, M., Fang, G.C., Main, D., Goicoechea, J.L., *et al.*, **2002**. New genomic resources for the honey bee (*Apis mellifera* L.): development of a deep-coverage BAC library and a preliminary STC database. *Genet. Mol. Res. 1*, 306–316.

Truman, J.W., Riddiford, L.M., **1999**. The origin of insect metamorphosis. *Nature 401*, 447–452.

Tschinkel, W.R., **1991**. Sociometry, a field in search of data. *Insectes Sociaux 34*, 143–164.

Tschinkel, W.R., Mikheyev, A.S., Storz, S.R., **2003**. Allometry of workers of the fire ant, *Solenopsis invicta*. *J. Insect Sci. 3.2*, 11.

Tyndale-Biscoe, M., **1996**. Australia's introduced dung beetles: original releases and redistribution. Technical Report No. 62. CSIRO, Division of Entomology, Canberra, ACT.

Uvarov, B.P., **1921**. A revision of the genus *Locusta* L. (=*Pachytylus* Fieb.), with a new theory as to periodicity and migrations of locusts. *Bull. Entomol. Res. 12*, 135–163.

Vargo, E.L., Laurel, M., **1994**. Studies on the mode of action of a queen primer pheromone of the fire ant *Solenopsis invicta*. *J. Insect Physiol. 40*, 601–610.

Vinson, S.B., Robeau, R., **1974**. Insect growth regulators: effects on colonies of the imported fire ant. *J. Econ. Entomol. 67*, 584–587.

Volny, V., Gordon, D.M., **2002**. Genetic basis for queen–worker dimorphism in a social insect. *Proc. Natl Acad. Sci. USA 99*, 6108–6111.

Watt, W.B., **1968**. Adaptive significance of pigment polymorphisms in *Colias* butterflies. 1. Variation of melanin pigment in relation to thermoregulation. *Evolution 22*, 437–458.

Watt, W.B., **1969**. Adaptive significance of pigment polymorphisms in *Colias* butterflies. 2. Thermoregulation and photoperiodically controlled melanin variation in *Colias eurytheme*. *Proc. Natl Acad. Sci. USA 63*, 767–774.

Weatherbee, S.D., Nijhout, H.F., Grunert, L.W., Haldeer, G., Galant, R., *et al.*, **1999**. Ultrabithorax function in butterfly wings and the evolution of insect wing patterns. *Curr. Biol. 9*, 109–115.

Wedekind-Hirschberger, S., Sickold, S., Dorn, A., **1999**. Expression of phase-specific haemolymph polypeptides in a laboratory strain and field catches of *Schistocerca gregaria*. *J. Insect Physiol. 45*, 1097–1103.

Weismann, A., **1875**. Studien zur Deszendenztheorie. 1. Über den Saisondimorphismus der Schmetterlinge. Engelmann, Leipzig.

West, D.A., Hazel, W.N., **1979**. Natural pupation sites of swallowtail butterflies (Lepidoptera: Papilionidae): *Papilio polyxenes* Fabr., *P. glaucus* L., and *Battus philenor* L. *Ecol. Entomol. 4*, 387–392.

West, D.A., Hazel, W.N., **1982**. An experimental test of natural selection for pupation site in swallowtail butterflies. *Evolution 36*, 152–159.

West, D.A., Hazel, W.N., **1985**. Pupal colour dimorphism in swallowtail butterflies: timing of the sensitive period and environmental control. *Physiol. Entomol. 10*, 113–119.

West, D.A., Hazel, W.N., **1996**. Natural pupation sites of three North American swallowtail butterflies: *Eurytides marcellus* (Cramer), *Papilio cresphontes* Cramer, and *P. troilus* L. (Papilionindae). *J. Lepidopt. Soc. 50*, 297–302.

West, D.A., Snelling, W.N., Herbeck, T.A., **1972**. Pupal colour dimorphism and its environmental control in *Papilio polyxenes asterias* Stoll (Lep. Papilionidae). *J. New York Entomol. Soc. 80*, 205–211.

West-Eberhard, M.J., **1996**. Wasp societies as microcosms for the study of development and evolution. In: Turillazzi, S., West-Eberhard, M.J. (Eds.), Natural History and Evolution of Paper Wasps. Oxford University Press, Oxford, pp. 290–317.

West-Eberhard, M.J., **2003**. Developmental Plasticity and Evolution. Oxford University Press, Oxford.

Wheeler, D.E., **1983**. Soldier determination in *Pheidole bicatinata*: effect of methoprene on caste and size within castes. *J. Insect Physiol. 29*, 847–854.

Wheeler, D.E., **1984**. Soldier determination in *Pheidole bicatinata*: inhibition by adult soldiers. *J. Insect Physiol. 30*, 127–135.

Wheeler, D.E., **1986**. Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. *Am. Nat. 128*, 13–34.

Wheeler, D.E., **1990**. The developmental basis of worker polymorphism in fire ants. *J. Insect Physiol. 36*, 315–322.

Wheeler, D.E., **1991**. The developmental basis of worker caste polymorphism in ants. *Am. Nat. 138*, 1218–1238.

Wheeler, D.E., **1994**. Nourishment in ants: patterns in individuals and societies. In: Hunt, J., Nalepa, C. (Eds.), Nourishment and Evolution in Insect Societies. Westview Press, Boulder, CO, pp. 245–278.

Whitfield, C.W., Band, M.R., Bonaldo, M.F., Kumar, C.G., Liu, L., *et al.*, **2002**. Annotated expressed sequence tags and cDNA microarrays for studies of brain and behaviour in the honey bee. *Genome Res. 12*, 555–566.

Wiklund, C., **1972**. Pupal colour polymorphism in *Papilio machaon* L. in response to wavelenght of light. *Naturwissenschaften 59*, 219.

Wiklund, C., **1975**. Pupal colour polymorphism in *Papilio machaon* L., and the survival of cryptic versus non-cryptic pupae. *Trans. Roy. Entomol. Soc. London 127*, 73–84.

Wiesel, G., Tappermann, S., Dorn, A., **1996**. Effects of juvenile hormone and juvenile hormone analogues on the phase behaviour of *Schistocerca gregaria* and *Locusta migratoria*. *J. Insect Physiology 42*, 385–395.

Wilson, E.O., **1971**. The Insect Societies. Belknapp Press of Harvard University Press, Cambridge, MA.

Wilson, E.O., **1987**. The earliest known ants: an analysis of the Cretaceous species and an inference on their social organization. *Paleobiology 13*, 44–53.

Windig, J.J., Brakefield, P.M., Reitsma, N., Wilson, J.G.M., **1994**. Seasonal polyphenism in the wild: survey of wing patterns in five species of *Bicyclus* butterflies in Malawi. *Ecol. Entomol. 19*, 285–298.

Winter, U., Buschinger, A., **1986**. Genetically mediated queen polymorphism and caste determination in the slave-making ant, *Harpagoxenus sublaevis* (Hymenoptera: Formicidae). *Entomol. Generalis 11*, 125–137.

Wood, T.W., **1867**. Remarks on the coloration of Chrysalides. *Proc. Roy. Soc. 1867*, 98–101.

Wourms, M.K., Wassermann, F.E., **1985**. Butterfly wing markings are more advantageous during handling than during the initial strike of an avian predator. *Evolution 39*, 845–851.

Yanega, C., **1989**. Caste determination and differential diapause within the first brood of *Halictus rubicundus*. *Behav. Ecol. Sociobiol. 24*, 97–107.

Zera, A.J., Denno, R.F., **1997**. Physiology and ecology of dispersal polymorphism in insects. *Annu. Rev. Entomol. 42*, 207–230.

Zera, A.J., Holtmeier, C.L., **1992**. *In vivo* and *in vitro* degradation of juvenile hormone-III in presumtive long-winged and short-winged *Gryllus rubens*. *J. Insect Physiol. 38*, 61–74.

Zera, A.J., Strambi, C., Tiebel, K.C., Strambi, A., Rankin, M.A., **1989**. Juvenile hormone and ecdysteroid titers during critical periods of wing determination in *Gryllus rubens*. *J. Insect Physiol. 35*, 501–511.

Zera, A.J., Tiebel, K.C., **1988**. Brachypterizing effect of group rearing, juvenile hormone III and methoprene in the wing-dimorphic cricket, *Gryllus rubens*. *J. Insect Physiol. 34*, 489–498.

Zera, A.J., Tiebel, K.C., **1989**. Differences in juvenile hormone esterase activity between presumptive macropterous and brachypterous *Gryllus rubens*: implications for the hormonal control of wing polymorphism. *J. Insect Physiol. 35*, 7–17.

Zera, A.J., Tobe, S.S., **1990**. Juvenile hormone-III biosynthesis in presumtive long-winged and short-winged *Gryllus rubens*: implications for the endocrine regulation of wing polymorphism. *J. Insect Physiol. 36*, 271–280.

Zera, A.J., Zhao, Z.W., **2003**. Life-history evolution and the microevolution of intermediary metabolism: activities of lipid-metabolizing enzymes in life-history morphs of a wing-dimorphic cricket. *Evolution 57*, 586–596.

Zhao, Z.W., Zera, A.J., **2002**. Differential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-dimorphic cricket. *Proc. Natl Acad. Sci. USA 99*, 16829–16834.

Zimmerer, E.J., Kallmann, K.D., **1989**. The genetic basis for alternative reproductive tactics in the pygmy swordtail, *Xiphophorus nigrensis*. *Evolution 43*, 1298–1307.

Zufelato, M.S., Bitondi, M.M.G., Simões, Z.L.P., Hartfelder, K., **2000**. The juvenile hormone analog pyriproxyfen affects ecdysteroid-dependent cuticle melanization and shifts the pupal ecdysteroid peak in the honey bee (*Apis mellifera*). *Arthropod Struct. Devel. 29*, 111–119.

3.14 Biochemistry and Molecular Biology of Pheromone Production

G J Blomquist, University of Nevada, Reno, NV, USA
R Jurenka, Iowa State University, Ames, IA, USA
C Schal, North Carolina State University, Raleigh, NC, USA
C Tittiger, University of Nevada, Reno, NV, USA

3.14.1. Introduction and Overview

The elucidation of the structure of the first insect sex pheromone, bombykol ((*E,Z*)-10,12-hexadecadien-1-ol) (Butenandt *et al*., 1959) (**Figure 1**), from the silkworm moth, *Bombyx mori*, spanned more than 20 years and required a half million female abdomens. A few years later, (*Z*)-7-dodecenyl acetate (**Figure 1**) was identified as the sex pheromone of the cabbage looper, *Trichoplusia ni* (Berger, 1966). At about the same time Silverstein *et al*. (1966) identified three terpenoid alcohols, ipsenol, ipsdienol, and verbenol (**Figure 1**), as the pheromone of the bark beetle, *Ips paraconfusus*. This latter finding led to the recognition that most insect pheromones consisted of multicomponent blends. This has since been shown to be true for most insects, while single-component pheromones are rare. Rapid improvements in analytical instrumentation and techniques reduced the number of insects needed for pheromone extracts from a half million or more to where now individual insects can sometimes provide sufficient material for chemical analysis. Over the last four decades, extensive research on insect pheromones has resulted in the chemical and/or behavioral elucidation of pheromone components from well over 3000 insect species, with much of the work concentrating on sex pheromones from economically important pests.

An early issue addressed in pheromone production was the origin of pheromone components. Ultimately, all precursors for pheromone biosynthesis can be traced through dietary intake. A question asked in several systems was whether pheromone components were derived from dietary components that were altered only minimally, or whether they were synthesized *de novo*. This simple question proved surprisingly difficult to answer, and different answers were obtained for different groups of insects. It is now clear that most insect pheromone components are synthesized by insect tissue, with a

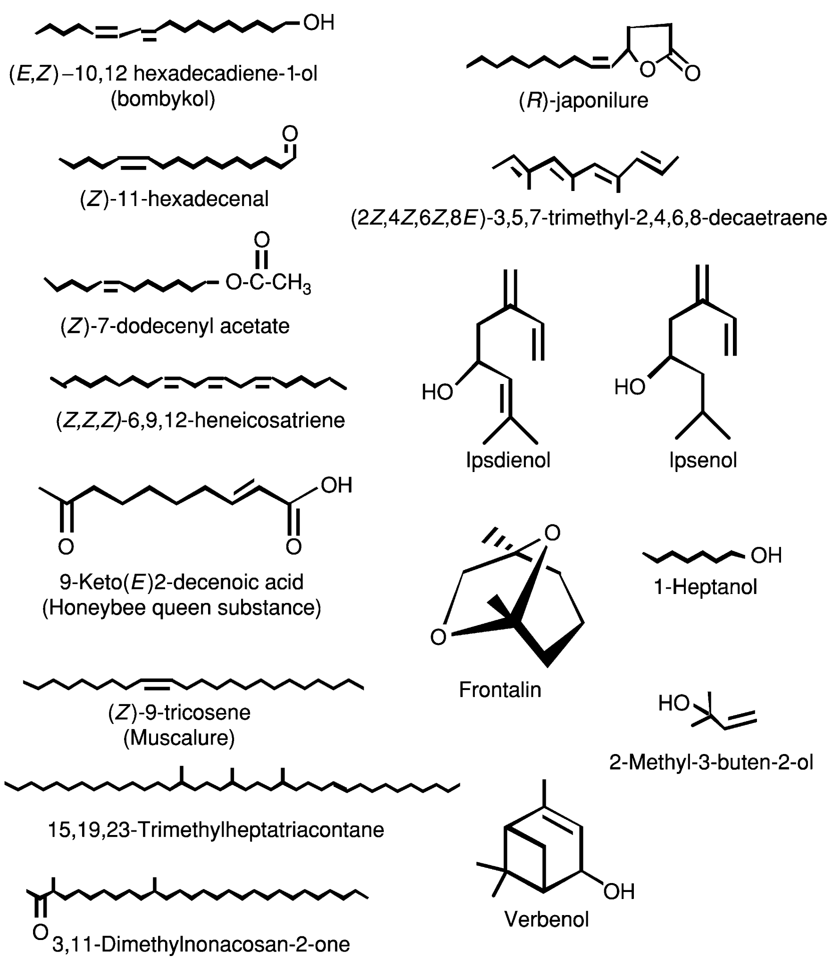


Figure 1 Selected pheromone components representing fatty acid, hydrocarbon, and isoprenoid-derived components. Components were selected based on historical interest and work performed on their biosynthesis.

number of notable exceptions (reviews: Tillman *et al.*, 1999; Eisner and Meinwald, 2003).

By the mid-1980s, it had became apparent that the products of normal metabolism, particularly those of the fatty acid and isoprenoid pathways, were modified by a few pheromone-tissue specific enzymes to produce the myriad of pheromone molecules. The elegant work of the Roelofs laboratory (review: Bjostad *et al.*, 1987) demonstrated that many of the lepidopteran pheromones could be formed by the appropriate interplay of highly selective chain shortening and unique Δ11 and other desaturases, followed by modification of the carboxyl carbon. This work has been extended, and there now exists a clear understanding of the biosynthetic pathways for many of the lepidopteran pheromones (review: Jurenka, 2003). The honeybee also uses highly specific chain shortening of fatty acids to produce the major component of the queen pheromone (Plettner *et al.*, 1996, 1998). In some insects, fatty acid elongation followed by decarboxylation produces the hydrocarbon and hydrocarbon-derived pheromones (review: Tillman *et al.*, 1999). Recent work in bark beetles has shown that *Ips* and *Dendroctonus* spp. produce their monoterpenoid-derived pheromones ipsenol, ipsdienol, and frontalin by modification of isoprenoid pathway products (review: Seybold and Tittiger, 2003).

The work on the biosynthesis and endocrine regulation of pheromone production has emphasized sex and aggregation pheromones in lepidopteran, coleopteran, dipteran, and blattodean models. Research on representative species from these orders was motivated by their economic importance, the relatively large amount of pheromone produced by some members, and extension of ongoing studies on hydrocarbon and fatty acid biosynthesis in some species.

The production and/or release of sex pheromones are influenced by a variety of environmental factors (Shorey, 1974). In general, insects do not release

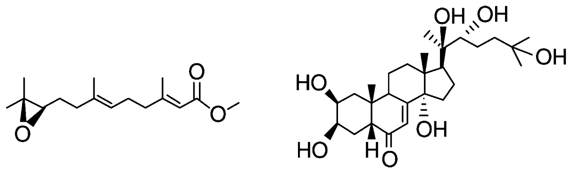


Figure 2 Structures of juvenile hormone (JH) III and 20-hydroxyecdysone (20E). The other major hormone that regulates pheromone production, PBAN, is shown in **Figure 10**.

pheromones until they are reproductively competent, although exceptions occur. Pheromone production is usually age related and coincides with the maturation of ovaries or testes, and in some cases with feeding. The observation that females of certain species have repeated reproductive cycles and that mating occurs only during defined periods of each cycle led to the proposal that pheromone production might be under hormonal control (Barth, 1965). Early work on cockroaches established that females require the presence of functional corpora allata (CA) in order to produce sex pheromone. It is now recognized that juvenile hormone (JH) (**Figure 2**) regulates pheromone production in a number of species, especially among beetles (review: Seybold and Vanderwel, 2003) and cockroaches (review: Schal *et al.*, 2003).

A unifying theme of this work on cockroaches and beetles was that the same hormone that regulated ovarian maturation (JH) also regulated pheromone production, coordinating sexual maturity with mating. Thus, in retrospect, it was not surprising that ovarian-produced 20-hydroxyecdysone (20E) (**Figure 2**), which plays an important role in reproduction in female Diptera, is also the key hormone inducing sex pheromone production in the female housefly, *Musca domestica* (review: Blomquist, 2003) and *Drosophila* (Wicker and Jallon, 1995a, 1995b).

It was recognized by the mid-1980s that female moths regulated pheromone production through a different mechanism than flies, cockroaches, and beetles (Raina and Klun, 1984), but it was not until 1989 that the structure of the pheromone biosynthesis activating neuropeptide (PBAN) was elucidated (Raina *et al.*, 1989; Raina, 1993) and work is ongoing deciphering its mode of action (review: Rafaeli and Jurenka, 2003). In some species, there does not appear to be a hormone regulating pheromone production.

3.14.2. Pheromone Chemistry

The pheromones of over 3000 insect species are now known. The website Pherolist is an up-to-date compilation of lepidopteran pheromones and lists over 500 compounds identified from over 1700 lepidopteran species. These numbers are a huge increase from the 80 pheromone components from about 120 lepidopterous species known in 1985 (Tamaki, 1985) and illustrate both the rapid growth in pheromone chemistry and the increased ease with which pheromones are identified. The book *Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants* (Hardie and Minks, 1999) discusses the pheromones of a number of pest species other than lepidopterans along with pheromones of beneficial insects. The *Handbook of Insect Pheromones and Sex Attractants* (Mayer and McLaughlin, 1991) contains a complete list of the pheromones identified from insects up to 1991.

The majority of lepidopteran pheromone components arise from fatty acids, and have carbon numbers from 7 to 29, with 12, 14, and 16 carbon components predominating. Of the 540 compounds included on the Pherolist website in 2003, 377 (70%) have an even number of carbons and 185 (34%) are acetate esters. The chemical classes alcohols, aldehydes, hydrocarbons, and epoxides each have between 65 and 85 components represented. Many of the lepidopteran pheromones have one or more double bonds with specific blends of (*Z*) and (*E*) isomers.

Many of the dipteran pheromones are hydrocarbons of 21 carbons and longer, and function as short range or contact pheromones. The pheromone of the housefly consists of (*Z*)-9-tricosene (**Figure 1**) (Carlson *et al.*, 1971), an epoxide and ketone derived from this hydrocarbon, and methyl-branched alkanes (review: Blomquist, 2003). Among the longest chain pheromones are the contact pheromones from the tsetse flies, which are often multimethyl branched hydrocarbons with up to 40 carbons (**Figure 1**) (Carlson *et al.*, 1978, 1984, 1998; Carlson and Schlein, 1991). The role of hydrocarbons in chemical communication in insects has become increasingly recognized over the past two decades (Howard, 1993).

A number of coleopteran pheromones are isoprenoid in origin (Francke *et al.*, 1995). The shortest chain pheromone described is 2-methyl-3-buten-2-ol from *Ips typographus* (Lanne *et al.*, 1989). Many of the components either contain multiples of 5 carbons or are derived from such precursors. **Figure 1** shows a number of pheromone components that are of isoprenoid origin, and include ipsdienol, ipsenol, and frontalin. Frontalin contains 8 carbons but is clearly isoprenoid in origin (Barkawi *et al.*, 2003). In addition to isoprenoids, the pheromones from coleopterans also consist of components derived from fatty acids and components of unknown orgin.

Many of the 4000+ described species of cockroaches use long-range volatile pheromones in mate-finding and cuticular contact pheromones in the final recognition process (Gemeno and Schal, 2004). Short-range volatile pheromones emitted by the male, coupled with nuptial tergal secretions, facilitate proper alignment of the pair prior to copulation (Nojima *et al.*, 1999). Pheromones also mediate intrasexual conflicts, especially when males establish dominance hierarchies and territories (Moore and Moore, 1999), and other behaviors. However, to date, few chemical structures have been elucidated, and the biosynthetic pathway of only one pheromone, the contact pheromone of the German cockroach, *Blattella germanica* has been described (major component is 3,11-dimethylnonacosan-2-one) (**Figure 1**) (Nishida *et al.*, 1974; Nishida and Fukami, 1983). Of particular note are the volatile sex pheromones of species in the genera *Periplaneta* and *Blatta* (periplanones) and 5-(2*R*,4*R*-dimethylheptyl)-3-methyl-2*H*-pyran-2-one (supellapyrone), the single-component sex pheromone of the brown-banded cockroach, *Supella longipalpa* (Charlton *et al.*, 1993; Leal *et al.*, 1995). Nevertheless, several cockroach pheromones have been investigated using behavioral and electrophysiological assays of crude body extracts. Interestingly, the pheromones of cockroach species that have served in important early studies on pheromone regulation (e.g., *Byrsotria fumigata*, *Blaberus* spp.) (see Barth, 1965; Barth and Lester, 1973) have yet to be identified.

3.14.3. Site of Pheromone Biosynthesis

There is much variability among insects in the anatomical location of pheromone production, just as there are many differences in the gross morphology and function of pheromone producing tissue. Complexity varies from simple unicellular glands distributed throughout the integument to elaborate internal cellular aggregates connected to a reservoir. Of the orders emphasized in this chapter (Lepidoptera, Coleoptera, Blattodea, and Diptera), the most common location for pheromone production is the abdomen. There are a number of excellent reviews of the ultrastructure of exocrine cells in general (Percy-Cunningham and MacDonald, 1987; Quennedey, 1998; Ma and Ramaswamy, 2003) and social insects in particular (Billen and Morgan, 1998). Definitive proof that pheromone production and release occur in certain tissues comes from studies where the isolated tissue has been shown to incorporate labeled precursors into pheromone components.

3.14.3.1. Location of Pheromone Production in Lepidoptera

The oxygenated lepidopteran pheromone components are usually produced and released from extrudable glands located between the 8th and 9th abdominal segments (Percy-Cunningham and MacDonald, 1987; Ma and Ramaswamy, 2003). The secretory cells in these glands typically contain a well-developed endoplasmic reticulum that is involved in fatty acid metabolism. Extensive studies examining the pheromone products and precursors from these glands in a number of species show that unusual specific fatty acids that had the same carbon number, double bond positions, and stereochemistry as the acetate, alcohol, or acetate ester pheromone components were present. The role of these glands in pheromone production has been clearly demonstrated with radiochemical studies (Bjostad *et al.*, 1987; Jurenka, 2003). Although a large number of moths utilize a gland located between the 8th and 9th segments, exceptions occur. It was shown in *Theresimima ampelophaga* (Zygaenidae) that the gland is located on the dorsal part of the 3–5th abdominal segments (Hallberg and Subchev, 1997).

In contrast, the site of synthesis of the hydrocarbon and hydrocarbon-derived pheromone components is more complicated. 2-Methylheptadecane is not synthesized in the pheromone gland of *Holomelina aurantiaca*, but rather is synthesized by epidermal tissue, transported by lipophorin, and then sequestered into and released from the pheromone gland (Schal *et al.*, 1998a). A similar situation exists for the gypsy moth, *Lymantra dispar*, in which the hydrocarbon precursor is synthesized in epidermal tissue, then transported by lipophorin to the pheromone gland where it is epoxidized and released (see Section 3.14.4.3.6) (Jurenka *et al.*, 2003).

3.14.3.2. Site of Pheromone Biosynthesis in Coleoptera

In many coleopteran species, pheromone production localizes to the abdomen, e.g., among the Scarabaeidae (Tada and Leal, 1997), Anobiidae (Levinson *et al.*, 1983), Dermestidae (Barak and Burkholder, 1977), and others (review: Plarre and Vanderwel, 1999; Tillman *et al.*, 1999). In some cases there are defined glands, whereas in others, groups of cells in defined anatomical locations may not form a gland, but are nevertheless a localized site of pheromone biosynthesis. Other anatomical locations for pheromone production have also been identified for some species. These include putative pheromone glands on the antennae of *Batrisodes oculatus* (de Mazo

and Vit, 1983) and on the forelimbs of *Tribolium castaneum* (Faustini *et al*., 1982).

Pheromone biosynthetic cells of many beetles are generally dermally derived, with one notable exception. Monoterpenoid pheromone components have been reported to be synthesized in the fat body of male boll weevils, *Anthonomus grandis* (Wiygul *et al*., 1990). The fat body is an unexpected location for boll weevil pheromone biosynthesis, because it requires transport of pheromone components into the lumen of the alimentary canal for release. This is unusual, because other pheromone glands and cells tend to be physically linked to release sites either by proximity or by specialized structures such as ducts or ductules (review: Ma and Ramaswamy, 2003). Recent work (Nural, Tittiger, Fu and Blomquist, unpublished data) demonstrate that isolated gut tissue from male *A. grandis* incorporated labeled acetate into components that coeluted on high-performance liquid chromatography (HPLC) with the four C_{10} pheromone components. It is difficult to obtain *A. grandis* gut tract tissue free from adhering fat body, making it impossible to conclude with certainity that fat body tissue is not involved. The clear demonstration that several bark beetles synthesize their frass-associated isoprenoid pheromone components in midgut tissue (see below) along with the data from *A. grandis* suggests that frass-associated pheromones in general may be produced in gut tissue.

The site of *de novo* monoterpenoid pheromone biosynthesis in bark beetles (especially *Ips* and *Dendroctonus* spp.) has been clearly demonstrated. Early work localized the pheromone and its release to the hindgut (Bordon *et al*., 1969; Byers, 1983), but did not determine the site of synthesis. Pheromone component precursors were localized in the abdomen–thorax border (Ivarsson *et al*., 1998), and the anterior midgut was determined as the site of *de novo* synthesis through the use of molecular probes to 3-hydroxy-3-methylglutaryl-CoA (HMG-R) reductase and radiotracer studies (**Figure 3**) (Hall *et al*., 2002a, 2002b). Electron microscopy studies show that pheromone-biosynthetic midgut cells have crystalline arrays of smooth endoplasmic reticulum, while nonpheromone-producing cells do not (Nardi *et al*., 2002). These studies also confirmed that bark beetle pheromones are synthesized by insect tissues, and not by microorganisms, as had been suggested by earlier studies (Brand *et al*., 1975, 1976). Pheromone components are secreted into the gut lumen through an apocrine mechanism, and all anterior midgut cells appear to be involved. Pheromone-biosynthetic cells are typically dedicated to that function, but anterior midgut cells of bark beetles apparently fulfill the dual roles of pheromone production and food digestion (Nardi *et al*., 2002).

Why midgut tissue? In considering the evolution of pheromone components, it is generally thought that pheromone molecules, as other signaling chemicals, originally had another purpose and were co-opted for signal function. Components were then shaped by selective forces acting on preexisting structures, and pheromones evolved so that the signal would be stronger and more easily discriminated. For bark beetles, it is thought that monoterpene components arose from the detoxification of oleoresin tree defensive components, and that many of the detoxification reactions involved hydroxylation reactions (Wood, 1982; Vanderwel and Oehlschlager, 1987). To lessen dependence upon host chemicals and increase host range, bark beetles then apparently evolved the ability to synthesize their monoterpenoid pheromones *de novo*, and with this, evolved the JH III regulation of pheromone production. As the ancient beetles chewed through the bark and phloem, they encountered tree terpenoids. Gut tissue was the first line of defense, and detoxification enzymes (cytochrome P450 hydoxylases) could have evolved to detoxify monoterpenes in this tissue. As *de novo* synthesis evolved, it is possible that production of pheromone arose in midgut tissue so that the hydroxylation reactions that were already present could be used.

3.14.3.3. Site of Pheromone Production in Diptera

The hydrocarbon pheromone components of many Diptera evolved from, and still function as, cuticular lipids. Thus, it is expected that these pheromone components are synthesized in the same specialized epidermal cells (oenocytes) that produce cuticular hydrocarbons. After synthesis, pheromone components are secreted into the hemolymph, where they are transported by lipophorin, as has been demonstrated in *Drosophila* (Ferveur *et al*., 1997) and the housefly (Schal *et al*., 2001), before being deposited on the cuticular surface. During the process of grooming, relatively large amounts of (*Z*)-9-tricosene accumulate on the legs of female houseflies (Dillwith and Blomquist, 1982). The mechanism of how hydrocarbon pheromones, and hydrocarbons in general, are unloaded from lipophorin and transported across the cuticle is not known.

3.14.3.4. Site of Pheromone Production in Blattodea

As in Coleoptera, early reports considered various organs in the head, thorax, and abdomen as possible sites of pheromone production in cockroaches, but

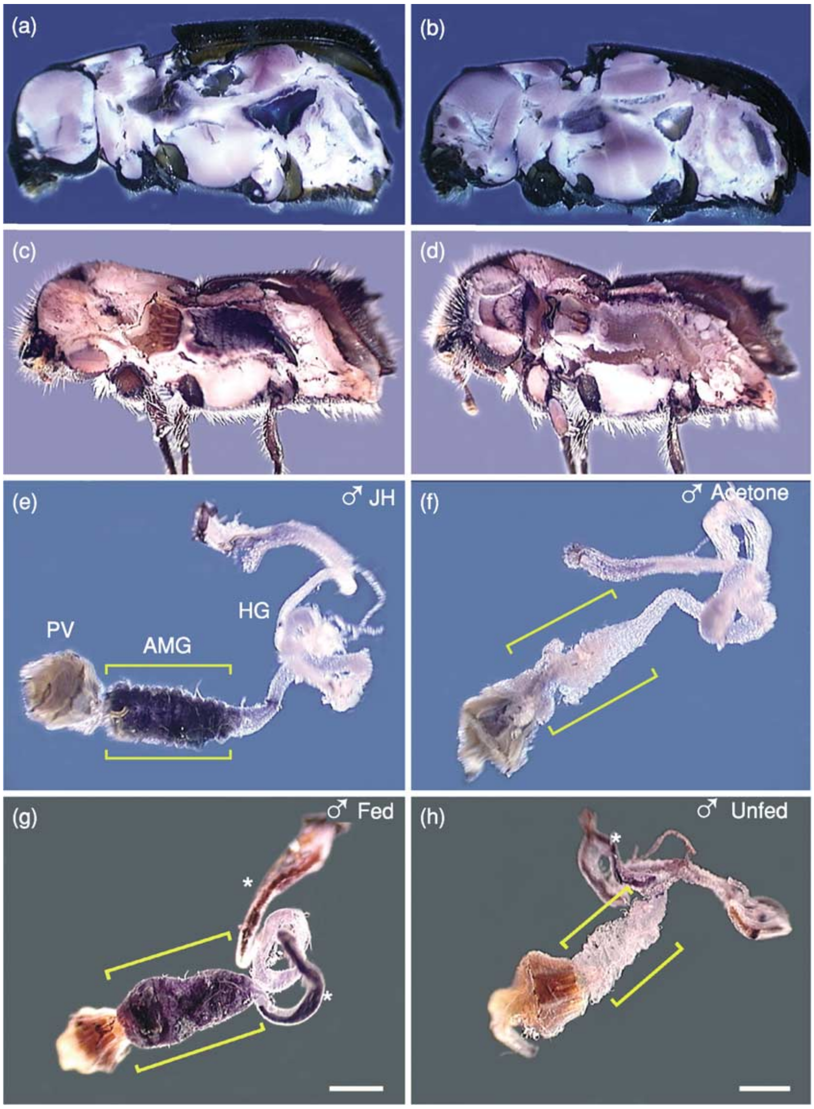


Figure 3 Tissue localization of *HMG-R* expression. Exposed whole mounts show that HMG-R mRNA is observed in the midgut of JH III-treated male *Dendroctonus jeffreyi* (a) and *Ips pini* (c), but not in untreated insects (b, d). Panels (e) through (h) show whole mount hybridizations of isolated *I. pini* alimentary canals. *HMG-R* expression in the anterior midgut (AMG, marked by yellow brackets) correlates with pheromone production in starved, JH III-treated males (e) and fed males (g), while starved and untreated males (f, g), which do not produce monoterpenoid pheromone components, do not strongly express *HMG-R*. Asterisks mark nonspecific signal in the hindguts. PV, proventriculus; HG, hindgut. Scale bar = 0.5 mm. (Modified from Hall *et al.*, 2002a, 2002b.)

most of the more recent research implicates specialized abdominal glands. Periplanone-A and periplanone-B, the sex pheromone components of the American cockroach, *Periplaneta americana*, appear to be concentrated in the midgut and released in feces. However, female calling involves opening the genital vestibulum (i.e., atrial gland), without excretion of feces, and this glandular tissue contains most of the periplanone-B, up to 60 ng (Abed *et al.*, 1993a). The epithelium of the atrial gland consists of class-1 glandular cells, in which the secretion passes directly to the cuticle and not through a duct. However, Yang *et al.* (1998) concluded that both periplanone-A and periplanone-B were most abundant in the colon and that this tissue produced the strongest electroantennogram (EAG) responses. To date, no definitive proof of the sites of pheromone biosynthesis is available through isotopic tracing of pheromone precursors in isolated tissues.

The volatile sex pheromones of other cockroaches have been localized to abdominal tergites and sternites. Females of the German cockroach, *B. germanica*, produce the pheromone in the 10th abdominal

tergite (pygidium) (Liang and Schal, 1993; Tokro *et al.*, 1993), *S. longipalpa* in the 4th and 5th tergites (Schal *et al.*, 1992), and *Parcoblatta lata* (wood cockroach) females produce the pheromone in tergites 1–7 (Gemeno *et al.*, 2003). In all three species, the tergal glands are composed of multiple class-3 secretory units, each leading through a long unbranched duct to a single cuticular pore. The secretory cells are characterized by abundant mitochondria, smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), a large nucleus, and numerous secretory vesicles that discharge through an end-apparatus with numerous long microvilli into the duct and to the cuticular surface. In all three species pheromone is released during a calling behavior.

Male cockroaches also release volatile sex pheromones from tergal or sternal glands. In *Nauphoeta cinerea* (lobster cockroach) glands on sternites 3–7 are exposed during calling, releasing a volatile male pheromone consisting of 3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethyl-2-methoxyphenol, and 2-methyl-2-thiazoline (Sreng, 1990; Sirugue *et al.*, 1992). In some species the male tergal glands form specialized regions on the cuticular surface and they produce a blend of close-range attractants, phagostimulants, and nutrients that the male deploys to place the female in a precopulatory position. For example, the tergal glands of *B. germanica* consist of transverse cuticular depressions on the 7th and 8th tergites, connected to numerous class-3 secretory cells (Sreng and Quennedey, 1976; Brossut and Roth, 1977).

Cuticular contact pheromones mediate species and sex recognition and, in most cases, they function as courtship-inducing pheromones. They are thought to be distributed throughout the epicuticular surface. The blend of hydrocarbon-derived ketones of *B. germanica* females is produced by oenocytes, which are localized within the abdominal integument, separated from the hemocoel by a basal lamina (Liang and Schal, 1993). Fan *et al.* (2003) enzymatically dissociated the integument underlying the sternites into a cell suspension. After further Percoll gradient centrifugation, each fraction was assayed for incorporation of $[^{14}C_1]$propionate into methyl-branched hydrocarbons. Only oenocytes produced hydrocarbons and methyl ketone pheromones, whereas the much larger population of epidermal cells did not (Fan *et al.*, 2003; Fan, unpublished data). As in the dipterans *M. domestica* and *Drosophila* and the moth *Holomelina*, the hydrocarbon-derived pheromones of *B. germanica* are produced by oenocytes and transported by lipophorin (see Section 3.14.5.4).

3.14.4. Biochemistry of Pheromone Production

3.14.4.1. Modification of "Normal Metabolism"

By the mid-1980s (Prestwich and Blomquist, 1987), the biosynthetic pathways of pheromones for a limited number of species had been determined, and work was progressing toward the characterization of some of the unique enzymes involved. It became apparent that the products and intermediates of normal metabolism, particularly those of the fatty acid and isoprenoid pathways, were modified by a few specific enzymes in pheromone gland tissue to produce the myriad of pheromone molecules. Many of the lepidopteran pheromones could be formed by the appropriate interplay of highly selective chain shortening and a unique Δ11 and other desaturases followed by modification of the carboxyl carbon (Bjostad *et al.*, 1987). This work has been extended, and a clear understanding of the biosynthetic pathways for many of the lepidopteran pheromones is now known (Jurenka, 2003). The Δ11 and other pheromone-specific desaturases in Lepidoptera have been characterized at the molecular level (Knipple and Roelofs, 2003). Chain shortening of fatty acids is also involved in producing the queen pheromone in honeybees (Plettner *et al.*, 1996, 1998). In some insects, fatty acid elongation followed by decarboxylation produces the hydrocarbon pheromones, and this process includes examples of lepidopterans (Jurenka, 2003), dipterans (Blomquist, 2003; Jallon and Wicker-Thomas, 2003), the German cockroach (Schal *et al.*, 2003), and the social insects (Blomquist and Howard, 2003). More recent work in bark beetles has shown that *Ips* and *Dendroctonus* spp. produce their monoterpenoid-derived pheromones ipsenol, ipsdienol, and frontalin by modifications of isoprenoid pathway products (Seybold and Vanderwel, 2003). Until that work, it was considered very rare for animals to produce monoterpenoids (C_{10} isoprenoids).

3.14.4.2. Pheromone Biosynthesis in Moths

Bjostad and Roelofs (1983) were the first to determine correctly how the major pheromone component for a particular moth was biosynthesized. They utilized the moth *T. ni* because the female produces a relatively large amount of pheromone (about 1 μg). They began by demonstrating that pheromone glands utilize acetate to produce the common fatty acids octadecanoate and hexadecanoate, which undergo Δ11 desaturation to produce *Z*11–18:acid and *Z*11–16:acid. However, the main pheromone component is *Z*7–12:OAc, which presumably is

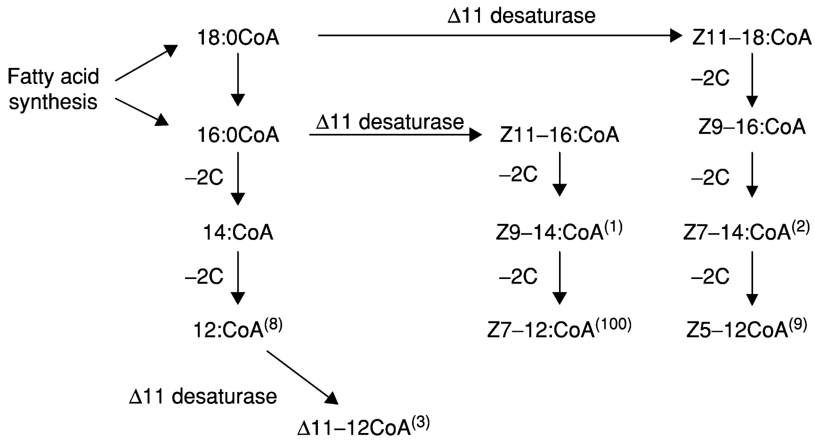


Figure 4 Biosynthetic pathways for producing the intermediate CoA derivatives of the pheromone blend of the cabbage looper, *Trichoplusia ni*. The CoA derivatives followed by the superscript number in parenthesis are reduced to an alcohol and acetylated to form the acetate esters that make up the pheromone blend. The superscript numbers indicate the approximate ratio of components found in the pheromone gland (Bjostad *et al.*, 1984). (Reprinted with permission from Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80; © Elsevier.)

made from *Z*7–12:acid. To demonstrate how the fatty acid precursor *Z*7–12:acid was produced, [$^3H_{16}$]*Z*11–16:acid was applied to glands and found to be incorporated into both *Z*7–12:acid and *Z*7–12:OAc. They concluded that limited chain-shortening of *Z*11–16:acid could account for this incorporation. The other minor components are produced in a similar way (**Figure 4**). Thus the pheromone components are produced through a fatty acid biosynthesis pathway involving a Δ11 desaturase and limited chain-shortening enzymes. The appropriate chain length fatty acid is then reduced and acetylated to form the acetate ester. Subsequent research has demonstrated that similar pathways occur in a wide variety of female moths (Bjostad and Roelofs, 1984; Roelofs and Wolf, 1988).

Most female moths produce their pheromone through modifications of fatty acid synthesis pathways and thus techniques in fatty acid research were utilized to determine biosynthetic pathways (Bjostad *et al.*, 1987; Morse and Meighen, 1987b). The main techniques include thin-layer chromatography, gas chromatography (GC), and GC-mass spectrometry (MS) with the latter being the primary method utilized. The advantage of using GC–MS is that the label (stable isotopes of deuterium or carbon-13) can be explicitly shown to be present in the compound of interest. By monitoring for diagnostic ions that correspond to unlabeled and labeled products, it can be determined with considerable certainty that the label is associated with a particular compound. By utilizing different proposed intermediate labeled fatty acids a biosynthetic pathway can be deduced. For example, a recent study determined that *Z*11–16:Ald occurs by Δ11-desaturation of 16:CoA followed by reduction in the moths *Helicoverpa zea* and *H. assulta* (Choi *et al.*, 2002). However, *Z*9–16:Ald, the major pheromone component in *H. assulta*, was produced by a Δ9-desaturase using 16:CoA as a substrate, but in *H. zea* *Z*9–16:Ald was produced by Δ11-desaturation of 18:CoA to produce *Z*11–18:CoA that is then chain-shortened to *Z*9–16:CoA (Choi *et al.*, 2002). These types of studies have shown that the key enzymes of sex pheromone biosynthetic pathways are fatty acid biosynthesis, desaturases, chain-shortening enzymes, and specific enzymes to produce a functional group.

3.14.4.3. Enzymes Involved in Lepidopteran Pheromone Production

3.14.4.3.1. Fatty acid synthesis A combination of acetyl-CoA carboxylase and fatty acid synthase produce saturated fatty acids. Although no direct enzymatic studies have been conducted using pheromone gland cells, these enzymes are presumably similar to enzymes found in other cell types. Labeling studies conducted with acetate indicated that pheromone glands produce 16:acid and 18:acid saturated products (Bjostad and Roelofs, 1984; Tang *et al.*, 1989; Jurenka *et al.*, 1991b, 1994).

3.14.4.3.2. Chain-shortening enzymes Insects in general have the ability to shorten long-chain fatty acids to specific shorter chain lengths (Stanley-Samuelson *et al.*, 1988). This chain-shortening pathway has not been characterized at the enzymatic level in insects. It presumably is similar to the characterized pathway as it occurs in vertebrates which

is essentially a partial β-oxidation pathway located in peroxisomes (Hashimoto, 1996). The evidence for limited chain-shortening enzymes in pheromone glands was originally demonstrated by Bjostad and Roelofs (1983) using the cabbage looper moth, *T. ni* in which it was shown that *Z*11–16:acid labeled the intermediate fatty acid *Z*7–12:OAc. A similar study using *Argyrotaenia velutinana* demonstrated that deuterium-labeled 16:acid was chain-shortened to 14:acid, which was used to make *Z* and *E*11–14:acid (Bjostad and Roelofs, 1984). Since then considerable evidence in a number of moths has accumulated to indicate that limited chain shortening occurs in a variety of pheromone biosynthetic pathways.

Radio and stable isotopes were topically applied directly to an intact pheromone gland to provide evidence for chain shortening. The position of the label was on the terminal methyl carbon making it difficult for any type of rearrangement to occur (Wolf and Roelofs, 1983; Rosell *et al.*, 1992). A more direct *in vitro* enzyme assay was utilized to demonstrate substrate preferences in a study using cabbage looper moths (Jurenka *et al.*, 1994). This study was prompted by the finding of a mutant line of cabbage loopers that produced a greatly increased amount of *Z*9–14:OAc (Haynes and Hunt, 1990), which is a minor component of normal cabbage loopers. Increased amounts of *Z*9–14:OAc indicate that chain shortening was affected in the mutant cabbage loopers. To determine if chain shortening was affected, substrate specificities for both normal and mutant cabbage loopers were determined using an *in vitro* enzyme assay (Jurenka *et al.*, 1994). Pheromone glands from normal cabbage loopers preferred to chain shorten *Z*11–16:CoA to *Z*7–12:CoA with two rounds of chain shortening, whereas pheromone glands from the mutant cabbage looper apparently have the ability to chain shorten by only one round. Therefore, *Z*11–16:CoA was chain shortened to *Z*9–14:CoA.

Changes in chain-shortening reactions have also been implicated in the alteration of pheromone ratios in several other species. In a laboratory selection pressure experiment using *A. velutinana*, the *Z*/*E* ratio of 11–14:OAc could not be changed much from a 92/8 ratio (Roelofs *et al.*, 1986). However, it was found that the ratio of *E*9–12:OAc/*E*11–14:OAc could be selected (Sreng *et al.*, 1989) and by comparing ratios of the 14-carbon/12-carbon pheromone components and *Z*/*E* isomers of each chain length it was determined that chain-shortening enzymes were selective for the *E* isomer (Roelofs and Jurenka, 1996). Another example where a change in chain shortening can account for a new pheromone blend is the larch budmoth, *Zeiraphera diniana*. The pheromone was first identified as *E*11–14:OAc (Roelofs *et al.*, 1971), but it was later determined that some populations utilize *E*9–12:OAc, which is made by chain shortening of *E*11–14:acid (Guerin *et al.*, 1984; Baltensweiler and Priesner, 1988). Another case where changes in chain shortening may have produced two populations of insects was found in the turnip moth, *Agrotis segetum*. A Swedish population has a ratio of *Z*9–14:OAc/*Z*7–12:OAc/*Z*5–10:OAc of 29/59/12, whereas, a Zimbabwean population has a ratio of 2/20/78. After conducting labeling studies, it was determined that chain-shortening enzymes could be affected to produce the alteration in pheromone ratios (Wu *et al.*, 1998). These studies indicate that alteration in chain-shortening enzymes can have a major effect on pheromone blends.

3.14.4.3.3. Desaturases Desaturases introduce a double bond into the fatty acid chain. A variety of desaturases have been described that are involved in the biosynthesis of female moth sex pheromones. The desaturases identified so far include enzymes that act on saturated and monounsaturated substrates. These include Δ5 (Foster and Roelofs, 1996), Δ9 (Löfstedt and Bengtsson, 1988; Martinez *et al.*, 1990), Δ10 (Foster and Roelofs, 1988), Δ11 (Bjostad and Roelofs, 1981, 1983), and Δ14 (Zhao *et al.*, 1990) desaturases that utilize saturated substrates. The combination of these desaturases along with chain shortening can account for the majority of double bond positions in the various chain-length monounsaturated pheromones so far identified (Roelofs and Wolf, 1988). **Figure 5** illustrates the large number of monounsaturated compounds that can be generated through a combination of desaturation and chain shortening. Addition of various functional groups, acetate esters, alcohols, and aldehydes, increases the potential number of pheromone components. Notice that some of the intermediate compounds could be produced in two different ways. Therefore, although the desaturation and chain-shortening steps occur in a wide variety of moths, the order in which they occur and the type of desaturase must still be determined experimentally.

Some pheromone components are dienes and these can be produced by either the action of two desaturases or one desaturase and isomerization around the double bond. Some dienes with a 6,9-double bond configuration are produced using linoleic acid. Desaturases that utilize monounsaturated acyl-CoA substrates include Δ5 (Ono *et al.*, 2002), Δ9 (Martinez *et al.*, 1990), Δ11 (Foster and Roelofs, 1990), Δ12 (Jurenka, 1997), and Δ13

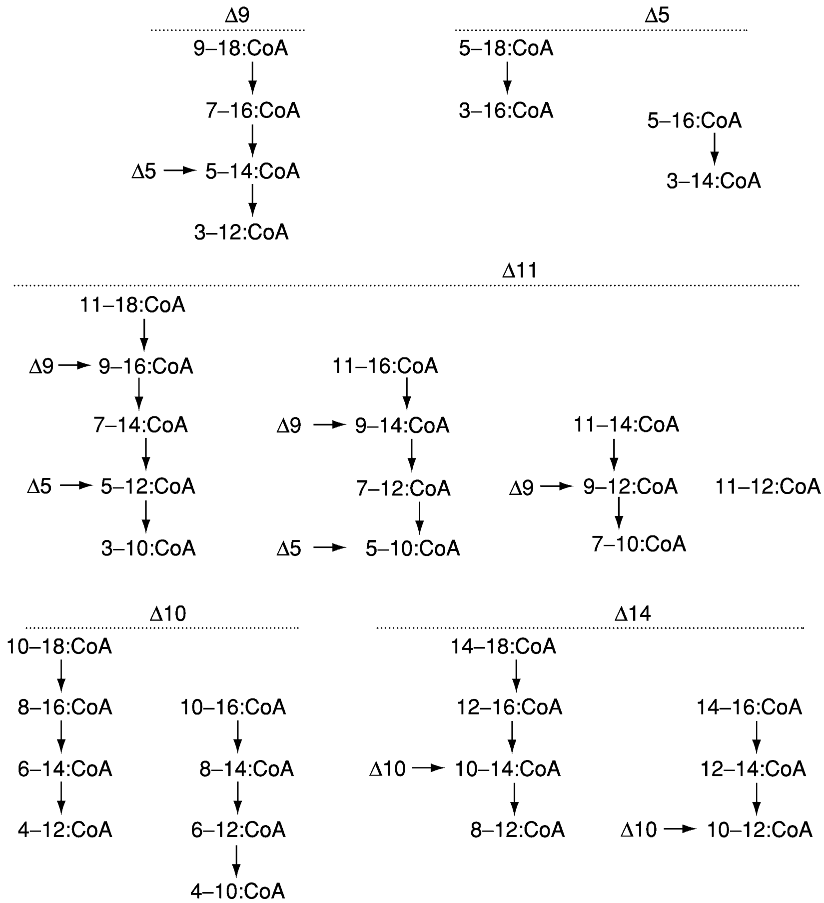


Figure 5 Combination of desaturation and chain shortening can produce a variety of monounsaturated acyl-CoA precursors that can be modified to form acetate esters, aldehydes, and alcohols. The number followed by the Δ sign indicates a desaturase that introduces a double bond into the first indicated chain length acyl-CoA. The arrow pointing down indicates limited chain-shortening by two carbons. The arrow pointing to the right indicates that desaturation could produce the compound found within a chain-shortening pathway. This indicates that certain compounds could be produced in two different ways. Modification of all 16-, 14-, 12-, and 10-carbon acyl-CoA derivatives on the carbonyl carbon can account for the majority of monounsaturated acetate esters, aldehydes, and alcohols identified as sex pheromones. (Reprinted with permission from Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80; © Elsevier.)

(Arsequell *et al*., 1990). These can act sequentially to produce the diene (Foster and Roelofs, 1990; Jurenka, 1997) or conjugated dienes could be produced by the action of one desaturase followed by isomerization (Ando *et al*., 1988; Löfstedt and Bengtsson, 1988; Fang *et al*., 1995a).

The biosynthesis of triene pheromone components has not been extensively investigated. Pheromones with a triene double bond system, that is *n*-3 (3,6,9-), are probably produced from linolenic acid (Millar, 2000). This was demonstrated in the saltmarsh caterpillar, *Estigmene acrea*, and the ruby tiger moth, *Phragmatobia fuliginosa* (Rule and Roelofs, 1989). Moths in the families Geometridae, Arctiidae, and Noctuidae apparently utilize linoleic and linolenic acid as precursors for their pheromones. Most of these pheromones are produced by chain elongation and decarboxylation to form hydrocarbons. Oxygen is added across one of the double bonds in the polyunsaturated hydrocarbon to produce an epoxide (Millar, 2000).

3.14.4.3.3.1. Molecular biology of the desaturases More recent research has identified a variety of desaturases at the gene level with the first gene encoding a Δ11 desaturase being identified in the cabbage looper, *T. ni* (Knipple *et al*., 1998). Since then a number of desaturase genes have been cloned and functionally expressed (Knipple and Roelofs, 2003). Expression in a strain of yeast lacking an endogenous desaturase was utilized to demonstrate functionality. This strain of yeast will not grow in a media lacking added unsaturated fatty acids but will grow if a functional desaturase is

inserted into the yeast genome. After growth, the fatty acids are analyzed to determine double bond positions and thus the desaturase can be characterized regarding double bond insertion and chain-length specificity.

Desaturase encoding cDNAs from pheromone glands that produce *Z*9, *Z*10, *Z*11, and *E*11 double bonds in 14- and 16-carbon acids have been identified so far. The *Z*9-desaturase is comparable to the metabolic desaturase found in the fat body. A *Z*10-desaturase was characterized from *Planotortrix octo* that produces Z10–16:acid (Hao *et al*., 2002), which would be chain shortened to produce the precursor to the pheromone *Z*8–14:OAc (Foster and Roelofs, 1988). Several Δ11-desaturases have been characterized including the ones from *T. ni* (Knipple *et al*., 1998) and *H. zea* (Rosenfield *et al*., 2001) that produce primarily *Z*11–16:acid. A single Δ11-desaturase was characterized from *A. velutinana* that produces both *Z*11– and *E*11–14:acid (Liu *et al*., 2002a). This desaturase is unique in that it produces both isomers and uses 14:acid as a substrate. Another unique Δ11-desaturase was identified from *Epiphyas postvittana* that not only produced *E*11–14:acid and *E*11–16:acid from saturated precursors, but also *E*9,*E*11–14:acid from a *E*9–14:acid precursor (produced by chain shortening *E*11–16:acid) (Liu *et al*., 2002b). Identification of desaturases in *Ostrinia nubilalis* and *O. furnicalis* produced the surprise finding that Δ11- and Δ14-desaturases are found in both moths (Roelofs *et al*., 2002). The Δ11-desaturase produced both *Z*11– and *E*11–14:acid and the Δ14-desaturase produced both *Z*14– and *E*14–16:acids. This finding has implications regarding the evolution of pheromone blends in moths (Baker, 2002).

3.14.4.3.4. Specific enzymes to produce functional group on carbonyl carbon Once a specific chain-length pheromone intermediate that has the appropriate double bonds is produced, the carbonyl carbon is modified to form a functional group. The majority of oxygenated pheromone components are acetate esters (or other esters), alcohols, and aldehydes. Production of these components requires the reduction of a fatty-acyl precursor to an alcohol which is a two-step reaction requiring a fatty acid reductase and an aldehyde reductase (Morse and Meighen, 1987b). Thus, alcohol formation goes through an aldehyde intermediate. Therefore, aldehydes could be produced by direct reduction of fatty acids. Another route for aldehyde formation is oxidation of alcohols. A cuticular oxidase has been characterized from pheromone glands of *H. zea* and *Manduca sexta* that produce aldehydes as pheromones (Teal and Tumlinson, 1988; Fang *et al*., 1995b). In those insects that utilize both an alcohol and an aldehyde as part of their pheromone, it is unclear how the production of both components occurs.

Production of acetate ester pheromone components utilizes an enzyme called acetyl-CoA:fatty alcohol acetyltransferase that converts a fatty alcohol to an acetate ester (Morse and Meighen, 1987a). Therefore, alcohols could be utilized as substrates for both aldehyde and acetate ester formation. Morse and Meighen (1987a) first demonstrated its presence in the spruce budworm, *Choristoneura fumiferana*, where it is involved in producing the acetate ester that serves as a precursor to the aldehyde pheromone (Morse and Meighen, 1987b). In some other tortricids, *A. velutinana*, *C. rosaceana*, and *Platynota idaeusalis,* an *in vitro* enzyme assay was utilized to demonstrate specificity of the acetyltransferase for the *Z* isomer of 11–14:OH (Jurenka and Roelofs, 1989). This specificity contributes to the final ratio of pheromone components. These results indicate that the family Tortricidae has members that have an acetyltransferase that is specific for the *Z* isomer of monounsaturated fatty alcohols. In contrast, several studies have shown no substrate preference for the acetyltransferase in other moths (Bestmann *et al*., 1987; Teal and Tumlinson, 1987; Jurenka and Roelofs, 1989). Therefore, this unique acetyltransferase apparently evolved within the Tortricidae.

3.14.4.3.5. Production of specific pheromone blends Most female moths utilize a blend of components produced in a specific ratio for pheromone attraction of conspecific males. A major question is how these species-specific ratios of components are produced. Research from several sources indicates that these ratios are produced by the inherent specificity of certain enzymes present in the biosynthetic pathways. The combination of these enzymes acting in concert produces the species-specific pheromone blend. Several examples will be utilized to illustrate this point.

The cabbage looper, *T. ni* uses *Z*7–12:OAc as the major sex pheromone component, which is produced by Δ11-desaturation of 16:CoA followed by two rounds of chain shortening, reduction, and acetylation. The Δ11-desaturase has been characterized (Wolf and Roelofs, 1986) and the gene isolated (Knipple *et al*., 1998). These studies indicate that 16:CoA and 18:CoA are substrates with 16:CoA as the preferred substrate and indeed *Z*11–16:acid is the most abundant monounsaturated fatty acid in pheromone glands (Bjostad *et al*., 1984). The next

step in the pathway is limited chain shortening and it was shown that *Z*11–16:CoA is the preferred substrate for these enzymes (Jurenka *et al*., 1994). After chain shortening the 14-carbon and 12-carbon intermediates are reduced to an alcohol and acetylated. The acetyltransferase enzyme is not specific and will accept a variety of substrates (Jurenka and Roelofs, 1989). From these observations, it can be inferred that the final ratio of pheromone components is produced by the specificity found within the Δ11-desaturase and chain-shortening enzymes.

A blend of seven acetate esters is used by *A. velutinana* that are produced in a biosynthetic pathway similar to the one just described, except that the Δ11-desaturase starts with 14:CoA as the substrate and produces both *Z* and *E* isomers of 11–14:CoA in about a 60/40 ratio (Wolf and Roelofs, 1987). Recent cloning of the Δ11-desaturase from redbanded leafroller (RBLR) females indicates that the expressed enzyme produces a ratio of *Z/E* of about 6/1 (Liu *et al*., 2002a). However, the final ratio of *Z*11– to *E*11–14:OAc is 92/8 (Miller and Roelofs, 1980). A selective increase in the *Z* isomer occurs within the biosynthetic pathway and it was determined that acetyl:CoA fatty alcohol acetyltransferase shows specificity for the *Z* isomer (Jurenka and Roelofs, 1989). Therefore, selective acetylation of *Z*11–14:OH and production of >60% *Z*11–14:CoA indicate that these enzymes have the inherent specificity to produce the 92/8 ratio of the major pheromone components *Z*11– and *E*11–14:OAc. Two minor pheromone components are produced by chain shortening *Z*11– and *E*11–14:OAc. The ratio of *Z*9– to *E*9–12:OAc is about 1 to 2. This indicates that the chain-shortening enzymes may prefer *E*11–14:CoA or that very little *Z*11–14:CoA is available to chain shorten. This combined information indicates that in *A. velutinana* pheromone glands, the final ratio of pheromone components can be produced through the concerted action of a Δ11-desaturase that produces at least a 60/40 ratio of *Z/E* intermediate isomers. The final ratio of acetate esters (92/8) is produced through the specificity for the *Z* isomer by the acetyltransferase. The minor components are produced by specificity in chain shortening.

Another insect that utilizes specific ratios of *Z*11– and *E*11–14:OAc is the European corn borer, *O. nubilalis*. Two strains are known in which one produces a ratio of *Z/E* of about 97/3 (*Z* strain) and the other produces an opposite ratio of *Z/E* of about 1/99 (*E* strain). Hybridization studies between the two strains indicated that offspring have an acetate ester ratio of *Z/E* of about 30/70 (Klun and Maini, 1982). The Δ11-desaturase from both strains produced a product with about 30/70 *Z/E* in an *in vitro* enzyme assay (Wolf and Roelofs, 1987). These results indicate that the final ratio of acetate ester isomers is produced after the desaturation step. The enzymes that follow the desaturase are a reductase to make an alcohol and an acetyltransferase to produce the acetate esters. Two studies have shown that the acetyltransferase is similar between the two strains (Jurenka and Roelofs, 1989; Zhu *et al*., 1996). However, labeled acids applied to glands *in vivo* were selectively incorporated into the correct pheromone ratio indicating that the reductase shows specificity (Zhu *et al*., 1996). Therefore, the final pheromone ratios produced by females of the European corn borer are made through the action of a Δ11-desaturase that can produce both *Z* and *E* isomers. The final acetate ester ratio is strain dependent and is produced through the specificity found in the reductase system.

The above three examples illustrate how a species-specific pheromone blend is produced by the concerted action of desaturases, chain-shortening enzymes, and a reductase and an acetyltransferase. The specificity inherent in certain enzymes in the pathway produces the final blend of pheromone components.

3.14.4.3.6. Hydrocarbon pheromones Moths in the families Geometridae, Arctiidae, Amatidae, Lymantriidae, Lyonetiidae, and some Noctuidae utilize hydrocarbons or epoxides of hydrocarbons as their sex pheromones. Biosynthesis of hydrocarbons occurs in oenocyte cells that are associated with either epidermal cells or fat body cells (Romer, 1991). Once the hydrocarbons are biosynthesized they are transported to the sex pheromone gland by lipophorin (Schal *et al*., 1998a). When the transport of hydrocarbon sex pheromones in arctiid moths was investigated in detail by Schal *et al.* (1998a), it was found that a very specific uptake was occurring at pheromone glands. Lipophorin was shown to contain both the sex pheromone and cuticular hydrocarbons; however, only the pheromone gland had the sex pheromone. Other studies have shown similar pathways in other moths (Jurenka and Subchev, 2000; Subchev and Jurenka, 2001; Wei *et al*., 2003).

Most moth sex pheromones that are straight chain hydrocarbons also usually have an odd number of carbons. Most of these are polyunsaturated with double bonds in the 3,6,9- or 6,9-positions, indicating that they are derived from linolenic or linoleic acid, respectively (Rule and Roelofs, 1989;

Millar, 2000). Linolenic and linoleic acid cannot be biosynthesized by moths so they must be obtained from the diet (Stanley-Samuelson *et al*., 1988). A few even-chain-length hydrocarbon sex pheromones have been identified that also have 3,6,9- or 6,9-double bond configurations (Millar, 2000), indicating that they too are derived from linolenic or linoleic acids; however, it is not known how these even-chain hydrocarbons are formed.

A major class of sex pheromones that are derived from hydrocarbons are the polyene monoepoxides (Millar, 2000). These usually have double bonds in the 3,6,9-positions or 6,9-positions, again indicating that they are biosynthesized from linolenic or linoleic acids, respectively. Although the production of hydrocarbon occurs in oenocytes the epoxidation step takes place in the pheromone gland. This has been demonstrated in several studies utilizing deuterium labeled precursors. In a study on the Japanese giant looper, *Ascotis selenaria cretacea*, that uses 6,9–19:3,4Epox as a sex pheromone component, deuterium-labeled hydrocarbon precursor, D3–3,6,9–19:Hc, was topically applied to pheromone glands and found to be converted to the epoxide, indicating that epoxidation takes place in pheromone glands (Miyamoto *et al*., 1999). By using a variety of polyene precursors, it was also determined that the monooxygenase regiospecifically attacked the *n*-3 double bond regardless of chain length or degree of unsaturation. This indicates that the epoxidation enzyme is regiospecific in this insect (Miyamoto *et al*., 1999).

A study using the gypsy moth, *L. dispar*, illustrates the overall pathways involved in production of epoxide pheromone components (**Figure 6**) (Jurenka *et al*., 2003). This insect uses disparlure, 2me-18:7,8Epox, as a pheromone component. Incubation of isolated abdominal epidermal tissue with deuterium-labeled valine resulted in incorporation into 2me-*Z*7–18:Hc. This indicates that the oenocyte cells associated with the epidermal tissues biosynthesize 2me-*Z*7–18:Hc using the carbons of valine to initiate the chain. The double bond is probably introduced by a Δ12-desaturase as determined by using specific deuterium-labeled intermediates. Hemolymph transport of 2me-*Z*7–18:Hc is indicated by the finding of this alkene in the hemolymph (Jurenka and Subchev, 2000). Demonstration that 2me-*Z*7–18:Hc is converted to the epoxide in the pheromone gland was shown by using deuterium-labeled 2me-*Z*7–18:Hc and incubation with isolated pheromone glands. Disparlure is a stereoisomer that has the 7*R*,8*S* or (+) configuration and chiral chromatography indicated that only the (+)-isomer was produced by pheromone glands (Jurenka *et al*., 2003). These results indicate that hydrocarbon pheromones and their epoxides are produced through a pathway outlined in **Figure 6**.

3.14.4.4. Pheromone Biosynthesis in Beetles

Pheromone biosynthesis in the Coleoptera is as diverse as the taxa and the pheromone structures, and the utilization of several types of pheromone biosynthetic pathways has been demonstrated (Vanderwel and Oehlschlager, 1987; Vanderwel, 1994; Seybold and Vanderwel, 2003; Tittiger, 2003). Extensive work has been done on the biosynthesis of coleopteran pheromones, and the major

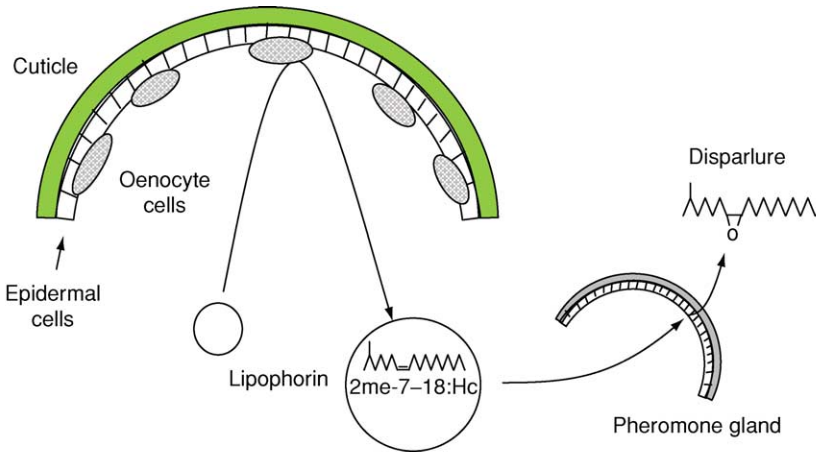


Figure 6 Production of the sex pheromone in the gypsy moth, *Lymantria dispar*. The oenocyte cells located in the abdomen biosynthesize the alkene hydrocarbon precursor to the pheromone, 2me-*Z*7–18:Hc. It is transported through the hemolymph by lipophorin. The alkene is taken up by pheromone gland cells where it is acted upon by an epoxidase to produce the pheromone disparlure, 2me-18:7,8Epox. (Reprinted with permission from Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80; © Elsevier.)

systems that have been investigated are described below. Beetles can generate pheromone either by modification of dietary host compounds or *de novo* biosynthesis, with the latter accounting for the majority of beetle pheromone components.

3.14.4.4.1. Isoprenoid pheromones from bark beetles Most of the knowledge about beetle pheromone biosynthesis and endocrine regulation (see Section 3.14.4.5.2) comes from studies of various bark beetles, especially *Ips* and *Dendroctonus* species (Scolytidae). Some bark beetles may modify fatty acyl or amino acid precursors (Vanderwel and Oehlschlager, 1987; Birgersson *et al*., 1990); however, the majority of pheromone components are isoprenoid (Schlyter and Birgarson, 1999: Seybold *et al*., 2000).

Understanding of the origin of bark beetle pheromone components has undergone a paradigm shift in the last decade. Until recently, it was widely accepted that bark beetles, in contrast to most other insects (Tillman *et al*., 1999), obtained their pheromone components by simple modification of of host tree dietary precursors (reviews: Bordon, 1985; Vanderwel and Oelschlager, 1987; Vanderwel, 1994). This model was based on various studies that showed the association of monoterpene synthesis with conifers (Croteau, 1981), the abundance of potential precursors in the host tree (Gershenzon and Croteau, 1991), the demonstration that monoterpenoid precursors such as myrcene and α-pinene could be converted to the pheromone components ipsdienol, ipsenol, and *cis* and *trans* verbenol (Hughes, 1974; Renwick *et al*., 1976a, 1976b; Byers, 1981), and the conclusive demonstration that deuterated myrcene was converted to ipsenol and ipsdienol in *I. paraconfusus* (Hendry *et al*., 1980).

In the past decade and a half, however, significant evidence has emerged supporting the *de novo* biosynthesis of most bark beetle pheromone components. First, doubts were raised about the role of myrcene in ipsdienol and ipsenol production when it was noted that myrcene may not be present in sufficient quantity in some host trees to account for the amount of pheromone that was produced (Byers, 1981; Byers and Birgersson, 1990). Second, *Ips* beetles treated with the HMG-R reductase inhibitor compactin show a marked decrease in ipsdienol production (Ivarsson *et al*., 1993). Third, myrcene-treated male *I. pini* produce ipsdienol with a racemic enantiomeric composition whereas JH III-treated males produce ipsdienol with a 87–96% (−) enantiomeric composition (Lu, Blomquist, and Seybold, unpublished data). The naturally occurring enantiomeric composition of ipsdienol from California *I. pini* is 95–98% (−) (Seybold *et al*., 1995a). Fourth, JH III-treated male *I. pini* incorporate labeled acetate and mevalonate into ipsdienol (Seybold *et al*., 1995b; Tillman *et al*., 1998). 2-Methyl-3-buten-2-ol is similarly synthesized *de novo* in *I. typographus* (Lanne *et al*., 1989). More recently, key genes in pheromone production, including HMG-R and HMG-CoA synthase (HMG-S) have expression patterns consistent with their roles in regulating *de novo* isoprenoid pheromone biosynthesis (Tittiger *et al*., 1999; Tillman, Blomquist, and Seybold, unpublished data). A geranyl diphosphate synthase (GPPS) cDNA from *I. pini* was also isolated, functionally expressed, and modeled (Gilg-Young, Welch, Tittiger, and Blomquist, unpublished results). The existence of this novel enzyme argues strongly for the evolution of *de novo* pheromone biosynthetic capacity in bark beetles. Taken together, the data emerging from *Ips* species overwhelmingly indicate the *de novo* production of monoterpenoid pheromone components.

These data are supported by studies in other beetles that similarly prove or imply *de novo* pheromone component biosynthesis. Radiotracer studies demonstrated the *de novo* biosynthesis of frontalin in a number of *Dendroctonus* species (Barkawi *et al*., 2003). Expression patterns of HMG-R and HMG-S in *D. jeffreyi* correlate tightly with frontalin production (Tittiger *et al*., 2000, 2003). The four monterpene alcohols and aldehydes in the boll weevil, *A. grandis*, are also synthesized from mevalonate and acetate (Mitlin and Hedin, 1974; Thompson and Mitlin, 1979).

The capacity for *de novo* biosynthesis does not preclude the conversion of host precursors to pheromone components. For example, the incorporation of acetate and mevalonolactone into ipsdienol and ipsenol may proceed through the conversion of geranyl diphosphate to myrcene, which could be directly hydroxylated to ipsdienol and *E*-myrcenol, and indirectly, perhaps through a ketone intermediate, to ipsenol (Martin *et al*., 2003). In this scheme, host myrcene ingested during feeding would enter the *de novo* pathway downstream of geranyl diphosphate. Similarly, cotton plant monoterpenes (myrcene and limonene) could enter a *de novo* biosynthetic pathway to grandlure in *A. grandis*. The question then arises as to whether *de novo* biosynthesis or host precursor conversion is the preferred route to pheromone production. Male *I. pini* exposed to myrcene vapors produce a racemic mixture of ipsdienol, whereas the naturally occurring pheromone of Western *I. pini* is about 95 : 5 (−)/(+) ratio (Lu, Blomquist, and Seybold, unpublished data). This suggests that myrcene is not the

direct precursor to ipsdienol, but that its hydroxylation is a true detoxification reaction. Perhaps a highly specific hydroxylase may synthesize ipsdienol *de novo*, while a hydroxylase with a different specificity detoxifies myrcene. Other possibilities also exist, particularly considering that ipsdienone appears to be a precursor to ipsdienol (Ivarsson *et al.*, 1997). To make ipsdienol, it is possible that geranyl diphosphate is hydroxylated and then dephosphorylated, bypassing myrcene as an intermediate. Thus, though the final biosynthetic steps remain uncharacterized, *de novo* biosynthesis is clearly the most important route to ipsdienol and ipsenol in *I. pini* and *I. paraconfusus*. Further examples should arise as other species are studied.

A proposed biosynthetic scheme for a number of coleopteran isoprenoid pheromone components is presented in **Figure 7**. Hemiterpene pheromone components of the bark beetles are similarly synthesized *de novo*. Lanne *et al.* (1989) demonstrated the incorporation of labeled acetate, glucose, and mevalonate into 2-methyl-3-buten-2-ol in *I. typographus*. This also argues for the *de novo* synthesis of 3-methyl-3-buten-1-ol and 3-methyl-2-buten-1-ol.

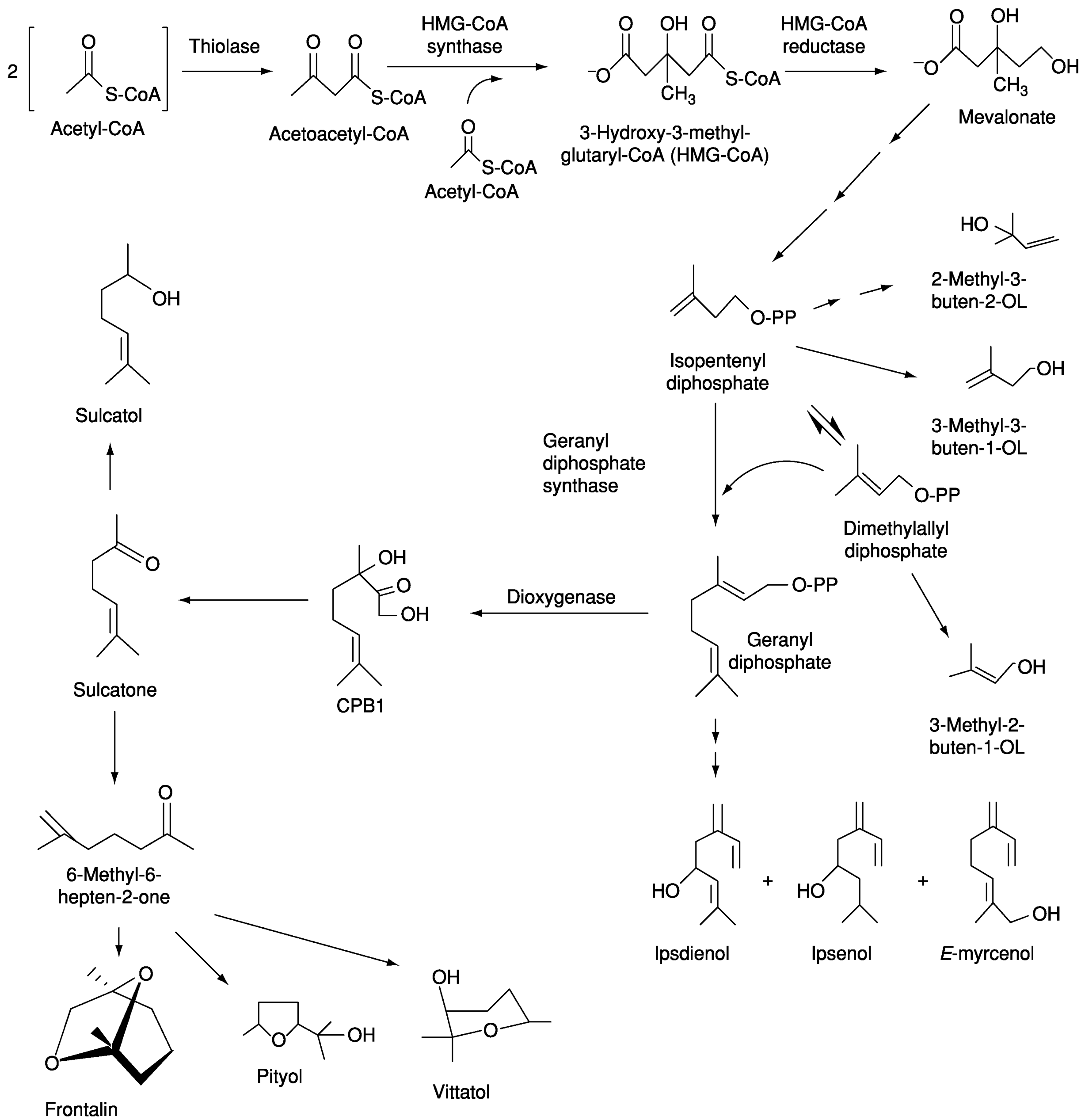


Figure 7 Proposed biosynthetic pathways for a number of hemi- and monoterpenoid pheromone components in the Coleoptera.

The mevalonate pathway intermediate, dimethylallyl diphosphate, likely provides the carbon skeleton for 3-methyl-2-buten-1-ol by dephosphorylation. The other five-carbon intermediate, isopentenyl diphosphate, could be directly converted to 3-methyl-3-buten-1-ol, and perhaps through several steps, to 2-methyl-3-buten-2-ol.

Ipsdienol and ipsenol production from geranyl diphosphate appears to involve relatively straightforward though not yet fully characterized biochemical modifications. The production of frontalin, and the recently identified male-produced pheromone of the Colorado potato beetle, *Leptinotarsa decemlineata* (*S*)-3,7-dimethyl-2-oxo-oct-6-ene-1,3-diol (CPB1) (Dickens *et al*., 2002), is less clear. Barkawi *et al*. (2003) demonstrated that acetate and mevalonolactone are precursors to frontalin, proving the isoprenoid origin of this common *Dendroctonus* pheromone component. he carbon skeleton of frontalin probably arises from geranyl disphosphate via a putative dioxygenase, which converts geranyl diphosphate to sulcatone. Sulcatone is an obvious precursor to sulcatol, which is a pheromone component of some bark beetles (*Gnathotrichus sulcatus*) (Byrne *et al*., 1974). In *Dendroctonus* spp., geranyl diphosphate-derived sulcatone may be converted to 6-methyl-6-hepten-2-one (6-MHO), a known intermediate to frontalin (Perez *et al*., 1996). Alternative cyclizations of 6-MHO in other beetles could lead to the pheromone components pityol and vittatol. Significant amounts of sulcatone are also found in Colorado potato beetle males during their synthesis of CPB1 (Dickens *et al*., 2002). It is easy to envision CPB1 as an intermediate in the dioxygenase reaction (**Figure 7**).

The picture emerging from these studies is that isoprenoid pheromone component production in beetles is mostly *de novo*, with carbon being diverted from the mevalonate pathway at geranyl diphosphate. Geranyl diphosphate may be directly modified through dephosphorylation and cyclizations (cotton boll weevil) or hydroxylations (*Ips* spp.). Alternatively, some bark beetles, such as *Dendroctonus* spp., apparently have a dioxygenase that oxidizes geranyl diphosphate or geraniol to produce a ketodiol (CPB1) or sulcatone, which acts as a precursor to some pheromone components such as sulcatol, pityol, and frontalin.

3.14.4.4.2. Fatty acid-derived pheromones Numerous beetle genera use modified fatty-acyl compounds as pheromone components. Less is known about their biosynthesis compared to that of isoprenoid pheromones, but the same general strategy of modifying or combining existing biosynthetic pathways is conserved.

For some beetles, the modifications are relatively minor. For example, *Attagenus* spp. (Dermestidae) myristic acid may be desaturated at the Δ5 and Δ7 positions to produce tetradecadienoic acid pheromone components. The stereochemistries of the double bonds apparently provide specificity between species (Fukui *et al*., 1977). It is unclear whether the short-chain fatty acid precursors to these pheromone components are synthesized through normal fatty acid elongations, or are the β-oxidation products of longer fatty acids. For other beetles, modifications can become more complex. Female *Tenebrio molitor* produce 4-methyl-1-nonanol from propionyl-, malonyl-, and methylmalonyl-precursors (Islam *et al*., 1999). This is an example of carbon being shunted away from fatty-acyl elongation before long fatty acids are completed. The use of methylmalonate to produce methyl-branched hydrocarbons is well established in other insect systems (Blomquist, 2003; Schal *et al*., 2003), though it is unknown if beetles have a secondary fatty acyl synthase which, similar to that in houseflies, incorporates methylmalonyl-CoA precursors efficiently.

The flexibility of the fatty acid biosynthetic pathway is extended in some nitidulid beetles (*Carpophilus* spp.), where males use propionate and butyrate (presumably as methylmalonate and ethylmalonate) to make methyl- and ethyl-branched triene and tetraene pheromone components (**Figure 1**), apparently also via the fatty acid biosynthetic pathway (Bartelt *et al*., 1992). The branched hydrocarbons generally have 10–12 carbon backbones with conjugated double bonds. In contrast to other systems, where pheromone component biosynthesis is highly specific, *Carpophilus* spp. males produce a mixture of related structures, some of which act as pheromones and some of which do not. Since di-substituted tetraenes are less abundant than mono- or unsubstituted tetraenes, it appears that nonacyl units placed in the growing hydrocarbon chains represent "mistakes" made by a synthesis machinery with a low stringency for substrate selection (Bartelt, 1999). Such nonspecific hydrocarbon biosynthesis may serve speciation, since changes in antennal receptivity may accommodate preexisting compounds (Bartelt, 1999). Interestingly, the desaturated nature of these hydrocarbons is not due to fatty acyl desaturases, but due to the inactivity of enoyl-ACP reductase during biosynthesis so that the enoyl-ACP intermediate formed during elongation is not reduced (Petroski *et al*., 1994). This

suggests that carbon is shunted out of the fatty acid biosynthetic pathway when the chains are of the correct length, similar to the situation in *T. molitor.*

Rather than modifying the normal biosynthetic pathway to produce pheromone components, some beetles modify normal products of the pathway. For example, lactone pheromone components of some scarab beetles are produced by the stereospecific alterations of long chain fatty acids. Female *Anomala japonica* (Scarabaeidae) are perhaps best studied among scarab beetles for the biosynthesis of japonilure and buibuilactone, which involves the successive Δ9 desaturation, hydroxylation, two rounds of β-oxidation to shorten the chain length, and cyclization of stearic and palmitic acids (Leal *et al.*, 1999). Of all these, only the hydroxylation step appears to be stereospecific (Leal, 1998). This step is important because different enantiomers have different functions in different *Anomala* species (Leal *et al.*, 1999).

3.14.4.4.3. Host precursor modifications While most bark beetle isoprenoid pheromones are clearly synthesized *de novo*, there is strong evidence that some pheromone components are indeed the result of modifying host precursor molecules. α-Pinene is produced naturally by pine trees, and can be hydroxylated to *cis-* and *trans-*verbenol (**Figure 1**) by *Ips* beetles (Renwick *et al.*, 1976a). A further oxidation of verbenol yields verbenone in *Dendroctonus ponderosae* (Hunt and Bordon, 1989). Similarly, Jeffrey pine trees contain relatively low levels of monoterpenoids, but high levels of heptane. Female *Dendroctonus jeffreyi* that attack these trees produce 1-heptanol and 2-heptanol, and 1-heptanol acts as a sex pheromone (Paine *et al.*, 1999).

3.14.4.5. Pheromone Biosynthesis in Diptera

3.14.4.5.1.1. Housefly pheromone biosynthesis: C23 sex pheromone components A combination of *in vivo* and *in vitro* studies using both radio- and stable-isotope techniques established the biosynthetic pathways for the major sex pheromone components in the housefly (**Figure 8**) (Dillwith *et al.*, 1981, 1982; Dillwith and Blomquist, 1982; Blomquist *et al.*, 1984b; Vaz *et al.*, 1988). (*Z*)-9-tricosene is formed by the microsomal elongation of 18:1-CoA to 24:1-CoA using malonyl-CoA and NADPH, and the elongated fatty acyl-CoA is then reduced to the aldehyde and converted to the hydrocarbon one carbon shorter (**Figure 8**) (Reed *et al.*, 1994, 1995; Mpuru *et al.*, 1996). A cytochrome P450 enzyme is involved in the metabolism of the alkene to the corresponding epoxide and ketone (Ahmad *et al.*, 1987). It appears that the same enzyme that catalyzes formation of an epoxide from the double bond between carbons 9 and 10 of the alkene of (*Z*)-9-tricosene also hydroxylates it at position *n*-10. The secondary alcohol thus formed is then converted to the unsaturated ketone (Guo *et al.*, 1991).

3.14.4.5.1.2. Mechanism of hydrocarbon formation: decarboxylation? The mechanism of hydrocarbon formation has proven elusive. In an elegant set of experiments in the 1960s and early 1970s, Kolattukudy and coworkers demonstrated that fatty acyl-CoAs were elongated and then converted to hydrocarbon by the loss of the carboxyl group (reviews: Kolattukudy *et al.*, 1976; Kolattukudy, 1980). In the 1980s and early 1990s, the hypothesis was put forward that very long chain fatty acyl-CoAs were reduced to aldehyde and then decarbonylated to hydrocarbon and carbon monoxide, and that this reaction did not require any cofactors or O_2. Evidence for this decarbonylation mechanism was obtained from a plant (Cheesbrough and Kolattukudy, 1984), an alga (Dennis and Kolattukudy, 1991, 1992), a vertebrate (Cheesbrough and Kolattukudy, 1988), and an insect (Yoder *et al.*, 1992).

In the work on hydrocarbon formation in the housefly, it was found that the acyl-CoA is reduced to the aldehyde, with the conversion of the aldehyde to hydrocarbon requiring NADPH and molecular oxygen, and that the products were hydrocarbon and carbon dioxide (demonstrated by radio-gas–liquid chromatography) (Reed *et al.*, 1994, 1995). Antibodies to housefly cytochrome P450 and to P450 reductase inhibited hydrocarbon formation in microsomes, as did exposure to CO, and the latter could be partially reversed by white light. GC-MS analyses of specifically deuterated substrates showed that the protons on positions 2,2 and 3,3 of the acyl-CoA were retained during conversion to hydrocarbon, and that the proton on position 1 of the aldehyde was transferred to the adjacent carbon and retained during hydrocarbon formation. Furthermore, several peroxides could substitute for O_2 and NADPH and support hydrocarbon production. All this evidence strongly supports a cytochrome P450 involvement in hydrocarbon synthesis (Reed *et al.*, 1994).

The mechanism of hydrocarbon formation remains controversial, with evidence obtained favoring both a decarbonylation and a decarboxylation mechanism. The resolution of the problem awaits cloning, expressing, and assaying the enzymes involved.

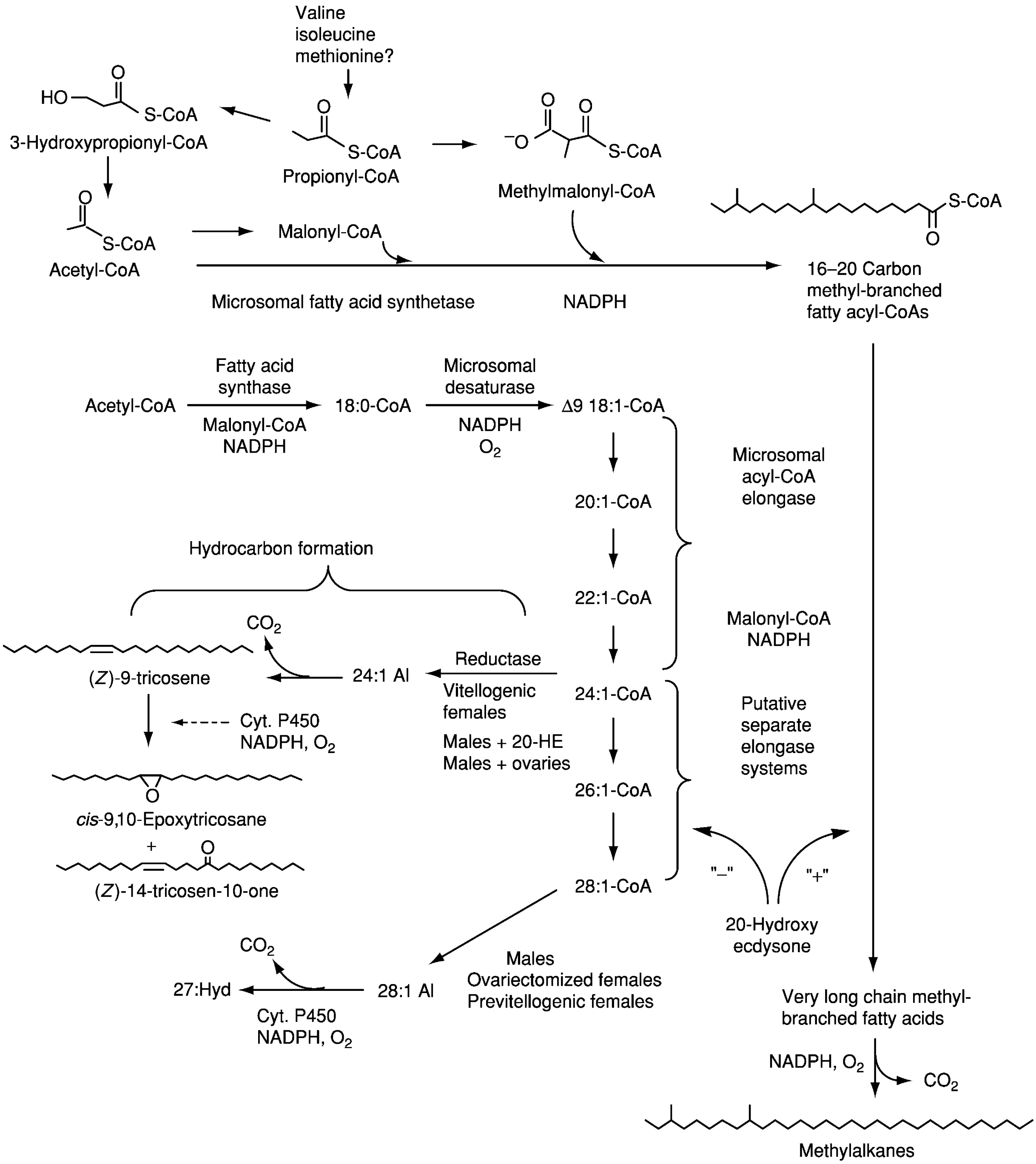


Figure 8 Biosynthetic pathways showing the putative steps at which ecdysteroids regulate the hydrocarbon and hydrocarbon-derived pheromone components of the female housefly, *Musca domestica*. (Reprinted with permission from Blomquist, G.J., **2003**. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, New York, pp. 131–252; © Elsevier.)

3.14.4.5.1.3. Biosynthesis of the methylalkane pheromone components Methylalkanes are formed by the substitution of methylmalonyl-CoA in place of malonyl-CoA at specific points during chain elongation. Carbon-13 nuclear magnetic resonance (NMR), mass spectrometry, and radiochemical studies (Dwyer *et al.*, 1981; Dillwith *et al.*, 1982; Chase *et al.*, 1990) demonstrated that the methylmalonyl-CoA was added during the initial steps of chain elongation in insects using what appears to be a novel microsomal fatty acid synthase (FAS) (Figure 8). A microsomal FAS was

first suggested from studies on *T. ni* for the formation of methyl-branched very long chain alcohols (de Renobales *et al*., 1989). In this insect, high rates of methyl-branched very long chain alcohol synthesis were observed in the mid-pupal stages at times when soluble FAS activity was very low or undetectable. The FAS of most organisms is soluble (cytoplasmic).

A microsomal FAS was also implicated in the biosynthesis of methyl-branched fatty acids and methyl-branched hydrocarbon precursors of the German cockroach contact sex pheromone (Juarez *et al*., 1992; Gu *et al*., 1993). A microsomal FAS present in the epidermal tissues of the housefly is a likely candidate responsible for methyl-branched fatty acid production (Blomquist *et al*., 1994). The housefly microsomal and soluble FASs were purified to homogeneity (Gu *et al*., 1997) and the microsomal FAS was shown to preferentially use methylmalonyl-CoA in comparison to the soluble FAS. GC–MS analyses showed that the methyl-branching positions of the methyl-branched fatty acids of the housefly (Blomquist *et al*., 1994) were in positions consistent with them being the precursors of the methyl-branched hydrocarbons.

The methylmalonyl-CoA unit that is the precursor to methyl-branched fatty acids and hydrocarbons arises from the carbon skeletons of valine and isoleucine, but not succinate (Dillwith *et al*., 1982). Propionate is also a precursor to methylmalonyl-CoA, and in the course of these studies, a novel pathway for propionate metabolism in insects was discovered. Many insect species, including the housefly, do not contain vitamin B_{12} (Wakayama *et al*., 1984), and therefore cannot catabolize propionate via methylmalonyl-CoA to succinate. Instead, as first demonstrated in the housefly (Dillwith *et al*., 1982), insects metabolize propionate to 3-hydroxypropionate and then to acetyl-CoA, with carbons 3 and 2 of propionate becoming carbons 1 and 2 of acetyl-CoA (**Figure 8**) (Halarnkar *et al*., 1986).

3.14.4.5.2. Pheromone biosynthesis in *Drosophila*
The other major dipteran system in which pheromone production has been extensively studied is that of *Drosophila*. Jallon and Wicker-Thomas (2003) provide an excellent and detailed review of the chemistry, biochemistry, molecular biology, and genetics of pheromone production in *Drosophila*. *Drosophila*, as do *Musca*, use long chain, sex-specific hydrocarbons as close range and contact sex pheromones. *Drosophila melanogaster* Canton-S mature females have abundant (*Z*,*Z*)-7,11-heptacosadiene, which is absent on males and was shown to effectively stimulate wing vibration in conspecific males when applied to a dummy (Antony and Jallon, 1982; Ferveur and Sureau, 1996). In some *Drosophila*, 7-tricosene is involved in chemical communication. A series of studies established that the hydrocarbon pheromones are produced in the oenocytes (Ferveur *et al*., 1997), transported through the hemolymph via lipophorin (Pho *et al*., 1996), and then deposited on the cuticle.

A series of experiments assaying the incorporation of various precursors into hydrocarbons established that *Drosophila* use an elongation–decarboxylation pathway for hydrocarbon production (Pennanec'h *et al*., 1997). An interesting question in *Drosophila* pheromone production is the origin of the 7,11 double bonds. Biochemical evidence suggested that either a Δ7-desaturase working on myristate, or a Δ9-desaturase working on palmitate give rise to the double bond in the 7-position, which could then be elongated to produce vaccenate (*n*-7, 18 : 1). Vaccenate could then be elongated and decarboxylated to produce 7-tricosene, and, with an additional desaturation step, to produce 7,11-27:2Hyd.

Wicker-Thomas *et al*. (1997) isolated a cDNA encoding a desaturase (*desat1*) in *D. melanogaster*. The expressed *desat1* protein is a Δ9-desaturase that preferentially used palmitate, resulting in *n*-7 fatty acids. A *desat2*, located close to *desat1*, appears to be responsible for the 5,9-dienes present in Tai females (Jallon and Wicker-Thomas, 2003). The identification of the desaturase genes responsible for pheromone production in fruit flies will greatly benefit from the characterization of the *D. melanogaster* genome. Understanding of the genetics combined with molecular biology undoubtedly will result in a more complete understanding of the mechanisms and genes involved and regulated in pheromone production in *Drosophila*.

3.14.4.6. Biosynthesis of Contact Pheromones in the German Cockroach

Upon antennal contact with a female, the male German cockroach rotates his body 180° and raises his wings, thus exposing specialized tergal glands that attract the female and place her into a precopulatory position (Nojima *et al*., 1999). The nonvolatile contact pheromone responsible for this behavior was identified as (3*S*,11*S*)-dimethylnonacosan-2-one (**Figure 1**) (Nishida *et al*., 1974), and an alcohol (29-hydroxy-3*S*,11*S*-dimethylnonacosan-2-one) and an aldehyde (29-oxo-3,11-dimethylnonacosan-2-one) derivatives with the same 3,11-dimethylketone skeleton (Nishida and Fukami, 1983). A fourth pheromone component, 3,11-dimethylheptacosan-2-one, is less active than its C_{29} homolog (Schal *et al*., 1990b).

The route of biosynthesis and its physiological regulation have been reviewed previously (Blomquist *et al.*, 1993; Tillman *et al.*, 1999; Schal *et al.*, 2003). Central to investigations of the biosynthetic pathway was the observation that the major cuticular hydrocarbon in all life stages of the German cockroach is an isomeric mixture of 3,7-, 3,9- and 3,11-dimethylnonacosane (Jurenka *et al.*, 1989). The presence of only the 3,11-isomer in the cuticular dimethyl ketone fraction and only in adult females prompted Jurenka *et al.* (1989) to propose that production of the pheromone might result from the sex-specific oxidation of its hydrocarbon analog only in adult females. This scheme follows the well-established conversion of hydrocarbons to methyl ketone and epoxide pheromones in the housefly (see above) (Blomquist *et al.*, 1984b; Ahmad *et al.*, 1987).

This model has since been validated with several independent approaches. Biochemical studies on the biosynthesis of methyl-branched alkanes showed that the methyl branches are added during the early stages of chain elongation (Chase *et al.*, 1990). Using carbon-13 labeling and NMR analyses, Chase *et al.* (1990) showed that carbons 1 and 2 of acetate are incorporated as the chain initiator, and that the carbon skeleton of propionate serves as the methyl branch donor (**Figure 9**). Further, propionate and succinate labeled methyl-branched hydrocarbons and the methyl ketone pheromone, as did the amino acids valine, isoleucine, and methionine, all of which can be metabolized to propionate. NMR studies confirmed that these substrates were metabolized to methylmalonyl-CoA for incorporation into the methyl branch unit of hydrocarbons (Chase *et al.*, 1990), as in the housefly (Dillwith *et al.*, 1982; Halarnkar *et al.*, 1986), American cockroach (Halarnkar *et al.*, 1985), cabbage looper moth (de Renobales and Blomquist, 1983), and the termite *Zootermopsis* (Chu and Blomquist, 1980).

Methyl-branched fatty acids are intermediates in branched alkane biosynthesis (Juarez *et al.*, 1992). Thus, [$^{14}C_1$]propionate labeled methyl-branched fatty acids of 16–20 carbons, but did not label straight-chain saturated and monounsaturated fatty acids (Chase *et al.*, 1990).

Chase *et al.* (1992) investigated the hypothesis that the 3,11-dimethyl ketone sex pheromone arises from the insertion of an oxygen into the preformed 3,11-dimethyl alkane. When high-specific activity, tritiated 3,11-dimethylnonacosane (mixture of stereoisomers), was topically applied on the cuticle of *B. germanica* females, it readily penetrated the cockroach and radioactivity from the alkane was detected in both 3,11-dimethylnonacosan-2-ol and 3,11-dimethylnonacosan-2-one. Likewise, when tritiated 3,11-dimethylnonacosan-2-ol was applied to the cuticle it was readily and highly efficiently converted to the corresponding methyl ketone pheromone. But, surprisingly, the dimethyl ketone pheromone was derived from the corresponding alcohol not only in females, as expected, but also in males. These results suggest that the sex pheromone of *B. germanica* arises via a female-specific hydroxylation of 3,11-dimethylnonacosane and a subsequent nonsex-specific oxidation, probably involving a polysubstrate monooxygenase system, to the (3*S*,11*S*)-dimethylnonacosan-2-one pheromone (**Figure 9**). Chase *et al.* (1992) also suggested that a similar hydroxylation and subsequent oxidation at the 29-position of 3,11-dimethylnonacosan-2-one might give rise to 29-hydroxy- and 29-oxo-(3,11)-dimethylnonacosan-2-one, the other components of the contact pheromone blend, but this hypothesis has yet to be tested. It is quite likely, as well, that the same mechanism converts 3,11-dimethylheptacosane to the corresponding methyl ketone pheromone, and perhaps its 27-hydroxy- and 27-oxo- analogs.

The contact sex pheromone of female *B. germanica* remains the only cockroach pheromone whose biosynthetic pathway has been investigated with radio- and stable-isotope tracers.

3.14.4.7. Biosythesis of the Honeybee Queen Pheromone

The queen substance used for "queen control" inside the nest is also the substance used by virgin queens to attract drones for mating. It is the best understood of the sexual pheromones of the social insects. Callow and Johnston (1960) and Barbier and Lederer (1960) identified ([*E*]-9-oxodec-2-enoic acid) (9-ODA) in queen mandibular glands. 9-Hydroxy-2(*E*)-decenoic acid (9-HDA) is also present (Callow *et al.*, 1964) and together both attract drones. Recent work (Keeling *et al.*, 2003) identified a number of additional compounds that function synergistically with the 9-ODA and 9-HDA, making this the most complex pheromone blend known for any organism.

In an elegant set of experiments, Plettner *et al.* (1996, 1998) elucidated the biosynthetic pathways for the honeybee queen mandibular pheromone (QMP) components 9-ODA and 9-HDA and compared their biosyntheses to that of worker produced 10-hydroxy-2(*E*)-decenoic acid and the corresponding diacid. Using carbon-13 and deuterated precursors, Plettner *et al.* (1996, 1998) demonstrated (1) the *de novo* synthesis of stearic acid in worker mandibular glands, (2) the hydroxylation of

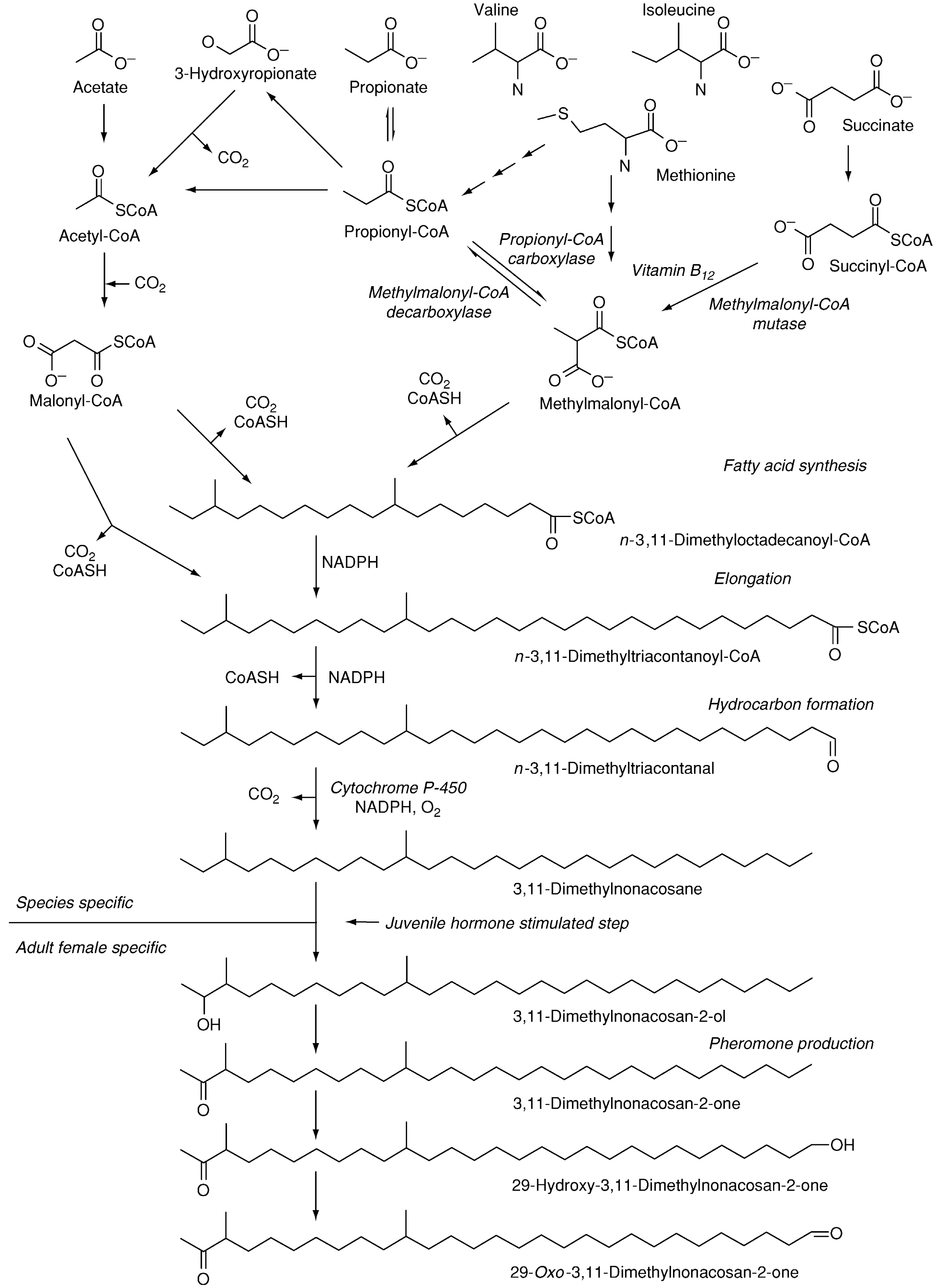


Figure 9 Proposed biosynthetic pathways for the major pheromone components of the German cockroach, *Blattella germanica*. The JH III regulated step appears to be the hydroxylation of the dimethylalkane.

stearic acid at the *n*-(workers) and *n*-1 (queens) positions, (3) chain shortening through β-oxidation to the 10- and 8-carbon hydroxy acids, and (4) oxidation of *n*- and *n*-1 hydroxy groups to give diacids and 9-keto-2(*E*)-decenoic acid, respectively. Stearic acid was shown to be the main precursor of the pheromone molecules as it was converted to C_{10} hydroxy acids and diacids more efficiently than either 16 or 14 carbon fatty acids.

3.14.5. Endocrine Regulation of Pheromone Production

3.14.5.1. Barth's Hypothesis

Juvenile hormone's central role in mate-finding was recognized in 1965 when Barth proposed that neuroendocrine control of pheromone production would be common in insects with a long-lived adult stage and with multiple reproductive cycles interrupted by periods during which sexual receptivity and mating are not appropriate or not even possible anatomically. Cockroaches and beetles are quintessential examples of this life-history syndrome. Conversely, in insects that eclose with mature oocytes, and live for only a few days as adults, Barth (1965) predicted that pheromone signaling would be part of the adult metamorphic process and not subject to neuroendocrine control. The discovery of PBAN in moths (see Section 3.14.4.5.2) appeared in conflict with this hypothesis, but Barth's model (Barth and Lester, 1973) clearly accounted for cases in moth species where adults feed and oocyte maturation requires the participation of JH or other neuroendocrine factors. Schal *et al.* (2003) proposed a reconsideration of the hypothesis, taking into account the coordination of reproductive developmental processes with mating-related events. Accordingly, in long-lived insects, such as cockroaches, pheromone production is expected to be synchronously regulated with other reproductive processes by the same hormone, usually JH. Cellular remodeling of the pheromone glands plays a prominent role in this group of insects, resulting in a slow stimulation of pheromone production. The cessation of pheromone production after mating is also slow, and precise control of pheromone signaling, therefore, is not at the level of pheromone production, but rather at the behavioral level through control of pheromone emission during calling. Conversely, in short-lived moths rapid modulation of rate-limiting enzymes in the pheromone biosynthetic pathway is much more prominent than developmental processes, and pheromone biosynthesis is turned on or off in coordination with activity cycles (day versus night) and sexual receptivity (virgin versus mated). Control of sexual signaling occurs at the level of pheromone production as well as emission, but these two events are usually regulated by different factors. Thus, both groups of insects exhibit neuroendocrine control of pheromone production. In cockroaches, pheromone production is coordinated with the gonotrophic cycle and the major gonadotropic hormone – JH – has been recruited to control both by acting at several target tissues. In most moths, alternatively, reproduction and pheromone production are regulated by different hormones. But here also, the hormones that control pheromone production (e.g., PBAN) also affect other target tissues such as myotropins, melanization agents, and diapause and pupariation factors. An interesting departure from the moth model occurs in migratory moth species in which reproduction is delayed by migration (low levels of JH production and sexual inactivity), and pheromone production and its release are JH dependent (Cusson *et al.*, 1994). All these observations are consistent with our interpretation of Barth's model.

The three hormones that regulate pheromone production in insects are shown in **Figure 2** and **Table 1**. PBAN has been studied in female moths and alters enzyme activity through second messengers at one or more steps during or subsequent to fatty acid synthesis during pheromone production (Rafaeli and Jurenka, 2003). In contrast, 20E and JH induce or repress the synthesis of specific enzymes at the transcription level. The action of JH has been studied most thoroughly in the German cockroach and in bark beetles, and this work is discussed below. Ecdysteroid regulation of pheromone production occurs in Diptera, and has been most extensively studied in the housefly, *M. domestica*.

3.14.5.2. PBAN Regulation in Moths

3.14.5.2.1. PBAN Most female moths release sex pheromones in a typical calling behavior in which the pheromone gland is extruded to release pheromone during a particular time of the photoperiod. In most cases pheromone biosynthesis coincides with calling behavior and the synchronization of these events is achieved by neuroendocrine mechanisms present in the female that in turn are influenced by various environmental and physiological events such as temperature, photoperiod, host plants, mating, hormones, neurohormones, and neuromodulators. We now know that the main neuroendocrine mechanism that regulates pheromone production in moths is pheromone biosynthesis activating neuropeptide (PBAN).

Table 1 Amino acid sequences of the pyrokinin/PBAN family of peptides; the FXPRLamide motif is shown in bold (X standing for S, T, G, or V)

Function	*Species*	*Peptide sequence*	*Reference*
PBAN	*Helicoverpa zea*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRL**amide[a]	Raina *et al.* (1989)
	Helicoverpa assulta	LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRL**amide[b]	Choi *et al.* (1998)
	Bombyx mori	LSEDMPATPADQEMYQPDPEEMESRTRY**FSPRL**amide[a]	Kitamura *et al.* (1989)
	Lymantria dispar	LADDMPATMADQEVYRPEPEQIDSRNKY**FSPRL**amide[a]	Masler *et al.* (1994)
	Agrotis ipsilon	LADDTPATPADQEMYRPDPEQIDSRTKY**FSPRL**amide[b]	Duportets *et al.* (1999)
	Mamestra brassicae	LADDMPATPADQEMYRPDPEQIDSRTKY**FSPRL**amide[b]	Jacquin-Joly *et al.* (1998)
	Spodoptera littoralis	LADDMPATPADQELYRPDPDQIDSRTKY**FSPRL**amide[b]	Iglesias *et al.* (2002)
Pheromontropic peptides	*Bombyx mori*	α II**FTP**K**L**amide[b]	Kawano *et al.* (1992)
	Helicoverpa zea	α VI**FTP**K**L**amide[b]	Ma *et al.* (1994)
	Helicoverpa assulta	α VI**FTP**K**L**amide[b]	Choi *et al.* (1998)
	Agrotis ipsilon	α VI**FTP**K**L**amide[b]	Duportets *et al.* (1999)
	Mamestra brassicae	α VI**FTP**K**L**amide[b]	Jacquin-Joly *et al.* (1998)
	Spodoptera littoralis	α VI**FTP**K**L**amide[b]	Iglesias *et al.* (2002)
	Bombyx mori	β SVAKPQTHESLE**F**I**PRL**amide[b]	Kawano *et al.* (1992)
	Helicoverpa zea	β SLAYDDKSFENVE**FTPRL**amide[b]	Ma *et al.* (1994)
	Helicoverpa assulta	β SLAYDDKSFENVE**FTPRL**amide[b]	Choi *et al.* (1998)
	Agrotis ipsilon	β SLSYEDKMFDNVE**FTPRL**amide[b]	Duportets *et al.* (1999)
	Mamestra brassicae	β SLAYDDKVFENVE**FTPRL**amide[b]	Jacquin-Joly *et al.* (1998)
	Pseudaletia separata	β KLSYDDKVFENVE**FTPRL**amide[b]	Matsumoto *et al.* (1992)
	Spodoptera littoralis	β SLAYDDKVFENVE**FTPRL**amide[b]	Iglesias *et al.* (2002)
	Bombyx mori	γ TMS**FSPRL**amide[b]	Kawano *et al.* (1992)
	Helicoverpa zea	γ TMN**FSPRL**amide[b]	Ma *et al.* (1994)
	Helicoverpa assulta	γ TMN**FSPRL**amide[b]	Choi *et al.* (1998)
	Agrotis ipsilon	γ TMN**FSPRL**amide[b]	Duportets *et al.* (1999)
	Mamestra brassicae	γ TMN**FSPRL**amide[b]	Jacquin-Joly *et al.* (1998)
	Spodoptera littoralis	γ TMN**FSPRL**amide[b]	Iglesias *et al.* (2002)
Diapause hormone	*Bombyx mori*	TDMKDESDRGAHSERGALC**FGPRL**amide[a]	Imai *et al.* (1991)
	Helicoverpa zea	NDVKDGAASGAHSDRLGLW**FGPRL**amide[b]	Ma *et al.* (1994)
	Helicoverpa assulta	NDVKDGAASGAHSDRLGLW**FGPRL**amide[b]	Choi *et al.* (1998)
	Agrotis ipsilon	NDVKDGGADRGAHSDRGGMW**FGPR**Iamide[b]	Duportets *et al.* (1999)
	Spodoptera littoralis	NEIKDGGSDRGAHSDRAGLW**FGPRL**amide[b]	Iglesias *et al.* (2002)
Pyrokinins	*Leucophaea maderae*	ETS**FTPRL**amide[a]	Holman *et al.* (1986)
	Locusta migratoria	(I) pEDSGDGWPQQP**FVPRL**amide[a]	Schoofs *et al.* (1991)
		(II) pESVPT**FTPRL**amide[a]	Schoofs *et al.* (1993)
Myotropins	*Locusta migratoria*	(I) GAVPAAQ**FSPRL**amide[a]	Schoofs *et al.* (1990a)
		(II) EGD**FTPRL**amide[a]	Schoofs *et al.* (1990b)
		(III) RQQP**FVPRL**amide[a]	Schoofs *et al.* (1992)
		(IV) RLHQNGMP**FSPRL**amide[a]	Schoofs *et al.* (1992)
	Schistocerca gregaria	MT1 GAAPAAQ**FSPRL**amide[a]	Veelaert *et al.* (1997)
		MT2 TSSLFPH**PRL**amide[a]	Veelaert *et al.* (1997)
	Periplaneta americana	PK1 HTAG**F**I**PRL**amide[a]	Predel *et al.* (1997)
		PK2 SPP**F**A**PRL**amide[a]	Predel *et al.* (1997)
		PK3 LVP**F**R**PRL**amide[a]	Predel *et al.* (1999)
		PK4 DHLPHDVY**SPRL**amide[a]	Predel *et al.* (1999)
		PK5 GGGGSGETSGMW**FGPRL**amide[a]	Predel *et al.* (1999)
		PK6 SESEVPGMW**FGPRL**amide[a]	Predel and Eckert (2000)
	Periplaneta fuliginosa	PK4 DHLSHDVY**SPRL**amide[a]	Predel and Eckert (2000)
	Drosophila melanogaster	CAP2b-3 TGPSASSGLW**FGPRL**amide[b]	Choi *et al.* (2001)
		PK-2 SVP**F**K**PRL**amide[b]	Choi *et al.* (2001)
		ETH-1 DDSSPGFFLKITKNV**PRL**amide[b]	Park *et al.* (1999)
		hug γ pELQSNGIPAYRVR**TPRL**amide[b]	Meng *et al.* (2002)
	Penaeus vannamei (Crustacea)	DFA**FSPRL**amide[a]	Torfs *et al.* (2001)
		ADFA**F**N**PRL**amide[a]	Torfs *et al.* (2001)

[a]Identified from the amino acid sequence of a purified peptide.
[b]Deduced from the cloned gene sequence.

The neuropeptide PBAN was localized to the subesophageal ganglion (SEG) (Raina and Menn, 1987), which facilitated purification and sequencing (Kitamura *et al.*, 1989; Raina *et al.*, 1989). The first PBAN identified had 33 amino acids with a C-terminal amidation and the core sequence FXPRLamide (where X represents S, T, G, or V) is required for activity, which places PBAN in a family of peptides with the C-terminal FXPRLamide motif (**Table 1**). The first member of this family to be identified was called leucopyrokinin based on its ability to stimulate hindgut contraction in the cockroach *Leucophaea maderae* (Holman *et al.*, 1986). Additional functions for this family include induction of embryonic diapause in *B. mori* (Imai *et al.*, 1991), induction of melanization in lepidopteran larvae (Matsumoto *et al.*, 1990), and acceleration of puparium formation in several flies (Zdarek *et al.*, 1998). In addition it was determined that the white shrimp, *Penaeus vannamei*, has two peptides that can induce myotropic activity (Torfs *et al.*, 2001). These results demonstrate the ubiquity and multifunctional nature of this family of peptides.

Localization of PBAN-like immunoreactivity in the central nervous system of adult moths indicated that several neurons in the SEG contain PBAN-like activity. These were found as clusters along the ventral midline, one each in the presumptive mandibular, maxillary, and labial neuromeres (Kingan *et al.*, 1992). All three groups of neurons have axons that project into the corpus cardiacum (CC) (Kingan *et al.*, 1992; Davis *et al.*, 1996). Two pairs of maxillary neurons send processes within the SEG that include posterior projections into the paired ventral nerve cord (VNC) and travel its entire length to terminate in the terminal abdominal ganglion (TAG). Arborizations arising from these paired projections were found in each segmental ganglion (Davis *et al.*, 1996). In addition the segmental ganglia have neurons that contain PBAN-like activity and these neurons can release peptides into the hemolymph (Ma and Roelofs, 1995c; Davis *et al.*, 1996; Ma *et al.*, 1996).

3.14.5.2.2. Molecular genetics of PBAN The gene encoding PBAN was first characterized from *H. zea* and *B. mori* (Imai *et al.*, 1991; Davis *et al.*, 1992; Kawano *et al.*, 1992; Sato *et al.*, 1993; Ma *et al.*, 1994). The full-length cDNA was found to encode PBAN plus four additional peptide domains with a common C-terminal FXPRL sequence motif including that of the diapause hormone of *B. mori*. Three additional peptides with the common C-termini and sequence homology to those of *H. zea* and *B. mori* have been deduced from cDNA isolated from pheromone glands of *Mamestra brassicae* (Jacquin-Joly and Descoins, 1996), *H. assulta* (Choi *et al.*, 1998), *Agrotis ipsilon* (Duportets *et al.*, 1999), *Spodoptera littoralis* (Iglesias *et al.*, 2002), *H. armigera* (Zhang *et al.*, 2001), and *Adoxophyes* sp. (Lee *et al.*, 2001). The posttranslational processed peptides can be found in the SEG (Sato *et al.*, 1993; Ma *et al.*, 1996). The matrix-assisted laser desorption ionization (MALDI) MS data indicated that PBAN was found to a greater extent in the mandibular and maxillary clusters than in the labial cluster (Ma *et al.*, 2000). The other neuropeptides were found in all clusters. In addition some larger peptide fragments were found indicating alternative processing of the precursor protein (Ma *et al.*, 2000).

3.14.5.2.3. PBAN mode of action Through the development of a sensitive *in vitro* bioassay, studies on *H. armigera* and *H. zea* demonstrated that brain extracts and synthetic *H. zea* PBAN could stimulate the production of the main pheromone component (Soroker and Rafaeli, 1989; Rafaeli *et al.*, 1990, 1991, 1993; Rafaeli, 1994; Rafaeli and Gileadi, 1996). The response obtained was specific to the pheromone gland and independent of other abdominal tissues (Rafaeli, 1994; Rafaeli *et al.*, 1997b). Evidence has since accumulated regarding several other species of moths including *B. mori*, *Spodoptera litura*, *O. nubilalis*, *Plodia interpunctella*, and *Thaumetopoea pityocampa* (Arima *et al.*, 1991; Fónagy *et al.*, 1992; Fabriàs *et al.*, 1995; Ma and Roelofs, 1995b; Rafaeli and Gileadi, 1995a; Jurenka, 1996). In addition, functional and viable pheromone gland cell-clusters were obtained from the intersegmental membrane of *B. mori* using papain enzymatic digestion (Fónagy *et al.*, 2000).

The signal transduction events that occur after PBAN binds to a receptor have been studied in several model moth species (**Figure 10**). The main difference found between these species so far is whether or not 3′,5′,cyclic-AMP (cAMP) is used as a second messenger. In the case of the heliothines and several others, cAMP is a second messenger. Alternatively, cAMP is thought not to act in pheromone gland cells of *B. mori* and *O. nubilalis* (Fónagy *et al.*, 1992; Ma and Roelofs, 1995b). Instead, in these insects, it is thought that an increase in cytosolic calcium directly activates downstream events leading to stimulation of the biosynthetic pathway.

Extracellular calcium is essential for pheromonotropic activity in all moths studied to date (Fónagy *et al.*, 1992, 1999; Ma and Roelofs, 1995a; Matsumoto *et al.*, 1995). It is suggested that free calcium,

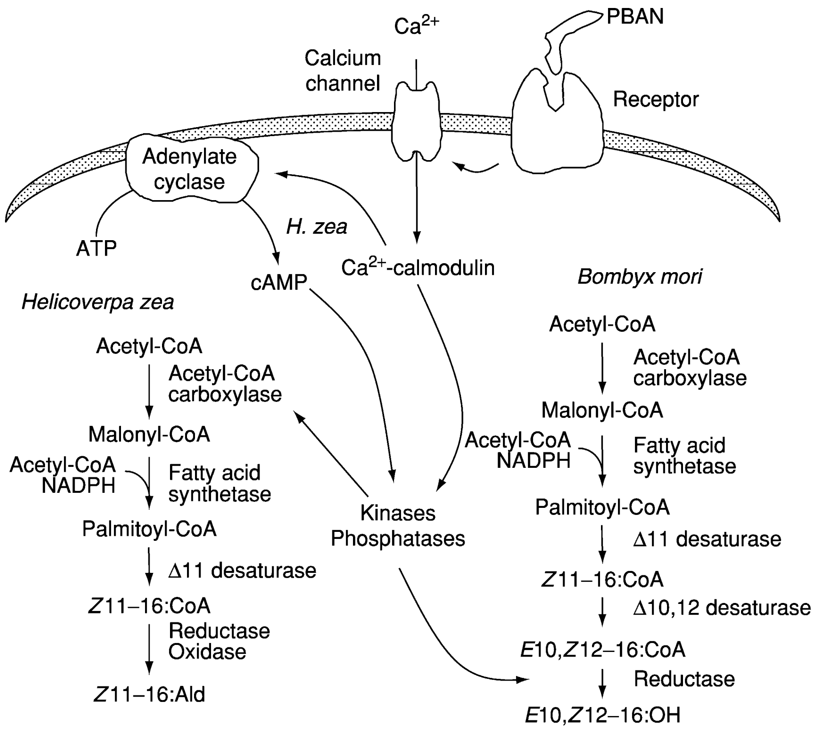


Figure 10 Proposed signal transduction mechanisms that stimulate the pheromone biosynthetic pathway in *Helicoverpa zea* and other heliothines as compared with that in *Bombyx mori*. It is proposed that PBAN binds to a receptor present in the cell membrane. Binding to the receptor somehow induces a receptor-activated calcium channel to open causing an influx of extracellular calcium. This calcium binds to calmodulin and in the case of *B. mori* will directly stimulate a phosphatase that will dephosphorylate and activate a reductase in the biosynthetic pathway. This activated reductase will then produce the pheromone bombykol. In *H. zea* and other heliothines like *H. armigera* the calcium-calmodulin will activate adenylate cyclase to produce cAMP that will then act through kinases and/or phosphatases to stimulate acetyl-CoA carboxylase in the biosynthetic pathway. (Reprinted with permission from Rafaeli, A., Jurenka, R.A., **2003**. PBAN regulation of pheromone biosynthesis in female moths. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 107–136; © Elsevier.)

entering the cell, binds to calmodulin to form a complex thereby activating adenylate cyclase and/or phosphoprotein phosphatases (**Figure 10**). Calmodulin was characterized from pheromone glands of *B. mori* (Iwanaga *et al.*, 1998) and was shown to have an identical amino acid sequence to *Drosophila* calmodulin (Smith *et al.*, 1987). In *B. mori*, it is suggested that the Ca^{2+}/calmodulin complex directly or indirectly activates a phosphoprotein phosphatase (Matsumoto *et al.*, 1995). This phosphatase will then activate an acyl-CoA reductase in the biosynthetic pathway. Two genes encoding calcineurin heterosubunits were identified from the pheromone gland of *B. mori* and were found to be homologous to the catalytic subunit and regulatory subunits of other animal calcineurins (Yoshiga *et al.*, 2002). The calcineurin complex will apparently dephosphorylate an acyl-CoA reductase, which catalyzes the formation of bombykol in *B. mori*.

3.14.5.2.4. Enzymes affected in the pheromone biosynthetic pathway PBAN has been shown to stimulate the reductase that converts an acyl-CoA to an alcohol precursor (**Figure 10**) in several moths including *B. mori* (Arima *et al.*, 1991; Ozawa *et al.*, 1993), *T. pityocampa* (Gosalbo *et al.*, 1994), *S. littoralis* (Martinez *et al.*, 1990; Fabriàs *et al.*, 1994), and *M. sexta* (Fang *et al.*, 1995b; Tumlinson *et al.*, 1997). In *A. velutinana* (Tang *et al.*, 1989), *H. zea* (Jurenka *et al.*, 1991a), *Cadra cautella*, *S. exigua* (Jurenka, 1997) and *M. brassicae* (Jacquin *et al.*, 1994), it was demonstrated that PBAN controls pheromone biosynthesis by regulating a step during or prior to fatty acid biosynthesis (**Figure 10**). Circumstantial evidence in *A. segetum* (Zhu *et al.*, 1995) and *H. armigera* (Rafaeli *et al.*, 1990) also points to the regulation of fatty acid synthesis by PBAN. In one study using the moth *Sesamia nonagrioides* it was shown that the acetyltransferase enzyme might be regulated by PBAN (Mas *et al.*, 2000). There appears to be no particular pattern as to which enzyme within the pheromone biosynthetic pathway will be regulated by PBAN. However, in the majority of moths studied it is either the reductase or fatty acid synthesis that is stimulated.

Several families of moths utilize hydrocarbons and/or their epoxides as sex pheromones (Millar, 2000). It is thought that PBAN does not regulate the production of hydrocarbon sex pheromones as demonstrated in *Scoliopteryx libatrix* (Subchev and Jurenka, 2001). However, PBAN is probably regulating the production of epoxide sex pheromones. This was demonstrated in *Ascotis selenaria cretacea* where decapitation resulted in pheromone decline and it could be restored by injecting PBAN (Miyamoto *et al.*, 1999). Decapitation also decreases the epoxide pheromone titer in the gypsy moth, *L. dispar*, and injection of PBAN can restore pheromone production (Thyagaraja and Raina, 1994). However, decapitation did not decrease the levels of the hydrocarbon precursor in the gypsy moth (Jurenka, unpublished data). These findings indicate that PBAN may regulate the epoxidation step in those moths that utilize epoxide pheromones but not the production of the alkene precursor or alkene pheromones.

3.14.5.2.5. Mediators and inhibitors of PBAN action Juvenile hormones play an important role in reproductive development of many moth species (see **Chapters 3.7** and **3.9**). Although JH probably does not regulate pheromone biosynthesis directly it has been shown to be involved in the release of PBAN in the migratory moths *Pseudaletia unipuncta* and *A. ipsilon* (Cusson and McNeil, 1989; Gadenne, 1993; Cusson *et al.*, 1994; Picimbon *et al.*, 1995). In addition, JH has been shown to prime the pheromone glands in pharate adults of the nonmigratory moth *H. armigera* (Fan *et al.*, 1999). JH II, in an *in vitro* assay, primed pheromone glands of pharate adults to respond to PBAN and induced earlier pheromone production by intact newly emerged females (Fan *et al.*, 1999). This induction could be mediated by JH upregulation of a putative PBAN receptor in pharate adults (Rafaeli *et al.*, 2003).

The corpus bursae has been implicated in the mediation of PBAN stimulation of pheromone biosynthesis in some tortricids (Jurenka *et al.*, 1991c). A peptide was partially purified from corpus bursae that could stimulate pheromone production (Fabriàs *et al.*, 1992). To date a bursal factor has only been demonstrated in *A. velutinana* and the related tortricids *C. fumiferana* and *C. rosaceana* (Delisle *et al.*, 1999).

The role of the nervous system in pheromone biosynthesis in moths is not clearly understood. In several moths including *L. dispar* (Tang *et al.*, 1987; Thyagaraja and Raina, 1994), *H. virescens* (Christensen *et al.*, 1991), *S. littoralis* (Marco *et al.*, 1996), and *M. brassicae* (Iglesias *et al.*, 1998) an intact VNC was reported as necessary for pheromone biosynthesis. Christensen and coworkers (Christensen *et al.*, 1991, 1992, 1994; Christensen and Hildebrand, 1995) proposed that the neurotransmitter octopamine might be involved as an intermediate messenger during the stimulation of sex pheromone production in *H. virescens*. These workers suggested that octopamine was involved in the regulation of pheromone production and that PBAN's role lies in the stimulation of octopamine release at nerve endings. However, contradicting results concerning VNC transection and octopamine-stimulated pheromone production were reported in the same species as well as in other moth species (Jurenka *et al.*, 1991c; Ramaswamy *et al.*, 1995; Rafaeli and Gileadi, 1996; Park and Ramaswamy, 1998; Delisle *et al.*, 1999). A modulatory role for octopamine was suggested by research conducted on *H. armigera* (Rafaeli and Gileadi, 1995b; Rafaeli *et al.*, 1997a, 1999). Octopamine and several octopaminergic analogs inhibited pheromone production in studies using both *in vitro* and *in vivo* bioassays in two species of moths (Rafaeli and Gileadi, 1995b, 1996; Rafaeli *et al.*, 1997, 1999; Hirashima *et al.*, 2001). The role of the VNC in pheromone production still requires clarification. It has been suggested, that in some moth species (*S. littoralis*) both humoral and neural regulation occurs (Marco *et al.*, 1996). Given the diversity of moths it may not be surprising to find several mechanisms regulating pheromone biosynthesis.

3.14.5.3. JH Regulation in Beetles

3.14.5.3.1. General In the Coleoptera, pheromone production or release is controlled by JH III (review: Tillman *et al.*, 1999). JH, or JH analogs, stimulate pheromone production in *T. molitor* (Menon, 1970), *T. castaneum* (both Tenebrionidae), some members of the Cucujidae (review: Plarre and Vanderwel, 1999), *A. grandis* (Curculionidae) (Wiygul *et al.*, 1990), and various Scolytidae (see Section 3.14.5.3.2). Various factors may work upstream as cues to stimulate JH biosynthesis. Feeding stimulates pheromone production or release in many species (Vanderwel and Oehlschlager, 1987). Sexual maturity and population density may also be important, e.g., for some Scolytidae (Byers, 1983) and Cucujidae (Plarre and Vanderwel, 1999). The effect of population density may be mediated by sensitivity to pheromone concentrations. Antennectomy can raise pheromone biosynthetic rates in *A. grandis* (Dickens *et al.*, 1988) and *L. decemlineata* (Dickens *et al.*, 2002), suggesting that detection of pheromone components may inhibit their production. Various combinations of physiological and

environmental factors therefore regulate JH titers, which in turn stimulate pheromone biosynthesis and/or release.

3.14.5.3.2. JH regulation in bark beetles (Scolytidae) Aggregation pheromone biosynthesis in bark beetles generally begins shortly after the beetle arrives at a new host tree (Wood, 1982; Seybold *et al*., 2000). Unfed beetles can be artificially stimulated to produce pheromones by treatment with JH III. For example, males are the pioneers of *Ips* spp., and starved male *I. paraconfusus* and *I. pini* can be induced to synthesize the pheromone components ipsenol and ipsdienol if JH III or a JH analog is applied topically (Bordon *et al*., 1969; Chen *et al*., 1988; Ivarsson and Birgersson, 1995; Tillman *et al*., 1998). Similarly, starved *Dendroctonus* spp. can be induced to synthesize pheromone components following treatment with JH (Bridges, 1982; Conn *et al*., 1984). Feeding apparently stimulates the synthesis of JH III in the CA (Tillman *et al*., 1998), resulting in elevated JH titers that trigger pheromone biosynthesis in midgut cells (Hall *et al*., 2002a, 2002b).

Pheromone biosynthesis requires a shift in metabolic priorities, particularly for those beetles that produce large quantities of pheromone. JH must actuate this shift, and since pheromone components are synthesized *de novo* (Seybold *et al*., 1995b), induction of the pheromone biosynthetic pathway involves elevated expression of at least some related genes. This was first demonstrated in *I. paraconfusus*, where JH-treated male beetles have increased HMG-R mRNA levels compared to controls (Tittiger *et al*., 1999). Male *I. pini* and *D. jeffreyi* HMG-R mRNA levels also rise in response to topical JH III applications (**Figure 11**) (Tittiger *et al*., 2003; Tillman, Lu, Tittiger, Blomquist, and Seybold, unpublished data). In all species studied, the response is dose and time dependent and correlates with pheromone component biosynthesis. This is consistent with the hypothesis that JH controls HMG-R, which functions as a regulator of the mevalonate (pheromone biosynthetic) pathway. HMG-S mRNA levels in male *D. jeffreyi* also respond to topical JH applications similarly to HMG-R, though at a more modest level (**Figure 11**) (Tittiger *et al*., 2000). In *I. pini*, mRNA for a GPPS gene is also elevated in JH III-treated beetles (Gilg-Young, Welch, Tittiger, and Blomquist, unpublished data). This enzyme diverts carbon from the normal mevalonate pathway into the pheromone biosynthetic pathway, and is thus also a likely regulatory step.

The HMG-S and HMG-R studies in *D. jeffreyi* show how putative regulatory enzymes are

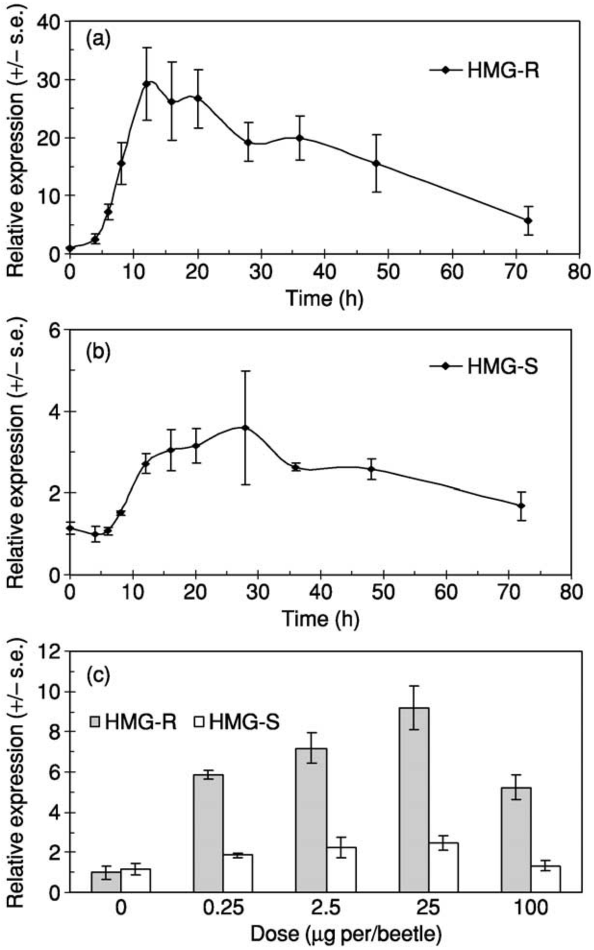


Figure 11 Regulation of *HMG-R* and *HMG-S* expression in male *Dendroctonus jeffreyi* by JH III. The time course (a, b) and dose response (c) for each gene in mature (emerged) males was investigated by Northern blotting. All values are relative to starved, untreated males. Each point represents the mean +/− standard error of three replicates, five isolated thoraces/sample. (Reprinted with permission from Tittiger, C., Barkawi, L.S., Bengoa, C.S., Blomquist, G.J., Seybold, S.J., **2003**. Structure and juvenile hormone-mediated regulation of the HMG-CoA reductase gene from the Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mol. Cell. Endocrinol.* *199*, 11–21; © Elsevier.)

controlled at the transcriptional level by JH III (though posttranscriptional regulation is probably also important; see below). Their coordinate induction implies that other mevalonate/pheromone biosynthetic genes may also be stimulated by JH III. This question has been addressed mostly in *I. pini*; indeed, more is known about pheromone biosynthetic gene regulation in this insect than in any other beetle, due in part to microarray-based expression profiling. Microarray analyses of male and female JH-treated and untreated *I. pini* give an idea of the profound change that male midgut cells undergo in order to synthesize pheromone, with numerous genes being upregulated or downregulated

following JH III treatment (Keeling and Tittiger, unpublished data). All identified mevalonate pathway genes in a recent expressed sequence tags (ESTs) project (BeetleBase, 2003; Eigenheer *et al.*, 2003) have elevated expression levels compared to controls (Keeling, Bearfield, Blomquist, and Tittiger, unpublished data). These include enzymes at predicted control points (HMG-R and GPPS) as well as others that would not be expected to have a strong regulatory role (e.g., isopentenyl diphosphate (IPP) isomerase). Thus, induction of identified pheromone-biosynthetic genes is apparently coordinate. By extension, genes encoding unidentified enzymes in the pheromone biosynthetic pathway are also likely JH regulated. The putative pheromone precursor, ipsdienone, was recovered from thoracic sections of JH-treated *I. paraconfusus* (Ivarsson *et al.*, 1998), and a male-specific myrcene synthase activity is also elevated in tissues of JH III-treated *I. pini* (Martin *et al.*, 2003). These data provide biochemical evidence that JH III affects enzyme activities converting geranyl diphosphate to ipsdienol, with myrcene and ipsdienone as probable intermediates. Confirmation that the corresponding genes are also JH regulated awaits their identification, and characterization of the relevant enzymes.

In the studies mentioned above, the amount of JH III penetrating the cuticle and acting on the midguts is unknown. A more biologically relevant question is whether feeding similarly stimulates pheromone biosynthetic gene expression. Recent real-time polymerase chain reaction (PCR) studies of RNA recovered from midguts from fed male and female *I. pini* confirm that HMG-R and HMG-S are induced in both sexes by feeding to levels similar to those observed in JH III-treated insects (Keeling and Tittiger, unpublished data). This is curious, because female midguts do not synthesize monoterpenoid pheromones, and female JH III titers do not naturally rise upon feeding (Tillman *et al.*, 1998). Other factors therefore must cause elevated *HMG-R* and *HMG-S* expression in female midguts. These experiments underscore the complexity of gene regulation in bark beetles, and remind us that mevalonate pathway genes almost certainly respond to other, possibly nonpheromone-related factors. Whatever the case, feeding clearly induces expression of certain genes in both male and female tissues, with males having an additional response characterized by increased rates of pheromone biosynthesis.

Juvenile hormone may act as a both trigger and stimulus for pheromone production. The basal HMG-R and HMG-S mRNA levels are from fivefold to eightfold higher in the midguts of males than of females, implying a greater capacity of mevalonate pathway flux in male cells compared to female cells (Keeling and Tittiger, unpublished data). Also, female GPPS mRNA levels, which are not clearly induced by feeding in female *I. pini* midguts, are approximately 16-fold lower than those in male midguts, and GPPS is induced by feeding and JH III treatment in males (Gilg-Young, Welch, Keeling, Bearfield, Blomquist, and Tittiger, unpublished data). This suggests that male midguts may be primed for pheromone biosynthesis, possibly due to an earlier, developmental cue, even before JH III triggers the process, and that pioneer beetles are ready to begin pheromone production when they arrive at a host tree. Subsequent feeding-induced JH III production thus both activates existing components of the pathway and stimulates continued production of pheromone pathway enzymes. Such priming might avoid a potentially dangerous "waiting" period, during which the pheromone-biosynthetic pathway is being stimulated, which would delay the arrival of other beetles to assist colonizing the host tree.

JH appears to be necessary for pheromone production, but it is not always sufficient. Similar gene responses to topically applied JH in male and female *I. pini* belie very different metabolic responses (Tillman, Lu, Seybold, Keeling, Bearfield, Blomquist, and Tittiger, unpublished data), suggesting a control mechanism for *I. pini* that is more complex than having JH III simply stimulate expression of pheromone biosynthetic genes. Other factors may be involved. For example, while all mevalonate pathway genes are induced in male *I. pini*, only genes corresponding to early steps (e.g., thiolase, HMG-R, HMG-S, IPP isomerase) are induced by JH III in females, while those for latter steps (GPPS, farnesyl diphosphate synthase (FPPS)) are not (Keeling *et al.*, 2004). Similarly, JH is clearly not sufficient to stimulate pheromone production in *I. paraconfusus* (Tillman, Lu, Tittiger, Blomquist, and Seybold, unpublished data). JH III-treated male *I. paraconfusus* have elevated HMG-R mRNA levels (the expression of other genes has not been studied in this insect), but neither HMG-R enzyme activity nor monoterpenoid pheromone component levels are significantly increased compared to controls. In contrast, male *I. paraconfusus* that have been allowed to feed on pine phloem have elevated HMG-R activity and, of course, elevated pheromone component levels. Decapitation studies confirm that an unknown ancillary hormone(s) is required in addition to JH III for pheromone biosynthesis (Lu, Blomquist, and Seybold, unpublished data). Presumably, one effect of this factor is in regulating the posttranscriptional activation of

HMG-R. Posttranscriptional regulation of pheromone biosynthetic activity has been demonstrated in other insect systems (e.g., PBAN-regulated activity of bombykol in silkmoths) (Moto *et al.*, 2003), and multilevel control of HMG-R activity is well documented (review: Hampton *et al.*, 1996). Future studies that concentrate on the effects of JH III on protein levels and enzyme activities should provide exciting new information of how JH III regulates the pheromone-biosynthetic machinery in bark beetles.

3.14.5.4. 20-Hydroxyecdysone Regulation of Pheromone Production in the Housefly

3.14.5.4.1. Ecdysteroid regulation Newly emerged female houseflies do not have detectable amounts of any C_{23} sex pheromone components. Sex pheromone production correlates with ovarian development and vitellogenesis. The C_{23} sex pheromone components first appear when ovaries mature to the early vitellogenic stages (Figure 12) and increase in amount until stages 9 and 10 (mature egg) (Dillwith *et al.*, 1983; Mpuru *et al.*, 2001). Females

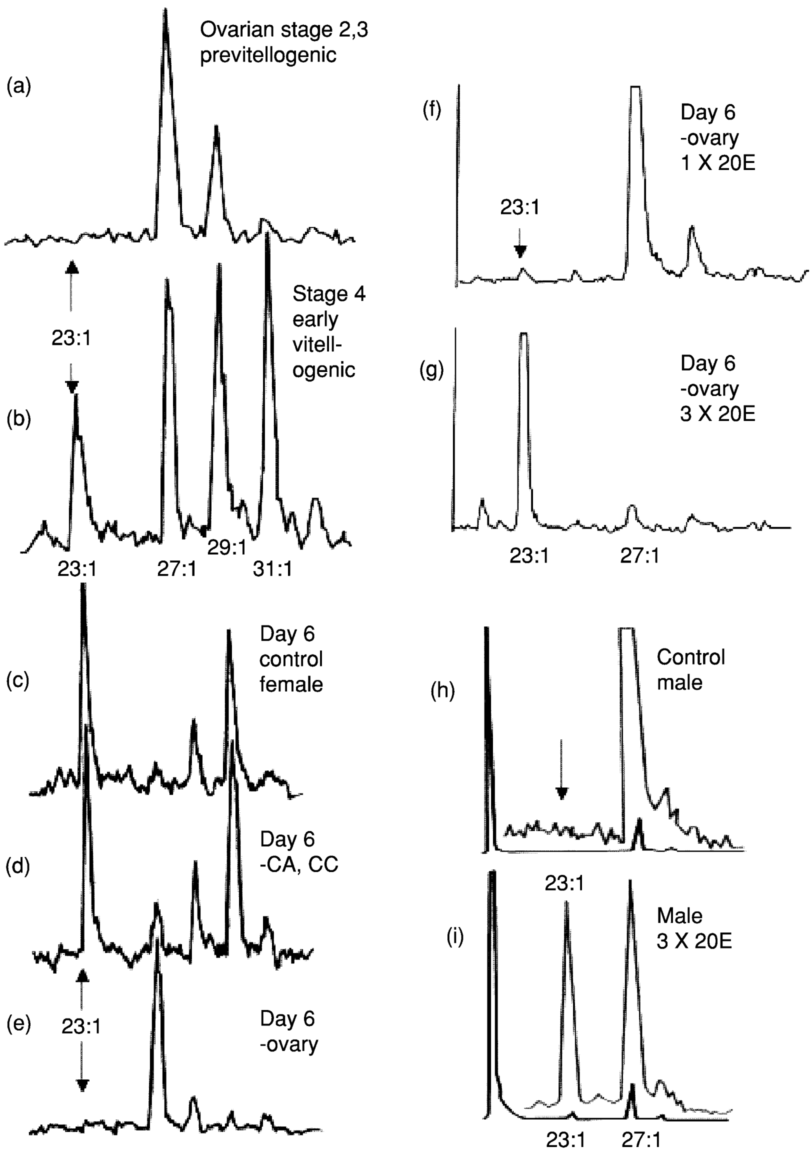


Figure 12 Effect of ovarian development (a, b), ovariectomy and allatectomy (c, d, e), and treatment with 20-hydroxyecdysone (20E) on ovariectomized females (f, g) and males (h, i) on (*Z*)-9-tricosene production. The results show that ovariectomy abolishes pheromone production, whereas treatment with 20E induces pheromone production in ovariectomized females and in males. (Reprinted with permission from Blomquist, G.J., **2003**. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, New York, pp. 131–252; © Elsevier.)

ovariectomized within 6 h of adult emergence do not produce any of the C_{23} sex pheromone components, whereas control and allatectomized females produced abundant amounts of (*Z*)-9-tricosene (**Figure 12c–e**). Ovariectomized insects that received ovary implants produced sex pheromone components in direct proportion to ovarian maturation. These data demonstrate that a hormone from the maturing ovary induced sex pheromone production (Blomquist *et al*., 1993, 1998).

Juvenile hormone regulates both vitellogenesis and pheromone production in some insect species (Tillman *et al*., 1999). In some Diptera, including the housefly, ovarian-produced ecdysteroids are involved in regulating vitellogenesis (Hagedorn, 1985; Adams *et al*., 1997) at the transcriptional level (Martin *et al*., 2001). Therefore, since ovariectomy abolished sex pheromone production whereas allatectomy (which abolishes JH production) had no effect on pheromone production (Blomquist *et al*., 1992), it was hypothesized that an ecdysteroid, and not JH, regulated sex pheromone production in the housefly. Injection of 20E at doses as low as 0.5 ng every 6 h induced sex pheromone production in ovariectomized houseflies in a time- and dose-dependent manner (**Figure 12f** and **g**) (Adams *et al*., 1984a, 1984b, 1995). Multiple injections of 20E into ovariectomized insects over several days resulted in as much 23 : 1 produced as in intact control females. Application of JH or JH analogs alone or in combination with ecdysteroids had no effect on pheromone production (Blomquist *et al*., 1992).

3.14.5.4.2. Induction of female sex pheromone production in male houseflies Male houseflies normally produce no detectable C_{23} sex pheromone components, but do produce the same C_{27} and longer alkenes as previtellogenic females. Implantation of ovaries into male houseflies resulted in a change in the chain length specificity of the alkenes such that (*Z*)-9-tricosene became a major component (Blomquist *et al*., 1984a, 1987). Likewise, injection of 20E into males induces sex pheromone production in a dose-dependent manner (**Figure 12h** and **i**). Thus, males possess the biosynthetic capability to produce sex pheromone, but normally do not produce the 20E necessary to induce sex pheromone production. This makes male houseflies a very convenient model to study the regulation of sex pheromone production, circumventing the need to ovariectomize a large number of female insects.

3.14.5.4.3. Ecdysteroids affect fatty acyl-CoA elongation enzymes There are two likely possibilities to account for the change in the chain length of the alkenes synthesized by the female housefly in the production of (*Z*)-9-trecosene. They are: (1) the chain-length specificity of the reductive conversion of acyl-CoAs to alkenes is altered such that 24:1-CoA becomes an efficient substrate, or (2) there is a change in the chain-length specificity of the fatty acyl-CoA elongation enzymes such that 24:1-CoA is not efficiently elongated, resulting in an accumulation of 24:1-CoA. To determine which enzyme activities are affected by 20E to regulate the chain length of the alkenes, experiments were performed to examine the chain-length specificity of the fatty acyl-CoA reductive conversion of acyl-CoAs to alkenes and elongation. Microsomal preparations from both males and females of all ages examined readily converted 24:1-CoA and the 24:1 aldehyde to (*Z*)-9-tricosene, indicating that 20E was not acting on this activity (Tillman-Wall *et al*., 1992; Reed *et al*., 1995). In contrast, microsomes from day-4 females (high ecdysteroid titer and production of (*Z*)-9 tricosene) did not elongate either 18:1-CoA or 24:1-CoA beyond 24 carbons, while microsomes from day-4 males or day-1 females (both of which produce alkenes of 27:1 and longer) readily elongated both 18:1-CoA and 24:1-CoA to 28:1-CoA (Tillman-Wall *et al*., 1992; Blomquist *et al*., 1995). Thus, 20E appears to regulate the fatty acyl-CoA elongases and not the enzymatic steps in the conversion of acyl-CoA to hydrocarbon.

3.14.5.4.4. Transport of pheromone The role of hemolymph in transporting hydrocarbons and hydrocarbon pheromones has only recently become fully appreciated. Older models of hydrocarbon formation showed epidermal-related cells (oenocytes) synthesizing and transporting hydrocarbons directly to the surface of the insect (Hadley, 1984). In the housefly, the role of hemolymph is most clearly seen when (*Z*)-9-tricosene production is initiated. (*Z*)-9-Tricosene first accumulates in the hemolymph, and then, after a number of hours, is observed on the surface of the insect. Modeling of the process (Mpuru *et al*., 2001) showed that the delay is surprisingly long; a period of more than 24 h is necessary for transport from site of synthesis to deposition on the surface of the insect.

In sexually mature females, (*Z*)-9-tricosene comprised a relatively large fraction of the hydrocarbon of the epicuticle and the hemolymph, but much smaller percentages of the hydrocabons in other tissues, including the ovaries. It appears that certain hydrocarbons were selectively partitioned to certain tissues such as the ovaries, from which pheromone was relatively excluded (Schal *et al*., 2001). Both KBr gradient ultracentrifugation and specific

immunoprecipitation showed that over 90% of the hemolymph hydrocarbon was associated with a high-density lipophorin (see **Chapter 4.6**). Lipophorin was composed of two aproproteins under denaturing conditions: apolipophorin I (≈240 kDa) and apolipophorin II (≈85 kDa) (Schal *et al.*, 2001). The data suggest that lipophorin may play an important role in an active mechanism that selectively delivers specific hydrocarbons to specific sites.

3.14.5.5. Regulation of Pheromone Production in Cockroaches

3.14.5.5.1. Development and cellular plasticity of pheromone glands In cockroaches, in striking contrast to many moths, pheromone glands acquire functional competence during an imaginal maturation period, and developmental regulation involves factors that also control adult reproductive readiness. Also, because reproduction in cockroaches is interrupted by periods of sexual inactivity (i.e., gestation), developmental regulation of the sex pheromone gland can result in alternating cycles of acquisition and subsequent waning of competence through maturation and retrogression, respectively, of cellular machinery. Consequently, in female cockroaches pheromone production is controlled by cyclic maturational changes in the gland in relation to the ovarian cycle.

Best exemplifying this phenomenon are the tergal and sternal glands of *N. cinerea* and *B. germanica* males. Both species possess class-3 glandular units, composed of two cells – a secretory cell and a duct cell (Quennedey, 1998). But after apolysis and before the imaginal molt the immature gland contains four concentric cells, including in addition to the two adult cells an enveloping cell and a ciliary cell (Sreng and Quennedey, 1976; Sreng, 1998). During several days after the adult molt the gland matures, in part by undergoing apoptosis (programmed cell death). The ciliary cell gives rise to a part of the microvillar end-apparatus, then dies, whereas the enveloping cell forms an upper portion of the duct, then it too dies (Sreng, 1998). In concert, before day 5 the immature sternal glands of *N. cinerea* males produce little pheromone, but after day 5 their pheromone content increases significantly (Sreng *et al.*, 1999). Decapitation or allatectomy of *N. cinerea* males completely blocked the apoptotic process, while JH III treatment restored apoptosis (Sreng *et al.*, 1999). Brain extracts or synthetic moth PBAN failed to restore gland differentiation or stimulate pheromone production.

Female *B. germanica* employ similar class-3 glands to produce a volatile sex pheromone that is yet to be identified. Ultrastructural, behavioral, and electrophysiological studies have shown that, as in males, pheromone gland cells mature as the female sexually matures (Abed *et al.*, 1993b; Liang and Schal, 1993; Tokro *et al.*, 1993; Schal *et al.*, 1996). The secretory cells of newly formed glands in the imaginal female are small and they contain little pheromone (determined by behavioral and EAG assays). As the female sexually matures, the size of pheromone-secreting cells increases, as does its pheromone content (Liang and Schal, 1993). The mature pheromone gland then undergoes cycles of cellular hypertrophy and retrogression in relation to the JH III titer in successive reproductive cycles. The gland becomes atrophied and its pheromone content declines during gestation, but as a new vitellogenic cycle begins after the egg case is deposited, the pheromone gland undergoes rapid regrowth and proliferation of cellular organelles and an increase in its pheromone content. Although this pattern corresponds well with the JH III titer in the hemolymph (Liang and Schal, 1993; Schal *et al.*, 1996), no experimental manipulations of hormone titers have been conducted to verify the hypothesis that JH III controls the cellular plasticity of the pheromone gland.

3.14.5.5.2. Pheromone production regulated by juvenile hormone Barth and Lester (1973) and Schal and Smith (1990) reviewed the early literature on hormone involvement in pheromone production in cockroaches. With the exception of *Nauphoeta* (detailed above), no studies on the regulation of volatile pheromone production are available that use analytical or biochemical approaches. The most detailed studies, with *B. germanica* and *S. longipalpa*, have employed behavioral and EAG responses of males to estimate the relative amount of pheromone in females or their pheromone glands. In both species, virgin females initiate pheromone production 4 days after the imaginal molt, in relation to increasing titers of JH III (Smith and Schal, 1990a; Liang and Schal, 1993). Ablation of the corpus allatum (CA) of newly emerged adult females prevents pheromone production in both species, and pheromone production is restored after reimplantation of active CA or by treatment with JH III or JH analogs. Interestingly, although growth of the vitellogenic oocytes is controlled by and highly correlated with JH III titers, direct or even intermediary involvement of the ovaries in regulating pheromone production and calling behavior in both species was excluded by ovariectomies (Smith and Schal, 1990a).

It is not known whether JH exerts its pheromonotropic effects directly on mature secretory cells of

the pheromone gland or if it acts indirectly by stimulating the synthesis and/or release of pheromonotropic neuropeptides. Although cockroach brain extracts induce PBAN-like pheromonotropic activity in moth pheromone glands (Raina *et al*., 1989), they fail to do so in allatectomized *Nauphoeta* males (Sreng *et al*., 1999) or *Supella* females (Schal, unpublished data). Moreover, lack of pheromone production in mated females that periodically produce large amounts of JH III suggests that JH plays a "permissive" role (Smith and Schal, 1990b; Schal *et al*., 1996). That is, its presence is required for pheromone to be produced, but even when the JH III titer is high pheromone production can be suppressed by neural or humoral pheromonostatic factors.

Blattella germanica has served as a useful model for delineating endocrine regulation of nonvolatile cuticular pheromones, namely (3*S*,11*S*)-dimethylnonacosan-2-one. Both the amount of pheromone on the cuticular surface and *in vivo* incorporation of radiolabel from [$^{14}C_1$]propionate into the sex pheromone coincide with active stages of vitellogenesis, suggesting the involvement of JH (Schal *et al*., 1990a, 1994; Sevala *et al*., 1999). Indeed, females treated so as to reduce their JH III titer (allatectomy, antiallatal drugs such as precocene, starvation, or implantation of an artificial egg case into the genital vestibulum, which inhibits JH biosynthesis) produce less pheromone (Schal *et al*., 1990a, 1994; Chase *et al*., 1992). Furthermore, pheromone production is greatly stimulated by treatments with JH III or with JH analogs. Because only the hydroxylation of 3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-ol is regulated in a sex-specific manner, it appears that this step is under JH III control (**Figure 11**) (Chase *et al*., 1992).

Normally, adult male cockroaches have a much lower titer of JH III in the hemolymph (Piulachs *et al*., 1992; review: Wyatt and Davey, 1996). Because males also produce 3,11-dimethylnonacosane, metabolism of the alkane to contact pheromone may be contingent upon high JH titers in the adult female. In response to exposure to the JH analog hydroprene, female pheromone increased sixfold in treated males (Schal, 1988), showing some capacity to express the putative female-specific polysubstrate monooxigenase. The parallels are striking with estrogen induction of vitellogenin synthesis in the male liver of oviparous vertebrates, JH induction of vitellogenin synthesis in male cockroaches (Mundall *et al*., 1983), and ecdysteroid induction of female pheromone production in houseflies (see Section 3.14.5.3.2) (Blomquist *et al*., 1984b, 1987).

Contact pheromone production in *B. germanica* is also regulated through the regulated production of its precursor, 3,11-dimethylnonacosane. Biosynthesis of this alkane drops dramatically when food intake declines at the end of each vitellogenic phase (Schal *et al*., 1994, 1996), suggesting that hydrocarbon biosynthesis is linked to food intake, as in nymphs (Young *et al*., 1999), and not directly to either the ecdysteroid or JH titers. Dietary intake also stimulates the production of JH III (Schal *et al*., 1993; Osorio *et al*., 1998), which in turn stimulates the conversion of the alkane to contact pheromone. In allatectomized females large amounts of hydrocarbons accumulate in the hemolymph because food intake is not suppressed (i.e., no gestation) and hydrocarbons are not provisioned into oocytes (i.e., no vitellogenesis) (Schal *et al*., 1994; Fan *et al*., 2002). As hydrocarbons accumulate in the hemolymph, the amount of cuticular pheromone also increases, suggesting that excess 3,11-dimethylnonacosane is metabolized to pheromone. These patterns suggest that under normal conditions, feeding in adult females is modulated in a stage-specific manner, regulating the amount of 3,11-dimethylnonacosane that is available for JH-mediated metabolism of 3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-one.

3.14.5.5.3. Transport and emission of pheromones Little is known of the cellular processes that deliver volatile pheromones from secretory cells to the cuticular surface, even in the intensively researched Lepidoptera. In cockroaches, electron microscopy studies often show accumulation of secretion in the end-apparatus, ducts, and around the cuticular pores of class-3 exocrine glands in both males and females. Recent studies in *L. maderae* have identified and sequenced an epicuticular protein, Lma-p54, that is expressed specifically in the tergites and sternites of adult males and females, but not in nymphs (Cornette *et al*., 2002). The sequence of this protein is closely related to aspartic proteases, but because it appears to be enzymatically inactive the authors speculate that it serves as a ligand-binding protein. Cornette *et al*. (2002) further hypothesize that Lma-p54, alone or together with a ligand, serves in sexual recognition. Other ligand-binding proteins, namely the lipocalins Lma-p22 and Lma-p18, have been isolated only from male tergal secretions of *L. maderae* (Cornette *et al*., 2001). These exciting findings suggest that carrier proteins might be involved in the transport of volatile pheromones to the cuticle, but functional studies will be needed to verify this hypothesis.

Attractant sex pheromones are usually emitted while the female or male cockroach performs a species-specific calling behavior (Gemeno and Schal, 2004; Gemeno *et al*., 2003). As in most lepidopterans, JH III regulates calling behavior in both species *B. germanica* and *S. longipalpa*. In both species, transection of the nerves connecting the CA to the brain, an operation that significantly accelerates the rate of JH III biosynthesis by the CA (Schal *et al*., 1993), also hastens the age when calling first occurs (Smith and Schal, 1990a; Liang and Schal, 1994). In *B. germanica*, the central role of JH has been confirmed by ablation of the CA and with rescue experiments with a JH analog (Liang and Schal, 1994). In this species, JH is also required for females to become sexually receptive and accept courting males (Schal and Chiang, 1995).

In *B. germanica*, the transfer of contact pheromones to the epicuticular surface is mediated by lipophorin, a high-density hemolymph lipoprotein (reviews: Schal *et al*., 1998b, 2003). As in *M. domestica* (see Section 3.14.5.4.4), newly biosynthesized pheromone appears first in the hemolymph before it is noted on the epicuticle (Gu *et al*., 1995). That hemolymph is required to transport the pheromone to the cuticular surface was demonstrated by severing the veins that enter the forewings. Subsequently, the amount of hydrocarbons and pheromone that appeared on the wings was significantly lower than on the intact forewings of the same insects.

In the cockroaches *P. americana* and *B. germanica*, virtually all newly synthesized hydrocarbons that enter the hemolymph are bound to lipophorin (Chino, 1985; Gu *et al*., 1995). Moreover, in both species newly synthesized hydrocarbons can only be transferred from the integument to an incubation medium if lipophorin is present, and other hemolymph lipoproteins, such as vitellogenin, cannot mediate this transfer (Katase and Chino, 1982, 1984; Fan *et al*., 2002). The mechanisms by which hydrocarbons and pheromones are taken up by lipophorin are poorly understood. Takeuchi and Chino (1993) demonstrated clearly in the American cockroach that a very-high-density lipid transfer particle (LTP) catalyzes the transfer of hydrocarbons between lipophorin particles. However, *in vitro* experiments with purified lipophorin of *P. americana* and *B. germanica* have shown that lipophorin accepts hydrocarbons from oenocytes, apparently without the involvement of LTP (Katase and Chino, 1982, 1984; Fan *et al*., 2002). It remains to be determined whether this is because sufficient LTP remains bound to dissected tissues, if LTP is produced by the dissected tissues, or whether it plays no significant role in hydrocarbon and pheromone uptake by lipophorin.

Interestingly, while the uptake of hydrocarbons and pheromones by lipophorin *in vitro* appears to lack molecular specificity (Katase and Chino, 1984; Fan and Schal, unpublished data), their delivery to pheromone gland cells is highly specific (Schal *et al*., 1998a, 1998b). Uptake of lipophorin and its ligands might involve receptor-mediated endocytosis, as demonstrated in mosquito oocytes (Cheon *et al*., 2001; see **Chapter 3.9**). This might explain why some 3,11-dimethylnonacosan-2-one has been isolated from mature ovaries of the German cockroach (Gu *et al*., 1995) and (*Z*)-9-tricosene is found in housefly ovaries (Schal *et al*., 2001). However, an endocytic process would fail to discriminate various ligands, suggesting that alternative mechanisms need to be investigated.

3.14.6. Concluding Remarks and Future Directions

Our increased understanding of the biochemistry and regulation of pheromone production over the last two decades has been most impressive. A 1983 review of the biochemistry and endocrine regulation of insect pheromone production (Blomquist and Dillwith, 1983) was 16 pages long. It was limited to early work on pheromone biosynthesis in moths and the housefly, and recognized that JH and ecdysteroids may play a role in the regulation of pheromone production. Since that time the discovery of PBAN and its role in the regulation of lepidopteran pheromone production have been elucidated, along with determining which enzymes are affected by JH and ecdysteroids to regulate pheromone production in model cockroaches/beetles and flies, respectively. The work in Lepidoptera has moved from simply demonstrating that pheromone components were synthesized *de novo* to the molecular characterization of the unique Δ11-desaturase and other desaturases that are involved in many female moths and their interplay with specific chain shortening steps. While it is still true that in no system do we have a complete understanding of both the biochemical pathways and their endocrine regulation, we do have a much better understanding of how pheromones are made and in some systems are developing an understanding of their regulation at the molecular level. The continued application of the powerful tools of molecular biology along with studies using genomics and proteomics will only increase the rate at which we increase our understanding of pheromone production. Ultimately, just as behavioral chemicals themselves have been extended into pest

control, research on pheromone production will be directed toward practical applications in insect control.

References

Abed, D., Cheviet, P., Farine, J.P., Bonnard, O., Le Quéré, J.L., *et al.*, **1993a**. Calling behaviour of female *Periplaneta americana*: behavioural analysis and identification of the pheromone source. *J. Insect Physiol. 39*, 709–720.

Abed, D., Tokro, P., Farine, J.-P., Brossut, R., **1993b**. Pheromones in *Blattella germanica* and *Blaberus craniifer* (Blaberoidea): glandular source, morphology and analyses of pheromonally released behaviours. *Chemoecol. 4*, 46–54.

Adams, T.S., Dillwith, J.W., Blomquist, G.J., **1984a**. The role of 20-hydroxyecdysone in housefly sex pheromone biosynthesis. *J. Insect Physiol. 30*, 287–294.

Adams, T.S., Gerst, J.W., Masler, E.P., **1997**. Regulation of ovarian ecdysteroid production in the housefly, *Musca domestica*. *Arch. Insect Biochem. Physiol. 35*, 135–148.

Adams, T.S., Holt, G.G., Blomquist, G.J., **1984b**. Endocrine control of pheromone biosynthesis and mating behavior in the housefly, *Musca domestica*. *Adv. Invert. Reprod. 3*, 441–456.

Adams, T.S., Nelson, D.R., Blomquist, G.J., **1995**. Effect of endocrine organs and hormones on (*Z*)-9-tricosene levels in the internal and external lipids of female house flies, *Musca domestica*. *J. Insect Physiol. 41*, 609–615.

Ahmad, S., Kirkland, K.E., Blomquist, G.J., **1987**. Evidence for a sex pheromone metabolizing cytochrome P-450 monooxygenase in the housefly. *Arch. Insect Biochem. Physiol. 6*, 121–140.

Ando, T., Hase, T., Arima, R., Uchiyama, M., **1988**. Biosynthetic pathway of bombykol, the sex pheromone of the female silkworm moth. *Agric. Biol. Chem. 52*, 473–478.

Antony, C., Jallon, J.-M., **1982**. The chemical basis for sex recognition in *Drosophila melanogaster*. *J. Insect Physiol. 28*, 873–880.

Arima, R., Takahara, K., Kadoshima, T., Numazaki, F., Ando, T., *et al.*, **1991**. Hormonal regulation of pheromone biosynthesis in the silkworm moth, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool. 26*, 137–147.

Arsequell, G., Fabriàs, G., Camps, F., **1990**. Sex pheromone biosynthesis in the processionary moth *Thaumetopoea pityocampa* by delta-13 desaturation. *Arch. Insect Biochem. Physiol. 14*, 47–56.

Baker, T.C., **2002**. Mechanism for saltational shifts in pheromone communication systems. *Proc. Natl Acad. Sci. USA 99*, 13368–13370.

Baltensweiler, W., Priesner, E., **1988**. A study of pheromone polymorphism in *Zeiraphera diniana* Gn. (Lep., Tortricidae). 3. Specificity of attraction to synthetic pheromone sources by different male response types from two host races. *J. Appl. Entomol. 106*, 217–231.

Barak, A.V., Burkholder, W.E., **1977**. Behavior and pheromone studies with *Attagenus elongatulus* Casey (Coleoptera: Dermestidae). *J. Chem. Ecol. 3*, 219–237.

Barbier, J., Lederer, E., **1960**. Structure chémique de la substance royale de la reine d'abeille (*Apis mellifica* L.). *C. R. Acad. Sci. Paris 251*, 1131–1135.

Barkawi, L.S., Francke, W., Blomquist, G.J., Seybold, S.J., **2003**. Frontalin: *de novo* synthesis of an aggregation pheromone component by *Dendroctonus* spp. bark beetles (Coleoptera: Scolytidae). *Insect Biochem. Mol. Biol. 33*, 773–788.

Bartelt, R.J., **1999**. Sap beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 69–89.

Bartelt, R.J., Weisleder, D., Dowd, P.F, Plattner, R.D., **1992**. Male-specific tetraene and triene hydrocarbons of *Carpophilus hemipterus*: structure and pheromonal activity. *J. Chem. Ecol. 18*, 379–402.

Barth, R.H., Jr., **1965**. Insect mating behavior: endocrine control of a chemical communication system. *Science 149*, 882–883.

Barth, R.H., Jr., Lester, L.J., **1973**. Neuro-hormonal control of sexual behavior in insects. *Annu. Rev. Entomol. 18*, 445–472.

BeetleBase, **2003**. Tittiger, C., Taylor, G., Gollery, M. University of Nevada, Reno Bioinformatics Core Facility. http://bioinformatics.unr.edu/beetle/.

Berger, R.S., **1966**. Isolation, identification, and synthesis of the sex attractant of the Cabbage Looper, *Trichoplusia ni*. *Ann. Entomol. Soc. America 59*, 767–771.

Bestmann, H.J., Herrig, M., Attygalle, A.B., **1987**. Terminal acetylation in pheromone biosynthesis by *Mamestra brassicae* L. (Lepidoptera: Noctuidae). *Experientia 43*, 1033–1034.

Billen, J., Morgan, E.D., **1998**. Pheromone communication in social insects: sources and secretions. In: Vander Meer, R.K., Breed, M.D., Winston, M.L., Espelie, K.E. (Eds.), Pheromone Communication in Social Insects. Westview Press, Boulder, CO, pp. 3–33.

Birgersson, G., Byers, J.A., Bergstrom, G., Lofqvist, J., **1990**. Production of pheromone components, chalcogran and methyl (*E,Z*)-2,4-decadienoate, in the spruce engraver *Pityogenes chalcographus*. *J. Insect Physiol. 36*, 391–395.

Bjostad, L.B., Linn, C.E., Du, J.-W., Roelofs, W.L., **1984**. Identification of new sex pheromone components in *Trichoplusia ni*, predicted from biosynthetic precursors. *J. Chem. Ecol. 10*, 1309–1323.

Bjostad, L.B., Roelofs, W.L., **1981**. Sex pheromone biosynthesis from radiolabeled fatty acids in the redbanded leafroller moth. *J. Biol. Chem. 256*, 7936–7940.

Bjostad, L.B., Roelofs, W.L., **1983**. Sex pheromone biosynthesis in *Trichoplusia ni*: key steps involve delta-11 desaturation and chain-shortening. *Science 220*, 1387–1389.

Bjostad, L.B., Roelofs, W.L., **1984**. Biosynthesis of sex pheromone components and glycerolipid precursors from sodium [1-^{14}C]acetate in redbanded leafroller moth. *J. Chem. Ecol. 10*, 681–691.

Bjostad, L.B., Wolf, W.A., Roelofs, W.L., **1987**. Pheromone biosynthesis in lepidopterans: desaturation and chain shortening. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, New York, pp. 77–120.

Blomquist, G.J., **2003**. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, New York, pp. 131–252.

Blomquist, G.J., Adams, T.S., Dillwith, J.W., **1984a**. Induction of female sex pheromone production in male houseflies by ovarian implants or 20-hydroxyecdysone. *J. Insect Physiol. 30*, 295–302.

Blomquist, G.J., Adams, T.S., Halarnkar, P.P., *et al.*, **1992**. Ecdysteroid induction of sex pheromone biosynthesis in the housefly, *Musca domestica*: are other factors involved? *J. Insect Physiol. 38*, 309–318.

Blomquist, G.J., Dillwith, J.W., **1983**. Pheromones: biochemistry and physiology. In: Downer, R.G.H., Laufer, H. (Eds.), Endocrinology of Insects. Alan R. Liss, New York, pp. 527–542.

Blomquist, G.J., Dillwith, J.W., Adams, T.S., **1987**. Biosynthesis and endocrine regulation of sex pheromone production in Diptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, New York, pp. 217–250.

Blomquist, G.J., Dillwith, J.W., Pomonis, J.G., **1984b**. Sex pheromone of the housefly: metabolism of (*Z*)-9-tricosene to (*Z*)-9,10-epoxytricosane and (*Z*)-14-tricosen-10-one. *Insect Biochem. 14*, 279–284.

Blomquist, G.J., Guo, L., Gu, P., Blomquist, C., Reitz, R.C., *et al.*, **1994**. Methyl-branched fatty acids and their biosynthesis in the housefly, *Musca domestica* L. (Diptera: Muscidae). *Insect Biochem. Mol. Biol. 24*, 803–810.

Blomquist, G.J., Howard, R.W., **2003**. Pheromone biosynthesis in social insects. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, New York, pp. 323–340.

Blomquist, G.J., Tillman, J.A., Mpuru, S., Seybold, S.J., **1998**. The cuticle and cuticular hydrocarbons of insects: structure, function and biochemistry. In: Vander Meer, R.K., Breed, M., Winston, M., Espelie, C. (Eds.), Pheromone Communication in Social Insects. Westview Press, Boulder, CO, pp. 34–54.

Blomquist, G.J., Tillman, J.A., Reed, J.R., Gu, P., Vanderwel, D., *et al.*, **1995**. Regulation of enzymatic activity involved in sex pheromone production in the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol. 25*, 751–757.

Blomquist, G.J., Tillman-Wall, J.A., Guo, L., Quilici, D., Gu, P., *et al.*, **1993**. Hydrocarbon and hydrocarbon derived sex pheromones in insects: biochemistry and endocrine regulation. In: Stanley-Samuelson, D.W., Nelson, D.R. (Eds.), Insect Lipids: Chemistry and Biology. University of Nebraska Press, Lincoln, NE, pp. 317–351.

Bordon, J.H., **1985**. Aggregation pheromones. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 9. Pergamon Press, Oxford, pp. 257–285.

Bordon, J.H., Nair, K.K., Slater, C.E., **1969**. Synthetic juvenile hormone: induction of sex pheromone production in *Ips confusus*. *Science 166*, 1626–1627.

Brand, J.M., Bracke, J.W., Britton, L.N., Markovetz, A.J., Barras, S.J., **1976**. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. *J. Chem. Ecol. 2*, 195–199.

Brand, J.M., Bracke, J.W., Markovetz, A.J., Wood, D.L., Browne, L.E., **1975**. Production of verbenol pheromone by a bacterium isolated from bark beetles. *Nature 254*, 136–137.

Bridges, J.R., **1982**. Effects of juvenile hormone on pheromone synthesis in *Dendroctonus frontalis*. *Environ. Entomol. 11*, 417–420.

Brossut, R., Roth, L.M., **1977**. Tergal modifications associated with abdominal glandular cells in the Blattaria. *J. Morphol. 151*, 259–297.

Butenandt, A., Beckmann, R., Stamm, D., Hecker, E., **1959**. Über dem sexual-lockstoff des seidenspinners *Bombyx mori*: Reindarstellung und konstitution. *Z. Naturforsch. A 14*, 283–284.

Byers, J.A., **1981**. Pheromone biosynthesis in the bark beetle, *Ips paraconfusus*, during feeding or exposure to vapours of host plant precursors. *Insect Biochem. 11*, 563–569.

Byers, J.A., **1983**. Influence of sex, maturity and host substances on pheromones in the guts of the bark beetles, *Ips paraconfusus* and *Dendroctonus brevicomis*. *J. Insect Physiol. 29*, 5–13.

Byers, J. A., Birgersson, G., **1990**. Pheromone production in a bark beetle independent of myrcene precursor in host pine species. *Naturwiss. 77*, 385–387.

Byrne, K.J., Swigar, A.A., Silverstein, R.M., Borden, J.H., Stokkink, E., **1974**. Sulcatol: population aggregation pheromone in the scolytid beetle, *Gnathotrichus sulcatus*. *J. Insect Physiol. 20*, 1895–1900.

Callow, R.K., Chapman, J.R., Paton, P.N., **1964**. Pheromones of the honey bee: chemical studies of the mandibular gland secretions of the queen. *J. Apicult. Res. 3*, 77–81.

Callow, R.K., Johnston, N.C., **1960**. The chemical constitution and synthesis of queen substance of honeybees (*Apis mellifera* L.). *Bee World 41*, 152–153.

Carlson, D.A., Langley, P.A., Huyton, P., **1978**. Sex pheromone of the tsetse fly: isolation, identification and synthesis of contact aphrodisiacs. *Science 201*, 750–753.

Carlson, D.A., Mayer, M.S., Sillhacek, D.L., James, J.D., Beroza, M., *et al.*, **1971**. Sex attractant pheromone of the housefly: isolation, identification and synthesis. *Science 174*, 76–78.

Carlson, D.A., Nelson, D.R., Langley, P.A., Coates, T.W., Leegwater-Vander Linden, M.E., **1984**. Contact sex pheromone in the tsetse fly *Glossina pallidipes* (Austen): identification and synthesis. *J. Chem. Ecol. 10*, 429–450.

Carlson, D.A., Offor, I.I., El Messoussi, S., Matsyyama, K., Mori, K., *et al.*, **1998**. Sex pheromone of *Glossina*

tachioides: isolation, identification and synthesis. *J. Chem. Ecol. 24*, 1563–1575.

Charlton, R.E., Webster, F.X., Zhang, A., Schal, C., Liang, D., *et al.*, **1993**. Sex pheromone for the brownbanded cockroach is an unusual dialkyl-substituted α-pyrone. *Proc. Natl Acad. Sci. USA 90*, 10202–10205.

Chase, J., Jurenka, R.J., Schal, C., Halarnkar, P.P., Blomquist, G.J., **1990**. Biosynthesis of methyl-branched hydrocarbons in the German cockroach *Blattella germanica* (L.) (Orthoptera, Blattellidae). *Insect Biochem. 20*, 149–156.

Chase, J., Touhara, K., Prestwich, G.D., Schal, C., Blomquist, G.J., **1992**. Biosynthesis and endocrine control of the production of the German cockroach sex pheromone, 3,11-dimethylnonacosan-2-one. *Proc. Natl Acad. Sci. USA 89*, 6050–6054.

Cheesbrough, T.M., Kolattukudy, P.E., **1984**. Alkane biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum sativum*. *Proc. Natl Acad. Sci. USA 81*, 6613–6617.

Cheesbrough, T.M., Kolattukudy, P.E., **1988**. Microsomal preparations from animal tissue catalyzes release of carbon monoxide from a fatty aldehyde to generate an alkane. *J. Biol. Chem. 263*, 2738–2743.

Chen, N.M., Bordon, J.H., Pierce, H.D., Jr., **1988**. Effect of juvenile hormone analog fenoxycarb, on pheromone production by *Ips paraconfusus* (Coleoptera: Scolytidae). *J. Chem. Ecol. 14*, 1087–1098.

Cheon, H.M., Seo, S.J., Sun, J., Sappington, T.W., Raikhel, A.S., **2001**. Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 31*, 753–760.

Chino, H., **1985**. Lipid transport: biochemistry of hemolymph lipophorin. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 115–135.

Choi, M.-Y., Han, K.S., Boo, K.S., Jurenka, R., **2002**. Pheromone biosynthetic pathway in *Helicoverpa zea* and *Helicoverpa assulta*. *Insect Biochem. Mol. Biol. 32*, 1353–1359.

Choi, M.-Y., Rafaeli, A., Jurenka, R., **2001**. Pyrokinin/PBAN-like peptides in the central nervous system of *Drosophila melanogaster. Cell Tissue Res. 306*, 459–465.

Choi, M.-Y., Tanaka, M., Kataoka, H., Boo, K.S., Tatsuki, S., **1998**. Isolation and identification of the cDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol. 28*, 759–766.

Christensen, T.A., Hildebrand, J.G., **1995**. Neural regulation of sex-pheromone glands in Lepidoptera. *Invert. Neurosci. 1*, 97–103.

Christensen, T.A., Itagaki, H., Teal, P.E.A., Jasensky, R.D., Tumlinson, J.H., *et al.*, **1991**. Innervation and neural regulation of the sex pheromone gland in female *Heliothis* moths. *Proc. Natl Acad. Sci. USA 88*, 4971–4975.

Christensen, T.A., Lashbrook, J.M., Hildebrand, J.G., **1994**. Neural activation of the sex-pheromone gland in the moth *Manduca sexta*: real-time measurement of pheromone release. *Physiol. Entomol. 19*, 265–270.

Christensen, T.A., Lehman, H.K., Teal, P.E.A., Itagaki, H., Tumlinson, J.H., *et al.*, **1992**. Diel changes in the presence and physiological actions of octopamine in the female sex-pheromone glands of heliothine moths. *Insect Biochem. Mol. Biol. 22*, 841–849.

Chu, A.J., Blomquist, G.J., **1980**. Biosynthesis of hydrocarbons in insects: succinate is a precursor of the methyl branched alkanes. *Arch. Biochem. Biophys. 201*, 304–312.

Conn, J.E., Bordon, J.H., Hunt, D.W.A., Holman, J., Whitney, H.S., *et al.*, **1984**. Pheromone production by axenically reared *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae). *J. Chem. Ecol. 10*, 281–290.

Cornette, R., Farine, J.-P., Quennedey, B., Brossut, R., **2001**. Molecular characterization of a new adult male putative calycin specific to tergal aphrodisiac secretion in the cockroach *Leucophaea maderae*. *FEBS Lett. 507*, 313–317.

Cornette, R., Farine, J.P., Quennedey, B., Riviere, S., Brossut, R., **2002**. Molecular characterization of Lma-p54, a new epicuticular surface protein in the cockroach *Leucophaea maderae* (Dictyoptera, Oxyhaloinae). *Insect Biochem. Mol. Biol. 32*, 1635–1642.

Croteau, R., **1981**. Biosynthesis of monoterpenes. In: Porter, J.W., Spurgeon, S.L. (Eds.), Biosynthesis of Isorpenoid Compounds, vol. 1. John Wiley, New York, pp. 225–282.

Cusson, M., McNeil, J.N., **1989**. Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. *Science 243*, 210–212.

Cusson, M., Tobe, S.S., McNeil, J.N., **1994**. Juvenile hormones: their role in the regulation of the pheromonal communication system of the armyworm moth, *Pseudaletia unipuncta*. *Arch. Insect Biochem. Physiol. 25*, 329–345.

Davis, M.B., Vakharia, V.N., Henry, J., Kempe, T.G., Raina, A.K., **1992**. Molecular cloning of the pheromone biosynthesis-activating neuropeptide in *Helicoverpa zea*. *Proc. Natl Acad. Sci. USA 89*, 142–146.

Davis, N.T., Homberg, U., Teal, P.E.A., Altstein, M., Agricola, H.-J., *et al.*, **1996**. Neuroanatomy and immunocytochemistry of the median neuroendocrine cells of the subesophageal ganglion of the tobacco hawkmoth, *Manduca sexta*: immunoreactivities to PBAN and other neuropeptides. *Microsc. Res. Tech. 35*, 201–229.

Delisle, J., Picimbon, J.-F., Simard, J., **1999**. Physiological control of pheromone production in *Choristoneura fummiferana* and *C. rosaceana*. *Arch. Insect Biochem. Physiol. 42*, 253–265.

De Mazo, L., Vit, S., **1983**. Contribution to the knowledge of Palearctic Batrisinae (Colopetera: Pselaphidae). Antennal male glands of *Batrisus* Aubé and *Batrisodes* Reitter: morponogy, histology and taxanomical implications. *Entomologica 18*, 77–110.

Dennis, M.W., Kolattukudy, P.E., **1991**. Alkane biosynthesis by decarbonylation of aldehyde catalyzed by a microsomal preparation from *Botryococcus brauni*. *Arch. Biochem. Biophys. 287*, 268–275.

Dennis, M.W., Kolattukudy, P.E., **1992**. A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. *Proc. Natl Acad. Sci. USA 89*, 5306–5310.

de Renobales, M., Blomquist, G.J., **1983**. A developmental study of the composition and biosynthesis of the cuticular hydrocarbons of *Trichoplusia ni*. *Insect Biochem. 13*, 493–502.

de Renobales, M., Nelson, D.R., Zamboni, A.C., Mackay, M.E., Dwyer, L.A., *et al.*, **1989**. Biosynthesis of very long-chain methyl branched alcohols during pupal development in the cabbage looper, *Trichoplusia ni*. *Insect Biochem. 19*, 209–214.

Dickens, J.C., McGovern, W.L., Wiygul, G., **1988**. Effects of antennectomy and a juvenile hormone analog on pheromone production in the boll weevil (Coleoptera: Curculionidae). *J. Entomol. Sci. 23*, 52–58.

Dickens, J.C., Oliver, J.E., Hollister, B., Davis, J.C., Klun, J.A., **2002**. Breaking a paradigm: male-produced aggregation pheromone for the Colorado potato beetle. *J. Exp. Biol. 205*, 1925–1933.

Dillwith, J.W., Adams, T.S., Blomquist, G.J., **1983**. Correlation of housefly sex pheromone production with ovarian development. *J. Insect Physiol. 29*, 377–386.

Dillwith, J.W., Blomquist, G.J., **1982**. Site of sex pheromone biosynthesis in the female housefly, *Musca domestica*. *Experientia 38*, 471–473.

Dillwith, J.W., Blomquist, G.J., Nelson, D.R., **1981**. Biosynthesis of the hydrocarbon components of the sex pheromone of the housefly, *Musca domestica* L. *Insect Biochem. 11*, 247–253.

Dillwith, J.W., Nelson, J.H., Pomonis, J.G., Nelson, D.R., Blomquist, G.J., **1982**. A 13C-NMR study of methyl-branched hydrocarbon biosynthesis in the housefly. *J. Biol. Chem. 257*, 11305–11314.

Duportets, L., Gadenne, C., Couillaud, F., **1999**. A cDNA, from *Agrotis ipsilon*, that encodes the pheromone biosynthesis activating neuropeptide (PBAN) and other FXPRL peptides. *Peptides 20*, 899–905.

Eigenheer, A.L., Keeling, C.I., Young, S., Tittiger, C., **2003**. Comparison of gene representation in midguts from two phytophagous insects, *Ips pini* and *Bombyx mori*, using expressed sequence tags. *Gene 316C*, 127–136.

Eisner, T., Meinwald, J., **2003**. Alkalid-derived pheromones and sexual selection in Lepidoptera. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 341–368.

Fabriàs, G., Barrot, M., Camps, F., **1995**. Control of the sex pheromone biosynthetic pathway in *Thaumetopoea pityocampa* by the pheromone biosynthesis activating neuropeptide. *Insect Biochem. Mol. Biol. 25*, 655–660.

Fabriàs, G., Jurenka, R.A., Roelofs, W.L., **1992**. Stimulation of sex pheromone production by proteinaceous extracts of the bursa copulatrix in the redbanded leafroller moth. *Arch. Insect Biochem. Physiol. 20*, 75–86.

Fabriàs, G., Marco, M.-P., Camps, F., **1994**. Effect of the pheromone biosynthesis activating neuropeptide on sex pheromone biosynthesis in *Spodoptera littoralis* isolated glands. *Arch. Insect Biochem. Physiol. 27*, 77–87.

Fan, Y., Chase, J., Sevala, V.L., Schal, C., **2002**. Lipophorin-facilitated hydrocarbon uptake by oocytes in the German cockroach, *Blattella germanica* (L.). *J. Exp. Biol. 205*, 781–790.

Fan, Y., Rafaeli, A., Gileadi, C., Applebaum, S.W., **1999**. Juvenile hormone induction of pheromone gland PBAN-responsiveness in *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol. 29*, 635–641.

Fan, Y., Zurek, L., Dykstra, M.J., Schal, C., **2003**. Hydrocarbon synthesis by enzymatically dissociated oenocytes of the abdominal integument of the German cockroach, *Blattella germanica*. *Naturwiss. 90*, 121–126.

Fang, N., Teal, P.E.A., Doolittle, R.E., Tumlinson, J.H., **1995a**. Biosynthesis of conjugated olefinic systems in the sex pheromone gland of female tobacco hornworm moths, *Manduca sexta* (L.). *Insect Biochem. Mol. Biol. 25*, 39–48.

Fang, N., Teal, P.E.A., Tumlinson, J.H., **1995b**. Characterization of oxidase(s) associated with the sex pheromone gland in *Manduca sexta* (L.) females. *Arch. Insect Biochem. Physiol. 29*, 243–257.

Faustini, D.L., Post, D.C., Burkholder, W.E., **1982**. Histology of aggregation pheromone gland in the red flour beetle. *Ann. Entomol. Soc. America 75*, 187–190.

Ferveur, J.F., Savarit, F., O'Kane, C.J., Jureau, G., Greenspan, R.J., *et al.*, **1997**. Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males. *Science 276*, 1555–1558.

Ferveur, J.F., Sureau, G., **1996**. Simultaneous influence on male courtship of stimulatory and inhibitory pheromones produced by life sex mosaic *Drosophila melangaster*. *Proc. Roy. Soc. B 263*, 967–973.

Fónagy, A., Matsumoto, S., Uchhiumi, K., Mitsui, T., **1992**. Role of calcium ion and cyclic nucleotides in pheromone production in *Bombyx mori*. *J. Pest Sci. 17*, 115–121.

Fónagy, A., Yokoyama, N., Okano, K., Tatsuki, S., Maeda, S., *et al.*, **2000**. Pheromone-producing cells in the silkmoth, *Bombyx mori*: identification and their morphological changes in response to pheromonotropic stimuli. *J. Insect Physiol. 46*, 735–744.

Fónagy, A., Yokoyama, N., Ozawa, R., Okano, K., Tatsuki, S., *et al.*, **1999**. Involvement of calcineurin in the signal transduction of PBAN in the silkworm, *Bombyx mori* (Lepidoptera). *Comp. Biochem. Physiol. B 124*, 51–60.

Foster, S.P., Roelofs, W.L., **1988**. Sex pheromone biosynthesis in the leafroller moth *Planotortrix excessana* by Δ10 desaturation. *Arch. Insect Biochem. Physiol. 8*, 1–9.

Foster, S.P., Roelofs, W.L., **1990**. Biosynthesis of a monoene and a conjugated diene sex pheromone component of the light brown apple moth by *E*11-desaturation. *Experientia 46*, 269–273.

Foster, S.P., Roelofs, W.L., **1996**. Sex pheromone biosynthesis in the tortricid moth, *Ctenopseustis herana* (Felder & Rogenhofer). *Arch. Insect Biochem. Physiol. 33*, 135–147.

Francke, W., Bartels, J., Meyer, H., Schroder, F., Kohnle, U., *et al*., **1995**. Semiochemicals from bark beetles: new results, remarks, and reflections. *J. Chem. Ecol. 21*, 1043–1063.

Fukui, H., Matsumura, F., Barak, A.V., Burkholder, W.E., **1977**. Isolation and identification of a major sex-attracting component of *Attagenus elongatus* (Casey) (Coleoptera: Dermestidae). *J. Chem. Ecol. 3*, 539–548.

Gadenne, C., **1993**. Effects of fenoxycarb, juvenile hormone mimetic, on female sexual behaviour of the black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae). *J. Insect Physiol. 39*, 25–29.

Gemeno, C., Schal, C., **2004**. Sex pheromones of cockroaches. In: Cardé, R.T., Millar, J. (Eds.), Advances in Insect Chemical Ecology. Cambridge University Press, New York, ch. 6, pp. 179–247.

Gemeno, C., Snook, K., Benda, N., Schal, C., **2003**. Behavioral and electrophysiological evidence for volatile sex pheromones in *Parcoblatta* wood cockroaches. *J. Chem. Ecol. 29*, 37–54.

Gershenzon, J., Croteau, R., **1991**. Terpenoids. In: Rosenthal, G.A., Berenbaum, M.A. (Eds.), Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York, pp. 225–282.

Gosalbo, L., Fabriàs, G., Camps, F., **1994**. Inhibitory effect of 10,11-methylenetetradec-10-enoic acid on a *Z*9-desaturase in the sex pheromone biosynthesis of *Spodoptera littoralis*. *Arch. Insect Biochem. Physiol. 26*, 279–286.

Gu, P., Welch, W.H., Blomquist, G.J., **1993**. Methyl-branched fatty acid biosynthesis in the German cockroach, *Blattella germanica*: kinetic studies comparing a microsomal and soluble fatty acid synthetases. *Insect Biochem. Mol. Biol. 23*, 263–271.

Gu, P., Welch, W.H., Guo, L., Schegg, K.M., Blomquist, G.J., **1997**. Characterization of a novel microsomal fatty acid synthetase (FAS) compared to a cytosolic FAS in the housefly, *Musca domestica*. *Comp. Biochem. Physiol. B 118*, 447–456.

Gu, X., Quilici, D., Juarez, P., Blomquist, G.J., Schal, C., **1995**. Biosynthesis of hydrocarbons and contact sex pheromone and their transport by lipophorin in females of the German cockroach (*Blattella germanica*). *J. Insect Physiol. 41*, 257–267.

Guerin, P.M., Baltensweiler, W., Arn, H., Buser, H.-R., **1984**. Host race pheromone polymorphism in the larch budmoth. *Experientia 40*, 892–894.

Guo, L., Latli, B., Prestwich, G.D., Blomquist, G.J., **1991**. Metabolically-blocked analogs of the housefly sex pheromone. 2. Metabolism studies. *J. Chem. Ecol. 17*, 1769–1782.

Hadley, N.F., **1984**. Cuticle: biochemistry. In: Bereiter-Hahn, J., Matoltsy, A.G., Richards, K.S. (Eds.), Biology of the Integument. Springer-Verlag, Berlin, pp. 685–702.

Hagedorn, H.H., **1985**. The role of ecdysteroids in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 205–262.

Halarnkar, P.P., Heisler, C.R., Blomquist, G.J., **1986**. Propionate catabolism in the housefly *Musca domestica* and the termite *Zootermopsis nevadensis*. *Insect Biochem. 16*, 455–461.

Halarnkar, P.P., Nelson, J.H., Heisler, C.R., Blomquist, G.J., **1985**. Metabolism of propionate to acetate in the cockroach *Periplaneta americana*. *Arch. Biochem. Biophys. 36*, 526–534.

Hall, G.M., Tittiger, C., Andrews, G., Mastick, G., Kuenzli, M., *et al*., **2002a**. Male pine engraver beetles, *Ips pini*, synthesize the monoterpenoid pheromone ipsdienol *de novo* in midgut tissue. *Naturwiss. 89*, 79–83.

Hall, G.M., Tittiger, C., Blomquist, G.J., Andrews, G.L., Mastick, G.S., *et al*., **2002b**. Male jeffrey pine beetle, *Dendroctonus jeffreyi*, synthesizes the pheromone component frontalin in anterior midgut tissue. *Insect Biochem. Mol. Biol. 32*, 1525–1532.

Hallberg, E., Subchev, M., **1997**. Unusual location and structure of female pheromone glands in *Theresimima* (=*Ino*) *ampelophaga* Bayle-Berelle (Lepidoptera: Zygaenidae). *Int. J. Insect Morphol. Embryol. 25*, 381–389.

Hampton, R., Dimster-Denk, D., Rine, J., **1996**. The biology of HMG-CoA reductase: the pros of contra-regulation. *Trends Biochem. Sci. 21*, 140–145.

Hao, G., Liu, W., O'Connor, M., Roelofs, W.L., **2002**. Acyl-CoA *Z*9- and *Z*10-desaturase genes from a New Zealand leafroller moth species, *Planotortrix octo*. *Insect Biochem. Mol. Biol. 32*, 961–966.

Hardie, J., Minks, A.K., **1999**. Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford.

Hashimoto, T., **1996**. Peroxisomal β-oxidation: enzymology and molecular biology. *Ann. New York Acad. Sci. 804*, 86–98.

Haynes, K.F., Hunt, R.E., **1990**. A mutation in pheromonal communication system of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol. 16*, 1249–1257.

Hendry, L.B., Piatek, B., Browne, L.E., Wood, D.L., Byers, J.A., *et al*., **1980**. *In vivo* conversion of a labelled host plant chemical to pheromones of the bark beetle, *Ips paraconfusus*. *Nature 284*, 485.

Holman, G.M., Cook, B.J., Nachman, R.J., **1986**. Isolation, primary structure and synthesis of a blocked neuropeptide isolated from the cockroach, *Leucophaea maderae*. *Comp. Biochem. Physiol. C 85*, 219–224.

Howard, R.W., **1993**. Cuticular hydrocarbons and chemical communication. In: Stanley-Samuelson, D.W., Nelson, D.R. (Eds.), Insect Lipids, Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln, NE, pp. 177–226.

Hughes, P.R., **1974**. Myrcene: a precursor of pheromones in *Ips* beetles. *J. Insect Physiol. 20*, 1271–1275.

Hunt, D.W.A., Bordon, J.H., **1989**. Terpene alcohol pheromone production by *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae) in the absence of readily culturable microorganisms. *J. Chem. Ecol. 15*, 1433–1463.

Iglesias, F., Marco, P., Francois, M.-C., Camps, F., Fabriàs, G., *et al.*, **2002**. A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualisation in scotophase hemolymph. *Insect Biochem. Mol. Biol. 32*, 901–908.

Iglesias, F., Marco, M.P., Jacquin-Joly, E., Camps, F., Fabriàs, G., **1998**. Regulation of sex pheromone biosynthesis in two noctuid species, *S. littoralis* and *M. brassicae*, may involve both PBAN and the ventral nerve cord. *Arch. Insect Biochem. Physiol. 37*, 295–304.

Imai, K., Konno, T., Nakazawa, Y., Komiya, T., Isobe, M., *et al.*, **1991**. Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. *Proc. Jap. Acad. B 67*, 98–101.

Islam, N., Bacala, R., Moore, A., Vanderwel, D., **1999**. Biosynthesis of 4-methyl-1-nonanol: female-produced sex pheromone of the yellow mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Insect Biochem. Mol. Biol. 29*, 201–208.

Ivarsson, P., Birgersson, G., **1995**. Regulation and biosynthesis of pheromone components in the double spined bark beetles, *Ips duplicatus* (Coleoptera: Scolytidae). *J. Insect Physiol. 41*, 843–849.

Ivarsson, P., Blomquist, G.J., Seybold, S.J., **1997**. *In vitro* production of the pheromone intermediates ipsdienone and ipsenone by the bark beetles *Ips pini* (Say) and *I. paraconfusus* Lanier (Coleoptera: Scolytidae). *Naturwiss. 84*, 454–457.

Ivarsson, P., Schlyter, F., Birgersson, G., **1993**. Demonstration of *de novo* pheromone biosynthesis in *Ips duplicatus* (Coleoptera: Scolytidae): inhibition of ipsdienol and *E*-myrcenol production by compactin. *Insect Biochem. Mol. Biol. 23*, 655–662.

Ivarsson, P., Tittiger, C., Borgeson, C.E., Seybold, S.J., Blomquist, G., *et al.*, **1998**. Pheromone precursor synthesis is localized in the metathorax of *Ips paraconfusus* Lanier (Coleoptera: Scolytidae). *Naturwiss. 85*, 507–511.

Iwanaga, M., Dohmae, N., Fonagy, A., Takio, K., Kawasaki, H., *et al.*, **1998**. Isolation and characterization of calmodulin in the pheromone gland of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol. B 120*, 761–767.

Jacquin, E., Jurenka, R.A., Ljungberg, H., Nagnan, P., Löfstedt, C., *et al.*, **1994**. Control of sex pheromone biosynthesis in the moth *Mamestra brassicae* by the pheromone biosynthesis activating neuropeptide. *Insect Biochem. Mol. Biol. 24*, 203–211.

Jacquin-Joly, E., Burnet, M., Francois, M., Ammar, D., Meillour, P., *et al.*, **1998**. cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of *Mamestra brassicae*: a new member of the PBAN family. *Insect Biochem. Mol. Biol. 28*, 251–258.

Jacquin-Joly, E., Descoins, C., **1996**. Identification of PBAN-like peptides in the brain–subesophageal ganglion complex of Lepidoptera using Western-blotting. *Insect Biochem. Mol. Biol. 26*, 209–216.

Jallon, J.-M., Wicker-Thomas, C., **2003**. Genetic studies on pheromone production in *Drosophila*. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Sex Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 253–281.

Juarez, P., Chase, J., Blomquist, G.J., **1992**. A microsomal fatty acid synthetase from the integument of *Blattella germanica* synthesizes methyl-branched fatty acids, precursors to hydrocarbon and contact sex pheromone. *Arch. Biochem. Biophys. 293*, 333–341.

Jurenka, R.A., **1996**. Signal transduction in the stimulation of sex pheromone biosynthesis in moths. *Arch. Insect Biochem. Physiol. 33*, 245–258.

Jurenka, R.A., **1997**. Biosynthetic pathway for producing the sex pheromone component (*Z*,*E*)-9,12-tetradecadienyl acetate in moths involves a Δ-12 desaturase. *Cell. Mol. Life Sci. 53*, 501–505.

Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80.

Jurenka, R.A., Fabriàs, G., Roelofs, W.L., **1991a**. Hormonal control of female sex pheromone biosynthesis in the redbanded leafroller moth, *Argyrotaenia velutinana*. *Insect Biochem. 21*, 81–89.

Jurenka, R.A., Haynes, K.F., Adlof, R.O., Bengtsson, M., Roelofs, W.L., **1994**. Sex pheromone component ratio in the cabbage looper moth altered by a mutation affecting the fatty acid chain-shortening reactions in the pheromone biosynthetic pathway. *Insect Biochem. Mol. Biol. 24*, 373–381.

Jurenka, R.A., Jacquin, E., Roelofs, W.L., **1991b**. Control of the pheromone biosynthetic pathway in *Helicoverpa zea* by the pheromone biosynthesis activating neuropeptide. *Arch. Insect Biochem. Physiol. 17*, 81–91.

Jurenka, R.A., Jacquin, E., Roelofs, W.L., **1991c**. Stimulation of sex pheromone biosynthesis in the moth *Helicoverpa zea* : action of a brain hormone on pheromone glands involves Ca^{2+} and cAMP as second messengers. *Proc. Natl Acad. Sci. USA 88*, 8621–8625.

Jurenka, R.A., Roelofs, W.L., **1989**. Characterization of the acetyltransferase involved in pheromone biosynthesis in moths: specificity for the *Z* isomer in Tortricidae. *Insect Biochem. 19*, 639–644.

Jurenka, R.A., Schal, C., Burns, E., Chase, J., Blomquist, G.J., **1989**. Structural correlation between cuticular hydrocarbons and female contact sex pheromone of German cockroach *Blattella germanica* (L.). *J. Chem. Ecol. 15*, 939–949.

Jurenka, R.A., Subchev, M., **2000**. Identification of cuticular hydrocarbons and the alkene precursor to the pheromone in hemolymph of the female gypsy moth,

Lymantria dispar. *Arch. Insect Biochem. Physiol. 43*, 108–115.

Jurenka, R.A., Subchev, M., Abad, J.-L., Choi, M.-Y., Fabriàs, G., **2003**. Sex pheromone biosynthetic pathway for disparlure in the gypsy moth, *Lymantria dispar. Proc. Natl Acad. Sci. USA 100*, 809–814.

Katase, H., Chino, H., **1982**. Transport of hydrocarbons by the lipophorin of insect hemolymph. *Biochim. Biophys. Acta 710*, 341–348.

Katase, H., Chino, H., **1984**. Transport of hydrocarbons by haemolymph lipophorin in *Locusta migratoria*. *Insect Biochem. 14*, 1–6.

Kawano, T., Kataoka, H., Nagasawa, H., Isogai, A., Suzuki, A., **1992**. cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun. 189*, 221–226.

Keeling, C.I., Blomquist, G.J., Tittiger, C., **2004**. Coordinated gene expression for pheromone biosynthesis in the pine engraver beetle, *Ipsini* (Coleoptera: Scolytidae). *Naturwissenschaften 91*, 324–328.

Keeling, C.I., Slessor, K.N., Higo, H.A., Winston, M.L., **2003**. New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. *Proc. Natl Acad. Sci. USA 100*, 4486–4491.

Kingan, T.G., Blackburn, M.B., Raina, A.K., **1992**. The distribution of PBAN immunoreactivity in the central nervous system of the corn earworm moth, *Helicoverpa zea*. *Cell Tissue Res. 270*, 229–240.

Kitamura, A., Nagasawa, H., Kataoka, H., Inoue, T., Matsumoto, S., *et al.*, **1989**. Amino acid sequence of pheromone-biosynthesis-activating neuropeptide (PBAN) of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun. 163*, 520–526.

Klun, J.A., Maini, S., **1982**. Genetic basis of an insect communication system: the European corn borer. *Environ. Entomol. 11*, 1084–1090.

Knipple, D.C., Roelofs, W.L., **2003**. Molecular biological investigations of pheromone desaturases. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 81–106.

Knipple, D.C., Rosenfield, C., Miller, S.J., Liu, W., Tang, J., *et al.*, **1998**. Cloning and functional expression of cDNA encoding a pheromone gland-specific acyl-CoA desaturase of the cabbage looper moth, *Trichoplusia ni*. *Proc. Natl Acad. Sci. USA 95*, 15287–15292.

Kolattukudy, P.E., **1980**. Cutin, suberin and waxes. In: Stumpf, P.K., Conn, E.V. (Eds.), The Biochemistry of Plants, vol. 4. Academic Press, New York, pp. 571–645.

Kolattukudy, P.E., Croteau, R., Buckner, J.S., **1976**. Biochemistry of plant waxes. In: Kolattukudy, P.E. (Ed.), Chemistry and Biochemistry of Natural Waxes. Elsevier, Amsterdam, pp. 289–347.

Lanne, B.S., Ivarrson, P., Johnsson, P., Bergström, G., Wassgren, A.-B., **1989**. Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera: Scolytidae). *Insect Biochem. 19*, 163–168.

Leal, W.S., **1998**. Chemical ecology of phytophagous scarab beetles. *Annu. Rev. Entomol. 43*, 39–61.

Leal, W.S., Shi, X., Liang, D., Schal, C., Meinwald, J., **1995**. Application of chiral gas chromatography with electroantennographic detection to the determination of the stereochemistry of a cockroach sex pheromone. *Proc. Natl Acad. Sci. USA 92*, 1033–1037.

Leal, W.S., Zarbin, P.H.G., Wojtasek, H., Ferreira, J.T., **1999**. Biosynthesis of scarab beetle pheromones: enantioselective 8-hydroxylation of fatty acids. *Eur. J. Biochem. 259*, 175–180.

Lee, J.M., Choi, M.Y., Han, K.S., Boo, K.S., **2001**. Cloning of the cDNA encoding pheromone biosynthesis activating neuropeptide in *Adoxophyes* sp. (Lepidoptera: Tortricidae): a new member of the PBAN family. GenBank accession number AF395670.

Levinson, H.Z., Levinson, A.R., Ren, Z., Mori, K., **1983**. Occurrence of a pheromone-producing gland in female tobacco beetles. *Experientia 39*, 1095–1097.

Liang, D., Schal, C., **1993**. Ultrastructure and maturation of a sex pheromone gland in the female German cockroach, *Blattella germanica*. *Tissue and Cell 25*, 763–776.

Liang, D., Schal, C., **1994**. Neural and hormonal regulation of calling behavior in *Blattella germanica* females. *J. Insect Physiol. 40*, 251–258.

Liu, W., Jiao, H., Murray, N.C., O'Connor, M., Roelofs, W.L., **2002b**. Gene characterized for membrane desaturase that produces (*E*)-11 isomers of mono- and diunsaturated fatty acids. *Proc. Natl Acad. Sci. USA 99*, 620–624.

Liu, W., Jiao, H., O'Connor, M., Roelofs, W.L., **2002a**. Moth desaturase characterized that produces both *Z* and *E* isomers of Δ11-tetradecenoic acids. *Insect Biochem. Mol. Biol. 32*, 1489–1495.

Löfstedt, C., Bengtsson, M., **1988**. Sex pheromone biosynthesis of (*E,E*)-8,10-dodecadienol in codling moth *Cydia pomonella* involves *E*9 desaturation. *J. Chem. Ecol. 14*, 903–915.

Ma, P.W.K., Garden, R.W., Niermann, J.T., O'Connor, M., Sweedler, J.V., *et al.*, **2000**. Characterizing the Hez-PBAN gene products in neuronal clusters with immunocytochemistry and MALDI MS. *J. Insect Physiol. 46*, 221–230.

Ma, P.W.K., Knipple, D.C., Roelofs, W.L., **1994**. Structural organization of the *Helicoverpa zea* gene encoding the precursor protein for pheromone biosynthesis-activating neuropeptide and other neuropeptides. *Proc. Natl Acad. Sci. USA 91*, 6506–6510.

Ma, P.W.K., Ramaswamy, S.B., **2003**. Biology and ultrastructure of sex pheromone producing tissue. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 19–51.

Ma, P.W.K., Roelofs, W.L., **1995a**. Calcium involvement in the stimulation of sex pheromone production by PBAN in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Insect Biochem. Mol. Biol. 25*, 467–473.

Ma, P.W.K., Roelofs, W.L., **1995b**. Sites of synthesis and release of PBAN-like factor in female European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol. 41*, 339–350.

Ma, P.W.K., Roelofs, W.L., **1995c**. Anatomy of the neurosecretory cells in the cerebral and subesophageal ganglia of the female European corn borer moth, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae). *Int. J. Insect Morphol. Embryol. 24*, 343–359.

Ma, P.W.K., Roelofs, W.L., Jurenka, R.A., **1996**. Characterization of PBAN and PBAN-encoding gene neuropeptides in the central nervous system of the corn earworm moth, *Helicoverpa zea*. *J. Insect Physiol. 42*, 257–266.

Marco, M.-P., Fabriàs, G., Lázaro, G., Camps, F., **1996**. Evidence for both humoral and neural regulation of sex pheromone biosynthesis in *Spodoptera littoralis*. *Arch. Insect Biochem. Physiol. 31*, 157–168.

Martin, D., Bohlmann, J., Gershenzon, J., Francke, W., Seybold, S.J., **2003**. A novel sex-specific and inducible monoterpene synthase activity associated with a pine bark beetle, the pine engraver, *Ips pini*. *Naturwiss. 90*, 173–179.

Martin, D., Wang, S.-F., Raikhel, A.S., **2001**. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol. Cell. Endocrinol. 173*, 75–86.

Martinez, T., Fabriàs, G., Camps, F., **1990**. Sex pheromone biosynthetic pathway in *Spodoptera littoralis* and its activation by a neurohormone. *J. Biol. Chem. 265*, 1381–1387.

Mas, E., Lloria, J., Quero, C., Camps, F., Fabriàs, G., **2000**. Control of the biosynthetic pathway of *Sesamia nonagrioides* sex pheromone by the pheromone biosynthesis activating neuropeptide. *Insect Biochem. Mol. Biol. 30*, 455–459.

Masler, E.P., Raina, A.K., Wagner, R.M., Kochansky, J.P., **1994**. Isolation and identification of a pheromonotropic neuropeptide from the brain–suboesophageal ganglion complex of *Lymantria dispar*: a new member of the PBAN family. *Insect Biochem. Mol. Biol. 24*, 829–836.

Matsumoto, S., Fónagy, A., Kurihara, M., Uchiumi, K., Nagamine, T., *et al.*, **1992.** Isolation and primary structure of a novel pheromonotropic neuropeptide structurally related to leucopyrokinin from the armyworm larvae, *Pseudaletia separata*. *Biochem. Biophys. Res. Commun. 182*, 534–539.

Matsumoto, S., Kitamura, A., Nagasawa, H., Kataoka, H., Orikasa, C., *et al.*, **1990**. Functional diversity of a neurohormone produced by the suboesophageal ganglion: molecular identity of melanization and reddish colouration hormone and pheromone biosynthesis activating neuropeptide. *J. Insect Physiol. 36*, 427–432.

Matsumoto, S., Ozawa, R.A., Nagamine, T., Kim, G., Uchiumi, K., *et al.*, **1995**. Intracellular transduction in the regulation of pheromone biosynthesis of the silkworm, *Bombyx mori:* suggested involvement of calmodulin and phosphoprotein phosphatase. *Biosci., Biotech. Biochem. 59*, 560–562.

Mayer, M.S., McLaughlin, J.R., **1991**. Handbook of Insect Pheromones and Sex Attractants. CRC Press, Boca Raton, FL.

Meng, X., Wahlstrom, G., Immonen, T., Kolmer, M., Tirronen, M., *et al.*, **2002**. The *Drosophila hugin* gene codes for myostimulatory and ecdysis-modifying neuropeptides. *Mechanisms Devel. 117*, 5–13.

Menon, M., **1970**. Hormone–pheromone relationships in the beetle *Tenebrio molitor*. *J. Insect Physiol. 16*, 1123–1139.

Millar, J.G., **2000**. Polyene hydrocarbons and epoxides: a second major class of lepidopteran sex attractant pheromones. *Annu. Rev. Entomol. 45*, 575–604.

Miller, J.R., Roelofs, W.L., **1980**. Individual variation in sex pheromone component ratios in two populations of the redbanded leafroller moth, *Argyrotaenia velutinana*. *Environ. Entomol. 9*, 359–363.

Mitlin, N., Hedin, P.A., **1974**. Biosynthesis of grandlure, the pheromone of the boll weevil, *Anthonomus grandis*, from acetate, mevalonate, and glucose. *J. Insect Physiol. 20*, 1825–1831.

Miyamoto, T., Yamamoto, M., Ono, A., Ohtani, K., Ando, T., **1999**. Substrate specificity of the epoxidation reaction in sex pheromone biosynthesis of the Japanese giant looper (Lepidoptera: Geometridae). *Insect Biochem. Mol. Biol. 29*, 63–69.

Moore, A.J., Moore, P.J., **1999**. Balancing sexual selection through opposing mate choice and male competition. *Proc. Roy. Soc. B 266*, 711–716.

Morse, D., Meighen, E.A., **1987a**. Biosynthesis of the acetate ester precursors of the spruce budworm sex pheromone by an acetyl CoA: fatty alcohol acetyltransferase. *Insect Biochem. 17*, 53–59.

Morse, D., Meighen, E.A., **1987b**. Pheromone biosynthesis: enzymatic studies in lepidoptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, San Diego, CA, pp. 121–158.

Moto, K., Yoshiga, T., Yamamoto, M., Takahashi, S., Okano, K., *et al.*, **2003**. Pheromone gland specific fatty-acyl reductase of the silk moth. *Bombyx mori. Proc. Natl Acad. Sci. USA 100*, 9156–9161.

Mpuru, S., Blomquist, G.J., Schal, C., Kuenzli, M., Dusticier, G., *et al.*, **2001**. Effect of age and sex on the production of internal and external hydrocarbons and pheromones in the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol. 31*, 139–155.

Mpuru, S., Reed, J.R., Reitz, R.C., Blomquist, G.J., **1996**. Mechanism of hydrocarbon biosynthesis from aldehyde in selected insect species: requirement for O_2 and NADPH and carbonyl group released as CO_2. *Insect Biochem. Mol. Biol. 2*, 203–208.

Mundall, E.C., Szibbo, C.M., Tobe, S.S., **1983**. Vitellogenin induced in adult male *Diploptera punctata* by juvenile hormone and juvenile hormone analogue: identification and quantitative aspects. *J. Insect Physiol. 29*, 201–207.

Nardi, J., Gilg-Young, A., Ujhelyi, E., Tittiger, C., Lehane, M., *et al.*, **2002**. Specialization of midgut cells for synthesis of male isoprenoid pheromone components in two scolytid beetles, *Dendroctonus jeffreyi* and *Ips pini*. *Tissue and Cell 34*, 221.

Nishida, R., Fukami, H., **1983**. Female sex pheromone of the German cockroach, *Blattella germanica*. *Mem. Coll. Agric. Kyoto Univ. 122*, 1–24.

Nishida, R., Fukami, H., Ishii, S., **1974**. Sex pheromone of the German cockroach (*Blattella germanica* L.) responsible for male wing-raising: 3,11-dimethyl-2-nonacosanone. *Experientia 30*, 978–979.

Nojima, S., Sakuma, M., Nishida, R., Kuwahara, Y., **1999**. A glandular gift in the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae): the courtship feeding of a female on secretions from male tergal glands. *J. Insect Behav. 12*, 627–640.

Ono, A., Imai, T., Inomata, S.-I., Watanabe, A., Ando, T., **2002**. Biosynthetic pathway for production of a conjugated dienyl sex pheromone of a plusiinae moth, *Thysanoplusia intermixta*. *Insect Biochem. Mol. Biol. 32*, 701–708.

Osorio, S., Piulachs, M.D., Bellés, X., **1998**. Feeding and activation of the corpora allata in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *J. Insect Physiol. 44*, 31–38.

Ozawa, R.A., Ando, T., Nagasawa, H., Kataoka, H., Suzuki, A., **1993**. Reduction of the acyl group: the critical step in bombykol biosynthesis that is regulated *in vitro* by the neuropeptide hormone in the pheromone gland of *Bombyx mori*. *Biosci., Biotech. Biochem. 57*, 2144–2147.

Paine, T.D., Millar, J.G., Hanlon, C.C., Hwang, J.-S., **1999**. Identification of semiochemicals associated with Jeffrey pine beetle, *Dendroctonus jeffreyi*. *J. Chem. Ecol. 25*, 433–453.

Park, Y.I., Ramaswamy, S.B., **1998**. Role of brain, ventral nerve cord and corpora cardiaca–corpora allata complex in the reproductive behavior of female tobacco budworm (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. America 91*, 329–334.

Park, Y., Zitnan, D., Gill, S.S., Adams, M.E., **1999**. Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. *FEBS Lett. 463*, 133–138.

Pennanec'h, M., Bricard, L., Kunesch, G., Jallon, J.M., **1997**. Incorporation of fatty acids into cuticular hydrocarbons of male and female *Drosophila melanogaster*. *J. Insect Physiol. 43*, 1111–1116.

Percy-Cunningham, J.E., MacDonald, J.A., **1987**. Biology and ultrastructure of sex pheromone-producing glands. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 27–75.

Perez, A.L., Gries, R., Gries, G., Oehlschlager, A.C., **1996**. Transformation of presumptive precursors to frontalin and *exo*-brevicomin by bark beetles and the West Indian sugarcane weevil (Coleoptera). *Bioorgan. Med. Chem. 4*, 445–450.

Petroski, R.J., Bartelt, R.J., Weisleder, D., **1994**. Biosynthesis of (2E,4E,6E)-5 ethyl-3-methyl-2,4,6-nonatriene: the aggregation pheromone of *Carpophilus freemani* (Coleoptera: Nitidulidae). *Insect Biochem. Mol. Biol. 24*, 69–78.

Pho, D.B., Pennanec'h, M., Jallon, J.M., **1996**. Purification of adult *Drosophila melanogaster* lipophorin and its role in hydrocarbon transport. *Arch. Insect Biochem. Physiol. 31*, 289–303.

Picimbon, J.-F., Becard, J.-M., Sreng, L., Clement, J.-L., Gadenne, C., **1995**. Juvenile hormone stimulates pheromonotropic brain factor release in the female black cutworm, *Agrotis ipsilon*. *J. Insect Physiol. 41*, 377–382.

Piulachs, M.D., Maestro, J.L., Bellés, X., **1992**. Juvenile hormone production and accessory gland development during sexual maturation of male *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Comp. Biochem. Physiol. A 102*, 477–480.

Plarre, R., Vanderwel, D.C., **1999**. Stored-product beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects associated with Agricultural Plants. CAB International, Wallingford, pp. 149–198.

Plettner, E., Slessor, K.N., Winston, M.L., **1998**. Biosynthesis of mandibular acids in honeybees (*Apis mellifera*): *de novo* synthesis, route of fatty acid hydroxylation and caste selective β-oxidation. *Insect Biochem. Mol. Biol. 28*, 31–42.

Plettner, E., Slessor, K.N., Winston, M.L., Oliver, J.E., **1996**. Caste-selective pheromone biosynthesis in honeybees. *Science 271*, 1851–1853.

Predel, R., Eckert, M., **2000**. Tagma-specific distribution of FXPRLamides in the nervous system of the American cockroach. *J. Comp. Neurol. 419*, 352–363.

Predel, R., Kellner, R., Kaufmann, R., Penzlin, H., Gäde, G., **1997**. Isolation and structural elucidation of two pyrokinins from the retrocerebral complex of the American cockroach. *Peptides 18*, 61–66.

Predel, R., Kellner, R., Nachman, R.J., Holman, G.M., Rapus, J., *et al.*, **1999**. Differential distribution of pyrokinin-isoforms in cerebral and abdominal neurohemal organs of the American cockroach. *Insect Biochem. Mol. Biol. 29*, 139–144.

Prestwich, G.D., Blomquist, G.J., **1987**. Pheromone Biochemistry. Academic Press, San Diego, CA.

Quennedey, A., **1998**. Insect epidermal gland cells: ultraxtructure and morphogenesis. In: Harrison, F.W., Locke, M. (Eds.), Microscopic Anatomy of Invertebrates, vol. 11A. Wiley–Liss, New York, pp. 177–207.

Rafaeli, A., **1994**. Pheromonotropic stimulation of moth pheromone gland cultures *in vitro*. *Arch. Insect Biochem. Physiol. 25*, 287–299.

Rafaeli, A., Gileadi, C., **1995a**. Factors affecting pheromone production in the stored product moth, *Plodia interpunctella*: a preliminary study. *J. Stored Prod. Res. 31*, 243–247.

Rafaeli, A., Gileadi, C., **1995b**. Modulation of the PBAN-stimulated pheromonotropic activity in *Helicoverpa armigera*. *Insect Biochem. Mol. Biol. 25*, 827–834.

Rafaeli, A., Gileadi, C., **1996**. Down regulation of pheromone biosynthesis: cellular mechanisms of pheromonostatic responses. *Insect Biochem. Mol. Biol. 26*, 797–808.

Rafaeli, A., Gileadi, C., Cao, M., **1997a**. Physiological mechanisms of pheromonostatic responses: effects of adrenergic agonists and antagonists on moth (*Helicoverpa armigera*) pheromone biosynthesis. *J. Insect Physiol. 43*, 261–269.

Rafaeli, A., Gileadi, C., Fan, Y., Cao, M., **1997**. Physiological mechanisms of pheromonostatic responses: effects of adrenergic agonists and antagonists on moth (*Heliocoverpa armigera*) pheromone biosynthesis. *J. Insect Physiol. 43*, 261–269.

Rafaeli, A., Gileadi, C., Hirashima, A., **1999**. Identification of novel synthetic octopamine receptor agonists which inhibit moth sex pheromone production. *Pesticide Biochem. Physiol. 65*, 194–204.

Rafaeli, A., Hirsch, J., Soroker, V., Kamensky, B., Raina, A.K., **1991**. Spatial and temporal distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa* (*Heliothis*) *armigera* using RIA and *in vitro* bioassay. *Arch. Insect Biochem. Physiol. 18*, 119–129.

Rafaeli, A., Jurenka, R.A., **2003**. PBAN regulation of pheromone biosynthesis in female moths. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 107–136.

Rafaeli, A., Soroker, V., Hirsch, J., Kamensky, B., Raina, A.K., **1993**. Influence of photoperiod and age on the competence of pheromone glands and on the distribution of immunoreactive PBAN in *Helicoverpa* spp. *Arch. Insect Biochem. Physiol. 22*, 169–180.

Rafaeli, A., Soroker, V., Kamensky, B., Gileadi, C., Zisman, U., **1997b**. Physiological and cellular mode of action of pheromone biosynthesis activating neuropeptide (PBAN) in the control of pheromonotropic activity of female moths. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, London, pp. 74–82.

Rafaeli, A., Soroker, V., Kamensky, B., Raina, A.K., **1990**. Action of pheromone biosynthesis activating neuropeptide on *in vitro* pheromone glands of *Heliothis armigera* females. *J. Insect Physiol. 36*, 641–646.

Rafaeli, A., Zakharova, T., Lapsker, Z., Jurenka, R.A., **2003**. The identification of an age- and female-specific putative PBAN membrane-receptor protein in pheromone glands of *Helicoverpa armigera*: possible up-regulation by juvenile hormone. *Insect Biochem. Mol. Biol. 33*, 371–380.

Raina, A.K., **1993**. Neuroendocrine control of sex pheromone biosynthesis in Lepidoptera. *Annu. Rev. Entomol. 38*, 329–349.

Raina, A.K., Jaffe, H., Kempe, T.G., Keim, P., Blacher, R.W., *et al.*, **1989**. Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science 244*, 796–798.

Raina, A.K., Klun, J.A., **1984**. Brain factor control of sex pheromone production in the female corn earworm moth. *Science 225*, 531–533.

Raina, A.K., Menn, J.J., **1987**. Endocrine regulation of pheromone production in Lepidoptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 159–174.

Ramaswamy, S.B., Jurenka, R.A., Linn, C.E., Roelofs, W.L., **1995**. Evidence for the presence of a pheromonotropic factor in hemolymph and regulation of sex pheromone production in *Helicoverpa zea*. *J. Insect Physiol. 41*, 501–508.

Reed, J.R., Quilici, D.R., Blomquist, G.J., Reitz, R.C., **1995**. Proposed mechanism for the cytochrome P450 catalyzed conversion of aldehyde to hydrocarbon in the house fly, *Musca domestica*. *Biochem. 34*, 16221–16227.

Reed, J.R., Vanderwel, D., Choi, S., Pomonis, J.G., Reitz, R.C., *et al.*, **1994**. Unusual mechanism of hydrocarbon formation in the housefly: cytochrome P450 converts aldehyde to the sex pheromone component (*Z*)-9-tricosene and CO_2. *Proc. Natl Acad. Sci. USA 91*, 10000–10004.

Renwick, J.A.A., Hughes, P.R., Krull, I.S., **1976a**. Selective production of *cis*- and *trans*-verbenol from (−)- and (+)-α-pinene by a bark beetle. *Science 191*, 199–201.

Renwick, J.A.A., Hughes, P.R., Pitman, G.B., Vita, J.B., **1976b**. Oxidation products of terpenes identified from *Dendroctonus* and *Ips* bark bectels. *J. Insect Physiol. 22*, 725–727.

Roelofs, W.L., Cardé, R.T., Benz, G., von Salis, G., **1971**. Sex attractant of the larch bud moth found by electroantennogram method. *Experientia 27*, 1438.

Roelofs, W.L., Du, J.-W., Linn, C.E., Glover, T.J., Bjostad, L.B., **1986**. The potential for genetic manipulation of the redbanded leafroller moth sex pheromone blend. In: Heuttel, M.D. (Ed.), Evolutionary Genetics of Invertebrate Behavior. Plenum Press, New York, pp. 263–272.

Roelofs, W.L., Jurenka, R.A., **1996**. Biosynthetic enzymes regulating ratios of sex pheromone components in female redbanded leafroller moths. *Bioorgan. Med. Chem. Lett. 4*, 461–466.

Roelofs, W.L., Liu, W., Hao, G., Jiao, H., Rooney, A.P., *et al.*, **2002**. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl Acad. Sci. USA 99*, 13621–13626.

Roelofs, W.L., Wolf, W.A., **1988**. Pheromone biosynthesis in Lepidoptera. *J. Chem. Ecol. 14*, 2019–2031.

Romer, F., **1991**. The oenocytes of insects: differentiation, changes during molting, and their possible involvement in the secretion of moulting hormone. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods, vol. 3. Rutgers University Press, New Brunswick, NJ, pp. 542–566.

Rosell, G., Hospital, S., Camps, F., Guerrero, A., **1992**. Inhibition of a chain shortening step in the biosynthesis of the sex pheromone of the Egyptian armyworm

Spodoptera littoralis. *Insect Biochem. Mol. Biol. 22*, 679–685.

Rosenfield, C., You, K.M., Marsella-Herrick, P., Roelofs, W.L., Knipple, D.C., **2001**. Structural and functional conservation and divergence among acyl-CoA desaturases of two noctuid species, the corn earworm, *Helicoverpa zea*, and the cabbage looper, *Trichoplusia ni*. *Insect Biochem. Mol. Biol. 31*, 949–964.

Rule, G.S., Roelofs, W.L., **1989**. Biosynthesis of sex pheromone components from linolenic acid in arctiid moths. *Arch. Insect Biochem. Physiol. 12*, 89–97.

Sato, Y., Oguchi, M., Menjo, N., Imai, K., Saito, H., *et al.*, **1993**. Precursor polyprotein for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. *Proc. Natl Acad. Sci. USA 90*, 3251–3255.

Schal, C., **1988**. Regulation of pheromone synthesis and release in cockroaches. In: Zabza, A., Sehnal, F., Denlinger, D.L. (Eds.), Endocrinological Frontiers in Physiological Insect Ecology. Technical University of Wrocław Press, Wrocław, pp. 695–700.

Schal, C., Burns, E.L., Blomquist, G.J., **1990a**. Endocrine regulation of female contact sex pheromone production in the German cockroach, *Blattella germanica*. *Physiol. Entomol. 15*, 81–91.

Schal, C., Burns, E.L., Jurenka, R.A., Blomquist, G.J., **1990b**. A new component of the female sex pheromone of *Blattella germanica* (L.) (Dictyoptera: Blattellidae) and interaction with other pheromone components. *J. Chem. Ecol. 16*, 1997–2008.

Schal, C., Chiang, A.-S., **1995**. Hormonal control of sexual receptivity in cockroaches. *Experientia 51*, 994–998.

Schal, C., Chiang, A.-S., Burns, E.L., Gadot, M., Cooper, R.A., **1993**. Role of the brain in juvenile hormone synthesis and oocyte development: effects of dietary protein in the cockroach *Blattella germanica* (L.). *J. Insect Physiol. 39*, 303–313.

Schal, C., Fan, Y., Blomquist, G.J., **2003**. Regulation of pheromone biosynthesis, transport and emission in cockroaches. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 283–322.

Schal, C., Gu, X., Burns, E.L., Blomquist, G.J., **1994**. Patterns of biosynthesis and accumulation of hydrocarbons and contact sex pheromone in the female German cockroach, *Blattella germanica*. *Arch. Insect Biochem. Physiol. 25*, 375–391.

Schal, C., Liang, D., Blomquist, G.J., **1996**. Neural and endocrine control of pheromone production and release in cockroaches. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 3–20.

Schal, C., Liang, D., Hazarika, L.K., Charlton, R.E., Roelofs, W.L., **1992**. Site of pheromone production in female *Supella longipalpa* (Dictyoptera: Blattellidae): behavioral, electrophysiological, and morphological evidence. *Ann. Entomol. Soc. America 85*, 605–611.

Schal, C., Sevala, V., Capurro, M.d.L., *et al.*, **2001**. Tissue distribution and lipophorin transport of hydrocarbon and sex pheromones in the house fly, *Musca domestica*. *J. Insect Sci. 1*, 12 Available online:. insectscience.org/1.12.

Schal, C., Sevala, V., Cardé, R.T., **1998a**. Novel and highly specific transport of a volatile sex pheromone by hemolymph lipophorin in moths. *Naturwiss. 85*, 339–342.

Schal, C., Sevala, V.L., Young, H.P., Bachmann, J.A.S., **1998b**. Synthesis and transport of hydrocarbons: cuticle and ovary as target tissues. *Am. Zool. 38*, 382–393.

Schal, C., Smith, A.F., **1990**. Neuroendocrine regulation of pheromone production in cockoraches. In: Huber, I., Masler, E.P., Rao, B.R. (Eds.), Cockroaches as Models for Neurobiology: Applications in Biomedical Research. CRC Press, Boca Raton, FL, pp. 179–200.

Schlyter, F., Birgersson, G.S., **1999**. Forest beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 113–148.

Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., DeLoof, A., **1991**. Isolation, primary structure and synthesis of locustapyrokinin: a myotropic peptide of *Locusta migratoria*. *Gen. Comp. Endocrinol. 81*, 97–104.

Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., Kochansky, J.P., *et al.*, **1992**. Isolation, identification and synthesis of locustamyotropin III and IV, two additional neuropeptides of *Locusta migratoria*: members of the locustamyotropin peptide family. *Insect Biochem. Mol. Biol. 22*, 447–452.

Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., Loof, A.D., **1990a**. Isolation, identification and synthesis of locustamyotropin II, an additional neuropeptide of *Locusta migratoria*: member of the cephalomyotropic peptide family. *Insect Biochem. 20*, 479–484.

Schoofs, L., Holman, G.M., Hayes, T.K., *et al.*, **1990b**. Isolation, identification and synthesis of locustamyotropin (Lom-MT), a novel biologically active insect neuropeptide. *Peptides 11*, 427–433.

Schoofs, L., Holman, G.M., Nachman, R., Proost, P., Vandamme, J., *et al.*, **1993**. Isolation, identification and synthesis of locustapyrokinin-II from *Locusta migratoria*, another member of the FXPRLamide peptide family. *Comp. Biochem. Physiol. C 106*, 103–109.

Sevala, V., Shu, S., Ramaswamy, S.B., Schal, C., **1999**. Lipophorin of female *Blattella germanica* (L.): characterization and relation to hemolymph titers of juvenile hormone and hydrocarbons. *J. Insect Physiol. 45*, 431–441.

Seybold, S.J., Bohlmann, J., Raffa, K.F., **2000**. Biosynthesis of coniferophagous bark beetle pheromones and conifer isoprenoids: evolutionary perspective and synthesis. *Can. Entomol. 132*, 697–753.

Seybold, S.J., Ohtsuka, T., Wood, D.L., Kubo, I., **1995a**. Enantiomeric composition of ipsdienol: a chemotaxonomic character for North American populations of *Ips* spp. in the *pini* subgeneric group (Coleoptera: Scolytidae). *J. Chem. Ecol. 21*, 995–1016.

Seybold, S.J., Quilici, D.R., Tillman, J.A., Vanderwel, D., Wood, D.L., *et al.*, **1995b**. *De novo* biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). *Proc. Natl Acad. Sci. USA 92*, 8393–8397.

Seybold, S.J., Tittiger, C., **2003**. Biochemistry and molecular biology of *de novo* isoprenoid pheromone production. 1. The Scolytidae. *Annu. Rev. Entomol. 48*, 425–453.

Seybold, S.J., Vanderwel, D., **2003**. Biosynthesis and endocrine regulation of pheromone production in the Coleoptera. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biochemistry. Elsevier, San Diego, CA, pp. 137–200.

Shorey, H.H., **1974**. Environmental and physiological control of insext sex pheromone behavior. In: Birch, M.C. (Ed.), Pheromones. Elsevier, New York, pp. 62–80.

Silverstein, R.M., Rodin, J.O., Wood, D.L., **1966**. Sex attractants in frass produced by male *Ips confusus* in ponderosa pine. *Science 154*, 509–510.

Sirugue, D., Bonnard, O., Le Quere, J.L., Farine, J.-P., Brossut, R., **1992**. 2-Methylthiazolidine and 4-ethylguaiacol, male sex pheromone components of the cockroach *Nauphoeta cinerea* (Dictyoptera, Blaberidae): a reinvestigation. *J. Chem. Ecol. 18*, 2261–2276.

Smith, A.F., Schal, C., **1990a**. Corpus allatum control of sex pheromone production and calling in the female brown-banded cockroach, *Supella longipalpa* (F.) (Dictyoptera: Blattellidae). *J. Insect Physiol. 36*, 251–257.

Smith, A.F., Schal, C., **1990b**. The physiological basis for the termination of pheromone-releasing behaviour in the female brown-banded cockroach, *Supella longipalpa* (F.) (Dictyoptera: Blattellidae). *J. Insect Physiol. 36*, 369–373.

Smith, V.L., Doyle, K.E., Maune, J.F., Munjaal, R.P., Beckingham, K., **1987**. Structure and sequence of the *Drosophila melanogaster* calmodulin gene. *J. Mol. Biol. 196*, 471–485.

Soroker, V., Rafaeli, A., **1989**. *In vitro* hormonal stimulation of [^{14}C]acetate incorporation by *Heliothis armigera* pheromone glands. *Insect Biochem. 19*, 1–5.

Sreng, L., **1990**. Seducin, male sex pheromone of the cockroach *Nauphoeta cinerea*: isolation, identification, and bioassay. *J. Chem. Ecol. 16*, 2899–2912.

Sreng, L., **1998**. Apostosis-inducing brain factors in maturation of an insect sex pheromone gland during differentiation. *Differentiation 63*, 53–58.

Sreng, I., Glover, T., Roelofs, W., **1989**. Canalization of the redbanded leafroller moth sex pheromone blend. *Arch. Insect Biochem. Physiol. 10*, 73–82.

Sreng, L., Leoncini, I., Clement, J.L., **1999**. Regulation of sex pheromone production in the male *Nauphoeta cinerea* cockroach: role of brain extracts, corpora allata (CA), and juvenile hormone (JH). *Arch. Insect Biochem. Physiol. 40*, 165–172.

Sreng, L., Quennedey, A., **1976**. Role of a temporary ciliary structure in the morphogenesis of insect glands. An electron microscope study of the tergal glands of male *Blattella germanica* L. (Dictyoptera, Blattellidae). *J. Ultrastruct. Res. 56*, 78–95.

Stanley-Samuelson, D.W., Jurenka, R.A., Cripps, C., Blomquist, G.J., deRenobales, M., **1988**. Fatty acids in insects: composition, metabolism and biological significance. *Arch. Insect Biochem. Physiol. 9*, 1–33.

Subchev, M., Jurenka, R.A., **2001**. Identification of the pheromone in the hemolymph and cuticular hydrocarbons from the moth *Scoliopteryx libatrix* L. (Lepidoptera: Noctuidae). *Arch. Insect Biochem. Physiol. 47*, 35–43.

Tada, S., Leal, W.S., **1997**. Localization and morphology of sex pheromone glands in scarab beetles. *J. Chem. Ecol. 23*, 903–915.

Takeuchi, N., Chino, H., **1993**. Lipid transfer particle in the hemolymph of the American cockroach: evidence for its capacity to transfer hydrocarbons between lipophorin particles. *J. Lipid Res. 34*, 543–551.

Tamaki, Y., **1985**. Sex pheromones. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 9. Pergamon Press, Oxford, pp. 145–191.

Tang, J.D., Charlton, R.E., Cardé, R.T., Yin, C.-M., **1987**. Effect of allatectomy and ventral nerve cord transection on calling, pheromone emission and pheromone production in *Lymantria dispar. J. Insect Physiol. 33*, 469–476.

Tang, J.D., Charlton, R.E., Jurenka, R.A., Wolf, W.A., Phelan, P.L., *et al.*, **1989**. Regulation of pheromone biosynthesis by a brain hormone in two moth species. *Proc. Natl Acad. Sci. USA 86*, 1806–1810.

Teal, P.E.A., Tumlinson, J.H., **1987**. The role of alcohols in pheromone biosynthesis by two noctuid moths that use acetate pheromone components. *Arch. Insect Biochem. Physiol. 4*, 261–269.

Teal, P.E.A., Tumlinson, J.H., **1988**. Properties of cuticular oxidases used for sex pheromone biosynthesis by *Heliothis zea. J. Chem. Ecol. 14*, 2131–2145.

Thompson, A.C., Mitlin, N., **1979**. Biosynthesis of the sex pheromone of the male boll weevil from monoterpene precursors. *Insect Biochem. 9*, 293–294.

Thyagaraja, B.S., Raina, A.K., **1994**. Regulation of pheromone production in the gypsy moth, *Lymantria dispar*, and development of an *in vitro* bioassay. *J. Insect Physiol. 40*, 969–974.

Tillman, J.A., Holbrook, G.L., Dallara, P., Schal, C., Wood, D.L., *et al.*, **1998**. Endocrine regulation of *de novo* aggregation pheromone biosynthesis in the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Insect Biochem. Mol. Biol. 28*, 705–715.

Tillman, J.A., Seybold, S.J., Jurenka, R.A., Blomquist, G.J., **1999**. Insect pheromones: an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol. 29*, 481–514.

Tillman-Wall, J.A., Vanderwel, D., Kuenzli, M.E., Reitz, R.C., Blomquist, G.J., **1992**. Regulation of sex pheromone biosynthesis in the housefly, *Musca domestica*: relative contribution of the elongation and reductive step. *Arch. Biochem. Biophys. 299*, 92–99.

Tittiger, C., **2003**. Molecular biology of bank beetle pheromone production and endocrine regulation. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 201–230.

Tittiger, C., Barkawi, L.S., Bengoa, C.S., Blomquist, G.J., Seybold, S.J., **2003**. Structure and juvenile hormone-mediated regulation of the HMG-CoA reductase gene from the Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mol. Cell. Endocrinol. 199*, 11–21.

Tittiger, C., Blomquist, G.J., Ivarsson, P., Borgeson, C.E., Seybold, S.J., **1999**. Juvenile hormone regulation of HMG-R gene expression in the bark beetle *Ips paraconfusus* (Coleoptera: Scolytidae): implications for male aggregation pheromone biosynthesis. *Cell. Mol. Life Sci. 55*, 121–127.

Tittiger, C., O'Keeffe, C., Bengoa, C.S., *et al.*, **2000**. Isolation and endocrine regulation of HMG-CoA synthase cDNA from the male Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Insect Biochem. Mol. Biol. 30*, 1203–1211.

Tokro, P.G., Brossut, R., Sreng, L., **1993**. Studies on the sex pheromone of female *Blattella germanica* L. *Insect Sci. Applic. 14*, 115–126.

Torfs, P., Nieto, J., Cerstiaens, A., Boon, D., Baggerman, G., *et al.*, **2001**. Pyrokinin neuropeptides in a crustacean: isolation and identification in the white shrimp *Penaeus vannamei*. *Eur. J. Biochem. 268*, 149–154.

Tumlinson, J.H., Fang, N., Teal, P.E.A., **1997**. The effect of PBAN on conversion of fatty acyls to pheromone aldehydes in female. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 54–55.

Vanderwel, D., **1994**. Factors affecting pheromone production in beetles. *Arch. Insect Biochem. Physiol. 25*, 347–362.

Vanderwel, D., Oehlschlager, A.C., **1987**. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 175–215.

Vaz, A.H., Jurenka, R.A., Blomquist, G.J., Reitz, R.C., **1988**. Tissue and chain length specificity of the fatty acyl-CoA elongation system in the American cockroach. *Arch. Biochem. Biophys. 267*, 551–557.

Veelaert, D., Schoofs, L., Verhaert, P., De Loof, A., **1997**. Identification of two novel peptides from the central nervous system of the desert locust, *Schistocerca gregaria*. *Biochem. Biophys. Res. Commun. 241*, 530–534.

Wakayama, E.J., Dillwith, J.W., Howard, R.W., Blomquist, G.J., **1984**. Vitamin B_{12} levels in selected insects. *Insect Biochem. 14*, 175–179.

Wei, W., Miyamoto, T., Endo, M., *et al.*, **2003**. Polyunsaturated hydrocarbons in the hemolymph: biosynthetic precursors of epoxy pheromones of geometrid and arctiid moths. *Insect Biochem. and Mol. Biol. 33*, 397–405.

Wicker, C., Jallon, J.M., **1995a**. Influence of ovary and ecdysteroids on pheromone biosynthesis in *Drosophila melanogaster* (Diptera, Drosophilidae). *Eur. J. Entomol. 92*, 197–202.

Wicker, C., Jallon, J.-M., **1995b**. Hormonal control of sex pheromone biosynthesis in *Drosophila melanogaster. J. Insect Physiol. 41*, 65–70.

Wicker-Thomas, C., Henriet, C., Dallerac, R., **1997**. Partial characterization of a fatty acid desaturase gene in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 11*, 963–972.

Wiygul, G., Dickens, J.C., Smith, J.W., **1990**. Effect of juvenile hormone and β-bisabolol on pheromone production in fat bodies of male boll weevils, *Anthonomus grandis* Boheman (Coleoptera; Curculionidae). *Comp. Biochem. Physiol. B 95*, 489–491.

Wolf, W.A., Roelofs, W.L., **1983**. A chain-shortening reaction in orange tortrix moth sex pheromone biosynthesis. *Insect Biochem. 13*, 375–379.

Wolf, W.A., Roelofs, W.L., **1986**. Properties of the Δ11-desaturase enzyme used in cabbage looper moth sex pheromone biosynthesis. *Arch. Insect Biochem. Physiol. 3*, 45–52.

Wolf, W.A., Roelofs, W.L., **1987**. Reinvestigation confirms action of Δ11-desaturase in spruce budworm moth sex pheromone biosynthesis. *J. Chem. Ecol. 13*, 1019–1027.

Wood, D.L., **1982**. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles Coleoptera. *Ann. Rev. Entomol. 27*, 411–446.

Wu, W.Q., Zhu, J.W., Millar, J., Löfstedt, C., **1998**. A comparative study of sex pheromone biosynthesis in two strains of the turnip moth, *Agrotis segetum*, producing different ratios of sex pheromone components. *Insect Biochem. Mol. Biol. 28*, 895–900.

Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of juvenile hormone. 2. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.

Yang, H.-T., Chow, Y.-S., Peng, W.-K., Hsu, E.-L., **1998**. Evidence for the site of female sex pheromone production in *Periplaneta americana*. *J. Chem. Ecol. 24*, 1831–1843.

Yoder, J.A., Denlinger, D.L., Dennis, M.W., Kolattukudy, P.E., **1992**. Enhancement of diapausing flesh fly puparia with additional hydrocarbons and evidence for alkane biosynthesis by a decarbonylation mechanism. *Insect Biochem. Mol. Biol. 22*, 237–243.

Yoshiga, T., Yokoyama, N., Imai, N., Ohnishi, A., Moto, K., *et al.*, **2002**. cDNA cloning of calcineurin heterosubunits from the pheromone gland of the silkmoth, *Bombyx mori*. *Insect Biochem. Mol. Biol. 32*, 477–486.

Young, H.P., Bachmann, J.A.S., Schal, C., **1999**. Food intake in *Blattella germanica* (L.) nymphs affects hydrocarbon synthesis and its allocation in adults between epicuticle and reproduction. *Arch. Insect Biochem. Physiol. 41*, 214–224.

Zdarek, J., Nachman, R.J., Hayes, T.K., **1998**. Structure–activity relationships of insect neuropeptides of the pyrokinin/PBAN family and their selective action on pupariation in fleshfly (*Neobelleria bullata*) larvae (Diptera, Sarcophagidae). *Eur. J. Entomol. 95*, 9–16.

Zhang, T., Zhang, L., Xu, W., Shen, J., **2001**. Cloning and characterization of the cDNA of diapause hormone–pheromone biosynthesis activating neuropeptide of *Helicoverpa armigera*. GenBank Direct Submission.

Zhao, C., Löfstedt, C., Wang, X., **1990**. Sex pheromone biosynthesis in the Asian corn borer *Ostrinia furnicalis* 2. Biosynthesis of (*E*) and (*Z*)-12-tetradecenyl acetate involves Δ14 desaturation. *Arch. Biochem. Physiol. 15*, 57–65.

Zhu, J., Millar, J., Löfstedt, C., **1995**. Hormonal regulation of sex pheromone biosynthesis in the turnip moth, *Agrotis segetum*. *Arch. Biochem. Physiol. 30*, 41–59.

Zhu, J., Zhao, C.H, Lu, F., Bengtsson, M., Löfstedt, C., **1996**. Reductase specificity and the ratio regulation of *E/Z* isomers in pheromone biosynthesis of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Insect Biochem. Mol. Biol. 26*, 171–176.

Relevant Website

http://www.nysaes.cornell.edu – The Phenolist, a listing of lepidopteran phenomones compiled at Cornell University.

3.15 Molecular Basis of Pheromone Detection in Insects

R G Vogt, University of South Carolina, Columbia, SC, USA

3.15.1. Introduction

The detection of environmental chemicals is surely one of the oldest senses, critical to bacteria, single-celled eukaryotes, plants, fungi, and animals. Chemical signatures carry enormously diverse information about the external world. Pheromones represent one class of environmental signals and are stringently defined as chemicals produced by individuals and detected by other members of the same species (Karlson and Lüscher, 1959). Pheromones have been categorized as primers, stimulating or modulating an immediate behavioral response, and releasers, stimulating or modulating a longer-lasting physiological state (Birch, 1974; Wyatt, 2003).

Pheromone detection is viewed as among the most specialized form of chemodetection, and the mechanisms underlying the detection and processing of pheromone signals have been viewed not merely as a comprising a special case of chemodetection, but often as a distinct process. However, chemicals produced outside the species may have effects as profound as pheromones on an individual, either short term or long term. Furthermore, the underlying mechanisms for detecting and processing pheromone and nonpheromone chemical signals are turning out to be quite similar (e.g., Christensen and Hildebrand, 2002). Certain chemical signals, including pheromones but also karimones (signals between species) and plant-derived oviposition cues, are components of processes that have special roles in the life of an organism, and as such, evolutionary selection has acted to elaborate preexisting detection/response pathways in ways appropriate for the processing of those chemical signals. So, while pheromone detection pathways are clearly under different kinds of selective pressure than say food-odor detection pathways, the specializations seen in pheromone pathways are most likely modifications of an overall common process. Nevertheless, pheromones are clearly unique among all chemical signals, in that selection has acted on different members of the same species to coordinate both the production and detection of these signals. And yet, remarkably much is still unexplored in the area of the physiological/hormonal regulation of these two distinct pheromonal processes.

How are pheromones detected? Insects have two major chemosensory systems, olfaction (smell) and gustation (taste) (Stocker, 1994). Pheromones might be detected by either system, though virtually all that is known about the mechanisms underlying pheromone detection involves olfaction.

Olfaction might be defined as the detection of volatile chemostimulants, but a more appropriate definition relates to the unique neuroanatomy of olfaction. In insects, odors are detected by olfactory sensilla which come in a variety of shapes, including long and short hair-like structures and plate-like

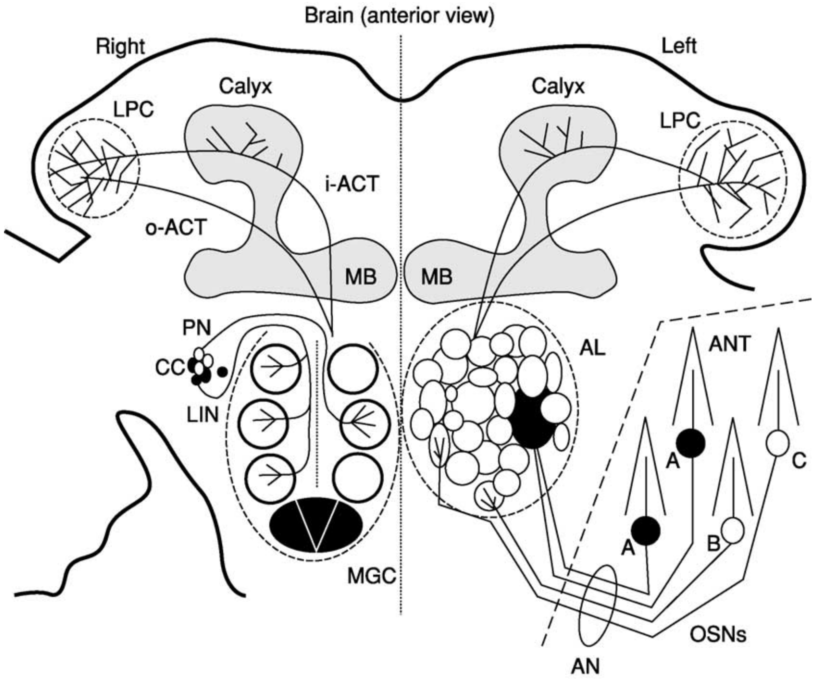


Figure 1 Frontal view of an adult insect brain detailing olfactory pathways through the brain. Left: Dendrites and cell bodies of olfactory sensory neurons (OSNs) are located in the antennas (ANT) and palps within sensilla; axons project, via the antennal nerve (AN), to spherical glomerulae in the antennal lobe (AL) where they synapse with local interneurons (LINs) and projection neurons (PNs). OSNs expressing the same OR project to the same glomerulus (e.g., neurons A) while OSNs expressing different receptors project to different glomerulae (e.g., neurons B, C) (Hansson *et al.*, 1992, 2003; Anton and Hansson, 1995; Joerges *et al.*, 1997; Galizia *et al.*, 1998, 1999, 2000; Gao *et al.*, 2000; Vosshall *et al.*, 2000; Galizia and Menzel, 2001; Carlsson *et al.*, 2002; Sadek *et al.*, 2002; Marin *et al.*, 2002; Carlsson and Hansson, 2003a, 2003b). Right: The AL contains multiple types of glomerulae, including specialized glomerulae such as the macroglomerular complex (MGC) (male specific in Lepidoptera) which comprises several glomerular units each receiving input from OSNs responding to specific components of a sex-pheromone blend (e.g., Christensen and Hildebrand, 2002; Hansson, 2002). LINs and PNs project from cell bodies located in one of several cell clusters (CC) located just outside the glomerular mass. LINs are multiglomerular, innervating all glomerulae, only the non-MGC glomerulae, or only the MGC (Matsumoto and Hildebrand, 1981; Hansson *et al.*, 1994). PNs are uniglomerular and projecting outward to the lateral protocerebrum (LPC) via one of two tracks. PNs of the inner antenno-cerebral tract (i-ACT) make synaptic connections with neurons of the mushroom bodies (MB) as well as in the LPC, while PNs of the outer antenno-cerebral tract (o-ACT) only make synaptic connections in the LPC (Homberg *et al.*, 1988; Kanzaki *et al.*, 1989, 2003; Hansson *et al.*, 1991; Sun *et al.*, 1997; McGuire *et al.*, 2001). Individual PNs innervate spatially restricted regions of the LPC, suggesting that there is a spatial conservation between glomerulus and LPC domain (Wong *et al.*, 2002).

structures, and which may have single or double cuticular walls; but all are multiporous, having many small holes penetrating the cuticle to provide odor molecules access to chemosensory neurons within (Steinbrecht, 1997, 1999; Boeckh *et al.*, 1965; see also Schneider, 1969; Kaissling, 1971). Adult olfactory neurons project to the olfactory lobes in the brain (Hildebrand, 1996; Hansson *et al.*, 2003) (**Figure 1**); larval olfactory neurons project to a location in the brain homologous to the adult olfactory lobe (Kent and Hildebrand, 1987; Python and Stocker, 2002). The dendrites of olfactory neurons are ensheathed within cuticular hairs, comprising olfactory sensilla which are restricted to the head (primarily antennas and maxillary palps) (Steinbrecht, 1997, 1999). While these olfactory sensilla certainly detect volatile chemicals for terrestrial insects, there are no data to say that homologous structures, as opposed to contact or gustatory sensilla, do not detect water-soluble molecules for aquatic insects. Nevertheless, it is the axonal projection pathway of the chemsensory neurons to the olfactory lobe that most consistently distinguishes the olfactory from the gustatory system.

Gustation might be defined as the detection of nonvolatile chemostimulants, but again, an anatomically based definition may better serve. Gustatory sensilla have a single pore at the tip (Steinbrecht, 1984); their frequent description as contact chemoreceptors confers the idea that these sensilla must make physical contact with a substrate permitting a chemostimulant to flow into the pore and to the sensory neurons within. Gustatory sensilla typically contain several chemosensory neurons tuned to

various modalities which may include nutrients, feeding stimulants or deterrents, water and/or salts, and may also include a mechanosensitive neuron (Hanson and Dethier, 1973; Steinbrecht, 1984; Kent and Hildebrand, 1987; Singh, 1997; Shanbhag *et al.*, 2001b). Neurons of these sensilla located on the head project to the subesophageal ganglia (Kent and Hildebrand, 1987; Dunipace *et al.*, 2001; Scott *et al.*, 2001). Gustatory sensilla are also situated elsewhere on the insect body, including legs, wings, and genitalia, and chemosensory axons from these locations project to the appropriate ganglion or ganglionic region serving that segment (Stocker, 1994). In general, neurons of gustatory sensilla do not project to the olfactory lobe, again providing a distinction between these two systems. However, a very small number of the gustatory receptor genes recently identified in *Drosophila melanogaster* have been shown to express in neurons that do project to the olfactory lobe (Scott *et al.*, 2001), emphasizing, perhaps, that in biology, no human-made distinctions should be set in stone.

There seems no reason that insect pheromones should be detected exclusively by olfactory rather than gustatory sensilla, although the majority of those pheromones that have been characterized are processed by the olfactory system. Nevertheless, pheromones run the gamut of volatility, including the relatively nonvolatile cuticular hydrocarbon pheromones of dipteran and various social insects (Blomquist *et al.*, 1998; Blomquist, 2003) (see **Chapter 3.14**). Also, there are numerous examples of insect courtship that employ both pheromones of low volatility and physical contact, suggesting that physical contact may be required for pheromone transfer, e.g. orthopteroids (Tregenza and Wedell, 1997; Rivault *et al.*, 1998) and social insects (review: Clement and Bagneres, 1998). *Drosophila* employs several types of pheromones. Several peptides are transferred with the spermatophore during mating which may enter the hemolymph and bind to olfactory axons to modulate olfactory response (Ottiger *et al.*, 2000) (see **Chapter 1.5**). A volatile aggregation pheromone, *cis*-vaccenyl acetate, is deposited on eggs and detected by trichoid sensilla on the antennas (Bartelt *et al.*, 1985; Clyne *et al.*, 1997). Various cuticular hydrocarbons also influence courtship behavior, including female specific *cis,cis*-7,11-heptacosadiene which alters male wing-beat frequency, and others which inhibit male–male interaction (Ferveur *et al.*, 1996, 1997; Ferveur, 1997; Savarit *et al.*, 1999).

The *Drosophila* cuticular hydrocarbon pheromones have been assumed to be detected by contact gustatory sensilla (Greenspan and Ferveur, 2000). In particular, *Drosophila* prothoracic legs show a sexual dimorphism in the number of gustatory sensilla, with males having the greater number (Nayak and Singh, 1983; Meunier *et al.*, 2000). This sexual asymmetry in sensilla number has led to suggestions that these sensilla may contain neurons that respond to the cuticular hydrocarbon pheromones (Robertson, 1983; Venard *et al.*, 1989; Stocker, 1994; de Bruyne, 2003). These sensilla have yet to be evaluated for the physiological response to cuticular hydrocarbons. However, they have been shown to respond to sugars, salts, and amino acids (the expected stimulants of the respective chemosensory neurons) and furthermore to show a corresponding sexual asymmetry in their response to these nonpheromonal stimulants (Meunier *et al.*, 2000), suggesting some sex-specific but nonpheromonal role for these gustatory sensilla. It may yet be demonstrated that *Drosophila* gustatory sensilla detect pheromones, but so far only olfactory sensilla of the antenna have been shown to participate in courtship behavior (Stocker and Gendre, 1989) or pheromone detection (Bartelt *et al.*, 1985; Clyne *et al.*, 1997). (Note: since this chapter was submitted, new data have been presented suggesting *Drosophila* leg gustatory sensilla are involved in pheromone detection, mediated by gustatory receptor gene Gr68a; see Bray and Amrein, 2003; Matsunami and Amrein, 2003.)

It remains less than definitive that any insect pheromones are in fact detected by gustatory sensilla. However, gustatory sensilla have been shown to function as pheromone detectors in noninsect arthropods such as ticks (Sonenshine, 1985; de Bruyne and Guerin, 1998). The lack of information from insects may merely be a sampling bias, with the vast majority of molecular and physiological research effort being focused on relatively few types of olfactory based pheromones.

This review covers the molecular biology of insect olfaction and to a lesser extent taste, with specific attention to those biochemical events, which led to the initial depolarization of chemosensory neurons. The discussion will be restricted to known gene products and the processes they mediate. In most cases, there are excellent examples of these processes working in the detection of pheromones. The exception to this is with odor receptors; as of early 2000s no pheromone receptor proteins have been reported, though this circumstance will likely change by the time this work is published, or very soon thereafter, as the genomes of the honeybee and several Lepidoptera become fully characterized.

3.15.2. Molecular Basis of Insect Chemodetection: General Schemes

Figure 2 summarizes our current general understanding of the biochemical events involved in the detection of pheromones and other odors by insect olfactory sensilla. Odor molecules enter the aqueous lumen of a sensillum via pores penetrating the cuticle (Steinbrecht, 1997; Steinbrecht and Stankiewicz, 1999). Once inside, odors are thought to bind to and be transported by soluble odorant binding proteins (OBPs) which deliver the odors to transmembrane olfactory receptor proteins (ORs). Odor molecules are subsequently degraded by a variety of enzymes that may be located within the sensillum lumen as well as within the cells lining the base of the sensillum. ORs so far identified are 7-transmembrane domain G-protein-coupled receptors (Vosshall and Keller, 2003); activation of these receptors is thought to generate a rapid increase in inositol triphosphate (IP3) which is thought to directly activate ion channels in the neuronal membrane. The *Drosophila* genome contains at least 61 OR genes and more than 50 OBP genes, providing the molecular basis for the detection of diverse odorants. For all but the ORs and arrestins, each of the biochemical events depicted in Figure 2 has examples in the processing of pheromone signals.

The biochemical processes at work in gustatory sensilla are less well characterized. Gustatory sensilla generally contain multiple sensory neurons including several chemosensory neurons and often one mechanosensory neuron; the chemosensory neurons are tuned to different modalities allowing insects, as is the case with vertebrates, to distinguish sweet, sour, salty, and bitter substances (review: Singh, 1997). However, the range of chemicals that insects detect with these sensilla is likely quite broad, e.g., ecdysteroid chemodetection (Descoins and Marion-Poll, 1999). A family of at least 55 G-protein coupled gustatory receptor (GR) genes has recently been identified in *Drosophila* (Clyne *et al*., 2000; Dunipace *et al*., 2001; Scott *et al*., 2001) and the malaria mosquito *Anopheles gambiae* (Hill *et al*., 2002). Of these, one (Gr5a) has been shown to interact with the sugar trehalose (Dahanukar *et al*., 2001; Ueno *et al*., 2001). An IP3 cascade, similar to that shown in Figure 2c, has also been described for the gustatory detection of sugars and amino acids (Koganezawa and Shimada, 2002). A member of the OBP family has also been reported to associate with certain gustatory sensilla of the blowfly *Phormia regina*, but participates in the detection of certain volatile compounds rather than those (e.g., sugars and amino acids) typically associated with gustatory sensilla (Ozaki *et al*., 1995, 2003).

The term "perireceptor events" was coined by Getchell *et al*. (1984) to describe processes in vertebrate olfaction that preceded the odor–OR interactions (see also Carr *et al*., 1990). The equivalent events in insect olfaction are depicted in Figure 2b, and are those involving OBPs, ODEs, and their interaction with ORs. Transductory events, then, are those depicted in Figure 2c, and involve ORs and the second messenger pathway(s) they regulate. While these two processes, perireception and transduction, clearly overlap at least at the level of the ORs, they remain useful distinctions. In particular, the transduction events that follow receptor activation appear relatively common for all odor–OR combinations. There may be more than one transduction pathway involved, as has been observed in the lobster (*Panulirus argus*) where both IP3 and cAMP pathways exist (Fadool and Ache, 1992; Michel and Ache, 1992; Boekhoff *et al*., 1994; Hatt and Ache, 1994). However, the immediate transduction elements (Figure 2c) do not show the genetic diversity seen for the perireceptor elements (Figure 2b). The OBPs, ODEs, and ORs are present in numbers and diverse sequences or types that suggest they are under strong evolutionary selective/diversification pressures characteristic of both the broad chemoreceptive needs and the divergent life histories of the species.

The majority of the elements depicted in Figure 2 have been identified since the publication of the previous edition of this work. In the early 1980s there were few laboratories studying the biochemistry of odor detection in insects. In 1981 the first identification of a pheromone binding protein (PBP) and a sensillar esterase (SE) was reported; both appeared uniquely expressed in the male antennas of the silk moth *Antheraea polyphemus* and were present in the extracellular fluid of the sensillum lumen (Vogt and Riddiford, 1981). In 1985 a scheme was proposed in which pheromone molecules first bound to PBPs for transport to ORs and were subsequently rapidly degraded by SEs (Vogt *et al*., 1985). The perceived need for such transport arose because pheromone molecules are hydrophobic odors that should not readily enter the aqueous interior of the sensillum lumen. This new scheme was a dramatic departure from the prevailing view that pore tubules (anatomical structures of the cuticle wall) served as conduits for odors from air to neuron (reviews: Vogt, 1987; Steinbrecht, 1997). The subsequent identification of general odorant binding proteins (GOBP1 and GOBP2) suggested that this new scheme might be extended to other

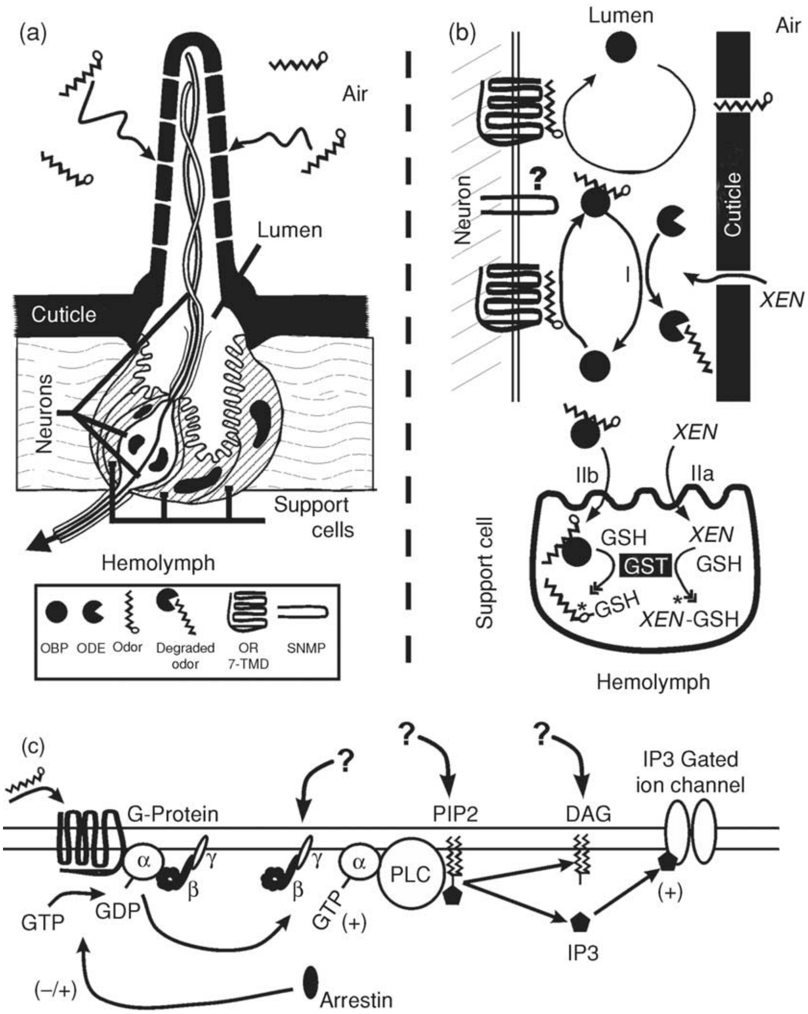


Figure 2 Schematic of an olfactory sensillum and a generalized biochemical pathway of odor reception. (a) Anatomy. An olfactory sensillum includes two or three neurons surrounded by three support cells; olfactory dendrites/cilia project up the fluid-filled lumen of a cuticular hair. The sensillum lumen is isolated from hemolymph by a cellular barrier. (Modified from Steinbrecht, R.A., **1969**. Comparative morphology of olfactory receptor. In: Pfaffmann, C. (Ed.), Olfaction and Taste III. Rockefeller University Press, New York, pp. 3–21.) Olfactory sensilla come in a range of shapes, including plate-like and long and short hair-like structures, and olfactory neurons may have branched or unbranched dendrites; sensilla classes are in part defined by the shape of the cuticular hair and the branched nature of the neurons (Steinbrecht, 1997, 1999). There are little data on the functional differences between different sensilla classes of a given individual, although in Lepidoptera, attractant sex pheromones are detected by the long trichoid sensilla, illustrated here. (b) Perireceptor events. Hydrophobic odor molecules enter the aqueous sensillum lumen via pores penetrating the cuticular hair wall. Hydrophilic odorant binding proteins (OBPs) are proposed to bind and transport odors to receptor proteins located in the neuronal membranes. Odor degrading enzymes (ODEs) (pathway I) in the sensillum lumen are proposed to degrade these odor molecules. Cytoplasm of support cells contains xenobiotic inactivating enzymes (pathway IIa), such as glutathione-*S*-transferase (GST), which may also serve to inactivate odor molecules (pathway IIb). Interactions between OBPs and ORs and the function of sensory neuron membrane proteins (SNMPs) are unclear. (Modified from Rogers, M.E., Jani, M.K., Vogt, R.G., **1999**. An olfactory specific glutathione *S*-transferase in the sphinx moth *Manduca sexta*. *J. Exp. Biol. 202*, 1625–1637.) (c) Transduction events. Odor–OR interaction stimulates an inositor triphosphate (IP3) second messenger cascade in which the α-subunit of a G-protein activates phospholipase C (PLC) to cleave the membrane lipid phosphotidyl inositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and IP3. IP3 is thought to bind directly to and activate transmembrane cation channels (IP3 receptors) (review: Krieger and Breer, 2003). Processes such as those mediated by phosphorylation (Schleicher *et al.*, 1994) or arrestins (Merrill *et al.*, 2002) may provide modulatory feedback on receptor–G-protein interactions. ‘‘?’’ pointing to Gβγ, PIP2, and DAG are to imply yet uncharacterized but possible modulatory roles for these signals.

olfactory modalities such as the detection of plant volatiles (Vogt and Lerner, 1989; Vogt *et al*., 1991a). The identification of an antennal specific but sex indifferent pheromone degrading aldehyde oxidase (AOX) in the moths *Manduca sexta* and *A. polyphemus* suggested that ODEs might also be a general feature of olfaction for both pheromones and plant volatiles (Rybczynski *et al*., 1989, 1990).

Prestwich joined these efforts in the mid-1980s synthesizing valuable chemical probes and pheromone analogs (Prestwich *et al*., 1984; Vogt *et al*., 1985, 1988) and elucidating the pheromone binding properties of PBPs (Prestwich, 1985, 1987; Du *et al*., 1994; Du and Prestwich, 1995; Plettner *et al*., 2000; Lazar *et al*., 2002). Many more laboratories became interested in OBPs beginning in the late 1980s, greatly expanding knowledge of the range of species possessing these proteins and knowledge of the size and diversity of the OBP gene family in single species (reviews: Pelosi and Maida, 1995; Krieger and Breer, 1999; Vogt *et al*., 1999; Leal, 2003; Nagnan-Le Meillour and Jacquin-Joly, 2003; Plettner, 2003; Vogt, 2003). The transduction pathway was identified as G-protein/IP3-based during the early 1990s (Boekhoff *et al*., 1990a, 1990b, 1993; Breer *et al*., 1990; Riesgo-Esgovar *et al*., 1995; Krieger and Breer, 2003). ORs were finally identified from the sequenced genome database of *Drosophila* (Clyne *et al*., 1999; Gao and Chess, 1999; Vosshall *et al*., 1999). Gustatory receptors were subsequently identified in a similar manner (Clyne *et al*., 2000; Dunipace *et al*., 2001; Scott *et al*., 2001). Now, having recently crossed the technological watershed of genome sequencing, we can expect to see this information expand rapidly across the Insecta.

3.15.3. Transduction Events

3.15.3.1. Receptors

3.15.3.1.1. Among noninsect species, odor molecules are detected by G-protein-coupled receptors The view that odor molecules are detected by membrane-bound receptor proteins was still being debated as recently as the early 1980s, and references to insect sensilla being light guides for infrared radiation being emitted from oscillating odor molecules still appear occasionally. But one really must credit Lancet for his seminal efforts in the early to mid-1980s, which firmly established the G-protein-coupled receptor (GPCR) model for odor detection. Working in vertebrates, Lancet and his group were the first to demonstrate odor-dependent increases in cAMP and the presence of G proteins in isolated olfactory neurons (Pace *et al*., 1985; Pace and Lancet, 1986). Work that followed established the existence of both cAMP and IP3 based G-protein transduction pathways in vertebrate olfaction (Jones and Reed, 1989; Dhallan *et al*., 1990; Bakalyar and Reed, 1991), setting the stage for the identification of 7-transmembrane domain GPCR odor receptors in the rat (Buck and Axel, 1991). The following decade saw the identification of odor receptors in vertebrates from fish, amphibians, birds, and mammals (Freitag *et al*., 1998; Mombaerts, 1999).

Vertebrate odor receptors comprise a large gene family, ranging from perhaps 100 members in fish to perhaps around 1300 in mammals (Zhang and Firestein, 2002; Young and Trask, 2002). These genes are quite conserved across the vertebrates; catfish receptors were initially identified using polymerase chain reaction (PCR) primers designed using rat OR sequences (Ngai *et al*., 1993), and a set of "universal" primers has been used to clone ORs from coelacanth to mammal (Freitag *et al*., 1998). Many details of this gene family, including its full size, are being revealed as full species genomes are made available. Interestingly, many OR genes have accumulated mutations rendering them nonfunctional pseudogenes, and the proportion of functional to nonfunctional genes varies considerably with the animal group and species (Frietag *et al*., 1998). For example, dolphins have no known functional OR genes (Frietag *et al*., 1998). Humans and mice have roughly similar number of OR genes, but in humans, nearly 70% are pseudogenes compared to perhaps 15% in mice (Young *et al*., 2002; Zhang and Firestein, 2002). The variation in functional members of this gene family can be considerable even among closely related species. Humans have as much as fourfold greater pseudogene content relative to other primates, a trend speculated to be related to differences in life history of the respective species (Rouquier *et al*., 2000; Gilad *et al*., 2003).

Vertebrate pheromone receptors are also GPCRs. The vertebrate vomeronasal organ (VNO), known in reptiles and mammals, is a distinct olfactory epithelium whose neuronal axons project to a distinct olfactory bulb (Halpern, 1987; Halpern *et al*., 1998; Tirindelli *et al*., 1998; Dulac, 2000; Takami, 2002). Three classes of unique GPCR genes have been identified as VNO receptors in mice: V1R, 80 genes; V2R, 50–100 genes; V3R, 100–120 genes (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Pantages and Dulac, 2000; Martini *et al*., 2001; Zufall *et al*., 2002). The V2R class of VNO receptors has also been identified in fish (Cao *et al*., 1998; Naito *et al*., 1998), and a V2R from goldfish

has been shown to interact with a variety of amino acids (Speca *et al.*, 1999). The V2R receptors, at least, are sufficiently conserved across the vertebrates to have permitted fish V2R genes to be identified using PCR primers based on mammalian V2R sequences (Cao *et al.*, 1998). An emerging view is that these VNO receptors may have originated as detectors of waterborne odor molecules, and have become increasingly specialized as pheromone receptors in higher vertebrates.

Chemoreceptor proteins of the nematode *Caenorhabditis elegans* are also GPCRs (Troemel *et al.*, 1995; Sengupta *et al.*, 1996; Wes and Bargmann, 2001). Analysis of the fully sequenced *C. elegans* genome indicates the presence of several families of functional GPCR chemoreceptors totaling nearly 800 genes (Robertson, 2000), which is remarkable considering that *C. elegans* has only 16 pairs of chemosensory neurons (Bargmann and Mori, 1997). Many nonfunctional *C. elegans* chemoreceptor genes are present in the genome; one gene family (*srh*) includes 214 functional genes and 90 pseudogenes (Robertson, 2000). One major difference between the chemoreceptor proteins of vertebrate and *C. elegans* is that vertebrate OR genes are more or less expressed one allele per neuron (Chess *et al.*, 1994) while *C. elegans* chemoreceptor genes may be expressed 40–50 per neuron (Robertson, 2000). Overall there seems to be remarkably little similarity between the chemoreceptor proteins of vertebrate and *C. elegans*, beyond the fact that they are GPCRs and they share sufficient structural features to suggest that both are 7-transmembrane domain proteins. Such extreme sequence divergence was discouraging to those hoping that common sequence domains between nematode and vertebrate OR genes would enable an expansion of these receptors to additional phyla, and suggests that rapid progress into other phyla may only be possible with the assistance of sequenced genomes.

3.15.3.1.2. Insect pheromones/odor molecules are detected by G-protein-coupled receptors The identification of GPCRs as odor receptors in vertebrates and nematodes (see Section 3.15.3.1.1), and the identification of a G-protein-coupled IP3 cascade in insect olfaction (see discussion below) set the stage for the identification of insect odor receptors in *Drosophila*. Efforts during the 1990s to identify ORs in a variety of insects using vertebrate or nematode OR sequences largely failed. One *Drosophila* gene that subsequently proved to be an OR was cloned by differential hybridization during the mid-1990s (DOR104/Or85e), but efforts to use this sequence to identify any additional genes were unsuccessful (Vosshall, 2003a). The problem was, and is, that it is difficult or impossible to recognize OR genes in an insect without the aid of the genome of that insect having been fully sequenced, obvious exceptions being close sibling species or recently duplicated gene pairs. *Drosophila* OR genes were identified from the partially sequenced genome, searching for any sequences that (1) were likely expressed, (2) encoded some degree of hydrophobicity, and (3) were otherwise unidentifiable. ORs emerged from the resulting pool, and were tentatively identified as such using histological methods (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999).

Analyses of the more or less fully sequenced genome of *Drosophila* have identified at least 61 candidate OR genes (*Dor*) (**Table 1**) (*Drosophila* Odorant Receptor Nomenclature Committee, 2000; Hill *et al.*, 2002; Vosshall, 2003a), and another 69 candidate GR genes (*Dgr*) a few of which are also likely involved in odor detection (Clyne *et al.*, 2000; Dunipace *et al.*, 2001; Scott *et al.*, 2001; Hill *et al.*, 2002); all appear to be GPCRs. The genome of *A. gambiae* contains at least 79 OR and 76 GR genes (Fox *et al.*, 2001, 2002; Hill *et al.*, 2002; Vosshall and Keller, 2003). Nine OR genes have been identified from a genome database of the moth *Heliothis virescens*, eight by comparison (BLAST) with the *Dor* sequences and one more from a low stringency cDNA library screen using the new *H. virescens* DNAs as probes; many more are presumably yet to be identified (Krieger *et al.*, 2002). Sequencing efforts are currently being applied to the genomes of several insects of diverse orders, both closely and distantly related to those above, which will greatly expand our knowledge of the insect OR gene family, as well as other olfactory gene families.

In adult *Drosophila*, olfactory neurons are restricted to the antennas (third segment) and maxillary palps. A total of about 1300 olfactory sensory neurons (OSNs) (1200 per antenna, 120 per palp) project axons to 43 glomeruli of the olfactory lobe (Venkatesh and Singh, 1984; Singh and Nayak, 1985; Stocker, 1994); all neurons expressing a given OR gene converge on one or in some cases a small subset of glomerulae (Gao *et al.*, 2000; Vosshall *et al.*, 2000; Bhalerao *et al.*, 2003). Overall there is little difference between male and female antennas/palps, though female antennas do have slightly more sensilla than males – 457 versus 419, primarily of the basiconic type (Shanbhag *et al.*, 1999). Of the 61 *Dor* genes, 39 are expressed in antennal neurons and nine are expressed in palp neurons (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*,

Table 1 *Drosophila* olfactory receptor (OR) gene names and accession numbers

Name[a]	*Accession number*
Or1a	CG17885
Or2a	CG3206
Or7a	CG10759
Or9a	CG15302
Or10a	CG17867
Or13a	CG12697
Or19a	CG18859
Or22a[b]	CG12193
Or22b[b]	CG4231
Or22c	CG15377
Or23a	CG9880
Or24a	CG11767
Or30a	CG13106
Or33a[b]	CG16960
Or33b[b]	CG16961
Or33c[b]	CG5006
Or35a	CG17868
Or42a[b]	CG17250
Or42b[b]	CG12754
Or43a	CG1854
Or43b	CG17853
Or45a	CG1978
Or45b	CG12931
Or46a[b]	CG17849
Or46b[b]	CG17848
Or47a	CG13225
Or47b	CG13206
Or49a	CG13158
Or49b	CG17584
Or56a[b]	CG12501
Or59a[b]	CG9820
Or59b[b]	CG3569
Or59c[b]	CG17226
Or63a	CG9969
Or65a[b]	NA
Or65b[b]	NA
Or65c[b]	NA
Or67a	CG12526
Or67b	CG14176
Or67c	CG14156
Or67d	CG14157
Or69a[b]	NA
Or69b[b]	CG17902
Or71a	CG17871
Or74a	CG13726
Or82a	NA
Or83a[b]	CG10612
Or83b[b]	CG10609
Or83c	CG15581
Or85a	CG7454
Or85b[b]	CG11735
Or85c[b]	CG17911
Or85d	CG11742
Or85e	CG9700
Or85f	CG16755
Or88a	CG14360
Or92a	CG17916
Or94a[b]	CG17241
Or94b[b]	CG6679
Or98a	CG5540
Or98b	CG1867

[a]*Drosophila* OR genes have been named after their map location. The *Drosophila* genome is artificially divided into 100 map regions: Chromosome 1 (C1), 1–20; C2, 21–60; C3, 61–99. Centromeres are located by map locations 40 and 80.
[b]OR genes residing near enough to define a gene cluster originating from probable ''recent'' gene duplications.
NA, not applicable.
Modified from Vosshall, L.B., **2003a**. Diversity and expression of odorant receptors in *Drosophila*. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 567–591.

1999, 2000); the remainder may be expressed at levels too low for the detection methods applied, or may express at specific developmental stages other than those examined, e.g., larval (Vosshall, 2003a), or may not be expressed at all. But clearly the majority of these OR genes are expressed and are apparently not pseudogenes. Each OSN is thought to express only one OR gene, though this is not fully confirmed; the one clear exception is *Dor83b* which actually appears to express in all olfactory neurons, coexpressing with other ORs (see below); on average, about 25 OSNs express any given OR gene (range 2–50 OSNs per OR gene) (Vosshall, 2003a). *Dor* genes encode proteins about 370–400 amino acids long; *Dor83b* is again an exception encoding a 486 amino acid protein. The presumptive 7-transmembrane domain of most Dor proteins contains a conserved motif Phe-Pro-X-Cys-Tyr-(X)20-Trp and the highest overall region of similarity among these proteins spans the 6- and 7-transmembrane domains (Vosshall, 2003a).

The insect OR genes are highly divergent. In general, the Dor proteins show about 17–26% sequence identity, with a few more similar (40–60%) presumably due to recent gene duplications (Vosshall, 2003a). A comparison of the *Anopheles* and *Drosophila* OR genes indicates that the majority of these dipteran genes segregate by species, the only clear ortholog being *Dor83b/AgamGPRor7* (Hill *et al.*, 2002). OR genes of the moth *H. virescens* are similarly divergent, sharing very low sequence identity with the Dor proteins and only 7–16% identity with each other (Krieger *et al.*, 2002). Analyses of all these proteins do suggest subgroupings based on sequence similarities (Hill *et al.*, 2002; Krieger *et al.*, 2002); whether these groupings reflect functional differences remains to be demonstrated.

The *Dor* genes are distributed throughout the *Drosophila* genome. Most reside in isolated locations, though about one-third of the *Dor* genes reside in clusters of two or three, presumably deriving from related gene duplications (Table 1). One extreme example of such a duplication is the OR*22a/22b* pair. These two genes are separated by

only about 650 bp, are 78% identical in their predicted amino acid sequences, and coexpress in the same OSNs (Dobritsa *et al.*, 2003).

The function of any of these genes as ORs is indirectly supported by their numbers, their divergent sequences, and their distributed expression among different OSNs (i.e., one OR per neuron). Dor43b has been immunologically localized to sensory dendrites (Elmore and Smith, 2001). Direct evidence that these genes encode ORs includes several electrophysiological studies. Ectopic expression of *Dor43a* in olfactory neurons using an OSN-specific driver gave increased responses in electroantennograms (EAGs) to several odor molecules including benzaldehyde, benzyl alcohol, cyclohexanol, and cyclohexanone (Störtkuhl and Kettler, 2001). Coexpression of Dor43a and a *Drosophila* G protein in frog oocytes made these oocytes sensitive to these same odor molecules (but not other odor molecules tested, and sham-injected oocytes were nonresponsive), and provided support that the ORs were indeed G protein coupled (Wetzel *et al.*, 2001).

Dor22a, Dor22b, and Dor47a have also recently been characterized (Dobritsa *et al.*, 2003). *Dor22a* and *Dor22b* are close gene neighbors (650 bp apart), share 78% amino acid sequence identity, and appear to coexpress in the same neuron in one class of basiconic sensilla (29 sensilla in female, 18 sensilla in male). Deletion/rescue electrophysiological studies suggest that Dor22a responds to ethyl butyrate, pentyl acetate, and ethyl acetate; however, no odor molecules (of 87 tested) were shown to stimulate Dor22b. This genetic system was shown to be useful for functionally characterizing other OR genes: expression of *Dor47a* using the *Dor22a* promoter and in a *Dor22a/Dor22b* null background demonstrated neuronal responsiveness to a set of odors novel to the *Dor22a*-expressing wild-type neuron. While these studies indicate that *Dor22a* and *Dor22b* coexpress, the only evidence presented that Dor22b might be functional is its identification in the sibling species *D. simulans*; observed conservation between the Dor22b orthologs in *D. melanogaster* and *D. simulans* was presumed possible only if selection was acting on the receptor genes, implying that the gene products are functionally active.

Dor83b is an OR gene that stands apart from the rest: it is expressed in all OSNs, it encodes a protein that is structurally distinct from other Dor proteins, and it is the only OR gene readily identified in diverse species: the fruit fly *D. melanogaster* (Dor83b: Vosshall *et al.*, 2000); the mosquito *A. gambiae* (AgamGPRor7: Hill *et al.*, 2002), the moth *H. virescens* (HR2: Krieger *et al.*, 2002), as well as the silk moths *Bombyx mori* and *A. polyphemus*, the honeybee *Apis mellifera,* the blowfly *Calliphor erythrocephala*, and the beetle *Tenebrio molitor* (Krieger *et al.*, 2003). These 83b/GPRor7/H2 proteins are 64–88% identical, differing from one another in a phylogenetically consistent manner (Krieger *et al.*, 2003). As this gene expresses in all OSNs, always coexpressing with one of the other OR genes, it is presumably not functioning as the other OR proteins do in interacting with an odor ligand. Several functions for this gene have been speculated including that (1) it forms a heterodimer with the ligand-sensitive OR, (2) that it activates a second messenger pathway in a ligand independent manner, (3) that it helps in the trafficking of OR proteins to the correct site in the dendritic membranes (discussed by Krieger *et al.*, 2003; Vosshall and Keller, 2003; Vosshall, 2003a). None of these functions has been verified as of early 2000s.

Insect OR genes partially define the phenotypes of OSNs, but they apparently do not contribute to the determination of these phenotypes. **Figure 1** summarizes general features of the brain neurocircuitry of the insect olfactory pathway and includes references to many elegant studies. Each glomerulus of the olfactory lobe receives axons belonging to OSNs of a single phenotype; all OSNs converging on an individual glomerulus are thought to express the same OR gene (Gao *et al.*, 2000; Vosshall *et al.*, 2000; Bhalerao *et al.*, 2003). The positions of these glomerulae are more or less constant from animal to animal, and in some cases the preservation of these positions may have a functional significance in coding (Hansson *et al.*, 1992, 2003; Carlsson and Hansson, 2003a, 2003b). Furthermore, a recent study has shown that the projection neurons emanating from these glomerulae terminate their axons in the lateral protocerebrum in a spatially restricted manner that to some degree maintains the spatial organization of the olfactory lobe (Wong *et al.*, 2002). In vertebrates, OR genes have been shown to influence the projection of OSNs to specific glomerulae; neurons missexpressing the appropriate OR gene miss or fail altogether to target a specific glomerulus (Mombaerts *et al.*, 1996; Wang *et al.*, 1998; reviews: Korsching, 2002; Vosshall, 2003a, 2003b). The *Drosophila* ORs, however, apparently do not have this capability. OSNs expressing Dor22a/Dor22b target an identifiable glomerulus; these OSNs still target this glomerulus in a Dor22a/Dor22b deletion mutant whether or not an alternative OR gene is expressed in these neurons (Dobritsa *et al.*, 2003). In insects, correct targeting of OSNs appears to function independent of the OR genes.

3.15.3.1.3. Gustatory neurons detect chemostimulants using G-protein-coupled receptors Perhaps 69 GPCRs have been identified as candidate gustatory receptors in the *Drosophila* genome (Clyne *et al.*, 2000; Dunipace *et al.*, 2001; Scott *et al.*, 2001; Hill *et al.*, 2002); 76 were identified in the *A. gambiae* genome (Hill *et al.*, 2002). The expression of a subset of the *Drosophila* GRs has been characterized; these are associated with a variety of tissues including the ventral pits, gut, mouth, dorsal, and terminal organs of larvae and labellum, cibarial organs, labral organ, legs, and wings of adults (Dunipace *et al.*, 2001; Scott *et al.*, 2001). One, *Gr21D1*, expresses in antennas, associating with a neuron that projects to a specific glomerulus in the olfactory lobe (Scott *et al.*, 2001) However, all others examined which express in head tissues associate with neurons that project to the subesophageal ganglion (SEG) (Stocker, 1994). Like the antennal lobe, the SEG is organized into a discrete number of glomerulae, i.e., seven (Shanbhag and Singh, 1992). However, unlike the antennal system, GR expressing neurons arborize into multiple SEG glomerulae (Singh and Nayak, 1985; Singh, 1997; Dunipace *et al.*, 2001; Scott *et al.*, 2001). Only one GR gene has been functionally characterized; *Gr5a* encodes a trehalose receptor (Dahanukar *et al.*, 2001; Ueno *et al.*, 2001). Interestingly, in both studies *Gr5a* was chosen for study because it is tightly linked to another gene, *tre* (Ishimoto *et al.*, 2000), which had previously been implicated as a trehalose receptor but has no sequence similarity to the putative *Drosophila* GR genes. *Gr5a* expresses in labellar sensilla. The GRs of *Drosophila* are highly divergent, ranging from 15% to 25% in sequence identity. Considering such divergence and the number of genes, these animals presumably “taste” a broad range of molecules that might further be quite different between larvae and adults. Insect taste is undoubtedly much more complex than may be implied by the understatement that taste discriminates the modalities of sweet, sour, salty, and bitter (Singh, 1997). As GRs are identified from a broad range of insects, they might be expected also to be highly divergent, driven so by the chemistry of host plants utilized by various insect groups. Whether any of these GRs are involved in pheromone detection remains to be determined.

3.15.3.2. Second Messenger Pathways

3.15.3.2.1. Pheromone/odor transduction is mediated by a G protein-based IP3 pathway The identification of GPCR odor receptors in both vertebrates and invertebrates was preceded by the identification of odor activated G protein/second messenger cascades. Two G-protein-coupled transduction pathways have been identified in olfactory systems of vertebrates and arthropods: (1) G protein dependent activation of phospholipase C (PLC) with the subsequent production of IP3; or (2) G protein dependent activation of adenylate cyclase with the subsequent production of cAMP (Krieger and Breer, 2003). Both pathways exist in vertebrates, and in lobsters both pathways are thought to coexist in the same olfactory chemosensory neurons (Fadool and Ache, 1992; Michel and Ache, 1992; Boekhoff *et al.*, 1994; Hatt and Ache, 1994). In insects, only the G-protein-coupled IP3 pathway illustrated in **Figure 2c** has been demonstrated for both pheromones and nonpheromonal odor molecules.

When pheromone or other odor molecules were applied to homogenates of cockroach or moth antennas, a rapid rise in IP3 was observed (Boekhoff *et al.*, 1990a, 1990b, 1993; Breer *et al.*, 1990). This rise in IP3 was assumed to be mediated by a receptor activated G protein. The Gα subunits Go and Gq have been identified in or cloned from antennas of cockroach, fly, and moth (Breer *et al.*, 1988; Boekhoff *et al.*, 1990b; Raming *et al.*, 1990; Talluri *et al.*, 1995), and Gq-like proteins have been immunohistologically visualized in the dendritic membranes of pheromone sensory neurons in the silk moths *B. mori* and *A. pernyi* (Laue *et al.*, 1997). To affect changes in IP3 levels, a receptor activated Gα subunit would activate the membrane-bound enzyme PLC, which in turn would cleave the membrane lipid phosphotidyl inositol 4,5-biphosphate (PIP2) to membrane-bound diacylglycerol (DAG) and cytosol soluble IP3 (see **Figure 2c**). A PLC *Drosophila* mutant, *norpA*, has been shown to be severely impaired in odor detection (Riesgo-Escovar *et al.*, 1995). PLC has also been immunologically identified by Western blot in homogenates of olfactory dendrites of the moth *A. polyphemus* (Maida *et al.*, 2000). The classic action of IP3 is to interact with Ca^{2+} channels, also referred to as IP3 receptors; such receptor/channels have been immunohistologically visualized in the dendritic membranes of pheromone sensory neurons of *B. mori* and *A. pernyi* (Laue and Steinbrecht, 1997). In olfactory neurons of the locust *Locusta migratoria*, extracellular application of odors or intracellular application of IP3 generated an increase in the inward membrane conductance of cations, including Ca^{2+} (Wegener *et al.*, 1997). In cultured adult olfactory neurons of the moth *M. sexta*, an IP3-dependent inward Ca^{2+} current was observed to precede general inward cation currents (Stengl, 1994). Thus, by 1999, all the components depicted in the **Figure 2c** scheme have been

identified, with the exception of the odor receptors themselves.

A limited number of studies have investigated the role of cAMP in insect olfactory transduction, but no increases in cAMP have been observed on stimulation with pheromone or other odor molecules in insects (Ziegelberger *et al.*, 1990; Boekhoff *et al.*, 1993). Curiously, cyclic nucleotide gated ion channels have been identified in insect antennas, and in fact is localized to olfactory receptor neurons (Baumann *et al.*, 1994; Krieger *et al.*, 1997). This may imply that a cAMP pathway does exist for certain odorants; cAMP olfactory pathways are known in lobsters (Michel and Ache, 1992; Hatt and Ache, 1994) and vertebrates (e.g., Jones and Reed, 1989; Bakalyar and Reed, 1990; Boekhoff and Breer, 1992; Rossler *et al.*, 2000b). Alternatively, these cyclic nucleotide gated channels may function in other roles, perhaps as part of neuromodulatory pathways in the neuronal cell bodies, or in the maintenance of background neural activity and sensitivity (Krieger *et al.*, 1997). The role of cGMP in pheromone transduction has also been demonstrated, but it appears to have a modulatory role in adaptation or desensitization rather than participating in primary transduction (see Section 3.15.3.2.2) (Ziegelberger *et al.*, 1990; Redkozubov, 2000; Stengl *et al.*, 2001; Dolzer *et al.*, 2003). As of now, there is little evidence that cyclic nucleotides act in a primary capacity in the transduction of pheromone or other odor signals in insects.

3.15.3.2.2. Pheromone/odor transduction is modulated by G-protein based second messenger processes Second messenger processes have dynamic properties due to the number of divergent pathways they can initiate; few of these potential pathways have been investigated. These might be forward pathways, where signal molecules such as IP3 might interact with secondary targets, perhaps altering the future response characteristics of the neuron, or they might be backward pathways, downregulating the activity of processes just initiated (e.g., the receptor mediated G protein-coupled pathway). The physiological dynamics of pheromone adaptation has recently been investigated in the moth *B. mori* with respect to stimulus concentration and duration (Dolzer *et al.*, 2003). Yet, few modulatory processes have been examined thoroughly in insect olfaction. Two loose ends depicted in **Figure 2c** are the βγ subunits of the G-protein (Gβγ) complex and DAG. "Gβγ is now known to directly regulate as many different protein targets as the Gα subunit" (Clapham and Neer, 1997). In vertebrates, Gβγ has been shown to activate G-protein-coupled inwardly rectifying K^+ channels (GIRKs) (Salvador *et al.*, 2003). Gβγ has been shown to activate regulators of G-protein signaling (RGS) proteins which in turn activate GAP protein (GTPase activating protein) which regulates the GTPase activity in the Gα subunit (turning off Gα activity) (Witherow and Slepak, 2003). Gβγ has been shown to regulate G-protein-coupled receptor kinases (GRKs) which phosphorylate and downregulate GPCRs (Inglese *et al.*, 1992; Pitcher *et al.*, 1998; Eichmann *et al.*, 2003). Gβγ has been shown to regulate the activities of certain PLC enzymes and the subsequent production of IP3 (Akgoz *et al.*, 2002). In vertebrate chemoreception, Gβγ has been shown to regulate PLC activity in mammalian vomeronasal neurons (Runnenburger *et al.*, 2002) and in bitter taste neurons (Rossler *et al.*, 2000a). Active Gβγ signaling in insect olfaction has yet to be reported.

Arrestins are proteins that bind to the phosphorylated state of a GPCR, and can mediate the uncoupling of GPCRs from G proteins (desensitization) (Inglese *et al.*, 1993; Pippig *et al.*, 1993; Freedman and Lefkowitz, 1996) as well as the internalization of GPCRs (with presumed subsequent dephosphorylation in microsomes with subsequent recycling of the receptor, i.e., resensitization) (Ferguson *et al.*, 1996). In an IP3 mediated pathway, GPCR phosphorylation may be mediated by GIRKs, which may be mediated by Gβγ (see above). Arrestins have been identified in insect antennas and shown to be critical in the olfactory transduction process in *Drosophila* and *A. gambiae* (Merrill *et al.*, 2002). In this study, an arrestin was cloned from *A. gambiae*, and was shown to express in both olfactory and visual sensory neurons. Of three *Drosophila* arrestins, *DmArr1* and *DmArr2* were shown to express in both visual and olfactory tissue while *DmKrz* was nonvisual but also expressed in olfactory tissue. A double mutant ($arr1^2; arr2^3$) showed reduced EAG response to butanol and ethyl acetate, suggesting that arrestins play a key role in olfactory transduction in these animals (Merrill *et al.*, 2002).

DAG is another product of the IP3 pathway that has potential targets. DAG is classically considered as a regulator of protein kinase C (PKC), but may also interact with membrane lipases to induce the release of polyunsaturated fatty acids (e.g., arachidonic acid) which may activate certain ion channels/membrane conductances. These ion channels may also be sensitive to levels of PIP2, regulated by PLC (Hardie, 2003; Krieger and Breer, 2003; Nilius *et al.*, 2003). Recently, a membrane permeable analog of

DAG, 1,2-dioctanol-*sn*-glycerol (DOG), was shown to elicit action potentials in pheromone sensitive neurons of the moth *B. mori* (Pophof, 2002; Pophof and van der Goes van Naters, 2002), suggesting an active role for DAG in insect olfactory transduction.

Prolonged but only moderately elevated cGMP levels in response to applied sex pheromone have been measured in the antennas of the moths *A. polyphemus* and *B. mori* (Ziegelberger *et al.*, 1990). Application of dibutyryl cGMP inhibited the response of *B. mori* pheromone sensitive neurons to pheromone (Redkozubov, 2000). These findings suggest a role for cGMP in modulating the response to sex pheromone. Pheromone-dependent elevation of cGMP levels was observed immunologically to occur in only a very small subpopulation of known pheromone sensitive sensilla (about 1% of sensilla trichoidea) of male antennas of the moth *M. sexta* (Stengl *et al.*, 2001). In these same studies, the location of NO-sensitive soluble guanylate cyclase was determined to be entirely restricted to a population of sensilla chaetica but absent from sensilla trichoidea. Thus, while there appears to be a role for cGMP in pheromone transduction, the mechanism underlying that role or its value to pheromone perception remains unresolved. However, cGMP appears to regulate olfactory transduction in a subpopulation of rat olfactory neurons that have behavioral significance (review: Zufall and Munger, 2001), suggesting that perhaps the subpopulation of neurons in *M. sexta* which shows a pheromone-dependent rise in cGMP may similarly serve a specialized behavioral function (Stengl *et al.*, 2001).

3.15.4. Perireception Events

Figure 2b summarizes a number of biochemical reactions that interact directly with pheromone and odor molecules in the extracellular space of the sensillum interior. Each sensillum contains at least three classes of protein with evolutionarily designed binding sites for these ligands: OBPs, ORs, and ODEs. Individual sensilla contain multiple members of each class, and the phenotypes of functionally distinct sensilla can be defined by the unique combinations of these gene products. These proteins comprise a biochemical network designed to process environmental signals. This network serves as a genetic interface between the animal and its environment, its components designed and organized by evolutionary selection; the single purpose of these gene products has allowed selection to proceed unconstrained from the need to balance requirements of other competing functions.

3.15.4.1. Odor Transport by Odorant Binding Proteins

Insect OBPs comprise a multigene family that includes a group of small hydrophilic proteins which bind and transport small hydrophobic ligands. Vertebrates also possess proteins that function as OBPs; however, vertebrate and insect OBPs are evolutionarily unrelated (Tegoni *et al.*, 2000; Ramoni *et al.*, 2001). The first insect OBP identified was the PBP of the silk moth *A. polyphemus* (Vogt and Riddiford, 1981). This 14 kDa protein appeared to be specific to the male antenna, was perhaps the most abundant soluble protein in the antenna, was located in the aqueous extracellular fluid that bathed the pheromone-sensitive neurons, and could bind sex pheromone. The concentration of *Apol*PBP within the sensillum fluid was estimated to be about 10 mM (Klein, 1987). Additional PBPs were subsequently identified by tissue specificity and N-terminal sequence from the moths *Hyalophora cecropia*, *B. mori*, *Lymantria dispar*, *M. sexta*, and *Orgyia pseudosugata* (Vogt, 1987; Vogt *et al.*, 1989, 1991a); the first full length OBP sequences were obtained for *M. sexta* PBP1 (Györgyi *et al.*, 1988) and *A. polyphemus* PBP1 (Raming *et al.*, 1989). The identification of two PBPs in *L. dispar* was the first indication of multiple PBP genes within a single species (Vogt *et al.*, 1989). The identification and cloning of the general odorant binding proteins GOBP1 and GOBP2 (Vogt and Lerner, 1989; Breer *et al.*, 1990; Vogt *et al.*, 1991a, 1991b) were the first indication that OBPs were members of a multigene family. These data on lepidopteran OBPs provided a basis for identifying the first OBPs in *Drosophila* (OS-E and OS-F: McKenna *et al.*, 1994; PBPRP1-5: Pikielny *et al.*, 1994; LUSH: Kim *et al.*, 1998). Collectively, the OBPs described above might be viewed as the "gold standard" OBPs against which all others are compared.

One hallmark feature of these proteins that is almost universally cited when presenting new OBP sequences is the positionally conserved presence of six cysteines (first noted by Breer *et al.*, 1990). In more recent genomic analyses, the presence of these six cysteines plus a similar size and an acceptable result from BLAST analysis has been viewed as sufficient to consider these genes members of an OBP gene family (Galindo and Smith, 2001; Graham and Davies, 2002; Hekmat-Scafe *et al.*, 2002).

OBPs have now been identified in at least 41 species from orders throughout the Neopterous insects (**Table 2**; **Figures 3** and **4**), suggesting that they are common elements in olfactory sensilla of

Table 2 Odorant-binding protein (OBP) related proteins with published sequences

Species	Protein/gene name	Accession number[a]
Lepidoptera		
Agrotis segetum	*Aseg*-PBP	AH007940
Antheraea pernyi	*Aper*-PBP1	X96773
	Aper-PBP2	X96860
	Aper-PBP3	AJ277265
	Aper-GOBP1	Y10970
	Aper-GOBP2	X96772
	Aper-ABPX	AJ002519
Antheraea polyphemus	*Apol*-PBP1	X17559
	Apol-PBP2	AJ277266
	Apol-PBP3	AJ277267
	Apol-GOBP2	P34169 (peptide)
Argyrotaenia velutinana	*Avel*-PBP	AF177641
Bombyx mori	*Bmor*-PBP	X94987
	Bmor-GOBP1	X94988
	Bmor-GOBP2	X94989
	Bmor-ABPX	X94990
Choristoneura fumiferana	*Cfum*-PBP	AF177643
Epiphyas postvittana	*Epos*-PBP1	AF416588
	Epos-PBP2	AF411459
	Epos-GOBP2	AF411460
Galleria mellonella	Sericotropin (nonolfactory)	L41640
Heliothis virescens	*Hvir*-PBP	X96861
	Hvir-GOBP1	X96862
	Hvir-GOBP2	X96863
	Hvir-ABPX	AJ002518
Helicoverpa armigera	*Harm*-PBP	AJ278992
	Harm-GOBP1	AY049739
	Harm-GOBP2	AJ278991
Helicoverpa zea	*Hzea*-PBP	AF090191
Hyalophora cecropia	*Hcec*-PBP	P34175 (peptide)
	Hcec-GOBP2	P34172 (peptide)
Lymantria dispar	*Ldis*-PBP1	AF007867
	Ldis-PBP2	AF007868
	Ldis-GOBP2	P34173 (peptide)
Mamestra brassicae	*Mbra*-PBP1	AF051143
	Mbra-PBP2	AF051142
	Mbra-PBP4	AF461143
	Mbra-GOBP2	AF051144
Manduca sexta	*Msex*-PBP1	M21798
	Msex-PBP1	AF323972
	Msex-PBP2	AF117589
	Msex-PBP3	AF117581
	Msex-GOBP1	M73797
	Msex-GOBP2	AF323972
	Msex-ABPX	AF117577
	Msex-ABP1	AF117591
	Msex-ABP2	AF393491
	Msex-ABP3	AF393488
	Msex-ABP4	AF393490
	Msex-ABP5	AF393498
	Msex-ABP6	AF393499
	Msex-ABP7	AF393500
Orgyia pseudosugata	*Opse*-PBP	P34178 (peptide)
Pectinophora gossypiella	*Pgos*-PBP	AF177656

Table 2 Continued

Species	Protein/gene name	Accession number[a]
Spodoptera exigua	*Spexi*-GOBP2	AJ294808
Synanthedon exitiosa	*Sexi*-PBP	AF177660
Yponomeuta agnagellus	*Ycag-PBP*	AF177661
Coleoptera		
Anomala cuprea	*Acup*-PBP1-	AB040980
	Acup-PBP2	AB040141
Anomala octiescostata	*Aoct*-PBP1	AB040143
	Aoct-PBP2	AB040981
Anomala osakana	*Aosa*-PBP1	AB040985
	Aosa-PBP2	AF031492
Exomala orientalis	*Eori*-PBP1	AB040144
	Eori-PBP2	AB040986
Holotrichia parallela	*Hpar*-OBP1	AB026554
	Hpar-OBP2	AB026556
Phyllopertha diversa	*Pdiv*-OBP1	AB026552
	Pdiv-OBP2	AB026553
Popillia japonica	*Pjap*-PRP1	AF031491
Rhynchophorus palmarum	*Rpal*-OBP2	AF139912
Tenebrio molitor	*Tmol*-B1	M97916
	Tmol-B2	M97917
	Tmol-THP12 (nonolfactory)	U24237
Diptera		
Aedes aegypti	*Aaeg*-OBPRP	AY062131
Anopheles gambiae	*Agam*-OBP1	AF393487
	Agam-OBP2	AF393485
	Agam-OBP-1 (OS-F)	AF437884
	Agam-OBP-2	AF437885
	Agam-OBP-3 (OS-E)	AF437886
	Agam-OBP-4	AF437887
	Agam-OBP-5	AF437888
	Agam-OBP-6	AF437889
	Agam-OBP-7	AF437890
	Agam-OBPRP-1	EAA00498
	Agam-OBPRP-2	EAA00779
	Agam-OBPRP-3	EAA00788
	Agam-OBPRP-4	EAA00801
	Agam-OBPRP-5	EAA01392
	Agam-OBPRP-6	EAA01491
	Agam-OBPRP-7	EAA03447
	Agam-OBPRP-8	EAA03742
	Agam-OBPRP-9	EAA03745
	Agam-OBPRP-10	EAA06799
	Agam-OBPRP-11	EAA06803
	Agam-OBPRP-12	EAA07741
	Agam-OBPRP-13	EAA07997
	Agam-OBPRP-14	EAA09324
	Agam-OBPRP-15	EAA12996
	Agam-OBPRP-16	EAA14622
Ceratitis capitata	*Ccap*-OBPRP	AJ252076
	Ccap-OBPRP	AJ252077
	serum protein	Y08954
	serum protein	Y19146
Culex quinquefasciatus	*Cqui*-OBPRP	AF468212

Continued

Table 2 Continued

Species	Protein/gene name	Accession number[a]
Drosophila melanogaster	*Dmel*-99D	CG7584
	Dmel-99C	CG12665
	Dmel-99B	CG7592
	Dmel-99A	CG18111
	Dmel-93A	CG17284
	Dmel-85A	CG11732
	Dmel-84A	PBPRP4
	Dmel-83F	CG15583
	Dmel-83E	CG15583
	Dmel-83D	CG15582
	Dmel-83C	CG15582
	Dmel-83B	OSE
	Dmel-83A	OSF
	Dmel-76A	LUSH
	Dmel-69A	PBPRP1
	Dmel-58A	CG13517
	Dmel-58B	CG13518
	Dmel-58C	CG13524
	Dmel-58D	CG13519
	Dmel-57E	CG13429
	Dmel-57D	AF457149
	Dmel-57C	CG13421
	Dmel-57B	AF457147
	Dmel-57A	AF457148
	Dmel-56I	AF457143
	Dmel-56H	CG13874
	Dmel-56G	CG13873
	Dmel-56F	AF457146
	Dmel-56E	CG8462
	Dmel-56D	CG11218
	Dmel-56C	CG15129a
	Dmel-56B	CG15129b
	Dmel-56A	CG11797
	Dmel-51A	AF457145
	Dmel-50A	??
	Dmel-50B	CG13940
	Dmel-50C	??
	Dmel-50D	??
	Dmel-50E	CG13939
	Dmel-49A	CG8769
	Dmel-47B	CG13208
	Dmel-47A	CG12944
	Dmel-46A	CG12905
	Dmel-44A	CG2297
	Dmel-28A	PBPRP5
	Dmel-22A	AF457144
	Dmel-19D	PBPRP2
	Dmel-19C	CG15457
	Dmel-19B	CG1670
	Dmel-19A	CG11748
	Dmel-18A	CG15883
	Dmel-8A	CG12665
Phormia regina	*Preg*-CSRBP (taste)	S78710
Hemiptera		
Lygus lineolaris	*Llin*-LAP	AF091118
Dictyoptera		
Leucophaea maderae (cockroach)	*Lmad*-PBP	AY116618

Table 2 Continued

Species	Protein/gene name	Accession number[a]
Zootermopsis nevadensis (termite)	*Znev*-OBP1	AY135381
	Znev-OBP2	AY135382
	Znev-OBP3	AY135383
Hymenoptera		
Apis mellifera (honeybee)	*Amel*-ASP1	AF166496
	Amel-ASP2	AF166497
	Amel-ASP4	AF393495
	Amel-ASP5	AF393497
	Amel-ASP6	AF393496
Solenopsis sp.	*S. amblychila*	AF427889
	S. aurea	AF427890
	S. geminata	AF427905
	S. globularia l.	AF427906
	S. interrupta	AF427891
	S. invicta (fireant) Gp-9	AF459414
	S. macdonaghi	AF427901
	S. quinquecuspis	AF427902
	S. richteri	AF427904
	S. saevissima	AF427892
Orthoptera		
Locusta migratoria	*Lmig*OBP	AF542076

[a]Accession numbers available as of August 2003.

at least 98% of all insect species (Vogt *et al.*, 1999). OBPs have yet to be identified in preneopterous lineages (e.g., Odonata – dragonflies, Ephemeroptera – mayflies, Thysanura – silverfish, Archaeognatha – bristletails). Direct cloning and genome analyses have identified more than 50 OBP-related genes in *Drosophila* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994; Kim *et al.*, 1998; Galindo and Smith, 2001; Graham and Davies, 2002; Hekmat-Scafe *et al.*, 2002; Vogt *et al.*, 2002). A similar number have been identified in *A. gambiae* based on genome analyses (Robertson *et al.*, 2001; Biessmann *et al.*, 2002; Vogt, 2002; Xu *et al.*, 2003). Most of these sequences are highly divergent, though some sequences are clearly the products of recent gene duplications based on sequence similarity, conserved exon structure, and clustered chromosomal location (e.g., *Drosophila* OS-E/OS-F: Hekmat-Scafe *et al.*, 2000, 2002; Vogt, 2002; Vogt *et al.*, 2002). Like the *Dors*, the *Drosophila* OBPs are distributed throughout the genome (see Table 2, where *DrosOBP* genes are named for their map location) (Vogt, 2003). The majority of OBPs that have been sequenced in the Lepidoptera are PBPs and GOBPs which are distinct but share common sequence motifs; however, sequencing efforts have identified 13 OBP genes in *M. sexta*, including three PBPs, GOBP1, GOBP2, and another eight that are as highly divergent as the dipteran OBPs (Györgyi

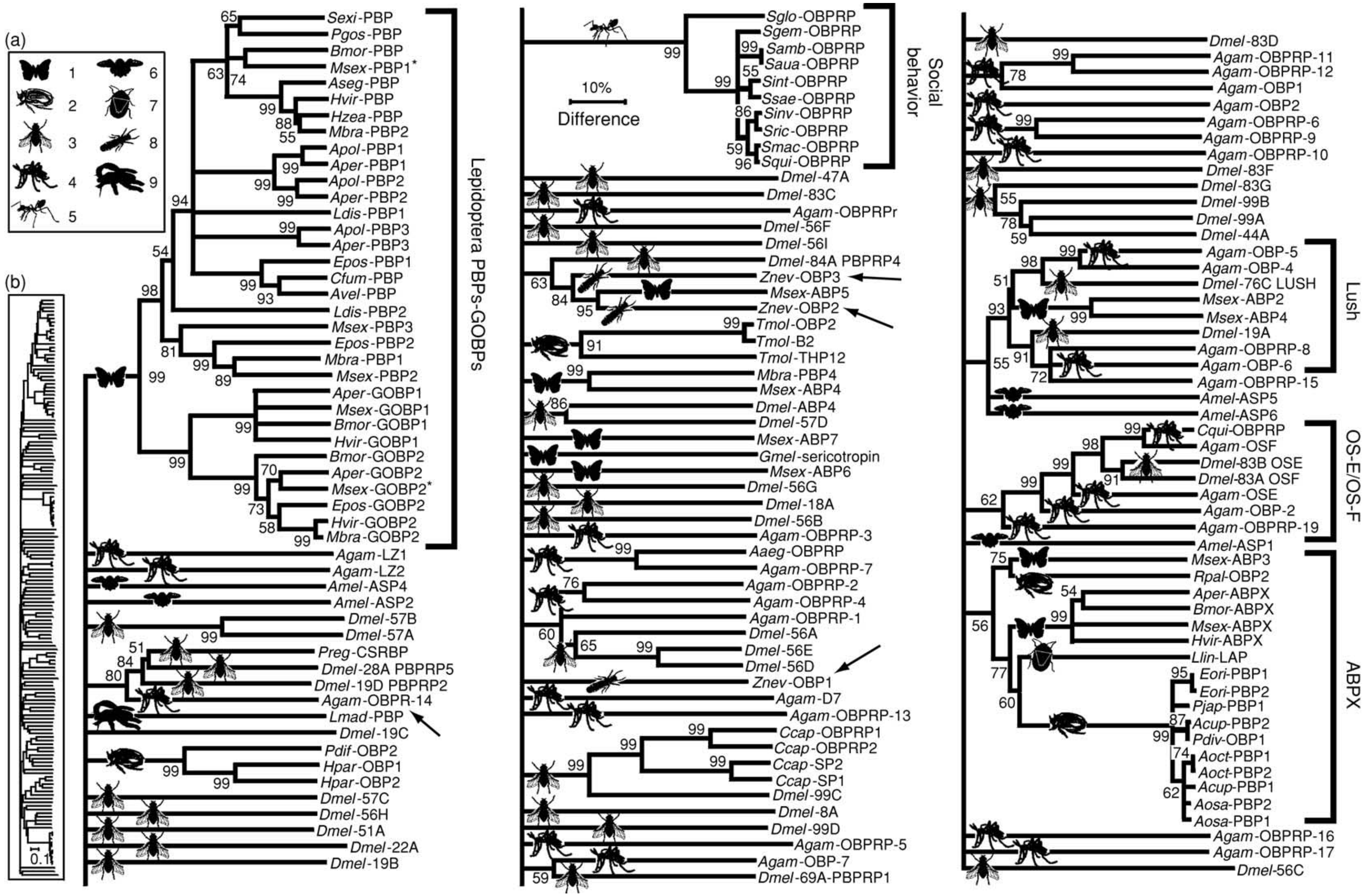


Figure 3 A neighbor-joining tree of most OBPs listed in Table 2; bootstrap support is based on 1000 replicates and only branches with >50% support are shown. A single tree is shown, broken into thirds to fit the page. Insert (a) is a key to the taxa. Insert (b) is the uncollapsed tree (taxa not indicated) to illustrate full branch lengths (percent sequence differences). A size bar indicating 10% sequence difference is shown. Named branch clusters (e.g., ABPX) are referred to in the text.

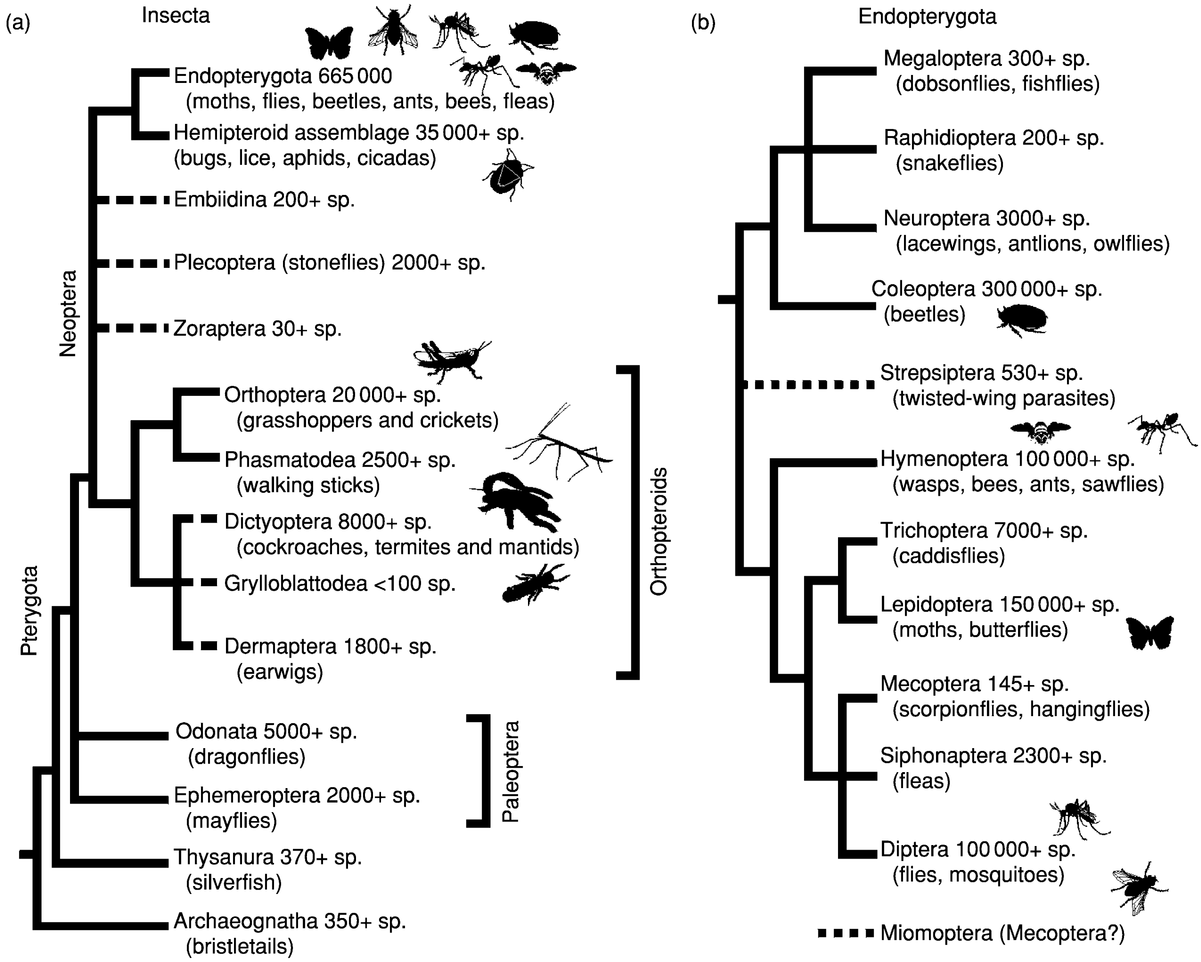


Figure 4 Overview of insect phylogeny (after Boudreaux, 1979; Hennig, 1981; Kristensen, 1991). In (a), the representation of a monophyletic orthopteroid group is considered debatable (Maddison and Maddison, 1998); this group was suggested by Boudreaux (1979) and Hennig (1981), but was not included by Kristensen (1991) due in part to uncertainties regarding the relationships of the Plecoptera. The monophyletic division of Endopterygota (holometabolous insects) (Kristensen, 1991; Whiting *et al.*, 1997) is supported by most authors in part because of the unique development in this group; all members undergo a complete metamorphosis from the nonreproductive larval stage to the reproductive adult stage. A shared common ancestor for the Endopterygota and the hemipteroid lineages is suggested by morphological (Hennig, 1981; Kristensen, 1991; Whiting *et al.*, 1997) and molecular data (Whiting *et al.*, 1997). In (b), two supported endopterygote lineages are shown, one including the Coleoptera and a second including Hymenoptera, Diptera, and Lepidoptera. The fossil record suggests that the orthopteroids are more ancient than most endopterygote orders (late Carboniferous vs. early Permian); among the Endopterygota, the Coleoptera (Permian) are more ancient than the Diptera/Lepidoptera lineage, and the Diptera (Triassic) are more ancient than the Lepidoptera (Jurassic) (Kukalová-Peck, 1991; Labandeira and Sepkoski, 1993). The greater part of molecular or biochemical based olfactory research has been done on a subset of these orders (noted by the insect symbols). Numbers of named species in each lineage are shown, taken from various entries in *The Insects of Australia* (Achterberg and CSIRO staff, 1991); these numbers are generally viewed as gross underestimates, but accurately reflect the relative success of the respective lineages.

et al., 1988; Vogt *et al.*, 1991b, 2002; Robertson *et al.*, 1999). Undoubtedly, full genome analysis of a lepidopteran species will identify many more.

Not all OBPs are involved in odor detection. The genes listed in Table 2 include serum proteins from the medfly *Ceratitis capitata* (Y08954 and Y19146: Thymianou *et al.*, 1998) and the beetle *T. molitor* (THP12: Rothemund *et al.*, 1999; Graham *et al.*, 2001); accessory gland proteins from *T. molitor* (B1 and B2: Paesen and Happ, 1995), and the *Drosophila* protein PBPRP2 which is localized in nonchemosensory tissues or spaces (Park *et al.*, 2000). Galindo and Smith (2001) examined the expression of 34 candidate *Drosophila* OBPs using a GAL4/UAS system, driving reporter gene expression using presumptive OBP promoters. Nine of these OBP-regulatory constructs expressed only in “chemosensory tissue” (sensilla of antennas and palps), four expressed only in “gustatory tissue” (sensilla of legs and wings), nine expressed in both tissues, five expressed

in nonsensillar tissue, and seven did not express at all, perhaps due to inappropriate choice of regulatory region or the genes in question being pseudogenes (Galindo and Smith, 2001). Another recent study identified seven OBP-related genes that express in gustatory sensilla of the fleshfly *Boettcherisca peregrina* (Koganezawa and Shimada, 2002 (not included in **Table 2**). Clearly, though many OBP genes are assumed to express proteins which have an important role in odor detection, assigned membership in this gene family does not necessarily imply an olfactory or even chemosensory function; although, at the very least such membership should imply common ancestry (i.e., the genes all derived by gene duplications originating from one common ancestral gene).

3.15.4.1.1. General properties of odorant binding proteins In general, OBPs are small, water-soluble, extracellular proteins around 14 kDa, and ranging between 120 and 150 amino acids long. OBPs presumably bind small ligands, and if involved in odor detection, those ligands are presumably odor molecules. OBPs are expressed with leader sequences which are removed during secretion. Most OBPs contain six cysteines in a certain asymmetric pattern, which has become a diagnostic feature of OBPs; these cysteines form disulfide bonds which stabilize the functionally relevant three-dimensional structure of the protein (Leal *et al*., 1999; Briand *et al*., 2001a, 2001b). OBPs involved in odor detection are expressed in sensilla support cells and secreted by these cells into the aqueous sensilla lumen (Vogt *et al*., 2002).

The structure of OBPs has been characterized by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. X-ray analysis of the moth *B. mori* PBP1 (*Bmor*PBP1) revealed six α-helix units stabilized by three disulfide bonds; a flask-shaped pheromone binding pocket is formed by four α-helices, and in an early study, another α-helical unit appeared to cover the narrow pocket opening (Sandler *et al*., 2000). Also, *Bmor*PBP1 formed a dimeric structure in the crystal (also suggested by Leal, 2000). Similar structures have been determined for antennal OBPs from the honeybee and cockroach (Lartigue *et al*., 2003a, 2003b), as well as a hemolymph OBP homolog (THP12) of unknown function from the beetle *T. molitor* (Rothemund *et al*., 1999; Graham *et al*., 2001). Disulfide bridges join cysteines 1–3, 2–5, 4–6 in *Bmor*PBP1 (Leal *et al*., 1999); the same disulfide bridge pattern has also been observed in the honeybee OBP ASP2 (Briand *et al*., 2001a, 2001b). Thus, even for OBP relatives with highly divergent sequences, the overall structural features appear remarkably conserved. Additional structural information is presented below (see Section 3.15.4.1.3).

3.15.4.1.2. Differential expression of odorant binding proteins The unique expression of PBPs in antennas, their localization within olfactory sensilla, and their ability to bind sex pheromones was strong evidence of their role in pheromone detection (e.g., Vogt and Riddiford, 1981; Vogt *et al*., 1989). The identification of PBPs and GOBPs with their sexual dimorphic expression patterns suggested that different OBPs may associate with sensilla of distinct odor phenotypes (Vogt *et al*., 1991a). For example, in the moth *M. sexta*, PBP1 and GOBP2 express in different functional classes of olfactory sensilla (Vogt *et al*., 2002) (**Figure 5**). Initially, PBPs were thought to be male specific, but it is now clear that while PBPs associate with pheromone sensitive sensilla in males, PBPs also express in female antennas where their role is unknown. It is possible that PBPs expressing in females may function in the monitoring of pheromone release (Callahan *et al*., 2000; Vogt *et al*., 2002). Certain families of Lepidoptera, especially the noctuids, display an almost total sex indifference in antennal levels of PBP expression.

The distribution of PBPs, GOBP1, GOBP2, and ABPX proteins have been characterized in several moth species using immunohistochemistry and electron microscopy; PBP proteins are present in the lumen of long trichoid sensilla, GOBP2 and ABPX proteins are present in the lumen of subsets of basiconic sensilla, and GOBP1 can be present in both trichoid and basiconic sensilla (Steinbrecht *et al*., 1995; Steinbrecht, 1996; Laue and Steinbrecht, 1997; Maida *et al*., 1999; Zhang *et al*., 2001). This broad expression of GOBP1 in both basiconic and trichoid sensilla was also observed by *in situ* hybridization in *M. sexta* (Vogt *et al*., 2002). Similar patterns have been described in Diptera. In *Drosophila*, OS-E, OS-F, and LUSH coexpress in trichoid sensilla, OS-E and OS-F but not LUSH coexpress in intermediate sensilla, and PBPRP5 expresses in a subset of basiconic sensilla (Hekmat-Scafe *et al*., 1997; Park *et al*., 2000; Shanbhag *et al*., 2001a). Galindo and Smith (2001) tested the expression patterns of 34 presumptive OBP promoters in *Drosophila*; nine drove expression in olfactory tissue alone (antenna and palps) while another nine drove expression in both olfactory and gustatory (wings and legs) tissues. These studies show that OBPs differentially express in sensilla that have diverse odor specificities, and therefore the OBPs contribute to the overall odor-specific phenotypes of the sensilla.

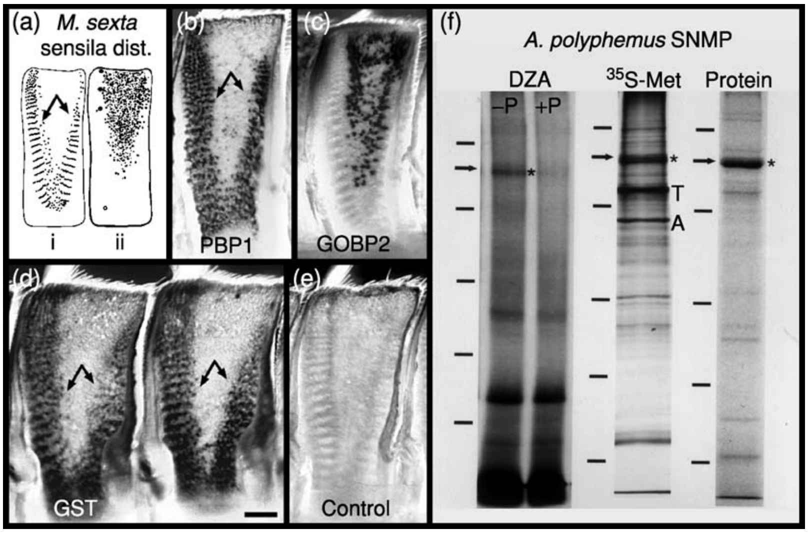


Figure 5 *In situ* hybridizations (wholemount) of *Msex*PBP1, *Msex*GOBP2, and *Msex*GSTolf in adult male *M. sexta* antenna (a–e) and visualizations of sensory membrane proteins of *Antheraea polyphemus*, including *Apol*SNMP1 (f). Expression of *M. sexta* PBP1, GOBP2, and GST. (a) Shows the nonoverlapping distribution of pheromone sensitive long trichoid sensilla (i) vs. other olfactory sensilla (after Lee and Strausfeld, 1990). (b) and (c) show the nonoverlapping expression of *Msex*PBP1 and *Msex*GOBP2 (Vogt *et al.*, 2002), and (d) shows the PBP-like expression of *Msex*GSTolf (Rogers *et al.*, 1999). Arrows point to pheromone-sensitive sensilla. (e) shows a control *in situ* using an noninsect probe. Scale bar = 100 μm. (Modified from Rogers, M.E., Jani, M.K., Vogt, R.G., **1999**. An olfactory specific glutathione *S*-transferase in the sphinx moth *Manduca sexta*. *J. Exp. Biol. 202*, 1625–1637.) (f) Visualizations of *A. polyphemus* SNMP1. Three experiments characterizing proteins of the receptor membranes of olfactory neurons of male *A. polyphemus*. DZA: dendrite membranes were photolabeled with [^{3}H]-DZA in the absence (−P) and presence (+P) of excess nonradioactive pheromone; asterisk marks SHMP69 thought to be a candidate pheromone receptor (fluorogram, from Vogt *et al.*, 1988). ^{35}S-Met: dendrite membrane proteins metabolically labeled with injected ^{35}S-Met, revealing a prominent band near 69 kDa (asterisk) as well as tubulin (T) and actin (a) (Vogt, unpublished fluorogram; for details of method see Vogt *et al.*, 1989). Protein: membranes were isolated from olfactory sensilla isolated from 800 male antennas of animals attracted to female moths releasing sex pheromone. One-tenth of this preparation was analyzed revealing an abundant protein near 69 kDa (asterisk) visualized by Coomassie Blue staining; this band (*Apol*SNMP1) was electroblotted and sequenced (Rogers *et al.*, 1997). Arrows identify the position of a 69 kDa marker in each gel.

3.15.4.1.3. Functional studies of odorant binding proteins The suggestion that OBPs function as odor transporters was first proposed (Vogt *et al.*, 1985) as an alternative to a model that was prevalent at the time in which pore tubules served as the pheromone conduit from sensillum surface to OSN membrane (Ernst, 1969; Steinbrecht and Müller, 1971; Kaissling, 1974, 1986; Kaissling and Thorson, 1980; Keil, 1982; Steinbrecht, 1997). Studies suggested that pore tubule to neuron contacts were quite rare (Keil, 1982), and their visualization highly dependent on tissue fixation in histological preparations (Steinbrecht and Müller, 1971; Steinbrecht, 1980; Keil, 1982). In the likely absence of such a pore tubule delivery system, the presence of a soluble binding protein and a potent pheromone degrading enzyme suggested the model presented in Figure 2.

In this new model, based on studies using the moth *A. polyphemus*, the hydrophobic pheromone molecules were solublized into the aqueous sensillum lumen by PBPs, transported by the PBPs to ORs in the OSN membranes, and subsequently degraded by ODEs (Vogt *et al.*, 1985). ODE kinetic properties were characterized, and the pheromone half-life within the sensillum was estimated at below 15 ms (Vogt *et al.*, 1985). An even shorter half-life was later estimated for pheromone within sensilla of the moth *M. sexta* (Rybczynski *et al.*, 1989). The ability of the *A. polyphemus* ODE to degrade pheromone was characterized at various pheromone concentrations and in the presence of various PBP concentrations (Vogt and Riddiford, 1986a). Results suggested that PBP did not protect pheromone from ODE through irreversible binding, but rather by mass action (i.e., PBP getting in the way of SE based on an estimated 1000-fold difference in concentrations for the two proteins). Results also suggested a pheromone-PBP K_D in the 1–10 μM range (Vogt and Riddiford, 1986a).

This function of PBPs as pheromone transporters in pheromone detection became generalized with the identification of PBPs from diverse species, and was extended to the detection of plant volatiles with the multispecies identification of GOBP1 and GOBP2 protein families (Vogt *et al.*, 1991a). The

identification of OBPs in *Drosophila* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994) expanded these roles beyond the Lepidoptera; the identification of an OBP in the hemipteran *Lygus lineolaris* suggested that OBPs were present throughout the holometabolous and hemipteran lineages (Vogt *et al.*, 1999), and the recent identification of OBPs in orthopteroid insects (Ishida *et al.*, 2002a; Ban *et al.*, 2003b; Rivière *et al.*, 2003) argues that OBPs function throughout the neopteran lineages, and thus presumably the vast majority of eukaryotic organisms (Erwin, 1982; Novotny *et al.*, 2002) (**Figure 4**). Finally, significant number of OBPs with divergent sequences have been identified in *M. sexta, D. melanogaster*, and *A. gambiae* (**Table 2**); such numbers are consistent with expectations for odor receptors, i.e., that there should be many odor receptors to deal with the number of odorous ligands an animal detects (Buck and Axel, 1991; Vosshall *et al.*, 1999). The number and diversity of OBP genes (**Table 2**, **Figure 3**) coupled with their differential expression in olfactory sensilla during a time when odors are being detected (**Figure 5**) argues that they play a central role in odor processing. But what is that role?

Pheromone binding has been demonstrated for PBPs from diverse species, including moths (Vogt and Riddiford, 1981; Vogt *et al.*, 1989; Maïbèche-Coisné *et al.*, 1997, 1998; Jacquin-Joly *et al.*, 2000, 2001), honeybee (Danty *et al.*, 1999), and beetle (Nikonov *et al.*, 2002). Binding constants between pheromone and PBP have been estimated as low as 60 nM (Kaissling, 1986) but more typically in the range of 0.5–10 μM (Vogt and Riddiford, 1986a; Du and Prestwich 1995; Plettner *et al.*, 2000; Kowcun *et al.*, 2001; Campanacci *et al.*, 2001a; Bette *et al.*, 2002). Values of K_D of 20–30 μM have also been determined for certain compounds and conditions (Kowcun *et al.*, 2001; Bette *et al.*, 2002); the K_D for a PBP of *Mamestra brassicae* was estimated at 0.2 μM (Campanacci *et al.*, 2001a). Two PBPs of the gypsy moth *L. dispar* were shown to differentially bind the two optical enantiomers of disparlure, the gypsy moth sex pheromone (Vogt *et al.*, 1989; Plettner *et al.*, 2000). Two PBPs of the silk moth *A. pernyi* were shown to differentially bind components of the sex pheromone of that species (Du *et al.*, 1994; Du and Prestwich, 1995). Specific ligand binding has also been demonstrated for the GOBP2 protein of *M. sexta* (Feng and Prestwich, 1997) and a GOBP of the moth *M. brassicae* (Bohbot *et al.*, 1998). *A. polyphemus* PBP1 (*Apol*PBP1) displayed different endogenous tryptophan based fluorescence in binding studies with three different pheromone components (Bette *et al.*, 2002). Differential specificities of the binding sites of PBPs of the moths *M. brassicae* and *A. polyphemus* have been demonstrated in competition studies examining the displacement of a fluorescent probe, 1-aminoanthracene (AMA) by pheromone components and nonpheromonal fatty acids (Campanacci *et al.*, 2001a). One OBP from *Drosophila*, Lush, has been shown to be necessary for the appropriate behavioral response to alcohol; *lush* mutants do not display the normal wild-type avoidance of alcohol (Kim *et al.*, 1998; Kim and Smith, 2001). *Lush* has also been shown to express in trichoid sensilla of the adult antenna, coexpressing with OS-E and OS-F (Shanbhag *et al.*, 2001a). Nevertheless, for all these studies, the vast majority of OBPs remain uncharacterized, either biochemically or genetically.

Pheromone binding may be regulated by pH dependent conformational changes in PBP. In *Bmor*PBP1 the pheromone binding pocket is formed by four α-helices (Sandler *et al.*, 2000). In crystals, a pheromone molecule interacts with diverse amino acids distributed throughout the protein but lining the binding pocket; both hydrophilic and electrostatic interactions are thus presumed to be critical for ligand binding specificity (Sandler *et al.*, 2000; Klusák *et al.*, 2003). It was initially suggested that an α-helix unit at the C-terminus covers the entry to the binding pocket and that this C-terminal α-helix must move to allow pheromone to enter or leave the binding pocket (Sandler *et al.*, 2000). Solution NMR studies suggest that *Bmor*PBP1 exists in two conformations which are pH dependent: a basic form (BmorPBP^B) above pH 6.0 and an acidic form (*Bmor*PBP^A) below pH 4.9 (Damberger *et al.*, 2000). More recent studies suggest that these conformational differences involve the C-terminus: at the higher pH range (i.e., physiological pH) the C-terminus is extended and lies on the surface of the protein (*Bmor*PBP^B) while at the lower pH range this same region forms a seventh α-helix which enters the binding pocket (*Bmor*PBP^A) and displaces a pheromone molecule bound within (Horst *et al.*, 2001). A pH dependent pheromone binding was observed for the *Bmor*PBP1; the pheromone–PBP complex was stable at pH 7.5 but significantly unstable at pH 5.5 (Wojtasek and Leal, 1999a). These studies have suggested that binding of pheromone at the pore tubule and release at the receptor may be driven by local pH differences within the sensillum, pH differences which alter the conformation of the PBP (i.e., from *Bmor*PBP^B to *Bmor*PBP^A) (Wojtasek and Leal, 1999a; Horst *et al.*, 2001; Leal, 2003). The *Bmor*PBP^B conformation would favor binding of pheromone at the pore tubule and transporting it to the OR, while the

*Bmor*PBP[A] conformation would favor releasing pheromone to the OR. The proposed mechanism for such a pH gradient within a sensillum is the neuron membrane. The membrane phospholipids carry a negative charge (at the pH of interest) and are thus presumed to create a locally acidic boundary layer by attracting free hydrogen ions (actually, any cation); the conformation of the PBP–pheromone complex is thus changed from a bound state to a released state as it approaches the membrane (Wojtasek and Leal, 1999a).

Other studies suggest alternative views regarding the mechanisms of odor–OBP binding. The crystal structure of an OBP from the cockroach *Leucophaea maderae* (*Lmad*OBP) has recently been characterized in the presence of either the pheromone component 3-hydroxy-butan-2-one or the fluorescent molecule amino-naphthalen sulfonate (Lartigue *et al*., 2003b). This protein is missing the C-terminal region equivalent to the seventh α-helix in *Bmor*PBP1, and therefore is missing the pH dependent opening/closing mechanism proposed for *Bmor*PBP1 (see above). The binding pocket of the *Lmad*OBP is also more hydrophilic than that of *Bmor*PBP1. The authors suggest that these changes, especially the hydrophilic pocket, may be adaptive (Lartigue *et al*., 2003b), although *Lmad*OBP is overall highly divergent from the other characterized OBPs. The lack of the C-terminal process in *Lmad*OBP is especially interesting and brings to mind the presence/absence of the N-terminal inactivation structure of different voltage-gated ion channels. Clearly it will be helpful to evaluate and compare the structural properties of divergent OBPs from one species, as well as more OBPs from diverse species, in order to determine which features have functional significance and which merely reflect phylogenetic difference.

Some studies also disagree with the pH-conformation hypothesis and to some degree with the crystallographic interpretations. The two PBPs of *L. dispar* show different K_D for the two (+ and −) optical enantiomers of the pheromone (PBP1: $K_D(+)$ 7 μM, $K_D(-)$ 2 μM; PBP2: $K_D(+)$, 1.8 μM, $K_D(-)$, 3 μM) (Plettner *et al*., 2000). However, this binding is not pH dependent over the ranges that would represent near-membrane conditions (pH 7.5 versus pH 5.5) (Kowcun *et al*., 2001). Modeling the membrane influence on the spatial distributions of K^+ and H^+ (i.e., pH) suggests that these influences only come into play at distances well under 2 nm from the cell membrane (Kowcun *et al*., 2001), which itself is only 8 nm thick. These studies suggest that pheromone offloading is not occurring by either pH or ionic strength gradients, but rather suggest that the driving force to offload pheromone is either mass action kinetics (i.e., ORs have higher affinity for pheromone than PBPs) or direct interaction between the pheromone–PBP complex and OR (or perhaps some other membrane protein). These studies also observed an increased PBP binding capacity at high pheromone concentrations, and suggest that the PBP may form dimers dynamically under these conditions to trap pheromone at high concentrations to mitigate against stimulus saturation (Honson *et al*., 2003).

The *Bmor*PBP1 structural studies describe a deep binding pocket with a narrow opening covered by a C-terminal lid, and the involvement of a large numbered amino acids which interact with the majority of the pheromone molecule (Sandler *et al*., 2000; Klusák *et al*., 2003). This seems a highly specific pocket and not terribly amenable to rapid release of pheromone; the conformational changes required would seem to require considerable energy, presumably provided by the proposed pH gradient, the presence of which has yet to be verified (it may be too shallow to have relevance, or it may be buffered by membrane proteins with high isoelectric points and thus be positively charged at pH 7.5). It was observed previously that PBP did not protect pheromone from degradation by a pheromone degrading esterase *in vitro*, though the PBP did maintain aqueous solubility of the pheromone, suggesting that a pheromone–PBP complex had formed (Vogt and Riddiford, 1986a). In contrast, the binding site described for *Bmor*PBP1 would seem well designed for protection from enzymes. The binding site would also seem well designed for a very high degree of specificity, considering the number of interacting amino acid side chains (Klusák *et al*., 2003). Yet studies examining the ability of various pheromone components and fatty acids to displace the fluorescent probe AMA suggest that the binding site may have relatively low specificity for its ligand(s) (Campanacci *et al*., 2001a). So, perhaps the binding site that is being described by crystallography is not the binding site used during rapid pheromone communication. And perhaps, though the PBPs may be conformationally sensitive to pH, these pH environments are not actually experienced *in vivo*. Clearly, one wants a system in which these hypotheses can be experimentally tested. Ideally one wants a genetic model, such as *Drosophila*, where OBPs can be modified or swapped and the result tested physiologically and/or behaviorally.

3.15.4.1.4. Evolutionary genomics of odorant binding proteins The OBP gene family was presumably derived through duplication of a gene with a func-

tion that was likely not chemosensory, and the family has expanded through subsequent gene duplications. Alleles of these duplicated OBP genes accumulated, and evolutionary selection acted on these alleles leading to changes in the properties and functions of the predominant alleles in the species. Speciation events allowed allelic selection to proceed in manners supporting unique olfactory behaviors (life histories) of individual species. Depending on the function (nonchemosensory or chemosensory) of the founder gene(s), subsequent lineages diverged to either chemosensory or nonchemosensory functions. The genes currently listed as OBPs of *D. melanogaster* and *A. gambiae* include sequences with four to twelve cysteines (Hekmat-Scafe *et al.*, 2002), differing significantly from the six cysteines of the gold standard OBPs, differences which assuredly influence the function of these proteins.

Structural relationships between different OBPs can be explored to some extent using phylogenetic analysis tools (**Figure 3**). Such analyses compare amino acid sequences emphasizing similarities and differences. Amino acid sequences are aligned using programs such as ClustalX (Thompson *et al.*, 1997), and trees are constructed using programs such as PAUP (Swofford, 2000) or MEGA (Kumar *et al.*, 2001) and methods such as neighbor joining (Saitou and Nei, 1987) or maximum likelihood (Strimmer and von Haeseler, 1996; Nei and Kumar, 2000) available within these programs. The results of such analyses, when applied to proteins rather than nucleic acids, usually do not imply any evolutionary history, but rather suggest properties (characters) that certain proteins may share. Other criteria must be considered to infer evolutionary relatedness and history such as a demonstrated positive selection (e.g., significant nonsynonymous differences in codon nucleotides) or conservation of gene structure (e.g., exon–intron boundaries, relative positions in chromosomes, identification of neighboring genes: Vogt, 2002; Vogt *et al.*, 2002).

The sequence similarities of most OBPs listed in **Table 2** are summarized in the neighbor joining tree illustrated in **Figure 3**. Overall, these OBPs are highly divergent, indicated by the relatively long lengths of most branches. Several branches include multiple taxa which define specific similarity groups; such groupings may suggest related functions. One such group is the Lepidoptera-specific PBP–GOBP gene family. Other similarity groups include a group of ant OBPs with a reputed role in governing the social behavior of *Solenopsis* spp. (fire ants and their relatives: Ross, 1997; Ross and Keller, 1998; Krieger and Ross, 2002), two groups that include the *Drosophila* OBPs LUSH and OS-E/OS-F, and a group that includes the lepidopteran ABPX proteins. Both the LUSH and ABPX groups include genes belonging to multiple insect orders, a feature that may suggest that these proteins have a functional role common to or of central importance to diverse insects. However, most branches do not include OBPs from multiple orders, suggesting that selection acting on these genes is strongly biased by features unique to the orders, families, or genera represented.

The PBPs and GOBPs of Lepidoptera represent a group that is not present in other insect orders, but these PBPs and GOBPs are clearly only a subgroup of lepidopteran OBPs. Their absence from Diptera, a sister lineage, and the relatively recent emergence of Lepidoptera implied by the fossil record, suggest that the PBP/GOBP genes may have arisen within Lepidoptera, or at least within the lepidopteran/trichopteran lineage (**Figure 4b**). PBPs, GOBP1s, and GOBP2s comprise three distinct subgroups; amino acid sequence identities between PBPs and GOBPs are consistently between 25% and 30%, and amino acid sequence identities between GOBP1s and GOBP2 are consistently about 50%. GOBP1s and GOBP2s are highly conserved (around 90% identical within each group) while PBPs are highly divergent (20% to 95% identical). Also, multiple PBPs have been identified in individual species while only single genes are yet known for GOBP1s and GOBP2s, suggesting that gene duplications are occurring and being retained for PBPs but are either not occurring or not being retained for GOBPs. Clearly, selection is acting very differently on these subgroups, as might be expected for systems supporting reproduction (PBPs) versus general maintenance (i.e., feeding) (GOBPs).

The genes encoding PBP1 and GOBP2 of *M. sexta* (*Msex*PBP1 and *Msex*GOBP2) are adjacent to one another, with coding regions separated by about 2000 bp, and both genes have identical exon–intron structures (Vogt *et al.*, 2002). Proximity and conserved gene structure argue strongly that PBP1 and GOBP2 are derived from a gene duplication, yet the two proteins have very different sequences and temporal and spatial expression patterns. *Msex*PBP1 and *Msex*GOBP2 express in different types of sensilla (**Figure 5a–c**); *Msex*PBP1 expresses only in adult moths while *Msex*GOBP2 expresses in both larvae and adults, but the expression of both is regulated by ecdysteroid hormones (Vogt *et al.*, 1993, 2002). If PBP1 and GOBP2 did derive from a gene duplication, then presumably GOBP1 derived from a subsequent duplication event involving GOBP2. The origins of multiple PBPs are less clear. Multiple PBPs may have been derived from

subsequent duplications involving PBP1, or the PBP1/GOBP2 pair may have been derived from a duplication involving one of these other PBPs. PBP diversification may have been driven by reproductive selective pressures within species, genera, or families (Merrit *et al.*, 1998); however, this does not appear to be the case with the GOBPs which are both highly conserved and broadly distributed among the lepidopteran families. An in-depth comparative study of the PBPs and GOBPs across the Lepidoptera might reveal much of the olfactory evolution of this insect order.

OS-E and OS-F of *Drosophila* are also presumably derived from a gene duplication based on the neighboring relationship of their genes (separated by about 1000 bp), similar exon–intron structure, and similar sequence. However, unlike *Msex*PBP1/*Msex*GOBP2 which differentially express, OS-E and OS-F coexpress in olfactory trichoid sensilla of the *Drosophila* antenna (Hekmat-Scafe *et al.*, 1997); at least a subset of these sensilla contains neurons responsive to the pheromone *cis*-vaccenyl acetate (Clyne *et al.*, 1997). Orthologs of OS-E and OS-F have been tentatively identified in *A. gambiae*, suggesting that the OS-E/OS-F gene duplication preceded the divergence of flies and mosquitoes within the Diptera (Vogt, 2002). A gene with significant sequence similarity to OS-E and OS-F has also been identified in the mosquito *Culex quinquefasciatus* (Ishida *et al.*, 2002b); the sequence conservation of these presumed OS-E/OS-F orthologs among different groups of Diptera suggests their singular importance in the olfactory biology of these species. Unlike in *Drosophila*, the presumptive *A. gambiae* OS-E/OS-F genes are separated by about 30 million nucleotides suggesting that a chromosomal translocation event has occurred. It will be interesting to learn whether this separation has also resulted in a divergence in expression or function between these *A. gambiae* genes.

The PBP/GOBP genes of Lepidoptera and OS-E/OS-F genes of Diptera are just two stories of duplication and expansion of olfactory genes; the previously described pair of *Drosophila* OR genes, Dor22a and Dor22b, are another (Dobritsa *et al.*, 2003) (see Section 3.15.3.1.2). A look at the distribution of OR, OBP, and SNMP homologs on the *Drosophila* chromosomes reveals a rich evolutionary history of gene duplication and translocation in the context of sensory biology and behavioral selection (Vogt, 2003). Increased access to genomes is permitting the identification of gene orthologs and the following of gene change across insect families and orders will allow us to hypothesize function in one species based on the characterized function of a gene ortholog in another.

3.15.4.1.5. Allelic variation of pheromone binding proteins Several efforts have examined allelic variation of PBPs between distinct populations. Electrophoretic variants (possible multiple alleles) were described for both *Apol*PBP1 and *Apol*SE between populations of the moth *A. polyphemus* from New York (Long Island) and Wisconsin. However, this apparent allelic variation appeared high within, but similar between, each population and nothing definitive could be concluded regarding how this variation might affect behavior (Vogt, 1987; Vogt and Prestwich, 1988). LaForest and colleagues (1999) characterized PBP genes from individuals of two populations of the noctuid moth *Agrotis segetum* which use the same pheromone components but in different ratios. However, only neutral sequence variation was observed in the PBP sequences and again nothing definitive could be concluded regarding how such variation might affect pheromone perception and behavior. Similar results were obtained from studies of distinct populations of the European corn borer *Ostrinia nubilalis* (Willett and Harrison, 1999a, 1999b). Positive selection was observed for PBPs of species using different pheromone structures, but it was not clear that this selection resulted from the changes in pheromone components (Willett, 2000a, 2000b). A limitation of all three studies is that each characterized only one PBP; closer examination has demonstrated consistently that lepidopteran species have several PBPs (**Table 2**). It is likely that multiple PBPs derived from relatively recent gene duplications which occurred within specific lepidopteran lineages rather than at the base of the lepidopteran lineage (Merritt *et al.*, 1998). If this is true, then it is also possible that, in certain species, PBP genes did not duplicate or that duplicated genes have become lost. But these possibilities are difficult to assess without a full genome sequence. If functionally relevant positive selection has occurred among the PBPs (which certainly seems likely), it may be discernable only by analyzing the larger repertoire of PBP genes within a species.

3.15.4.2. Odor Transport by OS-Ds, SAPs, or CSPs?

The proteins of another gene family, variously named OS-D, sensory appendage proteins (SAPs), or chemosensory proteins (CSPs), have been suggested frequently to have OBP-like properties in binding and transporting pheromones and other odors within sensilla (reviews: Picimbon, 2003;

Nagnan-Le Meillour and Jacquin-Joly, 2003). These proteins have a wide tissue distribution throughout the body, share no sequence similarity to the OBPs, contain about 100–110 amino acid residues (compared with 140 for OBPs), four conserved cysteines (versus six in OBPs) and six α-helices (similar number but in a very different configuration than OBPs), and a hydrophilic channel that passes through the protein (versus a single-opening pocket in OBPs) (Campanacci *et al*., 2001b, 2001c, 2003; Briand *et al*., 2002; Lartigue *et al*., 2002). **Table 3** lists members of this family reported to date. These proteins are remarkably conserved, showing consistently similar sequence identities independent of the phylogenetic distance of the organisms For example, CSPs share 50–60% identity between the two orthopteran species (*Schistocerca gregaria* and *L. migratoria*), 38–54% identity between the two lepidopteran groups CSPMbra A and CSPMbraB, and 37–50% identity between proteins of the Orthoptera versus Lepidoptera (Jacquin-Joly *et al*., 2001). Such conservation is in stark contrast to the high degree of divergence observed for ORs and OBPs.

Antennal CSPs were first identified through studies searching for antennal-specific genes. In their screen for antennal-specific proteins in *Drosophila*, Carlson's group identified six cDNAs, naming them OS-A through OS-F (McKenna *et al*., 1994). This study yielded OS-E and OS-F, which were among the first OBPs identified outside of Lepidoptera (McKenna *et al*., 1994); one of the non-OBP proteins was named OS-D and was also described that year and named A10 by Pikielny in his characterization of *Drosophila* antennal proteins (Pikielny *et al*., 1994). Two years later, Pelosi's group characterized antennal proteins of several species of phasmid (walking stick), identifying OS-D/A10 homologs in antenna and leg extracts of *Eurycantha calcarata* and *Extatosoma tiaratum* and antenna extracts of *Carausius morosus* (Mameli *et al*., 1996). Another 2 years later, Nagnan-Le Meillour's group reported on the pheromone binding properties of antennal proteins in the moth *M. brassicae* (Bohbot *et al*., 1998), demonstrating nonspecific odor binding to a moth homolog of OS-D/A10 and noting its relationship to the phasmid proteins as well as proteins CLP1 from the labile palps of the moth *Cactoblastis cactorum* (Maleszka and Stange, 1997) and DA6b from the antenna of *A. mellifera* (Danty *et al*., 1998). In 1999, Robertson's group reported their characterization of hundreds of antennal cDNAs of the moth *M. sexta*, including

Table 3 Chemosensory protein (CSP) related proteins with published sequences

Species	*Protein/ gene name*	*Accession number*[a]
Lepidoptera		
Bombyx mori	*Bmor*CSP1	AAM34276
	*Bmor*CSP2	AAM34275
Cactoblastis cactorum	*Ccac* CLP1	AAC47827
Helicoverpa armigera	*Harm*CSP	AAK53762
Heliothis virescens	*Hvir*CSP1	AAM77041
	*Hvir*CSP2	AAM77040
	*Hvir*CSP3	AAM77042
Mamestra brassicae	CSP*Mbra*A1	AAF19647
	CSP*Mbra*A2	AAF19648
	CSP*Mbra*A4	AAF19650
	CSP*Mbra*A5	AAF19651
	CSP*Mbra*A6/A3	AAF71289(A6)
	CSP*Mbra*B1	AAF19652
	CSP*Mbra*B2	AAF19653
	CSP*Mbra*B3	AAF71290
	CSP*Mbra*B4	AAF71291
Manduca sexta	*Msec*SAP1	AAF16696
	*Msec*SAP2	AAF16714
	*Msec*SAP3	AAF16707
	*Msec*SAP4	AAF16721
	*Msec*SAP5	AAF16716
Hymenoptera		
Linepithema humile (ant)	*Lhum*CSP	AAN01363
Diptera		
Anopheles gambiae	agCG50175	EAA12703
	agCG50200	EAA12591
	agCG50208	EAA12601
	agCG50210	EAA12338
	agCG50220	EAA12353
	agCP11484	EAA12322
	AgSAP1	AAL84186
Drosophila melanogaster	*DmelOS-D*/A10	AAA21358 (OS-D)
	CG30172	AAM68292
	CG9358	AAF47307
	PEBmeIII	AAA87058
	RH74005/ CG11390	AAM29645 (RH)
Dictyoptera		
Locusta migratoria	LmigOS-D1	CAB65177
	LmigOS-D2	CAB65178
	LmigOS-D3	CAB65179
	LmigOS-D4	CAB65180
	LmigOS-D5	CAB65181
Periplaneta americana	p10	AAB84283
Schistocerca gregaria	*CSPsg1*	AAC25399
	CSPsg2	AAC25400
	CSPsg3	AAC25401
	CSPsg4	AAC25402
	CSPsg5	AAC25403
Phasmatodea		
Eurycantha calcarata	CSPec1	AAD30550
	CSPec2	AAD30551
	CSPec3	AAD30552

[a]Accession numbers available as of August 2003.

five OS-D/A10 homologs which they named as sensory appendage proteins (SAPs) (Robertson *et al.*, 1999). In 2000, Pelosi's group characterized five OS-D/A10 homologs from the locust *S. gregaria*, referring to them as chemosensory proteins (CSPs) (Marchese *et al.*, 2000). **Table 3** lists the members of this gene family reported to date.

CSPs associate with a broad range of body tissues, including legs, heads, thorax, antennas, proboscis, subcuticular epithelium, pheromone glands, and ejaculatory duct (Nomura *et al.*, 1992; Dyanov and Dzitoeva, 1995; Nagnan-Le Meillour *et al.*, 2000; Picimbon *et al.*, 2000a, 2001; Jacquin-Joly *et al.*, 2001; Picone *et al.*, 2001; Ban *et al.*, 2003a). CSPs may be absent from the central nervous system (Picimbon *et al.*, 2000b). The name CSP (or SAP) is optimistically biased towards the chemosensory functions of these tissues, though clearly each tissue performs additional functions. Picimbon *et al.* (2000a, 2001) found that CSPs of the moths *B. mori* and *H. virescens* express in antennas, proboscis, legs, thorax, and head. The earliest identified member of the family, P10 of the cockroach *Periplaneta americana*, also expresses in legs, antennas, and heads (Nomura *et al.*, 1992; Kitabayashi *et al.*, 1998). In *H. virescens*, CSP expression in legs is initiated at least 5 days before adult eclosion, during a period when development is still ongoing, and expression persists into adult life; however, this time course does not match that of proteins where specific olfactory association is well established, such as OBPs and SNMP which tend to initiate expression only shortly before adult eclosion (Vogt *et al.*, 1989, 1993; Rogers *et al.*, 1997, 2001b; Picimbon *et al.*, 2001). In the cockroach, P10 expression was primarily associated with leg regeneration (Nomura *et al.*, 1992). While it may be true that most external insect tissues contain chemosensory organs, the mere association of CSPs with these tissues does not necessarily correlate with chemosensory function.

Several CSPs have been shown to associate with chemosensory sensilla, though not in a consistent pattern. In *Drosophila*, OS-D/A10 expression (*in situ* hybridization) was associated with the sacculus, an antennal structure rich in coeloconic sensilla, although actual association with these sensilla was not shown (Pikielny *et al.*, 1994). In the locust *S. gregaria*, antisera to CSPs purified from tarsi reacted with antigen in the lumen of gustatory sensilla of legs, palps, and antennas (Angeli *et al.*, 1999). In the moth *M. brassicae*, CSPMbraA6 expression (*in situ* hybridization) was associated with hair-like olfactory sensilla (trichogen or basiconic) but not with coeloconic sensilla (Jacquin-Joly *et al.*, 2001).

Several ligand binding studies have been reported for these proteins. CSPs from *M. brassicae* have been shown to interact with a wide range of hydrocarbons ranging in length from C9 to C18 and containing a variety of functional groups; K_D values for all of these molecules are close to 1 μM (Bohbot *et al.*, 1998; Jacquin-Joly *et al.*, 2001; Lartigue *et al.*, 2002; Campanacci *et al.*, 2003; Mosbah *et al.*, 2003). CSPs may be capable of receiving multiple ligands within their binding pocket; CSPMbraA6 was shown to bind ligands with a stoichiometry of 3 : 1 (Campanacci *et al.*, 2003). A CSP from the honeybee was shown to interact with C14–C18 compounds, including several compounds reported to be brood pheromones, with K_D values in the 1 μM range (Briand *et al.*, 2002). A broad range of ligands was observed to interact with a CSP from the locust *S. gregaria* in competition studies with the fluorochrome *N*-phenyl-1-naphthylamine; binding of the fluorochrome was heat stable (100 °C, 20 min) and had a K_D of 4 μM, and K_D values of competitors ranged from 10 to 150 μM (Ban *et al.*, 2002, 2003b) (such heat stability in a soluble, globular protein with α-helices and disulfide bonds seems somewhat surprising). CSPs from the wing of the locust *L. migratoria* were purified with the oleoamide bound to the protein, a compound interpreted as an endogenous ligand for this CSP (Ban *et al.*, 2003a); oleoamide is a C_{18} compound used as an industrial lubricant (recombinant PBPs have been isolated bound with spurious fatty acids) (Oldham *et al.*, 2001). Presumably addressing the remarkable lack of discrimination of these proteins, Lartigue *et al.* (2002) wrote "We hypothesize therefore that CSPMbraA6 should interact with membranes to extract pheromones or other lipidic compounds dispersed in the hydrophobic membrane matrix and bring them to their specific target."

It does appear that at least some CSPs associate with chemosensory sensilla, and that they are capable of binding odor-like molecules, if with relatively low selectivity. And their presence in a broad range of insects and tissues together with their small ligand carrying capacity marks them proteins of potentially significant physiological interest. Nevertheless, the importance of these proteins in chemodetection remains remarkably unclear.

3.15.4.3. Pheromone and Odor Degradation by Odor Degrading Enzymes

Signal termination plays a critical role in all chemically mediated biological processes, and this is no

less so in odor detection. The process of pheromone degradation has been studied for some years, at least as far back as Kasang (1971) in *B. mori* and Ferkovich *et al*. (1973a, 1973b) in the cabbage looper moth, *Trichoplusia ni*. Since then, a few pheromone degrading enzymes have been identified and characterized in detail and the general principal of pheromone degradation has become well established. Yet surprisingly little work is being done to investigate odor degrading enzymes (ODEs), in contrast with current efforts on ORs and OBPs. One reason to expand efforts on ODEs is their potential in insect control. If it is true that pheromones are perceived as precise mixtures, then the targeted inhibition of the ODE for a specific component should alter the blend ratio within a sensillum resulting in misperception of the pheromone. Of the three protein classes (ORs, OBPs, and ODEs), ODEs may be the least specific and thus the more generally targetable protein for behavioral inhibition.

ODEs are enzymes selectively evolved to degrade odor molecules (Prestwich, 1987). However, to call an enzyme an ODE it is not enough to merely demonstrate the ability of an enzyme to degrade an odor; that enzyme has to be shown to reside in a space that is relevant to odor detection. This principle should be applied to OBPs and ORs as well. In general, those ODEs studied have been ones that attack specific functional groups, such as acetate esters, aldehydes, alcohols, ketones, and epoxides. ODEs attacking such different functional groups presumably belong to different gene families. The 55 kDa antennal-specific esterase (*Apol*SE) (see Section 3.15.4.3.1.1), that degrades the acetate-ester pheromone component of the silk moth *A. polyphemus* (Vogt and Riddiford, 1981; Vogt *et al*., 1985), is a member of a gene family that encodes insect esterases, an assumption supported by the recent cloning of a candidate *Apol*SE using PCR primers based on conserved regions of insect esterase genes (Ishida and Leal, 2002a). Similarly, the 150 kDa antennal-specific AOXs that degrades the aldehyde components of the *A. polyphemus* and *M. sexta* pheromones (Rybczynski *et al*., 1989, 1990) must belong to a distinct gene family of insect AOXs. So far, only the antennal-specific *Apol*SE and glutathione-*S*-transferase (*Msex*GSTolf) have been cloned (Rogers *et al*., 1999; Ishida and Leal, 2002).

The following section organizes ODEs somewhat artificially into four categories, based on their location in the animal. To some degree this has functional significance, distinguishing between ODEs of the sensillum lumen (soluble or membrane bound), support cell cytosol and body surface.

3.15.4.3.1. Category 1: soluble extracellular ODEs of the sensillum lumen The early studies of the fate of pheromone within the antenna were among the first biochemical studies of insect odor detection. At the time, much seemed to be known about pheromone detection in moths: the pheromone of *B. mori* had been identified and radiolabeled (Butenandt *et al*., 1959; Kasang, 1968); pheromone detection had been characterized by electrophysiology in *B. mori* (Schneider, 1969); the ultrastructure of pheromone sensitive sensilla had been described for *B. mori* (Steinbrecht and Müller, 1971). Adam and Delbrück (1968) had used the *B. mori* pheromone system to justify their "reduction in dimension" hypothesis for ligand-receptor targeting. Steinbrecht and Müller (1971) had suggested that pore tubules might serve as conductive structures for the transport of pheromone molecules from the sensillum surface to receptors of the olfactory neurons (this view was later challenged, by Vogt *et al*. (1985), with the suggestion that OBPs performed odor transport). With this background, a series of studies were performed where radiolabled bombykol was applied to intact male *B. mori* antennas and the products analyzed following solvent extraction. These studies observed the general degradation of the alcohol bombykol to its acid metabolite, but identified no specific enzymes or enzyme activities (Kasang, 1971, 1973; Kasang and Kaissling, 1972; Kasang and Weiss, 1974; Kasang *et al*., 1989a, 1989b). About the same time an independent series of studies examined degradation of the *T. ni* pheromone from the active acetate ester to the inactive alcohol metabolite; these studies demonstrated esterase activities in the antenna but did not identify any antennal-specific enzymes (Ferkovich *et al*., 1973a, 1973b, 1980, 1982; Mayer, 1975; Taylor *et al*., 1981). Although no specific enzymes were identified, these efforts drew attention to the issues of pheromone degradation as an important component of the pheromone detection process.

3.15.4.3.1.1. Antennal-specific esterase The antennal-specific sensilla esterase (SE) of the moth *A. polyphemus* (*Apol*SE) was the first identified pheromone degrading enzyme (Vogt and Riddiford, 1981). The activity of *Apol*SE was visualized on nondenaturing polyacrylamide gel electrophoresis (PAGE), and was shown to be specific to the male antennas and to be located in the sensillar fluid (Vogt and Riddiford, 1981). *Apol*SE molecular mass was estimated at about 55 kDa (Klein, 1987). *Antheraea polyphyemus* was reported to have a two-component pheromone consisting of 9 : 1 ratio of acetate : aldehyde (Kochansky *et al*., 1975).

*Apol*SE was shown to degrade the acetate component and was subsequently purified and subjected to kinetic analysis; a spectrophotometric assay was established using α- and β-naphthyl acetate as substrates, and a radioactive thin-layer chromatography (TLC) assay was established using tritiated pheromone (Vogt *et al.*, 1985). *Apol*SE strongly preferred β- over α-naphthyl acetate, was inhibited by the volatile trifluoroketone 1,1,1-trifluoro-2-tetradecanone ($IC_{50} = 5$ nM), and, most importantly, degraded the sex pheromone with unexpected aggression. By making adjustments for the concentrations and volumes within a sensillum lumen, the *in vivo* half-life of pheromone was conservatively estimated to be 15 ms in the presence of this enzyme. The observation of rapid pheromone degradation prompted the proposal of a new model for pheromone reception, one in which OBPs served as pheromone transporters (replacing pore tubules in that role), and enzymes degraded pheromone molecules to rapidly terminate these odor signals within the sensillum (see **Figures 2 and 8**).

*Apol*SE was used to assess interactions between pheromone and PBP (Vogt and Riddiford, 1986a). *Apol*SE and *Apol*PBP1 were purified from antennas, and the ability of a fixed concentration of *Apol*SE to degrade pheromone was examined in various concentrations of PBP1 and pheromone. ApolSE activity was unaffected under conditions where PBP1 concentrations exceeded pheromone concentrations by 1000 times, suggesting that the PBP did not provide specific protection to pheromone from *Apol*SE, and did not bind pheromone irreversibly. Reduced activity was observed at all pheromone concentrations when PBP1 exceeded 1 μM. It was suggested that this transition was a consequence of protection during binding site-occupancy, but that occupancy must be transient, and that the K_D of the pheromone–PBP interaction must be in the range of 1–10 μM. The activity of *Apol*SE below 1 μM PBP further suggested that pheromone–PBP binding was either very slow or very ephemeral (binding and release occurred rapidly). These conclusions are currently being challenged by alternative views that the pheromone–PBP complex may be quite stable, requiring some secondary interaction to drive dissociation (see earlier discussions regarding OBP function).

An antennal-specific esterase was recently cloned from *A. polyphemus* and tentatively identified as *Apol*SE (accession no. AY091503) (Ishida and Leal, 2002). PCR primers were designed to conserved regions of other known insect esterases, and successfully used to amplify this cDNA. The cDNA encodes a protein with a predicted mass of 59 994 Da (553 amino acids, pI of 6.63) and three potential *N*-glycosylation sites. PCR studies confirmed that mRNA expression is male antenna specific. Kinetic analysis of expressed enzyme and histological localization should confirm the identity of this enzyme, but have not yet been reported. The apparent verification that pheromone degrading esterases are so readily identifiable, and the large number of species utilizing acetate ester pheromones suggest that a door has recently been opened wide for the future characterization of pheromone degrading esterases.

3.15.4.3.1.2. Antennal-specific aldehyde oxidase An antennal-specific aldehyde oxidase (AOX) of the moth *M. sexta* (*Msex*AOX) was the next identified pheromone degrading enzyme (Rybczynski *et al.*, 1989). The activity of *Msex*AOX was visualized on nondenaturing PAGE, and was shown to be antennal specific but present in sensilla of both male and female antennas. *Msex*AOX was observed as a dimer with a combined estimated molecular mass of 295 kDa. *Manduca sexta* uses a multicomponent pheromone consisting exclusively of aldehydes including bombykal (Starratt *et al.*, 1979; Tumlinson *et al.*, 1989, 1994); *Msex*AOX was shown to degrade bombykal to its carboxylic acid, in the absence of any additional cofactors. Both TLC and spectrophotometric assays were established and a variety of substrates and inhibitors were characterized. Making adjustments for the concentrations and volumes within a sensillum lumen, the *in vivo* half-life of pheromone was estimated at 0.6 ms in the presence of this enzyme (Rybczynski *et al.*, 1989).

Many species use pheromone components with chemically diverse functional groups, and thus presumably require multiple ODEs. The pheromone of *A. polyphemus* is a blend of acetate and aldehyde components (Kochansky *et al.*, 1975), and that of *B. mori* is a blend of alcohol and aldehyde (Kasang *et al.*, 1978). Both *A. polyphemus* and *B. mori* have antennal-specific AOXs; both proteins were identified in antennal extracts, and *Apol*AOX was identified in extracts of isolated pheromone sensilla of male antennas (Rybczynski *et al.*, 1990). *Apol*AOX and *Bmor*AOX are both high molecular weight proteins, similar to *Msex*AOX, and are present in male and female antennas. Both *Apol*AOX and *Bmor*AOX can degrade bombykal in the absence of any additional cofactors (Rybczynski *et al.*, 1990). *Bombyx mori* antennas thus contain both alcohol oxidase or dehydrogenase activity (Kasang *et al.*, 1989b) and antennal-specific AOX activities, and

A. polyphemus sensilla contain both antennal-specific SE and AOX activities (Rybczynski *et al.*, 1990). *Apol*SE is only found in male antennas, while *Apol*AOX is abundant in both male and female antennas, suggesting that *Apol*SE was selected to function specifically in pheromone degradation while *Apol*AOX (and presumably *Msex*AOX and *Bmor*AOX) has the multiple functions of inactivating both pheromone and plant volatile odorants (Rybczynski *et al.*, 1990).

Tasayco and Prestwich (1990a, 1990b, 1990c) studied AOX and aldehyde dehydrogenase (ALDH) activities in the antennas of noctuid moths *Heliothis virescens*, *H. subflexa*, and *Helicoverpa zea*. Antennal-specific AOXs were observed in both males and females; molecular weights were similar to those of *M. sexta*, *A. polyphemus*, and *B. mori*. The noctuid enzymes also converted aldehyde pheromone components to their corresponding carboxylic acid without the aid of additional cofactors. Antennnal-specific ALDH enzymes were detected through radiolabeling with a substrate analog, but the location of the enzymes within the antenna was not determined. These ALDH enzymes required the cofactor NAD(P^+). *Apol*SE and *Apol*AOX, as well as the other AOXs described above, function without the assistance of any cofactors, enabling them to act autonomously in the extracellular environment of the sensillum lumen where access to cellular metabolites might be limited. The ALDH requirement for cofactors may suggest that these enzymes are located in the cytoplasm of support cells where cofactors would be available. Antennal ALDH may serve as a biotransformation enzyme for pheromones and possibly xenobiotics in a manner similar to the antennal-specific GST of *M. sexta* (Rogers *et al.*, 1999) (see Section 3.15.4.3.3).

Several studies have reported slow rates of pheromone degradation on antennas (Kasang, 1971, 1974; Kasang and Kaissling, 1972; Kanaujia and Kaissling, 1985; Klun *et al.*, 1991, 1996, 1998). These studies typically applied high concentrations of pheromone to antennas and subsequently extracted metabolites by incubating treated intact antennas in solvent, and analyzed the resulting solvent extract. It is difficult to interpret many of these results as no efforts were made to identify the tissue location of observed enzymatic activity. Enzymes capable of degrading pheromone are present in the cuticle, the cytoplasm of cells, and the hemolymph, in addition to the lumen of sensilla; observed activities may have been from any of these tissue spaces. It was suggested previously that observed slow degradation rates may be due to significant portions of pheromone migrating into the cuticle matrix rather than directly entering sensilla, and that pheromone thus entering the cuticle matrix might exit the cuticle and encounter enzymes at slow rates and thus be degraded at slow rates (Vogt *et al.*, 1985; Vogt, 1987).

3.15.4.3.2. Category 2: membrane-bound ODEs While it might seem most efficient for ODEs to be soluble and evenly distributed throughout the aqueous sensillum lumen, certain classes of enzymes may only be available for selection in membrane-bound forms. Such may be the case with pheromone degrading epoxide hydrolases (EH). The epoxide pheromone disparlure can be degraded by EH activity that is antennal specific and associated with isolated sensilla; this activity is not water soluble, suggesting that it might instead be associated with neuronal or support cell membranes (Prestwich, 1987; Vogt, 1987; Graham and Prestwich, 1992, 1994). Similarly, but in a noninsect system, a membrane-bound ODE that degrades purinergic odor molecules has been characterized in the spiny lobster, *Panulirus argus* (Trapido-Rosenthal *et al.*, 1987, 1990; Grünert and Ache, 1988; Carr *et al.*, 1990; Gleeson *et al.*, 1992). Lobsters, arthropods by all rights, smell many things, including the purines ATP and ADP; the ratio of these molecules is thought to inform the lobster of how recently its food was alive (Zimmer-Faust *et al.*, 1988). Certain olfactory neurons are stimulated by ATP and ADP, and the membranes of these neurons contain enzymes which degrade these purinergic signals (Trapido-Rosenthal *et al.*, 1987, 1990; Gleeson *et al.*, 1992). These enzymes are localized to membrane regions at the base of the sensillum, with their activities oriented towards the lymph cavity.

3.15.4.3.3. Category 3: Cytosolic ODEs – multifunctional ODEs inactivating odors and xenobiotics Olfactory and respiratory tissues of animals are constantly under attack by toxic chemicals from the environment. These xenobiotic compounds have presumably driven the enrichment in such tissues of biotransformation enzymes such as glutathionine-*S*-transferases (GSTs), cytochrome P-450 oxygenases, and UDP-glucuronosyltransferases, and various dehydrogenases and oxidases (reviews: Dahl, 1988; Mannervik and Danielson, 1988; Clark, 1990; Feyereisen, 1999). Many of these enzymes can also attack odor molecules, and this has led to suggestions for mammals that biotransformation molecules may function in odor signal termination (Nef *et al.*, 1989; Lazard *et al.*, 1990, 1991; Burchell, 1991; Ding *et al.*, 1991; Ben-Arie *et al.*, 1993).

Several biotransformation enzymes have been identified in insects. These enzymes have features

suggesting they serve a dual function of attacking both xenobiotics and odor molecules. One of these, *Msex*GSTolf, is an antennal-specific GST in *M. sexta* capable of transforming aldehyde odorants (accession no. AF133268) (Rogers *et al*., 1999). GSTs often function as detoxification enzymes, complexing xenobiotics to endogenous glutathione (a cofactor) and thereby rendering them harmless (Yu, 1983, 1984, 1989; Fournier *et al*., 1992; Snyder *et al*., 1995). Though present in male and female antennas, in male antenna *Msex*GSTolf is restricted to cells underlying the pheromone sensitive trichoid sensilla (**Figure 5a**); this restricted expression to pheromone sensilla suggests that *Msex*GSTolf plays a role in pheromone inactivation (Rogers *et al*., 1999). *Msex*GSTolf mRNA encodes a 219 amino acid protein which lacks an encoded leader sequence; the absence of a leader sequence suggests that GSTolf is not extracellular but rather is retained in the cytoplasm of the cells expressing it. Pheromone molecules entering these cells, perhaps carried by PBPs, would be complexed and inactivated by *Msex*GSTolf and thus prevented from later stimulating the neurons (**Figures 2 and 8**). *Msex*GSTolf shares significant similarity with an antennal GST from *B. mor*i (accession no. AJ006502) (Krieger and Breer, 1998); *Msex*GSTolf and *Bmor*GSTolf are distinct from other insect GSTs and thus may define a unique class of multifunctional biotransformation enzymes (Rogers *et al*., 1999).

An antennal-specific cytochrome P-450 enzyme has been identified in the moth *M. brassicae*; like *Msex*GSTolf, this biotransformation enzyme was also observed to differentially associate with pheromone sensitive trichoid sensilla (accession no. AY063500) (Maïbèche-Coisné *et al*., 2002). Male antennal specific and pheromone degrading P-450 activity has also been observed in the beetle *Phyllopertha diversa*; this activity was membrane-bound, required the cofactor NAD(P)H, and was sensitive to the cytochrome P-450 inhibitors proadifen and metyrapone (Wojtasek and Leal, 1999b). Again, these are presumably enzymes strategically located for the inactivation of xenobiotics, but may have become secondarily selected for pheromone degradation. Discussion earlier in this section described noctuid ALDHs that require cofactors (Tasayco and Prestwich, 1990a, 1990b). This requirement for cofactors in enzymes such as GSTs, P-450s, and ALDHs could be difficult to satisfy in the extracellular space of the sensilla lumen and may therefore suggest a cytosolic location of these enzymes.

A cytosolic location of an ODE may seem incompatible with its function, i.e., once an odor/pheromone passes out of the sensillum lumen and enters a cytoplasm it would seem effectively inactivated and out of reach from the perspective of the ORs. Perhaps this is not so. The restricted association of *Msex*GSTolf or *M. brassicae* P-450 with pheromone sensilla may be the consequence of strong evolutionary selection acting on pheromone systems to maintain low signal backgrounds. Cytosolic signal degradation would ensure that pheromone molecules entering the support cells could not later diffuse back into the sensillum lymph or activate ORs located in the nearby cell bodies of the olfactory neurons, actions that might disrupt reproductive behavior.

3.15.4.3.4. Category 4: cuticular ODEs – surface catabolism of background noise Airborne pheromone and other odors are hydrophobic and tend to adsorb onto the waxy surface of the insect cuticle. Body surfaces thus can collect odors and become sources of background noise if these odors are later released. Degradation of these surface-bound odor molecules might significantly reduce such signal noise.

Pheromone degrading esterase activity associates with the scales covering the outer surface of the silk moth *A. polyphemus* (Vogt and Riddiford, 1986b). Isolated scales were loaded into small cartridges through which volatilized radioactive pheromone was blown ([^{3}H]6*E*,11*Z*-HDA); alcohol metabolites were subsequently extracted indicating that the scales degraded adsorbed pheromone. Isolated scales were suspended in detergent solution containing either α-naphthyl acetate or [^{3}H]-pheromone; both compounds were rapidly degraded. The degradative activity was apparently cross-linked to the scale cuticle as removal of the scales from the incubation solution reversibly removed the enzymatic activity as well. Boiling scales had only a partial effect on diminishing enzyme activity, suggesting that the presumed enzyme might be structurally stabilized by cross-linking to the cuticle. Activity was inhibited by trifluoroketone 1,1,1-trifluoro-2-tetradecanone and *O*-ethyl *S*-phenyl phosphoramidothiolate (EPPAT), the same compounds that inhibited the pheromone degrading of *Apol*SE (Vogt *et al*., 1985). Scales isolated from different body surfaces of *A. polyphemus* had somewhat different activities, and scales taken from other Lepidoptera had little or no activity compared to *A. polyphemus*, suggesting the activity was species specific for the *A. polyphemus* pheromone. These data suggested that the activity represented an essentially solid state and fairly indestructible enzyme selected to degrade sex pheromone adsorbed to the body surface, and that the *A. polyphemus* activity was

tuned to the *A. polyphemus* pheromone (assuming the other species tested had their own scale esterases). This enzyme may function to reduce the noise that adsorbed pheromone might create if later released at an inappropriate moment.

Surface catabolism of pheromones has been observed anecdotally but rarely studied with passion. Several studies have shown that homogenates or sonicates of general body parts of moths will degrade pheromone (Kasang, 1971; Mayer, 1975; Ferkovich *et al.*, 1982). Kasang (1971) suggesting that this activity was due to surface enzymes acting to prevent adsorbed pheromone serving as a false signal source. Ferkovich *et al.* (1982) suggested that activity associated with legs of *T. ni* might serve to clean the antennas of residual pheromone by using the legs as antennal combs. Catabolism of a beetle pheromone, for example, has been observed by placing beetles elytra vials containing volatilized radioactive pheromone. Such enzymes should be of interest as solid state enzymes in applications of non-solution chemistry or air filtration/detoxification. But they should also be interesting as examples of bizarre adaptive biochemistry underlying pheromone based behavior.

3.15.4.3.5. Inhibition of ODEs to inhibit pheromone behavior If ODEs can be differentially inhibited, then pheromone blend ratios might be altered within the sensillum and the pheromone thus misperceived and rendered unattractive. Several studies have used volatile trifluoroketones (TFKs) to probe the physiological and behavioral responses to lepidopteran pheromones; a TFK was shown to be a potent inhibitor of *Apol*SE (Vogt *et al.*, 1985). These studies have produced variable results. A TFK applied directly to the antennas of the pyralid moth *O. nubilalis* partially inhibited whole antennal esterase activity but had no significant effect on male attraction to a pheromone source in a wind tunnel (Klun *et al.*, 1991). However, attractant behavior was clearly inhibited in wind tunnel assays following pretreatment of the processionary moth (*Thaumetopoea pityocampa*) to volatilized TFKs (Quero *et al.*, 1995), direct application of TFKs to the antennas of the noctuid moths *Spodoptera littoralis* and *Sesamia nonagrioides* (Bau *et al.*, 1999), or copresentation with pheromone on a lure for the noctuid moth *M. brassicae* (Renou *et al.*, 1999). The effectiveness of TFKs seems to relate to their structural similarity to the pheromone (Renou and Guerrero, 2000); copresentation of TFK and pheromone in field studies significantly reduced the attraction to traps for *S. nonagrioides* (Riba *et al.*, 2001). The exact mode of action of TFKs is not clear; while they can clearly inhibit esterase activities (Vogt *et al.*, 1985; Vogt and Riddiford, 1986b), they may also interact with ORs (Klun *et al.*, 1991; Pophof 1998; Pophof *et al.*, 2000), and OBPs (untested).

3.15.4.4. Sensory Neuron Membrane Proteins

Sensory neuron membrane proteins (SNMPs) are antennal-specific proteins of Lepidoptera expressed in olfactory neurons and located in the receptive membranes of the dendrites (**Figures 5f and 6**). SNMPs belong to a larger gene family (CD36) that includes identified genes in other insects and other phyla (see below). SNMP (SNMP1) was first identified in the moth *A. polyphemus* (Rogers *et al.*, 1997), and subsequently in the moths *B. mori*, *H. virescens*, and *M. sexta* (Rogers *et al.*, 2001b); a second SNMP (SNMP2) was also identified in *M. sexta* (Robertson *et al.*, 1999; Rogers *et al.*, 2001b). These proteins express in both male and female antennas, although they are more abundant in male antennas, at least in *A. polyphemus* and *M. sexta* (not determined in *B. mori* or *H. virescens*). SNMPs are about 520 amino acids long; *Apol*SNMP1 is expressed as a 59 kDa peptide that is apparently posttranslationally modified to 69 kDa (Rogers *et al.*, 1997, 2001b). The proteins are thought to contain two presumptive transmembrane domains located near the C- and N-terminals and a single large extracellular loop which contains several *N*-glycosylation groups and presumed disulfide bridges (Rogers *et al.*, 1997, 2001b; Rasmussen *et al.*, 1998; Tabuchi *et al.*, 2000). The amino acid sequence identities between each SNMP1 range from 67% to 73%, and the identities between *Msex*SNMP2 and any of the SNMP1s are about 25%. Both SNMP1 and SNMP2 were recently identified in *M. brassicae* (*Mbra*SNMP1 accession no. AF462066); *Mbra*SNMP2 and *Msex* SNMP2 are about 65% identical (Jacquin-Joly, personal communication).

SNMP expression suggests these proteins play a central role in odor detection. *Apol*SNMP1, *Msex*SNMP1, *Msex*SNMP2, and *Bmor*SNMP1 are all known to be antennal specific (Rogers *et al.*, 1997, 2001b). *Apol*SNMP1, *Msex*SNMP1, *Msex*SNMP2, and *Hvir*SNMP1 are all known to express in olfactory neurons, and *Apol*SNMP1 protein has been visualized in the receptor membranes of these neurons (Rogers *et al.*, 1997, 2001a, 2001b; Krieger *et al.*, 2002) (**Figure 5**). *Apol*SNMP1 and *Msex*SNMP1 have been shown to be expressed late in adult development and into adult life, after morphogenesis has been completed but coincident with the expression of *M. sexta* OBPs and AOX and the onset of olfactory function (Vogt *et al.*, 1993;

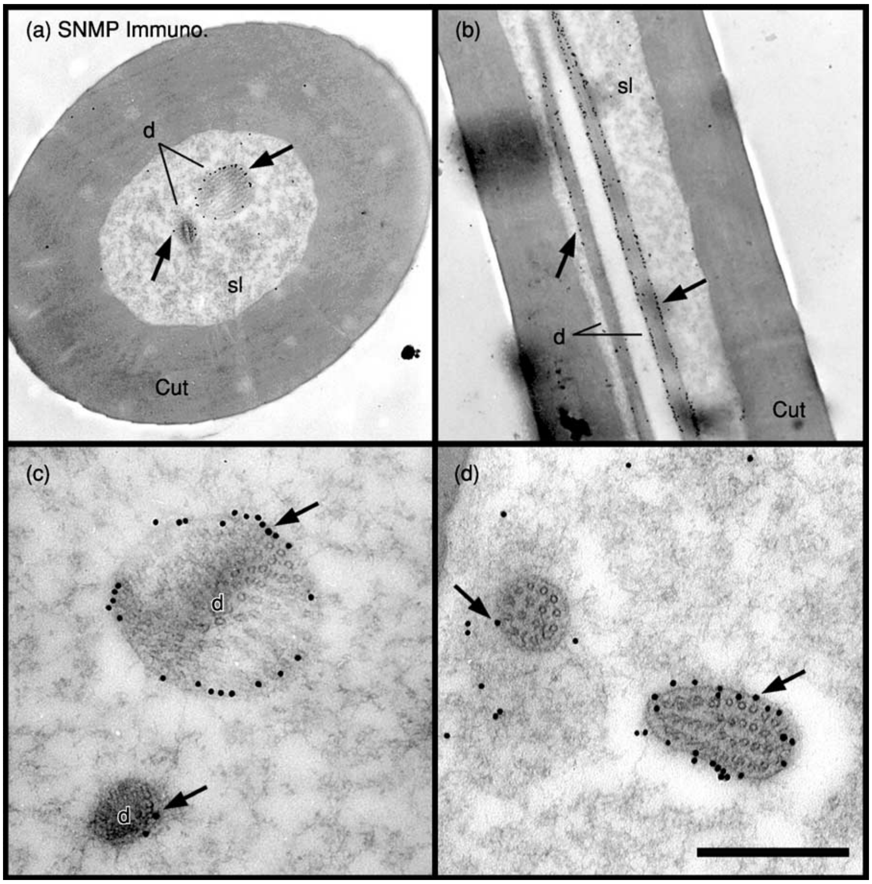


Figure 6 SNMP immunolabeling of electron micrograph sections of pheromone-sensitive trichoid sensilla. SNMP antibodies were visualized using secondary antibody conjugated to 10 nm colloidal gold particles. (a) and (b) include sensillum cuticle; (c) and (d) show only dendrites. Sensilla contained two neurons; one neuron consistently showed significantly greater labeling. Scale bars: (a) 1.25 µm, (b) 2.5 µm (c, d) 0.5 µm. (Modified from Rogers, M.E., Steinbrecht, R.A., Vogt, R.G., **2001a**. Expression of SNMP-1 in olfactory neurons and sensilla of male and female antennas of the silkmoth *Antheraea polyphemus*. *Cell Tissue Res. 303*, 433–446.)

Schweitzer *et al*., 1976; Rogers *et al*., 1997, 2001b). *Hvir*SNMP1 expression coincides with the expression of OR genes in adult male *H. virescens* (Krieger *et al*., 2002). The unique and abundant association of SNMPs with the receptor membrane of olfactory neurons at a time when these neurons are capable of detecting and responding to odors suggests that SNMP are involved in odor detection.

What do SNMPs do? The identification of *Apol*SNMP1 followed photoaffinity labeling studies that tentatively identified a 69 kDa protein as a pheromone receptor (Vogt *et al*., 1988) (**Figure 5f**); however, a role as pheromone receptor seems highly unlikely because SNMPs appear to associate with most olfactory neurons, and are neither 7-transmembrane domain receptors nor show the diversity expected for ORs. SNMPs certainly show no similarity to the presumed ORs identified in *D. melangaster*, *A. gambiae* and *H. virescens* (Clyne *et al*., 1999; Vosshall *et al*., 1999; Hill *et al*., 2002; Krieger *et al*., 2002). If SNMPs are not ORs, what are they?

SNMPs are homologous to a family of receptor proteins characterized by the human fatty acid transporter (FAT) CD36 (Oquendo *et al*., 1989; Greenwalt *et al*., 1992; Abumrad *et al*., 1993; see also Rogers *et al*., 2001a) (**Figure 7**). Among vertebrates, the CD36 family includes proteins known as scavenger receptors including LIMP II (Vega *et al*., 1991; Sandoval *et al*., 2000; Tabuchi *et al*., 2000), SRB1 (Acton *et al*., 1994, 1996; Krieger, 1999), and CLA 1 (Calvo and Vega, 1993). Members of this family have also been characterized in the insect *D. melanogaster* (Hart and Wilcox, 1993; Franc *et al*., 1999; Kiefer *et al*., 2002) and the slime mold *Dictyostelium discoideum* (Karakesisoglou *et al*., 1999; Janssen *et al*., 2001; Janssen and Schleicher, 2001).

The function of the vertebrate CD36 proteins is becoming clear. In mammals, these proteins are reported to be receptors for both high and low density lipoproteins (HDLs and LDLs), transporters of cholesterol and phospholipids, thrombospondin receptors, and cell recognition receptors for phago-

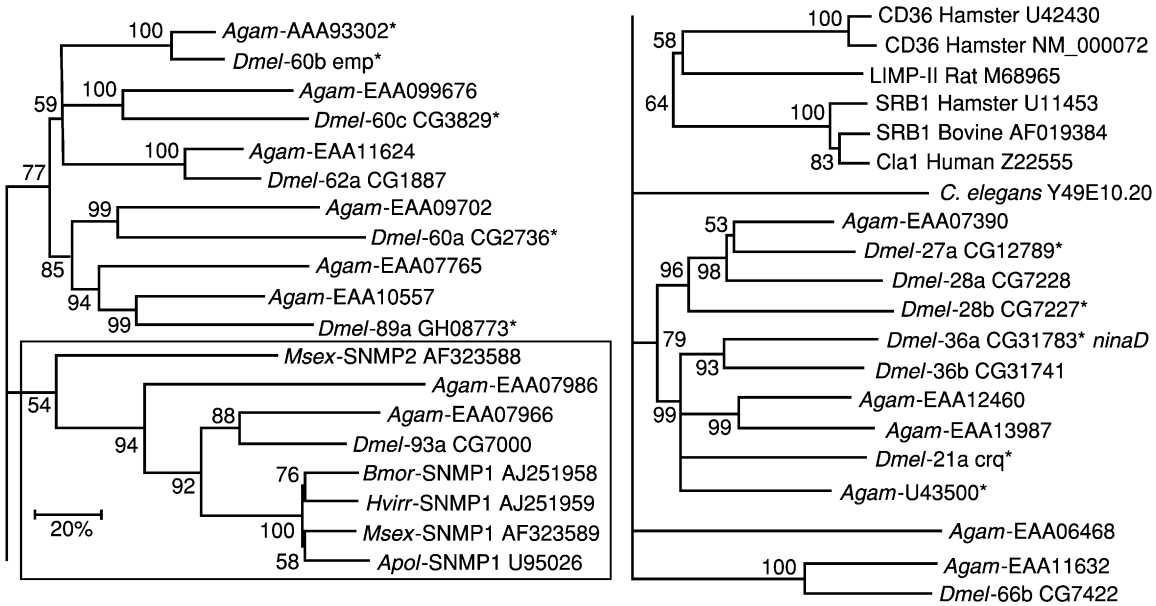


Figure 7 A neighbor-joining tree including lepidopteran SNMPs and their homolog in the *Drosophila melanogaster* and *Anopheles gambiae* genomes, as well as representative homolog from vertebrates (mammals) and a nematode (*Caenorhabditis elegans*). Box surrounds a similarity group that includes the olfactory specific lepidopteran SNMPs; *Dmel*-CG7000 is also known to be antennal specific (Vosshall, personal communication). Branches are collapsed to 50% bootstrap value or greater, based on 1000 replicates.

cytosis and recognition of *Plasmodium* (malaria) infected erythrocytes. Its role in malaria response was the first property ascribed to CD36 (Oquendo *et al.*, 1989); CD36 is expressed in endothelial cells of blood vessels and is responsible for cytoadherence of infected erythrocytes to these endothelial cells (Gamain *et al.*, 2002; Udomsangpetch *et al.*, 2002). But the role of these proteins most studied is their involvement in heart disease, and specifically the formation of vascular plaques in atherosclerosis. Plaque formation involves lipid/cholesterol uptake by macrophages; this uptake converts the macrophages to foam cells; both cell phenotypes secrete growth factors which stimulate the formation of plaques. CD36 and SRB1 are expressed in these macrophages and are responsible for the binding of HDLs and LDLs to the macrophage, as well as the transport of cholesterols (components of the HDLs and LDLs) both into and out of the macrophage, either by forming channels (Calvo *et al.*, 1998; Gu *et al.*, 1998; Coburn *et al.*, 2001; Reaven *et al.*, 2001; Frank *et al.*, 2002; Ibrahimi and Abumrad, 2002; Liu and Krieger, 2002; Miyazaki *et al.*, 2002; Moore *et al.*, 2002; Podrez *et al.*, 2002a, 2002b; Tsukamoto *et al.*, 2002; Kuniyasu *et al.*, 2002) or by endocytosis (e.g., Kuniyasu *et al.*, 2003). Similar activities of these proteins are now being examined in other contexts, including the mammalian nervous system with possible involvement in the mediation of microglial and macrophage responses to β-amyloid, playing roles in proinflammatory events associated with Alzheimer's disease (e.g., Panzenboeck *et al.*, 2002; El Khoury *et al.*, 2003; Srivastava, 2003). So, members of the vertebrate CD36 family can bind protein–lipid complexes and transport lipids across the cell membrane. These proteins have other less studied functions and activities as well. CD36 is involved in cytoadhesion and phagocytosis of rod outer segments in the eye (Ryeom *et al.*, 1996). LIMP II has some as yet unknown function in lysosomes (Kuronita *et al.*, 2002). SRB1 has recently been implicated as a receptor for hepatitis C virus (Scarselli *et al.*, 2002).

About 14 SNMP/CD36 homologs are present in the genomes of both *Drosophila melanogaster* and *A. gambiae* (Figure 7). Three of the *D. melanogaster* proteins have been characterized: emp (Hart and Wilcox, 1993), Croquemort (Franc *et al.*, 1996, 1999; Lee and Baehrecke, 2001), and the product of *ninaD* (CG31783) (Kiefer *et al.*, 2002). Emp (epithelial membrane protein) expresses in ectodermally derived tissues in the embryo and larva, including epithelial cells of wing imaginal discs; the authors suggested emp might have a role in development, but little more is known about this protein (Hart and Wilcox, 1993). Croquemort (Crq, catcher of death) expresses in circulating macrophages which engulf apoptotic cells by phagocytosis during

nonautophagic cell death; Crq serves, perhaps along with other receptors, to mediate this process (Franc *et al*., 1996, 1999; Lee and Baehrecke, 2001). Although the precise role of Crq in this process is not known, the similarity between this activity and the phagocytosis activity of CD36 in mammals suggests that functions of this gene family are conserved between these two distant phyla (Ryeom *et al*., 1996; Franc *et al*., 1999). *ninaD* is a blind mutant that is deficient in the dietary uptake of the visual pigment precursor carotenoids; the *ninaD* mutation is in the gene CG31783, a CD36 homolog; *P*-element mediated transformation with wild-type CG31783 rescues *ninaD* mutants (Kiefer *et al*., 2002). NinaD expression was detected in embryonic midgut primordia, in mesodermal tissue giving rise to hemocytes and macrophage, and in hemocytes. The authors suggest that NinaD functions in the uptake of carotenoids, in a manner similar to the uptake of cholesterol and phospholipids by mammalian CD36 and SRB1 proteins (Frank *et al*., 2002; Liu and Krieger, 2002).

The lepidopteran SNMPs are the only CD36 homologs clearly identified so far in neurons. Given what is known about the anatomy and physiology of olfactory sensilla (Steinbrecht, 1999), it is difficult to translate the specific described activities of the mammalian and dipteran members of the CD36 gene family to olfactory function. However, in a general sense, members of this gene family function as receptors in cell–cell interaction, as receptors that bind protein–lipid complexes, and as transporters of small hydrophobic molecules such as cholesterol and probably carotenoids. Translating these activities into olfactory activities to formulate hypotheses of SNMP function is feasible (**Figure 8**). One *Drosophila* and two *A. gambiae* proteins share notable sequence similarity with the lepidopteran SNMPs (**Figure 6**), and it may prove that studies of these dipteran proteins, and especially CG7000 of *Drosophila*, may prove fruitful in elucidating SNMP function.

3.15.4.5. Perireceptor Models

Figure 8 illustrates several versions of the perireception scheme presented earlier (**Figure 2**) and redrawn in **Figure 8a**. In these schemes, OBPs transport odors to receptors and ODEs degrade odors where and when they have the opportunity. A major concern regarding OBP function is the

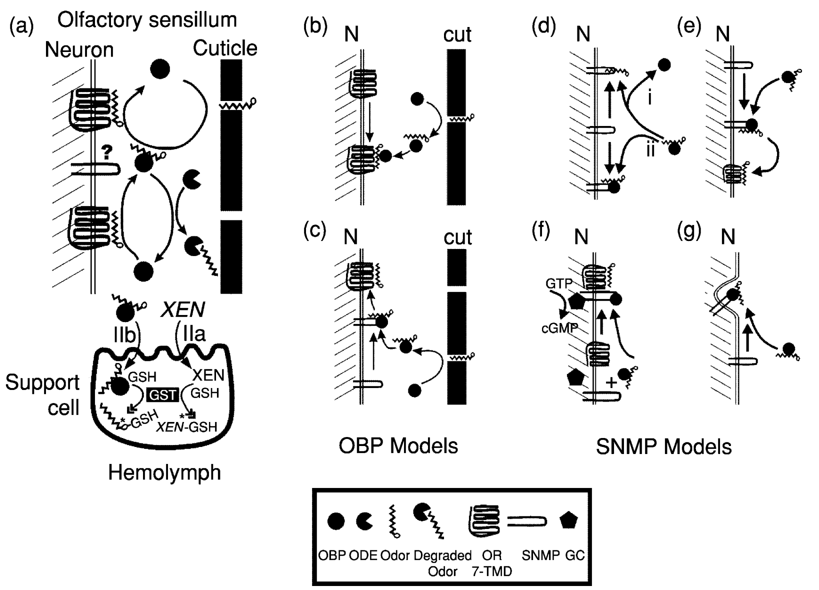


Figure 8 Models of ODE, OBP, and SNMP activities. (a) Depicts the general model for OBP and ODE activities proposed by Vogt *et al.* (1985); support cell activity is based on Rogers *et al.* (1999). Odor molecules are transported to ORs by OBPs and degraded by ODEs; odor molecules entering support cells are inactivated by dual function enzymes such as GSTs which also attack xenobiotics. (b) and (c) suggest alternative schemes for OBP activity (from Vogt *et al.*, 1999), where the ORs are stimulated by a stable odor–OBP complex (b), or by free odor after the odor–OBP complex is destabilized by some other process such as interaction with SNMP (c) or cell membrane (Wojtasek and Leal, 1999a). (d–g) Several possible functions for SNMP (from Rogers *et al.*, 2001a). SNMP may act as a novel odor receptor for either free odor (d-i) or odor–OBP complex (d-ii); or may destabilize the odor–OBP complex freeing odor to interact directly with ORs (e). Alternatively, SNMP may complex with ORs or cytosolic proteins to contribute in some manner to odor reception (f) or serve as an internalization process bringing odor or odor–OBP complexes into the cell (g). Further details on these models can be read in Rogers *et al.* (2001a, 2001b), and are discussed in the text.

reversibility of binding within a physiologically relevant period of time (10–500 ms); thus, receptor activation is represented as either a simple transfer of pheromone from OBP to OR (**Figure 8a**), involving the direct interaction of a odor–OBP complex (**Figure 8b**) or following destabilization of the odor–OBP complex mediated through interactions with the membrane or other membrane proteins such as SNMP or some OR partner such as Dor83b (**Figure 8c**). Some of these alternatives have been considered in a mathematical model developed by Kaissling (e.g., Kaissling, 2001).

Several schemes for SNMP action are shown in **Figure 8d–g**. **Figure 8d** considers the unlikely scheme that SNMP functions as an odor receptor, binding either (1) free odor or (2) the odor–OBP complex, while **Figure 8e** considers SNMP as a destabilizer of the odor–OBP complex. Perhaps more interesting is the scheme considered in **Figure 8f** where SNMP interacts with the OR or with intracelluar proteins (perhaps a guanylate cyclase, as described by Simpson *et al.* (1999)), or in some context similar to heterodimer receptor interactions observed in other systems (Vosshall and Keller, 2003). Finally, **Figure 8g** considers that SNMP might function to internalize odors or odor–OBP complexes, perhaps functioning as part of a signal termination pathway that removes odor accumulating near the membranes and thus maintains a concentration gradient for odors towards the neurons. This would be similar to the carotenoid transport function of NinaD in *Drosophila*, or the cholesterol transport function CD36 and SRB1 in mammals (Frank *et al.*, 2002; Kiefer *et al.*, 2002; Liu and Krieger, 2002; Kuniyasu *et al.*, 2003). Not shown is the possibility that SNMP1 and SNMP2 serve as phenotype determinants of the respective neurons, providing a communication link between the sensilla neurons (cell–cell interaction) that sustains the identity of the respective neurons. These schemes are described in greater detail in Rogers *et al.* (2001a).

3.15.5. Conclusions

3.15.5.1. Does the Detection of Pheromones Differ from the Detection of Other Odor Molecules?

For many years, detection of pheromones was viewed as a unique system, distinct from the detection of odor molecules. However, pheromones are odor molecules, and the detection of pheromone at the level of the sensillum (i.e., periphery) appears to be an adaptationally selected version of the detection of odors in general. Similarly, the central processing of pheromone and other odor signals has been argued to represent variations on a common theme (Christensen and Hildebrand, 2002). Nearly all of the elements illustrated in **Figure 2** have homologs identified in both pheromonal and nonpheromonal systems. OBPs have been identified in orthopteroid and holometabolous/hemipteran lineages suggesting they are present at least throughout the neopterous insects, though their rare encounter in orthopteroid insects suggests they may have expanded considerably in the holometabolous lineages. One lineage of OBP, the PBPs and GOBPs, appears to be specific to the Lepidoptera and represents by far the most abundant OBPs present in lepidopteran antennas despite evidence that there are many more OBP genes expressed in the antennas (Vogt *et al.*, 1991a; Robertson *et al.*, 1999). Pheromone-specific lineages of OBP genes may also exist in other species, as suggested by distinct PBP/OBP lineages in beetle (**Figure 3**). ODEs have also been identified associating with both pheromone and nonpheromone sensilla, though there is clearly an enhancement in these enzymes in pheromone sensilla, at least in the Lepidoptera. ORs so far identified are yet to be associated with pheromone ligands, but the transduction pathway illustrated in **Figure 2c** has been shown to support both pheromone and nonpheromonal transduction in insects spanning the neopteran lineages. Pheromone ORs may yet prove to be derived (distinct in sequence and structure) from other ORs, but they are strongly predicted to be GPCRs activating and to be activated by similar transduction and perireceptor pathways as the nonpheromone ORs that have been characterized in *Drosophila*. It therefore seems appropriate now to do away with the old distinction of specialist (ligand equals pheromone) and generalist (ligand equals plant volatile) olfactory receptors (i.e., sensilla and OSNs) and instead focus on the commonalities of these systems and how the various pathways have become selected to detect and pass on sensory information in the context of life history needs of the species.

3.15.5.2. Are OBPs Necessary for OR Activation?

The behavioral response to ethanol of the *Drosophila lush* mutant has been considered as a strong support for a critical role for OBPs in odor detection (Kim *et al.*, 1998; Kim and Smith, 2001). Assuming that Lush functions to transport ethanol to ORs, these experiments suggest OBPs are important in odor detection, but do not distinguish between (1) transport and (2) OBP–OR interaction. Certain-

ly, odor detection will be influenced by the effectiveness of any system delivering odor molecules to the ORs, and the diversity and selectivity of OBPs seems to suit them to this role. However, several studies have suggested, or at least considered the possibility, that the odor–OBP complex functions as an intact unit in stimulating ORs (Prestwich *et al.*, 1995; Ziegelberger, 1995; Plettner *et al.*, 2000; Kowcun *et al.*, 2001). Odor–OBP–OR interaction implies a high degree of stability in the odor–OBP complex, a view supported by several recent studies (Wojtasek and Leal, 1999a; Damberger *et al.*, 2000; Sandler *et al.*, 2000; Leal, 2003). Lazar *et al.* (2002) have gone so far as to suggest that such stability of the odor–OBP complex argues that OBPs are not deliverers of ligand (i.e., transporters), but rather are sequesters of ligand (inactivators), although this conjecture was based on a rather extreme extrapolation from elephant OBPs to moth OBPs, systems which share no homology in gene or species (beyond a divergence in their phylogenetic lineages perhaps at least 1 billion years ago) (Fortey *et al.*, 1997; Erwin and Davidson, 2002).

Several studies suggest OBPs may not be directly required for OR activation. In one study, olfactory neurons grown in primary culture responded to pheromone in the absence of applied PBP (Stengl *et al.*, 1992). These cultures were established by dissociating cells of an early developmental stage of adult male antennas of the moth *M. sexta*. The authors questioned the necessity of PBPs in pheromone transduction, but also observed that PBP secreting cells may have been present and that PBP thus may have been present in the culture. However, under somewhat analogous conditions, *Drosophila* OR Dor43a was expressed in frog oocytes and stimulated with applied odors (Wetzel *et al.*, 2001). These experiments were done without any applied OBP, and in the absence of any cells that could express an OBP, suggesting that the odor–OR interaction did not require a specific interaction with OBP. In a third study, the *Drosophila* OR Dor43a was misexpressed in olfactory sensilla lacking their endogenous ORs Dor22a/Dor22b (Dobritsa *et al.*, 2003). Neurons of Dor22a/Dor22b sensilla responded to Dor47a odor molecules, suggesting that the endogenous Dor22a/Dor22b OBP(s) was capable of transporting the Dor47a ligand. If Dor22a/Dor22b and Dor47a sensilla express different OBPs, which is not currently known, then presumably if the Dor22a/Dor22b OBP was transporting the Dor47a ligand, it was doing so without a high degree of specificity. Also, if OBP–OR interactions do occur, this experiment suggests they do not occur with a high degree of specificity. However, as there are as yet no correlations between the coexpression of ORs and OBPs, it remains possible that the Dor22a/Dor22b and Dor47a OBPs are the same, and that these assumptions are not supported.

3.15.5.3. Regulation of Pheromone Detection by Hormones, and the Regulation of Hormones by Pheromones

This subject falls into two broad areas: the hormonal regulation of olfactory development and gene expression, and the hormonal regulation of olfactory activity. Neither area has been heavily explored in insect olfactory systems, but sufficient work has been carried out to suggest there is much here to be studied. One can expect that development of the embryonic/larval olfactory system, modifications to the olfactory systems of hemimetabolous insects in the ultimate molt from juvenile to feeding adult, and metamorphosis of the olfactory systems of holometabolous insects offer rich landscapes to explore in an otherwise well-established background of endocrine studies (Truman and Riddiford, 2002). It is known that expression of the moth *M. sexta* PBP1, GOBP1, and GOBP2 are induced by declining levels of ecdysteroids late in adult development (Vogt *et al.*, 1993) and that *Msex*GOBP2 expression is turned off and on again during a larval molt as if it were being regulated by ecdysteroids (Vogt *et al.*, 2002). And it is known that several other olfactory genes including *MsexAOX*, *MsexGSTolf*, and *MsexSNMP1* turn on coincident with the *M. sexta* OBPs, suggesting they too may be under ecdysteroid regulation (Rybczynski *et al.*, 1990; Rogers *et al.*, 1999, 2001b). We also know that eversion of the *M. sexta* antennal imaginal disc during the fifth larval instar is ecdysteroid sensitive (Fernandez and Vogt, unpublished data) as is the proliferation of antennal sensory cells shortly after pupation and early during adult development (M.D. Franco and R.G. Vogt, unpublished data). These studies verify that the ecdysteroid (and juvenile hormone) regulation of olfactory development offer significant research opportunities.

The hormonal regulation of olfactory activity is largely unexplored territory. Insects display strong circadian behavioral shifts; moths, for example, have long been known to display sex pheromone-related behaviors at night (Fabre, 1916; Rau and Rau, 1929) (see **Chapter 3.11**). While it is well established in Lepidoptera that pheromone release and pheromone associated behaviors are strongly regulated in a circadian fashion, there are no reports of circadian changes in the OSN response to sex pheromones. There is, however, a study in *Drosophila* demonstrating circadian changes in the antennal

response to several odor molecules, though the function for this is only speculated to relate to feeding ecology (Krishnan *et al.*, 1999; also discussed by Zwiebel, 2003). There is also recent data from *A. gambiae* about changes in the expression of a number of olfactory-related gene products following a blood meal (Justice *et al.*, 2003). While these responses may be under local control, they may also be subject to endocrine pathways. Interestingly, the antenna, like most insect appendages, has a pulsatile organ at its base to move hemolymph through the antenna (Pass, 2000). In some insects at least, this also serves as neurohemal organ; hormones released from neurosecretory cells terminating in this structure are sent directly to the antenna, presumably to act in some as yet unknown fashion on antennal cells (Beattie, 1976; Pass *et al.*, 1988a, 1988b; Pass, 2000). Hormones can certainly come from other sources in the insect, but this proximity of neurosecretory cells relative to the antenna seems a strong, if indirect, suggestion that the olfactory activities are under hormonal regulation.

Pheromones have been categorized as releasers, stimulating or modulating an immediate behavioral response, and as primers, stimulating or modulating a longer-lasting physiological state (Wyatt, 2003). The best-characterized releaser pheromones may be the attractant sex pheromones of Lepidoptera which modulate visually guided flight of male moths (Kennedy *et al.*, 1980; David *et al.*, 1983; Preiss and Kramer, 1983; Willis and Arbas, 1991; Vickers *et al.*, 2001; Baker, 2002; review: Schneider, 1992). Primer pheromones regulate behavioral and developmental states in social insects (Vander Meer *et al.*, 1998) *Leptinotarsa decemlineata* (see **Chapter 3.13**). Pheromones may serve both categories, such as a male pheromone of the Colorado potato beetle, which serves as a female attractant (releaser), but may also modulate male pheromone production through an endocrine pathway (primer) (Dickens *et al.*, 2002). Activities of releaser pheromones are presumably mediated through the interaction of pheromone-sensitive neural pathways with other sensory pathways to modulate motor output (Olberg, 1983; Kanzaki and Shibuya, 1986; Olberg and Willis, 1990, Kanzaki *et al.*, 1991; Vickers and Baker, 1994; Kanzaki and Mishima, 1996; Kanzaki, 1998; Mishima and Kanzaki, 1998, 1999; Lei *et al.*, 2001).

Activities of primer pheromones are presumably mediated through the endocrine system. Unfortunately, there is currently no established neural, molecular, or genetic pathway between specific olfactory sensilla/OSNs/pheromones and any endocrine pathway. Nevertheless, this is a relatively easy pathway upon which to speculate. Neuroendocrine cells are distributed throughout the insect brain (Nässel, 2002), and projection neurons from the olfactory lobes arborize in regions rich in these cells (**Figure 1**); certainly the opportunity for olfactory-based signaling to influence the release of neurosecretory hormones is considerable. Given the state of genetically driven cell markers (Wong *et al.*, 2002), all that may be lacking is a well-established pheromone responsive OR and the knowledge of the specific hormone(s) that pheromone simulates in order to establish such a neural pathway.

New technologies drive the acquisition of new knowledge. Publicly accessible computer databases, N-terminal sequencing, cDNA cloning, PCR, and structural and spectroscopic biochemistry have generated much of the information presented here. The last few years have seen *Drosophila* emerge as a major tool in olfactory research, both because of its manipulatable genetics and its recently sequenced genome. The coming years will no doubt see these technologies spread to other insect models and the applications of profound new technologies to present models (Kalidas and Smith, 2002; Mori, 2002; Tomita *et al.*, 2003). Changing technologies not only provide changes in research approaches, but also change that laboratories and which intellectual assets are serving as the central players of the moment. But for all this, insect olfaction is an inherently biological phenomenon. The vast number of insect species with their divergent families of olfactory genes that serve to translate key environmental information supporting individual life histories and behavior makes insect olfaction a unique system in which to study the organization, actions, and evolution of genes and genetic pathways in an ecological context. At the conferences, it is the rule rather than the exception to hear cutting edge presentations on gene and protein structure and properties, on gene regulation, expression, and function, on olfactory neural circuitry, coding and behavior, on the most superb neural anatomy, on development at all levels, on the chemistry of pheromone production and the evolution of pheromone production and detection, on ecology of odor detection, on field application of pheromones and crop protection using pheromones, and recently, on remote sensing and military defense. Perhaps more than any other area of biology, insect olfaction is a system offering and generating enormous intellectual integration. For this aging scientist, studying insect olfaction has been highly stimulating, and I can only envy those younger scientists now embarking in this field.

References

Abumrad, N.A., El-Maghrabi, M.R., Amri, E., Lopez, E., Grimaldi, P., **1993**. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. *J. Biol. Chem. 268*, 17665–17668.

Achterberg, X.Y., CSIRO Staff, **1991**. The Insects of Australia: A Textbook for Students and Research Workers. Melbourne University Press, Melbourne.

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H., *et al.*, **1996**. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science 271*, 518–520.

Acton, S.L., Scherer, P.E., Lodish, H.F., Krieger, M., **1994**. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J. Biol. Chem. 269*, 21003–21009.

Adam, G., Delbrück, M., **1968**. Reduction of dimensionality in biological diffusion processes. In: Rich, A., Davidson, R. (Eds.), Structural Chemistry and Molecular Biology. Freeman W.H., San Francisco, CA, pp. 198–215.

Akgoz, M., Azpiazu, I., Kalyanaraman, V., Gautam, N., **2002**. Role of the G protein gamma subunit in beta gamma complex modulation of phospholipase C beta function. *J. Biol. Chem. 277*, 19573–19578.

Angeli, S., Ceron, F., Scaloni, A., Monti, M., Monteforti, G., *et al.*, **1999**. Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria*. *Eur. J. Biochem. 262*, 745–754.

Anton, S., Hansson, B.S., **1995**. Sex pheromone and plant-associated odour processing in antennal lobe interneurons of male *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A 176*, 773–789.

Bakalyar, H.A., Reed, R.R., **1990**. Identification of a specialized adenylyl cyclase that may mediate odorant detection. *Science 250*, 1403–1406.

Bakalyar, H.A., Reed, R.R, **1991**. The second messenger cascade in olfactory receptor neurons. *Curr. Opin. Neurobiol. 1*, 204–208.

Baker, T.C., **2002**. Mechanism for saltational shifts in pheromone communication systems. *Proc. Natl Acad. Sci. USA 99*, 13368–13370.

Ban, L., Scaloni, A., Brandazza, A., Angeli, S., Zhang, L., *et al.*, **2003a**. Chemosensory proteins of *Locusta migratoria*. *Insect Mol. Biol. 12*, 125–134.

Ban, L., Scaloni, A., D'Ambrosio, C., Zhang, L., Yan, Y., *et al.*, **2003b**. Biochemical characterization and bacterial expression of an odorant-binding protein from *Locusta migratoria*. *Cell Mol. Life Sci. 60*, 390–400.

Ban, L., Zhang, L., Yan, Y., Pelosi, P., **2002**. Binding properties of a locust's chemosensory protein. *Biochem. Biophys. Res. Commun. 293*, 50–54.

Bargmann, C.I., Mori, I., **1997**. Chemotaxis and thermotaxis. In: Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, J.R. (Eds.), *C. elegans II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 717–737.

Bartelt, R.J., Schaner, A.M., Jackson, L.L., **1985**. *Cis*-vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol. 11*, 1747–1756.

Bau, J., Martinez, D., Renou, M., Guerrero, A., **1999**. Pheromone-triggered orientation flight of male moths can be disrupted by trifluoromethyl ketones. *Chem. Senses 24*, 473–480.

Baumann, A., Frings, S., Godde, M., Seifert, R., Kaupp, U.B., **1994**. Primary structure and functional expression of a *Drosophila* cyclic nucleotide-gated channel present in eyes and antennae. *EMBO J. 13*, 5040–5050.

Beattie, T.M., **1976**. Autolysis in axon terminals of a new neurohaemal organ in the cockroach *Periplaneta americana*. *Tissue Cell 8*, 305–310.

Ben-Arie, N., Khen, M., Lancet, D., **1993**. Glutathione-*S*-transferase in rat olfactory epithelium: purification, molecular properties and odorant biotransformation. *Biochem. J. 292*, 379–384.

Bette, S., Breer, H., Krieger, J., **2002**. Probing a pheromone binding protein of the silkmoth *Antheraea polyphemus* by endogenous tryptophan fluorescence. *Insect Biochem. Mol. Biol. 32*, 241–246.

Bhalerao, S., Sen, A., Stocker, R., Rodrigues, V., **2003**. Olfactory neurons expressing identified receptor genes project to subsets of glomeruli within the antennal lobe of *Drosophila melanogaster*. *J. Neurobiol. 54*, 577–592.

Biessmann, H., Walter, M.F., Dimitratos, S., Woods, D., **2002**. Isolation of cDNA clones encoding putative odorant binding proteins from the antennae of the malaria-transmitting mosquito, *Anopheles gambiae*. *Insect Mol. Biol. 11*, 123–132.

Birch, M.C., **1974**. Pheromones. North-Holland, Amsterdam.

Blomquist, G.J., **2003**. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 231–252.

Blomquist, G.J., Tillman, J.A., Mpuru, S., Seybold, S.J., **1998**. The cuticle and cuticular hydrocarbons of insects: structure, function and biochemistry. In: Vander Meer, R.K., Breed, M.D., Winston, M.L., Espelie, K.E. (Eds.), Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites. Westview Press, Boulder, CO, pp. 34–54.

Boeckh, J., Kaissling, K.-E., Schneider, D., **1965**. Insect Olfactory Receptors. *Cold Spring Harbor Symposia on Quantitative Biology 30*, 263–280.

Boekhoff, I., Breer, H., **1992**. Termination of second messenger signaling in olfaction. *Proc. Natl Acad. Sci. USA 89*, 471–474.

Boekhoff, I., Michel, W.C., Breer, H., Ache, B.W., **1994**. Single odors differentially stimulate dual second messenger pathways in lobster olfactory receptor cells. *J. Neurosci. 14*, 3304–3309.

Boekhoff, I., Raming, K., Breer, H., **1990b**. Pheromone-induced stimulation of inositol-trisphosphate formation

in insect antennae is mediated by G-proteins. *J. Comp. Physiol. B 160*, 99–103.

Boekhoff, I., Seifert, E., Goggerle, S., Lindemann, M., Kruger, B.W., *et al.*, **1993**. Pheromone-induced second-messenger signaling in insect antennae. *Insect Biochem. Mol. Biol. 23*, 757–762.

Boekhoff, I., Strotmann, J., Raming, K., Tareilus, E., Breer, H., **1990a**. Odorant-sensitive phospholipase C in insect antennae. *Cell Signal 2*, 49–56.

Bohbot, J., Sobrio, F., Lucas, P., Nagnan-Le Meillour, P., **1998**. Functional characterization of a new class of odorant-binding proteins in the moth *Mamestra brassicae*. *Biochem. Biophys. Res. Commun. 253*, 489–494.

Boudreaux, H.B., **1979**. Arthropod Phylogeny with Special Reference to Insects. Wiley, New York.

Bray, S., Amrein, H., **2003**. A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. *Neuron 39*, 1019–1029.

Breer, H., Krieger, J., Raming, K., **1990**. A novel class of binding proteins in the antennae of the silkmoth *Antheraea pernyi*. *Insect Biochem. 20*, 735–740.

Breer, H., Raming, K., Boekhoff, I., **1988**. G-proteins in the antennae of insects. *Naturwissenschaft 75*, 627.

Briand, L., Nespoulous, C., Huet, J.C., Pernollet, J.C., **2001a**. Disulfide pairing and secondary structure of ASP1, an olfactory-binding protein from honeybee (*Apis mellifera* L). *J. Pept. Res. 58*, 540–545.

Briand, L., Nespoulous, C., Huet, J.C., Takahashi, M., Pernollet, J.C., **2001b**. Ligand binding and physico-chemical properties of ASP2, a recombinant odorant-binding protein from honeybee (*Apis mellifera* L.). *Eur. J. Biochem. 268*, 752–760.

Briand, L., Swasdipan, N., Nespoulous, C., Bezirard, V., Blon, F., *et al.*, **2002**. Characterization of a chemosensory protein (ASP3c) from honeybee (*Apis mellifera*, L.) as a brood pheromone carrier. *Eur. J. Biochem. 269*, 4586–4596.

Buck, L., Axel, R., **1991**. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell 65*, 175–187.

Burchell, B., **1991**. Turning on and turning off the sense of smell. *Nature 350*, 16–17.

Butenandt, A., Beckmann, R., Stamm, D., Hecker, E., **1959**. Über den Sexual-Lockstoff des Seidenspinners *Bombyx mori*: Reindanstellung und Konstitution. *Z. Naturforsch. 14b*, 283–284.

Callahan, F.E., Vogt, R.G., Tucker, M.L., Dickens, J.C., Mattoo, A.K., **2000**. High level expression of 'male specific' pheromone binding proteins (PBPs) in the antennae of female noctuid moths. *Insect Biochem. Mol. Biol. 30*, 507–514.

Calvo, D., Gómez-Coronado, D., Suárez, Y., Lasunción, M.A., Vega, M.A., **1998**. Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. *J. Lipid Res. 39*, 777–788.

Calvo, D., Vega, M.A., **1993**. Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. *J. Biol. Chem. 268*, 18929–18935.

Campanacci, V., Krieger, J., Bette, S., Sturgis, J.N., Lartigue, A., *et al.*, **2001a**. Revisiting the specificity of *Mamestra brassicae* and *Antheraea polyphemus* pheromone-binding proteins with a fluorescence binding assay. *J. Biol. Chem. 276*, 20078–20084.

Campanacci, V., Lartigue, A., Hallberg, B.M., Jones, T.A., Giudici-Orticoni, M.T., *et al.*, **2003**. Moth chemosensory protein exhibits drastic conformational changes and cooperativity on ligand binding. *Proc. Natl Acad. Sci. USA 100*, 5069–5074.

Campanacci, V., Mosbah, A., Bornet, O., Wechselberger, R., Jacquin-Joly, E., *et al.*, **2001b**. Chemosensory protein from the moth *Mamestra brassicae*: expression and secondary structure from 1H and 15N NMR. *Eur. J. Biochem. 268*, 4731–4739.

Campanacci, V., Spinelli, S., Lartigue, A., Lewandowski, C., Brown, K., *et al.*, **2001c**. Recombinant chemosensory protein (CSP2) from the moth *Mamestra brassicae*: crystallization and preliminary crystallographic study. *Acta Crystallogr. D Biol. Crystallogr. 57*, 137–139.

Cao, Y., Oh, B.C., Stryer, L., **1998**. Cloning and localization of two multigene receptor families in goldfish olfactory epithelium. *Proc. Natl Acad. Sci. USA 95*, 11987–11992.

Carlsson, M.A., Galizia, C.G., Hansson, B.S., **2002**. Spatial representation of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Chem. Senses 27*, 231–244.

Carlsson, M.A., Hansson, B.S., **2003a**. Dose-response characteristics of glomerular activity in the moth antennal lobe. *Chem. Senses 28*, 269–278.

Carlsson, M.A., Hansson, B.S., **2003b**. Plasticity and coding mechanisms in the insect antennal lobe. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 699–728.

Carr, W.E., Gleeson, R.A., Trapido-Rosenthal, H.G., **1990**. The role of perireceptor events in chemosensory processes. *Trends Neurosci. 13*, 212–215.

Chess, A., Simon, I., Cedar, H., Axel, R., **1994**. Allelic inactivation regulates olfactory receptor gene expression. *Cell 78*, 823–834.

Christensen, T.A., Hildebrand, J.G., **2002**. Pheromonal and host-odor processing in the insect antennal lobe: how different? *Curr. Opin. Neurobiol. 12*, 393–399.

Clapham, D.E., Neer, E.J., **1997**. G protein beta gamma subunits. *Annu. Rev. Pharmacol. Toxicol. 37*, 167–203.

Clark, A.G., **1990**. The glutathione *S*-transferases and resistance to insecticides. In: Hayes, J.D., Picket, C.B., Mantle, T.J. (Eds.), Glutathione *S*-Transferases and Drug Resistance. Taylor and Francis, London, pp. 369–378.

Clement, J.-L., Bagneres, A.-G., **1998**. Nestmate recognition in termites. In: Vander Meer, R.K., Breed, M.D., Winston, M.L., Espelie, K.E. (Eds.), Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites. Westview Press, Boulder, CO, pp. 126–155.

Clyne, P.J., Grant, A., O'Connell, R., Carlson, J.R., **1997**. Odorant response of individual sensilla on the *Drosophila* antenna. *Invert. Neurosci. 3*, 127–135.

Clyne, P.J., Warr, C.G., Carlson, J.R., **2000**. Candidate taste receptors in *Drosophila*. *Science 287*, 1830–1834.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., *et al*., **1999**. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron 22*, 327–338.

Coburn, C.T., Hajri, T., Ibrahimi, A., Abumrad, N.A., **2001**. Role of CD36 in membrane transport and utilization of long-chain fatty acids by different tissues. *J. Mol. Neurosci. 16*, 117–121; discussion 151–157.

Dahanukar, A., Foster, K., van der Goes van Naters, W.M., Carlson, J.R., **2001**. A Gr receptor is required for response to the sugar trehalose in taste neurons of *Drosophila*. *Nature Neurosci. 4*, 1182–1186.

Dahl, A.R., **1988**. The effect of cytochrome P-450-dependent metabolism and other enzyme activities on olfaction. In: Margolis, F., Getchel, T. (Eds.), Molecular Neurobiology of the Olfactory System. Plenum, New York, pp. 51–70.

Damberger, F., Nikonova, L., Horst, R., Peng, G., Leal, W.S., *et al*., **2000**. NMR characterization of a pH-dependent equilibrium between two folded solution conformations of the pheromone-binding protein from *Bombyx mori*. *Protein Sci. 9*, 1038–1041.

Danty, E., Arnold, G., Huet, J.-C., Huet, D., Masson, C., *et al*., **1998**. Separation, characterization and sexual heterogeneity of multiple putative odorant-binding proteins in the honeybee *Apis mellifera* L. (Hymenoptera: Apidea). *Chem. Senses 23*, 83–91.

Danty, E., Briand, L., Michard-Vanhee, C., Perez, V., Arnold, G., *et al*., **1999**. Cloning and expression of a queen pheromone-binding protein in the honeybee: an olfactory-specific, developmentally regulated protein. *J. Neurosci. 19*, 7468–7475.

David, C.T., Kennedy, J.S., Ludlow, A.R., **1983**. Finding of a sex pheromone source by gypsy moths released in the field. *Nature 303*, 804–806.

de Bruyne, M., **2003**. Physiology and genetics of odor perception in *Drosophila*. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 651–697.

de Bruyne, M., Guerin, P.M., **1998**. Contact chemostimuli in the mating behaviour of the cattle tick, *Boophilus microplus*. *Arch. Insect Biochem. Physiol. 39*, 65–80.

Descoins, C., Marion-Poll, F., **1999**. Electrophysiological responses of gustatory sensilla of *Mamestra brassicae* (Lepidoptera, Noctuidae) larvae to three ecdysteroids: ecdysone, 20-hydroxyecdysone and ponasterone A. *J. Insect Physiol. 45*, 871–876.

Dhallan, R.S., Yau, K.W., Schrader, K.A., Reed, R.R., **1990**. Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature 347*, 184–187.

Dickens, J.C., Oliver, J.E., Hollister, B., Davis, J.C., Klun, J.A., **2002**. Breaking a paradigm: male-produced aggregation pheromone for the Colorado potato beetle. *J. Exp. Biol. 205*, 1925–1933.

Ding, X., Porter, T.D., Peng, H-M., Coon, M.J., **1991**. cDNA and derived amino acid sequence of a rabbit nasal cytochrome p450NMB (p450IIG1), a unique isozyme possibly involved in olfaction. *Arch. Biochem. Biophys. 285*, 120–125.

Dobritsa, A.A., van der Goes van Naters, W., Warr, C.G., Steinbrecht, R.A., Carlson, J.R., **2003**. Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron 37*, 827–841.

Dolzer, J., Fischer, K., Stengl, M., **2003**. Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. *J. Exp. Biol. 206*, 1575–1588.

Drosophila Odorant Receptor Nomenclature Committee, **2000**. A unified nomenclature system for the *Drosophila* odorant receptors. *Cell 102*, 145–146.

Du, G., Ng, C.S., Prestwich, G.D., **1994**. Odorant binding by a pheromone binding protein: active site mapping by photoaffinity labeling. *Biochemistry 33*, 4812–4819.

Du, G., Prestwich, G.D., **1995**. Protein structure encodes the ligand binding specificity in pheromone binding proteins. *Biochemistry 34*, 8726–8732.

Dulac, C., **2000**. Sensory coding of pheromone signals in mammals. *Curr. Opin. Neurobiol. 10*, 511–518.

Dulac, C., Axel, R., **1995**. A novel family of genes encoding putative pheromone receptors in mammals. *Cell 83*, 195–206.

Dunipace, L., Meister, S., McNealy, C., Amrein, H., **2001**. Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system. *Curr. Biol. 11*, 822–835.

Dyanov, H.M., Dzitoeva, S.G., **1995**. Method for attachment of microscopic preparations on glass for *in situ* hybridization, PRINS and *in situ* PCR studies. *Biotechniques 18*, 822–824.

Eichmann, T., Lorenz, K., Hoffmann, M., Brockmann, J., Krasel, C., *et al*., **2003**. The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory beta gamma binding site. *J. Biol. Chem. 278*, 8052–8057.

El Khoury, J.B., Moore, K.J., Means, T.K., Leung, J., Terada, K., *et al*., **2003**. CD36 mediates the innate host response to beta-amyloid. *J. Exp. Med. 197*, 1657–1666.

Elmore, T., Smith, D.P., **2001**. Putative *Drosophila* odor receptor OR43b localizes to dendrites of olfactory neurons. *Insect Biochem. Mol. Biol. 31*, 791–798.

Ernst, K.-D., **1969**. Die Feinstruktor bon Reichsensillen auf der Antenne des Aakafers *Necrophorus* (Coleoptera). *Z. Zellforsch. 94*, 72–102.

Erwin, D.G., Davidson, E.H., **2002**. The last common bilaterian ancestor. *Development 129*, 3021–3032.

Erwin, T.L., **1982**. Tropical forests: their richness in coleoptera and other arthropod species. *Coleopt. Bull. 36*, 74–75.

Fabre, J.H., **1916**. XI: the great peacock. In: The Life of the Caterpillar. Dodd, Mead and Co., New York.

Fadool, D.A., Ache, B.W., **1992**. Plasma membrane inositol 1,4,5-trisphosphate activated channels mediate signal transduction in lobster olfactory receptor neurons. *Neuron 9*, 907–918.

Feng, L., Prestwich, G.D., **1997**. Expression and characterization of a lepidopteran general odorant binding protein. *Insect Biochem. Mol. Biol. 27*, 405–412.

Ferguson, S.S., Downey, W.E., III, Colapietro, A.M., Barak, L.S., Menard, L., *et al.*, **1996**. Role of beta-arrestin in mediating agonist-promoted G-protein-coupled receptor internalization. *Science 271*, 363–366.

Ferkovich, S.M., Mayer, M.S., Rutter, R.R., **1973a**. Conversion of the sex pheromone of the cabbage looper. *Nature 242*, 53–55.

Ferkovich, S.M., Mayer, M.S., Rutter, R.R., **1973b**. Sex pheromone of the cabbage looper: reactions with antennal proteins *in vitro*. *J. Insect Physiol. 19*, 2231–2243.

Ferkovich, S.M., Oliver, J.E., Dillard, C., **1982**. Pheromone hydrolysis by cuticular and interior esterases of the antennae, legs, and wings of the cabbage looper moth, *Trichoplusia ni* (Hubner). *J. Chem. Ecol. 8*, 859–866.

Ferkovich, S.M., Van Essen, F., Taylor, T.R., **1980**. Hydrolysis of sex pheromone by antennal esterases of the cabbage looper, *Trichoplusia ni*. *Chem. Senses Flavour 5*, 33–45.

Ferveur, J.F., **1997**. The pheromonal role of cuticular hydrocarbons in *Drosophila melanogaster*. *BioEssays 19*, 353–358.

Ferveur, J.F., Cobb, M., Boukella, H., Jallon, J.M., **1996**. World-wide vartiation in *Drosophila melanogaster* sex pheromone: behavioral effects, genetic bases and potential evolutionary consequences. *Genetica 97*, 73–80.

Ferveur, J.F., Savarit, F., O'Kane, C.J., Sureau, G., Greenspan, R.J., *et al.*, **1997**. Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males. *Science 276*, 1555–1558.

Feyereisen, R., **1999**. Insect P450 enzymes. *Annu. Rev. Entomol. 44*, 507–533.

Fortey, R.A., Briggs, D.E.G., Wills, M., **1997**. The Cambrian evolutionary 'explosion' recalibrated. *BioEssays 19*, 429–434.

Fournier, D., Bride, J.M., Poirie, M., Berge, J.B., Plapp, F.W., Jr., **1992**. Insect glutathione *S*-transferases: biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *J. Biol. Chem. 267*, 1840–1845.

Fox, A.N., Pitts, R.J., Robertson, H.M., Carlson, J.R., Zwiebel, L.J., **2001**. Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc. Natl Acad. Sci. USA 98*, 14693–14697.

Fox, A.N., Pitts, R.J., Zwiebel, L.J., **2002**. A cluster of candidate odorant receptors from the malaria vector mosquito, *Anopheles gambiae*. *Chem. Senses 27*, 453–459.

Franc, N.C., Dimarcq, J.-L., Lagueux, M., Hoffmann, J., Ezekowitz, R.A.B., **1996**. Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. *Immunity 4*, 431–443.

Franc, N.C., Heitzler, P., Ezekowitz, R.A., White, K., **1999**. Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*. *Science 284*, 1991–1994.

Frank, P.G., Marcel, Y.L., Connelly, M.A., Lublin, D.M., Franklin, V., *et al.*, **2002**. Stabilization of caveolin-1 by cellular cholesterol and scavenger receptor class B type I. *Biochemistry 41*, 11931–11940.

Freedman, N.J., Lefkowitz, R.J., **1996**. Desensitization of G-protein-coupled receptors. *Recent Progr. Hormone Res. 51*, 319–351.

Freitag, J., Ludwig, G., Andreini, I., Roessler, P., Breer, H., **1998**. Olfactory receptors in aquatic and terrestrial vertebrates. *J. Comp. Physiol. A 183*, 635–650.

Galindo, K., Smith, D.P., **2001**. A large family of divergent *Drosophila* odorant-binding proteins expressed in gustatory and olfactory sensilla. *Genetics 159*, 1059–1072.

Galizia, C.G., Menzel, R., **2001**. The role of glomeruli in the neural representation of odours: results from optical recording studies. *J. Insect Physiol. 47*, 115–130.

Galizia, C.G., Nägler, K., Hölldobler, B., Menzel, R., **1998**. Odour coding is bilaterally symmetrical in the antennal lobes of honeybees (*Apis mellifera*). *Eur. J. Neurosci. 10*, 2964–2974.

Galizia, G.G., Sachse, S., Mustaparta, H., **2000**. Calcium responses to pheromones and plant odours in the antennal lobe of the male and female moth *Heliothis virescens*. *J. Comp. Physiol. A 186*, 1049–1063.

Galizia, C.G., Sachse, S., Rappert, A., Menzel, R., **1999**. The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. *Nature Neurosci. 2*, 473–478.

Gamain, B., Gratepanche, S., Miller, L.H., Baruch, D.I., **2002**. Molecular basis for the dichotomy in *Plasmodium falciparum* adhesion to CD36 and chondroitin sulfate A. *Proc. Natl Acad. Sci. USA 99*, 10020–10024.

Gao, Q., Chess, A., **1999**. Identification of candidate *Drosophila* olfactory receptors from genomic DNA. *Genomics 60*, 31–39.

Gao, Q., Yuan, B., Chess, A., **2000**. Convergent projections of *Drosophila* olfactory neurons to specific glomeruli in the antennal lobe. *Nature Neurosci. 3*, 780–785.

Getchell, T.V., Margolis, F.L., Getchell, M.L., **1984**. Perireceptor and receptor events in vertebrate olfaction. *Progr. Neurobiol. 23*, 317–345.

Gilad, Y., Man, O., Paabo, S., Lancet, D., **2003**. Human specific loss of olfactory receptor genes. *Proc. Natl Acad. Sci. USA 100*, 3324–3327.

Gleeson, R.A., Trapido-Rosenthal, H.G., McDowell, L.M., Aldrich, H.C., Carr, W.E., **1992**. Ecto-ATPase/phosphatase activity in the olfactory sensilla of the spiny lobster, *Panulirus argus*: localization and characterization. *Cell Tissue Res. 269*, 439–445.

Graham, L.A., Davies, P.L., **2002**. The odorant-binding proteins of *Drosophila melanogaster*: annotation and

characterization of a divergent gene family. *Gene 292*, 43–55.

Graham, L.A., Tang, W., Baust, J.G., Liou, Y.C., Reid, T.S., *et al.*, **2001**. Characterization and cloning of a *Tenebrio molitor* hemolymph protein with sequence similarity to insect odorant-binding proteins. *Insect Biochem. Mol. Biol. 31*, 691–702.

Graham, S.M., Prestwich, G.D., **1992**. Tissue distribution and substrate specificity of an epoxide hydrase in the gypsy moth *Lymantria dispar. Experientia 48*, 19–21.

Graham, S.M., Prestwich, G.D., **1994**. Synthesis and inhibitory properties of pheromone analogs for the epoxide hydrolase of the gypsy moth. *J. Org. Chem. 59*, 2956–2966.

Greenspan, R.J., Ferveur, J.F., **2000**. Courtship in *Drosophila*. *Annu. Rev. Genet. 34*, 205–232.

Greenwalt, D.E., Lipsky, R.H., Ockenhouse, C.F., Ikeda, H., Tandon, N.N., *et al.*, **1992**. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood 80*, 1105–1115.

Grünert, U., Ache, B.W., **1988**. Ultrastructure of the aesthetase (olfactory) sensilla of the spiny lobster, *Panulirus argus*. *Cell Tissue Res. 251*, 95–103.

Gu, X.J., Trigatti, B., Xu, S.Z., Acton, S., Babitt, J., *et al.*, **1998**. The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. *J. Biol. Chem. 273*, 26338–26348.

Györgyi, T.K., Roby-Shemkovitz, A.J., Lerner, M.R., **1988**. Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: a tissue-specific developmentally regulated protein. *Proc. Natl Acad. Sci. USA 85*, 9851–9855.

Halpern, M., **1987**. The organization and function of the vomeronasal system. *Annu. Rev. Neurosci. 10*, 325–362.

Halpern, M., Jia, C., Shapiro, L.S., **1998**. Segregated pathways in the vomeronasal system. *Microsc. Res. Tech. 15*, 519–529.

Hanson, F.E., Dethier, V.G., **1973**. Role of gustation and olfaction in food plant discrimination in the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 19*, 1019–1034.

Hansson, B.S., **2002**. A bug's smell: research into insect olfaction. *Trends Neurosci. 25*, 270–274.

Hansson, B.S., Anton, S., Christensen, T.A., **1994**. Structure and function of antennal lobe interneurons in the male turnip moth, *Agrotis segetum* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A 175*, 547–562.

Hansson, B.S., Carlsson, M.A., Kalinovà, B., **2003**. Olfactory activation patterns in the antennal lobe of the sphinx moth, *Manduca sexta*. *J. Comp. Physiol. A 189*, 301–308.

Hansson, B.S., Christensen, T.A., Hildebrand, J.G., **1991**. Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta*. *J. Comp. Neurol. 312*, 264–278.

Hansson, B.S., Ljungberg, H., Hallberg, E., Löfstedt, C., **1992**. Functional specialization of olfactory glomeruli in a moth. *Science 256*, 547–562.

Hardie, R.C., **2003**. Regulation of TRP channels via lipid second messengers. *Annu. Rev. Physiol. 65*, 735–759.

Hart, K., Wilcox, M., **1993**. A *Drosophila* gene encoding an epithelial membrane protein with homology to CD36/LIMP II. *J. Mol. Biol. 234*, 249–253.

Hatt, H., Ache, B.W., **1994**. Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons. *Proc. Natl Acad. Sci. USA 91*, 6264–6268.

Hekmat-Scafe, D.S., Dorit, R.L., Carlson, J.R., **2000**. Molecular evolution of odorant-binding protein genes OS-E and OS-F in *Drosophila*. *Genetics 155*, 117–127.

Hekmat-Scafe, D.S., Scafe, C.R., McKinney, A.J., Tanouye, M.A., **2002**. Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster. Genome Res. 12*, 1357–1369.

Hekmat-Scafe, D., Steinbrecht, R.A., Carlson, J.R., **1997**. Coexpression of two odorant-binding protein homologs in *Drosophila*: implications for olfactory coding. *J. Neurosci. 17*, 1616–1624.

Hennig, W., **1981**. Insect Phylogeny. Wiley, New York.

Herrada, G., Dulac, C., **1997**. A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell 90*, 763–773.

Hildebrand, J.G., **1996**. Olfactory control of behavior in moth: central processing of odor information and functional significance of olfactory glomeruli. *J. Comp. Physiol. A 178*, 5–19.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, **2002**. G-protein-coupled receptors in *Anopheles gambiae*. *Science 298*, 176–178.

Homberg, U., Montague, R.A., Hildebrand, J.G., **1988**. Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta*. *Cell Tissue Res. 254*, 255–281.

Honson, N., Johnson, M.A., Oliver, J.E., Prestwich, G.D., Plettner, E., **2003**. Structure-activity studies with pheromone-binding proteins of the gypsy moth, *Lymantria dispar. Chem. Senses 28*, 479–489.

Horst, R., Damberger, F., Luginbuhl, P., Guntert, P., Peng, G., *et al.*, **2001**. NMR structure reveals intramolecular regulation mechanism for pheromone binding and release. *Proc. Natl Acad. Sci. USA 98*, 14374–14379.

Ibrahimi, A., Abumrad, N.A., **2002**. Role of CD36 in membrane transport of long-chain fatty acids. *Curr. Opin. Clin. Nutr. Metab. Care 5*, 139–145.

Inglese, J., Freedman, N.J., Koch, W.J., Lefkowitz, R.J., **1993**. Structure and mechanism of the G-protein-coupled receptor kinases. *J. Biol. Chem. 268*, 23735–23738.

Inglese, J., Koch, W.J., Caron, M.G., Lefkowitz, R.J., **1992**. Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature 359*, 147–150.

Ishida, Y., Chiang, V.P., Haverty, M.I., Leal, W.S., **2002a**. Primitive odorant-binding proteins. *J. Chem. Ecol. 28*, R21–R27.

Ishida, Y., Cornel, A.J., Leal, W.S., **2002b**. Identification and cloning of a female antenna-specific odorant-binding protein in the mosquito *Culex quinquefasciatus*. *J. Chem. Ecol. 28*, 867–871.

Ishida, Y., Leal, W.S., **2002**. Cloning of putative odorant-degrading enzyme and integumental estease cDNAs from the wild silkmoth, *Antheraea polyphemus*. *Insect Biochem. Mol. Biol. 32*, 1775–1780.

Ishimoto, H., Matsumoto, A., Tanimura, T., **2000**. Molecular identification of a taste receptor gene for trehalose in *Drosophila*. *Science 289*, 116–119.

Jacquin-Joly, E., Bohbot, J., François, M.C., Cain, A.H., Nagnan-Le Meillour, P., **2000**. Characterization of the general odorant-binding protein 2 in the molecular coding of odorants in *Mamestra brassicae*. *Eur. J. Biochem. 267*, 6708–6714.

Jacquin-Joly, E., Vogt, R.G., François, M.C., Nagnan-Le Meillour, P., **2001**. Functional and expression pattern analysis of chemosensory proteins expressed in antennae and pheromonal gland of *Mamestra brassicae*. *Chem. Senses 26*, 833–844.

Janssen, K.P., Rost, R., Eichinger, L., Schleicher, M., **2001**. Characterization of CD36/LIMPII homologues in *Dictyostelium discoideum*. *J. Biol. Chem. 276*, 38899–38910.

Janssen, K.P., Schleicher, M., **2001**. *Dictyostelium discoideum*: a genetic model system for the study of professional phagocytes. Profilin, phosphoinositides and the lmp gene family in *Dictyostelium*. *Biochim. Biophys. Acta 1525*, 228–233.

Joerges, J., Küttner, A., Galizia, C.G., Menzel, R., **1997**. Representation of odours and odour mixtures visualized in the honeybee brain. *Nature 387*, 285–288.

Jones, D.T., Reed, R.R., **1989**. Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. *Science 244*, 790–795.

Justice, R., Dimitratos, S., Walter, M.F., Woods, D.F., Biessmann, H., **2003**. Sexual dimorphic expression of putative antennal carrier protein genes in the malaria vector *Anopheles gambiae*. *Insect Mol. Biol. 12*, 581–594.

Kaissling, K.-E., **1971**. Insect olfaction. In: Beidler, L.M. (Ed.), Handbook of Sensory Physiology; Vol. IV Chemical Senses; Part 1, Olfaction. Springer, Berlin, pp. 351–431.

Kaissling, K.-E., **1974**. Sensory transduction in insect olfactory receptors. In: Jaenicke, L. (Ed.), Biochemistry of Sensory Function. Springer, Heidelberg, pp. 243–273.

Kaissling, K.-E., **1986**. Chemo-electrical transduction in insect olfactory receptors. *Annu. Rev. Neurosci. 9*, 121–141.

Kaissling, K.-E., **2001**. Olfactory perireceptor and receptor events in moths: a kinetic model. *Chem. Senses 26*, 125–150.

Kaissling, K.-E., Thorson, J., **1980**. Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. In: Sattelle, D.B., Hall, L.M., Hildebrand, J.G. (Eds.), Receptors for Neurotransmitters, Hormones and Pheromones in Insects. North-Holland, Amsterdam, pp. 261–282.

Kalidas, S., Smith, D.P., **2002**. Novel genomic cDNA hybrids produce effective RNA interference in adult *Drosophila*. *Neuron 33*, 177–184.

Kanaujia, A., Kaissling, K.-E., **1985**. Interactions of pheromone with moth antennae: adsorption, desorption and transport. *J. Insect Physiol. 31*, 71–81.

Kanzaki, R., **1998**. Coordination of wing motion and walking suggests common control of zigzag motor program in a male silkworm moth. *J. Comp. Physiol. A 182*, 267–276.

Kanzaki, R., Arbas, E.A., Hildebrand, J.G., **1991**. Physiology and morphology of descending neurons in pheromone-processing olfactory pathways in the male moth *Manduca sexta*. *J. Comp. Physiol. A 169*, 1–14.

Kanzaki, R., Arbas, E.A., Strausfeld, N.J., Hildebrand, J.G., **1989**. Physiology and morphology of projection neurons in the antennal lobe of the male moth *Manduca sexta*. *J. Comp. Physiol. A 165*, 427–453.

Kanzaki, R., Mishima, T., **1996**. Pheromone-triggered 'flipflopping' neural signals correlate with activities of neck motor neurons of a male moth, *Bombyx mori*. *Zool. Sci. 13*, 79–87.

Kanzaki, R., Shibuya, T., **1986**. Descending protocerebral neurons related to the mating dance of the male silkworm moth. *Brain Res. 377*, 378–382.

Kanzaki, R., Soo, K., Seki, Y., Wada, S., **2003**. Projections to higher olfactory centers from subdivisions of the antennal lobe macroglomerular complex of the male silkmoth. *Chem. Senses 28*, 113–130.

Karakesisoglou, I., Janssen, K.-P., Eichinger, L., Noegel, A.A., Schleicher, M., **1999**. Identification of a suppressor of the *Dictyostelium* profilin-minus phenotype as a CD36/LIMP-II homolog. *J. Cell Biol. 145*, 167–181.

Karlson, P., Lüscher, M., **1959**. "Pheromones": a new term for a class of biologically active substances. *Nature 183*, 55–56.

Kasang, G., **1968**. Tritium-Markierung des Sexuallockstoffes Bombykol. *Z. Naturforsch. 23b*, 1331–1335.

Kasang, G., **1971**. Bombykol reception and metabolism on the antennae of the silkmoth *Bombyx mori*. In: Ohloff, G., Thomas, A.F. (Eds.), Gustation and Olfaction. Academic Press, New York, pp. 245–250.

Kasang, G., **1973**. Physikomichemische Vorange beim Riechen des Seidenspinners. *Naturwissenschaften 60*, 95–101.

Kasang, G., **1974**. Uptake of the sex pheromone 3H-bombykol and related compounds by male and female *Bombyx* antennae. *J. Insect Physiol. 20*, 2407–2422.

Kasang, G., Kaissling, K.-E., **1972**. Specificity of primary and secondary olfactory processes in *Bombyx* antennae. *Int. Symp. Olfaction Taste 4*, 200–206.

Kasang, G., Kaissling, K.-E., Vostrowsky, O., Bestmann, H.J., **1978**. Bombykal, a second pheromone component of the silkworm moth *Bombyx mori*. *Angew. Chem. Int. Ed. Engl. 17*, 60.

Kasang, G., Nicholls, M., Keil, T., Kanaujia, S., **1989a**. Enzymatic conversion of sex pheromones in olfactory hairs of the male silkworm moth *Antheraea polyphemus*. *Z. Naturforsch. 44c*, 920–926.

Kasang, G., Nicholls, M., von Proff, L., **1989b**. Sex-pheromone conversion and degradation in antennae of the silkworm moth *Bombyx mori* (L.). *Experientia 45*, 81–87.

Kasang, G., Weiss, N., **1974**. Thin layer chromatographic analysis of radioactively labelled insect pheromones: metabolites of [^{3}H]bombykol. *J. Chromatogr. 92*, 401–417.

Keil, T.A., **1982**. Contacts of pore tubules and sensory dendrites in antennal chemosensilla of a silkmoth: demonstration of a possible pathway for olfactory molecules. *Tissue Cell 14*, 451–462.

Kennedy, J.S., Ludlow, A.R., Sanders, C.J., **1980**. Guidance system used in moth sex attraction. *Nature 288*, 475–477.

Kent, K.S., Hildebrand, J.G., **1987**. Cephalic sensory pathways in the central nervous system of larval *Manduca sexta* (Lepidoptera: Sphingidae). *Phil. Trans. Roy. Soc. London 315*, 1–36.

Kiefer, C., Sumser, E., Wernet, M.F., Von Lintig, J., **2002**. A class B scavenger receptor mediates the cellular uptake of carotenoids in *Drosophila*. *Proc. Natl Acad. Sci. USA 99*, 10581–10586.

Kim, M.S., Repp, A., Smith, D.P., **1998**. LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster. Genetics 150*, 711–721.

Kim, M.S., Smith, D.P., **2001**. The invertebrate odorant-binding protein LUSH is required for normal olfactory behavior in *Drosophila*. *Chem. Senses 26*, 195–199.

Kitabayashi, A.N., Arai, T., Kubo, T., Natori, S., **1998**. Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of *Periplaneta americana* (American cockroach). *Insect Biochem. Mol. Biol. 28*, 785–790.

Klein, U., **1987**. Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem. 17*, 1193–1204.

Klun, J.A., Khrimian, A.P., Oliver, J.E., **1998**. Evidence of pheromone catabolism via B-oxidation in the European corn borer (Lepidoptera: Crambidae). *J. Entomol. Sci. 33*, 400–406.

Klun, J.A., Potts, W.J.E., Oliver, J.E., **1996**. Four species of noctuid moths degrade sex pheromone by a common antennal metabolic pathway. *J. Entomol. Sci. 31*, 404–413.

Klun, J.A., Schwarz, M., Uebel, E.C., **1991**. European corn borer: pheromonal catabolism and behavioral response to sex pheromone. *J. Chem. Ecol. 17*, 317–334.

Klusák, V., Havlas, Z., Rulisek, L., Vondrásek, J., Svatos, A., **2003**. Sexual attraction in the silkworm moth. Nature of binding of bombykol in pheromone binding protein: an *ab initio* study. *Chem. Biol. 10*, 331–340.

Kochansky, J., Tette, J., Taschenberg, E.F., Cardé, R.T., Kaissling, K.E., *et al.*, **1975**. Sex pheromone of the moth *Antheraea polyphemus*. *J. Insect Physiol. 21*, 1977–1983.

Koganezawa, M., Shimada, I., **2002**. Inositol 1,4,5-trisphosphate transduction cascade in taste reception of the fleshfly, *Boettcherisca peregrina*. *J. Neurobiol. 51*, 66–83.

Korsching, S., **2002**. Olfactory maps and odor images. *Curr. Opin. Neurobiol. 12*, 387–392.

Kowcun, A., Honson, N., Plettner, E., **2001**. Olfaction in the gypsy moth, *Lymantria dispar*: effect of pH, ionic strength, and reductants on pheromone transport by pheromone-binding proteins. *J. Biol. Chem. 276*, 44770–44776.

Krieger, J., Breer, H., **1999**. Olfactory reception in invertebrates. *Science 286*, 720–723.

Krieger, J., Breer, H., **2003**. Transduction mechanisms of olfactory sensory neurons. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 593–607.

Krieger, J., Klink, O., Mohl, C., Raming, K., Breer, H., **2003**. A candidate olfactory receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. A 189*, 519–526.

Krieger, J., Mameli, M., Breer, H., **1997**. Elements of the olfactory signaling pathways in insect antennae. *Invert. Neurosci. 3*, 137–144.

Krieger, J., Raming, K., Dewer, Y.M., Bette, S., Conzelmann, S., *et al.*, **2002**. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci. 16*, 619–628.

Krieger, M., **1999**. Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu. Rev. Biochem. 68*, 523–558.

Krieger, M.J., Ross, K.G., **2002**. Identification of a major gene regulating complex social behavior. *Science 295*, 328–332.

Krishnan, B., Dryer, S.E., Hardin, P.E., **1999**. Circadian rhythms in olfactory responses of *Drosophila melanogaster. Nature 400*, 375–378.

Kristensen, N.P., **1991**. Phylogeny of extant hexapods. In: Achterberg, X.Y., *et al.* (Eds.), The Insects of Australia: A Textbook for Students and Research Workers. Melbourne University Press, Melbourne, pp. 125–140.

Kukalová-Peck, J., **1991**. Fossil history and the evolution of hexapod structures. In: Achterberg, X.Y., *et al.* (Eds.), The Insects of Australia: A Textbook for Students and Research Workers. Melbourne University Press, Melbourne, pp. 141–179.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., **2001**. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics 17*, 1244–1245.

Kuniyasu, A., Hayashi, S., Nakayama, H., **2002**. Adipocytes recognize and degrade oxidized low density lipoprotein through CD36. *Biochem. Biophys. Res. Commun. 295*, 319–323.

Kuniyasu, A., Ohgami, N., Hayashi, S., Miyazaki, A., Horiuchi, S., *et al.*, **2003**. CD36-mediated endocytic uptake of advanced glycation end products (AGE) in mouse 3T3-L1 and human subcutaneous adipocytes. *FEBS Lett. 537*, 85–90.

Kuronita, T., Eskelinen, E.L., Fujita, H., Saftig, P., Himeno, M., *et al.*, **2002**. A role for the lysosomal membrane protein LGP85 in the biogenesis and maintenance of endosomal and lysosomal morphology. *J. Cell Sci. 115*, 4117–4131.

Labandeira, C.C., Sepkoski, J.J., Jr., **1993**. Insect diversity in the fossil record. *Science 261*, 310–315.

LaForest, S.M., Prestwich, G.D., Lofstedt, C., **1999**. Intraspecific nucleotide variation at the pheromone binding protein locus in the turnip moth, *Agrotis segetum*. *Insect Mol. Biol. 8*, 481–490.

Lartigue, A., Campanacci, V., Roussel, A., Larsson, A.M., Jones, T.A., *et al.*, **2002**. X-ray structure and ligand binding study of a moth chemosensory protein. *J. Biol. Chem. 277*, 32094–32098.

Lartigue, A., Gruez, A., Briand, L., Pernollet, J.C., Spinelli, S., *et al.*, **2003a**. Optimization of crystals from nanodrops: crystallization and preliminary crystallographic study of a pheromone-binding protein from the honeybee *Apis mellifera* L. *Acta Crystallogr. D. Biol. Crystallogr. 59*, 919–921.

Lartigue, A., Gruez, A., Spinelli, S., Rivière, S., Brossut, R., *et al.*, **2003b**. The crystal structure of a cockroach pheromone binding protein suggests a new ligand binding and release mechanism. *J. Biol. Chem. 278*, 30213–30218.

Laue, M., Maida, R., Redkozubov, A., **1997**. G-protein activation, identification and immunolocalization in pheromone-sensitive sensilla trichodea of moths. *Cell Tissue Res. 288*, 149–158.

Laue, M., Steinbrecht, R.A., **1997**. Topochemistry of moth olfactory sensilla. *Int. J. Insect Morph. Embryol. 26*, 217–228.

Lazar, J., Greenwood, D.R., Rasmussen, L.E., Prestwich, G.D., **2002**. Molecular and functional characterization of an odorant binding protein of the Asian elephant, *Elephas maximus*: implications for the role of lipocalins in mammalian olfaction. *Biochemistry 41*, 11786–11794.

Lazard, D., Tal, N., Rubenstein, M., Khen, M., Lancet, D., *et al.*, **1990**. Identification and biochemical analysis of novel olfactory-specific cytochrome P-450IIA and UDP-glucuronosyl transferase. *Biochemistry 29*, 7433–7440.

Lazard, D., Zupko, K., Poria, Y., Nef, P., Lazarovits, J., *et al.*, **1991**. Odorant signal termination by olfactory UDP glucuronosyl transferase. *Nature 349*, 790–793.

Leal, W.S., **2000**. Duality monomer–dimer of the pheromone-binding protein from *Bombyx mori*. *Biochem. Biophys. Res. Commun. 268*, 521–529.

Leal, W.S., **2003**. Proteins that make sense. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 447–476.

Leal, W.S., Nikonova, L., Peng, G., **1999**. Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. *FEBS Lett. 468*, 85–90.

Lee, C.Y., Baehrecke, E.H., **2001**. Steroid regulation of autophagic programmed cell death during development. *Development 128*, 1443–1455.

Lee, K., Strausfeld, N.J., **1990**. Structure, distribution and number of surface sensilla and their receptor cells on the olfactory appendage of the male moth *Manduca sexta*. *J. Neurocytol. 19*, 519–538.

Lei, H., Anton, S., Hansson, B.S., **2001**. Olfactory protocerebral pathways processing sex pheromone and plant odor information in the male moth *Agrotis segetum*. *J. Comp. Neurol. 432*, 356–370.

Liu, B., Krieger, M., **2002**. Highly purified scavenger receptor class B, type I reconstituted into phosphatidylcholine/cholesterol liposomes mediates high affinity high density lipoprotein binding and selective lipid uptake. *J. Biol. Chem. 277*, 34125–34135.

Maddison, D.R., Maddison, W.P., **1998**. The Tree of Life: a multi-authored, distributed Internet project containing information about phylogeny and biodiversity. http://phylogeny.arizona.edu/ tree/phylogeny.html.

Maïbèche-Coisné, M., Jacquin-Joly, E., François, M.C., Nagnan-Le Meillour, P., **1998**. Molecular cloning of two pheromone binding proteins in the cabbage armyworm *Mamestra brassicae*. *Insect Biochem. Mol. Biol. 28*, 815–818.

Maïbèche-Coisné, M., Jacquin-Joly, E., François, M.C., Nagnan-Le Meillour, P., **2002**. cDNA cloning of biotransformation enzymes belonging to the cytochrome P450 family in the antennae of the noctuid moth *Mamestra brassicae*. *Insect Mol. Biol. 11*, 273–281.

Maïbèche-Coisné, M., Sobrio, F., Delaunay, T., Lettere, M., Dubroca, J., *et al.*, **1997**. Pheromone binding proteins of the moth *Mamestra brassicae*: specificity of ligand binding. *Insect Biochem. Mol. Biol. 27*, 213–221.

Maida, R., Mameli, M., Krieger, J., Breer, H., Ziegelberger, G., *et al.*, **1999**. Complex expression pattern of odorant-binding proteins in *Bombyx mori*. In: Elsner, N., Eysel, U. (Eds.), Gottingen Neurobiology Report. Georg Thieme, Stuttgart, p. 359.

Maida, R., Redkozubov, A., Ziegelberger, G., **2000**. Identification of PLC beta and PKC in pheromone receptor neurons of *Antheraea polyphemus*. *Neuroreport 11*, 1773–1776.

Maleszka, R., Stange, G., **1997**. Molecular cloning, by a novel approach, of a cDNA encoding a putative olfactory protein in the labial palps of the moth *Cactoblastis cactorum*. *Gene 202*, 39–43.

Mameli, M., Tuccini, A., Mazza, M., Petacchi, R., Pelosi, P., **1996**. Soluble proteins in chemosensory organs of phasmids. *Insect Biochem. Mol. Biol. 26*, 875–882.

Mannervik, B., Danielson, U.H., **1988**. Glutathione transferases-structure and catalytic activity. *CRC Crit. Review. Biochem. 23*, 283–337.

Marchese, S., Angeli, S., Andolfo, A., Scaloni, A., Brandazza, A., *et al*., **2000**. Soluble proteins from chemosensory organs of *Eurycantha calcarata* (Insects, Phasmatodea). *Insect Biochem. Mol. Biol. 30*, 1091–1098.

Marin, E.C., Jefferis, G.S., Komiyama, T., Zhu, H., Luo, L., **2002**. Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell 109*, 243–255.

Martini, S., Silvotti, L., Shirazi, A., Ryba, N.J.P., Tirindelli, R., **2001**. Coexpression of putative pheromone receptors in the sensory neurons of the vomeronasal organ. *J. Neurosci. 21*, 843–848.

Matsumoto, S.G., Hildebrand, J.G., **1981**. Olfactory mechanisms in the moth *Manduca sexta*: response characteristics and morphology of central neurons in the antennal lobes. *Proc. Roy. Soc. London B 213*, 249–277.

Matsunami, H., Amrein, H., **2003**. Taste and pheromone perception in mammals and flies. *Genome Biol. 4*, 220.0–220.9. (http://genomebiology.com/2003/4/7/220).

Matsunami, H., Buck, L.B., **1997**. A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell 90*, 775–784.

Mayer, M.S., **1975**. Hydrolysis of sex pheromone by the antennae of *Trichoplusiani*. *Experientia 31*, 452–454.

McGuire, S.E., Le Phuong, Davis, R.L., **2001**. The role of *Drosophila* mushroom body signaling in olfactory memory. *Science 293*, 1330–1333.

McKenna, M.P., Hekmat-Scafe, D.S., Gaines, P., Carlson, J.R., **1994**. Putative *Drosophila* pheromone-binding proteins expressed in a subregion of the olfactory system. *J. Biol. Chem. 269*, 16340–16347.

Merrill, C.E., Riesgo-Escovar, J., Pitts, R.J., Kafatos, F.C., Carlson, J.R., *et al*., **2002**. Visual arrestins in olfactory pathways of *Drosophila* and the malaria vector mosquito *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA 99*, 1633–1638.

Merritt, T.J., LaForest, S., Prestwich, G.D., Quattro, J.M., Vogt, R.G., **1998**. Patterns of gene duplication in lepidopteran pheromone binding proteins. *J. Mol. Evol. 46*, 272–276.

Meunier, N., Ferveur, J.F., Marion-Poll, F., **2000**. Sex-specific non-pheromonal taste receptors in *Drosophila*. *Curr. Biol. 10*, 1583–1586.

Michel, W.C., Ache, B.W., **1992**. Cyclic nucleotides mediate an odor-evoked potassium conductance in lobster olfactory receptor cells. *J. Neurosci. 12*, 3979–3984.

Mishima, T., Kanzaki, R., **1998**. Coordination of flip-flopping neural signals and head turning during pheromone-mediated walking in a male silkworm moth *Bombyx mori*. *J. Comp. Physiol. A 183*, 273–282.

Mishima, T., Kanzaki, R., **1999**. Physiological and morphological characterization of olfactory descending interneurons of the male silkworm moth, *Bombyx mori*. *J. Comp. Physiol. A 184*, 143–160.

Miyazaki, A., Nakayama, H., Horiuchi, S., **2002**. Scavenger receptors that recognize advanced glycation end products. *Trends Cardiovasc. Med. 12*, 258–262.

Mombaerts, P., **1999**. Molecular biology of odorant receptors in vertebrates. *Annu. Rev. Neurosci. 22*, 487–509.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., *et al*., **1996**. Visualizing an olfactory sensory map. *Cell 87*, 675–686.

Moore, K.J., El Khoury, J., Medeiros, L.A., Terada, K., Geula, C., *et al*., **2002**. A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. *J. Biol. Chem. 277*, 47373–47379.

Mori, H., **2002**. Transgenic insects: expressing green fluorescent protein-silk fibroin light chain fusion protein in transgenic silkworms. *Methods Mol. Biol. 183*, 235–243.

Mosbah, A., Campanacci, V., Lartigue, A., Tegoni, M., Cambillau, C., *et al*., **2003**. Solution structure of a chemosensory protein from the moth *Mamestra brassicae*. *Biochem. J. 369*, 39–44.

Nagnan-Le Meillour, P., Cain, A.H., Jacquin-Joly, E., François, M.C., Ramachandran, S., *et al*., **2000**. Chemosensory proteins from the proboscis of *Mamestra brassicae*. *Chem. Senses 25*, 541–553.

Nagnan-Le Meillour, P., Jacquin-Joly, E., **2003**. Biochemistry and diversity of insect odorant-binding proteins. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 509–537.

Naito, T., Saito, Y., Yamamoto, J., Noxaki, Y., Tomura, K., *et al*., **1998**. Putative pheromone receptors related to the Ca21-sensing receptor in *Fugu*. *Proc. Natl Acad. Sci. USA 95*, 5178–5181.

Nässel, D.R., **2002**. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Progr. Neurobiol. 68*, 1–84.

Nayak, S.V., Singh, R.N., **1983**. Sensilla of the tarsal segments and mouthparts of adult *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). *Int. J. Insect Morphol. Embryol. 12*, 273–291.

Nef, P., Heldman, J., Lazard, D., Margalit, T., Jaye, M., *et al*., **1989**. Olfactory-specific cytochrome P-450. *J. Biol. Chem. 264*, 6780–6785.

Nei, M., Kumar, S., **2000**. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Ngai, J., Dowling, M.M., Buck, L., Axel, R., Chess, A., **1993**. The family of genes encoding odorant receptors in the channel catfish. *Cell 12*, 657–666.

Nikonov, A.A., Peng, G., Tsurupa, G., Leal, W.S., **2002**. Unisex pheromone detectors and pheromone-binding proteins in scarab beetles. *Chem. Senses 27*, 495–504.

Nilius, B., Droogmans, G., Wondergem, R., **2003**. Transient receptor potential channels in endothelium: solving the calcium entry puzzle? *Endothelium 10*, 5–15.

Nomura, A., Kawasaki, K., Kubo, T., Natori, S., **1992**. Purification and localization of p10, a novel protein that increases in nymphal regenerating legs of *Periplaneta americana* (American cockroach). *Int. J. Devel. Biol. 36*, 391–398.

Novotny, V., Basset, Y., Miller, S.E., Weiblen, G.D., Bremer, B., *et al.*, **2002**. Low host specificity of herbivorous insects in a tropical forest. *Nature 416*, 841–844.

Olberg, R.M., **1983**. Pheromone-triggered flip-flopping interneurons in the ventral nerve cord of the silkworm moth, *Bombyx mori*. *J. Comp. Physiol. 152*, 297–307.

Olberg, R.M., Willis, M.A., **1990**. Pheromone-modulated optomotor response in male gypsy moths, *Lymantria dispar* L: directionally selective visual interneurons in the ventral nerve cord. *J. Comp. Physiol. A 167*, 707–714.

Oldham, N.J., Krieger, J., Breer, H., Fischedick, A., Hoskovec, M., *et al.*, **2001**. Detection and removal of an artifact fatty acid from the binding site of recombinant *Bombyx mori*. *Chem. Senses 26*, 529–531.

Oquendo, P., Hundt, E., Lawler, J., Seed, B., **1989**. CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitized erythrocytes. *Cell 58*, 95–101.

Ottiger, M., Soller, M., Stocker, R.F., Kubli, E., **2000**. Binding sites of *Drosophila melanogaster* sex peptide pheromones. *J. Neurobiol. 44*, 57–71.

Ozaki, M., Morisaki, K., Idei, W., Ozaki, K., Tokunaga, F., **1995**. A putative lipophilic stimulant carrier protein commonly found in the taste and olfactory systems. A unique member of the pheromone-binding protein superfamily. *Eur. J. Biochem. 230*, 298–308.

Ozaki, M., Takahara, T., Kawahara, Y., Wada-Katsumata, A., Seno, K., *et al.*, **2003**. Perception of noxious compounds by contact chemoreceptors of the blowfly, *Phormia regina*: putative role of an odorant-binding protein. *Chem. Senses 28*, 349–359.

Pace, U., Hanski, E., Salomon, Y., Lancet, D., **1985**. Odorant-sensitive adenylate cyclase may mediate olfactory reception. *Nature 316*, 255–258.

Pace, U., Lancet, D., **1986**. Olfactory GTP-binding protein: signal-transducing polypeptide of vertebrate chemosensory neurons. *Proc. Natl Acad. Sci. USA 83*, 4947–4951.

Paesen, G.C., Happ, G.M., **1995**. The B proteins secreted by the tubular accessory sex glands of the male mealworm beetle, *Tenebrio molitor*, have sequence similarity to moth pheromone-binding proteins. *Insect Biochem. Mol. Biol. 25*, 401–408.

Pantages, E., Dulac, C., **2000**. A novel family of candidate pheromone receptors in mammals. *Neuron 28*, 835–845.

Panzenboeck, U., Balazs, Z., Sovic, A., Hrzenjak, A., Levak-Frank, S., *et al.*, **2002**. ABCA1 and scavenger receptor class B, type I, are modulators of reverse sterol transport at an *in vitro* blood–brain barrier constituted of porcine brain capillary endothelial cells. *J. Biol. Chem. 277*, 42781–42789.

Park, S.-K., Shanbhag, S.R., Wang, Q., Hasan, G., Steinbrecht, R.A., *et al.*, **2000**. Expression patterns of two putative odorant-binding proteins in the olfactory organs of *Drosophila melanogaster* have different implications for their functions. *Cell Tissue Res. 300*, 181–197.

Pass, G., **2000**. Acessory pulsatile organs: evolutionary innovations in insects. *Annu. Rev. Entomol. 45*, 495–518.

Pass, G., Agricola, H., Birkenbeil, H., Penzlin, H., **1988a**. Morphology of neurons associated with the antennal heart of *Periplaneta americana* (Blattodea, Insecta). *Cell Tissue Res. 253*, 319–326.

Pass, G., Sperk, G., Agricola, H., Baumann, E., Penzlin, H., **1988b**. Octopamine in a neurohaemal area within the antennal heart of the American cockroach. *J. Exp. Biol. 135*, 495–498.

Pelosi, P., Maida, R., **1995**. Odorant-binding proteins in insects. *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 111*, 503–514.

Picimbon, J.F., **2003**. Biochemistry and evolution of OBP and CSP proteins. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 539–566.

Picimbon, J.F., Dietrich, K., Angeli, S., Scaloni, A., Krieger, J., *et al.*, **2000a**. Purification and molecular cloning of chemosensory proteins from *Bombyx mori*. *Arch. Insect Biochem. Physiol. 44*, 120–129.

Picimbon, J.F., Dietrich, K., Breer, H., Krieger, J., **2000b**. Chemosensory proteins of *Locusta migratoria* (Orthoptera: Acrididae). *Insect Biochem. Mol. Biol. 30*, 233–241.

Picimbon, J.F., Dietrich, K., Krieger, J., Breer, H., **2001**. Identity and expression pattern of chemosensory proteins in *Heliothis virescens* (Lepidoptera, Noctuidae). *Insect Biochem. Mol. Biol. 31*, 1173–1181.

Picone, D., Crescenzi, O., Angeli, S., Marchese, S., Brandazza, A., *et al.*, **2001**. Bacterial expression and conformational analysis of a chemosensory protein from *Schistocerca gregaria*. *Eur. J. Biochem. 268*, 4794–4801.

Pikielny, C.W., Hasan, G., Rouyer, F., Rosbash, M., **1994**. Members of a family of *Drosophila* putative odorant-binding proteins are expressed in different subsets of olfactory hairs. *Neuron 12*, 35–49.

Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M.G., *et al.*, **1993**. Overexpression of beta-arrestin and beta-adrenergic receptor kinase augment desensitization of beta 2-adrenergic receptors. *J. Biol. Chem. 268*, 3201–3208.

Pitcher, J.A., Freedman, N.J., Lefkowitz, R.L., **1998**. G-protein coupled receptor kinases. *Annu. Rev. Biochem. 67*, 653–692.

Plettner, E., **2003**. The peripheral pheromone olfactory system in insects: targets for species-selective insect control agents. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 477–507.

Plettner, E., Lazar, J., Prestwich, E.G., Prestwich, G.D., **2000**. Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth *Lymantria dispar*. *Biochemistry 39*, 8953–8962.

Podrez, E.A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., *et al.*, **2002a**. A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor CD36 and is enriched

in atherosclerotic lesions. *J. Biol. Chem. 277*, 38517–38523.

Podrez, E.A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., *et al.*, **2002b**. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J. Biol. Chem. 277*, 38503–38516.

Pophof, B., **1998**. Inhibitors of sensillar esterase reversibly block the responses of moth pheromone receptor cells. *J. Comp. Physiol. A 183*, 153–164.

Pophof, B., **2002**. Moth pheromone binding proteins contribute to the excitation of olfactory receptor cells. *Naturwissenschaften 89*, 515–518.

Pophof, B., Gebauer, T., Ziegelberger, G., **2000**. Decylthio-trifluoropropanone, a competitive inhibitor of moth pheromone receptors. *J. Comp. Physiol. A 186*, 315–323.

Pophof, B., van der Goes van Naters, W., **2002**. Activation and inhibition of the transductory process in silkmoth olfactory receptor neurons. *Chem. Senses 27*, 435–443.

Preiss, R., Kramer, E., **1983**. Stabilization of altitude and speed in tethered flying gypsy moth males: influence of (+)- and (−)-disparlure. *Physiol. Entomol. 8*, 55–68.

Prestwich, G.D., **1985**. Molecular communication of insects. *Qt. Rev. Biol. 60*, 437–456.

Prestwich, G.D., **1987**. Chemistry of pheromone and hormone metabolism in insects. *Science 237*, 999–1006.

Prestwich, G.D., Du, G., LaForest, S., **1995**. How is pheromone specificity encoded in proteins? *Chem. Senses 20*, 461–469.

Prestwich, G.D., Golec, F.A., Andersen, N.H., **1984**. Synthesis of a highly tritiated photoaffinity labeled pheromone analog for the moth *Antheraea polyphemus*. *J. Labelled Cmpnd Radiopharm. 21*, 593–601.

Python, F., Stocker, R.F., **2002**. Adult-like complexity of the larval antennal lobe of *D. melanogaster* despite markedly low numbers of odorant receptor neurons. *J. Comp. Neurol. 445*, 374–387.

Quero, C., Camps, F., Guerrero, A., **1995**. Behavior of processionary males (*Thaumetopoea pityocampa*) induced by sex pheromone and analogs in a wind tunnel. *J. Chem. Ecol. 21*, 1957–1969.

Raming, K., Krieger, J., Breer, H., **1989**. Molecular cloning of an insect pheromone-binding protein. *FEBS Lett. 256*, 215–218.

Raming, K., Krieger, J., Breer, H., **1990**. Molecular cloning sequencing and expression of cDNA encoding a G_0-protein from insect. *Cell Signal 2*, 311–321.

Ramoni, R., Vincent, F., Grolli, S., Conti, V., Malosse, C., *et al.*, **2001**. The insect attractant 1-octen-3-ol is the natural ligand of bovine odorant-binding protein. *J. Biol. Chem. 276*, 7150–7155.

Rasmussen, J.T., Berglund, L., Rasmussen, M.S., Petersen, T.E., **1998**. Assignment of disulfide bridges in bovine CD36. *Eur. J. Biochem. 257*, 488–494.

Rau, P., Rau, N., **1929**. The sex attraction and rhythmic periodicity in giant saturniid moths. *Trans. Acad. Sci. St. Louis (MO, USA) 26*, 83–221.

Reaven, E., Leers-Sucheta, S., Nomoto, A., Azhar, S., **2001**. Expression of scavenger receptor class B type 1 (SR-BI) promotes microvillar channel formation and selective cholesteryl ester transport in a heterologous reconstituted system. *Proc. Natl Acad. Sci. USA 98*, 1613–1618.

Redkozubov, A., **2000**. Guanosine 3′,5′-cyclic monophosphate reduces the response of the moth's olfactory receptor neuron to pheromone. *Chem. Senses 25*, 381–385.

Renou, M., Berthier, A., Desbarats, L., Van der Pers, J., Guerrero, A., **1999**. Actographic analysis of the effects of an esterase inhibitor on male moth responses to sex pheromone. *Chem. Senses 24*, 423–428.

Renou, M., Guerrero, A., **2000**. Insect parapheromones in olfaction research and semiochemical-based pest control strategies. *Annu. Rev. Entomol. 45*, 605–630.

Riba, M., Sans, A., Bau, P., Grolleau, G., Renou, M., *et al.*, **2001**. Pheromone response inhibitors of the corn stalk borer *Sesamia nonagrioides*: biological evaluation and toxicology. *J. Chem. Ecol. 27*, 1879–1897.

Riesgo-Escovar, J., Raha, D., Carlson, J.R., **1995**. Requirement for a phospholipase C in odor response: overlap between olfaction and vision in *Drosophila*. *Proc. Natl Acad. Sci. USA 28*, 2864–2868.

Rivault, C., Cloarec, A., Sreng, L., **1998**. Cuticular extracts inducing aggregation in the German cockroach, *Blattella germanica* (L.). *J. Insect Physiol. 44*, 909–918.

Rivière, S., Lartigue, A., Quennedey, B., Campanacci, V., Farine, J.-P., *et al.*, **2003**. A pheromone-binding protein from the cockroach *Leucophaea maderae:* cloning, expression and pheromone binding. *Biochem. J. 371*, 573–579.

Robertson, H.M., **1983**. Chemical stimuli eliciting courtship by males in *Drosophila melanogaster*. *Experientia 39*, 333–335.

Robertson, H.M., **2000**. The large srh family of chemoreceptor genes in *Caenorhabditis* nematodes reveals processes of genome evolution involving large duplications and deletions and intron gains and losses. *Genome Res. 10*, 192–203.

Robertson, H.M., Martos, R., Sears, C.R., Todres, E.Z., Walden, K.K., *et al.*, **1999**. Diversity of odourant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol. 8*, 501–518.

Rogers, M.E., Jani, M.K., Vogt, R.G., **1999**. An olfactory specific glutathione *S*-transferase in the sphinx moth *Manduca sexta*. *J. Exp. Biol. 202*, 1625–1637.

Rogers, M.E., Krieger, J., Vogt, R.G., **2001b**. Antennal SNMPs (sensory neuron membrane proteins) of Lepidoptera define a unique family of invertebrate CD36-like proteins. *J. Neurobiol. 49*, 47–61.

Rogers, M.E., Steinbrecht, R.A., Vogt, R.G., **2001a**. Expression of SNMP-1 in olfactory neurons and sensilla of male and female antennae of the silkmoth *Antheraea polyphemus*. *Cell Tissue Res. 303*, 433–446.

Rogers, M.E., Sun, M., Lerner, M.R., Vogt, R.G., **1997**. SNMP-1, a novel membrane protein of olfactory

neurons of the silk moth *Antheraea polyphemus* with homology to the CD36 family of membrane proteins. *J. Biol. Chem.* *272*, 14792–14799.

Ross, K.G., **1997**. Multilocus evolution in fire ants: effects of selection, gene flow and recombination. *Genetics* *145*, 961–974.

Ross, K.G., Keller, L., **1998**. Genetic control of social organization in an ant. *Proc. Natl Acad. Sci. USA* *95*, 14232–14237.

Rossler, P., Boekhoff, I., Tareilus, E., Beck, S., Breer, H., *et al.*, **2000a**. G protein beta-gamma complexes in circumvallate taste cells involved in bitter transduction. *Chem. Senses* *25*, 413–421.

Rossler, P., Kroner, C., Krieger, J., Lobel, D., Breer, H., *et al.*, **2000b**. Cyclic adenosine monophosphate signaling in the rat vomeronasal organ: role of an adenylyl cyclase type VI. *Chem. Senses* *25*, 313–322.

Rothemund, S., Liou, Y.C., Davies, P.L., Krause, E., Sonnichsen, F.D., **1999**. A new class of hexahelical insect proteins revealed as putative carriers of small hydrophobic ligands. *Struct. Fold Des.* *7*, 1325–1332.

Rouquier, S., Blancher, A., Giorgi, D., **2000**. The olfactory receptor gene repertoire in primates and mouse: evidence for reduction of the functional fraction in primates. *Proc. Natl Acad. Sci. USA* *97*, 2870–2874.

Runnenburger, K., Breer, H., Boekhoff, I., **2002**. Selective G protein beta gamma-subunit compositions mediate phospholipase C activation in the vomeronasal organ. *Eur. J. Cell Biol.* *81*, 539–547.

Ryba, N.J.P., Tirindelli, R., **1997**. A new multigene family of putative pheromone receptors. *Neuron* *19*, 371–379.

Rybczynski, R., Reagan, J., Lerner, M.R., **1989**. A pheromone-degrading aldehyde oxidase in the antennae of the moth *Manduca sexta*. *J. Neurosci.* *9*, 1341–1353.

Rybczynski, R., Vogt, R.G., Lerner, M.R., **1990**. Antennal-specific pheromone-degrading aldehyde oxidases from the moths *Antheraea polyphemus* and *Bombyx mori*. *J. Biol. Chem.* *32*, 19712–19715.

Ryeom, S.W., Sparrow, J.R., Silverstein, R.L., **1996**. CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium. *J. Cell Sci.* *109*, 387–395.

Sadek, M.M., Hansson, B.S., Rospars, J.P., Anton, S., **2002**. Glomerular representation of plant volatiles and sex pheromone components in the antennal lobe of the female *Spodoptera littoralis*. *J. Exp. Biol.* *205*, 1363–1376.

Saitou, N., Nei, M., **1987**. The neighbor joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* *4*, 406–425.

Salvador, C., Mora, S.I., Ordaz, B., Antaramian, A., Vaca, L., *et al.*, **2003**. Basal activity of GIRK5 isoforms. *Life Sci.* *72*, 1509–1518.

Sandler, B.H., Nikonova, L., Leal, W.S., Clardy, J., **2000**. Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. *Chem. Biol.* *7*, 143–151.

Sandoval, IV., Martinez-Arca, S., Valdueza, J., Palacios, S., Holman, G.D., **2000**. Distinct reading of different structural determinants modulates the dileucine-mediated transport steps of the lysosomal membrane protein LIMPII and the insulin-sensitive glucose transporter GLUt4. *J. Biol. Chem.* *275*, 39874–39885.

Savarit, F., Sureau, G., Cobb, M., Ferveur, J.F., **1999**. Genetic elimination of known pheromones reveals the fundamental chemical bases of mating and isolation in *Drosophila*. *Proc. Natl Acad. Sci. USA* *96*, 9015–9020.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R.M., Acali, S., *et al.*, **2002**. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* *21*, 5017–5025.

Schleicher, S., Boekhoff, I., Konietzko, U., Breer, H., **1994**. Pheromone-induced phosphorylation of antennal proteins from insects. *J. Comp. Physiol. B* *164*, 76–80.

Schneider, D., **1969**. Insect olfaction: deciphering system for chemical messages. *Science* *163*, 1031–1037.

Schneider, D., **1992**. 100 years of pheromone research: an essay on lepidoptera. *Naturwissenschaften* *79*, 241–250.

Schweitzer, E.S., Sanes, J.R., Hildebrand, J.G., **1976**. Ontogeny of electroantennogram responses in the moth, *Manduca sexta*. *J. Insect Physiol.* *2*, 955–960.

Scott, K., Brady, R., Jr., Cravchik, A., Morozov, P., Rzhetsky, A., *et al.*, **2001**. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* *104*, 661–673.

Sengupta, P., Chou, J.H., Bargmann, C.I., **1996**. *odr-10* encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* *84*, 899–909.

Shanbhag, S.R., Hekmat-Scafe, D., Kim, M.S., Park, S.K., Carlson, J.R., *et al.*, **2001a**. Expression mosaic of odorant-binding proteins in *Drosophila* olfactory organs. *Microsc. Res. Techn.* *55*, 297–306.

Shanbhag, S.R., Müller, B., Steinbrecht, R.A., **1999**. Atlas of olfactory organs of *Drosophila melanogaster*. 1. Types, external organization, innervation and distribution of olfactory sensilla. *Int. J. Insect Morphol. Embryol.* *28*, 377–397.

Shanbhag, S.R., Park, S.-K., Pikielny, C.W., Steinbrecht, R.A., **2001b**. Gustatory organs of *Drosophila melanogaster*: fine structure and expression of the putative odorant-binding protein PBPRP2. *Cell Tissue Res.* *304*, 423–437.

Shanbhag, S.R., Singh, R.N., **1992**. Functional implications of the projections of neurons from individual labellar sensillum of *Drosophila melanogaster* as revealed by neuronal marker horseradish peroxidase. *Cell Tissue Res.* *267*, 273–282.

Simpson, P.J., Nighorn, A., Morton, D.B., **1999**. Identification of a novel guanylyl cyclase that is related to receptor guanylyl cyclases but lacks extracellular and transmembrane domains. *J. Biol. Chem.* *274*, 4440–4446.

Singh, R.N., **1997**. Neurobiology of the gustatory systems of *Drosophila* and some terrestrial insects. *Microsc. Res. Techn. 39*, 547–563.

Singh, R.N., Nayak, S.V., **1985**. Fine structure and primary sensory projections of sensilla on the maxillary palp of *Drosophila melanogaster* Meigen (Diptera: Drosphilidae). *Int. J. Insect Morphol. Embryol. 14*, 291–306.

Snyder, M.J., Walding, J.K., Feyereisen, R., **1995**. Glutathione *S*-transferases from larval *Manduca sexta* midgut: sequence of two cDNAs and enzyme induction. *Insect Biochem. Mol. Biol. 25*, 455–465.

Sonenshine, D.E., **1985**. Pheromones and other semiochemicals of the acari. *Annu. Rev. Entomol. 30*, 1–28.

Speca, D.J., Lin, D.M., Sorensen, P.W., Isacoff, E.Y., Ngai, J., *et al.*, **1999**. Functional identification of a goldfish odorant receptor. *Neuron 23*, 487–498.

Srivastava, R.A., **2003**. Scavenger receptor class B type I expression in murine brain and regulation by estrogen and dietary cholesterol. *J. Neurol. Sci. 210*, 11–18.

Starratt, A.M., Dahm, K.H., Allen, N., Hildebrand, J.G., Payne, T.L., *et al.*, **1979**. Bombykal, a sex pheromone of the sphinx moth, *Manduca sexta*. *Z. Naturforsch. 34c*, 9–12.

Steinbrecht, R.A., **1969**. Comparative morphology of olfactory receptor. In: Pfaffmann, C. (Ed.), Olfaction and Taste III. Rockefeller University Press, New York, pp. 3–21.

Steinbrecht, R.A., **1980**. Cryofixation without cryoprotectants: freeze substitution and freeze etching of an insect olfactory receptor. *Tissue Cell 12*, 73–100.

Steinbrecht, R.A., **1984**. Chemo-, hygro-, and thermoreceptors. In: Bereiter-Hahn, J., Matoltsy, A.G., Richards, K.S. (Eds.), Biology of the Integument, vol. 1, Invertebrates. Springer, Berlin, pp. 523–553.

Steinbrecht, R.A., **1996**. Are odorant-binding proteins involved in odorant discrimination? *Chem. Senses 21*, 719–727.

Steinbrecht, R.A., **1997**. Pore structures in insect olfactory sensilla: a review of data and concepts. *Int. J. Insect Morphol. Embryol. 26*, 229–245.

Steinbrecht, R.A., **1999**. Olfactory receptors. In: Eguchi, E., Tominaga, Y. (Eds.), Atlas of Arthropod Sensory Receptors, Dynamic Morphology in Relation to Function. Springer, Tokyo, pp. 155–176.

Steinbrecht, R.A., Laue, M., Ziegelberger, G., **1995**. Immunolocalization of pheromone-binding protein and general odorant-binding protein in olfactory sensilla of the silk moths *Antheraea* and *Bombyx*. *Cell Tissue Res. 282*, 203–217.

Steinbrecht, R.A., Müller, B., **1971**. On the stimulus conducting structures in insect olfactory receptors. *Z. Zellforsch. 117*, 570–575.

Steinbrecht, R.A., Stankiewicz, B.A., **1999**. Molecular composition of the wall of insect olfactory sensilla: the chitin question. *J. Insect Physiol. 45*, 785–790.

Stengl, M., **1994**. Inositol-trisphosphate-dependent calcium currents precede cation currents in insect olfactory neurons *in vitro*. *J. Comp. Physiol. A 174*, 187–194.

Stengl, M., Zintl, R., De Vente, J., Nighorn, A., **2001**. Localization of cGMP immunoreactivity and of soluble guanylyl cyclase in antennal sensilla of the hawkmoth *Manduca sexta*. *Cell Tissue Res. 304*, 409–421.

Stengl, M., Zufall, F., Hatt, H., Hildebrand, J.G., **1992**. Olfactory receptor neurons from antennae of developing male *Manduca sexta* respond to components of the species-specific sex pheromone *in vitro*. *J. Neurosci. 12*, 2523–2531.

Stocker, R.F., **1994**. The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Res. 275*, 3–26.

Stocker, R.F., Gendre, N., **1989**. Courtship behavior of *Drosophila* genetically or surgically deprived of basiconic sensilla. *Behav. Genet. 19*, 371–385.

Störtkuhl, K.F., Kettler, R., **2001**. Functional analysis of an olfactory receptor in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 98*, 9381–9385.

Strimmer, K., von Haeseler, A., **1996**. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol. 13*, 964–969.

Sun, X.J., Tolbert, L.P., Hildebrand, J.G., **1997**. Synaptic organization of the uniglomerular projection neurons of the antennal lobe of the moth *Manduca sexta*: a laser scanning confocal and electron microscopic study. *J. Comp. Neurol. 379*, 2–20.

Swofford, D.L., **2000**. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), Version 4. Sinauer Associates, Sunderland, MA.

Tabuchi, N., Akasaki, K., Tsuji, H., **2000**. Two acidic amino acid residues, Asp(470) and Glu(471), contained in the carboxyl cytoplasmic tail of a major lysosomal membrane protein, LGP85/LIMP II, are important for its accumulation in secondary lysosomes. *Biochem. Biophys. Res. Commun. 270*, 557–563.

Takami, S., **2002**. Recent progress in the neurobiology of the vomeronasal organ. *Microsc. Res. Tech. 58*, 228–250.

Talluri, S., Bhatt, A., Smith, D.P., **1995**. Identification of a *Drosophila* G protein alpha subunit (dGq alpha-3) expressed in chemosensory cells and central neurons. *Proc. Natl Acad. Sci. USA 92*, 11475–11479.

Tasayco, M.L., Prestwich, G.D., **1990a**. Aldehyde oxidizing enzymes in an adult moth: *in vitro* study of pheromone degradation in *Heliothis virescens*. *Arch. Biochem. Biophys. 278*, 444–451.

Tasayco, M.L., Prestwich, G.D., **1990b**. A specific affinity reagent to distinguish aldehyde dehydrogenases and oxidases: enzymes catalyzing aldehyde oxidation in an adult moth. *J. Biol. Chem. 265*, 3094–4101.

Tasayco, M.L., Prestwich, G.D., **1990c**. Aldehyde oxidases and dehydrogenases in antennae of five moth species. *Insect Biochem. 20*, 691–700.

Taylor, T.R., Ferkovich, S.M., Van Essen, F., **1981**. Increased pheromone catabolism by antennal esterases after adult eclosion of the cabbage looper moth. *Experientia 37*, 729–731.

Tegoni, M., Pelosi, P., Vincent, F., Spinelli, S., Campanacci, V., *et al.*, **2000**. Mammalian odorant

binding proteins. *Biochim. Biophys. Acta 1482*, 229–240.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., **1997**. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res. 24*, 4876–4882.

Thymianou, S., Mavroidis, M., Kokolakis, G., Komitopoulou, K., Zacharopoulou, A., *et al.*, **1998**. Cloning and characterization of a cDNA encoding a male-specific serum protein of the Mediterranean fruit fly, *Ceratitis capitata*, with sequence similarity to odourant-binding proteins. *Insect Mol. Biol. 7*, 345–353.

Tirindelli, R., Mucignat-Caretta, C., Ryba, N.J.P., **1998**. Molecular aspects of pheromonal communication via the vomeronasal organ of mammals. *Trends Neurosci. 21*, 482–486.

Tomita, M., Munetsuna, H., Sato, T., Adachi, T., Hino, R., *et al.*, **2003**. Transgenic silkworms produce recombinant human type III procollagen in cocoons. *Nature Biotechnol. 21*, 52–56.

Trapido-Rosenthal, H.G., Carr, W.E., Gleeson, R.A., **1987**. Biochemistry of an olfactory purinergic system: dephosphorylation of excitatory nucleotides and uptake of adenosine. *J. Neurochem. 49*, 1174–1182.

Trapido-Rosenthal, H.G., Carr, W.E., Gleeson, R.A., **1990**. Ectonucleotidase activities associated with the olfactory organ of the spiny lobster. *J. Neurochem. 55*, 88–96.

Tregenza, T., Wedell, N., **1997**. Definitive evidence for cuticular pheromones in a cricket. *Anim. Behav. 54*, 979–984.

Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A., Bargmann, C.I., **1995**. Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell 83*, 207–218.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Tsukamoto, K., Kinoshita, M., Kojima, K., Mikuni, Y., Kudo, M., *et al.*, **2002**. Synergically increased expression of CD36, CLA-1 and CD68, but not of SR-A and LOX-1, with the progression to foam cells from macrophages. *J. Atheroscler. Thromb. 9*, 57–64.

Tumlinson, J., Brennan, M.M., Doolittle, R.E., Mitchell, E.R., Brabham, A., *et al.*, **1989**. Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. *Arch. Insect Biochem. Physiol. 10*, 255–271.

Tumlinson, J., Mitchell, E.R., Doolittle, R.E., Jackson, D., **1994**. Field tests of synthetic *Manduca sexta* sex pheromone. *J. Chem. Ecol. 20*, 579–591.

Udomsangpetch, R., Pipitaporn, B., Silamut, K., Pinches, R., Kyes, S., *et al.*, **2002**. Febrile temperatures induce cytoadherence of ring-stage *Plasmodium falciparum*-infected erythrocytes. *Proc. Natl Acad. Sci. USA 99*, 11825–11829.

Ueno, K., Ohta, M., Morita, H., Mikuni, Y., Nakajima, S., *et al.*, **2001**. Trehalose sensitivity in *Drosophila* correlates with mutations in and expression of the gustatory receptor gene *Gr5a*. *Curr. Biol. 11*, 1451–1455.

Vander Meer, R.K., Breed, M.D., Winston, M.L., Espelie, K.E. (Eds.), **1998**. Pheromone Communication in Social Insects: Ants, Wasps, Bees and Termites. Westview Press, Boulder, CO.

Vega, M.A., Sigui-Real, B., Garcia, J.A., Cales, C., Rodriguez, F., *et al.*, **1991**. Cloning, sequencing, and expression of a cDNA encoding rat LIMP II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36. *J. Biol. Chem. 266*, 16818–16824.

Venard, R., Antony, C., Jallon, J.M., **1989**. *Drosophila* chemoreceptors. In: Singh, R.N., Strausfeld, N.J. (Eds.), Neurobiology of Sensory Systems. Plenum, New York, pp. 377–385.

Venkatesh, S., Singh, R.N., **1984**. Sensilla on the third antennal segment of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). *Int. J. Insect Morphol. Embryol. 13*, 51–63.

Vickers, N.J., Baker, T.C., **1994**. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Natl Acad. Sci. USA 91*, 5756–5760.

Vickers, N.J., Christensen, T.A., Baker, T.C., Hildebrand, J.G., **2001**. Odour-plume dynamics influence the brain's olfactory code. *Nature 410*, 466–470.

Vogt, R.G., **1987**. The molecular basis of pheromone reception: its influence on behavior. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, New York, pp. 385–431.

Vogt, R.G., **2002**. Odorant binding proteins of the malaria mosquito *Anopheles gambiae*: possible orthologues of the OS-E and OS-F OBPs of *Drosophila melanogaster*. *J. Chem. Ecol. 28*, 2371–2376.

Vogt, R.G., **2003**. Biochemical diversity of odor detection: OBPs, ODEs and SNMPs. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 391–446.

Vogt, R.G., Callahan, F.E., Rogers, M.E., Dickens, J.C., **1999**. Odorant binding protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug *Lygus lineolaris* (Hemiptera, Heteroptera). *Chem. Senses 24*, 481–495.

Vogt, R.G., Köehne, A.C., Dubnau, J.T., Prestwich, G.D., **1989**. Expression of pheromone binding proteins during antennal development in the gypsy moth *Lymantria dispar*. *J. Neurosci. 9*, 3332–3346.

Vogt, R.G., Lerner, M.R., **1989**. Two groups of odorant binding proteins in insects suggest specific and general olfactory pathways. *Neurosci. Abstr. 15*, 1290.

Vogt, R.G., Prestwich, G.D., **1988**. Variation in olfactory proteins: evolvable elements encoding insect behavior. *Ann. New York Acad. Sci. 510*, 689–691.

Vogt, R.G., Prestwich, G.D., Lerner, M.R., **1991b**. Molecular cloning and sequencing of general-odorant binding proteins GOBP1 and GOBP2 from tobacco hawk moth *Manduca sexta*: comparisons with other

insect OBPs and their signal peptides. *J. Neurosci. 11*, 2972–2984.

Vogt, R.G., Prestwich, G.D., Lerner, M.R., **1991a**. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol. 22*, 74–84.

Vogt, R.G., Prestwich, G.D., Riddiford, L.M., **1988**. Sex pheromone receptor proteins, visualization using a radiolabeled photoaffinity analog. *J. Biol. Chem. 263*, 3952–3959.

Vogt, R.G., Riddiford, L.M., **1981**. Pheromone binding and inactivation by moth antennae. *Nature 293*, 161–163.

Vogt, R.G., Riddiford, L.M., **1986a**. Pheromone reception: a kinetic equilibirum. In: Payne, T.L., Birch, M.C., Kennedy, C.E.J. (Eds.), Mechanisms in Insect Olfaction. Clarendon Press, Oxford, pp. 201–208.

Vogt, R.G., Riddiford, L.M., **1986b**. Scale esterase: a pheromone degrading enzyme from the wing scales of the silk moth *Antheraea polyphemus*. *J. Chem. Ecol. 12*, 469–482.

Vogt, R.G., Riddiford, L.M., Prestwich, G.D., **1985**. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. Natl Acad. Sci. USA 82*, 8827–8831.

Vogt, R.G., Rogers, M.E., Franco, M.D., Sun, M., **2002**. A comparative study of odorant binding protein genes: differential expression of the PBP1-GOBP2 gene cluster in *Manduca sexta* (Lepidoptera) and the organization of OBP genes in *Drosophila melanogaster* (Diptera). *J. Exp. Biol. 205*, 719–744.

Vogt, R.G., Rybczynski, R., Cruz, M., Lerner, M.R., **1993**. Ecdysteroid regulation of olfactory protein expression in the developing antenna of the tobacco hawk moth, *Manduca sexta*. *J. Neurobiol. 22*, 581–597.

Vosshall, L.B., **2003a**. Diversity and expression of odorant receptors in *Drosophila*. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 567–591.

Vosshall, L.B., **2003b**. Putting smell on the map. *Trends Neurosci. 26*, 169–170.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., Axel, R., **1999**. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell 96*, 725–736.

Vosshall, L.B., Keller, A., **2003**. Decoding olfaction in *Drosophila*. *Curr. Opin. Neurobiol. 13*, 103–110.

Vosshall, L.B., Wong, A.M., Axel, R., **2000**. An olfactory sensory map in the fly brain. *Cell 102*, 147–159.

Wang, F., Nemes, A., Mendelsohn, M., Axel, R., **1998**. Odorant receptors govern the formation of a precise topographic map. *Cell 93*, 47–60.

Wegener, J.W., Hanke, W., Breer, H., **1997**. Second messenger-controlled membrane conductance in locust (*Locusta, migratoria*) olfactory neurons. *J. Insect Physiol. 43*, 595–605.

Wes, P.D., Bargmann, C.I., **2001**. *C. elegans* odour discrimination requires asymmetric diversity in olfactory neurons. *Nature 410*, 698–701.

Wetzel, C.H., Behrendt, H.-J., Gisselmann, G., Störtkuhl, K.F., Hovemann, B., *et al.*, **2001**. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. *Proc. Natl Acad. Sci. USA 98*, 9377–9380.

Whiting, M.F., Carpenter, J.C., Wheeler, Q.D., Wheeler, W.C., **1997**. The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *System. Bull. 46*, 1–68.

Willett, C.S., **2000a**. Evidence for directional selection acting on pheromone-binding proteins in the genus *Choristoneura*. *Mol. Biol. Evol. 17*, 553–562.

Willett, C.S., **2000b**. Do pheromone binding proteins converge in amino acid sequence when pheromones converge? *J. Mol. Evol. 50*, 175–183.

Willett, C.S., Harrison, R.G., **1999a**. Insights into genome differentiation: pheromone-binding protein variation and population history in the European corn borer (*Ostrinia nubilalis*). *Genetics 153*, 1743–1751.

Willett, C.S., Harrison, R.G., **1999b**. Pheromone binding proteins in the European and Asian corn borers: no protein change associated with pheromone differences. *Insect Biochem. Mol. Biol. 29*, 277–284.

Willis, M.A., Arbas, E.A., **1991**. Odor-modulated upwind flight of the sphinx moth, *Manduca sexta* L. *J. Comp. Physiol. A 169*, 427–440.

Witherow, D.S., Slepak, V.Z., **2003**. A novel kind of G protein heterodimer: the G beta5-RGS complex. *Recept. Channels 9*, 205–212.

Wojtasek, H., Leal, W.S., **1999a**. Conformational change in the pheromone-binding protein from *Bombyx mori* induced by pH and by interaction with membranes. *J. Biol. Chem. 274*, 30950–30956.

Wojtasek, H., Leal, W.S., **1999b**. Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllopertha diversa* (Coleoptera: Carabaeidae), by an insect olfactory cytochrome P450. *FEBS Lett. 458*, 333–336.

Wong, A.M., Wang, J.W., Axel, R., **2002**. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell 109*, 229–241.

Wyatt, T.D., **2003**. Pheromones and Animal Behavior: Communication by Smell and Taste. Cambridge University Press, Cambridge.

Xu, P.X., Zwiebel, L.J., Smith, D.P., **2003**. Identification of a distinct family of genes encoding atypical odorant-binding proteins in the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol. Biol. 12*, 549–560.

Young, J.M., Friedman, C., Williams, E.M., Ross, J.A., Tonnes-Priddy, L., *et al.*, **2002**. Different evolutionary processes shaped the mouse and human olfactory receptor gene families. *Hum. Mol. Genet. 11*, 535–546.

Young, J.M., Trask, B.J., **2002**. The sense of smell: genomics of vertebrate odorant receptors. *Hum. Mol. Genet. 11*, 1153–1160.

Yu, S.J., **1983**. Induction of detoxifying enzymes by allelochemicals and host plants in the fall armyworm. *Pest. Biochem. Physiol. 19*, 330–336.

Yu, S.J., **1984**. Interactions of allelochemicals with detoxication enzymes of insecticide susceptible and resistant fall armyworm. *Pest. Biochem. Physiol. 22*, 60–68.

Yu, S.J., **1989**. Purification and characterization of glutathione transferases from five phytophagous Lepidoptera. *Pest. Biochem. Physiol. 35*, 97–105.

Zhang, S.-G., Maida, R., Steinbrecht, R.A., **2001**. Immunolocalization of odorant-binding proteins in noctuid moths (Insecta, Lepidoptera). *Chem. Senses 26*, 885–896.

Zhang, X., Firestein, S., **2002**. The olfactory receptor gene superfamily of the mouse. *Nature Neurosci. 5*, 124–133.

Ziegelberger, G., **1995**. Redox shift of the pheromone-binding protein in the silkmoth *Antheraea polyphemus*. *Eur. J. Biochem. 232*, 706–711.

Ziegelberger, G., Van Den Berg, M.J., Kaissling, K.-E., Klumpp, S., Schultz, J.E., **1990**. Cyclic GMP levels and gyanylate cyclase activity in pheromone sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *J. Neurosci. 10*, 1217–1225.

Zimmer-Faust, R.K., Gleeson, R.A., Carr, W.E.S., **1988**. The behavioral response of spiny lobsters to ATP: evidence for mediation by P2-like chemosensory receptors. *Biol. Bull. 175*, 167–174.

Zufall, F., Kelliher, K.R., Leinders-Zufall, T., **2002**. Pheromone detection by mammalian vomeronasal neurons. *Microsc. Res. Techn. 58*, 251–260.

Zufall, F., Munger, S.D., **2001**. From odor and pheromone transduction to the organization of the sense of smell. *Trends Neurosci. 24*, 191–193.

Zwiebel, L.J., **2003**. The biochemistry of odor detection and its future prospects. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, London, pp. 371–390.

3.16 Endocrinology of Crustacea and Chelicerata

E S Chang, University of California, Bodega Bay, CA, USA
W R Kaufman, University of Alberta, Edmonton, AB, Canada

3.16.1. General Introduction

The Arthropoda comprise four subphyla (Mitchell *et al.*, 1988): Trilobita (about 4000 extinct species), Crustacea (about 40 000 species), Uniramia (about 1 million species, most of them insects), and Chelicerata (about 65 000 extant species). Although the focus of this series is on the insects, the editors of this volume decided that any thorough treatment of insect endocrinology should include a comparative discussion of the hormones of the other major extant arthropod groups.

Some of the hormones described here are ubiquitous among the Arthropoda (e.g., the ecdysteroids). There are several other hormones, however, that appear to be unique in these groups. Of course these groups cannot be treated as comprehensively in a single chapter as can the insects in an entire volume.

3.16.2. Crustacea

3.16.2.1. Introduction

The major crustacean classes are the Branchiopoda (water fleas and brine shrimp), the Maxillopoda (copepods and barnacles), the Ostracoda, and the Malacostraca (amphipods, isopods, and decapods). The decapods include the familiar crabs, crayfish, shrimp, and lobsters. Crustaceans are found in a wide variety of habitats that include marine, freshwater, and terrestrial environments and range from deep-sea thermal vents to freshwater pools in lightless caves. The adult head of crustaceans bears first and second antennae, mandibles, and first and second maxillae. Various types of appendages can be found on the thorax and abdomen. Although there are numerous orders of crustaceans, the greater part of the endocrinological work has been conducted on the Decapoda.

3.16.2.2. Molting and Metabolism

Arthropods must periodically shed their external, confining exoskeletons and take up air or water to expand their new and larger exoskeletons in order to grow in size. The problem of how to increase in body size is even more formidable for crustaceans due to their mineralized, relatively rigid exoskeletons. The molt cycle is defined as the interval between two successive molts. Drach (1939) divided the molt cycle into a series of stages associated with specific cuticular events from just after ecdysis (postmolt), through a lengthy intermolt period, to the period leading up to a subsequent ecdysis (premolt). These stages have been assigned a number of

substages, depending upon the author and the species of crustacean (e.g., Aiken, 1973, for the lobster *Homarus americanus*).

3.16.2.2.1. Ecdysteroids Horn and his colleagues (Hampshire and Horn, 1966; Horn *et al*., 1966) isolated and determined the structure of 20-hydroxyecdysone (20E) from the rock lobster *Jasus lalandei*. Although the hormone was originally called "crustecdysone" or "ecdysterone," Horn's group demonstrated the identity of 20E as the principal active form of the molting hormone from both insect and crustacean sources. In most species examined, ecdysone (E) is the prohormone secreted by the Y-organ and it is hydroxylated by target tissues to 20E (Chang and O'Connor, 1978). There are exceptions to this, however, as described below. Although 20E is the predominant molting hormone in all decapod species examined to date, other ecdysteroids (steroids structurally related to E that have molting hormone activity) have been characterized in hemolymph and tissues of various crustacean species. These include E; ponasterone A (25-deoxy-20-hydroxyecdysone); inokosterone (25-deoxy-20,26-dihydroxyecdysone); makisterone A (24-methyl-20-hydroxyecdysone); 20,26-dihydroxyecdysone; and 2-deoxyecdysone (reviews: Spindler *et al*., 1980; Skinner, 1985a; Watson *et al*., 1989; Chang, 1993). Comparisons in the absolute concentrations of various ecdysteroids between species are difficult to make due to different detection methods and the lack of standardization in the molt stage of the donor animals.

Classical morphological observations and endocrinological experiments (Gabe, 1953; Echalier, 1959) indicated that the molting gland in the crab *Carcinus maenas* was the thoracic Y-organ. Organ culture of the Y-organs from the crabs *Cancer antennarius* and *Pachygrapsus crassipes* (Chang and O'Connor, 1977) and from the crayfish *Orconectes limosus* (Keller and Schmid, 1979) resulted in the characterization of E as the primary secretory product of the Y-organ.

Other ecdysteroids are secreted by the Y-organs in other crab species: 3-dehydroecdysone from *Cancer antennarius* (Spaziani *et al*., 1989) and 25-deoxyecdysone from *Carcinus maenas* (Lachaise *et al*., 1989; see review by Watson *et al*., 1989). It is not clear why different types of ecdysteroids are secreted by the Y-organs of various species. In almost all cases examined, exogenous ecdysteroids promote progression through the molt cycle (Skinner, 1985b). Associated biological activities attributed to exogenous ecdysteroids include apolysis (separation from the exoskeleton from the underlying epidermis) (Webster, 1983), growth of regenerating limb buds (Rao *et al*., 1972; Hoarau, 1982), formation of gastroliths (McWhinnie *et al*., 1972), increased incorporation of labeled precursors into macromolecules (McWhinnie *et al*., 1972; Dall and Barclay, 1979), increased protein kinase activity (Christ and Sedlmeier, 1987), premolt behavior (Borowsky, 1980), and alterations in hemolymph osmolarity (Charmantier and Trilles, 1976).

Like vertebrate steroid hormones, ecdysteroids recognize target tissues by binding with nuclear receptors. The ecdysteroid receptor has been isolated and characterized from the fiddler crab *Uca pugilator* (Durica and Hopkins, 1996; Chung *et al*., 1998). It has been sequenced and has homologies with insect ecdysteroid receptors (see **Chapter 3.5**). Transcripts for the crab gene were isolated from limb buds and developing ovaries (Durica *et al*., 2002).

3.16.2.2.2. Molt-inhibiting hormone Hemolymph ecdysteroid concentration fluctuates dramatically during the molt cycle (e.g., from $<10\,\text{ng ml}^{-1}$ in postmolt to $>350\,\text{ng ml}^{-1}$ in premolt lobster) (Chang and Bruce, 1980) and these changes mediate the various biochemical and physiological processes that occur during the cycle. The rate of synthesis and/or secretion of E by the Y-organ varies during the molt cycle and partially explains these hemolymph fluctuations in ecdysteroid titer. Just prior to the substage of premolt in which the highest concentration of ecdysteroids was observed in the hemolymph, explanted Y-organs were found to secrete the greatest amount of E (Chang and O'Connor, 1978). Low hemolymph concentrations were correlated with low secretory rates.

Removal of both stalked eyes of *U. pugilator* resulted in a shortening of the molt interval (Zeleny, 1905). This observation lead to the postulation of an endocrine factor present in the eyestalks that normally inhibits molting – a molt-inhibiting hormone (MIH). Detailed microscopical examinations resulted in the description of a neurohemal organ in the eyestalk of several decapod crustaceans (Bliss and Welsh, 1952; Passano, 1953). This neurohemal organ is called the sinus gland and serves as a storage site for neurosecretory products. It consists of the enlarged endings of a group of neurosecretory neurons collectively called the X-organ (Hanström, 1939).

The shortened molt interval observed in eyestalk-ablated decapods is likely due to a rapid elevation in the concentration of circulating ecdysteroids, which is a result of X-organ/sinus gland removal (Chang *et al*., 1976; Chang, 1993). Conversely, the

demonstration of decreased ecdysteroid titers in eyestalk-ablated crabs that have been injected with eyestalk extracts (Hopkins, 1982; Keller and O'Connor, 1982) lends additional support to the paradigm of the MIH–Y-organ molt-controlling axis. Further evidence of this hypothesis was provided by Gersch *et al*. (1980), who demonstrated that if *O. limosus* were initially injected with sinus gland extracts, the subsequent culture of the crayfish Y-organs resulted in a decreased production of E compared to vehicle-injected animals. *In vitro* secretion of E by crab Y-organs could be inhibited when cultured with either conditioned medium that had previously been incubated with explanted sinus glands or by the addition of eyestalk extracts (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1985; Schoettker and Gist, 1990).

Callinectes sapidus MIH has been cloned and the cDNA was used as a probe to show that the amount of MIH mRNA in the eyestalk neural ganglia was negatively correlated with the hemolymph concentration of ecdysteroids; for example, there were low levels of MIH mRNA in the eyestalk during premolt (Lee *et al*., 1998). Similarly, Nakatsuji *et al*. (2000) observed an increased content of MIH in the premolt X-organ/sinus gland in *Procambarus clarkii*.

MIH from *C. maenas* was among the initial MIHs to be characterized (Webster, 1991) (**Figure 1**). It is a member of a novel neuropeptide family, representatives of which have so far been found only in arthropods (Edomi *et al*., 2002). This neuropeptide family regulates such diverse functions as molting, reproduction, and metabolism (Chang, 1993; De Kleijn and Van Herp, 1995; Webster, 1998; Watson *et al*., 2001; Böcking *et al*., 2002). MIH binds to hormone receptors on the membranes of Y-organ cells (Webster, 1993) and likely mediates its action via cyclic nucleotide second messengers (Saidi *et al*., 1994) or through alterations in calcium ion fluxes and activity of protein kinase C that phosphorylates enzymes involved in steroidogenesis (Spaziani *et al*., 2001). (See **Chapter 3.2** for the role of second messengers in the modulation of E synthesis in the insect prothoracic gland.)

3.16.2.2.3. Crustacean hyperglycemic hormone and related neuropeptides Crustacean hyperglycemic hormone (CHH), initially characterized from the crab *C. maenas* (Kegel *et al*., 1989; Weidemann *et al*., 1989), contains a high degree of homology with MIH (**Figure 1**). One important role of CHH is the maintenance of glucose homeostasis. Removal of the sinus glands from *O. limosus*, leaving the remainder of the eyestalk neural tissue and vision intact, resulted in a permanent decline in hemolymph glucose. The circadian rhythmicity of fluctuations in glucose concentration was attenuated (Hamann, 1974). Hyperglycemia is commonly observed under a variety of stressful conditions, and there is considerable indirect evidence to suggest that stress-induced hyperglycemia is caused by the release of CHH (Kleinholz and Keller, 1979; Keller and Sedlmeier, 1988; Webster, 1996). For example, under hypoxic conditions, glucose may be released by CHH from carbohydrate stores to serve as a substrate for glycolysis and lactate formation, as is typical for anaerobiosis of decapod crustaceans. After 4 h of emersion, which causes anoxia, the CHH concentration in lobsters rose from $<10\ \mathrm{fmol\ ml^{-1}}$ to approximately $200\ \mathrm{fmol\ ml^{-1}}$ (Chang *et al*., 1998). In addition to hyperglycemia, other demonstrated actions of CHH in crustaceans include lipid mobilization (Santos *et al*., 1997), secretory activity of the hepatopancreas (Sedlmeier 1988), and second-messenger responses in various tissues (Sedlmeier and Keller 1981; Goy *et al*., 1987).

	1										11										21				
Cam-CHH	pE	I	Y	D	T	S	C	K	G	V		Y	D	R	A	L	F	N	D	L	E	H	V	C	D	D
Cam-MIH	R	V	I	N	D	E	C	P	N	L	I	G	N	R	D	L	Y	K	K	V	E	W	I	C	E	D

						31										41									
Cam-CHH	C	Y	N	L	Y	R	T	S	Y	V	A	S	A	C	R	S	N	C	Y	S	N	L	V	F	R
Cam-MIH	C	S	N	I	F	R	K	T	G	M	A	S	L	C	R	R	N	C	F	F	N	E	D	F	V

	51										61										71						
Cam-CHH	Q	C	M	D	D	L	L	M	M	D	E	F	D	Q	Y	A	R	K	V	Q	M	V_{NH2}					
Cam-MIH	W	C	V	H	A	T	E	R	S	E	E	L	R	D	L	E	E	W	V	G	I	L	G	A	G	R	D

Figure 1 Amino acid sequence of *Carcinus maenas* crustacean hyperglycemic hormone (Cam-CHH) and molt-inhibiting hormone (Cam-MIH) (Kegel *et al*., 1989; Webster, 1991). In *C. maenas* (Weidemann *et al*., 1989) and *Orconectes limosus* (De Kleijn *et al*., 1994), the CHH preprohormone has a 26-amino acid signal peptide. At the C-terminus, the signal peptide is followed by a 38-amino acid CHH precursor-related peptide (CPRP) in *C. maenas*. The CPRP is 33 amino acids long in both *O. limosus* and *Homarus americanus* (Tensen *et al*., 1991; De Kleijn *et al*., 1995). The actual CHH peptide is located at the C-terminal end of the CPRP. MIH from *C. maenas* has 78 amino acid residues with six cysteines and unblocked N- and C-termini. The MIH precursor consists of a 35-amino acid residue signal peptide and the 78-amino acid mature MIH (Klein *et al*., 1993).

Chung *et al.* (1999) observed a dramatic rise in the late premolt concentration of CHH in *C. maenas* compared to intermolt values (over 100-fold). This surge in CHH triggers the massive ion and water uptake during ecdysis and mediates the body's expansion necessary for successful ecdysis. The mechanism of this uptake is unknown, but the source of this premolt surge in CHH is paraneurons in the foregut and hindgut (Webster *et al*., 2000).

Another member of this peptide family is mandibular organ-inhibiting hormone (MOIH). The mandibular organ synthesizes and secretes methyl farnesoate (MF). MF is discussed in detail below (see Section 3.16.2.2.4). MOIH prevents the synthesis and secretion of MF *in vitro*. MOIHs have been characterized from the crabs *Cancer pagurus* (Wainwright *et al*., 1996, 1998) and *Libinia emarginata* (Liu *et al*., 1997). There are indications that additional factors regulate MF synthesis by the mandibular organ. Although injected sinus gland extracts inhibited hemolymph levels of MF by 60–80% in *C. pagurus*, equivalent amounts of injected MOIH had little effect upon MF *in vivo* (Borst *et al*., 2002).

Another member of the CHH hormone family is the vitellogenesis-inhibiting hormone (VIH) from the lobster *H. americanus* (Soyez *et al*., 1991). This peptide was isolated with the use of a heterologous assay involving the inhibition of yolk synthesis in eyestalk-ablated shrimp *Palaemonetes varians*. A VIH was also characterized from an isopod (Grève *et al*., 1999). This peptide should perhaps be renamed "gonad-inhibiting hormone" because it can be found in both sexes of the lobster *Nephrops norvegicus* (Edomi *et al*., 2002).

It is clear that members of the MIH/CHH/MOIH/VIH neuropeptide family are closely related and regulate the major physiological processes of crabs and other crustaceans. This peptide family can be divided into two subgroups (review: Böcking *et al*., 2002). Peptides belonging to the CHH subgroup are encoded by exons that include a translated CHH precursor-related peptide (CPRP). This CPRP is cleaved from the CHH prohormone to produce mature CHH. Genes coding for peptides belonging to the MIH/MOIH/VIH subgroup do not code for a CPRP (**Figure 2**). These hormones may have physiological relevance beyond the process implied by the names given to these peptides.

The use of molecular techniques has permitted a number of studies on the structure and expression of the CHH family genes. In *Carcinus maenas* (Weidemann *et al*., 1989) and *O. limosus* (De Kleijn *et al*., 1994), the CHH preprohormone has a signal peptide at the N-terminus (**Figure 2**). The signal peptide is followed by a CPRP. The actual CHH peptide is located at the C-terminal end of the CPRP. Although the function of the CPRP is unknown, it can be found in the circulating hemolymph and hence may have an endocrine function that has yet to be identified (Wilcockson *et al*., 2002).

Two distinct genes for *O. limosus* CHH have been characterized by De Kleijn *et al.* (1994). The genes code for preprohormones that differ slightly in their signal peptides and their CPRPs, but are identical in their CHH coding regions. The two genes are

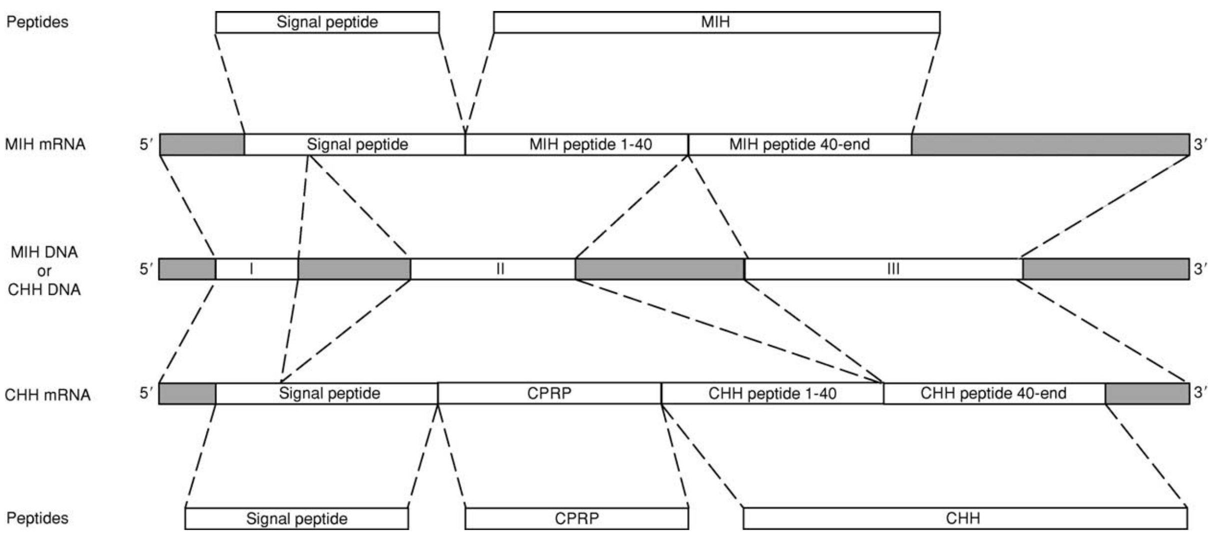


Figure 2 Schematic representation of the DNA, mRNA, and peptide structures of the CHH/MIH family of peptides. The mRNAs and peptides of MIH are shown above the central DNA. The mRNAs and peptides of CHH are shown below the central DNA. CHH, crustacean hyperglycemic hormone; CPRP, CHH precursor-related peptide; MIH, molt-inhibiting hormone. (Reproduced with permission from Böcking, D., Dircksen, H., Keller, R., 2002. The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. In: Wiese, K. (Ed.), The Crustacean Nervous System. Springer, Berlin, pp. 84–97; © Springer-Verlag.)

expressed in different amounts in different individuals. Since both genes code for the identical CHH peptide, the two observed CHH isoforms must be the result of posttranslational modification. In contrast to the ratio of the mRNAs from the two CHH genes, the ratio of the two CHH peptide isoforms is similar in individual crayfish (De Kleijn *et al.*, 1994). De Kleijn *et al.* (1995) also sequenced genes coding for CHH-A and CHH-B in *H. americanus*. They observed DNA sequence variations between the two genes that resulted in amino acid substitutions in the signal peptides, the CPRPs, and the CHH peptides.

Recent research indicates that some of the isoforms seen in the CHH family are due to the occurrence of isomers of specific amino acid residues. Soyez *et al.* (1994) observed two isoforms of both CHH-A and CHH-B and determined that these isoforms were due to the presence of either the L- or D-configuration of phenylalanine at the third position (Phe^3). A similar observation was made in another crayfish by Aguilar *et al.* (1995). Injection of the different isoforms resulted in different temporal responses. [L-Phe^3]CHH-A produced maximal hyperglycemia after 2 h; [D-Phe^3]CHH-A produced its maximal effect after 3–4 h (Soyez *et al.*, 1994). The levels of hyperglycemia produced by the two isoforms, however, were the same. The release rate of each of the isomers in *O. limosus* was similar under basal conditions (Ollivaux and Soyez, 2000).

Yasuda *et al.* (1994) observed a similar phenomenon in the CHH of *Procambarus clarkii* (Prc-CHH). Prc-CHH-I has an L-Phe^3, while Prc-CHH-II has a D-Phe^3. The two isoforms had similar hyperglycemic activities but Prc-CHH-II had ten-fold greater MIH activity in an *in vitro* Y-organ assay. The authors concluded that this isomerism could explain the differential biological effects of CHH (hyperglycemia and molt inhibition).

There are multiple copies of some members of the peptide family. There are at least two copies of the MIH gene and between three and ten copies of the MOIH gene in *Cancer pagurus* (Tang *et al.*, 1999; Lu *et al.*, 2000). The function of these multiple copies and the possibility of differential regulation remain to be determined. Sufficient sequence data have now been accumulated to permit the construction of a dendrogram of the family and the identification of conserved sequence motifs (Lacombe *et al.*, 1999). The arrangement and expression of genes of the CHH family have been reviewed in greater detail by De Kleijn and Van Herp (1995), Van Herp (1998), and Böcking *et al.* (2002).

As more DNA probes for the peptide family become available, researchers will be able to address the factors that influence transcription of the genes. Changing levels of peptide transcripts can be measured throughout the molt cycle (Reddy *et al.*, 1997; Lee *et al.*, 1998). Whether these differences during the molt cycle are due to differential transcription rates (as opposed to differential degradation or processing) remain to be determined.

Progress has been made in the synthesis and expression of recombinant CHH peptides. Using *Escherichia coli*, MIH from *Marsupenaeus japonicus* (Ohira *et al.*, 1999) and from *Metapenaeus ensis* (Gu *et al.*, 2001) were expressed successfully with biological activity. Other expression systems have used the yeast *Pichia pastoris* (Sun, 1997; Ohira *et al.*, 2003) and insect cells with a baculovirus (Lee and Watson, 2002). Crayfish (*Procambarus clarkii*) MIH has been chemically synthesized (Kawakami *et al.*, 2000). The availability of large quantities of biologically active peptides will certainly be important for more detailed physiological studies.

Release of CHH from the X-organ/sinus gland is stimulated by neurotransmitters such as serotonin. When 10 μM of serotonin was applied to crayfish eyestalk neural tissue *in vitro*, CHH secretion increased by 46% over control values (Escamilla-Chimal *et al.*, 2002). Enkephalins may act in an inhibitory manner upon CHH secretion. The application of 1.0 nM of Met-enkephalin and 0.1 μM of Leu-enkephalin resulted in a significant inhibition of CHH by crayfish eyestalk ganglia *in vitro* (Ollivaux *et al.*, 2002).

There are several reports indicating the synthesis and release of CHH family peptides from neurons from noneyestalk locations (Keller *et al.*, 1985; De Kleijn *et al.*, 1995; Chang *et al.*, 1999; Gu *et al.*, 2000; Dircksen *et al.*, 2001; Lu *et al.*, 2001; Yang and Rao, 2001). These locations include the subesophageal, thoracic, and abdominal ganglia, and the second roots of the thoracic ganglia. In the lobster, these ganglia or roots each contain <50 fmol of CHH, compared to the sinus glands, which each contain 1–5 pmol of CHH (Chang *et al.*, 1999). These extra-eyestalk sources of CHH may make small contributions to systemic glucose regulation but may be even more significant in terms of localized regulation of cellular glucose metabolism. For example, under periods of stress, localized release of CHH could be important in meeting elevated metabolic requirements of neural cells. It is also possible that the extra-eyestalk material could serve a neuromodulatory role by altering the response of nerves to transmitters or other hormones. Such a function has been ascribed to other "classical" crustacean eyestalk hormones, such as red pigment-concentrating hormone (RPCH).

Extra-eyestalk CHH may also mediate functions that are distinct from glucose metabolism. As introduced above, paraneurons have been described in distinct areas of the *Carcinus maenas* foregut and hindgut (Chung *et al.*, 1999; Webster *et al.*, 2000). These cells express CHH only during ecdysis and are likely the source of the CHH surge during ecdysis that mediates water uptake. In the crab *Pachygrapsus marmoratus*, CHH from the X-organ/sinus gland was observed to increase sodium flux in the gill (Spanings-Pierrot *et al.*, 2000). In the crayfish *Astacus leptodactylus*, the D-Phe^3 form of X-organ/sinus gland CHH had a higher level of biological activity compared to the L-Phe^3 form when assayed for an increase in hemolymph osmolality (Serrano *et al.*, 2003). An insect representative of the CHH peptide family has been identified in the locust *Schistocerca gregaria* as an ion-transport peptide that mediates ion and water balance (Audsley *et al.*, 1992a, 1992b).

Another extra-eyestalk source of a CHH-like peptide is the pericardial organs. Surprisingly, the C-terminal CHH from these thoracic organs has a different amino acid sequence from that of the X-organ/sinus gland CHH. The function of this peptide is not known since it does not have CHH or MIH activities (Dircksen *et al.*, 2001).

Much has been learned about the structure–function relationships of CHH peptides. The solution structures of CHH and MIH were elucidated by Katayama *et al.* (2003) and the α-helices have been mapped. The N-terminal pyroglutamate of *C. maenas* CHH does not seem to have a significant function compared to an unblocked glutamate, at least in regards to the known activities of CHH (Chung and Webster, 1996). In contrast, the C-terminal valine-amide of *M. japonicus* CHH had 10 times the hyperglycemic activity compared to the CHH with an unmodified C-terminal valine (Katayama *et al.*, 2002).

3.16.2.2.4. Methyl farnesoate The mandibular organ produces a sesquiterpenoid, MF (**Figure 3**), related to the insect juvenile hormone (JH) (see **Chapter 3.7**). Methyl farnesoate was initially isolated from *L. emarginata* (Laufer *et al.*, 1987) and from several other decapods (Borst *et al.*, 1987). Several different effects on crustacean development and reproduction have been attributed to MF (review: Homola and Chang, 1997), including control of the duration of the molt interval. When MF was added to the water in which lobster larvae were cultured (Borst *et al.*, 1987), there was a small but significant increase in the time to metamorphosis compared to control larvae (1.2 day increase with 1 μM MF). These observations are consistent with the hypothesis that MF is acting as a crustacean JH in that it promotes the retention of juvenile characteristics. There were, however, no significant changes in the morphology of the larval lobsters.

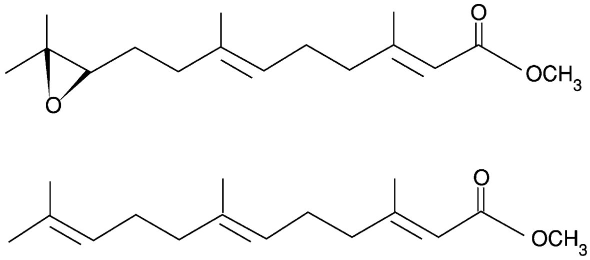


Figure 3 Structure of juvenile hormone III (top) and methyl farnesoate (MF) (bottom).

Yudin *et al.* (1980) observed that implantation of blue crab mandibular organs into white shrimp *Litopenaeus setiferus* resulted in shortened intermolt periods accompanied by more frequent molting and the molt stimulation was not due to ecdysteroid secretion by the mandibular organ. Tamone and Chang (1993) observed that application of MF increases the synthesis and/or secretion of ecdysteroids by the Y-organ. Y-organs from *Cancer magister* were incubated with various concentrations of MF. In the presence of MF, the organs secreted about 50% more ecdysteroids into the medium after 24 h when compared to controls. The magnitude of this stimulation increased with higher concentrations of MF and increasing incubation times. However, this ecdyisotropic effect has not been seen in other species (Spaziani *et al.*, 2001). In addition to molt interval effects, MF inhibits allometric growth of claws in crabs (*Libinia emarginata*). The mechanism of this inhibitory action is unknown (Laufer *et al.*, 2002).

Hemolymph MF-binding proteins have been characterized in the few crustacean species examined. Prestwich *et al.* (1990) characterized a 40 kDa protein in the hemolymph of *H. americanus* that bound a photoaffinity analog of MF (farnesyl diazomethyl ketone) (Ujváry and Prestwich, 1990). Binding of ^{3}H-farnesyl diazomethyl ketone was prevented in the presence of excess unlabeled MF. MF-binding proteins were also observed in the hemolymph of the shrimp *Sicyonia ingentis* (Chang *et al.*, 1992), the crayfish *Procambarus clarkii* (King *et al.*, 1995), and the crabs *L. emarginata* (Li and Borst, 1991) and *C. magister* (Tamone *et al.*, 1997). These binding proteins may facilitate the solubility and transport of MF in the hemolymph since the hormone is very hydrophobic (see **Chapter 3.7** for

studies on the insect juvenile hormone binding proteins).

Methyl farnesoate may also be involved in mediating various stress responses. Hemolymph levels of the hormone rise following challenges with heat stress, hypoxia, and hypo- and hypersalinity (Lovett *et al.*, 1997, 2001), but it is not known how MF helps the crustacean cope with these environmental stresses. Methyl farnesoate also acts as a sex determinant. In *Daphnia magna*, application of physiological amounts of exogenous MF (400 nM) to egg-maturing females resulted in all-male broods. There was a correlation with lower MF concentrations and increasing proportions of males in the broods (Olmstead and LeBlanc, 2002).

3.16.2.3. Pigmentary Hormones

There are two types of pigmentary effectors in the Crustacea: the chromatophores and the retinal pigment cells. The chromatophores are located primarily in the integument and their activities result in the body pigmentation of the individual animal. The retinal pigments are located in the compound eyes and they regulate the amount of light impinging on the rhabdom, the light-sensitive portion of each ommatidium (the functional unit) of the compound eye.

3.16.2.3.1. Color adaptation The chromatophores are pigment-containing cells that occur in the integument and internal organs. Their function is to adjust the body coloration to its surroundings, depending upon the situation (e.g., protection, mating behavior, antagonistic displays). When the pigment granules are spread out along the extensions of the chromatophore, they are described as being in a dispersed state. In this state, the granules are most visible and give the chromatophore its characteristic color. When the granules are withdrawn from the extensions of the chromatophore, they are in a concentrated state and are not as visible.

In addition to hastening the molt, eyestalk ablation of crustaceans results in a dramatic darkening or lightening of the body color (Koller, 1927). The fact that injection of eyestalk extracts could reverse the color changes induced by eyestalk ablation (Perkins, 1928) indicated that the regulation of body color is under hormonal control. Early observations indicated that the sinus gland was a storage site for neurosecretory material that regulated color change (chromatophorotropins). However, the chromatophorotropins are located not only in the X-organ/sinus gland complex, but in other neural tissue as well (see Rao (1985, 2001) and Fingerman (1988) for a more thorough discussion).

α-PDH N[1] S G M I N S I[11] L G I P R V M T E A_{NH2}

RPCH pE[1] L N F S P G W_{NH2}

Figure 4 Amino acid sequences of pigment-dispersing hormone (PDH) and red pigment-concentrating hormone (RPCH) from *Pandullas borealis* (Fernlund and Josefsson, 1968; Fernlund, 1976).

There are two general classes of pigmentary effector hormones: the RPCH and the pigment-dispersing hormone (PDH) (see Section 3.16.2.3.2) families. The first crustacean chromatophorotropin to be sequenced was RPCH from the eyestalks of the shrimp *Pandalus borealis* (Fernlund and Josefsson, 1968, 1972; Fernlund, 1974a, 1974b). Its amino acid sequence is shown in **Figure 4**. Some publications refer to this molecule as erythrophore (red chromatophore) concentrating hormone. In addition to the erythrophores, however, other types of chromatophores are concentrated with RPCH (Josefsson, 1975, 1983a, 1983b).

Red pigment-concentrating hormone is a member of a family of structurally related peptides which includes the adipokinetic hormone (AKH) of insects (see **Chapter 3.10**). Interestingly, although there is biological cross-reactivity between the insects and crustaceans (i.e., crustacean RPCH has AKH activity in insects and vice versa), the functions of the hormones are quite distinct in the two taxonomic groups (i.e., AKH does not appear to alter lipid mobilization in crustaceans).

3.16.2.3.2. Retinal pigments There are three retinal pigments in the compound eyes of shrimp and other decapod crustaceans. This distal retinal pigment is found within cells that extend from the distal ends of the retinular cells to the cornea. The proximal retinal pigment is contained within the retinular cells. The reflecting retinal pigment is contained within cells that are variously located between the neighboring ommatidia, depending upon the species.

These three pigments alter their cellular locations in concert such that their combined activities result in either more or less light impinging upon the rhabdom, depending upon the ambient light intensity. In dim light or darkness, the light-sensitive rhabdom is exposed by the movement of the distal and proximal pigments. In addition, the reflecting pigment is located distally to the basement membrane in a position that permits light to be reflected onto the rhabdom. In bright light, the distal and proximal pigments move to screen the rhabdom from excess light. In addition, the reflecting pigment is positioned adjacent to the basement membrane.

In this position, it does not reflect light onto the rhabdom.

Pigment-dispersing hormone was first isolated and its structure determined from the eyestalks of *P. borealis* (Fernlund, 1976) (**Figure 4**). It was initially referred to as the distal retinal pigment light-adapting hormone because of its action on the distal retinal pigment (Fernlund, 1971). Later studies (Kleinholz, 1975) demonstrated that this molecule was able to disperse the pigment granules in several types of chromatophores and it is now called PDH. Several additional members of the PDH family have been isolated and information has been obtained on the molecular organization of PDH genes. The PDH precursor contains a signal peptide, a precursor-related peptide, a cleavage site, the PDH peptide, a glycine, and one or two basic amino acids (Rao, 2001).

3.16.2.4. Reproduction

3.16.2.4.1. Female Ecdysteroids likely play a role in crustacean reproduction (review: Subramoniam, 2000). Various ecdysteroids have been identified in follicle cells and oocytes of brine shrimp (Spindler *et al.*, 1984), amphipods (Blanchet *et al.*, 1979), crabs (Lachaise *et al.*, 1981; Chaix and de Reggi, 1982; Lachaise and Hoffmann, 1982; Okazaki and Chang, 1991), crayfish (Spindler *et al.*, 1984), and shrimp (Spindler *et al.*, 1987; Wilder *et al.*, 1990). The function of these ovarian ecdysteroids in crustaceans remains uncertain although some correlations between vitellogenesis and hemolymph ecdysteroid titers have been reported (Lachaise *et al.*, 1981; Chaix and de Reggi, 1982). For unknown reasons, the effects of ecdysteroid injections or *in vitro* incubations have been either inhibitory or stimulatory (Adiyodi, 1985; Chang, 1989).

Varying levels of ecdysteroids have also been found in crustacean embryos. The published studies have shown either a general rise (Spindler *et al.*, 1987; Okazaki and Chang, 1991; Chang *et al.*, 1992) or decline (Wilder *et al.*, 1990; Okazaki and Chang, 1991) in ecdysteroids from ovulation to hatch, depending upon the species. In some studies, a greater sampling frequency has demonstrated peaks of ecdysteroids (Chaix and de Reggi, 1982; Lachaise and Hoffmann, 1982; Funke and Spindler, 1987; Spindler *et al.*, 1987; Goudeau *et al.*, 1990).

In addition to its effects upon the molt interval previously described, there are observations that MF is a gonadotropin (compare with the actions of JH in insects (see **Chapter 3.9**). Early, indirect studies involved eyestalk ablation and subsequent ovarian maturation. For example, mandibular organs (the source of MF) from immature female crabs secreted approximately 500 pg of MF per hour, the secretion rate being six times higher in female crabs with maturing oocytes (Laufer *et al.*, 1987; reviewed in Laufer and Biggers, 2001). Direct experiments involved the injection of exogenous MF into eyestalk-ablated crabs, *L. emarginata* (Vogel and Borst, 1989). Following injection, these crabs had an increased level of hemolymph yolk proteins. Enhanced egg production was observed in the shrimp *Litopenaeus vannamei* that had previously been administered exogenous MF (Laufer, 1992). Similar experiments resulted in increased ovarian maturation in the crayfish *Procambarus clarkii* (Laufer *et al.*, 1998; Rodriguez *et al.*, 2002) and the crab *Oziotelphusa senex* (Reddy and Ramamurthi, 1998).

3.16.2.4.2. Male Charniaux-Cotton (1954) proposed various actions of the androgenic gland in a male amphipod (*Orchestia gammarella*). This gland caused masculinization when it was implanted into a female and feminization or dedifferentiation when it was removed from a male (review: Charniaux-Cotton, 1985). Subsequent research used bioassays based upon the masculinization of young female isopods. Protein blotting experiments with polyclonal antisera raised against androgenic gland hormone (AGH) indicated the presence of a larger, biologically inactive protein. It was deduced that AGH was translated as a prohormone (Martin *et al.*, 1990; Hasegawa *et al.*, 1991). From the isopod *Armadillidium vulgare*, Martin *et al.* (1999) and Okuno *et al.* (1999) completed the DNA sequencing of the AGH gene. Pro-AGH consists of a B chain, a C peptide, and an A chain. This suggests that the structure of pro-AGH is analogous to that of vertebrate proinsulin. Recombinant isopod AGH has been produced (Okuno *et al.*, 2002). Secretions from the X-organ/sinus gland complex may regulate the androgenic gland since eyestalk ablation resulted in hypertrophied androgenic glands in crayfish (Khalaila *et al.*, 2002).

Other biologically active factors have been isolated from a crab (*Carcinus maenas*) androgenic gland e.g., the terpenes farnesylacetone and hexahydroxyfarnesylacetone were isolated. They inhibited the incorporation of radiolabeled leucine by ovaries *in vitro* (Ferezou *et al.*, 1977). Farnesylacetone also inhibited radiolabeled uridine incorporation, suggesting that it may inhibit transcription (Berreur-Bonnenfant and Lawrence, 1984). The

role of these terpene androgenic gland factors remains conjectural in decapods. They likely are not the AGH, however (review: Sagi and Khalaila, 2001).

3.16.2.5. Limb Autotomy and Regeneration

Decapods have an amazing ability to regenerate limbs, and crabs have been the primary experimental animals for research in this area. There is a direct correlation between the increase in circulating ecdysteroids during premolt and the growth of limb buds (Hopkins, 1989). Recent evidence indicates that retinoids may be involved in the regenerative process (Hopkins, 2001).

Skinner (1985b) observed that the temporal occurrence of autotomy (self-induced leg loss) during the molt cycle influenced the length of the molt interval. Using the crab *Gecarcinus lateralis*, she observed that loss (and hence initiation of regeneration) of five or more limbs during the intermolt stage resulted in a shortened molt interval. This has lead to the postulation of an endocrine factor that is synthesized/released upon multiple limb autotomy in anecdysis (intermolt) and that shortens the molt interval. This factor is called limb autotomy factor–anecdysial (LAF_{an}) (Holland and Skinner, 1976; Skinner, 1985b).

The other postulated factor, which is thought to be responsible for the proecdysial (premolt) lengthening of the molt interval, is called limb autotomy factor–proecdysial (LAF_{pro}). In *G. lateralis*, autotomy of a partially regenerated limb bud before a critical period during premolt delays molting. A secondary limb bud forms at the autotomized primary limb bud and molting is delayed such that this secondary limb bud is able to catch up in growth to any remaining primary limb buds (if present) and enables the crab to molt with a complete set of walking legs (Mykles, 2001). Yu *et al.* (2002) hypothesize that LAF_{pro} inhibits molting by suppressing Y-organ secretion of ecdysone. Autotomy of primary limb buds reduced the circulating ecdysteroid concentration by 73% after 1 week. Injection of secondary limb buds extracts, but not primary limb bud extracts, resulted in inhibition of primary limb bud growth. Not much is known about the chemical nature of these limb-related factors.

The vertebrate pineal hormone, melatonin, has been characterized in several invertebrate groups, including crustaceans. When it was added to the culture medium of regenerating crabs (*U. pugilator*), the rate of regeneration of their limb buds increased (Tilden *et al.*, 1997). Any relationship between melatonin and the limb autotomy factors is unknown.

3.16.2.6. Conclusions

This is an exciting time for research on crustacean endocrinology. There is increased interest in the topic due to aquaculture applications and the focus on crustaceans as keystone species in aquatic environments. Some key areas of future research include the following:

1. Y-organs secrete several different ecdysteroids. Does an individual species secrete only a single ecdysteroid during the molt cycle or is there a variety of secreted products? Why is there variation in the secreted ecdysteroid amongst species? In eyestalk-ablated crustaceans, which lack MIH, why do circulating levels of ecdysteroids continue to cycle with a peak in late premolt and basal levels in postmolt? There must be regulation of the Y-organ other than simple MIH inhibition.
2. Except for the ecdysteroid receptor, research on crustacean hormone receptors has been minimal. The characterization of MF and peptide hormone receptors will yield insight into the molecular action of these molecules.
3. New functions are being attributed to members of the CHH peptide family. Structure–function studies of the peptides will yield information about key amino acid positions and their resulting biological activities. An example of the significance of using this as a model system is seen with studies on the Phe^3 posttranslational isomerism.
4. Further research on the biological activities and mode of action of MF will be of comparative interest in relation to JH studies in insects. Both the developmental and reproductive aspects of MF need to be addressed.
5. There have been dozens of publications on various narrowly focused aspects of the hormonal control of reproduction. These studies examined neurotransmitters, VIH peptides, ecdysteroids, MF, or vertebrate-like steroids (such as estrogen or progesterone). The interactions between these factors will certainly be difficult to dissect experimentally. However, these experiments will need to be conducted in order to clarify what is currently a very confusing situation.
6. There is growing interest in the field of aquatic endocrine disrupters (deFur *et al.*, 1999). Since much is known about the endocrinology of crustaceans, and since crustaceans are often keystone species in aquatic food webs, there will likely be much more research devoted to the determination of the effects of exogenous chemicals upon

these various hormonal processes. Crustaceans will likely be useful indicator species for environmental contamination.

3.16.3. Chelicerata

3.16.3.1. Introduction

The major chelicerate classes are the Merostomata (horseshoe crabs), which are the closest living relatives of the trilobites (Shuster, 1982), the Pycnogonida (sea spiders), and the Arachnida. The latter is divided into seven minor, and three major, orders, the latter being: Scorpiones (scorpions), Araneae (spiders), and Acari (mites and ticks).

The chelicerate body is divided into an anterior prosoma, which bears the mouthparts and walking legs, and a posterior opisthosoma, which bears appendages in some groups (e.g., the Merostomata) or which lacks them entirely (e.g., the Arachnida). The chelicerates lack antennae, and their mouthparts include a pair of chelicerae, whence the name.

Most of the chelicerates have been neglected by endocrinologists. For example, the Merostomata require about 12 years (and up to 20 molts) to reach sexual maturity (Yamasaki *et al.*, 1988), and it has proven difficult to rear them from egg to adult in the laboratory (Sekiguchi *et al.*, 1988). The Pycnogonida are elusive creatures, found exclusively on the seabed, and very little in general is known about them (King, 1973). Although scorpions are relatively large, are of medical importance, and survive well in captivity, they have extraordinarily low metabolic rates and long generation times (Polis, 1990), characteristics not amenable to the design of endocrinological experiments. As for pseudoscorpions, there have been only about 200 entries in total on them between 1980 and 2004 in *Biological Abstracts*, and none on any aspect of their endocrinology.

Thus, our main focus will be on the endocrinology of spiders, mites, and ticks. Note also that sometimes the most recent endocrinological studies on these groups were published more than 10 years ago, which accounts for why this section of this chapter will seem more dated than most of the material presented in this series.

3.16.3.2. The Neuroendocrine Systems of the Chelicerata

3.16.3.2.1. Merostomata Jegla (1982) hypothesized that the median and/or lateral rudimentary eyes of the so-called "trilobite larva" of horseshoe crabs might be the merostomatan analog of the crustacean X-organ/sinus gland. However, the molt cycle was not significantly affected when he ablated them (laser irradiation) in a systematic manner and at various times between hatching and molting. He concluded:

> We do not know whether horseshoe crabs have a control system for molting that includes a neurohormone and a molting gland, let alone whether there may be an inhibitory control as in crustaceans or a stimulatory one as in insects.

The question remains unexplored since then.

3.16.3.2.2. Pycnogonida Some neurosecretory cells (NSCs) have been recognized in the sea spider brain, and a so-called "Sokolow's organ" has been found beneath the eyes and lateral to the optic lobes. This may be the pycnogonid counterpart of the X-organ/sinus gland of Crustacea, although any evidence for homology or function is absent (King, 1973). A series of paired, segmentally arranged "ventral organs" (possibly neurosecretory structures) develops in association with the neural ganglia. No other putative endocrine tissue has been identified (King, 1973; Arnaud and Bamber, 1987).

3.16.3.2.3. Arachnida: the "minor" orders Legendre (1985) has reviewed what is known about the neuroendocrine systems of seven minor orders of Arachnida: Ricinulei, Uropygi (whipscorpions), Palpigrada (microwhipscorpions), Solifugae (windscorpions), Amblypygi (whipspiders), Pseudoscorpiones, and Opiliones (harvestmen). In all cases, groups of NSCs have been observed (usually by paraldehyde fuchsin staining), at least in the cerebral ganglia. In some cases putative neurohemal organs have also been identified (Solifugae, Amblypygi, Pseudoscorpiones, and Opiliones). Details are provided by Legendre (1985). No further studies relating to their endocrinology were found.

3.16.3.2.4. Arachnida: Scorpiones The cephalothoracic nerve mass of scorpions contains numerous neurosecretory centers distributed in a way that reflects the metameric plan, but their specific functions have not been determined (Root, 1990). A factor isolated from the cephalothoracic mass of the Indian scorpion, *Heterometrus fulvipes*, raises sugar concentration in the hemolymph and in the hepatopancreas, although whether its source is from NSCs is not known (Root, 1990). An antiserum against *Homarus americanus* CHH (Hoa-CHHA) stains specific NSCs and associated structures in *Euscorpius carpathicus*, but the physiological

significance of this material is not known (Stockmann *et al.*, 1997).

Juberthie and Bonaric (1990) review the literature dealing with putative molting glands in scorpions, but for all such suggestions, direct evidence is lacking.

3.16.3.2.5. Arachnida: Araneae The following account is compiled from excellent descriptions of the spider neuroendocrine system by Babu (1973), Bonaric (1981), Legendre (1985), Bonaric *et al.* (1987), and Bonaric and Emerit (1995). The system comprises NSCs in the protocerebrum, two "Schneider's organs" (O.Sch.1 and O.Sch.2), the *Tropfenkomplex* (a neurohemal organ), and a molting gland. NSCs in the brain lead to O.Sch.1. Secretions originating in NSCs and O.Sch.1 enter the hemolymph via the *Tropfenkomplex*. At least one of these neurohormones probably stimulates the molting gland to secrete ecdysone (E) (see Section 3.16.3.3.4). Thus, the O.Sch.1 plus the *Tropfenkomplex* are at least analogous to the corpus cardiacum of insects. The O.Sch.1 may correspond to the intrinsic cells of the corpus cardiacum, but the role of the O.Sch.2 is not known (Bonaric, 1995).

Babu (1973) provided a detailed account of the morphology and secretory cycles of the neuroendocrine system in the orb web spider *Argiope aurantia*. Changes in stainability of the *Tropfenkomplex* generally mirrored those in the NSCs innervating it. During the intermolt of each stage, there is one peak of activity early in the instar, and a second toward the end, suggesting that the second peak is associated with molting. Moreover, NSCs in the cheliceral ganglia first become apparent only at the seventh stage, when the female attains maturity. By the 10th day of the ninth stage there occurs a prominent peak of staining in the cheliceral ganglia. As this is followed by gonad maturation and oviposition, the NSCs in the cheliceral ganglia were suggested to be involved in reproduction.

The cells of the molting gland display an ultrastructure characteristic of steroid-secreting tissue, and the secretory cycle of these cells can be correlated to the molt cycle (Bonaric and Juberthie, 1983; Juberthie and Bonaric, 1990). In nymphs of the Mediterranean spider *Pisaura mirabilis*, NSCs in the brain are active during the first half of the molt cycle, a time at which mitoses are common in the molting gland. The molting gland cells appear very active in the second half of the molt cycle, and this coincides with high levels of hemolymph ecdysteroids (Legendre, 1985). Finally, during the winter diapause, NSC activity is low in the brain and O.Sch.1, and molting does not occur (Legendre, 1985).

3.16.3.2.6. Arachnida: Acari Although the endocrinology of the Acari, especially ticks, is better known than that of any other chelicerate, our knowledge of their hormonal mechanisms remains decades behind that of the insect models covered in this volume. One reason is because not a single endocrine organ has been established with certainty in the Acari. Most mites weigh in the submilligram range, making surgery, ligation, and sampling of hemolymph virtually impossible. There has been much more success with ticks, although surgery remains difficult because the delicate gut frequently ruptures.

The ventral ganglia of insects echo the arthropod metameric pattern. In chelicerates, however, there is a considerable condensation of ganglia, and the extreme expression of this is among the Acari, where the complete central nervous system is represented by a single, anteroventral synganglion, from which all the nerve trunks radiate (Harris and Kaufman, 1984; Binnington, 1987; Evans, 1992).

3.16.3.2.6.1. Neurosecretory centers Neurosecretory centers have been well mapped in several tick species (Eisen *et al.*, 1973; Obenchain, 1974; Obenchain and Oliver, 1975; Binnington and Obenchain, 1982; Marzouk *et al.*, 1985; Binnington, 1986; Prullage *et al.*, 1992). In some cases, secretory activity has been correlated with molting, reproductive stage (virgin versus mated), oviposition and ovipositional diapause (Severino *et al.*, 1984; Hänel, 1986; Shanbaky *et al.*, 1990a). The system described by Prullage *et al.* (1992) is generally similar to those described for other ixodid ticks (Evans, 1992).

3.16.3.2.6.2. The "retrocerebral organ complex" The "retrocerebral organ complex" (ROC) has been postulated as a release site for neurosecretions in the Acari. Whereas the ROC of spiders comprises the two organs of Schneider, the *Tropfenkomplex*, and a number of nerves (Foelix, 1996), in the Acari, the ROC is a somewhat ill-defined anatomical complex of NSCs and glial matrix cells, closely associated with the esophagus as the latter emerges from the mid-posterior region of the synganglion. It is sometimes termed the "paraesophageal organ" (e.g., Roshdy *et al.*, 1973). In the mite *Dermanyssus gallinae*, the ROC is a small group of cells staining positively with paraldehyde fuchsin (Severino *et al.*, 1984). In *Dermacentor variabilis*, three major neurosecretory tracts terminate in the ROC (Obenchain and Oliver, 1975). Because of the presence of neurosecretory terminals and a close association between the sheath enveloping the ROC and the

periganglionic sinus, Obenchain and Oliver (1975) considered the ROC to be a neurohemal organ, a suggestion that has been repeated for other Acari. The staining properties of the ROC change with stages of the life cycle, but in no case is there evidence from bioassay of specific groups of cells to demonstrate a specific function.

Binnington (1987), however, could not find neurosecretory terminals in the ROC of *Boophilus microplus*. Instead, the primary sites of neurosecretory nerve terminals appear near the perineurial sheath and are generally dispersed around the circumference of the synganglion. He suggested that there is no need for a specific neurohemal organ because the aorta connects directly to the perineurial sinus which surrounds the synganglion. Thus, neurosecretory material released anywhere at the perineurial sheath will be carried directly into the main body of the hemocoel (Binnington and Obenchain, 1982). Zhu and Oliver (1991) noted that the insulin-like peptide in NSCs of *Ornithodoros parkeri* also seems to be released diffusely around the neurilemma.

3.16.3.2.6.3. The "lateral segmental organs" Binnington (1981) examined the ultrastructure of the "lateral segmental organs" (LSOs) of *B. microplus* throughout the adult stage. The LSOs present themselves as an anterior pair suspended from pedal nerve I, and a (larger) posterior pair suspended from a salivary gland (SG) nerve arising from the "lateral nerve plexus" (Harris and Kaufman, 1984). This arrangement occurs in many ticks, but in *D. variabilis* and *Argas persicus*, there are four pairs of LSOs. A few axons from the suspending nerves penetrate the LSOs (Binnington, 1981). Each organ consists of up to six similar cells. Golgi bodies are associated with an abundance of smooth endoplasmic reticulum (ER), with cisternae being generally dilated in feeding and freshly engorged females; rough ER is much less abundant (Binnington, 1981). LSOs are apparently absent in *Argas radiatus* (Marzouk *et al.*, 1985) and in *O. parkeri* (Pound and Oliver, 1982).

The LSO cells contain many lipid droplets, especially in ovipositing females (Binnington, 1981). The overall ultrastructure of these gland-like cells, and the apparent increase in activity with feeding and egg production suggest an endocrine function. In support of this hypothesis, the LSOs of engorged *Hyalomma dromedarii* are about double the diameter of those from unfed specimens (Marzouk *et al.*, 1985). Binnington (1981) suggested that the LSOs might secrete ecdysteroids or JH, and it is discussed below (see Section 3.16.3.3.6).

The LSOs and synganglion are stained by the vital dye, Janus Green B, and microsurgical procedures have been performed successfully on the synganglion of partially fed ticks (Kaufman and Harris, 1983; Harris and Kaufman, 1984). Thus, it should be possible to ablate the LSOs in newly engorged females and determine the effect on hemolymph ecdysteroid concentration, SG degeneration, vitellogenesis, egg development, or other physiological parameters.

3.16.3.2.7. Immunocytochemical identification of putative neurohormones There are three groups of NSCs in *O. parkeri*, which are detected by an antiserum to bovine insulin (Zhu and Oliver, 1991). Their function is not known, but they are detected in previtellogenic females and in third instar nymphs. So a general function in carbohydrate metabolism, or in ecdysteroidogenesis (e.g., Zhu *et al.*, 1991; Lomas *et al.*, 1997), rather than a specific role in reproduction, are reasonable possibilities. Davis *et al.* (1994) reported on similar cells in the synganglion of nymphal and adult *D. variabilis* (five of 18 groups of paraldehyde fuchsin positive NSCs are also positive to bovine insulin). The functions of these cells have not been determined although the contents of some of them change with feeding state.

Zhu *et al.* (1995) demonstrated FMRFamide immunoreactivity in many cells of the synganglion of *O. parkeri* and *D. variabilis*. FMRFamide-like material is also present near the perineurial sheath, which Binnington (1987) has suggested functions as a neurohemal organ (see Section 3.16.3.2.6.2). Thus, this material may be released into the hemolymph, but the specific function is not known (Zhu *et al.*, 1995).

3.16.3.3. Molting and Metamorphosis

3.16.3.3.1. Merostomata Horseshoe crabs hatch after undergoing four embryonic molts within 2 weeks postfertilization (Sekiguchi *et al.*, 1988). The (nonfeeding) trilobite larva molts within 20 days when held at 25 °C and a salinity of 30‰ (Jegla, 1982).

Krishnakumaran and Schneiderman (1968) were the first to test the effects of exogenous ecdysteroids on a chelicerate arthropod (mature *Limulus polyphemus*). All of the experimentals (injected with 40 μg 20E g^{-1} bodyweight (bw)) molted, though none completed ecdysis.

The so-called "*Limulus* bioassay," sometimes together with thin-layer chromatography, has been frequently used to detect ecdysteroids in horseshoe crabs. In this assay, the material of interest is

injected into a 1st stage larva, and a positive effect is indicated by apolysis, cuticulogenesis, or ecdysis. Sensitivity of the assay is approximately 200 pg 20E-equivalents (Jegla and Costlow, 1979a), and results are very similar to those achieved by radioimmunoassay (RIA) (Jegla and Costlow, 1979b).

Winget and Herman (1976) took blood from "juvenile" *Limulus* (life stages not specified, but in the weight range of 66–235 g) collected at the earliest stages of premolt. Using the *Limulus* bioassay following thin-layer chromatography, they detected bioactive material in the blood which comigrated with E, 20E, and inokosterone. Jegla and Costlow (1979b) demonstrated that extracts of *Limulus* larvae contained at least three times as much 20E equivalents during the premolt period than during intermolt.

Jegla (1982) described the histological changes of the integument occurring in the trilobite larva during the normal hatch–molt cycle of 20 days. He also reported a reduction of the premolt period by about 50%, as well as histological changes in the integument (apolysis and/or deposition of new cuticle) following injection of 20E. The amount of exogenous hormone necessary to induce molting was high (200 ng per larva) when injected 2–4 days after hatching, and mortality was high. When injected 10–12 days after hatching, however, a similar molting effect occurred at a dose of only 20 pg per larva, and mortality was low (Jegla, 1990).

Jegla (1982) extracted free ecdysteroids from the trilobite larva, resolved them by thin-layer chromatography and analyzed them by RIA and the *Limulus* bioassay. The most abundant ecdysteroid was 20E. The amount of this hormone fluctuated during the molt cycle, but achieved levels exceeding 30 ng g^{-1}.

Jegla (1982) treated eggs, embryos, and trilobite larvae of *Limulus* with farnesol, "*cecropia* oil" (crude extract from male abdomens of *Hyalophora cecropia*), synthetic *cecropia* JH, and two synthetic juvenile hormone analogs (JHAs). Larvae were injected, or treated topically, or immersed; eggs and embryos were immersed, the latter after removal of embryonic membranes. Trilobite larvae (6 days old) injected with an amount of *cecropia* oil known to be effective on *Tenebrio* pupae had no effect on development, and the synthetic JH, synthetic JHAs, and farnesol were toxic, although the doses tested were high.

In summary, ecdysteroid hormones have been detected in the Merostomata where they probably control molting. Evidence for the presence and functions of JH is lacking, and the endocrinology of reproduction has not been explored.

3.16.3.3.2. Pycnogonida Ecdysteroids have been authenticated in pycnogonid eggs, larval stages, and adults (Behrens and Bückmann, 1983; Bückmann *et al.*, 1986). Injection of 20E into 2nd stage larvae (*Pycnogonium litorale*) inhibited feeding and thus delayed molting (Bückmann and Tomaschko, 1992). So instead, the latter authors exposed larvae to 1–1000 ppm 20E in artificial sea water. Molting was accelerated when 20E was added to the sea water during days 5–9 of the larval period (Bückmann and Tomaschko, 1992).

Ecdysteroid levels in *P. litorale* are probably the highest levels recorded for any animal: up to 0.1% of body dry weight (equivalent to almost 600 μM if evenly distributed throughout the living animal) (Tomaschko, 1994). The ecdysteroids are concentrated in the cuticle and are secreted through cuticular pores when the animals are disturbed. Thus, crabs attempting to feed on pycnogonids trigger the release of ecdysteroids (mostly 20E and 20E-22-acetate) in amounts that are clearly distasteful to the predator (Tomaschko, 1994). It is not known why the high concentration in the integument does not disrupt the normal molting cycle of the pycnogonid itself.

3.16.3.3.3. Arachnida: Scorpiones Kumar (1980) injected 0.5–5.0 μg of a synthetic analog of E (3β,14α-dihydroxy-5,3-cholest-7-en-6-one) into the scorpion *Palamnaeus bengalensis* of various age classes (between 1 and 90 days; 0.2–4.5 g bw). In no case was there any sign of molting. El Bakary *et al.* (1987), using high-performance liquid chromatography (HPLC) followed by RIA, determined the whole body ecdysteroid titer in *Leiurus quinquestriatus* through the first and second larval molts. The ecdysteroid titer (mostly in the form of free 20E and more polar metabolites) fluctuated in a manner consistent with a putative role in molting. In adult *Leiurus*, it was possible to collect hemolymph. In males and females (age not specified) the ecdysteroid titer was 1.5 ng ml^{-1}. Ecdysteroid metabolites were also measured and only in the 1st stage larva did free 20E represent a majority of the ecdysteroid (67%). In the other developmental stages, 20E represented only 17–34% of the total ecdysteroid and free E was always lower than 20E. Fluctuating ecdysteroid titers were not correlated with reproductive state, and JH was not detected (El Bakary *et al.*, 1987).

3.16.3.3.4. Arachnida: Araneae and Opilionidae There is considerable evidence that molting in spiders is controlled by an ecdysteroid hormone. Krishnakumaran and Schneiderman (1968, 1970)

were the first to test the effects of exogenous ecdysteroids (~130 μg g^{-1} bw) on a spider (*Araneus cornutus*). Sixty-two percent of the experimentals (compared to 13% of controls) ceased web-spinning, entered apolysis, and secreted a new cuticle 4 weeks after injection, although all died prior to ecdysis (Krishnakumaran and Schneiderman, 1970). Hormonally treated spiders also showed significant cuticular abnormalities.

Krishnakumaran and Schneiderman (1970) performed similar experiments on the tarantula spider *Dugesiella hentzi* (16–25 μg 20E g^{-1}) 3 months after the normal season for molting. Although none molted within 60 days, three of them were injected again with 50 μg, and one injected again with 100 μg g^{-1} 20E. All either underwent apolysis, secretion of a new cuticle, and in one case, ecdysis. An attempt was also made to induce molting by injecting 20 μg 20E into a few isolated opisthosomata of *Araneus* and *Dugesiella*. Although the isolated opisthosomata survived for 7–10 days, none showed signs of molting.

There are 8–11 postembryonic stages in the spider *Pisaura mirabilis* (Bonaric, 1976). Bonaric (1976) injected nymphal stages 7–10 with 10–300 μg 20E g^{-1} bw under a variety of conditions, and determined the percentage of spiders that molted within 10 days of injection compared to controls. At 10–20 μg 20E g^{-1} bw there was a modest decrease of the intermolt period, with 63% molting within 10 days compared to 43 or 50% for the two control groups. At 100–200 μg 20E g^{-1} bw, mortality increased, but among those surviving, 81–86% molted within 10 days of injection. In no case did he observe supernumerary molts. Doses of 250–300 μg 20E g^{-1} bw were toxic.

Bonaric and de Reggi (1977) and Bonaric (1981) recorded whole body ecdysteroid levels (20E-equivalents by RIA) throughout the 22-day 8th nymphal stage of *P. mirabilis*. Ecdysteroid content averaged 150 pmol g^{-1} bw at emergence of the eighth instar, and remained in the range of 100–200 pmol g^{-1} bw for the next 16 days (Bonaric, 1981). Then the ecdysteroid content rose dramatically, peaking at 800 pmol g^{-1} bw on day 19 (2 days before ecdysis to the ninth instar) (Bonaric, 1981). All the immunoreactive material comigrated on a thin-layer chromatography with 20E (Bonaric and de Reggi, 1977). Also, injection of 20E (50 μg g^{-1} bw) at around day 10 of the molting cycle shortened the intermolt duration by several days (Bonaric, 1981).

Several tissues have been suggested as the endocrine source of E in spiders. One is the prosomal gland, an organ believed to be homologous to the prothoracic gland of insects (Bonaric *et al.*, 1984; Foelix, 1996). Another is a diffuse mass of tissue in the opisthosoma of mygalomorph spiders (Legendre, 1981). Similar tissues are found in various spiders, all appearing to be homologous organs (Bonaric, personal communication).

In the femurs of Opilionidae there are large cells resembling the oenocytes of insects (Romer and Gnatzy, 1981). As insect oenocytes synthesize ecdysteroids *in vitro* (Romer *et al.*, 1974; Romer, 1991), Romer and Gnatzy (1981) tested whether the femoral oenocytes of wild-caught harvestmen, *Opilio ravennae*, could do the same. In September and October (prior to winter dormancy), *in vitro* release of E and 20E ranged from 3.5 to 160 ng per sample. Rates of release were much lower (0.2–8.0 ng per sample) by tissues taken from animals that had entered winter diapause. From the experimental design it was not possible to prove that *de novo* synthesis of ecdysteroid had occurred. However, it is reasonable to hypothesize *de novo* synthesis because the epidermal cells of argasid ticks (Zhu *et al.*, 1991) and ixodid ticks (Lomas *et al.*, 1997) are the major source of ecdysteroid in those animals (see Section 3.16.3.3.6).

There has been an attempt to authenticate JH in spiders (Bonaric and Juberthie, 1990). Bonaric (1988), using an RIA following HPLC, found immunoreactive material (equivalent to about 20–80 pmol g^{-1} bw JH diol) during the intermolt period of eighth instar *P. mirabilis*. Although he could not confirm that any of his fractions corresponded to free JH I, JH II, or JH III, he did note the possibility that low levels of JH in the biological samples may not migrate similarly to larger quantities of authentic standard.

Bonaric (1979) injected 10 μg synthetic JH into the abdomens of crickets, which were then fed to 8th and 9th stage nymphs of wild-caught *P. mirabilis*. The crickets were offered to the spiders around the mid-period of the 22-day molt cycle. On consuming a whole cricket, the dose of JH to the spider would have been about 250 μg g^{-1} bw (Bonaric, 1979). After this treatment, neither supernumerary molts nor a delay in the appearance of secondary sexual characteristics was observed, however.

In summary, there is reasonably strong evidence for spiders that ecdysteroids play a normal role in molting, but no evidence that JH plays a morphogenetic role.

3.16.3.3.5. Arachnida: Acari: mites A large mass of the fungivorous mite *Tyrophagus putrescentiae*, at undefined stages of development, was analyzed

by HPLC and gas chromatography–mass spectrometry (GC–MS) (Sakagami *et al.*, 1992). 2-Deoxyecdysone occurred at 53 ng g^{-1} dried whole mites, and E occurred at 0.66 ng g^{-1}. Sakagami *et al.* (1992) also sprayed mites with an acetone solution of 20E (1000 ppm), or incorporated 20E in the yeast used to rear the mites. None of these treatments appeared to affect the molt cycle.

A more systematic study by Chambers *et al.* (1996) on the blood-sucking chicken mite, *Dermanyssus gallinae*, demonstrated a marked increase in ecdysteroid levels over 24 h of feeding (from 0.40 to 1.74 pg per mite in protonymphs, and from 0.98 to 4.01 pg per mite in deuteronymphs). The elevated titers were coincident with apolysis, and preceded ecdysis by less than 24 h. Content of 20E was five times higher than that of E. Ecdysteroid conjugates were present in both polar and apolar fractions, and hydrolysis with esterase and arylsulfatase enzymes increased the amount of RIA-positive material by three- to nine-fold depending on the particular fraction (Chambers *et al.*, 1996).

3.16.3.3.6. Arachnida: Acari: ticks The endocrinology of ticks has been better studied than that of any other chelicerate. In addition to their medical/veterinary importance, their relatively large size makes it possible to sample hemolymph and other tissues, and one can impose some physiological synchrony in an experimental group by feeding them simultaneously.

Ingested or injected ecdysteroids cause supermolting in argasid ticks (Connat *et al.*, 1983a; Diehl *et al.*, 1986), and the metabolic disposition of exogenous, radiolabeled ecdysteroids injected into the hemocoel has been described for several argasid and ixodid ticks (Bouvier *et al.*, 1982; Connat *et al.*, 1984, 1985, 1986, 1988; Wigglesworth *et al.*, 1985; Whitehead *et al.*, 1986; Connat and Dotson, 1988). Injected ecdysteroids are converted rapidly to polar and apolar conjugates, in which form they are stored (especially in the ovaries and eggs). In the hemolymph, the ecdysteroids tend to be mostly free 20E and E. The reader is referred to the foregoing references for further details on ecdysteroid metabolism.

As mentioned above (see Section 3.16.3.2.6.3), the LSOs have been suggested as the endocrine source for either ecdysteroids or JH (Binnington, 1981). Sonenshine *et al.* (1985) demonstrated the presence of ecdysteroids in most tissues of *Hyalomma dromedarii* and *Dermacentor variabilis*, with the highest concentrations appearing in the synganglion. It is noteworthy that when the synganglia were trimmed of the nerve plexi bearing the LSOs, ecdysteroid content was much reduced. When ^{14}C-cholesterol was injected into nymphs, some of it was converted to ecdysteroids or ecdysteroid metabolites. When tissues of the resultant adults were analyzed by autoradiography, the synganglion and lateral nerve plexi/LSOs contained more radioactivity than any other tissue (Sonenshine *et al.*, 1985).

Schreifer *et al.* (1987) demonstrated the synthesis/release of ecdysteroid by *D. variabilis* tissues, including synganglion/lateral plexus, held *in vitro*. They proposed that most of the ecdysteroid originated from the fat body, because this diffuse tissue adheres to virtually all internal organs. The foregoing results thus suggested that the LSOs and fat body are responsible for ecdysteroid synthesis in ixodid ticks, perhaps with the former secreting E and the latter converting it to 20E. Although a role for the fat body in converting E to 20E seems a reasonable conjecture, a major role for the LSOs seems less likely for the following reasons:

1. All ticks molt, but some species do not possess LSOs, e.g., *Argas radiatus* (Marzouk *et al.*, 1985) and *Ornithodoros parkeri* (Pound and Oliver, 1982).
2. Each LSO is composed of only four to six cells (Binnington, 1981), and so there is a maximum of only 25 such cells in a tick (most ticks possess two pairs of LSOs). But the adult female *Amblyomma hebraeum* produces approximately 3–4 μg of ecdysteroid during the gonotrophic cycle (Connat *et al.*, 1986). It seems difficult to imagine that such an amount of ecdysteroid could be produced by so few cells during this time.
3. Contrary to the report of Schreifer *et al.* (1987) on *Dermacentor* mentioned above, isolated synganglia of third instar *O. parkeri* (Zhu *et al.*, 1991) and adult *A. hebraeum* (Lomas *et al.*, 1997) do not synthesize ecdysteroid in culture, whereas the integument does so in both species (Zhu *et al.*, 1991; Lomas *et al.*, 1997). Thus there is autocrine control of molting in both families of ticks.
4. In the case of *A. hebraeum*, a neuropeptide is required to stimulate ecdysteroid synthesis by the integument of adult females *in vitro* (Lomas *et al.*, 1997). Nymphal integument of *O. parkeri*, however, does not require coincubation with a synganglion extract in order to synthesize ecdysteroid *in vitro* (Zhu *et al.*, 1991), even though molting in this species seems to be under neurosecretory control (Oliver and Dotson, 1993). Thus, the reason for this difference between *Ornithodoros* and *Amblyomma* is not obvious.

3.16.3.3.6.1. Family Argasidae The ingestion of ecdysteroids causes supermolting and inhibits egg development and oviposition in female *Ornithodoros moubata* (Mango *et al.*, 1976; Connat *et al.*, 1983a; Diehl *et al.*, 1986). This inhibitory effect on reproduction is usually reversed following the extra ecdysis. Pound *et al.* (1984) injected 20E into adult female *O. parkeri* and induced apolysis and cuticulogenesis.

Dotson *et al.* (1991) observed epidermal ultrastructure of *O. moubata* embryos at 8-h intervals throughout embryogenesis and larval development, and correlated the timing of embryonic and larval molts with whole body ecdysteroid titer in age-matched individuals. Free ecdysteroid content was 5 pg per embryo during the first few days of development, no peaks being associated with the first two embryonic molts. A peak of free ecdysteroid (20 pg per embryo) appeared during the deposition of the larval epicuticle and procuticle. At the larval hatch, ecdysteroid content had fallen back to 10 pg, but then rose during larval apolysis, peaking at 60 pg during deposition of the nymphal epicuticle, and falling back to 10 pg during ecdysis to the first nymph. Most of the esterified ecdysteroid arises from *de novo* synthesis rather than from maternal stores. A peak of ecdysteroid has also been associated with epicuticle formation in fifth instar nymphs (Germond *et al.*, 1982). Throughout embryogenesis most of embryonic ecdysteroid was esterified to fatty acid.

Zhu *et al.* (1994) measured whole body ecdysteroid content (by RIA) in fed third instar *O. parkeri* nymphs, and compared hormone titers to stages of the molt cycle. Most of the ecdysteroids were free E and 20E. Immediately following the meal there was a modest rise in ecdysteroid content from 1.0 to 6.2 $ng\,g^{-1}$ on day 3, at which level it remained for about a week. The ecdysteroid titer then rose again, reaching 23 $ng\,g^{-1}$ at the time of apolysis (day 13), and peaked at 33 $ng\,g^{-1}$ at the time of epicuticle deposition (day 15). The titer fell to 12 $ng\,g^{-1}$ at the time of procuticle deposition (day 17) and remained at that level at the time of ecdysis (day 19).

Fluctuating ecdysteroid concentrations in male *O. parkeri* nymphs correlate well with the molting cycle and spermatogenesis (Zhang *et al.*, 1995), and this will be discussed further below (see Section 3.16.3.4.5.4). In *O. moubata*, there is a peak of ecdysteroid in the middle of each of the five instars although the specific ecdysteroid composition varied somewhat between instars; in all instars, most ecdysteroid is esterified as apolar conjugates (Connat *et al.*, 1997).

In all studies in which immunoreactive material has been further analyzed by HPLC, the major component comigrates with E or 20E (e.g., Wigglesworth *et al.*, 1985; Dotson *et al.*, 1991; Zhu *et al.* 1994). In brief, evidence is compelling that 20E regulates molting in argasid ticks.

Connat *et al.* (1984) injected ^{3}H-ecdysone into female *O. moubata* at various times throughout the gonotrophic cycle of mated and virgin ticks. E was converted into two classes of apolar products (AP1 and AP2), only the second of which was incorporated in eggs. Free E and 20E could be recovered from AP2 following treatment with an esterase, suggesting that ecdysteroid-esters are the main storage form in the eggs. Although the function of these ecdysteroids is not known, the authors hypothesized that they are probably important hormones during embryogenesis, as is the case for some insects. Two later studies, however (Connat and Dotson, 1988; Connat *et al.*, 1988), provided evidence to suggest that AP2 is not normally metabolized to free 20E or E during embryonic development. They currently believe that the presence of AP2 in the egg may be due to nonspecific binding and may simply be one route by which the tick disposes of excess ecdysteroids.

3.16.3.3.6.2. Family Ixodidae Delbecque *et al.* (1978) were the first to authenticate the presence of ecdysteroids in ticks, by extracting ecdysteroids from nymphs at various times postfeeding. Using RIA, they found a peak of ecdysteroid at the beginning of cuticulogenesis (day 17). Using GC–MS, they confirmed the peak to consist of 95% 20E and 5% E.

In adult female *A. hebraeum*, there is a 4.3-fold increase in procuticle weight between the 4th and 7th day of feeding (Connat *et al.*, 1985). This synthesis of procuticle (first recorded by Lees (1952), precedes the rapid phase of engorgement, when the surface area of the integument must increase enormously in less than 24 h to accommodate a ten-fold increase in tick weight. Although whole body ecdysteroid content rises significantly at the time of cuticulogenesis, the hemolymph ecdysteroid titer remains low (10–20 $ng\,ml^{-1}$) (Connat *et al.*, 1985; Mao and Kaufman, 1999), so whether ecdysteroids control procuticle synthesis at this stage of the life cycle is not clear.

Stauffer and Connat (1990) carried out an elaborate study on *Amblyomma variegatum* nymphs through feeding and molting, comparing the ultrastructure of the integument with the ecdysteroid content of the ticks. A small peak of ecdysteroid was seen 3–4 days after dropping from the host

(~4 ng per tick) and a larger peak (~34 ng per tick) 10 days later. In *Amblyomma* nymphs, as just mentioned for adults, synthesis of the endocuticle occurred while ecdysteroid content was also low, again suggesting that cuticle synthesis at this time may be independent of ecdysteroids (Stauffer and Connat, 1990). The small ecdysteroid peak following the meal was coincident with mitoses in the integument and preceded apolysis. The large peak was coincident with epicuticle deposition of the pharate adult several days prior to endocuticle synthesis and ecdysis. Of particular interest in this study was the demonstration that mitosis and apolysis both began in the capitulum of the tick and followed progressively toward the prosoma and opisthosoma.

McDaniel and Oliver (1978) tested the effects of topically applied 20E and two JHAs on embryogenesis, nymphal development and spermatogenesis in *D. variabilis*, but observed mostly toxic effects. Similarly, when eggs of *Ixodes scapularis* were exposed to the JH agonist fenoxycarb, embryogenesis was not disrupted, and larvae hatched normally.

Fumigation of eggs with precocene 2 (P2), which disrupts JH synthesis in the insect corpus allatum, disrupted several parameters of development in *D. variabilis*, but there were no signs of precocious metamorphosis (Hayes and Oliver, 1981). Khalil *et al.* (1983) applied P2 to larvae and nymphs of *H. dromedarii*, either topically or by a type of fumigation by solutions in a petri dish containing ticks. Most nymphs were killed shortly after exposure, but among those that survived, the molting period was not altered, and adult structures were observed. P2 had similar effects on adults of this species (Gaber *et al.*, 1983) and of *D. variabilis* (Dees *et al.*, 1982). Khalil *et al.* (1984) applied JH I topically to *H. dromedarii* nymphs. High doses (50 μg) were toxic. The molting period was slightly prolonged (9–31%) in nymphs treated on the day of engorgement, but not when treated at other times.

In brief, treatment of several ixodid ticks with exogenous JH or precocenes does not influence molting or metamorphosis at less than toxic concentrations, and other attempts to authenticate JH in ticks have been equivocal (see Section 3.16.3.4.5.2). Nevertheless, a few studies demonstrating the presence of enzymes associated with JH metabolism have supported the concept of a JH system in ticks. For example, in *D. variabilis*, a JH-binding protein (Kulcsár *et al.*, 1989) and JH epoxide hydrolase and JH-specific esterase (JHE) (Venkatesh *et al.*, 1990) activities have been demonstrated. JHE activity in the nymphs of *D. variabilis* increased almost threefold 1 day after feeding (Roe *et al.*, 1993); JHE activity fell to low values by day 3, and remained low until molting to the adult. During this period, the ecdysteroid titer rose from barely detectable levels in newly fed nymphs to almost 1 ng per tick on days 10–12 (Dees *et al.*, 1984). Moreover, from Binnington's (1986) hypothesis that the LSOs are the most likely source for JH, Roe *et al.* (1993) tested the synganglion's ability to synthesize JH *in vitro*. They showed that synganglia of fed or unfed females incorporated ^{3}H-methionine into products that comigrated with JH III and the bis-epoxide of JH III (JHB_3) on thin-layer chromatography. From these and other data, Roe *et al.* (1993) hypothesized that elevated JHE activity early in the molting cycle of the nymph normally functions to reduce JH titer. Recalling that JH inhibits prothoracicotropic hormone (see **Chapter 3.2**) secretion in penultimate instar Lepidoptera (Nijhout, 1994), Roe *et al.* (1993) hypothesized further that the JHE-induced reduction of JH might function to trigger molting in the adult.

A more recent and exhaustive study from Roe's laboratory, however, has led to a complete reappraisal of the status of JH in *D. variabilis* and *O. parkeri*. The following results (Neese *et al.*, 2000) indicate strongly that neither of these ticks is able to synthesize a known insect JH:

1. None of seven organs (including synganglion) from several life stages of the two ticks was able to synthesize farnesol, MF, or JH (I, II, III, or JHB_3) *in vitro* from a number of radiolabeled precursors.
2. Using GC–MS, they could find no JH or JH-like compounds in extracts prepared from ticks at various stages of the life cycle, and could detect no juvenilizing activity in any of their tick extracts using a *Galleria* bioassay.
3. Connat *et al.* (1987) reported similar negative results for JH in *B. microplus* using GC–MS, even though their extracts expressed JH activity in two bioassays: inhibition of metamorphosis in pupae of *Tenebrio* and stimulation of oviposition in fed virgin *Ornithodoros*.
4. In all of the above experiments by Neese *et al.* (2000) in which there were negative results for the ticks, they showed positive results using corpora allata from three insects.

In brief, to account for the typical arthropod life stages of one or more larval and nymphal instars, it is assumed that a juvenilizing hormone is produced by ticks. But even though the extracts of at least some ticks do express JH bioactivity in insects, this hormone appears to be sufficiently different from known JHs to be recognized by current physicochemical assays.

3.16.3.4. Reproduction

3.16.3.4.1. Merostomata, Pycnogonida, Scorpiones, and the "Minor" Arachnida No work has been encountered specifically on the endocrinology of reproduction in any of these groups, perhaps for the reasons mentioned above (see Section 3.16.3.1). Only the morphology and histology of the reproductive system have been described for many members of these groups (see Kaufman (1997) for citations).

3.16.3.4.2. Arachnida: Araneae In common with those of other chelicerates, oocytes of spiders are not enveloped with a follicular epithelium, and oocyte development is asynchronous. Trabalon *et al.* (1992) describe the six stages of oocyte development of the spiders *Coelotes terrestris* and *Tegenaria domestica*: previtellogenic growth (stages 1 and 2), the vitellogenic phase (stages 3–5), and the postvitellogenic phase (stage 6). The oocytes of virgins never develop beyond stage 3 (small yolk vesicles only); mating is required to produce mature eggs (stage 6). In both spiders, the hemolymph ecdysteroid concentration (RIA) was low at stage 1 (6–7 ng ml^{-1}) and highest at around the transition between the previtellogenic and vitellogenic stages (25 ng ml^{-1} for *C. terrestris* and 18 ng ml^{-1} for *T. domestica*). Although this correlation is the only information suggesting a role for ecdysteroids in spider reproduction, at least it is in accord with what is known about the vitellogenic hormone in ticks (see Section 3.16.3.4.5.3).

Connat *et al.* (1988) conducted a study on the ability of five species of spider to metabolize ecdysteroids. Radiolabeled E or 20E was injected into the spider, or into a sample of its food (fly larvae), or into a drop of tissue culture Medium 199 for thirsty spiders (deprived of water for a week) to drink. The ecdysteroids were converted rapidly into polar and apolar metabolites. In the case of spiders which imbibed about 1 µg 20E in 20 µl Medium 199, all the hormones were metabolized within 15 min. However, spiders refused to eat larvae or drink fluid which had too high ecdysteroid content (>100 µM E) (Connat *et al.*, 1988): recall (see Section 3.16.3.3.2) that ecdysteroids are stored in the integument of pycnogonids as a feeding deterrent to crabs (Tomaschko, 1994).

3.16.3.4.3. Arachnida: Acari: mites Fecundity of the chicken mite, *Dermanyssus gallinae*, was much reduced following treatment with P2 for 4 days, but partially recovered following treatment with JH III (Oliver *et al.*, 1985). Fenoxycarb stimulated egg production in the grain mite *Acarus siro* (Thind and Edwards, 1990) but fenoxycarb, methoprene, and hydroprene were all toxic to the house-dust mite, *Dermatophagoides farinae* (Downing *et al.*, 1990).

It has been suggested that the gonotrophic cycle of mites that parasitize bee larvae may be influenced by the hormonal milieu of the host. *Varroa* mites produce eggs only when parasitizing the brood. Mites show a marked preference for drone broods and fecundity is higher in drone cells (Beetsma and Zonneveld, 1992; Boot *et al.*, 1992). Mites begin egg production a few days after the cell is capped, about five or six eggs being laid over 30 h (Beetsma and Zonneveld, 1992). The first egg (Beetsma and Zonneveld, 1992) or the second egg (Hänel, 1986) is usually a male, and the remainder are female. The male usually copulates with his sisters, and the bee and mites emerge from the cell.

Hänel (1986) correlated NSC activity in the *Varroa* synganglion with specific stages of the life cycle, and demonstrated that these changes could be induced by spraying mites with JH III. Moreover, there are correlations between mite fertility and hemolymph JH titer (RIA) in bee larvae (*Apis cerana indica* and *A. mellifera ligustica*) (Rosenkranz *et al.*, 1993):

- In worker larvae, hemolymph titer of JH (<10 pmol ml^{-1} at the time of cell capping) rose to 70–100 pmol ml^{-1} within 2 days. In the drones, hemolymph titer of JH was 50 pmol ml^{-1} at time of cell capping, and the peak value (170 pmol ml^{-1}) occurred at 3.5 days (see **Chapter 3.13**).
- There was a lower mite infestation of worker cells (4%) than drone cells (20%).
- None of the mites infesting the workers were fertile compared to 90% of the mites infesting drones.

Moreover, the sterol composition of *Varroa jacobsoni* (analyzed by GC–MS) reared on *A. mellifera* drones showed some similarities to that of the bee, with 24-methylenecholesterol being the major sterol in both species (Feldlaufer and Hartfelder, 1997). The fact that the major ecdysteroid in the bee drones is makisterone A, whereas E and 20E together account for 66% of the ecdysteroid in the mite, indicates that the latter can carry out independent synthesis of C-27 ecdysteroids from cholesterol (Feldlaufer and Hartfelder, 1997).

However, there is also the suggestion that the above correlations may be spurious. For example, Rosenkranz *et al.* (1990) examined two colonies of *A. mellifera* from Brazil and two from Germany, in which the *Varroa* infestation varied markedly.

There was little difference in JH titer among the colonies, however. In *A. mellifera carnica*, there are substantial caste differences for hemolymph JH and ecdysteroid titer in the last larval instar (Rachinsky *et al*., 1990). However, whereas the differences in JH occurred throughout the instar, those for the ecdysteroids occurred only during the spinning and prepupal stages. In brief, more direct evidence for the effect of the host's hormonal status on the endocrinology of *Varroa* reproduction is required.

3.16.3.4.4. Arachnida: Acari: ticks: family Argasidae In *O. moubata*, fertilization (which may occur before or after the meal) and blood-feeding (about 20–60 min) are both required to develop a normal clutch of eggs (Aeschlimann and Grandjean, 1973). Each female lays about 80–150 eggs following a preoviposition period of 10–15 days.

When virgin *O. moubata* engorge, vitellogenesis begins, but yolk resorption follows within 1 month, and the ovaries resemble the virgin condition by the third month; this regression is called "abortive vitellogenesis" (Germond and Aeschlimann, 1977). Such virgins will lay at least a small clutch of eggs, even if "delayed copulation" occurs up to 6 months following the original meal (Aeschlimann and Grandjean, 1973; Connat *et al*., 1986).

3.16.3.4.4.1. Neuroendocrine control of egg development Shanbaky and Khalil (1975) ligated ticks (*Argas* (*Persicargas*) *arboreus*) after feeding, such that the synganglion was isolated from the ovary. All mated females ligated 4 or 5 days after feeding developed their ova, but only 10% of ticks ligated 1 h after feeding did so. Thus, blood-feeding triggers the gradual release of a factor in the anterior region of the hemocoel which stimulates ovarian development. Ticks ligated 1–3 days after feeding showed intermediate values, indicating that the critical period for oocyte development is not narrowly defined in this tick. Oliver *et al.* (1992) arrived at a similar conclusion for *O. parkeri*.

Shanbaky and Khalil (1975) also tested the effect of injecting synganglion or hemolymph extracts (taken 1 h or 3 days after feeding) into the posterior compartment of ligated, mated females. Extracts of synganglion or hemolymph (3 days) stimulated oocyte development. Unfed, normal females did not respond to synganglion extracts, thus emphasizing the necessity of a blood meal for egg development in *A. arboreus*. In contrast, Chinzei *et al.* (1989a) and Taylor *et al.* (1991a) showed that egg development in *O. moubata* can occur in the absence of a blood meal.

Shanbaky *et al.* (1990b) monitored hemolymph vitellogenin (Vg) titer following injection of synganglion extracts into ligated female *Argas hermanni*. Normal vitellogenesis occurred even anterior to the ligature, indicating that the ovary itself does not participate in Vg synthesis. No Vg appeared in the hemolymph posterior to the ligature. Synganglion extracts also stimulate vitellogenesis when injected into fed virgin or mated *O. parkeri*, with activity occurring in both the supra- and subesophageal ganglia (Oliver *et al*., 1992).

The foregoing studies show that the synganglion contains a "vitellogenesis inducing factor" (VIF), but do not prove the NSCs as its source. Pyrethroid insecticides trigger the release of neurohormones in insects (Orchard and Osborne, 1979; Orchard, 1980; Ruight, 1985). Chinzei *et al.* (1989a) and Taylor *et al.* (1991a) showed that pyrethroid insecticides such as cypermethrin induce vitellogenesis and yolk uptake (but not oviposition) in unfed, mated, or virgin *O. moubata*. Cypermethrin induces Vg synthesis even in fourth instar nymphs (Taylor *et al*., 1991a). Indeed, the hemolymph Vg titer in cypermethrin-treated nymphs was higher than in similarly treated adult females, which the authors attribute to the inability of the prospective ovarian tissue to take up Vg. Cypermethrin stimulated Vg synthesis only when injected into the anterior of ligated females, further implicating the synganglion as its target (Chinzei *et al*., 1992).

3.16.3.4.4.2. The vitellogenic hormone Chinzei and Yano (1985) demonstrated that, as in insects, the fat body is the major site of vitellogenesis in *O. moubata* (see **Chapter 3.9**). Since then, several laboratories have described the course of vitellogenesis in argasid ticks e.g., *O. moubata* (Chinzei *et al*., 1989b; Chinzei and Taylor, 1990) and *A. hermanni* (Shanbaky *et al*., 1990c).

Not surprisingly, JH and related compounds have been tested as putative vitellogenic hormones in ticks. The following observations come from only a selection of studies that support a role for JH in argasid tick reproduction:

- The insect JHs and a number of related agonists all stimulated a normal degree of oviposition in fed virgin *O. moubata* (Connat *et al*., 1983b)
- The winter reproductive diapause of *A. arboreus* can be broken by a JHA (Bassal and Roshdy, 1974)
- P2 suppresses oogenesis in *O. parkeri*, an effect that is partially reversed by JH treatment (Pound and Oliver, 1979); P2 likewise induces sterility in

A. persicus and *O. coriaceus* (Leahy and Booth, 1980).

The following, however, do not support a role for JH in argasid tick reproduction (see also Connat and Nepa, 1990; Chinzei and Taylor, 1994):

- Although unfed *O. moubata* can express a normal degree of vitellogenesis when treated with cypermethrin (see Section 3.16.3.4.4.1), treatment with JH (I, II, or III) or any of four JHAs was totally ineffective in stimulating egg development (Chinzei *et al.*, 1991). Results for *O. parkeri* were similar (Taylor *et al.*, 1991b)
- P1 and P2 did not inhibit oocyte development nor did it reduce the hemolymph Vg titer in *O. moubata* (Taylor *et al.*, 1992). At best, high doses of P2 reduced oviposition in the surviving ticks.

Resolving these disparate conclusions will be no easy matter. Kaufman (1997) concluded:

> The only way [out] is to assume that JH acts on some system which becomes competent only following feeding, or that the primary role of JH in argasids is to stimulate oviposition only.

It can be seen below (Section 3.16.3.4.5.2) that studies with JH on ixodid ticks lead to similar discrepancies.

3.16.3.4.4.3. A model for hormonal control of egg production in argasid ticks VIF, a peptide from the synganglion, triggers vitellogenesis (see Section 3.16.3.4.4.1). Chinzei and Taylor (1990) showed that VIF acts somewhere in the posterior of the tick to produce a "fat body-stimulating factor," which in turn stimulates the fat body to synthesize and release Vg. The posterior compartment of ligated *O. moubata* can produce Vg if ligated 1 h after detachment, but in the anterior compartment does not produce Vg unless ligated 3 days after detachment. Thus, within an hour of detachment, VIF is present throughout the hemolymph. The fact that Vg appears in the posterior but not in the anterior compartment under these conditions indicates that an essential factor, produced by a tissue in the opisthosoma under stimulation by VIF, is required for vitellogenesis. Chinzei and Taylor (1994) and Taylor *et al.* (2000) speculate that the fat body-stimulating factor might be an ecdysteroid, because E and 20E increase Vg titer in the hemolymph of unfed mated females. Moreover, in mated *O. moubata*, there are peaks in the whole body and hemolymph ecdysteroid titer that correlate well with vitellogenesis and oviposition; in virgins, the ecdysteroid titer remains low throughout the 20-day postengorgement period (Ogihara, 2003). All this is in harmony with what is known for ixodid ticks (see Section 3.16.3.4.5.3). One potential problem with Chinzei and Taylor's (1994) model is that cypermethrin should not stimulate vitellogenesis if injected into either the anterior or posterior compartment of a ligated tick, as the posterior compartment lacks the synganglion, and the anterior compartment lacks the tissue source of a fat body-stimulating factor. And yet, Chinzei *et al.* (1992) reported that cypermethrin stimulated Vg synthesis when injected into the anterior compartment. To our knowledge, this anomaly has not yet been resolved.

3.16.3.4.5. Arachnida: Acari: ticks: family Ixodidae

3.16.3.4.5.1. Vitellogenin synthesis The fat body and some midgut cells are two major sites of Vg synthesis in *Dermacentor variabilis* and *Rhipicephalus appendiculatus* (Coons *et al.*, 1982, 1986, 1989; Rosell and Coons, 1990). In *D. variabilis*, synthesis of Vg begins early in feeding and the hemolymph Vg titer attains 430 μg ml^{-1} hemolymph when oviposition is under way (Rosell and Coons, 1991). The Vg concentration in the hemolymph of *Amblyomma hebraeum* peaks at almost 100 times this value (Friesen and Kaufman, 2002), a difference for which no firm explanation is available.

Recall that vitellogenesis in argasid ticks is triggered by a neuropeptide (VIF) (see Section 3.16.3.4.4.3). Oliver and Dotson (1993) state that fat body from fed virgin *H. dromedarii* increases Vg synthesis *in vitro* when exposed to synganglion extracts or to hemolymph from engorged females 2 days post detachment; this suggests that ixodids also use a neuropeptide to trigger vitellogenesis. Friesen and Kaufman (2003) tested this hypothesis by attempting to stimulate vitellogenesis in partially fed *A. hebraeum* using cypermethrin. Unlike what occurs in argasid ticks (see Section 3.16.3.4.4.1), however, cypermethrin failed to stimulate vitellogenesis at sublethal doses. Nevertheless, the strongest support for a neuropeptide triggering vitellogenesis in an ixodid tick comes from Lomas *et al.* (1997), who demonstrated that integument of adult female *A. hebraeum* does not synthesize ecdysteroids *in vitro* unless exposed to a neuropeptide (see Section 3.16.3.3.6); strong evidence that the vitellogenic hormone in ixodid ticks is an ecdysteroid is presented below (see Section 3.16.3.4.5.3).

Lunke and Kaufman (1993) demonstrated hormonal control of vitellogenesis by transplanting pieces of ovary into the hemocoels of engorged

females undergoing active vitellogenesis. The oocytes of those explanted ovaries which survived for 8 days were at an advanced stage of development. It is convenient to consider JH and ecdysteroids separately as the putative vitellogenic hormone.

3.16.3.4.5.2. Juvenile hormone and reproduction The earliest suggestions that JH might control vitellogenesis in ixodid ticks came from studies in which JH, JHAs, and precocenes were used to inhibit egg development. In most cases, however, the effects of these hormones and drugs may have been the result of nonspecific toxicity (Bassal, 1974; Mansingh and Rawlins, 1977; Hafez and Bassal, 1980; Leahy and Booth, 1980; Dees *et al.*, 1982; Gaber *et al.*, 1983; Khalil *et al.*, 1983, 1984; Connat, 1988; Slusser and Sonenshine, 1992). Alternatively, Hayes and Oliver (1981) fumigated eggs of *D. variabilis* with P2. This treatment caused a reduction in female-to-male ratio (from 1.1 : 1.0 for control versus 0.5 : 1.0 for P2), and an 85% reduction in the number of females completing the gonotrophic cycle. Such females treated with 1 μg JH III, 24 h prior to feeding (plus another 1 μg 6 days later) all completed the gonotrophic cycle, suggesting that P2 may have a specific anti-JH effect in this species. Moreover, the existence of a JH-binding protein (Kulcsár *et al.*, 1989) and JH esterase (Roe *et al.*, 1993) in ixodid ticks constitutes at least circumstantial evidence for JH. Finally, as mentioned above (see Section 3.16.3.3.6.2), hexane extracts of vitellogenic *B. microplus* possessed JH bioactivity, and hemolymph extracts from such females were recognized by antisera created against JH I, JH II, and JH III. But, similar to the findings of Neese *et al.* (2000), no JH 0, JH I, JH II, JH III, or MF were detected in the extracts by GC–MS (Connat *et al.*, 1987).

The following studies, however, diminish support that JH is the vitellogenic hormone:

1. Lunke and Kaufman (1993) could not induce egg development in partially fed female *A. hebraeum* by injections of JH, JHB_3, or MF. Nor could they inhibit egg development in fully engorged mated females by injections of precocene.
2. Methoprene does not stimulate the fat body of *D. variabilis* to synthesizes Vg *in vitro* (Sankhon *et al.*, 1999). Likewise, neither JH III nor triiodothyronine (a JH mimic in *Rhodnius*) (Davey and Gordon, 1996; Kim *et al.*, 1999) stimulated vitellogenesis when injected into partially fed *A. hebraeum* (Friesen and Kaufman, 2004).

In brief, the balance of evidence indicates that JH is not the vitellogenic hormone, but the nature of the substance in tick extracts having JH-like activity (Connat *et al.*, 1987), and what its role in reproduction might be, have not been adequately explained.

3.16.3.4.5.3. Effect of ecdysteroids on vitellogenesis There is reasonably firm evidence, however, that ecdysteroids regulate vitellogenesis in ixodid ticks:

1. The hemolymph ecdysteroid titer rises 10–100-fold during the first week postengorgement in female *A. hebraeum* (Connat *et al.*, 1985; Kaufman, 1991a; Friesen and Kaufman, 2002), coincident with maximum Vg synthesis. A similar profile occurs in *B. microplus*, although in this species, the peak ecdysteroid titer (70 ng ml^{-1}) is only about 12% that of *A. hebraeum* (600 ng ml^{-1}) (Connat *et al.*, 1986).
2. Ecdysteroids trigger SG degeneration within 4 days following engorgement (see Section 3.16.3.5.2), but hemolymph ecdysteroid levels continue to rise steeply for several days. The hormone titer peaks (at about five- to tenfold higher than the concentration necessary for SG degeneration) when Vg synthesis is most rapid (Kaufman, 1991a; Rosell and Coons, 1991; Lunke and Kaufman, 1993; Mao and Kaufman, 1999; Friesen and Kaufman, 2002).
3. James *et al.* (1997) monitored Vg synthetic activity of fat body *in vitro* and ecdysteroid content in whole body extracts of *I. scapularis* throughout the feeding and reproductive period. The observed rise and fall in ecdysteroid content, 1–2 days before the rise and fall of Vg synthetic activity in the fat body, is the pattern to be expected if vitellogenesis is controlled by an ecdysteroid.
4. 20E stimulates Vg synthesis by fat body of *D. variabilis* cultured *in vitro*; whereas the JH mimic methoprene had no such effect (Sankhon *et al.*, 1999). Friesen and Kaufman (2002) demonstrated that a physiological dose of 20E likewise triggers Vg synthesis in partially fed *A. hebraeum*, whereas JH does not.

Notwithstanding the strong evidence that an ecdysteroid serves as the vitellogenic hormone in ixodid ticks, why 20E is incapable of stimulating yolk uptake in partially fed ticks remains a puzzle. For example, Lunke and Kaufman (1993) infused 20E into partially fed females such that a high titer was maintained in the hemolymph over 24 h. Although the fat body became swollen (interpreted as a sign of active vitellogenesis), the oocytes did

not take up yolk. Treating engorged females with 20E (or 20E plus JH) did not hasten ovarian development compared to controls (Lunke and Kaufman, 1993). Although Friesen and Kaufman (2002) demonstrated that injected 20E stimulates Vg synthesis and release into the hemolymph, they confirmed Lunke and Kaufman's (1993) observation that oocyte development does not then follow. JH induces patency in the follicular epithelium of *Rhodnius* (Davey and Gordon, 1996), thus allowing the yolk direct access to the oocyte. Recall, however, that in chelicerates the eggs are not enveloped in a follicular epithelium (see Section 3.16.3.4.2), so there is unlikely to be a permeability barrier equivalent to the follicular epithelium that inhibits yolk uptake. Friesen and Kaufman (2004) considered the possibility that a Vg receptor may be downregulated in partially fed ticks. However, they identified an 86 kDa protein in the ovary as a putative Vg receptor, and found that it was present in partially fed females by the time they weighed 100 mg. Because 20E does not stimulate Vg uptake in ticks weighing 100–250 mg, downregulation of the Vg receptor also does not explain the inability of 20E to stimulate oocyte development. However, oocytes from partially fed females do take up yolk when ovaries are transplanted to the hemocoel of engorged females (Lunke and Kaufman, 1993). Taken together, all this suggests that some factor in engorged female hemolymph, distinct from the vitellogenic hormone, regulates yolk uptake.

The avermectins are broad-spectrum antiparasitic compounds that inhibit oviposition in ticks (Kaufman *et al.*, 1986). The avermectin analog MK-243 causes a marked reduction in hemolymph ecdysteroid titer and marked inhibition of ovarian development in engorged female *A. hebraeum* (Lunke and Kaufman, 1992). But whether MK-243 acts primarily by inhibiting ecdysteroid synthesis, Vg synthesis, or Vg uptake could not be determined until an enzyme-linked immunosorbent assay (ELISA) for Vg was available. Friesen *et al.* (2003) demonstrated that MK-243 did not significantly inhibit hemolymph Vg titer, and injections of 20E into MK-243-treated ticks did not restore measures of ovarian development. MK-243 may thus exert its major effect by inhibiting yolk uptake by the oocyte.

3.16.3.4.5.4. Hormonal control of spermatogenesis in ticks Hormonal control of spermatogenesis was reviewed by Oliver and Dotson (1993). Zhang *et al.* (1995) measured ecdysteroid content in extracts of fourth instar male *O. parkeri* nymphs throughout feeding and molting to the adult. There were two peaks of ecdysteroid, the first about 2 weeks after feeding (106 pg per tick; coincident with the late prophase of meiotic division I) and the second during the third week after feeding (277 pg per tick; coincident with epicuticle deposition). The later peak is undoubtedly associated with molting (ecdysis occurs around day 24–25). However, the authors suggest a role for 20E in spermatogenesis as well because (1) the earlier peak of ecdysteroid correlates with a key point in spermatogenesis and is not seen in female nymphs (Zhang *et al.*, 1995) and (2) 20E stimulates DNA synthesis in germ cells of unfed, male *D. variabilis* (Oliver, 1986).

3.16.3.5. Salivary Gland Development and Degeneration in Female Ixodid Ticks

Female ticks of the family Ixodidae feed to ~100 times their unfed weight (males barely double their weight during feeding). The meal is imbibed in two phases. During the slow phase (~7–10 days in *A. hebraeum*), tick weight increases ~10-fold; during the rapid phase (~1 day), tick weight increases a further tenfold.

3.16.3.5.1. Salivary gland development The SGs of unfed females secrete fluid *in vitro* very slowly (~4 nl min^{-1} in *Dermacentor andersoni*), but by the rapid phase of engorgement, the glands secrete at 217 nl min^{-1} (Kaufman, 1976). Coons and Kaufman (1988) transplanted SGs of unfed females into the hemocoels of partially fed females, and after several more days of feeding, ultrastructural changes characteristic of SG development were induced in the transplanted glands. Male SGs normally undergo much less development during feeding compared to female glands. When transplanted into the hemocoels of feeding females, however, development was similar to that of transplanted female glands. Thus, SG development in females seems to be controlled by a yet to be characterized humoral factor. It is interesting that the male SG seems to respond to a hormone that it normally would never encounter.

3.16.3.5.2. Salivary gland degeneration Following engorgement, the SGs undergo autolysis. Transplanting SGs from partially fed specimens to the hemocoels of engorged, but not partially fed, females induces SG degeneration in the transplanted tissue (Harris and Kaufman, 1981). Thus, SG degeneration is controlled by a hormone which Harris and Kaufman (1985) demonstrated to be an ecdysteroid. This hormone is released when females exceed a critical weight of approximately 10 times the unfed weight (Harris and Kaufman, 1984). Sensory

information from nerves in the opisthosoma is part of the pathway for the release of ecdysteroid (Harris and Kaufman, 1984).

Several studies indicate that the tick SG ecdysteroid receptor (EcR) is similar in many respects to the EcR of insects:

1. Only steroids that possess the structural requirements for ecdysteroid activity in insect systems (Horn and Bergamasco, 1985) (see **Chapter 3.3**) also trigger degeneration in tick SGs (Lindsay and Kaufman, 1988; Kaufman, 1991b).
2. Scatchard analysis of ^{3}H-ponasterone A binding to a cytosolic fraction of SGs from large partially fed ticks indicated a K_d and B_{max} of 0.72 nM and 175 fmol mg^{-1} protein, respectively (Mao *et al.*, 1995). Only steroids that meet the Horn and Bergamasco (1985) requirements are able to compete effectively for ponasterone A binding (Mao *et al.*, 1995).
3. Binding of the SG EcR of *A. hebraeum* (AheEcR) to *Drosophila* heatshock protein (hsp)27 EcR response element (EcRE) depends on heterodimerization with an ultraspiracle (USP)-like protein (see **Chapter 3.5**), and biologically effective ecdysteroids enhance this binding. Monoclonal antibodies against *Drosophila* EcR and USP reveal the tick equivalents on Western blots. Three bands react with the EcR antibody and, at any given time, between two to five react with the USP antibody (Mao and Kaufman, 1999). The hsp27 EcRE binding complex is shifted (gel mobility shift assay) by the *Drosophila* monoclonal antibody against USP (Mao and Kaufman, 1998).
4. Guo *et al.* (1997) isolated cDNAs from *Amblyomma americanum* that encode three EcR isoforms (AamEcRs) which have common DNA- and ligand-binding domains, but distinct amino termini that lack the F domain of insect EcRs. The common domains are 86 and 64% identical to the DNA- and ligand-binding domains, respectively, of insect EcRs. Guo *et al.* (1998) also isolated cDNAs encoding two retinoid X receptor (RXR) isoforms (AamRXR1 and AamRXR2), the DNA-binding domains sharing 95 and 87% identity with DNA-binding domains of insect USP and vertebrate RXR. The ligand-binding domains, however, share greater similarity with the vertebrate RXR (71%) than insect USP (52%). A functional receptor emerged when a monkey kidney cell line (containing luciferase reporter constructs and natural EcREs from *Drosophila*) was cotransfected with AamEcRA1 and either AamRXR1 or hRXR, and stimulated with muristerone A (Guo *et al.*, 1997).
5. Mao and Kaufman (1999), using Western blot analysis with *Drosophila* monoclonal antibodies, demonstrated the ontogeny of AheEcR and the USP-like protein throughout the feeding and reproductive period. EcR and USP-like protein were not detectable in the glands of unfed ticks. Three EcR bands and up to five USP bands were upregulated during the feeding period, reaching maximum density during the rapid phase of engorgement, and then falling away as the glands degenerated during the postengorgement period. This profile of upregulation correlated well with the peak hemolymph ecdysteroid titer during the rapid phase of feeding and, as mentioned above, exogenous 20E enhances the binding between the EcR/USP and hsp27 EcRE. Taken together, it appears that SG degeneration is triggered by ecdysteroid produced during the feeding period, and that 20E also upregulates the components of the functional receptor.

A *sine qua non* for identifying a hormone is that the putative substance must appear prior to the biological action it regulates. For the SGs of *A. hebraeum* to degenerate in culture, they must be exposed to a minimum of ~30 ng 20E ml^{-1}, and complete degeneration requires 100–150 ng 20E ml^{-1} (Harris and Kaufman, 1985). SG degeneration in normal engorged females is well under way within 1–2 days postengorgement (Harris and Kaufman, 1984), a time at which hemolymph ecdysteroid titers are below 20 ng 20E ml^{-1} (Kaufman, 1991a; Lomas and Kaufman, 1992b; Mao and Kaufman, 1999). Lomas *et al.* (1998) attempted to reconcile these disparities. One explanation may be that the glands require only 12 h (Harris and Kaufman, 1985) or 24 h (Lomas *et al.*, 1998) exposure to 20E, after which degeneration will proceed in its absence. Mao and Kaufman (1999) demonstrated a peak of hemolymph ecdysteroid (~50 ng 20E ml^{-1}) during the rapid phase of engorgement, after which the level fell back again to 20 ng 20E ml^{-1} during the first 2 days of postengorgement. It is possible that this peak during the rapid phase of engorgement constitutes the trigger for SG degeneration. Another possibility is that the SGs sequester ecdysteroid, such that the ecdysteroid concentration within the tissue is above the threshold for SG degeneration even though it is below that threshold in the hemolymph. Lomas *et al.* (1998) provide some evidence in support of this explanation.

Kaufman (1990) tested the effect of 20E on male SGs held in organ culture. Although 1 μg 20E ml^{-1}

induced a significant loss of fluid secretory competence, for reasons discussed in the paper, it was not possible to interpret this as SG degeneration.

3.16.3.6. A Role for 20-Hydroxyecdysone as a Reproductive Pheromone in Ixodid Ticks

The full repertoire of mating behavior in ticks is coordinated by a series of pheromones (Sonenshine, 1991). The three pheromones identified so far in ixodid ticks are (1) a volatile "attractive sex pheromone," which attracts males from some distance, (2) a "mounting sex pheromone," which induces the male to assume the copulatory position, and (3) a "genital sex pheromone," a nonvolatile secretion of the female genital tract which, when the male probes the female gonopore with his mouthparts, stimulates the male to produce the spermatophore and transfer it to the female. The first two pheromones are heterospecific, but only conspecific males respond to the third. 20E is reported to enhance the secretion of 2,6-dichlorophenol, the mounting sex pheromone of *Haemaphysalis longicornis* (Hai-Poong and Da-Xiang, 1999) and Taylor *et al.* (1991c) demonstrated that 20E is an important component of the genital sex pheromone, although presumably it is not the component that determines conspecific mating.

3.16.3.7. The Role of "Mating Factors" in Tick Reproduction

The sperm in any organism comprises the gametes plus secretions from various male accessory glands. Some of these male accessory gland secretions enhance fecundity and inhibit receptivity; often these are the same material in a given species (review: Gillott, 2003). A fecundity-enhancing substance is one that increases egg production, oviposition, or egg fertility. A receptivity-inhibiting substance is one that renders the female less likely to copulate with further males, and hence reduces their chances for paternity. Because such substances are produced by one individual in order to influence the physiology of another, strictly speaking they fall into the category of pheromones rather than hormones. Nevertheless, these mating factors merit a brief mention here because of their influence on reproductive endocrinology.

Although male ticks appear not to transfer a receptivity inhibiting substance to the female (relying instead on proximate mate-guarding for assuring paternity), a number of mating factors have been identified in ticks which enhance fecundity (review: Kaufman, 2004), as summarized below.

3.16.3.7.1. A "sperm capacitation factor" from argasid and ixodid ticks Spermatozoa must undergo capacitation before they are capable of fertilizing eggs. In both argasid and ixodid ticks, a 12.5 kDa protein originating from the male accessory gland stimulates capacitation following transfer to the female (Shepherd *et al.*, 1982). The sperm capacitation factor from argasid ticks is not active on the sperm of ixodid ticks and vice versa.

3.16.3.7.2. A "vitellogenesis-stimulating factor" from argasid ticks When virgin *O. moubata* feed, they begin vitellogenesis but then resorb the yolk within 3 months (abortive vitellogenesis) (Connat *et al.*, 1986). If mated at this time, vitellogenesis resumes and oviposition occurs, an effect caused by a "vitellogenesis-stimulating factor" (VSF). One or two large proteins (100 and 200 kDa), originating from within the spermatozoon itself, constitute VSF (Sahli *et al.*, 1985). Whether VSF acts directly on the fat body to stimulate Vg synthesis or on the neuroendocrine system is not known.

3.16.3.7.3. A "male factor" from ixodid ticks SG degeneration occurs in *A. hebraeum* which have exceeded the critical weight, a process regulated by 20E (see Section 3.16.3.5). Whereas the process takes only 4 days in mated females, it takes 8 days in virgins (Lomas and Kaufman, 1992a). This difference is due to a protein (20–100 kDa) from the testis/vas deferens, and is responsible for a number of other physiological and behavioral changes in the female which enhance fecundity (Kaufman and Lomas, 1996).

3.16.3.7.4. An "engorgement factor" from ixodid ticks Pappas and Oliver (1972) showed that female *Dermacentor variabilis* require a protein from the male gonad in order to feed to repletion. The authors recently extended these observations to *A. hebraeum*. Using a differential cross-screening approach to identify feeding-induced genes in the testis/vas deferens, 35 feeding-induced transcripts were identified (Weiss *et al.*, 2002). Recombinant proteins (*rec*proteins) were produced from these transcripts, and engorgement factor was identified among them using a specific bioassay. None of the other *rec*proteins had engorgement factor activity (Weiss and Kaufmann, 2004). The latter authors present strong circumstantial evidence that engorgement factor and male factor are the same substance. The site of action of these factors in the female has not been determined, but the working hypothesis is that it either stimulates

the synganglion to release the ecdysteroidogenic neuropeptide, or it acts directly on the epidermis to release E. It is also conceivable that the engorgement factor effect occurs at the synganglion and the male factor effect at the epidermis.

3.16.3.8. Conclusions

As the Pycnogonida and the minor arachnid orders have received only scant attention in general, it seems unlikely that we shall learn much about their endocrinology in the foreseeable future. In principle, the outlook should be brighter for the horseshoe crabs, scorpions, and spiders, because they are relatively large and a good start has already been made. One can only hope that the lack of research into their endocrinology during the past 15 years is an anomaly that will be reversed before too long. With mites, we are still restricted to look for endogenous hormones in extracts of whole organisms, and to test effects of exogenous hormones, hormone analogs, and antagonists by topical application. The limitations of these approaches are obvious. In contrast, the endocrinology of ticks has received the most attention by far among the chelicerates, and there are a number of obvious themes to pursue in the immediate future. This chapter concludes with four major ones:

1. The JH question (see Sections 3.16.3.3.6, 3.16.3.4.4, and 3.16.3.4.5) must be resolved. As in all arthropods, there are major morphogenetic differences between larva, nymph, and adult. For example, when the forelegs of larval *Ixodes ricinus* and *I. rubicundus* are amputated, they regenerate in the nymph (Belozerov, 1999). The regenerated "Haller's organ" (a sensory structure on each foreleg important for sensing host odors, etc.) (Sonenshine, 1991) displays larval rather than nymphal characteristics (Belozerov, 1999). In at least some ticks the reproductive system responds to JH or JHAs, and some tick extracts have juvenilizing activity in insect bioassays. Because the known JHs have not been detected in ticks by physicochemical assays, however, perhaps the time has come to isolate the "juvenilizing hormone" starting from first principles. One hopes that the anomalies mentioned in this review can be resolved when working with tick-derived material, rather than continuing to depend on the pharmacological tools used in insect studies.
2. The vitellogenic hormone in ticks is an ecdysteroid (probably 20E) (see Section 3.16.3.4.5.3). Whereas in argasid ticks 20E appears to stimulate Vg synthesis and uptake (Ogihara, 2003), in ixodid ticks there appears to be a distinct "Vg-uptake factor." The factor is present in the hemolymph of engorged females (Lunke and Kaufman, 1993). One should inject 20E into small partially fed females (to stimulate vitellogenesis) along with extracts of hemolymph from engorged females. Or, ovaries from day 0 engorged females could be incubated with purified Vg along with extracts of engorged female hemolymph. If either of these bioassays resulted in a significant degree of oocyte development, the way is open for characterizing the Vg-uptake factor from first principles.
3. The isolation and characterization of an engorgement factor (which the authors have recently named "voraxin") from *A. hebracum* (Weiss and Kaufman, 2004) is an exciting recent development (Section 3.16.3.7.4). A voraxin-ELISA should be developed in due course so that one can learn more about its pharmacodynamics. An antibody directed against voraxin may also be the basis for a vaccine to inhibit tick feeding, pathogen transmission, and reproduction (Weiss and Kaufman, 2004). The authors' working hypothesis is that voraxin exerts its effect via the female's synganglion.
4. Although it is known that the control of SG degeneration is mediated by 20E acting via a typical arthropod EcR, postreceptor events have barely been explored. The most striking ultrastructural feature of SG degeneration in *A. hebraeum* is the appearance of numerous autophagic vacuoles in the fluid secretory portion of the type III acinus (Harris and Kaufman, 1981), and 20E stimulates the appearance of autophagic vacuoles in the SGs of *A. americanum* (Lindsay and Kaufman, 1988) and in *I. ricinus* held *in vitro* (Bowman, personal communication). Although degenerating glands of *I. ricinus* exhibit no overt morphological signs characteristic of classical apoptosis (Type I programmed cell death), such as chromatin condensation and margination and blebbing of apoptotic bodies, there are clear biochemical indices of apoptosis such as DNA fragmentation and the presence of caspase-3 activity (L'Amoreaux *et al.*, 2003). Overall, it would appear that tick SGs are dismantled by a hybrid of both the classical Type I and Type II mechanisms of programmed cell death (Bowman, personal communication). The elucidation of the specific pathways to programmed cell death in the tick SG will undoubtedly suggest novel means for controlling ticks and the pathogens they transmit.

Acknowledgments

E.S.C. thanks the National Sea Grant College Program, National Oceanic and Atmospheric Administration, US Department of Commerce, under grant R/A-111A, project NA66RG0477, through the California Sea Grant College Program, and project LR/LR-1 through the Connecticut Sea Grant College Program for recent research support. Research in W.R.K.'s laboratory has been generously supported for many years by the Natural Sciences and Engineering Research Council (NSERC) of Canada. We are grateful to Ms S.A. Chang for editorial assistance and to Dr A. Bowman, University of Aberdeen, for permission to cite some of his unpublished observations.

References

Adiyodi, R.G., **1985**. Reproduction and its control. In: Bliss, D.E., Mantel, L.H. (Eds.), The Biology of Crustacea, vol. 9. Academic Press, Orlando, FL, pp. 147–215.

Aeschlimann, A.A., Grandjean, O., **1973**. Influence of natural and "artificial" mating on feeding, digestion, vitellogenesis and oviposition in ticks (Ixodoidea). *Folia Parasitol. (Praha) 20*, 67–74.

Aguilar, M.B., Soyez, D., Falchetto, R., Arnott, D., Shabanowitz, J., *et al.*, **1995**. Amino acid sequence of the minor isomorph of the crustacean hyperglycemic hormone (CHH-II) of the Mexican crayfish *Procambarus bouvieri* (Ortmann): presence of a D-amino acid. *Peptides 16*, 1375–1383.

Aiken, D.E., **1973**. Proecdysis, setal development, and molt prediction in the American lobster. *J. Fisheries Res. B Canada 30*, 1334–1337.

Arnaud, F., Bamber, R.N., **1987**. The biology of Pycnogonida. *Adv. Marine Biol. 24*, 1–96.

Audsley, N., McIntosh, C., Phillips, J.E., **1992a**. Actions of ion-transport peptide from locust corpus cardiacum on several hindgut transport processes. *J. Exp. Biol. 1173*, 275–288.

Audsley, N., McIntosh, C., Phillips, J.E., **1992b**. Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *J. Exp. Biol. 173*, 261–274.

Babu, K.S., **1973**. Histology of the neurosecretory system and neurohaemal organs of the spider, *Argiope aurantia* (Lucas). *J. Morphol. 141*, 77–98.

Bassal, T.T.M., **1974**. Biochemical and physiological studies of certain ticks (Ixodoidea): activity of juvenile hormone analogs during embryogenesis in *Hyalomma (H.) dromedarii* Koch (Ixodidae). *Z. Parasitenkund 45*, 85–89.

Bassal, T.T.M., Roshdy, M.A., **1974**. *Argas (Persicargas) arboreus*: juvenile hormone analog termination of diapause and oviposition control. *Exp. Parasitol. 36*, 34–39.

Beetsma, J., Zonneveld, K., **1992**. Observations on the initiation and stimulation of oviposition of the *Varroa* mite. *Exp. Appl. Acarol. 16*, 303–312.

Behrens, W., Bückmann, D., **1983**. Ecdysteroids in the pycnogonid *Pycnogonum litorale* (Arthropoda, Pantopoda). *Gen. Comp. Endocrinol. 51*, 8–14.

Belozerov, V., **1999**. Regenerative modifications in the external structure of Haller's organ in nymphs of *Ixodes ricinus* L. (Acari: Ixodidae). *Acarina 7*, 29–33.

Berreur-Bonnenfant, J., Lawrence, F., **1984**. Comparative effect of farnesylacetone on macromolecular synthesis in gonads of crustaceans. *Gen. Comp. Endocrinol. 54*, 462–468.

Binnington, K.C., **1981**. Ultrastructural evidence for the endocrine nature of the lateral organs of the cattle tick *Boophilus microplus*. *Tissue and Cell 13*, 475–490.

Binnington, K.C., **1986**. Ultrastructure of the tick neuroendocrine system. In: Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology and Behavioral Biology of Ticks. Ellis Horwood, Chichester, pp. 152–164.

Binnington, K.C., **1987**. Histology and ultrastructure of the acarine synganglion. In: Gupta, A.P. (Ed.), Arthropod Brain: Its Evolution, Development, Structure and Functions. John Wiley, New York, pp. 95–109.

Binnington, K.C., Obenchain, F.D., **1982**. Structure and function of the circulatory, nervous and neuroendocrine systems of ticks. In: Obenchain, F.D., Galun, R. (Eds.), Physiology of Ticks. Pergamon Press, Oxford, pp. 351–398.

Blanchet, M.-F., Porcheron, P., Dray, F., **1979**. Variations du taux des ecdystéroïdes au cours des cycles de mue et de vitellogenèse chez le Crustacé Amphipode, *Orchestia gammarellus*. *Int. J. Invert. Reprod. 1*, 133–139.

Bliss, D.E., Welsh, J.H., **1952**. The neurosecretory system of brachyuran Crustacea. *Biol. Bull. 103*, 157–169.

Böcking, D., Dircksen, H., Keller, R., **2002**. The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. In: Wiese, K. (Ed.), The Crustacean Nervous System. Springer, Berlin, pp. 84–97.

Bonaric, J.-C., **1976**. Effects of ecdysterone on the molting mechanisms and duration of the intermolt period in *Pisaura mirabilis* CL. *Gen. Comp. Endocrinol. 30*, 267–272.

Bonaric, J.-C., **1979**. Effets des ecdysones et de l'hormone juvénile sur la durée du cycle de mue chez l'araignée *Pisaura mirabilis* CL. (Araneae, Pisauridae). *Rev. Arachnol. 2*, 205–207.

Bonaric, J.-C., **1981**. La régulation hormonale des phénomènes de mue et de diapause hivernale chez les araignées. *Atti Soc. Toscana Sci. Nat. Proc. Verb. Mem. B 88*(suppl. 1981), 120–132.

Bonaric, J.-C., **1988**. Présence de composés apparentes aux hormones juveniles (JH) d'insectes chez les arachnides: détection et difficultés d'interpretation. *C. R. X^{ième} Colloq. Eur. Arachnol. Bull. Soc. Sci. Bretagne 59*, 27–36.

Bonaric, J.-C., **1995**. Le système rétrocérebral des araignées: structure et fonction. *Proc. 15th Eur. Colloq. Arachnol.* 27–34.

Bonaric, J.-C., de Reggi, M., **1977**. Changes in ecdysone levels in the spider *Pisaura mirabilis* nymphs (Araneae, Pisauridae). *Experientia 33*, 1664–1665.

Bonaric, J.-C., Emerit, M., **1995**. Le système neuroendocrine rétrocérebrale des araignées: généralisation du modèle "Pisaura" à l'ensemble des araignées labidognathes. *Rev. Arachnol. 11*, 29–34.

Bonaric, J.-C., Emerit, M., Legendre, R., **1984**. Le complexe neuroendocrine rétrocérébral et la "glande de mue" de *Filistata insidiatrix* Forsköl (Araneae, Filistatidae). *Rev. Arachnol. 5*, 301–310.

Bonaric, J.-C., Juberthie, C., **1983**. Ultrastructure of the retrocerebral neuroendocrine complex in *Pisaura mirabilis* CL (Araneae, Pisauridae). *Zool. Jb. Abteil. Allg. Zool. Physiol. Tiere 87*, 55–64.

Bonaric, J.-C., Juberthie, C., **1990**. Juvenile hormone-like compounds in terrestrial Chelicerata (Arachnida). In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods. Rutgers University Press, New Brunswick, NJ, pp. 61–72.

Bonaric, J.-C., Juberthie, C., Calderon, R., **1987**. Contribution a l'étude du système neuroendocrine rétrocérébral des araignées: les organes de Schneider et les formations neurohémales associées chez la mygale *Tryssothele pissii* Simon (Dipluridae). *Rev. Arachnol. 8*, 53–64.

Boot, W.J., Calis, J.N.M., Beetsma, J., **1992**. Differential periods of *Varroa* mite invasion into worker and drone cells of honey bees. *Exp. Appl. Acarol. 16*, 295–301.

Borowsky, B., **1980**. The physiological control of reproduction in *Microdeutopus gryllotalpa* (Crustacea: Amphipoda). 1. The effects of exogenous ecdysterone on the females' molt and behavioral cycles. *J. Exp. Zool. 213*, 399–403.

Borst, D.W., Laufer, H., Landau, M., Chang, E.S., Hertz, W.A., *et al.*, **1987**. Methyl farnesoate (MF) and its role in crustacean reproduction and development. *Insect Biochem. 17*, 1123–1127.

Borst, D.W., Wainwright, G., Rees, H.H., **2002**. *In vivo* regulation of the mandibular organ in the edible crab, *Cancer pagurus*. *Proc. Roy. Soc. B 269*, 483–490.

Bouvier, J., Diehl, P.A., Morici, M., **1982**. Ecdysone metabolism in the tick *Ornithodoros moubata* (Argasidae, Ixodoidea). *Rev. Suisse Zool. 89*, 967–976.

Bückmann, D., Starnecker, G., Tomaschko, K.H., Wilhelm, E., Lafont, R., *et al.*, **1986**. Isolation and identification of major ecdysteroids from the pycnogonid *Pycnogonum litorale* Ström (Arthropoda, Pantopoda). *J. Comp. Physiol. B 156*, 759–765.

Bückmann, D., Tomaschko, K.-H., **1992**. 20-Hydroxyecdysone stimulates molting in pycnogonid larvae. *Gen. Comp. Endocrinol. 88*, 261–266.

Chaix, J.C., de Reggi, M., **1982**. Ecdysteroid levels during ovarian development and embryogenesis in the spider crab *Acanthonyx lunulatus*. *Gen. Comp. Endocrinol. 47*, 7–14.

Chambers, C.M., Dotson, E.M., Oliver, J.H., Jr., **1996**. Ecdysteroid titers during postembryonic development of *Dermanyssus gallinae* (Acari: Dermanyssidae). *J. Med. Entomol. 33*, 11–14.

Chang, E.S., **1989**. Endocrine regulation of molting in Crustacea. *Rev. Aquatic Sci. 1*, 131–157.

Chang, E.S., **1993**. Comparative endocrinology of molting and reproduction: insects and crustaceans. *Annu. Rev. Entomol. 38*, 161–180.

Chang, E.S., Bruce, M.J., **1980**. Ecdysteroid titers of juvenile lobsters following molt induction. *J. Exp. Zool. 214*, 157–160.

Chang, E.S., Chang, S.A., Beltz, B.S., Kravitz, E.A., **1999**. Crustacean hyperglycemic hormone in the lobster nervous system: localization and release from cells in the subesophageal ganglion and thoracic second roots. *J. Comp. Neurol. 414*, 50–56.

Chang, E.S., Hertz, W.A., Prestwich, G.D., **1992**. Reproductive endocrinology of the shrimp *Sicyonia ingentis*: steroid, peptide, and terpenoid hormones. NOAA Technical Reports. *Nat. Marine Fish. Serv. 106*, 1–6.

Chang, E.S., Keller, R., Chang, S.A., **1998**. Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *Gen. Comp. Endocrinol. 111*, 359–366.

Chang, E.S., O'Connor, J.D., **1977**. Secretion of α-ecdysone by crab Y-organs *in vitro*. *Proc. Natl Acad. Sci. USA 74*, 615–618.

Chang, E.S., O'Connor, J.D., **1978**. *In vitro* secretion and hydroxylation of α-ecdysone as a function of the crustacean molt cycle. *Gen. Comp. Endocrinol. 36*, 151–160.

Chang, E.S., Sage, B.A., O'Connor, J.D., **1976**. The qualitative and quantitative determinations of ecdysones in tissues of the crab, *Pachygrapsus crassipes*, following molt induction. *Gen. Comp. Endocrinol. 30*, 21–33.

Charmantier, G., Trilles, J.P., **1976**. Ecdystérone premue et exuvation chez *Sphaeroma serratum* (Fabricius, 1787) (Crustacea, Isopoda, Flabellifera). *Gen. Comp. Endocrinol. 28*, 249–254.

Charniaux-Cotton, H., **1954**. Découverte chez un Crustacé Amphipode (*Orchestia gammarella*) glande endocrine responsable de la différenciation de caractères sexuels primaires et secondaires mâles. *C. R. Hebd. Acad. Sci. D 239*, 780–782.

Charniaux-Cotton, H., **1985**. Sexual differentiation. In: Bliss, D.E., Mantel, L.H. (Eds.), The Biology of Crustacea, vol. 9. Academic Press, Orlando, FL, pp. 217–299.

Chinzei, Y., Itoh, K., Ando, K., **1989a**. Cypermethrin induction of vitellogenesis and ovarian development in unfed adult female *Ornithodoros moubata* (Acari: Argasidae). *Invert. Reprod. Devel. 15*, 19–26.

Chinzei, Y., Okuda, T., Ando, K., **1989b**. Vitellogenin synthesis and ovarian development in nymphal and newly molted female *Ornithodoros moubata* (Acari: Argasidae). *J. Med. Entomol. 26*, 30–36.

Chinzei, Y., Taylor, D., **1990**. Regulation of vitellogenesis induction by engorgement in the soft tick, *Ornithodoros moubata*. *Adv. Invert. Reprod. 5*, 565–570.

Chinzei, Y., Taylor, D., **1994**. Hormonal regulation of vitellogenin biosynthesis in ticks. *Adv. Disease Vector Res. 10*, 1–22.

Chinzei, Y., Taylor, D., Ando, K., **1991**. Effects of juvenile hormone and its analogues on vitellogenin synthesis and ovarian development in *Ornithodoros moubata* (Acari: Argasidae). *J. Med. Entomol. 28*, 506–513.

Chinzei, Y., Taylor, D., Miura, K., Ando, K., **1992**. Vitellogenesis induction by synganglion factor in adult female tick, *Ornithodoros moubata* (Acari: Argasidae). *J. Acarol. Soc. Japan 1*, 15–26.

Chinzei, Y., Yano, I., **1985**. Fat body is the site of vitellogenin synthesis in the soft tick, *Ornithodoros moubata*. *J. Comp. Physiol. B 155*, 671–678.

Christ, B., Sedlmeier, D., **1987**. Variations in epidermal cyclic nucleotide-dependent protein kinase activity during moult cycle of the crayfish *Orconectes limosus* and hormonal control of kinase activity by 20-hydroxyecdysone. *Int. J. Biochem. 19*, 79–84.

Chung, A.C.-K., Durica, D.S., Hopkins, P.M., **1998**. Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X-receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol. 109*, 375–389.

Chung, J.S., Dircksen, H., Webster, S.G., **1999**. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc. Natl Acad. Sci. USA 96*, 13103–13107.

Chung, J.S., Webster, S.G., **1996**. Does the N-terminal pyroglutamate residue have any physiological significance for crab hyperglycemic neuropeptides? *Eur. J. Biochem. 240*, 358–364.

Connat, J.-L., **1988**. Effects of different anti-juvenile hormone agents on the fecundity of the female cattle tick *Boophilus microplus*. *Pesticide Biochem. Physiol. 30*, 28–34.

Connat, J.-L., Delbecque, J.-P., Alabouvette, J., Pitoizet, N., **1997**. Evolution of ecdysteroid and of their polar conjugates during the post-embryonic development of the tick, *Ornithodoros moubata*. *Arch. Insect Biochem. Physiol. 35*, 159–168.

Connat, J.-L., Diehl, P.A., Dumont, M., Carminati, S., Thompson, M.J., **1983a**. Effects of exogenous ecdysteroids on the female tick *Ornithodoros moubata*: induction of supermolting and influence on oogenesis. *Z. Angewandte Entomol. 96*, 520–530.

Connat, J.-L., Diehl, P.A., Gfeller, H., Morici, M., **1985**. Ecdysteroids in females and eggs of the ixodid tick *Amblyomma hebraeum*. *Int. J. Invert. Reprod. Devel. 8*, 103–116.

Connat, J.-L., Diehl, P.A., Morici, M., **1984**. Metabolism of ecdysteroids during vitellogenesis of the tick *Ornithodoros moubata* (Ixodoidea, Argasidae) accumulation of apolar metabolites in the eggs. *Gen. Comp. Endocrinol. 56*, 100–110.

Connat, J.-L., Dotson, E.M., **1988**. Comparative investigation of the egg ecdysteroids of ticks using radioimmunoassay and metabolic studies. *J. Insect Physiol. 34*, 639–645.

Connat, J.-L., Dotson, E.M., Diehl, P.A., **1988**. Apolar conjugates of ecdysteroids are not used as a storage form of molting hormone in the argasid tick *Ornithodoros moubata*. *Arch. Insect Biochem. Physiol. 9*, 221–235.

Connat, J.-L., Ducommun, J., Diehl, P.A., **1983b**. Juvenile hormone-like substances can induce vitellogenesis in the tick *Ornithodoros moubata* (Acarina: Argasidae). *Int. J. Invert. Reprod. Devel. 6*, 285–294.

Connat, J.-L., Ducommun, J., Diehl, P.A., Aeschlimann, A.A., **1986**. Some aspects of the control of the gonotrophic cycle in the tick *Ornithodoros moubata* (Ixodoidea, Argasidae). In: Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology and Behavioral Biology of Ticks. Ellis Horwood, Chichester, pp. 194–216.

Connat, J.-L., Mauchamp, B., Baehr, J.-C., **1987**. An attempt to identify juvenile hormone in the hemolymph of the cattle tick *Boophilus microplus* during vitellogenesis. Annexe 2 from Aspects Endocrinologiques de la Physiologie du Development et de la Reproduction chez les Tiques. Ph.D. thesis (J.-L. Connat), (87/DIJO/5005), Dijon, France.

Connat, J.-L., Nepa, M.-C., **1990**. Effects of different anti-juvenile hormone agents on the fecundity of the female tick, *Ornithodoros moubata*. *Pesticide Biochem. Physiol. 37*, 266–274.

Coons, L.B., Kaufman, W.R., **1988**. Evidence that developmental changes in type III acini in the tick, *Amblyomma hebraeum* Koch (Acari: Ixodidae) are initiated by a haemolymph-borne factor. *Exp. Appl. Acarol. 4*, 117–139.

Coons, L.B., Lamoreaux, W.J., Rosell-Davis, R., Tarnowski, B.I., **1989**. Onset of vitellogenin production and vitellogenesis, and their relationship to changes in the midgut epithelium and oocytes in the tick *Dermacentor variabilis*. *Exp. Appl. Acarol. 6*, 291–305.

Coons, L.B., Rosell-Davis, R., Tarnowski, B.I., **1986**. Bloodmeal digestion in ticks. In: Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology and Behavioral Biology of Ticks. Ellis Horwood, Chichester, pp. 248–279.

Coons, L.B., Tarnowski, B.I., Ourth, D., **1982**. *Rhipicephalus sanguineus*: localization of vitellogenin synthesis by immunological methods and electron microscopy. *Exp. Parasitol. 59*, 331–339.

Dall, W., Barclay, M.C., **1979**. The effect of exogenous 20-hydroxyecdysone on levels of epidermal DNA and RNA in the western rock lobster. *J. Exp. Marine Biol. Ecol. 36*, 103–110.

Davey, K.G., Gordon, D.R.B., **1996**. Fenoxycarb and thyroid hormones have JH-like effects on the follicle cells of *Locusta migratoria in vitro*. *Arch. Insect Biochem. Physiol. 32*, 613–622.

Davis, H.H., Dotson, E.M., Oliver, J.H., Jr., **1994**. Immunocytochemical localization of an insulin-like substance in the synganglion of various stages of the tick, *Dermacentor variabilis*. *Exp. Appl. Acarol. 18*, 111–122.

Dees, W.H., Sonenshine, D.E., Breidling, E., **1984**. Ecdysteroids in the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae), during different periods of tick development. *J. Med. Entomol. 21*, 514–523.

Dees, W.H., Sonenshine, D.E., Breidling, E., Buford, N.P., Khalil, G.M., **1982**. Toxicity of precocene-2 for the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae). *J. Med. Entomol. 19*, 734–742.

deFur, P.L., Crane, M., Ingersoll, C., Tattersfield, L., **1999**. Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment. SETAC Press, Pensacola, FL.

De Kleijn, D.P.V., de Leeuw, E.P.H., van den Berg, M.C., Martens, G.J.M., Van Herp, F., **1995**. Cloning and expression of two mRNAs encoding structurally different hyperglycemic hormone precursors in the lobster *Homarus americanus*. *Biochim. Biophys. Acta 1260*, 62–66.

De Kleijn, D.P.V., Janssen, K.P.C., Martens, G.J.M., Van Herp, F., **1994**. Cloning and expression of two crustacean hyperglycemic-hormone mRNAs in the eyestalk of the crayfish *Orconectes limosus*. *Eur. J. Biochem. 224*, 623–629.

De Kleijn, D.P.V., Van Herp, F., **1995**. Molecular biology of neurohormone precursors in the eyestalk of Crustacea. *Comp. Biochem. Physiol. B 112*, 573–579.

Delbecque, J.P., Diehl, P.A., O'Connor, J.D., **1978**. Presence of ecdysone and ecdysterone in the tick, *Amblyomma hebraeum* Koch. *Experientia 34*, 1379–1381.

Diehl, P.A., Connat, J.-L., Dotson, E., **1986**. Chemistry, function and metabolism of tick ecdysteroids. In: Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology and Behavioral Biology of Ticks. Ellis Horwood, Chichester, pp. 165–193.

Dircksen, H., Böcking, D., Heyn, U., Mandel, C., Chung, J.S., *et al.*, **2001**. Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. *Biochem. J. 356*, 159–170.

Dotson, E.M., Connat, J.-L., Diehl, P.A., **1991**. Cuticle deposition and ecdysteroid titers during embryonic and larval development of the argasid tick *Ornithodoros moubata* (Murray, 1877, *sensu* Walton, 1962) (Ixodoidea: Argasidae). *Gen. Comp. Endocrinol. 82*, 386–400.

Downing, A.S., Wright, C.G., Farrier, M.H., **1990**. Effects of five insect growth regulators on laboratory populations of the North American house-dust mite, *Dermatophagoides farinae*. *Exp. Appl. Acarol. 9*, 123–130.

Drach, P., **1939**. Mue et cycle d'intermue chez les Crustacés Décapodes. *Ann. Inst. Océanogr. 19*, 103–393.

Durica, D.S., Hopkins, P.M., **1996**. Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator. Gene 171*, 237–241.

Durica, D.S., Wu, X., Anilkumar, A., Hopkins, P.M., Chung, A.C.-K., **2002**. Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation. *Mol. Cell. Endocrinol. 189*, 59–76.

Echalier, G., **1959**. L'organe Y et le déterminisme de la croissance et de la mue chez *Carcinus maenas* (L.) Crustacé Décapode. *Ann. Sci. Nat. Zool. Biol. Animale 12*, 1–59.

Edomi, P., Azzoni, E., Mettulio, R., Pandolfelli, N., Ferrero, E.A., *et al.*, **2002**. Gonad-inhibiting hormone of the Norway lobster (*Nephrops norvegicus*): cDNA cloning, expression, recombinant protein production, and immunolocalization. *Gene 284*, 93–102.

Eisen, Y., Warburg, M.R., Galun, R., **1973**. Neurosecretory activity as related to feeding and oogenesis in the fowl-tick *Argas persicus* (Oken). *Gen. Comp. Endocrinol. 21*, 331–340.

El Bakary, Z., Porcheron, P., Morinière, M., Fuzeau-Braesch, S., **1987**. Mise en évidence et dosage d'ecdysteroïdes chez le scorpion *Leiurus quinquestriatus*. *C. R. Hebdom. Acad. Sci. D 304*, 453–456.

Escamilla-Chimal, E.G., Hiriart, M., Sanchez-Soto, M.C., Fanjul-Moles, M.L., **2002**. Serotonin modulation of CHH secretion by isolated cells of the crayfish retina and optic lobe. *Gen. Comp. Endocrinol. 125*, 283–290.

Evans, G.O., **1992**. Principles of Acarology. CAB International, Wallingford.

Feldlaufer, M.F., Hartfelder, K., **1997**. Relationship of the neutral sterols and ecdysteroids of the parasitic mite, *Varroa jacobsoni* to those of the honey bee, *Apis mellifera*. *J. Insect Physiol. 43*, 541–545.

Ferezou, J.P., Berreur-Bonnenfant, J., Meusy, J.J., Barbier, M., Suchy, M., *et al.*, **1977**. 6,10,14-Trimethylpentadecan-2-one and 6,10,14-trimethyl-5-*trans*, 13-pentadecatrien-2-one from the androgenic glands of the male crab *Carcinus maenas*. *Experientia 33*, 290.

Fernlund, P., **1971**. Chromactivating hormones of *Pandalus borealis*: isolation and purification of a light-adapting hormone. *Biochim. Biophys. Acta 237*, 519–529.

Fernlund, P., **1974a**. Structure of the red-pigment-concentrating hormone of the shrimp, *Pandalus borealis*. *Biochim. Biophys. Acta 371*, 304–311.

Fernlund, P., **1974b**. Synthesis of the red-pigment-concentrating hormone of the shrimp, *Pandalus borealis*. *Biochim. Biophys. Acta 371*, 312–322.

Fernlund, P., **1976**. Structure of a light-adapting hormone from the shrimp, *Pandalus borealis*. *Biochim. Biophys. Acta 439*, 17–25.

Fernlund, P., Josefsson, L., **1968**. Chromactivating hormones of *Pandalus borealis*: isolation and purification of the red-pigment-concentrating hormone. *Biochim. Biophys. Acta 158*, 262–273.

Fernlund, P., Josefsson, L., **1972**. Crustacean color change hormone: amino acid sequence and chemical synthesis. *Science 177*, 173–175.

Fingerman, M., **1988**. Pigment hormones in Crustacea. In: Laufer, H., Downer, R.G.H. (Eds.), Endocrinology of

Selected Invertebrate Types. Alan R. Liss, New York, pp. 357–374.

Foelix, R.F., **1996**. Biology of Spiders, 2nd edn. Oxford University Press, Oxford.

Friesen, K., Kaufman, W.R., **2002**. Quantification of vitellogenesis and its control by 20-hydroxyecdysone in the ixodid tick, *Amblyomma hebraeum*. *J. Insect Physiol. 48*, 773–782.

Friesen, K., Kaufman, W.R., **2003**. Cypermethrin inhibits egg development in the ixodid tick, *Amblyomma hebraeum*. *Pesticide Biochem. Physiol. 76*, 25–35.

Friesen, K., Kaufman, W.R., **2004**. Effects of 20-hydroxyecdysone and other hormones on egg development, and identification of a vitellin-binding protein in the ovary of the tick, *Amblyomma hebraeum*. *J. Insect Physiol. 50*, 519–529.

Friesen, K., Suri, R., Kaufman, W.R., **2003**. Effects of the avermectin, MK-243, on ovary development and salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*. *Pest. Biochem. Physiol. 76*, 82–90.

Funke, B., Spindler, K.-D., **1987**. Developmental changes of chitinolytic enzymes and ecdysteroid levels during the early development of the brine shrimp *Artemia*. In: Decleir, W., Moens, L., Slegers, H., Jaspers, E. (Eds.), Artemia Research and its Applications. Universa Press, Wetteren, pp. 67–78.

Gabe, M., **1953**. Sur l'éxistence, chez quelques Crustacés Malacostracés, d'un organe comparable à la glande de la mue des insects. *C. R. Hebdom. Acad. Sci. D 237*, 1111–1113.

Gaber, S.H.A., Khalil, G.M., Sonenshine, D.E., Moez, M.K.A., **1983**. Precocene-2 effects on the camel tick, *Hyalomma dromedarii* (Acari: Ixodidae). 1. Adult responses. *J. Medi. Entomol. 20*, 534–540.

Germond, J.-E., Aeschlimann, A.A., **1977**. Influence of copulation on vitellogenesis and egg-laying in *Ornithodoros moubata* Murray (Ixodoidea: Argasidae). *Adv. Invert. Reprod. 1*, 308–318.

Germond, J.E., Diehl, P.A., Morici, M., **1982**. Correlations between integument structure and ecdysteroid titers in fifth-stage nymphs of the tick, *Ornithodoros moubata* (Murray, 1877; *sensu* Walton, 1962). *Gen. Comp. Endocrinol. 46*, 255–266.

Gersch, M., Richter, K., Böhm, G.-A., Eibisch, H., **1980**. Experimentelle Untersuchungen zur saisonalen Abhangigkeit der neurohormonalen Steuerung der Gastrolithenbildung und der Ecdysteroidsynthese des Y-organs von *Orconectes limosus* (Crustacea, Decapoda). *Zool. Jb. Abteil. Allgemeine Zool. Physiol. Tiere 84*, 164–180.

Gillott, C., **2003**. Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annu. Rev. Entomol. 48*, 163–184.

Goudeau, M., Lachaise, F., Carpentier, G., Goxe, B., **1990**. High titers of ecdysteroids are associated with the secretory process of embryonic envelopes in the European lobster. *Tissue and Cell 22*, 269–281.

Goy, M.F., Mandelbrot, D.A., York, C.M., **1987**. Identification and characterization of a polypeptide from a lobster neurosecretory gland that induces cyclic GMP accumulation in lobster neuromuscular preparations. *J. Neurochem. 48*, 954–966.

Grève, P., Sorokine, O., Berges, T., Lacombe, C., VanDorsselaer, A., *et al.*, **1999**. Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). *Gen. Comp. Endocrinol. 115*, 406–414.

Gu, P.-L., Chu, K.H., Chan, S.-M., **2001**. Bacterial expression of the shrimp molt-inhibiting hormone (MIH): antibody production, immunocytochemical study and biological assay. *Cell and Tissue Res. 303*, 129–136.

Gu, P.-L., Yu, K.L., Chan, S.-M., **2000**. Molecular characterization of an additional shrimp hyperglycemic hormone: cDNA cloning, gene organization, expression and biological assay of recombinant proteins. *FEBS Lett. 472*, 122–128.

Guo, X., Harmon, M.A., Laudet, V., Mangelsdorf, D.J., Palmer, M.J., **1997**. Isolation of a functional ecdysteroid receptor homologue from the ixodid tick *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol. 27*, 945–962.

Guo, X., Xu, Q., Harmon, M.A., Jin, X., Laudet, V., *et al.*, **1998**. Isolation of two functional retinoid X receptor subtypes from the ixodid tick, *Amblyomma americanum* (L.). *Mol. Cell. Endocrinol. 139*, 45–60.

Hafez, M., Bassal, T.T.M., **1980**. Juvenilization of the brown dog tick *Rhipicephalus sanguineus* Latr. (Ixodidae, Acarina). *J. Egypt. Soc. Parasitol. 10*, 301–304.

Hai-Poong, L., Da-Xiang, S., **1999**. Relationship between ecdysteroids and sex pheromone, 2,6-DCP, of female *Haemaphysalis longicornis* (Acari: Ixodidae). *China-Korean J. Entomol. 29*, 65–67.

Hamann, A., **1974**. Sinus gland and neuroendocrine control of circadian blood sugar variations in the crayfish. *J. Comp. Physiol. 89*, 197–214.

Hampshire, F., Horn, D.H.S., **1966**. Structure of crustecdysone, a crustacean moulting hormone. *Chem. Commun. 1966*, 37–38.

Hänel, H., **1986**. Effect of juvenile hormone (III) from the host *Apis mellifera* (Insecta: Hymenoptera) on the neurosecretion of the parasitic mite *Varroa jacobsoni* (Acari: Mesostigmata). *Exp. Appl. Acarol. 2*, 257–271.

Hanström, B., **1939**. Hormones in Invertebrates. Oxford University Press, Oxford.

Harris, R.A., Kaufman, W.R., **1981**. Hormonal control of salivary degeneration in the ixodid tick *Amblyomma hebraeum*. *J. Insect Physiol. 27*, 241–248.

Harris, R.A., Kaufman, W.R., **1984**. Neural involvement in the control of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*. *J. Exp. Biol. 109*, 281–290.

Harris, R.A., Kaufman, W.R., **1985**. Ecdysteroids: possible candidates for the hormone which triggers salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*. *Experientia 41*, 740–742.

Hasegawa, Y., Haino-Fukushima, K., Katakura, Y., **1991**. An immunoassay for the androgenic gland hormone of the terrestrial isopod, *Armadillidium vulgare*. *Invert. Reprod. Devel. 20*, 59–66.

Hayes, M.J., Oliver, J.H., Jr., **1981**. Immediate and latent effects induced by the antiallatotropin precocene 2 (P2) on embryonic *Dermacentor variabilis* (Say) (Acari: Ixodidae). *J. Parasitol. 67*, 923–927.

Hoarau, F., **1982**. Influence des injections d'ecdystérone sur la régénération du péréiopode chez *Helleria brevicornis* Ebner (Isopode terrestre). *C. R. Hebdom. Acad. Sci. D 294*, 403–406.

Holland, C., Skinner, D., **1976**. Interactions between molting and regeneration in the land crab. *Biol. Bull. 150*, 222–240.

Homola, E., Chang, E.S., **1997**. Assay methods for methyl farnesoate esterases in crustaceans. *Arch. Insect Biochem. Physiol. 36*, 115–128.

Hopkins, P.M., **1982**. Effects of neurosecretory factors on *in vivo* levels of ecdysteroids in hemolymph of the fiddler crab, *Uca pugilator. Am. Zool. 22*, 938.

Hopkins, P.M., **1989**. Ecdysteroids and regeneration in the fiddler crab *Uca pugilator. J. Exp. Zool. 252*, 293–299.

Hopkins, P.M., **2001**. Limb regeneration in the fiddle crab, *Uca pugilator*: hormonal and growth factor control. *Am. Zool. 41*, 389–398.

Horn, D.H.S., Bergamasco, R., **1985**. Chemistry of ecdysteroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology, vol. 7. Pergamon, Oxford, pp. 185–248.

Horn, D.H.S., Middleton, E.J., Wunderlich, J.A., Hampshire, F., **1966**. Identity of the moulting hormones of insects and crustaceans. *Chem. Commun. 1966*, 339–340.

James, A.M., Zhu, X.X., Oliver, J.H., Jr., **1997**. Vitellogenin and ecdysteroid titers in Ixodes scapularis during vitellogenesis. *J. Parasitol. 83*, 559–563.

Jegla, T.C., **1982**. A review of the molting physiology of the trilobite larva of *Limulus*. In: Bonaventura, J., Bonaventura, C., Tesh, S. (Eds.), Physiology and Biology of Horseshoe Crabs. Alan R. Liss, New York, pp. 83–101.

Jegla, T.C., **1990**. Evidence for ecdysteroids as molting hormones in Chelicerata, Crustacea, and Myriapoda. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods, vol. 1. Rutgers University Press, New Brunswick, NJ, pp. 229–273.

Jegla, T.C., Costlow, J.D., **1979a**. The *Limulus* bioassay for ecdysteroids. *Biol. Bull. 156*, 103–114.

Jegla, T.C., Costlow, J.D., **1979b**. Ecdysteroids in *Limulus* larvae. *Experientia 35*, 554–555.

Josefsson, L., **1975**. Structure and function of crustracean chromatophorotropins. *Gen. Comp. Endocrinol. 25*, 199–202.

Josefsson, L., **1983a**. Chemical properties and physiological actions of crustacean chromatophorotropins. *Am. Zool. 23*, 507–515.

Josefsson, L., **1983b**. Invertebrate neuropeptide hormones. *Int. J. Peptide and Protein Res. 21*, 459–470.

Juberthie, C., Bonaric, J.-C., **1990**. Ecdysial glands and ecdysteroids in terrestrial Chelicerata. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods, vol. 1. Rutgers University Press, New Brunswick, NJ, pp. 270–305.

Katayama, H., Nagata, K., Ohira, T., Yumoto, F., Tanokura, M., *et al.*, **2003**. The solution structure of molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*. *J. Biol. Chem. 278*, 9620–9623.

Katayama, H., Ohira, T., Aida, K., Nagasawa, H., **2002**. Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone. *Peptides 23*, 1537–1546.

Kaufman, W.R., **1976**. The influence of various factors on fluid secretion by *in vitro* salivary glands of ixodid ticks. *J. Exp. Biol. 64*, 727–742.

Kaufman, W.R., **1990**. Effect of 20-hydroxyecdysone on the salivary glands of the male tick, *Amblyomma hebraeum*. *Exp. Appl. Acarol. 9*, 87–95.

Kaufman, W.R., **1991a**. Correlation between haemolymph ecdysteroid titre, salivary gland degeneration and ovarian development in the ixodid tick, *Amblyomma hebraeum* Koch. *J. Insect Physiol. 37*, 95–99.

Kaufman, W.R., **1991b**. Further investigations on the action of ecdysteroids on the salivary glands of the female tick *Amblyomma americanum*. *Exp. Appl. Acarol. 10*, 259–265.

Kaufman, W.R., **1997**. Arthropoda – Chelicerata. In: Adams, T.S. (Ed.), Progress in Reproductive Endocrinology. Oxford & IBH Publishing Co., Oxford, pp. 211–245.

Kaufman, W.R., **2004**. Assuring paternity in a promiscuous world: are there lessons for ticks among the insects? In: Bowman, A.S., Nuttall, P.A. (Eds.), Ticks, Diseases and Control (*Parasitology supplement*), vol. 128. Cambridge University Press, Cambridge.

Kaufman, W.R., Harris, R.A., **1983**. Neural pathways mediating salivary fluid secretion in the ixodid tick *Amblyomma hebraeum*. *Can. J. Zool. 61*, 1976–1980.

Kaufman, W.R., Lomas, L.O., **1996**. Male factors in ticks: their role in feeding and egg development. *Invert. Reprod. Devel. 30*, 191–198.

Kaufman, W.R., Ungarian, S.G., Noga, A.E., **1986**. The effect of avermectins on feeding, salivary fluid secretion, and fecundity in some ixodid ticks. *Exp. Appl. Acarol. 2*, 1–18.

Kawakami, T., Toda, C., Akaji, K., Nishimura, T., Nakatsuji, T., *et al.*, **2000**. Synthesis of a molt-inhibiting hormone of the American crayfish, *Procambarus clarkii*, and determination of the location of its disulfide linkages. *J. Biochem. 128*, 455–461.

Kegel, G., Reichwein, B., Weese, S., Gaus, G., Peter-Katalinic, J., *et al.*, **1989**. Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. *FEBS Lett. 255*, 10–14.

Keller, R., Jaros, P.P., Kegel, G., **1985**. Crustacean hyperglycemic neuropeptides. *Am. Zool. 25*, 207–221.

Keller, R., O'Connor, J.D., **1982**. Neuroendocrine regulation of ecdysteroid production in the crab *Pachygrapsus crassipes*. *Gen. Comp. Endocrinol. 46*, 384.

Keller, R., Schmid, E., **1979**. *In vitro* secretion of ecdysteroids by Y-organs and lack of secretion by mandibular organs of the crayfish following molt induction. *J. Comp. Physiol. 130*, 347–353.

Keller, R., Sedlmeier, D., **1988**. A metabolic hormone in crustaceans: the hyperglycemic neuropeptide. In: Laufer, H., Downer, R.G.H. (Eds.), Endocrinology of Selected Invertebrate Types. Alan R. Liss, New York, pp. 315–326.

Khalaila, I., Manor, R., Weil, S., Granot, Y., Keller, R., *et al.*, **2002**. The eyestalk–androgenic gland–testis endocrine axis in the crayfish *Cherax quadricarinatus*. *Gen. Comp. Endocrinol. 127*, 147–156.

Khalil, G.M., Gaber, S.H.A., Sonenshine, D.E., Gad, S.M., **1983**. Precocene-2 effects on the camel tick, *Hyalomma dromedarii* (Acari: Ixodidae). 2. Larval and nymphal responses. *J. Med. Entomol. 20*, 541–550.

Khalil, G.M., Sonenshine, D.E., Hanafy, H.A., Abdelmonem, A.E., **1984**. Juvenile hormone I effects on the camel tick, *Hyalomma dromedarii* (Acari: Ixodidae). *J. Med. Entomol. 21*, 561–566.

Kim, Y., Davari, E.D., Sevala, V., Davey, K.G., **1999**. Functional binding of a vertebrate hormone, L-3,5,3′-triiodothyronine (T_3) on insect follicle cell membranes. *Insect Biochem. Mol. Biol. 29*, 943–950.

King, L.E., Ding, Q., Prestwich, G.D., Tobe, S.S., **1995**. The characterization of a haemolymph methyl farnesoate binding protein and the assessment of methyl farnesoate metabolism by the haemolymph and other tissues from *Procambrus clarkii*. *Insect Biochem. Mol. Biol. 25*, 495–501.

King, P.E., **1973**. Pycnogonids. Hutchinson, London.

Klein, J.M., Mangerich, S., De Kleijn, D.P.V., Keller, R., Weidemann, W.M., **1993**. Molecular cloning of crustacean putative molt-inhibiting hormone (MIH) precursor. *FEBS Lett. 334*, 139–142.

Kleinholz, L., **1975**. Purified hormones from the crustacean eyestalk and their physiological specificity. *Nature 258*, 256–257.

Kleinholz, L.H., Keller, R., **1979**. Endocrine regulation in Crustacea. In: Barrington, E.J.W. (Ed.), Hormones and Evolution. Academic Press, London, pp. 159–213.

Koller, G., **1927**. Über Chromatophoren-system, Farbensinn und Farbwechsel bei *Crangon vulgaris*. *Z. Vergleich. Physiol. 5*, 191–246.

Krishnakumaran, A., Schneiderman, H.A., **1968**. Chemical control of moulting in arthropods. *Nature 220*, 601–603.

Krishnakumaran, A., Schneiderman, H.A., **1970**. Control of molting in mandibulate and chelicerate arthropods by ecdysones. *Biol. Bull. 139*, 520–538.

Kulcsár, P., Prestwich, G.D., Sonenshine, D.E., **1989**. Detection of binding proteins for juvenile hormone-like substances in ticks by photoaffinity labeling. In: Borovsky, D., Spielman, A. (Eds.), Host-Regulated Developmental Mechanisms in Vector Arthropods. University of Florida Press, Gainesville, FL, pp. 18–23.

Kumar, R., **1980**. Effect of administration of synthetic ecdysone on the moulting of *Palamnaeus bengalensis*. *Experientia 36*, 411–412.

Lachaise, F., Carpentier, G., Somme, G., Colardeau, J., Beydon, P., **1989**. Ecdysteroid synthesis by crab Y-organs. *J. Exp. Zool. 252*, 283–292.

Lachaise, F., Goudeau, M., Hetru, C., Kappler, C., Hoffmann, J.A., **1981**. Ecdysteroids and ovarian development in the shore crab, *Carcinus maenas*. *Hoppe-Seyler's Z. Physiol. Chem. 362*, 521–529.

Lachaise, F., Hoffmann, J.A., **1982**. Ecdysteroids and embryonic development in the shore crab, *Carcinus maenas*. *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1059–1067.

Lacombe, C., Grève, P., Martin, G., **1999**. Overview on the sub-grouping of the crustacean hyperglycemic hormone family. *Neuropeptides 33*, 71–80.

L'Amoreaux, W.J., Junaid, L., Trevidi, S., **2003**. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. *Tissue Cell 35*, 95–99.

Laufer, H., **1992**. Method for Increasing Crustacean Larval Production. US Patent and Trademark Office Patent No. 5,161,481.

Laufer, H., Ahl, J., Rotllant, G., Baclaski, B., **2002**. Evidence that ecdysteroids and methyl farnesoate control allometric growth and differentiation in a crustacean. *Insect Biochem. Mol. Biol. 32*, 205–210.

Laufer, H., Biggers, W.J., **2001**. Unifying concepts learned from methyl farnesoate for invertebrate reproduction and post-embryonic development. *Am. Zool. 41*, 442–457.

Laufer, H., Biggers, W.J., Ahl, J.S.B., **1998**. Stimulation of ovarian maturation in the crayfish *Procambarus clarkii* by methyl farnesoate. *Gen. Comp. Endocrinol. 111*, 113–118.

Laufer, H., Borst, D., Baker, F.C., Carrasco, C., Sinkus, M., *et al.*, **1987**. Identification of a juvenile hormone-like compound in a crustacean. *Science 235*, 202–205.

Leahy, M.G., Booth, K.S., **1980**. Precocene induction of tick sterility and ecdysis failure. *J. Med. Entomol. 17*, 18–21.

Lee, K.J., Watson, R.D., **2002**. Expression of crustacean (*Callinectes sapidus*) molt-inhibiting hormone in insect cells using recombinant baculovirus. *J. Exp. Zool. 292*, 41–51.

Lee, K.J., Watson, R.D., Roer, R.D., **1998**. Molt-inhibiting hormone mRNA levels and ecdysteroid titer during a molt cycle of the blue crab, *Callinectes sapidus*. *Biochem. Biophys. Res. Commun. 249*, 624–627.

Lees, A.D., **1952**. The role of cuticle growth in the feeding process of ticks. *Proc. Zool. Soc. London 121*, 759–772.

Legendre, R., **1981**. Les éléments endocrines de la glande de mue chez les araignées mygalomorphes. *Rev. Arachnol. 3*, 133–138.

Legendre, R., **1985**. The stomatogastric nervous system and neurosecretion. In: Barth, F.G. (Ed.), Neurobiology of Arachnids. Springer-Verlag, Berlin, pp. 38–49.

Li, H., Borst, D.W., **1991**. Characterization of a methyl farnesoate binding protein in hemolymph from *Libinia emarginata*. *Gen. Comp. Endocrinol. 81*, 335–342.

Lindsay, P.J., Kaufman, W.R., **1988**. Action of some steroids on salivary gland degeneration in the ixodid tick, *Amblyomma americanum* L. *J. Insect Physiol. 34*, 351–359.

Liu, L., Laufer, H., Wang, Y.J., Hayes, T., **1997**. A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. *Biochem. Biophys. Res. Commun. 237*, 694–701.

Lomas, L.O., Gelman, D., Kaufman, W.R., **1998**. Ecdysteroid regulation of salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*: a reconciliation of *in vivo* and *in vitro* observations. *Gen. Comp. Endocrinol. 109*, 200–211.

Lomas, L.O., Kaufman, W.R., **1992a**. The influence of a factor from the male genital tract on salivary gland degeneration in the female ixodid tick, *Amblyomma hebraeum*. *J. Insect Physiol. 38*, 595–601.

Lomas, L.O., Kaufman, W.R., **1992b**. An indirect mechanism by which a protein from the male gonad hastens salivary gland degeneration in the female ixodid tick, *Amblyomma hebraeum*. *Arch. Insect Biochem. Physiol. 21*, 169–178.

Lomas, L.O., Turner, P.C., Rees, H.H., **1997**. A novel neuropeptide–endocrine interaction controlling ecdysteroid production in ixodid ticks. *Proc. Roy. Soc. B 264*, 589–596.

Lovett, D.L., Clifford, P.D., Borst, D.W., **1997**. Physiological stress elevates hemolymph levels of methyl farnesoate in the green crab *Carcinus maenas*. *Biol. Bull. 193*, 266–267.

Lovett, D.L., Verzi, M.P., Clifford, P.D., Borst, D.W., **2001**. Hemolymph levels of methyl farnesoate increase in response to osmotic stress in the green crab, *Carcinus maenas*. *Comp. Biochem. Physiol. A 128*, 299–306.

Lu, W., Wainwright, G., Olohan, L.A., Webster, S.G., Rees, H.H., *et al.*, **2001**. Characterization of cDNA encoding molt-inhibiting hormone of the crab, *Cancer pagurus*; expression of *MIH* in non-X-organ tissues. *Gene 278*, 149–159.

Lu, W., Wainwright, G., Webster, S.G., Rees, H.H., Turner, P.C., **2000**. Clustering of mandibular organ-inhibiting hormone and moult-inhibiting hormone genes in the crab, *Cancer pagurus*, and implications for regulation of expression. *Gene 253*, 197–207.

Lunke, M.D., Kaufman, W.R., **1992**. Effects of the avermectin analogue MK-243 on vitellogenesis and reproduction in the ixodid tick, *Amblyomma hebraeum*. *Exp. Appl. Acarol. 13*, 249–259.

Lunke, M.D., Kaufman, W.R., **1993**. Hormonal control of ovarian development in the tick *Amblyomma hebraeum* Koch (Acari: Ixodidae). *Invert. Reprod. Devel. 23*, 25–38.

Mango, C., Odhiambo, T.R., Galun, R., **1976**. Ecdysone and the super tick. *Nature 260*, 318–319.

Mansingh, A., Rawlins, S.C., **1977**. Antigonadotropic action of insect hormone analogues on the cattle tick *Boophilus microplus*. *Naturwiss. 64*, 41.

Mao, H., Kaufman, W.R., **1998**. DNA binding properties of the ecdysteroid receptor in the salivary gland of the female ixodid tick, *Amblyomma hebraeum*. *Insect Biochem. Mol. Biol. 28*, 947–957.

Mao, H., Kaufman, W.R., **1999**. Profile of the ecdysteroid hormone and its receptor in the salivary gland of the adult female tick, *Amblyomma hebraeum*. *Insect Biochem. Mol. Biol. 29*, 33–42.

Mao, H., McBlain, W.A., Kaufman, W.R., **1995**. Some properties of the ecdysteroid receptor in the salivary gland of the ixodid tick, *Amblyomma hebraeum*. *Gen. Comp. Endocrinol. 99*, 340–348.

Martin, G., Juchault, P., Sorokine, O., Van Dorsselaer, A., **1990**. Purification and characterization of androgenic hormone from the terrestrial isopod *Armadillidium vulgare* Latr. (Crustacea, Oniscidea). *Gen. Comp. Endocrinol. 80*, 349–354.

Martin, G., Sorokine, O., Moniatte, M., Bulet, P., Hetru, C., *et al.*, **1999**. The structure of a glycosylated protein hormone responsible for sex determination in the isopod, *Armadillidium vulgare*. *Eur. J. Biochem. 262*, 727–736.

Marzouk, A.S., Mohamed, F.S.A., Khalil, G.M., **1985**. Neurohemal-endocrine organs in the camel tick, *Hyalomma dromedarii* (Acari: Ixodoidea: Ixodidae). *J. Med. Entomol. 22*, 385–391.

Mattson, M.P., Spaziani, E., **1985**. Characterization of molt-inhibiting hormone (MIH) action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH-activity. *J. Exp. Zool. 236*, 93–101.

McDaniel, R.S., Oliver, J.H., Jr., **1978**. Effects of two juvenile hormone analogs and β-ecdysone on nymphal development, spermatogenesis, and embryogenesis in *Dermacentor variabilis* (Say) (Acari: Ixodidae). *J. Parasitol. 64*, 571–573.

McWhinnie, M., Kirchenberg, R.J., Urbanski, R.J., Schwarz, J.E., **1972**. Crustecdysone mediated changes in crayfish. *Am. Zool. 12*, 357–372.

Mitchell, L.G., Mutchmor, J.A., Dolphin, W.D., **1988**. Zoology. Benjamin/Cummings, Menlo Park, CA.

Mykles, D.L., **2001**. Interactions between limb regeneration and molting in decapod crustaceans. *Am. Zool. 41*, 399–406.

Nakatsuji, T., Keino, H., Tamura, K., Yoshimura, S., Kawakami, T., *et al.*, **2000**. Changes in the amounts of the molt-inhibiting hormone in sinus glands during the molt cycle of the American crayfish, *Procambarus clarkii*. *Zool. Sci. 17*, 1129–1136.

Neese, P.A., Sonenshine, D.E., Kallapur, V.L., Apperson, C.S., Roe, R.M., **2000**. Absence of insect juvenile hormones in the American dog tick, *Dermacentor variabilis* (Say) (Acari: Ixodidae), and in *Ornithodoros parkeri* Cooley (Acari: Argasidae). *J. Insect Physiol. 46*, 477–490.

Nijhout, H.F., **1994**. Insect Hormones. Princeton University Press, Princeton, NJ.

Obenchain, F.D., **1974**. Neurosecretory system of the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae). *J. Morphol. 142*, 433–446.

Obenchain, F.D., Oliver, J.H., Jr., **1975**. Neurosecreory system of the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae). 2. Distribution of secretory cell types, axonal pathways and putative neurohemal–neuroendocrine associations: comparative histological and anatomical implications. *J. Morphol. 145*, 269–294.

Ogihara, K., **2003**. Ecdysteroid Hormone Titer and Expression of Ecdysone Receptor mRNA as Related to Vitellogenesis in the Soft Tick, *Ornithodoros moubata* (Acari: Argasidae). M. Agric. Sci. thesis. University of Tsukuba, Tsukuba, Japan.

Ohira, T., Katayama, H., Aida, K., Nagasawa, H., **2003**. Expression of a recombinant crustacean hyperglycemic hormone of the kuruma prawn *Penaeus japonicus* in methylotrophic yeast *Pichia pastoris*. *Fisheries Sci. 69*, 95–100.

Ohira, T., Nishimura, T., Sonobe, H., Okuno, A., Watanabe, T., *et al.*, **1999**. Expression of a recombinant molt-inhibiting hormone of the kuruma prawn *Penaeus japonicus* in *Escherichia coli*. *Biosci. Biotechn. Biochem. 63*, 1576–1581.

Okazaki, R.K., Chang, E.S., **1991**. Ecdysteroids in the embryos and sera of the crab, *Cancer magister* and *C. anthonyi*. *Gen. Comp. Endocrinol. 81*, 174–186.

Okuno, A., Hasegawa, Y., Nishiyama, M., Ohira, T., Ko, R., *et al.*, **2002**. Preparation of an active recombinant peptide of crustacean androgenic gland hormone. *Peptides 23*, 567–572.

Okuno, A., Hasegawa, Y., Ohira, T., Katakura, Y., Nagasawa, H., **1999**. Characterization and cDNA cloning of androgenic gland hormone of the terrestrial isopod *Armadillidium vulgare*. *Biochem. Biophys. Res. Commun. 264*, 419–423.

Oliver, J.H., Jr., **1986**. Relationship among feeding, gametogenesis, mating and syngamy in ticks. In: Borovsky, D., Spielman, A. (Eds.), Host-Regulated Developmental Mechanisms in Vector Arthropods, vol. 1. University of Florida Press, Gainesville, FL, pp. 93–99.

Oliver, J.H., Jr., Dotson, E.M., **1993**. Hormonal control of molting and reproduction in ticks. *Am. Zool. 33*, 384–396.

Oliver, J.H., Jr., Pound, J.M., Severino, G., **1985**. Evidence for a juvenile-hormone-like compound in the reproduction of *Dermanyssus gallinae* (Acari: Dermanyssidae). *J. Med. Entomol. 22*, 281–286.

Oliver, J.H., Jr., Zhu, X.X., Vogel, G.N., Dotson, E.M., **1992**. Role of synganglion in oogenesis of the tick *Ornithodoros parkeri* (Acari: Argasidae). *J. Parasitol. 78*, 93–98.

Ollivaux, C., Dircksen, H., Toullec, J.-Y., Soyez, D., **2002**. Enkephalinergic control of the secretory activity of neurons producing stereoisomers of crustacean hyperglycemic hormone in the eyestalk of the crayfish *Orconectes limosus*. *J. Comp. Neurol. 444*, 1–9.

Ollivaux, C., Soyez, D., **2000**. Dynamics of biosynthesis and release of crustacean hyperglycemic hormone isoforms in the X-organ-sinus gland complex of the crayfish *Orconectes limosus*. *Eur. J. Biochem. 267*, 5106–5114.

Olmstead, A.W., LeBlanc, G.A., **2002**. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool. 293*, 736–739.

Orchard, I., **1980**. The effect of pyrethroids on the electrical activity of neurosecretory cells from the brain of *Rhodnius prolixus*. *Pesticide Biochem. Physiol. 13*, 220–226.

Orchard, I., Osborne, M.P., **1979**. The action of insecticides on neurosecretory neurons in the stick insect, *Carausius morosus*. *Pesticide Biochem. Physiol. 10*, 197–202.

Pappas, P.J., Oliver, J.H., Jr., **1972**. Reproduction in ticks (Acari: Ixodoidea). 2. Analysis of the stimulus for rapid and complete feeding of female *Dermacentor variabilis* (Say). *J. Med. Entomol. 9*, 47–50.

Passano, L.M., **1953**. Neurosecretory control of molting in crabs by the X-organ sinus gland complex. *Physiol. Comp. Oecol. 3*, 155–189.

Perkins, E.B., **1928**. Color changes in crustaceans, especially in *Palaemonetes*. *J. Exp. Zool. 50*, 71–105.

Polis, G.A., **1990**. Introduction. In: Polis, G.A. (Ed.), The Biology of Scorpions. Stanford University Press, Stanford, CA, pp. 1–8.

Pound, J.M., Oliver, J.H., Jr., **1979**. Juvenile hormone: evidence of its role in the reproduction of ticks. *Science 206*, 355–357.

Pound, J.M., Oliver, J.H., Jr., **1982**. Synganglial and neurosecretory morphology of female *Ornithodoros parkeri* (Cooley) (Acari: Argasidae). *J. Morphol. 173*, 159–177.

Pound, J.M., Oliver, J.H., Jr., Andrews, R.H., **1984**. Induction of apolysis and cuticle formation in female *Ornithodoros parkeri* (Acari: Argasidae) by hemocoelic injection of β-ecdysone. *J. Med. Entomol. 21*, 612–614.

Prestwich, G.D., Bruce, M.J., Ujváry, I., Chang, E.S., **1990**. Binding proteins for methyl farnesoate in lobster tissues: detection by photoaffinity labeling. *Gen. Comp. Endocrinol. 80*, 232–237.

Prullage, J.P., Pound, J.M., Meola, S.M., **1992**. Synganglial morphology and neurosecretory centers of adult *Amblyomma americanum* (L.) (Acari: Ixodidae). *J. Med. Entomol. 29*, 1023–1034.

Rachinsky, A., Strambi, C., Strambi, A., Hartfelder, K., **1990**. Caste and metamorphosis: hemolymph titres of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen. Comp. Endocrinol. 79*, 31–38.

Rao, K.R., **1985**. Pigmentary effectors. In: Bliss, D.E. (Ed.), The Biology of Crustacea, vol. 9. Academic Press, Orlando, FL, pp. 395–462.

Rao, K.R., **2001**. Crustacean pigmentary-effector hormones: chemistry and functions of RPCH, PDH, and related peptides. *Am. Zool. 41*, 364–379.

Rao, K.R., Fingerman, M., Hays, C., **1972**. Comparison of the abilities of α-ecdysone and 20-hydroxyecdysone

to induce precocious proecdysis and ecdysis in the fiddler crab *Uca pugilator*. *Z. Vergleich. Physiol. 76*, 270–284.

Reddy, P.S., Prestwich, G.D., Chang, E.S., **1997**. Crustacean hyperglycemic hormone gene expression in the lobster *Homarus americanus*. In: Kawashima, S., Kikuyama, S. (Eds.), Advances in Comparative Endocrinology, vol. 1. Moduzzi Editore, Bologna, pp. 51–56.

Reddy, P.S., Ramamurthi, R., **1998**. Methyl farnesoate stimulates ovarian maturation in the freshwater crab *Oziotelphusa senex senex* Fabricius. *Curr. Sci. 74*, 68–70.

Rodriguez, E.M., Greco, L.S.L., Medesani, D.A., Laufer, H., Fingerman, M., **2002**. Effect of methyl farnesoate, alone and in combination with other hormones, on ovarian growth of the red swamp crayfish, *Procambarus clarkii*, during vitellogenesis. *Gen. Comp. Endocrinol. 125*, 34–40.

Roe, R.M., Kallapur, V.L., Majumder, C., Lassiter, M.T., Apperson, C.S., *et al.*, **1993**. Biochemical evidence for the presence of a juvenoid in ticks. In: Borovsky, D., Spielman, A. (Eds.), Host-Regulated Developmental Mechanisms in Vector Arthropods, vol. 1. University of Florida Press, Gainesville, FL, pp. 110–120.

Romer, F., **1991**. The oenocytes of insects: differentiation, changes during molting, and their possible involvement in the secretion of molting hormone. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods, vol. 3. Rutgers University Press, New Brunswick, NJ, pp. 542–566.

Romer, F., Emmerich, H., Nowock, A., **1974**. Biosynthesis of molting hormones in isolated prothoracic glands and oenocytes of *Tenebrio molitor in vitro*. *J. Insect Physiol. 20*, 1975–1987.

Romer, F., Gnatzy, W., **1981**. Arachnid oenocytes: ecdysone synthesis in the legs of harvestmen (Opilionidae). *Cell and Tissue Res. 216*, 449–453.

Root, T.M., **1990**. Neurobiology. In: Polis, G.A. (Ed.), The Biology of Scorpions. Stanford University Press, Stanford, CA, pp. 341–413.

Rosell, R., Coons, L.B., **1990**. Quantification of vitellogenin in the hemolymph and localization of vitellogenin in selected organs of adult female *Dermacentor variabilis*. *Adv. Invert. Reprod. 5*, 559–564.

Rosell, R., Coons, L.B., **1991**. Determination of vitellogenin titer in the hemolymph of *Dermacentor variabilis* (Acarina: Ixodidae) using an indirect enzyme-linked immunosorbent assay. *J. Med. Entomol. 28*, 41–44.

Rosenkranz, P., Rachinsky, A., Strambi, A., Strambi, C., Röpstorf, P., **1990**. Juvenile hormone titre in capped worker brood of *Apis mellifera* and reproduction in the bee mite *Varroa jacobsoni*. *Gen. Comp. Endocrinol. 78*, 189–193.

Rosenkranz, P., Tewarson, N.C., Rachinsky, A., Strambi, A., Strambi, C., *et al.*, **1993**. Juvenile hormone titre and reproduction of *Varroa jacobsoni* in capped brood stages of *Apis cerana indica* in comparison to *Apis mellifera ligustica*. *Apidologie 24*, 375–382.

Roshdy, M.A., Shoukrey, N.M., Coons, L.B., **1973**. The subgenus *Persicargas* (Ixodoidea: Argasidae: *Argas*). 17. A neurohemal organ in *A. (P.) arboreus* Kaiser, Hoogstraal, and Kohls. *J. Parasitol. 59*, 540–544.

Ruight, G.S.F., **1985**. Pyrethroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 12. Pergamon, Oxford, pp. 183–262.

Sagi, A., Khalaila, I., **2001**. The crustacean androgen: a hormone in an isopod and androgenic activity in decapods. *Am. Zool. 41*, 477–484.

Sahli, R., Germond, J.E., Diehl, P.A., **1985**. Spermateleosis and secretory activity of the sperm cell of the tick *Ornithodoros moubata*. *Exp. Parasitol. 60*, 383–395.

Saidi, B., de Besse, N., Webster, S.G., Sedlmeier, D., Lachaise, F., **1994**. Involvement of cAMP and cGMP in the mode of action of molt-inhibiting hormone (MIH) a neuropeptide which inhibits steroidogenesis in a crab. *Mol. Cell. Endocrinol. 102*, 53–61.

Sakagami, Y., Taki, K., Matsuhisa, T., Marumo, S., **1992**. Identification of 2-deoxyecdysone from the mite, *Tyrophagus putrescentiae*. *Experientia 48*, 793–795.

Sankhon, N., Lockey, T., Rosell, R.C., Rothschild, M., Coons, L., **1999**. Effect of methoprene and 20-hydroxyecdysone on vitellogenin production in cultured fat bodies and backless explants from unfed female *Dermacentor variabilis*. *J. Insect Physiol. 45*, 755–761.

Santos, E.A., Nery, L.E.M., Keller, R., Goncalves, A.A., **1997**. Evidence for the involvement of the crustacean hyperglycemic hormone in the regulation of lipid metabolism. *Physiol. Zool. 70*, 415–420.

Schoettker, P.J., Gist, D.H., **1990**. *In vitro* ecdysteroid production by Y-organs of the blue crab *Callinectes sapidus*. *J. Crustac. Biol. 10*, 487–491.

Schreifer, M.E., Beveridge, M., Sonenshine, D.E., Homsher, P.J., Carson, K.A., *et al.*, **1987**. Evidence of ecdysteroid production by tick (Acari: Ixodidae) fat-body tissues *in vitro*. *J. Med. Entomol. 24*, 295–302.

Sedlmeier, D., **1988**. The crustacean hyperglycemic hormone (CHH) releases amylase from the crayfish midgut gland. *Regulat. Peptides 20*, 91–98.

Sedlmeier, D., Keller, R., **1981**. The mode of action of the crustacean neurosecretory hyperglycemic hormone. 1. Involvement of cyclic nucleotides. *Gen. Comp. Endocrinol. 45*, 82–90.

Sekiguchi, K., Itow, T., Yamamichi, Y., **1988**. Experimental embryology. In: Sekiguchi, K. (Ed.), Biology of Horseshoe Crabs. Science House, Tokyo, pp. 225–287.

Serrano, L., Blanvillain, G., Soyez, D., Charmantier, G., Grousset, E., *et al.*, **2003**. Putative involvement of crustacean hyperglycemic hormone isoforms in the neuroendocrine mediation of osmoregulation in the crayfish *Astacus leptodactylus*. *J. Exp. Biol. 206*, 979–988.

Severino, G., Oliver, J.H., Jr., Pound, J.M., **1984**. Synganglial and neurosecretory morphology of the chicken mite *Dermanyssus gallinae* (DeGeer) (Mesostigmata: Dermanyssidae). *J. Morphol. 181*, 49–68.

Shanbaky, N.M., El-Said, A., Helmy, N., **1990a**. Changes in neurosecretory cell activity in female *Argas (Argas) hermanni* (Acari: Argasidae). *J. Med. Entomol. 27*, 975–981.

Shanbaky, N.M., Khalil, G.M., **1975**. The subgenus *Persicargas* (Ixodoidea: Argasidae: Argas). 22. The effect of feeding on hormonal control of egg development in *Argas (Persicargas) arboreus*. *Exp. Parasitol. 37*, 361–366.

Shanbaky, N.M., Mansour, M.M., Main, A.J., El-Said, A., Helmy, N., **1990b**. Hormonal control of vitellogenesis in *Argas (Argas) hermanni* (Acari: Argasidae). *J. Med. Entomol. 27*, 968–974.

Shanbaky, N.M., Mansour, M.M., Main, A.J., Helmy, N., **1990c**. Vitellogenic and non-vitellogenic proteins in haemolymph, ovaries and eggs of *Argas (Argas) hermanni* (Acari: Argasidae). *J. Med. Entomol. 27*, 986–992.

Shepherd, J., Oliver, J.H., Jr., Hall, J.D., **1982**. A polypeptide from male accessory glands which triggers maturation of tick spermatozoa. *Int. J. Invert. Reprod. 5*, 129–137.

Shuster, C.N., Jr., **1982**. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae. In: Bonaventura, J., Bonaventura, C., Tesh, S. (Eds.), Physiology and Biology of Horseshoe Crabs. Alan R. Liss, New York, pp. 1–52.

Skinner, D.M., **1985a**. Molting and regeneration. In: Bliss, D.E., Mantel, L.H. (Eds.), The Biology of Crustacea, vol. 9. Academic Press, Orlando, FL, pp. 43–146.

Skinner, D.M., **1985b**. Interacting factors in the control of the crustacean molt cycle. *Am. Zool. 25*, 275–284.

Slusser, J.H., Sonenshine, D.E., **1992**. Absence of ovicidal effects of fenoxycarb in the tick, *Ixodes dammini* as observed by light, scanning, and transmission electron microscopy. *J. Med. Entomol. 29*, 115–117.

Sonenshine, D.E., **1991**. Biology of Ticks, vol. 1. Oxford University Press, Oxford.

Sonenshine, D.E., Homsher, P.J., Beveridge, M., Dees, W.H., **1985**. Occurrence of ecdysteroids in specific body organs of the camel tick, *Hyalomma dromedarii*, and the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae), with notes on their synthesis from cholesterol. *J. Med. Entomol. 22*, 303–311.

Soumoff, C., O'Connor, J.D., **1982**. Repression of Y-organ secretory activity by molt inhibiting hormone in the crab *Pachygrapsus crassipes*. *Gen. Comp. Endocrinol. 48*, 432–439.

Soyez, D., Le Caer, J.P., Noel, P.Y., Rossier, J., **1991**. Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster *Homarus americanus*. *Neuropeptides 20*, 25–32.

Soyez, D., Van Herp, F., Rossier, J., Le Caer, J.-P., Tensen, C.P., *et al.*, **1994**. Evidence for a conformational polymorphism of invertebrate neurohormones: D-amino acid residue in crustacean hyperglycemic peptides. *J. Biol. Chem. 269*, 18295–18298.

Spanings-Pierrot, C., Soyez, D., Van Herp, F., Gompel, M., Skaret, G., *et al.*, **2000**. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol. 119*, 340–350.

Spaziani, E., Jegla, T.C., Wang, W.L., Booth, J.A., Connolly, S.M., *et al.*, **2001**. Further studies on signaling pathways for ecdysteroidogenesis by crustacean Y-organs. *Am. Zool. 41*, 418–429.

Spaziani, E., Rees, H.H., Wang, W.L., Watson, R.D., **1989**. Evidence that Y-organs of the crab *Cancer antennarius* secrete 3-dehydroecdysone. *Mol. Cell. Endocrinol. 66*, 17–25.

Spindler, K.-D., Dinan, L., Londershausen, M., **1984**. On the mode of action of ecdysteroids in crustaceans. In: Hoffmann, J., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, pp. 255–264.

Spindler, K.-D., Keller, R., O'Connor, J.D., **1980**. The role of ecdysteroids in the crustacean molt cycle. In: Hoffmann, J.A. (Ed.), Progress in Ecdysone Research. Elsevier/North-Holland, Amsterdam, pp. 247–280.

Spindler, K.-D., Van Wormhoudt, A., Sellos, D., Spindler-Barth, M., **1987**. Ecdysteroid levels during embryogenesis in the shrimp, *Palaemon serratus* (Crustacea Decapoda): quantitative and qualitative changes. *Gen. Comp. Endocrinol. 66*, 116–122.

Stauffer, A., Connat, J.-L., **1990**. Anteroposterior gradient during nymphal–adult moulting cycle of the tropical bont tick, *Amblyomma variegatum* (Acarina: Ixodidae): correlation between ecdysteroid titers and integument structure. *Roux's Arch. Devel. Biol. 198*, 309–321.

Stockmann, R., Laverdure, A.M., Breuzet, M., **1997**. Localization of a crustacean hyperglycemic hormone-like immunoreactivity in the neuroendocrine system of *Euscorpius carpathicus* (L.) (Scorpionida, Chactidae). *Gen. Comp. Endocrinol. 106*, 320–326.

Subramoniam, T., **2000**. Crustacean ecdysteroids in reproduction and embryogenesis. *Comp. Biochem. Physiol. C 125*, 135–156.

Sun, P.S., **1997**. Recombinant molt-inhibiting hormone-like neuropeptide produced in the yeast *Pichia pastoris*. *PACON International Proceedings 1997*, 509–518.

Tamone, S.L., Chang, E.S., **1993**. Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs *in vitro*. *Gen. Comp. Endocrinol. 89*, 425–432.

Tamone, S.L., Prestwich, G.D., Chang, E.S., **1997**. Identification and characterization of methyl farnesoate binding proteins from the crab, *Cancer magister. Gen. Comp. Endocrinol. 105*, 168–175.

Tang, C., Lu, W., Wainwright, G., Webster, S.G., Rees, H.H., *et al.*, **1999**. Molecular characterization and expression of mandibular organ-inhibiting hormone, a recently discovered neuropeptide involved in the regulation of growth and reproduction in the crab *Cancer pagurus*. *Biochem. J. 343*, 355–360.

Taylor, D., Chinzei, Y., Ito, K., Higuchi, N., Ando, K., **1991a**. Stimulation of vitellogenesis by pyrethroids in mated and virgin female adults and fourth instar

females of *Ornithodoros moubata* (Acari: Argasidae). *J. Med. Entomol. 28*, 322–329.

Taylor, D., Chinzei, Y., Miura, K., Ando, K., **1991b**. Vitellogenin synthesis, processing and hormonal regulation in the tick, *Ornithodoros parkeri* (Acari: Argasidae). *Insect Biochem. 21*, 723–733.

Taylor, D., Chinzei, Y., Miura, K., Ando, K., **1992**. Effects of precocenes on vitellogenesis in the adult female tick, *Ornithodoros moubata* (Acari: Argasidae). *Exp. Appl. Acarol. 14*, 123–136.

Taylor, D., Nakajima, Y., Chinzei, Y., **2000**. Ecdysteroids and vitellogenesis in the soft tick, *Ornithodoros moubata* (Acari: Argasidae). In: Proc. 3rd Int. Conf. "Ticks and Tick-borne Pathogens: Into the 21st Century," pp. 223–227.

Taylor, D., Phillips, J.S., Sonenshine, D.E., Hanson, F.E., **1991c**. Ecdysteroids as a component of the genital sex pheromone in two species of hard ticks, *Dermacentor variabilis* and *Dermacentor andersoni* (Acari: Ixodidae). *Exp. Appl. Acarol. 12*, 275–296.

Tensen, C.P., Verhoeven, A.H.M., Gaus, G., Janssen, P.C., Keller, R., *et al.*, **1991**. Isolation and amino acid sequence of crustacean hyperglycemic hormone precursor-related peptides. *Peptides 12*, 673–681.

Thind, B.B., Edwards, J.P., **1990**. Stimulation of egg production in the grain mite *Acarus siro* by the juvenile-hormone analogue fenoxycarb. *Exp. Appl. Acarol. 9*, 1–10.

Tilden, A.R., Rasmussen, P., Awantang, R.M., Furlan, S., Goldstein, J., *et al.*, **1997**. Melatonin cycle in the fiddler crab *Uca pugilator* and influence of melatonin on limb regeneration. *J. Pineal Res. 23*, 142–147.

Tomaschko, K.-H., **1994**. Ecdysteroids from *Pycnogonium litorale* (Arthropoda, Pantapoda) act as chemical defense against *Carcinus maenas* (Crustacea, Decapoda). *J. Chem. Ecol. 20*, 1445–1455.

Trabalon, M., Bautz, A.-M., Morinière, M., Porcheron, P., **1992**. Ovarian development and correlated changes in hemolymphatic ecdysteroid levels in two spiders, *Coelotes terrestris* and *Tegenaria domestica* (Araneae, Agelenidae). *Gen. Comp. Endocrinol. 88*, 128–136.

Ujváry, I., Prestwich, G.D., **1990**. An efficient synthesis of the crustacean hormone [12-^{3}H]-methyl farnesoate and its photolabile analog [13-^{3}H]-farnesyl diazomethyl ketone. *J. Labelled Compounds Radiopharm. 28*, 167–174.

Van Herp, F., **1998**. Molecular, cytological and physiological aspects of the crustacean hyperglycemic hormone family. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge, pp. 53–70.

Venkatesh, K., Roe, R.M., Apperson, C.S., Sonenshine, D.E., Schreifer, M.E., *et al.*, **1990**. Metabolism of juvenile hormone during adult development of *Dermacentor variabilis* (Acari: Ixodidae). *J. Med. Entomol. 27*, 36–42.

Vogel, J.M., Borst, D.W., **1989**. Spider crab yolk protein molecular characterization and the effect of methyl farnesoate (MF) on its hemolymph levels. *Am. Zool. 29*, 49A.

Wainwright, G., Webster, S.G., Rees, H.H., **1998**. Neuropeptide regulation of biosynthesis of the juvenoid, methyl farnesoate, in the edible crab, *Cancer pagurus*. *Biochem. J. 334*, 651–657.

Wainwright, G., Webster, S.G., Wilkinson, M.C., Chung, J.S., Rees, H.H., **1996**. Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*. *J. Biol. Chem. 271*, 12749–12754.

Watson, R.D., Lee, K.J., Qiu, S., Luo, M., Umphrey, H.R., *et al.*, **2001**. Molecular cloning, expression, and tissue distribution of crustacean molt-inhibiting hormone. *Am. Zool. 41*, 407–417.

Watson, R.D., Spaziani, E., Bollenbacher, W.E., **1989**. Regulation of ecdysone biosynthesis in insects and crustaceans: a comparison. In: Koolman, J. (Ed.), Ecdysone: From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, pp. 188–203.

Webster, S.G., **1983**. Effects of exogenous ecdysterone upon moulting, proecdysial development, and limb regeneration in the prawn *Palaemon elegans*. *Gen. Comp. Endocrinol. 49*, 459–469.

Webster, S.G., **1991**. Amino acid sequence of putative moult-inhibiting hormone from the crab *Carcinus maenas*. *Proc. Roy. Soc. B 244*, 247–252.

Webster, S.G., **1993**. High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane-bound receptors on the Y-organ of the shore crab *Carcinus maenas*. *Proc. Roy. Soc. B 251*, 53–59.

Webster, S.G., **1996**. Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *J. Exp. Biol. 199*, 1579–1585.

Webster, S.G., **1998**. Neuropeptides inhibiting growth and reproduction in crustaceans. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge, pp. 33–52.

Webster, S.G., Dircksen, H., Chung, J.S., **2000**. Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycaemic hormone and its precursor-related peptide. *Cell and Tissue Res. 300*, 193–205.

Weidemann, W., Gromoll, J., Keller, R., **1989**. Cloning and sequence analysis of cDNA for precursor of a crustacean hyperglycemic hormone. *FEBS Lett. 257*, 31–34.

Weiss, B.L., Kaufman, W.R., **2004**. Two feeding-induced proteins from the male gonad trigger engorgement of the female tick, *Amblyomma hebraeum*. *Proc. Natl Acad. Sci. USA 101*, 5874–5879.

Weiss, B.L., Stepczynski, J.M., Wong, P., Kaufman, W.R., **2002**. Identification and characterization of genes differentially expressed in the testis/vas deferens of the fed male tick, *Amblyomma hebraeum*. *Insect Biochem. Mol. Biol. 32*, 785–793.

Whitehead, D.L., Osir, E.W., Obenchain, F.D., Thomas, L.S., **1986**. Evidence for the presence of ecdysteroids

and preliminary characterization of their carrier protein in the eggs of the brown ear tick *Rhipicephalus appendiculatus* (Neumann). *Insect Biochem. 16*, 121–133.

Wigglesworth, K.P., Lewis, D., Rees, H.H., **1985**. Ecdysteroid titer and metabolism to novel apolar derivatives in adult female *Boophilus microplus* (Ixodidae). *Arch. Insect Biochem. Physiol. 2*, 39–54.

Wilcockson, D.C., Chung, J.S., Webster, S.G., **2002**. Is crustacean hyperglycaemic hormone precursor-related peptide a circulating neurohormone in crabs? *Cell and Tissue Res. 307*, 129–138.

Wilder, M.N., Okumura, T., Aida, K., Hanyu, I., **1990**. Ecdysteroid fluctuations during embryogenesis in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Gen. Comp. Endocrinol. 80*, 93–100.

Winget, R.R., Herman, W.S., **1976**. Occurrence of ecdysone in the blood of the chelicerate arthropod, *Limulus polyphemus*. *Experientia 32*, 1345–1346.

Yamasaki, T., Makioka, T., Saito, J., **1988**. Morphology. In: Sekiguchi, K. (Ed.), Biology of Horseshoe Crabs. Science House Co., Tokyo, pp. 69–132.

Yang, W.-J., Rao, K.R., **2001**. Cloning of precursors for two MIH/VIH-related peptides in the prawn, *Macrobrachium rosenbergii*. *Biochem. Biophys. Res. Comm. 289*, 407–413.

Yasuda, A., Yasuda, Y., Fujita, T., Naya, Y., **1994**. Characterization of crustacean hyperglycemic hormone from the crayfish (*Procambarus clarkii*): multiplicity of molecular forms by stereoinversion and diverse functions. *Gen. Comp. Endocrinol. 95*, 387–398.

Yu, X., Chang, E.S., Mykles, D.L., **2002**. Characterization of limb autotomy factor-proecdysis (LAF_{pro}), isolated from limb regenerates, that suspends molting in the land crab *Gecarcinus lateralis*. *Biol. Bull. 202*, 204–212.

Yudin, A.I., Diener, R.A., Clark, W.H., Chang, E.S., **1980**. Mandibular gland of the blue crab, *Callinectes sapidus*. *Biol. Bull. 159*, 760–772.

Zeleny, C., **1905**. Compensatory regulation. *J. Exp. Zool. 2*, 1–102.

Zhang, W.Y., Zhu, X.X., Oliver, J.H., Jr., **1995**. Ecdysteroids and spermatogenesis in the fourth instar nymph of the tick *Ornithodoros parkeri*. *Invert. Reprod. Devel. 27*, 1–9.

Zhu, X.X., Oliver, J.H., Jr., **1991**. Immunocytochemical localization of an insulin-like substance in the synganglion of the tick, *Ornithodoros parkeri* (Acari: Argasidae). *Exp. Appl. Acarol. 13*, 153–159.

Zhu, X.X., Oliver, J.H., Jr., Dotson, E.M., **1991**. Epidermis as the source of ecdysone in an argasid tick. *Proc. Natl Acad. Sci. USA 88*, 3744–3747.

Zhu, X.X., Oliver, J.H., Jr., Dotson, E.M., Ren, H.L., **1994**. Correlation between ecdysteroids and cuticulogenesis in nymphs of the tick *Ornithodoros parkeri* (Acari: Argaisdae). *J. Med. Entomol. 31*, 479–485.

Zhu, X.X., Zhang, W.Y., Oliver, J.H., Jr., **1995**. Immunocytochemical mapping of FMRFamide-like peptides in the argasid tick, *Ornithodoros parkeri* and the ixodid tick *Dermacentor variabilis*. *Exp. Appl. Acarol. 19*, 1–9.

4.1 Insect Cytochrome P450

R Feyereisen, INRA, Centre de Recherches de Sophia Antipolis, Sophia Antipolis, France

4.1.1. Introduction

4.1.1.1. Overview

Cytochromes P450 or *CYP* genes constitute one of the largest family of genes with representatives in virtually all living organisms, from bacteria to protists, plants, fungi, and animals (Werck-Reichhart and Feyereisen, 2000). An ever-growing number of P450 sequences are available, and the role of P450 enzymes is being documented in an ever-growing number of physiological processes. The human genome carries about 57 P450 genes and insect genomes carry about a hundred. Each P450 protein is the product of a distinct *CYP* gene and P450 diversity is the result of successive gene (or genome) duplications followed by sequence divergence. The typically 45–55 kDa P450 proteins are heme-thiolate enzymes that are known collectively to catalyze at least 60 chemically distinct reactions. Their essential common feature is the absorbance peak near 450 nm of their Fe^{II}–CO complex for which they are named. P450 enzymes are best known for their monooxygenase role, catalyzing the transfer of one atom of molecular oxygen to a substrate and reducing the other to water. The simple stoichiometry (eqn [1]) commonly describes the monooxygenase or mixed-function oxidase reaction of P450:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \quad [1]$$

However, oxygen atom transfer is not the only catalytic function of P450 enzymes. They also show activity as oxidases, reductases, desaturases, isomerases, etc. (Mansuy, 1998). There are soluble forms of P450 (in bacteria) and membrane-bound forms (in microsomes and mitochondria of eukaryotes). P450s are dependent on redox partners for their supply of reducing equivalents from NADH or NADPH (NADPH cyt P450 reductase and cyt b_5 in

microsomes; a ferredoxin and a ferredoxin reductase in mitochondrial and bacterial systems). Some bacterial P450s are fusion proteins with their redox partners, either P450 reductase (see below) or a phthalate family oxygenase reductase (PFOR) (De Mot and Parret, 2002). Because of their complex catalytic mechanism, P450 enzymes often, perhaps always, generate superoxide or hydrogen peroxide from "unsuccessful," or uncoupled reactions, leading to oxidative stress in cells.

Many P450 proteins are specialized in the metabolism of endogenous substrates (steroid hormones, lipids, etc.) but much of their notoriety has been associated with the metabolism or detoxification of xenobiotics (natural products, drugs, pesticides, etc.). In insects, they are involved in many cases of insecticide resistance. In well-studied cases, P450 enzymes generate more toxic compounds, e.g., carcinogenic metabolites of aryl hydrocarbons. Many foreign compounds, as well as endogenous metabolites, can induce the transcription of P450 genes through complex interactions with nuclear receptors and bHLH-PAS proteins, such as the *Ah* (aryl hydrocarbon) receptor of vertebrates. There are many excellent reviews and books clearly presenting the state of knowledge on P450s, generally with an emphasis on mammalian and bacterial enzymes (Omura *et al*., 1993; Schenkman and Greim, 1993; Ortiz de Montellano, 1995b). Reliance on the advances made in noninsect systems is both necessary and risky. Necessary because the structural and functional homology of P450 enzymes allows general principles to emerge. Risky because such principles may not always apply to peculiar aspects of insect P450 evolution. The chapter will certainly fail to be comprehensive in view of the enormous literature pertaining to P450 but it will try to convey the state of our current understanding as a basis for future research. Many aspects of P450 biology are intertwined and complex (structure, activity, induction) so the linear and compartmentalized treatment of the subject as done here will probably fail to pass muster with the P450 novice and specialist alike. Indeed, the interpretations and choices of examples cited are mine, and the reader is invited to browse through text and references repeatedly. To help fill the enormous gaps in our knowledge on insect P450, the reader is also invited to join the field of P450 research.

4.1.1.2. Historical Framework

This chapter humbly tries to update the exhaustive reviews of Hodgson on microsomal monooxygenases (Hodgson, 1985) and of Agosin on the role of microsomal oxidations in insecticide degradation (Agosin, 1985) published in the first edition of this series. The present chapter will focus on advances since 1985, and the earlier literature will be cited when needed for clarity or historical import. Both 1985 chapters had insect cytochromes P450 as their main focus and both revealed the enticing complexity of P450 catalysis and regulation. These two chapters, as well as insightful reviews that preceded them (Wilkinson and Brattsten, 1972; Brattsten, 1979b; Brooks, 1979), give a good account of the spirit of the times.

The great variety of reactions catalyzed by P450s was well recognized, but it was not really known how the multiplicity of enzymes and the suspected loose substrate specificity were blended to achieve this variety. The existence of multiple P450 forms was accepted but the true measure of this multiplicity was not foreseen. This multiplicity, once understood at the molecular genetic level, led to the early adoption of a common nomenclature in 1987 – the *CYP* genes. The substrate specificity of individual P450 enzymes was a matter of conjecture, because enzyme purification from insect sources and reconstitution of significant activity *in vitro* proved to be extremely difficult. Many cases of insecticide resistance were clearly attributable to alterations in the P450 system – a quantitative increase and a qualitative change. The molecular basis for this change was unknown, only a few resistance genes had been formally recognized at the genetic level, and the nature of the mutations leading to resistance was barely discussed (Terriere and Yu, 1974).

The role of insect P450 enzymes in the biosynthesis and degradation of endogenous compounds was becoming increasingly clear, but their relation to P450s involved in xenobiotic metabolism was unclear – were they truly the same class of enzymes? In contrast, an evolutionary link between the capacity to metabolize, and resist, synthetic insecticides and the ability of insects to thrive on chemically well-defended plants was recognized early on (Gordon, 1961). The metabolism of plant secondary compounds by P450s and the role these compounds played in regulating P450 activities brought insects forward as exquisite models to study the ecological role of P450 enzymes. Insects have now become equally exquisite models to study the evolution of P450s.

The first cloning and sequencing of a P450 cDNA (rat CYP2B1) was in 1982 (Fuji-Kuriyama *et al*., 1982) and that of an insect P450 (housefly CYP6A1) followed in 1989 (Feyereisen *et al*., 1989). The first crystal structure of a soluble bacterial P450, P450cam, was published in 1985 (Poulos

et al., 1985). The first structure of a mammalian microsomal P450 engineered for solubility followed in 2000 (Williams *et al.*, 2000a). After several heroic attempts at wresting activities from purified insect enzymes reconstituted *in vitro*, it was the heterologous expression of cloned insect P450 cDNAs in 1994 (Andersen *et al.*, 1994; Ma *et al.*, 1994) that allowed the rational study of individual P450 enzymology. Genome sequencing projects finally revealed the cast of P450 characters: insects have twice as many *CYP* genes as mammals, but only a third that of plants (David Nelson's cytochrome P450 homepage (web link)).

4.1.1.3. Vocabulary

Although P450 enzymes and *CYP* genes are displacing mixed-function oxidases (MFOs), microsomal oxidases, polysubstrate monooxygenases (PSMO) or simply monooxygenases in the vocabulary, words of caution are needed at the outset. The word "cytochrome" was initially given to the liver P450 pigment (Omura, 1993; Estabrook, 1996) and it is still associated with P450 enzymes. However, P450s are formally not cytochromes but rather heme-thiolate proteins (Mansuy, 1998). Not all P450-dependent reactions are monooxygenations, and not all monooxygenases are P450 enzymes (Mansuy and Renaud, 1995). In particular, flavin monooxygenases or FMOs are NADPH-dependent enzymes that catalyze some reactions similar or identical to those catalyzed by P450 enzymes. In mammalian species, FMOs are microsomal enzymes best known for their *N*- and *S*-oxidation activities (Ziegler, 2002). Until recently, FMOs had not been identified in insects. In the cinnabar moth, *Tyria jacobaea*, a soluble FMO specifically N-oxidizes pyrolizidine alkaloids such as senecionine, seneciphylline, monocrotaline, and axillarine (Lindigkeit *et al.*, 1997; Naumann *et al.*, 2002). Only two FMO genes of as yet unknown function have been recognized in the *Drosophila* genome, but there may well be many more in other species. Whether insect FMOs are involved in xenobiotic *N*- or *S*-oxidations is still unknown.

A word of caution, too, about the "microsomal" oxidases. This chapter will show that insect P450s can be found in microsomal membranes as well as in mitochondria, and that subcellular localization does not portend a particular physiological role or catalytic competence.

4.1.1.4. Nomenclature

A nomenclature of P450 genes and proteins was introduced when only 65 sequences were known (Nebert *et al.*, 1987). Gene families were initially designated by Roman numerals, but the proliferation of diverse sequences rapidly became discouraging, even to those versed in classics. The rules of nomenclature were then revised to their current form (Nebert *et al.*, 1991; Nelson *et al.*, 1993, 1996) where a CYP prefix, followed by an arabic numeral designates the family (all members nominally >40% identical), a capital letter designates the subfamily (all members nominally >55% identical) and an arabic numeral designates the individual gene (all italics) or message and protein (no italics) (**Figure 1**). Different P450 enzymes are generally products of different genes; they are not isozymes or isoforms. The identity (%) rules for family and subfamily designations are not strictly adhered to, but names once adopted are rarely changed. Initially, many insect P450s were arbitrarily lumped into the CYP6 and the CYP4 families even though they had less than 40% amino acid identity with CYP6A1 or with vertebrate CYP4 proteins. Naming genes in the lumper mode made the CYP6 and CYP4 families the largest ones in insects by a cascade effect. CYP6B1 is only 32% identical to CYP6A1 (Cohen *et al.*, 1992), so placing it in the CYP6 family "forced" many subsequent sequences into that family even if they did not meet the 40% criterion. The splitter mode prevailed at the completion of genome projects, which led to a new proliferation of CYP families in insects, the CYP300 series. A termite P450 claimed the welcoming designation of CYP4U2 (GenBank AF046011). Gotoh (1993) has introduced a useful nomenclature of higher order than CYP families: the E (for eukaryotic type) and B (for bacterial type) "classes" and subclasses (I, II, III, etc.) that regroup CYP families on the basis of phylogeny. Nelson (1998) has similarly introduced the notion of "clans," but the precise criteria for naming Gotoh's "classes" and Nelson's "clans" have not been defined.

Alleles of a gene are named as subscripts v1, v2 (e.g., *CYP6B1v2*, Cohen *et al.*, 1992). The human P450 polymorphisms are named according to a clear nomenclature. Pseudogenes are noted by the

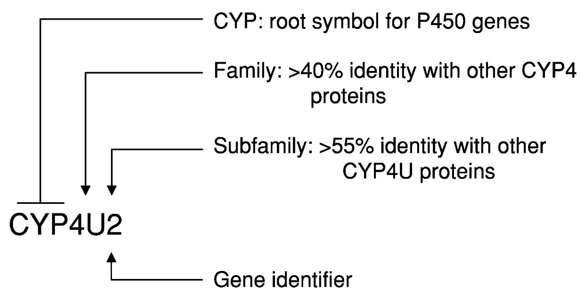


Figure 1 Scheme of the P450 nomenclature.

suffix P. This suffix ought to be added to the closest paralog that is an active gene, e.g., *CYP9E2* and *CYP9E2P1* in *Blattella germanica* (Wen *et al.*, 2001). However this is not always done, as the closest paralog is sometimes not easily recognized. In following the tradition that predates the CYP nomenclature, P450 enzymes can be named with a small suffix, such as P450cam, the camphor hydroxylase of *Pseudomonas putida* later named CYP101; P450BM3 the fatty acid hydroxylase of *Bacillus megaterium* (CYP102); or P450scc, the cholesterol side-chain cleavage enzyme (CYP11A1). In insects, few P450 enzymes have been named in this way. P450Lpr is the predominant P450 in the pyrethroid-resistant strain Learn-Pyr of the housefly, later identified as CYP6D1 (Tomita and Scott, 1995). P450hyd (Reed *et al.*, 1994) is the P450 forming hydrocarbons in the housefly. P450MA is a P450 purified from the Munsyana strain of the German cockroach (Scharf *et al.*, 1998). In the *Drosophila* gene nomenclature (Lindsley and Zimm, 1992), only the initial letter is capitalized, hence *CYP6A1* in the housefly and *Cyp6a2* in *Drosophila*.

The CYP nomenclature of Nebert *et al.* was clearly designed to reflect the evolutionary relationships between the genes as evidenced by the degree of sequence identity of the proteins they encoded. As such it is a Darwinian nomenclature. P450 proteins have been categorized into classes (Ravichandran *et al.*, 1993) that reflect the types of electron delivery to the active site. Class I proteins require both an FAD-flavoprotein reductase and a ferredoxin-type protein, class II P450s require only an FAD and FMN diflavin reductase, class III enzymes are self-sufficient, and class IV P450s receive electrons directly from NADPH. The utility of this nonevolutionary classification is debatable.

4.1.2. Diversity and Evolution of Insect P450 Genes

4.1.2.1. Sequence Diversity

4.1.2.1.1. P450 sequences from classical cloning techniques The first insect P450s cloned and sequenced were CYP6A1 from *Musca domestica* in 1989 (Feyereisen *et al.*, 1989), CYP4C1 from *Blaberus discoidalis* in 1991 (Bradfield *et al.*, 1991), CYP6A2 and CYP4D1 from *Drosophila* (Gandhi *et al.*, 1992; Waters *et al.*, 1992) as well as CYP6B1 from *Papilio polyxenes* in 1992 (Cohen *et al.*, 1992), and CYP4D2 from *Drosophila* in 1994 (Frolov and Alatortsev, 1994). The methods used in these early studies were screening of cDNA expression libraries with polyclonal (CYP6A1) or monoclonal (CYP6A2) antibodies to (partially) purified P450 proteins of insecticide-resistant flies (CYP6A1, CYP6A2). For CYP6B1, microsequencing of N-terminal and internal sequences of a P450-sized band on a SDS-PAGE gel of *P. polyxenes* larval midgut proteins led to the design of degenerate oligonucleotide probes for RT-PCR on midgut poly(A)$^+$ RNA. The cloned PCR product was used in turn as a probe to screen a xanthotoxin-induced midgut cDNA library. Significantly, two cDNAs representing alleles of the *CYP6B1* gene were thus isolated. Classical molecular approaches of this type have continued to yield new P450 sequences (Tomita and Scott, 1995; Wang and Hobbs, 1995; Winter *et al.*, 1999) and the initial P450 sequences have served as probes to isolate related sequences in the same species (Cohen and Feyereisen, 1995; Hung *et al.*, 1995a, 1996; Maitra *et al.*, 1996), or in phylogenetically close species (Li *et al.*, 2000a, 2001). Interestingly, CYP9A1 of *Heliothis virescens* was cloned by screening an expression library with monoclonal antibodies that also served to clone CYP6A2 of *Drosophila*, despite the fact that the two sequences are only 32.4% identical (Rose *et al.*, 1997).

4.1.2.1.2. Serendipity and P450 discovery In contrast to the targeted approaches, CYP4C1 was obtained in 1991 by differential screening of a cockroach fat body cDNA library (Bradfield *et al.*, 1991). The probes consisted of cDNA obtained from fat bodies of hypertrehalosemic hormone-treated roaches, or decapitated controls. The cloning and identification of *Drosophila Cyp4d1* and *Cyp4d2* were equally serendipitous, as investigators were interested in transcripts in the *prune* region at the tip of the X chromosome (Gandhi *et al.*, 1992; Frolov and Alatortsev, 1994). *Drosophila Cyp18* was initially cloned in a screen for ecdysone-inducible genes (Hurban and Thummel, 1993) and subsequently obtained as full-length cDNA (Bassett *et al.*, 1997). *Cyp4e1* deserves a special historical mention. Its sequence was discovered independently in a 9 kb genomic sequence of the 44D region of *Drosophila* encompassing a cluster of cuticle protein genes (GenBank Acc. K00045) by T. Holton and D. Nero in 1991, each searching databases with P450 sequences (personal communications). This partial sequence of insect *Cyp4e1* is thus the first (1983), though not annotated, record of an insect P450 in GenBank.

4.1.2.1.3. Exponential amplification of new P450 sequences by PCR By 1994, sufficient information about vertebrate and insect P450s allowed the

isolation of fragments of P450 cDNAs and genes by PCR methods with degenerate oligonucleotides corresponding to consensus sequences. A variety of approaches were taken, two of which being particularly successful: in the first, sequences in the I helix and surrounding the conserved cysteine (see Section 4.1.3.3.1) were used to isolate PCR products of about 450–500 bp that coded for about 130 amino acids or almost a third of the full-length P450. In the second, the sequence around the conserved cysteine was used to design a first primer, with oligo(dT) serving as anchor on the poly(A)$^+$ message. Fragments of varying length were obtained by this 3′-RACE strategy, the C-terminal 30–50 amino acids sequence of the P450 and a variable 3′ UTR sequence (review: Snyder *et al.*, 1996). The PCR approaches led to the description of 17 new *CYP4* genes in *A. albimanus* (Scott *et al.*, 1994); 5 *CYP4* genes from *Manduca sexta* (Snyder *et al.*, 1995); 8 *CYP* genes from *H. armigera* (Pittendrigh *et al.*, 1997); 4 genes of the new *CYP28* family from *Drosophila* species (Danielson *et al.*, 1997); 14 P450 fragments from *Ceratitis capitata* (Danielson *et al.*, 1999), 95 new sequences from 16 drosophilid species (Fogleman *et al.*, 1998), etc. (Amichot *et al.*, 1994; Stevens *et al.*, 2000; Tares *et al.*, 2000; Scharf *et al.*, 2001; Wen *et al.*, 2001; Ranson *et al.*, 2002b). The PCR method has often been used as a first step in the isolation of full-length P450 clones in insects (Snyder *et al.*, 1995; Danielson and Fogleman, 1997; Hung *et al.*, 1997; Danielson *et al.*, 1998, 1999; Guzov *et al.*, 1998; Kasai *et al.*, 1998a, 2000; Ranasinghe and Hobbs, 1998; Sutherland *et al.*, 1998; Stevens *et al.*, 2000; Wen *et al.*, 2001; Wen and Scott, 2001b; Liu and Zhang, 2002) and ticks (Crampton *et al.*, 1999; He *et al.*, 2002).

The large number of partial P450 sequences obtained by cloning and sequencing an even larger number of PCR products has led to two related problems. The first is that small sequence differences can be found between very closely related PCR products. Are these artifactual, or do they represent allelic variation? In the study on *A. albimanus* for instance, 64 clones were sequenced of which 47 encoded P450 fragments, describing 17 genes (Scott *et al.*, 1994). In some clones, up to seven nucleotide differences from the closest sequence were seen. A few nucleotides may not just differentiate two alleles of the same gene, but may be sufficient to differentiate two genes, as the complete sequence of the *Drosophila* and *A. gambiae* genomes subsequently showed. This causes a nomenclature problem, when two distinct genes are prematurely described as allelic variants of a single gene. The second problem is that the P450 fragments obtained by PCR, one third of the full sequence or less, make the calculation of percentage identity (the base of the nomenclature rules) difficult. In the *A. albimanus* study (Scott *et al.*, 1994) this problem was resolved by establishing a function that derives identity over the full length P450 from the percentage identity of the PCR product. Further validations of this approach have been presented (Danielson *et al.*, 1999; Fogleman and Danielson, 2000). Nonetheless, the practice of bestowing an official CYP designation to a short, partial P450 sequence should be abandoned.

4.1.2.1.4. Genome sequences: diversity revealed The *D. melanogaster* complete genome sequence was published in 2000 (Adams *et al.*, 2000). Annotation of the P450 sequences (Tijet *et al.*, 2001) was done by multiple Basic Local Alignment Search Tool (BLAST) searches, taking into account intronexon structure, pairwise and multiple alignments, EST sequences, and known features of well-characterized P450. This task gave 90 sequences, of which 83 appeared to code for potentially functional P450s. Seven sequences were either partial sequences or obvious pseudogenes. Forty genomic sequences were not represented by ESTs. With release 3 of the *Drosophila* genome (Celniker *et al.*, 2002), the "official count" of potentially functional P450 genes is 85 with five pseudogenes. A similar annotation of the P450 genes from the complete *A. gambiae* genome published in 2002 (Holt *et al.*, 2002) gave 111 genes (Ranson *et al.*, 2002a) of which five are thought to represent pseudogenes. The need for "manual" annotation remains critical because of the error-prone gene- and transcript-calling programs, as noted for P450s by Gotoh (1998). These difficulties remain a major challenge to the "completion" of a genome program (Misra *et al.*, 2002). Potentially confusing is the proliferation of GenBank accessions for transcripts identified *in silico*, with only scant notice that they represent nothing but software-digested genomic sequences. Some of the sources of errors noted for the *Drosophila* annotation (Misra *et al.*, 2002) were plainly evident with the *A. gambiae* genome sequence as well. They typically include the fusion of two neighboring P450 genes into one, or the truncation of a gene. Comparison of the intron/exon structure of closely related genes and alignments with EST sequences when these are available can facilitate P450 gene annotation in a majority of cases. Independent evidence, such as the cloning of full-length cDNAs, or functional expression is rarely available to resolve annotation problems, so that the complete description of the P450 gene complement of any

species remains an ongoing task. A website presents information on the P450 sequences from completely sequenced genomes (*D. melanogaster, A. gambiae*) as well as links to other P450 websites. The complete sequence of an increasing number of insect genomes (*Drosophila pseudoobscura, Apis mellifera, Bombyx mori, Tribolium castaneum*) and the continuous addition of P450 sequences in EST projects from various species is redefining the approach to P450 research.

4.1.2.2. Genomic Organization: Clusters

The presence of P450 genes in clusters was revealed in early studies with *Drosophila* and the housefly (Frolov and Alatortsev, 1994; Cohen and Feyereisen, 1995). Further evidence was obtained by *in situ* hybridization to polytene chromosomes of *Drosophila* (Dunkov *et al.*, 1996) and ultimately by the analysis of P450 genes in the complete genome sequences of *Drosophila* (Tijet *et al.*, 2001) and *A. gambiae* (Ranson *et al.*, 2002a, 2002b). The largest *Drosophila* P450 cluster carries nine genes (eight *CYP6A* genes and *CYP317A1*) at 51D on the right arm of chromosome 2. Six of these genes (*Cyp6a17* to *Cyp6a21*) are coordinately regulated during the circadian rhythm (Ueda *et al.*, 2002). In *A. gambiae*, the largest cluster carries 14 P450 genes of the *CYP6* family at 30A on the right arm of chromosome 3. A large cluster of *CYP325* genes in *A. gambiae* contains 12 genes and 2 pseudogenes. In *A. gambiae*, only 22 of the 111 genes are present as singletons, with 16 clusters of 4 or more genes (Ranson *et al.*, 2002a) (**Figure 2**). Gene clusters are thought to arise by sequential gene duplication events, the principal and initial mechanism of P450

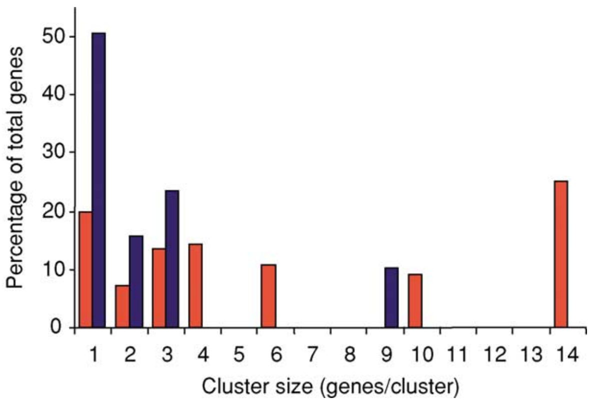


Figure 2 Clusters of P450 genes and singletons (cluster size = 1) genes in the *Drosophila melanogaster* (blue) and *Anopheles gambiae* (red) genomes. (Reproduced with permission from Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., *et al.*, **2002a**. Evolution of supergene families associated with insecticide resistance. *Science 298*, 179–181; © AAAS.)

diversification. In *Papilio glaucus, CYP6B4v2* and *CYP6B5v1* are clustered within 10 kb of each other (Hung *et al.*, 1996). They are 99.3% identical at the nucleotide level, with 98% identity of their single 732-bp intron and 95% identity over 616 bp of the promoter region. The proteins they encode differ by just one amino acid. *CYP6B4* and *CYP6B5* are thus recently duplicated genes that have not yet diverged substantially in sequence (Hung *et al.*, 1996). Similar close pairs are found in *Drosophila*, e.g., *Cyp12a4/Cyp12a5*; *Cyp9b1/Cyp9b2*; *Cyp28d1/Cyp28d2*. *Cyp12d1* and *Cyp12d2* are 2 kb apart and differ by only three nucleotides leading to three changes at the amino acid level. In *A. gambiae, CYP6AF1* and *CYP6AF2* are 99.8% identical at the nucleotide level and differ by just one amino acid (Ranson *et al.*, 2002a). *CYP6D1* and *CYP6D3* are clustered on chromosome 1 in the housefly (Kasai and Scott, 2001a). Although they are only 50% identical in their 500 nt UTR and their product 80% identical at the amino acid level, both genes are phenobarbital-inducible and are constitutively overexpressed in the LPR strain (Kasai and Scott, 2001b).

In the housefly, a cluster of six *CYP6* genes within 24 kb of each other on chromosome 5 shows evidence of both gene duplications and chromosomal inversions (Cohen and Feyereisen, 1995). Three genes are transcribed in one direction and the next three are transcribed in the opposite direction. The six genes have a short intron at the same position in the ETLR conserved region (see Section 4.1.3.3.1) as many *CYP6* genes. The *CYP6A6* gene located at one extremity of the cluster is in fact represented only by the second exon. The first exon was not found in 2 kb of DNA upstream of this exon boundary, although the intron length of the five other genes is only 57–125 bp. *CYP6A6* may therefore be a pseudogene generated during a chromosomal inversion whose breakpoint was located in the intron. The other five genes of the cluster are transcribed, with *CYP6A5* being predominantly expressed in larvae (Cohen and Feyereisen, 1995).

Two recently duplicated genes may undergo gene conversion, if they do not diverge fast enough (Walsh, 1987). Gene conversion at the 5′ end of the *CYP6B8* and *CYP6B28* genes of *Helicoverpa zea* (Li *et al.*, 2002b) has been suggested by the lower level of nt differences in the first half of the first exon, as compared to the rest of the sequence. Exon specific deficit in variation among pairs of P450 is indicative of a gene conversion event (Matsunaga *et al.*, 1990). Changes in regulatory patterns (induction, tissue specific expression) can be observed for recently duplicated genes (Li *et al.*, 2002c) and may

lead to their independent evolution. Extreme examples of gene conversion between duplicated P450 genes leading to their concerted evolution, of the kind seen with α-amylase genes (Hickey *et al.*, 1991), has not been reported to date.

Although events such as unequal crossing-over can lead to gene duplications, there are other mechanisms such as retrotransposition. Capture of a spliced mRNA by a retrotransposon can reintroduce an intronless sequence into the genome where it may evolve further or die as a pseudogene. This process generally occurs in germ cells, so it should be limited to genes that are expressed in those cells. The *Drosophila Cyp4g1* gene at the tip of the X chromosome (1B3) may be a case in point. It lacks introns and its closest paralog, *Cyp4g15*, has five introns and is located at 10C2-3. Furthermore, *Cyp4g1* is represented by the largest number of ESTs in *Drosophila*, and yet the apparent N-terminus of CYP4G1 is quite atypical of a microsomal P450 suggesting that an ancient 5′UTR is now part of the open reading frame. Further studies are needed to clarify the status of this gene.

There is little evidence for alternative splicing of insect P450 transcripts as additional means of generating diversity. The *Cyp4d1* gene seems to utilize two alternate first exons, and ESTs for each transcript type, CYP4D1 and CYP4D1*alt*, have been found. The first cDNA cloned (Gandhi *et al.*, 1992) uses a first exon (exon 1 prime) closest to the rest of the gene, whereas several ESTs use a more distal first exon (exon 1) instead. The two predicted proteins differ only from the N-terminal to the beginning of the first SRS (see Section 4.1.3.3.1 and **Figure 8**). The consequences of this alternative splicing in terms of catalytic competence thus remain to be examined.

4.1.2.3. Genomic Variation: Alleles, Pseudogenes

Apparent allelic variants of cloned P450 cDNAs and genes were already described in the earliest studies of insect P450s (Cohen *et al.*, 1992, 1994; Cohen and Feyereisen, 1995). Examples of this variation are most striking in *A. gambiae*, because the sequence released includes several "scaffolds," which are not included in the "golden path" used for genome assembly. These scaffolds represent large tracts of heterozygosity ("dual haplotype regions," Holt *et al.*, 2002), probably resulting from the mosaic nature (contributions from different *A. gambia*e cytotypes) of the strain that was sequenced. Interestingly, some of these scaffolds harbor P450 genes, even P450 gene clusters, and a comparison of the various haplotypes reveals not only considerable differences, but also variations in the total number of P450 genes. In other words, P450 gene duplication events have occured independently in different mosquito cytotypes. A gene recently converted to a pseudogene in one population or strain, as a result of a debilitating mutation or transposable element insertion (see **Chapter 4.12**) may still be active in another population. The total number of P450 genes in a species is therefore a relative number that is genotype dependent.

In *Blattella germanica*, three pseudogenes of *CYP4C21* and two pseudogenes of *CYP9E2* have been described (Wen *et al.*, 2001). These pseudogenes are characterized by deletions and/or the presence of several stop codons in the open reading frame. *CYP9E2P2*, for instance, has just 10 nucleotide differences from *CYP9E2*, but two base deletions render this gene nonfunctional. The processed pseudogene *CYP4W1P* in the cattle tick has a 191 bp deletion, but only three other nt changes in the open reading frame (Crampton *et al.*, 1999). Another tick pseudogene, *CYP319A1P*, contains two DNA insertions in the open reading frame and also appears to be of recent origin (He *et al.*, 2002).

The rate of gene duplication has been reported to be extremely high, making it an event almost as frequent as point mutations at the nucleotide level (Lynch and Conery, 2000). With a rate of 31 gene duplications per genome per million years in *Drosophila*, and a half life of less than 3 million years for a duplicated gene, one can estimate that there is a P450 gene duplication event on average every 5 million years. *Drosophila* is peculiar in its ability to delete genomic DNA at a high rate, and the proportion of pseudogenes in the genome is low – 1 pseudogene for 130 proteins as compared to 1 for 19 in *Caenorhabditis elegans* (Harrison *et al.*, 2003). However the proportion of pseudogenes in the P450 family is high (five pseudogenes) in *Drosophila*, another indication of rapid turnover of P450 genes.

4.1.2.4. P450 Gene Orthologs

With so much gene duplication, are there still any P450 orthologs in related species? (orthology = the "same" gene in different species that has only diverged as a result of speciation). The availability of two completely sequenced genomes made it possible to identify those members of the P450 family that were truly orthologous. This is not as easy as it seems, because in some borderline cases, two formally orthologous genes may be sufficiently related to their closest formal paralog in the same species as to make the evolutionary connection between the two pairs unclear. Nonetheless, it came as a surprise

to find a very small number of 1 : 1 pairs of orthologous P450 genes between *D. melanogaster* and *A. gambiae* (Ranson *et al.*, 2002a). Zdobnov *et al.* (2002) indicate a genome wide level of 44–47% orthologous genes, but for the P450 genes only ten orthologs were found, of which five are mitochondrial P450s (Ranson *et al.*, 2002a). When two P450 genes are recognized as orthologs even though the two species have diverged about 250 million years ago, the most likely explanation is that there is a strong evolutionary constraint that has maintained this orthologous relationship. A similar or identical physiological function for the orthologous pair of P450 enzymes may represent such a constraint. Three of the five mitochondrial P450s have indeed a recognized function that is predicted to be identical in *Drosophila* and *A. gambiae*: CYP302A1, CYP314A1, and CYP315A1 are hydroxylases of the ecdysteroid biosynthetic pathway (Warren *et al.*, 2002; Petryk *et al.*, 2003; and see Section 4.1.4.1.1). The number of pairs of orthologs between *Drosophila* and *A. gambiae* is however higher than the number of P450 enzymes thought to participate in ecdysteroid metabolism (see **Chapter 3.3**), so that several other insect-specific or dipteran-specific conserved functions are probably carried out by the remaining pairs of orthologs. There are several cases where one gene in one species has two "orthologs" in the other – this is the case when a gene duplication event occurred in just one of the two species after speciation and 250 million years of separate evolutionary history. These cases merit special attention because knowledge of the function of one of the P450s may quickly lead to understanding the function of its alter egos. The comparative analysis of the P450s of the two dipteran species showed that the deficit of true pairs of orthologs (10 versus the predicted ±40) was compensated by a most interesting alternative. Orthologous groups of P450 paralogs were seen, i.e., the phylogenetic analysis clearly identified several cases where one ancestral P450 gene underwent several duplication events in each of the two dipteran lineages. The *CYP6A* cluster on chromosome 5 of the housefly (Cohen and Feyereisen, 1995) and a cluster of *CYP6A* genes on the right arm of chromosome 2 in *Drosophila* are probably syntenic, in view of the synteny of these linkage groups (Weller and Foster, 1993), but the clusters have evolved separately for over 100 million years, and orthologous pairs of genes in each cluster can no longer be recognized.

Even when syntenic relationships are not maintained globally (Zdobnov *et al.*, 2002), cases of local microsynteny are observed. For example, *CYP302A1* and *CYP49A1* are very close on chromosome 2L in *A. gambiae*, but their orthologs in *Drosophila* are on 3L and 2R, respectively. Nonetheless, microsynteny is maintained around *CYP49A1*, which is close to the adrenodoxin reductase gene and is located in both species on the negative strand of the first intron of another gene, the G protein 0-α 47A (CG2204) in *Drosophila* and its ortholog in *A. gambiae*.

4.1.2.5. Intron/Exon Organization of *CYP* Genes

The intron/exon organization of P450 genes is a useful tool in the analysis of P450 phylogeny, as shown by the systematic studies in *C. elegans* (Gotoh, 1998), *Arabidopsis thaliana* (Paquette *et al.*, 2000), and *Drosophila* (Tijet *et al.*, 2001) (**Figure 3**). Intron sequence comparisons, as well as sequence comparisons of 5′ flanking sequences have also helped clarify the evolutionary relationships of very closely related *CYP6B* genes of *Papilio* species (Li *et al.*, 2002a). Multiple events of intron loss and gain can be deduced from a comparison of the intron/exon organization of orthologous pairs of P450 genes for *Drosophila* and *A. gambiae* (Ranson *et al.*, 2002a). Intron phase is nonbiased. *CYP* introns follow the GT/AG rule, except the first intron in the *Drosophila Cyp9c1* gene (Tijet *et al.*, 2001) and the first intron in the *Cyp6a8* gene (Maitra *et al.*, 2002). The latter was recognized by comparison with the full length cDNA, it does not conform to usual sequence patterns, and has an AT/TC splice junction. This intron is very short (36 bp) and potentially represents 12 additional in-frame codons (Maitra *et al.*, 2002).

4.1.3. The P450 Enzymatic Complexes

4.1.3.1. Classical Biochemical Approaches

4.1.3.1.1. Subcellular fractions The enzymology of insect P450 can be studied in different types of environments. These are enriched subcellular organelles from insects (microsomes, mitochondria) where multiple P450 interact *in situ* with their redox partners; membranes from cellular expression systems where a cloned recombinant P450 interacts with native or engineered redox partners; and ultimately a reconstituted system of purified recombinant P450 and its redox partners in a defined system devoid of biological membranes. Transgenic expression of P450 genes is another way to study P450 biochemistry, but also regulation.

The classical preparation of microsomal fractions from insect tissue homogenates by differential centrifugation has been described extensively by Hodgson (1985) and Wilkinson (1979). It remains, with minor modifications, the most widely used first step in the biochemical characterization of insect

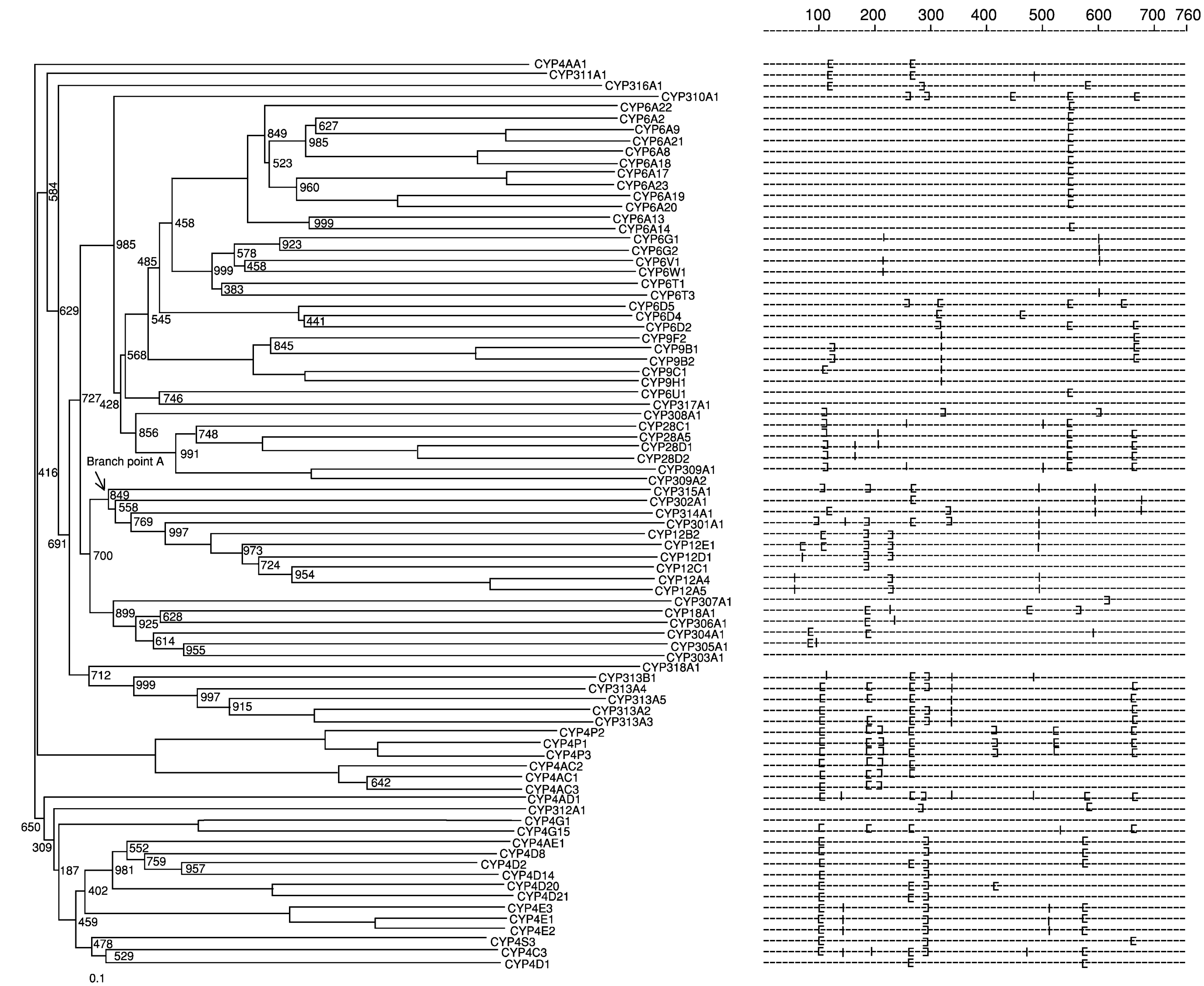


Figure 3 Neighbor-joining tree (with bootstrap value when different from 1000) and intron position of 83 P450 genes of *Drosophila melanogaster*. Branch point A indicates the mitochondrial P450 clade. Intron positions are shown schematically in the aligned open reading frames with their phase (| phase 0; [phase 1 and] phase 2). (Reprinted with permission from Tijet, N., Helvig, C., Feyereisen, R., **2001**. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene 262*, 189–198. For updated information see [http://P450.antibes.inra.fr].)

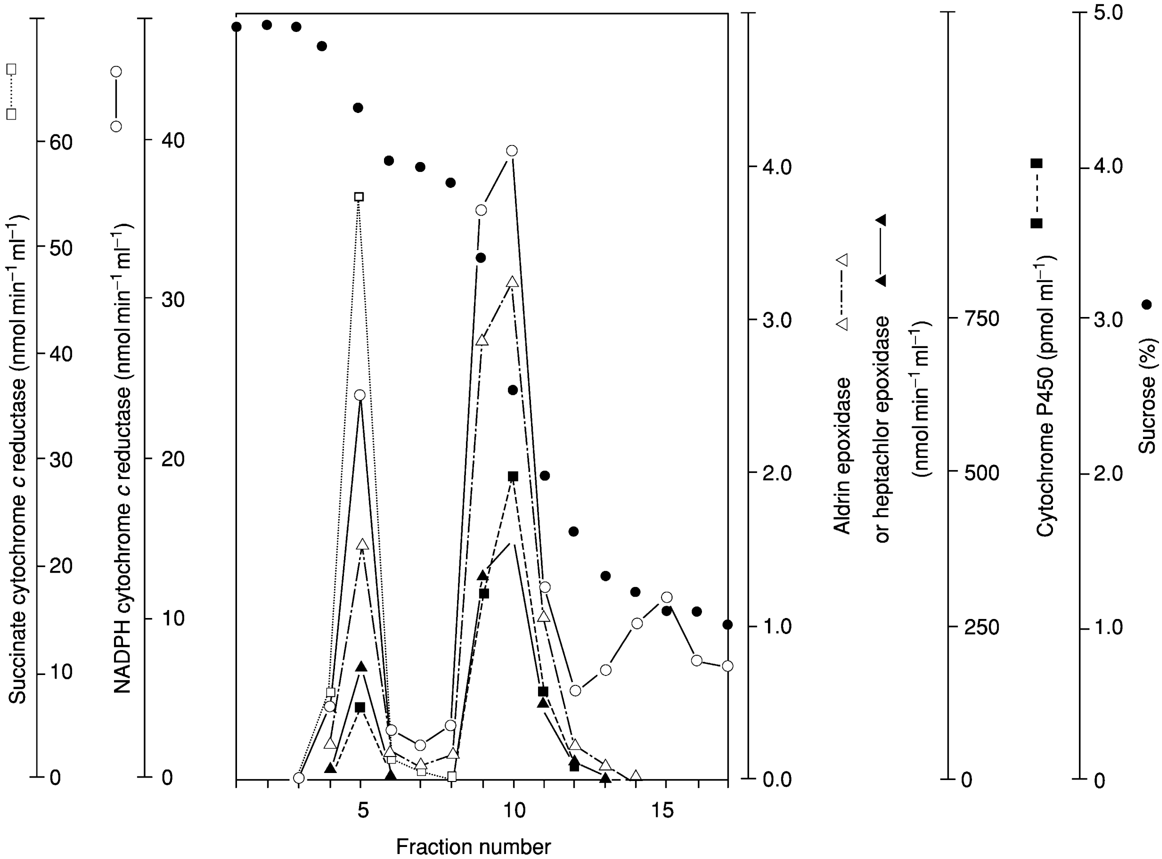


Figure 4 Sucrose density centrifugation separation of subcellular fractions of housefly larval homogenates showing the distribution of marker enzyme activities between mitochondrial and microsomal fractions. Note some P450 reductase activity in the top (soluble) fractions representing proteolytically cleaved enzyme. (Reprinted with permission from Feyereisen, R., **1983**. Polysubstrate monooxygenases (cytochrome P-450) in larvae of susceptible and resistant strains of house flies. *Pestic. Biochem. Physiol.* *19*, 262–269; © Elsevier.)

P450 enzymes. Linear or step gradients of sucrose for the centrifugal preparation of microsomes and mitochondria, or $CaCl_2$ precipitation of microsomes have been less favored. In all approaches, the careful use of marker enzymes is critical. A technique for the rapid preparation of microsomal fractions of small tissue samples relying on centrifugation at very high speed on sucrose layers in a vertical rotor has been described (Feyereisen *et al.*, 1985).

The well-documented sedimentation of P450-associated activities at low *g* forces in many early insect studies (reviews: Wilkinson and Brattsten, 1972; Wilkinson, 1979) has been considered a peculiar difficulty of insect biochemistry. At the time, the vertebrate toxicology and endocrinology literature had clearly identified P450 metabolism of xenobiotics as microsomal, whereas mitochondrial P450s were chiefly involved in hormone metabolism. There was therefore little incentive in probing the subcellular distribution of insect P450 activity more carefully. The discovery of insect CYP12 enzymes and their characterization as mitochondrial P450 enzymes capable of metabolizing xenobiotics (Guzov *et al.*, 1998) has shed a new light on the early difficulties in sedimenting insect P450 activities in the "correct" fractions. It is quite probable that at least a part of the P450 activities observed in "mitochondrial" fractions were indeed carried out by CYP12 enzymes. In housefly larvae, 15–20% of the aldrin and heptachlor epoxidase activities were associated with mitochondrial fractions after sucrose density centrifugation (Feyereisen, 1983) (**Figure 4**).

The insect midgut is a particularly rich source of P450 activity (Hodgson, 1985), but the external brush border membrane is a significant source of membrane vesicles (BBMV) upon homogenization of the tissue. Centrifugal methods to separate the BBMV fraction from the microsomes derived from the endoplasmic reticulum have been described (Neal and Reuveni, 1992).

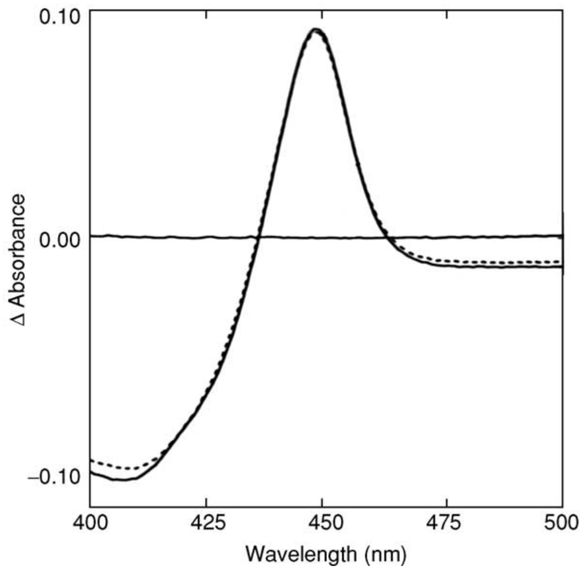


Figure 5 CO-difference spectrum of recombinant CYP12A1. The P450 was reduced with either sodium dithionite (solid line) or with bovine adrenodoxin, adrenodoxin reductase, and NADPH (dashed line).

4.1.3.1.2. Spectral characterization and ligand binding The analysis of P450 levels in subcellular fractions follows the original procedure of Omura and Sato (1964). A difference spectrum between reduced microsomes and reduced microsomes after gentle bubbling of CO readily displays the famous redshifted Soret peak at 450 nm (**Figure 5**). The concentration of P450 can be calculated from the ΔOD between 490 and 450 nm and Omura and Sato's extinction coefficient $\varepsilon = 91\,M^{-1}\,cm^{-1}$. This measure gives the total concentration of all forms of P450 present in the preparation. Individual P450 proteins may have peaks that are 1 or 2 nm off the 450 nm norm, and when they represent a large portion of the total P450, the total P450 peak may be shifted as a consequence. The degradation of P450 to the inactive P420 form may interfere with the measurement of P450, as already reviewed by Hodgson (1985), and the respiratory chain pigments interfere with the measurement of P450 in mitochondrial fractions. The classical Omura and Sato procedure remains the procedure of choice to measure the amount and purity of P450 proteins produced in heterologous systems (see below).

Ligand-induced spectral changes also follow classical procedures detailed in a useful review (Jefcoate, 1978). Type I spectra (peak at 380–390 nm, trough at 415–425 nm) result from ligand in the substrate binding site displacing water as sixth ligand to the heme iron (see Section 4.1.3.4.1, **Figure 6**). Type I spectra are concentration dependent, giving a spectral dissociation constant (Ks) and this titration is correlated with a shift of the iron from low spin to high spin. Not all type I ligands are substrates, and not all substrates are type I ligands, so this useful tool must be used with caution. A type I spectral Ks is not an enzymatic Kd.

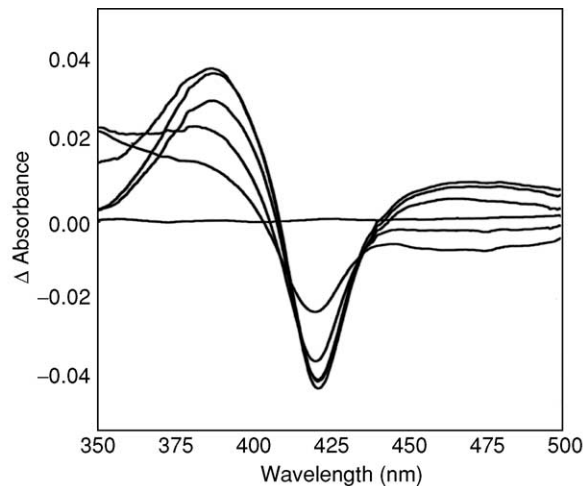


Figure 6 Type I substrate-induced difference spectrum of recombinant CYP12A1 with increasing concentrations of progesterone.

Type II spectra (peak at 425–435 nm, trough at 390–405 nm) result from the binding of a strong ligand to the heme iron, typically the nitrogen coordination of compounds such as pyrimidines, azoles, or n-octylamine. Type II spectral titration is correlated with a shift from high spin to low spin and is a hallmark of strong inhibitors such as imidazoles (**Figure 6**). Other, less frequently studied spectral changes induced by ligands or their metabolism will not be discussed here (e.g., type III spectra, Hodgson, 1985; spectra of phenyl–iron complexes, Andersen *et al.*, 1997).

4.1.3.1.3. Assays and substrates Measurement of P450 activity is a special challenge because of the large number of different P450 enzymes, each catalyzing the metabolism of a specific (broad or narrow) range of substrates. There is therefore a very large number of assays for P450 activity. Direct assays of product appearance or substrate disappearance rely on all the tools of analytical chemistry. Indirect assays (e.g., activity of a P450 product in an enzyme or bioassay) can be useful but the strength of the claim for P450 activity depends on the purpose of the assay. The assay of a P450 produced in a heterologous system can be straightforward, but the assay of a P450 in its native microsomal or mitochondrial membrane, where it is mixed with an undetermined number and amount of other P450s, is more problematic. Metabolism of compound *M*

to product *N* in microsomes is the sum of the contributions of all P450 enzymes that catalyze the *M* to *N* reaction (and sometimes non-P450 enzymes can catalyze the same reaction!). Selective inhibitors (chemicals or antibodies) of one P450 can, by substraction, indicate the relative contribution of that particular P450 to the reaction being measured (e.g., Wheelock and Scott, 1992a; Hatano and Scott, 1993; Korytko *et al.*, 2000b). This indirect inference is only as valid as the inhibitor is selective. Substrates that are selective for one P450 and that are easily assayed have been the object of considerable research in biomedical toxicology. The relative success of this quest (e.g., nifedipine as model probe for CYP3A4) is a result of both the limited number of major P450 expressed in human liver and the heavy investment in their study. The large number of insect P450s times the large number of insect species under study divided by the investment in their research makes a similar quest seem quixotic. By default then, but mostly by inertia of the historical development of insect P450 research, a certain number of assays have taken their place in the literature as some measure of "global" P450 activity. Most authors are now fully aware that the microsomal activity of, e.g., aldrin epoxidation, *p*-nitroanisole *O*-demethylation or 7-ethoxycoumarin *O*-deethylation is only a measure of those P450 enzymes catalyzing these reactions. But this awareness is only as recent as our understanding that there are really many P450 enzymes, and that their individual catalytic competence may be broad or narrow, overlapping with other P450 enzymes or not. Thus, the pioneers who used aldrin epoxidation as an assay did so at a time when P450 research was still strongly influenced by the dichotomy between steroid metabolism by specific P450 enzymes and drug metabolism by two major forms of liver P450. They used an analytical tool then readily available in pesticide toxicology laboratories (GC with electron capture detection for the sensitive detection of organochlorine pesticide residues), but they didn't use it without a caveat. Quoting the classical Krieger *et al.* (1971) study: "to the extent that the rate of epoxidation of aldrin to dieldrin typifies the activity of the enzymes toward a wider range of substrates…" The current and still widespread use of a "model" substrate to explore P450 activities in insect subcellular fractions can be useful. If the metabolism of a randomly chosen P450 substrate (e.g., aldrin, aminopyrine, 7-methoxyresorufin) is quantitatively different between insect strains, in different tissues, following induction, etc., then this substrate has provided a clue that the qualitative or quantitative complement of P450 enzymes is changing. It is up to the investigator to follow up on this clue. Alkoxycoumarins and alkoxyresorufins are useful substrates for sensitive fluorometric assays and have largely replaced organochlorines as model substrates. They can be used to "map" the catalytic competence of a heterologously expressed P450 (e.g., the preference of CYP12A1 for pentoxycoumarin, Guzov *et al.*, 1998) (**Figure 7**). Steroids such as testosterone have multiple sites of attack by P450 enzymes and can likewise be used to characterize the activity of subcellular fractions or of heterologously expressed P450s (Amichot *et al.*, 1998; Cuany *et al.*, 1990; M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data).

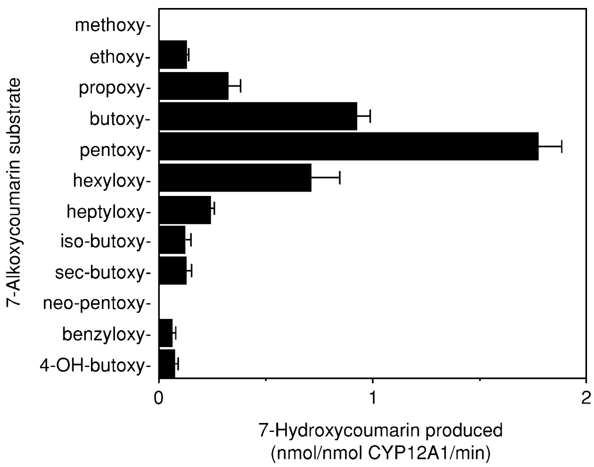


Figure 7 *O*-Dealkylation activity of *E. coli*-expressed recombinant housefly CYP12A1 in a reconstituted system. (Reprinted with permission from Guzov, V.M., Unnithan, G.C., Chernogolov, A.A., Feyereisen, R., **1998**. CYP12A1, a mitochondrial cytochrome P450 from the house fly. *Arch. Biochem. Biophys. 359*, 231–240; © Elsevier.)

Another approach to the study of P450 activity is to follow the consumption of the other substrates, O_2 or NADPH. This approach is certainly valid when the stoichiometry of the reaction (see eqn [1]) is under study, but its use to monitor metabolism by subcellular fractions is fraught with difficulties. Other enzymes consume O_2 and NADPH as well, and the background activity can be very high.

4.1.3.1.4. P450 assays in individual insects The design of assays suitable for assessing P450 activities in single insects has accompanied the need to study variations in P450 activities from the individual to the population level. Such assays allow the presentation of frequency histograms of activity levels in field-collected samples or in laboratory-selected populations and are ideally adapted to microtiter plate format. The NADPH-dependent conversion of *p*-nitroanisole to *p*-nitrophenol was followed in individual homogenates of *H. virescens* and

Pseudoplusia includens larvae (Kirby *et al*., 1994; Rose *et al*., 1995; Thomas *et al*., 1996). This assay has a relatively low sensitivity, but clearly distinguished individuals from susceptible and insecticide-resistant strains. Cut abdomens of adult *Drosophila* in buffer containing 7-ethoxycoumarin can be used to measure 7-hydroxycoumarin formation in a 96-well microtiter plate format (de Sousa *et al*., 1995). This rapid technique allows for instance the monitoring of individual variability over the course of a selection regime (Bride *et al*., 1997). In another example, 10 000 g supernatants of individual homogenates of *Chironomus riparius* larvae were assayed for 7-ethoxyresorufin *O*-deethylation activity (Fisher *et al*., 2003). The classical aldrin epoxidase assay adapted on single larvae of *Spodoptera frugiperda* has also been reported, but this assay is not adapted to the 96-well format (Yu, 1991, 1992).

A simple assay based on the peroxidase activity of the heme group with tetramethylbenzidine was developed for use in single mosquitoes (Brogdon, 1997). This assay, easily developed on a microtiter plate format, is an indirect assay measuring total heme content of the insect homogenate rather than P450 activity, and therefore needs to be carefully validated.

4.1.3.1.5. Solubilization and purification Solubilization and purification of insect P450 has generally followed the advances pioneered in the purification of vertebrate P450 (Agosin, 1985; Hodgson, 1985) and relatively a few studies since 1985 have pursued this difficult task (Ronis *et al*., 1988; Wheelock and Scott, 1989). P450 purification from microsomes of mixed tissues (e.g., fly abdomens) can be sufficient to obtain a protein fraction suitable for antibody generation or peptide sequencing (Feyereisen *et al*., 1989; Wheelock and Scott, 1990; Scott and Lee, 1993b).

Sequential chromatography on octylamino agarose, DEAE-cellulose, and hydroxyapatite was used to purify sodium cholate-solubilized P450s from *Drosophila* (Sundseth *et al*., 1990). The two protein fractions obtained, P450 A and B, had only a very low 7-ethoxycoumarin *O*-deethylase activity (0.01 nmol/nmol P450/min), but the proteins were useful in generating monoclonal antibodies that allowed the subsequent cloning of CYP6A2 (Waters *et al*., 1992).

An original approach was the purification of a locust P450 by affinity chromatography with type II and type I ligands (Winter *et al*., 2001) that led to cloning of CYP6H1 (Winter *et al*., 1999). In this approach, microsomes from larval *Locusta migratoria* Malpighian tubules were first treated with the detergent synperonic NP10 to solubilize P450. The extract was then chromatographed on ω-octylamino agarose then hydroxylapatite. The third and less classical step was chromatography on a triazole agarose affinity column. The affinity ligand was a derivative of the fungicide difenoconazole, which has an affinity for ecdysone 20-monoxygenase of the same level as that of the substrate ecdysone (0.5 versus 0.2 μM). This substituted triazole is a typical type II ligand (active site liganding of the heme) and its use on the affinity matrix led to the adsorption of all the P450 loaded on the column (Winter *et al*., 2001). Elution from the affinity column was done by replacing the immobilized type II ligand with a soluble type I ligand, ecdysone (see **Chapter 3.3**). A major protein band of 60 kDa was thus obtained in 4% yield, with a P450 specific activity of 13.1 nmol/mg protein. Unfortunately, biochemical evidence that this P450 is in fact an ecdysone 20-monooxygenase was not obtained in this study (Winter *et al*., 2001), so the nature of CYP6H1 (Winter *et al*., 1999) remains conjectural.

Another variant on the classical purification schemes has been the use of immobilized artificial membrane high performance liquid chromatography (IAM-HPLC) of microsomal proteins (Scharf *et al*., 1998). This technique allowed the 70-fold purification of a P450 from the German cockroach and the subsequent production of antibodies with this 49 kDa protein as antigen.

4.1.3.2. Heterologous Expression Systems

Biochemical characterization of a P450 protein and its substrate selectivity remains a *sine qua non* condition of its functional identification. Only a few P450 enzymes are characterized well enough (e.g., steroid metabolizing P450s in vertebrates) that sequence comparison can reasonably predict activity. For most other P450s, the sequence does not provide a clue to the activity, and there are now innumerable papers describing how one or a few mutations can change substrate selectivity (review: in Domanski and Halpert, 2001). Only three mutations are needed to confer to *Drosophila* CYP6A2 the ability to metabolize DDT (Bergé *et al*., 1998; Amichot *et al*., 2004). In the absence of significant studies on the activity of P450 proteins purified directly from insect tissues, it is the expression of P450 cDNAs in heterologous systems that has become the standard way of characterizing insect P450 proteins. A number of such expression systems have been developed over the last few years (**Table 1**), and the techniques are essentially similar to those used for the production of P450 proteins from mammalian or plant tissues.

Table 1 Heterologous expression systems for insect P450

Expression system	*P450 produced*	*Substrate metabolized*	*Reference*
Escherichia coli	CYP4C7	Sesquiterpenoids	Sutherland *et al.* (1998)
	CYP6A1	Aldrin, heptachlor	Andersen *et al.* (1994)
		Sesquiterpenoids	Andersen *et al.* (1997)
		Diazinon	Sabourault *et al.* (2001)
		7-Propoxycoumarin	[a]
		1-Bromochlordene, chlordene, 1-hydroxychlordene, isodrin	[b]
		Testosterone, progesterone, androstenedione	[c]
		Pisatin	[d]
		Chlorfenapyr	[e]
	CYP6A2	DDT, testosterone	Amichot *et al.* (2004)
	CYP6A5	Benzphetamine, *p*-chloro-*N*-methylanilin, methoxyresorufin	[f]
	CYP9E1	Sesquiterpenoids	[g]
	CYP12A1	Aldrin, heptachlor, diazinon, azinphosmethyl, amitraz, progesterone, testosterone, 7-alkoxycoumarins	Guzov *et al.* (1998)
	CYP15A1	*t t*, methyl farnesoate	Helvig *et al.* (2004)
Baculovirus	CYP6A2	Aldrin, heptachlor, diazinon	Dunkov *et al.* (1997)
	CYP6B1	Furanocoumarins	Ma *et al.* (1994), Hung *et al.* (1997), Wen *et al.* (2003)
	CYP6B4	Furanocoumarins, ethoxycoumarin	Hung *et al.* (1997), Li *et al.* (2003)
	CYP6B17	Furanocoumarins, ethoxycoumarin	Li *et al.* (2003)
	CYP6B21	Furanocoumarins, ethoxycoumarin	Li *et al.* (2003)
	CYP6B25	Furanocoumarins	Li *et al.* (2003)
Yeast	CYP6A2	AflatoxinB1, 7,12-dimethylbenz[a]anthracene, 3-amino-1-methyl-5H-pyrido[4,3-b]-indole	Saner *et al.* (1996)
	CYP6D1	Methoxyresorufin	Smith and Scott (1997)
Transfected S2 cells	CYP302A1	2,22-Dideoxyecdysone	Warren *et al.* (2002)
	CYP314A1	Ecdysone	Petryk *et al.* (2003)
	CYP315A1	2-Deoxyecdysone, 2,22-dideoxyecdysone	Warren *et al.* (2002)

[a] V.M. Guzov and R. Feyereisen, unpublished data.
[b] J. Walding, J.F. Andersen, and R. Feyereisen, unpublished data.
[c] M.B. Murataliev, V.M. Guzov, and R. Feyereisen, unpublished data.
[d] V.M. Guzov, H. VanEtten, and R. Feyereisen, unpublished data.
[e] V.M. Guzov, M. Kao, B.C. Black, and R. Feyereisen, unpublished data.
[f] J.L. Stevens, J.F. Andersen, and R. Feyereisen, unpublished data.
[g] J.F. Andersen and R. Feyereisen, unpublished data.

4.1.3.2.1. *Escherichia coli* Bacterial production (*Escherichia coli*) of insect P450s has required several modifications of the sequence. At the 5′ end of the cDNA, mutations are introduced to optimize expression (Barnes *et al.*, 1991). The second codon is replaced by Ala (Andersen *et al.*, 1994; Guzov *et al.*, 1998; Sutherland *et al.*, 1998) and silent substitutions are introduced to increase the A/T content (Sutherland *et al.*, 1998). In some cases, introduction of 4–6 His codons just before the stop codon directs the translational production of a C-terminal "histidine tag" (Guzov *et al.*, 1998; Sutherland *et al.*, 1998). P450 production can be enhanced by the addition of δ-aminolevulinic acid (a precursor for heme biosynthesis) to the culture broth (Sutherland *et al.*, 1998). The P450 produced in bacteria is found mostly in a membrane fraction, and sometimes in a fraction of inclusion bodies that are difficult to extract. In some cases (Andersen *et al.*, 1994), a significant amount of P450 is produced as a soluble form. The *E. coli* membrane fraction carrying the recombinant P450 protein is generally suitable for analysis by difference spectroscopy for either P450 content by the Omura and Sato (1964) procedure, or for ligand binding (type I binding for potential substrates or type II binding for azoles). Although some P450 proteins (e.g., CYP17) expressed in *E. coli* can utilize an endogenous flavodoxin

reductase/flavodoxin system for catalysis, none of the insect P450 proteins tested thus far have been catalytically active in *E. coli* membrane fractions in the absence of a P450 reductase. Therefore, P450 produced in bacteria needs to be solubilized and purified by classical methods. The proteins produced with a histidine tag, once solubilized, are purified by nickel chelate affinity chromatography. Extensive dialysis is needed in both procedures to remove excess detergent or imidazole used for elution from the nickel affinity column. The P450 obtained is then suitable for reconstitution with redox partners. These partners (microsomal or mitochondrial redox partners, see Section 4.1.3.3) are themselves produced in *E. coli* and purified (Guzov *et al*., 1998).

Reconstitution of a catalytically active enzyme system is then tedious or artistic, depending on one's degree of patience. It requires attention to the details of concentrations of phospholipids, detergents, proteins, and their order of addition, mixing and dilution (Sutherland *et al*., 1998). The advantages of bacterial expression are the low cost of production of large amounts of P450, and the possibility to work with a precisely defined *in vitro* system with highly purified enzymes and their partners. A thorough characterization of the enzyme can be undertaken. The disadvantage of this formal biochemical approach is that purification and reconstitution are difficult and time-consuming, and is probably not suitable for when the goal is simply a survey of the catalytic competence of the P450, or the comparison of a large number of P450s or P450 mutants. The host organism, *E. coli*, is a rare organism devoid of P450 genes of its own while other bacteria can carry over 20.

4.1.3.2.2. Baculovirus Expression of P450 in lepidopteran cells by the baculovirus system requires no modification of sequence and is a widely used method for the production of proteins in an eukaryotic system. It has the potential of producing large amounts of P450 proteins for subsequent purification, but studies with insect P450 expressed with this system (Ma *et al*., 1994; Dunkov *et al*., 1997; Hung *et al*., 1997; Chen *et al*., 2002; Wen *et al*., 2003) have relied instead on the advantage that the protein is present in a suitable milieu, the endoplasmic reticulum of an insect cell. Thus, cell lysates, briefly centrifuged to pellet cell debris, are used as enzyme source. Difference spectroscopy or immunological methods (Dunkov *et al*., 1997) can be used to assess the amount of P450 produced. The host cells provide their endogenous P450 reductase to support the activity of the heterologous P450 when the cell lysates are incubated with an NADPH regenerating system. Although the level of P450 reductase is sufficient to allow the measurement of a number of P450-dependent activities (Dunkov *et al*., 1997), the stoichiometry of endogenous P450 reductase, and cytochrome b_5, to heterologously expressed P450 is probably not optimal. The activities measured do not represent the full potential of the P450 under study. For instance, a thirty-fold increase in CYP6A2 activity was observed when purified housefly P450 reductase and cytochrome b_5 were added to lysates of cells expressing *Cyp6a2* (Dunkov *et al*., 1997). An improvement of the baculovirus expression system has therefore been designed, wherein the cells are coinfected with a virus engineered to carry the P450 and a virus engineered to carry a P450 reductase (housefly P450 reductase, Wen *et al*., 2003). Optimal conditions were sought, and a significant increase (33-fold) in CYP6B1 activity towards the substrate xanthotoxin was achieved with the improved P450 reductase/P450 ratio. In fact, the improved conditions allowed the measurement of angelicin metabolism that was barely detectable in the absence of additional P450 reductase. Thus, in the baculovirus system, insect P450s can be studied in an insect membrane environment, without need for purification. Those are great advantages over the *E. coli* expression system. However, the interactions with its redox partners are not manipulated as easily (Wen *et al*., 2003). The total amounts of P450 produced are also smaller, although addition of hemin to the culture medium can increase the amount of P450 produced (Dunkov *et al*., 1997; Wen *et al*., 2003). The total amount of P450 produced is less important in the baculovirus system than in the *E. coli* system as purification is not required for most applications, and as the highest activity of cell lysates is achieved at the correct P450/P450 reductase ratio, not at the maximal P450 production level (Wen *et al*., 2003). The level of endogenous P450 in the control experiments, i.e., uninfected cells or cells infected with a virus carrying a non-P450 "control" cDNA, are virtually undetectable.

4.1.3.2.3. Transfection in cell lines Heterologous expression in transfected mammalian COS cells was first established in 1986 for bovine CYP17 (Zuber *et al*., 1986), but it is not until later that an insect P450 was similarly expressed in an insect cell line. Thus, *Drosophila* Schneider 2 cells have been transfected with *Drosophila* P450 cDNAs (Warren *et al*., 2002). Expression under control of the actin 5C promoter produced sufficient P450 for activity measurements. The advantage of the method is its simplicity. When the expression of the P450 is coupled

with a very sensitive assay, the method can rapidly provide qualitative data on the catalytic competence of the enzyme. However, the quantitative determination of P450 levels is more difficult to achieve, and the interaction with redox partners cannot be optimized except by coinfection. It is interesting that the CYP302A1 and CYP315A1 expressed by this method are mitochondrial P450s and the S2 cell homogenates were able to provide adequate redox partners. As used so far, it has not allowed a measurement of the amounts of P450 produced, nor have the redox partners been characterized or optimized. Cell transfection does not have the potential of the baculovirus system for large-scale production of P450 proteins.

4.1.3.2.4. Yeast Yeast expression systems have only started to be exploited for the production of insect P450 proteins. *Saccharomyces cerevisiae* has three P450 genes that are fully characterized, and are expressed at low levels so that inducible expression of an exogenous P450 is not hindered by the endogenous P450. Coproduction of CYP6A2 from *Drosophila* and of human P450 reductase in yeast (Saner *et al*., 1996) generated a cell system capable of activating several procarcinogens to active metabolites that induced mitotic gene conversion or cytotoxicity. Housefly CYP6D1 was also produced in yeast but methoxyresorufin demethylation was the only marker activity obtained with microsomes of the transformed yeast (Smith and Scott, 1997). Insect P450 production in yeast has not yet achieved the success seen with plant P450 production in yeast (Schuler and Werck-Reichhart, 2003). P450 cDNAs may need to be engineered to recode the N-terminus of the protein. This has been done successfully with plant P450s to conform with the yeast codon usage (Hehn *et al*., 2002). The replacement of the yeast P450 reductase gene with an insect P450 reductase gene by homologous recombination (Pompon *et al*., 1996) should increase the usefulness of this yet underutilized expression system. Indeed, yeast combines the advantages of *E. coli* inducible production of large amounts of protein with the advantage of the eukaryotic cell system in which P450 enzymes can be studied in a normal membrane environment.

4.1.3.2.5. Transgenic insects The use of transgenic insects to study P450 function (or regulation, see Sections 4.1.4.5.3 and 4.1.5.2.2) has until now been restricted to *Drosophila*. In the first report, Gandhi *et al*. were unable to rescue by transgenesis the lethality of two complementation groups in the *Cyp4d1* region (Gandhi *et al*., 1992). Heterologous expression of vertebrate P450s was achieved in studies aimed at developing *Drosophila* as a genotoxicity model organism. The rat *CYP2B1* gene was expressed under control of the *Drosophila* LSP1a promoter (Jowett *et al*., 1991). This promoter ensures high levels of expression in third instar larvae. Transgenic flies expressed functional CYP2B1, as shown by increased CYP2B1-specific metabolism of 7-benzyloxyresorufin and by increased sensitivity to cyclophosphamide, a procarcinogenic drug activated by CYP2B1. In similar experiments, canine *CYP1A1* was expressed under the control of the *Drosophila* heatshock inducible *hsp70* promoter. Small amounts of CYP1A1 were produced after heatshock, sufficient to increase the sensitivity of the flies to 7,12-dimethylbenz[a]anthracene, a polycyclic aromatic hydrocarbon that is metabolized by CYP1A1 to a genotoxic metabolite (Komori *et al*., 1993). Housefly CYP6D1 was produced in *Drosophila* under control of the heatshock inducible *hsp70* promoter, and this led to a significant increase in benzo[a]pyrene hydroxylation (Korytko *et al*., 2000a), though heat shock decreased total P450 levels in whole body microsomes. Transgenic expression of CYP6G1 has been an important piece of evidence in demonstrating its role in DDT and neonicotinoid resistance (Daborn *et al*., 2002; Le Goff *et al*., 2003) as discussed below (Section 4.1.4.5.5). Transformation of other insects (see **Chapter 4.13**), notably with the piggyBac vector as in *Bombyx mori* (Tamura *et al*., 2000) will undoubtedly increase the applications of transgenesis to P450 research.

4.1.3.3. P450 Enzymes and Their Redox Partners

4.1.3.3.1. P450 proteins The sequence identity of distantly related P450 proteins can be as low as that predicted from the random assortment of two sets of 500 or so amino acids. This is because there are very few absolutely conserved amino acids. In insect sequences available to date, these are found in five conserved motifs of the protein (**Figure 8**), the WxxxR motif, the GxE/D **T**T/S motif, the **E**xL**R** motif, the PxxFxP**E**/D**R**F motif and the PFxxGxRx**C**xG/A motif. Despite this tremendous overall sequence diversity, the increasing number of crystal structures for P450 proteins, mostly soluble forms from bacteria (Poulos *et al*., 1995), reveals a quite high conservation of the three-dimensional structure. The description of this structure essentially follows the nomenclature of the P450cam protein, the camphor hydroxylase of *Pseudomonas putida* (Poulos *et al*., 1985). The first motif WxxxR is located in the C-helix, and the Arg is thought to form a charge pair with the propionate of the heme. This

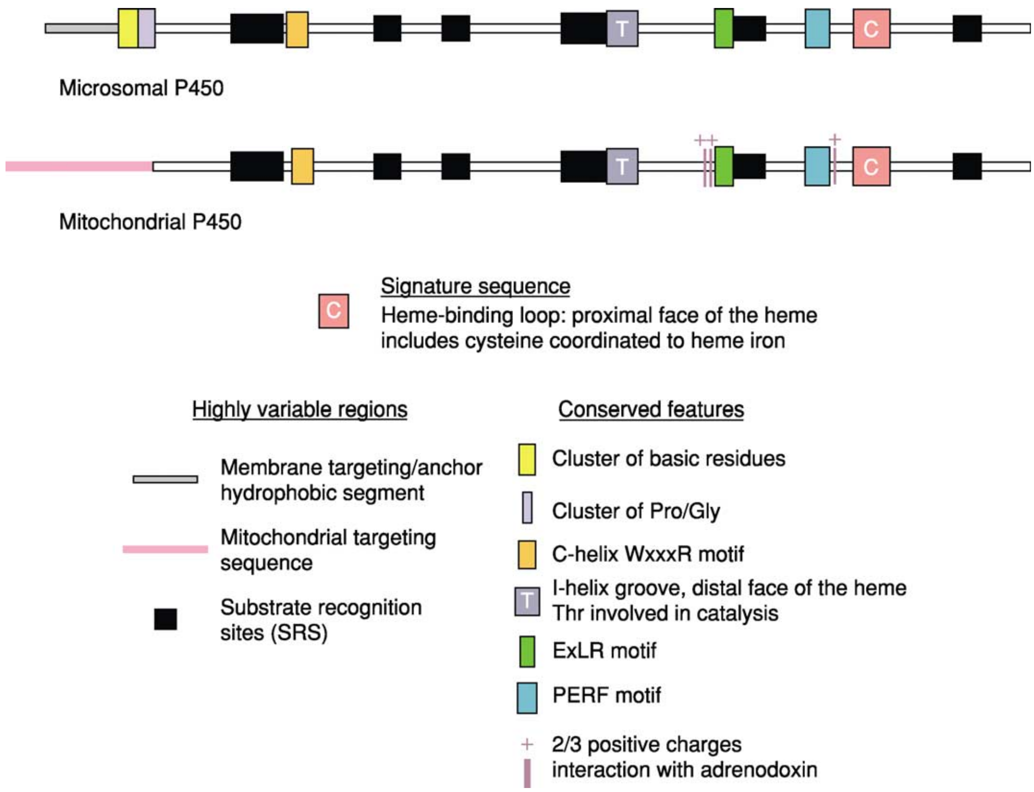


Figure 8 Conserved and variable regions of P450 proteins illustrated over their primary structure (sequence). (Adapted with permission from Werck-Reichhart, D., Feyereisen, R., **2000**. Cytochromes P450: a success story. *Genome Biol. 1*, 3003.1–3003.9; © GenomeBiology.)

motif is not easily discernible, except in multiple alignments. The second conserved motif GxE/D**T**T/S surrounds a conserved threonine in the middle of the long helix I that runs on top of the plane of the heme, over pyrrole ring B. The third conserved motif **E**xL**R** is located in helix K. It is thought to stabilize the overall structure through a set of salt bridge interactions (**E**-**R**-**R**) with the fourth conserved motif PxxFxPE/D**R**F (often PERF, but R is sometimes replaced by H or N) that is located after the K′ helix in the "meander" facing the **E**xL**R** motif (Hasemann *et al*., 1995). The fifth conserved motif PFxxGxRx**C**xG/A precedes helix L and carries the cysteine (thiolate) ligand to the heme iron on the opposite side of helix I. The cysteine ligand is responsible for the typical 450 nm (hence P450) absorption of the Fe^{II}–CO complex of P450 (Mansuy and Renaud, 1995). This heme binding loop is the most conserved portion of the protein, often considered as "signature" for P450 proteins. Deviations from the consensus sequences of these five motifs deserve special attention. For instance, the CYP301A1 of both *Drosophila* and *A. gambiae* has a very unusual Tyr instead of Phe in the canonical PFxxGxRx**C**xG/A motif around the Cys axial ligand to the heme. These deviations may denote an atypical catalytic function for the P450 enzyme, as seen in P450 enzymes that are not monooxygenases, such as plant allene oxide synthase (CYP74A) or vertebrate thromboxane synthase (CYP5A1) whose I helix lacks the conserved Thr. In the bacterial hydroxylase P450eryF (CYP107A), the Thr is replaced by Ala. A water molecule and a hydroxyl group of the substrate have become functional equivalents of the Thr hydroxyl (Poulos *et al*., 1995).

P450 proteins are also characterized by their N-terminal sequence (**Figure 9**). Those targeted to the endoplasmic reticulum have a stretch of about 20 hydrophobic amino acids. These precede one or two charged residues that serve as halt-transfer signal and a short motif of prolines and glycines. The latter serves as a "hinge" that slaps the globular domain of the protein onto the surface of the membrane while the N-terminus is anchored through it. The presence of the PGPP hinge is necessary for proper heme incorporation and assembly of functional P450s in the cell (Yamazaki *et al*., 1993; Chen *et al*., 1998). A hydrophobic region between helices F and G is thought to penetrate the lipid bilayer, thus increasing the contact of the P450 with the hydrophobic environment from which many substrates can enter the active site (Williams *et al*., 2000).

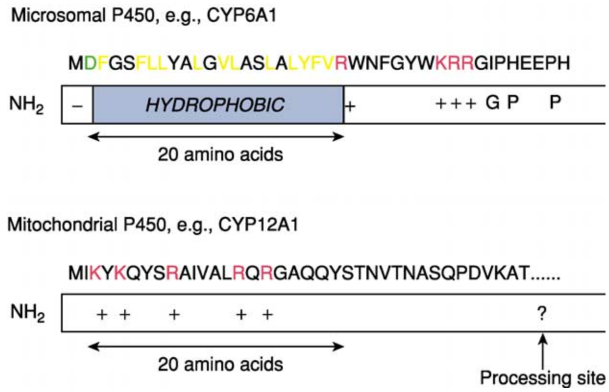


Figure 9 Scheme of the N-terminal sequence of microsomal and mitochondrial P450 proteins.

The N-terminal sequence of P450 proteins targeted to mitochondria is usually somewhat longer, and shows several charged residues (**Figure 9**). The mature mitochondrial protein is proteolytically cleaved at a position that has not been formally recognized for insect mitochondrial P450s to date, but is known for several mitochondrial P450s of vertebrate species. Mitochondrial P450 proteins are also characterized by a pair of charged amino acids in the K helix, R391 and K395 in CYP12A1. Homologous amino acids in mammalian P450scc (K377 and K381) are responsible for the high affinity to the ferrodoxin-type (adrenodoxin) electron donor (Wada and Waterman, 1992; Pikuleva *et al.*, 1999). An additional positively charged residue (R454 of CYP12A1) is homologous to R418 of CYP27A1 shown to increase affinity to adrenodoxin even further (Pikuleva *et al.*, 1999). The insect CYP12, 49, 301, 302, 314, and 315 proteins are most closely related to the mammalian mitochondrial P450 (CYP11, CYP24, and CYP27 families), to CYP44A1 from *C. elegans*, and to the pond snail CYP10. Subcellular localization of CYP12A1 by immunogold histochemistry with antibodies raised against the CYP12A1 protein produced in bacteria established the mitochondrial nature of CYP12A1 (Guzov *et al.*, 1998).

There is evidence that some vertebrate microsomal P450s, e.g., CYP1A1 and CYP2E1, are cleaved *in vivo* of their N-terminal anchor, thus revealing a cryptic mitochondrial targeting sequence, and the shortened protein is enzymatically active in mitochondria (Anandatheerthavarada *et al.*, 1999). Thus, the 1–44 residues of CYP1A1 serve a dual targeting role, with 1–32 targeting the protein to the ER cotranslationally, whereas cleavage and exposure of three basic amino acids in residues 33–44 direct the posttranslational transport to mitochondria (Bhagwat *et al.*, 1999). Whether some insect microsomal P450 proteins (e.g., CYP314A1, see Section 4.1.4.1.1) behave in this fashion is currently unknown.

Interspersed throughout the globular domain of the P450 proteins are six regions with a low degree of sequence similarity, covering about 16% of the total length of the protein (**Figure 8**). Initially recognized in CYP2 proteins by Gotoh (1992), these are called SRS (substrate recognition sites) and this designation has been generically extended to other P450s.

P450 enzymes, whether microsomal or mitochondrial, need to interact with redox partners for their supply of reducing equivalents from NADPH. **Figure 10** schematically illustrates the two types of electron transfer complexes thus formed, and the following sections provide a description of the redox partners.

4.1.3.3.2. NADPH cytochrome P450 reductase P450 reductase (EC 1.6.2.4) belongs to a family of flavoproteins utilizing both FAD and FMN as cofactors. These diflavin reductases emerged from the ancestral fusion of a gene coding for a ferredoxin reductase with its NADP(H) and FAD binding domains with a gene coding for a flavodoxin with its FMN domain. This origin of the enzyme was first proposed by Porter and Kasper (1986) based on their analysis of the rat P450 reductase sequence. The fusion is dramatically illustrated by the three-dimensional structure of P450 reductase (Wang *et al.*, 1997) where the domains are clearly distinguished (**Figure 11**). The architecture of this domain fusion has been found in a handful of other enzymes (Murataliev *et al.*, 2004a). In some cases, further fusion with a P450 gene has led to self-sufficient P450 proteins, e.g., the fatty acid hydroxylase of *Bacillus megaterium*, P450BM3 (Nahri and Fulco, 1986) and of *Fusarium oxysporum*, P450foxy (Nakayama *et al.*, 1996).

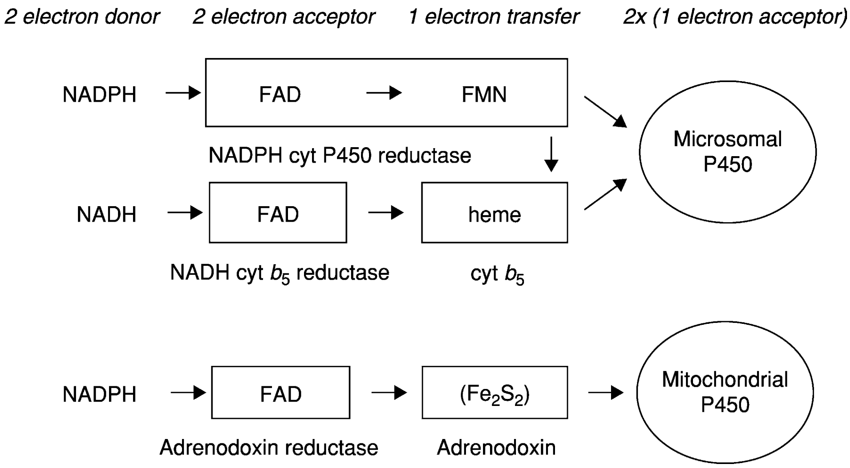


Figure 10 Mitochondrial and microsomal P450 redox partners.

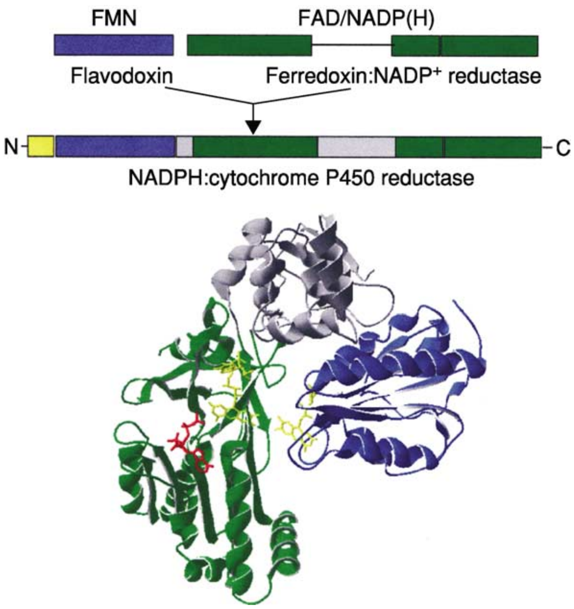


Figure 11 Structure of NADPH cytochrome P450 reductase. Top: evolutionary origin of the FMN (blue), FAD and NADP(H) (green) binding domains of the protein (yellow: membrane anchor; gray: connecting domain). Bottom: three-dimensional structure of the enzyme with the domains identified by color. The bound substrate NADPH (red) and cofactors FAD and FMN (yellow) are indicated. (Reprinted with permission from Murataliev, M.B., Feyereisen, R., Walker, F.A., **2004a**. Electron transfer by diflavin reductases. *Biochem. Biophys. Acta 1698*, 1–26; © Elsevier.)

The insect P450 reductases sequenced to date are clearly orthologous to the mammalian P450 reductases, with an overall amino acid sequence identity of 54% for the housefly P450 reductase, first cloned and sequenced in 1993 (Koener *et al.*, 1993). The housefly P450 reductase gene codes for a protein of 671 amino acids, and was mapped to chromosome III. The P450 reductases of other insects are very similar to the housefly enzyme – 82% identity for the *D. melanogaster* enzyme (Hovemann *et al.*, 1997), 57% identity for the *Bombyx mori* enzyme (Horike *et al.*, 2000), and 75% identity for the *A. gambiae* P450 reductase (Nikou *et al.*, 2003). The insect, mammalian, and yeast enzymes are functionally interchangeable in reconstituted systems of the purified proteins or in heterologous expression systems. However, no detailed study has documented how *well* a mammalian or yeast P450 reductase can support the activity of an insect P450 when compared to the cognate insect P450 reductase.

Early attempts to purify and characterize the enzyme from microsomes of housefly abdomens were hampered by the facile proteolytic cleavage of the N-terminal portion of the protein. This hydrophobic peptide anchors the reductase in the membrane, and its removal abolishes the ability of the remainder of the protein ("soluble" or "tryptic" reductase) to reduce P450s. The proteolytically cleaved reductase nonetheless retains the ability to reduce artificial electron acceptors such as cytochrome *c*, DCPIP or ferricyanide (Hodgson, 1985). Heterologous expression of the cloned P450 reductase has been achieved in *E. coli* (Andersen *et al.*, 1994) and in the baculovirus expression system (Wen *et al.*, 2003) and a purification scheme (Murataliev *et al.*, 1999) has produced quantities of enzyme sufficient for a detailed catalytic characterization of the enzyme's functioning and of its reconstitution with redox partners (**Figure 12**).

P450 reductase is an obligatory partner of microsomal P450 enzymes. Antisera to *Spodoptera eridania* or housefly P450 reductase inhibit all P450-dependent activities tested (Crankshaw *et al.*,

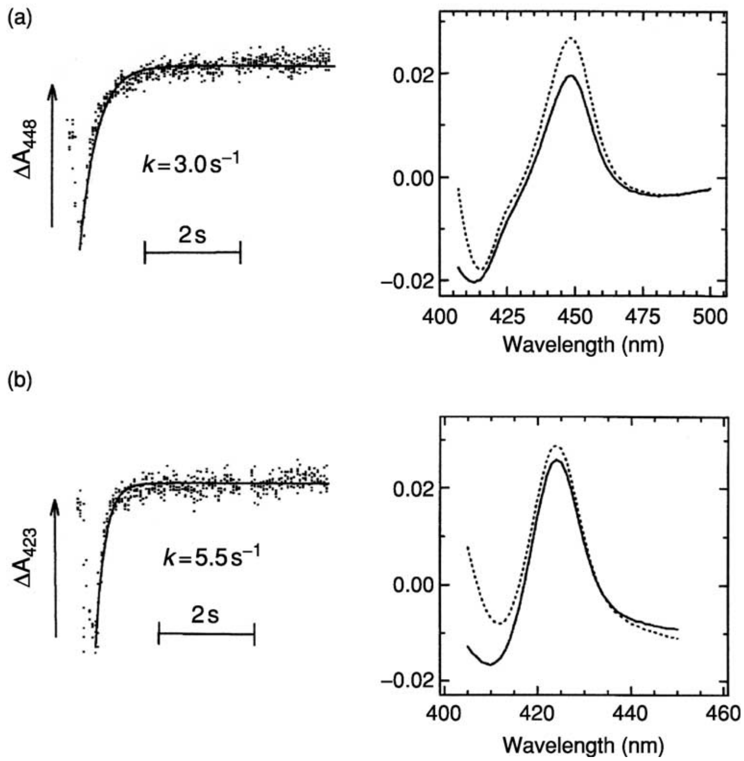


Figure 12 Reduction of housefly CYP6A1 (a) and cytochrome b_5 (b) by NADPH cytochrome P450 reductase. The recombinant proteins expressed in *E. coli* were reconstituted *in vitro*. On the left, the kinetics of reduction measured by stopped-flow spectrophotometry are shown with the calculated first-order rate constants. On the right, the end point difference spectra of CYP6A1 and cytochrome b_5 after reduction by P450 reductase (solid line) and sodium dithionite (dotted line). (Adapted from Guzov, V.M., Houston, H.L., Murataliev, M.B., Walker, F.A., Feyereisen, R., **1996**. Molecular cloning, overexpression in *Escherichia coli*, structural and functional characterization of house fly cytochrome b_5. *J. Biol. Chem. 271*, 26637–26645.)

1981; Feyereisen and Vincent, 1984). Immunoinhibition with P450 reductase antibodies serves as a strong indication of microsomal P450 involvement in NADPH-dependent activities, such as ecdysone 20-hydroxylation in the cockroach and in *B. mori* eggs (Halliday *et al.*, 1986; Horike and Sonobe, 1999; Horike *et al.*, 2000) and (*Z*)-9-tricosene biosynthesis in the housefly (Reed *et al.*, 1994). P450 reductase immunoinhibition could serve as a tool to distinguish P450 dependent activities from flavin monooxygenase (FMO) activities in microsomes from insect sources. P450 reductase also transfers electrons to cytochrome b_5 (see below) and to other microsomal enzymes such as heme oxygenase.

4.1.3.3.3. Cytochrome b_5 In contrast to P450 reductase, the role of cytochrome b_5 as partner in P450 dependent reactions is considerably more complex (see Section 4.1.3.3.3). The housefly cytochrome b_5 is a 134 amino acid protein (Guzov *et al.*, 1996) with 48% sequence identity with the orthologous rat cytochrome b_5. Its N-terminal domain of about 100 residues is the heme-binding domain that is about 60% identical to that of the vertebrate cytochrome b_5. Its C-terminal portion is a hydrophobic membrane anchor. A probable fatty acid desaturase-cyt b_5 fusion protein has been misidentified as the *Drosophila* cytochrome b_5 (Kula *et al.*, 1995; Scott, 1999; Kula and Rozek, 2000), but the correct ortholog is 76% identical to the housefly cytochrome b_5. The *H. armigera* cytochrome b_5 (Ranasinghe and Hobbs, 1999a) is 127 amino acids in length and 51% identical to the housefly cytochrome b_5, and the *A. gambiae* cytochrome b_5 is 54% identical (Nikou *et al.*, 2003). The known insect cytochrome b_5 sequences differ at their C-terminal from both the vertebrate microsomal and outer mitochonrial membrane cytochrome b_5 sequences, so that inferences about the subcellular targeting of the insect protein (Wang *et al.*, 2003) would seem premature.

The housefly cytochrome b_5 protein was produced in *E. coli*, purified and fully characterized (Guzov *et al.*, 1996). Absorption spectroscopy and EPR revealed properties very similar to cytochromes b_5 from vertebrates. NMR spectra indicated that the orientation of the heme in the protein relative to its α, γ *meso* axis is about 1 : 1. This means that the

protein is present in two forms of approximately equal abundance, that result from two modes of insertion of the noncovalently bound heme in the protein between the two coordinating histidines (face up and face down). Expression of the heme-binding domain in *E. coli* revealed that the heme is kinetically trapped in a 1.2 : 1 ratio of the two isomers, and that this orientation results from the selective binding of heme by the apoprotein (Wang *et al.*, 2003). A redox potential of −26 mV was measured by cyclic voltammetry on a treated gold electrode in the presence of hexamminechromium(III) chloride, and was verified by classical electrochemical titration. Stopped flow spectrophotometry showed that the cytochrome b_5 is reduced by housefly P450 reductase at a high rate ($5.5\,s^{-1}$) (Guzov *et al.*, 1996) (**Figure 12**).

Cytochrome b_5 can also be reduced by its own reductase, an NADH-dependent FAD flavoprotein, and can therefore provide either NADH- or NADPH-derived electrons to P450 enzymes. NADH-cytochrome b_5 reductase (EC 1.6.2.2) has been studied in *Ceratitis capitata* and *M. domestica* (Megias *et al.*, 1984; Zhang and Scott, 1996a). The N-terminus sequence of the purified housefly enzyme aligns to an internal sequence of the *Drosophila* enzyme (CG5946) indicating that it represents a proteolytically processed form. NADH-cytochrome b_5 reductase and cytochrome b_5 are also known to provide electrons to other acceptors, such as fatty acid desaturases and elongases.

4.1.3.3.4. Redox partners of mitochondrial P450 The redox partners of mitochondrial P450s are adrenodoxin reductase, an NADPH-dependent FAD flavoprotein and adrenodoxin, a [2Fe-2S] ferredoxin-type iron sulfur protein. These are named for the two redox partners of mammalian adrenal mitochondrial P450s, and this designation has been liberally bestowed on proteins from animals that don't have adrenals. Insect adrenodoxin reductase and adrenodoxin have not been functionally characterized, but their bovine orthologs are capable of supporting the activity of an insect mitochondrial P450, housefly CYP12A1 (Guzov *et al.*, 1998). The reduction of CYP12A1 is rapid and efficient with bovine adrenodoxin reductase/adrenodoxin while under the same conditions housefly microsomal P450 reductase is only marginally effective.

A fragment of a *Drosophila* adrenodoxin-like open reading frame is in GenBank on a stretch of DNA that also encodes a heatshock gene at 67B on the right arm of chromosome 3 (Pauli and Tonka, 1987). This stretch of 95 amino acids is similar (about 46%) to vertebrate adrenodoxin, and was initially identified as the *Drosophila* adrenodoxin ortholog (GenBank X06542). But the correct adrenodoxin ortholog was revealed by the complete genome sequence at 64B1 as a 152 amino acid protein, 45% identical to the bovine protein. EPR spectroscopic evidence for the presence of an adrenodoxin-like protein in fat body mitochondria of *Spodoptera littoralis* has been presented (Shergill *et al.*, 1995).

The *Drosophila* and *A. gambiae* adrenodoxin reductase and adrenodoxin genes have been annotated. The *Drosophila* P-element induced mutant *dare1* for *d*efective in the *a*voidance of *re*pellents was found to encode Drosophila *a*drenodoxin *re*ductase (Freeman *et al.*, 1999). Strong *dare* mutants undergo developmental arrest, and this phenotype is largely rescued by feeding 20-hydroxyecdysone. Decreasing by half the wild-type expression of *dare* blocks the olfactory response. The gene is expressed at low levels in all tissues of the adult fly, including the brain and the antennae. Highest expression is found in the prothoracic gland portion of the ring gland of third instar larvae, as well as in the nurse cells of adult ovaries. These tissues are known to require mitochondrial P450s for ecdysteroid production. The 55 kDa protein encoded by *dare* is 42% identical to the human enzyme.

4.1.3.4. Catalytic Mechanisms

4.1.3.4.1. P450 reactions Little work on insect P450 has focused on the catalytic cycle, and the mechanism derived from our understanding of the bacterial and mammalian P450 enzymes (Ortiz de Montellano, 1995b; Schlichting *et al.*, 2000) will be briefly summarized (**Figure 13**). The oxidized P450 is a mixture of two forms: a low spin (Fe^{III}) form with water as the sixth coordinated ligand on the opposite side of the Cys thiolate ligand, and a high spin (Fe^{III}) pentacoordinated form. Substrate binding displaces water from the sixth liganding position, leading to a shift to high spin. This shift can be observed (type I spectrum) and is accompanied by a decrease in the redox potential of P450. The P450–substrate complex receives a first electron from a redox partner (P450 reductase or adrenodoxin), and ferrous P450 (Fe^{II}) then binds O_2. At this step CO can compete with O_2 for binding to P450, its binding leads to a stable complex, with absorption maximum at 450 nm (**Figure 5**), that is catalytically inactive. CO can be displaced by light irradiation at 450 nm. The P450–O_2–substrate complex in the form of a ferric peroxide complex then accepts a second electron (from P450 reductase or in some cases cytochrome b_5, or from adrenodoxin). Different types of activated oxygen forms of the same

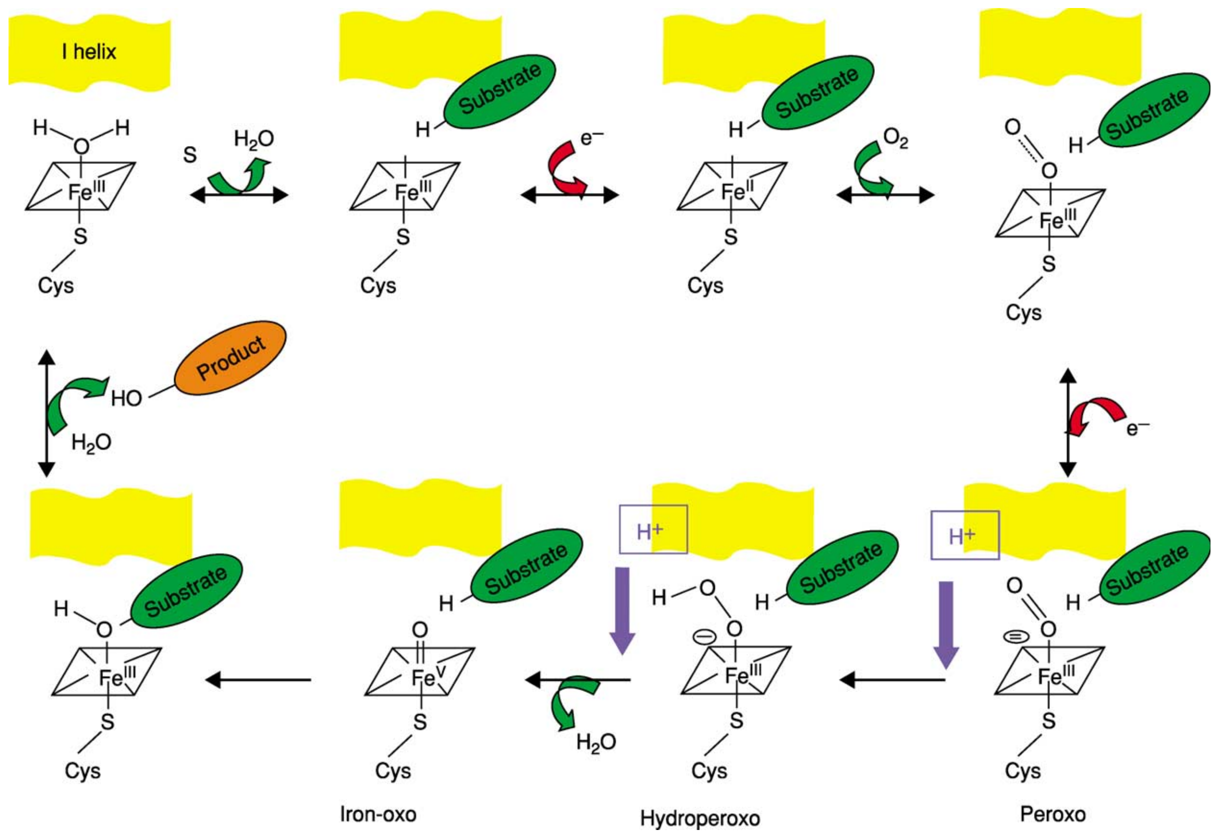


Figure 13 Catalytic cycle of P450 enzymes in monooxygenation reactions. Three possible forms of the activated oxygen species are shown. See text for details. Other reactions (reduction, isomerization, dehydration) can be catalyzed by oxygen-free forms of the enzyme. (Adapted with permission from Werck-Reichhart, D., Feyereisen, R., **2000**. Cytochromes P450: a success story. *Genome Biol. 1*, 3003.1–3003.9; © GenomeBiology.)

P450 enzyme can then be formed, depending on the protonation state of the complex and on the homolytic or heterolytic cleavage of the reduced dioxygen. Although the formal reaction is the insertion of an atom of oxygen into the substrate, the other atom being reduced to water (hence the term "mixed-function oxidase"), the nature of the oxidizing species can vary. A P450 $(Fe^{III}–O–O)^{2-}$ peroxo-iron form, a P450 $(Fe^{III}–O–OH)^{-}$ hydroperoxo form, and a P450 $(Fe^{V}=O)$ or P450 iron-oxo form are the preferred descriptions of the activated oxygen forms (Ortiz de Montellano, 1995b; Schlichting *et al.*, 2000; Newcomb *et al.*, 2003). The type of reaction catalyzed then depends on the substrate and substrate binding site and varies from hydroxylation to epoxidation, *O*-, *N*-, and *S*-dealkyation, *N*- and *S*-oxidations, or at least 60 different chemical reactions. The types of reactions currently known to be catalyzed by insect P450 enzymes are listed in **Table 2**. The $P450(Fe^{II})$–substrate complex can function as a reductase, and the P450–O_2–substrate complex can also function as an oxidase, releasing superoxide, hydrogen peroxide, or water. Under experimental conditions, NADPH and molecular oxygen can be substituted by organic hydroperoxides, sodium periodate, etc., in what is called the peroxide shunt (Ortiz de Montellano, 1995a).

The obligatory role of P450 reductase in catalysis of the microsomal P450 has been proven in reconstitution experiments, but the role of phospholipids is less clear and has not been specifically studied. The role of cytochrome b_5 is discussed below. Activity of the mitochondrial CYP12A1 also showed absolute dependence on reconstitution in the presence of (bovine) mitochondrial redox partners (Guzov *et al.*, 1998).

The stoichiometry (eqn [1])

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

that commonly describes the monooxygenase (*sensu* Hayaishi) or mixed-function oxidation (*sensu* Mason) reaction of P450 has not been confirmed experimentally for any insect P450. A more complex stoichiometry would take into account the "leakage" of activated oxygen species as superoxide, hydrogen

Table 2 Enzymatic reactions catalyzed by insect P450 enzymes

Reaction catalyzed	*P450*
Oxidase activity	
O_2 to H_2O, H_2O_2, O_2^{-}	CYP6A1 (and probably most P450)
Monooxygenations	
Aliphatic hydroxylation	
C–H hydroxylation	CYP4C7, CYP6A1, CYP6A2, CYP6A8, CYP12A1, CYP302A1, CYP312A1, CYP314A1, CYP315A1
O-dealkylation	CYP6A1, CYP6D1[a], CYP12A1, CYP6A5, CYP6B4, CYP6B17, CYP6B21
Dehalogenation	CYP6A2 (DDT to DDA, DDD)
Epoxidation	CYP6A1, CYP6A2, CYP12A1, CYP15A1, CYP9E1
Aromatic hydroxylation	CYP6D1[a]
Heteroatom oxidation and dealkylation	
Phosphorothioate ester oxidation	CYP6A1, CYP6A2, CYP12A1, CYP6D1[a]
N-dealkylation	CYP12A1, CYP6A5
N-oxidation	+ (nicotine)
S-oxidation	+ (phorate)
Aldehyde oxidation	+ (C-26 hydroxyecdysteroids)
Complex and atypical reactions	
Carbon–carbon cleavage	+? (sterols, ecdysteroid)
Decarbonylation with C–C cleavage	+ (P450hyd)
Aromatization	+ (defensive steroids)
Dehydrogenation	+ (cholesterol)
Dehydration	
Aldoxime dehydration	+ (R-CN biosynthesis)
Reduction	−
Endoperoxide isomerization	−

[a]Inference from immunoinhibition experiments.
+, indicates metabolism by microsomal P450, but specific enzyme not identified (substrate indicated), −, indicates no evidence to date.

peroxide, and water at the expense of NADPH during catalysis. In a "well coupled" reaction as in (eqn [1]) these by-products would not be formed. It is assumed, but not generally proven, that a specialized P450 metabolizing its favorite substrate would follow stoichiometry (eqn [1]). An approximate balance for CYP6A1 epoxidation of heptachlor (*Hept*) gives the following results: (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data).

$$1\ Hept + \underline{13\ O_2} + \underline{11.2\ \mathrm{NADPH}} \rightarrow \underline{1\ Hept\ epoxide} + 1.5\ H_2O + 11.2\ \mathrm{NADP}^+ + \underline{9.8\ H_2O_2} \quad [2]$$

and for testosterone (*Tst*) hydroxylation under the same experimental conditions:

$$1\ Tst + \underline{6\ O_2} + \underline{4.7\ \mathrm{NADPH}} \rightarrow \underline{1\ Tst\text{-}OH} + 0.5\ H_2O + 4.7\ \mathrm{NADP}^+ + \underline{4.2\ H_2O_2} \quad [3]$$

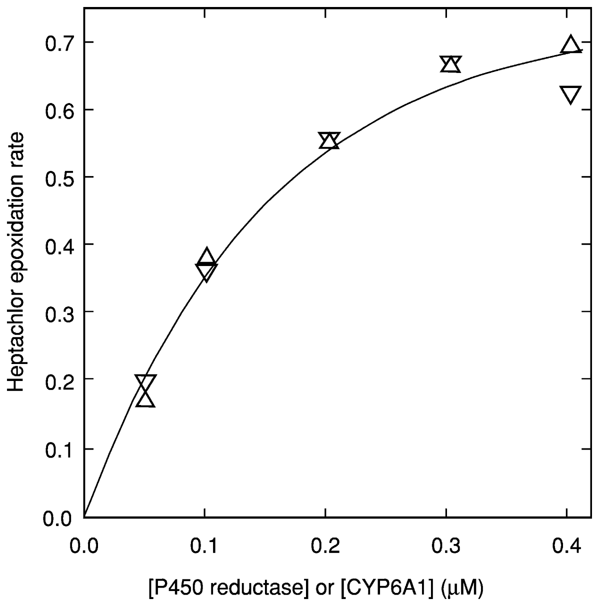
In eqns [2] and [3], the parameters that were measured are underlined. These stoichiometries show that a P450 such as CYP6A1 can be simultaneously an oxidase and a monooxygenase. These coupling stoichiometries (M.B. Murataliev, V.M. Guzov, R. Feyereisen, inpublished data) are dependent on the ratio of P450 and P450 reductase, as well as on the presence or absence of cytochrome b_5 (see below). Note that those stoichiometries are not balanced, reflecting experimental error in the measurements and that the addition of superoxide dismutase did not change the amount of H_2O_2, indicating either no superoxide production, or lack of success in measuring it. The uncoupling of monooxygenation is highly likely to be a common feature of insect P450 enzymes that metabolize xenobiotics of synthetic or plant origin. The generation of reactive oxygen species is a corrolary of active P450 metabolism.

4.1.3.4.2. P450 reductase

4.1.3.4.2.1. P450 interaction with P450 reductase

As seen above, the proper functioning of P450 enzymes depends on an efficient electron supply. In insect microsomes, the ratio of P450 enzymes to P450 reductase is about 6–18 to 1 (Feyereisen *et al.*, 1990). In this ratio, all P450 enzymes are summed, so that the actual ratio of one specific P450 enzyme to P450 reductase is probably smaller. The rate of the overall microsomal P450 reaction (two transfers of one electron) is relatively slow so that dissociation of the P450–P450 reductase complex is possible between the first and the second electron transfer. Indeed, cytochrome b_5 can replace P450 reductase

for the supply of the second electron in some cases (see below). The effect of varying the P450/P450 reductase ratio on catalytic rates was measured in a reconstituted system for heptachlor epoxidation by CYP6A1. The rate of epoxidation was determined by the concentration of the binary complex of P450 and P450 reductase, with the same high rate being observed in the presence of an excess of either protein (Figure 14). The half-saturating concentration of either protein was about 0.1 μM in the presence of cytochrome b_5 (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data). This is in good agreement with the Km of 0.14 and 0.5 μM for P450 reductase in the presence and absence of cytochrome b_5 measured previously (Guzov *et al.*, 1996). Coinfection of Sf9 cells with baculoviruses carrying CYP6B1 and P450 reductase has revealed that highest catalytic activity was achieved at an equivalent, moderate, multiplicities of infection for the two viruses (Wen *et al.*, 2003). Higher enzymatic activities of cell lysates towards furanocoumarins was not achieved when either protein was produced in excess. This result can be explained in part by documented limitations of the cell's ability to host, fold, and provide cofactors for both P450 and reductase (Wen *et al.*, 2003), but it also supports the idea that highest activity is achieved for the highest concentration of the binary complex of the two partners.



[Fixed protein] = 0.05 μM

Figure 14 Heptachlor epoxidation by *E. coli*-expressed recombinant housefly CYP6A1 and NADPH cytochrome P450 reductase. A reconstituted system containing variable concentration of CYP6A1 (△) or P450 reductase (▽) and a fixed concentration (0.05 μM) of the reciprocal partner, and a cytochrome b_5 concentration of 1.0 μM was incubated in the presence of NADPH. (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data)

4.1.3.4.2.2. P450 reductase functioning P450 reductase accepts two electrons from NADPH; more precisely, it accepts a hydride ion (one hydrogen plus one electron), and donates two electrons, one at a time, to P450 enzymes. P450 reductase is therefore an enzyme with two substrates: NADPH and the electron acceptor (P450 or artificial acceptor such as cyt *c*), and two products: $NADP^+$ and the reduced electron acceptor. With two bound flavins and a pathway of electron transfer NADPH > FAD > FMN > P450 (or cyt *c*), its reduction state during catalysis can theoretically vary between the fully oxidized state (0 el.) and the fully reduced state (4 el.). Studies with the purified recombinant housefly P450 reductase (Murataliev *et al.*, 1999; Murataliev and Feyereisen, 1999; Murataliev and Feyereisen, 2000; review: Murataliev *et al.*, 2004a) have shed light on two questions posed by this electron transfer function: what is the kinetic mechanism of this two-substrate enzyme (Ping-Pong or sequential Bi-Bi) and what are the respective reduction states of the two flavins during catalysis?

In the ping-pong mechanism, the first product of the reaction must be released before the second substrate binds to the enzyme, and no ternary complex is formed. In sequential Bi-Bi mechanisms both substrates bind to the enzyme to form a ternary complex. Although several kinetic mechanisms have been proposed (Hodgson, 1985), a careful study of the recombinant housefly P450 reductase clearly established a sequential random Bi-Bi mechanism (Murataliev *et al.*, 1999). The great sensitivity of the enzyme to ionic strength hampers the comparison of different studies (Murataliev *et al.*, 2004a). The formation of a ternary complex of NADPH, P450 reductase, and the electron acceptor suggested a role for reduced nucleotide binding in the catalysis of fast electron transfer. The rate of cytochrome *c* reduction was shown to equal the rate of hydride ion transfer from the nucleotide donor to FAD (Murataliev *et al.*, 1999). A faster electron transfer rate was observed with NADPH as compared to NADH (Murataliev *et al.*, 1999) and the 2′-phosphate was shown to contribute to more than half of the free energy of binding (Murataliev and Feyereisen, 2000). The affinity of the oxidized P450 reductase was ten times higher for NADPH than for $NADP^+$ (Murataliev *et al.*, 1999), and a conformational change induced by NADPH binding and important for fast catalysis was suggested by these studies.

The state of reduction of the flavins of P450 reductase during catalysis was deduced from kinetic

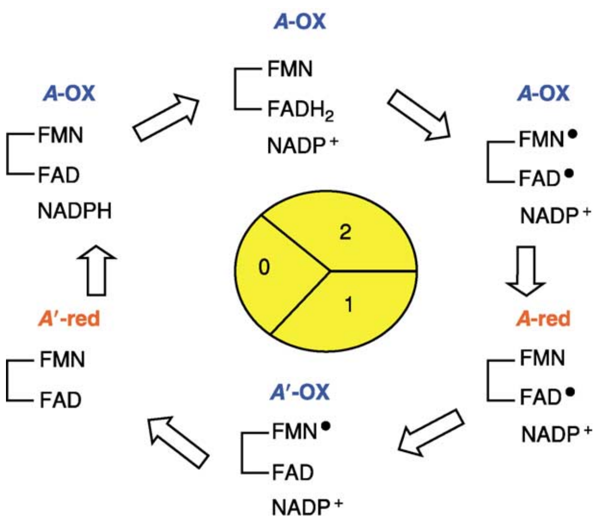


Figure 15 Reduction state of NADPH cytochrome P450 reductase during catalysis, the ''0-2-1-0'' cycle. The enzyme cycles between a fully oxidized state and a 2-electron reduced state. The electron acceptor (A and A′ = P450 or cytochrome *c*) receives one electron at a time from an FMN semiquinone form (FMN•) of the enzyme. The release of $NADP^+$ is shown here to occur at the last step but may occur earlier. Implicit additional steps are not shown, for clarity. See text for details and Murataliev *et al.* (2004a) for review.

experiments, rates of NADPH oxidation and EPR measurements of flavin semiquinone (free radical) levels. These revealed the existence of a catalytically competent FMN semiquinone, different from the "blue" neutral FMN semiquinone, known as the air-stable semiquinone that is not a catalytically relevant form of the enzyme (Murataliev and Feyereisen, 1999). Furthermore, the detailed studies of housefly P450 reductase led to a proposed catalytic cycle where the reduction state of the enzyme does not exceed 2 el., and where an FMN semiquinone, and not an FMN hydroquinone, serves as electron donor to the acceptor P450 or cytochrome *c*. This "0-2-1-0 cycle" (**Figure 15**) likely represents the general mechanism of P450 reductases, with strong evidence that it operates in P450BM3 and in the human P450 reductase as in the fly P450 reductase (Murataliev *et al.*, 2004a).

4.1.3.4.3. Role of cytochrome b_5 Depending on the P450 enzyme and on the reaction catalyzed, cytochrome b_5 may be either inhibitory, without effect, or its presence may be obligatory. Cytochrome b_5 can have a quantitative effect on overall reaction rates, and/or a qualitative role on the type of reaction catalyzed and the ratio of the reaction products. The role of cytochrome b_5 may or may not depend on its redox (electron transfer) properties. It can also influence the overall stoichiometry of the P450 reaction, in particular the "coupling rate," i.e., the utilization and fate of electrons from NADPH relative to monooxygenation. Cytochrome b_5 should therefore be regarded as an important *modulator* of microsomal P450 systems. General reviews of the role of cytochrome b_5 in P450 reactions are available (Porter, 2002; Schenkman and Jansson, 2003) and known examples of this modulator role in insect systems follow.

The relative contribution of NADH in P450 reactions, but more importantly the NADH synergism of NADPH-dependent reactions that is occasionally observed (e.g., Ronis *et al.*, 1988; Feng *et al.*, 1992), is probably attributable to cytochrome b_5 as redox partner. Indeed, the Km of the P450 reductase for NADH is a thousand-fold higher than for NADPH, and the V_{max} tenfold lower (Murataliev *et al.*, 1999), so that the contribution of NADH under normal conditions is probably channeled by NADH-cytochrome b_5 reductase and cytochrome b_5.

An anticytochrome b_5 antiserum severely inhibited (up to 90%) methoxycoumarin and ethoxycoumarin *O*-dealkylation and benzo[a]pyrene hydroxylation, but not methoxyresorufin and ethoxyresorufin *O*-dealkylation when assayed in microsomes of the housefly LPR strain (Zhang and Scott, 1994). This antiserum also inhibits cypermethrin 4′-hydroxylation by these CYP6D1-enriched microsomes (Zhang and Scott, 1996b).

Housefly cytochrome b_5 stimulates heptachlor epoxidation and steroid hydroxylation when reconstituted with cytochrome P450 reductase, housefly CYP6A1, and phospholipids (Guzov *et al.*, 1996; Murataliev *et al.*, 2004a). Stimulation of cyclodiene epoxidation and diazinon metabolism were also observed with *Drosophila* CYP6A2 expressed with the baculovirus system (Dunkov *et al.*, 1997). Cytochrome b_5 is efficiently reduced by P450 reductase (**Figure 12**), but it does not increase the rate of P450 reduction by P450 reductase. Because of its small redox potential (see above), cytochrome b_5 is unlikely to play an important role in delivering the first electron to P450 catalysis, and its stimulatory role probably involves an increased rate of transfer of the second electron. Cytochrome b_5 decreases the apparent Km for P450 reductase and increases the V_{max} for epoxidation at constant CYP6A1 concentrations (Guzov *et al.*, 1996). The results suggest a role for cytochrome b_5 in the P450 reductase–P450 interactions.

Whereas heptachlor epoxidation by CYP6A1 was increased two- to threefold by the addition of cytochrome b_5, the hydroxylation of testosterone, androstenedione, and progesterone was stimulated

seven- to tenfold. The addition of cytochrome b_5 increased the ratio of 2β-hydroxylation over 15β-hydroxylation of testosterone. This suggests that cytochrome b_5 can have an effect on CYP6A1 conformation, probably altering the interaction of the binding site with either the C-17 hydroxyl group (decreased) or the C-3 carbonyl (increased). Interestingly, the effect of cytochrome b_5 on hydroxylation regioselectivity was also obtained with apo-b_5 (cytochrome b_5 depleted of heme and therefore redox incompetent), whereas the effect on turnover number was only much smaller with apo-b_5. The effect of apo-b_5 is not due to heme transfer from P450 to apo-b_5 and, in fact, both apo-b_5 and (holo) cytochrome b_5 were shown to stabilize the ferrous–CO complex of CYP6A1, decreasing the rate of its conversion to P420 (M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data).

Cytochrome b_5 increases the coupling stoichiometry of CYP6A1 catalysis. In the heptachlor epoxidation assay, coupling (NADPH or O_2 used/heptachlor epoxide formed) increased from <8% to over 25%. This effect is even more pronounced for testosterone, where coupling efficiency in the presence of cytochrome b_5 can reach 84%. The effect of cytochrome b_5 results in a decrease in H_2O_2 production in both assays. The exact site of H_2O_2 production (P450 reductase or CYP6A1) is not known.

Coordinate induction and/or overexpression of cytochrome b_5 and P450 genes has been reported (Liu and Scott, 1996; Kasai *et al.*, 1998b; Ranasinghe and Hobbs, 1999a, 1999b; Nikou *et al.*, 2003) and this indicates that the effects of cytochrome b_5 seen *in vitro* may have significance *in vivo* as well.

4.1.3.4.4. Mechanisms and specificity of P450 inhibitors The common features of electron transfer, ligand binding, and catalysis described above are the features that determine the relative success of P450 inhibitors. Compounds that act as electron sinks and are readily autooxidizable can inhibit P450 reactions by inhibiting electron transfer by the respective redox partners. This mechanism is typical of the eye pigment xanthommatin that was identified as "endogenous inhibitor" in early studies (review: Hodgson, 1985). Several flavonoids may act in this way and care must be taken to distinguish P450 inhibition *per se* from inhibition of electron transfer.

Insecticide synergists (**Figure 16**) are among the most interesting inhibitors of P450 because of their widespread commercial use, in particular piperonyl butoxide. A landmark review paper on synergists remains that of Casida (1970). Synergists (Hodgson, 1985; Bernard and Philogene, 1993) as well as P450 inhibitors in general (Ortiz de Montellano and Correia, 1995) have been covered in other insightful reviews as well. The synergism of carbaryl by piperonyl butoxide has been used in a survey of 54 insect species to estimate P450 activity *in vivo* (Brattsten and Metcalf, 1970). Although the synergistic ratio is most often presented, the usefulness of a synergistic difference has also been proposed (Brindley, 1977). The mode of action of piperonyl butoxide and other related methylenedioxyphenyl (MDP) compounds (or benzodioxole compounds) involves an initial metabolic activation by the P450 enzyme, leading to the formation of a carbene–iron complex that is virtually irreversible (Ortiz de Montellano and Correia, 1995). It follows that those P450 enzymes with (1) low affinity for the MDP compound and/or (2) low capacity to metabolize it to the carbene inhibitory form will not be inhibited, and thus piperonyl butoxide and other MDP compounds are not universal inhibitors of all P450 enzymes. Self-catalyzed destruction of P450 enzymes by terminal acetylenes or olefins and other "suicide substrates" is also, and for the same reasons, not equally effective for all P450 enzymes. The phenylpropynyl ether synergists such as TCPPE fall into this category, as well as the newer synergist verbutin (Bertok *et al.*, 2003). This nongenerality of inhibition has been well documented *in vivo*. For instance, TCPPE can be an effective synergist when piperonyl butoxide

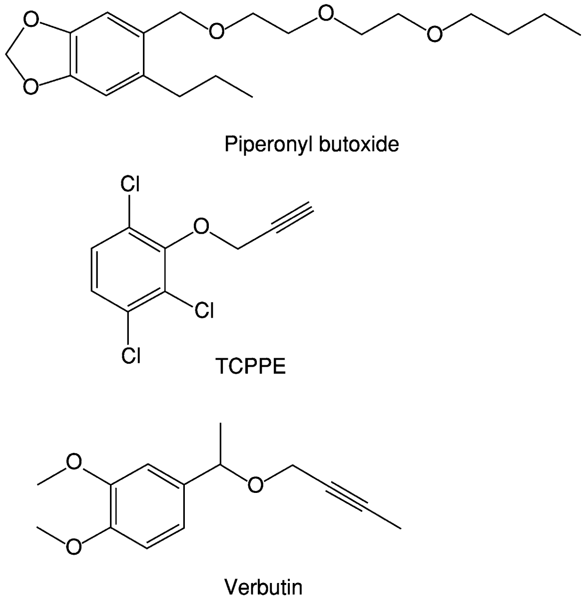


Figure 16 Structures of the synergists and P450 inhibitors piperonyl butoxide, a typical methylene dioxyphenyl (MDP) compound, TCPPE (trichlorophenylpropynyl ether) and verbutin.

cannot (Brown *et al.*, 1996; Zhang *et al.*, 1997). In the case of 1-aminobenzotriazole (ABT), which is metabolized to benzyne that covalently binds to the prosthetic heme, *in vitro* P450 destruction and formation of the porphyrin adduct have been measured in the housefly (Feyereisen *et al.*, 1984). P450 protein labeling by P450 inhibitors has also been achieved (Andersen *et al.*, 1995; Cuany *et al.*, 1995). The NADPH-dependent decrease in P450 caused by ABT, TCPPE, or piperonyl butoxide differs according to the induction status and fly strain, suggesting selectivity (Feyereisen *et al.*, 1984). This selectivity can be harnessed into the design of useful "suicide" inhibitors of, e.g., ecdysone biosynthesis (Luu and Werner, 1996). The synthesis of the MDP moiety seen in many plant natural products is itself dependent on a P450 activity (CYP719, Ikesawa *et al.*, 2003), and it is postulated that such compounds may be a legacy of evolutionary interactions with insect and other enemies (Berenbaum and Neal, 1987).

Another class of powerful P450 inhibitors are heterocyclic compounds with an sp2 hybridized nitrogen as in pyridines, azoles, and imidazoles. These compounds bind simultaneously to the heme iron (type II ligands) and to a hydrophobic binding site of the P450 for its substrate. This "two-point binding" has therefore an intrisinc potential for selectivity, with the substrate mimic moiety of the inhibitor targeting the specific P450 and the type II-ligand moiety coordinating the heme, and inhibiting the enzyme. This reasoning has led to the design of potent inhibitors and photoaffinity labels (Andersen *et al.*, 1995) for a specific insect P450 (see Section 4.1.4.1.2 below). The commercial importance of this type of P450 inhibitors is emphasized by the fungicides and CYP51 inhibitors miconazole and ketoconazole.

4.1.3.4.5. Substrate selectivity and structure/function of P450 enzymes The substrate recognition sites (SRS) of CYP2 proteins were first described by Gotoh (1992) as highly variable regions. Subsequently, a large number of site-directed mutagenesis studies have focused on these regions to explore substrate specificity of mammalian and bacterial P450 enzymes (e.g., review: Domanski and Halpert, 2001). Although multiple sequence alignments usually show that SRS1 is highly polymorphic, the SRS1 of the CYP6B sequences of Lepidoptera are highly conserved (Berenbaum *et al.*, 1996). This suggested that in this case SRS1 may contribute to the recognition of furanocoumarins – substrates of most CYP6B enzymes studied to date. Mutagenesis of six residues of CYP6B1v1 from *Papilio polyxenes* provided some evidence for this hypothesis (Chen *et al.*, 2002). Seven mutants at the Phe116 position in SRS1 led to incorrectly folded or assembled P450 proteins that were catalytically inactive and that had absorption maxima at 420 nm instead of 450 nm in their CO-difference spectrum. One mutant, Phe116 to Trp, had a severely reduced catalytic activity. The Phe116 to Tyr mutant (insertion of a hydroxyl group on the aromatic side chain) was catalytically active and showed an altered substrate specificity towards furanocoumarins. Xanthotoxin metabolism was drastically reduced, bergapten and isopimpinellin metabolism were cut in half whereas metabolism of trioxsalen or psoralen were not affected. Homology modeling of the CYP6B1v1 structure based on a CYP102 crystal structure indicated that the Phe116 side chain projects into the active site above the plane of the heme. Four mutants at the neighboring His117 were catalytically inactive as were nine mutants at the Phe484 position, which is located in SRS6. Val368 is located in SRS5, opposite Phe116 and also in the vicinity of the active site. Its replacement by Phe resulted in a dead enzyme, whereas the mutations of Val368 to Ala or Leu did not affect CYP6B1v1 activity or specificity (Chen *et al.*, 2002). Two further replacements were either lethal (Phe206 to Leu) or without effect (His204 to Leu). These residues are outside the SRS regions, but indicated by homology modeling to be located near the substrate access channel. The overall result of this extensive set of experiments on 33 variants indicates that CYP6B1v1 is very sensitive to changes at sites that control access and geometry of the active site. Only the Phe166 to Tyr mutation in SRS1 showed a clear effect on substrate selectivity towards furanocoumarins. Only three of the inactive mutants at the Phe484 position showed a normal CO difference spectrum, and thus may have lost activity towards furanocoumarins while retaining or gaining activity towards other substrates. This residue in SRS6 is not conserved among CYP6B enzymes, however (Chen *et al.*, 2002). Further analyses of *Helicoverpa* and *Papilio* CYP6B sequences have attempted to describe ancestral CYP6B sequences and to model the geometry of their active site (Li *et al.*, 2003). The crystal structure of a CYP6B protein would contribute enormously to anchor such studies on a firm basis.

Results obtained with naturally occurring mutations situated outside the SRS regions show that substrate specificity is not encoded in the SRS alone. Three point mutations in *Drosophila* CYP6A2 (Bergé *et al.*, 1998), when combined, confer to this P450 the ability to metabolize DDT

without modifying the metabolism of testosterone (Amichot *et al.*, 2004). These mutations found in a DDT-resistant strain (see Section 4.1.4.5.3 below) are located between SRS4 and SRS5 in the J helix (Arg335 to Ser and Leu336 to Val) and at the end of helix L, before SRS6 (Val476 to Leu). The three mutations are located towards the "top" of helix I and may together influence the positioning of this helix and hence access to the active site (Amichot *et al.*, 2004). Significantly, the recombinant enzyme carrying only the Arg335 to Ser mutation has only a fraction of the DDT metabolizing capacity of the triple (naturally occurring) mutant, and is unstable. A better understanding of the structure–activity relationships in insect P450 enzymes is still very distant, but the diversity of insect P450 sequences may prove useful in the engineering of P450 as biocatalysts. For instance, the replacement of portions of SRS1 of P450BM3 (CYP102, a fatty acid ω-1 hydroxylase) by the homologous portions of SRS1 from CYP4C7 (a terpenoid ω-hydroxylase, see Section 4.1.4.1.2) modifies the regioselectivity of hydroxylation of fatty acids and terpenoids of the P450BM3 enzyme (Murataliev *et al.*, 2004b).

Information on substrate access to the active site and active site topology can also be inferred from spectral studies (Section 4.1.3.1.2) and biochemical studies (**Figure 7**). The active site topology of CYP6A1 was studied by a technique developed in Ortiz de Montellano's group (Ortiz de Montellano and Graham-Lorence, 1993). The enzyme is first incubated with phenyldiazene to form a phenyl–iron complex. Ferricyanide-induced *in situ* migration of the phenyl group to the porphyrin nitrogens causes the formation of covalent adducts, which can then be separated by HPLC (**Figure 17**). The *N*-phenyl protoporphyrin IX adducts of CYP6A1 were formed in a 17:25:33:24 ratio of the $N_B:N_A:N_C:N_D$ isomers (Andersen *et al.*, 1997). Thus in the native protein, all four pyrrole groups are somewhat exposed, whereas in several other P450s, labeling is more specific. Specific labeling indicates that the protein encumbers more space on top of the heme prosthetic group, e.g., leaving only one pyrrole ring exposed as in P450scc (Pikuleva *et al.*, 1995). The type of labeling seen with CYP6A1 indicates less encumbrance by the protein on top of the heme as in CYP3A4 (Schrag and Wienkers, 2000). These experiments suggest that the active site of CYP6A1 is relatively accessible and not severely constrained. Indeed CYP6A1 metabolizes flat steroids, bulky cyclodiene insecticides, as well as a variety of sesquiterpenoids (**Table 1**) (Andersen *et al.*, 1994, 1997; M.B. Murataliev, V.M. Guzov, R. Feyereisen, unpublished data). High uncoupling of electron transfer relative to monooxygenation may be a result of this broad substrate specificity.

4.1.4. P450 Functions

Ever since the pioneering work of Agosin and of Terriere in the early sixties on microsomal enzymes that hydroxylate DDT and naphthalene (review: Agosin, 1985), the enzymes now recognized as P450 have been best known for their role in xenobiotic metabolism in insects. They are often denoted as "detoxification enzymes." This designation not only neglects the importance of P450 enzymes in basic physiological processes, as pointed out for drug-metabolizing enzymes in general by Nebert (1991). It also neglects the conceptual difference between metabolism (what the enzyme does to the chemical) and

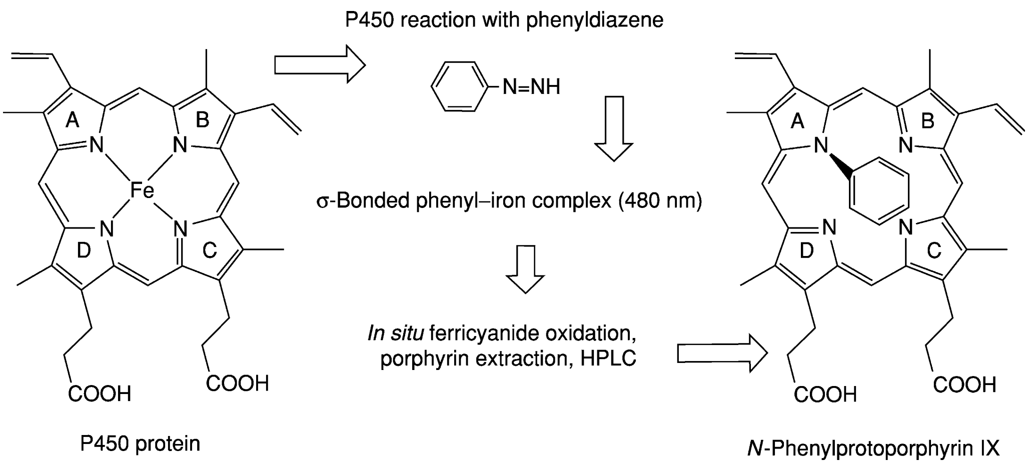


Figure 17 Active site topology of a P450 enzyme tested by formation of phenyl-porphyrin adducts. In this example, an adduct with pyrrole ring A is formed. The ratio of the four possible adducts indicates the degree of encumbrance in the native P450 protein.

toxicity (what the chemical does to the biological system). As noted before (Feyereisen, 1999):

> there are many cases where P450 enzymes act as anything but "detoxification enzymes." The easy dichotomy between biosynthetic and detoxification functions of P450s reflects more the teleological tendencies of the observer than the phylogeny or biochemistry of the enzymes.

In most studies, the P450 substrates tested are either endogenous compounds and their analogs, *or* xenobiotics. Rarely are both types of substrates tested on the same P450 and, indeed, the thermodynamic reality of enzyme–substrate interaction makes the quest for a formally complete description of the chemical diversity of substrates of any enzyme illusory. A future understanding of P450 functions perhaps will be based more on their evolutionary trajectories, some P450s being presently constrained by their proven physiological role (but how long have they played this role?) and some P450s being positively selected for their present adaptive role in detoxification (of plant chemicals in evolutionary time, or of insecticides in historical time). Our knowledge of insect P450 function is not sufficient to classify individual P450s or whole CYP families rationally along such evolutionary lines, so the following description may be found to be arbitrary.

4.1.4.1. Metabolism of Signal Molecules

4.1.4.1.1. Ecdysteroid metabolism

4.1.4.1.1.1. Ecdysone 20-monooxygenase activity (E20MO) The conversion of ecdysone to 20-hydroxyecdysone does not occur in the prothoracic glands but occurs in many peripheral tissues, such as the fat body, midgut, and Malpighian tubules (see **Chapter 3.3**). The P450 nature of the enzyme catalyzing the 20-hydroxylation of ecdysone was well established in 1985 (Smith, 1985) following the initial reports of 1977 (Bollenbacher *et al.*, 1977; Feyereisen, 1977; Johnson and Rees, 1977). An NADPH and O_2-dependent enzyme system, inhibited by typical pharmacological P450 inhibitors, was studied in several insect species. The evidence for P450 involvement was strengthened by the light-sensitive carbon monoxide inhibition observed in some of the most thorough studies (Feyereisen and Durst, 1978; Smith *et al.*, 1979; Greenwood and Rees, 1984). The agreement on the P450 nature of the reaction was accompanied by a lack of consensus on the subcellular localization of E20MO. Some studies showed a microsomal activity, other studies showed a mitochondrial activity, and yet other studies indicated the presence of both microsomal and mitochondrial activities in the same tissue (Smith, 1985). The differences in species, tissues, and experimental conditions, while real, did not permit glossing over the dual subcellular localization of E20MO (**Chapter 3.3**).

Studies past 1985 continued to present evidence for either the mitochondrial or microsomal, or both, localizations of the E20MO activity. For instance, it has been reported to be mostly microsomal in imaginal discs of *Pieris brassicae* (Blais and Lafont, 1986) and in *Gryllus bimaculatus* midgut (Liebrich and Hoffmann, 1991). In the midgut of *Diploptera punctata* (Halliday *et al.*, 1986) and in embryos of *Bombyx mori* (Horike and Sonobe, 1999), the activity is essentially microsomal, and can be inhibited by antibodies to the insect P450 reductase. This is clear evidence that the enzyme derives its reducing equivalents from the usual microsomal redox partner in those cases.

In *Spodoptera littoralis* fat body, E20MO activity is predominantly mitochondrial, with a small amount of microsomal activity (Hoggard and Rees, 1988). The mitochondrial E20MO activity was reportedly inhibited by antibodies to vertebrate P450scc (CYP11A), P45011β (CYP11B), adrenodoxin, and adrenodoxin reductase (Chen *et al.*, 1994), despite the considerable sequence divergence predicted between the vertebrate and insect proteins. The immunodetection of polypeptides significantly larger than predicted (e.g., P450 at 82 kDa and adrenodoxin at 73 kDa) was also a surprising feature of that study. In whole body homogenates of third-instar *Drosophila*, and in the midgut of larval *Spodoptera frugiperda*, the E20MO activity is distributed 1 : 3 between mitochondrial and microsomal fractions (Smith, 1985; Yu, 1995), and it is also distributed in both fractions in larvae of the housefly, and of the flesh fly *Neobellieria bullata* (Darvas *et al.*, 1993). Weirich *et al.* (1996) compared the apparent Km and specific activities of the mitochondrial and microsomal E20MO activities of *Manduca sexta* larval midgut. They concluded that at physiological ecdysone titers, despite a higher specific activity, the mitochondrial E20MO would contribute less than one-eighth the activity of the microsomal form.

The probable existence of several genes encoding P450s with E20MO activity was suggested (Feyereisen, 1999). This would account for the dual localization and complex regulation of the enzyme's activity. On the other hand, alternative splicing or posttranslational modifications of a single gene product could also lead to the microsomal and mitochondrial forms. Interestingly, both microsomal and mitochondrial activities of the *Spodoptera littoralis* fat body E20MO were reported to be

reversibly activated by phosphorylation (Hoggard and Rees, 1988; Hoggard *et al*., 1989). Cases of posttranslational modifications of P450 enzymes by reversible phosphorylation are not very common (Jansson, 1993; Oesch-Bartlomowicz and Oesch, 2003), and this observation would suggest that the *S. littoralis* fat body E20MOs are products of the same gene. The ecdysteroid 26-hydroxylases of *M. sexta* midgut and mitochondria are also both regulated by phosphorylation (Williams *et al*., 2000b).

Petryk *et al*. (2003) identified *Drosophila* CYP314A1 as the product of the *shade* (*shd*) gene, a member of the Halloween group of developmental mutants. Expression of CYP314A1 in *Drosophila* S2 cells enabled the NADPH-dependent hydroxylation of ecdysone to 20-hydroxyecdysone by cell homogenates. RNA *in situ* hybridization shows that the *shd* gene is not expressed in the ring glands, but expression is seen in the gut, fat body, and Malpighian tubules. In embryos, *shd* is expressed primarily in epidermal cells by the time of germ band extension. Embryonic lethality of *shd* mutants indicates that *Cyp314a1* encodes the only significant E20MO activity at that stage in *Drosophila*. A CYP314A1 protein modified at the C-terminus by the addition of three copies of the hemaglutinin epitope was targeted to mitochondria of S2 cells (Petryk *et al*., 2003). This study represents a breakthrough in our understanding of E20MO molecular genetics. *Cyp314a1* has a single clear ortholog in *A. gambiae*, but the sequence of both predicted proteins is unusual. They are clearly members of the mitochondrial P450 clade and have several intron positions in common with other mitochondrial P450 genes (Ranson *et al*., 2002a). However, they lack two of the three positively charged residues thought to confer high affinity to adrenodoxin (Pikuleva *et al*., 1999). Their exact N-terminal sequence is also somewhat unclear in the absence of EST sequences or proteomic data in either species to confirm the annotation prediction of the N-terminus.

Regulation of E20MO activity, developmental changes, induction, and inhibition is discussed by Lafont *et al*. in **Chapter 3.3**.

4.1.4.1.1.2. Ecdysone biosynthesis: biochemistry The hydroxylation of 2-deoxyecdysone to ecdysone in *Locusta migratoria* was observed in the prothoracic glands as expected but in the Malpighian tubules, midgut, fat body, and epidermal tissues as well (Kappler *et al*., 1986). The C-2 hydroxylation was characterized as a mitochondrial P450 activity in larval Malpighian tubules and in ovarian follicle cells of vitellogenic females (Kappler *et al*., 1986) as well as in the prothoracic glands (Kappler *et al*., 1988). This P450 activity is peculiar in its very low sensitivity to CO inhibition, but virtually stoichiometric incorporation of one atom of molecular oxygen was demonstrated (Kabbouh *et al*., 1987). The biochemical characterization of this P450 activity is supportive of the idea that the same gene product is responsible for C-2 hydroxylation in the Malphighian tubules and in the prothoracic glands. Two further hydroxylase activities have been characterized in *L. migratoria* prothoracic glands as typical P450 enzymes (Kappler *et al*., 1988). With 2,22,25-trideoxyecdysone as substrate for C-25 hydroxylation and 2,22-dideoxyecdysone as substrate for C-22 hydroxylation, these two activities were traced to the microsomal and mitochondrial fractions, respectively (Kappler *et al*., 1988). The specificity of the three enzymes is suggested by their low Km (0.5–2.5 μM) but significant competitive inhibition of the C-2- and C-22-hydroxylations by several ecdysteroids indicates that their place and substrate(s) in a grid or in a linear pathway is still conjectural, as noted by Rees (1995). Evidence towards the P450 nature of the C-25, C-22, and C-2 hydroxylases and their subcellular localization in *M. sexta* prothoracic glands has been presented (Grieneisen *et al*., 1993). That study also suggested that the 7,8-dehydrogenation of cholesterol was a microsomal, NADPH-dependent enzyme. Inhibition of the reaction in "mildly disrupted" prothoracic glands by fenarimol ($EC50 = 0.1$ mM) and carbon monoxide (63% inhibition at $CO:O_2$ of 19 : 1) was taken as evidence for P450 involvement. There is to date no convincing evidence for the involvement of P450 enzymes in the biosynthetic steps that occur in Dennis Horn's famed "black box" between the 7,8-dehydrogenation and the ultimate hydroxylations.

Furthermore, there is equally little biochemical information on the enzymes responsible for the dealkylation of phytosterols to cholesterol. The reactions involved (desaturation, epoxidation, C–C bond cleavage) have been elegantly described in terms of their chemistry (Ikekawa *et al*., 1993). They are well within the catalytic competence of P450 enzymes, and whether these dealkylation reactions are catalyzed by P450 enzymes or not, this remains a challenging area of research in view of their central role in the nutritional physiology of phytophagous species.

4.1.4.1.1.3. Ecdysone biosynthesis: molecular genetics The study of *Drosophila* Halloween mutants that identified CYP314A1 as an E20MO also identified the C-2 and the C-22 hydroxylase, and may identify further P450 enzymes of the

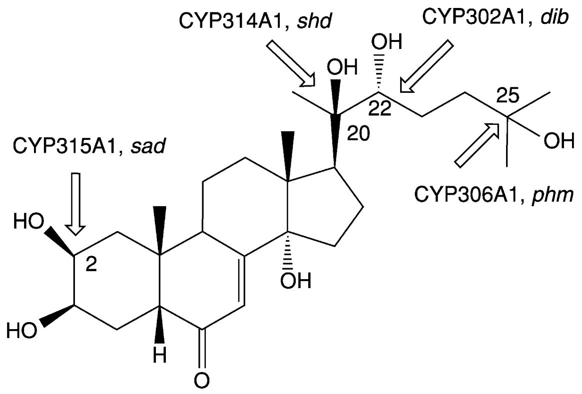


Figure 18 The insect molting hormone 20-hydroxyecdysone with the sites of P450 hydroxylation of its precursors identified to date in *Drosophila melanogaster*. P450 and gene names are shown.

ecdysteroid pathway (**Figure 18**). The *disembodied* (*dib*) gene encodes CYP302A1 identified as the C22 hydroxylase (Chavez *et al.*, 2000; Warren *et al.*, 2002). Similarly, the *shadow* (*sad*) gene encodes CYP315A1, the C2 hydroxylase (Warren *et al.*, 2002). The *dib* gene was identified by classical molecular genetics approaches starting from the genetic locus, ultimately leading to a mitochondrial P450 sequence (Chavez *et al.*, 2000). The identification of the *sad* gene (as that of *shd*, see above) exploited the match between genetic map and the sequence from the *Drosophila* genome project (Warren *et al.*, 2002). In all three cases, mutations leading to stop codons in the sequence confirmed the identification of the gene. Both *dib* and *sad* genes are expressed in embryos, as well as in the larval ring gland and follicle cells of adult ovaries. Transient expression in S2 cells coupled with the use of radiolabeled substrates, 2-deoxyecdysone and 2,22-dideoxyecdysone, was used to identify the reaction catalyzed (Warren *et al.*, 2002). Additional mutants such as *phantom* (*phm*) and *spook* (*spo*) that have a similar phenotype are thought to encode additional P450s involved in ecdysone biosynthesis, CYP306A1, a 25-hydroxylase, and CYP307A1, respectively (J.T. Warren *et al.*, personal communication). The *without children* (*woc*) mutant has identified a transcription factor that controls the 7,8-dehydrogenation of cholesterol or 25-hydroxycholesterol (Warren *et al.*, 2001) and may help identify the enzyme involved in this early step of ecdysone biosynthesis. Little is known of P450 expression in ecdysteroidogenic tissues of other insects. Interestingly, an RT-PCR fragment of a CYP4G-like transcript (GenBank AY635178) was isolated from *Manduca sexta* prothoracic glands. Northern hybridization shows high expression of a 2.3-kb message between days 2 and 5 of the last larval instar (before and during the first peak of ecdysteroid synthesis), with no detectable expression in fat body, midgut, nerve cord, or ovary (M.J. Snyder, V.M. Guzov, J.L. Stevens, and R. Feyereisen, unpublished data). The function of this member of the CYP4 family in prothoracic glands is unknown, but may be related to that of crayfish CYP4C15 (Aragon *et al.*, 2002) or *Drosophila* CYP4G15 (Maibeche-Coisne *et al.*, 2000).

4.1.4.1.1.4. Ecdysteroid catabolism The C-26 hydroxylation of 20-hydroxyecdysone has been characterized as a typical microsomal P450 activity in an epithelial cell line of *Chironomus tentans* (Kayser *et al.*, 1997). However, a dual localization (microsomal and mitochondrial) was reported for ecdysteroid 26-hydroxylase in *M. sexta* midgut (Williams *et al.*, 1997, 2000b). The reaction has a low Km for 20-hydroxyecdysone (~1 μM). The *C. tentans* cell line metabolizes 20, 26-dihydroxyecdysone further to two less polar metabolites produced in a constant 3 : 1 ratio. This metabolism is NADPH-dependent and inhibited by azole compounds. The two metabolites are diastereomers of a cyclic hemiacetal formed (nonenzymatically) by the reaction of the C-22 hydroxyl group with a C-26 aldehyde (Kayser *et al.*, 2002). It is not clearly established whether the P450 conversion of the C-26 hydroxyl to the C-26 aldehyde is carried out by the same enzyme that initially hydroxylates 20-hydroxyecdysone. The C-26 aldehyde is a presumed intermediate in the conversion of C-26 ecdysteroids to C-26 ecdysonoic acids, a common inactivation product of ecdysteroids, but phosphate conjugation of the C-26 hydroxyl is observed in some insects (Rees, 1995). P450-mediated hydroxylations followed by oxidations to the carboxylic acid are known, e.g. CYP701A3 of *A. thaliana* converting *ent*-kaurene to *ent*-kaurenoic acid or CYP27 in the biosynthesis of bile acids.

Side-chain cleavage of ecdysteroids has been reported as an inactivation route, but despite the analogy to the reaction catalyzed by vertebrate P450scc (CYP11A), i.e., the C–C bond cleavage at a vicinal C-20,C-22 diol, there is currently no information on the enzymology of this reaction. P450scc is a unique enzyme in that it catalyzes not just the C–C bond cleavage, but also the two preceding hydroxylations of cholesterol at C-20 and C-22 (in that order).

4.1.4.1.2. P450 and juvenile hormone metabolism (see also Chapter 3.7) The landmark chemical feature of juvenile hormones (JHs) is the presence of the epoxide group, and by 1985 it was already well

established that epoxidation of the JH precursors in the corpora allata (CA) was catalyzed by a P450 enzyme. The biochemical evidence was presented in two studies on the allatal enzyme, one with *Blaberus giganteus* CA homogenates (Hammock, 1975) and the other with *L. migratoria* CA (Feyereisen *et al*., 1981) that established its microsomal nature. A number of compounds that took advantage of the P450 nature of the epoxidase have been tested as inhibitors of juvenile hormone biosynthesis (Hammock and Mumby, 1978; Brooks *et al*., 1985; Pratt *et al*., 1990; Unnithan *et al*., 1995). The allatal epoxidase was viewed as an attractive new target for "biorational" insecticides, because it is an insect-specific target, and because its inhibition should lead to desirable effects – precocious metamorphosis and adult sterility akin to the effects of precocenes (Bowers *et al*., 1976). A serious pursuit of this goal would only be possible with a molecular characterization of the epoxidase and its heterologous expression that would permit screening at a scale not possible by the laborious dissection of the glands. Photoaffinity labeling was first developed as a technique that could facilitate purification and subsequent cloning of the epoxidase. Bifunctional compounds with a substituted imidazole to coordinate the heme iron and a diazirine or a benzophenone group to label the substrate binding site were synthesized and tested (Andersen *et al*., 1995). These compounds were potent inhibitors of methyl farnesoate epoxidation to JH III in the CA of *D. punctata*, and selectively labeled a 55 kDa protein.

4.1.4.1.2.1. CYP15A1 – the epoxidase However, eventual cloning of the epoxidase was achieved by a less subtle route, the sequencing of ESTs from *D. punctata* CA. Sequencing of >900 ESTs from the CA of vitellogenic females yielded three ESTs matching a P450 sequence by BLAST analysis. A full-length cDNA of this P450 was expressed in *E. coli* (Helvig *et al*., 2004). This P450 termed CYP15A1, has a high affinity for methyl farnesoate, showing a type I spectrum with a Ks of 6 μM. The enzyme, when reconstituted with fly P450 reductase, catalyzed the NADPH-dependent epoxidation of 2*E*, 6*E*-methyl farnesoate to JH III. The epoxidation is highly stereoselective (98 : 2) to the natural 10*R* enantiomer over its diastereomer. The enzyme also has a high substrate specificity, epoxidizing the 2*E*,6*E* isomer preferentially over the 2*Z*,6*E* isomer, and accepting no other substrate tested including farnesoic acid. CYP15A1 is expressed selectively in the CA, and the rank order of inhibition of CYP15A1 activity by substituted imidazoles is identical to that of JH biosynthesis by isolated CA (Helvig *et al*., 2004).

4.1.4.1.2.2. CYP4C7 – ω-hydroxylase Expression of the *CYP15A1* gene is high when JH synthetic levels are high, but another P450 gene, *CYP4C7*, also selectively expressed in the CA of *D. punctata* (Sutherland *et al*., 1998), has an expression pattern that mirrors the pattern of JH synthesis. The recombinant CYP4C7 enzyme produced in *E. coli* metabolized a variety of sesquiterpenoids but not mono- or diterpenes. In addition to metabolizing JH precursors, farnesol, farnesal, farnesoic acid, and methyl farnesoate, it also metabolized JH III to a major metabolite identified as (10*E*)-12-hydroxy-JH III (Sutherland *et al*., 1998). Although this ω-hydroxylated JH III has not been identified as a product of the CA, *L. migratoria* CA are known to produce several hydroxylated JHs in the radiochemical assay *in vitro* (Darrouzet *et al*., 1997; Mauchamp *et al*., 1999). These hydroxy-JHs, 8′-OH-, 12′-OH (Darrouzet *et al*., 1997), and 4′-OH-JH III (Mauchamp *et al*., 1999) may be major products of the CA after JH III itself and their role is unknown. Their presence suggests that locust CA have a P450 homologous to cockroach CYP4C7 that has a lower regioselectivity. Indeed, the hydroxy-JHs can be synthesized by locust CA from JH III, and this hydroxylation is inhibited by CO and piperonyl butoxide (Couillaud *et al*., 1996).

The tight physiological regulation of *CYP4C7* expression in adult female *D. punctata* (Sutherland *et al*., 2000) indicates that the terpenoid ω-hydroxylase has an important function to play at the end of vitellogenesis and at the time of impending chorionation and ovulation. It was hypothesized (Sutherland *et al*., 1998, 2000) that this hydroxylation was a first step in the inactivation of the very large amounts of JH and JH precursors present in the CA of this species after the peak of JH synthesis.

The study of JH metabolism has long been dominated by esterases and epoxide hydrolases (see **Chapter 3.7**), but early work on insecticide-resistant strains of the housefly revealed oxidative metabolism as well (review: Hammock, 1985). Evidence that housefly CYP6A1 efficiently metabolizes sesquiterpenoids, including JH to its 6,7-epoxide, confirms these early studies (Andersen *et al*., 1997). Hydroxylation and epoxidation of JHs are thus confirmed P450-mediated metabolic pathways for these hormones.

A comparison of four P450 enzymes that metabolize methyl farnesoate (**Table 3**) shows their

Table 3 Specificity of insect P450 enzymes towards methyl farnesoate (MF)

P450	*Substrate*	*Reaction*	*Product formed*	*Reference*
CYP15A1	2*E*, 6*E*-MF	Epoxidation	10*R*-epoxide	Helvig *et al.* (2004)
CYP4C7	Sesquiterpenoids	ω-Hydroxylation	12*E*-OH	Sutherland *et al.* (1998)
CYP9E1	2*Z* > 2*E*-MF (1.5 : 1)	Epoxidation	10-Epoxide 10R : 10S (1 : 1)	[a]
CYP6A1	MF or JH all 4 isomers	Epoxidation	6 or 10-epoxide 10S : 10R (3 : 1)	Andersen *et al.* (1997)

[a]J.F. Andersen, G.C. Unnithan, J.F. Koener, and R. Feyereisen, unpublished data. Data from Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., **2004**. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. *Proc. Natl Acad. Sci. USA 101*, 4024–4029; © National Academy of Sciences, USA.

catalytic versatility. One is extremely substrate specific (CYP15A1), another is not (CYP6A1). Three of them are epoxidases, one is a hydroxylase on this substrate (CYP6A1 also has activity as a hydroxylase – but not on this substrate). One is a stereoselective epoxidase (CYP15A1), another lacks product enantioselectivity (CYP9E1).

4.1.4.1.3. Biosynthesis of long-chain hydrocarbons The cuticle of insects can be characterized by its hydrocarbon composition, and this can serve as a subtle tool in chemical taxonomy (Lockey, 1991). In some insects, cuticular or exocrine alkanes and alkenes can serve as allomones, or even pheromones (see **Chapter 3.14**). To cite but one example, the Dufour gland secretions of the leaf-cutting ant *Atta laevigata* are deposited on foraging trails. This trail pheromone comprises n-heptadecane, (*Z*)-9-nonadecene, 8,11-nonadecadiene, and (*Z*)-9-tricosene (Salzemann *et al.*, 1992). Despite their apparently simple structure, the biosynthesis of these hydrocarbons is complex and the enzymes involved are still under intense study. Schematically (**Figure 19**), long chain (18–28 carbons) fatty acid CoA esters are first reduced to their aldehyde by an acyl-CoA reductase, and they are subsequently shortened to form C_{n-1} hydrocarbons (Reed *et al.*, 1994). The conversion of (*Z*)-tetracosenoyl-CoA (from a C24 : 1 fatty acid) to (*Z*)-9-tricosene (a C23 : 1 alkene) has been characterized in housefly epidermal microsomes. The role of the aldehyde tetracosenal as an intermediate is evidenced by trapping experiments with hydroxylamine. The next step is NADPH- and O_2-dependent, and is truly an oxidative decarbonylation reaction, which releases the terminal carbon as CO_2 and not as CO (Reed *et al.*, 1994). Inhibition by carbon monoxide and anti-P450 reductase antibodies are strongly indicative of a P450 reaction, and this most peculiar enzyme was called P450hyd (Reed *et al.*, 1994). The reaction does not involve a terminal desaturation, because both C-2 protons of the aldehyde are retained in the hydrocarbon product, and the aldehydic proton is transfered to the product (Reed *et al.*, 1995). NADPH and molecular oxygen can be replaced by "peroxide shunt" donors, suggesting that this reaction involves a perferryl iron-oxene species of activated oxygen (Ortiz de Montellano, 1995a), and that it proceeds through an alkyl radical intermediate (Reed *et al.*, 1995). This type of oxidative decarbonylation reaction may be widespread in insects because a mechanistically identical conversion of octadecanal to heptadecane has been documented in flies, cockroaches, crickets, and termites (Mpuru *et al.*, 1996).

4.1.4.1.4. Pheromone metabolism In female houseflies, the (*Z*)-9-tricosene produced by P450hyd is a major component of the sex pheromone, being responsible for inducing the courtship ritual and the males' striking activity (see **Chapter 3.14**). Additional components of the pheromone are the sex recognition factors (*Z*)-9,10-epoxytricosane and (*Z*)-14-tricosen-10-one. These compounds are obviously derived from (*Z*)-9-tricosene by oxidative metabolism (Blomquist *et al.*, 1984). A microsomal P450 was shown to oxidize the alkene from either side at a distance of 9/10 carbons in-chain (Ahmad *et al.*, 1987), but the structural requirements of the enzyme for its substrate are otherwise strict (Latli and Prestwich, 1991). This P450 activity is found in various tissues of the male and female, including the epidermis and fat body, but the highest specific activity is found in male antennae (Ahmad *et al.*, 1987; **Chapter 3.15**). The C23 epoxide and ketone are absent internally in females, but accumulate on the surface of the cuticle (Mpuru *et al.*, 2001), suggesting the localization of this P450 activity in epidermal cells of female flies.

More generally, P450 enzymes are probably involved in the biosynthesis of many insect pheromones and allomones, e.g., epoxides of polyunsaturated hydrocarbons in arctiid moths, disparlure of the gypsy moth (Brattsten, 1979a), or monoterpenes

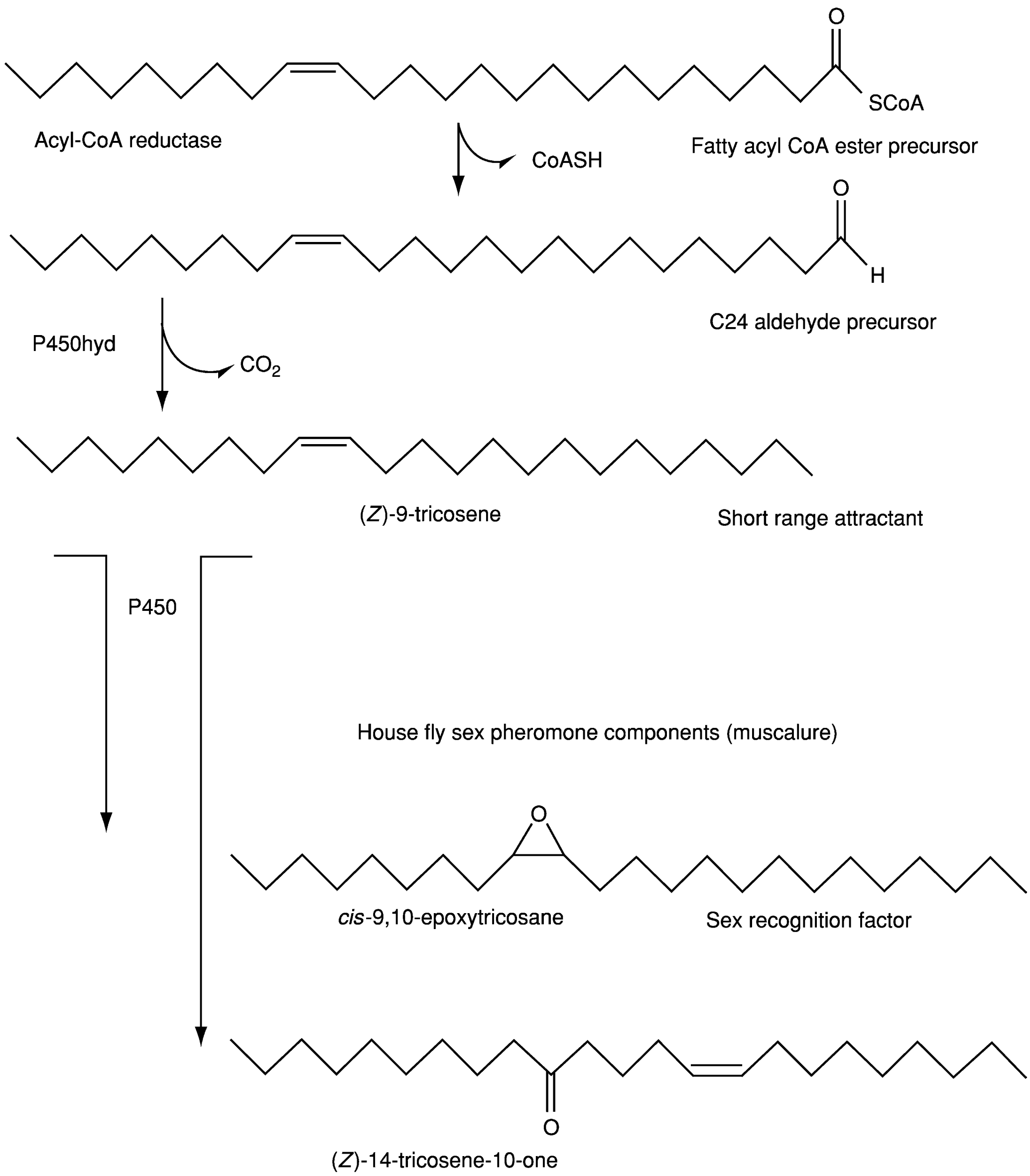


Figure 19 Biosynthesis of (*Z*)-9-tricosene and components of the housefly sex pheromone. P450hyd acts as a decarbonylase to produce (*Z*)-9-tricosene from an aldehyde precursor. Another P450 attacks (*Z*)-9-tricosene from either side to give the two components of muscalure.

in bark beetles (Brattsten, 1979a; White *et al*., 1979; Hunt and Smirle, 1988). In the honeybee, caste-specific ω and ω-1 hydroxylations of fatty acids to mandibular pheromones (see **Chapter 3.13**) have all the characteristics of P450 reactions (Plettner *et al*., 1998). These P450s involved in pheromone biosynthesis are likely to be exquisitely specific.

Evidence for pheromone catabolism by P450 enzymes is also accumulating. As shown for the metabolism of (*Z*)-9-tricosene in the housefly, a distinction between biosynthesis and catabolism can be purely semantic in the case of a biogenetic succession of chemicals that have different signaling functions. Compound B that is a metabolite of compound A may have less or none of compound A's activity, but may have its own specific biological activity. Nonetheless, pheromones as signal molecules need to be metabolically inactivated and this catabolism may occur in the antennae themselves. Several P450 mRNAs have been identified in insect antennae. In *Drosophila*, a partial P450 cDNA, along with a UDP-glycosyltransferase and a short chain dehydrogenase/reductase, were found by northern blot analysis to be preferentially expressed in the third antennal segments, with lower expression in legs (Wang *et al*., 1999). This P450 cDNA

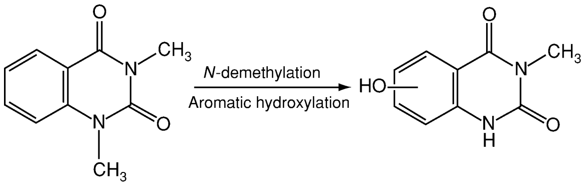


Figure 20 P450-dependent *N*-demethylation and ring hydroxylation of the *Phyllopertha diversa* sex pheromone by antennal microsomes of male scarab beetles. See text and Wojtasek and Leal (1999) for details.

corresponds to *Cyp6w1* for which many ESTs have been identified in a head cDNA library. In *Mamestra brassicae*, two P450 cDNAs were cloned from antennae, CYP4L4 and CYP4S4 (Maibeche-Coisne *et al.*, 2002). Both genes are strongly expressed in the sensilla trichodea as shown by *in situ* hybridization. Whereas *CYP4S4* expression is restricted to the antennae, *CYP4L4* is also expressed in proboscis and legs. Five P450 ESTs representing three P450 genes were found in a small EST project using male *Manduca sexta* antennae (Robertson *et al.*, 1999), which is further evidence that antennae may harbor several P450 enzymes. High levels of P450 reductase expression have been noted in *Drosophila* antennae (Hovemann *et al.*, 1997), and adrenodoxin reductase is also expressed in antennae (Freeman *et al.*, 1999). Antennal P450s are therefore an active enzyme system, complete with their redox partners. The physiological role of the P450s thus found in antennae is still formally unknown, but the presence of other enzymes generally associated with detoxification processes (Robertson *et al.*, 1999; Wang *et al.*, 1999) are indeed suggestive of a large category of odorant-metabolizing enzymes. In addition to the presence of P450 transcripts in antennae, there is at least one report where a P450 reaction has been characterized in antennae, but the CYP gene coding for the P450 catalyzing this reaction is still unknown. Antennal microsomes of the pale brown chafer, *Phyllopertha diversa*, metabolize the alkaloid sex pheromone by a P450 enzyme (Wojtasek and Leal, 1999). This enzyme is specifically produced in male antennae, and its activity is not detected in other scarab beetles or lepidopteran species (**Figure 20**). P450 enzymes may have found in insects a role as odorant degrading enzymes (ODE) along with esterases, aldehyde oxidases, glucosyl-transferases, etc. (review: **Chapter 3.15**).

4.1.4.1.5. Metabolism of fatty acids and related compounds P450-catalyzed fatty acid ω-hydroxylation has been reported in insects (Feyereisen and Durst, 1980; Ronis *et al.*, 1988; Clarke *et al.*, 1989; Cuany *et al.*, 1990; Rose *et al.*, 1991). Clofibrate selectively induces ω-hydroxylation (and not ω-1) of lauric acid by housefly and *Drosophila* microsomes, just as it induces the rat CYP4A1 ω-hydroxylase (Clarke *et al.*, 1989; Amichot *et al.*, 1998). No clear peroxisome proliferator activated receptor (PPAR, Issemann and Green, 1990) gene ortholog but several paralogs are present in the *Drosophila* genome. In contrast to clofibrate, phenobarbital induces *Drosophila* ω, ω-1, and ω-2 hydroxylations (Amichot *et al.*, 1998), indicating the presence of several fatty acid hydroxylases. By homology to vertebrate CYP4 enzymes it is thought that cockroach CYP4C1 has a ω-hydroxylase function as well, but evidence is lacking. CYP4C7, CYP6A1, and CYP12A1 lack lauric acid hydroxylase activity (Andersen *et al.*, 1997; Guzov *et al.*, 1998; Sutherland *et al.*, 1998), but CYP6A8 has laurate ω-1 hydroxylase activity (C. Helvig, personal communication). Although the role of vertebrate P450 enzymes in the metabolism of arachidonic acid and eicosanoids is well established (Capdevila *et al.*, 2002), there is currently no indication for a similar function of P450 in insects.

The biosynthesis of volicitin, an elicitor of plant volatile production found in the oral secretions of caterpillars occurs in the insect from the plant-derived fatty acid linolenic acid (Pare *et al.*, 1998). This biosynthesis involves C-17 hydroxylation and glutamine conjugation. It is likely that this hydroxylation will be shown to be catalyzed by a P450 enzyme.

4.1.4.1.6. Defensive compounds The tremendous variety of chemicals used for defense of insects against predation is well documented, but the enzymes involved in their *de novo* synthesis or in their transformation from ingested precursors are less studied. It is very likely that P450 enzymes may have found there a fertile ground for their chemical prowess. Just two examples are described.

The biosynthesis of cyanogenic compounds found as defensive compounds in many insect species (Nahrstedt, 1988) may well involve P450 enzymes. For instance, the cyanogenic glucosides linamarin and lotaustralin found in *Heliconius* butterflies and *Zygaena* moths, are clearly derived from the amino acids valine and isoleucine. The pathway probably involves N-hydroxylation of the amino acids, further metabolism to the aldoximes and nitriles, and final C-hydroxylation before conjugation to the glucoside (**Figure 21**). The aldoximes and nitriles are efficiently incorporated *in vivo* (Davis and Nahrstedt, 1987; Holzkamp and Nahrstedt, 1994).

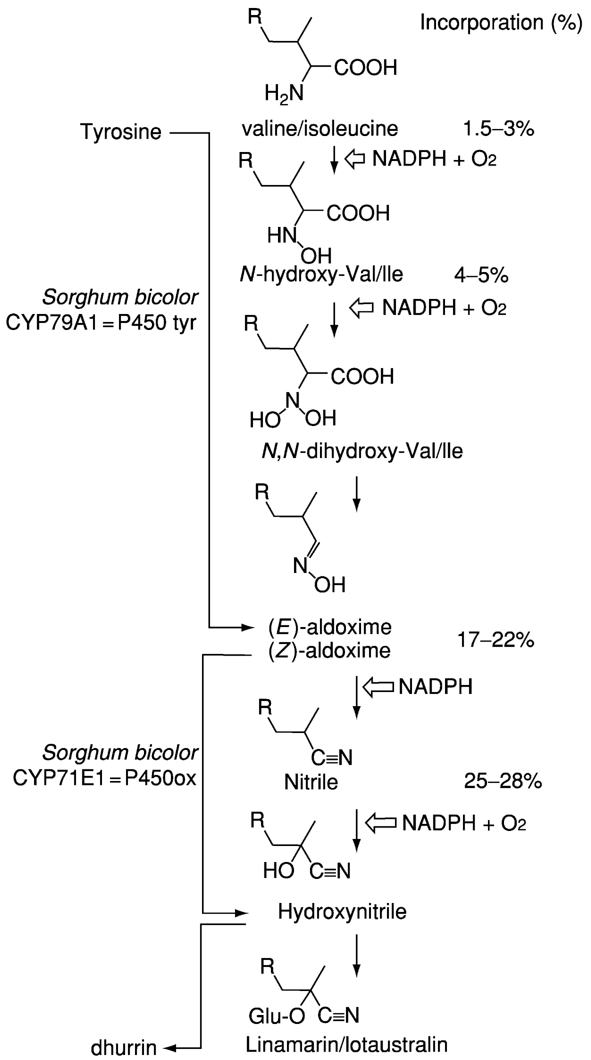


Figure 21 Biosynthesis of linamarin and lotaustralin from valine (R=H) and isoleucine (R=CH_3), indicating possible sites of P450 metabolism. The efficiency of incorporation of intermediates *in vivo* is shown on the right. The homologous reactions catalyzed by two multifunctional Sorghum P450 enzymes converting tyrosine to dhurrin are shown on the left.

The pathway resembles that found in plants, where two multifunctional P450 enzymes are sufficient to convert the amino acid to the hydroxynitrile substrate of the conjugating enzyme (**Figure 21**). For dhurrin biosynthesis from tyrosine in sorghum, these are CYP79A1 and CYP71E1 (Kahn *et al.*, 1997). Whereas the plant pathway appears to "channel" the substrates through the two P450s with little escape of the aldoxime intermediate, nothing is known of the number and functioning of the cyanogenic pathway enzymes used by Lepidoptera. Characterization of the P450 enzymes involved should allow a comparison of the plant and insect solutions to this biosynthetic challenge.

Another comparison of interest will be that of enzymes involved in the biosynthesis of insect defensive steroids with the well characterized steroid metabolizing enzymes of vertebrates. A number of aquatic Coleoptera (Dysticidae) and Hemiptera (Belastomatidae) synthesize a variety of steroids, mostly pregnanes (Scrimshaw and Kerfoot, 1987). With cholesterol as the presumed precursor in these carnivorous insects, one may envisage the evolution of an insect side-chain cleavage enzyme, of a C-21 hydroxylase and of a C17-C21 lyase that would catalyze reactions identical to those of CYP11A, CYP21, and CYP17. Some defensive steroids also have an aromatic A-ring, 7α, or 15α hydroxyl groups. A pregnene-3β, 20β-diol glucoside is synthesized from cholesterol in female pupae of *M. sexta* (Thompson *et al.*, 1985). The role of this compound is unknown, but its synthesis strongly suggests the existence of a C20-C22 side chain cleavage enzyme in Lepidoptera as well.

4.1.4.2. Xenobiotic Metabolism: Activation and Inactivation

4.1.4.2.1. Natural products The metabolism of plant toxins by insects has been reviewed extensively (see, e.g., Brattsten, 1979b; Dowd *et al.*, 1983; Ahmad, 1986; Ahmad *et al.*, 1986; Mullin, 1986; Yu, 1986). Relatively a few studies have directly assessed the role of P450 enzymes in the metabolism of natural compounds by more than one or two criteria such as microsomal localization, NADPH and O_2 dependence, and inhibition by piperonyl butoxide. In most cases, metabolism is associated with detoxification, e.g., the metabolism of xanthotoxin in *Papilio polyxenes*, *S. frugiperda*, and *Depressaria pastinacella* (Bull *et al.*, 1986; Nitao, 1990), the metabolism of α-terthienyl in larvae of three lepidopteran species (Iyengar *et al.*, 1990), and the metabolism of nicotine in *Manduca sexta* larvae (Snyder *et al.*, 1993). Alpha- and beta-thujones are detoxified by P450 as evidenced by synergism of their toxicity by three P450 inhibitors in *Drosophila* and by the lower toxicity of six of their metabolites (Hold *et al.*, 2001). Studies with flavone (Wheeler *et al.*, 1993), monoterpenes (Harwood *et al.*, 1990) and the alkaloid carnegine (Danielson *et al.*, 1995) show clearly however that evidence for metabolism by P450 *in vitro* may not be sufficient to define an *in vivo* toxicological outcome.

Natural products in the diet can act as inducers of P450 as well of other enzymes (e.g., glutathione S-transferases) and as a result of this induction (see below), the metabolism of the inducing compound or of coingested plant compounds can change dramatically over time (Brattsten *et al.*, 1977).

Differences between acute and chronic toxicity are thus often the result of altered expression patterns (quantitative and qualitative) of P450 genes. This was demonstrated, for instance, in studies on *Spodoptera* larvae (Brattsten, 1983; Gunderson *et al.*, 1986). The monoterpene pulegone and its metabolite menthofuran are more acutely toxic to *S. eridania* than to *S. frugiperda*, but the reverse is true for chronic toxicity.

A study by Yu (1987) compared the metabolism of a large number of plant chemicals of different chemical classes by *S. frugiperda* and *Anticarsia gemmatalis* (velvetbean caterpillar) microsomes. Two indirect methods were used, on the one hand, the NADPH-dependent decrease in substrate and on the other hand, the substrate-induced NADPH oxidation. This metabolism is inhibited by piperonyl butoxide and by carbon monoxide, and induced by a number of chemicals, particularly indole-3-carbinol, strongly suggesting P450 involvement. Such indirect methods are very useful as screening tools, as a first step towards a more thorough characterization of metabolism. However, they give no qualitative indication of the chemical fate of the substrate, nor quantitative indication of the levels of metabolism, as NADPH consumption is correlated to the coupling rate of the reaction, rather than to the rate of product formation (see Section 4.1.3.4.1). Clearly, insect P450 enzymes as a whole are capable of metabolizing a tremendous variety of naturally occurring chemicals, but the role of individual enzymes and their catalytic competence still needs a better description.

Heterologously expressed P450 enzymes of the CYP6B subfamily from *Papilio* species (see Section 4.1.4.3 and **Table 1**) are well characterized for their ability to metabolize furanocoumarins (Hung *et al.*, 1997; Li *et al.*, 2003; Wen *et al.*, 2003). They fit the description of enzymes with "broad and overlapping specificity" towards these compounds. Their range of catalytic competence is quite variable. For instance, CYP6B21 and CYPB25 metabolize the angular furanocoumarin angelicin at a similar rate (0.4–0.5 nmol/min/nmol P450), but whereas CYP6B21 also metabolizes 7-ethoxycoumarin at a similar rate (0.5 nmol/min/nmol P450), CYP6B25 does not have appreciable 7-dealkylation activity (Li *et al.*, 2003).

Natural products are not just an endless catalog of P450 substrates and inducers, but they also comprise a varied and complex set of inhibitors of P450 enzymes. These inhibitors range from "classical" reversible inhibitors to substrates that are activated to chemically reactive, cytotoxic forms (e.g., Neal and Wu, 1994).

4.1.4.2.2. Insecticides and other xenobiotics The metabolism of insecticides by P450 enzymes is very often a key factor in determining toxicity to insects and to nontarget species, but it can also represent a key step in the chain of events between contact, penetration, and interaction at the target site. The classical example is probably the metabolism of phosphorothioate insecticides. In many cases, the active ingredients of organophosphorus insecticides are phosphorothioate (P=S) compounds (a.k.a. phosphorothionates), whereas the molecule active at the acetylcholinesterase target site is the corresponding phosphate (P=O). It has long been recognized that the P=S to P=O conversion is a P450-dependent reaction. In the case of diazinon, this desulfuration has been studied for three heterologously expressed insect P450 enzymes (Dunkov *et al.*, 1997; Guzov *et al.*, 1998; Sabourault *et al.*, 2001). All three P450s metabolized diazinon not just to diazoxon, the metabolite resulting from desulfuration, but also to a second metabolite resulting from oxidative ester cleavage. Similarly, antibodies to housefly CYP6D1 inhibit the microsomal desulfuration of chlorpyriphos as well as its oxidative ester cleavage (Hatano and Scott, 1993). The mechanism of P450-dependent desulfuration is believed to involve the initial attack of the P=S bond by an activated oxygen species of P450, leading to an unstable and therefore hypothetical phosphooxythiirane product (**Figure 22**). The collapse of this product can lead to two possible outcomes: (1) the replacement of sulfur by oxygen in the organophosphate product with the release of a reactive form of sulfur; and (2) the cleavage of the phosphate ester (or thioester) link with the substituent of highest electron-withdrawing properties, the "leaving group."

Outcome (1) can be viewed as "activation" because the P=S to P=O desulfuration produces an inhibitor of acetylcholinesterase often several orders of magnitude more potent than the P=S parent compound. However, the fate of this product of "activation" depends on the histological proximity to the target, sequestration, excretion, and further metabolism of the phosphate (by oxidative or hydrolytic enzymes). Kinetic evidence with the heterologously expressed CYP6A1, CYP6A2, and CYP12A1, as well as immunological evidence with CYP6D1 indicate that these P450 enzymes do not metabolize the P=O product of the parent P=S compound they metabolize.

Outcome (2) or "dearylation" is without question a detoxification because the oxidative cleavage of the "leaving group" yields compounds unable to inhibit acetylcholinesterase, the dialkylphosphorothioate,

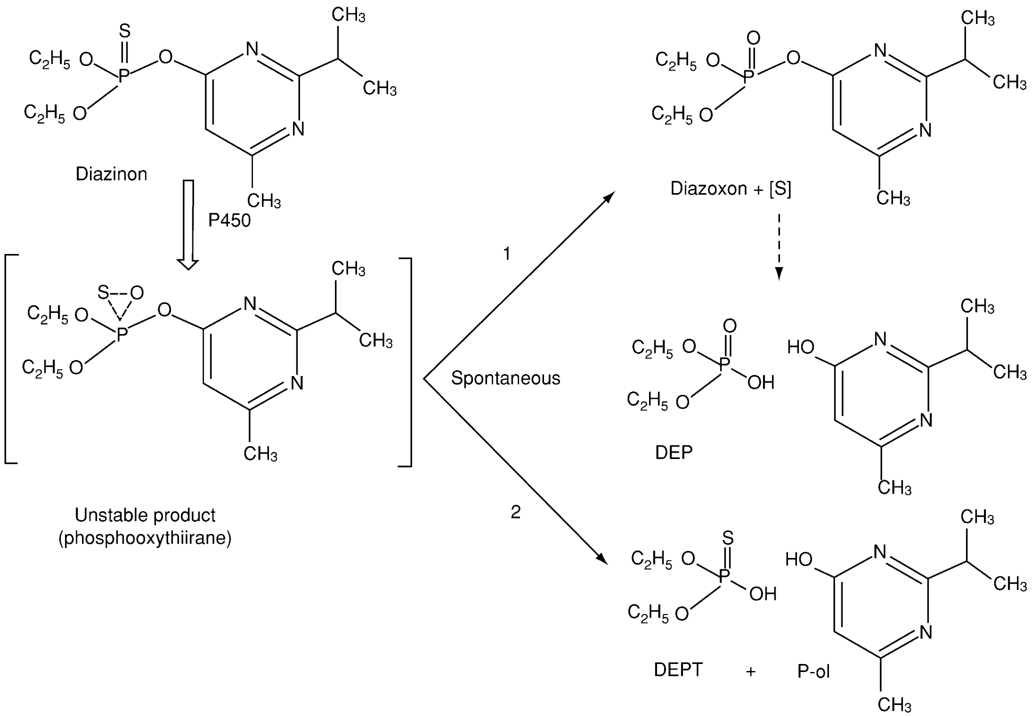


Figure 22 Metabolism of diazinon by cytochrome P450. Following an insertion of oxygen into the substrate, a reactive intermediate collapses (1) by desulfuration or (2) by cleavage of the ester linkage (dearylation). DEP: diethylphosphate, DEPT: diethylphosphorothioate, P-ol: 2-isopropopoxy-4-methyl-6-hydroxypyrimidine, [S]: reactive form of sulfur released during the reaction. Diazoxon can be further converted to DEP and P-ol by the same or another P450 in a subsequent reaction, or by a phosphotriester hydrolase. DEP may also be formed by spontaneous degradation of the initial product of diazinon monooxygenation. The ratio of outcomes 1 and 2 and the fate of the reactive sulfur depends on the P450 enzyme and on the type of OP substrate (see **Table 4**).

and/or dialkylphosphate. The ratio of outcome (1) and (2) appears P450-specific and substrate specific (**Table 4**) suggesting that the collapse of the unstable initial product of P450 attack is influenced by the active site environment. Theoretically, some P450 enzymes may very strongly favor outcome (1) or (2) or vice versa, thus qualifying as relatively "clean" activators or detoxifiers, but there is to date little direct evidence from the insect toxicological literature for such P450 enzymes (see however Oi *et al.*, 1990).

Furthermore, the sulfur released by the reaction can bind covalently either to neighboring proteins thus leading to cellular damage, or to the P450 protein itself (at least in vertebrate liver where this specific aspect has been studied, Kamataki and Neal, 1976). Parathion causes NADPH-dependent inhibition of methoxyresorufin *O*-demethylation activity (a P450Lpr-selective activity) in the housefly whereas chlorpyriphos does not (Scott *et al.*, 2000). Therefore, it is not just the fate of the initial P450 metabolite of the P=S compound that depends on the P450 and the OP, but it is also the fate of the sulfur released that can vary.

Changes in the level of expression of P450 genes or P450 point mutations may be sufficient to change this delicate balance between activation and inactivation *in vivo*. For instance, fenitrothion resistance in the Akita-f strain of the housefly is related to an increase in oxidative ester cleavage over desulfuration measured in abdominal microsomes (Ugaki *et al.*, 1985). In *H. virescens*, methyl parathion resistance in the NC-86 strain, which has an unchanged level of total P450, is related to a replacement of a set of P450 enzymes with high desulfuration activity by a set of P450 enzymes that metabolize less parathion, and do so with a lower desulfuration/oxidative ester cleavage ratio (Konno and Dauterman, 1989) (see **Table 4**).

P450 enzymes that metabolize OPs can metabolize other insecticides as well and this sometimes leads to potentially useful interactions. Thus, enhanced detoxification of dicofol in spider mites can lead to enhanced chlorpyriphos activation, hence negative cross-resistance (Hatano *et al.*, 1992). Similarly, permethrin resistance in horn flies is suppressible by piperonyl butoxide and negatively related to diazinon toxicity (Cilek *et al.*, 1995). In *H. armigera*

Table 4 Desulfuration and oxidative ester cleavage of organophosphorus insecticides by P450 enzymes and microsomes

P450 enzyme	*Ratio of OP desulfuration/oxidative ester cleavage*	*Reference*
Human CYP2C19	8.5 d, 1.30 p, 0.14 c	Kappers *et al.* (2001), Tang *et al.* (2001), Mutch *et al.* (2003)
Human CYP3A4	3.0 d, 0.50 p, 0.66 c	Kappers *et al.* (2001), Mutch *et al.* (2003)
Human CYP2B6	0.7 d, 0.01 p, 3.38 c	Kappers *et al.* (2001), Mutch *et al.* (2003)
Human liver microsomes	0.29 d, 0.37 p, 0.57c	Kappers *et al.* (2001), Mutch *et al.* (2003)
Housefly CYP6A1	0.37 d	Sabourault *et al.* (2001)
Drosophila CYP6A2	0.92 d	Dunkov *et al.* (1997)
Housefly CYP12A1	0.69 d	Guzov *et al.* (1998)
Housefly CYP6D1	2.0 c	Hatano and Scott (1993)
Housefly *CSMA* microsomes	0.95 f	Ugaki *et al.* (1985)
Housefly *Akita-f*[a] microsomes	0.59 f	Ugaki *et al.* (1985)
Heliothis virescens microsomes	1.90 mp	Konno and Dauterman (1989)
Heliothis virescens NC-86[a] microsomes	1.32 mp	Konno and Dauterman (1989)

[a]Resistant strain.
d, diazinon; p, ethyl parathion; c, chlorpyriphos; f, fenitrothion; mp, methyl parathion.

populations from West Africa, triazophos shows negative cross-resistance with pyrethroids, and in this case the synergism shown by the OP towards the pyrethroids appears to be due to an enhanced activation to the oxon form (Martin *et al.*, 2003). These interactions were observed *in vivo* or with microsomes, but it is likely that they do reflect the properties of single P450 enzymes with broad substrate specificity rather than the fortuitous coordinate regulation of different P450 enzymes with distinct specificities.

Organophosphorus compounds such as disulfoton and fenthion can also be activated by thioether oxidation (formation of sulfoxide and sulfone), but it is not clear whether these reactions are catalyzed in insects by a P450 or by a flavin monooxygenase (FMO). Further examples of oxidative bioactivation of organophosphorus compounds have been discussed (Drabek and Neumann, 1985).

The toxicity of fipronil to house flies is increased sixfold by the synergist piperonyl butoxide, whereas the desulfinyl photodegradation product is not detoxified substantially by P450 (Hainzl and Casida, 1996; Hainzl *et al.*, 1998). Conversion of fipronil to its sulfone appears to be catalyzed by a P450 enzyme in *Ostrinia nubilalis* (Durham *et al.*, 2002) and in *Diabrotica virgifera* (Scharf *et al.*, 2000). In the latter, the toxicity of fipronil sulfone is about the same as that of the parent compound, and piperonyl butoxide has only a marginal effect as synergist. In contrast, synergists antagonize the toxicity of fipronil in *Blattella germanica*, suggesting that oxidation to the sulfone represents an activation step in this species (Valles *et al.*, 1997).

The now banned cyclodiene insecticides aldrin, heptachlor, and isodrin are epoxidized by P450 enzymes to environmentally stable, toxic epoxides, dieldrin, heptachlor epoxide, and endrin (Brooks, 1979; Drabek and Neumann, 1985). Recombinant CYP6A1, CYP6A2, and CYP12A1 can catalyze these epoxidations (see **Table 1**). Examples of proinsecticide metabolism include the activation of chlorfenapyr by *N*-dealkylation (Black *et al.*, 1994) and of diafenthiuron by *S*-oxidation (Kayser and Eilinger, 2001). In each case, the insect P450-dependent activation is a key in the selective toxicity of these proinsecticides that target mitochondrial respiration. Recombinant housefly CYP6A1 catalyzes the activation of chlorfenapyr (V.M. Guzov, M. Kao, B.C. Black, and R. Feyereisen, unpublished data). In *H. virescens*, toxicity of chlorfenapyr is negatively correlated with cypermethrin toxicity (Pimprale *et al.*, 1997). Genetic analysis indicates that a single factor is involved so the same P450 that activates chlorfenapyr may also detoxify cypermethrin in this species (**Figure 23**). A similar case of negative cross-resistance of chlorfenapyr in a pyrethroid-resistant strain has been reported in the hornfly *Haematobia irritans* (Sheppard and Joyce, 1998).

The metabolism of imidacloprid is also of interest in this respect. Although not extensively studied to date, there is evidence that piperonyl butoxide can synergize the toxicity of imidacloprid, but P450-dependent metabolism can also lead to several bioactive metabolites in some insects. How these are further metabolized and how resistance can be caused by P450 attack on this molecule remains unclear (see however Section 4.1.4.5.5).

In vivo synergism by piperonyl butoxide, a typical inhibitor of P450 enzymes (see Section 4.1.4.5.1), is often used to implicate a P450-mediated detoxification, and there are innumerable such examples in

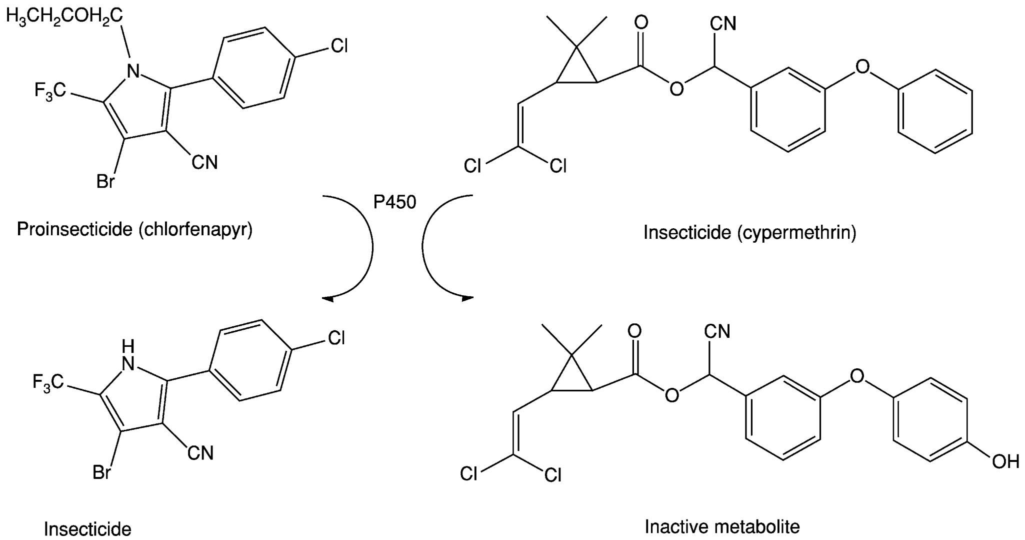


Figure 23 Chlorfenapyr and cypermethrin metabolism. The same P450 in *Heliothis virescens* probably activates the pyrrole and inactivates the pyrethroid, resulting in negative cross-resistance.

the literature. The inference is much stronger when two unrelated synergists are used *in vitro*, and when metabolites of the pesticide are identified. For instance, pyriproxifen is hydroxylated by fat body and midgut microsomes of larval house flies to 4′-OH-pyriproxyfen and 5″-OH-pyriproxyfen and these activities are inhibited by PB and TCPPE (Zhang *et al.*, 1998).

The study of xenobiotic metabolism by individual P450 enzymes expressed in heterologous systems has barely begun (**Table 1**). Whereas the CYP6 enzymes clearly comprise some enzymes with "broad and overlapping" substrate specificity, even closely related enzymes of this family can differ substantially in their catalytic competence. The task of predicting which xenobiotic or natural product will be metabolized by which type of P450 is currently not possible.

4.1.4.3. P450 and Host Plant Specialization

4.1.4.3.1. The Krieger hypothesis and beyond The interactions of plants and insects, and more specifically the role of plant chemistry on the specialization of phytophagous insects have generated a vast literature. "Secondary" plant substances are variously seen to regulate insect behavior and/or to serve as weapons in a coevolutionary "arms race" (Dethier, 1954; Fraenkel, 1959; Ehrlich and Raven, 1964; Jermy, 1984; Bernays and Graham, 1988). In chemical ecology alone, "no other area is quite so rife with theory" (Berenbaum, 1995). Many of the theories and some of the experiments implicitly or explicitly deal with the insect's ability to metabolize plant secondary substances by P450 and other enzymes. In the case of behavioral cues, we are far from understanding the true importance of P450 enzymes in the integration of chemosensory information, e.g., as "odorant degrading enzymes." In the case of detoxification, however, the landmark paper of Krieger *et al.* (1971) can be seen as echoing the Fraenkel (1959) paper, by exposing the *raison d'être* of P450 enzymes. They stated that "higher activities of midgut microsomal oxidase enzymes in polyphagous than in monophagous species indicates that the natural function of these enzymes is to detoxify natural insecticides present in the larval food plants." In that 1971 study, aldrin epoxidation was measured in gut homogenates of last instar larvae from 35 species of Lepidoptera. Polyphagous species had on average a 15 times higher activity than monophagous species. This trend was seen in sucking insects as well, with a 20-fold lower aldrin epoxidase activity in the oleander aphid *Aphis nerii* when compared to the potato aphid *Myzus euphorbiae* or to the green peach aphid *M. persicae* (Mullin, 1986). The former is a specialist feeder on two plant families, Asclepiadaceae and Apocyanaceae, whereas the latter two are generalists found on 30–72 plant families. The concept extended to other detoxification enzymes and was broadened to cover prey/predator, e.g., in mites where the predatory mite has a five times lower aldrin epoxidase activity than its herbivorous prey (Mullin *et al.*, 1982). The toxicity of the natural phototoxin α-terthienyl is inversely propotional to the level of its metabolism in Lepidoptera and is related to diet breadth. Metabolism (4.0 nmol/min/nmol P450) is highest in *O. nubilalis* that feeds on numerous

phototoxic Asteraceae, lower in *H. virescens* that has a broad diet, including some Asteraceae that are nonphototoxic, and lowest in *M. sexta,* a specialist of Solanaceae (Iyengar *et al.*, 1990).

The conceptual framework of Krieger *et al.* has been challenged (Gould, 1984) and defended (Ahmad, 1986). An alternative view (Berenbaum *et al.*, 1992) proposes that aldrin epoxidation represents "P450s with broad substrate specificity [that] are most abundant in insects that encounter a wide range of host plant metabolites." A careful repetition of the Krieger experiments on lepidopteran larvae from 58 species of New South Wales failed to show significant differences in aldrin epoxidation between monophagous and polyphagous species (Rose, 1985). High activity in both monophagous and polyphagous species was invariably linked to the presence of monoterpenes in the host diet. The evidence presented in the sections below indicates that polyphagous and oligophagous species alike rely on the ability to draw on a great diversity of P450 genes, encoding a great diversity of specific and less specific enzymes and regulated by a great diversity of environmental sensing mechanisms – induction. The ability to induce P450 enzymes and deal with a wide range of toxic chemicals in the diet has been thought to present a "metabolic load" for polyphagous species, with specialists restricting their "detoxification energy" to one or a few harmful substrates (e.g., Whittaker and Feeny, 1971). However, careful studies in both oligophagous and polyphagous species have refuted this concept of metabolic load (e.g., Neal, 1987; Appel and Martin, 1992).

4.1.4.3.2. Host plant chemistry and herbivore P450 Cactophilic *Drosophila* species from the Sonoran desert are specialized to specific columnar cactus hosts by their dependency on unusual sterols (*D. pachea*) or by their unique ability to detoxify their host's allelochemicals, notably isoquinoline alkaloids and triterpene glycosides (Frank and Fogleman, 1992; Fogleman *et al.*, 1998). P450-mediated detoxification was shown by the loss of larval viability in media that contained both allelochemicals and piperonyl butoxide, and by the induction of total P450 or alkaloid metabolism by the cactus allelochemicals or by phenobarbital (Frank and Fogleman, 1992; Fogleman *et al.*, 1998). Several P450s of the *CYP4*, *CYP6*, *CYP9*, and *CYP28* families are induced by cactus-derived isoquinoline alkaloids and by phenobarbital, but not by triterpene glycosides; only a *CYP9* gene was induced by alkaloids and not by phenobarbital (Danielson *et al.*, 1997, 1998; Fogleman *et al.*, 1998). The capacity to detoxify isoquinoline alkaloids was not related to DDT or propoxur tolerance, and while phenobarbital induced P450s capable of metabolizing the alkaloid carnegine in *D. melanogaster*, this was not sufficient to produce *in vivo* tolerance (Danielson *et al.*, 1995). Selection of *D. melanogaster* with Saguaro alkaloids over 16 generations, however, led to P450-mediated resistance to the cactus alkaloids (Fogleman, 2000). These studies suggest the evolution of specific responses in the cactophilic species involving the recruitment of a phylogenetically unrelated subset of P450 genes in each instance of specialization of a fly species on its host cactus.

The oligophagous tobacco hornworm (*M. sexta*) feeds essentially on Solanaceae and its adaptation to the high levels of insecticidal nicotine found in tobacco depends largely on metabolic detoxification, although other tolerance mechanisms may be contributing as well (Snyder and Glendinning, 1996). Hornworm larvae fed an nicotine-free artificial diet (naive insects) are rapidly poisoned by the ingestion of a nicotine-supplemented diet, but this diet is not deterrent. Poisoning is evidenced by convulsions and inhibition of feeding. The small amount of ingested nicotine induces its own metabolism, so that approximately 36 h later the larvae resume feeding normally, without further signs of poisoning. The inhibition of feeding and its resumption after nicotine exposure is directly related to P450 induction. Indeed, treatment with piperonyl butoxide, which itself has no effect on feeding, inhibits the increase in nicotine-diet consumption that occurs once nicotine metabolism has been induced (Snyder and Glendinning, 1996). Naive insects metabolize nicotine to nicotine 1-N-oxide at a low level, whereas nicotine-fed insects metabolize it further to cotinine-N-oxide at a higher level (Snyder *et al.*, 1994). These reactions are catalyzed by one or more P450 enzymes (Snyder *et al.*, 1993). The effects of nicotine on marker P450 activities are complex: the metabolism of three substrates is induced at low nicotine levels, seven are only induced at higher levels, and three are unaffected (Snyder *et al.*, 1993). *CYP4M1* and *CYP4M3* are moderately induced in the midgut but not in the fat body, but *CYP4M3* and *CYP9A2* are not affected by nicotine (Snyder *et al.*, 1995; Stevens *et al.*, 2000).

P450 induction has also been inferred in the polyphagous spider mite *Tetranychus urticae*, where the performance of a bean-adapted population on tomato was severely compromised by piperonyl butoxide (Agrawal *et al.*, 2002). The P450 inhibitor did not reduce acceptance of tomato as a host, nor did it reduce the performance of the bean-adapted population on bean, strongly suggesting a postingestive induction of P450 as a mechanism of acclimation

to the novel host. In the polyphagous noctuid *Spodoptera frugiperda*, ingestion of indole 3-carbinol increases once the continuous exposure to this toxic compound has induced P450 enzymes (Glendinning and Slansky, 1995).

4.1.4.3.3. *Papilio* species and furanocoumarins The adaptation of specialist herbivores to toxic components of their host plants is best documented in the genus *Papilio*. The black swallowtail, *P. polyxenes*, feeds on host plants from just two families, the Apiaceae (Umbelliferae) and the Rutaceae. These plants are phytochemically similar, particularly in their ability to synthesize furanocoumarins. Biogenetically derived from umbelliferone (7-hydroxycoumarin) the linear furanocoumarins (related to psoralen) and angular furanocoumarins (related to angelicin) are toxic to nonadapted herbivores (Berenbaum, 1990). This toxicity is enhanced by light as furanocoumarins are best known for their UV photoreactivity leading to adduct formation with macromolecules, particularly DNA. *Papilio polyxenes* has become a model in the study of adaptation to dietary furanocoumarins. Xanthotoxin, a linear furanocoumarin, induces its own metabolism in a dose-dependent fashion when added to the diet of *P. polyxenes* larvae (Cohen *et al.*, 1989). This P450-dependent metabolism proceeds probably by an initial epoxidation of the furan ring followed by further oxidative attack and opening of the ring, leading to nontoxic hydroxylated carboxylic acids (Ivie *et al.*, 1983; Bull *et al.*, 1986). Inducible xanthotoxin metabolism is observed in all leaf-feeding stages of *P. polyxenes*, and is higher in early instars (Harrison *et al.*, 2001). Xanthotoxin induces its own metabolism in the midgut, but also in the fat body and integument (Petersen *et al.*, 2001). The metabolism of bergapten and sphondin is also induced by dietary xanthotoxin. Levels of total midgut microsomal P450s are unaffected by xanthotoxin, and photoactivation is not required for induction (Cohen *et al.*, 1989). The metabolism of xanthotoxin is 10 times faster in *P. polyxenes* than in the nonadapted *S. frugiperda* (Bull *et al.*, 1986). *Papilio polyxenes* microsomal P450s are also less sensitive to the inhibitory effects of xanthotoxin. NADPH-dependent metabolism of xanthotoxin leads to an uncharacterized reactive metabolite that can covalently bind P450 or neighboring macromolecules, i.e., xanthotoxin can act as a "suicide substrate" (Neal and Wu, 1994). This NADPH-dependent covalent labeling of microsomal proteins is seven times higher in *M. sexta* than in *P. polyxenes*. Inhibition of aldrin epoxidation and *p*-nitroanisole *O*-demethylation by xanthotoxin is also 6- and 300-fold higher, respectively, in *M. sexta* than in *P. polyxenes* (Zumwalt and Neal, 1993). Myristicin, a methylene dioxyphenyl compound (see Section 4.1.3.4.4) found in the host plant parsnip is less inhibitory to *P. polyxenes* than to *H. zea* (Berenbaum and Neal, 1985; Neal and Berenbaum, 1989).

A distinct protein band of 55 kDa appears in midgut microsomes of xanthotoxin-treated *P. polyxenes* larvae and its microsequencing led to the cloning of *CYP6B1*, a P450 shown to be inducible by xanthotoxin or parsnip (Cohen *et al.*, 1992). Several variants of *CYP6B1* have been cloned that presumably represent different alleles, v1, v2, and v3. The three variants differ from each other at 3, 6, or 9 amino acid positions (Cohen *et al.*, 1992; Prapaipong *et al.*, 1994). The *CYP6B1* gene is selectively induced by linear furanocoumarins. Initial studies suggested that additional, related P450 transcripts were present in *P. polyxenes* and inducible by angular furanocoumarins (Hung *et al.*, 1995b). Expression in the baculovirus system revealed that CYP6B1 v1 and v2 metabolize the linear furanocoumarins bergapten, xanthotoxin, isopimpinellin, and psoralen (Ma *et al.*, 1994). Little metabolism of the angular furanocoumarin angelicin was observed in this early study but improvements in the heterologous expression system by coexpression of insect P450 reductase increased rates of metabolism sufficiently to confirm the role of CYP6B1 in the metabolism of angelicin as well (Wen *et al.*, 2003). The furanocoumarins were metabolized in the improved expression system with the following preference: xanthotoxin > psoralen > angelicin. The latter is less efficiently metabolized *in vivo* (Li *et al.*, 2003) and *P. polyxenes* is less adapted to it (Berenbaum and Feeny, 1981).

A second P450 was cloned from *P. polyxenes*; it encodes CYP6B3 that is 88% identical to CYP6B1 (Hung *et al.*, 1995a). *CYP6B3* is expressed at lower basal levels than *CYP6B1*, but both *CYP6B1* and *CYP6B3* are inducible by xanthotoxin, sphondin, angelicin, and bergapten in the midgut. *CYP6B3* responds more readily to the angular furanocoumarins than *CYP6B1* (Hung *et al.*, 1995a), and *CYP6B1* is more inducible than *CYP6B3* (Harrison *et al.*, 2001). A later study showed that *CYP6B1* is induced by xanthotoxin in the midgut, fat body, and integument, but *CYP6B3* is induced by xanthotoxin only in the fat body (Petersen *et al.*, 2001).

The presence of CYP6B-like transcripts in species related to *P. polyxenes* was suggested early on by positive signals on northern blots with RNA from *Papilio brevicauda* and *Papilio glaucus* that were treated with xanthotoxin (Cohen *et al.*, 1992). *Papilio brevicauda* is like *P. polyxenes*, a species

that feeds on furanocoumarin-containing Apiaceae, but *Papilio glaucus* is a generalist that encounters furanocoumarin-containing plants (e.g., hoptree, *Ptelea trifoliata*) only occasionally. Xanthotoxin, nevertheless, induces its own metabolism in all three species (Cohen *et al.*, 1992). *Papilio glaucus* is highly polyphagous and is reported to feed on over 34 plant families, and therefore offers an interesting contrast to *P. polyxenes*. Esterase, glutathione *S*-transferase, and P450 activities are highly variable and dependent on the species of deciduous tree foliage that this species feeds on (Lindroth, 1989). *Papilio glaucus* has significant levels of linear and angular furanocoumarin metabolism, that are highly inducible by xanthotoxin (Hung *et al.*, 1997). A series of nine *CYP6B* genes and some presumed allelic variants were cloned from *P. glaucus* (Hung *et al.*, 1996, 1997; Li *et al.*, 2001, 2002a). The first two genes *CYP6B4* and *CYP6B5* are products of a recent gene duplication event, and their promoter region is very similar (Hung *et al.*, 1996). Six additional and closely related members of the *CYP6B* subfamily were cloned from *Papilio canadensis*, another generalist closely related to *P. glaucus* but not known to feed on plants containing furanocoumarins (Li *et al.*, 2001, 2002a). Xanthotoxin induced *CYP6B4*-like and *CYP6B17*-like genes in both species, but the level of furanocoumarin metabolism was lower in *P. canadensis* (Li *et al.*, 2001). This wide spectrum of CYP6B enzymes represents a wide range of activities towards furanocoumarin substrates. Whereas CYP6B4 of *P. glaucus* expressed in the baculovirus system efficiently metabolizes these compounds (Hung *et al.*, 1997), CYP6B17 of *P. glaucus*, and CYP6B21 and CYP6B25 from *P. canadiensis* have a more modest catalytic capacity (Li *et al.*, 2003). *Papilio troilus*, a relative of *P. glaucus* that specializes on Lauraceae that lack furanocoumarins, has undetectable basal or induced xanthotoxin metabolism (Cohen *et al.*, 1992).

The genus *Papilio* thus offers a complete range of situations: (1) specialists that deal efficiently with furanocoumarins by inducible expression of *CYP6B* genes; (2) generalists that also carry related *CYP6B* genes, but whose inducibility and metabolism are less efficient; and (3) nonadapted specialists that appear to have lost the inducible *CYP6B* panoply (Berenbaum *et al.*, 1996; Berenbaum, 1999, 2002; Li *et al.*, 2003).

4.1.4.3.4. Furanocoumarins and other insects The metabolism of furanocoumarins or the inducibility of P450 by these compounds is not restricted to Papilionidae. Xanthotoxin induces its own metabolism in the parsnip webworm, *Depressaria pastinacella* (Nitao, 1989). This species belongs to the Oecophoridae, and is a specialist feeder on three genera of furanocoumarin-containing Apiaceae. It is highly tolerant to these compounds and metabolizes them not just by opening the furan ring, but in the case of sphondin, it is also capable of *O*-demethylation (Nitao *et al.*, 2003). Although furanocoumarin metabolism is inducible, the basal (uninduced) activity is high (Nitao, 1989), and the response is a general one, with little discrimination of the type of furanocoumarin inducer or the type of furanocoumarin metabolized (Cianfrogna *et al.*, 2002). The P450 enzymes involved in furanocoumarin metabolism by *D. pastinacella* are unknown. Low stringency northern hybridization failed to elicit a signal with a CYP6B1 probe (Cohen *et al.*, 1992).

A species that does not encounter furanocoumarins, the solanaceous oligophage *M. sexta*, responds to xanthotoxin by inducing *CYP9A4* and *CYP9A5* (Stevens *et al.*, 2000). The generalist *S. frugiperda* also induces P450 as well as glutathione *S*-transferases in response to xanthotoxin (Yu, 1984; Kirby and Ottea, 1995). It has low basal P450-mediated xanthotoxin metabolism, but this metabolism is inducible by a variety of compounds including terpenes and flavone (Yu, 1987). In the highly polyphagous *H. zea*, a similar situation is encountered. Xanthotoxin metabolism is low, but inducible by itself as well as by phenobarbital and α-cypermethrin (Li *et al.*, 2000b). A number of *CYP6B* genes have been cloned from these *Helicoverpa* species. *CYP6B8* of *H. zea* is very close in sequence to *CYP6B7* from *H. armigera*, and it is inducible by xanthotoxin and phenobarbital (Li *et al.*, 2000a). The high conservation of sequence in the SRS1 region suggests that the CYP6B enzymes of *Helicoverpa* are competent in furanocoumarin metabolism as indeed, these species occasionally encounter furanocoumarins in their diet. The *CYP6B9/B27* and *B8/B28* genes are pairs of recently duplicated genes (Li *et al.*, 2002b). Their tissue and developmental pattern of expression is subtly different as is their pattern of induction by a variety of chemicals (Li *et al.*, 2002c).

4.1.4.4. Host Plant, Induction and Pesticides

The adaptive plasticity conferred by the inducibility of P450 enzymes on different diets can have important consequences for insect control and the bionomics of pest insects. It is far from being just an ecological oddity or an interesting set of tales of insect natural history. It is well recognized that the same insect species fed different (host) plants will show differences in their response to pesticides

(Ahmad, 1986; Yu, 1986; Lindroth, 1991), and that these differences often reflect the induction of P450 enzymes, as well as of other enzymes, glutathione *S*-transferases, epoxide hydrolases, etc. The complexity of plant chemistry makes it difficult to account for the contribution of each individual chemical to this response and key components are often analyzed first (e.g., Moldenke *et al.*, 1992). Similarly, the multiplicity of P450 genes and the range of P450 enzyme specificity makes it difficult to predict the outcome of exposure to a plant chemical. The toxicological importance of the plant diet on the herbivore's P450 status (induction, inhibition) is well recognized in pharmacology where the joint use of chemical therapy and traditional herbs can have unpredicted outcomes (Zhou *et al.*, 2003).

Larvae of the European corn borer, *Ostrinia nubilalis*, fed leaves from corn varieties with increasing DIMBOA content and thus increasing levels of resistance to leaf damage had correspondingly increased levels of total midgut P450 and *p*-nitroanisole *O*-demethylation activity (Feng *et al.*, 1992). These studies suggest that constitutive host plant resistance may affect the insect response to xenobiotics. In addition, the induction of host plant defense by insect damage may itself be a signal for induction of herbivore P450 enzymes, as shown in *H. zea*. The plant defense signal molecules jasmonate and salicylate induce *CYP6B8*, *B9*, *B27*, and *B28* (Li *et al.*, 2002d) in both fat body and midgut. The response to salicylate is relatively specific as *p*-hydroxybenzoate, but not methylparaben, also acts as an inducer.

Treatment with 2-tridecanone, a toxic allelochemical from trichomes of wild tomato, protects *H. zea* larvae against carbaryl toxicity (Kennedy, 1984) and *H. virescens* larvae against diazinon toxicity (Riskallah *et al.*, 1986b). In *H. virescens* larvae, the compound caused both qualitative and quantitative changes in P450 spectral properties (Riskallah *et al.*, 1986a), an induction confirmed by its effect on specific P450 genes in the gut of *M. sexta* larvae (Snyder *et al.*, 1995; Stevens *et al.*, 2000). Larvae of *H. virescens* with a genetic resistance to 2-tridecanone have increased P450 levels and P450 marker activities (benzphetamine demethylation, benzo[a]-pyrene hydroxylation, phorate sulfoxidation), and these can be further increased by feeding 2-tridecanone (Rose *et al.*, 1991). A laboratory population of *H. zea* can rapidly display increased tolerance to α-cypermethrin by selection of an increased P450 detoxification ability with a high dose of dietary xanthotoxin (Li *et al.*, 2000b).

Beyond the host plant, it is the whole biotic and chemical environment that determines the response of an insect to pesticide exposure. Herbicides and insecticide solvents can serve as inducers (Brattsten and Wilkinson, 1977; Kao *et al.*, 1995; Miota *et al.*, 2000). Aquatic larvae are exposed to natural or anthropogenic compounds that alter their P450 detoxification profile (David *et al.*, 2000, 2002; Suwanchaichinda and Brattsten, 2002). Virus infection affects P450 levels (Brattsten, 1987), and the expression of several P450 genes is affected during the immune response (see Section 4.1.5.1.2).

4.1.4.5. Insecticide Resistance

4.1.4.5.1. Phenotype, genotype, and causal relationships Insecticide resistance is achieved in a selected strain or population by: (1) an alteration of the target site; (2) an alteration of the effective dose of insecticide that reaches the target; or (3) a combination of the two. The resistance phenotypes have long been analyzed according to these useful biochemical and physiological criteria. At the molecular genetic level, several classes of mutations can account for these phenotypes (Taylor and Feyereisen, 1996) and a causal relationship between a discrete mutation and resistance has been clearly established for several cases of target site resistance (ffrench-Constant *et al.*, 1999). The molecular mutations responsible for P450-mediated insecticide resistance are only beginning to be explored. In contrast to CYP51, which is a target for a major class of fungicides, no insect P450 has been recognized as a primary target for a commercial insecticide. Thus, biochemical changes in P450 structure or activity can lead to changes in insecticide sequestration, activation, or inactivation, so that all the classes of molecular mutations (structural, up- or downregulation, see Taylor and Feyereisen, 1996) can be theoretically involved in P450-mediated resistance. When the number of P450 genes is taken into account, it is little wonder that P450 enzymes are so often involved in insecticide resistance, and that it has been so difficult finding, and establishing, the role of resistance mutations for P450 genes.

Traditionally, the first line of evidence for a role of a P450 enzyme in resistance has been the use of an insecticide synergist (e.g., piperonyl butoxide), a suppression or decrease in the level of resistance by treatment with the synergist being diagnostic. In cases too many to list here, this initial and indirect evidence is probably correct, however there are cases where piperonyl butoxide synergism has not been explained by increased detoxification (Kennaugh *et al.*, 1993). Piperonyl butoxide may also be a poor inhibitor of the P450(s) responsible for resistance, so that the use of a second, unrelated synergist may be warranted (Brown *et al.*, 1996; Zhang *et al.*,

1997). In addition, the synergist as P450 inhibitor can decrease the activation of a proinsecticide, so that lack of resistance suppression can be misleading. Chlorpyriphos resistance in *D. melanogaster* from vineyards in Israel maps to the right arm of chromosome 2 (see Section 4.1.4.5.5) and is enhanced by piperonyl butoxide rather than suppressed (Ringo *et al.*, 1995).

An independent and additional line of evidence is the measurement of total P450 levels or metabolism of selected model substrates. An increase in either or both being viewed as diagnostic. Again, such evidence is tantalizing but indirect, and the absence of change uninformative. The validation of a model substrate for resistance studies requires substantial knowledge about the P450(s) involved, and is therefore best assessed a posteriori.

An increase in the metabolism of the insecticide itself in the resistant strain is more conclusive. For instance, permethrin metabolism to 4′-hydroxypermethrin was higher in microsomes from *Culex quinquefasciatus* larvae that are highly resistant to permethrin (Kasai *et al.*, 1998b) than in their susceptible counterparts. Total P450 and cytochrome b_5 levels were 2.5 times higher in the resistant strain. Both permethrin toxicity and metabolism were inhibited by two unrelated synergists, TCPPE and piperonyl butoxide. A similarly convincing approach was taken to show P450 involvement in the resistance of housefly larvae of the YPPF strain to pyriproxifen. Gut and fat body microsomes were shown to metabolize the IGR to 4′-OH-pyriproxyfen and 5″-OH-pyriproxyfen at higher rates than microsomes of the susceptible strains and this metabolism was synergist-suppressible (Zhang *et al.*, 1998). The major, dominant resistance factor was linked to chromosome 2 in that strain (Zhang *et al.*, 1997).

Increased levels of transcripts for one or more P450 genes in insecticide-resistant strains has now been reported in many cases (see **Table 5**). This suggests that overexpression of one or more P450 genes is a common phenomenon of metabolic resistance but does not by itself establish a causal relationship with resistance. In some cases, the increased mRNA levels have been related to increased transcription (Liu and Scott, 1998), or increased protein levels (Liu and Scott, 1998; Sabourault *et al.*, 2001). Genetic linkage between increased mRNA or protein levels for a particular P450 and resistance has been obtained to the chromosome level (*CYP6A1*, *Cyp6a2*, *Cyp6a8*, *CYP6D1*, *CYP9A1*, *CYP12A1*: Cariño *et al.*, 1994; Liu and Scott, 1996; Rose *et al.*, 1997; Guzov *et al.*, 1998; Maitra *et al.*, 2000), and closer to marker genes (*Cyp6g1*, *CYP6A1*: Daborn *et al.*, 2001; Sabourault *et al.*, 2001). Linkage is just the first step in establishing a causal link between a P450 gene and resistance.

The following is a discussion of specific cases of P450 genes associated with insecticide resistance that have been studied in greater detail. Evidence for mutations causing constitutive overexpression in *cis* and *trans*, as well as an example of point mutations in a P450 coding sequence are currently available. The variety of mechanisms, even in a single species in response to the same insecticide, is striking. The paucity of available data on the molecular definition of the resistant genotype and on its causal relationship to resistance is also striking when compared to the wealth of data on target site resistance (ffrench-Constant *et al.*, 1999).

4.1.4.5.2. CYP6A1 and diazinon resistance in the housefly Rutgers strain CYP6A1 was the first insect P450 cDNA to be cloned, and the gene was shown to be phenobarbital-inducible and constitutively overexpressed in the multiresistant Rutgers strain (Feyereisen *et al.*, 1989). A survey of 15 housefly strains (Cariño *et al.*, 1992) showed that *CYP6A1* is constitutively overexpressed at various degrees in eight resistant strains, but not in all resistant strains, notably R-Fc known to possess a P450-based resistance mechanism. Thus, the first survey with a P450 molecular probe confirmed the results of the first survey of housefly strains with marker P450 activities (aldrin epoxidation and naphthalene hydroxylation; Schonbrod *et al.*, 1968): there is no simple relationship between resistance and a molecular marker, here the level of expression of a single P450 gene. That different P450 genes would be involved in different cases of insecticide resistance was a sobering observation (Cariño *et al.*, 1992), even before the total number of P450 genes in an insect genome was known. The constitutive overexpression of *CYP6A1* was observed in larvae and in adults of both sexes. Overexpression was shown in both developmental stages to be linked to a semidominant factor on chromosome 2 (Cariño *et al.*, 1994), but the *CYP6A1* gene was mapped to chromosome 5 (Cohen *et al.*, 1994). The gene copy number being identical between Rutgers and a standard susceptible strain (*sbo*), gene amplification could not be invoked to explain overexpression (Cariño *et al.*, 1994), and the existence of a chromosome 2 *trans*-acting factor(s) differentially regulating *CYP6A1* expression in the Rutgers and *sbo* strains was implied. Competitive ELISA using purified recombinant CYP6A1 protein as standard showed that the elevated mRNA levels were indeed translated into elevated protein levels (Sabourault

Table 5 P450 overexpression in insecticide-resistant strains

P450 overexpressed	*Strain*	*Resistance pattern*	*Reference*
Musca domestica			
CYP6A1	Rutgers and other strains	OP, carbamates, IGR	Feyereisen *et al.* (1989), Cariño *et al.* (1992)
CYP6A1[a]	Rutgers		Sabourault *et al.* (2001)
CYP6D1	LPR	Pyrethroids	Liu and Scott (1996)
P450Lpr/CYP6D1[a]	LPR		Liu and Scott (1996)
CYP6D1/CYP6D1[a]	NG98, Georgia	Pyrethroids	Kasai and Scott (2000)
CYP6D1	YPER	Pyrethroids	Shono *et al.* (2002)
CYP6D3	LPR		Kasai and Scott (2001b)
CYP12A1	Rutgers		Guzov *et al.* (1998)
Drosophila melanogaster			
Cyp4e2	RaleighDDT	DDT	Amichot *et al.* (1994)
Cyp6a2	91R	DDT	Waters *et al.* (1992)
	RaleighDDT	DDT	Amichot *et al.* (1994)
	MHIII-D23	Malathion	Maitra *et al.* (2000)
CYP6A2[a]	Several strains	DDT, pyrethroids	Bride *et al.* (1997)
Cyp6a8	91R		Maitra *et al.* (1996)
	MHIII-D23		Maitra *et al.* (2000)
	Wis-1lab	DDT	Le Goff *et al.* (2003)
Cyp6g1	Hikone R and 20 strains	DDT	Daborn *et al.* (2001, 2002)
	WC2	Lufenuron, propoxur	Daborn *et al.* (2002)
	EMS1	Imidacloprid	Daborn *et al.* (2002)
	Wisconsin-1, 91-R	DDT	Brandt *et al.* (2002)
Cyp12d1/2	Wisconsin-1, 91-R	DDT	Brandt *et al.* (2002), Le Goff *et al.* (2003)
Drosophila simulans			
CYP6G1[b]	OV1	DDT, imidacloprid, malathion	Le Goff *et al.* (2003)
Heliothis virescens			
CYP9A1	Macon Ridge	Thiodicarb	Rose *et al.* (1997)
Helicoverpa armigera			
CYP4G8		Pyrethroids	Pittendrigh *et al.* (1997)
CYP6B7		Pyrethroids	Ranasinghe and Hobbs (1998)
Lygus lineolaris			
CYP6X1		Permethrin	Zhu and Snodgrass (2003)
Anopheles gambiae			
CYP6Z1	PSP	Pyrethroids	Nikou *et al.* (2003)
Culex quinquefasciatus			
CYP6F1	JPal-per	Pyrethroids	Kasai *et al.* (2000)
Culex pipiens pallens			
CYP4H21, H22, H23 CYP4J4, CYP4J6	RR	Deltamethrin	Shen *et al.* (2003)
Diabrotica virgifera			
CYP4		Me-parathion, carbaryl	Scharf *et al.* (2001)
Blattella germanica			
P450MA[a]		Chlorpyriphos	Scharf *et al.* (1999)

[a]P450 protein level increased.
[b]Presumed CYP6G1 ortholog of *D. simulans*.

et al., 2001; see Table 6 for comparison of Rutgers and *sbo*). A comparison of the coding sequence of CYP6A1 between Rutgers and two susceptible strains showed no (*sbo*) or little (*aabys*) sequence variation. Five amino acid changes were noted in aabys, two at the far N-terminus and three at the far C-terminus, well outside the regions (SRS) thought to influence enzyme activity (Cohen *et al.*, 1994). The lack of CYP6A1 sequence difference between Rutgers and *sbo* indicated that if CYP6A1 was implicated in diazinon resistance in the Rutgers strain, it was through elevation of enzyme activity alone. Reconstitution of recombinant CYP6A1 expressed in *E. coli* with its redox partners (Sabourault *et al.*, 2001) provided the conclusive evidence for its role in diazinon resistance, as CYP6A1 metabolizes the insecticide with a high turnover (18.7 pmol/pmol CYP6A1/min), and a

Table 6 Comparison of a susceptible and a resistant strain of the housefly

Fly strain	*sbo* (*S*)	*Rutgers (R)*
Diazinon contact toxicity: LC50 (μg/pint jar)	4.4	167.8
Resistance ratio	1	37.8
Diazinon topical toxicity: LD50 (μg/fly)	0.059[a]	7.1
Resistance ratio	1	120
P450 level (nmol/mg protein)	0.14	0.29
Aldrin epoxidation (pmol/min/pmol P450)	4.4[a]	15.6
CYP6A1 mRNA relative level	1	27.5
CYP12A1 mRNA relative level	1	15
CYP6A1 protein level (fmol/abdomen)	36	565
Diazinon metabolized by CYP6A1		
Oxidative cleavage (pmol/min/fly)	0.5	7.7
Desulfuration to oxon (pmol/min/fly)	0.2	2.9
OP oxon metabolized by mutant ali-esterase (pmol/min/fly)	0.0	2.2–2.5
NADPH-dep. diazinon metabolism by microsomes (pmol/min/abdomen)	2.9[a]	15.5

[a]NAIDM susceptible strain.

favorable ratio (2.7) between oxidative ester cleavage and desulfuration (see Section 4.1.4.2.2 and Table 4).

The nature of the chromosome 2 *trans*-acting factor and of the mutation leading to resistance in the Rutgers strain has long remained enigmatic despite considerable circumstantial evidence for a major resistance factor on chromosome 2 (Plapp, 1984). Diazinon resistance and high CYP6A1 protein levels could not be separated by recombination in the short distance between the *ar* and *car* genes (3.3–12.4 cM). This region carries an ali-esterase gene (*MdαE7*) implicated as its *Lucilia cuprina* ortholog in organophosphorus insecticide resistance (Newcomb *et al*., 1997; Claudianos *et al*., 1999). A Gly137 to Asp mutation in this ali-esterase abolishes carboxylesterase activity towards model compounds such as methylthiobutyrate, and confers a measurable phosphotriester hydrolase activity towards an organophosphate ("P=O"), chlorfenvinphos. Chromosome 2 of the Rutgers strain carries this Gly137 to Asp mutation, and low CYP6A1 protein levels are correlated with the presence of at least one wild-type (Gly137) allele of *MdαE7*. Recombination in the *ar-car* region could not dissociate diazinon susceptibility, low CYP6A1 protein level, and the presence of a Gly137 allele of the ali-esterase (Sabourault *et al*., 2001). It was therefore hypothesized that the wild-type ali-esterase metabolizes an (unknown) endogenous substrate into a negative regulator of *CYP6A1* transcription. Removal of this regulator (by loss-of-function of the ali-esterase) would increase CYP6A1 production and, hence, diazinon metabolism. Nature seems to have found the optimal loss-of-function mutation (Gly137 to Asp) as the Rutgers haplotype has swept through global populations of the housefly (C. Claudianos, J. Brownlie, V. Taskin, M. Kence, R.J. Russell, J.G. Oakeshott, personal communication). The mutant ali-esterase probably helps clearing the activated form (P=O) of the insecticide (Sabourault *et al*., 2001). The negative regulation by the Gly137 allele and the diazinon resistance-enhancing effect of the Asp137 allele may explain the incomplete dominance of the diazinon resistance trait. Housefly strains that are susceptible or that are not known/shown to overexpress *CYP6A1* predictably carry at least one Gly137 allele (Scott and Zhang, 2003). The LPR strain that overexpresses *CYP6A1* (Cariño *et al*., 1992) and has increased OP metabolism (Hatano and Scott, 1993), as well as another resistant strain (NG98) carry other alleles (Scott and Zhang, 2003) that have impaired ali-esterase activity, Trp251 to Ser or Leu (Campbell *et al*., 1998; Claudianos *et al*., 2001). Corroborating, but indirect evidence for the hypothesis is the predicted pleiotropic effect of a *trans*-acting regulator. Constitutive overexpression of *CYP12A1* whose product metabolizes diazinon as well (Guzov *et al*., 1998) and *GST-1* in the Rutgers strain are also controlled by a chromosome 2 factor, possibly the same as the one controlling *CYP6A1* expression.

Although alternative hypotheses can be advanced, such as the fortuitous genetic closeness between the ali-esterase and a factor that increases the level of CYP6A1, a diazinon metabolizing enzyme, such hypotheses do not have the benefit of elegance. The mutant ali-esterase cannot account for the carbamate and JHA resistance so is not the sole factor of resistance seen in many housefly strains. Diazinon-resistant *L. cuprina* have provided evidence for Oppenoorth's mutant ali-esterase hypothesis (Newcomb *et al*., 1997), but the role of P450 in OP resistance cannot be ignored. Indeed, the Q strain of the sheep blowfly is more resistant to parathion than to paraoxon (Hughes and Devonshire, 1982) and indirect evidence for a P450 involvement in *L. cuprina* diazinon resistance has also been presented (Kotze, 1995; Kotze and Sales, 1995).

4.1.4.5.3. *Cyp6a2* in *Drosophila:* overexpression and mutant enzyme Insecticide-resistant strains of *D. melanogaster* have been studied at the molecular genetic level and the *Cyp6a2* gene has been implicated in the metabolic resistance of several of

them. *Cyp6a2* is constitutively overexpressed in the 91R strain that is resistant to malathion and DDT by a factor of 20–30 relative to the susceptible 91C strain (Waters *et al*., 1992). DDT resistance maps to 56 cM on the left arm of chromosome 2 in the 91R strain (Dapkus, 1992), which is at or near the chromosomal location of *Cyp6a2* (43A1-2). Initially, the presence of a 96 bp insertion in the 3′ UTR of the gene was proposed to confer a low level of expression to the *Cyp6a2* gene (Waters *et al*., 1992), but this insertion (or rather, the lack of it) was neither correlated with DDT resistance (Delpuech *et al*., 1993) nor confirmed to be linked to overexpression in resistant strains (Dombrowski *et al*., 1998). *Cyp6a8* is also constitutively overexpressed in the 91R strain (Maitra *et al*., 1996) and the expression of both *Cyp6a2* and *Cyp6a8* is repressed in 91R/91C hybrids (Maitra *et al*., 1996; Dombrowski *et al*., 1998; Maitra *et al*., 2000). Flies ($rosy^{506}$, insecticide susceptible and with constitutively low expression of *Cyp6a2*) were transformed with a P-element carrying the *Cyp6a2* gene of the 91R strain driver by its own promoter. These flies express the transgene at higher levels than the endogenous *Cyp6a2* but at lower levels than in their native 91R background (Dombrowski *et al*., 1998). The expression of both *Cyp6a2* and *Cyp6a8* is also constitutively higher in the MHIII-D23 strain initially selected for malathion resistance (Maitra *et al*., 2000). Genetic crosses and chromosome substitution experiments conclusively showed that the expression of both genes (located on the 2R chromosome) is repressed by factors on the third chromosome of the insecticide-susceptible 91C and $rosy^{506}$ strains. In contrast, the third chromosome of the MHIII-D23 and 91R strains carries a loss-of-function mutation for this negative *trans* regulator, allowing the constitutive overexpression of the two genes (Maitra *et al*., 2000). Further careful examination of the promoter activity of the *Cyp6a8* gene by fusion with a luciferase reporter gene in transgenic flies identified a −11/−761 bp region of the *Cyp6a8* gene of the 91R strain that was sufficient to respond to the negative regulation by the $rosy^{506}$ (wild-type) *trans* acting factor (Maitra *et al*., 2002). CYP6A2, similar to CYP6A1, appears to have a broad substrate specificity (see **Table 1**). Whether CYP6A2 of the 91R strain is capable of metabolizing DDT is unknown, as its sequence differs from that of the baculovirus produced CYP6A2 from the *iso-1* strain, which does not metabolize DDT, and from that of the Raleigh-DDT strain, which does (see below). Nonetheless, the studies with the 91R and MHIII-D23 strains are clear indications for loss-of-function mutations in gene(s) encoding negative regulators of P450 gene expression on chromosome 3. Genetic analyses of malathion resistance and of P450 expression (electrophoretic bands and marker activities) (Hallstrom, 1985; Hallstrom and Blanck, 1985; Houpt *et al*., 1988; Waters and Nix, 1988) have pointed to one or more loci between 51 and 61 cM on the right arm of chromosome 3, and it is interesting that this chromosome arm is thought to be orthologous to chromosome 2 of the housefly (Weller and Foster, 1993). The *Drosophila* ortholog of the *MdαE7* gene (*Est23*) is located at 84D9.

Resistance to DDT in the RaleighDDT strain offers a different picture. This strain has very high DDT resistance (>10 000-fold; Cuany *et al*., 1990). Its piperonyl butoxide-suppressible resistance is polyfactorial but the major, dominant resistance factor maps to 55 cM on the second chromosome as in the 91R strain. At least two P450 genes, *Cyp6a2* and *Cyp4e2*, are constitutively overexpressed in this strain (Amichot *et al*., 1994). The genetic localization of resistance matches the locus of *Cyp6a2* (A. Brun-Barale, S. Tares, J.M. Bride, A. Cuary, J.B. Bergé, M. Amichot, personal communication), and the RaleighDDT allele was sequenced. Three point mutations, Arg335 to Ser, Leu336 to Val, and Val476 to Leu, were found (Bergé *et al*., 1998). Overexpression was separated from the point mutations by repeated backcrossing to a marked susceptible strain and DDT selection. The resulting strain called 152 retained the mutant *Cyp6a2* gene and high monofactorial resistance to DDT (>1000-fold), which also maps to 54.4 cM. Both RaleighDDT and 152 flies are characterized by elevated *in vitro* DDT metabolism, as well as elevated ethoxycoumarin and ethoxyresorufin *O*-deethylase activities. Strain 152 lost the constitutive expression of *Cyp6a2*, which was therefore caused by an unlinked *trans*-acting factor (A. Brun-Barale, S. Tares, J.M. Bride, A. Cuary, J.B. Bergé, M. Amichot, personal communication).

The wild-type CYP6A2 and five engineered versions (each individual mutation, a double mutant carrying the first two and a triple mutant corresponding to the RaleighDDT allele) were expressed in *E. coli* and assayed for activity. The mutant enzymes were all characterized by a decreased stability when compared to the wild-type enzyme. The enzyme production in *E.coli* was significantly lower and the ratio of holoenzyme produced (measured by the Fe^{II}–CO complex) to apoenzyme produced (measured with an anti-CYP6A2 antibody) was also lower. However, the triple mutant was uniquely capable of metabolizing DDT to DDA, DDD, and dicofol at rates that were

9–13 times higher than the wild-type enzyme whose DDT metabolism was barely measurable. In addition to the triple mutant, only the Arg335 to Ser single mutant had the capacity to hydroxylate DDT to dicofol at a rate significantly different from wild-type. In contrast to DDT metabolism, the 16β hydroxylation of testosterone was not affected in the various single and multiple mutants. Thus, the three mutations found in *Cyp6a2* of the RaleighDDT (and its derivative 152) strain collectively confer DDT-metabolizing ability to the mutant CYP6A2 enzyme. Genetic localization of DDT resistance to the *Cyp6a2* gene locus is therefore explained. When the point mutations of the 152 strain are combined with overexpression as in the parent RaleighDDT strain, the very high level of resistance can be rationalized. This is the first example of point mutations in an insect P450 enzyme that contribute to insecticide resistance.

4.1.4.5.4. CYP6D1, the housefly LPR strain, and pyrethroid resistance The LPR strain of the housefly is highly resistant to pyrethroids (see **Chapter 6.1**) with a phenoxybenzyl moiety. This permethrin-selected strain has several resistance mechanisms, with P450-based detoxification as a major contributor. An abundant form of P450 (P450Lpr) was purified from abdomens of adult LPR flies, and immunological data indicated that P450Lpr represents 67% of the P450 in microsomes from LPR flies, a tenfold (Wheelock and Scott, 1990) increase over the reference-susceptible strain. Peptide sequences from the purified protein allowed PCR amplification and cloning of the P450Lpr gene, *CYP6D1* (Tomita and Scott, 1995). This gene is located on chromosome 1 of the housefly (Liu *et al.*, 1995), and is constitutively overexpressed by about tenfold in the LPR strain. This overexpression is not caused by gene amplification, but by increased transcription. It has been claimed that increased transcription of *CYP6D1* causes insecticide resistance (Liu and Scott, 1998), but transgenic expression of *CYP6D1* in *Drosophila* (Korytko *et al.*, 2000a) has not been reported to confer resistance, and CYP6D1 produced in yeast (Smith and Scott, 1997) has not been reported to metabolize pyrethroids. Instead, the evidence for the role of CYP6D1 in pyrethroid resistance is based on the inhibition of microsomal deltamethrin and cypermethrin metabolism by anti-P450Lpr antibodies (Wheelock and Scott, 1992b; Korytko and Scott, 1998). The metabolism of pyrethroids by CYP6D1 has been studied in its microsomal environment of the LPR strain. Deltamethrin is metabolized (<0.5pmol/min/pmol CYP6D1) preferentially at the gem-dimethyl group on the acid moiety (Wheelock and Scott, 1992b) whereas cypermethrin is mainly hydroxylated at the 4′ position on the alcohol moiety, at a very low rate (4.17 pmol/min/pmol CYP6D1, or less than 1 CYP6D1 turnover per 2 h) (Zhang and Scott, 1996b). Whether overexpression or point mutations of *CYP6D1* or both (as for *Cyp6a2* of *Drosophila* RaleighDDT) are involved in pyrethroid resistance in the LPR strain is yet unknown. Indeed, the *CYP6D1* gene sequence from 5 strains shows a high polymorphism, with 57 variable sites in the coding region alone, of which 12 are nonsilent (Tomita *et al.*, 1995). Six amino acid changes are specific to the LPR strain (CYP6D1v1) when compared to pyrethroid-susceptible strains and several of these mutations appear to align with SRS3.

The characterization of the resistance mutation(s) is made difficult by its almost completely recessive nature, and by the important contribution of the genes *pen* (for reduced penetration) and *kdr* (for target site resistance) of chromosome 3 (Liu and Scott, 1995). The absence of this resistant chromosome 3 decreases permethrin resistance from 16 000-fold to 170-fold. The remaining resistance is entirely suppressible by piperonyl butoxide, and is conferred by a combination of the resistant chromosomes 1 and 2 from the parent LPR strain in the homozygous condition (Liu and Scott, 1995). There is no substantial resistance or CYP6D1 overproduction conferred by isolated LPR chromosomes 1 or 2, or by their subsequent combination (Liu and Scott, 1995, 1996). Thus, permethrin selection of a P450-mediated resistance requires both copies of the LPR chromosomes 1 and 2. The resistance and *CYP6D1* overexpression linked to chromosome 1 are dominant, whereas the contributions of chromosome 2 are mostly recessive (Liu and Scott, 1996, 1997a). Furthermore, cytochrome b_5 is essential for CYP6D1-metabolism of cypermethrin (Zhang and Scott, 1996b), and the overexpression of cytochrome b_5 in LPR maps to the same chromosomes as permethrin resistance and *CYP6D1* overexpression (Liu and Scott, 1996). These data suggest a unique combination in the LPR strain of chromosome 2 *trans*-acting factor(s) with at least a matched *cis*-factor on chromosome 1. Sequence differences between the 5′UTR of the *CYP6D1* gene of LPR and of other strains have been documented (Scott *et al.*, 1999).

CYP6D1 is also overexpressed, but cytochrome b_5 is not, in the permethrin-resistant strain NG98 from Georgia, USA (Kasai and Scott, 2000). These permethrin-resistant flies from Georgia carry virtually the same *CYP6D1v1* haplotype as LPR, with

only 2 nt changes in introns in a sequence spanning from 658 nt upstream of the ATG to the stop codon (Seifert and Scott, 2002). However, in a strain (ALHF) from neighboring Alabama with high permethrin resistance, chromosomes 1 and 2 play little role but chromosome 5 plays a major piperonyl butoxide-suppressible role (Liu and Yue, 2001). *CYP6D1* is overexpressed by about 2.4-fold and cytochrome b_5 by about 1.5-fold in the Japanese strain YPER (Shono *et al*., 2002). This strain does not carry the *CYP6D1v1* allele, and chromosome 2 has a major role in this permethrin-resistant strain. Permethrin resistance thus appears largely recessive in several housefly strains, with multiple mutations, both regulatory and structural, potentially playing a role in the P450 contribution to the multifactorial resistance.

A gene closely linked to *CYP6D1* on chromosome 1 codes for a similar (78% identity) P450, CYP6D3 (Kasai and Scott, 2001a). It is 12-fold overexpressed in adult flies of the LPR strain, but it is also expressed in larvae (Kasai and Scott, 2001b), as opposed to *CYP6D1*, which has an adult-specific pattern of expression.

4.1.4.5.5. *Drosophila Cyp6g1*, the *Rst(2)DDT* gene at 64.5 cM on chromosome 2 The power of *Drosophila* genetics coupled with the tools made possible by the complete genome sequence has provided the most detailed, yet complex molecular genetic detail about a P450-based insecticide resistance mechanism. The resistance gene *Rst(2)DDT* has been genetically characterized for over 40 years (reviews: Daborn *et al*., 2001; Wilson, 2001). The position of this gene around 64.5 cM on the left arm of chromosome 2 has become almost mythical, as a number of phenotypes were linked to this locus, from dominant DDT resistance to phenylthiourea susceptibility, from organophosphorus to carbamate resistance, from various P450-dependent activities to vinyl chloride activation. EMS mutagenesis of a wild-type stock and selection with imidacloprid led to two strains with moderate imidacloprid resistance and moderate cross-resistance to DDT (Daborn *et al*., 2001). Conversely, two DDT-resistant strains (Hikone-R and Wisconsin-1) were shown to be cross-resistant to imidacloprid. Fine scale mapping of this dominant resistance localized *Rst(2)DDT* to a region from 48D5-6 to 48F3-6 on the polytene chromosome map. Of three candidate P450 genes in this region, *Cyp6g1*, *Cyp6g2*, and *Cyp6t3*, only the first one showed constitutive overexpression in the DDT- and imidacloprid-resistant strains tested (Daborn *et al*., 2001). A DNA microarray comprising probes for all the *Drosophila* P450 genes was addressed with target cDNAs from susceptible strains and from the DDT-resistant Hikone-R strain and the propoxur-resistant WC2 strain. In both cases, *Cyp6g1* was the only P450 gene showing constitutive overexpression (Daborn *et al*., 2002). Microarray data thus offer, at the transcriptional level, a complete analysis of all P450 genes, thereby facilitating the further rational study of one or more P450 genes (**Figure 24**). This is in constrast to the classical approaches that address single genes simply because they are the only ones for which a molecular probe is available. Overexpression of *Cyp6g1* was confirmed by quantitative (RT)PCR in 20 strains, and DDT, imidacloprid, nitenpyram, and lufenuron resistances were all independently mapped to the *Cyp6g1* locus in the Hikone-R and WC2 strains. The insertion of a terminal direct repeat of the transposable element *Accord* was systematically found in the 5′ UTR of 20 different resistant strains from across the globe. Phylogenetic analysis of the first intron sequence of the gene showed a unique haplotype in resistant strains versus a large diversity of susceptible haplotypes, suggesting that a selective sweep had occurred in global *Drosophila* populations (Daborn *et al*., 2002). Transgenic flies producing CYP6G1 under control of a heatshock promoter in the GAL4/UAS system showed both increased transcription of *Cyp6g1* and survival to a discriminating dose of DDT after heatshock. Similarly, transgenic expression under the tubulin promoter showed overexpression of only the *Cyp6g1* gene, and larval survival to discriminating doses of acetamiprid, imidacloprid, and nitenpyram (Le Goff *et al*., 2003). *Cyp6g1* overexpression was confirmed in the DDT-resistant Wisconsin and 91R strains, and the adjacent *Cyp12d1* (or *Cyp12d2*) gene is overexpressed, as well as DDT-inducible in both strains (Brandt *et al*., 2002). In the Wisconsin-1 strain, *Cyp6g1* and *Cyp12d1/2* are the only P450 genes that are overexpressed, whereas in the Wis1lab strain, in which DDT selection was applied after genetic removal of the *Cyp6g1* region, only *Cyp6a8* is overexpressed (Le Goff *et al*., 2003). In all the strains discussed above, DDT resistance is significant but low compared to the RaleighDDT strain suggesting that while *Cyp6g1* (overexpressed in the RaleighDDT strain; S. Tares, personal communication) may constitute a first line of defense seen in field populations, further insecticide pressure in the laboratory may select additional mechanisms.

In a Brazilian strain of *D. simulans* resistant to DDT, imidacloprid, and malathion, only the *Cyp6g1* probable ortholog is overexpressed (Le Goff *et al*., 2003). In a California population of

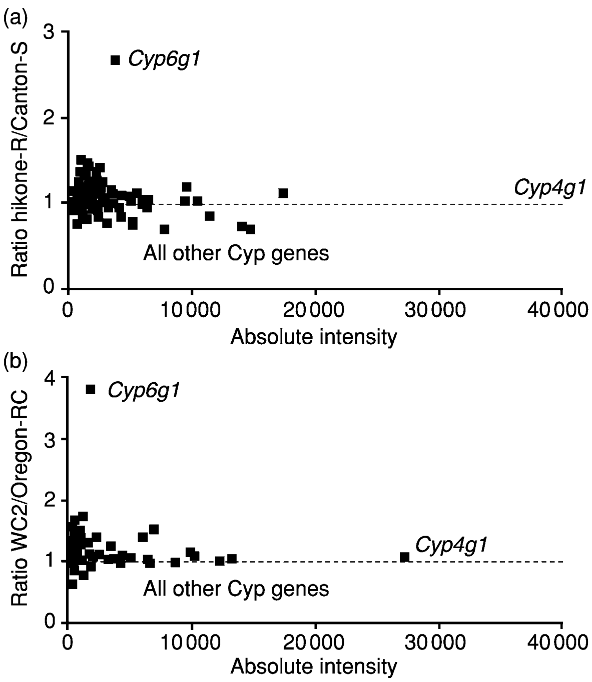


Figure 24 *Cyp6g1* overexpression in two insecticide-resistant strains, Hikone-R(a) and WC2(b), of *Drosophila melanogaster*. DNA microarray analysis revealed overexpression of just one P450 gene. (Reproduced with permission from Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., *et al.*, **2002**. A single P450 allele associated with insecticide resistance in *Drosophila*. *Science 297*, 2253–2256; © AAAS.)

D. simulans the 5′-flanking sequence of the *Cyp6g1* ortholog is nearly fixed for a *Doc* transposable element insertion. This insertion is absent from African populations and is associated with increased transcript abundance of *Cyp6g1* and resistance in a remarkable analogy with the Accord case of *D. melanogaster Cyp6g1* (Schlenke and Begun, 2004).

4.1.4.5.6. P450-mediated resistance: evolution Resistance-conferring mutations in insecticide target sites (Rdl, AchE, sodium channel) have a remarkable pattern of orthology in widely divergent species. The hypothesis that a few paralogous, perhaps orthologous P450 genes would repeatedly be found to be responsible for metabolic resistance in various insect species has not been confirmed, beyond the single example of *Cyp6g1* in *D. melanogaster* and *D. simulans* (that are only 2.5 million years apart). Furthermore, when resistance due to constitutive overexpression of a P450 is caused by a mutation is *trans*, the effect can be pleiotropic with more than one P450 gene from more than one family being overexpressed (e.g., *CYP12A1* and *CYP6A1*). In Diptera as well as in Lepidoptera, members of the *CYP4* family also appear to be involved in resistance, and there is evidence for involvement of *CYP9* genes in the latter (Rose *et al.*, 1997). Therefore, an alternative hypothesis is that the multitude of P450 genes, whose expression is inducible and therefore not strongly expressed in most developmental stages/tissues, constitute a "reservoir" in which mutations affecting expression levels can be selected by insecticide exposure. In the field, this can lead to selective "sweeps" of these most adapted mutations as seen for the global predominance of the Rutgers and *Cyp6g1*/Accord haplotypes in house flies and fruit flies. These mutations would typically be loss-of-function mutations, which inactivate the fine level regulation of expression and therefore increase overall expression of a random P450 gene. If its product happens to metabolize the insecticide, even marginally, this may constitute a selective advantage for the organism. Loss-of-function mutations in the large target of negative regulatory sequences are predicted to be more frequent than gain-of-function mutations (in the smaller open reading frame) that would improve the catalytic efficiency of a P450 enzyme towards the insecticide (Taylor and Feyereisen, 1996). However, polymorphisms in the P450 sequence may be less detrimental to fitness than (often pleiotropic) changes in expression (see, e.g., Halpern and Morton, 1987), so that more examples of point mutations in P450 coding sequences are likely to emerge. The study of insecticide resistance has

clearly entered the age of genomics (Oakeshott *et al*., 2003) and much progress is expected in the coming years.

4.1.5. Regulation of P450 Gene Expression

4.1.5.1. Spatial and Temporal Patterns of P450 Gene Expression

4.1.5.1.1. Enzyme activities and P450 expression P450-dependent enzyme activities had been detected in virtually all insect tissues and developmental stages studied as early as the first edition of this series (Hodgson, 1985). Many studies have continued to document these patterns and changes (e.g., Feyereisen and Farnsworth, 1985; Ahmad, 1986; Gunderson *et al*., 1986; Scott and Lee, 1993a). With molecular probes, the expression patterns of individual P450 genes have now been presented in different tissues, developmental stages or induction regimes with varying degrees of detail, and with probes of varying degree of specificity. Early studies relied on northern blots and dilution dot-blots for quantification (e.g., Cariño *et al*., 1992, 1994), with later ones relying on the highly specific ribonuclease protection assays (e.g., Hung *et al*., 1995b; Sutherland *et al*., 1998). **Table 7** shows a summary of those studies reporting a specific pattern of expression. The many studies in which the expression of a particular P450 was studied episodically or by simply rounding up the usual suspects (midgut, fat body, etc.) are not specifically listed.

In situ mRNA hybridization for the *Cyp6a2* gene has been used in *Drosophila* to show that in adults treated with phenobarbital, this gene is transcribed in the midgut, the pericuticular fat bodies, and the Malpighian tubules (Brun *et al*., 1996). *Cyp6a2* expression is seen in a large number of tissues by immunohistochemistry (Saner *et al*., 1996), and peaks in the third larval and pupal stages as shown by northern blots. The *Cyp6a2* promoter linked to a GFP reporter gene has been used in transgenic flies to document the expression of this gene (G.C. Unnithan, unpublished data). *In situ* hybridization has been used to document the expression patterns of *sad*, *dib*, and *shd* (Warren *et al*., 2002; Petryk *et al*., 2003). Systematic surveys (Tomancak *et al*., 2002) will eventually document the expression patterns of all P450 genes.

Many unanswered questions remain about P450 expression patterns. For instance, sublethal infection by cytoplasmic polyhedrosis viruses (see **Chapter 6.10**) in *H. virescens*, *M. sexta*, and *S. frugiperda* depresses P450 levels and P450-dependent activities, and increases the toxicity of insecticides (Brattsten, 1987). What are the mechanisms, the P450 genes involved and the significance of these observations? Changes in P450 levels are observed after a blood meal in *Culex pipiens* (Baldridge and Feyereisen,

Table 7 Specific spatial or temporal expression of P450 genes

P450	*Species*	*Tissue/comments*	*Reference*
CYP4C7	*Diploptera punctata*	Corpora allata	Sutherland *et al*. (1998)
CYP4G15	*Drosophila melanogaster*	Larval brain	Maibeche-Coisne *et al*. (2000)
CYP4D21	*Drosophila melanogaster*	Head fat cells in males under control of *transformer* and *doublesex*	Fujii and Amrein (2002)
CYP4L4	*Mamestra brassicae*	Antennae, proboscis, legs	Maibeche-Coisne *et al*. (2002)
CYP4S4	*Mamestra brassicae*	Antennae	Maibeche-Coisne *et al*. (2002)
CYP6B2	*Helicoverpa armigera*	Larval specific	Ranasinghe *et al*. (1997)
CYP6D1	*Musca domestica*	Adult specific	Scott *et al*. (1996)
CYP6D1	*Musca domestica*	Thoracic ganglia	Korytko and Scott (1998)
CYP6L1	*Blattella germanica*	Adult male (probably accessory glands, may be testes as well)	Wen and Scott (2001a)
CYP6Z1	*Anopheles gambiae*	Adult specific	Nikou *et al*. (2003)
CYP15A1	*Diploptera punctata*	Corpora allata	Helvig *et al*. (2004)
CYP302A1	*Drosophila melanogaster*	(Embryo) prothoracic gland portion of the ring gland, follicle cells	Chavez *et al*. (2000); Warren *et al*. (2002)
CYP310A1	*Drosophila melanogaster*	Overexpressed in embryos of Toll1oB mutants (Dorsal target in the mesoderm)	Stathopoulos *et al*. (2002)
CYP312A1	*Drosophila melanogaster*	Male specific	Kasai and Tomita (2003)
CYP314A1	*Drosophila melanogaster*	(Embryo) ovary, gut, Malpighian tubules, fat body	Petryk *et al*. (2003)
CYP315A1	*Drosophila melanogaster*	(Embryo) prothoracic gland portion of the ring gland, follicle cells, nurse cells	Warren *et al*. (2002)
CYP315A1	*Drosophila melanogaster*	One of 30 significantly downregulated genes in eyes overexpressing TIGR/MYOC	Borras *et al*. (2003)

1986), but too little is known of P450 expression patterns in blood-feeding or phloem-feeding insects. What is the significance of the patterns of expression seen in *Drosophila* where about 20% of the genes are in groups of 10–30 genes spread over 20–200 kb that are coordinately expressed (Spellman and Rubin, 2002). Are the P450s present in some of these 200 groups, for instance the *CYP6A* cluster at 55A on chromosome 2R (Ueda *et al*., 2002), functionally linked?

4.1.5.1.2. DNA microarrays and P450 gene expression The genomic approach to biology has transformed the study of genes one by one to a study of very large numbers of genes in parallel, and ultimately to the study of the whole transcriptome of an organism (see **Chapters 4.12** and **4.13**). This approach, using tools such as DNA microarrays and SAGE, will rapidly change our understanding of the role of P450 in the life of insects. The interpretation of transcriptome studies is in its infancy and "data mining" will require a considerable interplay of experimental design and *in silico* analysis. In the case of P450 genes, the initial EST-based DNA microarrays (e.g., White *et al*., 1999; Arbeitman *et al*., 2002) were biased by the poor representation of P450 genes in the early EST collections (Tijet *et al*., 2001). Careful attention to the potential for cross hybridization between closely related genes is needed (Xu *et al*., 2001), but the design and validation of thematic arrays (e.g., with all the P450 genes) can allay this concern. The massive amount of data can reveal a link between a P450 gene and a physiological response; it is easy to overstate the relationship that may be a remote effect in a cascade of events. Genomic physiology has nonetheless provided much descriptive information on P450 gene expression already, and a few *Drosophila* examples are listed here. An exhaustive, P450-focused study will be possible in the near future, so these examples are just an exciting preview.

The earliest developmental study in which 534 sequences showed developmental variations (White *et al*., 1999, Arbeitman *et al*., 2002) revealed four genes repressed by premature expression of the ecdysone-inducible DHR3 nuclear receptor (*Cyp12a2, Cyp6a2, Cyp4d2*, and *Cyp4ad1*). The link between *Cyp6a2* and the ecdysteroid cascade confirms earlier experimental (Spiegelman *et al*., 1997) and *in silico* (Dunkov *et al*., 1997; Dombrowski *et al*., 1998) data. Two other genes, *Cyp28d1* and *Cyp9f2*, were repressed at the onset of metamorphosis (White *et al*., 1999). *Cyp310a1* was overexpressed in Toll10B mutant embryos (Furlong *et al*., 2001), identified as a Dorsal target (Stathopoulos *et al*., 2002) and its pattern of expression confirmed in a high-throughput *in situ* hybridization project (Tomancak *et al*., 2002). The Toll pathway represses seven P450 genes (*Cyp316a1, Cyp4ac1, Cyp6g1, Cyp18a1, Cyp28d1, Cyp6w1*, and *Cyp4d14*) in response to bacterial infection in adults, while *Cyp4e2* is also repressed in the acute response (De Gregorio *et al*., 2001, 2002). Profiles of circadian rhythms (see **Chapter 4.11**) have revealed the coregulation of six *Cyp6a* genes that are adjacent in a cluster at 51D5 on chromosome 2R (Ueda *et al*., 2002) as well as the control of *Cyp4e2* expression by the *Clk* gene. A number of other P450 genes including *Cyp18a1* and *Cyp4p1* are also cycling with different phases (Claridge-Chang *et al*., 2001; McDonald and Rosbash, 2001), with *Cyp4d21, Cyp6a21*, and *Cyp304a1* identified in both studies (**Figure 25**). Aging and oxidative stress (paraquat treatment) have revealed the responsiveness of a number of P450 genes, with *Cyp6a17* and *Cyp28a5* upregulated by paraquat and *Cyp6g1*

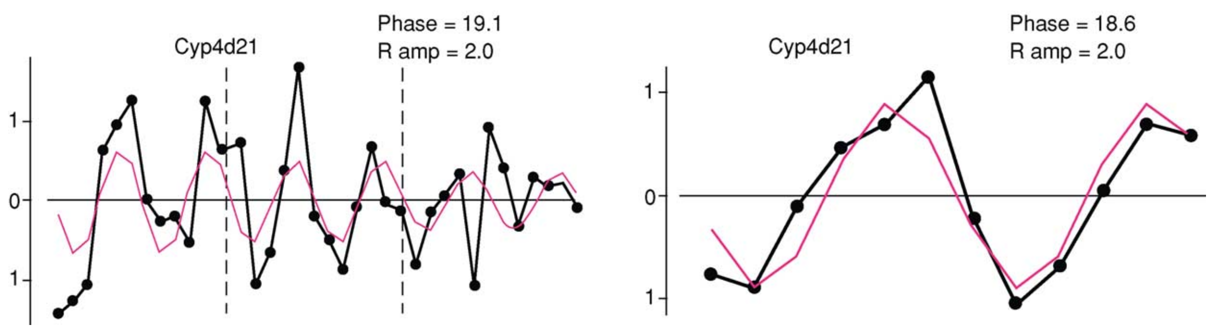


Figure 25 Circadian rhythmicity of *Cyp4d21* expression in *Drosophila melanogaster* expressed as a log ratio of change. Left: 36 time points collected over 6 days (Affymetrix microarray data); right: 12 time points collected over 2 days. Estimated phases and log ratio amplitudes are indicated. Red lines are 24-h guidelines. (Reprinted with permission from Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., *et al*., **2001**. Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron.* *32*, 657–671; © Elsevier.)

downregulated under those conditions (Zou *et al.*, 2000). In another study, 18 P450 genes were found to respond to paraquat treatment by either increased or decreased expression. Those increased by paraquat treatment were also increased by H_2O_2 treatment. Four genes are increased by treatment with tunicamycin that blocks N-glycosylation and thus causes a stress at the level of the endoplasmic reticulum (ER). *Cyp28a5* is strongly induced by the three treatments, suggesting it may be a general stress-responsive gene (F. Girardot, V. Monnier, and H. Tricoire, personal communication). The subtle interactions of sex, age, and genotype have been discussed (Jin *et al.*, 2001), with *Cyp4c3* and *Cyp6g1* as two examples. *Cyp4c3* is upregulated by starvation in larvae, an effect that can be attributed to lack of sugar (Zinke *et al.*, 2002). This effect points to a conserved physiological function of some *CYP4C* genes, as cockroach *CYP4C1* is also induced by starvation and is upregulated by hypertrehalosemic hormone (Lu *et al.*, 1995).

DNA microarray analysis of the transcriptome of transgenic flies has also led to the validation of *Drosophila* as a model for human disease (Borras *et al.*, 2003). Flies transformed with a human gene associated with glaucoma show a phenotype of distorted ommatidia and fluid discharge. Fifty transcripts have an altered expression profile in the head and, interestingly, *Cyp315a1* is suppressed significantly in these experiments (Borras *et al.*, 2003). Specific or thematic microarrays have also started to change research in insecticide resistance (Oakeshott *et al.*, 2003; see Section 4.1.4.5.5).

Serial analysis of gene expression (SAGE) analysis identified *Cyp4d21* as one of three genes expressed preferentially in the fat cells of the head, and specifically expressed in males under control of the sex determination genes *transformer* and *doublesex* (Fujii and Amrein, 2002). The male-specific expression of *Cyp312a1* in microarray experiments has also been reported and confirmed by real-time PCR (Kasai and Tomita, 2003).

4.1.5.2. P450 Induction, Inducers and Signal Transduction

4.1.5.2.1. Induction by hormones *Drosophila Cyp18* was discovered as the ecdysone-inducible *Eig17-1* gene (Hurban and Thummel, 1993). The expression of this gene clearly pulses closely after each ecdysteroid peak (Bassett *et al.*, 1997) (**Figure 26**), but the mechanism of its ecdysone inducibility has not been studied in detail. The *Cyp6a2* gene is also inducible by ecdysone. The arrest of ecdysone production decreases CYP6A2 levels and slightly increases DDT toxicity

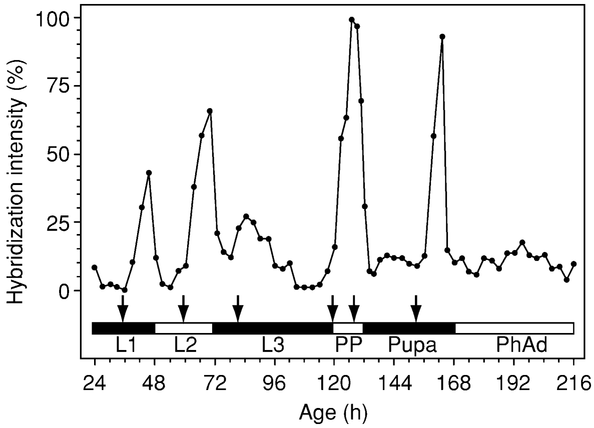


Figure 26 Developmental pattern of *Cyp18* expression in *Drosophila*. Peaks of *Cyp18* expression follow each surge in endogenous molting hormone level. (Reprinted with permission from Bassett, M.H., McCarthy, J.L., Waterman, M.R., Sliter, T.J. **1997**. Sequence and developmental expression of Cyp18, a member of a new cytochrome P450 family from *Drosophila*. *Mol. Cell. Endocrinol.* *131*, 39–49; © Elsevier.)

(Spiegelman *et al.*, 1997). Ecdysone inducibility of *Cyp6a2* may be mediated by Broad-Complex Z1 and Z4 transcription factor binding sites or ecdysone response elements (EcRE) (see **Chapter 3.5**) seen in the promoter of the gene (Dunkov *et al.*, 1997; Dombrowski *et al.*, 1998).

CYP4C1 is inducible by hypertrehalosemic hormone (HTH) (see **Chapter 3.10**) and starvation in the fat body of *Blaberus discoidalis* (Bradfield *et al.*, 1991). The dose-response for *CYP4C1* induction (ED50 = 3 pmol/insect, 8 h after injection) corresponds to the physiological range of HTH-dependent biosynthesis of trehalose and this induction appears to be a direct effect of the hormone (Lu *et al.*, 1995). JH inhibits *CYP4C1* expression in adult females, but not in males; this JH inhibition controls the decrease in *CYP4C1* transcript levels in the fat body. Yet HTH can override the JH inhibition (Lu *et al.*, 1999). The dual regulation of *CYP4C1* expression is indicative of a fine regulation of this P450, presumed to be involved in fatty acid ω-hydroxylation (by analogy to the function of mammalian CYP4 enzymes). If confirmed, such a function might be related to gluconeogenesis from fatty acid oxidation (an HTH stress and starvation response) as opposed to (and thus inhibited by) the JH-controlled metabolism of the fat body cell towards vitellogenesis (Lu *et al.*, 1999) (see **Chapters 3.7** and **3.9**).

4.1.5.2.2. Xenobiotic-inducible genes One remarkable feature of P450 genes is that the transcription rate of many of them (sometimes gene batteries

that also include genes other than P450s) is induced by foreign chemicals or "xenobiotics." Genetic models have provided much of our current understanding of induction of the mammalian *CYP1* genes by aryl hydrocarbons, such as dioxin, through the Ah receptor pathway (Denison and Nagy, 2003). For instance, dioxin and other *a*romatic *h*ydrocarbons bind to the Ah receptor, which itself dimerizes with a protein called Arnt. The dimer of these two bHLH-PAS proteins is thought to induce a battery of genes including the *CYP1A1* gene. However, the molecular mechanism is not well understood for many other classes of inducers. Nuclear receptors play a key role at the interface between physiological responses and environmental responses (Chawla *et al*., 2001; Honkakoski *et al*., 2003) (see **Chapter 3.6**).

Our understanding of the molecular genetics of induction in insects will benefit from the power of the *Drosophila* model. Phenobarbital (see below) is a known inducer of many P450 activities in *Drosophila* (Hallstrom and Grafstrom, 1981; Bigelow *et al*., 1985; Fuchs *et al*., 1994; Amichot *et al*., 1998). Other inducers of one or more marker activities in *Drosophila* include: β-naphthoflavone or polychlorinated biphenyls (Hallstrom and Grafstrom, 1981), but not benzo[a]anthracene or dioxin (TCDD) (Bigelow *et al*., 1985); butylated hydroxytoluene and rifampicin (Zijlstra *et al*., 1984); *trans*-stilbene oxide, triphenyldioxane, benzo[a]pyrene, but not TCPOBOP, a potent inducer of *CYP2B* genes in mice but not in rats (Fuchs *et al*., 1994); prochloraz, aminopyrine and clofibrate (Amichot *et al*., 1998). Thus, the major types of inducers known in the vertebrate toxicology literature, if not each specific chemical, have shown to be active as inducers in *Drosophila*. Specific genes induced are shown in **Table 8**. Biochemical techniques for the measurement of an Ah receptor in *Drosophila* have detected characteristic TCDD binding to a cytosolic fraction (Bigelow *et al*., 1985, but see Hahn, 2002). The *Drosophila* genome carries 12 paralogs of the vertebrate *Ah* receptor gene and a probable ortholog, *spineless* (Hahn, 2002). The mechanism of aryl hydrocarbon induction in insects remains uncertain.

4.1.5.2.3. Phenobarbital-inducible genes Phenobarbital-like inducers include a variety of chemicals with widely divergent physicochemical properties. In mammals, these inducers are mostly tumor promoters (phenobarbital, polychlorinated biphenyls, chlordane) but they are not genotoxic. They must affect some important homeostatic process in cells, as phenobarbital induction is observed in plants, nematodes, insects and mammals, and some bacteria. Thus phenobarbital may not be an ecologically relevant inducer of environmental response genes *sensu* Berenbaum (2002), but a physiologically relevant inducer *sensu* Nebert (1991). It highlights a pathway shared by many chemically unrelated compounds.

In vitro studies with vertebrate phenobarbital-inducible genes have identified a phenobarbital-responsive enhancer module (PBREM) in the distal regulatory element of *CYP2B* genes. Heterodimers of the nuclear receptor CAR and of the retinoid X receptor activate the PBREM of the mouse *Cyp2b10* gene (Honkakoski and Negishi, 2000; Wei *et al*., 2000). Both the CAR and the PXR nuclear receptors are involved in the induction of drug metabolizing enzymes in vertebrates. In the case of phenobarbital induction in *Drosophila*, the definition of *cis*- and *trans*-regulatory elements has begun. Transfection experiments with *Cyp6a2*/luciferase reporter constructs showed that dose-dependent phenobarbital induction can be studied in this system. Promoter elements sufficient for directing both basal and phenobarbital-inducible expression are located within 428 bp of 5′ of the start codon (Dunkov *et al*., 1997). Elements further upstream (984 and 1328 bp) are needed for higher basal activity. *In vivo* experiments with transgenic flies showed that (pheno)barbital induction was functional with 1331 or 985 bp of 5′ upstream DNA, whereas 129 bp upstream of the start codon, while conferring a low level of basal expression, does not support induction (Dombrowski *et al*., 1998). Further experiments with transgenic flies carrying the luciferase reporter gene driven by promoter sequences of the *Cyp6a8* gene showed low basal activity of a −11/−199 bp construct, with higher basal activities seen with 761 and 3100 bp of upstream DNA (Maitra *et al*., 2002). Phenobarbital inducibility was apparent with the three types of constructs, the highest level of induced activity being achieved with the −11/−761 bp promoter region. These studies thus consistently identify a small upstream region of the promoter that appears necessary and sufficient for phenobarbital inducibility, and all point to the presence of barbie box-like sequences in the promoter region of these inducible genes (Dunkov *et al*., 1997; Dombrowski *et al*., 1998; Maitra *et al*., 2002). Barbie box sequences are difficult to define structurally in insects as the only functionally verified sequences are bacterial (Shaw and Fulco, 1993). However, their distribution is not random, and has been observed upstream of phenobarbital-inducible P450 genes of other species

Table 8 Inducers of P450 genes in insects

Inducers	*P450 induced*	*Reference*
Hormones		
HTH	*CYP4C1*	Bradfield *et al.* (1991); Lu *et al.* (1995, 1999)
Ecdysteroids	*Cyp6a2, Cyp18*	Hurban and Thummel (1993); Spiegelman *et al.* (1997)
Ovarian hormone[a]	*CYP4C7*	Sutherland *et al.* (2000)
Alkaloids		
Nicotine	*CYP4M1, CYP4M3*	Snyder *et al.* (1995a)
Senita/saguaro cactus alkaloids	*CYP28A1, A2, A3, CYP4D10*	Danielson *et al.* (1997, 1998); Fogleman *et al.* (1998)
Terpenoids		
Monoterpenes (peppermint oil)	*CYP6B2*	Ranasinghe *et al.* (1997)
α-Pinene	*CYP6B2, CYP6B7*	Ranasinghe *et al.* (1997); Ranasinghe and Hobbs (1999b)
Menthol	*CYP6B2*	Ranasinghe *et al.* (1997)
Gossypol	*CYP6B27*	Li *et al.* (2002c)
Derived from phenylpropanoid pathway		
Salicylic acid	*CYP6B8, B9, B27, B28*	Li *et al.* (2002d)
Chlorogenic acid	*CYP6B8, B9, B27, B28*	Li *et al.* (2002c)
Coumarins		
Coumarin	*CYP6B27*	Li *et al.* (2002c)
Xanthotoxin	*CYP6B1, B3, B4, B8, B9, B17, B27, B28, CYP9A2, A4, A5*	Cohen *et al.* (1992); Hung *et al.* (1995b); Stevens *et al.* (2000); Li *et al.* (2000a, 2001, 2002c)
Bergapten	*CYP6B3*	Hung *et al.* (1995a)
Angelicin	*CYP6B3*	Hung *et al.* (1995a)
Sphondin	*CYP6B3*	Hung *et al.* (1995a)
Flavonoids		
Flavone	*CYP6B8, B9, B27, B28*	Li *et al.* (2002c)
Quercetin	*CYP6B8*	Li *et al.* (2002c)
Rutin	*CYP6B8, B27, B28*	Li *et al.* (2002c)
Various natural products		
Ethanol	*CYP6A1*	Cariño *et al.* (1992)
2-tridecanone	*CYP4M3, CYP6A2*	Snyder *et al.* (1995); Stevens *et al.* (2000)
2-undecanone	*CYP4M1, M3, CYP9A2, A4, A5*	Snyder *et al.* (1995); Stevens *et al.* (2000)
Jasmonic acid	*CYP6B8, B9, B27, B28*	Li *et al.* (2002d)
Indole-3-carbinol	*CYP6B8, B9, B27, B28, CYP9A2*	Stevens *et al.* (2000); Li *et al.* (2002c)
Synthetic chemicals		
Phenobarbital	*CYP4D10, Cyp4e2, CYP4L2, CYP4M1, CYP4M3, CYP6A1, Cyp6a2, CYP6B7, CYP6B8, CYP6B9, CYP6B27, CYP6B28, CYP6D1, CYP6D3, CYP9A2, CYP12A1, CYP28A1, A2, A3*	Snyder *et al.* (1995); Brun *et al.* (1996); Dunkov *et al.* (1996); Danielson *et al.* (1997, 1998); Guzov *et al.* (1998); Ranasinghe and Hobbs (1999a); Li *et al.* (2000a, 2002c), Kasai and Scott (2001b)
Barbital	*Cyp6a2, Cyp6a8, Cyp6a9*	Maitra *et al.* (1996), Dombrowski *et al.* (1998)
DDT	*Cyp12d1/2*	Brandt *et al.* (2002)
Alkylbenzenes (pentamethyl benzene)	*CYP4*	Scharf *et al.* (2001)
Butylated hydroxyanisole	*CYP6B2*	Ranasinghe *et al.* (1997)
Piperonyl butoxide	*CYP6A1, CYP6B2*	Cariño *et al.* (1992); Ranasinghe *et al.* (1997)
Clofibrate	*CYP4M1, M3, CYP9A2, 4*	Snyder *et al.* (1995); Stevens *et al.* (2000)
p-Hydroxybenzoate	*CYP6B8, B9, B27, B28*	Li *et al.* (2002d)
Permethrin	*CYP6X1*	Zhu and Snodgrass (2003)
Fenvalerate	*CYP6B7*	Ranasinghe and Hobbs (1999b)
Cypermethrin	*CYP6B7, B27, B28*	Ranasinghe and Hobbs (1999b); Li *et al.* (2002c)

[a]Identity of ovarian hormone unknown.

such as *CYP6A1, CYP6D1* (Dunkov *et al.*, 1997; Scott *et al.*, 1999), and of *CYP6B* genes (Hung *et al.*, 1996), of which some are phenobarbital-inducible (Li *et al.*, 2002c). The role of these sequences in regulating insect P450 genes thus remain conjectural. Microarray experiments show that less than 10% of the Drosophila P450 genes are inducible by phenobarbital, and this approach will facilitate the rational study of P450 induction in this model species.

Inducibility is under genetic control (Hallstrom, 1987), but is not trivial to quantify. Several pioneers have noted that induction in insecticide-resistant strains was modified (e.g., Terriere and Yu, 1974), often lower (in fold induction) than in susceptible strains in the housefly and in *Drosophila*. Non-inducibility maps to the resistance gene (Hallstrom *et al.*, 1982). Resistant strains have thus been called "constitutive mutants" (Hallstrom, 1985). It has been claimed that phenobarbital induction of *CYP6D1* is *due* to a *trans* acting factor on chromosome 2 of the housefly (Liu and Scott, 1997b). However, this claim is based on genetic crosses between an inducible multimarker strain *aabys*, and the pyrethroid-resistant strain LPR. In that strain, CYP6D1 transcript levels are higher than those achieved in phenobarbital-treated *aabys* flies, so that lack of further induction cannot be characterized as refractoriness to induction (a term which should be reserved for a low, basal level that is not changed by phenobarbital treatment – a volunteer is not refractory to the draft). That study therefore has no relevance to the genetic control of phenobarbital inducibility, but merely confirms that a specific interaction of LPR chromosomes 1 and 2 is needed for high expression of *CYP6D1v1* (Liu and Scott, 1995, 1996).

4.1.5.2.4. Furanocoumarin-inducible genes The inducibility of *CYP6B* genes by furanocoumarins has been studied extensively in *Papilio* species (see Section 4.1.4.3.3). The *CYP6B1* and *CYP6B3* genes are inducible by the linear furanocoumarin xanthotoxin, and variably induced by bergapten, angelicin, and sphondin (Prapaipong *et al.*, 1994; Hung *et al.*, 1995a, 1995b). This variability may be the result of individual polymorphism in inducibility. The 5′ flanking sequence of the *CYP6B1v3* gene comprising nt −838 to +22 (relative to the transcription start site) was fused to the reporter chloramphenicol acetyl transferase (CAT) gene and this construct was transfected into Sf9 cells (Prapaipong *et al.*, 1994). In this system, the promoter region of the *CYP6B3* gene had a low basal activity, and this was maximally induced (about twofold) by 2 μg xanthotoxin/ml culture. Thus, at least some of the sequences required for induction are present in this upstream region, and the xanthotoxin signaling cascade is present in these cells from the generalist herbivore *Spodoptera frugiperda*. Analysis of the 5′-upstream region of the *CYP6B1, B3, B4*, and *B5* genes from *P. polyxenes* and *P. glaucus* indicated a high degree of similarity, with several putative regulatory elements being noted (Hung *et al.*, 1996). These include sequences similar to the XRE (xenobiotic response element) of the Ah receptor of vertebrates, the barbie box, or the ARE (antioxidant RE) of rodent GST genes. With the cloning of additional *CYP6B* genes, a number of additional sequences, conserved among *CYP6B* genes and with similarities to known regulatory sequences of other genes, have been recorded (Petersen *et al.*, 2001; Li *et al.*, 2002a; Petersen *et al.*, 2003). A region (−136 to −119 of the *CYP6B1* gene) named XRE-xan (for XRE-xanthotoxin) has reportedly been identified as a being required for basal transcription and xanthotoxin inducibility and is conserved among *CYP6B* genes (results quoted by Hung *et al.*, 1996; Berenbaum, 2002). Evidence for the function of this XRE-xan was presented in an extensive series of experiments with the promoter region of the *CYP6B1v3* gene driving expression of CAT in Sf9 cells (Petersen *et al.*, 2003). Serial deletion experiments from −5 kb to −97 indicated the presence of a negative regulatory element necessary for basal transcription between nt −228 and −146 and a strong positive element between −146 and +22. Xanthotoxin inducibility was similarly mapped to a region between −146 and −97. This region includes the CAAT box basal promoter element. Substitution mutagenesis of tracts of DNA within this region identified nt −136 to −119 (TGACTGGCAATTTTTTTT) as the element called XRE-xan. Sequences similar to the ecdysone RE (EcRE) and an ARE overlap on the 5′ end of the XRE-xan. A region −37 to +22 was found to be necessary for both basal and induced activity and thus forms part of the core promoter of the *CYP6B1* gene. Mutagenesis of the EcRE portion 5′ of the XRE-xan element slightly diminished basal and induced expression, but mutagenesis of the TGAC sequence common to the EcRE, ARE, and XRE-xan abolished basal and induced expression. However, this sequence is not conserved in the same region of the xanthotoxin-inducible *CYP6B3* gene. The induction pattern of the *CYP6B1v3* promoter fragment (−380 to +22) driving CAT expression in Sf9 cells revealed a significant induction by flavone, a coumarin, and angular furanocoumarins, in contrast to the pattern seen for the *CYP6B1* gene *in vivo* (Petersen *et al.*, 2003). This lack of fidelity may be caused by a different specificity of the receptor(s)

expressed in Sf9 cells from that of the *P. polyxenes* midgut receptor(s). It may also be due to the absence of additional regulatory elements upstream of −380 in the test system. The definition of xenobiotic-responsive elements of well-characterized P450 genes may facilitate their detection in other inducible genes (e.g., lepidopteran *CYP9A* genes; Stevens *et al.*, 2000), and will help identify the DNA-binding proteins that bind to them.

4.1.6. Conclusion and Prospects

Some examples of the multiple functions of insect P450 enzymes, of their complex biochemistry and of their toxicological and physiological importance have been presented. Is there a common thread and does the hopeless chaos of this chapter simply reflect our fragmentary knowledge? With evolution as the guide, we can now summarize the relative positions of the insect P450s that have been discussed on the phylogenetic tree of the P450 superfamily. Insect P450 sequences can be distinguished in four major clades, which fall into four subclasses of Gotoh's classification of P450 families (Gotoh, 1993) and this is schematically illustrated in **Figure 27**:

1. A large group of insect P450s comprising the CYP6, CYP9, CYP28 families as well as the CYP308–310 and CYP321 families is most closely related to vertebrate CYP3 and CYP5 families. Because of the initial "lumping" of CYP6B1 into the CYP6 family and later "splitting" of the CYP300s, this group is rather heterogeneous with regard to CYP families and subfamilies. Few of the CYP6 enzymes have been characterized, the CYP6A1 and 2 of Diptera are capable of metabolizing xenobiotics, and several CYP6B of Lepidoptera are known to metabolize a variety of furanocoumarins. Genes from this group appear to share the characteristics of "environmental response genes" as defined by Berenbaum (2002), specifically: (1) very high diversity; (2) proliferation by duplication events; (3) rapid rates of evolution; (4) occurrence in gene clusters; and (5) tissue- or temporal-specific expression. Of course these characteristics are not independent of each other, and they are difficult to measure objectively. Nonetheless, the multiple paralogs from these families can be dated to no more than 150–200 MYA and this gives a good idea of the dynamic nature of this clade's evolution.
2. Another clade includes the large CYP4 family that has members from vertebrates and insects, but also several P450 families from *C. elegans*, as well as the insect CYP311, 312, 313, 316, and 325 families initially discovered with the sequence of the *Drosophila* and *Anopheles* genomes. This group of sequences is highly diversified, perhaps even more so than the CYP6/9/28 group, and some *CYP4* genes are clearly inducible by xenobiotics. However, specialized physiological functions are recognized (Sutherland *et al.*, 1998) or proposed (Bradfield *et al.*, 1991) for some enzymes of this group.
3. The mitochondrial P450s of vertebrates and insects (as well as CYP10 of *Lymnea stagnalis* and CYP44A1 of *C. elegans*, both presumed mitochondrial P450 sequences) are monophyletic. Within the mitochondrial clade, the CYP12 family appears to behave like the CYP6/9/28 clade, as a rapidly evolving group of paralogous genes. Some of the other families of mitochondrial P450s are now clearly linked to the ecdysteroid metabolism pathway. Mitochondrial P450s of insects thus evolved differently from the vertebrate mitochondrial P450s and subcellular localization in mitochondria can no longer be considered as evidence for a role in endocrine physiology. These sequences are all derived from an ancestral microsomal P450, and are only distantly related to soluble bacterial P450s that rely on a similar tandem of redox partners (i.e., adrenodoxin and adrenodoxin reductase and their paralogs). It is likely that mitochondrial P450s evolved as a result of the mistargeting of a microsomal P450 after mutations affected the N-terminal sequence. Drastic changes in the redox partner interactions were probably not necessary as rabbit CYP2B4 activity is supported by the bacterial ferredoxin and ferredoxin reductases of the P450cam and P450lin systems (Bernhardt and Gunsalus, 1992). Also, truncated CYP1A1 targeted to mitochondria interacts productively with either adrenodoxin, P450 reductase, or bacterial flavodoxin (Anandatheerthavarada *et al.*, 2001).
4. In fact, mitochondrial sequences are more closely related to a group of sequences that include vertebrate microsomal CYP1, CYP2, CYP17, and CYP21 as well as insect CYP15, CYP18, and the CYP303–307 series. Several of these insect sequences represent enzymes involved in essential physiological functions (e.g., CYP15), as are CYP17 and CYP21 of vertebrates. The CYP2, however, are widely considered as "environmental response genes" in mammalian species.

No clear pattern emerges from this current knowledge of insect P450 evolution. There is not one

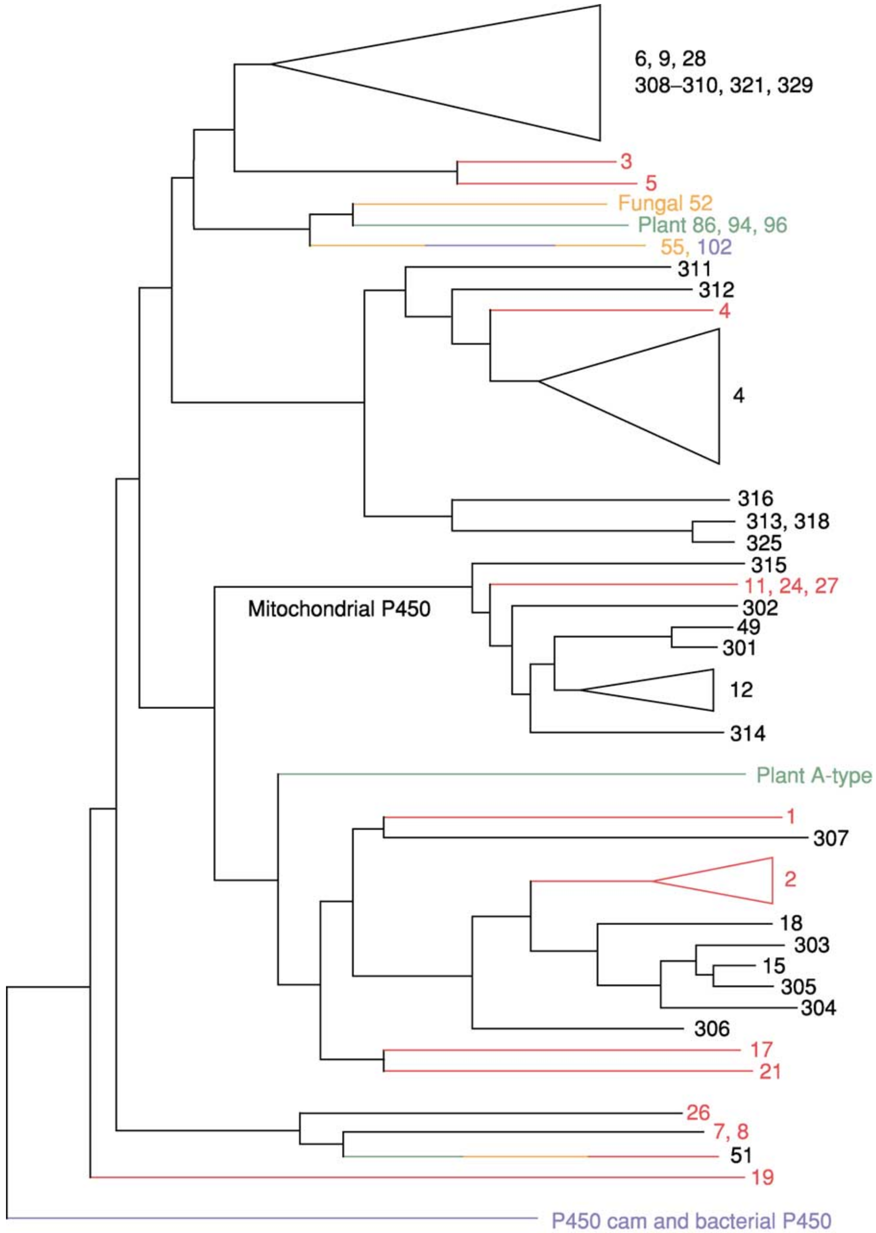


Figure 27 Schematic phylogeny of insect P450 genes and relationships with major P450 families from other organisms. The color code is: black, insects; red, vertebrates; brown, fungi; green, plants; blue, bacteria. Triangles represent families with large numbers of genes. Plant A-type P450 genes are found only in plants. CYP55 and CYP102 are P450foxy and P450BM3, fatty acid hydroxylases fused with a P450 reductase domain. CYP51 is the sterol 14α-demethylase, the only P450 ortholog found in different phyla. The phylogeny is not drawn to scale. The deep branch topology may be revised and there are two main sources of bias: most P450 sequences are from Diptera, and the molecular clock is not constant. This is seen for instance in the deep branching of the steroid aromatase (CYP19).

class of P450s involved in physiological processes and another distinct class involved mostly in xenobiotic metabolism. Physiological functions are not restricted to one branch of the P450 evolutionary tree, and the ramifications that seem typical of "environmental response genes" are found in both microsomal and mitochondrial P450 encoding genes. Will this fuzzy image clear up with the availability of more insect genome sequences, or will it be clouded even more by the discovery of dispersed physiological functions throughout the phylogeny?

There are many physiological functions for P450s that are not known or even suspected. In humans, mutations in the *CYP1B1* gene were linked to congenital glaucoma (Stoilov *et al.*, 1997), a clear physiological effect, still unexplained by what is known

of CYP1B1 biochemistry. In insects, CYP4C7 was shown to be selectively expressed in the corpora allata and to metabolize JH and its precursors to new metabolites (Sutherland *et al.*, 1998). Both the presence of this P450 in corpora allata and the existence of these metabolites were unexpected findings. Consequently, the search for new functions of P450 enzymes should involve broad, rational screens, and the technology is now available to perform such functional screens.

When significantly more data are obtained on the catalytic competence of a wide variety of insect P450 enzymes, it will become easier to understand the way in which insects maintain a wide repertoire of P450 genes. If positive selection of a few P450 genes can lead to specialized enzymes in oligophagous species (Li *et al.*, 2003), is this an evolutionary dead-end? Do the P450 enzymes with "broad and overlapping" specificity serve as a perpetual reservoir where some genes, because of their pattern of expression or inducibility or catalytic competence, can then serve as templates for the evolution of a new branch of specialized enzymes? Does this "primordial soup" perpetuate itself simply by a neutral process of intense gene duplication or are new chemical insults of the environment frequent enough to positively select for a minimal number of "jack-of-all-trades" P450 enzymes? How do P450 genes get recruited into physiological networks and biosynthetic pathways? The gap between "endogenous" and "xenobiotic" is being filled with new data and insights from P450 research (Nebert, 1991). Every 20 years or so researchers are discovering and rediscovering that treatments with inducers and inhibitors that cause major imbalances in P450 levels are deleterious to fitness in insects (Mitlin and Konecky, 1955; Yu and Terriere, 1974; Darvas *et al.*, 1992; Fuchs *et al.*, 1993). It is unlikely that these effects result solely from an interference with the handful of P450 enzymes involved in ecdysteroid and JH metabolism. Other regulatory pathways and other signal molecules remain to be discovered through a better understanding of insect P450 enzymes.

At the time of the first edition of this series, it seemed difficult enough to fully comprehend the function and role, the catalytic competence and the regulation of a single insect P450 enzyme. In the era of genomics, this task has now been multiplied by a hundred. It is hoped that interest in insect P450s will also grow by as much, and that new knowledge on insect P450 will continue to contribute to all branches of entomology, from toxicology to physiology and ecology.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Agosin, M., **1985**. Role of microsomal oxidations in insecticide degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 12. Pergamon, Oxford, pp. 647–712.

Agrawal, A.A., Vala, F., Sabelis, M.W., **2002**. Induction of preference and performance after acclimation to novel hosts in a phytophagous spider mite: adaptive plasticity? *Am. Nat. 159*, 553–565.

Ahmad, S., **1986**. Enzymatic adaptations of herbivorous insects and mites to phytochemicals. *J. Chem. Ecol. 12*, 533–560.

Ahmad, S., Brattsten, L.B., Mullin, C.A., Yu, S.J., **1986**. Enzymes involved in the metabolism of plant allelochemicals. In: Brattsten, L.B., Ahmad, S. (Eds.), Molecular Aspects of Insect–Plant Interactions. Plenum, New York, pp. 73–151.

Ahmad, S., Kirkland, K.E., Blomquist, G.J., **1987**. Evidence for a sex pheromone metabolizing cytochrome P-450 mono-oxygenase in the housefly. *Arch. Insect Biochem. Physiol. 6*, 21–40.

Amichot, M., Brun, A., Cuany, A., Helvig, C., Salaun, J.P., *et al.*, **1994**. Expression study of CYP genes in *Drosophila* strains resistant or sensitive to insecticides. In: Lechner, M.C. (Ed.), Cytochrome P450. John Libbey Eurotext, Paris, pp. 689–692.

Amichot, M., Brun, A., Cuany, A., Souza, G.D., Mouel, T.L., *et al.*, **1998**. Induction of cytochrome P450 activities in *Drosophila melanogaster* strains susceptible or resistant to insecticides. *Comp. Biochem. Physiol. 121C*, 311–319.

Amichot, M., Tares, S., Brun-Barale, A., Arthaud, L., Bride, J.M., *et al.*, **2004**. Point mutations associated with insecticide resistance in the *Drosophila* cytochrome P450 CYP6A2 enable DDT metabolism. *Eur. J. Biochem. 271*, 1250–1257.

Anandatheerthavarada, H.K., Amuthan, G., Biswas, G., Robin, M.A., Murali, R., *et al.*, **2001**. Evolutionary divergent electron donor proteins interact with P450MT2 through the same helical domain but different contact points. *EMBO J. 20*, 2394–2403.

Anandatheerthavarada, H.K., Vijayasarathy, C., Bhagwat, S.V., Biswas, G., Mullick, J., *et al.*, **1999**. Physiological role of the N-terminal processed P4501A1 targeted to mitochondria in erythromycin metabolism and reversal of erythromycin-mediated inhibition of mitochondrial protein synthesis. *J. Biol. Chem. 274*, 6617–6625.

Andersen, J.F., Ceruso, M., Unnithan, G.C., Kuwano, E., Prestwich, G.D., *et al.*, **1995**. Photoaffinity labeling of methyl farnesoate epoxidase in cockroach corpora allata. *Insect Biochem. Mol. Biol. 25*, 713–719.

Andersen, J.F., Utermohlen, J.G., Feyereisen, R., **1994**. Expression of housefly CYP6A1 and NADPH-cytochrome P450 reductase in *Escherichia coli* and

reconstitution of an insecticide-metabolizing P450 system. *Biochemistry 33*, 2171–2177.
Andersen, J.F., Walding, J.K., Evans, P.H., Bowers, W.S., Feyereisen, R., **1997**. Substrate specificity for the epoxidation of terpenoids and active site topology of housefly cytochrome P450 6A1. *Chem. Res. Toxicol. 10*, 156–164.
Appel, H.M., Martin, M., **1992**. Significance of metabolic load in the evolution of host specificity of *Manduca sexta*. *Ecology 73*, 216–228.
Aragon, S., Claudinot, S., Blais, C., Maibeche, M., Dauphin-Villemant, C., **2002**. Molting cycle-dependent expression of CYP4C15, a cytochrome P450 enzyme putatively involved in ecdysteroidogenesis in the crayfish, *Orconectes limosus*. *Insect Biochem. Mol. Biol. 32*, 153–159.
Arbeitman, M.N., Furlong, E.E., Imam, F., Johnson, E., Null, B.H., *et al.*, **2002**. Gene expression during the life cycle of *Drosophila melanogaster. Science 297*, 2270–2275.
Baldridge, G.D., Feyereisen, R., **1986**. Blood meal and cytochrome P-450 monooxygenases in the northern house mosquito, *Culex pipiens*. *Pestic. Biochem. Physiol. 25*, 407–413.
Barnes, H.J., Arlotto, M.P., Waterman, M.R., **1991**. Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in *Escherichia coli*. *Proc. Natl Acad. Sci. USA 88*, 5597–5601.
Bassett, M.H., McCarthy, J.L., Waterman, M.R., Sliter, T.J., **1997**. Sequence and developmental expression of Cyp18, a member of a new cytochrome P450 family from *Drosophila*. *Mol. Cell. Endocrinol. 131*, 39–49.
Berenbaum, M.R., **1990**. Evolution of specialization in insect-umbellifer associations. *Annu. Rev. Entomol. 35*, 319–343.
Berenbaum, M.R., **1995**. The chemistry of defense: theory and practice. *Proc. Natl Acad. Sci. USA 92*, 2–8.
Berenbaum, M.R., **1999**. Animal–plant warfare: molecular basis for cytochrome P450-mediated natural adaptation. In: Puga, A., Wallace, K.B. (Eds.), Molecular Biology of the Toxic Response. Taylor & Francis, Philadelphia, pp. 553–571.
Berenbaum, M.R., **2002**. Postgenomic chemical ecology: from genetic code to ecological interactions. *J. Chem. Ecol. 28*, 873–896.
Berenbaum, M.R., Cohen, M.B., Schuler, M.A., **1992**. Cytochrome P450 monooxygenase genes in oligophagous lepidoptera. *ACS Symp. Ser. 505*, 114–124.
Berenbaum, M.R., Favret, C., Schuler, M.A., **1996**. On defining key innovations in an adaptive radiation – cytochrome P450s and Papilionidae. *Am. Nat. 148*, S139–S155.
Berenbaum, M.R., Feeny, P., **1981**. Toxicity of angular furanocoumarins to swallowtails: escalation in the coevolutionary arms race. *Science 212*, 927–929.
Berenbaum, M.R., Neal, J.J., **1985**. Synergism between myristicin and xanthotoxin, a naturally co-occurring plant toxicant. *J. Chem. Ecol. 11*, 1349–1358.
Berenbaum, M.R., Neal, J.J., **1987**. Interactions among allelochemicals and insect resistance in crop plants. *ACS Symp. Ser. 330*, 416–430.
Bergé, J.B., Feyereisen, R., Amichot, M., **1998**. Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci. 353*, 1701–1705.
Bernard, C.B., Philogene, B.J., **1993**. Insecticide synergists: role, importance, and perspectives. *J. Toxicol. Environ. Health 38*, 199–223.
Bernays, E., Graham, M., **1988**. On the evolution of host specificity in phytophagous arthropods. *Ecology 69*, 886–892.
Bernhardt, R., Gunsalus, I.C., **1992**. Reconstitution of cytochrome P4502B4 (LM2) activity with camphor and linalool monooxygenase electron donors. *Biochem. Biophys. Res. Commun. 187*, 310–317.
Bertok, B., Pap, L., Arvai, G., Bakonyvari, I., Kuruczne Ribai, Z., **2003**. Structure-activity relationship study of alkynyl ether insecticide synergists and the development of MB-599 (verbutin). *Pestic. Manag. Sci. 59*, 377–392.
Bhagwat, S.V., Biswas, G., Anandatheerthavarada, H.K., Addya, S., Pandak, W., *et al.*, **1999**. Dual targeting property of the N-terminal signal sequence of P4501A1. Targeting of heterologous proteins to endoplasmic reticulum and mitochondria. *J. Biol. Chem. 274*, 24014–24022.
Bigelow, S.W., Zijlstra, J.A., Vogel, E.W., Nebert, D.W., **1985**. Measurements of the cytosolic Ah receptor among four stains of *Drosophila melanogaster. Arch. Toxicol. 56*, 219–225.
Black, B.C., Hollingworth, R.M., Ahammadsahib, K.I., Kukel, C.D., Donovan, S., **1994**. Insecticidal action and mitochondrial uncoupling activity of AC303,630 and related halogenated pyrroles. *Pestic. Biochem. Physiol. 50*, 115–128.
Blais, C., Lafont, R., **1986**. Ecdysone 20-hydroxylation in imaginal wing discs of *Pieris brassicae* (Lepidoptera): correlations with ecdysone and 20-hydroxyecdysone titers in pupae. *Arch. Insect Biochem. Physiol. 3*, 501–512.
Blomquist, G.J., Dillwith, J.W., Pomonis, J.G., **1984**. Sex pheromone of the housefly. Metabolism of (Z)-9-tricosene to (Z)-9,10-epoxytricosane and (Z)-14-tricosen-10-one. *Insect Biochem. 14*, 279–284.
Bollenbacher, W.E., Smith, S.L., Wielgus, J.J., Gilbert, L.I., **1977**. Evidence for an α-ecdysone cytochrome P-450 mixed function oxidase in insect fat body mitochondria. *Nature 268*, 660–663.
Borras, T., Morozova, T.V., Heinsohn, S.L., Lyman, R.F., Mackay, T.F., *et al.*, **2003**. Transcription profiling in *Drosophila* eyes that overexpress the human glaucoma-associated trabecular meshwork-inductible glucocorticoid response protein/myocilin (TIGR/MYOC). *Genetics 163*, 637–645.
Bowers, W.S., Ohta, T., Cleere, J.S., Marsella, P.A., **1976**. Discovery of insect anti-juvenile hormones in plants. *Science 193*, 542–547.

Bradfield, J.Y., Lee, Y.H., Keeley, L.L., **1991**. Cytochrome P450 family 4 in a cockroach: molecular cloning and regulation by hypertrehalosemic hormone. *Proc. Natl Acad. Sci. USA 88*, 4558–4562.

Brandt, A., Scharf, M., Pedra, J.H., Holmes, G., Dean, A., *et al*., **2002**. Differential expression and induction of two *Drosophila* cytochrome P450 genes near the Rst(2)DDT locus. *Insect Mol. Biol. 11*, 337–341.

Brattsten, L.B., **1979a**. Ecological significance of mixed-function oxidations. *Drug Metab. Rev. 10*, 35–58.

Brattsten, L.B., **1979b**. Biochemical defense mechanisms in herbivores against plant allelochemicals. In: Rosenthal, G.A., Janzen, D.H. (Eds.), Herbivores, their Interaction with Secondary Plant Metabolites. Academic Press, New York, pp. 199–270.

Brattsten, L.B., **1983**. Cytochrome P-450 involvement in the interactions between plant terpenes and insect herbivores. In: Hedin, P.A. (Ed.), Plant Resistance to Insects. American Chemical Society, pp. 173–195.

Brattsten, L.B., **1987**. Sublethal virus infection depresses cytochrome P-450 in an insect. *Experientia 43*, 451–454.

Brattsten, L.B., Metcalf, R.L., **1970**. The synergistic ratio of carbaryl with piperonyl butoxide as an indicator of the distribution of multifunction oxidases in the insecta. *J. Econ. Entomol. 63*, 101–104.

Brattsten, L.B., Wilkinson, C.F., **1977**. Insecticide solvents: interference with insecticidal action. *Science 196*, 1211–1213.

Brattsten, L.B., Wilkinson, C.F., Eisner, T., **1977**. Herbivore–plant interactions: mixed-function oxidases and secondary plant substances. *Science 196*, 1349–1352.

Bride, J.M., Cuany, A., Amichot, M., Brun, A., Babault, M., *et al*., **1997**. Cytochrome P-450 field insecticide tolerance and development of laboratory resistance in grape vine populations of *Drosophila melanogaster* (Diptera: Drosophilidae). *J. Econ. Entomol. 90*, 1514–1520.

Brindley, W.A., **1977**. Synergist differences as an alternate interpretation of carbaryl-piperonyl butoxide toxicity data. *Environ. Entomol. 6*, 885–888.

Brogdon, W.G., **1997**. Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. *J. Am. Mosq. Control Assoc. 13*, 233–237.

Brooks, G.T., **1979**. The metabolism of xenobiotics in insects. In: Bridges, J.W., Chasseaud, L. (Eds.), Progress in Drug Metabolism. Wiley, London, pp. 151–214.

Brooks, G.T., Pratt, G.E., Mace, D.W., Cocks, J.A., **1985**. Inhibitors of juvenile hormone biosynthesis in *corpora allata* of the cockroach *Periplaneta amaericana* (L.) *in vitro*. *Pestic. Sci. 16*, 132–142.

Brown, T.M., Bryson, P.K., Payne, G.T., **1996**. Synergism by propynyl aryl ethers in permethrin-resistant tobacco budworm larvae. *Pestic. Sci. 46*, 323–331.

Brun, A., Cuany, A., Le Mouel, T., Berge, J., Amichot, M., **1996**. Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, CYP6A2, by phenobarbital in insecticide susceptible or resistant strains. *Insect Biochem. Mol. Biol. 26*, 697–703.

Bull, D.L., Ivie, G.W., Beier, R.C., Pryor, N.W., **1986**. In vitro metabolism of a linear furanocoumarin (8-methoxypsoralen, xanthotoxin) by mixed-function oxidases of larvae of black swallowtail butterfly and fall armyworm. *J. Chem. Ecol. 12*, 885–892.

Campbell, P.M., Newcomb, R.D., Russell, R.J., Oakeshott, J.G., **1998**. Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 28*, 139–150.

Capdevila, J.H., Harris, R.C., Falck, J.R., **2002**. Microsomal cytochrome P450 and eicosanoid metabolism. *Cell. Mol. Life Sci. 59*, 780–789.

Cariño, F., Koener, J.F., Plapp, F.W., Jr., Feyereisen, R., **1992**. Expression of the cytochrome P450 gene CYP6A1 in the housefly, *Musca domestica*. *ACS Symp. Ser. 505*, 31–40.

Cariño, F.A., Koener, J.F., Plapp, F.W., Jr., Feyereisen, R., **1994**. Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a housefly strain with metabolic resistance to insecticides. *Insect Biochem. Mol. Biol. 24*, 411–418.

Casida, J.E., **1970**. Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J. Agric. Food Chem. 18*, 753–771.

Celniker, S.E., Wheeler, D.A., Kronmiller, B., Carlson, J.W., Halpern, A., *et al*., **2002**. Finishing a whole-genome shotgun: Release 3 of the *Drosophila melanogaster* euchromatic genome sequence. *Genome Biol. 3*, 79.1–79.14.

Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., *et al*., **2000**. The *Drosophila* disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126.

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., **2001**. Nuclear receptors and lipid physiology: opening the X-files. *Science 294*, 1866–1870.

Chen, J.S., Berenbaum, M.R., Schuler, M.A., **2002**. Amino acids in SRS1 and SRS6 are critical for furanocoumarin metabolism by CYP6B1v1, a cytochrome P450 monooxygenase. *Insect Mol. Biol. 11*, 175–186.

Chen, C.D., Doray, B., Kemper, B., **1998**. A conserved proline-rich sequence between the N-terminal signal-anchor and catalytic domains is required for assembly of functional cytochrome P450 2C2. *Arch. Biochem. Biophys. 350*, 233–238.

Chen, J.H., Hara, T., Fisher, M.J., Rees, H.H., **1994**. Immunological analysis of developmental changes in ecdysone 20-monooxygenase expression in the cotton leafworm, *Spodoptera littoralis*. *Biochem. J. 299*, 711–717.

Cianfrogna, J.A., Zangerl, A.R., Berenbaum, M.R., **2002**. Dietary and developmental influences on induced

detoxification in an oligophage. *J. Chem. Ecol. 28*, 1349–1364.

Cilek, J.E., Dahlman, D.L., Knapp, F.W., **1995**. Possible mechanism of diazinon negative cross-resistance in pyrethroid-resistant horn flies (Diptera: Muscidae). *J. Econ. Entomol. 88*, 520–524.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., *et al.*, **2001**. Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron 32*, 657–671.

Clarke, S.E., Brealey, C.J., Gibson, G.G., **1989**. Cytochrome P-450 in the housefly: induction, substrate specificity and comparison to three rat hepatic isoenzymes. *Xenobiotica 19*, 1175–1180.

Claudianos, C., Crone, E., Coppin, C., Russell, R., Oakeshott, J.G., **2001**. A genomics perspective on mutant aliesterases and metabolic resistance to organophosphates. In: Clark, J.M., Yamaguchi, I. (Eds.), Agrochemical Resistance: Extent, Mechanism and Detection. Oxford Press, Washington, pp. 90–101.

Claudianos, C., Russell, R.J., Oakeshott, J.G., **1999**. The same amino acid substitution in orthologous esterases confers organophosphate resistance on the housefly and a blowfly. *Insect Biochem. Mol. Biol. 29*, 675–686.

Cohen, M.B., Berenbaum, M.R., Schuler, M.A., **1989**. Induction of cytochome P450-mediated detoxification of xanthotoxin in the black swallowtail. *J. Chem. Ecol. 15*, 2347–2355.

Cohen, M.B., Feyereisen, R., **1995**. A cluster of cytochrome P450 genes of the CYP6 family in the housefly. *DNA Cell Biol. 14*, 73–82.

Cohen, M.B., Koener, J.F., Feyereisen, R., **1994**. Structure and chromosomal localization of CYP6A1, a cytochrome P450-encoding gene from the housefly. *Gene 146*, 267–272.

Cohen, M.B., Schuler, M.A., Berenbaum, M.R., **1992**. A host-inducible cytochrome P-450 from a host-specific caterpillar: molecular cloning and evolution. *Proc. Natl Acad. Sci. USA 89*, 10920–10924.

Couillaud, F., Debernard, S., Darrouzet, E., Rossignol, F., **1996**. Hidden face of juvenile hormone metabolism in the African locust. *Arch. Insect Biochem. Physiol. 32*, 387–397.

Crampton, A.L., Baxter, G.D., Barker, S.C., **1999**. Identification and characterisation of a cytochrome P450 gene and processed pseudogene from an arachnid: the cattle tick, *Boophilus microplus*. *Insect Biochem. Mol. Biol. 29*, 377–384.

Crankshaw, D.L., Hetnarski, K., Wilkinson, C.F., **1981**. The functional role of NADPH-cytochrome *c* reductase in southern armyworm (*Spodoptera eridania*) midgut microsomes. *Insect Biochem. 11*, 515–522.

Cuany, A., Helvig, C., Amichot, M., Pflieger, P., Mioskowski, C., *et al.*, **1995**. Fate of a terminal olefin with *Drosophila* microsomes and its inhibitory effects on some P-450 dependent activities. *Arch. Insect Biochem. Physiol. 28*, 325–338.

Cuany, A., Pralavorio, M., Pauron, D., Berge, J.B., Fournier, D., *et al.*, **1990**. Characterization of microsomal oxidative activities in a wild-type and in a DDT resistant strain of *Drosophila melanogaster. Pestic. Biochem. Physiol. 37*, 293–302.

Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., ffrench-Constant, R., **2001**. DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genomics 266*, 556–563.

Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., *et al.*, **2002**. A single P450 allele associated with insecticide resistance in *Drosophila*. *Science 297*, 2253–2256.

Danielson, P.B., Fogleman, J.C., **1997**. Isolation and sequence analysis of cytochrome P450 12B1: the first mitochondrial insect P450 with homology to 1 alpha, 25 dihydroxy-D3 24-hydroxylase. *Insect Biochem. Mol. Biol. 27*, 595–604.

Danielson, P.B., Foster, J.L., Cooper, S.K., Fogleman, J.C., **1999**. Diversity of expressed cytochrome P450 genes in the adult Mediterranean fruit fly, *Ceratitis capitata*. *Insect Mol. Biol. 8*, 149–159.

Danielson, P.B., Foster, J.L., McMahill, M.M., Smith, M.K., Fogleman, J.C., **1998**. Induction by alkaloids and phenobarbital of Family 4 Cytochrome P450s in *Drosophila*: evidence for involvement in host plant utilization. *Mol. Gen. Genet. 259*, 54–59.

Danielson, P.B., Letman, J.A., Fogleman, J.C., **1995**. Alkaloid metabolism by cytochrome P-450 enzymes in *Drosophila melanogaster. Comp. Biochem. Physiol. 110B*, 683–688.

Danielson, P.B., MacIntyre, R.J., Fogleman, J.C., **1997**. Molecular cloning of a family of xenobiotic-inducible drosophilid cytochrome P450s: evidence for involvement in host–plant allelochemical resistance. *Proc. Natl Acad. Sci. USA 94*, 10797–10802.

Dapkus, D., **1992**. Genetic localization of DDT resistance in *Drosophila melanogaster* (Diptera: Drosophilidae). *J. Econ. Entomol. 85*, 340–347.

Darrouzet, E., Mauchamp, B., Prestwich, G.D., Kerhoas, L., Ujvary, I., *et al.*, **1997**. Hydroxy juvenile hormones: new putative juvenile hormones biosynthesized by locust corpora allata *in vitro*. *Biochem. Biophys. Res. Commun. 240*, 752–758.

Darvas, B., Rees, H.H., Hoggard, N., **1993**. Ecdysone 20-monooxygenase systems in flesh-flies (Diptera: Sarcophagidae), *Neobellieria bullata* and *Parasarcophaga argyrostoma*. *Comp. Biochem. Physiol. 105B*, 765–773.

Darvas, B., Rees, H.H., Hoggard, N., Tag El-Din, M.H., Kuwano, E., *et al.*, **1992**. Cytrochrome P-450 inducers and inhibitors interfering with ecdysone 20-monooxygenases and their activities during postembryonic development of *Neobellieria bullata* Parker. *Pestic. Sci. 36*, 135–142.

David, J.P., Rey, D., Cuany, A., Amichot, M., Meyran, J.C., **2000**. Comparative ability to detoxify alder leaf

litter in field larval mosquito collections. *Arch. Insect Biochem. Physiol. 44*, 143–150.

David, J.P., Rey, D., Cuany, A., Bride, J.M., Meyran, J.C., **2002**. Larvicidal properties of decomposed leaf litter in the subalpine mosquito breeding sites. *Environ. Toxicol. Chem. 21*, 62–66.

Davis, R.H., Nahrstedt, A., **1987**. Biosynthesis of cyanogenic glucosides in butterflies and moths. *Insect Biochem. 17*, 689–693.

De Gregorio, E., Spellman, P.T., Rubin, G.M., Lemaitre, B., **2001**. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microrrays. *Proc. Natl Acad. Sci. USA 98*, 12590–12595.

De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., Lemaitre, B., **2002**. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J. 21*, 2568–2579.

Delpuech, J.M., Aquadro, C.F., Roush, R.T., **1993**. Non-involvement of the long terminal repeat of transposable element 17.6 in insecticide resistance in *Drosophila*. *Proc. Natl Acad. Sci. USA 90*, 5643–5647.

De Mot, R., Parret, A.H.A, **2002**. A novel class of self-sufficient cytochrome P450 monooxygenases in prokaryotes. *Trends Microbiol. 10*, 502–508.

Denison, M.S., Nagy, S.R., **2003**. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Ann. Rev. Pharmacol. Toxicol. 43*, 309–334.

de Sousa, G., Cuany, A., Brun, A., Amichot, M., Rahmani, R., *et al.*, **1995**. A microfluorometric method for measuring ethoxycoumarin-O-deethylase activity on individual *Drosophila melanogaster* abdomens: interest for screening resistance in insect populations. *Anal. Biochem. 229*, 86–91.

Dethier, V.G., **1954**. Evolution of feeding preferences in phytophagous insects. *Evolution 8*, 33–54.

Domanski, T.L., Halpert, J.R., **2001**. Analysis of mammalian cytochrome P450 structure and function by site-directed mutagenesis. *Curr. Drug Metab. 2*, 117–137.

Dombrowski, S.M., Krishnan, R., Witte, M., Maitra, S., Diesing, C., *et al.*, **1998**. Constitutive and barbital-induced expression of the *Cyp6a2* allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain. *Gene 221*, 69–77.

Dowd, P.F., Smith, C.M., Sparks, T.C., **1983**. Detoxification of plant toxins by insects. *Insect Biochem. 13*, 453–468.

Drabek, J., Neumann, R., **1985**. Proinsecticides. In: Hutson, D.H., Roberts, T.R. (Eds.), Insecticides. Wiley, London, pp. 35–86.

Dunkov, B.C., Guzov, V.M., Mocelin, G., Shotkoski, F., Brun, A., *et al.*, **1997**. The *Drosophila* cytochrome P450 gene *Cyp6a2*: structure, localization, heterologous expression, and induction by phenobarbital. *DNA Cell Biol. 16*, 1345–1356.

Dunkov, B.C., Rodriguez-Arnaiz, R., Pittendrigh, B., ffrench-Constant, R.H., Feyereisen, R., **1996**. Cytochrome P450 gene clusters in *Drosophila melanogaster*. *Mol. Gen. Genet. 251*, 290–297.

Durham, E.W., Siegfried, B.D., Scharf, M.E., **2002**. *In vivo* and *in vitro* metabolism of fipronil by larvae of the European corn borer *Ostrinia nubilalis*. *Pest Manag. Sci. 58*, 799–804.

Ehrlich, P.R., Raven, P.H., **1964**. Butterflies and plants: a study in coevolution. *Evolution 18*, 586–608.

Estabrook, R.W., **1996**. The remarkable P450s: a historical overview of these versatile hemeprotein catalysts. *FASEB J. 10*, 202–204.

Feng, R., Houseman, J.G., Downe, A.E.R., **1992**. Effect of ingested meridic diet and corn leaves on midgut detoxification processes in the European corn borer, *Ostrinia nubilalis*. *Pestic. Biochem. Physiol. 42*, 203–210.

Feyereisen, R., **1977**. Cytochrome P-450 et hydroxylation de l'ecdysone en ecdysterone chez *Locusta migratoria*. *C.R. Acad. Sci. Paris 284*, 1831–1834.

Feyereisen, R., **1983**. Polysubstrate monooxygenases (cytochrome P-450) in larvae of susceptible and resistant strains of house flies. *Pestic. Biochem. Physiol. 19*, 262–269.

Feyereisen, R., **1999**. Insect P450 enzymes. *Annu. Rev. Entomol. 44*, 507–533.

Feyereisen, R., Baldridge, G.D., Farnsworth, D.E., **1985**. A rapid method for preparing insect microsomes. *Comp. Biochem. Physiol. 82B*, 559–562.

Feyereisen, R., Durst, F., **1978**. Ecdysterone biosynthesis: a microsomal cytochrome-P-450-linked ecdysone 20-monooxygenase from tissues of the African migratory locust. *Eur. J. Biochem. 88*, 37–47.

Feyereisen, R., Durst, F., **1980**. Development of microsomal cytochrome P-450 monooxygenases during the last larval instar of the locust, *Locusta migratoria*: correlation with the hemolymph 20-hydroxyecdysone titer. *Mol. Cell. Endocrinol. 20*, 157–169.

Feyereisen, R., Farnsworth, D.E., **1985**. Developmental changes of microsomal cytochrome P-450 monooxygenases in larval and adult *Diploptera punctata*. *Insect Biochem. 15*, 755–761.

Feyereisen, R., Koener, J.F., Cariño, F.A., Daggett, A.S., **1990**. Biochemistry and molecular biology of insect cytochrome P450. In: Hagedorn, H.H., Hildebrand, J.G., Kidwell, M.G., Law, J.H. (Eds.), Molecular Insect Science. Plenum, New York, pp. 263–272.

Feyereisen, R., Koener, J.F., Farnsworth, D.E., Nebert, D.W., **1989**. Isolation and sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant strain of the housefly, *Musca domestica*. *Proc. Natl Acad. Sci. USA 86*, 1465–1469.

Feyereisen, R., Langry, K.C., Ortiz de Montellano, P.R., **1984**. Self-catalyzed destruction of insect cytochrome P-450. *Insect Biochem. 14*, 19–26.

Feyereisen, R., Pratt, G.E., Hamnett, A.F., **1981**. Enzymic synthesis of juvenile hormone in locust corpora allata: evidence for a microsomal cytochrome P-450 linked methyl farnesoate epoxidase. *Eur. J. Biochem. 118*, 231–238.

Feyereisen, R., Vincent, D.R., **1984**. Characterization of antibodies to housefly NADPH-cytochrome P-450 reductase. *Insect Biochem. 14*, 163–168.

ffrench-Constant, R.H., Park, Y., Feyereisen, R., **1999**. Molecular biology of insecticide resistance. In: Puga, A., Wallace, K.B. (Eds.), Molecular Biology of the Toxic Response. Taylor & Francis, Philadelphia, pp. 533–551.

Fisher, T., Crane, M., Callaghan, A., **2003**. Induction of cytochrome P450 activity in individual *Chironomus riparius* Meigen larvae exposed to xenobiotics. *Ecotoxicol. Environ. Saf. 54*, 1–6.

Fogleman, J.C., **2000**. Response of *Drosophila melanogaster* to selection for P450-mediated resistance to isoquinoline alkaloids. *Chem. Biol. Interact. 125*, 93–105.

Fogleman, J.C., Danielson, P.B., **2000**. Analysis of fragment homology among DNA sequences from cytochrome P450 families 4 and 6. *Genetica 110*, 257–265.

Fogleman, J.C., Danielson, P.B., MacLntyre, R.J., **1998**. The molecular basis of adaptation in *Drosophila*: the role of cytochrome P450s. In: Hecht, M., MacIntyre, R., Clegg, M. (Eds.), Evolutionary Biology, vol. 30. Plenum, New York, pp. 15–77.

Fraenkel, G.S., **1959**. The raison d'etre of secondary plant substances. *Science 129*, 1466–1470.

Frank, M.R., Fogleman, J.C., **1992**. Involvement of cytochrome P450 in host–plant utilization by Sonoran Desert *Drosophila*. *Proc. Natl Acad. Sci. USA 89*, 11998–12002.

Freeman, M.R., Dobritsa, A., Gaines, P., Segraves, W.A., Carlson, J.R., **1999**. The dare gene: steroid hormone production, olfactory behavior, and neural degeneration in *Drosophila*. *Development 126*, 4591–4602.

Frolov, M.V., Alatortsev, V.E., **1994**. Cluster of cytochrome P450 genes on the X chromosome of *Drosophila melanogaster. DNA Cell Biol. 13*, 663–668.

Fuchs, S., Spiegelman, V.S., Belitsky, G.A., **1993**. The effect of the cytochrome P-450 system inducers on the development of *Drosophila melanogaster. J. Biochem. Toxicol. 8*, 83–88.

Fuchs, S.Y., Spiegelman, V.S., Belitsky, G.A., **1994**. Inducibility of various cytochrome P450 isozymes by phenobarbital and some other xenobiotics in *Drosophila melanogaster. Biochem. Pharmacol. 47*, 1867–1873.

Fujii, S., Amrein, H., **2002**. Genes expressed in the *Drosophila* head reveal a role for fat cells in sex-specific physiology. *EMBO J. 21*, 5353–5363.

Fuji-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., Muramatsu, M., **1982**. Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. *Proc. Natl. Acad. Sci. USA 79*, 2793–2797.

Furlong, E.E., Andersen, E.C., Null, B., White, K.P., Scott, M.P., **2001**. Patterns of gene expression during *Drosophila* mesoderm development. *Science 293*, 1629–1633.

Gandhi, R., Varak, E., Goldberg, M.L., **1992**. Molecular analysis of a cytochrome P450 gene of family 4 on the *Drosophila* X chromosome. *DNA Cell Biol. 11*, 397–404.

Glendinning, J.I., Slansky, F., Jr., **1995**. Consumption of a toxic food by caterpillars increases with dietary exposure: support for a role of induced detoxification enzymes. *J. Comp. Physiol. A 176*, 337–345.

Gordon, H.T., **1961**. Nutritional factors in insect resistance to chemicals. *Annu. Rev. Entomol. 6*, 27–54.

Gotoh, O., **1992**. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem. 267*, 83–90.

Gotoh, O., **1993**. Evolution and differentiation of P-450 genes. In: Omura, T., Ishimura, Y., Fuji-Kuriyama, Y. (Eds.), Cytochrome P-450, 2nd edn. Kodansha, Tokyo, pp. 255–272.

Gotoh, O., **1998**. Divergent structures of *Caenorhabditis elegans* cytochrome P450 genes suggest the frequent loss and gain of introns during the evolution of nematodes. *Mol. Biol. Evol. 15*, 1447–1459.

Gould, F., **1984**. Mixed function oxidases and herbivore polyphagy: the devil's advocate position. *Ecol. Entomol. 9*, 29–34.

Greenwood, D.R., Rees, H.H., **1984**. Ecdysone 20-monooxygenase in the desert locust, *Schistocerca gregaria*. *Biochem. J. 223*, 837–847.

Grieneisen, M.L., Warren, J.T., Gilbert, L.I., **1993**. Early steps in ecdysteroid biosynthesis: evidence for the involvement of cytochrome P-450 enzymes. *Insect Biochem. Mol. Biol. 23*, 13–23.

Gunderson, C.A., Brattsten, L.B., Fleming, J.T., **1986**. Microsomal oxidase and glutathione transferase as factors influencing the effects of pulegone in southern and fall armyworm larvae. *Pestic. Biochem. Physiol. 26*, 238–249.

Guzov, V.M., Houston, H.L., Murataliev, M.B., Walker, F.A., Feyereisen, R., **1996**. Molecular cloning, overexpression in *Escherichia coli*, structural and functional characterization of housefly cytochrome b_5. *J. Biol. Chem. 271*, 26637–26645.

Guzov, V.M., Unnithan, G.C., Chernogolov, A.A., Feyereisen, R., **1998**. CYP12A1, a mitochondrial cytochrome P450 from the housefly. *Arch. Biochem. Biophys. 359*, 231–240.

Hahn, M.E., **2002**. Aryl hydrocarbon receptors: diversity and evolution. *Chem. Biol. Interact. 141*, 131–160.

Hainzl, D., Casida, J.E., **1996**. Fipronil insecticide: novel photochemical desulfinylation with retention of neurotoxicity. *Proc. Natl Acad. Sci. USA 93*, 12764–12767.

Hainzl, D., Cole, L.M., Casida, J.E., **1998**. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem. Res. Toxicol. 11*, 1529–1535.

Halliday, W.R., Farnsworth, D.E., Feyereisen, R., **1986**. Hemolymph ecdysteroid titer and midgut ecdysone 20-monooxygenase activity during the last larval stage of *Diploptera punctata*. *Insect Biochem. 16*, 627–634.

Hallstrom, I., **1985**. Genetic regulation of the cytochrome P-450 system in *Drosophila melanogaster*: II. Localization of some genes regulating cytochrome P-450 activity. *Chem. Biol. Interact. 56*, 173–184.

Hallstrom, I., **1987**. Genetic variation in cytochrome P-450-dependent demethylation in *Drosophila melanogaster*. *Biochem. Pharmacol. 36*, 2279–2282.

Hallstrom, I., Blanck, A., **1985**. Genetic regulation of the cytochrome P-450 system in *Drosophila melanogaster*: I. Chromosomal determination of some cytochrome P-450-dependent reactions. *Chem. Biol. Interact. 56*, 157–171.

Hallstrom, I., Grafstrom, R., **1981**. The metabolism of drugs and carcinogens in isolated subcellular fractions of *Drosophila melanogaster*. II. Enzyme induction and metabolism of benzo[*a*]pyrene. *Chem. Biol. Interact. 34*, 145–159.

Hallstrom, I., Magnusson, J., Ramel, C., **1982**. Relation between the somatic toxicity of dimethylnitrosamine and a genetically determined variation in the level and induction of cytochrome P450 in *Drosophila melanogaster*. *Mutat. Res. 92*, 161–168.

Halpern, M.E., Morton, R.A., **1987**. Reproductive and developmental defects in a malathion-resistant laboratory-selected population of *Drosophila melanogaster*. *Pestic. Biochem. Physiol. 28*, 44–56.

Hammock, B.D., **1975**. NADPH dependent epoxidation of methyl farnesoate to juvenile hormone in the cockroach *Blaberus giganteus* L. *Life Sci. 17*, 323–328.

Hammock, B.D., **1985**. Regulation of juvenile hormone titer: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 431–472.

Hammock, B.D., Mumby, S.M., **1978**. Inhibition of epoxidation of methyl farnesoate to juvenile hormone III by cockroach corpus allatum homogenates. *Pestic. Biochem. Physiol. 9*, 39–47.

Harrison, P.M., Milburn, D., Zhang, Z., Bertone, P., Gerstein, M., **2003**. Identification of pseudogenes in the *Drosophila melanogaster* genome. *Nucleic Acids Res. 31*, 1033–1037.

Harrison, T.L., Zangerl, A.R., Schuler, M.A., Berenbaum, M.R., **2001**. Developmental variation in cytochrome P450 expression in *Papilio polyxenes* in response to xanthotoxin, a hostplant allelochemical. *Arch. Insect Biochem. Physiol. 48*, 179–189.

Harwood, S.H., Moldenke, A.F., Berry, R.E., **1990**. Toxicity of peppermint monoterpenes to the variegated cutworm, *Peridroma saucia* Hubner (Lepidoptera: Noctuidae). *J. Econ. Entomol. 83*, 1761–1767.

Hasemann, C.A., Kurumbail, R.G., Boddupalli, S.S., Peterson, J.A., Deisenhofer, J., **1995**. Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure 3*, 41–62.

Hatano, R., Scott, J.G., **1993**. Anti-P450lpr antiserum inhibits the activation of chlorpyrifos to chlorpyrifos oxon in housefly microsomes. *Pestic. Biochem. Physiol. 45*, 228–233.

Hatano, R., Scott, J.G., Dennehy, T.J., **1992**. Enhanced activation is the mechanism of negative cross-resistance to chlorpyriphos in the Dicofol-IR strain of *Tetranychus urticae* (Acari: Tetranychidae). *J. Econ. Entomol. 85*, 1088–1091.

He, H., Chen, A.C., Davey, R.B., Ivie, G.W., **2002**. Molecular cloning and nucleotide sequence of a new P450 gene, *CYP319A1*, from the cattle tick, *Boophilus microplus*. *Insect Biochem. Mol. Biol. 32*, 303–309.

Hehn, A., Morant, M., Werck-Reichhart, D., **2002**. Partial recoding of P450 and P450 reductase cDNAs for improved expression in yeast and plants. *Methods in Enzymol. 357*, 343–351.

Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., **2004**. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. *Proc. Natl Acad. Sci. USA 101*, 4024–4029.

Hickey, D.A., Bally-Cuif, L., Abukashawa, S., Patyant, V., Benkel, B.F., **1991**. Concerted evolution of duplicated protein coding genes in *Drosophila*. *Proc. Natl Acad. Sci. USA 88*, 1611–1615.

Hodgson, E., **1985**. Microsomal monooxygenases. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 11. Pergamon, Oxford, pp. 225–331.

Hoggard, N., Fisher, M.J., Rees, H.H., **1989**. Possible role for covalent modification in the reversible activation of ecdysone 20-monooxygenase activity. *Arch. Insect Biochem. Physiol. 10*, 241–253.

Hoggard, N., Rees, H.H., **1988**. Reversible activation-inactivation of mitochondrial ecdysone 20-mono-oxygenase: a possible role for phosphorylation-dephosphorylation. *J. Insect Physiol. 34*, 647–653.

Hold, K.M., Sirisoma, N.S., Casida, J.E., **2001**. Detoxification of alpha- and beta-Thujones (the active ingredients of absinthe): site specificity and species differences in cytochrome P450 oxidation in vitro and in vivo. *Chem. Res. Toxicol. 14*, 589–595.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Holzkamp, G., Nahrstedt, A., **1994**. Biosynthesis of cyanogenic glucosides in the lepidoptera. Incorporation of [U-14C]-2-methylpropanealdoxime, 2S-[U-14C]-nethylbutanealdoxime and -[U-14C]-N-hydroxyisoleucine into linamarin and lotaustralin by the larvae of *Zygaena trifolli*. *Insect Biochem. Mol. Biol. 24*, 161–165.

Honkakoski, P., Negishi, M., **2000**. Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem. J. 347*, 321–337.

Honkakoski, P., Sueyoshi, T., Negishi, M., **2003**. Drug-activated nuclear receptors CAR and PXR. *Ann. Med. 35*, 172–182.

Horike, N., Sonobe, H., **1999**. Ecdysone 20-monooxygenase in eggs of the silkworm, *Bombyx mori*: enzymatic properties and developmental changes. *Arch. Insect Biochem. Physiol. 41*, 9–17.

Horike, N., Takemori, H., Nonaka, Y., Sonobe, H., Okamoto, M., **2000**. Molecular cloning of NADPH-

cytochrome P450 oxidoreductase from silkworm eggs. Its involvement in 20-hydroxyecdysone biosynthesis during embryonic development. *Eur. J. Biochem. 267*, 6914–6920.

Houpt, D.R., Pursey, J.C., Morton, R.A., **1988**. Genes controlling malathion resistance in a laboratory-selected population of *Drosophila melanogaster. Genome 30*, 844–853.

Hovemann, B.T., Sehlmeyer, F., Malz, J., **1997**. *Drosophila melanogaster* NADPH-cytochrome P450 oxidoreductase: pronounced expression in antennae may be related to odorant clearance. *Gene 189*, 213–219.

Hughes, P.B., Devonshire, A.L., **1982**. The biochemical basis of resistance to organophosphorus insecticides in the sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol. 18*, 289–297.

Hung, C.F., Berenbaum, M.R., Schuler, M.A., **1997**. Isolation and characterization of CYP6B4, a furanocoumarin-inducible cytochrome P450 from a polyphagous caterpillar (Lepidoptera:papilionidae). *Insect Biochem. Mol. Biol. 27*, 377–385.

Hung, C.F., Harrison, T.L., Berenbaum, M.R., Schuler, M.A., **1995a**. CYP6B3: a second furanocoumarin–inducible cytochrome P450 expressed in *Papilio polyxenes*. *Insect Mol. Biol. 4*, 149–160.

Hung, C.F., Holzmacher, R., Connolly, E., Berenbaum, M.R., Schuler, M.A., **1996**. Conserved promoter elements in the CYP6B gene family suggest common ancestry for cytochrome P450 monooxygenases mediating furanocoumarin detoxification. *Proc. Natl Acad. Sci. USA 93*, 12200–12205.

Hung, C.F., Prapaipong, H., Berenbaum, M.R., Schuler, M.A., **1995b**. Differential induction of cytochrome P-450 transcripts in *Papilio polyxenes* by linear and angular furanocoumarins. *Insect Biochem. Mol. Biol. 25*, 89–99.

Hunt, D.W.A., Smirle, M.J., **1988**. Partial inhibition of pheromone production in *Dendroctonus ponderosae* (Coleoptera: Scolytidae) by polysubstrate monooxygenase inhibitors. *J. Chem. Ecol. 14*, 529–536.

Hurban, P., Thummel, C.S., **1993**. Isolation and characterization of fifteen ecdysone-inducible *Drosophila* genes reveal unexpected complexities in ecdysone regulation. *Mol. Cell Biol. 13*, 7101–7111.

Ikezawa, N., Morisaki, M., Fujimoto, Y., **1993**. Sterol metabolism in insects: dealkylation of phytosterol to cholesterol. *Acc. Chem. Res. 26*, 139–146.

Ikezawa, N., Tanaka, M., Nagayoski, M., Shinkyo, R., Sakaki, T., *et al.*, **2003**. Molecular cloning and characterization of CYP719, a methylenedioxy bridge-forming enzyme that belongs to a novel P450 family, from cultured *Coptis japonica* cells. *J. Biol. Chem. 278*, 38557–38565.

Issemann, I., Green, S., **1990**. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature 347*, 645–650.

Ivie, G.W., Bull, D.L., Beier, R.C., Pryor, N.W., Oertli, E.H., **1983**. Metabolic detoxification: mechanism of insect resistance to plant psoralens. *Science 221*, 374–376.

Iyengar, S., Arnason, J.T., Philogene, B.J.R., Werstiuk, N.H., Morand, P., **1990**. Comparative metabolism of the phototoxic allelochemical α-terthienyl in three species of Lepidopterans. *Pestic. Biochem. Physiol. 37*, 154–164.

Jansson, I., **1993**. Posttranslational modification of cytochrome P450. In: Schenkman, J.B., Greim, H. (Eds.), Cytochrome P450. Springer, Berlin, pp. 561–580.

Jefcoate, C.F., **1978**. Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. *Methods Enzymol. 52*, 258–279.

Jermy, T., **1984**. Evolution of insect/host plant relationships. *Am. Naturalist. 124*, 609–630.

Jin, W., Riley, R.M., Wolfinger, R.D., White, K.P., Passador-Gurgel, G., *et al.*, **2001**. The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster. Nat. Genet. 29*, 389–395.

Johnson, P., Rees, H.H., **1977**. The mechanism of C-20 hydroxylation of α-ecdysone in the desert locust, *Schistocerca gregaria*. *Biochem. J. 168*, 513–520.

Jowett, T., Wajidi, M.F., Oxtoby, E., Wolf, C.R., **1991**. Mammalian genes expressed in *Drosophila*: a transgenic model for the study of mechanisms of chemical mutagenesis and metabolism. *EMBO Journal 10*, 1075–1081.

Kabbouh, M., Kappler, C., Hetru, C., Durst, F., **1987**. Further characterization of the 2-deoxyecdysone C-2 hydroxylase from *Locusta migratoria*. *Insect Biochem. 17*, 1155–1161.

Kahn, R.A., Bak, S., Svendsen, I., Halkier, B.A., Moller, B.L., **1997**. Isolation and reconstitution of cytochrome P450ox and in vitro reconstitution of the entire biosynthetic pathway of the cyanogenic glucosidedhurrin from sorghum. *Plant Physiol. 115*, 1661–1670.

Kamataki, T., Neal, R.A., **1976**. Metabolism of diethyl p-nitrophenyl phosphorothionate (parathion) by a reconstituted mixed-function oxidase enzyme system: studies of the covalent binding of the sulfur atom. *Mol. Pharmacol. 12*, 933–944.

Kao, L.M., Wilkinson, C.F., Brattsten, L.B., **1995**. In vivo effects of 2,4–D and atrazine on cytochrome P-450 and insecticide toxicity in southern armyworm (*Spodoptera eridania*) larvae. *Pestic. Sci. 45*, 331–334.

Kappers, W.A., Edwards, R.J., Murray, S., Boobis, A.R., **2001**. Diazinon is activated by CYP2C19 in human liver. *Toxicol. Appl. Pharmacol. 177*, 68–76.

Kappler, C., Kabbouh, M., Durst, F., Hoffmann, J.A., **1986**. Studies on the C-2 hydroxylation of 2-deoxyecdysone in *Locusta migratoria*. *Insect Biochem. 16*, 25–32.

Kappler, C., Kabbouh, M., Hetru, C., Durst, F., Hoffmann, J.A., **1988**. Characterization of three hydroxylases involved in the final steps of biosynthesis of the steroid hormone ecdysone in *Locusta migratoria* (Insecta, Orthoptera). *J. Steroid Biochem. 31*, 891–898.

Kasai, S., Scott, J.G., **2000**. Overexpression of cytochrome P450 CYP6D1 is associated with monoxygenase-mediated pyrethroid resistance in house flies from Georgia. *Pestic. Biochem. Physiol. 68*, 34–41.

Kasai, S., Scott, J.G., **2001a**. Cytochrome P450s CYP6D3 and CYP6D1 are part of a P450 gene cluster on autosome 1 in the housefly. *Insect Mol. Biol. 10*, 191–196.

Kasai, S., Scott, J.G., **2001b**. Expression and regulation of CYP6D3 in the housefly, *Musca domestica* (L.). *Insect Biochem. Mol. Biol. 32*, 1–8.

Kasai, S., Shono, T., Yamakawa, M., **1998a**. Molecular cloning and nucleotide sequence of a cytochrome P450 cDNA from a pyrethroid-resistant mosquito, *Culex quinquefasciatus* say. *Insect Mol. Biol. 7*, 185–190.

Kasai, S., Tomita, T., **2003**. Male specific expression of a cytochrome P450 (*Cyp312a1*) in *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 300*, 894–900.

Kasai, S., Weerasinghe, I.S., Shono, T., **1998b**. P450 monooxygenases are an important mechanism of permethrin resistance in *Culex quinquefasciatus* Say larvae. *Arch. Insect Biochem. Physiol. 37*, 47–56.

Kasai, S., Weerashinghe, I.S., Shono, T., Yamakawa, M., **2000**. Molecular cloning, nucleotide sequence and gene expression of a cytochrome P450 (CYP6F1) from the pyrethroid-resistant mosquito, *Culex quinquefasciatus* Say. *Insect Biochem. Mol. Biol. 30*, 163–171.

Kayser, H., Eilinger, P., **2001**. Metabolism of diafenthiuron by microsomal oxidation: procide activation and inactivation as mechanisms contributing to selectivity. *Pest Manag. Sci. 57*, 975–980.

Kayser, H., Ertl, P., Eilinger, P., Spindler-Barth, M., Winkler, T., **2002**. Diastereomeric ecdysteroids with a cyclic hemiacetal in the side chain produced by cytochrome P450 in hormonally resistant insect cells. *Arch. Biochem. Biophys. 400*, 180–187.

Kayser, H., Winkler, T., Spindler-Barth, M., **1997**. 26-Hydroxylation of ecdysteroids is catalyzed by a typical cytochrome P-450-dependent oxidase and related to ecdysteroid resistance in an insect cell line. *Eur. J. Biochem. 248*, 707–716.

Kennaugh, L., Pearce, D., Daly, J.C., Hobbs, A.A., **1993**. A piperonyl butoxide synergizable resistance to permethrin in *Helicoverpa armigera* which is not due to increased detoxification by cytochrome P450. *Pestic. Biochem. Physiol. 45*, 234–241.

Kennedy, G.G., **1984**. 2-Tridecanone, tomatoes and *Heliothis zea*: potential incompability of plant antibiosis with insecticidal control. *Entomol. Exp. Appl. 35*, 305–311.

Kirby, M.L., Ottea, J.A., **1995**. Multiple mechanisms for enhancement of glutathione S-transferase activities in *Spodoptera frugiperda* (Lepidopter: Noctuidae). *Insect Biochem. Mol. Biol. 25*, 347–353.

Kirby, M.L., Young, R.J., Ottea, J.A., **1994**. Mixed-function oxidase and glutathione S-transferase activities from field-collected larval and adult tobacco budworms, *Heliothis virescens* (F.). *Pestic. Biochem. Physiol. 49*, 24–36.

Koener, J.F., Cariño, F.A., Feyereisen, R., **1993**. The cDNA and deduced protein sequence of housefly NADPH-cytochrome P450 reductase. *Insect Biochem. Mol. Biol. 23*, 439–447.

Komori, M., Kitamura, R., Fukuta, H., Inoue, H., Baba, H., *et al.*, **1993**. Transgenic *Drosophila* carrying mammalian cytochrome P-4501A1: an application to toxicology testing. *Carcinogenesis 14*, 1683–1688.

Konno, T.E.H., Dauterman, W.C., **1989**. Studies on methyl parathion resistance in *Heliothis virescens*. *Pestic. Biochem. Physiol. 33*, 189–199.

Korytko, P.J., MacLntyre, R.J., Scott, J.G., **2000a**. Expression and activity of a house-fly cytochrome P450, CYP6D1, in *Drosophila melanogaster. Insect Mol. Biol. 9*, 441–449.

Korytko, P.J., Scott, J.G., **1998**. CYP6D1 protects thoracic ganglia of houseflies from the neurotoxic insecticide cypermethrin. *Arch. Insect Biochem. Physiol. 37*, 57–63.

Korytko, P.J., Quimby, F.W., Scott, J.G., **2000b**. Metabolism of phenanthrene by housefly CYP6D1 and dog liver cytochrome P450. *J. Biochem. Mol. Toxicol. 14*, 20–25.

Kotze, A.C., **1995**. Induced insecticide tolerance in larvae of *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) following dietary phenobarbital treatment. *J. Austral. Entomol. Soc. 34*, 205–209.

Kotze, A.C., Sales, N., **1995**. Elevated in vitro monooxygenase activity associated with insecticide resistances in field-strain larvae of the Australian sheep blowfly (Diptera: Calliphoridae). *J. Econ. Entomol. 88*, 782–787.

Krieger, R.I., Feeny, P.P., Wilkinson, C.F., **1971**. Detoxication enzymes in the guts of caterpillars: an evolutionary answer to plant defenses? *Science 172*, 579–581.

Kula, M.E., Rozek, C.E., **2000**. Expression and translocation of *Drosophila* nuclear encoded cytochrome b(5) proteins to mitochondria. *Insect Biochem. Mol. Biol. 30*, 927–935.

Kula, M.E., Allay, E.R., Rozek, C.E., **1995**. Evolutionary divergence of the cytochrome b_5 gene of *Drosophila*. *J. Mol. Evol. 41*, 430–439.

Latli, B., Prestwich, G.D., **1991**. Metabolically blocked analogs of housefly sex pheromone: I. Synthesis of alternative substrates for the cuticular monooxygenases. *J. Chem. Ecol. 17*, 1745–1768.

Le Goff, G., Boundy, S., Daborn, P.J., Yen, J.L., Sofer, L., *et al.*, **2003**. Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*. *Insect Biochem. Mol. Biol. 33*, 701–708.

Li, X., Berenbaum, M.R., Schuler, M.A., **2000a**. Molecular cloning and expression of CYP6B8: a xanthotoxin-inducible cytochrome P450 cDNA from *Helicoverpa zea*. *Insect Biochem. Mol. Biol. 30*, 75–84.

Li, X., Berenbaum, M.R., Schuler, M.A., **2002c**. Plant allelochemicals differentially regulate *Helicoverpa zea* cytochrome P450 genes. *Insect Mol. Biol. 11*, 343–351.

Li, W., Berenbaum, M.R., Schuler, M.A., **2001**. Molecular analysis of multiple CYP6B genes from polyphagous

Papilio species. *Insect Biochem. Mol. Biol. 31*, 999–1011.

Li, X., Berenbaum, M.R., Schuler, M.A., **2002b**. Cytochrome P450 and actin genes expressed in *Helicoverpa zea* and *Helicoverpa armigera*: paralogy/orthology identification, gene conversion and evolution. *Insect Biochem. Mol. Biol. 32*, 311–320.

Li, W., Petersen, R.A., Schuler, M.A., Berenbaum, M.R., **2002a**. CYP6B cytochrome P450 monooxygenases from *Papilio canadensis* and *Papilio glaucus*: potential contributions of sequence divergence to host plant associations. *Insect Mol. Biol. 11*, 543–551.

Li, X., Schuler, M.A., Berenbaum, M.R., **2002d**. Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes. *Nature 419*, 712–715.

Li, W., Schuler, M.A., Berenbaum, M.R., **2003**. Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: specificity and substrate encounter rate. *Proc. Natl Acad. Sci. USA 100*, 14593–14598.

Li, X., Zangerl, A.R., Schuler, M.A., Berenbaum, M., **2000b**. Cross-resistance to α-cypermethrin after xanthotoxin ingestion in *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Econ. Entomol. 93*, 18–25.

Liebrich, W., Hoffmann, K.H., **1991**. Ecdysone 20-monooxygenase in a cricket, *Gryllus bimaculatus* (Ensifera, Gryllidae): characterization of the microsomal midgut steroid hydroxylase in adult females. *J. Comp. Physiol. B 161*, 93–99.

Lindigkeit, R., Biller, A., Buch, M., Schiebel, H.M., Boppre, M., *et al.*, **1997**. The two facies of pyrrolizidine alkaloids: the role of the tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. *Eur. J. Biochem. 245*, 626–636.

Lindroth, R.L., **1989**. Host plant alteration of detoxication activity in *Papilio glaucus glaucus*. *Entomol. Exp. Appl. 50*, 29–35.

Lindroth, R.L., **1991**. Differential toxicity of plant allelochemicals to insects: roles of enzymatic detoxication systems. In: Bernays, E.A. (Ed.), Insect–Plant Interactions, vol. 3. CRC Press, Boca Raton, pp. 1–33.

Lindsley, D.L., Zimm, G.G., **1992**. The Genome of *Drosophila melanogaster.* Academic Press, San Diego.

Liu, N., Scott, J.G., **1995**. Genetics of resistance to pyrethroid insecticides in the housefly, *Musca domestica*. *Pestic. Biochem. Physiol. 52*, 116–124.

Liu, N., Scott, J.G., **1996**. Genetic analysis of factors controlling high-level expression of cytochrome P450, CYP6D1, cytochrome b_5, P450 reductase, and monooxygenase activities in LPR house flies, *Musca domestica*. *Biochem. Genet. 34*, 133–148.

Liu, N., Scott, J.G., **1997a**. Inheritance of CYP6D1-mediated pyrethroid resistance in housefly (Diptera: Muscidae). *J. Econ. Entomol. 90*, 1478–1481.

Liu, N., Scott, J.G., **1997b**. Phenobarbital induction of CYP6D1 is due to a trans acting factor on autosome 2 in house flies, *Musca domestica*. *Insect Mol. Biol. 6*, 77–81.

Liu, N., Scott, J.G., **1998**. Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in housefly. *Insect Biochem. Mol. Biol. 28*, 531–535.

Liu, N., Tomita, T., Scott, J.G., **1995**. Allele-specific PCR reveals that CYP6D1 is on chromosome 1 in the housefly, *Musca domestica*. *Experientia 51*, 164–167.

Liu, N., Yue, X., **2001**. Genetics of pyrethroid resistance in a strain (ALHF) of house flies (Diptera: Muscidae). *Pestic. Biochem. Physiol. 70*, 151–158.

Liu, N., Zhang, L., **2002**. Identification of two new cytochrome P450 genes and their 5′-flanking regions from the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol. 32*, 755–764.

Lockey, K.H., **1991**. Insect hydrocarbon classes: implications for chemotaxonomy. *Insect Biochem. 21*, 91–97.

Lu, K.H., Bradfield, J.Y., Keeley, L.L., **1995**. Hypertrehalosemic hormone-regulated gene expression for cytochrome P4504C1 in the fat body of the Cockroach, *Blaberus discoidalis*. *Arch. Insect Biochem. Physiol. 28*, 79–90.

Lu, K.H., Bradfield, J.Y., Keeley, L.L., **1999**. Juvenile hormone inhibition of gene expression for cytochrome P4504C1 in adult females of the cockroach, *Blaberus discoidalis*. *Insect Biochem. Mol. Biol. 29*, 667–673.

Luu, B., Werner, F., **1996**. Sterols that modify moulting in insects. *Pestic. Sci. 46*, 49–53.

Lynch, M., Conery, J.S., **2000**. The evolutionary fate and consequences of duplicate genes. *Science 290*, 1151–1155.

Ma, R., Cohen, M.B., Berenbaum, M.R., Schuler, M.A., **1994**. Black swallowtail (*Papilio polyxenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins. *Arch. Biochem. Biophys. 310*, 332–340.

Maibeche-Coisne, M., Jacquin-Joly, E., Francois, M.C., Nagnan-Le Meillour, P., **2002**. cDNA cloning of biotransformation enzymes belonging to the cytochrome P450 family in the antennae of the noctuid moth *Mamestra brassicae*. *Insect Mol. Biol. 11*, 273–281.

Maibeche-Coisne, M., Monti-Dedieu, L., Aragon, S., Dauphin-Villemant, C., **2000**. A new cytochrome P450 from *Drosophila melanogaster*, CYP4G15, expressed in the nervous system. *Biochem. Biophys. Res. Commun. 273*, 1132–1137.

Maitra, S., Dombrowski, S.M., Basu, M., Raustol, O., Waters, L.C., *et al.*, **2000**. Factors on the third chromosome affect the level of *Cyp6a2* and *Cyp6a8* expression in *Drosophila melanogaster. Gene 248*, 147–156.

Maitra, S., Dombrowski, S.M., Waters, L.C., Ganguly, R., **1996**. Three second chromosome-linked clustered Cyp6 genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster. Gene 180*, 165–171.

Maitra, S., Price, C., Ganguly, R., **2002**. *Cyp6a8* of *Drosophila melanogaster*: gene structure, and sequence and

functional analysis of the upstream DNA. *Insect Biochem. Mol. Biol. 32*, 859–870.

Mansuy, D., **1998**. The great diversity of reactions catalyzed by cytochrome P450. *Comp. Biochem. Physiol. 121C*, 5–14.

Mansuy, D., Renaud, J.P., **1995**. Heme-thiolate proteins different from cytochromes P450 catalyzing monooxygenations. In: Ortiz de Montellano, P.R. (Ed.), Cytochrome P450. Plenum, New York and London, pp. 537–574.

Martin, T., Ochou, O.G., Vaissayre, M., Fournier, D., **2003**. Oxidases responsible for resistance to pyrethroids sensitize *Helicoverpa armigera* (Hubner) to triazophos in West Africa. *Insect Biochem. Mol. Biol. 33*, 883–887.

Matsunaga, E., Umeno, M., Gonzalez, F.J., **1990**. The rat P450 IID subfamily: complete sequences of four closely linked genes and evidence that gene conversions maintained sequence homogeneity at the heme-binding region of the cytochrome P450 active site. *J. Mol. Evol. 30*, 155–169.

Mauchamp, B., Darrouzet, E., Malosse, C., Couillaud, F., **1999**. 4′-OH-JH-III: an additional hydroxylated juvenile hormone produced by locust corpora allata in vitro. *Insect Biochem. Mol. Biol. 29*, 475–480.

McDonald, M.J., Rosbash, M., **2001**. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell 107*, 567–578.

Megias, A., Saborido, A., Muncio, A.M., **1984**. NADH-cytochrome b_5 reductase from the insect *Ceratitis capitata*. Enzyme properties and membrane binding capacity. *Comp. Biochem. Physiol. 77B*, 679–685.

Miota, F., Siegfried, B.D., Scharf, M.E., Lydy, M.J., **2000**. Atrazine induction of cytochrome P450 in *Chironomus tentans* larvae. *Chemosphere 40*, 285–291.

Misra, S., Crosby, M.A., Mungall, C.J., Matthews, B.B., Campbell, K.S., *et al.*, **2002**. Annotation of the *Drosophila melanogaster* euchromatic genome: a systematic review. *Genome Biol. 3*, 83.1–83.22.

Mitlin, N., Konecky, M.S., **1955**. The inhibition of development in the housefly by piperonyl butoxide. *J. Econ. Entomol. 48*, 93–94.

Moldenke, A.F., Berry, R.E., Miller, J.C., Kelsey, R.G., Wernz, J.G., *et al.*, **1992**. Carbaryl susceptibility and detoxication enzymes in gypsy moth (Lepidoptera: Lymantriidae): influence of host plant. *J. Econ. Entomol. 85*, 1628–1635.

Mpuru, S., Blomquist, G.J., Schal, C., Roux, M., Kuenzli, M., *et al.*, **2001**. Effect of age and sex on the production of internal and external hydrocarbons and pheromones in the housefly, *Musca domestica*. *Insect Biochem. Mol. Biol. 31*, 139–155.

Mpuru, S., Reed, J.R., Reitz, R.C., Blomquist, G.J., **1996**. Mechanism of hydrocarbon biosynthesis from aldehyde in selected insect species: requirement for O_2 and NADPH and carbonyl group released as CO_2. *Insect Biochem. Mol. Biol. 26*, 203–208.

Mullin, C.A., **1986**. Adaptive divergence of chewing and sucking arthropods to plant allelochemicals. In: Brattsten, L.B., Ahmad, S. (Eds.), Molecular Aspects of Insect–Plant Interactions. Plenum, New York, pp. 175–209.

Mullin, C.A., Croft, B.A., Strickler, K., Matsumura, F., Miller, J.R., **1982**. Detoxification enzyme differences between a herbivorous and predatory mite. *Science 217*, 1270–1272.

Murataliev, M.B., Ariño, A., Guzov, V.M., Feyereisen, R., **1999**. Kinetic mechanism of cytochrome P450 reductase from the housefly (*Musca domestica*). *Insect Biochem. Mol. Biol. 29*, 233–242.

Murataliev, M.B., Feyereisen, R., **1999**. Mechanism of cytochrome P450 reductase from the housefly: evidence for an FMN semiquinone as electron donor. *FEBS Lett. 453*, 201–204.

Murataliev, M.B., Feyereisen, R., **2000**. Interaction of NADP(H) with oxidized and reduced P450 reductase during catalysis. Studies with nucleotide analogues. *Biochemistry 39*, 5066–5074.

Murataliev, M.B., Feyereisen, R., Walker, F.A., **2004a**. Electron transfer by diflavin reductases. *Biochem. Biophys. Acta 1698*, 1–26.

Murataliev, M.B., Trinh, L.N., Moser, L.V., Bates, R.B., Feyereisen, R., *et al.*, **2004b**. Chimeragenesis of the fatty acid binding site of cytochrome P450BM3. Replacement of residues 73–84 with the homologous residues from the insect cytochrome P450 CYP4C7. *Biochemistry 43*, 1771–1780.

Mutch, E., Daly, A.K., Leathart, J.B., Blain, P.G., Williams, F.M., **2003**. Do multiple cytochrome P450 isoforms contribute to parathion metabolism in man? *Arch. Toxicol. 77*, 313–320.

Nahri, L.O., Fulco, A.J., **1986**. Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem. 261*, 7160–7169.

Nahrstedt, A., **1988**. Cyanogenesis and the role of cyanogenic compounds in insects. *Ciba Found. Symp. 140*, 131–150.

Nakayama, N., Takemae, A., Shoun, H., **1996**. Cytochrome P450foxy, a catalytically self-sufficient fatty acid hydroxylase of the fungus *Fusarium oxysporum*. *J. Biochem. 119*, 435–440.

Naumann, C., Hartmann, T., Ober, D., **2002**. Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxication of host plant-acquired pyrrolizidine alkaloid-defended arctiid moth *Tyria Jacobaeae*. *Proc. Natl Acad. Sci. USA 99*, 6085–6090.

Neal, J.J., **1987**. Metabolic costs of mixed-function oxidase induction in *Heliothis zea*. *Entomol. Exp. Appl. 43*, 175–179.

Neal, J.J., Berenbaum, M.R., **1989**. Decreased sensitivity of mixed-function oxidases from *Papilio polyxenes* to inhibitors in host plants. *J. Chem. Ecol. 15*, 439–446.

Neal, J.J., Reuveni, M., **1992**. Separation of cytochrome P450 containing vesicles from the midgut microsomal fraction of *Manduca sexta*. *Comp. Biochem. Physiol. 102C*, 77–82.

Neal, J.J., Wu, D., **1994**. Inhibition of insect cytochromes P450 by furanocoumarins. *Pestic. Biochem. Physiol. 50*, 43–50.

Nebert, D.W., **1991**. Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. *Mol. Endocrinol. 5*, 1203–1214.

Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., *et al.*, **1987**. The P450 gene superfamily: recommended nomenclature. *DNA 6*, 1–11.

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., *et al.*, **1991**. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature [published erratum appears in DNA Cell Biol. 1991 Jun;10(5):397–398]. *DNA Cell Biol. 10*, 1–14.

Nelson, D.R., **1998**. Metazoan cytochrome P450 evolution. *Comp. Biochem. Physiol. 121C*, 15–22.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., *et al.*, **1993**. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol. 12*, 1–51.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., *et al.*, **1996**. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics 6*, 1–42.

Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J., *et al.*, **1997**. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc. Natl Acad. Sci. USA 94*, 7464–7468.

Newcomb, M., Hollenberg, P.F., Coon, M.J., **2003**. Multiple mechanisms and multiple oxidants in P450-catalyzed hydroxylations. *Arch. Biochem. Biophys. 409*, 72–79.

Nikou, D., Ranson, H., Hemingway, J., **2003**. An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene 318*, 91–102.

Nitao, J.K., **1989**. Enzymatic adaptation in a specialist herbivore for feeding on furanocoumarin-containing plants. *Ecology 70*, 629–635.

Nitao, J.K., **1990**. Metabolism and excretion of the furanocoumarin xanthotoxin by parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol. 16*, 417–428.

Nitao, J.K., Berhow, M., Duval, S.M., Weisleder, D., Vaughn, S.F., *et al.*, **2003**. Characterization of furanocoumarin metabolites in parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol. 29*, 671–682.

Oakeshott, J.G., Horne, I., Sutherland, T.D., Russell, R.J., **2003**. The genomics of insecticide resistance. *Genome Biol. 4*, 202.

Oesch-Bartlomowicz, B., Oesch, F., **2003**. Cytochrome P450 phosphorylation as a functional switch. *Arch. Biochem. Biophys. 409*, 228–234.

Oi, M., Dauterman, W.C., Motoyama, N., **1990**. Biochemical factors responsible for an extremely high level of diazinon resistance in a housefly strain. *J. Pestic. Sci. 15*, 217–224.

Omura, T., **1993**. History of cytochrome P450. In: Omura, T., Ishimura, Y., Fujii-Kuriyama, Y. (Eds.), Cytochrome P-450. Kodansha, Tokyo, pp. 1–15.

Omura, T., Ishimura, Y., Fujii-Kuriyama, Y. (Eds.), **1993**. Cytochrome P-450. Kodansha, Tokyo.

Omura, T., Sato, R., **1964**. The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemoprotein nature. *J. Biol. Chem. 239*, 2370–2378.

Ortiz de Montellano, P.R., **1995a**. Oxygen activation and reactivity. In: Ortiz de Montellano, P.R. (Ed.), Cytochrome P450. Plenum, New York and London, pp. 245–303.

Ortiz de Montellano, P.R. (Ed.), **1995b**. Cytochrome P450, Structure, Mechanism, and Biochemistry. Plenum, New York and London.

Ortiz de Montellano, P.R., Correia, M.A., **1995**. Inhibition of cytochrome P450 enzymes. In: Ortiz de Montellano, P.R. (Ed.), Cytochrome P450. Plenum, New York and London, pp. 305–364.

Ortiz de Montellano, P.R., Graham-Lorence, S.E., **1993**. Structure of cytochrome P450: heme-binding site and heme reactivity. In: Schenkman, J.B., Greim, H. (Eds.), Cytochrome P450. Springer, Berlin, pp. 169–181.

Paquette, S.M., Bak, S., Feyereisen, R., **2000**. Intron-exon organization and phylogeny in a large superfamily, the paralogous cytochrome P450 genes of *Arabidopsis thaliana*. *DNA Cell Biol. 19*, 307–317.

Pare, P.W., Alborn, H.T., Tumlinson, J.H., **1998**. Concerted biosynthesis of an insect elicitor of plant volatiles. *Proc. Natl Acad. Sci. USA 95*, 13971–13975.

Pauli, D., Tonka, C.H., **1987**. A *Drosophila* heat shock gene from locus 67B is expressed during embryogenesis and pupation. *J. Mol. Biol. 198*, 235–240.

Petersen, R.A., Niamsup, H., Berenbaum, M.R., Schuler, M.A., **2003**. Transcriptional response elements in the promoter of CYP6B1, an insect P450 gene regulated by plant chemicals. *Biochimica et Biophysica Acta (BBA) – General Subjects 1619*, 269–282.

Petersen, R.A., Zangerl, A.R., Berenbaum, M.R., Schuler, M.A., **2001**. Expression of CYP6B1 and CYP6B3 cytochrome P450 monooxygenases and furanocoumarin metabolism in different tissues of *Papilio polyxenes* (Lepidoptera: Papilionidae). *Insect Biochem. Mol. Biol. 31*, 679–690.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., *et al.*, **2003**. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778.

Pikuleva, I.A., Cao, C., Waterman, M.R., **1999**. An additional electrostatic interaction between adrenodoxin and P450c27 (CYP27A1) results in tighter binding than between adrenodoxin and P450scc (CYP11A1). *J. Biol. Chem. 274*, 2045–2052.

Pikuleva, I.A., Mackman, R.L., Kagawa, N., Waterman, M.R., Ortiz de Montellano, P.R., **1995**. Active-site

topology of bovine cholesterol side-chain cleavage cytochrome P450 (P450scc) and evidence for interaction of tyrosine 94 with the side chain of cholesterol. *Arch. Biochem. Biophys. 322*, 189–197.

Pimprale, S.S., Besco, C.L., Bryson, P.K., Brown, T.M., **1997**. Increased susceptibility of pyrethroid-resistant tobacco budworm (Lepidoptera: Noctuidae) to chlorfenapyr. *J. Econ. Entomol. 90*, 49–54.

Pittendrigh, B., Aronstein, K., Zinkovsky, E., Andreev, O., Campbell, B., *et al.*, **1997**. Cytochrome P450 genes from *Helicoverpa armigera*: expression in a pyrethroid-susceptible and resistant strain. *Insect Biochem. Mol. Biol. 27*, 507–512.

Plapp, F.W., Jr., **1984**. The genetic basis of insecticide resistance in the housefly: evidence that a single locus plays a major role in metabolic resistance to insecticides. *Pestic. Biochem. Physiol. 22*, 94–201.

Plettner, E., Slessor, K.N., Winston, M.L., **1998**. Biosynthesis of mandibular acids in honey bees (*Apis mellifera*): De novo synthesis, route of fatty acid hydroxylation and caste selective β-oxidation. *Insect Biochem. Mol. Biol. 28*, 31–42.

Pompon, D., Louerat, B., Bronine, A., Urban, P., **1996**. Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol. 272*, 51–64.

Porter, T.D., **2002**. The roles of cytochrome b_5 in cytochrome P450 reactions. *J. Biochem. Mol. Toxicol. 16*, 311–316.

Porter, T.D., Kasper, C.B., **1986**. NADPH-cytochrome P-450 oxidoreductase: flavin mononucleotide and flavin adenine dinucleotide domains evolved from different flavoproteins. *Biochemistry 25*, 1682–1687.

Poulos, T.L., Cupp-Vickery, J., Li, H., **1995**. Structural studies on prokaryotic cytochromes P450. In: Ortiz de Montellano, P.R. (Ed.), Cytochrome P450. Plenum, New York and London, pp. 125–150.

Poulos, T.L., Finzel, B.C., Gunsalus, I.C., Wagner, G.C., Kraut, J., **1985**. The 2.6-A crystal structure of *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem. 260*, 16122–16130.

Prapaipong, H., Berenbaum, M.R., Schuler, M.A., **1994**. Transcriptional regulation of the *Papilio polyxenes* CYP6B1 gene. *Nucleic Acids Res. 22*, 3210–3217.

Pratt, G.E., Kuwano, E., Farnsworth, D.E., Feyereisen, R., **1990**. Structure/activity studies on 1,5-disubstituted imidazoles as inhibitors of juvenile hormone biosynthesis in isolated corpora allata of the cockroach, *Diploptera punctata*. *Pestic. Biochem. Physiol. 38*, 223–230.

Ranasinghe, C., Headlam, M., Hobbs, A.A., **1997**. Induction of the mRNA for CYP6B2, a pyrethroid inducible cytochrome P450, in *Helicoverpa armigera* (Hubner) by dietary monoterpenes. *Arch. Insect Biochem. Physiol. 34*, 99–109.

Ranasinghe, C., Hobbs, A.A., **1998**. Isolation and characterization of two cytochrome P450 cDNA clones for CYP6B6 and CYP6B7 from *Helicoverpa armigera* (Hubner): possible involvement of CYP6B7 in pyrethroid resistance. *Insect Biochem. Mol. Biol. 28*, 571–580.

Ranasinghe, C., Hobbs, A.A., **1999a**. Isolation and characterisation of a cytochrome b_5 cDNA clone from *Helicoverpa armigera* (Hubner): possible involvement of cytochrome b_5 in cytochrome P450 CYP6B7 activity towards pyrethroids. *Insect Biochem. Mol. Biol. 29*, 145–151.

Ranasinghe, C., Hobbs, A.A., **1999b**. Induction of cytochrome P450 CYP6B7 and cytochrome b_5 mRNAs from *Helicoverpa armigera* (Hubner) by pyrethroid insecticides in organ culture. *Insect Mol. Biol. 8*, 443–447.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., *et al.*, **2002a**. Evolution of supergene families associated with insecticide resistance. *Science 298*, 179–181.

Ranson, H., Nikou, D., Hutchinson, M., Wang, X., Roth, C.W., *et al.*, **2002b**. Molecular analysis of multiple cytochrome P450 genes from the malaria vector, *Anopheles gambiae*. *Insect Mol. Biol. 11*, 409–418.

Ravichandran, K.G., Boddupalli, S.S., Haseman, C.A., Peterson, J.A., Deisenhofer, J., **1993**. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science 261*, 731–736.

Reed, J.R., Quilici, D.R., Blomquist, G.J., Reitz, R.C., **1995**. Proposed mechanism for the cytochrome P450-catalyzed conversion of aldehydes to hydrocarbons in the housefly, *Musca domestica*. *Biochemistry 34*, 16221–16227.

Reed, J.R., Vanderwel, D., Choi, S., Pomonis, J.G., Reitz, R.C., *et al.*, **1994**. Unusual mechanism of hydrocarbon formation in the housefly: cytochrome P450 converts aldehyde to the sex pheromone component (Z)-9-tricosene and CO_2. *Proc. Natl Acad. Sci. USA 91*, 10000–10004.

Rees, H.H., **1995**. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol. 92*, 9–39.

Ringo, J., Jona, G., Rockwell, R., Segal, D., Cohen, E., **1995**. Genetic variation for resistance to chlorpyrifos in *Drosophila melanogaster* (Diptera: Drosophilidae) infesting grapes in Israel. *J. Econ. Entomol. 88*, 1158–1163.

Riskallah, M.R., Dauterman, W.C., Hodgson, E., **1986a**. Nutritional effects on the induction of cytochrome P-450 and glutathione transferase in larvae of the tobacco budworm, *Heliothis virescens* (F.). *Insect Biochem. 16*, 491–499.

Riskallah, M.R., Dauterman, W.C., Hodgson, E., **1986b**. Host plant induction of microsomal monooxygenases activity in relation to diazinon metabolism and toxicity in larvae of the tobacco budworm *Heliothis virescens* (F.). *Pestic. Biochem. Physiol. 25*, 233–247.

Robertson, H.M., Martos, R., Sears, C.R., Todres, E.Z., Walden, K.K., *et al.*, **1999**. Diversity of odourant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol. 8*, 501–518.

Ronis, M.J.J., Hodgson, E., Dauterman, W.C., **1988**. Characterization of multiple forms of cytochrome P-450 from an insecticide resistant strain of housefly (*Musca domestica*). *Pestic. Biochem. Physiol. 32*, 74–90.

Rose, H.A., **1985**. The relationship between feeding specialization and host plants to aldrin epoxidase activities of midgut homogenates in larval Lepidoptera. *Ecol. Entomol. 10*, 455–467.

Rose, R.L., Barbhaiya, L., Roe, R.M., Rock, G.C., Hodgson, E., **1995**. Cytochrome P450-associated insecticide resistance and the development of biochemical diagnostic assays in *Heliothis virescens*. *Pestic. Biochem. Physiol. 51*, 178–191.

Rose, R.L., Goh, D., Thompson, D.M., Verma, K.D., Heckel, D.G., *et al.*, **1997**. Cytochrome P450 (CYP)9A1 in *Heliothis virescens*: the first member of a new CYP family. *Insect Biochem. Mol. Biol. 27*, 605–615.

Rose, R.L., Gould, F., Levi, P.E., Hodgson, E., **1991**. Differences in cytochrome P450 activities in tobacco budworm larvae as influenced by resistance to host plant allelochemicals and induction. *Comp. Biochem. Physiol. 99B*, 535–540.

Sabourault, C., Guzov, V.M., Koener, J.F., Claudianos, C., Plapp, F.W., Jr., *et al.*, **2001**. Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (MdαE7) gene in resistant house flies. *Insect Mol. Biol. 10*, 609–618.

Salzemann, A., Nagnan, P., Tellier, F., Jaffe, K., **1992**. Leaf-cutting ant *Atta laevigata* (Formicidae: Attini) marks its territory with colony-specific Dufour gland secretion. *J. Chem. Ecol. 18*, 183.

Saner, C., Weibel, B., Wurgler, F.E., Sengstag, C., **1996**. Metabolism of promutagens catalyzed by *Drosophila melanogaster* CYP6A2 enzyme in *Saccharomyces cerevisiae*. *Environ. Mol. Mutagen. 27*, 46–58.

Scharf, M.E., Lee, C.Y., Neal, J.J., Bennett, G.W., **1999**. Cytochrome P450 MA expression in insecticide-resistant German cockroaches (Dictyoptera: Blattellidae). *J. Econ. Entomol. 92*, 788–793.

Scharf, M.E., Neal, J.J., Marcus, C.B., Bennett, G.W., **1998**. Cytochrome P450 purification and immunological detection in an insecticide resistant strain of German cockroach (*Blattella germanica*, L.). *Insect Biochem. Mol. Biol. 28*, 1–9.

Scharf, M.E., Parimi, S., Meinke, L.J., Chandler, L.D., Siegfried, B.D., **2001**. Expression and induction of three family 4 cytochrome P450 (CYP4)* genes identified from insecticide-resistant and susceptible western corn rootworms, *Diabrotica virgifera virgifera*. *Insect Mol. Biol. 10*, 139–146.

Scharf, M., Siegrfried, B.D., Meinke, L.J., Chandler, L.D., **2000**. Fipronil metabolism, oxidative sulfone formation and toxicity among organophosphate- and carbamate-resistant and susceptible western corn rootworm populations. *Pest Manag. Sci. 56*, 757–766.

Schenkman, J.B., Greim, H. (Eds.), **1993**. Cytochrome P450. Springer, Berlin.

Schenkman, J.B., Jansson, I., **2003**. The many roles of cytochrome b_5. *Pharmacol. Ther. 97*, 139–152.

Schlenke, T.A., Begun, D.J., **2004**. Strong selective sweep associated with a transposon insertion in *Drosophila simulans*. *Proc. Natl Acad. Sci. USA 101*, 1626–1631.

Schlichting, I., Berendzen, J., Chu, K., Stock, A.M., Maves, S.A., *et al.*, **2000**. The catalytic pathway of cytochrome P450cam at atomic resolution. *Science 287*, 1615–1622.

Schonbrod, R.D., Khan, M.A.Q., Terriere, L.C., Plapp, F.W., Jr., **1968**. Microsomal oxidases in the housefly: a survey of fourteen strains. *Life Sci. 7*, 681–688.

Schrag, M.L., Wienkers, L.C., **2000**. Topological alteration of the CYP3A4 active site by the divalent cation $Mg^{(2+)}$. *Drug Metab. Disposition 28*, 1198–1201.

Schuler, M.A., Werck-Reichhart, D., **2003**. Functional genomics of P450s. *Ann. Rev. Plant Biol. 54*, 629–667.

Scott, J.G., **1999**. Cytochromes P450 and insecticide resistance. *Insect Biochem. Mol. Biol. 29*, 757–777.

Scott, J.A., Collins, F.H., Feyereisen, R., **1994**. Diversity of cytochrome P450 genes in the mosquito, *Anopheles albimanus*. *Biochem. Biophys. Res. Commun. 205*, 1452–1459.

Scott, J.G., Foroozesh, M., Hopkins, N.E., Alefantis, T.G., Alworth, W.L., **2000**. Inhibition of cytochrome P450 6D1 by alkynylarenes, methylenedioxyarenes, and other subsituted aromatics. *Pestic. Biochem. Physiol. 67*, 63–71.

Scott, J.G., Lee, S.S., **1993a**. Tissue distribution of microsomal cytochrome P-450 monooxygenases and their inducibility by phenobarbital in the insecticide resistant LPR strain of housefly, *Musca domestica* L. *Insect Biochem. Mol. Biol. 23*, 729–738.

Scott, J.G., Lee, S.S., **1993b**. Purification and characterization of a cytochrome P-450 from insecticide susceptible and resistant strains of housefly, *Musca domestica* L., before and after phenobarbital exposure. *Arch. Insect Biochem. Physiol. 24*, 1–19.

Scott, J.G., Liu, N., Wen, Z., Smith, F.F., Kasai, S., *et al.*, **1999**. House-fly cytochrome P450 CYP6D1: 5′ flanking sequences and comparison of alleles. *Gene 226*, 347–353.

Scott, J.G., Sridhar, P., Liu, N., **1996**. Adult specific expression and induction of cytochrome P450lpr in house flies. *Arch. Insect Biochem. Physiol. 31*, 313–323.

Scott, J.G., Zhang, L., **2003**. The housefly aliesterase gene (MdαE7) is not associated with insecticide resistance or P450 expression in three strains of housefly. *Insect Biochem. Mol. Biol. 33*, 139–144.

Scrimshaw, S., Kerfoot, W.C., **1987**. Chemical defenses of freshwater organisms: beetles and bugs. In: Kerfoot, W.C., Sih, A. (Eds.), Predation: Direct and Indirect Impacts on Aquatic Communities. University Press of New England, London, pp. 240–262.

Seifert, J., Scott, J.G., **2002**. The CYP6D1v1 allele is associated with pyrethroid resistance in the housefly, *Musca domestica*. *Pestic. Biochem. Physiol. 72*, 40–44.

Shaw, G.C., Fulco, A.J., **1993**. Inhibition by barbiturates of the binding of Bm3R1 repressor to its operator site

on the barbiturate-inducible cytochrome P450BM-3 gene of *Bacillus megaterium*. *J. Biol. Chem. 268*, 2997–3004.

Shen, B., Dong, H.Q., Tian, H.S., Ma, L., Li, X.L., *et al.*, **2003**. Cytochrome P450 genes expressed in the deltamethrin-susceptible and -resistant strains of *Culex pipiens pallens*. *Pestic. Biochem. Physiol. 75*, 19–26.

Sheppard, D.G., Joyce, J.A., **1998**. Increased susceptibility of pyrethroid-resistant horn flies (Diptera: Muscidae) to chlorfenapyr. *J. Econ. Entomol. 91*, 398–400.

Shergill, J.K., Cammack, R., Chen, J.H., Fisher, M.J., Madden, S., *et al.*, **1995**. EPR spectroscopic characterization of the iron-sulphur proteins and cytochrome P-450 in mitochondria from the insect *Spodoptera littoralis* (cotton leafworm). *Biochem. J. 307*, 719–728.

Shono, T., Kasai, S., Kamiya, E., Kono, Y., Scott, J.G., **2002**. Genetics and mechanisms of permethrin resistance in the YPER strain of housefly. *Pestic. Biochem. Physiol. 73*, 27–36.

Smith, S.L., **1985**. Regulation of ecdysteroid titer: synthesis. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 295–341.

Smith, S.L., Bollenbacher, W.E., Cooper, D.Y., Schleyer, H., Wielgus, J.J., *et al.*, **1979**. Ecdysone 20-monooxygenase: characterization of an insect cytochrome P-450 dependent steroid hydroxylase. *Mol. Cell. Endocrinol. 15*, 111–133.

Smith, F.F., Scott, J.G., **1997**. Functional expression of housefly (*Musca domestica*) cytochrome P450 CYP6D1 in yeast (*Saccharomyces cerevisiae*). *Insect Biochem. Mol. Biol. 27*, 999–1006.

Snyder, M.J., Glendinning, J.I., **1996**. Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A 179*, 255–261.

Snyder, M.J., Hsu, E.-L., Feyereisen, R., **1993**. Induction of cytochrome P450 activities by nicotine in the tobacco hornworm, *Manduca sexta*. *J. Chem. Ecol. 19*, 2903–2916.

Snyder, M.J., Scott, J.A., Andersen, J.F., Feyereisen, R., **1996**. Sampling P450 diversity by cloning polymerase chain reaction products obtained with degenerate primers. *Methods Enzymol. 272*, 304–312.

Snyder, M.J., Stevens, J.L., Andersen, J.F., Feyereisen, R., **1995**. Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*. *Arch. Biochem. Biophys. 321*, 13–20.

Snyder, M.J., Walding, J.K., Feyereisen, R., **1994**. Metabolic fate of the allelochemical nicotine in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 837–846.

Spellman, P.T., Rubin, G.M., **2002**. Evidence for large domains of similarly expressed genes in the *Drosophila* genome. *J. Biol. 1*, 5.

Spiegelman, V.S., Fuchs, S.Y., Belitsky, G.A., **1997**. The expression of insecticide resistance-related cytochrome P450 forms is regulated by molting hormone in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun. 232*, 304–307.

Stathopoulos, A., Van Drenth, M., Erives, A., Markstein, M., Levine, M., **2002**. Whole-genome analysis of dorsal–ventral patterning in the *Drosophila* embryo. *Cell 111*, 687–701.

Stevens, J.L., Snyder, M.J., Koener, J.F., Feyereisen, R., **2000**. Inducible P450s of the CYP9 family from larval *Manduca sexta* midgut. *Insect Biochem. Mol. Biol. 30*, 559–568.

Stoilov, I., Akarsu, A.N., Sarfarazi, M., **1997**. Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum. Mol. Genet. 6*, 641–647.

Sundseth, S.S., Nix, C.E., Waters, L.C., **1990**. Isolation of insecticide resistance-related forms of cytochrome P-450 from *Drosophila melanogaster*. *Biochem. J. 265*, 213–217.

Sutherland, T.D., Unnithan, G.C., Andersen, J.F., Evans, P.H., Murataliev, M.B., *et al.*, **1998**. A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. *Proc. Natl Acad. Sci. USA 95*, 12884–12889.

Sutherland, T.D., Unnithan, G.C., Feyereisen, R., **2000**. Terpenoid omega-hydroxylase (CYP4C7) messenger RNA levels in the corpora allata: a marker for ovarian control of juvenile hormone synthesis in *Diploptera punctata*. *J. Insect Physiol. 46*, 1219–1227.

Suwanchaichinda, C., Brattsten, L.B., **2002**. Induction of microsomal cytochrome P450s by tire-leachate compounds, habitat components of *Aedes albopictus* mosquito larvae. *Arch. Insect Biochem. Physiol. 49*, 71–79.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., *et al.*, **2000**. Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. *Nat. Biotechnol. 18*, 81–84.

Tares, S., Berge, J., Amichot, M., **2000**. Cloning and expression of cytochrome P450 genes belonging to the CYP4 family and to a novel family, CYP48, in two hymenopteran insects, *Trichogramma cacoeciae* and *Apis mellifera*. *Biochem. Biophys. Res. Commun. 268*, 677–682.

Taylor, M., Feyereisen, R., **1996**. Molecular biology and evolution of resistance of toxicants. *Mol. Biol. Evol. 13*, 719–734.

Terriere, L.C., Yu, S.J., **1974**. The induction of detoxifying enzymes in insects. *J. Agric. Food Chem. 22*, 366–373.

Thomas, J.D., Ottea, J.A., Boethel, D.J., Ibrahim, S., **1996**. Factors influencing pyrethroid resistance in a permethrin-resistant strain of the soybean looper, *Pseudoplusia includens* (Walker). *Pestic. Biochem. Physiol. 55*, 1–9.

Thompson, M., Svoboda, J., Lusby, W., Rees, H., Oliver, J., *et al.*, **1985**. Biosynthesis of a C21 steroid conjugate in an insect. The conversion of [^{14}C]cholesterol

to 5-[^{14}C]pregnen-3 beta, 20 beta-diol glucoside in the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem. 260*, 15410–15412.

Tijet, N., Helvig, C., Feyereisen, R., **2001**. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene 262*, 189–198.

Tomancak, P., Beaton, A., Weiszmann, R., Kwan, E., Shu, S., *et al.*, **2002**. Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol. 3*, 88.1–88.14.

Tomita, T., Liu, N., Smith, F.F., Sridhar, P., Scott, J.G., **1995**. Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. *Insect Mol. Biol. 4*, 135–140.

Tomita, T., Scott, J.G., **1995**. cDNA and deduced protein sequence of CYP6D1: the putative gene for a cytochrome P450 responsible for pyrethroid resistance in housefly. *Insect Biochem. Mol. Biol. 25*, 275–283.

Ueda, H.R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., *et al.*, **2002**. Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. *J. Biol. Chem. 277*, 14048–14052.

Ugaki, M., Shono, T., Fukami, J.I., **1985**. Metabolism of fenitrothion by organophosphorus-resistant and susceptible house flies, *Musca domestica* L. *Pestic. Biochem. Physiol. 23*, 33–40.

Unnithan, G.C., Andersen, J.F., Hisano, T., Kuwano, E., Feyereisen, R., **1995**. Inhibition of juvenile hormone biosynthesis and methyl farnesoate epoxidase activity by 1,5-disubstituted imidazoles in the cockroach, *Diploptera punctata*. *Pestic. Sci. 43*, 13–19.

Valles, S.M., Koehler, P.G., Brenner, R.J., **1997**. Antagonism of fipronil toxicity by piperonyl butoxide and *S,S,S*-tributyl phosphorotrithioate in the German cockroach (Dictyoptera: Blattelliidae). *J. Econ. Entomol. 90*, 1254–1258.

Wada, A., Waterman, M.R., **1992**. Identification by site-directed mutagenesis of two lysine residues in cholesterol side chain cleavage cytochrome P450 that are essential for adrenodoxin binding. *J. Biol. Chem. 267*, 22877–22882.

Walsh, J.B., **1987**. Sequence-dependent gene conversion: can duplicated genes diverge fast enough to escape conversion? *Genetics 117*, 543–557.

Wang, L., Bieber Urbauer, R.J., Urbauer, J.L., Benson, D.R., **2003**. Housefly cytochrome b_5 exhibits kinetically trapped hemin and selectivity in hemin binding. *Biochem. Biophys. Res. Commun. 305*, 840–845.

Wang, Q., Hasan, G., Pikielny, C.W., **1999**. Preferential expression of biotransformation enzymes in the olfactory organs of *Drosophila melanogaster*, the antennae. *J. Biol. Chem. 274*, 10309–10315.

Wang, X.P., Hobbs, A.A., **1995**. Isolation and sequence analysis of a cDNA clone for a pyrethroid inducible cytochrome P450 from *Helicoverpa armigera*. *Insect Biochem. Mol. Biol. 25*, 1001–1009.

Wang, M., Roberts, D.L., Paschke, R., Shea, T.M., Masters, B.S., *et al.*, **1997**. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc. Natl Acad. Sci. USA 94*, 8411–8416.

Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048.

Warren, J.T., Wismar, J., Subrahmanyam, B., Gilbert, L.I., **2001**. Woc (without children) gene control of ecdysone biosynthesis in *Drosophila melanogaster*. *Mol. Cell. Endocrinol. 181*, 1–14.

Waters, L.C., Nix, C.E., **1988**. Regulation of insecticide resistance-related cytochrome P-450 expression in *Drosophila melanogaster*. *Pestic. Biochem. Physiol. 30*, 214–227.

Waters, L.C., Zelhof, A.C., Shaw, B.J., Ch'ang, L.Y., **1992**. Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila*. [published erratum appears in Proc. Natl Acad. Sci. USA 1992, 89, 12209]. *Proc. Natl Acad. Sci. USA 89*, 4855–4859.

Wei, P., Zhang, J., Egan-Hafley, M., Liang, S., Moore, D.D., **2000**. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature 407*, 920–923.

Weirich, G.F., Williams, V.P., Feldlaufer, M.F., **1996**. Ecdysone 20-hydroxylation in *Manduca sexta* midgut: kinetic parameters of mitochondrial and microsomal ecdysone 20-monooxygenases. *Arch. Insect Biochem. Physiol. 31*, 305–312.

Weller, G.L., Foster, G.G., **1993**. Genetic maps of the sheep blowfly *Lucilia cuprina*: linkage-group corelations with other dipteran genera. *Genome 36*, 495–506.

Wen, Z., Horak, C.E., Scott, J.G., **2001**. CYP9E2, CYP4C21 and related pseudogenes from German cockroaches, *Blattella germanica*: implications for molecular evolution, expression studies and nomenclature of P450s. *Gene 272*, 257–266.

Wen, Z., Pan, L., Berenbaum, M.B., Schuler, M.A., **2003**. Metabolism of linear and angular furanocoumarins by *Papilio polyxenes* CYP6B1 co-expressed with NADPH cytochrome P450 reductase. *Insect Biochem. Mol. Biol. 33*, 937–947.

Wen, Z., Scott, J.G., **2001a**. Cytochrome P450 CYP6L1 is specifically expressed in the reproductive tissues of adult male German cockroaches, *Blattella germanica* (L.). *Insect Biochem. Mol. Biol. 31*, 179–187.

Wen, Z., Scott, J.G., **2001b**. Cloning of two novel P450 cDNAs from German cockroaches, *Blattella germanica* (L.): CYP6K1 and CYP6J1. *Insect Mol. Biol. 10*, 131–137.

Werck-Reichhart, D., Feyereisen, R., **2000**. Cytochromes P450: a success story. *Genome Biol. 1*, 3003.1–3003.9.

Wheeler, G.S., Slansky, F., Jr., Yu, S.J., **1993**. Fall armyworm sensitivity to flavone: limited role of constitutive

and induced detoxifying enzyme activity. *J. Chem. Ecol. 19*, 645–667.

Wheelock, G.D., Scott, J.G., **1989**. Simultaneous purification of a cytochrome P-450 and cytochrome b_5 from the housefly, *Musca domestica* L. *Insect Biochem. 19*, 481–488.

Wheelock, G.D., Scott, J.G., **1990**. Immunological detection of cytochrome P450 from insecticide resistant and susceptible house flies (*Musca domestica*). *Pestic. Biochem. Physiol. 38*, 130–139.

Wheelock, G.D., Scott, J.G., **1992a**. Anti-P450lpr antiserum inhibits specific monooxygenase activities in LPR housefly microsomes. *J. Exp. Zool. 264*, 153–158.

Wheelock, G.D., Scott, J.G., **1992b**. The role of cytochrome P450 in deltamethrin metabolism by pyrethroid-resistant and susceptible strains of house flies. *Pestic. Biochem. Physiol. 43*, 67–77.

White, J.R.A., Franklin, R.T., Agosin, M., **1979**. Conversion of α-pinene to α-pinene oxide by rat liver and the bark beetle *Dendroctonus terebrans* microsomal fractions. *Pestic. Biochem. Physiol. 10*, 233–242.

White, K.P., Rifkin, S.A., Hurban, P., Hogness, D.S., **1999**. Microarray analysis of *Drosophila* development during metamorphosis. *Science 286*, 2179–2184.

Whittaker, R.H., Feeny, P.P., **1971**. Allelochemics: chemical interactions between species. *Science 171*, 757–770.

Wilkinson, C.F., **1979**. The use of insect subcellular components for studying the metabolism of xenobiotics. *ACS Symp. Ser. 97*, 249–284.

Wilkinson, C.F., Brattsten, L.B., **1972**. Microsomal drug metabolizing enzymes in insects. *Drug Metab. Rev. 1*, 153–228.

Williams, D.R., Chen, J.H., Fisher, M.J., Rees, H.H., **1997**. Induction of enzymes involved in molting hormone (ecdysteroid) inactivation by ecdysteroids and an agonist, 1,2-dibenzoyl-1-*tert*-butylhydrazine (RH-5849). *J. Biol. Chem. 272*, 8427–8432.

Williams, P.A., Cosme, J., Sridhar, V., Johnson, E.F., McRee, D.E., **2000a**. Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. *Mol. Cell 5*, 121–131.

Williams, D.R., Fisher, M.J., Rees, H.H., **2000b**. Characterization of ecdysteroid 26-hydroxylase: an enzyme involved in molting hormone inactivation. *Arch. Biochem. Biophys. 376*, 389–398.

Wilson, T.G., **2001**. Resistance of *Drosophila* to toxins. *Annu. Rev. Entomol. 46*, 545–571.

Winter, J., Bilbe, G., Richener, H., Sehringer, B., Kayser, H., **1999**. Cloning of a cDNA encoding a novel cytochrome P450 from the insect *Locusta migratoria*: CYP6H1, a putative ecdysone 20-hydroxylase. *Biochem. Biophys. Res. Commun. 259*, 305–310.

Winter, J., Eckerskorn, C., Waditschatka, R., Kayser, H., **2001**. A microsomal ecdysone-binding cytochrome P450 from the insect *Locusta migratoria* purified by sequential use of type-II and type-I ligands. *Biol. Chem. 382*, 1541–1549.

Wojtasek, H., Leal, W.S., **1999**. Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllopertha diversa* (Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450. *FEBS Lett. 458*, 333–336.

Xu, W., Bak, S., Decker, A., Paquette, S.M., Feyereisen, R., *et al.*, **2001**. Microarray-based analysis of gene expression in very large gene families: the cytochrome P450 gene superfamily of *Arabidopsis thaliana*. *Gene 272*, 61–74.

Yamazaki, S., Sato, K., Suhara, K., Sakaguchi, M., Mihara, K., *et al.*, **1993**. Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. *J. Biochem. (Tokyo) 114*, 652–657.

Yu, S.J., **1984**. Interactions of allelochemicals with detoxication enzymes of insecticide-susceptible and resistant fall armyworms. *Pestic. Biochem. Physiol. 22*, 60–68.

Yu, S.J., **1986**. Consequences of induction of foreign compound-metabolizing enzymes in insects. In: Brattsten, L.B., Ahmad, S. (Eds.), Molecular Aspects of Insect–Plant Interactions. Plenum, New York, pp. 211–255.

Yu, S.J., **1987**. Microsomal oxidation of allelochemicals in generalist (*Spodoptera frugiperda*) and semispecialist (*Anticarsia gemmatalis*) insects. *J. Chem. Ecol. 13*, 423–436.

Yu, S.J., **1991**. Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol. 39*, 84–91.

Yu, S.J., **1992**. Detection and biochemical characterization of insecticide resistance in fall armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 85*, 675–682.

Yu, S.J., **1995**. Allelochemical stimulation of ecdysone 20-monoxygenase in fall armyworm larvae. *Arch. Insect Biochem. Physiol. 28*, 365–375.

Yu, S.J., Terriere, L.C., **1974**. A possible role for microsomal oxidases in metamorphosis and reproduction in the housefly. *J. Insect Physiol. 20*, 1901–1912.

Zdobnov, E.M., von Mering, C., Letunic, I., Torrents, D., Suyama, M., *et al.*, **2002**. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster. Science 298*, 149–159.

Zhang, L., Harada, K., Shono, T., **1997**. Genetic analysis of pyriproxifen resistance in the housefly, *Musca domestica* L. *Appl. Ent. Zool. 32*, 217–226.

Zhang, L., Kasai, S., Shono, T., **1998**. In vitro metabolism of pyriproxyfen by microsomes from susceptible and resistant housefly larvae. *Arch. Insect Biochem. Physiol. 37*, 215–224.

Zhang, M., Scott, J.G., **1994**. Cytochrome b_5 involvement in cytochrome P450 monooxygenase activities in housefly microsomes. *Arch. Insect Biochem. Physiol. 27*, 205–216.

Zhang, M., Scott, J.G., **1996a**. Purification and characterization of cytochrome b_5 reductase from the housefly, *Musca domestica*. *Comp. Biochem. Physiol. 113B*, 175–183.

Zhang, M., Scott, J.G., **1996b**. Cytochome b_5 is essential for cytochome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pestic. Biochem. Physiol. 55*, 150–156.

Zhou, S., Gao, Y., Jiang, W., Huang, M., Xu, A., *et al.*, **2003**. Interactions of herbs with cytochrome P450. *Drug Metab. Rev. 35*, 35–98.

Zhu, Y.C., Snodgrass, G.L., **2003**. Cytochrome P450 CYP6X1 cDNAs and mRNA expression levels in three strains of the tarnished plant bug *Lygus lineolaris* (Heteroptera: Miridae) having different susceptibilities to pyrethroid insecticide. *Insect Mol. Biol. 12*, 39–49.

Ziegler, D.M., **2002**. An overview of the mechanism, substrate specificities, and structure of FMOs. *Drug Metab. Rev. 34*, 503–511.

Zijlstra, J.A., Vogel, E.W., Breimer, D.D., **1984**. Strain-differences and inducibility of microsomal oxidative enzymes in *Drosophila melanogaster* flies. *Chem. Biol. Interact. 48*, 317–338.

Zinke, I., Schutz, C.S., Katzenberger, J.D., Bauer, M., Pankratz, M.J., **2002**. Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *EMBO J. 21*, 6162–6173.

Zou, S., Meadows, S., Sharp, L., Yan, L.Y., Jan, Y.N., **2000**. Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 97*, 13726–13731.

Zuber, M.X., Simpson, E.R., Waterman, M.R., **1986**. Expression of bovine 17α-hydroxylase cytochrome P450 cDNA in non-steroidogenic (COS1) cells. *Science 234*, 1258–1261.

Zumwalt, J.G., Neal, J.J., **1993**. Cytochromes P450 from *Papilio polyxenes*: adaptations to host plant allelochemicals. *Comp. Biochem. Physiol. 106C*, 111–118.

Relevant Websites

http://www.imm.ki.se – Human cytochrome P450 (CYP) allele nomenclature committee. A complete referenced and annotated list of human P450 alleles.

http://P450.antibes.inra.fr – The insect P450 site at INRA. A searchable annotation of insect cytochrome P450 sequences.

http://dinelson.utmem.edu – David Nelson's cytochrome P450 homepage. Listing of P450 genes from a variety of organisms.

4.2 Cuticular Proteins

J H Willis, University of Georgia, Athens, GA, USA
V A Iconomidou, University of Athens, Athens, Greece
R F Smith, Northern Arizona University, Flagstaff, AZ, USA
S J Hamodrakas, University of Athens, Athens, Greece

4.2.1. Introduction

In the previous edition of this series, Silvert (1985) outlined several major areas of uncertainty regarding cuticular proteins. The questions raised were: Were proteins extracted from cuticle authentic cuticular proteins or might some be contaminants of adhering cells and hemolymph? Was the epidermis the sole site of synthesis of cuticular proteins or were some synthesized in other tissues and transported to the cuticle? What was the relation among cuticular proteins of various developmental stages? Did cuticular proteins share common structural features?

That article presented all the cuticular protein sequence data then available – four complete and three partial sequences from *Drosophila melanogaster* and one partial sequence from *Sarcophaga bullata*. The *considerable* sequence similarity seen with those limited data indicated that cuticular protein genes belonged to multigene families, and the even more limited genomic information revealed that similar genes were adjacent on a chromosome.

Progress in less than two decades has been spectacular, but not surprising given the advances in relevant techniques. Elegant immunolocalization analyses have solved the problem of the sources of cuticular proteins. Over 300 cuticular protein sequences are now available from six orders and over 20 species of insects. Listed in **Tables 1 and 2** are all but those that come exclusively from the annotation of the genomes of *D. melanogaster* and *Anopheles gambiae* or SilkBase, an extensive expressed sequence tag (EST) project in *Bombyx mori* (Mita *et al*., 1999; SilkBase, 2003). Throughout this chapter, proteins are referred to by the names used in **Tables 1 and 2**. These tables also provide a gi number or an identifier from SwissProt that provides access to the complete sequence and the relevant references. Recombinant proteins have

Table 1 Characteristics of ''structural'' cuticular proteins that have been completely sequenced

Order/species	*Protein name*	*Number of amino acids* [a]	*Type*	*AAP(A/V) Repeats* [b]	*Other features* [c]	*Sequence method* [d]	*Identifier* [e]
Coleoptera							
Apriona germari	LCP10.7	87	RR-1			CT	16226511
	LCP12.3	114	RR-1			CT	21617523
	LCP12.6	120	RR-1			CT	21617525
Tenebrio molitor	TM-LCP-A1A	174	RR-2	2N 1C	2[AAP(I/L)]	DS	1706191
	TM-LCP-A2B	117	RR-2	1N 1C		DS	1706192
	TM-LCP-A3A	134	RR-2	1N 1C	1[AAP(I/L)]	DS	1706194
	TM-PCP-C1B	161		9	51 aa motif 1[AAP(I/L)]	DS	1706197
	TM-E1A	243		22		DS	913040
	TM-F1A	154		3	5 [AAP(I/L)]	DS	998953
	TM1-F1B	158		4	4 [AAPL]	DS	998954
	TM-F1C	162		4	5 [AAP(I/L)]	DS	998955
	TM-PCP-G1A	211		5	51 aa motif	DS	1706198
	TM-H1C = TMLPCP22	195		4	51 aa motif 1[AAP(I/L)]	CT DS	3123202*
	PCP5.8	53				DS	7511760
	PCP9.2	92				DS	7511761
	PCP15.6	161		5	1[AAP(I/L)]	DS	7441995
	PCP16.7	166				DS	7511759
	TMLPCP-23	214		2	51 aa motif 1[AAP(I/L)]	CT	3121955*
	TMLPCP29	276	RR-3	11N	1 (18 residue motif) 1[AAP(I/L)]	CT	2275132*
	ACP17	167		2	High G	CT	1078986*
	ACP20	191	RR-2		High G	CT	102879
	ACP-22	180	RR-2		2(6G), 1(8G), 1(15G)	CT	113012*
Dictyoptera							
Blaberus craniifer	BC-NCP1	87			Has 6 Cys, forming 3 potential S-S	DS	3023587
	BC-NCP2	99			8 As; 2 (18 residue motif)	DS	3023589
	BC-NCP4	127			2 (18 residue motif)	DS	3023588
	BC-NCP5	145			2 (18 residue motif)	DS	P82118
	BC-NCP6	139	RR-3		1 (18 residue motif)	DS	P82119
	BC-NCP7	145	RR-3		1 (18 residue motif)	DS	P82120
	BC-NCP8	195	RR-2			DS	P82121
	BC-NCP9	34				DS	P82122
Diptera							
Anopheles gambiae	AGCP2A	214	RR-2	1N	4 [(S/A)APIAH]	CT	2961109*
	AGCP2B	222	RR-2	1N	5 [(S/A)APIAH]	CT	2961110*
	AGCP2C	214	RR-2	2N	5 [(S/A)APIAH]	CT	2961111*
	AGCP2D	215	RR-2	2N	4 [(S/A)APIAH]	CT	2961113
Drosophila melanogaster	Dacp-1	116		3		CT	7580461*
	LCP-1	114	RR-1			DS CT	17380379*
	LCP-2	110	RR-1			DS CT	117634*
	LCP-3	96	RR-1			DS CT	117635*
	LCP-4	96	RR-1			DS CT	117636*
	PCP = GART INTRON	166	RR-1			CT	157483*
	ACP65A	85	RR-1			CT	1857602*
	LCP65Aa	83	RR-1			CT	1857600*
	LCP65Ab1	86	RR-1			CT pDS	1857597*
	LCP65Ab2	86	RR-1			CT pDS	1857595*
	LCP65Ac	91	RR-1			CT	1857593*
	LCP65Ad	90	RR-1			CT	1857495*
	LCP65Ae	83	RR-1			CT	1857604*
	LCP65Af	84	RR-1			CT	1857606*
	LCP65Ag1	87	RR-1			CT pDS	1857608*
	LCP65Ag2	87	RR-1			CT pDS	1857610*
	EDG-78	106	RR-1			CT	117639*
	EDG-84	171	RR-2	1C		CT	117640*

Table 1 Continued

Order/species	*Protein name*	*Number of amino acids*[a]	*Type*	*AAP(A/V) Repeats*[b]	*Other features*[c]	*Sequence method*[d]	*Identifier*[e]
	EDG91	138			8 (2G) 6(3G) 3 (4G)	CT	17380419*
	Ccp84Aa	188	RR-2	1N 6C		CT	4389433*
	Ccp84Ab	204	RR-2	1N 7C		CT	4389434*
	Ccp84Ac	199	RR-2	2C		CT	4389435*
	Ccp84Ad	182	RR-2	1N 6C		CT	4389436*
	Ccp84Ae	191	RR-2	1N 3C		CT	4389437*
	Ccp84Af	134	RR-2	1N		CT	4389438*
	Ccp84Ag	173	RR-2	6C		CT	4389439*
	Cry	457	RR-2		7Q, internal M	CT	22946279*
Drosophila miranda	LCP-1	122	RR-1			CT	3023591*
	LCP-2	110	RR-1			CT pDS	3023592
	LCP-3	96	RR-1			CT	231917*
	LCP-4	96	RR-1			CT	231917*
	LCP-3Y	96	RR-1			CT pDS	386246
Drosophila pseudoobscura	CP = GART INTRON	177	RR-1			CT	435017*
Drosophila simulans	DS-06238.4-like	188	RR-2			CT	9966434*
Drosophila yakuba	DS-06238.4-like	194	RR-2			CT	9966436*
Lucilia cuprina	CUT1	102	RR-1			CT	2565392
	CUT12	89	RR-1			CT	2565394
Hemiptera							
Myzus persicae	CP	208	RR-2	1N 4C	[(S/K)APAY] 1N 10C	CT	16798648
Aphis fabae	CP	208	RR-2	3C	[(S/K)APAY] 1N 12C	CT	29124938
Aphis gossypii	CP	203	RR-2	1N 3C	[(S/K)APAY] 10C	CT	29124934
Brevicoryne brassicae	CP	208	RR-2	1N 2C	[(S/K)APAY] 1N 12C	CT	29124932
Lipaphis erysimi	CP	208	RR-2	1N 3C	[(S/K)APAY] 1N 11C	CT	29124930
Rhopalosiphum maidis	CP	210	RR-2	2N 2C	[(S/K)APAY] 13C	CT	29124936
Lepidoptera							
Bombyx mori	PCP	235		3	3 (18 residue motif)	CT	1169137*
	BMWCP1A	205	RR-2	5N		CT	12862579
	BMWCP1B	205	RR-2	5N		CT	12862581
	BMWCP2	231	RR-2	9N		CT	12862583
	BMWCP3	215	RR-2	7N		CT	12862585
	BMWCP4	226	RR-2	3N		CT	12862587
	BMWCP5	274	RR-2	2N 1C		CT	12862589
	BMWCP6	186	RR-2	2N 2C		CT	
	BMWCP7A	157	RR-2	1N		CT	12862593
	BMWCP7B	157	RR-2	1N		CT	12862595
	BMWCP8	221	RR-2			CT	12862597
	BMWCP9	158	RR-1			CT	12862599
	BMWCP10	295	RR-1		Internal M	CT	23096118
	EDG84A homologue	180	RR-2	1N 1C		CT	3608259
	BMCP17	127	RR-1	1N		CT pDS	2204069
	BMCP18	89	RR-1	1N		CT pDS	5360249*
	BMCP22	158	RR-1			CT pDS	2204071
	BMCP30	223	RR-1			CT pDS	6634056*
	GCP1	149			17(GGY)	CT	15146344
Galleria melonella	PCP-52	338		1	Has 2 Cys; 21% Ala	CT	1086154
Helicoverpa armigera	LCP-1	95	RR-1		VVVV	CT	3913261
Hyalophora cecropia	HCCP12	89	RR-1			CT pDS	1169129*

Continued

Table 1 Continued

Order/species	Protein name	Number of amino acids[a]	Type	AAP(A/V) Repeats[b]	Other features[c]	Sequence method[d]	Identifier[e]
	HCCP66	112	RR-2			CT pDS	1169133*
Manduca sexta	LCP-14	109	RR-1			CT	117623
	CP14.6	90	RR-1			CT	3121956*
	LCP16/17	123	RR-1			CT	3121953*
	CP20	182	RR-1		7(GG) 3(GGG); glycosylated	CT DS	19548965
	CP27	165	RR-1		Glycosylated	CT DS	19743772
	CP36	327	RR-1		32(GG) 1(GGG); glycosylated	CT DS	22000820
Orthoptera							
Locusta migratoria	LM-ACP-abd4	116	RR-1		3 glycosylated forms	DS	461860
	LM-ACP-abd5	82	RR-1		Glycosylated	DS	3913394
	LM-ACP7	131	RR-2	2N 3C		DS	998751
	LM-ACP8	148	RR-2	5N		DS	84730
	LM-ACP19	157	RR-2	6N 1C	GYL motif	DS	1345864
	LM-ACP21	169	RR-2	6N 3C		DS	3287770
	LM-ACP38	163		14	GYL motif	DS	72263
	LM-NCP55	33		2		DS	446069
	LM-NCP62	88				DS	446070
	LM-ACP63	157		8	GYL motif	DS	1169130
	LM-ACP64	152		7	GYL motif	DS	1169131
	LM-ACP65	145		7	GYL motif	DS	1169132
	LM-ACP67a	98		6	51 aa motif	DS	416850
	LM-ACP67b	104		6	51 aa motif	DS	542520
	LM-ACP70	88		4	GYL motif	DS	416852
	LM-ACP76	139		6	GYL motif; 51 aa motif	DS	1169134
	LM-ACP79a	131		3	5 [GGG(L/Y)]	DS	1168721
	LM-ACP79b	131		3	5 [GGG(L/Y)]	DS	1168722
	LM-NCP4.9	46		1		DS	P82168
	LM-NCP5.1	48		1		DS	P82169
	LM-NCP6.4	62				DS	P82170
	LM-NCP9.5	90				DS	P82171
	LM-NCP18.7	193			4 (18 residue motif)	DS	P82165
	LM-NCP21.3	200		10		DS	P82167
	LM-NCP19.8	200	RR-2	3N 5C		DS	P82166
Schistocerca gregaria	SGAbd-1	184	RR-1		Glycosylated; (PPPPPPP)	DS	7511754
	SGAbd-2	135	RR-1		Glycosylated	DS	7511755
	SGAbd-3	119	RR-1		Glycosylated	DS	7441999
	SGAbd-4	116	RR-1		Glycosylated	DS	7441997
	SGAbd-5	82	RR-1		Glycosylated	DS	3913395
	SGAbd-6	82	RR-1		Glycosylated	DS	7511756
	SGAbd-8	139	RR-1		Glycosylated; internal M	DS	7511757
	SGAb-9	129	RR-1		Glycosylated	DS	7441998

[a]Sequence length of mature peptide; signal peptides were deleted using data from authors or SignalP V2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/).
[b]If a protein has an R&R Consensus, location of the AAP(A/V) repeats is given relative to the consensus.
[c]See Section 4.2.3.2.2 for description of these features.
[d]DS, direct sequencing of protein, a p indicates only a partial (generally N-terminal) sequence was obtained; CT, conceptual translation of a cDNA, genomic region, or EST product.
[e]Protein sequences and additional annotation can be found at http://www3.ncbi.nlm.nih.gov/Entrez/index.html. Sequences that have an identifier that begins with a letter can be found at http://us.expasy.org. An asterisk indicates that genomic sequence information is available.

Table 2 Characteristics of some nonstructural proteins that have been found in cuticle

Species	*Protein name*	*Number of amino acids*[a]	*Function*	*Sequence method*[b]	*Identifier*[c]
Schistocerca gregaria	Putative carotene binding protein	250	Transfers carotene into cuticle	DS	13959527
Caliphora vicinia	ARYLPHORIN A4	743	Found in cuticle	CT	114232
	ARYLPHORIN C223	743		CT	114236
Drosophila melanogaster	YELLOW	520	Positions melanin pigment in cuticle	CT	140623
Bombyx mori	CECROPIN A	41	Defense protein	CT	2493573
	CECROPIN B	41	Defense protein	CT	1705754
	PROPHENOLOXIDASE	675	Melanization enzyme	CT	13591614
Calpodes ethlius	CECP22	169	Cuticle digestion	CT	4104409
Manduca sexta	ARYLPHORINα	684		CT	114240
	ARYLPHORINβ	687		CT	1168527
	INSECTICYANIN A	189	Blue pigment	CT	102968
	INSECTICYANIN B	189	Blue pigment	CT	124527
	SCOLEXIN A	279	Serine protease immune protein	CT	4262357
	SCOLEXIN B	279	Serine protease immune protein	CT	4262359

[a]Sequence length of mature peptide; signal peptides were deleted using data from authors or SignalP V2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/).
[b]DS, direct sequencing of protein; CT, conceptual translation of a cDNA, genomic region, or EST product.
[c]Protein sequences and additional annotation can be found at: http://www3.ncbi.nlm.nih.gov/Entrez/index.html.

revealed a function for a highly conserved domain that was present in those first protein sequences discussed by Silvert. Structural predictions have elucidated the basis for this function. It is these developments that will be the focus of this review.

4.2.2. Cuticle Structure and Synthesis

4.2.2.1. Cuticle Morphology

4.2.2.1.1. Terminology The descriptive terms used here to describe the regions of cuticle have been simplified according to Locke's (2001) cogent suggestions for new nomenclature. He proposes the use of the term "envelope" to describe the outermost layer of cuticle, rather than the previous term "cuticulin." At the start of each molt cycle, the smooth apical plasma membrane forms microvilli with plaques at their tips where the new envelope assembles. This discrete layer of 10–30 nm not only serves to protect the underlying epidermis from molting fluid enzymes that begin to digest the old cuticle, but, as Locke points out, affects "resistance to abrasion and infection, penetration of insecticides, permeability, surface reflectivity, and physical colors." The sequences and properties of its constituent proteins remain unknown.

Next formed is the epicuticle, about 1 μm in thickness. This chitin-free layer (but see Section 4.2.2.1.3) is stabilized by quinones. It was formerly referred to as the "inner epicuticle" with cuticulin being the outer.

Former arguments about the precise distinction between *exo*- and *endo*-cuticle are eliminated by Locke's lumping of the inner regions of the cuticle under the term "procuticle," encompassing both preecdysial and postecdysial secretions. The procuticle, then, is the region that combines chitin and cuticular proteins in various combinations and becomes sclerotized (see **Chapter 4.4**) and pigmented to varying degrees. This is the region depicted in electron micrographs showing stacks of precisely oriented lamellae. According to Locke (2001), apical microvilli bend in concert across the epithelial sheet and this movement serves to orient the laminae that will form lamellae. While knowledge of the process of secreting and assembling such a highly ordered structure is limited, details about the proteins associated with the lamellae are now voluminous.

4.2.2.1.2. Growth of the cuticle within an instar Central to the issue of cuticle structure is the important fact that considerable cuticle growth can occur during an intermolt period (Williams, 1980), some of it by a smoothing out of macro- and microscopic folds and pleats (Carter and Locke, 1993). During intrainstar growth, new cuticular proteins are interspersed among the old, necessitating a model of chitin–protein and protein–protein

interactions that will permit such intussusception (Condoulis and Locke, 1966; Wolfgang and Riddiford, 1986).

4.2.2.1.3. Localization of cuticular proteins within the cuticle Precise localization of cuticular proteins within the cuticle and even within cellular organelles has been made possible with immunogold labeling of electron microscopic sections. Here a specific primary antibody is bound to the sections and visualized with a secondary antibody conjugated to colloidal gold particles.

Antibodies have been raised against extracts of whole cuticle or isolated electrophoretic bands and the specificity of each antibody ascertained with Western blots. While each polyclonal antibody raised against a single band was specific for the immunizing protein, monoclonals raised against cuticular extracts frequently reacted with more than one electrophoretic band.

One concern with immunolocalization is that as cuticular proteins become modified in the cuticle by binding to chitin or by becoming sclerotized, the immunizing epitopes might become masked, a problem that should be more serious with monoclonal than polyclonal antibodies. All groups recognized that while the presence of an antigen is significant, its absence may reflect no more than such masking.

This concern is significant when one considers results of immunolocalization in the assembly zone, the region of cuticle directly above the microvilli. It is here that chitin secreted from the tips of the microvilli interacts with cuticular proteins secreted into the perimicrovillar space. Immunolocalization studies revealed only a few of the cuticular proteins within the perimicrovillar space but the same ones and others were abundant in the assembly zone directly above it (Locke *et al.*, 1994; Locke, 1998). The authors' conclusion was that the assembly zone "is where we should expect proteins to unravel and expose most epitopes in preparation for assuming a new configuration as they stabilize in the maturing cuticle." Wolfgang *et al.* (1986, 1987) found two *D. melanogaster* cuticular proteins exclusively in this zone and suggested they might function in cuticle assembly. Locke *et al.* (1994) point out that it was common for antibodies raised against *Calpodes ethlius* proteins to react more strongly with the assembly zone than with more mature regions of cuticle where sclerotization and chitin binding might mask epitopes. Thus more substantial evidence than the failure to detect a protein in more mature regions is needed to confirm that it belonged exclusively to the assembly zone.

It was known from earlier work on protein and mRNA distribution that cuticles from different metamorphic stages and different anatomical regions had different cuticular proteins and that there may be a change in cuticular proteins synthesized by a single cell within a molt cycle (review: Willis, 1996). Such a transition in proteins synthesized is especially apparent at the time of ecdysis, and, in some insects, late in the instar. Consistent with this, immunolocalization revealed different proteins in morphologically distinct early and late lamellae in *D. melanogaster* pupae, and *Tenebrio molitor* and *Manduca sexta* larvae (Doctor *et al.*, 1985; Fristrom *et al.*, 1986; Wolfgang and Riddiford, 1986; Wolfgang *et al.*, 1986; Lemoine *et al.*, 1989, 1993; Bouhin *et al.*, 1992a, 1992b; Rondot *et al.*, 1996). Only two proteins with known sequence are among this group, TMACP22 and TMLPCP22.

Csikos *et al.* (1999) have used immunohistochemistry to follow some of *Manduca*'s cuticular proteins throughout the molt cycle. These proteins are obviously in a dynamic state as they move from epidermis to cuticle to molting fluid to fat body and then apparently back to cuticle via the hemolymph. More detailed studies are needed to learn if the same molecules make the return trip, and whether their initial passage from molting fluid into the hemolymph is solely via uptake and then basal secretion by the epidermis or whether the midgut plays a role, since lepidopteran larvae drink their molting fluid (Cornell and Pan, 1983).

The findings with epicuticle, the first region to be secreted beneath the envelope, were complex. None of the monoclonal antibodies that recognized *Tenebrio* cuticular proteins reacted with epicuticle (Lemoine *et al.*, 1990). On the other hand, arylphorin from *Calpodes* has been localized to epicuticle and no other cuticular region (Leung *et al.*, 1989) and several proteins, of unknown sequence, were found both in the epicuticle and in the lamellar regions of the procuticle in *D. melanogaster* (Fristrom *et al.*, 1986) and *Calpodes* (Locke *et al.*, 1994). This finding of cuticular proteins in both epicuticle and lamellar regions was surprising, since the epicuticle had always been described as lacking chitin (cf. Fristrom *et al.*, 1986; Fristrom and Fristrom, 1993) and thus was expected to have unique proteins.

In addition to temporal differences in the secretion of cuticular proteins by single cells, there may be regional differences in the cuticle secreted by single cells. Individual epidermal cells of the articulating membranes (intersegmental membranes) in *Tenebrio* secrete a cuticle with sclerotized cones

embedded in softer cuticle. Two of the classes of monoclonal antibodies raised against *Tenebrio*'s larval and pupal cuticular proteins recognized proteins in these cones. The same antibodies recognized proteins in cuticles in other regions that were destined to be sclerotized. Different antibodies recognized the proteins in the softer cuticle (Lemoine *et al.*, 1990, 1993).

Locke *et al.* (1994) were able, using carefully reconstructed sections of *Calpodes* larval cuticle, to distinguish one protein (C36) that was found with the same distribution as the chitin microfibrils that had been visualized with wheat germ agglutinin, a lectin that recognizes *N*-acetylglucosamine, while other antigens failed to show this distribution. Notably, only C36 isolated from cuticle reacted with wheat germ agglutinin on lectin blots. Based on this evidence Locke *et al.* (1994) suggest that the isolated protein may have obtained its *N*-acetylglucosamine from chitin.

4.2.2.1.4. Cuticles formed following disruption of normal metamorphosis Treatment of many insects with juvenile hormone (JH) causes them to resynthesize a cuticle with a morphology characteristic of the current metamorphic stage, rather than the next (see **Chapter 3.7**). Thus, in *Tenebrio*, treatment of pupae with JH prior to pupal–adult apolysis causes the formation of a second pupa rather than an adult. Earlier work revealed that these second pupae had proteins with the same electrophoretic mobility as those extracted from normal pupae (Roberts and Willis, 1980b; Lemoine *et al.*, 1989). A combination of Northern analysis and *in situ* hybridization demonstrated that second pupae have the same cuticular protein mRNAs and protein localization as normal pupae (Lemoine *et al.*, 1993; Rondot *et al.*, 1996). Adult cuticular proteins are not deposited in these cuticles and the adult mRNAs do not appear (Lemoine *et al.*, 1989, 1993; Bouhin *et al.*, 1992a, 1992b; Charles *et al.*, 1992). Some JH-treated *Tenebrio* pupae form two cuticles, the first pupalike in morphology and the second with adult features. The adultlike cuticle was shown with immunolocalization to have ACP22 (Bouhin *et al.*, 1992a). If JH is applied too late to form a perfect second pupa, the next cuticle formed will be a composite with morphological features of two metamorphic stages (Willis *et al.*, 1982). Bouhin *et al.* (1992b) found that all the epidermal cells laying down such a composite cuticle had mRNAs for ACP22.

Zhou and Riddiford (2002) used Northern analysis to characterize the somewhat nondescript cuticles made by *D. melanogaster* that had been manipulated by misexpressing the gene, *broad*, that codes for a transcription factor that appears before the larval–pupal molt in flies and moths. By following mRNAs for the adult cuticular protein ACP65A or the pupal cuticular protein Edg78E, they were able to demonstrate the essential role of *broad* in directing pupal development and thereby helped clarify the perplexing action of juvenoids in the higher Diptera.

4.2.2.2. The Site of Synthesis of Cuticular Proteins

One of the unresolved issues addressed in Silvert's (1985) review was the site of synthesis of cuticular proteins. This might appear to be a trivial issue, for one would expect that the epidermis that underlies the cuticle would synthesize the cuticular proteins. There are, however, reports in the literature that proteins found in the hemolymph were present in cuticle and even that labeled proteins injected into the hemolymph would appear in cuticle. Silvert discussed the possibility that the injected protein had been broken down and resynthesized so that the cuticular protein was labeled solely because its constituent amino acids had come from a labeled pool.

Five methods have now provided data that address the site of synthesis of cuticular proteins. The most common is to use Northern analysis to learn in what tissues and at which stages mRNA is present for a particular cuticular protein. This method is so common that specific examples will not be given. The second method is to incubate epidermis or integument *in vitro* with radioactive amino acids, separate the proteins, and compare the electrophoretic mobility of the labeled proteins to proteins isolated from cleaned cuticles. A third method is to isolate mRNAs from tissues and translate these *in vitro* with commercially available wheat germ extracts or rabbit reticulocytes and compare the translation products to known cuticular proteins. The fourth method is *in situ* hybridization, and the fifth immunolocalization to visualize proteins within the endoplasmic reticulum and Golgi apparatus.

The first three methods suffer from the possibility that tracheae and adhering tissues, fat body, muscles, hemocytes, contribute to the mRNA pool. Both labeling methods suffer from the problem that cuticular proteins are notoriously sensitive to solubilizing buffer and gel conditions (pH, urea concentration) (Cox and Willis, 1987a) and unless cuticular protein standards and labeled translation products are mixed prior to electrophoresis, they may not show identical electrophoretic mobility even in adjacent lanes. Some workers have precipitated labeled

translation products with antibodies raised against extracts of cuticle or individual cuticular proteins, then solublized the precipitate, run it on a gel, and detected the labeled product with fluorography. Csikos *et al*. (1999) used Western blots of translation products to identify cuticular proteins. Since cuticular proteins are destined for secretion from cells, they have a signal peptide that is cleaved before the protein is secreted into the cuticle. Hence, translation products made *in vitro* will be larger than the protein extracted from cuticle. There are two methods to circumvent this problem. The translation products can have their signal peptides cleaved by adding a preparation of canine microsomes, or antibodies against cuticular proteins (specific or against an extract) can be used to precipitate the translation products before they are solubilized and run on a gel. Either method allows some certainty in the comparison of these *in vitro* translation products with authentic cuticular proteins. It was also found that some commercial preparations of wheat germ extract have endogenous signal peptide processing activity (Binger and Willis, 1990).

Frequently, ^{35}S-methionine was used for metabolic labeling of integument and for *in vitro* translation. This is an unfortunate choice as almost all mature cuticular proteins lack methionine residues (see Section 4.2.3.2.1). The initiator methionine will be lost along with the entire signal peptide. Clear differences in labeling patterns with ^{35}S-methionine and ^{3}H-leucine have been found, with none of the major proteins from pharate adult cuticle of *D. melanogaster* or from larval cuticles of *Hyalophora cecropia* showing methionine labeling (Roter *et al*., 1985; Willis, 1999). Why then did several studies find all of the known cuticular proteins labeled with methionine? Perhaps the finding that ^{35}S-methionine can donate its label to a variety of amino acids in preformed proteins (Browder *et al*., 1992; Kalinich and McClain, 1992) explains its appearance and suggests that it needs to be used with caution for such studies with cuticular proteins.

The fourth method is *in situ* hybridization, where specific mRNAs can be identified in the epidermis. Results from several studies are summarized in **Table 3**. *In situ* hybridization allows one to be somewhat more discerning about the site of synthesis of a cuticular protein because it is possible to monitor the presence or absence of a particular mRNA at the level of an individual cell. With this technique, integument is fixed and sectioned and then probed with a labeled cDNA or cRNA allowing the identification of particular regions of the epidermis by examining the morphology of the overlying cuticle. With most detection methods, contaminating tissues and precise regions of the epidermis can be identified and the presence of the particular mRNA in them can be assessed. Thus this technique identifies the location of the mRNAs recognized by the specific probe used. It was this technique that revealed the precision with which mRNAs are produced, for abrupt boundaries of expression occur between sclerites and intersegmental membranes (Rebers *et al*., 1997) or at muscle insertion zones (Horodyski and Riddiford, 1989) or next to specialized epidermal cells (Horodyski and Riddiford, 1989; Rebers *et al*., 1997). This technique even revealed the presence of mRNA for cuticular proteins in epithelia of imaginal disc from young larvae (Gu and Willis, 2003). A limitation of the technique is that cRNA probes sometimes bind to the cuticle itself, possibly obscuring detection of mRNA in the underlying epidermis (Fechtel *et al*., 1989; Gu and Willis, 2003). Fechtel *et al*. (1989) found this artifact to be cuticle-type as well as strand- and probe-specific. Results from several species are summarized in **Table 3**.

The fifth method, immunolocalization, was described earlier in conjunction with localization of specific proteins within the cuticle, but it can also be used to identify the site of synthesis by looking for a particular protein within the endoplasmic reticulum or Golgi apparatus (Sass *et al*., 1994a, 1994b).

The results from Northern analyses, metabolic tissue labeling, and *in vitro* translations reveal that all cuticular proteins with known sequences or for which specific probes are available are synthesized by the integumental preparations. Different proteins are synthesized at different times in a molt cycle and in different anatomical regions and there are some cuticular proteins whose synthesis is stage-specific. Differences in the presence of mRNA parallel the appearance of labeled proteins indicating that much of the temporal and spatial control of cuticular protein synthesis is at the level of transcription. As mentioned above, however, all three of these methods are limited by the possible contamination of tissues by nonepidermal cells and by their inability to address heterogeneity of cell types within the epidermis.

Studies that have combined tissue labeling or *in vitro* translations with immunolocalization have at last clarified the relationship between hemolymph and cuticular proteins with identical electrophoretic and immunological properties. The most comprehensive studies of protein trafficking, carried out in *Calpodes*, revealed four classes of exported proteins that are handled by the epidermis.

Table 3 Evidence for the association of location or type of cuticle and sequence class of some cuticular proteins

Species	*Protein*	*Sequence class*	*Localization*[a]	*Nature of evidence*[b]	*When deposited*	*Reference*
Bombyx mori	BMLCP18	RR-1	Imaginal discs	EST		Gu and Willis (2003)
Drosophila melanogaster	EDG-78	RR-1	Larval and imaginal cells of prepupa	ISH		Fechtel *et al.* (1989)
Drosophila melanogaster	EDG-84	RR-2	Imaginal disc cells	ISH		Fechtel *et al.* (1989)
Drosophila melanogaster	PCP	RR-1	Prepupal thorax and abdomen	ISH		Henikoff *et al.* (1986)
Hyalophora cecropia	HCCP12	RR-1	Soft cuticle; imaginal discs	CD and ISH		Cox and Willis (1985), Gu and Willis (2003)
Hyalophora cecropia	HCCP66	RR-2	Hard cuticle	CD and ISH		Cox and Willis (1985), Gu and Willis (2003)
Locusta migratoria	LM-ACP7	RR-2	Hard cuticle	CD		Andersen *et al.* (1995a)
Locusta migratoria	LM-ACP8	RR-2	Hard cuticle	CD		Andersen *et al.* (1995a)
Locusta migratoria	LM-ACP19	RR-2	Hard cuticle	CD		Andersen *et al.* (1995a)
Manduca sexta	CP14.6	RR-1	Soft cuticle	ISH		Rebers *et al.* (1997)
Manduca sexta	LCP16/17	RR-1	Soft cuticle	ISH		Horodyski and Riddiford (1989)
Tenebrio molitor	ACP17	Glycine-rich	Hard cuticle	ISH	Strongest post-ecdysis	Mathelin *et al.* (1995, 1998)
Tenebrio molitor	ACP20	RR-2	Hard cuticle	ISH	Primarily pre-ecdysis	Charles *et al.* (1992)
Tenebrio molitor	ACP-22	RR-2	Hard cuticle	ISH, mAB	Pre-ecdysis	Bouhin *et al.* (1992a, 1992b)
Tenebrio molitor	TMLPCP22	51 aa motif	Hard and soft cuticle pre-ecdysis, then only soft cuticle	ISH, mAB	Primarily pre-ecdysis	Rondot *et al.* (1998)
Tenebrio molitor	TMLPCP23	51 aa motif	Hard and soft cuticle	ISH	Only pre-ecdysis	Rondot *et al.* (1998)
Tenebrio molitor	TMLPCP29	RR-3 and 18-residue motif	Hard and soft cuticle, except not posterior borders of sclerites	ISH	Post-ecdysis	Mathelin *et al.* (1998)

[a]For *in situ* hybridization, cuticle type was determined by nature of cuticle overlying the epidermis.
[b]CD, careful dissection prior to extraction of proteins; ISH, *in situ* hybridization used to localize mRNA; mAB, monoclonal antibody immunolocalization; EST, from *Bombyx* EST project (Mita *et al.* 1999).

These findings are so important that the experimental methodology is worth discussing. The first approach used was to seal sheets of final instar integument into a bathing chamber so there could be no leakage from the cut edges of the tissue and then find what proteins were made in a 2 h exposure to ^{35}S-methionine. Three classes of proteins were identified with this procedure. One was secreted exclusively into the cuticle (C class), a second appeared in the bathing fluid, hence has been secreted basally (B class) while the third was secreted in both directions (BD class) (Palli and Locke, 1987). Immunolocalization of numerous other *Calpodes* proteins (of unknown sequence) confirmed the existence of these three routing classes of epidermal proteins. A fourth, T class, for proteins transported into cuticle, but not synthesized by the epidermis, was identified. Its presence eliminated any concerns that the classes might be artifacts from labeling with ^{35}S-methionine (Sass *et al.*, 1993).

One member of the T class (T66) was studied in more detail. It was localized by immunogold throughout the cuticle, and although found in epidermal cells was not found in association with the Golgi apparatus, confirming its transcellular transport, rather than synthesis by the epidermis. A subsequent study identified the exclusive site of its synthesis as spherulocytes (Sass *et al.*, 1994a).

Whether the BD proteins are secreted from both apical and basal borders of epidermal cells is still not clear. Locke (1998, 2003) now favors the possibility that all secretion is apical, where the Golgi are concentrated, and that the secreted proteins are subsequently taken back into the cell from the perimicrovillar space and transported in vesicles to the basal surface where the contents are released into the hemolymph.

In conclusion, it is now clear that the epidermis can synthesize both cuticular and hemolymph proteins. It can also transport proteins made in tissues other than epidermis from hemolymph to cuticle.

4.2.2.3. Tracheal Cuticular Proteins

An often-neglected source of cuticle in insects is the tracheal system. Since tracheae are associated with all insect tissues, caution is needed in interpreting the significance of the presence of mRNAs or cuticular proteins from nonintegumental tissues. Cox and Willis (1985) recognized that some of the proteins from tracheae had the same isoelectric points as proteins isolated from integumentary cuticle. A further study was carried out a decade later by Sass *et al*. (1994b), combining electrophoretic analysis with immunogold labeling. Chitin was localized with wheat germ agglutinin and found in all regions of tracheae and tracheoles except the taenidial cushion. Antibodies that had been raised against individual electrophoretic bands from integumentary extracts represented proteins from all four classes of integumentary peptides. Some C proteins, those from the surface cuticle, were found associated with chitin but only in taenidia, other C proteins were in the general matrix with and without chitin. The B and BD peptides were only found in the taenidial cushion, the region lacking chitin. It appears that hemolymph peptides that are synthesized by the epidermis may be tracheal cuticle precursors. The one T protein studied (T66, made in spherulocytes) was also found in the general matrix. An important insight from this study was the conclusion that: "The extremely thin tracheal epithelium suggests that transepithelial transport might supply proteins to the tracheal cuticle more evenly than Golgi complex secretions" (Sass *et al*., 1994b).

4.2.3. Classes of Proteins Found in Cuticles

4.2.3.1. Nonstructural Proteins

Nonstructural proteins that have been identified in cuticle are listed in **Table 2**.

4.2.3.1.1. Pigments Proteins from three classes of pigments used in cuticle – insecticyanins and two different yellow proteins – have been sequenced. The insecticyanins are blue pigments made by the epidermis and secreted into both hemolymph and cuticle. They are easily extracted from cuticle with aqueous buffers. Members of the lipocalin family, they are present as tetramers with the gamma isomer of biliverdin IX situated in a hydrophobic pocket (see **Chapter 4.8**). In the cuticle, in cooperation with carotenes, they confer green coloration. Their structure has been determined to 2.6 Å by X-ray diffraction (Holden *et al*., 1987), making them structurally the best characterized cuticular proteins. Two genes code for insecticyanins in *Manduca* (Li and Riddiford, 1992).

The yellow protein in *D. melanogaster* has been localized with immunocytochemistry in cuticles destined to become melanized (Kornezos and Chia, 1992). Thus it was found in association with larval mouth hooks, denticle belts, and Keilin's organs. Mutants of the gene *yellow* lack black pigment in the affected cuticular region. Mutant analysis revealed two classes of mutants, those that affect all types of cuticle at all stages, and those affecting only particular areas of specific stages. At least 40 different adult cuticular structures could express their color independently (Nash, 1976), and the regulatory regions responsible for some of the stage and regional specificity have been identified (Geyer and Corces, 1987). The yellow protein has been described as a structural component of the cuticle that interacts with products from the gene *ebony*, a β-alanyl-dopamine synthase, to allow melanin to be deposited. Flybase (2003) reports that 740 different alleles of *yellow* have been described, in 542 references beginning in 1916. The complete sequence of *yellow* has been determined for 13 species of *Drosophila* in addition to *D. melanogaster.* An examination of *yellow* expression revealed that both *cis*- and *trans*-regulation are responsible for differences in pigmentation patterns among different species (Wittkopp *et al*., 2002). There is no evidence for a known chitin-binding domain in the yellow protein; the only domain recognized is pfam03022 (major royal jelly protein). Although the sequence for yellow is 37% identical and 56% similar to a dopachrome conversion enzyme from *Aedes aegypti* that is involved in the melanotic encapsulation immune response, yellow itself evidently is devoid of enzyme activity (Han *et al*., 2002) (see **Chapter 4.4**).

Another cuticular protein implicated in pigmentation, putatively β-carotene binding, has been isolated from extracts of cuticle from mature adult

Schistocerca gregaria using column chromatography to isolate a protein that was yellow in color. It bears significant sequence similarity to various insect JH-binding proteins (see **Chapter 3.7**), as well as odorant-binding proteins (see **Chapter 3.15**). Wybrandt and Andersen (2001) suggest that it is involved in the transport of carotenes into epidermis and then the cuticle.

4.2.3.1.2. Enzymes Some of the enzymes involved in sclerotization have been identified in cuticle. Since they are discussed by Andersen (see **Chapter 4.4**) they will not be considered here.

Some enzymes that belong to the molting fluid become evident as the electrophoretic banding pattern of cuticular proteins changes as *Calpodes* initiates molting at the end of the fifth instar, with the most conspicuous change being the appearance of a band of 19 kDa. Antibodies raised against this protein were used to isolate a cDNA from a library cloned in an expression vector. The conceptual translation revealed a protein (CECP22). Its sequence suggested it might have amidase activity. Further analysis revealed that the protein was present in the cuticle before each molt, and was also found in molting fluid. Marcu and Locke (1998, 1999) present evidence that this protein may be activated by proteolysis and speculate that it may function to cleave an amidic bond between *N*-acetylglucosamine from chitin and amino acids in cuticular proteins.

Enzymes involved in digesting the old cuticle are temporary residents in cuticle. These include proteases and chitinases. Their interaction is discussed by Marcu and Locke (1998).

4.2.3.1.3. Defense proteins Also found in the cuticle are components of the insect defense system. In one study, cuticle was removed from *Bombyx* larvae 24 h after they had been abraded with emery paper and exposed to bacteria. The antibacterial peptide cecropin was purified from the cuticles (Lee and Brey, 1994). Both prophenoloxidase and a zymogen form of a serine protease capable of activating it have been extracted from *Bombyx* larval cuticle. Colloidal gold secondary antibodies revealed that the prophenoloxidase was localized throughout the epicuticle and procuticle, and in a conspicuous orderly array on the basal side of the helicoidal chitin lamellae. An extraepidermal source is likely for this enzyme since no labeling was found in the epidermis, nor was mRNA detected in the epidermal cells. It is assumed to function in the melanization that occurs in response to injury (Ashida and Brey, 1995).

Molnar *et al.* (2001) presented immunological evidence for a protein related to the defense protein scolexin in the cuticle of *Manduca*. This protein exists in two forms in *Manduca*, but the antibody used did not distinguish between them.

4.2.3.1.4. Arylphorins The final class of nonstructural proteins is the arylphorins, proteins with high content of aromatic amino acids and some lipid. These proteins, assumed to be hemolymph proteins, have been of special interest since the discovery by Scheller *et al.* (1980) that although calliphorin (the arylophorin from *Calliphora*) was found in cuticle, it seemed to come from the hemolymph, because radioactively labeled calliphorin injected into the hemolymph appeared in cuticle. But there is also evidence that the epidermis is capable of synthesizing arylphorins, for Riddiford and Hice (1985) had detected arylphorin mRNA in the epidermis of *Manduca*.

Palli and Locke (1987) used an anti-arylphorin antibody to identify an 82 kDa protein made in *Calpodes* integumental sheets *in vitro* that appeared in both cuticle and media. Thus arylphorin appeared to be a bidirectionally secreted integumentary protein. Next, colloidal gold secondary antibodies were used to visualize the location of anti-arylphorin in ultrathin sections of various tissues (Leung *et al.*, 1989). The resolution afforded by this method made it possible to recognize arylphorin in epicuticle (but not lamellar cuticle) and in the Golgi complexes of the fat body, and to show by quantifying gold particles that it was also found in Golgi complexes of epidermis, midgut, pericardial cells, and hemocytes as well as the meshwork of fibrous cuticle in tracheae. Thus, while the possibility remains that some arylphorin is transported from hemolymph to cuticle, it need not be, for the epidermis itself is capable of synthesizing and secreting this protein. These studies further demonstrated that a given protein can be synthesized by multiple tissues. Whether it is the same gene that functions in all tissues remains to be determined.

The role of arylphorin remains unknown. It is generally assumed to be participating in sclerotization because of its high tyrosine content. Is it degraded in the cuticle so that its constituent amino acids are released or does it remain an integral part of the cuticle? The latter is favored by the available evidence because calliphorin has been shown to bind strongly to chitin *in vitro* (Agrawal and Scheller, 1986) and no breakdown products were detected after injection of labeled calliphorin (Konig *et al.*, 1986).

4.2.3.2. Structural Proteins

4.2.3.2.1. Overview Slightly less than a decade ago, a comprehensive and insightful review of cuticular proteins presented the complete sequence and full citation for all 40 cuticular proteins known at that time and identified features that remain their hallmarks (Andersen *et al.*, 1995a). As of June 2003, in addition to the nonstructural cuticular proteins discussed above, there are now 139 sequences available for what are postulated to be structural proteins. These numbers do not include almost 200 more that have been identified by protein prediction programs used to annotate the *D. melanogaster* and *Anopheles gambiae* genomes. These have been omitted because their annotation is still in a state of flux. All 139 sequences and some of their key features are listed in **Table 1**. Marcu and Locke (1998) have also published tabular summaries of a smaller number of cuticular proteins.

Unfortunately, cuticular protein terminology is not uniform. Most workers have included the initials of the genus and species. Some have named their proteins based on their molecular mass, others on the order in which they obtained them. Many *D. melanogaster* proteins have been designated by the chromosomal band to which a gene-specific probe hybridized. Some have been named after their sequence similarity to a particular cuticular protein from another species. Capitalization and the use of hyphens are erratic. Two different groups have worked on proteins from *Tenebrio*, and given two different names to one protein. Some of the names have included a designator for genus, species, and metamorphic stage (e.g., TMLCP-A1A). Although this designation is an accurate indication of the stage from which the protein was purified, it can inadvertently support the misconception that a particular cuticular protein is stage-specific. In *Tenebrio*, larval and pupal cuticular proteins are indistinguishable electrophoretically (Andersen, 1975; Roberts and Willis, 1980a; Lemoine and Delachambre, 1986; Andersen *et al.*, 1995b), and molecular analyses of several *Tenebrio* cDNAs found that all expressed in pupae are also expressed in larvae (Mathelin *et al.*, 1998; Rondot *et al.*, 1998). Furthermore, proteins that are a major component of the cuticle of one stage can be a minor component of another (Cox and Willis, 1985; Willis, 1986). A final complication is whether two almost identical proteins are allelic variants or distinct proteins. In some cases an "isoform" has been described. Genomic sequences, however, have revealed that stretches coding for proteins with very similar or indeed identical sequence may be linked on a chromosome (Charles *et al.*, 1997; Dotson *et al.*, 1998) (see Section 4.2.4.2). Thus the finding of "isoforms" may reflect distinct genes and hence distinct proteins.

Table 1 includes proteins from discrete genes even when two or more may have the same amino acid sequence. In **Table 1**, proteins isolated from cleaned cuticles were counted as cuticular proteins, as were proteins whose nucleic acid sequences were obtained using partial protein sequences or antibodies raised against cuticular protein to select corresponding cDNAs. In addition, **Table 1** contains numerous proteins that had been classified as cuticular proteins by their "discoverers" because their sequence, or a part thereof, was similar to a cuticular protein already in the databases. For many of those in the latter category, the source of the cDNA that led to the sequence came from integumental epidermis or imaginal discs or a cDNA hybridized to epidermal RNA in a Northern analysis. For some, especially those from *Tenebrio* studied by Delachambre's group, confirmation came from *in situ* hybridization of specific probes (see Section 4.2.2.1.3 and **Table 3**). But for many, sequence similarity served as the sole criterion.

Most of the structural cuticular proteins whose sequences were known in 1995 came from the efforts of Svend Andersen and his group, and a significant fraction (42%) still does. All of their sequences come from direct sequencing of purified cuticular proteins. Most of the other protein sequences come from sequencing cDNA or genomic DNA. For these, the length of the mature proteins can only be deduced by subtracting the amino acids of the signal peptide. In a few cases, N-terminal sequence data is available to assure that the signal peptide has been correctly identified. In cases where this information was not available, or when the original submissions did not provide this information, it was determined using the program SignalP (Nielsen *et al.*, 1997; Nielsen and Krogh, 1998). All lengths in **Table 1** represent the mature, processed protein.

One notable feature of the structural cuticular proteins is that almost all lack cysteine and methionine residues in the mature protein; the five exceptions to this are indicated in **Table 1** and **Figure 4**. Andersen (see **Chapter 4.4**) suggests that the reactivity of cystine and cysteine with *ortho*-quinones could interfere with sclerotization.

Most of the cuticular proteins identified to date are quite short. Those less than 100 amino acids account for 27% of the 139 sequences in **Table 1**, while those between 100 and 199 account for an additional 52%. Only three proteins have more than 300 amino acids. The largest is the gene for

a *D. melanogaster* corneal lens protein (Cry, drosocrystallin) with 457 amino acids, and a perfect RR-2 consensus (Janssens and Gehring, 1999). (See Section 4.2.3.2.3 for discussion of this consensus.) A cDNA of the appropriate size has been described (gi:2143072). The next largest is for the only cuticular protein characterized from *Galleria* (Kollberg *et al*., 1995). This protein is unusual in that its only resemblance to known cuticular proteins is an abundance of alanine residues, and it is unique in having two cysteine residues. Yet its cDNA was selected with a polyclonal antibody raised against pupal cuticular proteins. The third largest protein is MSCP36; this is a high glycine protein that has a RR-1 consensus.

4.2.3.2.2. Motifs found in cuticular proteins The review by Andersen *et al*. (1995a) was the first to assemble a variety of motifs found in cuticular proteins. The occurrence of such motifs is given in **Table 1** and summarized in **Table 4**. Most common of these is a 28-residue region, first recognized by Rebers and Riddiford (1988) in seven cuticular proteins that is commonly referred to as the R&R Consensus. The original R&R Consensus is part of a longer conserved sequence – pfam00379 – and it is now apparent that there are three distinct forms of the extended R&R Consensus. These matters are discussed in detail below (see Section 4.2.3.2.3).

After the R&R Consensus, the next most common motifs were repeats of A-A-P-(A/V). These repeats were found in cuticular proteins both with and lacking the R&R Consensus; in sequences with the R&R Consensus they may occur N- or C-terminal to the extended Consensus. They are found in 46% of the sequences in **Table 1**. Thus, while abundant in cuticular proteins, they certainly are not diagnostic for this class of protein.

Andersen *et al*. (1995a) recognized several sequences with stretches of glycine, leucine, and tyrosine, beginning G-Y-G-L- or G-L-L-G. In **Table 1**, they are combined under the designation, G(Y/L) motifs. Other cuticular proteins are also high in glycine, but with less regular motifs; these are designated by the number of consecutive Gs. Proteins enriched in glycine residues are found in a variety of structures such as plant cell walls, cockroach ootheca, and silk (see Bouhin *et al*. (1992a) for discussion). Subsequent to their 1995 review, Andersen and his colleagues recognized two additional motifs. There is an 18-residue motif found in seven cuticular proteins from four orders of insects (and two crustaceans), and its consensus has been described (Andersen, 2000). It occurs in proteins with and without the R&R Consensus. Also reported was a 51-residue motif so far identified only in cuticular proteins from *Locusta* and *Tenebrio*. It has not been found in proteins with the R&R Consensus (Andersen *et al*., 1997). Other short repeats have been found in a limited number of proteins, from a single species. Proteins with the various motifs are identified in **Table 1**.

The basic sequences of the three long repeats are:

Original R&R Consensus: [G-x(8)-G-x(6)-Y-x(2)-A-x-E-x-G-F-x(7)-P-x-P.]
18 amino acid repeat: [(PV)-x-D-T-P-E-V-A-A-A-(KR)-A-A-(HF)-x-A-A-(HY).]
51 amino acid repeat: [x(6)-A-x(9)-R-S-x-G-x(4)-V-S-x-Y-x-K-x(2)-D-x(3)-S-S-V-x-K-x-D-x-R-x(2)-N-x(3).]

With this nomenclature, x is any amino acid, the number in parentheses represents the number of amino acids, and multiple letters in parentheses indicate that either of two amino acids may be present. This is the format used by MOTIF (2003), a resource that lets you search a given motif against various databases.

Andersen (2000) presented a model where proteins with the R&R Consensus bind to chitin and the other structural proteins remain free in the interfilament space.

4.2.3.2.3. Proteins with the R&R consensus The R&R Consensus is a common feature of cuticular proteins from all six orders of insects examined to date and it has also been recognized in cuticular proteins from arachnids and crustaceans (review: Willis, 1999).

Three distinct forms of the consensus have been recognized and named by Andersen (1998, 2000) RR-1, RR-2, and RR-3 (**Figures 1–3**). RR-1 is present in 51 (37%) of the proteins in **Table 1**.

Table 4 Summary characteristics of the 139 ''structural'' cuticular proteins that have been completely sequenced

	Number with AAP(AV)	*Number lacking AAP(AV)*	*Number 18-residue motif*	*Number 51-residue motif*	*Mean number H+K in extended R&R region (range)*	*Number with Met/Cys residues*	*Total proteins in class*
RR-1	2	49	0	0	3.7 (0–9)	3/0	51
RR-2	36	8	0	0	7.4 (2–19)	0/0	44
RR-3	1	2	3	0	6.3 (5–8)	0/0	3
Not RR	26	15	4	7		0/2	41

RR-1-bearing proteins have been isolated from flexible cuticles, while RR-2 proteins have been associated with hard cuticle. This generalization, based on relatively few cases (**Table 3**), has been used to link proteins to cuticle types in the absence of any other evidence.

The RR-1 proteins have the essential features of the original consensus (**Figure 1**). The vast majority have the short sequence -Y-x-A-x-E-x-G-(FY)-x(7)-P. N-terminal to this region the sequences diverge, but most have a series of three aromatic residues, such as Y-x-F-x-Y, that begins about 32 amino acids N-terminal to the start of the R&R Consensus (**Figure 1**). Another distinguishing feature defined by Andersen (1998) is a glutamic acid residue found in a conserved position [Y-x-A-x-**E**-G-(FY)] in 90% of the sequences in **Figure 1**.

The RR-2 proteins have a considerably extended consensus, first recognized by Bouhin *et al.* (1992a) and Charles *et al.* (1992). What is extraordinary about the RR-2 consensus is its conservation across six orders of insects. Only two single amino acid gaps are required to accommodate all 44 RR-2 sequences with this variant listed in **Table 1**. Twenty-two of the 70 residues in the extended consensus (31%) are virtually invariant and an additional 21 are represented by a single amino acid in over half of the proteins (**Figure 2**). The first few RR-2 sequences identified suggested that the residues G-F-N-A-V-V would be diagnostic (Andersen, 1998). The identification of more RR-2 sequences revealed that that region of the consensus is not perfectly conserved. Rather, all of the sequences have G-F-x-A-x-V, a configuration found in none of the RR-1 sequences.

There are other differences between RR-1- and RR-2-bearing proteins. Most of the RR-2 (82 %) have at least one A-A-P-(AV) motif, while only 2 (4%) of the RR-1 type have this motif (**Table 1**). Histidine and lysine residues can be more abundant in the extended consensus region of RR-2 proteins (**Table 4**, **Figures 1** and **2**). Only one RR-1 protein has been found with more than six histidine plus lysines in this region, while seven or more were present in 20 of the 44 RR-2 sequences. There is an invariant lysine in all RR-2 sequences and several other positions appear to be favorable for either of these amino acids. Over half of the RR-2 proteins, but only a quarter of the RR-1 proteins, have histidine as their final or penultimate C-terminal amino acid. Histidines and lysines are known to be reactive sites for sclerotizing agents (see **Chapter 4.4**), so it is possibly significant that proteins from "hard" sclerotized cuticles would have these amino acids in abundance. The number of potential sclerotization sites may also be related to whether a cuticle can grow by intussusception, something that would be impossible if the proteins were extensively cross-linked. The six aphid cuticular proteins, while all of the RR-2 type, have relatively few histidines plus lysines (only four or five); this paucity may reflect the need for cuticular expansion with the type of feeding and brooding of progeny that occurs in these animals.

An RR-3 form of the consensus has been based on three sequences from insects and two from other arthropods (Andersen, 2000). A tentative consensus (**Figure 3**) was constructed from the sequence alignment in Andersen (2000).

Whole genome sequencing has led to the need to classify annotated sequences. A valuable website, Pfam (2003a) has used hidden Markov modeling to define motifs characteristic of particular classes of proteins (Bateman *et al.*, 2002). When a protein sequence is searched against all known (and predicted) proteins using the BLAST server (Blast, 2003), the first information that is presented is an indication of matches to Pfam entries. The Pfam sequence that allows annotators to classify a protein as a cuticular protein is Pfam00379, a 68 amino acid sequence that includes the extended R&R Consensus. It also goes under the name "chitin_bind_4," for reasons that will become apparent later (see Section 4.2.5.4). The pfam00379 was obviously based on proteins of both RR-1 and RR-2 classes, for it matches neither particularly well (**Figure 3**). This makes it particularly useful for a preliminary classification of a putative cuticular protein sequence.

The pfam00379 is found in 70% of the 139 cuticular proteins in **Table 1**, i.e., all the RR-1, RR-2, and RR-3 sequences. A complete listing of all sequences with pfam00379 can be found at Pfam (2003b) or at ENTREZ (2003), where you search Domains for pfam00379. There are no nonarthropod sequences with this consensus. Now that two species of insects (*D. melanogaster* and *A. gambiae*) have had their genomes completely sequenced and pfam00379 is being used to recognize cuticular proteins, the representation of proteins bearing this motif will be disproportionate. As of August 2003, 90 *D. melanogaster* sequences beyond those listed in **Table 1** have been found to have pfam00379, and using the ENTREZ site, *A. gambiae* had over 100 beyond those in **Table 1**.

Pfam00379 so far has been found to occur only once in a given protein, with the notable exception of a protein from the tailfin of the prawn *Penaeus japonicus*. The entire sequence of this protein is made up of 14 consecutive pfam00379 motifs (Ikeya *et al.*, 2001). In the insect proteins, this motif has been found near the N- or C-terminus, or within the protein.

```
L HALCP-1        PEGSYQFGFETADGISRSETGDVKEALDEENKPHKVVVVRGSYSYTDKEGNPETVNYFAD-ETGYHAEGSSIP
L MSLCP16/17     PEGSYVFGFETEDGISRDETGEVKEALDEDNKPHSVVVVRGQYSYVDPDGNPQVIKYYAD-ETGYHAEGDSIP
O LM/SG-abd-4    PDGSYQWNYETGNGIKADETGTLKKGSKPDEGDFIVAQGSFSYTGPDGTAYQVQYSADDENGFVPQGAHFP
O SGAbd-1        DGSYRWNYETENGIAADETGALKAIAPNEDGTAAQGFYSYTAPDGTPIRVTYTAD-ENGFQAQGDHFP
O SGAbd-2        DGSYAYSYQTGNGIAAQEQGYLKNPGQRDLEAENVQGTFSYTAPDGTPISLRYVAD-ENGFRAEGAHLP
O SGAbd-8        PDGSYAWSYETGNGIAADESGALENPGQKDLEAMRAQGSFSYTAPDGSPISVRYVAD-RDGFHPEGAHLP
L BMWCP9         PDGSYKWSYETGNGISAEEQGYIKNQGIPEQEAQTAQGQYQYTAPDGQVIHVQYLAD-ENGFQPQGAHLP
L MSCP20         GDGSYHFSYETGNGISAQESGAPRAPGPEGLAVTAEGAFSYRTPDGQQIALTYTAD-ENGFRAEGAHLP
O SGAbd-3        DGSYRYSFETSDGQRASQEGALKQVSAPGPDGDTLGEAVRGDFSYTDDAGNQFAIQYTAD-ENGYVPQGAHLP
D DMLCP65Aa      SYSYKFETSDGTKQEQHGSLKSLGPEEDALQVAGSFSFVGDDGQTHAISYVAD-ENGFQPQGEDIP
L MSCP36         FAYDFETSNGIRADAQGVATNGVQSQGSFAYKGDDGQDYSITYTAD-ENGFVPQGAHLP
L MSCP27         GNYHFGFETSNGIRAEEAGGPEQAQGGYSYKGDDGQTYTLIYTSG-EGGFKPQGEHLP
O LMAbd-5        GQYNFAYRTSDGIARQEQGALKNAGSENEALEVQGSYTYKGVDGKDYTVTFVAN-ENGYQPRVQS
O SGAbd-5        GQYNFAYRTSDGIARQEQGALKNAGSENEAIEVQGSYTYKGVDGKDYTVTFVAN-ENGYQPRVQS
O SGAbd-6        GQYTFGFKTSDGLIRQEQGVVKNQGTENEALEVRGTITWLGADGKDYSINFVAD-ENGFQPQYTQ
O SGAbd-9        VDGSYTFSYESADGSARQESGVVNAPGTPLEAQAVQGSYTYVGTDGVPVQVNYVAD-ENGFQPVGNVVA
D LCCUT12        SFQYSYATSDGVEAEAQGQLKNVGTDEEAIVVKGSFSFVADDGQTYTVNYVAD-ENGFQPQGAHLP
D DMLCP65Af      GPVSFNYGYETSDGSSAQAAGQLKNVGTDEEALNVKGTYSFVADDGQTYSIAYTAD-ENGYQPQGAHLP
D DMLCP65Ag1/g2  SFKYDWETSDGQAAQAVGQLNDIGTENEAISVSGSYRFIADDGQTYQVNYIAD-KNGFQPQGAHLP
D DMLCP65Ae      NFQWSFETSDGQAANAKGQLKYPNTDHESLAVQGSFRFVADDGQTYEVNYIAD-ENGFQPQGAHLP
D DMLCP65Ac      YNFALETSDGKKHEEQGQLKNVGTEQEAIVVRGSYSFVADDGQTYTVNYIAD-ENGFQPEGAHLP
D DMLCP65Ad      YKFAVETSDGKSHQEEGQLKDVGTDHEAIVVRGSYAYVGDDGQTYSIQYLAD-ENGFQPEGAHLP
D DMLCP65Ab1/b2  ETSDGTSIKQEGVLKNAGTDNEAAVVHGSFTWVDEKTGEKFTITYVAD-ENGYQPQGAHLP
D DMACP65A       GGYKFSYKLSDGTSRTEEGVVNNAGTDNESISIRGSVTWVAPDGQTYTINFVAD-ENGFQPEGAHLP
L MSCP14.6       DPQHYSYSVETSNGIAFSEEGALKNVGSENEANSVRGSYAYVGPDGVTYSVVYIAD-ENGFQPQGAHLP
L HCCP12         FQYGYETSNGIQHQESGQLNNVGTENEGIEVRGQFSYVGPDGVTYSVTYTAG-QEGFKPVGAHIP
L BMCP18         YNTGYETSNGIKAQETGQLKNIGTENEALEVRGEFAYIGPDGVTYAVTYVAN-EGGFQPSAPHIP
C ApGLCP10.7     YNYAYDTSNGISAQENGQVINAGTDNEAIAARGQFTYTGPDGVQYSVSYTAD-ENGFQPVGAHIP
L BMCP30         YHYLYETENKILAEEAGKVENIGTENEGIKVKGFYEYVGPDGVTYRVDYTAD-ENGFVADGAHIP
D DMPCP          DGKYRYAYETSNGISASQEGLGGVAVQGGSSYTSPEGEVISVNYVAD-EFGYHPVGAHIP
D DPCP           DGNYRYAYETSNGISATQEGLGGVSVQGGSSYTSPEGSVISVSYVAD-ETGYHPVGDSIP
C ApGLCP12.3     EDGNFHYSYETGDGIKAHEEGTLKKVNDQLVESVSGGFEYTAPDGKPISISYVAD-ETGYHPVGDSIP
D DMEDG-78       AEGNYQYAYETSNGIQIQEAGNANGARGAVAYVSPEGEHISLTYTAD-EEGYHPVGDHLP
D DMLCP-1        FDSSLHTSNGIEQAASGDAHGNIHGNFGWISPEGEHVEVKYVAN-ENGYQPSGAWIP
D DMLCP-2        FDSSLHTSNGIEQAASGDAHGNIHGNFGWISPEGEHVEVKYVAN-ENGYQPSGAWIP
D LCCUT1         FDSVLDTSNHIHQAASGDEHGNIHGDFEWVSPEGQHVAVKYVAD-ENGYQPSGDVVP
D DMiLCP-1       FDADLLVSNSIQQASSGDVHGNIHGSFSWISPEGEHVEIKYVAD-ENGYQPVGAVLP
D DMiLCP-2       NSIQQAASGDIHGNAHGSFSWISPEGEHVDIKYVAD-ENGYQPVGAVLP
D DMLCP-3        PDGFVSKLVLDDGSASSATGDIHGNIDGVFEWISPEGVHVRVSYKAD-ENGYQPQSDLLP
D DMLCP-4        FVSKLVLDNGSAASATGDVHGNIDGVFEWVSPEGEHVRVSYKAD-ENGYQPQSDLLP
D DmiLCP-3/4     PDGFKTVVSLSDGSASQASGDVHGNIDGVFEWVSPEGVHVRVAYKAD-ENGYQPSSDLLP
D DmiLCP-3Y      PDGFKTVVSLSDGSASQASGDVHGNIDGVFEWVSPEGVHVRVAYKAD-ENGYQPTSDLLP
C ApGLCP12.6     PDGTYQFNYETENGISAQESGVPKSIDPAEPPAVVANGNFGYQSPEGVPVHISYVAD-ENGYQPVGDVLP
L BMCP22         PDGSYTYFYETNNGIAAQEQGVPRNLGGNPPAVPVVAQGSFSWTSPEGVPISVNYVAD-ENGYQPTGNAIP
L BMCP17         PEGHFQFNYETGNGIYAQAEGAVKNVNSEYPAIEVKGAYKYTSPDGQPIDLAYVAD-ENGYQPQGSHLP
L MSLCP-14       PEGSYNYAFESNNGISGQAEGKFKVFDKDSAAVVVAGSSQYKGSDGKVYSLTYVAD-ENGYQPQADFLP
L BMWCP10        DNGSYKYEYQIADGTHVGEEGYFTNPNTEEASLVKKGWYSYTGADGKVYTVHYWAD-KTGYHAYGDHLP

RR-1 Consensus   GxFxYxxPDGxxxxVxYxAD-ENGYQPxGAHLP

RR-2 Consensus   xEYDAxPxYxFxYxVxDxHTGDxKSQxExRDGDVVxGxYSLxExDGxxRTVxYTADxxNGFNAVVxxEx
```

Figure 1 Alignment of the pfam00379 regions of 51 proteins with the RR-1 consensus. The pfam 00379 regions for RR-1 proteins were aligned with ClustalW (http://clustalw.genome.ad.jp/); only one internal gap was used to allow direct comparison with the RR-2 consensus. Orders of insects are: (**C**) Coleoptera, (**D**) Diptera, (**L**) Lepidoptera, (**O**) Orthoptera. Abbreviations for proteins as in **Table 1**. Four pairs of identical sequences are each presented on a single line. Red represents amino acids present in at least 95% of the proteins, green in the majority. Histidines are shown in yellow, lysines in light gray. A common triad of aromatic residues is shown in light blue. Bolded and underlined are several residues from HCCP12 that are shown in **Figure 5a–c**. An RR-1 consensus based on these sequences is given. The bottom line gives the consensus for RR-2 proteins from **Figure 2**, except that two single amino acid gaps needed to accommodate three atypical sequences were eliminated.

The wealth of information on cuticular protein sequences and the unraveling of how the structure of some contributes to the interaction of chitin and protein (see Section 4.2.5) is only a beginning. Essential properties of cuticle remain to be explained, and important questions raised in the older literature about various means of achieving cuticle plasticity and the importance of hydration in cuticle stabilization must not be forgotten (Vincent, 2002 and references therein).

```
D  DS/DY          DHHDSHAEYDFEYGVKDHKTGDVKSQSESRHGH-TVTGHYELIDADG-HKRTVHYTADKHKGFEAHVHREK
Dy BC-NCP8        PQYDPNPQYTFSYNVDDPETGDSKSQEETRNGD-NVQGRYSVIESDG-SRRVVEYSADAVSGFNAVVHREA
D  DMCry          EDYDTRPQYSFAYDVRDSLTGDDKRQEEKRDGD-LVKGQYSLIEPDG-TRRIVEYTADDVSGFNAIVSKQR
O  LM-ACP21       AEYDPNPQYSYAYNVQDALTGDSKAQQETRDGD-VVQGSYSLVEPDG-SIRTVDYTADPVNGFNAVVHKEA
O  LM-NCP19.8     AEYDPHPQYSYGYSVNDALTGDSKSQQESRDGD-VVQGSYSLVEPDG-SVRTVDYTADPVNGFNAVVHKEP
C  TM-LCP-A1A     DEYDPNPQYSFGYDVQDGLTGDSKNQVESRSGD-VVQGSYSLVDPDG-TRRTVEYTADPINGFNAVVHREP
C  TM-LCP-A2B     DEYDPHPQYQYGYDVQDGLTGDSKSQIESRSGD-VVQGSYSLVDPDG-TRRTVEYTADPINGFNAVVHREP
C  TM-LCP-A3A     DEYDPHPQYSYGYDIQDGLTGDSKNQQETRDGD-VVQGSYSLVDPDG-TRRTVEYTADPINGFNAVVHREP
L  BMWCP6         EEYDAHPQYSFAYDVQDSLTGDSKTQHETRDGD-VVQGSYSVVDPDG-TKRTVDYTADPHNGFNAVVHKEP
D  DMCcp84Aa      EEYDPHPQYRFSYGVDDKLTGDNKGQVEERDGD-VVRGEYSLIDADG-YKRIVQYTADPINGFNAVVNREP
D  DMCcp84Ab      EEYDPHPQYRFSYGVDDKLTGDNKGQVEERDGD-VVRGEYSLIDADG-YKRTVQYTADPINGFNAVVNREP
D  DMCcp84Ae      EEVDPHPQYTYSYDVQDTLSGDNKGHVEERDGD-VVRGEYSLIDADG-FKRTVTYTADSINGFNAVVRREP
D  DMCcp84Ad      EEYDPHPQYKYAYDVQDSLSGDSKSQVEERDGD-VVRGEYSLIDADG-YKRTVQYTADPINGFNAVVNREP
D  DMCcp84Af      EEYDPHPQYKFAYDVQDSLSGDSKSQVEERDGD-VVHGEYSLIDSDG-YKRIVQYTSDPVNGFNAVVNRVP
D  DMCcp84Ag      EEYDPHPQYTYGYDVKDAISGDSKTQVETREGD-VVQGQYSLNDADG-YRRIVDYTADPINGFNAVVRREP
D  DMCcp84Ac      PDDDPHPKYNFAYDVQDALSGDSKSQVESRDGD-VVQGEYSLDDADG-FRRTVKYTADSVNGFNAVVHREP
D  DMEDG84        DTYDSHPQYSFNYDVQDPETGDVKSQSESRDGD-VVHGQYSVNDADG-YRRTVDYTADDVRGFNAVVRREP
D  AnGCP2b,c,d    VEHHAPANYEFSYSVHDEHTGDIKSQHETRHGD-EVHGQYSLLDSDG-HQRIVDYHADHHTGFNAVVRREP
D  AnGCP2a        VEHHAPANYEFSYSVHDEHTGDIKNQHETRHGD-EVHGQYSLLDSDG-HQRIVDYHADHHTGFNAVVRPEP
L  HCCP66            SDFSSFSYGVADPSTGDFKSQIESRLGD-NVQGQYSLLESDG-TQRTVDYAAGSE-GFNAVVRKDP
L  BMWCP1B,2      EEEYAHPKYDFAYSVADGHSGDNKSQHESRDGD-AVHGEYTLLEADG-SVRKVEYTADDHHGFNAVVSNSA
L  BMWCP1A        EEEYAHPKYDFAYSVADGHSGDNKSQHESRDGD-AVHGEYTLVEADG-SVRKVEYTADDHHGFNAIVSNTA
L  BMEDG84A       HDTYAHPKNDYAYSVADPHTGGHKSQHENRDGG-AVHGSYSLVEPDG-SVRKVDNTADDHHGFNAVVHKTP
L  BMWCP3         AEEIAYPKYEFNYSVADGHSGVNKSQQEVRDGD-AVKGSYSFHEADG-SIRTVEYTADAHNGFNAVVHNTA
L  BMWCP4         VDEYAHPKYGYSYSVEDPHTGDHKSQHETRDGD-VVKGEYSLLQPDG-SFRKVTYTADHHNGFNAVVHNTP
L  BMWCP5         VEDHAPAKYEFSYSVEDPHTGDHKSQHETRDGD-VVKGEYSLLQPDG-SIRKVEYTADHHNGFNAIVHNSE
L  BMWCP7A,B      EDYDAHPKYAFEYKIEDPHTGDLKSQHETRDGD-VVKGYYSLHEADG-SIRVVEYSADKHNGFNAVVKHTA
L  BMWCP8         EDHYAYPKYAFEYKIEDPHTGDNKYQHEIRDGD-VVKGEYSLHEADG-SIRTVKYTADKKSGFNAEVINSG
O  LM-ACP19       VDYYSYPKYAFEYGVNDPHTGDVKRQWEERDGD-VVRGEYSLLEPDG-TTRTVTYTADAHNGFNAVVHRSG
O  LM-ACP7        IEYDPNPHYSFEYSVSDAHTGDQKAQHETREGD-VVQGSYSLVEPDG-SVRTVEYTADPHNGFNAVVHRQA
O  LM-ACP8        AEPVAYPKYEFNYGVHDAHTGDIKQQSEARDGD-VVKGSYSLVEPDG-STRTVEYQADDHNGFNAVVHRTP
H  5 sp.          ESYDAPAPYNFEYSVNDPHTYDVKSQSEYADGNGYVKGSYSLVEPDG-STRTVEYTADDYNGFNAVVKKEG
H  AGCP           ESYDAPAPYNFEYSVNDPHTYDVKSQSEYADGNGYVKGSYSLVEPDG-STRTVEYTAEDYNGFNAVVKKEG
C  TMACP20        VDLHTPAHYQFKYGVEDHRTGDRKQQAEVRVGD-VVKGEYSLAEPDG-TVRVVKYTADDHNGFNAVVSRVG
C  TMACP22        IHLKAHPEYHSDYHVADHKTKDFKSKHEVRDGY-KVKGTYSLLEPDHKTVRVVDYVSDKKRGFIARVSYRK

RR-2 Consensus    xEYDAxPxYxFxYxVxDxHTGDxKSQxExRDGD-VVxGxYSLxExDG-xxRTVxYTADxxNGFNAVVxxEx
```

Figure 2 Alignment of the pfam00379 region of 44 cuticular proteins with the RR-2 consensus. Abbreviations as in **Figure 1** plus Dictyoptera (**Dy**) and Hemiptera (**H**). All hemipteran proteins except the one from *Aphis gossypii* are indicated by [***H*** 5 sp]. The order of the proteins was based on alignment by ClustalW. Red represents amino acids present in at least 95% of the proteins, green in the majority. Histidines are shown in yellow, lysines in light gray. The seven histidines in AGCP2b that are discussed in the text (see Section 4.2.5.4) and modeled in **Figure 5d** are bolded and underlined, beginning with residue 99. An RR-2 consensus is given.

```
pfam00379:
    PDGSYNYAYETSNGIADQETGDLKSQGEERDGDVVQGSYSYVDPDGQTRTVTYTAD-ENGFQPVGAHLP
RR-1 Consensus:
                                        GxFxYxxPDGxxxxVxYxAD-ENGYQPxGAHLP
RR-2 Consensus:
    xEYDAxPxYxFxYxVxDxHTGDxKSQxExRDGDVVxGxYSLxExDGxxRTVxYTADxxNGFNAVVxxEx
RR-3 Consensus:
V-xVxTxYHAQDxLGQxSFGHxxxxQxRxExxDAAGNKxGSYxYVDPxGKVxxxxYVAD-AxGFRVAxx-NLPVxP
```

Figure 3 Consensus regions from the three types of RR cuticular proteins plus pfam00379. For pfam00379, the three aromatic residues found in RR-1 and RR-2 sequences (**Figures 1** and **2**) are shown in light blue. Indicated in red is the original R&R Consensus; the dash (-) was inserted to facilitate alignment with the other sequences. The green F was a Y in the original. RR-1 and RR-2 consensuses are from **Figures 1** and **2**, respectively. Red residues were found in at least 95% of the sequences, green in at least 50%. RR-3 was taken from Andersen (2000) using the sequences from the three insects and two arthropods arachnid and crustacean, that was identified as RR-3. Red residues were found in all five RR-3 sequences, green in three or four. The first and third dashes were inserted to allow alignment among all RR-3 sequences; the second was necessary to allow alignment with the RR-2 sequence.

4.2.3.2.4. Resilin The name resilin has been given to the rubberlike proteins responsible for the elasticity of jumping fleas and vibrating wings. Resilins are characterized by a high percentage of glycine (35–40%) and proline (7–10%). They are cross-linked with di- and tri-tyrosine residues (Andersen and Weis-Fogh, 1964). An intact protein corresponding to resilin has never been isolated from cuticle, presumably reflecting its insolublility after cross-linking. Ardell and Andersen (2001) used short peptide sequences that they had obtained from resilin-bearing regions of *Schistocerca* cuticle to probe the annotated *D. melanogaster* genome. Two candidate proteins had good matches to the locust peptides and to some that Lombardi and Kaplan (1993) had obtained from resilin in *Periplaneta americana*.

Ardell and Andersen concluded that predicted protein CG15920 (gi:24654243) of 620 amino acids was most likely to be a true proresilin (the non-cross-linked version), for in addition to the peptide matches, it had 35% glycine and 11% proline. Its 18 N-terminal copies of a 15-residue repeat and 13 C-terminal copies of a 13-residue repeat were predicted to contribute to a β-spiral, a common form for proteins with elastic properties (Ardell and Andersen, 2001). A cDNA (gi:27820115) is available that corresponds to most of the genomic sequence, but it lacks 45 internal amino acids. These correspond to the predicted second exon in the genomic sequence and contain almost the entire match to the locust proresilin peptides as well as most of the match to pfam00379. All of the repeat regions, however, are present. Thus, in order for CG15920 to match the locust resilin, it would have to be coded by an alternatively spliced form.

The second *D. melanogaster* gene that had "resilin peptides" was CG9036. Ardell and Andersen considered it to be a less likely candidate because it lacked both a predicted signal peptide and features expected for elastic properties. The original version they described has been replaced (gi:19922620) and a cDNA identical to this new sequence has been obtained. The new version has a predicted signal peptide of 19 amino acids. The mature protein of 198 amino acids has a pfam00379 region (occupying one-third of the mature protein) and is 20% glycine and 10% proline; it has neither of the repeats found in the other candidate protein.

Proof that either of these sequences is proresilin will require localization of the mRNA or protein to the tendons shown to have resilin in Diptera (Andersen and Weis-Fogh, 1964). The special properties of resilin justify further work to establish its sequence.

4.2.3.2.5. Glycosylation of cuticular proteins Glycosylation of cuticular proteins was first reported by Trim (1941) and in limited subsequent reports (review: Cox and Willis, 1987b). In recent years, posttranslational modifications of cuticular proteins have been determined by staining gels with periodic acid Schiff (PAS), by using labeled lectins to probe blots of electrophoretically separated proteins or by discovering discrepancies in masses of peptide fragments experimentally determined by matrix-assisted laser desorption ionization – mass spectrometry (MALDI-MS) analysis and calculated from Edman sequencing.

Most of the major cuticular proteins seen on gels stained with Coomassie Blue are not recognized by PAS or lectins, while some minor ones are glycosylated. This was true for *H. cecropia* where PAS staining revealed glycosylated proteins in extracts of flexible cuticles and a screen with eight lectins revealed the presence of mannose and *N*-acetylgalactosamine, with more limited binding to *N*-acetylglucosamine, galactose, and fucose, in a few of the proteins from all stages (Cox and Willis, 1987b). A comparable study in *Tenebrio* revealed one major band of water-soluble larval and pupal cuticular proteins that had *N*-acetylglucosamine; a few other bands were weakly visualized with lectins; none of the proteins from adult cuticle reacted with the lectins (Lemoine *et al.*, 1990). In another coleopteran, *Anthonomus grandis*, glycosylation was found in cuticular proteins extracted from all three metamorphic stages (Stiles, 1991). In *Calpodes*, all the BD peptides (see Section 4.2.2.2) extracted from the cuticle were associated with α-D-glucose and α-D-mannose, just like most of the hemolymph proteins but very few of the C class proteins. Some of each class appeared to be modified with *N*-acetylglucosamine. T66, a protein synthesized in spherulocytes, transported to epidermis, and then secreted into the cuticle, however, was not glycosylated. In none of these species is the amino acid sequence of a glycosylated protein known.

Sequence-related information about glycosylation is available for cuticular proteins isolated from locusts and *Manduca* (see **Table 1**) where the direct analysis of residues had been used. In *Locusta migratoria*, one to three threonine residues were modified in the protein LM-ACP-abd4. In each case, the modification was with a moiety with a mass of 203, identified as *N*-acetylglucosamine (Talbo *et al.*, 1991). Each of the three threonine residues occurred in association with proline (FPTPPP, LATLPPTPE). All eight of the cuticular proteins that have been sequenced from *Schistocerca gregaria* nymphs had evidence for glycosylation with a moiety with a mass of 203, all at a threonine residue found in a cluster of prolines (Andersen, 1998). Three proteins recently isolated from *Manduca* were similarly shown to be

glycosylated on threonines also in proline-rich regions. Surprisingly in these cases, masses of the adducts were varied (184, 188, and 189) and their nature was not determined (Suderman *et al*., 2003). In all of these cases, the available evidence indicates that the threonine residues had been O-glycosylated. The significance of such glycosylation awaits further elucidation.

4.2.4. Genomic Information

4.2.4.1. Introduction

The first four cuticular proteins whose complete sequences were determined were also the first to have their genes described (Snyder *et al*., 1982). The wealth of experimental detail and thoughtful discussion in that paper make it a classic in the cuticular protein literature. These four genes were for *D. melanogaster* cuticular proteins LCP-1, -2, -3, and -4, and were found to occupy 7.9 kb of DNA, along with what appeared to be a pseudogene. Each gene had a single intron and that intron interrupted the protein-coding region between the third and fourth amino acid. *LCP-1* and *-2* were in the opposite orientation of *LCP-3* and *-4*. The nucleic acid sequences in the protein coding regions for LCP-1 and -2 were 91% identical, for LCP-3 and -4, 85%, with similarity between the two groups about 60%. For the noncoding regions of the mRNAs, the 5′ upstream regions had more sequence similarity than the 3′ downstream. A consensus poly(A) addition site, AATAAA, was found for two of the genes, 110 bp from the stop codon, while similar but not identical sequences (AATACA, AGTAAA), were found for the other two. The four genes were all expressed in the third instar and several short, shared elements were found in their 5′ regions upstream from the transcription start site. Snyder *et al.* (1982) also speculated on the origin of the cluster through gene duplication and inversion. These features of those four genes (coding for RR-1 proteins) have turned out to be the common elements of most of the cuticular protein genes that are known – hence linkage, shared and divergent orientation, an unusually placed intron that interrupts the signal peptide, presence of a pseudogene in the cluster, atypical poly(A) addition sites, and divergence of 3′-untranslated regions have been found for cuticular protein genes in Diptera, Lepidoptera, and Coleoptera.

4.2.4.2. Chromosomal Linkage of Cuticular Proteins Genes

In addition to the four *D. melanogaster* genes discussed in the previous section, several more instances of linked cuticular proteins genes were described prior to sequencing entire genomes. In some cases, the evidence for these genes was restricted to cross-hybridization of the genomic fragment, and complete sequences are not known for all the members. A summary of such linked genes is presented in Table 5. Many more instances will become known as annotation of the *A. gambiae* and *D. melanogaster* genomes is completed.

A detailed analysis of the cluster of genes at 65A allowed Charles *et al.* (1997, 1998) to describe important features that most likely contributed to the multiplication and diversification of cuticular protein genes. Twelve genes (Table 5) were identified in a stretch of 22 kb with the direction of transcription, or more accurately the strand used, was

><<<<<<   >>>>> .

The third gene in the cluster appeared to be a pseudogene. Several important features were found. First, the number of *Lcp-b* genes within the cluster was variable among different strains of *D. melanogaster*, two in the original line (with identical coding sequences), one in another, and three in a third. On the other hand, three copies of the *Lcp-g*

Table 5 Linked cuticular protein genes

Species	*Length of DNA examined*	*Number of genes found*	*Protein names*	*Reference*
Anopheles gambiae	17.4 kb	3	AGCP2a, 2b, 2c	Dotson *et al.* (1998)
Drosophila melanogaster	9 kb	6	DM-LCP1, 1ψ, 2, 3, 4 and one other	Snyder *et al.* (1982)
Drosophila melanogaster	20.5 kb	8	EDG-84, 84Aa, Ab, Ac, Ad, Ae, Af, Ag	Apple and Fristrom (1991), Kaufman *et al.* (1990), genome annotation
Drosophila melanogaster	22 kb	12	ACP65A, LCP65Aa, aψ, b1, b2, c, d, e, f, g1, g2, g3	Charles *et al.* (1997, 1998)
Manduca sexta	20 kb	3	LCP16/17 + 2 not named	Horodyski and Riddiford (1989)
Tenebrio molitor	3.9 kb	2	TMLPCP 22, 23	Rondot *et al.* (1998)

genes (also with 100% sequence identity) were found in all three strains. Comparison of cDNA sequences and genes revealed that *Lcp-b1* and *-b2* lacked introns. Both *Lcp-b1* and *-b2* had tracts of As at the 3′ end of the genes, as well as short flanking direct repeats. These features are consistent with the *Lcp-b* genes arising in this cluster by retrotransposition. The sequence data also indicated that *Acp* and *Lcp-a* lacked introns. The rest of the genes had introns but not the common one interrupting the signal peptide (see Section 4.2.4.3).

Evidence for gene conversion between the *Lcp-c* gene and those on the right side of the cluster was also found after a careful analysis of the sequences (Charles *et al.*, 1997).

The consequences of gene duplication in terms of gene expression are an important issue. It could be that duplicated genes were preserved to boost the amount of product made in the short period that the single-layer epidermis is secreting cuticle. Alternatively, duplication may allow for precise regulation of expression of genes both spatially and temporally. Subtle difference in protein sequence may be advantageous for particular structures. A detailed analysis of mRNA levels with Northern blot analysis demonstrated that some members of the *65A* cuticular protein cluster have quite different patterns of expression. *Acp* was expressed only in adults. Expression was not detected for *Lcp-a;* all other *Lcp* genes were expressed in all larval stages, and all but *Lcp-b* and *-f* also contributed to pupal cuticle (Charles *et al.*, 1998).

4.2.4.3. Intron Structure of Cuticular Protein Genes

Genomic sequence data is available for 45 cuticular proteins in **Table 1**. None of these has more than two introns; most have only one, and these introns are in a very conserved positions. Thirty-five of these proteins have an intron that interrupts the signal peptide. In 25 of these sequences, interruption occurs after four amino acids (12 bp); in the remainder, two to eight amino acids are coded for before the intron begins. The PSORT tool (Psort, 2003) calculates the location of discrete regions of a potential signal peptide using modifications of McGeoch's method (McGeoch, 1985) and reports this information as "PSG" data. These short stretches, confined to the first exon, were shown to be identical to the N-terminal positively charged region in 20 cases; all but four of the rest were but one amino acid longer. Whether this correlation of the coding region of the first exon with the N-region of the signal peptide is because it is so short, or because it reflects something more fundamental awaits further exploration. Most of the putative cuticular protein genes in the annotated *A. gambiae* database are missing their initiator methionine, probably because it resides in a short exon, coding for these few amino acids of the signal plus 5′ untranslated nucleotides. The programs, unfortunately, are not yet trained to recognize a configuration with such a short open reading frame.

Genes for four of the proteins listed in **Table 1** (HCCP12 and MSLCP 16/17, MSCP14.6, and TMACP22) have two introns, one interrupting the signal peptide, the other occurring shortly after the beginning of the pfam00379 region. The intron-bearing *D. melanogaster* genes in the cluster at 65A (see above) have their sole intron at the internal position. This led Charles *et al.* (1997) to postulate that the primitive condition for introns in insect cuticular proteins would be two; over time, some genes lost one, some the other, and some lost both or arrived in the genome by retrotransposition.

There is also a *Drosophila* cuticular protein whose gene is located within the region corresponding to the first intron of *Gart*, a gene that encodes three proteins involved in the purine pathway. The gene for this RR-1 protein (*Gart Intron*) is read off the opposite strand and has its own intron, conventionally placed interrupting the signal peptide (Henikoff *et al.*, 1986). A comparably placed gene with 70% amino acid sequence identity is found in *D. pseudoobscura* (Henikoff and Eghtedarzadeh, 1987).

4.2.4.4. Regulatory Elements

One of the attractions of studying cuticular proteins is that they are secreted at precise times in the molt cycle and are thus candidates for genes under hormonal control (Riddiford, 1994). It would be expected, therefore, that some might have hormone response elements (see **Chapter 3.5**). Imperfect matches to ecdysteroid response elements (EcREs) from *D. melanogaster* have been found on two of its cuticular protein genes: EDG78 and EDG84 (Apple and Fristrom, 1991). These genes are activated in imaginal discs exposed to a pulse of ecdysteroids, but if exposed to continuous hormone, no message appears. The two cuticular protein genes that have been studied in *H. cecropia* have regions close to their transcription start sites that resemble EcREs (Binger and Willis, 1994; Lampe and Willis, 1994) and upstream from MSCP14.6 are also two regions that match (Rebers *et al.*, 1997).

Both *Bombyx* PCP and *H. cecropia* HCCP66 have response elements for members of the POU

family of receptors (Nakato *et al*., 1992; Lampe and Willis, 1994). POU proteins are transcription factors used for tissue-specific regulation in mammals (Scholer, 1991). Gel mobility shift experiments established that there was a protein in epidermal cells that could bind to this element (Lampe and Willis, 1994).

As more genomic sequence information becomes available, identification of regulatory elements and verification of their action is certain to be productive.

4.2.5. Interaction of Cuticular Proteins with Chitin

Ever since the R&R Consensus was recognized in 1988, it has been predicted that it must be playing an important role in cuticle. As more and more sequences were discovered with the Consensus, and as it was learned that it also is present in cuticles formed by arachnids and crustaceans, this prediction became more likely. Several workers suggested that the role of the R&R Consensus might be to bind to chitin (Bouhin *et al*., 1992a; Charles *et al*., 1992; Andersen *et al*., 1995a).

Four complementary routes have been followed to learn more about the function of this consensus region. The first was to analyze it with appropriate programs to generate predictions of secondary structure. The second approach was to use spectroscopic techniques on cuticular components to gain information about the conformation of their protein constituents *in situ*. Third, the tertiary structure of the extended Consensus has been modeled, and the fourth route was a direct experimental approach to test whether the extended Consensus could bind to chitin.

4.2.5.1. Secondary Structure Predictions

Prediction of secondary structure was carried out on the extended R&R Consensus region (67–68 amino acids, the pfam00379 region) of cuticular proteins representing different metamorphic stages and four different orders (Iconomidou *et al*., 1999). For each protein, individual predictions of α-helix, β-sheet, and β-turn/coil/loops were carried out using several different predictions programs. These predictions on individual proteins were combined to produce joint prediction histograms for the two classes of proteins. (See Iconomidou *et al*. (1999) for details of proteins analyzed, programs used, and pictorial representation of results.)

The results indicated that the extended R&R domain of cuticular proteins has a considerable proportion of β-pleated sheet structure and a total absence of α-helix. There appeared to be four β-strands in the RR-2 proteins and only three in the RR-1. Three other features were immediately apparent:

1. The three invariant glycines of the original R&R Consensus correspond exactly at the maxima of β-turn/loop predictions, and it is well known that glycines are good turn/loop formers (Chou and Fasman, 1974a, 1974b).
2. With both classes of cuticular proteins, the sheets showed an amphipathic character, i.e., one face is polar, the other nonpolar. Alternating residues along a strand point in the opposite direction on the two faces of a β-sheet. With these proteins, it is the aromatic or hydrophobic amino acids that alternate with other, sometimes hydrophilic, residues. The aromatic rings are thus positioned to stack against faces of the saccharide rings of chitin. This type of interaction is fairly common in protein–saccharide complexes (Vyas, 1991; Hamodrakas *et al*., 1997; Tews *et al*., 1997).
3. The turn/loop regions frequently contained histidines. This would place them "exposed" at the "edges" of a β-pleated sheet. Histidines are involved in cuticular sclerotization (see **Chapter 4.4**) and are involved in the variations of the water-binding capacity of cuticle and the interactions of its constituent proteins. This occurs because small changes of pH can affect the ionization of their imidazole group (Andersen *et al*., 1995a).

The suggestion that cuticular proteins adopt a β-sheet configuration is not new. Fraenkel and Rudall (1947) provided evidence from X-ray diffraction that the protein associated with chitin in insect cuticle has a β-type of structure.

4.2.5.2. Experimental Studies of Cuticular Protein Secondary Structure

The next step in probing the structure of cuticular proteins involved direct measurements on intact cuticles, on proteins extracted from them with a strong denaturing buffer with 8 M guanidine hydrochloride, and on the extracted cuticle. The cuticles came from the flexible abdominal cuticle of larvae of *H. cecropia*, and extracts have HCCP12, a RR-1 protein, as a major constituent (Cox and Willis, 1985). The same prediction programs described above were used on the sequence for HCCP12, and it indicated that the entire protein had a considerable proportion of β-pleated sheet and total absence of α-helix. Fourier-transform Raman spectroscopy

(FT-Raman), attenuated total reflectance infrared spectroscopy (ATR-FT-IR), and circular dichroism spectroscopy (CD) were carried out on these preparations (Iconomidou *et al.*, 2001). These techniques eliminated problems that had been found previously with more conventional laser-Raman spectra due to the high fluorescent background associated with cuticle.

The FT-Raman spectra of both the intact and extracted cuticle were dominated by the contribution of bands due to chitin. Certain features of the Raman spectrum of the intact cuticle signified the presence of proteins. The protein contribution to the spectrum of intact cuticle was revealed by subtracting the spectrum of the extracted cuticle, after scaling the discrete chitin bands of both preparations. The comparison of this difference spectra to that from the isolated proteins revealed striking similarities suggesting that the former gave a reliable physical picture of the cuticle protein vibrations in the native state. While Iconomidou *et al.* (2001) presented a detailed analysis of the spectra and the basis for each assignment, only a few features will be reviewed here. Several of the bands could be attributed to side-chain vibrations of amino acids with aromatic rings, tyrosine, phenylalanine, and tryptophan. Bands in the amide I region (1600–1700 cm^{-1}) of the Raman spectra of the extracted cuticle proteins and of the difference spectrum exhibited a well-defined maximum at 1669 cm^{-1}, typical of β-sheet structure. The absence of bands at ~1650 cm^{-1} indicates that α-helical structures are not favored. The amide III range (1230–1320 cm^{-1}) is relatively free from side group vibrations and, thus, highly diagnostic of secondary structure. The extracted proteins had a doublet at 1241 and 1268 cm^{-1}; the former can be assigned to β-sheet and the latter to β-turns or coil.

Results from ATR-FT-IR spectra from the extracted proteins were in good agreement with their FT-Raman spectra. These spectra had been obtained on lyophilized samples: the CD spectrum, on the other hand, was obtained with proteins solubilized in water. Detailed analysis of the CD spectrum indicated a high percentage (54%) of β-sheet conformation with a small contribution of α-helix (~13%). The contributions of β-turns/loops and random coil were estimated as 24% and 9% respectively (Iconomidou *et al.*, 2001). These results demonstrated that the main structural element of cuticle protein is the antiparallel β-pleated sheet. Comparable results were obtained from lyophilized proteins and intact cuticles and from proteins in solutions, thus negating the concern that lyophilization might increase the β-sheet content of proteins as discussed by Griebenow *et al.* (1999). These direct measurements confirm the results from secondary structure prediction discussed above (see Section 4.2.5.1).

These findings are in accord with the prediction of Atkins (1985) that the antiparallel β-pleated sheet part of cuticular proteins would bind to α-chitin. His proposal was based mainly on a two-dimensional lattice matching between the surface of α-chitin and the antiparallel β-pleated sheet structure of cuticular proteins.

There seem to have been several independent solutions in nature whereby chitin binds to protein; in all surface aromatic residues appear to be significant (Shen and Jacobs-Lorena, 1999). In several cases β-sheets have been implicated. The chitin-binding motifs of two lectins studied at atomic resolution contain a two-stranded β-sheet (Suetake *et al.*, 2000). In bacterial chitinases, an antiparallel β-sheet barrel has also been postulated to play an important role in "holding" the chitin chain in place to facilitate catalysis. Four conserved tryptophans on the surface of the β-sheet are assumed to interact firmly with chitin, "guiding" the long chitin chains towards the catalytic "groove" (Perrakis *et al.*, 1997; Uchiyama *et al.*, 2001).

4.2.5.3. Modeling of Chitin-Binding Domains of Cuticular Proteins

Secondary structure prediction and experimental data summarized above (see Sections 4.2.5.1 and 4.2.5.2) indicated that β-pleated sheet is most probably the underlying molecular conformation of a large part of the extended R&R Consensus, especially the part which contains the R&R Consensus itself, and that this conformation is most probably involved in β-sheet/chitin–chain interactions of the cuticular proteins with the chitin filaments (Iconomidou *et al.*, 1999, 2001). Can this information be translated into a three-dimensional model?

Unexpectedly, a distant (20%) sequence similarity was found between RR-1-bearing cuticular proteins and the crystallographically determined C-terminal, β-sheet barrel portion, of bovine plasma retinol-binding protein (RBP). When, following alignment, both conservative substitutions and identities were combined, the similarity rises to 60% of the total HCCP12 sequence (Hamodrakas *et al.*, 2002). This similarity allowed the construction, by "homology" modeling, of a structural model of the "extended R&R consensus" (Hamodrakas *et al.*, 2002). This modeling was successful even though it is clear that RBP and the R&R Consensus-bearing cuticular

proteins are not strictly homologous, for HCCP12 lacks the N-terminal region that is conserved in members of the lipocalin superfamily to which RBP belongs. The original model (**Figure 5a**) comprises the C-terminal 66 residues (out of 89 in total) of HCCP12 and corresponds to the "extended R&R consensus" (see Section 4.2.3.2.3).

Does this model fit both major classes of RR proteins? Stereo plots of this model of HCCP12 (**Figure 4a**) can be compared to comparable models of two RR-2 proteins (HCCP66 and AGCP2b) (**Figure 4b** and **c**). These models demonstrated that the extended R&R Consensus of both "soft" and "hard" cuticle proteins may easily adopt the proposed conformation.

How would this proposed structure interact with chitin? A low-resolution docking experiment of an extended *N*-acetylglucosamine tetramer to the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996) revealed that the proposed model for cuticle proteins accommodates, rather comfortably, at least one extended chitin chain (**Figure 4d**) (Hamodrakas *et al.*, 2002). The features revealed by secondary structure predictions (see Section 4.2.5.1) and by experimental spectroscopic analysis (see Section 4.2.5.2) work exceedingly well with this model. It is an antiparallel β-sheet structure with a "cleft" full of conserved aromatic residues that form "flat" hydrophobic surfaces on one "face," perfectly positioned to stack against faces of the saccharide rings of chitin. One unpredicted feature in the model is a short (seven-residue) two-turn α-helix at the C-terminus of the extended R&R Consensus of HCCP12, starting and ending with two proline residues, present in 60% of the "soft" cuticle proteins in **Figure 1**. This C-terminal part of the model is reminiscent in some respects of the chitin-binding domain of an invertebrate chitin-binding lectin, a two-stranded β-sheet followed by a helical turn (Suetake *et al.*, 2000). The structures of these two different chitin-binding proteins cannot be superimposed, however, and show no sequence similarity.

For this review, the proposed half-barrel model (see **Chapter 4.8**). has been used as a basis for more detailed docking experiments. A "high-resolution" docking experiment with the same tetramer was performed and the results are displayed and discussed in **Figure 5**. A new possibility emerges from this "high-resolution" experiment: the chitin chains can run either parallel to the β-strands of the half β-barrel model (**Figure 5b** and **c**), in good agreement with the observations of Atkins (1985), or perpendicular to the β-strands (**Figure 5a**). **Figure 5b** and **c** also provide an instructive view as to how a twisted helicoidal structure might arise from a close packing interaction of half-β-barrel models of cuticle

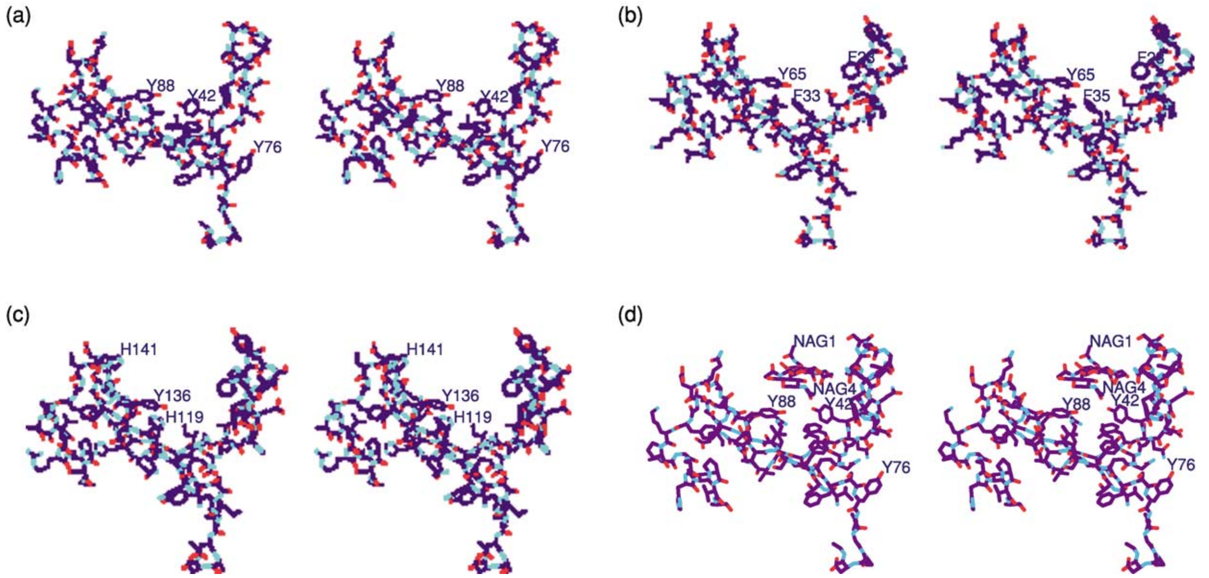


Figure 4 Stero pairs of cuticular proteins and their interaction with chitin. Stero pairs of cuticular proteins drawn with the program O (Jones *et al.*, 1991). The numbering scheme used is that of the unprocessed proteins. (a) View of the ''soft'' cuticle protein HCCP12. (b) View of the ''hard'' cuticle protein HCCP66. The terminal Ile83 residue could not be modeled and is not shown. (c) View of ''hard'' cuticle protein AGCP2b. His140 and the terminal Val155 residues could not be modeled and are not shown. (d) HCCP12 shown with an *N*-acetyl glucosamine (NAG) tetramer in an extended conformation. The complex was derived from a ''low-resolution'' docking experiment of a NAG tetramer, in an extended conformation, with the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996) and the default parameters of the program. (Reprinted with permission from Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., **2002.** A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem. Mol. Biol. 32*, 1577–1583, © Elsevier.)

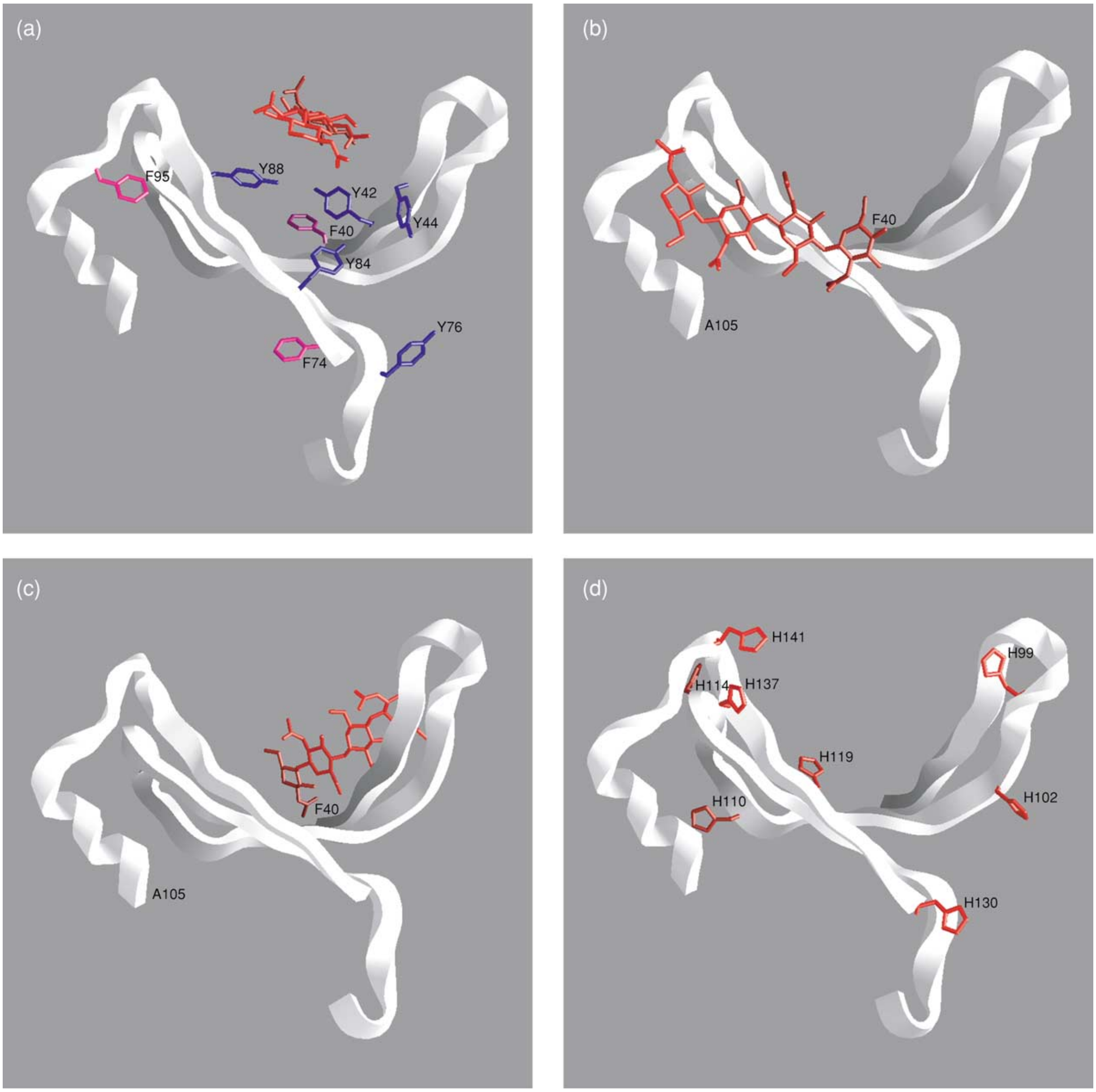


Figure 5 Ribbon models of cuticular proteins derived from homology modeling. (a) A ribbon model of cuticle protein structure, displayed using GRASP (Nicholls *et al.*, 1991). The structure of the representative ''soft'' cuticle protein HCCP12 was modeled on that of bovine retinol binding protein (RBP; PDB code 1FEN) (Zanotti *et al.*, 1994) utilizing the program WHAT IF (Vriend, 1990). Further details are in Hamodrakas *et al.* (2002). The side chains of several aromatic residues are shown and numbered, following the numbering scheme of the unprocessed HCCP12 sequence. These are: F40, Y42, Y44, H52, F74, Y76, Y84, Y88, and F95, underlined and bolded in **Figure 1**. The model structure has a ''cleft'' full of aromatic residues, which form ''flat'' surfaces of aromatic rings (upper side), ideally suited for cuticle protein–chitin chain interactions, and an outer surface (lower side) which should be important for protein–protein interactions in cuticle. The model is a complex of HCCP12 with an *N*-acetyl glucosamine (NAG) tetramer in an extended conformation. The complex was derived from a ''low-resolution'' docking experiment of a NAG tetramer, in an extended conformation, with the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996) and the default parameters of the program (a view similar to **Figure 4d**). (b) and (c) Two more possible complexes of HCCP12 with an NAG tetramer in an extended conformation derived from a ''high-resolution'' docking experiment, utilizing the program GRAMM (Vakser, 1996) and the default parameters of the program for ''high resolution.'' The two models presented in (b) and (c) are the two ''top on the list,'' most favorable complexes, whereas third on the list is a structure similar to that of (a). The one in (b) has the NAG tetramer more or less parallel to the last β-strand of the HCCP12 half β-barrel model, whereas that in (c) has the NAG tetramer more or less parallel to the first β-strand of the HCCP12 half β-barrel model. Note that, both in (b) and (c) the chitin chain runs parallel to the β-strands, whereas in (a) the chain is arranged perpendicular to the β-strands. (d) A display of a model of the ''hard cuticle'' protein AGCP2b. The numbering is that of the unprocessed protein. Histidine (H) side chains are shown as ''ball and sticks,'' in red, with their corresponding numbering. The corresponding residues are underlined in the sequence for AGCP2b in **Figure 2**.

proteins with chitin chains. It can be seen that the chitin chains, although more or less parallel to the β-strands, are forming an angle of the order of 10–15 degrees with the β-strands, and this, together with the inherent twist of the β-strands in the β-barrel, could provide the basis for the twisted helicoidal structure of the cuticle in general. Thus, both the inherent twist of the half-barrel β-sheet of the cuticle proteins and its packing arrangement at an angle with the chitin chains may provide a molecular basis for the morphological observation of a helicoidal twist in cuticle.

The model proposed by Hamodrakas *et al.* (2002) was subjected to a further test, namely that it should provide for the right positioning of histidine residues in the "hard cuticle" proteins, so that these histidines might play a significant role in cuticle sclerotization (Neville, 1975; Andersen *et al.*, 1995a (see **Chapter 4.4**). Histidines are a common feature of the extended R&R Consensus of many RR-2 proteins and many of their positions are conserved (**Figure 2**) (see Section 4.2.3.2.3). If they are to function in protein cross-linking by sclerotizing agents, they must reside on opposite faces to the aromatic residues that were postulated to interact with chitin (Iconomidou *et al.*, 1999; Hamodrakas *et al.*, 2002). In **Figure 5d**, a model of the "hard cuticle" protein AGCP2b is shown, similar in orientation to **Figure 5a**, indicating the positions of the histidine side chains. The relevant histidines are underlined and in bold in the sequence for AGCP2b in **Figure 2**. Three histidines, H102, H110, and H141 (the second, third, and last) are at sites where histidines are common. Such an interspecies conservation of these histidines, most probably signifies their very important structural and functional role (see below).

All the bolded and underlined histidines occupy "exposed" positions either in turns (like H99, H114, H137, H141), or at the "edges" of the half-β-barrel or its periphery (like H102, H110, H130), in excellent positions to be involved in cuticular sclerotization, readily reacting with activated *N*-acetyldopamine residues. Alternatively, they could be involved in the variations of the water-binding capacity of cuticle and the interactions of its constituent proteins, because small changes of pH can affect the ionization of their imidazole groups (Andersen *et al.*, 1995a).

These observations are in excellent agreement with the predictions made several years ago for the role of histidines from secondary structure predictions (Iconomidou *et al.*, 1999) and strengthen further the value of the model previously proposed both for "soft" and "hard" cuticle proteins (Hamodrakas *et al.*, 2002).

4.2.5.4. Fusion Proteins Establish a Role for the Extended R&R Consensus

Predictions of secondary and tertiary structure and experimental evidence supporting them (discussed above in Sections 4.2.5.1–4.2.5.3) established that the extended R&R Consensus has the properties to serve as a chitin-binding motif. In particular, the planar surfaces of the predicted β-sheets will expose aromatic residues positioned for protein–chitin interaction. The ultimate test of these predictions would be to show that the extended consensus region is sufficient to confer chitin binding on a protein.

Rebers and Willis (2001) investigated this possibility by creating fusion proteins using the extended R&R Consensus from the *A. gambiae* putative cuticular protein, AGCP2b. First they expressed this protein in *Escherichia coli* and isolated it from cell lysates. The construct used coded for the complete protein minus the predicted signal peptide and had a histidine-tag added to the N-terminus to facilitate purification (Dotson *et al.*, 1998). AGCP2b is a protein of 222 amino acids, with a RR-2 type of consensus. The purified protein bound to chitin beads and could be eluted from these beads with 8 M urea or boiling SDS. This established unequivocally that AGCP2b was a chitin-binding protein. Chitin binding previously had been obtained with mixtures of protein extracted from cuticles of two beetles and *D. melanogaster* (Hackman, 1955; Fristrom *et al.*, 1978; Hackman and Goldberg, 1978).

The next, and essential, step was to create a fusion protein uniting a protein that did not bind to chitin with the extended R&R Consensus region. Such a fusion was created between glutathione-*S*-transferase (GST) and 65 amino acids for AGCP2b, covering the region of pfam00379, as shown in **Figure 6**.

The GST and the fusion protein were each affinity purified using a glutathione–sepharose column. GST alone did not bind to chitin but the fusion protein did, requiring denaturing agents for release.

Other experiments defined in more detail the requirements for converting GST into a chitin-binding protein. A shorter fragment of AGCP2b,

APANYEFSYSVHDEHTGDIKSQHET<u>RHGDEVH*GQYSLLDSDGHQRIVDYHADHHTGFNAVVRREP*</u>

Figure 6 The pfam00379 region of AGcP26 used to construct fusion proteins. For details, see text.

40 amino acids (underlined in Figure 6) with the strict R&R Consensus (shown in italics) did not bind chitin. Nor did the full construct when either the Y and F (bolded and highlighted) of the strict R&R Consensus or the T and D (highlighted) of the extended consensus were "mutated" to alanine (Rebers and Willis, 2001).

These experiments established, at last, that the extended R&R Consensus is sufficient to confer chitin-binding properties on a protein and thereby resolved years of speculation on the importance of this region.

Chitinase, some lectins, and proteins from peritrophic membranes all bind chitin (review: Shen and Jacobs-Lorena, 1999). What is unique about the extended R&R Consensus is that it lacks cysteine residues. These residues serve essential roles in the other types of chitin-binding proteins, forming disulfide bonds that hold the protein in the proper configuration for binding. While these other chitin-binding proteins have weak sequence similarities to one another, they do not approach the sequence conservation seen in the R&R Consensus throughout the arthropods. Rebers and Willis (2001) suggested that this conservation (see Figures 1 and 2) could well be due to the need to preserve a precise conformation of the chitin-binding domain in the absence of stabilizing disulfide bonds.

In addition to establishing a function of the extended R&R Consensus, these experiments also provided confirmation of key elements in the models discussed above (see Section 4.2.5.4). Substitution of the two conserved aromatic residues abolished chitin binding. With the TD "mutations," alanines were substituted for two other conserved residues. These flank a glycine that is conserved in position in the "extended consensus" of all hard and many soft cuticles (Iconomidou *et al.*, 1999). According to the proposed model (Figure 5a), these two polar residues would point away from the hydrophobic "cleft" and thus should not participate in chitin binding. It should be noted, however, that this glycine is located at a sharp turn, at the end of the second β-strand (in the vicinity of H102 of Figure 5d). The substitution of two polar residues by two alanines may result in destruction of this turn and to improper folding, thus leading to a structure not capable of binding chitin.

4.2.5.5. Summary

Four different types of data have been presented above (see Section 4.2.5) analyzing the extended R&R Consensus: secondary structure predictions of antiparallel β-sheets (see Section 4.2.5.1), experimental spectroscopic evidence from cuticles and cuticle extracts for the predominance of such β-sheets in cuticular protein conformation (see Section 4.2.5.2), models showing organization of the consensus into a half β-barrel with a groove that can accommodate chitin (see Section 4.2.5.3), and direct demonstration that the extended consensus is sufficient to confer chitin binding on a protein (see Section 4.2.5.4). These four types of data are all in agreement that the highly conserved amino acid sequence of the extended R&R Consensus forms a novel chitin-binding domain, albeit one that displays an essential feature of other proteins that interact with chitin, namely the presentation of aromatic residues in a planar surface. Crystal structures of the cuticular protein–chitin complex are needed to assure that these inferences are correct.

4.2.6. Comparison of Cuticle and Chorion: Structure and Proteins

Silkmoth and fish chorions (eggshells) and cuticle are known to have a helicoidal architecture (Neville, 1975; Hamodrakas, 1992). Excellent reviews on helicoidal architecture and its appearance in biological systems have been made by Bouligand (1972, 1978a, 1978b) and Neville (1975, 1981, 1986). These works describe, in a beautiful and most comprehensive way, how helicoids are identified, how widespread they are, and the basic molecular principles of their formation as well as their geometrical, physical, and biological properties. The close analogy between the helicoidal structures of (usually extracellular) biological materials and the structure of cholesteric liquid crystals suggests that these structures self-assemble according to a mechanism that is very similar to the process allowing materials to form liquid crystals. Apparently, helicoids should pass through a liquid crystalline phase before solidifying. It is assumed that this occurs in the assembly zone during cuticle formation. Self-assembling systems are important in biology, as they are economical in energy terms, requiring neither enzymatic control nor the expenditure of energy-rich bonds. They are particularly appropriate for building extracellular skeletal structures outside of the cells that secrete the components (Bouligand, 1978a, 1978b; Neville, 1986).

Silkmoth chorion is produced by the follicular cells that surround the oocyte (Regier and Kafatos, 1985 and references therein). Fish eggshell is mainly produced by the oocyte, with minor contributions from the follicular cells (Hamodrakas, 1992 and references therein) and cuticle is produced by the epidermis.

Natural helicoidal composites occur in several combinations such as polysaccharide fibers in a polysaccharide matrix (plant cell walls), polysaccharide fibers in a protein matrix (arthropod cuticle), and protein fibers in a protein matrix (insect and fish eggshells). In all cases, principles of molecular recognition and weak intermolecular interactions should govern the self-assembly mechanisms (Neville, 1986).

In silkmoth chorion, disulfide bonds, and in fish eggshell, isopeptide bonds between the side chains of R-K and D-E, are major contributors to stabilization. These covalent bonds, however, are totally absent in cuticle, where stabilization occurs via protein–chitin interaction and by cross-linking by sclerotization compounds (see **Chapter 4.4**).

It is clear that the main characteristic of chorion proteins is the presence of exact, tandemly repeating hexapeptide motifs that adopt a characteristic antiparallel β-pleated sheet structure. This is the main structural unit of silkmoth chorion fibrils and, apparently, the molecular denominator, which dictates formation of the helicoidal architecture (Hamodrakas, 1992). The ellipsoidal shape of silkmoth chorions is, most probably, due to the fact that the basic building-blocks, chorion protein fibrils, are so uniform in shape. By contrast, in cuticle, despite the fact that there are regions of the molecules rich in tandem repeats of certain motifs (see Section 4.2.3.2.2), the sequences are mainly characteristic of globular proteins, and cuticle may adopt all sorts of shapes depending on the local needs of the arthropods producing it. The majority of cuticular proteins contain a conserved domain, rich in a characteristic antiparallel β-pleated sheet structure, a half β-barrel (see Section 4.2.5.3) which again should serve as the molecular denominator determining the helicoidal structure of cuticle, interacting with chitin crystalline chains and giving rise to a plethora of architectural plans as needed locally.

Apparently, an antiparallel β-pleated sheet type of structure is the common molecular denominator, that dictates the helicoidal architecture adopted by the chorion of Lepidoptera and fish and also by the arthropod cuticle.

4.2.7. Summary and Future Challenges

This review has summarized the wealth of information about cuticular proteins amassed since Silvert's review in 1985. Most striking is that the 35-fold increase in sequences for structural cuticular proteins has revealed that the majority has a conserved domain (pfam00379) that is an extended version of the R&R Consensus. A group of proteins that appears to contribute to hard cuticles have a highly conserved extended consensus (RR-2). It is now known that RR-2 proteins interact with chitin and we can predict in some detail the features of their sequence that confer this property. It is not known whether the RR-1 proteins are as effective in binding chitin. We have not yet begun to analyze how the regions outside the consensus contribute to cuticular properties, nor have we learned how the proteins lacking the consensus but with other conserved features contribute to cuticle structure.

Cuticular proteins with pfam00379 are one of the largest multigene families found in *Drosophila* (Lespinet *et al.*, 2002). We need to learn whether this multiplicity serves to allow rapid synthesis of cuticle or whether different genes are used to construct cuticles in different regions. If the latter, the question becomes whether subtle differences in sequence are important for different cuticular properties, or if gene multiplication has been exploited to allow precise temporal and spatial control. The elegant immunolocalization studies that have been carried out were done with antibodies against proteins whose sequences for the most part are unknown. Now that we recognize that several genes may have almost identical sequences, we have to be very careful in designing specific probes for use in Northern analyses, for *in situ* hybridization, and for immunolocalization, if our goal is to learn the use to which each individual gene is put.

Cuticular protein sequences are certain to be described in ever-increasing numbers as more insect genomes are analyzed. Describers need to be careful to submit to databases an indication of whether assignment as a cuticular protein is based on sequence alone or on some type of corroborating evidence. It would be helpful if there were a more consistent system for naming cuticular proteins. At the very least, each protein should have a designation of genus and species and a unique number.

A wealth of information is available already but many challenges lie ahead for those who wish to continue to further our understanding of how the diverse forms and properties of cuticle are constructed extracellularly as these proteins self-assemble in proximity to chitin.

Acknowledgments

We appreciate the helpful comments of John Rebers, Augustine Dunn, and John S. Willis.

References

Agrawal, O.P., Scheller, K., **1986**. The formation of the chitin-arylphorin complex *in vitro*. In: Muzzarelli, R., Jeuniaux, C., Gooday, G.W. (Eds.), Chitin in Nature and Technology. Plenum Press, New York, pp. 316–320.

Andersen, S.O., **1975**. Cuticular sclerotization in the beetles *Pachynoda epphipiata* and *Tenebrio molitor. J. Insect Physiol. 21*, 1225–1232.

Andersen, S.O., **1998**. Amino acid sequence studies on endocuticular proteins form the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 28*, 421–434.

Andersen, S.O., **2000**. Studies on proteins in post-ecdysial nymphal cuticle of locust, *Locusta migratoria*, and cockroach, *Blaberus cranifer. Insect Biochem. Mol. Biol. 30*, 569–577.

Andersen, S.O., Hojrup, P., Roepstorff, P., **1995a**. Insect cuticular proteins. *Insect Biochem. Mol. Biol. 25*, 153–176.

Andersen, S.O., Rafn, K., Krogh, T.N., Hojrup, P., Roepstorff, P., **1995b**. Comparison of larval and pupal cuticular proteins in *Tenebrio molitor. Insect Biochem. Mol. Biol. 25*, 177–187.

Andersen, S.O., Rafn, K., Roepstorff, P., **1997**. Sequence studies of proteins from larval and pupal cuticle of the yellow meal worm, *Tenebrio molitor. Insect Biochem. Mol. Biol. 27*, 121–131.

Andersen, S.O., Weis-Fogh, T., **1964**. Resilin: a rubber-like protein in arthropod cuticle. *Adv. Insect Physiol. 2*, 1–65.

Apple, R.T., Fristrom, J.W., **1991**. 20-Hydroxyecdysone is required for, and negatively regulates, transcription of *Drosophila* pupal cuticle protein genes. *Devel. Biol. 146*, 569–582.

Ardell, D.H., Andersen, S.O., **2001**. Tentative identification of a resilin gene in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 31*, 965–970.

Ashida, M., Brey, P.T., **1995**. Role of the integument in insect defense: pro-phenol oxidase cascade in the cuticular matrix. *Proc. Natl Acad. Sci. USA 92*, 10698–10702.

Atkins, E.D.T., **1985**. Conformations in polysaccharides and complex carbohydrates. *Proc. Int. Symp. Biomol. Struct. Interactions, Suppl. J. Biosci. 8*, 375–387.

Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., *et al.*, **2002**. The Pfam protein families database. *Nucleic Acids Res. 30*, 276–280.

Binger, L.C., Willis, J.H., **1990**. *In vitro* translation of epidermal RNAs from different anatomical regions and metamorphic stages of *Hyalophora cecropia*. *Insect Biochem. 20*, 573–583.

Binger, L.C., Willis, J.H., **1994**. Identification of the cDNA, gene and promoter for a major protein from flexible cuticles of the giant silkmoth *Hyalophora cecropia*. *Insect Biochem. Mol. Biol. 24*, 989–1000.

BLAST, **2003**. http://www.ncbi.nlm.nih.gov/BLAST/

Bouhin, H., Charles, J.-P., Quennedey, B., Courrent, A., Delachambre, J., **1992a**. Characterization of a cDNA clone encoding a glycine-rich cuticular protein of *Tenebrio molitor*: developmental expression and effect of a juvenile hormone analogue. *Insect Mol. Biol. 1*, 53–62.

Bouhin, H., Charles, J.-P., Quennedey, B., Delachambre, J., **1992b**. Developmental profiles of epidermal mRNAs during the pupal–adult molt of *Tenebrio molitor* and isolation of a cDNA encoding an adult cuticular protein: effects of a juvenile hormone analogue. *Devel. Biol. 149*, 112–122.

Bouligand, Y., **1972**. Twisted fibrous arrangements in biological materials and cholesteric mesophases. *Tissue and Cell 4*, 189–217.

Bouligand, Y., **1978a**. Cholesteric order in biopolymers. *ACS Symposium Series 74*, 237–247.

Bouligand, Y., **1978b**. Liquid crystalline order in biological materials. In: Blumstein, A. (Ed.), Liquid Crystalline Order in Polymers. Academic Press, London, pp. 261–297.

Browder, L.W., Wilkes, J., Rodenhiser, D.I., **1992**. Preparative labeling of proteins with [^{35}S]methionine. *Anal. Biochem. 204*, 85–89.

Carter, D., Locke, M., **1993**. Why caterpillars do not grow short and fat. *Int. J. Insect Morphol. Embryol. 22*, 81–102.

Charles, J.-P., Bouhin, H., Quennedey, B., Courrent, A., Delachambre, J., **1992**. cDNA cloning and deduced amino acid sequence of a major, glycine-rich cuticular protein from the coleopteran *Tenebrio molitor*: temporal and spatial distribution of the transcript during metamorphosis. *Eur. J. Biochem. 206*, 813–819.

Charles, J.-P., Chihara, C., Nejad, S., Riddiford, L.M., **1997**. A cluster of cuticle protein genes of *Drosophila melanogaster* at 65A: sequence, structure and evolution. *Genetics 147*, 1213–1226.

Charles, J.-P., Chihara, C., Nejad, S., Riddiford, L.M., **1998**. Identification of proteins and developmental expression of RNAs encoded by the 65A cuticle protein gene cluster in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 28*, 131–138.

Chou, P., Fasman, G.D., **1974a**. Conformational parameters for amino acids in helical, β-sheet and random coil regions calculated from proteins. *Biochemistry 13*, 211–221.

Chou, P., Fasman, G.D., **1974b**. Prediction of protein conformation. *Biochemistry 13*, 222–245.

Condoulis, W.V., Locke, M., **1966**. The deposition of endocuticle in an insect, *Calpodes ethlius* Stoll (Lepidoptera, Hesperiidae). *J. Insect Physiol. 12*, 311–323.

Cornell, J.C., Pan, M.L., **1983**. The disappearance of moulting fluid in the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol. 107*, 501–504.

Cox, D.L., Willis, J.H., **1985**. The cuticular proteins of *Hyalophora cecropia* from different anatomical regions and metamorphic stages. *Insect Biochem. 15*, 349–362.

Cox, D.L., Willis, J.H., **1987a**. Analysis of the cuticular proteins of *Hyalophora cecropia* with two dimensional electrophoresis. *Insect Biochem. 17*, 457–468.

Cox, D.L., Willis, J.H., **1987b**. Post-translational modifications of the cuticular proteins of *Hyalophora cecropia*

from different anatomical regions and metamorphic stages. *Insect Biochem. 17*, 469–484.

Csikos, G., Molnar, K., Borhegyi, N.H., Talian, G.C., Sass, M., **1999**. Insect cuticle, an *in vivo* model of protein trafficking. *J. Cell Sci. 112*, 2113–2124.

Doctor, J., Fristrom, D., Fristrom, J.W., **1985**. The pupal cuticle of *Drosophila*: biphasic synthesis of pupal cuticle proteins *in vivo* and *in vitro* in response to 20-hydroxyecdysone. *J. Cell Biol. 101*, 189–200.

Dotson, E.M., Cornel, A.J., Willis, J.H., Collins, F.H., **1998**. A family of pupal-specific cuticular protein genes in the mosquito *Anopheles gambiae*. *Insect Biochem. Mol. Biol. 28*, 459–472.

ENTREZ, **2003**. http://www3.ncbi.nlm.nih.gov/Entrez/index.html

Fechtel, K., Fristrom, D.K., Fristrom, J.W., **1989**. Prepupal differentiation in *Drosophila*: distinct cell types elaborate a shared structure, the pupal cuticle, but accumulate transcripts in unique patterns. *Development 106*, 649–656.

Flybase, **2003**. Genetic data on *Drosophila*. http://flybase.bio.indiana.edu/

Fraenkel, G., Rudall, K.M., **1947**. The structure of insect cuticles. *Proc. Roy. Soc. B 134*, 111–143.

Fristrom, D., Doctor, J., Fristrom, J.W., **1986**. Procuticle proteins and chitin-like material in the inner epicuticle of the *Drosophila* pupal cuticle. *Tissue and Cell 18*, 531–543.

Fristrom, D., Fristrom, J.W., **1993**. The metamorphic development of the adult epidermis. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 843–897.

Fristrom, J.W., Hill, R.J., Watt, F., **1978**. The procuticle of *Drosophila*: heterogeneity of urea-soluble proteins. *Biochemistry 17*, 3917–3924.

Geyer, P.G., Corces, V.G., **1987**. Separate regulatory elements are responsible for the complex pattern of tissue-specific and developmental transcription of the *yellow* locus in *Drosophila melanogaster. Genes and Devel. 1*, 996–1004.

Griebenow, K., Santos, A.M., Carrasquillo, K.G., **1999**. Secondary structure of proteins in the amorphous dehydrated state probed by FTIR spectroscopy: dehydration-induced structural changes and their prevention. *Int. J. Vibr. Spectrosc. 3*, 1–34.

Gu, S., Willis, J.H., **2003**. Distribution of cuticular protein mRNAs in silk moth integument and imaginal discs. *Insect Biochem. Mol. Biol. 33*, 1177–1188.

Hackman, R.H., **1955**. Studies on chitin. 3. Absorption of proteins to chitin. *Austral. J. Biol. Sci. 8*, 530–536.

Hackman, R.H., Goldberg, M., **1978**. The non-covalent binding of two insect cuticular proteins by a chitin. *Insect Biochem. 8*, 353–357.

Hamodrakas, S.J., **1992**. Molecular architecture of helicoidal proteinaceous eggshells. In: Case, S.T. (Ed.), Results and Problems in Cell Differentiation, vol. 19. Springer-Verlag, Berlin, pp. 116–186.

Hamodrakas, S.J., Kanellopoulos, P.N., Pavlou, K., Tucker, P.A., **1997**. The crystal structure of the complex of concanavalin A with 4-methylumbelliferyl-α-D-glucopyranoside. *J. Struct. Biol. 118*, 23–30.

Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., **2002**. A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem. Mol. Biol. 32*, 1577–1583.

Han, Q., Fang, J., Ding, H., Johnson, J.K., Christensen, B.M., *et al.*, **2002**. Identification of *Drosophila melaogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. *Biochem. J. 368*, 333–340.

Henikoff, S., Eghtedarzadeh, M.K., **1987**. Conserved arrangement of nested genes at the *Drosophila Gart* locus. *Genetics 117*, 711–725.

Henikoff, S., Keene, M.A., Fechtel, K., Fristrom, J.W., **1986**. Gene within a gene: nested *Drosophila* genes encode unrelated proteins on opposite DNA strands. *Cell 44*, 33–42.

Holden, H.M., Rypniewski, W.R., Law, J.H., Rayment, I., **1987**. The molecular structure of insecticyanin from the tobacco hornworm *Manduca sexta* L. at 2.6 A resolution. *EMBO J. 6*, 1565–1570.

Horodyski, F.M., Riddiford, L.M., **1989**. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. *Devel. Biol. 132*, 292–303.

Iconomidou, V.A., Chryssikos, G.D., Gionis, V., Willis, J.H., Hamodrakas, S.J., **2001**. "Soft"-cuticle protein secondary structure as revealed by FT-Raman, ATR-FT-IR and CD spectroscopy. *Insect Biochem. Mol. Biol. 31*, 877–885.

Iconomidou, V.A., Willis, J.H., Hamodrakas, S.J., **1999**. Is β-pleated sheet the molecular conformation which dictates formation of helicoidal cuticle? Insect Biochem. *Mol. Biol. 29*, 285–292.

Ikeya, T., Persson, P., Kono, M., Watanabe, T., **2001**. The *DD5* gene of the decapod crustacean *Penaeus japonicus* encodes a putative exoskeletal protein with a novel tandem repeat structure. *Comp. Biochem. Physiol. B 128*, 379–388.

Janssens, H., Gehring, W.J., **1999**. Isolation and characterization of *drosocrystallin*, a lens crystallin gene of *Drosophila melanogaster. Devel. Biol. 207*, 204–214.

Jones, T.A., Zou, J.Y., Cowan, S.W., Kjeldgaard, M., **1991**. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A47*, 110–119.

Kalinich, J.F., McClain, D.E., **1992**. An *in vitro* method for radiolabeling proteins with ^{35}S. *Anal. Biochem. 205*, 208–212.

Kaufman, T.C., Seeger, M.A., Olsen, G., **1990**. Molecular and genetic organization of the Antennapedia gene complex of *Drosophila melanogaster. Adv. Genet. 27*, 309–362.

Kollberg, U., Obermaier, B., Hirsch, H., Kelber, G., Wobert, P., **1995**. Expression cloning and characterization of a pupal cuticle protein cDNA of *Galleria mellonella* L. Insect Biochem. *Mol. Biol. 25*, 355–363.

Konig, M., Agrawal, O.P., Schenkel, H., Scheller, K., **1986**. Incorporation of calliphorin into the cuticle of the developing blowfly, *Calliphora vicinia*. *Roux's Arch. Devel. Biol. 195*, 296–301.

Kornezos, A., Chia, W., **1992**. Apical secretion and association of the *Drosophila yellow* gene product with developing larval cuticle structures during embryogenesis. *Mol. Gen. Genet. 235*, 397–405.

Lampe, D.J., Willis, J.H., **1994**. Characterization of a cDNA and gene encoding a cuticular protein from rigid cuticles of the giant silkmoth, *Hyalophora cecropia*. *Insect Biochem. Mol. Biol. 24*, 419–435.

Lee, W.-J., Brey, P.T., **1994**. Isolation and identification of cecropin antibacterial peptides from the extracellular matrix of insect integument. *Anal. Biochem. 217*, 231–235.

Lemoine, A., Delachambre, J., **1986**. A water-soluble protein specific to the adult cuticle in *Tenebrio*: its use as a marker of a new programme expressed by epidermal cells. *Insect Biochem. 16*, 483–489.

Lemoine, A., Millot, C., Curie, G., Delachambre, J., **1989**. A monoclonal antibody against an adult-specific cuticular protein of *Tenebrio molitor* (Insecta, Coleoptera). *Devel. Biol. 136*, 546–554.

Lemoine, A., Millot, C., Curie, G., Delachambre, J., **1990**. Spatial and temporal variations in cuticle proteins as revealed by monoclonal antibodies, immunoblotting analysis and ultrastructural immunolocalization in a beetle, *Tenebrio molitor. Tissue and Cell 22*, 177–189.

Lemoine, A., Millot, C., Curie, G., Massonneau, V., Delachambre, J., **1993**. Monoclonal antibodies recognizing larval- and pupal-specific cuticular proteins of *Tenebrio molitor* (Insecta, Coleoptera). *Roux's Arch. Devel. Biol. 203*, 92–99.

Lespinet, O., Wolf, Y.I., Koonin, E.V., Aravind, L., **2002**. The role of lineage-specific gene family expansion in the evolution of eukaryotes. *Genome Res. 12*, 1048–1059.

Leung, H., Palli, S.R., Locke, M., **1989**. The localization of arylphorin in an insect, *Calpodes ethlius*. *J. Insect Physiol. 35*, 223–231.

Li, W., Riddiford, L.M., **1992**. Two distinct genes encode two major isoelectric forms of insecticyanin in the tobacco hornworm, *Manduca sexta*. *Eur. J. Biochem. 205*, 491–499.

Locke, M., **1998**. Epidermis. In: Harrison, F.W., Locke, M. (Eds.), Microscopic Anatomy of Invertebrates, vol. 11A. Wiley–Liss, New York, pp. 75–138.

Locke, M., **2001**. The Wigglesworth Lecture: Insects for studying fundamental problems in biology. *J. Insect Physiol. 47*, 495–507.

Locke, M., **2003**. Surface membranes, golgi complexes and vacuolar systems. *Annu. Rev. Entomol. 48*, 1–27.

Locke, M., Kiss, A., Sass, M., **1994**. The cuticular localization of integument peptides from particular routing categories. *Tissue and Cell 26*, 707–734.

Lombardi, E.C., Kaplan, D.L., **1993**. Preliminary characterization of resilin isolated from the cockroach, *Periplanata americana*. *Mat. Res. Soc. Proc. 292*, 3–7.

Marcu, O., Locke, M., **1998**. A cuticular protein from the moulting stages of an insect. *Insect Biochem. Mol. Biol. 28*, 659–669.

Marcu, O., Locke, M., **1999**. The origin, transport and cleavage of the molt-associated cuticular protein CECP22 from *Calpodes ethlius* (Lepidoptera, Hesperridae). *J. Insect Physiol. 45*, 861–870.

Mathelin, J., Bouhin, H., Quennedey, B., Courrent, A., Delachambre, J., **1995**. Identification, sequence and mRNA expression pattern during metamorphosis of a cDNA encoding a glycine-rich cuticular protein in *Tenebrio molitor. Gene 156*, 259–264.

Mathelin, J., Quennedey, B., Bouhin, H., Delachambre, J., **1998**. Characterization of two new cuticular genes specifically expressed during the post-ecdysial molting period in *Tenebrio molitor. Gene 211*, 351–359.

McGeoch, D.J., **1985**. On the predictive recognition of signal peptide sequences. *Virus Res. 3*, 271–286.

Mita, K., Morimyo, M., Okano, K., Shimada, T., Maeda, S., **1999**. The construction of EST database for genome analysis of *Bombyx mori*. *RIKEN Rev. 22*, 63–67.

Molnar, K., Borhegyi, N.H., Csikos, G., Sass, M., **2001**. The immunoprotein scolexin and its synthesizing sites: the midgut epithelium and the epidermis. *Acta Biol. Hungarica 52*, 473–484.

MOTIF, **2003**. http://motif.genome.ad.jp/MOTIF2.html

Nakato, H., Izumi, S., Tomino, S., **1992**. Structure and expression of gene coding for a pupal cuticle protein of *Bombyx mori*. *Biochim. Biophys. Acta 1132*, 161–167.

Nash, W.G., **1976**. Patterns of pigmentation color states regulated by the *y* locus in *Drosophila melanogaster. Devel. Biol. 48*, 336–343.

Neville, A.C., **1975**. Biology of the Arthropod Cuticle. Springer-Verlag, Berlin.

Neville, A.C., **1981**. Cholesteric proteins. *Mol. Cryst. Liq. Cryst. 76*, 279–286.

Neville, A.C., **1986**. The physics of helicoids: multidirectional "plywood" structures in biological systems. *Phys. Bull. 37*, 74–76.

Nicholls, A., Sharp, K.A., Honig, B., **1991**. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins: Struct. Funct. Genet. 11*, 281–296.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., **1997**. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng. 10*, 1–6.

Nielsen, H., Krogh, A., **1998**. Prediction of signal peptides and signal anchors by a hidden Markov model. *Proc. Int. Conf. Intell. Syst. Mol. Biol. 6*, 122–130.

Palli, S.R., Locke, M., **1987**. The synthesis of hemolymph proteins by the larval epidermis of an insect *Calpodes ethlius* (Lepidoptera: Hesperiidae). *Insect Biochem. 17*, 711–722.

Perrakis, A., Ouzounis, C., Wilson, K.S., **1997**. Evolution of immunoglobulin-like modules in chitinases: their structural flexibility and functional implications. *Folding and Design 2*, 291–294.

Pfam, **2003a**. http://www.sanger.ac.uk/Software/Pfam/

Pfam, **2003b**. http://pfam.wustl.edu/cgi-bin/getdesc?acc=PF00379

Psort, **2003**. http://psort.ims.u-tokyo.ac.jp/

Rebers, J.E., Niu, J., Riddiford, L.M., **1997**. Structure and spatial expression of the *Manduca sexta* MSCP14.6 cuticle gene. *Insect Biochem. Mol. Biol. 27*, 229–240.

Rebers, J.F., Riddiford, L.M., **1988**. Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. *J. Mol. Biol. 203*, 411–423.

Rebers, J.E., Willis, J.H., **2001**. A conserved domain in arthropod cuticular proteins binds chitin. *Insect Biochem. Mol. Biol. 31*, 1083–1093.

Regier, J.C., Kafatos, F.C., **1985**. Molecular aspects of chorion formation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 1. Pergamon Press, Oxford, pp. 113–151.

Riddiford, L.M., **1994**. Cellular and molecular actions of juvenile hormone I: general considerations and premetamorphic actions. *Adv. Insect Physiol. 24*, 213–274.

Riddiford, L.M., Hice, R.H., **1985**. Developmental profiles of the mRNAs for *Manduca* arylphorin and two other storage proteins during the final larval instar of *Manduca sexta*. *Insect Biochem. 15*, 489–502.

Roberts, P.E., Willis, J.H., **1980a**. The cuticular proteins of *Tenebrio molitor*. 1. Electrophoretic banding patterns during postembryonic development. *Devel. Biol. 75*, 59–69.

Roberts, P.E., Willis, J.H., **1980b**. Effects of juvenile hormone, ecdysterone, actinomycin D, and mitomycin C on the cuticular proteins of *Tenebrio molitor. J. Embryol. Exp. Morph. 56*, 107–123.

Rondot, I., Quennedey, B., Courrent, A., Lemoine, A., Delachambre, J., **1996**. Cloning and sequencing of a cDNA encoding a larval–pupal-specific cuticular protein in *Tenebrio molitor* (Insecta, Coleoptera): developmental expression and effect of a juvenile hormone analogue. *Eur. J. Biochem. 235*, 138–143.

Rondot, I., Quennedey, B., Delachambre, J., **1998**. Structure, organization and expression of two clustered cuticle protein genes during the metamorphosis of an insect *Tenebrio molitor. Eur. J. Biochem. 254*, 304–312.

Roter, A.H., Spofford, J.B., Swift, H., **1985**. Synthesis of the major adult cuticle proteins of *Drosophila melanogaster* during hypoderm differentiation. *Devel. Biol. 107*, 420–431.

Sass, M., Kiss, A., Locke, M., **1993**. Classes of integument peptides. *Insect Biochem. Mol. Biol. 23*, 845–857.

Sass, M., Kiss, A., Locke, M., **1994a**. Integument and hemocyte peptides. *J. Insect Physiol. 40*, 407–421.

Sass, M., Kiss, A., Locke, M., **1994b**. The localization of surface integument peptides in tracheae and tracheoles. *J. Insect Physiol. 40*, 561–575.

Scheller, K., Zimmermann, H.-P., Sekeris, C.E., **1980**. Calliphorin, a protein involved in the cuticle formation of the blowfly, *Calliphora vicinia*. *Z. Naturforsch. 35c*, 387–389.

Scholer, H.R., **1991**. Octamania: the POU factors in murine development. *Trends Genet. 7*, 323–328.

Shen, Z., Jacobs-Lorena, M., **1999**. Evolution of chitin-binding proteins in invertebrates. *J. Mol. Evol. 48*, 341–347.

SilkBase, **2003**. Expressed sequence tags in *Bombyx mori*. http://www.ab.a.u-tokyo.ac.jp/silkbase

Silvert, D.J., **1985**. Cuticular proteins during postembryonic development. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 2. Pergamon Press, Oxford, pp. 239–254.

Snyder, M., Hunkapiller, M., Yuen, D., Silvert, D., Fristrom, J., *et al.*, **1982**. Cuticle protein genes of *Drosophila*: structure, organization and evolution of four clustered genes. *Cell 29*, 1027–1040.

Stiles, B., **1991**. Cuticle proteins of the boll weevil, *Anthonomus grandis*, abdomen: structural similarities and glycosylation. *Insect Biochem. 21*, 249–258.

Suderman, R.J., Andersen, S.O., Hopkins, T.L., Kanost, M.R., Kramer, K.J., **2003**. Characterization and cDNA cloning of three major proteins from pharate pupal cuticle of *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 331–343.

Suetake, T., Tsuda, S., Kawabata, S., Miura, K., Iwanaga, S., *et al.*, **2000**. Chitin-binding proteins in invertebrates and plants comprise a common chitin-binding structural motif. *J. Biol. Chem. 275*, 17929–17932.

Talbo, G., Hojrup, P., Rahbek-Nielsen, H., Andersen, S.O., Roepstorff, P., **1991**. Determination of the covalent structure of an N- and C-terminally blocked glycoprotein from endocuticle of *Locusta migratoria*: combined use of plasma desorption mass spectrometry and Edman degradation to study post-translationally modified proteins. *Eur. J. Biochem. 195*, 495–504.

Tews, I., Scheltiga, T., Perrakis, A., Wilson, K.S., Dijkstra, B.W., **1997**. Substrate-assisted catalysis unifies 2 families of chitinolytic enzymes. *J. Am. Chem. Soc. 119*, 7954–7959.

Trim, A.R.H., **1941**. Studies in the chemistry of insect cuticle. 1. Some general observations on certain arthropod cuticles with special reference to the characterization of the proteins. *Biochem. J. 35*, 1088–1098.

Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., *et al.*, **2001**. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. *J. Biol. Chem. 276*, 41343–41349.

Vakser, I.A., **1996**. Low-resolution docking: prediction of complexes for undetermined structures. *Biopolymers 39*, 455–464.

Vincent, J.F.V., **2002**. Arthropod cuticle: a natural composite shell system. *Composites: Part A 33*, 1311–1315.

Vriend, G., **1990**. WHAT IF: A molecular modeling and drug design package. *J. Mol. Graph. 8*, 52–56.

Vyas, N.K., **1991**. Atomic features of protein–carbohydrate interactions. *Curr. Opin. Struct. Biol. 1*, 723–740.

Williams, C.M., **1980**. Growth in insects. In: Locke, M., Smith, D.S. (Eds.), Insect Biology in the Future. Academic Press, London, pp. 369–383.

Willis, J.H., **1986**. The paradigm of stage-specific gene sets in insect metamorphosis: time for revision! *Arch. Insect Biochem. Physiol.* (Suppl. 1), 47–57.

Willis, J.H., **1996**. Metamorphosis of the cuticle, its proteins, and their genes. In: Gilbert, L.I., Atkinson, B.G., Tata, J. (Eds.), Metamorphosis/Post-Embryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, London, pp. 253–282.

Willis, J.H., **1999**. Cuticular proteins in insects and crustaceans. *Amr. Zool. 39*, 600–609.

Willis, J.H., Rezaur, R., Sehnal, F., **1982**. Juvenoids cause some insects to form composite cuticles. *J. Embryol. Exp. Morph. 71*, 25–40.

Wittkopp, P.J., Vaccaro, K., Carroll, S.B., **2002**. Evolution of *yellow* gene regulation and pigmentation in *Drosophila*. *Curr. Biol. 12*, 1547–1556.

Wolfgang, W.J., Fristrom, D., Fristrom, J.W., **1986**. The pupal cuticle of *Drosophila*: differential ultrastructural immunolocalization of cuticle proteins. *J. Cell Biol. 102*, 306–311.

Wolfgang, W.J., Fristrom, D., Fristrom, J.W., **1987**. An assembly zone antigen of the insect cuticle. *Tissue and Cell 19*, 827–838.

Wolfgang, W.J., Riddiford, L.M., **1986**. Larval cuticular morphogenesis in the tobacco hornworm, *Manduca sexta*, and its hormonal regulation. *Devel. Biol. 113*, 305–316.

Wybrandt, G.B., Andersen, S.O., **2001**. Purification and sequence determination of a yellow protein from sexually mature males of the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 31*, 1183–1189.

Zanotti, G., Marcello, M., Malpeli, G., Folli, C., Sartori, G., *et al.*, **1994**. Crystallographic studies on complexes between retinoids and plasma retinol-binding protein. *J. Biol. Chem. 269*, 29613–29620.

Zhou, X., Riddiford, L.M., **2002**. Broad specifies pupal development and mediates the "status quo" action of juvenile hormone on the pupal–adult transformation in *Drosophila* and *Manduca*. *Development 129*, 2259–2269.

Relevant Website

http://bioinformatics2.biol.uoa.gr – A relational database of arthropod cuticular proteins established by C.K. Magkrioti, I.C. Spyropoulos, V.A. Iconomidou, J.H. Willis, and S.J. Hamodrakas.

4.3 Chitin Metabolism in Insects

K J Kramer, US Department of Agriculture,
Manhattan, KS, USA
S Muthukrishnan, Kansas State University,
Manhattan, KS, USA

4.3.1. Introduction

"Chitin Metabolism in Insects" was the title of one of the chapters in the original edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* series published in 1985 (Kramer *et al.*, 1985). Since that time substantial progress in gaining an understanding of this topic has occurred, primarily through the application of techniques of molecular genetics and biotechnology to assorted studies on insect chitin metabolism. Several other reviews have also been published, which have reported on some of the advances that have taken place (Kramer and Koga, 1986; Cohen, 1987, 2001; Koga *et al.*, 1999; Fukamizo, 2000). Thus, in this chapter we will highlight some of the more important findings since 1985, with an emphasis on results obtained from studies conducted on the two enzymes primarily responsible for chitin synthesis and degradation, namely chitin synthase (CHS) and chitinase (CHI).

4.3.2. Chitin Structure and Occurrence

Chitin is widely distributed in animals and represents the skeletal polysaccharide of several phyla such as the Arthropoda, Annelida, Mollusca, and Coelenterata. In several groups of fungi, chitin replaces cellulose as the structural polysaccharide. In insects, it is found in the body wall, gut lining, cuticle, salivary glands, trachea, mouth parts, and muscle attachment points.

In the course of evolution, insects have made excellent use of the rigidity and chemical stability of the polymeric chitin to assemble extracellular structures such as the cuticle (exoskeleton) and gut lining (peritrophic membrane (PM)), both of which enable insects to be protected from the environment while allowing growth, mobility, respiration, and communication. Several genes and gene products are involved in chitin metabolism in insects. In general there are two primary extracellular structures in

which chitin deposition occurs. Those are the cuticle and the PM where both synthesis and degradation of chitin take place at different developmental stages.

Chitin is the major polysaccharide present in insects and many other invertebrates and several microbes. Structurally, it is the simplest of the glycosaminoglycans, being a β (1→4) linked linear homopolymer of *N*-acetylglucosamine (GlcNAc, $(C_8H_{13}O_5N)_{n\gg1}$). It is usually synthesized as the old endocuticle and PM are resorbed and the digested materials are recycled. Because of the intractable nature of insect sclerotized structures such as cuticle, there was very little quantitative data available about chemical composition until recently when solid-state nuclear magnetic resonance (NMR) was utilized for analyses. The cuticle and PM are composed primarily of a mixture of protein and chitin, with the former usually predominating (Kramer *et al*., 1995). Chitin contents vary substantially depending on the type of cuticle. For example, in the sclerotized puparial cuticle from the housefly, *Musca domestica*, the chitin content is approximately 45% of the wet weight, whereas in the mineralized puparial cuticle of the face fly, *Musca autumnalis*, the chitin content is only about 19% (Roseland *et al*., 1985; Kramer *et al*., 1988). In larval, pupal, and adult cuticles of the tobacco hornworm, *Manduca sexta*, the chitin content is approximately 14%, 25%, and 7%, respectively (Kramer *et al*., 1995). In newly ecdysed pupal cuticle, there is only about 2% chitin prior to sclerotization, but that amount increases more than 10-fold after sclerotization. When cuticular protein and chitin are mixed, they form a matrix in which the components of lower abundance, such as water, catechols, lipids, and minerals, are interspersed. The PM of the tobacco hornworm is made up primarily of protein (60%) and chitin (40%) (Kramer *et al*., 1995). Although primarily composed of poly-GlcNAc, chitin also can contain a small percentage of unsubstituted (or *N*-deacetylated) glucosamine (GlcN) residues (Fukamizo *et al*., 1986). When the epidermal and gut cells synthesize and secrete a particular form of chitin consisting of antiparallel chains, α-chitin, the chains are formed into sheets. As layers are added, the sheets become cross-oriented to one another, which can contribute to the formation of an extremely strong plywood-like material. The origin of proteins in the cuticle is unknown, but some hemolymph proteins are deposited in cuticle. Thus, apparently the epidermal cells do not need to supply all of the component parts of the exoskeleton. The cells lining the gut produce some of the PM-associated proteins and these proteins are referred to as the peritrophins (Tellam *et al*., 1999; Wang and Granados, 2000a; Bolognesi *et al*., 2001; Eisemann *et al*., 2001). Analysis of expressed sequence tags in the cat flea, *Ctenocephalides felis*, demonstrated that some peritrophins are produced exclusively by hindgut and Malpighian tubule tissues (Gaines *et al*., 2002).

The last step in cuticle formation, tanning, involves modification of the free amino acid tyrosine that is sequestered as a conjugate with glucose in the hemolymph. Tyrosine is first hydroxylated to 3,4-dihydroxyphenylalanine (DOPA), and then decarboxylated to 3,4-dihydroxyphenethylamine (dopamine) (Hopkins and Kramer, 1992). Dopamine is *N*-acylated with acetate or β-alanine in the epidermal cells and sequestered in the hemolymph as conjugates with glucose, sulfate, or another hydrophilic compound. The *N*-acylated dopamine conjugates then are delivered through pore canals to the epicuticle where the conjugates are hydrolyzed and then converted by phenoloxidases to very highly reactive quinones and quinone methides. These transient compounds then cross-link proteins to form tanned proteins in a process known as sclerotization (see **Chapter 4.4**). These cross-linked proteins and chitin make up most of the exocuticle. Chitin chains also may become cross-linked with cuticular proteins, but the evidence for that is not definitive.

Chitin oligosaccharides that are produced during degradation of chitin by chitinases appear to play an important role in insect immunity towards microorganisms. The basic immune strategy against microbial infection in insects appears to be similar to the strategy used by plants against fungal infection. These oligosaccharides are known to activate chitinase genes in plants, which are actively involved in the plant defense response against fungal infection (Nichols *et al*., 1980). In the silkworm, *Bombyx mori*, chitin oligomers trigger expression of three different antibacterial proteins – cecropin, attacin, and lebocin – in the fat body and hemocytes (Furukawa *et al*., 1999).

4.3.3. Chitin Synthesis

Relatively little additional biochemical data on the enzymes of the chitin biosynthetic pathway have been generated since the previous review was published (Kramer *et al*., 1985). The paucity of information concerning the biochemical properties of these enzymes is due to the inability to obtain soluble preparations of CHSs and the instability of the glutamine-fructose-6-phosphate aminotransferase (GFAT), the enzyme that provides

the GlcN precursor of the chitin biosynthetic pathway. However, CHSs have been identified in a variety of organisms, including nematodes, fungi, and insects. Amino acid sequence similarities have been the principal tools used for identifying CHSs, which form a subfamily within a larger group (family GT2) of the glycosyltransferases that catalyze the transfer of a sugar moiety from an activated sugar donor onto saccharide or nonsaccharide acceptors (Coutinho and Henrissat, 1999; Coutinho *et al*., 2003; CAZY, 2004). During the past 3 years, there has been a sudden increase in research in the area of chitin synthesis. The impetus for this enhanced interest has come predominantly from cloning of genes for the two key enzymes of the pathway, GFAT and CHS, from insects.

CHS has not been an easy enzyme to assay, which has made its study rather difficult. Traditionally, CHS activity was measured by a radioactive assay using [^{14}C]UDP-GlcNAc as the substrate followed by quantification of insoluble ^{14}C-labeled chitin after acid precipitation. Recently, however, a high throughput nonradioactive assay has been developed (Lucero *et al*., 2002). The procedure involves binding of synthesized chitin to a wheat germ agglutinin (WGA)-coated surface followed by detection of the polymer with a horseradish peroxidase–WGA conjugate. This nonradioactive assay should facilitate greater progress in CHS studies in the future.

4.3.3.1. Precursors of the Chitin Biosynthetic Pathway

Early studies on chitin synthesis using whole insects or isolated tissues demonstrated that in addition to whole animals, a variety of tissues including larval and pupal epidermis, abdomen, integument, gut, imaginal discs, leg regenerates, hypodermis, and oocytes were capable of synthesizing chitin (review: Kramer *et al*., 1985). An assortment of compounds, including glycogen, glucose, glucosamine, fructose, and GlcNAc could serve as biosynthetic precursors of chitin in these tissues. These early studies also identified several compounds that inhibited the pathway. This list includes substrate analogs such as tunicamycin, polyoxin D, nikkomycin, and uridine diphosphate (UDP), as well as several compounds belonging to the benzoylphenylurea class of insect growth regulators whose exact mode of action has not yet been established. Results of these studies also indicated that ecdysone may influence chitin synthesis either directly or indirectly. However, the details of such a regulation remain unclear.

4.3.3.2. Sites of Chitin Biosynthesis

The epidermis and the midgut are two major tissues where chitin synthesis occurs in insects. Epidermal cells are responsible for the deposition of new cuticle during each molt and the midgut cells are generally associated with the formation of the PM during feeding. Chitin is associated with other tissues as well, including the foregut, hindgut, trachea, wing hinges, salivary gland, and mouth parts of adults and/or larvae (Wilson and Cryan, 1997). In general, it is assumed that the cells closest to the site where chitin is found are responsible for its biosynthesis. However, this interpretation is complicated by the fact that assembly of chitin microfibrils occurs in the extracellular space and is influenced by the presence or absence of associated proteins. This is particularly true in the gut where some cells around the cardia may be contributing to chitin synthesis and secretion, whereas other cells in different parts of the gut may be responsible for synthesis of PM-associated proteins (Wang and Granados, 2000a). Visible PM may appear at sites remote from the original site of synthesis of either chitin or PM proteins.

4.3.3.3. Light and Electron Microscopic Studies of Peritrophic Membrane Synthesis

The most detailed picture of chitin synthesis and its association with proteins to form the composite PM has emerged from observations using light microscopy as well as transmission and scanning electron microscopy (SEM) of PM synthesis in the three lepidopteran insects, *Ostrinia nubilalis* (European corn borer), *Trichoplusia ni* (cabbage looper), and *M. sexta* (Harper and Hopkins, 1997; Harper *et al*., 1998; Harper and Granados, 1999; Wang and Granados, 2000a; Hopkins and Harper, 2001). The presence of chitin in nascent PM can be followed by staining with gold-labeled WGA, which binds to GlcNAc residues in chitin and glycoproteins. This method was used to show that chitin-containing fibrous material appears first at the tips of the microvilli of the midgut epithelial cells of *O. nubilalis* just past the stomadeal valves and is rapidly assimilated into a thin PM surrounding the food bolus (Harper and Hopkins, 1997). The PM becomes thicker and multilayered in the middle and posterior regions of the mesenteron. The orthogonal lattice of chitin meshwork is slightly larger than the diameter of the microvilli. SEM and light microscopic studies revealed that the PM delaminates from the tips of the microvilli. This observation suggests that microvilli serve as sites and possibly as templates for the organization of the PM by laying down a matrix of chitin microfibrils onto

which some PM proteins are deposited. A similar pattern of delamination of PM containing both chitin and intestinal mucins was demonstrated in larvae of *T. ni* (Harper and Granados, 1999; Wang and Granados, 2000a).

Incorporating WGA into the diet can interrupt formation of the PM. WGA-fed *O. nubilalis* larvae had an unorganized PM, which was multilayered and thicker than the normal PM (Hopkins and Harper, 2001). WGA was actually associated with the PM as well as with the microvillar surface as revealed by immunostaining with antibodies specific for WGA. Because there was very little WGA within the epithelial cells, the action of WGA appears to be extracellular. Presumably, WGA interferes with the formation of the organized chitin network and/or the association of PM proteins with the chitin network, leading to a reduced protein association with the PM (Harper *et al*., 1998). There was also extensive disintegration of the microvilli and the appearance of dark inclusion bodies as well as apparent microvillar fragments within the thickened multilayered PM. Insects such as *M. sexta*, which secrete multiple and thickened PMs that are somewhat randomly organized, tolerated WGA better and sequestered large amounts of WGA within the multilayered PM (Hopkins and Harper, 2001).

4.3.3.4. *In Situ* Hybridization and Immunological Studies

In situ hybridizations with a DNA probe for the catalytic domain of a CHS revealed that high levels of transcripts for this gene are present in apical regions of the columnar cells of the anterior midgut of *M. sexta* larvae (Zimoch and Merzendorfer, 2002). Lesser amounts of CHS transcripts were detected in the posterior midgut. An antibody to the catalytic domain of *M. sexta* CHS also detected the enzyme in midgut brush border membranes at the extreme apical ends of microvilli, suggestive of some special compartment or possibly apical membrane-associated vesicles. Staining was also seen in apical membranes of tracheal and salivary gland cells. Materials reacting with CHS antibody also were detected underneath the epidermal cuticle, even though it could not be specifically assigned to the apical membrane of epidermal cells due to loss of structural integrity of these cells during cryosectioning. These *in situ* hybridization and immunochemical studies are in agreement with earlier observations about chitin synthesis in *Calpodes ethlius* (larger canna leafroller), which indicated the involvement of specialized structures called plasma membrane plaques found in apical portions of epidermal cells (Locke and Huie, 1979). Comparable electron microscope (EM) and immunological localization of CHS associated with epidermis during cuticle deposition have not been reported primarily because of technical difficulties with the handling of cuticular samples. In *Drosophila melanogaster* the chitin synthase gene (*kkv*) is expressed predominantly in developmental stages 13-14 in the embryonic ventral and dorsal epidermis, foregut and in the larval tracheal system (see the "Patterns of gene expression in *Drosophila* embryogenesis" at the Berkeley Drosophila Genome Project (BDGP)).

4.3.3.5. Chitin Biosynthetic Pathway

It has been assumed that the pathway of chitin biosynthesis in insects would be similar or identical to the pathway that has been worked out extensively in fungi and other microbes (**Figure 1**). This appears to be the case except for some minor details (Palli and Retnakaran, 1999). The source of the sugar residues for chitin synthesis can be traced to fat body glycogen, which is acted upon by glycogen phosphorylase. Glucose-1-P produced by this reaction is converted to trehalose, which is released into the hemolymph. Trehalose, the extracellular source of sugar in many insects, is acted upon by a trehalase, which is widely distributed in insect tissues including the epidermis and gut to yield intracellular glucose (Becker *et al*., 1996). The conversion of glucose to fructose-6-P needed for chitin synthesis involves two glycolytic enzymes present in the cytosol. These enzymes are hexokinase and glucose-6-P isomerase, which convert glucose to fructose-6-P. From the latter, the chitin biosynthetic pathway branches off, with the first enzyme catalyzing this branch being GFAT, which might be thought of as the first committed step in amino sugar biosynthesis. The conversion of fructose-6-P to GlcNAc phosphate involves amination, acetyl transfer, and an isomerization step, which moves the phosphate from C-6 to C-1 (phosphoacetylglucoasmine mutase). The conversion of this compound to the nucleotide sugar derivative follows the standard pathway and leads to the formation of a UDP-derivative of GlcNAc, which serves as the substrate for CHS. The entire chitin biosynthetic pathway is outlined in **Figure 1**.

The involvement of dolichol-linked GlcNAc as a precursor for chitin was proposed quite some time ago (Horst, 1983), but it has received very limited experimental support (Quesada-Allue, 1982). At this point, this possibility remains unproven. Similarly, the requirement for a primer to which the

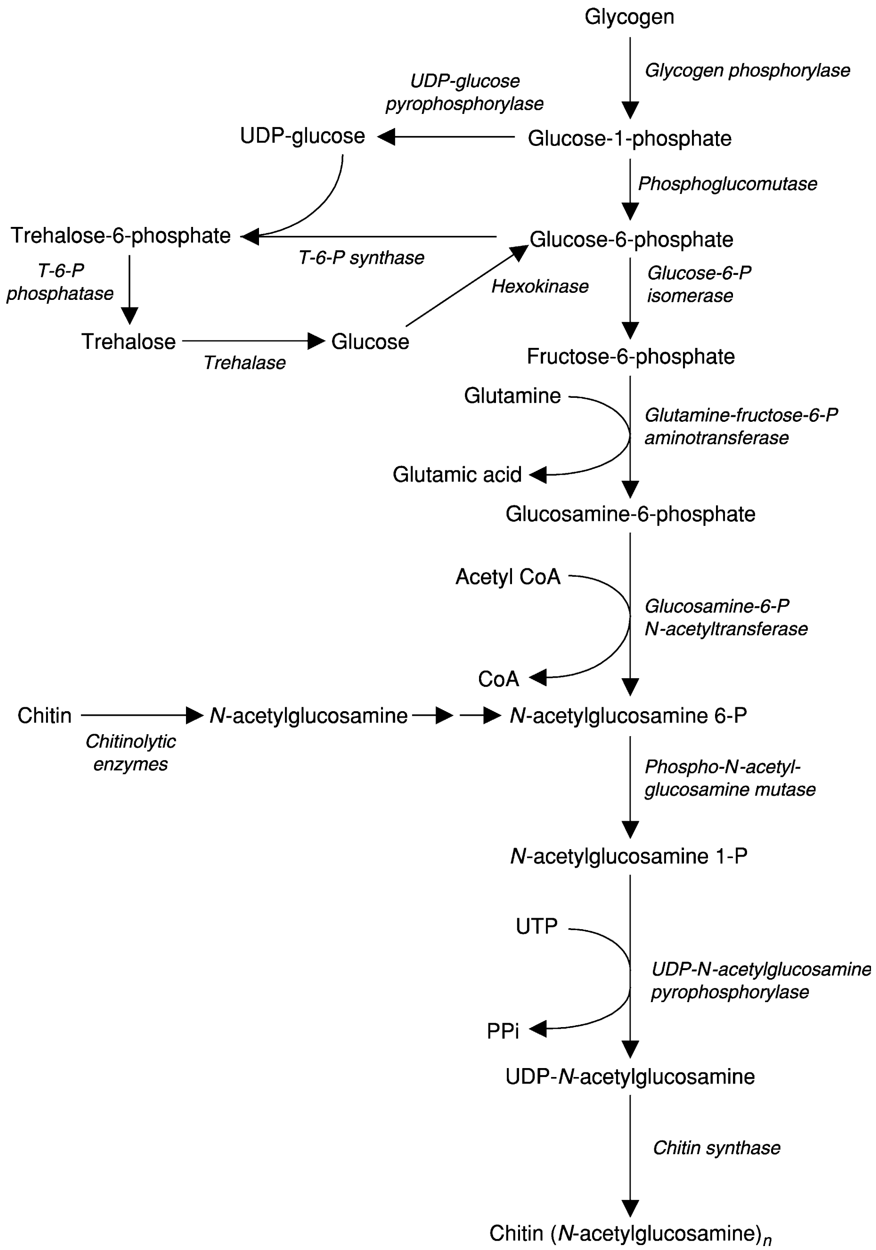


Figure 1 Biosynthetic pathway for chitin in insects starting from glycogen, trehalose, and recycled chitin.

GlcNAc residues can be transferred also remains speculative. Based on the model for glycogen biosynthesis, which requires glycogenin as the primer (Gibbons *et al.*, 2002), CHS or an associated protein may fulfill this priming function. Because each sugar residue in chitin is rotated ~180° relative to the preceding sugar, which requires CHS to accommodate a alternating "up/down" configuration, another precursor, UDP-chitobiose, has been proposed to be a disaccharide donor during biosynthesis (Chang *et al.*, 2003). However, evaluation of radiolabeled UDP-chitobiose as a CHS substrate in yeast revealed that it was not a viable one. Even at elevated concentrations, no incorporation of radioactivity above background was observed using membranous preparations of CHS from the yeast *Saccharomyces cerevisiae* (Chang *et al.*, 2003).

4.3.3.5.1. Key enzymes The biosynthetic pathway of chitin can be thought of as consisting of two segments. The first set of reactions leads to the formation of the amino sugar, GlcNAc, and the second set of reactions leads to the synthesis of the polymeric chitin from the amino sugar. The

rate-limiting enzyme in the first segment appears to be GFAT (also known as glucosamine-fructose-6-phosphate aminotransferase (GFAT, EC 2.6.1.16), which is found in the cytosol. The critical enzyme in the second segment is CHS (EC 2.4.1.16), which is localized in the plasma membrane. Not surprisingly, these two enzymes appear to be major sites of regulation of chitin synthesis.

4.3.3.5.2. Regulation of glutamine-fructose-6-phosphate aminotransferase synthesis

4.3.3.5.2.1. Drosophila GFAT Two genes encoding GFAT (*Gfat1* and *Gfat2*) have been identified in *Drosophila* (Adams *et al.*, 2000; Graack *et al.*, 2001). Both of these genes are on chromosome 3, but they are at different locations. Their intron–exon organizations are different as are the amino acid sequences of the encoded proteins. GFAT consists of two separate domains, an N-terminal domain that has both glutamine binding and aminotransferase motifs identified in GFATs from other sources and a C-terminal domain with both fructose-6-phosphate binding and isomerase motifs. *Gfat1* is expressed in embryos in the developing trachea and in cuticle-forming tissues including the chitinous mouth armature of the developing first instar larva. In the last larval stadium, *Gfat1* is expressed in the corpus cells of salivary glands, but this synthesis may be related to the production of the highly glycosylated Sgs glue proteins (Graack *et al.*, 2001). The major regulation of GFAT1 appears to be posttranslational. When *Gfat1* was expressed in yeast cells, the resulting enzyme was feedback inhibited by UDP-GlcNAc and was stimulated by protein kinase A. Even though it has not been demonstrated that there is a phosphorylated form of GFAT1 that is susceptible to feedback inhibition by UDP-GlcNAc, this possibility remains viable. The expression and regulation of the other GFAT isozyme (GFAT2) has not yet been reported.

4.3.3.5.2.2. Aedes aegypti GFAT The gene and cDNA for the mosquito *Aedes aegypti* GFAT1 have been cloned (Kato *et al.*, 2002). The mosquito gene has no introns and the promoter appears to contain sequences related to ecdysteroid response elements (EcRE) as well as E74 and Broad complex Z4 elements. E74 and Broad complex Z4 proteins are transcription factors known to be upregulated by ecdysone (Thummel, 1996). Two *Gfat1* transcripts with different sizes were observed in Northern blot analyses of RNA from adult females and their levels increased further after blood-feeding (Kato *et al.*, 2002). Since ecdysteroid titers increase following blood-feeding, it is possible that this gene is under the control of ecdysteroid either directly or indirectly. Feedback inhibition by UDP-GlcNAc has not been reported, but the *Aedes* enzyme is likely to be regulated in a manner similar to the *Drosophila* enzyme by this effector and possibly by a phosphorylation/dephosphorylation mechanism as well.

4.3.3.5.3. CHS gene number and organization CHS genes from numerous fungi have been isolated and characterized (Munrow and Gow, 2001). However, the complete sequence of a cDNA clone for an insect CHS (sheep blowfly, *Lucilia cuprina*) was reported only recently (Tellam *et al.*, 2000). Since then, the sequences of several other full-length cDNAs and genes for CHSs from other insects and nematodes have been reported. The nematode CHSs were from two filarial pathogens, *Brugia malayi*, and *Dirofilaria immitis*, and the plant parasite *Meloidogyne artiellia* (Harris *et al.*, 2000; Veronico *et al.*, 2001; Harris and Fuhrman, 2002). The other insect species from which CHS cDNAs have been isolated are *A. aegypti* (Ibrahim *et al.*, 2000), *M. sexta* (Zhu *et al.*, 2002) and the red flour beetle, *Tribolium castaneum* (Arakane *et al.*, 2004). DNA sequencing of polymerase chain reaction (PCR)-amplified fragments encoding a highly conserved region in the catalytic domains of insect CHSs indicates a high degree of sequence conservation (Tellam *et al.*, 2000). In addition, a search of the databases in light of the sequence data from these cDNAs has allowed identification of open reading frames (ORFs) from CHS genes from *Drosophila*, *Anopheles, Aedes* and the nematode *Caenorhabditis elegans* (Tellam *et al.*, 2000; Gagou *et al.*, 2002; Arakane *et al.*, 2004). **Table 1** lists the properties of insect CHSs encoded by these genes/cDNAs. Insect species typically have two genes for CHSs. Among the nematodes, the *C. elegans* genome contains two CHS genes, but so far there is evidence for only one gene in the plant parasitic nematode *M. artiellia*, and in the filarial nematodes *B. malayi* and *D. immitis* (Harris *et al.*, 2000; Veronico *et al.*, 2001; Harris and Fuhrman, 2002). Fungi, on the other hand, exhibit a wide range in the number of genes for CHS (Munrow and Gow, 2001).

The two *Tribolium* CHS genes, *TcCHS1* and *TcCHS2*, have ten and eight exons, respectively (Arakane *et al.*, 2004). The organizations of the two genes in *Tribolium* are quite different, with some introns occurring in identical positions in both genes, whereas others are at variable positions. The introns ranged in length from 46 bp to more than 3000 bp. The most interesting difference between the two genes was the presence of two

Table 1 Properties of insect chitin synthases and their genes

Species	Number of amino acids	Expressed in	Alt. Exon	Coiled-coil	CHS class	GI no.	Reference
Lucilia cuprina	1592	Epidermis	Yes[a]	Yes	A	9963823	Tellam *et al.* (2000)
Drosophila melanogaster	1615	Epidermis/gut/tracheal	Yes	Yes	A	24644218	Adams *et al.* (2000); Fly base – http://www.flybase.bio.indiana.edu; Berkeley Drosophila genome project (Drosophila EST database) – http://www.fruitfly.org
	1674		Yes	Yes	A	24644220	
	1416	ND	No	No	B	24668460	
Anopheles gambiae	1578		Yes	Yes	A		
	1583		No	No	B		
Aedes aegypti	1564	Midgut	No	No	B	22773456	Ibrahim *et al.* (2000)
Tribolium castaneum	1558	ND	Yes	Yes	A		
	1558	ND	Yes	Yes	A		Arakane *et al.* (2004)
	1464	ND	No	No	B		
Manduca sexta	1563	Epidermis/gut	Yes	Yes	A	24762312	Zhu *et al.* (2002)
	1563	Epidermis/gut	Yes	Yes	A		H. Merzendorfer (unpublished data)
	1524	Gut	No	No	B		D. Hogenkamp *et al.* (unpublished data)

[a]Predicted.
ND, not determined.

nonidentical copies of exon 8 (named 8a and 8b) in *TcCHS1*, whereas *TcCHS2* has only one copy of this region as a part of exon 6. An analysis of genomic sequences from the *D. melanogaster* and *Anopheles gambiae* genome projects, partial sequencing of cDNAs available as separate sequence files submitted to GenBank, and "TBLASTN" queries were used to determine the organization of CHS genes in these insects (**Figure 2**). These analyses revealed that the sequences and organization of CHS genes of *D. melanogaster* (Tellam *et al.*, 2000) and *A. gambiae* were similar to those of *TcCHS1* and *TcCHS2* (Arakane *et al.*, 2004). One major difference between the two exons that are alternately spliced is that all of the B forms code for segments that have a site for *N*-linked glycosylation just before the transmembrane helix, whereas none of the A forms do. The physiological significance of alternate exon usage and potential glycosylation in *CHS* expression is unknown even though it is clear that there is developmental regulation of alternate exon usage (see Section 4.3.3.5.6).

4.3.3.5.4. Modular structure of chitin synthases CHSs are members of family GT2 of the glycosyltransferases (Coutinho *et al.*, 2003), which generally utilize a mechanism where inversion of the anomeric configuration of the sugar donor occurs. The protein fold (termed GT-A) for this family is considered to be two associated β/α/β domains that form a continuous central sheet of at least eight β-strands. The GT-A enzymes share a common ribose/metal ion-coordinating motif (termed DxD motif) as well as another carboxylate residue that acts as a catalytic base. The general organization of CHSs has been deduced from a comparison of amino acid sequences of these enzymes from several insects, nematodes and yeasts (Zhu *et al.*, 2002; Arakane *et al.*, 2004). These enzymes have three distinguishable domains: an N-terminal domain with moderate sequence conservation among different species and containing several transmembrane segments, a middle catalytic domain that is highly conserved even among CHSs from different kingdoms, and a C-terminal module with multiple transmembrane segments (**Figure 3**). The catalytic domain contains several stretches of highly conserved amino acid sequences including the following: CATMWHXT at the beginning of the catalytic domain, FEYAIGHW and VQYDDQGEDRW in the middle of the catalytic domain, and the presumed catalytic site, EFYNQRRRW, at the end of the catalytic domain. While the transmembrane segments in the N-terminal domain show different patterns among different insect species, the transmembrane segments in the C-terminal domain are remarkably conserved both with respect to their location and the spacing between adjacent transmembrane segments. Particularly striking is the fact that five such transmembrane segments are found in a cluster immediately following the catalytic domain and two more segments are located closer to the

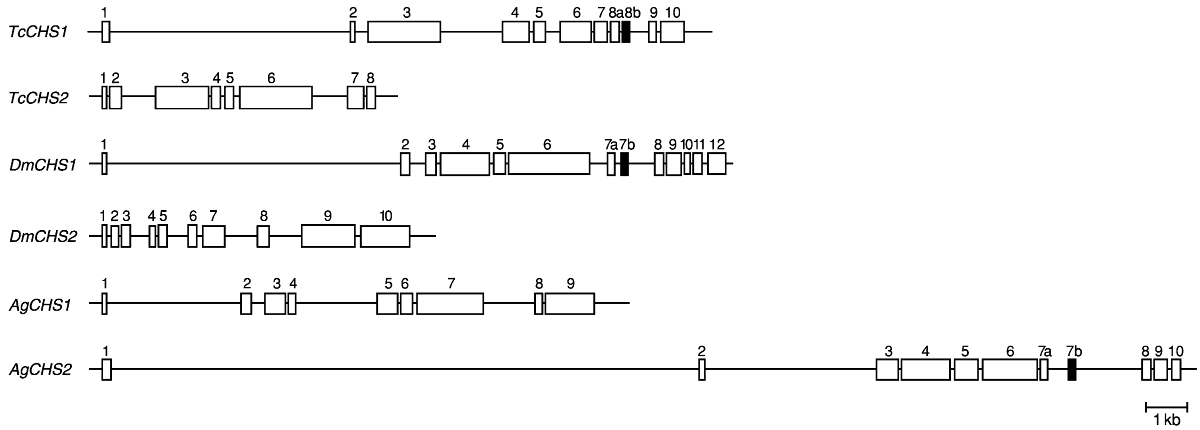


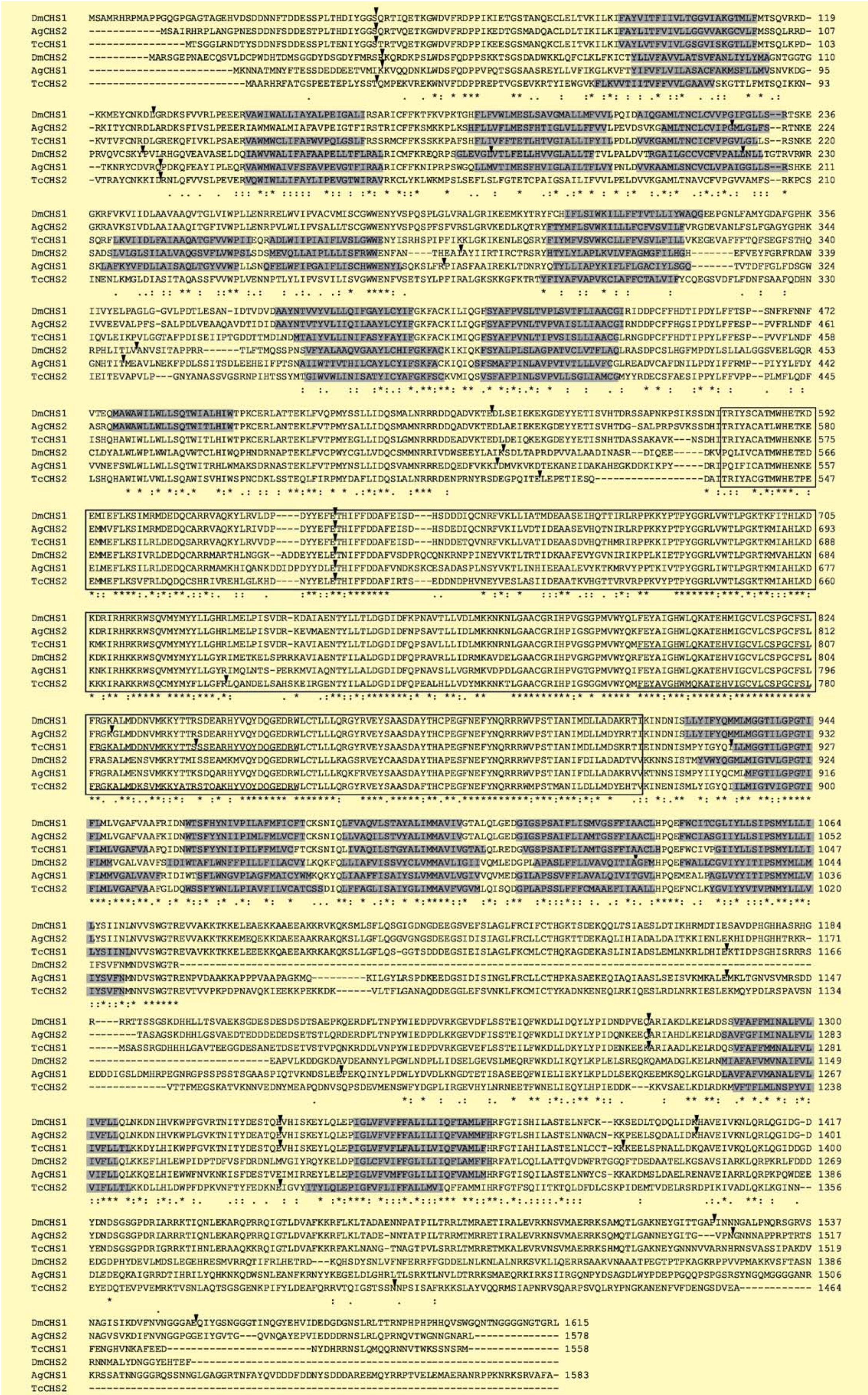
Figure 2 Schematic diagram of the organization of the *TcCHS1*, *TcCHS2*, *DmCHS1, DmCHS2, AgCHS1*, and *AgCHS2* genes. Boxes indicate exons. Lines indicate introns. The second of the two alternative exons (8b) of *TcCHS1*, *DmCHS1 (7b),* and *AgCHS2 (6b)* are indicated as closed boxes. About 9 kb of the *TcCHS1* and *TcCHS2* gDNA sequences were compared to their respective cDNA sequences to define the exons and introns. The exon–intron organization of the other four CHS genes was deduced partially from comparisons of available cDNA and genomic sequences. (Reprinted with permission from Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, **2004**. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol. 34*, 291–304.)

C-terminus. The 5-transmembrane cluster, known as 5-TMS, has been suggested to be involved in the extrusion of the polymerized chitin chains across the plasma membrane to the exterior of the cell as proposed for extrusion of cellulose (Richmond, 2000).

The CHSs of insects characterized so far can be broadly grouped into two classes, A and B, based on amino acid sequence identities. The class A proteins were predicted to have a coiled-coil region immediately following the 5-TMS region (Zhu *et al.*, 2002; Arakane *et al.*, 2004). Also, all of the genes encoding the class A CHSs have two alternate exons (corresponding to alternate exon 7 of *D. melanogaster*, exon 8 of *T. castaneum*, exon 6 of *A. gambiae*, and an unnumbered exon of *M. sexta* CHS-A gene) (see **Table 1**). The alternate exons are located on the C-terminal side of the 5-TMS region and encode the next transmembrane segment and flanking sequences. The alternate exon-encoded regions of the CHS proteins differ in sequence by as much as 30% and most of these differences are in the regions flanking the transmembrane segment. This finding suggests that the proteins may differ in their ability to interact with cytosolic or extracellular proteins, which might regulate chitin synthesis and/or transport. An attractive hypothesis is that these flanking sequences may influence the plasma membrane location of a CHS by interacting with cytoskeletal elements or perhaps by generation of extracellular vesicles involved in chitin assembly.

4.3.3.5.5. Regulation of chitin synthase gene expression The two insect genes encoding CHSs appear to have different patterns of expression during development. The high degree of sequence identity of the catalytic domains and the absence of antibodies capable of discriminating between the two isoforms have complicated the interpretation of experimental data to some extent. In some cases, the technical difficulties associated with isolation of specific tissues free of other contaminating tissues have precluded unambiguous assignment of tissue specificity of expression. Nonetheless, the following conclusions can be reached from the analyses of expression of CHS genes in several insect species. CHS genes are expressed at all stages of insect growth including embryonic, larval, pupal, and adult stages. *CHS1* genes (coding for class A CHS proteins) are expressed over a wider range of developmental stages (Tellam *et al.*, 2000; Zhu *et al.*,

Figure 3 Alignment of deduced amino acid sequences of TcCHS1, TcCHS2, DmCHS1, DmCHS2, AgCHS1, and AgCHS2 using ClustalW software. Transmembrane regions predicted using TMHMM software (v. 2.0) are shaded. Shaded arrowheads indicate the positions in the protein sequences of TcCHS1 and TcCHS2 where coding regions are interrupted by introns. Intron 1 of *TcCHS1* lies in the 5′-UTR region two nucleotides 5′ of the translation start site and is not indicated in this figure. The putative catalytic domains are boxed. Symbols below the aligned amino acid sequences indicate identical (*), highly conserved (:), and conserved residues (.). The regions in TcCHS1 and TcCHS2 corresponding to the PCR probe made from two degenerate primers representing two highly conserved sequences in CHSs are underlined. (Reprinted with permission from Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, **2004**. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol. 34*, 291–304.)



2002). *CHS2* genes (coding for class B CHSs) are not expressed in the embryonic or pupal stages but are expressed in the larval stages, especially during feeding in the last instar and in the adults including blood-fed mosquitoes (Ibrahim *et al*., 2000; Zimoch and Merzendorfer, 2002; Arakane *et al*., 2004). The finding that both classes of CHS genes are expressed at high levels 3 h after pupariation in *Drosophila* suggests that both enzymes are required for postpuparial development (Gagou *et al*., 2002).

CHS genes also show tissue-specific expression patterns. In *L. cuprina*, *CHS1* (coding for a class A CHS) is expressed only in the carcass (larva minus internal tissues) and trachea but not in salivary gland, crop, cardia, midgut or hindgut (Tellam *et al*., 2000). In blood-fed female mosquitoes, a CHS gene encoding a class B CHS is expressed in the epithelial cells of the midgut (Ibrahim *et al*., 2000). In *M. sexta*, *CHS1* (coding for a class A CHS) is expressed in the epidermal cells of larvae and pupae (Zhu *et al*., 2002). Transcripts specific for class B CHS were detected only in the gut tissue (D. Hogenkamp *et al*., unpublished data). As discussed above, in *Drosophila,* both classes of CHS genes were shown to be upregulated after the ecdysone pulse had ceased in the last larval instar, but the tissue specificity of expression of each gene was not determined. In *T. castaneum*, the *CHS1* gene (coding for a class A CHS) was expressed in embryos, larvae and pupae, and in young adults, but not in mature adults (Arakane *et al*., 2004). Even though unequivocal data are not available for each of these insect species, the following generalizations may be made. Class A CHS proteins are synthesized by epidermal cells when cuticle deposition occurs in embryos, larvae, pupae, and young adults, whereas the class B CHS proteins are expressed by the midgut columnar epithelial cells facing the gut lumen in the larval and adult stages and is probably limited to feeding stages.

4.3.3.5.6. Developmental control of alternate exon usage Insect CHS genes characterized so far have eight or more exons. The genes encoding *Drosophila*, *Anopheles*, *Tribolium*, and *Manduca* class A CHSs, but not the genes encoding class B CHSs, have two alternate exons, each encoding a 59 amino acid long segment following the 5-TMS region (**Table 1**). This segment contains a 20 amino acid long transmembrane region and flanking sequences. In addition, the presence of a predicted coiled-coil region immediately following the 5-TMS region in the CHSs encoded by those genes that have the alternate exons suggests a link between these two structural features and the possibility of regulation of alternate exon usage. In agreement with this idea, transcripts containing either one of these exons have been detected in *T. castaneum* and *M. sexta* (Arakane *et al*., 2004; D. Hogenkamp *et al*., unpublished data). In *T. castaneum* embryos, transcripts with either exon 8a or 8b were detected, whereas in last instar larvae and prepupae, only exon 8a transcripts were present. By the pupal stage, however, transcripts with exon 8a or exon 8b were abundant along with trace amounts of a transcript with both exons. In mature adults, none of these transcripts was detectable, whereas *TcCHS2* transcripts were easily detected especially in females (Arakane *et al*., 2004). In *Drosophila*, transcripts containing either exon 7a or both exons 7a and 7b (but not those containing exon 7b alone) have been reported (Drosophila EST Database).

It appears that the TcCHS1 with the exon 8b-encoded segment is needed during cuticle deposition in the pupal and embryonic stages but not at other stages of development. Similar results were observed with fifth instar *M. sexta* larvae (Hogenkamp *et al*., unpublished data). The biochemical basis for a specific requirement of the TcCHS1 with the exon 8b-encoded segment is unknown.

4.3.3.6. Chitin Synthesis during Development

4.3.3.6.1. Effect of chitin inhibitors Chitin synthesis occurs during embryonic, larval, pupal, and adult stages for cuticle deposition and for production of the PM in larvae and adults. The inhibition of chitin synthesis using chemical inhibitors or by introduction of mutations affects insect development at different developmental stages and to varying degrees. Studies with "chitin inhibitors" have provided some insights concerning the role of chitin in development and its biological function. The use of lufenuron, a member of the class of insecticides known as benzoylphenylureas, has provided substantial information on chitin synthesis during *Drosophila* development (Wilson and Cryan, 1997). The effects of this insect growth regulator were complex and variable depending on the developmental stage and dose at which the insects were exposed to this agent. When newly hatched larvae were reared on a diet containing very low concentrations of lufenuron, the larvae did not die until the second or third instar and usually pupariated even though the pupae were abnormally compressed. Pharate adults either failed to eclose or died shortly after emergence and had deformed legs. The flight ability of the emerged adults was also affected when the larvae were exposed to very low concentrations of lufenuron. First and second instar larvae fed higher concentrations of lufenuron

had normal growth and physical activity for several hours, but the insects died at about the time of the next ecdysis. Third instar larvae fed high concentrations of lufenuron underwent pupariation, but the puparia had an abnormal appearance. The anterior spiracles failed to evert. Thus, insect development is affected by lufenuron at all stages when chitin synthesis occurs. Another aspect of insect development affected by this compound was egg hatching even though oviposition was normal. The embryos completed development but failed to rupture the vitelline membrane. These results indicated that maternally derived lufenuron can affect egg hatching, which requires the use of chitinous mouth parts by the newly ecdysed larvae. The adults showed no mortality and had no flight disability even when fed high levels of lufenuron, indicating that once all chitin-containing structures had been formed, this "chitin inhibitor" had very little effect on adult morphology and function. However, the benzoylphenylureas may not be affecting CHS activity directly because diflubenzuron did not inhibit incorporation of UDP-GlcNAc into chitin microfibrils in an *in vitro* assay using a microsomal preparation from *T. castaneum* (Cohen and Casida, 1980). It is more likely that the benzoylphenylurea class of insecticides interferes with a step in the assembly of the cuticle and/or PM rather than chitin synthesis *per se*.

4.3.3.6.2. Genetics of chitin synthesis Several *Drosophila* genes involved in controlling cuticle morphology have been characterized (Jurgens *et al*., 1984; Nusslein-Volhard *et al*., 1984; Wiechaus *et al*., 1984; Ostrowski *et al*., 2002). These genes are *krotzkopf verkehrt* (*kkv*), *knickkopf* (*knk*), *grainy head (grh), retroactive (rtv)*, and *zepellin* (*zep*). All of these mutations result in poor cuticle integrity and reversal of embryo orientation in the egg to varying degrees. The homozygous mutant embryos failed to hatch. When these mutant embryos were mechanically devitellinized, the cuticles became grossly enlarged, yielding the "blimp" phenotype. Ostrowski *et al.* (2002) characterized the *kkv* gene and identified it as a *CHS*-like gene. Interestingly, embryos derived from wild-type females treated with high concentrations of lufenuron displayed a similar "blimp" phenotype when devitellinized, indicating that either genetic or chemical disruption of chitin deposition leads to this phenotype. The *knk* gene codes for a protein with sequence similarity to a protein component of the nuclear spindle matrix and is located on chromosome 3 close to the *kkv* gene near the centromere. The *knk* and *kkv* functions are not additive and *kkv* appears to be epistatic to *knk*, which is expressed at very low levels compared to the *kkv* gene as indicated by mRNA levels. The *knk* and *zep* genes appear to function in the epidermis prior to cuticle deposition because they exacerbate the effect of a heterozygous *shotgun* (*shg*) mutation, which codes for an E-cadherin-like protein. The *shg* gene is recessive, but in a *knk/knk* or *zep/zep* background, the cuticle is fragmented suggesting that the protein products of these genes interact with cadherin to reinforce the cuticle by promoting adhesion of the epithelia. Thus, products of all of the "blimp" class of genes, including *kkv*, control the integrity of the embryonic cuticle. It is also possible that some of these genes, whose functions have not been identified yet, may be involved directly or indirectly in extrusion or polymerization of chitin microfibrils. Alternatively, these proteins may reinforce chitin–chitin or chitin–protein interactions. For example, the *grh* gene encodes a GATA family transcription factor that regulates the expression of a DOPA decarboxylase needed for the production of precursors of cuticular protein cross-linking agents (Bray and Kafatos, 1991). It is also possible that some of these proteins are involved in vesicular trafficking and/or targeting CHS to plasma membrane plaques that are associated with chitin synthesis (Locke and Huie, 1979).

4.3.4. Chitin Degradation

Chitinases are among a group of proteins that insects use to digest the structural polysaccharide in their exoskeletons and gut linings during the molting process (Kramer *et al*., 1985; Kramer and Koga, 1986; Kramer and Muthukrishnan, 1997; Fukamizo, 2000). Chitin is digested in the cuticle and PM to GlcNAc by a binary enzyme system composed of a chitinase (CHI) and a β-*N*-acetylglucosaminidase (Fukamizo and Kramer, 1985; Filho *et al*., 2002). The former enzyme from molting fluid hydrolyzes chitin into oligosaccharides, whereas the latter, which is also found in the molting fluid, further degrades the oligomers to the monomer from the nonreducing end. This system also probably operates in the gut during degradation of chitin in the PM or in digestion of chitin-containing prey.

Chitinase (EC 3.2.1.14, endochitinase) is defined as an enzyme that catalyzes the random hydrolysis of *N*-acetyl-β-D-glucosaminide β-1,4-linkages in chitin and chitodextrins. Chitinases are found in a variety of organisms besides insects including bacteria, fungi, plants, and marine and land animals (Watanabe and Kono, 2002). Many genes encoding chitinolytic enzymes including several from insects

(Table 2) have been cloned and characterized. Some chitinases are now being used for biotechnological applications in agriculture and healthcare (Patil *et al.*, 2000).

Chitinases are members of the superfamily of *O*-glycoside hydrolases, which hydrolyze the glycosidic bond in polysaccharides or between a sugar and a noncarbohydrate moiety. The International Union for Biochemistry and Molecular Biology enzyme nomenclature of glycoside hydrolases is based on their substrate specificity and occasionally based on their molecular mechanism. Such a classification, however, does not reflect the structural features of these enzymes. Another classification of glycoside hydrolases into families is based on amino acid sequence similarities. This classification is expected to: (1) reflect the structural features of these enzymes better than their sole substrate specificity; (2) help to reveal the evolutionary relationships between these enzymes; and (3) provide a convenient tool to derive mechanistic information (Henrissat and Bairoch, 1996). There are 91 families of glycosylhydrolases and to date all mechanistically characterized insect chitinases belong to family 18 (Coutinho and Henrissat, 1999; CAZY, 2004). Unlike family 19 chitinases that are found almost exclusively in plants, members of family 18 have been found in a wide variety of sources including bacteria, yeasts and other fungi, nematodes, arthropods, and even vertebrates such as mice, chickens, and humans (Nagano *et al.*, 2002). The vertebrate proteins probably function as defensive proteins against chitin-containing pathogenic organisms.

4.3.4.1. Insect *N*-Acetylglucosaminidases

Beta-*N*-acetylglucosaminidases (EC 3.2.1.30) have been defined as enzymes that release β-*N*-acetylglucosamine residues from the nonreducing end of chitooligosaccharides and from glycoproteins with terminal *N*-acetylglucosamine. Insect β-*N*-acetylglucosaminidases are members of family 20 of the glycosylhydrolases (Coutinho and Henrissat, 1999; CAZY, 2004). These enzymes have been detected in the molting fluid, hemolymph, integument, and gut tissues of several species of insects (Kramer and Koga, 1986 and references therein). A β-*N*-acetylglucosaminidase also has been detected in the gut of *A. aegypti*, where its activity increased dramatically after blood feeding (Filho *et al.*, 2002). Beta-*N*-acetylglucosaminidases also hydrolyze synthetic substrates such as *p*-nitrophenyl *N*-acetylglucosamine and 4-methylumbelliferyl oligo-β-*N*-acetylglucosamines. These two substrates have proven to be very useful in assays of these enzymes.

During development, β-*N*-acetylglucosaminidase activities are the highest in hemolymph a few days prior to larval or pupal ecdysis and in molting fluid from pharate pupae (Kimura, 1976, 1977; Turner *et al.*, 1981). Two different enzymes with different physical and kinetic properties have been purified from the lepidopterans *B. mori* and *M. sexta*. The first enzyme (EI), which is found in larval and pharate pupal molting fluid and in pupal hemolymph, is probably involved in the turnover of chitobiose and possibly chitooligosaccharides because it has a lower K_m for these substrates than does the second (EII) enzyme. EII is found in larval and pupal hemolymph and has a lower K_m for pNpGlcNAc. The role of the enzyme (EII) is unclear, but its natural substrates may be glycoproteins containing terminal *N*-acetylglucosamines. However, this specificity remains to be proven.

4.3.4.2. Catalytic Mechanism of Insect *N*-Acetylglucosaminidases

N-acetylglucosaminidases have lower K_m values for substrates containing *N*-acetylglucosamine than those with *N*-acetylgalactosamine residues. They release monosaccharides from the nonreducing end by an exocleavage mechanism. Two ionizable groups with pKa values of 3.8 and 8.1 are involved in catalysis (Koga *et al.*, 1982). Studies with competitive inhibitors such as δ-lactone derivatives of *N*-acetylglucosamine and *N*-acetylgalactosamine suggested that the active site of enzyme EI consists of subsites that bind larger substrates than does the active site of the EII enzyme. EI has a lower K_m than EII for the chitooligosaccharides and a larger K_m for pNpβGlcNAc, properties that are consistent with the two enzymes having different endogenous substrate specificities.

4.3.4.3. Cloning of cDNAs for Insect *N*-Acetylglucosaminidases

cDNAs for epidermal β-*N*-acetylglucosaminidases of *B. mori* (GenBank accession no. S77548), *B. mandarina* (accession no. AAG48701), *T. ni* (accession no. AAL82580), and *M. sexta* (accession no. AY368703) have been isolated and characterized (Nagamatsu *et al.*, 1995; Zen *et al.*, 1996; Goo *et al.*, 1999). A search of the *Drosophila* and *Anopheles* genome databases also revealed the presence of closely related genes encoding β-*N*-acetylglucosaminidases. These genes encode closely related proteins (70-75% amino acid sequence identity between the *Manduca* and *Bombyx* enzymes) of approximately 68 kDa. The conceptual proteins contain leader peptides of 22-23 amino acids followed by stretches of

Table 2 Properties of insect chitinases

Species	*Common name*	*Tissue source*	*Number of amino acids*	*Domain structure*[a]	*GI no.*	*Reference*
Aedes aegypti	Yellow fever mosquito	ND	574	Cat-linker-ChBD 3ChBDs-3Cats	2564719	de la Vega *et al.* (1998)
Anopheles gambiae	Malaria mosquito	Gut	525	Cat-linker-ChBD	2654602	Shen and Jacobs-Lorena (1997)
Bombyx mori	Silkworm	Epidermis/gut	565	Cat-linker-ChBD	1841851, 10119784	Kim *et al.* (1998), Mikitani *et al.* (2000), Abdel-Banat and Koga (2001)
Chelonus sp. venom	Wasp	Venom gland	483	Cat-linker-ChBD	1079185	Krishnan *et al.* (1994)
Chironomus tentans	Midge	Cell line	475	Cat	2113832	Feix *et al.* (2000)
Choristoneura fumiferana	Spruce budworm	Epidermis/fat body	557	Cat-linker-ChBD	21913148	Zheng *et al.* (2002)
Drosophila melanogaster	Fruit fly	ND	508	Cat	17647257	de la Vega *et al.* (1998), Adams *et al.* (2000)
		ND	484	Cat	24655584	
		ND	458	ChBD-Cat	17647259	
Glossina morsitans	Tsetse fly	Fat body	460	Cat-ChBD	18201665	Yan *et al.* (2002)
Hyphantria cunea	Fall webworm	Epidermis	553	Cat-linker-ChBD	1841853	Kim *et al.* (1998)
Lutzomyia longipalpis	Sand fly	Midgut	474	Cat-linker-ChBD	28863959	Ramalho-Ortigão and Traub-Csekö (2003)
Manduca sexta	Tobacco hornworm	Epidermis/gut	554	Cat-linker-ChBD	1079015	Kramer *et al.* (1993), Choi *et al.* (1997)
Phaedon cochleariae	Mustard beetle	Gut	405	Cat	4210812	Girard and Jouanin (1999)
Spodoptera litura	Common cutworm	Epidermis	552	Cat-linker-ChBD	9971609	Shinoda *et al.* (2001)
Tenebrio molitor	Yellow mealworm	ND	2838	5 Cats+5 linkers+4 ChBDs+2 Mucs	21038943	Royer *et al.* (2002)

[a]Cat, catalytic domain; linker, linker region; ChBD, chitin-binding domain; Muc, mucin-like domain.
ND, not determined.

mature N-terminal amino acid sequences experimentally determined from *N*-acetylglucosaminidases purified from either the molting fluid or integument of these two species. The amino acid sequences include two regions that are highly conserved among *N*-acetylglucosaminidases from a variety sources including bacteria, yeast, mouse, and humans (Zen *et al.*, 1996). The *M. sexta* gene was expressed most abundantly in epidermal and gut tissues prior to metamorphosis and was induced by 20-hydroxyecdysone. The inductive effect of molting hormone was suppressed by juvenoids (Zen *et al.*, 1996).

4.3.4.4. Cloning of Genes Encoding Insect Chitinases

A chitinase from *M. sexta*, which is a 535 amino acid long glycoprotein (Chi535), as well as the cDNA and gene that encode it (*MsCHI*, accession no. AAC04924) were the first insect chitinase and gene to be isolated and characterized (Koga *et al.*, 1983; Kramer *et al.*, 1993; Choi *et al.*, 1997; Kramer and Muthukrishnan, 1997). They represent the most extensively studied chitinase enzyme–gene system in any insect species and they have become a model for study of other insect chitinases and their genes. Since the cloning of the *M. sexta* gene in 1993, cDNAs or genomic clones for several other insect chitinases have been isolated and sequenced (**Table 2**). The organization of most of these genes is very similar to that of *M. sexta* and most of the proteins display a domain architecture consisting of catalytic, linker, and/or chitin-binding domains similar to MsCHI. These genes/enzymes include epidermal chitinases from the silkworm, *B. mori* (Kim *et al.*, 1998; Abdel-Banat and Koga, 2001), the fall webworm, *Hyphantria cunea* (Kim *et al.*, 1998), wasp venom (*Chelonus* sp.) (Krishnan *et al.*, 1994), the common cutworm, *Spodoptera litura* (Shinoda *et al.*, 2001), a molt-associated chitinase from the spruce budworm, *Choristoneura fumiferana* (Zheng *et al.*, 2002), and midgut-associated chitinases from the malaria mosquito, *A. gambiae* (Shen and Jacobs-Lorena, 1997), yellow fever mosquito, *A. aegypti* (de la Vega *et al.*, 1998), the beetle *Phaedon cochleariae* (Girard and Jouanin, 1999), and the sand fly, *Lutzomyia longipalpis* (Ramalho-Ortigão and Traub-Csekö, 2003), and several deduced from the *Drosophila* genome data. A smaller linkerless fatbody-specific chitinase from the tsetse fly, *Glossina morsitans* (Yan *et al.*, 2002) and a very large epidermal chitinase with five copies of the catalytic-linker-chitin binding domain from the yellow mealworm, *Tenebrio molitor* (Royer *et al.*, 2002) have also been described.

Recently, a gene encoding another type of chitinase from the silkworm, *BmChi-h*, has been reported (Daimon *et al.*, 2003). The encoded chitinase shared extensive similarities with microbial and baculoviral chitinases (73% amino acid sequence identity to *Serratia marcescens* chitinase and 63% identity to *Autographa californica* nuclear polyhedrosis virus chitinase). Even though this enzyme had the signature sequence characteristic of family 18 chitinases, it had a rather low percentage of sequence identity with the family of insect chitinases listed in **Table 2**. It was suggested that an ancestral species of *B. mori* acquired this chitinase gene via horizontal gene transfer from *Serratia* or a baculovirus. Unlike the chitinases listed in **Table 2**, which typically have a leader peptide, catalytic domain, a serine/threonine(S/T)-rich domain and a C-terminal chitin-binding domain, BmChi-h chitinase has a leader peptide, one copy of module w1 domain that is found only in bacterial and baculoviral chitinases (Perrakis *et al.*, 1994; Henrissat, 1999), and a catalytic domain. Apparently, *B. mori* is not alone among insects possessing such a chitinase of bacterial origin. A protein in the molting fluid of *M. sexta*, which cross-reacted with an antibody to *M. sexta N*-acetylglucosaminidase, was found to have an N-terminal amino acid sequence closely resembling that of *Serratia* chitinase (Zen *et al.*, 1996). The N-terminal sequence of this protein was identical to that of BmChi-h up to the 25th amino acid residue, which strongly suggested that an ortholog of this chitinase gene exists in *M. sexta* as well. It will be interesting to investigate in the future whether this enzyme is widespread and found in other insect species. A search of the *Drosophila* and *Anopheles* genome databases, however, failed to identify any chitinase-like protein with an amino acid sequence identity to BmChi-h of greater than 40% (S. Muthukrishnan *et al.*, unpublished data).

Reports of multiple forms of insect chitinases, which can be generated by several mechanisms, have appeared. Some of these proteins are no doubt products of multiple genes as described in the previous paragraph. Others are likely the result of posttranslational modifications that are caused by glycosylation and/or proteolysis, which can lead to larger glycosylated forms and smaller truncated forms (Koga *et al.*, 1983; Wang *et al.*, 1996; Gopalakrishnan *et al.*, 1995; Arakane *et al.*, 2003). Another cause can be alternative splicing of mRNA. In *B. mori*, alternative splicing of the primary transcript from a single chitinase gene generates heterogeneity within the products (Abdel-Banat and Koga, 2002). Larger chitinase-like proteins have been observed in the mosquito *Anopheles* and

it has been proposed that these zymogenic proteins are activated via proteolysis by trypsin (Shen and Jacobs-Lorena, 1997). However, Filho *et al.* (2002) found no evidence for such activation in the mosquito *Aedes* because high levels of chitinase activity were observed early after a blood meal and even in the guts of unfed insects. Putative zymogenic forms have been reported in other insects as well (Koga *et al.*, 1992; Bhatnagar *et al.*, 2003). However, the existence of a chitinase zymogen is still speculative in most cases because all of the fully characterized cDNAs encoding full-length insect chitinases apparently have the mature catalytic domains immediately following their leader peptides and there is no indication of the presence of pre-proproteins (**Table 2**). Preliminary evidence suggests that most, if not all, of the larger proteins reacting with chitinase antibodies are multimeric forms that are enzymatically inactive and produced as a result of intermolecular disulfide pairing. These larger forms appear after long periods of storage of the monomeric enzyme and they can be reconverted to enzymatically active monomeric forms by treatment with thiol reagents (Y. Arakane *et al.*, unpublished data).

4.3.4.5. Modular Structure of Insect Chitinases

A multidomain structural organization is generally observed in polysaccharide-degrading enzymes where one or more domains are responsible for hydrolysis and other domains are responsible for associating with the solid polysaccharide substrate. In addition, there usually are linker regions between the two types of domains, which also may be responsible, at least in part, for some functional properties of the enzymes. For example, the first chitinases shown to contain catalytic, linker, and chitin-binding or fibronectin-like domains were isolated from the bacterium *Bacillus circulans* (Watanabe *et al.*, 1990), the yeast *S. cereviseae* (Kuranda and Robbins, 1991), and the parasitic nematode *B. malayi* (Venegas *et al.*, 1996). Insect chitinases possess a similar structural organization, as do some other nematode, microbial, and plant chitinases as well as fungal cellulases. Observed in all of these enzymes is a multidomain architecture that may include a signal peptide and one or more of the following domains: catalytic domains, cysteine-rich chitin-binding domains, fibronectin-like domains, mucin-like domains, and S/T-rich linker domains, with the latter usually being rather heavily glycosylated (Tellam, 1996; Henrissat, 1999; Suzuki *et al.*, 1999). For example, chitinases from the bacterium *S. marcescens*, fall into three classes with sizes ranging from 36 to 52 kDa, which are composed of different combinations of catalytic domains, fibronectin type-III-like domains, and N- or C-terminal chitin-binding domains (Suzuki *et al.*, 1999). A novel multidomain structure exhibited by an insect chitinase is that of the yellow mealworm beetle, *T. molitor* (Royer *et al.*, 2002). This protein is unusually large, with a calculated molecular mass of approximately 320 kDa. It contains five catalytic domains, five S/T-rich linker domains, four chitin-binding domains, and two mucin-like domains. Gene duplication and domain deletion mechanisms have probably generated the diversity and multiplicity of chitinase genes in insects, as was demonstrated previously in bacteria (Saito *et al.*, 2003).

The structure of the catalytic domain of insect chitinase is a $(\beta\alpha)_8$ TIM (triose phosphate isomerase) barrel fold, which is one of the most common folds found in proteins (Nagano *et al.*, 2001, 2002). During protein evolution, domain shuffling has allowed this fold to acquire a large number of specific catalytic functions such as enzymes with a glycosidase activity like insect chitinase. The presence of additional domains such as linker and chitin-binding domains appears to further enhance the catalytic properties of these enzymes.

Figure 4 shows a phylogenetic tree of 16 insect chitinases inferred from an amino acid sequence alignment. All five of the lepidopteran enzymes and only one dipteran chitinase reside in the upper portion of the tree, whereas the other seven dipteran, one hymenopteran, and two coleopteran enzymes appear in the lower part.

Manduca sexta CHI is much smaller than the *Tenebrio* enzyme and much less complex in domain structure with only a single N-terminal catalytic domain (376 amino acids long), a linker domain (about 100 amino acids long), and a C-terminal chitin-binding domain (ChBD, 58 amino acids long) (Arakane *et al.*, 2003). Alternate domain arrangements occur in other glycosylhydrolases. For example, class I, class IV, and class VII plant chitinases contain an N-terminal ChBD and a G/P-rich linker preceding the catalytic domain (Raikhel *et al.*, 1993; Neuhaus, 1999), whereas fungal cellulases, like insect chitinase, possess a threonine/serine/proline-rich linker between the N-terminal catalytic domain and the C-terminal cellulose-binding domain (Srisodsuk *et al.*, 1993). The *Manduca* CHI linker region that is rich in T and S residues is also rich in P, D, and E residues, which qualifies it as a PEST sequence-containing protein according to Rogers *et al.* (1986). That composition suggested that insect chitinase might be rapidly degraded via the intracellular ubiquitin-conjugating enzymes/proteosome system, which recognizes the PEST

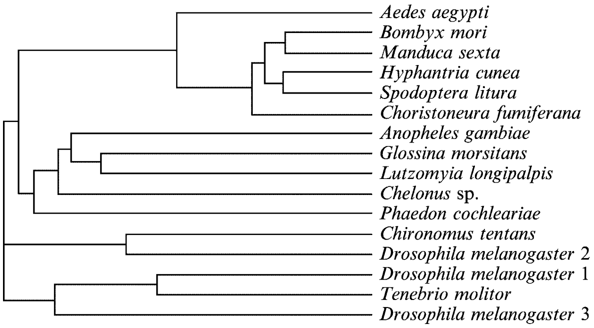


Figure 4 Phylogenetic tree of insect chitinases inferred from an amino acid sequence alignment of 16 enzymes from *Aedes aegypti*, *Bombyx mori*, *Manduca sexta*, *Hyphantria cunea*, *Spodoptera litura*, *Choristoneura fumiferana*, *Anopheles gambiae*, *Glossina morsitans*, *Lutzomyia longipalpis*, *Chelonus* sp., *Phaedon cochleariae*, *Chironomus tentans*, *Drosophila melanogaster*, and *Tenebrio molitor*. The GI numbers are listed in **Table 2**. Multiple sequence alignment was performed using Clustal W software (Thompson *et al.*, 1994) and the tree was built using the neighbor-joining method (Saitou and Nei, 1987).

sequence so that proteosomes can digest the conjugated protein when it is localized intracellularly. However, since insect chitinase is a secreted protein, it would be exposed to intracellular proteases or the ubiquitin-conjugating system only for a relatively short period of time. Instead, the linker apparently helps to optimize interactions with the insoluble substrates and to stabilize proteins, and perhaps also helps to protect protease-susceptible bonds in the catalytic domains from hydrolysis. Recombinant chitinases that contain this linker region were more stable in the presence of midgut digestive proteases than recombinant proteins lacking the linker region (Arakane *et al.*, 2003). The linker domain also may have another function involving protein trafficking. Recombinant forms of *Manduca* CHI lacking amino acid residues beyond position 376 accumulated intracellularly during expression in the baculovirus-insect cell line, whereas all of the forms that had an additional ten amino acids or longer stretches of the linker domain were secreted into the media (Arakane *et al.*, 2003). We concluded, therefore, that for secretion of recombinant protein to the outside of the insect cells to occur, the N-terminal portion of the linker region (residues 377–386) must be present, in addition to the 19 amino acid long N-terminal leader peptide. For secretion, the linker region may also need to be *O*-glycosylated because when glycosylation was inhibited by the addition of tunicamycin, insect chitinase accumulated intracellularly in an insect cell line (Gopalakrishnan *et al.*, 1995). Some of the critical residues for secretion/glycosylation, therefore, may involve residues between amino acids 376 and 386 (which includes two threonines) because the truncated Chi376 accumulated intracellularly, whereas Chi386 was secreted. Site-directed mutagenesis of these residues might help to answer the question about what residues in the linker region are required for secretion.

Peptides linking protein domains are very common in nature and some, unlike the insect chitinase linker, are believed to join domains rather passively without disturbing their function or affecting their susceptibility to cleavage by host proteases (Argos, 1990; Gilkes *et al.*, 1991). Linker peptides with G, T, or S residues are most common, perhaps because those residues are relatively small with G providing flexibility and T and S being uncharged but polar enough to interact with solvent or by their ability to hydrogen bond to water or to the protein backbone to achieve conformational and energetic stability. The interdomain linker peptide of a fungal cellobiohydrolase apparently has a dual role in providing the necessary distance between the two functional domains and also facilitating the dynamic adsorption process led by the cellulose-binding domain (Srisodsuk *et al.*, 1993). Solution conformation studies of a fungal cellulase with two domains revealed that its linker exhibited an extended conformation leading to maximum distance between the two domains and that heterogeneous glycosylation of the linker was likely a key factor defining its extended conformation (Receveur *et al.*, 2002). Since the domain structure of *M. sexta* CHI is similar to that of this fungal cellulase, these two enzymes may have similar global structural characteristics. Circular dichroism (CD) spectra of the wild-type and truncated insect chitinases were consistent with the hypothesis that whereas the catalytic and ChBDs possess secondary structure, the linker region itself does not (Arakane *et al.*, 2003).

Mammalian chitinase is similar in structure to *M. sexta* chitinase in both the catalytic domain and ChBD, but it lacks a linker domain (Tjoelker *et al.*, 2000). The absence of the ChBD does not affect the ability of the human enzyme to hydrolyze soluble oligosaccharides but does abolish hydrolysis of the insoluble substrate, a result consistent with the hypothesis that the function of the ChBD is to facilitate heterogeneous catalysis on insoluble substrates. One of the basic functions of carbohydrate-binding domains (CBD) is thought to be to help localize the enzyme on the insoluble substrate to enhance the efficiency of degradation (Linder and Teeri, 1997). These domains aid in recognition and hydrolysis of substrates that can exist in several physical states, i.e., contain both crystalline and noncrystalline forms. In general, for many glycosylhydrolases,

the binding specificity of the carbohydrate-binding domain mirrors that of the catalytic domain and these two domains are usually in relatively close association. Such is not the case for *Manduca* CHI, which has a very long linker of over several hundred angstroms.

Like their cognate catalytic domains, CBDs are classified into families of related amino acid sequences. The ChBD of insect chitinases belongs to carbohydrate-binding module family 14, which consists of approximately 70 residues (Coutinho and Henrissat, 1999; CAZY, 2004). Only three subfamilies of chitin-binding modules have been identified to date and the ChBD of *M. sexta* CHI is a member of subfamily 1 (Henrissat, 1999). Such a carbohydrate-binding function has been demonstrated in several other carbohydrolases and carbohydrate-binding proteins. Other CBD families, family 17 and family 28, both of which recognize cellulose, have been found to act in a cooperative manner either by modifying the action of the catalytic module or by targeting the enzyme to areas of cellulose that differ in susceptibility to hydrolysis (Boraston *et al.*, 2003). ChBDs may play a similar role in chitinases. These domains are attached not only to catalytic domains but also to chitinase-like proteins devoid of enzyme activity. The ChBDs can be either N- or C-terminal and may be present as a single copy or as multiple repeats. They are cysteine-rich and have several highly conserved aromatic residues (Shen and Jacobs-Lorena, 1999). The cysteine residues help to maintain protein folding by forming disulfide bridges and the aromatic residues interact with saccharides in the ligand-binding pocket. The PM proteins, mucins, which have affinity for chitin, also have a six-cysteine-containing peritrophin-A/mucin consensus sequence that is similar to ChBD sequences in chitinases (Tellam *et al.*, 1999; Morlais and Severson, 2001).

When fused with the catalytic domain of *M. sexta* CHI, both insect and rice ChBDs promoted the binding to and hydrolysis of chitin (Arakane *et al.*, 2003). The influence of extra substrate-binding domains has been examined previously using a fungal chitinase that was constructed to include plant and fungal carbohydrate-binding domains (Limón *et al.*, 2001). The addition of those domains increased the substrate-binding capacity and specific activity of the enzyme toward insoluble substrates of high molecular mass such as ground chitin or chitin-rich fungal cell walls. On the other hand, removal or addition of cellulose-binding domains can reduce or enhance, respectively, the ability of cellulases to degrade crystalline cellulose (Chhabra and Kelly, 2002). When a second cellulose-binding domain was fused to *Trichoderma reesei* cellulase, the resulting protein had a much higher affinity for cellulose than the protein with only a single binding domain (Linder *et al.*, 1996). Likewise, the *M. sexta* CHI catalytic domain fused with two ChBDs associated with chitin more strongly than any of the single ChBD-containing proteins or the protein devoid of a ChBD (Arakane *et al.*, 2003). This domain apparently helps to target the secreted enzyme to its insoluble substrate.

The chitin-binding domain of insect chitinase not only has the function of associating with insoluble chitin, but it may also help to direct the chitin chain into the active site of the catalytic domain in a manner similar to the processive hydrolysis mechanism proposed for *S. marcescens* chitinase A (ChiA), which has a very short ChBD (Uchiyama *et al.*, 2001). However, whether such an extended linker like that of *M. sexta* chitinase can direct the substrate into the active site in a manner similar to that proposed for a shorter linker is unknown. Catalytically, the full-length *M. sexta* CHI was two- to fourfold more active in hydrolyzing insoluble colloidal chitin than any of the other truncated enzymes with an intact catalytic domain, but all of the enzymes were comparable in turnover rate when two soluble substrates, carboxymethyl-chitin-remazol-brilliant-violet (CM-chitin-RBV), which is a chromogenic chitin derivative that is *O*-carboxymethylated, and MU-$(GlcNAc)_3$, a fluorogenic oligosaccharide substrate, were hydrolyzed (Arakane *et al.*, 2003). A moderate increase in catalytic efficiency of hydrolysis of insoluble substrate was observed when the catalytic domain was fused with the ChBD. When the C-terminal ChBD was deleted from a bacterial chitinase (*Aeromonas caviae*), this truncated chitinase was active also, but it liberated longer oligosaccharide products than did the full-length enzyme (Zhou *et al.*, 2002). Thus, as was observed with other carbohydrolases such as xylanases (Gill *et al.*, 1999), the ChBD of insect chitinase facilitates hydrolysis of insoluble, but not soluble, substrates, and also influences the size of the oligosaccharide products generated. The linker region also can influence the functionality of the carbohydrate-binding domain. When a fungal cellulose-binding domain was fused with a fungal S/T-rich linker peptide, the fusion protein adsorbed to both crystalline and amorphous cellulose. However, deletion of the linker peptide caused a decrease in cellulose adsorption and a higher sensitivity to protease digestion (Quentin *et al.*, 2002). The addition of a carbohydrate-binding module to a catalytic domain via a linker domain may increase the catalytic efficiency for degradation of the insoluble

polysaccharide and may modify the finely tuned binding specificity of the enzyme (McLean *et al*., 2002; Lehtio *et al*., 2003).

Figure 5 shows a theoretical model structure for *M. sexta* chitinase that is complexed with chitin oligosaccharides in both the catalytic domain and ChBD at a time subsequent to hydrolysis of a larger oligosaccharide. What is perhaps most striking is the very long linker (>200 Å) between the other domains. Apparently, the enzyme is tethered to the cuticle by the ChBD, which anchors the catalytic domain to the insoluble substrate and localizes the hydrolysis of chitin to an area with a radius of several hundred angstroms. The use of such a tethered enzyme would help to prevent diffusion of the soluble enzyme from the insoluble polysaccharide. In the case of *Tenebrio* chitinase, which consists of five catalytic, five linker, and six chitin-binding domains (Royer *et al*., 2002), one could envision a situation where a much wider area of the chitin–protein matrix undergoes intensive degradation by a much larger tethered enzyme.

4.3.4.6. Mechanism of Catalysis

Insect chitinases are members of family 18 of the glycosylhydrolases (CAZY, 2004), which generally utilize a mechanism where retention of the anomeric configuration of the sugar donor occurs via a substrate-assisted catalysis, rather than a mechanism similar to lysozyme, which involves a proton donor and an electrostatic stabilizer (Fukamizo, 2000). However, a recent kinetic study of bacterial family 18 chitinases demonstrated that substrates lacking the *N*-acetyl group and thus incapable of anchimeric assistance were nevertheless hydrolyzed, suggesting that the reaction mechanism of family 18 chitinases cannot be fully explained by the substrate-assisted catalysis model (Honda *et al*., 2003). Therefore, additional studies are still required to understand fully the reaction mechanism of family 18 chitinases.

The interaction of insect chitinases with insoluble chitin in the exoskeleton and PM is rather complex and believed to be a dynamic process that involves adsorption via a substrate-binding domain, hydrolysis, desorption, and repositioning of the catalytic domain on the surface of the substrate. This degradative process apparently requires a coordinated action of multiple domains by a mechanism that is not well understood. In addition to the catalytic events, the mechanism of binding of the enzyme onto the heterogeneous surface of native chitin is poorly characterized. Hydrolysis of chitin to GlcNAc is accomplished by a binary enzyme system composed of a chitinase and a β-*N*-acetylglucosaminidase (Fukamizo and Kramer, 1985;

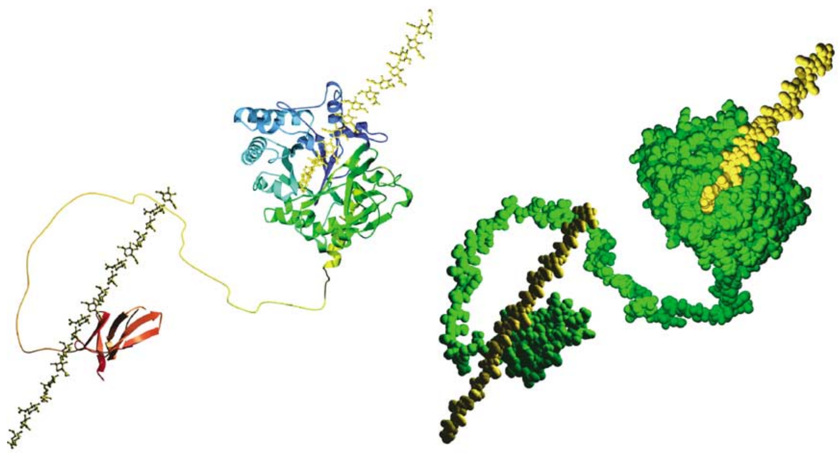


Figure 5 Ribbon (left) and space-filling (right) model structures of *Manduca sexta* chitinase with the catalytic and chitin-binding domains shown in complexes with chitin oligosaccharides (yellow). In the ribbon representation, the polypeptide chain is color-coded, beginning with blue at the N-terminus and proceeding through the rainbow to red at the C-terminus. The catalytic domain structure (top) was modeled using the program SOD (Kleywegt *et al*., 2001) with human chitotriosidase (PDB entry code 1LG1) (Fusetti *et al*., 2002) serving as the template. The chitin-binding domain (bottom) was similarly obtained using tachychitin (PDB entry 1DQC) (Suetake *et al*., 2000) as the template. The linker region is shown as a random coil as predicted by secondary structure prediction software and supported by circular dichroism data. The oligosaccharides are shown as stick models (left) and space-filling models (right). Substrate binding to the catalytic domain was modeled using the available structures of complexes from glycosyl hydrolase family 18, while binding to the chitin-binding domain was modeled based on sequence conservation within the subfamily. *M. sexta* chitinase is a glycoprotein that is glycosylated especially in the linker region; however, no carbohydrate is shown in the model. (The model was constructed by Wimal Ubhayasekera and Dr. Sherry Mowbray, Swedish University of Agricultural Sciences, Uppsala.)

Filho *et al.*, 2002). The former enzyme hydrolyzes the insoluble polymer into soluble oligosaccharides, whereas the latter further degrades the oligomers to the monomer from the nonreducing end. Mechanistically, chitinases of family 18 hydrolyze chitin with retention of the anomeric configuration at the cleavage site, involving a double-displacement mechanism where a substrate-assisted catalysis occurs (Tomme *et al.*, 1995; Henrissat, 1999; Zechel and Withers, 2000; Brameld *et al.*, 2002). *B. mori* chitinase utilizes a retaining mechanism, yielding products that retain the β-anomeric configuration (Abdel-Banat *et al.*, 1999). All of the enzymes of this family are inhibited by allosamidin, a transition state analog inhibitor which apparently is diagnostic for enzymes that utilize the retaining mechanism (Koga *et al.*, 1987; Bortone *et al.*, 2002; Brameld *et al.*, 2002; Lu *et al.*, 2002). Analysis of the products from the hydrolysis of chitin oligosaccharides by the family 18 chitinase from *S. marcescens* revealed variable subsite binding preferences, anomeric selectivity, and the importance of individual binding sites for the processing of short oligosaccharides compared to the cumulative recognition and processive hydrolysis mechanism used to digest the polysaccharide (Aronson *et al.*, 2003).

Polysaccharide-hydrolyzing enzymes are known to exhibit nonideal kinetic behavior because they often are susceptible to inhibition by both substrates and products (Väljamäe *et al.*, 2001). All insect chitinases examined were found to be susceptible to inhibition by oligosaccharide substrates but to varying extents (Fukamizo and Kramer, 1985; Fukamizo *et al.*, 1995; Fukamizo, 2000). Apparently, the oligosaccharide substrate molecules can bind to these enzymes in such a manner that none of the target bonds is properly exposed to the functional groups of catalytic amino acids or the substrate may bind in only noncatalytic subsites of the larger active site, forming nonproductive instead of productive complexes. Cellulose is also degraded by the synergistic action of cellulolytic enzymes, which also display this characteristic substrate inhibition (Väljamäe *et al.*, 2001). Site-directed mutagenesis studies involving amino acids present in the putative catalytic site of *M. sexta* CHI have identified residues required for catalysis (Huang *et al.*, 2000; Lu *et al.*, 2002; Zhang *et al.*, 2002). Aspartic acids 142 and 144, tryptophan 145, and glutamic acid 146 were identified as residues very important for catalysis and also for extending the pH range of enzyme activity into the alkaline pH range. Acidic and aromatic residues in other family 18 chitinases also are important for substrate binding and catalysis (Watanabe *et al.*, 1993, 1994; Uchiyama *et al.*, 2001; Bortone *et al.*, 2002). Some of these residues are essential only for crystalline chitin hydrolysis, whereas others are important not only for crystalline chitin hydrolysis but for other substrates as well (Watanabe *et al.*, 2003).

4.3.4.7. Glycosylation of Insect Chitinases

Manduca sexta CHI is moderately *N*-glycosylated in the catalytic domain and heavily *O*-glycosylated in the linker region (Arakane *et al.*, 2003). The insect cell line TN-5B1-4 (Hi 5), which is routinely used for expression of recombinant foreign glycoprotein, synthesizes proteins with both *N*- and *O*-linked oligosaccharides (Davidson *et al.*, 1990; Davis and Wood, 1995; Jarvis and Finn, 1995; Hsu *et al.*, 1997). Results of experiments investigating the effects of the *N*-glycosylation inhibitor tunicamycin on recombinant expression of insect chitinases in these cells indicated that the proteins were glycosylated prior to being secreted by the cells (Gopalakrishnan *et al.*, 1995; Zheng *et al.*, 2002). Direct chemical and enzymatic analyses confirmed that *M. sexta* CHI was both *N*- and *O*-glycosylated. Prolonged deglycosylation with a mixture of *N*- and *O*-glycosidases resulted in a protein that was smaller by about 6 kDa accounting for about 30 sugar residues per mole of protein (Arakane *et al.*, 2003). Because *N*-linked oligosaccharides in insects typically have six or seven residues, two of which are GlcNAc (Paulson, 1989; Kubelka *et al.*, 1995), the best estimate of the distribution of *N*-glycosylation indicated a single or possibly two sites of *N*-glycosylation in the catalytic domain and *O*-glycosylation of between 10 and 20 serine or threonine residues in the linker region. *O*-glycosylation may involve mainly addition of galactose and *N*-acetylgalactosamine.

The chitinase from *B. mori* also is probably glycosylated because this protein and its breakdown product (65 kDa) stain with periodic acid–Schiff reagent. Further, the apparent mobility of the protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is 88 kDa, whereas the molecular weight of the mature protein predicted from the cDNA sequence is only 60 kDa (Koga *et al.*, 1997). This protein has an S/T-rich linker similar to the *M. sexta* chitinase. On the other hand, the chitinase from wasp venom which has only a short linker region and is low in serine and threonine has nearly the same molecular weight as the one predicted from the cDNA sequence, suggesting that this protein may not be glycosylated (Krishnan *et al.*, 1994). Thus, there is a good

correlation between the presence of an S/T-rich linker and extensive glycosylation (predominantly *O*-glycosylation) of the chitinolytic proteins.

Glycosylation of the linker region may help to prevent proteolytic cleavage(s) at sites between the catalytic and chitin-binding domains. Such a functional role of glycosylated regions has been observed in some bacterial cellulases (Langsford *et al.*, 1987). The full-length and near full-length *O*-glycosylated forms of *M. sexta* CHI were the most stable proteins when incubated with the midgut proteinases of the hornworm (Arakane *et al.*, 2003). Protein modeling studies using the crystal structures of other family 18 glycosylhydrolases as templates suggested that the catalytic domain of *M. sexta* CHI has a $(\beta\alpha)_8$-triose phosphate isomerase (TIM) barrel structure (Kramer and Muthukrishnan, 1997; Nagano *et al.*, 2002). The ChBD probably exhibits a multistranded β-sheet structure based on similarity to tachycitin (Suetake *et al.*, 2000). We know of no structures computed or proposed for linker domains, which may be very hydrophilic and rather flexible as well as potentially susceptible to proteolytic degradation unless they are protected by glycosylation. The CD spectrum of the linker domain was consistent with the lack of any secondary structure in this domain (**Figure 5**). It is conceivable that during the developmental period of maximum chitinase activity, the enzyme is fully glycosylated. When required, a glycosidase(s) could be produced that would remove sugar residues, thus exposing several more peptide bonds for proteolytic cleavage. Alternatively, proteolytic cleavage may be reduced because of glycosylation. Consistent with this notion is the finding that analysis of molting fluid from *M. sexta* and *B. mori* revealed the presence of truncated forms of catalytically active chitinases with sizes ranging from 50 to 60 kDa (Kramer and Koga, 1986; Koga *et al.*, 1997; Abdel-Banat *et al.*, 1999). We also detected similar truncated forms in our insect cell recombinant chitinase expression system, especially several days subsequent to infection with the recombinant baculovirus (Gopalakrishnan *et al.*, 1995).

4.3.4.8. Antigenicity of Insect Chitinases

Invertebrate chitinases have been reported to elicit allergies in mammals. For example, a high prevalence of IgE antibodies to a tick chitinase was identified in canine atopic dermatitis with the chitinase formally designated Der f 15 (McCall *et al.*, 2001). In ticks, this chitinase was localized in the proventriculus and intestine, indicating that it has a digestive, rather than molting-related, function. Like insect chitinase, tick chitinase is extensively *O*-glycosylated on multiple sites along the 84 amino acid long S/T-rich sequence in the molecule. The transmission blocking antibody MF1 from the blood of gerbils infected with the nematode *B. malayi* was found to be directed against a microfilarial chitinase (Fuhrman *et al.*, 1992). This antibody mediates the clearance of peripheral microfileremia in gerbils, indicating that chitinase is indeed a potent antigen. Even though it is unclear which region of the nematode chitinase is highly antigenic, the most probable one is the S/T–rich region known to be *O*-glycosylated.

The primary epitope recognized by antibodies elicited by *Manduca* chitinases is the highly glycosylated S/T-rich linker region (Arakane *et al.*, 2003). Other highly immunogenic insect proteins that also are extensively *O*-glycosylated in S/T-rich domains similar to the linker region of *Manduca* CHI are peritrophins-55 and -95 from the sheep blowfly, *L. cuprina* (Tellam *et al.*, 2000, 2003). The sera of sheep vaccinated with these peritrophins exhibited a strong immune response that also inhibited growth of blowfly larvae (Casu *et al.*, 1997; Tellam *et al.*, 2003).

4.3.4.9. Other Possible Enzymes of Chitin Metabolism

Chitin deacetylases and chitosanases are two other enzymes that play major roles in chitin catabolism in other types of organisms. Chitin deacetylase catalyzes the removal of acetyl groups from chitin. This enzyme is widely distributed in microorganisms and may have a role in cell wall biosynthesis and in counteracting plant defenses (Tsigos *et al.*, 2000). There is one report of an insect chitin deacetylase in physogastric queens of the termite *Macrotermetes estherae* (Sundara Rajulu *et al.*, 1982). However, there have been no follow-up studies about this enzyme in other insect species. To our knowledge, there are no reports of chitosanases present in insects.

4.3.5. Nonenzymatic Proteins That Bind to Chitin

There are approximately 32 families of CBDs that are defined as contiguous amino acid sequences within a carbohydrate-active enzyme or noncatalytic analogs, which exhibit a discrete fold having carbohydrate-binding activity (CAZY, 2004). One or more members in families 1, 2, 3, 5, 12, 14, 16, 18, and 19 are reported to bind chitin. Most, if not all, of the insect ChBDs, however, belong only to family 14.

Several chitinase-related proteins have been identified in insects, which are catalytically inactive because they are missing an amino acid residue critical for hydrolytic activity but nonetheless are carbohydrate-binding proteins with either a single copy or multiple repeats of ChBDs. These proteins may act as growth factors or play a defensive function as anti-inflammatory proteins. A chitinase homolog glycoprotein HAIP (hemolymph aggregation inhibiting protein) occurs in hemolymph of the lepidopteran *M. sexta*, which inhibits hemocyte aggregation (Kanost *et al.*, 1994). A similar immunoreactive protein was detected in hemolymph of three other lepidopterans, *B. mori*, *Heliothis zea*, and *Galleria mellonella*. These proteins may have a role in modulating adhesion of hemocytes during defensive responses. Another glycoprotein, Ds47, which is produced *in vitro* by a *Drosophila* embryo-derived cell line and by fat body and hemocytes, may play a role in promoting the growth of imaginal discs (Kirkpatrick *et al.*, 1995; Bryant, 2001). Another chitinase-related protein is induced together with a chitinase and β-*N*-acetylglucosaminidase by ecdysteroid in the anterior silk gland of *B. mori* at molting and at metamorphosis (Takahashi *et al.*, 2002). The former is rather large in size and has a novel structure consisting of tandemly repeated catalytic domain-like plus linker sequences, but it has only one ChBD located in the middle of the protein. All of these proteins are evolutionarily related to chitinases, but they apparently have acquired a new growth-promoting or infection-resistance function that does not require catalytic activity. Evidently, chitinases have evolved into these lectin-like proteins by mutation of key residues in the active site, which abolishes enzyme activity and fine tunes the ligand-binding specificity.

Chitin-binding proteins in vertebrates, invertebrates, and plants share a common structural motif composed of one to eight disulfide bonds and several aromatic residues, apparently the result of convergent evolution (Shen and Jacobs-Lorena, 1999; Suetake *et al.*, 2000). A chitin-binding antifungal peptide from the coconut rhinoceros beetle, *Oryctes rhinoceros*, scarabaecin, is only 36 residues in length and contains only one disulfide bond (Hemmi *et al.*, 2003). It shares significant tertiary structural similarity with ChBDs of other invertebrates and plants that have multiple disulfide bonds, even though there is no overall sequence similarity. Other invertebrate proteins that contain one or more ChBDs include the peritrophins (Tellam *et al.*, 1999), mucins (Casu *et al.*, 1997; Wang and Granados, 1997; Tellam *et al.*, 1999; Rayms-Keller *et al.*, 2000; Sarauer *et al.*, 2003), and tachycitin (Suetake *et al.*, 2000).

Other proteins that bind to chitin include several lectins and cuticular proteins (see **Chapter 4.2**). The lectins are related to ChBDs found in PM and chitinases. Many insect cuticular proteins contain an amino acid sequence motif of approximately 35 residues known as the R&R consensus sequence (Rebers and Willis, 2001). This sequence, however, has no similarity to the cysteine-rich ChBDs found in chitinases, some PM proteins, and lectins. There are no or very few cysteine residues in the cuticular protein ChBDs (noncysChBD). Thus, there are two distinct classes of invertebrate ChBDs, those with the chitin-binding domain found in lectins, chitinases, and PM proteins (cysChBDs) and those with the cuticular protein chitin-binding domain (noncysChBDs). Homology modeling of insect cuticle proteins using the bovine plasma retinol binding protein as a template predicted an antiparallel β-sheet half-barrel structure as the basic folding motif where an almost flat surface consisting of aromatic amino acid side chains interacts with the polysaccharide chains of chitin (Hamodrakas *et al.*, 2002).

In mammals there are several nonenzymatic members of the chitinase protein family. Oviduct-specific glycoprotein (OGP), a member of this family, is believed to be involved in the process of fertilization such as sperm function and gamete interactions (Araki *et al.*, 2003). However, OGP was not essential for *in vitro* fertilization in mice, and so the functionality of OGP remains unknown. The human cartilage protein HCgp-39 is a chitin-specific lectin (Renkema *et al.*, 1998; Houston *et al.*, 2003) that is overexpressed in articular chondrocytes and certain cancers. It is thought to be an anti-inflammatory-response protein and/or to play a role in connective tissue remodeling. In contrast to chitinases, which bind and hydrolyze chitin oligosaccharides but do not undergo large conformational changes, HCgp-39 exhibits a large conformational change upon ligand binding, which appears to signal the presence of chitinous pathogens such as fungi and nematodes (van Aalten, 2003). The murine *Ym1* gene belongs to a family of mammalian genes encoding nonenzymatic proteins that are homologous to the chitinases from lower organisms, such as insects, nematodes, bacteria, and plants (Sun *et al.*, 2001). YKL-40 is a nonenzymatic member of the mammalian family 18 glycosylhydrolases, which is a growth factor for connective tissue cells and stimulates migration of endothelial cells (Johansen *et al.*, 2003). It is secreted in large amounts by

human osteosarcoma cells and murine mammary tumors, and it is also elevated in patients with metastatic breast cancer and colorectal cancer. These homologous mammalian proteins have no demonstrable chitinase activity and, therefore, cannot be considered chitinases. The biological functions of these proteins remain obscure. However, these proteins likely function through binding to carbohydrate polymers and since they are secreted from activated hemocytes, they may have a function in immunity such as a hemocyte inhibition (Falcone *et al.*, 2001). Sequence comparison of these nonenzymatic and enzymatic proteins indicates that the enzymatic proteins have evolved into these lectins by the mutation of key residues in the active site and optimization of the substrate-binding specificity (Fusetti *et al.*, 2002).

4.3.6. Regulation of Chitin Degradation

The *M. sexta* chitinase and *N*-acetylglucosaminidase genes were shown to be upregulated by ecdysteroid (see **Chapter 3.5**) and down-regulated by the juvenile hormone mimic (see **Chapter 3.7**), phenoxycarb, in larval abdomens cut off from their hormonal sources (Fukamizo and Kramer, 1987; Koga *et al.*, 1991; Kramer *et al.*, 1993; Zen *et al.*, 1996). Differential display was used to show that chitinase expression was regulated not only by ecdysteroid but also by juvenile hormone in the beetle *T. molitor* (Royer *et al.*, 2002). Northern blot analysis of RNA from epidermis and 20-hydroxyecdysone-injected pupae showed that chitinase transcripts were correlated with molting hormone levels during metamorphosis. In addition, topical application of a juvenile hormone (JH) analog indirectly induced expression of chitinase mRNA. Thus, the *Tenebrio* chitinase gene is an early direct ecdysteroid-responsive one at the transcriptional level, but unlike *M. sexta* chitinase, it is apparently a direct target of JH as well. In the former case, the level at which JH regulates chitinase mRNA levels remains to be determined. The 20-hydroxyecdysone agonist, tebufenozide, induced expression of *C. fumiferana* chitinase when it was injected into mature larvae. The enzyme was produced 24 h post treatment in both the epidermis and molting fluid (Zheng *et al.*, 2003).

4.3.7. Chitin Metabolism and Insect Control

Chitinases have been used in a variety of ways for insect control and other purposes (Kramer *et al.*, 1997; Gooday, 1999). Several chitinase inhibitors with biological activity have been identified based on natural products chemistry (Spindler and Spindler-Barth, 1999), such as allosamidin (Rao *et al.*, 2003) which mimics the carbohydrate substrate, and cyclic peptides (Houston *et al.*, 2002). Although useful for biochemical studies, none of these chitin catabolism inhibitors have been developed for commercial use primarily because of their high cost of production and potential side effects. As we learn more details about chitinase catalysis, it might become more economically feasible to develop and optimize chitinase inhibitors for insect pest management.

Additional uncharacterized steps in chitin synthesis and/or assembly of chitin microfibrils, on the other hand, have proved to be important for developing control chemicals that act selectively on economically important groups of insect pests (Verloop and Ferrell, 1977; Ishaaya, 2001). The benzoylphenylureas have been developed as commercial compounds for controlling agricultural pests. These antimolting insecticides are relatively nontoxic to mammals due to their strong protein binding and extensive metabolization to less toxic compounds (Bayoumi *et al.*, 2003). Studies using imaginal discs and cell-free systems indicated that benzoylphenylureas inhibit ecdysteroid-dependent GlcNAc incorporation into chitin (Mikolajczyk *et al.*, 1994; Oberlander and Silhacek, 1998). Those results suggest that benzoylphenylureas affect ecdysone-dependent sites, which leads to chitin inhibition. However, the site of action of the benzoylphenylureas still is not well known. Recently, several heteryl nucleoside nonhydrolyzable transition state analogs of UDP-GlcNAc were synthesized and evaluated for fungicidal activity, but they were not assayed for insecticidal activity (Behr *et al.*, 2003).

Entomopathogens secrete a plethora of extracellular proteins with potential activity in insect hosts. One of these proteins is chitinase, which is used by fungi such as *Metarhizium anisopliae* to help penetrate the host cuticle and render host tissues suitable for consumption (St. Leger *et al.*, 1996; Krieger de Moraes *et al.*, 2003). Among the 10 most frequent transcripts in a strain of *M. anisopliae* are three encoding chitinases and one a chitosanase, presumably reflecting a greater propensity to produce chitinases for host cuticle penetration (Freimoser *et al.*, 2003a). Expressed sequence tag analysis of *M. anisopliae* may hasten gene discovery to enhance development of improved mycoinsecticides. However, when *M. anisopliae* was transformed to overexpress its native chitinase, the pathogenicity to the tobacco hornworm was unaltered, suggesting that

wild-type levels of chitinase are not limiting for cuticle penetration (Screen *et al.*, 2001). Another fungal species, *Conidiobolus coronatus*, also produces both endo- and exo-acting chitinolytic enzymes during growth on insect cuticle (Freimoser *et al.*, 2003b). Apparently, both *M. anisopliae* and *C. coronatus* produce a chitinolytic enzyme system to degrade cuticular components.

Both microbial and insect chitinases have been shown to enhance the toxicity of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) (Regev *et al.*, 1996; Tantimavanich *et al.*, 1997; Ding *et al.*, 1998; Sampson and Gooday, 1998; Wiwat *et al.*, 2000). For example, when the chitinolytic activities of several strains of *B. thuringiensis* were compared with their insecticidal activity, it was determined that the enzyme could enhance the toxicity of Bt to *Spodoptera exigua* larvae by more than twofold (Liu *et al.*, 2002). Microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic microorganisms (review: Kramer *et al.*, 1997). Synergistic effects between chitinolytic enzymes and microbial insecticides have been reported as early as the 1970s. Bacterial chitinolytic enzymes were first used to enhance the activity of Bt and a baculovirus. Larvae of *C. fumiferana* died more rapidly when exposed to chitinase–Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff and Valero, 1972; Morris, 1976; Lysenko, 1976). Mortality of gypsy moth, *Lymantria dispar*, larvae was enhanced when chitinase was mixed with Bt relative to a treatment with Bt alone in laboratory experiments (Dubois, 1977). The toxic effect was correlated positively with enzyme levels (Gunner *et al.*, 1985). The larvicidal activity of a nuclear polyhedrosis virus toward *L. dispar* larvae was increased about fivefold when it was administered with a bacterial chitinase (Shapiro *et al.*, 1987). Chitin synthesis-inhibiting antifungal agents such as flufenoxuron and nikkomycin were used to promote the infection of silkworms with *B. mori* nucleopolyhedrovirus (Arakawa, 2002, 2003; Arakawa and Sugiyama, 2002; Arakawa *et al.*, 2002). The mechanism of viral infection enhancement by these agents is not established, but it may involve destruction of PM structure, which would facilitate tissue invasion.

Inducible chitinolytic enzymes from bacteria cause insect mortality under certain conditions. These enzymes may compromise the structural integrity of the PM barrier and improve the effectiveness of Bt toxin by enhancing contact of the toxin molecules with their epithelial membrane receptors. For example, five chitinolytic bacterial strains isolated from midguts of *Spodoptera littoralis* induced a synergistic increase in larval mortality when combined with Bt spore-crystal suspensions relative to either an individual bacterial strain or a Bt suspension alone (Sneh *et al.*, 1983). An enhanced toxic effect toward *S. littoralis* also resulted when a combination of low levels of a truncated recombinant Bt toxin and a bacterial endochitinase was incorporated into a semisynthetic insect diet (Regev *et al.*, 1996). Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* toward diamondback moth larvae (Wiwat *et al.*, 1996). Liu *et al.* (2002) recently reported that several strains of Bt produced their own chitinases, which had synergistic larvicidal activity with the endotoxins.

In biopesticide development research, we used a family 18 insect chitinase as an enhancer protein for baculovirus toxicity and as a host plant resistance factor in transgenic plants. Introduction of an insect chitinase cDNA into *A. californica* multiple nuclear polyhedrosis viral (AcMNPV) DNA accelerated the rate of killing of fall armyworm compared to the wild-type virus (Gopalakrishnan *et al.*, 1995). Baculoviral chitinases themselves play a role in liquefaction of insect hosts (Hawtin *et al.*, 1997; Thomas *et al.*, 2000). A constitutively expressed exochitinase from *B. thuringiensis* potentiated the insecticidal effect of the vegetative insecticidal protein Vip when they were fed to neonate larvae of *S. litura* (Arora *et al.*, 2003). Some granuloviruses, on the other hand, do not utilize chitinases in a similar manner, which helps to explain why some granulovirus-infected insects do not lyse at the end of the infection process (Wormleaton *et al.*, 2003). Mutagenesis of the AcMNPV chitinase gene resulted in cessation of liquefaction of infected *T. ni* larvae, supporting a role of chitinase in virus spread (Thomas *et al.*, 2000). However, the insecticidal activity of insect chitinase was not substantial enough for commercial development. We have attempted with little success to improve the catalytic efficiency and stability of this enzyme so that its pesticidal activity would be enhanced (Lu *et al.*, 2002; Zhang *et al.*, 2002; Arakane *et al.*, 2003). Nevertheless, tobacco budworms were killed when reared on transgenic tobacco expressing a truncated, enzymatically active form of insect chitinase (Ding *et al.*, 1998). We also discovered a synergistic interaction between insect chitinase expressed in transgenic tobacco plants and Bt (applied as a spray at sublethal levels) using the tobacco hornworm as the test insect. In contrast to results obtained with the tobacco budworm, studies with the hornworm revealed no consistent differences in larval growth or foliar damage when the insects were reared on

first-generation transgenic chitinase-positive tobacco plants as compared to chitinase-negative control plants. When Bt toxin was applied at levels where no growth inhibition was observed on control plants, chitinase-positive plants had significantly less foliar damage and lower larval biomass production. These results indicated that the insect chitinase transgene did potentiate the effect of sublethal doses of Bt toxin and vice versa (Ding *et al*., 1998). Tomato plants have been transformed with fungal chitinase genes with concomitant enhancement in resistance to insect pests (Gongora *et al*., 2001). Effects observed include reduced growth rates and increased mortality, as well as a decrease in plant height and flowering time with an increase in the number of flowers and fruits (Gongora and Broadway, 2002). Chitinase-secreting bacteria have been used to suppress herbivorous insect pests. A chitinase gene-transformed strain of *Enterobacter cloacae* digested the chitinous membranes of phytophagous ladybird beetles, *Epilachna vigintioctopunctata*, and also suppressed leaf-feeding and oviposition when the beetles ingested transformed bacteria entrapped in alginate microbeads sprayed on tomato seedlings (Otsu *et al*., 2003).

Several GlcNAc-specific lectins from plants have been evaluated for insect toxicity (Harper *et al*., 1998; Macedo *et al*., 2003). These proteins appear to disrupt the integrity of the PM by binding to chitin or glycan receptors on the surface of cells lining the insect gut. They also may bind to glycosylated digestive enzymes and inhibit their activity. Another type of plant chitin-binding protein is the seed storage protein, vicilin, which is actually a family of oligomeric proteins with variable degrees of glycosylation (Macedo *et al*., 1993; Shutov *et al*., 1995). Some vicilins are insecticidal to bruchid beetles and stalk borers (Sales *et al*., 2001; Mota *et al*., 2003). Apparently, these proteins bind to the PM, causing developmental abnormalities and reduced survival rates. To date no carbohydrate-binding protein derived from an insect has been evaluated for biocidal activity. A novel approach has been proposed to develop strategies for insect control by utilizing chitin-binding molecules to specifically target formation of the PM. Calcofluor, a chemical whitener with chitin-binding properties, was used as a model compound in the diet to inhibit PM formation in *T. ni* and also to increase larval susceptibility to baculovirus infection (Wang and Granados, 2000b). It also was effective in suppressing PM formation in *Spodoptera frugiperda* and at the same time in preventing the establishment of a decreasing gradient of proteinases along the midgut tissue (Bolognesi *et al*., 2001).

Another type of hydrolytic enzyme with a ChBD has been shown to exhibit insecticidal activity in plants. Maize accumulates a 33 kDa cysteine protease containing a ChBD in response to insect feeding (Perchan *et al*., 2002). This enzyme apparently damages the insect's PM by utilizing the ChBD to localize itself at the chitin-protein-rich PM, where the PM proteins are digested, rendering the PM dysfunctional. Another protease with a chitin-binding domain has been described from *A. gambiae*, which may be involved in insect defense (Danielli *et al*., 2000). This 147 kDa protein, sp22D, is expressed in a variety of tissues, most strongly in hemocytes, and is secreted into the hemolymph. Upon bacterial infection, the transcripts for this protein increase by about twofold suggesting a role in insect defense. This protein has a multidomain organization that includes two copies of an N-terminal ChBD, a C-terminal protease domain, and additional receptor domains. It binds strongly to chitin and undergoes complex proteolytic processing during pupal to adult metamorphosis. It has been proposed that exposure of this protease to chitin may regulate its activity during tissue remodeling or wounding.

Recently, two synthetic peptides were found to inhibit *A. gambiae* midgut chitinase and also to block sporogonic development of the human malaria parasite, *Plasmodium falciparum*, and avian malaria parasite, *P. gallinaceum*, when the peptides were fed to infected mosquitoes (Bhatnagar *et al*., 2003). The design of these peptides was based on the putative proregion sequence of mosquito midgut chitinase. The results indicated that expression of chitinase inhibitory peptides in transgenic mosquitoes might alter the vectorial capacity of mosquitoes to transmit malaria.

4.3.8. Concluding Remarks

Although chitin was discovered nearly two centuries ago, it remains a biomaterial in waiting because, unlike other natural materials such as collagen and hyaluronic acid, very few technological uses have been developed (Khor, 2002; Tharanathan and Kittur, 2003). There are many unanswered questions about chitin morphology and chitin deposition in the insect cuticle and PM. We do not know how or whether chitin forms covalent interactions with other components in these extracellular matrices. Chitosan, on the other hand, does react with quinones (Muzzarelli and Muzzarelli, 2002; Muzzarelli *et al*., 2003). Thus, if there were any free amino groups in insect chitin, C–N linkages between chitin and catechols would be expected (Schaefer *et al*.,

1987). We do not yet understand how factors such as metal ions affect chitin metabolism. In fungi, ions such as zinc were found to alter chitin deposition and morphology (Lanfranco *et al*., 2002). Perhaps, in insects there is an ionic effect on differential expression of CHS isozymes.

We know much more about insect chitinolytic enzymes than about insect chitin biosynthetic enzymes. Many questions remain about the biosynthesis of insect chitin, not the least of which are why insects have multiple genes for CHS, how many CHSs are required to make an insect, at what developmental stages are the various CHSs produced, and what are the unique properties and functions of each CHS. Of particular interest is the role of alternate splicing in generating different isoforms of CHSs from the same gene. The developmental cues that control alternate splicing and how they affect chitin synthesis and/or deposition will be subjects of future studies. The cloning of CHS genes should soon lead to availability of large amounts of recombinant enzymes or subdomains thereof using appropriate expression systems. Studies with pure proteins and the availability of molecular probes will provide a better understanding of the chitin biosynthetic pathway and its regulation in the future.

Two other major questions about insect chitin biosynthesis are: what is the mechanism of the initiation phase and is there an autocatalytic initiator. Like glycogen synthesis, chitin synthesis probably includes both initiation and elongation phases. As the initiator of glycogen synthesis, glycogenin transfers glucose from UDP-glucose to itself to form an oligosaccharide–protein primer for elongation (Gibbons *et al*., 2002). Like chitin synthase, glycogenin is a glycosyltransferase, which raises the question of whether chitin synthase has an autocatalytic function similar to glycogenin and whether there is a chitinogenin-like protein. Another possibility is the participation of a lipid primer for chitin synthesis. Recently, cellulose synthesis in plants was found to involve the transfer of lipid-linked cellodextrins to a growing glucan chain (Read and Bacic, 2002). The lipid in this case was sitosterol-β-glucoside.

Little is known about the catalytic mechanism of any insect CHS. Once insect CHS-related recombinant proteins are obtained, site-directed mutagenesis can be used to probe for essential residues in the catalytic and regulatory domains. It is likely that acidic amino acids play critical roles in CHS catalysis in a manner comparable to those identified in other glycosyltransferases (Hefner and Stockigt, 2003) and in yeast chitin synthases (Nagahashi *et al*., 1995).

Chitinolytic enzymes are gaining importance for their biotechnological applications in agriculture and healthcare (Patil *et al*., 2000). Additional success in using chitinases for different applications depends on a better understanding of their biochemistry and regulation so that their useful properties can be optimized through genetic and biochemical engineering. Reasons for the rather high multiplicity of domain structures for insect and other chitinases are not fully understood. So far little success has occurred in using chitinase in pest control applications, but it may prove more useful as an enhancer protein in a cocktail with other biopesticides targeted at the cuticle or gut. Also, only a few catalytic domains or chitin-binding domains or various combinations thereof have been evaluated for biocidal activity and thus, further toxicological experimentation is warranted.

Although substantial progress in studies of insect chitin metabolism has occurred since the first edition of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* was published in 1985, we still do not know much about how chitin is produced and transported across the membrane so that it can interact perfectly with other components for assembly of the supramolecular extracellular structures called the exoskeleton and PM. These materials are still very much biochemical puzzles in which we do not understand well how the various components come together during morphogenesis or are digested apart during the molting process. Hopefully, this chapter will stimulate more effort to understand how insects utilize chitin metabolism for growth and development, and to develop materials that may perturb insect chitin metabolism for pest management purposes.

Acknowledgments

The authors are grateful to Sherry Mowbray, Wimal Ubhayasekera, Yasuyuki Arakane, Qingsong Zhu, David Hogenkamp, Renata Bolognesi, Tamo Fukamizo, Daizo Koga, Walter Terra and Clelia Terra for their help with the preparation and/or comments on various aspects of this review. Supported in part by National Science Foundation grant no. 0316963. Mention of a proprietary product does not constitute a recommendation or endorsement by the US Department of Agriculture. The US Department of Agriculture is an equal opportunity/affirmative action employer and all agency services are available without discrimination. This is contribution no. 04-058-C of the Kansas Agricultural Experiment Station.

References

Abdel-Banat, B.M.A., Kameyama, Y., Yoshioka, T., Koga, D., **1999**. Purification and characterization of a 54 kDa chitinase from *Bombyx mori*. *Insect Biochem. Mol. Biol. 30*, 107–117.

Abdel-Banat, B.M.A., Koga, D., **2001**. A genomic clone for a chitinase gene from the silkworm, *Bombyx mori*: structural organization identifies functional motifs. *Insect Biochem. Mol. Biol. 31*, 497–508.

Abdel-Banat, B.M.A., Koga, D., **2002**. Alternative splicing of the primary transcript generates heterogeneity within the products of the gene for *Bombyx mori* chitinase. *J. Biol. Chem. 277*, 30524–30534.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Arakane, Y., Hogenkamp, D., Zhu, Y.C., Kramer, K.J., Specht, C.A., *et al.*, **2004**. Chitin synthase genes of the red flour beetle, *Tribolium castaneum*: characterization, expression, linkage mapping and alternate exon usage. *Insect Biochem. Mol. Biol. 34*, 291–304.

Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S., Kramer, K.J., **2003**. Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem. Mol. Biol. 33*, 631–648.

Arakawa, T., **2002**. Promotion of nucleopolyhedrovirus infection in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) by flufenoxuron. *Appl. Entomol. Zool. 37*, 7–11.

Arakawa, T., **2003**. Chitin synthesis inhibiting antifungal agents promote nucleopolyhedrovirus infection in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) larvae. *J. Invert. Pathol. 83*, 261–263.

Arakawa, T., Furuta, Y., Miyazawa, M., Kato, M., **2002**. Flufenoxuron, an insect growth regulator, promotes peroral infection by nucleopolyhedrovirus (BmNPV) budded particles in the silkworm, *Bombyx mori* L. *J. Virol. Methods 100*, 141–147.

Arakawa, T., Sugiyama, M., **2002**. Promotion of nucleopolyhedrovirus infection in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) by an antibiotic, nikkomycin Z. *Appl. Entomol. Zool. 37*, 393–397.

Araki, Y., Nohara, M., Yoshida-Komiya, H., Kuramochi, T., Ito, M., *et al.*, **2003**. Effect of a null mutation of the oviduct-specific glycoprotein gene on mouse fertilization. *Biochem. J. 374*, 551–557.

Argos, P., **1990**. An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion. *J. Mol. Biol. 211*, 943–958.

Aronson, N.N., Halloran, B.A., Alexyev, M.F., Amable, L., Madura, J.D., *et al.*, **2003**. Family 18 chitinase-oligosaccharide substrate interaction: subsite preference and anomer selectivity of *S. marcescens* chitinase A. *Biochem. J. 376*, 87–95. Immediate Publication, doi:10.1042/BJ20030273.

Arora, N., Ahmad, T., Rajagopal, R., Bhatnagar, R.K., **2003**. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1. *Biochem. Biophys. Res. Commun. 307*, 620–625.

Bayoumi, A.E., Rerez-Pertejo, Y., Zidan, H.Z., Balana-Fouce, R., Ordonez, C., *et al.*, **2003**. Cytotoxic effects of two antimolting insecticides in mammalian CHO-K1 cells. *Ecotoxicol. Environ. Safety 55*, 19–23.

Becker, A., Schloder, P., Steele, J.E., Wegener, G., **1996**. The regulation of insect metabolism in insects. *Experientia 52*, 433–439.

Behr, J.B., Gourlain, T., Helimi, A., Guillerm, G., **2003**. Design, synthesis and biological evaluation of heteryl-nucleoside derivatives as inhibitors of chitin synthase. *Biorgan. Medicinal Chem. Lett. 13*, 1713–1716.

Bhatnagar, R.K., Arora, N., Sachidanand, S., Shahabuddin, M., Keister, D., *et al.*, **2003**. Synthetic propeptide inhibits mosquito midgut chitinase and blocks sporogonic development of malaria parasite. *Biochem. Biophys. Res. Comm. 304*, 783–787.

Bolognesi, R., Ribeiro, A.F., Terra, W.R., Terra, C., **2001**. The peritrophic membrane of *Spodoptera frugiperda*: secretion of peritrophins and role in immobilization and recycling digestive enzymes. *Arch. Insect Biochem. Physiol. 47*, 62–75.

Boraston, A.B., Kwan, E., Chiu, P., Warren, A.J., Kilburn, D.G., **2003**. Recognition and hydrolysis of noncrystalline cellulose. *J. Biol. Chem. 278*, 6120–6127.

Bortone, K., Monzingo, A.F., Ernst, S., Robertus, J.D., **2002**. The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. *J. Mol. Biol. 320*, 293–302.

Brameld, K.A., Shrader, W.D., Imperiali, B., Goddard, W.A., **2002**. Substrate assistance in the mechanism of family 18 chitinases: theoretical studies of potential intermediates and inhibitors. *J. Mol. Biol. 280*, 913–923.

Bray, S.J., Kafatos, F.C., **1991**. Developmental function of *Elf-1*: an essential transcription factor during embryogenesis in *Drosophila*. *Genes Devel. 5*, 1672–1683.

Bryant, P., **2001**. Growth factors controlling imaginal disc growth in *Drosophila*. In: Bock, G., Cardew, G., Goode, J.A. (Eds.), The Cell Cycle and Development. John Wiley, New York, pp. 182–199.

Casu, R., Eisemann, C., Pearson, R., Riding, G., East, I., *et al.*, **1997**. Antibody-mediated inhibition of the growth of larvae from an insect causing cutaneous myiasis in a mammalian host. *Proc. Natl Acad. Sci. USA 94*, 8939–8944.

CAZY, **2004**. Carbohydrate-active enzymes family server: http://afmb.cnrs mrs.fr/~pedro/CAZY/db.html.

Chang, R., Yeager, A.R., Finney, N.S., **2003**. Probing the mechanism of fungal glycosyltransferase essential for cell wall synthesis: UDP-Chitobiose is not a substrate for chitin synthase. *Org. Biomol. Chem. 1*, 39–41.

Chhabra, S.R., Kelly, R.M., **2002**. Biochemical characterization of *Thermotoga maritima* endoglucanase Cel74

with and without a carbohydrate-binding module (CBM). *FEBS Lett. 531*, 375–380.

Choi, H.K., Choi, K.H., Kramer, K.J., Muthukrishnan, S., **1997**. Isolation and characterization of a genomic clone for the gene of an insect molting enzyme, chitinase. *Insect Biochem. Mol. Biol. 27*, 37–47.

Cohen, E., **1987**. Chitin biochemistry: synthesis and inhibition. *Annu. Rev. Entomol. 32*, 71–93.

Cohen, E., **2001**. Chitin synthesis and inhibition: a revisit. *Pest Mgt Sci. 57*, 946–950.

Cohen, E., Casida, J.E., **1980**. Inhibition of *Tribolium* gut chitin synthetase. *Insecticide Biochem. Physiol. 13*, 129–136.

Coutinho, P.M., Deleury, E., Davies, G.J., Henrissat, B., **2003**. An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol. 328*, 307–317.

Coutinho, P.M., Henrissat, B., **1999**. Carbohydrate-active enzymes: an integrated database approach. In: Gilbert, H.H., Davies, G.J., Henrissat, H., Svensson, B. (Eds.), Recent Advances in Carbohydrate Bioengineering. Royal Society of Chemistry, Cambridge, pp. 3–12.

Daimon, T., Hamada, K., Mita, K., Okano, K., Suzuki, M.G., *et al.*, **2003**. A *Bombyx mori* gene, *BmChi-h*, encodes a protein homologous to bacterial and baculovirus chitinases. *Insect Biochem. Mol. Biol. 33*, 749–759.

Danielli, A., Loukeris, T.G., Lagueux, M., Muller, H.M., Richman, A., *et al.*, **2000**. A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA 97*, 7136–7141.

Davidson, D.J., Fraser, M.J., Castellino, F.J., **1990**. Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochemistry 19*, 5584–5590.

Davis, T.R., Wood, H.A., **1995**. Intrinsic glycosylation potentials of insect cell lines and insect larvae. *In Vitro Cell. Devel. Biol. Anim. 31*, 659–663.

de la Vega, H., Specht, C.A., Liu, Y., Robbins, P.W., **1998**. Chitinases are a multi-gene family in *Aedes*, *Anopheles* and *Drosophila*. *Insect Mol. Biol. 7*, 233–239.

Ding, X., Gopalakrishnan, B., Johnson, L., White, F.F., Wang, X., *et al.*, **1998**. Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Res. 7*, 77–84.

Dubois, N.R., **1977**. Pathogenicity of selected resident microorganisms of *Lymantria dispar* after induction for chitinase. Ph.D. Dissertation, Univ. Massachusetts, Amherst.

Eisemann, C., Wijffels, G., Tellam, R.L., **2001**. Secretion of type 2 peritrophic matrix protein, peritrophin-15, from the cardia. *Arch. Insect Biochem. Physiol. 47*, 76–85.

Falcone, F.H., Loke, P., Zang, X., MacDonald, A.S., Maizels, R.M., *et al.*, **2001**. A *Brugia malayi* homolog of macrophage migration inhibitory factor reveals an important link between macrophages and eosinophil recruitment during nematode infection. *J. Immunol. 167*, 5348–5354.

Feix, M., Gloggler, S., Londershausen, M., Weidemann, W., Spindler, K.D., *et al.*, **2000**. A cDNA encoding a chitinase from the epithelial cell line of *Chironomus tentans* (Insecta, Diptera) and its functional expression. *Arch. Insect Biochem. Physiol. 45*, 24–36.

Filho, B.P.D., Lemos, F.J.A., Secundino, N.F.C., Pascoa, V., Pereira, S.T., *et al.*, **2002**. Presence of chitinase and beta-*N*-acetylglucosaminidase in the *Aedes aegypti* chitinolytic system involving peritrophic matrix formation and degradation. *Insect Biochem. Mol. Biol. 32*, 1723–1729.

Fly Base, **2004**. http://www.flybase.bio.indiana.edu/

Freimoser, F.M., Screen, S., Bagga, S., Hu, G., St. Leger, R.J., **2003a**. Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology 149*, 239–247.

Freimoser, F.M., Screen, S., Hu, G., St. Leger, R.J., **2003b**. EST analysis of genes expressed by the zygomycete pathogen *Conidiobolus coronatus* during growth on insect cuticle. *Microbiology 149*, 1893–1900.

Fuhrman, J.A., Lane, W.S., Smith, R.F., Piessens, W.F., Perler, F.B., **1992**. Transmission-blocking antibodies recognize microfilarial chitinase in brugian lymphatic filariasis. *Proc. Natl Acad. Sci. USA 89*, 1548–1552.

Fukamizo, T., **2000**. Chitinolytic enzymes: catalysis, substrate binding, and their application. *Curr. Protein Peptide Sci. 1*, 105–124.

Fukamizo, T., Koga, D., Goto, S., **1995**. Comparative biochemistry of chitinases: anomeric form of the reaction products. *Biosci. Biotech. Biochem. 59*, 311–313.

Fukamizo, T., Kramer, K.J., **1985**. Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. *Insect Biochem. 15*, 141–145.

Fukamizo, T., Kramer, K.J., **1987**. Effect of 20-hydroxyecdysone on chitinase and β-*N*-acetylglucosaminidase during the larval-pupal transformation in *Manduca sexta* (L.). *Insect Biochem. 17*, 547–550.

Fukamizo, T., Kramer, K.J., Mueller, D.D., Schaefer, J., Garbow, J., *et al.*, **1986**. Analysis of chitin structure by nuclear magnetic resonance spectroscopy and chitinolytic enzyme digestion. *Arch. Biochem. Biophys. 249*, 15–26.

Furukawa, S., Taniai, K., Yang, J., Shono, T., Yamakawa, M., **1999**. Induction of gene expression of antibacterial proteins by chitin oligomers in the silkworm, *Bombyx mori*. *Insect Mol. Biol. 8*, 145–148.

Fusetti, F., von Moeller, H., Houston, D., Rozeboom, H.J., Dijkstra, B.W., *et al.*, **2002**. Structure of human chitotriosidase: implications for specific inhibitor design and function of mammalian chitinase-like lectins. *J. Biol. Chem. 277*, 25537–25544.

Gagou, M.E., Kapsetaki, M., Turberg, A., Kafetzopoulos, D., **2002**. Stage-specific expression of the chitin synthase *DmeChSA* and *DmeChSB* genes during the onset of *Drosophila* metamorphosis. *Insect Biochem. Mol. Biol. 32*, 141–146.

Gaines, P.J., Brandt, K.S., Eisele, A.M., Wagner, W.P., Bozic, C.M., *et al.*, **2002**. Analysis of expressed

sequence tags from *Ctenocephalides felis* hindgut and Malpighian tubule cDNA libraries. *Insect Mol. Biol. 11*, 299–306.

Gibbons, B.J., Roach, P.J., Hurley, T.D., **2002**. Crystal structure of the autocatalytic initiator of glycogen biosynthesis, glycogenin. *J. Mol. Biol. 319*, 463–477.

Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., Warren, R.A.J., **1991**. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev. 55*, 303–315.

Gill, J., Rixon, J.E., Bolam, D.N., McQueen-Mason, S., Simpson, P.J., *et al.*, **1999**. The type II and X cellulose-binding domains of *Pseudomonas* xylanase A potentiate catalytic activity against complex substrates by a common mechanism. *Biochem. J. 342*, 473–480.

Girard, C., Jouanin, L., **1999**. Molecular cloning of a gut-specific chitinase cDNA from the beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol. 29*, 549–556.

Gongora, C.E., Broadway, R.M., **2002**. Plant growth and development influenced by transgenic insertion of bacterial chitinolytic enzymes. *Mol. Breeding 9*, 123–135.

Gongora, C.E., Wang, S., Barbehenn, R.V., Broadway, R.M., **2001**. Chitinolytic enzymes from *Streptomyces albidoflavus* expressed in tomato plants: effects on *Trichoplusia ni*. *Entomol. Experimentia Appl. 99*, 193–204.

Goo, T.-W., Hwang, J.-S., Sung, G.-B., Yun, E.-Y., Bang, H.-S., *et al.*, **1999**. Molecular cloning and characterization of a gene encoding a beta-*N*-acetylglucosaminidase homologue from *Bombyx mandarina*. *Korean J. Sericult. Sci. 41*, 147–153.

Gooday, G.W., **1999**. Aggressive and defensive roles for chitinases. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Basel, pp. 157–169.

Gopalakrishnan, B., Muthukrishnan, S., Kramer, K.J., **1995**. Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem. Mol. Biol. 25*, 255–265.

Graack, H., Cinque, U., Kress, H., **2001**. Functional regulation of glutamine: fructose-6-phosphate aminotransferase 1 (GFAT1) of *Drosophila melanogaster* in a UDP-*N*-acetylglucosamine and cAMP-dependent manner. *Biochem. J. 360*, 401–412.

Gunner, H.P., Met, M.Z., Berger, S., **1985**. In: Gamble, D.G., Lewis, F.B. (Eds.), Microbial Control of Spruce Budworms. US Forest Service GTR-NE-100, Northeastern Forest Experiment Station, Broomall, PA, p. 102.

Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., **2002**. A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem. Mol. Biol. 32*, 1577–1583.

Harper, M.S., Granados, R.R., **1999**. Peritrophic membrane structure and formation of larval *Trichoplusia ni* with an investigation on the secretion patterns of a PM mucin. *Tissue and Cell 31*, 201–211.

Harper, M.S., Hopkins, T.L., **1997**. Peritrophic membrane structure and secretion in European corn borer larvae (*Ostrinia nubilalis*). *Tissue and Cell 29*, 461–475.

Harper, M.S., Hopkins, T.L., Czapla, T.H., **1998**. Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. *Tissue and Cell 30*, 166–176.

Harris, M.T., Fuhrman, J.A., **2002**. Structure and expression of chitin synthase in the parasitic nematode *Dirofilaria immitis*. *Mol. Biochem. Parasitol. 122*, 231–234.

Harris, M.T., Lai, K., Arnold, K., Martinez, H.F., Specht, C.A., *et al.*, **2000**. Chitin synthase in the filarial parasite, *Brugia malayi*. *Mol. Biochem. Parasitol. 111*, 351–362.

Hawtin, R.E., Zarkowska, T., Arnold, K., Thomas, C.J., Gooday, G.W., *et al.*, **1997**. Liquefaction of *Autographa californica* nucleopolyhedrosis-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. *Virology 238*, 243–253.

Hefner, T., Stockigt, J., **2003**. Probing suggested catalytic domains of glycosyltransferases by site-directed mutagenesis. *Eur. J. Biochem. 270*, 533–538.

Hemmi, H., Ishibashi, J., Tomie, T., Yamakawa, M., **2003**. Structural basis for new pattern of conserved amino acid residues related to chitin binding in the antifungal peptide from the coconut rhinoceros beetle *Oryctes rhinoceros*. *J. Biol. Chem. 278*, 22820–22827.

Henrissat, B., **1999**. Classification of chitinase modules. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Basel, pp. 137–156.

Henrissat, B., Bairoch, A., **1996**. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J. 316*, 695–696.

Honda, Y., Kitaoka, M., Tokuyasu, K., Sasaki, C., Fukamizo, T., *et al.*, **2003**. Kinetic studies on the hydrolysis of *N*-acetylated and *N*-deacetylated derivatives of 4-methylumbelliferyl chitobioside by the family 18 chitinases ChiA and ChiB from *Serratia marcescens*. *J. Biochem. (Tokyo) 133*, 253–258.

Hopkins, T.L., Harper, M.S., **2001**. Lepidopteran peritrophic membranes and effects on dietary wheat germ agglutinin on their formation and structure. *Arch. Insect Biochem. Physiol. 47*, 100–109.

Hopkins, T.L., Kramer, K.J., **1992**. Insect cuticle sclerotization. *Annu. Rev. Entomol. 37*, 273–302.

Horst, M.N., **1983**. The biosynthesis of crustacean chitin: isolation and characterization of polyprenol-linked intermediates from brine shrimp microsomes. *Arch. Biochem. Biophys. 223*, 254–263.

Houston, D.R., Reeklies, A.D., Krupa, J.C., van Aalten, D.M.F., **2003**. Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes. *J. Biol. Chem. 278*, 30206–30212.

Houston, D.R., Shiomi, K., Arai, N., Omura, S., Peter, M.G., *et al.*, **2002**. High-resolution structures of a chitinase complexed with natural product cyclopentapeptide inhibitors: mimicry of carbohydrate substrate. *Proc. Natl Acad. Sci. USA 99*, 9127–9132.

Hsu, T.A., Takahashi, N., Tsukamoto, Y., Kato, K., Shimada, I., *et al.*, **1997**. Differential *N*-glycan patterns of secreted and intracellular IgG produced in *Trichoplusia ni* cells. *J. Biol. Chem. 272*, 9062–9070.

Huang, X., Zhang, H., Zen, K.C., Muthukrishnan, S., Kramer, K.J., **2000**. Homology modeling of the insect chitinase catalytic domain-oligosaccharide complex and the role of a putative active site tryptophan in catalysis. *Insect Biochem. Mol. Biol. 30*, 107–117.

Ibrahim, G.H., Smartt, C.T., Kiley, L.M., Christensen, B.M., **2000**. Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 30*, 1213–1222.

Ishaaya, I., **2001**. Biochemical processes related to insecticide action: an overview. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer-Verlag, New York, pp. 1–16.

Jarvis, D.L., Finn, E.E., **1995**. Biochemical analysis of the *N*-glycosylation pathway in baculovirus-infected lepidopteran insect cells. *Virology 212*, 500–511.

Johansen, J.S., Christensen, I.J., Riisbro, R., Greenall, M., Han, C., *et al.*, **2003**. High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival. *Breast Cancer Res. and Treatmt 80*, 15–21.

Jurgens, G., Wieschaus, E., Nusslein-Volhard, C., Kluding, H., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* 2. Zygotic loci on the third chromosome. *Roux's Arch. Devel. Biol. 193*, 283–295.

Kanost, M.R., Zepp, M.K., Ladendorff, N.E., Andersson, L.A., **1994**. Isolation and characterization of a hemocyte aggregation inhibitor from hemolymph of *Manduca sexta* larvae. *Arch. Insect Biochem. Physiol. 27*, 123–136.

Kato, N., Dasgupta, R., Smartt, C.T., Christensen, B.M., **2002**. Glucosamine: fructose-6-phosphate aminotransferase: gene characterization, chitin biosynthesis and peritrophic matrix formation in *Aedes aegypti*. *Insect Mol. Biol. 11*, 207–216.

Khor, E., **2002**. Chitin: a biomaterial in waiting. *Curr. Opin. Solid State Materials Sci. 2*, 313–317.

Kim, M.G., Shin, S.W., Bae, K.S., Kim, S.C., Park, H.Y., **1998**. Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori*, and the fall webworm, *Hyphantria cunea*. *Insect. Biochem. Mol. Biol. 28*, 163–171.

Kimura, S., **1976**. Insect haemolymph exo-β-*N*-acetylglucosaminidase from *Bombyx mori*. *Biochim. Biophys. Acta 446*, 399–406.

Kimura, S., **1977**. Exo-β-*N*-acetylglucosaminidase and chitobiase in *Bombyx mori*. *Insect Biochem. 7*, 237–245.

Kirkpatrick, R.B., Matico, R.E., McNulty, D.E., Strickler, J.E., Rosenberg, M., **1995**. An abundantly secreted glycoprotein from *Drosophila melanogaster* is related to mammalian secretory proteins produced in rheumatoid tissues and by activated macrophages. *Gene 153*, 147–154.

Kleywegt, G.J., Zou, J.Y., Kjeldgaard, M., Jones, T.A., **2001**. In: Around, O., Rossmann, M.G., Arnold, E. (Eds.), International Tables for Crystallography. vol. F. Kluwer Academic, Dordrecht, The Netherlands, pp. 353–356.

Koga, D., Funakoshi, T., Fujimoto, H., Kuwano, E., Eto, M., *et al.*, **1991**. Effects of 20-hydroxyecdysone and KK-42 on chitinase and beta-*N*-acetylglucosaminidase during the larval–pupal transformation of *Bombyx mori*. *Insect Biochem. 21*, 277–284.

Koga, D., Funakoshi, T., Mizuki, K., Ide, A., Kramer, K.J., *et al.*, **1992**. Immunoblot analysis of chitinolytic enzymes in integument and molting fluid of the silkworm, *Bombyx mori* and the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 22*, 305–311.

Koga, D., Isogai, A., Sakuda, S., Matsumoto, S., Suzuki, A., *et al.*, **1987**. Specific inhibition of *Bombyx mori* chitinase by allosamidin. *Agric. Biol. Chem. 51*, 471–476.

Koga, D., Jilka, J., Kramer, K.J., **1983**. Insect endochitinases: glycoproteins from moulting fluid, integument and pupal haemolymph. *Insect Biochem. 13*, 295–305.

Koga, D., Mai, M.S., Dziadik-Turner, C., Kramer, K.J., **1982**. Kinetics and mechanism of exochitinase and β-*N*-acetylglucosaminidase from the tobacco hornworm, *Manduca sexta* L. (*Lepidoptera: Sphingidae*). *Insect Biochem. 12*, 493–499.

Koga, D., Mitsutomi, M., Kono, M., Matsumiya, M., **1999**. Biochemistry of chitinases. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Basel, pp. 111–123.

Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., *et al.*, **1997**. Purification and characterization of *Bombyx mori* chitinase. *Insect Biochem. Mol. Biol. 27*, 757–767.

Kramer, K.J., Corpuz, L., Choi, H., Muthukrishnan, S., **1993**. Sequence of a cDNA and expression of the genes encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem. Mol. Biol. 23*, 691–701.

Kramer, K.J., Dziadik-Turner, C., Koga, D., **1985**. Chitin metabolism in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 3. Pergamon Press, Oxford, pp. 75–115.

Kramer, K.J., Hopkins, T.L., Schaefer, J., **1988**. Insect cuticle structure and metabolism. *Adv. Chem. 379*, 160–185.

Kramer, K.J., Hopkins, T.L., Schaefer, J., **1995**. Applications of solids NMR to the analysis of insect sclerotized structures. *Insect Biochem. Mol. Biol. 25*, 1067–1080.

Kramer, K.J., Koga, D., **1986**. Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem. 16*, 851–877.

Kramer, K.J., Muthukrishnan, S., **1997**. Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem. Mol. Biol. 27*, 887–900.

Kramer, K.J., Muthukrishnan, S., Johnson, L., White, F., **1997**. Chitinase for insect control. In: Carozzi, N., Koziel, M. (Eds.), Advances in Insect Control: The Role of Transgenic Plants. Taylor and Francis, London, pp. 185–193.

Krieger de Moraes, C., Schrank, A., Vainstein, M.H., **2003**. Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. *Curr. Microbiol. 46*, 205–210.

Krishnan, A., Nair, P.N., Jones, D., **1994**. Isolation, cloning, and characterization of new chitinase stored in active form in chitin-lined venom reservoir. *J. Biol. Chem. 269*, 20971–20976.

Kubelka, V., Altmann, F., Marz, L., **1995**. The asparagine-linked carbohydrate of honeybee venom hyaluronidase. *Glycoconjugate J. 12*, 77–83.

Kuranda, M.J., Robbins, P.W., **1991**. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem. 266*, 19758–19767.

Lanfranco, L., Balsamo, R., Martino, E., Perotto, S., Bonfante, P., **2002**. Zinc ions alter morphology and chitin deposition in an ericoid fungus. *Eur. J. Histochem. 46*, 341–350.

Langsford, M.L., Gilkes, N.R., Singh, B., Moser, B., Miller, R.C., *et al.*, **1987**. Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. *FEBS Lett. 25*, 163–167.

Lehtio, J., Sugiyama, J., Gustavsson, M., Fransson, L., Linder, M., *et al.*, **2003**. The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. *Proc. Natl Acad. Sci. USA 100*, 484–489.

Limón, M.C., Margolles-Clark, E., Benítez, T., Penttilä, M., **2001**. Addition of substrate-binding domains increases substrate-binding capacity and specific activity of a chitinase from *Trichoderma harzianum*. *FEMS Microbiol. Lett. 198*, 57–63.

Linder, M., Salovuori, I., Ruohonen, L., Teeri, T.T., **1996**. Characterization of a double cellulose-binding domain: synergistic high affinity binding to crystalline cellulose. *J. Biol. Chem. 271*, 21268–21272.

Linder, M., Teeri, T.T., **1997**. The roles and function of cellulose-binding domains. *J. Biotechnol. 57*, 15–28.

Liu, M., Cai, Q.X., Liu, H.Z., Zhang, B.H., Yan, J.P., *et al.*, **2002**. Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. *J. Appl. Microbiol. 93*, 374–379.

Locke, M., Huie, P., **1979**. Apolysis and the turnover of plasma membrane plaques during cuticle formation in an insect. *Tissue and Cell 11*, 277–291.

Lu, Y., Zen, K.C., Muthukrishnan, S., Kramer, K.J., **2002**. Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase. *Insect Biochem. Mol. Biol. 32*, 1369–1382.

Lucero, H.A., Kuranda, M.J., Bulik, D.A., **2002**. A nonradioactive, high throughput assay for chitin synthase activity. *Anal. Biochem. 305*, 97–105.

Lysenko, O., **1976**. Chitinase of *Serratia marcescens* and its toxicity to insects. *J. Invert. Pathol. 27*, 385–386.

Macedo, M.L.R., Andrade, L.B.S., Moraes, R.A., Xavier-Filho, J., **1993**. Vicilin variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). *Comp. Biochem. Physiol. C 105*, 89–94.

Macedo, M.L.R., Damico, D.C.S., Freire, M. D. G. M., Toyama, M.H., Marangoni, S., *et al.*, **2003**. Purification and characterization of an *N*-acetylglucosamine-binding lectin from *Koelreuteria paniculata* seeds and its effect on the larval development of *Callosobruchus maculatus* (Coleoptera: Bruchidae) and *Anagasta kuehniella* (Lepidoptera: Pyralidae). *J. Agric. Food Chem. 51*, 2980–2986.

McCall, C., Hunter, S., Stedman, K., Weber, E., Hillier, A., *et al.*, **2001**. Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. *Veter. Immunol. Immunopathol. 78*, 231–247.

McLean, B.W., Boraston, A.B., Brouwer, D., Sanaie, N., Fyfe, C.A., *et al.*, **2002**. Carbohydrate-binding modules recognize fine substructures of cellulose. *J. Biol. Chem. 277*, 50245–50254.

Mikitani, K., Sugasaki, T., Shimada, T., Kobayashi, M., Gustafsson, J.A., **2000**. The chitinase gene of the silkworm, *Bombyx mori*, contains a novel Tc-like transposable element. *J. Biol. Chem. 275*, 37725–37732.

Mikolajczyk, P., Oberlander, H., Silhacek, D., Ishaaya, I., Shaaya, E., **1994**. Chitin synthesis in *Spodoptera frugiperda* wing imaginal discs. 1. Chlorfluzuron, diflubenzuron, and teflubenzuron inhibit incorporation but not uptake of [^{14}C]-*N*-acetyl-D-glucosamine. *Arch. Insect Biochem. Physiol. 25*, 245–258.

Morlais, I., Severson, D.W., **2001**. Identification of a polymorphic mucin-like gene expressed in the midgut of the mosquito, *Aedes aegypti*, using an integrated bulked segregant and differential display analysis. *Genetics 158*, 1125–1136.

Morris, O.N., **1976**. A 2-year study of the efficacy of *Bacillus thuringiensis*–chitinase combinations in spruce budworm (*Choristoneura fumiferana*) control. *Can. Entomol. 108*, 225.

Mota, A.C., DaMatta, R.A., Filho, M.L., Silva, C.P., Xavier-Filho, J., **2003**. Cowpea (*Vigna unguiculata*) vicilins bind to the peritrophic membrane of larval sugarcane stalk borer (*Diatraea saccharalis*). *J. Insect Physiol. 49*, 873–880.

Munrow, C.A., Gow, N.A., **2001**. Chitin synthesis in human pathogenic fungi. *Med. Mycol. 39*(Suppl. 1), 41–53.

Muzzarelli, C., Muzzarelli, R.A.A., **2002**. Reactivity of quinones towards chitosans. Trends Glycosci. *Glycotech. 14*, 223–229.

Muzzarelli, R.A.A., Littarru, G., Muzzarelli, C., Tosi, G., **2003**. Selective reactivity of biochemically relevant quinones towards chitosans. *Carbohydrate Polymers 53*, 109–115.

Nagahashi, S., Sudoh, M., Ono, N., Sawada, R., Yamaguchi, E., *et al.*, **1995**. Characterization of chitin synthase 2 of *Saccharomyces cereviseae*: implications of two highly conserved domains as possible catalytic sites. *J. Biol. Chem. 270*, 13961–13967.

Nagamatsu, Y., Yanagisawa, I., Kimoto, M., Okamoto, M., Koga, D., **1995**. Purification of a chitooligosaccharidolytic β-*N*-acetylglucosaminidase from *Bombyx mori* larvae during metamorphosis and the nucleotide sequence of its cDNA. *Biosci. Biotech. Biochem. 59*, 219–225.

Nagano, N., Orengo, C.A., Thornton, J.M., **2002**. One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. *J. Mol. Biol. 321*, 741–765.

Nagano, N., Porter, C.T., Thornton, J.M., **2001**. The $(\beta\alpha)_8$ glycosidases: sequence and structure analyses suggest distant evolutionary relationships. *Protein Eng. 14*, 845–855.

Neuhaus, J.M., **1999**. Plant chitinases (PR-3, PR-4, PR-8, PR-11). In: Datta, S.K., Muthukrishnan, S. (Eds.), Pathogenesis-Related Proteins in Plants. CRC Press, New York, pp. 77–106.

Nichols, E.J., Beckman, J.M., Hadwiger, L.A., **1980**. Glycosidic enzyme activity in pea tissue and pea–*Fusariun solani* interactions. *Plant Physiol. 66*, 199–204.

Nusslein-Volhard, C., Wieschaus, E., Kluding, H., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* 1. Zygotic loci on the second chromosome. *Roux's Arch. Devel. Biol. 193*, 267–282.

Oberlander, H., Silhacek, D., **1998**. New perspectives on the mode of action of benzoylphenylurea insecticides. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer-Verlag, New York, pp. 92–105.

Ostrowski, S., Dierick, H.A., Bejsovec, A., **2002**. Genetic control of cuticle formation during embryonic development of *Drosophila melanogaster. Genetics 161*, 171–182.

Otsu, Y., Mori, H., Komuta, K., Shimizu, H., Nogawa, S., *et al.*, **2003**. Suppression of leaf feeding and oviposition of phytophagous ladybird beetles (Coleoptera: Coccinellidae) by chitinase gene-transformed phylloplane bacteria and their specific bacteriophages entrapped in alginate gel beads. *J. Econ. Entomol. 96*, 555–563.

Palli, S.R., Retnakaran, A., **1999**. Molecular and biochemical aspects of chitin synthesis inhibition. In: Jolles, P, Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Basel, pp. 85–98.

Patil, R.S., Ghormade, V., Deshpande, M.V., **2000**. Chitinolytic enzymes: an exploration. *Enzyme Microbiol. Technol. 26*, 473–483.

Paulson, J.C., **1989**. Glycoproteins: what are the sugar chains for? *Trends Biochem. Sci. 14*, 272–276.

Perchan, T., Cohen, A., Williams, W.P., Luthe, D.S., **2002**. Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. *Proc. Natl Acad. Sci. USA 99*, 13319–13323.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., *et al.*, **1994**. Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure 2*, 1169–1180.

Quentin, M., Ebbelaar, M., Derksen, J., Mariani, C., Van der Valk, H., **2002**. Description of a cellulose-binding domain and a linker sequence from *Aspergillus* fungi. *Appl. Microbiol. Biotechnol. 58*, 658–662.

Quesada-Allue, L.A., **1982**. The inhibition of insect chitin biosynthesis by tunicamycin. *Biochem. Biophys. Res. Commun. 105*, 312–319.

Raikhel, N.V., Lee, H.-I., Broekaert, W.F., **1993**. Structure and function of chitin-binding proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol. 44*, 591–615.

Ramalho-Ortigão, J.M., Traub-Csekö, Y.M., **2003**. Molecular characterization of *Llchit1*, a midgut chitinase cDNA from the leishmaniasis vector *Lutzomyia longipalpis*. *Insect Biochem. Mol. Biol. 33*, 279–287.

Rao, F.V., Houston, D.R., Boot, R.G., Aerts, J. M. F. G., Sakuda, S., van Aalten, D.M.F., **2003**. Crystal structures of allosamidin derivatives in complex with human macrophage chitinase. *J. Biol. Chem. 278*, 20110–20116.

Rayms-Keller, A., McGaw, M., Oray, C., Carlson, J.O., Beaty, B.J., **2000**. Molecular cloning and characterization of a metal responsive *Aedes aegypti* mucin cDNA. *Insect Mol. Biol. 9*, 419–426.

Read, S.M., Bacic, T., **2002**. Prime time for cellulose. *Science 295*, 59–60.

Rebers, J.E., Willis, J.H., **2001**. A conserved domain in arthropod cuticular proteins binds chitin. *Insect Biochem. Mol. Biol. 31*, 1083–1093.

Receveur, V., Czjzek, M., Schülein, M., Panine, P., Heinrissat, B., **2002**. Dimension, shape, and conformational flexibility of a two domain fungal cellulase in solution probed by small angle X-ray scattering. *J. Biol. Chem. 277*, 40887–40892.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., *et al.*, **1996**. Synergistic activity of a *Bacillus thuringiensis* delta-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Appl. Environ. Microbiol. 62*, 3581–3586.

Renkema, G.H., Boot, R.G., Au, F.L., Donker-Koopman, W.E., Strijland, A., *et al.*, **1998**. Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur. J. Biochem. 251*, 504–509.

Richmond, T., **2000**. Higher plant cellulose synthases. *Genome Biol. 1*, 3001–3006.

Rogers, S., Wells, R., Rechsteiner, M., **1986**. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science 234*, 364–368.

Roseland, C.R., Grodowitz, M.J., Kramer, K.J., Hopkins, T.L., Broce, A.B., **1985**. Stabilization of mineralized and sclerotized puparial cuticle of muscid flies. *Insect Biochem. 15*, 521–528.

Royer, V., Fraichard, S., Bouhin, H., **2002**. A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis. *Biochem. J. 366*, 921–928.

Saito, A., Fujii, T., Miyashita, K., **2003**. Distribution and evolution of chitinase genes in *Streptomyces* species: involvement of gene-duplication and domain-deletion. *Antonie van Leeuwenhoek 84*, 7–16.

Saitou, N., Nei, M., **1987**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol. 4*, 406–425.

Sales, M.P., Pimenta, P.P., Paes, N.S., Grossi-de-Sá, M.F., Xavier-Filho, J., **2001**. Vicilins (7S storage globulins) of cowpea (*Vigna unguiculata*) seeds bind to chitinous structures of the midgut of *Callosobruchus maculatus* (Coleoptera: Bruchidae) larvae. *Brazil. J. Med. Biol. Res. 34*, 27–34.

Sampson, M.N., Gooday, G.W., **1998**. Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiology 144*, 2189–2194.

Sarauer, B.L., Gillott, C., Hegedus, D., **2003**. Characterization of an intestinal mucin from the peritrophic matrix of the diamondback moth, *Plutella xylostella*. *Insect Mol. Biol. 12*, 333–343.

Schaefer, J., Kramer, K.J., Garbow, J.R., Jacob, G.S., Stejskal, E.O., *et al.*, **1987**. Aromatic cross-links in insect cuticle: detection by solid-state ^{13}C and ^{15}N NMR. *Science 235*, 1200–1204.

Screen, S.E., Hu, G., St. Leger, R.J., **2001**. Transformants of *Metarhizium anisopliae* sf. *anisopliae* over expressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. *J. Invert. Pathol. 78*, 260–266.

Shapiro, M., Preisler, H.K., Robertson, J.L., **1987**. Enhancement of baculovirus activity on gypsy moth (Lepidoptera: Limantidae) by chitinase. *J. Econ. Entomol. 80*, 1113–1115.

Shen, Z., Jacobs-Lorena, M., **1997**. Characterization of a novel gut-specific gene from the human malaria vector *Anopheles gambiae*. *J. Biol. Chem. 272*, 28895–28900.

Shen, Z., Jacobs-Lorena, M., **1999**. Evolution of chitin-binding proteins in invertebrates. *J. Mol. Evol. 48*, 341–347.

Shinoda, T., Kobayashi, J., Matsui, M., Chinzei, Y., **2001**. Cloning and functional expression of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using a baculovirus lacking the virus-encoded chitinase gene. *Insect Biochem. Mol. Biol. 31*, 521–532.

Shutov, A.D., Kakhovskaya, I.A., Braun, H., Bäumlein, H., Müntz, K., **1995**. Legumin-like and vicilin-like seed storage proteins: evidence for a common single-domain ancestral gene. *J. Mol. Evol. 41*, 1057–1069.

Smirnoff, W.A., Valero, R., **1972**. Perturbations métaboliques chez *Choristoneura fumiferana* Clemens au cours de l'infection par *Bacillus thuringiensis* seul ou en présence de chitinase. *Rev. Con. Biol. 31*, 163.

Sneh, B., Schuster, S., Gross, S., **1983**. Biological control of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) by *Bacillus thuringiensis* subsp. *entomocidus* and *Bracon hebetor* Say (Hymenoptera:Braconidae). Z. *Ang. Entomol. 96*, 77–83.

Spindler, K.D., Spindler-Barth, M., **1999**. Inhibitors of chitinases. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Basel, pp. 201–209.

Srisodsuk, M., Reinikainen, T., Pentilä, M., Teeri, T., **1993**. Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. *J. Biol. Chem. 268*, 20756–20761.

St. Leger, R.J., Joshi, L., Bidochka, M.L., Rizzo, N.W., Roberts, D.W., **1996**. Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. *Appl. Environ. Microbiol. 62*, 907–912.

Suetake, T., Tsuda, S., Kawabata, S., Miura, K., Iwanaga, S., *et al.*, **2000**. Chitin-binding proteins in invertebrates and plants comprise a common chitin-binding motif. *J. Biol. Chem. 275*, 17929–17932.

Sun, Y.J., Chang, N.C., Hung, S.I., Chang, A.C., Chou, C.C., *et al.*, **2001**. The crystal structure of a novel mammalian lectin, Ym1, suggests a saccharide-binding site. *J. Biol. Chem. 276*, 17507–17514.

Sundara Rajulu, G., Aruchami, A., Gowri, N., **1982**. Natural deacetylation of chitin to chitosan in the abdominal cuticle of the physogastric queen of *Macrotermetes estherae*. In: Proc. 2nd Int. Conf. Chitin and Chitosan, cited in Gooday, G. W., 1985. Chitin deacetylases in invertebrates. In: Muzzerelli, R., Jeuniaux, C., Gooday, G.W. (Eds.), Chitin in Nature and Technology. Plenum Press, New York, pp. 263–268.

Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Hernissat, B., *et al.*, **1999**. The third chitinase gene (*ChiC*) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. *Biochem. J. 343*, 587–596.

Takahashi, M., Kiuchi, M., Kamimura, M., **2002**. A new chitinase-related gene, *BmChiR1*, is induced in the *Bombyx mori* anterior silk gland at molt and metamorphosis by ecdysteroid. *Insect Biochem. Mol. Biol. 32*, 147–151.

Tantimavanich, S., Pantuwatana, S., Bhumiratana, A., Panbangred, W., **1997**. Cloning of a chitinase gene into *Bacillus thuringiensis* subsp. *aizawai* for enhanced insecticidal activity. *J. Gen. Appl. Microbiol. 43*, 341–347.

Tellam, R.L., **1996**. Protein motifs in filarial chitinases: an alternative view. *Parasitol. Today 12*, 291–292.

Tellam, R.L., Vuocolo, T., Eisemann, C., Briscoe, S., Riding, G., *et al.*, **2003**. Identification of an immunoprotective mucin-like protein, peritrophin-55, from the peritrophic matrix of *Lucilia cuprina* larvae. Insect Biochem. *Mol. Biol. 33*, 239–252.

Tellam, R.L., Vuocolo, T., Johnson, S.E., Jarmey, J., Pearson, R.D., **2000**. Insect chitin synthase cDNA sequence, gene organization and expression. *Eur. J. Biochem. 267*, 6025–6043.

Tellam, R.L., Wijffels, G., Willadsen, P., **1999**. Peritrophic matrix proteins. *Insect Biochem. Mol. Biol. 29*, 87–101.

Tharanathan, R.N., Kittur, F.S., **2003**. Chitin: the undisputed biomolecule of great potential. *Crit. Rev. Food Sci. Nutri. 43*, 61–87.

Thomas, C.J., Gooday, G.W., King, L.A., Possee, R.D., **2000**. Mutagenesis of the active site coding region of the *Autographa californica* nucleopolyhedrovirus *chiA* gene. *J. Gen. Virol. 81*, 1403–1411.

Thompson, J.D., Higgins, D.G., Gibson, T.J., **1994**. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res. 22*, 4673–4680.

Thummel, C.S., **1996**. Flies on steroids: *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet. 12*, 306–310.

Tjoelker, L.W., Gosting, L., Frey, S., Hunter, C.L., Trong, H.L., *et al.*, **2000**. Structural and functional definition of the human chitinase chitin-binding domain. *J. Biol. Chem. 275*, 514–520.

Tomme, P., Warren, R.A., Miller, R.C., Kilburn, D.G., Gilkes, N.R., **1995**. In: Saddler, J.N., Penner, M. (Eds.), Enzymatic Degradation of Insoluble Polysaccharides. American Chemical Society, Washington, DC, pp. 142–163.

Tsigos, I., Martinou, A., Kafetzopoulos, D., Bouriotis, V., **2000**. Chitin deacetylases: new, versatile tools in biotechnology. *Trends Biotechnol. 18*, 305–312.

Turner, C.D., Koga, D., Mai, M.S., Kramer, K.J., **1981**. Purification and characterization of two β-*N*-acetylhexosaminidases from the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Arch. Biochem. Biophys. 212*, 546–560.

Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., *et al.*, **2001**. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. *J. Biol. Chem. 276*, 41343–41349.

Väljamäe, P., Pettersson, G., Johansson, G., **2001**. Mechanism of substrate inhibition in cellulose synergistic degradation. *Eur. J. Biochem. 268*, 4520–4526.

van Aalten, D. 2003. Ligand-induced signaling and conformational change of the 39 kD glycoprotein from human chondrocytes. Proc. 9th Int. Chitin Chitosan Conf., Montreal, Abstract Ha. 1, p. 6.

Venegas, A., Goldstein, J.C., Beauregard, K., Oles, A., Abdulhayoglu, N., *et al.*, **1996**. Expression of recombinant microfilarial chitinase and analysis of domain function. *Mol. Biochem. Parasitol. 78*, 149–159.

Verloop, A., Ferrell, C.D., **1977**. Benzoylphenylureas: a new group of insecticides interfering with chitin deposition. *Pesticide Chem. 20th Cent. 37*, 237–270.

Veronico, P., Gray, L.J., Jones, J.T., Bazzicalupo, P., Arbucci, S., *et al.*, **2001**. Nematode chitin synthases: gene structure, expression and function in *Caenorhabditis elegans* and the plant parasitic nematode *Meloidogyne artiellia*. *Mol. Genet. Genomics 266*, 28–34.

Wang, P., Granados, R.R., **1997**. Molecular cloning and sequencing of a novel invertebrate intestinal mucin cDNA. *J. Biol. Chem. 272*, 16663–16669.

Wang, P., Granados, R.R., **2000a**. Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol. 47*, 110–118.

Wang, P., Granados, R.R., **2000b**. Calcofluor disrupts the midgut defense system in insects. *Insect Biochem. Mol. Biol. 30*, 135–143.

Wang, X., Ding, X., Gopalakrishnan, B., Morgan, T.D., Johnson, L., *et al.*, **1996**. Characterization of a 46 kDa insect chitinase from transgenic tobacco. *Insect Biochem. Mol. Biol. 26*, 1055–1064.

Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, M., *et al.*, **2003**. Aromatic residues within the substrate-binding cleft of Bacillus circulans chitinase A1 are essential for crystalline chitin hydrolysis. *Biochem. J. 376*, 237–244. Immediate Publication, doi:10.1042/BJ20030419.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., *et al.*, **1993**. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem. 268*, 18567–18572.

Watanabe, T., Kono, M., **2002**. Molecular studies of family 18 chitinases in animals: structural and functional diversity. *Chitin Chitosan Res. 8*, 95–104.

Watanabe, T., Oyangi, W., Suzuki, K., Tanaka, H., **1990**. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol. 172*, 4017–4022.

Watanabe, T., Uchida, M., Kobori, K., Tanaka, H., **1994**. Site-directed mutagenesis of the Asp-197 and Asp-202 residues in chitinase A1 of *Bacillus circulans* WL-12. *Biosci. Biotechnol. Biochem. 58*, 2283–2285.

Wiechaus, E., Nusslein-Volhard, C., Jurgens, G., **1984**. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* 1 Zygotic loci on the X chromosome and the fourth chromosome. *Roux's Arch. Devel. Biol. 193*, 296–307.

Wilson, T.G., Cryan, J.R., **1997**. Lufenuron, a chitin-synthesis inhibitor, disrupts development of *Drosophila melanogaster. J. Exp. Biol. 278*, 37–44.

Wiwat, C., Lertcanawanichkul, M., Siwayapran, P., Pantuwatana, S., Bhumiratan, A., **1996**. Expression of chitinase encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* in *Bacillus thuringiensis* subsp. *israelensis*. *Gene 179*, 119–126.

Wiwat, C., Thaithanun, S., Pantuwatana, S., Bhumiratana, A., **2000**. Toxicity of chitinase-producing *Bacillus thuringiensis* sp. *kurstaki* HD-1 toward *Plutella xylostella*. *J. Invert. Path. 76*, 270–277.

Wormleaton, S., Kuzio, J., Winstanley, D., **2003**. The complete sequence of the *Adoxophyes orana* granulovirus genome. *Virology 311*, 350–365.

Yan, J., Cheng, Q., Narashimhan, S., Li, C.B., Aksoy, S., **2002**. Cloning and functional expression of a fat

body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol. 32*, 979–989.

Zechel, D.L., Withers, S.G., **2000**. Glycosidase mechanisms: anatomy of a finely tuned catalyst. *Acc. Chem. Res. 33*, 11–18.

Zen, K.C., Choi, H.K., Nandegama, K., Muthukrishnan, S., Kramer, K.J., **1996**. Cloning, expression and hormonal regulation of an insect β-*N*-acetylglucosaminidase gene. *Insect Biochem. Mol. Biol. 26*, 435–444.

Zhang, H., Huang, X., Fukamizo, T., Muthukrishnan, S., Kramer, K.J., **2002**. Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase. *Insect Biochem. Mol. Biol. 32*, 1477–1488.

Zheng, Y., Zheng, S., Cheng, X., Ladd, T., Lingohr, E.J., *et al.*, **2002**. A molt-associated chitinase cDNA from the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol. 32*, 1813–1823.

Zheng, Y.P., Retnakaran, A., Krell, P.J., Arif, B.M., Primavera, M., *et al.*, **2003**. Temporal, spatial and induced expression of chitinase in the spruce budworm, *Choristoneura fumiferana*. *J. Insect Physiol. 49*, 241–247.

Zhou, Y., Wang, F., Xiao, X., **2002**. Structures of the cloned chitinase and its truncant from *A. caviae*. *Prog. Nat. Sci. 12*, 587–592.

Zhu, Y.C., Muthukrishnan, S., Specht, C.A., Dittmer, N., Kanost, M.R., *et al.*, **2002**. Sequence of a cDNA and expression of the gene encoding a putative epidermal chitin synthase of *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 1497–1506.

Zimoch, L., Merzendorfer, H., **2002**. Immunolocalization of chitin synthase in the tobacco hornworm. *Cell Tissue Res. 308*, 287–297.

4.4 Cuticular Sclerotization and Tanning

S O Andersen, University of Copenhagen,
Copenhagen, Denmark

4.4.1. Introduction

The cuticle covers the complete body of the insects as an effective barrier between the animal and its surroundings; it provides protection against desiccation, microorganisms, and predators, and as an exoskeleton it serves as attachment sites for muscles. Cuticle is typically divided into relatively hard and stiff regions, the sclerites, separated by more flexible and pliable regions, the arthrodial membranes, which make the various forms of locomotion possible. Marked differences in mechanical properties may also be present on the microscopical level; two neighboring epidermal cells can produce cuticle with highly contrasting properties, indicating a precise control of cuticular composition. The mechanical properties of individual cuticular regions correspond closely to their functions and the forces to which they are exposed during the normal life of the animal. Proper flight can thus only be sustained when all wing regions have near-optimal balance between stiffness and flexibility; if the wing material locally is too soft or too stiff, the varying air pressure during the wing strokes will not cause the wings to bend to the shapes needed for generating optimal lift.

The mechanical properties of cuticle are determined by the interplay of many factors, such as cuticular thickness, relative amounts of chitin and proteins, chitin architecture, protein composition, water content, intracuticular pH, and degree of sclerotization and other secondary modifications. Sclerotization of insect cuticle has been reviewed several times in recent years (Sugumaran, 1988, 1998; Andersen, 1990; Hopkins and Kramer, 1992; Andersen *et al.*, 1996), but many aspects of the process are still rather poorly understood. Besides giving an overview of the present knowledge of the sclerotization process attention is drawn to some problems that need to be investigated in more detail.

Cuticular sclerotization is a chemical process whereby certain regions of insect cuticle are transformed irreversibly from a pliant material into a stiffer and harder structure, characterized by decreased deformability, decreased extractability of the matrix proteins, and increased resistance towards enzymatic degradation. During sclerotization the color of the cuticle may change from nearly colorless over various brown and black shades to the completely black. The term tanning is often used synonymously with sclerotization, but sometimes it is specifically used for the processes resulting in light brown (tan) cuticles. Sclerotization often takes place in connection with molting, starting just after the new, yet unsclerotized cuticle has been expanded to its final size and shape, but some specialized cuticular regions are sclerotized while the insect is still in its pharate state inside the old cuticle. Such pre-ecdysially sclerotized regions cannot be expanded post-ecdysially, but may help the insect to escape from the exuvium. The dipteran puparium is an example of a soft larval cuticle, which is sclerotized at the end of the last larval instar to form a hard protective case inside which metamorphosis to pupa and adult can take place. Sclerotization of structural materials in insects is not restricted to cuticle; other materials as well, such as egg cases and silks, may be stabilized by chemical processes closely related to cuticular sclerotization.

4.4.2. Current Model for Cuticular Sclerotization

During the years several models have been proposed for the chemical reactions which occur in the insect cuticle during the sclerotization process, and although many details of the individual steps in the reactions are still controversial or unexplored, there is general agreement concerning the main features of the process. The currently accepted sclerotization model is shown in Figures 1 and 2, and its main features are: the amino acid tyrosine (**1**) is hydroxylated to 3,4-dihydroxyphenylalanine (DOPA, **2**), which by decarboxylation is transformed to dopamine (**3**), a compound of central importance for both sclerotization and melanine formation. Dopamine can be *N*-acylated to either *N*-acetyldopamine (NADA, **4**) or *N*-β-alanyldopamine (NBAD, **5**), and both can serve as precursors in the sclerotization process. They are enzymatically oxidized to the corresponding *o*-quinones (**6**), which can react with available nucleophilic groups, whereby the catecholic structure is regained and the nucleophile is linked to the aromatic ring (**11**). The *o*-quinones of NADA and NBAD may also be enzymatically isomerized to the corresponding *p*-quinone methides (**7**), and the β-position of their side chain react readily with nucleophiles (**12**). The *p*-quinone methides may be enzymatically isomerized to side chain unsaturated catechol derivatives (**8**), dehydro-NADA

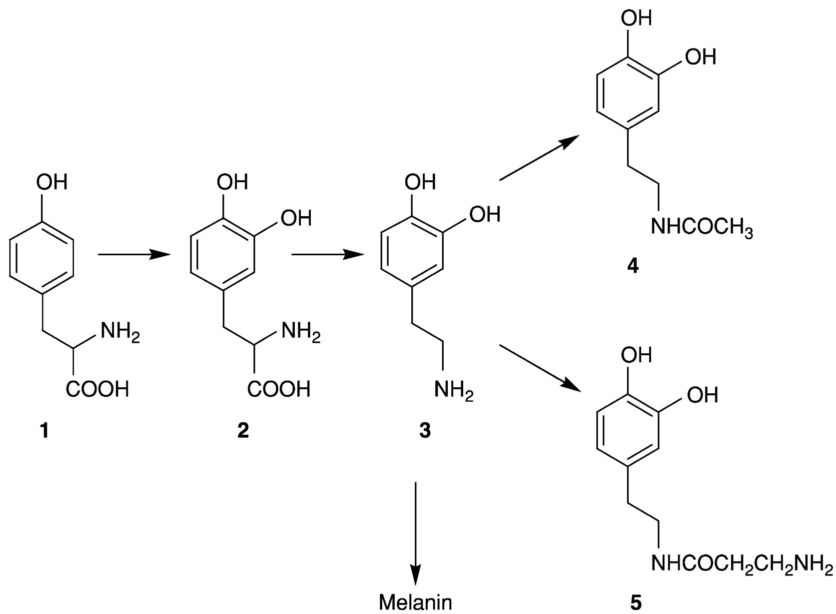


Figure 1 Biosynthesis of the sclerotization precursors NADA and NBAD from tyrosine. The enzyme tyrosine hydroxylase hydroxylates tyrosine (**1**) to 3,4-dihydroxyphenylalanine (DOPA, **2**), which is decarboxylated to dopamine (**3**) by the enzyme dopa-decarboxylase. Dopamine can be enzymatically acylated to either *N*-acetyldopamine (NADA, **4**) or *N*-β-alanyldopamine (NBDA, **5**). Surplus dopamine can be used for melanin synthesis.

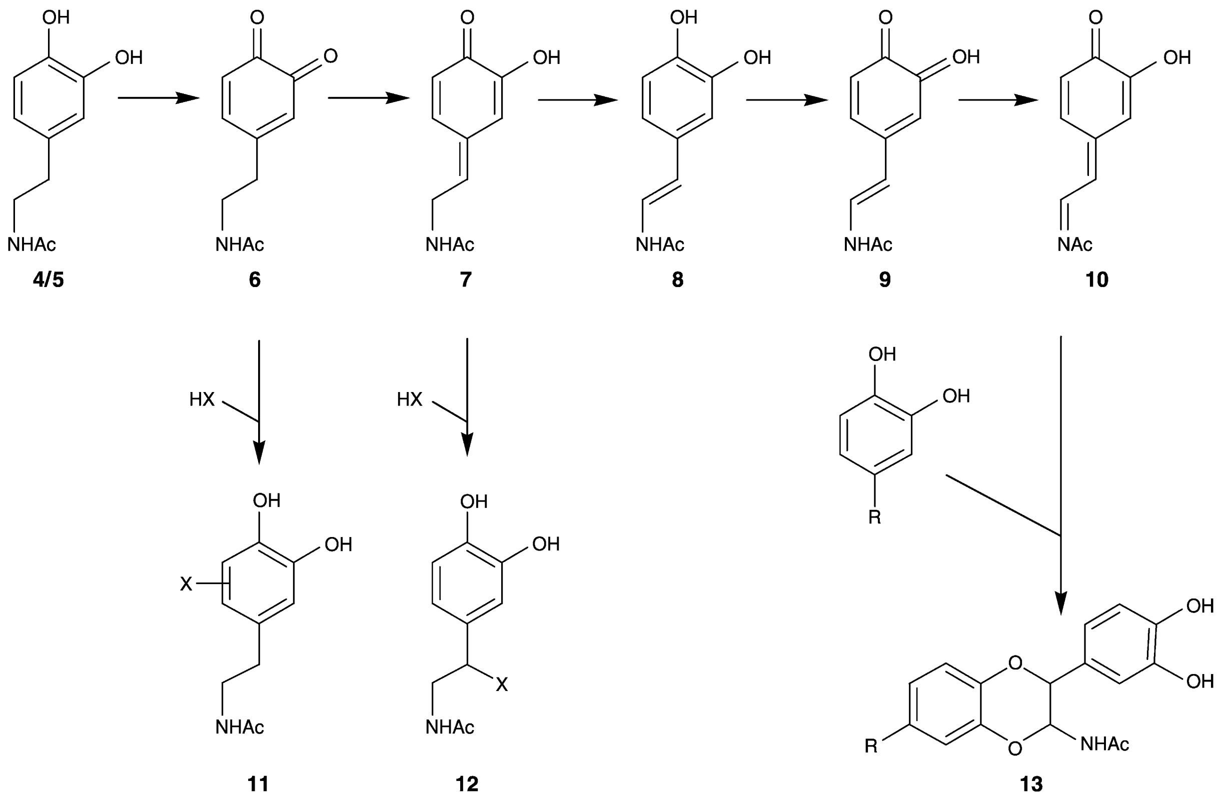


Figure 2 Cuticular oxidation of NADA and NBAD to various sclerotization agents. The acyldopamines are enzymatically oxidized to *o*-quinones (**6**), which can react spontaneously with nucleophilic compounds (HX) to give ring-substituted adducts (**11**), or they can be isomerized to the more reactive *p*-quinone methides (**7**), which can react with nucleophiles to give side chain substituted adducts (**12**). The quinone methides may also be isomerized to dehydro-acyldopamines (**8**), which after oxidation to quinones (**9** and **10**) can react with catechols to give dihydroxyphenyl-dihydrobenzodioxine derivatives (**13**).

and dehydro-NBAD, which after oxidation to unsaturated quinones can react with catechols to form dihydroxyphenyl-dihydrobenzodioxine derivatives (**13**) and with other nucleophilic groups to give yet unidentified compounds.

The *o*-quinones and *p*-quinone methides react preferably with the imidazole group in the cuticular proteins, but may also react with free amino groups, such as terminal amino groups in proteins and ε-amino groups in lysine residues, with catechols, with water, and probably also with hydroxyl groups in the *N*-acetylglucosamine residues in chitin. Depending upon the degree of sclerotization the various reactions between quinones and nucleophilic residues in the cuticular matrix proteins will result in the proteins being more or less covered by aromatic residues; some of these residues may be involved in cross-linking the cuticular proteins and maybe also forming links between proteins and chitin; some will be linked to only a single protein molecule, thereby increasing its hydrophobicity without being part of a covalent cross-link. During sclerotization most of the water-filled spaces between the matrix proteins in the presclerotized cuticle will become filled with polymerized catecholic material. As a result of these processes the interactions between the cuticular components have become stronger, the peptide chains more difficult to deform, and the proteins cannot be moved relative to each other or to the chitin filament system. Together, all these changes contribute to make the material stiffer and more resistant towards degradation.

The various reactions involved in the model will be discussed in more detail in the following sections, with main emphasis on aspects where the evidence is insufficient or missing or where some observations disagree with the scheme, to indicate areas where more research is needed. The appearance and properties of cuticle from different body regions of the same animal can vary widely, and a considerable part of this variation, such as the different coloration and mechanical properties, is probably due to quantitative or qualitative differences in the sclerotization process. There is no compelling reasons to believe that exactly the same stabilization process is used for sclerotization in all types of solid cuticle,

and generalizations based upon results obtained with a single or a few insect species can easily be misleading. A number of cuticular types will have to be analyzed to determine how the individual steps involved in sclerotization are modulated to give the local variation between cuticular regions of a given insect and between cuticles from different insect species. It will also be important to study how the reactions are controlled to give an optimal degree of sclerotization. Most of the results and ideas presented in this chapter have been obtained by studies involving material from only a few insect species, such as cuticle of blowfly larvae, pupae of *Manduca sexta*, and locust femurs, and it is to be expected that detailed studies of cuticle from more species will give a much more varied and fascinating picture of the complexity of cuticular sclerotization.

4.4.3. Sclerotization (Tanning) Precursors

The terms sclerotization agents and tanning agents were originally used for the compounds which are secreted from the epidermal cells into the cuticle, where they are oxidized by enzymes to become sufficiently reactive to form covalent linkages to proteins and chitin. There is now a tendency to restrict the term sclerotization agents to the reactive species directly involved in forming links to the cuticular components. The compounds secreted from the epidermis to be activated in the cuticle shall accordingly be called sclerotization or tanning precursors (Sugumaran, 1998).

4.4.3.1. *N*-Acetyldopamine and *N*-β-Alanyldopamine

The first discovered and most common precursor for cuticular sclerotization is NADA (**4**), which is synthesized by *N*-acetylation of dopamine. The central role of NADA in sclerotization was demonstrated by Karlson's research group during studies of tyrosine metabolism in insects (review: Karlson and Sekeris, 1976). They showed that NADA is incorporated into the puparial cuticle of the blowfly *Calliphora vicina* during its sclerotization, and that radioactively labeled tyrosine was metabolized to NADA when injected into last instar larvae shortly before puparium formation, and degraded when injected into younger larvae. NADA was also shown to be involved in cuticular sclerotization in several other insect species, such as the desert locust, *Schistocerca gregaria* (Karlson and Schlossberger-Raecke, 1962; Schlossberger-Raecke and Karlson, 1964). Incorporation of NADA into cuticle can be a very efficient process; after injection of radioactive NADA into young adult locusts about 80% of the total radioactivity was later recovered from the sclerotized cuticle (Andersen, 1971). NADA appears to be involved in cuticular sclerotization in all insect species investigated.

The amino acid β-alanine has been reported as a constituent of several types of sclerotized cuticle, and was suspected to participate somehow in the sclerotization process (Andersen, 1979a). Hopkins *et al*. (1982) showed that the β-alanyl derivative of dopamine, NBAD (**6**), is a sclerotizing precursor in the cuticle of *M. sexta* pupae, thus accounting for the presence of β-alanine in hydrolysates of the fully sclerotized cuticle. NBAD is also a sclerotization precursor in other cuticles from which β-alanine is released by acid hydrolysis, such as the cuticle of the red flour beetle, *Tribolium castaneum* (Kramer *et al*., 1984). The synthesis and utilization of NBAD during pupation of *M. sexta* has been reported (Krueger *et al*., 1989). An enzyme system in the medfly, *Ceratitis capitata* which can catalyze β-alanylation of dopamine to give NBAD has been partially characterized (Pérez *et al*., 2002).

The two sclerotizing compounds, NADA and NBAD, are used together in many types of cuticles; but the cuticle of some insects, such as the locusts, *S. gregaria* and *Locusta migratoria*, appears to be exclusively sclerotized by NADA, as no β-alanine has been obtained from their acid hydrolysates. No cuticles have yet been reported to be sclerotized exclusively by NBAD. A correlation appears to exist between the intensity of brown color of the fully sclerotized cuticle and the amounts of NBAD taking part in the sclerotization process: cuticles that are sclerotized exclusively by NADA are colorless or very lightly straw-colored, and the more NBAD dominates in the process the darker brown will the cuticle become (Brunet, 1980; Hopkins *et al*., 1984). Czapla *et al*. (1990) reported that cuticular strength in five differently colored strains of the cockroach *Blattella germanica* correlated well with their concentrations of β-alanine and NBANE, whereas dopamine concentration correlated with melanization. Cuticular strength as well as cuticular concentrations of β-alanine and NBAD increased more rapidly in the rust-red wild-type of *T. castaneum* than in the black mutant strain, whereas cuticular dopamine increased more rapidly in the black strain than in the wild-type (Roseland *et al*., 1987).

Significant amounts of sclerotization precursors are often present as conjugates before the onset of sclerotization. The conjugates, which can be glucosides, phosphates, or sulfates (Brunet, 1980; Kramer and Hopkins, 1987), are not easily oxidized, and

have to be hydrolyzed to free catechols before they can take part in sclerotization. It is assumed that the catechol conjugates serve as a storage reservoir of catecholamines ready to be used when the need for sclerotization arises (Brunet, 1980). A dopamine conjugate, identified as the 3-O-sulfate ester, is present in the hemolymph of newly ecdysed cockroaches, and its concentration decreases rapidly as the cockroach cuticle sclerotizes. The sulfate moiety is not transferred into the cuticle, and removal of sulfate and acylation of the liberated dopamine to NADA and/or NBAD most likely occurs in the epidermal cells (Bodnaryk and Brunet, 1974; Czapla *et al.*, 1988, 1989).

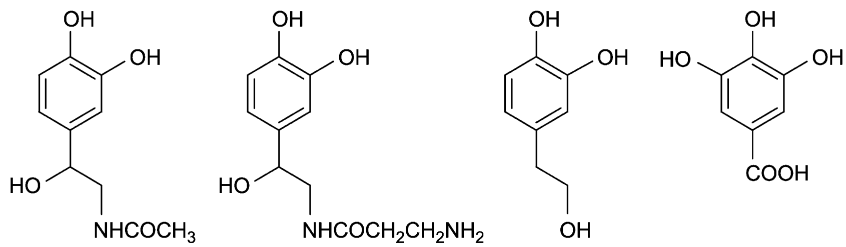
Hopkins *et al.* (1984) reported that a large fraction of the various catecholamines in *M. sexta* hemolymph and cuticle is present as acid labile conjugates. In larval and pupal hemolymph these conjugates are mainly 3-O-glucosides together with small amounts of the 4-O-glucosides, whereas adult hemolymph contains more of the 4-O-glucoside than of the 3-O-glucoside (Hopkins *et al.*, 1995). Both conjugated and unconjugated forms of NADA and NBAD were extracted from *M. sexta* cuticle (Hopkins *et al.*, 1984), indicating that the epidermal cells possess transporting systems for conjugated as well as unconjugated catecholamines. It is also likely that a β-glucosidase activity is present inside the *Manduca* cuticular matrix, to catalyze the release of the sclerotization precursors from their conjugates, as the conjugated forms cannot be used directly for sclerotization.

4.4.3.2. Putative Sclerotization Precursors

So far, convincing evidence that they function as cuticular sclerotization precursors has only been obtained for NADA and NBAD, but several other compounds have been described as likely sclerotization precursor candidates, such as dopamine (**3**), *N*-acetyl-norepinephrine (NANE) (**14**), *N*-β-alanyl-norepinephrine (NBANE) (**15**), and 3,4-dihydroxyphenylethanol (DOPET) (**16**) (Figure 3). It is likely that they all have some role to play in sclerotization.

4.4.3.2.1. Dopamine Dopamine is precursor for both NADA and NBAD synthesis, and it can also be precursor for black, insoluble melanins. Melanin-like materials are of common occurrence in cuticular structures, and they can be present either in microscopic granules or homogeneously distributed within the cuticular matrix (Kayser-Wegmann, 1976; Kayser-Wegmann and Kayser, 1983; Hiruma and Riddiford, 1988). The granules are produced within epidermal cells and transported to the subepicuticular space via long cellular projections (Curtis *et al.*, 1984; Kayser, 1985), and apparently the melanin in the granules is linked to granular proteins, but not to proteins in the cuticular matrix. The diffusely distributed melanins appears to be formed *in situ* within the cuticular matrix, and covalent links are probably formed between the polymeric melanin and the matrix proteins, thereby rendering the proteins more stable and insoluble, contributing to both darkening and increased mechanical stiffness of the cuticle. It will therefore be difficult in all cases to discern between the process of melanization of cuticle and the process of sclerotization. The observation that cuticular incorporation of radioactive tyrosine is nearly the same in wild-type and albino mutant of *S. gregaria* (Karlson and Schlossberger-Raecke, 1962) shows that melanization does not play a major role in sclerotization, but handling of small samples of melanized or albino cuticle indicates that it may have a minor role, as the melanized samples appear to be more brittle than the unmelanized (S.O. Andersen, unpublished data). A quantitative comparison of the mechanical properties of the two cuticular samples, to see to what extent melanization influences the physical properties of the material, would be interesting.

Melanin can be formed from either DOPA or dopamine by slightly different routes. DOPA can via dopachrome and 5,6-dihydroxyindole carboxylic acid be transformed to 5,6-dihydroxyindole,



14 **15** **16** **17**

Figure 3 Putative precursors for cuticular sclerotization. **14**, *N*-acetylnorepinephrine (NANE); **15**, *N*-β-alanylnorepinephrine (NBANE); **16**, 3,4-dihydroxyphenylethanol (DOPET); **17**, gallic acid.

which after enzymatic oxidation polymerizes to melanin, and transformation of dopamine to 5,6-dihydroxyindole goes via dopamine chrome. The formation of the two intermediates, dopachrome and dopamine chrome, is catalyzed by different, but related enzymes. Dopamine appears to be a better substrate than DOPA for formation of cuticular melanin, and the melanins formed in hemolymph during defense reactions appear to be formed mainly via the DOPA pathway. In *Drosophila melanogaster* the products of two of the *yellow* genes, *yellow*-f and *yellow*-f2, are dopachrome-conversion enzymes, which have low activities towards dopamine chrome. The product of the gene *yellow*-y, involved in wing and cuticle melanization, has significant sequence similarity to yellow-f and yellow-f2 proteins, but is apparently devoid of dopachrome-conversion activity (Han *et al*., 2002). Whether the yellow-y protein can catalyze the conversion of dopamine chrome to 5,6-dihydroxyindole was not reported.

The black body color of the *D. melanogaster* mutants *black* and *ebony* is due to the inability of these mutants to produce sufficient NBAD for cuticular sclerotization: *black* is defective in the synthesis of β-alanine, and can be rescued by injection of β-alanine, and *ebony* is defective in the enzyme NBAD-synthetase, and cannot be rescued by injection of β-alanine (Wright, 1987). The result is that in both mutants some of the dopamine not used for NBAD synthesis is channeled into cuticular melanin production. Both stiffness and puncture resistance of the cuticle are decreased in the mutants, and electron microscope studies show that the cuticular chitin lamellae are abnormally wide and diffuse (Jacobs, 1978, 1980, 1985), indicating that even if dopamine-derived melanin can take part in cuticular stabilization, the result is inferior to the material obtained by NBAD sclerotization.

The *tan* mutant of *D. melanogaster,* which is characterized by absence of the wild-type cuticular melanin pattern, has low activity of the enzyme *N*-β-alanyldopamine-hydrolase, catalyzing the hydrolysis of NBAD to β-alanine and dopamine (Wright, 1987). The enzyme systems, responsible for NBAD synthesis and NBAD hydrolysis, respectively, are probably located in different compartments, and the presence of melanin in some but not all cuticular regions of wild-type fruitflies could be explained by the local presence of the NBAD-hydrolase within the matrix of the melanizing regions, where it will hydrolyze some of the NBAD secreted from the epidermal cells, creating a dopamine concentration sufficient to stimulate a localized melanin production.

4.4.3.2.2. *N*-acetylnorepinephrine (NANE) and *N*-β-alanylnorepinephrine (NBANE) NANE and NBANE are special cases among the cuticular catechols, as they can be considered both as byproducts of the sclerotization process and precursors for sclerotization. They have been reported to occur both free and as an *O*-glucoside in hemolymph and integument in several insects (Hopkins *et al*., 1984, 1995; Morgan *et al*., 1987; Czapla *et al*., 1989). NANE and NBANE can be generated within the cuticle, when the enzymatically produced *p*-quinone methides of NADA and NBAD react with water instead of reacting with cuticular proteins or isomerizing to dehydro-derivatives, but they may also be produced by hydrolysis of some unidentified products of the sclerotization process. Mild acid treatment of sclerotized cuticles can release some NANE and NBANE from the cuticular structure, probably due to hydrolysis of a bond between the β-position of the catechols and some cuticular constituent. The nature of the bond is uncertain, but it could well be an ether linkage connecting the acyldopamine side chain to chitin. Formation of an ether, β-methoxy-NADA, occurs when isolated pieces of cuticle or extracted cuticular enzymes act upon NADA in the presence of methanol; the compound is acid labile and is readily hydrolyzed to free NANE (Andersen, 1989c; Sugumaran *et al*., 1989b).

NANE can be covalently incorporated into the cuticular matrix during sclerotization, indicating that the compound can serve as a sclerotization precursor (Andersen, 1971), and this is probably also the case for NBANE. When radioactively labeled NANE was injected into newly ecdysed locusts a significant fraction of the radioactivity (about 10%) was incorporated into the cuticle, and hydrolysis of the cuticle was needed to release the activity. Acid hydrolysis of cuticle from locusts injected with labeled norepinephrine resulted in the release of both labeled norepinephrine and arterenone, whereas little radioactivity was present in the neutral ketocatechol fraction. This is in contrast to parallel experiments when labeled dopamine was injected and nearly all the radioactivity was recovered as neutral ketocatechols, indicating that the cuticular enzymes can catalyze the incorporation of norepinephrine and NANE into the cuticular matrix, but not as efficiently and not by the same route as the incorporation of dopamine and NADA.

4.4.3.2.3. Dihydroxyphenylethanol (DOPET) The third putative sclerotization precursor, 3,4-dihydroxyphenylethanol (DOPET), is also present in the hemolymph and integument of insects; it has been obtained from cuticle of the cockroach *Periplaneta*

americana (Atkinson *et al*., 1973b; Czapla *et al*., 1988) and the beetle *Pachynoda sinuata* (Andersen and Roepstorff, 1978), and it can function as substrate for the cuticular phenoloxidases. Extraction and acid hydrolysis of sclerotized cuticles have yielded various DOPET derivatives, suggesting that DOPET is transported into the cuticle and incorporated into the cuticular matrix during sclerotization. Adducts of DOPET and histidine have been obtained by acid hydrolysis of sclerotized *Manduca* pupal cuticle and identified by means of mass spectrometry (Kerwin *et al*., 1999). A dihydroxyphenyl-dihydrobezodioxine-type adduct of DOPET and NADA has also been extracted from sclerotized beetle (*P. sinuata*) cuticle (Andersen and Roepstorff, 1981), but the metabolism of DOPET in insects has not been studied in much detail.

The relative roles of dopamine, NANE, NBAD, and DOPET compared to the two major sclerotization precursors, NADA and NBAD, have never been properly established. The compounds are probably only of minor importance for the mechanical properties of sclerotized cuticles, but their involvement in the sclerotization process may play a role in fine-tuning of the cuticular properties.

4.4.3.2.4. Other sclerotization precursors It has been reported that improved growth of the tree locust, *Anacridium melanorhodon* can be obtained by addition of gallic acid (**17**) and other plant phenols to its food, and that the ingested plant phenols are incorporated into the cuticle and may contribute to its stabilization (Bernays *et al*., 1980; Bernays and Woodhead, 1982).

4.4.3.3. Noncuticular Sclerotization

Although the sclerotization process has mainly been studied in the cuticle of insects, related processes are used for stabilization of other insect materials, such as silks, egg capsules, and chorions, and their structures are shown in **Figure 4**. Pryor (1940a) showed that 3,4-hydroxybenzoic acid (**18**) is a sclerotization precursor for egg capsules of the cockroach *Blatta orientalis*, and Pau and Acheson (1968) reported that other cockroach species use 3,4-dihydroxybenzyl alcohol (**19**) as a precursor for egg capsule sclerotization. The praying mantid, *Hierodula patellifera* uses various catechol derivatives for stabilizing the egg capsules; besides NADA and NBAD the following catechols have been reported: *N*-malonyldopamine (**20**), *N*-(*N*-acetyl-β-alanyl) dopamine (**21**), and *N*-(*N*-malonyl-β-alanyl)dopamine (**22**) (Kawasaki and Yago, 1983; Yago and Kawasaki, 1984; Yago *et al*., 1984). The cockroach catechols are stored in the left colleterial gland as 4-*O*-glucosides (Brunet, 1980), and the catechols in praying mantids are stored as 3-*O*-glucosides (Yago

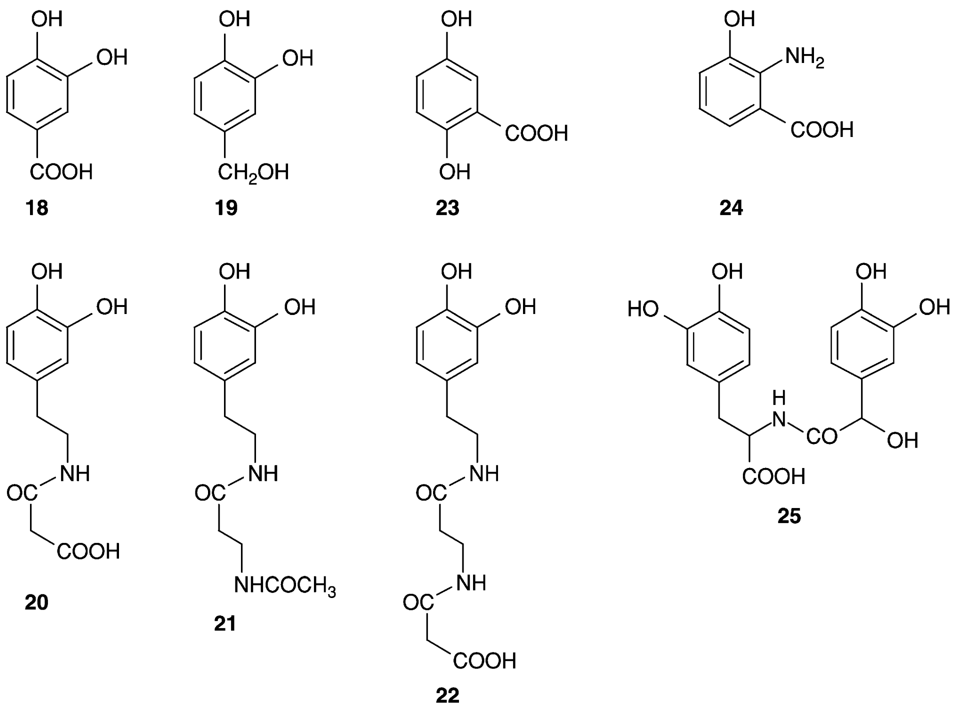


Figure 4 Precursors for noncuticular sclerotization. **18**, 3,4-dihydroxybenzoic acid (protocatechuic acid); **19**, 3,4-dihydroxybenzyl alcohol; **20**, *N*-malonyldopamine; **21**, *N*-(*N*-acetyl-β-alanyl)dopamine; **22**, *N*-(*N*-malonyl-β-alanyl)dopamine; **23**, gentisic acid; **24**, 3-hydroxyanthranilic acid; **25**, *N*-(3,4-dihydroxyphenyllactyl)DOPA.

et al., 1984). The right colleterial gland in both species contains a glucosidase, and when the secretions from the two glands are mixed, the glucosides are hydrolyzed, and the liberated catechols are enzymatically oxidized to *o*-quinones, which in mantids may be further converted to a highly reactive intermediates, presumably *p*-quinone methides (Yago *et al.*, 1990).

The eggs of the grasshopper *Melanoplus sanguinipes* are deposited in soil and covered by a frothy proteinaceous material produced in the female accessory glands. An acid-labile conjugate of 3,4-dihydroxybenzoic acid was extracted from the accessory glands, and unconjugated 3,4-dihydroxybenzoic acid was obtained from the egg pods shortly after their deposition, indicating that it may be involved in sclerotization of the material (Hopkins *et al.*, 1999).

Some moths, such as saturniid silkmoths (*Actias selene, Antheraea pernyi, Hyalophora cecropia, H. gloveri,* and *Samia cynthia*), produce silk cocoons that are sclerotized after spinning. The silks are white when spun and remain white as long as they are in a dry atmosphere: When exposed to humid conditions a sclerotization process is initiated and the cocoons become brown. Glucosides of gentisic acid (**23**) and 3-hydroxyanthranilic acid (**24**) have been obtained from newly spun silks, and β-glucosidases and diphenoloxidases are present, indicating that silk sclerotization follow a pattern similar to that of the other sclerotization processes (Brunet and Coles, 1974). Manthey *et al.* (1992) reported that oxidation of 3-hydroxyanthranilic acid in the presence of proteins leads to formation of adducts between 3-hydroxyanthranilic acid and tyrosine residues in the proteins, and such adducts were isolated from cocoons of *H. gloveri* and *S. cynthia*. It appears that the adducts are formed via a radical–radical coupling mechanism.

The newly spun silk of the Japanese giant silkmoth, *Dictyoploca japonica* contains a diphenoloxidase and a catecholic derivative, which was tentatively identified as *N*-(3,4-dihydroxyphenyllactyl)DOPA (**25**) (Kawasaki and Sato, 1985). It is apparently not glycosylated and no glucosidase activity was found in the silk. The presence of two catecholic groups in this sclerotization precursor may make it a more effective cross-linking agent than precursors with only a single catechol group.

4.4.4. Cuticular Enzymes and Sclerotization

The study of cuticular enzymes has to a large extent been concerned with characterization of enzymes assumed to play a role in cuticular sclerotization, mainly those involved in catechol oxidation and quinone isomerization, and there has been a tendency to neglect the possible presence of other types of enzymes that may play a role in cuticular stabilization. Catechol oxidation is an important step in sclerotization, wound healing, and immune responses in insects, and it is often difficult to decide whether a given cuticular phenoloxidase activity is of importance for sclerotization or whether its main role is to take part in defense reactions. It is therefore necessary to be extremely careful in interpreting the observations reported for cuticular enzymes.

4.4.4.1. *ortho*-Diphenoloxidases

After being transferred from the epidermal cells into the cuticle the catecholic sclerotization precursors may encounter various enzymes, such as *o*-diphenoloxidases, laccases, and peroxidases, capable of oxidizing them to quinones, but the relative roles of these activities are still uncertain. Insects contain inactive proenzymes for *o*-diphenoloxidases both in hemolymph and in the cuticle, and the proenzymes can be activated by a process initiated by wounds or the presence of small amounts of microbial cell wall components and involving limited proteolysis (Ashida and Dohke, 1980; Ashida and Brey, 1995). The *o*-diphenoloxidases can oxidize a wide range of *o*-diphenols, but not *p*-diphenols; they possess low hydroxylating activity towards monophenols, such as tyrosine and tyramine; and they are copper-containing enzymes readily inhibited by thioureas and Na-diethyldithiocarbamate. They have been isolated and characterized from both soft, nonsclerotizing cuticles, such as larval cuticle of *Bombyx mori* (Ashida and Brey, 1995; Asano and Ashida, 2001a), and from blowfly larval cuticles (Barrett, 1987a, 1987b, 1991) and sclerotizing pupal cuticle of *Manduca sexta* (Aso *et al.*, 1984; Morgan *et al.*, 1990). The amino acid sequences of *o*-diphenoloxidases from various insect species have been deduced from the corresponding DNA sequences (Fujimoto *et al.*, 1995; Hall *et al.*, 1995; Kawabata *et al.*, 1995). The insect *o*-diphenoloxidases differ from diphenoloxidases (tyrosinases) from other organisms with regard to both substrate specificity and amino acid sequence (Sugumaran, 1998; Chase *et al.*, 2000).

According to the established sequences of insect prophenoloxidase genes, their gene products do not possess an N-terminal signal peptide sequence (Sugumaran, 1998), which is in contrast to what is commonly observed for proteins destined for export from the cells. The silk moth *B. mori* has genes for two *o*-diphenoloxidase proenzymes, and in the larvae the products of both genes are present in both

hemolymph and cuticle, the only difference between the cuticular and hemolymphal forms being that several methionine residues are intact in the hemolymphal proenzymes, and they are oxidized to methionine sulfoxides in the cuticular proenzymes. The activated cuticular enzymes have nearly the same substrate specificity as the hemolymph enzymes; the main difference between the proenzymes is that in contrast to the unmodified hemolymph form the oxidized cuticular form cannot be transported across the epidermal cell layer, indicating that the epidermal transport of the proenzymes is a one-way traffic, from hemolymph to cuticle (Asano and Ashida, 2001a, 2001b).

The enzymatic properties of the diphenoloxidases purified from hemolymph and from pharate pupal cuticle of *M. sexta* are very similar, suggesting a close relationship between the enzymes (Aso *et al*., 1985; Morgan *et al*., 1990), and it seems probable that they, like the *B. mori* enzymes, are derived from the same gene(s).

When activated, both cuticular and hemolymph *o*-diphenoloxidases are very sticky i.e., they tend to aggregate and stick to any available surface and macromolecule, which will hinder diffusion of the active enzymes from the site where they became activated. The ultrastructural localization of *o*-diphenoloxidase activity in the larval cuticle of the blowfly *Lucilia cuprina* was described by Binnington and Barrett (1988) who observed activity in the epicuticular filaments. Activity was also observed in the procuticle, but only when the cuticle had been damaged beforehand, and the activity was limited to the close neighborhood of the wound, indicating that wounding is needed to activate the enzyme, and that the active enzyme remains in the vicinity of the wound.

The prophenoloxidase in larval cuticle of *B. mori* was localized by immunocytochemical methods to the nonlamellate endocuticle, where it was randomly distributed, and to an orderly arrayed pattern in the lamellate endocuticle, but it appeared to be absent from the cuticulin layer and from the epidermal cells (Ashida and Brey, 1995).

The role of the various cuticular *o*-diphenoloxidases in cuticular sclerotization is problematic; their presence as inactive proenzymes, which have to be activated, their close relationship to the hemolymphal phenoloxidases, and their abundance in nonsclerotizing cuticle indicate that their role is to take part in defense against wounding and microorganisms and not to be involved in sclerotization. It is, however, difficult to state with certainty that they do not play some role in sclerotization.

4.4.4.2. Laccases

Laccase-type phenoloxidases have been reported to be present in dipteran larval cuticles shortly before and during puparium sclerotization, such as *Drosophila virilis* (Yamazaki, 1969), *D. melanogaster* (Sugumaran *et al*., 1992), *Calliphora vicina* (Barrett and Andersen, 1981), *Sarcophaga bullata* (Barrett, 1987a), and *L. cuprina* (Barrett, 1987b), and such enzymes have also been described from pupal cuticles of *B. mori* (Yamazaki, 1972) and *M. sexta* (Thomas *et al*., 1989) as well as from adult cuticle of the locust *Schistocerca gregaria* (Andersen, 1978). The nucleotide sequences for two laccase genes from *M. sexta* and a laccase gene from the mosquito *Anopheles gambiae* have recently been deposited in the GenBank, and the accession numbers for the corresponding proteins are: AAN1706, AAN1707, and AAN17505, respectively. The insect laccases are structurally related to laccases of plant or fungal origin. In contrast to the insect diphenoloxidases the laccase gene products contain a typical signal peptide sequence, indicating that the enzymes are secreted into the extracellular space.

In larval cuticles of *D. virilis* (Yamazaki, 1969) and *L. cuprina* (Binnington and Barrett, 1988) laccase activity makes its appearance shortly before pupariation. In both species the enzyme activity decreases gradually as puparial sclerotization progresses. Laccase activity can be demonstrated a few days before ecdysis in pharate cuticle of adult locusts, *S. gregaria*; it remains at high levels for at least 2 weeks after ecdysis, and activity has also been demonstrated in nymphal exuviae, indicating that the locust enzyme is not inactivated by sclerotization (S.O. Andersen, unpublished data).

Laccases are active towards a broad spectrum of *o*- and *p*-diphenols: NBAD and NADA are among the best *o*-phenolic substrates tested, and methylhydroquinone is the best *p*-diphenolic substrate. Insect laccases are not inhibited by compounds, such as thiourea, phenylthiourea, and Na-diethyldithiocarbamate, which are effective inhibitors of *o*-diphenoloxidases, but they are inhibited by carbon monoxide and millimolar concentrations of fluorides, cyanides, and azides (Yamazaki, 1972; Andersen, 1978; Barrett and Andersen, 1981; Barrett, 1987a). The laccases are resistant towards treatments inactivating many enzyme activities; the *S. gregaria* laccase remains active after blocking available amino and phenolic groups by dinitrophenylation or dansylation, and it survives temperatures up to about 70 °C, but it is inactivated by treatment with tetranitromethane, which nitrates tyrosine residues (Andersen, 1979b). The laccases

appear to be firmly linked to the cuticular structure; typically they cannot be extracted by conventional protein extractants, but are readily extracted after limited tryptic digestion of the yet-unhardened cuticle (Yamazaki, 1972; Andersen, 1978). The enzyme was obtained from *C. vicina* larval cuticle by prolonged extraction at pH 8 without addition of any protease, but as latent protease activity is present in the cuticle the release of laccase from the cuticular residue may be due to proteolysis (Barrett and Andersen, 1981). The enzyme is not released by tryptic digestion of already sclerotized cuticle.

The ultrastructural localization of laccase activity has been studied in the *L. cuprina* larval cuticle (Binnington and Barrett, 1988) and enzyme activity was observed in the inner epicuticle of late third instar larvae (about to pupariate), but not in epicuticle of younger larvae. The laccase activity in *L. cuprina* larval cuticle could be demonstrated without prior activation, in contrast to the cuticular *o*-diphenoloxidases, indicating that the laccase is not deposited as an inactive precursor in this insect, and neither is an inactive proenzyme likely to be present in pharate locust cuticle since enzyme activity could be demonstrated without any activating treatment. A pro-laccase has been purified and partially characterized from cuticle of newly pupated pupae of *B. mori* (Ashida and Yamazaki, 1990). The inactive pro-laccase could be activated by treatment with various proteolytic enzymes, and the substrate specificities of the laccase variants obtained depended upon the protease used for activation.

4.4.4.3. Cuticular Peroxidases

Several different routes for the oxidation of catechols to *o*-quinones may be of advantage for an insect, especially if they can be regulated independently in various cuticular regions, and peroxidase activity may provide such an alternative route. Peroxidase activity has been demonstrated by histochemical methods in proleg spines of *Calpodes ethlius* larvae (Locke, 1969) and in larval and pupal cuticle of *Galleria mellonella* and *Protophormia terraenovae* (Grossmüller and Messner, 1978; Messner and Janda, 1991; Messner and Kerstan, 1991), and such activity is also observed intracellularly in different cell types in insects. It is not known whether the cuticular peroxidase activities are identical to the intracellular enzymes, as the cuticular activity has never been properly characterized. Proteins can be cross-linked by means of the peroxidase system, and it has been suggested that the enzyme could be involved in cuticular sclerotization (Hasson and Sugumaran, 1987). A peroxidase is likely to be involved in the cross-linking of the rubberlike elastic cuticular protein resilin (Andersen, 1966; Coles, 1966). This cuticular protein is cross-linked by oxidative coupling of tyrosine residues during its extracellular deposition, and the tyrosine radicals needed for the coupling may be formed by a peroxidase catalyzed oxidation process. Peroxidases can also oxidize catechols to semiquinone radicals, two of which readily dismutate to form an *o*-quinone and a catechol. The enzyme needs hydrogen peroxide as one of its substrates, and Candy (1979) reported that locust cuticle contains a glucose oxidase activity, which oxidizes glucose to D-gluconate with concomitant production of hydrogen peroxide. It was suggested that the hydrogen peroxide produced may participate in sclerotization reactions. Candy (1979) also reported the presence in locust cuticle of several other enzymes involved in hydrogen peroxide metabolism, such as peroxidase, catalase, and superoxide dismutase.

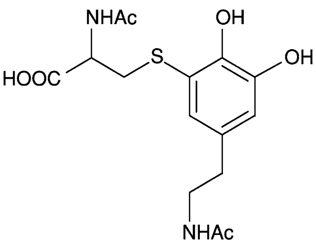
Peroxidase activity in solid cuticle may be involved in the production of dityrosine cross-links and in oxidizing catechols to quinones for sclerotization. Dityrosine has so far not been demonstrated in sclerotized cuticle from insects, but dityrosine as well as brominated dityrosines have been obtained from the hardened exocuticle of the crab *Cancer pagurus* (Welinder *et al*., 1976). The eggshells of *D. melanogaster* are stabilized by formation of dityrosine cross-links between the protein chains (Petri *et al*., 1976; Mindrinos *et al*., 1980), and the hardening of *Aedes aegypti* egg chorion includes both peroxidase-mediated protein cross-linking through dityrosine formation and diphenoloxidase-catalyzed chorion melanization (Li *et al*., 1996). The hydrogen peroxide necessary for dityrosine formation in *A. aegypti* chorion is produced by an enzymatic process by which NADH is oxidized with concomitant reduction of molecular oxygen to hydrogen peroxide. The necessary supply of NADH for this process is provided by enzyme-catalyzed oxidation of malate coupled to reduction of NAD^+ (Han *et al*., 2000a, 2000b). It is unknown whether a similar system for providing hydrogen peroxide operates during sclerotization of insect cuticles.

4.4.4.4. *ortho*-Quinones and *para*-Quinone Methides

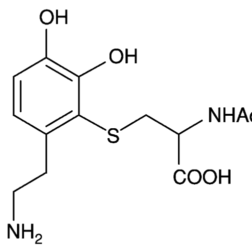
The three types of oxidases, *o*-diphenoloxidases, laccases, and peroxidases, can all oxidize NADA and NBAD to their respective *o*-quinones. The *o*-quinones are reactive compounds, they can spontaneously form adducts by reaction with available nucleophilic groups, and they can serve as substrates for other cuticular enzymes, *o*-quinone and *p*-quinone

methide isomerases, catalyzing isomerization to *p*-quinone methides, which also react readily with nucleophilic compounds. In the traditional sclerotization scheme, suggested by Pryor (1940a, 1940b), *o*-quinones were supposed to react preferably with ε-amino groups from lysine residues, but solid state nuclear magnetic resonance (NMR) studies have shown that the imidazole group in histidine residues is the preferred cuticular target for reaction with quinones (Schaefer *et al*., 1987; Christensen *et al*., 1991).

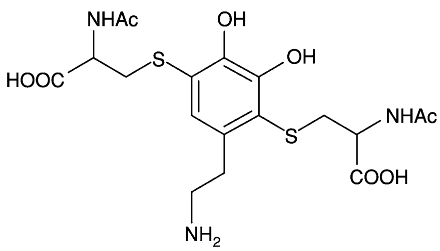
Incubation of blowfly (*Sarcophaga bullata)* larval cuticle with NADA and *N*-acetylcysteine resulted in formation of an adduct (**26**) where the sulfur atom is linked to the 5-position of the NADA moiety (Sugumaran *et al*., 1989a), indicating that SH-groups are good acceptors for oxidized NADA (**Figure 5**). Electrochemical oxidation of dopamine to dopamine quinone in the presence of *N*-acetylcysteine gave a mixture of C-5 and C-2 (**27**) monoadducts together with some of the disubstituted product, 2,5-S,S′-di(*N*-acetylcysteinyl)dopamine (**28**) (Xu *et al*., 1996a; Huang *et al*., 1998). Since the monoadducts are more readily oxidized to quinones than is the parent catechol, a monoadduct formed between an oxidized catechol and a protein-linked cysteine will be more prone than a free catechol to be reoxidized to quinone, which can react with a cysteine residue in neighboring protein chain to form a covalent cross-link between the proteins. Thus cysteine–catechol based cross-links are possible,



26



27



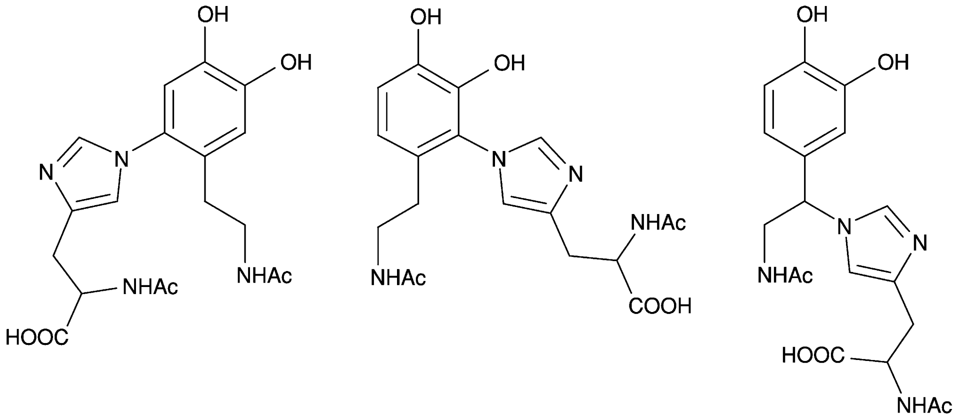
28

Figure 5 Adducts formed by reaction of *N*-acetylcysteine with the *o*-quinones of NADA and dopamine. **26**, 5-S-(*N*-acetylcysteinyl)-*N*-acetyldopamine; **27**, 2-S-(*N*-acetylcysteinyl)-dopamine; **28**, 2,5-S,S′-di-(*N*-acetylcysteinyl)-dopamine.

but they appear not to play an important role in cross-linking cuticular proteins since these proteins typically contain neither cysteine nor cystine. Among all the cuticular proteins that have been sequenced so far, only a few contain residues of sulfur-containing amino acids, and it seems unlikely that sulfur can have an important role in sclerotization. Perhaps the scarcity of cystine and cysteine in cuticular proteins is related to the readiness with which they react with *o*-quinones. When locust cuticle is incubated with NADA and benzenesulfinic acid, the oxidized NADA is trapped by adduct formation with the sulfinic acid, and before all sulfinic acid has been consumed by adduct formation no *o*-quinone is available for reaction with cuticular proteins or isomerization and further metabolism to polymeric compounds (S.O. Andersen, unpublished data). The presence of significant amounts of free SH-groups in the cuticular matrix proteins could in a similar way delay further metabolism of the *o*-quinones and thereby cause suboptimal sclerotization.

Electrochemical oxidation of NADA to NADA-quinone in the presence of *N*-acetylhistidine gave monoadducts (**Figure 6**), where a nitrogen atom in the imidazole ring is linked to either the C-6 (**29**) or the C-2 ring position (**30**) in NADA, the C-6 position being the preferred position (Xu *et al*., 1996b). Electrochemical characterization of the C-6 and C-2 *N*-acetylhistidine-NADA adducts showed that both adducts are more difficult to oxidize than NADA itself (Xu *et al*., 1996b), indicating that formation of adducts involving two *N*-acetylhistidine residues linked to the same NADA residue is not very likely.

Oxidation of a mixture of NADA plus *N*-acetylhistidine by means of larval cuticle from *H. cecropia* gave a mixture of products, and adduct formation to the C-6 ring position as well as to the β-position of the side chain (**31**) was observed (**Figure 6**) (Andersen *et al*., 1991, 1992c). The formation of a side chain adduct indicates activation of the β-position, probably due to formation of the *p*-quinone methide, demonstrating that cuticular oxidation of catechols is more complex than electrochemical oxidation. From acid hydrolysates of *M. sexta* pupal cuticle, adducts have been obtained where histidine is linked to either the C-6 ring position or the β-position of dopamine or to the corresponding positions in DOPET, demonstrating that adduct formation to histidine residues occurs during *in vivo* sclerotization (Xu *et al*., 1997; Kerwin *et al*., 1999), and confirming that DOPET has a role as a sclerotization precursor. Direct evidence that covalent bonds are formed between acyldopamines and

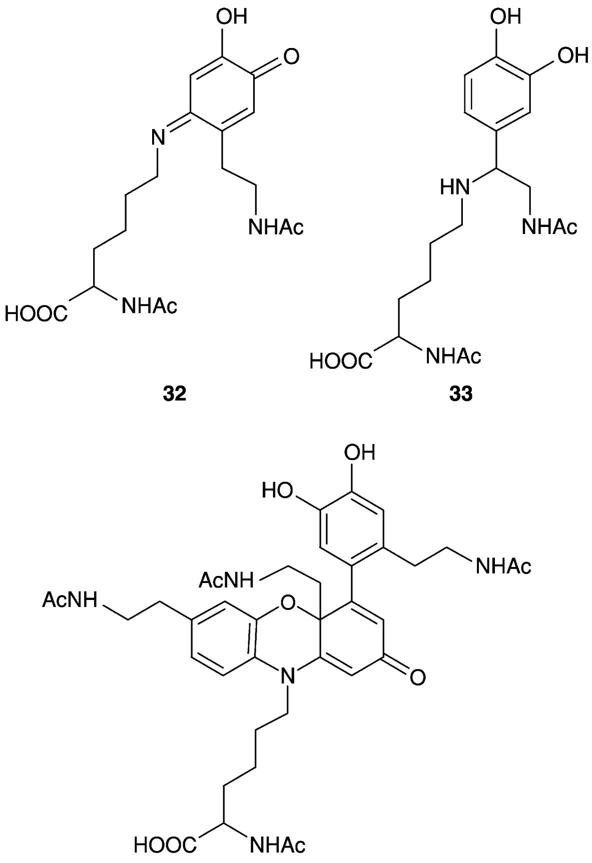


29 **30** **31**

Figure 6 Adducts formed by reaction of *N*-acetylhistidine with NADA-*o*-quinone or NADA-*p*-quinone methide. **29**, 6-(*N*-acetylhistidyl)-*N*-acetyldopamine; **30**, 2-(*N*-acetylhistidyl)-*N*-acetyldopamine; **31**, 7-(*N*-acetylhistidyl)-*N*-acetyldopamine.

histidine residues during sclerotization had previously been obtained by solid state NMR studies, utilizing incorporation of isotopically labeled dopamine, histidine, and β-alanine into sclerotizing pupal cuticle of *M. sexta* (Schaefer *et al.*, 1987; Christensen *et al.*, 1991). The spectra demonstrated the presence of bonds between nitrogen atoms in the imidazole ring of histidine and ring positions and β-position of the side chain of dopamine. The formation of covalent bonds involving the amino group of β-alanine and the ε-amino group of lysine was also indicated. Furthermore, catecholamine-containing proteins have been purified and partially characterized from sclerotizing *M. sexta* pupal cuticle, and NBANE was released from these proteins on mild acid hydrolysis, indicating the presence of a bond between the β-position of the side chain of NBAD and some amino acid residues in the proteins (Okot-Kotber *et al.*, 1996).

Incubation of larval cuticle of *H. cecropia* with NADA together with compounds containing a free amino group, α-*N*-acetyllysine or β-alanine, resulted in the formation of a number of products (Figure 7), and adducts were identified with the amino groups linked to the 6-position of the ring in NADA (**32**). These adducts have a quinoid structure, indicating that the primary catecholic product is readily oxidized, in contrast to the adducts formed to the β-position of the side chain (**33**), which are stable in their catecholic form (Andersen *et al.*, 1992b). Adduct formation to either histidine and lysine residues during cuticular oxidation of NADA appears to be so much slower than adduct formation to cysteine that a significant fraction of the *o*-quinones obtained from NADA or NBAD will be available for the isomerase catalyzing further metabolism of the quinones. Prolonged incubation of NADA and α-*N*-acetyllysine together with cuticle resulted in the formation of 4-phenylphenoxazin-2-ones (**34**), which are composed of three NADA residues joined to one α-*N*-acetyllysine residue (Peter *et al.*, 1992).



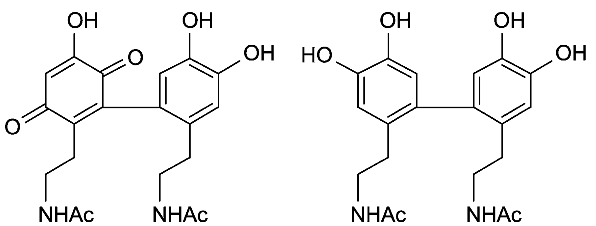
32 **33** **34**

Figure 7 Adducts formed by cuticular oxidation of a mixture of NADA and α-*N*-acetyllysine.

The structure of one of the products formed during incubation of insect cuticles with NADA plus *N*-acetylamino acids indicated that *o*-quinone of NADA can react with water to form *N*-acetyl-3,4,6-trihydroxyphenylethylamine (6-hydroxy-NADA), which then couples oxidatively with another NADA residue to give the dimeric compound (**35**) (**Figure 8**) (Andersen *et al*., 1992a). Corresponding derivatives indicating formation of 6-hydroxy-NADA have so far not been reported from naturally sclerotized cuticle, and its formation *in vitro* may be due to the large excess of water in incubation experiments, in contrast to the relatively low water content in sclerotizing cuticle (30–40% of the cuticular wet weight). The incubation of cuticle together with NADA resulted also in the production of small amounts of a product (**36**) consisting of two NADA-quinones linked together via their 6-positions (Andersen *et al*., 1992a). A corresponding 2,6′-linked dimer of NADA was suggested as intermediate in the formation of the above mentioned 4-phenylphenoxazin-2-ones (**34**) (Peter *et al*., 1992).

The side chain β-position in the *p*-quinone methides reacts readily with water, and the lack of stereospecificity in the reaction indicates that the addition of water is nonenzymatic (Peter and Vaupel, 1985). The relative high yields of NANE and NBANE obtained by extraction of various cuticles are probably due to both this reaction and hydrolysis of labile adducts formed with other nucleophiles, such as hydroxyl groups in chitin (see Section 4.4.3.2.2).

The enzyme responsible for quinone isomerization has been partially characterized from larval cuticles from *H. cecropia* (Andersen, 1989c) and the flesh fly *S. bullata* (Sugumaran, 1987; Saul and Sugumaran, 1988), both cuticles belonging to the soft, pliant type. The enzyme is also present in the hemolymph of *S. bullata* (Saul and Sugumaran, 1989a, 1990), where it participates in defense reactions. The enzyme has thus been described from both sclerotizing and nonsclerotizing cuticles and from insect hemolymph, leading to the question whether the same enzyme is involved in both defense reactions and sclerotization. It would be worthwhile



35 **36**

Figure 8 Products obtained by cuticular oxidation of NADA.

to study the temporal and regional distribution of the enzyme in various insect systems.

4.4.4.5. Dehydro-NADA and Dehydro-NBAD

The *p*-quinone methide formed by isomerization of NADA-*o*-quinone can be further isomerized to dehydro-NADA, a NADA derivative carrying a double bond between the α- and β-carbon atoms in the side chain. The enzyme responsible for this isomerization has been called *N*-acetyldopamine quinone methide/1,2-dehydro-*N*-acetyldopamine tautomerase (Saul and Sugumaran, 1989c). The activity has been reported to be present in larval cuticle of *S. bullata* (Saul and Sugumaran, 1989b, 1989c) and *D. melanogaster* (Sugumaran *et al*., 1992). A related enzyme activity catalyzing the isomerization of NBAD quinone methide to dehydro-NBAD has been demonstrated in extracts of *C. vicina* larval cuticle (Ricketts and Sugumaran, 1994). It is probably the same enzyme which leads to the formation of dehydro-NADA and dehydro-NBAD. Locust cuticle catalyzes formation of dehydro-NADA from NADA, whereas NBAD is a poor substrate for the locust enzyme (Andersen, 1989d). It appears that several cuticles, which are sclerotized by mixtures of NADA and NBAD, readily convert NADA to the dehydro-derivative, while conversion of NBAD to dehydro-NBAD only occurs to a minor extent, if at all (Andersen, 1989d; Andersen *et al*., 1996).

It is uncertain whether formation of dehydro-NADA in locust cuticle occurs only via NADA-quinone methide or whether it can also be formed directly from NADA by a route circumventing quinone formation. Inhibition of oxidation of NADA to NADA-quinone resulted in accumulation of dehydro-NADA in locust cuticle, and since it seemed unlikely that accumulation of a product can be caused by decreased production of its precursors, the presence of a specific "NADA-desaturase" was suggested (Andersen and Roepstorff, 1982; Andersen *et al*., 1996).

The dehydro-NADA formed during cuticular sclerotization can be oxidized by both the *o*-diphenoloxidases and laccases present in cuticle, and the resulting unsaturated quinones react spontaneously with other catechols to give substituted dihydroxyphenyl-dihydrobenzodioxines (XIII) (Andersen and Roepstorff, 1982; Sugumaran *et al*., 1988). It has not been established whether the unsaturated quinones will also react with nucleophilic residues in cuticular proteins. Locust cuticle will catalyze *in vitro* formation of adducts between dehydro-NADA and catechols, and dihydroxyphenyl-dihydrobenzodioxine derivatives, corresponding to those extracted from naturally sclerotized cuticle, can be formed in this

way (Andersen and Roepstorff, 1982; Andersen, 1985). It appears that the presence of dihydroxyphenyl-benzodioxine derivatives in sclerotized cuticles can be taken as an indication for the presence of a dehydro-NADA forming activity.

It has been suggested that the cuticular diphenoloxidases, isomerases, and tautomerases occur together in large enzyme complexes, enabling one enzyme to deliver its products directly to the following enzyme for further processing (Andersen *et al.*, 1996; Sugumaran, 1998). The evidence for such complexes is insufficient, but the observation that during *in vitro* incubation with NADA some cuticles convert NADA into benzodioxine dimers with very little formation of NANE, while other cuticles preferably produce NANE, may indicate that the former cuticles contain enzyme complexes with tight coupling between the individual enzymes, so the intermediates will have little chance of reacting with water or the surrounding proteins before they are used as substrates for the next enzyme in the sequence. The sclerotization of some types of cuticle may thus be dominated by reactions involving the dehydro-NADA *o*-quinone, while sclerotization of other types of cuticle is dominated by reactions between the initially generated quinones and the matrix proteins.

The final outcome of the sclerotization process probably depends upon a carefully controlled balance between various enzyme activities and available substrates, a balance that differs between the local cuticular regions according to their mechanical properties. It would be interesting to have quantitative determinations of the relevant enzyme activities in different types of cuticle, and to be able to specifically inhibit the individual enzymes participating in sclerotization to study their individual roles in the total process. Quantitative enzyme activity determinations on pieces of insoluble materials are problematic, due both to slow diffusion of substrates into, and products out of, the pieces and because it is difficult to obtain homogeneous samples for analysis. However, it should be possible to obtain relative values for the activities to be used for comparison of various cuticular regions.

4.4.4.6. Various Catechol Derivatives Obtained from Cuticles

An indication of the quantitative role of the possible sclerotization reaction pathways may be obtained by identification and quantification of the low molecular weight catecholic compounds, which can be extracted from sclerotized cuticles after more or less extensive degradation of the cuticular material. The structures of such compounds may suggest whether they are likely to be products derived from the sclerotizing precursors and perhaps give some indication of the reactions responsible for their generation. The compounds extracted from sclerotized cuticle could represent unused sclerotization precursors, intermediates in the sclerotization process, by-products from the process, and degradation products of protein-bound cross-links and polymers. It is also possible that the extracted compounds have their own distinct functions in the cuticle, quite unrelated to sclerotization; i.e., catechols can thus be precursors for pigments, such as papiliochromes (Umebachi, 1993), or they can function as antioxidants protecting the epicuticular lipids from autoxidation (Atkinson *et al.*, 1973a). 3,4-Dihydroxyphenylacetic acid, which is present in the solid cuticle of many beetle species (Andersen, 1975), could serve the latter purpose, as it apparently takes no part in the sclerotization process (Barrett, 1984, 1990). A detailed study of the formation and fate of the various cuticular compounds is necessary for deciding whether they are related to the sclerotization process.

The mixture of compounds obtained from cuticles by the use of mild extraction methods, such as extraction in distilled water or neutral salt solutions at moderate temperatures, contains compounds that are less modified than those obtained by acidic extraction at elevated temperatures, but a critical and careful interpretation of their structures will be necessary in all cases. Extraction with dilute acids tends to give higher yields of catechols than extraction with water, but the compounds identified in the extracts are often the same (Atkinson *et al.*, 1973b). The higher yield obtained with acids may be due partly to swelling of the cuticular material at low pH values, resulting in easier liberation of trapped compounds, and partly to hydrolysis of acid-labile bonds. Typical extraction products are the sclerotization precursors, NADA and NBAD, and their hydroxylated derivatives, NANE and NBANE, and they can also occur as *O*-glucosyl derivatives. The *O*-glycoside linkage is acid-labile and may not survive prolonged extraction, and the β-hydroxyl group in NANE and NBANE may either be produced by reaction of the *p*-quinone methides of NADA and NBAD with water, or can result from the hydrolysis of the products formed by reaction of a *p*-quinone methide with cuticular proteins and chitin.

3,4-Dihydroxybenzoic acid (**18**) and 3,4-dihydroxybenzaldehyde have been extracted from several types of sclerotized cuticle; they are probably formed by extensive oxidative degradation of the side chain of the sclerotizing precursors, NADA and NBAD. The precise reaction pathway for their

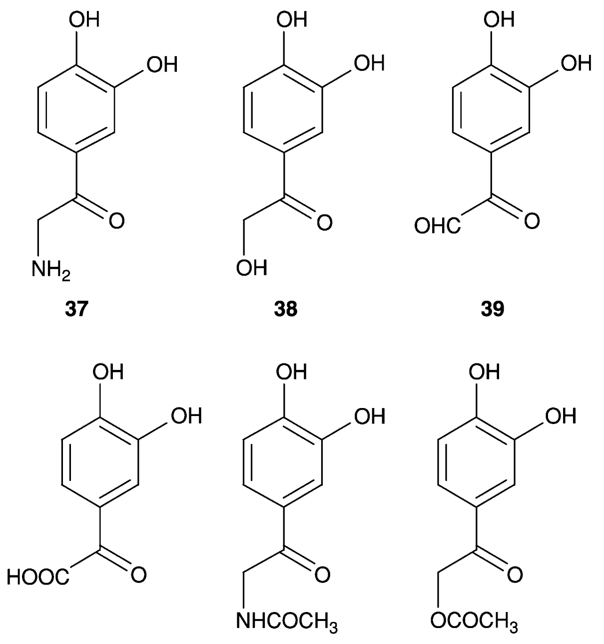


Figure 9 Ketocatechols obtained by mild acid hydrolysis of sclerotized insect cuticle. **37**, arterenone; **38**, 3,4-dihydroxyphenylketoethanol (DOPKET); **39**, 3,4-dihydroxyphenylglyoxal; **40**, 3,4-dihydroxyphenylglyoxylic acid; **41**, *N*-acetylarterenone; **42**, 3,4-dihydroxyphenylketoethylacetate.

formation is not known, but their common occurrence in sclerotized cuticle indicates that the intracuticular environment is highly oxidative during the sclerotization process. The presence of dopamine and norepinephrine in cuticular extracts may be due to deacylation of the *N*-acylated forms, or they may have been transferred directly from epidermal cells to cuticle, either by active transport across the apical cell membrane or by passive leakage through the cell membrane. The black cuticle of the fruitfly mutants *black* and *ebony* contains elevated levels of dopamine (Wright, 1987), which probably have been transferred to the cuticle in the nonacylated state.

Quite large amounts of ketocatechols (**Figure 9**), such as arterenone (37), DOPKET (38), and *N*-acetylarterenone (**41**), as well as 3,4-dihydroxyphenylglyoxal (**39**), 3,4-dihydroxyglyoxylic acid (**40**), and 3,4-dihydroxyphenylketoethylacetate (**42**), can be obtained from sclerotized cuticle by treatment with dilute acids (Andersen, 1970, 1971; Andersen and Barrett, 1971; Andersen and Roepstorff, 1978). The yields of ketocatechols can amount to several percent of the cuticular dry weight (Andersen, 1975; Barrett, 1977), and the type of ketocatechols released depends upon the exact conditions of hydrolysis. It has been argued that they are all

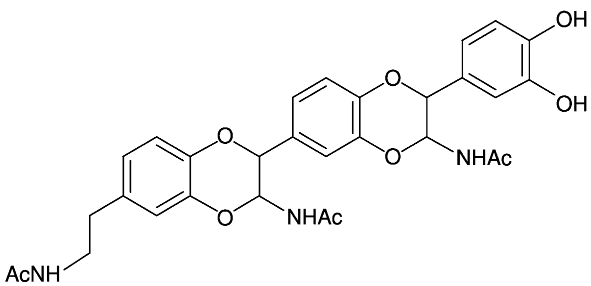


Figure 10 **43**, Trimeric dihydrobenzodioxine derivative from sclerotized locust cuticle.

degradation products of a common precursor in the cuticle (Andersen, 1971).

More complex catechol derivatives can be obtained by extracting sclerotized cuticle at relatively mild conditions, such as concentrated formic acid at ambient temperature or boiling dilute acetic acid. From such extracts a number of dimeric compounds of the dihydroxyphenyl-dihydrobenzodioxine type were isolated and identified (Andersen and Roepstorff, 1981; Roepstorff and Andersen, 1981), and later a trimeric compound (**44**) (**Figure 10**) was obtained by formic acid extraction of sclerotized locust cuticle (Andersen *et al.*, 1992c). Ketocatechols are readily formed when such dimers and oligomers are hydrolyzed with acid (Andersen and Roepstorff, 1981), but the extractable material accounts for only a minor fraction of ketocatechols formed by acid hydrolysis of sclerotized cuticle, and the main fraction of ketocatechols obtainable from cuticle is presumably derived from catecholic material covalently linked to the cuticular proteins and chitin. Since the various dimers can be formed *in vitro* by reaction between oxidized dehydro-NADA and catechols, it is likely that oxidized dehydro-NADA will react with catechols (NADA or NBAD) linked to cuticular proteins to form protein-linked dimers and higher oligomers.

4.4.5. Control of Sclerotization

Sclerotization of insect cuticles varies regionally both with respect to time of initiation, intensity, duration, and balance between NADA- and NBAD-sclerotization, indicating that the process must be controlled locally to give the optimal result.

4.4.5.1. Pre-Ecdysial Sclerotization

In some cuticular regions sclerotization starts in the pharate stage, and these regions attain their final size and shape during the pre-ecdysial deposition of cuticular materials (Cottrell, 1964). Pre-ecdysial

sclerotization may be limited to small, local regions, such as mandibles and spines, or larger regions of cuticle, covering thorax, head, and legs, may be stabilized before ecdysis. In the presclerotized regions sclerotization continues after ecdysis, whereby the already stiffened material is further strengthened. Pre-ecdysial sclerotization has been observed in several cuticular regions in adult honeybees (*Apis mellifera adansonii*) (Andersen *et al.*, 1981), and the matrix proteins in these regions resist extraction with solvents that do not degrade the cuticle. Acid hydrolysis of pre-ecdysial sclerotized honeybee pharate cuticle yielded significant amounts of ketocatechols, and still more was obtained by hydrolysis of the corresponding cuticular regions from mature worker bees where endocuticle deposition was complete, indicating that the same type of sclerotization is involved in both pre- and post-ecdysial stabilization. The wings were the only body region where no pre-ecdysial sclerotization was observed. Their sclerotization occurred rapidly after ecdysis and was nearly complete when the bees left the cell in which they had pupated, confirming that insect wings are not stabilized until they have been expanded to their proper size after emergence.

4.4.5.2. Post-Ecdysial Sclerotization

Many regions of the cuticle are often expanded to a new and bigger size after the insect has emerged from the old cuticle (Cottrell, 1964). In some insects the period from ecdysis to fully expanded cuticle can be rather prolonged, for instance in flies where the newly emerged adult has to dig its way through the substratum in which it pupariated, but many insects can start cuticular expansion as soon as they have escaped from the exuvium. Release of the neurohormone bursicon from the central nervous system is a signal for initiating general sclerotization after ecdysis. Bursicon has a pronounced influence on the activities of the epidermal cells. It has been reported that lack of bursicon results in the failure of endocuticle deposition as well as melanin production and sclerotization of the cuticle (Fraenkel and Hsiao, 1965; Fogal and Fraenkel, 1969), and it appears that bursicon is involved in the control of tyrosine hydroxylation to DOPA (Seligman *et al.*, 1969).

In some insects sclerotization stops when the pre-ecdysially deposited cuticle has become sclerotized to form exocuticle, although deposition of endocuticle continues for several days, resulting in a sclerotized exocuticle and a nonsclerotized endocuticle. In other insects cuticular sclerotization continues during endocuticle deposition with the result that both exo- and endocuticle become sclerotized, but not to the same degree. Sclerotization of femur cuticle in adult locusts (*Schistocerca gregaria*) continues for at least 12 days after ecdysis, and both exo- and endocuticle are sclerotized (Andersen and Barrett, 1971), in contrast to sclerotization of the femur cuticle of fifth instar nymphs of the same species which lasts for only 1 day, and deposition of unsclerotized endocuticle continues for about 4–5 days (Andersen, 1973). Accordingly, the endocuticular proteins are readily extractable from femurs of mature nymphs, but little protein can be extracted from the femurs of mature adult locusts. The difference in duration of sclerotization in locust nymphs and adults is probably related to the different fate of these two types of cuticle. The nymphal cuticle will to a large extent be degraded in preparation for the next ecdysis, and sclerotized cuticle is more resistant to enzymatic degradation than nonsclerotized cuticle. The adult cuticle has to last for the rest of the life of the animal, and there is no apparent advantage in having an easily degraded endocuticle. The leg cuticle of adult locusts is also exposed to stronger mechanical forces than the cuticle of nymphal legs, and it may therefore be an advantage for adult locusts to have both layers of the leg cuticle stabilized, but not to the same degree. A similar sclerotization difference between adult cuticle and cuticle in younger instars is probably present in other insect species. It has not yet been established how the duration of the post-ecdysial sclerotization is regulated.

4.4.5.3. Puparial Sclerotization

During puparium formation in the higher Diptera the soft, pliable cuticle of the last larval instar is modified to a hard and nondeformable material, its function being mainly to protect the animal during pupal and adult development and not to allow movements. Pronounced regional differentiation in cuticular sclerotization is apparently not essential for puparia. The chemical processes of puparium sclerotization are very similar to those involved in the sclerotization of adult cuticle, but the two systems differ in the way they are controlled (Sekeris, 1991). Puparium sclerotization begins with an increase in ecdysteroid titer, which induces the expression of the enzyme DOPA decarboxylase, catalyzing the decarboxylation of DOPA to dopamine. The latter is then acylated to the sclerotization precursors, NADA and NBAD, a process catalyzed by the enzymes acetyl transferase and β-alanyl transferase, respectively. The precursors are oxidized to *o*-quinones, which are then isomerized to *p*-quinone methides which in turn may be converted to the corresponding dehydro-derivatives (Saul and Sugumaran, 1989a; Sugumaran *et al.*, 1992). The flies *Musca autumnalis* and *M. fergusoni* harden

their puparia by the deposition of calcium and magnesium phosphates and not by phenolic sclerotization (Gilby and McKellar, 1976; Darlington *et al*., 1983).

4.4.5.4. Transport of Sclerotization Precursors to the Cuticle

Cuticle of actively moving insects is constantly exposed to external forces which vary in intensity and direction, and to keep the resulting local deformations of the cuticle within functionally acceptable ranges, the mechanical properties of each of the cuticular regions will probably have to be rather precisely regulated, suggesting that the degree of sclerotization of the individual regions is controlled by local mechanisms. In all likelihood such local sclerotization control resides in the underlying epidermal cells. It could operate by regulating the supply of sclerotization precursors to the cuticle, for instance by controlling local synthesis of precursors and/or their uptake from hemolymph into epidermis or by controlling the transport of precursors from epidermal cells to the cuticular matrix where they will be exposed to the enzymes converting them to sclerotization agents. Precursors for sclerotization can be synthesized in the epidermal cells, but it is likely that they can also be produced by other cell types, such as hemocytes and/or fat body cells. Precursors, such as dopamine, NADA, and NBAD, injected into the hemocoel shortly before or during cuticular sclerotization, are rapidly taken up by the epidermal cells and transported into the cuticle, whereas precursors injected several days before the start of sclerotization are mainly degraded or modified by glycosylation or phosphorylation. Such precursor conjugates may either remain in the animal and serve as a reserve pool of sclerotizing material, or they may be excreted via the Malphigian tubules by standard detoxification mechanism.

The *white pupa* mutant of the Mediterranean fruit fly, *Ceratitis capitata,* fails to sclerotize the puparium, but develops normal larval and adult cuticles. The concentrations of the various catecholamines were very low in the mutant puparial cuticle compared to the wild-type strain, whereas the concentrations in the hemolymph of NADA, NBAD, and dopamine were about ten times higher in the mutant than in the wild-type, indicating that the mutant is defective in the system transporting catecholamines from hemolymph to puparial cuticle (Wappner *et al*., 1995).

To control the transport of sclerotizing precursors into the cuticle in the right amounts at the right time the epidermal cells must possess specific transport systems both in the basolateral cell membrane to facilitate uptake from the hemolymph and in the apical cell membrane to control the transport of sclerotizing precursors from the cells into the cuticle. Active diphenoloxidases (laccases) are, at least in some insects, present in unsclerotized pharate cuticle before or at ecdysis (Andersen, 1972, 1979b, and unpublished data), and it appears unlikely that the precursors are transported into these cuticles before sclerotization is initiated following ecdysis. Radioactive NADA injected into fifth instar locust nymphs 1–2 days before ecdysis, when deposition of the pharate adult cuticle occurs, is partly incorporated into the old nymphal cuticle, soon to be discarded, partly retained to be incorporated into the new adult cuticle after ecdysis, and partly excreted as an *O*-glucoside (Andersen, 1974b and unpublished data). The labeled NADA, which after ecdysis was incorporated into the new cuticle, was in the meantime probably stored in the epidermal cells, but other locations, such as the fat body or hemocytes, cannot be disregarded, and the mechanism for transferring NADA into the cuticle is unexplored.

It has been reported that proteins to which catechol derivatives are attached can be transported intact from the hemolymph to the cuticular structure to serve as combined matrix components and sclerotization precursors (Koeppe and Mills, 1972; Koeppe and Gilbert, 1974; Bailey *et al*., 1999), indicating that receptors able to recognize such proteins must be present in the basolateral membrane of the epidermal cells.

4.4.5.5. Balance between Cuticular Enzymes

The type of sclerotization occurring in the local regions will depend in part upon the relative amounts of the two precursor compunds, NADA and NBAD, imported from the epidermal cells, and partly upon the balance between the various enzyme activities in the cuticle. An attempt to study some of these questions was made 30 years ago (Andersen, 1974a, 1974b), but the means available at that time were insufficient to give conclusive answers. NADA labeled with tritium either on the aromatic ring or on the β-position of the side chain was used to study the extent to which the ring and the side chain were involved in adduct formation, based on the assumption that sclerotization via an *o*-quinone will result in tritium release from the ring and that release of tritium from the NADA side chain will occur without intermediate quinone formation. Different types of cuticle were found to differ in their ability to release tritium from the two positions; lightly colored, sclerotized cuticles preferentially released tritium from the side chain, and dark-brown cuticles

released tritium preferentially from the ring system (Andersen, 1974a). It is now evident that tritium release from the β-position of the side chain will occur during formation of the *p*-quinone methide of NADA and will be a measure of the *o*-quinone isomerase activity present, and that tritium release from the aromatic ring will occur during adduct formation between an *o*-quinone and some nucleophile. This will be a measure of the fraction of available NADA that is oxidized by the cuticular diphenoloxidase, but escapes being isomerized to a *p*-quinone methide. Release of tritium from NADA specifically labeled as the α-position of the side chain can presumably be used for obtaining an estimate of the relative importance of the formation of dehydro-NADA during cuticular sclerotization (Andersen, 1991).

Locust (*S. gregaria*) femur cuticle was found to release little tritium from the ring system of NADA, but significant and nearly equal amounts of tritium from the α- and β-positions of the NADA side chain (Andersen, 1974a and unpublished data), indicating that almost all NADA used for sclerotization by this insect is converted to 1,2-dehydro-NADA. Since NADA and NBAD appear to be treated differently during cuticular sclerotization, it will be necessary to use α- as well as β-tritiated forms of both NADA and NBAD to determine the quantitative importance of *p*-quinone methide sclerotization relative to sclerotization involving the dehydro-derivatives. Such determinations have, to the best of our knowledge, not been reported.

4.4.5.6. Intensity of Cuticular Sclerotization

It is difficult to obtain a useful measure of the degree of sclerotization of a given piece of cuticle, but due to its importance for the mechanical properties of cuticles, it would be an advantage to have some means for determining how much NADA and NBAD are incorporated during sclerotization. It is possible that solid state NMR studies can be used for the measurements (Schaefer *et al*., 1987; Christensen *et al*., 1991), but such techniques are not generally available. Attempts to determine the degree of sclerotization of various cuticular samples have been made by measuring the amounts of ketocatechols released by acid hydrolysis from cuticular samples (Andersen, 1974b, 1975; Barrett, 1977, 1980), but this method only determines that part of total sclerotization which is due to reactions involving activated dehydro-NADA and dehydro-NBAD. A useful measure for the quantitative role of NBAD in sclerotization might be obtained by determining the amounts of β-alanine released by acid hydrolysis; it appears that some of the amino groups in β-alanine take part in the sclerotization process and cannot be released by hydrolysis (Christensen *et al*., 1991), but this will probably only represent a minor fraction of the total amount of β-alanine incorporated. Although analysis for ketocatechols as well as β-alanine cannot give absolute values for the types of sclerotization they represent, relative values can be useful for comparing different cuticular samples.

Ketocatechols were not obtained from adult femur cuticle of the locust *S. gregaria* when the samples were taken just after the animals had emerged from their exuvium, as no sclerotization had yet occurred. Later, during maturation of the locusts, a steady increase in the yield of ketocatechols was observed, reaching a constant level after about 1 week, when the ketocatechol yield was about 0.2 $\mu mol\,mg^{-1}$ dry cuticle (Andersen and Barrett, 1971), indicating a close relationship between ketocatechol formation and sclerotization in locust cuticle.

Determination of ketocatechol release from various types of cuticle from mature locusts (*S. gregaria*) showed that all cuticular types yielded some ketocatechols upon hydrolysis: from 10–12 days old locusts the lowest yields were obtained from abdominal intersegmental membranes (0.20% of the dry weight), abdominal sclerites gave 0.38% of the dry weight, and the highest values were obtained from the dorsal mesothorax (5.25% of the dry weight) and the mandibles (3.36% of the dry weight) (Andersen, 1974b), in agreement with the expectation that the regions where strength and hardness are essential for proper function are also the regions giving the highest yields of ketocatechols.

Schistocerca gregaria mature nymphal cuticle gave much lower amounts of ketocatechols than corresponding samples from mature adults. The only exception was the nymphal mandibles; 3.74% of their dry weight was recovered as ketocatechols, comparable to what was obtained from the adult mandibles (Andersen, 1974b). The relative low yield of ketocatechols obtained from nymphal cuticle are probably related to the nymphs weighing less than adults, their leg cuticle being exposed to smaller forces during walking and jumping than the legs of adults, and the nymphal wings and thorax cuticle not being exposed to deforming forces comparable to those for which adult cuticle is exposed to during flight.

Quantitative ketocatechol determinations have been performed on a few other insect species, such as the beetles *Tenebrio molitor* and *Pachynoda epphipiata* (Andersen, 1975). Various parts of the exuviae of a cicada, *Tibicen pruinosa*, have also been analyzed (Barrett, 1977), and most regions

gave ketocatechol values between 6% and 7% of the exuvial dry weight. The exuvial cuticle from the cicada compound eyes gave a ketocatechol yield (11.8% of the dry weight) that was much higher than that obtained from any of the other cuticular regions. The high values obtained from exuviae agree with the notion that sclerotization is generally more pronounced in exocuticle than in endocuticle. The very high values obtained from exuvial cornea of cicada taken together with the low, but significant values from cornea and intersegmental membrane from adult locusts (Andersen, 1974b) indicate that these samples of soft cuticles, often considered to be unsclerotized, are sclerotized in the epicuticle and maybe also in the procuticular layer immediately beneath the epicuticle.

An attempt has been made to obtain a measure of the relative degree of sclerotization by determining the amounts of radioactivity incorporated into the various cuticular regions after injection of a single dose of labeled dopamine or NADA (Andersen, 1974b). This can give a picture of how the various regions compete for the available sclerotization precursor at the time of injection, but it cannot measure the total sclerotization occurring in the regions. Soon after ecdysis labeled dopamine was injected into nymphal and adult *S. gregaria*, and good agreement was observed between the amounts of radioactivity incorporated into the various cuticular regions and the amounts of ketocatechols recovered after acid hydrolysis of corresponding regions from noninjected animals. This indicates that yield of ketocatechols can be a useful measure of sclerotization of locust cuticle. The only exception was that significantly less ketocatechol was obtained from the adult mandibles than expected considering their ability to incorporate radioactive dopamine.

The regional pattern of incorporation of labeled NADA into locust cuticle was the same as that observed for labeled dopamine, and both patterns changed similarly during maturation of the animals (Andersen, 1974b). No convincing correlation was observed between the rate at which the various cuticular regions released tritium from β-labeled NADA *in vitro* and either the uptake of labeled dopamine and NADA *in vivo* or the yield of ketocatechols obtained by hydrolysis of these regions. These results suggest that it is not the amounts of sclerotizing enzymes which are the main determining factor for the degree of sclerotization, but the local availability of sclerotizing precursors. Similar attempts to determine the rate limiting factors for cuticular sclerotization have, to the best of our knowledge, not been performed on other insect species.

4.4.6. Comparative Aspects

4.4.6.1. Cuticular Darkening

It is an old observation that cuticular sclerotization often is accompanied by a darkening of the cuticle, resulting in various shades of brown or black coloration. The black colors are presumably always due to deposition of melanins formed by oxidation of dopamine or DOPA (see Section 4.4.3.2.1), and melanin formation can apparently be blocked without interference with sclerotization. Melanins are not deposited in the cuticle of an albino mutant of the locust *S. gregaria*, but cuticular sclerotization appears not to be affected (Malek, 1957; Karlson and Schlossberger-Raecke, 1962). Further, injection of the phenoloxidase inhibitor phenylthiourea into larvae of *Protophormia terraenovae* prevents melanin deposition during puparium formation but does not affect hardening or the appearance of brown color (Dennell, 1958).

The formation of brown colors appears to be more directly linked to cuticular sclerotization, and various suggestions have been put forward to explain why some cuticles become brown during sclerotization while other cuticles remain colorless. It has been suggested that a correlation exists between the use of NBAD as a sclerotization precursor and the intensity of brown color of the sclerotized cuticle (Brunet, 1980; Morgan *et al.*, 1987; Hopkins and Kramer, 1992), and it has also been suggested that a dark-brown cuticular color indicates that the ring system of the sclerotization precursors is linked directly to cuticular proteins via quinone formation, whereas formation of links between the NADA side chain and the proteins results in a colorless cuticle (Andersen, 1974a). That suggestion was based upon the observation that colorless or lightly colored cuticles preferentially release tritium from the side chain of NADA and that dark-brown cuticles preferentially release tritium from the ring positions.

The colorless benzodioxine-type compounds, which on acid hydrolysis yield ketocatechols, are preferentially formed from NADA and only to a minor extent from NBAD, as NBAD appears to be a poor substrate for the enzymatic activities responsible for introducing a double bond into the side chain (Andersen, 1989d). During sclerotization NBAD can be expected to form links to cuticular proteins via both ring positions and the β-position of the side chain, and some of the β-alanyl amino groups may react with quinones, while most of the NADA residues will be processed to dehydro-NADA, resulting in formation of colorless, protein-linked dihydroxyphenyl-benzodioxine derivatives. The formation of

brown color during sclerotization can thus depend both on the amounts of NBAD available and the balance between quinone-forming enzymes and the enzyme activity catalyzing formation of a double bond in the side chain (Andersen, 1989b).

4.4.6.2. Cuticular Sclerotization in Insects Compared to That in Other Arthropods

The release of ketocatechols during acid hydrolysis can be used as an indication of the involvement of dehydro-NADA in sclerotization, and makes it possible to determine how widespread the occurrence of this variant of cuticular sclerotization is. Ketocatechols have been obtained in varying amounts from cuticular samples of all pterygote insects studied so far, and especially the wings were found to be a good source of ketocatechols. None of the apterygote insects analyzed, representing Thysanura, Collembola, and Diplura, gave any measurable amounts of ketocatechols, and neither did the sclerotized cuticle of noninsectan arthropods, such as Decapoda, Isopoda, Araneae, Xiphosura, and Acarina (Andersen, 1985). The distribution of ketocatechol-yielding material among cuticles indicates that development of the ability to fly occurred in parallel with the development of the use of dehydro-NADA in sclerotization, resulting in a form of sclerotin which combines strength, toughness, and lightness to an optimal degree for flight purposes. The suggestion needs to be investigated in much more detail, and a detailed characterization of the sclerotization process(es) in cuticle of noninsectan arthropods is also needed. Little is known of sclerotization in crustaceans or most other arthropod groups.

4.4.7. Unsolved Problems

The model shown in **Figures 1 and 2** for sclerotization of insect cuticle can account for most of the observations and experimental results which have been published during the years of cuticular studies. It appears likely that we have now obtained an understanding of the main features of the chemical processes occurring during sclerotization, but some observations are difficult to reconcile with the suggested scheme. This may be due to faulty observations or errors in interpretation, but could also represent variations of the scheme developed to serve specialized demands in some types of cuticle, or may represent essential, but unrecognized elements in the general sclerotization scheme. In any case, these observations deserve critical study before we can consider our understanding of the chemistry of sclerotization complete. A weakness in the suggested sclerotization scheme is that it is a combination of individual reactions that have been characterized in different types of cuticles, and we have not yet obtained sufficient evidence that all these reactions, and only these reactions, are of general occurrence in cuticular sclerotization.

4.4.7.1. Alternative Pathway for Dehydro-NADA Formation?

In **Figure 2** the sclerotization process is depicted as a linear reaction chain, where reactive intermediates formed at different steps in the chain can react with cuticular proteins, and it is supposed that the sclerotization patterns in different cuticular types depend partly upon the absolute and relative amounts of the two sclerotization precursors, NADA and NBAD, entering the chain at the beginning of the process and partly upon the extent to which the intermediate products will react with cuticular proteins or will continue along the reaction chain. If the *o*-quinone isomerase is present in larger amounts than the *p*-quinone methide isomerase one should expect that the quinone methide will be produced more rapidly than it is isomerized, favoring reaction between the quinone methide and proteins, whereas if the opposite is the case only a small fraction of the NADA *p*-quinone methide produced will have an opportunity to react with proteins before being isomerized to dehydro-NADA.

Inhibition of laccase in locust cuticle by Na-azide results in a decrease in the consumption of NADA and an accumulation of dehydro-NADA in the incubation medium. As dehydro-NADA is a better substrate for the cuticular laccase than NADA, it was unexpected that Na-azide inhibits the laccase-catalyzed oxidation of dehydro-NADA much more than oxidation of NADA (Andersen, 1989a). One possible explanation could be that dehydro-NADA can be formed not only by isomerization of the *p*-quinone methide, but directly from NADA by means of a special enzyme, a desaturase, as suggested by Andersen *et al*. (1996). Another possible explanation could be that different enzymes catalyze the oxidation of NADA and dehydro-NADA, and that the dehydro-NADA oxidizing enzyme is much more sensitive to azide inhibition than the NADA oxidizing enzyme. This could result in accumulation of dehydro-NADA, but will demand a strict compartmentalization for the two enzyme activities, as the surplus of dehydro-NADA should not have access to the NADA-oxidizing laccase.

4.4.7.2. Extracuticular Synthesis of Catechol–Protein Conjugates for Sclerotization?

Another question to be studied in more detail is to what extent protein-bound catecholic derivatives

are transferred from hemolymph to cuticle to participate in sclerotization. It has been reported that the epidermal cells can transfer proteins, arylphorins, from hemolymph to the cuticular compartment (Scheller *et al*., 1980; Schenkel *et al*., 1983; König *et al*., 1986; Peter and Scheller, 1991), and apparently such transfer can also occur for proteins to which catechols are covalently bound (Koeppe and Mills, 1972; Koeppe and Gilbert, 1974; Bailey *et al*., 1999). If the protein-bound catechols are oxidized to quinones inside the cuticle during sclerotization, it is reasonable to assume that they will react with residues in the other proteins present in the cuticle and thus participate in sclerotin formation, but so far it is not known for certain how, where, and whether such protein–catechol conjugates are formed, and whether such conjugates after transfer to the cuticle will take part in sclerotization. Diffusion problems and steric hindrance may make it difficult for the catecholic residues to access the cuticular diphenoloxidases, but it is possible that the catechols are oxidized by encountering small, easily diffusable quinones formed by enzyme catalyzed oxidation of free catechols.

4.4.7.3. Importance of Cuticular Dehydration?

Fraenkel and Rudall (1940) reported a significant decrease in cuticular water content in connection with puparium sclerotization in blowflies, and it was later argued that formation of covalent cross-links cannot fully explain the changes in mechanical properties occurring during sclerotization and that controlled dehydration of the cuticular matrix may be the most important factor in the stabilization of cuticle (Hillerton and Vincent, 1979; Vincent and Hillerton, 1979; Vincent, 1980). Dehydration may be caused by increased hydrophobicity of cuticular proteins due to reaction with the enzymatically formed quinones, by filling the initially water-filled interstices between proteins with polymerized catechols, and by water being actively transported out of the cuticle by some transport system residing in the epidermal apical cell membrane. All three mechanisms are probably involved in cuticular dehydration. Water transport coupled to active transport of ions appears to be the process which can be most precisely controlled and may be the most important dehydration mechanism.

4.4.7.4. Lipids and Sclerotization?

Lipids may play an essential role in cuticular sclerotization (Wigglesworth, 1985, 1988), a possibility that should be studied in more detail. The epicuticle consists mainly of proteins and lipids connected to each other to form a thin, extremely resistant, and inextractable layer. It is not known how the lipids and proteins are linked together, but the resistance towards hydrolytic degradation indicates that it is not only ester bonds which are involved, and that stable carbon–carbon bonds between lipid molecules and between lipids and proteins may play an important role. Semiquinones and other free radicals can easily be formed during enzyme catalyzed oxidation of catechols to quinones, and free radicals may react with unsaturated lipids resulting in stable lipophenolic complexes. Such reactions could be part of the stabilization of the epicuticle, and they could also contribute to making the connections between epicuticle and the underlying procuticle more stable and secure.

References

Andersen, S.O., **1966**. Covalent cross-links in a structural protein, resilin. *Acta Physiol. Scand. 66* (Suppl. 263), 1–81.

Andersen, S.O., **1970**. Isolation of arterenone (2-amino-3′,4′-dihydroxyacetophenone) from hydrolysates of sclerotized insect cuticle. *J. Insect Physiol. 16*, 1951–1959.

Andersen, S.O., **1971**. Phenolic compounds isolated from insect hard cuticle and their relationship to the sclerotization process. *Insect Biochem. 1*, 157–170.

Andersen, S.O., **1972**. An enzyme from locust cuticle involved in the formation of cross-links from *N*-acetyldopamine. *J. Insect Physiol. 18*, 527–540.

Andersen, S.O., **1973**. Comparison between the sclerotization of adult and larval cuticle in *Schistocerca gregaria*. *J. Insect Physiol. 19*, 1603–1614.

Andersen, S.O., **1974a**. Evidence for two mechanisms of sclerotization in insect cuticle. *Nature 251*, 507–508.

Andersen, S.O., **1974b**. Cuticular sclerotization in larval and adult locusts, *Schistocerca gregaria*. *J. Insect Physiol. 20*, 1537–1552.

Andersen, S.O., **1975**. Cuticular sclerotization in the beetles, *Pachynoda epphipiata* and *Tenebrio molitor*. *J. Insect Physiol. 21*, 1225–1232.

Andersen, S.O., **1978**. Characterization of trypsin-solubilized phenoloxidase from locust cuticle. *Insect Biochem. 8*, 143–148.

Andersen, S.O., **1979a**. Biochemistry of insect cuticle. *Annu. Rev. Entomol. 24*, 29–61.

Andersen, S.O., **1979b**. Characterization of the sclerotization enzyme(s) in locust cuticle. *Insect Biochem. 9*, 233–239.

Andersen, S.O., **1985**. Sclerotization and tanning of the cuticle. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 3. Pergamon, Oxford, pp. 59–74.

Andersen, S.O., **1989a**. Enzymatic activities in locust cuticle involved in sclerotization. *Insect Biochem. 19*, 59–67.

Andersen, S.O., **1989b**. Enzymatic activities involved in incorporation of *N*-acetyldopamine into insect cuticle during sclerotization. *Insect Biochem. 19*, 375–382.

Andersen, S.O., **1989c**. Investigation of an *ortho*-quinone isomerase from larval cuticle of the American silkmoth, *Hyalophora cecropia*. *Insect Biochem. 19*, 803–808.

Andersen, S.O., **1989d**. Oxidation of *N*-beta-alanyldopamine by insect cuticles and its role in cuticular sclerotization. *Insect Biochem. 19*, 581–586.

Andersen, S.O., **1990**. Sclerotization of insect cuticle. In: Ohnishi, E., Ishizaki, H. (Eds.), Molting and Metamorphosis. Japan Scientific Societies Press, Tokyo, pp. 133–155.

Andersen, S.O., **1991**. Sclerotization. In: Binnington, K., Retnakaran, A. (Eds.), Physiology of the Insect Epidermis. CSIRO, Melbourne, pp. 123–140.

Andersen, S.O., Barrett, F.M., **1971**. The isolation of ketocatechols from insect cuticle and their possible role in sclerotization. *J. Insect Physiol. 17*, 69–83.

Andersen, S.O., Jacobsen, J.P., Bojesen, G., Roepstorff, P., **1992a**. Phenoloxidase catalyzed coupling of catechols: identification of novel coupling products. *Biochim. Biophys. Acta 1118*, 134–138.

Andersen, S.O., Jacobsen, J.P., Roepstorff, P., **1992b**. Coupling reactions between amino compounds and *N*-acetyldopamine catalyzed by cuticular enzymes. *Insect Biochem. Mol. Biol. 22*, 517–527.

Andersen, S.O., Jacobsen, J.P., Roepstorff, P., Peter, M.G., **1991**. Catecholamine–protein conjugates: isolation of an adduct of *N*-acetylhistidine to the side chain of *N*-acetyldopamine from an insect-enzyme catalyzed reaction. *Tetrahedron Lett. 32*, 4287–4290.

Andersen, S.O., Peter, M.G., Roepstorff, P., **1992c**. Cuticle-catalyzed coupling between *N*-acetylhistidine and *N*-acetyldopamine. *Insect Biochem. Mol. Biol. 22*, 459–469.

Andersen, S.O., Peter, M.G., Roepstorff, P., **1996**. Cuticular sclerotization in insects. *Comp. Biochem. Physiol. B 113*, 689–705.

Andersen, S.O., Roepstorff, P., **1978**. Phenolic compounds released by mild acid hydrolysis from sclerotized cuticle: purification, structure, and possible origin from cross-links. *Insect Biochem. 8*, 99–104.

Andersen, S.O., Roepstorff, P., **1981**. Sclerotization of insect cuticle. 2. Isolation and identification of phenolic dimers from sclerotized insect cuticle. *Insect Biochem. 11*, 25–31.

Andersen, S.O., Roepstorff, P., **1982**. Sclerotization of insect cuticle. 3. An unsaturated derivative of *N*-acetyldopamine and its role in sclerotization. *Insect Biochem. 12*, 269–276.

Andersen, S.O., Thompson, P.R., Hepburn, H.R., **1981**. Cuticular sclerotization in the honeybee (*Apis mellifera adansonii*). *J. Comp. Physiol. 145*, 17–20.

Asano, T., Ashida, M., **2001a**. Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori*: purification and demonstration of its transport from hemolymph. *J. Biol. Chem. 276*, 11100–11112.

Asano, T., Ashida, M., **2001b**. Transepithelially transported pro-phenoloxidase in the cuticle of the silkworm, *Bombyx mori*: identification of its methionyl residues oxidized to methionine sulfoxides. *J. Biol. Chem. 276*, 11113–11125.

Ashida, M., Brey, P.T., **1995**. Role of the integument in insect defense: pro-phenol oxidase cascade in the cuticular matrix. *Proc. Natl Acad. Sci. USA 92*, 10698–10702.

Ashida, M., Dohke, K., **1980**. Activation of pro-phenoloxidase by the activating enzyme of the silk worm, *Bombyx mori*. *Insect Biochem. 10*, 37–47.

Ashida, M., Yamazaki, H.I., **1990**. Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In: Ohnishi, E., Ishizaki, H. (Eds.), Molting and Metamorphosis. Japan Scientific Societies Press, Tokyo, pp. 239–265.

Aso, Y., Kramer, K.J., Hopkins, T.L., Lookhart, G.L., **1985**. Characterization of haemolymph protyrosinase and a cuticular activator from *Manduca sexta* (L.). *Insect Biochem. 15*, 9–17.

Aso, Y., Kramer, K.J., Hopkins, T.L., Whetzel, S.Z., **1984**. Properties of tyrosinase and DOPA quinone imine conversion factor from pharate pupal cuticle of *Manduca sexta* L. *Insect Biochem. 14*, 463–472.

Atkinson, P.W., Brown, W.V., Gilby, A.R., **1973a**. Autoxidation of insect cuticular lipids: stabilization of alkyl dienes by 3,4-dihydricphenols. *Insect Biochem. 3*, 103–112.

Atkinson, P.W., Brown, W.V., Gilby, A.R., **1973b**. Phenolic compounds from insect cuticle: identification of some lipid antioxidants. *Insect Biochem. 3*, 309–315.

Bailey, W.D., Kimbrough, T.D., Mills, R.R., **1999**. Catechol conjugation with hemolymph proteins and their incorporation into the cuticle of the American cockroach, *Perplaneta americana*. *Comp. Biochem. Physiol. C 122*, 139–145.

Barrett, F.M., **1977**. Recovery of ketocatechols from exuviae of last instar larvae of the cicada, *Tibicen pruinosa*. *Insect Biochem. 7*, 209–214.

Barrett, F.M., **1980**. Recovery of phenolic compounds from exuviae of the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Can. Entomol. 112*, 151–157.

Barrett, F.M., **1984**. Metabolic origin of 3,4-dihydroxyphenylacetic acid in *Tenebrio molitor* and differences in its content in larval, pupal, and adult cuticle. *Can. J. Zool. 62*, 1005–1010.

Barrett, F.M., **1987a**. Characterization of phenoloxidases from larval cuticle of *Sarcophaga bullata* and a comparison with cuticular enzymes from other species. *Can. J. Zool. 65*, 1158–1166.

Barrett, F.M., **1987b**. Phenoloxidases from larval cuticle of the sheep blowfly, *Lucilia cuprina:* characterization, developmental changes, and inhibition by antiphenoloxidase antibodies. *Arch. Insect Biochem. Physiol. 5*, 99–118.

Barrett, F.M., **1990**. Incorporation of various putative precursors into cuticular 3,4-dihydroxyphenylacetic acid of adult *Tenebrio molitor. Insect Biochem. 20*, 645–652.

Barrett, F.M., **1991**. Phenoloxidases and the integument. In: Binnington, K., Retnakaran, A. (Eds.), Physiology of the Insect Epidermis. CSIRO, Melbourne, pp. 195–212.

Barrett, F.M., Andersen, S.O., **1981**. Phenoloxidases in larval cuticle of the blowfly, *Calliphora vicina*. *Insect Biochem. 11*, 17–23.

Bernays, E.A., Chamberlain, D.J., McCarthy, P., **1980**. The differential effects of ingested tannic acid on different species of Acridoidea. *Entomol. Experiment. Applic. 28*, 158–166.

Bernays, E.A., Woodhead, S., **1982**. Incorporation of dietary phenols into the cuticle in the tree locust *Anacridium melanorhodon*. *J. Insect Physiol. 28*, 601–606.

Binnington, K.C., Barrett, F.M., **1988**. Ultrastructural localization of phenoloxidases in cuticle and haemopoietic tissue of the blowfly *Lucilia cuprina*. *Tissue Cell 20*, 405–419.

Bodnaryk, R.P., Brunet, P.C.J., **1974**. 3-*O*-Hydrosulphato-4-hydroxyphenethylamine (dopamine 3-*O*-sulphate), a metabolite involved in the sclerotization of insect cuticle. *Biochem. J. 138*, 463–469.

Brunet, P.C.J., **1980**. The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem. 10*, 467–500.

Brunet, P.C.J., Coles, B.C., **1974**. Tanned silks. *Proc. R. Soc. B 187*, 133–170.

Candy, D.J., **1979**. Glucose oxidase and other enzymes of hydrogen peroxide metabolism from cuticle of *Schistocerca gregaria*. *Insect Biochem. 9*, 661–665.

Chase, M.R., Raina, K., Bruno, J., Sugumaran, M., **2000**. Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. *Insect Biochem. Mol. Biol. 30*, 953–967.

Christensen, A.M., Schaefer, J., Kramer, K.J., Morgan, T.D., Hopkins, T.L., **1991**. Detection of cross-links in insect cuticle by REDOR NMR spectroscopy. *J. Am. Chem. Soc. 113*, 6799–6802.

Coles, G.C., **1966**. Studies on resilin biosynthesis. *J. Insect Physiol. 12*, 679–691.

Cottrell, C.B., **1964**. Insect ecdysis with particular emphasis on cuticular hardening and darkening. *Adv. Insect Physiol. 2*, 175–218.

Curtis, A.T., Hori, M., Green, J.M., Wolfgang, W.J., Hiruma, K., *et al.*, **1984**. Ecdysteroid regulation of the onset of cuticular melanization in allatectomized and *black* mutant *Manduca sexta* larvae. *J. Insect Physiol. 30*, 597–606.

Czapla, T.H., Hopkins, T.L., Kramer, K.J., **1989**. Catecholamines and related *o*-diphenols in the hemolymph and cuticle of the cockroach *Leucophaea maderae* (F.) during sclerotization and pigmentation. *Insect Biochem. 19*, 509–515.

Czapla, T.H., Hopkins, T.L., Kramer, K.J., **1990**. Cuticular strength and pigmentation of five strains of adult *Blattella germanica* (L.) during sclerotization: correlations with catecholamines, beta-alanine and food deprivation. *J. Insect Physiol. 36*, 647–654.

Czapla, T.H., Hopkins, T.L., Kramer, K.J., Morgan, T.D., **1988**. Diphenols in hemolymph and cuticle during development and cuticle tanning of *Periplaneta americana* (L.) and other cockroach species. *Arch. Insect Biochem. Physiol. 7*, 13–28.

Darlington, M.V., Meyer, H.J., Graf, G., Freeman, T.P., **1983**. The calcified puparium of the face fly, *Musca autumnalis* (Diptera: Muscidae). *J. Insect Physiol. 29*, 157–162.

Dennell, R., **1958**. The amino acid metabolism of a developing insect cuticle: the larval cuticle and puparium of *Calliphora vomitoria*. 3. The formation of the puparium. *Proc. R. Soc. B 148*, 176–183.

Fogal, W., Fraenkel, G., **1969**. The role of bursicon in melanization and endocuticle formation in the adult fleshfly, *Sarcophaga bullata*. *J. Insect Physiol. 15*, 1235–1247.

Fraenkel, G., Hsiao, C., **1965**. Bursicon, a hormone which mediates tanning of the cuticle in the adult fly and other insects. *J. Insect Physiol. 11*, 513–556.

Fraenkel, G., Rudall, K.M., **1940**. A study of the physical and chemical properties of insect cuticle. *Proc. R. Soc. B 129*, 1–35.

Fujimoto, K., Okino, N., Kawabata, S., Iwagana, S., Ohnishi, E., **1995**. Nucleotide sequence of the cDNA encoding the proenzyme of phenoloxidase A_1 of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 92*, 7769–7773.

Gilby, A.R., McKellar, J.W., **1976**. The calcified puparium of a fly. *J. Insect Physiol. 22*, 1465–1468.

Grossmüller, M., Messner, B., **1978**. Zur quantitativen Verteilung der Peroxidase- und Phenoloxidase-Aktivität in der Kutikula von *Galleria mellonella*-Larven und -Puppen: Ein Beitrag zur Sklerotisierung der Insektenkutikula. *Zool. Jb. Physiol. 82*, 16–22.

Hall, M., Scott, T., Sugumaran, M., Söderhall, K., Law, J.H., **1995**. Proenzyme of *Manduca sexta* phenoloxidase: purification, activation, substrate specificity of the active enzyme and molecular cloning. *Proc. Natl Acad. Sci. USA 92*, 7764–7768.

Han, Q., Fang, J., Ding, H., Johnson, J.K., Christensen, B.M., *et al.*, **2002**. Identification of *Drosophila melanogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. *Biochem. J. 368*, 333–340.

Han, Q., Li, G., Li, J., **2000a**. Chorion peroxidase-mediated $NADH/O_2$ oxidoreduction cooperated by chorion malate dehydrogenase-catalyzed NADH production: a feasible pathway leading to H_2O_2 formation during chorion hardening in *Aedes aegypti* mosquitoes. *Biochim. Biophys. Acta 1523*, 246–253.

Han, Q., Li, G., Li, J., **2000b**. Purification and characterization of chorion peroxidase from *Aedes aegypti* eggs. *Arch. Biochem. Biophys. 378*, 107–115.

Hasson, C., Sugumaran, M., **1987**. Protein cross-linking by peroxidase: possible mechanism for sclerotization of insect cuticle. *Arch. Insect Biochem. Physiol. 5*, 13–28.

Hillerton, J.E., Vincent, J.F.V., **1979**. The stabilization of insect cuticles. *J. Insect Physiol. 25*, 957–963.

Hiruma, K., Riddiford, L.M., **1988**. Granular phenoloxidase involved in cuticular melanization in the tobacco hornworm: regulation of its synthesis in the epidermis by juvenile hormone. *Devel. Biol. 130*, 87–97.

Hopkins, T.L., Kramer, K.J., **1992**. Insect cuticle sclerotization. *Annu Rev. Entomol. 37*, 273–302.

Hopkins, T.L., Morgan, T.D., Aso, Y., Kramer, K.J., **1982**. *N*-β-alanyldopamine: major role in insect cuticle tanning. *Science 217*, 364–366.

Hopkins, T.L., Morgan, T.D., Kramer, K.J., **1984**. Catecholamines in haemolymph and cuticle during larval, pupal and adult development of *Manduca sexta* (L.). *Insect Biochem. 14*, 533–540.

Hopkins, T.L., Morgan, T.D., Mueller, D.D., Tomer, K.B., Kramer, K.J., **1995**. Identification of catecholamine β-glucosides in the hemolymph of the tobacco hornworm, *Manduca sexta* (L.), during development. *Insect Biochem. Mol. Biol. 25*, 29–37.

Hopkins, T.L., Starkey, S.R., Xu, R., Merritt, M.E., Schaefer, J., *et al.*, **1999**. Catechols involved in sclerotization of the grasshopper, *Melanoplus sanguinipes,* and their interactions with cuticular proteins. *Arch. Insect Biochem. Physiol. 40*, 119–128.

Huang, X., Xu, R., Hawley, M.D., Hopkins, T.L., Kramer, K.J., **1998**. Electrochemical oxidation of *N*-acyldopamines and regioselective reactions of their quinones with *N*-acetylcysteine and thiourea. *Arch. Biochem. Biophys. 352*, 19–30.

Jacobs, M.E., **1978**. β-Alanine tanning of *Drosophila* cuticles and chitin. *Insect Biochem. 8*, 37–41.

Jacobs, M.E., **1980**. Influence of β-alanine on ultrastructure, tanning, and melanization of *Drosophila melanogaster* cuticles. *Biochem. Genet. 18*, 65–76.

Jacobs, M.E., **1985**. Role of β-alanine in cuticular tanning, sclerotization, and temperature regulation in *Drosophila melanogaster. J. Insect Physiol. 31*, 509–515.

Karlson, P., Schlossberger-Raecke, I., **1962**. Zum Tyrosinstoffwechsel der Insekten. 8. Die Sklerotisierung der Cuticula bei der Wildform und der Albinomutante von *Schistocerca gregaria* Forsk. *J. Insect Physiol. 8*, 441–452.

Karlson, P., Sekeris, C.E., **1976**. Control of tyrosine metabolism and cuticle sclerotization by ecdysone. In: Hepburn, H.P. (Ed.), The Insect Integument. Elsevier, London, pp. 145–156.

Kawabata, T., Yasuhara, Y., Ochiai, M., Matsuura, S., Ashida, M., **1995**. Molecular cloning of insect pro-phenoloxidase: a copper-containing protein homologous to arthropod hemocyanin. *Proc. Natl Acad. Sci. USA 92*, 7774–7778.

Kawasaki, H., Sato, H., **1985**. The tanning agent in the silk of the Japanese giant silkmoth, *Dictyoploca japonica* Butler. *Insect Biochem. 15*, 681–684.

Kawasaki, H., Yago, M., **1983**. The identification of two *N*-acyldopamine glucosides in the left colleterial gland of the praying mantid, *Tenodera aridifolia sinensis* Saussure, and their role in the oothecal sclerotization. *Insect Biochem 13*, 267–271.

Kayser, H., **1985**. Pigments. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 10. Pergamon, Oxford, pp. 367–415.

Kayser-Wegmann, I., **1976**. Differences in black pigmentation in lepidopteran cuticles as revealed by light and electron microscopy. *Cell Tissue Res. 171*, 513–521.

Kayser-Wegmann, I., Kayser, H., **1983**. Black pigmentation of insect cuticles: a view based on microscopic and autoradiographic studies. In: Scheller, K. (Ed.), The Larval Serum Proteins of Insects: Function, Biosynthesis, Genetics. Georg Thieme Verlag, Stuttgart, pp. 151–167.

Kerwin, J.L., Turecek, F., Xu, R., Kramer, K.J., Hopkins, T.L., *et al.*, **1999**. Mass spectrometric analysis of catechol-histidine adducts from insect cuticle. *Anal. Biochem. 268*, 229–237.

Koeppe, J.K., Gilbert, L.I., **1974**. Metabolism and protein transport of a possible pupal tanning agent in *Manduca sexta*. *J. Insect Physiol. 20*, 981–992.

Koeppe, J.K., Mills, R.R., **1972**. Hormonal control of tanning by the American cockroach: probable bursicon mediated translocation of protein-bound phenols. *J. Insect Physiol. 18*, 465–469.

König, M., Agrawal, O.P., Schenkel, H., Scheller, K., **1986**. Incorporation of calliphorin into the cuticle of the developing blowfly, *Calliphora vicina*. *Roux's Arch. Devel. Biol. 195*, 296–301.

Kramer, K.J., Hopkins, T.L., **1987**. Tyrosine metabolism for insect cuticle tanning. *Arch. Insect Biochem. Physiol. 6*, 279–301.

Kramer, K.J., Morgan, T.D., Hopkins, T.L., Roseland, C.R., Aso, Y., *et al.*, **1984**. Catecholamines and β-alanine in the red flour beetle, *Tribolium castaneum*: roles in cuticle sclerotization and melanization. *Insect Biochem. 14*, 293–298.

Krueger, R.A., Kramer, K.J., Hopkins, T.L., Speirs, R.D., **1989**. *N*-β-alanyldopamine levels and synthesis in integument and other tissues of *Manduca sexta* (L.) during the larval–pupal transformation. *Insect Biochem. 19*, 169–175.

Li, J., Hodgeman, B.A., Christensen, B.M., **1996**. Involvement of peroxidase in chorion hardening in *Aedes aegypti*. *Insect Biochem. Mol. Biol. 26*, 309–317.

Locke, M., **1969**. The localization of a peroxidase associated with hard cuticle formation in an insect, *Calpodes ethlius* Stoll, Lepidoptera, Hesperiidae. *Tissue and Cell 1*, 555–574.

Malek, S.R.A., **1957**. Sclerotization and melanization: two independent processes in the cuticle of the desert locust. *Nature 180*, 237.

Manthey, M.K., Pyne, S.G., Truscott, R.J.W., **1992**. Involvement of tyrosine residues in the tanning of proteins by 3-hydroxyanthranilic acid. *Proc. Natl Acad. Sci. USA 89*, 1954–1957.

Messner, B., Janda, V., **1991**. Der Peroxidase-Nachweis in der Kutikula von *Galleria mellonella* (Insecta,

Lepidoptera) und *Protophormia terraenovae* (Insecta, Diptera): Ein Beitrag zur Sklerotisierung der Insektenkutikula. *Zool. Jb. Physiol. 95*, 31–37.

Messner, B., Kerstan, U., **1991**. Die Peroxidase im Sklerotisierungsprozess von Hartteilen verschiedener wirbelloser Tiere. *Zool. Jb. Physiol. 95*, 23–29.

Mindrinos, M.N., Petri, W.H., Galanopoulos, V.K., Lombard, M.F., Margaritis, L.H., **1980**. Crosslinking of the *Drosophila* chorion involves a peroxidase. *Roux's Arch. Devel. Biol. 189*, 187–196.

Morgan, T.D., Hopkins, T.L., Kramer, K.J., Roseland, C.R., Czapla, T.H., *et al.*, **1987**. *N*-β-Alanylnorepinephrine: biosynthesis in insect cuticle and possible role in sclerotization. *Insect Biochem. 17*, 255–263.

Morgan, T.D., Thomas, B.R., Yonekura, M., Czapla, T.H., Kramer, K.J., *et al.*, **1990**. Soluble tyrosinases from pharate pupal integument of the tobacco hornworm, *Manduca sexta* (L.). *Insect Biochem. 20*, 251–260.

Okot-Kotber, B.M., Morgan, T.D., Hopkins, T.L., Kramer, K.J., **1996**. Catecholamine-containing proteins from the pharate pupal cuticle of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 26*, 475–484.

Pau, R.N., Acheson, R.M., **1968**. The identification of 3-hydroxy-4-O-β-D-glucosidobenzyl alcohol in the left colleterial gland of *Blaberus discoidalis*. *Biochim. Biophys. Acta 158*, 206–211.

Pérez, M., Wappner, P., Quesada-Allué, L.A., **2002**. Catecholamine-β-alanyl ligase in the medfly *Ceratitis capitata*. *Insect Biochem. Mol. Biol. 32*, 617–625.

Peter, M.G., Andersen, S.O., Hartmann, R., Miessner, M., Roepstorff, P., **1992**. Catechol–protein conjugates: isolation of 4-phenylphenoxazin-2-ones from oxidative coupling of *N*-acetyldopamine with aliphatic amino acids. *Tetrahedron 48*, 8927–8934.

Peter, M.G., Scheller, K., **1991**. Arylphorins and the integument. In: Binnington, K., Retnakaran, A. (Eds.), Physiology of the Insect Epidermis. CSIRO, Melbourne, pp. 113–122.

Peter, M.G., Vaupel, W., **1985**. Lack of stereoselectivity in the enzymatic conversion of *N*-acetyldopamine into *N*-acetylnoradrenaline in insect cuticle. *J. Chem. Soc., Chem. Commun.* 848–850.

Petri, W.H., Wyman, A.R., Kafatos, F.C., **1976**. Specific proteins synthesis in cellular differentiation. 3. The egg shell proteins in *Drosophila melanogaster* and their program of synthesis. *Devel. Biol. 49*, 185–199.

Pryor, M.G.M., **1940a**. On the hardening of the ootheca of *Blatta orientalis*. *Proc. R. Soc. B 128*, 378–392.

Pryor, M.G.M., **1940b**. On the hardening of cuticle of insects. *Proc. R. Soc. B 128*, 393–407.

Ricketts, D., Sugumaran, M., **1994**. 1,2-Dehydro-*N*-β-alanyldopamine as a new intermediate in insect cuticular sclerotization. *J. Biol. Chem. 269*, 22217–22221.

Roepstorff, P., Andersen, S.O., **1981**. Electron impact, chemical ionization and field desorption mass spectrometry of substituted dihydroxyphenyl-benzodioxins isolated from insect cuticle: occurrence of thermal decomposition and oligomerization reactions in the mass spectrometer. *Biomed. Mass Spectrom. 8*, 174–178.

Roseland, C.R., Kramer, K.J., Hopkins, T.L., **1987**. Cuticular strength and pigmentation of rust-red and black strains of *Tribolium castaneum*: correlation with catecholamine and β-alanine content. *Insect Biochem. 17*, 21–28.

Saul, S.J., Sugumaran, M., **1988**. A novel quinone: quinone methide isomerase generates quinone methides in insect cuticle. *FEBS Lett. 237*, 155–158.

Saul, S.J., Sugumaran, M., **1989a**. *o*-Quinone/quinone methide isomerase: a novel enzyme preventing the destruction of self-matter by phenoloxidase-generated quinones during immune response in insects. *FEBS Lett. 249*, 155–158.

Saul, S.J., Sugumaran, M., **1989b**. Characterization of a new enzyme system that desaturates the side chain of *N*-acetyldopamine. *FEBS Lett. 251*, 69–73.

Saul, S.J., Sugumaran, M., **1989c**. *N*-acetyldopamine quinone methide/1,2-dehydro-*N*-acetyldopamine tautomerase: a new enzyme involved in sclerotization of insect cuticle. *FEBS Lett. 255*, 340–344.

Saul, S.J., Sugumaran, M., **1990**. 4-Alkyl-*o*-quinone/2-hydroxy-*p*-quinone methide isomerase from the larval hemolymph of *Sarcophaga bullata*. 1. Purification and characterization of enzyme-catalyzed reaction. *J. Biol. Chem. 265*, 16992–16999.

Schaefer, J., Kramer, K.J., Garbow, J.R., Jacob, G.S., Stejskal, E.O., *et al.*, **1987**. Aromatic cross-links in insect cuticle: detection by solid-state ^{13}C and ^{15}N NMR. *Science 235*, 1200–1204.

Scheller, K., Zimmermann, H.-P., Sekeris, C.E., **1980**. Calliphorin, a protein involved in the cuticle formation of the blowfly, *Calliphora vicina*. *Z. Naturforsch. C 35*, 387–389.

Schenkel, H., Myllek, C., König, M., Hausberg, P., Scheller, K., **1983**. Calliphorin: studies on its biosynthesis and function. In: Scheller, K. (Ed.), The Larval Serum Proteins of Insects: Function, Biosynthesis, Genetics. Georg Thieme Verlag, Stuttgart, pp. 18–39.

Schlossberger-Raecke, I., Karlson, P., **1964**. Zum Tyrosinstoffwechsel der Insekten. 13. Radioautographische Lokalisation von Tyrosinmetaboliten in der Cuticula von *Schistocerca gregaria* Forsk. *J. Insect Physiol. 10*, 261–266.

Sekeris, C.E., **1991**. The role of molting hormone in sclerotization in insects. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods: Roles in Histogenesis, Organogenesis and Morphogenesis, vol. 1, part 3. Rutgers University Press, New Brunswick, NJ, pp. 150–212.

Seligman, M., Friedman, S., Fraenkel, G., **1969**. Bursicon mediation of tyrosine hydroxylation during tanning of the adult cuticle of the fly, *Sarcophaga bullata*. *J. Insect Physiol. 15*, 553–561.

Sugumaran, M., **1987**. Quinone methide sclerotization: a revised mechanism for β-sclerotization of insect cuticle. *Bioorgan. Chem. 15*, 194–211.

Sugumaran, M., **1988**. Molecular mechanisms for cuticular sclerotization. *Adv. Insect Physiol. 21*, 179–231.

Sugumaran, M., **1998**. Unified mechanism for sclerotization of insect cuticle. *Adv. Insect Physiol. 27*, 229–334.

Sugumaran, M., Dali, H., Semensi, V., **1989a**. Chemical and cuticular phenoloxidase-mediated synthesis of cysteinyl-catechol adducts. *Arch. Insect Biochem. Physiol. 11*, 127–137.

Sugumaran, M., Giglio, L., Kundzicz, H., Saul, S.P., Semensi, V., **1992**. Studies on the enzymes involved in puparial cuticle sclerotization in *Drosophila melanogaster. Arch. Insect Biochem. Physiol. 19*, 271–283.

Sugumaran, M., Hennigan, B., Semensi, V., Dali, H., **1988**. On the nature of nonenzymatic and enzymatic oxidation of the putative sclerotizing precursor, 1,2-dehydro-*N*-acetyldopamine. *Arch. Insect Biochem. Physiol. 8*, 89–100.

Sugumaran, M., Saul, S.P., Semensi, V., **1989b**. Trapping of transiently formed quinone methide during enzymatic conversion of *N*-acetyldopamine to *N*-acetylnorepinephrine. *FEBS Lett. 252*, 135–138.

Thomas, B.R., Yonekura, M., Morgan, T.D., Czapla, T.H., Hopkins, T.L., *et al.*, **1989**. A trypsin-solubilized laccase from pharate pupal integument of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. 19*, 611–622.

Umebachi, Y., **1993**. The third way of dopamine. *Trends Comp. Biochem. Physiol. 1*, 709–720.

Vincent, J.F.V., **1980**. Insect cuticle: a paradigm for natural composites. *Symp. Soc. Exp. Biol. 34*, 183–209.

Vincent, J.F.V., Hillerton, J.E., **1979**. The tanning of insect cuticle: a critical review and a revised mechanism. *J. Insect Physiol. 25*, 653–658.

Wappner, P., Kramer, K.J., Hopkins, T.L., Merritt, M., Schaefer, J., *et al.*, **1995**. *White pupa:* a *Ceratitis capitata* mutant lacking catecholamines for tanning the puparium. *Insect Biochem. Mol. Biol. 25*, 365–373.

Welinder, B.S., Roepstorff, P., Andersen, S.O., **1976**. The crustacean cuticle. 4. Isolation and identification of cross-links from *Cancer pagurus* cuticle. *Comp. Biochem. Physiol. B 53*, 529–533.

Wigglesworth, V.B., **1985**. Sclerotin and lipid in the waterproofing of the insect cuticle. *Tissue and Cell 17*, 227–248.

Wigglesworth, V.B., **1988**. The source of lipids and polyphenols for the insect cuticle: the role of fat body, oenocytes and oenocytoids. *Tissue and Cell 20*, 919–932.

Wright, T.R.F., **1987**. The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster. Adv. Genet. 24*, 127–222.

Xu, R., Huang, X., Hopkins, T.L., Kramer, K.J., **1997**. Catecholamine and histidyl protein cross-linked structures in sclerotized insect cuticle. *Insect Biochem. Mol. Biol. 27*, 101–108.

Xu, R., Huang, X., Kramer, K.J., Hawley, M.D., **1996a**. Characterization of products from the reactions of dopamine quinone with *N*-acetylcysteine. *Bioorgan. Chem. 24*, 110–126.

Xu, R., Huang, X., Morgan, T.D., Prakash, O., Kramer, K.J., *et al.*, **1996b**. Characterization of products from the reactions of *N*-acetyldopamine quinone with *N*-acetylhistidine. *Arch. Biochem. Biophys. 329*, 56–64.

Yago, M., Kawasaki, H., **1984**. The identification of five *N*-acyldopamine glucosides in the left colleterial gland of the praying mantid, *Hierodula patellifera* Serville. *Insect Biochem. 14*, 487–489.

Yago, M., Sato, H., Kawasaki, H., **1984**. The identification of *N*-acyldopamine glucosides in the left colleterial gland of the praying mantids, *Mantis religiosa* L., *Statilia maculata* Thunberg and *Tenodera angustipennis* Saussure. *Insect Biochem. 14*, 7–9.

Yago, M., Sato, H., Oshima, S., Kawasaki, H., **1990**. Enzymic activities involved in the oothecal sclerotization of the praying mantid, *Tenodera sinensis* Saussure. *Insect Biochem. 20*, 745–750.

Yamazaki, H.I., **1969**. The cuticular phenoloxidase in *Drosophila virilis*. *J. Insect Physiol. 15*, 2203–2211.

Yamazaki, H.I., **1972**. Cuticular phenoloxidase from the silkworm *Bombyx mori*: properties, solubilization, and purification. *Insect Biochem. 2*, 431–444.

4.5 Biochemistry of Digestion

W R Terra and C Ferreira, University of São Paulo,
São Paulo, Brazil

4.5.1. Introduction

Most reviews start with the statement that the field under study has undergone remarkable progress over the last decade and the same can be said about the biochemistry of insect digestion. This growth is a characteristic of science as a whole that on average doubles in size every 15 years (Price, 1963). The growth of knowledge in the biochemistry of insect digestion had a bright start during the first decades of the last century, but slowed down after the development of synthetic chemical insecticides in the 1940s. Later on, with the environmental problems caused by chemical insecticides, new approaches for insect control were investigated. Midgut studies were particularly stimulated after the realization that the gut is a very large and relatively unprotected interface between the insect and its environment. Hence, an understanding of gut function was thought to be essential when developing methods of control that act through the gut,

such as the use of transgenic plants to control phytophagous insects.

Applebaum (1985) in his review on the biochemistry of digestion for the first edition of this series recognized the beginning of the renewed growth of the field. He discussed contemporary research showing that most insect digestive enzymes are similar to their mammalian counterparts, but that insect exotic diets require specific enzymes. In the next decade it became apparent that even enzymes similar to those of mammals have distinct characteristics because each insect taxon deals with food in a special way (Terra and Ferreira, 1994). Since then, the field of digestive physiology and biochemistry has progressed dramatically at the molecular level.

The aim of this chapter is to review the recent and spectacular progress in the study of insect digestive biochemistry. To provide a broad coverage while keeping the chapter within reasonable size limits, only a brief account with key references is given for work done prior to 1994. Papers after 1994 have been selected from those richer in molecular details, and, when they were too numerous, representative papers were chosen, especially when rich in references to other papers. Throughout, the focus is on providing a coherent picture of phenomena and highlighting further research areas. Amino acid residues are denoted by the one-letter code, if in peptides, for the sake of brevity. When mentioned in text with a position number, amino acid residues are denoted by the three-letter code to avoid ambiguity. For consistency, traditional abbreviations, like BAPA for benzoyl-arginine *p*-nitroanilide, have been changed, in the example to B-R-pNA, because the one-letter code for arginine is R.

The chapter is organized into four parts. The first part (Sections 4.5.2 and 4.5.3) tries to establish uniform parameters for studying insect digestive enzymes, providing an overview of the biochemistry of insect digestion, and discusses factors affecting digestive enzymes *in vivo*. The second part (Sections 4.5.4–4.5.6) reviews digestive enzymes with emphasis on molecular aspects, whereas the third part (Sections 4.5.7 and 4.5.8) describes the details of the digestive biochemical process along insect evolution. Finally, the fourth part (Section 4.5.9) discusses data on digestive enzyme secretion mechanisms and control.

4.5.2. Overview of the Digestive Process

4.5.2.1. Initial Considerations

Digestion is the process by which food molecules are broken down into smaller molecules that are absorbed by cells in the gut tissue. This process is controlled by digestive enzymes and is dependent on their localization in the insect gut.

4.5.2.2. Characterization of Digestive Enzymes

Enzyme kinetic parameters are meaningless unless assays are performed in conditions in which enzymes are stable. If researchers adopt uniform parameters and methods, comparisons among similar and different insect species will be more meaningful. A rectilinear plot of product formation (or substrate disappearance) versus time will ensure that enzymes are stable in a given condition. Activities (velocities) calculated from this plot are reliable parameters. According to the International Union of Biochemistry and Molecular Biology, the assay temperature should be 30 °C, except when the enzyme is unstable at this temperature or altered for specific purposes. Owing to partial inactivation, the optimum temperature is not a true property of enzymes and therefore should not be included in the characterization. Enzyme pH optimum should be determined using different buffers to discount the effects of chemical constituents of the buffers and their ionic strength on enzyme activity. The number of molecular forms of a given enzyme should be evaluated by submitting the enzyme preparation to a separation process (gel permeation, ion-exchange chromatography, electrophoresis, gradient ultracentrifugation, etc.), followed by assays of the resulting fractions. Substrate specificity of each molecular form of a given enzyme should be evaluated and substrate preference quantified by determining Vm/Km ratios for each substrate, keeping the amount of each enzyme form constant. Substrate preference expressed as the percentage activity towards a given substrate in relation to the activity upon a reference substrate may be misleading because, in this condition, enzyme activities are determined at different substrate saturations. The isoelectric points of many enzymes can be determined after staining with specific substrates following the separation of the native enzymes on isoelectrofocusing gels.

If enzyme characterization is performed as part of a digestive physiology study, emphasis should be given to enzyme compartmentalization, substrate specificity, and substrate preference, in order to discover the sequential action of enzymes during the digestive process. Knowledge of the effect of pH on enzyme activity is useful in evaluating enzyme action in gut compartments (**Figure 1**) with different pH values. Finally, the determination of the molecular masses of digestive enzymes, associated with the ability of enzymes to pass through the peritrophic membrane, allows estimation of the pore sizes of the peritrophic membrane. Molecular

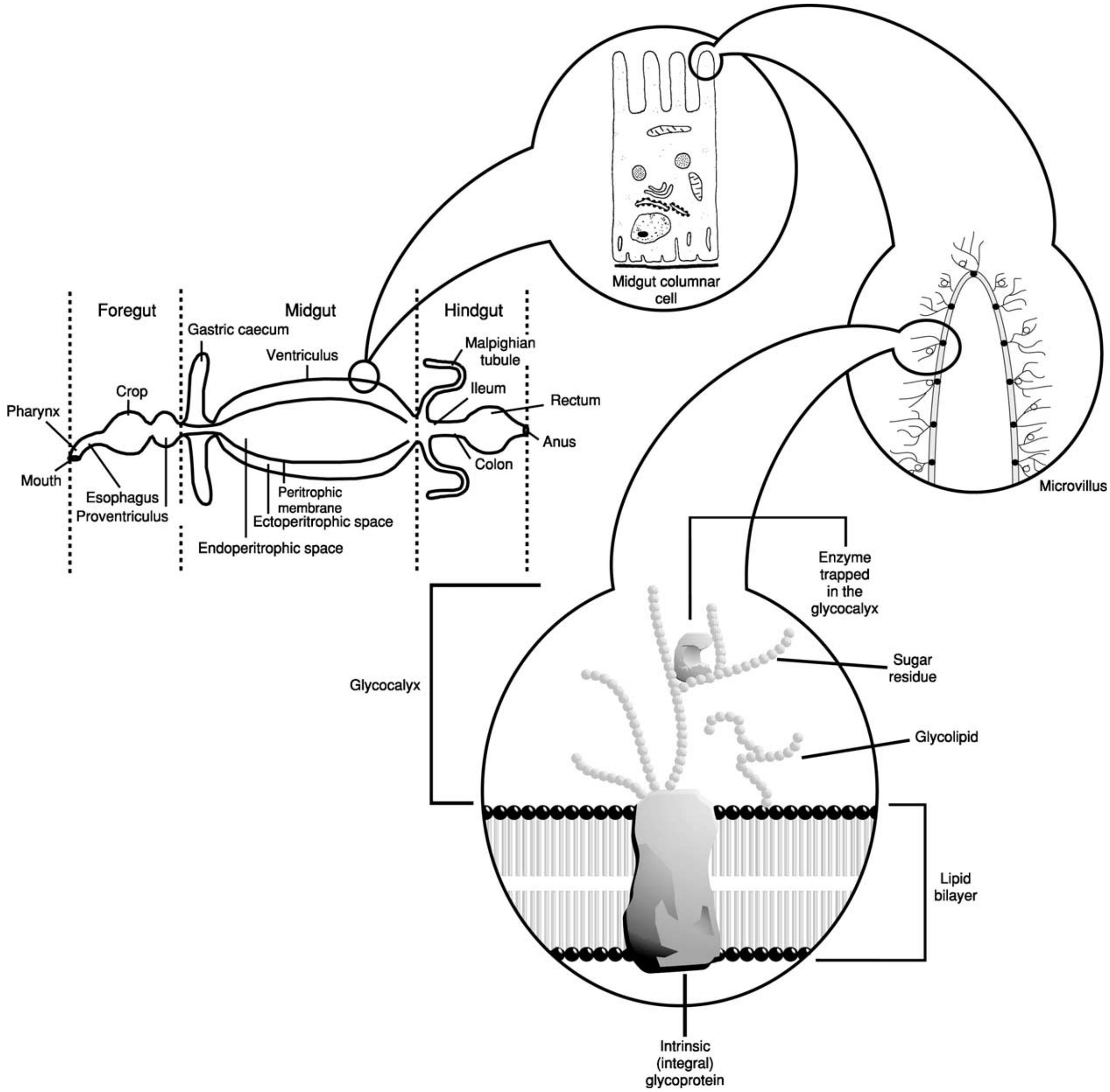


Figure 1 Diagrammatic representation of insect gut compartments. Glycocalyx: the carbohydrate moiety of intrinsic proteins and glycolipids occurring in the luminal face of microvillar membranes.

masses determined in nondenaturing conditions are preferred, since in these conditions the enzymes should maintain their *in vivo* aggregation states (not only their quaternary structures if present). The method of choice in this case is gradient ultracentrifugation.

Complete enzymological characterization requires purification to homogeneity and sequencing. Furthermore, details of the catalytic mechanisms, including involvement of amino acid residues in catalysis and substrate specificity, should be determined. This permits the classification of insect digestive enzymes into catalytic families, and discloses the structural basis of substrate specificities; it will also enable us to establish evolutionary relationships with enzymes from other organisms.

Cloning cDNA sequences encoding digestive enzymes enables the expression of large amounts of recombinant enzymes that may be crystallized or used for the production of antibodies. Antibodies are used in Western blots to identify a specific enzyme in protein mixtures or to localize the enzyme in tissue sections in a light or electron microscope. Enzyme crystals used for resolving three-dimensional (3D) structures (via X-ray diffraction or nuclear magnetic resonance (NMR)) need amounts

of purified enzymes that frequently are difficult to isolate from insects by conventional separation procedures. However, detailed 3D structures are necessary to understand enzyme mechanisms and the binding of inhibitors to enzyme molecules. Alternatively, cDNA may be amenable to site-directed mutagenesis for structure/function studies. Site-directed mutagenesis tests the role of individual amino acid residues in enzyme function or structure. Such knowledge is a prerequisite in developing new biotechnological approaches to control insects via the gut.

4.5.2.3. Classification of Digestive Enzymes

Digestive enzymes are hydrolases. The enzyme classification and numbering system used here is that recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Enzyme Commission).

Peptidases (peptide hydrolases, EC 3.4) are enzymes that act on peptide bonds and include the proteinases (endopeptidases, EC 3.4.21–24) and the exopeptidases (EC 3.2.4.11–19). Proteinases are divided into subclasses on the basis of catalytic mechanism, as shown with specific reagents or effect of pH. Specificity is only used to identify individual enzymes within subclasses. Serine proteinases (EC 3.4.21) have a serine and a histidine in the active site. Cysteine proteinases (EC 3.4.22) possess a cysteine in the active site and are inhibited by mercurial compounds. Aspartic proteinases (EC 3.4.23) have a pH optimum below 5, due to the involvement of a carboxyl residue in catalysis. Metalloproteinases (EC 2.3.24) need a metal ion in the catalytic process. Exopeptidases include enzymes that hydrolyze single amino acids from the N-terminus (aminopeptidases, EC 3.4.11) or from the C-terminus (carboxypeptidases, EC 3.4.16–18) of the peptide chain and those enzymes specific for dipeptides (dipeptide hydrolases, EC 3.4.13) (Figure 2).

Glycosidases (EC 3.2) are classified according to their substrate specificities. They include the enzymes that cleave internal bonds in polysaccharides and are usually named from their substrates, e.g., amylase, cellulase, pectinase, and chitinase. They also include the enzymes that hydrolyze oligosaccharides and disaccharides. Oligosaccharidases and disaccharidases are usually named based on the monosaccharide that gives its reducing group to the glycosidic bond and on the configuration (α or β) of this bond (Figure 2).

Lipids are a large and heterogeneous group of substances that are relatively insoluble in water but readily soluble in apolar solvents. Some contain fatty acids (fats, phospholipids, glycolipids, and waxes) and others lack them (terpenes, steroids, and carotenoids). Ester bonds are hydrolyzed in lipids containing fatty acids before they are absorbed. The enzymes that hydrolyze ester bonds comprise: (1) carboxylic ester hydrolases (EC 3.1.1), e.g., lipases, esterases, and phospholipases A and B; (2) phosphoric monoester hydrolases (EC 3.1.3), which are the phosphatases; and (3) phosphoric diester hydrolases (EC 3.1.4), including phospholipases C and D (Figure 2).

4.5.2.4. Phases of Digestion and Their Compartmentalization in the Insect Gut

Most food molecules to be digested are polymers such as proteins and starch and are digested sequentially in three phases. Primary digestion is the dispersion and reduction in molecular size of the polymers and results in oligomers. During intermediate digestion, these undergo a further reduction in molecular size to dimers; in final digestion, they become monomers. Digestion usually occurs under the action of digestive enzymes from the midgut, with little or no participation of salivary enzymes.

Any description of the spatial organization of digestion in an insect must relate the midgut compartments (cell, ecto-, and endoperitrophic spaces) to each phase of digestion and, hence, to the corresponding enzymes. To accomplish this, enzyme determinations must be performed in each midgut luminal compartment and in the corresponding tissue. Techniques of sampling enzymes from midgut luminal compartments and for identifying microvillar enzymes, and enzymes trapped in cell glycocalyx have been reviewed elsewhere (Terra and Ferreira, 1994).

Frequently, initial digestion occurs inside the peritrophic membrane (see Sections 4.5.7.1 and 4.5.7.2), intermediate digestion in the ectoperitrophic space, and final digestion at the surface of midgut cells by integral microvillar enzymes or by enzymes trapped into the glycocalyx (Figure 1). Exceptions to this rule, and the procedures for studying the organization of the digestive process, will be detailed below.

4.5.2.5. Role of Microorganisms in Digestion

Most insects harbor a substantial microbiota including bacteria, yeast, and protozoa. Microorganisms might be symbiotic or fortuitous contaminants from the external environment. They are found in the lumen, adhering to the peritrophic membrane, attached to the midgut surface, or within cells. Intracellular bacteria are usually found in special cells, the mycetocytes, which may be organized in groups, the

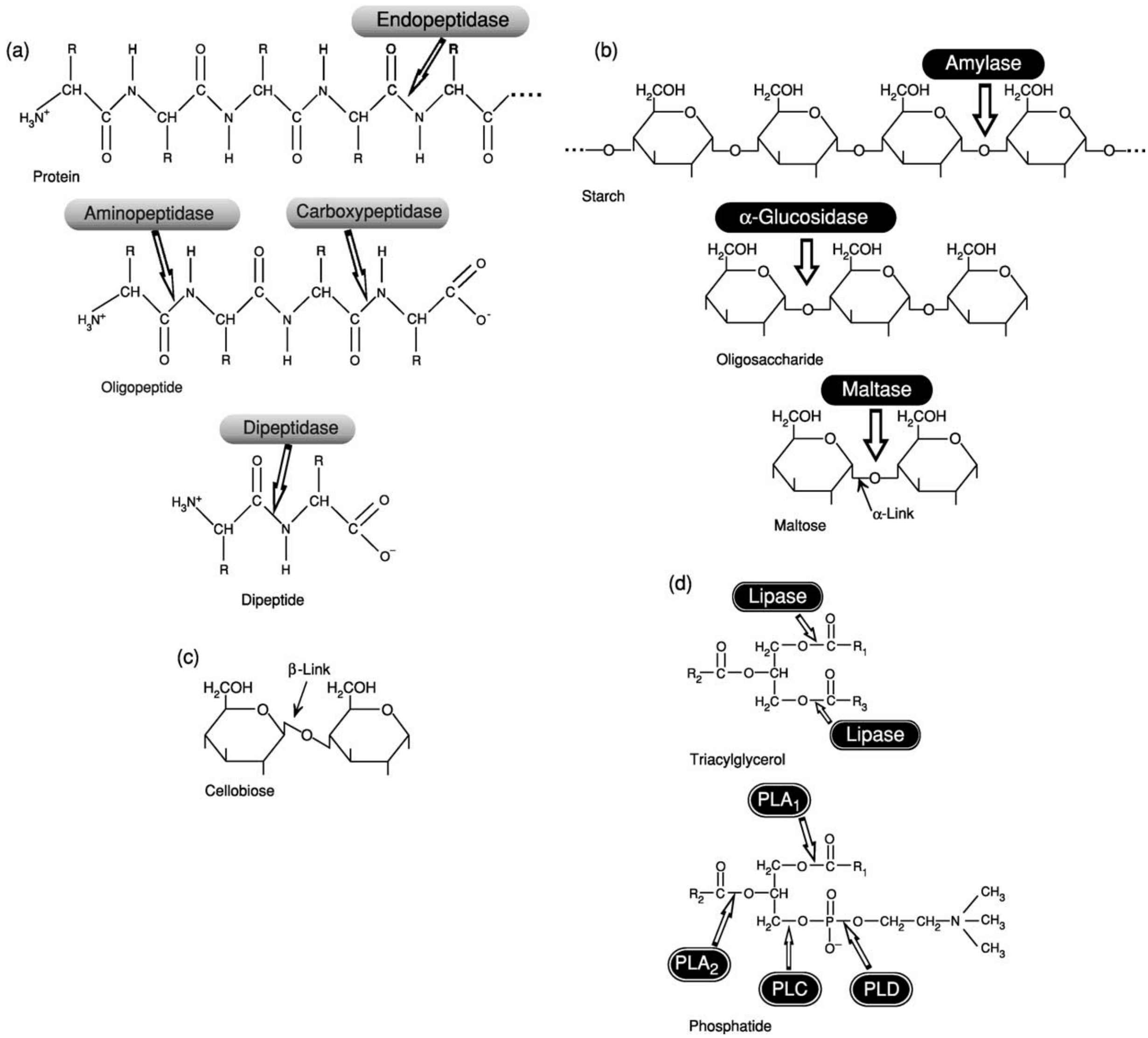


Figure 2 Digestion of important nutrient classes. Arrows point to bonds cleaved by enzymes. (a) Protein digestion; R, different amino acid moieties; (b) starch digestion; (c) β-linked glucoside; (d) lipid digestion; PL, phospholipase; R, fatty acyl moieties. (Reprinted with permission from Terra, W.R., **2003**. Digestion. In: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects. Academic Press, San Diego, CA, pp. 310–313; © Elsevier.)

mycetomes. Microorganisms produce and secrete their own hydrolases and cell death will result in the release of enzymes into the intestinal milieu. Any consideration of the spectrum of hydrolase activity in the midgut must include the possibility that some of the activity may derive from microorganisms. Despite the fact that digestive enzymes of some insects are thought to be derived from the microbiota, there are relatively few studies that show an unambiguous contribution of microbial hydrolases. Best examples are found among wood- and humus-feeding insects like termites, tipulid fly larvae, and scarabid beetle larvae. Although these insects may have their own cellulases (see Section 4.5.4.3.1), only fungi and certain filamentous bacteria developed a strategy for the chemical breakdown of lignin. Lignin is a phenolic polymer that forms an amorphous resin in which the polysaccharides of the secondary plant cell wall are embedded, thus becoming protected from enzymatic attack (Terra *et al*., 1996; Brune, 1998; Dillon and Dillon, 2004).

Microorganisms play a limited role in digestion, but they may enable phytophagous insects to overcome biochemical barriers to herbivory (e.g., detoxifying flavonoids and alkaloids). They may also provide complex-B vitamins for blood-feeders and essential amino acids for phloem feeders, produce pheromone components, or withstand the colonization of the gut by nonindigenous species (including pathogens) (Dillon and Dillon, 2004).

4.5.3. Midgut Conditions Affecting Enzyme Activity

The pH of the contents of the midgut is one of the important internal environmental properties that affect digestive enzymes. Although midgut pH is hypothesized to result from adaptation of an ancestral insect to a particular diet, its descendants may diverge, feeding on different diets, while still retaining the ancestral midgut pH condition. Thus there is not necessarily a correlation between midgut pH and diet. In fact midgut pH correlates well with insect phylogeny (Terra and Ferreira, 1994; Clark, 1999).

The pH of insect midgut contents is usually in the 6–7.5 range. Major exceptions are the very alkaline midgut contents (pH 9–12) of Lepidoptera, scarab beetles and nematoceran Diptera larvae, the very acid (pH 3.1–3.4) middle region of the midgut of cyclorrhaphous Diptera, and the acid posterior region of the midgut of heteropteran Hemiptera (Terra and Ferreira, 1994; Clark, 1999). pH values may not be equally buffered along the midgut. Thus, midgut contents are acidic in the anterior midgut and nearly neutral or alkaline in the posterior midgut in Dictyoptera, Orthoptera, and most families of Coleoptera. Cyclorrhaphan Diptera midguts have nearly neutral contents in the anterior and posterior regions, whereas in middle midgut the contents are very acid (Terra and Ferreira, 1994).

The high alkanity of lepidopteran midgut contents is thought to allow these insects to feed on plant material rich in tannins, which bind to proteins at lower pH, reducing the efficiency of digestion (Berenbaum, 1980). This explanation may also hold for scarab beetles and for detritus-feeding nematoceran Diptera larvae that usually feed on refractory materials such as humus. Nevertheless, mechanisms other than high gut pH must account for the resistance to tannin displayed by some locusts (Bernays *et al*., 1981) and beetles (Fox and Macauley, 1977). One possibility is the effect of surfactants, such as lysolecithin which is formed in insect fluids due to the action of phospholipase A on cell membranes (**Figure 2**), and which occurs widely in insect digestive fluids (De Veau and Schultz, 1992). Surfactants are known to prevent the precipitation of proteins by tannins even at pH as low as 6.5 (Martin and Martin, 1984). Present knowledge is not sufficient to relate midgut detergency to diet or phylogeny or to both.

Tannins may have deleterious effects other than precipitating proteins. Tannic acid is frequently oxidized in the midgut lumen, generating peroxides, including hydrogen peroxide, which readily diffuses across cell membranes and is a powerful cytotoxin. In some insects, e.g., *Orgyia leucostigma,* tannic acid oxidation and the generation of peroxides are suppressed by the presence of high concentrations of ascorbate and glutathione in the midgut lumen (Barbehenn *et al*., 2003). Dihydroxy phenolics in an alkaline medium are converted to quinones that react with lysine ε-amino groups. This leads to protein aggregation and a decrease in lysine availability for the insect. Other compounds, e.g., oleuropein, alkylate lysine residues in proteins, causing the same problems as dihydroxy phenolics. These phenomena are inhibited in larvae of several lepidopteran species by secreting glycine into the midgut lumen. Glycine competes with lysine residues in the denaturating reaction (Konno *et al*., 2001).

A high midgut pH may also be of importance, in addition to its role in preventing tannin binding to proteins, in freeing hemicelluloses from plant cell walls ingested by insects. Hemicelluloses are usually extracted in alkaline solutions for analytical purposes (Blake *et al*., 1971) and insects, such as the caterpillar *Erinnyis ello*, are able to digest hemicelluloses efficiently without affecting the cellulose from the leaves they ingest to any degree (Terra, 1988). This explanation is better than the previous one in accounting for the very high pH observed in several insects, since a pH of about 8 is sufficient to prevent tannin binding to proteins (Terra, 1988).

The acid region in the cyclorrhaphous Diptera midgut is assumed to be involved in the process of killing and digesting bacteria, which may be an important food for maggots. This region is retained in Muscidae that have not diverged from the putative ancestral bacteria-feeding habit, as well as in the flesh-feeding Calliphoridae and in the fruit-feeding Tephritidae (Terra and Ferreira, 1994). The acid posterior midgut of Hemiptera may be related to their lysosome-like digestive enzymes (cysteine and aspartic proteinase) (see Sections 4.5.5.3 and 4.5.5.4).

Few papers have dealt with midgut pH buffering mechanisms. The early unsuccessful attempts to relate midgut buffering activity to the large amounts of phosphate frequently found in insect midguts, as well as other unsuccessful attempts to describe buffering mechanisms, are reviewed by House (1974). The results of more recent research on midgut buffering mechanisms are more encouraging. Dow (1992) showed that the lepidopteran larval midgut transports equal amounts of K^+ and alkali from blood to the midgut lumen. Based on this and other data he described a carbonate secretion system, which may be responsible for the high pH found in Lepidoptera midguts (**Figure 3**). Phosphorus NMR

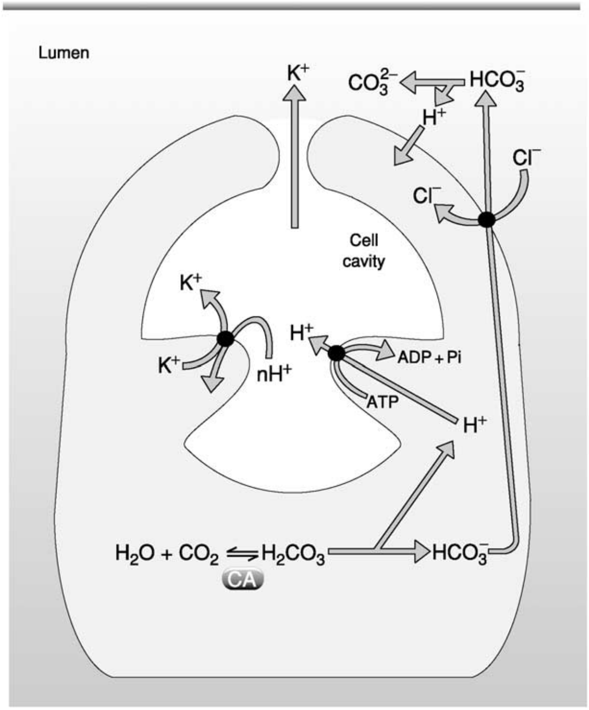


Figure 3 A model for generation of high gut pH by the goblet cells of lepidopteran larvae. Carbonic anhydrase (CA) produces carbonic acid that dissociates into bicarbonate and a proton. The proton is pumped by a V-ATPase into the goblet cell cavity, from where it is removed in exchange with K^+ that eventually diffuses into lumen. Bicarbonate is secreted in exchange with chloride and loses a proton due to the intense field near the membrane, forming carbonate and raising the gut pH. (Data from Dow, J.A.T., **1992**. pH gradients in lepidopteran midgut. *J. Exp. Biol. 172*, 355–375.)

microscopy has been used to show that valinomycin leads to a loss of alkalinization in the midgut of *Spodoptera litura* (Skibbe *et al.*, 1996). As valinomycin is known to transport K^+ down its concentration gradient, this result gives further support to the model described in **Figure 3**. It is not known whether midgut alkalinization in scarab beetles and nematoceran Diptera occurs by mechanisms similar to those of lepitopteran larvae. Terra and Regel (1995) determined pH values and concentrations of ammonia, chloride, and phosphate in the presence or absence of ouabain and vanadate in *Musca domestica* midguts. From the results they proposed that middle midgut acidification is accomplished by a proton pump of mammalian-like oxyntic cells, whereas the neutralization of posterior midgut contents depends on ammonia secretion (**Figure 4**).

Redox conditions in the midgut are regulated and may be the result of phylogeny, although data are scarce. Reducing conditions are observed in clothes moth, sphinx moths, owlet moths, and dermestid beetles (Appel and Martin, 1990) and in Hemiptera (Silva and Terra, 1994). Reducing conditions are important to open disulfide bonds in keratin ingested by some insects (clothes moths, dermestid beetles) (Appel and Martin, 1990), to maintain the activity of the major proteinase in Hemiptera (see Section 4.5.5.3), and to reduce the impact of some plant allelochemicals, such as phenol, in some herbivores (Appel and Martin, 1990). In spite of this, the artificial lowering of *in vivo* redox potentials did not

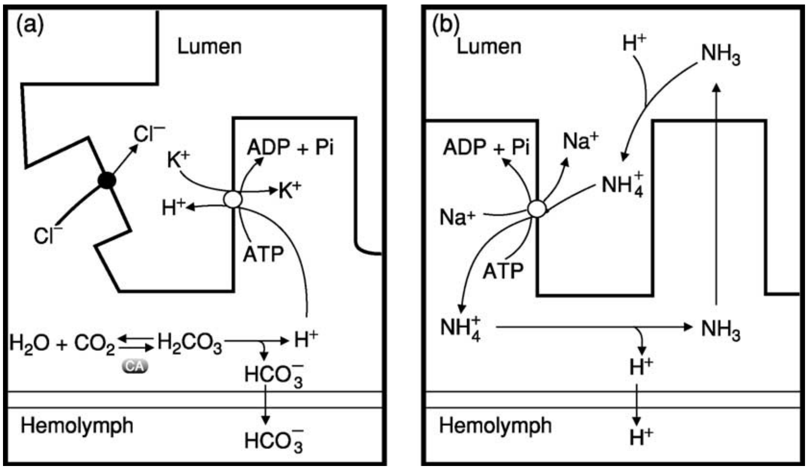


Figure 4 Diagrammatic representation of ion movements, proposed as being responsible for maintenance of pH in the larval midgut contents of *Musca domestica*. Carbonic anhydrase (CA) in cup-shaped oxyntic cells in the middle of the midgut (a) produces carbonic acid which dissociates into bicarbonate and a proton. Bicarbonate is transported into the hemolymph, whereas the proton is actively translocated into the midgut lumen acidifying its contents to pH 3.2. Chloride ions follow the movement of protons. NH_3 diffuses from anterior and posterior midgut cells (b) into the midgut lumen, becoming protonated and neutralizing their contents to pH 6.1–6.8. NH_4^+ is then exchanged for Na^+ by a microvillar Na^+/K^+-ATPase. Inside the cells, NH_4^+ forms NH_3, which diffuses into midgut lumen, and proton that is transferred into the hemolymph. (Reprinted with permission from Terra, W.R., Regel, R., **1995**. pH buffering in *Musca domestica* midguts. *Comp. Biochem. Physiol. A 112*, 559–564; © Elsevier.)

significantly impact digestive efficiency of the herbivore *Helicoverpa zea*, although the reducing agent used (dithiothreitol) inhibited some proteinases *in vitro* (Johnson and Felton, 2000).

Although several allelochemicals other than phenols may be present in the insect gut lumen, including alkaloids, terpene aldehydes, saponins, and hydroxamic acids (Appel, 1994), there are no sufficient data on their effect on digestion.

4.5.4. Digestion of Carbohydrates

4.5.4.1. Initial Considerations

Polysaccharides are major constituents of cell walls and energy reserves such as starch granules within plant cells and glycogen within animal cells. For phytophagous insects, disruption of plant cell walls is necessary in order to expose storage polymers in cell contents to polymer hydrolases. Cell wall breakdown may be achieved by mastication, but more frequently it is the result of the action of digestive enzymes. Thus, even insects unable to obtain nourishment from the cellulosic and noncellulosic cell wall biochemicals would profit from having enzymes active against these structural components. Cell walls are disrupted by β-glucanases, xylanases, and pectinases (plant cells), lysozyme (bacterial cells), or chitinase and β-1,3-glucanase (fungal cells). The carbohydrates associated with cellulose are frequently named "hemicelluloses" and the enzymes that attack them "hemicellulases." Thus, the hemicellulases include β-glucanases other than cellulases, xylanases, and pectinases. Following the loss of cell wall integrity, starch digestion is initiated by amylases. A complex of carbohydrases converts the oligomers resulting from the action of the polymer hydrolyzing enzymes into dimers (such as sucrose, cellobiose, and maltose that also occur free in some cells) and finally into monosaccharides like glucose and fructose.

4.5.4.2. Amylases

α-Amylases (EC 3.2.1.1) catalyze the endohydrolysis of long α-1,4-glucan chains such as starch and glycogen. Amylases are usually purified by glycogen–amylase complex formation followed by precipitation in cold ethanol or, alternatively, by affinity chromatography in a gel matrix linked to a protein amylase inhibitor. In sequence, isoamylases can be resolved by anion exchange chromatography (Terra and Ferreira, 1994).

Most insect amylases have molecular weights in the range 48–60 kDa, pI values of 3.5–4.0, and Km values with soluble starch around 0.1%. pH optima generally correspond to the pH prevailing in midguts from which the amylases were isolated. Insect amylases are calcium-dependent enzymes and are activated by chloride with displacement of the pH optimum. Activation also occurs with anions other than chloride, such as bromide and nitrate, and it seems to depend upon the ionic size (Terra and Ferreira, 1994).

The best-known insect α-amylase, and the only one whose 3D structure has been resolved, is the midgut α-amylase of *Tenebrio molitor* larvae. The enzyme has three domains. The central domain (domain A) is an $(\beta/\alpha)_8$-barrel that comprises the core of the molecule and includes the catalytic amino acid residues (Asp 185, Glu 222, and Asp 287) (*T. molitor* α-amylase numbering throughout). Domains B and C are almost opposite to each other, on each side of domain A. The substrate-binding site is located in a long V-shaped cleft between domains A and B. There, six saccharide units can be accommodated, with the sugar chain being cleaved between the third and fourth bound glucose residues. A calcium ion is placed in domain B and is coordinated by Asn 98, Arg 146, and Asp 155. This ion is important for the structural integrity of the enzyme and seems also to be relevant due its contact with His 189. This residue interacts with the fourth sugar of the substrate bound in the active site, forming a hinge between the catalytic site and the Ca^{2+}-binding site. A chloride ion is coordinated by Arg 183, Asn 285, and Arg 321 in domain A (Strobl *et al*., 1998a). Domain C is placed in the C-terminal part of the enzyme, contains the so-called "Greek key" motif and has no clear function (**Figure 5**). These structural features are shared by all known α-amylases (Nielsen and Borchert, 2000), although not all α-amylases have a chloride-binding site (Strobl *et al*., 1998a). The most striking difference between mammalian and insect α-amylases is the presence of additional loops in the vicinity of the active site of the mammalian enzymes (Strobl *et al*., 1998a).

Asp 287 is conserved in all α-amylases. Comparative studies have shown that Glu 222 is the proton donor and Asp 185 the nucleophile, and that Asp 287 is important but not a direct participant in catalysis. It is proposed that its role is to elevate the pK_a of the proton donor (Nielsen and Borchert, 2000).

Chloride ion is an allosteric activator of α-amylases, leading to a conformation change in the enzyme that changes the environment of the proton donor. This change causes an increase in the pK_a of the proton donor, thus displacing the pH optimum of the enzyme and increasing its V_{max} (Levitzki and Steer, 1974). According to Strobl *et al*. (1998a), the increase in V_{max} is a consequence of chloride ion being close to the water molecule that has been

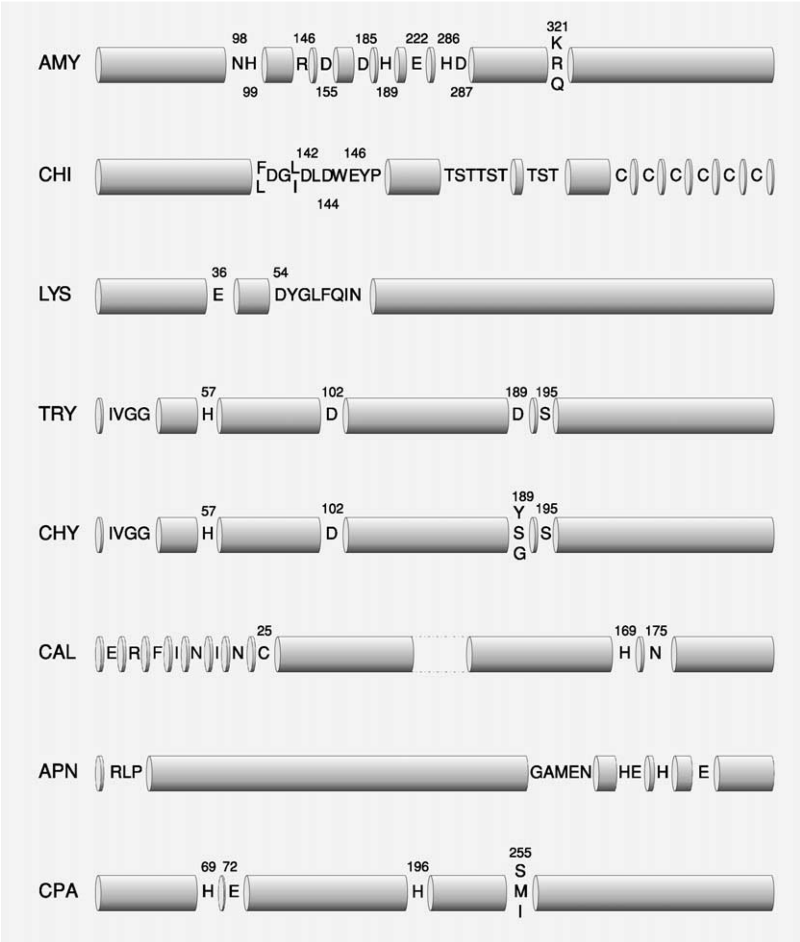


Figure 5 Conserved residues in the primary structures of major insect digestive enzymes. AMY (amylase) follows *Tenebrio molitor* amylase numbering; CHI (chitinase), molting-fluid *Manduca sexta* chitinase numbering. TRY (trypsin) and CHY (chymotrypsin) follow the bovine chymotrypsin numbering; CAL (cathepsin L), papain numbering: APN (aminopeptidase N) does not have a consensual numbering; CPA (carboxypeptidase A), mammalian CPA numbering.

suggested to initiate the cleavage of the substrate chain. The nucleophilicity of this water molecule might be enhanced by the negative charge of the ion.

There are 21 complete sequences of insect α-amylases registered in the GenBank (as of May 2003) excluding all of the sequences of *Drosophila*. The sequences correspond to 18 species found in four orders. Examples may be found among Hymenoptera (Ohashi *et al*., 1999; Da Lage *et al*., 2002), Coleoptera (Strobl *et al*., 1997; Titarenko and Chrispeels, 2000), Diptera (Grossmann *et al*., 1997; Charlab *et al*., 1999), and Lepidoptera (Da Lage *et al*., 2002). All the sequences have the catalytic triad (Asp 185, Glu 222, and Asp 287), the substrate-binding histidine residues (His 99, His 189, and His 286), and the Ca^{2+}-coordinating residues (Asn 98, Arg 146, and Asp 155) (Figure 5). From the residues found to be involved in chloride binding, Arg 183 and Asn 285 are conserved, whereas position 321 varies. According to D'Amico *et al*. (2000), all known chloride-activated α-amylases have an arginine or lysine residue at position 321. Insect α-amylase sequences have arginine at position 321, except those of *Zabrotes subfasciatus* and *Anthonomus grandis*, which have lysine, and the lepidopteran α-amylases which have glutamine. This agrees with the observation that most insect α-amylases are activated by chloride with the remarkable exception of lepidopteran amylases (Terra and Ferreira, 1994). The few coleopteran and hymenopteran α-amylases reported to be not affected by chloride (Terra and Ferreira, 1994) deserve reinvestigation. It is possible that another anion is replacing chloride as an activator, as shown for some hemipteran amylases (Hori, 1972).

Action pattern refers to the number of bonds hydrolyzed during the lifetime of a particular enzyme–substrate complex. If more than one bond

is hydrolyzed after the first hydrolytic step, the action pattern is said to be processive. The degree of multiple attack is the average number of hydrolyzed bonds after the first bond is broken. *Rhynchosciara americana* amylase has a degree of multiple attack between that of the amylase of *Bacillus subtilis* (1.7) and porcine pancreas (6). Amylases from larvae and adults of *Sitophilus zeamais*, *S. granarius*, and *S. oryzae* and larvae of *Bombyx mori* have action patterns similar to that of porcine pancreas amylase (Terra and Ferreira, 1994). These studies need to be taken further, including the determination of the affinities corresponding to each subsite in the active center. Such studies, specially if combined with crystallographic data, may describe in molecular detail the reasons why amylases act differently toward starches of distinct origins.

There is a variety of natural compounds that affect amylases, including many plant protein inhibitors (Franco *et al.*, 2002). Crystallographic data have shown that these protein inhibitors always occupy the amylase active site (Strobl *et al.*, 1998b; Nahoum *et al.*, 1999). In the case of the *Amaranth* α-amylase inhibitor, a comparison of *T. molitor* amylase–inhibitor complex with a modeled complex between porcine pancreatic α-amylase and the inhibitor identified six hydrogen bonds that can be formed only in the *T. molitor* amylase–inhibitor complex (Pereira *et al.*, 1999). This was the first successful explanation of how a protein inhibitor specifically inhibits α-amylases from insects, but not from mammalian sources. As will be discussed with details for trypsins (see Section 4.5.5.2.1), specific amylases are induced when insect larvae are fed with α-amylase inhibitor-containing diets (Silva *et al.*, 2001). The mechanisms underlying this induction are unknown and are presently under study (C.P. Silva, personal communication).

4.5.4.3. β-Glucanases

β-Glucanases are enzymes acting on β-glucans. These are major polysaccharide components of plant cell walls and include β-1,4-glucans (cellulose), β-1,3-glucans (callose), and β-1,3;1,4-glucans (cereal β-glucans) (Bacic *et al.*, 1988). The cell walls of certain groups of fungi have β-1,3;1,6-glucans (Bacic *et al.*, 1988).

4.5.4.3.1. Cellulases Cellulose is by far the most important β-glucan. It occurs in the form of β-1,4-glucan chains packed in an ordered manner to form compact aggregates which are stabilized by hydrogen bonds. The resulting structure is crystalline and not soluble. According to work done with microbial systems, cellulose is digested by a combined action of two enzymes. An *endo*-β-1,4-glucanase (EC 3.2.1.4), with an open substrate-binding cleft, cleaves bonds located within chains in the amorphous regions of cellulose, creating new chain ends. An *exo*-β-1,4-glucanase (EC 3.2.1.91) processively releases cellobiose from the ends of cellulose chains in a tunnel-like active site. Surface loops in cellobiohydrolase prevent the dislodged cellulose chains from readhering to the crystal surface, as the enzyme progresses into crystalline cellulose (Rouvinen *et al.*, 1990; Kleywegt *et al.*, 1997). Cellobiohydrolase structure is modular, comprising a catalytic domain linked to a distinct cellulose-binding domain, which enhances the activity of the enzyme towards insoluble cellulose (Linder and Teeri, 1997).

Cellulose digestion in insects is rare, presumably because the dietary factor that usually limits growth in plant feeders is protein quality. Thus, cellulose digestion is unlikely to be advantageous to an insect that can meet its dietary requirements using more easily digested constituents. Nevertheless, cellulose digestion occurs in several insects that have, as a rule, nutritionally poor diets (Terra and Ferreira, 1994). The role of symbiotic organisms in insect cellulose digestion is less important than initially believed (Slaytor, 1992), although symbiotic nitrogen-fixing organisms are certainly involved in increasing the nutritive value of diets of many insects (Terra, 1990).

Few insect cellulases have been purified and characterized. Two *endo*-β-1,4-glucanases (41 and 42 kDa) were isolated from the lower termite *Reticulitermes speratus* (Watanabe *et al.*, 1997). The cDNA that encodes this protein was cloned (Watanabe *et al.*, 1998) and the protein was shown to be secreted from the salivary glands (Tokuda *et al.*, 1999). The cDNAs that encode the *endo*-β-1,4-glucanase secreted by the midgut of the higher termite *Nasutitermes takasagoensis* (Tokuda *et al.*, 1999) and the two *endo*-β-1,4-glucanases from the woodroach *Panestria cribrata* of 47 kDa (Tokuda *et al.*, 1997) and 54 and 49 kDa (Scrivener and Slaytor, 1994), respectively, were also cloned (Tokuda *et al.*, 1999; Lo *et al.*, 2000).

Alignments of the sequences of termite and woodroach endoglucanases from data banks showed that they belong to family 9 of glycoside hydrolases (Coutinho and Henrissat, 1999). The paradigm of this family is the endo/exocellulase from the bacteria *Thermomonospora fusca*, whose catalytic center binds a cellopentaose residue and cleaves it into cellotetraose plus glucose or cellotriose plus cellobiose, and has Asp 55 as a nucleophile and Glu 424 as a proton donor (Sakon *et al.*, 1997).

The active site groups are conserved in the termite and woodroach endoglucanases although these enzymes lack the cellulose-binding domains that improve the binding and facilitate the activity of the catalytic domain on crystalline cellulose (Linder and Teeri, 1997). The conclusions drawn from sequence alignments were confirmed by the 3D structure resolution of the *N. takasagoensis* endoglucanase, which also revealed a Ca^{2+}-binding site near its substrate binding cleft (Khadeni *et al.*, 2002). According to Slaytor (1992), the large production of endoglucanases in termites and woodroaches would compensate their low efficiency on crystalline cellulose.

The phytophagous beetle *Phaedon cochleariae* has cellulase activity in its midgut (Girard and Jouanin, 1999a). A cDNA that encodes one cellulase was cloned and shown to belong to the glycoside hydrolase family 45 and to consist only of a catalytic domain. The purification of an *exo*-β-1,4-glucanase (52 kDa) from the termite *Macrotermes mulleri* (Rouland *et al.*, 1988) and the partial resolution from *Ergates faber* (Coleoptera) midgut extracts of another *exo*-β-1,4-glucanase (Chararas *et al.*, 1983) suggest that these insects, in contrast to the others, have cellulases with cellulose-binding domains.

4.5.4.3.2. Laminarinases and licheninases Licheninases (EC 3.2.1.73) digest only β-1,3;1,4-glucans whereas laminarinases may hydrolyze β-1,3;1,4-glucans and also β-1,3-glucans (EC 3.2.1.6) or only the last polymer (EC 3.2.1.39).

In spite of laminarinase activities being widespread among insects (Terra and Ferreira, 1994), only the laminarinases of *Periplaneta americana* (LAM, LIC 1, and LIC 2) have been purified and studied in detail (Genta *et al.*, 2003). The enzymes are secreted by salivary glands, stabilized by calcium ions, and have pH optima around 6. LAM (46.2 kDa) is an *endo*-β-1,3-glucanase that processively releases mainly glucose from laminarin and shows lytic activity on fungal cells. LIC 1 (24.6 kDa) is an *endo*-β-1,3-(4)-glucanase highly active on sequences of β-1,3-linked cellotetraoses, releasing cellotetraose from lichenin and is also able to lyse fungal cells. The specificities of *P. americana* β-glucanases agree with the omnivorous detritus-feeding habit of this insect, as they are able to help the cellulases in attacking plant cell walls (mainly those from cereals) and also in opening the cell walls of fungi, usually present in detritus.

No insect licheninase has been characterized in detail to date.

4.5.4.4. Xylanases and Pectinases

Xylans constitute the major noncellulosic polysaccharides (hemicelluloses) of primary walls of grasses and secondary walls of all angiosperms, accounting for one-third of all renewable organic carbon available on earth (Bacic *et al.*, 1988). Chemically, xylans are a family of linear β-1,4-xylans with a few branches. *Endo*-β-1,4-xylanase activities (EC 3.2.1.8) were found in several insects (Terra and Ferreira, 1994). One of these enzymes was cloned from a beetle and shown to correspond to a protein of 22 kDa, with high sequence identity to fungal xylanases, and conserving the usual two catalytic regions (Girard and Jouanin, 1999a). An *exo*-β-1,4-xylanase (EC 3.2.1.37) was partially purified from termites (Matoub and Rouland, 1995) and thought to act synergistically with an *endo*-β-1,4-xylanase originating from fungus ingested by the termites. Much more work is needed on this class of enzymes that may be important mainly for detritivorous insects.

Pectin is a linear chain of a D-galacturonic acid units with α-1,4-linkages in which varying proportions of the acid groups are present as methyl esters. It is the main component of the rhamnogalacturonan backbone of the structure formed by the pectin polysaccharides. Pectin is hydrolyzed by pectinases (polygalacturonases, EC 3.2.1.15) described in many insects (Terra and Ferreira, 1994).

Pectinases are thought to be important for hemipterans, as they would facilitate penetration of their stylets through plant tissues into sap-conducting structures and for insects boring plant parts. Accordingly, pectinases have been found in hemipteran saliva (Vonk and Western, 1984) and have been isolated and characterized from two weevils (Shen *et al.*, 1996; Dootsdar *et al.*, 1997) and cloned from a phytophagous beetle (Girard and Jouanin, 1999a).

The pectinases from the weevils *S. oryzae* (Shen *et al.*, 1996) and *Diaprepes abbreviatus* (Dootsdar *et al.*, 1997) were purified to electrophoretical homogeneity from whole-body extracts and gut homogenates, respectively. Purification of the pectinases was achieved by affinity chromatography through cross-linked pectate in addition to two ion exchange chromatographic steps. The enzymes have molecular masses of 35–45 kDa, pH optimum 5.5 and Km values of 1–4 mg ml^{-1} for pectic acid. The *D. abbreviatus* pectinase is inhibited by a polygalacturonase-inhibitor protein that may be associated with plant resistance to insects (Dootsdar *et al.*, 1997). Although the weevil pectinases may originate from endosymbiotic bacteria (Campbell *et al.*, 1992), the finding that the cDNA-coding pectinase

of the beetle *P. cochleariae* has a poly(A) tail (Girard and Jouanin, 1999a) argues against this hypothesis. The beetle pectinase belongs to family 28 of the glycoside hydrolases and is most related to fungal endopolygalacturonases, conserving the active site signature centered on the His 223 catalytic residue (Girard and Jouanin, 1999a; Markovic and Janecek, 2001).

4.5.4.5. Chitinases and Lysozymes

Chitin, the simplest of the glycosaminoglycans, is a β-1,4-homopolymer of *N*-acetylglucosamine (see **Chapter 4.3**). Chitinolytic enzymes (Kramer and Koga, 1986) include: chitinase (EC 3.2.1.14), which catalyzes the random hydrolysis of internal bonds in chitin forming smaller oligosaccharides, and β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.52), which liberates *N*-acetylglucosamine from the nonreducing end of oligosaccharides (Kramer and Koga, 1986). Lysozyme, as described below, also has some chitinase activity, whereas chitinase has no lysozyme activity. Chitinolytic enzymes associated with the ecdysial cycle have been studied and demonstrated to act synergistically in cuticular chitin degradation (Kramer and Koga, 1986). Nevertheless, these enzymes may also have a digestive role. Chitinase assays with midguts of several insects showed that there is a correlation between the presence of chitinase and a diet rich in chitin (Terra and Ferreira, 1994).

The best-known insect chitinase is the molting fluid chitinase from the lepidopteran *Manduca sexta* (**Figure 5**). The enzyme has a multidomain architecture that includes a signal peptide, an N-terminal catalytic domain, with the consensus sequence (F/L)DG(L/I)DLDWEYP, and a C-terminal cysteine-rich chitin-binding domain, which are connected by the interdomain serine/threonine-rich *O*-glycosylated linker. The residues Asp 142, Asp 144, Trp 145, and Glu 146 of the consensus sequence have been shown by site-directed mutagenesis to be involved in catalysis. Glu 146 functions as a proton donor in the hydrolysis like homologous residues in other glycoside hydrolases. Asp 144 apparently functions as an electrostatic stabilizer of the positively charged transition state, whereas Asp 142, and perhaps also Trp 145, influences the pK_a values of Asp 144 and Glu 146. The chitin-binding domains have 6 cysteines (with the consensus sequence CXnCXnCXnCXnCXnC, where Xn stands for a variable number of residues) and include several highly conserved aromatic residues (Tellam *et al*., 1999). The three disulfide bonds in the domain may constrain the polypeptide to present the aromatic amino acids on the protein surface for interactions with the ring structures of sugars. Thus, the chitin-binding domains enhance activity toward the insoluble polymer, whereas the linker region facilitates secretion from the cell and helps to stabilize the enzyme in the presence of proteolytic enzymes (Kramer *et al*., 1993; Lu *et al*., 2002; Zhang *et al*., 2002; Arakane *et al*., 2003).

Chitinase in molting fluid of the beetle *T. molitor* is a large (about 320 kDa) multidomain protein with five catalytic domains, five serine/threonine-rich linker domains, four chitin-binding domains, and two mucin-like domains. There is evidence that the enzyme is secreted as a zymogen activated by trypsin (Royer *et al*., 2002).

The *Anopheles gambiae* gut chitinase is secreted upon blood-feeding as an inactive proenzyme that is later activated by trypsin. Sequencing a cDNA coding the gut chitinase showed that the enzyme comprises a putative catalytic domain at the N-terminus, a putative chitin-binding domain at the C-terminus, and a serine/threonine/proline-rich amino acid stretch between them (Shen and Jacobs-Lorena, 1997). The mosquito chitinase seems to modulate the thickness and permeability of the chitin-containing peritrophic membrane (see Section 4.5.7.1). Supporting this conjecture the authors found that the peritrophic membrane is stronger and persisted longer when the mosquitoes were fed diets containing chitinase inhibitor.

The beetle *P. cochleariae* has one group of chitinases of 40–70 kDa active at pH 5.0 and detected in guts and another group of 40–70 kDa that are more active at pH 7.0 and that are associated with molting. A cDNA encoding a gut chitinase showed this enzyme has an active site centered on the catalytic residues Asp 146 and Glu 150 (*M. sexta* chitinase numbering), but lacks the C-terminal chitin-binding domain and the serine/threonine-rich interdomain (Girard and Jouanin, 1999b). The *T. molitor* midgut chitinase (GenBank accession no. AY325895) is similar to that of *P. cochleariae*. The putative role of *P. cochleariae* chitinase is in turnover of the peritrophic membrane (Girard and Jouanin, 1999b), as proposed above for *A. gambiae*. It is not clear, however, why the chitinase from *A. gambiae* in contrast to that of *P. cochleariae* has a chitin-binding domain if their putative role is to affect the same type (type I) (see Section 4.5.7.1) of peritrophic membrane. The *T. molitor* midgut chitinase may have the same proposed function, although it may also digest the cell walls of fungi usually present in its food. More research is necessary to clarify this point.

Lysozyme (EC 3.2.1.17) catalyzes the hydrolysis of the 1,4-β-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the

peptidoglycan present in the cell wall of many bacteria, causing cell lysis. Lysozyme is part of an immune defense mechanism against bacteria and has been described in most animals, including insects (Hultmark, 1996). Lysozyme has also been implicated in the midgut digestion of bacteria by organisms which ingest large amounts of them, such as marine bivalves (McHenery *et al.*, 1979), or that harbor a bacterial culture in their guts, as exemplified by ruminants (Stewart *et al.*, 1987).

Among insects, the capacity of digesting bacteria in the midgut seems to be an ancestral trait of Diptera Cyclorrhapha (Lemos and Terra, 1991a; Regel *et al.*, 1998), which agrees with the fact that most Diptera Cyclorrhapha larvae are saprophagous, feeding largely on bacteria (Terra, 1990). These insects have midgut lysozymes (Lemos *et al.*, 1993; Regel *et al.*, 1998) similar to those of vertebrate fermenters. Thus, these enzymes have low pI values, are more active at pH values 3–4, when present in media with physiological ionic strengths, and are resistant to the cathepsin D-like aspartic proteinase present in midguts (Lemos *et al.*, 1993; Regel *et al.*, 1998).

Sequence analyses (Kylsten *et al.*, 1992; Daffre *et al.*, 1994; Ito *et al.*, 1995) showed that cyclorrhaphan (*Drosophila melanogaster* and *Musca domestica)* digestive lysozymes have the same conserved residues as vertebrate lysozymes (Imoto *et al.*, 1972) (numbering according to Regel *et al.*, 1998): positions 55–61, Glu 36, and Asp 54. Glu 36 is believed to act as a general acid in catalysis, whereas Asp 54 is postulated to stabilize the resulting metastable oxocarbonium intermediate (Imoto *et al.*, 1972). More recently, Asp 54 has been implicated more strongly in catalysis of the hydrolysis of chitin-derived substrates (Matsumura and Kirsch, 1996). The ability of *D. melanogaster* and *M. domestica* purified lysozymes in hydrolyzing chitosan favors this view. The most remarkable sequence convergence of cyclorrhaphan digestive lysozymes with that of vertebrate foregut fermenters are Ser 104 and a decrease in the number of basic amino acids, suggesting that these features are necessary for a digestive role in an acid environment (Regel *et al.*, 1998). These hypotheses must be tested by site-directed mutagenesis and by the resolution of the 3D structure of at least one of these insect digestive lysozymes.

Lysozyme is also found in the salivary glands of *R. speratus*. This insect is a termite that feeds mainly on dead wood, which tends to lack nitrogen. Fujita *et al.* (2001) suggested, on the basis of the distribution and activity of lysozyme in this termite, that wood-feeding termites use lysozyme secreted from the salivary gland to digest their hindgut bacteria, which are transferred by proctodeal trophallaxis. The termite lysozyme is active in neutral pH and has no serine in position 104 (Fujita *et al.*, 2002), thus differing from the digestive cyclorrhaphan lysozymes.

Chitin and the bacterial peptidoglycan resemble one another chemically and structurally. Because of this one might anticipate that chitinases and lysozymes would be structurally related enzymes. Indeed, some lysozymes, exemplified by cyclorrhaphan digestive lysozymes (Regel *et al.*, 1998) are very good chitinases. Structurally well-known chitinases and lysozymes share no significant amino acid sequence similarities, but have a structurally invariant core consisting of two helices and a three-stranded β-sheet, which form the substrate-binding catalytic cleft. These enzymes are considered to represent a superfamily of hydrolases which are likely to have arisen by divergent evolution (Monzingo *et al.*, 1996). However, this picture does not take into account insect enzymes, for which 3D structural data are lacking.

4.5.4.6. α-Glucosidases

α-Glucosidases (EC 3.2.1.20) catalyze the hydrolysis of terminal, nonreducing α-1,4-linked glucose residues from aryl (or alkyl)-glucosides, disaccharides, or oligosaccharides. α-glucosidases are frequently named maltases, although some of them may have weak activity on maltose. Insect α-glucosidases occur as soluble forms in the midgut lumen or are trapped in the midgut cell glycocalyx. They are also bound to microvillar membranes (Terra and Ferreira, 1994), to perimicrovillar membranes (lipoprotein membranes ensheathing the midgut cell microvillar membranes in most hemipterans) (Silva and Terra, 1995), or to the modified perimicrovillar membranes of aphid midgut cells (Cristofoletti *et al.*, 2003). The last two membrane-bound α-glucosidases, as well as the soluble enzyme from bee midguts (Nishimoto *et al.*, 2001), were purified to electrophoretic homogeneity. *Culex pipiens* microvillar α-glucosidase is the primary target of the binary toxin of *Bacillus sphericus* and, although not purified, cDNA sequencing data suggest it is bound by a glycosyl phosphatidyl inositol anchor (Darboux *et al.*, 2001). α-Glucosidase is a major protein in dipteran midgut microvillae (Terra and Ferreira, 1994) and probably because of that it is the receptor of endotoxins, similar to that observed with aminopeptidase N in lepidopteran midgut cells (see Section 4.5.5.5).

Although biochemical properties of many crude, partially or completely purified gut α-glucosidases

are known, including molecular masses (range 60–80 kDa or a multiple of these values), pH optima (range 5–6.5, irrespective of the corresponding midgut pH value), pI values (range 5.0–7.2), and inhibition by tris(hydroxylmethyl)aminomethane (Tris), only few studies report on α-glucosidases specificities. These studies showed that insect α-glucosidases hydrolyze oligosaccharides up to at least maltopentaose (Terra and Ferreira, 1994), although there are exceptions. The perimicrovillar α-glucosidase from *Dysdercus peruvianus* prefers oligosaccharides up to maltotetraose (Silva and Terra, 1995) and the midgut bee α-glucosidase, up to maltotriose (Nishimoto *et al.*, 2001). The purified midgut α-glucosidase of the pea aphid, *Acyrthosiphon pisum* catalyzes transglycosylation reactions in the presence of excess sucrose, thus freeing glucose from sucrose without increasing the osmolarity of the medium (Cristofoletti *et al.*, 2003). This phenomenon associated with a quick fructose absorption (Ashford *et al.*, 2000) explains why the midgut luminal osmolarity decreases as the ingested sucrose-containing phloem sap passes along the aphid midgut.

Three digestive α-glucosidases from dipterans have been sequenced: one salivary (James *et al.*, 1989) and two midgut (Zheng *et al.*, 1995; Darboux *et al.*, 2001) enzymes. All the sequences have the invariant residues: Asp 123, His 128, Asp 206, Arg 221, Glu 271, His 296, and Asp 297 (numbers are relative to the positions in the sequence of *C. pipiens* α-glucosidases) (Darboux *et al.*, 2001) that are involved in the active site of α-amylase family of enzymes, and the three residues Gly 69, Pro 77, and Gly 323, that have a structural role for some α-glucosidases (Janecek, 1997).

4.5.4.7. β-Glucosidases and β-Galactosidases

β-Glycosidases (EC 3.2.1) catalyze the hydrolysis of terminal, nonreducing β-linked monosaccharide residues from the corresponding glycoside. Depending on the monosaccharide that is removed, the β-glycosidase is named β-glucosidase (glucose), β-galactosidase (galactose), β-xylosidase (xylose), and so on. Frequently, the same β-glycosidase is able to hydrolyze several different monosaccharide residues from glycosides. In this case, β-glucosidase (EC 3.2.1.21) is used to name all enzymes that remove glucose efficiently. The active site of these enzymes is formed by subsites numbered from the glycosidic linkage to be broken with negative (towards the nonreducing end of the substrate) or positive (towards the reducing end of the substrate) integers (Davies *et al.*, 1997). The nonreducing monosaccharide residue binds at the glycone (−1) subsite, whereas the rest of the molecule accommodates at the aglycone subsite, which actually may correspond to several monosaccharide residue-binding subsites.

Some insects have three or four digestive β-glycosidases with different substrate specificity. In others only two of these enzymes are found, that are able to hydrolyze as many different β-glycosides as the other three or four enzymes together (Ferreira *et al.*, 1998; Azevedo *et al.*, 2003).

Insect β-glycosidases best characterized have molecular masses of 30–150 kDa, pH optima of 4.5–6.5, and pI values of 3.7–6.8, whereas Km values with cellobiose or *p*-nitrophenyl β-glucoside (NpβGlu) as substrates, are found in the range of 0.2–2 mM. Although hydrolyzing several similar substances, insect digestive β-glycosidases have different specificities, preferring β-glucosides or β-galactosides as substrates, with hydrophobic or hydrophilic moieties in the aglycone part of the substrate (Terra and Ferreira, 1994; Azevedo *et al.*, 2003).

A few insect digestive β-glycosidases are more active on galactosides than on glucosides. Such enzymes are found in *Locusta migratoria* adults (Morgan, 1975), *Abracris flavolineata* adults (Marana, Terra, and Ferreira, unpublished data). In *Rhynchosciara americana* (Ferreira and Terra, 1983) and *T. molitor* (Ferreira *et al.*, 2003) larvae, there is a β-glycosidase that hydrolyzes galactosides but not glucosides.

Based on relative catalytical efficiency on several substrates, insect β-glycosidases can be divided into two classes. Class A includes the enzymes that efficiently hydrolyze substrates with hydrophilic aglycones, such as disaccharides and oligosaccharides. Class B comprises enzymes that have high activity only on substrates with hydrophobic aglycones, such as alkyl-, *p*-nitrophenyl-, and methylumbelliferyl-glycosides. Based on the same properties, β-glycosidases were previously divided into three classes (Terra and Ferreira, 1994), but the characterization of more enzymes showed that the previously proposed class 2 of β-glycosidases does not exist. Enzymes from the former class 2 comprised β-glycosidases supposed to have high activity only on di- and oligosaccharides. Former classes 2 plus 1 are now grouped in class A. Former class 3 are now named class B.

Enzymes from class A are more abundant than β-glycosidases from class B. Class A β-glycosidases hydrolyze di- and oligosaccharides and have four subsites for glucose binding in the active site: one in the glycone (−1) and three in the aglycone (+1, +2, +3) position (Ferreira *et al.*, 2001, 2003;

Marana *et al.*, 2001; Azevedo *et al.*, 2003). Some enzymes seem to be adapted to use disaccharides besides oligosaccharides as substrates. Optimal hydrolysis of disaccharides relies on high affinities to glucose moieties in −1 and primarily in the +1 subsite (Ferreira *et al.*, 2003). The enzymes highly active against oligosaccharides have subsites −1, +1, and +2 with similar affinities to glucose moieties (Ferreira *et al.*, 2001, 2003).

The affinities of some β-glycosidases for alkyl-glucosides were determined and binding energies for each methylene group to the active site were calculated. Values obtained for class A enzymes from *Spodoptera frugiperda* βGly50 (Marana *et al.*, 2001) and *T. molitor* βGly1 (Ferreira *et al.*, 2001) are 1.3 kJ mol^{-1} and −0.97 kJ mol^{-1}, respectively. Surprisingly, the binding energy in the case of *A. flavolineata* alkyl-glycosidase, which is an enzyme from class B, is only 0.47 kJ mol^{-1} (calculated from Marana *et al.*, 1995). Since the enzymes from *T. molitor* and *S. frugiperda* can hydrolyze di- and oligosaccharides and the *A. flavolineata* alkyl β-glycosidase can only use synthetic and more hydrophobic compounds as substrates, it seems that the hydrophobicity of the aglycone region is not directly related to the type of substrate that is hydrolyzed by the enzyme. Probably the extension of the aglycone site plays a major role.

Class A β-glycosidases are able to hydrolyze β-1,3, β-1,4, and β-1,6 glycoside bonds from di- and oligosaccharides. These enzymes are likely to be involved in the intermediate and terminal digestion of cellulose, hemicellulose, and glycoproteins present in food.

Class B β-glycosidases such as *D. saccharalis* βGly2 (Azevedo *et al.*, 2003) and *T. molitor* βGly4 seem to have two active sites (Ferreira *et al.*, 2003). These were detected by using alternative substrates as inhibitors. Two active sites are also found in βGly47 from *S. frugiperda* (Marana *et al.*, 2000) which is a class A enzyme, but one of its active site has properties of class B β-glycosidase. The three enzymes with two putative active sites are the main enzymes responsible for β-galactoside hydrolysis in the insect gut. The enzymes from the lepidopteran *S. frugiperda* and *D. saccharalis* hydrolyze mainly galactosides in one active site and glucosides in the other. The presence of two active sites in mammalian lactase-phlorizin hydrolase (the digestive mammalian β-glycosidase) has been known for a long time. The difference between the enzymes is that insect β-glycosidases with two active sites have the size of only one of the two mammalian β-glycosidase domains. Unfortunately, none of these types of enzymes has yet been cloned in insects. It is interesting to note that *T. molitor* βGly4 is activated by the detergent Triton X-100.

Class B β-glycosidases (or active site) with high activity against hydrophobic substrates may have the physiological role of hydrolyzing glycolipids, mainly galactolipidis that are found in high amount in vegetal tissues. The main galactolipids in plants are 2,3-diacyl β-galactoside D-glycerol (mono galactosyl diglyceride) and 2,3-diacyl 1-(α-galactosyl 1,6 β-galactosyl)-D-glycerol (digalactosyl diglyceride) (Harwood, 1980). These enzymes may act directly against the monogalactosyl diglyceride or on digalactosyl diglyceride after the removal of one of the galactose residues by α-galactosidase. The activation by amphipatic substances (as Triton X-100; see above) may be a mechanism to maintain high enzyme activity only in the neighborhood of plant cell membranes undergoing digestion in the insect midgut. Those membranes are the source of glycolipid substrates and activating detergent-like molecules. Distant from membranes, the β-glycosidase would be less active, thus hydrolyzing plant glucosides (see below) ingested by the insect with decreased efficiency.

In agreement with the hypothesis presented above, βGly47 from *S. frugiperda* can hydrolyze glycosylceramide, although with low activity (Marana *et al.*, 2000). In mammals, sphingosine and ceramide hydrolysis are dependent on enzyme activation by proteins called saposins (Harzer *et al.*, 2001). Given that genome sequences similar to saposins were found in *Drosophila melanogaster*, insect β-glycosidases active on glycolipids may also need the same kind of activators, and their absence in the assay reaction may explain why the activity against ceramides is low or not detected at all.

Free energy relationships (Withers and Rupitz, 1990) were used to compare the specificity of insect β-glycosidase subsites (Azevedo *et al.*, 2003). The enzymes with more similar active sites are *Diatraea saccharalis* βGly3 and *T. molitor* βGly1, followed by *D. saccharalis* βGly1 and *T. molitor* βGly3. *Diatraea saccharalis* βGly1 and βGly3 have active sites somewhat similar to the *S. frugiperda* βGly50K and *T. molitor* βGly1 with *T. molitor* βGly3. *D. saccharalis* βGly2, *T. molitor* βGly4 (Ferreira *et al.*, 2001), and *S. frugiperda* βGly47K (Marana *et al.*, 2000) are similar in some aspects. The latter three enzymes seem to have two active sites and are, in each insect, primarily responsible for β-galactoside digestion. Since each of the three *D. saccharalis* β-glycosidases has a counterpart in *T. molitor*, Azevedo *et al.* (2003) speculated that insects with the same number of β-glycosidases could have similar enzymes.

The only insect β-glycosidases sequenced to date have been those from the moth *S. frugiperda* midgut (βGly50) (Marana *et al*., 2001), the beetle *T. molitor midgut* (βGly1) (Ferreira *et al*., 2001), and termite *Neotermes koshunensis* salivary glands (Tokuda *et al*., 2002). The three enzymes share about 50% identity with one another and to other enzymes from glycosyl hydrolases from family 1, which have two glutamic acid residues responsible for catalysis and a structure of $(\beta/\alpha)_8$-barrel. In the βGly50 from *S. frugiperda* the pK_a of the nucleophile (Glu 399) is 4.9 and of the proton donor (Glu 187) is 7.5. In this enzyme, residue Glu 451 seems to be a key residue in determining the enzyme preference for glucosides versus galactosides. This is due to its interaction, in the glycone site, with substrate equatorial or axial hydroxyl 4, which is the only position where glucose differs from galactose. The steric hindrance of the same residue with hydroxyl 6 probably also explains why fucosides are the best substrate for many β-glycosidases (Marana *et al*., 2002b).

Besides having a role in digestion, β-glycosidases are important in insect–plant relationships. To avoid herbivory plants synthesize a large number of toxic glucosides (**Figure 6**) which may be present in concentrations from 0.5% to 1% (Spencer, 1988). The presence of these glucosides in some insect diets may explain the variable number of β-glycosidases with different specificities present in their guts. Most plant glucosides have a hydrophobic aglycone and are β-linked *O*-glycosyl compounds. Since aglycones are usually more toxic than the glycosides themselves, intoxication may be avoided by decreasing the activity of the enzyme most active on toxic glucosides, without affecting the final digestion of hemicellulose and cellulose carried out by the other enzymes. This is exemplified by *D. saccharalis* larvae, which have three β-glycosidases in their midgut, feeding on diets containing the cyanogenic glucoside amygdalin. In this condition, the activity of the enzyme responsible for the hydrolysis of prunasin is decreased (Ferreira *et al*., 1997). Prunasin is the saccharide resulting after the removal from amygdalin, and that forms the cyanogenic mandelonitrille upon hydrolysis (**Figure 6**). Resistance to toxic glucosides may also be achieved by the lack of enzymes able to hydrolyze toxic β-glucosides,

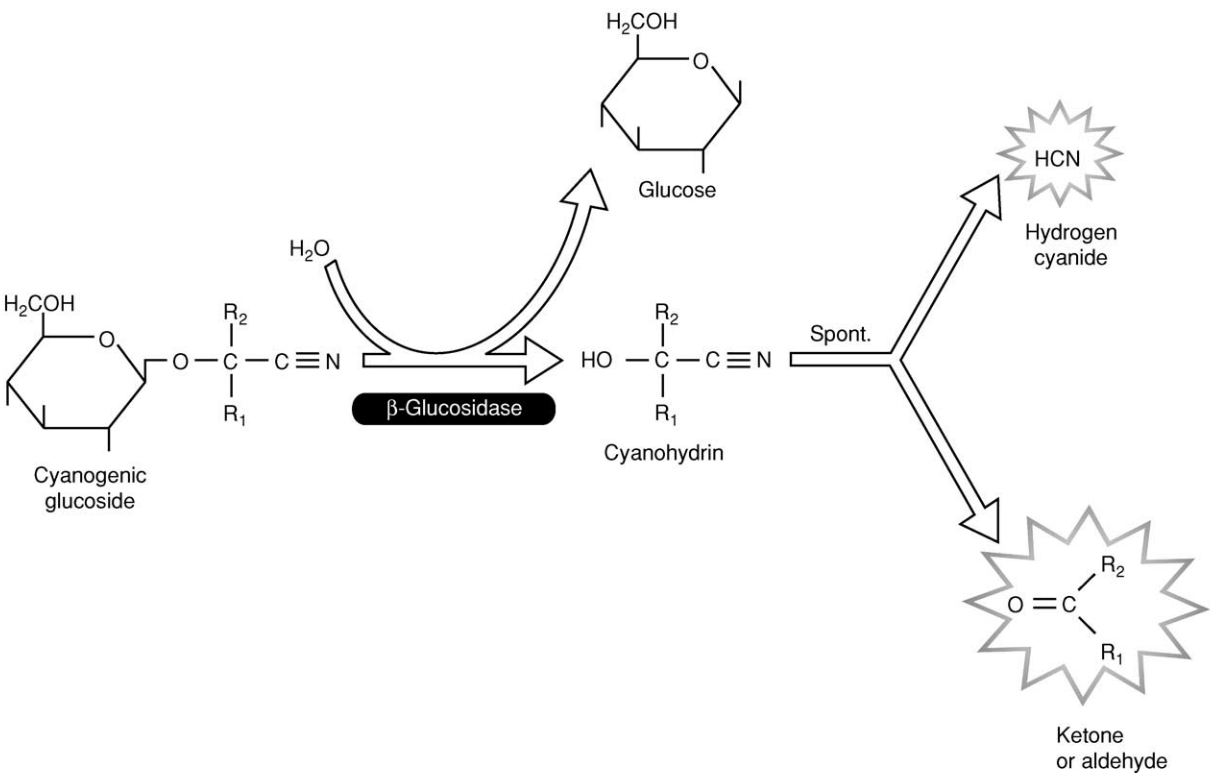


Figure 6 β-Glucosidase acting on a cyanogenic glucoside releases glucose and cyanohydrin that spontaneously decomposes, producing a ketone (or an aldehyde) and hydrogen cyanide. If $R_1 = R_2 = CH_3$, the glucoside is linamarin and the resulting ketone is acetone. If R_1 = H and R_2 = phenyl, the glucoside is prunasin and the resulting aldehyde is benzaldehyde (see more examples in Vetter (2000)).

as observed with *S. frugiperda* larvae, which have two β-glycosidases unable to efficiently hydrolyze prunasin (Marana *et al.*, 2001; S.R. Marana, personal communication).

Progress in this field will require disclosing the mechanisms by which the presence of toxic β-glucosides differentially affects the midgut β-glycosidases and knowing the details of the active site architecture responsible for the specificity of these enzymes.

4.5.4.8. Trehalases

Trehalase (EC 3.2.1.28) hydrolyzes α,α′-trehalose into two glucose molecules and is one of the most widespread carbohydrases in insects, occurring in most tissues. Trehalase is very important for insect metabolism, since trehalose is the main circulating sugar in these organisms. Apical and basal trehalases can be distinguished in insect midguts. The apical trehalase may be soluble (glycocalyx-associated or secreted into the midgut lumen) or microvillar, whereas the midgut basal trehalase is an integral protein of the basal plasma membrane. The apical midgut trehalase is a true digestive enzyme. The midgut basal trehalase probably plays a role in the midgut utilization of hemolymph trehalose (Terra and Ferreira, 1994). In spite of the importance of trehalase, it is poorly studied in insects and also in other sources. There is not a single trehalase with known catalytical groups or with its 3D structure resolved.

Trehalases partially or completely purified from insect guts have pH optima from 4.8 to 6.0, Km from 0.33 to 1.1 mM, pI around 4.6, and molecular masses from 60 to 138 kDa (Terra and Ferreira, 1994).

Tris is usually a competitive inhibitor of trehalase. Inhibition of midgut trehalases at their optimum pH was reported in *Apis mellifera*, *R. americana*, and *B. mori*, with Kis of 50, 74, and 47 mM, respectively, (Terra and Ferreira, 1994). Tris inhibited *R. americana* trehalase at pH 9.0 with a higher Ki (182 mM) than at pH 6.0 (74 mM), suggesting the presence of a negative charge at the active site to which the protonated Tris binds (Terra *et al*., 1978). In the Lepidoptera *Lymantria dispar* (Valaitis and Bowers, 1993) and *S. frugiperda* (Silva, Terra, and Ferreira, unpublished data) Tris up to 100 mM does not inhibit the enzymes near the pH optimum. Contrasting to what was found in trehalase from *R. americana*, in *S. frugiperda* Tris is a competitive inhibitor with a small Ki (0.55 mM) at pH 9.0. The results may be related to differences in the active site of midgut trehalases from Lepidoptera.

There have been a few attempts to identify important groups in the midgut trehalase active site. Terra *et al*. (1978, 1979, 1983) determined the pK_a values of the catalytical groups of the *R. americana* midgut trehalase. The pK_a value of the nucleophile was 5.0 (kinetic data) or 5.3 (carbodiimide modification results), whereas the pK_a value of the proton donor was 8.3 (kinetic data) or 7.7 (carbodiimide modification). Since there was a disagreement between the pK_a values determined for the proton donor, and taking into account that carbodiimide modification is only partially protected by trehalose, the authors suggested that the proton donor is near, but not at the active site, and that it participates in the reaction through another amino acid residue, like histidine (Terra *et al*., 1979).

Lee *et al*. (2001) with the same approach as Terra *et al*. (1979) found pK_a values of 5.3 and 8.5 for the *A. mellifera* trehalase. These authors and Valaitis and Bowers (1993), who worked with *L. dispar* trehalase, showed that the trehalases were inactivated by diethyl pyrocarbonate (DPC), but the work did not progress further. Purified midgut *S. frugiperda* trehalase is modified by DPC only in the presence of 2 × Ki of α-methyl glucoside, a linear competitive inhibitor of the enzyme, indicating that there is a conformational change in the enzyme following inhibitor binding. The modification achieved with DPC affects an imidazole group and only inhibits 60% of the enzyme activity. The pK_a values obtained from kinetic data are 4.8 and 7.6, and the pK_a values calculated from carbodiimide and phenyl glyoxal modification are 4.9 and 7.8, respectively. Whereas 10 Km trehalose competitively protect the enzyme from phenyl glyoxal modification, only partial protection is seen when the modifier is carbodiimide. These and other results suggest that a carboxyl group is the nucleophile and an Arg residue is the proton donor, which pK_a is affected by a nearby imidazole group (Silva, Terra, and Ferreira, unpublished data).

The only insect midgut trehalase sequenced up till now is that from *B. mori* pupae (Su *et al*., 1993). The corresponding mRNA is actively transcribed in midgut and Malpighian tubules, but not in the other tissues (Su *et al*., 1994). It is interesting to note that, in mammals, the same trehalases are present in the microvillar membranes of small intestine and kidney. The midgut trehalase from *B. mori* has identity of only 46% with trehalase from the male accessory gland from *T. molitor*. Since similar low identities are found for *B. mori* trehalase (known to be from midgut) and the other insect trehalases deposited in the GenBank, these probably do not have a midgut origin.

Plant toxic β-glucosides and their aglycones can inhibit, with varied efficiency, some or all trehalases

from Malpighian tubules, fat body, midgut, and body wall of *P. americana*, *M. domestica*, *S. frugiperda*, and *D. saccharalis* (Silva, Terra, and Ferreira, unpublished data). Toxic β-glucosides are produced by many plant species and are present in high concentrations (see Section 4.5.4.7). It is not known whether those glucosides or aglycones are absorbed by the insect gut and interact with trehalases in tissues other than the midgut. It would be interesting to know if this happens and how insects resistant to toxic β-glucosides avoid the damage they can cause.

4.5.4.9. Acetylhexosaminidases, β-Fructosidases, and α-Galactosidases

An enzyme related to chitinolytic enzymes is β-*N*-acetyl-D-hexosaminidase (EC 3.2.1.52), which differs from β-*N*-acetyl-D-glucosaminidase in having a rather wide substrate specificity. The enzyme is found in many insects and its presumed physiological role is the hydrolysis of *N*-acetylglucosamine β-linked compounds such as glycoproteins (Terra and Ferreira, 1994). Detailed studies of this enzyme are lacking.

Sucrose hydrolysis is catalyzed by enzymes that are specific for the α-glucosyl (α-glucosidase, EC 3.2.1.20; see above) or for the β-fructosyl residue (β-fructosidase, EC 3.2.1.26) of the substrate. β-Fructosidase is characterized by its activity toward sucrose and raffinose and lack of activity upon maltose and melibiose. In insect midguts, sucrose hydrolysis generally occurs by action of the conspicuous α-glucosidase rather than by β-fructosidase. Only a small number of reports verify the presence of β-fructosidase in insects (Terra and Ferreira, 1994; Scrivener *et al.*, 1997) and there has been only one attempt (Santos and Terra, 1986a) at characterization. Larvae and adults of the moth *Erinnyis ello* have a midgut β-fructosidase with pH optimum 6.0, Km 30 mM (sucrose), pI 5.2, and molecular mass of 78 kDa. The physiological role of this enzyme is to hydrolyze sucrose, the major leaf (larvae) or nectar (adults) carbohydrate, which is not efficiently digested by *E. ello* midgut α-glucosidase (Santos and Terra, 1986b).

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) catalyzes the hydrolysis of α-D-galactosidic linkages in the nonreducing end of oligosaccharides, galactomannans, and galactolipids and is widely distributed in nature (Dey and Pridham, 1972). Galactooligosaccharides, such as melibiose, raffinose, and stachiose are common in plants, mainly in lipid-rich seeds (Shiroya, 1963), whereas galactolipids are widespread among leaves. The major lipids in chloroplast membranes are monogalactosyldiglyceride and digalactosyldiglyceride (Harwood, 1980).

There have been few attempts to resolve insect midgut α-galactosidases. Gel filtration and heat inactivation suggested that there is a single α-galactosidase activity (30 kDa, pH optimum 5.0) in *Dysdercus peruvianus* midgut that is more efficient on raffinose than on melibiose and NPαGal (Silva and Terra, 1997). There are two α-galactosidases in *Abracris flavolineata* midguts: the major (112 kDa, pH optimum 5.4) is active on melibiose and raffinose in addition to NPαGal, whereas the minor (70 kDa, pH optimum 5.7) hydrolyzes only NPαGal (Ferreira *et al.*, 1999). In the case of *Psacothea hilaris*, gel filtration gave evidence of the presence of multiple overlapping α-galactosidases more active on NPαGal than on melibiose (Scrivener *et al.*, 1997). There are three midgut luminal α-galactosidases (TG1, TG2, and TG3) from *T. molitor* larvae that are partially resolved by ion-exchange chromatography (Grossmann and Terra, 2001). The enzymes have approximately the same pH optimum (5.0), pI value (4.6), and molecular mass (46–49 kDa). TG2 hydrolyzes α-1,6-galactosaccharides, exemplified by raffinose, whereas TG3 acts on melibiose and apparently also on digalactosyldiglyceride, the most important compound in thylacoid membranes of chloroplast, converting it into monogalactosyldiglyceride. *Spodoptera frugiperda* larvae have three midgut α-galactosidases (SG1, SG2, and SG3) partially resolved by ion-exchange chromatography (Grossmann and Terra, 2001). The enzymes have similar pH optimum (5.8), pI value (7.2), and molecular mass (46–52 kDa). SG1 and SG3 hydrolyze melibiose and SG3 digests raffinose and, perhaps, also digalactosyldiglyceride.

4.5.5. Digestion of Proteins

4.5.5.1. Initial Considerations

The initial digestion of proteins is carried out by proteinases (endopeptidases) that break internal bonds in proteins. Different proteinases are necessary to do this because the amino acid residues vary along the peptide chain. There are three subclasses of proteinases involved in digestion classified according to their active site group (and hence by their mechanism): serine, cysteine, and aspartic proteinases. In each of the mentioned subclasses, there are several proteinases differing in substrate specificities. The oligopeptides resulting from proteinase action are attacked from the N-terminal end by aminopeptidases and from the C-terminal end by carboxypeptidases, both enzymes liberating one amino acid residue at each catalytic step. Finally, the dipeptides formed are hydrolyzed by dipeptidases.

4.5.5.2. Serine Proteinases

Serine proteinases (EC 3.4.21) (MEROPS) have serine, histidine, and aspartic acid residues (called the catalytic triad) in the active site. The family of enzymes homologous to chymotrypsin (Barrett *et al.*, 1998) includes the major digestive enzymes trypsin, chymotrypsin, and elastase. These enzymes differ in structural features that are associated with their different substrate specificities, as detailed below. The numbering of residues in enzyme polypeptide chains is referred to that of bovine chymotrypsin.

4.5.5.2.1. Trypsins Trypsins (EC 3.4.21.4) preferentially cleave protein chains on the carboxyl side of basic L-amino acids such as arginine and lysine. Most insect trypsins have molecular masses in the range 20–35 kDa, pI values 4–5, and pH optima 8–10. These enzymes occur in the majority of the insects, with the remarkable exception of hemipteran species and some taxa belonging to the series Cucujiformia of Coleoptera like Curculionidae (Terra and Ferreira, 1994). Nevertheless, some heteropteran Hemiptera have trypsin in the salivary glands (Zeng *et al.*, 2002).

Trypsin is usually identified in insect midgut homogenates using benzoyl-arginine *p*-nitroanilide (B-R-pNA, often referred to as BApNA) or benzoyl-arginine 7-amino-4-methyl coumarin (B-R-MCA) as substrates and with *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF), or diisopropylfluorophosphate (DFP) as inactivating compounds. The substrates of choice for assaying insect trypsins are those in Figure 7. Trypsins from Orthoptera, Dictyoptera, and Coleoptera are usually purified by ion-exchange chromatography, whereas those from Diptera and Lepidoptera, by affinity chromatography, either in benzamidine-agarose (elution with benzamidine or by change in pH) or in soybean trypsin inhibitor (SBTI)-Sepharose (elution by change in pH). Due to significant autolysis, lepidopteran trypsins are more frequently purified by chromatography on benzamidine-Agarose with elution with benzamidine.

A total of 109 insect trypsin sequences were registered in GenBank in March 2003, corresponding to 34 species pertaining to five orders. Examples may be found among Hemiptera (Zeng *et al.*, 2002), Coleoptera (Girard and Jouanin, 1999a; Zhu and Baker, 1999), Diptera (Davis *et al.*, 1985; Barillas-Mury *et al.*, 1993), Siphonaptera (Gaines *et al.*, 1999), and Lepidoptera (Peterson *et al.*, 1994; Zhu *et al.*, 2000). Several sequences were not complete and some insect species are represented by many sequences that probably include enzymes other than digestive enzymes. The complete sequences have signal and activation

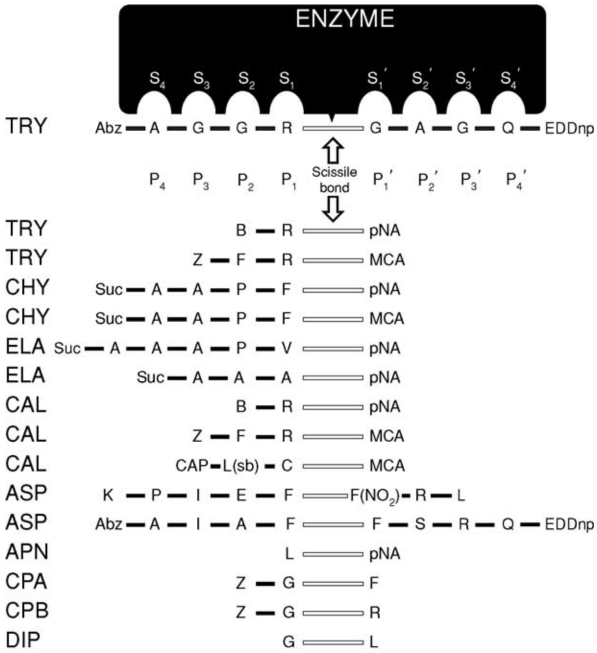


Figure 7 Substrates used in the assay of enzymes involved in protein digestion. S_n are subsites in the enzymes and P_n are amino acid residues in substrates. The arrows point to bonds cleaved by the different enzymes. Abz-Xn-EDDnp is a class of peptides with quenching (EDDnp) and fluorescent (Abz) groups at the C- and N-terminal ends, respectively, so that after hydrolysis the peptides become fluorescent. Substrates with C-terminal MCA are fluorescent and those with pNA are colorimetric. Contrary to GL, LG is also hydrolyzed by APN in addition to dipeptidase. For further details, see text. TRY, trypsin; CHY, chymotrypsin; ELA, elastase; CAL, cathepsin L-like enzyme; ASP, aspartic proteinase; APN, aminopeptidase N; CPA, carboxypeptidase A; CPB carboxypeptidase B; DIP, dipeptidase.

peptides and the features typical of trypsin-like enzymes, including the conserved N-terminal residues IVGG, the catalytic amino acid triad of serine proteinase active sites (His 57, Asp 102, and Ser 195), three pairs of conserved cysteine residues for disulfide bonds, and the residue Asp 189 that determines specificity in trypsin-like enzymes (see Figure 5).

In spite of having structural features resembling vertebrate trypsins, insect trypsins differ from these because they are not activated or stabilized by calcium ions and frequently are unstable in acidic pH (Terra and Ferreira, 1994). Another contrasting aspect is the almost lack of reports on the isolation of inactive insect trypsinogens, that is, trypsin molecules still having the activation peptide (Reeck *et al.*, 1999). The activation of insect trypsinogen to trypsin deserves a closer look. Finally, other differences between vertebrate and insect trypsins include their substrate specificities and their interaction with protein inhibitors.

Amino acyl residues in proteinase substrates are numbered from the hydrolyzed peptide bonds as P_1, $P_2, P_3, \ldots, P_n$ in the direction of the N-terminus and $P'_1, P'_2, P'_3, \ldots, P'_n$ in the direction of the peptide C-terminus, whereas the corresponding enzyme subsites are numbered $S_1, S_2, S_3, \ldots, S_n$ and S'_1, S'_2, S'_3, $\ldots$, S'_n (Schechter and Berger, 1967) (**Figure 7**). Mammalian trypsin preferably cleaves substrates having arginine rather than lysine at P_1 (primary specificity) (Craik *et al.*, 1985). The same primary specificity was found for insect trypsins, except those from lepidopterans, which prefer lysine at P_1 (Lopes *et al.*, 2004). This will be discussed below in relation to trypsin insensitivity to protein inhibitors. In order to characterize the trypsin specificity at subsites other than S_1, quenched fluorescent substrates like *o*-aminobenzoyl-GGRGAGQ-2,4-dinitrophenyl-ethylene diamine (where R stands for arginine at P_1 position) were synthesized with 15 amino acid replacements at each of the positions P'_1, P'_2, P_2, and P_3. The results suggested that trypsin subsites are more hydrophobic in trypsins from the more evolved insects (A.R. Lopes *et al.*, unpublished data). Trypsin from different insects also differ in the strength their subsites bind the substrate or the transition state (high-energy intermediate of the reaction). In other words, trypsin subsites differ in how they favor substrate binding or catalysis (Marana *et al.*, 2002a).

Plants have protein inhibitors (PIs) of insect midgut serine proteinases that affect insect development (Ryan, 1990). Insects may adapt to the presence of PIs in diet by overexpressing proteinases (Bown *et al.*, 1997; Broadway, 1997; Gatehouse *et al.*, 1997), by proteolytical inactivation of PIs mediated by the insect's own proteinases (Giri *et al.*, 1998), or by expressing new proteinases that are resistant to the inhibitor (Mazumbar-Leighton and Broadway, 2001a, 2001b). Current research is investigating the molecular basis of the difference between sensitive and inhibitor-insensitive trypsins, as well as the regulation of these enzymes.

PIs produced by plants have a region, named the reactive site, that interacts with the active site of their target enzymes. The reactive sites of many PIs are hydrophilic loops with a lysine residue at P_1 (Lopes *et al.*, 2004). As lepidopteran trypsins have hydrophobic subsites and prefer lysine rather than arginine at P_1 (see above), they are usually more resistant to PIs than the other insect trypsins. In this respect, it is interesting to note that PI-insensitive trypsins from *Heliothis virescens* bind more tightly to a hydrophobic chromatographic column than sensitive trypsins do (Brito *et al.*, 2001). These observations lead to the hypothesis that the molecular differences between sensitive and insensitive trypsins must rely on the interactions of PIs with residues in and around the enzyme active site.

An interesting approach to study insect–PI interactions was introduced by Volpicella *et al.* (2003). They compared the sequence of a sensitive trypsin from *Helicoverpa armigera* with the insensitive trypsin from the closely related species *H. zea*. The 57 different amino acids observed between the two enzymes were superimposed on the porcine trypsin crystal structure, where the residues known to be in contact to a Kunitz-type inhibitor (Song and Suh, 1998) were identified. The residues at positions (chymotrypsin numbering) 41, 57, 60, 95, 99, 151, 175, 213, 217, and 220 were considered by Volpicella *et al.* (2003) to be important in *H. zea* trypsin–PI interaction. However, some of the interacting residues may have been misidentified because trypsins from different species were compared. In a similar approach, Lopes *et al.* (2004) aligned all available trypsin sequences characterized as sensitive or insensitive to Kunitz-type inhibitor (Bown *et al.*, 1997; Mazumbar-Leighton and Broadway, 2001a) with porcine trypsin. After discounting conserved positions and positions not typical of sensitive or insensitive trypsins, the remaining positions that agree with those involved in porcine trypsin–PI (Bowman-Birk type, Lin *et al.*, 1993; Kunitz type, Song and Suh, 1998) or substrate (Koepke *et al.*, 2000) interactions were: 60, 94, 97, 98, 99, 188, 190, 213, 215, 217, 219, 228. These positions support the tree branches in a neighbor-joining analysis of sensitive (I, III) and insensitive (II) trypsin sequences (Lopes *et al.*, 2004) (**Figure 8a**). Site-directed mutagenesis of trypsin, followed by the determination of the binding constants of mutated trypsins with PIs, may help to resolve the discrepancy.

The mechanism by which PIs in diet induces the synthesis of insensitive trypsin in responsive insects remains completely unknown, as well as the regulatory element that may be involved.

4.5.5.2.2. Chymotrypsins Chymotrypsin (EC 3.4.21.1) preferentially cleaves protein chains at the carboxyl side of aromatic amino acids. Insect chymotrypsins usually have molecular masses of 20–30 kDa and pH optima of 8–11, and they differ from their vertebrate counterparts in their instability at acidic pH, inhibition pattern with SBTI (Terra and Ferreira, 1994) and, finally, in reacting with *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (see below). The distribution of chymotrypsin among insect taxa is similar to that of trypsin (Terra and Ferreira, 1994), including the occurrence in the salivary glands of some heteropteran bugs

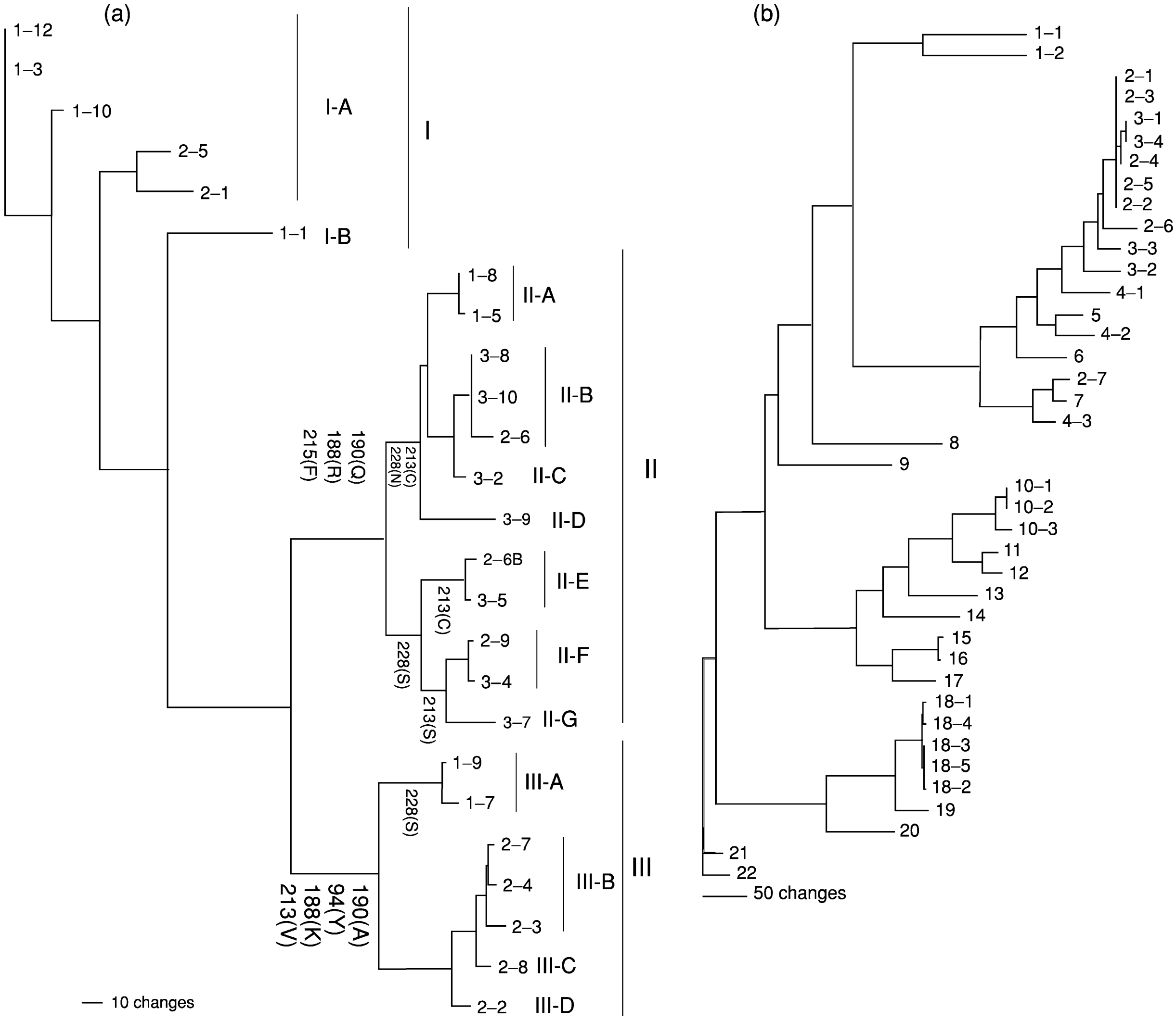


Figure 8 Neighbor-joining distance analysis tree of insect trypsin and chymotrypsin sequences. (a) Clusters formed by sequences of Lepidoptera trypsins sensitive (I, III) or insensitive (II) to protein inhibitors according to Bown *et al.* (1997) and Mazumbar-Leighton and Broadway (2001b). The amino acid positions important in defining the clusters are indicated. (b) Clusters formed by sequences of insect chymotrypsins. The sequences are identified by two digits: the first denotes the insect species (see below) and the second identify the different sequences of the same insect. Trypsin sequences: 1, *Agrotis ipsilon*; 2, *Helicoverpa zea*; 3, *H. armigera*. Chymotrypsin sequences: 1, *Plodia interpunctella*; 2, *H. armigera*; 3, *H. zea;* 4, *A. ipsilon*; 5, *Spodoptera frugiperda*; 6, *Manduca sexta*; 7, *Heliothis virescens*; 8, *Phaedon cochleariae*; 9, *Scirpophaga incertulas*; 10, *Anopheles gambiae*; 11, *A. aquasalis*; 12, *A. darlingi*; 13, *Rhyzopertha dominica*; 14, *Ctenocephalides felis*; 15, *Vespa crabro*; 16, *V. orientalis*; 17, *Solenopsis invicta*; 18, *Aedes aegypti*; 19, *Culex pipiens pallens*; 20, *Glossina morsitans morsitans*; 21, cow; 22, rat. (Courtesy of A.R. Lopes.)

(Colebatch *et al*., 2002). The earlier failure to detect chymotrypsin activity in insect midguts was a consequence of using the mammalian chymotrypsin substrates, like benzoyl-tyrosine *p*-nitroanilide (B-Y-pNA) or benzoyl-tyrosine ethyl ester (B-Y-ee), in the assays. Insect chymotrypsins have an extended active site and larger substrates, like succinyl-AAPF-*p*-nitroanilide (Suc-AAPF-pNA), are necessary for their detection (Lee and Anstee, 1995; Lopes *et al*., 2004) (**Figure** 7). Insect chymotrypsins are usually purified by affinity chromatography in phenyl butylamina-Sepharose (elution with phenyl butylamina) or in SBTI-Sepharose (elution with benzamidine) for enymes from lepidopterans, and by ion-exchange chromatography for those from dictyopterans, orthopterans, hymenopterans, and dipterans.

There are 63 complete sequences of insect chymotrypsins registered in the GenBank (as of March 2003) corresponding to 22 species pertaining to six different orders. Examples are found among Hemiptera (Colebatch *et al*., 2002), Coleoptera (Girard and Jouanin, 1999a; Zhu and Baker, 2000), Hymenoptera (Jany *et al*., 1983; Whitworth *et al*., 1998),

Siphonaptera (Gaines *et al*., 1999), Diptera (Casu *et al*., 1994; de Almeida *et al*., 2003), and Lepidoptera (Peterson *et al*., 1995; Bown *et al*., 1997). All the sequences have a signal peptide, an activation peptide (ending with an arginine residue), the catalytic triad (His 57, Asp 102, and Ser 195), three pairs of conserved cysteine residue, conserved N-terminal sequence IVGG and Ser/Gly/Tyr 189 which confers specificity to chymotrypsin-like enzymes (Figure 5).

The insect digestive chymotrypsin that has been most thoroughly studied is that of *Manduca sexta* (Lepidoptera: Sphingidae) (Peterson *et al*., 1995). In this enzyme, the activation peptide is longer and has a net charge different from that of bovine chymotrypsinogen, leading the authors to suggest that the insect enzyme is activated by a peculiar mechanism. The mammalian chymotrypsin has a pH optimum around 8 with two catalytic important pK_as of 6.8 and 9.5, corresponding to the active-site histidine, and N-terminal leucine, respectively. In contrast, the *M. sexta* chymotrypsin has pH optimum 10.5–11 and a single kinetically significant pK_a at pH 9.2. This pK_a may represent the active-site histidine in an appropriate environment, although several other hypotheses were discussed (Peterson *et al*., 1995). It is not clear whether the insect chymotrypsin active-site changes associated with TPCK resistance (see below) may also be the cause of the putative histidine pK_a displacement.

The resolution of the 3D structure of the fire ant digestive chymotrypsin led to the conclusion that it is strikingly similar to mammalian chymotrypsin, but has differences beyond those found among homologs from different mammalian systems (Botos *et al*., 2000). The similarities include a conserved backbone scaffold and structural domains. Differences include the activation mechanism and substitutions in the subsite S_1 and mainly in the other subsites (S_4–S'_4) that suggest different substrate specificities and interactions with PIs. In agreement with this, different insect chymotrypsins are sensitive to distinct PIs and like trypsins, PI-insensitive chymotrypsins may be induced in insects ingesting PI-containing diets (Bown *et al*., 1997; Mazumbar-Leighton and Broadway, 2001b).

Chymotrypsin sequences form several branches in a neighbor-joining analysis that correspond to phylogenetic groups (**Figure 8b**). Lepidoptera species are clustered in the two branches supported by Trp 59 (all sequences have a conserved tryptophan at position 59), except for the oligophagous pyralid *Scirpophaga incertulas* (9 in **Figure 8b**). One of the major lepidopteran branches corresponds to sequences from the pyralid *Plodia interpunctella* (1 in **Figure 8b**) and the other two sequences from noctuids and sphingids, all of them polyphagous. The data suggest that Trp 59 is related to a polyphagous habit.

Chymotrypsins from insects that routinely ingest ketone-releasing compounds (like several plant glycosides) (see **Figures 6 and 9**) are not affected much by these compounds and others that react with His 57. Thus, in comparison with bovine chymotrypsin, the chymotrypsin from polyphagous lepidopteran insects reacts slowly with chloromethyl ketones, whereas those of oligophagous pyralid insects react rapidly (A.R. Lopes *et al*., unpublished data). Modeling *Spodoptera frugiperda* (Noctuidae) chymotrypsin, based on its sequence and on crystallographic data of bovine chymotrypsin, showed that His 57 is probably protected from alkylation by a bulky (tryptophan) residue at position 59 (A.R. Lopes *et al*., unpublished data). Bovine chymotrypsin has at position 59 Gly, thus rendering His 57 exposed to the medium. These adaptations are new examples of the interplay between insects and plants during their evolutionary arms race and deserve more attention through site-directed mutagenesis of recombinant chymotrypsins.

4.5.5.2.3. Elastases Since Christeller *et al*. (1990) described an elastase (EC 3.4.21.36)-like enzyme in

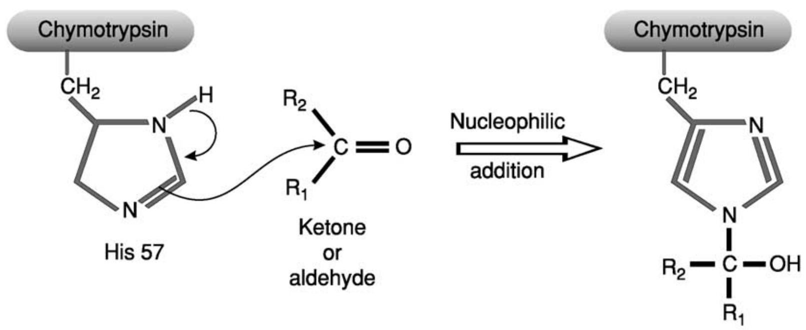


Figure 9 Ketones or aldehydes formed after the action of β-glucosidases on cyanogenic glucosides (**Figure 6**) may react with His 57 of chymotrypsin, inactivating it.

the cricket *Teleogryllus commodus*, this enzyme has been described in many other insects, including in homogeneous form (Terra and Ferreira, 1994; Whitworth *et al.*, 1998). Usually elastase is identified with the substrate Suc-AAPL-pNA (**Figure 7**), combined with the observation of lack of activity on B-Y-pNA or B-Y-ee and resistance to TPCK. Since the mentioned substrate may also be hydrolyzed by chymotrypsin and lack of activity on B-Y-pNA and/or resistance to TPCK are usual among chymotrypsins (see Section 4.5.5.2.2), most described elastase may actually be chymotrypsins. True elastases were isolated from gypsy moth midguts (Valaitis, 1995) and from whole larvae of *Solenopsis invicta* (Whitworth *et al.*, 1998). The last-mentioned enzymes hydrolyze Suc-APA-pNA, but not substrates with phenylalanine at P_1. Although the specific substrate for elastase (Suc-AAA-pNA) (Bieth *et al.*, 1974) was not tested, the hydrolysis of Suc-AAAPV-pNA and the lack of hydrolysis of substrates with phenylalanine in P_1 discount a chymotrypsin. One of the *S. invicta* elastases (E2) was cloned, sequenced, and shown to be more similar to chymotrypsin than to elastase (Whitworth *et al.*, 1999). This work confirms the occurrence of elastase in insect midgut. Further work is necessary to evaluate the extent of this enzyme in insect midguts and the importance in digestion.

4.5.5.3. Cysteine Proteinases

Cysteine proteinase is usually assayed in insect midgut contents or midgut homogenates at pH 5–6 with B-R-pNA, B-R-NA, casein, or hemoglobin as substrate. Activation by sulfhydryl agents (dithiothreitol (DTT) or cysteine) and inhibition by trans-epoxysuccinyl-L-leucyl-amido (4-guanidinobutane) (E-64) are usually indicative of the presence of the enzyme. The observation of inhibition of hydrolytic activity on any of the mentioned substrates by E-64 is insufficient for a positive identification of cysteine proteinase. Trypsin hydrolyzes the same substrates and may be reversibly inhibited by E-64 (Novillo *et al.*, 1997). The identification of cysteine proteinase was made easier with the substrate ε-amino-caproyl-leucyl-(*S*-benzyl)-cysteinyl-MCA, which is hydrolyzed by cysteine proteinase but not by serine proteinases (Alves *et al.*, 1996). Using such criteria, cysteine proteinases were described in Hemiptera Heteroptera and in species belonging to the series Cucujiformia of Coleoptera (Terra and Ferreira, 1994). Exceptions to this rule are the identification of cysteine proteinase in Hemiptera Auchenorrhyncha (aphids) (Cristofoletti *et al.*, 2003) and the lack of this enzyme in cucujiform cerambycid beetles (Johnson and Rabosky, 2000).

Insect midgut cysteine proteinases were at first denoted as cathepsin B (EC 3.4.22.1)-like enzymes, because cathepsin B was the first animal cysteine proteinase described. Later on it became known that cathepsin B is more important as a peptidyl dipeptidase, rather than as an endopeptidase, because of the existence of an extended loop that forms a cap to the active-site cleft, and carries a pair of histidine residues that are thought to bind to the C-terminal carboxylate of the substrate (Barrett *et al.*, 1998). Cathepsin L (EC 3.4.22.15) is a true endopeptidase that preferentially cleaves peptide bonds with hydrophobic amino acid residues in P_2 (cathepsin B prefers arginine at the same position) (Barrett *et al.*, 1998). Thus, by using substrates like carbobenzoxy (Z)-FR-MCA and Z-RR-MCA it is possible to distinguish between the two enzymes (see **Figure 7**). Current research revealed that cathepsin L-like enzymes are the only insect cysteine proteinase quantitatively important. Much more difficult to ascertain is that a cathepsin L-like enzyme assayed in insect midguts has been secreted into midgut contents, and hence may be considered as a truly digestive enzyme. As in other animals (Barrett *et al.*, 1998), cathepsin L-like enzymes in insects are expected to occur in lysosomes and never leave the cells. The same difficulties arise in trying to relate digestion to cathepsin L-like enzymes encoded by cDNAs cloned from midgut cells.

The problems that may arise during cathepsin L-like enzyme characterization are well illustrated in a study with *T. molitor* larvae. Three cathepsin L-like sequences were recognized in a cDNA library prepared from *T. molitor* midguts. One sequence after being expressed and used to raise antibodies was found to correspond to a lysosomal cathepsin L immunolocalized mainly at hemocytes and fat body cells. The second sequence was not related yet with any enzyme active in midgut. Finally, the third one corresponds to a cathepsin L-like enzyme purified from midgut contents (Cristofoletti *et al.*, unpublished data).

Digestive cathepsin L-like enzymes have been purified to homogeneity only from *Diabrotica virgifera* (Coleoptera: Cucujiformia) (Koiwa *et al.*, 2000), *Acyrthosiphon pisum* (Hemiptera: Auchenorrhyncha) (Cristofoletti *et al.*, 2003), and *T. molitor* (Coleoptera: Cucujiformia) (Cristofoletti *et al.*, unpublished data). The *A. pisum* enzyme is cell membrane-bound and faces the luminal contents, whereas *D. virgifera* and *T. molitor* ones are soluble enzymes secreted into midgut contents. The complete purification of those enzymes was achieved by affinity chromatography with soyacystatin as a ligand or by a combination of ion-exchange

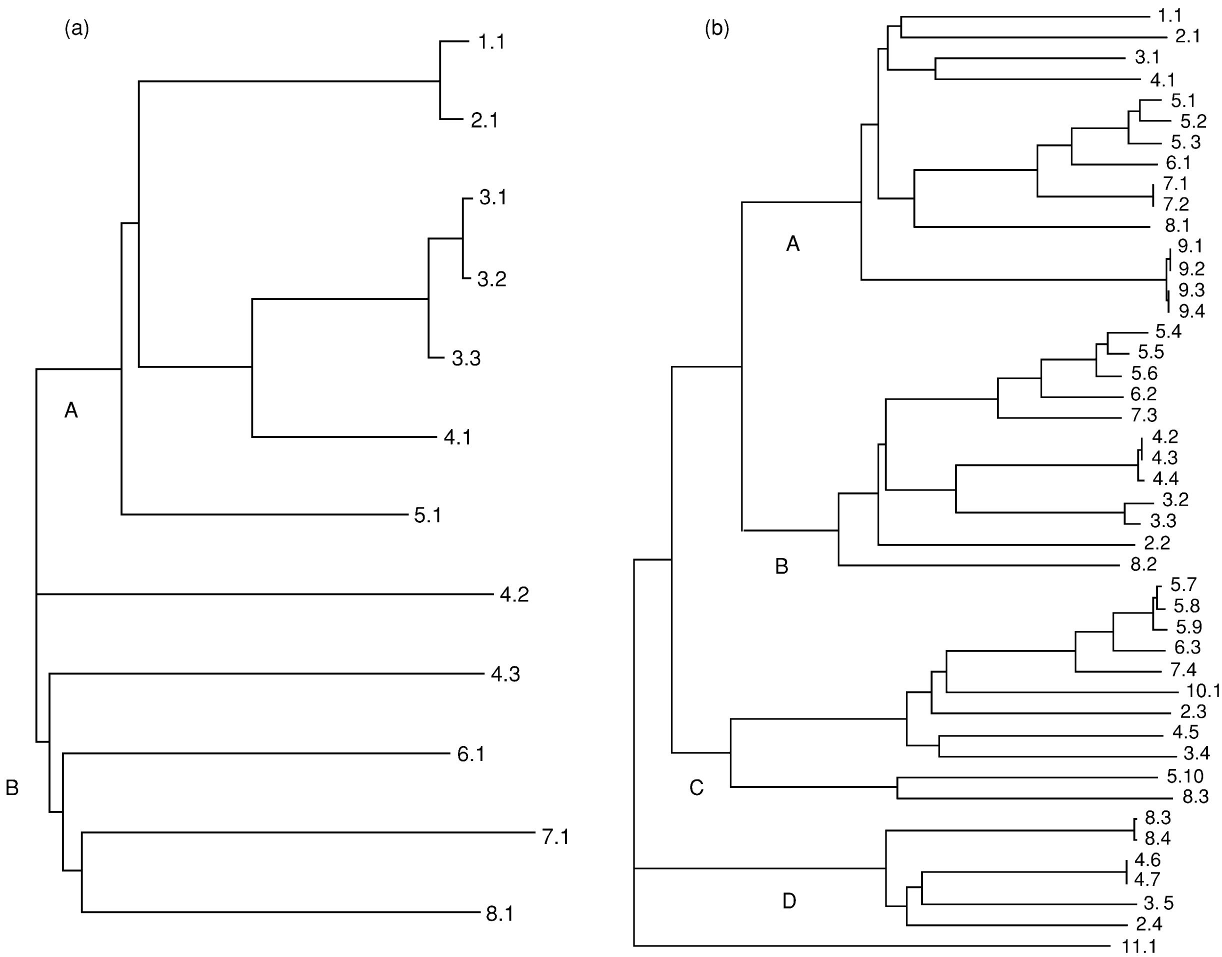


Figure 10 Neighbor-joining distance analysis tree of insect cathepsin L and aminopeptidase N sequences. (a) Clusters formed by cathepsin L-like sequences of insects known to have digestive cathepsins. (b) Clusters formed by coleopteran and lepidopteran aminopeptidases N. Sequences are identified as described in **Figure 8**. Cathepsin L-like sequences: 1, *Myzus persicae*; 2, *Aphis gossypii*; 3, *Sitophilus zeamais*; 4, *Tenebrio molitor; 5, Rhodnius prolixus*; 6, *Diabrotica virgifera*; 7, *Phaedon cochleariae*; 8, *Hypera postica*. Aminopeptidase N sequences: 1, *Epiphyas postvittana*; 2, *Lymantria dispar*; 3, *Bombyx mori*; 4, *Manduca sexta*; 5, *Helicoverpa armigera*; 6, *Heliothis punctigera*; 7, *H. virescens*; 8, *Plutella xylostella*; 9, *Plodia interpunctella*; 10, *Spodoptera littura*; 11, *Tenebrio molitor*. (Courtesy of P. T. Cristofoletti.)

chromatographies. The enzymes have pH optima of 5–6, molecular masses of 20–40 kDa, prefer Z-FR-MCA over Z-RR-MCA, are inhibited by E-64, and activated by cysteine or DTT. At least the *A. pisum* enzyme is also inhibited by chymostatin (completely) and elastatinal (partly) (Cristofoletti *et al*., 2003).

Twelve cathepsin L-like sequences corresponding to eight species of coleopterans and hemipterans (those known to have digestive cathepsins) are registered in GenBank (as of March 2003). The sequences demonstrate the features characteristic of family 1 of cysteine proteinases (Barrett *et al*., 1998): N-terminal propeptide that must be removed to activate the enzyme and the catalytic triad, Cys 25, His 169, and Asn 175 (papain numbering) and the ERFININ motif (Figure 5). The sequences form two major branches in a neighbor-joining analysis (Figure 10a) (P.T. Cristofoletti *et al*., unpublished data). The data suggest that the Hemiptera digestive cathepsin is close to the lysosomal one from *T. molitor* (sequence 1), whereas those from the coleopterans (except from *Sitophilis oryzae*) are similar to the *T. molitor* digestive enzyme (sequence 3) (Figure 10a). It is probable that the *S. oryzae* enzymes are not true digestive but lysosomal, like *T. molitor* sequence 1 (P.T. Cristofoletti *et al*., unpublished data). More work is needed to clarify the role of cathepsin L-like enzymes in insect digestion.

4.5.5.4. Aspartic Proteinases

Aspartic proteinases are active at acid pH, hydrolyze internal peptide bonds in proteins, and attack some synthetic substrates, either chromophoric (Dunn

et al., 1986) or internally quenched fluorescent substrates (Pimenta *et al.*, 2001) (**Figure 7**). Mammalian cathepsin D has a substrate-binding cleft that can accommodate up to seven amino acids and prefers to cleave between two hydrophobic residues (Barrett *et al.*, 1998).

The first report of aspartic proteinases in insects was made by Greenberg and Paretsky (1955), who found a strong proteolytic activity at pH 2.5–3.0 in homogenates of whole bodies of *Musca domestica*. Lemos and Terra (1991b) showed that the enzyme occurs in midguts and is cathepsin D-like. An aspartic proteinase similar to cathepsin D was found in families of Hemiptera, Heteroptera and in several families belonging to the cucujiform series of Coleoptera (Terra and Ferreira, 1994). Thus, it is possible that aspartic proteinases occur together with cysteine proteinase in Hemiptera and in most Coleoptera.

The aspartic proteinase isolated from *Callosobruchus maculatus* (pH optimum 3.3, 62 kDa) (Silva and Xavier-Filho, 1991) and *Tribolium castaneum* (pH optimum 3.0, 22 kDa) (Blanco-Labra *et al.*, 1996) were partially purified and shown to be similar to cathepsin D. These studies need to be extended, so that the origin, specificity, and structure of insect cathepsin D-like enzymes be clarified.

4.5.5.5. Aminopeptidases

Aminopeptidases sequentially remove amino acids from the N-terminus of peptides and are classified on the basis of their dependence on metal ions (usually Zn^{2+} or Mn^{2+}) and substrate specificity. Aminopeptidase N (EC 3.4.11.2) has a broad specificity, although it removes preferentially alanine and leucine residues from peptides, whereas aminopeptidase A (EC 3.4.11.7) prefers aspartyl (or glutamyl)-peptides as substrates. Both are metalloenzymes (Norén *et al.*, 1986).

In insect midguts, major amounts of soluble aminopeptidases are found in less evolved insects (e.g., Orthoptera, Hemiptera, Coleoptera Adephaga), whereas in more evolved insects (e.g., Coleoptera Polyphaga, Diptera, and Lepidoptera) aminopeptidase is found mainly bound to the microvillar membranes of midgut cells (Terra and Ferreira, 1994). Insect midgut aminopeptidases are metalloenzymes (ethylenediaminetetraacetic acid (EDTA) inhibition) and have pH optima of 7.2–9.0, irrespective of the pH of the midgut lumen from the different species, Km values (L-pNA) of 0.13–0.78 mM and molecular masses of 90–130 kDa. With a single exception (see below), all known insect aminopeptidases have a broad specificity, hydrolyzing a variety of amino acyl β-naphthylamides (except acidic amino acyl β-naphthylamides), indicating they are aminopeptidases N (Terra and Ferreira, 1994) (**Figure 7**). The exception is a soluble glycocalyx-associated midgut aminopeptidase from *R. americana*. This enzyme is an aminopeptidase removing N-terminal aspartic acid or glutamic acid residues from peptides that are not efficiently attacked by the other aminopeptidases (Klinkowstrom *et al.*, 1994).

In addition to a midgut aminopeptidase A, the dipteran *R. americana* has three midgut aminopeptidases N (one soluble and two membrane-bound). The soluble aminopeptidase N (115.7 kDa) prefers tetrapeptides over tripeptides (Ferreira and Terra, 1984), like the minor 107 kDa membrane-bound enzyme, whereas the contrary is true for the major 169 kDa membrane-bound aminopeptidase (Ferreira and Terra, 1985, 1986a, 1986b). The single midgut aminopeptidase N of the coleopterans *Attagenus megatoma* (Baker and Woo, 1981) and *Tenebrio molitor* (Cristofoletti and Terra, 1999) resemble the 115.7 kDa and 107 kDa aminopeptidase of *R. americana*. Approximately the same substrate specificity was observed with the two midgut aminopeptidases of the lepidopteran *Tineola bisselliella* (Ward, 1975a, 1975b). The data suggest that panorpoid insects (Diptera and Lepidoptera) present multiple aminopeptidases with different substrate specificities, in contrast with the single aminopeptidase of coleopterans. However, much more data are needed to support this hypothesis.

There have been few attempts to characterize the active site of insect midgut aminopeptidases. Using multiple inhibition analysis and observing the protection against EDTA inactivation that different competitive inhibitors conferred to the enzyme, two subsites were proposed to occur in the active center of *R. americana* microvillar aminopeptidase: a hydrophobic subsite, to which isoamyl alcohol binds exposing the metal ion, and a polar subsite, to which hydroxylamine binds. Exposure of the metal ion after isoamyl alcohol binding may be analogous to the situation that results when part of the substrate occupies the hydrophobic subsite, causing conformational changes associated with the catalytic step (Ferreira and Terra, 1986b). The effect of pH at different temperatures on kinetic parameters of *T. molitor* midgut aminopeptidase and its inactivation by different compounds were studied (Cristofoletti and Terra, 2000). The data showed that *T. molitor* aminopeptidase catalysis depends on a metal ion, a carboxylate, and a protonated imidazole group and is, somehow, influenced by an arginine residue in the neighborhood of the active site. The catalytic metal binding depends on at least a deprotonated imidazole. In addition to the

above-mentioned groups involved in catalysis, at least one phenol group and one carboxylate are associated with substrate binding. Thus, *T. molitor* aminopeptidase shares common features with those of other zinc metallopeptidases, especially with mammalian aminopeptidase N, but it differs in some details. An imidazole group seems to be involved in catalysis in *T. molitor* aminopeptidase; this is not observed in mammalian aminopeptidase N, which has an imidazole group participating in substrate binding.

Aminopeptidase N sequences are available for the following lepidopterans: *Epiphyas postvittana, B. mori, Heliothis virescens, H. punctigera, Helicoverpa armigera, L. dispar, Manduca sexta, P. interpuctella*, and *Plutella xylostella* (Gill *et al*., 1995; Knight *et al*., 1995; Denolf *et al*., 1997; Hua *et al*., 1998; Chang *et al*., 1999; Oltean *et al*., 1999; Yaoi *et al*., 1999; Garner *et al*., 1999; Emmerling *et al*., 2001; Rajagopal *et al*., 2003). The sequences have a signal peptide, a conserved RLP motif near the N-terminal, a zinc binding/gluzincin motif $HEXXHX_{18}E$, a GAMEN conserved motif and a long hydrophobic C-terminal containing a glycosyl phosphatidyl inositol anchor (**Figure 5**). Based on the crystal structure of leukotriene A_4 hydrolase, the two histidine residues and the distant glutamic acid residue of the gluzincin motif are zinc ligands, the glutamic acid residue between the histidine residues is involved in catalysis (Hooper, 1994; Rawlings and Barrett, 1995), and the glutamic acid residue of the GAMEN motif binds the substrate N-terminal amino acid (Luciani *et al*., 1998). In contrast to the situation in mammals, insect aminopeptidase N is membrane bound at the C-terminal. No soluble insect aminopeptidase N has been sequenced.

Dendrograms derived from alignments of coleopteran and lepidopteran midgut aminopeptidases suggest that there are at least four groups of lepidopteran aminopeptidases, with the isoforms of the same animal distributed among the groups (Chang *et al*., 1999; Emmerling *et al*., 2001; Nakanishi *et al*., 2002; Rajagopal *et al*., 2003; P. T. Cristofoletti *et al*., unpublished data) (**Figure 10b**, clusters A, B, C, and D). The existence of a number of different aminopeptidases in lepidopterans could be explained by the need for enzymes with different substrate specificities (as shown above for *R. americana*) or different susceptibilities to inhibitors, similar to serine proteinases (see Section 4.5.5.2).

Probably associated with the fact that aminopeptidases are major proteins in some microvillar membranes (55% of *T. molitor* midgut microvillar proteins) (Cristofoletti and Terra, 1999), they are targets of insecticidal *Bacillus thuringiensis* crystal δ-endotoxins. These toxins, after binding to aminopeptidases and receptor molecules called cadherins, form channels through which cell contents leak leading to death of the insect (Gill *et al*., 1995; Knight *et al*., 1995; Denolf *et al*., 1997). Although data on substrate specificity for lepidopteran aminopeptidase isoforms are lacking, there is evidence that the isoforms may have differences in toxin binding (Valaitis *et al*., 1997; Zhu *et al*., 2000; Nakanishi *et al*., 2002; Rajagopal *et al*., 2003).

Cloning and sequencing dipteran aminopeptidases, for which differences in substrate specificity are known, and a study of substrate specificities of lepidopteran aminopeptidases, may clarify the selective advantages of the evolution of aminopeptidase groups. Furthermore, this study may support the hypothesis that aminopeptidase gene duplications have occurred in the panorpoid ancestor, before differentiation between dipterans and lepidopterans.

4.5.5.6. Carboxypeptidases and Dipeptidases

Carboxypeptidases hydrolyze single amino acids from the C-terminus of the peptide chain and are divided into classes on the basis of their catalytic mechanism. There are two digestive metallocarboxypeptidases in mammals: carboxypeptidase A (EC 3.4.17.1), which hydrolyzes, in alkaline medium, C-terminal amino acids, except arginine, lysine, and proline, and carboxypeptidase B (EC 3.4.1.7.2), which releases, in alkaline conditions, C-terminal lysine and arginine preferentially. Insect digestive carboxypeptidases have been classified as carboxypeptidase A or B depending on activity in alkaline medium against Z-GF (or hippuryl β-phenyllactic acid) or Z-GR (or hippuryl-L-arginine), respectively (**Figure 7**). Digestive insect carboxypeptidase A-like enzymes are widespread among insects and most of them have pH otima of 7.5–9.0 and molecular masses of 20–50 kDa (Terra and Ferreira, 1994). They have been cloned and sequenced from Diptera (Ramos *et al*., 1993; Edwards *et al*., 1997) and Lepidoptera (Bown *et al*., 1998) and the enzyme from the lepidopteran *H. armigera* was also submitted to crystallographic studies (Estébanez-Perpiñá *et al*., 2001). The sequences have signal and activation peptides and the features typical of carboxypeptidases A, including the residues His 69, Glu 72, and His 196, which bind the catalytic zinc ion, and Arg 71, Asn 144, Arg 145, and Tyr 248 responsible for substrate binding and Arg 127 and Glu 270 for catalysis. In spite of the overall similarity of *H. armigera* procarboxypeptidase with human procarboxypeptidase A2, there are differences in the loops between the conserved secondary structures, including the loop where the activation processing occurs.

Another important difference is the residue 255 (bottom of the S′$_1$ pocket) that defines the enzyme specificity. In mammalian sequences Asp 255 is found in carboxypeptidase B and Ile 255 in carboxypeptidase A. In insect carboxypeptidases A, this residue varies (but never is an acid residue) (**Figure 5**).

Carboxypeptidases B-like enzymes have been detected in insect midguts (Terra and Ferreira, 1994) but none has been characterized in detail, because they are much less active than carboxypeptidases A.

Dipeptidases hydrolyze dipeptides and are classified according to their substrate specificities. Dipeptidases comprise the poorest known of the insect peptide hydrolases. There have been few studies in which dipeptidase assays were performed and even fewer attempts to characterize the enzymes (Terra and Ferreira, 1994). The larval midgut of *R. americana* has three dipeptidases (two soluble with 63 kDa and 73 kDa, respectively, and one membrane-bound) that hydrolyze Gly-Leu, resembling dipeptide hydrolase (dipeptidase, E.C. 3.4.13.18), although in contrast to the mammalian enzyme they are very active upon Pro-Gly (**Figure 7**). *Rhynchosciara americana* also seems to have an amino acyl-histidine dipeptidase (carnosinase, EC 3.4.13.3) (Klinkowstrom *et al.*, 1995). More work on insect digestive dipeptidases is urgently needed.

4.5.6. Digestion of Lipids and Phosphates

4.5.6.1. Overview

Lipids that contain fatty acids comprise storage lipids and membrane lipids. Storage lipids, such as oils present in seeds and fats in adipose tissue of animals, are triacylglycerols (triglycerides) and are hydrolyzed by lipases. Membrane lipids include phospholipids and glycolipids like mono- and digalactosyldiglycerides (see Section 4.5.4.7). Phospholipids are digested by phospholipases. A combination of α- and β-galactosidases may remove galactose residues from mono- and digalactosyldiglyceride to leave a diacylglycerol which may be hydrolyzed by a triacylglycerol lipase.

Phosphate moieties need to be removed from phosphorylated compounds prior to absorption. This is accomplished by nonspecific phosphatases. The phosphatases may be active in an alkaline (alkaline phosphatase, EC 3.1.3.1) or acid (acid phosphates, EC 3.1.3.2) medium.

4.5.6.2. Lipases

Triacylglycerol lipases (EC 3.1.1.3) are enzymes that preferentially hydrolyze the outer links of triacylglycerols and act only on the water–lipid interface. Activity of the lipase is increased as the interface becomes larger due to lipid emulsification caused by emulsifiers (surfactants). Insects lack emulsifiers comparable to the bile salts of vertebrates, but surfactant phospholipids, including lysolecithin, occur in their midguts in sufficient concentration to alter the surface tension of midgut contents (De Veau and Schultz, 1992). Lysolecithin, and other surfactants, may be formed by the action of phospholipase A on ingested phospholipids (see below and **Figure 1**).

Current research on insect lipases is focused on triacylglycerol lipase from the fat body and its interplay with flight muscles (Ryan and Van der Horst, 2000). Insect midgut triacylglycerol lipases have been studied in few insects and only in crude preparations. The data suggested that the enzyme preferentially releases fatty acids from the α-positions, prefers unsaturated fatty acids and is activated by calcium ions, thus resembling the action of mammalian pancreatic lipase. The resulting 2-monoacylglycerol may be absorbed or hydrolyzed (Terra *et al.*, 1996). Hydrolysis of 2-monoacylglycerol may be accomplished by the triacylglycerol lipase, following migration of the fatty acid to the 1-position, which seems to be favored by the alkaline midgut pH, at least in *M. sexta* (Tsuchida and Wells, 1988).

Esterases, which are usually named the carboxylesterases (ali-esterases, EC 3.1.1.1) catalyze the hydrolysis of carboxyl ester into alcohol and carboxylate. This enzyme, in contrast to lipases, attacks molecules that are completely dissolved in water. It also hydrolyzes water-insoluble long-chain fatty acid esters in the presence of surfactants, but at a rate much slower than that of triacylglycerol lipase. A role for esterases in digestion is unclear and because of this they are not reviewed in detail here.

In spite of the fact that a requirement for essential fatty acids is probably universal in insects, progress has been limited in the study of lipid digestion. Presumably, the lack of comparatively simple, sensitive assays and the complexities of digestion related to lipid solubilization have hindered work in this area. Another reason to study enzymes associated with lipid digestion is that they might be important in limiting the development of pathogens and parasites. Hydrolysis of membrane lipids might cause cellular lysis and fatty acid products of digestion may possess antibiotic effects.

4.5.6.3. Phospholipases

Phospholipase A_2 (EC 3.1.1.4) and phospholipase A_1 (EC 3.1.1.32) remove from phosphatides the fatty acid attached to the 2-position and 1-position,

respectively, resulting in a lysophosphatide (**Figure 2**). Lysophosphatide is more stable in micellar aggregates than on membranes. Thus, the action of phospholipase A on the membrane phosphatides causes the solubilization of cell membranes, rendering the cell contents free to be acted upon by the appropriate digestive enzymes. Phospholipase is widespread among insects (Terra *et al.*, 1996; Nor Aliza *et al.*, 1999). Phospholipase A_2 partially purified from the midgut of adult beetle *Cincindella circumpicta* has a molecular mass of 22 kDa and pH optimum 9.0, is calcium dependent, and is inhibited by the site-specific inhibitor oleyoxyethyl phosphorylcholine. Unfed beetles did not express the phospholipase in the midgut contents (Uscian *et al.*, 1995). Although lysophosphatide may be further hydrolyzed by a lysophospholipase (phospholipase B, EC 3.1.1.5), evidence suggests it is absorbed intact by insects (Turunen and Kastari, 1979; Weiher and Komnick, 1997). Phosphatides may also be hydrolyzed by phospholipase C (EC 3.1.4.3) yielding the phosphoryl base moiety and diacylglycerol, or by phospholipase D (EC 3.1.4.4), resulting phosphatidate and the base (**Figure 2**). Both enzymes have been found in insect midgut (Turunen, 1993), but have not studied in detail.

4.5.6.4. Phosphatases

Alkaline phosphatase is usually a midgut microvillar membrane marker in dipteran and lepidopteran species, although it may also occur in midgut basolateral membranes and even as a secretory enzyme. Acid phosphatase is usually soluble in the cytosol of midgut cells in many insects and may also appear in midgut contents or be found membrane-bound in midgut cells (Terra and Ferreira, 1994).

The best-known alkaline phosphatases are those from *B. mori* (Lepidoptera: Bombycidae) larval midgut. The major membrane-bound and the minor soluble alkaline phosphatases were purified and shown to be monomeric enzymes with the following properties: (1) soluble enzyme, molecular mass of 61 kDa, pH optimum 9.8; (2) membrane-bound enzyme, molecular mass of 58 kDa, pH optimum 10.9. Both enzymes have wide substrate specificity and are inhibited by cysteine. The membrane-bound alkaline phosphatase occurs in the microvillar membranes of columnar cells, whereas the soluble enzyme is loosely attached to the goblet cell apical membrane facing the cell cavity (Eguchi, 1995). The determination of the complete sequence of the membrane-bound alkaline phosphatase led to the finding of putative regions for phosphatidylinositol anchoring, zinc-binding site but not for *N*-glycosylation, despite the fact that the enzyme contains *N*-linked oligosaccharides (Itoh *et al.*, 1991). The sequence of the soluble alkaline phosphatase was also determined and has high identity with the membrane-bound enzyme (Itoh *et al.*, 1999).

Acid phosphatases have been characterized in some detail only in *Rhodnius prolixus* (Hemiptera: Reduvidae). The major enzyme activity is soluble and has the following properties: wide specificity, a molecular mass of 82 kDa, Km for *p*-nitrophenyl phosphate 0.7 mM, and is inhibited by fluoride, tartrate, and molybdate. The minor enzyme activity is membrane-bound and is resolved into two enzymes (123 and 164 kDa) which are resistant to fluoride and tartrate (Terra *et al.*, 1988).

4.5.7. The Peritrophic Membrane

4.5.7.1. The Origin, Structure, and Formation of the Peritrophic Membrane

There is a film surrounding the food bolus in most insects that occasionally is fluid (peritrophic gel) but more frequently is membranous (peritrophic membrane, PM). The PM is made up of a matrix of proteins (peritrophins) and chitin to which other components (e.g., enzymes, food molecules) may associate (see **Chapter 4.3**). This anatomical structure is sometimes called peritrophic matrix, but this term should be avoided because it does not convey the idea of a film and suggests it is the fundamental substance of some structure, usually filling a space as the mitochondrial matrix. The argument that membrane means a lipid bilayer is not valid because the PM is not a cell part, but an anatomical structure, like the nictitating membrane of birds and reptiles.

Peritrophins, the integral PM proteins, are made of several domains. The major domain (peritrophin A-domain), is a cysteine-rich domain with chitin-binding properties having the consensus sequence: $CX_{13-20}CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}C$ (where X is any amino acid except cysteine) that includes several conserved aromatic amino acids (see Section 4.5.4.5). Variations of this chitin-binding domain are peritrophin-B and peritrophin-C domains with consensus sequences:

$CX_{12-13}CX_{20-21}CX_{10}CX_{12}CX_{2}CX_{8}CX_{7-12}C$ and $CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}CX_{11}C$, respectively. Another kind of domain occurring in peritrophins are proline/threonine-rich domains that are heavily glycosylated and similar to mucins. Peritrophins may have one (e.g., Cb-peritrophin-15 from *Lucilia cuprina*) to several (e.g., peritrophin-44 from

L. cuprina) chitin-binding domains or chitin-binding domains with small (e.g., Ag-AperI from *Anopheles gambiae*) or very large mucin-like domains (e.g., IIM from *Trichoplusia ni*) (Wang and Granados, 1997; Shen and Jacobs-Lorena, 1998; Tellam *et al.*, 1999, 2003).

The 3D structure of PM is thought to result from chitin fibrils being interlocked with the chitin-binding domains of peritrophins. Mucin-like domains of peritrophins are thought to face the ectoperitrophic and endoperitrophic sides of the PM. As these domains are highly hydrated they lubricate the surface of the PM, easing the movement of food inside the PM and of the ectoperitrophic fluid outside the PM. Furthermore, the glucan chains associated with peritrophin mucin-like domains may assure high proteinase resistance to PM (see **Figure 9** in Schorderet *et al.*, 1998; **Figure 5** in Wang and Granados, 2001).

The structure of peritrophins prompted Terra (2001) to develop a speculative model of the origin and evolution of the PM. According to this model, ancestral insects had their midgut cells covered with a mucus similar to that found in most animals. This gastrointestinal mucus, at least in vertebrates, is a gel-like substance composed of mucins (Allen, 1983; Forstner and Forstner, 1986). It was proposed that evolutionary processes led to the development of the PM from the gastrointestinal mucus. According to this hypothesis, the peritrophins, the major PM proteins, evolved from mucins by acquiring chitin–binding domains. The concomitant evolution of chitin secretion by midgut cells permitted the formation of the chitin–protein network characteristic of PM structure described above. Later on in evolution, some peritrophins lost their mucin-like domains. If the hypothesis that the PM is derived from the gastrointestinal mucus is correct, it should have originally been synthesized by midgut cells along the whole midgut and should have had the properties of the mucus. Later in evolution, insect species would have appeared with a chitin–protein network resulting in PM formation. Therefore, the formation of the PM by the whole midgut epithelium is the ancestral condition, whereas the restriction of PM production to midgut sections, or the lack of a PM and its replacement by the peritrophic gel, are derived conditions.

PMs are classified into two types (Peters, 1992). Type I PM is found in cockroaches (Dictyoptera), grasshoppers (Orthoptera), beetles (Coleoptera), bees, wasps, and ants (Hymenoptera), moths and butterflies (Lepidoptera), and in hematophagous adult mosquitoes (Diptera). Type II PM occurs in larval and adult (except hematophagous ones) mosquitoes and flies (Diptera), and in a few adult Lepidoptera.

Type I PM is formed either by the whole midgut epithelium, or by part of it (anterior or posterior regions). PM produced by the whole or anterior midgut epithelium envelops the food along the whole midgut. When PM is produced only by the posterior part, the anterior midgut epithelium is usually covered with a viscous material, the peritrophic gel, as observed in carabid beetles (Ferreira and Terra, 1989) and bees (Jimenez and Gilliam, 1990). This gel is also observed in the anteriorly placed midgut caeca of some insects and along the whole midgut of others (Terra, 2001).

It has been shown with light microscopy, as well as transmission and scanning electron microscopy, that during the formation of type I PM chitin-containing fibrous material appears first at the tips of the microvilli of anterior midgut cells and then is rapidly included into a thin PM surrounding the food bolus (Harper and Hopkins, 1997). Chitin is synthesized outside the cells by a chitin synthase bound to microvillar membranes using precursors formed inside the cells (see **Chapter 4.3**). In agreement with this, antibodies raised against a translated product of a cDNA fragment coding chitin synthase immunolabeled only the apical ends of midgut microvilli (Zimoch and Merzendorfer, 2002). Chitin, after being self-organized into fibers, is interlocked by peritrophins. These are released by microapocrine secretion (Bolognesi *et al.*, 2001). The formation of these PMs is frequently induced by the distension of the gut caused by food ingestion.

Type II PM is secreted by a few rows of cells at the entrance of the midgut (cardia) and usually is found in insects irrespective of food ingestion. Peritrophins are secreted by exocytosis (Eisemann *et al.*, 2001).

Although a PM is found in most insects, it does not occur in Hemiptera and Thysanoptera, which have perimicrovillar membranes in their cells (see below). The other insects that apparently do not have a PM are adult Lepidoptera, Phthiraptera, Psocoptera, Zoraptera, Strepsiptera, Raphidioptera, Megaloptera, adult Siphonaptera, bruchid beetles, and some adult ants (Hymenoptera) (Peters, 1992). These insects may have a peritrophic gel instead of PM, one example being bruchid beetles (Terra, 2001), or may have had their PMs overlooked, because the insects were unfed.

Another possibility is that minute hematophagous insects (like Siphonaptera and Phthiraptera) have lost their PM because the blood clot assures countercurrent flows (see Section 4.5.7.2.2.3) and their

small size makes easy the efficient diffusion of digestion products up to the midgut surface.

The PM may have a large range of pore sizes: some small or very large and most of them in the middle range. The average pore sizes of PM may be determined by comparing molecular masses of enzymes restricted to the ectoperitrophic fluid (**Figure 1**) with those of enzymes present inside PM. This method of pore size estimation is probably the most accurate one since it reflects *in vivo* conditions. Pore sizes have also been determined by feeding insects with colloidal gold particles or fluorescent dextran molecules of known molecular masses and recording passage through the PM *in vivo* or using PM mounted as a sac and measuring diffusing rates. Determinations performed with these techniques by different authors (Zhuzhikov, 1964; Terra and Ferreira, 1983; Espinoza-Fuentes *et al.*, 1984; Peters and Wiese, 1986; Santos and Terra, 1986; Wolfersberger *et al.*, 1986; Miller and Lehane, 1990; Ferreira *et al.*, 1994a) found pores in the range 7–9 nm for insects pertaining to different orders. Other authors described pores in the range 17–36 nm (Barbehenn and Martin, 1995; Edwards and Jacobs-Lorena, 2000). Pore sizes in the range 17–36 nm were obtained with fluorescent dextran molecules in conditions able to detect very small amounts of substances traversing the PM and, for this, they probably correspond to the large pores occurring at low frequency in PMs. Although these large pores are supposed to be of no importance regarding digestive events, they set the size limits that an infecting particle must have to successfully pass through the PM.

As a consequences of its small pores, the PM hinders the free movement of molecules, dividing the midgut lumen into two compartments (**Figure 1**) with different molecules. The functions of this structure include those of the mucus (protection against food abrasion and invasion by microorganisms) and several roles associated with the compartmentalization of the midgut. These roles result in improvements in digestive physiology efficiency thereby leading to decreased digestive enzyme excretion, and restrict the production of the final products of digestion close to their transporters, thus facilitating absorption. These roles will be detailed below (see Section 4.5.7.2).

In the light of current research, the distinction between PM and peritrophic gel may be more important than the traditional distinction between type I and type II PMs, in spite of the fact that the two types may have somewhat different structures (Tellam *et al.*, 1999). Other major points needing clarification are how the chemical nature of peritrophic gel and PM define their strength, elasticity and porosity and how these structures are self-assembled in the midgut lumen.

4.5.7.2. The Physiological Role of the Peritrophic Membrane

4.5.7.2.1. Protection against food abrasion and invasion by microorganisms As mentioned before, gut cells in most animals are covered with a gel-like coating of mucus, which has been most thoroughly studied in mammals (Forstner and Forstner, 1986). In these animals, the mucus is supposed to lubricate the mucosa, protecting it from mechanical damage, and to trap bacteria and parasites. Since the insect midgut epithelium lacks a mucus coating, PM functions were supposed to be analogous to that of mucus. Thus, insects deprived of PM may have the midgut cells damaged by coarse food and may be liable to microorganism invasion in some reported cases (Peters, 1992; Tellam, 1996; Lehane, 1997).

The PM as a barrier against invasion by microorganisms has particular relevance in insects that transmit viruses and parasites to human beings, as these microorganisms may have specific developmental phases in insect tissues (Tellam, 1996; Lehane, 1997). Microorganisms invade the insect midgut cells after disrupting the PM with the use of chitinase (Shahabuddin, 1995) or by using a proteinase such as enhancin that affects specifically the peritrophins (Peng *et al.*, 1999; Ivanova *et al.*, 2003).

A barrier against microorganism invasion is probably less important for the majority of insects that feed on plants, as exemplified by observations carried out with the moth *T. ni*. Larvae of this insect deprived of PM by Calcofluor treatment show high mortality. Examination of dead larvae showed no signs of microbial infection or cell damage by Calcofluor, although these larvae were more susceptible to experimental infection (Wang and Granados, 2000). The results may be interpreted as Calcofluor killing larvae by affecting PM functions in digestion.

In the same direction goes the observation that some plants respond to herbivorous insect attack by producing a unique 33 kDa cysteine proteinase with chitin-binding activity. This proteinase damages the PM, resulting in significant reduction in caterpillar growth caused by impaired nutrient utilization (Pechan *et al.*, 2002).

4.5.7.2.2. Enhancing digestive efficiency

4.5.7.2.2.1. Overview The proposal of roles for the PM in digestion has benefited from studies on the organization of the digestive process. These studies (reviews: Terra, 1990; Terra and Ferreira,

1994, 2003) revealed that in most insects initial digestion occurs in the endoperitrophic space (Figure 1), intermediate digestion in the ectoperitrophic space, and final digestion at the surface of midgut cells. Such studies led to the formulation of the hypothesis of the endo–ectoperitrophic circulation of digestive enzymes. It was suggested that there is a recycling mechanism (Figure 11), where food flows inside the PM from the anterior midgut to the posterior, whereas in the ectoperitrophic space water flows from the posterior midgut to the caeca. When the polymeric food molecules become sufficiently

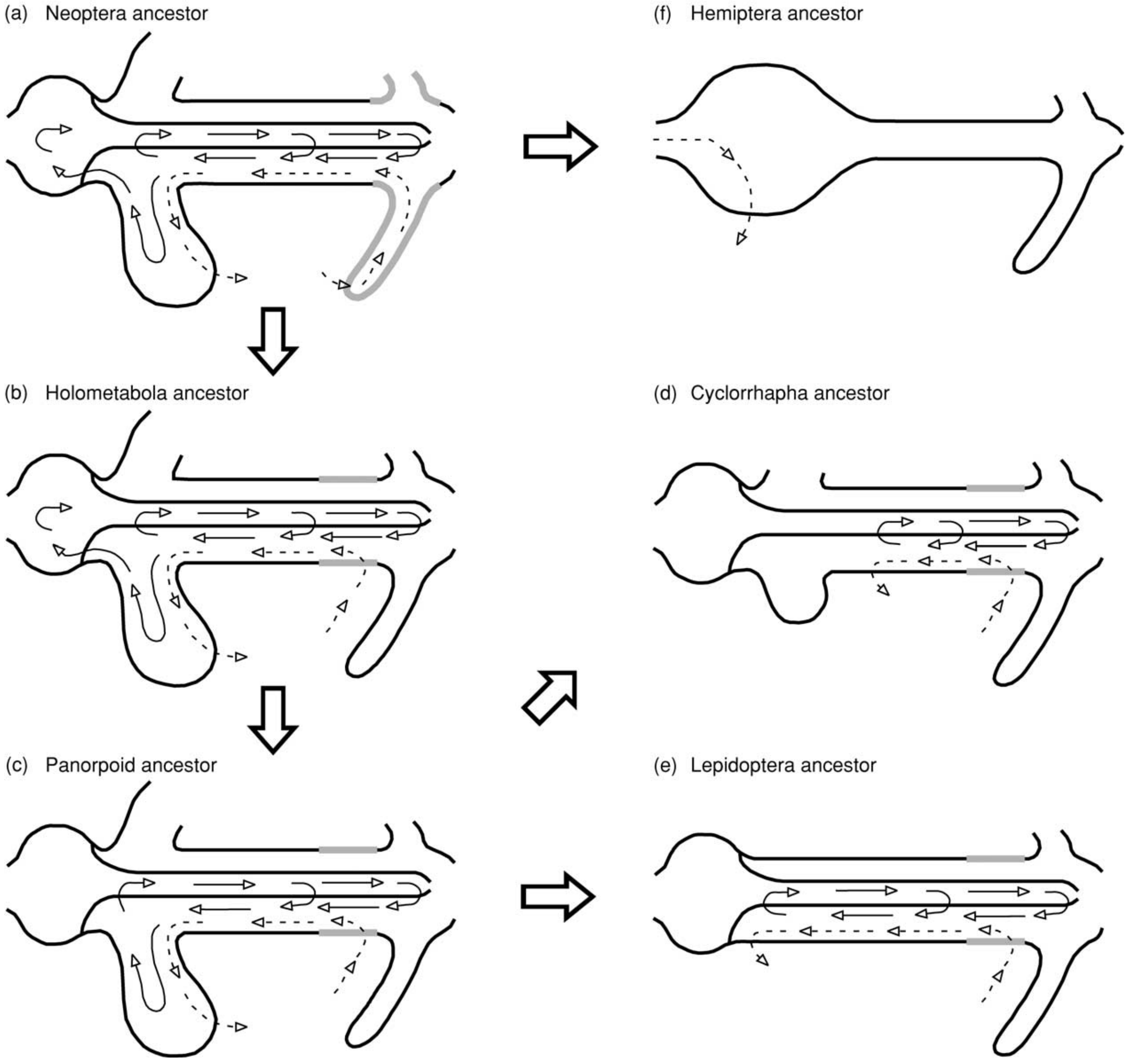


Figure 11 Diagrammatic representation of water fluxes (dotted arrows) and of the circulation of digestive enzymes (solid arrows) in putative insect ancestors that correspond to the major basic gut plans. In Neoptera ancestors (a), midgut digestive enzymes pass into the crop. Countercurrent fluxes depend on the secretion of fluid by the Malpighian tubules and its absorption by the caeca. Enzymes involved in initial, intermediate, and final digestion circulate freely among gut compartments. Holometabola ancestors (b) are similar except that secretion of fluid occurs in posterior ventriculus. The ancestors of hymenopteran and panorpoid (Lepidoptera and Diptera assemblage) insects (c) display countercurrent fluxes like Holometabola ancestors, midgut enzymes are not found in the crop, and only the enzymes involved in initial digestion pass through the peritrophic membrane. Enzymes involved in intermediate digestion are restricted to the ectoperitrophic space and those responsible for terminal digestion are immobilized at the surface of midgut cells. Cyclorrhapha ancestors (d) have a reduction in caeca, absorption of fluid in middle midgut, and anterior midgut playing a storage role. Lepidoptera ancestors (e) are similar to panorpoid ancestors, except that the anterior midgut replaces the caeca in fluid absorption. Hemiptera ancestors (f) have lost crop, caeca, and fluid-secreting regions. Fluid is absorbed in anterior midgut. (Reprinted with permission from Terra, W.R., Ferreira, C., **2003**. Digestive system. In: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects. Academic Press, San Diego, CA, pp. 313–323; © Elsevier.)

small to pass through the PM (with the accompanying polymer hydrolases) the flow patterns result in the carriage towards the caeca or the anterior midgut where intermediate and final digestion occurs.

Terra *et al.* (reviews: Terra and Ferreira, 1994; Terra, 2001) hypothesized that as a consequence of the compartmentalization of digestive events, there is an increase in the efficiency of digestion of polymeric food by allowing the removal of the oligomeric molecules from the endoperitrophic space, which is powered by the recycling mechanism associated with the midgut fluxes. Because oligomers may be substrates or inhibitors for some polymer hydrolases, their presence should decrease the rate of polymer degradation. Fast polymer degradation ensures that polymers are not excreted and hence increases their digestibility. Another possible consequence of compartmentalization is an increase in the efficiency of oligomeric food hydrolysis due to the transference of oligomeric molecules to the ectoperitrophic space and restriction of oligomer hydrolases to this compartment. In these conditions, oligomer hydrolysis occurs in the absence of probable partial inhibition (because of nonproductive binding) by polymer food and presumed nonspecific binding by nondispersed undigested food. This process should lead to the production of food monomers in the vicinity of midgut cell surface, causing an increase in the concentration of the final products of digestion close to their transporters, thus facilitating absorption. Experimental evidence supporting the adaptations for increasing digestive efficiency proposals are discussed in the following sections.

4.5.7.2.2.2. Prevention of nonspecific binding A model system was used to test the hypothesis that the PM prevents nonspecific binding of undigested material onto midgut cell surface (R. Bolognesi, W.R. Terra, and C. Ferreira, unpublished data). Aminopeptidase-containing microvillar membranes from *Spodoptera frugiperda* midgut cells were purified and assayed in the absence and presence of a concentration of midgut contents resembling *in vivo* conditions. The activity of the aminopeptidase in the presence of the midgut contents was about 50% of the activity in their absence, favoring the idea that undigested material in contact with microvillar enzymes negatively affects their activity.

4.5.7.2.2.3. Prevention of enzyme excretion This function was at first proposed based on results obtained with dipteran larvae (reviews: Terra, 1990; Terra and Ferreira, 1994). Both *R. americana* and *Musca domestica* present a decreasing trypsin gradient along midgut contents (putatively generated by the recycling mechanism) and excreted less than 15% of midgut luminal trypsin after each gut emptying. When the larvae were fed a diet with excess protein, the trypsin gradient along midgut contents becomes less discernible and trypsin excretion increases to 40%. This is exactly what would be expected if the recycling mechanism existed and an increase in undigested dietary protein prevents trypsin from diffusing into the ectoperitrophic space and moving into anterior midgut by the countercurrent flux of fluid. Subsequently, dye experiments showed the existence of the appropriate fluid fluxes.

More recently, experimental evidence that a recycling mechanism also occurs in Lepidoptera and Coleoptera was described. As predicted by the model, most trypsin activity is found in the lumen of *Manduca sexta* (Lepidoptera, type I PM) anterior midgut, whereas trypsin mRNA predominates in middle midgut (Peterson *et al.*, 1994). Furthermore, immunocytochemical data showed the occurrence in the anterior midgut of significant amounts of a 41 kDa protein and a β-glycosidase secreted by the posterior midgut of *M. sexta* (Borhegyi *et al.*, 1999) and middle midgut in *Tenebrio molitor* (Coleoptera, type I PM) (Ferreira *et al.*, 2002), respectively. Finally, the decreasing trypsin and chymotrysin gradient along *S. frugiperda* midgut contents disappeared in Calcofluor-treated larvae lacking a peritrophic membrane (Bolognesi *et al.*, 2001) and the excretory rate increased from 0.1% to 0.9% of midgut contents at each gut emptying (R. Bolognesi, W. R. Terra, and C. Ferreira, unpublished data).

4.5.7.2.2.4. Increase in the efficiency of digestion of polymeric food A model system was used to test this hypothesis (R. Bolognesi, W.R. Terra, and C. Ferreira, unpublished data). Midgut contents from *S. frugiperda* larvae were placed into dialysis bags suspended in stirred and unstirred media. Trypsin activities in stirred and unstirred bags were 210% and 160%, respectively, over the activities of similar samples maintained in a test tube. The results suggested that the diffusion of products from the trypsin reaction media favors enzyme action and that stirring (an *in vitro* model of the ectoperitrophic countercurrent flux) enhances the effect.

4.5.7.2.2.5. Increase in the efficiency of oligomeric food hydrolysis This putative function is supported by the experiments of Bolognesi *et al.* (R. Bolognesi, W.R. Terra, and C. Ferreira, unpublished data). They collected ectoperitrophic fluid from the large midgut caeca of *R. americana*. Aminopeptidase A, *N*-acetylglucosaminidase, and carboxypeptidase A are enzymes restricted to the

ectoperitrophic space. When those enzymes were put in the presence of PM contents their activities decreased in relation to controls as follows: aminopeptidase A, 46%; *N*-acetylglucosaminidase, 56%; carboxypeptidase A, 92%. These decreases in activity probably result from oligomer hydrolase competitive inhibition by luminal polymers.

4.5.7.2.2.6. Restriction of food monomer production at cell surface This is a consequence of restricting oligomer hydrolases to the ectoperitrophic space (see Section 4.5.7.2.2.5) and causes an increase in the concentration of the final products of digestion close to the carriers responsible for their absorption. A model system should be developed to test this hypothesis.

4.5.7.2.2.7. Enzyme immobilization Midgut luminal enzymes, in addition to occurring in the endoperitrophic and ectoperitrophic spaces, may be associated with the PM. For example, results obtained with *S. frugiperda* larvae showed that PM may contain up to 13% and 18% of the midgut luminal activity of amylase and trypsin, respectively (Ferreira *et al*., 1994b). Hence, enzyme immobilization may play a role in digestion, although a minor one. The attachment mechanism of enzymes in PM is not well known. Nevertheless, there is evidence, at least in *S. frugiperda*, that trypsin, amylase, and microvillar enzymes are incorporated into the jelly-like substance associated with PM when the enzymes, still bound to membranes, are released from midgut cells by a microaprocrine process (Jordão *et al*., 1999; Bolognesi *et al*., 2001).

4.5.7.2.2.8. Toxin binding Although potentially toxic dietary tannins are attached to and excreted with *Schistocerca gregaria* PM (Bernays and Chamberlain, 1980), toxin binding by the PM seems to be a less widespread phenomenon than previously suggested. Thus, tannins in *M. sexta* (Barbehenn and Martin, 1998) and lipophilic and amphiphilic noxious substances in *Melanoplus sanguinipes* (Barbehenn, 1999) are maintained in the endoperitrophic space because they form high molecular weight complexes, not because of PM binding.

4.5.7.2.2.9. Peritrophic membrane functions and insect phylogeny Current data detailed below suggest that PMs of all insects have functions (see Sections 4.5.7.2.1, 4.5.7.2.2.2–4.5.7.2.2.4), whereas functions (see Sections 4.5.7.2.2.5 and 4.5.7.2.2.6) are demonstrable only in PMs of Panorpodea (the taxon that includes Diptera and Lepidoptera) and of the hymenopteran sawflies. PM function (see Section 4.5.7.2.2.7) may occur in all insects but this needs further confirmation. Function (see Section 4.5.7.2.2.8), although it may be important for some insects, should be viewed as opportunistic. In other words, the PM probably evolved from a protective role (see Section 4.5.7.2.1) to more sophisticated functions (see Sections 4.5.7.2.2.2–4.5.7.2.2.7) under selective pressures and, due to the chemical properties of their constituents, the PM also developed the ability to bind different compounds including toxins.

4.5.8. Organization of the Digestive Process

4.5.8.1. Evolutionary Trends of Insect Digestive Systems

After studying the spatial organization of the digestive events in insects of different taxa and diets, it was realized that insects may be grouped relative to their digestive physiology, assuming they have common ancestors.Those putative ancestors correspond to basic gut plans from which groups of insects may have evolved by adapting to different diets (Terra and Ferreira, 1994, 2003).

The basic plan of digestive physiology for most winged insects (Neoptera ancestors) is summarized in **Figure 11**. In these ancestors, the major part of digestion is carried out in the crop by digestive enzymes propelled by antiperistalsis forward from the midgut. Saliva plays a variable role in carbohydrate digestion. After a while following ingestion, the crop contracts transferring digestive enzymes and partly digested food into the ventriculus. The anterior ventriculus is acid and has high carbohydrase activity, whereas the posterior ventriculus is alkaline and has high proteinase activity. This differentiation along the midgut may be an adaptation to instability of ancestral carbohydrases in the presence of proteinases. The food bolus moves backward in the midgut of the insect by peristalsis. As soon as the polymeric food molecules are digested to become sufficiently small to pass through the peritrophic membrane, they diffuse with the digestive enzymes into the ectoperitrophic space (**Figure 1**). The enzymes and nutrients are then displaced toward the caeca with a countercurrent flux caused by secretion of fluid at the Malpighian tubules and its absorption back by cells at the caeca (**Figure 11**), where final digestion is completed and nutrient absorption occurs. When the insect starts a new meal, the caeca contents are moved into the crop. As a consequence of the countercurrent flux,

digestive enzymes occur as a decreasing gradient in the midgut and their excretion is lowered.

The Neoptera basic plan gave origin to that of the Polyneoptera orders, which include Blattodea, Isoptera, and Orthoptera, and evolved to the basic plans of Paraneoptera and Holometabola. The characteristics of the Paraneoptera ancestors cannot be inferred because midgut function data are available only for Hemiptera.

The basic gut plan of the Holometabola (Figure 11b) (which include Coleoptera, Megaloptera, Hymenoptera, Diptera, and Lepidoptera) is similar to that of Neoptera, except that fluid secretion occurs by the posterior ventriculus, instead of by the Malpighian tubules. Because the posterior midgut fluid does not contain wastes, as is the case for Malpighian tubular fluid, the accumulation of wastes in caeca is decreased. Caeca loss probably further decreases the accumulation of noxious substances in the midgut, which would be more serious in insects that have high relative food consumption rates, as is common among Holometabola.

The basic plan of Coleoptera did not evolve dramatically from the holometabolan ancestor, whereas the basic plan of Hymenoptera, Diptera, and Lepidoptera ancestor (hymenopteran–panorpoid ancestor, Figure 11c) presents important differences. Thus, hymenopteran–panorpoid ancestors have countercurrent fluxes like holometabolan ancestors, but differ from these in the lack of crop digestion, midgut differentiation in luminal pH, and in which compartment is responsible for each phase of digestion. In holometabolan ancestors, all phases of digestion occur in the endoperitrophic space (Figure 1), whereas in hymenopteran–panorpoid ancestors only initial digestion occurs in that region. In the latter ancestors, intermediate digestion is carried out by free enzymes in the ectoperitrophic space and final digestion occurs at the midgut cell surface by immobilized enzymes. The free digestive enzymes do not pass through the PM because they are larger than the PM's pores. As a consequence of the compartmentalization of digestive events in hymenopteran and panorpoid insects, there is an increase in the efficiency of digestion of polymeric food as discussed before.

The evolution of insect digestive systems summarized above and in Figure 11 was proposed, as discussed before, from studies carried out in 12 species pertaining to four insect orders. To give further support for the hypothesis that the characteristics of gut function and morphology depend more on phylogeny than on diet, another approach was used. A total of 29 gut morphology and digestive physiology characteristics (e.g., luminal pH, ratio of gut

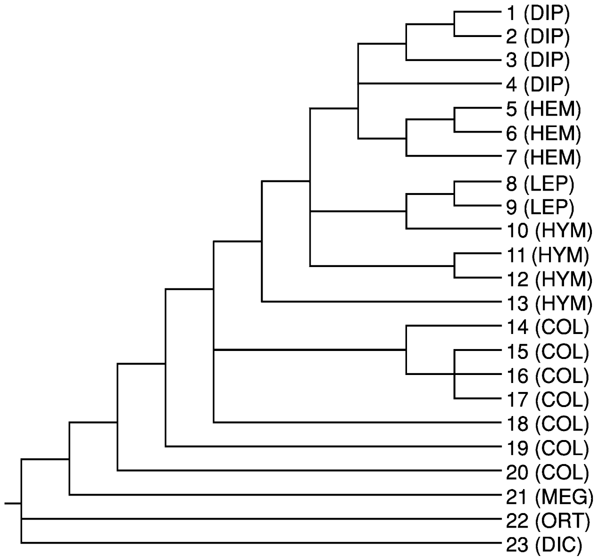


Figure 12 Cladogram of representative insects based on 29 gut morphology and digestive physiology characteristics. Insects: 1, *Trichosia pubescens*; 2, *Rhynchosciara americana*; 3, *Musca domestica*; 4, *Anopheles* spp.; 5, *Rhodnius prolixus*; 6, *Dysdercus peruvianus*; 7, *Acyrthosiphon pisum*; 8, *Erinnyis ello*; 9, *Spodoptera frugiperda*; 10, *Themos malaisei*; 11, *Camponotus rufipes*; 12, *Scaptotrigona bipunctata*; 13, *Bracon hebetor*; 14, *Tenebrio molitor*; 15, *Migdolus fryanus*; 16, *Sphenophorus levis*; 17, *Cyrtomon solana*; 18, *Dermestes maculatus*; 19, *Pyrearinus termitilluminans*; 20, *Pheropsophus aequinoctialis*; 21, *Corydalus* sp.; 22, *Abracris flavolineata*; 23, *Periplaneta americana*. (Courtesy of A.B. Dias.)

section volumes, type of peritrophic membrane, presence of special gut cells, distribution of digestive enzymes along the gut, major proteinase) were identified in 23 species from eight different insect orders. Making use of these characteristics, a cladogram was constructed putting together the data from studied species (Figure 12). The data confirmed that the morphological and functional traits associated with the digestive system are more dependent on taxon than on dietary habits of the different insects (Dias, Vanin, Marques, and Terra, unpublished data). There are two insect species that do not apparently fit the model: *Anopheles* spp. and *Themos malaisei*. *Anopheles* spp. is an adult, whereas the other Diptera is larval. *Themos malaisei* is an unexpected finding that will be discussed below (see Section 4.5.8.2.8).

4.5.8.2. Digestion in the Major Insect Orders

4.5.8.2.1. Initial comments Applebaum (1985) in his review for the first edition of this series divided the insects according to their diet when describing their digestive processes. Recent data (see Section 4.5.8.1) support the view that functional digestive traits of insects are linked with their phylogenetic

position. The organization of the digestive process in the different insect orders have been reviewed several times (Terra, 1988, 1990; Terra and Ferreira, 1994, 2003). The following section is therefore an abridged version of those texts, highlighting new findings and trying to identify points that deserve more research, especially in relation to molecular aspects. Only key references before 1994 are cited and the reader should find more references in the above-mentioned reviews.

4.5.8.2.2. Blattodea Cockroaches are usually omnivorous. It is thought that digestion in cockroaches occurs as described for the Neoptera ancestor (**Figure 11a**), except that part of the final digestion of proteins occurs on the surface of midgut cells (Terra and Ferreira, 1994). This was confirmed by the finding in *P. americana* that most trypsin, maltase, and amylase are found in the crop, whereas aminopeptidase predominates in the microvillar membranes of posterior midgut. There is a decreasing gradient of trypsin, maltase, and amylase along the midgut contents and less than 5% of trypsin and maltase (amylase, 27%) are excreted during each midgut emptying. This suggests the existence of midgut digestive enzyme recycling, with amylase excretion increased probably due to excess dietary starch. The recycling mechanism is thought to be powered by water fluxes as in the Neoptera ancestor, although there are no data supporting this. Major digestive proteinases are trypsin and chymotrypsin (Dias and Terra, unpublished data).

The differentiation of pH along the midgut (acid anterior midgut and alkaline posterior midgut) is not conserved among some cockroaches like *P. americana*, but it was maintained in others exemplified by the blaberid *Nauphoeta cinerea* (Elpidina *et al.*, 2001a). The organization of digestion in this cockroach seems similar to that in *P. americana*, although data on enzyme excretion are lacking. At least blaberoid cockroaches possess proteinase inhibitory proteins active in the anterior midgut. These inhibitors are thought to be a primitive device to decrease the proteolytic inactivation of the animal's own carbohydrases, which are thus expected to be more active in the anterior midgut. The digestive carbohydrases from more evolved insects are stable in the presence of their own proteinases (Terra, 1988). Recently several proteinase inhibitors have been partially purified from *N. cinerea* (Elpidina *et al.*, 2001b).

Another difference between cockroaches and the Neoptera ancestor is the enlargement of hindgut structures, noted mainly in wood-feeding cockroaches. These hindgut structures harbor bacteria producing acetate and butyrate from ingested wood or other cellulose-containing materials. Acetate and butyrate are absorbed by the hindgut of all cockroaches, but this is more remarkable with woodroaches (Terra and Ferreira, 1994). Cellulose digestion may be partly accomplished by bacteria in the hindgut of *P. americana* or protozoa in *Cryptocercus punctulatus* (Bignell, 1981). Nevertheless, now it is clear that *P. americana* saliva contains two cellulases and three laminarinases that may open plant cells and lyze fungal cells (Genta *et al.*, 2003). This agrees with the omnivorous detritus feeding habit of the insect. The woodroach *Panestria cribrata* also has its own cellulase (Scrivener *et al.*, 1989).

4.5.8.2.3. Isoptera Termites may be seen as insects derived from, and more adapted than, woodroaches in dealing with refractory material as wood and humus. Associated with this specialization, they lost the crop and midgut caeca, and enlarged their hindgut structures. Both lower and higher termites digest cellulose with their own cellulase, despite the occurrence of cellulose-producing protozoa in the paunch, an enlarged region of the anterior hindgut in lower termites. The products of cellulose digestion pass from the midgut into the hindgut, where they are converted into acetate and butyrate by hindgut bacteria as in woodroaches. Symbiotic bacteria are also responsible for nitrogen fixation in the hindgut, resulting in bacterial protein. This is incorporated into the termite body mass after being expelled in feces by one individual and being ingested and digested by another. This explains the ability of termites to develop successfully in diets very poor in protein. Both lower and higher feeding termites seem to have an endo–ectoperitrophic circulation of digestive enzymes (Terra and Ferreira, 1994; Nakashima *et al.*, 2002; see also Section 4.5.4.3.1).

4.5.8.2.4. Orthoptera Grasshoppers feed mainly on grasses and their digestive physiology has clearly evolved from the Neoptera ancestor. Carbohydrate digestion occurs mainly in the crop, under the action of midgut enzymes, whereas protein digestion and final carbohydrate digestion take place at the anterior midgut caeca. The abundant saliva (devoid of significant enzymes) produced by grasshoppers saturate the absorbing sites in the midgut caeca, thus hindering the countercurrent flux of fluid. This probably avoids excessive accumulation of noxious wastes in the caeca, coming from Malpighian tubule secretion, and makes possible the high relative food consumption observed among locusts

in their migratory phases. Starving grasshoppers present midgut countercurrent fluxes. Cellulase found in some grasshoppers is believed to facilitate the access of digestive enzymes to the plant cells ingested by the insects by degrading the cellulose framework of cell walls (Dow, 1986; Terra and Ferreira, 1994; Marana *et al*., 1997).

4.5.8.2.5. Hemiptera The Hemiptera comprise insects of the major suborders Auchenorrhyncha (cicadas, spittlebugs, leafhoppers, and planthoppers) and Sternorrhyncha (aphids and white flies) that feed almost exclusively on plant sap, and Heteroptera (e.g., assassin bugs, plant bugs, stink bugs, and lygaeid bugs) that are adapted to different diets.

The ancestor of the entire order Hemiptera is supposed to have been a sap-sucker similar to present day Auchenorrhyncha. Sap-sucking Hemiptera may suck phloem or xylem sap. These food sources have very low contents of proteins (with the exception of few phloem saps; see below) and carbohydrate polymers and are relatively poor in free essential amino acids. In contrast to xylem sap, phloem sap is very rich in sucrose (Terra, 1990). Thus, except for dimer (sucrose) hydrolysis, no food digestion is usually necessary in sap-suckers. Upon adapting to dilute phloem and/or xylem sap, hemipteran ancestors would lose the enzymes involved in initial and intermediate digestion and lose the peritrophic membrane (**Figure 11f**). These changes are associated with the lack of luminal digestion.

The major problem facing a sap-sucking insect (specially on dilute phloem or xylem sap) is to absorb nutrients, such as essential amino acids, that are present in very low concentration in sap. Whichever mechanism is used, xylem feeders may absorb as much as 99% of dietary amino acids and carbohydrate (Andersen *et al*., 1989). Amino acids may be absorbed according to a hypothesized mechanism that depends on perimicrovillar membranes, which are membranes ensheathing the midgut microvilli with a dead end (**Figure 13**). A role in midgut amino acid absorption depends on the presence of amino acid–K^+ symports on the surface of the perimicrovillar membranes and of amino acid carriers and potassium pumps on the microvillar membranes. Although amino acid carriers have been found in the microvillar membranes of several insects (Wolfersberger, 2000), no attempts have been made to study the other postulated proteins. Thus, in spite of the model provided an explanation for the occurrence of these peculiar cell structures in Hemiptera, it is supported only by: (1) evidence that amino acids are absorbed with potassium ions in *Dysdercus peruvianus* (Silva and Terra, 1994); (2) occurrence of particles studying the cytoplasmic face of the midgut microvillar membranes of *D. peruvianus*. These might be ion pumps responsible for the putative potassium ion transport, like similar structures in several epithelia (Silva *et al*., 1995).

Another problem that deserves more attention regarding perimicrovillar membranes is their origin. Immunolocalization of the perimicrovillar enzyme marker, α-glucosidase, suggests that these membranes are formed when double membrane vesicles fuse their outer membranes with the microvillar membranes and their inner membranes with the perimicrovillar membranes. A double membrane Golgi cisterna (on budding) forms the double membrane vesicles (Silva *et al*., 1995).

Organic compounds in xylem sap need to be concentrated before they can be absorbed by the perimicrovillar system. This occurs in the filter chamber of Cicadoidea and Cercopoidea, which concentrates xylem sap tenfold. The filter chamber consists of a thin-walled, dilated anterior midgut in close contact with the posterior midgut and the proximal ends of the Malpighian tubules. This arrangement enables water to pass directly from the anterior midgut to the Malpighian tubules, concentrating food in midgut and eliminating excess water. The high permeability of the filter chamber membrane to water results from the occurrence of specific channels formed by proteins named aquaporins. These were characterized as membrane proteins with 15–26 kDa and were immunolocalized in the filter chamber of several xylem sap feeders (Le Cahérec *et al*., 1997).

Sternorrhyncha, as exemplified by aphids, may suck more or less continuously phloem sap of sucrose concentration up to 1.0 M and osmolarity up to three times that of the insect hemolymph. This results in a considerable hydrostatic pressure caused by the tendency of water to move from the hemolymph into midgut lumen. To withstand these high hydrostatic pressures, aphids have developed several adaptations. Midgut stretching resistance is helped by the existence of links between apical lamellae (replacing usual midgut cell microvilli) that become less conspicuous along the midgut. As a consequence of the links between the lamellae, the perimicrovillar membranes could no longer exist and were replaced by membranes seen associated with the tips of the lamellae, the modified perimicrovillar membranes (Ponsen, 1991; Cristofoletti *et al*., 2003). A modified perimicrovillar membrane-associated α-glucosidase frees fructose from sucrose without increasing the osmolarity by promoting transglycosylations. As the fructose is quickly absorbed, the osmolarity decreases, resulting in a

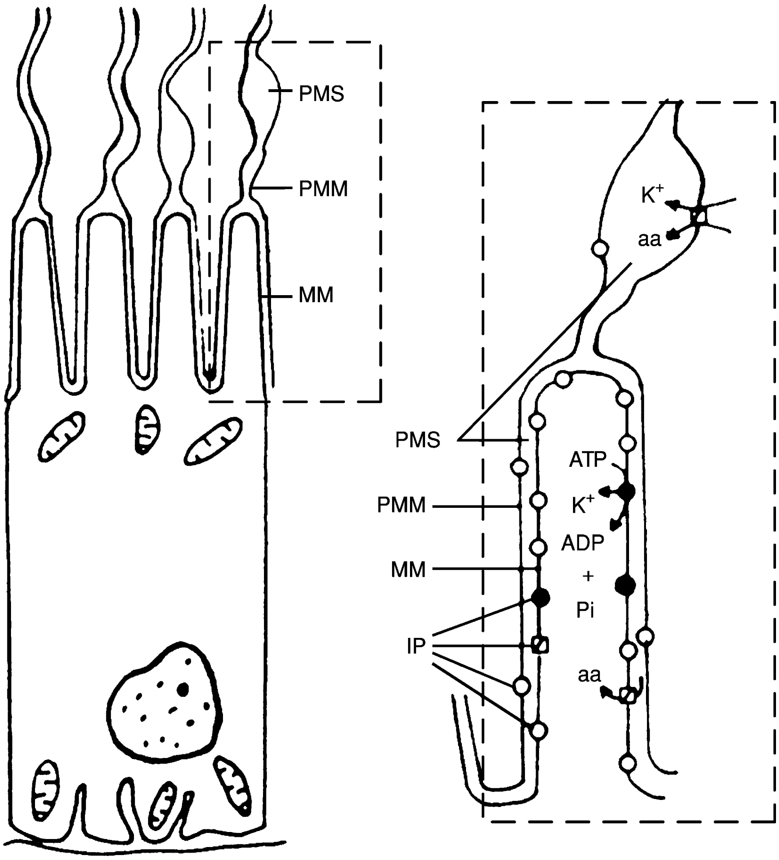


Figure 13 Model for the structure and physiological role of the microvillar border of midgut cells from Hemiptera. The left figure is a diagrammatic representation of a typical Hemiptera midgut cell and the right figure details its apex. The microvillar membrane (MM) is ensheathed by the perimicrovillar membrane (PMM), which extends toward the luminal compartment with a dead end. The microvillar and perimicrovillar membranes delimit a closed compartment, i.e., the perimicrovillar space (PMS). The microvillar membrane is rich and the perimicrovillar membrane is poor in integral proteins (IP). Microvillar membranes actively transport potassium ions (the most important ion in sap) from PMS into the midgut cells, generating a concentration gradient between the gut luminal sap and the PMS. This concentration gradient may be a driving force for the active absorption of organic compounds (amino acids, aa, for example) by appropriate carriers present in the PMM. Organic compounds, once in the PMS, may diffuse up to specific carriers on the microvillar surface. This movement is probably enhanced by a transfer of water from midgut lumen to midgut cells, following (as solvation water) the transmembrane transport of compounds and ions by the putative carriers. (Reprinted with permission from Terra, W.R., Ferreira, C., **1994**. Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol. B 109*, 1–62; © Elsevier.)

honeydew isoosmotic with hemolymph (Ashford *et al*., 2000; Cristofoletti *et al*., 2003). Another interesting adaptation is observed in whiteflies, where a trehalulose synthase forms trehalulose from sucrose, thus making available less substrate for an α-glucosidase that otherwise would increase the osmolarity of ingested fluid on hydrolyzing sucrose (Salvucci, 2003).

A cathepsin L (see Section 4.5.5.3) bound to the modified perimicrovillar membranes of *Acyrthosiphon pisum* (Cristofoletti *et al*., 2003) may explain the capacity of some phloem sap feeders to rely on protein found in some phloem saps (Salvucci *et al*., 1998) and the failure of other authors to find an active proteinase in sap feeders. They worked with homogenate supernatants or supernatants of Triton X-100-treated samples, under which conditions the cathepsin L would remain in the pellet.

Amino acid absorption in *A. pisum* midguts is influenced by the presence of the bacteria *Buchnera* in the mycetocytes of the mycetomes occurring in the aphid hemocoel (Prosser *et al*., 1992). The molecular mechanisms underlying this phenomenon are not known, in spite of the fact that there is strong evidence showing that *Buchnera* uses the nonessential amino acids absorbed by the host in the synthesis of essential amino acids (Prosser and Douglas, 1992; Shigenobu *et al*., 2000). It is likely that amino acid absorption through apical lamellar carriers depends on the amino acid concentration gradient between midgut lumen and hemolymph, whereas hemolymph titers vary widely according

to *Buchnera* metabolic activity (Liadouze *et al*., 1995).

The evolution of Heteroptera was associated with regaining the ability to digest polymers. Because the appropriate digestive enzymes were lost, they instead used enzymes derived from lysosomes. Lysosomes are cell organelles involved in intracellular digestion carried out by special proteinases referred to as cathepsins. Compartmentalization of digestion was maintained by the perimicrovillar membranes, as a substitute for the absent peritrophic membrane. Digestion in the two major Heteroptera taxa, Cimicomorpha (exemplified by the blood-feeder *Rhodnius prolixus*), and Pentatomorpha (exemplified by the seed-sucker *Dysdercus peruvianus*), are similar. The dilated anterior midgut stores food and absorbs water and, at least in *D. peruvianus*, also absorbs glucose. Digestion of proteins and absorption of amino acids occurs in the posterior ventriculus. Most protein digestion occurs in lumen with the aid of a cysteine proteinase, and ends in the perimicrovillar space under the action of aminopeptidases and dipeptidases (Terra and Ferreira, 1994). Symbiont bacteria may occur in blood-feeders putatively to provide vitamins (see Section 4.5.2.5). At least in *R. prolixus*, the neuroendocrine system has factors important for maintaining the ultrastructural organization of the midgut epithelial cells (Gonzales *et al*., 1998).

4.5.8.2.6. Megaloptera Megaloptera include alderflies and dobsonflies and are often considered to be the most primitive group of insects with complete metamorphosis. All their larvae are aquatic predators feeding on invertebrates (Theischinger, 1991). Megaloptera ancestors are like Holometabola ancestors except that for the anterior midgut caeca, which were lost and replaced in function by the anterior midgut. Thus, in *Corydalus* sp. larvae, most digestion occurs in the crop under the action of soluble amylase, maltase, aminopeptidase, and trypsin (major proteinase). Digestive enzyme recycling should occur, as less than 10% of midgut amylase, maltase, and aminopeptidase are lost at each midgut emptying. The higher excretory rate of trypsin (27%) probably results from excess dietary protein (Dias and Terra, unpublished data).

4.5.8.2.7. Coleoptera Coleoptera ancestors are like Megaloptera ones. Nevertheless, there are evolutionary trends leading to a great reduction or loss of the crop and, as in the panorpoid orders, occurrence of at least final digestion of proteins at the surface of midgut cells. Thus, in predatory Carabidae most of the digestive phases occur in the crop by means of midgut enzymes, whereas in predatory larvae of Elateridae initial digestion occurs extraorally by the action of enzymes regurgitated onto their prey. The preliquified material is then ingested by the larvae and its digestion is finished at the surface of midgut cells (Terra and Ferreira, 1994).

The entire digestive process occurs in the dermestid larval endoperitrophic space that is limited by a peritrophic gel in anterior midgut and a peritrophic membrane in posterior midgut. There is a decreasing gradient along the midgut of amylase, maltase, trypsin (major proteinase), and aminopeptidase suggesting the occurrence of digestive enzyme recycling (Terra and Ferreira, 1994; Caldeira, Dias, Terra, and Ribeiro, unpublished data).

Like dermestid beetles, the larvae of *Migdolus fryanus* (Cerambycidae) and *Sphenophorus levis* (Curculionidae) have a peritrophic gel and a peritrophic membrane in the anterior and posterior midgut, respectively, and a decreasing gradient of amylase, maltase, and proteinase along the midgut. In contrast to dermestids, aminopeptidase is a microvillar enzyme in both insects (Dias and Terra, unpublished data). These data do not confirm the earlier suggestion (Terra and Ferreira, 1994) that the final digestion of all nutrients occurs on the surface of midgut cells of Curculionidae.

Tenebrionid larvae also have aminopeptidase as a microvillar enzyme and the distribution of enzymes in gut regions of adults is similar to that in the larvae (Terra and Ferreira, 1994). This suggests that the overall pattern of digestion in larvae and adults of Coleoptera is similar, despite the fact that (in contrast to adults) beetle larvae usually lack a crop.

Insects of the series Cucujiformia (which includes Tenebrionidae, Chrysomelidae, Bruchidae, and Curculionidae) have cysteine proteinases (see Section 4.5.5.3) in addition to (or in place of) serine proteinases as digestive enzymes, suggesting that the ancestors of the whole taxon were insects adapted to feed on seeds rich in serine proteinase inhibitors. The occurrence of trypsin as the major proteinase in *M. fryanus* (Dias and Terra, unpublished data) confirmed the preliminary work (Murdock *et al*., 1987) according to which cerambycid larvae reacquired serine proteinases.

Scarabaeidae and several related families are relatively isolated in the series Elateriformia and evolved considerably from the Coleoptera ancestor. Scarabid larvae, exemplified by dung beetles, usually feed on cellulose materials undergoing degradation by a fungus-rich flora. Digestion occurs in the midgut, which has three rows of caeca, with a ventral groove between the middle and posterior row. The alkalinity of gut contents increase to almost

pH 12 along the midgut ventral groove. This high pH probably enhances cellulose digestion, which occurs mainly in the hindgut fermentation chamber, through the probable action of bacterial cell-bound enzymes. The final product of cellulose degradation is mainly acetic acid, which is absorbed through the hindgut wall. There is controversy as to whether scarabid larvae ingest feces to obtain nitrogen compounds, as described above for termites (Terra and Ferreira, 1994; Biggs and McGregor, 1996).

4.5.8.2.8. Hymenoptera The organization of the digestive process is variable among hymenopterans and to understand its peculiarities it is necessary to review briefly their evolution. The hymenopteran basal lineages are phytophagous as larvae, feeding both ecto- and endophytically and include several superfamilies like Xyeloidea and Tenthredinoidea, all known as sawflies. Close to these are the Siricoidea (wood wasps) that are adapted to ingest fungus-infected wood. Wood wasp-like ancestors gave rise to the Apocrita (wasp-waisted Hymenoptera) that are parasitoids of insects. They use their ovipositor to injure or kill their host which represents a single meal for their complete development. A taxon sister of Ichneumonoidea in Apocrita gave rise to Aculeata (bees, ants, and wasps with thin waist) (Quicke, 2003).

The digestive systems of Hymenoptera ancestors are like the panorpoid ancestors (**Figure 11c**). However, there are evolutionary trends leading to the loss of midgut caeca (replaced in function by the anterior midgut) and changes in midgut enzyme compartmentalization. These trends appear to be associated with the development of parasitoid habits and were maintained in Aculeata, as described below.

The sawfly *T. malaisei* (Tenthredinoidea: Argidae) larva has a midgut with a ring of anterior caeca that forms a U at the ventral side. Luminal pH is above 9.5 in the first two-thirds of the midgut. Trypsin (major proteinase) and amylase have a decreasing activity along the endoperitrophic space, suggesting enzyme recycling. Maltase predominates in the anterior midgut tissue as a soluble glycocalyx-associated enzyme, whereas aminopeptidase is a microvillar enzyme in posterior midgut (Dias, Ribeiro, and Terra, unpublished data). These characteristics (except the presence of caeca) are similar to those of lepidopteran larvae (see Section 4.5.8.2.10) and explain the fact that this insect is close to the lepidopterans in **Figure 12**. Otherwise, Aculeata with their less sophisticated midgut (see below) branches closer to coleopterans (**Figure 12**).

Wood wasp larvae of the genus *Sirex* are believed to be able to digest and assimilate wood constituents by acquiring cellulase and xylanase, and possibly other enzymes, from fungi present in wood on which they feed (Martin, 1987).

The larvae of Apocrita present a midgut which is closed at its rear end, and which remains unconnected with the hindgut until the time of pupation. It is probable that this condition evolved as an adaptation of endoparasitoid Apocrita ancestors to avoid the release of toxic compounds into the host in which they lived (Terra, 1988).

In larval bees, most digestion occurs in the endoperitrophic space. Countercurrent fluxes seem to occur but there is no midgut luminal pH gradient. Adult bees ingest nectar and pollen. Sucrose from nectar is hydrolyzed in the crop by the action of a sucrase from the hypopharyngeal glands. After ingestion, pollen grains extrude their protoplasm in the ventriculus, where digestion occurs. As in larvae there is also evidence of an endo–ectoperitrophic circulation of digestive enzymes (Jimenez and Gilliam, 1990; Terra and Ferreira, 1994).

Although many authors favor the view that pollen grains are digested in bees after their extrusion by osmotic shock, this subject is controversial not only in bees but also among pollen-feeder beetles (Human and Nicholson, 2003).

Worker ants feed on nectar, honeydew, plant sap, or on partly digested food regurgitated by their larva. Thus, they frequently were said to lack digestive enzymes or display only those enzymes associated with intermediate and (or) final digestion (Terra and Ferreira, 1994). Although this seems true for leaf-cutting ants that appear to rely only on monosaccharides, produced by fungal enzymes acting on plant polysaccharides (Silva *et al*., 2003), this is not widespread. Thus, adult *Camponotus rufipes* (Formicinae) have soluble amylase, trypsin (major proteinase), maltase, and aminopeptidase enclosed in a type I PM in their midguts. As only 14% of amylase and less than 7% of the other digestive enzymes are excreted during the midgut emptying, these insects may have a digestive enzyme recycling mechanism (Dias and Terra, unpublished data).

4.5.8.2.9. Diptera The Diptera evolved along two major lines: an assemblage (early Nematocera) of suborders corresponding to the mosquitoes, including the basal Diptera, and the suborder Brachycera that includes the most evolved flies (Cyclorrhapha). The Diptera ancestor is similar to the panorpoid ancestor (**Figure 11c**) in having the enzymes involved in intermediate digestion free in the ectoperitrophic fluid (mainly in the large caeca), whereas the enzymes of terminal digestion are

membrane bound at the midgut cell microvilli (Terra and Ferreira, 1994). Although these characteristics are observed in most nonbrachyceran larvae, the more evolved of these larvae may show reduction in size of midgut caeca (e.g., Culicidae). Nonhematophagous adults store liquid food (nectar or decay products) in their crops. Digestion occurs in their midgut as in larvae. Nectar ingested by mosquitoes (males and females) is stored in the crop, and is digested and absorbed at the anterior midgut. Blood, which is ingested only by females, passes to the posterior midgut, where it is digested and absorbed (Billingsley, 1990; Terra and Ferreira, 1994).

Adult *Aedes aegypti* midgut surface is covered in a large proportion by tubular bilayers with diameter fourfold smaller than microvilli. They fuse and branch forming bundles that seem to originate in the intercellular crypts and seem to be fused with the microvillar surface (Ziegler *et al*., 2000). These structures are not related with the perimicrovillar membranes of Hemiptera. The latter envelop the microvilli before extending into the lumen in structures that may resemble the tubular membrane bilayers of *A. aegypti* (see Section 4.5.8.2.5 and **Figure 13**). The puzzling structures of *A. aegypti* should be further studied to discover their relationships with digestion.

The Cyclorrhapha ancestor (**Figure 11d**) evolved dramatically from the panorpoid ancestor (**Figure 11c**), apparently as a result of adaptations to a diet consisting mainly of bacteria. Digestive events in Cyclorrhapha larvae are exemplified by larvae of the housefly *Musca domestica*. These ingest food rich in bacteria. In the anterior midgut there is a decrease in the starch content of the food bolus, facilitating bacterial death. The bolus now passes into the middle midgut where bacteria are killed by the combined action of low pH, a special lysozyme (see Section 4.5.4.5) and an aspartic proteinase (see Section 4.5.5.4). Finally, the material released by bacteria is digested in the posterior midgut, as is observed in the whole midgut of insects of other taxa. Countercurrent fluxes occur in the posterior midgut powered by secretion of fluid in the distal part of the posterior midgut and its absorption back in middle midgut. The middle midgut has specialized cells for buffering the luminal contents in the acidic zone (**Figure 4**), in addition to those functioning in fluid absorption. Cyclorrhaphan adults, except for a few blood-suckers, feed mainly on liquids associated with decaying material (rich in bacteria) in a way similar to housefly *M. domestica* adults. These salivate (or regurgitate their crop contents) onto their food. After the dispersed material is ingested, starch digestion is accomplished primarily in the crop by the action of salivary amylase. Digestion is followed in the midgut, essentially as described for larvae (Terra and Ferreira, 1994).

The stable fly *Stomoxys calcitrans* stores and concentrates the blood meal in the anterior midgut and gradually passes it to the posterior midgut, where digestion takes place, resembling what occurs in larvae. These adults lack the characteristic cyclorrhaphan middle midgut and the associated luminal low pH. Stable flies occasionally take nectar (Jordão *et al*., 1996a).

4.5.8.2.10. Lepidoptera Lepidopteran ancestors (**Figure 11e**) differ from panorpoid ancestors because they lack midgut caeca, have all their digestive enzymes (except those of initial digestion) immobilized at the midgut cell surface, and present long-neck goblet cells and stalked goblet-cells in the anterior and posterior larval midgut regions, respectively. Goblet cells excrete K^+ ions that are absorbed from leaves ingested by larvae. Goblet cells also seem to assist anterior columnar cells in water absorption and posterior columnar cells in water secretion (Terra and Ferreira, 1994; Ortego *et al*., 1996).

Although most lepidopteran larvae have a common pattern of digestion, species that feed on unique diets generally display some adaptations. *Tineola bisselliella* (Tineidae) larvae feed on wool and display a highly reducing midgut for cleaving the disulfide bonds in keratin to facilitate proteolytic hydrolysis of this otherwise insoluble protein (Terra and Ferreira, 1994). Similar results were obtained with *Hofmannophila pseudospretella* (Christeller, 1996). Wax moths (*Galleria mellonella*) infest beehives and digest and absorb wax. The participation of symbiotic bacteria in this process is controversial. Another adaptation has apparently occurred in lepidopteran adults which feed solely on nectar. Digestion of nectar only requires the action of an α-glucosidase (or a β-fructosidase) to hydrolyze sucrose, the major component present. Many nectar-feeding lepidopteran adults have amylase in salivary glands and several glycosidases and peptidases in the midgut (Terra and Ferreira, 1994).

Woods and Kingsolver (1999) developed a chemical reactor model of the caterpillar midgut and used the model as a framework for generating hypotheses about the relationship between feeding responses to variable dietary protein and the physical and biochemical events in the midgut and body. They concluded that absorption (or postabsorptive processes) is limiting in a caterpillar maintained in artificial diets. Caterpillars eating leaves may not have

the same limiting step and this deserves a similar detailed study. Another interesting study would be the development of a model for the beetle midgut. This would determine whether beetles have digestion as the limiting step or consumption to compensate for their less sophisticated midguts.

4.5.9. Digestive Enzyme Secretion Mechanisms and Control

Digestive enzyme secretory mechanisms and control probably are the least understood areas in insect digestion. Studies of the secretory mechanisms have only described major differences, which seem to include unique aspects not seen in other animals.

Insects are continuous (e.g., Lepidoptera and Diptera larvae) or discontinuous (e.g., predators and many hematophagous insects) feeders. The synthesis and secretion of digestive enzymes in continuous feeders seem to be constitutive, that is, they occur continuously (at least between molts), whereas in discontinuous feeders they are regulated (Lehane *et al.*, 1996). Digestive enzymes, as with all animal proteins, are synthesized in the rough endoplasmic reticulum and processed in the Golgi complex, and are packed into secretory vesicles (Figure 14). There are several mechanisms by which the contents of the secretory vesicles are freed in the midgut lumen. In holocrine secretion, secretory vesicles are stored in the cytoplasm until they are released, at which time the whole secretory cell is lost to the extracellular space. During exocytic secretion, secretory vesicles fuse with the midgut cell apical membrane emptying their contents without any loss of cytoplasm (Figure 14a). In contrast, apocrine secretion involves the loss of at least 10% of the apical cytoplasm following the release of secretory vesicles (Figure 14b). These have previously undergone fusions originating larger vesicles that after release eventually free their contents by solubilization (Figure 14b). When the loss of cytoplasm is very small, the secretory mechanism is called microapocrine. Microapocrine secretion consists in releasing budding double-membrane vesicles (Figure 14c) or, at least in insect midguts, pinched-off vesicles that may contain a single or several secretory vesicles (Figure 14d). In both cases the secretory vesicle contents are released by membrane fusion and/or by membrane solubilization caused by high pH contents or by luminal detergents.

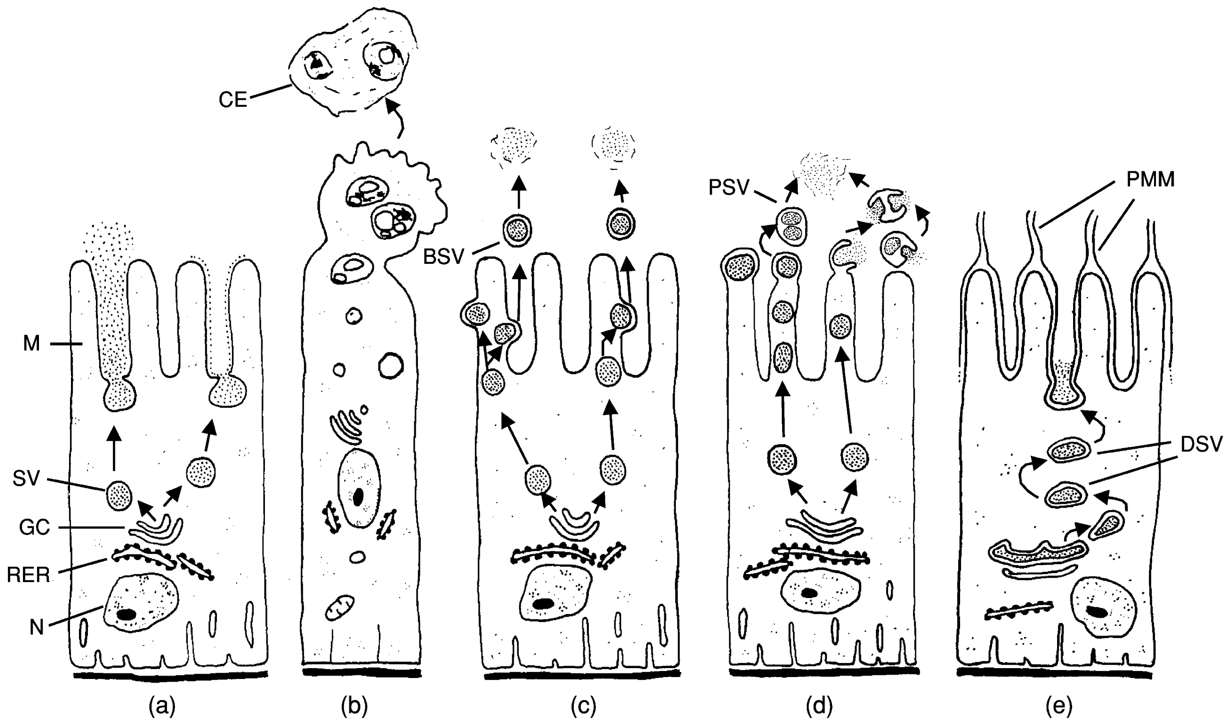


Figure 14 Models for secretory processes of insect digestive enzymes. (a) Exocytic secretion; (b) apocrine secretion; (c) microapocrine secretion with budding vesicles; (d) microapocrine secretion with pinched-off vesicles; (e) modified exocytic secretion in hemipteran midgut cell. BSV, budding secretory vesicle; CE, cellular extrusion; DSV, double-membrane secretory vesicle; GC, Golgi complex; M, microvilli; N, nucleus; PMM, perimicrovillar membrane; PSV, pinched-off secretory vesicle; RER, rough endoplasmatic reticulum; SV, secretory vesicle. (Reprinted with permission from Terra, W.R., Ferreira, C., **2003**. Digestive system. In: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects. Academic Press, San Diego, CA, pp. 313–323; © Elsevier.)

The secretory mechanisms of insect midgut cells reviewed below are based on immunocytolocalization data or on data combining biochemical procedures and electron micrographs. Studies based only on traditional cytology have been reviewed elsewhere (Terra and Ferreira, 1994; Lehane *et al.*, 1996).

Holocrine secretion is usually described on histological grounds mainly in midgut of insects other than higher Holometabola. These insects have large number of regenerative cells in their midguts. Thus, it is probable that cell renewal in these insects is being misinterpreted as holocrine secretion (Terra and Ferreira, 1994). In spite of this, immunocytochemical data showed that trypsin-containing vesicles along with cell organelles are discharged by opaque zone cells of adult stable flies, suggesting holocrine secretion (Jordão *et al.*, 1996a).

Exocytic, apocrine, and microaprocrine secretory mechanisms depend largely on midgut regions. Digestive enzymes are usually secreted by exocytosis in the posterior midgut, whereas alternate mechanisms may be observed in anterior midgut. Thus, trypsin is secreted by the posterior midgut of adult mosquitoes (Graf *et al.*, 1986), larval flies (Jordão *et al.*, 1996b), and caterpillars (Jordão *et al.*, 1999) by exocytosis, as well as, β-glycosidase by *Tenebrio molitor* middle midguts (Ferreira *et al.*, 2002). Trypsin is secreted by the anterior midgut of caterpillars using a microapocrine route (Santos *et al.*, 1986; Jordão *et al.*, 1999), whereas in the anterior midgut of *T. molitor* amylase secretion occurs by an apocrine mechanism (Cristofoletti *et al.*, 2001). Based only on morphological evidence, one may say that, in addition to *E. ello* and *Spodoptera frugiperda*, microapocrine secretion occurs in other lepidopteran species, such as *Manduca sexta* (Cioffi, 1979), whereas apocrine secretion is observed in some Orthoptera (Heinrich and Zebe, 1973) and in many coleopteran species other than *T. molitor* (Bayon, 1981; Silva and Souza, 1981; Baker *et al.*, 1984).

Immunocytolocalization data (Silva *et al.*, 1995) showed that secretion by hemipteran midgut cells displays special features, as the cells have perimicrovillar membranes, in addition to microvillar ones (**Figure 14e**). In this case, double membrane vesicles bud from modified (double membrane) Golgi structures (**Figure 14e**). The double membrane vesicles move to the cell apex, their outer membranes fuse with the microvillar membrane, and their inner membranes fuse with the perimicrovillar membranes, emptying their contents (**Figure 14e**).

Control of digestive enzyme synthesis and secretion in insects has been extensively investigated but the results are often difficult to interpret. It is generally hypothesized that short-term variations in enzymatic activity are controlled by a secretagogue mechanism, whereas long-term changes are regulated hormonally (Lehane *et al.*, 1996).

An example of the long-term effects of hormones is the transcription of the early trypsin gene which starts a few hours after mosquito emergence and is under the control of juvenile hormone. However, the early trypsin mRNA is stored in the midgut epithelium and remains untranslated until stimulated by a secretagogue mechanism (Noriega and Wells, 1999) as described below. Other examples are known in molecular detail. A decapeptide trypsin modulating oostatic factor (TMOF) was isolated from the ovaries of *A. aegypti*. It is an ovarian signal that terminates trypsin biosynthesis in the midgut cells after the blood has been digested and its amino acids have been utilized for egg yolk protein synthesis (Borovsky *et al.*, 1994). A TMOF-like factor was found in *Heliothis virescens* hemolymph that seems to depress trypsin synthesis at the end of each larval instar (Naven *et al.*, 2001).

The presence of food in the midgut is necessary to stimulate synthesis and secretion of digestive enzymes in some insects (Lehane *et al.*, 1996). This secretagogue mechanism is known in molecular detail only in mosquitoes. On feeding, these insects express small amounts of early trypsin, using stored early trypsin mRNA (see above). This generates free amino acids and small peptides from blood proteins. These compounds are the initial signals that induce the synthesis and secretion of large amounts of late trypsins that complete digestion (**Figure 15**) (Noriega and Wells, 1999).

The involvement of putative endocrine cells in the control of synthesis and secretion of digestive enzymes has frequently been proposed (Lehane *et al.*, 1996). In support of an endocrine role for these cells, the contents of their secretory granules have been shown immunocytochemically to share epitopes with vertebrate neuropeptides. Furthermore, feeding has been shown to cause quantitative changes in the levels of these putative peptide hormones in mosquitoes (Sehnal and Zitman, 1996). However, there is no clear evidence to show what role these putative endocrine cells play in control of midgut events.

4.5.10. Concluding Remarks

In spite of numerous gaps demanding further research, already indicated in this review, it is clear that insect digestive biochemistry is becoming a

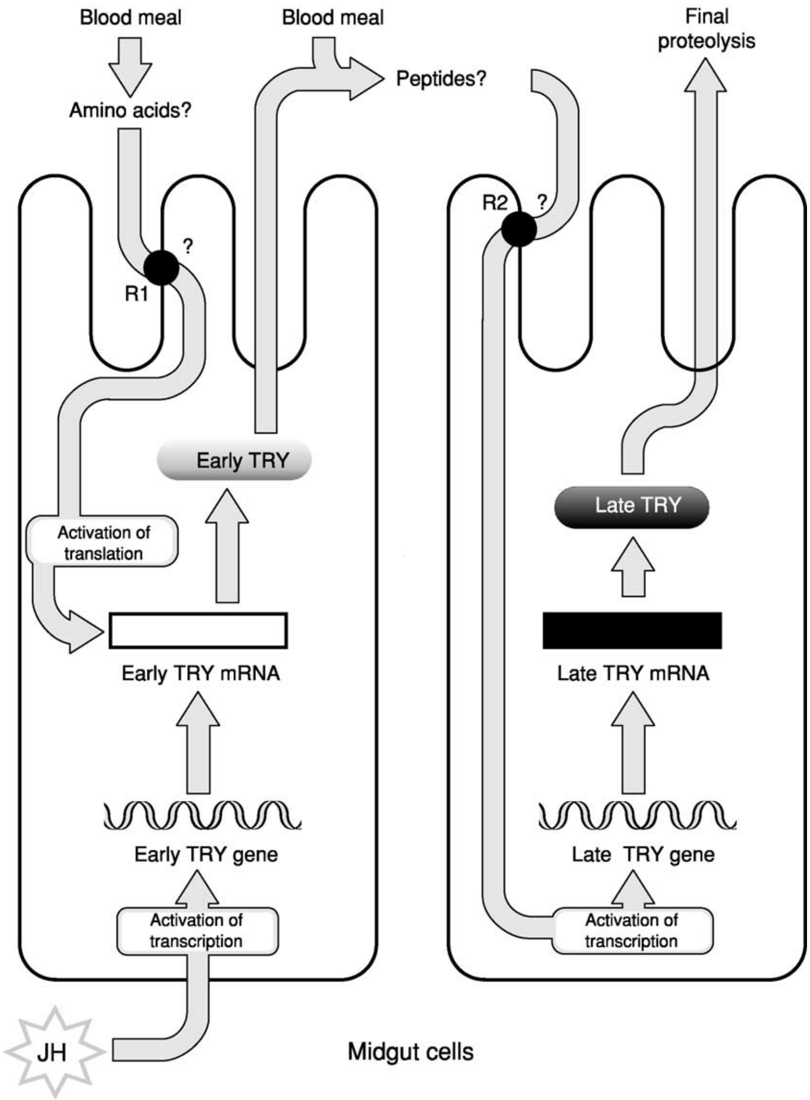


Figure 15 Model for regulation of trypsin (TRY) synthesis following a blood meal in the mosquito midgut. After emergence, juvenile hormone (JH) activates early trypsin gene transcription. Amino acids originating soon after the blood meal (unknown process) enter the cell (R1, amino acid–Na^+ symporter?) and activate early trypsin translation. Early trypsin causes limited proteolysis on blood meal proteins producing unidentified peptides. These probably bind at a receptor (R2) and somehow activate late trypsin transcription. Late trypsin carries out complete proteolysis of blood meal proteins. As digestion of the blood meal nears completion, the mRNA for late trypsin disappears from the midgut. The mechanisms underlying this phenomenon are unknown (Noriega and Wells, 1999).

developed science and that its methods are powerful enough to lead to steady progress. It is conceivable that, in the next few decades, knowledge of the structural biology and function of digestive enzymes and of the control of expression of alternate digestive enzymes and their secretory mechanisms, as well as on microvillar biochemistry, will support the development of more effective and specific methods of insect control. “Quem viver, verá”–Brazilian proverb which has a similar meaning to “Whoever is alive, will see”.

Acknowledgments

The work on our laboratory greatly benefited from the long collaboration with our friend A.F. Ribeiro (Instituto de Biociencias, University of São Paulo) on cell biology. Also instrumental for our work were the collaboration with our friends J.R.P. Parra and M.C. Silva-Filho (Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo) on insect nutrition and insect–plant interactions, respectively. We are indebted to Drs. J.E. Baker and B. Oppert

(US Department of Agriculture, Agricultural Research Station, Kansas, USA), R.J. Dillon (University of Bath, UK), and S.R. Marana (University of São Paulo) for critical review of the manuscript. We are also grateful to our former students and now colleagues and friends A.R. Lopes and P.T. Cristofoletti for help with the literature and enzymes sequence trees and to A.B. Dias for the tree of midgut characters and for preparing all drawings. Our work was supported by Brazilian research agencies Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) (Temático and SMOLBnet programs) and CNPq. The authors are staff members of the Biochemistry Department and research fellows of CNPq.

References

Allen, A., **1983**. Mucus: a protective secretion of complexity. *Trends Biochem. Sci. 8*, 169–173.

Alves, L.C., Almeida, P.C., Franzoni, L., Juliano, L., Juliano, M.A., **1996**. Synthesis of *N*-α-protected aminoacyl 7-amino-4-methyl-coumarin amide by phosphorus oxychloride and preparation of specific fluorogenic substrates for papain. *Peptide Res. 9*, 92–96.

Andersen, P.C., Brodbeck, B.V., Mizell, R.F., **1989**. Metabolism of aminoacids, organic acids and sugars extracted from the xylem fluid of four host plants by adult *Homalodisca coagulata*. *Entomol. Exp. Appl. 50*, 149–159.

Appel, H.M., **1994**. The chewing herbivore gut lumen: physicochemical conditions and their impact on plant nutrients, allelochemicals, and insect pathogens. In: Bernays, E.A. (Ed.), Insect–Plant Interactions, vol. 5. CRC Press, Boca Raton, FL, pp. 203–223.

Appel, H.M., Martin, M.M., **1990**. Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol. 16*, 3277–3290.

Applebaum, S.W., **1985**. Biochemistry of digestion. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 4. Pergamon, Oxford, pp. 279–312.

Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S., Kramer, K.J., **2003**. Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem. Mol. Biol. 33*, 631–648.

Ashford, D.A., Smith, W.A., Douglas, A.E., **2000**. Living on a high sugar diet: the fate of sucrose ingested by a phloem-feeding insect, the pea aphid *Acyrthosiphon pisum*. *J. Insect Physiol. 46*, 335–341.

Azevedo, T.M., Terra, W.R., Ferreira, C., **2003**. Purification and characterization of three β-glycosidases from midgut of the sugar cane borer, *Diathraea saccharalis*. *Insect Biochem. Mol. Biol. 33*, 81–92.

Bacic, A., Harris, P.J., Stone, B.A., **1988**. Structure and function of plant cell walls. In: Stumpf, P.K., Conn, E.E. (Eds.), The Biochemistry of Plants, vol. 14. Academic Press, New York, pp. 297–371.

Baker, J.E., Woo, S.M., **1981**. Properties and specificities of a digestive aminopeptidase from larvae of *Attagenus megatoma* (Coleoptera: Dermestidae). *Comp. Biochem. Physiol. B 69*, 189–193.

Baker, J.E., Woo, S.M., Byrd, R.V., **1984**. Ultrastructure of the gut of *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) with notes on distribution of proteinases and amylases in crop and midgut. *Can. J. Zool. 62*, 1251–1259.

Barbehenn, R.V., **1999**. Non-absorption of ingested lipophilic and amphiphilic allelochemicals by generalist grasshoppers: the role of extractive ultrafiltration by the peritrophic envelope. *Arch. Insect Biochem. Physiol. 42*, 130–137.

Barbehenn, R.V., Martin, M.M., **1995**. Peritrophic envelope permeability in herbivorous insects. *J. Insect Physiol. 4*, 303–311.

Barbehenn, R.V., Martin, M.M., **1998**. Formation of insoluble and colloidally dispersed tannic acid complexes in the midgut fluid of *Manduca sexta* (Lepidoptera: Sphingidae): an explanation for the failure of tannic acid to cross the peritrophic envelopes of Lepidopteran larvae. *Arch. Insect Biochem. Physiol. 39*, 109–117.

Barbehenn, R.V., Poopat, U., Spencer, B., **2003**. Semiquinone and ascorbyl radicals in the gut fluids of caterpillars with EPR spectrometry. *Insect Biochem. Mol. Biol. 33*, 125–130.

Barillas-Mury, C., Graf, R., Hagedorn, H.H., Wells, M.A., **1993**. cDNA and deduced amino acid sequence of a blood meal-induced trypsin from the mosquito, *Aedes aegypti*. *Insect Biochem. 2*, 7–12.

Barrett, A.J., Rawlings, N.D., Wasner, J.F., **1998**. Handbook of Proteolytic Enzymes. Academic Press, London.

Bayon, C., **1981**. Ultrastrucuture de l'épithelium intestinal et flore pariétale chez la larve xylophage d' *Oryctes nasicornis* L. (Coleoptera: Scarabaeidae). *Int. J. Insect Morphol. Embryol. 10*, 359–371.

Berenbaum, M., **1980**. Adaptive significance of midgut pH in larval Lepidoptera. *Am. Natural 115*, 138–146.

Bernays, E.A., Chamberlain, D.J., **1980**. A study of tolerance of ingested tannin in *Schistocerca gregaria*. *J. Insect Physiol. 26*, 415–420.

Bernays, E.A., Chamberlain, D.J., Leather, E.M., **1981**. Tolerance of acridids to ingested condensed tannin. *J. Chem. Ecol. 7*, 247–256.

Bieth, J., Spiess, B., Wermuth, C.G., **1974**. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem. Med. 11*, 350–357.

Biggs, D.R., McGregor, P.G., **1996**. Gut pH and amylase and protease activity PM larvae of the New Zealand grass grub (*Costelytra zealandica*; Coleoptera: Scarabaeidae) as a basis for selecting inhibitors. *Insect Biochem. Mol. Biol. 26*, 69–75.

Bignell, D.E., **1981**. Nutrition and digestion. In: Bell, W.J., Adiyodi, N.G. (Eds.), The American Cockroach. Chapman and Hall, London, pp. 57–86.

Billingsley, P.F., **1990**. The midgut ultrastructure of hematophagous insects. *Annu. Rev. Entomol. 35*, 219–248.

Blake, J.D., Murphy, P.T., Richards, G.N., **1971**. Isolation and A/B classification of hemicelluloses. *Carbohydr. Res. 16*, 49–57.

Blanco-Labra, A., Martinez-Gallardo, N.A., Sandoval-Cardoso, L., Delano-Frier, J., **1996**. Purification and characterization of a digestive cathepsin D proteinase isolated from *Tribolium castaneum* larvae (Herbst). *Insect Biochem. Mol. Biol. 26*, 95–100.

Bolognesi, R., Ribeiro, A.F., Terra, W.R., Ferreira, C., **2001**. The peritrophic membrane of *Spodoptera frugiperda*: secretion of peritrophins and role in immobilization and recycling digestive enzymes. *Arch. Insect Biochem. Physiol. 47*, 62–75.

Borhegyi, N.H., Molnár, K., Csikós, G., Sass, M., **1999**. Isolation and characterization of an apically sorted 41-kDa protein from the midgut of tobacco hornworm (*Manduca sexta*). *Cell Tissue Res. 297*, 513–525.

Borovsky, D., Powell, C.A., Nayar, J.K., Blalock, J.E., Hayes, T.K., **1994**. Characterization and localization of mosquito-gut receptors for trypsin modulating oostatic factor using a complementary peptide and immunocytochemistry. *FASEB J. 8*, 350–355.

Botos, I., Meyer, E., Nguyen, M., Swanson, S.M., Koomen, J.M., *et al.*, **2000**. The structure of an insect chymotrypsin. *J. Mol. Biol. 298*, 895–901.

Bown, D.P., Wilkinson, H.S., Gatehouse, J.A., **1997**. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. *Insect Biochem. Mol. Biol. 27*, 625–638.

Bown, D.P., Wilkinson, H.S., Gatehouse, J.A., **1998**. Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidopetra: Noctuidae) larvae: enzyme characterization, cDNA cloning and expression. *Insect Biochem. Mol. Biol. 28*, 739–749.

Brito, L.O., Lopes, A.R., Parra, J.R.P., Terra, W.R., Silva-Filho, M.C., **2001**. Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitor may be mediated by the synthesis of new proteinases. *Comp. Biochem. Physiol. B 128*, 365–375.

Broadway, P., **1997**. Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. *J. Insect Physiol. 43*, 855–874.

Brune, A., **1998**. Termite guts: the world's smallest bioreactors. *Trends Biotechnol. 16*, 16–21.

Campbell, B.C., Bragg, T.S., Turner, C.E., **1992**. Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16s ribosomal DNA. *Insect Biochem. Mol. Biol. 22*, 415–421.

Casu, R.E., Jarmey, J.M., Elvin, C.M., Eisemann, C.H., **1994**. Isolation of a trypsin-like serine protease gene family from the sheep blowfly *Lucilia cuprina*. *Insect Mol. Biol. 3*, 159–170.

Chang, W.X.Z., Gahan, L.J., Tabashnik, B.E., Heckel, D.G., **1999**. A new aminopeptidase from diamondback moth provides evidence for a gene duplication event in Lepidoptera. *Insect Mol. Biol. 8*, 171–177.

Chararas, C., Eberhard, R., Courtois, J.E., Petek, F., **1983**. Purification of three cellulases from the xylophagous larvae of *Ergates faber* (Coleoptera: Cerambycidae). *Insect Biochem. 13*, 213–218.

Charlab, R., Valenzuela, J.G., Rowton, E.D., Ribeiro, J.M., **1999**. Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly *Lutzomyia longipalpis*. *Proc. Natl Acad. Sci. USA 96*, 15155–15160.

Christeller, J.T., **1996**. Degradation of wool by *Hofmannophila pseudospretella* (Lepidoptera: Oecophoridae) larval midgut extracts under condition simulating the midgut environment. *Arch. Insect Biochem. Physiol. 33*, 99–119.

Christeller, J.T., Laing, W.A., Shaw, B.D., Burgess, E.P.J., **1990**. Characterization and partial purification of the digestive proteases of the black field cricket, *Teleogryllus commodus* (Walker): elastase is a major component. *Insect Biochem. 20*, 157–164.

Cioffi, M., **1979**. The morphology and fine structure of the larval midgut of a moth (*Manduca sexta*) in relation to active ion transport. *Tissue Cell 11*, 467–479.

Clark, T.M., **1999**. Evolution and adaptive significance of larval midgut alkalinization in the insect superorder Mecopterida. *J. Chem. Ecol. 25*, 1945–1960.

Colebatch, G., Cooper, P., East, P., **2002**. cDNA cloning of a salivary chymotrypsin-like protease and the identification of a six additional cDNAs encoding putative digestive proteases from the green mirid, *Creontiades dilutus* (Hemiptera: Miridae). *Insect Biochem. Mol. Biol. 32*, 1065–1075.

Coutinho, P.M., Henrissat, B., **1999**. Carbohydrate-active enzymes server. http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html.

Craik, C.S., Largman, C., Fletcher, T., Roczmiak, S., Barr, P.J., *et al.*, **1985**. Redesigning trypsin: alteration of substrate specificity. *Science 228*, 291–297.

Cristofoletti, P.T., Ribeiro, A.F., Deraison, C., Rahbé, Y., Terra, W.R., **2003**. Midgut adaptation and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrthosiphon pisum*. *J. Insect Physiol. 49*, 11–24.

Cristofoletti, P.T., Ribeiro, A.F., Terra, W.R., **2001**. Apocrine secretion of amylase and exocytosis of trypsin along the midgut of *Tenebrio molitor*. *J. Insect Physiol. 47*, 143–155.

Cristofoletti, P.T., Terra, W.R., **1999**. Specificity, anchoring, and subsites in the active center of a microvillar aminopeptidase purified from *Tenebrio molitor* (Coleoptera) midgut cells. *Insect Biochem. Mol. Biol. 29*, 807–819.

Cristofoletti, P.T., Terra, W.R., **2000**. The role of amino acid residues in the active site of a midgut microvillar aminopeptidase from the beetle *Tenebrio molitor*. *Biochim. Biophys. Acta 1479*, 185–195.

Daffre, S., Kylsten, P., Samakovlis, C., Hultmark, D., **1994**. The lysozyme locus in *Drosophila melanogaster*:

an expanded gene family adapted for expression in the digestive tract. *Mol. Gen. Genet. 242*, 152–162.

Da Lage, J.L., Von Wormhoudt, A., Cariou, M.L., **2002**. Diversity and evolution of the alpha-amylase genes in animals. *Biologia 57*, 181–189.

D'Amico, S., Gerday, C., Feller, G., **2000**. Structural similarities and evolutionary relationships in chloride-dependent α-amylases. *Gene 253*, 95–105.

Darboux, I., Nielsen-LeRoux, C., Charles, J.F., Pauron, D., **2001**. The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: molecular cloning and expression. *Insect Biochem. Mol. Biol. 31*, 981–990.

Davies, G.J., Wilson, K.S., Henrisat, B., **1997**. Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J. 321*, 557–559.

Davis, C.A., Riddell, D.C., Higgins, M.J., Holden, J.J., White, B.N., **1985**. A gene family in *Drosophila melanogaster* coding for trypsin-like enzymes. *Nucleic Acids Res. 13*, 6605–6619.

de Almeida, R.W., Tovar, F.J., Ferreira, I.I., Leoncini, O., **2003**. Chymotrypsin genes in the malaria mosquitoes, *Anopheles aquasalis* and *Anopheles darlingi*. *Insect Biochem. Mol. Biol. 33*, 307–315.

Denolf, P., Hendrickx, K., Van Damme, J., Jansens, S., Peferoen, M., *et al.*, **1997**. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. *Eur. J. Biochem. 248*, 748–761.

De Veau, E.J.I., Schultz, J.C., **1992**. Reassessment of interaction between gut detergents and tannins in Lepidoptera and significance for gypsy moth larvae. *J. Chem. Ecol. 18*, 1437–1453.

Dey, P.M., Pridham, J.B., **1972**. Biochemistry of α-galactosidases. *Adv. Enzymol. 36*, 91–130.

Dillon, R.J., Dillon, V.M., **2004**. The insect gut bacteria: an overview. *Annu. Rev. Entomol. 49*, 71–92.

Dootsdar, H., McCollum, T.G., Mayer, R.T., **1997**. Purification and characterization of an endo-polygalacturonase from the gut of West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* L.) larvae. *Comp. Biochem. Physiol. B 118*, 861–867.

Dow, J.A.T., **1986**. Insect midgut function. *Adv. Insect Physiol. 19*, 187–328.

Dow, J.A.T., **1992**. pH gradients in lepidopteran midgut. *J. Exp. Biol. 172*, 355–375.

Dunn, B.M., Jimenez, M., Parten, B.F., Valler, M.J., Rolph, C.E., *et al.*, **1986**. A systematic series of synthetic chromophoric substrates for aspartic proteinases. *Biochem. J. 237*, 899–906.

Edwards, M.J., Jacobs-Lorena, M., **2000**. Permeability and disruption of the peritrophic matrix and caecal membrane from *Aedes aegypti* and *Anopheles gambiae* mosquito larvae. *J. Insect Physiol. 46*, 1313–1320.

Edwards, M.J., Lemos, F.J.A., Donnelly-Doman, M., Jacobs-Lorena, M., **1997**. Rapid induction by a blood meal of a carboxypeptidase gene in the gut of the mosquito *Anopheles gambiae*. *Insect Biochem. Mol. Biol. 27*, 1063–1072.

Eguchi, M., **1995**. Alkaline phosphatase isozymes in insects and comparison with mammalian enzyme. *Comp. Biochem. Physiol. B 111*, 151–162.

Eisemann, C., Wijffels, G., Tellam, R.L., **2001**. Secretion of type 2 peritrophic matrix protein, peritrophin-15, from the cardia. *Arch. Insect Biochem. Physiol. 472*, 76–85.

Elpidina, E.N., Vinokurov, K.S., Gromenko, V.A., Rudenskoya, Y.A., Dunaevsky, Y.E., *et al.*, **2001a**. Compartmentalization of proteinases and amylases in *Nauphoeta cinerea* midgut. *Arch. Insect Biochem. Physiol. 48*, 206–216.

Elpidina, E.N., Vinokurov, K.S., Rudenskaya, Y.A., Dunoevsky, Y.E., Zhuzhikov, D.P., **2001b**. Proteinase inhibitors in *Nauphoeta cinerea* midgut. *Arch. Insect Biochem. Physiol. 48*, 217–222.

Emmerling, M., Chandler, D., Sandeman, M., **2001**. Molecular cloning of three cDNAs encoding aminopeptidases from the midgut of *Helicoverpa punctigera*, the Australian native budworm. *Insect Biochem. Mol. Biol. 31*, 899–907.

Espinoza-Fuentes, F.P., Ferreira, C., Terra, W.R., **1984**. Spatial organization of digestion in the larval and imaginal stages of the sciarid fly *Trichosia pubescens*. *Insect Biochem. 14*, 631–638.

Estébanez-Perpiñá, E., Bayés, A., Vendrell, J., Jongsma, M.A., Bown, D.P., *et al.*, **2001**. Crystal structure of a novel mid-gut procarboxypeptidase from the cotton pest *Helicoverpa armigera*. *J. Mol. Biol. 313*, 629–638.

Ferreira, A.H.P., Marana, S.R., Terra, W.R., Ferreira, C., **2001**. Purification, molecular cloning, and properties of a β-glycosidase isolated from midgut lumen of *Tenebrio molitor* (Coleoptera) larvae. *Insect Biochem. Mol. Biol. 31*, 1065–1076.

Ferreira, A.H.P., Ribeiro, A.F., Terra, W.R., Ferreira, C., **2002**. Secretion of β-glycosidase by middle midgut cells and its recycling in the midgut of *Tenebrio molitor* larvae. *J. Insect Physiol. 48*, 113–118.

Ferreira, A.H.P., Terra, W.R., Ferreira, C., **2003**. Characteization of a β-glycosidase highly active on disaccharides and of a β-galactosidase from *Tenebrio molitor* midgut lumen. *Insect Biochem. Mol. Biol. 33*, 253–265.

Ferreira, C., Capella, A.N., Sitnik, R., Terra, W.R., **1994a**. Properties of the digestive enzymes and the permeability of the peritrophic membrane of *Spodoptera frugiperda* (Lepidoptera) larvae. *Comp. Biochem. Physiol. A 107*, 631–640.

Ferreira, C., Capella, A.N., Sitnik, R., Terra, W.R., **1994b**. Digestive enzymes in midgut cells, endo- and ectoperitrophic contents and peritrophic membranes of *Spodoptera frugiperda* (Lepidoptera) larvae. *Arch. Insect Biochem. Physiol. 26*, 299–313.

Ferreira, C., Parra, J.R.P., Terra, W.R., **1997**. The effect of dietary plant glycosides on larval midgut β-glycosidases from *Spodoptera frugiperda* and *Diatraea saccharalis*. *Insect Biochem. Mol. Biol. 27*, 55–59.

Ferreira, C., Terra, W.R., **1983**. Physical and kinetic properties of a plasma membrane bound β-D-glucosidase (cellobiase) from midgut cells of an insect (*Rhynchosciara americana* larva). *Biochem. J. 213*, 43–51.

Ferreira, C., Terra, W.R., **1984**. Soluble aminopeptidases from cytosol and luminal contents of *Rhynchosciara americana* midgut caeca: properties and phenanthroline inhibition. *Insect Biochem. 14*, 145–150.

Ferreira, C., Terra, W.R., **1985**. Minor aminopeptidases purified from the plasma membrane of midgut caeca cells of an insect (*Rhynchosciara americana*) larva. *Insect Biochem. 15*, 619–625.

Ferreira, C., Terra, W.R., **1986a**. The detergent form of the major aminopeptidase from the plasma membrane of midgut caeca cells of *Rhynchosciara americana* (Diptera) larva. *Comp. Biochem. Physiol. B 84*, 373–376.

Ferreira, C., Terra, W.R., **1986b**. Substrate specificity and binding loci for inhibitors in an aminopeptidase purified from the plasma membrane of midgut cells of an insect (*Rhynchosciara americana*) larva. *Arch. Biochem. Biophys. 244*, 478–485.

Ferreira, C., Terra, W.R., **1989**. Spatial organization of digestion, secretory mechanisms and digestive enzyme properties in *Pheropsophus aequinoctialis* (Coleoptera: Carabidae). *Insect Biochem. 19*, 383–391.

Ferreira, C., Torres, B.B., Terra, W.R., **1998**. Substrate specificities of midgut β-glycosidases from insects of different orders. *Comp. Biochem. Physiol. B 119*, 219–225.

Ferreira, C., Marana, S.R., Silva, C., Terra, W.R., **1999**. Properties of digestive glycosidases and peptidases and the permeability of the peritrophic membranes of *Abracris flavolineata* (Orthoptera : Acrididae). *Comp. Biochem. Physiol. B 123*, 241–250.

Forstner, G.G., Forstner, J.F., **1986**. Structure and function of the gastrointestinal fluid. In: Desnuelle, P., Sjostrom, H., Noren, O. (Eds.), Molecular and Cellular Basis of Digestion. Elsevier, Amsterdam, pp. 125–143.

Fox, L.R., Macauley, B.J., **1977**. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia 29*, 145–162.

Franco, O.L., Rigden, D.J., Melo, F.R., Grossi-de-Sá, M.F., **2002**. Plant α-amylase inhibitors and their interaction with insect α-amylases: structure, function and potential for crop protection. *Eur. J. Biochem. 269*, 397–412.

Fujita, A., Minamoto, T., Shimizu, I., Abe, T., **2002**. Molecular cloning of lysozyme-encoding cDNAs expressed in the salivary gland of a wood-feeding termite, *Reticulitermes speratus*. *Insect Biochem. Mol. Biol. 32*, 1615–1624.

Fujita, A., Shimizu, I., Abe, T., **2001**. Distribution of lysozyme and protease, and amino acid concentration in guts of a wood-feeding termite, *Reticulitermes speratus* (Kolbe): possible digestion of symbiont bacteria transferred by trophallaxis. *Physiol. Entomol. 26*, 116–123.

Gaines, P.J., Sampson, C.M., Rushlow, K.E., Stiegler, G.L., **1999**. Cloning of a family of serine protease genes from the cat flea *Ctenocephalides felis*. *Insect Mol. Biol. 81*, 11–22.

Garner, K.J., Hiremath, S., Lehtoma, K., Valaitis, A.P., **1999**. Cloning and complete sequence characterization of two gypsy moth aminopeptidase-N cDNAs, including the receptor for *Bacillus thuringiensis* Cry 1Ac toxin. *Insect Biochem. Mol. Biol. 29*, 527–535.

Gatehouse, L.N., Shannon, A.L., Burgess, E.P.J., Christeller, J.T., **1997**. Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in response to four proteinase inhibitors in the diet. *Insect Biochem. Mol. Biol. 27*, 929–944.

Genta, F.A., Terra, W.R., Ferreira, C., **2003**. Action pattern, specifity, lytic activities, and physiological role of five digestive β-glucanases isolated from *Periplaneta americana*. *Insect Biochem. Mol. Biol. 33*, 1085–1097.

Gill, S.S., Cowles, E., Francis, V., **1995**. Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry 1ac-toxin binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem. 270*, 27277–27282.

Girard, C., Jouanin, L., **1999a**. Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol. 29*, 1129–1142.

Girard, G., Jouanin, L., **1999b**. Molecular cloning of a gut-specific chitinase cDNA from the beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol. 29*, 549–556.

Giri, A.P., Harsulkar, A.M., Deshpande, V.V., Sainani, M.N., Gupta, V.S., *et al.*, **1998**. Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. *Plant Physiol. 116*, 393–401.

Gonzales, M.S., Nogueira, N.F.S., Feder, D., de Souza, W., Azambuja, P., *et al.*, **1998**. Role of the head in the ultrastructural midgut organization in *Rhodnius prolixus* larvae: evidence from head transplantation experiments and acdysone therapy. *J. Insect Physiol. 44*, 553–560.

Graf, R., Raikhel, A.S., Brown, M.R., Lea, A.O., Briegel, H., **1986**. Mosquito trypsin: immunocytochemical localization in the midgut of blood-fed *Aedes aegyti* (L.). *Cell Tissue Res. 245*, 19–27.

Greenberg, B., Paretsky, D., **1955**. Proteolytic enzymes in the housefly, *Musca domestica* (L.). *Ann. Entomol. Soc. America 48*, 46–50.

Grossmann, G.A., Terra, W.R., **2001**. Alpha-galactosidases from the larval midgut of *Tenebrio molitor* (Coleoptera) and *Spodoptera frugiperda* (Lepidoptera). *Comp. Biochem. Physiol. B 128*, 109–122.

Grossmann, G.L., Campos, Y., Senerson, D.W., James, A.A., **1997**. Evidence for two distinct members of the amylase gene family in the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 27*, 769–781.

Harper, M.S., Hopkins, T.L., **1997**. Peritrophic membrane structure and secretion in European corn borer larvae (*Ostrinia nubilalis*). *Tissue Cell 29*, 461–475.

Harwood, J.L., **1980**. Plant acyl lipids: structure, distribution and analysis. In: Stumpf, P.K. (Ed.), The Biochemistry of Plants: A Comprehensive Treatise, vol. 4, Lipids: Structure and Function. Academic Press, New York, pp. 1–56.

Harzer, K., Hiraiwa, M., Paton, B.C., **2001**. Saposins (sap) A and C activate the degradation of galactosylsphingosine. *FEBS Lett. 508*, 107–110.

Heinrich, D., Zebe, E., **1973**. Zur Feinstruktur der Mitteldarmzellen von *Locusta migratoria* in verschiedenen Phasen der Verdauung. *Cytobiologie 7*, 315–326.

Hooper, N.M., **1994**. Families of zinc metalloproteases. *FEBS Lett. 354*, 1–6.

Hori, K., **1972**. Comparative study of a property of salivary amylase among various heteropterous insects. *Comp. Biochem. Physiol. B 42*, 501–508.

House, H.L., **1974**. Digestion. In: Rockstein, M. (Ed.), The Physiology of Insecta, 2nd edn., vol. 5. Academic Press, New York, pp. 63–117.

Hua, G., Tsukamoto, K., Ikezawa, H., **1998**. Cloning and sequence analysis of the aminopeptidase N isozyme (APN2) from *Bombyx mori* midgut. *Comp. Biochem. Physiol. B 121*, 213–232.

Hultmark, D., **1996**. Insect lysozymes. In: Jollés, P. (Ed.), Lysozymes: Model Enzymes in Biochemistry and Biology. Birkhäuser Verlag, Basel, pp. 87–102.

Human, H., Nicolson, S.W., **2003**. Digestion of maize and sunflower pollen by the spotted maize *Astylus atromaculatus* (Melyridae): is there a role for osmotic shock? *J. Insect Physiol. 49*, 633–643.

Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C., Ruppley, J.A., **1972**. Vertebrate lysozymes. In: Boyer, P.D. (Ed.), The Enzymes, vol. 7. Academic Press, New York, pp. 665–868.

Ito, Y., Nakamura, M., Hotani, T., Imoto, T., **1995**. Insect lysozyme from house fly (*Musca domestica*) larvae: possible digestive function based on sequence and enzymatic properties. *J. Biochem. Tokyo 118*, 546–551.

Itoh, M., Kanamori, Y., Takao, M., Eguchi, M., **1999**. Cloning of soluble alkaline phosphatase cDNA and molecular basis of the polymorphic nature in alkaline phosphatases isozymes of *Bombyx mori* midgut. *Insect Biochem. Mol. Biol. 29*, 121–129.

Itoh, M., Takeda, S., Yamamoto, H., Izumi, S., Tomino, S., *et al.*, **1991**. Cloning and sequence analysis of membrane-bound alkaline phosphatase cDNA of the silkworm, *Bombyx mori*. *Biochim. Biophys. Acta 1129*, 135–138.

Ivanova, N., Sorokin, A., Anderson, L., Galleron, N., Candellon, B., *et al.*, **2003**. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature 423*, 87–91.

James, A.A., Blackmer, K., Racioppi, J.V., **1989**. A salivary gland-specific, maltase-like gene of the vector mosquito, *Aedes aegypti*. *Gene 75*, 73–83.

Janecek, S., **1997**. Alpha-amylase family: molecular biology and evolution. *Progr. Biophys. Mol. Biol. 67*, 67–97.

Jany, K.D., Bekeler, K., Pfleiderer, G., Ishay, J., **1983**. Amino acid sequence of an insect chymotrypsin from the larvae of the hornet, *Vespa orientalis*. *Biochem. Biophys. Res. Commun. 110*, 1–7.

Jimenez, D.R., Gilliam, M., **1990**. Ultrastructure of ventriculus of the honey bee *Apis mellifera* (L): cytochemical localization of the acid phosphatase, alkaline phosphatase, and non-specific esterase. *Cell Tissue Res. 261*, 431–443.

Johnson, K.S., Felton, G.W., **2000**. Digestive proteinase activity in corn earworm (*Helicoverpa zea*) after molting and in response to lowered redox potential. *Arch. Insect Biochem. Physiol. 44*, 151–161.

Johnson, K.S., Rabosky, D., **2000**. Phylogenetic distribution of cysteine proteinases in beetles: evidence for an evolutionary shift to an alkaline digestive strategy in Cerambycidae. *Comp. Biochem. Physiol. B 126*, 609–619.

Jordão, B.P., Capella, A.N., Terra, W.R., Ribeiro, A.F., Ferreira, C., **1999**. Nature of the anchors of membrane-bound aminopeptidase, amylase, and trypsin and secretory mechanisms in *Spodoptera frugiperda* (Lepidoptera) midgut cells. *J. Insect Physiol. 45*, 29–37.

Jordão, B.P., Lehane, M.J., Terra, W.R., Ribeiro, A.F., Ferreira, C., **1996a**. An immunocytochemical investigation of trypsin secretion in the midgut of the stablefly *Stomoxys calcitrans*. *Insect Biochem. Mol. Biol. 26*, 445–453.

Jordão, B.P., Terra, W.R., Ribeiro, A.F., Lehane, M.J., Ferreira, C., **1996b**. Trypsin secretion in *Musca domestica* larval midguts: a biochemical and immunocytochemical study. *Insect Biochem. Mol. Biol. 26*, 337–346.

Khadeni, S., Guarino, L.A., Watenabe, H., Tokuda, G., Meyer, E.F., **2002**. Structure of an endoglucanase from termite, *Nasutitermes takasagoensis*. *Acta Cryst. D 58*, 653–658.

Kleywegt, G.J., Zou, J.Y., Divne, C., Davies, G.J., Sinning, I., *et al.*, **1997**. The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 angstrom resolution, and a comparison with related enzymes. *J. Mol. Biol. 272*, 383–397.

Klinkowstrom, A.M., Terra, W.R., Ferreira, C., **1994**. Aminopeptidase A from *Rhynchosciara americana* (Diptera) larval midguts: properties and midgut distribution. *Arch. Insect Biochem. Physiol. 27*, 301–305.

Klinkowstrom, A.M., Terra, W.R., Ferreira, C., **1995**. Midgut dipeptidases from *Rhynchosciara americana* (Diptera) larvae: properties of soluble and membrane bound forms. *Insect Biochem. Mol. Biol. 25*, 303–310.

Knight, P.J.K., Knowles, B.H., Eller, D.J., **1995**. Molecular cloning of an insect aminopeptidase N that serves as a receptor of *Bacillus thuringiensis* Cry 1a (c) toxin. *J. Biol. Chem. 270*, 17765–17770.

Koepke, J., Ermler, V., Warkentin, E., Wenzi, G., Flecker, P., **2000**. Crystal structure of cancer chemopreventive Bowman–Birk inhibition in ternary complex with bovine trypsin at 2–3 Å resolution: structural basis of

Janus-faced serine protease inhibitor specificity. *J. Mol. Biol. 298*, 477–491.

Koiwa, H., Shade, R.E., Zhu-Salzman, K., D'Urzo, M.P., Murdock, L.L., *et al.*, **2000**. A plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases (DuCALs) in the larval midgut of the western corn rootworm (*Diabrotica virgifera virgifera*). *FEBS Lett. 471*, 67–70.

Konno, K., Okada, S., Hirayama, C., **2001**. Selective secretion of free glycine, a neutralizer against a plant defense chemical, in the digestive juice of the privet moth larvae. *J. Insect Physiol. 47*, 1451–1457.

Kramer, K.J., Corpuz, L., Choi, H., Muthukrishnan, S., **1993**. Sequence of a cDNA and expression of the genes encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem. Mol. Biol. 23*, 691–701.

Kramer, K.J., Koga, D., **1986**. Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem. 16*, 851–877.

Kylsten, P., Kimbrell, D.A., Daffre, S., Samakovlis, C., Hultmark, D., **1992**. The lysozyme locus in *Drosophila melanogaster*: different genes are expressed in midgut and salivary glands. *Mol. Gen. Genet. 232*, 335–343.

Le Cahérec, F., Guillam, M.T., Beuron, F., Cavalier, A., Thomas, D., *et al.*, **1997**. Aquaporin-related proteins in the filter chamber of homopteran insects. *Cell Tissue Res. 290*, 143–151.

Lee, J.H., Tsuji, M., Nakamura, M., Nishimoto, M., Okuyama, M., *et al.*, **2001**. Purification and identification of the essential ionizable groups of honeybee, *Apis mellifera* L., trehalase. *Biosci. Biotechnol. Biochem. 65*, 2657–2665.

Lee, M.J., Anstee, J.H., **1995**. Endoproteases from the midgut of *Spodoptera littoralis* include a chymotrypsin-like enzyme with an extended binding site. *Insect Biochem. Mol. Biol. 25*, 49–61.

Lehane, M.J., **1997**. Peritrophic matrix structure and function. *Annu. Rev. Entomol. 42*, 525–550.

Lehane, M.J., Müller, H.M., Crisanti, A., **1996**. Mechanisms controlling the synthesis and secretion of digestive enzymes in insects. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, pp. 195–205.

Lemos, F.J.A., Ribeiro, A.F., Terra, W.R., **1993**. A bacteria-digesting midgut lysozyme from *Musca domestica* (Diptera) larvae: purification, properties and secretory mechanism. *Insect Biochem. Mol. Biol. 23*, 533–541.

Lemos, F.J.A., Terra, W.R., **1991a**. Digestion of bacteria and the role midgut lysozyme in some insect larvae. *Comp. Biochem. Physiol. B 100*, 265–268.

Lemos, F.J.A., Terra, W.R., **1991b**. Properties and intracellular distribution of a cathepsin D-like proteinase active at the acid region of *Musca domestica* midgut. *Insect Biochem. 21*, 457–465.

Levitzki, A., Steer, M.L., **1974**. The allosteric activation of mammalian α-amylase by chloride. *Eur. J. Biochem. 41*, 171–180.

Liadouze, I., Febvay, G., Guillaud, J., Bonnot, G., **1995**. Effect of diet on the free amino acid pools of symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. *J. Insect Physiol. 41*, 33–40.

Lin, G., Bode, W., Huber, R., Chi, C., Engh, R.A., **1993**. The 0.25-nm X-ray structure of Bowman–Birk-type inhibitor from mung bean in ternary complex with porcine trypsin. *Eur. J. Biochem. 212*, 549–555.

Linder, M., Teeri, T.T., **1997**. The roles and function of cellulose-binding domains. *J. Biotechnol. 57*, 15–28.

Lo, N., Tokuda, G., Watanabe, H., Rose, H., Slaytor, M., *et al.*, **2000**. Evidence from multiple gene sequences indicates that termites evolved from wood-feeding cockroaches. *Current Biol. 10*, 801–804.

Lopes, A.R., Juliano, M.A., Juliano, L., Terra, W.R., **2004**. Coevolution of insect trypsins and inhibitors. *Arch. Insect Biochem. Physiol. 55*, 140–152.

Lu, Y., Zen, K.-C., Muthukrishnan, S., Kramer, K.J., **2002**. Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase. *Insect Biochem. Mol. Biol. 32*, 1369–1382.

Luciani, V., Marie-Claire, C., Ruffet, E., Beaumont, A., Roques, B.P., *et al.*, **1998**. Characterization of Glu^{350} as a critical residue involved in the N-terminal amine binding site of aminopeptidase N (EC 3.4.11.2): insight into its mechanism of action. *Biochemistry 37*, 686–692.

Marana, S.R., Jacobs-Lorena, M., Terra, W.R., Ferreira, C., **2001**. Amino acid residues involved in substrate binding and catalysis in an insect digestive β-glycosidase. *Biochim. Biophys. Acta 1545*, 41–52.

Marana, S.R., Lopes, A.R., Juliano, L., Juliano, M.A., Ferreira, C., *et al.*, **2002a**. Subsites of trypsin active site favor catalysis or substrate binding. *Biochem. Biophys. Res. Commun. 290*, 494–497.

Marana, S.R., Ribeiro, A.F., Terra, W.R., Ferreira, C., **1997**. Ultrastructure and secretory activity of *Abracris flavolineata* (Orthoptera: Acrididae) midguts. *J. Insect Physiol. 43*, 465–473.

Marana, S.R., Terra, W.R., Ferreira, C., **1995**. Midgut β-D-glucosidases from *Abracris flavolineata* (Orthoptera: Acrididae): physical properties, substrate specificities and function. *Insect Biochem. Mol. Biol. 25*, 835–843.

Marana, S.R., Terra, W.R., Ferreira, C., **2000**. Purification and properties of a β-glycosidase purified from midgut cells of *Spodoptera frugiperda* (Lepidoptera) larvae. *Insect Biochem. Mol. Biol. 30*, 1139–1146.

Marana, S.R., Terra, W.R., Ferreira, C., **2002b**. The role of amino acid residues Q39 and E451 in the determination of substrate specificity of the *Spodoptera frugiperda* β-glycosidase. *Eur. J. Biochem. 269*, 3705–3714.

Markovic, O., Janecek, S., **2001**. Pectin degrading glycoside hydrolases of family 28: sequence-structural features, specificities and evolution. *Protein Eng. 14*, 615–631.

Martin, M.M., **1987**. Invertebrate–Microbial Interactions: Ingested Fungal Enzymes in Anthropod Biology. Cornell University Press, Ithaca, NY.

Martin, M.M., Martin, J.S., **1984**. Surfactants: their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia 61*, 342–345.

Matoub, M., Rouland, C., **1995**. Purification and properties of the xylanases from the termite *Macrotermes bellicosus* and its symbiotic fungus *Termitomyces* sp. *Comp. Biochem. Physiol. B 112*, 629–635.

Matsumura, I., Kirsch, J.F., **1996.** Is aspartate 52 essential for catalysis by chicken egg white lysozyme? The role of natural substrate-assisted hydrolysis. *Biochemistry 35*, 1881–1889.

Mazumbar-Leighton, S., Broadway, R.M., **2001a**. Transcriptional induction of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. *Insect Biochem. Mol. Biol. 31*, 645–657.

Mazumbar-Leighton, S., Broadway, R.M., **2001b**. Identification of six chymotrypsin cDNAs from larval midgut of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. *Insect Biochem. Mol. Biol. 31*, 633–644.

McHenery, J.G., Birkbeck, T.H., Allen, S.A., **1979**. The occurrence of lysozyme in marine bivalves. *Comp. Biochem. Physiol. B 63*, 25–28.

Miller, N., Lehane, M.J., **1990**. *In vitro* perfusion studies on the peritrophic membrane of the tsetse fly *Glossina morsitans morsitans* (Diptera, Glossinidae). *J. Insect Physiol. 36*, 813–818.

Monzingo, A.F., Marcotte, E.M., Hart, P.J., Roberts, J.D., **1996**. Chitinases, chitosanases, and lysozymes can be divided into prokaryotic and eucaryotic families sharing a conserved core. *Nature Struct. Biol. 3*, 133–140.

Morgan, M.R.J., **1975**. Relationship between gut cellobiase, lactose, aryl β-glucosidase, and aryl β-galactosidase activities of *Locusta migratoria*. *Insect Biochem. 5*, 609–617.

Murdock, L.L., Brookhart, G., Dunn, P.E., Foard, D.E., Kelley, S., *et al.*, **1987**. Cysteine digestive proteinases in Coleoptera. *Comp. Biochem. Physiol. B 87*, 783–787.

Nahoum, V., Farisei, F., Lê-Berre-Anton, V., Egloff, M.P., Rougé, P., *et al.*, **1999**. A plant-seed inhibitor of two classes of α-amylases: X-ray analysis of *Tenebrio molitor* larval α-amylases in complex with the bean *Phaseolus vulgaris* inhibitor. *Acta Crystallogr. D 55*, 360–362.

Nakanishi, K., Yaoi, K., Nagino, Y., Hara, H., Kitami, M., *et al.*, **2002**. Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella*: their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry 1A toxin. *FEBS Lett. 519*, 215–220.

Nakashima, K., Watanabe, H., Saitoh, H., Tokuda, G., Azuma, J.I., **2002.** Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochem. Mol. Biol. 32*, 777–784.

Naven, R., Sorge, D., Sterner, A., Borovsky, D., **2001**. TMOF-like factor controls the biosynthesis of serine proteases in the larval gut of *Heliothis virescens*. *Arch. Insect Biochem. Physiol. 47*, 169–180.

Nielsen, J.E., Borchert, T.U., **2000**. Protein engineering of bacterial α-amylases. *Biochim. Biophys. Acta 1543*, 253–274.

Nishimoto, M., Kubota, M., Tsuji, M., Mori, H., Kimura, A., *et al.*, **2001**. Purification and substrate specificity of honeybee, *Apis mellifera* L., alpha-glucosidase III. *Biosci. Biotech. Biochem. 65*, 1610–1616.

Nor Aliza, A.R., Rana, R.L., Skoda, S.R., Berkebile, D.R., Stanley, D.W., **1999**. Tissue polyunsaturated fatty acids and a digestive phospholipase A_2 in the primary screwworm, *Cochliomya hominivorax*. *Insect Biochem. Mol. Biol. 29*, 1029–1038.

Norén, O., Sjostrom, H., Danielsen, E.M., Cowell, G.M., Skovbjerg, H., **1986**. The enzymes of the enterocyte plasma membrane. In: Desnuelle, P., Sjostrom, H., Norén, O. (Eds.), Molecular and Cellular Basis of Digestion. Elsevier, Amsterdam, pp. 355–365.

Noriega, F.G., Wells, M.A., **1999**. A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*. *J. Insect Physiol. 45*, 613–620.

Novillo, C., Castañera, P., Ortego, F., **1997**. Inhibition of digestive trypsin-like proteases from larvae of several lepidopteran species by the diagnostic cysteine protease inhibitor E-64. *Insect Biochem. Mol. Biol. 27*, 247–254.

Ohashi, K., Natori, S., Kubo, T., **1999**. Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.). *Eur. J. Biochem. 265*, 127–133.

Oltean, D.I., Pullikuth, A.K., Lee, H.K., Gill, S.S., **1999**. Partial purification and characterization of *Bacillus thuringiensis* Cry 1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. *Appl. Environ. Microbiol. 65*, 4760–4766.

Ortego, F., Novillo, C., Catañera, P., **1996**. Characterization and distribution of digestive proteases of the stalk corn borer, *Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae). *Arch. Insect Biochem. Physiol. 33*, 136–180.

Pechan, T., Cohen, A., Williams, W.P., Luthe, D.S., **2002**. Insect feeding mobilizes a unique defense protease that disrupts the peritrophic matrix of caterpillars. *Proc. Natl Acad. Sci. USA 99*, 13319–13323.

Peng, J., Zhong, J., Granados, R.R., **1999**. A baculovirus enhancin alters the permeability of a mucosal midgut peritrophic matrix from lepidopteran larvae. *J. Insect Physiol. 45*, 159–166.

Pereira, P.J.B., Lozanov, V., Patthy, A., Huber, R., Bode, W., *et al.*, **1999**. Specific inhibition of insect α-amylases: yellow mealworm α-amylase in complex with the Amaranth at 2.0 Å resolution. *Structure 7*, 1079–1088.

Peters, W., **1992**. Peritrophic Membranes. Springer, Berlin.

Peters, W., Wiese, B., **1986**. Permeability of the peritrophic membranes of some Diptera to labelled dextrans. *J. Insect Physiol. 32*, 43–49.

Peterson, A.M., Barillas-Mury, C.V., Wells, M.A., **1994**. Sequence of three cDNAs encoding an alkaline midgut trypsin from *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 463–471.

Peterson, A.M., Fernando, G.J.P., Wells, M., **1995**. Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 765–774.

Pimenta, D.C., Oliveira, A., Juliano, M.A., Juliano, L., **2001**. Substrate specificity of human cathepsin D using internally quenched fluorescent peptides derived from reactive site loop of kallistatin. *Biochim. Biophys. Acta 1544*, 113–122.

Ponsen, M.B., **1991**. Structure of the digestive system of aphids, in particular *Hyalopterus* and *Coloradoa*, and its bearing on the evolution of filter chambers in Aphidoidea. Wageningen Agricultural University Press, Wageningen 91–95, pp. 3–61.

Price, D.J.S., **1963**. Little Science, Big Science. Columbia University Press, New York.

Prosser, W.A., Douglas, A.E., **1992**. A test of the hypotheses that nitrogen is upgraded and recycled in aphid (*Acyrthosiphon pisum*) symbiosis. *J. Insect Physiol. 38*, 93–99.

Prosser, W.A., Simpson, S.J., Douglas, A.E., **1992**. How an aphid (*Acyrthosiphon pisum*) symbiosis responds to variation in dietary nitrogen. *J. Insect Physiol. 38*, 301–307.

Quicke, D.L.J., **2003**. Hymenoptera (ants, bees, wasps). In: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects. Academic Press, San Diego, CA, pp. 534–546.

Rajagopal, R., Agrawal, N., Selvapandiyan, A., Sivakumar, S., Ahmad, S., *et al.*, **2003**. Recombinantly expressed isoenzymic aminopeptidases from *Helicoverpa armigera* (American cotton bollworm) midgut display differential interaction with closely related *Bacillus thuringiensis* insecticidal proteins. *Biochem. J. 379*, 971–978.

Ramos, A., Mahowald, A., Jacobs-Lorena, M., **1993**. Gut-specific genes from the black-fly *Simulium vittatum* encoding trypsin-like and carboxypeptidase-like proteins. *Insect Mol. Biol. 1*, 149–163.

Rawlings, N.D., Barrett, A.J., **1995**. Evolutionary families of metallopeptidases. *Methods Enzymol. 248*, 183–228.

Reeck, G., Oppert, B., Denton, M., Kanost, M., Baker, J., *et al.*, **1999**. Insect proteinases. In: Turk, V. (Ed.), Proteases. Birkhäuser Verlag, Basel, pp. 125–148.

Regel, R., Matioli, S.R., Terra, W.R., **1998**. Molecular adaptation of *Drosophila melanogaster* lysozymes to a digestive function. *Insect Biochem. Mol. Biol. 28*, 309–319.

Rouland, C., Civas, A., Renoux, J., Petek, F., **1988**. Purification and properties of cellulases from the termite *Macrotermes mulleri* (Termitidae: Macrotermitinae) and its symbiotic fungus *Termitomyces* sp. *Comp. Biochem. Physiol. B 91*, 449–458.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C., Jones, T.A., **1990**. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. *Science 249*, 380–386.

Royer, V., Fraichard, S., Bouhin, H., **2002**. A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis. *Biochem. J. 366*, 921–928.

Ryan, C.A., **1990**. Proteinase inhibitors in plants: genes for improving defense against insects and pathogens. *Annu. Rev. Phytopathol. 28*, 425–449.

Ryan, R.O., Van der Horst, D.J., **2000**. Lipid transport biochemistry and its role in energy production. *Annu. Rev. Entomol. 45*, 233–260.

Sakon, J., Irwin, D., Wilson, D.B., Karplus, P.A., **1997**. Structure and mechanism of endo/exocellulase E4 from *Thermomonospora fusca*. *Nature Struct. Biol. 4*, 810–818.

Salvucci, M.E., **2003**. Distinct sucrose isomerases catalyze trehalulose synthesis in whiteflies, *Bemisia argentifolii*, and *Erwinia rhapontici*. *Comp. Biochem. Physiol. B 135*, 385–395.

Salvucci, M.E., Rosell, R.C., Brown, J.K., **1998**. Uptake and metabolism of leaf proteins by the silverleaf whitefly. *Arch. Insect Biochem. Physiol. 39*, 155–165.

Santos, C.D., Ribeiro, A.F., Terra, W.R., **1986**. Differential centrifugation, calcium precipitation and ultrasonic disruption of midgut cells of *Erinnyis ello* caterpillars: purification of cell microvilli and inferences concerning secretory mechanisms. *Can. J. Zool. 64*, 490–500.

Santos, C.D., Terra, W.R., **1986a**. Distribution and characterization of oligomeric digestive enzymes from *Erinnyis ello* caterpillars and inferences concerning secretory mechanisms and the permeability of the peritrophic membrane. *Insect Biochem. 16*, 691–700.

Santos, C.D., Terra, W.R., **1986b**. Midgut alpha-glucosidase and beta-fructosidase from *Erinnyis ello* larvae and imagoes: physical and kinetic-properties. *Insect Biochem. 16*, 819–824.

Schechter, I., Berger, A., **1967**. On the size of active site in proteases. *Biochem. Biophys. Res. Commun. 27*, 157–162.

Schorderet, S., Pearson, R.D., Vuocolo, T., Eisemann, C., Riding, G.A., *et al.*, **1998**. cDNA and deduced amino acid sequences of a peritrophic membrane glycoprotein, "Peritrophin-48," from the larvae of *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 28*, 99–111.

Scrivener, A.M., Slaytor, M., **1994**. Properties of the endogenous cellulase from *Panestia cribrata* Saussure and purification of major endo-β-1,4-glucanase components. *Insect Biochem. Mol. Biol. 24*, 223–231.

Scrivener, A.M., Slaytor, M., Rose, H.A., **1989**. Symbiont-independent digestion of cellulose and starch in *Panesthia cribrata* Saussure, an Australian wood-eating cockroach. *J. Insect Physiol. 35*, 935–941.

Scrivener, A.M., Watanabe, H., Noda, H., **1997**. Diet and carbohydrate digestion in the yellow-spotted longicorn beetle *Psacothea hilaris*. *J. Insect Physiol. 43*, 1039–1052.

Sehnal, F., Zitman, D., **1996**. Midgut endocrine cells. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, pp. 55–85.

Shahabuddin, M., **1995**. Chitinase as a vaccine. *Parasitol. Today 11*, 46–47.

Shen, Z., Jacobs-Lorena, M., **1997**. Characterization of a novel gut-specific chitinase gene from the human malaria vector *Anopheles gambiae*. *J. Biol. Chem. 272*, 28895–28900.

Shen, Z., Jacobs-Lorena, M., **1998**. A type I peritrophic matrix protein from the malaria vector *Anopheles gambiae* binds to chitin: cloning, expression and characterization. *J. Biol. Chem. 273*, 17665–17670.

Shen, Z., Reese, J.C., Reeck, G.R., **1996**. Purification and characterization of polygalacturonase from the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Insect Biochem. Mol. Biol. 26*, 427–433.

Shigenobu, S., Watanabe, H., Hattori, M., Sasaki, Y., Ishikawa, H., **2000**. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature 407*, 81–86.

Shiroya, T., **1963**. Metabolism of raffinose in cotton seeds. *Phytochemistry 2*, 23–46.

Silva, A., Bacci, M., Jr., Siqueira, C.Q., Bueno, O.L., Pagnoca, F.C., *et al.*, **2003**. Survival of *Atta sexdens* workers on different food sources. *J. Insect Physiol. 49*, 307–313.

Silva, C.P., Ribeiro, A.F., Gulbenkian, S., Terra, W.R., **1995**. Organization, origin and function of the outer microvillar (perimicrovillar) membranes of *Dysdercus peruvianus* (Hemiptera) midgut cells. *J. Insect Physiol. 41*, 1093–1103.

Silva, C.P., Terra, W.R., **1994**. Digestive and absortive sites along the midgut of the cotton seed sucker bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). *Insect Biochem. Mol. Biol. 24*, 493–505.

Silva, C.P., Terra, W.R., **1995**. An α-glucosidase from perimicrovillar membranes of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) midgut cells: purification and properties. *Insect Biochem. Mol. Biol. 25*, 487–494.

Silva, C.P., Terra, W.R., **1997**. Alpha-galactosidase activity in ingested seeds and in the midgut of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). *Arch. Insect Biochem. Physiol. 34*, 443–460.

Silva, C.P., Terra, W.R., de Sá, M.F.G., Samuels, R.I., Isejima, E.M., *et al.*, **2001**. Induction of digestive α-amylases in larvae of *Zabrotes subfasciatus* (Coleoptera: Bruchidae) in response to ingestion of common bean α-amylase inhibitor 1. *J. Insect Physiol. 47*, 1283–1290.

Silva, C.P., Xavier-Filho, J., **1991**. Comparison between the levels of aspartic and cysteine proteinase of the larval midguts of *Callosobruchus maculatus* (F.) and *Zabrotes subfasciatus* (Boh.) (Coleoptera: Bruchidae). *Comp. Biochem. Physiol. B 99*, 529–533.

Silva, I., Souza, V.B.V., **1981**. Ultrastructural aspects of the anterior midgut of *Oncideres s. saga* (Dalman, 1823) larva (Coleoptera: Cerambycidae). *Rev. Brasil. Entomol. 25*, 103–112.

Skibbe, U., Christeller, J.T., Callaghan, P.T., Eccles, C.D., Laing, W.A., **1996**. Visualization of pH gradients in the larval midgut of *Spodoptera litura* using ^{31}P-NMR microscopy. *J. Insect Physiol. 42*, 777–790.

Slaytor, M., **1992**. Cellulose digestion in termites and cockroaches: what role do symbionts play. *Comp. Biochem. Physiol. B 103*, 775–784.

Song, H.K., Suh, S.W., **1998**. Kunitz-type soybean trypsin inhibitor revisited: refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from *Erythrina caffra* and tissue-type plasminogen activator. *J. Mol. Biol. 275*, 347–363.

Spencer, K.C., **1988**. Chemical mediation of coevolution in the *Passiflora–Heliconius* interaction. In: Spencer, K.C. (Ed.), Chemical Mediation of Coevolution. Academic Press, London, pp. 167–240.

Stewart, C.B., Schilling, J.W., Wilson, A.C., **1987**. Adaptive evolution in the stomach lysozymes of foregut fermenters. *Nature 330*, 401–404.

Strobl, S., Gomis-Ruth, F.X., Maskos, K., Frank, G., Huber, R., *et al.*, **1997**. The alpha-amylase from the yellow mealworm: complete primary structure, crystallization and preliminary X-ray analysis. *FEBS Lett. 409*, 109–114.

Strobl, S., Maskos, K., Betz, M., Wiegand, G., Huber, R., *et al.*, **1998a**. Crystal structure of yellow meal worm α-amylase at 1.64 Å resolution. *J. Mol. Biol. 278*, 617–628.

Strobl, S., Maskos, K., Wiegand, G., Huber, R., Gomis-Rüth, F.X., *et al.*, **1998b**. A novel strategy for inhibition of α-amylases: yellow mealworm α-amylases in complex with the *Ragi* bifunctional inhibitor at 2.5 Å resolution. *Structure 6*, 911–921.

Su, Z.H., Ikeda, M., Sato, Y., Saito, H., Imai, K., *et al.*, **1994**. Molecular characterization of ovary trehalase of the silkworm, *Bombyx mori*, and its transcriptional activation by diapause hormone. *Biochim. Biophys. Acta 1218*, 366–374.

Su, Z.H., Sato, Y., Yamashita, O., **1993**. Purification, cDNA cloning and Northern blot analysis of trehalase of pupal midgut of the silkworm, *Bombyx mori*. *Biochim. Biophys. Acta 1173*, 217–224.

Tellam, R.L., **1996**. The peritrophic matrix. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, pp. 86–114.

Tellam, R.L., Vuocolo, T., Eisemann, C., Briscoe, S., Riding, G., *et al.*, **2003**. Identification of an immunoprotective mucin-like protein, peritrophin-55, from the peritrophic matrix of *Lucilia cuprina* larvae. *Insect Biochem. Mol. Biol. 33*, 239–252.

Tellam, R.L., Wijffels, G., Willadsen, P., **1999**. Peritrophic matrix proteins. *Insect Biochem. Mol. Biol. 29*, 87–101.

Terra, W.R., **1988**. Physiology and biochemistry of insect digestion: an evolutionary perspective. *Braz. J. Med. Biol. Res. 21*, 675–734.

Terra, W.R., **1990**. Evolution of digestive systems of insects. *Annu. Rev. Entomol. 35*, 181–200.

Terra, W.R., **2001**. The origin and functions of the insect peritrophic membrane and peritrophic gel. *Arch. Insect Biochem. Physiol. 47*, 47–61.

Terra, W.R., **2003**. Digestion. In: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects. Academic Press, San Diego, CA, pp. 310–315.

Terra, W.R., Ferreira, C., **1994**. Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol. B 109*, 1–62.

Terra, W.R., Ferreira, C., **1983**. Further evidence that enzymes involved in the final stages of digestion by *Rhynchosciara americana* do not enter the endoperitrophic space. *Insect Biochem. 13*, 143–150.

Terra, W.R., Ferreira, C., **2003**. Digestive system. In: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects. Academic Press, San Diego, CA, pp. 313–323.

Terra, W.R., Ferreira, C., De Bianchi, A.G., **1978**. Physical properties and Tris inhibition of an insect trehalase and a thermodynamic approach to the nature of its active site. *Biochim. Biophys. Acta 524*, 131–141.

Terra, W.R., Ferreira, C., Garcia, E.S., **1988**. Origin, distribution, properties and functions of the major *Rhodnius prolixus* midgut hydrolases. *Insect Biochem. 18*, 423–434.

Terra, W.R., Ferreira, C., Jordão, B.P., Dillon, R.J., **1996**. Digestive enzmes. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, pp. 153–194.

Terra, W.R., Regel, R., **1995**. pH buffering in *Musca domestica* midguts. *Comp. Biochem. Physiol. A 112*, 559–564.

Terra, W.R., Terra, I.C.M., Ferreira, C., **1983**. Inhibition of an insect midgut trehalase by dioxane and δ-gluconolactone: enzyme pK_a values and geometric relationships at the active site. *Int. J. Biochem. 15*, 143–146.

Terra, W.R., Terra, I.C.M., Ferreira, C., de Bianchi, A.G., **1979**. Carbodiimide-reactive carboxyl groups at the active site of an insect midgut trehalase. *Biochim. Biophys. Acta 571*, 79–85.

Theischinger, G., **1991**. Megaloptera. In: Naumann, I.D., Carne, P.B., Lawrence, J.F., Nielsen, E.S., Spradbery, J.P., Taylor, R.W., Whitten, M.J., Littlejohn, M.J. (Eds.), The Insects of Australia. 2nd edn. Melbourne University Press, Melbourne, pp. 516–520.

Titarenko, E., Chrispeels, M.J., **2000**. cDNA cloning, biochemical characterization and inhibition by plant inhibitors of the alpha-amylases of the Western corn rootworm, *Diabrotica virgifera virgifera*. *Insect Biochem. Mol. Biol. 30*, 979–990.

Tokuda, G., Lo, N., Watanabe, H., Slaytor, M., Matsumoto, T., *et al.*, **1999**. Metazoan cellulase genes from termites: intron/exon structures and sites of expression. *Biochim. Biophys. Acta 1447*, 146–159.

Tokuda, G., Saito, H., Watanabe, H., **2002**. A digestive β-glycosidase from the salivary glands of the termite, *Neotermes koshunensis* (Shiraki): distribution, characterization and isolation of its precursor cDNA by 5′- and 3′-RACE amplifications with degenerate primers. *Insect Biochem. Mol. Biol. 32*, 1681–1689.

Tokuda, G., Watanabe, H., Matsumoto, T., Noda, H., **1997**. Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagoensis* (Shiraki): distribution of cellulases and properties of endo-β-1,4-glucanase. *Zool. Sci. 14*, 83–93.

Tsuchida, K., Wells, M.A., **1988**. Digestion, absorption, transport and storage of fat during the last stadium of *Manduca sexta*: changes in the role lipophorin in the delivery of dietary lipids to the fat body. *Insect Biochem. 18*, 263–268.

Turunen, S., **1993**. Metabolic pathways in the midgut epithelium of *Pieris brassicae* during carbohydrate and lipid assimilation. *Insect Biochem. Mol. Biol. 23*, 681–689.

Turunen, S., Kastari, T., **1979**. Digestion and absorption of lecithin in larvae of the cabbage butterfly, *Pieris brassicae*. *Comp. Biochem. Physiol. A 62*, 933–937.

Uscian, J.M., Miller, J.S., Sarath, G., Stanley-Samuelson, D.W., **1995**. A digestive phospholipase A_2 in the tiger beetle *Cincindella circumpicta*. *J. Insect Physiol. 41*, 135–141.

Valaitis, A.P., **1995**. Gypsy moth midgut proteinases: purification and characterization of luminal trypsin, elastase and the brush-border membrane leucine aminopeptidase. *Insect Biochem. Mol. Biol. 25*, 139–149.

Valaitis, A.P., Bowers, D.F., **1993**. Purification and properties of the soluble midgut trehalase from the gypsy moth, *Lymantria dispar. Insect Biochem. Mol. Biol. 23*, 599–606.

Valaitis, A.P., Mazza, A., Brousseau, R., Masson, L., **1997**. Interaction analyses of *Bacillus thuringiensis* Cry 1A toxins with two aminopeptidases from gipsy moth midgut brush border membranes. *Insect Biochem. Mol. Biol. 27*, 529–539.

Vetter, J., **2000**. Plant cyanogenic glycosides. *Toxicon 38*, 11–36.

Volpicella, M., Ceci, L.R., Cordewenwe, J., America, T., Gallerani, R., *et al.*, **2003**. Properties of purified gut trypsin from *Helicoverpa zea*, adapted to proteinase inhibitors. *Eur. J. Biochem. 270*, 10–19.

Vonk, H.J., Western, J.R.H., **1984**. Comparative Biochemistry and Physiology of Enzymatic Digestion. Academic Press, New York.

Wang, P., Granados, R.R., **1997**. Molecular cloning and sequencing of a novel invertebrate intestinal mucin. *J. Biol. Chem. 272*, 16663–16669.

Wang, P., Granados, R.R., **2000**. Calcofluor disrupts the midgut defense system in insects. *Insect Biochem. Mol. Biol. 30*, 135–143.

Wang, P., Granados, R.R., **2001**. Molecular structure of the peritrophic membrane (PM): identification of

potential PM target sites for insect control. *Arch. Insect Biochem. Physiol. 47*, 110–118.

Ward, C.W., **1975a**. Aminopeptidases in webbing clothes moth larvae: properties and specificities of the enzymes of intermediate electrophoretic mobility. *Biochim. Biophys. Acta 410*, 361–369.

Ward, C.W., **1975b**. Aminopeptidases in webbing clothes moth larvae: properties and specificities of enzymes of highest electrophoretic mobility. *Austral. J. Biol. Sci. 28*, 447–455.

Watanabe, H., Nakamura, M., Tokuda, G., Yamaoka, I., Scrivener, A.M., *et al.*, **1997**. Site of secretion and properties of endogenous endo-β-1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. *Insect Biochem. Mol. Biol. 27*, 305–313.

Watanabe, H., Noda, H., Tokuda, G., Lo, N., **1998**. A cellulase gene of termite origin. *Nature 394*, 330–331.

Weiher, B., Komnick, H., **1997**. Digestion of phosphatidylcholines, absorption, and esterification of lipolytic products by *Aeshna cyanea* larvae as studied *in vivo* and *in vitro*. *Arch Insect Biochem. Physiol. 36*, 273–293.

Whitworth, S.T., Blum, M.S., Travis, J., **1998**. Proteolytic enzymes from larvae of the fire ant, *Solenopsis invicta*: isolation and characterization of four serine endopeptidases. *J. Biol. Chem. 273*, 14430–14434.

Whitworth, S.T., Kordula, T., Travis, J., **1999**. Molecular cloning of Soli EC: an elastase-like serine proteinase from the imported red fire ant (*Solenopsis invicta*). *Insect Biochem. Mol. Biol. 29*, 249–254.

Withers, S.G., Rupitz, K., **1990**. Measurement of active-site homology between potato and rabbit muscle α-glucan phosphorylases through use of a free energy relationship. *Biochemistry 29*, 6405–6409.

Wolfersberger, M.G., **2000**. Amino acid transport in insects. *Annu. Rev. Entomol. 45*, 111–120.

Wolfersberger, M.G., Spaeth, D.D., Dow, J.A.T., **1986**. Permeability of the peritrophic membrane of tobacco hornworm larval midgut. *Am. Zool. 26*, 356.

Woods, H.A., Kingsolver, J.G., **1999**. Feeding rate and the structure of protein digestion and absorption in Lepidoptera midguts. *Arch. Insect Biochem. Physiol. 42*, 74–87.

Yaoi, K., Nakanishi, K., Kadotani, T., Imamura, M., Koizumi, N., *et al.*, **1999**. cDNA cloning and expression of *Bacillus thuringiensis* Cry 1 Aa toxin binding 120 kDa aminopeptidase N from *Bombyx mori*. *Biochim. Biophys. Acta 1444*, 131–137.

Zeng, F., Zhu, Y.C., Cohen, A.C., **2002**. Molecular cloning and partial characterization of a trypsin-like protein in salivary glands of *Lygus hesperus* (Hemiptera: Miridae). *Insect Biochem. Mol. Biol. 32*, 455–464.

Zhang, H., Huang, X., Fukamizo, T., Muthukrishnan, Kramer, K.J., **2002**. Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase. *Insect Biochem. Mol. Biol. 32*, 1477–1488.

Zheng, L., Whang, L.H., Kumar, V., Kafatos, F.C., **1995**. Two genes encoding midgut-specific maltase-like polypeptides from *Anopheles gambiae*. *Exp. Parasitol. 81*, 272–283.

Zhu, Y.C., Baker, J.E., **1999**. Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Insect Biochem. Mol. Biol. 29*, 1053–1063.

Zhu, Y.C., Baker, J.E., **2000**. Molecular cloning and characterization of a midgut chymotrypsin-like enzyme from the lesser grain borer, *Rhyzopertha dominica*. *Arch. Insect Biochem. Physiol. 43*, 173–184.

Zhu, Y.C., Kramer, K.K., Oppert, B., Dowdy, A.K., **2000**. cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins. *Insect Biochem. Mol. Biol. 30*, 215–224.

Zhuzhikov, D.P., **1964**. Function of the peritrophic membrane in *Musca domestica* L. and *Calliphora erythrocephala* Meig. *J. Insect Physiol. 10*, 273–278.

Ziegler, H., Garon, C.F., Fischer, E.R., Shahabuddin, M., **2000**. A tubular network associated with the brush-border surface of the *Aedes aegypti* midgut: implications for pathogen transmission by mosquitoes. *J. Exp. Biol. 203*, 1599–1611.

Zimoch, L., Merzendorfer, H., **2002**. Immunolocalization of chitin synthase in the tabacco hornworm. *Cell Tissue Res. 308*, 287–297.

Relevant Websites

http://www.chem.gmw.ac.uk/iubmb – International Union of Biochemistry and Molecular Biology, Enzyme Commission.

http://www.merops.ac.uk – MEROPS.

4.6 Lipid Transport

D J Van der Horst, Utrecht University, Utrecht, The Netherlands
R O Ryan, Children's Hospital Oakland Research Institute, CA, USA

4.6.1. Historical Perspective

4.6.1.1. Lipophorin Structure and Morphology

Lipophorin was discovered 40 years ago as a major hemolymph component and key transport vehicle for water insoluble metabolites (Beenakkers *et al.*, 1985; Chino, 1985). Lipophorin is generally regarded as a multifunctional carrier because it displays a broad ability to accommodate hydrophobic biomolecules. In essence, lipophorin can be described as a noncovalent assembly of lipids and proteins, organized as a largely spherical particle. The core of the particle is made up of hydrophobic lipid molecules, such as diacylglycerol (DAG), hydrocarbons, and carotenoids. DAG, which serves as the transport form of neutral glycerolipid in hemolymph, provides an energy source for various tissues through oxidative metabolism of its fatty acid constituents. Hydrocarbons, in the form of long-chain aliphatic alkanes and alkenes, are extremely hydrophobic lipid molecules that are deposited on the cuticle where they serve to prevent desiccation and may function as semiochemicals. Carotenoids are plant-derived pigments used for coloration and as a precursor to visual pigments (Canavoso *et al.*, 2001). Another important lipid component of lipophorin is phospholipid. In general, the major glycerophospholipids present are phosphatidylcholine and phosphatidylethanolamine (Wang *et al.*, 1992). These amphiphilic lipids exist as a monolayer at the lipophorin particle surface, positioned in such a way that their fatty acyl chains interact with the hydrophobic core of the particle while their polar head groups are presented to the aqueous milieu. In this manner, the phospholipid moieties of lipophorin serve a key structural role. The other major structural component of lipophorin is protein. All lipophorin particles possess two apolipoproteins, termed apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II). ApoLp-I and apoLp-II are integral components of the lipophorin particle and cannot be removed without destruction of lipophorin particle integrity. It is recognized that apoLp-I and apoLp-II are the product of the same gene and that the two proteins arise from a post-translational proteolytic processing event (Weers *et al.*, 1993). This finding is consistent with the fact that apoLp-I and apoLp-II are found in a 1 : 1

molar ratio in lipophorin particles. At present it is not known if one or the other apoLp possesses additional functions aside from its primary role in stabilizing lipophorin particle integrity although a role in receptor interactions is implied (see below). The structural role is fulfilled by the capacity of apoLp-I and apoLp-II to interact with lipid and create an interface between the nonpolar core of the particle and the external environment. In this capacity, apoLp-I and apoLp-II function in a manner similar to that proposed for apolipoprotein B in vertebrate plasma. Indeed, it is now recognized that the genes encoding these proteins are derived from a common ancestor (Babin *et al.*, 1999).

4.6.1.2. Lipophorin Subspecies

One of the hallmark features of lipophorin-mediated lipid transport relates to the dynamic nature of the particle. Lipophorin isolated from various life stages is generally of a unique density and lipid composition. For example, lipophorin from *Manduca sexta* fifth instar larvae displays a density of 1.15 g ml^{-1} with a particle diameter in the range of 16 nm. By contrast, lipophorin isolated from adult hemolymph is of lower density and larger diameter. Indeed, in *M. sexta* a broad array of unique lipophorin subspecies has been identified, each with characteristic properties (Prasad *et al.*, 1986). On the basis of this diversity, a nomenclature system has been adopted that distinguishes various lipophorin subspecies based on their density. Since most particles fall within the density limits 1.21 and 1.07 g ml^{-1}, the term high-density lipophorin (HDLp) is commonly used. Because many lipophorin subspecies are present at well-defined developmental stages, a suffix may be added to denote this. Hence, HDLp-P and HDLp-A may be used to distinguish HDLp from pupal and adult hemolymphs, respectively.

One of the features of HDLp-A is its ability to associate with a third apolipophorin, apoLp-III. In insect species that use lipid as a fuel for flight (such as *Locusta migratoria* and *M. sexta*), apoLp-III is present in abundance in adult hemolymph as a lipid free protein. Whereas a small amount of apoLp-III may be associated with HDLp under resting conditions, flight activity induces association of large amounts of apoLp-III with the lipophorin particle surface (Van der Horst *et al.*, 1979). This process, which is dependent upon the uptake of DAG by the lipophorin particle, leads to the conversion of HDLp into low-density lipophorin (LDLp). LDLp has a larger diameter, a significantly increased DAG content, and a lower density. In studies of this conversion, it has been shown that apoLp-III associates with the surface of the expanding lipophorin particle as a function of DAG enrichment (Soulages and Wells, 1994b; Ryan and Van der Horst, 2000). Thus, it has been hypothesized that apoLp-III serves to stabilize the DAG-enriched particle, providing an interface between surface localized hydrophobic DAG molecules and the external aqueous medium. It is envisioned that continued DAG accumulation by HDLp results in partitioning of DAG between the hydrophobic core of the particle and the surface monolayer (Wang *et al.*, 1995). The presence of DAG in the surface monolayer exerts a destabilizing effect on the particle structure and, if allowed to persist, would result in deleterious particle fusion and aggregation. By "sensing" the presence of DAG in the lipophorin surface monolayer, apoLp-III is attracted to the particle surface and forms a stable binding interaction. This event is fully reversible and, upon removal of DAG from the particle, apoLp-III dissociates, leading to regeneration of HDLp. Importantly, it is recognized that lipophorin particles can then bind additional DAG, forming a cycle of transport. It is noteworthy that these concepts about apoLp-III association/dissociation from lipophorin emerged from physiological studies of flight activity in *L. migratoria* conducted in the late 1970s and early 1980s in The Netherlands and England (Mwangi and Goldsworthy, 1977, 1981; Van der Horst *et al.*, 1979, 1981). A cartoon depicting metabolic and biochemical processes related to induction of flight-related lipophorin conversions and the accompanying increase in neutral lipid transport capacity is presented in **Figure 1**. Elaboration of various aspects of this central scheme will occur in the next sections. At this point, however, it should be noted that this generalized mechanism differs fundamentally from metabolic processes in vertebrates, where lipoproteins do not have a function in the transport of energy substrates during exercise (Van der Horst *et al.*, 2002). That being said, it is evident that novel insight into structural and functional aspects of vertebrate lipid transport processes can be gained from the study of insect lipid transport. A vivid example of this emerged recently with the identification and functional characterization of the *Drosophila* homolog of the vertebrate microsomal lipid transfer protein (MTP) (Sellers *et al.*, 2003). Analysis of the *Drosophila* genome revealed the existence of an expressed sequence tag with 23% sequence identity to vertebrate MTP. Coexpression of this gene with that encoding a truncated version of apolipoprotein B in African green monkey kidney (COS) cells afforded the ability to assemble and secrete lipoproteins. Compared to mammalian MTP, the *Drosophila* MTP homolog possesses unique structural and catalytic properties that appear

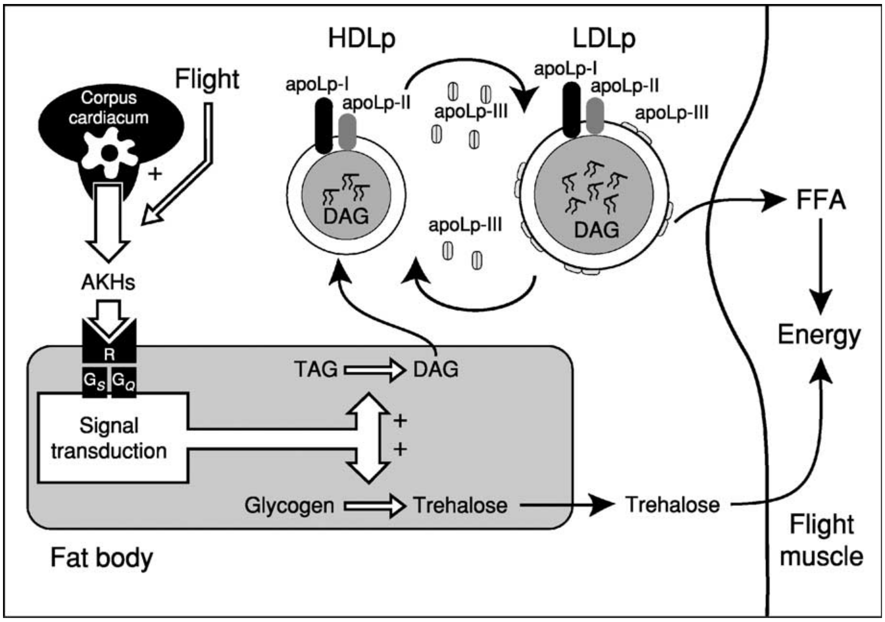


Figure 1 Molecular basis of the lipophorin lipid shuttle: AKH-controlled DAG mobilization from insect fat body during flight activity results in the reversible alternation of lipophorin from a relatively lipid-poor (HDLp) to a lipid-rich (LDLp) state, and apoLp-III from a lipid-free in a lipid-bound state. The reversible conformational change in apoLp-III induced by DAG loading of lipophorin is schematically visualized. AKHs, adipokinetic hormones; R, receptor; G, G protein; HDLp, high-density lipophorin; LDLp, low-density lipophorin; apoLp-I, -II, and -III, apolipophorin I, II, and III; TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acids. (Based on data from several insect species, particularly *Locusta migratoria* and *Manduca sexta*, reviewed in Ryan and Van der Horst (2000) and Van der Horst *et al*. (2001); and this review.)

to be related to distinct strategies of lipid transport operative in insects.

4.6.2. Flight-Related Processes

4.6.2.1. Adipokinetic Hormone

Insect flight involves the mobilization, transport, and utilization of endogenous energy reserves at extremely high rates. In insects that engage in long-distance flight, the demand for fuel, particularly lipids, by the flight muscles can remain elevated for extended periods of time. Peptide adipokinetic hormones (AKHs), synthesized and stored in neuroendocrine cells, play a crucial role in this process as they integrate flight energy metabolism. Insect AKHs are short peptides consisting of 8–11 amino acid residues. To date the structures of over 35 different AKHs are known from representatives of most insect orders; in spite of considerable variation in their structures they are clearly related (reviews: Gäde, 1997; Van der Horst *et al*., 2001; Oudejans and Van der Horst, 2003). All AKHs are N-terminally blocked by a pyroglutamate (pGlu) residue and all but one (Köllisch *et al*., 2000) are C-terminally amidated. Initiation of flight activity induces the release of AKHs from the intrinsic AKH-producing cells (adipokinetic cells) in the glandular lobes of the corpus cardiacum, a neuroendocrine gland located caudal to the insect brain and physiologically equivalent to the pituitary of mammals. The fat body plays a fundamental role in lipid storage, as well as in the process of lipolysis controlled by the AKHs. Binding of these hormones to their G protein-coupled receptors at the fat body target cells triggers a number of coordinated signal transduction processes that ultimately result in the mobilization of carbohydrate and lipid reserves as fuels for flight activity (see Figure 1). Energy-yielding metabolites are transported via the hemolymph to the contracting flight muscles. Carbohydrate (trehalose) in the circulation provides energy for the initial period of flight and is replenished from glycogen reserves. However, similar to sustained activity in many other animal species, flight activity of insects covering vast distances nonstop is powered principally by mobilization of endogenous reserves of triacylglycerol (TAG), the most concentrated form of energy available to biological tissues. As a result of TAG mobilization, the concentration of *sn*-1,2-DAG in the hemolymph increases progressively and gradually constitutes the principal fuel for flight. The mechanism for hormonal activation of glycogen phosphorylase, the enzyme determining

the rate of glycogen breakdown and trehalose biosynthesis, has been well established. In contrast, little is known of the mechanism by which the pivotal enzyme TAG lipase catalyzes AKH-controlled production of the DAG on which long-distance flight is dependent.

For a considerable part, the success of insects in long-distance flights is attributable to their system of neuropeptide AKHs integrating flight energy metabolism, involving the transfer of energy substrates, particularly lipids, to the flight muscles as discussed above. Therefore, recent advances in the strategy of adipokinetic cells in hormone storage and release will be discussed along with the effects of the AKHs on lipid mobilization.

4.6.2.2. Strategy of the Adipokinetic Cells

In view of their involvement in the regulation and integration of extremely intense metabolic processes, the AKH-producing cells (adipokinetic cells) of the corpus cardiacum constitute an appropriate model system for studying neuropeptide biosynthesis and processing, as well as the coherence between biosynthesis, storage, and release of these neurohormones (reviews: Ryan and Van der Horst, 2000; Van der Horst *et al*., 2001; Diederen *et al*., 2002). These processes have been particularly studied in two locust species notorious for their long-range flight capacity, *L. migratoria* and *Schistocerca gregaria*, which similarly to several other insect species mobilize more than one AKH. The three AKHs synthesized in the adipokinetic cells of *L. migratoria* consist of a decapeptide AKH-I and two octapeptides (AKH-II and -III). AKH-I is by far the most abundant peptide; the ratio of AKH-I : -II : -III in the corpus cardiacum is approximately 14 : 2 : 1 (Oudejans *et al*., 1993). All three AKHs are involved in the mobilization of both lipids and carbohydrates, although their action is differential (reviews: Vroemen *et al*., 1998; Van der Horst and Oudejans, 2003). In addition, several other effects of AKH are known, such as inhibition of the synthesis of proteins, fatty acids, and RNA (reviews: Gäde, 1996; Gäde *et al*., 1997).

The transport of these hydrophobic peptides in the circulation occurs independently of a carrier (Oudejans *et al*., 1996). The AKHs of *L. migratoria* appear to be catabolized differentially after their release; turnover half-times of AKH-I and -II during flight are relatively slow (35 and 37 min, respectively), whereas the hemolymph half-time of AKH-III is very rapid (3 min) (Oudejans *et al*., 1996). Degradation of the (single) AKH in the hemolymph of adult females of the cricket *Gryllus bimaculatus*, which do not fly well, was estimated to be remarkably short (half-life approximately 3 min) in the resting state (Woodring *et al*., 2002). A recent study in which AKH concentrations were measured by radioimmunoassay shows that the hemolymph concentration of two AKHs from *S. gregaria* (AKH-I and -II) increases within 5 min of initiation of flight and are maintained at approximately 15-fold (AKH-I) and 6-fold (AKH-II) the resting levels over flights of at least 60 min (Candy, 2002). The increase in hormone level preceded an increase in hemolymph lipid content. Furthermore, a rapid release of the AKHs over the first few minutes was followed by a slower release, maintaining the elevated hormone levels.

The AKH peptides are derived from preprohormones that are translated from separate mRNAs and subsequently enzymatically processed. Cotranslational cleavage of the signal sequences generates the AKH-I, -II, and -III prohormones, consisting of a single copy of AKH, a GKR or GRR processing site, and an AKH-associated peptide (AAP). AKH-I and -II prohormones are structurally very similar whereas AKH-III is remarkably different (Bogerd *et al*., 1995) (**Figure 2**). Prior to further processing, the AKH-I and -II prohormones dimerize at random by oxidation of their (single) cysteine residues in the AAP, giving rise to two homodimers and one heterodimer. Proteolytic processing of these dimeric products at their processing sites, involving removal of the two basic amino acid residues and amidation, using glycine as the donor, yields the bioactive hormones as well as three (two homodimeric and one heterodimeric) AKH-precursor related peptides (APRPs) with as yet unknown functions (reviews: Van der Horst *et al*., 2001; Diederen *et al*., 2002; Oudejans and Van der Horst, 2003). Recent data from capillary liquid chromatography-tandem mass spectrometry analysis indicate that these APRPs are further processed to form smaller peptides, designed AKH joining peptide 1 (AKH-JP I) and 2 (AKH-JP II), respectively (Baggerman *et al*., 2002) (**Figure 2**). The biosynthesis of AKH-III from its prohormone has only very recently been disclosed (Huybrechts *et al*., 2002). By the use of sophisticated techniques including capillary high-performance liquid chromatography (HPLC) and nanoflow electrospray ionization quantitative time-of-flight (Q-TOF) mass spectrometry, another (fourth) APRP was identified, a homodimer resulting from the cross-linking of two AKH-III prohormone molecules (in a parallel and/or antiparallel fashion) by two disulfide bridges formed between their (two) cysteine residues and subsequent proteolytic cleavage of the AKH-III molecules. This finding indicates that

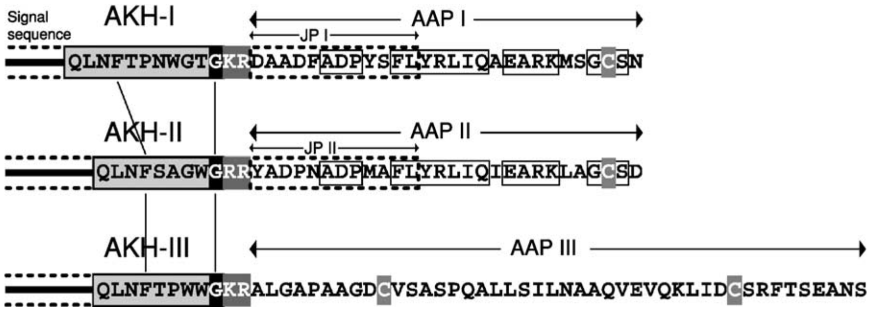


Figure 2 Sequence and proteolytic processing of *Locusta migratoria* AKH prohormones. The AKH sequence is followed by a processing site (GKR or GRR); identical residues in the AKH-associated peptides (AAPs) I and II are boxed. The cysteine residues forming disulfide bridges prior to proteolytic processing of all AKH prohormones are shown in white. JP, joining peptide. (Based on data from Bogerd, J., Kooiman, F.P., Pijnenburg, M.A.P., Hekking, L.H.P., Oudejans, R.C.H.M., *et al.*, **1995**. Molecular cloning of three distinct cDNAs, each encoding a different adipokinetic hormone precursor, of the migratory locust, *Locusta migratoria*: differential expression of the distinct adipokinetic hormone precursor genes during flight activity. *J. Biol. Chem. 270*, 23038–23043; Baggerman, G., Huybrechts, J., Clynen, E., Hens, K., Harthoorn, L., *et al.*, **2002**. New insights in adipokinetic hormone (AKH) precursor processing in *Locusta migratoria* obtained by capillary liquid chromatography-tandem mass spectrometry. *Peptides 23*, 635–644; and Huybrechts, J., Clynen, E., Baggerman, G., De Loof, A., Schoofs, L., **2002**. Isolation and identification of the AKH III precursor-related peptide from *Locusta migratoria*. *Biochem. Biophys. Res. Commun. 296*, 1112–1117.)

processing of AKH-III prohormone occurs similar to that of the AKH-I and -II prohormones. In contrast to the APRPs derived from AKH-I and -II prohormones, however, no evidence was found for further processing of the APRP generated along with AKH-III production.

In situ hybridization showed that the mRNA signals encoding the three different AKH preprohormones are colocalized in the cell bodies of the glandular lobes of the corpus cardiacum (Bogerd *et al.*, 1995). Following their synthesis in the rough endoplasmic reticulum in the cell bodies, the AKH prohormones are transported to the Golgi complex and packaged into secretory granules at the *trans*-Golgi network, whereas proteolytic processing of the prohormones to bioactive AKHs is presumed to take place in the secretory granules (reviews: Van der Horst *et al.*, 2001; Diederen *et al.*, 2002; Oudejans and Van der Horst, 2003). The intracellular location of the AKHs was probed with antibodies specific for the corresponding associated peptides (AAP I, II, and III), the amino acid sequences of which differ to a larger degree from each other than those of the AKHs. All three (dimeric) AAPs were shown to be colocalized in the same secretory granules, which implies that these three AKHs colocalize in these granules and are released simultaneously during flight (Harthoorn *et al.*, 1999). Since the membranes of exocytosed secretory granules fuse with the plasma membrane, the total content of the granules is released into the hemolymph. Consequently, in addition to bioactive AKHs, the APRPs, and possibly other products, are released. Whether the AKH-JPs are released is not yet clear (Baggerman *et al.*, 2002; Huybrechts *et al.*, 2002); data on AKH-JP I and II indicate that these peptides do not stimulate lipid release from the fat body nor activate fat body glycogen phosphorylase, both key functions of the AKHs (Baggerman *et al.*, 2002).

The AKH cells continuously synthesize AKHs, resulting in a steady increase in the amounts of the three hormones in the corpus cardiacum with age. Concurrently, the number of the AKH-containing secretory granules (diameter ~300 nm) also increases. In addition, particularly in older adults, intracisternal granules (ICGs) are produced. ICGs are present in both exocrine and endocrine cells, and originate from premature condensation of peptidergic products within cisternae of the rough endoplasmic reticulum (review: Diederen *et al.*, 2002). In the locust adipokinetic cells, these granules, which may attain diameters up to 5 μm and even more, appear to function as a store for AKH-I and -II prohormones, as shown immunocytochemically with specific anti-AAPs (Harthoorn *et al.*, 1999, 2000). The prohormone for AKH-III is absent, which points to differences in physiological function between AKH-III and the other two AKHs.

The secretory activity of the adipokinetic cells, which has been investigated *in vitro* primarily for AKH-I, is subject to many regulatory substances including neurogenic locustatachykinins and humoral crustacean cardioactive peptide (CCAP) as initiating factors, trehalose as an inhibitor, and several positive and negative modulators (reviews: Van der Horst *et al.*, 1999; Vullings *et al.*, 1999). Recent data on the release of AKH from the corpora cardiaca *in vitro* show that regulatory substances

(including CCAP) affect the release of all three AKHs in proportion to their concentration in the corpus cardiacum (Harthoorn *et al.*, 2001). However, the only natural stimulus for the release of the AKHs is flight activity, and the relative contributions of all known substances effective in the process of release of these neurohormones remain to be established *in vivo*.

The amount of AKHs released during flight represents only a few percent of the huge stores harbored in the adipokinetic cells. On the other hand, only a limited part of these AKH stores appear to be actually releaseable. In studies in which young secretory granules were specifically labeled, these newly formed secretory granules were preferentially released (last in, first out) (reviews: Van der Horst *et al.*, 2001; Diederen *et al.*, 2002; Oudejans and Van der Horst, 2003). Following the biosynthesis of new AKH prohormones, their packaging into secretory granules and their processing to bioactive AKHs, which takes less than 1 h, granules containing newly synthesized AKHs appeared to be available for release during a restricted period of approximately 8 h before they are supposed to enter a pool of older secretory granules that appear to be unable to release their content upon secretory stimulation. This indicates that only a relatively small readily releasable pool of new secretory granules exists. Therefore, an important question is whether the secretory output of AKHs during flight would induce a stimulation of the rate of AKH biosynthesis. The mRNA levels of all three AKH preprohormones, however, did not appear to be affected by flight activity, while the rate of synthesis of AKH prohormones and AKHs was not affected either (Harthoorn *et al.*, 2001). Apparently, a coupling between release and biosynthesis of AKHs is absent. Inhibition of AKH biosynthesis *in vitro* by Brefeldin A, a specific blocker of the transport of newly synthesized secretory proteins from the endoplasmic reticulum to the Golgi complex, resulted in a considerable decrease in the release of AKHs induced by CCAP, and highlighted once more that the regulated secretion of AKHs is completely dependent on the existence of a readily releasable pool of newly formed secretion granules (Harthoorn *et al.*, 2002). Therefore, we conclude that the strategy of the adipokinetic cells to cope with variations in secretory output of AKHs apparently is to rely on the continuous biosynthesis of AKHs, which produces a readily releasable pool that is sufficiently large and constantly replenished.

An important question remaining unanswered is, what might be the rationale for the storage of such large quantities of hormones that are not accessible for secretory release? In addition, the possible function of the prohormones for AKH-I and -II in the ICGs in providing an additional source of AKH prohormones when called upon remains to be established.

4.6.2.3. Effect of Adipokinetic Hormones on Lipid Mobilization

Binding of the AKHs to their plasma membrane receptor(s) at the fat body cells is the primary step to the induction of signal transduction events that ultimately lead to the activation of target key enzymes and the mobilization of lipids as a fuel for flight. Although the AKHs constitute extensively studied neurohormones and their actions have been shown to occur via G protein-coupled receptors (reviews: Van Marrewijk and Van der Horst, 1998; Vroemen *et al.*, 1998), the general properties of which are remarkably well conserved during evolution (review: Vanden Broeck, 2001), the identification of these receptors has not been successful. Very recently, however, the first insect AKH receptors have been identified at the molecular level, namely those of the fruitfly *Drosophila melanogaster* and the silkworm *Bombyx mori* (Staubli *et al.*, 2002). They appear to be structurally related to mammalian gonadotropin-releasing hormone (GnRH) receptors. These data promise to elucidate the nature of AKH receptors from other insects; it is envisaged that insects such as the locust, that produce two or more different types of AKH, may have two or more different AKH receptors.

The signal transduction mechanism of the three locust AKHs has been studied extensively, and involves stimulation of cAMP production, which is dependent on the presence of extracellular Ca^{2+}. Additionally, the AKHs enhance the production of inositol phosphates including inositol 1,4,5-trisphosphate (IP_3), which may mediate the mobilization of Ca^{2+} from intracellular stores. This depletion of Ca^{2+} from intracellular stores stimulates the influx of extracellular Ca^{2+}, indicative of the operation of a capacitative (store-operated) calcium entry mechanism. The interactions between the AKH signaling pathways ultimately result in mobilization of stored reserves as fuel for flight (reviews: Van Marrewijk and Van der Horst, 1998; Vroemen *et al.*, 1998; Ryan and Van der Horst, 2000; Van der Horst *et al.*, 2001; Van Marrewijk, 2003). The concentration of DAG in the hemolymph increases progressively at the expense of stored TAG reserves in the fat body, which implies hormonal activation of the key enzyme, fat body TAG lipase. In a bioassay, all three AKHs are able to stimulate lipid mobilization, although their

relative potencies are different. This recalls the concept of a hormonally redundant system involving multiple regulatory molecules with overlapping actions (reviews: Goldsworthy *et al*., 1997; Vroemen *et al*., 1998). Results obtained with combinations of two or three AKHs, which are likely to occur together in locust hemolymph under physiological conditions *in vivo*, revealed that the maximal responses for the lipid-mobilizing effects were much lower than the theoretically calculated responses based on dose-response curves for the individual hormones. In the lower (probably physiological) range, however, combinations of the AKHs were more effective than the theoretical values calculated from the responses elicited by the individual hormones (review: Van Marrewijk and Van der Horst, 1998).

The mechanism by which TAG lipase catalyzes AKH-controlled production of the DAG on which long-distance flight depends is only poorly understood, mainly due to technical problems in isolating or activating the lipase. In vertebrates, hormone-sensitive lipase (HSL) controls mobilization of TAG stores in adipose tissue, and although contrary to insects, free fatty acids (FFA) are released into the blood for uptake and oxidation in muscle, there is a clear functional similarity between vertebrate adipose tissue HSL and insect fat body TAG lipase (reviews: Ryan and Van der Horst, 2000; Van der Horst *et al*., 2001; Van der Horst and Oudejans, 2003).

4.6.3. Apolipophorin III

4.6.3.1. Lipid Free Helix Bundle Structure

ApoLp-III was discovered in the late 1970s and early 1980s by research groups in Europe and North America (reviews: Blacklock and Ryan, 1994; Ryan and Van der Horst, 2000). ApoLp-III was first isolated from hemolymph of *L. migratoria* (Van der Horst *et al*., 1984) and the tobacco hawkmoth, *M. sexta* (Kawooya *et al*., 1984). *Manduca sexta* apoLp-III is a 166 amino acid protein that lacks tryptophan and cysteine (Cole *et al*., 1987). However, the well-characterized apoLp-III from *L. migratoria* is 164 residues long and lacks cysteine, methionine, and tyrosine (Kanost *et al*., 1988; Smith *et al*., 1994). *Manduca sexta* apoLp-III is nonglycosylated while *L. migratoria* apoLp-III contains two complex carbohydrate chains (Hård *et al*., 1993). Sequence analysis predicts that all apoLp-IIIs are composed of predominantly amphipathic α-helix secondary structure, consistent with far ultraviolet circular dichroism (CD) studies (Ryan *et al*., 1993; Weers *et al*., 1998). An important breakthrough in our understanding of the structure of apoLp-III occurred with determination of the X-ray crystal structure of *L. migratoria* apoLp-III (Breiter *et al*., 1991). These authors showed that apoLp-III exists as a globular, up-and-down amphipathic α-helix bundle in the absence of lipid. The molecule is composed of five discrete α-helix segments that orient their hydrophobic faces toward the center of the bundle. Using a convenient method for bacterial overexpression, recombinant *M. sexta* apoLp-III was enriched with stable isotopes (Ryan *et al*., 1995; Wang *et al*., 1997b). Application of heteronuclear multidimensional nuclear magnetic resonance (NMR) techniques to isotopically enriched *M. sexta* apoLp-III yielded a complete assignment of this protein (Wang *et al*., 1997a). Structure calculations revealed a five-helix bundle molecular architecture, representing the first full-length apolipoprotein whose high resolution solution structure has been determined in the absence of detergent (Wang *et al*., 2002) (**Figure 3**). In keeping with the X-ray structure of *L. migratoria* apoLp-III, this structure also reveals an up-and-down bundle of five amphipathic α-helices. Interesting, however, Wang and coworkers identified a distinct short segment of α-helix that connects helix 3 and helix 4 in the bundle (termed helix 3′). This sequence segment ($P_{95}DVEKE_{100}$) aligns perpendicular to the long axis of the bundle and, as discussed below, has been shown to play a role in the initiation of apoLp-III lipid interaction. More recently, Fan *et al*. (2001, 2003) employed multidimensional NMR techniques to obtain a complete assignment and solution structure determination for *L. migratoria* apoLp-III. This work is significant in that it permits direct comparison between the X-ray crystal structure and the NMR structure. Interestingly, Fan *et al*. provide previously unreported structural evidence for a solvent exposed short helix that is positioned perpendicular to the long axis of the helix bundle. These authors propose that this short helix can serve as a recognition helix for initiation of apoLp-III lipid interaction, leading to conformational opening of the helix bundle. Contrary to the model presented by Breiter *et al*. (1991), Fan *et al*. (2003) suggest an alternate opening mechanism. Further studies will be required to elucidate the precise mechanism whereby apoLp-III recognizes and binds to available lipid surfaces (see below).

4.6.3.2. Lipid Induced Conformation Change

The up-and-down antiparallel organization of helical segments in apoLp-III allows for a simple opening of the bundle about putative "hinge" loops that connect the helices as originally proposed

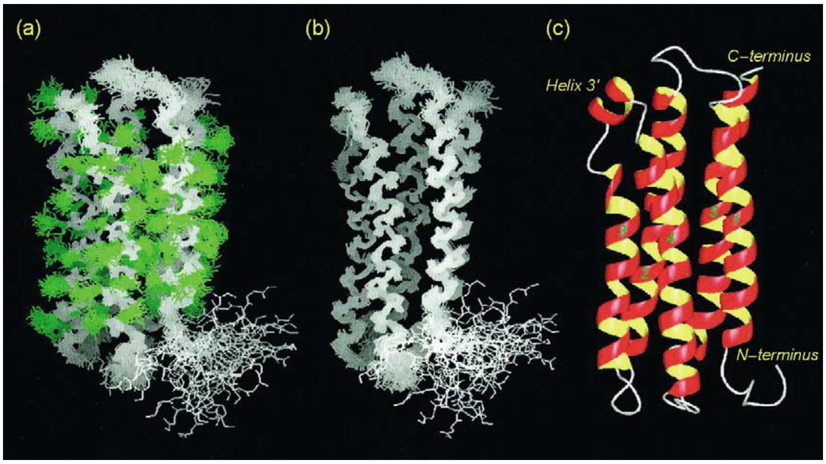


Figure 3 Nuclear magnetic resonance (NMR) visualizations of structure of lipid-free *Manduca sexta* apoLp-III. (a, b) Superposition of 40 NMR-derived structures of apoLp-III, with backbone atoms displayed in white and side chain heavy atoms displayed in green. (c) Ribbon representation of an energy-minimized, average structure of apoLp-III (PDB code 1EQ1). (Reproduced with permission from Wang, J., Sykes, B.D., Ryan, R.O., **2002**. Structural basis for the conformational adaptability of apolipophorin III, a helix bundle exchangeable apolipoprotein. *Proc. Natl Acad. Sci. USA 99*, 1188–1193; © by the National Academy of Sciences of the United States of America.)

by Breiter *et al.* (1991). The model suggests that apoLp-III initiates contact with lipid surfaces via one end of the helix bundle. Conformational opening could then occur with retention of helix boundaries present in the bundle configuration. Such an event would result in substitution of helix–helix interactions in the bundle conformation for helix–lipid interactions. Current evidence suggests that this conformational transition is triggered by availability of a suitable lipid surface and is reversible (Singh *et al.*, 1992; Liu *et al.*, 1993; Soulages and Wells, 1994a; Soulages *et al.*, 1995, 1996). Thus, it is conceivable that helix 3 and helix 4 move away from helices 1, 2, and 5 in concert as the bundle opens about the loop segments connecting helix 2 and helix 3 and helix 4 and helix 5 (Breiter *et al.*, 1991; Narayanaswami *et al.*, 1996b).

A well-known property of amphipathic exchangeable apolipoproteins in general is an ability to disrupt phospholipid bilayer vesicles and transform them into apolipoprotein–phospholipid disk complexes (Pownall *et al.*, 1978). This property represents a useful method to investigate aspects of the proposed lipid-induced helix bundle molecular switch process. The disk-shaped complexes formed between apoLp-III and dimyristoylphosphatidylcholine (DMPC) are of uniform size and composition, permitting detailed analysis of their structural organization (Wientzek *et al.*, 1994). Attenuated total reflectance Fourier transformed infrared spectroscopy has been employed to characterize helix orientation in apoLp-III–DMPC disk complexes (Raussens *et al.*, 1995, 1996). This analysis, and more recent studies (Soulages and Arrese, 2001) reveal that apoLp-III helical segments interact with phospholipid fatty acyl chains around the perimeter of the disk complex.

Several independent studies have provided convincing evidence that apoLp-III undergoes a significant conformational change upon association with lipid. Kawooya *et al.* (1986) used a monolayer balance to investigate apoLp-III behavior at the air–water interface, while Narayanaswami *et al.* (1996a) studied the unique fluorescence properties of the lone tyrosine in *M. sexta* apoLp-III. Near-ultraviolet CD analysis of *L. migratoria* apoLp-III indicates that helix realignment and reorientation occurs upon interaction with phospholipid vesicles (Weers *et al.*, 1994). Sahoo *et al.* (2000) used pyrene excimer fluorescence spectroscopy to investigate lipid binding induced realignment of helix 2 and helix 3 in *M. sexta* apoLp-III. In this study cysteine residues were introduced into the protein by site-directed mutagenesis (N40C and L90C). These sites were selected for introduction of cysteine residues based on the fact that they reside in close proximity in the helix bundle conformation. Covalent modification of the cysteine thiol groups with pyrene maleimide yielded a double pyrene labeled apoLp-III. In the absence of lipid, pyrene labeled apoLp-III adopts a helix bundle conformation. Fluorescence spectroscopy experiments revealed normal pyrene emission at 375 and 395 nm (excitation 345 nm) as well as excimer (excited state dimer) fluorescence at longer wavelengths (~460 nm). Control experiments verified that the excimer peak arose from intramolecular pyrene–pyrene interactions in the

labeled protein and was not due to intermolecular interactions. Because it is known that excimer fluorescence is manifest only when pyrene moieties are within 10 Å of one another, this property was used to assess the effect of lipid binding. The observation that excimer fluorescence was greatly reduced when apoLp-III was complexed with DMPC was taken as evidence for a conformational change in the protein upon lipid binding that results in relocation of helix 2 away from helix 3.

In fluorescence studies of apoLp-III, carried out by Soulages and Arrese (2000a, 2000b), site-directed mutagenesis was used to create various mutant apoLp-IIIs with a single tryptophen residue in each of the five helical segments of the protein. Data obtained in this study suggests that apoLp-III undergoes a conformational change that brings helices 1, 4, and 5 into contact with the lipid surface, while others (helices 2 and 3) appear to behave differently. In other studies Soulages *et al.* (2001) used disulfide bond engineering to show that conformational flexibility of helices 1 and 5 of *L. migratoria* apoLp-III play an important role in the lipid binding process. In other studies Dettloff *et al.* (2001b) reported that a C-terminal truncated apoLp-III from the wax moth *Galleria mellonella*, comprising the first three helical segments of the protein, retains structural integrity and an ability to interact with lipid surfaces. More recently, Dettloff *et al.* (2002) expanded this work to encompass two additional three helix mutants derived from *G. mellonella* apoLp-III, a C-terminal fragment comprising helices 3–5 and a core fragment comprising helices 2–4. All three truncation mutants retained their ability to solubilize bilayer vesicles of DMPC, an event that led to large increases in their α-helix content. The N-terminal and core fragment, but not the C-terminal fragment, were able to interact with phospholipase C modified human low-density lipoprotein, thereby preventing its aggregation. This result suggests that impairment of the lipid interaction properties of the C-terminal fragment has occurred as a result of removal of N-terminal helix segments. Taken together, it appears that the minimal essential elements required for apoLp-III lipid binding function is less than the intact five-helix bundle. Recent experiments have provided evidence that opening of the helix bundle is even more dramatic than originally postulated. It is now proposed that the protein adopts a fully extended belt-like conformation (Garda *et al.*, 2002; Sahoo *et al.*, 2002) (**Figure 4**). Garda *et al.* (2002) employed fluorescence resonance energy transfer methods while Sahoo *et al.* (2002) used pyrene excimer fluorescence to probe aspects of helix repositioning upon interaction with DMPC. In both of these studies, knowledge of the three-dimensional structure of the apoLp-III in the absence of lipid (i.e., the helix bundle conformation) allowed for structure guided site-directed mutagenesis to introduce strategically placed cysteine residues to which fluorescent reporter groups could be covalently attached. Subsequent characterization studies yielded a unifying model of apoLp-III conformation

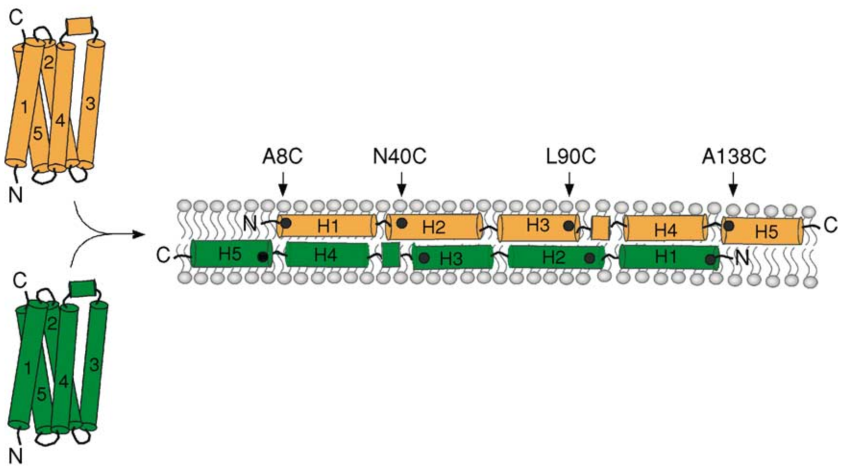


Figure 4 Model of apoLp-III bound to phospholipid discoidal complexes. ApoLp-III complexes with phospholipids on a discoidal particle adopting an extended α-helical conformation. Lipid-triggered association involves extension of H1 away from H5, helix bundle opening and repositioning of H2 and H3. The positions of cysteine substitution mutations employed in this and previous analyses are indicated: A8C, N40C, L90C, and A138C; H1, H2, H3, and H5. Apolp-III adopts an extended helical conformation around the periphery of discoidal phospholipid bilayer complexes, with neighboring molecules aligned antiparallel with respect to each other, and shifted by one helix. (Reprinted with permission from Sahoo, D., Weers, P.M.M., Ryan, R.O., Narayanaswami, V., **2002**. Lipid-triggered conformational switch of apolipophorin III helix bundle to an extended helix organization. *J. Mol. Biol. 321*, 201–214; © Elsevier.)

on disk complexes wherein the resulting structure resembles concepts and models that describe the organization of human apolipoprotein A-I on nascent high-density lipoproteins (Klon *et al.*, 2002).

4.6.3.3. Initiation of ApoLp-III Lipid Binding

Analysis of the structure of *L. migratoria* apoLp-III indicates the presence of solvent exposed leucine residues at one end of the protein (Breiter *et al.*, 1991). These authors proposed that this region of the molecule functions as a "hydrophobic sensor", which recognizes potential lipid surface binding sites. Surface plasmon resonance spectroscopy studies revealed that small amounts of DAG induce binding of apoLp-III to a phospholipid bilayer with its long molecular axis normal to the lipid surface (Soulages *et al.*, 1995). This interaction is proposed to be the first step in formation of a stable binding interaction. Site-directed mutagenesis was performed to determine whether alteration in the hydrophobicity of the putative sensor region of *L. migratoria* apoLp-III affects its ability to initiate contact with lipid surfaces (Weers *et al.*, 1999). In this study three partially exposed leucine residues, located at the end of the protein containing the loop segments that connect helix 1 and helix 2 and helix 3 and helix 4, were mutated to arginine. Three single arginine to leucine substitution mutants and a triple mutant were expressed in *Escherichia coli* and characterized in terms of their structural and stability properties. The effect of these mutations on phospholipid bilayer vesicle transformation into disk complexes versus lipoprotein binding suggests that the former binding interaction has an electrostatic component. Taken together, the data support the view that the end of the molecule bearing Leu 32, 34, and 95 is responsible for initiating contact with potential lipid surface binding sites.

The solution structure of *M. sexta* apoLp-III revealed the presence of helix 3′ at one end of the protein globule (Wang *et al.*, 1997a, 2002). One possibility is that helix 3′ reorientation facilitates contact with a lipid surface by exposing the hydrophobic interior of the helix bundle. The lipid surface could then trigger a molecular switch to induce conformational opening of the helix bundle and formation of a stable binding interaction. To investigate this, protein engineering was employed to remove helix 3′ and replace it with a sequence that has a high probability of forming a β-turn (Narayanaswami *et al.*, 1999). Characterization of the lipid binding properties of this "helix-to-turn" mutant apoLp-III revealed defective lipid binding properties. In more refined site-directed mutagenesis studies it was determined that Val 97, located in the center of helix 3′, is a critical residue for initiation of apoLp-III lipid binding. As described above, a similar short helix was identified in *L. migratoria* apoLp-III based on its NMR determined solution structure (Fan *et al.*, 2003). This helix, however, is present as the opposite end of the apoLp-III helix bundle suggesting that, if it is a recognition helix, bundle opening is different from that proposed for *M. sexta* apoLp-III by Narayanaswami *et al.* (1999) and Wang *et al.* (2002). It is conceivable that an experimental approach similar to that employed by Narayanaswami *et al.* (1999) will permit direct experimental assessment of the role of this short helix in *L. migratoria* apoLp-III lipid interaction.

Studies of the effect of the glycosyl moieties of *L. migratoria* apoLp-III on its lipid binding properties have also been investigated. Soulages *et al.* (1998) showed that recombinant apoLp-III, which lacks covalently bound carbohydrate, displayed a much stronger interaction with phospholipid vesicles than natural insect-derived apoLp-III. From the X-ray structure of *L. migratoria* apoLp-III in the absence of lipid, it is known that both glycosylation sites (at residues 18 and 85) are localized in the central region of the long axis of the bundle. Further study of this phenomenon revealed that apoLp-III sugar moieties interfere with helix bundle penetration into the bilayer surface during disruption and transformation into disk complexes (Weers *et al.*, 2000). Thus, it is apparent that structural aspects of the helix bundle as well as the composition of the lipid surface influence the ability of apoLp-III to initiate and form a stable lipid-binding interaction.

4.6.3.4. ApoLp-III Alternate Functions

Based on the observed developmentally timed upregulation of its mRNA, apoLp-III has been implicated in muscle and neuron programmed cell death (Sun *et al.*, 1995). When considered in light of its known lipid interaction properties, it is conceivable that it serves a function in membrane dissolution and/or lipid reabsorption during metamorphosis. Others have reported apoLp-III functions in insect immunity (Wiesner *et al.*, 1997). Indeed, recent reports suggest that it is lipid associated apoLp-III that manifests this biological activity (Dettloff *et al.*, 2001a, 2001c). These authors hypothesize that LDLp, formed *in vivo*, serves as an endogenous signal for immune activation, specifically mediated by lipid-associated apoLp-III interaction with hemocyte membrane receptors. From a structural standpoint, the truncated variants of *G. mellonella* apoLp-III (see above) that retain functional ability,

represent useful tools to probe the structural and physiological role of apoLp-III in innate immunity. Support for this general concept has emerged from studies of *G. mellonella* apoLp-III variants wherein point mutations were introduced at residues 66 and 68 (Niere *et al.*, 2001). The observation that mutation-induced decreases in apoLp-III lipid interaction properties correlate with decreased immune inducing activity is consistent with the hypothesis that apoLp-III immune activation is related to the conformational change that accompanies lipid interaction of this protein. On a broader scale it is important to understand the molecular details of this emerging group of proteins (Narayanaswami and Ryan, 2000) because the property of reversible interconversion between water-soluble and lipid bound states could have applications beyond their natural biological settings. Indeed, as work on this system continues, it is evident that apoLp-III and analogous helix bundle apolipoproteins represent novel biosurfactants with potentially useful properties, including biodegradability.

4.6.4. Lipophorin Receptor Interactions

4.6.4.1. The Low-Density Lipoprotein Receptor Family

In the concept of lipid transport during intense lipid utilization in insects, a major difference between the functioning of lipoproteins of mammals and insects is the selective mechanism by which insect lipoproteins transfer their hydrophobic cargo. Circulating HDLp particles may serve as a DAG donor or acceptor, dependent on the physiological situation, and function as a reusable lipid shuttle without additional synthesis or increased degradation of the apolipoprotein matrix, as discussed above. In apparent contrast to this concept, in fat body tissue of larval and young adult locusts, receptor-mediated uptake of HDLp was demonstrated (Dantuma *et al.*, 1997). A receptor has been cloned and sequenced from locust fat body cDNA, and identified as a novel member of the LDL receptor (LDLR) family, that is particularly expressed in fat body, oocytes, and the brain (Dantuma *et al.*, 1999). When stably transfected in an LDLR-deficient Chinese hamster ovary cell line, the locust receptor mediated endocytic uptake of fluorescently labeled HDLp that was absent in mock-transfected cells, suggesting that the receptor may function *in vivo* as an endocytic receptor for HDLp (Dantuma *et al.*, 1999). Domain organization of this insect lipophorin receptor (iLR; in this review LpR is adopted) shows a high similarity to mammalian LDLR. However, the ligand binding domain of LpR contains one additional cysteine-rich repeat compared to the seven repeats in LDLR, and is therefore identical to that of the human very low-density lipoprotein (VLDL) receptor (VLDLR), which also contains eight consecutive cysteine-rich repeats in this domain, as is schematically depicted in Figure 5. The amino acid sequence of the longer cytoplasmic tail of LpR is unique for insect lipophorin receptors: the 12 C-terminal amino acid residues of LDLR are completely different from those of LpR, whereas the C-terminal tail of LpR contains an additional 10 amino acid residues (Van Hoof *et al.*, 2002). A similar VLDLR homolog was identified in mosquito oocytes and shown to bind lipophorin (Cheon *et al.*, 2001). Three-dimensional models of the elements representing both the ligand binding

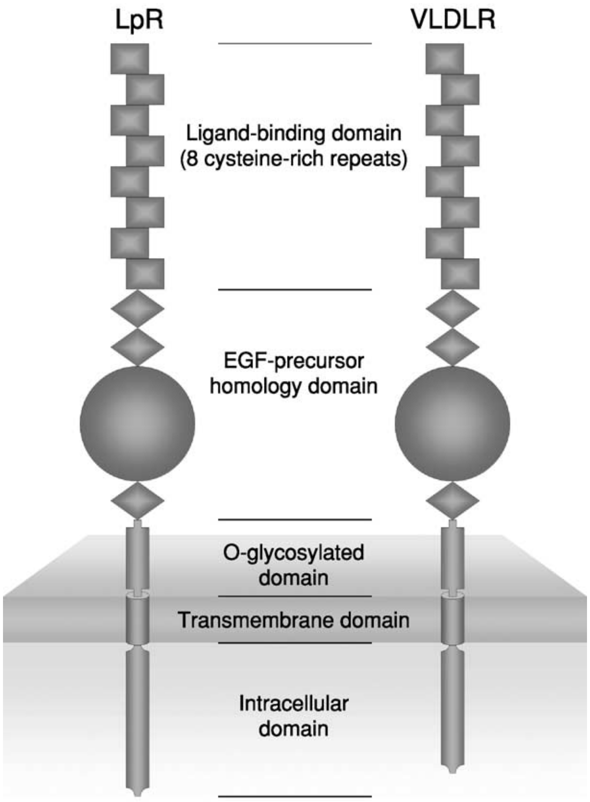


Figure 5 Schematic representation of the insect lipophorin receptor (LpR) and the mammalian VLDL receptor (VLDLR), indicating the identical domain organization. The mammalian LDL receptor has the same organization, but one ligand binding repeat less. EGF, epidermal growth factor. (Based on data from Dantuma, N.P., Potters, M., De Winther, M.P.J., Tensen, C.P., Kooiman, F.P., *et al.*, **1999**. An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins. *J. Lipid Res. 40*, 973–978.)

domain and the epidermal growth factor precursor homology domain of locust LpR bear a striking resemblance to those of mammalian LDLR (Van der Horst *et al.*, 2002). Despite their pronounced structural similarity, however, the ligand specificity of LpR and LDLR for lipophorin and LDL, respectively, is mutually exclusive (Van Hoof *et al.*, 2002). Additionally, the functioning of both receptors in lipid transport in the two animal groups seems to be intriguingly different, as discussed below.

Interaction of HDLp with a specific high-affinity binding site or receptor in the cell membrane of the fat body and other tissues of several insect species has been well documented (review: Ryan and Van der Horst, 2000; see also the recent data by Pontes *et al.*, 2002; Van Hoof *et al.*, 2003). In the binding of human LDL to its receptor, the most C-terminal 1000 amino acids in apoB are involved (Borén *et al.*, 1998). Remarkably, although both the sequence and domain structure of the precursor protein of insect apoLp-I and apoLp-II (apoLp-II/I) resemble that of apoB100 (Babin *et al.*, 1999; Van der Horst *et al.*, 2002), apoLp-II/I does not show homology to this C-terminal part of apoB100, leaving the receptor-binding domain of apoLp-II/I to be disclosed in the future.

Immunocytochemical localization of HDLp has demonstrated the presence of the lipoprotein in endosomes of fat body of the larval dragonfly *Ashna cyanea* (Bauerfeind and Komnick, 1992) and in developing mosquito oocytes (Sun *et al.*, 2000), suggesting endocytosis of circulating HDLp. Uptake of HDLp in the fat body of young adult locusts was shown to be receptor mediated (Dantuma *et al.*, 1997). A recent study presents evidence for the involvement of LpR in the endocytic uptake mechanism for HDLp in the locust that is temporally present during specific periods of development (Van Hoof *et al.*, 2003). Shortly after ecdysis, when lipid reserves are depleted, LpR is expressed in fat body tissue of young adult locusts as well as larvae, and fat body cells are able to endocytose the complete HDLp particle. On the fourth day after (larval or imaginal) ecdysis, however, expression of LpR is downregulated and drops below detectable levels; consequently, HDLp is no longer internalized. Downregulation of LpR was postponed by experimental starvation of adult locusts immediately after ecdysis. Moreover, by starving adult locusts after downregulation of LpR, expression of the receptor was induced. These data suggest that LpR expression is regulated by the demand of fat body tissue for lipid components (Van Hoof *et al.*, 2003).

4.6.4.2. Ligand Recycling Hypothesis

Receptor-mediated uptake of HDLp in newly ecdysed adult and larval locusts may provide a mechanism for the uptake of specific lipid components separate from the mechanism of selective unloading of HDLp lipid cargo at the cell surface. At this time, however, the simultaneous existence of the two distinct mechanisms cannot be excluded. Downregulation of LpR in fat body cells suggests that this receptor is not involved in the lipophorin shuttle mechanism operative in the flying insect. Nevertheless, an endocytic uptake of HDLp seems to conflict with the selective process of lipid transport between HDLp and fat body cells without degradation of the lipophorin matrix. However, the pathway followed by the internalized HDLp may be different from the classical receptor-mediated lysosomal pathway typical of LDLR-internalized ligands. Therefore, an intriguing question is whether this novel LpR, in contrast to all other members of the LDLR family, is able to recycle its ligand after intracellular trafficking. In mammalian cells, LDL and diferric transferrin have been used extensively to study intracellular transport of ligands that are internalized by receptor-mediated endocytosis (Goldstein *et al.*, 1985; Brown and Goldstein, 1986; Mellman, 1996; Mukherjee *et al.*, 1997). Whereas LDL dissociates from its receptor and is completely degraded in lysosomes, transferrin remains attached to its receptor and is eventually resecreted from the cells (Ghosh *et al.*, 1994). LDLR-expressing Chinese hamster ovary cells transfected with LpR cDNA were used to study the endocytic uptake and intracellular pathways of locust HDLp simultaneously with either human LDL or transferrin. Intracellular trafficking of multiple fluorescently labeled ligands was visualized by multicolor confocal laser scanning microscopy, and provided evidence for different intracellular routes followed by the mammalian and insect lipoproteins (Van Hoof *et al.*, 2002) (**Figure 6**). In contrast to LDL, which is degraded in lysosomes after dissociating from its receptor, HDLp remained coupled to LpR and was transported to a nonlysosomal juxtanuclear compartment. Colocalization of HDLp with transferrin identified this organelle as the endocytic recycling compartment, from which internalized HDLp was eventually resecreted (half-time ~13 min), in a manner similar to that operative in the transferrin recycling pathway.

The above data indicate that, in mammalian cells, endocytosed insect HDLp, in contrast to human LDL, follows a recycling pathway mediated by LpR. This promises to elucidate new aspects of

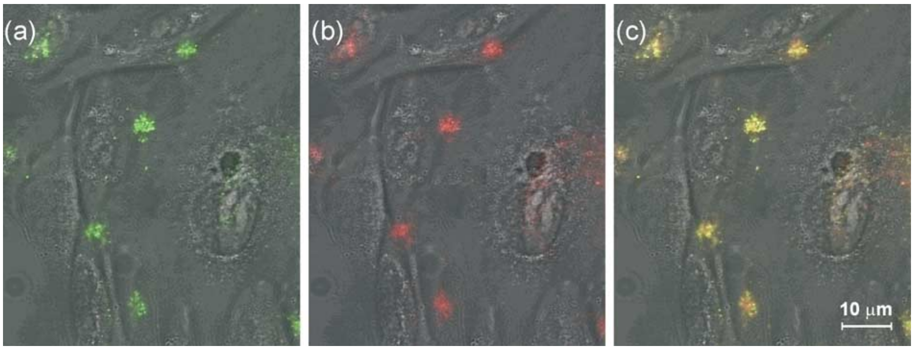


Figure 6 Confocal laser microscopic digital image of Chinese hamster ovary cells incubated with fluorescently labeled HDLp (a) and transferrin (b) after a chase period of 20 min. Colocalization of both ligands is visualized in yellow when images (a) and (b) are merged (c). (Reproduced with permission from Van der Horst, D.J., Van Hoof, D., Van Marrewijk, W.J.A., Rodenburg, K.W., **2002**. Alternative lipid mobilization: the insect shuttle system. *Mol. Cell. Biochem. 239*, 113–119; © Kluwer Academic Publishers.)

LDLR functions, although recycling of endocytosed HDLp in insect fat body cells remains to be shown. Additionally, although the acidic endosomal environment of endocytosed HDLp has been postulated to facilitate the transfer of lipid components other than DAG or cholesterol (Dantuma *et al.*, 1997), both the function of the process of receptor-mediated endocytosis and the rationale for its occurrence during specific stages of insect development remain to be elucidated.

4.6.5. Other Lipid Binding Proteins

4.6.5.1. Lipid Transfer Particle

The concept that specialized proteins exist, which function in redistribution of hydrophobic lipid molecules, is well documented in the mammalian literature. A wide variety of distinct lipid transfer proteins have been characterized and their metabolic roles investigated. In 1986 a lipid transfer particle (LTP) was isolated from *M. sexta* larvae and shown to facilitate vectorial redistribution of lipids among plasma lipophorin subspecies (Ryan *et al.*, 1986a, 1986b). In subsequent studies LTP was implicated in formation of LDLp from HDLp in response to AKH (Van Heusden and Law, 1989). The concept that LTP functions in flight-related lipophorin conversions correlates well with observed increases in LTP concentration in adult hemolymph compared with other developmental stages (Van Heusden *et al.*, 1996; Tsuchida *et al.*, 1998). When compared to other lipid transfer proteins, however, LTP displays unique structural characteristics. For example, it exists as a high molecular weight complex of three apoproteins (apoLTP-I, ~320 000 kDa; apoLTP-II, 85 000 kDa; and apoLTP-III, 55 000 kDa) and 14% noncovalently associated lipid (Ryan *et al.*, 1988). LTPs exhibiting similar structural properties have been isolated from *L. migratoria*, *Periplaneta americana*, *B. mori*, and *Rhodnius prolixus* hemolymph (Hirayama and Chino, 1990; Takeuchi and Chino, 1993; Tsuchida *et al.*, 1997; Golodne *et al.*, 2001). The large size of LTP permitted examination of its structural properties by negative stain electron microscopy (Ryan *et al.*, 1990a; Takeuchi and Chino, 1993). LTP from these two distinct species displays a highly asymmetric morphology with two major structural features, a quasispherical head region and an elongated cylindrical tail, which appears to possess a central hinge. The lipid component resembles that of lipophorin in that it contains predominantly phospholipid and DAG (Ryan, 1990). An important question arising from these physical characteristics relates to the requirement of the lipid component as a structural entity and/or its involvement in catalysis of lipid transfer. Studies employing lipoproteins containing radiolabeled lipids in incubations with LTP have revealed that the lipid component of the particle is in dynamic equilibrium with that of lipoprotein substrates (Ryan *et al.*, 1988). Thus, it is evident that the lipid moiety is not merely a static structural component of LTP. Rather, it can be considered as a functional element in the mechanism of lipid transfer.

Very recently, studies of the *Drosophila* genome revealed the presence of a homolog of mammalian microsomal lipid transfer protein, MTP. Sellers *et al.* (2003) provided evidence that *Drosophila* MTP possesses the functional ability to transfer TAG onto nascent apolipoprotein B-containing lipoproteins in transfected COS cells, although it does not facilitate transfer of TAG among vesicles *in vitro*. Future studies, designed to elucidate the physiological role of this newly identified insect MTP and its participation in lipophorin assembly,

interconversions or some other aspect of lipid transport, represents a fruitful direction for future research. Given the size similarity between *Drosophila* MTP and apoLTP-II from other insect species, as well as their apparent functional similarities (see below), it is conceivable that apoLTP-II may be related to insect MTP.

4.6.5.1.1. Lipid substrate specificity Experiments have been conducted to examine the ability of LTP to utilize various substrate lipids. As reviewed earlier (Ryan and Van der Horst, 2000; Arrese *et al.*, 2001) LTP catalyses the exchange and net transfer of DAG, in keeping with its proposed role in lipophorin interconversions *in vivo*. Extending this concept, Canavoso and Wells (2001) incubated radiolabeled midgut sacs with lipophorin containing medium. These authors found that transfer of DAG from the midgut sacs to lipophorin was blocked by preincubation with antibody against LTP, supporting the view that LTP functions in DAG export from the midgut to lipophorin. In a similar manner, LTP was shown by Jouni *et al.* (2003) to be required for DAG transfer from lipophorin to *B. mori* ovarioles.

In studies of other potential lipid substrates, Singh and Ryan (1991) used [$^{14}C_1$]acetate to label the DAG and hydrocarbon moiety of lipophorin *in vivo*. Subsequent lipid transfer experiments revealed that LTP is capable of facilitating transfer of hydrocarbon among lipoprotein substrates, suggesting LTP plays a role in movement of these extremely hydrophobic, specialized lipids from their site of synthesis to their site of deposition at the cuticle (Takeuchi and Chino, 1993). Interestingly, the rate of LTP-mediated hydrocarbon transfer was slower than DAG transfer. In other work, *B. mori* LTP was employed in studies of LTP-mediated carotene transfer among lipophorin particles (Tsuchida *et al.*, 1998). Again, compared to DAG transfer, the rate of LTP-mediated carotene redistribution was much slower. Taken together, these results suggest that LTP may have a preference for DAG versus hydrocarbon or carotenes. Alternatively, the observed preference for DAG may be a function of the relative accessibility of the substrates within the donor lipoprotein. The ability of LTP to facilitate phospholipid transfer was studied by Golodne *et al.* (2001). These authors observed that LTP mediated a time-dependent transfer of phospholipid that was nonselective. In contrast to the requirement for LTP to mediate transfer of DAG, hydrocarbon, carotenoids, and phospholipids, Yun *et al.* (2002) provided evidence that LTP does not function in cholesterol transfer or redistribution in *M. sexta*. Rather, cholesterol is proposed to diffuse among tissues via mass action, freely transferring between lipophorin and tissues, depending on the physiological need. In keeping with this interpretation, Jouni *et al.* (2003) found that cholesterol transfer from lipophorin to *B. mori* ovarioles was unaffected by antibodies directed against LTP, whereas DAG transfer was inhibited.

4.6.5.1.2. Mechanism of facilitated lipid transfer In general lipid transfer catalysts may act as carriers of lipid between donor and acceptor lipoproteins or transfer may require formation of a ternary complex between donor, acceptor, and LTP. Based on the observed LTP-mediated net transfer of DAG from HDLp to human LDL (Ryan *et al.*, 1990b), a strategy was developed to address this question experimentally (Blacklock *et al.*, 1992). [^{3}H]-DAG–HDLp and unlabeled LDL were covalently bound to Sepharose matrices and packed into separate columns connected in series, followed by circulation of LTP or buffer. Circulation of LTP, but not buffer, resulted in a concentration-dependent increase in the amount of radiolabeled DAG recovered in the LDL fraction, revealing that LTP facilitates net lipid transfer via a carrier-mediated mechanism.

Blacklock and Ryan (1995) employed LTP apolipoprotein specific antibodies to probe the structure and catalytic properties of *M. sexta* LTP, obtaining evidence that apoLTP-II is a catalytically important apoprotein. In a similar manner Van Heusden *et al.* (1996) employed LTP antibody inhibition experiments to demonstrate that all three LTP apoproteins are important for lipid transfer activity. These authors found that, unlike apoLp-III, apoLTP-III is not found as a free protein in hemolymph (Van Heusden *et al.*, 1996) despite the fact that it dissociates from the complex following exposure to nonionic detergent (Blacklock and Ryan, 1995).

4.6.5.2. Carotenoid Binding Proteins

In insects, the involvement of lipophorin in the hemolymph transport of dietary carotenoids is well documented (Tsuchida *et al.*, 1998). Lipophorin may selectively deposit these isoprenoid lipid components at specific tissues, which is in keeping with the general function of the insect lipoprotein to selectively accept and deliver its hydrophobic cargo. The mechanism involved in this selective delivery is not fully understood, and may involve LTP and/or the lipophorin receptor, as discussed above. Carotenoids fulfill several important roles in insects. Certain carotenoids are the provitamins for vitamin A, which is required as the visual

pigment chromophore in the animal kingdom, including *D. melanogaster* (Giovannucci and Stephenson, 1999). Another example is the production of a yellow cocoon in the silkworm, *B. mori*, which is dependent on the availability of carotenoids in the silk gland (Tabunoki *et al*., 2002). On both aspects, novel data illustrate both the presence and function of carotenoid binding proteins in insects.

Recently, a carotenoid binding protein (CBP) from the silk gland of *B. mori* larvae has been identified (Tabunoki *et al*., 2002). The function of the 33 kDa protein was studied using several phenotypes of *B. mori* mutants; only in mutants carrying the dominant *Y* (yellow hemolymph) gene CBP is present in the villi of the midgut epithelium, suggesting that CBP may be involved in absorption of carotenoids. Similarly, using another mutant, it was inferred that CBP is involved in the uptake of carotenoids by the silk gland. The deduced amino acid sequence from CBP cDNA revealed the protein to be a novel member of the steroidogenic acute regulatory (StAR) protein family; CBP binds carotenoids rather than cholesterol.

Another protein involved in the cellular uptake of dietary carotenoids was discovered in *Drosophila*. Recent studies demonstrate that the molecular basis for blindness of the *ninaD* visual mutant fly is a defect in the cellular uptake of carotenoids (Kiefer *et al*., 2002). The *ninaD* gene encodes a class B scavenger receptor with significant sequence identity to the mammalian class B scavenger receptors, SR-BI and CD36; the loss of this function abolishes carotenoid uptake and results in a vitamin A-deficient phenotype. In mammals, class B scavenger receptors, particularly SR-BI, are involved in cholesterol homeostasis and mediate the bidirectional flux of unesterified cholesterol between target cells and lipoproteins (Jian *et al*., 1998; Yancey *et al*., 2000). As lipophorin is structurally related to mammalian lipoproteins, it was speculated that the *ninaD* scavenger receptor mediates the transfer of carotenoid from lipophorin in a mechanistically similar manner (Kiefer *et al*., 2002). *ninaD* mRNA levels were particularly high in pupae, which may indicate that NinaD has an important role in the redistribution of zeaxanthin, the larval storage form of carotenoids in the fat body, to the developing eyes during pupation (Giovannucci and Stephenson, 1999). Interestingly, in 1–2-day-old adults, *ninaD* mRNA levels were equally high, in contrast to older adults (>10 days) where *ninaD* mRNA was hardly visible. This suggests a similarity to the expression of the lipophorin receptor in the adult locust (Van Hoof *et al*., 2003), and may indicate that both proteins function in specific lipid metabolic processes in the young adult stage that are no longer required in the older adult.

4.6.5.3. Fatty Acid Binding Proteins

Hydrolysis of LDLp-carried DAG by a lipophorin lipase at the flight muscles (review: Van der Horst *et al*., 2001) results in the extracellular production of FFAs. After uptake by the flight muscle cells, these FFAs are oxidized for energy generation. The mechanism by which the extracellularly liberated FFAs are translocated across the plasma membrane is as yet unknown, but may involve membrane fatty acid transporter proteins similar to those identified in mammals, i.e., fatty acid translocase (FAT)/CD36, fatty acid transport protein (FATP), and plasma membrane fatty acid binding protein ($FABP_{pm}$) (reviews: Glatz and Van der Vusse, 1996; Brinkmann *et al*., 2002). The intracellular transport of FFAs in insect flight muscle is mediated by a fatty acid binding protein (FABP) (review: Haunerland, 1997). This insect FABP belongs to the cytoplasmic FABPs that comprise a family of 14–15 kDa proteins that bind fatty acid ligands with high affinity and are involved in shuttling fatty acids to cellular compartments, modulating intracellular lipid metabolism, and regulating gene expression (review: Boord *et al*., 2002). Intriguingly, in contrast to FABP in mammals, locust FABP is an adult-specific protein, the expression of which is directly linked to metamorphosis; to accomplish the extremely high metabolic rate encountered during migratory flight, the concentration of FABP in locust flight muscle cytosol is over three times that as in the mammalian heart (review: Haunerland, 1997). The high amino acid sequence similarity (82%) between the FABP of *L. migratoria* flight muscle and that of human skeletal muscles (Maatman *et al*., 1994) is reflected in a strong similarity in their three-dimensional structures (Van der Horst *et al*., 2002).

4.6.5.4. Lipases

Similar to those in mammals, the lipids that are oxidized during sustained physical exercise in insects are derived from stored TAG reserves by the action of a lipase. In mammals, however, FFAs are mobilized from adipose tissue and transported in the blood bound to serum albumin for uptake and oxidation in muscle, whereas in insects, DAG is released from the fat body and transported to the flight muscles by lipophorin as discussed above, requiring hydrolysis of DAG at the flight muscles to liberate the fatty acids that are eventually taken up. The lipase catalyzing the hydrolysis of TAG

stores in both mammals and insects plays a crucial role in energy metabolism by providing the source of energy for the working muscles, and thus between mammalian adipose tissue lipase (HSL) and insect fat body TAG lipase there is a clear functional similarity. However, there is an essential difference in the mode of action of both lipolytic enzymes, as TAG hydrolysis by HSL results in the release of FFAs, whereas that of the insect TAG lipase results in release of DAG.

HSL is regulated posttranscriptionally by phosphorylation; structural studies of the enzyme have identified several amino acids and regions of the molecule that are critical for enzymatic activity and regulation of HSL (reviews: Holm *et al*., 1997; Kraemer and Shen, 2002). In addition, other lipid-droplet associated proteins such as perilipins and lipoptransin may be involved in the interaction of HSL with the stored lipid (reviews: Ryan and Van der Horst, 2000; Van der Horst *et al*., 2001). Recent data in which the acute changes in HSL activity of human adipose tissue were monitored during physical exercise by assaying the phosphorylation state of the enzyme show that lipase activity increased over sixfold above baseline at 5 min of exercise and subsequently decreased gradually despite continuation of the exercise stimulus (Petridou and Mougios, 2002).

4.6.5.4.1. Regulation of lipolysis In spite of the importance of lipid mobilization for sustained insect flight, the regulation of lipolysis in insect fat body is largely unknown. The involvement of AKHs in lipolysis is without debate and was demonstrated both *in vivo* from enhanced levels of DAG in hemolymph of insects injected with the hormones and *in vitro* by the accumulation of DAG in isolated *L. migratoria* fat body tissue that was incubated in the presence of AKH; both cAMP and Ca^{2+} were shown to play an important role in the effect of AKH on lipolysis (reviews: Ryan and Van der Horst, 2000; Van der Horst *et al*., 2001; Van der Horst and Oudejans, 2003).

In two insect species that rely on lipid mobilization during flight activity, *L. migratoria* and *M. sexta*, it has been shown that the DAG, which is released from the fat body by the action of AKH, is stereospecific and has the *sn*-1,2 configuration (review: Ryan and Van der Horst, 2000). The pathway for the stereospecific synthesis of this *sn*-1,2-DAG is not well understood. Although a number of experimental data suggest that a stereospecific TAG lipase is involved (Arrese and Wells, 1997; Arrese *et al*., 2001) other data obtained by the same authors are conflicting with this view (review: Ryan and Van der Horst, 2000). For instance, direct stereospecific hydrolysis of TAG into *sn*-1,2-DAG would involve enzymatic removal of the *sn*-3 fatty acid. As the latter fatty acid was not accumulated in the fat body nor in the hemolymph (Arrese *et al*., 2001), conclusive data demonstrating unambiguously the occurrence of this mode of TAG lipase action is still lacking.

In summary, unlike the functional similarity between HSL in vertebrate adipose tissue and TAG lipase in insect fat body, there is a discrepancy in the mode of action of both lipolytic enzymes. The vertebrate HSL catalyzes nonstereospecific hydrolysis of TAG (as well as of DAG and monoacylglycerol) (review: Kraemer and Shen, 2002), leading to the release of fatty acids, whereas the action of the insect TAG lipase results in the formation and release of *sn*-1,2-DAG. So far, the reason for this discrepancy is unknown, but both structural and regulatory aspects may be involved. Elucidation of the mechanism of TAG lipase action and the regulation of its activity is essential for a better insight in the as yet poorly understood mechanism of flight-directed lipolysis in insects.

4.6.5.5. Vitellogenin

Oocyte development in adult females involves the accumulation of large amounts of lipid, most of which is extraovarian in origin and is delivered by lipophorin. Another lipid binding protein that serves a role in lipid transport to the oocyte is vitellogenin; although its overall contribution to the oocyte lipid accumulation is relatively modest (about 5%) (Sun *et al*., 2000). While the structural properties of insect vitellogenins are diverse, they generally possess ~10% lipid, primarily phospholipid and glycerolipid. Vitellogenin is synthesized and assembled in the fat body, secreted into hemolymph, and taken up by oocytes. Vitellogenin uptake is facilitated by members of a subfamily of the LDLR family that have been characterized in *Drosophila* (Schonbaum *et al*., 1995) and in the mosquito *Aedes aegypti* (Sappington *et al*., 1996). Recently, it was demonstrated that the ovarian vitellogenin receptor (VgR) is only distantly related to the lipophorin receptor (LpR), another ovarian LDLR homolog with a different ligand (Cheon *et al*. 2001). These data imply that the receptor-mediated mechanisms involved in the uptake of lipid and the accumulation of yolk protein precursors (which provide a key nutrient source for the developing oocyte), utilize two separate receptors (VgR and LpR) which are specific for their respective ligands, vitellogenin and lipophorin. Considerable early work was performed to characterize

the lipid transport properties of vitellogenin (review: Kunkel and Nordin, 1985), while more recent work has focused on molecular and evolutionary aspects of vitellogenin proteins and receptor-mediated endocytosis. These aspects, which are beyond the scope of the present treatise, have been comprehensively reviewed elsewhere (Sappington and Raikhel, 1998; Raikhel *et al*., 2002) With respect to lipophorin internalization, it is important to note that immunocytochemical data in the mosquito (Sun *et al*., 2000) revealed that only a small amount of lipophorin accumulates in developing oocytes as yolk protein, comprising ~3% of total ovarian proteins upon completion of protein internalization. Since lipid accounts for 35–40% of the insect egg dry weight (Kawooya and Law, 1988), Sun *et al*. (2000) proposed that internalization of lipophorin is unlikely to be the major route of lipid delivery to the developing oocyte. A dual mechanism for lipophorin-mediated lipid delivery to oocytes (a lipophorin shuttle mechanism involving LDLp and internalization of HDLp, with stripping of most of its lipid) has been demonstrated earlier (Kawooya and Law, 1988; Kawooya *et al*., 1988; Liu and Ryan, 1991). However, considering that the LpR receptor involved in uptake of HDLp by mosquito oocytes bears a high structural similarity to the LpR discovered in locust fat body cell membranes (Dantuma *et al*., 1999; Cheon *et al*., 2001), the precise mechanism of LpR-mediated endocytosis in the oocyte, and the fate of HDLp, remain open questions. In light of the ligand recycling hypothesis discussed earlier for lipid delivery to the fat body, the possibility exists that LpR recycles its ligand after intracellular trafficking, providing another mechanism for the uptake of specific lipid components by the oocyte.

References

Arrese, E.L., Canavoso, L.E., Jouni, Z.E., Pennington, J.E., Tsuchida, K., *et al*., **2001**. Lipid storage and mobilization in insects: current status and future directions. *Insect Biochem. Mol. Biol. 31*, 7–17.

Arrese, E.L., Wells, M.A., **1997**. Adipokinetic hormone-induced lipolysis in the fat body of an insect, *Manduca sexta*: synthesis of *sn*-1,2-diacylglycerols. *J. Lipid Res. 38*, 68–76.

Babin, P.J., Bogerd, J., Kooiman, F.P., Van Marrewijk, W.J.A., Van der Horst, D.J., **1999**. Apolipophorin II/I, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. *J. Mol. Evol. 49*, 150–160.

Baggerman, G., Huybrechts, J., Clynen, E., Hens, K., Harthoorn, L., *et al*., **2002**. New insights in adipokinetic hormone (AKH) precursor processing in *Locusta migratoria* obtained by capillary liquid chromatography-tandem mass spectrometry. *Peptides 23*, 635–644.

Bauerfeind, R., Komnick, H., **1992**. Immunocytochemical localization of lipophorin in the fat body of dragonfly larvae (*Aeshna cyanea*). *J. Insect Physiol. 38*, 185–198.

Beenakkers, A.M.T., Van der Horst, D.J., Van Marrewijk, W.J.A., **1985**. Insect lipids and lipoproteins, and their role in physiological processes. *Prog. Lipid Res. 24*, 19–67.

Blacklock, B.J., Ryan, R.O., **1994**. Hemolymph lipid transport. *Insect Biochem. Mol. Biol. 24*, 855–873.

Blacklock, B.J., Ryan, R.O., **1995**. Structural studies of *Manduca sexta* lipid transfer particle with apolipoprotein-specific antibodies. *J. Lipid Res. 35*, 108–116.

Blacklock, B.J., Smillie, M., Ryan, R.O., **1992**. *Manduca sexta* lipid transfer particle can facilitate lipid transfer via a carrier-mediated mechanism. *J. Biol. Chem. 267*, 14033–14037.

Bogerd, J., Kooiman, F.P., Pijnenburg, M.A.P., Hekking, L.H.P., Oudejans, R.C.H.M., *et al*., **1995**. Molecular cloning of three distinct cDNAs, each encoding a different adipokinetic hormone precursor, of the migratory locust, *Locusta migratoria*: differential expression of the distinct adipokinetic hormone precursor genes during flight activity. *J. Biol. Chem. 270*, 23038–23043.

Boord, J.B., Fazio, S., Linton, M.F., **2002**. Cytoplasmic fatty acid-binding proteins: emerging roles in metabolism and atherosclerosis. *Curr. Opin. Lipidol. 13*, 141–147.

Borén, J., Lee, I., Zhu, W., Arnold, K., Taylor, S., *et al*., **1998**. Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *J. Clin. Invest. 101*, 1084–1093.

Breiter, D.R., Kanost, M.R., Benning, M.M., Wesenberg, G., Law, J.H., *et al*., **1991**. Molecular structure of an apolipoprotein determined at 2.5-Å resolution. *Biochemistry 30*, 603–608.

Brinkmann, J.F.F., Abumrad, N.A., Ibrahimi, A., Van der Vusse, G.J., Glatz, J.F.C., **2002**. New insights into long-chain fatty acid uptake by heart muscle: a crucial role for fatty acid translocase/CD36. *Biochem. J. 367*, 561–570.

Brown, M.S., Goldstein, J.L., **1986**. A receptor-mediated pathway for cholesterol homeostasis. *Science 232*, 34–47.

Canavoso, L.E., Jouni, Z.E., Karnas, K.J., Pennington, J.E., Wells, M.A., **2001**. Fat metabolism in insects. *Annu. Rev. Nutr. 21*, 23–46.

Canavoso, L.E., Wells, M.A., **2001**. Role of lipid transfer particle in delivery of diacylglycerol from midgut to lipophorin in larval *Manduca sexta*. *Insect Biochem. Mol. Biol. 31*, 783–790.

Candy, D.J., **2002**. Adipokinetic hormones concentrations in the haemolymph of *Schistocerca gregaria*, measured by radioimmunoassay. *Insect Biochem. Mol. Biol. 32*, 1361–1367.

Cheon, H.-M., Seo, S.-J., Sun, J., Sappington, T.W., Raikhel, A.S., **2001**. Molecular characterization of the

VLDL receptor homolog mediating uptake of lipophorin in oocytes of the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 31*, 753–760.

Chino, H., **1985**. Lipid transport: biochemistry of hemolymph lipophorin. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 115–134.

Cole, K.D., Fernando-Warnakulasuriya, G.J.P., Boguski, M.S., Freeman, M., Gordon, J.I., *et al.*, **1987**. Primary structure and comparative sequence analysis of an insect apolipoprotein: apolipophorin III from *Manduca sexta*. *J. Biol. Chem. 262*, 11794–11800.

Dantuma, N.P., Pijnenburg, M.A.P., Diederen, J.H.B., Van der Horst, D.J., **1997**. Developmental down-regulation of receptor-mediated endocytosis of an insect lipoprotein. *J. Lipid Res. 38*, 254–265.

Dantuma, N.P., Potters, M., De Winther, M.P.J., Tensen, C.P., Kooiman, F.P., *et al.*, **1999**. An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins. *J. Lipid Res. 40*, 973–978.

Dettloff, M., Kaiser, B., Wiesner, A., **2001a**. Localization of injected apolipophorin III *in vivo*: new insights into the immune activation process directed by this protein. *J. Insect Physiol. 47*, 789–797.

Dettloff, M., Niere, M., Ryan, R.O., Kay, C.M., Wiesner, A., *et al.*, **2002**. Differential lipid binding of truncation mutants of *Galleria mellonella* apolipophorin III. *Biochemistry 41*, 9688–9695.

Dettloff, M., Weers, P.M.M., Niere, M., Kay, C.M., Ryan, R.O., *et al.*, **2001b**. An N-terminal three-helix fragment of an exchangeable insect apolipoprotein, apolipophorin III, conserves the lipid binding properties of the full length protein. *Biochemistry 40*, 3150–3157.

Dettloff, M., Wittwer, D., Weise, C., Wiesner, A., **2001c**. Lipophorin of a lower density is formed during immune responses in the lepidopteran insect *Galleria mellonella*. *Cell Tissue Res. 306*, 449–458.

Diederen, J.H.B., Oudejans, R.C.H.M., Harthoorn, L.F., Van der Horst, D.J., **2002**. Cell biology of the adipokinetic hormone-producing neurosecretory cells in the locust corpus cardiacum. *Microsc. Res. Tech. 56*, 227–236.

Fan, D., Reese, L., Ren, X., Weers, P.M.M., Ryan, R.O., *et al.*, **2001**. Complete ^{1}H, ^{15}N, and ^{13}C assignments of an exchangeable apolipoprotein, *Locusta migratoria* apolipophorin III. *J. Biomol. NMR 19*, 83–84.

Fan, D., Zheng, Y., Yang, D., Wang, J., **2003**. NMR solution structure and dynamics of an exchangeable apolipoprotein, *Locusta migratoria* apolipophorin III. *J. Biol. Chem. 278*, 21210–21220.

Gäde, G., **1996**. The revolution in insect neuropeptides illustrated by the adipokinetic hormone/red pigment-concentrating hormone family of peptides. *Z. Naturforsch. 51C*, 607–617.

Gäde, G., **1997**. The explosion of structural information on insect neuropeptides. *Prog. Chem. Organ. Nat. Prod. 71*, 1–128.

Gäde, G., Hoffmann, K.H., Spring, J.H., **1997**. Hormonal regulation in insects: facts, gaps and future directions. *Physiol. Rev. 77*, 963–1032.

Garda, H.A., Arrese, E.L., Soulages, J.L., **2002**. Structure of apolipophorin III in discoidal lipoproteins: interhelical distances in the lipid-bound state and conformational change upon binding to lipid. *J. Biol. Chem. 262*, 11794–11800.

Ghosh, R.N., Gelman, D.L., Maxfield, F.R., **1994**. Quantification of low density lipoprotein and transferrin endocytic sorting HEp2 cells using confocal microscopy. *J. Cell Sci. 107*, 2177–2189.

Giovannucci, D.R., Stephenson, R.S., **1999**. Identification and distribution of dietary precursors of the *Drosophila* visual pigment chromophore: analysis of carotenoids in wild type and *ninaD* mutants by HPLC. *Vision Res. 39*, 219–229.

Glatz, J.F.C., Van der Vusse, G.J., **1996**. Cellular fatty acid binding proteins: their function and physiological significance. *Prog. Lipid Res. 35*, 243–282.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W., Schneider, W.J., **1985**. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol. 1*, 1–39.

Goldsworthy, G.J., Lee, M.J., Luswata, R., Drake, A.F., Hyde, D., **1997**. Structures, assays and receptors for locust adipokinetic hormones. *Comp. Biochem. Physiol. B 117*, 483–496.

Golodne, D.M., Van Heusden, M.C., Gondim, K.C., Masuda, H., Atella, G.C., **2001**. Purification and characterization of a lipid transfer particle in *Rhodnius prolixus*: phospholipid transfer. *Insect Biochem. Mol. Biol. 31*, 563–571.

Hård, K., Van Doorn, J.M., Thomas-Oates, J.E., Kamerling, J.P., Van der Horst, D.J., **1993**. Structure of the Asn-linked oligosaccharides of apolipophorin III from the insect *Locusta migratoria*: carbohydrate-linked 2-amino-ethylphosphonate as a constituent of a glycoprotein. *Biochemistry 32*, 766–775.

Harthoorn, L.F., Diederen, J.H.B., Oudejans, R.C.H.M., Van der Horst, D.J., **1999**. Differential location of peptide hormones in the secretory pathway of insect adipokinetic cells. *Cell Tissue Res. 298*, 361–369.

Harthoorn, L.F., Diederen, J.H.B., Oudejans, R.C.H.M., Verstegen, M.M.A., Vullings, H.G.B., *et al.*, **2000**. Intracisternal granules in the adipokinetic cells of locusts are not degraded and apparently function as supplementary stores of secretory material. *Eur. J. Cell Biol. 79*, 27–34.

Harthoorn, L.F., Oudejans, R.C.H.M., Diederen, J.H.B., Van der Horst, D.J., **2002**. Coherence between biosynthesis and secretion of insect adipokinetic hormones. *Peptides 23*, 629–634.

Harthoorn, L.F., Oudejans, R.C.H.M., Diederen, J.H.B., Van de Wijngaart, D.J., Van der Horst, D.J., **2001**. Absence of coupling between release and biosynthesis of peptide hormones in insect neuroendocrine cells. *Eur. J. Cell Biol. 80*, 451–457.

Haunerland, N.H., **1997**. Transport and utilization of lipids in insect flight muscle. *Comp. Biochem. Physiol. B 117*, 475–482.

Hirayama, Y., Chino, H., **1990**. Lipid transfer particle in locust hemolymph: purification and characterization. *J. Lipid Res. 31*, 793–799.

Holm, C., Langin, D., Manganiello, V., Belfrage, P., Degerman, E., **1997**. Regulation of hormone-sensitive lipase activity in adipose tissue. *Methods Enzymol. 286*, 45–67.

Huybrechts, J., Clynen, E., Baggerman, G., De Loof, A., Schoofs, L., **2002**. Isolation and identification of the AKH III precursor-related peptide from *Locusta migratoria*. *Biochem. Biophys. Res. Commun. 296*, 1112–1117.

Jian, B., de la Llera-Moya, M., Ji, Y., Wang, N., Phillips, M.C., *et al.*, **1998**. Scavenger receptor class B type I as mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J. Biol. Chem. 273*, 5599–5606.

Jouni, Z.E., Takada, N., Gazard, J., Maekawa, H., Wells, M.A., *et al.*, **2003**. Transfer of cholesterol and diacylglycerol from lipophorin to *Bombyx mori* ovarioles *in vitro*: role of lipid transfer particle. *Insect Biochem. Mol. Biol. 33*, 145–153.

Kanost, M.R., Boguski, M.S., Freeman, M., Gordon, J.I., Wyatt, G.R., *et al.*, **1988**. Primary structure of apolipophorin-III from the migratory locust, *Locusta migratoria*. *J. Biol. Chem. 263*, 10568–10573.

Kawooya, J.K., Keim, P.S., Ryan, R.O., Shapiro, J.P., Samaraweera, P., *et al.*, **1984**. Insect apolipophorin III: purification and properties. *J. Biol. Chem. 259*, 10733–10737.

Kawooya, J.K., Law, J.H., **1988**. Role of lipophorin in lipid transport to the insect egg. *J. Biol. Chem. 263*, 8748–8753.

Kawooya, J.K., Meredith, S.C., Wells, M.A., Kézdy, F.J., Law, J.H., **1986**. Physical and surface properties of insect apolipophorin III. *J. Biol. Chem. 261*, 13588–13591.

Kawooya, J.K., Osir, E.O., Law, J.H., **1988**. Uptake of the major lipoprotein and its transformation in the insect egg. *J. Biol. Chem. 263*, 8740–8747.

Kiefer, C., Sumser, E., Wernet, M.F., von Lintig, J., **2002**. A class B scavenger receptor mediates the cullular uptake of carotenoids in *Drosophila*. *Proc. Natl Acad. Sci. USA 99*, 10581–10586.

Klon, A.E., Segrest, J.P., Harvey, S.C., **2002**. Comparative models for human apolipoprotein A-I bound to lipid in discoidal high-density lipoprotein particles. *Biochemistry 41*, 10895–10905.

Köllisch, G.V., Lorenz, M.W., Kellner, R., Verhaert, P.D., Hoffman, K.H., **2000**. Structure elucidation and biological activity of an unusual adipokinetic hormone from corpora cardiaca of the butterfly, *Vanessa cardui*. *Eur. J. Biochem. 267*, 5502–5508.

Kraemer, F.B., Shen, W.-J., **2002**. Hormone-sensitive lipase: control of intracellular tri- (di-)acylglycerol and cholesteryl ester hydrolysis. *J. Lipid Res. 43*, 1585–1594.

Kunkel, J.G., Nordin, J.H., **1985**. Yolk proteins. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 1. Pergamon Press, Oxford, pp. 83–111.

Liu, H., Ryan, R.O., **1991**. Role of lipid transfer particle in transformation of lipophorin in insect oocytes. Biochim. Biophys. *Acta 1085*, 112–118.

Liu, H., Scraba, D.G., Ryan, R.O., **1993**. Prevention of phospholipase-C induced aggregation of low density lipoprotein by amphipathic apolipoproteins. *FEBS Lett. 316*, 27–33.

Maatman, R.G.H.J., Degano, M., Van Moerkerk, H.T.B., Van Marrewijk, W.J.A., Van der Horst, D.J., *et al.*, **1994**. Primary structure and binding characteristics of locust and human muscle fatty-acid-binding proteins. *Eur. J. Biochem. 221*, 801–810.

Mellman, I., **1996**. Endocytosis and molecular sorting. *Annu. Rev. Cell Devel. Biol. 12*, 575–625.

Mukherjee, S., Ghosh, R.N., Maxfield, F.R., **1997**. Endocytosis. *Physiol. Rev. 77*, 759–803.

Mwangi, R.W., Goldsworthy, G.J., **1977**. Diglyceride-transporting lipoproteins in *Locusta*. *J. Comp. Physiol. 114*, 177–190.

Mwangi, R.W., Goldsworthy, G.J., **1981**. Diacylglycerol-transporting lipoproteins and flight in *Locusta*. *J. Insect Physiol. 27*, 47–50.

Narayanaswami, V., Frolov, A., Schroeder, F., Oikawa, K., Kay, C.M., *et al.*, **1996a**. Fluorescence studies of lipid-association induced conformational adaptations of an exchangeable amphipathic apolipoprotein. *Arch. Biochem. Biophys. 334*, 143–150.

Narayanaswami, V., Ryan, R.O., **2000**. The molecular basis of exchangeable apolipoprotein function. *Biochim. Biophys. Acta 1483*, 15–36.

Narayanaswami, V., Wang, J., Kay, C.M., Scraba, D.G., Ryan, R.O., **1996b**. Disulfide bond engineering to monitor conformational opening of apolipophorin III during lipid binding. *J. Biol. Chem. 271*, 26855–26862.

Narayanaswami, V., Wang, J., Schieve, D., Kay, C.M., Ryan, R.O., **1999**. A molecular trigger of lipid-binding induced opening of a helix bundle exchangeable apolipoprotein. *Proc. Natl Acad. Sci. USA 96*, 4366–4371.

Niere, M., Dettloff, M., Maier, T., Ziegler, M., Wiesner, A., **2001**. Insect immune activation by apolipophorin III is correlated with the lipid-binding properties of this protein. *Biochemistry 40*, 11502–11508.

Oudejans, R.C.H.M., Mes, T.H.M., Kooiman, F.P., Van der Horst, D.J., **1993**. Adipokinetic peptide hormone content and biosynthesis during locust development. *Peptides 14*, 877–881.

Oudejans, R.C.H.M., Van der Horst, D.J., **2003**. Adipokinetic hormones: structure and biosynthesis. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, vol. 1. Academic Press, London, pp. 38–42.

Oudejans, R.C.H.M., Vroemen, S.F., Jansen, R.F.R., Van der Horst, D.J., **1996**. Locust adipokinetic hormones: carrier-independent transport and differential inactivation at physiological concentrations during rest and flight. *Proc. Natl Acad. Sci. USA 93*, 8654–8659.

Petridou, A., Mougios, V., **2002**. Acute changes in triacylglycerol lipase of human adipose tissue during exercise. *J. Lipid Res. 43*, 1331–1334.

Pontes, E.G., Grillo, L.A.M., Gondim, K.C., **2002**. Characterization of lipophorin binding to the fat body of *Rhodnius prolixus*. *Insect Biochem. Mol. Biol. 32*, 1409–1417.

Pownall, H.J., Massey, J.B., Kusserow, S.K., Gotto, A.M., Jr., **1978**. Kinetics of lipid–protein interactions: interaction of apolipoprotein A-I from human plasma high density lipoproteins with phosphatidylcholines. *Biochemistry 17*, 1183–1188.

Prasad, S.V., Ryan, R.O., Wells, M.A., Law, J.H., **1986**. Changes in lipoprotein composition during larval–pupal metamorphosis of an insect, *Manduca sexta*. *J. Biol. Chem. 261*, 558–562.

Raikhel, A.S., Kokoza, V.A., Zhu, J., Martin, D., Wang, S.F., *et al.*, **2002**. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochem. Mol. Biol. 32*, 1275–1286.

Raussens, V., Goormaghtigh, E., Narayanaswami, V., Ryan, R.O., Ruysschaert, J.M., **1995**. Alignment of apolipophorin III α-helices in complex with dimyristoylphosphatidylcholine: a unique spatial orientation. *J. Biol. Chem. 270*, 12542–12547.

Raussens, V., Narayanaswami, V., Goormaghtigh, E., Ryan, R.O., Ruysschaert, J.M., **1996**. Hydrogen/deuterium exchange kinetics of apolipophorin III in lipid free and phospholipid bound states: an analysis by Fourier transform infrared spectroscopy. *J. Biol. Chem. 271*, 23089–23095.

Ryan, R.O., **1990**. The dynamics of insect lipophorin metabolism. *J. Lipid Res. 31*, 1725–1739.

Ryan, R.O., Howe, A., Scraba, D.G., **1990a**. Studies of the morphology and structure of the plasma lipid transfer particle from the tobacco hornworm, *Manduca sexta*. *J. Lipid Res. 31*, 871–879.

Ryan, R.O., Oikawa, K., Kay, C.M., **1993**. Conformational, thermodynamic and stability properties of *Manduca sexta* apolipophorin III. *J. Biol. Chem. 268*, 1525–1530.

Ryan, R.O., Prasad, S.V., Henriksen, E.J., Wells, M.A., Law, J.H., **1986a**. Lipoprotein interconversions in an insect, *Manduca sexta*: evidence for a lipid transfer factor in the hemolymph. *J. Biol. Chem. 261*, 563–568.

Ryan, R.O., Schieve, D.S., Wientzek, M., Narayanaswami, V., Oikawa, K., *et al.*, **1995**. Bacterial expression and site directed mutagenesis of a functional recombinant apolipoprotein. *J. Lipid Res. 36*, 1066–1072.

Ryan, R.O., Senthilathipan, K.R., Wells, M.A., Law, J.H., **1988**. Facilitated diacylglycerol exchange between insect hemolymph lipophorins: properties of *Manduca sexta* lipid transfer particle. *J. Biol. Chem. 263*, 14140–14145.

Ryan, R.O., Van der Horst, D.J., **2000**. Lipid transport biochemistry and its role in energy production. *Annu. Rev. Entomol. 45*, 233–260.

Ryan, R.O., Wells, M.A., Law, J.H., **1986b**. Lipid transfer protein from *Manduca sexta* hemolymph. *Biochem. Biophys. Res. Commun. 136*, 260–265.

Ryan, R.O., Wessler, A.N., Ando, S., Price, H.M., Yokoyama, S., **1990b**. Insect lipid transfer particle catalyzes bidirectional vectorial transfer of diacylglycerol from lipophorin to human low density lipoprotein. *J. Biol. Chem. 265*, 10551–10555.

Sahoo, D., Narayanaswami, V., Kay, C.M., Ryan, R.O., **2000**. Pyrene excimer fluorescence: a spatially sensitive probe to monitor lipid induced rearrangement of apolipophorin III. *Biochemistry 39*, 6594–6601.

Sahoo, D., Weers, P.M.M., Ryan, R.O., Narayanaswami, V., **2002**. Lipid-triggered conformational switch of apolipophorin III helix bundle to an extended helix organization. *J. Mol. Biol. 321*, 201–214.

Sappington, T.W., Kokoza, V.A., Cho, W.L., Raikhel, A.S., **1996**. Molecular characterization of the mosquito vitellogenin receptor reveals unexpected high homology to the *Drosophila* yolk protein receptor. *Proc. Natl Acad. Sci. USA 93*, 8934–8939.

Sappington, T.W., Raikhel, A.S., **1998**. Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem. Mol. Biol. 28*, 277–300.

Schonbaum, C.P., Lee, S., Mahowald, A.P., **1995**. The *Drosophila* yolkless gene encodes a vitellogenin receptor belonging to the low-density lipoprotein receptor superfamily. *Proc. Natl Acad. Sci. USA 92*, 1485–1489.

Sellers, J.A., Hou, L., Athar, H., Hussain, M.M., Shelness, G.S., **2003**. A *Drosophila* microsomal triglyceride transfer protein homolog promotes the assembly and secretion of human apolipoprotein B: implications for human and insect lipid transport and metabolism. *J. Biol. Chem. 278*, 20367–20373.

Singh, T.K.A., Ryan, R.O., **1991**. Lipid transfer particle catalyzed transfer of lipoprotein-associated diacylglycerol and long chain aliphatic hydrocarbons. *Arch. Biochem. Biophys. 286*, 376–382.

Singh, T.K.A., Scraba, D.G., Ryan, R.O., **1992**. Conversion of human low density lipoprotein into a very low density lipoprotein like particle *in vitro*. *J. Biol. Chem. 267*, 9275–9280.

Smith, A.F., Owen, L.M., Strobel, L.M., Chen, H., Kanost, M.R., *et al.*, **1994**. Exchangeable apolipoproteins of insects share a common structural motif. *J. Lipid Res. 35*, 1976–1984.

Soulages, J.L., Arrese, E.L., **2000a**. Dynamics and hydration of the α-helices of apolipophorin III. *J. Biol. Chem. 275*, 17501–17509.

Soulages, J.L., Arrese, E.L., **2000b**. Fluorescence spectroscopy of single tryptophan mutants of apolipophorin-III in discoidal lipoproteins of dymyristoylphosphatidylcholine. *Biochemistry 39*, 10574–10580.

Soulages, J.L., Arrese, E.L., **2001**. Interaction of the α-helices of apolipophorin III with phospholipid acyl chains in discoidal lipoprotein particle: a fluorescence quenching study. *Biochemistry 40*, 14279–14290.

Soulages, J.L., Arrese, E.L., Chetty, P.S., Rodriguez, V., **2001**. Essential role of the conformational flexibility of helices 1 and 5 on the lipid binding activity of apolipophorin III. *J. Biol. Chem. 276*, 34162–34166.

Soulages, J.L., Pennington, J., Bendavid, O., Wells, M.A., **1998**. Role of glycosylation in the lipid-binding activity of the exchangeable apolipoprotein, apolipophorin-III. *Biochem. Biophys. Res. Commun. 243*, 372–376.

Soulages, J.L., Salamon, Z., Wells, M.A., Tollin, G., **1995**. Low concentrations of diacylglycerol promote the binding of apolipophorin III to a phospholipid bilayer: a surface plasmon resonance spectroscopy study. *Proc. Natl Acad. Sci. USA 92*, 5650–5654.

Soulages, J.L., van Antwerpen, R., Wells, M.A., **1996**. Role of diacylglycerol and apolipophorin-III in regulation of physicochemical properties of the lipophorin surface: metabolic implications. *Biochemistry 35*, 5191–5198.

Soulages, J.L., Wells, M.A., **1994a**. Effect of diacylglycerol content on some physicochemical properties of the insect lipoprotein, lipophorin: correlation with the binding of apolipophorin-III. *Biochemistry 33*, 2356–2362.

Soulages, J.L., Wells, M.A., **1994b**. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Adv. Protein Chem. 45*, 371–415.

Staubli, F., Jørgensen, T.J.D., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99*, 3446–3451.

Sun, D., Ziegler, R., Milligan, C.E., Fahrbach, S., Schwartz, L.M., **1995**. Apolipophorin III is dramatically up-regulated during the programmed cell death of insect skeletal muscle and neurons. *J. Neurobiol. 26*, 119–129.

Sun, J., Hiraoka, T., Dittmer, N.T., Cho, K.-H., Raikhel, A.S., **2000**. Lipophorin as a yolk protein in the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 30*, 1161–1171.

Tabunoki, H., Sugiyama, H., Tanaka, Y., Fujii, H., Banno, Y., *et al.*, **2002**. Isolation, characterization, and cDNA sequence of a carotenoid binding protein from the silk gland of *Bombyx mori* larvae. *J. Biol. Chem. 277*, 32133–32140.

Takeuchi, N., Chino, H., **1993**. Lipid transfer particle in the hemolymph of the American cockroach: evidence for its capacity to transfer hydrocarbons between lipophorin particles. *J. Lipid Res. 34*, 543–551.

Tsuchida, K., Arai, M., Tanaka, Y., Ishihara, R., Ryan, R.O., *et al.*, **1998**. Lipid transfer particle catalyzes transfer of carotenoids between lipophorins of *Bombyx mori*. *Insect Biochem. Mol. Biol. 28*, 927–934.

Tsuchida, K., Soulages, J.L., Moribayashi, A., Suzuki, K., Maekawa, H., *et al.*, **1997**. Purification and properties of a lipid transfer particle from *Bombyx mori*: comparison to the lipid transfer particle from *Manduca sexta*. *Biochim. Biophys. Acta 1337*, 57–65.

Vanden Broeck, J., **2001**. Insect G protein-coupled receptors and signal transduction. *Arch. Insect Biochem. Physiol. 48*, 1–12.

Van der Horst, D.J., Oudejans, R.C.H.M., **2003**. Adipokinetic hormones and lipid mobilization. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, vol. 1. Academic Press, London, pp. 34–38.

Van der Horst, D.J., Van Doorn, J.M., Beenakkers, A.M.T., **1979**. Effects of the adipokinetic hormone on the release and turnover of hemolymph diglycerides and on the function of the diglyceride-transporting lipoprotein system during locust flight. *Insect Biochem. 9*, 627–635.

Van der Horst, D.J., Van Doorn, J.M., Beenakkers, A.M.T., **1984**. Hormone induced rearrangements of locust hemolymph lipopoproteins: the involvement of glycoprotein C_2. *Insect Biochem. 14*, 495–504.

Van der Horst, D.J., Van Doorn, J.M., De Keijzer, A.N., Beennakkers, A.M.T., **1981**. Interconversions of diacylglycerol-transporting lipoproteins in the haemolymph of *Locusta migratoria*. *Insect Biochem. 11*, 717–723.

Van der Horst, D.J., Van Hoof, D., Van Marrewijk, W.J.A., Rodenburg, K.W., **2002**. Alternative lipid mobilization: the insect shuttle system. *Mol. Cell. Biochem. 239*, 113–119.

Van der Horst, D.J., Van Marrewijk, W.J.A., Diederen, J.H.B., **2001**. Adipokinetic hormones of insects: release, signal transduction, and responses. *Int. Rev. Cytol. 211*, 179–240.

Van der Horst, D.J., Van Marrewijk, W.J.A., Vullings, H.G.B., Diederen, J.H.B., **1999**. Metabolic neurohormones: release, signal transduction and physiological responses of adipokinetic hormones in insects. *Eur. J. Entomol. 96*, 299–308.

Van Heusden, M.C., Law, J.H., **1989**. An insect lipid transfer particle promotes lipid loading from fat body to lipoprotein. *J. Biol. Chem. 264*, 17287–17292.

Van Heusden, M.C., Yepiz-Plascencia, G.M., Walker, A.M., Law, J.H., **1996**. *Manduca sexta* lipid transfer particle: synthesis by fat body and occurrence in hemolymph. *Arch. Insect Biochem. Physiol. 31*, 39–51.

Van Hoof, D., Rodenburg, K.W., Van der Horst, D.J., **2002**. Insect lipoprotein follows a transferrin-like recycling route that is mediated by the insect LDL receptor homologue. *J. Cell Sci. 115*, 4001–4012.

Van Hoof, D., Rodenburg, K.W., Van der Horst, D.J., **2003**. Lipophorin receptor-mediated lipoprotein endocytosis in insect fat body cells. *J. Lipid Res. 44*, 1431–1440.

Van Marrewijk, W.J.A., **2003**. Adipokinetic hormones and carbohydrate metabolism. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, vol. 1. Academic Press, London, pp. 29–34.

Van Marrewijk, W.J.A., Van der Horst, D.J., **1998**. Signal transduction of adipokinetic hormone. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge, pp. 172–188.

Vroemen, S.F., Van der Horst, D.J., Van Marrewijk, W.J.A., **1998**. New insights into adipokinetic hormone signaling. *Mol. Cell. Endocrinol. 141*, 7–12.

Vullings, H.G.B., Diederen, J.H.B., Veelaert, D., Van der Horst, D.J., **1999**. Multifactorial control of the release of hormones from the locust retrocerebral complex. *Microsc. Res. Tech. 45*, 142–153.

Wang, J., Gagne, S., Sykes, B.D., Ryan, R.O., **1997a**. Insight into lipid surface recognition and reversible conformational adaptations of an exchangeable apolipoprotein by multidimensional heteronuclear NMR techniques. *J. Biol. Chem. 272*, 17912–17920.

Wang, J., Liu, H., Sykes, B.D., Ryan, R.O., **1992**. ^{31}P-NMR study of the phospholipid moiety of lipophorin subspecies. *Biochemistry 31*, 8706–8712.

Wang, J., Liu, H., Sykes, B.D., Ryan, R.O., **1995**. Localization of two distinct microenvironments for the diacylglycerol component of lipophorin particles by 13C-NMR. *Biochemistry 34*, 6755–6761.

Wang, J., Sahoo, D., Schieve, D., Gagne, S., Sykes, B.D., *et al.*, **1997b**. Multidimensional NMR studies of an exchangeable apolipoprotein and its interaction with lipids. *Tech. Protein Chem. 8*, 427–438.

Wang, J., Sykes, B.D., Ryan, R.O., **2002**. Structural basis for the conformational adaptability of apolipophorin III, a helix bundle exchangeable apolipoprotein. *Proc. Natl Acad. Sci. USA 99*, 1188–1193.

Weers, P.M.M., Kay, C.M., Oikawa, K., Wientzek, M., Van der Horst, D.J., *et al.*, **1994**. Factors affecting the stability and conformation of *Locusta migratoria* apolipophorin III. *Biochemistry 33*, 3617–3624.

Weers, P.M.M., Narayanaswami, V., Kay, C.M., Ryan, R.O., **1999**. Interaction of an exchangeable apolipoprotein with phospholipid vesicles and lipoprotein particles: role of leucines 32, 34, and 95 in *Locusta migratoria* apolipophorin III. *J. Biol. Chem. 274*, 21804–21810.

Weers, P.M.M., Van der Horst, D.J., Ryan, R.O., **2000**. Interaction of locust apolipophorin III with lipoproteins and phospholipid vesicles: effect of glycosylation. *J. Lipid Res. 41*, 416–423.

Weers, P.M.M., Van Marrewijk, W.J.A., Beenakkers, A.M.T., Van der Horst, D.J., **1993**. Biosynthesis of locust lipophorin: apolipophorins I and II originate from a common precursor. *J. Biol. Chem. 268*, 4300–4303.

Weers, P.M.M., Wang, J., Van der Horst, D.J., Kay, C.M., Sykes, B.D., *et al.*, **1998**. Recombinant locust apolipophorin III: characterization and NMR spectroscopy. *Biochim. Biophys. Acta 1393*, 99–107.

Wientzek, M., Kay, C.M., Oikawa, K., Ryan, R.O., **1994**. Binding of insect apolipophorin III to dimyristoylphosphatidylcholine vesicles: evidence for a confirmational change. *J. Biol. Chem. 269*, 4605–4612.

Wiesner, A., Losen, S., Kopacek, P., Weise, C., Gotz, P., **1997**. Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect. *J. Insect Physiol. 43*, 383–391.

Woodring, J., Lorenz, M.W., Hoffmann, K.H., **2002**. Sensitivity of larval and adult crickets (*Gryllus bimaculatus*) to adipokinetic hormone. *Comp. Biochem. Physiol. A 133*, 637–644.

Yancey, P.G., de la Llera-Moya, M., Swarnakar, S., Monzo, P., Klein, S.M., *et al.*, **2000**. High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J. Biol. Chem. 275*, 36596–36604.

Yun, H.K., Jouni, Z.E., Wells, M.A., **2002**. Characterization of cholesterol transport from midgut to fat body in *Manduca sexta* larvae. *Insect Biochem. Mol. Biol. 32*, 1151–1158.

4.7 Proteases

M R Kanost and T E Clarke, Kansas State University, Manhattan, KS, USA

4.7.1. Introduction and History

Proteases (peptidases) are enzymes that hydrolyze peptide bonds in proteins. Exopeptidases cleave a terminal amino acid residue at the end of a polypeptide; endopeptidases cleave internal peptide bonds. Hooper (2002) provides a useful introduction to the general properties of proteases. Proteases can be classified based on the chemical groups that function in catalysis. In serine proteases the hydroxyl group in the side chain of a serine residue in the active site acts as a nucleophile in the reaction that hydrolyzes a peptide bond, whereas in cysteine proteases the sulfhydryl group of a cysteine side chain performs this function. In aspartic acid proteases and metalloproteases, a water molecule in the active site (positioned by interacting with an aspartyl group or a metal ion, respectively) functions as the nucleophile that attacks the peptide bond. Proteases are classified on this basis of catalytic mechanism in a system developed by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/). However, proteases can have the same catalytic mechanism but will be unrelated in amino acid sequence, as products of convergent evolution. The MEROPS classification system groups proteases into families based on sequence similarity (Rawlings and Barrett, 1999) (http://merops.sanger.ac.uk).

A protease cleaves a peptide bond, called the scissile bond, between two amino acid residues named P1 and P1′ (Schechter and Berger, 1967). Residues on the N-terminal side of the scissile bond are numbered in the C to N direction, whereas residues on the C-terminal side of the scissile bond (the "prime" side) are numbered in the N to C direction (**Figure 1**). The substrate specificity of most endopeptidases is highly dependent on the nature of the side chain of the P1 residue, but the sequence of other residues near the scissile bond can also affect binding of the substrate to the active site and thus influence substrate specificity.

Insects produce abundant proteases that function in digestion of dietary proteins in the gut. Such proteases are thoroughly discussed in 00053. This chapter focuses on nondigestive proteases, which have many diverse roles in insect biology. These proteases often function in cascade pathways, in which one protease activates the zymogen form of another protease, leading to amplification of an initial signal that may involve a few molecules and finally generating a very large number of effector molecules at the end of the pathway. The complement and blood coagulation pathways in mammals are well-understood examples of this type of protease cascade, which also occur in insect embryonic development and insect immune responses (Jiang and Kanost, 2000; Krem and DiCerra, 2002; see **Chapter 4.5**). Details of the organization and regulation of such pathways in insect biology are beginning to be understood. This chapter will include an

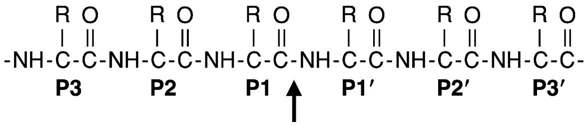


Figure 1 The Schechter and Berger (1967) notation for protease cleavage sites. The arrow designates the scissile peptide bond between amino acid residues P1 and P1′.

emphasis on the current state of knowledge in this rapidly developing area.

Insect proteases have previously been reviewed by Law *et al.* (1977), Applebaum (1985), Terra *et al.* (1996), and Reeck *et al.* (1999). These reviews deal primarily with proteases as they function in the digestion of food. Only quite recently has much detailed information appeared about proteases with other functions in insect biology. An exception is cocoonase, the first insect protease that was purified and well-characterized biochemically. Cocoonase is a serine protease from silk moths that functions to hydrolyze silk proteins in the cocoon, enabling the adult moth to emerge (Kafatos *et al.*, 1967a, 1967b). It digests sericin, the silk protein that cements fibroin threads together (see **Chapter 2.11**). A specialized tissue called the galea, derived from modified mouthparts, synthesizes and secretes the zymogen form, prococoonase (Kafatos, 1972). On the surface of the galea, prococoonase is activated by cleavage at a specific site by an unknown protease in the molting fluid (Berger *et al.*, 1971). Sequencing of an amino terminal fragment and the peptide containing the active site Ser residue indicated that the activation and catalytic mechanisms of coccoonase were quite similar to those of mammalian trypsin (Felsted *et al.*, 1973; Kramer *et al.*, 1973). It is surprising that no molecular cloning has apparently yet been carried out for this historically important insect protease.

4.7.2. Proteases in Eggs and Embryos

4.7.2.1. Proteases That Digest Egg Yolk Proteins

Vitellin and a few other egg-specific proteins stored in yolk granules of insect eggs are digested by proteases to release amino acids for use in embryonic development (Raikhel and Dhadialla, 1992; **Chapter 3.9**). Such enzymes in eggs represent several different protease families and mechanistic classes. In *Bombyx mori*, a serine protease that degrades vitellin was purified from *B. mori* eggs (Indrasith *et al.*, 1988), and its cDNA was cloned (Ikeda *et al.*, 1991). This protease cleaves after Arg or Lys P1 residues and is a member of the S1 (chymotrypsin-like) family of serine proteases. It is synthesized in ovaries as a zymogen and is activated during embryogenesis. A second serine protease from the S1 family specifically degrades the 30 kDa yolk proteins present in *B. mori* eggs (Maki and Yamashita, 1997, 2001). This protease, which is synthesized at the end of embryogenesis, has elastase-like specificity, cleaving after P1 residues with small side chains. A serine carboxypeptidase is synthesized in the fat body of a mosquito, *Aedes aegypti*, transported through the hemolymph, and taken up by oocytes (Cho *et al.*, 1991). This protease is synthesized as a zymogen and activated within eggs during embryogenesis.

Cysteine proteases have been characterized from eggs of several insect species. Those that have been sequenced are from the C1 (papain-like) family of cysteine proteases. They typically have acidic pH optima and have biochemical properties similar to mammalian cysteine proteases known as cathepsins (although not all proteases called cathepsins are cysteine proteases). A 47 kDa cysteine protease that can digest vitelllin has been purified from *B. mori* eggs (Kageyama and Takahashi, 1990; Yamamoto and Takahashi, 1993), and its cDNA has been cloned (Yamamoto *et al.*, 1994). It has sequence similarity to mammalian cathepsin L and a preference for cleaving at sites that have hydrophobic residues at the P2 and P3 positions. It is synthesized as a zymogen in ovary and fat body as a maternal product and taken up into oocytes (Yamamoto *et al.*, 2000). This cysteine protease is self-activated at low pH by proteolytic processing, apparently by a weak activity of the proenzyme under acidic conditions (Takahashi *et al.*, 1993).

Cysteine proteases with sequence similarity to mammalian cathepsin B have also been identified as enzymes that digest insect egg yolk proteins. In *Drosophila melanogaster*, a cysteine protease is associated with yolk granules (Medina *et al.*, 1988). Its zymogen is apparently activated by a serine protease during embryonic development, and the active cathepsin-B then digests yolk proteins. A cysteine protease that digests yolk proteins has also been identified in another higher dipteran, *Musca domestica* (Ribolla and De Bianchi, 1995). In *A. aegypti* a “vitellogenic cathepsin B” is synthesized in adult female fat body after the insect has taken a blood meal, and the zymogen is transported through the hemolymph and taken up by oocytes (Cho *et al.*, 1999). The enzyme is activated by proteolytic processing when embryonic development begins and then probably functions to digest vitellin. A cathepsin B-like protease that can digest vitellin is also synthesized in the fat body and ovaries of a

lepidopteran insect, *Helicoverpa armigera* (Zhao *et al*., 2002). However, its gene is expressed in fat body of males and females and in larvae and pupae, and thus is not coordinated with vitellogenesis as is the mosquito cathepsin-B.

4.7.2.2. The Dorsal Pathway in Embryonic Development

A signal transduction system that regulates dorsal/ventral patterning in *D. melanogaster* embryonic development is activated by an extracellular serine protease cascade (Morisato and Anderson, 1995; LeMosy *et al*., 1999). The members of this cascade are produced maternally and deposited in the space between the vitellin membrane and the embryo. The pathway was elucidated by genetic analysis, and recently the recombinant forms of the proteases have been studied. This pathway involves a serine protease cascade (**Figure 2**) that eventually cleaves an inactive protein called spätzle, making it competent to bind to a transmembrane receptor named Toll (Belvin and Anderson, 1996). Toll is homologous to the mammalian interleukin-1 receptor. Binding of spätzle to Toll initiates a signal transduction pathway that leads to activation of a transcription factor from the rel family named Dorsal.

A large (350 kDa) multi-domain protein called nudel, containing a serine protease domain, regions of LDL receptor repeats (see **Chapter 4.6**), and an N-terminal glycosaminoglycan modification, is secreted by the ovarian follicle cells into the perivitelline space (Hong and Hashimoto, 1995; Turcotte and Hashimoto, 2002). The nudel protease is autoactivated by a mechanism not yet understood (LeMosy *et al*., 1998, 2000) and is thought to activate the second protease in the pathway, "gastrulation defective" by specific proteolysis. Mutations in nudel's protease domain produce a dorsalizing phenotype and can also result in fragile eggshells, suggesting an additional function for the protease activity (Hong and Hashimoto, 1996; LeMosy *et al*., 1998, 2000; LeMosy and Hashimoto, 2000).

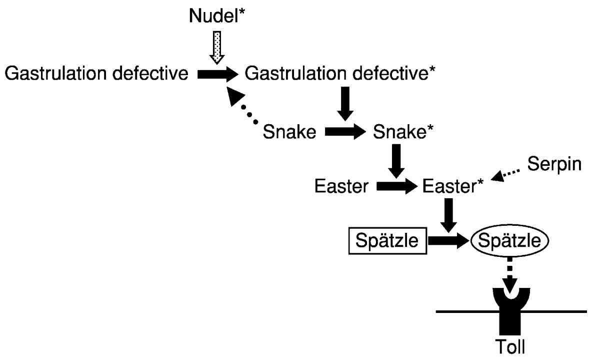


Figure 2 A model of the protease cascade that activates the Dorsal signal transduction pathway in *D. melanogaster* embryonic development. Nudel, gastrulation defective, snake, and easter are serine proteases that are synthesized as zymogens. The active forms of the proteases are indicated with an asterisk. Solid arrows indicate proteolytic activation steps that have been demonstrated by biochemical studies. A dotted arrow indicates that interaction between the snake and gastrulation defective zymogens can leads to activation of gastrulation defective. Easter* cleaves spätzle to produce an active ligand that binds to Toll, a transmembrane receptor. Easter is negatively regulated by interaction with an inhibitor from the serpin family.

Gastrulation defective is a serine protease (Konrad *et al*., 1998; Han *et al*., 2000), with sequence similarity to mammalian complement factors C2 and B (DeLotto, 2001). Experiments with recombinant proteins have demonstrated that the gastrulation defective zymogen can be autoactivated when it interacts with the protease snake zymogen and that active gastrulation defective can in turn proteolytically activate the snake zymogen (Dissing *et al*., 2001; LeMosy *et al*., 2001). Computer modeling studies indicate that the zymogen activation site of gastrulation defective is a good fit in the active site of nudel and that the snake zymogen activation site can dock in the active site of gastrulation defective, consistent with the proposed functions of these enzymes in the cascade pathway (Rose *et al*., 2003). A potential lower affinity interaction of the gastrulation defective active site with its own zymogen activation sequence may explain gastrulation defective's autoactivation in the absence of nudel when it is overexpressed in embryos or at high concentration *in vitro*.

The final two proteases in this cascade, snake and easter, contain C-terminal serine protease domains and N-terminal clip domains similar to horseshoe crab proclotting enzyme (DeLotto and Spierer, 1986; Chasan and Anderson, 1989; Gay and Keith, 1992; Smith and DeLotto, 1992, 1994). Clip domains, thought to function in protein–protein interactions, are also present in some hemolymph proteases of insects (Jiang and Kanost, 2000) (see Section 4.7.3.1 below). Mutations that eliminate the protease activity of snake (Smith *et al*., 1994) or easter (Jin and Anderson, 1990) have abnormal dorsoventral phenotypes, indicating that a functional protease domain is essential for their roles in embryonic development. *In vitro* experiments with recombinant snake and easter zymogens confirm their order in the cascade indicated by genetic analysis: snake cleaves and activates easter, which cleaves prospätzle (Smith *et al*., 1995; DeLotto and DeLotto, 1998; Dissing *et al*., 2001; LeMosy *et al*., 2001). These results are consistent with predictions

based on computer modeling of the snake and easter three-dimensional structures and substrate interactions sites (Rose *et al.*, 2003). Active easter is converted *in vivo* to a high molecular mass form that is probably a complex with a protease inhibitor that regulates its activity (Misra *et al.*, 1998; Chang and Morisato, 2002). Female flies with a mutation in the gene for a serine protease inhibitor, serpin 27A, produce embryos that show defects in dorsal–ventral polarity, suggesting that this inhibitor is a maternal product that regulates at least one of the proteases in the pathway (Hashimoto *et al.*, 2003; Ligoxygakis *et al.*, 2003).

4.7.3. Hemolymph Plasma Proteases

4.7.3.1. Serine Proteases

Serine proteases in hemolymph have several types of physiological functions in defense against infection or wounding. An unusual phenomenon, perhaps related to protection against predation, involves serine proteases in the hemolymph of South American saturniid caterpillars of the genus *Lonomia* that are toxic to mammals. Contact with these caterpillars can result in acquired bleeding disorders due to potent fibrinolytic activity of these hemolymph proteases (Amarant *et al.*, 1991; Arocha-Pinango and Guerrero, 2000).

Extracellular serine protease cascades mediate rapid responses to infection and wounding in vertebrate and invertebrate animals. Biochemical and genetic evidence indicates that activation of serine proteases in arthropod hemolymph is a component of several immune responses, including coagulation, melanotic encapsulation, activation of antimicrobial peptide synthesis, and modulation of hemocyte function (Yoshida and Ashida, 1986; Katsumi *et al.*, 1995; Kawabata *et al.*, 1996; Ashida and Brey, 1998; Levashina *et al.*, 1999; Jiang and Kanost, 2000; Gorman and Paskewitz, 2001; Kanost *et al.*, 2001). Microbial challenge induces expression of many nondigestive serine proteases (Dimopoulos and Della Torre, 1996; Oduol *et al.*, 2000; Irving *et al.*, 2001; Dimopoulos *et al.*, 2002; Zhu *et al.*, 2003a). In *Anopheles gamibiae*, a large, multidomain serine protease, Sp22D, is expressed in hemocytes, fat body, and midgut and is secreted into the hemolymph, with a low level of induced expression in response to infection (Danielli *et al.*, 2000; Gorman *et al.*, 2000a). Sp22D contains chitin-binding domains, lipoprotein receptor class A domains, scavenger receptor domains, and a C-terminal serine protease domain. This complex domain architecture with multiple domains that might function in binding to proteins or polysaccharides suggests that Sp22D may participate in the formation of protein complexes in defense responses or perhaps in tissue remodeling at metamorphosis.

Most of the hemolymph proteases are expressed in fat body or hemocytes, but a bacteria-induced protease, scolexin, from *Manduca sexta* is expressed in epidermis (Kyriakides *et al.*, 1995; Finnerty *et al.*, 1999). Another novel serine protease expressed in pupal yellow body of *Sarcophaga peregrina* has antibacterial activity distinct from its protease activity (Nakajima *et al.*, 1997; Tsuji *et al.*, 1998).

Serine proteases that contain a C-terminal protease domain and an N-terminal clip domain are known to act in cascade pathways in arthropod hemolymph (Jiang and Kanost, 2000). Among clip domain proteases with known function are horseshoe crab proclotting enzyme and clotting factor B (Kawabata *et al.*, 1996), *D. melanogaster* snake and easter, and phenoloxidase-activating proteases described below. Clip domains are 35–55 amino acid residue sequences that contain three conserved disulfide bonds. They may function to mediate interaction of members of protease cascade pathways. Proteases may contain one or more N-terminal clip domains, followed by a 20–100 residue linking sequences connecting them to the catalytic protease domain.

Insects that have been investigated in some detail are known to contain a large number of genes for clip domain proteases. Among the 204 genes with homology to the S1 serine protease family in the *D. melanogaster* genome, 24 are clip domain proteases, most of whose functions are unknown (Ross *et al.*, 2003). The *A. gambiae* genome contains 41 clip domain proteases (Christophides *et al.*, 2002) but only a few have been studied in detail (Han *et al.*, 1997; Paskewitz *et al.*, 1999; Gorman *et al.*, 2000b; review: Gorman and Paskewitz, 2001). In *M. sexta*, more than 20 clip domain proteases expressed in fat body or hemocytes have been identified (Jiang *et al.*, 1999; Jiang, Y. Wang and M.R. Kanost, unpublished results; Kanost *et al.*, 2001).

Melanization, a response to wounding and infection in insects and crustaceans, involves activation of a cascade of serine protease zymogens (**Figure 3**). This pathway leads to rapid activation of a protease that in turn activates a phenoloxidase zymogen (prophenoloxidase; proPO) (Söderhäll *et al.*, 1996; Ashida and Brey, 1998). Oxidation of phenols by phenoloxidase leads to production of quinones that polymerize to form melanin (see **Chapters 4.3** and **4.4**). Melanization of encapsulated parasites is believed to be an important defensive response in insects, including insect vectors of human diseases

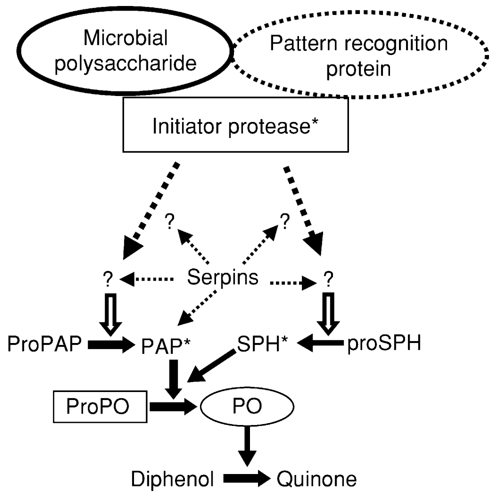


Figure 3 A model of the protease cascade that activates prophenoloxidase in response to infection. Hemolymph plasma proteins known as pattern recognition proteins bind to polysaccharides on the surface of microorganisms. This interaction leads to activation of initiator protease(s) by a mechanism not yet understood, which triggers a protease cascade. The number of proteases in the pathway is not yet known (indicated by dashed arrows). Activation of uncharacterized proteases leads to activation of activator of prophenoloxidase activating protease (PAP) and serine protease homologs (SPH) that function together to form a prophenoloxidase activating enzyme, which cleaves prophenoloxidase (ProPO) to form active phenoloxidase (PO). PO catalyzes the oxidation of hemolymph catecholic phenols to corresponding quinones, which can undergo further reactions to form melanin. Proteases in the pathway are regulated by serine protease inhibitors known as serpins.

(Gillespie *et al.*, 1997; Paskewitz and Gorman, 1999). Serine proteases that activate prophenoloxidase have been purified, and corresponding cDNAs have been cloned from two lepidopteran insects: *M. sexta* (Jiang *et al.*, 1998, 2003a, 2003b) and *B. mori* (Satoh *et al.*, 1999); a beetle, *Holotrichia diomphalia* (Lee *et al.*, 1998a, 1998b); and a crayfish (Wang *et al.*, 2001). All of these enzymes contain a C-terminal serine protease catalytic domain and one or two N-terminal clip domains, similar to horseshoe crab clotting enzyme and *D. melanogaster* easter (Jiang and Kanost, 2000). These proteases are synthesized as zymogens, which must be activated by a protease upstream in the pathway. However, it is not known how many steps occur between recognition of a microorganism by a hemolymph protein and activation of the terminal protease in the pathway. Furthermore, it is not yet understood how the first protease in the cascade is activated. Proteins that bind to microbial surfaces (pattern recognition proteins) are probably involved in this step, through interaction with microbial polysaccharides and an initiating protease. Plasma proteins that bind to bacterial lipopolysaccharide, peptidoglycan, or fungal glucans can stimulate activation of the prophenoloxidase cascade (Ochiai and Ashida, 1988, 1999, 2000; Söderhäll *et al.*, 1988; Yu *et al.*, 1999, 2002; Ma and Kanost 2000; Yu and Kanost, 2000).

Initial characterization of a proPO-activating proteinase in *M. sexta* indicated that the purified protease could not efficiently activate proPO, but required participation of a nonproteolytic protein fraction (Jiang *et al.*, 1998). This protein cofactor was identified in *H. diomphalia* (Kwon *et al.*, 2000) and *M. sexta* (Yu *et al.*, 2003) as a protein with a clip domain and a serine protease domain, in which the active site serine residue is changed to glycine. There are 13 such clip domain serine protease homolog genes in the *D. melanogaster* genome (Ross *et al.*, 2003). These serine protease homologs lack protease activity due to the incomplete catalytic triad and must therefore have other functions. One *D. melanogaster* serine protease homolog, masquerade, functions in nerve and muscle development (Morugasu-Oei *et al.*, 1995, 1996). A serine protease homolog in crayfish hemolymph has a role in immune responses, indicating evolutionary conservation of function in these unusual proteins (Lee and Söderhäll, 2001). The active form of the serine protease homologs that function as cofactors for proPO activation are themselves activated through specific cleavage by a serine protease in hemolymph (Kim *et al.*, 2002; Lee *et al.*, 2002b; Yu *et al.*, 2003) adding additional complexity to this pathway. The serine protease homologs from *M. sexta* that stimulate proPO activation bind to a hemolymph lectin, which is a recognition protein for lipopolysaccharides from Gram-negative bacteria, and to proPO and prophenoloxidase activating protease (Yu *et al.*, 2003). The interaction between the lectin and a proPO activation complex may serve to localize melanin synthesis to the surface of invading bacteria.

Serine proteases are also involved in other insect immune responses. The signal transduction system that regulates dorsal/ventral development in *D. melanogaster* embryos also regulates expression of the gene for an antifungal peptide in larvae and adults (review: Hoffmann, 2003). In embryonic development, this pathway involves an extracellular serine protease cascade that eventually cleaves an inactive protein, spätzle, making it competent to bind to a transmembrane receptor named Toll. This same receptor–ligand interaction activates a signal pathway that leads to activation of rel family transcription factors that stimulate expression of drosomycin, an antifungal peptide normally synthesized by the fat body after microbial challenge.

Mutants deficient in Toll or spätzle exhibit decreased induction of drosomycin (Lemaitre *et al.*, 1996). These results suggest that infection leads to proteolytic processing of spätzle in the hemolymph. The activated spätzle binds to the Toll receptor in fat body membranes, leading to activation of the Dif pathway and expression of drosomycin. Mutants of gastrulation defective, snake, or easter do not have an impaired antifungal response, indicating that a different set of proteases functions in the immune response protease cascade. Like prophenoloxidase activation, this pathway is initiated by interactions of pattern recognition proteins with microbial surface polysaccharides (Kim *et al.*, 2000; Leulier *et al.*, 2003), presumably stimulating activation of the first protease in the cascade. A clip domain protease known as persephone has been shown to participate in this pathway (Ligoxygakis *et al.*, 2002a), but its position in the cascade is not yet known.

4.7.3.2. Protease Inhibitors

Insect hemolymph contains high concentrations of serine protease inhibitors from several different gene families (reviews: Kanost and Jiang, 1996; Polanowski and Wilusz, 1996; Kanost, 1999). Protease cascade pathways in mammalian blood are regulated by ~45 kDa protease inhibitors known as serpins (Silverman *et al.*, 2001; Gettins, 2002). Serpins in arthropod hemolymph also function to regulate protease cascades, preventing detrimental effects of uncontrolled immune responses. For example, each of the proteases in the horseshoe crab coagulation pathway is regulated by serpins produced by hemocytes (Kawabata *et al.*, 1996). Fourteen serpin genes have been identified in the *A. gambiae* genome (Christophides *et al.*, 2002) and 26 serpin genes have been annotated in the *D. melanogaster* genome. In *M. sexta*, 7 serpin genes have been identified (Jiang *et al.*, 1996; Gan *et al.*, 2001; Zhu *et al.*, 2003b). However, the physiological target proteases of only a few insect serpins have been determined (Ashida and Sasaki, 1994; Jiang *et al.*, 2003b; Zhu *et al.*, 2003a).

The reactive site in a serpin protein that interacts with the target protease is part of an exposed loop near the C-terminal end of the serpin sequence. Some insect serpin genes have a unique structure in which mutually exclusive alternate splicing of an exon that encodes the reactive site loop results in production of several inhibitors with different specificity. This was first observed in the gene for *M. sexta* serpin-1, which contains 12 copies of its 9th exon. Each version of exon 9 encodes a different reactive site loop sequence and inhibits a different spectrum of proteases (Jiang *et al.*, 1996; Jiang and Kanost, 1997). Structures of two of the *M. sexta* serpin-1 variants have been determined by X-ray crystallography (Li *et al.*, 1999; Ye *et al.*, 2001). Serpin genes with alternate exons in the same position as in *M. sexta* serpin-1 have been identified in other insect species, including the lepidopterans *B. mori* (Sasaki, 1991; Narumi *et al.*, 1993) and *Mamestra configurata* (Chamankhah *et al.*, 2003), in the dipterans *D. melanogaster* (Kruger *et al.*, 2002) and *A. gambiae* (Danielli *et al.*, 2003), and in the cat flea, *Ctenocephalides felis* (Brandt *et al.*, 2004).

Like blood clotting, phenoloxidase activation is normally regulated *in vivo* as a local reaction of brief duration. This regulation involves serine protease inhibitors in plasma (Kanost and Jiang, 1996; Kanost, 1999). Two serpins from the hemolymph of *M. sexta* (serpin-1J and serpin-3) can inhibit proPO activating proteases (Jiang *et al.*, 2003a, 2003b; Zhu *et al.*, 2003a, 2003b). In *D. melanogaster*, serpin 27A (an apparent ortholog of *Manduca* serpin-3) is involved in regulation of melanization (DeGregorio *et al.*, 2002; Ligoxygakis *et al.*, 2002b). Similarly, a mutation in *D. melanogaster* serpin 43Ac (Necrotic) leads to constitutive expression of drosomycin, indicating that this serpin regulates a protease in the cascade that processes spätzle (**Figure 4**) (Levashina *et al.*, 1999; Green *et al.*, 2000). It is not yet known whether serpin 43Ac inhibits persephone or some other protease in the pathway.

In addition to serpins, lower molecular weight inhibitors from the Kunitz family (Sugumaran *et al.*, 1985; Saul and Sugumaran, 1986; Aso *et al.*, 1994) and a family of 4 kDa inhibitors from locusts (Boigegrain *et al.*, 1992) can interfere with proPO activation (review: Kanost, 1999), although it is not yet known which proteases in the pathway they can inhibit.

4.7.4. Cellular Proteases

4.7.4.1. Serine Proteases

Serine proteases appear to function in tissue remodeling and extracellular matrix degradation required for cell movements in metamorphosis. Evagination of *D. melanogaster* imaginal discs is blocked by serine protease inhibitors, and the discs release serine proteases (Pino-Heiss and Schubiger, 1989). The *Stubble-stubbloid* gene encodes an integral membrane protein with an extracellular serine protease domain. It is expressed in imaginal discs under control of 20-hydroxyecdysone (see

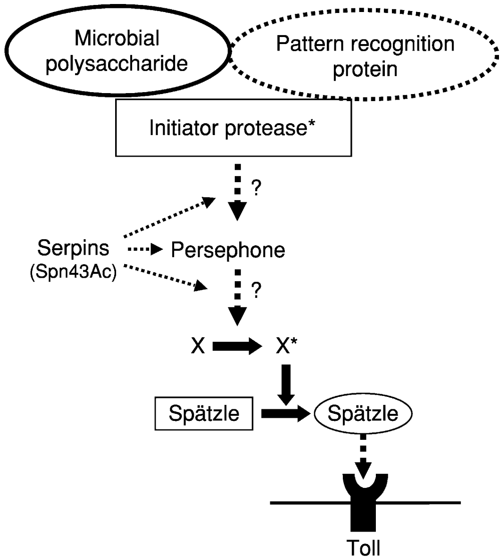


Figure 4 A model of the protease cascade that activates the *D. melanogaster* Toll pathway for synthesis of antimicrobial peptides. Hemolymph plasma proteins known as pattern recognition proteins bind to polysaccharides on the surface of microorganisms. This interaction leads to activation of initiator protease(s) by a mechanism not yet understood, which triggers a protease cascade. The number of proteases in the pathway is not yet known (indicated by dashed arrows). A clip domain protease called Persephone is known from genetic data to be a component in the pathway. The cascade leads to activation of a protease (designated as X in this figure) that cleaves spätzle to produce an active ligand that binds to Toll, a transmembrane receptor. A serpin, Spn43Ac negatively regulates at least one protease in the pathway.

Chapter 3.3) and may function to detach imaginal discs from extracellular matrices (Appel *et al.*, 1993).

4.7.4.2. Cathepsin-Type Cysteine Proteases

Cysteine proteases related to cathepsin B and cathepsin L have been identified as proteins produced by hemocytes that participate in tissue remodeling in metamorphosis of several insects. In *S. peregrina*, a 26/29 kDa protease synthesized in hemocytes was identified as a cathepsin B (Saito *et al.*, 1992; Kurata *et al.*, 1992a; Takahashi *et al.*, 1993; Fujimoto *et al.*, 1999). This protease may be released from pupal hemocytes to cause dissociation of fat body at metamorphosis (Kurata *et al.*, 1992b). A cathepsin B from hemocytes of *B. mori* may also function in tissue degradation during metamorphosis, including histolysis of silk glands (Shiba *et al.*, 2001; Xu and Kawasaki, 2001; **Chapter 2.11**).

Cysteine proteases classified as cathepsin L have also been identified as participants in tissue remodeling at metamorphosis. A cathepsin L from *S. peregrina* appears to function in the differentiation of imaginal discs (Homma *et al.*, 1994; Homma and Natori, 1996). A similar cathepsin L from another dipteran, *Delia radicum*, is expressed highly in midgut beginning in late third instar and may function in metamorphosis of the midgut (Hegedus *et al.*, 2002). A cathepsin L-like protease expressed in a *D. melanogaster* haemocyte cell line is present in granules and may be a lysosomal enzyme functioning to degrade phagocytosed material (Tryselius and Hultmark, 1997).

4.7.4.3. Caspases

Programmed cell death, known as apoptosis, is an essential process in development (Richardson and Kumar, 2002; **Chapter 2.5**) and is also a response by insect cells to viral infection (Clarke and Clem, 2003). An intracellular cascade of cysteine proteases called caspases (MEROPS family C14) is a key pathway in initiating apoptosis. Caspases are conserved in structure and function in animal species. They cleave target protein substrates after Asp or Glu P1 residues. Caspases are synthesized as inactive zymogens. They are activated by specific proteolytic cleavage, yielding a small and a large subunit that together form an active site. These heterodimers further dimerize to form a hetrotetramer with two active sites (Earnshaw *et al.*, 1999).

Caspases are divided into two groups, initiator caspases that interact with apoptosis-initiating proteins, and effector caspases, which are activated by the initiators. Initiator caspases contain a C-terminal cysteine protease domain and a long N-terminal region that contains death effector domains (DEDs) or caspase recruitment domains (CARDs), which interact with proteins that stimulate apoptosis. Dimerization of initiator caspases occurs when they associate with apoptosis-promoting proteins, causing caspase activation through conformational change in the absence of proteolytic cleavage (Boatright *et al.*, 2003). The initiator caspases then activate the effector caspases through specific proteolytic cleavage. Effector caspases, which lack a long N-terminal domain, cleave structural components of the cytoskeleton and nucleus and proteins involved in signaling pathways, resulting in death of the cell. Cleavage by effector caspases activates death-promoting enzymes, including certain kinases and nucleases (Earnshaw *et al.*, 1999).

Apoptosis and caspases have been investigated in detail in *D. melanogaster* (**Figure 5**). The *D. melanogaster* genome contains seven caspase genes. Three genes encode caspases with long prodomains (*dronc*, *dcp-2/dredd*, and *strica/dream*) and four genes encode caspases with short prodomains (*dcp-1*, *drICE*, *decay*, and *damm/daydream*) (Vernooy *et al.*, 2000; Kumar and Doumanis, 2000).

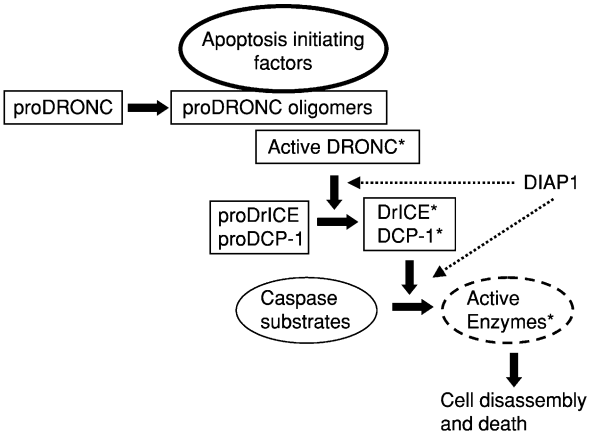


Figure 5 A model for a caspase cascade pathway in apoptosis in *D. melanogaster*. Cellular proapoptotic protein factors initiate oligomerization of the caspase zymogen proDRONC, which results in its self-processing and activation. Active DRONC cleaves the effector caspases DrICE and DcP-1, which then become active and cleave protein substrates including inactive forms of nucleases and kinases. The resulting active enzymes then catalyze reactions that result in progressive disassembly of the nucleus and cytoskeleton and cell death. A caspase inhibitor DIAP1 inhibits DRONC, DCP-1, and DrICE to regulate their activities.

Dronc contains an N-terminal CARD, and its overexpression in flies or in cultured cells stimulates apoptosis (Dorstyn *et al.*, 1999a; Quinn *et al.*, 2000). Dronc expression is stimulated by ecdysteroid, and it appears to function in apoptosis in tissue remodeling at metamorphosis (Dorstyn *et al.*, 1999a; Cakouros *et al.*, 2002; Lee *et al.*, 2002b; **Chapter 2.5**). Depletion of Dronc by RNA interference results in a decrease in normal programmed cell death in embryogenesis (Quinn *et al.*, 2000). Dronc can cleave substrates with P1 Glu or Asp residues. It can autoactivate by cleaving following a glutamate residue, but it activates drICE by cutting at an aspartate residue (Hawkins *et al.*, 2000). Dredd is an initiator caspase that participates in apoptosis (Chen *et al.*, 1998). Dredd also functions in a signal transduction pathway that leads to activation of immune responses (Elrod-Erickson *et al.*, 2000; Leulier *et al.*, 2000; Georgel *et al.*, 2001) through its cleavage and activation of a rel family transcription factor called relish (Stoven *et al.*, 2000, 2003). The third initiator caspase in *D. melanogaster*, STRICA, lacks either a CARD or a DED and instead contains a serine/threonine rich prodomain, suggesting that STRICA functions in an as yet unidentified pathway (Doumanis *et al.*, 2001).

The effector caspases Dcp-1 and DrICE are processed by active DRONC and then cleave target proteins that result in the disassembly of the cell. Both caspases induce apoptosis when overexpressed, have preferred target cleavage sites very similar to those of mammalian caspase-3, and can be inhibited by the baculovirus protein P35, a powerful inhibitor of effector caspases (Fraser and Evan, 1997; Fraser *et al.*, 1997; Song *et al.*, 1997; Meier *et al.*, 2000). Dcp-1 and DrICE have slight differences in specificity. Active DCP-1 can activate other proDCP-1 proteins and proDrICE, whereas active DrICE can not cleave and activate proDrICE (Song *et al.*, 2000). A third effector caspase, DECAY, shares similarity with DrICE and DCP-1 in sequence and preferred cleavage site, and can induce apoptosis when ectopically expressed in cultured cells (Dorstyn *et al.*, 1999b). In contrast, the short-prodomain caspase DAMM appears to have an accessory role in apoptosis, sensitizing the cell to prodeath signals rather then directly killing the cell. DAMM shares strong sequence similarity with STRICA, has a preferred cleavage site similar to mammalian caspases -1, -4, and -5, and is not processed *in vitro* by DAMM, DRONC, DECAY, DCP-1, or DrICE. DAMM mRNA is upregulated in apoptotic tissues, and DAMM over-expression in eye tissue sensitizes the cells to apoptosis from either developmental signals or radiation (Harvey *et al.*, 2001).

The caspase pathways in other insects may be even more complex. For example, the *A. gambiae* genome contains 12 caspase genes, compared to 7 in *D. melanogaster.* An effector caspase has been identified in the lepidopteran *Spodptera frugiperda* (Ahmad *et al.*, 1997; Seshagiri and Miller, 1997). This enzyme, Sf-caspase-1, is activated by an initiator caspase equivalent to DRONC (Manji and Friesen, 2001). The three-dimensional structure of Sf-caspase-1 has been determined (the first structure of a nonhuman caspase) by X-ray crystallography (Forsyth *et al.*, 2004) in the presence of a tetrapeptide inhibitor. The Sf-caspase-1 fold is very similar to that of human effector caspases, but it has unique features in its N-terminal prodomain that may be important in regulating its activation.

Caspases are regulated by specific inhibitor proteins that are present in the cytoplasm. In *D. melanogaster*, cells appear to be constitutively primed for caspase activation, and removal of caspase inhibition is sufficient to cause cell death. Three inhibitors of apoptosis (IAP) proteins, which inhibit caspases, have been identified in *D. melanogaster* (Hay *et al.*, 1995; Jones *et al.*, 2000). DIAP1 inhibits Dronc, DrICE, and DCP-1 (Kaiser *et al.*, 1998; Hawkins *et al.*, 1999; Meier *et al.*, 2000; Wilson *et al.*, 2002). *D. melanogaster* exhibits a constitutive proapoptotic predisposition, resulting in rapid apoptosis of cultured cells or embryos in the event of

the decreased expression of DIAP1 (Igaki *et al.*, 2002; Muro *et al.*, 2002; Rodriguez *et al.*, 2002).

Iap genes, similar to *D. melanogaster* diap1 and sharing the same capability of inhibiting initiator caspases, have been identified from three lepidopterans, *Trichoplusia ni* (Liao *et al.*, 2002; Seshagiri *et al.*, 1999), *B. mori* (Huang *et al.*, 2001), and *S. frugiperda* (Huang *et al.*, 2000). RNA interference depletion of Sf-IAP from *S. frugiperda* cell lines results in apoptosis, suggesting that, like *Drosophila* cells, *Spodoptera* cells are maintained in a state predisposed to cell death (Muro *et al.*, 2002).

Baculoviruses have evolved two types of caspase inhibitors that can block the lepidopteran apoptotic program. AcMNPV expresses its gene for the anti-apoptotic protein P35 at both early and late times during an infection, thus preventing infected cells from prematurely terminating viral replication through apoptosis (Clem *et al.*, 1991). P35 is a globular protein with an extended loop (Fisher *et al.*, 1999; Zoog *et al.*, 1999) that contains a caspase cleavage site recognized by a wide range of effector caspases, including human caspases-1, -3, -6, -7, -8, -10 (Zhou *et al.*, 1998), Sf-caspase-1 (Ahmad *et al.*, 1997), and DrICE (Fraser *et al.*, 1997), but not by initiator caspases such as human caspase-9 (Vier *et al.*, 2000), DRONC (Meier *et al.*, 2000), or Sf-caspase-X (LaCount *et al.*, 2000). When an effector caspase cleaves in the reactive site loop of P35, a thioester bond forms between the active site cysteine of the caspase and the P1 aspartic acid residue of P35, causing a conformational change in P35 that blocks access to water molecules, thus preventing completion of the peptide bond hydrolysis (Bertin *et al.*, 1996; dela Cruz *et al.*, 2001; Riedl *et al.*, 2001; Xu *et al.*, 2001).

Baculoviruses also contain genes for IAP proteins that block apoptosis by inhibiting initiator caspases (Huang *et al.*, 2000b; Birnbaum *et al.*, 1994; LaCount *et al.*, 2000; Means *et al.*, 2003) suggesting that, at some point during their evolution, baculoviruses co-opted the cell's own mechanism for regulating caspase activity. In fact, the first IAP protein to be discovered was Cp-IAP from the baculovirus *Cydia pomonella* granulovirus (Crook *et al.*, 1993).

4.7.4.4. Metalloproteases

Three families of metalloproteases that are not involved in digestion of food have been targets of fairly limited investigation in insects. They appear to function in remodeling of the extracellular matrix or in degradation of peptide hormones.

Matrix metalloproteases (MMP) are integral membrane proteins, present on the outer surface of cells. They are multidomain proteins that include a catalytic domain that incorporates a zinc ion in the active site. In mammals, these enzymes regulate processes involving morphogenesis in development and tissue remodeling by digesting protein components of the extracellular matrix (Nagase and Woessner, 1999). In *D. melanogaster* there are two MPP genes. Dm1-MMP is expressed strongly in embryos and may have a role in remodeling of the extracellular matrix in development of the central nervous system (Llano *et al.*, 2000). Dm2-MMP is expressed at a low level at all developmental stages, but with strong expression in regions of the brain and the eye imaginal discs (Llano *et al.*, 2002). Mutants in Dm1-MMP have defects in larval tracheal development and pupal head eversion, whereas mutants in Dm2-MMP do not undergo proper tissue remodeling during metamorphosis. However, normal embryonic development was observed even in double mutants lacking both MMPs (Page-McCaw *et al.*, 2003). It appears that these proteases are required primarily for remodeling of the extracellular matrix in metamorphosis. In mammals, the MMPs are regulated by proteins called tissue inhibitors of metalloproteases (TIMP). A homolog of these proteins in *D. melanogaster* has been shown to inhibit its MMPs (Wei *et al.*, 2003).

A different class of metalloproteases known as ADAMs (because they contain a disintegrin and metalloprotease domain) are also integral membrane proteins, with the zinc-containing protease domain on the extracellular surface. In mammals, they participate in growth factor processing, cell adhesion, cell fusion, and tissue remodeling processes, although their physiological functions, particularly of the protease domain, are still not well understood (Seals and Courtneidge, 2003). A *D. melanogaster* ADAM called kuzbanian has been shown to function in axon extension in nervous system development (Fambrough *et al.*, 1996; Rooke *et al.*, 1996). Kuzbanian is involved in initiation of a signal transduction pathway by a transmembrane receptor called Notch (Sotillos *et al.*, 1997). The metalloprotease domain of Kuzbanian may exert its physiological effect through cleavage of Notch (Lieber *et al.*, 2002) or the Notch ligand, Delta (Bland *et al.*, 2003).

A third family of zinc metalloproteases identified in insects contains members that may function in the degradation of peptide hormones. These transmembrane proteins are similar to mammalian neprilysins (also called neutral endopeptidases), which cleave oligopeptides on the amino side of hydrophobic residues. They cleave physiologically active signaling peptides with functions in nervous, cardiovascular,

inflammatory, and immune systems and have a wide tissue distribution (Turner *et al*., 2001). Metalloprotease activities with properties similar to neprylins have been identified as enzymes that can degrade tachykinin-related peptides in a cockroach, *Leucophaea maderae*, a locust, *Locusta migratoria,* a dipteran*, D. melanogaster,* and a lepidopteran, *Lacanobia oleracea* (Isaac *et al*., 2002; Isaac and Nassel, 2003). Similar activities that degrade adipokinetic hormone have been identified in the lepidopterans *Lymantria dispar* (Masler *et al*., 1996) and *M. sexta* (Fox and Reynolds, 1991), and a dipteran, *M. domestica* (Lamango and Isaac, 1993). cDNA clones for proteases with sequence similarity to neprilysin have been described in *B. mori* (Zhao *et al*., 2001), *M. sexta* (Zhu *et al*., 2003a), and *L. migratoria* (Macours *et al*., 2003). The *D. melanogaster* genome contains 24 neprilysin-like genes (Coates *et al*., 2000). Further study is needed to determine the substrates and physiological roles of these proteases, but it seems likely that they function as negative regulators of peptide hormones.

4.7.4.5. Aspartic Acid Proteases

The aspartic acid proteases from the MEROPS families A1 and A2 (similar to human pepsin) are a group that has received little study in insects. The *D. melanogaster* genome contains 34 genes that encode proteins similar to these enzymes, but none appear to have been investigated at the molecular level. A cathepsin D-like aspartic acid protease from *A. aegypti* has been identified as a lysosomal enzyme, which accumulates in fat body during vitellogenin synthesis (Cho and Raikhel, 1992; Dittmer and Raikhel, 1997).

An enzymatically inactive protein from the A1 family is an important allergen from a cockroach (*Blattella germanica*) that triggers asthmatic responses in humans (Pomés *et al*., 2002). It is present at highest concentration in the gut (Arruda *et al*., 1995), but since it apparently lacks proteolytic activity, its function in the insect is unknown.

4.7.4.6. Proteasomes

Proteasomes are complex intracellular proteases that function in regulated degradation of cellular proteins. Turnover of proteins by the proteasome regulates many processes including the cell cycle, circadian cycles (see **Chapter 4.11**), transcription, growth, development, as well as removal of abnormal proteins. Proteins are targeted for degradation by the proteasome by attachment of polyubiquitin chains to an amino group on a lysine side chain. Eukaryotic proteasomes are composed of four stacked heptameric rings that form a cylinder with multiple protease catalytic sites in its interior. This structure, the 20S proteasome, is composed of 28 subunits from multiple homologous gene products and has a mass of ~700 kDa. The 20S proteasome has little activity unless it is activated by another 700 kDa, 20 subunit protein, PA700, that can bind to one or both ends of the cylinder. When both ends of the 20S proteasome are capped by a PA700, the resulting complex is the 26S proteasome, which is active in degrading ubiquitinated proteins, an ATP-dependent process (DeMartino and Slaughter, 1999).

Proteasomes in insects have been studied primarily in *D. melanogaster* and *M. sexta* (Mykles, 1997, 1999). They have physical and catalytic properties similar to those of proteasomes from other eukaryotic species (Uvardy, 1993; Haire *et al*., 1995; Walz *et al*., 1998). Mutations in genes for subunits of *D. melanogaster* proteasomes or proteins, that regulate proteasome activity, result in lethal or otherwise complex phenotypes involving the disruption of multiple aspects of physiology and development (Schweisguth, 1999; Ma *et al*., 2002; Watts *et al*., 2003). Some mutations alter subunit composition or disrupt proteasome assembly (Covi *et al*., 1999; Smyth and Belote, 1999; Szlanka *et al*., 2003). Recent studies have used double-stranded RNA interference to study disruptions caused by reduced expression of individual proteasome components (Wojcik and DeMartino, 2002; Lundgren *et al*., 2003). In *M. sexta*, programmed cell death of intersegmental muscles at metamorophosis is accompanied by marked changes in proteasome activity and subunit composition (Dawson *et al*., 1995; Jones *et al*., 1995; Takayanagi *et al*., 1996; Low *et al*., 1997, 2000, 2001; Hastings *et al*., 1999).

4.7.5. Conclusions and Future Prospects

Tremendous advances have been made in the last 10 years in our knowledge of the existence, structure, and function of insect proteases that have biological roles unrelated to digestion of food. Much more remain to be discovered. Through examination of the genome sequences of *D. melanogaster* and *A. gambiae*, we can see that these insects have an enormous number of genes encoding proteases and that most of them are unstudied and have unknown functions. Nearly all of the research in this area has focused on a few dipteran and lepidopteran species. It is to be expected that detailed investigation of a broader range of species will reveal complex and diverse functions for proteases

in regulating intracellular and extracellular processes. A common feature of proteases is that they are synthesized as inactive zymogens, activated by proteolysis when the time is right, and then rapidly inhibited by specific inhibitors. Better understanding of molecular mechanisms of this tight regulation of multiple and varied protease cascade pathways will impact many areas of insect biology.

References

Ahmad, M., Srinivasula, S.M., Wang, L., Litwack, G., Fernandes-Alnemri, T., *et al.*, **1997**. *Spodoptera frugiperda* caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein p35. *J. Biol. Chem. 272*, 1421–1424.

Amarant, T., Burkhart, W., LeVine, H., 3rd, Arocha-Pinango, C.L., Parikh, I., **1991**. Isolation and complete amino acid sequence of two fibrinolytic proteinases from the toxic Saturnid caterpillar *Lonomia achelous*. *Biochem. Biophys. Acta 1079*, 214–221.

Appel, L.F., Prout, M., Abu-Shumays, R., Hammonds, A., Garbe, J.C., *et al.*, **1993**. The *Drosophila Stubble-stubbloid* gene encodes an apparent transmembrane serine protease required for epithelial morphogenesis. *Proc. Natl Acad. Sci. USA 90*, 4937–4941.

Applebaum, S.W., **1985**. Biochemistry of digestion. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry, and Pharmacology, vol. 4. Pergamon, Oxford, pp. 279–311.

Arocha-Pinango, C.L., Guerrero, B., **2000**. *Lonomia* genus caterpillar toxins: biochemical aspects. *Biochemie 82*, 937–942.

Arruda, L.K., Vailes, L.D., Mann, B.J., Shannon, J., Fox, J.W., *et al.*, **1995**. Molecular cloning of a major cockroach (*Blattella germanica*) allergen, Bla g 2. Sequence homology to the aspartic proteases. *J. Biol. Chem. 270*, 19563–19568.

Ashida, M., Brey, P.T., **1998**. Recent advances on the research of the insect prophenoloxidase cascade. In: Brey, P.T., Hultmark, D. (Eds.), Molecular Mechanisms of Immune Responses in Insects. Chapman and Hall, London, pp. 135–172.

Ashida, M., Sasaki, T., **1994**. A target protease activity of serpins in insect hemolymph. *Insect Biochem. Molec. Biol. 24*, 1037–1041.

Aso, Y., Yamashita, T., Meno, K., Murakami, M., **1994**. Inhibition of prophenoloxidase-activating enzyme from *Bombyx mori* by endogenous chymotrypsin inhibitors. *Biochem. Mol. Biol. International 33*, 751–758.

Belvin, M.P., Anderson, K.V., **1996**. A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. *Annu. Rev. Cell Devel. Biol. 12*, 393–416.

Berger, E., Kafatos, F.C., Felsted, R.L., Law, J.H., **1971**. Cocoonase III. Purification, preliminary characterization, and activation of the zymogen of an insect protease. *J. Biol. Chem. 246*, 4131–4137.

Bertin, J., Mendrysa, S.M., LaCount, D.J., Gaur, S., Krebs, J.F., *et al.*, **1996**. Apoptotic suppression by baculovirus P35 involves cleavage by and inhibition of a virus-induced CED-3/ICE-like protease. *J. Virol. 70*, 6251–6259.

Birnbaum, M.J., Clem, R.J., Miller, L.K., **1994**. An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J. Virol. 68*, 2521–2528.

Bland, C.E., Kimberly, P., Rand, M.D., **2003**. Notch-induced proteolysis and nuclear localization of the Delta ligand. *J. Biol. Chem. 278*, 13607–13610.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., *et al.*, **2003**. A unified model for apical caspase activation. *Mol. Cell. 11*, 529–541.

Boigegrain, R., Mattras, H., Brehélin, M., Paroutaud, P., Coletti-Previero, M., **1992**. Insect immunity: two proteinase inhibitors from hemolymph of *Locusta migratoria*. *Biochem. Biophys. Res. Commun. 189*, 790–793.

Brandt, K.S., Silver, G.M., Becher, A.M., Gaines, P.J., Maddux, J.D., *et al.*, **2004**. Isolation, characterization, and recombinant expression of multiple serpins from the cat flea, *Ctenocephalides felis*. *Arch. Insect Biochem. Physiol. 55*, 200–214.

Cakouros, D., Daish, T., Martin, D., Baehrecke, E.H., Kumar, S.J., **2002**. Ecdysone-induced expression of the caspase DRONC during hormone-dependent programmed cell death in *Drosophila* is regulated by Broad-Complex. *Cell Biol. 157*, 985–995.

Chamankhah, M., Braun, L., Visal-Shah, S., O'Grady, M., Baldwin, D., *et al.*, **2003**. *Mamestra configurata* serpin-1 homologues: cloning, localization and developmental regulation. *Insect Biochem. Mol. Biol. 33*, 355–369.

Chang, A.J., Morisato, D., **2002**. Regulation of Easter activity is required for shaping the Dorsal gradient in the *Drosophila* embryo. *Development 129*, 5635–5645.

Chasan, R., Anderson, K., **1989**. The role of easter, an apparent serine protease, in organizing the dorsal–ventral pattern of the *Drosophila* embryo. *Cell 56*, 391–400.

Chen, P., Rodriguez, A., Erskine, R., Thach, T., Abrams, J.M., **1998**. Dredd, a novel effector of the apoptosis activators REAPER, Grim, and Hid in *Drosophila*. *Devel. Biol. 201*, 202–216.

Cho, W.-L., Deitsch, K.W., Raikhel, A.S., **1991**. An extra-ovarian protein accumulated in mosquito oocytes is a carboxypeptidase activated in embryos. *Proc. Natl Acad. Sci. USA 88*, 10821–10824.

Cho, W.-L., Raikhel, A., **1992**. Cloning of cDNA for mosquito lysosomal aspartic protease. *J. Biol. Chem. 267*, 21823–21829.

Cho, W.-L., Tsao, S.-M., Hays, A.R., Walter, R., Chen, J.-S., *et al.*, **1999**. Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *J. Biol. Chem. 274*, 13311–13321.

Christophides, G.K, Evgeny, Z., Barillas-Mury, C., Birney, E., Blandin, S., *et al.*, **2002**. Immunity-related genes

and gene families in *Anopheles gambiae*. *Science 298*, 159–165.

Clarke, T.E., Clem, R.J., **2003**. Insect defenses against virus infection: the role of apoptosis. *Int. Rev. Immunol. 22*, 401–424.

Clem, R.J., Fechheimer, M., Miller, L.K., **1991**. Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science 254*, 1388–1390.

Coates, D., Siviter, R., Isaac, R.E., **2000**. Exploring the *Caenorhabditis elegans* and *Drosophila melanogaster* genomes to understand neuropeptide and peptidase function. *Biochem. Soc. Trans. 28*, 464–469.

Covi, J.A., Belote, J.M., Mykles, D.L., **1999**. Subunit compositions and catalytic properties of proteasomes from developmental temperature-sensitive mutants of *Drosophila melanogaster. Arch. Biochem. Biophys. 368*, 85–97.

Crook, N.E., Clem, R.J., Miller, L.K., **1993**. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol. 67*, 2168–2174.

Danielli, A., Kafatos, F.C., Loukeris, T.G., **2003**. Cloning and characterization of four *Anopheles gambiae* serpin isoforms, differentially induced in the midgut by *Plasmodium berghei* invasion. *J. Biol. Chem. 278*, 4184–4193.

Danielli, A., Loukeris, T.G., Lagueux, M., Muller, H.-M., Richman, A., *et al.*, **2000**. A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA 97*, 7136–7141.

Dawson, S.P., Arnold, J.E., Mayer, N.J., Reynolds, S.E., Billett, M.A., *et al.*, **1995**. Developmental changes of the 26 S proteasome in abdominal intersegmental muscles of *Manduca sexta* during programmed cell death. *J. Biol. Chem. 270*, 1850–1858.

dela Cruz, W.P., Friesen, P.D., Fisher, A.J., **2001**. Crystal structure of baculovirus P35 reveals a novel conformational change in the reactive site loop after caspase cleavage. *J. Biol. Chem. 276*, 32933–32939.

De Gregorio, E., Han, S.J., Lee, W.J., Baek, M.J., Osaki, T., *et al.*, **2002**. An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Devel. Cell 3*, 581–592.

DeLotto, R., **2001**. Gastrulation defective, a complement factor C2/B-like protease interprets a ventral prepattern in *Drosophila*. *EMBO Rep. 2*, 721–726.

DeLotto, Y., DeLotto, R., **1998**. Proteolytic processing of the *Drosophila* Spätzle protein by easter generates a dimeric NGF-like molecule with ventralising activity. *Mech. Devel. 72*, 141–148.

DeLotto, R., Spierer, P., **1986**. A gene required for the specification of dorsal–ventral pattern in *Drosophila* appears to encode a serine protease. *Nature 323*, 688–692.

DeMartino, G.N., Slaughter, C.A., **1999**. The proteasome, a novel protease regulated by multiple mechanisms. *J. Biol. Chem. 274*, 22123–22126.

Dimopoulos, G., Christophides, G.K., Meister, S., Schultz, J., White, K.P., *et al.*, **2002**. Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. *Proc. Natl Acad. Sci. USA 99*, 8814–8819.

Dimopoulos, G., Richman, A., della Torre, A., Kafatos, F.C., Louisn, C., **1996**. Identification and characterization of differentially expressed cDNAs of the vector mosquito, *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA 93*, 13066–13071.

Dissing, M., Giordano, H., DeLotto, R., **2001**. Autoproteolysis and feedback in a protease cascade directing *Drosophila* dorsal–ventral cell fate. *EMBO J. 20*, 2387–2393.

Dittmer, N.T., Raikhel, A.S., **1997**. Analysis of the mosquito lysosomal aspartic protease gene: an insect housekeeping gene with fat body-enhanced expression. *Insect Biochem. Mol. Biol. 27*, 323–335.

Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H., Kumar, S., **1999a**. DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl Acad. Sci. USA 96*, 4307–4312.

Dorstyn, L., Read, S.H., Quinn, L.M., Richardson, H., Kumar, S., **1999b**. DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. *J. Biol. Chem. 274*, 30778–30783.

Doumanis, J., Quinn, L., Richardson, H., Kumar, S., **2001**. STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. *Cell. Death Differ. 8*, 387–394.

Earnshaw, W.C., Martins, L.M., Kaufmann, S.H., **1999**. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem. 68*, 383–424.

Elrod-Erickson, M., Mishra, S., Schneider, D., **2000**. Interactions between the cellular and humoral immune responses in *Drosophila*. *Curr. Biol. 10*, 781–784.

Fambrough, D., Pan, D., Rubin, G.M., Goodman, C.S., **1996**. The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in *Drosophila*. *Proc. Natl Acad. Sci. USA 93*, 13233–13238.

Felsted, R.L., Kramer, K.J., Law, J.H., Berger, E., Kafatos, F.C., **1973**. Cocoonase IV. Mechanism of activation of prococoonase from *Antheraea polyphemus*. *J. Biol. Chem. 248*, 3021–3028.

Finnerty, C.M., Karplus, P.A., Granados, R.R., **1999**. The insect immune protein scolexin is a novel serine proteinase homolog. *Protein Sci. 8*, 242–248.

Fisher, A.J., Cruz, W., Zoog, S.J., Schneider, C.L., Friesen, P.D., **1999**. Crystal structure of baculovirus P35: role of a novel reactive site loop in apoptotic caspase inhibition. *EMBO J. 18*, 2031–2039.

Forsyth, C.M., Lemongello, D., LaCount, D.J., Friesen, P.D., Fisher, A.J., **2004**. Crystal structure of an invertebrate caspase. *J. Biol. Chem. 279*, 7001–7008.

Fox, A.M., Reynolds, S.E., **1991**. Degradation of adipokinetic hormone family peptides by a circulating endopeptidase in the insect *Manduca sexta*. *Peptides 12*, 937–944.

Fraser, A.G., Evan, G.I., **1997**. Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. *EMBO J. 16*, 2805–2813.

Fraser, A.G., McCarthy, N.J., Evan, G.I., **1997**. drICE is an essential caspase required for apoptotic activity in *Drosophila* cells. *EMBO J. 16*, 6192–6199.

Fujimoto, Y., Kobayashi, A., Kurata, S., Natori, S., **1999**. Two subunits of the insect 26/29-kDa proteinase are probably derived from a common precursor protein. *J. Biochem.* (*Tokyo*) *125*, 566–573.

Gan, H., Wang, Y., Jiang, M., Mita, K., Kanost, M.R., **2001**. A bacteria-induced, intracellular serpin in granular hemocytes of *Manduca sexta*. *Insect Biochem. Molec. Biol. 31*, 887–898.

Gay, N.J., Keith, F.J., **1992**. Regulation of translation and proteolysis during the development of embryonic dorso–ventral polarity in *Drosophila*. Homology of easter proteinase with Limulus proclotting enzyme and translational activation of Toll receptor synthesis. *Biochim. Biophys. Acta 1132*, 290–296.

Georgel, P., Naitza, S., Kappler, C., Ferrandon, D., Zachary, D., *et al.*, **2001**. *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Devel. Cell. 1*, 503–514.

Gettins, P.G., **2002**. Serpin structure, mechanism, and function. *Chem. Rev. 102*, 4751–4804.

Gillespie, J.P., Kanost, M.R., Trenczek, T., **1997**. Biological mediators of insect immunity. *Ann. Rev. Entomol. 42*, 611–643.

Gorman, M.J., Andreeva, O.V., Paskewitz, S.M., **2000a**. Sp22D: a multidomain serine protease with a putative role in insect immunity. *Gene 251*, 9–17.

Gorman, M.J., Andreeva, O.V., Paskewitz, S.M., **2000b**. Molecular characterization of five serine protease genes cloned from *Anopheles gambiae* hemolymph. *Insect Biochem. Mol. Biol. 30*, 35–46.

Gorman, M., Paskewitz, S.M., **2001**. Serine proteases as mediators of mosquito immune responses. *Insect Biochem. Molec. Biol. 31*, 257–262.

Green, C., Levashina, E., McKimmie, C., Dafforn, T., Reichhart, J.-M., *et al.*, **2000**. The necrotic gene in *Drosophila* corresponds to one of a cluster of three serpin transcripts mapping at 43A1.2. *Genetics 156*, 1117–1127.

Haire, M.F., Clark, J.J., Jones, M.E., Hendil, K.B., Schwartz, L.M., *et al.*, **1995**. The multicatalytic protease (proteasome) of the hawkmoth, *Manduca sexta*: catalytic properties and immunological comparison with the lobster enzyme complex. *Arch. Biochem. Biophys. 318*, 15–24.

Han, J.-H., Lee, S.H., Tan, Y.-Q., Lemosy, E.K., Hashimoto, C., **2000**. Gastrulation defective is a serine protease involved in activating the receptor Toll to polarize the *Drosophila* embryo. *Proc. Natl Acad. Sci. 97*, 9093–9097.

Han, Y., Salazar, C., Reese-Stardy, S., Cornel, A., Gorman, M., *et al.*, **1997**. Cloning and characterization of a serine protease from the human malaria vector, *Anopheles gambiae*. *Insect Molec. Biol. 6*, 385–395.

Harvey, N.L., Daish, T., Mills, K., Dorstyn, L., Quinn, L.M., *et al.*, **2001**. Characterization of the *Drosophila* caspase, DAMM. *J. Biol. Chem. 276*, 25342–25350.

Hashimoto, C., Kim, D.R., Weiss, L.A., Miller, J.W., Morisato, D., **2003**. Spatial regulation of developmental signaling by a serpin. *Devel. Cell. 5*, 945–950.

Hastings, R.A., Eyheralde, I., Dawson, S.P., Walker, G., Reynolds, S.E., *et al.*, **1999**. A 220-kDa activator complex of the 26 S proteasome in insects and humans. A role in type II programmed insect muscle cell death and cross-activation of proteasomes from different species. *J. Biol. Chem. 274*, 25691–25700.

Hawkins, C.J., Wang, S.L., Hay, B.A., **1999**. A cloning method to identify caspases and their regulators in yeast: identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. *Proc. Natl Acad. Sci. USA 96*, 2885–2890.

Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y., *et al.*, **2000**. The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem. 275*, 27084–27093.

Hay, B.A., Wassarman, D.A., Rubin, G.M., **1995**. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell 83*, 1253–1262.

Hegedus, D., O'Grady, M., Chamankhah, M., Baldwin, D., Gleddie, S., *et al.*, **2002**. Changes in cysteine protease activity and localization during midgut metamorphosis in the crucifer root maggot (*Delia radicum*). *Insect Biochem. Molec. Biol. 32*, 1585–1596.

Hoffmann, J.A., **2003**. The immune response of *Drosophila*. *Nature 426*, 33–38.

Homma, K., Kurata, S., Natori, S., **1994**. Purification, characterization, and cDNA cloning of procathepsin L from the culture medium of NIH-SAPE-4, an embryonic cell line of *Sarcophaga peregrina* (flesh fly), and its involvement in the differentiation of imaginal disks. *J. Biol. Chem. 269*, 15258–15264.

Homma, H., Natori, N., **1996**. Identification of substrate proteins for cathepsin L that are selectively hydrolyzed during the differentiation of imaginal disks of *Sarcophaga peregrina*. *Eur. J. Biochem. 240*, 443–447.

Hong, C., Hashimoto, C., **1995**. An unusual mosaic protein with a protease domain, encoded by the nudel gene, is involved in defining embryonic dorsoventral polarity in *Drosophila*. *Cell 82*, 785–794.

Hong, C.C., Hashimoto, C., **1996**. The maternal nudel protein of *Drosophila* has two distinct roles important for embryogenesis. *Genetics 143*, 1653–1661.

Hooper, N.M., **2002**. Proteases: a primer. In: Hooper, N.M. (Ed.), Essays in Biochemistry: Proteases in Biology and Medicine. Portland Press, London, pp. 1–8.

Huang, Q., Deveraux, Q.L., Maeda, S., Salvesen, G.S., Stennicke, H.R., *et al.*, **2000**. Evolutionary conservation of apoptosis mechanisms: lepidopteran and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalian caspase-9. *Proc. Natl Acad. Sci. USA 97*, 1427–1432.

Huang, Q., Deveraux, Q.L., Maeda, S., Stennicke, H.R., Hammock, B.D., *et al.*, **2001**. Cloning and characterization of an inhibitor of apoptosis protein (IAP) from *Bombyx mori*. *Biochim. Biophys. Acta 1499*, 191–198.

Igaki, T., Yamamoto-Goto, Y., Tokushige, N., Kanda, H., Miura, M., **2002**. Down-regulation of DIAP1 triggers a novel *Drosophila* cell death pathway mediated by Dark and DRONC. *J. Biol. Chem. 277*, 23103–23106.

Ikeda, M., Yaginuma, T., Kobayashi, M., Yamashita, O., **1991**. cDNA cloning, sequencing and temporal expression of the protease responsible for vitellin degradation in the silkworm *Bombyx mori. Comp. Biochem. Physiol. B 99*, 405–411.

Indrasith, L.S., Sasaki, T., Yamashita, O., **1988**. A unique protease responsible for selective degradation of a yolk protein in *Bombyx mori*. Purification, characterization, and cleavage profile. *J. Biol. Chem. 263*, 1045–1051.

Irving, P., Troxler, L., Heuer, T.S., Belvin, M., Kopczynski, C., *et al.*, **2001**. A genome-wide analysis of immune responses in *Drosophila*. *Proc. Natl Acad. Sci. USA 98*, 15119–15124.

Isaac, R.E., Nassel, D.R., **2003**. Identification and localization of a neprilysin-like activity that degrades tachykinin-related peptides in the brain of the cockroach, *Leucophaea maderae*, and locust, *Locusta migratoria*. *J. Comp. Neurol. 457*, 57–66.

Isaac, R.E., Parkin, E.T., Keen, J.N., Nassel, D.R., Siviter, R.J., *et al.*, **2002**. Inactivation of a tachykinin-related peptide: identification of four neuropeptide-degrading enzymes in neuronal membranes of insects from four different orders. *Peptides 23*, 725–733.

Jiang, H., Kanost, M.R., **1997**. Characterization and functional analysis of twelve naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. *J. Biol. Chem. 272*, 1082–1087.

Jiang, H., Kanost, M.R., **2000**. The clip-domain family of serine proteinases in arthropods. *Insect Biochem. Molec. Biol. 30*, 95–105.

Jiang, H., Wang, Y., Huang, Y., Mulnix, A.B., Kadel, J., *et al.*, **1996**. Organization of serpin gene-1 from *Manduca sexta*: evolution of a family of alternate exons encoding the reactive site loop. *J. Biol. Chem. 271*, 28017–28023.

Jiang, H., Wang, Y., Kanost, M.R., **1998**. Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila* easter. *Proc. Natl Acad. Sci. USA 95*, 12220–12225.

Jiang, H., Wang, Y., Kanost, M.R., **1999**. Four serine proteinases expressed in *Manduca sexta* hemocytes. *Insect Mol. Biol. 8*, 39–53.

Jiang, H., Wang, Y., Yu, X.-Q., Kanost, M.R., **2003a**. Prophenoloxidase-activating proteinase-2 from hemolymph of *Manduca sexta*. A bacteria-inducible serine proteinase containing two clip domains. *J. Biol. Chem. 278*, 3552–3561.

Jiang, H., Wang, Y., Yu, X.-Q., Zhu, Y., Kanost, M.R., **2003b**. Prophenoloxidase-activating proteinase-3 (PAP-3) from *Manduca sexta* hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem. Mol. Biol. 33*, 1049–1060.

Jin, Y., Anderson, K.V., **1990**. Dominant and recessive alleles of the *Drosophila* easter gene are point mutations at conserved sites in the serine protease catalytic domain. *Cell 60*, 873–881.

Jones, M.E., Haire, M.F., Kloetzel, P.M., Mykles, D.L., Schwartz, L.M., **1995**. Changes in the structure and function of the multicatalytic proteinase (proteasome) during programmed cell death in the intersegmental muscles of the hawkmoth, *Manduca sexta*. *Devel. Biol. 169*, 436–447.

Jones, G., Jones, D., Zhou, L., Steller, H., Chu, Y., **2000**. Deterin, a new inhibitor of apoptosis from *Drosophila melanogaster*. *J. Biol. Chem. 275*, 22157–22165.

Kafatos, F.C., **1972**. The cocoonase zymogen cells of silk moths: a model of terminal cell differentiation for specific protein synthesis. *Curr. Top. Devel. Biol. 7*, 125–191.

Kafatos, F.C., Law, J.H., Tartakoff, A.M., **1967b**. Cocoonase II. Substrate specificity, inhibitors, and classification of the enzyme. *J. Biol. Chem. 424*, 1488–1494.

Kafatos, F.C., Tartakoff, A.M., Law, J.H., **1967a**. Cocoonase I. Preliminary characterization of a proteolytic enzyme from silk moths. *J. Biol. Chem. 242*, 1477–1487.

Kageyama, T., Takahashi, S.Y., **1990**. Purification and characterization of a cysteine proteinase from silkworm eggs. *Eur. J. Biochem. 193*, 203–210.

Kaiser, W.J., Vucic, D., Miller, L.K., **1998**. The *Drosophila* inhibitor of apoptosis D-IAP1 suppresses cell death induced by the caspase drICE. *FEBS Lett. 440*, 243–248.

Kanost, M.R., **1999**. Serine proteinase inhibitors in arthropod immunity. *Devel. Comp. Immunol. 23*, 291–301.

Kanost, M.R., Jiang, H., **1996**. Proteinase inhibitors in invertebrate immunity. In: Söderhäll, K., Iwanga, S., Vasta, G. (Eds.), New Directions in Invertebrate Immunology. SOS Publications, Fair Haven, CT, pp. 155–173.

Kanost, M.R., Jiang, H., Wang, Y., Yu, X.-Q., Ma, C.C., *et al.*, **2001**. Hemolymph proteinases in immune responses of *Manduca sexta*. *Adv. Exp. Med. Biol. 484*, 319–328.

Katsumi, Y., Kihara, H., Ochiai, M., Ashida, M., **1995**. A serine protease zymogen in insect plasma. Purification and activation by microbial cell components. *Eur. J. Biochem. 228*, 870–877.

Kawabata, S., Muta, T., Iwanaga, S., **1996**. The clotting cascade and defense molecules found in the hemolymph of the horseshoe crab. In: Söderhäll, K., Iwanaga, S., Vasta, G. (Eds.), New Directions in Invertebrate Immunology. SOS Publications, Fair Haven, CT, pp. 255–283.

Kim, M.S., Baek, M.J., Lee, M.H., Park, J.W., Lee, S.Y., *et al.*, **2002**. A new easter-type serine protease cleaves a masquerade-like protein during prophenoloxidase activation in *Holotrichia diomphalia* larvae. *J. Biol. Chem. 277*, 39999–40004.

Kim, Y.S., Ryu, J.H., Han, S.J., Choi, K.H., Nam, K.B., *et al.*, **2000**. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the

induction of innate immune genes in *Drosophila melanogaster* cells. *J. Biol. Chem. 275*, 32721–32727.

Konrad, K.D., Goralski, T.J., Mahowald, A.P., Marsh, J.L., **1998**. The gastrulation defective gene of *Drosophila melanogaster* is a member of the serine protease superfamily. *Proc. Natl Acad. Sci. USA 95*, 6819–6824.

Kramer, K.J., Felsted, R.L., Law, J.H., **1973**. Cocoonase V. Structural studies on an insect serine protease. *J. Biol. Chem. 248*, 3021–3028.

Krem, M.M., Di Cerra, E., **2002**. Evolution of enzyme cascades from embryonic development to blood coagulation. *Trends Biochem. Sci. 27*, 67–74.

Kruger, O., Ladewig, J., Koster, K., Ragg, H., **2002**. Widespread occurrence of serpin genes with multiple reactive centre-containing exon cassettes in insects and nematodes. *Gene 293*, 97–105.

Kryiakides, T.R., McKillip, J.L., Spence, K.D., **1995**. *Arch. Insect Biochem. Physiol. 29*, 269–280.

Kumar, S., Doumanis, J., **2000**. The fly caspases. *Cell Death Differ. 7*, 1039–1044.

Kurata, S., Saito, H., Natori, S., **1992a**. Purification of a 29 kDa proteinase of *Sarcophaga peregrina*. *Eur. J. Biochem. 204*, 911–914.

Kurata, S., Saito, H., Natori, S., **1992b**. The 29-kDa hemocyte proteinase dissociates fat body at metamorphosis of *Sarcophaga*. *Devel. Biol. 153*, 115–121.

Kwon, T.H., Kim, M.S., Choi, H.W., Loo, C.H., Cho, M.Y., *et al.*, **2000**. A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem. 267*, 6188–6196.

LaCount, D.J., Hanson, S.F., Schneider, C.L., Friesen, P.D., **2000**. Caspase inhibitor P35 and inhibitor of apoptosis Op-IAP block *in vivo* proteolytic activation of an effector caspase at different steps. *J. Biol. Chem. 275*, 15657–15664.

Lamango, N.S., Isaac, R.E., **1993**. Metabolism of insect neuropeptides: properties of a membrane-bound endopeptidase form heads of *Musca domestica*. *Insect Biochem. Mol. Biol. 23*, 801–808.

Law, J.H., Dunn, P.E., Kramer, K.J., **1977**. Insect proteases and peptidases. *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 389–425.

Lee, C.Y., Cooksey, B.A., Baehrecke, E.H., **2002a**. Steroid regulation of midgut cell death during *Drosophila* development. *Devel. Biol. 250*, 101–111.

Lee, K.Y., Zhang, R., Kim, M.S., Park, J.W., Park, H.Y., *et al.*, **2002b**. A zymogen form of masquerade-like serine proteinase homologue is cleaved during prophenoloxidase activation by Ca^{2+} in coleopteran and *Tenebrio molitor* larvae. *Eur. J. Biochem. 269*, 4375–4383.

Lee, S.Y., Cho, M.Y., Hyun, J.H., Lee, K.M., Homma, K., *et al.*, **1998b**. Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine proteinase is induced by lipopolysaccharide or 1,3-β-glucan in a coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem. 257*, 615–621.

Lee, S.Y., Kwon, T.H., Hyun, J.H., Choi, J.S., Kawabata, S.I., *et al.*, **1998a**. *In vitro* activation of pro-phenoloxidase by two kinds of pro-phenol-oxidase activating factors isolated from hemolymph of coleopteran *Holotrichia diomphalia* larvae. *Eur. J. Biochem. 15*, 50–57.

Lee, S.Y., Söderhäll, K., **2001**. Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. *J. Immunol. 166*, 7319–7326.

Lemaitre, B., Nocolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., **1996**. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell 86*, 973–983.

LeMosy, E.K., Hashimoto, C., **2000**. The nudel protease of *Drosophila* is required for eggshell biogenesis in addition to embryonic patterning. *Devel. Biol. 217*, 352–361.

LeMosy, E.K., Hong, C.C., Hashimoto, C., **1999**. Signal transduction by a protease cascade. *Trends Cell Biol. 9*, 102–107.

LeMosy, E.K., Kemler, D., Hashimoto, C., **1998**. Role of Nudel protease activation in triggering dorsoventral polarization of the *Drosophila* embryo. *Development 125*, 4045–4053.

LeMosy, E.K., Leclerc, C.L., Hashimoto, C., **2000**. Biochemical defects of mutant *nudel* alleles causing early developmental arrest or dorsalization of the *Drosophila* embryo. *Genetics 154*, 247–257.

LeMosy, E.K., Tan, Y.-Q., Hashimoto, C., **2001**. Activation of a protease cascade involved in patterning the *Drosophila* embryo. *Proc. Natl Acad. Sci. 98*, 5055–5060.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., *et al.*, **2003**. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nat. Immunol. 4*, 478–484.

Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., Lemaitre, B., **2000**. The *Drosophila* caspase Dredd is required to resist gram-negative bacterial infection. *EMBO Rep 1*, 353–358.

Levashina, E.A., Langley, E., Green, C., Gubb, D., Ashburner, M., *et al.*, **1999**. Constitutive activation of Toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science. 285*, 1917–1919.

Li, J., Wang, Z., Canagarajah, B., Jiang, H., Kanost, M.R., *et al.*, **1999**. The structure of active serpin K from *Manduca sexta* and a model for serpin-protease complex formation. *Structure 7*, 103–109.

Liao, W.T., Yang, Y., Wu, X.F., **2002**. Expression and functional analysis of an inhibitor of apoptosis protein from *Trichoplusia ni*. *Biochem. Biophys. Res. Commun. 293*, 675–679.

Lieber, T., Kidd, S., Young, M.W., **2002**. Kuzbanian-mediated cleavage of *Drosophila* Notch. *Genes Devel. 16*, 209–221.

Ligoxygakis, P., Pelte, N., Hoffmann, J.A., Reichhart, J.M., **2002a**. Activation of *Drosophila* Toll during fungal infection by a blood serine protease. *Science 297*, 114–116.

Ligoxygakis, P., Pelte, N., Ji, C., Leclerc, V., Duvic, B., *et al.*, **2002b**. A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. *EMBO J. 21*, 6330–6337.

Ligoxygakis, P., Roth, S., Rechhart, J.M., **2003**. A serpin regulates dorsal–ventral axis formation in the *Drosophila* embryo. *Curr. Biol. 13*, 2097–2102.

Llano, E., Adam, G., Pendas, A.M., Quesada, V., Sanchez, L.M., *et al.*, **2002**. Structural and enzymatic characterization of *Drosophila* Dm2-MMP, a membrane-bound matrix metalloproteinase with tissue-specific expression. *J. Biol. Chem. 277*, 23321–23329.

Llano, E., Pendas, A.M., Aza-Blanc, P., Kornberg, T.B., Lopez-Otin, C., **2000**. Dm1-MMP, a matrix metalloproteinase from *Drosophila* with a potential role in extracellular matriz remodeling during neural development. *J. Biol. Chem. 275*, 35978–35985.

Low, P., Bussell, K., Dawson, S.P., Billett, M.A., Mayer, R.J., *et al.*, **1997**. Expression of a 26S proteasome ATPase subunit, M73, in muscles that undergo developmentally programmed cell death, and its control by ecdysteroid hormones in the insect *Manduca sexta*. *FEBS Lett. 400*, 345–349.

Low, P., Hastings, R.A., Dawson, S.P., Sass, M., Billett, M.A., *et al.*, **2000**. Localisation of 26S proteasomes with different subunit composition in insect muscles undergoing programmed cell death. *Cell Death Differ. 7*, 1210–1217.

Low, P., Reynolds, S.E., Sass, M., **2001**. Proteolytic activity of 26s proteasomes isolated from muscles of the tobacco hornworm, *Manduca sexta*: differences between surviving muscles and those undergoing developmentally programmed cell death. *Acta Biol. Hung. 52*, 435–442.

Lundgren, J., Masson, P., Realini, C.A., Young, P., **2003**. Use of RNA interference and complementation to study the function of the *Drosophila* and human 26S proteasome subunit S13. *Mol. Cell. Biol. 23*, 5320–5330.

Ma, C., Kanost, M.R., **2000**. A β-1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. *J. Biol. Chem. 275*, 7505–7514.

Ma, J., Katz, E., Belote, J.M., **2002**. Expression of proteasome subunit isoforms during spermatogenesis in *Drosophila melanogaster. Insect Mol. Biol. 11*, 627–639.

Macours, N., Poels, J., Hens, K., Luciani, N., De Loof, A., *et al.*, **2003**. An endothelin-converting enzyme homologue in the locust, *Locusta migratoria*: functional activity, molecular cloning and tissue distribution. *Insect Mol. Biol. 12*, 233–240.

Maki, N., Yamashita, O., **1997**. Purification and characterization of a protease degrading 30 kDa yolk proteins of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol. 27*, 721–728.

Maki, N., Yamashita, O., **2001**. The 30 k protease A responsible for 30-kDa yolk protein degradation of the silkworm, *Bombyx mori*: cDNA structure, developmental change and regulation by feeding. *Insect Biochem. Mol. Biol. 31*, 407–413.

Manji, G.A., Friesen, P.D., **2001**. Apoptosis in motion. An apical, P35-insensitive caspase mediates programmed cell death in insect cells. *J. Biol. Chem. 276*, 16704–16710.

Masler, E.P., Wagner, R.M., Kovaleva, E.S., **1996**. *In vitro* metabolism of an insect neuropeptide by neural membrane preparations from *Lymantria dispar. Peptides 17*, 321–326.

Means, J.C., Muro, I., Clem, R.J., **2003**. Silencing of the baculovirus Op-iap3 gene by RNA interference reveals that it is required for prevention of apoptosis during *Orgyia pseudotsugata* M nucleopolyhedrovirus infection of Ld652Y cells. *J. Virol. 77*, 4481–4488.

Medina, M., Leon, P., Vallejo, C.G., **1988**. *Drosophila* cathepsin B-like proteinase: a suggested role in yolk degradation. *Arch. Biochem. Biophys. 263*, 355–363.

Meier, P., Silke, J., Leevers, S.J., Evan, G.I., **2000**. The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J. 19*, 598–611.

Misra, S., Hecht, P., Maeda, R., Anderson, K.V., **1998**. Positive and negative regulation of Easter, a member of the serine protease family that controls dorsal-ventral patterning in the *Drosophila* embryo. *Development 125*, 1261–1267.

Morisato, D., Anderson, K.V., **1995**. Signaling pathways that establish the dorsal–ventral pattern of the *Drosophila* embryo. *Annu. Rev. Genet. 29*, 371–399.

Morugasu-Oei, B., Balakrishnan, R., Yang, X., Chia, W., Rodrigues, V., **1996**. Mutations in masquerade, a novel serine-protease-like molecule, affect axonal guidance and taste behavior in *Drosophila*. *Mech. Devel. 57*, 91–101.

Morugasu-Oei, B., Rodrigues, V., Yang, X., Chia, W., **1995**. Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the *Drosophila* embryo. *Genes Devel. 9*, 139–154.

Muro, I., Hay, B.A., Clem, R.J., **2002**. The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J. Biol. Chem. 277*, 49644–49650.

Mykles, D.L., **1997**. Biochemical properties of insect and crustacean proteasomes. *Mol. Biol. Rep. 24*, 133–138.

Mykles, D.L., **1999**. Structure and functions of arthropod proteasomes. *Mol. Biol. Rep. 26*, 103–111.

Nagase, H., Woessner, F.J., **1999**. Matrix metalloproteinases. *J. Biol. Chem., 274*, 21491–21494.

Nakajima, Y., Tsuji, Y., Homma, K., Natori, S., **1997**. A novel protease in the pupal yellow body of *Sarcophaga peregrina* (flesh/fly). Its purification and cDNA cloning. *J. Biol. Chem. 272*, 23805–23810.

Narumi, H., Hishida, T., Sasaki, T., Feng, D.F., Doolittle, R.F., **1993**. Molecular cloning of silkworm (*Bombyx mori*) antichymotrypsin. A new member of the serpin superfamily of proteins from insects. *Eur. J. Biochem. 214*, 181–187.

Ochiai, M., Ashida, M., **1988**. Purification of a β-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem. 263*, 12056–12062.

Ochiai, M., Ashida, M., **1999**. A pattern recognition protein for peptidoglycan. Cloning the cDNA and the gene of the silkworm, *Bombyx mori*. *J. Biol. Chem. 274*, 11854–11858.

Ochiai, M., Ashida, M., **2000**. A pattern recognition protein for β-1,3-glucan. *J. Biol. Chem. 275*, 4995–5002.

Oduol, F., Xu, J., Niare, O., Natarajan, R., Vernick, K.D., **2000**. Genes identified by an expression screen of the vector mosquito *Anopheles gambiae* display differential molecular immune response to malaria parasites and bacteria. *Proc. Natl Acad. Sci. USA 97*, 11394–11402.

Page-McCaw, A., Serano, J., Sante, J.M., Rubin, G.M., **2003**. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. *Devel. Cell 4*, 95–106.

Paskewitz, S.M., Gorman, M.J., **1999**. Mosquito immunity and malaria parasites. *M. Entomol. 45*, 80–94.

Paskewitz, S.M., Reese-Stardy, S., Gorman, M.J., **1999**. An easter-like serine protease from *Anopheles gambiae* exhibits changes in transcript abundance following immune challenge. *Insect Mol. Biol. 8*, 329–337.

Pino-Heiss, S., Schubiger, G., **1989**. Extracellular protease production by *Drosophila* imaginal disks. *Devel. Biol. 132*, 282–291.

Polanowski, A., Wilusz, T., **1996**. Serine proteinase inhibitors from insect hemolymph. *Acta Biochimica. Polonica. 43*, 445–454.

Pomés, A., Chapman, M.D., Vailes, L.D., Blundell, T.L., Dhanaraj, V., **2002**. Cockroach allergen Bla g2: structure, function, and implications for allergic sensitization. *Am. J. Respir. Crit. Care Med. 165*, 391–397.

Quinn, L.M., Dorstyn, L., Mills, K., Colussi, P.A., Chen, P., *et al.*, **2000**. An essential role for the caspase dronc in developmentally programmed cell death in *Drosophila*. *J. Biol. Chem. 275*, 40416–40424.

Raikhel, A.S., Dhadialla, T.S., **1992**. Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol. 37*, 217–251.

Rawlings, N.D., Barrett, A.J., **1999**. MEROPS: the peptidase database. *Nucleic Acids Res. 27*, 325–331.

Reeck, G., Oppert, B., Denton, M., Kanost, M., Baker, J., *et al.*, **1999**. Insect proteinases. In: Turk, V. (Ed.), Proteases: New Perspectives. Birkhauser, Basel, pp. 125–148.

Ribolla, P.E., De Bianchi, A.G., **1995**. Processing of procathepsin from *Musca domestica* eggs. *Insect Biochem. Mol. Biol. 25*, 1011–1017.

Richardson, H., Kumar, S., **2002**. Death to flies: *Drosophila* as a model system to study programmed cell death. *J. Immunol. Methods 265*, 21–38.

Riedl, S.J., Renatus, M., Snipas, S.J., Salvesen, G.S., **2001**. Mechanism-based inactivation of caspases by the apoptotic suppressor p35. *Biochemistry 40*, 13274–13280.

Rodriguez, A., Chen, P., Oliver, H., Abrams, J.M., **2002**. Unrestrained caspase-dependent cell death caused by loss of Diap1 function requires the *Drosophila* Apaf-1 homolog, Dark. *EMBO J. 21*, 2189–2197.

Rooke, J., Pan, D., Xu, T., Rubin, G.M., **1996**. KUZ, a conserved metalloprotease-disintegrin protein with two roles in *Drosophila* neurogenesis. *Science 273*, 1227–1231.

Rose, T., LeMosy, E.K., Cantwell, A.M., Banerjee-Roy, D., Skeath, J.B., *et al.*, **2003**. Three-dimensional models of proteases involved in patterning of the *Drosophila* embryo. *Crucial role of predicted cation binding sites. J. Biol. Chem. 278*, 11320–11330.

Ross, J., Jiang, H., Kanost, M.R., Wang, Y., **2003**. Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships. *Gene 304*, 117–131.

Saito, H., Kurata, S., Natori, S., **1992**. Purification and characterization of a hemocyte proteinase of *Sarcophaga*, possibly participating in elimination of foreign substances. *Eur. J. Biochem. 209*, 939–944.

Sasaki, T., **1991**. Patchwork-structure serpins from silkworm (*Bombyx mori*) larval hemolymph. *Eur. J. Biochem. 202*, 255–161.

Satoh, D., Horii, A., Ochiai, M., Ashida, M., **1999**. Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*: purification, characterization and cDNA cloning. *J. Biol. Chem. 274*, 7441–7453.

Saul, S., Sugumaran, M., **1986**. Protease inhibitor controls prophenoloxidase activation in *Manduca sexta*. *FEBS Lett. 208*, 113–116.

Schechter, I., Berger, A., **1967**. On the size of the active site in proteases. *I. Papain. Biochem. Biophys. Res. Commun. 27*, 157–162.

Schweisguth, F., **1999**. Dominant-negative mutation in the β2 and β6 proteasome subunit genes affect alternate cell fate decisions in the Drosophila sense organ lineage. *Proc. Natl Acad. Sci. USA 96*, 11382–11386.

Seals, D.F., Courtneidge, S.A., **2003**. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Devel. 17*, 7–30.

Seshagiri, S., Miller, L.K., **1997**. Baculovirus inhibitors of apoptosis (IAPs) block activation of Sf-caspase-1. *Proc. Natl Acad. Sci. USA 94*, 13606–13611.

Seshagiri, S., Vucic, D., Lee, J., Dixit, V.M., **1999**. Baculovirus-based genetic screen for antiapoptotic genes identifies a novel IAP. *J. Biol. Chem. 274*, 36769–36773.

Shiba, H., Uchida, D., Kobayashi, H., Natori, M., **2001**. Involvement of cathepsin B- and L-like proteinases in silk gland histolysis during metamorphosis of *Bombyx mori*. *Arch. Biochem. Biophys. 390*, 28–34.

Silverman, G.A., Bird, P.I., Carrell, R.W., Church, F.C., Coughlin, P.B., *et al.*, **2001**. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J. Biol. Chem. 276*, 33293–33296.

Smith, C., DeLotto, R., **1992**. A common domain within the proenzyme regions of the *Drosophila* snake and easter proteins and *Tachypleus* proclotting enzyme

defines a new subfamily of serine proteases. *Protein Sci. 11*, 1225–1226.

Smith, C., DeLotto, R., **1994**. Ventralizing signal determined by protease activation in *Drosophila* embryogenesis. *Nature 368*, 548–551.

Smith, C., Giordano, H., DeLotto, R., **1994**. Mutational analysis of the *Drosophila* snake protease: an essential rule for domains within the proenzyme polypeptide chain. *Genetics 136*, 1355–1365.

Smith, C.L., Giordano, H., Schwartz, M., DeLotto, R., **1995**. Spatial regulation of Drosophila snake protease activity in the generation of dorsal–ventral polarity. *Development 121*, 4127–4135.

Smyth, K.A., Belote, J.M., **1999**. The dominant temperature-sensitive lethal DTS7 of *Drosophila melanogaster* encodes an altered 20S proteasome β-type subunit. *Genetics 151*, 211–220.

Söderhäll, K., Derenius, L., Johansson, M.W., **1996**. The prophenoloxidase activating system in invertebrates. In: Söderhäll, K., Iwanaga, S., Vasta, G. (Eds.), New Directions in Invertebrate Immunology. SOS Publications, Fair Haven, CT, pp. 229–253.

Söderhäll, K., Rogener, W., Söderhäll, I., Newton, R.P., Ratcliffe, N.A., **1988**. The properties and purification of a *Blaberus craniifer* plasma protein which enhances the activation of haemocyte prophenoloxidase by a β-1,3-glucan. *Insect Biochem. 18*, 323–330.

Song, Z., Guan, B., Bergman, A., Nicholson, D.W., Thornberry, N.A., *et al*., **2000**. Biochemical and genetic interactions between *Drosophila* caspases and the proapoptotic genes rpr, hid, and grim. *Mol. Cell. Biol. 20*, 2907–2914.

Song, Z., McCall, K., Steller, H., **1997**. DCP-1, a *Drosophila* cell death protease essential for development. *Science 275*, 536–540.

Sotillos, S., Roch, F., Campuzano, S., **1997**. The metalloprotease-disintintegrin Kuzbanian participates in Notch activation during growth and patterning of Drosophila imaginal disks. *Development 124*, 4769–4779.

Stoven, S., Ando, I., Kadalayil, L., Engström, Y., Hultmark, D., **2000**. Activation of the *Drosophila* NF-kappaB factor Relish by rapid endoproteolytic cleavage. *EMBO Rep. 1*, 347–352.

Stoven, S., Silverman, N., Junell, A., Hedengren-Olcott, M., Erturk, D., *et al*., **2003**. Caspase-mediated processing of the *Drosophila* NF-kappaB factor Relish. *Proc. Natl Acad. Sci. USA 100*, 5991–5996.

Sugumaran, M., Saul, S., Ramesh, N., **1985**. Endogenous protease inhibitors prevent undesired activation of prophenolase in insect hemolymph. *Biochem. Biophys. Res. Commun. 132*, 1124–1129.

Szlanka, T., Haracska, L., Kiss, I., Deak, P., Kurucz, E., *et al*., **2003**. Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in *Drosophila melanogaster. J. Cell Sci. 116*, 1023–1033.

Takahashi, N., Kurata, S., Natori, S., **1993**. Molecular cloning of cDNA for the 29 kDa proteinase participating on decomposition of the larval fat body during metamorphosis of *Sarcophaga peregrina* (flesh fly). *FEBS Lett. 334*, 153–157.

Takahashi, S.Y., Yamamoto, Y., Shionoya, Y., Kageyama, T., **1993**. Cysteine proteinase from the eggs of the silkmoth, *Bombyx mori*: identification of a latent enzyme and characterization of activation and proteolytic processing *in vivo* and *in vitro*. *J. Biochem.* (*Tokyo*) *114*, 267–272.

Takayanagi, K., Dawson, S., Reynolds, S.E., Mayer, R.J., **1996**. Specific developmental changes in the regulatory subunits of the 26 S proteasome in intersegmental muscles preceding eclosion in *Manduca sexta*. *Biochem. Biophys. Res. Commun. 228*, 517–523.

Terra, W.R., Ferreira, C., Jordão, B.P., Dillon, R.J., **1996**. Digestive enzymes. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, pp. 153–194.

Tryselius, Y., Hultmark, D., **1997**. Cysteine proteinase 1 (CP1), a cathespin L-like enzyme expressed in the *Drosophila melanogaster* haemocyte cell line mbn-2. *Insect Mol. Biol. 6*, 173–181.

Tsuji, Y., Nakajima, Y., Homma, K.-I., Natori, S., **1998**. Antibacterial activity of a novel 26-kDa serine protease in the yellow body of *Sarcophaga peregrina* (flesh fly) pupae. *FEBS Lett. 425*, 131–133.

Turcotte, C.L., Hashimoto, C., **2002**. Evidence for a glycosaminoglycan on the nudel protein important for dorsoventral patterning of the Drosophila embryo. *Devel. Dyn. 224*, 51–57.

Turner, A.J., Isaac, R.E., Coates, D., **2001**. The neprilysin (NEP) family of zinc metalloendopeptidases, genomics and function. *BioEssays 23*, 261–269.

Uvardy, A., **1993**. Purification and characterization of a multiprotein component of the *Drosophila* 26S (1500 kDa) proteolytic complex. *J. Biol. Chem. 268*, 9055–9062.

Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J., *et al*., **2000**. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol. 150*, F69–F75.

Vier, J., Furmann, C., Hacker, G., **2000**. Baculovirus P35 protein does not inhibit caspase-9 in a cell-free system of apoptosis. *Biochem. Biophys. Res. Commun. 276*, 855–861.

Walz, J., Erdmann, A., Kania, M., Typke, D., Koster, A.J., *et al*., **1998**. 26S proteasome structure revealed by three-dimensional electron microscopy. *J. Struct. Biol. 121*, 19–29.

Wang, R., Lee, S.Y., Cerenius, L., Söderhall, K., **2001**. Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. *Eur. J. Biochem. 268*, 895–902.

Watts, R.J., Hoopfer, E.D., Luo, L., **2003**. Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron. 38*, 871–887.

Wei, S., Xie, Z., Filenova, E., Brew, K., **2003**. *Drosophila* TIMP is a potent inhibitor of MMPs and TACE:

similarities in structure and function to TIMP-3. *Biochemistry 42*, 12200–12207.

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., *et al.*, **2002**. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nature Cell Biol. 4*, 445–450.

Wojcik, C., DeMartino, G.N., **2002**. Analysis of *Drosophila* 26S proteasome using RNA interference. *J. Biol. Chem. 277*, 6188–6197.

Xu, G., Cirilli, M., Huang, Y., Rich, R.L., Myszka, D.G., *et al.*, **2001**. Covalent inhibition revealed by the crystal structure of the caspase-8/p35 complex. *Nature 410*, 494–497.

Xu, Y., Kawasaki, H., **2001**. Isolation and expression of cathepsin B cDNA in hemocytes during metamorphosis of *Bombyx mori*. *Comp. Biochem. Physiol. B 130*, 393–399.

Yamamoto, Y., Takahashi, S.Y., **1993**. Cysteine proteinase from *Bombyx* eggs: role in programmed degradation of yolk proteins during embryogenesis. *Comp. Biochem. Physiol. B 106*, 35–45.

Yamamoto, Y., Takimoto, K., Izumi, S., Toriyama-Sakurai, M., Kageyama, T., *et al.*, **1994**. Molecular cloning and sequencing of cDNA that encodes cysteine proteinase in the eggs of the silkmoth, *Bombyx mori*. *J. Biochem.* (*Tokyo*) *116*, 1330–1335.

Yamamoto, Y., Yamahama, Y., Katou, K., Watabe, S., Takahashi, S.Y., **2000**. *Bombyx* acid cysteine protease (BCP): hormonal regulation of biosynthesis and accumulation in the ovary. *J. Insect Physiol. 46*, 783–791.

Ye, S., Cech, A.L., Belmares, R., Bergstrom, R.C., Tong, Y., *et al.*, **2001**. The structure of a Michaelis serpin-protease complex. *Nature Struct. Biol. 8*, 979–983.

Yoshida, H., Ashida, M., **1986**. Microbial activation of two serine enzymes and prophenoloxidase in the plasma fraction of hemolymph of the silkworm, *Bombyx mori*. *Insect Biochem. 20*, 539–545.

Yu, X.-Q., Gan, H., Kanost, M.R., **1999**. Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. *Insect Biochem. Molec. Biol. 29*, 585–597.

Yu, X.-Q., Jiang, H., Wang, Y., Kanost, M.R., **2003**. Non-proteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 197–208.

Yu, X.-Q., Kanost, M.R., **2000**. Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to Gram-negative bacteria. *J. Biol. Chem. 275*, 37373–37381.

Yu, X.-Q., Zhu, Y.-F., Ma, C., Fabrick, J.A., Kanost, M.R., **2002**. Pattern recognition proteins in *Manduca sexta*-plasma. *Insect Biochem. Molec. Biol. 32*, 1287–1293.

Zhao, X., Mita, K., Shimada, T., Okano, K., Quan, G.X., *et al.*, **2001**. Isolation and expression of an ecdysteroid-inducible neutral endopeptidase 24.11-like gene in wing disks of *Bombyx mori*. *Insect Biochem. Mol. Biol. 31*, 1213–1219.

Zhao, X.F., Wang, J.X., Xu, X.L., Schid, R., Wieczorek, H., **2002**. Molecular cloning and characterization of the cathepsin B-like proteinase from the cotton boll worm, *Helicoverpa armigera*. *Insect Mol. Biol. 11*, 567–575.

Zhou, Q., Krebs, J.F., Snipas, S.J., Price, A., Alnemri, E.S., *et al.*, **1998**. Interaction of the baculovirus anti-apoptotic protein P35 with caspases. Specificity, kinetics, and characterization of the caspase/p35 complex. *Biochemistry 37*, 10757–10765.

Zhu, Y., Johnson, T.J., Myers, A.A., Kanost, M.R., **2003a**. Identification by subtractive suppression hybridization of bacteria-induced genes expressed in *Manduca sexta* fat body. *Insect Biochem. Mol. Biol. 33*, 541–559.

Zhu, Y., Wang, Y., Gorman, M.J., Jiang, H., Kanost, M.R., **2003b**. Manduca sexta serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *J. Biol. Chem. 278*, 46556–46564.

Zoog, S.J., Bertin, J., Friesen, P.D., **1999**. Caspase inhibition by baculovirus P35 requires interaction between the reactive site loop and the beta-sheet core. *J. Biol. Chem. 274*, 25995–26002.

4.8 Lipocalins and Structurally Related Ligand-Binding Proteins

H Kayser, University of Ulm, Germany

4.8.1. Introduction

Studies of insects have significantly contributed to the establishment of the lipocalins as a new family of proteins. Members of the lipocalin family are defined by a specific three-dimensional motif of polypeptide folding and by the binding of small lipophilic molecules in their interior. Starting about 20 years ago, the number of lipocalins has increased enormously through the structural analysis of known proteins as well as the discovery of new proteins showing the lipocalin structural motif. Today, the lipocalins represent a superfamily of proteins including members from vertebrates, insects, mites, ticks, crustaceans, other invertebrates, and, more recently, from plants and even prokaryotes. This wide occurrence of lipocalins is thought to reflect a common ancestry and a long evolutionary history suggesting also the involvement of these proteins in many vital processes (Ganfornina *et al.*, 2000).

The relatively late discovery of the lipocalins as a structural family of proteins is likely due to a low level of homology of their amino acid sequences. In pairwise comparisons of sequences, the identity between lipocalins may be below 20%, which is regarded as the lowest level for reliable alignments. Alternatively, lipocalins share a few sequence motifs in structurally conserved regions besides their overall highly conserved three-dimensional structure. Since sequence homology is of only limited value in identifying a new protein as a lipocalin, crystal structures have to be established for a definite assignment to this family, if modeling of its sequence into a known lipocalin structure is not successful. Hence, progress in the molecular characterization of

lipocalins depends on the availability of the protein of interest in sufficient quantity obtained either from a biological source or by heterologous expression of its cDNA. It depends further on the feasibility of obtaining well-diffracting crystals and, of course, on technical advancement in the crystallography field. The rapid growth of knowledge of the lipocalins with respect to structure and function in the past decade is accounted for by a recent special edition of *Biochimica Biophysica Acta* (vol. 1482, nos. 1–2, 2000) devoted entirely to this family of proteins. This issue offers a collection of relatively recent overviews of the lipocalin field dealing with general aspects of structures and functions as well as individual lipocalins including some from insects. The progress and growing importance of the lipocalins is further demonstrated by the first international Lipocalin Conference, which was held in 2003 in Copenhagen (Denmark). Those readers interested in the world of lipocalins are also referred to the lipocalin website.

The significant contributions from insect studies to the discovery and understanding of the lipocalins have not yet been comprehensively documented. The intention, therefore, of this chapter is to present an overview of the lipocalins identified in insects to date, including the fatty acid-binding proteins as structurally closely related ligand-binding proteins. Since all these diverse proteins represent variations of a common core structural theme, called an antiparallel β-barrel, this chapter describes the group-specific polypeptide folding and the underlying primary structures followed by the biological context, which comprises the occurrence of these proteins and the expression patterns of the corresponding genes, as well as the established and putative functions of each type of protein. The few lipocalins and structurally related proteins from ticks, mites, and crustaceans are included to cover all arthropods. Two well-known families of insect ligand-binding proteins that turned out not to be constructed as β-barrels, however, are also described to demonstrate their alternate folding. The lipocalins and fatty acid-binding proteins referred to in this chapter are listed in **Table 1**, including their accession codes. All multiple alignments of protein sequences shown in this chapter were performed using Clustal W, version 1.82, accessed via ExPASy. Graphical representations of crystal structures were prepared with Programme O (Jones *et al.*, 1991).

4.8.2. The β-Barrel Structural Motif

The characteristic lipocalin fold was first described for the human retinol-binding protein 20 years ago (Newcomer *et al.*, 1984), which thus became a kind of reference or prototypic lipocalin. Retinol-binding protein was soon followed by another vertebrate protein, β-lactoglobulin, the crystal structure of which showed high similarity to that of retinol-binding protein, though their sequences were not closely related (Papiz *et al.*, 1986). At this very early stage of the analysis of the lipocalin family, two insect proteins were studied independently for their crystal structures and, again, the close similarities in the folding motifs to that of retinol-binding protein were recognized instantly (Holden *et al.*, 1987; Huber *et al.*, 1987a, 1987b). These insect proteins were two lepidopteran biliproteins, which are discussed in detail below (see Section 4.8.3.1). The discovery of the architectural principle of retinol-binding protein in two evolutionarily distant insect proteins immediately suggested this new folding type to be very common across the phyla, though obviously not restricted to a specific functional class of proteins.

Typically, lipocalins are small extracellular, i.e., secreted, proteins of a size in the 20 kDa range, corresponding to about 180 amino acid residues, with affinity for specific small lipophilic molecules. Therefore, lipocalins are often referred to as extracellular lipid-binding proteins (eLBPs) to differentiate them from the related intracellular type (iLBPs) (see Section 4.8.6). The remarkably conserved lipocalin tertiary protein structure is called a β-barrel with a repeated +1 topology. This means it is made up of eight sequentially arranged antiparallel β-strands, assigned A to H, which are hydrogen-bonded to form a β-sheet that is folded back on itself to form an orthogonal stacking of two layers. The result is a barrel-like structure of a spherical shape. The eight β-strands are linked by seven loops, L1 to L7, which are all very short β-hairpins, except L1 that forms a large Ω loop covering partially the barrel opening with the internal ligand binding site. The other end of the barrel is closed by an N-terminal short 3_{10}-helical structure, which is followed immediately by strand A. One side of the barrel is lined with the C-terminal sequence, which includes a conserved α-helix preceding the short extra β-strand I, which does not contribute to the barrel structure. There are typically three conserved motifs, called structurally conserved regions (SCRs), each of them being characterized by a more or less extended invariant sequence motif. Two of the conserved regions, SCR1 and SCR3, which are present in all lipocalins, are located within the N- and C-terminal sequences comprising the 3_{10}-helix + strand A and strand H + the loop to the C-terminal α-helix, respectively. Another region, SCR2, that may be less

Table 1 Arthropod lipocalins and fatty acid-/retinoic acid-binding proteins referred to in the text, and their accession codes

Name of protein	*Source*	*Sequence (SwissProt/ TrEMBL)*	*Crystal structure (PDB)*
Lipocalins			
Bilin-binding protein	*Pieris brassicae*	P09464	1BBP
Insecticyanin a, mature form	*Manduca sexta*	P00305	coord. not in data bank
Insecticyanin a	*Manduca sexta*	Q00629	
Insecticyanin b	*Manduca sexta*	Q00630	
Biliverdin-binding protein I	*Samia cynthia ricini*	Q8T118	
Biliverdin-binding protein II	*Samia cynthia ricini*	Q8T119	
Nitrophorin 1	*Rhodnius prolixus*	Q26239	1NP1; 2NP1; 3NP1; 4NP1
Nitrophorin 2	*Rhodnius prolixus*	Q26241	1EUO
Nitrophorin 3	*Rhodnius prolixus*	Q94733	
Nitrophorin 4	*Rhodnius prolixus*	Q94734	1NP4; 1ERX; 1IKE; 1KOI; 1IKJ
Triabin	*Triatoma pallidipennis*	Q27049	1AVG
Pallidipin 2	*Triatoma pallidipennis*	Q27042	
Salivary platelet aggregation inhibitor 1	*Rhodnius prolixus*	Q94731	
Salivary platelet aggregation inhibitor 2	*Rhodnius prolixus*	Q94732	
Biogenic amine-binding protein	*Rhodnius prolixus*	Q86PT9	
Histamine-binding protein 1, female specific	*Rhipicephalus appendiculatus*	O77420	
Histamine-binding protein 2, female specific	*Rhipicephalus appendiculatus*	O77421	1QFT, 1QFV
Histamine-binding protein 3, male specific	*Rhipicephalus appendiculatus*	O77422	
Serotonin-histamine-binding protein	*Dermacentor reticulatus*	Q8WSK7	
Procalin	*Triatoma protracta*	Q9U6R6	
Bla g 4	*Blattella germanica*	P54962	
Lazarillo	*Schistocerca americana*	P49291	
glial Lazarillo	*Drosophila melanogaster*	Q9NAZ3 (Q9V6K5)	
neural Lazarillo	*Drosophila melanogaster*	Q9NAZ4 (Q8SXR1)	
Gallerin	*Galleria mellonella*	Q24996	
Bombyrin	*Bombyx mori*	Q95P97	
Hyphantrin	*Hyphantria cunea*	Q8T5Q9	
Tergal gland protein Lma-P22	*Leucophaea maderae*	O46130	
Tergal calycin p18	*Leucophaea maderae*	Q95VP9	
Crustacyanin A_1 subunit	*Homarus gammarus*	P58989	1GKA (holoA_1/A_2-dimer)
Crustacyanin A_1 subunit	*Homarus gammarus*		1H91 (apoA_1/A_1-dimer)
Crustacyanin A_2 subunit	*Homarus gammarus*	P80007	
Crustacyanin C_1 subunit	*Homarus gammarus*	P80029	1I4U (apoC_1/C_1-dimer)
Fatty acid-/retinoic acid-binding proteins			
Fatty acid-binding protein MFB1	*Manduca sexta*	P31416	
Fatty acid-binding protein MFB2	*Manduca sexta*	P31417	1MDC
Fatty acid-binding protein	*Schistocerca gregaria*	P41496	1FTP
Fatty acid-binding protein	*Locusta migratoria*	P41509	
Fatty acid-binding protein	*Heliothis zea*	O76515	
Fatty acid-binding protein	*Drosophila melanogaster*	Q9VGM2	
Fatty acid-binding protein	*Anopheles gambiae*	Q17017	
Fatty acid-binding protein	*Apis mellifera*	Q9Y1C6	
Fatty acid-binding protein Blo t 13	*Blomia tropicalis*	Q17284	
Fatty acid-binding protein Lep d 13	*Lepidoglyphus destructor*	Q9U5P1	
Fatty acid-binding protein Aca s 13	*Acarus siro*	O76821	
Cellular retinoic acid-binding protein	*Manduca sexta*	O61236	

PDB, protein data bank.

well conserved, covers parts of the strands F and G and the connecting loop L6 at the bottom of the barrel. All SCRs are located at the closed end of the β-barrel which may provide a docking site for other proteins. Furthermore, lipocalin sequences display typically four cysteine residues that form two disulfide bridges at comparable positions. The typical arrangement of secondary structures and structurally

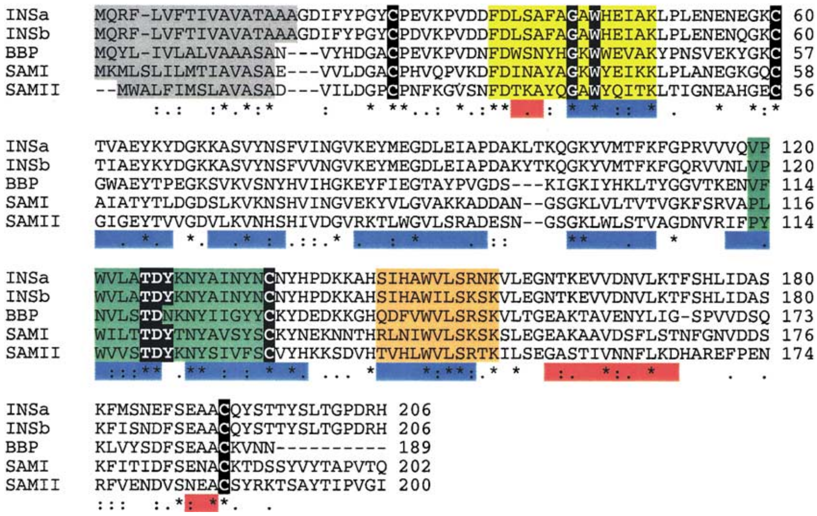


Figure 1 Alignment of the biliproteins with known full sequences. The proteins are insecticyanin a (INSa) and insecticyanin b (INSb) from *Manduca sexta*, the bilin-binding protein (BBP) from *Pieris brassicae*, and the biliverdin-binding proteins I and II (SAMI and SAMII) from *Samia cynthia ricini*. Black boxes show residues typically conserved in lipocalins. N-terminal secretion signals are boxed in gray and the structurally conserved regions SCR1, SCR2, and SCR3 are in yellow, green, and orange, respectively. Below the sequences, the approximate positions of helices and β-strands are labeled as red and blue bars, respectively. Asterisks indicate identical residues, double and single points more or less conserved substitutions.

conserved regions is illustrated in Figure 1 with sequences from biliproteins representing prototypic lipocalins (see Section 4.8.3).

The interior of the β-barrel harbors the binding site for a (usually single) molecule of ligand, which can be very different in shape, size, and physicochemical properties depending on the structure of the loop scaffold and the cavity of the individual lipocalin. Ligands can be retinoids, bilins, heme, carotenoids, and odorants (not in insects), depending on the physiological context of the carrier protein. However, the physiological ligands are not known from all lipocalins; some may also function without any bound ligand (see Section 4.8.5). The collective name "lipocalin," proposed in 1987 (Pervais and Brew, 1987), denotes these proteins as carriers of lipophilic ligands that are harbored within a "calyx" (from Greek, meaning cup). Though lipocalins may be found as monomers *in vivo* and *in vitro*, they have a tendency to form homomeric oligomers, as frequently observed in the crystalline state. Lipocalins may also complex to other soluble proteins (e.g., retinol-binding protein combines with transthyretin in vertebrate blood) or to specific cell surface receptors. The latter aspect is not yet well documented, however.

There are several other groups of proteins that share a repeated +1 topology β-barrel with the lipocalins but display significant differences in topology and the construction of the barrel. The lipocalin-related proteins are represented by the superfamily of fatty acid-binding proteins (see Section 4.8.6), which were structurally discovered nearly one decade later, and triabin, an unusual single lipocalin that was isolated from an insect even more recently (see Section 4.8.4.3). The specific modifications of the β-barrel structure, as described above for the lipocalins, will be described in the appropriate sections. Two other protein families, the biotin-binding avidins and the bacterial metalloproteinases, which are structurally related to the lipocalins, fatty acid-binding proteins, and triabin, are outside the scope of this chapter dealing with insect proteins. All these variants of β-barrel proteins are part of a structural superfamily, called "calycins," as they are all characterized by a cuplike cavity as a real or potential internal binding site for a ligand (reviews: Flower *et al*., 1993, 2000; LaLonde *et al*., 1994).

4.8.3. Biliproteins: Prototypic Lipocalins

4.8.3.1. Biliproteins with Known Crystal Structure

The green coloration of many insect larvae, thought to provide camouflage at their feeding sites on plants, has been subject to many biological and chemical studies in the past. This green color is known to result from a mixture of yellow carotenoids derived from the food and blue bile pigments synthesized by the insect. Both pigments are usually associated with different proteins, the chromatographic separation of which demonstrates nicely the two underlying coloring principles (review: Kayser, 1985). Insect larvae that are fed artificial diets, usually very poor in carotenoids,



Figure 2 Last instar larvae of *Manduca sexta* fed tobacco leaves (green form) and artificial diet (blue form), respectively. The difference in color results from the lack of yellow carotenoids in the artificial diet. The blue coloration is due to the presence of insecticyanin, a biliprotein synthesized by the larvae.

consequently exhibit not a green but a blue coloration. Insect scientists working with a laboratory strain of the tobacco hornworm, *Manduca sexta*, for example, may have rarely seen the "wild" green coloration of the insect in nature (**Figure 2**). Blue chromoproteins, all most likely representing biliproteins, are present in many insect orders (Kayser, 1985). The eye-catching colors of biliproteins have certainly contributed to the fact they were studied early at the biochemical level and thus had a good chance to contribute to the discovery of the lipocalins. As mentioned above, the third and fourth lipocalin, the structures of which were independently solved by X-ray crystallography, were not only two proteins from insects but also two rather similar ones: the biliproteins from the butterfly *Pieris brassicae* and from the moth *M. sexta* (see below). Long before, both were known as proteins with an attractive sky-blue color, which is due to the presence of a bilatrien bile pigment, biliverdin IXγ. Both proteins were studied for their crystal structures in the expectation of finding a predominantly α-helical folding like that established for the light-harvesting cyanobacterial C-phycocyanins that represent biliproteins with a covalently bound derivative of biliverdin IXα (Schirmer *et al*., 1987).

4.8.3.1.1. Bilin-binding protein from *Pieris brassicae* The biliprotein from *P. brassicae*, which was given the specific name bilin-binding protein (BBP) (Huber *et al*., 1987a), was isolated from whole butterflies, where it is located predominantly in the wings, and characterized in the author's laboratory as a monomeric, nonglycosylated protein that is noncovalently associated with biliverdin IXγ at a 1 : 1 stoichiometry (Zipfel, 1982; Kayser, unpublished data). A molecular mass of ~20 kDa was obtained for purified BBP by both gel filtration and sodium dodecylsulfate (SDS)-gel electrophoresis consistent with its monomeric status. Alignment of the BBP sequence revealed closest homology to the biliprotein from *M. sexta*, known as insecticyanin (see Section 4.8.3.1.2), and to human apolipoprotein D with identities of 43% and 31%, respectively. The mature protein, as isolated from the butterflies, comprises 174 amino acid residues corresponding to a molecular mass of 19 790 Da in agreement with the chromatographic results (Suter *et al*., 1988). An N-terminal signal sequence of 15 amino acids is found in the precursor, which has been cloned from larvae (Schmidt and Skerra, 1994). In the crystal state BBP was found as a tetramer consisting of two dimers (Huber *et al*., 1987a, 1987b). The folding of the monomers was that of the typical eight-stranded β-barrel with orthogonal arrangement in the two sheets (**Figure 3**), as was first described for the retinol-binding protein. A typical short 3_{10}-helix is located in the N-terminal sequence just before the first β-strand. A long α-helix is attached along one side of the barrel in the C-terminal region that terminates in a short helical sequence. BBP contains two cysteine bridges that are believed to support this folding structure. Though the sequence homology of these two lipocalins is only ~10%, their tertiary structures match almost perfectly in the central and lower segments, while the loop region around the open end of the barrel is more varied. The calyx-like cavity of BBP harbors

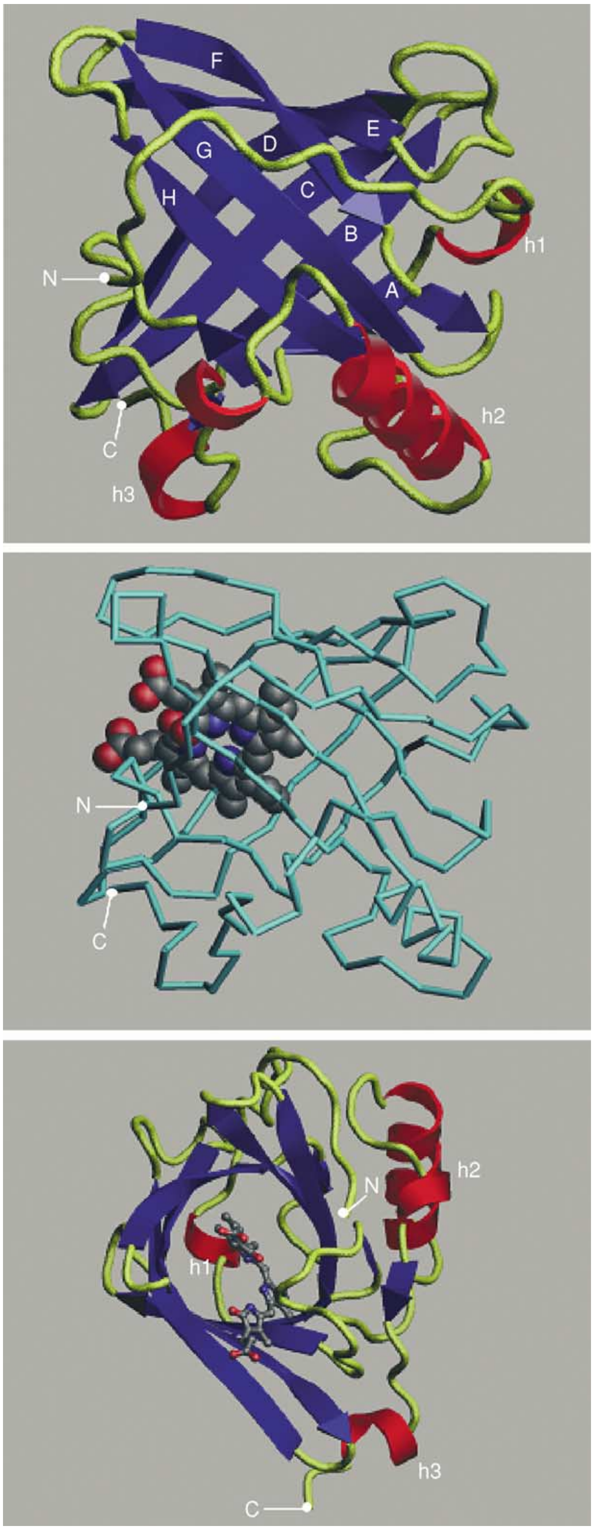


Figure 3 Crystal structure of the bilin-binding protein (BBP) from *Pieris brassicae*. Upper and lower panels: ribbon drawing with eight β-strands (blue arrows, labeled A–H) and three helices (red, labeled h1–h3). Middle panel: Cα-backbone structure (light blue) of the protein (same orientation as in the upper panel) with bound biliverdin-IXγ represented as stick-and-ball model. Oxygens are in red and nitrogens in blue. The N- and C-termini are marked with white dots.

the chromophore ligand, which is not deeply buried in the cavity but located close to the open end of the barrel with the two carboxyl groups of the heme propionate side chains pointing to the solvent. This implies only little contribution of these polar groups to the binding of the tetrapyrrole. The pyrrole nitrogens of the bilin are complexed to water molecules and in contact with the protein via a few hydrogen bonds.

The identity of the ligand of BBP was confirmed by its crystal structure as the γ-isomer of biliverdin IX. The bilin shows a cyclic helical structure characterized by all-*Z* configuration and all-*syn* conformation (**Figure 3**). This geometry is adopted also by free bilatrienes in solution (Kayser, 1985) and in agreement with the visible absorption spectrum of the bound chromophore (see below). The cyclic helical geometry of a bilatrien gives rise to two enantiomers with opposite helical sense, which are rapidly interconverted in solution. The crystallographic analysis revealed that the protein binds only one enantiomer of the ligand (**Figure 3**), thus inducing chirality in a compound that is achiral by structure (Huber *et al*., 1987b; Scheer and Kayser, 1988). The induced optical activity of the *P. brassicae* BBP is nearly as strong as that in the cyanobacterial phycocyanins, which has a different basis, however. The circular dichroism (CD) spectrum of BBP shows a positive Cotton effect in the visible range, which could be correlated with the right-handed helix of the bilin for the first time (Huber *et al*., 1987b). Treatment of the holoprotein with urea completely abolishes the optical activity of the holoprotein due to the unfolding of the protein that destroys the specific ligand binding site (Scheer and Kayser, 1988). As shown with the recombinant BBP apoprotein derived from larval cDNA, the holoprotein can be reconstituted by incubation with free biliverdin IXγ to yield an apparently native product as judged from its characteristic absorption spectrum (Schmidt and Skerra, 1994). However, no CD spectrum was recorded, hence the correct binding of the chromophore as the right-handed helical enantiomer has not been demonstrated. As indicated above, the visible spectrum of the bound chromophore is similar to that of the free form, as both are in a cyclic helical conformation. Characteristic differences, however, are seen at the long-wave absorption peak with a bathochromic shift of ~25 nm and a significant hyperchromic effect to produce an absorbance plateau around 670 nm; the sharp short-wave peak (the typical Soret band of tetrapyrroles) is at 383 nm (Zipfel, 1982; Scheer and Kayser, 1988). The tight fitting of ligand binding to its carrier lipocalin, as is obvious in case of BBP, may

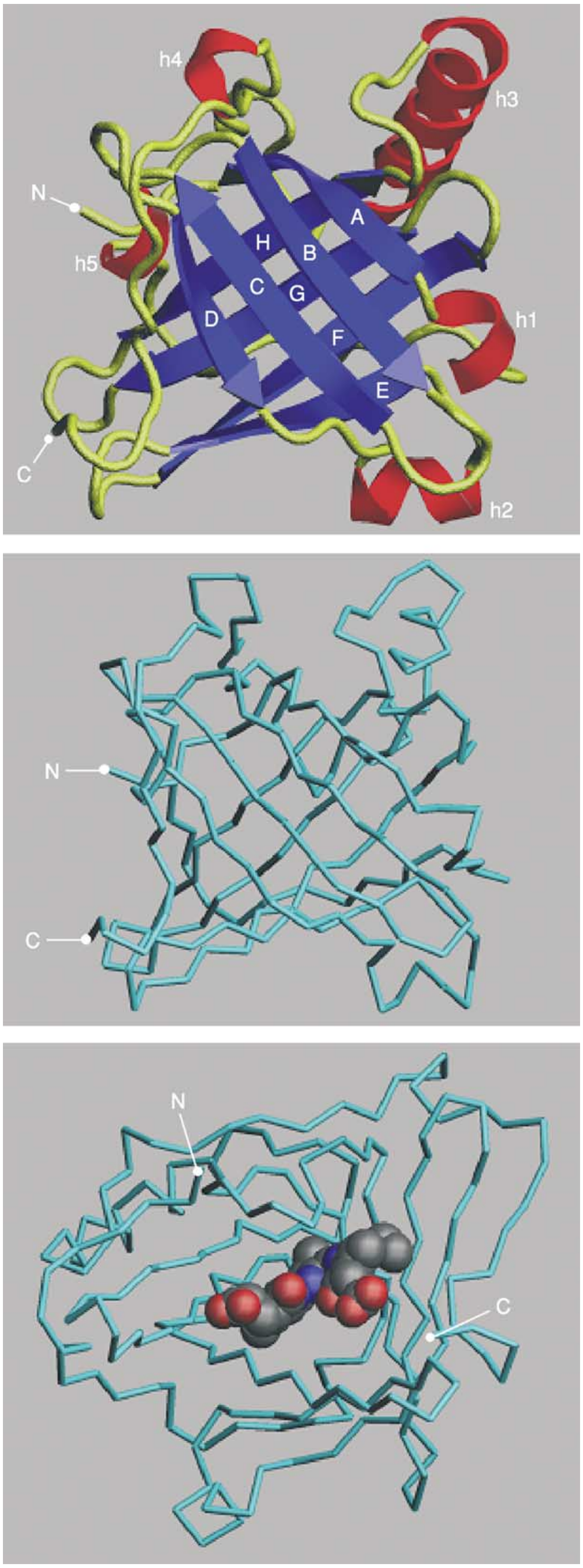


Figure 4 Crystal structure of insecticyanin from *Manduca sexta*. Upper panel: ribbon drawing with eight β-strands (blue arrows, labeled A–H) and several helices (red, labeled h1–h5). Middle and lower panels: Cα-backbone structure (light blue) of the protein with bound biliverdin-IXγ in the lower panel. The ligand is represented as stick-and-ball model with oxygens in red and nitrogens in blue. The upper and middle panels show the protein in the same orientation. The N- and C-termini are marked with white dots.

generally result in some electronic interaction between the bound chromophore and the protein. Conversely, a biliprotein with a fairly unchanged absorption spectrum versus the free bilin is unlikely to represent a lipocalin.

4.8.3.1.2. Insecticyanin from *Manduca sexta*
Insecticyanin from larvae of the tobacco hornworm, *M. sexta*, was the first insect biliprotein that was purified and studied at the biochemical level (Cherbas, 1973). It can be easily obtained from larval hemolymph, where it may account for up to 5% of the total protein. Insecticyanin is very similar to BBP from *P. brassicae* with respect to its molecular size (189 amino acids for the protein as isolated; molecular mass of 21 378 Da), the presence of two disulfide bridges, the absorbance spectrum, the non-covalent binding of one molecule of biliverdin IXγ per monomer as well as the absence of bound carbohydrate and lipid (Riley *et al*., 1984; Goodman *et al*., 1985). The crystal structure of insecticyanin, solved independently and published only weeks after that of BBP, revealed very close similarity to that of retinol-binding protein (Holden *et al*., 1987). This finding extends the overall similarity between the biliproteins from *M. sexta* and *P. brassicae* to the level of their crystal structures, though the sequence identity is only 40%. Moreover, both biliproteins crystallized as tetramers.

As shown in Figure 4, insecticyanin is folded as a barrel made up of eight antiparallel β-strands with an orthogonal arrangement of the two sheets, comparable to BBP. The N- and C-terminal sequences with several conserved helical structures are wrapped near the opening of the barrel. There is an extra helical structure inserted between the fourth and fifth β-strand (labeled h2 in Figure 4). The biliverdin ligand is located near the opening with the two propionate side chains directed to the solvent. The ligand of insecticyanin is in contact with hydrophobic residues on both sides, one of which is more polar than the other one in contrast to the situation in the related BBP. Like in the latter, biliverdin IXγ in insecticyanin adopts a porphyrin-like cyclic helical conformation, which is also right-handed like that in BBP (cf. Figures 3 and 4). There is no mention of the helical sense in the publication by Holden *et al*. (1987). As expected from the study of the *P. brassicae* protein, the chromophore of

insecticyanin is optically active, producing a CD spectrum that is consistent with a right-handed helical sense (Kayser, unpublished data).

4.8.3.2. Biliproteins with Unknown Crystal Structure

Due to their widespread occurrence in insects and easy visibility, a number of biliproteins have been isolated, mostly from lepidopteran species, and biochemically characterized in more or less detail (Table 2). The biliproteins from *P. brassicae* and *M. sexta* have been studied most thoroughly including their crystal structures, as described above. The possible classification of the other biliproteins as lipocalins depends on the results of future crystallographic analysis or successful modeling of amino acid sequences into the crystal structures of one of the known biliproteins. The full-length amino acid sequences are also known for the two biliproteins from the silk moth *Samia cynthia ricini* and from two other proteins from the butterfly *P. rapae*. The latter two proteins, which differ from each other by

Table 2 Purified insect biliproteins

Insects	*Name of protein*[a]	*Subunit size (kDa)*	*Oligomeric state*	*Bilin type (bilin/subunit)*	*Glyco/ Lipo protein*	*Reference*
Lepidoptera						
Pieris brassicae	Bilin-binding protein BBP[b,c]	19.8	Monomer	BV IXγ (1 : 1)	−/−	Suter *et al.* (1998)
Pieris rapae	Bilin-binding proteins BBP-1, BBP-2	∼18	?	BV IXγ ? (?)	?/?	Yun (personal communication)
Manduca sexta	Insecticyanin[b,c]	21.4	Tetramer	BV IXγ (1 : 1)	−/−	Riley *et al.* (1984)
Agrius convolvuli	*A. convolvuli* insecticyanin[d]	21.2	Trimer (?)	BV IXγ? (1 : 1)	?/?	Saito and Shimoda (1997)
Attacus atlas	Biliverdin-binding protein (BBP)[d]	22.4	Dimer	BV IXγ? (1 : 1)	?/?	Saito (1997)
Samia cynthia ricini	Biliverdin-binding proteins BBP-I,[c] BBP-II[c]	I: 20.5 II: 22.7	Monomer Dimer	BV IXγ (1 : 1) BV IXγ (1 : 1)	?/? ?/?	Saito (1998a)
Antheraea yamamai	Biliprotein BP = P-II[d]	21.5	Monomer	Phorcabilin (?)	?/?	Saito *et al.* (1998)
Rhodinia fugax	Biliproteins BP-I,[d] BP-II[d]	I: 22.6 II: 22.9	I: Monomer II: Dimer	I: phorcbilin (?) II: BV IXγ (?)	?/? ?/?	Saito (1998b)
Cerura vinula	*Cerura (vinula)* biliprotein	∼80	Tetramer ?	unknown (?)	?/?	Scheer and Kayser (1988)
Heliothis zea	Blue chromoprotein	∼150	Tetramer	BV (?)	+/+	Haunerland and Bowers (1986)
Trichoplusia ni	Chromoprotein	∼150	Dimer	BV (?)	+/+	G. Jones *et al.* (1988)
Spodoptera litura	Biliverdin-binding proteins BP-1–BP-4	∼165	Dimers	BV (?)	+/+	Yoshiga and Tojo (1995)
Spodoptera litura	Biliverdin-binding vitellogenin	∼188	Dimer	BV (?)	?/+	Maruta *et al.* (2002)
Orthoptera						
Locusta migratoria	Cyanoprotein	∼83	Tetramer	BV (2 : 1)	+/−	Chino *et al.* (1983)
Heteroptera						
Podisus maculiventris	Blue lipophorin	∼250/∼80/∼20	>700 kDa	BV IXγ (?)	?/+	Haunerland *et al.* (1992b)
Riptortus clavatus	Cyanoproteins CP1–CP4	∼76	Hexamers	BV IXγ (4 : 1)	+/−	Chinzei *et al.* (1990)

[a]As referred to in the reference.
[b]Crystal structure available; see **Table 1**.
[c]Full sequence available; see **Table 1**.
[d]N-terminal sequence available.
BV, biliverdin.

a few residues only, are of the same size as BBP from *P. brassicae* to which they show ~94% identity (Yun, personal communication). Hence, it is most likely that the biliproteins from *P. rapae* have crystal structures almost identical to that of BBP from the closely related *P. brassicae*. N-terminal sequences are known from several moth biliproteins (see **Table 2**).

The chromophores of most insect biliproteins have been described on the basis of their visible absorption spectra as either biliverdin IXγ or "biliverdin(-like)" without further specification (**Table 2**). A more rigorous approach by, for example, microchemical degradation has not been performed with any of these chromoproteins. On the other hand, the identification of biliverdin IXγ, though tentative so far, in most of the lepidopteran proteins is not surprising since this biliverdin isomer is typical for this insect order (review: Kayser, 1985).

Phorcabilin, a derivative of biliverdin IXγ with extended chromophore geometry, seems to be associated with proteins in two saturniid moths only, in accordance with the restrictive occurrence of this bilin (Kayser, 1985). The ligand of the biliprotein from the moth *Cerura vinula* has not yet been identified. Preliminary results suggest a new chromophore, possibly a derivative of biliverdin IXγ (Scheer and Kayser, 1988; Kayser, unpublished data).

In many cases, the names of the insect biliproteins that have been described to date may give rise to confusion, because they are used for different proteins, or they simply refer to the blue color, as documented in **Table 2**. Though this is no attempt to propose a nomenclature for biliproteins, it should be kept in mind that the name "bilin-binding protein" or "BBP" has been given specifically to the biliprotein(s) from *P. brassicae* (Huber *et al.*, 1987a, 1987b). Similarly, "insecticyanin" has been coined for the biliprotein from *M. sexta* (Cherbas, 1973). These names should not be used for biliproteins from other species. Furthermore, a protein may be denoted as "biliverdin-binding" only if the chromophore has been definitely identified as one of the isomers of biliverdin IX. If the chemical class of the (blue) ligand is unknown, the protein may be described just as, for example, a "blue chromoprotein," "blue lipophorin," or "cyanoprotein," as in the past. To avoid confusion in future work it is proposed to use the generic term "biliprotein," abbreviated as "BP," linked to the name of the species from which it was isolated.

At least two groups of biliproteins may be discriminated on the basis of their overall characteristics (**Table 2**). One group is characterized by small proteins of ~20 kDa, lack of sugar and lipid constituents, binding of a single ligand per subunit, and occurrence as monomers or as homo-oligomers. This group may represent lipocalins, as typified by BBP from *P. brassicae* and insecticyanin from *M. sexta*. The second group comprises proteins characterized by much larger subunits in the 80 kDa or even 150 kDa range, the presence of sugar and/or lipid and the aggregation to complexes with sizes of up to 600 kDa and more. Some proteins of this type, such as the biliverdin-binding vitellogenin from *Spodoptera litura* (Maruta *et al.*, 2002), the blue lipophorin from *Podisus maculiventris* (Haunerland *et al.*, 1992b), and the cyanoprotein from *Riptortus clavatus* (Chinzei *et al.*, 1990) show properties that relate them to the storage proteins which are used during the embryo and metamorphosis stages (see Haunerland, 1996). In most of the biliproteins listed in **Table 2**, the absorption spectrum of the bound chromophore differs more or less significantly from that of the free ligand obtained after extraction with an organic solvent. As in the *Pieris* BBP, the long-wave absorption maximum of the bilin is usually redshifted (bathochromic effect) and increased (hyperchromic effect), which implies some electronic interaction between the ligand and the protein. Whether such spectral changes indicate that the ligand is specifically bound with respect to its structure and the binding site is not clear. It may well be that less specific binding of a ligand to a site on the protein surface or to the lipid moiety of a protein also results in a shift in the absorption spectrum. In any case, the binding cavity of the known lipocalins would not be large enough to harbor more than one bilin per monomer. So, the nature of the ligand-binding site of the large-size biliprotein remains conjecture. However, the crystal structure of the ~80 kDa subunit biliprotein from *Cerura vinula*, isolated in the author's laboratory, may be solved in the near future if current attempts to obtain high-quality crystals are successful.

4.8.3.3. Molecular Variants of Biliproteins

In a number of species, multiple biliproteins have been isolated and characterized by their amino acid composition, N-terminal sequence, and molecular mass (**Table 2**). Examples are found among the small-size (~20 kDa) proteins from two saturniid moths. In *Samia cynthia ricini*, the two biliverdin-binding proteins (BBP-I and BBP-II) from larval hemolymph share only 48% identity in their N-terminal 50 amino acid residues. BBP-II is likely identical to the biliprotein isolated from the molting fluid at pupation from the same insect (Saito, 1993, 1998a). The identity of the predominant BBP-I from this moth with BBP from *P. brassicae*

and insecticyanin from *M. sexta* is 46% and 52%, respectively. The corresponding value for BBP-II is 42% for both comparisons. In *Rhodinia fugax*, the biliproteins BP-I from larval hemolymph and cuticle and BP-II from epidermis are similar to one another and to those from *P. brassicae* and *M. sexta*, respectively (Saito, 1998b).

An example for multiple large-size (~165 kDa) proteins has been found in *Spodoptera litura*, from which four dimeric hemolymph biliverdin-binding proteins, differing in their isoelectric point, have been isolated indicating the presence of several non-identical subunits of about equal size (Yoshiga and Tojo, 1995). Unfortunately, partial sequences are not available to support this conclusion, but immunological differences have been recognized. As mentioned above, another biliverdin-binding protein from the same moth has been identified as a vitellogenin as it is female-specific and shows significant sequence homology with lepidopteran vitellogenins (Maruta *et al*., 2002).

The oligomeric situation with the cyanoproteins from the bug *Riptortus clavatus* is different (Chinzei *et al*., 1990). Here, two distinct subunits of about equal size combine to form four biliverdin-associated hexameric complexes which have been isolated as CP1 to CP4. According to their cDNA-deduced amino acid sequences, these proteins are closely related to arthropod hemocyanins and phenol oxidases, which are members of the superfamily of hexamerins (Miura *et al*., 1998). Their hexameric state and the high ratio of chromophore per subunit of 4 : 1, as calculated for CP1, support the conclusion that these bug cyanoproteins are not lipocalins.

Full data at the gene level on molecular variants of biliproteins have been obtained for the established lipocalin from *M. sexta* (Kiely and Riddiford, 1985; Riddiford *et al*., 1990; Li and Riddiford, 1992, 1994, 1996). In this insect, two major isoelectric forms of insecticyanin, described as a more acidic INS-a (pI 5.5) and a more basic INS-b (pI 5.7), have been identified. Both forms are present in the larval epidermis and cuticle, whereas only INS-b is found in the hemolymph. It is this form which has been studied as the first insect biliprotein by Cherbas (1973). The protein sequences encoded by the cDNAs for INS-a and INS-b, respectively, differ in 13 of the 189 amino acid residues of the mature protein; the N-terminal signal sequences are identical. The 3′ noncoding regions of the two cDNAs contain a sequence stretch that is unique for each gene and led to specific probes for expression studies of the two duplicated insecticyanin genes.

Comparable to the results on insecticyanin in *M. sexta*, two isoelectric forms of the BBP were purified from *P. brassicae* with pI values of 6.4 (BBP-I) and 6.2 (BBP-II), respectively (Kayser, unpublished data). The two proteins differ only in the N-terminal residue, which is asparagine in the predominant form BBP-I and aspartate in the minor variant BBP-II (Huber *et al*., 1987b). This single difference in sequence fully accounts for the observed difference in the isoelectric point of the two forms. As expected, the crystal structures of the two BBP variants are identical, but BBP-II does not dimerize. In contrast to the two isoforms obtained as proteins from adult insects, only one cDNA sequence coding for BBP (174 amino acid residues of the mature protein plus an N-terminal signal peptide of 15 amino acids) has been found in last instar larvae, which are actively expressing this gene (Schmidt and Skerra, 1994), and accumulate most of the BBP holoprotein during the insects' life (Kayser, 1984; Kayser and Krull-Savage, 1984). Hence, BBP-II may arise artifactually by deamidation of BBP-I during the process of purification. The presence of the minor form BBP-II *in vivo* has yet to be confirmed.

Two isoelectric forms of biliproteins are also known from the related butterfly, *P. rapae*, which differ in 12 nucleotides in their cDNA sequences (Yun, personal communication). Hence, there are obviously separate genes for the two biliproteins in *P. rapae*, in contrast to only a single gene in *P. brassicae*.

4.8.3.4. Developmental Expression and Biosynthesis of Biliproteins

Though biliproteins are widespread among insects and may share a number of physicochemical features, there seems to be no clear common pattern with respect to their location in the body, their site of synthesis, and their fate during development. Detailed studies of the transcription of genes coding for apobiliproteins and the corresponding synthesis of proteins have been performed for insecticyanin in *M. sexta* (Riddiford, 1982; Trost and Goodman, 1986; Riddiford *et al*., 1990; Li and Riddiford, 1994). Insecticyanin is present in the larval epidermis and hemolymph up to the adult stage. The highest concentration is found in the hemolymph of early fourth instar larvae and in adults after eclosion (up to 0.8 mg ml^{-1}), while the levels decrease after each larval molt. The genes *ins*-a and *ins*-b coding for the two isoelectric forms of insecticyanin are expressed during all larval instars with significant cyclic variations, followed by corresponding increases and decreases of the protein titers. Since the expression of the *ins* genes is restricted to the larva, insecticyanin present in the adult insect must

be retained from the larval stage. Both insecticyanin variants are mainly synthesized in the larval epidermis and to a lesser extent in the fat body. While the epidermis stores both proteins in granular form and secretes them into the cuticle, it delivers only INS-b to the hemolymph. INS-b is also produced as the predominant isoform in the fat body, but obviously released quickly and not stored there, as this tissue lacks blue coloration. The epidermal expression of *ins*-a lasts until pupal commitment, while that of *ins*-b continues up to the wandering stage. This differential control of gene expression is due to a small peak in the ecdysteroid titer occurring after the disappearance of juvenile hormone (Riddiford *et al*., 1990; Li and Riddiford, 1994). Thus, the developmental regulation of insecticyanin synthesis is apparently part of the overall endocrine program that controls the larval–pupal transition. In the black mutant of *M. sexta*, which is characterized by a lack of juvenile hormone (see **Chapter 3.7**), the expression of both *ins* genes is reduced in the epidermis, while it is enhanced in the fat body, suggesting a direct, tissue-specific effect of juvenile hormone on the expression of these genes (Li and Riddiford, 1996).

Insecticyanin is present in mature eggs of *M. sexta* at a concentration exceeding that in the hemolymph, though the *ins* genes are not expressed in any cell type in pupae and adults. The protein persisting from the larval stage is taken up from the hemolymph into the developing oocytes by a saturable and specific membrane-bound mechanism that requires the presence of calcium, as found by Kang *et al*. (1995, 1997). An apparently multimeric receptor with an estimated size of ~185 kDa that selectively binds insecticyanin with high affinity (K_d ~17 nM) has been characterized in detergent extracts from oocyte membranes. Thus, insecticyanin in the *Manduca* egg behaves like the storage proteins vitellogenin and lipophorin despite the differences in protein architecture, molecular size and mechanism of uptake into the egg (review: Haunerland, 1996) (see **Chapter 3.9**).

The expression of the BBP in *P. brassicae* is under developmental and tissue-specific control. Its mRNA can be demonstrated in penultimate and last instar larvae with a depression during the last larval and larval–pupal molts (Schmidt and Skerra, 1994). This kind of cyclic expression resembles that seen for insecticyanin in *M. sexta*. On the other hand, the BBP gene is not expressed in the larval epidermis but in the fat body, predominantly in the dorsolateral sheets and in the fat body tissue underlying the epidermis. This is not surprising since this tissue is of bluish coloration. Only little BBP mRNA is seen in the ventrolateral fat body, which is colorless indicating that the holoprotein is not stored there to a significant extent. Furthermore, the gene seems to be active also in the larval "gonads" (testes, according to this author). In marked contrast to the situation in *M. sexta*, the expression of the BBP gene in *P. brassicae* is again strong in the late pupa (pharate adult) and in young adults before it ceases almost completely (Schmidt and Skerra, 1994). This developmental pattern of BBP expression is in accordance with results from biochemical studies on the protein titer and the incorporation of specific precursors into the bilin and the apoprotein, respectively (Kayser, 1984; Kayser and Krull-Savage, 1984). The most active phases of holoprotein synthesis are in the last larval instar before the wandering stage and in late pupae following the increased ecdysteroid titer, which triggers adult development (see **Chapter 3.5**). The BBP synthesis ceases 2 days after adult emergence. The strong synthesis of BBP in developing adults is surprising as it is not sequestered into the eggs of *P. brassicae*, in contrast to the fate of insecticyanin in *M. sexta*. Most of the BBP in the butterfly is located in the wings, which are blue underneath the layer of white scales containing large amounts of pterin pigments in granular form (review: Kayser, 1985). Recent studies in the author's laboratory revealed that this wing BBP is synthesized *de novo* as holoprotein in the wings during adult development, while the BBP gene is switched off in the rest of the body (Sehringer, 1999; Sehringer and Kayser, unpublished data). These studies also demonstrated that the wings are capable of synthesizing the bilin ligand of BBP from the specific heme precursor 5-aminolevulinate (see below). Results from a developmental study of the activity of one of the key enzymes of tetrapyrrole synthesis, porphobilinogen synthase, are in accordance with the prominent phases of BBP synthesis and with the wings as the sites of BBP synthesis in the pupal stage (Kayser and Rilk-van Gessel, unpublished data).

There is an interesting difference between *P. brassicae* and *P. rapae* relating to the regulation of their biliproteins during development. In *P. rapae*, the isoform BBP-1 is abundant in the larval hemolymph, while there is more BBP-2 in the pupal stage (Yun, personal communication). The site of synthesis of the two *P. rapae* biliproteins remains to be studied. No isoforms with differential temporal expression are known from the closely related *P. brassicae*, as mentioned above.

In the larvae of *Spodoptera litura*, the four biliverdin-binding proteins that are composed of two ~165 kDa lipoprotein subunits (**Table 2**) also

show hemolymph levels that fluctuate cyclically in the course of larval development (Yoshiga *et al*., 1998), as described above for insecticyanin. The timing of BP-4 differs from that of the others, as it appears in earlier instars and has a low titer in the last instar, when the levels of the others are high. Synthesis of BP-4 seems to be triggered by juvenile hormone (Yoshiga and Tojo, 2001). At pupation, the biliproteins are taken up by the fat body, which turns blue in the pupa. A comparable sequestering of large-size biliproteins as glycolipoproteins by the fat body from the hemolymph at pupation is observed in the moth *Heliothis zea* (Haunerland and Bowers, 1986) and in the bug *Riptortus clavatus* (Chinzei *et al*., 1990). These biliproteins with subunits of ~150 kDa and ~76 kDa, respectively, represent high-density lipoproteins (see **Chapter 4.6**) and behave like storage proteins that are used up for adult development and egg formation. It is remarkable that the *Manduca* insecticyanin, a typical ~20 kDa lipocalin, also behaves like a storage protein in the embryo, as described above. This demonstrates that the presence of a bilin ligand does not denote a specific functional class of proteins.

As for the biosynthesis of the bilin chromophore of biliproteins, labeling studies in a number of insects have demonstrated that the heme precursor 5-aminolevulinate is efficiently incorporated into the bilin (review: Kayser, 1985). More detailed experiments, also in relation to the synthesis of the apoprotein, seem to be available only for the BBP from *P. brassicae* (Kayser, 1984; Kayser and Krull-Savage, 1984). These studies revealed that the syntheses of the bilin and the apoprotein are coordinated but independent from each other, since cycloheximide has a strong inhibitory effect on the synthesis of the apoprotein, while the incorporation of radiolabel from 5-aminolevulinate into protein-bound bilin was only weakly reduced. This suggests the presence of a relatively large pool of apoprotein available for binding of the heme derivative. A study of the time course of BBP synthesis in young adults of *P. brassicae* demonstrated further that label from [^{14}C]5-aminolevulinate appeared in the bilin almost immediately with no lag phase, comparable to the results for the apoprotein labeled with [^{3}H]leucine (Kayser and Krull-Savage, 1984). This result is consistent with a straightforward synthesis of the bilin, presumably via a heme precursor. Obviously, the bilin is not accumulated like a degradation product of some relatively stabile heme protein, for example like hemoglobin in vertebrates. The formation of bilins from heme has not yet been studied at the enzyme level in insects, though the action mechanism of heme oxygenase is fairly well understood and evolutionarily conserved (reviews: Maines, 1997; Wilks, 2002). It is furthermore unknown whether apobiliproteins or their precursors could serve as heme oxygenases, or whether the open chain tetrapyrrole is synthesized independently to associate with the apoprotein, as studies in *P. brassicae* suggested (Kayser and Krull-Savage, 1984). This possibility gains some support from the apparently successful reconstitution of BBP from its recombinant apoprotein (Schmidt and Skerra, 1994).

4.8.3.5. Putative Functions of Biliproteins

Usually, biliproteins are considered to contribute primarily to camouflage coloration of insects, in particular to reduce the risk for larvae feeding on (green) plants of being preyed upon. This is due to their strong blue color, which combines with the yellow color of carotenoids derived from the food plant to yield green coloration that may be modified by, e.g., dark pigmentation, to further enhance any camouflage effect (review: Kayser, 1985). This role is certainly true and evident in many insects, as in the example shown in **Figure 5**. However, there are also examples where biliproteins are present in epidermis, hemolymph, and/or fat body of the larva but are masked by other pigmentation of the cuticle. One obvious example is the butterfly *P. brassicae*. The larval integument displays a pattern of black and yellow pigments that mask the biliprotein underneath, and in the adults, where the bulk of



Figure 5 Fourth instar larva of *Cerura vinula* as an example for camouflage coloration based on yellow carotenoids and a blue biliprotein present in epidermis and hemolymph, combined with ''lytic'' dark pigmentation in the cuticle.

the biliprotein is located in the wings, the scales are bright white, except for a few black patches. Presumably, the camouflage potential is a secondary property of biliproteins that is successfully utilized in many insects.

A major, more general role of biliproteins is inferred from several considerations. The high cost of establishing the complex multienzyme pathway of heme synthesis followed by heme cleavage seems to be inadequate for biliproteins to be limited to just a role in camouflage, which might be achieved by other, "cheaper" means. Furthermore, the strong synthesis of heme products without any known need for a corresponding large quantity of a heme protein, such as hemoglobin or cytochrome P450 (see **Chapter 4.1**), suggests a genuine and vital role for the biliproteins as end (not waste) products of the heme pathway. In newly emerged adults of *P. brassicae*, for example, about 90% of the heme precursor 5-aminolevulinate is allocated directly to the synthesis of BBP, even at the time of peak formation of mitochondrial cytochromes during flight muscle development (Kayser, 1984).

The photochemical properties of the cyanobacterial biliproteins, which function as accessory light-harvesting complexes, led to a study of the photochemical behavior of an insect representative, BBP from *P. brassicae* (Scheer and Kayser, 1988; Schneider *et al*., 1988). The fluorescence intensity of BBP was very weak, compared to that of the cyanobacterial counterparts, and there was no indication of an intermolecular energy transfer in accordance with the monomeric nature of this protein. Overall, the photochemical properties of BBP are much too weak to support any speculation about a possible role based on the use of light for energy conversion or signaling. Most likely, this is true for insect (animal) biliproteins in general.

Concerning possible metabolic roles of biliproteins, it must be recalled that the cleavage of heme yields an open-chain tetrapyrrole, which is a biliverdin in all studied organisms, as well as carbon monoxide and iron in equimolar quantities. All these products are biologically active. A role for the released iron is difficult to reconcile since it has to be introduced into the porphyrin for its cleavage via its Fe-complex, heme. The production of carbon monoxide is possibly more relevant, as evidence is being accumulated for this gas to function as a second messenger comparable to the well-established role of nitric oxide which regulates a wide spectrum of biochemical and physiological processes by binding to the heme moiety of soluble guanylyl cyclases. Moreover, the pathways of nitric oxide and carbon monoxide signaling have been shown to interact (reviews: Barañano and Snyder, 2001; Hartsfield, 2002; Ryter *et al*., 2002). Thus, heme oxygenase is in a position to potentially control a number of cellular reactions. Isoforms of heme oxygenases with different functions and tissue specificity are known from vertebrates. Insects have been widely neglected in this field so far.

There are more speculative roles for bilins and their carrier proteins. The cleavage of the heme ring to an open-chain bilin is unlikely to be just a means to control the cellular levels of porphyrins because of the high investment to establish such a mechanism. Multistep biosynthetic pathways are usually regulated at the level of initial (key) enzymes, not at the terminal ones. Porphyrins are cytotoxic due to their photochemical properties and therefore have to be inactivated or eliminated. Their cleavage to a linear tetrapyrrole requires the insertion of ferrous iron for the activation of molecular oxygen and reduction equivalents, usually NADPH. However, bilins may also have regulatory roles according to recent studies in vertebrates. For example, biliverdin has been shown to inhibit soluble guanylyl cyclase *in vivo* and *in vitro* (Koglin and Behrends, 2002), to play a role in embryonic development (Falchuk *et al*., 2002), and, as a redox partner of bilirubin, to play a vital role in the prevention of cellular damage by reactive oxygen and nitrogen species (Barañano *et al*., 2002; Kaur *et al*., 2003). Apparently, a variety of actions of linear tetrapyrroles in biological systems are just emerging.

4.8.4. Nitrophorins and Related Proteins

4.8.4.1. The Problem of Blood Feeders

The basic lipocalin fold has evolved as a universal scaffold that can be modified to achieve affinity for chemically diverse ligands involved in a variety of biological functions. This universality becomes strikingly evident with the nitrophorins and functionally related proteins that have evolved independently in several arthropods specialized in taking blood meals. General aspects of this special feeding behavior have been reviewed very recently (Ribeiro and Francischetti, 2003). Blood-sucking arthropods, such as a number of bugs and ticks, have developed biochemical means to maintain a continuous blood flow at the feeding site and to prevent or delay any counteraction of the host's body that would reduce feeding success. This common requirement of blood feeders obviously guided the evolution of proteins specialized to interact at different sites and in different ways with the complex

process of blood coagulation in vertebrates. Obviously, the lipocalin fold provided a versatile basis to create binding sites for powerful ligands acting together to support the insects' needs. The basic studies on this group of functionally highly specialized lipocalins have been performed in *Rhodnius prolixus*, a South American bug known to transmit *Trypanosoma cruzi*, the vector of Chagas' disease. A general overview with a focus on the structures of the specialized proteins of blood-feeding insects has been given by Montfort *et al.* (2000). The chemical and physical properties of nitrophorins have been reviewed by Walker *et al.* (1999).

4.8.4.2. Crystal Structures of Nitrophorins from *Rhodnius prolixus*

The nitrophorins of the bug *R. prolixus* are stored in the salivary gland, which therefore looks red, and are injected, together with a number of other proteins of related functions, into the blood of the host. There are four nitrophorins, NP1 to NP4, that have been purified from the insect tissue, cloned, and produced as recombinant proteins for crystallization studies (Champagne *et al.*, 1995; Ribeiro *et al.*, 1995; Andersen *et al.*, 1997; Sun *et al.*, 1998). The numbering of the nitrophorins is in the order of their relative abundance with NP1 representing the predominant one. Each protein has a mass of ~20 kDa as is typical for members of the lipocalin family. In pairwise comparisons, NP1 and NP4 show ~90% sequence identity, while this is ~80% for NP2 and NP3. The identities between the two pairs are much lower, about 45%. This demonstrates different degrees of relatedness suggesting that the four nitrophorins originated from two gene duplications. The overall sequence identity of the nitrophorins is 38%.

The crystal structures of the nitrophorins, which are available from NP1, NP4, and NP2 in various complexes, are largely identical (Andersen *et al.*, 1998; Weichsel *et al.*, 1998, 2000). All of them show the typical conserved lipocalin folding of a β-barrel consisting of eight antiparallel β-strands with orthogonal orientation in the two sheets (**Figure 6**), as described in detail for the biliproteins from *P. brassicae* and *M. sexta* (see Section 4.8.3.1). Moreover, the nitrophorins share with the biliproteins the conserved long helix (actually two helical regions here, labeled h2 and h3 in **Figure 6**) at the extended C-terminal sequence that does not contribute to the barrel structure. Furthermore, two disulfide bridges are present at positions comparable to those in the biliproteins. The main difference between the two types of lipocalins refers to the ligand, which is heme in the ferric state in each of the nitrophorins (**Figure 7**). The heme is held in place via 10 hydrophobic amino acid side chains and a histidine (His59) as the fifth iron ligand. This detail is reminiscent of the hemoglobins in which the heme is in the ferrous state, however. Even more, these two types of hemoproteins represent quite different protein structures: β-barrels in the nitrophorins and α-helical globular folds in the hemoglobins, demonstrating that they are evolutionarily unrelated. In the nitrophorins, the structure of the heme is very unusual as it is distorted and nonplanar. The carboxyl groups of the two propionate side chains of heme are hydrogen-bonded to the same lysine, and one of the propionates is also linked by an unusual carboxylate–carboxylate hydrogen bond to an aspartate side chain. Both heme propionate groups point to the solvent (**Figure 7**). The orientation of the heme in the nitrophorins is thus comparable to that of the cyclic but open-chain bilins in the biliproteins, as is

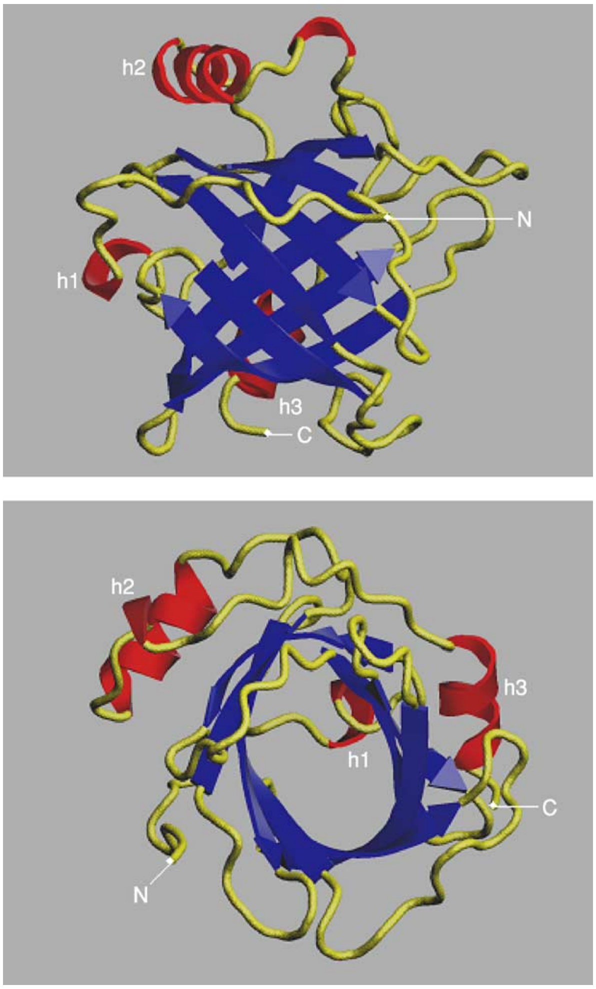


Figure 6 Crystal structure of nitrophorin 1 from *Rhodnius prolixus*. Ribbon drawings with eight β-strands (blue arrows) and several helices (red, labeled h1–h3). The N- and C-termini are marked with white dots. For the heme ligand, see **Figure 7**.

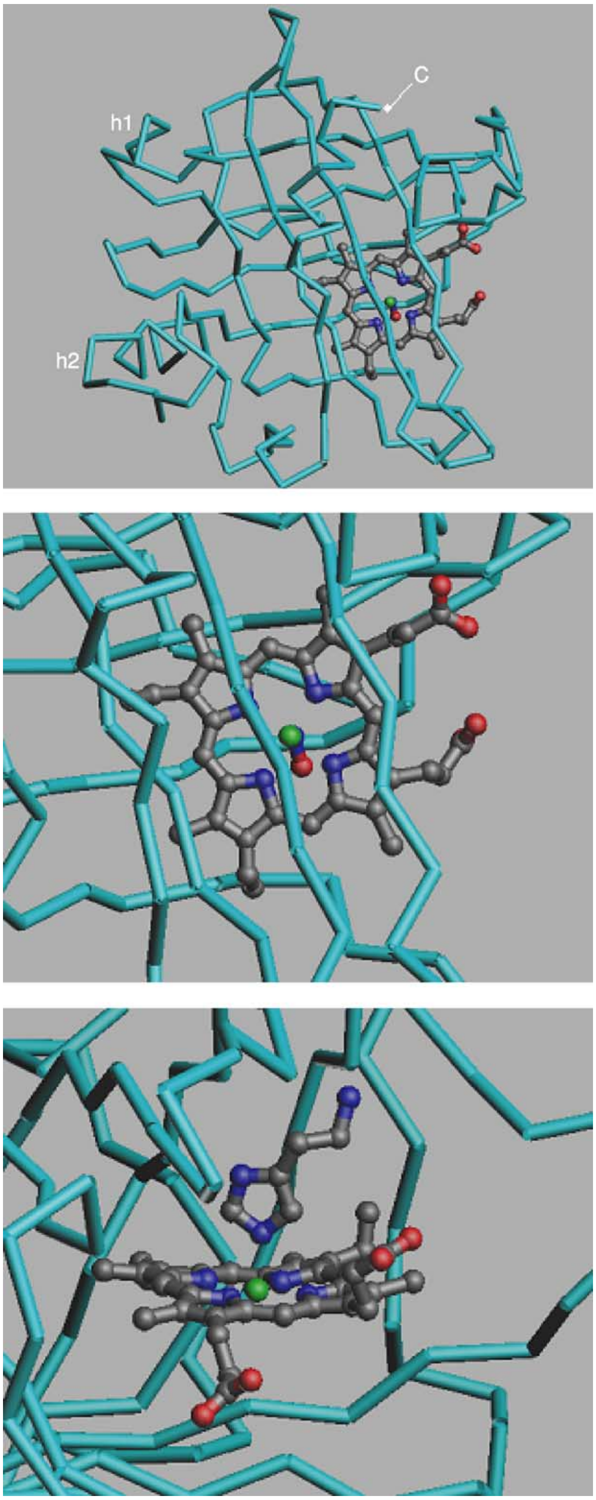


Figure 7 Crystal structure of nitrophorin 1 from *Rhodnius prolixus*. Cα-backbone structures (light blue) with the heme ligand in complex with nitric oxide (upper and middle panels) and histamine (lower panel), respectively. Heme is represented as stick-and-ball model with oxygen in red, nitrogen in blue, and iron in green. Two helices (h1 and h2) and the C-terminus are labeled. For further orientation, compare to **Figure 6**.

the location of the heme near the opening of the barrel. Though the nitrophorins show a higher degree of sequence identity with the biliproteins, their core lipocalin structures fit better to the vertebrate lipocalins. On the other hand, the positions of the disulfide bridges are identical in the two groups of insect lipocalins, while they are different from the mammalian lipocalins. Evolutionary relatedness in this case may hence be better reflected by the disulfide pattern than by identical positions of the core atoms of the β-barrel.

As can be seen from the crystal structure depicted in Figure 7, the nitrophorins are isolated as complexes of the heme iron with nitric oxide, which can be exchanged by histamine (Weichsel *et al*., 1998). The heme is in the low-spin state in the complexes with nitric oxide and histamine (Soret maximum at 419 nm and 413 nm, respectively), and in the high-spin state when these ligands are replaced by water (Soret maximum at 404 nm). The dissociation constants (K_d values) of nitric oxide in complex with the various ferric-heme nitrophorins is in the 10×10^{-9} M to 1000×10^{-9} M range, depending on pH and the protein isoform. The NP2/NP3 pair shows higher affinities than the NP1/NP4 isoforms. In NP4, binding of nitric oxide changes the conformation of two loops at the β-barrel's opening so that nitric oxide gets enclosed by hydrophobic residues after several water molecules have been expelled from the cavity (Weichsel *et al*., 2000). Keeping the heme iron in the oxidized (ferric) state is very important since the nitric oxide complex with ferrous iron is more stable by about six orders of magnitude, which means practically irreversible binding. The ferric state of heme is stabilized by the protein structure. The affinity for the heme ligands is modulated by pH in such a way that at the weakly acidic pH (~5) of the salivary gland the nitric oxide–heme complex is more stable than at the higher pH (~7.5) at the host's feeding site. This pH difference facilitates both the release of nitric oxide into the host's blood and the binding of histamine, due to its higher affinity, in place of nitric oxide. Histamine binds with a K_d of $\sim 20 \times 10^{-9}$ M at the host's pH, which is a ~100-fold higher affinity compared to that of nitric oxide in the same environment. NP2, also known as prolixin-S, also exerts anticoagulant activity in addition to its nitric oxide and histamine binding properties. Though details are not known, NP2 interferes at an early step with the multilevel cascade of blood coagulation.

Very recently, two additional proteins most likely representing new nitrophorins have been identified in the salivary gland of *R. prolixus*, and named NP5 and NP6 (Moreira *et al*., 2003). One of these new

proteins, NP5, has been partially characterized as a heme protein, according to its absorption spectrum, with an N-terminal sequence revealing high similarity to NP4. Furthermore, NP5 binds nitric oxide and shows a spectral shift of the Soret peak from 404 nm to 422 nm upon binding similar to that of the other nitrophorins. The other protein, NP6, is only tentatively described as a nitrophorin so far. Interestingly, the relative abundance of the nitrophorins in the salivary gland depends on the life cycle stage of this bug (see Section 4.8.4.5).

It may be of interest to note that the bedbug *Cimex lectularius*, a blood feeder like *R. prolixus* but a member of a different family of Hemiptera, also has a heme-based donor protein for nitric oxide (Valenzuela and Ribeiro, 1998). By this property, it may also be called a nitrophorin. However, its amino acid sequence is unrelated to those of the *Rhodnius* nitrophorins and shows no lipocalin signature. It will therefore not be discussed further here. These nonhomologous but functionally equivalent proteins have likely evolved independently to meet the common constraints of blood feeding.

4.8.4.3. Other Lipocalins from Blood-Feeding Insects

The anticoagulant activity of the nitrophorin NP2 from *R. prolixus* has already been mentioned above. Another protein supporting the action of the nitrophorins as donors of nitric oxide to the host has been isolated and cloned from the salivary gland of this bug (Francischetti *et al.*, 2000). This protein, called *Rhodnius prolixus* aggregation inhibitor 1 (RPAI-1) or salivary platelet aggregation inhibitor 1, comprises 155 amino acids in its mature form and has a molecular mass of ~19 kDa. Though its crystal structure is not known, it obviously represents also a lipocalin as suggested by its sequence homology with some other insect proteins believed to have a lipocalin structure. These are pallidipin and triabin (see below) from the bug *Triatoma pallidipennis,* which both interfere with blood coagulation (Noeske-Jungblut *et al.*, 1994, 1995). RPAI-1 has a nucleotide binding site specific for ADP (and other adenine nucleotides) that is known to potentiate platelet aggregation induced by agonists such as collagen (Francischetti *et al.*, 2002). RPAI-1 is suggested to be most efficient in scavenging small concentrations of ADP that would otherwise induce the formation of large platelet aggregates. The sequence of a related salivary platelet aggregation inhibitor 2 has been reported also from *R. prolixus* (Champagne *et al.*, 1996).

Quite recently, another protein has been detected in the saliva of *R. prolixus* that apparently supports the bug in taking a blood meal. The new protein binds biogenic amines that are known to promote platelet aggregation in concert with ADP (Andersen *et al.*, 2003). This biogenic amine-binding protein (ABP) binds norepinephrine with highest affinity (K_d ~25 nM), followed by serotonin (K_d ~100 nM) and epinephrine (K_d ~350 nM). Alignment of the amino acid sequence of ABP with those of the four nitrophorins revealed significant similarity, a little lower than that between the nitrophorins. The lipocalin nature of ABP is further stressed by four cysteines at comparable positions (**Figure 8**). ABP is not associated with heme, which is explained by the absence of the proximal histidine that acts as fifth ligand of the heme iron in the nitrophorins. Hence, ABP lacks the structural basis for nitric oxide binding.

Triabin from *Triatoma pallidipennis*, a blood-sucking bug like *R. prolixus*, is a 16 kDa protein consisting of 142 amino acids in the mature form that inhibits thrombin by forming an equimolar noncovalent complex with this key compound for blood coagulation (Noeske-Jungblut *et al.*, 1995). Its structure is remarkable (**Figure 9**), as it turned out to be an unusual lipocalin with an eight-stranded β-barrel in which, however, the strands B and C of the first sheet are exchanged resulting in an "up–up–down–down" topology instead of the strict antiparallel "up–down–up–down" strand orientation (Fuentes-Prior *et al.*, 1997). Triabin lacks the long C-terminal α-helix that terminates in a short β-strand in typical lipocalins, which accounts for its lower size. Instead short helices are found at both termini. Like the retinol-binding proteins but unlike the biliproteins and nitrophorins, triabin contains three disulfide bridges. A sequence aligment of triabin and nitrophorins, together with related proteins, is shown in **Figure 8**.

4.8.4.4. Lipocalins from Blood-Feeding Ticks

The common feeding biology of blood-feeding insects and ticks has resulted in the convergent evolution of proteins serving the same goal, which is to support blood feeding by counteracting the hosts' responses to the attack. These functions are obviously best performed on the basis of a common protein structure, which turned out to be the conserved lipocalin fold. The protein structures as well as their ligands have been studied in two ticks, *Rhipicephalus appendiculatus*, which prefers cattle, and *Dermacentor reticulatus*, feeding on rodents.

Three histamine-binding proteins (HBP1 to HBP3) were identified in salivary glands of *R. appendiculatus* and cloned, and the crystal structure of one of them was solved (review: Paesen *et al.*, 2000).

```
NP1    MKSYTALLAVAILCLFAAVGVSGKCTKNALAQTGFNKDKYFNGDVWYVTDY-LDLEPDDV 59
NP2    MELYTALLAVTILCLTSTMGVSGDCSTNISPKQGLDKAKYFSG-KWYVTHF-LDKDPQ-V 57
ABP    MRAYAALVLFVVALWMSEAEGASGCST-VDTVKDFNKDNFFTG-SWYITHYKLGDSTLEV 58
TRI    MKTIIAVTIFGILTCAYAAEGD-DCSIEKAMG-DFKPEEFFNG-TWYLAHG-----PGVT 52
Bg4    ---------AVLALCATDTLANEDCFRHESLVPNLDYERFRGS--WIIAAG--------- 40
                  :            *        .:.  .:  .  * ::

NP1    PKRYCAALAAG-TASGKLKEALYHYDPKTQDTFYDVSELQE---ESPG-KYTANFKKVEK 114
NP2    TDQYCSSFTPR-ESDGTVKEALYHYNANKKTSFYNIGEGKL---ESSGLQYTAKYKTVDK 113
ABP    GDKNCTKFLHQKTADGKIKEVFSNYNPNAKTYSYDISFAKVSDFDGNNGKYTAKNVIVEK 118
TRI    SPAVCQKFTTS-GSKGFTQIVEIGYNKFESNVKFQCNQVDN----KNGEQYSFKCKSS-- 105
Bg4    TSEALTQYKCWIDRFSYDDALVSKYTDSQGKNRTTIRGRTK----FEGNKFTIDYNDK-- 94
                      .  .     *                      . ::: .

NP1    NGNVKVDVTSGNYYTFTVMYADDSSALIHTCLHKGN---KDLGDLYAVLNRNKDTNAGDK 171
NP2    KKAVLKEADEKNSYTLTVLEADDSSALVHICLREGS---KDLGDLYTVLTHQKDAEPSAK 170
ABP    DG---RKIDERTLQVSYIDTDYSKYSVVHVCDPAAP---DYYLYAVQSRTENVKEDVKSK 172
TRI    ------DNTEFEADFTFISVSYDNFALVCRSITFTS---QPKEDDYLVLERTKSDTDPDA 156
Bg4    -------GKAFSAPYSVLATDYENYAIVEGCPAAANGHVIYVQIRFSVRRFHPKLGDKEM 147
                        :    .. :::  .                      .

NP1    VKGAVTAASLKFSDFISTKDNK--CEYDNVSLKSLLTK------- 207
NP2    VKSAVTQAGLQLSQFVGTKDLG--CQYDDQ-FTSL---------- 202
ABP    VEAALGKVGLKLSGLFDATTLGNKCQYDDETLQKLLKQSFPNYEK 217
TRI    KEIC----------------------------------------- 160
Bg4    IQHYTLDQVNQHKKAIEEDLKHFNLKYEDLHSTCH---------- 182
        :
```

Figure 8 Aligment of the nitrophorins 1 and 2 (NP1, NP2) and biogenic amine-binding protein (ABP) from *Rhodnius prolixus*, triabin (TRI) from *Triatoma pallidipennis* and Bla g 4 (Bg 4) from *Blattella germanica*. N-terminal secretion signals are boxed in gray. Residues typically conserved in lipocalins are boxed in black. In NP1 and NP2, the heme-binding His59 are also boxed in black. Asterisks indicate identical residues, double and single points more or less conserved substitutions.

Remarkably, these proteins are sex-specific, as two forms, HBP1 and HBP2, are found only in females, while HBP3 is restricted to males. Moreover, while the female-specific proteins are present only in adult ticks and produced only during the early phase of the single feeding period of females, the male-specific form is not restricted to the adult stage but occurs also in nymphs and larvae, and is synthesized over the entire feeding period involving several attacks of a host. These differences suggest that the three forms of HBPs are adapted to the sex-specific feeding biology of this tick species and may serve different needs. According to alignment studies of the amino acid sequences, the two female-specific isoforms, HBP1 and HBP2, are more closely related to each other (66% identity) than to the male protein, HBP3 (32% and 39% identity, respectively). The HBP sequences are unrelated to those of the nitrophorins and other lipocalins. The three structurally conserved regions of the lipocalins are also found in the HBPs, though as substantially modified motifs not readily recognized in the sequences. The molecular masses, based on the sequences without the N-terminal signals, are 19.5 kDa for both HBP1 and HBP2, and 21 kDa for HBP3. There are more sex-specific differences between these proteins: the male protein HBP3 is glycosylated and secreted as a dimer apparently linked by a disulfide bond, while HBP1 and HBP2 from females are monomeric and not posttranscriptionally modified. All HBPs, studied as recombinant proteins, bind histamine with high specificity but different affinities that are not related to sex, however. HBP1 binds histamine with a K_d of 18×10^{-9} M, while the values for HBP2 and HBP3 are in the $1–2 \times 10^{-9}$ M range.

The crystal structure of HBP2 has been reported (Paesen *et al.*, 1999). The overall structure matches the lipocalin fold with eight-stranded antiparallel sheets arranged to a fairly spherical β-barrel (**Figure 10**). This basic fold is modified by several structural details that are unique to this protein. Nevertheless, the structure of HBP2 is most similar to that of the BBP from the butterfly, *P. brassicae* (see Section 4.8.3.1.1). The N-terminal sequence of HBP2, comprising two helical structures, is extended and attached to the β-barrel via hydrogen bonds. The second helix, labeled h1 in **Figure 10**, occludes the open end of the barrel. The long C-terminal region comprises another helix, labeled h2, which is fixed to the barrel via two disulfide bridges. The most characteristic feature of HBP2 refers to the barrel cavity, which offers two distinct binding sites for two molecules of histamine (**Figure 10**), whereas a single ligand is usually present in classical lipocalins. The two sites are located at opposite ends of the cavity, which is separated into two parts due to the side chains of a tyrosine, a glutamate, and a tryptophane that are conserved in all three HBPs. The two histamine ligands are bound with different affinities and exhibit different orientations at their sites. At the high-affinity site, the histamine is perpendicular to the long axis of the β-barrel, while the other ligand at the low-affinity site is oriented parallel to that axis (**Figure 10**). The histamine at the high-affinity site of HBP2 is in a position comparable to that of heme in the

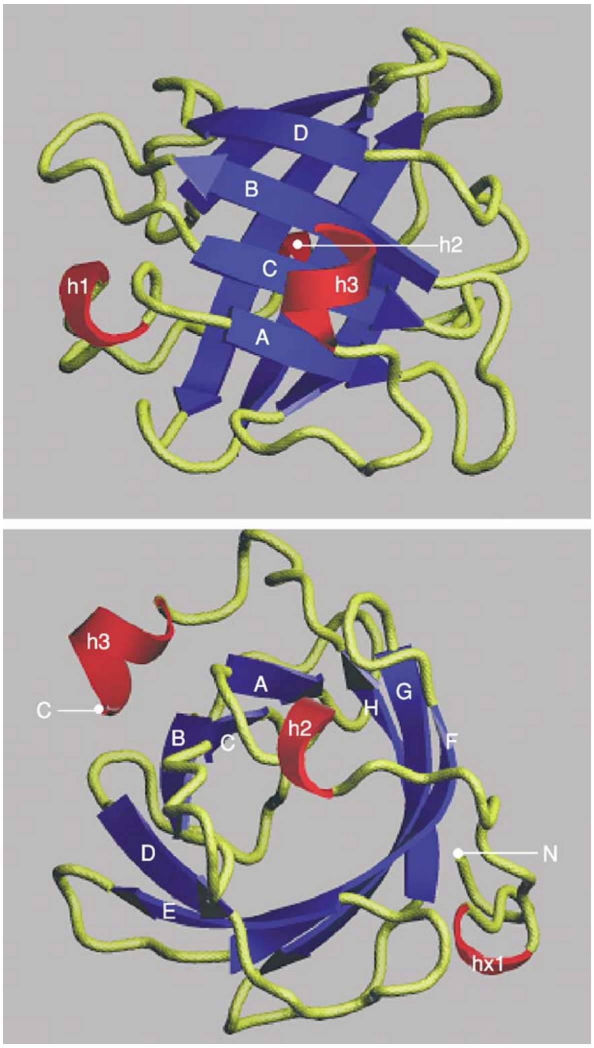


Figure 9 Crystal structure of triabin from *Triatoma pallidipennis*. Ribbon drawings with eight β-strands (blue arrows, labeled A–H) and several helices (red, labeled h1–h3). The N- and C-termini are marked with white dots. Note the unusual topology of the strands A–C–B–D.

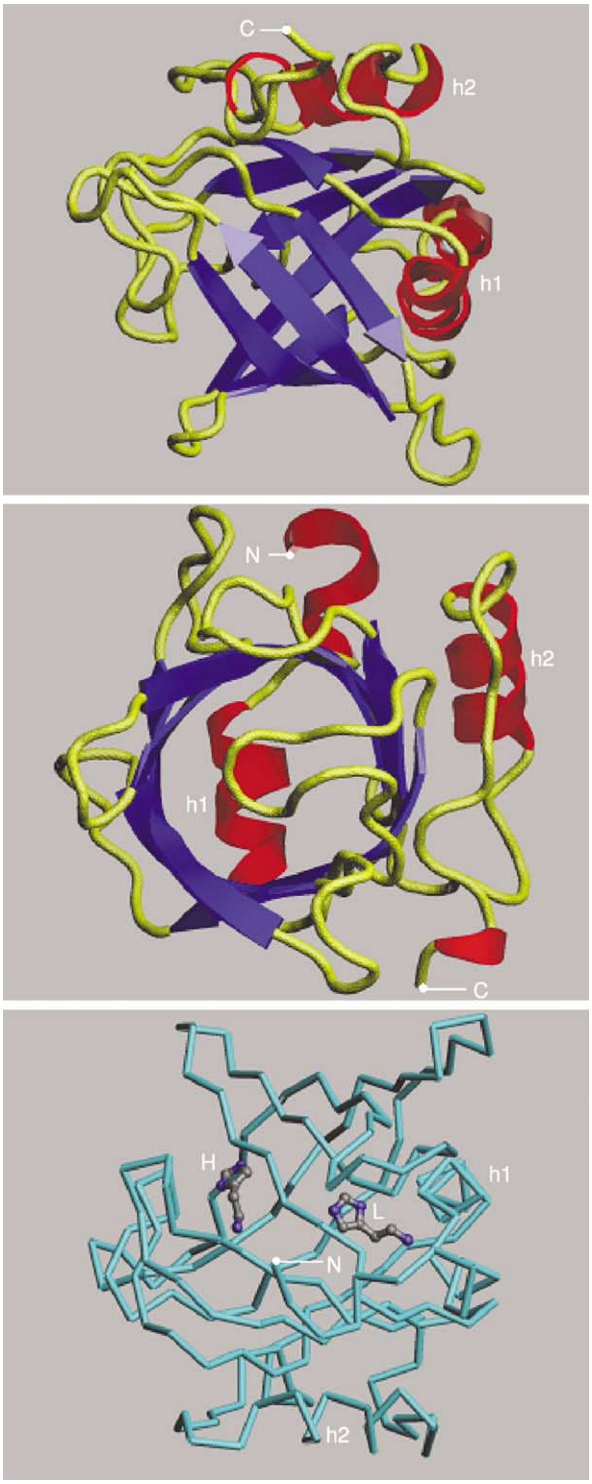


Figure 10 Crystal structure of the histamine-binding protein HBP2 from *Rhipicephalus appendiculatus*. Upper and middle panels: ribbon drawings with eight β-strands (blue arrows) and several helical regions (red, the major ones labeled h1 and h2). Lower panel: Cα-backbone structure (light blue) with two histamines bound at the high (H) and low (L) affinity sites. The N- and C-termini are marked with white dots.

nitrophorins; the low-affinity site is buried in the protein. How the histamines access the two different sites is not clear. To accommodate the hydrophilic histamine, which is positively charged in a physiological environment, both binding sites in the cavity are lined with a number of negatively charged and therefore polar amino acid residues. This is in contrast to typical lipocalins, which usually harbor hydrophobic ligands. In addition to the acidic residues, aromatic side chains contribute to the binding of histamine at the high-affinity site.

An interesting variant of a HBP has been recently found in the salivary gland of another tick, *Dermacentor reticulatus* (Sangamnatdej *et al*., 2002). cDNA

cloning provided a sequence of 192 amino acids for the mature protein, corresponding to a molecular mass of ~22 kDa. The expressed protein exists as a dimer. It is glycosylated, and four putative glycosylation sites are derived from the sequence, which is 36–40% identical with those of the HBPs from *R. appendiculatus*, described above. Binding experiments with histamine revealed two specific binding sites in the recombinant protein from *D. reticulatus*. This is in accordance with the HBPs from the other tick, *R. appendiculatus*. The histamine at one of the sites can be competed by serotonin, which binds with high affinity (K_d of 6×10^{-10} M) to this single site. Thus, this salivary protein is a serotonin-histamine-binding protein (SHBP) with separate binding sites for the two distinct ligands. Though SHBP has not been crystallized, modeling of its sequence into the structure of the HBP2 revealed that the high-affinity site is well conserved, while the low-affinity site is enlarged mainly due to the substitution of an aspartate by glycine. This more spaceous site obviously serves as the binding site for serotonin that is larger than histamine.

With respect to the dependence of the synthesis of the HBPs on sex and sex-specific feeding behavior in *R. appendiculatus* (see above), it is of interest to note that the corresponding SHBP of *D. reticulatus* is expressed in both sexes. Both types of proteins are produced at increased rates over the feeding period though the two ticks differ in their feeding biology.

4.8.4.5. Developmental Expression of Nitrophorins in *Rhodnius prolixus*

The four well-studied nitrophorins have been isolated from the salivary glands of the adults of *Rhodnius prolixus*, where they are stored at different concentrations with NP1 as the dominant isoform, followed by NP2 and NP3, and NP4. This isoform pattern is not constant during the insect's life cycle, according to recent studies (Moreira *et al.*, 2003). In the first instar, NP2 represents the only major nitrophorin that is accompanied by two recently discovered nitrophorins, named NP5 and NP6, which have been only partially studied so far. At each instar, one additional nitrophorin is acquired, which is NP4 in the second instar and NP1 in the third instar. NP3 appears in the fifth instar and accumulates up to the adult stage. During larval development, the amounts of NP5 and NP6 decrease steadily; they are practically absent in the adult insect. In conclusion, the isoform pattern of the nitrophorins in the salivary gland of *R. prolixus* is development-specific. It is interesting to note that the nitrophorin form appearing earliest is NP2, the only protein of this group with anticoagulant activity in addition to its capability to reversibly bind nitric oxide and histamine. So, this nitrophorin variant provides the first instar nymphs with the full spectrum of activities that are relevant to blood feeding.

It should be mentioned that *R. prolixus* synthesizes in the fat body another heme protein that is released into the hemolymph and taken up by the developing oocytes. This protein with a molecular mass of ~15 kDa has a high content of α-helices. Therefore, this *Rhodnius* heme-binding protein is not a lipocalin. This is further stressed by the lack of any sequence similarity to the nitrophorins (Oliveira *et al.*, 1995; Paiva-Silva *et al.*, 2002).

4.8.4.6. Functions of Nitrophorins and Related Proteins

The main roles of the nitrophorins and functionally related proteins have been described in the context of their crystal structures, which allowed a molecular understanding of their function (see Section 4.8.4.2). So, this paragraph mainly summarizes the functional architectures of proteins adapted to a specific feeding biology. A study of these proteins has revealed a sophisticated molecular concept that enables insects and ticks to feed on vertebrate blood. The overall strategy is to prevent the host's response to the feeders' attack in several independent ways that act together in a concerted action.

In blood-sucking insects, the nitrophorins act as heme-based stable stores of nitric oxide, which is released into the blood after dilution and as a consequence of the higher pH at the feeding site (Nussenzveig *et al.*, 1995). The nitric oxide is distributed rapidly into the neighboring cells where it binds to the heme of soluble guanylyl cyclase for their activation that finally results in smooth muscle relaxation and vasodilatation. In exchange for nitric oxide, the nitrophorins bind histamine with high affinity that is produced by the host's mast cells to induce inflammation, immune response, and wound healing. All four nitrophorins studied in detail (NP1 to NP4) exert both activities as nitric oxide donors and as histamine scavengers. Nitrophorin NP2 exhibits anticoagulation activity in addition (Ribeiro *et al.*, 1995). Several other proteins of salivary glands reduce platelet aggregation by binding either ADP (RPAI-1), biogenic amines (ABP), or thrombin (triabin), which all promote blood coagulation. In conclusion, blood-sucking insects have developed a most remarkable battery of lipocalins targeted at diverse steps of the complex blood coagulation cascade in order to maintain the host's blood flow at the feeding site.

Ticks have developed a similar strategy to support their blood-feeding by using the same versatile protein fold, that of the lipocalins, to provide binding sites for histamine, but without employing heme, in contrast to the molecular concept of the nitrophorins of bugs. The tick proteins do not transport nitric oxide because they lack heme. The binding of serotonin in addition to histamine in the protein (SHBP) from *D. reticulatus* is not surprising since biogenic amines in general are established potentiators of blood coagulation and the anti-inflammation response, which are also targeted by the biogenic ABP from the bug *R. prolixus*.

4.8.5. Lipocalins with Unknown Ligands

4.8.5.1. Lipocalins Putatively Related to Development

As described in the foregoing, lipocalins are typically secreted, hence extracellular, proteins found in fluids of insects, like hemolymph (e.g., biliproteins) and saliva (e.g., nitrophorins), although they are also frequently stored as holoproteins within the cells, where they are synthesized (e.g., in the epidermis or fat body) or taken up (e.g., into the eggs). All these lipocalins are typified as carriers of specific ligands that are known, or at least presumed, to serve some metabolic roles in the insects. Well-studied examples of ligands are porphyrin products, nitric oxide, histamine, and biogenic amines. However, a number of proteins have been isolated or cloned from cDNA that were identified as lipocalins on the basis of sequence similarities and the presence of typical motifs in their secondary structures without knowing any real or potential ligands. It is possible that not all lipocalins carry a ligand but may function by, for example, docking to some other proteins as members of a signaling pathway. Under this view, it is interesting to note that several lipocalins without known ligands have been found to be associated with embryonic and postembryonic phases of development, frequently linked to that of the nervous system.

4.8.5.1.1. Lazarillo from *Schistocerca americana* Studies of the development of the nervous system uncovered a most unusual form of a lipocalin, as it is not free but covalently bound to the outer cell surface. There is no other eukaryotic lipocalin firmly anchored to a membrane (bacterial lipocalins are bound to the outer membrane). Playing a role as a guide to developing neurons, this protein was named Lazarillo after "Lazarillo de Tomes, a crafty boy who guided a blind man," as explained by Ganfornina *et al.* (1995), who discovered this lipocalin by the use of a monoclonal antibody in embryos of the grasshopper *Schistocerca americana*. A recent overview of Lazarillo has been published by Sánchez *et al.* (2000a).

Lazarillo is highly glycosylated, thus behaving like a ~45 kDa protein as affinity-isolated with the monoclonal antibody used to detect this lipocalin. From its cDNA, a molecular mass of ~20 kDa was predicted for the mature protein and seven potential *N*-glycosylation sites, however. The amino acid sequence of Lazarillo is further characterized by hydrophobic sequences at both ends. The N-terminal sequence likely represents a secretion signal, and the C-terminal sequence serves as an anchor for the cell surface attachment via a glycosylphosphatidylinositol (GPI) linkage. As can be seen from **Figure 11**, the Lazarillo sequence shows four cysteine residues which are most likely oxidized to form disulfide bridges corresponding to the two conserved disulfide bridges in other lipocalins. Moreover, the structurally conserved regions (SCRs) that characterize members of the lipocalin superfamily of proteins (Flower *et al.*, 1993) are well conserved in Lazarillo. At the amino acid sequence level, Lazarillo is about 30% identical with the other lipocalins, a value comparable to other members of this superfamily. A more detailed comparison places Lazarillo in the neighborhood of the biliproteins from *P. brassicae* and *M. sexta*, together with various forms of apolipoprotein D from mammals. Based on its relationship to the biliproteins, a homology model for Lazarillo was built using the atomic coordinates of insecticyanin from *M. sexta* (Sánchez *et al.*, 2000a). This model (**Figure 12**) shows a close match of the two structures. Remarkably, six of the seven potential glycosylation sites are located on the same side of the protein and close to the C-terminal GPI attachment site, thus resulting in a very polarized glycoprotein. The modeled cavity of Lazarillo also compares well with that of the biliprotein with respect to the distribution of hydrophobic and polar sites. It should be clearly stated at this point that no natural or artificial ligand of Lazarillo is known, though the binding cavity could accommodate a tetrapyrrole, for example.

4.8.5.1.2. Lazarillo-like lipocalins from *Drosophila melanogaster* The sequencing of the *Drosophila* genome provided two novel sequences with similarities to Lazarillo. These novel lipocalins were called *Drosophila* neural Lazarillo (DNLaz) and *Drosophila* glial Lazarillo (DGLaz), respectively (Sánchez *et al.*, 2000b). As deduced from their sequences, DNLaz is an acidic protein (pI 4.3), while DGLaz

```
GAL     ----------MLRMVLFAFVAAASASAVHEGKCPDFKPVDNFNLTAYQGVWYEISKTPND 50
BOM     ----------MLRLVLLTLLAAATAEVIHEGTCPELKPVNNFNLTAYQGIWYEISKFPNE 50
DNL     MNHHSSSHLLLLISVVFGAVWVAHAQVPFPGKCPDVKLLDTFDAEAYMGVWYEYAAYPFA 60
DGL     MMSGQPLGSRVWLLSGVLLVTSAGTDAYGFGRCPNYPSMPKFNMSRVLGHWYEVERSFYL 60
HYP     ----------MWRLYLLKFLATASAQIPSLGWCPDFQAMANFNMDRFLGTWYEAERYFTV 50
LAZ     -----MIRRGLLSVTAALVLLSVSCSAQETMGCADRTAINDFNATLYMGKWYEYAKMGSM 55
                  :        :  .  .      *.:   :  *:     * ***

GAL     -AEKNGKCGQAEYKLEG---------EVVKVKNSHV--VDGVQKYVEGTAKFAEDANKSA 98
BOM     -SEKNGKCSSAEYKLEG---------DVVKVKNVHI--IDGVKKYIEGTAKLTDDANKAA 98
DNL     -FEIGKKCIYANYSLIDN--------STVSVVNAAINRFTGQPSNVTGQAKVLGPG---- 107
DGL     -PEIASGCTTFQFEPYNKGEQSKFSNSKLAVAIKNINRITGNPNVNIGYATPENSRSSIM 119
HYP     -SELGTRCVTTHYTATPE--------GRILITNEITNSITGFKRLMEGHLQMVGREGEGR 101
LAZ     PYEEGGVCVTAEYSMSSN---------NITVVNSMKDNTTHEVNTTTGWAEFASELHTDG 106
          *    *   .:               : :                *

GAL     KLLVTLTYGAVNR-----ESPLNVIATDYQNYAIAYTCKYDEKSKSHNDSIWILSRAKKL 153
BOM     KLTVTFKFGEISR-----DGSVQVLATDYNNYAIAYNCKYDDKKKSHQVFVWILSRNKKL 153
DNL     QLAVAFYPTQPLT-----KANYLVLGTDYESYAVVYSCTS-VTPLANFKIVWILTRQREP 161
DGL     DFKFTTRFPDVIARLLPGSGKYQVLYTDYENFAILWSCGS-IGSLGHSDQIWILGRDRDF 178
HYP     VLVKYSSLPLPYD------FEYSILDTDYDTYAVMWACSG--IGPVHTQNTWLLSRERLP 153
LAZ     KLSVHFPNSPSVG-------NYWILSTDYDNYSIVWSCVKRPDSAASTEISWILLRSRNS 159
         :                     ::  ***:.::: : *             *:* * :

GAL     EGDAKTAVDNYLKEH----AKEIDASK--LVQTDFSEEACKFT----------STSAVTE 197
BOM     EGDAKTAVDNFIKEH----SKEIDSSK--LVHTDFSEEACKFT----------ISSVITE 197
DNL     SAEAVDAARKILEDNDVSQAFLIDTVQ--KNCPRLDGNGTGLTGEDGLDVDDFVSTTVPN 219
DGL     EVDIRSKVYDVLKRLS------LDPER--LIISKNKQCPEAL------------------ 212
HYP     SMSVMQNAYAVLDKFKISRTFFQKTNQ--ADCQFYQHRLPQMN----------------- 194
LAZ     SNMTLERVEDELKNLQLDLNKYTKTEQSAKYCAGAEHVVGAMLS---------VAIASLF 210
        .      .   :.          .. :        .     :

GAL     PQTKKQ 203
BOM     HGKH-- 201
DNL     AIEKA- 224
DGL     ------
HYP     ------
LAZ     ALLH-- 214
```

Figure 11 Alignment of lipocalins putatively related to development. The proteins are gallerin (GAL) from *Galleria mellonella*, bombyrin (BOM) from *Bombyx mori*, neural and glial Lazarillo-like proteins (DNL and DGL, respectively) from *Drosophila melanogaster*, hyphantrin (HYP) from *Hyphantria cunea*, and Lazarillo (LAZ) from *Schistocerca americana*. N-terminal secretion signals are boxed in gray. Residues typically conserved in lipocalins are boxed in black. Stars indicate identical residues, double and single points more or less conserved substitutions.

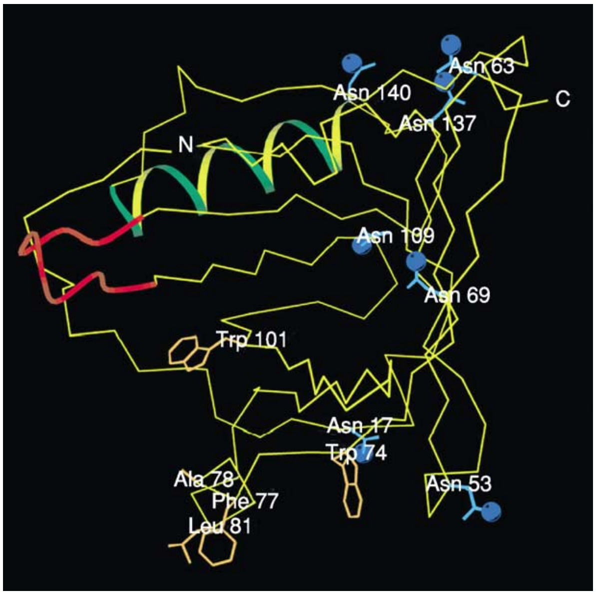


Figure 12 Homology model of Lazarillo from *Schistocerca americana* using the atomic coordinates of insecticyanin. The C-terminal helix (green) and the helical region with hydrophobic side chains (orange) might be involved in protein–protein interactions. The seven Asn glycosylation sites are labeled with blue balls. (Courtesy of Dr. Diego Sánchez.)

is basic (pI 8.6). Both proteins are ~21 kDa in size, exhibit the four conserved cysteines of lipocalins and represent secretory products according to their N-terminal signal sequences. Both may also be glycosylated; there are four potential sites in DNLaz, but only a single one in DGLaz. The two *Drosophila* proteins are different from other lipocalins and also from each other due to the presence of extra sequences (Figure 11). DGLaz has two of them of different lengths in the middle part of the coding region, which are located in two loops at the bottom of the lipocalin β-barrel. DNLaz shows an extra long C-terminal sequence, which, however, is hydrophilic in contrast to that of Lazarillo. No GPI tail is detected in the two Lazarillo-like proteins from *Drosophila*.

4.8.5.1.3. Gallerin from *Galleria mellonella*

The sequence of gallerin was deduced from a brain cDNA library from the wax moth, *Galleria mellonella* (Filippov *et al.*, 1995). Gallerin comprises 203 amino acid residues including a signal sequence of 15 residues. According to homology searches, gallerin is related to BBP, the bilin-binding protein

from *P. brassicae*, and to the related insecticyanin from *M. sexta*. (see Section 4.8.3.1). Gallerin shows 40% identity with the two biliproteins, the four cysteines are at comparable positions, and also the three structurally conserved regions (SCRs) of typical lipocalins are present (**Figure 11**). Unfortunately, gallerin was not compared with the more known neuronal lipocalin Lazarillo, although mentioned in the respective publication (Filippov *et al.*, 1995). The same publication also reports the sequence of another protein, called sericotropin, which was used to pick the gallerin sequence. According to a homology search performed by this author using BLAST, sericotropin is not a lipocalin as claimed by Filippov *et al.* (1995) since all the reference proteins are in fact of mainly helical structure. This correction is in agreement with the current view of the senior author of this publication (Sehnal, personal communication).

4.8.5.1.4. Bombyrin from *Bombyx mori* Bombyrin was identified in a brain cDNA library from silkworm, *Bombyx mori* (Sakai *et al.*, 2001). The sequence of bombyrin was assigned to a lipocalin-type protein based on typical signatures like the four cysteines and the structurally conserved regions (**Figure 11**). A sequence alignment study, performed by this author, revealed that bombyrin is closely related to gallerin (68% identity), while no significant overall homologies (18–23% identity) were found to Lazarillo from the locust and the two Lazarillo-like proteins from the fruit fly.

4.8.5.1.5. Hyphantrin from *Hyphantria cunea* Another putatively developmental lipocalin, named hyphantrin, was cloned from the fall webworm *Hyphantria cunea* (Seo and Cheon, 2003). The sequence of hyphantrin was first assumed to represent a BBP though it is not associated with any blue pigment (Seo, personal communication). Only recently has hyphantrin been recognized as a protein with homology to the two Lazarillo-like proteins, to bombyrin, gallerin and Lazarillo (Seo, personal communication) (**Figure 11**). Hyphantrin is similarly related to various forms of apolipoprotein D and biliproteins (range of 25–30% identity, according to this author).

4.8.5.1.6. Expression and putative functions of developmental lipocalins By the use of the monoclonal antibody that led to the discovery of Lazarillo in *S. americana*, this protein was not ubiquitious in the nervous system of the embryo but only in subsets of neurons of the central nervous system (CNS), in the enteric nervous system, and in all sensory cells of the peripheral nervous system (Sánchez *et al.*, 1995). Lazarillo is also expressed in a number of cells at the tips of the Malpighian tubules, in a group of nephrocytes, and in mesodermal cells. Lazarillo is expressed during the entire life cycle from the embryo up to the adult stage. In any case, the occurrence of this lipocalin is restricted to the cell surface, where it seems to be evenly distributed. What role Lazarillo could play in such diverse cell types is presently unclear. Studies of embryos of *S. americana* suggest that Lazarillo guides axon outgrowth during the development of the nervous system, as axon growth was no longer directed to make the correct contacts in the presence of an antibody against Lazarillo. Similar studies have been performed on the developing brain in the related species, *S. gregaria*, confirming the essential role of Lazarillo for directed axon pathfinding (Graf *et al.*, 2000). Several hypotheses have been put forward on the mode of action of Lazarillo taking into account typical properties of lipocalins: to be designed as vehicles or receptors of mostly hydrophobic small ligands and to interact with other proteins as a means of intercellular communication (Sánchez *et al.*, 2000a). In the latter speculative role, the large sugar moiety of Lazarillo could play an important role in cellular recognition.

In *Drosophila*, DNLaz and DGLaz are both expressed during embryogenesis in the CNS and in some nonneuronal tissues, but not in the peripheral and enteric nervous systems. The temporal patterns of expression of DNLaz and DGLaz are different during embryogenesis but similar thereafter with a low expression in the larvae and a high expression in pupae and adult flies (Sánchez *et al.*, 2000b). This compares well with Lazarillo that is also mainly expressed in embryos and adults of the locust (Ganfornina *et al.*, 1995). While a hypothesis on the role of Lazarillo in the locust has been formulated on experimental ground, the functions of the Lazarillo-like lipocalins in the fly are completely conjectural. The analysis of loss-of-function mutants, which is now under way, may provide some answer in the near future (Sánchez, personal communication).

According to Filippov *et al.* (1995), gallerin is expressed in the CNS of larvae, pupae, and adults of the wax moth. Gallerin expression was also found in the larval fat body, not in any other tissues. In contrast to the Lazarillo-type proteins and to gallerin, hyphantrin is found only in the pupal stage of *H. cunea* during a few days (days 4–6). Being mainly expressed in the epidermis, hyphantrin may not represent a typical neuronal lipocalin, although positive Northern plots were obtained with brain and fat body (Seo, personal communication).

4.8.5.2. Lipocalins Putatively Related to Reproduction

While the above developmental lipocalins, as they are tentatively grouped here, play only a speculative role in the (growing) insect, i.e., between cells, some other proteins, also belonging to the lipocalin superfamily, seem to be involved in the interactions between the (adult) insect and the environment. As in the developmental forms, ligands are also yet unknown for these lipocalins described below.

4.8.5.2.1. A lipocalin related to sexual behavior A lipocalin apparently involved in sexual behavior has been identified in the cockroach *Leucophaea maderae* (Korchi *et al.*, 1999). This protein, Lma-P22, is specific for adult males, where it is synthesized only in the epidermis of the tergites 2, 3, and 4 and becomes a constituent of the secretion of the dermal gland. The products of this gland are ingested by the female during courtship to act as an aphrodisiac. Partial sequences of the isolated Lma-P22 allowed the preparation of a cDNA encoding a protein with a molecular mass of 19.7 kDa. It consisted of 178 amino acids including an N-terminal signal sequence of 20 residues. Two putative sites for *N*-glycosylation were identified. Sequence alignments revealed an overall identity of 17–26% between Lma-P22 and several established and putative lipocalins including insecticyanin and gallerin. Moreover, the predicted secondary structure of Lma-P22 was in agreement with the lipocalin family. Whether Lma-P22 carries a ligand under physiological conditions is unknown, though a pheromone or another hydrophobic compound may qualify as candidates. Another protein with high homology to Lma-P22 has also been identified in the tergal secretion of male *L. maderae* (Cornette *et al.*, 2001).

4.8.5.2.2. A lipocalin related to oviposition A female-specific protein, referred to as Jf23, has been identified in the tarsi of the forelegs of the swallowtail butterfly, *Atrophaneura alcinous* (Tsuchihara *et al.*, 2000). Jf23 can be extracted from the tarsi as a ~23 kDa protein. The sequence of Jf23, as deduced from its cDNA, revealed a preprotein consisting of 203 amino acid residues, including a leader peptide of 15 amino acids. The calculated molecular mass of 20 296 Da of the Jf23 precursor is in good agreement with the estimated size of the isolated protein. Nearly half of the amino acids are hydrophobic. With a sequence identity of 38%, Jf23 is most closely related to BBP, the bilin-binding protein from *P. brassicae*, followed by insecticyanin, gallerin, and some mammalian lipocalins. A lipocalin structure of Jf23 is further supported by the presence of the conserved cysteines and of two structurally conserved regions at the corresponding locations. The presence of Jf23 in the female tarsi suggests that it may play a role in the transduction of plant chemical signals in the context of egg deposition. In fact, in electrophysiological experiments with the sensilla of female tarsi the response to compounds of a host plant was partially suppressed after pretreatment of the sensilla with antiserum against Jf23.

4.8.6. Fatty Acid-Binding Proteins

4.8.6.1. Variation of the Lipocalin Structure

Fatty acid-binding proteins (FABPs) are ubiquitous in animals. Members of this superfamily have been isolated from numerous vertebrates including humans, and from a variety of tissues such as intestine, liver, and heart muscle. While lipocalins are said to function typically as transporters of a variety of lipophilic molecules between cells, i.e., in the extracellular space (e.g., biliproteins in insect hemolymph or retinol-binding protein in vertebrate serum), FABPs are intracellular proteins and specialized as an almost single class of ligands, as their name indicates. FABPs are acidic proteins (pI ~5) and smaller than lipocalins, being composed of about 130 amino acid residues, corresponding to ~15 kDa proteins. On the other hand, FABPs share the overall structure of a β-barrel and a repeated +1 topology with the lipocalins. Because of some differences in the construction of the β-barrel, however, they are grouped as a separate superfamily within the calycin structural superfamily of proteins. Two other groups of intracellular proteins exhibit the same structural motif as the FABPs, which therefore all belong to the same superfamily. These proteins are the cellular retinol- and retinoic acid-binding proteins (CRBPs and CRABPs), respectively, reviewed by Banaszak *et al.* (1994) and Newcomer (1995). Only one representative has been identified in an insect to date (see Section 4.8.6.3). Because of their location in distinct compartments, the FABPs/CRBPs/CRABPs and the lipocalins are also referred to as intracellular and extracellular lipid-binding proteins, iLBPs and eLBPs, respectively. As a further difference between the two types of proteins, the orientation of the ligand is different. In the iLBPs, the polar group of the ligand (e.g., the carboxyl group of a fatty acid) is directed to the interior of the cavity, while it points to the barrel opening in the eLBPs (e.g., the carboxyl groups of

the tetrapyrroles). The crystal structure of the first representative of the FABPs was described in 1988 for the P2 myelin protein from the vertebrate peripheral nervous system (Jones *et al.*, 1988). To date, the crystal structures of only two insects FABPs have been resolved, as described below. General overviews of the two major forms of β-barrel ligand-binding proteins, the lipocalins and the FABPs, have been written by Flower *et al.* (1993) and LaLonde *et al.* (1994), while the excellent review by Banaszak *et al.* (1994) is much more comprehensive and detailed.

Overall, the β-barrel of the FABPs is flatter and more clamlike in comparison to the more spherical lipocalin barrel. The major difference in the folding pattern is that the β-barrel of the FABPs is constructed not by eight, but by 10 antiparallel β-strands that are hydrogen-bonded to form two sheets of four and six strands, respectively. Like in the lipocalins, the strands forming the two sheets are in orthogonal orientation to one another. A characteristic feature of the general folding of the FABPs is the gap between the fourth and the fifth strand, assigned βD and βE. The first two strands are separated by a helix–turn–helix motif that is not present in the lipocalins. A scheme of the sequential arragement of secondary structures of the FABPs is depicted in **Figure 13**. The helical parts of the N-terminal sequence occlude the β-barrel similarly to the first loop, the large Ω loop, in the lipocalins (see Section 4.8.2). FABPs lack the C-terminal helix of the lipocalins. The cavity of the β-barrel is mostly lined with hydrophobic side chains, but polar groups are also present. It is considerably more spacious than is required to bind a single molecule of fatty acid. In contrast to the situation in the lipocalins, it is unknown where or how the ligand enters the cavity of a FABP. It is speculated that the loading and unloading of the ligand may require dynamic changes in the protein folding that could be induced by the fatty acid itself. Nuclear magnetic resonance (NMR) studies at least indicate some flexibility in the region of the helices possibly serving as a portal for the ligand. The gap between the strands βD and βE is apparently not suitable for this role.

Despite the widespread occurrence of FABPs, only two representatives from insects have been purified, crystallized, and studied for their structure to date. These FABPs have been isolated from *M. sexta* and *S. gregaria*. Several other proteins have been identified as FABPs on the basis of sequence similarity with established FABPs, as described below (see Section 4.8.6.3). A number of FABPs from insects and mites are well-known allergens, as will be discussed separately (see Section 4.8.7). Those readers with particular interest in muscle-type FABPs from mammals and insects may consult the comparative article by Zanotti (1999). More comprehensive reviews of the FABPs from vertebrates are from Veerkamp and Maatman (1995) and Glatz and van der Vusse (1996). For more details and an overall view of lipid transport in insects, see **Chapter 4.6**.

4.8.6.2. Fatty Acid-Binding Proteins with Known Crystal Structure

4.8.6.2.1. Fatty acid-binding proteins from *Manduca sexta* Two FABPs, referred to as MFB1 and MFB2, have been isolated from the midgut cytosol of fifth instar larvae of *M. sexta*, as the most

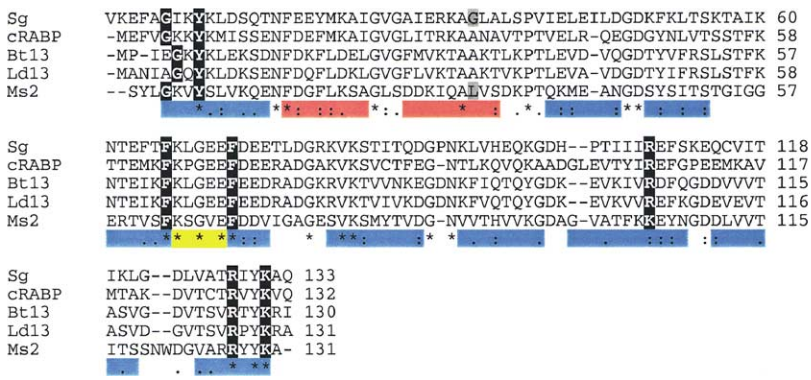


Figure 13 Aligment of fatty acid-/retinoic acid-binding proteins. The proteins are the fatty acid-binding proteins from *Schistocera gregaria* (Sg), *Manduca sexta* (Ms2: MFB2), Blo t 13 from *Blomia tropicalis* (Bt13), Lep d 13 (Ld13) from *Lepidoglyphus destructor*, and the cellular retinoic acid-binding protein (CRABP) from *M. sexta*. Black boxes show residues typically conserved in fatty acid-binding proteins. The residues boxed in gray (Gly in Sg and Leu in Ms2) are apparently responsible for the conformation of the bound fatty acid (see text for details). Below the sequences, the approximate positions of helices and β-strands are labeled as red and blue bars, respectively. The gap between the strands D and E is marked in yellow. Asterisks indicate identical residues, double and single points more or less conserved substitutions.

abundant proteins amounting to 2% and 12%, respectively, of the total soluble protein (Smith *et al.*, 1992). The predominant form, MFB2, has been crystallized and its structure determined as the first representative of an insect FABP (Benning *et al.*, 1992). The crystal structure of MFB2 (**Figure 14**) agrees with the folding motif of vertebrate FABPs. The β-barrel is made up of 10 up-and-down β-strands that are combined to form one sheet with four and one sheet with six strands. The N-terminal sequence is characterized by two helices that link strand A and strand B with a helix–turn–helix motif giving rise to the conserved clamlike overall structure of the barrel. The amino acid side chains defining the cavity for binding a fatty acid are hydrophobic, and most of them are uncharged. The aliphatic chain of the ligand is in contact with these hydrophobic sites, while the polar carboxyl group is hydrogen-bonded mainly to a conserved arginine (Arg127) and a tyrosine (Tyr129) located close to the C-termius that lacks the lipocalin-typical helix. The carboxyl group of the ligand is additionally bound to a sulfate which may be an artifact since the protein was crystallized from an ammonium sulfate solution. It is unknown whether a polar small molecule plays a role in the binding of a fatty acid in a physiological environment. While the position of the carboxyl group of the fatty acid in MFB2 is comparable to that in the vertebrate proteins, the conformation of the aliphatic chain is different. This is obviously due to a difference in the amino acid sequences which has an impact on size and shape of the cavity. In MFB2, a bulky leucine (Leu32) residue, corresponding to a glycine or alanine in the vertebrate proteins, forces the aliphatic tail of the fatty acid beyond C6 to an opposite orientation compared to the vertebrate proteins.

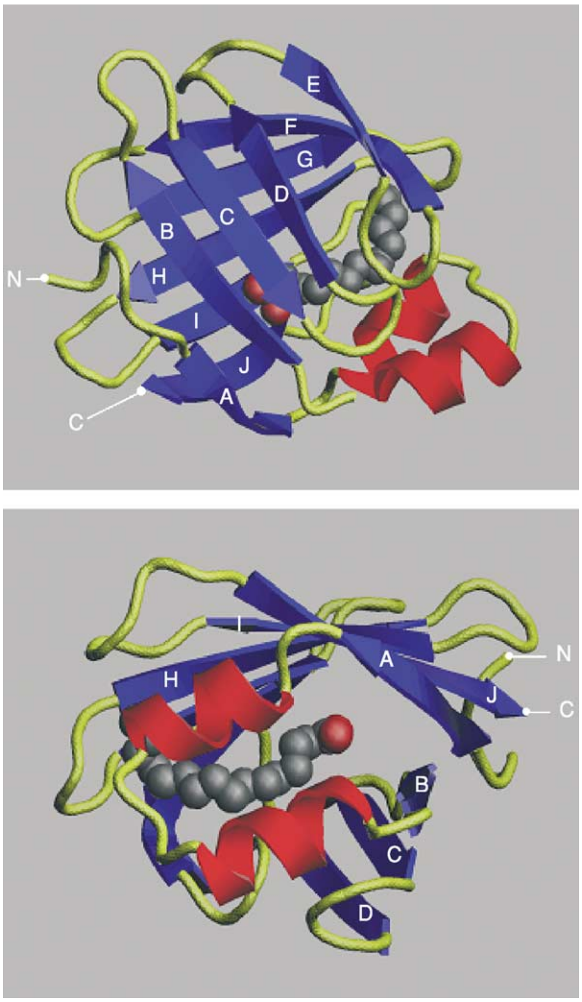


Figure 14 Crystal structure of the fatty acid-binding protein MFB2 from *Manduca sexta*. Ribbon drawings with ten β-strands (blue arrows, labeled A–J) and two helices (red) located between strands A and B (cf. **Figure 13**). The N- and C-termini are marked with white dots. The bound fatty acid is shown in space-filling representation with the carboxyl oxygens in red. Note the gap between strands D and E.

The minor form, MFB1, of the FABPs from the midgut of *M. sexta* has not been crystallized but its three-dimensional structure is thought to be very similar to that of MFB2. As derived from their cDNA sequences, MFB1 and MFB2 are of identical length comprising 131 amino acid residues, corresponding to molecular masses of 14 834 Da and 14 081 Da, respectively. As in most other FABPs, the N-termini are blocked also in the two *Manduca* proteins. The sequence identity between MFB1 and MFB2 is 56%, which is low for proteins of the same type and from the same tissue and species. The identities of the FABPs from the moth to those from various vertebrates are around 30% or lower. Remarkably, the two moth FABPs are not evenly distributed along the midgut. MFB1 is more concentrated in the anterior part but also present in the posterior part, while MFB2 is limited the posterior part of the midgut. Neither of the two proteins could be detected in extracts from fat body, muscles, and eggs. Like the lipocalins, MFB1 and MFB2 bind ligands in a 1 : 1 molar ratio. As isolated from the midgut, both proteins were loaded with a mixture of saturated and unsaturated fatty acids among which $C_{16:0}$ and $C_{18:2}$ fatty acids were predominant. The bound fatty acid can be easily exchanged in MFB1, but not in MFB2. Saturation binding experiments performed with MFB1 and radiolabeled oleic acid yielded an apparent dissociation constant of 14 μM for the exchange reaction. This is, of course, not a true measure for the affinity of the ligand to the apoprotein.

4.8.6.2.2. Fatty acid-binding protein from *Schistocerca gregaria* The first invertebrate FABP was isolated from *S. gregaria*. It was discovered in extracts from the flight muscles from adult locusts where it makes up about 18% of the total cytosolic protein (Haunerland and Chrisholm, 1990; Haunerland *et al*., 1993). As a soluble acidic protein (pI 5.2) with a molecular mass of ~15 kDa and fatty acids bound in a 1 : 1 molar ratio, this locust protein fits quite well the overall characteristics of other FABPs (see Section 4.8.6.1). By contrast, however, the N-terminus of the locust FABP was not blocked. The profile of the fatty acids, bound to the FABP from *S. gregaria* as isolated, was very similar to that of the two proteins from *M. sexta*. The locust FABP crystallized as a dimer in which the monomers are connected via the conserved helix–turn–helix motif that occludes the barrel like a lid (Haunerland *et al*., 1994). Overall, the protein shows the same conserved tertiary structure that is characteristic of members of this superfamily (**Figure 15**). These features are the highly conserved β-barrel with 10 antiparallel up-and-down β-strands with a gap between the βD and βE strands (**Figure 15**). The interior of the barrel contains 23 ordered water molecules, five of which are conserved between the proteins from *S. gregaria* and *M. sexta*. The locust FABP was obviously studied in the apoprotein form, as no fatty acid-ligand was found in the cavity suggesting its loss during the purification of the protein. However, the conserved C-terminal arginine and tyrosine residues that provide binding sites for the carboxyl group of the fatty acid in the moth MFB2, are also present in the locust FABP. While this suggests a similar mode of ligand binding, the conformation of the hydrocarbon tail of the ligand in the locust protein is expected to be opposite to that in MFB2, as the relevant bulky leucine (Leu32) in MFB2 corresponds to a small glycine (Gly34) in the locust FABP. This ligand conformation would be comparable to that in the vertebrate FABPs.

The FABP from *S. gregaria*, which comprises 133 amino acids (**Figure 13**), shows unexpected low similarity to the two proteins from *M. sexta* and to those from vertebrates (about 30% sequence identity), while it is closer related (41% identity) to the FABP from human cardiac muscle (Price *et al*., 1992). Generally, FABPs from the same type of tissue but from different species turned out to be more similar to each other than those from different tissues in the same species. A comparison between the FABPs from insect flight muscles and midgut, for example, would be very interesting in this respect. Not surprisingly, however, the amino acid sequences of the FABPs from the flight muscles of the two locusts *S. gregaria* and *Locusta migratoria* are nearly identical (see Section 4.8.6.2.3).

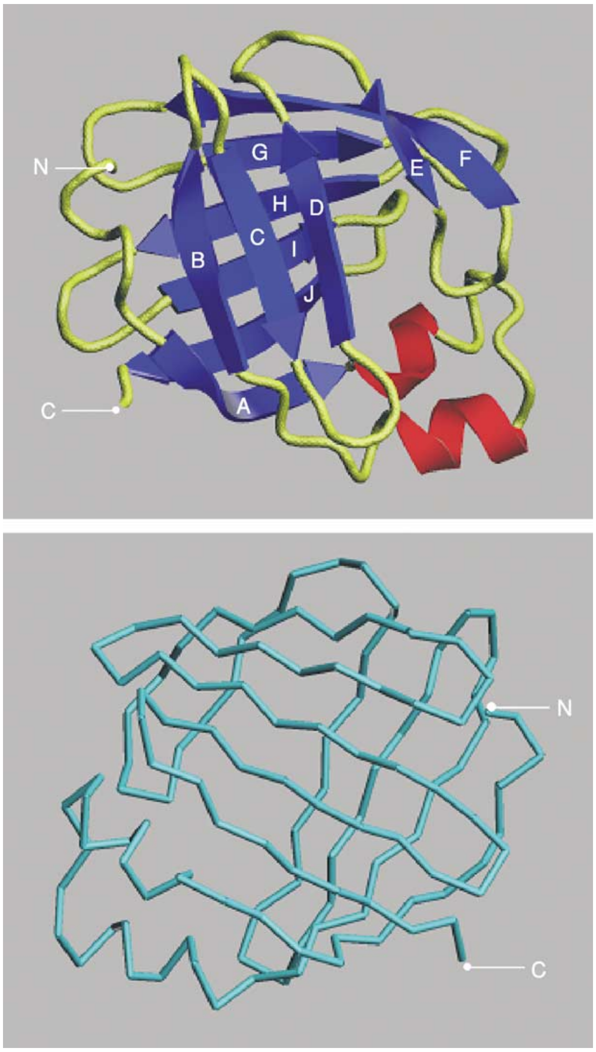


Figure 15 Crystal structure of the fatty acid-binding protein from *Schistocerca gregaria*. Upper panel: ribbon drawing showing ten β-strands (blue arrows, labeled A–J) and two helices (red) located between strands A and B (cf. **Figure 13**). The N- and C-termini are marked with white dots. Lower panel: Cα-backbone structure of the upper representation flipped around a vertical axis. The protein was isolated in the apo form.

The FABP from *S. gregaria* has also been studied at the gene level (Wu and Haunerland, 2001; Wu *et al*., 2001). In mammals, the FABP genes are characterized by three introns of varied length inserted at identical positions of the coding sequence. The locust gene shows only two introns, exactly corresponding to the first and third mammalian introns. This suggests a common evolutionary orgin of the FABPs. The promoter region contains a palindrome with a unique sequence of 19 bp that is essential for the induced expression of the gene by fatty acids. How this transcriptional effect is

mediated is unknown. It has been hypothesized that the conserved helix–turn–helix motif of a FABP could play a role in gene regulation, as it is reminiscent of a known transcription factor motif.

4.8.6.2.3. Fatty acid-binding protein from *Locusta migratoria* Another locust FABP is known from the flight muscles of *L. migratoria* (Maatman *et al.*, 1994). Cloning and heterologous expression of its cDNA has provided data on its sequence as well as binding of various ligands to the recombinant FABP. Although crystals of the protein were obtained, structural data have not yet been published. The *L. migratoria* FABP comprises 133 amino acids, corresponding to a molecular mass of 14 935 Da, and has a nonacetylated N-terminus, like in the other locust FABP. The sequence of the FABP from *L. migratoria* shows 98% identity with that of the *S. gregaria* homolog and 42% identity to that of the human muscle protein (Maatman *et al.*, 1994). According to the conserved features of its primary structure, the FABP from *L. migratoria* is expected to also posses the conserved tertiary structure known for this protein superfamily. Binding experiments, using the recombinant protein, provided a K_d for oleic acid of ~0.5 μM and confirmed the 1 : 1 stoichiometry of ligand binding. The FABP from *L. migratoria* is less acidic (pI 6.1) than that from *S. gregaria* (pI 5.2).

4.8.6.3. Fatty Acid-Binding Proteins with Unknown Crystal Structure

The sequence of a FABP from the moth *Heliothis zea*, the corn earworm, has been deposited in the databases (Heilmann, 1998). It has been identified on the basis of homology of its cDNA-derived sequence with that of established members of the FABP superfamily. Sequence alignment studies with insect FABPs, performed by this author using the Clustal W program, revealed a relatively high identity of the muscle protein from *H. zea* with the two midgut proteins from *M. sexta* (44% and 39% for MFB1 and MFB2, respectively), while the corresponding values for almost all other FABPs, including those from flight muscles of the two locusts, were in the 20% range. Another FABP has been reported (as a DNA sequence fragment) from the honeybee, *Apis mellifera*, where it is expressed only in larvae of workers, not of queens (Evans and Wheeler, 1999). A 14-kDa protein with a free N-terminus was purified from the flight muscles of the bug *Dipetalogaster maximus*, and identified as a heart-type FABP based on its N-terminal sequence (Cavagnari *et al.*, 2000). The sequencing of the genome of *Drosophila melanogaster* has revealed several genes and gene fragments obviously coding for FABPs. Similarly, one gene coding for a FABP of 141 amino acids and a calculated molecular mass of 15 071 Da has been cloned from the mosquito *Anopheles gambiae* (Favia *et al.*, 1996). There are other FABPs that are discussed subsequently as they have been identified as important allergens, a property also shared by a number of lipocalins (see Section 4.8.7).

The FABPs are not the only intracellular ligand- or lipid-binding proteins characterized by a β-barrel of ten antiparallel strands. The same folding-type is also found in a family of proteins called cellular retinol- and retinoic acid-binding proteins (CRBPs and CRABPs), respectively, as mentioned above (see Section 4.8.6.1). Their presence in vertebrates is well known, and the crystal structures of several forms have been described (reviews: Banaszak *et al.*, 1994; Newcomer, 1995). Only one insect member of the CRABP family has been identified to date. This protein isolated from *M. sexta* exhibits 71% identity with bovine and murine CRABP I and could therefore well be modeled into the crystal structures of the vertebrate homologs (Mansfield *et al.*, 1998). The sequence of this moth protein matches well the conserved FABP pattern (**Figure 13**). The natural ligand is unknown but the binding cavity could accommodate retinoic acid, for example.

4.8.6.4. Functions and Developmental Expression of Fatty Acid-Binding Proteins

As their name indicates, FABPs are intracellular transporters specialized for fatty acids as ligands. Though it seems evident that fatty acids require a carrier system that is mobile in the aqueous cytosol while offering a suitable site for the hydrophobic ligand, the implicated uptake and release of fatty acids by these binding proteins in a physiological environment has not yet been demonstrated clearly (Haunerland, 1997). As discussed above (see Section 4.8.6.1), the conserved folding structure of the FABPs does not suggest an opening of the cavity through which the fatty acid could access or exit the protein cavity. This is quite in contrast to the lipocalins with their calyx structure (see Section 4.8.2). While experiments *in vitro* with the *M. sexta* protein MFB1 demonstrated that a bound ligand can readily be exchanged, this was not possible in the case of the homolog MFB2 (Smith *et al.*, 1992). Hence, there may be significant differences between the various FABPs with respect to the ease and the rate of ligand exchange, as well as to the affinities for potential ligands. It is presumed that these different binding properties can be traced back to differences in the amino acid sequences translating

into differences in the ligand-binding pocket of the proteins. It would be of interest to compare the two FABPs from *M. sexta* in this respect and to relate their structural differences to the physiology of those parts of the midgut, where they are predominantly localized. The same considerations may be applied to the various tissue-specific isoforms of FABPs that are known from vertebrates.

In spite of the lack of understanding of the mechanisms of ligand uptake and release in the FABPs at the level of their crystal structure, these proteins are apparently tightly linked to lipid metabolism, specifically to the utilization of fatty acids for energy production (Van der Horst *et al.*, 1993). This seems to hold for insects as well as vertebrates. In the latter, the heart muscle is a typical tissue specialized on lipid oxidation to generate energy, and it contains a high concentration of tissue-specific FABP. In insects, migratory species similarly depend on the use of fatty acids to fuel long-distance flight. The flight muscles of locusts, for example, are an exceptionally rich source of FABP (Haunerland *et al.*, 1993). Under the conditions of extended flight, lipids are released from the fat body and transported *via* specialized hemolymph proteins to the flight muscles (see Section 4.8.8.2), where the fatty acids are taken up for β-oxidation (reviews: Haunerland, 1997; Ryan and Van der Horst, 2000) (see **Chapter 4.6**). The FABPs are supposed to be responsible for the intracellular transport of the fatty acids from the cell membrane to the mitochondria. It has been proposed that FABPs may also play a role in regulating the intracellular concentration of free fatty acids to prevent damage due to their detergent effect. In *S. gregaria*, FABP was localized in the cytosol as well as in the nucleus but not in mitochondria. The nuclear presence might suggest a genomic action, possibly on the expression of the FABP gene itself, as the transcription of the FABP gene can be enhanced by fatty acids. Thus, this transporter protein could be regulated according to physiological needs (Haunerland *et al.*, 1992a; Chen and Haunerland, 1994).

The FABP from *S. gregaria* flight muscle is present only in the adult. Only mature locusts are able to perform extended flight that requires the presence of this type of protein. The concentration of FABP starts to rise a few days after adult ecdysis reaching a plateau after about 10 days. This increase in FABP protein follows a transient but strong increase in its mRNA before it returns to a constant, low level (Haunerland *et al.*, 1992a; Haunerland, 1997). The high capacity to utilize fatty acids for energy production develops only several days after the molt to the adult stage, in parallel with the ability to perform extended flight. Overall, FABP synthesis appears to be part of the developmental program of an adult-specific muscle. This is also demonstrated by the application of inhibitors of metamorphosis that prevent the development of mature flight muscles and, in consequence, the appearance of the FABPs (Haunerland *et al.*, 1993).

4.8.7. Lipocalins and Fatty Acid-Binding Proteins as Allergens

Proteins with a β-barrel structure are of growing medical interest after several major respiratory allergens of mammalian and insect origin were identified as lipocalins and fatty acid-binding proteins, respectively (reviews: Mäntyjärvi *et al.*, 2000; Arlian, 2002). Though these calycins share the conserved protein architecture, conserved sequence regions, and some overall sequence homology, the structural determinants of their allergenicity have not yet been identified. It is evident, however, that it is not the β-barrel per se since by far not all lipocalins induce an allergic immune response. Well-studied examples of allergenic lipocalins from vertebrates are Bos d 2 from cow dander and Mus m 1 from mouse urine. The major sources for arthropod allergens from the superfamilies of lipocalins and fatty acid-binding proteins (FABPs), respectively, are species belonging to cockroaches, bugs, and mites.

Among the numerous allergens from cockroaches, Bla g 4 from *Blattella germanica* has been studied at the structural level and recognized as a typical lipocalin (Arruda *et al.*, 1995). The sequence of Bla g 4 comprises 182 amino acids with a calculated molecular mass of 20 904 Da, as deduced from its cDNA. The N-terminal 12 residues suggest that the encoded protein is secreted. The sequence contains one potential *N*-glycosylation site. The allergenic nature of the protein was confirmed using its recombinant form. A gene coding for Bla g 4 was also identified in *Periplaneta americana*, but its expression could not be demonstrated in this species, however. Searches for similarity to the Bla g 4 sequence revealed several established lipocalins from insects and vertebrates as related proteins. This cockroach allergen contains all three structurally conserved regions (SCRs) and the four conserved cysteines that define membership in the lipocalin family (**Figure 8**). Though the overall identity is low, as typically found among lipocalins, the sequence of Bla g 4 was successfully modeled into the crystal structure of the bilin-binding protein (BBP) from *P. brassicae*. The biological function of

Bla g 4 is unknown as is the binding of any ligand, although a role as a carrier of pheromones has been proposed.

Another group of insects with high allergenic potency are hematophagous bugs. The allergenic activity is stored in the salivary glands in many *Triatoma* species. In *T. protracta*, most of this activity is associated with a 19 kDa protein, named procalin, that comprises, as predicted from its cDNA, 169 amino acids with an N-terminal hydrophobic signal sequence (Paddock *et al.*, 2001). Unlike Bla g 4, procalin has no potential glycosylation site. An analysis of the procalin sequence predicts that procalin adopts a typical lipocalin fold with eight β-strands and a C-terminal helix. In fact, the closest homologs of procalin are the salivary platelet aggregation inhibitors from *R. prolixus* and triabin from *T. pallidipennis* with its unusual lipocalin fold (see Section 4.8.4.3). Moreover, the similarity of procalin to several established lipocalins, such as the moth biliproteins, retinol-binding protein, and β-lactoglobulin, has already been noted.

One of the allergens of the dust mite *Blomia tropicalis*, a most prevalent species in tropical areas, shows ~40% sequence identity with several FABPs from vertebrates and a flatworm. In fact, this allergen with the official name Blo t 13 represents a typical FABP with respect to size (130 amino acids; molecular mass of 14 800 Da) and binding specificity for fatty acids. Moreover, Blo t 13 is characterized by ten up-and-down β-strands, and its sequence could well be fitted into the conserved crystal structure of FABPs (Caraballo *et al.*, 1997; Puerta *et al.*, 1999). Further FABP allergens from mites are Lep d 13 from *Lepidoglyphus destructor* and Aca s 13 from *Acarus siro* (Eriksson *et al.*, 1999, 2001). The sequences of Blo t 13 and Lep d 13 are presented in **Figure 13** in comparison to those of other FABPs.

4.8.8. Ligand-Binding Proteins That Are Not β-Barrels

4.8.8.1. Odorant-Binding Proteins

Olfaction plays a major role in the lifes of insects and vertebrates (see **Chapter 3.15**). It is based on air-borne chemicals that could be pheromones (see **Chapter 3.14**), plant-derived compounds or other signaling substances. Volatile compounds are usually small and hydrophobic, requiring a carrier system that takes them from the cuticular surface of insects or from the nasal epithelium of vertebrates to the olfactory receptors. This carrier function is supposed to rely on a specialized set of proteins, called odorant-binding proteins (OBPs). They comprise the general odorant-binding proteins that occur in both sexes and have no clear ligand preferences and the pheromone(sex attractant)-binding proteins, which are male-specific and specialized in binding a defined ligand, the female pheromone. Many of these OBPs from insects and vertebrates have been purified, cloned, and studied for their functional properties (Pelosi and Maida, 1995; Field *et al.*, 2000). OBPs from insects and vertebrates share several features: they are small (15 to 20 kDa), acidic (pI ~5) and secreted in high concentrations. However, there is no sequence similarity between insect and vertebrate OBPs. This is reflected in their three-dimensional structures: the OBPs from vertebrates have been clearly identified as members of the lipocalin superfamily with all the features of β-barrel proteins, while those from insects are rich in α-helical structures and therefore not lipocalins. The protein folds of two OBPs from insects have recently been elucidated by NMR spectroscopy and X-ray diffraction, respectively: these are a hemolymph protein from the beetle *Tenebrio molitor* (Rothemund *et al.*, 1999) and a pheromone-binding protein in the complex with bombykol from *Bombyx mori* (Sandler *et al.*, 2000). Both studies revealed a new structural class of proteins defined by six α-helices forming a small hydrophobic core that provides a ligand-binding site. Three disulfide bridges are also conserved. Recently, an X-ray study of a chemosensory protein from the moth *Mamestra brassicae* demonstrated another novel fold consisting of six α-helices linked by α–α loops (Lartigue *et al.*, 2002). Evidence for a predominant helical structure has also been obtained for a chemosensory protein from the honeybee, studied by CD spectroscopy (Briand *et al.*, 2002). Furthermore, the genome of *Drosophila* has been searched for OBP genes; all identified 38 genes clearly featured a six-helical fold (Graham and Davies, 2002). It is remarkable from an evolutionary standpoint that insects and vertebrates developed completely different protein structures to achieve functionally equivalent tools for chemical communication.

4.8.8.2. Lipophorins

Hydrophobic molecules in general need amphiphilic transport systems operating in the extracellular space between the tissues as well as in the intracellular milieu (see **Chapter 4.6**). This aspect has already been discussed in the intracellular transport of fatty acids by a specialized set of binding proteins, the fatty acid-binding proteins (FABPs), which represent a variation of the β-barrel structure as it is found in the lipocalins (see Section 4.8.6.1). Insects performing extended flight like migratory

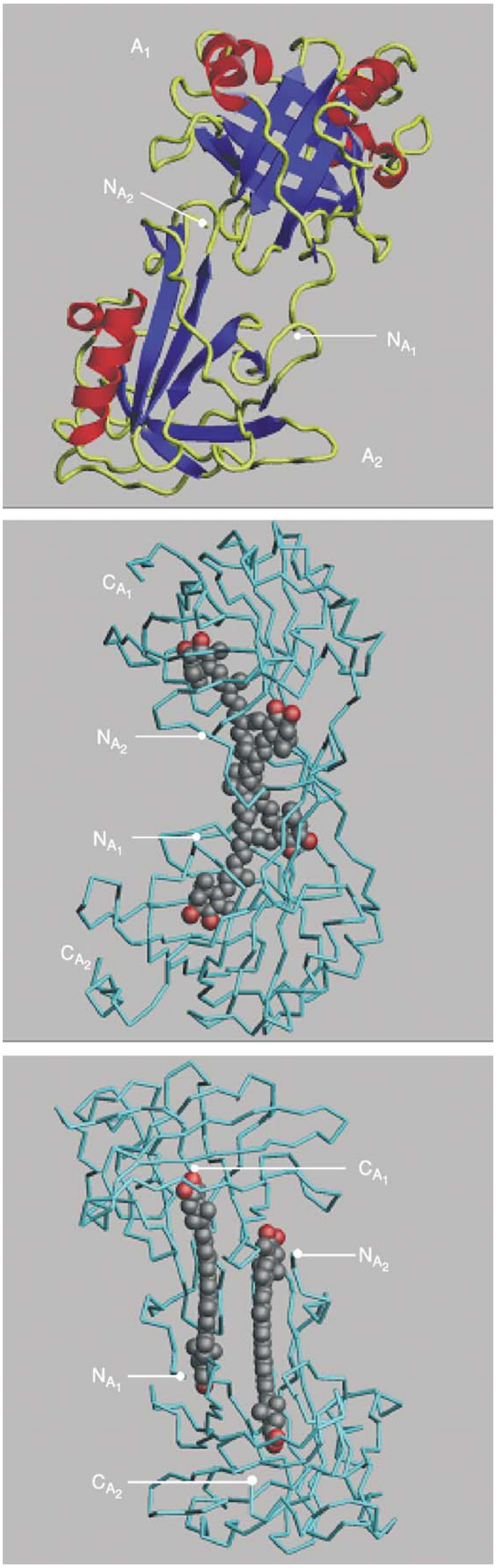
locusts and sphingid moths rely on the oxidation of lipids to produce energy. The lipids are stored in the fat body and activated to fuel flight by the conversion to diacylglycerols, representing their transport form. The transport of lipids to the cells, which finally β-oxidize the fatty acids, is performed by a special carrier system, called lipophorin. Lipophorin represents a large lipoprotein complex composed of two integral units of different size, apolipophorin I and apolipophorin II, with molecular masses of ~250 kDa and ~80 kDa, respectively. Lipophorin is charged with a variable amount of lipid depending on the intensity of lipid utilization by the insect and can also be associated with steroids, carotenoids, or other hydrophobic compounds. Another protein reversibly associates with the core lipophorin to stabilize this carrier complex and to allow more diacylglycerol to bind. This additional protein, apolipophorin III, with a molecular mass of ~19 kDa is highly abundant in the hemolymph as a lipid-free monomer, according to studies in *L. migratoria* and *M. sexta*. While the amino acid sequences of apolipophorin III are known from a number of insects, the only available crystal structure is that of the protein from *L. migratoria* (Breiter *et al.*, 1991). The locust apolipophorin III does not represent a lipocalin or a structural variant thereof like the fatty acid-binding proteins (FABPs). By contrast, it consists of five long α-helices connected by short loops. The helices show a clear amphiphilic arrangement of hydrophobic and hydrophilic amino acid residues, thus fitting the physicochemical requirements to interface the lipid and aqueous compartments. The sequence homology between the apolipophorins III from *L. migratoria* and *M. sexta* suggests a fairly conserved structure of this lipophorin partner. For more details on lipophorin in the overall context of lipid transport (see **Chapter 4.6**).

4.8.9. Crustacean Lipocalins with Established Crystal Structure

Lobster (*Homarus gammarus*) is of long-standing interest not only for gourmets but also for scientists interested in the secrets of the blue coloration of its shell turning into appetizing red upon cooking. The red color is due to astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), a carotenoid metabolically derived from β-carotene by oxidation at both cyclohexene end rings to produce an extended chromophore with a light absorption maximum in the range of 470–490 nm, depending on the solvent (review: Kayser, 1985). In lobster, astaxanthin is noncovalently bound to protein in a complex, called crustacyanin, with an absorption maximum of the carotenoid at ~630 nm, resulting in a blue protein. This huge bathochromic shift of more than 100 nm upon binding is due to a specific arrangement of the red carotenoid in the native protein complex (i.e., before cooking), as described below.

Crustacyanin or, more specifically, α-crustacyanin is a multimeric protein complex, which comprises eight heterodimeric units, called β-crustacyanins. Each of the β-crustacyanin dimers is composed of two types of monomers (the known monomers are A_{1-3} and C_{1-2}) of similar size (~20 kDa) each with one bound molecule of astaxanthin. Thus, the holoprotein with a molecular mass of ~320 kDa contains 16 molecules of astaxanthin. The amino acid sequences of the 20 kDa subunit show homology to members of the lipocalin family (e.g., retinol-binding protein; biliproteins from *P. brassicae* and *M. sexta*; see Section 4.8.3.1), and the predicted tertiary structure is consistent with the antiparallel β-barrel fold. Moreover, two of the sequence regions and the two disulfide bridges that are conserved in the lipocalins are also present in the crustacyanin subunits. On the other side, the homology between subunits may be surprisingly low (for example, <40% between A_1 and A_2) for functionally identical proteins from the same species (Keen *et al.*, 1991).

The predicted lipocalin fold has been confirmed with crystals of the apo-C_1 subunit, which forms homodimers by packing the open ends of the two monomeric barrels face-to-face together (Gordon *et al.*, 2001). Comparable results have been obtained for the dimer of the apo A_1 subunit (Cianci *et al.*, 2001). The positions of the two astaxanthins were recently identified in a β-crustacyanin heterodimer that could be crystallized and its structure determined (Cianci *et al.*, 2002). As presented in **Figure 16**, the subunits A_1 and A_2 also dimerize in a face-to-face way and each subunit exhibits the typical lipocalin fold comparable to that in the previously studied apo-C_1 homodimer. Most surprisingly, however, the astaxanthin ligand of each subunit is not enclosed in the cavity formed by each barrel but extends into the other subunit by about half of its length. This unusual feature explains the tight binding of the chromophores in the dimeric β-crustacyanins in contrast to the labile complex in the monomeric state. In the dimer, the polyene chains of the two carotenoids are parallel to each other and close together. This position is fixed by the two central methyl groups (C20 and C20′) of the chain that are strongly bound to the protein by hydrophobic interaction. The cyclohexenone end rings are twisted to come into plane with the all-*trans* polyene chain, unlike in the free carotenoid.



This brings the double bonds of the end rings into full conjugation with the polyene chain resulting in an extension of the chromophore, and thus in a significant bathochromic shift of the absorption spectrum of the bound carotenoid. Further structural details, thought to enhance this effect, are the way of binding of the carbonyl groups of the end rings to the protein, the electronic interaction between the amino acids and the conjugated chain, and the formation of the finite α-crustacyanin aggregate (Cianci *et al.*, 2002).

Astaxanthin is typically encountered in crustaceans and other marine animals and provides the basis for similar camouflage colorations like in lobster. This red carotenoid is also found in insects from several orders where it most likely is also associated with protein (review: Kayser, 1985). However, an astaxanthin-based blue carotenoprotein has not yet been described from insects. The highly lipophilic carotenoids are presumed to be in general associated with proteins and, in fact, carotenoproteins have been reported from numerous insects, but none of these has been characterized with respect to the mode of ligand binding and protein architecture to date.

4.8.10. Lipocalin Engineering: An Insect Protein Takes the Lead

The preceding paragraphs have provided numerous examples of the evolutionary highly conserved core structure of the calycins as a structural superfamily, which comprises the lipocalins as the most diverse members (review: Flower *et al.*, 2000). The structural concept of a barrel constructed from an orthogonal arrangement of two sheets of antiparallel β-strands provides a remarkable rigid type of protein that is small, water-soluble, and resistant to heat and proteases. Its interior provides a binding site that is adapted in size, shape, and distribution of hydrophobic and polar amino acid residues to the specific binding of typically hydrophobic molecules. The lipocalin ligands enter the barrel at one end through a wide opening that is surrounded by four

Figure 16 Crystal structure of a β-crustacyanin dimer composed of the subunits A_1 and A_2 from lobster (*Homarus gammarus*). Upper panel: ribbon drawing with β-strands (blue arrows) and helices (red). Middle and lower panels: Cα-backbone structures in different orientations with one molecule of bound astaxanthin per monomer. The oxygens at the ionone end rings of the astaxanthin ligands are shown in red in the space-filling representations. The N- and C-termini are marked with white dots. The suffix A_1 and A_2, respectively, specifies the termini of the respective subunit.

loops connecting the strands. The amino acid residues in these loops play a major role in the recognition of the specific ligand. This is reflected by the variability in loop sequence and length in the lipocalins adapted to different ligands.

Based on these features, the lipocalins were early recognized as a potential scaffold to construct new versions of binding proteins for nonphysiological ligands. This concept for protein design has been successfully applied by Skerra and co-workers (reviews: Skerra, 2000; Weiss and Lowman, 2000) to the bilin-binding protein (BBP) from *P. brassicae*, which was one of the first lipocalins established by its crystal structure at high resolution (see Section 4.8.3.1.1). This insect protein was preferred to other members of this superfamily, such as the vertebrate retinol-binding protein, because of its more spacious binding cavity and the presence of only two, instead of three, disulfide cross-links. By mutating a number of amino acids in all four loops at the open end of the barrel, which were selected on the basis of the known crystal structure of BBP, variants of the wild-type lipocalin were created with affinity and selectivity for fluorescein and digoxigenin as new ligands, which bind with affinities in the nanomolar range. These engineered lipocalins were termed "anticalins," because they have similar molecular recognition properties as antibodies (Beste *et al*., 1999; Skerra, 2001). The crystal structure of the fluorescein-binding anticalin, which was solved very recently (**Figure 17**), demonstrated that the loops and even the core of the β-barrel are tolerant to sequence changes and hence offer possibilities for the design of novel receptor proteins for various targets (Korndörfer *et al*., 2003). Moreover, fusion proteins were generated between a BBP variant with a binding site for digoxigenin and a functional reporter enzyme, linked N- or C-terminally, or between two different engineered BBPs with affinity for fluorescein and digoxigenin, respectively. These fused lipocalins carried two functionalities and were hence called "duocalins" (Schlehuber and Skerra, 2001).

Furthermore, in earlier studies by the same group, a binding site for Zn(II) and other metal ions was introduced into the mammalian retinol-binding protein via three histidine side chains. The metal binding site was located at the outer surface of the β-barrel and bound Zn(II) in a 1 : 1 stoichiometry in the nanomolar range without affecting the binding of retinol in the interior of the engineered lipocalin (Skerra, 2000).

The potential of lipocalins to provide a scaffold for the design of numerous binding sites is comparable to that of antibodies while offering several

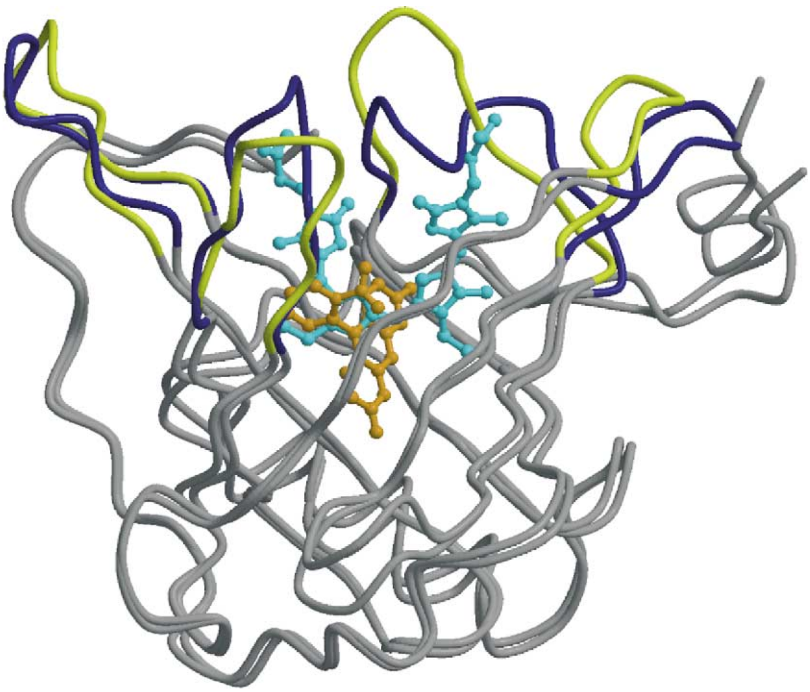


Figure 17 Crystal structure of engineered bilin-binding protein (BBP) from *Pieris brassicae* with high affinity for fluorescein. The structures of BBP (blue loop region) and the fluorescein-binding construct (yellow loop region) are superposed via the Cα-backbone structure of the β-barrel. The bound biliverdin IXγ (green) in BBP and fluorescein (brown) in the engineered protein are represented as stick-and-ball models. (Courtesy of Prof. A. Skerra.)

advantages (for details, see Skerra, 2001). Such "anticalins" may be used as new tools in medicine, biotechnology, bioanalytics, cosmetics, pest control, and other fields in the future. These novel approaches in the design of new proteins represent also a convincing example for the successful transformation of results from basic research on insects into a variety of potential applications that could not be foreseen at the beginning.

4.8.11. Lipocalins – One Fold, Many Roles: Summary and Outlook

The objective of the present overview of lipocalins and structurally related proteins, taken together as the calycins, was to demonstrate the functional versatility of an ancient motif of protein folding dating back to the roots of eukaryotes or even earlier periods of evolution (Ganfornina *et al*., 2000). Most remarkably, the core structure of the calycins, the β-barrel with antiparallel stands, has been highly conserved, while amino acid residues have been allowed to exchange to tailor the protein to the specific and, in most cases, high-affinity binding of small, hydrophobic ligands (Flower, 1995). The very low sequence homologies between members of this structural superfamily are a consequence of this evolutionary strategy.

Due to this wide variation of an ingenious theme, calycins got involved into many roles within cells and between cells. In most cases, however, the functions of the various types of calycins are far from being thoroughly understood, even in proteins characterized by a specific ligand. In lipocalins that lack a ligand, assigning a function may be even more speculative. This is not the case for the insect nitrophorins representing a group of highly specialized lipocalins with obviously well-defined tasks in the context of blood-feeding. The fatty acid-binding proteins are similarly specialized for a single chemical class of ligands; however, it is not clear yet whether their role is limited to the transfer of fatty acids from the cell membrane to the mitochondria, or whether they may also directly regulate gene expression and other vital cellular processes.

According to an increasing number of reports, lipocalins seem to serve not only specialized roles but are also involved in general cellular processes. While several lipocalins have long been implicated in various aspects of cell regulation (Flower, 1994), recent studies suggested that, for example, a lipocalin might trigger apoptosis in leukocytes (Yousefi and Simon, 2002). Results with the human tear lipocalin, which accepts a variety of ligands, indicate that this protein may be able to protect cells from oxidative damage by scavenging potentially harmful products (Lechner *et al*., 2001).

As mentioned at the beginning (see Section 4.8.1), a few lipocalins have also been identified in plants in the past few years. The first plant lipocalins were two enzymes of the xanthophyll cycle, violaxanthin de-epoxidase and zeaxanthin epoxidase (Bugos *et al*., 1998). These proteins are larger than the typical lipocalins with a β-barrel as a central core structure, as deduced from the sequences. Other proteins from *Arabidopsis thaliana* and wheat were found to show the typical lipocalin signature and homology to established members of this family, mainly to locust Lazarillo and vertebrate apolipoprotein D (Charron *et al*., 2002). These plant lipocalins are induced by heat-shock and cold acclimation. It may be of interest to look for similar effects in insects. Another protein from *A. thaliana* shows homology to insect biliproteins and vertebrate lipocalins (Hieber *et al*., 2000).

Before the identification of the two plant enzymes as lipocalins, the lipocalin-type of prostaglandin D synthase (the other type of prostaglandin D synthase, called the hematopoetic type, is unrelated to the lipocalin-type by sequence and protein structure), which is abundant in vertebrate cerebrospinal fluid, was the only lipocalin with enzymatic activity (Urade and Hayashi, 2000). *In vitro*, the lipocalin-type of prostaglandin D synthase also binds the nonsubstrate compounds biliverdin and bilirubin with high affinity; thyroid hormones and retinoids show markedly less affinity (Beuckmann *et al*., 1999). In conclusion, the lipocalin-type of prostaglandin D synthase may act like a typical, though nonspecific, lipocalin in addition to its role as an enzyme. The biological significance of this dual role is not known. Since the synthesis of prostaglandins has also been documented for insects (Büyükgüzel *et al*., 2002) (see **Chapter 4.9**), as study of the corresponding synthase(s) may be rewarding also from the lipocalin point of view.

Finally, it is worth mentioning apolipoprotein D, a classical mammalian lipocalin that is present in many tissues. This protein is unrelated to the apolipophorin family but shows close homology with the insect biliproteins insecticyanin and BBP. Moreover, the crystal structures of apolipoprotein D and the insect biliproteins are superposable and the distribution of hydrophobic and polar sites of the binding cavities is comparable among the three proteins (Peitsch and Boguski, 1990). Hence, apolipoprotein D has been also viewed as a mammalian bilin-binding protein. A detailed phylogenetic analysis of the lipocalin superfamily performed by Ganfornina *et al*. (2000) demonstrated a close

relationship between the arthropod lipocalins and the various forms of apolipoprotein D, clustering together in the same clade.

The insect biliproteins represent a kind of mystery due to their tight coupling to a strong heme pathway that is not understood from the relatively low requirement of heme products in insects compared to the hemoglobin-dependent vertebrates. Biliproteins are frequently referred to as the blue basis for camouflage coloration in insects (see Section 4.8.3.5). This role is certainly true for a number of insects, especially for larval stages. However, there is no clear correlation between the presence of biliproteins and their contribution to the visible coloration of a given insect. Moreover, the synthesis of open-chain bilins along the porphyrin pathway via heme is a costly undertaking that is unlikely to be repaid by a simple visible protection effect. Cryptic coloration might be achieved with much less effort by utilizing, for example, chlorophyll-derived products simply sequestered from the plant food. In conclusion, the primary roles of biliproteins in insects are likely still to be discovered.

Another most interesting property of lipocalins in general is binding to other proteins, in particular to receptors that may function in cellular signaling. Though cell surface receptors have been claimed for several lipocalins there is only little experimental proof for their presence and identity even in the case of the prototypic lipocalin retinol-binding protein (review: Flower, 2000). A well-studied example even at the crystal level, however, is the 2 : 1 complex of the retinol-binding protein with the tetrameric transthyretin as it occurs in vertebrate blood. In insects, studies of the uptake of insecticyanin into developing oocytes of *M. sexta* provided preliminary data for a membrane receptor that is likely involved in uptake or internalization of this biliprotein (see Section 4.8.3.4). Another relation of lipocalins to membranes is seen in those proteins firmly associated with the cell surface like for Lazarillo that plays a still enigmatic role in developing neuronal networks (see Section 4.8.5.1.6). It is not known whether Lazarillo requires a specific ligand to be active. It is tempting to speculate about retinoic acid (or a related retinoid) as a potential ligand as this compound is known as a powerful mediator of cell differentiation and embryonic development. This would place Lazarillo near the cellular retinoic acid-binding proteins (CRABPs) that are members of the FABP superfamily, however (see Section 4.8.6.3).

Taken together, lipocalins seem to act everywhere and in a variety of ways in living systems. Research in insects has contributed significantly to the lipocalin field from the beginning and will hopefully do so in the future. There are many intriguing aspects of lipocalins insects may provide an ideal platform to focus on. This chapter may be helpful in this respect in attempting to provide an encouraging overview of this field.

Acknowledgments

The author is grateful to Lars Prade (Basel, Switzerland) for preparing all the basic figures of the crystal structures for this chapter. Hazel M. Holden (Madison, USA) kindly provided the atomic coordinates for insecticyamin. He further likes to thank Diego Sánchez (Valladolid, Spain), Arne Skerra (Munich, Germany), Chi-Young Yun (Deajeon, Korea), Sook Jae Seo (Gyeongnam, Korea), Hitoshi Saito (Tokyo, Japan), and Frantisek Sehnal (Ceské Budejovice, Czech Republic) for providing figures, reprints, accepted manuscripts, data of unpublished research, and other useful information.

References

Andersen, J.F., Champagne, D.E., Weichsel, A., Ribeiro, J.M.C., Balfour, C.A., *et al.*, **1997**. Nitric oxide binding and crystallization of recombinant nitrophorin I, a nitric oxide transport protein from the blood-sucking bug *Rhodnius prolixus*. *Biochemistry 36*, 4423–4428.

Andersen, J.F., Francischetti, I.M.B., Valenzuela, J.G., Schuck, P., Ribeiro, J.M.C., **2003**. Inhibition of hemostasis by a high affinity biogenic amine-binding protein from the saliva of a blood-feeding insect. *J. Biol. Chem. 278*, 4611–4617.

Andersen, J.F., Weichsel, A., Balfour, C.A., Champagne, D.E., Montfort, W.R., **1998**. The crystal structure of nitrophorin 4 at 1.5 Å resolution: transport of nitric oxide by a lipocalin-based heme protein. *Structure 15*, 1315–1327.

Arlian, L.G., **2002**. Arthropod allergens and human health. *Annu. Rev. Entomol. 47*, 395–433.

Arruda, L.K., Vailes, L.D., Hayden, M.L., Benjamin, D.C., Chapman, M.D., **1995**. Cloning of cockroach allergen, Bla g 4, identifies ligand binding proteins (or calycins) as a cause of IgE antibody responses. *J. Biol. Chem. 270*, 31196–31201.

Banaszak, L., Winter, N., Xu, Z., Bernlohr, D.A., Cowan, S., *et al.*, **1994**. Lipid-binding proteins: a family of fatty acid and retinoid transport proteins. *Adv. Protein Chem. 45*, 89–151.

Barañano, D.E., Rao, M., Ferris, C.D., Snyder, S.H., **2002**. Biliverdin reductase: a major physiologic cytoprotectant. *Proc. Natl Acad. Sci. USA 99*, 16093–16098.

Barañano, D.E., Snyder, S.H., **2001**. Neural roles for heme oxygenase: contrasts to nitric oxid synthase. *Proc. Natl Acad. Sci. USA 98*, 10996–11002.

Benning, M.M., Smith, A.F., Wells, M.A., Holden, H.M., **1992**. Crystallization, structure determination and least-squares refinement to 1.75 Å resolution of the fatty-acid-binding protein isolated from *Manduca sexta* L. *J. Mol. Biol. 228*, 208–219.

Beste, G., Schmidt, F.S., Stibora, T., Skerra, A., **1999**. Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc. Natl Acad. Sci. USA 96*, 1898–1903.

Beuckmann, C.T., Aoyagi, M., Okazaki, I., Hiroike, T., Toh, H., *et al.*, **1999**. Binding of biliverdin, bilirubin, and thyroid hormones to lipocalin-type prostaglandin D synthase. *Biochemistry 38*, 8006–8013.

Breiter, D.R., Kanost, M.R., Benning, M.M., Wesenberg, G., Law, J.H., *et al.*, **1991**. Molecular structure of an apolipoprotein determined at 2.5-Å resolution. *Biochemistry 30*, 603–608.

Briand, L., Swasdipan, N., Nespoulous, C., Bézirard, V., Blon, F., *et al.*, **2002**. Characterization of a chemosensory protein (ASP3c) from honeybee (*Apis mellifera* L.) as a brood pheromone carrier. *Eur. J. Biochem. 269*, 4586–4596.

Bugos, R.C., Hieber, A.D., Yamamoto, H.Y., **1998**. Xanthophyll cycle enzymes are members of the lipocalin family, the first identified in plants. *J. Biol. Chem. 273*, 15321–15324.

Büyükgüzel, K., Tunaz, H., Putnam, S.M., Stanley, D., **2002**. Prostaglandin biosynthesis by midgut tissue isolated from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 435–443.

Caraballo, L., Puerta, L., Jimenez, S., Martinez, B., Mercado, D., *et al.*, **1997**. Cloning and IgE binding of a recombinant allergen from the mite *Blomia tropicalis*, homologous with fatty acid-binding proteins. *Int. Arch. Allergy Immunol. 112*, 341–347.

Cavagnari, B.M., Scaraffia, P.Y., Haller, J.F., Gerez de Burgos, N.M., Santomé, J.A., **2000**. Presence of a fatty acid-binding protein and lipid stores in flight muscles of *Dipetalogaster maximus* (Hemiptera: Reduviidae). *J. Med. Entomol. 37*, 938–944.

Champagne, D.E., Andersen, J.F., Ribeiro, J.M.C., **1996**. Sequence of salivary platelet aggregation inhibitor 2 submitted to EMBL/GenBank/DDBJ databases (URL for: GenBank – www.ncbi.nlm.nib.gov; EMBL – www.ebi.ac.uk/embl; DDBJ –www.ddbj.nig.ac.jp).

Champagne, D.E., Nussenzveig, R.H., Ribeiro, J.M.C., **1995**. Purification, partial characterization, and cloning of nitric oxide-carrying heme proteins (nitrophorins) from salivary glands of the blood-sucking insect *Rhodnius prolixus*. *J. Biol. Chem. 270*, 8691–8695.

Charron, J.-B.F., Breton, G., Badawi, M., Sarhan, F., **2002**. Molecular and structural analysis of a novel temperature stress-induced lipocalin from wheat and *Arabidopsis*. *FEBS Lett. 517*, 129–132.

Chen, X., Haunerland, N.H., **1994**. Fatty acid binding protein expression in locust flight muscle: induction by flight, adipokinetic hormone, and low density lipoprotein. *Insect Biochem. Mol. Biol. 24*, 573–579.

Cherbas, P., **1973**. Biochemical studies of insecticyanin. Ph.D. thesis, Harvard University, Cambridge, MA.

Chino, H., Abe, Y., Takahashi, K., **1983**. Purification and characterization of a biliverdin-binding cyanoprotein from the locust hemolymph. *Biochim. Biophys. Acta 748*, 109–115.

Chinzei, Y., Haruna, T., Miura, K., Numata, H., Nakayama, S., **1990**. Purification and characterization of biliverdin-associated cyanoprotein from eggs and hemolymph of the bean bug, *Riptortus clavatus* (Heteroptera: Alydidae). *Insect Biochem. 20*, 545–555.

Cianci, M., Rizkallah, P.J., Olczak, A., Raftery, J., Chayen, N.E., *et al.*, **2001**. Structure of lobster apocrustacyanin A_1 using softer X-rays. *Acta Crystallog. D57*, 1219–1229.

Cianci, M., Rizkallah, P.J., Olczak, A., Raftery, J., Chayen, N.E., *et al.*, **2002**. The molecular basis of the coloration mechanism in lobster shell: β-crustacyanin at 3.2-Å resolution. *Proc. Natl Acad. Sci. USA 99*, 9795–9800.

Cornette, R., Farine, J.-P., Quennedey, B., Brossut, R., **2001**. Molecular characterization of a new adult male putative calycin specific to tergal gland aphrodisiac secretion in the cockroach *Leucophaea maderae*. Submitted to the EMBL/GenBank/DDBJ databases (URL for: GenBank – www.ncbi.nlm.nib.gov; EMBL – www.ebi.ac.uk/embl; DDBJ – www.ddbj.nig.ac.jp).

Eriksson, T.L.J., Rasool, O., Huecas, S., Whitley, P., Crameri, R., *et al.*, **2001**. Cloning of three new allergens from the dust mite *Lepidoglyphus destructur* using phage surface display technology. *Eur. J. Biochem. 268*, 287–294.

Eriksson, T.L.J., Whitley, P., Johansson, E., van Hage-Hamsten, M., Gafvelin, G., **1999**. Identification and characterization of two allergens from the dust mite *Acarus siro*, homologous with fatty acid-binding proteins. *Int. Arch. Allergy Immunol. 119*, 275–281.

Evans, J.D., Wheeler, D.E., **1999**. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. *Proc. Natl Acad. Sci. USA 96*, 5575–5580.

ExPASy, **2003**. http://us.expasy.org.

Falchuk, K.H., Contin, J.M., Dziedzik, T.S., Feng, Z., French, T.C., *et al.*, **2002**. A role for biliverdin IXα in dorsal axis development of *Xenopus laevis* embryo. *Proc. Natl Acad. Sci. USA 99*, 251–256.

Favia, G., Mariotti, G., Coluzzi, M., Mathiopoulos, K.D., **1996**. Sequence submitted to EMBL/GenBank/DDBJ databases (URL for: GenBank – www.ncbi.nlm.nib.gov; EMBL – www.ebi.ac.uk/embl; DDBJ – www.ddbj.nig.ac.jp).

Field, L.M., Pickett, J.A., Wadhams, L.J., **2000**. Molecular studies in insect olfaction. *Insect Mol. Biol. 9*, 545–551.

Filippov, V.A., Filippova, M.A., Kodrík, D., Sehnal, F., **1995**. Two lipocalin-like peptides of insect brain. In: Suzuki, A., Kataoka, H., Matsumoto, S. (Eds.), Molecular Mechanisms of Insect Metamorphosis and

Diapause. Industrial Publishing and Consulting, Tokyo, pp. 35–43.

Flower, D.R., **1994**. The lipocalin protein family: a role in cell regulation. *FEBS Lett. 354*, 7–11.

Flower, D.R., **1995**. Multiple molecular recognition properties of the lipocalin protein family. *J. Mol. Recogn. 8*, 185–195.

Flower, D.R., **2000**. Beyond the superfamily: the lipocalin receptors. *Biochim. Biophys. Acta 1482*, 327–336.

Flower, D.R., North, A.C.T., Attwood, T.K., **1993**. Structure and sequence relationships in the lipocalins and related proteins. *Protein Sci. 2*, 753–761.

Flower, D.R., North, A.C.T., Sansom, C.E., **2000**. The lipocalin protein family: structural and sequence overview. *Biochim. Biophys. Acta 1482*, 9–24.

Francischetti, I.M.B., Andersen, J., Ribeiro, J.M.C., **2002**. Biochemical and functional characterization of recombinant *Rhodnius prolixus* platelet aggregation inhibitor 1 as a novel lipocalin with high affinity for adenosine diphosphate and other adenine nucleotides. *Biochemistry 41*, 3810–3818.

Francischetti, I.M.B., Ribeiro, J.M.C., Champagne, D., Andersen, J., **2000**. Purification, cloning, expression, and mechanism of action of a novel platelet aggregation inhibitor from the salivary gland of the blood-sucking bug, *Rhodnius prolixus*. *J. Biol. Chem. 275*, 12639–12650.

Fuentes-Prior, P., Noeske-Jungblut, C., Donner, P., Schleuning, W.-D., Huber, R., *et al.*, **1997**. Structure of the thrombin complex with triabin, a lipocalin-like exosite-binding inhibitor derived from a triatomine bug. *Proc. Natl Acad. Sci. USA 94*, 11845–11850.

Ganfornina, M.D., Gutiérrez, G., Bastiani, M., Sánchez, D., **2000**. A phylogenetic analysis of the lipocalin protein family. *Mol. Biol. Evolution 17*, 114–126.

Ganfornina, M.D., Sánchez, D., Bastiani, M.J., **1995**. Lazarillo, a new GPI-linked surface lipocalin, is restricted to a subset of neurons in the grasshopper embryo. *Development 121*, 123–134.

Glatz, J.F.C., van der Vusse, G.J., **1996**. Cellular fatty acid-binding proteins: their function and physiological significance. *Prog. Lipid Res. 35*, 243–282.

Goodman, W.G., Adams, B., Trost, J.T., **1985**. Purification and characterization of a biliverdin-associated protein from the hemolymph of *Manduca sexta*. *Biochemistry 24*, 1168–1175.

Gordon, E.J., Leonard, G.A., McSweeney, S., Zagalsky, P.F., **2001**. The C_1 subunit of α-crustacyanin: the *de novo* phasing of the crystal structure of a 40 kDa homomeric protein using the anomalous scattering from S atoms combined with the direct methods. *Acta Crystallog. D57*, 1230–1237.

Graf, S., Ludwig, P., Boyan, G., **2000**. Lazarillo expression reveals a subset of neurons contributing to the primary axon scaffold of the embryonic brain of the grasshopper *Schistocerca gregaria*. *J. Comp. Neurol. 419*, 394–405.

Graham, L.A., Davies, P.L., **2002**. The odorant-binding proteins of *Drosophila melanogaster*: annotation and characterization of a divergent gene family. *Gene 292*, 43–55.

Hartsfield, C.L., **2002**. Cross talk between carbon monoxide and nitric oxide. *Antiox. Redox Signal. 4*, 301–307.

Haunerland, N.H., **1996**. Insect storage proteins: gene families and receptors. *Insect Biochem. Mol. Biol. 26*, 755–765.

Haunerland, N.H., **1997**. Transport and utilization of lipids in insect flight muscles. *Comp. Biochem. Physiol. B 117*, 475–482.

Haunerland, N.H., Andolfatto, P., Chrisholm, J.M., Wang, Z., Chen, X., **1992a**. Fatty-acid-binding protein in locust flight muscle: developmental changes of expression, concentration and intracellular distribution. *Eur. J. Biochem. 210*, 1045–1051.

Haunerland, N.H., Bowers, W.S., **1986**. A larval specific lipoprotein: purification and characterization of a blue chromoprotein from *Heliothis zea*. *Biochem. Biophys. Res. Commun. 134*, 580–586.

Haunerland, N.H., Chen, X., Andolfatto, P., Chrisholm, J.M., Wang, Z., **1993**. Developmental changes of FABP concentration, expression, and intracellular distribution in locust flight muscle. *Mol. Cell. Biochem. 123*, 153–158.

Haunerland, N.H., Chrisholm, J.M., **1990**. Fatty acid binding protein in flight muscle of the locust, *Schistocera gregaria*. *Biochim. Biophys. Acta 1047*, 233–238.

Haunerland, N.H., Jacobson, B.L., Wesenberg, G., Rayment, I., Holden, H.M., **1994**. Three-dimensional structure of the muscle fatty-acid-binding protein isolated from the desert locust *Schistocera gregaria*. *Biochemistry 33*, 12378–12385.

Haunerland, N.H., Ortego, F., Strausfeld, C.M., Bowers, W.S., **1992b**. Blue lipophorin from *Podisus maculaventris*. *Arch. Insect Biochem. Physiol. 20*, 49–59.

Heilmann, L.J., **1998**. Fatty acid binding protein of *Helicoverpa zea*. Submitted to the EMBL/GenBank/DDBJ databases (URL for: GenBank – www.ncbi.nlm.nib.gov; EMBL – www.ebi.ac.uk/embl; DDBJ – www.ddbj.nig.ac.jp).

Hieber, A.D., Bugos, R.C., Yamamoto, H.Y., **2000**. Plant lipocalins: violaxanthin de-epoxidase and zeaxanthin epoxidase. *Biochim. Biophys. Acta 1482*, 84–91.

Holden, H.M., Rypniewski, W.R., Law, J.H., **1987**. The molecular structure of insecticyanin from the tobacco hornworm *Manduca sexta* L. at 2.6 Å resolution. *EMBO J. 6*, 1565–1570.

Huber, R., Schneider, M., Epp, O., Mayr, I., Messerschmidt, A., *et al.*, **1987a**. Crystallization, crystal structure analysis and preliminary molecular model of the bilin binding protein from the insect *Pieris brassicae*. *J. Mol. Biol. 195*, 423–434.

Huber, R., Schneider, M., Mayr, I., Müller, R., Deutzmann, R., *et al.*, **1987b**. Molecular structure of the bilin binding protein (BBP) from *Pieris brassicae* after refinement at 2.0 Å resolution. *J. Mol. Biol. 198*, 499–513.

Jones, G., Ryan, R.O., Haunerland, N.H., Law, J.H., **1988**. Purification and characterization of a very high

density chromolipoprotein from the hemolymph of *Trichoplusia ni* (Hübner). *Arch. Insect Biochem. Physiol. 7*, 1–11.

Jones, T.A., Bergfors, T., Sedzik, J., Unge, T., **1988**. The three-dimensional structure of P2 myelin protein. *EMBO J. 7*, 1597–1604.

Jones, T.A., Zou, J.-Y., Cowan, S.W., Kjeldgaard, M., **1991**. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallog. A47*, 110–119.

Kang, Y., Kulakosky, P.C., van Antwerpen, R., Law, J.H., **1995**. Sequestration of insecticyanin, a blue hemolymph protein, into the egg of the hawkmoth *Manduca sexta*. Evidence for receptor-mediated endocytosis. *Insect Biochem. Mol. Biol. 25*, 503–510.

Kang, Y., Ziegler, R., van Antwerpen, R., Law, J.H., **1997**. Characterization of the solubilized oocyte membrane receptor for insecticyanin, a biliprotein of the hawkmoth, *Manduca sexta*. *Biochem. Biophys. Acta 1324*, 285–295.

Kaur, H., Hughes, M.N., Green, C.J., Naughton, P., Foresti, R., *et al.*, **2003**. Interaction of bilirubin and biliverdin with reactive nitrogen species. *FEBS Lett. 543*, 113–119.

Kayser, H., **1984**. *De novo* synthesis and levels of cytochrome *c* and a biliprotein during pupal–adult development of a butterfly, *Pieris brassicae*. *Z. Naturforsch. 39c*, 938–947.

Kayser, H., **1985**. Pigments. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Biochemistry, Physiology and Pharmacology, vol. 10. Pergamon Press, Oxford, pp. 367–415.

Kayser, H., Krull-Savage, U., **1984**. Development-specific incorporation of [^{14}C]5-aminolevulinate and [^{3}H]leucine into cytochrome *c* and biliprotein in the butterfly, *Pieris brassicae*: correlation with the ecdysteroid titer in the pupa. *Z. Naturforsch. 39c*, 948–957.

Keen, J.N., Caceres, I., Eliopoulos, E.E., Zagalsky, P.F., Findlay, J.B.C., **1991**. Complete sequence and model for the C_1 subunit of the carotenoprotein, crustacyanin, and model for the dimer, β-crustacyanin, formed from the C_1 and A_2 subunits with astaxanthin. *Eur. J. Biochem. 202*, 31–40.

Kiely, M.L., Riddiford, L.M., **1985**. Temporal programming of epidermal cell protein synthesis during the larval–pupal transformation of *Manduca sexta*. *Roux's Arch. Devel. Biol. 194*, 325–335.

Koglin, M., Behrends, S., **2002**. Biliverdin IX is an endogenous inhibitor of soluble guanylyl cyclase. *Biochem. Pharmacol. 64*, 109–116.

Korchi, A., Brossut, R., Bouhin, H., Delachambre, J., **1999**. cDNA cloning of an adult male putative lipocalin specific to tergal gland aphrodisiac secretion in an insect (*Leucophaea maderae*). *FEBS Lett. 449*, 125–128.

Korndörfer, I.P., Beste, G., Skerra, A., **2003**. Crystallographic analysis of an “anticalin” with tailored specificity for fluorescein reveals high structural plasticity of the lipocalin loop region. *Proteins 53*, 121–129.

LaLonde, J.M., Bernlohr, D.A., Banaszak, L.J., **1994**. The up-and-down β-barrel proteins. *FASEB J. 8*, 1240–1247.

Lartigue, A., Campanacci, V., Roussel, A., Larsson, A.M., Jones, T.A., *et al.*, **2002**. X-ray structure and ligand binding study of a moth chemosensory protein. *J. Biol. Chem. 277*, 32094–32098.

Lechner, M., Wojnar, P., Redl, B., **2001**. Human tear lipocalin acts as an oxidative-stress-induced scavenger of potentially harmful lipid peroxidation products in a cell culture system. *Biochem. J. 356*, 129–135.

Li, W.-C., Riddiford, L.M., **1992**. Two distinct genes encode two major isoelectric forms of insecticyanin in the tobacco hornworm, *Manduca sexta*. *Eur. J. Biochem. 205*, 491–499.

Li, W.-C., Riddiford, L.M., **1994**. The two duplicated insecticyanin genes, *ins*-a and *ins*-b are differentially expressed in the tobacco hornworm, *Manduca sexta*. *Nucl. Acids Res. 22*, 2945–2950.

Li, W.-C., Riddiford, L.M., **1996**. Differential expression of the two duplicated insecticyanin genes, *ins*-a and *ins*-b, in the black mutant of *Manduca sexta*. *Arch. Biochem. Biophys. 330*, 65–70.

Lipocalin, **2004**. www.jenner.ac.uk/lipocalin.htm.

Maatman, R.G.H.J., Degano, M., Van Moerkerk, H.T.B., Van Marrewijk, W.J.A., Van der Horst, D.J., *et al.*, **1994**. Primary structure and binding characteristics of locust and human muscle fatty-acid-binding proteins. *Eur. J. Biochem. 221*, 801–810.

Maines, M.D., **1997**. The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol. 37*, 517–554.

Mansfield, S.G., Cammer, S., Alexander, S.C., Muehleisen, D.P., Gray, R.S., *et al.*, **1998**. Molecular cloning and characterization of an invertebrate cellular retinoic acid binding protein. *Proc. Natl Acad. Sci. USA 95*, 6825–6830.

Mäntyjärvi, R., Rautiainen, J., Virtanen, T., **2000**. Lipocalins as allergens. *Biochim. Biophys. Acta 1482*, 308–317.

Maruta, K., Yoshiga, T., Katagiri, C., Ochiai, M., Tojo, S., **2002**. Purification and characterization of biliverdin-binding vitellogenin from the hemolymph of the common cutworm, *Spodoptera litura*. *Arch. Insect Biochem. Physiol. 50*, 97–106.

Miura, K., Shinoda, T., Yura, M., Nomura, S., Kamiya, K., *et al.*, **1998**. Two hexameric cyanoprotein subunits from an insect, *Riptortus clavatus*: sequence, phylogeny and developmental and juvenile hormone regulation. *Eur. J. Biochem. 258*, 929–940.

Montfort, W.R., Weichsel, A., Andersen, J.F., **2000**. Nitrophorins and related antihemostatic lipocalins from *Rhodnius prolixus* and other blood-sucking arthropods. *Biochim. Biophys. Acta 1482*, 110–118.

Moreira, M.F., Coelho, H.S.L., Zingali, R.B., Oliveira, P.L., Masuda, H., **2003**. Changes in salivary nitrophorin profile during the life cycle of the blood-sucking bug *Rhodnius prolixus*. *Insect Biochem. Mol. Biol. 33*, 23–28.

Newcomer, M.E., **1995**. Retinoid-binding proteins: structural determinants important for function. *FASEB J. 9*, 229–239.

Newcomer, M.E., Jones, T.A., Aqvist, J., Sundelin, J., Eriksson, U., *et al.*, **1984**. The 3-dimensional structure of retinol-binding protein. *EMBO J. 3*, 1451–1454.

Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L., *et al.*, **1995**. Triabin, a highly potent exosite inhibitor of thrombin. *J. Biol. Chem. 270*, 28629–28634.

Noeske-Jungblut, C., Kratzschmar, J., Haendler, B., Alagon, A., Possani, L., *et al.*, **1994**. An inhibitor of collagen-induced platelet aggregation from the saliva of *Triatoma pallidipennis*. *J. Biol. Chem. 269*, 5050–5053.

Nussenzveig, R.H., Bentley, D.L., Ribeiro, J.M.C., **1995**. Nitric oxide loading of the salivary nitric-oxide-carrying hemoproteins (nitrophorins) in the blood sucking bug *Rhodnius prolixus*. *J. Exp. Biol. 198*, 1093–1098.

Oliveira, P.L., Kawooya, J.K., Ribeiro, J.M.C., Meyer, T., Poorman, R., *et al.*, **1995**. A heme-binding protein from hemolymph and oocytes of the blood-sucking insect, *Rhodnius prolixus*. *J. Biol. Chem. 270*, 10897–10901.

Paddock, C.D., McKerrow, J.H., Hansell, E., Foreman, K.W., Hsieh, I., *et al.*, **2001**. Identification, cloning, and recombinant expression of procalin, a major Triatomine allergen. *J. Immunol. 167*, 2694–2699.

Paesen, G.C., Adams, P.L., Harlos, K., Nuttall, P.A., Stuart, D.I., **1999**. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. *Mol. Cell 3*, 661–671.

Paesen, G.C., Adams, P.L., Nuttall, P.A., Stuart, D.I., **2000**. Tick histamine-binding proteins: lipocalins with a second binding site. *Biochim. Biophys. Acta 1482*, 92–101.

Paiva-Silva, G.O., Sorgine, M.H.F., Benedetti, C.E., Meneghini, R., Almeida, I.C., *et al.*, **2002**. On the biosynthesis of *Rhodnius prolixus* heme-binding protein. *Insect Biochem. Mol. Biol. 32*, 1533–1541.

Papis, M.Z., Sawyer, L., Eliopoulos, E.E., North, A.C.T., Findlay, J.B.C., *et al.*, **1986**. The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature 324*, 383–385.

Peitsch, M.C., Boguski, M.S., **1990**. Is apolipoprotein D a mammalian bilin-binding protein? *New Biol. 2*, 197–206.

Pelosi, P., Maida, R., **1995**. Odorant-binding proteins in insects. *Comp. Biochem. Physiol. B 111*, 503–514.

Pervais, S., Brew, K., **1987**. Homology and structure-function correlations between alpha 1-acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J. 1*, 209–214.

Price, H.M., Ryan, R.O., Haunerland, N.H., **1992**. Primary structure of locust flight muscle fatty acid binding protein. *Arch. Biochem. Biophys. 297*, 285–290.

Puerta, L., Kennedy, M.W., Jimenez, S., Caraballo, L., **1999**. Structural and ligand binding analysis of recombinant blo t 1 allergen from *Blomia tropicalis* mite, a fatty acid binding protein. *Int. Arch. Allergy Immunol. 119*, 181–184.

Ribeiro, J.M.C., Francischetti, I.M.B., **2003**. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu. Rev. Entomol. 48*, 73–88.

Ribeiro, J.M.C., Schneider, M., Guimarães, J.A., **1995**. Purification and characterization of prolixin S (nitrophorin 2), the salivary anticoagulant of the blood-sucking bug *Rhodnius prolixus*. *Biochem. J. 308*, 243–249.

Riddiford, L.M., **1982**. Changes in translatable mRNAs during the larval–pupal transformation of the epidermis of the tobacco hornworm. *Devel. Biol. 92*, 330–342.

Riddiford, L.M., Palli, S.R., Hiruma, K., Li, W.-C., Green, J., *et al.*, **1990**. Developmental expression, synthesis, and secretion of insecticyanin by the epidermis of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 14*, 171–190.

Riley, C.T., Barbeau, B.K., Keim, P.S., Kézdy, F.J., Heinrikson, R.L., *et al.*, **1984**. The covalent protein structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm, *Manduca sexta* L. *J. Biol. Chem. 259*, 13159–13165.

Rothemund, S., Liou, Y.-C., Davies, P.L., Krause, E., Sönnichsen, F.D., **1999**. A new class of hexahelical insect proteins revealed as putative carriers of small hydrophobic ligands. *Structure 7*, 1325–1332.

Ryan, R.O., van der Horst, D.J., **2000**. Lipid transport biochemistry and its role in energy production. *Annu. Rev. Entomol. 45*, 233–260.

Ryter, S.W., Otterbein, L.E., Morse, D., Choi, A.M.K., **2002**. Heme oxygenase/carbon monoxide signalling pathways: regulation and functional significance. *Mol. Cell. Biochem. 234/235*, 249–263.

Saito, H., **1993**. Purification and characterization of a biliverdin-binding protein from the molting fluid of the eri silkworm, *Samia cynthia ricini* (Lepidoptera: Saturniidae). *Comp. Biochem. Physiol. B 105*, 473–479.

Saito, H., **1997**. Purification and characterization of a biliverdin-binding protein from the larval cuticle of the saturniid silkworn, *Attacus atlas*. *Int. J. Wild Silkmoth Silk 3*, 1–6.

Saito, H., **1998a**. Purification and characterization of two insecticyanin-type proteins from the larval hemolymph of the Eri-silkworm, *Samia cynthia ricini*. *Biochim. Biophys. Acta 1380*, 141–150.

Saito, H., **1998b**. Purification and properties of two blue biliproteins from the larval hemolymph and integument of *Rhodinia fugax* (Lepidoptera: Saturniidae). *Insect Biochem. Mol. Biol. 28*, 995–1005.

Saito, H., Shimoda, M., **1997**. Insecticyanin of *Agrius convolvuli*: purification and characterization of the biliverdin-binding protein from the larval hemolymph. *Zool. Sci. 14*, 777–783.

Saito, H., Yamada, H., Kato, Y., **1998**. Isolation and partial characterization of chromoprotein from the larval hemolymph of the Japanese oak silkworm (*Antheraea yamamai*). *Comp. Biochem. Physiol. B 119*, 625–630.

Sakai, M., Wu, C., Suzuki, K., **2001**. Nucleotide and deduced amino acid sequences of a cDNA encoding a lipocalin protein in the central nervous system of *Bombxy mori*. *Nippon Sanshi-gaku Zasshi 70*, 105–111.

Sánchez, D., Ganfornina, M.D., Bastiani, M.J., **1995**. Developmental expression of the lipocalin Lazarillo and its role in axonal pathfinding in the grasshopper embryo. *Development 121*, 135–147.

Sánchez, D., Ganfornina, M.D., Bastiani, M.J., **2000a**. Lazarillo, a neuronal lipocalin in grasshoppers with a role in axon guidance. *Biochim. Biophys. Acta 1482*, 102–109.

Sánchez, D., Ganfornina, M.D., Torres-Schumann, S., Speese, S.D., Lora, J.M., *et al.*, **2000b**. Characterization of two novel lipocalins expressed in the *Drosophila* embryonic nervous system. *Int. J. Devel. Biol. 44*, 349–359.

Sandler, B.H., Nikonowa, L., Leal, W.S., Clardy, J., **2000**. Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. *Chem. Biol. 7*, 143–151.

Sangamnatdej, S., Paesen, G.C., Slovak, M., Nuttall, P.A., **2002**. A high affinity serotonin- and histamine-binding lipocalin from tick saliva. *Insect Mol. Biol. 11*, 79–86.

Scheer, H., Kayser, H., **1988**. Conformational studies of biliproteins from the insects *Pieris brassicae* and *Cerura vinula*. *Z. Naturforsch. 43c*, 84–90.

Schirmer, T., Bode, W., Huber, R., **1987**. Refined three-dimensional structures of two cyanobacterial C-phycocyanins at 2.1 and 2.5 Å resolution. *J. Mol. Biol. 196*, 677–695.

Schlehuber, S., Skerra, A., **2001**. Duocalins: engineered ligand-binding proteins with dual specificity derived from the lipocalin fold. *Biol. Chem. 382*, 1335–1342.

Schmidt, F.S., Skerra, A., **1994**. The bilin-binding protein of *Pieris brassicae*: cDNA sequence and regulation of expression reveal distinct features of this insect pigment protein. *Eur. J. Biochem. 219*, 855–863.

Schneider, S., Baumann, F., Geiselhart, P., Kayser, H., Scheer, H., **1988**. Biliproteins from the butterfly *Pieris brassicae* studied by time-resolved fluorescence and coherent anti-stokes Raman spectroscopy. *Photochem. Photobiol. 48*, 239–242.

Sehringer, B., **1999**. Bilin-bindendes protein von *Pieris brassicae*: untersuchungen zur regulation des mRNA-gehalts und der proteinbiosynthese dieses pigmentproteins während der puppenentwicklung. Diploma thesis, University of Freiburg, Germany.

Seo, S.-J., Cheon, H.-M., **2003**. Molecular characterization of hyphantrin from *Hyphantria cunea*. Submitted to the EMBL/GenBank/DDBJ databases (URL for: GenBank – www.ncbi.nlm.nib.gov; EMBL – www.ebi.ac.uk/embl; DDBJ – www.ddbj.nig.ac.jp).

Skerra, A., **2000**. Lipocalins as a scaffold. *Biochim. Biophys. Acta 1482*, 337–350.

Skerra, A., **2001**. "Anticalins": a new class of engineered ligand-binding proteins with antibody-like properties. *Rev. Mol. Biotechnol. 74*, 257–275.

Smith, A.F., Tsuchida, K., Hanneman, E., Suzuki, T.C., Wells, M.A., **1992**. Isolation, characterization, and cDNA sequence of two fatty acid-binding proteins from the midgut of *Manduca sexta* larvae. *J. Biol. Chem. 267*, 380–384.

Sun, J., Yuda, M., Miura, K., Chinzei, Y., **1998**. Characterization and cDNA cloning of a hemoprotein in the salivary glands of the blood-sucking insect, *Rhodnius prolixus*. *Insect Biochem. Mol. Biol. 28*, 191–200.

Suter, F., Kayser, H., Zuber, H., **1988**. The complete amino-acid sequence of the bilin-binding protein from *Pieris brassicae* and its similarity to a family of serum transport proteins like the retinol-binding proteins. *Biol. Chem. Hoppe-Seyler 369*, 497–505.

Tsuchihara, K., Ueno, K., Yamanaka, A., Isono, K., Endo, K., *et al.*, **2000**. A putative binding protein for lipophilic substances related to butterfly oviposition. *FEBS Lett. 478*, 299–303.

Trost, J.T., Goodman, W.G., **1986**. Hemolymph titers of the biliprotein, insecticyanin, during development of *Manduca sexta*. *Insect Biochem. 16*, 353–358.

Urade, Y., Hayashi, O., **2000**. Prostaglandin D synthase: structure and function. *Vitam. Hormones 58*, 89–120.

Valenzuela, J.G., Ribeiro, J.M.C., **1998**. Purification and cloning of the salivary nitrophorin from the hemipteran *Cimex lectularius*. *J. Exp. Biol. 201*, 2659–2664.

Van der Horst, D.J., van Doorn, J.M., Passier, P.C.C.M., Vork, M.M., Glatz, J.F.C., **1993**. Role of fatty acid-binding protein in lipid metabolism of insect flight muscle. *Mol. Cell. Biochem. 123*, 145–152.

Veerkamp, J.H., Maatman, R.G.H.J., **1995**. Cytoplasmic fatty acid-binding proteins: their structure and genes. *Prog. Lipid Res. 34*, 17–52.

Walker, F.A., Ribeiro, J.M.C., Montfort, W.R., **1999**. Novel nitric oxide-liberating heme proteins from the saliva of bloodsucking insects. *Metal Ions Biol. Syst. 36*, 621–663.

Weichsel, A., Andersen, J.F., Champagne, D.E., Walker, F.A., Montfort, W.R., **1998**. Crystal structures of a nitric oxide transport protein from a blood-sucking insect. *Nature Struct. Biol. 5*, 304–309.

Weichsel, A., Andersen, J.F., Roberts, S.A., Montfort, W.R., **2000**. Nitric oxide binding to nitrophorin 4 induces complete distal pocket burial. *Nature Struct. Biol. 7*, 551–554.

Weiss, G.A., Lowman, H.B., **2000**. Anticalins versus antibodies: made-to-order binding proteins from small molecules. *Chem. Biol. 7*, R177–R184.

Wilks, A., **2002**. Heme oxygenase: evolution, structure, and mechanism. *Antiox. Redox Signal. 4*, 603–614.

Wu, Q., Andolfatto, P., Haunerland, N.H., **2001**. Cloning and sequence of the gene encoding the muscle fatty acid binding protein from the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol. 31*, 553–562.

Wu, Q., Haunerland, N.H., **2001**. A novel fatty acid response element controls the expression of the flight muscle FABP gene of the desert locust, *Schistocerca gregaria*. *Eur. J. Biochem. 268*, 5894–5900.

Yoshiga, T., Maruta, K., Tojo, S., **1998**. Developmental changes of storage proteins and biliverdin-binding proteins in the haemolymph and fat body of the common cutworm, *Spodoptera litura*. *J. Insect Physiol. 44*, 67–76.

Yoshiga, T., Tojo, S., **1995**. Purification and characterization of four biliverdin-binding proteins from larval haemolymph of the common cutworm, *Spodoptera litura*. *Insect Biochem. Mol. Biol. 25*, 575–581.

Yoshiga, T., Tojo, S., **2001**. Effect of a juvenile hormone analog, methoprene, on the hemolymph titers of biliverdin-binding proteins in the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Appl. Entomol. Zool. 36*, 337–343.

Yousefi, S., Simon, H.U., **2002**. Granulocyte apoptosis: death by a secreted lipocalin? *Cell Death Different. 9*, 595–597.

Zanotti, G., **1999**. Muscle fatty acid-binding protein. *Biochim. Biophys. Acta 1441*, 94–105.

Zipfel, D., **1982**. Isolierung und Charakterisierung eines Biliproteins von *Pieris brassicae* L. Diploma thesis, University of Ulm, Germany.

Relevant Websites

http://us.expasy.org – ExPASy, site for various software including Clustal W.

http://www.jenner.ac.uk – database for lipocalins.

http://www.ncbi.nlm.nih.gov – database for GenBank.

http://www.ebi.ac.uk – database for EMBL.

http://www.ddbj.nig.ac.jp – database for DDBJ.

http://www.rcsb.org – Protein Data Bank.

4.9 Eicosanoids

D W Stanley, University of Nebraska, Lincoln, NE, USA

4.9.1. Introduction

Dadd's chapter in the first edition of *Comprehensive Insect Biochemistry, Physiology, and Pharmacology* (Dadd, 1985) treated organismal nutrition, an inquiry into the requirements, metabolism, and cellular functions of the chemicals which make up the natural diets of animals. Among the many classes of nutrients, Dadd reviewed what were then recent revelations of the requirements, presence and biological significance of arachidonic acid (AA) in insect nutritional science. He drew on the biomedical background to speculate that one of the main functions of AA in insect biology would be its role as direct precursor for prostaglandin (PG) biosynthesis, although there was very little idea of meaning which could be attached to the presence of PGs in insect tissues. In the early 1980s, it was known that PGE_2 results in egg-laying behavior in two cricket species (Loher *et al.*, 1981) and that mosquito larvae required dietary AA to complete the developmental excursion through pupation and successful adult emergence (Dadd and Kleinjan, 1979). In the years since Dadd's seminal review of insect nutrition, considerablely more insight into the biological significance of PGs and other eicosanoids in insects has come to light. Thus, this chapter is regarded (in the second edition of the comprehensive series) as an update on progress in the wake of Dadd's speculation.

The term "eicosanoid" was originally coined by Corey *et al.* (1980), who drew upon the Greek word "eikosi" meaning twenty, to formulate a single, encompassing term for all biologically active, oxygenated metabolites of AA and two other C_{20} polyunsaturated fatty acids (PUFAs). Three groups of eicosanoids: PGs, epoxyeicosatrienoic acids, and various lipoxygenase (LOX) products are recognized. Most of the known eicosanoids were first discovered in research into the physiology of humans and other mammals. The first known eicosanoids, PGs, were discovered in the 1930s. PGs, so named because they were first associated with the human prostate gland, stimulated contractions in some, but not all, mammalian smooth-muscle preparations (Goldblatt, 1933; von Euler, 1936). Von Euler (whose father Hans von Euler shared the 1929 Nobel Prize in Chemistry for work on the enzymology of sugar metabolism) eventually took part in the 1970 Nobel Prize in Medicine or Physiology for his discoveries of hormone-like substances, including PGs and adrenalin. The early research revealed the presence of a stimulating substance in human semen, which had an acidic and lipoidal nature. Further research into the substance was stimulated by the isolation and determination of the chemical structures of three PGs (Bergstrom *et al.*, 1962a, 1962b). Inspection of these structures immediately revealed the close similarity between

AA and PGs, from which it became apparent that PGs were formed by enzymatic oxygenation of AA.

Biosynthesis of PGs and other eicosanoids from AA might not have attracted the enormous attention it received were it not for the work of Sir John Vane, who discovered that the analgesic properties of aspirin and indomethacin were based on inhibition of PG biosynthesis (Ferreira *et al.*, 1971; Vane, 1971). Discovery that a range of discomforts associated with fever, inflammation, and dysmenorrhea (among other ailments) could be eased by inhibition of the enzymes involved in PG biosynthesis had immediate and obvious pharmacological implications. A very large research venture in government, university, and corporate laboratories, which continues unabated, ensued. Sune Bergstrom, his student Bengt Samuelsson, and John Vane were awarded the 1982 Nobel Prize in Medicine or Physiology for their work on the chemistry and pharmacology of PGs (Oates, 1982).

It would be difficult to overstate the biological and pathophysiological significance of PGs and other eicosanoids in human and veterinary medicine. These compounds are present and responsible for important biological actions in virtually all mammalian tissues. Among mammals, eicosanoids are responsible for physiological events registered at the organismal (fever), tissue (smooth-muscle contraction or relaxation), cellular (downregulation of mast cell function), and molecular (gene expression) levels of biological organization. Many eicosanoid-regulated events relate directly to pathophysiology and other aspects of human health and well-being. The slow-reacting substance of anaphylaxis, for example, is a mixture of eicosanoids. Virtually all over-the-counter (and some prescription) pain relievers act by inhibiting PG biosynthesis. Again, taken as a group of biologically active compounds, eicosanoids exert profound influences at several levels of mammalian biology.

The biological significance of PGs and other eicosanoids seems all the more interesting when viewed in the broader context of nonmammalian vertebrates and invertebrates. Stanley and Howard (1998) and Stanley (2000) expressed this as the biological paradigm of eicosanoids, in distinction to what might be regarded as a more traditional biomedical model. This view arises from the observation that PGs and other eicosanoids are present and biologically active in representatives of all major animal phyla, including single celled organisms (Stanley, 2000). Eicosanoids probably comprise one of the earliest signaling systems in cell biology. Throughout animal – particularly invertebrate – evolution, eicosanoids have been recruited into a large number of diverse biological roles. Some of these roles, such as modulation of ion transport physiology or cellular immune defense reactions, may be fundamental eicosanoid actions which occur in all animal cells, while others appear to be rather specific to certain evolutionary or ecological contexts. For example, PGE_2 releases egg-laying behavior in newly mated females of a few, but only a few, insect species (Stanley-Samuelson and Loher, 1986). The large diversity of eicosanoid actions in animal biology suggests considerable explanatory power. That is to say, curiosity about eicosanoids can reveal new information on the regulation and mediation of physiological events in animals, and for our purposes, in the Class Insecta.

The goal of this chapter is to provide an overview of eicosanoids as they relate to insect biology. As mentioned earlier, eicosanoids occur in virtually all invertebrates and this broader information has been reviewed elsewhere (Stanley-Samuelson, 1987, 1991; Stanley and Howard, 1998; Stanley, 2000). Discussion begins with a brief overview of the chemistry and biochemistry of eicosanoids, to which we now turn.

4.9.2. Definitions, Structures, and Biosynthesis of Eicosanoids

4.9.2.1. Introduction

Our understanding of eicosanoids was developed from research on the chemistry, biochemistry, and biology of these molecules in mammals. Due to its clinical significance, this research has produced a very large corpus of literature, which for convenience is called the "biomedical model." Probably because research on eicosanoid actions in mammals preceded inquiry into invertebrates by a couple of decades, a great deal of the research on eicosanoids in invertebrate systems followed the protocols as well as the language of the biomedical model.

Eicosanoids are biosynthesized by enzymatic oxygenation of AA or two other C_{20} PUFAs. Information on the presence, metabolism, and nutritional requirements of PUFAs in insects can be taken from from several reviews (Dadd, 1973, 1977, 1981, 1983, 1985; Stanley-Samuelson *et al.*, 1988; Stanley, 2000).

Three PUFAs, $C_{20:3n-6}$, $C_{20:4n-6}$, and $C_{20:5n-3}$, are potential direct substrates for eicosanoid biosynthesis, although most of the available information on eicosanoid biosynthesis is focused on metabolism of AA. Indeed, eicosanoid biosynthesis is often taken to be synonymous with "arachidonate metabolism," sometimes incorrectly referred to as the

"arachidonate cascade." AA does not actually enter a cascade; instead, it enters a range of metabolic pathways. Perhaps the sheer number of potential metabolic fates gives the sense of a cascade, but, this is not consistent with the meaning of the word. Eicosanoid biosynthesis, or arachidonate metabolism, more accurately describe the processes, and are the appropriate terms.

4.9.2.2. The Biomedical Model of Eicosanoid Biosynthesis

The following paragraphs outline the main pathways of eicosanoid biosynthesis, as understood in mammals. This outline represents our understanding of eicosanoid biosynthesis in insects and other invertebrates as well. Nonetheless, we can expect insects will express considerable departures from this model, particularly in biochemical details.

4.9.2.2.1. Phospholipase A_2 (PLA_2) is the first step in eicosanoid biosynthesis Eicosanoid biosynthesis is thought to begin with hydrolysis of AA from the *sn*-2 position of cellular phospholipids (PLs) (Dennis, 1994, 1997; Balsinde *et al*., 1999). PUFAs are generally associated with the *sn*-2 position of PLs, while saturated or monounsaturated fatty acids are associated with the *sn*-1 position. This asymmetry in the molecular localization of fatty acids within PLs was partly responsible for suggesting that PLA_2 might be a regulatory step in eicosanoid biosynthesis.

The number of recognized types of PLA_2s seem to steadily increase (Dennis, 1994, 1997; Balsinde *et al*., 1999), but there are two major PLA_2 classes. The secretory PLA_2s include those found in snake and arthropod venoms, those found in synovial fluids in some cases of pathology, and those associated with digestion. Most of these enzymes are low molecular mass (about 14 kDa) proteins stabilized by five to seven disulfide bridges. They require the presence of mM concentrations of Ca^{2+} for full catalytic activity. The other major class consists of the intracellular, or cytosolic PLA_2s ($cPLA_2$s). These are high molecular mass (>85 kDa) proteins that achieve full catalytic activity in the presence of µM calcium concentrations. Some of the $cPLA_2$s are of particular interest because they show a marked preference for PL substrate with AA in the *sn*-2 position. These enzymes are thought to mediate the first step in eicosanoid biosynthesis.

$cPLA_2$s have been purified from several mammalian sources (Dennis, 1994, 1997; Balsinde *et al*., 1999), including rat mesangial cells, human monocytes, human platelets, leukocytes, Swiss mouse 3T3 cells, glomerular mesangial cells, mouse keratinocytes, and mouse peritoneal macrophages. These enzymes are variously stimulated by proinflammatory cytokines, tumor necrosis factor, lipopolysaccharides (LPSs), and mitogens. The stimulations result in the translocation of the enzyme from the cytosol to the cellular membranes, where AA is selectively released from PLs. While the concentration of free AA is maintained at submicromolar levels by a reacylation pathway, stimulation of PLA_2 activity produces a rapid increase in free AA, which can then be available for eicosanoid biosynthesis.

The $cPLA_2$s are upregulated by several mechanisms. One is a calcium-dependent translocation to the membrane fractions of cells, as just mentioned. Alternatively, the enzymes are activated and deactivated inphosphorylation/dephosphorylation cycles. In other cases, a receptor-associated G protein activates $cPLA_2$s. Finally, some of these enzymes are activated by transcriptional activation, resulting in increased levels of $cPLA_2$ protein. The key point is that $cPLA_2$s represent the first step in eicosanoid biosynthesis.

Figure 1 provides an overview of the major eicosanoid biosynthetic pathways. The cyclooxygenase (COX) pathways yield the PGs and thromboxanes and the LOX pathways convert AA into various hydroperoxyeicosatetranoic acids (HPETEs) and hydroxyeicosatetranoic acids (HETEs). These species are themselves biologically active; they are also potential substrates for further metabolism to leukotrienes and still other biologically active products. The epoxygenases are cytochromes P450 (see **Chapter 4.1**), which yield the various epoxyeicosatrienoic acids.

4.9.2.2.2. The cyclooxygenase pathways PGs are C_{20} carboxylic acids with a five-membered ring variously substituted at C9 and C11, and two aliphatic chains featuring a substitution at C15 and one, two, or three double bonds. Three PUFAs, $C_{20:3n-6}$, $C_{20:4n-6}$, and $C_{20:5n-3}$, are potential substrates for the COX pathways, although AA is the commonest substrate among mammals. The PGs always have two fewer double bonds than their parental PUFAs, giving rise to the 1-, 2-, and 3-series PGs (Figure 2). PGs are defined by the substitutions at C9 and C11. PGE, for example, features a keto function at C9 and a hydroxyl function at C11. Specific PGs are identified by combining the number and letter designations. PGE_1, to continue the example, features one double bond at C13. The parental fatty acid is $C_{20:3n-6}$.

Figure 3 indicates that PG biosynthesis requires three enzyme steps. First is the COX step, which catalyzes the bis-oxygenation of AA to form the endoperoxide PGG_2. Second, the endoperoxide

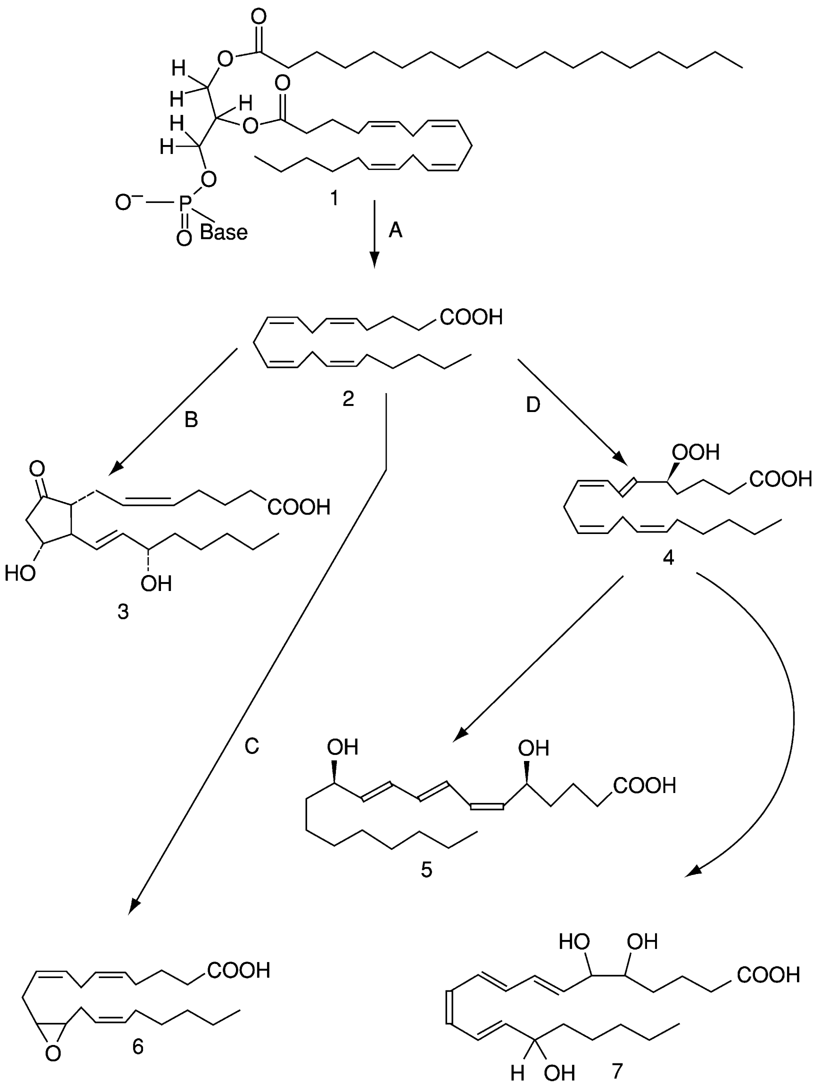


Figure 1 A sketch of arachidonic acid (AA) metabolism based on the biomedical background. Three fatty acids, $C_{20:3n-6}$, AA, and $C_{20:5n-3}$ are potential substrates for eicosanoid biosynthesis. Chemical structures are denoted by numerals: 1, cellular phospholipid; 2, hydrolyzed AA; 3, prostaglandin E_2; 4, 5-hydroperoxyeicosatetraenoic acid; 5, leukotriene B_4; 6, 11,12-epoxyeicosatrienoic acid; 7, lipoxin A. Capital letters indicate major enzyme systems responsible for eicosanoid biosynthesis: A, phospholipase A_2; B, cyclooxygenase (COX); C, cytochrome P450 epoxygenase; D, lipoxygenase (LOX). (Reproduced with permission from Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ; © Princeton University Press.)

undergoes a two-electron reduction at C15, catalyzed by a peroxidase activity. The peroxidase activity yields PGH_2, from which other biologically active PGs are produced. The COX and peroxidase activities are juxtaposed in a single protein, known for many years as PG endoperoxide synthase, or PGH synthase. It is now termed COX.

A couple of interesting features of COX help us understand PG biosynthesis. For one, the COX undergoes "suicide" inactivation, thought to be an intrinsic property of the enzyme. This was first recognized because COX is inactivated before all available substrate is converted into product, in mammalian cells after about 1300–1400 catalytic operations (Smith and Marnett, 1991; Smith *et al.*, 1991). The suicide step is a feature of the COX component of the protein because the peroxidase activity remains after the COX has faded. Suicide inactivation may set an upper limit on cellular capacity for PG biosynthesis.

A second interesting feature of mammalian COXs is the intracellular localization of the protein. COX is a glycoprotein associated with membrane fractions, mainly endoplasmic reticulum and to some extent the nuclear membrane. The enzyme was originally thought to feature transmembrane domains,

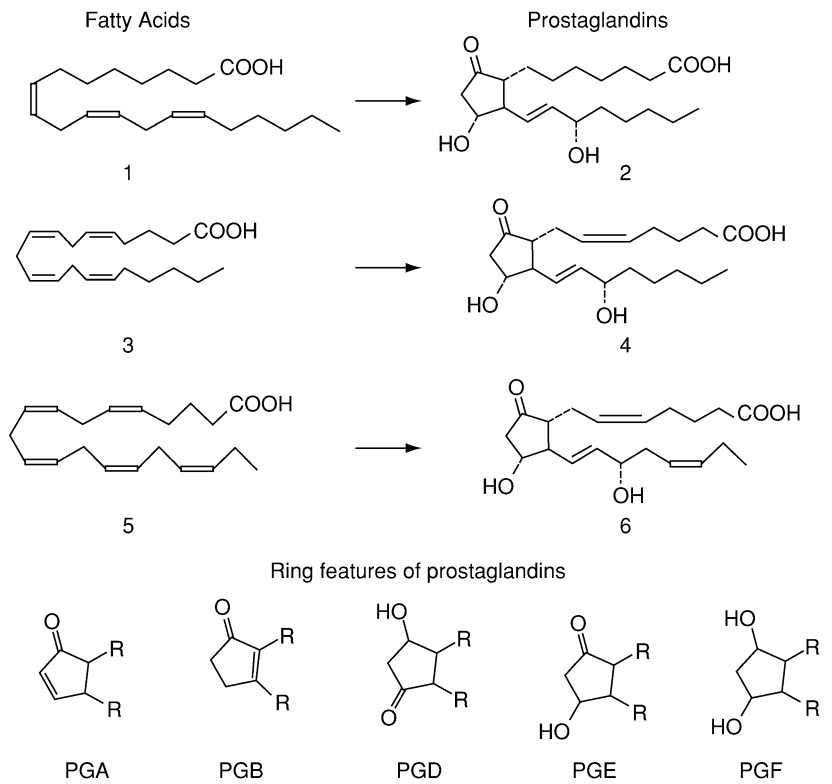


Figure 2 Cyclooxygenase (COX) converts certain C_{20} PUFAs into their respective 1-, 2-, and 3-series prostaglandin (PG) products. Each PG series features two fewer double bonds than its corresponding precursor PUFA. 1, $C_{20:3n-6}$; 2, PGE_1; 3, $C_{20:4n-6}$; 4, PGE_2; 5, $C_{20:5n-3}$; 6, PGE_3. The lower panel presents the ring features of five prostaglandins, where R represents the aliphatic chains that appear on the complete structures. (Reproduced with permission from Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ; © Princeton University Press.)

but current thinking places the enzyme entirely within the lumen of the endoplasmic reticulum and nuclear membranes (Otto and Smith, 1995).

This model held sway until early 1991 when it became clear that some cells express another form of COX. The two forms are now called COX-1 and COX-2. All the preceding remarks are based on our understanding of COX-1, which is thought to serve as a housekeeping isozyme. That is, it is responsible for biosynthesizing PGs active in physiological homeostasis. This enzyme is constitutively expressed in most mammalian tissues, although not in all cells within a tissue. COX-1 is thought to release PGH_2 into the cytosol where it is converted by other enzymes into biologically active PGs. The active PGs may act within the cell or may exit the cells, probably assisted by a PG transporter (Kanai *et al.*, 1995).

COX-2 mediates the biosynthesis of PGs for inflammatory processes, ovulation, and mitogenesis. In contrast to COX-1, COX-2 is not expressed in most mammalian cells (Smith *et al.*, 1996). However, it can be induced rapidly in many cells, including fibroblasts, endothelial cells, monocytes, and ovarian follicles. COX-2 is an inducible, rather than constitutive enzyme, and its expression is increased tremendously, from 10- to 80-fold by proinflammatory or mitogenetic factors, such as cytokines and tumor-promoting phorbol esters. While also associated with the membrane fractions of cells, COX-2 is mainly associated with the luminal surface of nuclear membranes. It may release PGH_2 into the nucleus, and the PGH_2 or another PG derived from it may interact with nuclear proteins to influence gene expression. Hence, COX-1 and COX-2 may represent two unrelated pools of active enzyme within the same cell, each with separate biological functions (Otto and Smith, 1995). While previously noted, solid information on the forms of the enzymes responsible for PG biosynthesis in invertebrates is not yet available.

Recently, the possibility of additional COX forms has arisen, surfaced by efforts to understand the pharmacology of acetaminophen (Tylenol in the USA and Paracetamol in the UK). While acetaminophen is an effective analgesic and antipyretic, it is a poor antiinflammatory drug and weak inhibitor of COX-1 and COX-2. The possibility of a COX-3 was first raised by Simmons and his colleagues

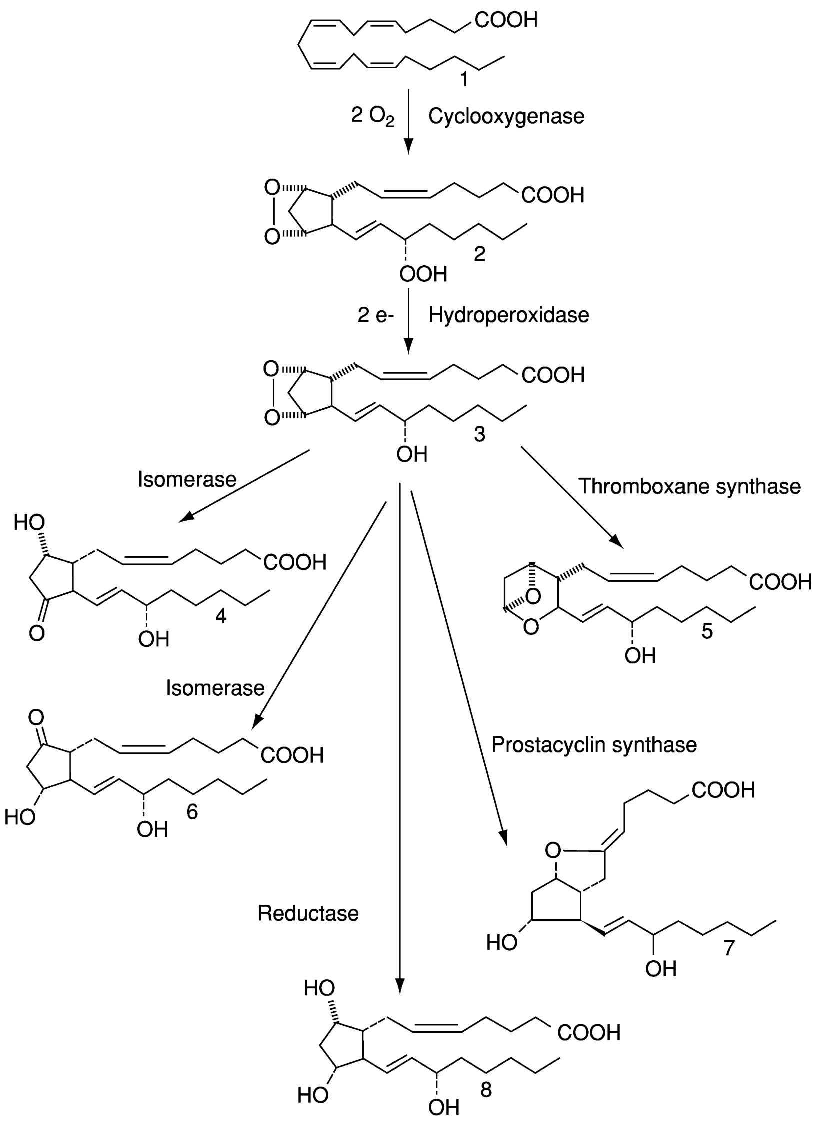


Figure 3 Prostaglandin (PG) biosynthesis requires three enzymatic steps. First, AA (structure 1) is oxygenated to the unstable endoperoxide, PGG_2 (structure 2). Second, a hydroperoxidase step reduces PGG_2 to the more stable PGH_2 (structure 3). Both of these enzymatic steps are catalyzed within a single protein, COX. PGH_2 is converted into the classical prostaglandins, including PGE_2 (4), thromboxane B_2 (5), PGD_2 (6), PGI_2 (prostacyclin, structure 7), and $PGF_{2\alpha}$ (8) by actions of cell-specific enzymes, as indicated. (Reproduced with permission from Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ; © Princeton University Press.)

(Simmons *et al.*, 1999), who found that treating a murine macrophage line (J774.2) with any of a variety of nonsteroidal antiinflammatory drugs induced expression of COX-2. The key finding was that, within the same cell line, the COX-2 induced by antiinflammatory drugs was more sensitive to inhibition with acetaminophen than COX-2 induced by LPSs. The authors postulated two enzyme activities with different properties. In another paper the authors suggested a COX-3, which is expressed as a variant of the COX-1 gene (Chandrasekharan *et al.*, 2002). The story is further complicated by the suggestion that COX-3 is a variant of COX-2 (Botting, 2002). However, in all cases, the new COX expressed increased sensitivity to acetaminophen. This work opens the possibility of multiple isoforms of COX, all derived from just two separate genes (Warner and Mitchell, 2002) and also for the eventual discovery of rather specific therapeutics which act on specific tissues. Again, we have no information on COX isoforms in insects, although studies in this area may contribute important new knowledge on the enzymes responsible for PG biosynthesis.

Figure 3 also shows the third enzymatic step in the biosynthesis of PGs. PGH_2 is a substrate for several

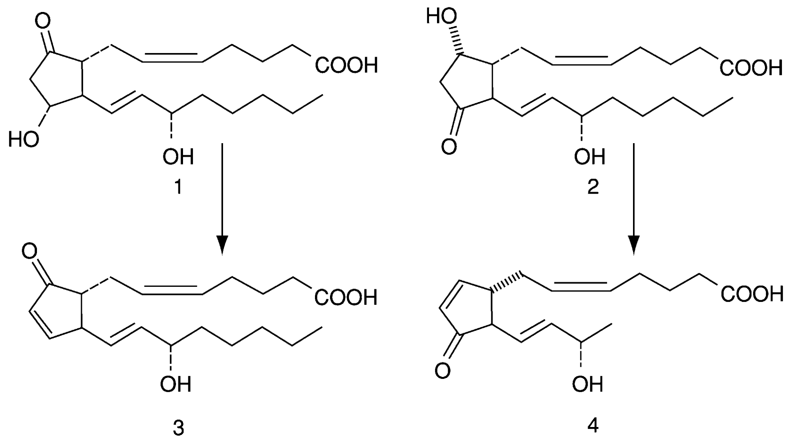


Figure 4 Two prostaglandins (PGs) are formed by nonenzymatic rearrangements of other PGs, rather than by enzymatic treatment of PGH_2. PGA_2 (3) is formed from PGE_2 (1). Δ^{12}-PGJ_2 (4) is formed by a nonenzymatic dehydration of PGD_2 (2). (Reproduced with permission from Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ; © Princeton University Press.)

enzymes responsible for converting PGH_2 into the active PGs. The PGs D, E, and F are formed by three groups of enzymes, respectively, the PGD synthases, the PGE synthases, and the PGF synthases, (Urade *et al.*, 1995). Two other PGs are formed directly from PGH_2, thromboxane A_2 and prostacyclin, also called PGI_2 (**Figure 3**). Both of these compounds act in platelet aggregation, among other things. Thromboxase strongly induces platelet aggregation while PGI_2 exerts the opposite action. PGI_2 synthase is responsible for converting PGH_2 into PGI_2, and this enzyme is also a member of the cytochrome P450 superfamily (Tanabe and Ullrich, 1995). The structures of thromboxane A_2 and PGI_2 are a little different from the other PGs, and for this reason we see the term “prostanoid” used to describe all COX products.

Two other PGs, Δ^{12}-PGJ_2 and PGA_2 (**Figure 4**), are not formed directly from PGH_2 (Negishi *et al.*, 1995a). PGA_2 is produced through a nonenzymatic rearrangement of PGE_2. PGJ_2 is a nonenzymatic dehydration product of PGD_2, which is converted to the Δ^{12}-PGJ_2 in the presence of serum albumin. Most PGs, including PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane A_2, express their actions through specific receptors located on cell surfaces. PGA_2 and Δ^{12}-PGJ_2 operate through a different mechanism. These PGs are thought to be actively moved into cells via a transporter protein. An intracellular carrier molecule facilitates transport into the nucleus, where the PGs bind with thiol groups of nuclear proteins. The PG–protein complex then interacts with DNA, resulting in the expression of genes. Among their other biological actions, the A and J series PGs induce expression of genes for heatshock proteins in many normal and tumor cells.

4.9.2.2.3. The lipoxygenase (LOX) pathways Polymorphonuclear leukocytes are central to many mammalian inflammatory events. Samuelsson and his colleagues used these cells to investigate the possibility that AA could be metabolized into products other than PGs. They discovered that the main AA oxygenation pathways in leukocytes yielded 5-HETE and a series of products later called leukotrienes (Oates, 1982; Samuelsson, 1983).

The mammalian AA LOXs produce a series of six products (**Figure 5**). These are 5-, 8-, 9-, 11-, 12-, and 15-HPETEs. Each of the LOXs is named according to the carbon that is oxygenated. Thus, 5-LOX yields 5-HPETE, and so forth (Pace-Asciak and Asotra, 1989). The LOX products are quickly taken into various metabolic systems, which yield other biologically active products. In one of the most common fates, the hydroperoxide products are quickly reduced to the corresponding HETE by various glutathione peroxidases, which are abundant in most mammalian cells. The HETEs are biologically active (Spector *et al.*, 1988). For example, two products, 5- and 12-HETE, induce degranulation of human neutrophils. Similarly, 12-HETE is a potent chemoattractant for polymorphonuclear leukocytes. Several HETEs are active in various pathophysiological events, including proinflammatory processes.

One of the LOX products, 5-HPETE, serves as substrate for biosynthesis of the LTs (**Figure 6**). The root LT is LTA_4, an unstable epoxide of 5-HPETE. The enzyme responsible for this is called LTA_4 synthase. The 5-LOXs from human leukocytes and mouse mast cells also express LTA_4 synthase activity, and many workers believe both enzymatic steps are carried out by the same bifunctional enzyme.

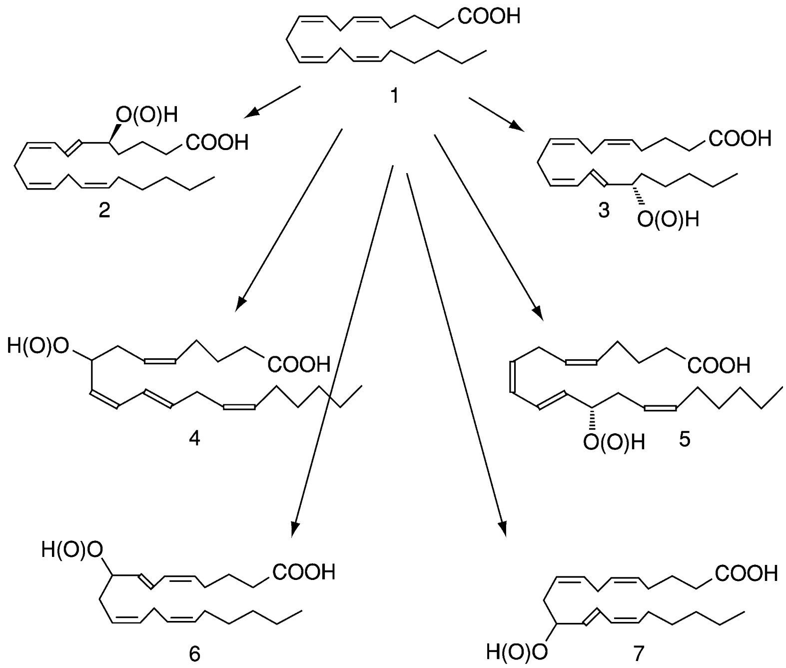


Figure 5 Lipoxygenase (LOX) is responsible for converting AA (1) into various hydroperoxyeicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs). Each of these compounds are formed by specific LOXs, identified by the positional specificity of the introduced oxygen. For example, 5-LOX yields 5-HPETE (2). The hydroperoxy acids may be reduced to their corresponding hydroxy acids by glutathione peroxidases. The remaining structures are identified by the position of the introduced oxygen: (3), 15-HPETE and 15-HETE; (4), 8-HPETE and 8-HETE; (5), 12-HPETE and 12-HETE; (6), 9-HPETE and 9-hydroxyeicosatetraenoic acid; (7), 11-HPETE and 11-HETE. (Reproduced with permission from Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ; © Princeton University Press.)

LTA_4 is an unstable substance whose main function is to serve as a substrate for the biosynthesis of other LTs. There are two main pathways (Pace-Asciak and Asotra, 1989). The first is catalyzed by LTA_4 hydrolase, which yields LTB_4 (**Figure 6**). This LT is a proinflammatory mediator of host defense reactions in mammals. It activates polymorphonuclear leukocytes, myeloid cells, and mast cells. LTB_4 also induces neutrophils to adhere to endothelial cell walls (Metters, 1995).

Alternatively, LTA_4 can be modified into the cysteinyl LTs or peptidoLTs (**Figure 6**). LTC_4 synthetase catalyzes addition of glutathione, in covalent linkage, to C6, yielding LTC_4. This LT can be converted to LTD_4 by a single transpeptidase step which catalyzes hydrolysis of the terminal amino acid residue from LTC_4. LTD_4 undergoes another transformation to LTE_4 by hydrolyzing the glycine residue from LTD_4. This step is catalyzed by a dipeptidase. LTF_4 can be formed by adding an amino acid residue.

The cysteinyl LTs make up the slow-reacting substance of anaphylaxis (Samuelsson, 1983). The major biological action of these compounds is contraction of smooth muscles associated with respiratory and vascular systems, and with the alimentary canals of mammals. These actions are mediated through specific receptors, of which two types are known (Metters, 1995). These are designated cys-LT_1 and cys-LT_2 receptors. Unlike the PG receptors, which exhibit marked specificity for each PG, the receptors for the cysteinyl LTs are much less fastidious. Both types of receptors have equal affinity for LTC_4 and LTD_4 and less affinity for LTE_4. We have no information on receptors for LTF_4. The LTs have not yet been considered in insect physiology.

Beside the HPETEs, HETEs, and LTs, the LOX pathways can yield another suite of compounds, none of which is known from invertebrates. Similarly, products of the epoxygenase pathway mentioned in **Figure 1** have not been discovered for invertebrates. These are considered in detail elsewhere (Stanley, 2000).

Gerwick (1993) noted that the term eicosanoid is limited to oxygenated metabolites of a limited group of fatty acids, specifically C_{20} PUFAs. He suggested that a new term, "oxylipin," was required to serve as a broader term for all oxygenated compounds formed from fatty acids of any chain length by reactions involving at least one step of a monooxygenase- or dioxygenase-dependent oxygenation. This broad word informs our appreciation of many fatty acid-derived products in various animal systems. Another

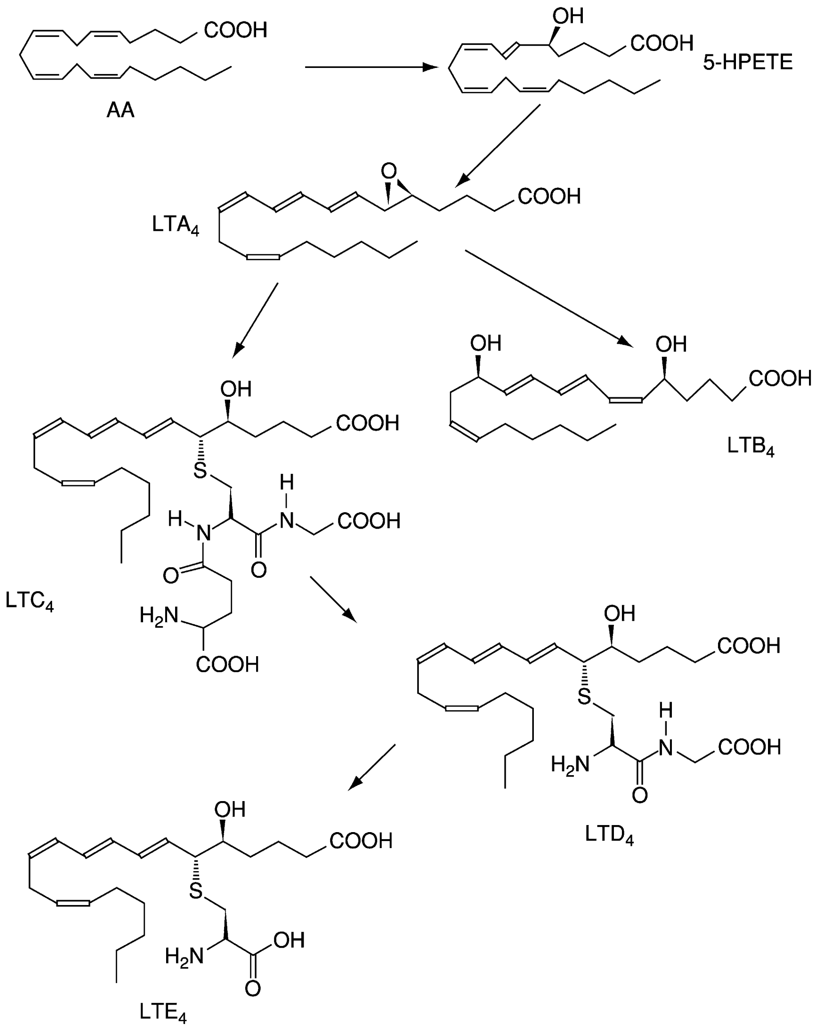


Figure 6 The biosynthesis and structures of leukotrienes. The pathways begin with conversion of AA to 5-HPETE by a 5-LOX. Leukotriene A_4 synthase yields the unstable epoxide leukotriene A_4 (LTA_4). LTA_4 may serve as substrate for leukotriene biosynthesis. In a one-step pathway, leukotriene hydrolyase yields leukotriene B_4 (LTB_4). The alternative pathway yields peptidoleukotrienes: leukotrienes C_4 (LTC_4), D_4 (LTD_4), and E_4 (LDE_4). (Reproduced with permission from Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ; © Princeton University Press.)

term, phytooxylipins, similarly describes a very wide array of oxygenated fatty acids that serve important roles in plant defense reactions (Blee, 1998). We may infer that the various forms of oxygenated fatty acids are crucial mediators in the life histories of most organisms. One area of most fundamental importance is reproduction, discussed in the next section.

4.9.3. Reproduction

Appreciation of the biological significance of eicosanoids began with the discovery that a substance in unfractionated human semen causes contractions of uterine smooth muscle (Kurzrok and Lieb, 1930). Although we now know eicosanoids occur and exert biological actions in virtually every mammalian tissue and body fluid, their influence on uterine muscle contraction, a function vital in reproductive biology, marks the discovery of the first known eicosanoid action.

Release of egg-laying behavior in newly mated house crickets, *Acheto domesticus*, another action in reproductive biology, also marks discovery of the first known biological action of PGs in insects and other invertebrates (Destephano and Brady, 1977). Although only coincidental, it is quite intriguing because at a superficial glance, it appears that PGs mediate events in reproduction of mammals and a very distantly related invertebrate species. The occurrence and actions of PGs in insect reproductive systems have been reviewed (Stanley-Samuelson and Loher, 1986; Stanley-Samuelson, 1994a; Miller and Stanley, 1998a; Stanley 2000). These reviews

remain useful because this is an area in which new information has been slow coming. This section presents an abbreviated synopsis of known information.

The most detailed studies on the role of PGs in releasing egg-laying behavior come from the work of Werner Loher on the Australian field cricket, *Teleogryllus commodus*. Loher and his colleagues (Loher *et al*., 1981; Stanley-Samuelson *et al*., 1987) developed an "enzyme-transfer" model to account for transfer of a still-unidentified form of COX (along with its substrate, AA) from males to females via spermatophores. This model seems to hold for some, but certainly not all, cricket species. PGs may release egg-laying behavior in newly mated silkmoths, *Bombyx mori*, although some of the data on this species are not entirely convincing (Yamaja Setty and Ramaiah, 1979, 1980).

PGs also may release egg-laying behavior in a few other insect species. Buprofezin is an insect growth regulator, which functions by inhibiting chitin biosynthesis and cuticle deposition in larvae of some insect species. Izawa *et al*. (1986) found that this compound inhibited egg-laying behavior in adults of the 28-spotted ladybird, *Henosepilachna vigintioctopunctata*. Similarly, Uchida *et al*. (1987) discovered that buprofezin inhibited egg-laying behavior and PG biosynthesis in adults of the brown rice planthopper, *Nilaparvata lugens*. The PG biosynthesis inhibiting action of the growth regulator suggested that PGs may release egg-laying behavior in these species.

The occurrence among insect species of PG-mediated egg-laying behaviors cannot be estimated in a reliable way. The model seems to apply to a few orthopterans, a lepidopteran, a coleopteran, and a hemipteran. Several points suggest that this is not a general model for insects. First, there are several insect species in which PG titers increase in female reproductive tracts after mating, but they do not release egg-laying behavior. Second, the role of PGs in releasing egg-laying behavior needs to be regarded in the context of individual mating systems (Thornhill and Alcock, 1983). In many species, eggs are formed, fertilized, and oviposited in separate time frames, and mating, *per se*, does not result in immediate egg-laying activities. Moreover, among those species in which mating does release egg-laying behavior, there are several mechanisms of releasing egg-laying behavior not involving PGs or other eicosanoids. Mechanical stimulation of egg-laying behavior is one example.

PGs or PG biosynthetic activity are transferred from males to females of several insect species in which PGs do not influence egg-laying behavior (Stanley, 2000). Our question is: what is the meaning of this information? In general terms, one could speculate that the PGs act in modulating and coordinating various details in reproductive physiology. The recent work by Medeiros and colleagues provides a specific example (Medeiros *et al*., 2002). As seen in other insect species, the ovaries of *Rhodnius prolixus* incorporate vitellogenin as well as several other specific proteins (see **Chapter 3.9**). Medeiros *et al*. (2002) tested the idea that eicosanoids influence the ovarian incorporation of *Rhodnius* heme-binding protein (RHBP). Ovaries of vitellogenic females were incubated in the presence of iodinated RHBP and either PGE_2 or indomethacin, a COX inhibitor. Their results indicated that incubations in the presence of PGE_2 downregulated RHBP uptake by up to 35%, while incubations in the presence of indomethacin upregulated RHBP uptake by up to 50%, both in comparison to control incubations. The authors also showed that the *Rhodnius* ovaries secreted PGE_2 into the culture medium and that the amount of secreted PGE_2 was reduced significantly in incubations conducted in the presence of indomethacin. Although the indomethacin did not influence the surface area nor the patency of follicle cells, indomethacin treatments did influence dephosphorylation of two ovarian proteins, an 18 kDa and a 25 kDa protein. Medeiros *et al*. (2002) concluded that eicosanoids influence the reproductive physiology of *Rhodnius* ovaries. Phosphorylation signal transduction pathways may be involved in PG modulation of RHBP uptake. The significance of this paper lies in its direct illumination of a previously unrecognized role of PGs in insect reproduction. Perhaps more important, however, the paper illustrates the point that many more-or-less subtle physiological steps in the overall process of producing progeny may be influenced by PGs and other eicosanoids.

Aside from the roles of PGs in releasing egg-laying behavior in a few insect species and in modulating RHBP endocytosis in *Rhodnius* ovaries, eicosanoids act in various other aspects in reproduction of many invertebrates and lower invertebrates. This work on animals outside of Insecta has been treated elsewhere (Stanley-Samuelson, 1994a; Miller and Stanley, 1998; Stanley, 2000).

It is worth noting that eicosanoids influence reproduction by acting at several levels of biological organization, as mentioned earlier for mammals. Actions such as modulating insect ovarian endocytosis of a protein (Medeiros *et al*., 2002) and many other eicosanoid actions in invertebrate reproduction (Stanley, 2000) are expressed at the cellular level, i.e., the eicosanoids influence cellular events. Other actions, including release of the egg-laying behavioral program are registered at the organismal level, even though they are probably driven by PG

actions at the level of a few select neurons (Loher *et al*., 1981). Some eicosanoid actions take place at the population level. Sorensen *et al*. (1988), for example, reported that PGs of the F series act as the postovulatory pheromone in goldfish. There remains much to be discovered in the roles of eicosanoids in insect reproductive biology.

4.9.4. Eicosanoids in Ion Transport Physiology

It is generally thought that cellular life evolved in aqueous environments. Cells live in more or less aqueous environments, and virtually all cells have evolved physiological mechanisms to maintain homeostasis of ion and water balance. Terrestrial insects tend to lose water to their environments, and they have mechanisms to restrict and offset water losses (Kirschner, 1991). Freshwater animals tend to maintain their extracellular fluid compartments hyperosmotic to their environments. Still other animals live in water subject to rapid changes in osmotic concentration, such as estuaries and rocky pools in intertidal zones. The osmotic homeostasis of all animals is subject to frequent challenges at the cellular and organismal levels.

Many homeostatic mechanisms are expressed at the organismal level. The respiratory surfaces of all terrestrial animals, including insects, are internalized. The internalization of these surfaces is regarded as a major adaptation to terrestrial life. Terrestrial insects have a small layer of hydrocarbons and other lipids on their integument which helps reduce water loss. Some insects drink water to offset losses while other species can absorb water from humid atmospheres. Finally, some insects are able to sustain extreme dehydration. Larvae of the midge *Polypedilum vanderplanki*, which live in ephemeral pools on rocks in Africa, provide the best example of this. These pools evaporate in the dry season, and the larvae can tolerate nearly complete dehydration until the following rainy season (Hinton, 1960). Cellular mechanisms for maintaining water balance are integrated into organismal-level reactions to osmotic challenge. Vertebrate kidneys and the Malpighian tubules of insects and certain other invertebrates, for example, are influenced by diuretic and antidiuretic hormones which act to eliminate or conserve body water or solutes.

To appreciate eicosanoid actions in ion transport, a brief overview of some mechanisms of solute transport is given (Alberts *et al*., 1994). The lipid bilayers of cellular membranes are fairly permeable to water and relatively impermeable to biologically important ions, such as chloride, sodium, potassium, magnesium, and calcium. These ions are transported across membranes by members of two classes of membrane-associated proteins, channels and carrier proteins. Channels are transmembrane proteins that form hydrophilic pores through membranes. The pores are generally selective, allowing specific ions, such as potassium or calcium, to pass through. Carrier proteins also bind specific solutes, which are transported due to conformational changes in the carriers. All channel-mediated and some carrier-mediated transport actions are driven solely by local electrochemical gradients known as passive transport or facilitated diffusion. Some carrier proteins are associated with an energy source, usually an ATPase. These carriers sometimes transport solutes against steep electrochemical gradients. Other carrier proteins act as coupled transporters in which a transport protein is responsible for movement of one solute with simultaneous movement of another. Symports move the two solutes in the same direction; antiports move the two solutes in opposite directions. For an example, the ubiquitous sodium–potassium antiport is responsible for actively pumping of sodium ions out of cells and potassium ions into cells. Passive and active transport mechanisms also are responsible for moving water across membranes. This brief glimpse allows us to regard the homeostasis of water and solute concentrations at the organismal and cellular levels as the outcome of regulated actions of specific intracellular, and often intramembrane, proteins. Eicosanoids are among the molecules which modulate homeostasis of water and solute concentrations.

As usual, most of our knowledge of the roles of eicosanoids in the physiology of water and solute homeostasis comes from the studies on vertebrate systems, particularly various kidney and toad preparations. Nephrons are the operative cells in kidney water and solute transport, and eicosanoid biosynthesis and actions vary along the nephrons, as detailed by Bonvalet *et al*. (1987).

Dalton (1977a, 1977b) provided the first recognition that eicosanoids act in the physiology of fluid secretion or ion transport in invertebrates. Fluid secretion by salivary glands isolated from the blow fly *Calliphora erythrocephala* can be stimulated by treating the glands with serotonin, a biogenic amine. In this system, the serotonin acts as an external hormone, and it is the natural ligand for salivary gland cell surface receptors. Serotonin-receptor interactions stimulate increased concentrations of intracellular cAMP in the salivary gland preparations (Berridge, 1970; Prince *et al*., 1972) and these increased intracellular cAMP concentrations lead to increased secretion of an isosmotic potassium-rich

fluid. Dalton (1977a) investigated the influence of PGE_1 on salivary gland physiology, showing that treating isolated salivary glands with low doses (10^{-7}–10^{-9} M) of PGE_1 did not alter their basal fluid secretion rates. Alternatively, these low doses of PGE_1 attenuated the usual stimulatory influence of serotonin on fluid secretion rates. Dalton (1977b) suggested that PGE_1 reduces intracellular cAMP concentrations by downregulating adenylate cyclase, with no influence on phosphodiesterase. It appeared that at least one eicosanoid, PGE_1, plays an important physiological role in fluid secretion physiology in an invertebrate. In another early line of work with the locust rectum, Phillips and his colleagues suggested that eicosanoids may stimulate increased intracellular cAMP concentrations, which in turn lead to increased chloride transport (Phillips, 1980).

Petzel and Stanley-Samuelson (1992) suggested that PGs modulate basal fluid secretion rates in Malpighian tubules of female yellow fever mosquitoes, *Aedes aegypti*. This hypothesis was tested in a series of fluid secretion assays based on the Ramsey protocol (Petzel, 1993). In each experiment Malpighian tubules were incubated in the presence of physiological buffer. After an equilibration period, half of the buffer was exchanged with the same volume of buffer containing an inhibitor of eicosanoid biosynthesis. First, the influence of eicosatetraynoic acid (ETYA), an AA analog with triple bonds in place of the usual double bonds, was assessed. ETYA inhibits many enzymes which process AA, including PLA_2, LOX, and COX. In the presence of ETYA, basal fluid secretion decreased from about $1.0\,nl\,min^{-1}$ in the pretreatment period to $0.55\,nl\,min^{-1}$ in the experimental period. These findings supported the idea that PGs are involved in modulating basal fluid secretion rates in mosquito Malpighian tubules.

Results of similar experiments with selective LOX and expoxygenase inhibitors indicated that products of these two pathways do not influence basal fluid secretion rates in Malpighian tubule preparations. Experiments with the COX inhibitor indomethacin, however, showed that PGs modulate basal fluid secretion rates. In the presence of $100\,\mu M$ indomethacin, fluid secretion decreased from 0.9 to $0.5\,nl\,min^{-1}$ in a dose-dependent manner. It was inferred from this work that PGs, but not LOX or epoxygenase products, are involved in maintaining basal fluid secretion rates in mosquito Malpighian tubules (Petzel and Stanley-Samuelson, 1992).

The idea that PGs act in mosquito Malpighian tubules raised the issue of the occurrence and metabolism of AA in these tissues. Petzel *et al.* (1993) determined the presence of AA and PGE_2 in mosquito Malpighian tubules. They used immunohistochemistry to detect PGs (Howard *et al.*, 1992; Petzel *et al.*, 1993). The repeated dark-brown staining pattern showed the presence of PGE_2 in principal, but not stellate, cells of the tubule. The principal cells are responsible for secreting fluid into the lumen of the tubule. A similar staining pattern was observed for PGE_2 in Malpighian tubules of the yellow mealworm, *Tenebrio molitor* (Howard *et al.*, 1992). $PGF_{2\alpha}$ is also present in Malpighian tubules from the mosquito and the mealworm, shown by similar histological procedures. The $PGF_{2\alpha}$ staining patterns are quite different from the patterns obtained for PGE_2. For $PGF_{2\alpha}$, the staining is not restricted to the principal cells, but seems to be more or less evenly distributed among both major Malpighian tubule cell types.

Hypothesizing that PGs act through G protein-coupled receptors, Petzel also conducted a preliminary investigation of the possibility that PGs stimulate increased cAMP biosynthesis in mosquito Malpighian tubules (D.H. Petzel, personal communication). The tubules were isolated from adult female mosquitoes, then incubated in mosquito saline. PGE_2 was added to the saline and after selected incubation periods the tubules were frozen in liquid nitrogen and cAMP concentrations in the tubules were determined. This work showed that PGE_2 stimulated a four- to fivefold increase in intracellular cAMP concentrations. Petzel also recorded the influence of PGE_2 on fluid secretion rates. In these experiments, tubules were equilibrated as usual in mosquito saline, then PGE_2 was added to the saline. PGE_2 stimulated increased fluid secretion in mosquito Malpighian tubules, suggesting that PGs are among the regulatory elements in mosquito Malpighian tubule physiology. Van Kerkhove *et al.* (1995) conducted a similar line of work on Malpighian tubules from workers of the ant *Formica polyctena*. These findings are similar to the data with mosquito Malpighian tubules and support the notion that PGs act in insect Malpighian tubule physiology.

Phillips (1980) implicated PGs in the physiology of the locust rectum. Radallah *et al.* (1995) continued this work using an everted sac preparation of the locust rectum to assess the influence of AA and PGE_2 on water resorption. The everted sac is formed by tying one end of the rectum onto a catheter tube. Test compounds could then be injected into the sac, thereby exposing the hemolymph side of the preparation to the compounds. PGE_2 stimulated increased fluid resorption in a dose-dependent manner. Maximal stimulation, 59% greater than controls, was obtained at about 10^{-9} M PGE_2. They could also stimulate increased fluid resorption by adding AA, which resulted in a 79% increase in resorption at

10^{-6} M AA. Similar experiments with aspirin and indomethacin also stimulated dose-dependent increases in fluid resorption. These data suggest that PGs and inhibitors of PG biosynthesis exert similar influences on the rectal preparations. Several possibilities help resolve this apparent paradox. For one, indomethacin restricts biosynthesis of all COX products, some of which exert inhibitory influences on cellular processes. The possible inhibitory PG may exert greater influence on overall fluid transport dynamics than the stimulatory PG. If so, inhibiting the biosynthesis of a possible inhibitory PG may create an apparent stimulation of fluid transport. The idea of considering possible inhibitory and stimulatory PG actions in the same system has not been explored.

Radallah *et al.* (1995) determined the influence of AA and PGE_2 on selected intracellular signal transduction messengers. To record the influence of AA, PGE_2, and indomethacin on the influx of calcium into rectal cells they used a microfluorimetric technique. PGE_2 and AA stimulated large increases in calcium influx, which decreased to baseline within minutes. As in the fluid transport experiments, indomethacin similarly provoked increased calcium influx. Pretreating the tissues with nifedipine, which blocks L-type calcium channels, completely blocked the effects of AA and PGE_2 on calcium transport. This paper also reported that treating the locust rectum with AA or PGE_2 stimulated substantial increases in phospholipase C (PLC) activity, which indirectly influences calcium transport (Berridge, 1993). Once again, aspirin and indomethacin similarly induced increased PLC activity. This apparently depends on the influx of calcium because an L-type calcium channel blocker strongly attenuated the influences of AA and PGE_2 on PLC activity. They also showed that the PLC activity is associated with the release of inositol phosphates.

Radallah *et al.* (1995) present a convincing postulate that AA and at least one eicosanoid, PGE_2, are involved in modulating fluid transport in the locust rectum. The locust rectum is probably under primary regulation of the antidiuretic hormone, neuroparsin, which stimulates fluid resorption in the rectum. The hormone apparently acts through a G protein-coupled receptor, and it stimulates increased PLC activity. But it seems that the hormone also stimulates the release of AA from cellular PLs, which leads to increased PGE_2. The PGE_2 probably acts by an autocoidal mechanism to coordinate the cellular reactions to the central hormone. If so, PGE_2 acts similarly in the insect rectum as in segments of the mammalian kidney (Bonvalet *et al.*, 1987).

PGs also modulate fluid secretion rates in salivary glands of the lone star tick, *Amblyomma americanum* (Qian *et al.*, 1997). Tick salivary glands are osmoregulatory organs during host–parasite interactions. The salivary glands concentrate the nutrients associated with vertebrate blood and in the process they form a copious salt-rich saliva which is injected back into their hosts during feeding. The salivary glands are under nervous control and the neurotransmitter dopamine stimulates fluid secretion in isolated salivary glands (Sauer *et al.*, 1995). Qian *et al.* (1997) investigated the influence of PGs on dopamine-stimulated fluid secretion. Compared to controls, treatments with the PLA_2 inhibitor oleyloxyethyl phosphorylcholine (OOPC) and the COX inhibitor aspirin resulted in about 30–40% reductions in dopamine-stimulated fluid secretion rates. Longer incubations produced greater reductions and the influence of both inhibitors was expressed in a dose-dependent manner. The influence of OOPC was reversed by incubating the inhibitor-treated glands with 100 μM PGE_2 or its stable analog 17-phenyl trinor PGE_2. The PG treatments did not reverse the inhibitory influence of the COX inhibitors aspirin and diclofenac.

Dopamine stimulates fluid secretion through G protein-coupled receptors resulting in increased intracellular cAMP concentrations (Sauer *et al.*, 1995). Qian *et al.* (1997) determined the influence of PG biosynthesis inhibitors and of PGs on salivary gland cAMP concentrations. They found that the PLA_2 inhibitor OOPC and the COX inhibitor indomethacin inhibited dopamine-stimulated increases in intracellular cAMP concentrations by about 25%. In the presence of OOPC, PGE_2 and its analog stimulated 20–40% increases in the OOPC-treated cAMP concentrations.

The eicosanoid system influenced neither fluid secretion nor intracellular cAMP concentrations in the absence of dopamine stimulation. The authors' results provide strong support for their hypothesis that eicosanoids modulate dopamine-stimulated fluid secretion rates in tick salivary glands. Moreover, this work marks the first identification of physiologically functional PG receptors in an invertebrate system. Their data from binding studies support the view that the salivary gland receptor is functionally coupled to a stimulatory G protein. The authors concluded that the tick salivary gland expresses a functional PGE_2 receptor that does not directly regulate adenylate cyclase. They speculated that the PGE_2 receptor may influence calcium mobilization, which they confirmed in a subsequent paper (Qian *et al.*, 1998). The biological significance of the salivary gland PGE_2 receptor is linked

to secretion, or exocytosis, of anticoagulant proteins, which are thought to facilitate blood-feeding. Qian *et al*. (1998) demonstrated this by incubating dispersed salivary gland tissue in the presence of PGE_2, after which they recorded increased release of anticoagulant proteins.

Taken together, work on blowfly salivary glands, locust rectum, insect Malpighian tubules, and tick salivary glands provide quite convincing evidence for the biological actions of eicosanoids in water and solute transport in representatives of arthropods. Eicosanoids similarly act in other invertebrates, lower vertebrates, and mammals (Stanley, 2000). It is suggested that regulating the actions of specific proteins involved in ion transport is a fundamental biological action of PGs and other eicosanoids in animals.

4.9.5. Insect Immunity

4.9.5.1. Introduction

The ability of organisms to defend themselves from parasites and pathogens is a fundamental aspect of biology. Many microbes produce and secrete enzymes and toxins that provide a measure of protection from other microbes. Plants elaborate sophisticated defense reactions to wounding as well as invasions by bacteria and fungi. Metazoan animals thrive in constant contact with pathogenic and nonpathogenic microbes, fungi, and parasites. An animal's ability to defend itself from microbial invasions is known as immunity.

Immunologists recognize separable categories of immune reactions, innate immunity and acquired (or adaptive) immunity. Innate immunity is made up of several protective mechanisms that function in a nonspecific way. In mammals and other higher vertebrates, clonal biosynthesis of antibodies to specific invading antigens is a main line of acquired, or adaptive, immunity. Invertebrates lack lymphocytes and immunoglobulins, and therefore do not produce specific antibody reactions to infections. On this basis invertebrates are often said, incorrectly, to lack immune systems. Rowley (1996) registered this incongruity, noting that some of the very important early experiments in immunology were conducted on invertebrates.

Invertebrates and vertebrates express several forms of passive immunity, so named because they do not entail directed protective actions. These might include killing ingested organisms due to relatively harsh pH conditions (either strongly acid as in the mammalian stomach or very alkaline as in many insect midguts) in the alimentary canal. Foodborne microbes may be killed by hydrolytic actions of digestive enzymes. The integument serves as a very effective barrier to invading microbes.

Animals also express innate immunity to infections. Armstrong *et al*. (1996) recognized several expressions of innate immunity, some of which are seen in vertebrates and in invertebrates. Inducible antibacterial peptides of insects, defensins in insects and mammals, and the α_2-macroglobulins, known in mammals and some arthropods, are elements of innate immunity.

The innate immunity of insects is often divided into two major categories, cellular and humoral immunity. Cellular immunity involves direct interactions between circulating hemocytes and invading organisms (reviews: Brehelin, 1986; Dunn, 1986; Gupta, 1986, 1991; Boman and Hultmark, 1987; Lackie, 1988; Strand and Pech, 1995; Gillespie *et al*., 1997; Levine and Strand, 2002). Cellular defense reactions include phagocytosis, a form of endocytosis; nodulation, entrapping bacterial cells into clusters of hemocytes; and encapsulation of organisms too large for phagocytosis, such as eggs of parasitoid insects. Following capture, the internalized microorganisms are destroyed by intracellular killing mechanisms. Formation of oxygen radicals and nitric oxide are killing mechanisms within invertebrate cells (Conte and Ottaviani, 1995). These immune reactions create a general inflammatory response to injury, infection, and parasitization.

Insect humoral immunity involves induced biosynthesis of antibacterial proteins (Tauszig *et al*., 2000; Hoffmann and Reichart, 2002). This very important area of inquiry has revealed a large number of antibacterial proteins and has generated new understanding of fundamental aspects of biology, including gene regulation. The chapter by Hultmark is the best entry point to literature on humoral immunity.

Insects respond to large bacterial infections by nodule formation, the predominant response to bacterial infections (Dunn and Drake, 1983; Horohov and Dunn, 1983). Nodulation begins with entrapment of bacterial cells by granule-containing hemocytes. The granule-containing cells undergo degranulation, which releases proinflammatory chemicals. The nodulation process is completed by attaching layers of flattened phagocytes to the mature nodule. The last stage is a melanization process, leaving darkened, easily visible nodules attached to the inner sides of the body wall or various organs. Invading bacterial cells are topologically removed from circulation by effectively forming an impermeable wall between the bacterial mass and the remainder of the organism.

Nodulation can be regarded as a form of cellular encapsulation, seen in response to infection by organisms larger than bacterial cells, such as parasites and other foreign materials. Wounds to the integument also evoke encapsulation reactions. As in nodulation, encapsulation begins with release of proinflammatory chemicals by degranulation of granule-containing cells. Again, the ending stages involve attaching layers of phagocytes, which flatten and melanize the capsule.

4.9.5.2. Eicosanoids in Insect Immunity

The inflammatory defense reactions just described have been studied quite extensively in many invertebrate species, particularly arthropods (Levine and Strand, 2002), although we have relatively little information on the biochemical molecules that mediate inflammation in invertebrates. Some mediators known from mammalian host defense reactions operate in invertebrates. Proinflammatory cytokines, or cytokine-like molecules, exist in invertebrates, including a bivalve mollusc, *Mytilus edulis*, a sea star, *Asterias forbesi,* and a urochordate, *Styele clava*, all reviewed by Rowley (1996). Downer and his colleagues showed that biogenic amines stimulate hemocytic inflammatory reactions in cockroaches (Baines *et al*., 1992; Baines and Downer, 1994) and waxmoths (Dunphy and Downer, 1994; Diehl-Jones *et al*., 1996). Similarly, it appears that eicosanoids, to which we now turn attention, also serve as crucial mediators of insect inflammatory reactions to bacterial infections.

4.9.5.2.1. Immune reactions to bacterial infection Eicosanoids exert many influences, in some cases stimulatory and in others inhibitory, on mammalian host defense systems. Based on this background, Stanley-Samuelson considered the possible actions of eicosanoids in insect immunity (Stanley-Samuelson *et al*., 1991). The results of these experiments, all using fifth instar *Manduca sexta* larvae, strongly supported the idea that eicosanoids mediate one or more cellular reactions to bacterial infections (Stanley-Samuelson *et al*., 1991).

This paper prompted the question: which of the hemocytic defense reactions to bacterial infections are mediated by eicosanoids? Stanley and his colleagues hypothesized that eicosanoids mediate insect nodulation responses to bacterial infections (Miller *et al*., 1994). Nodulation can be assessed by counting numbers of melanized nodules within insect hemocoels following infection (described in detail by Miller and Stanley, 1998b). It was inferred from the results of these experiments that eicosanoids mediate one or more of the early steps in the nodulation reaction to bacterial infection (Miller *et al*., 1994).

These two investigations with tobacco hornworms were the first experiments on eicosanoid actions in invertebrate immune systems. The assays for microaggregation and nodulation reactions are simple, and they facilitate investigation of a broader hypothesis. Specifically, do eicosanoids similarly act in nodulation reactions in other insect species? This question was tested in a series of similar exercises with other species. These include the tenebrionid beetle, *Zophobas atratus* (Miller *et al*., 1996), black cutworms, *Agrotis ipsilon*, and true armyworms, *Pseudaletia unipuncta* (Jurenka *et al*., 1997), the silkworm, *B. mori* (Stanley-Samuelson *et al*., 1997), and caterpillars of the butterfly, *Colias eurytheme* (Stanley *et al*., 1999). The results of all the experiments with these species supported the hypothesis. Similar findings emerged from work with adults of the cricket *Gryllus assimilis* (Miller *et al*., 1999), adult cockroaches *Periplaneta americana* (Tunaz and Stanley, 1999) and adult 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini* (Tunaz *et al*., 1999). Although these species do not establish an exhaustive representation of the Class Insecta, they make up a sufficient sampling of insects to suggest that PGs and other eicosanoids are key mediators of insect cellular immunity.

Beyond the work described here, several other laboratories have investigated the influence of eicosanoids in insect immunity. Mandato *et al*. (1997) carried out a detailed investigation of eicosanoid actions in three discrete cellular processes within the overall nodulation reaction of the waxmoth larvae, *Galleria mellonella*. They found that eicosanoids mediate phagocytosis, cell spreading, and prophenyloxidase (PPO) activation in waxmoth larvae. Their work on PPO activation is quite interesting, because other groups found that eicosanoids do not influence PPO activation. Morishima *et al*. (1997) suggested a completely new role for eicosanoids in insect immunity. As mentioned earlier, the immune reactions of insects and other invertebrates to bacterial infections include humoral and cellular responses. The humoral responses include induced synthesis of antibacterial proteins, including cecropins and lysozymes. Cecropins are not found in the hemolymph in unchallenged insects, and the gene for this protein is somehow activated upon bacterial infection. Lysozyme occurs at low, constitutive levels in hemolymph, and expression of the lysozyme gene is upregulated following stimulation with bacterial cells or components of the bacterial cells. Morishima *et al*. (1997) suggested that eicosanoids mediate induction of the genes for cecropin

and lysozyme in fat body of silkworm, *B. mori*. This work marks the recognition of a newly discovered eicosanoid action in invertebrate immunity, made all the more interesting in light of the recent discovery of a functional coupling between eicosanoid biosynthetic pathways and the immune deficiency (imd) pathway in *Drosophila* (Yajima *et al*., 2003). These authors reported that treatments with either of the two PLA_2 inhibitors, dexamethasone or *p*-bromophenacyl bromide, inhibited activation of the imd pathway. The inhibitory effects of these two inhibitors were attenuated by additional treatments with eicosanoid biosynthesis precursor fatty acids. Yajima *et al*. (2003) also found that AA alone did not activate the imd pathway, which indicated that eicosanoids participate in the activation of the imd pathway, but requires further LPS stimulation.

4.9.5.2.2. Immune reactions to pathogenic fungal infection All the work just mentioned is based on insect cellular defense reactions to bacterial infections. Dean *et al*. (2002) broadened the scope of this work with their hypothesis that eicosanoids mediate insect cellular reactions to the fungal pathogen *Metarhizium anisopliae*. They showed that treating tobacco hornworms with dexamethasone prior to challenge with fungal spores resulted in reduced number of nodules in reaction to the spores and that the influence of the drug could be reversed by treating experimental hornworms with AA. This was the first suggestion that eicosanoids mediate insect cellular reactions to fungal challenge. Lord *et al.* (2002) carried out a similar investigation using the fungal pathogen *Beauveria bassiana*. They reported that treating experimental tobacco hornworms with dexamethasone or the LOX inhibitors caffeic acid or esculetin or the COX inhibitor ibuprofen resulted in substantial reduction in number of nodules formed in reaction to fungal challenge. They also conducted a series of revealing rescue experiments. They found that the dexamethasone effect on nodulation could be reversed using the LOX product 5-HPETE, but not with the COX product PGH_2. Moreover, the influence of caffeic acid and esculetin was reversed with 5-HPETE but the ibuprofen effect was not changed by treating experimental hornworms with PGH_2. The authors inferred that products of the LOX pathways, but not the COX pathways, act in mediating hornworm immune reactions to *B. bassiana*. Lord *et al*. (2002) also noted that eicosanoids did not influence PPO activation, as seen in waxmoth larvae (Mandato *et al*., 1997). These two findings with different insect fungal pathogens add important new information to understanding signaling mechanisms in insect cellular immunity.

4.9.5.2.3. Immune reactions to parasitoid eggs Carton *et al*. (2002) added a new insight into insect immunity with their report that dexamethasone treatments inhibited cellular reactions to parasitoid invasion. In these experiments, larvae of the fruitfly *Drosophila melanogaster* were injected with dexamethasone, then exposed to adults of the parasitoid wasp *Leptopilina boulardi*. Compared to control larvae, in which approximately 90% of the parasitized larvae encapsulated the wasp eggs, about one-third of the larvae treated with 5 mg of dexamethasone encapsulated eggs and about 14% of larvae treated with 8 mg of dexamethasone encapsulated eggs. These results demonstrated a substantial dose-dependent effect of dexamethasone on encapsulation of parasitoid eggs, from which the authors inferred that eicosanoids mediate cellular encapsulation reactions to parasitoid eggs.

The information reviewed here may suggest that eicosanoids mediate cellular immune reactions to bacterial, fungal, and parasitoid challenge. It is suggested that the roles of eicosanoids in immunity may be another fundamental eicosanoid action in animals. More to the point of understanding insect immunity, however, it has been suggested that the immune system of *D. melanogaster* discriminates between bacterial and fungal challenge, seen with respect to differential activation of signal transduction pathways, which lead to expression of genes for antimicrobial peptides (Lemaitre *et al*., 1997). In light of this, it is noted that *M. sexta* cellular reactions to challenge by one fungal pathogen apparently depend on LOX, rather than COX, pathways, while reactions to bacterial challenge may be mediated by products of both pathways. As seen with the pathways for expression of genes for antimicrobial proteins, fungal and bacterial challenges seem to activate different pathways of eicosanoid biosynthesis.

4.9.5.2.4. Interactions between insect immune and neuroendocrine systems It was noted earlier that the signal transduction systems in insect cellular immunity include other important biomolecules, including peptides and biogenic amines. Beyond this, recent evidence points to interactions between the immune and endocrine systems in insect cellular immune reactions to infection. Wiesner *et al*. (1997) reported that apolipophorin-III stimulates immune reactions in *G. mellonella*. Halwani and Dunphy (1999) also noted that apolipophorin-III potentiates immune reactions in *G. mellonella*. Goldsworthy *et al.* (2003) considered another element of the neuroendocrine system in their study of adipokinetic hormone-I, apolipophorin-III, and eicosanoids in the locust,

Locusta migratoria. They found that adipokinetic hormone and eicosanoids are important in signaling nodulation reactions while adipokinetic hormone and apolipophoren-III are crucial for stimulating PPO activation. In line with the report by Lord *et al.* (2002), eicosanoids do not seem to influence PPO activation in locusts. As noted elsewhere, there is room for many mediators in insect cellular immunity.

Cellular reactions to immune challenge involve an unknown, albeit conceivably large, number of discrete cellular events (Miller *et al.*, 1994, 1996; Stanley, 2000). Nodulation, in particular, undoubtedly involves many discrete cellular activities, only a few of which have been identified. While not meant to be an exhaustive list, these may include recognition of microbial cell wall components, producing and secreting various signal moieties which attract other hemocytes toward the site of infection, cell migration, various cell adhesion actions (microbe–hemocyte and hemocyte–hemocyte), cell spreading, and activation of prophenoloxidase. One of the major gains of investigating the roles of eicosanoids in cellular immunity is the possibility of recognizing additional cell actions and identifying which of many possible eicosanoids are responsible for signaling particular cell actions.

4.9.5.3. Testing the Eicosanoid Hypothesis

The idea that eicosanoids mediate cellular immune reactions to microbial and parasitoid challenge is a robust notion, which requires testing from a number of approaches. By and large, the evidence supporting the hypothesis has been circumstantial in nature. On one hand, we see that treating insects with pharmaceutical inhibitors of eicosanoid biosynthesis impairs cellular defense reactions to immune challenge. On the other hand, the ability of insect tissues, including immunity-conferring tissues, to produce PGs and other eicosanoids has been documented. However, this biochemical work does not, in itself, forge a link between eicosanoids and cellular immune signaling. In the following paragraphs, a few experiments designed to more directly test the eicosanoid hypothesis are reviewed.

The outcomes of experiments with inhibitors of eicosanoid biosynthesis uniformly indicate that insects which had been treated with the inhibitors were impaired in their ability to clear bacterial cells from hemolymph circulation and in their ability to form microaggregates and nodules following infection. If the eicosanoid hypothesis makes sense, the impairments are due to the inability of hemocytes or other tissues to biosynthesize eicosanoids in response to microbial challenge. It should follow, then, that microbial infections stimulate production of various eicosanoids.

Jurenka *et al.* (1999) investigated this possibility in a series of experiments with black cutworms. Cutworms were artificially infected by injecting heat-killed bacteria, *Serratia marcescens*, into their hemocoels. After 30 min incubations, hemolymph was collected and processed for eicosanoid extraction. The eicosanoids were derivatized with the fluorescent compound ADAM, then analyzed on high-performance liquid chromatography (HPLC) equipped with a flow-through fluorescence detector. This work revealed large increases in the titer of one PG, $PGF_{2\alpha}$, in hemolymph of experimental, but not vehicle-injected control insects. Moreover, pretreating experimental cutworms with selected COX inhibitors blocked the infection-stimulated increases in $PGF_{2\alpha}$. This work strongly supports the eicosanoid hypothesis because it indicates that insects increase PG production following bacterial challenge.

Miller and Stanley (2001) considered the issue of signaling among immune tissues by investigating microaggregation reactions to bacterial challenge in isolated hemocyte populations. Hemocytes were prepared from tobacco hornworms, then challenged with lyophilized bacteria, *S. marcescens*. After selected incubation periods, generally 2 h, number of microaggregates were determined. These experiments documented the point that isolated hemocytes preparations are able to form microaggregates when challenged with bacteria. Results of similar experiments in which hemocytes were pretreated with various inhibitors of eicosanoid biosynthesis indicated that the microaggregation reactions also depend on eicosanoids.

This work led to the suggestion that isolated hemocytes are competent to biosynthesize and secrete eicosanoids in reaction to bacterial challenge. This idea was confirmed by experiments with hemocyte culture media that had been conditioned by challenging the hemocytes with bacteria (Miller and Stanley, 2001). Hemocyte preparations were challenged with bacteria and the hemocyte media were filtered through microfilters to produce "conditioned medium." Naive hemocyte preparations were then treated with the conditioned medium, and hemocyte microaggregation was determined. These experiments showed that exposing naive hemocyte preparations to conditioned medium was sufficient to generate microaggregation reactions in the naive cells. In another control experiment, it was observed that media, which was conditioned using hemocytes, that have been pretreated with dexamethasone or other inhibitors of eicosanoid biosynthesis, did not generate microaggregation reactions.

However, this attractive view suggesting rapidly expanding discoveries of eicosanoid actions in

invertebrate immunity is best viewed with skepticism (Stanley-Samuelson, 1994b). Several important issues should be addressed before the biological actions of eicosanoids in immunity are accepted. First, as mentioned elsewhere (Stanley-Samuelson and Dadd, 1983; Stanley-Samuelson *et al.*, 1988; Stanley, 2000), the presence and significance of eicosanoid-precursor PUFAs is not well appreciated in insect biochemistry. Second, although there are several reports on the topic, there is very little information on eicosanoid biosynthesis in insects and other invertebrates. Third, the pharmacological fates of COX and LOX inhibitors in invertebrates is not known with certainty. All of these issues relate to various aspects of the biochemistry of eicosanoids, to which we now turn.

4.9.5.4. The Biochemistry of Eicosanoid Systems in Immune Tissues

The fat body and hemocytes are thought to be the immunity-conferring tissues of insects, and the eicosanoid systems in these tissues were of prime interest. Soon after the hypothesis that eicosanoids mediate clearance of invading bacterial cells from hemolymph circulation was tested, an investigation into the main points of eicosanoid biosynthesis in these two tissues from the tobacco hornworm, *M. sexta* was launched.

4.9.5.4.1. Polyunsaturated fatty acids The fatty acid compositions of total lipids and selected PL fractions prepared from hemocytes isolated from fifth instar tobacco hornworms were determined (Ogg *et al.*, 1991). For these experiments, hemolymph was prepared by pericardial puncture, to prevent inadvertent activation of hemocytes. The hemocytes were pelleted by centrifugation, washed to eliminate hemolymph contamination, then processed for lipid analysis. As expected (Stanley-Samuelson *et al.*, 1988), only traces (less than 0.1% of the fatty acids) of $C_{20:3n-6}$, AA, or $C_{20:5n-3}$ were associated with total hemocyte lipids, or with total hemocyte PLs. This finding opened the possibility that AA or other eicosanoid-precursor components are sequestered into particular PL fractions. Two major glycerophospholipids, phosphatidylcholine and phosphatidylethanolamine, were isolated. Analysis of the fatty acids associated with these PLs again revealed only traces of the eicosanoid-precursor components. With the idea that these low levels might be related to an environmental constraint, the artificial culture medium was analyzed, which revealed higher proportions of AA in the medium than in the hemocytes. Hence, it was concluded the presence of low proportions of AA in hornworm hemocytes is the usual biological condition (Ogg *et al.*, 1991).

Certainly there are exceptions to this notion. One of the long-standing pillars of the field of animal lipids is that PUFAs are mostly associated with PLs, and less so with neutral lipids. This is generally true for insects, as well (Stanley-Samuelson and Dadd, 1983), but such ideas mut be tested. The triacylglycerol fractions prepared from tobacco hornworm heads yielded the highest proportions of AA ever recorded in a lepidopteran, i.e., AA constituted about 12% of the triacylglyerol fatty acids (Ogg and Stanley-Samuelson, 1992). A few other such pools of AA and $C_{20:5n-3}$ have been recorded in insect studies, one of which certainly relates to PG biosynthesis. Stanley-Samuelson and Loher (1983) found AA in a special pool in spermatophores of the cricket *T. commodus*. The AA was present at about 24% of phosphatidylcholine fatty acids, and virtually absent from phosphatidylethanolamine fatty acids. The authors suggested that the AA was specially sequestered for transfer from male to female crickets during mating. Perhaps the high AA proportions in hornworm heads represents a pool of storage AA that can be drawn upon as needed. Obviously, this is a speculation which requires more concrete examination. More recently, Nor Aliza *et al.* (2001) reported the presence of high proportions of AA and $C_{20:5n-3}$ in tissue PLs of adult fireflies, *Photinus pyralis*. They recorded AA at about 25% of PLs fatty acids in light organ and fat body from males, 13% for testes, and about 8% for midgut epithelia. Although such high proportions might indicate a very high potential for PG biosynthesis, preliminary experiments indicated that firefly tissues produced PGs at rates no different from other studied insect systems.

Recording very low proportions of AA in *M. sexta*, fat body and hemocytes is consistent with the general picture of PUFAs in terrestrial insects. These low proportions elicit questions about insect fatty acid biochemistry. Do hemocytes actively maintain low proportions of AA and other eicosanoid-precursor PUFAs? This was investigated by tracing the incorporation and remodeling of radioactive fatty acids into hemocyte lipids (Gadelhak and Stanley-Samuelson, 1994).

Four radioactive fatty acids, $C_{18:1n-9}$, $C_{18:2n-6}$, $C_{20:4n-6}$, and $C_{20:5n-3}$, were used in separate incorporation experiments. Consistent with the general background of animal lipid biochemistry, the *Manduca* hemocytes incorporated all four of the radioactive fatty acids into cellular complex lipids. Of the four, $C_{18:2n-6}$ was most efficiently incorporated into PLs, while $C_{18:1n-9}$ and $C_{18:2n-6}$ were efficiently incorporated into triacylglycerols. About 1–3% of the starting radioactivity in $C_{18:1n-9}$ was recovered

in diacylglycerols and monoacylglycerols. Very little of the other fatty acids was incorporated into these two fractions. The incorporation patterns generally agreed with the fatty acid composition of hemocytes.

The incorporated fatty acids were redistributed among lipid fractions in hornworm hemocytes during longer incubation periods (Gadelhak and Stanley-Samuelson, 1994). This redistribution is due to selected hydrolysis of some components from PLs, allowing incorporation of other components. Chilton and Murphy (1986) documented this remodeling process in human neutrophils, showing that after initial incorporation into ester-linked PLs, radioactive AA was selectively remodeled into ether- and plasmalogen-linked PL pools over time. These PL fractions were not considered in our studies because they have not yet been sufficiently detailed in insect systems.

Remodeling of incorporated fatty acids from PLs to triacylglyerols was recorded (Gadelhak and Stanley-Samuelson, 1994). After longer incubations, the radioactivity recovered in the PL fraction declined, with concomitant increases in radioactivity in the triacylglycerols. For AA, the radioactivity recovered in PLs declined to about 97% at 20 min, and to about 83% by 120 min. This directional shift was not seen with incorporated $C_{18:2n-6}$ and $C_{18:1n-9}$, although some of the radioactivity associated with these fatty acids was shifted between these two major cellular lipid fractions.

A similar picture emerged from the analysis of selected PL fractions. After 5 min incubation periods, most of the radioactivity associated with AA was recovered in phosphatidylcholine. With longer incubations, the proportions of radioactivity recovered in phosphatidylcholine declined, with attending increased radioactivity in phosphatidylethanolamine. After 120 min incubations, slightly more radioactivity associated with AA was detected in phosphatidylethanolamine, rather than phosphatidylcholine. Again, these results support the idea that PUFAs are selectively remodeled among the complex lipid fractions in hornworm hemocytes. Similar analyses of other insect tissues have not yet been carried out, but nonetheless, remodeling dynamics of this nature are to be expected. Again, these processes help understand the low proportions of AA and other eicosanoid-precursor PUFAs in terrestrial insects.

AA is present in fat body from larvae of the beetle *Z. atratus* (Howard and Stanley-Samuelson, 1996; Miller *et al*., 1996). Similarly, AA is present in trace levels in black cutworms, *A. ipsilon*, true armyworms, *P. unipuncta* (Jurenka *et al*., 1997), in silkworms, *B. mori* (Stanley-Samuelson *et al*., 1997), and in adults of the cricket *G. assimilis* (Miller *et al*., 1999). Relative to the work on waxmoth hemocytes (Mandato *et al*., 1997), AA and $C_{20:5n-3}$ were confirmed in these insects during early studies of fatty acid nutritional metabolism (Stanley-Samuelson and Dadd, 1984; Stanley-Samuelson *et al*., 1988). Hence, one element required for eicosanoid biosynthesis in invertebrate immune tissues is present, albeit at low levels, in all systems in which eicosanoids are present.

4.9.5.4.2. Phospholipase A_2 is the first step in eicosanoid biosynthesis Two of the eicosanoid biosynthesis inhibitors used in the immune studies, namely dexamethasone and *p*-bromophenacylbromide, are thought to act at the level of PLA_2. These two reagents do not directly inhibit eicosanoid-biosynthetic enzymes, but inhibit the overall process of eicosanoid biosynthesis by arresting the release of AA or other PUFA from cellular PLs. In the absence of free substrate, eicosanoid biosynthesis cannot proceed. Because these two inhibitors attenuate cellular and humoral immune reactions to bacterial infections and the influence of one of these, dexamethasone, can be reversed by treating experimental insects with free substrate, AA, it was proposed that an intervening PLA_2 step must be operative in the eicosanoid-mediated immune reactions to bacterial infections.

The PLA_2s are responsible for hydrolyzing the acyl moiety from the *sn*-2 position of glycerophospholipids and an intracellular PLA_2 in *Manduca* fat body has been characterized (Uscian and Stanley-Samuelson, 1993). In brief, phosphatidylcholine with radioactive AA esterified at the *sn*-2 position was prepared in the form of substrate vesicles to facilitate enzyme activity. *Manduca* fat body homogenates were centrifuged to produce microsomal-enriched fractions used as enzyme sources. The enzyme was incubated with substrate vesicles, then total lipids were extracted from the reaction mixtures. The lipid extracts were separated and fractions associated with unprocessed substrate, free fatty acids, and diacylglycerol were radioassayed. PLA_2 activity was calculated from the amount of radioactivity associated with the free fatty acid fraction.

The fat body PLA_2 was sensitive to the usual biophysical parameters and the calcium requirements for this enzyme were assessed. For this experiment, the fat body was homogenized and centrifuged in calcium-free buffer containing the calcium chelator ethylene glycol bis (B-aminoethyl/ether)-*N*,*N*,*n*′, *N*′-tetraacetic acid (EGTA). Reactions run in the presence or absence of calcium yielded similar results, from which we inferred that the *Manduca*

fat body PLA_2 is a calcium-independent enzyme, or possibly one with very low stringency calcium requirements. To test this hypothesis, enzyme preparations were dialyzed against 200 volumes of EGTA buffer and enzyme activity was assessed. Again, rigorous removal of calcium did not reduce enzyme activity.

A similar characterization of a PLA_2 in hemocytes from fifth instar larvae was reported by Schleusener and Stanley-Samuelson (1996). For these experiments, hemolymph was collected by pericardial puncture, diluted in buffer, the hemocytes rinsed in fresh buffer, and the cells were homogenized by sonication. Microsomal-enriched fractions were prepared from the sonicates and these were used as the enzyme source.

Again, the hemocyte PLA_2 was sensitive to substrate concentration, protein concentration, and pH. As seen in virtually all mammalian preparations, the hemocyte enzyme was inhibited in the presence of 5–50 μM OOPC. The calcium requirements of the hemocyte PLA_2 were determined, and as in the fat body preparations, all hemocyte preparation steps were carried out in buffer containing EGTA. Enzyme activity increased with increasing concentrations of EGTA, which suggested that the chelator removes from the reaction medium an ion that downregulates the hemocyte PLA_2 (Schleusener and Stanley-Samuelson, 1996).

Based on findings with some mammalian $cPLA_2$s, the idea that the hemocyte PLA_2 exhibits a selectivity for arachidonyl-associated PLs was considered. In these experiments, enzyme sources were prepared as usual, then incubated with either of the two substrates, one a palmitoyl-associated phosphatidylcholine and the other an arachidonyl-linked phosphatidylcholine. Enzyme activity with the palmitoyl-associated substrate was reduced by about 40% relative to activity with the arachidonyl-associated substrate. It is more appropriate to assess substrate specificity using purified, or at least highly enriched, enzyme preparations since most animal cells have several forms of cytosolic PLA_2, and experiments meant to detect the substrate preferences of a subset of these enzymes can yield ambiguous results. In this case, however, the 40% reduction in enzyme activity indicates that at least one PLA_2 in *Manduca* hemocytes has a fairly strong preference for arachidonyl-linked PL substrate. This enzyme may regulate eicosanoid biosynthesis in hemocytes.

These preliminary investigations serve to document both the presence of a cytosolic PLA_2 in hornworm fat body and the fact that hemocytes can hydrolyze AA from the *sn*-2 position of cellular PLs. These results are preliminary because documenting the presence of an enzyme in fat body and hemocytes does not demonstrate unequivocally that the enzyme is an integral part of the eicosanoid-mediated cellular reactions to bacterial infections, although recent work supports the idea that PLA_2 is a key step in eicosanoid biosynthesis following bacterial infection. The circumstantial evidence on the influence of PLA_2 inhibitors on cellular and humoral immune reactions has been bolstered with more direct experiments. Given the very low levels of AA in *Manduca* immune tissues, some of these experiments could be better designed using other invertebrate systems. For now, however, it can be said that the tobacco hornworm expresses $cPLA_2$s, and some forms of these enzymes may be involved in immune reactions to bacterial infections.

4.9.5.4.3. Eicosanoid biosynthesis in immune tissues While eicosanoid biosynthesis has been demonstrated in a number of invertebrate systems, the idea that eicosanoids mediate cellular and humoral immune reactions to bacterial infections in insects opens a window of opportunity for investigation. Eicosanoid biosynthesis in *Manduca* fat body and hemocyte preparations has been recorded, but before considering this work, an important issue should be considered. The methodology of tracing PG biosynthesis in invertebrates has not yet matured to a series of standard techniques. Although most methods in essence amount to reacting an enzyme source with radioactive AA, there are a few subtle points which deserve attention because they can influence the outcome of such experiments.

In work with the fat body (Stanley-Samuelson and Ogg, 1994), the eicosanoid biosynthesis reaction mixtures included radioactive AA emulsified in buffer containing a cofactor cocktail (2.4 mM reduced glutathione, 0.25 mM hydroquinone, and 25 μg hemoglobin in each reaction). The reactions were preceded by 3 min preincubations with all reaction components except the enzyme sources. The reactions were started by adding the microsomal-enriched enzyme source, and stopped by acidification and extraction of products.

The radioactive PGs were separated by thin-layer chromatography (TLC) (Hurst *et al.*, 1987). Chromatography with this system differs from usual procedures because the plates are placed into the solvent immediately after the solvent is transferred to the developing chamber. There is no equilibration period in this system, which requires about 2.5 h to develop. In some experiments, the reaction products were also separated by two-dimensional TLC. Bands corresponding to authentic standards were transferred to vials, and the radioactivity in each fraction

was determined. PG biosynthesis was calculated from the amount of radioactivity in each fraction.

PUFAs are fairly unstable molecules in oxygen atmospheres and experimental artifacts can result from spontaneous oxygenation reactions, or autoxidation, during reactions and subsequent work up steps. Autoxidation was determined by conducting "zero-time" reactions. In these control experiments, the substrate was preincubated in parallel with the experimental tubes. At time zero, when the enzyme source would ordinarily be added, the control preincubations were stopped and extraction and chromatographic separation followed. Zero-time reactions were done in every experiment, and values from these control experiments were used to correct for autoxidation in the biosynthesis reactions.

The fat body preparations yielded four PGs: $PGF_{2\alpha}$, PGE_2, PGD_2, and PGA_2. Under most experimental conditions, PGA_2 was the predominant fat body product. The enzyme preparation was sensitive to routine biophysical parameters although the literature on PG biosynthesis in invertebrates presents an ambiguous picture with respect to optimal reaction times. Drawing on the mammalian data (Smith *et al*., 1996), the first step in PG biosynthesis is catalyzed by COX, which undergoes "suicide" inactivation after about 1400 catalytic operations. The suicide inactivation is regarded as an autoregulatory mechanism, which imposes an upper limit on cellular potential to biosynthesize PGs. PG biosynthesis therefore occurs in short, very rapid bursts of enzyme activity. However, the overall dynamics of maintaining appropriate PG titers within cells includes PG biosynthesis and degradation. This could produce two phases of PG biosynthesis in *in vitro* reactions and possibly in intact cells. Early in the reactions, the rapid bursts of biosynthesis would exceed PG degradation, thereby favoring product accumulation. During the second phase of the reaction, the suicide inactivation of COX would lead to decreased PG biosynthesis and product degradation would exceed product formation. The overall result of this asymmetry in the reaction progress would be registered as higher product accumulation in shorter reaction periods which decreases during longer reaction periods.

In work with the *Manduca* fat body, an initial burst of PG biosynthesis which peaked at about 1 min was observed. Thereafter, product accumulation decreased over the next 9 min. These findings agree with mammalian data, and also with the time course of PG biosynthesis seen in housefly preparations. Wakayama *et al*. (1986) also observed rapid PG biosynthesis during 2 min incubations. After the first 2 min, there was a very gradual increase in product formation over the following 58 min. In contrast, Brenner and Bernasconi (1989) reported a linear increase in PGE_2 biosynthesis over a 60 min time course. In their characterization of PG biosynthesis by spermatophore content from males of the Australian field cricket, *T. commodus*, Tobe and Loher (1983) also found a linear increase in synthesis over 60 min reaction periods. The systems in which longer reaction times promote increased product formation may point to important differences between the well-established mammalian studies and the emerging information on invertebrate eicosanoid systems.

All of the biological investigations into the roles of eicosanoids in cellular and humoral immunity described in this section were based on the use of pharmacological inhibitors of eicosanoid biosynthesis. Some of these inhibitors designed for studies on mammalian tissues reduce PG biosynthesis in hornworm tissues. In these experiments, the fat body preparations were incubated in the presence of selected doses of the pharmacological reagents, and the reaction products were then extracted and analyzed. The fat body preparations were very sensitive to the COX inhibitors, indomethacin and naproxin. At the low dosage of 0.1 μM, indomethacin and naproxin reduced total PG biosynthesis by about 80%. Higher dosages virtually abolished PG biosynthesis. Along with inhibition of PG biosynthesis, reactions with naproxin yielded substantial levels of a radioactive product, tentatively identified as the LOX product 15-HETE. The influence of naproxin on increased LOX activity occurred in a dose-dependent manner. In the presence of increasing naproxin concentrations, the decreasing COX activity was accompanied by increasing LOX activity. While indomethacin effectively inhibited COX activity, there was no accompanying increase in LOX activity. It may be presumed that the indomethacin also inhibits the insect LOX, as it does in some mammalian preparations.

Tobacco hornworm hemocyte preparations also are capable of eicosanoid biosynthesis (Gadelhak *et al*., 1995). Pools of hemocytes were washed, then homogenized by sonification, microsomal-enriched preparations were prepared by centrifugation, and eicosanoid biosynthesis assayed. The hemocyte preparations yielded two major products, the COX product PGA_2 and the LOX product 15-HETE. The identification of 15-HETE is based on a single TLC step, and undoubtedly that fraction contains more than one LOX product. For this reason, this product is simply noted as total LOX activity.

Two inhibitors of eicosanoid biosynthesis influenced eicosanoid biosynthesis by the hemocyte

preparations. The COX inhibitor naproxin reduced COX activity by 88% at 0.1 mM and by 96% at 1.0 mM. Contrary to results with the fat body, the hemocyte LOX activity was not enhanced in the presence of naproxin. Reactions carried out in the presence of the LOX inhibitor esculetin also yielded reduced COX and LOX product formation. At 0.1 mM, esculetin nearly inhibited all eicosanoid biosynthesis. These studies support the view that eicosanoid biosynthesis observed on TLC is not an artifact of substrate autoxidation. However, the influence of these eicosanoid-biosynthesis inhibitors is quite instructive. While these are well-characterized pharmacological agents in mammalian systems, their actions in invertebrate systems may differ in important ways. For example, indomethacin, a COX inhibitor, potently inhibited cellular defense reactions and COX activity in *Manduca* fat body preparations. This compound did not inhibit PG biosynthesis in preparations of male reproductive tracts from the cricket *A. domesticus* (Destephano *et al*., 1976). More to the point, naproxin is accepted as a specific COX inhibitor. Results with the hornworm fat body preparation would suggest that this is so in invertebrates as well, because low doses of naproxin (0.1 μM) effectively inhibited COX, but not LOX, activity. These results suggest that the influence of eicosanoid-biosynthesis inhibitors should be investigated in each species, and each tissue within a species, to ensure that the inhibitors act as they do in vertebrate experiments.

This comment is quite relevant to work on immunity. On the basis of a single experiment with naproxin, one might conclude that COX activity is essential to cellular immune reactions to bacterial challenge. However, the results of our biochemical experiments with hemocyte preparations make it clear that naproxin may act by inhibiting LOX as well as COX activities. This is one of the reasons for conducting experiments with several separate inhibitors.

Eicosanoid biosynthesizing enzymes are rather uniformly distributed within mammalian cellular fractions, with COXs exclusively associated with endomembrane fractions of cells. The subcellular localization of COX and LOX activities in hemocytes can be assessed by determining eicosanoid biosynthesis in the mitochondrial, microsomal, and cytosolic fractions. The COX activity was unevenly distributed among the three cellular fractions, about 5% in the mitochondrial fraction, 58% in the microsomal fraction, and about 36% in the cytosolic fraction. Most of the LOX activity (87%) was recovered in the cytosolic fraction, and the remaining 13% in the microsomal fraction. These data indicate that insect COXs do not have the same subcellular distribution as one finds in mammals.

Although the broad themes are similar, there is substantial variation in eicosanoid systems among mammalian species and among tissues within a mammalian species. Relative to invertebrates, much more variation will emerge as research activities which continue to generate new information. Therefore, it is important to investigate at least some aspects of eicosanoid biosynthesis in several insect species in which eicosanoids play a role. AA and other C_{20} PUFAs as well as PG biosynthesis capacity is present in tissues from the tenebrionid beetle *Z. atratus* (Miller *et al*., 1996). Similarly, AA is present in fat body PLs from true armyworms and black cutworms (Jurenka *et al*., 1997). The fat body from these larvae also expressed an intracellular PLA_2 that can hydrolyze AA from cellular PLs. Fat body preparations from both species are able to convert radioactive AA into PGA_2, PGD_2, PGE_2, and $PGF_{2\alpha}$. A similar line of documentation experiments showed the presence of eicosanoid-biosynthesizing enzymes in fat body from silkworms, *B. mori* (Stanley-Samuelson *et al*., 1997). Miller *et al*. (1999) recorded traces of AA in the fat body of adult crickets, *G. assimilis*, as well as biosynthesis of three eicosanoids by fat body preparations, PGA_2, PGE_2, and a hydroxyeicosatetraenoic acid. As in the silkworm fat body preparations, the LOX product(s) was the major product produced by the cricket fat body preparations.

The point of these exercises is to document the presence of an eicosanoid-biosynthesizing system in insects thought to use eicosanoids in cellular immune signal transduction mechanisms. Even at this superficial level of analysis, however, substantial differences are evident in the eicosanoid systems among these few insect species. More detailed characterizations of the elements of eicosanoid biosynthesis in these species will undoubtedly reveal more differences. The biochemistry of these systems merits considerably more research attention.

4.9.5.4.4. The pharmacology of eicosanoid biosynthesis inhibitors There is very little knowledge concerning the movements or metabolic fates of eicosanoid biosynthesis inhibitors in invertebrates. Murtaugh and Denlinger (1982) maintained a group of house crickets on diets with indomethacin. After several days on these diets, they found reduced levels of PGE_2 and $PGF_{2\alpha}$ in testes from the experimental males and spermathecae from mated females. These findings suggest that indomethacin can move from the alimentary canal to at least two tissues in house crickets and also suggest the possibility of substantial biochemical differences between

the action of these compounds in whole animals and enzyme preparations.

These issues motivated the study of pharmacology of indomethacin in *Manduca*. Using fifth instar larvae, radioactive indomethacin was injected into the hemocoels of the animals and its movement, excretion, and metabolism examined. Over 99% of the radioactivity associated with injected indomethacin was cleared from the hemolymph circulation within the first 3 min after injection. The indomethacin was rapidly taken up by hornworm tissues. Radioactive indomethacin was recovered from all tissues analyzed, including integument, ventral nerve cord, salivary gland, fat body, Malpighian tubules, and gut epithelium. The greatest amount of radioactivity was recovered from fat body, the largest tissue. However, when normalized to wet tissue weight, most radioactivity was recovered from the salivary glands. These results indicate that indomethacin is probably distributed among all tissues in this insect, but not uniformly, as seen in mammals. Less radioactivity was recovered from integument and nerve cord, while more was recovered from fat body, silk gland, and Malpighian tubules. This is also consistent with the pharmacology of indomethacin in mammals.

The extraction system used to recover indomethacin and its metabolites from hornworm tissues provided information on the metabolic fate of indomethacin. Indomethacin is highly soluble in chloroform and other organic solvents, and is virtually insoluble in water. Rigorous multiple extraction procedures yielded about 90% of the recovered radioactivity in the collected organic phases. The remaining 10% of the radioactivity was detected in the aqueous phases. This 10% represents polar metabolites of indomethacin, taken to indicate that about 10% of the injected indomethacin was metabolized to water-soluble products.

There was no evidence for the metabolism of indomethacin in most tissues, including integument, nerve cord, fat body, Malpighian tubules, and gut epithelium. Virtually all of the radioactivity recovered from these tissues cochromatographed with authentic indomethacin on TLC. Contrary to results with other tissues, the salivary gland produced at least one polar product of indomethacin, which made up no more than 25% of the starting material. Hence, the material taken up into virtually all hornworm tissues is present as indomethacin.

The excretion of radioactive indomethacin was recorded by collecting frass. In this experiment, radioactive indomethacin was injected into the hemocoel of fifth instar larvae. The insects were held in individual cups, and frass pellets were collected every 2 h for the following 40 h. About 56% of the injected material was recovered over the 40 h incubation period. A trace of radioactivity appeared in the frass as early as 2 h after injection and almost half of the injected radioactivity was recovered between 4 and 12 h. These data reveal that substantial amounts of injected indomethacin remain in the hornworm tissues in its original form during the first 12 h after treatments. For purposes of experimental design, the inhibitor was present in the hornworm throughout the time course of our immunology experiments.

The products extracted from the frass were analyzed and three to five indomethacin metabolites were present in frass, accounting for about 30% of the recovered radioactivity. Since enteric microbes may have been responsible for the apparent metabolism, radioactive indomethacin was incubated with freshly collected frass pellets and the data showed that about 10% of the starting material was metabolized into more polar products after 40 h. This result suggests that one or more factors in hornworm frass are responsible for indomethacin metabolism prior to excretion.

The composite data indicate that indomethacin is an appropriate probe for assessing the roles of eicosanoid biosynthetic pathways in the cellular immunity of tobacco hornworms, rather than insects or invertebrates, because there are substantial differences in the pharmacology of indomethacin among mammalian species. For example, dogs and guinea pigs require about 20 min to clear 50% of injected indomethacin doses from the circulation (Yesair *et al*., 1970), whereas rats require about 4 h (Hucker *et al*., 1966). The distribution of indomethacin among tissues within mammals also differs among species. Rats maintain higher concentrations of indomethacin in blood circulation than in tissues at all times after injection. In guinea pigs, indomethacin is concentrated from blood into liver, kidney, and small intestine. The excretion of indomethacin also differs among mammals (Hucker *et al*., 1966; Yesair *et al*., 1970). Nearly all injected indomethacin is excreted in feces in dogs, while rabbits excrete most injected indomethacin in the urine. Guinea pigs and humans excrete about half of the injected indomethacin in urine, the remainder in feces. These findings emphasize differences in distribution, metabolism, and excretion of injected indomethacin among mammals. Similar differences are to be expected among invertebrates. Indeed, the different biochemical effects of indomethacin on eicosanoid biosynthesis in insect systems have been discussed. Overall, this work points to the importance of understanding the pharmacology of eicosanoid biosynthesis inhibitors in the work on assessing the roles of eicosanoids

in invertebrates, and the pharmacology of inhibitors in general.

4.9.6. Emerging Topics

During the time Dadd (1985) was developing his review of insect nutrition, appreciation of the biological significance of PGs and other eicosanoids in insects or other invertebrates was an emerging topic in insect biochemistry and physiology. There was virtually no background literature on the presence or meaning of AA in insect tissue lipids (Stanley-Samuelson and Dadd, 1983) and very little idea of PG action in insects. Understanding of eicosanoids in insect biology has grown in the last two decades, mostly due to studies that revealed new – or emerging – information. In this section several emerging areas have been highlighted.

4.9.6.1. An Insect Pathogen Inhibits Eicosanoid-Mediated Immunity

The significance of the hypothesis that eicosanoids mediate insect cellular immunity is the identification of a previously unrecognized and crucial signal transduction system in insect immunity. The biological actions of eicosanoids involve a suite of enzymes responsible for eicosanoid biosynthesis and, presumably, specific receptors and intracellular receptor interactions with effector systems. These enzymes, receptors, and intracellular proteins all represent potential novel targets that may ultimately be exploited in the ongoing development of new insecticides. Given the diversity of microbial organisms, it is not surprising to see that at least one insect pathogen has already exploited the inhibition of eicosanoid biosynthesis as a mechanism to downregulate insect immunity.

The nematode *Steinernema carpocapsae* lives in a symbiotic relationship with the bacterium *Xenorhabdus nematophilus* from which both partners gain advantage. The nematode serves as a productive environment for growth of the bacterium and *X. nematophilus* serves the nematode by killing newly invaded insect hosts. The biology of this and other bacterium–nematode–insect systems has been well documented (Kaya and Gaugler, 1993). Park and Kim (2000) recently illuminated a new aspect of these systems with their report that eicosanoids attenuate the lethality of *X. nematophilus* in the insect *Spodoptera exigua*. The authors inferred that the bacterium somehow inhibits eicosanoid biosynthesis in newly infected host insects, thereby impairing the insect cellular immune reactions to the presence of the bacterium.

It was hypothesized that the bacterium inhibited eicosanoid biosynthesis in insect immune tissues by restraining the action of PLA_2, the first step in eicosanoid biosynthesis. Testing this hypothesis began by showing that bacterial infections stimulate increased PLA_2 activity in hemocytes from tobacco hornworms (Tunaz *et al.*, 2003). The significance of this work is that it supports the view that bacterial infections lead to increased eicosanoid biosynthesis (as shown by Jurenka *et al.* (1999)) by stimulating hemocytic PLA_2 activity. It was subsequently found that, as Park and Kim (2000) originally suggested for *S. exigua*, *X. nematophilus* impairs nodulation reactions to bacterial infection in tobacco hornworms by inhibiting eicosanoid biosynthesis (Park *et al.*, 2003). This work showed that hornworms challenged with living bacteria produced far fewer nodules than did hornworms challenged with heat-killed bacteria. The immunity-impairing influence of challenge with living bacteria could be off-set by treating experimental hornworms with AA. It may be inferred that *X. nematophilus* inhibits eicosanoid biosynthesis in all of its host insect species.

It appears from this work (Park *et al.*, 2003) that the bacterium *X. nematophilus* secretes a specific product that inhibits eicosanoid biosynthesis. To investigate this, *X. nematophilus* cells were cultured, then separated from their culture medium by centrifugation; the medium was then fractionated into an aqueous and an organic fraction. After concentrating the extracts, each fraction was tested for its influence on nodulation. The organic fraction inhibited nodulation reactions to living bacteria while the aqueous fraction had no influence on nodulation. The organic fraction was fractionated into five discrete fractions by column chromatography. After testing each of the five, nodulation-inhibiting activity was present in only one. Moreover, results indicate *X. nematophilus* cells and its immunity-impairing product specifically inhibits PLA_2 in tobacco hornworm hemocytes (Y. Park *et al.*, unpublished data). The identity of the inhibitory factor should be determined in the near future.

This work revealed important new information on the biochemical mechanisms, which underlie the relationships among certain entomopathogenic nematodes, their symbiotic bacteria, and their insect hosts. It also strongly supported the hypothesis that eicosanoids mediate insect cellular defense reactions to microbial infection. Another aspect of the significance of these findings is that they indicate the potentials for discovery of mediating mechanisms in insect physiology and biochemistry.

4.9.6.2. Bioactive Oxylipids from Linoleic Acid

In addition to oxygenation of AA via the COX or LOX pathways, biomedical research on mammalian systems has revealed over a dozen of oxygenated

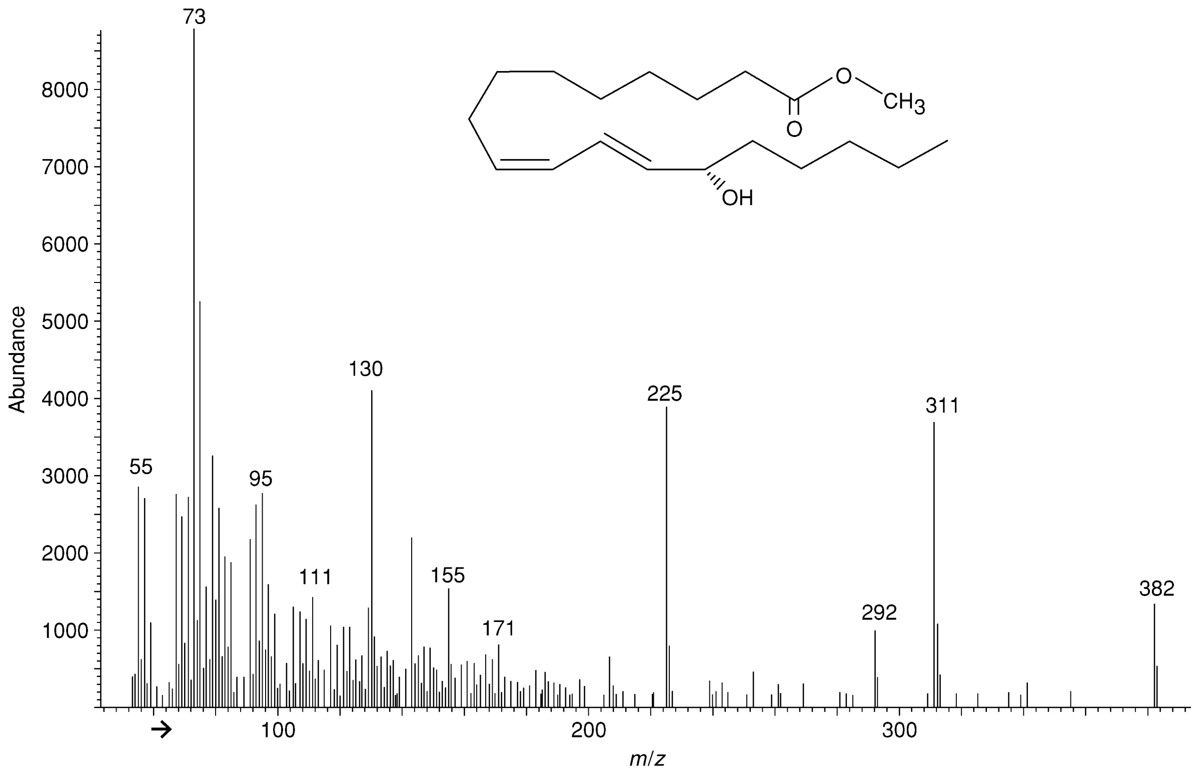


Figure 7 The structure of 13-hydroxyoctadecanoic acid methyl ester and an electron impact mass spectrum of the trimethylsilyl derivative of 13-hydroxyoctadecanoic acid methyl ester synthesized by a microsomal-enriched preparation of *Manduca sexta* fat body.

metabolites of linoleic acid, some of which exert important biological influence on cells. Products of linoleic acid metabolism include 9-hydroxyoctadecadienoic acid (9(S)-HODE) and 13(S)-HODE. Glasgow and Eling (1990) reported that epidermal growth factor stimulates linoleic acid metabolism in fibroblasts of BALB/c 3T3 mice. The same group later reported that 13(S)-HODE augments the epidermal growth factor receptor signaling pathway (Glasgow *et al.*, 2002).

Based on information in the biomedical literature, Putnam and Stanley (unpublished data) considered the hypothesis that insect tissues, also, are able to form potentially bioactive products of linoleic acid. Using fat body from tobacco hornworms, the biosynthesis of two bioactive lipids from radioactive $C_{18:2n-6}$, specifically 9- and 13-HODE, was recorded. The structures of these compounds were determined by gas chromatography-mass spectrometry (Figure 7). The possibility that metabolites of $C_{18:2n-6}$ may play important regulatory roles in insect cellular physiology is now emerging.

4.9.6.3. PG Receptors

Turning attention to another emerging topic, recall the broad claim of the eicosanoid hypothesis, i.e., eicosanoids somehow mediate insect cellular immune reactions to challenge. While the general theme of the hypothesis is strongly supported, information on the mechanisms of eicosanoid actions in insect biology remains the most prominent gap in our current knowledge. In contemporary models of eicosanoid action with respect to immunity (Stanley-Samuelson and Pedibhotla, 1996; Stanley *et al.*, 2002), circulating hemocytes recognize an immune challenge, most likely by recognition of specific cell components, such as LPS in the case of infection with Gram-negative bacteria. Upon stimulation by an invader, hemocytes produce and secrete eicosanoids which enter the hemolymph and influence the action and behavior of other hemocytes.

There are important implications in this model. First, the secreted eicosanoids are thought to act on other hemocytes, presumably through functional receptors which have been well characterized in mammalian systems. Second, hemocyte eicosanoid receptors are not uniformly expressed in all hemocytes. To the contrary, because eicosanoids mediate various cell defense actions – such as microaggregation, phagocytosis, cell spreading, nodulation, melanization, and encapsulation – it is possible

that subpopulations of hemocytes express receptors for particular eicosanoids.

The most important implication is that an understanding of eicosanoid receptors can lead to a more detailed understanding of hemocytic immunity and other areas of insect physiology because it will become possible to establish a direct linkage between particular eicosanoids and specific cell actions (Stanley, 2000).

4.9.7. Biochemical Mechanisms of Prostaglandin Actions

4.9.7.1. Background on G Protein-Coupled Receptors

The physiological actions of most PGs are mediated by G protein-coupled receptors (GPCRs) (Breyer *et al.*, 2001). The exceptions are PGA and other cyclopentenone PGs, which readily traverse cellular membranes and interact with intracellular receptors (Negishi *et al.*, 1995b). GPCRs are seven-transmembrane proteins responsible for transducing extracellular signals into intracellular compartments, where cellular responses to the signal are initiated. There are literally hundreds of GPCRs, which are specific for a wide range of ligands, including those involved in vision, olfaction, taste, neurotransmission, neuromodulation, and hormone actions. All multicellular animals have GPCRs, and the genes that encode these receptors make up substantial proportions of their entire genomes. GPRCs make up about 6% of the *Caenorhabditis elegans* genome, possibly 2% of mammalian genomes, and about 1% of the *D. melanogaster* genome (Brody and Cravchik, 2000; Vanden Broeck, 2001). The many known GPCRs are sorted into families, including the olfactory receptor family, a glutamate receptor family, a biogenic amine receptor family, and a diuretic hormone receptor family. PGs interact with GPCRs of the rhodopsin family. GPCRs transduce extracellular signals through selective coupling with intracellular G proteins, which in turn regulate the activity of cellular effector proteins, such as adenylyl cyclase, phospholipase C, or ion channels (Blenau and Baumann, 2001).

Receptors for most PGs are localized in the plasma membrane, although nuclear PG receptors have been reported (Bhattacharya *et al.*, 1999). PG receptors are classified on the basis of amino acid sequences and the effects of synthetic ligands which interact with the receptors (Coleman *et al.*, 1990). PGs D, E, F, I, and thromboxane A have corresponding receptors, denoted as DP, EP, FP, IP, and TP. So far, four subclasses of PGE receptors have been described, EP1, EP2, EP3, and EP4. These receptors affect changes in intracellular signal moieties by coupling with various G proteins. For example, EP1 receptors stimulate increased intracellular Ca^{2+}, and EP2 and EP4 receptors stimulate increased intracellular cAMP. EP3 receptors are unique as so far understood because they exist in variants which differ only in their intracellular C-termini, due to splice variations (Hatae *et al.*, 1989). This allows the different splice variants to couple with multiple G proteins, producing different physiological responses. For example, the EP3A can stimulate increased cAMP concentrations, while EP3B can exert the opposite effect.

4.9.7.2. PG Receptors in Insects

While many GPCRs have been identified and cloned from various insect sources (Roeder, 1999; Blenau and Baumann, 2001; Torfs *et al.*, 2001; Vanden Broeck, 2001), PG receptors have not been reported for insects. Indeed, information on the biochemical and molecular features of insect PG receptors remains the most prominent gap in understanding eicosanoid action in insect physiology (Stanley, 2000). Tunaz and Stanley (unpublished data) now have preliminary data on PGE_2 receptors in tobacco hornworm hemocytes. They used classical ligand binding studies, drawn from Negishi *et al.* (1993) and from Qian *et al.* (1997), who reported on PGE_2 receptors in salivary glands from the lone star tick, to register the presence of PGE_2 receptors in membrane preparations made from tobacco hornworm hemocytes. Data from this work indicate the presence of saturable, specific PGE_2 receptors associated with membrane preparations of hemocytes from tobacco hornworms. Analysis of the binding data indicted a single binding site model with a K_D of approximately 35 nM and B_{max} of approximately 7.5 fmol mg^{-1} protein.

It should be borne in mind that analysis of insect PG receptors by radioligand binding studies can be quite problematic. For example, insect tissues are small and they seem to express a small number of PG binding proteins relative to mammalian cells while binding studies require a great deal of tissue. Second, because PGs and other eicosanoids are themselves lipids, they present potentially serious problems with nonspecific or low-affinity binding. Finally, it is very difficult to isolate and study separate subpopulations of insect hemocytes or other subpopulations of cells in other tissues. The difficulties inherent in classical radioligand binding studies have been addressed in work on mammalian PG receptors. The contemporary approach is to clone the genes for the receptors, stably express the genes in established cell lines, and use the cell lines to characterize the receptors. This strategy

has yielded a very detailed picture of PG receptors in the biomedical context of mammalian systems.

Information from the *Drosophila* and the *Anopheles* genomes indicates that sequences are present in both insect genomes, which are quite similar to mammalian sequences for PG receptors. In their analysis of the *Anopheles* genome, Hill *et al.* (2002) identified a total of 276 GPCRs. Of these, the authors predicted the presence of five classes of PGCRs, including rhodopsin-like receptors. In mammals, PG receptors are members of the rhodopsin-like receptor family. Within the mosquito rhodopsin-like receptors, 22 were identified as "orphan receptors," that is, receptors for which the physiological ligand is unknown. The predicted number of orphan rhodopsin-like receptors in the *Drosophila* genome is also 22.

Haas and Stanley (unpublished data) used the orphan receptors 1 through 21 from *A. gambiae* (drawn from the supporting online material in Hill *et al.* (2002)) to conduct BLAST searches for similar receptor sequences in SWISSPROT. Two of these orphans, GPRorpha5 and GPRorpha11, are very similar to sequences representing receptors for various PGs, with over 100 hits with at least some similarity to mammalian PG receptors and over 40 hits with very high similarity (bit scores >44; E values <0.001).

Analysis of the two *Drosophila* orthologs to the *A. gambiae* orphans yielded similar findings. Again, there were over 100 hits with some similarity and over 40 hits with very high similarity, judged by the same statistical criteria. It may be inferred from these two series of *in silico* searches that at least some of the rhodopsin-like GPCRs, now recognized as orphans, may turn out to be specific PG receptors which mediate crucial physiological actions in insects.

Of course, it cannot be known whether these are, indeed, PG receptors of physiological significance until they have been analyzed in detail. Such analysis is now under way in the laboratory of Jozef Vanden Broeck with his student Vanessa Franssens in Leuven, Belgium. One of the *Drosophila* gene sequences (GC 4794) has been cloned from an expressed sequence tag and ligated into an expression vector. The expression vector was transvected into established *Drosophil*a cell lines for detailed biochemical studies. The results of this work should add a great deal of new information on the biological actions of PGs in insects.

4.9.8. Conclusions: The Potentials of Eicosanoids in Insect Biology

A great deal of new information on the presence and biological actions of PGs and other eicosanoids in insects, as well as in other invertebrates, has come to light in the nearly 20 years since publication of the first edition of *Comprehensive Insect Biochemistry, Physiology, and Pharmacology.* Work on the biological significance of eicosanoids in insects is rewarding because there is tremendous possibility for discovery. On one hand, research in this area has revealed biochemical signal mechanisms responsible for mediating crucial events in the lives of insects, such as cellular immune reactions to infection, ovarian development, and releasing egg-laying behavior in some insect species. Plainly, continued interest in this topic will yield new knowledge about how insects modulate a large variety of physiological actions.

Analysis of eicosanoid biosynthesis suggests that eicosanoids act in most insect tissues. For example, Büyükgüzel *et al.* (2002) characterized PG biosynthesis in midgut tissue from tobacco hornworms, showing that the alimentary canals of at least some insects are able to produce PGs. It is reasonable to now question the roles of these PGs in insect midguts.

Beyond gaining new appreciation of insect function, most invertebrate groups are far older than mammals. Although eicosanoids were first discovered in mammals, the long evolutionary history of eicosanoid actions in invertebrates has provided ample opportunity to recruit eicosanoids into many biological roles, most of which remain to be discovered. For example at least one PG serves as a post-ovulatory pheromone in some fish species. Do PGs serve as pheromones in any other animal group? Another example is this: it is thought that some corals produce very high levels of tissue PGs, which serve to reduce herbivory by inducing vomiting in fish which feed on the coral. In these cases it appears that PGs mediate some protective ecological interactions. There are other examples of PG actions in invertebrates and lower vertebrates which are not seen in mammals, and their study should lead to the elucidation of new roles for PGs in the future.

Work on invertebrate eicosanoid systems also will produce new information on fundamental issues. Efforts to clone a gene encoding COX from insect tissues (collaboration between David Stanley and Kulliki Varvas in Tallinn, Estonia) have been frustrated by the substantial differences among the mammalian genes that encode COX and their insect counterparts. Work on insects and other invertebrates will likely turn up a large number of COX isoforms, all of which are unknown at present. This work will enable scientists to better understand the organization and evolution of vertebrate and invertebrate COXs. Similarly, molecular studies of receptors for PGs and other eicosanoids

should enhance our understanding of intracellular signal transduction mechanisms substantially.

Research into insect eicosanoid systems may also have important practical applications. In the early days of work on PGs in insects, it was not at all clear how this information might be integrated into insect pest management schemes. The work by Park and Kim (Park and Kim, 2000; Park *et al.*, 2003) opens possibilities for the discovery of natural products that impair insect immunity by inhibiting eicosanoid biosynthesis. Moreover, their work suggests mechanisms for the delivery of the products in a targeted way to pest populations. This may be the only first inkling of future opportunity to apply our understanding of eicosanoid systems.

Studies of PGs may teach us a great deal about how insects work, may generate information on fundamental aspects of eicosanoid biosynthesis and mechanisms of action, and may yield information of practical importance in agricultural and medical entomology. Young scientists are entering this emerging field, and these investigators will surely make truly exciting discoveries in the future. I look forward to discussing these discoveries in the future editions of this series.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., *et al.*, **1994**. Molecular Biology of the Cell, 3rd edn. Garland, New York.

Armstrong, P.B., Melchior, R., Quigley, J.P., **1996**. Humoral immunity in long-lived arthropods. *J. Insect Physiol. 42*, 53–64.

Baines, D., DeSantis, T., Downer, R.G.H., **1992**. Octopamine and 5-hydroxy-tryptamine enhance the phagocytic and nodule formation activities of cockroach (*Periplaneta americana*) haemocytes. *J. Insect Physiol. 11*, 905–914.

Baines, D., Downer, R.G.H., **1994**. Octopamine enhances phagocytosis in cockroach hemocytes: involvement of inositol triphosphate. *Arch. Insect Biochem. Physiol. 22*, 249–261.

Balsinde, J., Balboa, M.A., Insel, P.A., Dennis, E.A., **1999**. Regulation and inhibition of phospholipase A_2. *Annu. Rev. Pharmacol. Toxicol. 39*, 175–198.

Bergstrom, S., Dressler, F., Krabisch, C., Ryhage, R., Sjovall, J., **1962a**. The isolation and structure of a smooth muscle simulating factor in normal sheep and pig lungs. *Ark. Kem. 20*, 63–66.

Bergstrom, S., Ryhage, R., Samuelsson, B., Sjovall, J., **1962b**. The structure of prostaglandin E, F_1 and F_2. *Acta Chem. Scandin. 16*, 501–502.

Berridge, M.J., **1970**. The role of 5-hydroxytryptamine and cyclic AMP in the control of fluid secretion by isolated salivary glands. *J. Exp. Biol. 53*, 171–186.

Berridge, M.J., **1993**. Inositol triphosphate and calcium signalling. *Nature 361*, 315–325.

Bhattacharya, M., Varma, D.R., Chemtob, S., **1999**. Nuclear prostaglandin receptors. *Gene Therapy Mol. Biol. 4*, 323–338.

Blee, E., **1998**. Phytooxylipins and plant defense reactions. *Prog. Lipid Res. 37*, 33–72.

Blenau, W., Baumann, A., **2001**. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol. 48*, 13–38.

Boman, H.G., Hultmark, D., **1987**. Cell-free immunity in insects. *Annu. Rev. Microbiol. 41*, 103–126.

Bonvalet, J.-P., Pradelles, P., Farman, N., **1987**. Segmental synthesis and actions of prostaglandins along the nephron. *Am. J. Physiol. 253*, F377–F387.

Botting, R.M., **2002**. Mechanism of action of acetaminophen: is there a COX 3? *Clin. Infect. Diseases 31*, S202–S210.

Brehelin, M. (Ed.) **1986**. Immunity in Invertebrates. Springer-Verlag, New York.

Brenner, X., Bernasconi, Z., **1989**. Prostaglandin biosynthesis in the gonads of the hematophagous insect *Triatoma infestans*. *Comp. Biochem. Physiol. 93B*, 1–4.

Breyer, R.M., Bagdassarian, C.K., Myers, S.A., Breyer, M.D., **2001**. Prostanoid receptors: subtypes and signaling. *Annu. Rev. Pharmacol. Toxicol. 41*, 661–690.

Brody, T., Cravchik, A., **2000**. *Drosophila melanogaster* G protein-coupled receptors. *J. Cell Biol. 150*, F83–F88.

Büyükgüzel, K., Tunaz, H., Putnam, S.M., Stanley, D.W., **2002**. Prostaglandin biosynthesis by midgut tissue isolated from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 435–443.

Carton, Y., Frey, F., Stanley, D.W., Vass, E., Nappi, A.J., **2002**. Dexamethasone inhibition of the cellular immune response of *Drosophila melanogaster* against a parasitoid. *J. Parasitol. 288*, 405–407.

Chandrasekharan, N.V., Dai, H., Roos, K.L.T., Evanson, N.K., Tomsik, J., *et al.*, **2002**. COX-3, a COX-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure and expression. *Proc. Natl Acad. Sci. USA 99*, 13926–13931.

Chilton, F.H., Murphy, R.C., **1986**. Remodeling of arachidonate-containing phosphoglycerides within the human neutrophil. *J. Biol. Chem. 261*, 7771–7777.

Coleman, R.A., Kennedy, L., Humphrey, P.P.A., Bunce, K., Lumley, P., **1990**. Prostanoids and their receptors. In: Transch, C., Sammes, F.G., Taylor, J. (Eds.), Comprehensive Medicinal Chemistry, vol. 3. Pergamon Press, Oxford, p. 643.

Conte, A., Ottaviani, E., **1995**. Nitric oxide synthase activity in molluscan hemocytes. *FEBS Lett. 365*, 120–124.

Corey, E.J., Albright, J.O., Barton, A.E., Hashimoto, S., **1980**. Chemical and enzymic syntheses of 5-HPETE, a key biological precursor of slow-reacting substance of anaphylaxis (SRS) and 5-HETE. *J. Am. Chem. Soc. 102*, 1435–1436.

Dadd, R.H., **1973**. Insect nutrition: current developments and metabolic implications. *Annu. Rev. Entomol. 18*, 331–420.

Dadd, R.H., **1977**. Quantitative requirements and utilization of nutrients: insects. In: Rechcigl, M. (Ed.), Handbook Series in Nutrition and Food, vol. 1. CRC Press, Boca Raton, FL, p. 305.

Dadd, R.H., **1981**. Essential fatty acids for mosquitoes, other insects and vertebrates. In: Bhaskaran, G., Friedman, S., Rodrigue, J.G. (Eds.), Current Topics in Insect Endocrinology and Nutrition. Plenum Press, New York, pp. 184–214.

Dadd, R.H., **1983**. Essential fatty acids: insects and vertebrates compared. In: Mittler, T.E., Dadd, R.H. (Eds.), Metabolic Aspects of Lipid Nutrition in Insects. Westview Press, Boulder, pp. 107–147.

Dadd, R.H., **1985**. Nutrition: organisms. In: Kerkut, G. A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 4. Pergamon Press, Oxford, pp. 313–390.

Dadd, R.H., Kleinjan, J.E., **1979**. Essential fatty acid for the mosquito *Culex pipiens*: arachidonic acid. *J. Insect Physiol. 25*, 495–502.

Dalton, T., **1977a**. Threshold and receptor reserve in the action of 5-hydroxytryptamine on the salivary glands of *Calliphora erythrocephala*. *J. Insect Physiol. 23*, 625–631.

Dalton, T., **1977b**. The effect of prostaglandin E_1 on cyclic AMP production in the salivary glands of *Calliphora erythrocephala*. *Experientia 33*, 1329–1330.

Dean, P., Gadsden, J.C., Richards, E.H., Edwards, J.P., Charnley, A.K., *et al.*, **2002**. Modulation by eicosanoids of immune responses by the insect *Manduca sexta* to the pathogenic fungus *Metarhizium anisopliae*. *J. Invert. Pathol. 79*, 93–101.

Dennis, E.A., **1994**. Diversity of group types, regulation, and function of phospholipase A_2. *J. Biol. Chem. 269*, 13057–13060.

Dennis, E.A., **1997**. The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends Biochem. Sci. 22*, 1–2.

Destephano, D.B., Brady, U.E., **1977**. Prostaglandin and prostaglandin synthetase in the cricket, *Acheta domesticus*. *J. Insect Physiol. 23*, 905–911.

Destephano, D.B., Brady, U.E., Woodall, L.B., **1976**. Partial characterization of prostaglandin synthetase in the reproductive tract of the male house cricket, *Acheta domesticus*. *Prostaglandins 11*, 261–273.

Diehl-Jones, W.L., Mandato, C.A., Whent, G., Downer, R.G.H., **1996**. Monoaminergic regulation of hemocyte activity. *J. Insect Physiol. 42*, 12–19.

Dunn, P.E., **1986**. Biochemical aspects of insect immunity. *Annu. Rev. Entomol. 31*, 321–339.

Dunn, P.E., Drake, D.R., **1983**. Fate of bacteria injected into naive and immunized larvae of the tobacco hornworm *Manduca sexta*. *J. Invert. Pathol. 41*, 77–85.

Dunphy, G.B., Downer, R.G.H., **1994**. Octopamine: a modulator of the haemocytic nodulation response of non-immune *Galleria mellonella* larvae. *J. Insect Physiol. 40*, 267–272.

Ferreira, S.H., Moncada, S., Vane, J.R., **1971**. Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature New Biol. 231*, 237–239.

Gadelhak, G.G., Pedibhotla, V.K., Stanley-Samuelson, D.W., **1995**. Eicosanoid biosynthesis by hemocytes from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 743–749.

Gadelhak, G.G., Stanley-Samuelson, D.W., **1994**. Incorporation of polyunsaturated fatty acids into phospholipids of hemocytes from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 775–785.

Gerwick, W.H., **1993**. Carbocyclic oxylipins of marine origin. *Chem. Rev. 93*, 1807–1823.

Gillespie, J.P., Kanost, M.R., Trenczek, T., **1997**. Biological mediators of insect immunity. *Annu. Rev. Entomol. 42*, 611–643.

Glasgow, W.C., Eling, T.E., **1990**. Epidermal growth factor stimulates linoleic acid metabolism in BALBL/c 3T3 fibroblasts. *Mol. Pharmacol. 38*, 503–510.

Glasgow, W.C., Hui, R., Kameda, H., Eling, T.E., **2002**. 13(S)-HpODE modulates mitogenic signal transduction through enhancing the phosphorylation and association of EGF receptor with the tyrosine phosphatase SHP-2. *Adv. Exp. Med. Biol. 507*, 463–467.

Goldblatt, M.W., **1933**. A depressor substance in seminal fluid. *Chem. Industry 52*, 1056–1057.

Goldsworthy, G., Mullen, L., Opoku-Ware, K., Chandrakant, S., **2003**. Interactions between the endocrine and immune systems in locusts. *Physiol. Entomol. 28*, 54–61.

Gupta, A.P. (Ed.) **1986**. Hemocytic and Humoral Immunity in Arthropods. John Wiley, New York.

Gupta, A.P. (Ed.) **1991**. Immunology of Insects and Other Arthropods. CRC Press, Boca Raton, FL.

Halwani, A.E., Dunphy, G.B., **1999**. Apolipophorin-III in *Galleria mellonella* potentiates haemolymph lytic activity. *Devel. Comp. Immunol. 23*, 563–570.

Hatae, N., Sugimoto, Y., Ichikawa, A., **1989**. Prostglandin receptors: advances in the study of EP3 receptor signaling. *J. Biochem. (Tokyo) 131*, 781–784.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, **2002**. G protein-coupled receptors in *Anopheles gambiae*. *Science 298*, 176–178.

Hinton, H.E., **1960**. Cryptobiosis in the larva of *Polypedilum vanderplanki* Hint. (Chironomidae). *J. Insect Physiol. 5*, 286–300.

Hoffmann, J.A., Reichart, J.M., **2002**. *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunol. 3*, 121–126.

Horohov, D.W., Dunn, P.E., **1983**. Phagocytosis and nodule formation by hemocytes of *Manduca sexta* larvae following injection of *Pseudomonas aeruginosa*. *J. Invert. Pathol. 41*, 203–213.

Howard, R.W., Stanley-Samuelson, D.W., **1996**. Fatty acid composition of the fat body and Malpighian tubules of the tenebrionid beetle, *Zophobas atratus*:

significance in eicosanoid-mediated physiology. *Comp. Biochem. Phyiosol. B 115*, 429–437.

Howard, R.W., Witters, N.A., Stanley-Samuelson, D.W., **1992**. Immunohistochemical localization of PGE_2 and $PGF_{2\alpha}$ and fatty acid compositions of Malpighian tubule phospholipids from laboratory and feral populations of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). *Ann. Entomol. Soc. America 85*, 489–498.

Hucker, H.B., Zacchei, A.G., Cox, S.V., Brodie, D.A., Cantwell, N.H.R., **1966**. Studies on the absorption, distribution and excretion of indomethacin in various species. *J. Pharmacol. Exp. Therapy 153*, 237–249.

Hurst, J.S., Flatman, S., McDonald-Gibson, R.G., **1987**. Thin-layer chromatography (including radio thin-layer chromatography and autoradiography) of prostaglandins and related compounds. In: Benedetto, C., McDonald-Gibson, R.G., Nigam, S., Slater, T.F. (Eds.), Prostaglandins and Related Substances: A Practical Approach. IRL Press, Oxford, pp. 53–73.

Izawa, Y., Uchida, M., Yasui, M., **1986**. Mode of action of buprofezin on the twenty-eight spotted ladybird, *Henosepilachna vigintioctopunctata* Fabricius. *Agric. Biol. Chem. 50*, 1369–1371.

Jurenka, R.A., Miller, J.S., Pedibhotla, V.K., Rana, R.L., Stanley-Samuelson, D.W., **1997**. Eicosanoids mediate microaggregation and nodulation responses to bacterial infections in black cutworms, *Agrotis ipsilon*, and true armyworms, *Pseudaletia unipuncta*. *J. Insect Physiol. 43*, 125–133.

Jurenka, R.A., Pedibhotla, V.K., Stanley, D.W., **1999**. Prostaglandin production in response to a bacterial infection in true armyworm larvae. *Arch. Insect Biochem. Physiol. 41*, 225–232.

Kanai, N., Lu, R., Satrian, J.A., Bao, Y., Wolkoff, A.W., *et al.*, **1995**. Identification and characterization of a prostaglandin transporter. *Science 268*, 866–869.

Kaya, H.K., Gaugler, R., **1993**. Entomopathogenic nematodes. *Annu. Rev. Entomol. 38*, 181–206.

Kirschner, L.B., **1991**. Water and ions. In: Prosser, C. L. (Ed.), Comparative Animal Physiology, 4th edn: Environmental and Metabolic Animal Physiology. Wiley–Liss, New York, pp. 13–107.

Kurzrok, R., Lieb, C.C., **1930**. Biochemical studies of human semen. 2. The action of semen on the human uterus. *Proc. Soc. Exp. Biol. Med. 28*, 268–272.

Lackie, A.M., **1988**. Hemocyte behaviour. *Adv. Insect Physiol. 21*, 85–178.

Lemaitre, B., Reichhart, J.-M., Hoffmann, J.A., **1997**. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl Acad. Sci. USA 94*, 14614–14619.

Levine, M.D., Strand, M.R., **2002**. Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol. 32*, 1295–1309.

Loher, W., Ganjian, I., Kubo, I., Stanley-Samuelson, D.W., Tobe, S.S., **1981**. Prostaglandins: their role in egg-laying in the cricket *Teleogryllus commodus*. *Proc. Natl Acad. Sci. USA 78*, 7835–7838.

Lord, J.C., Anderson, S., Stanley, D.W., **2002**. Eicosanoids mediate *Manduca sexta* cellular response to the fungal pathogen *Beauveria bassiana*: a role for the lipoxygenase pathway. *Arch. Insect Biochem. Physiol. 51*, 46–54.

Mandato, C.A., Diehl-Jones, W.L., Moore, S.J., Downer, R.G.H., **1997**. The effects of eicosanoid biosynthesis inhibitors on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*. *J. Insect Physiol. 43*, 1–8.

Medeiros, M.N., Oliveira, M.P., Paiva-Silva, G.O., Silva-Neto, M.A.C., Romeiro, A., *et al.*, **2002**. The role of eicosanoids on *Rhodnius* heme-binding protein (RHBP) endocytosis by *Rhodnius prolixus* ovaries. *Insect Biochem. Mol. Biol. 32*, 537–545.

Metters, K.M., **1995**. Leukotriene receptors. *J. Lipid Mediators Cell Signal. 12*, 413–427.

Miller, J.S., Howard, R.W., Nguyen, T., Nguye, A., Rosario, R.M.T., *et al.*, **1996**. Eicosanoids mediate nodulation responses to bacterial infections in larvae of the tenebrionid beetle, *Zophobas atratus*. *J. Insect Physiol. 42*, 3–12.

Miller, J.S., Howard, R.W., Rana, R.L., Tunaz, H., Stanley, D.W., **1999**. Eicosanoids mediate nodulation reactions to bacterial infections in adults of the cricket, *Gryllus assimilis*. *J. Insect Physiol. 45*, 75–83.

Miller, J.S., Nguyen, T., Stanley-Samuelson, D.W., **1994**. Eicosanoids mediate insect nodulation responses to bacterial infections. *Proc. Natl Acad. Sci. USA 91*, 12418–12422.

Miller, J.S., Stanley, D.W., **1998a**. Eicosanoids in animal reproduction: what can we learn from invertebrates? In: Rowley, A.F., Kuhn, H., Schewe, T. (Eds.), Eicosanoids and Related Compounds in Plants and Animals. Portland Press, London, pp. 183–196.

Miller, J.S., Stanley, D.W., **1998b**. The nodule formation reaction to bacterial infection: assessing the role of eicosanoids. In: Wiesner, A., Dunphy, G.B., Marmaras, V.J., Morishima, I., Sugumaran, M., Yamakawa, M. (Eds.), Techniques in Insect Immunology. SOS Publications, Fair Haven, pp. 265–270.

Miller, J.S., Stanley, D.W., **2001**. Eicosanoids mediate microaggregation reactions to bacterial challenge in isolated insect hemocyte preparations. *J. Insect Physiol. 47*, 61–69.

Morishima, I., Yamano, Y., Inoue, K., Matsuo, N., **1997**. Eicosanoids mediate induction of immune genes in the fat body of the silkworm, *Bombyx mori*. *FEBS Lett. 419*, 83–86.

Murtaugh, M.P., Denlinger, D.L., **1982**. Prostaglandin E and $F_{2\alpha}$ in the house cricket and other insects. *Insect Biochem. 12*, 599–603.

Negishi, M., Koizumi, T., Ichikawa, A., **1995a**. Biological actions of Δ^{12}-prostaglandin J_2. *J. Lipid Mediators Cell Signal. 12*, 443–448.

Negishi, M., Sugimoto, Y., Ichikawa, A., **1993**. Prostanoid receptors and their biological actions. *Prog. Lipid Res. 32*, 414–434.

Negishi, M., Sugimoto, Y., Ichikawa, A., **1995b**. Molecular mechanisms of diverse actions of prostanoid receptors. *Biochim. Biophys. Acta 1259*, 109–120.

Nor Aliza, A.R., Bedick, J.C., Rana, R.L., Tunaz, H., Hoback, W.W., *et al.*, **2001**. Arachidonic and eicosapentaenoic acids in tissues of the firefly, *Photinus pyralis* (Insecta: Coleoptera). *Comp. Biochem. Physiol. A 128*, 251–257.

Oates, J.A., **1982**. The 1982 Nobel Prize in physiology or medicine. *Science 218*, 765–768.

Ogg, C.L., Howard, R.W., Stanley-Samuelson, D.W., **1991**. Fatty acid composition and incorporation of arachidonic acid into phospholipids of hemocytes from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. 21*, 109–114.

Ogg, C.L., Stanley-Samuelson, D.W., **1992**. Phospholipid and triacylglycerol fatty acid compositions of the major life stages and selected tissues of the tobacco hornworm *Manduca sexta*. *Comp. Biochem. Physiol. B 101*, 345–351.

Otto, J.C., Smith, W.L., **1995**. Prostaglandin endoperoxide synthases-1 and -2. *J. Lipid Mediators Cell Signal. 12*, 139–156.

Pace-Asciak, C.R., Asotra, S., **1989**. Biosynthesis, catabolism, and biological properties of HPETEs, hydroperoxide derivatives of arachidonic acid. *Free Radical Biol. Med. 7*, 409–433.

Park, Y., Kim, Y., **2000**. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. *J. Insect Physiol. 46*, 1469–1476.

Park, Y., Kim, Y., Putnam, S.M., Stanley, D.W., **2003**. The bacterium *Xenorhabdus nematophilus* depresses nodulation reactions to infection by inhibiting eicosanoid biosynthesis in tobacco hornworms, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 52*, 71–80.

Petzel, D.H., **1993**. Prostanoids and fluid balance in insects. In: Stanley-Samuelson, D.W., Nelson, D.R. (Eds.), Insect Lipids, Chemisry, Biochemistry and Biology. University of Nebraska Press, Lincoln, NE, pp. 139–178.

Petzel, D.H., Parrish, A.K., Ogg, C.L., Witters, N.A., Howard, R.W., *et al.*, **1993**. Arachidonic acid and prostaglandin E_2 in Malpighian tubules of female yellow fever mosquitoes. *Insect Biochem. Mol. Biol. 25*, 431–437.

Petzel, D.H., Stanley-Samuelson, D.W., **1992**. Inhibition of eicosanoid biosynthesis modulates basal fluid secretion in the Malpighian tubules of the yellow fever mosquito (*Aedes aegypti*). *J. Insect Physiol. 38*, 1–8.

Phillips, J.E., **1980**. Epithelial transport and control of recta of terrestrial insects. In: Locke, M.L., Smith, D.E. (Eds.), Insect Biology of the Future. Academic Press, New York, pp. 145–177.

Prince, W.T., Berridge, M.J., Ramussen, H., **1972**. Role of calcium and adenosine 3′-5′cyclic monophosphate in controlling fly salivary gland secretion. *Proc. Natl Acad. Sci. USA 69*, 553–557.

Qian, Y., Essenberg, R.C., Dillwith, J.W., Bowman, A.S., Sauer, J.R., **1997**. A specific prostaglandin E_2 receptor and its role in modulating salivary secretion in the female tick, *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol. 27*, 387–395.

Qian, Y., Yuan, J., Essenberg, R.C., Bowman, A.S., Shook, A.L., *et al.*, **1998**. Prostaglandin E_2 in the salivary glands of the female tick, *Amblyomma americanum* (L.): calcium mobilization and exocytosis. *Insect Biochem. Mol. Biol. 28*, 221–228.

Radallah, D., Nogaro, M., Fournier, B., **1995**. Arachidonic acid and prostaglandin E_2 stimulate phospholipase C activity in the rectum of the African locust, *Locusta migratoria migratorioides*. *J. Insect Physiol. 41*, 7–16.

Roeder, T., **1999**. Octopamaine in invertebrates. *Prog. Neurobiol. 59*, 533–561.

Rowley, A.F., **1996**. The evolution of inflammatory mediators. *Mediat. Inflamm. 5*, 3–13.

Samuelsson, B., **1983**. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science 220*, 568–575.

Sauer, J.R., McSwain, J.L., Bowman, A.S., Essenberg, R.C., **1995**. Tick salivary gland physiology. *Annu. Rev. Entomol. 40*, 245–267.

Schleusener, D.R., Stanley-Samuelson, D.W., **1996**. Phospholipase A_2 in hemocytes of the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol. 33*, 63–74.

Simmons, D.L., Botting, R.M., Robertson, P.M., Madsen, M.L., Vane, J.R., **1999**. Induction of an acetaminopohen-sensitive COX with reduced sensitivity to nonsteroid anti-inflammatory drugs. *Proc. Natl Acad. Sci. USA 96*, 3275–3280.

Smith, W.L., Garavito, R.M., DeWitt, D.L., **1996**. Prostaglandin endoperoxide H synthases (COXs)-1 and -2. *J. Biol. Chem. 271*, 33157–33160.

Smith, W.L., Marnett, L.J., **1991**. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta. 1083*, 1–17.

Smith, W.L., Borgeat, P., Fitzpatrick, F.A., **1991**. The eicosanoids: cyclooxygenase, lipoxygenase, and epoxygenase pathways. In: Vance, D.E., Vance, J.E. (Eds.), Biochemistry of Lipids, Lipoproteins, and Membranes. Elsevier, Amsterdam, pp. 297–325.

Sorensen, P.W., Hara, T.J., Stacey, N.E., Goetz, F.W., **1988**. F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. *Biol. Reprod. 39*, 1039–1050.

Spector, A.A., Gordon, J.A., Moore, S.A., **1988**. Hydroxyeicosatetraenoic acids (HETEs). *Prog. Lipid Res. 27*, 271–323.

Stanley, D.W., **2000**. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ.

Stanley, D.W., Hoback, W.W., Bedick, J.C., Tunaz, H., Rana, R.L., *et al.*, **1999**. Eicosanoids mediate nodulation reactions to bacterial infections in larvae of the butterfly, *Colias eurytheme*. *Comp. Biochem. Physiol. C 123*, 217–223.

Stanley, D.W., Howard, R.W., **1998**. The biology of prostaglandins and related eicosanoids in invertebrates:

cellular, organismal and ecological actions. *Am. Zool. 38*, 369–381.
Stanley, D.W., Nor Aliza, A.R., Tunaz, H., Putnam, S.M., Park, Y., *et al.*, **2002**. Eicosanoids in insect biology. *Neotrop. Entomol. 31*, 341–350.
Stanley-Samuelson, D.W., **1987**. Physiological roles of prostaglandins and other eicosanoids in invertebrates. *Biol. Bull. 173*, 92–109.
Stanley-Samuelson, D.W., **1991**. Comparative eicosanoid physiology in invertebrate animals. *Am. J. Physiol. 260*, R849–R853.
Stanley-Samuelson, D.W., **1994a**. Prostaglandins and related eicosanoids in insects. *Adv. Insect Physiol. 24*, 115–212.
Stanley-Samuelson, D.W., **1994b**. Assessing the significance of prostaglandins and other eicosanoids in insect physiology. *J. Insect Physiol. 40*, 3–11.
Stanley-Samuelson, D.W., Dadd, R.H., **1983**. Long-chain polyunsaturated fatty acids: patterns of occurrence in insects. *Insect Biochem. 13*, 549–558.
Stanley-Samuelson, D.W., Dadd, R.H., **1984**. Polyunsaturated fatty acids in the lipids from adult *Galleria mellonella* reared on diets to which only one unsaturated fatty acid had beed added. *Insect Biochem. 14*, 321–327.
Stanley-Samuelson, D.W., Jensen, E., Nickerson, K.W., Tiebel, K., Ogg, C.L., *et al.*, **1991**. Insect immune response to bacterial infection is mediated by eicosanoids. *Proc. Natl Acad. Sci. USA 88*, 1064–1068.
Stanley-Samuelson, D.W., Jurenka, R.A., Blomquist, G.J., Loher, W., **1987**. Sexual transfer of prostaglandin precursor in the field cricket, *Teleogryllus commodus*. *Physiol. Entomol. 12*, 347–354.
Stanley-Samuelson, D.W., Jurenka, R.A., Cripps, C., Blomquist, G.J., de Renobales, M., **1988**. Fatty acids in insects: composition, metabolism, and biological significance. *Arch. Insect Biochem. Physiol. 9*, 1–33.
Stanley-Samuelson, D.W., Loher, W., **1983**. Arachidonic and other long-chain polyunsaturated fatty acids in spermatophores and spermathecae of *Teleogryllus commodus*: significance in prostaglandin-mediated reproductive behavior. *J. Insect Physiol. 29*, 41–45.
Stanley-Samuelson, D.W., Loher, W., **1986**. Prostaglandins in insect reproduction. *Ann. Entomol. Soc. America 79*, 841–853.
Stanley-Samuelson, D.W., Ogg, C.L., **1994**. Prostaglandin biosynthesis by fat body from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 24*, 481–491.
Stanley-Samuelson, D.W., Pedibhotla, V.K., **1996**. What can we learn from prostaglandins and related eicosanoids in insects? *Insect Biochem. Mol. Biol. 26*, 223–234.
Stanley-Samuelson, D.W., Pedibhotla, V.K., Rana, R.L., Nor Aliza, A.R., Hoback, W.W., *et al.*, **1997**. Eicosanoids mediate nodulation responses to bacterial infections in larvae of the silkmoth, *Bombyx mori*. *Comp. Biochem. Physiol. A 118*, 93–100.
Strand, M.R., Pech, L.L., **1995**. Immunological basis for compatibility in parasitoid–host relationships. *Annu. Rev. Entomol. 40*, 31–56.
Tanabe, T., Ullrich, V., **1995**. Prostacyclin and thromboxane synthases. *J. Lipid Mediators Cell Signal. 12*, 243–255.
Tauszig, S., Jauanguy, E., Hoffmann, J.A., Imler, J.L., **2000**. Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*. *Proc. Natl Acad. Sci. USA 97*, 10520–10525.
Thornhill, R., Alcock, R., **1983**. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge, MA.
Tobe, S.S., Loher, W., **1983**. Properties of the prostaglandin synthetase complex in the cricket *Teleogryllus commodus*. *Insect Biochem. 13*, 137–141.
Torfs, H., Oonk, H.B., Vanden Broeck, J., Poels, J., Van Poyer, W., *et al.*, **2001**. Pharmacological characterization of STKR, an insect G protein-coupled receptors for tachykinin-like peptides. *Arch. Insect Biochem. Physiol. 48*, 39–49.
Tunaz, H., Bedick, J.C., Miller, J.S., Hoback, W.W., Rana, R.L., *et al.*, **1999**. Eicosanoids mediate nodulation reactions to bacterial infections in adults of two 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini*. *J. Insect Physiol. 45*, 923–931.
Tunaz, H., Park, Y., Büyükgüzel, K., Bedick, J.C., Nor Aliza, A.R., *et al.*, **2003**. Eicosanoids in insect immunity: bacterial infection stimulates hemocytic phospholipase A_2 activity in tobacco hornworms. *Arch. Insect Biochem. Physiol. 52*, 1–6.
Tunaz, H., Stanley, D.W., **1999**. Eicosanoids mediate nodulation reactions to bacterial infections in adults of the American cockroach, *Periplaneta americana* (L.). *Proc. Entomol. Soc. Ontario 130*, 97–108.
Uchida, M., Izawa, Y., Sugimoto, T., **1987**. Inhibition of prostaglandin biosynthesis and oviposition by an insect growth regulator, Buprofezin, in *Nilaparvata lugens* Stal. *Pesticide Biochem. Physiol. 27*, 71–75.
Urade, Y., Watanabe, K., Hayaishi, O., **1995**. Prostaglandin D, E, and F synthases. *J. Lipid Mediators Cell Signal. 12*, 257–273.
Uscian, J.M., Stanley-Samuelson, D.W., **1993**. Phospholipase A_2 activity in the fat body of the tobacco hornworm *Manduca sexta*. *Arch. Insect Biochem. Physiol. 24*, 187–201.
Vanden Broeck, J., **2001**. Insect G protein-coupled receptors and signal transduction. *Arch. Insect Biochem. Physiol. 48*, 1–12.
Vane, J.R., **1971**. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol. 231*, 232–235.
Van Kerkhove, E., Pirotte, P., Petzel, D.H., Stanley-Samuelson, D.W., **1995**. Eicosanoid biosynthesis inhibitors modulate basal fluid secretion rates in the Malpighian tubules of the ant, *Formica polyctena*. *J. Insect Physiol. 41*, 435–441.
von Euler, U.S., **1936**. On the specific vasodilating and plain muscle stimulating substances from accessory genital glands in men and certain animals (prostaglandin and vesiglandin). *J. Physiol. (London) 88*, 213–234.
Wakayama, E.J., Dillwith, J.W., Blomquist, G.J., **1986**. Characterization of prostaglandin synthesis in the housefly, *Musca domestica* (L.). *Insect Biochem. 16*, 903–909.

Warner, T.D., Mitchell, J.A., **2002**. COX-3 (COX-3): filling in the gaps toward a COX continuum? *Proc. Natl Acad. Sci. USA 99*, 13371–13373.

Wiesner, A., Losen, S., Kopacek, P., Weise, C., Gotz, P., **1997**. Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect. *J. Insect Physiol. 43*, 383–391.

Yajima, M., Takada, M., Takahashi, N., Kikuchi, H., Natori, S., *et al.*, **2003**. A newly established *in vitro* ulture using transgenic *Drosophila* reveals functional coupling between the phospholipase A_2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. *Biochem. J. 371*, 205–210.

Yamaja Setty, B.N., Ramaiah, T.R., **1979**. Isolation and identification of prostaglandins from the reproductive organs of male silkmoth, *Bombyx mori* (L.). *Insect Biochem. 9*, 613–617.

Yamaja Setty, B.N., Ramaiah, T.R., **1980**. Effect of prostaglandins and inhibitors of prostaglandin biosynthesis on oviposition in the silkmoth *Bombyx mori*. *Ind. J. Exp. Biol. 18*, 539–541.

Yesair, D.W., Callahan, M., Remington, L., Kensler, C.J., **1970**. Comparative effects of salicylic acid, phenylbutazone, probenecid and other anions on the metabolism, distribution and excretion of indomethacin by rats. *Biochem. Pharmacol. 19*, 1591–1600.

4.10 Ferritin

J J Winzerling, University of Arizona, Tucson, AZ, USA
D Q-D Pham, University of Wisconsin-Parkside, Kenosha, WI, USA

4.10.1. Importance of Ferritin

Scientific interest in iron lies in the fact that it is both an essential nutrient and a potential toxin. Iron is required for many metabolic processes as a cofactor of numerous proteins. However, in the presence of oxygen, ferrous is readily oxidized to ferric rendering it insoluble at physiological pH. Ferrous oxidation also can result in formation of hydroxyl radical anions by the Haber–Weiss and Fenton reactions:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet}$$
$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

Hydroxyl radical anions can damage many biological molecules and produce a variety of reactive oxygen species (ROS) (Kehrer, 2000; Aisen *et al.*, 2001). Organisms must have adequate supplies of iron to meet nutritional requirements, and at the same time, prevent this potential cytotoxic effect. This balance is met, in part, by storing iron inside ferritin.

Ferritin is the primary iron storage protein of mammals. It is found predominately in the cell cytoplasm, and influences iron availability and response to oxidative stress (Aisen *et al.*, 2001). In mammalian cells, the loss of ferritin expression is associated with susceptibility to iron-dependent toxicity and increased oxidative stress (DeRusso *et al.*, 1995; Kakhlon *et al.*, 2001). Ferritin overexpression is associated with increased iron deposition, changes in cell growth and improved response to oxidative challenge (Corsi *et al.*, 1998; Epsztejn *et al.*, 1999; Kakhlon *et al.*, 2002; Kato and Niitsu, 2002).

The study of iron metabolism of insects was initiated by the work of Michael Locke and his colleagues (Locke and Leung, 1984; Nichol and Locke, 1989; Huebers *et al.* (1988); Bartfeld and Law, 1990). Since then much progress has been made and several reviews have been written on the subject of insect iron metabolism (Locke and Nichol, 1992; Winzerling and Law, 1997; Nichol *et al.*, 2002). This chapter will focus on the composition, structure, expression, and putative roles of ferritin in insects.

4.10.2. Ferritin Structure and Iron Storage

Insect ferritins are usually compared with the well-characterized vertebrate cytoplasmic ferritins. Vertebrate cytoplasmic ferritins have a mass ≥450 kDa and consist of 24 subunits of ~19 kDa (light chain, L) and ~21 kDa (heavy chain, H) configured as a spherical polymer wherein iron is stored (Harrison and Arosio, 1996; Chasteen and Harrison, 1999). Vertebrate H subunits are characterized by a ferroxidase center which facilitates ferrous oxidation and uptake (Lawson *et al.*, 1989, 1991; Levi *et al.*, 1994; Santambrogio *et al.*, 1996). The vertebrate L subunits have glutamic acid residues that constitute a nucleation site that allows iron deposition within the ferritin core, as well as other residues that form salt bridges that contribute to the structural stability of this large molecule (Trikha *et al.*, 1995; Gallois *et al.*, 1997; Hempstead *et al.*, 1997).

Apoferritin becomes holoferritin when ferrous is oxidized, moved inside the molecule, and forms a crystalline mineral. *In vitro* studies show that Fe^{2+} binds to the glutamate and histidine residues of the ferroxidase center of the H subunits and is rapidly oxidized to Fe^{3+} (Aisen *et al*., 2001). Fe^{3+} is then released from the oxidase site and transported to the nucleation site. Mineralization proceeds as the surface of the growing mineral core catalyzes autooxidation of joining iron molecules. Iron is stored in ferritin as hydroxide polymers:

$$n\mathrm{Fe}^{3+}(\mathrm{OH})_3 \xrightarrow{-n\mathrm{H_2O}} -(\mathrm{FeOOH})_{n-}$$

Although ferritin can accommodate 4500 hydroxyl-bridged iron atoms, isolated mammalian ferritins usually contain only 2000–2500 iron atoms (Aisen *et al*., 2001). Iron is released from ferritin *in vitro* by strong chelators.

How iron is incorporated and released from ferritin *in vivo* has yet to be elucidated. *In vitro* iron loading by the ferroxidase site produces H_2O_2 and oxidative damage to the H subunit (Welch *et al*., 2002). For this reason, it is suggested that *in vivo* iron loading occurs enzymatically and that the ferroxidase site is used only when this process is exceeded (Welch *et al*., 2002). Treatment of ferritin with the 20S proteosome is accompanied by the release of iron (Aisen *et al*., 2001), whether this serves to provide iron for cellular needs is not known.

Insect ferritins have a mass of 450–660 kDa with subunits that range in size from 21 to 36 kDa (Nichol and Locke, 1989; Dunkov *et al*., 1995; Winzerling *et al*., 1995; Charlesworth *et al*., 1997; Du *et al*., 2000). Although crystal structure studies of insect ferritins have not been done, several lines of evidence support the hypothesis that the structure of insect ferritins is similar to that of vertebrate cytoplasmic ferritin. Insect ferritins assume a rosette appearance on electron microscopic analysis that is characteristic of vertebrate ferritins (Nichol and Locke, 1989). Further, the mass of the protein predicted by a combination of 24 subunits agrees with the respective size of the native insect ferritin (Winzerling *et al*., 1995; Nichol and Locke, 1999). In addition, the structure of the insect ferritin subunits is similar to that of the vertebrate ferritin subunits.

4.10.2.1. Comparative Structure of Ferritin Subunits

The terminology identifying the insect ferritin subunits is varied. The subunits with greatest structural similarity to the vertebrate H subunit are referred to in the literature as the heavy chain homolog (HCH) (Dunkov *et al*., 1995; Zhang *et al*., 2001a), S (Nichol and Locke, 1999), Fer-1 (Charlesworth *et al*., 1997), and Fersub1 (Du *et al*., 2000). The amino acid residues that constitute the ferroxidase center of vertebrate H subunits are conserved in all of these subunits (**Table 1**). Insect subunits that retain this feature will be referred to as an HCH.

A second type of insect ferritin subunit that lacks the residues of the ferroxidase site is referred to in the literature as the light chain homolog (LCH) (Pham *et al*., 1996; Georgieva *et al*., 2002b), G (Nichol and Locke, 1999), and Fersub2 (Du *et al*., 2000). We will refer to subunits of this type as an LCH. Although the LCH nomenclature has been used, the LCH subunits show no greater similarity to vertebrate L subunits than to vertebrate H subunits (**Table 2**) and are actually larger than the insect HCHs.

Nucleotide sequences of HCH subunits are available for *Manduca sexta* (hawkmoth, Lepidoptera (Zhang *et al*., 2001a)), *Calpodes ethlius* (skipper butterfly, Lepidoptera (Nichol and Locke, 1999)), *Galleria mellonella* (wax moth, Lepidoptera (Kim *et al*., 2001)), *Nilaparvata lugens* (plant hopper, Hemiptera (Du *et al*., 2000)), *Aedes aegypti* (yellow fever mosquito, Diptera (Dunkov *et al*., 1995)), *Drosophila melanogaster* (fruit fly, Diptera (Charlesworth *et al*., 1997)), *Bombyx mori* (silk moth, Lepidoptera (Nichol *et al*., 2002)), and *Anopheles gambiae* (malaria mosquito, Diptera (Holt *et al*., 2002)). LCH subunit sequences are reported for *M. sexta* (Pham *et al*., 1996), *C. ethlius* (Nichol and Locke, 1999), *G. mellonella* (Kim *et al*., 2002), *B. mori* (Nichol *et al*., 2002), *N. lugens* (Du *et al*., 2000), *D. melanogaster* (Dunkov and Georgieva, 1999; Georgieva *et al*., 2002b), *A. gambiae* (Holt *et al*., 2002) and *A. aegypti* (Geiser *et al*., 2003). A comparison of the deduced amino acid sequences of the HCH and LCH insect subunits shows a much lower percentage of identity for subunits of the same species than for the same subunit among species (**Table 2**).

The structure of the insect ferritin subunits is similar to that of vertebrates (Pham, 2000). Vertebrate ferritin subunits consist of five α-helices (A–E) and an internal loop (Lawson *et al*., 1991; Trikha *et al*., 1994; Gallois *et al*., 1997; Hempstead *et al*., 1997). The modeling programs PHD (Rost and Sander, 1993, 1994a, 1994b; Rost *et al*., 1995) and Swiss-PdbViewer (Peitsch, 1995, 1996; Guex and Peitsch, 1997; Guex *et al*., 1999) detect the first four α-helices (A, B, C, and D) in the HCHs and LCHs from lepidopterans and dipterans (Pham,

Table 1 Characteristics of the insect ferritin subunits

Species (strain)	Subunit name	Deduced mature peptide (~kDa)	Matched N-terminal sequence (~kDa)	Ferroxidase residues	Glycosylation site[a] (ConA)	Iron-responsive element	GenBank accession number	Reference
Aedes aegypti (Bahaman)	HCH	21	24 26	+	–	+	L37082	Dunkov *et al.* (1995)
Aedes aegypti	LCH	23	28	–	+(+)	–	AY171561	Geiser *et al.* (2003)
Calpodes ethlius	HCH (S)	22	24	+	+(–)	+	AF161707	Nichol and Locke, (1999)
Calpodes ethlius	LCH (G)	24	31	–	+(+)	+	AF161709	Nichol and Locke, (1999)
Drosophila melanogaster	HCH	21	25 26	+	–	+	Y15629 U91524	Charlesworth *et al.* (1997)
Drosophila melanogaster	LCH	23	28	–	–	–	AF145124	Georgieva *et al.* (2002b)
Galleria mellonella	HCH	22	26	+	–	+	AF142340	Kim *et al.* (2001)
Galleria mellonella	LCH	27	32	–	+	+	AF329683	Kim *et al.* (2002)
Manduca sexta	HCH	22		+	–	+	AY032659	Zhang *et al.* (2001b)
Manduca sexta	LCH	25		–	+(+)	+	AF270492	Pham *et al.* (1996)
Nilaparvata lugens	HCH (Fersub1)	24		+	+(–)		AJ251148	Du *et al.* (2000)
Nilaparvata lugens	LCH (Fersub2)	24	26	–	+(+GNA)	+	AJ251147	Du *et al.* (2000)

[a]Putative glycosylation site by computer analysis; conA, Concanaolin A; GNA, *Galanthus nivalis*.

Table 2 Comparative identities of insect ferritin subunits

	Species														
	Subunit	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
1	Human H														
2	Human L	55													
3	*Aa* HCH	35	23												
4	*Aa* LCH	17	17	17											
5	*Ce* HCH	36	23	41	16										
6	*Ce* LCH	19	19	16	23	16									
7	*Dm* HCH	35	31	50	18	49	17								
8	*Dm* LCH	27	24	17	28	27	32	23							
9	*Gm* HCH	27	18	39	18	74	19	50	19						
10	*Gm* LCH	21	21	18	26	18	69	35	22	19					
11	*Ms* HCH	43	42	42	20	70	19	52	18	83	22				
12	*Ms* LCH	26	23	24	27	17	67	40	23	20	78	25			
13	*Nl* HCH	30	29	40	16	48	23	42	22	46	14	45	15		
14	*Nl* LCH	21	21	15	29	14	34	34	16	14	34	35	18	14	

Human H, human heavy chain, human H chain (GenBank assession number L20941); Human L, human light chain, human L chain (GenBank assession number M10119); HCH, heavy chain homolog; LCH, light chain homolog; *Aa, Aedes aegypti*, *Ce, Calpodes ethlius; Dm, Drosophila melanogaster; Gm, Galleria mellonella; Ms, Manduca sexta; Nl, Nilaparvata lugens*. Assession numbers are listed in **Table 1**. Reprinted, with permission from the Annual Review of Entomology, Volume 47, © 2002 by Annual Reviews www.annualreviews.org.

2000). However, only the Swiss model recognizes with certainty the fifth helix (E) in both orders of insects.

Although the dipteran and lepidopteran HCHs have diverged from their vertebrate counterparts, they still retain a significant structural similarity. Specifically, the tracing of the HCH carbon backbone is easily superimposed with the human H subunit backbone and the α-helices align as do the different types of amino acid residues (such as nonpolar, polar, acidic, and basic). Further, the seven amino acid residues (four glutamates, tyrosine, histidine, and glutamine) involved in the ferroxidase center maintain the same three-dimensional structure as the vertebrate ferroxidase center (Pham, 2000). Iron loading of ferritin *in vitro* using the ferroxidase site results in free radical-mediated damage of residue C90 of the H subunit and protein aggregation (Welch *et al*., 2002). Interestingly, this residue is not conserved in insect HCH subunits, suggesting that the ferroxidase site could be used for iron oxidation without damage to the subunit at this residue.

Although the α-helices in the insect LCHs are identified by modeling programs, these subunits exhibit less similarity to their vertebrate counterparts than the HCHs (**Table 2**) and lack important features associated with the vertebrate L subunits (Trikha *et al*., 1994; Gallois *et al*., 1997; Hempstead *et al*., 1997). Vertebrate L subunits have a porphyrin-binding pocket associated with ferrihydrite nucleation and residues that form salt bridges that contribute to structural integrity (Andrews *et al*., 1992; Trikha *et al*., 1994; Gallois *et al*., 1997; Hempstead *et al*., 1997). Sequence alignment indicates that the cluster of glutamic acids that functions as the porphyrin-binding pocket in the vertebrate L subunits is missing in insect LCHs (Pham, 2000). The loss of this cluster suggests that nucleation for iron storage occurs differently in insect ferritins. Further, although some amino acid residues that permit salt bridge formation appear to be semiconserved, the substitutions at these sites in insect LCHs make bridge formation unlikely because the salt interaction is lost. Since these bridges maintain the stability of vertebrate ferritins, the loss of these sites suggests that either the insect ferritins are less stable or they are stabilized by other forces. Current data do not support the former hypothesis because insect ferritins have been shown to be quite stable (Nichol and Locke, 1989; Dunkov *et al*., 1995; Winzerling *et al*., 1995). Finally, insect LCHs have a unique attribute: all contain tyrosine kinase phosphorylation sites (Pham, 2000).

In summary, the insect HCHs retain many structural features relevant to iron uptake and storage, whereas, the LCHs maintain neither the porphyrin ring nor the salt bridges that are found in vertebrate L subunits. In addition, significant differences exist in primary structure between the insect LCHs and the vertebrate L subunits, suggesting that species-specific activities of insect ferritins are a function of the LCH.

Most of the insect ferritin subunits identified to date have hydrophobic leader sequences that signal secretion, and secreted ferritin appears to serve important roles in insects. However, an insect ferritin subunit that lacks a secretion signal sequence was identified in the *Drosophila* genome (Adams *et al.*, 2000). The deduced amino acid sequence indicates that this subunit retains the ferroxidase center and is most similar to a vertebrate H subunit (Nichol *et al.*, 2002). A cytosolic ferritin also was described for *Philaenus spumarius* (spittle bug, Homoptera), which consists of a single subunit that is much smaller than other insect ferritin subunits (Collin *et al.*, 1988). The sequence of this subunit has not been reported.

4.10.3. The Biology of Insect Ferritin

4.10.3.1. Roles of Ferritin in Insects

How insects absorb iron and maintain iron homeostasis is unknown. Plant-feeders are exposed to a variety of iron compounds and plant ferritins (Theil, 1987; Andrews *et al.*, 1992; Briat, 1996). Alternatively, hematophagous insects are exposed to an iron load in the forms of hemoglobin and ferric-transferrin. Since various sources of iron are available to insects, the mechanisms of iron absorption and loss among insects are likely to be noticeably different.

Lepidopterans express high levels of ferritin messages in gut tissues (Pham *et al.*, 1996; Nichol and Locke, 1999; Kim *et al.*, 2001, 2002). Iron administration increases gut mRNA expression and, in *C. ethlius* larvae, provokes secretion of holoferritin into the posterior midgut lumen (Locke and Leung, 1984). Locke and colleagues have suggested that iron homeostasis is preserved in these insects by secreting holoferritin into the gut lumen for excretion in the feces (Locke and Leung, 1984; Nichol *et al.*, 2002). Like the lepidopterans, ferritin mRNA and protein are increased in the gut tissues of the dipterans *D. melanogaster* and *Musca domestica* (housefly) in response to iron (Capurro *et al.*, 1996; Georgieva *et al.*, 2002b), and in *A. aegypti* females following a blood meal (Dunkov *et al.*, 2002). However in contrast to lepidopterans, Dunkov *et al.* (2002) found no evidence of ferritin in the gut contents of *A. aegypti* after blood feeding. Others have shown that substantial amounts of heme are bound to the peritrophic matrix in *A. aegypti* and lost when the matrix is shed into lumen at the end of blood digestion (Pascoa *et al.*, 2002). The absence of ferritin in the gut contents in these insects probably reflects this alternate mechanism of iron disposal. Interestingly, yet another mechanism limiting iron absorption is found in *Rhodnius prolixus* (kissing bug, Hemiptera), where heme from the blood meal is sequestered in the gut as hemozoin (Oliveira *et al.*, 2000). Despite limiting heme absorption, blood-feeding insects would still receive substantial iron from ferric-transferrin. Iron from this source could account for the increase in gut ferritin expression observed in *A. aegypti*. In any case, insects appear to have developed a variety of mechanisms for maintaining iron homeostasis by limiting iron uptake or by enhancing iron excretion. The secretion of holoferritin into the hindgut, the upregulation of ferritin expression in gut tissues, and the sequestering of heme all represent mechanisms that would protect insects against iron overload and iron-mediated oxidative challenge.

Other roles of insect ferritin in iron metabolism are not clear. In the majority of insects, ferritin is found in the hemolymph suggesting that this protein could be involved in iron movement among body tissues. In *C. ethlius*, apoferritin secreted from fat body becomes holoferritin in the hemolymph (Nichol and Locke, 1999; Locke, 2003). Further, when *M. sexta* larvae are injected with iron, holoferritin in the hemolymph increases and constitutes about 1% of the total protein (Winzerling *et al.*, 1995; Zhang *et al.*, 2001b). Similarly, in *D. melanogaster* (Georgieva *et al.*, 2002b) and *A. aegypti* (Dunkov *et al.*, 2002), hemolymph ferritin is increased following exposure to iron. Together these findings indicate that iron exposure increases ferritin in the hemolymph, that ferritin can be loaded with iron in this tissue, and that ferritin serves as the major iron-sequestering protein in insects. By sequestering iron in hemolymph, ferritin also could serve to protect insects from iron overload and subsequent oxidative stress, as well as to limit the iron available to invading pathogens.

The tissue source of hemolymph ferritin could vary with the insect species. In lepidopterans, hemolymph ferritin appears to originate in the fat body (Locke, 1991; Locke and Nichol, 1992; Nichol and Locke, 1999). Ferritin expression is increased in fat body in response to iron (Huebers *et al.*, 1988; Locke *et al.*, 1991; Pham *et al.*, 1996; Nichol and Locke, 1999) and apoferritin is secreted from the fat body into the hemolymph (Nichol and Locke, 1999; Locke, 2003). In *Apis mellifera* (honey bee, Hymenoptera (Keim *et al.*, 2002)), as well as several other insects, holoferritin also is found in the secretory pathway of fat body tissue (Locke and Leung, 1984; Locke and Nichol, 1992; Locke, 2003). However, in *D. melanogaster*, ferritin mRNA and protein are very low in fat body, but are high in gut tissues (Georgieva *et al.*, 2002b). Georgieva *et al.* (2002b)

have suggested that in these insects hemolymph ferritin could originate from the gut. Regardless of the source, the secretion of ferritin from insect cells into the hemolymph appears crucial to insect iron metabolism.

Locke has reviewed the secretory pathway in insects (Locke, 2003). In dictyopterans, dipterans, coleopterans, hemipterans, and lepidopterans, intracellular holoferritin is rarely seen in the cytosol or the nuclear compartment, but is consistently observed in the secretory pathway (Nichol and Locke, 1990; Locke and Nichol, 1992). Only in *P. spumarius* are similar levels of cytosolic and nuclear holoferritin observed (Nichol and Locke, 1990). Insect holoferritin has been found in the rough endoplasmic reticulum (RER), Golgi complex, and secretory vesicles (Locke and Leung, 1984; Locke and Nichol, 1992; Nichol *et al.*, 2002). Apparently, ferritin assembles in the RER, but is not secreted immediately. How 24 subunits come together in an orderly manner in the lumen of the RER remains unknown. The subunits from several insects have cysteine residues in the N-terminus that could serve as acylation sites (Nichol and Locke, 1999; Nichol *et al.*, 2002). Nichol and Locke (1999) showed that ferritin subunits from *C. ethlius* can be acylated *in vitro*. In addition, computational analysis predicts that some of the subunits have myristoylation sites, as well as tyrosine kinase phosphorylation sites that could be involved in ferritin assembly and secretion (Pham, 2000). Reversible associations of myristoylated proteins with plasma membranes have been observed (Trowler *et al.*, 1988; Peitzsch and McLaughlin, 1993). Perhaps some subunits are reversibly associated with the inner membrane through a myristoylated site allowing ferritin assembly, and in some cases, iron loading. Once this process is finished, ferritin detaches and is secreted. Possibly, conformational changes occur with phosphorylation/dephosphorylation of the tyrosine kinase sites of the LCH subunits that results in detachment from the membrane. Protein detachment from membranes following conformational changes caused by phosphorylation/dephosphorylation at tyrosine kinase sites has been documented (Hubbard *et al.*, 1998).

Another potential role for ferritin in insects could be the provision of iron for the developing embryo. In vertebrates, iron is required for growth and deficiency results in developmental defects. In dipterans, ferritin is expressed in the ovaries and is found in eggs (Dunkov *et al.*, 2002; Georgieva *et al.*, 2002b) and its presence suggests that iron stored in ferritin can be used by the developing embryo (Dunkov *et al.*, 2002). This appears to differ for lepidopterans, since ferritin is not found in the ovaries of *G. mellonella* (Kim *et al.*, 2001).

In summary, the roles of ferritin in insects are only beginning to be elucidated. The increase in holoferritin in the cell secretory pathway and in hemolymph following iron administration shows that insects load ferritin with iron and suggests that ferritin could be involved in the movement of iron among insect tissues. These findings, as well as the increase in ferritin expression following iron administration in insects from several orders, suggest that ferritin is the primary iron sequestering protein in these animals. Further, the consistent finding that iron elicits ferritin expression in insect gut tissues suggests that gut ferritin could serve roles in iron homeostasis, in iron excretion, and in the protection of some insects from iron overload. Finally, the increase in ferritin expression in eggs and ovaries of some insects indicates a role for ferritin in providing iron for the developing embryo.

4.10.3.2. Ferritin Subunit Messages and Protein Expression

Ferritin purified from *M. sexta* (Winzerling *et al.*, 1995) and *C. ethlius* (Nichol and Locke, 1999) has a mass of ~660 kDA and contains multiple subunits of 24–36 kDa. N-terminus sequencing data indicate that these subunits are the products of two different genes and that the various size differences result from posttranslational processing. The lepidopteran LCH subunits contain *N*-glycosylation sites (Pham *et al.*, 1996; Nichol and Locke, 1999; Kim *et al.*, 2002), and those from *M. sexta* and *C. ethlius* bind concanavalin A (Nichol and Locke, 1989; Pham *et al.*, 1996). Northern blot analyses indicate that LCH mRNA in lepidopterans is expressed in the fat body (Pham *et al.*, 1996; Nichol and Locke, 1999; Kim *et al.*, 2002), hemocytes (Pham *et al.*, 1996), and midgut (Pham *et al.*, 1996; Nichol and Locke, 1999; Kim *et al.*, 2002), with greatest expression in the midgut (Pham *et al.*, 1996; Kim *et al.*, 2002). In *G. mellonella*, LCH message is expressed in the fat body and midgut and is increased with iron treatment (50 mM $FeCl_3$ with an artificial diet). However, an increase in the LCH protein is observed only in the midgut (Kim *et al.*, 2001, 2002). The HCH mRNA also is expressed in these tissues (Kim *et al.*, 2001) and increases after iron feeding. Neither mRNA is detectable in the ovaries or testes.

Purified midgut *N. lugens* (Hemiptera) ferritin has a molecular mass ~440 kDa and dissociates into three subunits of 20, 26, and 27 kDa (Du *et al.*, 2000). N-terminus sequencing is successful for the 26 kDa, but not for the 20 or 27 kDa subunits. The

cDNA clone for the 26 kDa subunit encodes a protein of 236 amino acids with highest homology with the *M. sexta* LCH subunit (Table 2). The sequence has an *N*-glycosylation site and shows strong binding affinity to the lectin *Galanthus nivalis* agglutinin (GNA). The fact that this ferritin subunit is one of the most abundant GNA-binding proteins from the *N. lugens* midgut is of interest since GNA is toxic to *N. lugens* as shown by artificial diet bioassays. Perhaps one plant defense mechanism against insects involves interference with insect iron metabolism by the binding of GNA with ferritin. A second ferritin subunit cDNA clone was identified as part of a strategy to obtain nonabundant GNA-binding proteins from *N. lugens* midgut. This second clone encodes a 227 deduced amino acid sequence with a predicted molecular weight of 24 kDa and a glycosylation site.

In dipterans, ferritin is composed of at least four subunits. The HCH subunits are 24 kDa (Dunkov *et al.*, 1995), 25 kDa (Charlesworth *et al.*, 1997), and 26 kDa (Dunkov *et al.*, 1995), and the cDNA sequences have been obtained for both *Drosophila* (Charlesworth *et al.*, 1997) and *Aedes* (Dunkov *et al.*, 1995). Although the native subunits are greater in mass than predicted by their respective cDNA sequences, none has been shown to be glycosylated.

The expression of the HCH in *Drosophila* is low. It is found in second instar and wandering larvae gut tissues and a trace is seen in adults; it is not expressed in early pupae (Charlesworth *et al.*, 1997; Georgieva *et al.*, 2002b). In contrast, the HCH in *A. aegypti* is highly expressed. The 26 kDa HCH is expressed at all life stages and in all tissues and is responsive to iron administration (Dunkov *et al.*, 2002). Expression is increased in adult females fed an artificial meal with iron supplements or a blood meal. Blood feeding increases HCH expression in the gut wall, fat body, hemolymph, thorax, ovaries, and eggs. The 24 kDa HCH is expressed in mosquito larvae and pupae, increases at these stages following iron administration and increases in adult females following either an artificial meal supplemented with iron or blood feeding (Dunkov *et al.*, 2002). In adult females, expression of the 24 kDa subunit appears limited to the ovaries and eggs. In contrast to the 26 kDa subunit, expression of the 24 kDa subunit is minimal in sugar-fed adult females.

Although the HCH predominates in mosquitoes, the LCH is highly expressed in flies at all life stages (Georgieva *et al.*, 2002b). The cDNA sequence for the *Drosophila* LCH encodes a deduced amino acid sequence that predicts a mature polypeptide of ~23 kDa with an N-terminus that matches that of a 28 kDa subunit (Georgieva *et al.*, 2002b). Since the 28 kDa subunit binds concanavalin A, glycosylation could account for the mass of the native polypeptide. LCH mRNA is expressed in larvae, in light and dark pupae, and in adults. With the exception of dark pupae, the mRNA quantity is responsive to iron treatment. The protein is also present at all stages and, with the exception of eggs and third instar larvae, expression is enhanced modestly with iron treatment. In imagos, the LCH subunit is present in the hemolymph and fat body, gut, ovaries, and testes. Although LCH mRNA content is very low in the larval fat body, the protein is found in this tissue. Both the LCH message and protein are expressed in gut tissues and are strongly upregulated in response to iron (Georgieva *et al.*, 2002b).

A genomic clone of the *A. aegypti* LCH subunit was recently sequenced (Geiser *et al.*, 2003). Similar to the LCH of *Drosophila*, the mosquito cDNA sequence encodes a protein with an N-terminus that matches the *A. aegypti* 28 kDa subunit and the deduced amino acid sequence predicts a mature polypeptide of ~23 kDa. When *A. aegypti* embryonic cultured cells (Aag2 cells) are treated with iron, LCH mRNA is induced and this induction is sustained at 16 h. The LCH message is also expressed in larvae and elicited in blood-fed females in contrast to sugar-fed females. Dunkov *et al.* (2002) reported that the LCH protein is expressed in larvae and pupae only following iron or hemin treatment. Adult females express low levels of this subunit; however, expression increases in female hemolymph and thorax after a blood meal (Dunkov *et al.*, 2002).

Clearly, the expression of ferritin subunits in dipterans differs; perhaps iron exposure could help explain these differences. Flies are rarely exposed to high iron intake, whereas female mosquitoes are exposed to an iron load in a blood meal. In mosquitoes, the HCH subunit could be more highly expressed because rapid iron uptake into ferritin is a priority. In contrast, perhaps the LCH subunit predominates in flies because longer-term iron storage is desirable.

4.10.4. Control of Ferritin Expression

Several mechanisms are involved in the control of ferritin expression in insects. These include transcriptional control, alternative splicing, polyadenylation, and translational control.

4.10.4.1. Transcriptional Control

The limited knowledge available for the transcriptional control of vertebrate ferritin genes comes

from studies of the H genes (Torti and Torti, 2002). In mammalian cells, iron salts stimulate a threefold increase in H subunit mRNA and a fourfold increase in the synthesis of the protein (Coccia *et al.*, 1995). In addition to iron, other agents, such as hormones, cytokines, and certain chemicals, upregulate the transcription of the H gene (Chou *et al.*, 1986; Chazenbalk *et al.*, 1990b; Wei *et al.*, 1990; Miller *et al.*, 1991; Yokomori *et al.*, 1991; Tsuji *et al.*, 1995).

In *A. aegypti*, transcriptional control is relevant in the regulation of HCH gene expression (Pham *et al.*, 1999). HCH mRNA levels increase in Aag2 cells following exposure to iron as shown by Northern blot analyses. This could be attributed to an increase in the transcription of the HCH gene, a decrease in the degradation rate of HCH mRNA, or both. Since pretreatment of Aag2 cells with actinomycin D prevents upregulation of HCH message, the increase in this mRNA in response to iron is likely due to induced transcription rather than decreased mRNA degradation.

For vertebrate H genes, all known *cis*-regulatory elements are found upstream of the transcriptional start sites (Ponka *et al.*, 1998). The proximal promoter region of the vertebrate H gene contains a TATA box, a CCAAT box (C/EBP binding site), and one or more SP1 binding sites (Wei *et al.*, 1990; Miller *et al.*, 1991; Bevilacqua *et al.*, 1992; Beaumont *et al.*, 1994a, 1994b; Kwak *et al.*, 1995; Tsuji *et al.*, 1995; Ponka *et al.*, 1998). Current data indicate that additional regulatory elements located further upstream from the transcriptional start site are necessary for full activation (Ponka *et al.*, 1998). These elements include a 180 bp hemin response element located at 4.5 kb upstream of the transcriptional start site (Beaumont *et al.*, 1994b), an NF-E2 binding site that regulates the expression of genes involved in the heme biosynthetic pathway (Beaumont *et al.*, 1994a), and a Fer-2 site that is recognized by tumor necrosis factor-α (TNF-α) (Kwak *et al.*, 1995).

In *A. aegypti*, transient transfection expression assays of progressive deletions of the HCH gene promoter region reveal that the minimal promoter sequence necessary to sustain transcription *in vitro* lies between positions −40 and +50 (Pham *et al.*, 2003). These assays also show that the promoter of this gene is strong (30-fold stronger than the promoter of cytomegalovirus and 100-fold stronger than the SV40 promoter) and that, unlike vertebrate H genes, the *Aedes* HCH gene contains regulatory elements both upstream and downstream of the transcriptional start site. Results from DNase footprinting assays and MatInspector predictions suggest that, like the vertebrate H genes, the promoter of the mosquito ferritin gene contains a TATA-box and a C/EBP site (**Figure 1**). However, in the *Aedes* HCH gene, the C/EBP binding site is found downstream of the transcriptional start site. A putative element common in the promoters of cell cycle genes, E2F, also is present in the mosquito HCH gene. This element could be the analog of an E1A repressor site found in the vertebrate ferritin H genes (Tsuji *et al.*, 1999). Regulatory elements known for

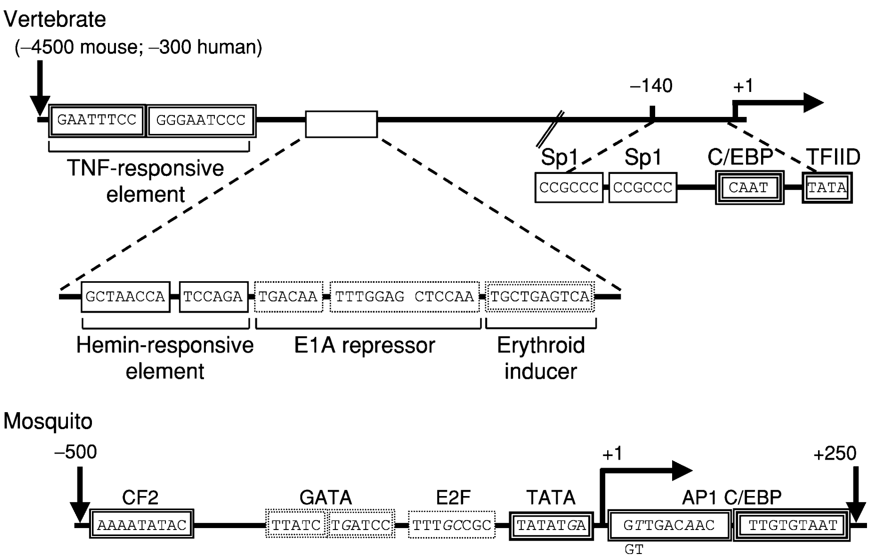


Figure 1 A diagram of the promotor regions of the genes for the vertebrate heavy chain subunit and the *Aedes* heavy chain homolog. The diagram for the vertebrate heavy chain gene promoter was adapted from information provided by Ponka *et al.* (1998) and Torti and Torti (2002). Boxes with the same border represent putative analogous *cis*-regulatory elements. +1, transcription start site; the nucleotide sequence inside the boxes are binding sites; numbers give positions using +1 as the transcriptional start site.

their involvement with erythropoiesis, GATA-binding sites and two cell-signaling sites, CF2 and AP-1, are also found in the mosquito HCH gene. These two latter putative sites might be the equivalents of the vertebrate Fer-2 site. Data from DNase footprinting assays and MatInspector predictions reveal that the regulatory elements described above are noncanonical.

Interestingly, the TATA box (TATAtga) of the *Aedes* HCH promotor does not match the known consensus sequence (TATAWAW) perfectly and the affinity of the TATA box binding protein (TBP) to TATA at this site is weak. Nonetheless, primer extension analysis indicates that transcription for the HCH gene initiates at one site in untreated cells, but switches to two sites in iron-treated cells (Pham, 2000; Pham *et al.*, 2003). These data suggest that TBP recognizes the noncanonical TATA box under normal conditions and directs RNA pol II to initiate at a single start site. However, when cells are exposed to high levels of iron, TBP loses its accuracy in directing RNA pol II, resulting in RNA pol initiating at two start sites. The situation observed for iron treatment parallels transcriptional initiation seen for TATA-less promoters, where multiple transcriptional start sites are observed, perhaps as a result of a random response to the lack of a strong selector (Sitzler *et al.*, 1991; Ince and Scotto, 1995; Lin *et al.*, 2001). Since DNase footprinting analyses show that the recognition ability of TBP to TATA is not significantly altered under iron treatment, these data suggest that TBP can still recognize TATA in iron overload conditions, but loses the ability to direct RNA pol with accuracy. Thus, cellular iron concentration could alter transcription regulation by modifying interactions among *trans*-factors rather than between *trans*-factors and *cis*-elements.

For many years, the prevailing view in eukaryotic gene expression studies has been that response elements, specifically enhancers and silencers, are distance and orientation independent. However, recent work has challenged this tenet. Many newly found enhancers and silencers are both orientation and distance dependent (Surinya *et al.*, 1998; Guevara-Garcia *et al.*, 1999; Coughlin *et al.*, 2000; Givogri *et al.*, 2000; Lin and Chang, 2000; Wei and Brennan, 2000; Sun *et al.*, 2001; Takahashi *et al.*, 2001). Some elements even have the unusual property of switching activity with orientation. These elements act as an enhancer in one orientation and as a silencer in the other, as well as operating at dual promoters of two adjacent genes (Guevara-Garcia *et al.*, 1999; Givogri *et al.*, 2000). In the dipterans, the ferritin genes are located adjacent to, and in opposite directions from, each other (Dunkov and Georgieva, 1999; Holt *et al.*, 2002). The enhancers and silencers of the HCH gene are orientation dependent (D.Q.-D. Pham, unpublished data). Preliminary data suggest that the response elements of these genes could play a role in the control of the expression of both LCH and HCH genes. Since the genes are located adjacent to each other, a switch in activity of some response elements could allow the elements to exert a dual and/or directional control over the ferritin genes.

4.10.4.2. Posttranscriptional Control: Alternative Splicing and Polyadenylation

Available evidence indicates that posttranscriptional control of ferritin synthesis also occurs in insects (Zhang *et al.*, 2001b). Possible mechanisms for posttranscriptional control include alternative splicing (Lind *et al.*, 1998; Georgieva *et al.*, 1999), use of multiple polyadenylation sites (Lind *et al.*, 1998; Georgieva *et al.*, 1999, 2002b; Dunkov *et al.*, 2002), and translational repression by an iron-responsive element (IRE) (Zhang *et al.*, 2001a, 2002).

In mammals, the IRE is a *cis*-acting, stem–loop sequence that allows iron-mediated repression of ferritin translation (Leibold and Munro, 1988; Theil, 1994). A single IRE is found in the 5′-untranslated region (UTR) of mammalian ferritin mRNA (Caughman *et al.*, 1988). An IRE also is present in the 5′-UTR of the ferritin HCH message of *D. melanogaster* (Charlesworth *et al.*, 1997), *A. aegypti* (Dunkov *et al.*, 1995), *A. gambiae* (Georgieva *et al.*, 2002a), *N. lugens* (Du *et al.*, 2000), and *G. mellonella* (Kim *et al.*, 2001), as well as the HCH and LCH messages of *M. sexta* (Pham *et al.*, 1996; Zhang *et al.*, 2001a) and *C. ethlius* (Nichol and Locke, 1999).

In contrast to these ferritin messages, the LCH message of the dipterans, *Drosophila* (Georgieva *et al.*, 2002b) and *Aedes* (Geiser *et al.*, 2003), lacks a canonical IRE. Although not seen in mammals, ferritin subunits without IREs are found in other organisms (Dietzel *et al.*, 1992). The LCH message in both dipterans is increased in response to iron. However, the protein does not appear to increase in proportion to message upregulation, suggesting that in addition to transcriptional control, other mechanisms control the synthesis of these subunits. This is supported by the finding that the *Drosophila* LCH is encoded by two mRNAs that differ in length as a result of using alternative polyadenylation sites (Georgieva *et al.*, 2002b). Iron loading significantly increases the shorter message and induces both transcripts at all life stages except dark pupae. Vertebrate ferritin mRNA levels can be increased through the binding of proteins to conserved polypyrimidine

regions in the 3′-UTR that increase ferritin mRNA stability (Ai and Chau, 1999), but whether this is the case for these messages in this insect is unknown.

In contrast to the *Drosophila* LCH message, the *Drosophila* HCH message has a canonical IRE (Charlesworth *et al.*, 1997). Both Lind *et al.* (1998) and Georgieva *et al.* (1999) reported that this IRE could be removed by alternative splicing. In fact, eight messages for the ferritin HCH exist in *Drosophila* that originate from three alternative splicing sites coupled with either of the two polyadenylation sites (Lind *et al.*, 1998). In all cases, alternative splicing removes the IRE. Iron treatment increases splicing activity in a tissue-specific manner with the greatest increase in spliced messages occurring in the gut (Georgieva *et al.*, 1999). These authors suggest that when a high level of HCH is desirable for the rapid storage of iron, alternative splicing of the IRE allows unhindered expression of this subunit.

In contrast to the *Drosophila* HCH message, Dunkov *et al.* (2002) reported no alternative splicing of the *Aedes* HCH message. They found only two messages for *Aedes* HCH that occur as a result of using different polyadenylation sites. Both messages increase in females following iron supplementation or blood feeding (Dunkov *et al.*, 2002). The longer mRNA increases in concentration progressively after blood feeding and is sustained for 7 days. While the shorter mRNA increases progressively, it exceeds the levels of the longer message at 48 h and then declines. Both mRNAs are present in ovaries, midgut, and thorax, with greatest expression noted in midgut. Only the larger mRNA is observed in Malpighian tubules and fat body.

4.10.4.3. Translational Control

In mammals, repression of ferritin synthesis occurs when intracellular iron levels are low because an iron regulatory protein, IRP1 or IRP2, binds to the ferritin mRNA IRE and prevents translation (Gray and Hentze, 1994; Muckenthaler *et al.*, 1998a). When intracellular iron levels increase and iron storage is desirable, ferritin synthesis increases as IRP1 binding activity declines (Eisenstein, 2000) and IRP2 is rapidly degraded (Guo *et al.*, 1995). Although no measurable difference in IRP1 message or protein levels generally occurs in response to iron administration (Tang *et al.*, 1992; Chen *et al.*, 1997), binding activity declines because a cubane (4Fe–4S) iron sulfur cluster forms in the IRP1 core that prevents IRP1–IRE interaction (Beinert *et al.*, 1996; Haile, 1999). Iron sulfur cluster assembly allows IRP1 to respond to iron levels, changes in oxidative stress, and redox potential (Cairo *et al.*, 1995; Bouton, 1999; Eisenstein, 2000). When the cluster is present, IRP1 becomes cytoplasmic aconitase (Beinert *et al.*, 1996), and recent work indicates that this enzyme is important in the generation of cellular NADPH (Narahari *et al.*, 2000).

In 1990, a protein with binding activity to transcripts of the vertebrate ferritin IRE was detected in cytoplasmic extracts of *D. melanogaster* Schneider cells (Rothenberger *et al.*, 1990). IRP1 was subsequently cloned and sequenced from *Drosophila* (Muckenthaler *et al.*, 1998b), *Aedes* (Zhang *et al.*, 2002), and *Manduca* (Zhang *et al.*, 2001b). The predicted proteins all show high homology to the mammalian IRP1 (Zhang *et al.*, 2001b). The residues necessary for iron sulfur cluster formation and aconitase activity are universally conserved, whereas a 72 amino acid insert that distinguishes the mammalian IRP2 from IRP1 is missing from the insect IRP sequences. The *Drosophila* IRP1 is expressed throughout the embryo and at all developmental stages (Muckenthaler *et al.*, 1998b) and the mosquito IRP1 is expressed at all developmental stages (Zhang *et al.*, 2002). No IRP2 has been identified in insects despite an exhaustive search.

Current data indicate that the insect IRP1 can regulate mRNA translation by binding to the insect IRE. Recombinant *M. sexta* or *A. aegypti* IRP1 (Zhang *et al.*, 2001a, 2002) binds to transcripts of the insect or vertebrate ferritin IRE, and both repress ferritin translation *in vitro* by specific interaction with the IRE (Zhang *et al.*, 2001a; D. Zhang and J.J. Winzerling, unpublished data). Recently, we found that an iron sulfur cluster forms in the recombinant *M. sexta* IRP1 exposed to iron and sulfide *in vitro* (Gailer, George, Pickering, and Winzerling, unpublished data). When the cluster is present, the IRP1 will not prevent the translation of *M. sexta* ferritin HCH *in vitro* (D. Zhang and J.J. Winzerling, unpublished data). *In vivo* data also support the postulation that IRP–IRE interaction is involved in iron-mediated control of ferritin synthesis. When *M. sexta* larvae are injected with iron, fat body IRP1–IRE binding activity declines without a change in IRP1 mRNA or protein expression and hemolymph ferritin increases (Winzerling *et al.*, 1995; Zhang *et al.*, 2001b). Similarly, blood-fed *A. aegypti* females show increased ferritin synthesis without a change in IRP1 mRNA expression (Dunkov *et al.*, 1995; Zhang *et al.*, 2002).

The mechanism whereby IRP–IRE interaction allows repression of ferritin translation in insects was studied recently (Nichol and Winzerling, 2002). In vertebrates, the ferritin IRE is within 60 nucleotides of the mRNA cap site; complete repression of ferritin translation occurs because

IRP–IRE interaction prevents recruitment of the full ribosomal apparatus (Gray and Hentze, 1994; Muckenthaler *et al.*, 1998a). In constructs where the IRE is positioned more distant from the cap site, the ribosomal apparatus assembles and scans the message, but pauses at the region of IRP1–IRE interaction resulting in only partial repression of translation. Insect ferritin mRNA IREs are located distant from the cap site, yet complete repression of translation occurs. Current evidence suggests that the secondary structure of the insect ferritin mRNA could bring the IRE sufficiently close to the cap site to allow complete translational repression by the IRP1 (Nichol and Winzerling, 2002).

Interestingly, a 5′ UTR IRE also is found in the mRNA of the succinate dehydrogenase subunit b (SDhb, *D. melanogaster*) (Kohler *et al.*, 1995; Gray *et al.*, 1996; Melefors, 1996). *In vivo* synthesis of SDhb is increased following iron treatment of *Drosophila* cells indicating that the IRE is an active control site (Gray *et al.*, 1996; Melefors, 1996). Under low iron conditions, the SDhb message is distributed to a nonpolysomal pool (Kohler *et al.*, 1995). The SDhb IRE also confers iron-mediated translational control of the synthesis of a reporter protein in transfected cells (Kohler *et al.*, 1995; Gray *et al.*, 1996). These findings support the concept that the IRE is an active site of iron-mediated translational control in insects.

4.10.5. Iron, Oxidative Stress, and Ferritin

Ferritin synthesis in vertebrates is responsive to several factors in addition to intracellular iron concentration. These factors include some hormones (Chazenbalk *et al.*, 1990a, 1990b), infection, oxidative stress (Beinert and Kiley, 1999; Bouton, 1999; Epsztejn *et al.*, 1999; Haile, 1999; Cheng *et al.*, 2000; Tsuji *et al.*, 2000; Mueller *et al.*, 2001), and hemin (Lin *et al.*, 1990; Coccia *et al.*, 1992; Beaumont *et al.*, 1994a).

Free heme is a powerful generator of ROS that can damage biological molecules (Oliveira *et al.*, 2002). Oliveira and colleagues found that, in *R. prolixis,* heme from the blood meal is transported in the hemolymph bound to a heme-binding protein (Oliveira *et al.*, 2000; Braz *et al.*, 2001, 2002). The relationship of heme to ferritin synthesis in blood-feeding insects is of considerable interest because in mammals ferritin serves as a protective agent against oxidative stress (Galvani *et al.*, 1995; Cairo *et al.*, 1996), and exposure to ROS alters ferritin expression (Cairo *et al.*, 1995; Lobreaux *et al.*, 1995; Haile, 1999; Tsuji *et al.*, 2000).

Treatment of Aag2 cells with hemin increases LCH and HCH messages significantly in a time-dependent manner (Geiser *et al.*, 2003). In *A. aegypti* females, hemin added to an artificial meal also increases expression of the LCH subunit in pupae and the HCH 24 kDa subunit in larvae, pupae, and adult females (Dunkov *et al.*, 2002). Interestingly, although treatment with hemin elicits expression of the HCH 26 kDA subunit in pupae, expression of the 26 kDa subunit is not induced in larvae and in adult females it is decreased. Taken together, these data show that heme induces ferritin expression in mosquitoes in a stage-specific manner and suggest that the processing of the 24 kDa subunit is influenced by hemin. The mechanisms responsible for these changes are not known, but could involve induction by both iron and oxidative stress.

Oxidative stress imposed by exposure of Aag2 cells to H_2O_2 upregulates HCH and LCH mRNAs significantly; induction is time and dose dependent and follows a similar pattern to that observed when the cells are treated with hemin (Geiser *et al.*, 2003). Treatment of mammalian cells with H_2O_2 also results in upregulation of ferritin message by acting at the transcriptional level (Tsuji *et al.*, 2000). It seems probable that the increase in LCH and HCH messages observed for mosquito cells is mediated at the transcriptional level as well. In mammals, although oxidative stress enhances ferritin mRNA expression, it simultaneously downregulates ferritin synthesis at the translational level by increased IRP1–IRE interaction (Tsuji *et al.*, 2000). The overall change in ferritin levels that results from these two opposing effects appears to be related to the type and strength of the stimulus. Both nitric oxide (NO) and H_2O_2 enhance IRP–IRE interaction by different mechanisms (Pantopoulos and Hentze, 1995) that result in disassembly of the iron sulfur cluster (Weiss *et al.*, 1993, 1998; Pantopoulos and Hentze, 1995; Wardrop *et al.*, 2000). Whether similar mechanisms exist in insects is unknown.

In mammals, infection results in increased IRP1–IRE binding activity without a change in IRP1 protein (Phillips *et al.*, 1996; Weiss *et al.*, 1997). This appears to be the case in insects as well. Mosquito cells exposed to lipopolysaccaharide show an immune response accompanied by an increase in IRP1–IRE binding activity (Zhang *et al.*, 2002). Injection of lepidopteran larvae with water results in a significant increase in fat body IRP1–IRE binding activity accompanied by a significant fall in hemolymph ferritin (Zhang *et al.*, 2001b). Interestingly, NO levels are increased in mosquitoes in response to a blood meal infected with parasites (Luckhart *et al.*, 1998). The increase in IRP1–IRE

interaction in insects in response to infection or injury could be related to an effect of NO on the IRP1. Alternatively, when *A. gambiae* cells are exposed to H_2O_2, thioredoxin reductase also is induced (Dimopoulos *et al.*, 2002). Work with mammalian cells indicates that NO and thioredoxin reductase cooperate to modulate changes in IRP1–IRE interaction. If infection of insects with a blood meal induces both NO and thioredoxin reductase, these compounds could work together to enhance IRP1–IRE interaction and reduce ferritin synthesis at a time when iron is supplied in the blood meal.

Recent work on the effects of infection and oxidative stress in *A. gambiae* shows that these two factors primarily influence expression of different gene clusters (Dimopoulos *et al.*, 2002). Moreover, different types of infectious agents provoke expression of different gene clusters. Such information provides the opportunity to identify the role of the genes involved in these important response mechanisms. It will be interesting to see if genes regulated by iron overlap with genes of these clusters.

Acknowledgments

Research for this publication was supported by the US Public Health Service (GM 56812, GM55886, and GM 65861), the Agricultural Experiment Station and the College of Agriculture and Life Sciences and the Center for Insect Science of the Arizona Research Laboratories at the University of Arizona, and the University of Wisconsin-Parkside. The authors thank E. Kohlhepp, Dawn Geiser, and Jonathan Mayo for helpful comments on the manuscript; and acknowledge that parts of the text are reworked from Nichol *et al.* (2002).

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster*. *Science 287*, 2185–2195.

Ai, L.S., Chau, L.Y., **1999**. Post-transcriptional regulation of H-ferritin mRNA. *J. Biol. Chem. 274*, 30209–30214.

Aisen, P., Enns, C., Wessling-Resnick, M., **2001**. Chemistry and biology of eukaryotic iron metabolism. *Int. J. Biochem. Cell Biol. 33*, 940–959.

Andrews, S.C., Arosio, P., Bottke, W., Briat, J.-F., Von Darl, M., *et al.*, **1992**. Structure, function, and evolution of ferritins. *J. Inorg. Biochem. 47*, 161–174.

Bartfeld, N.S., Law, J.H., **1990**. Isolation and molecular cloning of transferrin from the tobacco hornworm, *Manduca sexta*. *J. Biol. Chem. 265*, 1–8.

Beaumont, C., Jones, R., Seyhan, A., Grandchamp, B., **1994a**. A hemin-inducible enhancer lies 4.5 Kb upstream of the mouse ferritin H subunit gene. *Adv. Exp. Med. Biol. 356*, 211–218.

Beaumont, C., Seyhan, A., Yachou, A.K., Grandchamp, B., Jones, R., **1994b**. Mouse ferritin H subunit gene: functional analysis of the promoter and identification of an upstream regulatory element active in erythroid cells. *J. Biol. Chem. 269*, 20281–20288.

Beinert, H., Kennedy, M.C., Stout, C.D., **1996**. Aconitase as iron–sulfur protein, enzyme, and iron-regulatory protein. *Chem. Rev. 96*, 2335–2373.

Beinert, H., Kiley, P.J., **1999**. Fe–S proteins in sensing and regulatory functions. *Curr. Opin. Chem. Biol. 3*, 152–157.

Bevilacqua, M.A., Giordano, M., D'Agostino, P., Santoro, C., Cimino, F., *et al.*, **1992**. Promoter for the human ferritin heavy chain-encoding gen (*FERH*): structure and functional characterization. *Gene 111*, 255–260.

Bouton, C., **1999**. Nitrosative and oxidative modulation of iron regulatory proteins. *Cell. Mol. Life Sci. 55*, 1043–1053.

Braz, G.R., Abreu, L., Masuda, H., Oliveira, P.L., **2001**. Heme biosynthesis and oogenesis in the blood-sucking bug, *Rhodnius prolixus*. *Insect Biochem. Mol. Biol. 31*, 359–364.

Braz, G.R., Moreira, M.F., Masuda, H., Oliveira, P.L., **2002**. *Rhodnius* heme-binding protein (RHBP) is a heme source for embryonic development in the blood-sucking bug *Rhodnius prolixus* (Hemiptera, Reduviidae). *Insect Biochem. Mol. Biol. 32*, 361–367.

Briat, J.F., **1996**. Roles of ferritin in plants. *J. Plant Nutr. 19*, 1331–1342.

Cairo, G., Castrusini, E., Minotti, G., Bernellizazzera, A., **1996**. Superoxide and hydrogen peroxide-dependent inhibition of iron regulatory protein activity: a protective stratagem against oxidative injury. *FASEB J. 10*, 1326–1335.

Cairo, G., Tacchini, L., Pogliaghi, G., Anzon, E., Tomasi, A., *et al.*, **1995**. Induction of ferritin synthesis by oxidative stress: transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. *J. Biol. Chem. 270*, 700–703.

Capurro, M.d.L., Iughetti, P., Ribolla, P.E., de Bianchi, A.G., **1996**. *Musca domestica* hemolymph ferritin. *Arch. Insect Biochem. Physiol. 32*, 197–207.

Caughman, S.W., Hentze, M.W., Rouault, T.A., Harford, J.B., Klausner, R.D., **1988**. The iron-responsive element is the single element responsible for iron-dependent translation regulation of ferritin biosynthesis. *J. Biol. Chem. 263*, 19048–19052.

Charlesworth, A., Georgieva, T., Gospodov, I., Law, J.H., Dunkov, B.C., *et al.*, **1997**. Isolation and properties of *Drosophila melanogaster* ferritin: molecular cloning of a cDNA that encodes one subunit, and localization of the gene on the third chromosome. *Eur. J. Biochem. 247*, 470–475.

Chasteen, N.D., Harrison, P.M., **1999**. Mineralization in ferritin: an efficient means of iron storage. *J. Struct. Biol. 126*, 182–194.

Chazenbalk, G.D., Wadsworth, H.L., Foti, D., Rapoport, B., **1990a**. Thyrotropin and adenosine 3′,5′-monophosphate stimulate the activity of the ferritin-H promoter. *Mol. Endocrinol. 4*, 1117–1124.

Chazenbalk, G.D., Wadsworth, H.L., Rapoport, B., **1990b**. Transcriptional regulation of ferritin H messenger RNA levels in FRTL rat thyroid cells by thyrotropin. *J. Biol. Chem. 265*, 666–670.

Chen, O.S., Schalinske, K.L., Eisenstein, R.S., **1997**. Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver. *J. Nutr. 127*, 238–248.

Cheng, Q., Gonzalez, P., Zigler, J.S., Jr., **2000**. High level of ferritin light chain mRNA in lens. *Biochem. Biophys. Res. Commun. 270*, 349–355.

Chou, C.C., Gatti, R.A., Fuller, M.L., Concannon, P., Wong, A., *et al.*, **1986**. Structure and expression of ferritin genes in a human promyelocytic cell line that differentiates *in vitro*. *Mol. Cell. Biol. 6*, 566–573.

Coccia, E.M., Profita, V., Fiorucci, G., Romeo, G., Affabris, E., *et al.*, **1992**. Modulation of ferritin H-chain expression in Friend erythroleukemia cells: transcriptional and translational regulation by hemin. *Mol. Cell. Biol. 12*, 3015–3022.

Coccia, E.M., Stellacci, E., Orsatti, R., Testa, U., Battistini, A., **1995**. Regulation ferritin H-chain expression in differentiating Friend leukemia cells. *Blood 86*, 1570–1579.

Collin, O., Thomas, D., Flifla, M., Quintana, C., Gouranton, J., **1988**. Characterization of a ferritin isolated from the midgut epithelial cells of a homopteran insect, *Philaenus spumarious* L. *Biol. Cell 63*, 297–305.

Corsi, B., Perrone, F., Bourgeois, M., Beaumont, C., Panzeri, M.C., *et al.*, **1998**. Transient overexpression of human H- and L-ferritin chains in COS cells. *Biochem. J. 330*, 315–320.

Coughlin, B.C., Teixeira, S.M.R., Kirchhoff, L.V., Donelson, J.E., **2000**. Amastin mRNA abundance in *Trypanosoma cruzi* is controlled by a 3′-untranslated region position-dependent *cis*-element and an untranslated region-binding protein. *J. Biol. Chem. 275*, 12051–12060.

DeRusso, P.A., Philpott, C.C., Iwai, K., Mostowski, H.S., Klausner, R.D., *et al.*, **1995**. Expression of a constitutive mutant of iron regulatory protein 1 abolishes iron homeostasis in mammalian cells. *J. Biol. Chem. 270*, 15451–15454.

Dietzel, J., Hirzmann, M., Preis, D., Symmons, P., Kunz, W., **1992**. Ferritins of *Schistosoma mansoni*: sequence comparison and expression in female and male worms. *Mol. Biochem. Parasitol. 50*, 245–254.

Dimopoulos, G., Christophides, G.K., Meister, S., Schultz, J., White, K.P., *et al.*, **2002**. Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. *Proc. Natl Acad. Sci. USA 99*, 8814–8819.

Du, J., Foissac, X., Carss, A., Gatehouse, A.M.R., Gatehouse, J.A., **2000**. Ferritin acts as the most abundant binding protein for snowdrop lectin in the midgut of rice brown planthoppers (*Nilaparvata lugens*). *Insect Biochem. Mol. Biol. 30*, 297–305.

Dunkov, B.C., Georgieva, T., **1999**. Organization of the ferritin genes in *Drosophila melanogaster*. *DNA Cell Biol. 18*, 937–944.

Dunkov, B.C., Georgieva, T., Yoshiga, T., Hall, M., Law, J.H., **2002**. *Aedes aegypti* ferritin heavy chain homologue: feeding of iron or blood influences message levels, lengths and subunit abundance. *J. Insect Sci. 2*, 7–17.

Dunkov, B.C., Zhang, D., Choumarov, K., Winzerling, J.J., Law, J.H., **1995**. Isolation and characterization of mosquito ferritin and cloning of a cDNA that encodes one subunit. *Arch. Insect Biochem. Physiol. 29*, 293–307.

Eisenstein, R.S., **2000**. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu. Rev. Nutr. 20*, 627–662.

Epsztejn, S., Glickstein, H., Picard, V., Slotki, I.N., Breuer, W., *et al.*, **1999**. H-ferritin subunit overexpression in erythroid cells reduces the oxidative stress response and induces multidrug resistance properties. *Blood 94*, 3593–3603.

Gallois, B., d'Estaintot, B.L., Michaux, M.-A., Dautant, A., Granier, T., *et al.*, **1997**. X-ray structure of recombinant horse L-chain apoferritin at 2.0 Å resolution: implication for stability and functions. *J. Biol. Inorg. Chem. 2*, 360–367.

Galvani, P., Colleoni, M., Origgi, M., Santagostino, A., **1995**. Mitochondrial toxicity of iron and the protective role of ferritin on dopaminergic PC12 cell line. *Tox. In Vitro 9*, 365–368.

Geiser, D.L., Chavez, C.A., Flores-Manguia, R.F., Winzerling, J.J., Pham, D.Q., **2003**. *Aedes aegypti* ferritin A cytotoxic protector against iron and oxidative challenge? *Eur. J. Biochem. 270*, 1–8.

Georgieva, T., Dunkov, B.C., Dimov, S., Ralchev, K., Law, J.H., **2002b**. *Drosophila melanogaster* ferritin: cDNA encoding a light chain homologue, temporal and tissue specific expression of both subunit types. *Insect Biochem. Mol. Biol. 32*, 295–302.

Georgieva, T., Dunkov, B.C., Harazanova, N., Ralchev, K., Law, J.H., **1999**. Iron availability dramatically alters the distribution of ferritin subunit messages in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 96*, 2716–2721.

Georgieva, T., Dunkov, B., Law, J.H., **2002a**. *Anopheles gambia* secreted ferritin: characterization of cDNAs encoding two subunits, mRNA expression patterns, and gene organization. In: 4th Int. Symp. on Molecular Insect Science, Tuscon, AZ.

Givogri, M.I., Kampf, K., Schonmann, V., Campagnoni, A.T., **2000**. Identification of a novel silencer that regulates the myelin basic protein gene in neural cells. *Gene 252*, 183–193.

Gray, N.K., Hentze, M.W., **1994**. Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J. 13*, 3882–3891.

Gray, N.K., Pantopoulous, K., Dandekar, T., Ackrell, B.A., Hentze, M.W., **1996**. Translational regulation of mammalian and *Drosophila* citric acid cycle enzymes via iron-responsive elements. *Proc. Natl Acad. Sci. USA 93*, 4925–4930.

Guevara-Garcia, A., Lopez-Bucio, J., Herrera-Estrella, L., **1999**. The mannopine synthase promoter contains vectorial *cis*-regulatory elements that act as enhancers and silencers. *Mol. Gen. Genet. 262*, 608–617.

Guex, N., Diemand, A., Peitsch, M.C., **1999**. Protein modeling for all. *Trends Biochem. Sci. 24*, 364–367.

Guex, N., Peitsch, M.C., **1997**. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis 18*, 2714–2723.

Guo, B., Phillips, J.D., Yu, Y., Leibold, E.A., **1995**. Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J. Biol. Chem. 270*, 21645–21651.

Haile, D.J., **1999**. Regulation of genes of iron metabolism by the iron-response proteins. *Am. J. Med. Sci. 318*, 230–240.

Harrison, P.M., Arosio, P., **1996**. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochem. Biophys. Acta 1275*, 161–203.

Hempstead, P.D., Yewdall, S.J., Fernie, A.R., Lawson, D.M., Artymiuk, P.J., *et al.*, **1997**. Comparison of the three-dimensional structures of recombinant human H and horse L ferritins at high resolution. *J. Mol. Biol. 268*, 424–448.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Hubbard, S.R., Mohammadi, M., Schlessinger, J., **1998**. Autoregulatory mechanisms in protein-tyrosine kinase. *J. Biol. Chem. 273*, 11987–11990.

Huebers, H.A., Huebers, E., Finch, C., Webb, B.A., Truman, J.W., *et al.*, **1988**. Iron binding proteins and their roles in the tobacco hornworm *Manduca sexta* (L). *J. Comp. Physiol. Biochem. 158*, 291–300.

Ince, T.A., Scotto, K.W., **1995**. Differential utilization of multiple transcription start points accompanies the overexpression of the P-glycoprotein-encoding gene in Chinese hamster lung cells. *Gene 156*, 287–290.

Kakhlon, O., Gruenbaum, Y., Cabatchik, Z.L., **2001**. Repression of ferritin expression increases the labile iron pool, oxidative stress and short-term growth of human erythroleukemia cells. *Blood 97*, 2863–2871.

Kakhlon, O., Gruenbaum, Y., Cabantchik, Z.I., **2002**. Ferritin expression modulates cell cycle dynamics and cell responsiveness to H-ras-induced growth via expansion of the labile iron pool. *Biochem. J. 363*, 431–436.

Kato, J., Niitsu, Y., **2002**. Recent advances in molecular iron metabolism: translational disorders of ferritin. *Int. J. Hematol. 76*, 1–5.

Kehrer, J.P., **2000**. The Haber–Weiss reaction and mechanisms of toxicity. *Toxicology 149*, 43–50.

Keim, C.N., Cruz-Landim, C., Carneiro, F.G., Farina, M., **2002**. Ferritin in iron containing granules from the fat body of the honeybees *Apis mellifera* and *Scaptotrigona postica*. *Micron 33*, 53–59.

Kim, B.S., Lee, C.S., Seol, J.Y., Yun, C.Y., Kim, H.R., **2002**. Cloning and expression of a 32 kDa ferritin from *Galleria mellonella*. *Arch. Biochem. Physiol. 51*, 80–90.

Kim, B.S., Yun, C.Y., Yeo, S.M., Lee, H.J., Kim, H.R., **2001**. Cloning and expression of a ferritin subunit for *Galleria mellonella*. *Arch. Biochem. Physiol. 47*, 8–17.

Kohler, S.A., Henderson, B.R., Kuhn, L.C., **1995**. Succinate dehydrogenase b mRNA of *Drosophila melanogaster* has a functional iron-responsive element in its 5′-untranslated region. *J. Biol. Chem. 270*, 30781–30786.

Kwak, E.L., Larochelle, D.A., Beaumont, C., Torti, S.V., Torti, F.M., **1995**. Role for NF-kappa B in the regulation of ferritin H by tumor necrosis factor-alpha. *J. Biol. Chem. 270*, 15285–15293.

Lawson, D.M., Treffry, A., Artymiuk, P.J., Harrison, P.M., Yewdall, S.J., *et al.*, **1989**. Identification of the ferroxidase centre in ferritin. *FEBS Lett. 254*, 207–210.

Lawson, D.M., Artymiuk, P.J., Yewdall, S.J., Smith, J.M.A., Livingston, J.C., *et al.*, **1991**. Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts. *Nature 349*, 541–544.

Leibold, E.A., Munro, H.N., **1988**. Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs. *Proc. Natl Acad. Sci. USA 85*, 2171–2175.

Levi, S., Corsi, B., Rovida, E., Cozzi, A., Santambrogio, P., *et al.*, **1994**. Construction of a ferroxidase center in human ferritin L-chain. *J. Biol. Chem. 269*, 30334–30339.

Lin, D.-L., Chang, C., **2000**. Identification of an essential *cis*-acting element (TR2-PACE) in the 5′ promoter of human TR2 orphan receptor gene. *Endocrine 12*, 89–95.

Lin, J.J., Daniels-McQueen, S., Patino, M.M., Gaffield, L., Walden, W.E., *et al.*, **1990**. Derepression of ferritin messenger RNA translation by hemin *in vitro*. *Science 247*, 74–77.

Lin, Y., Ince, T.A., Scotto, K.W., **2001**. Optimization of a versatile *in vitro* transcription assay for the expression of multiple start site TATA-less promoters. *Biochemistry 40*, 12959–12966.

Lind, M.I., Ekengren, S., Melefors, O., Soderhall, K., **1998**. *Drosophila* ferritin mRNA: alternative RNA splicing regulates the presence of the iron-responsive element. *FEBS Lett. 436*, 476–482.

Lobreaux, S., Thoiron, S., Briat, J.F., **1995**. Induction of ferritin synthesis in maize leaves by an iron-mediated oxidative stress. *Plant J. 8*, 443–449.

Locke, M., **1991**. Apoferritin in the vacuolar system of insect hemocytes. *Tissue Cell 23*, 367–375.

Locke, M., **2003**. Surface membranes, Golgi complexes, and vaculoar systems. *Annu. Rev. Entomol. 48*, 1–27.

Locke, M., Ketola-Pirie, C., Leung, H., Nichol, H., **1991**. Vacuolar apoferritin synthesis by the fat body of an insect (*Calpodes ethlius*). *J. Insect Physiol. 37*, 297–309.

Locke, M., Leung, H., **1984**. The induction and distribution of an insect ferritin: a new function for the endoplasmic reticulum. *Tissue Cell 16*, 739–766.

Locke, M., Nichol, H., **1992**. Iron economy in insects. *Annu. Rev. Entomol. 37*, 195–215.

Luckhart, S., Vodovotz, Y., Cui, L., Rosenberg, R., **1998**. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc. Natl Acad. Sci. USA 95*, 5700–5705.

Melefors, O., **1996**. Translational regulation *in vivo* of the *Drosophila melanogaster* mRNA encoding succinate dehydrogenase iron protein via iron responsive elements. *Biochem. Biophys. Res. Commun. 221*, 437–441.

Miller, L.L., Miller, S.C., Torti, S.V., Tsuji, Y., Torti, F.M., **1991**. Iron-independent induction of ferritin H chain by tumor necrosis factor. *Proc. Natl Acad. Sci. USA 88*, 4946–4950.

Muckenthaler, M., Gray, N.K., Hentze, M.W., **1998a**. IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol. Cell 2*, 383–388.

Muckenthaler, M., Gunkel, N., Frishman, D., Cyrklaff, A., Tomancak, P., *et al.*, **1998b**. Iron-regulatory protein-1 (IRP-1) is highly conserved in two invertebrate species: characterization of IRP-1 homologues in *Drosophila melanogaster* and *Caenorhabditis elegans*. *Eur. J. Biochem. 254*, 230–237.

Mueller, S., Pantopoulos, K., Hubner, C.A., Stremmel, W., Hentze, M.W., **2001**. IRP1 activation by extracellular oxidative stress in the perfused rat liver. *J. Biol. Chem. 276*, 23192–23196.

Narahari, J., Ma, R., Wang, M., Walden, W.E., **2000**. The aconitase function of iron regulatory protein 1. Genetic studies in yeast implicate its role in iron-mediated redox regulation. *J. Biol. Chem. 275*, 16227–16234.

Nichol, H., Law, J.H., Winzerling, J.J., **2002**. Iron metabolism in insects. *Annu. Rev. Entomol. 47*, 535–559.

Nichol, H.K., Locke, M., **1989**. The characterization of ferritin in an insect. *Insect Biochem. Mol. Biol. 19*, 587–602.

Nichol, H.K., Locke, M., **1990**. The localization of ferritin in insects. *Tissue Cell 22*, 767–777.

Nichol, H., Locke, M., **1999**. Secreted ferritin subunits are of two kinds in insects molecular cloning of cDNAs encoding two major subunits of secreted ferritin from *Calpodes ethlius*. *Insect Biochem. Mol. Biol. 29*, 999–1013.

Nichol, H., Winzerling, J.J., **2002**. Structured RNA upstream of insect cap distal iron responsive elements enhances iron regulatory protein-mediated control of translation. *Insect Biochem. Mol. Biol. 32*, 1699–1710.

Oliveira, M.F., Silva, J.R., Dansa-Petretski, M., de Souza, W., Braga, C.M., *et al.*, **2000**. Haemozoin formation in the midgut of the blood-sucking insect *Rhodnius prolixus*. *FEBS Lett. 477*, 95–98.

Oliveira, M.F., Timm, B.L., Machaldo, E.A., Miranda, E.A., Attias, M., *et al.*, **2002**. On the pro-oxidant effect of haemozoin. *FEBS Lett. 512*, 139–144.

Pantopoulos, K., Hentze, M.W., **1995**. Nitric oxide signaling to iron-regulatory protein: direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. *Proc. Natl Acad. Sci. USA 92*, 1267–1271.

Pascoa, V., Oliveira, P.L., Dansa-Petretski, M., Silva, J.R., Alvarenga, P.H., *et al.*, **2002**. *Aedes aegypti* peritrophic matrix and its interaction with heme during blood digestion. *Insect Biochem. Mol. Biol. 32*, 517–533.

Peitsch, M.C., **1995**. Protein modeling by E-mail. *Biotechnology 13*, 658–666.

Peitsch, M.C., **1996**. ProMod and Swiss-Model: internet-based tools for automated comparative protein modelling. *Biochem. Soc. Trans. 24*, 274–279.

Peitzsch, R.M., McLaughlin, S., **1993**. Binding of acylated peptides and fatty acids to phospholipid-vesicles pertinence to myristoylated proteins. *Biochemistry 32*, 10436–10443.

Pham, D.Q.-D., **2000**. Molecular modeling of insect ferritins. *In Silico Biol.,* 531–544.

Pham, D.Q.-D., Shaffer, J.J., Chavez, C.A., Douglass, P.L., **2003**. Identification and mapping of the promoter for the gene encoding the ferritin heavy-chain homologue of the yellow fever mosquito *Aedes aegypt*i. *Insect Biochem. Mol. Biol. 33*, 51–62.

Pham, D.Q.-D., Winzerling, J.J., Dodson, M.S., Law, J.H., **1999**. Transcriptional control is relevant in the modulation of mosquito ferritin synthesis by iron. *Eur. J. Biochem. 266*, 236–240.

Pham, D.Q.-D., Zhang, D., Hufnagel, D.H., Winzerling, J.J., **1996**. *Manduca sexta* hemolymph ferritin: cDNA sequence and mRNA expression. *Gene 172*, 255–259.

Phillips, J.D., Kinikini, D.V., Yu, Y., Guo, B., Leibold, E.A., **1996**. Differential regulation of IRP1 and IRP2 by nitric oxide in rat hepatoma cells. *Blood 87*, 2983–2992.

Ponka, P., Beaumont, C., Richardson, D.R., **1998**. Function and regulation of transferrin and ferritin. *Sem. Hem. 35*, 35–54.

Rost, B., Casadio, R., Fariselli, P., Sander, C., **1995**. Transmembrane helices predicted at 95% accuracy. *Protein Sci. 4*, 521–533.

Rost, B., Sander, C., **1993**. Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol. 232*, 584–599.

Rost, B., Sander, C., **1994a**. Conservation and prediction of solvent accessibility in protein families. *Proteins 20*, 216–226.

Rost, B., Sander, C., **1994b**. Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins 19*, 55–72.

Rothenberger, S., Mullner, E.W., Kuhn, L.C., **1990**. The mRNA-binding protein which controls ferritin and

transferrin receptor expression is conserved during evolution. *Nucleic Acids Res. 18*, 1175–1179.

Santambrogio, P., Levi, S., Cozzi, A., Corsi, B., Arosio, P., **1996**. Evidence that the specificity of iron incorporation into homopolymers of human ferritin L- and H-chains is conferred by the nucleation and ferroxidase centres. *Biochem. J. 314*, 139–144.

Sitzler, S., Oldenburg, I., Petersen, G., Bautz, E.K.F., **1991**. Analysis of the promoter region of the housekeeping gene *DmRP140* by sequence comparison of *Drosophila melanogaster* and *Drosophila virilis*. *Gene 100*, 155–162.

Sun, G., Tomita, H., Shakkottai, V., Gargus, J.J., **2001**. Genomic orgnization and promoter analysis of human KCNN3 gene. *J. Hum. Genet. 46*, 463–470.

Surinya, K., Cox, T.C., May, B.K., **1998**. Identification and characterization of a conserved erythroid-specific enhancer located in intron 8 of the human 5–aminolevulinate synthase 2 gene. *J. Biol. Chem. 273*, 16798–16809.

Takahashi, K., Nishiyama, C., Nishiyama, M., Okumura, K., Ra, C., *et al.*, **2001**. A complex composed of USF1 and USF2 activates the human FcεRI α chain expression *via* a CAGCTG element in the first intron. *Eur. J. Immunol. 31*, 590–599.

Tang, C.K., Chin, J., Harford, J.B., Klausner, R.D., Rouault, T.A., **1992**. Iron regulates the activity of the iron-responsive element binding protein without changing its rate of synthesis or degradation. *J. Biol. Chem. 267*, 24466–24470.

Theil, E.C., **1987**. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem. 56*, 289–315.

Theil, E.C., **1994**. Iron regulatory elements (IREs): a family of mRNA non-coding sequences. *Biochem. J. 304*, 1–11.

Torti, F.M., Torti, S.V., **2002**. Regulation of ferritin genes and protein. *Blood 99*, 3505–3516.

Trikha, J., Theil, E.C., Allewell, N.M., **1995**. High resolution crystal structures of amphibian red-cell L ferritin: potential roles for structural plasticity and solvation in function. *J. Mol. Biol. 248*, 949–967.

Trikha, J., Waldo, G.S., Lewandowski, F.A., Ha, Y., Theil, E.C., *et al.*, **1994**. Crystallization and structural analysis of bullfrog red cell L-subunit ferritins. *Proteins 18*, 107–118.

Trowler, D.A., Gordon, J.I., Adams, S.P., Glaser, L., **1988**. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem. 57*, 69–99.

Tsuji, Y., Akebi, N., Lam, T.K., Nakabeppu, Y., Torti, S.V., *et al.*, **1995**. FER-1, an enhancer of the ferritin H gene and a target of E1A-mediated transcriptional repression. *Mol. Cell. Biol. 15*, 5152–5164.

Tsuji, Y., Ayaki, H., Whitman, S.P., Morrow, C.S., Torti, S.V., *et al.*, **2000**. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol. Cell. Biol. 20*, 5818–5827.

Tsuji, Y., Moran, E., Torti, S.V., Torti, F.M., **1999**. Transcriptional regulation of the mouse ferritin H gene: involvement of p300/CBP adaptor proteins in FER-1 enhancer activity. *J. Biol. Chem. 274*, 7501–7507.

Wardrop, S.L., Watts, R.N., Richardson, D.R., **2000**. Nitrogen monoxide activates iron regulatory protein 1 RNA-binding activity by two possible mechanisms: effect on the [4Fe–4S] cluster and iron mobilization from cells. *Biochemistry 39*, 2748–2758.

Wei, W., Brennan, M.D., **2000**. Polarity of transcriptional enhancement revealed by an insulator element. *Proc. Natl Acad. Sci. USA 97*, 14518–14523.

Wei, Y., Miller, S.C., Tsuji, Y., Torti, S.V., Torti, F.M., **1990**. Interleukin 1 induces ferritin heavy chain in human muscle cells. *Biochem. Biophys. Res. Commun. 169*, 289–296.

Weiss, G., Bogdan, C., Hentze, M.W., **1997**. Pathways for the regulation of macrophage iron metabolism by the anti-inflammatory cytokines IL-4 and IL-13. *J. Immunol. 158*, 420–425.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., *et al.*, **1993**. Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *EMBO J. 12*, 3651–3657.

Weiss, G., Widner, B., Zoller, H., Schobersberger, W., Fuchs, D., **1998**. Immune response and iron metabolism. *Br. J. Anaesth. 81*(Suppl. 1), 6–9.

Welch, K.D., Reilly, C.A., Aust, S.D., **2002**. The role of cysteine residues in the oxidation of ferritin. *Free Rad. Biol. Med. 33*, 399–408.

Winzerling, J.J., Law, J.H., **1997**. Comparative nutrition of iron and copper. *Annu. Rev. Nutr. 17*, 501–526.

Winzerling, J.J., Nez, P., Porath, J., Law, J.H., **1995**. Rapid and efficient isolation of transferrin and ferritin from *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 217–224.

Yokomori, N., Iwasa, Y., Aida, K., Inoue, M., Tawata, M., *et al.*, **1991**. Transcriptional regulation of ferritin messenger ribonucleic acid levels by insulin in cultured rat glioma cells. *Endocrinology 128*, 1474–1480.

Zhang, D., Albert, D.W., Kohlhepp, P., Pham, D.Q.-D., Winzerling, J.J., **2001a**. Repression of *Manduca sexta* ferritin synthesis by IRP1/IRE interaction. *Insect Mol. Biol. 10*, 531–539.

Zhang, D., Dimopoulos, G., Wolf, A., Minana, B., Kafatos, F.C., *et al.*, **2002**. Cloning and molecular characterization of two mosquito iron regulatory proteins. *Insect Biochem. Mol. Biol. 32*, 579–589.

Zhang, D., Ferris, C., Gailer, J., Kohlhepp, P., Winzerling, J.J., **2001b**. *Manduca sexta* IRP1: molecular characterization and *in vivo* response to iron. *Insect Biochem. Mol. Biol. 32*, 85–96.

4.11 Tick-Talk, the Cellular and Molecular Biology of *Drosophila* Circadian Rhythms

P H Taghert and Y Lin, Washington University Medical School, Saint Louis, MO, USA

4.11.1. Introduction

Circadian rhythms describe daily cycles of behavioral, physiological, or biochemical events that display a precise period, and that can persist without reference to environmental cues. They arise from endogenous biological clocks, the gears of which are driven by cycling genes and proteins, and whose free-running periods approximate 24 h. Environmental cues that register predictable time changes, such as the daily light/dark (LD) or temperature cycles (see **Chapter 3.11**), normally adjust the phase and period of the clock to maintain precise synchrony with the rotation of the earth. The registration of internal clocks with external events is termed "entrainment" (Pittendrigh, 1974).

Forward mutagenesis screens in *Drosophila* have identified a small handful of proteins whose principles of structural design subserve the central clock function, a daily rhythm of changes in their levels. Mutations in each of these genes result in circadian rhythm abnormalities, ranging from alterations in circadian period to complete arrhythmicity. The analysis of these proteins and their cognate genes has produced a wealth of information and insight. In sum, these findings have translated into detailed biochemical and genetic interactions. They have also begun to define general principles underlying the fundamental clock mechanisms. This subject has been reviewed extensively (e.g., recently by Young and Kay, 2001; Ashmore and Sehgal, 2003; Hall,

2003; Helfrich-Förster, 2003; Van Gelder *et al.*, 2003). That level of overview testifies to the sustained successes of circadian biology researchers, and to the general interest the field is able to stimulate.

In circadian biology, work on *Drosophila* has contributed the most to mechanistic studies of the central circadian pacemaker (the clockworks). However, there are two other important aspects of circadian biology that have begun to receive increasing attention from researchers in the past few years, and which also merit continued review: first, how circadian rhythms are synchronized with environmental cycles (the input end); and second, how they organize more distal rhythmic events (the output end). Therefore, this review is divided into sections that consider in turn clock mechanisms, their inputs, and their outputs. Separating circadian rhythms in this way facilitates presentation, but it also creates an impression of seams that likely do not correspond to biological reality. For example and as reviewed below, recent work has indicated that "input factors" can also act as central clockwork components, depending on the pacemakers studied. Likewise, *bona fide* "output factors" can also act as input factors, depending on which cells receive the timing signal. These issues reflect and represent complexities that presently are challenging to consider, and that eventually will be resolved and rationalized. At such a time, circadian biology will probably be presented and reviewed more seamlessly. In the meantime, the canonical three-part organization serves the present purposes well, and we have adopted it once more.

4.11.2. The Central Oscillator Described by Studies of *Drosophila*

4.11.2.1. An Overview of the Circadian Clockwork Mechanism in *Drosophila*

A brief description of the clockwork mechanism is as follows. *period* and *timeless* transcription are driven via a common *cis*-promoter element (the E-box) by the helix–loop–helix transcription factors *Clock* and *cycle*, working *in trans*. In the early night, Period (PER) and Timeless (TIM) accumulate in the cytoplasm; however, as individual proteins they are rapidly degraded. By a process of posttranslational modifications and protein–protein interaction, the TIM and PER are stabilized and their concentrations increase. Those increases promote the dimerization of TIM and PER, and the phosphorylation of TIM by Shaggy (SGG); the PER : TIM complex is imported (along with the Double-time (DBT)) kinase into the nucleus in the late night, where it binds to and inactivates the Clock : Cycle (CLK : CYC) dimer, causing transcription to halt. The PER : TIM dimer is destabilized by DBT phosphorylation, and PER and TIM are degraded via the proteosome. Their declining levels permit CLK : CYC dimers once again to bind to E-boxes and activate *per* and *tim* transcription. In the sections immediately following, these events and some of the evidence underlying the model are reviewed in greater detail.

4.11.2.2. The *period* and *timeless* Genes: Identification and Molecular Rhythms

4.11.2.2.1. *period* – the founding member of the clock genes The *period* gene was identified in pioneering genetic screens for mutations that affect circadian period (Konopka and Benzer, 1971). These and subsequent screens (e.g., Hamblen-Coyle *et al.*, 1989; Konopka *et al.*, 1994) recovered *per* alleles that either shorten or lengthen the period of rhythmicity, or eliminate it altogether (Baylies *et al.*, 1987; Yu *et al.*, 1987). Following its molecular cloning (Jackson *et al.*, 1986; Citri *et al.*, 1987), a conceptual translation related PER to the transcription factor proteins ARNT, AHR, and SIM by a novel sequence domain (Crews *et al.*, 1988; Hoffman *et al.*, 1991; Burbach *et al.*, 1992; Reyes *et al.*, 1992). That region was called the PAS domain, a name that acknowledges its founding members (*P*ER, *A*RNT, and *S*IM). Biochemical studies showed that the PER PAS domain mediates homodimerization (Huang *et al.*, 1993) and heterodimerization with the TIM protein (Gekakis *et al.*, 1995). PER lacks motifs typical of DNA-binding proteins (Jackson *et al.*, 1986; Citri *et al.*, 1987). *per* RNA and protein levels display robust diurnal and circadian rhythms (Siwicki *et al.*, 1988; Hardin *et al.*, 1990, 1992a; Zerr *et al.*, 1990; Edery *et al.*, 1994a). During a 24-h cycle under LD conditions, *per* RNAs are detectable between ZT11 and ZT18 (ZT = zeitgeber time where ZT0 = time of lights-on and ZT12 = time of lights-off). The *per* RNA rhythm displays a peak early in the evening, during ZT13–16. The trough to peak amplitude of this rhythm is about 10-fold. The levels of PER protein also display a circadian rhythm, but their accumulation and peak lag behind the RNA cycle by 6–8 h (Hardin *et al.*, 1990; Zerr *et al.*, 1990). The mechanisms and significance of separated peaks in *per* RNA versus PER protein has been a key point of analysis, which is described below.

4.11.2.2.2. *timeless* – the second circadian clock gene The second central clock gene isolated in flies was called *timeless* (*tim*). As with *per*, a null

mutation at *tim* results in arrhythmic individuals, indicating that *tim* is necessary to generate rhythmic behavior (Sehgal *et al.*, 1994). There are at least 10 reported *tim* alleles including arrhythmic, short, long, and ultralong phenotypes (Sehgal *et al.*, 1994; Rutila *et al.*, 1996; Matsumoto *et al.*, 1999; Rothenfluh *et al.*, 2000a, 2000b). *tim* encodes a large protein of novel structure whose only recognizable domains were separate acidic and basic regions (Myers *et al.*, 1995). *tim* RNA cycles roughly in phase with *per* RNA (Sehgal *et al.*, 1995); TIM protein levels peak around ZT17–19 and PER protein levels peak a short while later, at about ZT19–21 (Zeng *et al.*, 1996). The *tim* gene's function supports *per* RNA cycling (Sehgal *et al.*, 1994) and PER protein cycling (Price *et al.*, 1995). Together these observations provided important early clues that TIM and PER probably interact in their time-keeping functions.

Are the observed molecular rhythms of *per* and *tim* integral to the manifestation of behavioral rhythms? If so, it would follow that they should "track" alterations of behavioral rhythms that are observed in period-altering mutant stock. That prediction was borne out repeatedly. For example, PER protein in the per^S stock displayed altered peak times corresponding to the altered period of behavior in both LD and in constant darkness (DD; Zerr *et al.*, 1990). Significantly, when alterations in PER or TIM levels were experimentally induced, the phase of behavioral rhythms was shifted (Edery *et al.*, 1994b; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996; Suri *et al.*, 1999). Those combined correlations and causal effects demonstrated that circadian behavioral rhythms are true read-outs of *per* and *tim* molecular rhythms.

4.11.2.3. The Autoregulatory Feedback Loop Model

The earliest genetic evidence suggested that the *per* gene product operates close to the heart of the circadian cycling machinery, but at what level it participates remained a mystery until a seminal discovery about a decade ago by Hardin *et al.* (1990). They showed that the *period* gene product regulates its own expression: this observation provided the essential clue to developing a model of the circadian clock mechanism. The PER protein feedbacks to affect *per* mRNA levels as indicated by the activity of a wild-type *per* transgene in a *per* null mutant. In the per^{01} stock, *per* RNA does not cycle and is stable at about 50% of its normal peak amplitude. Transgenic *per* activity restored cycling properties to that of per^{01} RNA – the mutant *per* RNA now displayed up to fourfold cycle amplitude (from a normal ~10-fold range). The findings led directly to a model in which PER protein affects a negative regulation on its own transcription, hence, a negative autoregulatory feedback loop. This concept was extended to incorporate the fundamental influence of TIM (Sehgal *et al.*, 1995), and together these results suggested that fluctuating PER and TIM protein levels are a consequence of, and contribute to, the fluctuating levels of *per* and *tim* RNA (Hardin *et al.*, 1990; Sehgal *et al.*, 1995; Marrus *et al.*, 1996). Cycles of *per* and *tim* gene expression therefore form the central elements of the *Drosophila* pacemaker – a feedback loop that meets the criteria set for a self-sustaining oscillator (Hardin *et al.*, 1992a).

4.11.2.4. Mechanisms of Clock Gene Expression

4.11.2.4.1. Clock gene cycling largely reflects changes in transcription The initial observations of *per* RNA cycles were pursued to establish whether such fluctuations reflect transcriptional or posttranscriptional mechanisms. While both explanations have validity, evidence suggests that fluctuations in *per* gene transcription are the primary source for *per* mRNA oscillations. So and Rosbash (1997) showed that the rates of both *per* and *tim* transcription were circadianly regulated through the use of run-on assays. Further evidence of transcriptional control of the *per* gene was revealed by demonstrations that its upstream sequences confer circadian cycling onto heterologous (reporter) genes (Hardin *et al.*, 1992a; Brandes *et al.*, 1996). Hao *et al.* (1997, 1999) pursued the definition of *per* regulatory sequences by progressively reducing test fragments; they identified a 69-bp fragment capable of driving high-amplitude mRNA cycling under both LD and constant dark DD conditions. That transcriptional activity is *per* protein (PER)-dependent, but is not dependent on a conserved E-box found within the 69-bp fragment (Hao *et al.*, 1997). Even more impressive was the demonstration that this small regulatory fragment, and even its isolated E-box, could produce correct spatial "*per*-like" expression (Hao *et al.*, 1999; Darlington *et al.*, 2000), and drive wild-type *per* sequences in a regulated manner sufficient to rescue behavioral arrhythmicity in per^{01} flies (Hao *et al.*, 1999). Similar promoter analyses, including identification of relevant E-boxes, have also been reported for *tim* (McDonald *et al.*, 2001).

4.11.2.4.2. Clock gene cycling also reflects posttranscriptional regulation Not only does *per* RNA rely upon transcriptional mechanisms, it also depends in part on a circadian regulation of *per* RNA stability (So and Rosbash, 1997; Stanewsky

et al., 1997; Chen *et al.*, 1998; Suri *et al.*, 1999). For example, the rate of *per* transcription rises faster than does the rate of *per* mRNA accumulation, suggesting phase-dependent differences in *per* mRNA stability (So and Rosbash, 1997). Modeling of the data indicates that the posttranscriptional effect contributes 20–40% of the total amplitude of the rhythm. Both TIM and PER proteins are likely to contribute to this stabilization of *per* RNAs (Suri *et al.*, 1999). A posttranscriptional mechanism regulating *per* RNA was also invoked to explain phase differences in expression between PER-LUC fusion proteins versus PER-LUC fusion genes (the latter lacking PER amino acids; Stanewsky *et al.*, 1997). The indicated *cis*-sequences map to the 5′ untranslated region of the *per* mRNA, within the first intron (Stanewsky *et al.*, 2002). In all, there is good evidence to include posttranscriptional mechanisms in modeling the daily fluctuations in clock gene cycling, specifically, a clock-regulated temporal stabilization of *per* mRNA during its daily upswing in the morning.

4.11.2.5. *per* and *tim* RNA Cycles are not Required for the Circadian Autoregulatory Loop

The evidence discussed above showed that oscillations in PER are necessary for oscillation in *per* RNA (Hardin *et al.*, 1990), but is the opposite also true, i.e., is the cycling of *per* RNA needed for PER protein rhythms and for rhythmic behavior? Several observations indicate the answer to this is no. Frisch *et al.* (1994) partially rescued behavioral rhythmicity using a *per* rescue construct that contained no 5′ upstream sequence. The resultant PER levels clearly cycled, while transgenic *per* RNA displayed only a low amplitude (~twofold) rhythm. Cheng and Hardin (1998) addressed the question by driving *per* with a constitutive promoter (*rhodopsin1*) in the eye. They rescued PER cycling in that tissue, with no evidence of cycling by the transgenic *per* RNA. Likewise, Vosshall and Young (1995) also rescued *per* arrhythmicity using a *glass* promoter that is known to be constitutively active, although transgenic *per* RNA levels were not directly measured in that study. Finally, Yang and Sehgal (2001) extended this idea to show that *tim* RNA cycles also are not required for behavioral rhythmicity. Even in the case of a per^{01}; tim^{01} double null mutant fly, behavioral rhythms could be partially rescued by constitutive expression of both *tim* and *per* genes. Each of these studies supports the conclusion that circadian oscillators arise through some posttranscriptional mechanism(s). Together they deny an absolute necessity for *per* or *tim* RNA cycles. A prediction from this general conclusion is that if PER or TIM protein cycles are swamped, then behavioral rhythms and pacemaker protein cycling should be attenuated or lost. That proved to be true following the use of very strong, constitutive promoters to drive PER and TIM to high levels in wild-type stocks (e.g., Zeng *et al.*, 1994; Yang and Sehgal, 2001; Blanchardon *et al.*, 2001).

per and *tim* RNA cycles, though not strictly required for a manifestation of rhythmicity, probably serve several useful functions (Cheng and Hardin, 1998). The strength and/or phasing of rhythmicity, in the absence of high amplitude clock gene RNA cycling, are often abnormal (e.g., Frisch *et al.*, 1994; Vosshall and Young, 1995; Yang and Sehgal, 2001). Clock gene RNA cycling is also likely to be critical for the mechanisms that reset circadian phase according to light. Current models of that reset mechanism suggest different outcomes, depending on *tim* RNA levels (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996; see Section 4.11.3.2.1 for a description of these models).

4.11.2.6. Nuclear Translocation

4.11.2.6.1. PER and TIM nuclear translocation In considering the potential functions of PER and TIM in the clock mechanism, their subcellular locations were a good basis on which to formulate hypotheses. If the proteins remain cytoplasmic, the putative negative feedback on *per* RNA (Section 4.11.2.3) must have agency via other intermediary proteins. If, on the other hand, PER and/or TIM had a nuclear distribution, that would allow for consideration of their potential direct and/or indirect roles in such transcriptional regulation. All the available evidence suggests PER and TIM are nuclear proteins for at least a significant portion of the daily cycle. Using specific antibodies, PER and TIM were localized to the cytoplasm, but also to the nucleus at predictable times *in vivo* (Saez and Young, 1988; Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Liu *et al.*, 1992; Curtin *et al.*, 1995; Shafer *et al.*, 2002).

4.11.2.6.2. The importance of PER and TIM coexpression for nuclear translocation PER and TIM require each other's presence for nuclear translocation to occur normally. Evidence for these requirements came from genetic observations: PER is absent from the nucleus without TIM (Vosshall *et al.*, 1994; Price *et al.*, 1995; Saez and Young, 1996) and TIM's nuclear localization is likewise dependent on *per* function (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996). This mutual requirement was strikingly illustrated in cultured (S2) *Drosophila* cells. When either TIM or PER proteins were

expressed independently, they accumulated in the cytoplasm; however, when coexpressed, both proteins translocated to the nuclei (Saez and Young, 1996).

The requirement for coexpression was most easily explained by a direct interaction between the PER and TIM proteins. Several observations *in vivo* and *in vitro* supported this model. Vosshall *et al.* (1994) studied PER–β-galactosidase (PER–β-gal) fusion proteins *in vivo* and noted that fusion proteins lacking PER amino acids 95 and 529 localized to nuclei in both wild-type and tim^{01} strains of flies. In contrast, fusion proteins containing this region of PER were nuclear in wild-type, but cytoplasmic in tim^{01} flies. This suggested that sequences mapping in the deleted section of PER normally inhibit its own nuclear localization, in the absence of *tim* activity (Vosshall *et al.*, 1994). By the simplest model, TIM binds PER in this region, or permits some other factor to do so, and thus facilitates PER nuclear translocation.

4.11.2.6.3. The importance of PER and TIM heterodimerization for nuclear translocation Evidence for physical interactions between PER and TIM were first demonstrated *in vitro*. The *tim* gene was isolated in a two-hybrid screen for PER-interacting proteins and, in that same yeast interaction assay, TIM binds the PAS domain of PER (Gekakis *et al.*, 1995). By deletion analysis, specific regions were identified in PER and TIM that are required for (1) nuclear entry and that (2) promote cytoplasmic retention (Saez and Young, 1996). These results indicated that each protein contains a nuclear localization signal (NLS) and a cytoplasmic localization domain (CLD). Remarkably, TIM also binds to the PER CLD region, as indicated by GST pull-down assays (Saez and Young, 1996). The same PER region that promotes cytoplasmic retention is also the site of interaction between the two proteins. In addition, the PER[L] protein isoform, which is defective in TIM binding, exhibits a delay in nuclear entry *in vivo* (Gekakis *et al.*, 1995). Thus, suppression of cytoplasmic localization is accomplished by direct interaction of PER and TIM via sites that otherwise discourage nuclear entry. These observations helped establish the hypothesis that a critical checkpoint in the circadian cycle is the requirement for cytoplasmic assembly of a PER/TIM heterodimer (or complex) as a condition for nuclear transport of either protein. A consequence of this mechanism is that rates of PER and TIM synthesis and accumulation should influence the efficiency of PER/TIM heterodimerization and therefore the timing of nuclear localization.

In vivo, TIM and PER also interact in a predictable manner as a function of time of day, and PER appears to be the rate-limiting factor. By employing the technique of coimmunoprecipitation, Lee *et al.* (1996) and Zeng *et al.* (1996) showed that PER and TIM form a 1 : 1 heterodimer. Remarkably, at ZT20, $\geq$80% of the total amount of PER is bound to TIM in a 1 : 1 stoichiometric relationship. The evidence suggests that TIM is usually present in excess (1.5–1.8 times more TIM than PER) and is thus not rate-limiting; further, TIM is present in PER immunodepleted extracts (Zeng *et al.*, 1996; Lee *et al.*, 1998). Genetic evidence agrees with this conclusion: Smith and Konopka (1981) showed that decreasing *per* dosage lengthens circadian period, while increasing *per* dosage shortens it. PER is a circadian state variable in that its levels tell the organism the time of day. In contrast, TIM is not a state variable, as the clock is not sensitive to *tim* dosage (Rothenfluh *et al.*, 2000a; Ashmore *et al.*, 2003).

4.11.2.6.4. A recent revision to the model of the obligate PER : TIM heterodimer The simplest model predicts that PER : TIM dimers begin to enter the nucleus late in the day, and accumulate to a peak late at night. By several criteria heterodimer formation promotes that stable translocation and was therefore considered a requirement. But, in revision to the original model, PER is found in the nucleus of the principal brain pacemaker neurons 3–4 h prior to the nuclear detection of TIM (Shafer *et al.*, 2002). The brain pacemakers in question are the small lateral cells (vLNs; see Section 4.11.4.2.1) and PER arrives in the nucleus at a time consistent with its participation in transcriptional repression (ZT18). This observation calls into the question the hypothesis of a requirement for the obligate PER : TIM heterodimer formation to enter the nucleus. That hypothesis was based primarily on biochemical analyses of head extracts, which presents a primary contribution by events occurring in photoreceptor pacemakers. An alternative mechanism (at least within LN pacemaker neurons) suggests that a PER : TIM heterodimer may enter the nucleus, but that TIM specifically may not be retained as efficiently as PER. Supporting observations were made by Ashmore *et al.* (2003). In S2 cells, TIM, but not PER, is subject to active nuclear export, as evidenced by the fact that TIM can enter the nucleus without PER, if nuclear export is blocked. Together these observations suggest that PER in the vLN pacemaker neurons may initially function to keep TIM in the cytoplasm as dimer formation progresses. It is then imported into the nucleus at a net rate faster than TIM and, later

in the cycle, it is required to permit TIM nuclear accumulation.

4.11.2.7. Posttranslational Modifications – Phosphorylation

If the PER : TIM dimer were able to accumulate in the nucleus directly following translation, negative regulation of *per* and *tim* gene expression would be exerted before the *per* and *tim* RNA levels had reached maximal levels, thereby dampening or eliminating oscillations (e.g., Sehgal *et al*., 1994; Leloup and Goldbeter, 1998). PER and TIM stability in the cytoplasm during the early evening therefore appears to be a major point of regulation to effect a lag in protein accumulation. The rate at which these proteins enter and/or accumulate in the nucleus is also likely to be critical. These points of regulation are processes likely to involve post-translational modifications, and the one that has received the most experimental attention to date is phosphorylation.

4.11.2.7.1. PER and TIM phosphorylation PER displays a substantial decrease in gel mobility between ZT15 and ZT24 and that cycle continues in DD (Edery *et al*., 1994a). Phosphorylation underlies that mobility change, as the shift is entirely sensitive to phosphatases. During the period of phosphorylation, mobility changes appear continuously or repeatedly, which suggested the occurrence of multiple sequential phosphorylation events, perhaps in an interdependent fashion (Edery *et al*., 1994a). The most highly phosphorylated forms of PER occur at times of night when the proteins are predominantly nuclear. The functional significance of the phosphorylation of relevant clock proteins was further indicated by the demonstration of altered periodicities of phosphorylation rhythms that occur in per^S and per^L flies. The directions of the altered phosphorylation rhythms matched the directions of the altered behavioral rhythms. TIM also displays phosphorylation-dependent mobility shifts as a function of time of day, although the extent is not as dramatic as the case of PER, as indicated by the percentage mobility change (Myers *et al*., 1996; Zeng *et al*., 1996). To date, three kinases have been implicated in the modifications of PER and TIM, and their analysis has greatly informed the current models of circadian mechanisms.

4.11.2.7.2. Double-time A principal determinant of the subcellular movements and stability of PER over the course of the circadian cycle is the protein kinase encoded by the *double-time* (*dbt*) locus (Price *et al*., 1998). DBT is a kinase related to the vertebrate delta and epsilon isoforms of casein kinase I (Kloss *et al*., 1998). A mammalian ortholog (CK1 ε) is also known to have a circadian function, corresponding to the naturally occurring *tau* mutation of hamsters, which displays a short period (Lowrey *et al*., 2000). Genetic analyses of *dbt* functions in *Drosophila* suggest that DBT contributes to the generation of circadian rhythmicity by regulating multiple steps of clock biochemistry. To date, three main effects have been suggested. First, DBT helps to determine the ~8-h lag normally occurring between peaks of *per* RNA and protein. It binds to monomeric PER in the cytoplasm and slows its accumulation via phosphorylation, and later degradation (Kloss *et al*., 1998; Price *et al*., 1998). Second, a role for *dbt* in the turnover of nuclear PER has been proposed. PER is lost from wild-type nuclei but persists longer in *dbt* mutant nuclei (Price *et al*., 1998). Hypo-phosphorylated PER thus accumulates to high levels in *dbt* mutants and becomes a constitutive repressor in DD (Price *et al*., 1998; Rothenfluh *et al*., 2000b; Bao *et al*., 2001; Kloss *et al*., 2001; Suri *et al*., 2001). Third, DBT appears to affect the rate of nuclear accumulation of PER by delaying the rate at which PER translocates or is stabilized upon translocation (Bao *et al*., 2001; Suri *et al*., 2001).

Several alleles of *dbt* have been recovered, based mostly on chemical mutagenesis screens to detect circadian period alterations in heterozygous mutant flies (Price *et al*., 1998; Rothenfluh *et al*., 2000b; Suri *et al*., 2001). The semidominant alleles dbt^S and dbt^L display strong effects on circadian period (shortened and lengthened, respectively). Like per^S, the dbt^S mutation increases the amplitude of the phase-response curve (PRC) and shortens its period (Bao *et al*., 2001). These alleles influence the rate of PER phosphorylation in concert with their effects on behavior: phosphorylation is advanced in dbt^S and delayed in dbt^L. The rate of TIM phosphorylation appears much less affected. The basis for dbt^S and dbt^L semidominance is unknown: extra copies of wild-type *dbt* do not appreciably affect circadian period (Price *et al*., 1998). Kloss *et al*. suggest their semidominance may reflect a stable stoichiometric interaction between PER and DBT *in vivo*.

Cyclic associations of PER with DBT are critical in producing stereotyped changes in PER phosphorylation state and its stability. The *dbt* gene is highly pleiotropic and is allelic to the gene *discs large* (Zilian *et al*., 1999); homozygous mutants display pupal lethality. Antibody localization of DBT indicates that in the brain it is widely expressed, and found in those neurons that also express PER and TIM (Kloss *et al*., 2001). *dbt* RNA and DBT protein

levels do not cycle; however, DBT displays a robust circadian translocation from cytoplasm to nucleus in the LN pacemaker neurons (Kloss *et al.*, 2001). DBT nuclear accumulation depends on expression of PER and not of TIM, which implies a physical association between DBT and PER. That inference is supported by genetic evidence that *per* short alleles specifically suppress the arrhythmic phenotype of the dbt^{AR} allele (Rothenfluh *et al.*, 2000b). Furthermore, DBT interacts directly with PER *in vitro* and also within S2 cells. DBT : PER interactions *in vivo* can be detected by coimmunoprecipitations in the cytoplasm of tim^{01} heads (these are interactions with the residual PER that remains in that mutant). DBT is also detected in PER : TIM complexes, but does not appear to interact directly with TIM, as indicated by measurements in per^{01} heads (Kloss *et al.*, 2001). Together these data suggest DBT is closely associated with monomeric PER, and later in the nucleus DBT is again closely associated with PER when it is complexed to TIM. One model has TIM inhibiting DBT phosphorylation of PER in the cytoplasm (Kloss *et al.*, 2001). Then, TIM is slowly lost from the nuclear complex, which allows greater repression, but also more PER phosphorylation by DBT. Hyperphosphorylated PER is targeted for degradation.

Thus, DBT appears to promote good temporal order in the circadian system by delaying PER's normal trafficking and thus enforcing the lag of several hours normally seen between the times of peak *per* RNA versus peak PER protein. Promoting the instability of monomeric PER proteins, DBT thus allows PER accumulation only in conjunction with high titers of TIM. Lack of DBT function leads to a continuous, rather than a stably periodic, nuclear transport of PER : TIM complexes. The result of a continuous transport process would be a feedback loop at equilibrium rather than a dynamic molecular oscillator (Kloss *et al.*, 1998). Accordingly, high amplitude cycles of *per* and *tim* RNA can only be achieved with normal DBT functions. Interestingly, in several *dbt* alleles, the lag between *per* RNA and protein peaks is nearly lost under constant conditions, despite the retention of behavioral rhythmicity (Bao *et al.*, 2001; Suri *et al.*, 2001). Both sets of authors conclude that the lag of total PER accumulation may not be especially critical, in comparison to the relative amount of phosphorylated PER in the nucleus.

A broader role for DBT in the definition of PER's spatial pattern of expression throughout the brain was suggested by observations reported by Price *et al.* (1998). PER expression in the hypomorphic allele dbt^{P} was widened to include regions of the brain not significantly stained in a wild-type background. They suggest that the effect is likely not due to changes in *per* transcription or RNA stability. Rather, they infer that normally there is a low level of *per* expression in a set of neurons, which is larger than that normally described. Diminution in the rate of PER degradation (here by loss of DBT activity) permits its visualization. Specifically, they relate this inferred pattern to similar ones seen with PER-BGAL fusion proteins (Kaneko *et al.*, 1997 and see Section 4.11.4.2.1).

4.11.2.7.3. Shaggy The involvement of a second kinase called Shaggy (SGG)/Glycogen Synthase Kinase-3 was revealed in a gain-of-function screen by Martinek *et al.* (2001). They screened thousands of stocks for possible defects in circadian period when single genes were overexpressed in clock cells (using *tim*-GAL4). Overexpression of *sgg* produced a strong reduction in circadian period, and in complimentary experiments, reducing *sgg* levels lengthened period. SGG overexpression also shortened the cycle of TIM and PER nuclear entry to an extent predicted by the shortened behavioral rhythms. In addition, SGG overexpression also shortened the delay phase of the PRC, suggesting SGG affects a cytoplasmic event in the molecular oscillation mechanism. The primary biochemical event dependent on SGG appears to be TIM phosphorylation: hypomorphic *sgg* mutations alter TIM phosphorylation, and *sgg* overexpression elevates TIM phosphorylation *in vivo* (Martinek *et al.*, 2001). The mammalian ortholog GSK3b phosphorylates TIM *in vitro*.

Martinek *et al.* suggest that, like DBT, SGG's principal influence is on the nuclear translocation of the TIM : PER heterodimer. By that scenario, these two kinases are prime actors, working in concert but at cross-purposes, to help determine the rate at which PER's negative feedback regulation occurs. While DBT retards that translocation to the nucleus, SGG accelerates it. Remarkably, in vertebrates, the ortholog of DBT and SGG coparticipate in the Wnt signaling pathway (Peifer *et al.*, 1994; Siegfried *et al.*, 1994; Sakanaka *et al.*, 1999), signaling that appears otherwise unrelated to circadian periodicity.

4.11.2.7.4. Casein kinase II The analysis of PER phosphorylation in *dbt* mutants suggested the participation by other kinases (Price *et al.*, 1998; Suri *et al.*, 2001). Recently, two groups reported genetic evidence to implicate separate subunits of casein kinase II (CK2) in that pacemaker phosphorylation pathway (J.M. Lin *et al.*, 2002; Akten *et al.*, 2003).

The CK2 holoenzyme is a tetramer composed of two α (catalytic) and two β (regulatory) subunits. In *Drosophila*, CK2-α and CK2-β mutants both lengthen circadian period in loss-of-function states, while overexpression of the β form leads to a shortened period (Akten *et al*., 2003). CK2-β corresponds to a mutant called *Andante* that was originally identified in a genetic screen for alterations in circadian period (Konopka *et al*., 1991). Based on analogy to biochemical studies with the human enzyme, the β mutation is predicted to diminish the rate of association between CK2 subunits; experimentally, it leads to lowered levels of the β subunit but not the α subunit (Akten *et al*., 2003). A dominant mutation called *Timekeeper* (*Tik*), corresponding to CK2-α was also identified due to a period-altering phenotype (J.M. Lin *et al*., 2002), and it displays a genetic interaction with *per*. A revertant strain *TikR* behaved like a strong hypomorph. CK2 enzyme levels were reduced in *Tik* and *TikR* heterozygotes.

Bacterially expressed CK2-α directly phosphorylates PER *in vitro*, and to a lesser extent TIM also (Zeng *et al*., 1996; J.M. Lin *et al*., 2002). However, PER and TIM phosphorylation levels (mobilities) were not appreciably different in CK2-β mutant tissues (Akten *et al*., 2003). There are two molecular phenotypes associated with loss of CK2-β function. The first is increased PER/TIM abundance (Akten *et al*., 2003), consistent with a hypothesis that CK2 activity promotes PER and TIM degradation, akin to the actions of *dbt* and *sgg* (Zeng *et al*., 1996; Price *et al*., 1998; cf. Rothenfluh *et al*., 2000b; Martinek *et al*., 2001). The second phenotype is a delayed nuclear translocation of both PER and TIM proteins (J.M. Lin *et al*., 2002; Akten *et al*., 2003). It is likely that the mechanism by which CK2 affects circadian period involves this delayed PER/TIM nuclear entry. Both the molecular and behavioral oscillations are phase delayed by about 2 h. Interestingly, CK2 delays PER nuclear entry only in the small vLNs, but not in the large vLNs (Akten *et al*., 2003). Both groups suggest that CK2 might act both in the cytoplasm and nucleus of clock cells to determine the timing of nuclear entry and/or stability of clock proteins.

Both enzyme subunits are expressed preferentially in vLN pacemaker neurons and both display constitutive cytoplasmic expression throughout the day. Intriguingly, CK2 immunosignals are not restricted to the vLN cell bodies, but can also be found at high level in their axonal terminals (J.M. Lin *et al*., 2002). Those authors speculate that the enzyme has roles in addition to PER phosphorylation, perhaps in the processing or release of the neuropeptide PDF (described in Section 4.11.4.2.1).

4.11.2.7.5. PER degradation Phosphorylated PER is targeted for degradation by DBT and perhaps by other kinases (Price *et al*., 1998; Bao *et al*., 2001; Kloss *et al*., 2001; Suri *et al*., 2001). Two studies have shown that hyperphosphorylated PER is targeted to the proteasome by interactions with Slimb, an F-box/WD40-repeat protein functioning in the ubiquitin-proteasome pathway (Grima *et al*., 2002; Ko *et al*., 2002). In S2 cells, Slimb interacts preferentially with phosphorylated PER; overexpression of Slimb *in vivo* produces circadian behavioral phenotypes.

4.11.2.7.6. TIM degradation TIM is degraded in the proteasome via a tyrosine kinase-dependent step (Naidoo *et al*., 1999). This process is discussed below in the context of circadian photosensitivity (Section 4.11.3.2.1).

4.11.2.8. The Positive Clock Factors

4.11.2.8.1. Clock and cycle In flies, the *Clock* and *cycle* genes encode basic helix–loop–helix (bHLH) transcription factors and are critical for circadian rhythmicity and transcription of the *per* and *tim* genes (Allada *et al*., 1998; Bae *et al*., 1998; Darlington *et al*., 1998; Rutila *et al*., 1998). They are both members of the PER-ARNT-SIM (PAS) superfamily of transcription factors and their mammalian orthologs appear to play comparable functional roles (reviews: Stanewsky, 2003; Van Gelder *et al*., 2003). In both *Clk* and *cyc* mutants, nuclear run-on assays indicate that *per* transcription is severely reduced (Allada *et al*., 1998; Rutila *et al*., 1998). In those backgrounds, *per* and *tim* levels are as low as their normal trough levels, and much lower than the depressed amounts found in *per* or *tim* mutants, indicating that *Clk* and *cyc* are "epistatic to and upstream of *per* and *tim*" (Rutila *et al*., 1998). Of the two molecules, the sequence attributes of CLK suggest that it is likely to contain the functional transcriptional activator domains (Rutila *et al*., 1998).

4.11.2.8.2. The CLK : CYC dimer activates clock gene expression CLK and CYC activate *per* and *tim* transcriptions as a heterodimer, primarily through binding to *cis*-regulatory sequences, specifically termed as E-boxes, and to neighboring sequences. In S2 cells, *Clk* increased transcription of *tim-luciferase or per-luciferase* reporters in a manner that depended on the presence of the E-box in each promoter (Darlington *et al*., 1998). Lyons *et al*. (2000) used deletion analysis to scan the E-box containing 69 bp *per* regulatory fragment defined by Hao *et al*. (1997) and their results suggested

that the CLK–CYC target site actually extends beyond the E-box.

In S2 cells, transcriptional activity by CLK : CYC was repressed by coexpression of both *per* and *tim*, but not by either alone. In addition, *per* and *tim* expression without concomitant *Clk* expression did not affect *tim-luciferase* levels. CLK and CYC proteins bind to the E-box in a manner that depends on the presence of both proteins (Lee *et al.*, 1999). PER, TIM, or both were able to block such binding, and did so efficiently when applied in a 1 : 1 molar ratio. Those data suggested that inhibition is due to binding of the inhibitors to one or both components of the CLK : CYC dimer. The available evidence suggests that repression by PER : TIM does not involve destabilization of the CLK : CYC heterodimer (Lee *et al.*, 1999). *In vivo,* the rhythmic activity of the 69-bp *per* promoter fragment described by Hao *et al.* (1997) was dependent on both CLK and CYC (Allada *et al.*, 1998; Rutila *et al.*, 1998). These and other observations help to describe a model of a negative feedback loop in greater detail: CLK : CYC heterodimers transcriptionally activate the *per* and *tim* genes, whose protein products form a complex that subsequently represses that transcriptional activity (cf. Huang *et al.*, 1993).

Clk RNA cycles in antiphase to those of *per* and *tim* RNAs; its levels peak late at night to early in the morning (ZT23 to ZT4), while *cyc* RNA levels do not exhibit rhythmicity (Bae *et al.*, 1998). In the absence of either PER or TIM, *Clk* rhythms are abolished and its levels set at approximately trough values, suggesting that PER and TIM can function (directly or indirectly) as transcriptional activators (see Section 4.11.2.9.1). In agreement with the feedback model, the CLOCK protein undergoes circadian fluctuations in abundance, in phase with Cryptochrome (CRY) and in antiphase with PER and TIM. It is phosphorylated throughout a daily cycle, and interacts with PER, TIM, and/or the PER–TIM complex during the night, but not during most of the day (Lee *et al.*, 1998). The time course for the accumulation of CLK and its RNA products is similar – this is in contrast to the phase lag seen for PER and TIM versus their RNAs. The peak to trough amplitudes of *Clk* cycles are higher in LD than DD (Lee *et al.*, 1998), similar to differences seen for *per* and *tim* (Bae *et al.*, 1998). These data all reflect an influence of light on the amplitude of clock gene expression rhythms (cf. Y. Lin *et al.*, 2002; Section 4.11.4.1.2).

4.11.2.8.3. In the PER : TIM complex, which protein represses the CLK : CYC heterodimer? Most evidence to date suggests that PER is the agent of repression (Marrus *et al.*, 1996; So and Rosbash, 1997; Lee *et al.*, 1998; Rothenfluh *et al.*, 2000c). A range of possible interactions are observed between TIM, PER, and CLK (Lee *et al.*, 1998). Depending on the time in a daily cycle, PER, TIM, and preferentially the PER : TIM complex, can interact directly with CLK or a CLK-containing complex. In anti-CLK coimmunoprecipitations, PER and TIM are first detected at ZT12, followed by increases in amounts that reach peak values at ZT23.9. Also, compared to PER and TIM, CLK is present in limiting amounts during the night. Thus, the interaction of PER and TIM with CLK is restricted mainly to nighttime hours. At ZT20, the majority of the PER and TIM proteins that interact with CLK are in the form of a heterodimeric PER : TIM complex.

In per^{01}, TIM is still found in anti-CLK immunoprecipitants; in contrast, no PER is found associated with CLK in tim^{01}. Either there is too little PER in that mutant or PER requires TIM to bind CLK. Do PER or TIM provide repressing activity or do they only do so when working in concert? TIM is not able to repress CLK : CYC transcription without PER in S2 cells, although, *in vitro* TIM disrupts CLK : CYC binding to an E-box (Lee *et al.*, 1999). Evidence that PER, and not TIM, is the transcriptional repressor of *Clk* came from studies of per^{S}. In that mutant, PER levels in the nucleus fall more quickly than do TIM levels in the nucleus (Marrus *et al.*, 1996). This "per^{S} effect," which advances the subsequent *per* RNA peak, is consistent with the notion that monomeric PER might be the major transcriptional repressor. Likewise, PER cycling, but not TIM cycling, is strongly affected (i.e., displayed lowered amplitude) in the per^{01} background (So and Rosbash, 1997), suggesting that TIM levels are less tightly coupled to the transcriptional feedback loop and that the PER monomer or the PER–TIM heterodimer provides the relevant agent of negative feedback. In agreement with that general conclusion, Ashmore *et al.* (2003) showed that when TIM is localized to the nucleus of S2 cells in the absence of PER (by blocking nuclear export), it was not able to inhibit CLK : CYC transcriptional activation of an E-box reporter.

4.11.2.8.4. CLK levels and the phase of gene activation If CLK and CYC represent the positively acting factors in the circadian oscillation, the simplest model predicts that CLK levels should determine the rising phase of *per* and *tim* RNA cycles. In other words, CLK levels "should" commence at ZT5 and "should" peak between ZT10 and ZT12. However, and paradoxically, the levels of CLK decrease precisely when *per* and *tim* transcripts

accumulate; they are in roughly antiphase temporal alignment in LD (cf. So and Rosbash, 1997). Therefore, the CLK abundance cycle cannot be the main determinant of time at which *per* and *tim* expression increases. An explanation for this apparent paradox is suggested by Lee *et al.* (1998) invoking the importance of preexisting PER : TIM complexes. By this scenario, PER : TIM are extant at early phases of the CLK abundance cycle, and can therefore depress the transcriptional activity of the CLK : CYC heterodimer when CLK levels begin to rise.

4.11.2.9. A Second "Interlocked" Circadian Transcriptional Loop

4.11.2.9.1. Evidence for the second loop There are two interlocked negative feedback loops underlying circadian cycling (Glossop *et al.*, 1999). In the first loop (introduced in Section 4.11.2.3), genes that are activated by CLK are turned off by the PER : TIM complex; for example, the transcription of *per* and *tim* is turned off by PER : TIM repression of CLK : CYC activity (Darlington *et al.*, 1998; Lee *et al.*, 1999). In the second loop, genes that are repressed by CLK are turned on by the PER : TIM complex; for example, *Clk* transcription is activated by PER : TIM repression of CLK : CYC activity (Glossop *et al.*, 1999). The loops are interlocked in that both involve PER : TIM attenuation of CLK regulatory activity. *Clk* RNA levels peak just after dawn, roughly in antiphase with the peaks of *per* and *tim* RNAs (Bae *et al.*, 1998). The findings that underlie definition of the second loop involve *Clk* RNA measurements in single versus double mutant backgrounds. For example, *Clk* RNA levels are constitutively low in per^{01} and tim^{01} suggesting that the PER : TIM complex is required to activate *Clk* transcription (Bae *et al.*, 1998). However, *Clk* RNA levels are surprisingly high in Clk^{Jrk} and in cyc^{01}, indicating negative feedback by CLK on its own expression. Because *Clk* RNA levels are high even in per^{01}; Clk^{Jrk} or per^{01}; cyc^{01} double mutants, PER : TIM must act to activate *Clk* by derepressing CLK-mediated repression (Glossop *et al.*, 1999). In addition, the high levels of *Clk* mRNA in the double mutant stocks imply that a separate *Clk* activator is present (Glossop *et al.*, 1999). Blau, Young, Hardin, and colleagues have produced a series of reports that implicate two related proteins, VRILLE (VRI) and PDP1, as transcription factors that are targets of CLK, and that help to close this second circadian loop (Glossop *et al.*, 1999, 2003; Kim *et al.*, 2002; Cyran *et al.*, 2003).

VRI and PDP1 are hypothesized to have opposite actions: VRI is a repressor and PDP1 is an activator of *Clk* transcription. In doing so, they contribute to a sharpening of rhythmic transcriptional regulation for *Clk*, and possibly also for other clock-controlled genes. VRI levels peak 3–6 h before that of PDP1, and that phase difference is essential to the model of iterated actions by a repressor of *Clk*, then by an activator of *Clk*. In addition, the phenotypic consequences of increasing or decreasing *vri* levels genetically are complimentary to those manipulating *pdp1* levels. For example, raising *vri* levels coupled with a decrease in *pdp1* gene dosage leads to a synergistic lengthening of the circadian period (Cyran *et al.*, 2003). Significantly, VRI and PDP1 compete for access to a binding site on the *Clk* promoter, on which they act as predictable repressor and activator *in vitro*, respectively. These and other data strongly support a model whereby *Clk* activates *vri* and *pdp1*, and these two targets feedback with gene specific delays to generate a proper phasing for *Clk* transcriptional activity.

In further comment on the larger scope of the circadian mechanism, Allada *et al.* (2003) put forward the hypothesis that circadian rhythm amplitude and circadian rhythm period or phase are controlled separately. The posttranscriptional protein phosphorylation feedback loop (involving PER, TIM, SGG, DBT, and CK2) is primarily responsible for period determination. Its alleles display large period changes, whereas *Clk* and *cyc* do not. cyc^{0} does produce increased periods as a heterozygote (Rutila *et al.*, 1998) but that could be ascribed to a dominant negative gain of function. However, a *cyc* deletion/+phenotype is identical, indicating that the cyc^{0}/+phenotype is not due to a dominant mutant effect but rather to a dosage sensitive effect on period. A similar effect of a *Clk* deletion on circadian period is problematic due to its removal of neighboring clock-relevant genes, specifically *pdp1* (Allada *et al.*, 2003). So this proposition is challenging, but the issue remains unresolved.

4.11.2.9.2. The phase of the *Clk* RNA peaks is not a critical feature In spite of the exquisite control evidently in place to ensure the proper phasing of *Clk* RNA expression, there is good evidence that such RNA cycling is not strictly required for normal circadian periodicity in molecular cycling or in behavioral rhythmicity (Kim *et al.*, 2002). The evidence rests on misexpression of *Clk* using the *per*-GAL4 system to drive *Clk* at an inappropriate phase. Such a manipulation does not cause a disturbance in the period or phasing of molecular cycles or of behavioral rhythms. Analogous to the arguments that *per* and *tim* RNA cycles are not strictly required for behavioral or molecular

rhythmicity (cf., Section 4.11.2.5), these observations further the hypothesis that posttranscriptional events are central to the operation of a functional circadian clock. It is noteworthy that swamping *Clk* RNA cycles did produce alterations in certain aspects of light sensitivity (e.g., the phase-altering responses to light pulses delivered in the night). Such observations led to the surprising conclusion that *Clk* regulates the direct response of the locomotor system to light.

4.11.2.10. *In Situ* Expression of Clock Proteins

A great deal has been learned about the functions of clock proteins like PER by examining the cells and tissues in which they are expressed, and by analyzing their subcellular localization. Numerous studies over the years have detailed such information for PER and other clock proteins (Liu *et al*., 1988; Saez and Young, 1988; Siwicki *et al*., 1988; Zerr *et al*., 1990; Ewer *et al*., 1992; Liu *et al*., 1992; Kaneko *et al*., 1997; Rachidi *et al*., 1997; Kloss *et al*., 1998; Price *et al*., 1998; Blau and Young, 1999; Kaneko *et al*., 2000a; Cyran *et al*., 2003). PER is found in various cell types in the brain, typically associated with TIM (review: Helfrich-Förster, 2003), and also in diverse tissues (e.g., Hege *et al*., 1997; Plautz *et al*., 1997; Giebultowicz 1999; Giebultowicz *et al*., 2000; Myers *et al*., 2003). However, only in the cases of the antennal chemosensory cells (see **Chapter 3.15**) and prothoracic glands (see **Chapter 3.11**) have these peripheral oscillators been linked to functional outputs (Krishnan *et al*., 1999; Myers *et al*., 2003). PER's prominent nuclear localization was noted early on (Saez and Young, 1988; Liu *et al*., 1992) and are described in greater detail below, in considering both input pathways to the circadian pacemaking centers (Sections 4.11.3.3.2 and 4.11.3.4.1) and the output pathways (Sections 4.11.4.2.1 and 4.11.4.3). In a related vein, the issue of timing regarding when clock gene action "is required" was addressed by a conditional rescue protocol (Ewer *et al*., 1990). For adult behavioral rhythmicity, it was determined that *period* gene action is needed during the performance of the behavior, and not during antecedent developmental stages.

4.11.2.11. How to Build a Pacemaker: Lessons from the Misexpression of Clock Proteins

The proteins of the clock mechanism are, for the most part, limited in their spatial expression (e.g., Blau and Young, 1999; Kaneko and Hall, 2000; Kloss *et al*., 2001; Helfrich-Förster, 2003; Zhao *et al*., 2003). Recent experiments have asked whether the normal differentiation of a pacemaker cell is triggered by expression of a single "master" clock gene (Zhao *et al*., 2003). Zhao *et al*. misexpressed *Clk* and *per* (separately) and monitored for subsequent ectopic pacemaker activity. In most cases, misexpression of UAS-*Clk* was lethal (driven by promoters like *tim*-GAL4 or *per*-GAL4); these results may reflect the fact that such drivers likely produce patterns of expression greater than the "normal" patterns of their proteins (cf. Section 4.11.4.2.1). However, some drivers produced viable adults: when their brains were examined for other circadian gene expression, new patterns of pacemaker activity were observed (Zhao *et al*., 2003). Moreover, these ectopic pacemakers show diurnal variation of clock gene expression with phases similar to those displayed by normal pacemakers. These results were found using either a "circadian gene" driver (*cry*-GAL4; see Section 4.11.3.2.2) or a "noncircadian gene" driver (MJ162a; primarily expressed in mushroom bodies). Ectopic cycling was maintained in DD and locomotor rhythms in LD were disrupted, indicating that the ectopic clocks were autonomous, functionally activated, and somehow had access to locomotor centers.

Are these ectopic sites truly naïve for circadian gene expression or do they represent latent clocks that have, for example, low levels of *per* gene expression (cf. Price *et al*., 1998) and hence may be biased towards display of pacemaker function? The answer would lead to different conclusions regarding how pacemaker cells are organized. If the ectopic cells were truly naïve, then the selective expression of an additional regulatory molecule (e.g., CLK) could tip the balance and start a cascade that leads to an organized pacemaker function. If that scenario were true, then perhaps *Clk* does so in normal pacemaker cells as well during their differentiation. Because similar misexpression of *per* did not have this effect, *Clk* appears to have special properties in this regard (Zhao *et al*., 2003). Thus, if there are "master" clock genes, *Clk* may be unique in this respect, or it may simply be the first to be demonstrated. Further misexpression studies will help to resolve this significant point.

4.11.2.12. An Obligate Role for Membrane Excitability to Maintain Molecular Cycling in Pacemaker Cells

Physiological studies of cultured pacemaker neurons have measured rhythmic changes in membrane properties. In the snail *Bulla*, isolated retinal pacemakers display a robust rhythm in membrane conductance (Michel *et al*., 1993), which underlies a circadian rhythm of optic nerve activity. Electrical events at the membrane do more than reflect

an underlying oscillation: manipulations of the neurons' membrane potential affect both the period and phase of that rhythm (Block *et al.*, 1995). That observation suggested that modulation of electrical activity in pacemakers represents both a circadian output and circadian input.

New data from genetic manipulations in *Drosophila* corroborate that hypothesis, and suggest that the effects of membrane excitability on pacemaker function *in vivo* may indeed be substantial (Nitabach *et al.*, 2002). Nitabach *et al.* misexpressed a dominant negative form of a potassium channel (a "constitutively active" open rectifier channel). Such a molecule is predicted to enforce "electrical silence" by clamping the membrane at or near the K^+ equilibrium potential. Significantly, the neurons did not appear to be adversely affected by such treatment. As expected, when the critical vLN pacemaker neurons were silenced by driving UAS-*dORK* with *pdf*-GAL4, rhythmic behavior in DD was abolished (see Section 4.11.4.2.1 for a description of vLN cells). More surprising was the disappearance of molecular oscillations in those vLNs in DD and the retention of their oscillations in LD (Nitabach *et al.*, 2002). These results indicate an essential role for electrical activity in the cycling of the clock, at least in the absence of environmental cues. This method permits designs to silence select neuronal populations to study their selective contributions to synaptic circuits. Further, the specific results force a reconsideration of the concept of a cell-autonomous circadian oscillator. Depending on the underlying mechanism, an absolute requirement for electrical activity may indicate that circadian pacemaker neurons depend on each other not just for synchronization, but also for sustained activity.

4.11.3. Molecular and Cellular Pathways for Circadian Photosensitivity

4.11.3.1. Introduction

The biological clock must be synchronized to local time, and it gains much of that information by sampling the intensity, spectral composition, and total daily duration of ambient light conditions. These features vary predictably as a function of time of day, with intensity high in midday, while low at dusk and dawn. Ambient light is also enriched for blue wavelengths during transition times, but for reds and greens at midday (Hall, 2000). It is likely therefore, that several mechanisms (including different photopigments) contribute to behavioral synchronization.

Studies of the earliest events in the fly's circadian photic sensitivity has now circumscribed a focus around the action of the blue-light photopigment CRY. That molecule acts primarily to change the status or activity of TIM. In addition, other photopigments collude with CRY. There is also now considerable genetic and anatomical evidence to implicate numerous parallel neural pathways as functional routes by which photic input, directly or indirectly, accesses critical pacemaking neurons. This section outlines recent progress made that addresses these critical aspects of clock function in *Drosophila*. This subject has been reviewed carefully over the past few years (Hall, 2000; Foster and Helfrich-Förster, 2001; Shafer, 2001; Ashmore and Sehgal, 2003) and the description here will be brief.

4.11.3.2. Light and Cryptochrome

4.11.3.2.1. A model for the light resetting mechanism Identification of the principal clock proteins permitted consideration of which molecular aspects display light sensitivity, and so may contribute to resetting and subsequent entrainment. There is now strong consensus that the TIM protein levels are lost rapidly upon exposure to light (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). While TIM protein levels fall abruptly with light onset, PER levels decline at a much slower pace (Zerr *et al.*, 1990; Edery *et al.*, 1994a; Zeng *et al.*, 1996). Neither *tim* nor *per* RNAs display such light sensitivity. TIM levels are light-sensitive within the first hour of the light signal, which is consistent with the pace at which light resets *Drosophila* behavioral rhythms. Decreases in TIM levels induced by pulses of light likely reflect a mechanism similar to that underlying the constitutive low TIM levels in flies kept in constant light (Price *et al.*, 1995). Naidoo *et al.* (1999) reported that TIM degradation in response to light in the vLN pacemakers is mediated by the proteasome, and that TIM is phosphorylated and ubiquitinated prior to its degradation. Ubiquitination of TIM in response to light can also be observed in cultured S2 cells, despite the fact that TIM levels are nevertheless maintained.

The molecular significance of losing TIM in response to light is based on observations indicating that PER and TIM influence each other's appearance in the nucleus. PER and TIM display a reciprocal dependence in order to efficiently accumulate in the nucleus (Vosshall and Young, 1995 Hunter-Ensor *et al.*, 1996;; Ashmore *et al.*, 2003). The effect of light on TIM levels establishes a link between an environmental Zeitgeber to an alteration in a critical clock component, and that linkage is manifest as an

altered behavioral rhythm. A light pulse experienced early at night delays the onset of the subsequent cycle, whereas light experienced late in the night advances phase (Pittendrigh, 1974). According to the model, phase advances result from a premature decrement in TIM levels, which can only be reversed with sufficient time to allow additional *tim* translation. Likewise, light-triggered destruction of TIM late at night is predicted to hasten the rate at which PER levels decline and so hasten the onset of the next cycle.

A second model to explain the effects of light on the circadian machinery highlights negative regulation by light on the activity of a (presumed) PER : TIM heterodimer in repressing CLK : CYC transcriptional activity (Ceriani *et al*., 1999). This hypothesis is based on studies of the relevant clock molecules following their functional expression in S2 tissue culture cells. The authors found that the proposed photopigment CRY (see below) is associated with PER : TIM-containing complexes, and that it blocked PER : TIM derepression of CLK : CYC activity. This CRY effect was light-dependent and, importantly, it was efficient in the absence of TIM degradation, which is a feature not seen in S2 cells. Thus, in this cell line, photic regulation of the negative feedback loop (and by inference, the resetting of circadian phase) occurs despite the lack of TIM degradation. Lin *et al.* (2001) argue that such a degradation-independent phenomenon, because it may be specific to S2 cells, may not reflect the mechanistic details that occur within endogenous pacemaker cells (like the vLNs). Further, they contend that a substantial resetting only occurs by a process that changes a clock component (e.g., TIM levels) with a duration that outlasts the stimulus.

4.11.3.2.2. TIM's light sensitivity reflects activation by CRY Having identified a light-sensitive component of the negative feedback loop, efforts to dissect the initial events in circadian photoreception turned to the definition of the photopigments. Experimental evidence suggests that TIM is not directly light-sensitive, and that light-triggered degradation reflects an indirect action via the molecule CRY. Cryptochromes absorb light and transmit electromagnetic signals, using pterin and flavin adenine dinucleotide (FAD) as chromophore/cofactors (reviews: Sancar, 2000; Van Gelder, 2002). Cryptochromes are evolutionarily conserved and structurally related to the DNA repair enzyme photolyases – light-dependent enzymes that utilize flavin cofactors to repair DNA. In flies, CRY were studied by genomic methods: details of *cry* expression and its activities were reported by several groups (Emery *et al*., 1998; Egan *et al*., 1999; Ishikawa *et al*., 1999). Additionally, the *cry* locus was revealed by mutational analysis of loci that affected a *per-luciferase* daily rhythm (Stanewsky *et al*., 1998).

Quoting here from Sancar's (2000) review of the field, an explanation of the origin of the curious name is as follows: "The pigment system(s)... has been nicknamed 'cryptochrome' because of [its] importance in cryptogamic plants and its cryptic nature." Cryptochromes have 20–25% sequence identity with microbial photolyases and 40–60% sequence identity with the (6–4) photolyase. The sequence similarities between CRYs from various sources is remarkably high: human, *Drosophila*, and *Arabidopsis* CRYs share about 60% sequence identity (Todo, 1999). However, CRYs from different sources have nonhomologous C-terminal extensions ranging from very short ones in *Drosophila* to as many as 240 amino acids in plants. The two human CRYs are 73% identical to each other, but exhibit no sequence similarity within the C-terminal 75 amino acids. It is thought that this domain may bind to regulatory molecules (Cashmore *et al*., 1999; Todo, 1999).

Because declines in TIM levels are largely shaped by light, the absence of a *tim-luc* rhythm in cry^b suggested that CRY may normally contribute to TIM's photosensitivity. In addition, *per-luc* and *tim-luc* cycling was restored when cry^b mutants were exposed to rhythmic temperature cycles (Stanewsky *et al*., 1998). That result suggested that the basic clock mechanism is intact in such mutants, and that the behavioral defects derive from an inability to synchronize pacemaker activity to light cycles. Ceriani *et al.* (1999) reported that CRY and TIM interact in yeast in a light-dependent manner. Further, they showed that CRY is found in molecular complexes within S2 tissue culture cells that also contain TIM, and that CRY prevents TIM : PER transcriptional repression in S2 cells in a light-dependent manner. When expressed in S2 cells, CRY is degraded in response to light by the proteasome through a mechanism that requires electron transport (Lin *et al*., 2001). Various CRY mutant proteins (including CRY-B) were not so degraded. Does light promote TIM degradation by promoting or attenuating its interactions with CRY? TIM degradation is blocked by coexpression with mutant CRY (CRY-B), and TIM ubiquitination precedes CRY degradation. Thus, it appears more likely that in response to light, CRY transmits a signal that leads to TIM ubiquitination and degradation (Lin *et al*., 2001).

4.11.3.2.3. A second model of CRY activation and its interactions CRY may also interact directly with PER. Rosato *et al.* (2001) found genetic and molecular levels of interaction between CRY and PER. First they found that per^S; cry^b double mutant flies are able to detect light, but are deficient in the transmission of light information to the clock mechanism in a temperature-dependent manner. In contrast to the findings of Ceriani *et al.* (1999), they also showed that CRY and a truncated (TIM-interacting) PER interact in yeast cells, in a light-dependent manner. Furthermore, CRY can be coimmunoprecipitated along with full-length PER from S2 cells. Because CRY can interact with PER in the cytoplasm of S2 cells kept in the dark, it is possible that CRY can adopt "active forms" without the benefit of light. Therefore light activation, according to this model, involves derepression of an additional CRY-binding factor that prevents CRY from interactions with TIM or PER in the nucleus. There is evidence to suggest that CRY is not its own repressor, and that repression is exerted by other effectors acting at the CRY C-terminus (Rosato *et al.*, 2001).

4.11.3.2.4. The redox-based mechanisms of CRY activation Flavoenzymes like CRY catalyze redox processes because flavin itself is a reducing agent and can exist in different redox states (Sancar, 2000). Lin *et al.* (2001) tested the hypothesis that CRY has similar properties and that these may be important for circadian photoreception. CRY signaling in plants requires redox activity and is mediated, at least in part, by the flavin moiety bound to CRY. This is based on the finding that diphenylene iodonium (DPI), which inhibits the transport of electrons from reduced flavin (i.e., a reagent that blocks the transfer of electrons from reduced flavin prevents CRY degradation by light), was effective in blocking CRY-mediated photic signaling in *Arabidopsis* (Long and Jenkins, 1998). DPI also attenuated light-dependent CRY degradation in S2 cells, suggesting that an intramolecular conversion is required for this light response. Light-induced TIM ubiquitination precedes CRY degradation and is increased when electron transport is blocked. Thus, Lin *et al.* (2001) proposed that inhibition of electron transport may "lock" CRY in an active state by preventing signaling required either to degrade CRY or to convert it to an inactive form.

Further support for a role of the redox model of CRY action came from studies by Froy *et al.* (2002). Based on the crystal structures of photolyases from *E. coli* and *A. nidulans* (Park *et al.*, 1995; Tamada *et al.*, 1997), they targeted for mutagenesis conserved CRY residues predicted to participate in flavin binding. They then tested CRYs ability to block PER : TIM repression of CLK : CYC activated transcription in S2 cells. Alteration of three of the four flavin-binding residues abrogated light responsiveness in CRY. Additionally, two Trp residues, which are predicted to participate in intramolecular reduction, were also implicated in CRY function by similar criteria. These data support a multistep redox model for CRYs light sensitivity. In contrast, alterations of these same residues were without effect on the transcriptional repressive activity displayed by mouse CRY1, which to date has not been demonstrated to show photic sensitivity in its role in the SCN (Froy *et al.*, 2002).

4.11.3.3. What is CRY Doing?

4.11.3.3.1. CRY is a circadian photoreceptor In wild-type flies, *cry* RNA cycles in phase with *Clk* in LD and weakly in DD; CRY protein levels also cycle but only in LD (Emery *et al.*, 1998). From analysis in various mutant backgrounds, it was learned that *cry* transcription is under clock control and is also light-regulated at the translational or posttranslational levels. Additional copies of *cry*, in an otherwise wild-type background, enhances photosensitivity to phase shifting by short pulses of light at night (Emery *et al.*, 1998; Ishikawa *et al.*, 1999). In particular, the delay portion of the PRC is more affected than is the advance portion.

The cry^b mutant is a strong hypomorph, with a mutation that maps to a conserved flavin-binding site; CRY protein is absent or at very low levels in the cry^b mutant (Stanewsky *et al.*, 1998). In these CRY-depleted *Drosophila*, the entrainment by short light pulses is impaired (Stanewsky *et al.*, 1998). However, the clock still entrains to light–dark cycles, probably due to light input from the visual system. In addition, it can follow ~8-h shifts in LD phase (jet lag experiments) with only slight impairment. Significantly, under constant light conditions these animals are still rhythmic, unlike wild-type flies, which rapidly become arrhythmic (Emery *et al.*, 2000a). The site of *cry*s actions maps anatomically to the brain and not the eye: *cry* behavioral phenotypes are largely rescued by expressing *cry* under the control of a *tim*-GAL4 promoter, while a photoreceptor-specific GAL4 does not confer such a rescue (Emery *et al.*, 2000b). Significantly, restricting *cry* expression to just PDF neurons (with *pdf*-GAL4, see Section 4.11.4.2) partially rescues the LL phenotype (Emery *et al.*, 2000b) suggesting that CRY photosensitivity is principally required in just those few neurons for normal entrainment.

4.11.3.3.2. For CRY action(s), location may be everything A full description of CRY expression is still lacking, as no antibody-based localization has yet been reported. By *in situ* hybridization *cry* appears to be expressed in the vLNs and perhaps other pacemakers (Egan *et al.*, 1999). *cry*-GAL4 promoter patterns indicate the *cry* gene expression is normally found within pacemaker neurons (Emery *et al.*, 2000b), as well as in numerous peripheral tissues including the gut, antennae, and Malpighian tubules (Ivanchenko *et al.*, 2001; Levine *et al.*, 2002a), and in other neurons not traditionally considered to be pacemakers (Zhao *et al.*, 2003). In spite of the fact that PER and TIM cycling is heavily damped in head extracts, animals are paradoxically rhythmic in DD. A likely explanation lies in the fact that PER and TIM in fact still cycle in vLNs and dLNs in both LD and DD (Stanewsky *et al.*, 1998): this persistent rhythmicity in the pacemaker neurons that are critical for behavior probably explains the persistent behavioral rhythmicity.

The hypothesis that CRY is a circadian photoreceptor in *Drosophila* contrasts with the current conception of the two mammalian CRYs' roles as components of the core negative feedback loop (e.g., Froy *et al.*, 2002). It is worth noting, therefore, that the photoreceptor function for CRY is a phenotype ascribed to its role in the central vLN pacemaker neurons. It remains possible that CRY plays different roles in different pacemaking tissues of the fly and several observations support that possibility. First, the differences in PER and TIM rhythmicity of *cry* mutant LNs versus photoreceptors proves that different pacemaker cells can display significant differences in the details of their molecular oscillations. Second, autonomous clocks present in peripheral tissues can be studied with isolated body parts (e.g., legs) and by measuring rhythms in *per-luc* or *tim-luc* (Plautz *et al.*, 1997). The phenotype of the circadian oscillator present within those peripheral tissues often appears more severely affected by the *cry* mutation than the central oscillator. Those observations have led to the generalization that CRY may be a remote photosensor for the central clock, but may have a role closer to the actual clockworks in other oscillators.

The strongest evidence to suggest that CRY can be a clockwork component derives from observations in antennae, which display a circadian rhythm of sensitivity (Krishnan *et al.*, 1999), and a tissue-autonomous *per-luc* rhythm (Plautz *et al.*, 1997). In *cry* mutants, the amplitude of antennal *per-luc* cycles was severely diminished (Levine *et al.*, 2002a; Krishnan *et al.*, 2001). Surveying other, isolated peripheral tissues with the *per-luc* reporter, Levine *et al.* (2000a) reported that the cry^b mutation significantly decreased the number of rhythmic specimens in most cases, and also decreased the amplitude and altered the phases of rhythmicity. Likewise, Ivanchenko *et al.* (2001) examined a *tim-luc* reporter and found that the cycling of TIM was abolished in larval Malpighian tubules in DD, but it persisted in larval LNs of cry^b flies. From these and other observations, they concluded that CRY is involved in TIM-mediated entrainment of both central and peripheral clocks, but that it is also an indispensable component of peripheral oscillators. Dissecting these different roles for CRY, depending on cellular location, represents a substantial challenge for future research.

4.11.3.4. Cells Mediating Circadian Photoreception

4.11.3.4.1. Neuroanatomy In *Drosophila*, the photosensitive cells that feed light information to pacemaking centers are widespread. The presence or function of the compound eyes is not necessary to synchronize the fly's rhythms by light input (e.g., Wheeler *et al.*, 1993; Yang *et al.*, 1998), and the insect circadian system is sensitive to phototransduction from extraocular sources (Truman, 1976). However, the compound eyes are involved, as flies lacking them or lacking functional components (e.g., *norpA* – Pearn *et al.*, 1996) display lowered sensitivity to entrainment by light (Helfrich-Förster, 1997; Stanewsky *et al.*, 1998; Yang *et al.*, 1998). In the retina, some or all of the eight retinal photoreceptors may signal via synapses in the optic lobes to the large vLNs, which form a tangential projection across the distal medulla (Helfrich-Förster, 1995; Chen *et al.*, 1999; Taghert *et al.*, 2000).

Candidate extraocular photoreceptors have now been identified and functionally implicated (Helfrich-Förster *et al.*, 2002). In the periphery, the candidates include two extraretinal groups, the ocelli and the H-B Eyelet (Hofbauer and Buchner, 1989). The Eyelet resides at the posterior margin of the compound eye in a subretinal position and forms a specialized pigmented organ with cells that have numerous microvilli arranged into coherent rhabdomeres (Yasuyama and Meinertzhagen, 1999). The candidate photoreceptive cells in the brain include the LNs that likely express CRY (Emery *et al.*, 2000b), and the PER-expressing dorsal neurons that likely express CRY and perhaps also a different photopigment (Malpel *et al.*, 2002; Veleri *et al.*, 2003).

Both Bolwig's neurons and Eyelet neurons express Rh5- and Rh6-opsin-like immunoreactivity

(Yasuyama and Meinertzhagen, 1999; Malpel *et al*., 2002), and they express a phospholipase (Malpel *et al*., 2002) encoded by *norpA* (Pearn *et al*., 1995). The Bolwig's neurons do not stain for antihistamine (Pollack and Hofbauer, 1991), in contrast to Eyelet cells (Yasuyama and Meinertzhagen, 1999), which also stain for anticholine acetyltransferase (Yasuyama and Meinertzhagen, 1999). The hypothesis that Eyelet neurons are cholinergic is further supported by pharmacological experiments on isolated LNs (Wegener *et al*., 2004). A circadian photoreceptor function for Eyelet was indicated in flies lacking compound eyes but retaining Eyelet (*sine oculis, so* – Helfrich-Förster *et al*., 2002); the action spectrum of light entraining the residual rhythmicity in *so* flies displayed a peak at 480 nm, different from the peak sensitivity of CRY. Although Eyelet photoreceptors alone were able to cause entrainment in the absence of compound eyes and of CRY, such entrainment was not robust under the circumstances tested.

4.11.3.4.2. Development of Eyelet The developmental origins of the Eyelet were subject to speculation until recently. It appeared likely that it was derived from Bolwig's organ, as suggested by the common positions of their axonal terminations in the accessory medulla (Gibbs and Truman, 1998; Yasuyama and Meinertzhagen, 1999). In larvae, Bolwig's neuron axons are in close proximity to the dendrites of PDF-expressing LNs (Kaneko *et al*., 1997; Malpel *et al*., 2002) and are likely to mediate circadian entrainment (Kaneko *et al*., 2000b). Helfrich-Förster *et al*. (2002) established a direct link to Eyelet neurons following expression of a *Kruppel-lacZ* reporter that is specific to the Bolwig's organ neurons. The metamorphic reorganization of Eyelet is not complete until adult eclosion – this suggests that Eyelet functions to help entrain locomotor rhythms, but not eclosion rhythms (see **Chapter 3.1**). Thus, the adult circadian system is dependent on the photoreceptive structures of the larva with the added contributions of photoreceptors derived from metamorphic tissues.

The hypothesis that multiple photoreceptors combine to help entrain the *Drosophila* circadian clock was indicated by analyses of various single and double mutant stocks (e.g., *cry*; *norpA*). The double mutant stock lacks function of both CRY and the compound eyes and ocelli. Such flies display less photic sensitivity than did either single mutant stock. Nevertheless, these double mutants maintained some sensitivity to entraining photic inputs (Stanewsky *et al*., 1998). Only when all known ocular and extraocular photoreceptors were genetically eliminated (by a *cry; glass* double mutation) were flies unable to respond at all to LD cycles (Helfrich-Förster *et al*., 2001). The *glass* gene is required for normal development of the compound eyes, ocelli, H-B Eyelet, and DN1 neurons. The recent research mentioned above has established the range of photoreceptors and photopigments involved in circadian photoreception in flies and begun to indicate their individual roles. Of course, new information always produces a generation of new questions (e.g., what are the qualitative and quantitative contributions of the various photoreceptors?) and much remains to be resolved to give a clear description of the parallel functions. This point was suggested by Helfrich-Förster *et al*. (2001): "Apparently, cryptochrome and rhodopsins of Eyelet and the compound eyes collude in a complex manner to entrain the adult fruit fly's circadian activity to the 24 h day."

4.11.3.5. Transmitter Sensitivity of Pacemaker Neurons

Two recent studies have indicated the identities of small molecule transmitters that are likely to provide critical inputs to the vLN pacemaker neurons. Ionotropic GluR-IB receptor subunits are preferentially expressed in vLNs of the adult head (Volkner *et al*., 2000), suggesting a role for glutamate signaling (see **Chapter 5.2**) in mediating entrainment mechanisms. In addition, isolated vLNs display pharmacological properties that are consistent with the expression of nicotinic Ach receptors that signal via calcium (Wegener *et al*., 2004). The latter results are congruent with the demonstration of cholinergic signaling (see **Chapter 6.2**) by Eyelet cells (Yasuyama and Meinertzhagen, 1999). In the cockroach, Petri *et al*. (2002) have shown that injections of GABA and of the neuropeptide Mas-allatostatin both resulted in stable phase-dependent resetting of the circadian locomotor activity. The PRCs for the transmitter effects matched those of the light PRC, suggesting that these substances may contribute to neural signaling underlying photic entrainment in this insect.

4.11.3.6. Other Entrainment Mechanisms: Sociality

Environmental inputs in addition to light can synchronize internal clocks: the most prominent is temperature (review: Rensing and Ruoff, 2002). A recent report indicated that *Drosophila* clocks are also set by social interactions (Levine *et al*., 2002b). The authors determined that the phase coherence of daily activity (a measure of synchronicity, where phase peak equaled the peak of daily

locomotion) was stronger among flies that had been jointly housed prior to testing, versus those housed individually. In effect, social experience had promoted the resetting of the activity clock. Likewise, the phase coherence of wild-type flies was diminished by the interactions (co-housing) with arrhythmic *per*^{o} flies (at a 4 : 1 and 2 : 1 ratio). Interestingly, the effect was lost when the populations were at equal strength. Levine *et al.* (2002b) favor the hypothesis that the inferred social interactions predominantly reflect chemosensory sensations. Genetic analyses and "conditioned air" tests were both consistent with a mechanism whereby short-lived volatile substances signal to con-specifics (see **Chapter 3.13**) and represent social experiences that help to reset the biological clock.

4.11.4. Output Studies

At some level, clock information morphs into output signals by which rhythms are synchronized and/or evoked in downstream centers. The substance of circadian output can take many forms (reviews: Jackson *et al*., 2001; Taghert, 2001; Park, 2002), among which three levels can be described. At the most proximal level, circadian output regulates rhythmic processes in the pacemaker cell itself, and is represented by cycling gene expression within that cell. Estimates of the percentage of the genome that is subject to circadian control have ranged from one to many per cent (review: by Dunlap, 1999). Leaving the bounds of single cells, circadian output also describes cellular interactions that take place between pacemakers. Because pacemakers influence each other's rhythms, this circadian output transforms into substantial circadian input, affecting the phase and/or amplitude of subsequent cycles. Finally, circadian output is translated to signals that affect cells and tissues which are not themselves capable of generating pacemaker activities. The latter rely on timing information from the biological clock for their normal rhythmic physiology.

The literature in this field is reviewed here, with special emphasis on *Drosophila*, using the three-part categorization. To begin with, the results of several microarray studies of *Drosophila* circadian rhythmicity are summarized. Next, cellular descriptions of circadian pacemakers, their hierarchies, and the nature of signals used to coordinate their interactions are disscussed. Finally, several emerging areas of research activity (e.g., eclosion, drug sensitivity, and sleep), in which new information has become available to understand how circadian output is manifested as behavioral rhythms, are outlined.

4.11.4.1. Microarray Studies

4.11.4.1.1. Whole genome analysis of cycling gene expression Because many of the known circadian genes display regular fluctuations in their transcript levels, a method for discovering new components of the molecular clock is to search for rhythmic transcripts – transcripts which, in the absence of environmental cues, continue to display identical, daily waveform patterns. Such screens have been performed previously, including the examination of anonymous cDNAs by time-series Northern blots (Van Gelder *et al*., 1995), enhancer trap screening of rhythmic signals with the luciferase reporter (Stempfl *et al*., 2002), and a subtracted cDNA library strategy to identify time-dependent, differentially expressed transcripts (Rouyer *et al*., 1997). With the advent of microarrays and the ability to perform, in effect, thousands of Northern blots in parallel, the circadian field quickly adopted microarray technology to continue this search.

Within the period of a year, five published studies described the extent of transcript rhythms in *Drosophila* (MacDonald *et al*., 2001; Claridge-Chang *et al*., 2001; Ceriani *et al*., 2002; Y. Lin *et al*., 2002; Ueda *et al*., 2002). These studies have generated a treasure-trove of data, which is amenable to future meta-analysis by virtue of the commonalities in their experimental designs. All groups extracted transcripts specifically from fly heads, used the Affymetrix oligonucleotide microarray platform to monitor the activity of ~13 500 genes, and performed temporal sampling at a resolution of 4-h intervals. Most of the studies performed light-entrained (LD) microarray experiments and all studies also monitored transcript levels under constant conditions (DD). Some differences such as the use of fly strains with differing genetic backgrounds were unlikely to result in material differences with respect to the identification of novel clock candidates, as the underlying molecular components should be identical between strains.

4.11.4.1.2. A current lack of consensus on the number of cycling genes Even with so many common elements spanning these studies, the results of the analysis were in sharp disagreement. One study identified 378 rhythmic genes under DD conditions, yet of these 378, only 28 were identified by at least three studies, and only five were agreed upon by all five studies. These numbers illustrate the degree to which false positives may populate the various lists of rhythmic genes in *Drosophila*. Two salient issues distinguished these five reports and may have resulted in such disparities. One was the extent of

time in which flies were kept under constant conditions before the collection of DD data. Light-driven effects in flies may take several days to extinguish, as evidenced, for example, by the residual behavioral rhythms seen in the first 2 DD days of the otherwise arrhythmic *disco*[1] and *pdf*[01] mutants (Hardin *et al*., 1990; Helfrich-Förster, 1998; Renn *et al*., 1999). Most studies collected data on the first day of constant darkness, whereas Ceriani *et al*. collected data from the first two consecutive DD days and Y. Lin *et al*. collected replicate cycles on the third day of DD. In contrast to the >115 genes identified by the other studies, Ceriani *et al*. found that 63 genes remained rhythmic 2 days into DD, and Y. Lin *et al*. found 22 genes that cycled on the third day of DD. The correlation between more cycles of free-run conditions and fewer identified rhythmic genes is notable. This time-dependent decrement in rhythmicity may reflect a damping effect on molecular rhythms when pacemakers become de-synchronized, as observed in peripheral clocks (Plautz *et al*., 1997). However, that explanation is not valid in this case as Y. Lin *et al*. found that all of the canonical clock genes – *per, tim, vri*, and *Clk* – continued to cycle robustly 3 days into DD.

The other significant difference between the studies was the treatment of statistics and methods of analysis. To identify rhythmic waveforms, the methods ranged from spectral analysis (Claridge-Chang *et al*., 2001) to idealized cosine curve fitting (McDonald *et al*., 2001; Ceriani *et al*., 2002; Ueda *et al*., 2002) to direct measurement of intercycle reproducibility of waveforms with autocorrelation analysis (Y. Lin *et al*., 2002). A concise treatment on the strengths and weaknesses of each strategy was recently detailed by Levine *et al*. (2002c).

It is reasonable to assume that all five studies have correctly identified at least some unambiguous, robustly cycling transcripts. Therefore, until a unified, meta-analysis is performed on the pooled data, a measure of confidence may be afforded by relying upon genes commonly identified across multiple studies. To balance between the degree of agreement amongst the various studies, and the inclusion of the canonical rhythmic genes *per, tim, takeout, vri*, and *Clk*, **Table 1** lists the 28 genes that were identified by at least three groups to display rhythms in DD. No functional patterns among the known or predicted gene functions seem yet to emerge from the list.

Table 1 The list of *Drosophila* genes that were commonly identified by at least three microarray studies as circadianly cycling

Gene	*Location*	*Known/predicted function*
Clk	66A12-66A12	RNA polymerase II transcription factor
Cyp18a1	17D1-17D1	Cytochrome P450, CYP18A1; cytochrome P45; EC:1.14.14.1
Cyp4d21	28A6-28B1	Cytochrome P450, CYP4D21; cytochrome P45
Cyp6a21	51D2-51D2	Cytochrome P450, CYP6A21; cytochrome P45
Pdh	72F1-72F1	Enzyme
Slob	28B1-28B3	Signal transduction
Ugt35b	86D5-86D5	UDP-glucuronosyltransferase; UDP-glucuronosyltransferase; EC:2.4.1.17
per	3B4-3B4	Transcription factor
tim	23F3-23F5	RNA polymerase II transcription factor
vri	25D4-25D4	RNA polymerase II transcription factor
BcDNA:GH02901	13A5-13A5	Long-chain-fatty-acid-CoA-ligase
CG1441	46C5-46C5	
CG4784	72F1-72F1	Cuticle protein; structural protein
CG4919	94C3-94C3	
CG5156	21F2-21F2	
CG5798	93C1-93C1	Ubiquitin thiolesterase; endopeptidase
CG5945	34A11-34A11	
CG9645	88B3-88B3	Endopeptidase
CG9649	88B3-88B3	Endopeptidase
CG10513	96C7-96C7	
CG10553	96C8-96C8	
CG11407	92B3-92B3	Luciferase-like; enzyme
CG11796	77C1-77C1	
CG11853	96C4-96C4	
CG11891	96C6-96C7	
CG14275	29B1-29B1	
CG15093	55F1-55F1	3-Hydroxyisobutyrate dehydrogenase-like; enzyme
CG17386	51A1-51A1	RNA binding

4.11.4.1.3. The clock also controls the basal level of expression for thousands of genes To control against the false-positive identification of rhythms, all groups performed time-series experiments with arrhythmic flies bearing mutations in clock components such as per^{01} and Clk^{Jrk}. A surprising additional observation from these data was that such mutations produced broad effects on noncycling, basal expression of genes across the *Drosophila* genome. MacDonald *et al*., Claridge-Change *et al*., Ueda *et al*., and Ceriani *et al*. observed significant differences in gene expression between Clk^{Jrk} mutants and control flies. These changes are likely to represent the absence of normal clock-controlled processes, but may also include gain-of-function consequences that reflect the dominant-negative character of the Clk^{Jrk} allele (Allada *et al*., 2003). Claridge-Change *et al*. and Y. Lin *et al*. found that normal expression levels of large swaths of the fly genome depended on *per* and *tim* functions. Furthermore, Y. Lin *et al*. observed that light influenced the expression levels of hundreds of genes when comparing expression levels of control flies under LD versus DD conditions. These light effects were largely dependent upon *per* function, as per^{01} mutants did not display comparable changes between LD and DD conditions.

Although the power of microarrays to generate candidates for future pursuit cannot be disputed, some caveats should be noted. Several limitations have been enumerated in the reports, such as the heavy dependence of probe sets upon the quality of genome annotation, and the relative insensitivity of microarrays to low-abundance and tissue-specific transcripts. Related to computational annotation is the issue of alternative splicing, a phenomenon thought to contribute heavily to the complexity of multicellular organisms (Harrison *et al*., 2002). Current generations of microarrays do not yet distinguish between splice variant forms of genes, thus limiting the accuracy of the circadian landscape as seen through a microarray filter. This point was exemplified recently, with the demonstration by Cyran *et al*. (2003) that only one of six splice variants of the new clock component *pdp1* displays transcript rhythms.

Is the limited manifestation of transcript rhythms as suggested by Y. Lin *et al*. and as indicated by the small group of genes coincident between the studies, a meaningful result? One possibility is that despite the existence of a limited number of rhythmic transcripts, a greater population of rhythmic proteins may exist. For example, rhythms in the Lark protein, a protein required for eclosion rhythms, are generated from a nonrhythmic transcript (McNeil *et al*., 1998). Another possibility is that beyond the immediate proximity of the core molecular clock, rhythms exist primarily at the physiological level. Perhaps this is demonstrated by the neurotransmitter PDF, which is neither transcriptionally nor translationally rhythmic, but is rhythmic in its release from axon terminals (J.H. Park *et al*., 2000; and see below). Likewise, several antecedent studies suggest that even high-amplitude rhythms of transcription in *per* and *tim* are not absolutely required for the production of rhythmic behavior (e.g., Frisch *et al*., 1994; Cheng and Hardin, 1998; Yang and Sehgal, 2001). In sum, these observations all point to the primacy of posttranscriptional events in defining circadian molecular oscillations. Thus, the "limited manifestation of transcript rhythms" may be a reasonable initial description of rhythmicity in the *Drosophila* transcriptome. Perhaps with future high-throughput techniques such as quantitative mass spectrometry, and the accumulation of more laborious, time-series immunostain-tracking of individual proteins, a broader sense of the *Drosophila* circadian clock will be achieved.

4.11.4.2. Pacemaker Neurons in the Fly Brain: Anatomy and Roles

4.11.4.2.1. Pacemaker cell types PER expression is found in numerous cells in numerous tissues (Liu *et al*., 1988, 1992; Siwicki *et al*., 1988; Zerr *et al*., 1990; Ewer *et al*., 1992; Kaneko *et al*., 1997; Rachidi *et al*., 1997) and the scale of the pattern depends on the method of visualization. PER immunostaining and *per* RNA *in situ* tend to feature small clusters of neurons in the lateral and dorsal brain of the adult, photoreceptors, as well as numerous glia cells in the optic lobes (e.g., Zerr *et al*., 1990, Ewer *et al*., 1992, Helfrich-Förster, 1995; Kaneko *et al*., 1997). In contrast, *per*- and *tim*-promoter fusions also display these same groups, plus many others not otherwise associated with circadian pacemaking (e.g., Kaneko and Hall, 2000). Price *et al*. (1998) observed that PER immunostaining was greatly increased in level and distribution (beyond the normal) in a strongly hypomorphic allele of *dbt*. That result is consistent with the hypothesis that PER is expressed normally, but highly unstable, in a large population of neurons in the larval brain.

The canonical PER neuronal groups comprise the lateral cells (LNs) which include the small and large vLNs (s-vLNs and l-vLNs), and dorsal cells (dLNs). There are four to five s-vLNs, of which four express the neuropeptide PDH/PDF (Helfrich-Förster, 1995; Kaneko *et al*., 1997; and see below). There are four to five l-vLNs and approximately seven dLNs.

In the dorsal brain, there are three additional groups: the DN1 cell group contains ~10 neurons; the DN2 group is composed of a pair of cells; and the DN3 group contains ~30 neurons. The axonal projections of all these neurons appear to target the neuropils of the dorsal protocerebrum, where they intermingle and may interact (Kaneko and Hall, 2000; Helfrich-Förster, 2003; Veleri *et al.*, 2003). In addition, a pair of DN1s and some DN3s project to the accessory medulla and appear to provide reciprocal innervation to the s-vLNs. Other DN1s project caudally towards the suboesophageal region and perhaps to the ventral nerve cord.

4.11.4.2.2. Which PER neurons are necessary for rhythmic behavior? Given the several different pacemaker cell groups, which are necessary and which are sufficient to act as pacemakers to drive rhythmic behavior? Leak and Moore (2000) argue that the different rhythmic systems in mammals are controlled by separate sources of SCN efferents ("core" versus "shell"). Similar questions can now be applied to the insect brain, where smaller numbers of neurons may facilitate the cellular analysis. Several different genetic designs have tried to define the relative participation of the different pacemaker cells groups in *Drosophila*. Of these, the study of *disconnected* mutant flies was among the first and most substantive.

The *disconnected* (*disco*) gene in *Drosophila* encodes a widely expressed transcription factor that is required to establish normal sensory connections in the central nervous system (CNS) (Steller *et al.*, 1987; Glossop and Sheperd, 1998). Approximately 5–10% of mutant animals display some retinal-brain connections. While Dushay *et al.* (1989) found that *disco* flies are arrhythmic for circadian behaviors, Hardin *et al.* (1992b) showed that PER levels fluctuate normally in homogenates of *disco* heads. Those findings suggested that *disco* acts on the output level because while the mutation eliminated behavioral rhythms, it left the "clock mechanism" intact. An alternative emphasis follows from considering abnormalities of PER expression in *disco* flies (Helfrich-Förster, 1998). PER is found in photoreceptors, DN pacemakers, and putative glia in *disco*, but is largely absent from LN pacemaker neurons. Blanchardon *et al.* (2001) subsequently reported that many LNs do in fact survive this mutant background, as indicated by expression of a P{GAL4} reporter line. Together these observations suggest that *disco* animals retain many functional clock centers (e.g., the retina, the DNs), but display poor behavioral rhythmicity due to the lack of functional clock centers (e.g., LNs) that normally organize daily locomotion. These animals were very useful to learn specific facts concerning output mechanisms, especially by restricting analysis to mutant animals displaying quasi-normal behavior. Helfrich-Förster (1998) monitored the behavior of hundreds of mutant animals: the few that displayed some rhythmic behavior were the only ones to also retain at least one s-vLN. Thus a strong correlation was made between the presence of a s-vLN and circadian locomotor behavior.

Mosaic analysis of *per* expression has also been influential in deciphering the hierarchies of pacemaker cells (Ewer *et al.*, 1992). Frisch *et al.* (1994) showed that, in complimentary fashion to the *disco* phenotype, PER expression in just the LNs (both ventral and dorsal) was sufficient to display some rhythmic behavior. Evidence that the DNs also participate in rhythmic output comes from two studies. When PER was returned only to LNs and not to DNs of *per* mutant flies, the restored rhythms were abnormal in period and strength (Frisch *et al.*, 1994): by inference, DNs may contribute the difference. Similarly, PER expression in accessory medulla neurons under the *glass* promoter in *per* mutant flies produced rhythmic behavior (Vosshall and Young, 1995). More recently, Veleri *et al.* (2003) described a *per-luc* transgene fusion that restored rhythmicity to *per* mutant flies under LD cycles, although not under constant conditions. In such flies, the only site of molecular oscillations was in the DN3 populations: strong evidence to suggest these DN neurons are sufficient to provide pacemaker activity for behavior, at least in LD.

Peng *et al.* (2003) and Allada *et al.* (2003) addressed the same question by asking whether limited expression of transgenic *cyc* or *Clk* could rescue either mutant phenotype. Peng *et al.* (2003) reported that in *cycle* mutant flies now expressing UAS-*cycle* under control of *pdf*-GAL4, PDF neurons alone now displayed proper rhythms of *tim* RNA, suggesting that UAS-*cyc* had indeed rescued molecular rhythmic in mosaic fashion. However, the behavior was not rescued, suggesting that other activity in other pacemaker neurons is required. It should be remembered that *cyc* mutant flies display aberrant PDF neuronal morphology (J.H. Park *et al.*, 2000), and that aberrant PDF neuronal morphology is correlated with arrhythmic behavior (Helfrich-Förster, 1998). Allada *et al.* (2003) used UAS-*Clk* to rescue two different mutant alleles of *Clk*. They found that a *cry*-GAL4 line that is expressed in both vLNs and dLNs (Emery *et al.*, 2000b; but see also Zhao *et al.*, 2003) partially rescued the behavioral rhythmicity of *Clk* mutants. *pdf*-GAL4 : UAS-*Clk* did not provide such rescue. These results, while not yet

definitive, are consistent with the hypothesis that pacemaker actions of the ventral and dorsal LNs are both necessary and sufficient for *Drosophila* to display behavioral rhythms.

Within the vLN group, five lines of evidence (all indirect) suggest that the s-vLNs may be especially important and the l-vLNs largely unimportant as circadian pacemakers. First, the daily fluctuation in PDF immunostaining of vLN terminals occurs only in the case of the small cell subset (J.H. Park *et al*., 2000; see further discussion below). Second, the effects of *Clock* and *cyc* mutations on *pdf* RNA levels are seen only in small and not l-vLNs (J.H. Park *et al*., 2000). Third, in a study of the effects of *disco* mutations on rhythmicity, Helfrich-Förster (1998) confirmed that most or all *per*-expressing neurons (and hence all *pdf* vLNs) were typically undetected in *disco* animals (cf. Blanchardon *et al*., 2001). She reported a small minority of animals ($n = 4$) that retained rhythmicity: PDF-expressing s-vLN were visible (as few as one neuron) only in this minority. A l-vLN was also found in a single rhythmic individual, but its processes projected in a s-vLN fashion, to the dorsal protocerebrum. Fourth, Yang and Sehgal (2001) and Shafer *et al*. (2002) reported fluctuations in PER and TIM immunostaining levels under constant darkness in small, but not in large, vLNs. Finally, *cry*^{b} mutants fail to maintain rhythms of PER or TIM in DD, in any pacemakers but the s-vLNs (Stanewskey *et al*., 1998). These diverse and consistent observations form a compelling hypothesis to indicate a special role for the s-vLNs. Direct confirmation of that hypothesis would be a useful step in narrowly defining the pacemaker cell hierarchies within the brain. To do so will require further and more precise manipulation of these neuronal populations.

4.11.4.2.3. PER expression in other insects Several groups have used *period* gene expression as a method of surveying the number and position of potential circadian pacemakers in other insects. In addition, several of these comparative studies have addressed the potential conservation of circadian pacemaker cell types across insect phylogeny by examining possible coexpression of PER with PDH/PDF. Frisch *et al*. (1994) used anti-PER antibodies (against a conserved region of the *Drosophila* PER) to stain the brain of a beetle and reported finding many immunoreactive neuronal groups, some of which had nuclear labeling. In addition to the neurons, they also found many labeled glia in the optic lobes, reminiscent of glial *per* expression in the *Drosophila* brain (cf. Ewer *et al*., 1992). Comparisons of anti-PER and anti-PDH stains suggested there was overlap in stained populations, but that many PER-positive did not express PDF. Reppert *et al*. (1994) cloned a *per* ortholog from the silkmoth *A. pernyii*, and reported that antibodies to the moth PER protein labeled the cytoplasm of eight neurosecretory neurons in the protocerebrum (Sauman and Reppert, 1996). Surprisingly, the nuclei of these neurons were never so labeled, the cells were distinct from the PDF-immunoreactive neurons, and no other cells displayed immunostaining. Interestingly, this anti-moth PER antibody when used in *Drosophila* produced a pattern of PER-like immunoreactivity that was highly similar to that previously found with antibodies to the fly PER (Levine *et al*., 1995). In addition, the moth protein was functional within *Drosophila*: it could rescue the *per* behavioral phenotype when expressed as a transgene in *per* mutant *Drosophila* (Levine *et al*., 1995).

The theme of differences between the patterns of PER in *Drosophila* versus other insects has been repeated in more recent studies, specifically in the moth *Manduca* (Wise *et al*., 2002) and the honeybee (Bloch *et al*., 2003). In both studies, the endogenous PER proteins were used to raise specific antibodies. In *Manduca*, widespread *per* expression was found in numerous neurons and glia. Many expressing cells displayed both nuclear and cytoplasmic staining, although evidence for rhythmic expression was only found in glia. Analogous to the observations in *A. pernyii*, four neurosecretory cells in the *pars lateralis* of each brain hemisphere exhibited both nuclear and cytoplasmic staining with anti-PER antibodies. These cells were identified as Ia(1) neurosecretory cells that express neuropeptide corazonin immunoreactivity. The accessory medulla contained ~100 neurons expressing *per* RNA but no immunoreactivity. No correspondence of *per* expression to PDH/PDF expression was evident in any part of the brain.

Likewise, in the honeybee brain, PER immunosignals were prominently found in small sets of protocerebral neurosecretory cells, but not within PDH-immunoreactive neurons of the medulla. The latter appeared to have dendrites within the accessory medulla and to project to dorsal protocerebrum, but to lack clock (e.g., *period*) protein expression. Bloch *et al*. concluded that: "... although clock proteins are conserved across insect groups, there is no universal pattern of co-expression that allows ready identification of pacemaker neurons within the insect brain." Finally, using an anti-*Drosophila* PER antiserum and anti-PDH, Zavodska *et al*. (2003) examined several insect

orders and found consistent expression of both immunosignals in most representatives. However, they reported no correspondence between PER and PDH immunoreactivities. Examples of the lack of nuclear PER staining may reflect a noncircadian function in those cells, or perhaps offer instances when PER's negative feedback functions may not involve its direct participation in the nucleus (cf. So and Rosbash, 1997). Nevertheless, it remains a challenge for the field to compare the different PER-expressing neuronal populations in different insects, and to rationalize these in terms of neuronal circuits underlying rhythmic behavior.

4.11.4.3. PDF: A Circadian Transmitter

4.11.4.3.1. Expression Interest in the pigment dispersing factor (PDF) by circadian biologists stems directly from the demonstration of its expression within specific circadian pacemaker neurons of *Drosophila* (Helfrich-Förster, 1995). Its expression within insect tissues, especially in areas associated with circadian pacemakers, was first described several years earlier (Homberg *et al*., 1991; Nässel *et al*., 1991, 1993; Helfrich-Förster and Homberg, 1993) using antibodies to crustacean pigment dispersing hormone (PDH, discussed below in Section 4.11.4.3.2). PDH immunosignals are normally found in a limited set of brain and ventral nerve cord cell types. Fundamental elements of the cellular pattern are found throughout insect orders, with salient differences also noted (e.g., Helfrich-Förster *et al*., 1998; Zavodska *et al*., 2003). Based on its hormonal activities in crustacea (see **Chapter 3.16** and see below), there is good evidence to hypothesize that PDF in *Drosophila* is a secreted neuropeptide and hence a true output signal for certain clock neurons (review: Taghert and Veenstra, 2003). However, recent studies in the cricket indicate that PDH/PDF may also have a nuclear location in some cell types (Chuman *et al*., 2002). These careful *in vivo* observations were also supported by studies of PDF transfected into mammalian cells, where the functionality of a putative nuclear localization signal on the PDF precursor was tested. This work underscores the importance of testing all assumptions rigorously and the need to address a range of plausible hypotheses.

In *Drosophila*, PDF expression is limited to the CNS. Within the CNS, there are two PDF cell types in the larva and three cell types in the adult. In larvae, the brain contains four to five LNs that display molecular pacemaker properties (Kaneko *et al*., 1997). LNs are the likely precursors of the s-vLNs of adults (Helfrich-Förster, 1997). In addition, the larval ventral nerve cord contains a prominent set of four to six large neuroendocrine neurons in the terminal abdominal segments that appear to release PDF into the hemolymph (cf. Persson *et al*., 2001). The abdominal cells do not display clock properties. In the adult, the vLN group is enlarged by the differentiation of the l-vLNs, which project axons tangentially across a distal layer of the medulla. The abdominal neuroendocrine PDF cell group is maintained. Finally, the third group in adults is a transiently occurring population: a pair of cells in the suboesophageal ganglion of the adult that do not express clock properties and disappear by the second day of adult life. Despite the fact that larvae produce no known circadian output, larval LNs display all the molecular hallmarks of functional pacemakers (e.g., Price *et al*., 1998). Using anti-PDH (crustacean PDH), an additional two to three cells in the larval protocerebrum and three to five cells in the adult protocerebrum are found. These appear to represent crossreactivity with another, so far unidentified, substance as these cells do not stain with *pdf in situ* methods (J.H. Park *et al*., 2000) and they retain immunostaining in *pdf* mutant animals (Renn *et al*., 1999).

4.11.4.3.2. The PDH/PDF family of peptides PDH peptides were first studied in crustacea where they cause diurnal movements of pigment granules in retinal cells and their dispersion in epithelial chromatophores. In 1971, a factor that caused the dispersion of distal retinal pigment was purified from eyestalk extracts of the prawn *Pandalus borealis* (Fernlund, 1971). Upon sequencing, the factor was revealed to be an 18 amino acid peptide with an amidated C-terminus and a free N-terminus (Fernlund, 1976). Originally called light adapting distal retinal pigment hormone (DRPH), it was renamed pigment-dispersing hormone (thus, Panbo-α-PDH), because it also translocates the pigments in the chromatophores centrifugally (Kleinholz, 1975). About a decade later, a second PDH was chemically identified from the eyestalks of the fiddler crab *Uca pugilator*, a so-called β-PDH that differs from α-PDH in six positions (Rao *et al*., 1985). To date, PDHs from 15 crustacean species are known (see **Chapter 3.16**).

Extracts from heads of insects were able to elicit a dispersion of pigments in the epidermis of eyestalkless fiddler crabs (Rao *et al*., 1987). This bioassay then served to isolate the active factor from the grasshopper *Romalea microptera*, a modified β-PDH. Pigment-dispersing factors (PDFs) have since been identified from several insect groups and orthologs identified in the genomes of *D. melanogaster*

(Park and Hall, 1998), and in the mosquito *A. gambiea* (Riehle *et al.*, 2002). The distribution of PDF/PDH appears limited to arthropods to date, but there is one report of immunoreactivity in a mollusk (Elekes and Nässel, 1999).

The PDH/PDF family of peptides displays a large amount of structural conservation; including the length (18 amino acid residues), the N-terminal Asn and C-terminal amidated Ala residues, as well as conserved amino acids at several internal positions. Interestingly, *pdh* genes in crustaceans have undergone gene duplication and up to three forms, for example, two α-PDHs and one β-PDH, are found in *Pandalus jordani* (Ohira *et al.*, 2002). *Drosophila* contains only a single *pdf* gene and there is no evidence to suggest that PDFs in *Drosophila* affect pigment dispersion. In various animals including *Drosophila*, the PDF peptide is predicted to be synthesized following posttranslational processing of a ~100 amino acid preproPDF precursor. Nothing is known about the actual biosynthesis of PDF. The general organization of PDF precursors (Ohira *et al.*, 2002) features a signal peptide that is followed immediately by a precursor-associated peptide (PAP) of unknown function, a di- or tri-basic cleavage site and the PDH/PDF octadecapeptide with an N-terminal Gly for amidation, and a mono- or di- or tri-basic cleavage site prior to the translation stop signal. The PAP does not display evolutionary conservation in either its length or primary sequence.

4.11.4.3.3. Cellular release of PDH/PDF There is a rhythm in antibody staining for PDF in the terminals of the s-vLNs of the adult *Drosophila* brain (J.H. Park *et al.*, 2000 – discussed further below). This observation is thought to reflect a daily rhythm of release. In addition, Kaneko *et al.* (2000a) and Blanchardon *et al.* (2001) reported that overexpression of an active tetanus toxin in *pdf* neurons of *Drosophila* (with which to cleave synaptobrevin and so reduce evoked release; Sweeney *et al.*, 1995) was not effective in disrupting circadian behavior. It was predicted that disruption of transmitter release by those neurons would have a strong behavioral effect due to results seen when either the *pdf* gene was mutant or the cells ablated (see below). The lack of a behavioral phenotype in these experiments may be explained by a lack of sensitivity by the PDF peptidergic release system to tetanus toxin. It may be that *Drosophila* peptidergic release relies on molecules different from synaptobrevin. However, there are two points that suggest caution in accepting that interpretation. For another peptidergic system of *Drosophila*, release of the eclosion hormone by two identified neurons is sensitive to this toxin (McNabb *et al.*, 1997) (see **Chapter 3.1**). That result suggests at least some *Drosophila* peptidergic neurons are likely to employ synaptobrevins in the exocytosis of peptidergic secretory granules. In addition, the *UAS-tetanus toxin* system produces only incomplete cleavage of synaptobrevin (Sweeney *et al.*, 1995), leaving open the possibility that the levels of toxin expression were not sufficient to effect a complete block of release in this instance.

4.11.4.3.4. Control of PDF by the clockworks In mammals, several SCN transmitters display diurnal and/or circadian variation in their expression reflecting control by the clockworks (e.g., Jin *et al.*, 1999). To what extent is PDF expression controlled by clockwork genes? Given a circadian role for PDF signaling, it is now important to ask how the circadian clock produces a diurnal PDF signal. Several cellular phenomena represent potential points of clock regulation: these include *pdf* transcription and translation, the electrical activity of *pdf*-expressing neurons, and the sensitivity of PDF-receptive neurons. We now know that PDF expression is regulated by components of the circadian clock, but the details of that regulation are still emerging and they reveal a number of unexpected features. Genetic observations show that *pdf* RNA is positively regulated by the transcription factors *Clock* and *cyc* (J.H. Park *et al.*, 2000) and is also regulated by *vrille* (Blau and Young, 1999). The effect of the dominant negative Clk^{Jrk} allele appeared more severe than that of *cyc* alleles, consistent with the hypothesis that *Clk* may have additional partners in mediating its control on *pdf* (J.H. Park *et al.*, 2000). As yet, no factors have been shown to regulate *pdf* expression by direct transcriptional activation assays. In the specific case of regulation by CLK : CYC, their actions appear to be indirect (J.H. Park *et al.*, 2000). The regulators *Clk* and *vri* produce distinct effects on PDF expression: continuous expression of *vri* produced a decrease in PDF levels in larval pacemaker neurons (vLNs) but no effect on *pdf* RNA levels (Blau and Young, 1999).

A remarkable feature of this clockwork regulation of PDF expression is its exquisite cell type specificity: it is seen only in clock neurons (not in the abdominal PDF neurons) and, more specifically, only in some clock neurons (the s-vLNs). In spite of its evident clock-controlled transcriptional regulation, *pdf* RNA does not fluctuate on a daily basis, nor does PDF immunoreactivity vary in large-scale fashion (Park and Hall, 1998). This conclusion was subsequently supported by many microarray experiments (op. cit. Section 4.11.4.1). *Clk* and *cyc*

mutants also displayed aberrations in PDF s-vLN axonal processes, consistent with a normal role in those cells' morphological differentiation (J.H. Park *et al.*, 2000). Such coordinate control of transmitter expression and axonal morphology by a single transcription factor may reflect a broader developmental theme, akin to similar demonstrations for other nonclock *Drosophila* neurons (Thor and Thomas, 1997; Allan *et al.*, 2003). In sum, the available data suggest substantial clockwork control of PDF expression. It also indicates that neither *pdf* transcription nor its translation contribute extensively to the principal gating mechanism within the s-vLN neurons.

There is substantial evidence for a daily rhythm of PDF release. In houseflies, PDH injections cause a swelling of L1 and L2 (lamina) axonal diameters that mimics a diurnal change of axon caliber these neurons normally display (Pyza and Meinertzhagen, 1996): an effect consistent with a daytime release of PDF. *Drosophila* exhibits similar diurnal and circadian changes in laminar axonal diameters and shape (Pyza and Meinertzhagen, 1999). Pyza and Meinertzhagen (1997) also reported that PDH varicosities in the optic lobes display a circadian rhythm in size and spacing: fewer in the subjective day, again consistent with a daily, daytime PDF release. PDF immunoreactivity within s-vLN terminals of *Drosophila* varies in a diurnal and circadian fashion (J.H. Park *et al.*, 2000). It is high in the subjective day (with a peak ~1 h after the start of the subjective day) and low in the subjective night. These findings are also consistent with a hypothesis of release of PDF during the subjective day. This predicted daily PDF release event displays clock influence, in that the period of the staining variation is sensitive to a period length-altering allele of *per* (J.H. Park *et al.*, 2000). By way of speculation, the daily changes in PDF neurite morphology of the larger flies may find a parallel in the phenotype of the fragile X-related protein gene (*dFMR-1*: Dockendorf *et al.*, 2002; Inoue *et al.*, 2002; Morales *et al.*, 2002). As discussed below, *dFMR-1* mutant animals display both circadian phenotypes and alterations in PDF cell branching.

The *per* and *tim* mutations did not affect *pdf* RNA levels, but did affect the level of PDF expression in the s-vLN terminals. Surprisingly, they have opposite effects in this regard. A per^0 allele caused consistently low PDF staining, while a tim^0 allele caused consistently high PDF staining (J.H. Park *et al.*, 2000). In sum, observations to date indicate multiple levels of control by the clockworks on PDF, specifically within the s-vLNs. *Clk* and *cyc* appear to affect *pdf* gene expression and PDF cell differentiation, while *vri, per*, and *tim* appear to affect a later step(s) in PDF expression, perhaps involving rhythmic transport, processing and/or release. A more complete definition of *pdf* regulation and an understanding of how its fluctuations contribute to gated PDF signaling represent important future goals.

4.11.4.3.5. PDF physiology and genetics *Drosophila* mutants for *pdf* were discovered resident among laboratory stocks of long standing (Renn *et al.*, 1999). These animals contain a nonsense mutation in the signal sequence of preproPDF and are protein nulls. The mutant animals appear and behave normally in most respects. PDF neurons are present and appear fully differentiated in the mutant background. However, the circadian clock-regulated behavior of *pdf* mutant animals is highly abnormal: while they entrain to light signals, a large majority of the population displays arrhythmicity under constant darkness (DD). The arrhythmicity of *pdf* mutants is not evident for 1 to 3 cycles of DD. This is unlike the phenotype of animals bearing mutations in clock genes like *period* or *timeless* for which arrhythmic behavior is evident as soon as the animal is placed in DD. Transgenic expression of wild-type *pdf* sequences restored expression of PDF peptide and rhythmic behavior to a great extent. Ablation of the PDF neurons (affected by genetic targeting) in an otherwise wild-type background produced a behavioral phenotype that was in all ways comparable to that produced by *pdf* mutant flies. A similar conclusion was reached by Blanchardon *et al.* (2001) who also ablated *pdf* neurons genetically using a specific GAL4 insertion that prominently features that cell group. Additionally, Helfrich-Förster *et al.* (2000) reported that overexpression of PDF by the UAS : GAL4 system in neurons projecting to the dorsal brain resulted in severe arrhythmicity. That result is consistent with the hypothesis that activation of PDF receptor(s) affects circadian locomotor activity, and that the timing or level of such activation is important to the signaling.

Together these observations indicate that the phenotypic deficits exhibited by *pdf* mutant animals could be attributed in large part to the absence of the *pdf* gene product, and they lead to several conclusions. First, they contribute directly to the hypothesis that the PDF-expressing vLNs are the primary pacemakers underlying control of daily locomotion (cf. Frisch *et al.*, 1994; Vosshall and Young, 1995). Second, they indicate that *pdf* is the sole functional output of vLN pacemakers and that PDF is the principal circadian transmitter in

Drosophila. Third, they predict that other neurons provide auxiliary pacemaking mechanisms and that they must release other (e.g., non-PDF) transmitters (cf. Taghert *et al*., 2001; Allada *et al*., 2003; Helfrich-Förster, 2003; Peng *et al*., 2003; Veleri *et al*., 2003).

4.11.4.3.6. PDF effects on pacemaker synchronization The phenotype of *pdf* mutant flies is consistent with either of two extreme cellular models. First, *pdf* signaling may be required to maintain synchronization and/or oscillation among/in pacemaker neurons. Additionally, *pdf* may be a factor that couples the pacemaker network to premotor centers that govern rhythmic behaviors. These models are not mutually exclusive. Three sets of studies have addressed the first possibility. Petri and Stengl (1997) injected PDH into cockroach brains and reported a significant effect on the phase of subsequent cycles of locomotor activity. PDH caused a phase-dependent ~3-h phase delay consistent with it being an input signal that helps sharpen the synchronization of circadian pacemaker neurons. The maximal effect was during the late subjective day, and the shape of the phase-response curve suggested that PDH presents a nonphotic input to the clock, perhaps especially important with respect to coupling the clock outputs from both sides of the brain. In similar fashion, the *Drosophila* mutant for *pdf* entrained to LD cycles, but with a phase-advanced activity peak (Renn *et al*., 1999). One interpretation of the latter observation is that *pdf* normally acts to delay the phase of the wild-type activity rhythm. That would be congruent with its pharmacological activity in the larger insects. In this regard, an important role for PDF may therefore be as clock input – to help synchronize the phases of disparate clock neurons. These studies suggest that other transmitters are likely to play similar input roles, promoting either clock phase advances or delays (cf. Volkner *et al*., 2000; Petri *et al*., 2002; Wegener *et al*., 2004).

More recently, Peng *et al*. (2003) analyzed the pattern of *tim* RNA *in situ* in the *Drosophila* brain as a measure of molecular pacemaker function in control versus *pdf* mutant animals. On DD5, control brains displayed a strong rhythm of *tim in situ* signals in each of the several sites. In contrast, the amplitude of cycling was much diminished in *pdf* mutant brains. The authors concluded that *pdf* was required to maintain high amplitude, molecular rhythms as well as synchronized activity among different pacemaker groups, akin to a recent hypothesis describing the role of neuropeptide VIP receptors in the mammalian SCN (Harmar *et al*., 2002). They speculate that the effect they observe may be related to the demonstration that membrane electrical activity is required by *Drosophila* pacemaker neurons in order to maintain molecular rhythms (Nitabach *et al*., 2002). A different conclusion was reached by Blanchardon *et al*. (2001) who monitored PER immunostaining on DD2-3 in brains of flies that had had the PDF-expressing vLNs (and several other) neurons genetically ablated. That group reported normal PER staining fluctuations in dLN and DN neuronal groups. Two technical differences between these studies are notable and may explain the differences – the amount of time flies were in DD before analysis and the nature of the probes (*tim in situ* probe versus anti-PER antibodies).

4.11.4.3.7. *pdf* and geotaxis Toma *et al*. (2002) reported on studies of highly inbred *Drosophila* stocks that display either a positive or negative geotaxic bias. Flies called "Hi" typically distribute at higher perches of a vertical maze; the opposite is true of flies called "Lo." Control animals typically occupy intermediate positions. The authors reasoned that such behavioral biases likely reflect polygenic differences and so they employed microarray technology to determine which genes showed average level differences between the two stocks. Surprisingly, *pdf* was among the genes found to be reliably different. Further, when tested in the same geotaxic maze device, *pdf* nulls displayed a negative geotaxic bias as great as that of "Hi" flies. Other clock mutants did not display a similar phenotype. Remarkably, the geotaxic test score was a function of *pdf* gene dosage; this presumably suggests that the behavior is sensitive to several quantitative levels of PDF peptide. Given the limited sites of PDF expression in the adult animal (essentially two sets of neurons, see above), it should be possible to narrow down the cellular sites of action using molecular genetic techniques.

4.11.4.3.8. PDF receptors There is currently no direct information on the identity of receptors that mediate PDF effects in any organism. Such data would help address many outstanding questions regarding the sites and mechanisms of PDF actions. Peng *et al*. (2003) reported recently on the distribution of biotinylated PDF in the *Drosophila* CNS. They applied the labeled probe to fixed brains in whole mount and described a pattern of binding that almost entirely overlapped the pattern of *tim-GAL4* gene expression. That result is consistent with an intriguing hypothesis proposed by those authors – that within the CNS, PDF receptors are

largely associated with neuronal pacemakers and support their synchronization. However, the report also indicated a need for more characterization of these putative PDF receptor sites: the molecular specificity was uncertain, as the staining effects could not be competed out by unlabeled PDF, even when applied at a 5000-fold excess. In addition, the staining patterns displayed an inconsistent character.

Structure-activity studies of the crustacean PDH have revealed possible facts about its interactions with a putative receptor and/or with degradative enzymes (Riehm and Rao, 1982). N-terminal deletions of PDH, removing as many as five amino acids, still resulted in the retention of some (weak) biological activity. Progressively re-extending the molecule back towards the N-terminus restores activity incrementally. Loss of the C-terminal amide resulted in ~300-fold loss of activity (Riehm *et al.*, 1985).

Because all small neuropeptides act via G protein-coupled receptors (GPCRs; see **Chapter 5.5**), it is reasonable to think that PDF receptors also act in this manner. Nery and Castrucci (1997) suggested that PDH effects in crustacean chromatophores reflect G's activation following binding to a GPCR. Hewes and Taghert (2001) found evidence for 44 (±1) peptide GPCRs in the *Drosophila* genome. Those assignments rested on the assumption that *Drosophila* peptide GPCRs are related to those of other animals by sequence similarities within the highly conserved seven transmembrane domains. Pharmacological study of many of these molecules following functional expression has proceeded using different methods, including signaling assays that rely on a promiscuous G protein (e.g., Cazzamali and Grimmelikhuijzen, 2002; Meeusen *et al.*, 2002), assays of membrane currents in *Xenopus* (e.g., Park *et al.*, 2002), assays of competitive radioligand binding (e.g., Johnson *et al.*, 2003a), and assays of ligand-dependent translocation of β-arrestin-GFP (Johnson *et al.*, 2003b). To date, roughly 26 of the 44 indicated peptide receptors have been "de-orphaned" by one or more methods. Hopefully, identification and analysis of the PDF receptor(s) will occur in the near future.

4.11.4.4. Second Messenger Systems Mediating PDF and/or Circadian Output

4.11.4.4.1. cAMP Only a few studies have been conducted on the signaling of PDH in crustaceans. It is suggested that pigment dispersion is achieved by ligand binding to a G_s protein-coupled receptor, resulting in the activation of adenylate cyclase and the increase of the intracellular concentration of cAMP, which in turn activates a cAMP-dependent protein kinase (Nery and Castrucci, 1997) (see also **Chapter 3.2**). There is genetic evidence supporting the role of the catalytic subunit of protein kinase A (PKA – called *DCO*) in circadian output controlling locomotor rhythms (Majercak *et al.*, 1997), as well as for a type II PKA regulatory subunit (S.K. Park *et al.*, 2000). However, there is no information yet available to indicate at what cellular/synaptic level PKA may be acting, nor whether this action reflects PDF action. Finally, Belvin *et al.* (1999) reported that *dCREB2* cycles in circadian fashion, and that it may help sustain rhythmicity of *period* gene expression by affecting both circadian period and amplitude. These observations suggest that cAMP signaling is likely important at several levels in the circadian system.

4.11.4.4.2. Nf1/MAP kinase Williams *et al.* (2001) showed an involvement by several putative signaling components that act downstream of PDF in mediating *Drosophila* circadian output. The *Drosophila neurofibromatosis 1* (*Nf1*) gene product neurofibromin regulates both Ras and cyclic AMP (cAMP) (The *et al.*, 1997). *Nf1* mutants exhibited virtually no rhythmic behavior under constant conditions. However, PER and TIM protein, and *tim* RNA levels in the head continued to oscillate normally in those flies, suggesting a defect downstream of the clockworks. Likewise, PER and TIM proteins also continued to oscillate in the larval LN pacemaker neurons. Furthermore, attempts at phenotypic rescue by restoring wild-type *Nf1* only to the *pdf*-expressing vLN neurons was not effective. Reduced Ras activity partially rescued the circadian rhythm defect of *Nf1* mutants, suggesting that NF1 normally regulates Ras in control of this behavior. A link to PDF action was made by demonstrating that phospho-MAP kinase levels were substantially elevated in *Nf1* mutants, that such kinase levels cycled diurnally, and that such cycling was reduced in *pdf* mutants. The authors concluded that PDF driven circadian output is mediated at least in part by NF1 activity through a Ras/MAP kinase-dependent signaling pathway (Williams *et al.*, 2001) (see also **Chapter 3.2**).

4.11.4.5. Contributions to Circadian Output by Other Transmitters

4.11.4.5.1. Other neuropeptides It follows from the chemical heterogeneity of pacemaker neurons (PDF is only expressed by a subset of them) that other transmitters must play important roles in the circuits underlying circadian output. Taghert *et al.* (2001) presented evidence to suggest that these

include other amidated peptides different from PDF. In particular, certain peptidylglycine alpha-hydroxylating monooxygenase (*PHM*) mosaic animals displayed a more severe locomotor defect than did *pdf* mutant animals. PHM is an enzyme responsible for the initial step in C-terminal α-amidation, a posttranslational modification displayed by more than 90% of all known *Drosophila* neuropeptides (Hewes and Taghert, 2001). Importantly, amidation is typically required for full biological activity of neuropeptides (see Riehm *et al*., 1985, for an example focusing on PDH), and it is specific to secretory peptides. The amidated peptide corazonin is a good candidate for further consideration as it is found in many insects to be co-localized with PER proteins (e.g., Wise *et al*., 2002; Bloch *et al*., 2003).

Ecdysial behaviors, which are gated by the circadian clock in some cases (e.g., adult eclosion; Truman, 1992) (see also **Chapter 3.1**), are regulated by several neuropeptides that act in a complex fashion (Ewer and Reynolds, 2002). Release of these factors (including eclosion hormone and CCAP) may be closely (perhaps directly) controlled by clock neurons (Park *et al*., 2003).

4.11.4.5.2. Other transmitters Two sets of studies highlight circadian changes in the conventional transmitter signaling in the fly. Wide field serotinergic neurons (and PDH-containing neurons) have both been proposed to modulate visual input in the optic ganglia (Nässel *et al*., 1991; Pyza and Meinertzhagen, 1997). To investigate possible rhythms in functional aspects of the visual system, Chen *et al*. (1999) described a diurnal and circadian change in the amplitude of ON and OFF transients in the electroretinogram (ERG) of the fly retina. Consistent with its circadian-regulated release, serotonin injections enhanced the amplitude of ERG transients, and antagonists decreased them. Andretic *et al*. (1999) found a clock-controlled change in dopamine receptor expression linked to locomotor output (also discussed below in Section 4.11.4.6.5).

4.11.4.6. Evidence for Additional Processes Related to Circadian Output

4.11.4.6.1. The clock controlling rhythmic eclosion Truman and Riddiford (1970) first described the control of adult eclosion by a circadian clock located within the silkmoth brain. They used brain extirpation and reimplantation techniques to demonstrate that an endocrine signal (since identified as the peptide "eclosion hormone," EH; see **Chapter 3.1**) is released by the brain according to a signal gated by a photoperiod-sensitive clock. Recent studies of the moth have identified specific EH neurons, permitting their comparison to putative sites of *period* gene expression (Sauman and Reppert, 1996). EH neurons do not appear to be intrinsic circadian pacemakers; instead they are likely driven by higher order pacemakers, which may themselves include other neurosecretory neurons. Myers *et al*. (2003) re-examined the cellular basis for rhythmic eclosion. They found that circadian oscillations in both the vLNs and the prothoracic gland (PG, a peripheral endocrine organ; see **Chapter 3.3**) were required for normal rhythmic eclosion. Further, the action of LNs on eclosion and on the rhythms of PER and TIM in the PG was shown to be mediated by its (presumed) secretion of the neuropeptide PDF. The manipulations used affected both clock PDF neurons (vLNs) and nonclock neurons (abdominal neuroendocrine neurons). It is therefore possible that the vLN PDF brain neurons regulate the PG directly (via hemolymph-borne PDF) or indirectly via the neurons themselves controlled by the vLNs.

4.11.4.6.2. A humoral signal controlling daily locomotion Handler and Konopka (1979) performed brain transplants similar to those performed by Truman and Riddiford (1970), but in the much smaller *Drosophila* to examine the possible influence of brain secretions on rhythmic activity. They found that in a small number of instances, brains from per^S animals could restore rhythmicity when transplanted into animals that were otherwise arrhythmic due to their carrying a per^{01} allele. The implicated molecule(s) remain unidentified. In summary, there is evidence of diffusible (hormonal) signals normally influencing rhythmic locomotor and eclosion behaviors in insects.

4.11.4.6.3. Fragile X-related protein Several groups have recently implicated the *dFMR-1* gene product as a component of circadian output. *dFMR-1* encodes an RNA-binding protein related to the mammalian fragile X protein. In *Drosophila*, it is a determinant of neuronal morphology (Zhang *et al*., 2001; Dockendorf *et al*., 2002; Morales *et al*., 2002; Lee *et al*., 2003; Schenck *et al*., 2003). Each group determined that *dFMR-1* mutant flies were largely arrhythmic in constant darkness (Dockendorf *et al*., 2002; Inoue *et al*., 2002; Morales *et al*., 2002). Inoue *et al*. (2002) found that the mutants had normal eclosion rhythms, while the other two groups reported either phase (Dockendorf *et al*., 2002), or phase and amplitude (Morales *et al*., 2002)

disturbances in the eclosion rhythm. The vLNs display specific axonal branching defects (Morales *et al*., 2002), although Dockendorf *et al*. (2002) reported that these were not observed uniformly. Rhythms of CREB activity were much reduced in mutants (Morales *et al*., 2002), but rhythmic *per* expression was maintained (Morales *et al*., 2002). Interestingly, the double mutant *futsch, dFMR-1* suppressed *dFMR-1* phenotypes in motor neurons (synaptic overgrowth and defects in neurotransmission; Zhang *et al*., 2001). However, that genotype did not suppress the *dFMR-1* circadian phenotype (Dockendorf *et al*., 2002). In sum, *dFMR-1* mutants maintained clock gene expression, but displayed alterations in behavioral rhythms and in the morphology of pacemaker neurons, and alterations in at least one molecular index of clock output. Determining where and how *dFMR-1* acts will provide valuable information concerning which circadian output mechanisms may be defined further.

4.11.4.6.4. *takeout* Further downstream, the *takeout* gene contributes to the animals' starvation responses: this gene responds to starvation with an upregulation of transcription that is dependent on clock gene expression (Sarov-Blat *et al*., 2000). Its transcription is dependent on expression of most of the major clock genes, but with a distinct delayed phase (So *et al*., 2000). Additional information concerning *takeout* functions comes from Dauwalder *et al*. (2002) who report that the gene is controlled by the sex determination pathway (see **Chapter 1.7**), and that a loss-of-function allele affects male courtship. Further, they argue that *takeout* is a member of a large family of genes encoding secreted factors that bind small lipophilic molecules. There exist apparent differences in the reported patterns of *takeout* gene expression in these various reports. Further studies are needed to help form a consensus regarding *takeout* functions, and so better understand its role in the circadian system.

4.11.4.6.5. *Lark* Several lines of evidence demonstrate that the Lark protein participates in construction of a circadian gate for rhythmic eclosion behavior (Newby and Jackson, 1996; McNeil *et al*., 1998). *Lark* heterozygotes alter the phase of the eclosion gate, but not its period (*Lark* homozygotes die during embryogenesis, likely due to loss of other functions). Furthermore, *Lark* rhythm defects are behavior specific in that they affect eclosion, but not daily locomotor activity. This protein has the molecular signature of an RNA binding protein and is widely expressed in the nucleus of most cells. Significantly, the protein accumulates in the cytoplasm of specific peptidergic (CCAP) neurons (Zhang *et al*., 2000). In other insects, these neurons have been implicated in triggering eclosion behavior motor patterns (Ewer and Truman, 1996; Park *et al*., 2003). Identifying the signal transduction pathways regulated by circadian transmitters and relating Lark actions to the elaboration of a circadian gate are challenges for the near future.

4.11.4.6.6. Circadian regulation of drug sensitivity in *Drosophila* Hirsh and colleagues have related circadian outputs to drug sensitivity using a decapitated fly assay that allows drug application directly to the CNS. Decapitated flies retain some CNS structures, i.e., the ventral nerve cord, which directly controls somatic musculature and gut motility. Quinpirole, a D2-like dopamine receptor agonist, induces reflexive locomotion, and is most effective during the subjective night (Andretic and Hirsh, 2000). Those studies indicated that dopamine receptor responsiveness is under circadian control and depends on the normal function of the *period* gene. Flies also show behavioral responsiveness to free base cocaine and sensitization to repeated cocaine doses. Four of five circadian genes tested (*per*, *Clk*, *cyc*, and *dbt*) altered cocaine sensitization responsiveness (Andretic *et al*., 1999). Similar phenomena were later described in the mouse (Abarca *et al*., 2002). Mutant flies also show a lack of tyrosine decarboxylase induction normally seen with cocaine administration. Interestingly, tim^{01} mutants displayed normal cocaine responses. Andretic *et al*. (1999) deduce that an unidentified PER binding partner is specifically involved in regulation of drug responsiveness. This result also suggests that drug responsiveness is likely regulated by *per* expression in a set of cells distinct from those involved in circadian function.

4.11.4.6.7. Circadian output and sleep The biological clock controls many rhythmic output processes, some overt and some obscure. Among these, sleep is arguably the most obvious. For many, it is also the most mysterious. Studies that focus on *Drosophila* rest as a model began in earnest several years ago (Hendricks *et al*., 2000; Shaw *et al*., 2000). Those empirical findings have provided a conceptual framework for which rest in flies can formally correspond to mammalian sleep (reviews: Hendricks, 2003; Shaw, 2003). A significant element in that concept is the demonstration that flies display a robust sleep homeostat – a mechanism to increase the duration of sleep (sleep rebound) within a daily cycle in response to some sleep deprivation experienced in past cycles.

Given the rhythmic nature of sleep, the possible effects of circadian clock mutations on *Drosophila* sleep has been an early and enduring point of interest. Two sets of observations describe the current understanding of the relationship between the circadian and sleep mechanisms in *Drosophila*. First, sleep rebound following a 3-, 6-, or 9-h deprivation protocol is displayed in the cycle immediately following, and it occurs principally during the subjective day (Hendricks *et al*., 2000; Shaw *et al*., 2000). Hence, sleep homeostasis is normally gated by the circadian clock. Beyond this permissive feature, does the clock provide regulatory control over sleep mechanisms? The second set of observations suggests the answer is yes. Clock gene mutants display predictable alterations in the dynamics and extent of sleep homeostasis (Shaw *et al*., 2002; Hendricks *et al*., 2003). There is clear consensus from observing mutant phenotypes that at least some of the core clock elements may directly regulate aspects of sleep. For example, *per* and *Clk* mutants display 100% recovery from sleep deprivation with one daily cycle, whereas control stocks only recover ~40% in that same time period. Additionally, *cyc* and *tim* mutant flies display increased or decreased amounts of sleep rebound after deprivation that reflect the amount of deprivation experienced. The phenotype of *cyc* mutant flies in response to sleep deprivation is especially intriguing, and can include exaggerated sleep rebound, increases in baseline sleep amount (set-point), and lethality. That syndrome suggests that not all components of the molecular pathway, as understood in a circadian context, participate equally in a sleep context. Nevertheless, these data together strongly imply that there is a close mechanistic association between elements of the circadian clock and the sleep homeostat. With this strong foundation, we can anticipate that further genetic analysis in *Drosophila* will make contributions to help define hypotheses by which to describe underlying mechanisms.

Acknowledgments

We are very grateful to Erik Johnson, Russ Van Gelder, and Paul Shaw for helpful discussions, and for reading sections of earlier drafts. We apologize to colleagues whose work was not covered for lack of space or oversight. We thank David Van Essen whose pun we refashioned to compose a title for this chapter. YL is supported by the Washington University Medical School MSTP Program. Work in PT's laboratory is supported by grants from the NINCDS (NS21749) and by the NIMH (MH067122).

References

Abarca, C., Albrecht, U., Spanagel, R., **2002**. Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc. Natl Acad. Sci. USA 99*, 9026–9030.

Akten, B., Jauch, E., Genova, G.K., Kim, E.Y., Edery, I., *et al*., **2003**. A role for CK2 in the *Drosophila* circadian oscillator. *Nature (Lond.) Neurosci. 6*, 251–257.

Allada, R., Kadener, S., Nandakumar, N., Rosbash, M., **2003**. A recessive mutant of *Drosophila Clock* reveals a role in circadian rhythm amplitude. *Eur. Mol. Biol. Org. J. 22*, 3367–3375.

Allada, R., White, N.E., So, W.V., Hall, J.C., Rosbash, M., **1998**. A mutant *Drosophila* homolog of mammalian clock disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell 93*, 791–804.

Allan, D.W., St. Pierre, S.E., Miguel-Aliaga, I., Thor, S., **2003**. Specification of neuropeptide cell identity by the integration of retrograde BMP signaling and a combinatorial transcription factor code. *Cell 113*, 73–86.

Andretic, R., Chaney, S., Hirsh, J., **1999**. Requirement of circadian genes for cocaine sensitization in *Drosophila*. *Science (Wash.) 285*, 1066–1068.

Andretic, R., Hirsh, J., **2000**. Circadian modulation of dopamine receptor responsiveness in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 97*, 1873–1878.

Ashmore, L.J., Sathyanarayanan, S., Silvestre, D.W., Emerson, M.M., Schotland, P., *et al*., **2003**. Novel insights into the regulation of the timeless protein. *J. Neurosci. 23*, 7810–7819.

Ashmore, L.J., Sehgal, A., **2003**. A fly's eye view of circadian entrainment. *J. Biol. Rhyth. 18*, 206–216.

Bae, K., Lee, C., Sidote, D., Chuang, K.Y., Edery, I., **1998**. Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators. *Mol. Cell. Biol. 18*, 6142–6151.

Bao, S., Rihel, J., Bjes, E., Fan, J.Y., Price, J.L., **2001**. The *Drosophila double-timeS* mutation delays the nuclear accumulation of period protein and affects the feedback regulation of *period* mRNA. *J. Neurosci. 21*, 7117–7126.

Baylies, M.K., Bargiello, T.A., Jackson, F.R., Young, M.W., **1987**. Changes in abundance or structure of the *per* gene product can alter periodicity of the *Drosophila* clock. *Nature (Lond.) 326*, 390–392.

Belvin, M.P., Zhou, H., Yin, J.C., **1999**. The *Drosophila dCREB2* gene affects the circadian clock. *Neuron 22*, 777–787.

Blanchardon, E., Grima, B., Klarsfeld, A., Chelot, E., Hardin, P.E., *et al*., **2001**. Defining the role of *Drosophila* lateral neurons in the control of circadian rhythms in motor activity and eclosion by targeted genetic ablation and PERIOD protein overexpression. *Eur. J. Neurosci. 13*, 871–888.

Blau, J., Young, M.W., **1999**. Cycling *vrille* expression is required for a functional *Drosophila* clock. *Cell 99*, 661–671.

Block, G., Geusz, M., Khalsa, S., Michel, S., Whitmore, D., **1995**. Cellular analysis of a molluscan retinal biological clock. *Ciba Found. Symp. 183*, 51–60.

Bloch, G., Solomon, S.M., Robinson, G.E., Fahrbach, S.E., **2003**. Patterns of PERIOD and pigment-dispersing hormone immunoreactivity in the brain of the European honeybee (*Apis mellifera*), age- and time-related plasticity. *J. Comp. Neurol. 464*, 269–284.

Brandes, C., Plautz, J.D., Stanewsky, R., Jamison, C.F., Straume, M., *et al.*, **1996**. Novel features of *Drosophila period* transcription revealed by real-time luciferase reporting. *Neuron 16*, 687–692.

Burbach, K.M., Poland, A., Bradfield, C.A., **1992**. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl Acad. Sci. USA 89*, 8185–8189.

Cashmore, A.R., Jarillo, J.A., Wu, Y.J., Liu, D., **1999**. Cryptochromes, blue light receptors for plants and animals. *Science (Wash.) 284*, 760–765.

Cazzamali, G., Grimmelikhuijzen, C.J., **2002**. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 12073–12078.

Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., *et al.*, **1999**. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science (Wash.) 285*, 553–556.

Ceriani, M.F., Hogenesch, J.B., Yanovsky, M., Panda, S., Straume, M., *et al.*, **2002**. Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. *J. Neurosci. 22*, 9305–9319.

Chen, B., Meinertzhagen, I.A., Shaw, S.R., **1999**. Circadian rhythms in light-evoked responses of the fly's compound eye, and the effects of neuromodulators 5-HT and the peptide PDF. *J. Comp. Physiol. (A) 185*, 393–404.

Chen, Y., Hunter-Ensor, M., Schotland, P., Sehgal, A., **1998**. Alterations of *per* RNA in noncoding regions affect periodicity of circadian behavioral rhythms. *J. Biol. Rhyth. 13*, 364–379.

Cheng, Y., Hardin, P.E., **1998**. *Drosophila* photoreceptors contain an autonomous circadian oscillator that can function without *period* mRNA cycling. *J. Neurosci. 18*, 741–750.

Chuman, Y., Matsushima, A., Sato, S., Tomioka, K., Tominaga, Y., *et al.*, **2002**. cDNA cloning and nuclear localization of the circadian neuropeptide designated as pigment-dispersing factor PDF in the cricket *Gryllus bimaculatus*. *J. Biochem. (Tokyo) 131*, 895–903.

Citri, Y., Colot, H.V., Jacquier, A.C., Yu, Q., Hall, J.C., *et al.*, **1987**. A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. *Nature (Lond.) 326*, 42–47.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., *et al.*, **2001**. Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron 32*, 657–671.

Crews, S.T., Thomas, J.B., Goodman, C.S., **1988**. The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell 52*, 143–151.

Curtin, K.D., Huang, Z.J., Rosbash, M., **1995**. Temporally regulated entry of the *Drosophila period* protein contributes to the circadian clock. *Neuron 14*, 365–372.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R., *et al.*, **2003**. vrille, Pdp1, and dClock form a second feedback loop in the *Drosophila* circadian clock. *Cell 112*, 329–341.

Darlington, T.K., Lyons, L.C., Hardin, P.E., Kay, S.A., **2000**. The period E-box is sufficient to drive circadian oscillation of transcription *in vivo*. *J. Biol. Rhyth. 15*, 462–471.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., *et al.*, **1998**. Closing the circadian loop, CLOCK-induced transcription of its own inhibitors per and tim. *Science (Wash.) 280*, 1599–1603.

Dauwalder, B., Tsujimoto, S., Moss, J., Mattox, W., **2002**. The *Drosophila takeout* gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. *Genes Devel. 16*, 2879–2892.

Dockendorf, T.C., Su, H.S., McBride, S.M., Yang, Z., Choi, C.H., *et al.*, **2002**. *Drosophila* lacking *dfmr1* activity show defects in circadian output and fail to maintain courtship interest. *Neuron 34*, 973–984.

Dunlap, J., **1999**. Molecular bases for circadian clocks. *Cell 96*, 271–290.

Dushay, M.S., Rosbash, M., Hall, J.C., **1989**. The *disconnected* visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. *J. Biol. Rhyth. 4*, 1–27.

Edery, I., Rutila, J.E., Rosbash, M., **1994b**. Phase shifting of the circadian clock by induction of the *Drosophila period* protein. *Science (Wash.) 263*, 237–240.

Edery, I., Zwiebel, L.J., Dembinska, M.E., Rosbash, M., **1994a**. Temporal phosphorylation of the *Drosophila* period protein. *Proc. Natl Acad. Sci. USA 91*, 2260–2264.

Egan, E.S., Franklin, T.M., Hilderbrand-Chae, M.J., McNeil, G.P., Roberts, M.A., *et al.*, **1999**. An extraretinally expressed insect cryptochrome with similarity to the blue light photoreceptors of mammals and plants. *J. Neurosci. 19*, 3665–3673.

Elekes, K., Nässel, D.R., **1999**. Pigment-dispersing hormone-like immunoreactive neurons in the central nervous system of the gastropods, *Helix pomatia* and *Lymnaea stagnalis*. *Cell Tiss. Res. 295*, 339–348.

Emery, P., So, W.V., Kaneko, M., Hall, J.C., Rosbash, M., **1998**. CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell 95*, 669–679.

Emery, P., Stanewsky, R., Hall, J.C., Rosbash, M., **2000a**. A unique circadian-rhythm photoreceptor. *Nature (Lond.) 404*, 456–457.

Emery, P., Stanewsky, R., Helfrich-Förster, C., Emery-Le, M., Hall, J.C., *et al.*, **2000b**. *Drosophila* CRY is a deep brain circadian photoreceptor. *Neuron 26*, 493–504.

Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbash, M., Hall, J.C., **1992**. Expression of the *period* clock gene

within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci. 12*, 3321–3349.

Ewer, J., Hamblen-Coyle, M., Rosbash, M., Hall, J.C., **1990**. Requirement for *period* gene expression in the adult and not during development for locomotor activity rhythms of imaginal *Drosophila melanogaster. J. Neurogenet. 7*, 31–73.

Ewer, J., Reynolds, S.E., **2002**. Neuropeptide control of molting in insects. In: Pfaff, D.W., Arnold, A.P., Fahrbach, S.E., Etgen, A.M., Rubin, R.T. (Eds.), Hormones, Brain and Behavior, vol. 35. Academic Press, San Diego, pp. 1–92.

Ewer, J., Truman, J.W., **1996**. Increases in cyclic 3′,5′-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide-immunoreactive insect neuronal network. *J. Comp. Neurol. 370*, 330–341.

Fernlund, P., **1971**. Chromactivating hormones of *Pandalus borealis*, isolation and purification of a light-adapting hormone. *Biochim. Biophys. Acta 237*, 519–529.

Fernlund, P., **1976**. Structure of a light-adapting hormone from the shrimp, *Pandalus borealis*. *Biochim. Biophys. Acta 439*, 17–25.

Foster, R.G., Helfrich-Förster, C., **2001**. The regulation of circadian clocks by light in fruitflies and mice. *Philos. Trans. R. Soc. Lond. B Biol. Sci. 356*, 1779–1789.

Frisch, B., Fleissner, G., Brandes, C., Hall, J.C., **1994**. Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock-gene product, labeling of cells with possible importance for the beetle's circadian rhythms. *Cell Tiss. Res. 286*, 411–429.

Frisch, B., Hardin, P.E., Hamblen-Coyle, M.J., Rosbash, M., Hall, J.C., **1994**. A promoterless *period* gene mediates behavioral rhythmicity and cyclical per expression in a restricted subset of the *Drosophila* nervous system. *Neuron 12*, 555–570.

Froy, O., Chang, D.C., Reppert, S.M., **2002**. Redox potential, differential roles in CRY and mCRY1 functions. *Curr. Biol. 12*, 147–152.

Gekakis, N., Saez, L., Delahaye-Brown, A.-M., Myers, M.P., Sehgal, A., *et al.*, **1995**. Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER^L. *Science (Wash.) 270*, 811–815.

Gibbs, S.M., Truman, J.W., **1998**. Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of *Drosophila*. *Neuron 20*, 83–93.

Giebultowicz, J.M., **1999**. Insect circadian clocks, is it all in their heads? *J. Insect Physiol. 45*, 791–800.

Giebultowicz, J.M., Stanewsky, R., Hall, J.C., Hege, D.M., **2000**. Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. *Curr. Biol. 10*, 107–110.

Glossop, N.R., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M., *et al.*, **2003**. VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator. *Neuron 37*, 249–261.

Glossop, N.R., Lyons, L.C., Hardin, P.E., **1999**. Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science (Wash.) 286*, 766–768.

Glossop, N.R., Shepherd, D., **1998**. *Disconnected* mutants show disruption to the central projections of proprioceptive neurons in *Drosophila melanogaster. J. Neurobiol. 36*, 337–347.

Grima, B., Lamouroux, A., Chelot, E., Papin, C., Limbourg-Bouchon, B., *et al.*, **2002**. The F-box protein slimb controls the levels of clock proteins period and timeless. *Nature (Lond.) 420*, 178–182.

Hall, J.C., **2000**. Cryptochromes, sensory reception, transduction and clock functions subserving circadian systems. *Curr. Opin. Neurobiol. 10*, 456–466.

Hall, J.C., **2003**. Genetics and molecular Biology of rhythms in *Drosophila* and other insects. *Adv. Genet. 48*, 1–280.

Hamblen-Coyle, M., Konopka, R.J., Zwiebel, L.J., Colot, H.V., Dowse, H.B., *et al.*, **1989**. A new mutation at the *period* locus of *Drosophila melanogaster* with some novel effects on circadian rhythms. *J. Neurogenet. 5*, 229–256.

Handler, A.M., Konopka, R.J., **1979**. Transplantation of a circadian pacemaker in *Drosophila*. *Nature (Lond.) 279*, 236–238.

Hao, H., Allen, D.L., Hardin, P.E., **1997**. A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster. Mol. Cell. Biol. 17*, 3687–3693.

Hao, H., Glossop, N.R., Lyons, L., Qiu, J., Morrish, B., *et al.*, **1999**. The 69 bp circadian regulatory sequence (CRS) mediates *per*-like developmental, spatial, and circadian expression and behavioral rescue in *Drosophila*. *J. Neurosci. 19*, 987–994.

Hardin, P.E., Hall, J.C., Rosbash, M., **1990**. Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature (Lond.) 343*, 536–540.

Hardin, P.E., Hall, J.C., Rosbash, M., **1992a**. Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. *Proc. Natl Acad. Sci. USA 89*, 11711–11715.

Hardin, P.E., Hall, J.C., Rosbash, M., **1992b**. Behavioral and molecular analyses suggest that circadian output is disrupted by *disconnected* mutants in *D. melanogaster. EMBO J. 11*, 1–6.

Harmar, A.J., Marston, H.M., Shen, S., Spratt, C., West, K.M., *et al.*, **2002**. The VPAC 2 receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell 10*, 497–508.

Harrison, P.M., Kumar, A., Lang, N., Snyder, M., Gerstein, M., **2002**. A question of size: the eukaryotic proteome and the problems in defining it. *Nucleic Acids Res. 30*, 1083–1090.

Hege, D.M., Stanewsky, R., Hall, J.C., Giebultowicz, J.M., **1997**. Rhythmic expression of a PER-reporter in

the Malpighian tubules of decapitated *Drosophila*, evidence for a brain-independent circadian clock. *J. Biol. Rhyth. 12*, 300–308.

Helfrich-Förster, C., **1995**. The *period* clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 92*, 612–616.

Helfrich-Förster, C., **1997**. Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster. J. Comp. Neurol. 380*, 335–354.

Helfrich-Förster, C., **1998**. Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons, a brain-behavioral study of *disconnected* mutants. *J. Comp. Physiol. (A) 182*, 435–453.

Helfrich-Förster, C., **2003**. The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster. Microsc. Res. Tech. 62*, 94–102.

Helfrich-Förster, C., Edwards, T., Yasuyama, K., Wisotzki, B., Schneuwly, S., *et al.*, **2002**. The extraretinal eyelet of *Drosophila*, development, ultrastructure, and putative circadian function. *J. Neurosci. 22*, 9255–9266.

Helfrich-Förster, C., Homberg, U., **1993**. Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. *J. Comp. Neurol. 337*, 177–190.

Helfrich-Förster, C., Stengl, M., Homberg, U., **1998**. Organization of the circadian system in insects. *Chronobiol. Int. 15*, 567–594.

Helfrich-Förster, C., Tauber, M., Park, J.H., Muhlig-Versen, M., Schneuwly, S., *et al.*, **2000**. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster. J. Neurosci. 20*, 3339–3353.

Helfrich-Förster, C., Winter, C., Hofbauer, A., Hall, J.C., Stanewsky, R., **2001**. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron 30*, 249–261.

Hendricks, J.C., **2003**. Genetic Models in Applied Physiology, Invited Review, Sleeping flies don't lie, the use of *Drosophila melanogaster* to study sleep and circadian rhythms. *J. Appl. Physiol. 94*, 1660–1672.

Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., *et al.*, **2000**. Rest in *Drosophila* is a sleep-like state. *Neuron 25*, 129–138.

Hendricks, J.C., Lu, S., Kume, K., Yin, J.C., Yang, Z., *et al.*, **2003**. Gender dimorphism in the role of *cycle* (*BMAL1*) in rest, rest regulation, and longevity in *Drosophila melanogaster. J. Biol. Rhyth. 18*, 12–25.

Hewes, R.S., Taghert, P.H., **2001**. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res. 11*, 1126–1142.

Hofbauer, A., Buchner, E., **1989**. Does *Drosophila* have seven eyes? *Naturwissen 76*, 335–336.

Hoffman, E.C., Reyes, H., Chu, F.F., Sander, F., Conley, L.H., *et al.*, **1991**. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science (Wash.) 252*, 954–958.

Homberg, U., Davis, N.T., Hildebrand, J.G., **1991**. Peptide-immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta. J. Comp. Neurol. 303*, 35–52.

Huang, Z.J., Edery, I., Rosbash, M., **1993**. PAS is a dimerization domain common to *Drosophila* period and several transcription factors. *Nature (Lond.) 364*, 259–262.

Hunter-Ensor, M., Ousley, A., Sehgal, A., **1996**. Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. *Cell 84*, 677–685.

Inoue, S., Shimoda, M., Nishinokubi, I., Siomi, M.C., Okamura, M., *et al.*, **2002**. A role for the *Drosophila fragile X-related* gene in circadian output. *Curr. Biol. 12*, 1331–1335.

Ishikawa, T., Matsumoto, A., Kato, T., Jr., Togashi, S., Ryo, H., *et al.*, **1999**. CRY is a *Drosophila* photoreceptor protein implicated in light entrainment of circadian rhythm. *Genes Cells 4*, 57–65.

Ivanchenko, M., Stanewsky, R., Giebultowicz, J.M., **2001**. Circadian photoreception in *Drosophila*, functions of cryptochrome in peripheral and central clocks. *J. Biol. Rhyth. 16*, 205–215.

Jackson, F.R., Bargiello, T.A., Yun, S.H., Young, M.W., **1986**. Product of *per* locus of *Drosophila* shares homology with proteoglycans. *Nature (Lond.) 320*, 185–188.

Jackson, F.R., Schroeder, A.J., Roberts, M.A., McNeil, G.P., Kume, K., *et al.*, **2001**. Cellular and molecular mechanisms of circadian control in insects. *J. Insect Physiol. 47*, 822–842.

Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., de Vries, G.J., *et al.*, **1999**. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell 96*, 57–68.

Johnson, E.C., Bohn, L.M., Barak, L.S., Birse, R.T., Nässel, D.R., *et al.*, **2003b**. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. *J. Biol. Chem. 278*, 52172–52178.

Johnson, E.C., Garczynski, S.F., Park, D., Crim, J.W., Nässel, D.R., *et al.*, **2003a**. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 100*, 6198–6203.

Kaneko, M., Hall, J.C., **2000**. Neuroanatomy of cells expressing clock genes in *Drosophila*, transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol. 422*, 66–94.

Kaneko, M., Hamblen, M.J., Hall, J.C., **2000b**. Involvement of the *period* gene in developmental time-memory: effect of the *per^{Short}* mutation on phase shifts induced by light pulses delivered to *Drosophila* larvae. *J. Biol. Rhyth. 15*, 13–30.

Kaneko, M., Helfrich-Förster, C., Hall, J.C., **1997**. Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*, newly identified pacemaker candidates and novel features of clock gene product cycling. *J. Neurosci. 17*, 6745–6760.

Kaneko, M., Park, J.H., Cheng, Y., Hardin, P.E., Hall, J.C., **2000a**. Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of *Drosophila* cause abnormal behavioral rhythms. *J. Neurobiol. 43*, 207–233.

Kim, E.Y., Bae, K., Ng, F.S., Glossop, N.R., Hardin, P.E., *et al.*, **2002**. *Drosophila* CLOCK protein is under posttranscriptional control and influences light-induced activity. *Neuron 34*, 69–81.

Kleinholz, L.H., **1975**. Purified hormones from the crustacean eyestalk and their physiological specificity. *Nature (Lond.) 258*, 256–257.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., *et al.*, **1998**. The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase I epsilon. *Cell 94*, 97–107.

Kloss, B., Rothenfluh, A., Young, M.W., Saez, L., **2001**. Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the *Drosophila* clock. *Neuron 30*, 699–706.

Ko, H.W., Jiang, J., Edery, I., **2002**. Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. *Nature (Lond.) 420*, 673–678.

Konopka, R.J., Benzer, S., **1971**. Clock mutants of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 68*, 2112–2116.

Konopka, R.J., Hamblen-Coyle, M.J., Jamison, C.F., Hall, J.C., **1994**. An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. *J. Biol. Rhyth. 9*, 189–216.

Konopka, R.J., Smith, R.F., Orr, D., **1991**. Characterization of *Andante*, a new *Drosophila* clock mutant, and its interactions with other clock mutants. *J. Neurogenet. 7*, 103–114.

Krishnan, B., Dryer, S.E., Hardin, P.E., **1999**. Circadian rhythms in olfactory responses of *Drosophila melanogaster. Nature (Lond.) 400*, 375–378.

Krishnan, B., Levine, J.D., Lynch, M.K., Dowse, H.B., Funes, P., *et al.*, **2001**. A new role for *cryptochrome* in a *Drosophila* circadian oscillator. *Nature (Lond.) 411*, 313–317.

Leak, R.K., Moore, R.Y., **2000**. Topographic organization of suprachiasmatic nucleus projection neurons. *J. Comp. Neurol. 433*, 312–334.

Lee, C., Bae, K., Edery, I., **1998**. The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. *Neuron 21*, 857–867.

Lee, C., Bae, K., Edery, I., **1999**. PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer, a basis for circadian transcription. *Mol. Cell. Biol. 19*, 5316–5325.

Lee, A., Li, W., Xu, K., Bogert, B.A., Su, K., *et al.*, **2003**. Control of dendritic development by the *Drosophila fragile X-related* gene involves the small GTPase Rac1. *Development 130*, 5543–5552.

Lee, C., Parikh, V., Itsukaichi, T., Bae, K., Edery, I., **1996**. Resetting the *Drosophila* clock by photic regulation of PER and a PER-TIM complex. *Science (Wash.) 271*, 1740–1744.

Leloup, J.C., Goldbeter, A., **1998**. A model for circadian rhythms in *Drosophila* incorporating the formation of a complex between the PER and TIM proteins. *J. Biol. Rhyth. 13*, 70–87.

Levine, J.D., Funes, P., Dowse, H.B., Hall, J.C., **2002a**. Advanced analysis of a *cryptochrome* mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. *BMC Neurosci. 3*, 5.

Levine, J.D., Funes, P., Dowse, H.B., Hall, J.C., **2002b**. Resetting the circadian clock by social experience in *Drosophila melanogaster. Science (Wash.) 298*, 2010–2012.

Levine, J.D., Funes, P., Dowse, H.B., Hall, J.C., **2002c**. Signal analysis of behavioral and molecular cycles. *BMC Neurosci. 3*, 1.

Levine, J.D., Sauman, I., Imbalzano, M., Reppert, S.M., Jackson, F.R., **1995**. Period protein from the giant silkmoth *Antheraea pernyi* functions as a circadian clock element in *Drosophila melanogaster. Neuron 15*, 147–157.

Lin, F.J., Song, W., Meyer-Bernstein, E., Naidoo, N., Sehgal, A., **2001**. Photic signaling by cryptochrome in the *Drosophila* circadian system. *Mol. Cell. Biol. 21*, 7287–7294.

Lin, J.M., Kilman, V.L., Keegan, K., Paddock, B., Emery-Le, M., *et al.*, **2002**. A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature (Lond.) 420*, 816–820.

Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T.M., *et al.*, **2002**. Influence of the *period*-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 99*, 9562–9567.

Liu, X., Lorenz, L., Yu, Q.N., Hall, J.C., Rosbash, M., **1988**. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster. Genes Devel. 2*, 228–238.

Liu, X., Zwiebel, L.J., Hinton, D., Benzer, S., Hall, J.C., *et al.*, **1992**. The *period* gene encodes a predominantly nuclear protein in adult *Drosophila*. *J. Neurosci. 12*, 2735–2744.

Long, J.C., Jenkins, G.I., **1998**. Involvement of plasma membrane redox activity and calcium homeostasis in the UV-B and UV-A/blue light induction of gene expression in *Arabidopsis*. *Plant Cell 10*, 2077–2086.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., *et al.*, **2000**. Positional syntenic cloning and functional characterization of the mammalian circadian mutation *tau*. *Science (Wash.) 288*, 483–492.

Lyons, L.C., Darlington, T.K., Hao, H., Houl, J., Kay, S.A., *et al.*, **2000**. Specific sequences outside the E-box are required for proper *per* expression and behavioral rescue. *J. Biol. Rhyth. 15*, 472–482.

Majercak, J., Kalderon, D., Edery, I., **1997**. *Drosophila melanogaster* deficient in protein kinase A manifests behavior-specific arrhythmia but normal clock function. *Mol. Cell. Biol. 17*, 5915–5922.

Malpel, S., Klarsfeld, A., Rouyer, F., **2002**. Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. *Development 129*, 1443–1453.

Marrus, S.B., Zeng, H., Rosbash, M., **1996**. Effect of constant light and circadian entrainment of per^{s} flies, evidence for light-mediated delay of the negative feedback loop in *Drosophila*. *Eur. Mol. Biol. Org. J. 15*, 6877–6886.

Martinek, S., Inonog, S., Manoukian, A.S., Young, M.W., **2001**. A role for the segment polarity gene *shaggy/GSK-3* in the *Drosophila* circadian clock. *Cell 105*, 769–779.

Matsumoto, A., Tomioka, K., Chiba, Y., Tanimura, T., **1999**. tim^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. *Mol. Cell. Biol. 19*, 4343–4354.

McDonald, M.J., Rosbash, M., **2001**. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell 107*, 567–578.

McDonald, M.J., Rosbash, M., Emery, P., **2001**. Wild-type circadian rhythmicity is dependent on closely spaced E boxes in the *Drosophila timeless* promoter. *Mol. Cell. Biol. 21*, 1207–1217.

McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., Riddiford, L.M., *et al.*, **1997**. Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron 19*, 813–823.

McNeil, G.P., Zhang, X., Genova, G., Jackson, F.R., **1998**. A molecular rhythm mediating circadian clock output in *Drosophila*. *Neuron 20*, 297–303.

Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., *et al.*, **2002**. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 15363–15368.

Michel, S., Geusz, M.E., Zaritsky, J.J., Block, G.D., **1993**. Circadian rhythm in membrane conductance expressed in isolated neurons. *Science (Wash.) 259*, 239–241.

Morales, J., Hiesinger, P.R., Schroeder, A.J., Kume, K., Verstreken, P., *et al.*, **2002**. *Drosophila* fragile X protein, DFXR, regulates neuronal morphology and function in the brain. *Neuron 34*, 961–972.

Myers, M.P., Wager-Smith, K., Wesley, C.S., Young, M.W., Sehgal, A., **1995**. Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. *Science (Wash.) 270*, 805–808.

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., Young, M.W., **1996**. Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science (Wash.) 271*, 1736–1740.

Myers, E.M., Yu, J., Sehgal, A., **2003**. Circadian control of eclosion. Interaction between a central and peripheral clock in *Drosophila melanogaster. Curr. Biol. 13*, 526–533.

Naidoo, N., Song, W., Hunter-Ensor, M., Sehgal, A., **1999**. A role for the proteasome in the light response of the timeless clock protein. *Science (Wash.) 285*, 1737–1741.

Nässel, D.R., Shiga, S., Mohrherr, C.J., Rao, K.R., **1993**. Pigment-dispersing hormone-like peptide in the nervous system of the flies *Phormia* and *Drosophila*, immunocytochemistry and partial characterization. *J. Comp. Neurol. 331*, 183–198.

Nässel, D.R., Shiga, S., Wikstrand, E.M., Rao, K.R., **1991**. Pigment-dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual system. *Cell Tiss. Res. 266*, 511–523.

Nery, L.E., Castrucci, A.M., **1997**. Pigment cell signaling for physiological color change. *Comp. Biochem. Physiol. A 118*, 1135–1144.

Newby, L.M., Jackson, F.R., **1996**. Regulation of a specific circadian clock output pathway by lark, a putative RNA-binding protein with repressor activity. *J. Neurobiol. 31*, 117–128.

Nitabach, M.N., Blau, J., Holmes, T.C., **2002**. Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. *Cell 109*, 485–495.

Ohira, T., Nagasawa, H., Aida, K., **2002**. Molecular cloning of cDNAs encoding two pigment-dispersing hormones and two corresponding genes from the Kuruma Prawn (*Penaeus japonicus*). *Mar. Biotechnol. (NY) 4*, 463–470.

Park, H.W., Kim, S.T., Sancar, A., Deisenhofer, J., **1995**. Crystal structure of DNA photolyase from *Escherichia coli*. *Science (Wash.) 268*, 1866–1872.

Park, J., **2002**. Downloading central clock information in *Drosophila*. *Mol. Neurobiol. 26*, 217–233.

Park, J.H., Hall, J.C., **1998**. Isolation and chronobiological analysis of a neuropeptide *pigment-dispersing factor* gene in *Drosophila melanogaster. J. Biol. Rhyth. 13*, 219–228.

Park, J.H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M., *et al.*, **2000**. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl Acad. Sci. USA 97*, 3608–3613.

Park, J.H., Schroeder, A.J., Helfrich-Förster, C., Jackson, F.R., Ewer, J., **2003**. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. *Development 130*, 2645–2656.

Park, S.K., Sedore, S.A., Cronmiller, C., Hirsh, J., **2000**. Type II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. *J. Biol. Chem. 275*, 20588–20596.

Park, Y., Kim, Y.J., Adams, M.E., **2002**. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. *Proc. Natl Acad. Sci. USA 99*, 11423–11428.

Pearn, M.T., Randall, L.L., Shortridge, R.D., Burg, M.G., Pak, W.L., **1995**. Molecular, biochemical, and electrophysiological characterization of *Drosophila norpA* mutants. *J. Biol. Chem. 271*, 4937–4945.

Pearn, M.T., Randall, L.L., Shortridge, R.D., Burg, M.G., Pak, W.L., **1996**. Molecular, biochemical, and electrophysiological characterization of *Drosophila norpA* mutants. *J. Biol. Chem. 271*, 4937–4945.

Peifer, M., Pai, L.M., Casey, M., **1994**. Phosphorylation of the *Drosophila* adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. *Dev. Biol. 16*, 543–556.

Peng, Y., Stoleru, D., Levine, J.D., Hall, J.C., Rosbash, M., **2003**. *Drosophila* free-running rhythms require intercellular communication. *PLoS Biological 1*, E13. Epub 2003 Sep 15.

Persson, M.G., Eklund, M.B., Dircksen, H., Muren, J.E., Nässel, D.R., **2001**. Pigment-dispersing factor in the locust abdominal ganglia may have roles as circulating neurohormone and central neuromodulator. *J. Neurobiol. 48*, 19–41.

Petri, B., Homberg, U., Loesel, R., Stengl, M., **2002**. Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol. 205*, 1459–1469.

Petri, B., Stengl, M., **1997**. Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci. 17*, 4087–4093.

Pittendrigh, C.S., **1974**. Circadian oscillations in cells and circadian organization of multicellular systems. In: Schmitt, F.O., Worden, F.G. (Eds.), The Neurosciences Third Study Program. MIT Press, Cambridge, MA, pp. 437–458.

Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A., **1997**. Independent photoreceptive circadian clocks throughout *Drosophila*. *Science (Wash.) 278*, 1632–1635.

Pollack, I., Hofbauer, A., **1991**. Histamine-like immunoreactivity in the visual system and brain of *Drosophila melanogaster*. *Cell Tiss. Res. 266*, 391–398.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., *et al*., **1998**. *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell 94*, 83–95.

Price, J.L., Dembinska, M.E., Young, M.W., Rosbash, M., **1995**. Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. *Eur. Mol. Biol. Org. J. 14*, 4044–4049.

Pyza, E., Meinertzhagen, I.A., **1996**. Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobe. *J. Comp. Physiol. A 178*, 33–45.

Pyza, E., Meinertzhagen, I.A., **1997**. Neurites of period-expressing PDH cells in the fly's optic lobe exhibit circadian oscillations in morphology. *Eur. J. Neurosci. 9*, 1784–1788.

Pyza, E., Meinertzhagen, I.A., **1999**. Daily rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*. *J. Neurobiol. 40*, 77–88.

Rachidi, M., Lopes, C., Benichou, J.-C., Rouyer, F., **1997**. Analysis of *period* circadian expression in the *Drosophila* head by *in situ* hybridization. *J. Neurogenet. 11*, 255–263.

Rao, K.R., Mohrherr, C.J., Riehm, J.P., Zahnow, C.A., Norton, S., *et al*., **1987**. Primary structure of an analog of crustacean pigment-dispersing hormone from the lubber grasshopper *Romalea microptera*. *J. Biol. Chem. 262*, 2672–2675.

Rao, K.R., Riehm, J.P., Zahnow, C.A., Kleinholz, L.H., Tarr, G.E., *et al*., **1985**. Characterization of a Pigment-Dispersing Hormone is eyestalks of the Fiddler crab *Uca pugilator*. *Proc. Natl Acad. Sci. USA 82*, 5319–5322.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., Taghert, P.H., **1999**. A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell 99*, 791–802.

Rensing, L., Ruoff, P., **2002**. Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiol. Int. 19*, 807–864.

Reppert, S.M., Tsai, T., Roca, A.L., Sauman, I., **1994**. Cloning of a structural and functional homolog of the circadian clock gene *period* from the giant silkmoth *Antheraea pernyi*. *Neuron 13*, 1167–1176.

Reyes, H., Reisz-Porszasz, S., Hankinson, O., **1992**. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science (Wash.) 256*, 1193–1195.

Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., **2002**. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science (Wash.) 298*, 172–175.

Riehm, J.P., Rao, K.R., **1982**. Structure-activity relationships of a pigment-dispersing crustacean neurohormone. *Peptides 3*, 643–647.

Riehm, J.P., Rao, K.R., Semmes, O.J., Jorenby, W.H., Hintz, M.F., *et al*., **1985**. C-terminal deletion analogs of a crustacean pigment-dispersing hormone. *Peptides 6*, 1051–1056.

Rosato, E., Codd, V., Mazzotta, G., Piccin, A., Zordan, M., *et al*., **2001**. Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. *Curr. Biol. 11*, 909–917.

Rothenfluh, A., Abodeely, M., Young, M.W., **2000a**. Isolation and analysis of six *timeless* alleles that cause short- or long-period circadian rhythms in *Drosophila*. *Genetics 156*, 665–675.

Rothenfluh, A., Abodeely, M., Young, M.W., **2000b**. Short-period mutations of *per* affect a *double-time*-

dependent step in the *Drosophila* circadian clock. *Curr. Biol. 10*, 1399–1402.

Rothenfluh, A., Young, M.W., Saez, L., **2000c**. A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron 26*, 505–514.

Rouyer, F., Rachidi, M., Pikielny, C., Rosbash, M., **1997**. A new gene encoding a putative transcription factor regulated by the *Drosophila* circadian clock. *Eur. Mol. Biol. Org. J. 16*, 3944–3954.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., *et al.*, **1996**. The *timSL* mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. *Neuron 17*, 921–929.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., *et al.*, **1998**. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell 93*, 805–814.

Saez, L., Young, M.W., **1988**. *In situ* localization of the per clock protein during development of *Drosophila melanogaster. Mol. Cell. Biol. 8*, 5378–5385.

Saez, L., Young, M.W., **1996**. Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless. *Neuron 17*, 911–920.

Sakanaka, C., Leong, P., Xu, L., Harrison, S.D., Williams, L.T., **1999**. Casein kinase iepsilon in the *wnt* pathway: regulation of beta-catenin function. *Proc. Natl Acad. Sci. USA 96*, 12548–12552.

Sancar, A., **2000**. CRYPTOCHROME, The second photoactive pigment in the eye and its role in circadian photoreception. *Annu. Rev. Biochem. 69*, 31–67.

Sarov-Blat, L., So, W.V., Liu, L., Rosbash, M., **2000**. The *Drosophila takeout* gene is a novel molecular link between circadian rhythms and feeding behavior. *Cell 101*, 647–656.

Sauman, I., Reppert, S.M., **1996**. Circadian clock neurons in the silkmoth *Antheraea pernyi*, novel mechanisms of Period protein regulation. *Neuron 17*, 889–900.

Schenck, A., Bardoni, B., Langmann, C., Harden, N., Mandel, J.L., *et al.*, **2003**. CYFIP/Sra-1 controls neuronal connectivity in *Drosophila* and links the Rac1 GTPase pathway to the fragile X protein. *Neuron 38*, 887–898.

Sehgal, A., Price, J.L., Man, B., Young, M.W., **1994**. Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science (Wash.) 263*, 1603–1606.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., *et al.*, **1995**. Rhythmic expression of *timeless*, a basis for promoting circadian cycles in *period* gene autoregulation. *Science (Wash.) 270*, 808–810.

Shafer, O.T., **2001**. Blind clocks reveal elusive light input pathway in *Drosophila*. *Trends Neurosci. 24*, 627–628.

Shafer, O.T., Rosbash, M., Truman, J.W., **2002**. Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of *Drosophila melanogaster. J. Neurosci. 22*, 5946–5954.

Shaw, P., **2003**. Awakening to the behavioral analysis of sleep in *Drosophila*. *J. Biol. Rhyth. 18*, 4–11.

Shaw, P.J., Cirelli, C., Greenspan, R.J., Tononi, G., **2000**. Correlates of sleep and waking in *Drosophila melanogaster. Science (Wash.) 287*, 1834–1837.

Shaw, P.J., Tononi, G., Greenspan, R.J., Robinson, D.F., **2002**. Stress response genes protect against lethal effects of sleep deprivation in *Drosophila*. *Nature (Lond.) 417*, 287–291.

Siegfried, E., Wilder, E.L., Perrimon, N., **1994**. Components of wingless signalling in *Drosophila*. *Nature (Lond.) 367*, 76–80.

Siwicki, K.K., Eastman, C., Petersen, G., Rosbash, M., Hall, J.C., **1988**. Antibodies to the period gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron 1*, 141–150.

Smith, R.F., Konopka, R.J., **1981**. Circadian clock phenotypes of chromosome aberrations with a breakpoint at the *per* locus. *Mol. Gen. Genet. 183*, 243–251.

So, W.V., Rosbash, M., **1997**. Post-transcriptional regulation contributes to *Drosophila clock* gene mRNA cycling. *EMBO J. 16*, 7146–7155.

So, W.V., Sarov-Blat, L., Kotarski, C.K., McDonald, M.J., Allada, R., *et al.*, **2000**. *takeout*, a novel *Drosophila* gene under circadian clock transcriptional regulation. *Mol. Cell. Biol. 20*, 6935–6944.

Stanewsky, R., **2003**. Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. *J. Neurobiol. 54*, 111–147.

Stanewsky, R., Jamison, C.F., Plautz, J.D., Kay, S.A., Hall, J.C., **1997**. Multiple circadian-regulated elements contribute to cycling *period* gene expression in *Drosophila*. *EMBO J. 16*, 5006–5018.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., *et al.*, **1998**. The *cryb* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell 95*, 681–692.

Steller, H., Fischbach, K.F., Rubin, G., **1987**. *disconnected,* a locus required for neuronal pathway formation in the visual system of *Drosophila*. *Cell 50*, 1139–1153.

Stempfl, T., Vogel, M., Szabo, G., Wulbeck, C., Liu, J., *et al.*, **2002**. Identification of circadian-clock-regulated enhancers and genes of *Drosophila melanogaster* by transposon mobilization and luciferase reporting of cyclical gene expression. *Genetics 160*, 571–593.

Suri, V., Hall, J.C., Rosbash, M., **2001**. Two novel *doubletime* mutants alter circadian properties and eliminate the delay between RNA and protein in *Drosophila*. *J. Neurosci. 20*, 7547–7555.

Suri, V., Lanjuin, A., Rosbash, M., **1999**. TIMELESS-dependent positive and negative autoregulation in the *Drosophila* circadian clock. *EMBO J. 18*, 675–686.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., O'Kane, C.J., **1995**. Targeted expression of tetanus toxin lighr chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron 14*, 341–351.

Taghert, P.H., **2001**. How does the circadian clock send timing information to the brain? *Sem. Cell. Devel. Biol. 12*, 329–341.

Taghert, P.H., Hewes, R.S., Park, J.H., O'Brien, M.A., Han, M., *et al.*, **2001**. Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in *Drosophila. J. Neurosci. 21*, 6673–6686.

Taghert, P.H., Roberts, M.E., Renn, S.C., Jacobs, P.S., **2000**. Metamorphosis of tangential visual system neurons in *Drosophila. Devel. Biol. 222*, 471–485.

Taghert, P.H., Veenstra, J.A., **2003**. Neuropeptide signaling in *Drosophila. Adv. Genet. 49*, 1–65.

Tamada, T., Kitadokoro, K., Higuchi, Y., Inaka, K., Yasui, A., *et al.*, **1997**. Crystal structure of DNA photolyase from *Anacystis nidulans. Nat. Struct. Biol. 4*, 887–891.

The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Z., *et al.*, **1997**. Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. *Science (Wash.) 276*, 791–794.

Thor, S., Thomas, J.B., **1997**. The *Drosophila islet* gene governs axon pathfinding and neurotransmitter identity. *Neuron 18*, 397–409.

Todo, T., **1999**. Functional diversity of the DNA photolyase/blue light receptor family. *Mutat. Res. 434*, 89–97.

Toma, D.P., White, K.P., Hirsch, J., Greenspan, R.J., **2002**. Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait. *Nature (Lond.) Genetics 31*, 349–353.

Truman, J.W., **1976**. Extraretinal photoreception in insects. *Photophysiology 23*, 215–225.

Truman, J.W., **1992**. The eclosion hormone system of insects. *Prog. Brain Res. 92*, 361–374.

Truman, J.W., Riddiford, L.M., **1970**. Neuroendocrine control of ecdysis in silkmoths. *Science (Wash.) 167*, 1624–1626.

Ueda, H.R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., *et al.*, **2002**. Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila. J. Biol. Chem. 277*, 14048–14052.

Van Gelder, R.N., **2002**. Tales from the crypt(ochromes). *J. Biol. Rhyth. 17*, 110–120.

Van Gelder, R.N., Herzog, E.D., Schwartz, W.J., Taghert, P.H., **2003**. Circadian rhythms, in the loop at last. *Science (Wash.) 300*, 1534–1535.

Van Gelder, R.N., von Zastrow, M.E., Yool, A., Dement, W.C., Barchas, J.D., *et al.*, **1995**. Extent and character of circadian gene expression in *Drosophila melanogaster*, identification of twenty oscillating mRNAs in the fly head. *Curr. Biol. 5*, 1424–1436.

Veleri, S., Brandes, C., Helfrich-Förster, C., Hall, J.C., Stanewsky, R., **2003**. A self-sustaining, light-entrainable circadian oscillator in the *Drosophila* brain. *Curr. Biol. 13*, 1758–1767.

Volkner, M., Lenz-Bohme, B., Betz, H., Schmitt, B., **2000**. Novel CNS glutamate receptor subunit genes of *Drosophila melanogaster. J. Neurochem. 75*, 1791–1799.

Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., Young, M.W., **1994**. Block in nuclear localization of period protein by a second clock mutation, *timeless. Science (Wash.) 263*, 1606–1609.

Vosshall, L.B., Young, M.W., **1995**. Circadian rhythms in *Drosophila* can be driven by *period* expression in a restricted group of central brain cells. *Neuron 15*, 345–360.

Wegener, C., Hamasaka, Y., Nässel, D.R., **2004**. Acetylcholine increases intracellular Ca^{2+} via nicotinic receptors in cultured PDF-containing clock neurons of *Drosophila. J. Neurophysiol. 91*, 912–923.

Wheeler, D.A., Hamblen-Coyle, M.J., Dushay, M.S., Hall, J.C., **1993**. Behavior in light–dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. *J. Biol. Rhythms 8*, 67–94.

Williams, J.A., Su, H.S., Bernards, A., Field, J., Sehgal, A., **2001**. A circadian output in *Drosophila* mediated by neurofibromatosis-1 and Ras/MAPK. *Science (Wash.) 293*, 2251–2256.

Wise, S., Davis, N.T., Tyndale, E., Noveral, J., Folwell, M.G., *et al.*, **2002**. Neuroanatomical studies of *period* gene expression in the hawkmoth, *Manduca sexta. J. Comp. Neurol. 447*, 366–380.

Yang, Z., Emerson, M., Su, H.S., Sehgal, A., **1998**. Response of the timeless protein to light correlates with behavioral entrainment and suggests a nonvisual pathway for circadian photoreception. *Neuron 21*, 215–223.

Yang, Z., Sehgal, A., **2001**. Role of molecular oscillations in generating behavioral rhythms in *Drosophila. Neuron 29*, 453–467.

Yasuyama, K., Meinertzhagen, I.A., **1999**. Extraretinal photoreceptors at the compound eye's posterior margin in *Drosophila melanogaster. J. Comp. Neurol. 412*, 193–202.

Young, M.W., Kay, S.A., **2001**. Time zones: a comparative genetics of circadian clocks. *Nat. Rev. Genet. 2*, 702–715.

Yu, Q., Jacquier, A.C., Citri, Y., Hamblen, M., Hall, J.C., *et al.*, **1987**. Molecular mapping of point mutations in the *period* gene that stop or speed up biological clocks in *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 84*, 784–788.

Zavodska, R., Sauman, I., Sehnal, F., **2003**. Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *J. Biol. Rhyth. 18*, 106–122.

Zeng, H., Hardin, P.E., Rosbash, M., **1994**. Constitutive overexpression of the *Drosophila* period protein inhibits period mRNA cycling. *Eur. Mol. Biol. Org. J. 13*, 3590–3598.

Zeng, H., Qian, Z., Myers, M.P., Rosbash, M., **1996**. A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature (Lond.) 380*, 129–135.

Zerr, D.M., Hall, J.C., Rosbash, M., Siwicki, K.K., **1990**. Circadian rhythms of period protein immunoreactivity in the CNS and the visual system of *Drosophila. J. Neurosci. 10*, 2749–2762.

Zhang, X., McNeil, G.P., Hilderbrand-Chae, M.J., Franklin, T.M., Schroeder, A.J., *et al.*, **2000**. Circadian regulation of the lark RNA-binding protein within identifiable neurosecretory cells. *J. Neurobiol. 45*, 14–29.

Zhang, Y.Q., Bailey, A.M., Matthies, H.J., Renden, R.B., Smith, M.A., *et al.*, **2001**. *Drosophila fragile X-related* gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell 107*, 591–603.

Zhao, J., Kilman, V.L., Keegan, K.P., Peng, Y., Emery, P., *et al.*, **2003**. *Drosophila* clock can generate ectopic circadian clocks. *Cell 113*, 755–766.

Zilian, O., Frei, E., Burke, R., Brentrup, D., Gutjahr, T., *et al.*, **1999**. *double-time* is identical to *discs overgrown*, which is required for cell survival, proliferation and growth arrest in *Drosophila* imaginal discs. *Development 126*, 5409–5420.

4.12 Insect Transposable Elements

Z Tu, Virginia Tech, Blacksburg, VA, USA

4.12.1. Introduction

More than half a century ago, Barbara McClintock's observation of unstable mutations in maize led to the discovery of two mobile genetic elements, *Activator* (*Ac*) and *Dissociator* (*Ds*) (McClintock, 1948, 1950). The discovery of these mobile segments of DNA, later named as transposable elements (TEs), set forth the revolutionary concept of a fluid and dynamic genome. Five decades later as biology is entering the genomic era, the tremendous diversity of TEs and their potential impact are just being appreciated.

Being mobile, TEs have the ability to replicate and spread in the genome as primarily "selfish" genetic units (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). They tend to occupy significant portions of the eukaryotic genome. For example, at least 46% of the human genome (Lander *et al*., 2001) and 16% of the euchromatic portion of the newly reported malaria mosquito genome (Holt *et al*., 2002) are TE-derived sequences. The relative abundance and diversity of TEs have contributed to the differences in the structure and size of eukaryotic genomes (Kidwell, 2002). Recent evidence suggests

that the "selfish" property may have enabled TEs to provide the genome with potent agents to generate genetic and genomic plasticity (Kidwell and Lisch, 2000). For example, TEs may have reshaped the human genome by ectopic rearrangements, by creating new genes, and by modifying and shuffling existing genes (Lander *et al*., 2001). In some cases, TEs had been co-opted to perform critical functions in the biology of their host. One well-documented example is the generation of the extensive array of immunoglobulins and T cell receptors by V(D)J recombination, which is believed to have evolved from an ancient transposition system (Gellert, 2002). Another example is the maintenance of telomeric structures in *Drosophila melanogaster* by site-specific insertions of two TEs (Pardue and DeBaryshe, 2002). An emerging view is that genomes are dynamic systems, within which diverse TEs evolve. The "selfish" TEs could evolve a wide spectrum of relationships with their hosts, ranging from "junk parasites" to "molecular symbionts" (Brookfield, 1995; Kidwell and Lisch, 2000).

The intricate dynamic between TEs and their host genomes is further complicated by the fact that some TEs are capable of crossing species barriers to spread in a new genome. Such a process is referred to as horizontal (or lateral) transfer, which is distinct from the vertical transmission of genetic material from ancestral species/organisms to their offspring. Horizontal transfer may be an important part of the life cycle of some TEs and it may contribute to their continued success during evolution (Silva *et al*., 2004).

From an applied perspective, TEs have been used as tools to genetically manipulate cells/organisms, taking advantage of their ability to integrate cognate DNA in the genome. A well-known example is the transformation system derived from the *D. melanogaster P* transposable element, which has been instrumental to our understanding of this model genetic organism by providing transformation and mutagenesis tools (see **Chapter 4.13**). In addition, some TEs have been used as genetic markers for mapping and population studies, taking advantage of their dimorphic insertion states (presence and absence of an insertion) and their interspersed distribution in the genome. For example, the human *Alu* elements have been shown to be useful population genetic markers (Batzer *et al*., 1994; Batzer and Deininger, 2002; Salem *et al*., 2003). The presence and absence of insertions of short interspersed TEs at different genomic loci have also been used as molecular systematics markers to trace the explosive speciation of the cichlid fishes and other vertebrates (Shedlock and Okada, 2000; Terai *et al*., 2003).

The focus of this chapter is on recent advances in the study of insect TEs. A brief introduction on TE classification and transposition mechanisms will be provided, followed by sections that describe the current approaches to study insect TEs and sections that highlight the diversity and evolutionary dynamics of TEs in insect genomes. Applications of TEs in genetic and molecular analysis of insects will be discussed in the end. Readers may consult recent reviews (Silva *et al*., 2004) (see **Chapter 4.13**) and the second edition of a book on mobile DNA (Craig *et al*., 2002) for details on related topics.

4.12.2. Classification and Transposition Mechanisms of Eukaryotic TEs

TEs can be categorized as Class I RNA-mediated or Class II DNA-mediated elements according to their transposition mechanisms (Finnegan, 1992). The transposition of RNA-mediated TEs involves a reverse transcription step, which generates cDNA from RNA molecules (Eickbush and Malik, 2002). The cDNA molecules are integrated in the genome, allowing replicative amplification. The transposition of DNA-mediated elements is directly from DNA to DNA and does not involve an RNA intermediate (Craig, 2002). In most cases, both classes of TEs will create target site duplication (TSD) upon their insertion in the genome (**Figure 1**). Both classes can be further categorized into different groups and all groups of TEs discussed here have been found in various species of insects. There have been several recent reviews on different groups of TEs in both classes (Deininger and Roy-Engel, 2002; Eickbush and Malik, 2002; Feschotte *et al*., 2002; Robertson, 2002).

4.12.2.1. Class I RNA-Mediated TEs

RNA-mediated TEs include long terminal repeat (LTR) retrotransposons, non-LTR retrotransposons, and short interspersed repetitive/nuclear elements (SINEs). Non-LTR retrotransposons are also referred to as retroposons or long interspersed repetitive/nuclear elements (LINEs). The structural features of the three groups of RNA-mediated TEs are illustrated in **Figure 2** using representatives from different insects. All RNA-mediated TEs produce RNA transcripts that are reverse transcribed into cDNA to be integrated in the genome (Eickbush and Malik, 2002). Detailed mechanisms used by LTR and non-LTR retrotransposons are elegantly described in two recent reviews (Eickbush, 2002; Voytas and Boeke, 2002).

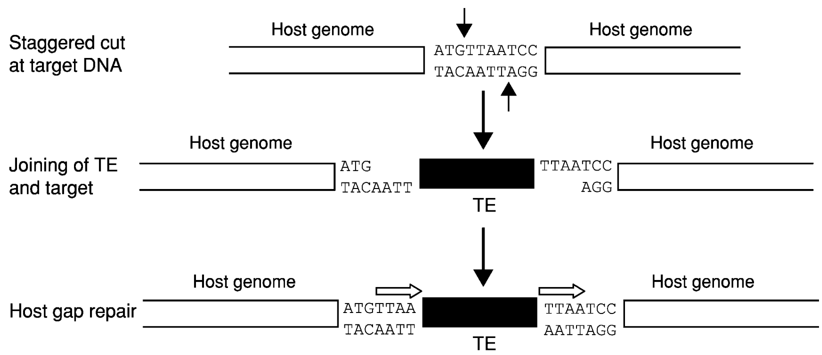


Figure 1 Mechanism of generating target site duplication (TSD). The TSDs, which are marked by open arrows, are not part of the TE sequence. They are target sequences duplicated upon a TE insertion. Most TEs create TSDs although the *Helitron* transposons and some non-LTR retrotransposons do not. See recent reviews (Kapitonov and Jurka, 2001; Craig, 2002; Eickbush and Malik, 2002) for illustrations of the transposition mechanisms of different types of TEs.

4.12.2.1.1. LTR retrotransposons LTR retrotransposons transpose through a mechanism much like that used by retroviruses. The LTRs in the LTR retrotransposons are generally 200 to 500 bp long and are involved in all aspects of their life cycle that includes providing promoter sequences and transcription termination signals (Eickbush and Malik, 2002). As shown in **Figure 2**, LTR retrotransposons encode a *pol*(polymerase)-like protein that contains reverse transcriptase (RT), ribonuclease H (RNase H), protease (PR), and integrase (IN) domains that are important for their retrotransposition. The RT domain performs the key function of reverse transcription and its sequence has been used for phylogenetic classification of LTR retrotransposons into four clades including Ty1/*copia*, Ty3/*gypsy*, BEL, and DIRS (Eickbush and Malik, 2002). The IN domain is responsible for inserting the cDNA copy into the host genome. In addition to the *pol*-like protein, LTR retrotransposons encode an additional protein related to the retroviral *gag* (group-associated antigene or group-specific antigen) protein that binds nucleic acid or forms the nucleocapsid shell. Some LTR retrotransposons also have an *env*(envelope)-like fragment that encodes a transmembrane receptor-binding protein that allows the transmission of retroviruses. Some of the LTR retrotransposons that encode an *env* protein are in fact retroviruses (Eickbush and Malik, 2002).

4.12.2.1.2. Non-LTR retrotransposons Non-LTR retrotransposons, or LINEs, or retroposons are generally 3 to 8 kb long and have been found in virtually all eukaryotes studied. Like the LTR retrotransposons, most non-LTR retrotransposons also have a *pol*-like protein that includes a RT domain that is essential for their retrotransposition. The RT domain has been used for phylogenetic classification of non-LTR retrotransposons into 17 clades, most of which probably date back to the Precambrian era, approximately 600 million years ago (Malik *et al*., 1999; Eickbush and Malik, 2002; Biedler and Tu, 2003). Some elements also have an RNase H and/or AP endonuclease (APE) domain encoded in the *pol*-like open reading frame (ORF). In addition to the *pol*-like protein, many non-LTR retrotransposons encode a protein that is related to the retroviral *gag* protein. Studies of a *gag*-like protein from L1 retrotransposon in mice show that it acts as a nucleic acid chaperone (Martin and Bushman, 2001). Other typical structural characteristics found in various non-LTR retrotransposons are internal Pol II promoters and 3′ ends containing AATAAA polyadenylation signals, poly(A) tails, or simple tandem repeats. Target primed reverse transcription has been proposed as the mechanism of retrotransposition for R2 of *Bombyx mori* and may be generally true for all non-LTR elements (Luan *et al*., 1993; Eickbush, 2002). Because they transpose by target primed reverse transcription, some non-LTR retrotransposons could rely rather heavily on host DNA repair mechanisms, and this relationship with the host may give non-LTR retrotransposons some flexibility with regard to the domains required in an autonomous element (Eickbush and Malik, 2002). Some non-LTR retrotransposons such as R2 are site-specific because their endonucleases make precise cleavage at specific targets (Xiong and Eickbush, 1988; Eickbush, 2002).

4.12.2.1.3. SINEs SINEs are generally between 100 and 500 bp long and they do not have any coding potential. SINEs may have been borrowing the

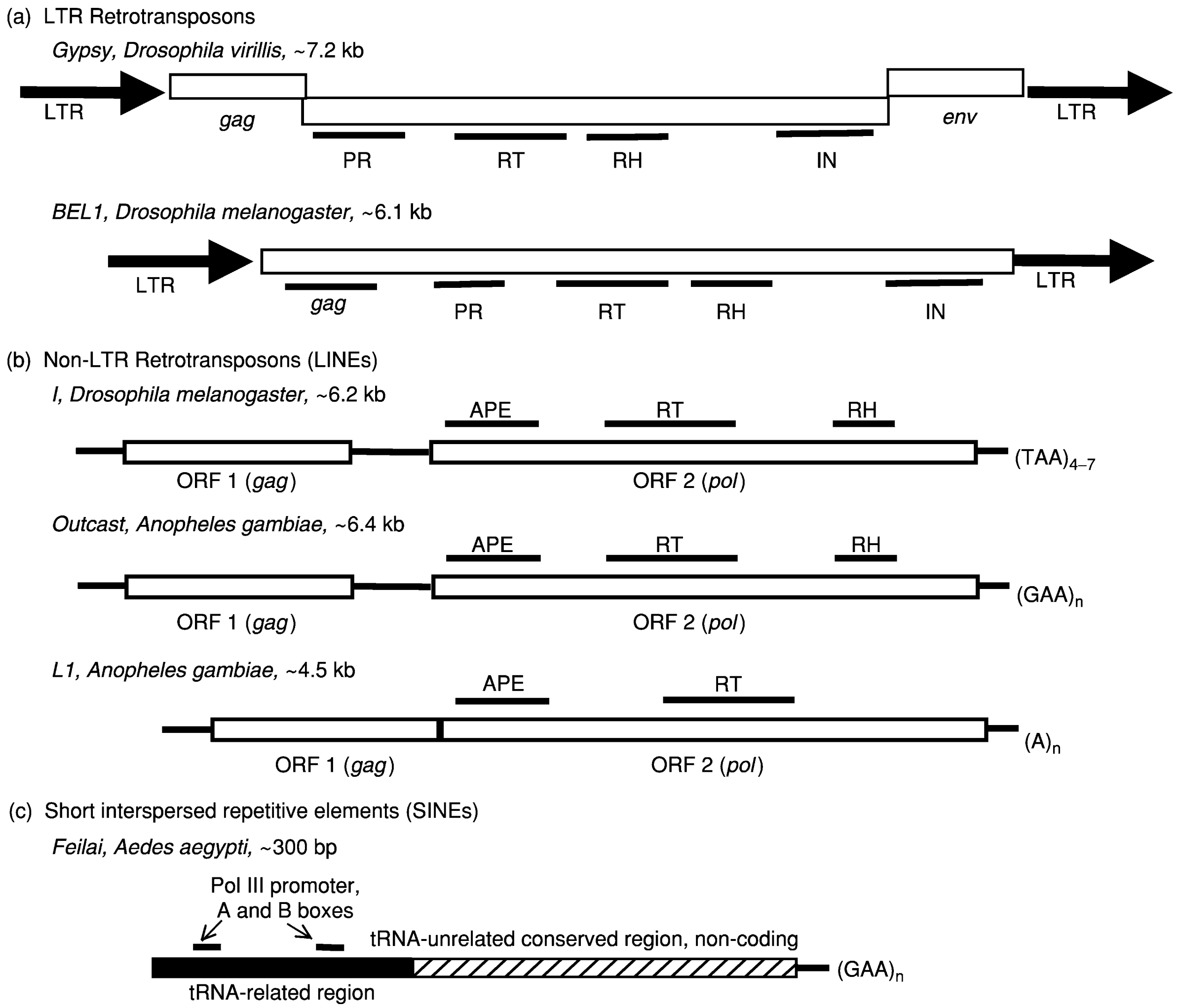


Figure 2 Structural characteristics of representative Class I RNA-mediated TEs in insects. Representatives are shown from three major groups: (a) long terminal repeat (LTR) retrotransposons, (b) non-LTR retrotransposons, and (c) short interspersed repetitive elements (SINEs). The name of each representative element, its host species, and its approximate length are shown as the heading. Open reading frames (ORFs) are shown as open boxes. The elements are not drawn to scale. References for information on these RNA-mediated elements are the following. *BEL1*, Davis and Judd (1995); *Feilai*, Tu (1999); *Gypsy*, Mizrokhi and Mazo (1991); *I*, Fawcett *et al.* (1986); *L1*, Biedler and Tu (2003); *Outcast*, Biedler and Tu (2003). *env*, envelope protein; *gag*, group-associated antigene or group-specific antigen; *pol*, polymerase-like protein; APE, apurinic-apyrimidinic endonuclease; IN, integrase; LINEs, long interspersed repetitive elements; LTR, long terminal repeat; PR, protease; RH, RNase H; RT, reverse transcriptase.

retrotransposition machinery from autonomous non-LTR retrotransposons, which may be facilitated by similar sequences or structures at the 3′ ends of a SINE and its “partner” non-LTR retrotransposon (Ohshima *et al.*, 1996; Okada and Hamada, 1997). Unlike non-LTR retrotransposons that use internal Pol II promoters, SINE transcription is directed from their own Pol III promoters that are similar to those found in small RNA genes. SINEs can be further divided into three groups based on similarities of their 5′ sequences to different types of small RNA genes. Elements such as the primate *Alu* family share sequence similarities with 7SL RNA (Jurka, 1995) while most other SINEs belong to a different group that shares sequence similarities to tRNA molecules (Adams *et al.*, 1986; Okada, 1991; Tu, 1999). Recently, a new group of SINEs named *SINE3* have been discovered in the zebrafish genome, which share similarities to 5S rRNA (Kapitonov and Jurka, 2003a). Some non-LTR retrotransposons tend to generate truncated copies due to incomplete reverse transcription during cDNA synthesis. Although these short copies of RNA-mediated TEs are also called SINEs (Malik and Eickbush, 1998), they should not be confused with the true SINEs that use Pol III promoters.

4.12.2.2. Class II DNA-Mediated TEs

DNA-mediated TEs include cut-and-paste DNA transposons (**Figure 3**), miniature inverted-repeat TEs (MITEs) (**Figure 4**), and a recently discovered group called *Helitrons* (Kapitonov and Jurka, 2001, 2003b). Their transposition is directly from DNA to DNA, and does not involve an RNA intermediate (Craig, 2002).

4.12.2.2.1. Cut-and-paste DNA transposons DNA transposons such as *P*, *hobo*, and *mariner* are usually characterized by 10–200 bp terminal inverted repeats (TIRs) flanking one or more ORFs that encode a transposase. They usually transpose by a cut-and-paste mechanism and their copy number can be increased through a repair mechanism (Finnegan, 1992; Craig, 2002). As shown in **Figure 3**, cut-and-paste DNA transposons can be subdivided into several families or superfamiles according to their transposase sequences and these families/superfamilies are also characterized by TSDs of specific sequence or length. The families/superfamilies that have been found in insects include *IS630-Tc1-mariner, hAT, piggyBac, PIF/Harbinger, P,* and *Transib* (Shao and Tu, 2001; Robertson, 2002; Kapitonov and Jurka, 2003b).

4.12.2.2.2. MITEs MITEs are widely distributed in plants, vertebrates, and invertebrates (Oosumi *et al.*, 1995; Wessler *et al.*, 1995; Smit and Riggs, 1996; Tu, 1997, 2001a). Most MITEs share common structural characteristics such as TIRs, small size, lack of coding potential, AT richness, and the potential to form stable secondary structures (Wessler *et al.*, 1995). MITEs may have been "borrowing" the transposition machinery of autonomous DNA transposons by taking advantage of shared TIRs (MacRae and Clegg, 1992; Feschotte and Mouches, 2000b; Zhang *et al.*, 2001). However, an alternative hypothesis suggests that they may transpose by a hairpin DNA intermediate produced from the folding back of single-stranded DNA during replication, which may better explain how MITEs could achieve immensely high copy numbers in some genomes (Izsvak *et al.*, 1999). The evolutionary origin of MITEs is still not clear (see Section 4.12.4.3.3). One obvious source of MITEs would be internal deleted autonomous DNA transposons (Feschotte and Mouches, 2000b). In this case, MITEs are basically nonautonomous deletion derivatives of DNA transposons. Recent studies show that the similarities between many MITEs and their putative autonomous partners are restricted to the TIRs (Feschotte *et al.*, 2003) (**Figure 4**). Although subsequent loss of autonomous partners in the genome remains a possible explanation for the lack of internal sequence similarity between MITEs and autonomous DNA transposons, two other explanations are perhaps more plausible. First, MITEs could originate *de novo* from chance mutation or recombination events resulting in the association of TIRs flanking unrelated segments of DNA (MacRae and Clegg, 1992; Tu, 2000; Feschotte *et al.*, 2003). Alternatively, MITEs could originate from abortive gap-repair following the transposition of DNA transposons, which has been shown to occasionally introduce transposon-unrelated sequences (Rubin and Levy, 1997).

4.12.2.2.3. *Helitrons*: the rolling-circle DNA transposons *Helitrons* and related transposons have recently been discovered in insects and plants, which appear to use a rolling-circle mechanism of transposition (Le *et al.*, 2000; Kapitonov and Jurka, 2001; 2003b). Instead of cut-and-paste transposase, *Helitrons* encode proteins similar to helicase, ssDNA-binding protein, and replication initiation protein. These proteins facilitate the rolling-circle replication of *Helitrons*, a mechanism previously described for the bacterial *IS91* transposons (Garcillan-Barcia *et al.*, 2002).

4.12.2.3. Related Topics

4.12.2.3.1. *Foldback* elements *Drosophila Foldback* elements are characterized by very long inverted repeats (Truett *et al.*, 1981). It is not known how *Foldback* elements transpose, although the presence of long inverted repeats indicates a possible DNA-mediated mechanism. Some researchers group *Foldback* elements as a distinct class, namely Class III (Kaminker *et al.*, 2002).

4.12.2.3.2. What is a family? Before we move ahead with discussions on the discovery and diversity of insect TEs in the next two sections, it may be helpful to clarify the use of the term "family" in the context of TEs. The term "family" is often used to refer to a group of related TEs in diverse organisms that usually share conserved amino acid sequences in their transposase or RT and one such example is the *mariner* family. A TE also consists of many copies generated by transposition events in a genome, and therefore these related copies are sometimes also referred to as a family. Some families consist of multiple distinct groups that are subdivided into subfamilies. Obviously, relatedness is a relative concept in evolution and thus a working definition is needed in each case until a universal family definition is developed.

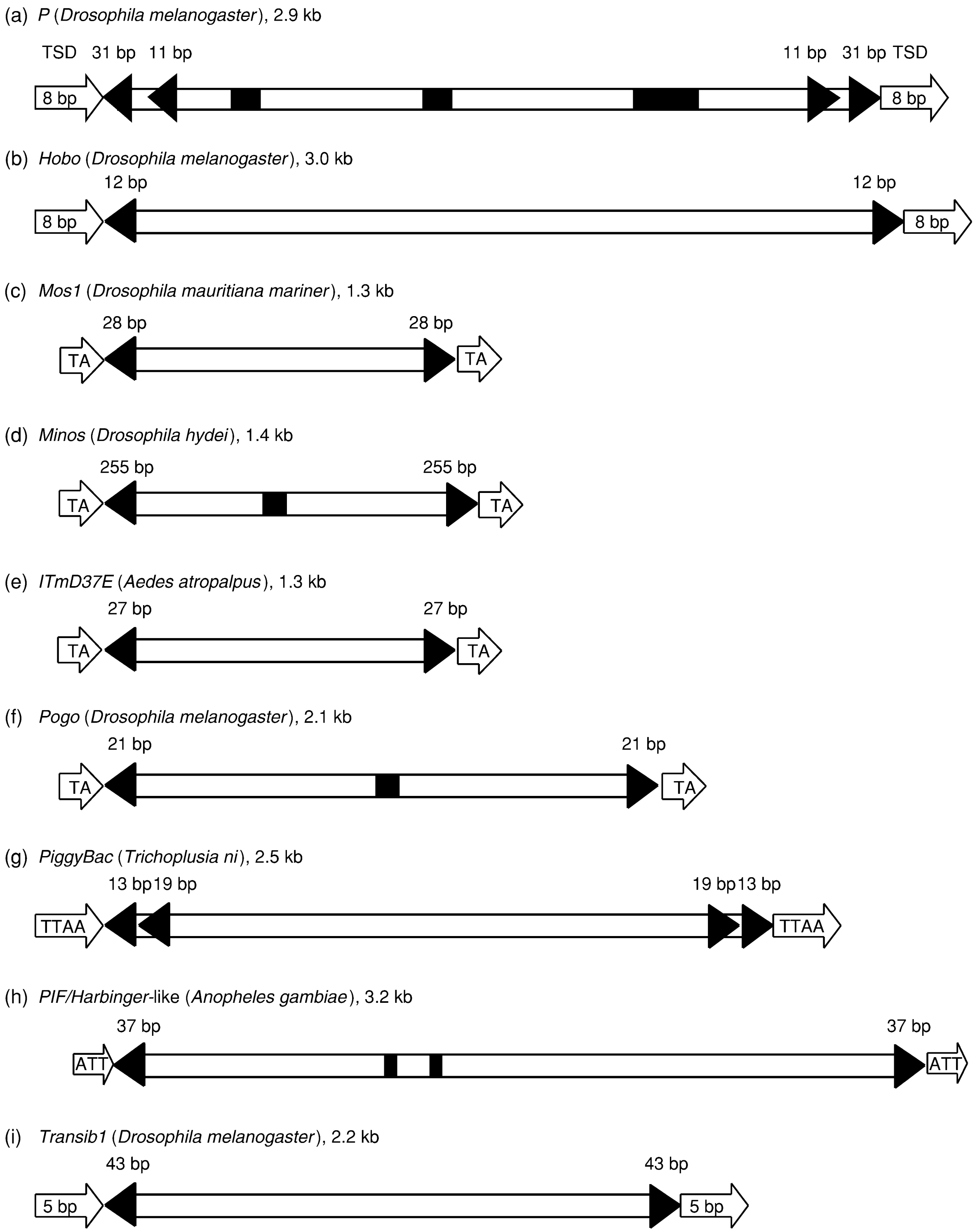


Figure 2 Structural characteristics of representative cut-and-paste DNA transposons in insects. The name of each representative transposon, its host species, and its approximate length are shown as the heading of each panel. Open arrows indicate target site duplications (TSDs). Filled triangles indicate terminal and subterminal inverted repeats. The lengths of these inverted repeats are marked. Exons are shown as open boxes and introns are shown as filled black boxes. The 5′ and 3′ untranslated regions are not shown. The elements are not drawn to scale. References for information on these transposons are the following: *P*, Engels (1989) and Rio (2002); *hobo*, Streck *et al.* (1986); *mariner Mos1*, Jacobson *et al.* (1986); *Minos*, Franz and Savakis (1991); *ITmD37E*, Shao and Tu (2001); *Pogo*, Tudor *et al.* (1992) and Feschotte and Mouches (2000b); *piggyBac*, Cary *et al.* (1989); *PIF/Harbinger*-like, Biedler *et al.* unpublished data; *Transib1*, Kapitonov and Jurka (2003b).

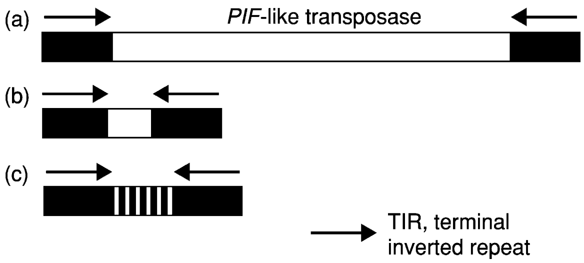


Figure 4 Relationship between *PIF/Harbinger*-like DNA transposons and related MITEs in *Anopheles gambiae*. (a) Structural features of *PIF/Harbinger*-like DNA transposons. There are multiple *PIF/Harbinger*-like DNA transposon families in *A. gambiae*, all of which have the characteristic AT-rich 3 bp TSDs (Biedler *et al.*, unpublished data). Although the TIRs are highly conserved between different copies of a family, they are variable between different *PIF/Harbinger*-like transposon families. (b) Structural features of deletion derivatives of *PIF/Harbinger*-like DNA transposons. Only two elements are apparent deletion derivatives of two different *PIF/Harbinger*-like transposons. (c) Structural features of *PIF/Harbinger*-related MITEs. These putative MITEs share similar TSDs and TIRs with *PIF/Harbinger*-like DNA transposons, although there are no internal sequence similarities between them. Twenty families of *PIF/Harbinger*-realted MITEs belong to this category. Note that the term ''family'' is used here to refer to a group of similar copies.

4.12.3. Methods to Uncover and Characterize Insect TEs

4.12.3.1. Early Discoveries and General Criteria

Before the availability of the large amount of genomic sequence data, TEs were often discovered by serendipitous observations during genetic experiments. As described above (see Section 4.12.1), McClintock's observation of the unstable mutations in maize led to the discovery of two mobile genetic elements *Ac* and *Ds*, although the molecular characterization of these elements came many years later (review: Fedoroff, 1989). Similarly, the observation of an unstable *white-peach* eye-color mutation in *Drosophila mauritiana* led to the discovery of the *mariner* transposon (Hartl, 1989). The *piggyBac* transposon was discovered as an insertion in a baculovirus after passage through a cell line of the cabbage looper *Trichoplusia ni* (review: Fraser, 2000). In a slightly different vein, the *D. melanogaster P* and *I* elements were discovered because of their association with a genetic phenomenon called hybrid dysgenesis, which refers to a group of abnormal traits including high mutation rates and sterility in crosses of certain strains (Kidwell, 1977; Finnegan, 1989). The genetic mutations described above, albeit rare, tend to identify active transposition events that resulted from active TEs in the genome.

The repetitive nature of TEs can also be used for their discovery and isolation, although not all repetitive elements are TEs. When DNA sequences are available, TEs can be identified on the basis of either similarity to known TEs, or common structural characteristics. In some cases, evidence for past TE insertion events could be identified on the basis of sequence analysis, which further supports the mobile history of a particular element. Although the criteria and methods described in this section are not unique to insects, it may be necessary to visit this topic here because of the lack of a systematic review on these issues and because of the growing interests in TE analysis in the current genomic environment.

4.12.3.2. Experimental Methods to Isolate and Characterize TEs

Several experimental methods have been used to discover TEs on the basis of their repetitive nature. Although relatively straightforward, these methods may not clearly distinguish between TEs and other repetitive sequences in the genome. In other words, the repeats discovered using these methods are not always TEs. One way to discover repeats in the genome is to isolate visible bands in an agarose gel running a sample of restriction enzyme-digested genomic DNA. This is based on the assumption that only highly reiterated sequences containing two or more conserved recognition sites for the restriction enzyme will produce a visible band among the smear of digested genomic DNA, and the bands can be cut out from the gel and purified for cloning and sequencing. Another approach to search for repeats is to screen a genomic library using labeled genomic DNA as a probe. This approach can be used effectively to identify abundant or highly repetitive sequences in the genome, which is based on the principle that only the repetitive fraction of the genome will produce a sufficient amount of labeled fragments that will generate hybridization signals during the screening (Gale, 1987; Cockburn and Mitchell, 1989). A third method is to use *Cot* analysis to help identify repetitive sequences in the genome, which is based on DNA reassociation kinetics (Adams *et al.*, 1986; Peterson *et al.*, 2002). For example, *Cot* analysis of genomic DNA can be performed to isolate the moderately repetitive portion of the genome that tends to contain TEs, and one can construct a subgenomic library using this fraction of genomic DNA to search for potential TEs. Several methods can be used to identify and isolate TEs on the basis of information derived from related TEs. For example, homologous TE probes may be used in Southern blotting and genomic library

screening experiments to identify related TEs. Polymerase chain reaction (PCR) analysis using primers that are conserved between related TEs can also be used to isolate different members of a TE family.

4.12.3.3. Computational Approaches to Discover and Analyze TEs

Insect science is rapidly entering a new era as demonstrated by the publication of the *D. melanogaster* genome sequence (Adams *et al*., 2000), the report of the genome assembly of the African malaria mosquito *Anopheles gambiae* (Holt *et al*., 2002), and the recent progress in genome projects of a number of other insects including the yellow fever mosquito *Aedes aegypti*, the honeybee *Apis mellifera*, the silkworm *Bombyx mori*, and the tobacco budworm *Heliothis virescens* (Kaufman *et al*., 2002; Transgenesis and Genomics of Invertebrate Organisms, 2003). This genome revolution is producing an ever-expanding sea of data that can be explored using a bioinformatics approach to identify interspersed TEs. As described below, a few new tools have been developed which represent a shift from merely masking TEs (e.g., RepeatMasker; review: Jurka, 2000) to the discovery, annotation, and genomic analysis of TEs. The use of bioinformatics tools provides great advantages by allowing analysis of TEs in the entire genome and by allowing quick surveys of a large number of TE families to identify the most promising candidates for discovering active TEs (see Section 4.12.5) and for population analysis (see Section 4.12.9). It should be noted that these approaches are not limited to fully sequenced genomes. Because of the repetitive nature of TEs, sequences from a small fraction of a genome tend to contain a large number of TEs that may be discovered using the bioinformatics approaches described here. Of course, greater numbers of sequences and longer assembly would be beneficial in analyzing low copy number or long TE sequences. It should also be mentioned that no high-end computing facilities are required for the majority of these bioinformatics programs.

4.12.3.3.1. Homology-dependent approaches Searching for TEs in a genome on the basis of similarities to known elements discovered in different species is relatively straightforward. However, given the diversity and abundance of TEs, systematic computational approaches are necessary for efficient and comprehensive analysis. One such program was reported that uses profile hidden Markov models to find all sequences matching the full-length RT with the conserved FYXDD motif common to all reverse transcriptases (Berezikov *et al*., 2000). A BLAST-based systematic approach to simultaneously identify and classify TEs is developed recently (**Figure 5**). This approach incorporates multiquery BLAST (Altschul *et al*., 1997) and a few computer program modules (freely available at jaketu.biochem.vt.edu) that organize BLAST output, retrieve sequence fragment, and mask database for identified TEs. The method was successfully used to discover and characterize non-LTR retrotransposons in the *A. gambiae* genome assembly (Biedler and Tu, 2003). The strategy is explained using the example of a representative non-LTR retrotransposon as shown in **Figure 5a**. The potentially comprehensive nature of this approach was demonstrated as two new clades were identified (Biedler and Tu, 2003) (see Section 4.12.4). The inclusive nature of this approach was further indicated when observed that non-LTRs across all existing clades were identified using the reiterative approach with a single *D. melanogaster* representative in the *Jockey* clade as the starting query. The reiterative feature of the strategy described in this study provides an opportunity for further automation by linking the modules described in **Figure 5a**. An early version of a fully automated program was tested, which was named TEpipe, to identify all non-LTR retrotransposon families in the *A. gambiae* genome. Preliminary results showed that 102 of the 104 non-LTR retrotransposon families were identified in one run of the program which takes less than 2 h on a Linux workstation. The robustness of the family classification assigned by the program was confirmed using a few independent tests. Alignment of the nucleotide sequences plus flanking genomic sequence of each family was performed with ClustalW (Thompson *et al*., 1994) to determine transposon boundaries, full-length elements, and TSDs (**Figure 5b**). The alignment process has recently been automated. The TEpipe approach should work for any TE with coding capacity in any genome.

4.12.3.3.2. Homology-independent approaches A suite of computer programs has been developed recently to search large databases rapidly for sequences with characteristics of MITEs (Tu, 2001a). The key program, FINDMITE1, searches the database for inverted repeats flanked by user-defined direct repeats within a specified distance range (**Figure 6**). The program uses the idea of the Knuth–Morris–Pratt string-matching algorithm (Tu, 2001a) to speed up the pattern match shifts. FINDMITE1 was used to uncover eight novel families of MITEs in *A. gambiae* (Tu, 2001a). Improvements have been made so that the new version of

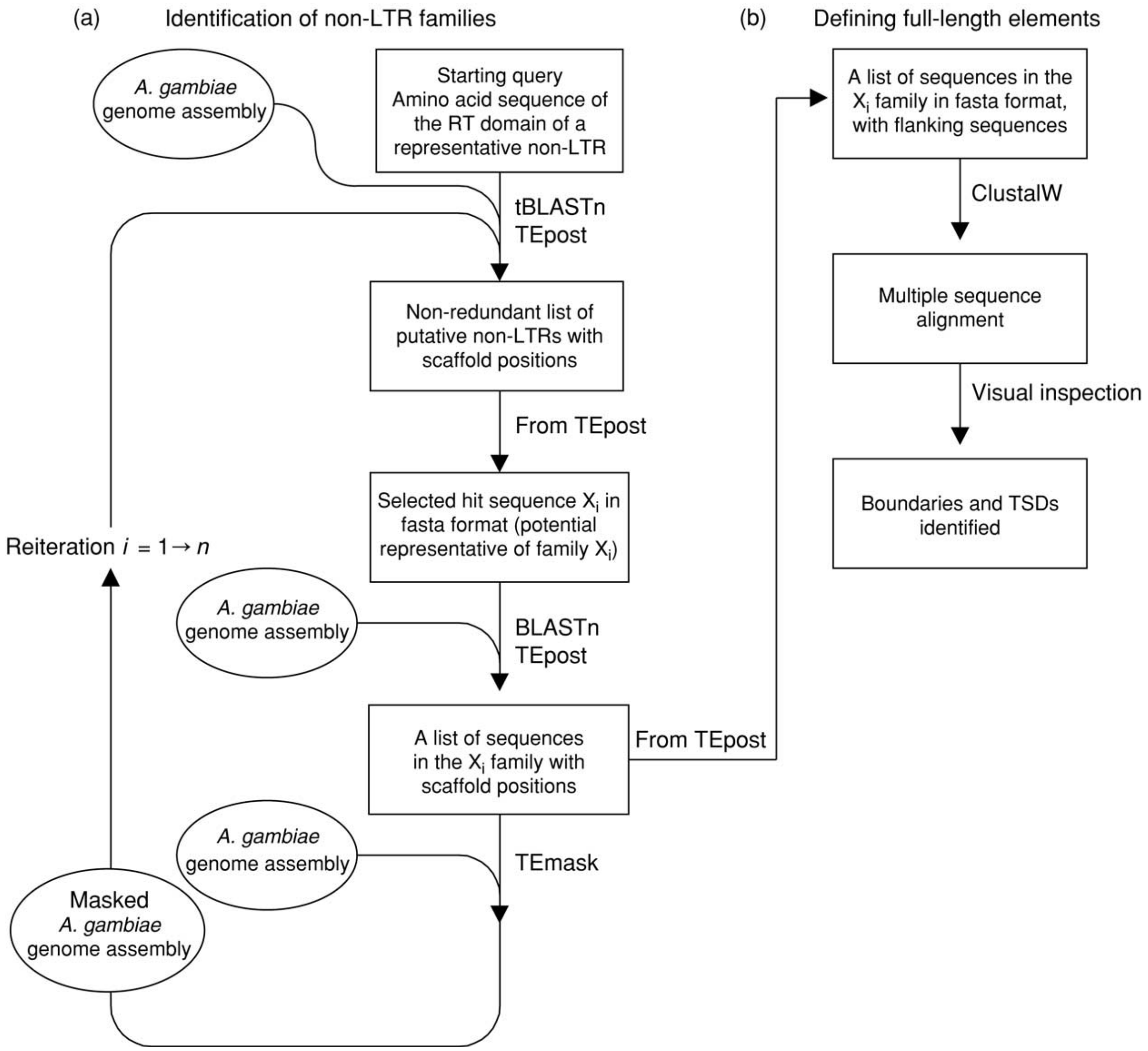


Figure 5 Design of TEpipe, a pipeline program for simultaneous identification and classification of TEs on the basis of sequence similarities. The example shown here is the strategy used to analyze all non-LTR retrotransposons in the *Anopheles gambiae* genome (Biedler and Tu, 2003). (a) Strategy to identify non-LTR retrotransposon families. (b) Strategy to define full-length elements. Ovals indicate databases used for searches. Rectangles indicate input/output files. Program modules are in bold beside arrows. Modules written in our laboratory are available at the website jaketu.biochem.vt.edu. (Biedler and Tu, 2003). TSDs, target site duplications. (Reproduced with permission from Biedler, J., Tu, Z., **2003**. Non-LTR retrotransposons in the African malaria mosquito, *Anopheles gambiae*: unprecedented diversity and evidence of recent activity. *Mol. Biol. Evol. 20*, 1811–1825; © Oxford University Press.)

FindMITE handles whole-genome sequence databases better, incorporates downstream analysis, and produces fewer false positive results which could be overwhelming in some cases. A program module AlignMITE has been developed to identify candidate MITEs that share the same TIRs and automatically align them by calling ClustalW (Thompson *et al.*, 1994) to determine MITE boundaries and full-length elements. Another program module, MITEInsertion, has been developed to uncover evidence of MITE insertion. Because a whole-genome database is not required, this systematic approach could have broad applications for the analysis of the model genomes as well as the vast majority of the less sequenced genomes. Although FINDMITE1 was originally designed to discover and analyze MITEs, it is possible to use it for the identification and characterization of DNA transposons that also contain TIRs. It is possible to use TEpipe to survey for the transposase-encoding sequences and use FINDMITE to analyze the TIRs and TSDs.

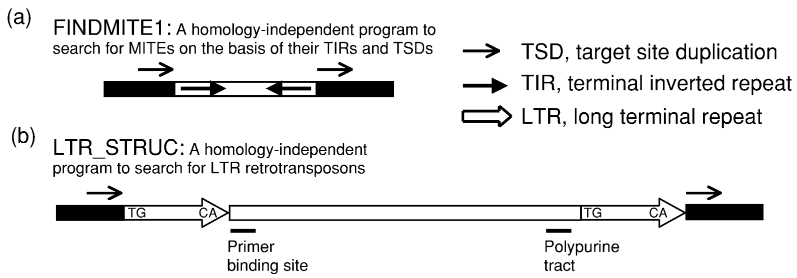


Figure 6 Design of two programs to search for different groups of TEs on the basis of shared structural features. (a) FINDMITE1 is a C program designed to rapidly search a database for sequences that have the characteristics of MITEs (Tu, 2001a). The program searches sequences in the database for inverted repeats flanked by user-defined direct repeats within a specified distance range. FINDMITE1 is available at our website (jaketu.biochem.vt.edu). The newly developed MITEpipe program, which further reduces false positive signals and incorporates FINDMITE1 with downstream analysis, will be available soon at the same website. (b) LTR_STRUC is a program that identifies LTR retrotransposons on the basis of the presence of long terminal repeats (most LTRs contain TG...CA termini), target site duplications (TSDs), and additional information such as primer binding site and polypurine tract (McCarthy and McDonald, 2003). The program is available as a console application from its authors.

LTR_STRUC is a program that identifies LTR retrotransposons on the basis of the presence of LTRs (most LTRs contain TG...CA termini), TSDs, and additional information such as primer binding site and polypurine tract (McCarthy and McDonald, 2003) (**Figure 6b**). Although it is not designed to uncover solo LTRs or truncated LTR retrotransposons, the program offers a rapid and efficient approach to systematically identify and characterize LTR retrotransposons in a given genome and it can be used as a discovery tool for new families of LTR retrotransposons. The program is available as a console application from its authors.

Recently, an automated program named RECON has been reported (Bao and Eddy, 2002), which identifies TE sequences on the basis of their repetitive nature in the genome. RECON uses a multiple sequence alignment algorithm which represents a significant improvement to previous methods based on the same strategy. Because it does not rely on sequence homology or structural information, RECON is potentially comprehensive and may be able to identify all repetitive sequences. By the same token, RECON is computationally intensive as the size of the genome database increases. A two-step approach in which a large number of repeats are identified and masked first using a small fraction of the genome sequence may resolve this potential problem.

4.12.4. Diversity and Characteristics of Insect TEs

4.12.4.1. Overview

Virtually all classes and types of eukaryotic TEs have been found in insects. Insect TEs such as *copia, gypsy, I, R1, P, mariner, hobo, piggyBac*, and *transib* are the founding members of several diverse families/superfamilies that were later shown to have broad distributions in eukaryotes. In addition, recent studies revealed a few novel and intriguing TEs in insects that are described in detail below. Tables 1 and 2 provide a relatively extensive, but by no means exhaustive, compilation of the two classes of TEs in insects. Figures 2 and 3 depict the characteristics of representatives of different RNA-mediated TEs and the cut-and-paste DNA transposons. In addition to the two "fully sequenced" dipterans, a few other dipteran species and a lepidopteran species (*Bombyx mori*) represent the sources from which the majority of TEs have been discovered and analyzed. As more insect genome projects move ahead and studies on insect TEs expand, more TEs from diverse species will be discovered which will expand our horizon and offer new insights from a comparative genomics perspective. In this section, a detailed account of the characteristics of all different families of TEs will not be discussed. Instead the focus will be on recent advances and interesting features of some novel insect TEs. In anticipation of the explosion of TE discovery in the near future, my laboratory will periodically upload updated Tables 1 and 2 on a website (jaketu. biochem.vt.edu).

4.12.4.2. RNA-Mediated TEs

4.12.4.2.1. **LTR retrotransposons** The distribution of LTR retrotransposons in insects is shown in Table 1. The structural characteristics of representatives of three major groups found in insects, Ty1/*copia*, Ty3/*gypsy*, and BEL, are shown in Figure 2. In addition to the LTRs that contain the

Table 1 RNA-mediated transposable elements in insects

Superfamily (clade)	*Element and reference*[b]	*Order*	*Organism*[c,d]
I. LTR retrotransposons[a]			
Ty1/copia	*Mosqcopia* (Tu, unpublished data), *Zebedee* (Warren *et al.*, 1997)	Diptera	*Aedes aegypti*
	copia-like (Holt *et al.*, 2002), *mtanga* (Rohr *et al.*, 2002)	Diptera	*Anopheles gambiae*
	1731, copia, Dm88, frogger	Diptera	*Drosophila melanogaster*
	Yokozuna (Ohbayashi *et al.*, 1998)	Lepidoptera	*Bombyx mori*
Ty3/gypsy	*Woot* (Beeman *et al.*, 1996)	Coleoptera	*Tribolium castaneum*
	Beagle, gypsy-like, Cruiser, Osvaldo, Springer (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
	Ozymandias (Hill *et al.*, 2001)	Diptera	*Anopheles gambiae*
	yoyo (Zhou and Haymer, 1998)	Diptera	*Ceratitis capitata*
	17.6, 297, 412, accord, blastopia, blodd, Burdock, Circe, gtwin, gypsy, HMS Beagle, Idefix, invader, McClintock, mdg1, mag3, micropia, opus, qbert, Quasimodo, rover, springer, Stalker, Tabor, Tirant, Transpac, Zam	Diptera	*Drosophila melanogaster*
	Judo, Karate (Abe *et al.*, 2002)	Lepidoptera	*Bombyx mandarina*
	Kabuki (Abe *et al.*, 2000), *Mag* (Garel *et al.*, 1994)	Lepidoptera	*Bombyx mori*
	Lydia (Pfeifer *et al.*, 2000)	Lepidoptera	*Lymantria dispar*
	TED (Friesen and Nissen, 1990)	Lepidoptera	*Trichoplusia ni*
BEL	*MosqNinja* (Tu, unpublished data)	Diptera	*Aedes aegypti*
	Moose, Pao-like (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
	aurora, Bel, diver, GATE, roo, rooA	Diptera	*Drosophila melanogaster*
	Yamato (Abe *et al.*, 2002)	Lepidoptera	*Bombyx mandarina*
	Pao (Xiong *et al.*, 1993), *Kamikaze, Yamato* (Abe *et al.*, 2001)	Lepidoptera	*Bombyx mori*
II. Non-LTR retrotransposons[a]			
R4	*Ag-R4_* 1 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
	Dong (Xiong and Eickbush, 1993)	Lepidoptera	*Bombyx mori*
R2	*R2* (Eickbush and Malik, 2002)	Diptera	*Drosophila melanogaster*
	R2Bm (Xiong and Eickbush, 1988)	Lepidoptera	*Bombyx mori*
L1	*Ag-L1_* 1–5 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
L2	*Ag-L2_* 1–3 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
RTE	*JAM1* (GenBank Z86117)	Diptera	*Aedes aegypti*
	*Ag-Jammin_* 1–2 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
R1	*RT1, RT2, Ag-R1* _1–11 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
	R1, RT1 (Waldo)	Diptera	*Drosophila melanogaster*
	R1Bm (Xiong and Eickbush, 1988), *SART1, TRAS1* (Anzai *et al.*, 2001)	Lepidoptera	*Bombyx mori*
LOA	*Lian* (Tu *et al.*, 1998)	Diptera	*Aedes aegypti*
	Bilbo, Baggins1 (Kapitonov and Jurka, 2003b)	Diptera	*Drosophila melanogaster*
	LOA (Felger and Hunt, 1992)	Diptera	*Drosophila silvestris*
I	*MosqI* (Tu and Hill, 1999)	Diptera	*Aedes aegypti*
	*Ag-I_* 1–7 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
	I, I2, You	Diptera	*Drosophila melanogaster*
Jockey	*JuanA* (Mouches *et al.*, 1992)	Diptera	*Aedes aegypti*
	*Ag-Jockey_* 1–13, *Ag-Jen* _1–12, (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
	NCR1Cth1 (Blinov *et al.*, 1997)	Diptera	*Chironomus thummi*
	JuanC (Agarwal *et al.*, 1993)	Diptera	*Culex pipiens*
	BS, Doc, F, G, Helena, Het-A, Jockey, Juan, TART, X	Diptera	*Drosophila melanogaster*
	AMY (*BMC1, L1Bm*; Abe *et al.*, 1998)	Lepidoptera	*Bombyx mori*
	LDT1 (Garner and Slavicek, 1999)	Lepidoptera	*Lymantria dispar*
CR1	*T1, Q, Ag-CR1* _1–29 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
	DMCR1A (Kapitonov and Jurka, 2003b)	Diptera	*Drosophila melanogaster*
Loner	*Ag-Loner_* 1–3 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
Outcast	*Ag-Outcast_* 1–11 (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
Not classified	*Sponge* (Biedler and Tu, 2003)	Diptera	*Anopheles gambiae*
	CM-gag (Bensaadi-Merchermek *et al.*, 1997)	Diptera	*Culex pipiens*
	Kurosawa, Kendo (Abe *et al.*, 2002)	Lepidoptera	*Bombyx mandarina*
III. SINEs			
tRNA-related SINEs	*Feilai* (Tu, 1999)	Diptera	*Aedes aegypti*
	SINE200 (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
	Cp1 (Liao *et al.*, 1998)	Diptera	*Chironomus pallidivittatus*

Continued

Table 1 Continued

Superfamily (clade)	*Element and reference*[b]	*Order*	*Organism*[c,d]
	Twin (Feschotte *et al.*, 2001)	Diptera	*Culex pipiens*
	Bm1 (Adams *et al.*, 1986)	Lepidoptera	*Bombyx mori*
	Lm1 (Bradfield *et al.*, 1985)	Orthoptera	*Locusta migratoria*
Unclassified SINEs?	*Maque* (Tu, 2001b)	Diptera	*Anopheles gambiae*
	DINE-1 (Locke *et al.*, 1999)	Diptera	*Drosophila melanogaster*
	IE (Sun *et al.*, 1991; Lampe and Willis, 1994)	Lepidoptera	*Hyalophora cecropia*
IV. Penelope-like retrotransposons			
Penelope	*Penelope-like* (Biedler and Tu, unpublished data)	Diptera	*Aedes aegypti*
	Penelope (Evgen'ev *et al.*, 1997)	Diptera	*Drosophila virilis*

[a]Classifications of LTR and non-LTR retrotransposons are according to Eickbush and Malik (2002).
[b]The citation is not always the original report of a TE. In some cases a review or an article describing the phylogenetic placement of the TE is cited instead. References for all *D. melanogaster* TEs are from Kaminker *et al.* (2002) unless otherwise noted. There are several PCR surveys of a few RNA-mediated TEs in different insects that are not listed in the table (Booth *et al.*, 1994; Rongnoparut *et al.*, 1998; Cook *et al.*, 2000).
[c]TEs in *Drosophila* species other than *D. melanogaster* are not listed unless they are the founding member of a group of TEs.
[d]In some cases, TEs from only one species are listed although they were found in several closely related species.

promoter and transcription termination sequences, the LTR retrotransposons have a flexible structure that allows gain, loss, and perhaps rearrangement of functional domains (Eickbush and Malik, 2002). While the RT and a few other protein domains perform the key function of making double-stranded cDNA, the acquisition of the IN activity allowed the integration of the cDNA in a way much like the mechanisms employed by DNA transposons. In fact, the IN domain and some of the prokaryotic and eukaryotic DNA transposases are believed to share a common origin (Capy *et al.*, 1997; and see below). The acquisition of the *env*-like protein by some LTR retrotransposons such as *gypsy* confers the ability to leave the cell and become infectious retroviruses (Eickbush and Malik, 2002).

4.12.4.2.2. Non-LTR retrotransposons Twelve of the 17 clades of non-LTR retrotransposons have been found in insects (Eickbush and Malik, 2002; Biedler and Tu, 2003) (**Table 1** and **Figure 7**). In fact, the founding members of many of these clades were discovered in insects. The characterization and classification of 104 families of non-LTR retrotransposons in *A. gambiae* have been recently reported (Biedler and Tu, 2003). The 104 *A. gambiae* families represent divergent lineages in eight previously established clades (*R4*, *L1*, *RTE*, *R1*, *L2*, *CR1*, *Jockey*, and *I*) and two new clades, *Loner* and *Outcast*. Representation appears to be biased toward the most derived clades in non-LTR retrotransposon evolution, especially the *CR1* and *Jockey* clades, with 31 and 25 families respectively. All of the 31 *A. gambiae CR1* elements are grouped together with the *Drosophila CR1* being a sister branch. On the other hand, there appear to be three groups (I, II, and III) of the *A. gambiae Jockey* elements, which may have different sister elements either from other mosquitoes or other dipterans (Biedler and Tu, 2003).

4.12.4.2.3. SINEs SINEs have not been extensively investigated in insects. Only a small number of them have been found as shown in **Table 1**. Insect SINEs characterized so far all belong to the tRNA-related group. The structural features of *Feilai*, a SINE discovered in *Aedes aegypti*, include a tRNA-related promoter region, a tRNA-unrelated conserved region, and a triplet tandem repeat at its 3′ end (**Figure 2**). The *Twin* SINE family, which was discovered in *Culex pipiens* (Feschotte *et al.*, 2001), consists of two tRNA-related regions separated by a 39 bp spacer. *SINE200* from *A. gambiae* contains only one of the two conserved boxes found in tRNA-related Pol III promoters (Tu, unpublished data). *DINE-1*, a SINE from *D. melanogaster*, lacks the structural features of typical SINEs (Locke *et al.*, 1999). *Feilai*, *SINE200*, and two other SINEs *BM1* and *Lm1* (**Table 1**) are all highly repetitive in their respective genomes. *Twin* is only moderately repetitive with a copy number of 500. *Cp1*, a *Chironomus pallidivittatus* SINE, inserts specifically to centromeric tandem repeats (Liao *et al.*, 1998).

4.12.4.2.4. Two intriguing families: *Maque* and *Penelope* A family of very short interspersed repetitive elements named *Maque* has recently been found in *A. gambiae*. There are approximately 220

Table 2 DNA-mediated transposable elements in insects

Superfamily/family	*Defining features*	*Element and reference*[a]	*Order*	*Organism*[b,c]
I. Cut-and-paste transposons				
P	8 bp TSD, conserved transposase	*AgaP* (8 subfamilies: Sarkar *et al.*, 2003)	Diptera	*Anopheles gambiae*
		P (Canonical, M, O, T), *ProtoP (Hoppel), ProtoP_B* (Kapitonov and Jurka, 2003b)	Diptera	*Drosophila melanogaster*
		Lu-P1, Lu-P2 (Perkins and Howells, 1992)	Diptera	*Lucilia cuprina*
		P (Lee *et al.*, 1999)	Diptera	*Musca domestica*
hAT (hobo-Ac-Tam3)	8 bp TSD, conserved transposase	*hAT*-type (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
		hopper (Handler, 2003)	Diptera	*Bactrocera dorsalis*
		Homer (Pinkerton *et al.*, 1999)	Diptera	*Bactrocera tryoni*
		hobo (Calvi *et al.*, 1991)	Diptera	*Drosophila melanogaster*
		Hermit (Coates *et al.*, 1996)	Diptera	*Lucilia cuprina*
		Hermes (Warren *et al.*, 1994)	Diptera	*Musca domestica*
IS630-Tc1-mariner	TA TSD, DDE(D) catalytic triad			
Tc1	DD34E	*Quetzal* (Ke *et al.*, 1996)	Diptera	*Anopheles albimanus*
		Crusoe (Hill *et al.*, 2001), *DD34E, Tiang, Topi, Tsessebe* (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
		Minos (Franz and Savakis, 1991)	Diptera	*Drosophila hydei*
		Bari1-2, HB, S, S2, Tc1 (Kaminker *et al.*, 2002); *Tc3*-like (Shao *et al.*, 2001)	Diptera	*Drosophila melanogaster*
		Unnamed element (Mikitani *et al.*, 2000)	Lepidoptera	*Bombyx mori*
mariner[d]	DD34D	*mariner* (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
		D.mauritiana.mar1 (Robertson, 2002)	Diptera	*Drosophila mauritiana*
		mariner2 (Kaminker *et al.*, 2002)	Diptera	*Drosophila melanogaster*
		A.mellifera.mar1 (Robertson, 2002)	Hymenoptera	*Apis mellifera*
		H.cecropia.mar1 (Robertson, 2002)	Lepidoptera	*Hyalophora cecropia*
ITmD37D (maT)	DD37D	*ITmD37D* (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
		MdmaT1 (Claudianos *et al.*, 2002)	Diptera	*Musca domestica*
		Bmmar1 (Robertson and Asplund, 1996), *Bmmar6* (Robertson and Walden, 2003)	Lepidoptera	*Bombyx mori*
ITmD41D	DD41D	*Crmar2* (Gomulski *et al.*, 2001)	Diptera	*Ceratitis rosa*
		Tcp3.2 (Arends and Jehle, 2002; Robertson and Walden, 2003)	Lepidoptera	*Cydia pomonella*
ITmD37E	DD37E	*A.aegypti.ITmD37E* (Shao and Tu, 2001)	Diptera	*Aedes aegypti*
		A.gambiae.ITmD37E (Shao and Tu, 2001)	Diptera	*Anopheles gambiae*
Pogo[e]	DDxD, long C-terminus	*Pogo*-like (Holt *et al.*, 2002)	Diptera	*Anopheles gambiae*
		Pogo (Tudor *et al.*, 1992)	Diptera	*Drosophila melanogaster*
piggyBac	TTAA TSD, conserved transposase	*piggyBac* (Holt *et al.*, 2002; Sarkar *et al.*, 2003)	Diptera	*Anopheles gambiae*
		piggyBac (Handler and McCombs, 2000)	Diptera	*Bactrocera dorsalis*
		looper1 (Kapitonov and Jurka, 2002)	Diptera	*Drosophila melanogaster*
		piggyBac (Sarkar *et al.*, 2003)	Lepidoptera	*Bombyx mori*
		piggyBac (Cary *et al.*, 1989)	Lepidoptera	*Trichoplusia ni*

Continued

Table 2 Continued

Superfamily/family	*Defining features*	*Element and reference*[a]	*Order*	*Organism*[b,c]
PIF-harbinger	3 bp TSD, conserved transposase	*PIF/harbinger*-like (Biedler *et al.*, unpublished data)	Diptera	*Anopheles gambiae*
Transib	5 bp TSD, conserved transposase	*Transib1_AG* (Kapitonov and Jurka, 2003b)	Diptera	*Anopheles gambiae*
		Transib1-4 (Kapitonov and Jurka, 2003b), *Hopper* (Bernstein *et al.*, 1995)	Diptera	*Drosophila melanogaster*
Not classified		*Ikirara* (Romans *et al.*, 1998)	Diptera	*Anopheles gambiae*
		TECth1 (Wobus *et al.*, 1990)	Diptera	*Chironomus thummi*
II. Rolling circle transposons				
Helitron	No TSD, similarity to helicase	*Helitron1_AG, Helitron 2_AG* (Kapitonov and Jurka, 2003b)	Diptera	*Anopheles gambiae*
		Helitron (Kapitonov and Jurka, 2003b)	Diptera	*Drosophila melanogaster*
III. MITEs				
mTATA TSD	*DEC* (Braquart *et al.*, 1999)	Cleoptera	*Tenebrio molitor*	
		Pony (Tu, 2000), *Wujin* (Tu, 1997); *mTA_1–8* (Mao and Tu, unpublished data)	Diptera	*Aedes aegypti*
		TA-I-Ag, TA-II-Ag, TA-III-Ag, TA-IV-Ag, TA-V-Ag (Tu, 2001a)	Diptera	*Anopheles gambiae*
		Mikado, Milord, Mimo, Mirza, Nemo (Feschotte *et al.*, 2002)	Diptera	*Culex pipiens*
		mPogo (Feschotte *et al.*, 2002)	Diptera	*Drosophila melanogaster*
m3bp (*Tourist*-like MITEs)	3 bp TSD	*m3bp_1–10* (Mao and Tu, unpublished data)	Diptera	*Aedes aegypti*
		Joey, TAA-I-Ag, TAA-II-Ag (Tu, 2001a), *m3bp_1–19* (Biedler *et al.*, unpublished data)	Diptera	*Anopheles gambiae*
		A MITE in a NOS gene (Luckhart and Rosenberg, 1999)	Diptera	*Anopheles stephensi*
m4bp	4 bp (often TTAA) TSD	*Wukong, Wuneng* (Tu, 1997), *m4bp_1–6* (Mao and Tu, unpublished data)	Diptera	*Aedes aegypti*
m7bp	7 bp TSD	*m7bp_1–3* (Mao and Tu, unpublished data)	Diptera	*Aedes aegypti*
m8bp	8 bp TSD	*m8bp_1–10* (Mao and Tu, unpublished data)	Diptera	*Aedes aegypti*
		8bp-I-Ag (Tu, 2001a), *Pegasus* (Besansky *et al.*, 1996)	Diptera	*Anopheles gambiae*
		Mar, Vege (Holyoake and Kidwell, 2003)	Diptera	*Drosophila willistoni*
m9bp	9 bp TSD	*m9bp_1–2* (Mao and Tu, unpublished data)	Diptera	*Aedes aegypti*
Not classified		*Microuli* (TTAA TSD, SIR: Tu and Orphanidis, 2001)	Diptera	*Aedes aegypti*
		MEC (5 bp TSD: Blinov *et al.*, 1991)	Diptera	*Chironomus thummi*
		Mint (CA TSD, SIR: Feschotte *et al.*, 2002)	Diptera	*Culex pipiens*
		SGM-IS (SIR: Miller *et al.*, 2000)	Diptera	*Drosophila obscura*

[a]The citation is not always the original report of a TE. In some cases a review or an article describing the phylogenetic placement of the TE is cited instead. *Drosophila Foldback* elements are not listed. There are several PCR surveys of a few DNA-mediated TEs in different insects that are not listed in the table (Daniels *et al.*, 1990; Robertson, 1993; Bigot *et al.*, 1994; Clark and Kidwell, 1997; Imwong *et al.*, 2000; Green and Frommer, 2001).
[b]TEs in *Drosophila* species other than *D. melanogaster* are not listed unless they are the founding member of a group of TEs.
[c]In some cases, TEs from only one species are listed although they were found in several closely related species.
[d]*mariner* sequences, full-length or fragment, have been found in a wide range of insects. The DD34D mariners consist of 6 subfamilies briggase, cecropia, elegans, irritans, mauritiana, and mellifera. See Shao and Tu (2001) for a reclassification of the *IS630-Tc1-mariner* superfamily. See Robertson (1993, 2002) for reviews on *mariner* evolution.
[e]The status of *Pogo* is not certain. It is either considered a member of the *IS630-Tc1-mariner* superfamily or a separate superfamily (Shao and Tu, 2001; Robertson, 2002).

copies of *Maque*. Only approximately 60 bp long, *Maque* has the appearance of a distinct transposition unit. The majority of *Maque* elements were flanked by 9–14 bp TSDs. *Maque* has several characteristics of non-LTR retrotransposons such as TSDs of variable length, imprecise 5′ terminus, and CAA simple repeats at the 3′ end. The evolutionary origin of *Maque* and the differences between *Maque* and other known retro-elements including SINEs is not yet known. The 5′ end of *Maque* represents a strong stop position that caused frequent premature termination of reverse transcription is suggested (Tu, 2001b). Although no autonomous non-LTR retrotransposons have been found that share similar 3′ sequences with *Maque*, there is a family of non-LTR retrotransposons, *Ag-I-2* (Biedler and Tu, 2003) that has the same CAA tandem repeats at their 3′ termini. It is possible that short sequences such as *Maque* that contain just the RT recognition signal could potentially contribute to the genesis of some primordial SINEs (Tu, 2001b).

Penelope, another intriguing family, was discovered as a TE involved in the hybrid dysgenesis of crosses between field-collected and laboratory strains of *D. virilis* (Evgen'ev *et al*., 1997). It has a RT that is grouped with the RT from telomerase (Arkhipova *et al*., 2003). More strikingly, members of the *Penelope* family in bdelloid rotifers are able to retain their introns, which is inconsistent with a transposition mechanism involving an RNA intermediate. It was proposed that the Uri endonuclease domain found in all *Penelope*-like elements may allow them, at least in part, to use a DNA-mediated mechanism similar to that used by group I introns (Arkhipova *et al*., 2003). On the basis of these unique features, *Penelope* was classified as a unique group that is distinct from LTR and non-LTR retrotransposons.

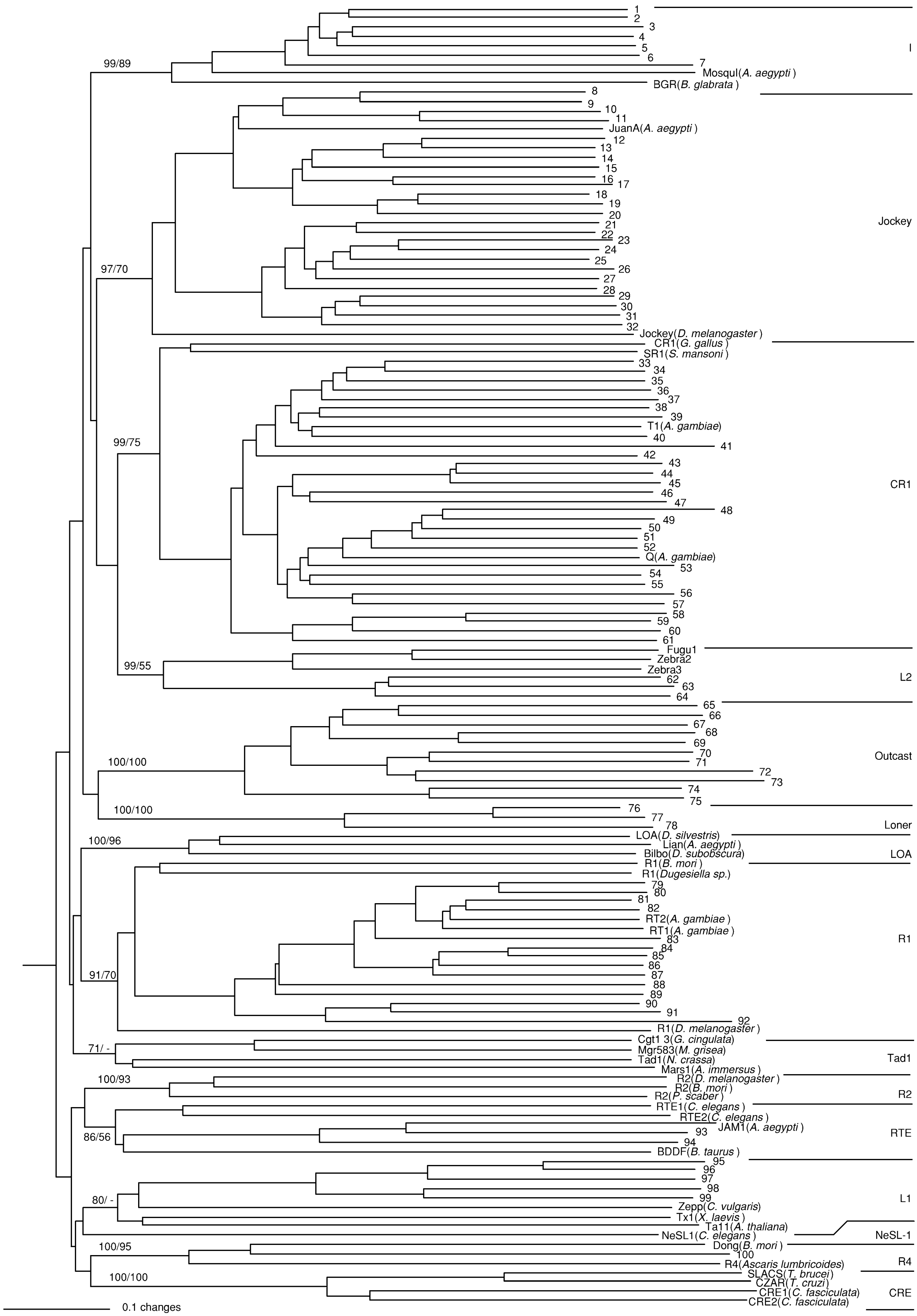
4.12.4.3. DNA-Mediated TEs

4.12.4.3.1. Cut-and-paste DNA transposons The majority of DNA-mediated TEs are believed to transpose by a cut-and-paste mechanism. This group of elements is characterized by 10–200 bp TIRs flanking one or more ORFs that encode a transposase, the enzyme that performs the excision and integration (cut and paste) of the cognate TE DNA. Their copy number may be increased through a repair mechanism, or by transposing ahead of a replication fork (Finnegan, 1992; Craig, 2002).

Different families/superfamilies of insect TEs in this group are listed in **Table 2**. Again, several insect TEs are the founding members of their respective families/superfamilies that have broad distributions. The families/superfamilies that have been found in insects include *IS630-Tc1-mariner, hAT, piggyBac, PIF/Harbinger, P*, and *Transib* (Shao and Tu, 2001; Robertson, 2002; Kapitonov and Jurka, 2003b). Conserved transposase sequences and TSDs of specific sequence or length are the hallmarks of each family/superfamily. *In vitro* experiments have shown that, for a few cut-and-paste DNA transposons characterized so far, the transposase alone is sufficient to direct the transposition reactions by interacting with the TIRs and the insertion target (Plasterk *et al*., 1999). However, the transposition of *P* elements requires a host protein (inverted repeat binding protein, IRBP) that binds to its TIRs (Badge and Brookfield, 1997; Rio, 2002). This requirement may explain the lack of success in the early attempts to use *P* elements to transform insects other than *Drosophila*.

The structural characteristics of representative elements from each family are shown in **Figure 3** (see **Chapter 4.13**). A few TE families including *PIF/Harbinger* (Biedler *et al*., unpublished data) and *Transib* (Kapitonov and Jurka, 2003b), which were discovered in insects only recently, will be highlighted in this section. Recent expansion and reclassification of the *IS630-Tc1-mariner* superfamily will also be discussed (Shao and Tu, 2001). In a separate section on MITEs (see Section 4.12.4.3.2), two new groups of mosquito MITEs will be described that have 7 and 9 bp TSDs respectively (Mao and Tu, unpublished data). The discovery of MITEs with 9 bp TSDs provides a preliminary indication of the possible existence of *Mutator*-like transposons in insects, which have so far been only found in plants (Walbot and Rudenko, 2002). On the other hand, the 7 bp TSD MITEs could potentially lead to the discovery of an entirely new family of eukaryotic DNA transposons because 7 bp TSD elements have until now only been found in bacteria (Krebs *et al*., 1990; Mahillon and Chandler, 1998) (see Section 4.12.4.3.2.2).

4.12.4.3.1.1. Discovery of the* ITmD37E *transposon and the reclassification of the* IS630-Tc1-mariner (ITm) *superfamily It was shown previously that some prokaryotic *IS* elements, eukaryotic *Tc1* and *mariner* transposons, and eukaryotic retrotransposons and retroviruses form a megafamily which shares similar signature sequences or motifs in the catalytic domain of their respective transposase and IN (Capy *et al*., 1996, 1997). The common motif for this transposase–integrase megafamily is a conserved D(Asp)DE(Glu) or DDD catalytic triad. The distance between the first two Ds is variable while the distance between the last two residues in the catalytic triad is mostly invariable


1
2
3
4
5
6
7
Mosqui(*A. aegypti*)
BGR(*B. glabrata*)
8
9
10
11
JuanA(*A. aegypti*)
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
Jockey(*D. melanogaster*)
CR1(*G. gallus*)
SR1(*S. mansoni*)
33
34
35
36
37
38
39
T1(*A. gambiae*)
40
41
42
43
44
45
46
47
48
49
50
51
52
Q(*A. gambiae*)
53
54
55
56
57
58
59
60
61
Fugu1
Zebra2
Zebra3
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
LOA(*D. silvestris*)
Lian(*A. aegypti*)
Bilbo(*D. subobscura*)
R1(*B. mori*)
R1(*Dugesiella sp.*)
79
80
81
82
RT2(*A. gambiae*)
RT1(*A. gambiae*)
83
84
85
86
87
88
89
90
91
92
R1(*D. melanogaster*)
Cgt1 3(*G. cingulata*)
Mgr583(*M. grisea*)
Tad1(*N. crassa*)
Mars1(*A. immersus*)
R2(*D. melanogaster*)
R2(*B. mori*)
R2(*P. scaber*)
RTE1(*C. elegans*)
RTE2(*C. elegans*)
JAM1(*A. aegypti*)
93
94
BDDF(*B. taurus*)
95
96
97
98
99
Zepp(*C. vulgaris*)
Tx1(*X. laevis*)
Ta11(*A. thaliana*)
NeSL1(*C. elegans*)
Dong(*B. mori*)
100
R4(*Ascaris lumbricoides*)
SLACS(*T. brucei*)
CZAR(*T. cruzi*)
CRE1(*C. fasciculata*)
CRE2(*C. fasciculata*)
99/89
97/70
99/75
99/55
100/100
100/100
100/96
91/70
71/ -
100/93
86/56
80/ -
100/95
100/100
I
Jockey
CR1
L2
Outcast
Loner
LOA
R1
Tad1
R2
RTE
L1
NeSL-1
R4
CRE
0.1 changes

for a given transposon family in eukaryotes, indicating functional importance. Within this megafamily, the eukaryotic DNA transposon families *Tc1* and *mariner* and the bacterial *IS630* element and its relatives in prokaryotes and ciliates comprise a superfamily, the *IS630-Tc1-mariner* superfamily, which is based on overall transposase similarities and a common TA dinucleotide insertion target (Henikoff, 1992; Doak *et al*., 1994; Robertson and Lampe, 1995; Capy *et al*., 1996; Shao and Tu, 2001). *Tc1*-like elements identified in fungi, invertebrates, and vertebrates all contain a DD34E motif while most *mariner* elements identified in flatworms, insects, and vertebrates contain a DD34D motif. A few TEs that contain DD37D and DD39D motifs were previously regarded as basal subfamilies, the max subfamily and mori subfamily respectively, of the *mariner* family (Robertson, 2002). A novel transposon, *ITmD37E*, was recently reported in a wide range of mosquito species (Shao and Tu, 2001). The *ITmD37E* transposases contain a conserved DD37E catalytic motif, which is unique among the reported transposons of the ITm superfamily. Sequence comparisons and phylogenetic analyses suggest that *ITmD37E* is a novel family (**Figure 8**). In addition, our phylogenetic analyses show that the mori subfamily (DD37D) and max subfamily (DD39D) of *mariner* may also be classified as two distinct families, namely the *ITmD37D* and *ITmD39D* families. In fact, *ITmD37D* (previously mori subfamily of *mariner*) is more closely related to *Tc1* (DD34E) than other *mariner* elements (**Figure 8**). The recognition of the three new families *ITmD37E, ITmD37D*, and *ITmD39D* is consistent with the fact that they share family-specific catalytic motifs and similar TIRs. Claudianos and colleagues also noticed the need for reclassification of the DD37D transposons and named them the *maT* family (Claudianos *et al*., 2002). Finally, a group of transposons that contain a DD41D catalytic motif have been found in the medfly *Ceratitis rosa*, establishing yet another family (Gomulski *et al*., 2001; Robertson and Walden, 2003), namely the *ITmD41D* family. In summary, according to recent analyses, the ITm superfamily can be organized in seven families including *ITmD37E*, *ITmD37D*, *ITmD39D*, *ITmD41D*, *Tc1*, *mariner*, *pogo*, and an unresolved clade which includes bacterial *IS630*-like elements and some fungal and ciliate transposons (**Figure 8**). *pogo* is an interesting case as it has a unique N-terminal DNA-binding domain and a long C-terminal domain rich in acidic residues, although it contains a DDxD catalytic domain related to the ITm transposons (Smit and Riggs, 1996).

4.12.4.3.1.2. Two new families: **PIF/Harbinger** *and* **Transib** *PIF/Harbinger* and *Transib* are two newly discovered families of DNA transposons in insects. *PIF* and related TEs *Harbinger* and *Tc8* have previously been found in plants and nematodes (Kapitonov and Jurka, 1999; Le *et al*., 2001; Zhang *et al*., 2001) and they may have mobilized *Tourist*, one of the most abundant MITEs in plants (Zhang *et al*., 2001; Jiang *et al*., 2003). Analysis of the *A. gambiae* genome uncovered approximately 30 families of *PIF/Harbinger*-like transposons, although the majority of them are "fossil"-like sequences (Biedler *et al*., unpublished data). Like all other *PIF/Harbinger*-like transposons, the *A. gambiae PIF/Harbinger*-like elements are characterized by 3 bp TSDs and transposase sequences similar to that of the bacterial *IS5* elements (Zhang *et al*., 2001). A second new family, the *Transib* transposons, are so far found only in *D. melanogster* and *A. gambiae* (Kapitonov and Jurka, 2003b). They use a unique transposase and generate 5 bp TSDs. *Transib* transposons have resided in these two genomes for a long time as extensive *in silico* reconstruction had to be employed to trace back the ancestral full-length copies (Kapitonov and Jurka, 2003b).

Figure 7 Phylogenetic analysis of diverse non-LTR retrotransposons found in *Anopheles gambiae*. Phylogenetic analysis classifies 104 families of *A. gambiae* elements into two new clades and eight previously defined clades. The two new clades, *Loner* and *Outcast*, are in bold. Shown here is the neighbor-joining tree constructed using alignment of approximately 260 amino acids of the reverse transcriptase (RT) domain from non-LTR retrotransposons of *A. gambiae* and representative elements in different clades. The tree was rooted using RTs of three prokaryotic Group II introns (not shown). Maximum parsimony was also used which produced a similar phylogenetic tree (not shown). Confidence of the groupings was estimated using 500 bootstrap replications for both methods. The first and second numbers at a particular node represent the bootstrap values derived from 500 neighbor-joining and maximum parsimony analysis, respectively. Only the values for the major groupings (clades) that are above 50% are shown. The scale at bottom left indicates amino acid divergence. The names of elements from previously established clades are given but names of new *A. gambiae* non-LTR families are omitted to save space (see Biedler and Tu, 2003). Previously reported *A. gambiae* non-LTRs in the tree are *Q*, *T1*, *RT1*, and *RT2* (Besansky, 1990; Besansky *et al*., 1992, 1994). (Reproduced with permission from Biedler, J., Tu, Z., **2003**. Non-LTR retrotransposons in the African malaria mosquito, *Anopheles gambiae*: unprecedented diversity and evidence of recent activity. *Mol. Biol. Evol. 20*, 1811–1825; © Oxford University Press.)

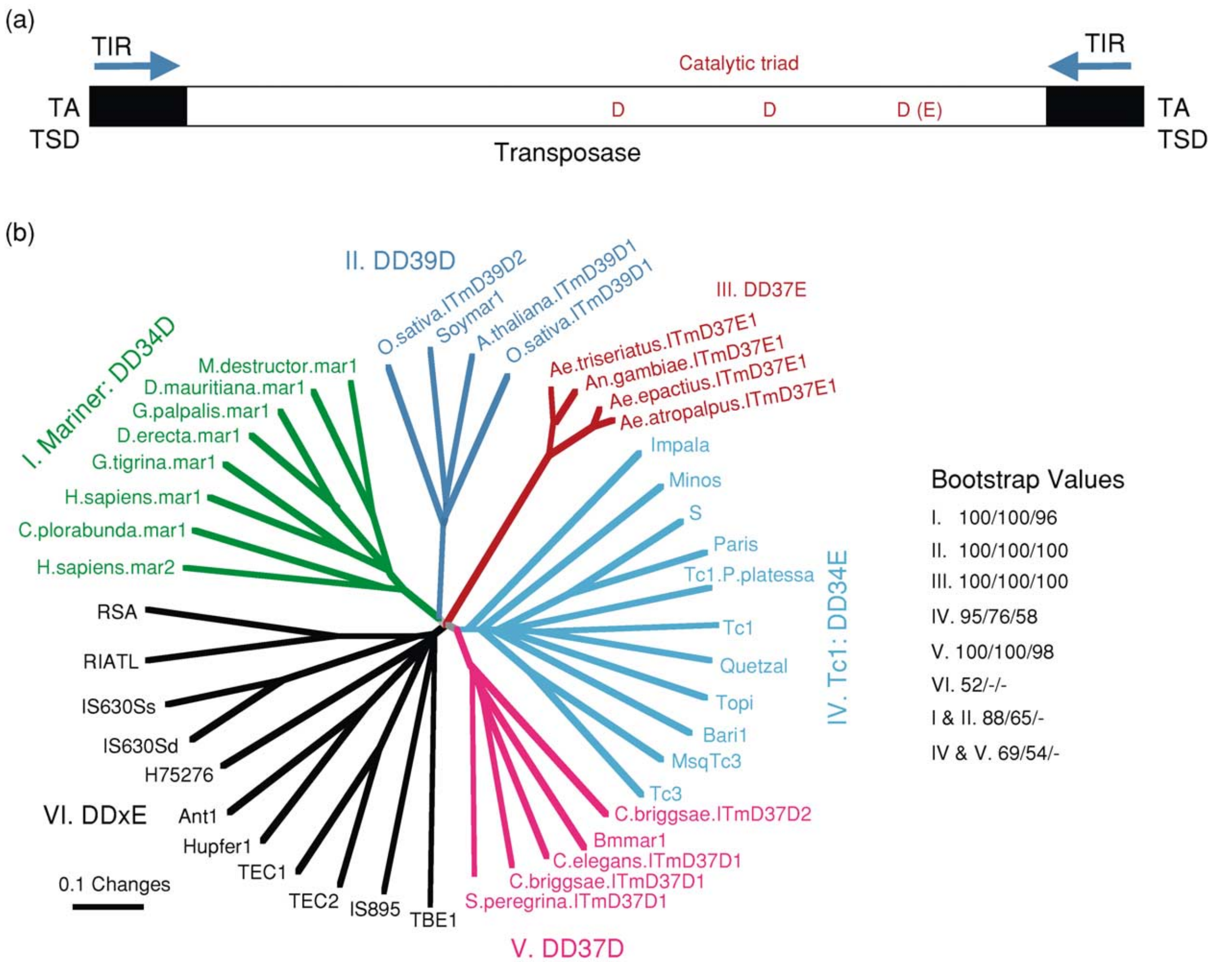


Figure 8 Structural features and classification of the *IS630-Tc1-mariner* superfamily. (a) Structural features. The catalytic triad in the transposase is highlighted. The characteristic TA target site duplications (TSDs) flanking an *IS630-Tc1-mariner* are shown. The terminal inverted repeats (TIRs) specify the boundries of the element. Possible introns are not shown. (b) Phylogenetic relationship between members of the *IS630-Tc1-mariner* superfamily on the basis of the catalytic domain. The alignment used here was previously described (Shao and Tu, 2001). The tree shown here is an unrooted phylogram constructed using a minimum evolution algorithm. Two additional methods, neighbor-joining and maximum parsimony, were also used. Confidence of the groupings was estimated using 500 bootstrap replications. The bootstrap value represents percent of times that branches were grouped together at a particular node. The first, second, and third numbers represent the bootstrap value derived from minimum evolution, neighbor-joining, and maximum parsimony analysis, respectively. Only the values for major groupings are shown. Various colors indicate different clades. All phylogenetic analyses were conducted using PAUP 4.0 b8 (Swofford, 2001). Note that a recently described group of transposons that contain a DD41D catalytic triad was not included here. They are a distinct group related to the DD37D transoposons (Gomulski *et al*., 2001). (Modified from Shao, H., Tu, Z., **2001**. Expanding the diversity of the *IS630-Tc1-mariner* superfamily: discovery of a unique DD37E transposon and reclassification of the DD37D and DD39D transposons. *Genetics 159*, 1103–1115.)

4.12.4.3.2. MITEs As shown in Table 2, MITEs that share similar TSDs and TIRs with DNA transposons in the *IS630-Tc1-mariner, hAT, piggyBac, PIF/Harbinger* families have been found in three species of mosquitoes. A MITE that generates a specific TA TSD has also been reported in a coleopteran (Braquart *et al*., 1999). Two *hAT*-like MITEs have been recently found in *D. willistoni* (Holyoake and Kidwell, 2003) and a deletion-derivative of the *pogo* transposon has been found in *D. melanogaster* (Feschotte *et al*., 2002).

The relationship between *PIF/Harbinger*-like DNA transposons and related *Tourist*-like MITEs in *A. gambiae* is interesting (Biedler *et al*., unpublished data). There are multiple *PIF/Harbinger*-like DNA transposon families in *A. gambiae*, all of which have the characteristic AT-rich 3 bp TSDs. Although the TIRs are highly conserved between different copies of a family, they are often variable between different *PIF/Harbinger*-like transposon families. Only two MITE families were found to be apparent deletion derivatives of two *PIF/Harbinger*-like

transposons (**Figure 4**). On the other hand, 20 families of the *Tourist*-like MITEs were found to share similar TSDs and TIRs with their respective *PIF/Harbinger*-like transposons although no similarities were found between the internal sequences of these MITEs and any of the *PIF/Harbinger*-like transposons. The implication of these results on the evolution of MITEs is discussed elsewhere (see Sections 4.12.2 and 4.12.6).

4.12.4.3.2.1.* m9bp*, MITEs with 9 bp TSDs Two novel groups of MITEs have been discovered in *Aedes aegypti*. One group, *m9bp*, includes two families of putative MITEs that have 9 bp TSDs (Mao and Tu, unpublished data). These two families are moderately repetitive and evidence of insertion has been found for both families. Several plant MITEs have 9 bp TSDs (Charrier *et al*., 1999; Feschotte *et al*., 2002) and they may be mobilized by *Mutator*-like transposons only found in plants (Walbot and Rudenko, 2002). The discovery of 9 bp TSD MITEs in *Aedes aegypti* is the first in animals, which may indicate a broader distribution of the *Mutator*-like transposons.

4.12.4.3.2.2.* m7bp*, MITEs with 7 bp TSDs Three families of putative MITEs that have 7 bp TSDs (*m7bp_1, m7bp_2, m7bp_3*) have been found in *Aedes aegypti* (Mao and Tu, unpublished data). These families are moderately repetitive and evidence of insertion has been found for all three families. These 7 bp TSD MITEs could potentially lead to the discovery of an entirely new family of eukaryotic DNA transposons, as 7 bp TSD elements have until now only been found in bacteria (Krebs *et al*., 1990; Mahillon and Chandler, 1998). No full-length protein-encoding DNA transposons that produce 7 bp TSDs have been identified in *A. aegypti* yet. However, this is within expectation considering the fragmented nature of the BAC-end sequences in the current *Aedes aegypti* database.

4.12.4.3.2.3.* Microuli*, a miniature subterminal inverted-repeat TE *Microuli* is a family of small (~200 bp) and highly AT rich (68.8–72.6%) TEs found in *Aedes aegypti* that do not have any coding capacity (Tu and Orphanidis, 2001). There is a 61 to 62 bp internal subterminal inverted repeat as well as a 7 bp subterminal inverted repeat 11 bp from the two termini. In addition, there are three imperfect subterminal direct repeats near the 5′ end. All of the above characteristics clearly resemble the structural features of MITEs. The only feature that separates *Microuli* from MITEs is that *Microuli* elements lack TIRs. Therefore, we use the phrase "miniature subterminal inverted-repeat transposable elements," or MSITEs, to refer to the structural characteristics of the *Microuli* elements. Short insertion sequences that contain subterminal inverted repeats but lack TIRs have been identified in the genomes of rice and a *Culex* mosquito (Song *et al*., 1998; Feschotte and Mouches, 2000a). Fourteen of the 19 nucleotides at the 5′ (and only 5′) terminus of *Microuli* are identical to the TIR of *Wuneng*, a previously characterized MITE in *Aedes aegypti* (Tu, 1997). Both *Microuli* and *Wuneng* insert specifically into the TTAA target. It has been suggested that MITEs and the autonomous DNA transposons share the same transposition machinery based on common TIRs (Feschotte *et al*., 2002). Then how did *Microuli* transpose without the TIRs? The three subterminal direct repeats could potentially be the binding sites for transposases because subterminal inverted repeats and subterminal direct repeats have been shown to bind transposases in several autonomous DNA transposons (Morgan and Middleton, 1990; Beall and Rio, 1997; Becker and Kunze, 1997). It remains unclear how the termini of *Microuli* are determined at the strand cleavage step without the TIR. The TTAA target duplication plus a 3 bp TIR are essential for the excision of the autonomous transposon *piggyBac* (Bauser *et al*., 1999). Therefore, it is possible that *Microuli* may also be able to use the TTAA target sequence as part of the signal for recombination. It is tempting to hypothesize that some MITEs could evolve from MSITEs through mutation and/or recombination events at the termini which would result in TIRs.

4.12.4.3.3. *Helitrons* *Helitrons* have been found in *D. melanogaster* and *Anopheles gambiae*, which use a rolling-circle mechanism of transposition (Kapitonov and Jurka, 2001; Kapitonov and Jurka, 2003b) (**Table 2**). Insect *Helitrons* has several characteristics including short specific terminal sequences (5′ TC and 3′ CTAG), a 3′ hairpin, and the lack of TSDs. Instead of a cut-and-paste transposase, *Helitron1* in *A. gambiae* encodes an intronless protein including domains similar to helicase and replication initiation protein. There are approximately 100 copies of *Helitron* elements in *A. gambiae* that form 10 distinct families (Kapitonov and Jurka, 2003b).

In summary, the diversity demonstrated by insect TEs encompasses all classes and types of eukaryotic elements. Analyses of insect TEs have more than once led to discoveries that broadly impacted the field of TE research. Because of extensive genetic studies, *D. melanogaster* has long served as the launching pad for the discovery of novel families

of TEs in eukaryotes. The advent of genomics has provided the opportunity for the discovery of novel TEs in a wide range of organisms including non-drosophilid insects. As demonstrated above, greater TE diversity may be revealed and new insights may be gained from these genomic analyses.

4.12.5. Search for Active TEs in Insects

Active TEs may be used as tools for genetic manipulation of insects in basic and applied research (see Section 4.12.9). In addition, the behavior of TEs in host genomes and their spread in natural populations may be studied by monitoring active TE families. It is therefore highly desirable to isolate active copies of TEs. As described above (see Section 4.12.3), TEs discovered from observations of genetic mutations tend to result from active transposition events. Although several active TEs were discovered in this manner, this discovery process relies heavily on fortuitous events. Several methods that, when used in concert, could provide a systematic approach to uncover active TEs in insect genomes are described below.

4.12.5.1. Identification of Potentially Active TEs on the Basis of Bioinformatics Analysis

As discussed above, the ongoing genome revolution has produced an immense amount of sequence data from which diverse TEs can be identified in various insect genomes. The computational programs described above (see Section 4.12.3) can greatly facilitate the discovery and characterization of a large number of TE families. Unfortunately, the vast majority of TEs have accumulated inactivating mutations during evolution, rendering the discovery of active TEs the difficult task of “finding needles in a haystack.” Bioinformatics analysis can provide leads to potentially active candidates that can be studied further. For example, using a semi-automated reiterative search strategy, many potentially active families of non-LTR retrotransposons in the *A. gambiae* genome were identified (Biedler and Tu, 2003). Here candidate families were identified based on sequence characteristics, which include the presence of full-length elements, intact ORFs, multiple copies with high nucleotide identity, and the presence of TSDs. High nucleotide identity indicates recent amplification from a source element, without enough time for divergence caused by nucleotide substitution and other mutations. It should be emphasized that sequence analysis can only provide leads for further analysis. For example, high sequence identity between copies of a TE family may not always indicate recent transposition activity because it can also result from gene conversion events.

4.12.5.2. Detection of TE Transcription

Transcription is a required step during transposition of the RNA-mediated TEs. Although DNA-mediated TEs do not use RNA as an intermediate, transcription is required for production of transposase proteins. Therefore, the detection of transcription may offer further support for an active family in both classes of TEs. Transcription can be inferred if a match was found in an expressed sequence tag (EST) database to a TE sequence from the same organism. For example, 21 families of non-LTR retrotransposons had significant hits when BLAST searches were carried out against over 94 000 *A. gambiae* ESTs downloaded from NCBI (Biedler and Tu, 2003). Transcription of TEs can also be detected experimentally by real time polymerase chain reaction (RT-PCR), Northern blot, and even microarray. The source of mRNA may affect the outcome of these experiments because the activity of some TEs may be temporally and spatially controlled. It has been shown that TE activity can be elevated during the culturing of mammalian and plant cells (Wessler, 1996; Grandbastien, 1998; Liu and Wendel, 2000; Kazazian and Goodier, 2002). Different cell lines are available for a number of insect species. One caveat of the above approach is that, transcripts shown by either experimental detection or EST analysis could arise from spurious transcription. These transcripts could originate by transcription from a nearby host promoter.

4.12.5.3. Detection of Transposition Events by TE Display

TE display (Van den Broeck *et al*., 1998; Casa *et al*., 2000; Biedler *et al*., 2003) is a sensentive and reproducible experimental method to detect TE insertions. We have used it recently to study insertion site polymorphisms of endogenous insect TEs (Biedler *et al*., 2003) (see Sections 4.12.7 and 4.12.9). It is a modified form of amplified fragment length polymorphism (AFLP), the difference being that one primer is designed according to a TE sequence (**Figure 9**). First, genomic DNA is digested using a four-base restriction enzyme such as BfaI and then ligated to an adapter sequence. This is followed by two rounds of PCR. During the second PCR, a radioactive-labeled nested primer specific for the TE sequence is used following a touch-down protocol. After the second PCR, products are run on a sequencing gel and visualized by autoradiography. TE display is a powerful tool for genome-wide analysis of TE insertions and for detection of new insertions due to transposition (De Keukeleire *et al*.,

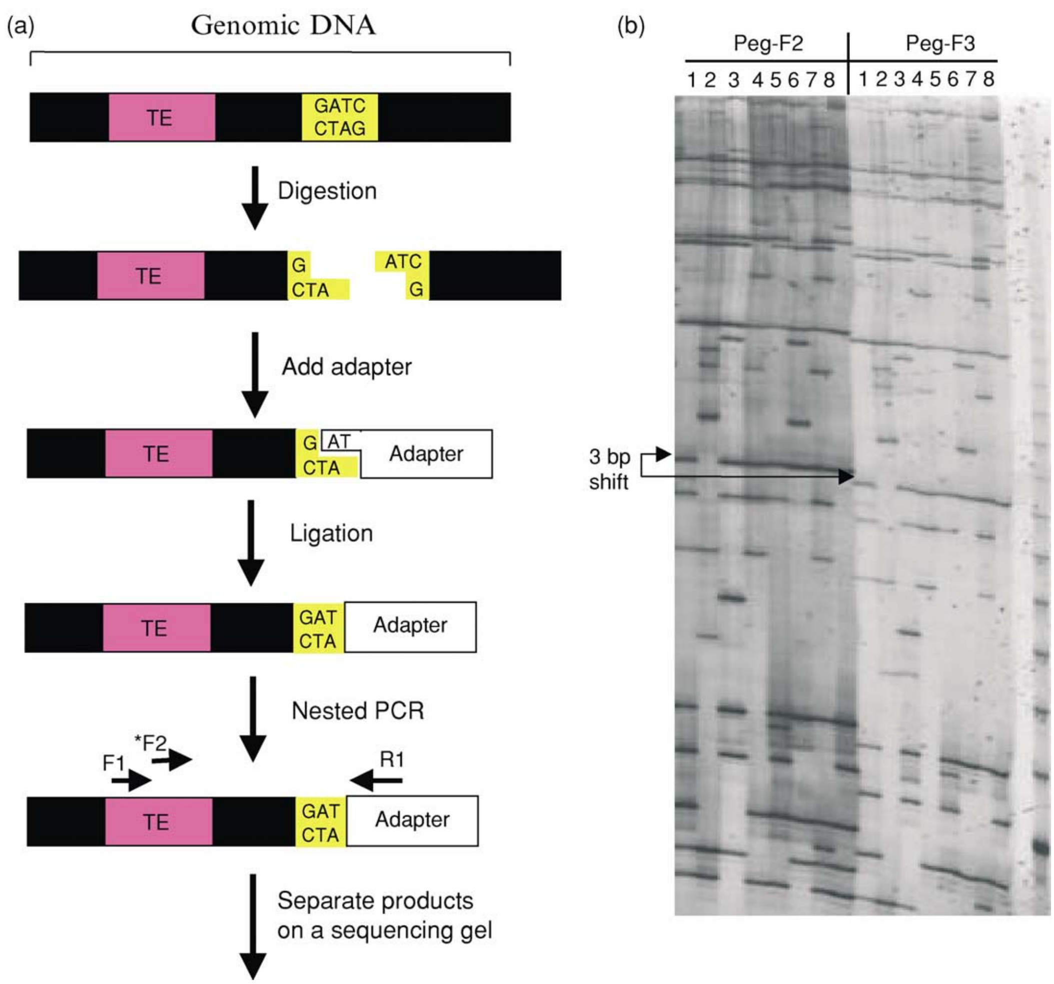


Figure 9 TE display, a method to scan multiple insertion sites of a TE in the genome. (a) Principle of TE display, which is a modified form of amplified fragment length polymorphism (AFLP). The difference is that TE-specific primers (F1 and F2) are used in addition to the adaptor primer (R1). F2 is labeled as shown by the asterisk. (b) Partial image of a TE display using primers for the *Pegasus* element with eight female individuals from an *Anopheles gambiae* colony (GAMCAM) originally collected from Cameroon (Biedler *et al.*, 2003). The eight samples on the left are amplified with a *Pegasus*-specific primer Peg-F2. The eight samples on the right are the same as those on the left except they were amplified with primer Peg-F3, which is designed to amplify a product smaller by three bases. The three base shift is clearly observable. A 10 bp ladder is shown on the right. Bands from a TE display gel were reamplified and sequenced, showing that they contained *Pegasus* sequences as well as flanking genomic and adapter sequences in the expected order (not shown). Comigrating bands among different individuals had the same flanking genomic sequence, indicating that they were from the same genomic locus. TE displays have been also developed using two highly reiterated SINEs, *SINE200* in *A. gambiae* and *Feilai* in *Aedes aegypti* (not shown). (Modified from Biedler, J., Qi, Y., Holligan, D., Della Torre, A., Wessler, S., *et al.*, **2003**. Transposable element (TE) display and rapid detection of TE insertion polymorphism in the *Anopheles gambiae* species complex. *Insect Mol. Biol. 12*, 211–216.)

2001). It offers a higher degree of sensitivity and resolution than genomic Southern blot analysis.

TE display has been used to detect somatic cell transposition (De Keukeleire *et al.*, 2001), simply by looking for the presence of new bands that represent newly transposed copies of a TE. A caveat of this approach is that a change in a restriction site may also result in new TE display bands. The same method may also be used to identify germline transposition by comparing TE display patterns of parent insects with the patterns of a large number of offspring. Alternatively, one could take advantage of the possibility that some TEs are activated in cell culture. Using TE display, one may be able to identify active families by comparing the relative abundance of a TE in cultured cells with that in individuals from different strains of the same species (Jiang *et al.*, 2003). This approach is based on the assumption that some TE families may be more active in cultured cells than in live organisms.

After TE display, one side of the new insertional copy and its associated flanking sequence can be recovered because the band can be reamplified and sequenced. To recover the entire sequence of the

newly inserted copy, one has to rely on mapping the insertion site to the genome if the genome sequence is available. Otherwise, further PCR or screening of a genomic library is necessary to recover the entire copy and both sides of the flanking sequence. The identified insertional copy itself may or may not be active because it could have been mobilized by a *trans*-acting protein that is encoded by an active TE in the genome. However, the experiment described above can demonstrate *in vivo* or *ex vivo* transposition events and the recovered insertional copy may lead to the identification of the autonomous active TE on the basis of shared *cis*-acting sequences such as TIRs.

4.12.5.4. Detection of Transposition Events by Inverse PCR

Actively transposing DNA-mediated TEs can be identified as extrachromsomal DNA in the form of linear or circular intermediates or byproducts (Arca *et al.*, 1997; Gorbunova and Levy, 1997) (see **Chapter 4.13**). Using a set of outward-orienting primers within the TE, the circular extrachromosomal copies may be amplified, which could serve as evidence of active excision or transposition. However, head-to-head copies of the same TE in the genome could also produce PCR products when outward-orienting primers are used, which must be ruled out by sequencing and further analysis. Extrachromosomal circles of *Hermes* (see **Chapter 4.13**) and *Pegasus* (Coy and Tu, unpublished data) have been identified. Imprecise excision had occurred when *Pegasus* is excised from its genomic location within the *A. gambiae* genome.

4.12.5.5. Transposition Assay, Reconstruction, and Genetic Screen

Transposition assays can be used to directly assess the functionalities of both the *cis*-(TIRs) and the *trans*- (transposase) components of a DNA transposon, allowing the demonstration of autonomous transposition events (see **Chapter 4.13**). In addition, transposition assays have also been established for the detection of retrotransposition of non-LTR retrotransposons (Jensen *et al.*, 1994; Ostertag *et al.*, 2000). Recently, a molecular reconstruction approach has been developed to restore inactivated copies of a vertebrate transposon, *Sleeping Beauty* (Ivics *et al.*, 1997), but such an approach requires extensive phylogenetic analysis and elaborate reconstructions (Ivics *et al.*, 1997). As an alternative, a genetic screen based on a bacterial system has been developed to identify hyperactive copies of an insect *mariner* transposon among randomly mutated copies (Lampe *et al.*, 1999). This approach can potentially be used to screen for active copies of transposons that do not require specific host factors. In summary, the progress in insect genome projects and the development and application of the methods described in this section will greatly facilitate searches for active TEs. The task of "finding needles in a haystack" could potentially be replaced by targeted and more efficient investigations.

4.12.6. Evolution of Insect TEs

The evolutionary dynamics of TEs are complex, which is in part due to their replication and their interactions with the host genome. The intricate dynamics between TEs and their host genomes are further complicated by the ability of some TEs to cross species barriers and spread in a new genome by horizontal transfer. Horizontal transfer may be an important part of the life cycle of some TEs and contribute to their continued success during evolution (Silva *et al.*, 2004). While the broad distribution of both RNA-mediated TEs and DNA-mediated TEs in all eukaryotic groups is evidence of the long-term evolutionary success of TEs, the two TE classes may have adopted different strategies, for which several insect TEs in both classes provide good examples.

4.12.6.1. Genomic Considerations of TE Evolution

It has been hypothesized that TE insertions may present three types of potentially deleterious effects including: (1) insertional mutagenesis which may disrupt gene function and/or regulation; (2) transcriptional/translational cost of the production of TE transcripts and proteins; and (3) ectopic recombination between homologous copies of TEs in different chromosomal locations that may result in duplication, deletion, and a new linkage relationship between genes (Nuzhdin, 1999; Bartolome *et al.*, 2002; Kidwell and Lisch, 2002; Rizzon *et al.*, 2002; Petrov *et al.*, 2003). The costs of having TEs may also include the cost associated with DNA replication when TEs occupy a large fraction of the genome. Obviously, these hypotheses are not mutually exclusive. This section discusses the intragenomic dynamics of TE–host interaction. The population dynamics affecting the spread of TEs in insects, which is also important for TE evolution, will be discussed below (see Section 4.12.7).

4.12.6.1.1. Self-regulation of insect TEs Both TE-driven mechanisms (self-regulation) and host-driven mechanisms (host control) have been shown to affect TE activities in insects. Self-regulation has been shown for *mariner* and *P* elements in *Drosophila*

(Hartl *et al.*, 1997; Kidwell and Lisch, 2001). In the case of the *Drosophila P* element, self-regulation is achieved through the activities of at least two types of element-encoded repressors. In the case of *mariner*, several mechanisms may be involved including overproduction inhibition (an increase in the amount of transposase results in a decrease in net transposase activity), missense mutation effects (defective transposase encoded by missense copies interfering with functional transposase), and titration effects by inactive copies. In this regard, it is interesting to note that several hyperactive mutants of an active *mariner*, originally discovered in the horn fly, have been isolated (Lampe *et al.*, 1999). This suggests that the horn fly *mariner* has not evolved for maximal activity.

4.12.6.1.2. Host control of TEs in insects Host control of TE activity can either be targeted at a particular family of TEs or a large group of TEs in general. Family-specific host control has been demonstrated in several cases (Labrador and Corces, 2002). The best example so far is the control of *gypsy* activity by a genetic locus *flamenco* in *D. melanogaster* (Bucheton, 1995). Homozygous female mutants of *flamenco*, which is an X-linked recessive gene that represses the transposition of *gypsy*, produce progeny that show high rates of *gypsy* transposition. The relief of the suppression of *gypsy* activity in *flamenco* mutants, which are also called permissive mutants, functions through maternal factors. Another example of host control is the *P* element which only transposes in the germline because correct splicing of the *P* transcript only occurs in germ cells. The tissue-specificity of the *P* element allows it to transmit efficiently to the next generation while minimizing potential damage to the host. Two host genes have been implicated in the inhibition of *P* activity in somatic cells (Siebel *et al.*, 1992, 1995).

RNA interference (RNAi), a mechanism that confers posttranscriptional degradation of mRNA on the basis of homology to small fragments of double-stranded RNA, has been implicated as a host defense mechanism against a broad spectrum of TEs in the nematode *Caenorhabditis elegans* (Ketting *et al.*, 1999; Tabara *et al.*, 1999). RNAi has shown to function in *Drosophila* and a few other insects as well (Misquitta *et al.*, 1999; Hammond *et al.*, 2000). The potential role of RNAi as a general control method against TE activity in *Drosophila* has recently been proposed on the basis of cosuppression of the *I* element by an increasing number of *I*-related transgenes (Jensen *et al.*, 1999; Labrador and Corces, 2002). It has been proposed that RNAi has evolved as a host defense mechanism against the invasion by TEs and viruses (review: Fedoroff, 2002). In addition to posttranscriptional degradation, this defense system may also be involved in the formation of transcriptionally inactive heterochromatin where TEs and other repeats concentrate (Couzin, 2002).

4.12.6.1.3. Nonrandom distribution of insect TEs Patterns of nonrandom TE distribution have been shown in both *D. melanogaster* and *A. gambiae* (Bartolome *et al.*, 2002; Holt *et al.*, 2002; Kapitonov and Jurka, 2003b). TEs tend to accumulate in heterochromatin. Such a distribution bias could result either from preferential TE insertion, or selection against insertions in euchromatic regions, or both. Bartolome and colleagues suggested that the abundance of TEs is more strongly associated with local recombination rates (Bartolome *et al.*, 2002), which are low in heterochromatic regions, rather than with gene density. They argue that this association is consistent with the hypothesis that selection against harmful effects of ectopic recombination is a major force opposing TE spread. However, selection against insertional mutagenesis is also at work as shown by the absence of insertions in coding regions. The insertional bias of *P* elements has been demonstrated recently during genome-scale *P* mutagenesis analysis (Spradling *et al.*, 1995, 1999). Therefore insertion bias may contribute to the biased pattern of TE distribution in insects. A related topic here is the suggestion that concentrations of TE insertions in the *Drosophila* Y chromosome may have contributed to the evolutionary process leading to its inactivation (Labrador and Corces, 2002). It should be noted that not all TEs have a bias towards heterochromatic or recombination-deprived regions. On the basis of analysis of limited gene sequences, it was shown that *Aedes aegypti* MITEs tend to be associated with the noncoding regions within or near genes (Tu, 1997), which is similar to what has been observed for plant MITEs (Zhang *et al.*, 2000).

4.12.6.1.4. Autonomous and nonautonomous TEs In addition to the genomic interactions described above (see Section 4.12.6.1.3), most TEs have to contend with the fact that defective copies are often generated during or after transposition. This process could contribute to self-regulation as discussed above (see Section 4.12.6.1.1). It can also lead to total inactivation and ultimate extinction of a TE as the inactive TE population eventually overwhelms the active copies (Eickbush and Malik, 2002). Therefore, the replicative ability that is responsible for the success of the TE may also lead

to its inactivation in a genome. Interestingly, some nonautonomous TEs have been very successful with regard to amplification, although the mechanisms that contribute to their success are not entirely clear. For example, SINEs found in insect genomes including *Aedes aegypti*, *Anopheles gambiae*, and *Bombyx mori* (**Table 1**) all contain thousands of copies. Similarly, most of the MITEs found in insects are also highly reiterated although low-copy-number families are also found (Tu, 1997, 2000, 2001a). The small size of MITEs and SINEs may confer less deleterious effects on the host, either because they are less efficient substrates for homologous recombination (Petrov *et al*., 2003) or because their impact on neighboring genes may be less severe. Therefore reduced selection pressure as well as other properties inherent to MITEs and SINEs could contribute to their apparent success. It is a fascinating question as to how SINEs and MITEs affect the evolution of the autonomous TEs that mobilize them.

4.12.6.2. Vertical Transmission and Horizontal Transfer of Insect TEs

As described above (see Section 4.12.6.1), the replicative ability that is responsible for the success of a TE may in some cases lead to its inactivation by generating defective copies or by activating host control mechanisms. This is especially true for DNA transposons. Therefore, the ability to escape the above vertical inactivation by invading a new genome would greatly enhance the evolutionary success of DNA transposons. However, not all TEs have adopted this life cycle of invasion, amplification, senescence, and new invasion (Hartl *et al*., 1997; Eickbush and Malik, 2002; Robertson, 2002) (Figure 10).

4.12.6.2.1. Detection of horizontal transfer The occurrence of horizontal transfer can be supported in various degrees by three types of evidence (Silva *et al*., 2004). First, detection of elements with a high level of sequence similarity in divergent taxa will offer strong support for horizontal transfer although variable rates of sequence change should be considered. Second, detection of phylogenetic incongruence between TEs and their hosts will also provide relatively strong support for horizontal transfer. However, this alone will not be convincing, especially in light of the high levels of intragenomic diversity of TE families observed in insects. In other words, the existence of multiple TE lineages could confound the phylogenetic analysis as paralogous lineages may be treated as orthologous ones. Finally, horizontal transfer may be inferred when "patchy" distribution of a TE among closely related taxa is observed. This type of support is weak as loss of

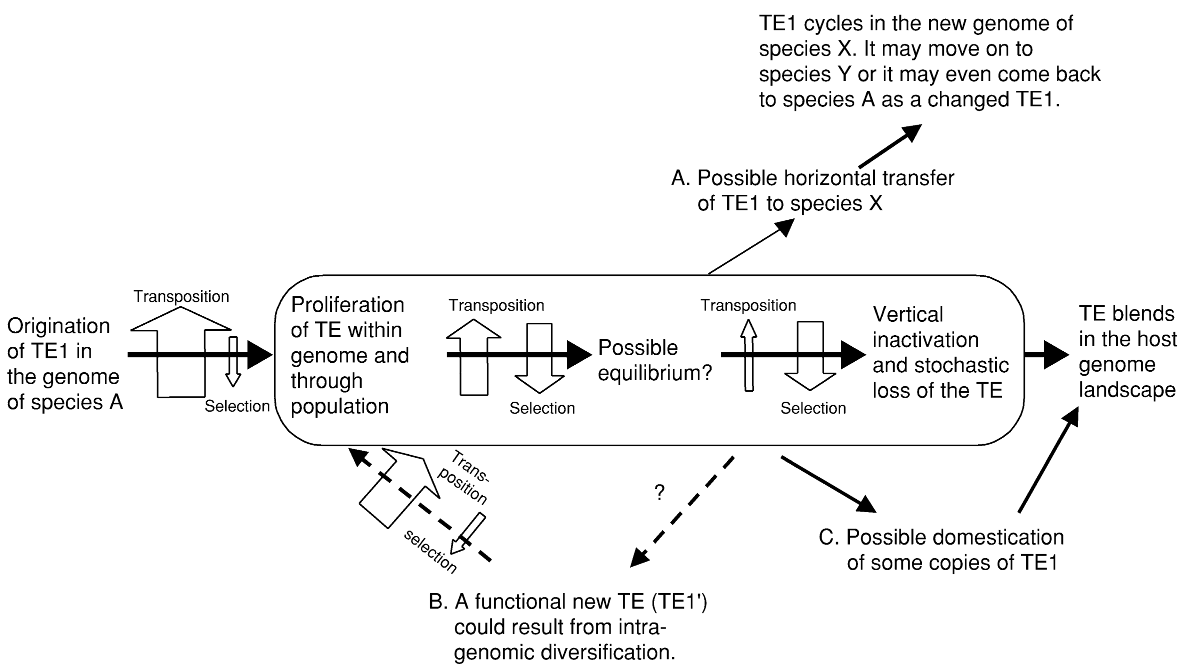


Figure 10 A model of the evolutionary dynamics of TEs in eukaryotic genomes. This hypothetical model incorporates recent work by several groups (Hartl *et al*., 1997; Lampe *et al*., 2001; Silva *et al*., 2004). Some aspects of this model are better suited for DNA transposons that generally have a high propensity for horizontal transfer. Three possible alternatives to inactivation and stochostic loss are highlighted (A, B, and C).

TEs from sister taxa may result from a phenomenon similar to assortment of an ancestral polymorphism (Silva *et al.*, 2004).

4.12.6.2.2. Horizontal transfer and vertical transmission in insects: differences between different groups of TEs The first case of eukaryotic horizontal transfer was reported in *Drosophila*, where the *P* element was shown to have invaded the *D. melanogaster* genome during the last century from a species in the *D. willistoni* group (review: Kidwell, 1992). Evidence for this lateral event includes all three types of support described above and is therefore widely accepted (Silva *et al.*, 2004). Further analyses of a large number of *P* element sequences from a number of *Drosophila* species showed that many horizontal transfer events must have occurred to account for the current distribution pattern of *P* in *Drosophila* (Silva and Kidwell, 2000; Silva *et al.*, 2004). Another spectacular case of horizontal transfer, which was also initially discovered in insects, involves the *mariner* transposons. The *mariner* transposon family has been implicated in hundreds or more horizontal transfer events among a wide range of animal species including a large number of insects across different orders (Robertson, 1993, 2002). It has been shown that the newly discovered *ITmD37E* transposons have also been involved in relatively frequent horizontal transfer events among different mosquito species (Shao and Tu, 2001, unpublished data). Finally, horizontal transfers of *hobo* and *piggyBac* has been shown in flies and moths (Bonnivard *et al.*, 2000; Handler and McCombs, 2000). It has been proposed that horizontal transfer may be a key link in the life cycle of those DNA TEs that are not intricately associated with their host biology, allowing them to escape the vertical inactivation and stochastic loss (Hartl *et al.*, 1997). Thus, these types of TEs may be regarded as "resident aliens" in a host (Plasterk *et al.*, 1999). It has been shown that selection of some *mariner* transposases acts only during horizontal transfer, which is consistent with the invasion–inactivation–escape model (Lampe *et al.*, 2003).

Horizontal transfer has also been shown for LTR retrotransposons in insects. A clear example involves the *copia* element in *Drosophila* (Jordan *et al.*, 1999). The *copia* elements in *D. melanogaster* and *D. willistoni*, two divergent species that separated more than 40 million years ago, showed less than 1% nucleotide difference. It appears that *copia* jumped from *D. melanogaster* to *D. willistoni*. Horizontal transfer of the *Drosophila gypsy* element has also been reported (Terzian *et al.*, 2000; Vazquez-Manrique *et al.*, 2000).

Possible horizontal transfer of the *Drosophila* non-LTR retrotransposon *Jockey* was suggested on the basis of phylogenetic incongruence (Mizrokhi and Mazo, 1990). However, Malik and colleagues recently showed that an analysis that takes into account the rates of evolution was consistent with vertical transmission of the *Jockey* elements in *Drosophila* (Malik *et al.*, 1999). Eickbush and Malik further suggest that no convincing evidence exists for the horizontal transfer of any non-LTR retrotransposon during the past 600 million years (Eickbush and Malik, 2002). In addition, strict vertical transmission of two non-LTR retrotransposons *R1* and *R2* have been shown in *Drosophila* (Eickbush and Eickbush, 1995). In these cases, the TE phylogenies well reflect those of the host species. Furthermore, it was shown that the relationship between *R2* elements from several insect orders including Diptera, Lepidoptera, Coleoptera, and Hymenoptera is consistent with vertical transmission (Eickbush, 2002).

4.12.6.2.3. Possible reasons for differing propensities for horizontal transfer Two reasons have been proposed to explain the apparent differences in the prevalence of horizontal transfer events between DNA transposons and non-LTR retrotransposons (Eickbush and Malik, 2002). The first is that DNA transposons need horizontal transfer for their long-term survival but non-LTR retrotransposons appear not to be dependent on such rare evolutionary events. Defective copies of DNA transposons retain the ability to be transposed as long as they have the *cis*-acting signals such as the TIRs. This indiscrimination leads to the inevitable fate of inactivation of the entire transposon family. Therefore, horizontal transfer offers a much-needed escape from the above vertical inactivation, which greatly enhances the evolutionary success of DNA transposons. On the other hand, it has been shown that the RT of non-LTR retrotransposons tends to associate with the mRNA molecules from which they were translated (Wei *et al.*, 2001). This *cis*-preference would bias transposition events in favor of the active elements, thus providing a mechanism to sustain the non-LTR retrotransposons. However, the *cis*-preference is not enough to prevent the highly successful retrotransposition of SINEs in insects (Adams *et al.*, 1986; Tu, 1999) and other organisms (Lander *et al.*, 2001), which presumably borrows the retrotransposition machinery from non-LTR retrotransposons. It will be interesting to see how SINEs affect the evolution of their

non-LTR retrotransposon "partners." The second explanation is that DNA transposons may be more predisposed to horizontal transfer than non-LTR retrotransposons. The transposition process of DNA transposons involves an extrachromosomal DNA intermediate, which may facilitate horizontal transfer (Eickbush and Malik, 2002) (see Section 4.12.6.2.4). DNA transposons use their transposase for integration, which may be less dependent on host repair machinery than non-LTR retrotransposons and thus not as restricted to its original host.

LTR retrotransposons form an extrachromosomal DNA intermediate and use transposase-like IN for integration. Therefore, LTR retrotransposons have access to the same horizontal transfer mechanisms as the DNA transposons although their life cycle may not require horizontal transfer because defective copies are not thought to be a major factor (Eickbush and Malik, 2002). It should be noted that the above are general statements and that the propensity for horizontal transfer may vary among individual families within the three groups discussed here.

4.12.6.2.4. Mechanisms of horizontal transfer Mechanisms of horizontal transfer are poorly understood although direct transfer of the extrachromosomal DNA intermediate and indirect transfer through a viral vector have been proposed as possible mechanisms (Eickbush and Malik, 2002; Silva *et al*., 2004). Geographical and temporal overlap between the donor and recipient host species may be essential. An intriguing case of horizontal transfer of a *mariner* element between a parasitoid wasp and its lepidopteran host offers a good example of such overlap (Yoshiyama *et al*., 2001).

4.12.6.3. Other Possible Evolutionary Strategies

In addition to horizontal transfer and vertical extinction, recent studies suggest that there might be a third way, or an alternative strategy, which may be adopted by some TEs (Lampe *et al*., 2001). On the basis of the loss of interaction between *mariner* transposons of slightly changed TIRs, it was proposed that intraspecific or intragenomic diversification of *mariner* transposons may allow the newly diverged *mariner* to start a new lineage. Although this requires the coevolutionary events to occur in both the transposase and the TIRs, this scenario would provide the transposon the opportunity to escape vertical inactivation because it is now virtually a brand new element in a virgin genome because of the loss of interaction between itself and its relatives in the genome. Genome sequencing has provided increasing opportunity to survey the diversity of different families of TEs. Our recent analysis showed a large number of lineages of non-LTR retrotransposons of the *CR1* and *Jockey* clades in *A. gambiae* (Biedler and Tu, 2003) (**Figure 7**). Given the presence of multiple recently active lineages within the *CR1* and *Jockey* clades, it is tempting to speculate that the observed diversity may be driven by competition among different non-LTR families or by attempts to escape suppressive mechanisms imposed by the host.

On the other hand, some TEs are recruited for host functions and thus become "domesticated" (Lander *et al*., 2001; Kidwell and Lisch, 2002). This type of molecular domestication is the ultimate case of trading "freedom" for "security." It allows TEs to sustain and positively impact the host, examples of which will be discussed below (see Section 4.12.8). Strictly speaking, these domesticated TEs are no longer TEs. However, it is theoretically possible that these "domesticated" TEs could revert back to their "old ways" on rare occasions.

4.12.6.4. Understanding the Intragenomic Diversity of Insect TEs

High levels of TE diversity have been reported in the two insect genomes in which large-scale systematic analyses have been carried out (Holt *et al*., 2002; Kaminker *et al*., 2002; Kapitonov and Jurka, 2003b; Biedler and Tu, 2003) (**Figures 4** and **7**). The evolutionary process that generated this diversity may also be quite diverse. It is possible that the evolution of some TEs may be a complex mix of both vertical transmission and horizontal transfer events. Parsing out the results of intragenomic diversification from those of horizontal transfer events may require additional data from related species. Understanding the process responsible for the intragenomic diversity of insect TEs and the potential interactions between different TE families in insect genomes will be both challenging and rewarding. A summary of the current hypothesis on TE evolution is illustrated in **Figure 10**. Some aspects of this model are better suited for DNA transposons that have a high propensity for horizontal transfer.

4.12.7. TEs in Insect Populations

4.12.7.1. Fundamental Questions and Practical Relevance

In general, the increase of TE copy number through transposition is balanced by selective forces against the potential genetic load of TEs on host fitness (Nuzhdin, 1999). The control of transposition rate of TEs and other mechanisms to minimize their deleterious effects has been discussed (see Section 4.12.6). The population dynamics affecting the

spread of TEs in insect genomes is described. Earlier work on TEs in *Drosophila* populations suggest that the copy numbers in euchromatic regions are low and most euchromatic copies exist at very low frequency (<5%) in the population (Petrov *et al.*, 2003). This is interpreted as evidence of selection against individual TE copies in nature. It has been hypothesized that this selection is against three types of potentially overlapping deleterious effects by TEs including insertional mutagenesis, transcriptional or translational cost, and ectopic recombinations between similar copies of TEs in different chromosomal regions (Nuzhdin, 1999; Bartolome *et al.*, 2002; Kidwell and Lisch, 2002; Petrov *et al.*, 2003). One of the major questions in this field has been parsing out the main factors containing the spread of TEs in natural populations. From an applied perspective, TEs have been proposed as tools to genetically drive the spread of beneficial genes through insect populations to control infectious diseases (e.g., Ashburner *et al.*, 1998; Alphey *et al.*, 2002). For such a sophisticated approach to work, it is important to understand the population dynamics of TEs in their insect hosts.

4.12.7.2. Experimental Approaches

In situ hybridization of the *Drosophila* polytene chromosomes has been the main workhorse in studies of the population dynamics of different TE families (Charlesworth and Langley, 1989). Although extremely useful, this method obviously only works in species with accessible polytene chromosomes and it is not efficient in detecting short regions of sequence similarities to the probe (Petrov *et al.*, 2003). An alternative method, genomic Southern blotting, was also used to study TE insertions and excisions in *Drosophila* (Maside *et al.*, 2001). Although Southern blotting is a good method to estimate TE copy numbers, it is not reliable when the numbers are high. In addition, it may not be able to detect low-frequency sites in cases where multiple small insects have to be pooled to obtain enough high-quality genomic DNA. However, a single *Drosophila* can provide enough DNA for a Southern blot experiment.

TE display, a genome-scale detection method for TE insertions (see Section 4.12.5.3), has been used recently in *Drosophila* and two sibling species of mosquitoes *A. gambiae* and *A. arabiensis* (Biedler *et al.*, 2003; Yang and Nuzhdin, 2003). Because it is a PCR-based method, TE display allows investigation of multiple TE families using genomic DNA isolated from an individual insect. Although extremely powerful, this method cannot reliably distinguish homozygous from heterozygous insertions. However, TE display will allow recovery of a specific insertion site by sequencing the corresponding band. The sequence flanking the TE copy can be used to locate the specific site by searching the genome database if available or by inverse PCR. Therefore, sequences flanking a TE at a specific locus can be used as primers to amplify genomic DNA isolated from an individual sample (**Figure 11**). When the PCR products are run on an agarose gel, individuals with insertions at both alleles will show a single high molecular mass band while individuals with no insertions at either allele will give a single low molecular mass band (**Figure 11**). Individuals that have heterozygous alleles will yield both bands. Thus this locus-specific approach is codominant. When the genome sequence or a bulk of genomic sequences are available for an insect species, it is not absolutely necessary to couple locus-specific PCR with TE display because a number of TE insertion sites will already have been available for analysis (Petrov *et al.*, 2003). However, TE display can facilitate the investigation by providing a rapid scan of a large number of loci and by providing initial assessment of the level of polymorphism.

4.12.7.3. Recent Advances

Population studies, mainly of *Drosophila* TEs, suggest that selection against ectopic recombination may be the major factor in containing TE copy number in nature (Bartolome *et al.*, 2002; Kidwell and Lisch, 2002; Rizzon *et al.*, 2002; Petrov *et al.*, 2003). However, this issue apparently is quite complex (Biemont *et al.*, 1997). Although there appears to be evidence rejecting the effect of TE expression as a selection mechanism (Yang and Nuzhdin, 2003), the relative importance of insertional mutagenesis as a force to contain TE spread in *Drosophila* populations is still debated (Biemont *et al.*, 1997; Bartolome *et al.*, 2002; Petrov *et al.*, 2003).

TE insertions were shown to be generally at very low frequencies in natural populations of *D. melanogaster* (Charlesworth and Langley, 1989), which is consistent with the hypothesis of natural selection acting against the TE copies. However, recent surveys in *D. melanogaster* revealed an unexpected number of sites that are either fixed or of high frequency (Petrov *et al.*, 2003; Yang and Nuzhdin, 2003). In addition, an analysis of a retrotransposon *Osvaldo* in *D. buzzatii* (Labrador *et al.*, 1998) has shown a number of euchromatic sites with high occupancy in all populations from the Iberian Peninsula. The authors suggest that genetic drift is

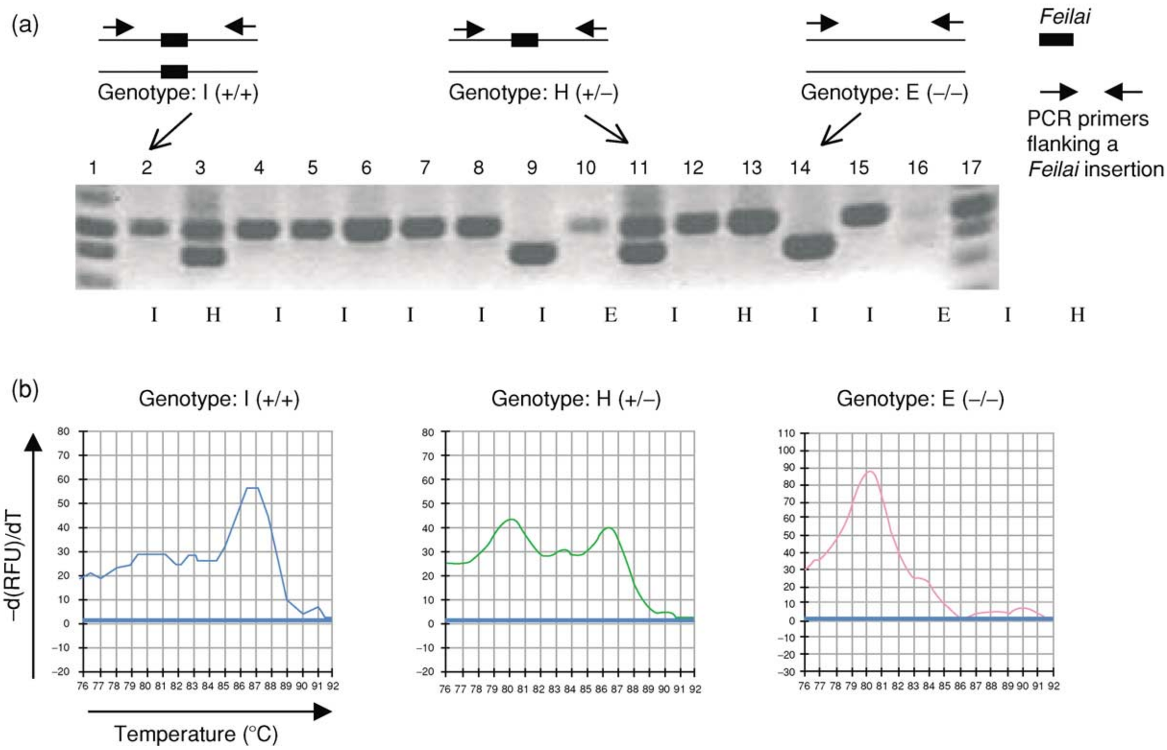


Figure 11 Locus-specific PCR for the detection of SINE insertion polymorphism. Shown here are images from analysis of a *Feilai* insertion in the yellow fever mosquito, *Aedes aegypti*. (a) Different genotypes revealed by PCR and agarose gel electrophoresis. Lanes 1 and 17 are size markers. Lanes 2–16 are PCR products from 15 individual mosquitoes collected in Thailand. Three genotypes and their corresponding banding patterns are shown. I, homozygous insertion (+/+); E, homozygous empty (−/−); H, heterozygotes (+/−). (b) The same three genotypes at the same *Feilai* insertion site detected using melt-curve analysis on an iCycler from Bio-Rad (Hercules, CA). The peaks represent PCR products. Higher melting temperature reflect larger and/or GC-rich PCR products. −d(RFU)/dT represents the rate of change of the relative fluorescence units (RFU) with time (T).

the main force responsible for the high frequency distribution of *Osvaldo* as a result of the founder effect. Using TE display, it has been found that fixed and high-occupancy sites of MITEs and SINEs are common in *A. gambiae* and *Aedes aegypti* strains and natural populations (Qi and Tu, unpublished data). The small size of MITEs and SINEs may confer less deleterious effects on the host either because they are less efficient substrates for ectopic recombination (Petrov *et al.*, 2003) or because their impact on neighboring genes may be less severe. Further analyses of different types of TEs in the natural populations of a wide range of insects will allow us to examine the validity and applicability of some of the fundamental conclusions drawn from the analysis of *Drosophila* TEs. The use of TE display and the advent of insect genomics provide an exciting opportunity for such investigations.

It may be reasonable to assume that TE copy number and insertion frequency in a population are highly dynamic parameters that can be influenced by TE-specific factors such as their intrinsic ability for transposition and self-regulation, by species- or genome-specific factors such as deletion and recombination rate and genomic control of transposition, and of course by effective population size and other ecological factors. As most population surveys only reflect a cross-section during evolution, the relative stage of a TE in its life cycle (**Figure 10**) is also an important consideration (Vieira *et al.*, 1999).

4.12.8. Impact of TEs in Insects

4.12.8.1. TEs and Genome Size and Organization

TEs are integral and significant components of eukaryotic genomes (Berg and Howe, 1989; Sherratt, 1995; Kidwell and Lisch, 2000; Craig *et al.*, 2002). For example, at least 46% of the human genome is TE-derived sequences (Lander *et al.*, 2001). In the two "completed" insect genomes, TEs occupy approximately 22% in *D. melanogaster* (Kapitonov and Jurka, 2003b) and at least 16% of the euchromatic region in *Anopheles gambiae* (Holt *et al.*, 2002). It has been proposed that the differing TE abundance may account for the "C value

paradox," which reflects the discrepancy between genome size or DNA content of an organism (as indicated by the C value) and its biological complexity (Kidwell, 2002). In other words, organisms with similar genetic/biological complexity may have huge variations in genome size due to differences in TE content. In higher eukaryotes, the interspersed repetitive sequences such as TEs are often organized in two distinct patterns relative to single copy DNA. One pattern of organization is the "short period interspersion" where single copy DNA is often interrupted every 1–3 kb by repetitive elements (Davidson *et al.*, 1975). The other is the "long period interspersion" in which single copy DNA in the euchromatic region is less interrupted by repetitive sequences (Davidson *et al.*, 1975). These two different patterns are probably shaped by the diversity, varied distribution, and abundance of repetitive sequences in the genome.

As noted above, TEs occupy approximately 22% of the *D. melanogaster* genome, which is threefold higher than previously reported (Kapitonov and Jurka, 2003b). Most TEs in *D. melanogaster* are relatively young (<20 million years old) and they consitute a major component of the paracentromeric regions. Detailed comparisons of TE age, distribution, and abundance are under way between the *D. melanogaster* and the *A. gambiae* genomes. Further comparisons that include other genomes such as *Aedes aegypti*, *Apis mellifera*, and *Bombyx mori* will be extremely informative. Some preliminary information gleaned from a comparative analysis of different species of mosquitoes are briefly described. Genome size and organization vary significantly between different mosquitoes. The genome of *Anopheles gambiae* is 270 Mb in size, and it is organized in a pattern of "long period interspersion" (Rai and Black, 1999). In contrast, the *Aedes aegypti* genome is 800 Mb in size, and it is organized in a pattern of mainly "short period interspersion" (Warren and Crampton, 1991). The relative abundance of MITEs and SINEs in these two mosquito genomes is interesting. The copy number of *Anopheles gambiae* MITEs ranges from 40 to 1340, much lower compared with 400 to 10 000 copies of MITEs in *Aedes aegypti* (Tu, 1997, 2000, 2001a). Similarly, the *Aedes aegypti* SINE family *Feilai* has 60 000 copies while *SINE200* in *Anopheles gambiae* has fewer than 6000 copies (Tu, 1999, unpublished data). The difference in the relative abundance of MITEs and SINEs may have contributed to the different organizations of the mosquito genomes and reflect different types of interactions between the hosts and these widespread TEs. Because of their small size, the copy number differences in MITEs may not explain the bulk of differences in the size of these two mosquito genomes. The relative abundance of other TEs, different deletion rates, simple repeat expansion, and large-segment duplication are among other possible contributing factors (Petrov, 2001). In *Anopheles gambiae*, TEs including MITEs are significantly more concentrated in heterochromatic regions (Holt *et al.*, 2002). Alternatively, indications that a few families of MITEs are associated with the noncoding regions of genes in *Aedes aegypti* are presented (Tu, 1997). It is possible that unlike many other TEs, MITEs may be tolerated in gene-rich euchromatic regions in *A. aegypti*. MITEs have been found near plant genes where they could potentially affect gene regulation and/or define chromatin domains through structures such as matrix attachment regions (Wessler *et al.*, 1995; Tikhonov *et al.*, 2000). Varied amounts of repetitive elements have also been shown to be a major factor for the nearly threefold intraspecific differences of genome size in populations of the Asian tiger mosquito, *A. albopictus* (Rai and Black, 1999). Such an intraspecific variation provides a rare opportunity to study the contribution of TEs to genome evolution at the initial stage of the evolutionary process.

As noted before, TEs can induce chromosomal rearrangements through ectopic recombination and other mechanisms (Gray, 2000). Such rearrangements could result in gross reorganizations of the chromosomes, which may have a significant evolutionary impact as suggested by McClintock (McClintock, 1984). A DNA TE named *Odysseus* was found adjacent to the distal breakpoint of a naturally occurring paracentric chromosomal inversion that is characteristic of *Anopheles arabiensis*, one of the cryptic species in the *A. gambiae* complex (Mathiopoulos *et al.*, 1998). Similar evidence of TE involvement in chromosomal rearrangement has been reported in *Drosophila* (Lim and Simmons, 1994; Caceres *et al.*, 2001).

4.12.8.2. Evolutionary Impact

The tremendous potential for TEs to generate genetic diversity has long been recognized (McClintock, 1956). They can cause spontaneous mutation, recombination, chromosomal rearrangement, and hybrid dysgenesis (Bregliano *et al.*, 1980; Engels, 1989; Shiroishi *et al.*, 1993; Mathiopoulos *et al.*, 1998). However, the long-term evolutionary impact of TEs is less clear. TEs have been generally regarded as "selfish" DNA since the early 1980s (Doolittle and Sapienza, 1980; Orgel and Crick, 1980) as opposed to the original "controlling elements"

hypothesis stating that TEs provide the physical basis controlling gene action and mutation (McClintock, 1956). This change of attitude was mainly due to the realization that TEs are somewhat "independent" genetic units and that their replicative ability allows them to spread even when they are not beneficial to the host organism. The question of whether the "selfish" TEs are just "junk DNA" to the host, or whether they can play important and even adaptive roles in organismal evolution is at the heart of the debate. Thanks in part to recent studies of insect TEs, it is increasingly clear that there may be a middle ground between the "junk DNA" and the "controlling elements" hypotheses. The host genome can be viewed as an ecological community with complex host–TE and TE–TE interactions (Brookfield, 1995; Kidwell and Lisch, 2000). Given the opportunistic nature of the evolutionary process, a "selfish" element could develop a wide spectrum of relationships with their host. They could be a "junk parasite," a "molecular symbiont," or something in between.

TE insertions are often under negative selection and their activities are under tight control by themselves and their host genomes (Bucheton, 1995; Hartl *et al*., 1997; Lin and Avery, 1999). Therefore the mere presence of TEs may represent a powerful genetic force with which the genome has been evolving. The possibility that RNAi has evolved as a host defense mechanism against the invasion by TEs and viruses and the potential involvement of RNAi in the formation of transcriptionally inactive heterochromatin are good examples of the host reacting to TE activities (Couzin, 2002). DNA transposons such as the *P* element in *Drosophila* can induce mutations by insertion as well imprecise excision in the coding or regulatory regions of genes (Kidwell and Lisch, 2002). On a larger scale, TE insertions can serve as substrates for homologous recombination that can result in chromosomal deletion, duplication, and inversion. Polymorphic chromosomal inversions are common in *Drosophila* and *A. gambiae*. TEs are often implicated in the generation of these inversions (Kidwell and Lisch, 2002) (see Section 4.12.8.1). It is proposed that these natural chromosomal inversions may result in reproductive isolation and perhaps genetic changes that allow *A. gambiae* to exploit a range of ecological niches (Mathiopoulos *et al*., 1999).

Recent evidence also indicates that some TEs may be co-opted to contribute to organismal biology. One of the early examples supporting this concept was the discovery that the telomere in *Drosophila* was maintained by site-specific insertions of two retrotransposons *HET-A* and *TART* (Biessmann *et al*., 1992; Levis *et al*., 1993). In addition, some TEs such as MITEs are often found near genes where they may change the expression profile of nearby host genes (Tu, 1997). The recruitment of TE insertions to produce diversity in gene expression profiles may have a better chance to confer a selective advantage when it occurs in duplicated genes. A fascinating genome-wide analysis showed that retrotransposition was responsible for the creation of a significant number of new functional genes in *Drosophila* (Betran *et al*., 2002). Finally, Some TE copies can be "domesticated" and take on a host function (see Section 4.12.6.3). For example, one of the *P* element repeats appears to have evolved the function of transcription factors (Miller *et al*., 1995). Other examples of "domesticated" TEs found in noninsect species include the *RAG1* and *RAG2* genes involved in V(D)J recombination in vertebrate lymphocytes and the more than 40 new genes derived from TEs in the human genome (Kidwell and Lisch, 2000; Lander *et al*., 2001; Gellert, 2002). On the basis of their broad distribution in bacteria, archea, and eukaryotes (Craig *et al*., 2002), it is safe to assume that TEs have long been, and will continue to be, evolving together with the immensely diverse life forms on this planet.

4.12.9. Applications of Insect TEs

4.12.9.1. Endogenous TEs and Genetic Manipulation of Insects

P element-based transgenic and mutagenesis tools in *D. melanogaster* have played a major role in the tremendous success of this tiny fly as a model organism for genetic analysis. A limited number of DNA transposons such as *hobo*, *mariner, minos,* and *piggyBac*, which have a broader host range than *P*, are being developed as tools in insects (see **Chapter 4.13**). Further analysis of TEs in insect genomes may expand the pool of active DNA transposons, which may be used to generate a set of tools with diverse features that can be used collectively for a variety of genetic analysis in different insects. In addition to simply transforming an insect, active TEs mentioned above are used to construct specific vectors to be used in gene trapping, enhancer trapping, and genome-wide insertional mutagenesis studies (Spradling *et al*., 1999; Klinakis *et al*., 2000; Horn *et al*., 2003). These analyses are powerful new ways to investigate gene function and regulation on a genome scale.

In addition to providing new active TEs to be used as tools for genetic manipulations of insects, a better understanding of endogenous TEs will allow

better-informed usage of current transposon-based genetic tools. Interactions between exogenous and endogenous transposons that share similar TIRs have been shown to be a potential problem (Sundararajan *et al.*, 1999; Jasinskiene *et al.*, 2000). Such interactions could be significant in light of the discovery of a diverse range of DNA TEs in a few insects in which genetic manipulation is actively pursued (Tu, 2001a; Holt *et al.*, 2002; Mao and Tu, unpublished data). For example, the diverse families of endogenous MITEs and DNA transposons could act as potential substrates for transposition if the introduced transposon uses similar TIRs. Thus, analyses of endogenous insect TEs will lead to better-informed design of transposon-based transformation tools that reduce instability resulting from interactions with endogenous TEs (Ashburner *et al.*, 1998; Atkinson *et al.*, 2001). It is also hoped that the non-Mendelian inheritance of TEs could help beneficial transgenes sweep through insect populations as the *P* element did in *Drosophila* (Ribeiro and Kidwell, 1994; Engels, 1997). Such a strategy is being investigated in the context of driving refractory genes into mosquito populations to control mosquito-borne infectious diseases (Ashburner *et al.*, 1998; Alphey *et al.*, 2002). A better understanding of endogenous TEs may be important to help achieve sustained success of such sophisticated genetic approaches.

4.12.9.2. SINE Insertion Polymorphism as Genetic Markers

The genetic differences and the pattern of gene flow between insect populations are of fundamental importance to a number of entomological questions ranging from evolution to practical applications. Single nucleotide polymorphisms (SNPs) are being developed as powerful markers for population genetic analysis, especially for insects with a large amount of sequence data available (Berger *et al.*, 2001). However, for most other insects, markers such as microsatellite, restriction fragment length polymorphism (RFLP) (Severson *et al.*, 1993; Yan *et al.*, 1999), cDNA-based single strand conformational polymorphism (SSCP) (Fulton *et al.*, 2001), mitochondrial (mtDNA), and ribosomal genes (rDNA) are still the tools of choice. For many insect species, finding a useful set of genetic markers is not trivial (Fagerberg *et al.*, 2001). It is clear that additional genome-wide markers are needed. Polymorphic insertion sites of interspersed TEs are potentially rich sources of genetic markers for population and genetic mapping studies. SINEs, which are especially useful for reasons described below are discussed. In population studies, sequences flanking a SINE at a specific locus are used as primers to amplify genomic DNA isolated from an individual sample. When the PCR products are run on an agarose gel, the genotype of an individual will be revealed on the basis of the number and size of bands (**Figure 11**) (see Section 4.12.7.2). Thus this locus-specific PCR assay may be used as codominant markers that reveal the dimorphism (insertion versus noninsertion) at a specific site. SINEs including the human *Alu* elements have been shown to be powerful genetic markers using the above method (Batzer *et al.*, 1994; Stoneking *et al.*, 1997; Carroll *et al.*, 2001; de Pancorbo *et al.*, 2001; Nasidze *et al.*, 2001; Roy-Engel *et al.*, 2001; Batzer and Deininger, 2002; Salem *et al.*, 2003). The ability of SINE insertion polymorphic markers to differentiate recently separated human populations is a good indication of their power (de Pancorbo *et al.*, 2001; Nasidze *et al.*, 2001; Watkins *et al.*, 2001). The locus-specific PCR assay of SINE insertions (**Figure 11**) has a few potential advantages over the popular microsatellite markers. The same SINE insertions are identical by descent. The probability that different SINEs of the same size independently insert into the same chromosomal location may be negligible (Stoneking *et al.*, 1997; York *et al.*, 1999; de Pancorbo *et al.*, 2001; Watkins *et al.*, 2001). However, the same signals in microsatellite, RFLP, SSCP, and melting curve single nucleotide polymorphism (McSNP) can only be considered identical by state, with the possibility of different origins. Moreover, the ancestral state of the SINE insertion polymorphism is likely to be the absence of a SINE because of general lack of excision, although exceptions do exist (Medstrand *et al.*, 2002). The ability to distinguish the ancestral versus derived states provides additional resolving power to address population genetic questions (York *et al.*, 1999). Because of the significant size difference of the PCR products and the dimorphic nature of the alleles, SINE insertions can be easily scored using agarose gels or melt-curve analysis, which can be developed for high-throughput analysis. Finally, a minimal amount of template is required for the PCR assay, allowing the use of a single insect for hundreds if not thousands of assays. One potential limitation of this approach could be the removal of a SINE insertion by rare recombination or gene conversion events, which may be confused with the noninsertion state. Such events may be revealed during the PCR analysis. Sequence analysis at the insertion site will also allow the investigation of this possibility. A better understanding of the evolutionary and population dynamics of SINEs will further provide theoretical foundations for this

potentially powerful method (Batzer and Deininger, 2002).

The recent development of a TE-anchored PCR approach, or TE display (see Section 4.12.5.3), has made it possible to directly screen for TE insertion polymorphism in a few species including insects (Roy *et al.*, 1999; Casa *et al.*, 2000, 2002; De Keukeleire *et al.*, 2001; Biedler *et al.*, 2003; Yang and Nuzhdin, 2003) (**Figure 9**). TE display efficiently scans a large number of loci in the genome, which makes it a very good tool for genotyping. However, as a population genetic tool, it has a major limitation common for dominant markers such as AFLP and randomly amplified polymorphic DNA (RAPD), namely the inability to distinguish between heterozygous and homozygous insertions, rendering the detection of population genetic structure difficult. However, TE display can be used as a direct screen to identify potential polymorphic insertion sites. Therefore TE display in conjunction with the development of locus-specific PCR markers that are codominant (**Figure 11**) will help TE insertion polymorphism markers reach their full potential as population genomic tools for insects. The use of TE insertion polymorphic markers in insect population studies is at an early stage. TE display and TE-based locus-specific PCR are used as genome-wide markers to investigate the genetic differences between different populations of *A. gambiae sensu stricto*, a species in the *A. gambiae* complex, and to study its ongoing speciation. These population genomic studies also have significant practical implications because genetic heterogeneity between mosquito populations can affect both the efficiency of disease transmission and the relative value of mosquito control measures (Coluzzi *et al.*, 2002; della Torre *et al.*, 2002). It was shown that different regions of the *A. gambiae* genome behave very differently with regard to introgression and gene flow (della Torre *et al.*, 2002). One important advantage of TE-based tools is the ability to scan a large number of TE insertions across different regions of the genome. Our initial TE display using *A. gambiae SINE200* showed significant differences at multiple sites between two forms of *A. gambiae sensu stricto*, namely the M and S forms (Coluzzi *et al.*, 2002). Polymorphic insertion sites for locus-specific PCR are isolated. Similar assays are also developed for *Aedes aegypti* using a SINE named *Feilai* (Tu, 1999). The development of TE-based population genomic tools for *A. aegypti* will be especially significant considering the lack of useful microsatellite markers in this species. The likely broad distribution of SINEs in insects makes them potentially rich sources of genetic markers for many different species.

4.12.9.3. SINE Insertions as Phylogenetic Markers

TE insertions, more specifically SINE insertions, have recently been used as molecular systematic tools to trace the evolutionary relationship between whales and Artiodactyla (Shimamura *et al.*, 1997; Nikaido *et al.*, 1999) and between salmonid fishes (Murata *et al.*, 1993). Perhaps the most impressive use of SINE insertions is the resolution of the evolutionary relationship of one of the major tribes of the African cichlid fishes which have evolved through an explosive adaptive radiation (Takahashi *et al.*, 1998; Terai *et al.*, 2003). To obtain TE insertion information for molecular systematics, locus-specific PCR described above is used. Here an RNA-mediated TE such as a SINE is again better suited because its transposition does not involve excison. Therefore, the ancestral state is known to be the absence of a SINE (Shedlock and Okada, 2000). The basic concept of this approach is illustrated in **Figure 12**. The insertion state can be easily determined using an agarose gel or melt-curve analysis as shown in **Figure 11**. When a fixed insertion is found at a particular site in species 1 and 2 but not in species 3, it can be inferred that species 1 and 2 are sister taxa. One of the requirements of the above approach is that the TE insertion site should be fixed within a species. TE display, a fingerprinting method described earlier (**Figure 9**), may be used to search for such sites. Potential problems such as nonspecific deletions, gene conversions, and sorting of ancestral polymorphisms can either be detected during the PCR and gel electrophoresis analysis or mitigated by surveying multiple loci (Shedlock and Okada, 2000). Sequences of the SINEs themselves in the loci used for the systematic analysis may provide further

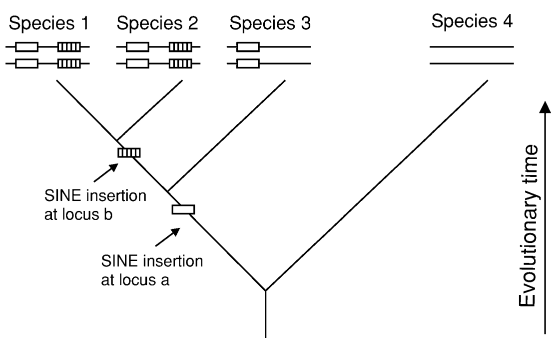


Figure 12 A schematic illustration of the principle of using SINE insertions as molecular systematic markers. Monophyletic relationship between species may be inferred on the basis of shared SINE insertions. See Section 4.12.9 for detailed explanations.

phylogenetic information. The usefulness of a particular SINE family in molecular systematics studies is dependent on its distribution and lifespan in the taxonomic group of interest. The tremendous diversity of insect species offers interesting challenges to evolutionary biologists. For example, a number of medically and economically important insect organisms exist as cryptic species complexes (Munstermann and Conn, 1997; Krzywinski and Besansky, 2003). New phylogenetic tools that can be integrated with methods using conventional characters such as morphology and DNA sequences will undoubtedly be of significance. Although SINEs have not been extensively studied in insects, highly repetitive SINEs have already been characterized in different orders including many medically and economically important species. Therefore SINEs may provide useful markers for molecular systematic analysis of insects, one of the most diverse groups of life forms on this planet.

In summary, TEs have been successfully used as vectors for genetic manipulation of insects and other organisms. A better understanding of insect TEs will allow better-informed usage of the currently available TE-based tools. The application of TEs as population and phylogenetic markers is at an early stage. Although these markers are promising tools, their scope of application, their resolving power and reliability depend on a better understanding of the population and evolutionary dynamics of the TEs. Therefore the fundamental studies dicussed in previous sections also have significant implications for the applications of TE-based molecular tools.

4.12.10. Summary

The last few years have witnessed an explosive growth both in the development of TE-based genetic and molecular tools and in our fundamental understanding of the diversity and impact of TEs in insects. Studies of TEs in insects, especially in *D. melanogaster*, have more than once led to discoveries that broadly impacted the field of TE research. The availability of new bioinformatics and experimental tools and the expanding genome revolution provide an exciting opportunity for the discovery of novel TEs in a wide range of insects and for the in-depth molecular and genomic analysis of these mobile genetic elements. Only through comparative genomic approaches, can we achieve a full appreciation of the complex and intricate dynamics governing the evolution of diverse TEs in insect genomes. From an applied perspective, systematic analysis of TEs in many insect species has significant economic and health implications because of the importance of these insects in disease transmission and agriculture.

Acknowledgments

I thank F. Collins, G. Gasperi, L. Gomulski, A. Handler, M. Kidwell, D. Lampe, H. Robertson, A. Sarkar, and J. Silva for sharing their manuscripts before publication. I thank H. Hagedorn, A. Handler, M. Kidwell, D. Lisch, S. Nuzhdin, and J. Silva for helpful comments. I thank J. Biedler, Monique Coy, Senay Sengul, and Rui Yang for help with the manuscript and for interesting discussions. Work in the author's laboratory is supported by NIH grants AI42121 and AI53203, Jeffress Foundation, and the Virginia Agricultural Experimental Station.

References

Abe, H., Ohbayashi, F., Shimada, T., Sugasaki, T., Kawai, S., *et al.*, **1998**. A complete full-length non-LTR retrotransposon, BMC1, on the W chromosome of the silkworm *Bombyx mori*. *Genes Genet. Syst. 73*, 353–358.

Abe, H., Ohbayashi, F., Shimada, T., Sugasaki, T., Kawai, S., *et al.*, **2000**. Molecular structure of a novel gypsy-Ty3-like retrotransposon (Kabuki) and nested retrotransposable elements on the W chromosome of the silkworm *Bombyx mori*. *Mol. Gen. Genet. 263*, 916–924.

Abe, H., Ohbayashi, F., Sugasaki, T., Kanehara, M., Terada, T., *et al.*, **2001**. Two novel Pao-like retrotransposons (Kamikaze and Yamato) from the silkworm species *Bombyx mori* and *B. mandarina*: common structural features of Pao-like elements. *Mol. Genet. Genomics 265*, 375–385.

Abe, H., Sugasaki, T., Kanehara, M., Ohbayashi, F., Shimada, T., *et al.*, **2002**. Nested retrotransposons on the W chromosome of the wild silkworm *Bombyx mandarina*. *Insect Mol. Biol. 11*, 307–314.

Adams, D.S., Eickbush, T.H., Herrera, R.J., Lizardi, P.M., **1986**. A highly reiterated family of transcribed oligo(A)-terminated, interspersed DNA elements in the genome of *Bombyx mori*. *J. Mol. Biol. 187*, 465–478.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster*. *Science 287*, 2185–2195.

Agarwal, M., Bensaadi, N., Salvado, J.C., Campbell, K., Mouches, C., **1993**. Characterization and genetic organization of full-length copies of a LINE retroposon family dispersed in the genome of *Culex pipiens* mosquitoes. *Insect Biochem. Mol. Biol. 23*, 621–629.

Alphey, L., Beard, C.B., Billingsley, P., Coetzee, M., Crisanti, A., *et al.*, **2002**. Malaria control with genetically manipulated insect vectors. *Science 298*, 119–121.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., *et al.*, **1997**. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res. 25*, 3389–3402.

Anzai, T., Takahashi, H., Fujiwara, H., **2001**. Sequence-specific recognition and cleavage of telomeric repeat (TTAGG)(n) by endonuclease of non-long terminal repeat retrotransposon TRAS1. *Mol. Cell Biol. 21*, 100–108.

Arca, B., Zabalou, S., Loukeris, T.G., Savakis, C., **1997**. Mobilization of a *Minos* transposon in *Drosophila melanogaster* chromosomes and chromatid repair by heteroduplex formation. *Genetics 145*, 267–279.

Arends, H.M., Jehle, J.A., **2002**. Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of *Cydia pomonella* granulovirus. *J. Gen. Virol. 83*, 1573–1578.

Arkhipova, I.R., Pyatkov, K.I., Meselson, M., Evgen'ev, M.B., **2003**. Retroelements containing introns in diverse invertebrate taxa. *Nat. Genet. 33*, 123–124.

Ashburner, M., Hoy, M.A., Peloquin, J.J., **1998**. Prospects for the genetic transformation of arthropods. *Insect Mol. Biol. 7*, 201–213.

Atkinson, P.W., Pinkerton, A.C., O'Brochta, D.A., **2001**. Genetic transformation systems in insects. *Annu. Rev. Entomol. 46*, 317–346.

Badge, R.M., Brookfield, J.F., **1997**. The role of host factors in the population dynamics of selfish transposable elements. *J. Theoret. Biol. 187*, 261–271.

Bao, Z., Eddy, S.R., **2002**. Automated *de novo* identification of repeat sequence families in sequenced genomes. *Genome Res. 12*, 1269–1276.

Bartolome, C., Maside, X., Charlesworth, B., **2002**. On the abundance and distribution of transposable elements in the genome of *Drosophila melanogaster. Mol. Biol. Evol. 19*, 926–937.

Batzer, M.A., Deininger, P.L., **2002**. *Alu* repeats and human genomic diversity. *Nat. Rev. Genet. 3*, 370–379.

Batzer, M.A., Stoneking, M., Alegria-Hartman, M., Bazan, H., Kass, D.H., *et al.*, **1994**. African origin of human-specific polymorphic *Alu* insertions. *Proc. Natl Acad. Sci. USA 91*, 12288–12292.

Bauser, C.A., Elick, T.A., Fraser, M.J., **1999**. Proteins from nuclear extracts of two lepidopteran cell lines the ends of TTAA-specific transposons *piggyBac* and *tagalong*. *Insect Mol. Biol. 8*, 223–230.

Beall, E.L., Rio, D.C., **1997**. *Drosophila P*-element transposase is a novel site-specific. *Genes Devel. 11*, 2137–2151.

Becker, H.A., Kunze, R., **1997**. Maize *Activator* transposase has a bipartite DNA binding domain recognizes subterminal sequences and the terminal inverted. *Mol. Gen. Genet. 254*, 219–230.

Beeman, R.W., Thomson, M.S., Clark, J.M., DeCamillis, M.A., Brown, S.J., *et al.*, **1996**. *Woot*, an active gypsy-class retrotransposon in the flour beetle, *Tribolium castaneum*, is associated with a recent mutation. *Genetics 143*, 417–426.

Bensaadi-Merchermek, N., Cagnon, C., Desmons, I., Salvado, J.C., Karama, S., *et al.*, **1997**. *CM-gag*, a transposable-like element reiterated in the genome of *Culex pipiens* mosquitoes, contains only a *gag* gene. *Genetica 100*, 141–148.

Berezikov, E., Bucheton, A., Busseau, I., **2000**. A search for reverse transcriptase-coding sequences reveals new non-LTR retrotransposons in the genome of *Drosophila melanogaster. Genome Biol. 1*, RESEARCH0012.

Berg, D.E., Howe, M.J. (Eds.), **1989**. Mobile DNA. American Society for Microbiology Press, Washington, DC.

Berger, J., Suzuki, T., Senti, K.A., Stubbs, J., Schaffner, G., *et al.*, **2001**. Genetic mapping with SNP markers in *Drosophila*. *Nat. Genet. 29*, 475–481.

Bernstein, M., Lersch, R.A., Subrahmanyan, L., Cline, T.W., **1995**. Transposon insertions causing sex-lethal activity in *Drosophila melanogaster* affect Sx1 sex-specific transcript splicing. *Genetics 139*, 631–648.

Besansky, N.J., **1990**. A retrotransposable element from the mosquito *Anopheles gambiae*. *Mol. Cell Biol. 10*, 863–871.

Besansky, N.J., Bedell, J.A., Mukabayire, O., **1994**. *Q*: a new retrotransposon from the mosquito *Anopheles gambiae*. *Insect Mol. Biol. 3*, 49–56.

Besansky, N.J., Mukabayire, O., Bedell, J.A., Lusz, H., **1996**. *Pegasus*, a small terminal inverted repeat transposable element found in the *white* gene of *Anopheles gambiae*. *Genetica 98*, 119–129.

Besansky, N.J., Paskewitz, S.M., Hamm, D.M., Collins, F.H., **1992**. Distinct families of site-specific retrotransposons occupy identical positions in the rRNA genes of *Anopheles gambiae*. *Mol. Cell Biol. 12*, 5102–5110.

Betran, E., Thornton, K., Long, M., **2002**. Retroposed new genes out of the X in *Drosophila*. *Genome Res. 12*, 1854–1859.

Biedler, J., Qi, Y., Holligan, D., Della Torre, A., Wessler, S., *et al.*, **2003**. Transposable element (TE) display and rapid detection of TE insertion polymorphism in the *Anopheles gambiae* species complex. *Insect Mol. Biol. 12*, 211–216.

Biedler, J., Tu, Z., **2003**. Non-LTR retrotransposons in the African malaria mosquito, *Anopheles gambiae*: unprecedented diversity and evidence of recent activity. *Mol. Biol. Evol. 20*, 1811–1825.

Biemont, C., Tsitrone, A., Vieira, C., Hoogland, C., **1997**. Transposable element distribution in *Drosophila*. *Genetics 147*, 1997–1999.

Biessmann, H., Valgeirsdottir, K., Lofsky, A., Chin, C., Ginther, B., *et al.*, **1992**. HeT-A, a transposable element specifically involved in "healing" broken chromosome ends in *Drosophila melanogaster. Mol. Cell. Biol. 12*, 3910–3918.

Bigot, Y., Hamelin, M.H., Capy, P., Periquet, G., **1994**. *Mariner*-like elements in hymenopteran species: insertion site and distribution. *Proc. Natl Acad. Sci. USA 91*, 3408–3412.

Blinov, A.G., Sobanov, Y.V., Gaidamakova, E.K., Bogachev, S.S., Kolesnikov, N.N., *et al.*, **1991**. MEC: a transposable element from *Chironomus thummi* (Diptera). *Mol. Gen. Genet. 229*, 152–154.

Blinov, A.G., Sobanov, Y.V., Scherbik, S.V., Aimanova, K.G., **1997**. The *Chironomus* (*Camptochironomus*) *tentans* genome contains two non-LTR retrotransposons. *Genome 40*, 143–150.

Bonnivard, E., Bazin, C., Denis, B., Higuet, D., **2000**. A scenario for the *hobo* transposable element invasion, deduced from the structure of natural populations of *Drosophila melanogaster* using tandem TPE repeats. *Genet. Res. 75*, 13–23.

Booth, D.R., Ready, P.D., Smith, D.F., **1994**. Isolation of non-LTR retrotransposon reverse transcriptase-like sequences from phlebotomine sandflies. *Insect Mol. Biol. 3*, 89–96.

Bradfield, J.Y., Locke, J., Wyatt, G.R., **1985**. An ubiquitous interspersed DNA sequence family in an insect. *DNA 4*, 357–363.

Braquart, C., Royer, V., Bouhin, H., **1999**. *DEC*: a new miniature inverted-repeat transposable element from the genome of the beetle *Tenebrio molitor. Insect Mol. Biol. 8*, 571–574.

Bregliano, J.C., Picard, G., Bucheton, A., Pelisson, A., Lavige, J.M., *et al.*, **1980**. Hybrid dysgenesis in *Drosophila melanogaster. Science 207*, 606–611.

Brookfield, J.F., **1995**. Transposable elements as selfish DNA. In: Sherratt, D.J. (Ed.), Mobile Genetic Elements. Oxford University Press, Oxford, pp. 130–153.

Bucheton, A., **1995**. The relationship between the *flamenco* gene and *gypsy* in tame a retrovirus. *Trends Genet. 11*, 349–353.

Caceres, M., Puig, M., Ruiz, A., **2001**. Molecular characterization of two natural hotspots in the *D. buzzatii* genome induced by transposon insertions. *Genome Res. 11*, 1353–1364.

Calvi, B.R., Hong, T.J., Findley, S.D., Gelbart, W.M., **1991**. Evidence for a common evolutionary origin of inverted repeat transposons in *Drosophila* and plants: *hobo*, *Activator*, and *Tam3*. *Cell 66*, 465–471.

Capy, P., Langin, T., Higuet, D., Maurer, P., Bazin, C., **1997**. Do the integrases of LTR-retrotransposons and class II element transposases have a common ancestor? *Genetica 100*, 63–72.

Capy, P., Vitalis, R., Langin, T., Higuet, D., Bazin, C., **1996**. Relationships between transposable elements based upon the integrase-transposase domains: is there a common ancestor? *J. Mol. Evol. 42*, 359–368.

Carroll, M.L., Roy-Engel, A.M., Nguyen, S.V., Salem, A.H., Vogel, E., *et al.*, **2001**. Large-scale analysis of the *Alu Ya5* and *Yb8* subfamilies and their contribution to human genomic diversity. *J. Mol. Biol. 311*, 17–40.

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E., *et al.*, **1989**. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon *IFP2* insertions within the *FP*-locus of nuclear polyhedrosis viruses. *Virology 172*, 156–169.

Casa, A.M., Brouwer, C., Nagel, A., Wang, L., Zhang, Q., *et al.*, **2000**. Inaugural article: the MITE family *heartbreaker* (*Hbr*): molecular markers in maize. *Proc. Natl Acad. Sci. USA 97*, 10083–10089.

Casa, A.M., Mitchell, S.E., Smith, O.S., Register, J.C., III, Wessler, S.R., *et al.*, **2002**. Evaluation of *Hbr* (MITE) markers for assessment of genetic relationships among maize (*Zea mays* L.) inbred lines. *Theoret. Appl. Genet. 104*, 104–110.

Charlesworth, B., Langley, C.H., **1989**. The population genetics of *Drosophila* transposable elements. *Annu. Rev. Genet. 23*, 251–287.

Charrier, B., Foucher, F., Kondorosi, E., d'Aubenton-Carafa, Y., Thermes, C., *et al.*, **1999**. *Bigfoot*, a new family of MITE elements characterized from the genus. *Plant J. 18*, 431–441.

Clark, J.B., Kidwell, M.G., **1997**. A phylogenetic perspective on *P* transposable element evolution in *Drosophila*. *Proc. Natl Acad. Sci. USA 94*, 11428–11433.

Claudianos, C., Brownlie, J., Russell, R., Oakeshott, J., Whyard, S., **2002**. *maT*: a clade of transposons intermediate between *mariner* and *Tc1*. *Mol. Biol. Evol. 19*, 2101–2109.

Coates, C.J., Johnson, K.N., Perkins, H.D., Howells, A.J., O'Brochta, D.A., *et al.*, **1996**. The hermit transposable element of the Australian sheep blowfly, *Lucilia cuprina*, belongs to the *hAT* family of transposable elements. *Genetica 97*, 23–31.

Cockburn, A.F., Mitchell, S.E., **1989**. Repetitive DNA interspersion patterns in Diptera. *Arch. Insect Biochem. Physiol. 10*, 105–113.

Coluzzi, M., Sabatini, A., della Torre, A., Di Deco, M.A., Petrarca, V., **2002**. A polytene chromosome analysis of the *Anopheles gambiae* species complex. *Science 298*, 1415–1418.

Cook, J.M., Martin, J., Lewin, A., Sinden, R.E., Tristem, M., **2000**. Systematic screening of *Anopheles mosquito* genomes yields evidence for a major clade of *Pao*-like retrotransposons. *Insect Mol. Biol. 9*, 109–117.

Couzin, J., **2002**. Breakthrough of the year: small RNAs make big splash. *Science 298*, 2296–2297.

Craig, N., **2002**. Mobile DNA: an introduction. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 3–11.

Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), **2002**. Mobile DNA II. American Society for Microbiology Press, Washington, DC.

Daniels, S.B., Chovnick, A., Boussy, I.A., **1990**. Distribution of *hobo* transposable elements in the genus *Drosophila*. *Mol. Biol. Evol. 7*, 589–606.

Davidson, E.H., Galau, G.A., Angerer, R.C., Britten, R.J., **1975**. Comparative aspects of DNA organization in Metazoa. *Chromosoma 51*, 253–259.

Davis, P.S., Judd, B.H., **1995**. Molecular characterization of the 3C region between white and roughest loci of *Drosophila melanogaster. Drosophila Inf. Serv. 76*, 130–134.

De Keukeleire, P., Maes, T., Sauer, M., Zethof, J., Van Montagu, M., *et al.*, **2001**. Analysis by transposon display of the behavior of the *dTph1* element family during ontogeny and inbreeding of *Petunia hybrida*. *Mol. Genet. Genomics 265*, 72–81.

de Pancorbo, M.M., Lopez-Martinez, M., Martinez-Bouzas, C., Castro, A., Fernandez-Fernandez, I., *et al.*, **2001**. The Basques according to polymorphic *Alu* insertions. *Hum. Genet. 109*, 224–233.

Deininger, P., Roy-Engel, A., **2002**. Mobile elements in plant and animal genomes. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 1074–1092.

della Torre, A., Costantini, C., Besansky, N.J., Caccone, A., Petrarca, V., *et al.*, **2002**. Speciation within *Anopheles gambiae*: the glass is half full. *Science 298*, 115–117.

Doak, T.G., Doerder, F.P., Jahn, C.L., Herrick, G., **1994**. A proposed superfamily of transposase genes: transposon-like ciliated protozoa and a common "D35E" motif. *Proc. Natl Acad. Sci. USA 91*, 942–946.

Doolittle, W.F., Sapienza, C., **1980**. Selfish genes, the phenotype paradigm and genome evolution. *Nature 284*, 601–603.

Eickbush, D.G., Eickbush, T.H., **1995**. Vertical transmission of the retrotransposable elements *R1* and *R2* during the evolution of the *Drosophila melanogaster* species subgroup. *Genetics 139*, 671–684.

Eickbush, T., **2002**. *R2* and related site-specific non-long terminal repeat retrotransposons. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 813–835.

Eickbush, T., Malik, H., **2002**. Origins and evolution of retrotransposons. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 1111–1144.

Engels, W.R., **1989**. *P* elements in *Drosophila melanogaster*. In: Berg, D.E., Howe, M.J. (Eds.), Mobile DNA. American Society for Microbiology Press, Washington, DC, pp. 437–484.

Engels, W.R., **1997**. Invasions of *P* elements. *Genetics 145*, 11–15.

Evgen'ev, M.B., Zelentsova, H., Shostak, N., Kozitsina, M., Barskyi, V., *et al.*, **1997**. *Penelope*, a new family of transposable elements and its possible role in hybrid dysgenesis in *Drosophila virilis*. *Proc. Natl Acad. Sci. USA 94*, 196–201.

Fagerberg, A.J., Fulton, R.E., Black, W.C., **2001**. Microsatellite loci are not abundant in all arthropod genomes: analyses in the hard tick, *Ixodes scapularis* and the yellow fever mosquito, *Aedes aegypti*. *Insect Mol. Biol. 10*, 225–236.

Fawcett, D.H., Lister, C.K., Kellett, E., Finnegan, D.J., **1986**. Transposable elements controlling I-R hybrid dysgenesis in *D. melanogaster* are similar to mammalian LINEs. *Cell 47*, 1007–1015.

Fedoroff, N., **1989**. Maize transposable elements. In: Berg, D.E., Howe, M.J. (Eds.), Mobile DNA. American Society for Microbiology Press, Washington, DC, pp. 375–411.

Fedoroff, N., **2002**. Control of mobile DNA. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 997–1007.

Felger, I., Hunt, J.A., **1992**. A non-LTR retrotransposon from the Hawaiian *Drosophila*: the *LOA* element. *Genetica 85*, 119–130.

Feschotte, C., Fourrier, N., Desmons, I., Mouches, C., **2001**. Birth of a retroposon: the *Twin* SINE family from the vector mosquito *Culex pipiens* may have originated from a dimeric tRNA precursor. *Mol. Biol. Evol. 18*, 74–84.

Feschotte, C., Mouches, C., **2000a**. Recent amplification of miniature inverted-repeat transposable elements in the vector mosquito *Culex pipiens*: characterization of the *Mimo* family. *Gene 250*, 109–116.

Feschotte, C., Mouches, C., **2000b**. Evidence that a family of miniature inverted-repeat transposable elements (MITEs) from the *Arabidopsis thaliana* genome has arisen from a *pogo*-like DNA transposon. *Mol. Biol. Evol. 17*, 730–737.

Feschotte, C., Swamy, L., Wessler, S.R., **2003**. Genome-wide analysis of *mariner*-like transposable elements in rice reveals complex relationships with stowaway miniature inverted repeat transposable elements (MITEs). *Genetics 163*, 747–758.

Feschotte, C., Zhang, X., Wessler, S., **2002**. Miniature inverted-repeat transposable elements and their relationship to established DNA transposons. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 1147–1158.

Finnegan, D.J., **1989**. The *I* factor and *I-R* hybrid dysgenesis in *Drosophila melanogaster*. In: Berg, D.E., Howe, M.J. (Eds.), Mobile DNA. American Society for Microbiology Press, Washington, DC, pp. 503–517.

Finnegan, D.J., **1992**. Transposable elements. *Curr. Opin. Genet. Devel. 2*, 861–867.

Franz, G., Savakis, C., **1991**. *Minos*, a new transposable element from *Drosophila hydei*, is a member of the *Tc1*-like family of transposons. *Nucl. Acids Res. 19*, 6646.

Fraser, M.J., **2000**. The TTAA-specific family of transposable elements: identification, functional characterization, and utility for transformation of insects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, p. 249.

Friesen, P.D., Nissen, M.S., **1990**. Gene organization and transcription of TED, a lepidopteran retrotransposon integrated within the baculovirus genome. *Mol. Cell Biol. 10*, 3067–3077.

Fulton, R.E., Salasek, M.L., DuTeau, N.M., Black, W.C.T., **2001**. SSCP analysis of cDNA markers provides a dense linkage map of the *Aedes aegypti* genome. *Genetics 158*, 715–726.

Gale, K.R., **1987**. Characterization of the mosquito, *Aedes aegypti*, at the molecular level of genetics. Ph.D. thesis, University of Liverpool.

Garcillan-Barcia, M.P., Bernales, I., Mendiola, M.V., De La Cruz, F., **2002**. IS91 rolling-circle transposition. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 891–904.

Garel, A., Nony, P., Prudhomme, J.C., **1994**. Structural features of *mag*, a *gypsy*-like retrotransposon of *Bombyx mori*, with unusual short terminal repeats. *Genetica 93*, 125–137.

Garner, K.J., Slavicek, J.M., **1999**. Identification of a non-LTR retrotransposon from the gypsy moth. *Insect Mol. Biol. 8*, 231–242.

Gellert, M., **2002**. V(D)J recombination. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 705–729.

Gomulski, L.M., Torti, C., Bonizzoni, M., Moralli, D., Raimondi, E., *et al.*, **2001**. A new basal subfamily of *mariner* elements in *Ceratitis rosa* and other tephritid flies. *J. Mol. Evol. 53*, 597–606.

Gorbunova, V., Levy, A.A., **1997**. Circularized *Ac*/*D*s transposons: formation, structure and fate. *Genetics 145*, 1161–1169.

Grandbastien, M.A., **1998**. Activation of plant retrotransposons under stress condiitons. *Trends Plant Sci. 3*, 181–187.

Gray, Y.H., **2000**. It takes two transposons to tango: transposable-element-mediated chromosomal rearrangements. *Trends Genet. 16*, 461–468.

Green, C.L., Frommer, M., **2001**. The genome of the Queensland fruit fly *Bactrocera tryoni* contains multiple representatives of the *mariner* family of transposable elements. *Insect Mol. Biol. 10*, 371–386.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., **2000**. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature 404*, 293–296.

Handler, A.M., **2003**. Isolation and analysis of a new *hopper* hAT transposon from the dorsalis white eye strain. *Genetica 118*, 17–24.

Handler, A.M., McCombs, S.D., **2000**. The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. *Insect Mol. Biol. 9*, 605–612.

Hartl, D., **1989**. Transposable element *mariner* in *Drosophila* species. In: Berg, D.E., Howe, M.J. (Eds.), Mobile DNA. American Society for Microbiology Press, Washington, DC, pp. 531–536.

Hartl, D.L., Lozovskaya, E.R., Nurminsky, D.I., Lohe, A.R., **1997**. What restricts the activity of *mariner*-like transposable elements. *Trends Genet. 13*, 197–201.

Henikoff, S., **1992**. Detection of *Caenorhabditis* transposon homologs in diverse. *New Biol. 4*, 382–388.

Hill, S.R., Leung, S.S., Quercia, N.L., Vasiliauskas, D., Yu, J., *et al.*, **2001**. Ikirara insertions reveal five new *Anopheles gambiae* transposable elements in islands of repetitious sequence. *J. Mol. Evol. 52*, 215–231.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Holyoake, A.J., Kidwell, M.G., **2003**. *Vege* and *Mar*: two novel *hAT* MITE families from *Drosophila willistoni*. *Mol. Biol. Evol. 20*, 163–167.

Horn, C., Offen, N., Nystedt, S., Hacker, U., Wimmer, E.A., **2003**. *piggyBac*-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. *Genetics 163*, 647–661.

Imwong, M., Sharpe, R.G., Kittayapong, P., Baimai, V., **2000**. Distribution of the transposable element *mariner* in anopheline mosquitoes. *Heredity 85*, 271–276.

Ivics, Z., Hackett, P.B., Plasterk, R.H., Izsvak, Z., **1997**. Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells. *Cell 91*, 501–510.

Izsvak, Z., Ivics, Z., Shimoda, N., Mohn, D., Okamoto, H., *et al.*, **1999**. Short inverted-repeat transposable elements in teleost fish and implications for a mechanism of their amplification. *J. Mol. Evol. 48*, 13–21.

Jacobson, J.W., Medhora, M.M., Hartl, D.L., **1986**. Molecular structure of a somatically unstable transposable element in *Drosophila*. *Proc. Natl Acad. Sci. USA 83*, 8684–8688.

Jasinskiene, N., Coates, C.J., James, A.A., **2000**. Structure of *hermes* integrations in the germline of the yellow fever mosquito, *Aedes aegypti*. *Insect Mol. Biol. 9*, 11–18.

Jensen, S., Cavarec, L., Dhellin, O., Heidmann, T., **1994**. Retrotransposition of a marked *Drosophila* LINE-like I element in cells in culture. *Nucl. Acids Res. 22*, 1484–1488.

Jensen, S., Gassama, M.P., Heidmann, T., **1999**. Cosuppression of *I* transposon activity in *Drosophila* by *I*-containing sense and antisense transgenes. *Genetics 153*, 1767–1774.

Jiang, N., Bao, Z., Zhang, X., Hirochika, H., Eddy, S.R., *et al.*, **2003**. An active DNA transposon family in rice. *Nature 421*, 163–167.

Jordan, I.K., Matyunina, L.V., McDonald, J.F., **1999**. Evidence for the recent horizontal transfer of long terminal repeat retrotransposon. *Proc. Natl Acad. Sci. USA 96*, 12621–12625.

Jurka, J., **1995**. Origin and evolution of *Alu* repetitive elements. In: Maraia, R.J. (Ed.), The Impact of Short Interspersed Elements (SINEs) on the Host Genome. R. G. Landes, Austin, TX, pp. 25–41.

Jurka, J., **2000**. Repbase update: a database and an electronic journal of elements. *Trends Genet. 16*, 418–420.

Kaminker, J.S., Bergman, C.M., Kronmiller, B., Carlson, J., Svirskas, R., *et al.*, **2002**. The transposable elements of the *Drosophila melanogaster* genomics perspective. *Genome Biol. 3*, RESEARCH0084–0084.

Kapitonov, V.V., Jurka, J., **1999**. Molecular paleontology of transposable elements from *Arabidopsis*. *Genetica 107*, 27–37.

Kapitonov, V.V., Jurka, J., **2001**. Rolling-circle transposons in eukaryotes. *Proc. Natl Acad. Sci. USA 98*, 8714–8719.

Kapitonov, V.V., Jurka, J., **2002**. *Looper1_DM*, a family of *Looper/piggyBac* DNA transposons in *D. melanogaster*. *Repbase Reps. 2*, 6.

Kapitonov, V.V., Jurka, J., **2003a**. A novel class of SINE elements derived from 5S rRNA. *Mol. Biol. Evol. 20*, 694–702.

Kapitonov, V.V., Jurka, J., **2003b**. Molecular paleontology of transposable elements in the *Drosophila melanogaster* genome. *Proc. Natl Acad. Sci. USA 100*, 6569–6574.

Kaufman, T.C., Severson, D.W., Robinson, G.E., **2002**. The *Anopheles* genome and comparative insect genomics. *Science 298*, 97–98.

Kazazian, H.H., Jr., Goodier, J.L., **2002**. LINE drive, retrotransposition and genome instability. *Cell 110*, 277–280.

Ke, Z., Grossman, G.L., Cornel, A.J., Collins, F.H., **1996**. *Quetzal*: a transposon of the *Tc1* family in the mosquito *Anopheles albimanus*. *Genetica 98*, 141–147.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G., Plasterk, R.H., **1999**. *Mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell 99*, 133–141.

Kidwell, M.G., **1977**. Reciprocal differences in female recombination associated with dysgenesis in *Drosophila melanogaster. Genet. Res. 30*, 77–88.

Kidwell, M.G., **1992**. Horizontal transfer of *P* elements and other short inverted repeat transposons. *Genetica 86*, 275–286.

Kidwell, M.G., **2002**. Transposable elements and the evolution of genome size in eukaryotes. *Genetica 115*, 49–63.

Kidwell, M.G., Lisch, D.R., **2000**. Transposable elements and host genome evolution. *Trends Ecol. Evol. 15*, 95–99.

Kidwell, M.G., Lisch, D.R., **2001**. Perspective: transposable elements, parasitic DNA, and genome evolution. *Int. J. Org. Evol. 55*, 1–24.

Kidwell, M.G., Lisch, D.R., **2002**. Transposable elements as sources of genomic variation. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 59–90.

Klinakis, A.G., Loukeris, T.G., Pavlopoulos, A., Savakis, C., **2000**. Mobility assays confirm the broad host-range activity of the transposable element and validate new transformation tools. *Insect Mol. Biol. 9*, 269–275.

Krebs, M.P., RajBhandary, U.L., Khorana, H.G., **1990**. Nucleotide sequence of ISH11, a new *Halobacterium halobium* element isolated from the plasmid pGRB1. *Nucl. Acids Res. 18*, 6699.

Krzywinski, J., Besansky, N.J., **2003**. Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annu. Rev. Entomol. 48*, 111–139.

Labrador, M., Corces, V., **2002**. Interactions between transposable elements and the host genome. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 1008–1023.

Labrador, M., Seleme, M.C., Fontdevila, A., **1998**. The evolutionary history of *Drosophila buzzatii*. XXXIV. The distribution of the retrotransposon *Osvaldo* in original and colonizing populations. *Mol. Biol. Evol. 15*, 1532–1547.

Lampe, D.J., Akerley, B.J., Rubin, E.J., Mekalanos, J.J., Robertson, H.M., **1999**. Hyperactive transposase mutants of the Himar1 *mariner* transposon. *Proc. Natl Acad. Sci. USA 96*, 11428–11433.

Lampe, D.J., Walden, K.K., Robertson, H.M., **2001**. Loss of transposase-DNA interaction may underlie the divergence of mariner family transposable elements and the ability of more than one mariner to occupy the same genome. *Mol. Biol. Evol. 18*, 954–961.

Lampe, D.J., Willis, J.H., **1994**. Characterization of a cDNA and gene encoding a cuticular protein from rigid cuticles of the giant silkmoth, *Hyalophora cecropia*. *Insect Biochem. Mol. Biol. 24*, 419–435.

Lampe, D.J., Witherspoon, D.J., Soto-Adames, F.N., Robertson, H.M., **2003**. Recent horizontal transfer of mellifera subfamily mariner transposons into insect lineages representing four different orders shows that selection acts only during horizontal transfer. *Mol. Biol. Evol. 20*, 554–562.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., *et al.*, **2001**. Initial sequencing and analysis of the human genome. *Nature 409*, 860–921.

Le, Q.H., Turcotte, K., Bureau, T., **2001**. *Tc8*, a *Tourist*-like transposon in *Caenorhabditis elegans*. *Genetics 158*, 1081–1088.

Le, Q.H., Wright, S., Yu, Z., Bureau, T., **2000**. Transposon diversity in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA 97*, 7376–7381.

Lee, S.H., Clark, J.B., Kidwell, M.G., **1999**. A *P* element-homologous sequence in the house fly, *Musca domestica*. *Insect Mol. Biol. 8*, 491–500.

Levis, R.W., Ganesan, R., Houtchens, K., Tolar, L.A., Sheen, F.M., **1993**. Transposons in place of telomeric repeats at a *Drosophila* telomere. *Cell 75*, 1083–1093.

Liao, C., Rovira, C., He, H., Edstrom, J.E., **1998**. Site-specific insertion of a SINE-like element, *Cp1*, into centromeric tandem repeats from *Chironomus pallidivittatus*. *J. Mol. Biol. 280*, 811–821.

Lim, J.K., Simmons, M.J., **1994**. Gross chromosome rearrangements mediated by transposable elements in *Drosophila melanogaster. BioEssays 16*, 269–275.

Lin, R., Avery, L., **1999**. RNA interference: policing rogue genes. *Nature 402*, 128–129.

Liu, B., Wendel, J.F., **2000**. Retrotransposon activation followed by rapid repression in introgressed rice plants. *Genome 43*, 874–880.

Locke, J., Howard, L.T., Aippersbach, N., Podemski, L., Hodgetts, R.B., **1999**. The characterization of DINE-1, a short, interspersed repetitive element present on chromosome and in the centric heterochromatin of *Drosophila melanogaster. Chromosoma 108*, 356–366.

Luan, D.D., Korman, M.H., Jakubczak, J.L., Eickbush, T.H., **1993**. Reverse transcription of R2Bm RNA is primed by a nick at the chromosomal target site: a mechanism for non-LTR retrotransposition. *Cell* 72, 595–605.

Luckhart, S., Rosenberg, R., **1999**. Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. *Gene* 232, 25–34.

MacRae, A.F., Clegg, M.T., **1992**. Evolution of *Ac* and *Dsl* elements in select grasses (Poaceae). *Genetica* 86, 55–66.

Mahillon, J., Chandler, M., **1998**. Insertion sequences. *Microbiol. Mol. Biol. Rev.* 62, 725–774.

Malik, H.S., Burke, W.D., Eickbush, T.H., **1999**. The age and evolution of non-LTR retrotransposable elements. *Mol. Biol. Evol.* 16, 793–805.

Malik, H.S., Eickbush, T.H., **1998**. The RTE class of non-LTR retrotransposons is widely distributed in animals and is the origin of many SINEs. *Mol. Biol. Evol.* 15, 1123–1134.

Martin, S.L., Bushman, F.D., **2001**. Nucleic acid chaperone activity of the ORF1 protein from the mouse LINE-1 retrotransposon. *Mol. Cell Biol.* 21, 467–475.

Maside, X., Bartolome, C., Assimacopoulos, S., Charlesworth, B., **2001**. Rates of movement and distribution of transposable elements in *Drosophila melanogaster*: *in situ* hybridization vs. Southern blotting data. *Genet. Res.* 78, 121–136.

Mathiopoulos, K.D., della Torre, A., Predazzi, V., Petrarca, V., *et al.*, **1998**. Cloning of inversion breakpoints in the *Anopheles gambiae* complex traces a transposable element at the inversion junction. *Proc. Natl Acad. Sci. USA* 95, 12444–12449.

Mathiopoulos, K.D., della Torre, A., Santolamazza, F., Predazzi, V., Petrarca, V., *et al.*, **1999**. Are chromosomal inversions induced by transposable elements? A paradigm from the malaria mosquito *Anopheles gambiae*. *Parasitologia* 41, 119–123.

McCarthy, E.M., McDonald, J.F., **2003**. LTR_STRUC: a novel search and identification program for LTR retrotransposons. *Bioinformatics* 19, 362–367.

McClintock, B., **1948**. Mutable loci in maize. *Carnegie Inst. Wash. Yb* 47, 155–169.

McClintock, B., **1950**. The origin and behavior of mutable loci in maize. *Proc. Natl Acad. Sci. USA* 36, 344–355.

McClintock, B., **1956**. Controlling elements and the gene. *Cold Spring Harbor Symp. Quant. Biol.* 8, 58–74.

McClintock, B., **1984**. The significance of responses of the genome to challenge. *Science* 226, 792–801.

Medstrand, P., van de Lagemaat, L.N., Mager, D.L., **2002**. Retroelement distributions in the human genome: variations associated with age and proximity to genes. *Genome Res.* 12, 1483–1495.

Mikitani, K., Sugasaki, T., Shimada, T., Kobayashi, M., Gustafsson, J.A., **2000**. The chitinase gene of the silkworm, *Bombyx mori*, contains a novel *Tc*-like transposable element. *J. Biol. Chem.* 275, 37725–37732.

Miller, W.J., Nagel, A., Bachmann, J., Bachmann, L., **2000**. Evolutionary dynamics of the SGM transposon family in the *Drosophila obscura* species group. *Mol. Biol. Evol.* 17, 1597–1609.

Miller, W.J., Paricio, N., Hagemann, S., Martinez-Sebastian, M.J., Pinsker, W., *et al.*, **1995**. Structure and expression of clustered *P* element homologues in *Drosophila subobscura* and *Drosophila guanche*. *Gene* 156, 167–174.

Misquitta, L., Paterson, B.M., **1999**. Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc. Natl Acad. Sci. USA* 96, 1451–1456.

Mizrokhi, L.J., Mazo, A.M., **1990**. Evidence for horizontal transmission of the mobile element *jockey* between distant *Drosophila* species. *Proc. Natl Acad. Sci. USA* 87, 9216–9220.

Mizrokhi, L.J., Mazo, A.M., **1991**. Cloning and analysis of the mobile element *gypsy* from *D. virilis*. *Nucl. Acids Res.* 19, 913–916.

Morgan, G.T., Middleton, K.M., **1990**. Short interspersed repeats from *Xenopus* that contain multiple motifs are related to known transposable elements. *Nucl. Acids Res.* 18, 5781–5786.

Mouches, C., Bensaadi, N., Salvado, J.C., **1992**. Characterization of a LINE retroposon dispersed in the genome of three non-sibling *Aedes* mosquito species. *Gene* 120, 183–190.

Munstermann, L.E., Conn, J.E., **1997**. Systematics of mosquito disease vectors (Diptera, Culicidae): impact of molecular biology and cladistic analysis. *Annu. Rev. Entomol.* 42, 351–369.

Murata, S., Takasaki, N., Saitoh, M., Okada, N., **1993**. Determination of the phylogenetic relationships among Pacific salmonids by using short interspersed elements (SINEs) as temporal landmarks of evolution. *Proc. Natl Acad. Sci. USA* 90, 6995–6999.

Nasidze, I., Risch, G.M., Robichaux, M., Sherry, S.T., Batzer, M.A., *et al.*, **2001**. *Alu* insertion polymorphisms and the genetic structure of human from the Caucasus. *Eur. J. Hum. Genet.* 9, 267–272.

Nikaido, M., Rooney, A.P., Okada, N., **1999**. Phylogenetic relationships among cetartiodactyls based on insertions of short and long interpersed elements: hippopotamuses are the closest extant relatives of whales. *Proc. Natl Acad. Sci. USA* 96, 10261–10266.

Nuzhdin, S.V., **1999**. Sure facts, speculations, and open questions about the evolution of transposable element copy number. *Genetica* 107, 129–137.

Ohbayashi, F., Shimada, T., Sugasaki, T., Kawai, S., Mita, K., *et al.*, **1998**. Molecular structure of the *copia*-like retrotransposable element *Yokozuna* on the W chromosome of the silkworm, *Bombyx mori*. *Genes Genet. Syst.* 73, 345–352.

Ohshima, K., Hamada, M., Terai, Y., Okada, N., **1996**. The 3′ ends of tRNA-derived short interspersed repetitive elements are derived from the 3′ ends of long interspersed repetitive elements. *Mol. Cell Biol.* 16, 3756–3764.

Okada, N., **1991**. SINEs. *Curr. Opin. Genet. Devel.* 1, 498–504.

Okada, N., Hamada, M., **1997**. The 3′ ends of tRNA-derived SINEs originated from the 3′ ends of LINEs: a new example from the bovine genome. *J. Mol. Evol. 44* (Suppl. 1), S52–56.

Oosumi, T., Garlick, B., Belknap, W.R., **1995**. Identification and characterization of putative transposable DNA elements in solanaceous plants and *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA 92*, 8886–8890.

Orgel, L., Crick, F., **1980**. Selfish DNA: the ultimate parasite. *Nature 284*, 604–607.

Ostertag, E.M., Prak, E.T., DeBerardinis, R.J., Moran, J.V., Kazazian, H.H., Jr., **2000**. Determination of L1 retrotransposition kinetics in cultured cells. *Nucl. Acids Res. 28*, 1418–1423.

Perkins, H.D., Howells, A.J., **1992**. Genomic sequences with homology to the *P* element of *Drosophila melanogaster* occur in the blowfly *Lucilia cuprina*. *Proc. Natl Acad. Sci. USA 89*, 10753–10757.

Peterson, D.G., Schulze, S.R., Sciara, E.B., Lee, S.A., Bowers, J.E., *et al.*, **2002**. Integration of Cot analysis, DNA cloning, and high-throughput facilitates genome characterization and gene discovery. *Genome Res. 12*, 795–807.

Petrov, D.A., **2001**. Evolution of genome size: new approaches to an old problem. *Trends Genet. 17*, 23–28.

Petrov, D.A., Aminetzach, Y.T., Davis, J.C., Bensasson, D., Hirsh, A.E., **2003**. Size matters: non-LTR retrotransposable elements and ectopic recombination in *Drosophila*. *Mol. Biol. Evol. 20*, 880–892.

Pfeifer, T.A., Ring, M., Grigliatti, T.A., **2000**. Identification and analysis of *Lydia*, a LTR retrotransposon from *Lymantria dispar*. *Insect Mol. Biol. 9*, 349–356.

Pinkerton, A.C., Whyard, S., Mende, H.A., Coates, C.J., O'Brochta, D.A., *et al.*, **1999**. The Queensland fruit fly, *Bactrocera tryoni*, contains multiple members of the *hAT* family of transposable elements. *Insect Mol. Biol. 8*, 423–434.

Plasterk, R.H., Izsvak, Z., Ivics, Z., **1999**. Resident aliens: the *Tc1/mariner* superfamily of transposable elements. *Trends Genet. 15*, 326–332.

Rai, K.S., Black, W.C.T., **1999**. Mosquito genomes: structure, organization, and evolution. *Adv. Genet. 41*, 1–33.

Ribeiro, J.M., Kidwell, M.G., **1994**. Transposable elements as population drive mechanisms: specification of critical parameter values. *J. Med. Entomol. 31*, 10–16.

Rio, D., **2002**. *P* transposable elements in *Drosophila melanogaster.* In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 484–518.

Rizzon, C., Marais, G., Gouy, M., Biemont, C., **2002**. Recombination rate and the distribution of transposable elements *Drosophila melanogaster* genome. *Genome Res. 12*, 400–407.

Robertson, H.M., **1993**. The *mariner* transposable element is widespread in insects. *Nature 362*, 241–245.

Robertson, H.M., **2002**. Evolution of DNA transposons in eukaryotes. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 1093–1110.

Robertson, H.M., Asplund, M.L., **1996**. Bmmar1: a basal lineage of the *mariner* family of transposable elements in the silkworm moth, *Bombyx mori*. *Insect Biochem. Mol. Biol. 26*, 945–954.

Robertson, H.M., Lampe, D.J., **1995**. Distribution of transposable elements in arthropods. *Annu. Rev. Entomol. 40*, 333–357.

Robertson, H.M., Walden, K.K., **2003**. Bmmar6, a second mori subfamily *mariner* transposon from *Bombyx mori*. *Insect Mol. Biol. 12*, 167–171.

Rohr, C.J., Ranson, H., Wang, X., Besansky, N.J., **2002**. Structure and evolution of *mtanga*, a retrotransposon actively expressed on the Y chromosome of the African malaria vector *Anopheles gambiae*. *Mol. Biol. Evol. 19*, 149–162.

Romans, P., Bhattacharyya, R.K., Colavita, A., **1998**. *Ikirara*, a novel transposon family from the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol. Biol. 7*, 1–10.

Rongnoparut, P., Sirichotpakorn, N., Rattanarithikul, R., Linthicum, K.J., **1998**. Sequence heterogeneity in *copia*-like retrotransposons in *Anopheles* (Diptera: Culicidae) in Thailand. *J. Med. Entomol. 35*, 771–777.

Roy, A.M., Carroll, M.L., Kass, D.H., Nguyen, S.V., Salem, A.H., *et al.*, **1999**. Recently integrated human *Alu* repeats: finding needles in the haystack. *Genetica 107*, 149–161.

Roy-Engel, A.M., Carroll, M.L., Vogel, E., Garber, R.K., Nguyen, S.V., *et al.*, **2001**. *Alu* insertion polymorphisms for the study of human genomic diversity. *Genetics 159*, 279–290.

Rubin, E., Levy, A.A., **1997**. Abortive gap repair: underlying mechanism for *Ds* element formation. *Mol. Cell Biol. 17*, 6294–6302.

Salem, A.H., Kilroy, G.E., Watkins, W.S., Jorde, L.B., Batzer, M.A., **2003**. Recently integrated *Alu* elements and human genomic diversity. *Mol. Biol. Evol. 20*, 1349–1361.

Sarkar, A., Sengupta, R., Krzywinski, J., Wang, X., Roth, C., *et al.*, **2003**. *P* elements are found in the genomes of nematoceran insects of the genus *Anopheles*. *Insect Biochem. Mol. Biol. 33*, 381–387.

Sarkar, A., Sin, C., Hong, Y.S., Hogan, J.R., Fraser, M.J., *et al.*, **2003**. Molecular evolutionary analysis of the widespread *piggyBac* transposon family and related "domesticated" sequences. *Mol. Genet. Genomics 270*, 173–180.

Severson, D.W., Mori, A., Zhang, Y., Christensen, B.M., **1993**. Linkage map for *Aedes aegypti* using restriction fragment length polymorphisms. *J. Hered. 84*, 241–247.

Shao, H., Qi, Y., Tu, Z., **2001**. *MsqTc3*, a *Tc3*-like transposon in the yellow fever mosquito *Aedes*. *Insect Mol. Biol. 10*, 421–425.

Shao, H., Tu, Z., **2001**. Expanding the diversity of the *IS630-Tc1-mariner* superfamily: discovery of a unique DD37E transposon and reclassification of the DD37D and DD39D transposons. *Genetics 159*, 1103–1115.

Shedlock, A.M., Okada, N., **2000**. SINE insertions: powerful tools for molecular systematics. *BioEssays 22*, 148–160.

Sherratt, D.J., **1995**. Mobile Genetic Elements. Oxford University Press, Oxford.

Shimamura, M., Yasue, H., Ohshima, K., Abe, H., Kato, H., *et al.*, **1997**. Molecular evidence from retroposons that whales form a clade within even-toed ungulates. *Nature 388*, 666–670.

Shiroishi, T., Sagai, T., Moriwaki, K., **1993**. Hotspots of meiotic recombination in the mouse major histocompatibility complex. *Genetica 88*, 187–196.

Siebel, C.W., Admon, A., Rio, D.C., **1995**. Soma-specific expression and cloning of PSI, a negative regulator of *P* element pre-mRNA splicing. *Genes Devel. 9*, 269–283.

Siebel, C.W., Fresco, L.D., Rio, D.C., **1992**. The mechanism of somatic inhibition of *Drosophila P*-element pre-mRNA splicing: multiprotein complexes at an exon pseudo-5′ splice site control U1 snRNP binding. *Genes Devel. 6*, 1386–1401.

Silva, J.C., Kidwell, M.G., **2000**. Horizontal transfer and selection in the evolution of *P* elements. *Mol. Biol. Evol. 17*, 1542–1557.

Silva, J.C., Loreto, E.L., Clark, J.B., **2004**. Factors that affect the horizontal transfer of transposable elements. *Curr. Issues Mol. Biol. 6*, 57–71.

Smit, A.F., Riggs, A.D., **1996**. Tiggers and DNA transposon fossils in the human genome. *Proc. Natl Acad. Sci. USA 93*, 1443–1448.

Song, W.Y., Pi, L.Y., Bureau, T.E., Ronald, P.C., **1998**. Identification and characterization of 14 transposon-like noncoding regions of members of the Xa21 family of disease genes in rice. *Mol. Gen. Genet. 258*, 449–456.

Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., *et al.*, **1999**. The Berkeley Drosophila Genome Project gene disruption project: single *P*-element insertions mutating 25% of vital *Drosophila* genes. *Genetics 153*, 135–177.

Spradling, A.C., Stern, D.M., Kiss, I., Roote, J., Laverty, T., *et al.*, **1995**. Gene disruptions using *P* transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl Acad. Sci. USA 92*, 10824–10830.

Stoneking, M., Fontius, J.J., Clifford, S.L., Soodyall, H., Arcot, S.S., *et al.*, **1997**. Alu insertion polymorphisms and human evolution: evidence for a larger population size in Africa. *Genome Res. 7*, 1061–1071.

Streck, R.D., MacGaffey, J.E., Beckendorf, S.K., **1986**. The structure of *hobo* transposable elements and their insertion. *EMBO J. 5*, 3615–3623.

Sun, S.C., Asling, B., Faye, I., **1991**. Organization and expression of the immunoresponsive lysozyme gene in the giant silk moth, *Hyalophora cecropia*. *J. Biol. Chem. 266*, 6644–6649.

Sundararajan, P., Atkinson, P.W., O'Brochta, D.A., **1999**. Transposable element interactions in insects: cross-mobilization of *hobo* and *Hermes*. *Insect Mol. Biol. 8*, 359–368.

Swofford, D.L., **2001**. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates, Sunderland, MA.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., *et al.*, **1999**. The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell 99*, 123–132.

Takahashi, K., Terai, Y., Nishida, M., Okada, N., **1998**. A novel family of short interspersed repetitive elements (SINEs) from cichlids: the patterns of insertion of SINEs at orthologous loci support the proposed monophyly of four major groups of cichlid fishes in Lake Tanganyika. *Mol. Biol. Evol. 15*, 391–407.

Terai, Y., Takahashi, K., Nishida, M., Sato, T., Okada, N., **2003**. Using SINEs to probe ancient explosive speciation: "hidden" radiation of African cichlids? *Mol. Biol. Evol. 20*, 924–930.

Terzian, C., Ferraz, C., Demaille, J., Bucheton, A., **2000**. Evolution of the *Gypsy* endogenous retrovirus in the *Drosophila melanogaster* subgroup. *Mol. Biol. Evol. 17*, 908–914.

Thompson, J.D., Higgins, D.G., Gibson, T.J., **1994**. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res. 22*, 4673–4680.

Tikhonov, A.P., Bennetzen, J.L., Avramova, Z.V., **2000**. Structural domains and matrix attachment regions along colinear chromosomal segments of maize and sorghum. *Plant Cell 12*, 249–264.

Transgenesis and Genomics of Invertebrate Organisms, **2003**. Reports of the 4th International Workshop, Pacific Grove, CA, 11–15 May 2003. http://www.nises.affrc.go.jp/sgp/SGP.htm; www.genoptera.com/press-release/PR20030403.html.

Truett, M.A., Jones, R.S., Potter, S.S., **1981**. Unusual structure of the FB family of transposable elements in *Drosophila*. *Cell 24*, 753–763.

Tu, Z., **1997**. Three novel families of miniature inverted-repeat transposable elements are associated with genes of the yellow fever mosquito, *Aedes aegypti*. *Proc. Natl Acad. Sci. USA 94*, 7475–7480.

Tu, Z., **1999**. Genomic and evolutionary analysis of *Feilai*, a diverse family of highly reiterated SINEs in the yellow fever mosquito, *Aedes aegypti*. *Mol. Biol. Evol. 16*, 760–772.

Tu, Z., **2000**. Molecular and evolutionary analysis of two divergent subfamilies of a novel miniature inverted repeat transposable element in the yellow fever mosquito, *Aedes aegypti*. *Mol. Biol. Evol. 17*, 1313–1325.

Tu, Z., **2001a**. Eight novel families of miniature inverted repeat transposable elements in the African malaria mosquito, *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA 98*, 1699–1704.

Tu, Z., **2001b**. *Maque*, a family of extremely short interspersed repetitive elements: characterization, possible mechanism of transposition, and evolutionary implications. *Gene 263*, 247–253.

Tu, Z., Hill, J.J., **1999**. *MosquI*, a novel family of mosquito retrotransposons distantly related to the *Drosophila I* factors, may consist of elements of more than one origin. *Mol. Biol. Evol. 16*, 1675–1686.

Tu, Z., Isoe, J., Guzova, J.A., **1998**. Structural, genomic, and phylogenetic analysis of *Lian*, a novel family of non-LTR retrotransposons in the yellow fever mosquito, *Aedes aegypti*. *Mol. Biol. Evol. 15*, 837–853.

Tu, Z., Orphanidis, S.P., **2001**. *Microuli*, a family of miniature subterminal inverted-repeat transposable elements (MSITEs): transposition without terminal inverted repeats. *Mol. Biol. Evol. 18*, 893–895.

Tudor, M., Lobocka, M., Goodell, M., Pettitt, J., O'Hare, K., **1992**. The *pogo* transposable element family of *Drosophila melanogaster*. *Mol. Gen. Genet. 232*, 126–134.

Van den Broeck, D., Maes, T., Sauer, M., Zethof, J., De Keukeleire, P., *et al.*, **1998**. Transposon display identifies individual transposable elements in high copy number lines. *Plant J. 13*, 121–129.

Vazquez-Manrique, R.P., Hernandez, M., Martinez-Sebastian, M.J., de Frutos, R., **2000**. Evolution of *gypsy* endogenous retrovirus in the *Drosophila obscura* species group. *Mol. Biol. Evol. 17*, 1185–1193.

Vieira, C., Lepetit, D., Dumont, S., Biemont, C., **1999**. Wake up of transposable elements following *Drosophila simulans* worldwide colonization. *Mol. Biol. Evol. 16*, 1251–1255.

Voytas, D., Boeke, J., **2002**. Ty1 and Ty5 of *Saccharomyces cerevisiae*. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 631–662.

Walbot, V., Rudenko, G., **2002**. *MuDR/Mu* transposable elements of maize. In: Craig, N., Craigie, R., Gellert, M., Lambowitz, A. (Eds.), Mobile DNA II. American Society for Microbiology Press, Washington, DC, pp. 533–564.

Warren, A.M., Crampton, J.M., **1991**. The *Aedes aegypti* genome: complexity and organization. *Genet. Res. 58*, 225–232.

Warren, A.M., Hughes, M.A., Crampton, J.M., **1997**. Zebedee: a novel *copia*-Ty1 family of transposable elements in the genome of the medically important mosquito *Aedes aegypti*. *Mol. Gen. Genet. 254*, 505–513.

Warren, W.D., Atkinson, P.W., O'Brochta, D.A., **1994**. The *Hermes* transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the *hobo, Ac*, and *Tam3* (*hAT*) element family. *Genet. Res. 64*, 87–97.

Watkins, W.S., Ricker, C.E., Bamshad, M.J., Carroll, M.L., Nguyen, S.V., *et al.*, **2001**. Patterns of ancestral human diversity: an analysis of *Alu*-insertion and restriction-site polymorphisms. *Am. J. Hum. Genet. 68*, 738–752.

Wei, W., Gilbert, N., Ooi, S.L., Lawler, J.F., Ostertag, E.M., *et al.*, **2001**. Human L1 retrotransposition: *cis* preference versus *trans* complementation. *Mol. Cell Biol. 21*, 1429–1439.

Wessler, S.R., **1996**. Turned on by stress: plant retrotransposons. *Curr. Biol. 6*, 959–961.

Wessler, S.R., Bureau, T.E., White, S.E., **1995**. LTR-retrotransposons and MITEs: important players in the evolution of plant genomes. *Curr. Opin. Genet. Devel. 5*, 814–821.

Wobus, U., Baumlein, H., Bogachev, S.S., Borisevich, I.V., Panitz, R., *et al.*, **1990**. A new transposable element in *Chironomus thummi*. *Mol. Gen. Genet. 222*, 311–316.

Xiong, Y., Burke, W.D., Eickbush, T.H., **1993**. Pao, a highly divergent retrotransposable element from *Bombyx mori* containing long terminal repeats with tandem copies of the putative R regions. *Nucleic Acids Res. 21*, 2117–2123.

Xiong, Y., Eickbush, T.H., **1988**. The site-specific ribosomal DNA insertion element *R1Bm* belongs to a class of non-long-terminal-repeat retrotransposons. *Mol. Cell Biol. 8*, 114–123.

Xiong, Y., Eickbush, T.H., **1993**. *Dong*, a non-long terminal repeat (non-LTR) retrotransposable element from *Bombyx mori*. *Nucl. Acids Res. 21*, 1318.

Yan, G., Romero-Severson, J., Walton, M., Chadee, D.D., Severson, D.W., **1999**. Population genetics of the yellow fever mosquito in Trinidad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. *Mol. Ecol. 8*, 951–963.

Yang, H.P., Nuzhdin, S.V., **2003**. Fitness costs of Doc expression are insufficient to stabilize its copy number in *Drosophila melanogaster*. *Mol. Biol. Evol. 20*, 800–804.

York, D.S., Blum, V.M., Low, J.A., Rowold, D.J., Puzyrev, V., *et al.*, **1999**. Phylogenetic signals from point mutations and polymorphic *Alu* insertions. *Genetica 107*, 163–170.

Yoshiyama, M., Tu, Z., Kainoh, Y., Honda, H., Shono, T., *et al.*, **2001**. Possible horizontal transfer of a transposable element from host to parasitoid. *Mol. Biol. Evol. 18*, 1952–1958.

Zhang, Q., Arbuckle, J., Wessler, S.R., **2000**. Recent, extensive, and preferential insertion of members of the miniature inverted-repeat transposable element family *Heartbreaker* into genic regions of maize. *Proc. Natl Acad. Sci. USA 97*, 1160–1165.

Zhang, X., Feschotte, C., Zhang, Q., Jiang, N., Eggleston, W.B., *et al.*, **2001**. *P* instability factor: an active maize transposon system associated with the amplification of *Tourist*-like MITEs and a new superfamily of transposases. *Proc. Natl Acad. Sci. USA 98*, 12572–12577.

Zhou, Q., Haymer, D.S., **1997**. Molecular structure of *yoyo*, a *gypsy*-like retrotransposon from the mediterranean fruit fly, *Ceratitis capitata*. *Genetica 101*, 167–178.

Relevant Websites

http://jaketu.biochem.vt.edu – programs for TE searches from Virginia Tech.

http://www.genetics.wustl.edu – includes program RECON for identifying TE sequences.

4.13 Transposable Elements for Insect Transformation

A M Handler, US Department of Agriculture,
Agricultural Research Service, Gainesville, FL, USA
D A O'Brochta, University of Maryland
Biotechnology Institute, College Park, MD, USA

Published by Elsevier BV.

4.13.1. Introduction

The ability to create genetically transformed organisms has played a central role in the history of modern genetics, in particular, to our understanding of gene expression and development. Indeed, the pioneering transformation experiments of *Pneumococcus* by Griffith (1928) and subsequent systematic analyses by Avery *et al.* (1944), that showed transformation from a "rough" to a "smooth" bacterial cell wall phenotype, were instrumental in defining DNA as the inherited genetic material. The importance of these initial transformation experiments to prokaryotic genetic analysis was widely appreciated, and continued studies by many other laboratories laid the foundation for modern molecular biology.

The importance of "transformation" technology to eukaryotic genetic studies was apparent, and several of the initial attempts to create transgenic animals were performed in insects, though the means of achieving and assessing insect transformation were not straightforward. The primary reasons why these early attempts to create transgenic insects were largely unsuccessful were the inability to isolate and reproduce individual genetic elements that could be used as transformation vectors and markers, and the lack of efficient means of introducing DNA into germ cells. Most of the initial studies of insect transformation relied on soaking embryos or larvae, with visible mutant phenotypes, in solutions of total wild-type genomic DNA in hopes of reverting the mutant phenotype. The first experiments performed on *Bombyx* and *Ephestia* met with some success where mutant wing color pattern phenotypes were reverted in some organisms, though inheritance was inconsistent and transformation events could not be confirmed unequivocally

(Caspari and Nawa, 1965; Nawa and Yamada, 1968; Nawa *et al.*, 1971). Similar results were obtained in *Drosophila* studies (Fox and Yoon, 1966, 1970; Fox *et al.*, 1970), and for all of these initial experiments, it is most likely that the observed phenotypic changes resulted from extra-chromosomal maintainance of introduced DNA in the somatic tissue by an unknown mechanism. A different approach involving the microinjection of wild-type genomic DNA into embryos homozygous for a recessive eye color mutation (*vermilion*), resulted in transformants with a reversion to the normal red eye color phenotype. While the reversion event was genetically mapped away from the mutant locus, a thorough molecular analysis to verify a transformation event, before the lines were lost, was not achieved and so the nature of the phenomenon observed in this experiment remains unexplained (Germeraad, 1976).

In the mid-1970s a turning point in insect science occurred with the extension of molecular genetic analysis to *Drosophila melanogaster.* These early studies and subsequent studies not only provided many of the tools and reagents necessary for developing and critically assessing genetic transformation in insects, but they also emphasized the need for a technology that would facilitate a more complete understanding of the genes being isolated using recombinant DNA methods. One technology that was clearly needed was a means to integrate DNA molecules into the chromosomes of germ cells where it remained stable, resulting in heritable germline transformation. The simple introduction of raw linearized DNA into preblastoderm embryos in the hope of fortuitous recombination into host chromosomes was clearly not reliable. Interest was growing, however, in the use of mobile genetic elements as vectors for DNA integration, including retrotransposons and transposons that were being isolated in *Drosophila* for the first time. Foremost among these was the *P* transposable element, isolated from certain mutant alleles of the *white* gene. The subsequent testing and success of transformation mediated by the *P* element in the *Drosophila* germline proved to be a dramatic turning point in the genetic analysis of an insect species. The eventual impact of this technology on understanding genetic mechanisms in all eukaryotic systems cannot be understated. The success with *P* in *Drosophila* gave hope that this system could be straightforwardly extended to genetic manipulation of other insect species, and especially those highly important to agriculture and human health. While there was reason for optimism, we now realize that this was a naive expectation given what we now understand about the natural history of *P* elements relative to other Class II transposable elements, in particular its extremely limited distribution and its dependence on species-specific host factors. The inability of *P* to function in non-drosophilids, however, was a motivating force to more completely understand transposon regulation and the identification and testing of new vector systems. These included other transposable elements, as well as viral and bacterial vectors.

The development of routine methods for insect gene-transfer was probably delayed by a decade due to attention being focused exclusively on the *P* element. Yet, this delay has resulted in a more varied toolbox of vectors and markers that now allow nearly routine transformation for many important species, and the potential for transformation of most insects (see Handler, 2001). Indeed, some of the tools developed for testing the *P* element, in particular embryonic mobility assays, are now routinely used for initial tests for function of other vectors in an insect species before more laborious and time-consuming transformation experiments are attempted.

The creation of this varied toolbox was first related to the potential need for different vector and marker systems for different insect species. We now realize that the future of genetic analysis will depend on multiple vector and marker systems for each of these species, since genomics and functional genomics studies will require multiple systems for DNA integration and reporters for gene expression. Indeed, germline transformation is essential for the insertional mutagenesis and functional genomics studies that are critical underpinnings for both assessing genomic architecture and relating sequences to gene expression. Notably, the continuing functional analysis of the *Drosophila* genome now relies on the vectors and markers, described in this chapter, that were first developed for nondrosophilid insect species.

4.13.2. *P* Element Transformation

4.13.2.1. *P* Element

The use of transposable element-based vectors for *Drosophila* transformation followed the discovery of short inverted terminal repeat-type elements similar to the *Activator* (*Ac*) element discovered in maize by McClinotck (see Federoff, 1989). The first such element to be discovered in insects was the *P* element, the factor responsible for hybrid dysgenesis that occurred in crosses of males from a *P* strain (containing *P* factor) with females from an

M strain (devoid of *P* factor) females (Kidwell *et al*., 1977). The identification of *P* sequences resulted from the molecular analysis of *P*-induced *white* mutations that occurred in dysgenic hybrids (Rubin *et al*., 1982). While the initial *P* elements isolated as insertion sequences were incomplete, nonautonomous elements, complete functional elements were later isolated and characterized by O'Hare and Rubin (1983).

P is 2907 bp in length with 31 bp inverted terminal repeats (ITRs) and 11 bp subterminal inverted repeats that occur approximately 125 bp from each terminus (**Figure 1**). Other repeat sequences exist within *P*, but their functional significance, if any, remains unknown. A defining signature for *P*, as with other transposable elements, is the nature of its insertion site which consists of an 8 bp direct repeat duplication. The extensive use of *P* for transformation and transposon mutagenesis has shown the element to have a distinctly nonrandom pattern of integration. It is now clear that *P* elements are blind to a significant fraction of the genome and new gene vectors are being employed in *Drosophila* to complement these limitations. *P* elements and all transposable elements currently used as insect gene vectors belong to a general group of transposable elements known as Class II short inverted terminal repeat transposons (see Finnegan, 1989). These elements transpose via a DNA intermediate and generally utilize a cut-and-paste mechanism that creates a duplication of the insertion site. These are distinguished from Class I elements, or retrotransposons, that have long direct terminal repeats (LTRs) and transpose via reverse transcription an RNA intermediate.

The original use of *P* for germline transformation was accomplished by inserting a marker gene within the element so that it did not disrupt activity of the terminal sequences or the transposase gene. The *rosy*$^+$ gene was inserted at the 3′ end of the transposase-coding region, but upstream of the 3′ subterminal inverted repeat sequence. Plasmids containing this vector were injected into preblastoderm (syncytial) embryos homozygous for *ry*$^-$ so the *P* vector could transpose into germ cell nuclei. Germline transformation events were identified in the following generation (G_1) by virtue of reversion of the mutant *ry*$^-$ eye color phenotype to wild-type. These experiments not only proved the feasibility of transposon-mediated transformation, but also permitted structure–function relationships within the *P* element to be defined (Karess and Rubin, 1984). The *P* transcriptional unit was found to be composed of four exons separated by three introns. Further analysis determined that the *P* transposase function is cell-type specific owing to differential transcript splicing of the third intron that is limited to the germline. The lack of splicing in the soma results in production of nonfunctional truncated polypeptides in this tissue (Rio *et al*., 1986).

While the original *P* vector allowed efficient transformation, the presence of a functional transposase gene within the vector made the system self-mobilizable (autonomous) and inherently unstable, allowing potential excision or transposition of the original insertion event. Subsequent vector development resulted in a binary system in which the vector transposase was deleted or made defective by insertion of a marker gene. The ability of the transposase

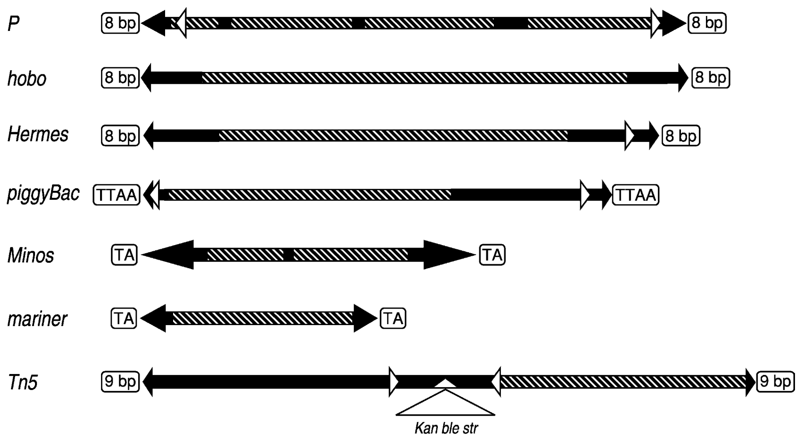


Figure 1 Diagram of transposable elements currently in use for the germline transformation of insect species. The left arms represent the 5′ termini and right arms represent the 3′ termini. Transposon sizes and specific internal elements are shown in relative positions but are not at precise scale. Major structural elements include duplicated insertion sites (open boxes); inverted terminal repeat sequences (black arrowheads); internal subterminal repeat sequences (white arrowheads); transposase coding region (boxed diagonals); and intron sequences (black boxes). The *Tn5* element is a composite transposable element consisting of two functional elements flanking three antibiotic resistance genes. Refer to text for specific details on nucleotide lengths and relative positions.

to act *in trans* allowed transposase to be provided by a separate plasmid (helper), that could facilitate vector integrations when cointroduced with the vector-containing plasmid into the same nucleus (Rubin and Spradling, 1982). Integrations would remain stable if the helper did not integrate, but the original helpers, such as pπ25.1, were autonomous *P* elements themselves that could integrate along with the vector. While helper integration was diminished by injecting much higher concentrations of vector plasmid, this possibility was only eliminated with the creation of defective helpers having one or both of their terminal sequences deleted (known as "wings-clipped" helpers). The first of these was pπ25.7wc, which was immobilized by deletion of 3′ terminal sequences (Karess and Rubin, 1984). This prototype vector system served as a model for binary systems of nonautonomous vector: helper elements used for all the transposon-based transformation systems currently in use (**Table 1**).

A notable characteristic of *P* elements was not only their discontinuous distribution within the species (*P* and *M* strains), but their discontinuous interspecific distribution. Based on its distribution patterns it has become apparent that *P* was recently introduced into *D. melanogaster* from *D. willistoni* by an unknown mechanism (Daniels and Strausbaugh, 1986). Regardless of the mechanism since the 1950s, *P* elements have thoroughly invaded wild populations of *D. melanogaster* (Anxolabéhère *et al*., 1988), and without the existence of *M* strain laboratory stocks that were removed from nature before this time, the development of *P* vectors might never have been realized. This is due to the repression of *P* mobility in *P*-containing strains that was first observed in hybrid dysgenesis studies, which also showed that movement was not repressed in *M* strains devoid of *P*. The basis for *P* strain repression appears to be due to a number of factors including repressor protein synthesis, transposase titration by resident defective elements, and regulation of transposase gene transcription (Handler *et al*., 1993b; Simmons *et al*., 2002). As will be discussed further on, other vector systems in use have thus far been shown to be widely functional in several orders of insects, and the presence of the same or related transposon in a host insect does not necessarily repress vector transposition. In this and several other aspects, the *P* vector system appears to be the exception rather than the rule for transposon-mediated gene transfer in insects.

4.13.2.2. *P* Vectors and Markers

Regardless of regulatory differences between *P* and other transposon vector systems currently in use, methods developed for *P* transformation of *Drosophila* serve as a paradigm for all other insect vector systems. Those familiar with *Drosophila* transformation will be in the best position to attempt these methods in other insects. Current techniques developed for other insect species are variations on a theme, although as we describe, considerable modifications have been made. Several comprehensive reviews are available for more specific details on the structure, function, and use of *P* for transformation in *Drosophila*, which are highly relevant to the understanding and use of other vector systems (see Karess, 1985; Spradling, 1986; Engels, 1989; Handler and O'Brochta, 1991). Particularly useful are the books and method manuals by Ashburner (1989a, 1989b) that review the various vectors, markers, and methodologies used for *Drosophila* transformation, as well as early techniques used to manipulate *Drosophila* embryos. This information is especially applicable to other insect systems.

The first consideration for transformation is the design of vector and helper plasmids, and the marker system used for transformant selection. The first *P* vectors and helpers were actually autonomous vectors, which was probably a useful starting point since the actual sequence requirements for vector mobility and transposase function were unknown. As noted, the first nonautonomous helper had a 3′ terminal deletion that prevented its transposition, providing greater control over vector stability. However, this source of transposase was inefficient, until it was placed under *hsp70* regulation which allowed transposase induction by heat shock (Steller and Pirrotta, 1986). All other vector system helper constructs have similarly taken advantage of heat shock promoters, mostly from the *D. melanogaster hsp70* gene, but other *hsp* promoters have been tested including those from the host species being transformed. Other constitutive promoters such as those from the genes for *actin* and *α1-tubulin* have proven successful for helper transposase regulation, and will be discussed further on.

While sufficient transposase production is critical for transposition, the structure of the vector is equally important, and for some, very subtle changes from the autonomous vector can dramatically decrease or eliminate mobility. These variations include critical sequences (typically in the termini and subtermini), and placement and amount of exogenous DNA inserted within the termini. For some vectors the amount of plasmid DNA external to the vector can affect transposition rates. Subsequent to the initial test of several *P* vectors, the terminal sequence requirements for *P* mobility were determined to

Table 1 Transposon-mediated germline transformation

Transposon	*Host species*	*Marker*	*Reference*
Hermes	*Aedes aegypti*	*Dm-cinnabar*$^+$	Jasinskiene *et al.* (1998)
		actin5C-EGFP	Pinkerton *et al.* (2000)
	Culex quinquefasciatus	actin5C-EGFP	Allen *et al.* (2001)
	Ceratitis capitata	*Cc-white*$^+$	Michel *et al.* (2001)
	Drosophila melanogaster	*Dm-white*$^+$	O'Brochta *et al.* (1996)
		actin5C-EGFP	Pinkerton *et al.* (2000)
		3xP3-EGFP	Horn *et al.* (2000)
	Stomoxys calcitrans	actin5C-EGFP	O'Brochta *et al.* (2000)
	Tribolium castaneum	3xP3-EGFP	Berghammer *et al.* (1999)
hobo	*Drosophila melanogaster*	*Dm*-mini-*white*$^+$	Blackman *et al.* (1989)
	Drosophila virilis	*Dm*-mini-*white*$^+$	Lozovskaya *et al.* (1996); Gomez and Handler (1997)
mariner (*Mos1*)	*Aedes aegypti*	*Dm-cinnabar*$^+$	Coates *et al.* (1998)
	Drosophila melanogaster	*Dm-white*$^+$	Garza *et al.* (1991); Lidholm *et al.* (1993)
		3xP3-EGFP	Horn *et al.* (2000)
	Drosophila virilis	*Dm-white*$^+$	Lohe and Hartl (1996a)
Minos	*Anopheles stephensi*	actin5C-EGFP	Catteruccia *et al.* (2000b)
	Ceratitis capitata	*Cc-white*$^+$	Loukeris *et al.* (1995b)
	Drosophila melanogaster	*Dm-white*$^+$	Loukeris *et al.* (1995a)
P	*Drosophila melanogaster*	*Dm-rosy*$^+$	Rubin and Spradling (1982)
		Dm-white$^+$	Hazelrigg *et al.* (1984); Pirrotta *et al.* (1985)
		Dm-hsp70-mini-*white*$^+$	Klemenz *et al.* (1987)
		pUChsneo	Steller and Pirrotta (1985)
	Drosophila simulans	*Dm-rosy*$^+$	Scavarda and Hartl (1984)
piggyBac	*Aedes aegypti*	*Dm-cinnabar*$^+$	Lobo *et al.* (2002)
		3xP3-EGFP	Kokoza *et al.* (2001)
	Anastrepha suspensa	PUb-nls-EGFP	Handler and Harrell (2000)
	Anopheles albimanus	PUb-nls-EGFP	Perera *et al.* (2002)
	Anopheles gambiae	hr5-ie1:EGFP	Grossman *et al.* (2001)
	Anopheles stephensi	actin5C-DsRed	Nolan *et al.* (2002)
	Athalia rosae	*Bm*A3-EGFP, *hsp70*-GFP	Sumitani *et al.* (2003)
	Bactrocera dorsalis	*Cc-white*$^+$	Handler and McCombs (2000)
		PUb-nls-EGFP	Handler and McCombs (unpublished data)
	Bombyx mori	*Bm*A3-EGFP	Tamura *et al.* (2000)
		3xP3-EGFP	Thomas *et al.* (2002); Uhlirova *et al.* (2002)
	Ceratitis capitata	*Cc-white*$^+$	Handler *et al.* (1998)
		PUb-nls-EGFP	Handler and Krasteva (unpublished data)
		PUb-DsRed1	Handler and Krasteva (unpublished data)
	Cochliomyia hominivorax	PUb-nls-EGFP	Allen *et al.* (2004)
	Drosophila melanogaster	*Dm-white*$^+$, PUb-nls-EGFP	Handler and Harrell (1999)
		PUb-DsRed1	Handler and Harrell (2001)
		3xP3-EGFP	Horn *et al.* (2000)
		3xP3-EYFP	Horn and Wimmer (2000)
		3xP3-ECFP	Horn and Wimmer (2000)
		3xP3-DsRed	Horn *et al.* (2002)
	Lucilia cuprina	PUb-nls-EGFP	Heinrich *et al.* (2002)
	Musca domestica	3xP3-EGFP	Hediger *et al.* (2000)
	Pectinophora gossypiella	*Bm*A3-EGFP	Peloquin *et al.* (2000)
	Tribolium castaneum	3xP3-EGFP	Berghammer *et al.* (1999)
			Lorenzen *et al.* (2003)
Tn5	*Aedes aegypti*	3xP3-DsRed	O'Brochta (unpublished data)

EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein.

include 138 bp of the 5′ end and 216 bp of the 3′ end. While the inverted repeat sequences within these terminal regions are identical, the adjacent sequences were found not to be interchangeable in terms of vector mobility (Mullins *et al*., 1989). Of interest was the discovery that the strongest binding affinity for the *P* transposase was at sequences approximately 50 bp internal to the terminal repeats (Rio and Rubin, 1988; Kaufman *et al*., 1989). While the minimal sequences required for mobility may be used in vectors, typically the rate of mobility decreases with the decreased length of terminal sequence. Specific sequences may be required for binding of transposase or other nuclear factors, and conformational changes needed for recombination may be dependent upon sequence length and position.

P vector mobility was also found to be influenced by the amount of exogenous DNA inserted between the termini, with transformation frequency diminishing with increasing size. Initial tests with 8 kb vectors marked with *rosy* yielded transformation frequencies of approximately 50% per fertile G_0, while use of 15 kb vectors resulted in 20% frequencies (see Spradling, 1986). Larger vectors could transpose but frequencies approached 1% or less.

Of equal importance to creating an efficient vector system is having marker genes and appropriate host strains that will allow efficient and unambiguous identification or selection of transgenic individuals. Indeed, the genetic resources available for *Drosophila* also provided cloned wild-type DNA and appropriate mutant hosts for use in visible mutant-rescue marker systems that made testing *P* transformation possible. As noted, the first of these used the ry^+ eye color gene, but this required a relatively large genomic fragment of nearly 8 kb. The *white* (*w*) eye color gene was then tested, but this required a genomic sequence that was longer than *ry*, and resultant transformation frequencies were relatively low (Hazelrigg *et al*., 1984; Pirrotta *et al*., 1985). New *w* markers, known as mini-*white*, that had the large first intron deleted decreased the marker insert to 4 kb resulting in much more efficient transformation, and placing the mini-*white* marker under *hsp70* regulation increased efficiencies further (Klemenz *et al*., 1987). Use of *w* markers, especially in CaSpeR vectors (Pirrotta, 1988), has been a mainstay of *Drosophila* transformation, yet expression of the *w* gene in particular is subject to position effect variegation/suppression (PEV) that typically diminishes eye pigmentation. PEV, indeed, was originally discovered as a result of translocating w^+ proximal to heterochromatin (Green, 1996), and it routinely manifests itself in w^- flies transformed with w^+. This effect has been observed with the use of eye pigmentation markers in several other insect species as well.

Other markers based upon chemical selections or enzymatic activity were also developed for *Drosophila*, though none has found routine use. These included alcohol dehydrogenase (*Adh*) (Goldberg *et al*., 1983) and dopa decarboxylase (*Ddc*) (Scholnick *et al*., 1983) that complemented existing mutations, and neomycin phosphotransferase (NPT or *neo*) (Steller and Pirrotta, 1985), β-galactosidase (Lis *et al*., 1983), organophosphorus dehydrogenase (*opd*) (Benedict *et al*., 1995), and dieldrin resistance (*Rdl*) (ffrench-Constant *et al*., 1991) which are dominant selections not requiring preexisting mutations (see ffrench-Constant and Benedict, 2000).

4.13.2.3. *P* Transformation of Non-Drosophilids

Given the straightforward procedures for transforming *Drosophila* with *P* elements, there were high expectations that the system would function in other insects. The ability to test this was facilitated by the development of the neomycin (G418)-resistance marker system (Steller and Pirrotta, 1985), and neomycin resistance-containing *P* vectors were widely tested in tephritid flies and mosquitoes (see Walker, 1990; Handler and O'Brochta, 1991). Unfortunately, the neomycin resistance system was generally unreliable, and recovery of resistant individuals that were not transgenic was common. In three mosquito species, however, neomycin resistant transgenic insects were recovered but they arose from rare transposition-independent recombination events (Miller *et al*., 1987; McGrane *et al*., 1988; Morris *et al*., 1989). Other dominant chemical resistance markers, including *opd* and *Rdl*, which had had some success in *Drosophila*, were also tested, but no transformation events could be verified in other insects. A major limitation of these experiments was that, given the numerous variables involved, it was impossible to determine which components in the system were failing. This limitation led to efforts to determine systematically whether the transposon vector system was, indeed, functional in host embryos, which resulted in the development of rapid transposon mobility assays as described below. The first of these assays tested *P* excision in drosophilid and non-drosophilid embryos, revealing that *P* function decreased in drosophilids as a function of relatedness to *D. melanogaster*, with no function evident in non-drosophilids (O'Brochta and Handler, 1988; Handler *et al*., 1993a). These results were the first indication that for transposon-mediated germline transformation to succeed in non-drosophilids, new vector

systems would have to be created from existing and newly discovered transposon systems.

4.13.3. Excision and Transposition Assays for Vector Mobility

Assessing the ability of an insect gene vector to function in a particular species can be challenging. The procedures required to create a transgenic insect using transposable element-based gene vectors require a great deal of technical skill and the ability to perform basic genetic manipulations. Depending on the insect, its generation time, and its amenability to being reared in the laboratory, the process of genetic transformation can be quite lengthy. At the early stages of developing non-drosophilid transformation technology there was little experience in manipulating and injecting the embryos of the various non-drosophilid species of insects. In addition, the genetic markers available to select for, or recognize, transgenic insects were limited and none could be confidently expected to function optimally in the species being tested at that time. Consequently, early efforts to test the functionality of potential gene vectors, by attempting to create transgenic insects, required simultaneous success in dealing with a number of daunting challenges. The failure of these efforts to yield a transgenic insect could not, unfortunately, be ascribed to the failure of any one particular step in the process (see Handler and O'Brochta, 1991). These efforts, therefore, did not represent an isolated test of the gene vector since failure to obtain a transgenic insect might have been due to a failure in DNA delivery, expression of the genetic marker, or the failure of the transposable element vector system. Technology development under these conditions was very difficult. What was needed was an experimental system that permitted the activity of the transposable element system to be assessed in the species of interest independent of any prospective genetic marker system and DNA delivery system. Such a system was developed for investigating the mobility properties of the *D. melanogaster* *P* element, and was very adaptable to other transposable element and insect systems (Figure 2).

The system developed for *P* elements involved transfecting *Drosophila* cells with a mixture of two plasmids – one containing a *P* element inserted into the coding region of the LacZα peptide of a common cloning vector, and a second containing the *P* element transposase gene under the regulatory control

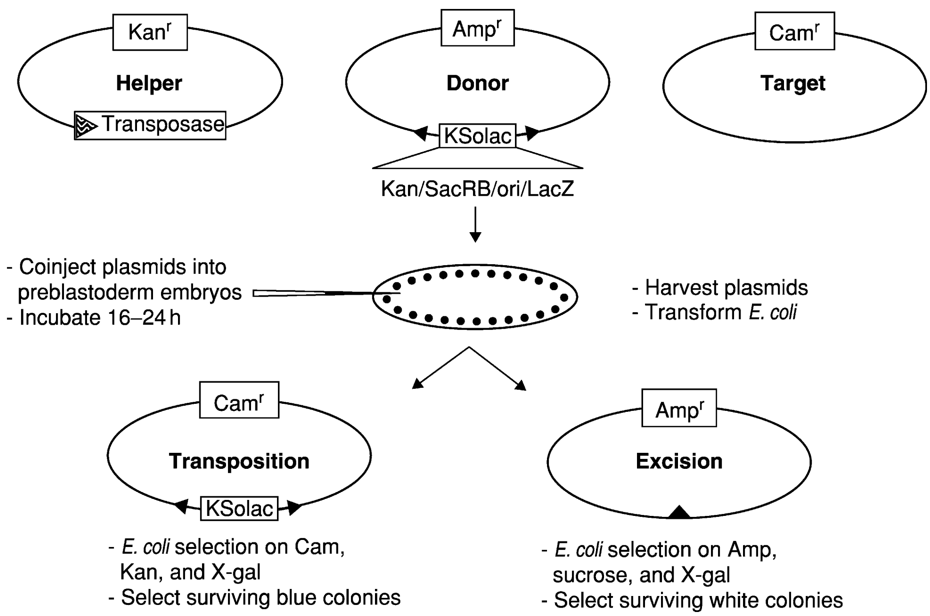


Figure 2 Plasmid-based transposable element mobility assays. A mixture of three plasmids is coinjected into preblastoderm embryos to insure incorporation into nuclei. After approximately 24 h the plasmids are extracted from the embryos and introduced into *E. coli*. Transient expression of the transposase gene on the helper plasmid in the developing embryos results in the production of functional transposase. If the transposase catalyzes excision and transposition of the element, excision will result in the loss of element-specific markers on the donor plasmid. In the example shown sucrose sensitivity, β-galactosidase activity, and kanamycin resistance are lost, and others could be used. Transposition results in the target plasmid acquiring all of the element-specific markers. In this example the target plasmid is from a Gram-positive bacteria and incapable of replicating in *E. coli* unless it acquires the origin of replication present on the element. Assays can be completed in 3 days and rates of movement of 0.001% or greater are routinely detectable.

of a strong promoter (Rio *et al*., 1986). Transient expression of the transposase gene resulted in the production of transposase, catalyzing the excision of *P* elements from the "excision indicator plasmids." Subsequent recovery of the injected plasmids from the cells, followed by their introduction into an appropriate strain of *Escherichia coli,* permitted plasmids that had lost the *P* element through excision to be recognized by virtue of their restored LacZα peptide coding capacity. This transient *P* element excision assay was readily adaptable to use in *Drosophila* embryos through the process of direct microinjection of preblastoderm embryos, and it played a critical role in assessing the functionality of the *P* element system in a variety of drosophilid and non-drosophilid insect systems (O'Brochta and Handler, 1988).

As originally configured the excision assay only permitted the identification and recovery of excision events that resulted in the restoration of the open reading frame of the LacZα peptide reporter gene. Various modifications in this basic assay were adopted that permitted precise and imprecise excisions to be identified and recovered (O'Brochta *et al*., 1991). For example, marker genes such as the *E. coli* LacZα peptide coding region, *E. coli supF*, sucrase (*SacRB*) from *Bacillus subtilis*, and streptomycin sensitivity, were incorporated into the transposable element (O'Brochta *et al*., 1991; Coates *et al*., 1997; Sundararajan *et al*., 1999). Plasmids recovered that lacked marker gene expression were usually excision events. Further refinements of the excision assay involved the use of transposable element-specific restriction endonuclease sites as a means for selecting for excision events. Digesting plasmids recovered from embryos with restriction endonucleases with sites only in the transposable element was a very powerful method of physically removing plasmids that had not undergone excision from the pool of plasmids recovered from embryos and used to transform *E. coli*. Each restriction site was essentially a single dominant genetic marker and therefore transposable elements with multiple restriction sites provided a very powerful system for selecting against plasmids that have not undergone excision (O'Brochta, unpublished data).

Continued development of element mobility assays lead to assays in which interplasmid transposition could be measured. These assays involved the coinjection of a transposase–encoding helper plasmid, an element "donor" plasmid and a "target" plasmid. Typically the target plasmid contains a gene whose inactivation results in a selectable phenotype. For example, the *SacRB* gene has been used since its inactivation eliminates sucrose sensitivity. If the donor element also contains unique genetic markers then transposition events would lead to a recombinant plasmid with a new combination of a variety of markers (Saville *et al*., 1999). Perhaps the most powerful transposition assay developed for assessing transposable elements in insect embryos involved the use of a genetic marker cassette containing a plasmid origin of replication, an antibiotic resistance marker, and the LacZα peptide coding region, in combination with a target consisting of a Gram-positive plasmid (pGDV1) (Sarkar *et al*., 1997a). pGDV1 contains an origin of replication that cannot function in *E. coli,* although it does have a chloramphenicol resistance gene that is functional in this species. Transposition of the marked transposable element into pGDV1 converts it into a functional replicon in *E. coli*. Because of the absolute *cis*-dependence of origins of replication, and the complete inability of pGDV1 to replicate in *E. coli,* transposition events can be readily detected even at low frequencies.

Transient mobility assays are now a standard for defining vector competence in insect embryos, and in particular, when assessing a vector in a species for the first time. For this application, transposition assays provide the most information relevant to the potential for successful germline transformation, and can be used as a system to test helper construct function. As noted below, however, there may be differing constraints on plasmid and chromosomal transpositions for particular transposons. The use of these assays for analyzing transposon function is discussed in more detail in the relevant sections below.

Embryonic assays also provide an essential test system for assessing potential transgene instability by mobilizing or cross-mobilizing systems within a host genome, which is a critical information for the risk analysis of transgenic insects being considered for release. The importance of excision assays for this purpose became evident by the *hobo* excision assays in *Musca domestica* that revealed the existence of the *Hermes* element (Atkinson *et al*., 1993), and the subsequent assays that defined the interaction between the two transposons (Sundararajan *et al*., 1999). Since cross-mobilizing systems do not always promote precise exicisions, assays that reveal imprecise as well as precise excisions are most sensitive for this purpose. Since successful transposition may depend on precise excision, transposition assays may only reveal the existence of mobilizing systems that have a high level of functional relatedness.

4.13.4. Transformation Marker Systems

The availability and development of selectable marker systems has played a large part in recent

advancements in insect transformation, that have been equal to the importance of vector development. The rapid implementation and expansion of *P* transformation in *Drosophila* was possible, in large part, due to the availability of several eye color mutant-rescue systems. These systems depend on the transgenic expression of the dominant-acting wild-type gene for an eye color mutation present in the host strain (see Sarkar and Collins, 2000). Successful transformation of non-drosophilid species was similarly dependent upon the development of analogous systems, with the first transformations in the medfly, *Ceratitis capitata*, and a mosquito, *Aedes aegypti*, relying on *white* and *cinnabar* mutant-rescue systems. While chemical resistance markers were used initially for non-drosophilid transformation, and can be highly useful for specific applications, their inefficiency and inconsistency when used alone provided ambiguous results for several species (see ffrench-Constant and Benedict, 2000). Eye color marker systems are generally efficient and reliable, and cloned wild-type genes from *Drosophila* often complement orthologous mutant alleles in other insects. However, only a handful of species have stable mutant strains that can serve as suitable hosts for mutant-rescue strategies. The most significant advancement in marker gene development for the wide use of insect transformation has been the development of fluorescent protein markers (Higgs and Sinkins, 2000; Horn *et al*., 2002). As dominant-acting neomorphs, that do not depend on preexisting mutations, they are directly useful in almost all host strains. When compared to the *white* eye color marker in *Drosophila*, the enhanced green fluorescent protein (EGFP) gene seemed to be less affected by position effect suppression, and thus has the additional advantage of more reliable detection (Handler and Harrell, 1999). Certainly for the forseeable future, fluorescent protein markers will continue to be the markers of choice for most insect transformation strategies.

4.13.4.1. Eye Color Markers

The first insect transformations used mutant-rescue systems to identify transformant individuals, but in these experiments total genomic DNA was used, rather unreliably, to complement mutations in the respective host strains. The most reliable of these, however, was reversion of the *vermilion* eye color mutation in *D. melanogaster* (Germeraad, 1976). The success of the initial *P* element transformations in *Drosophila* also depended on reversion of eye color mutant strains, but the use of cloned *rosy* and *white* genomic DNA within the vector plasmid allowed for much greater efficiency and reliability. The first non-drosophilid transformations in medfly (Loukeris *et al*., 1995b; Handler *et al*., 1998; Michel *et al*., 2001) similarly relied on use of the wild-type medfly *white* gene cDNA that was placed under *Drosophila hsp70* regulation (Zwiebel *et al*., 1995). This gene complemented a mutant allele in a *white eye* medfly host strain that was isolated more than 20 years earlier. The medfly *white* gene also complemented the orthologous gene mutation in the oriental fruit fly, yielding in one line a nearly complete reversion (Handler and McCombs, 2000). The first transformations in *A. aegypti* used a kynurenine hydroxylase-*white* mutant host strain, but for these tests the complementing marker was the *D. melanogaster cinnabar* gene (Cornel *et al*., 1997). The *D. melanogaster vermilion* gene, that encodes tryptophan oxygensase (*to*) has also been used to complement the orthologous *green* eye color mutation in *M. domestica* (White *et al*., 1996), and the *Anopheles gambiae* tryptophan oxygensase gene complements *vermilion* in *Drosophila* (Besansky *et al*., 1997). The *vermilion* and *cinnabar* orthologs have also been cloned from *Tribolium*, and while the *white* mutation in this species is complemented by tryptophan oxygensase, no preexisting eye color mutation is complemented by kynurenine hydroxylase (Lorenzen *et al*., 2002). The use of eye color mutant-rescue systems has certainly been critical to initial advances in insect transformation, and these markers should have continued utility for those species that have been successfully tested. The use of these markers, however, for the development of insect transformation in other species will be limited by the availability of suitable mutant host strains.

4.13.4.2. Chemical Selections

Previous to the development of mutant-rescue marker systems, transformant selections in non-drosophilid insects focused on genes that could confer resistance to particular chemicals or drugs. Importantly, these types of selections could be used for screening transformants en masse by providing the selectable chemical or drug in culture media. Ideally, only transformant individuals would survive the selection, allowing the rapid screening of large numbers of G_1 insects. For vectors that are inefficient and insects that are difficult to rear, the efficient screening of populations can be essential to identifying transformant individuals. The first drug resistance selection tested used the bacterial neomycin phosphotransferase gene (NPT II or *neomycin*^{r}) that conferred resistance by inactivation of the neomycin analog G418 (or Geneticin) (Steller

and Pirrotta, 1985). This seemed straightforward since the selection and *hsneo* marker system (putting NPT II under heat shock regulation) was already developed and tested in *Drosophila* for mass transformant screens, and the bacterial resistance gene was thought to be functional in most eukaryotes. The initial *P* transformations in *Drosophila* using the pUChsneo vector were generally reliable; however, the marker was not easily transferable to other species. G418 resistance was highly variable, most likely due to species differences in diet, physiology, and symbiotic bacteria, and indeed, variations in resistance in transformed *Drosophila* have been attributed to strains of yeast used in culture media (Ashburner, 1989a). Other chemical resistance markers, including *opd* conferring resistance to paraoxan (Phillips *et al*., 1990; Benedict *et al*., 1995), and the gene for dieldrin resistance (*Rdl*) (ffrench-Constant *et al*., 1991), that were initially tested in *Drosophila* were also problematic when tested in other species. These failures were due in large part to ineffective vector systems, but a common attribute in these studies was the selection of individuals having nonvector related or natural resistance to the respective chemical. While naturally resistant insects could be selected out by molecular tests in primary transformant screeens, the recurrence of resistant insects in subsequent generations would make use of the transgenic strains highly impractical.

While the problems cited made chemical resistance selections frustrating for several species, and they have not been used for any recent transformation experiments, some successes were reported and the need for mass screening still exists. The initial tests for *P* transformation in several mosquito species used the pUChsneo vector with G418 resistant transformants being selected, though transformation frequencies were low and all of them resulted from fortuitous recombination events and not *P*-mediated transposition (Miller *et al*., 1987; McGrane *et al*., 1988; Morris *et al*., 1989). Nonetheless, chemical selections can be very powerful, and if reliable, they would dramatically improve the efficiency of transformation screens for most insects. It is quite possible that many species will not be amenable to current transformation techniques without markers that allow selection en masse. A potential means of increasing the reliability of chemical resistance screens would be to link a resistance marker to a visible marker within the vector. Initial G_1 transformants could be screened en masse by chemical resistance, with surviving individuals verified as transformants and maintained in culture by use of the visible marker. This type of marking is tested by linking the *hsneo* construct with a red fluorescent protein marker in the *piggyBac* vector. Thus far initial results in *Drosophila* are highly encouraging (Handler and Harrell, unpublished data).

Of the enzyme systems tested for chemical selection in *Drosophila* that might be extended to other insects, the *Adh* system might have the most promise (Goldberg *et al*., 1983). An *Adh* marker gene can complement the *adh* mutation in *Drosophila*, eliminating lethal sensitivity to ethanol treatment in mutant hosts. An *adh* gene has been cloned from the medfly, and a strategy has been developed to use it for genetic sexing by male-specific overexpression (Christophides *et al*., 2001). Conceivably a similar strategy could be extended to transformant selections, though its use would be limited to medfly and possibly other tephritid species.

4.13.4.3. Fluorescent Protein Markers

The dramatic advancement of insect transformation in recent years has been due, primarily, to the development of fluorescent protein markers which are dominant-acting neomorphs that do not depend on preexisting mutations. The first of these to be tested was the green fluorescent protein (GFP) gene that was isolated from the jellyfish *Aequorea victoria* (Prasher *et al*., 1992) and which initially exhibited heterologous function in the nematode *Caenorhabditis elegans* (Chalfie *et al*., 1994). GFP expression was then tested in transformant *Drosophila* where it was used as a reporter for gene expression (Plautz *et al*., 1996; Hazelrigg *et al*., 1998), and several other species for both *in vivo* and *in vitro* studies. GFP was first tested in non-drosophilid insects as marker for Sindbis viral infection in *Aedes aegypti* (Higgs *et al*., 1996), and the dramatic somatic expression of GFP in adults was highly encouraging for the further use of GFP for germline transformants.

This possibility was first tested in *Drosophila* using a construct that linked EGFP to a *polyubiquitin* promoter and nuclear localizing sequence (Lee *et al*., 1988; Davis *et al*., 1995; Handler and Harrell, 1999). A control for transformation, that also allowed a direct comparison of EGFP expression to that from the visible mini-*white* marker, was the initial use of a *piggyBac* vector, pB[Dm*w*, PUbnlsEGFP], that linked the two markers in a mutant *white* strain. The results from this experiment indicated that not only was the PUbnslEGFP marker efficient and easily detectable under epifluorescense optics, but that many of the G_1 transformants that expressed GFP did not express a detectable level

of *white*$^{+}$. Presumably the chromosomal position effects that suppressed *white* had a negligible effect on GFP expression. This result was highly encouraging for the use of GFP as a marker in nondrosophilids and several subsequent transformation experiments used EGFP regulated by a variety of promoters in *piggyBac, Hermes*, and *Minos* vectors. Notably, this allowed germline transformation to be tested in several species that otherwise have no visible marker systems, such as the Caribbean fruit fly, *Anastrepha suspensa*, which was transformed with pB[PUbnlsEGFP] (Handler and Harrell, 2000). This vector was subsequently tested in the Australian sheep blowfly, *Lucilia cuprina* (Heinrich *et al*., 2002) and the mosquito *Anopheles albimanus* (Perera *et al*., 2002). Similarly, a *Hermes* vector marked with EGFP regulated by the *Drosophila actin5C* promoter was first tested in *Drosophila* (Pinkerton *et al*., 2000), and was used then to efficiently select transformants in *Aedes aegypti* (Pinkerton *et al*., 2000), *Stomoxys calcitrans* (O'Brochta *et al*., 2000), and *Culex quinquefasciatus* (Allen *et al*., 2001). A *Minos* vector marked with *actin5C*-EGFP was used to select *Anopheles stephensi* transformants (Catteruccia *et al*., 2000b), and a *piggyBac* vector marked with EGFP under *Bombyx actin 3A* promoter regulation was used to transform the lepidopteran species *Bombyx mori* (Tamura *et al*., 2000) and *Pectinophora gossypiella* (Peloquin *et al*., 2000).

Both the *polyubiquitin* and *actin* promoters have activity in all tissues throughout development, making insects marked in this fashion particularly useful for some applications such as the marking of insects used in biocontrol release programs (see Handler, 2002b). However, the detection of these markers can be limited due to quenching or obstruction by melanized cuticle or scales, and fluorescent protein expression regulated by a strong tissue-specific promoter can be valuable for difficult insects and particular applications. Foremost among these markers have been a series of fluorescent protein constructs regulated by the artificial 3xP3 promoter derived from the *Drosophila eyeless* gene (Sheng *et al*., 1997; Horn *et al*., 2000). The markers express strongly from the larval nervous system and adult eyes and ocelli. A 3xP3–EGFP marker within *piggyBac* was first used to transform *D. melanogaster* and *Tribolium castaneum* (Berghammer *et al*., 1999) and has since been used in *M. domestica* (Hediger *et al*., 2000), *Aedes aegypti* (Kokoza *et al*., 2001), and the sawfly *Athalia rosae* (Sumitani *et al*., 2003). The particular strengths and weaknesses for a marker construct such as 3xP3–EGFP is evident from experiments where it allowed transformant selection in *Bombyx* embryos prior to larval hatching (Thomas *et al*., 2002), while it was undetectable in *Aedes aegypti* adults having normal eye pigmentation (Kokoza *et al*., 2001). It must, therefore, be recognized that the utility of fluorescent protein markers must be considered in the context of the host insect's structure and physiology during development.

GFP expression is less sensitive to position effect suppression than eye color markers, yet there is much evidence for quantitative and qualitative variabilities in fluorescent protein expression from transgenes. It is likely that tissue-specific variations are primarily due to position effects, while expression of new tissue phenotypes are due to proximal enhancer effects. Polyubiquitin-regulated EGFP expression is most intense in the thoracic flight mucles in *Drosophila* and tephritid fruit fly adults. In Caribbean fruit fly transgenic adult lines, EGFP was only observed in the thorax, but spectrofluorometric assays revealed as much as fivefold differences in fluorescence between lines having the same number of vector integrations (Handler and Harrell, 1999), and differences in expression that are stable within lines are often observed by inspection. In contrast to typical thoracic expression in tephritid flies, adult PUb-EGFP expression in *Lucilia* was limited to female ovaries (Heinrich *et al*., 2002), and a PUb-DsRed transgenic medfly line exhibits most intense expression in the tarsi while another line expresses in abdominal tracheal apertures at the dorsal–ventral midline (Handler and Krasteva, unpublished data). In *Tribolium*, 3xP3–EGFP expresses typically from the eyes and brain, though several lines are atypical with one having muscle-specific expression throughout development (Lorenzen *et al*., 2003). In *Anopheles stephensi*, the 3xP3–EGFP marker showed atypical expression in the pylorus, epidermal cells and in a subset of cells in the rectum (O'Brochta, Kim, and Koo, unpublished data).

The use of GFP will certainly be continued for transformant identification in many, if not most, other species where transformation is tested. Yet continuing studies in species already transformed will require multiple marking systems that are distinguishable from one another, especially when coexpressed. This will allow the detection of multiple independent transgenes when used in concert for conditional gene expression systems and gene discovery methods such as enhancer traps (Bellen *et al*., 1989; Wilson *et al*., 1989; Brand *et al*., 1994). After testing 3xP3–EGFP, the 3xP3 promoter was linked to the GFP redshifted variants that emit blue (BFP), cyan (CFP), and yellow (YFP) fluorescence; these were

tested in *Drosophila*, and have proven useful individually as reporters and for identifying transformants (Horn and Wimmer, 2000). BFP and GFP have distinct enough emission spectra to be used together, though BFP photobleaches quickly and is not useful for many applications. While use of EGFP with ECFP is also problematic, ECFP and EYFP can be distinguished when using appropriate filter sets. For details on appropriate filter sets for particular applications see Horn *et al*. (2002) and the website for Chroma Technology Corp. (Chroma, 2004) which manufactures filters for most of the stereozoom fluorescence microscopes used for insect studies.

The most spectrally distinct fluorescent protein distinct from GFP and its variants is a red fluorescent protein, known as DsRed, isolated from the Indo-Pacific sea coral *Discosoma striata* (Matz *et al*., 1999). It was first tested in insects by linking it to the *polyubiquitin* promoter in a *piggyBac* vector (pB[PUb-DsRed1]) and tested in *Drosophila*, where it exhibited highly intense expression (Handler and Harrell, 2001). Importantly, DsRed expression was completely distinguishable from EGFP when the two transgenic lines were interbred, and when coexpressed as an hsp70-Gal4/UAS-DsRed reporter in lines having vectors marked with EGFP. DsRed has since been incorporated into several mosquito and fruit fly species (Nolan *et al*., 2002; Handler, unpublished data).

Both EGFP and DsRed are highly stable and generally resistant to photobleaching, and could be detected in tephritid flies several weeks after death, though DsRed1 was relatively the more stable of the two. This is highly advantageous for the use of these genes as markers for released insects that might only be retrieved several weeks after death in traps. A drawback for fluorescent proteins, and DsRed in particular, is that they require oligomerization and slow maturation that can take up to 48 h, resulting in low intensity in early development. However, variants of DsRed with shorter maturation times (Campbell *et al*., 2002), and new fluorescent proteins with enhanced properties for specific applications are becoming available on a consistent basis (see Matz *et al*., 2002).

4.13.4.3.1. Detection methods for fluorescent proteins Once heterologous expression of GFP in nematodes was discovered it was realized that use of the marker for whole-body analysis of gene expression would require an optical system allowing a large depth of field and a stage with working space for culture plates. Up to this time, most epifluoresence systems were linked to compound or inverted microscopes that had limited field depth and capability to manipulate organisms under observation. This led to the development of an epifluorescence module using a mercury lamp that could be attached to a Leica stereozoom microscope system. Most major microscope manufacturers now market integrated epifluorescent stereozoom microscopes with capabilities for several filter systems.

A lower cost alternative for GFP screening is use of a lamp module using ultra bright blue light emitting diodes (LEDs) with barrier filters that attaches to the objective lens of most stereozoom microscopes (BLS Ltd., Budapest, Hungary). It costs considerably less than a mercury lamp system, but at present, it only has capabilities for detecting GFP and YFP.

The use of fluorescent protein markers, and especially multiple markers will be greatly aided by the use of fluorescence activated embryo sorters. A device first developed to sort *Drosophila* embryos expressing GFP (Furlong *et al*., 2001) has been modified and commercially marketed for *Drosophila* and other organisms by Union Biometrica (Somerville, MA, USA). The latest sorting machines are highly sensitive having the ability not only to distinguish different fluorescent proteins, but also to discriminate between levels of fluoresence from the same protein. Thus, these systems may have enormous importance to the straightforward screening for transgenics, and more sophisticated assays such as those for enhancer traps. Practical applications could include the screening of released transgenic insects caught in traps (in systems adapted for adults), or for genetic-sexing of embryos having a Y-linked or male-specific fluorescent marker.

4.13.5. Transposon Vectors

4.13.5.1. *Hermes*

4.13.5.1.1. Discovery, description, and characteristics *Hermes* is a member of the *hAT* family of transposable elements and is related to the *hobo* element of *D. melanogaster*, the *Ac* element from maize, *Zea mays*, and the *Tam3* element from *Antirrhinum majus* (Warren *et al*., 1994). The initial interest in this family of elements by those interested in creating new insect gene vectors stemmed from two observations. First, during the middle and late 1980s the mobility characteristics of the *Ac/Ds* element system were being extensively studied because the element was recognized as having great potential to serve as a gene-analysis and gene-finding tool in maize and other plants. In addition, the mobility properties of *Ac/Ds* were being extensively tested in species of plants other than maize and in almost

every case evidence for *Ac/Ds* mobility was obtained (Fedoroff, 1989). *Ac/Ds* appeared to be a transposable element with a very broad host range, unlike, for example, the *P* element from *D. melanogaster*, which only functions in closely related species (O'Brochta and Handler, 1988). Because transposable elements with broad host ranges were of interest to those attempting to develop insect transformation technology, *Ac*-like elements warranted attention. The second significant observation at this time was that the *hobo* element from *D. melanogaster* had notable DNA sequence similarity to *Ac/Ds*, suggesting that it was a distant relative of this broadly active element (Calvi *et al*., 1991). Investigation into the host range of *hobo* using plasmid-based mobility assays (as described above) ensued (O'Brochta *et al*., 1994). It was during the investigation of *hobo* that *Hermes* was discovered (Atkinson *et al*., 1993). Atkinson *et al*. (1993) performed plasmid-based *hobo* excision assays in embryos of *M. domestica* as part of an initial attempt to assess the host range of *hobo*. Assays were performed in the presence of *hobo*-encoded transposase and *hobo* excision events were recovered suggesting that *hobo*, like *Ac/Ds*, would have a broad host range. However, when the assays were performed without providing *hobo*-encoded transposase *hobo*, excision events were still recovered in *M. domestica*. The movement of *hobo* in the absence of *hobo*-transposase was completely dependent upon the inverted terminal repeats of *hobo* and the resulting excision events had all of the characteristics of a transposase-mediated process. It was proposed that *M. domestica* embryos contained a *hobo* transposase activity and that this activity arose from the transposase gene of an endogenous *hobo*-like transposable element (Atkinson *et al*., 1993). These investigators were eventually able to confirm their hypothesis and the element they discovered was called *Hermes* (Warren *et al*., 1994).

Hermes is 2749 bp in length and is organized like other Class II transposable elements in that it contains ITRs and a transposase-coding region (**Figure 1**). It contains 17 bp ITRs with 10 of the distal 12 nucleotides being identical to the 12 bp ITRs of *hobo*. *Hermes* encodes for a transposase with a predicted size of 72 kDa and based on the amino acid sequence is 55% identical and 71% similar to *hobo* transposase (Warren *et al*., 1994). The cross-mobilization of *hobo* by *Hermes* transposase that was proposed by Atkinson *et al*. (1993) was tested directly by Sundararajan *et al*. (1999). These investigators used plasmid-based excision assays in *D. melanogaster* embryos to show that *hobo* transposase could mobilize *Hermes* elements and that *Hermes* transposase could mobilize *hobo* elements (Sundararajan *et al*., 1999). The phenomenon of cross-mobilization has important implications for the future use of transposable element-based gene vectors in non-drosophilid insects and will be discussed below. As is typical of transposable elements, *Hermes* is present as a middle repetitive sequence within the genomes of multiple strains of *M. domestica* and in all populations examined there appeared to be full-length copies of the element. The natural history of this element within *M. domestica* has not been investigated and its mobility properties within this species and the existence of any regulatory system remain unknown.

4.13.5.1.2. Patterns of integration The integration behavior of *Hermes* has been examined in a variety of contexts. Sarkar *et al*. (1997a, 1997b) tested the ability of *Hermes* to transpose, using a plasmid-based assay, in five species of Diptera. They recovered transpositions of *Hermes* in the target plasmid at a frequency of approximately 10^{-3} in all species tested. In addition they examined the distribution of 127 independent transposition events into the 2.8 kb plasmid used as a target in their assay and observed a distinctly nonrandom pattern of integrations. Most notable was the existence of three sites that were targets for *Hermes* integration 10 or more times each. In an experiment in which any site used two or more times was considered a hot spot for integration, the three sites used 10 or more times constitute sites with unusual characteristics. The precise nature of those characteristics however could not be defined. The sites shared four of eight nucleotides of the target site in common (GTNNNNAC); however, other sites with this nucleotide composition were not equally attractive as integration sites indicating that other factors must be influencing target choice. Saville *et al*. (1999) demonstrated that sequences flanking *hobo* integration hot spots were critical for determining the targeting characteristics of a site. These investigators were able to move an 8 bp *hobo* target site from plasmid to plasmid without losing its target characteristics as long as they included 20 bp of flanking sequence on each side of the target. It was suggested that proximity to a preferred integration site increased the likelihood of a site being used as a target (Sarkar *et al*., 1997a). They found that sites 80 and 160 bp flanking the integration hot spot were also preferred integration sites. The authors suggested that nucleosomal organization of the target contributes significantly to the target site selection process and contributed to the local juxtaposition of hot spots and flanking DNA.

4.13.5.1.3. Structure–function relationships Many Class II transposable elements contain a distinct amino acid motif within their catalytic domains consisting of two aspartate residues and a glutamate. This DD35E motif can be found in many but not all Class II transposable elements. The presence of this motif in *Hermes* transposase has been unclear. Bigot *et al.* (1996) proposed the existence of a DDE motif among members of the *hAT* family. However, they proposed that the second aspartate was replaced by a serine in *Ac*, *hobo*, and *Hermes*. Capy *et al.* (1996) concluded that *hAT* elements, like *P* elements from *Drosophila*, do not contain the DDE motif based on sequence alignments, and Lerat *et al.* (1999) supported this conclusion based on the lack of similarity in predicted secondary structure of the transposase of members of the *mariner/Tc* superfamily and *hobo* transposase. Michel *et al.* (2002) examined experimentally the importance of D402, S535, and E572 to the proper functioning of *Hermes* transposase. They found that mutations D402N and E572Q abolished transposase activity while the mutations S535A and S535D had no effect on transposase activity. The work of Michel *et al.* (2002) provided the first experimental data to support the hypothesis that the positive charge of residues D402 and E572 are required for transposition. The authors concluded, based on these data, that D402, S535, and E572 do not constitute the catalytic center of *Hermes* transposase because one of the residues was not essential for activity. Therefore, *Hermes* (and *hAT* elements in general) do not appear to be of the DD35E type of transposable elements, making them distinct from *mariner/Tc* elements.

Because *Hermes* transposase acts within the nucleus it is expected to contain a nuclear localization signal to direct the mature transposase from the ribosome to the nucleus. Deletion and site-directed mutagenesis analysis were performed that demonstrated that the *Hermes* nuclear localization signal is located at the amino acid end of the protein and divided among three domains (Michel and Atkinson, 2003).

The ITRs of transposable elements play an essential role in their mobility. Altering the sequence of ITRs can, depending on the element, lead to loss of function, hyperactivity of the element, or switching the mode of transposition from a cut-and-paste mechanism to a replicative mechanism. *Hermes* contains imperfect ITRs with a 2 bp mismatch within the ITR (Warren *et al.*, 1994). In addition, a naturally occurring polymorphism in the terminal nucleotide of the right 3′ ITR exists. Elements with a cytidine in the terminal position of the right ITR have no activity within *D. melanogaster* but are capable of undergoing an aberrant form of transposition in mosquitoes. Small pentanucleotide motifs in the subterminal regions of both *Hermes* and *hobo* have been found to be important for the mobilization of *Hermes* and *hobo*. The sequences GTGGC and GTGAC are interspersed throughout the subterminal region of the element, and similar repeats are present in the subterminal regions of *Ac* and are known to be transposase binding sites. In *Hermes*, altering a single repeat can eliminate transpositional activity (Atkinson *et al.*, 2001).

Hermes transposase is capable of dimerizing and one region of the protein critical for dimerization is located in the C-terminus of the protein including amino acids 551–569. This region is not only essential for dimerization but is also required for transposition activity. A second region that affects dimerization is located in the N-terminus of the protein within the first 252 amino acids of the transposase. However, this region apparently plays a nonspecific role in dimerization (Michel *et al.*, 2003).

4.13.5.1.4. Host range of *Hermes* *Hermes* has a wide insect host range and has been found to function (as measured by either plasmid-based mobility assays or by germline transformation) in at least 13 species of insects including 11 flies, one beetle, and one moth (Atkinson *et al.*, 2001). *Hermes* functions rather efficiently in *D. melanogaster* and transforms this species at rates of 20–40% (O'Brochta *et al.*, 1996). In all other species tested the efficiency of transformation was considerably lower and tended to be less than 10%. For example, *T. castenaeum* was transformed at a rate of 1%, *A. aegypti* at 5%, *C. quinquefasciatus* at 11%, *C. capitata* at 3%, and *S. calcitrans* at 4% (Atkinson *et al.*, 2001). In all insects except mosquitoes, *Hermes* appeared to use a standard cut-and-paste type mechanism as is typical of most Class II transposable elements. Such integrations are characterized by the movement of only those sequences delimited by the ITRs, and the integrated elements are flanked by direct duplications of 8 bp. Integration of *Hermes* into the germline of *A. aegypti* and *C. quinquefasciatus* appears to occur by a noncanonical mechanism resulting in the integration of DNA sequences originally flanking the element on the donor plasmid. The amount of flanking DNA that accompanies the integration of *Hermes* in these mosquito species varies. In some cases two tandem copies of the *Hermes* element were transferred to the chromosome and each copy was separated by plasmid DNA sequences (Jasinskiene *et al.*, 2000). Although

these transposition reactions are unusual they are dependent upon *Hermes* transposase since the introduction of *Hermes*-containing plasmid DNA in the absence of *Hermes* transposase failed to yield transformation events. The germline integration behavior of *Hermes* in mosquitoes is not unique; however, other elements being used as gene vectors such as *mariner* and *piggyBac* have occasionally shown similar behavior in *A. aegypti* (O'Brochta, unpublished data). Transposition assays performed with plasmids in developing mosquito embryos and in mosquito cell lines showed that *Hermes* could transpose via a canonical cut-and-paste type mechanism under these conditions (Sarkar *et al*., 1997b). The basis for the difference in types of integration events between plasmid-based transposition assays and chromosomal integrations is unknown but may reflect differences in somatic and germ cells. In *Aedes*, canonical cut-and-paste transposition has been readily detected in the somatic tissues of insects containing an autonomous element. Germline transposition in these same insects has not been detected. It has been suggested that mosquitoes might contain endogenous *hAT* elements that affect the ability of *Hermes* elements to be integrated precisely. An alternative suggestion is that *Hermes* may have a second mode of transposition as do the transposable elements *Tn7*, *IS903*, and *Mu*, which utilize a replicative mechanism of integration. Such a mechanism would result in integration products that resemble those observed in the germline of *A. aegypti* and *C. quinquefasciatus*. Replicative transposition of *Hermes* has not been demonstrated experimentally and direct tests of the "alternate mechanism" hypothesis have not been reported.

4.13.5.1.5. Postintegration behavior Once integrated into the genome of *D. melanogaster*, *Hermes* maintains its ability to be remobilized and has shown mobility characteristics that are similar to other transposable elements. Following the introduction of an autonomous *Hermes* element in which the transposase gene was under *hsp70* promoter regulation, and also contained an EGFP marker gene under constitutive regulatory control of the a*ctin5C* promoter, Guimond *et al*. (2003) found that the element continued to transpose in the germline at a rate of 0.03 jumps per element per generation. The element used in this study was also active in the somatic tissue and they used this as a means of collecting approximately 250 independent transposition events. Analysis of somatic integration events revealed a number of interesting patterns. First, they found that transpositions were clustered around the original integration event. On average 39% of the *Hermes* transpositions recovered were intrachromosomal and 17% were within the same numbered polytene chromosome division. Ten percent of the new insertions were at sites within 2 kb of the donor element, indicating that *Hermes*, like other transposable elements, shows the characteristic of local hopping. Local hopping refers to the tendency of some elements to preferentially integrate into closely linked sites. Local hopping has been described for a number of elements and is likely to be a general characteristic of Class II transposable elements although the mechanistic basis for this behavior is unknown. Certain regions of the *D. melanogaster* genome, as defined by numbered divisions of the polytene chromosomes, are preferred as integration sites, with these regions being repeatedly targeted by *Hermes*. The observed clustering of independent transposition events in regions of the chromosome seems to reflect undefined aspects of the transposition process that might be influenced by the chromatin landscape. With one exception, the clustering observed by Guimond *et al*. (2003) was not correlated with any common feature of the chromosomes or the genes within a region. This type of nonrandom pattern of integration with regional differences has also been reported for other elements. Interestingly, there does not seem to be any strong correlation between the preferred insertion-site regions of the elements *P*, *hobo*, and *Hermes*, at least with respect to chromosome 3 of *D. melanogaster* (see figure 7 from Guimond *et al*., 2003). Guimond *et al*. (2003) also observed a notable clustering of integrations in polytene chromosome division 5. Eight of the 11 integration events recovered from division 5 (3.2% of all the transposition events examined) were within the 2.7 kb segment of DNA upstream of the cytoplasmic actin gene, *actin5C*. This same 2.7 kb segment of the 5′ regulatory region of a*ctin5C* was also present within the autonomous *Hermes* element, as a promoter for the EGFP marker, which the investigators tracked as it jumped within the genome.

The strong clustering of transpositions in a target sequence that is homologous to a sequence contained within the vector has been referred to as "homing." This type of target site selection bias was first described for *P* elements and has been reported on a number of occasions. It was initially reported as a strong bias in the integration site distribution of a number of primary germline integration events in which a *P* element containing the *engrailed* gene preferentially integrated into the *engrailed* region of the host genome (Hama *et al*., 1990; Kassis *et al*., 1992). A similar biasing of integration site selection was also observed with *P* elements containing *Bithorax*

and *Antennapedia* regulatory sequences (Engstrom *et al*., 1992; Bender and Hudson, 2000). Taillebourg and Dura (1999) reported a remarkable example of homing of a remobilized *P* element in *D. melanogaster.* This element contained either an 11 kb or 1.6 kb fragment of the 5′ region of the *linotte* gene, and it was found that 20% of the remobilized elements integrated into the 5′ region of the *linotte* gene. Insertions in this case were highly localized and most occurred within a 36 bp fragment of the *linotte* regulatory region. *Hermes* homing indicates that the phenomenon is not element specific, but may be a general characteristic of Class II elements. Guimond *et al*. (2003) suggested that homing was a special case of local hopping, and the physical proximity between donor elements and target sites seems to underlie the phenomenon of local hopping. The presence of transgene regulatory sequences (e.g., *actin5C* 5′ region) may promote tethering of the donor elements to similar regulatory regions via proteins with common DNA binding sites. Deliberate tethering or transposable elements to selected sequences may be a means to regulate target site selection and to minimize the detrimental mutagenic effects of transposable element integration (Bushman, 1994; Kaminski *et al*., 2002).

The postintegration behavior of the same autonomous *Hermes* element described above in *A. aegypti* had quite different characteristics. In this case germline transposition of the autonomous *Hermes* element was never detected, and it should be noted that the primary integration events in the germline involved the integration of DNA sequences flanking the element (Jasinskiene *et al*., 1998, 2000). Despite the fact that the element was intact and that functional transposase was expressed, the element was immobile in the germline. This was not the case, however, in the soma of *A. aegypti* where *Hermes* excision and cut-and-paste transpositions were readily detected. Transposition events in the soma had all of the hallmarks of Class II cut-and-paste integration. Only those sequences precisely delimited by the ITRs moved and integration resulted in the creation of 8 bp direct duplications at the target site. Excision of *Hermes* was imprecise and led, in some cases, to the creation of small deletions. The basis for the difference in behavior of the *Hermes* element in the germline versus the somatic tissue of *A. aegypti* is unknown. Clearly the postintegration behavior of *Hermes* in this species will influence how this element will be employed, and in situations were germline stability is essential, *Hermes* will be particularly useful. It will not be useful in its present form for constructing gene-finding tools such as enhancer and promoter traps that rely heavily on transposable element vector remobilization to be effective.

4.13.5.1.6. Extrachromosomal forms of *Hermes* Excision of *Hermes* in *M. domestica*, and autonomous *Hermes* integrations in *D. melanogaster* and *A. aegypti*, lead to the formation of circularized *Hermes* elements in which the terminal inverted repeats are jointed end-to-end in various ways following the excision reaction (Atkinson and O'Brochta, unpublished data). The most common configuration results in the ends being joined end-to-end with a short spacer sequence between them. The spacer sequence was most often 1, 3, or 4 bp but could also be as much as 200 bp. The extrachromosomal *Hermes* elements found in *M. domestica* are particularly interesting because they have been found in all populations tested and in great abundance in somatic tissue. These data provide evidence for the somatic activity of *Hermes* in the insect from which it was originally isolated. Circularized forms of excised transposable elements of a number of types have been reported in the past (Sundraresan and Freeling, 1987). For example, circularized forms of *Ac/Ds* have been described as well as *Minos* (Arca *et al*., 1997; Gorbunova and Levy, 1997), yet the significance of extrachromosomal forms of transposable elements has remained unclear. In some cases the circularized elements do not contain intact ITRs and consequently the elements are not expected to be integration competent. Based on rather limited data it has generally been concluded that such forms represent byproducts of aborted or interrupted transposition reactions. A study of the extrachromosomal forms of *Hermes* suggests that these elements may have some biological significance. Circularized *Hermes* elements with intact ITRs are integration-competent, potentially allowing them to contribute to forward transposition (Atkinson and O'Brochta, unpublished data). The ability of circularized forms of excised *Hermes* elements to reintegrate may have an impact on the transmission potential of this element. The existence of a large pool of functional extrachromosomal transposable elements may have implications for the ability of the element to be transferred horizontally and may provide an additional means of vertical transmission (e.g., maternally inherited), both of which will potentially enhance the elements ability to increase in frequency within a population. The biology of extrachromosomal *Hermes* elements needs to be investigated further.

4.13.5.1.7. *hAT* elements have been found in other insects The Queensland fruit fly, *Bactrocera tryoni*, contains members of at least two distinct *hAT*-like transposable elements (Pinkerton *et al.*, 1999). *Homer* is a 3789 kb element whose sequence is 53% identical to *Hermes* and 54% identical to *hobo*. The transposase coding region is approximately 53% identical and 71% similar to the transposases of *Hermes* and *hobo*. Similarly, the ITRs of *Homer*, which are 12 bp in length, are identical to those of the *hobo* and *Hermes* elements at 10 of 12 positions. There are also *Homer*-like elements within *B. tryoni*. There are fewer than ten copies per genome, and while these elements have not been fully characterized, a conceptual translation of the transposase of this *Homer*-like element reveals 48% identity and 66% similarity to the transposase of *Homer*. These *Homer*-like elements are as similar to *hobo* as they are to *Homer*. Although *Homer* appears to be weakly functional in *D. melanogaster* based on plasmid-based excision assays, all *Homer*-like elements contain inactivating frameshift mutaions.

The blowfly *L. cuprina* contains a nonfunctional *hAT* element called *hermit*. *Hermit* was initially found by low stringency hybridization screening of an *L. cuprina* genomic library using a DNA probe homologous to *hobo* (Coates *et al.*, 1996). *Hermit* is 2716 bp and contains perfect 15 bp ITRs, the distal 12 of which are identical to the *hobo* ITRs at 10 of 12 positions. Although inactive because of frameshift mutations within the transposase coding region, its amino acid sequence is 42% identical and 64% similar to *hobo* transposase. *Hermit* is unusual in that it is present as a unique sequence within *L. cuprina*, in contrast to multiple copies that exist for most transposons. Although present only once within this species it does appear to have arisen within the genome as a result of transposition since the existing copy of the element is flanked by an 8 bp direct duplication of a sequence that is similar to the consensus target site duplication derived from other *hAT* elements. *Hermit* appears to have become inactivated soon after integrating into the *L. cuprina* genome.

Several *hAT* elements have been discovered in tephritid fruit flies using a polymerase chain reaction (PCR) approach similar to that used to discover *Hermes* (Handler and Gomez, 1996). Of these elements, a complete *hAT* transposon (*hopper*) was isolated from a genomic library of the wild Kahuku strain of the Oriental fruit fly, *B. dorsalis*, using the *B. dorsalis hobo*-related element (Bd-HRE) PCR product as a hybridization probe (Handler and Gomez, 1997). A complete 3120 kb element was isolated having 19 bp ITRs. However, the putative transposase-coding region was frameshifted and it did not have a duplicated 8 bp insertion site, suggesting that it had accumulated mutations and was nonfunctional. The Kahuku sequence was used to isolate additional *hopper* elements using an inverse and direct PCR approach, and a new 3131 bp *hopper* was isolated from the *B. dorsalis white eye* strain (Handler, 2003). This element has an uninterrupted coding region and an 8 bp duplicated insertion site. Notably, *hopper* is highly diverged from all other known insect *hAT* elements and its transposase is distantly yet equally related to the coding regions of *hobo* and *Ac*. Of the terminal 12 nucleotides only five are identical to those of *hobo*, while six are identical to the ITRs of *Homer* (*B. tryoni*). *hopper* also exists in the melonfly, *B. cucurbitae*, and another *hAT* element originally discovered in the melonfly, that is closely related to *hobo* and *Hermes*, also exists in *B. dorsalis* (Handler and Gomez, 1996).

hAT elements have been also reported in the human malaria vector *A. gambiae*. Approximately 25 copies of sequences that resemble *hAT* transposases were discovered although none appeared to be part of an intact transposable element. More recently, however, search criteria were used based on unique aspects of *hAT* transposable elements such as length and spacing of ITRs and the characteristics of *hAT* element target sites. This search revealed a *hAT* element in *A. gambiae* that contained perfect 12 bp ITRs flanked by 8 bp direct duplications and a 603 amino acid transposase open reading frame that appeared to contain no internal stop codons. This element (*Herves*) is most closely related to *hopper* and the ability of this element to excise and transpose in *A. gambiae* or other species has not been determined (Atkinson and Arenburger, personal communication).

4.13.5.2. *piggyBac*

4.13.5.2.1. Discovery of *piggyBac* and other TTAA-specific elements Similar to several other insect transposable element systems, the *piggyBac* element was discovered fortuitously in association with a mutant phenotype. However, unlike all the other transposons used for insect transformation, the mutant phenotype was the result of a functional element that had transposed into an infectious organism. Fraser and colleagues (see Fraser, 2000) discovered several Few Polyhedra (FP) mutations in the baculoviruses, *Autographa californica* nucleopolyhedrovirus (AcNPV), and *Galleria mellonella* nucleopolyhedrovirus (GmNPV), after passage through the *Trichoplusia ni* cell line TN-368 (Fraser *et al.*, 1983, 1985). Among these elements that inserted specifically into tetranucleotide TTAA sites

was *piggyBac* (then named IFP2), which transposed into AcNPV. Although it might be assumed that IFP2 was an autonomous functional element based on its mobility, another TTAA insertion-site element, *tagalong* (then called TFP3), discovered in AcNPV and GmNPV, was later found not to have a transposase coding region and thus had to be mobilized by another TFP3 or related element. Autonomous functional elements have not yet been found for *tagalong*, though the original IFP2 *piggyBac* element was indeed functional (Wang *et al.*, 1989; Wang and Fraser, 1993). All the *piggyBac* elements discovered in TN-368 were found to be identical, having a length of 2472 kb with 13 bp perfect ITRs and 19 bp subterminal repeats located 31 bp from the 5′ ITR and 3 bp from the 3′ ITR (Cary *et al.*, 1989) (**Figure 1**). The transposase coding region exists as a single reading frame of 2.1 kb that encodes a protein with a predicted molecular mass of 64 kDa. The functionality of *piggyBac* and the precise nature of its transposition was further verified by a series of viral and plasmid transposition and excision assays. A *piggyBac* indicator plasmid was marked with *polh/lacZ*, and assays in the fall armyworm, *Spodoptera frugiperda* cell line SF21AE showed that the original *piggyBac* element, within the p3E1.2 plasmid, could mobilize the marked element. These assays proved that the 3E1 *piggyBac* element encoded a functional transposase, and defined the element's TTAA insertion-site specificity and the precise nature of its transposition. Importantly, these assays also showed directly that *piggyBac* could be mobilized in other lepidopteran species (Fraser *et al.*, 1995), indicating that it might function similarly as a vector for germline transformation. This was a critical realization given the failure of *P* to be mobilized in non-drosophilids, which was consistent with its failure as a vector in these species.

4.13.5.2.2. *piggyBac* transformation The failure of *P* vectors to transform non-drosophilid species made the testing of other available transposon systems a high priority. The other systems found to be functional in non-drosophilids, however, were first tested successfully for gene transfer vector function in *Drosophila*. For *piggyBac*, germline transformation was first attempted in the Mediterranean fruit fly, *Ceratitis capitata*. This was possible due to the availability of a marker system that had been tested previously by medfly transformation with the *Minos* transposon vector. The medfly *white* gene cDNA was linked to the *Drosophila hsp70* promoter, and was used as a mutant-rescue system in a white eye host strain (Loukeris *et al.*, 1995b; Zwiebel *et al.*, 1995). In the absence of data for the minimal sequence requirements for *piggyBac* mobility, the first *piggyBac* vector was constructed by insertion of the 3.6 kb *hsp-white* cDNA marker into the unique *Hpa*I site within *piggyBac* in the p3E1.2 plasmid. None of the *piggyBac* sequence was deleted though the insertion interrupted the coding region eliminating transposase function. Construction of the first helper was a simple deletion of the 5′ ITR sequence resulting from a *Sac*I digestion and religation of p3E1.2. There is some uncertainty as to whether the upstream *Sac*I site cuts within the *piggyBac* promoter (Cary *et al.*, 1989), yet transposase expression was indeed sufficient to support germline transpositions from the vector plasmid. The first experiment with this helper in the medfly resulted in one transgenic line at a transformation frequency of 5% per fertile G_0. However, sibling sublines exhibited two and three independent integrations (Handler *et al.*, 1998). This experiment with a *piggyBac*-regulated helper was repeated with five additional G_1 lines isolated, but at approximately the same frequency. These attempts at *piggyBac* transformation yielded relatively low transformation frequencies, but it was notable that a lepidopteran transposon vector system had autonomous function in a dipteran species.

Subsequent to the medfly transformation, *piggyBac* transformation was tested in *Drosophila* using the mini-*white* marker from that species (Handler and Harrell, 1999). Using the self-regulated helper, transformants were isolated at a similar frequency of 1–3%, but tests with a *hsp70*-regulated transposase increased the frequency to above 25%, consistent with *P* and *hobo* transformations using heat shock promoted transposase.

Given that *piggyBac* was first isolated from a lepidopteran species, there was some optimism that it would be functional as a vector in other moth species. Function was first tested by transposition assays in the pink bollworn, *Pectinophora gossypiella* (Thibault *et al.*, 1999), which then led to successful germline transformation of this species using the phspBac helper and a vector marked with EGFP regulated by the *Bombyx actinA3* promoter (Peloquin *et al.*, 2000). Concurrent experiments were also performed in the silkmoth *B. mori* using a similar *actinA3*-regulated EGFP marker, but for this species transformation was achieved with an *actinA3*-regulated transposase helper (Tamura *et al.*, 2000). While these are the only moth species reported to be transformed with *piggyBac*, several other dipteran species have been transformed, as well as species in the orders Coleoptera and Hymenoptera. The dipteran species transformed include

several of medical and agricultural importance, such as the mosquitoes *Aedes aegypti* (Kokoza *et al*., 2001; Lobo *et al*., 2002), *A. gambiae* (Grossman *et al*., 2001), *A. albimanus* (Perera *et al*., 2002), and *A. stephensi* (Nolan *et al*., 2002), and the tephritid fruit flies *Anastrepha suspensa* (Handler and Harrell, 2000) and *Bactrocera dorsalis* (Handler and McCombs, 2000). Other transformed dipterans include *M. domestica* (Hediger *et al*., 2000) and *L. cuprina* (Heinrich *et al*., 2002). Of particular importance at this time, has been the use of *piggyBac* to transform a coleopteran, the red flour beetle, *Tribolium castaneum* (Berghammer *et al*., 1999; Lorenzen *et al*., 2003), and a hymenopteran, the sawfly *Athalia rosae* (Sumitani *et al*., 2003). Notably, all of these species were primarily transformed using a helper regulated by the *Drosophila hsp70* promoter, and with vectors marked with EGFP, though other fluorescent proteins have since been used for some as well.

Although most of these transformations occurred at frequencies between 3% and 5% per fertile G_0, dramatic differences between species have been observed as well, and in some of the same species performed by different laboratories. A single transformant line was reported for *Anopheles gambiae*, at a frequency of approximately 1% (Grossman *et al*., 2001), while transformation in *A. albimanus* occurred at frequencies ranging from 20% to 40% (Perera *et al*., 2002). The first transformations of *Tribolium* occurred at an unusually high frequency of 60% (Berghammer *et al*., 1999).

Many of the transformations were preceded by testing *piggyBac* function by embryonic transposition assays that were first developed for *piggyBac* mobility in the pink bollworm (Thibault *et al*., 1999). As discussed previously, these assays can rapidly assess the relative mobility of *piggyBac* in a specific host species in a few days. Positive results from these assays provided some assurance that more tedious and time-consuming transformation experiments had some likelihood of success. For some studies the assays also were used to test promoter function in helper plasmids, or provided insights into insertion site specificity, or determined the likelihood of a particular vector construct retaining function in the absence of specific sequences (Lobo *et al*., 2001). For example, *piggyBac* helper promoters were tested by transposition assays and germline transformation in *D. melanogaster* and *L. cuprina* (Li *et al*., 2001a; Heinrich *et al*., 2002). It was found that in *Drosophila*, an *hsp70*-regulated helper yielded the highest transposition frequency, while a constitutive *α1-tubulin*-regulated helper was more effective for germline transformation. By comparison, in *Lucilia* the *hsp70* helper was most effective for both plasmid and germline transpositions, while the *Drosophila α1-tub* helper failed to support transformation. Transposition assays have also shown target site preferences among the TTAA sites within the pGDV1 target plasmid, and assays in *Drosophila* indicated a bias for sites having A or T nucleotides at positions −3, −1, +1, and +3 relative to TTAA (Li *et al*., 2001a). However, a sequence analysis of 45 genomic integrations sites in *Tribolium*, after *piggyBac* vector remobilization, failed to show this bias (Lorenzen *et al*., 2003), which may be an indication of species specificity for insertion site preference.

Mobility assays also provide a rapid means of testing sequence requirements for vector mobility, which allow modifications for more efficient vector function. Since vector mobility is known to be affected negatively with increasing size, this information should allow minimal vectors to be created that retain optimal function. However, minimal sequence requirements for plasmid transpositions may differ from those for chromosomal transposition. For example, excision and transposition assays performed in *Trichoplusia ni* embryos showed that the *piggyBac* inverted terminal repeat and subterminal repeat sequences were sufficient for transposition (35 bp from the 5′ terminus and 63 bp from the 3′ terminus), but that an outside spacer region between the ITRs of greater than 40 bp is necessary for optimal transposition from a plasmid (Li *et al*., 2001b). Use of similar vectors in *Drosophila*, however, did not result in germline transformants (Handler, unpublished data). The minimal sequence requirements for *piggyBac* transformation verified thus far for *Drosophila* are 300 bp from the 5′ terminus and 250 bp from the 3′ terminus (Li, Fraser, and Handler, unpublished data).

4.13.5.2.3. Phylogenetic distribution of *piggyBac* and implications for transgene stability Unlike most other transposons used for transformation, *piggyBac* is not an apparent member of a larger family, or superfamily of related elements such as the *mariner*/*Tc* or *hAT* families. Until recently, the only *piggyBac* elements known were the functional elements orginally discovered in *T. ni* (Fraser *et al*., 1983). Thus, an unexpected finding from the Southern analysis of *B. dorsalis* transformants was that 8 to 10 *piggyBac*-related elements exist in the host strain genome (Handler and McCombs, 2000). PCR analysis of internal coding sequence indicated that these were nearly identical elements, though none has been found to be identical to *piggyBac*, nor are their coding regions consistent with transposase

functionality. The isolation of complete *B. dorsalis* *piggyBac* elements as genomic clones and by inverse PCR indicate that complete elements exist with conserved terminal and subterminal sequences that are integrated into duplicated TTAA insertion sites. *Bactrocera dorsalis* is part of a larger species complex and PCR analysis indicated that, indeed, *piggyBac* exists throughout the complex having nucleotide sequence identities of 92% among all the elements sequenced, with individual identities of 96–99% to one another and the *T. ni* 3E1 *piggyBac* (Handler, unpublished data).

4.13.5.2.4. *piggyBac* in other insects The evolutionary distance between *T. ni* and *Bactrocera* strongly suggests that the transposon moved between these species by recent horizontal transmission, and the separation of their geographical habitats raises the possibility that this movement may have been mediated by intermediary species. A Southern blot survey for *piggyBac* in more than 50 species showed the most clear evidence for multiple *piggyBac* elements in *S. frugiperda*, but hybridization patterns suggested that most of the elements are defective and nonfunctional (Handler, unpublished data; see Handler, 2002a). Evidence for *piggyBac* in other insects and other organisms, including mosquitoes and humans, comes from recent sequence data from genome projects. Although discrete sequence similarities suggests that *piggyBac* has an ancient history, there is little evidence at present to indicate that *piggyBacs* have coevolved as functional elements, and related complete elements have yet to be discovered.

The first functional *piggyBac* elements were discovered in a *T. ni* cell line, but little analysis has been done to characterize *piggyBac* in the organismal genome. Recent hybridization analysis of *piggyBac* from larval *T. ni* genomes indicates that *piggyBac* exists, and that its general structure is consistent with full-length functional elements. However, a PCR survey of these genomic sequences has only identified nonfunctional elements, having a level of identity no greater than many of the elements found in *Bactrocera* (>96%) (Zimowska and Handler, unpublished data). If functional *piggyBacs* do not exist *in vivo*, this could be explained by genomic instability that may arise from a highly active transposon. Functional elements may create a genetic load resulting in organismal lethality, which is more easily withstood in cell lines. If this is the case, it is therefore intriguing to consider how the functional element arose in the cell line.

It is also intriguing to consider how horizontal transmission of *piggyBac* may have occurred, considering that the element was originally discovered by virtue of its transposition into an infectious baculovirus. This could potentially explain a distribution among lepidopterans, but not the apparent recent movement between moths and flies. Understanding the interspecies movement of *piggyBac*, as well as all other vectors used for practical application, will be critical to undertanding and eliminating risk associated with the release of transgenic insects.

4.13.5.3. *mariner*

4.13.5.3.1. Discovery, description, and characteristics The *mariner* element was first discovered as an insertion element responsible for the *white*-peach (w^{pch}) mutant allele of *D. mauritiana* (Haymer and Marsh, 1986; Jacobson *et al.*, 1986). This particular allele was interesting when discovered because it was highly unstable with reversions to wild type occurring at a frequency of approximately 10^{-3} per gene per generation. *white*-peach individuals also had a high frequency of mosaic eyes, at an approximate frequency of 10^{-3}, suggesting somatic instability. Molecular analysis of the w^{pch} allele indicated that it was the result of a 1286 bp transposable element insertion into the 5′ untranslated leader region of the *white* gene (Jacobson *et al.*, 1986) (**Figure 1**). The *mariner* element is a Class II type transposable element with 28 bp imperfect inverted repeats with four mismatches. The element recovered from w^{pch} contained a single open reading frame capable of encoding a 346 amino acid polypeptide (Jacobson *et al.*, 1986). While the original w^{pch} was highly unstable, another strain of *D. mauritiana* was discovered in which mosaicism of the eyes occurred in every fly (Bryan *et al.*, 1987). This mosacism factor was found to be heritable and was referred to as *Mos1* (Mosaic eyes). *Mos1* was a dominant autosomal factor on chromosome 3 and was subsequently found to be identical to *mariner* except for six amino acid differences in the putative transposase coding region (Medhora *et al.*, 1988). *Mos1* encodes for a functional transposase while the 346 amino acid polypeptide of the w^{pch} *mariner* element was not a functional transposase.

One of the most notable characteristics of *mariner* and *mariner*-like elements (*MLE*s) is their widespread distribution. *MLE*s are found not only in insects and invertebrates but also in vertebrates and plants (Robertson, 2000; Robertson and Zumpano, 1997). Not long after the *D. mauritiana* *mariner* elements were described, a related element was discovered in the *cecropin* gene of the moth *Hyalophora cecropia* (Lidholm *et al.*, 1991). Based on the sequence comparison between the *mariner* elements from *D. mauritiana* and *H. cecropia*, Robertson (1993) designed degenerate PCR primers

and surveyed 404 species of insects for the presence of related sequences. He found that 64 of the genomes examined contained *MLE*s, and within this group are five subgroups referred to as the *mauritiana, cecropia, mellifera, irritans*, and *capitata* subgroups (Robertson and MacLeod, 1993). Since that original analysis insect *MLE*s have continued to be discovered and currently there are two additional subgroups recognized, known as *mori* and *briggsae* (Lampe *et al*., 2000). Additional subgroups are likely to be recognized in the future as additional representatives of this family of elements are found. Elements from different subgroups are typically about 50% identical at the nucleotide sequence level while the transposases encoded by elements from different subgroups are usually between 25% and 45% identical at the amino acid level. A notable feature of the phylogenetic relationships of the *MLE*s is their incongruence with the phylogenetic relationships of the insects from which they were isolated. The implication is that many of these elements were introduced into their host genome via a horizontal gene transfer event (Robertson and Lampe, 1995a). The abundant examples of horizontal transfer of *mariner* elements have led to the conclusion that such transfers occur relatively frequently. Hartl *et al*. (1997) estimated that the rate of horizontal transmission of *MLE*s is about the same as the rate of speciation, at least within the *D. melanogaster* species subgroup. The widespread occurrence of horizontal transmission of *MLE*s has been proposed to be critical for the long-term survival of these elements. Horizontal transmission provides a means for invading naive genomes where element proliferation can occur before inactivating influences of mutation and host regulation can occur (Hartl *et al*., 1997).

Although hundreds of *MLE*s have been reported, only two (*Mos1* from *D. mauritiana* and *Himar1* from *Haematobia irritans*) have been demonstrated to be functional or active. *Haemotobia irritans* contains approximately 17 000 copies of *Himar1*, although all of the copies examined were highly defective. Functional elements could be reconstructed based on the consensus sequence of *Himar1* and then constructed by modifying the closely related *Cpmar1* element from the green lacewing, *Chrysoperla plorabunda,* to match the *Himar* consensus sequence (Robertson and Lampe, 1995b; Lampe *et al*., 1998). Purification of the transposase from a bacterial expression system and its use in an *in vitro* mobility assay demonstrated the functionality of the *Himar1* protein and the ITRs of the element (Lampe *et al*., 1996).

4.13.5.3.2. Structure–function relationships The transposases of *MLE*s belong to a large group of integrases and transposases that share a significant feature of their catalytic domains. Specifically, *MLE*s contain the highly conserved DD35E motif within the active site of the protein (Robertson, 2000). This part of the active site interacts with a divalent cation that is essential for catalysis. Transposase binds to the ITRs of the element, and gel retardation assays were used to assess the binding activity of eight mutant transposases with deletions at the N- or C-termini (Auge-Gouillou *et al*., 2001a). It was possible to show that amino acids 1–141 were sufficient for binding to the ITRs. The ITR binding domain of *Mos1* transposase differs somewhat from that of *Tc1* elements in that it is composed of two different structural motifs, a helix–turn–helix motif and an α-helical region (Auge-Gouillou *et al*., 2001a).

The ITRs of *Mos1* are not identical and differ in sequence at four positions, which have effects on the activity of the element *in vitro*. Auge-Gouillou *et al*. (2001b) reported a 10-fold higher affinity of *Mos1* transposase for the 3′ ITR compared to the 5′ ITR. In addition, modified 5′ ITRs that were made to resemble 3′ ITRs at one of the four variable positions resulted in an increase in transposase binding. These investigators also showed that a *Mos1* element with two 3′ ITRs had 104 times the transposition activity of the native ITRs (Auge-Gouillou *et al*., 2001b). This hyperactive double-ended configuration has not been tested *in vivo*.

Hyperactive transposase mutants of the *Himar1* transposase have been reported (Lampe *et al*., 1999) and one of the mutants contains two amino acid changes (at positions 131 and 137) in the ITR binding domain of the protein. Although not tested directly, it is possible that these hyperactive mutants result in increased binding of the transposase and consequently higher rates of movement. Paradoxically, neither *Himar1* nor any of the hyperactive muatants shows any transpositional activity in insects (Lampe *et al*., 2000).

4.13.5.3.3. Host range of *mariner* The widespread distribution of *MLE*s in nature and the frequent examples of their horizontal transfer between species suggest that these elements have a broad host range. Empirical studies in which *Mos1* has been employed as a gene vector in a wide variety of organisms supports this conclusion. *Mos1* has been used successfully to create transgenic *D. melanogaster* (Lidholm *et al*., 1993), *D. virilis* (Lohe and Hartl, 1996a), and *Aedes aegypti* (Coates *et al*.,

1998). In each of these species the frequency of transformation was approximately 5%. This element has also been used to create transgenic *B. mori* cells in culture (Wang *et al*., 2000). In addition to transgenic insects, *Mos1* has been used to create transgenic *Leishmania* (Gueiros-Filho and Beverley, 1997), *Plasmodium* (Mamoun *et al*., 2000), zebrafish (Fadool *et al*., 1998), and chickens (Sherman *et al*., 1998). Similarly the *Himar1* element has been shown to function in *E. coli* (Rubin *et al*., 1999), Archaebacteria (Zhang *et al*., 2000), and human cells (Zhang *et al*., 1998). However, this element has not been shown to be active in *D. melanogaster* or any other insect species, for reasons that are not clear (Lampe *et al*., 2000).

4.13.5.3.4. Postintegration behavior The postintegration behavior of *Mos1* has been investigated in *D. melanogaster* and *A. aegypti*. *mariner* gene vectors used to create transgenic *D. melanogaster* have been found to be uncommonly stable even in the presence of functional transposase. Lidholm *et al*. (1993) created two lines of transgenic *D. melanogaster* with a *mariner* vector derived from *Mos1* and containing the mini-*white* gene as a genetic marker. When these lines were crossed to *Mos1* transposase-expressing lines, eye mosaicism was found in only 1% of the progeny, while these same *Mos1* expressing lines resulted in 100% mosaicism of the w^{pch} element. Similarly, germline transposition occurred at rates of less than 1% (Lidholm *et al*., 1993), and Lohe *et al*. (1995) reported similar evidence for postintegration stability of *mariner* vectors. Lozovsky *et al*. (2002) suggested, after investigating the postintegration mobility of a number of *mariner* vectors containing different genetic markers in different locations within the element, that *mariner* mobility is highly dependent upon critical spacing of subterminal sequences and ITRs. They found that vectors with simple insertions of exogenous DNA of varying lengths and in varying positions showed levels of somatic and germline excision that were at least 100-fold lower than that observed with uninterrupted *mariner* elements. Only vectors consisting of two, almost complete, elements flanking the marker gene showed detectable levels of both somatic and germline mobility. Approximately 10% of the insects with these composite vectors had mosaic eyes when transposase was present. Germline excision rates of approximately 0.04% were observed in these same insects. Again, these values are considerably less than those reported for uninterrupted elements. In addition to the potential importance of subterminal sequence spacing (Lozovsky *et al*., 2002), Lohe and Hartl (2002) suggested that efficient mobilization of *mariner in vivo* also depends on the presence of critical sequences located quite distant from the ITRs. Based on the mobility characteristics of about 20 *mariner* elements with a wide range of internal deletions, they concluded that there are three regions within the element that play an important role in *cis*. Region I is approximately 350 bp in length and is located 200 bp from the left 5′ ITR. Region II is approximately 50 bp in length and located approximately 500 bp from the right 3′ ITR. Region III is about 125 nucleotides in length and located approximately 200 bp from the right ITR (Lohe and Hartl, 2002). While the presence of subterminal sequences that play a critical role in the movement of many Class II transposable elements is not unusual, what is uncommon in the case of *mariner* is the location of these *cis*-critical sequences. Their dispersed distribution within the element is unique, and consequently, manipulating the element for the purposes of creating gene vectors and associated tools without disrupting these important relationships may be difficult.

The postintegration mobility of *Mos1* can also be regulated by nonstructural aspects of the system including “overproduction inhibition” and “dominant-negative complementation.” Increasing the copy number of *Mos1* in the genome resulted in a 25% decrease in the rate of germline excision. Copy number increases in *Mos1* presumably lead to increased transposase levels and, by an unknown mechanism, to the inhibition of excision (Lohe and Hartl, 1996b). High concentrations of transposase may lead to nonspecific associations of the protein resulting in inactive oligomers of transposase. In addition, the presence of mutated forms of *Mos1* transposase can repress the activity of functional transposase. Because the transposases of other transposable elements act as dimers or multimers it is thought that mutated *Mos1* transposases may become incorporated into multimers with functional transposases, thereby inactivating the entire complex (Lohe and Hartl, 1996b).

The possibility that transposase overproduction may negatively affect its own activity is a highly important concept in terms of vector system development. Most systems have the helper transposase under strong promoter regulation to optimize transpositional activity, though this may, indeed, be counterproductive. For *mariner* vectors, and potentially other systems, optimal transformation may require testing various helper promoters and a range of plasmid concentrations.

The postintegration mobility properties of *mariner* were also examined in *A. aegypti* (Wilson *et al*.,

2003). As part of an effort to create an enhancer trapping and gene discovery technology for *A. aegypti*, they created nonautonomous *mariner*-containing lines and lines expressing *Mos1* transposase. By creating heterozygotes between these two lines, they attempted to detect and recover germline transposition events, but only a single germline transposition event was recovered after screening 14 000 progeny. Somatic transpositions were detected, and while precise estimates of rates of somatic transposition were not possible because of the detection method, the authors observed fewer than one event per individual which they estimated to be an indication of a very low rate of movement. The vectors used by Wilson *et al*. (2003) resembled the simple vectors reported by Lozovsky *et al*. (2002) which had apparently disrupted spacing of the ITRs, and partial deletions of *cis*-critical sequences described by Lohe and Hartl (2002).

While the postintegration stability of *mariner* has been described in two species and appears to be a general mobility characteristic of this element, and not a reflection of a species-specific host effect, paradoxical observations remain to be explained. First, the use of *mariner* as a primary germline transformation vector in non-drosophilid insects and in non-insect systems is an effective means for creating transgenic organisms. Indeed, the host range of *mariner* as a gene transformation vector is unrivaled by any of the other gene vectors currently employed for insect transformation. *mariner* has been used as a gene vector in microbes, protozoans, insects, and vertebrates. The rates of germline transformation using *mariner*-based vectors in insects is approximately 10% or less, and is comparable to the efficiency of *Hermes*, *Minos*, and *piggyBac* gene vectors. This raises the question of whether *mariner* vectors present on plasmids behave the same as *mariner* vectors integrated into insect chromosomes. Given the rates of germline integration from plasmids it appears that the *mariner* vectors being used are not suffering from "critical spacing/critical sequence" defects. In addition, the *in vitro* behavior of *mariner* also differs from the behavior of chromosomally integrated elements. Tosi and Beverly (2000) demonstrated that only 64 nucleotides from the left end, and 33 nucleotides from the right end, of *mariner* were essential for transposition of a 1.1 kb vector *in vitro*. The rate of transposition of a minimal *mariner* vector *in vitro* was only two-fold less than that of a vector containing essentially a complete *mariner* element. These results suggest that *mariner* mobility has relatively simple sequence requirements and that the role of subterminal sequences is minimal *in vitro*. These apparently conflicting data suggest that host factors may play an important role in the transposition process *in vivo*, and may influence the relative importance of *cis* sequences in the *mariner* transposition process. The broad distribution of *MLE*s and host range of *mariner/Mos1* suggest, however, that host factors play little role in the movement of these elements.

The postintegration behavior of *mariner/Mos1* seems to indicate that this element will not be a good candidate for developing gene-finding tools such as promoter/enhancer trapping and transposon tagging systems in *A. aegypti* or perhaps other insects. On the other hand, if a high level of post-integration stability is desired, then *mariner* is an appropriate element to consider in insects. The potential of this element to be lost through excision or transposition is low, even in the presence of functional *mariner* transposase. As currently configured and used, *mariner* vectors may be considered as suicide vectors in insects since they essentially become dysfunctional upon integration.

4.13.5.3.5. *MLE*s have been found in other insects While hundreds of *MLE*s have been described, few have been shown to be functional. The original *mariner* element from the *white*-peach allele was transpositionally competent although it did not produce a functional transposase. *Mos1* is a functional autonomous element and has been the basis for constructing all *mariner* gene vectors that function in insects. *Himar1* is a functional element from the *irritans* subgroup that was reconstructed based on multiple sequence comparisons of elements within this group. It has not been shown to be functional in insects despite significant efforts to do so. Lampe *et al*. (2000) report that at least eight other elements from the other subgroups are likely to be active or made active by minor modifications.

4.13.5.4. *Minos*

The first germline transformation of a non-drosophilid insect mediated by a transposon-based vector system was achieved with the *Minos* element. *Minos* was originally isolated as a fortuitous discovery in *D. hydei* during the sequencing of the non-coding region of a ribosomal gene (Franz and Savakis, 1991). *Minos* was found to be a 1.4 kb element having, unlike the other Class II transposons used as vectors, relatively long ITRs of 255 bp, with its transcriptional unit consisting of two exons (**Figure 1**). Additional *Minos* elements were isolated from *D. hydei* having small variations of one or two nucleotides, though the new elements had a transition change that restored the normal reading frame allowing translation of a functional transposase.

The sequence homology, general structure, and TA insertion-site specificty placed *Minos* within the *Tc* transposon family (Franz *et al.*, 1994). *Minos* was first used to transform *D. melanogaster* with *Minos*-mediated events demonstrated by sequencing insertion sites and remobilization of integrations (Loukeris *et al.*, 1995a). The first non-drosophilid transformation with *Minos* was achieved in a medfly *white eye* host strain using a cDNA clone for the medfly *white* gene as a marker (Zwiebel *et al.*, 1995), at an approximate frequency of 1–3% per fertile G_0 (Loukeris *et al.*, 1995b). *Minos* transposition was subsequently demonstrated in dipteran and lepidopteran cell lines (Klinakis *et al.*, 2000; Catteruccia *et al.*, 2000a), with germline transformation reported for *Anopheles stephensi* (Catteruccia *et al.*, 2000b) and *D. virilis* (Megna and Cline, personal communication). Recently transformation frequencies have been substantialy increased in *Drosophila* and medfly by the use of *in vitro* synthesized transposase mRNA as helper (Kapetanaki *et al.*, 2002).

Although *Minos* has not been widely used for insect transformation, embryonic and cell line mobility assays in several insect species in the Diptera, Lepidoptera, and Orthoptera have indicated a broad range of function. Notably, *Minos* transposition in the cricket *Gryllus bimaculatus* was driven by transposase regulated by a *Gryllus* actin gene promoter, and not by the *Drosophila hsp70* promoter that has been widely used in dipterans (Zhang *et al.*, 2002). The broad function of the *Minos* vector is further supported by its ability to transpose in a mouse germline (Drabek *et al.*, 2003).

Minos structure places it within the *mariner*/*Tc* transposon superfamily, though knowledge of the distribution of *Minos* is thus far limited to the genus *Drosophila* (Arca and Savakis, 2000). In *Drosophila*, *Minos* is clearly widely distributed in the *Drosophila* and *Sophophora* subgenera, though discontinuously in the *Sophophora*. As noted for the *hAT*, *mariner*, and *piggyBac* elements, *Minos* may have also undergone horizontal transfer between *Drosophila* species.

4.13.5.5. *Tn5*

Tn5 is one of a number of very well-characterized transposable elements from prokaryotes. Recently, hyperactive forms of this element have been created in the laboratory that have proven to be the basis for the development of a number of commercially useful genomics tools (Goryshin and Reznikoff, 1998; Epicentre, 2004). *Tn5*-based genomics tools can be used in a wide variety of bacterial species and given the system's independence from host-encoded factors, might be applicable to eukaryotic systems as well (Goryshin *et al.*, 2000). Efforts to use *Tn5* as an insect gene vector have been successful.

Tn5 is a prokaryotic transposon 5.8 kb in length, and it is often referred to as a composite transposon because it consists of five independently functional units (review: Reznikoff, 2000) (**Figure 1**). It contains three antibiotic resistance genes that are flanked by 1.5 kb inverted repeat sequences. Each inverted repeat is actually a copy of an *IS50* insertion sequence that are themselves functional transposons. Each *IS50* element contains 19 bp terminal sequences known as outside end (OE) and inside end (IE), and while OE and IE are very similar, they are not identical. *IS50* also encodes for two proteins: transposase (Tnp) is 476 amino acids long and catalyzes transposition while the second protein is an inhibitor of transposition (Inh). The *IS50* elements present at each end of *Tn5* are not identical and only *IS50R* is fully functional. *IS50L* contains an ochre codon that prematurely terminates the Tnp and Inh proteins resulting in a loss of function of both proteins.

The transposition reaction and all of the components involved in the reaction have been studied in great detail (Reznikoff *et al.*, 1999). Transposition proceeds by a cut-and-paste process involving binding of Tnp to the end sequences followed by dimerization of the bound Tnp to form a synaptic complex. Cleavage at the ends of the element results in an excised transposon with bound transposase that interacts with a target DNA molecule. Strand transfer results in the integration of the element into the target, and *in vitro*, this reaction requires only a donor element, a target DNA molecule, transposase, and Mg^{2+} (Goryshin and Reznikoff, 1998). Modifications of both the transposase and the terminal 19 bp sequences have lead to the creation of *Tn5* elements consisting of little more than two copies of end sequences that can be mobilized a 1000-fold more efficiently than an unmodified *Tn5* element. This hyperactive *Tn5* system has been developed into a powerful tool for genetic analysis of a variety of organisms. *Tn5* has been attractive as a broad host range genomics tool because its pattern of integration is random and its biochemical requirements very simple. *Tn5* has been shown to function in a variety of bacterial and nonbacterial systems.

Current insect transformation protocols consist of microinjecting a mixture of two plasmids into preblastoderm embryos (see Section 4.13.6.3). One plasmid contains a nonautonomous transposable element with the transgenes and genetic markers of interest while the second plasmid contains a copy of the transposase gene. Transient expression of the

transposase gene is required postinjection and is followed by element excision and integration. Previous experiments examining the frequency of element excision of elements, such as *Hermes*, *mariner*, *Minos*, and *piggyBac*, from plasmids injected into insect embryos along with helper plasmids indicated that only one plasmid per thousand injected underwent an excision event. Therefore, 99.9% of the donor plasmids introduced into insect embryos will contribute nothing to the transformation efforts. The introduction of preexcised elements configured as active intermediates, such as synaptic complexes, was considered a means to permit higher integration rates and overall efficiency of transformation.

Transgenic *Aedes aegypti* were created using a *Tn5* vector containing DsRed under the regulatory control of the 3xP3 promoter (Rowan *et al*., 2004). Preexcised vectors in the form of synaptic complexes were injected into preblastoderm embryos. Nine hundred adults were obtained from the injected embryos and families consisting of approximately 10 G_0 individuals were established. Two families of G_0 individuals produced transgenic progeny for an estimated transformation frequency of 0.22% (2/900). Analysis of the transgenic progeny showed that multiple integrations of *Tn5* occurred in each line. The patterns of integrations were complex with evidence of the *Tn5* vector integrating into *Tn5* vector sequences. The integration of the vector into copies of itself followed by the integration of the resulting concatamers was very unusual, and in no case was a simple cut-and-paste integration of the *Tn5* vector found with characteristic 9 bp direct duplications flanking the element. The complex pattern of *Tn5* integration was thought to be a direct consequence of injecting preassembled intermediates, that were inactive in the absence of Mg^{2+}. Therefore, as soon as the synaptic complexes were injected they became activated and the first target sequences the elements were likely to encounter were other *Tn5* synaptic complexes. At the time of injection, *A. aegypti* embryos only contain approximately four to eight nuclei making genomic target DNA relatively rare. Furthermore, the synaptic complexes injected were expected to have a very short half-life. Therefore, although active intermediates were being introduced, a number of factors contributed to the inefficiency observed with this system including a short half-life of the active intermediate and low numbers of genomic target sequences. Injecting binary plasmid systems (as is done with *Hermes*, *mariner*, *Minos*, and *piggyBac*), while relatively inefficient in producing active transposition intermediates, achieves persistence over an extended period of time. Consequently, more target genomes are exposed to active vectors over a longer period of time, resulting in higher transformation rates. The limitations of injecting synaptic complexes is unlikely to be specific to the *Tn5* system and similar approaches with other insect gene vectors are likely to encounter similar problems. It should be noted, however, that the results of Rowan *et al*. (2004) demonstrate that *Tn5* is functional in insects and, while injecting active intermediates is not recommended, using *Tn5* in a more conventional binary plasmid system consisting of a donor and helper plasmids is likely to be a viable option for creating transgenic insects.

4.13.6. Transformation Methodology

The technical methodology for insect transformation has largely remained the same or only slightly modified from the techniques originally used to transform *Drosophila*. The references cited for *P* transformation are relevant to this, as well as several recent articles that focus on methods for non-drosophilid transformation (Handler and O'Brochta, 1991; Morris, 1997; Ashburner *et al*., 1998; Handler, 2000; Handler and James, 2000). The most variable aspect of this method is the preparation of embryos for DNA microinjection, though arguably, the lack of new techniques for DNA introduction has been the primary limitation in the more widespread use of the technology. While all successful insect transformations have utilized microinjection, variations on this method have been necessary for different types of embryos, and most of the procedures must be tested empirically and modified for particular insect species. This may be extended to different strains and for a variety of local ambient conditions including temperature and humidity. The apparatus for microinjection is usually the same for all species, though a wide variety of variations and modifications are possible and sometimes required. The basic equipment includes an inverted microscope or a stereozoom microscope with a mechanical stage having a magnification up to 60 to 80×; a micromanipulator that is adjustable in three axes with an appropriate needle holder; and a means to transmit the DNA into the egg. For dechorionated eggs, transmitted light allows precise positioning of the needle within the egg posterior, while direct illumination is needed for nondechorionated eggs that typically include mosquitoes and moths.

The standard for gene transfer methodology in general, and embryo microinjection in particular, was originally developed for *Drosophila*. The standard method involves collecting preblastoderm embryos

within 30 min of oviposition, and dechorionating them either manually or chemically. The timing of egg collection and DNA injection is related to the need to inject into preblastoderm embryos during a phase of nuclear divisions previous to cellularization. This allows the injected DNA to be taken up into the nuclei, and specifically into the primordial germ cell nuclei that are the gamete progenitors. For *Drosophila*, cellularization of the pole cells begins at approximately 90 min after fertilization at 25 °C, with blastoderm formation occurring about 30 min afterwards. The timing of these events and location of the pole cells varies among insects, and thus some knowledge of early embryogenesis in the desired host insect is highly advantageous. In the absence of this information for a particular species, the most prudent time of injection would be the earliest time after oviposition that does not compromise viability.

4.13.6.1. Embryo Preparation

Manual dechorionation of *Drosophila* eggs is achieved by gently rolling the eggs on double-stick tape with a forcep until the chorions peel off. While gentle on the eggs and requiring little desiccation time, manual dechorionation is tedious and has not been applicable to any other insect. Chemical dechorionation is typically achieved by soaking eggs in a 50% bleach solution (2.5% hypochlorite) for 2–4 min and washing at least three times in 0.02% Triton X-100. Tephritid fruit fly eggs usually have thinner chorions that can be dechorionated in 30% bleach (1.25% hypochlorite) in 2–3 min, but this must be determined empirically since they are easily overbleached resulting in death, either directly or after injection. Some species, such as *M. domestica*, can be only partially dechorionated, but bleached eggs can be released from the chorion by agitation. We have found the simplest and most precise method for bleach dechorionation with rapid washes is by using a 42 mm Buchner funnel with a filter flask attached to a water vacuum. Eggs can be washed into the funnel on filter paper and swirled within the funnel with the solution gently sucked out by regulating the water flow or the seal between the funnel and flask. The last wash is done on black filter paper that allows the eggs to be easily detected, which facilitates their mounting for injection (see below).

Many insects eggs cannot be dechorionated without a high level of lethality, and must be injected without dechorionation. These include most moth and mosquito species. *Drosophila* and tephritid flies can, similarly, be injected without dechorionation, and while embryo viability after injection is often lower than for dechorionated eggs, the frequency of transformation in surviving embryos is often higher. It is more difficult to determine a precise site for injection in nondechorionated eggs, though this can be aided by adding food coloring to the DNA injection mix.

After dechorionation, fruit fly embryos are typically placed on a thin strip (~1 mm) of double stick tape placed on a microscope slide or 22 × 30 mm cover slip, though use of a cover slip is more adaptable for subsequent operations. A thin strip of tape is suggested due to anecdotal reports of toxic solvents from the tape affecting survival, though some particular tapes are considered to be nontoxic (3M Double Coated Tape 415; 3M, St. Paul, MN, USA) and some are useful for particular applications such as aqueous conditions needed for mosquito eggs. Adhesives resistant to moisture include Toupee tape (TopStick™, Vapon Inc.) and Tegaderm (3M). When eggs are injected under oil, the tape strip is placed within a thick rectangle created with a wax pencil that can retain the oil. It is important that the wax fence not be breached by oil when overlaying the eggs, since the loss of oil will result in embryo death.

Where possible, eggs are placed on the tape in an orientation having their posterior ends facing outwards towards the needle, but at a slight angle. All fruit fly eggs must be desiccated to some extent before injection. The interior of the egg is normally under positive pressure, and yolk and injected DNA will invariably flow out after injection without desiccation. This will result in lethality, sterility (from loss of pole plasm), or the lack of transformation if the plasmid DNA is lost. The time and type of desiccation, however, must be evaluated empirically, and sometimes varied during the course of an injection period. A major factor for dechorionated eggs is the length of time they are kept on moist filter paper before being placed on the tape. Typically we desiccate embryos on one strip of tape (15–20 embryos) for 8 to 10 min. Depending on the ease of injection the time can be varied by 1–2 min. In ambient conditions that are humid, it may be necessary to desiccate in a closed chamber with a drying agent (e.g., drierite), with or without a gentle vacuum. An important consideration is that a very short variation in the time for desiccation can be the difference between perfect desiccation and overdesiccation resulting in death, and that the optimal desiccation time will vary for different eggs on the tape. Thus, it is unlikely that all the eggs will respond well to the set conditions, which must be modified so that the majority of eggs can be injected with DNA at a high level of survival and fertility. After the determined time for desiccation, the eggs

must be placed immediately under Halocarbon 700 oil, or oil of similar density, to stop the desiccation process. Desiccation of most nondechorionated eggs is more challenging and one approach is to soak eggs in 1 M NaCl for several minutes. In contrast, for nondechorionated mosquito eggs, desiccation can occur within 1–2 min after removal from water, which is evidenced by slight dimpling of the egg surface, and this must be observed to avoid overdesiccation. Due to the rapidity of desiccation, mosquito eggs are typically arranged on moist filter paper and blotted together onto a taped cover slip from above, and after desiccation, the eggs are submerged in Halocarbon oil. Nondechorionated eggs from many species do not require oil, and it may be lethal for some insects such as moths, yet oil submersion was helpful for the survival of *Drosophila* and tephritid flies.

4.13.6.2. Needles

The type of needle and its preparation is possibly the most important component of successful embryo injections. Most dechorionated fruit fly eggs can be injected easily with borosilicate needles, which are drawn out to a fine tip and broken off to a 1 to 2 μm opening. Opening the tip is typically achieved by scraping the needle against the edge of the slide carrying the eggs to be injected. Opening the needle by beveling, however, creates consistently sharp tips that are much more important for nondechorionated eggs, and stronger alumina-silicate and quartz needles also provide an improvement to easily pierce chorions or tough vitelline membranes. Beveled needles are also critical when a large tip opening is required for large plasmids that are susceptible to shearing. Preparation of borosilicate needles, pulled from 25 μl capillary stock that has been silanized, can be achieved with several types of vertical or horizontal needle pullers, and we find the Sutter Model P-30 (Sutter Instruments, Novato, CA, USA) vertical micropipette puller to be highly effective. Alumina-silicate needles, and certainly quartz needles, require more sophisticated pullers that allow for fine programmable adjustment of high filament temperatures and pulling force, and the Sutter Models P-97 and P-2000 fulfill this need. Several needle bevelers are available, with the Sutter BV-10 used by many laboratories.

4.13.6.3. DNA Preparation and Injection

A mixture of highly purified vector and helper plasmid DNA is essential to embryo survival. This is achieved most optimally by purifying plasmid twice through cesium chloride gradients or a solid-phase anion exchange chromatography column. These have the advantage of high yields of DNA, but the disadvantage of specialized equipment and long preparation times. Successful transformation has been achieved with plasmids prepared with silica-gel membrane kits from Qiagen Corp. (Valencia, CA, USA), but their successful use has been inconsistent, with failures possibly related to the type of host bacteria and its growth conditions. The Qiagen Endotoxin-free plasmid preparation systems allow additional purity, and this system is routinely used for successful plasmid injection.

Purified plasmid concentration must be titered accurately and verified by gel electrophoresis previous to injection mix preparation. Appropriate amounts of vector and helper plasmid are ethanol precipitated, washed several times in 70% ethanol, and resuspended in injection buffer. Injection buffer has typically been the same as that originally used for *Drosophila* (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8), though this may not be optimal for other insects and embryo survival should be assessed by control injections. Total DNA concentration for injection should not exceed 1 mg ml^{-1}, using two- to fourfold higher concentration of vector to helper (e.g., 600 ng μl^{-1} vector to 200 ng μl^{-1} helper). Higher DNA concentrations are inadvisable since they are subject to shearing during injection and may clog the needle, and the nucleic acids and/or contaminants can be toxic to the embryo. High transposase levels may also have a negative effect on transposition, as with the overproduction–inhibition phenomenon observed with *mariner* (Lohe and Hartl, 1996b).

Previous to injection the DNA mixture should be filtered through a 0.45 μm membrane, or centrifuged before loading into the injection needle. Typically, DNA is back-filled into the injection needle using a drawn-out silanized 100 μl microcapillary, and a microliter of DNA should be sufficient for injecting hundreds of eggs.

4.13.6.3.1. DNA injection The microinjection of DNA into embryos requires a system that forces a minute amount of DNA through the needle in a highly controllable fashion. Remarkably, many *Drosophila* laboratories simply use a mounted syringe and tubing filled with oil connected to a needle holder, with manual pressure applied. This system is successful due to accumulated expertise and the efficiency of transformation in the species, but would probably be less useful for injecting more sensitive embryos that transform less easily. Regulated air-pressure systems are available that are economical and allow highly controlled and rapid DNA injection. We use the PicoPump from WPI that

is most versatile in allowing positive and negative (with vacuum) pressure, and a hold capability that prevents back flow into the needle resulting in clogging (especially by yolk). A less expensive system can be constructed from Clippard components (Clippard Instrument Laboratory, Inc., Cincinnati, OH, USA) that uses a simple air-pressure regulator and electronic valve and switch (see Handler, 2000). Needle holders from WPI can be used with both systems (MPH-3 and MPH-1, respectively).

All embryo injections are performed on a microscope with a mechanical stage, with the injection needle mounted on a micromanipulator. Microscopes first used for *Drosophila* transformation were inverted or compound microscopes, but the availability of a useful mechanical stage and stage adaptor for the Olympus SZ stereozoom microscopes makes this the most versatile choice (the Olympus stage can be mounted on most stereomicroscopes). The micromanipulator can be free-standing next to the stage or mounted on the microscope base. It allows the precise positioning of the needle at the desired point of entry into the egg, while the actual injection occurs by using the mechanical stage to push the egg onto the needle. Piezo Translators that were developed for rapid and automatic intracellular injection may be more efficient for some embryos, and will obviate the need for a mechanical stage (Peloquin *et al*., 1997). The WPI MPM20 translator used with the PV820 PicoPump allows a fully automated system for egg penetration, DNA injection, and needle withdrawal.

4.13.6.4. Postinjection Treatment

After injection the cover slip can be placed in a covered petri dish (but not sealed) with moist filter paper. The use of square dishes with black filter paper seems to be most suitable for up to six cover slips and simple observation of the embryos and hatched larvae. For injected embryos submerged in oil, oxygen concentration may be a limiting factor for development, if not viability. This can be ameliorated by reducing the crowding of eggs on the cover slip, or by incubation in a portable hatbox tissue culture chamber that is humidified and under slight positive pressure with oxygen. For eggs without oil, oxygen saturation without pressure is advisable.

Most helper constructs have the transposase gene under heat shock regulation. The *Drosophila hsp70* promoter is a constitutive promoter that is active in the absence of heat shock (but also responds to anoxia which may occur in embryos under oil), and transformation is possible with most vectors with or without heat shock treatment. If heat shock is desirable, it should be noted that the optimal temperature varies for different species. For example, *hsp70* responds optimally at 37 °C in *Drosophila*, but at 39 °C in medfly (Papadimitriou *et al*., 1998). Injected embryos should be incubated for at least 4–6 h after injection before heat shock, or after overnight incubation. Optimal temperatures for insect development vary, but the lowest temperatures possible can be beneficial to survival, and the injection process can slow development by 50% or more. Thus, larval hatching may be delayed considerably and hatching should be monitored for several days after the expected time before discarding embryos.

Hatched larvae can be placed on normal culture media, though they may be weak and require careful handling and soft diet. Rearing of putative transgenic lines is typically achieved by backcrossing to the parental line in small group matings, or individual mating if a determination of transformation frequency is required. Inbreeding of G_0s can minimize rearing efforts, but this may be complicated by high rates of infertility which is typically close to 50% after fruit fly injections.

4.13.7. Research Needs for Improved Transgenesis

4.13.7.1. DNA Delivery

Dramatic progress has been made in transformation technology for non-drosophilid insects, and it appears that the vectors and markers in use should be widely applicable. Nevertheless, transformation of many other insect species will be highly challenging, primarily due to limitations in the delivery of DNA into preblastoderm embryos. As noted, to date all successful non-drosophilid transformations have resulted from embryonic microinjection of DNA, but for many species current injection techniques are likely to result in high levels of lethality or sterility. Experimentation with alternative methods has been reported, though arguably, none has been tested exhaustively for germline transformation, or vector systems were used that are now known to be ineffective. The most promising method is biolistics where eggs are bombarded with micropellets encapsulated by DNA, which was first developed as a ballistics method to transform plant cells (Klein *et al*., 1987). Ballistics is based upon a “shotgun” technique for bombardment, and it is the only non-injection method successfully used to transform an insect. This was a *P* transformation of *Drosophila*, though only a single transformant line was created

and the technique never gained wide applicability (Baldarelli and Lengyel, 1990). This was most likely due to the high efficiency of *P* transformation of *Drosophila* by microinjection, eliminating the need for an alternative technique. Mosquito eggs are considerably more difficult to inject, and a significant effort was made to modify a biolistics approach to DNA delivery in *A. gambiae*, using a burst of pressurized helium for bombardments (Miahle and Miller, 1994). This technique was effective in introducing plasmid DNA into mosquito eggs, yielding high levels of transient expression of a reporter gene. Biolistics was subsequently used for transient expression in specific tissues, allowing the testing of fibroin gene promoters in the *B. mori* silk gland (Horard *et al.*, 1994; Kravariti *et al.*, 2001). Recent advances have included the use of a rigid macrocarrier in the Bio-Rad PDS/1000-Helium biolistics apparatus, which minimizes the blast effect in soft tissue (Thomas *et al.*, 2001). This allows greater micropellet penetration into insect tissues with improved survival. Despite these advances in delivering DNA into eggs and tissue, biolistics has yet to yield a germline transformant.

The only other method reported for DNA delivery is electroporation, which, like biolistics, has resulted in high levels of transient expression of plasmid-encoded genes in *Drosophila* (Kamdar *et al.*, 1992), as well as in *Helicoverpa zea* and *M. domestica* (Leopold *et al.*, 1996). Though transformation has not been reported, as with biolistics, it is not apparent that this was seriously tested or if functional vectors systems were used (certainly for non-drosophilids). Electroporation techniques have also advanced in recent years, with DNA transferred into many different tissue types from a variety of organisms using new electroporation chamber designs and electric field paramenters.

These recent advances with both biolistics and electroporation are highly encouraging that new efforts will have greater chances for success, and they deserve a high priority for testing. Both methods also have the advantage, if successful, of delivering DNA simultaneously to multiple embryos, ranging from hundreds to thousands depending on the species. This would be highly beneficial to all transformation experiments, but especially so for species that transform at low frequencies. These methods could also be used in cellularized embryos after blastoderm formation in insects having embryos that cannot be handled easily or collected in the preblastoderm stage.

Other approaches to DNA delivery can include the incorporation of vector/helper DNA into bacterial or viral carriers, that may be delivered by maternal injection or feeding. Variations on microinjection that might be required for ovoviviparous insects include maternal injection into ovaries or abdominal hemocoel (Presnail and Hoy, 1994), and the use of liposomes might allow injection into cellularized embryos (Felgner *et al.*, 1987). All of these techniques should be reevaluated with the use of vectors and markers now known to be highly efficient in non-drosophilid systems.

4.13.7.2. Gene Targeting

The ability to target genes to specific or desired integration sites in the genome would be highly advantageous to the basic and applied uses of transgenic strains. The expression of transgenes in most vector integrations is affected negatively by chromosomal position effects, so target sites known to be devoid of, or insulated from, suppression elements could be utilized for optimal or consistent transgene expression. Target sites positioned in innocuous genomic regions could also eliminate random integrations into genes necessary for viability and fertility, eliminating costs to fitness in host strains. It will also be highly important to gene expression studies to achieve reliable methods for gene replacement or targeted transposition, which is especially important for systems where preexisting null mutations, or gene "knockouts," do not exist.

Gene targeting can be achieved, generally, in two ways. First is homologous recombination where an endogenous genomic sequence is replaced by recombination with homologous sequences within or surrounding the transgene. This results in targeted transposition which can be used for gene replacement, or for targeting to an innocuous genomic region. This approach has been effective in transforming lower eukaryotes, and plant and vertebrate systems (see Bollag *et al.*, 1989) and has been reported to occur in *Drosophila* (Cherbas and Cherbas, 1997) and mosquito (Eggleston and Zhao, 2000) cell line studies. Homologous recombination can also occur in insects *in vivo*, but this is not routine and thus far, must be facilitated. In *B. mori*, female moths were infected with a modified AcNPV baculovirus that had its polyhedrin gene replaced with fibroin light chain-GFP gene fusion (Yamao *et al.*, 1999). Progeny of the infected moths exhibited stable integration of the gene fusion into the genomic fibroin gene, with resulting GFP expression. This particular method relies on host susceptibility to baculovirus infection, though conceivably other pantropic or species-specific viruses could be used for a wider range of insects.

Homologous recombination was also achieved in *Drosophila* where linearized extrachromosomal

DNA was found to be recombinogenic with homologous sequences in the genome (Rong and Golic, 2000). This was achieved by using the *FRT*–FLP recombination system to create DNA circles, which were linearized at a rare endonuclease recognition site within the *FRT* sequences, resulting in a *yellow* marker gene integrating into its homologous chromosomal site. This system has potential application in any insect species that can be stably transformed, but it requires the integration of three components which can be tedious to achieve and may present fitness costs to the host. Though its use could have major importance to genetic studies in non-drosophilid insects, thus far it is not routinely used in *Drosophila*.

4.13.7.2.1. Site-specific recombination Site-specific recombination systems such as the *FRT*–FLP system from the 2 μm circle of yeast (Senecoff *et al.*, 1985) mentioned above, and the bacteriophage Cre/*lox* system (Hoess *et al.*, 1985) can be used for various types of gene targeting and chromosomal manipulation. Both systems function in *Drosophila* in which recombination occurs between specific sequences in the presence of a recombinase enzyme (Golic and Lindquist, 1989; Siegal and Hartl, 1996). For *FRT*, the recombination site consists of two 13 bp inverted repeats separated by an 8 bp spacer that specifically recombines with identical *FRT* sites in the presence of FLP recombinase. Depending upon the orientation of the *FRT* sites, the intervening sequence between them can be inverted or deleted by recombination (Golic and Golic, 1996; Golic *et al.*, 1997). When placed within a vector, such *FRT* rearrangements can allow several types of vector manipulation after genomic integration. Genes or sequences within the vector necessary for the initial transformation or selection, but deleterious to use of the transformed strain, can be deleted or inactivated (Dale and Ow, 1991; see Handler, 2002b). This may include the marker system used for selection (e.g., chemical resistance system) or even a transposase gene used in a single plasmid autonomous vector system. Expression of genes of interest can be similarly manipulated by placing *FRT* sites outside the gene and within an internal noncoding region. Of particular importance to transgene stability would be the rearrangement of vector sequences required for mobility, that typically would include the terminal and subterminal ITRs. If *FRT* site placement between the terminal sequences does not hinder the primary transposon vector integration, then subsequent subterminal ITR deletion could eliminate any secondary vector mobilization. Such mobilization or cross-mobilziation would have serious consequences for strain stability and function, as well as ecological risks due to unintended transmission of the vector into other organisms. Eliminating this possibility would provide a major advancement to the applied use of transgenic insects.

The ultimate use of recombination systems for improved transformant stability and transgene expression would be their development into a second generation of vectors that use an integrated recombination site as a stable chromosomal target. Plasmids having the same recombination site and a marker gene would be used as vectors that integrate by recombination in the presence of recombinase. The expectation is that these systems would be highly stable in eukaryotes, and specific target site loci could be selected that are minimally affected by position effect variegation/suppression. The internal 8 bp spacer sequence within the *FRT* can be varied, but only identical *FRT* sequences will recombine with one another. Thus, multiple independent *FRT* target sites can be incorporated into the same genome. Importantly, manipulations by *FRT* recombination will depend upon a controllable source of FLP recombinase, which can be provided as a separate transgene integration, or exogenously by DNA, RNA, or protein injection.

4.13.8. Summary

After concerted efforts for more than 30 years to achieve gene transfer in non-drosophilid insects, only in the last decade have these efforts been fruitful. Since 1995 the germline of nearly 20 species in four orders of insects have been transformed, and this number may be only limited by the insects of current experimental and applied interest. Unlike plant and vertebrate animal systems that allow relatively efficient genomic integration of introduced DNA, insect systems have generally relied on vector-mediated integrations, and the only vectors found reliable for germline transformation are those based on transposable elements. Curiously, the two main vector systems developed for routine use in *D. melanogaster*, and originally discovered in that species, *P* and *hobo*, have not been applicable as vectors to any other species. Yet, four other transposons found in non-*melanogaster* or non-drosophilid species are widely functional in insects, and for some, other organisms. Their discovery has been of enormous importance to the wider use of transformation technology, since little progress would have been made if most vector systems were specific to a particular host. Equal in importance to the advancements in vector development, have been concurrent progress in genetic marker discovery and development. This began with the finding

that cloned eye color genes from *Drosophila* could complement existing mutations in other insects, and has continued with the more recent use of several fluorescent protein genes that are widely applicable as markers for transformation and reporters for gene expression.

The advancement of these techniques comes at a fortuitous time when genomics is providing a wealth of genetic infomation and resources that might be used to create transgenic strains of pest and beneficial insects to control their population size and behavior. As part of these efforts, genetic transformation is also critical to functional genomics studies that will provide information essential to understanding the biological function of genetic material, and relating specific genomic elements to those functions. Techniques such as enhancer traps and transposon tagging, which rely on remobilizable insertional mutagenesis, are only possible with transposon-based vector systems, and other techniques such as RNA interference (RNAi) are greatly facilitated by these systems. Together, routine methods for transposon-mediated germline transformation and genomics analysis should provide the tools for dramatic progress in our understanding and control of insect species.

Acknowledgments

We gratefully acknowledge support from the USDA-NRI Competetitive Grants Program and National Institutes of Health. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

Allen, M.L., O'Brochta, D.A., Atkinson, P.W., Levesque, C.S., **2001**. Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol. 38*, 701–710.

Allen, M.L., Handler, A.M., Berkebile, D.R., Skoda, S.R., **2004**. *piggyBac* transformation of the New World screwworm, *Cochliomyia hominivorax*, produces multiple distinct mutant strains. *Med. Vet. Entomol. 18*, 1–9.

Anxolabéhère, D., Kidwell, M., Periquet, G., **1988**. Molecular characteristics of diverse populations are consistent with the hypothesis of a recent invasion of *Drosophila melanogaster* by mobile *P* elements. *Mol. Biol. Evol. 5*, 252–269.

Arca, B., Savakis, C., **2000**. Distribution of the transposable element *Minos* in the genus *Drosophila*. *Genetica 108*, 263–267.

Arca, B., Zabolou, S., Loukeris, T.G., Savakis, C., **1997**. Mobilization of a *Minos* transposon in *Drosophila melanogaster* chromosomes and chromatid repair by heteroduplex formation. *Genetics 145*, 267–279.

Ashburner, M., **1989a**. *Drosophila*: A Laboratory Handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ashburner, M., **1989b**. *Drosophila*: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ashburner, M., Hoy, M.A., Peloquin, J., **1998**. Transformation of arthropods: research needs and long term prospects. *Insect Mol. Biol. 7*, 201–213.

Atkinson, P.W., Pinkerton, A.C., O'Brochta, D.A., **2001**. Genetic transformation systems in insects. *Annu. Rev. Entomol. 46*, 317–346.

Atkinson, P.W., Warren, W.D., O'Brochta, D.A., **1993**. The *hobo* transposable element of *Drosophila* can be cross-mobilized in houseflies and excises like the *Ac* element of maize. *Proc. Natl Acad. Sci. USA 90*, 9693–9697.

Auge-Gouillou, C., Hamelin, M.-H., Demattei, M.-V., Periquet, G., Bigot, Y., **2001a**. The ITR binding domain of the mariner Mos-1 transposase. *Mol. Gen. Genomics 265*, 58–65.

Auge-Gouillou, C., Hamelin, M.-H., Demattei, M.-V., Periquet, G., Bigot, Y., **2001b**. The wild-type conformation of the Mos-1 inverted terminal repeats is suboptimal for transposition in bacteria. *Mol. Gen. Genomics 265*, 51–57.

Avery, T., Macleod, C.M., McCarty, M., **1944**. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. I. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med. 79*, 137–158.

Baldarelli, R.M., Lengyel, J.A., **1990**. Transient expression of DNA after ballistic introduction into *Drosophila* embryos. *Nucl. Acids Res. 18*, 5903–5904.

Bellen, H.J., O'Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R.K., *et al.*, **1989**. *P*-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Development 3*, 1288–1300.

Bender, W., Hudson, A., **2000**. *P* element homing in the *Drosophila* bithorax complex. *Development 127*, 3981–3992.

Benedict, M.Q., Salazar, C.E., Collins, F.H., **1995**. A new dominant selectable marker for genetic transformation: *Hsp70-opd*. *Insect Biochem. Mol. Biol. 25*, 1061–1065.

Berghammer, A.J., Klingler, M., Wimmer, E.A., **1999**. A universal marker for transgenic insects. *Nature 402*, 370–371.

Besansky, N.J., Mukabayire, O., Benedict, M.Q., Rafferty, C.S., Hamm, D.M., *et al.*, **1997**. The *Anopheles gambiae* tryptophan oxygenase gene expressed from a baculovirus promoter complements *Drosophila melanogaster vermilion*. *Insect Biochem. Mol. Biol. 27*, 803–805.

Bigot, Y., Auge-Gouillou, C., Periquet, G., **1996**. Computer analyses reveal a *hobo*-like element in the nematode *Caenorhabditis elegans*, which presents a conserved transposase domain common with the *Tc1-mariner* transposon family. *Gene 174*, 265–271.

Blackman, R.K., Macy, M., Koehler, D., Grimaila, R., Gelbart, W.M., **1989**. Identification of a fully functional *hobo* transposable element and its use for germ-line transformation of *Drosophila*. *EMBO J. 8*, 211–217.

Bollag, R.J., Waldman, A.S., Liskay, R.M., **1989**. Homologous recombination in mammalian cells. *Annu. Rev. Genet. 23*, 199–225.

Brand, A.H., Manoukian, A.S., Perrimon, N., **1994**. Ectopic expression in *Drosophila*. *Methods Cell Biol. 44*, 635–654.

Bryan, G.J., Jacobson, J.W., Hartl, D.L., **1987**. Heritable somatic excision of a *Drosophila* transposon. *Science 235*, 1636–1638.

Bushman, F.D., **1994**. Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. *Proc. Natl Acad. Sci. USA 91*, 9233–9237.

Calvi, B.R., Hong, T.J., Findley, S.D., Gelbart, W.M., **1991**. Evidence for a common evolutionary origin of inverted repeat transposons in *Drosophila* and plants: *hobo*, *Activator*, and *Tam*3. *Cell 66*, 465–471.

Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., *et al*., **2002**. A monomeric red fluorescent protein. *Proc. Natl Acad. Sci. USA 99*, 7877–7882.

Capy, P., Vitalis, R., Langin, T., Higuet, D., Bazin, C., **1996**. Relationships between transposable elements based upon the integrase–transposase domains: is there a common ancestor? *J. Mol. Evol. 42*, 359–368.

Cary, L.C., Goebel, M., Corsaro, H.H., Wang, H.H., Rosen, E., *et al*., **1989**. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-Locus of nuclear polyhedrosis viruses. *Virology 161*, 8–17.

Caspari, E., Nawa, S., **1965**. A method to demonstrate transformation in *Ephestia*. *Z. Naturforsch. 206*, 281–284.

Catteruccia, F., Nolan, T., Blass, C., Muller, H.-M., Crisanti, A., *et al*., **2000a**. Toward *Anopheles* transformation: *Minos* element activity in anopehline cells and embryos. *Proc. Natl Acad. Sci. USA 97*, 2157–2162.

Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, C., *et al*., **2000b**. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature 405*, 959–962.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., Prasher, D.C., **1994**. Green fluorescent protein as a marker for gene expression. *Science 263*, 802–805.

Cherbas, L., Cherbas, P., **1997**. “Parahomologous” gene targeting in *Drosophila* cells: an efficient, homology-dependent pathway of illegitimate recombination near a target site. *Genetics 145*, 349–358.

Christophides, G.K., Savakis, C., Mintzas, A.C., Komitopoulou, K., **2001**. Expression and function of the *Drosophila melanogaster* ADH in male *Ceratitis capitata* adults: a potential strategy for medfly genetic sexing based on gene-transfer technology. *Insect Mol. Biol. 10*, 249–254.

Chroma, **2004**. Chroma Technology Corp., Brattleboro, VT, USA. http://www.chroma.com.

Coates, C.J., Jasinskiene, N., Miyashiro, L., James, A.A., **1998**. *Mariner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc. Natl Acad. Sci. USA 95*, 3742–3751.

Coates, C.J., Johnson, K.N., Perkins, H.D., Howells, A.J., O’Brochta, D.A., *et al*., **1996**. The *hermit* transposable element of the Australian sheep blowfly, *Lucilia cuprina*, belongs to the *hAT* family of transposable elements. *Genetica 97*, 23–31.

Coates, C.J., Turney, C.L., Frommer, M., O’Brochta, D.A., Atkinson, P.W., **1997**. Interplasmid transposition of the *mariner* transposable element in non-drosophilid insects. *Mol. Gen. Genet. 253*, 728–733.

Cornel, A.J., Benedict, M.Q., Rafferty, C.S., Howells, A.J., Collins, F.H., **1997**. Transient expression of the *Drosophila melanogaster cinnabar* gene rescues eye color in the white eye (WE) strain of *Aedes aegypti*. *Insect. Biochem. Mol. Biol. 27*, 993–997.

Dale, E.C., Ow, D.W., **1991**. Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl Acad. Sci. USA 88*, 10558–10562.

Daniels, S.B., Strausbaugh, L.D., **1986**. The distribution of *P*-element sequences in *Drosophila*: the *willistoni* and *saltans* species groups. *J. Mol. Evol. 23*, 138–148.

Davis, I., Girdham, C.H., O’Farrell, P.H., **1995**. A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. *Devel. Biol. 170*, 726–729.

Drabek, D., Zagoraiou, L., deWit, T., Langeveld, A., Roumpaki, C., *et al*., **2003**. Transposition of the *Drosophila hydei Minos* transposon in the mouse germ line. *Genomics 81*, 108–111.

Eggleston, P., Zhao, Y., **2000**. Targeted transformation of the insect genome. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 29–52.

Engels, W.R., **1989**. *P* elements in *Drosophila melanogaster*. In: Berg, D.E., Howe, M.M. (Eds.), Mobile DNA. American Society of Microbiology, Washington, DC, pp. 437–484.

Engstrom, Y., Schneuwly, S., Gehring, W., **1992**. Spatial and temporal expression of an *Antennapedia LacZ* gene construct integrated into the endogenous *Antennapedia* gene of *Drosophila melanogaster. Roux’s Arch. Devel. Biol. 201*, 65–80.

Epicentre, **2004**. Epicentre, Madison, WI, USA. http://www.epicentre.com.

Fadool, J.M., Hartl, D.L., Dowling, J.E., **1998**. Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish. *Proc. Natl Acad. Sci. USA 95*, 5182–5186.

Federoff, N., **1989**. Maize transposable elements. In: Berg, D.E., Howe, M.M. (Eds.), Mobile DNA. American Society for Microbiology, Washington, DC, pp. 375–411.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., *et al*., **1987**. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. *Natl Acad. Sci. USA 84*, 7413–7417.

ffrench-Constant, R.H., Benedict, M.Q., **2000**. Resistance genes as candidates for insect transgenesis. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 109–121.

ffrench-Constant, R.H., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J., Roush, R.T., **1991**. Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: an invertebrate $GABA_A$ receptor locus. *Proc. Natl Acad. Sci. USA 88*, 7209–7213.

Finnegan, D.J., **1989**. Eucaryotic transposable elements and genome evolution. *Trends Genet. 5*, 103–107.

Fox, A.S., Duggleby, W.F., Gelbart, W.M., Yoon, S.B., **1970**. DNA-induced transformation in *Drosophila*: evidence for transmission without integration. *Proc. Natl Acad. Sci. USA 67*, 1834–1838.

Fox, A.S., Yoon, S.B., **1966**. Specific genetic effects of DNA in *Drosophila melanogaster. Genetics 53*, 897–911.

Fox, A.S., Yoon, S.B., **1970**. DNA-induced transformation in *Drosophila*: locus specificity and the establishment of transformed stocks. *Proc. Natl Acad. Sci. USA 67*, 1608–1615.

Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R.L., Savakis, C., **1994**. Mobile *Minos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. *Proc. Natl Acad. Sci. USA 91*, 4746–4750.

Franz, G., Savakis, C., **1991**. *Minos*, a new transposable element from *Drosophila hydei*, is a member of the Tc-1-like family of transposons. *Nucl. Acids Res. 19*, 6646.

Fraser, M.J., **2000**. The TTAA-specific family of transposable elements: identification, functional characterization, and utility for transformation of insects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 249–268.

Fraser, M.J., Brusca, J.S., Smith, G.E., Summers, M.D., **1985**. Transposon-mediated mutagenesis of a baculovirus. *Virology 145*, 356–361.

Fraser, M.J., Cary, L., Boonvisudhi, K., Wang, H.G., **1995**. Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. *Virology 211*, 397–407.

Fraser, M.J., Smith, G.E., Summers, M.D., **1983**. Acquisition of host-cell DNA sequences by baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. *J. Virol. 47*, 287–300.

Furlong, E.E., Profitt, D., Scott, M.P., **2001**. Automated sorting of live transgenic embryos. *Nature Biotechnol. 19*, 153–156.

Garza, D., Medhora, M., Koga, A., Hartl, D.L., **1991**. Introduction of the transposable element *mariner* into the germline of *Drosophila melanogaster. Genetics 128*, 303–310.

Germeraad, S., **1976**. Genetic transformation in *Drosophila* by microinjection of DNA. *Nature 262*, 229–231.

Goldberg, D.A., Posakony, J.W., Maniatis, T., **1983**. Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germ line. *Cell 34*, 59–73.

Golic, K.G., Golic, M.M., **1996**. Engineering the *Drosophila* genome: chromosome rearrangements by design. *Genetics 144*, 1693–1711.

Golic, K.G., Lindquist, S.L., **1989**. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell 59*, 499–509.

Golic, M.M., Rong, Y.S., Petersen, R.B., Lindquist, S.L., Golic, K.G., **1997**. FLP-mediated DNA mobilization to specific target sites in *Drosophila* chromosomes. *Nucl. Acids Res. 25*, 3665–3671.

Gomez, S.P., Handler, A.M., **1997**. A *Drosophila melanogaster hobo-white*$^+$ vector mediates low frequency gene transfer in *D. virilis* with full interspecific *white*$^+$ complementation. *Insect Mol. Biol. 6*, 1–8.

Gorbunova, V., Levy, A.A., **1997**. Circularized *Ac/Ds* transposons: formation, structure and fate. *Genetics 145*, 1161–1169.

Goryshin, I.Y., Jendrisak, J., Hoffman, L.M., Meis, R., Reznikoff, W.S., **2000**. Insertional transposon mutagenesis by electroporation of released *Tn5* transposition complexes. *Nature Biotechnol. 18*, 97–100.

Goryshin, I.Y., Reznikoff, W.S., **1998**. *Tn5 in vitro* transposition. *J. Biol. Chem. 273*, 7367–7374.

Green, M.M., **1996**. The "Genesis of the White-Eyed Mutant" in *Drosophila melanogaster*: a reappraisal. *Genetics 142*, 329–331.

Griffith, F., **1928**. Significance of pneumococcal types. *J. Hyg., Camb. 27*, 113–159.

Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O., *et al.*, **2001**. Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. *Insect Mol. Biol. 10*, 597–604.

Gueiros-Filho, F.J., Beverley, S.M., **1997**. Trans-kingdom transposition of the *Drosophila* element *mariner* within the protozoan *Leishmania*. *Science 276*, 1716–1719.

Guimond, N., Bideshi, D.K., Pinkerton, A.C., Atkinson, P.W., O'Brochta, D.A., **2003**. Patterns of *Hermes* transposition in *Drosophila melanogaster. Mol. Gen. Genet. 268*, 779–790.

Hama, C., Ali, Z., Kornberg, T.B., **1990**. Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. *Genes Devel. 4*, 1079–1093.

Handler, A.M., **2000**. An introduction to the history and methodology of insect gene transfer. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 3–26.

Handler, A.M., **2001**. A current perspective on insect gene transfer. *Insect Biochem. Mol. Biol. 31*, 111–128.

Handler, A.M., **2002a**. Use of the *piggyBac* transposon for germ-line transformation of insects. *Insect Biochem. Mol. Biol. 32*, 1211–1220.

Handler, A.M., **2002b**. Prospects for using genetic transformation for improved SIT and new biocontrol methods. *Genetica 116*, 137–149.

Handler, A.M., **2003**. Isolation and analysis of a new *hopper hAT* transposon from the *Bactrocera dorsalis* white eye strain. *Genetica 118*, 17–24.

Handler, A.M., Gomez, S.P., **1996**. The *hobo* transposable element excises and has related elements in tephritid species. *Genetics 143*, 1339–1347.

Handler, A.M., Gomez, S.P., **1997**. A new *hobo*, *Activator*, *Tam3* transposable element, *hopper*, from *Bactrocera dorsalis* is distantly related to *hobo* and *Ac*. *Gene 185*, 133–135.

Handler, A.M., Gomez, S.P., O'Brochta, D.A., **1993a**. A functional analysis of the *P*-element gene-transfer vector in insects. *Arch. Insect Biochem. Physiol. 22*, 373–384.

Handler, A.M., Gomez, S.P., O'Brochta, D.A., **1993b**. Negative regulation of *P* element excision by the somatic product and terminal sequences of *P* in *Drosophila melanogaster. Mol. Gen. Genet. 237*, 145–151.

Handler, A.M., Harrell, R.A., **1999**. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Mol. Biol. 8*, 449–458.

Handler, A.M., Harrell, R.A., **2000**. Transformation of the Caribbean fruit fly with a *piggyBac* transposon vector marked with polyubiquitin-regulated GFP. *Insect Biochem. Mol. Biol. 31*, 199–205.

Handler, A.M., Harrell, R.A., **2001**. Polyubiquitin-regulated DsRed marker for transgenic insects. *Biotechniques 31*, 820–828.

Handler, A.M., James, A.A. (Eds.), **2000**. Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL.

Handler, A.M., McCombs, S.D., **2000**. The *piggyBac* transposon mediates germ-line transformation in the Oriental fruit fly and closely related elements exist in its genome. *Insect Mol. Biol. 9*, 605–612.

Handler, A.M., McCombs, S.D., Fraser, M.J., Saul, S.H., **1998**. The lepidopteran transposon vector, *piggyBac*, mediates germline transformation in the Mediterranean fruitfly. *Proc. Natl Acad. Sci. USA 95*, 7520–7525.

Handler, A.M., O'Brochta, D.A., **1991**. Prospects for gene transformation in insects. *Annu. Rev. Entomol. 36*, 159–183.

Hartl, D.L., Lohe, A.R., Lozovskaya, E.R., **1997**. Modern thoughts on an ancyent marinere: function, evolution, regulation. *Annu. Rev. Genet. 31*, 337–358.

Haymer, D.S., Marsh, J.L., **1986**. Germ line and somatic instability of a *white* mutation in *Drosophila mauritiana* due to a transposable element. *Devel. Genet. 6*, 281–291.

Hazelrigg, T., Levis, R., Rubin, G.M., **1984**. Transformation of *white* locus DNA in *Drosophila*: dosage compensation, zeste interaction, and position effects. *Cell 64*, 1083–1092.

Hazelrigg, T., Liu, N., Hong, Y., Wang, S., **1998**. GFP expression in *Drosophila* tissues: time requirements for formation of a fluorescent product. *Devel. Biol. 199*, 245–249.

Hediger, M., Niessen, M., Wimmer, E.A., Dübendorfer, A., Bopp, D., **2000**. Genetic transformation of the housefly *Musca domestica* with the lepidopteran derived transposon *piggyBac*. *Insect Mol. Biol. 10*, 113–119.

Heinrich, J.C., Li, X., Henry, R.A., Haack, N., Stringfellow, L., *et al.*, **2002**. Germ-line transformation of the Australian sheep blowfly *Lucilia cuprina*. *Insect Mol. Biol. 11*, 1–10.

Higgs, S., Sinkins, D.L., **2000**. Green fluorescent protein (GFP) as a marker for transgenic insects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 93–108.

Higgs, S., Traul, D., Davis, B.S., Kamrud, K.I., Wilcox, C.L., *et al.*, **1996**. Green fluorescent protein expressed in living mosquitoes: without the requirement of transformation. *Biotechniques 21*, 660–664.

Hoess, R., Wierzbicki, A., Abremski, K., **1985**. Formation of small circular DNA molecules via an *in vitro* site-specific recombination system. *Gene 40*, 325–329.

Horard, B., Mange, A., Pelissier, B., Couble, P., **1994**. *Bombyx* gene promoter analysis in transplanted silk gland transformed by particle delivery system. *Insect Mol. Biol. 3*, 261–265.

Horn, C., Jaunich, B., Wimmer, E.A., **2000**. Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. *Devel. Genes Evol. 210*, 623–629.

Horn, C., Schmid, B., Pogoda, F.S., Wimmer, E.A., **2002**. Fluorescent transformation markers for insect transgenesis. *Insect Biochem. Mol. Biol. 32*, 1221–1235.

Horn, C., Wimmer, E.A., **2000**. A versatile vector set for animal transgenesis. *Devel. Genes Evol. 210*, 630–637.

Jacobson, J.W., Medhora, M.M., Hartl, D.L., **1986**. Molecular structure of somatically unstable transposable element in *Drosophila*. *Proc. Natl Acad. Sci. USA 83*, 8684–8688.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S., *et al.*, **1998**. Stable transposon mediated transformation of the yellow fever mosquito, *Aedes aegypti*, using the *Hermes* element from the housefly. *Proc. Natl Acad. Sci. USA 95*, 3743–3747.

Jasinskiene, N., Coates, C.J., James, A.A., **2000**. Structure of *Hermes* integrations in the germline of the yellow fever mosquito, *Aedes aegypti*. *Insect Mol. Biol. 9*, 11–18.

Kamdar, P., Von Allmen, G., Finnerty, V., **1992**. Transient expression of DNA in *Drosophila* via electroporation. *Nuc. Acids Res. 11*, 3526.

Kaminski, J.M., Huber, M.R., Summers, J.B., Ward, M.B., **2002**. Design of a nonviral vector for site-selective, efficient integration into the human genome. *FASEB J. 16*, 1242–1247.

Kapetanaki, M.G., Loukeris, T.G., Livadaras, I., Savakis, C., **2002**. High frequencies of *Minos* transposon mobilization are obtained in insects by using *in vitro*

synthesized mRNA as a source of transposase. *Nucl. Acids Res. 30*, 3333–3340.

Karess, R.E., **1985**. *P* element mediated germ line transformation of *Drosophila*. In: Glover, D.M. (Ed.), DNA Cloning, vol. 2. A Practical Approach. IRL Press, Oxford, pp. 121–141.

Karess, R.E., Rubin, G.R., **1984**. Analysis of *P* transposable element functions in *Drosophila*. *Cell 38*, 135–146.

Kassis, J.A., Noll, E., VanSickle, E.P., Odenwald, W.F., Perrimon, N., **1992**. Altering the insertional specificity of a *Drosophila* transposable element. *Proc. Natl Acad. Sci. USA 89*, 1919–1923.

Kaufman, P.K., Doll, R.F., Rio, D.C., **1989**. *Drosophila P* element transposase recognizes internal *P* element DNA sequences. *Cell 59*, 359–371.

Kidwell, M.G., Kidwell, J.F., Sved, J.A., **1977**. Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility, and male recombination. *Genetics 86*, 813–833.

Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C., **1987**. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature 327*, 70–73.

Klemenz, R., Weber, U., Gehring, W.J., **1987**. The white gene as a marker in a new *P*-element vector for gene transfer in *Drosophila*. *Nucl. Acids Res. 15*, 3947–3959.

Klinakis, A.G., Loukeris, T.G., Pavlopoulos, A., Savakis, C., **2000**. Mobility assays confirm the broad host-range activity of the *Minos* transposable element and validate new transformation tools. *Insect Mol. Biol. 9*, 269–276.

Kokoza, V., Ahmed, A., Wimmer, E.A., Raikhel, A.S., **2001**. Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the *piggyBac* transposable element vector pBac[3xP3–EGFP afm]. *Insect Biochem. Mol. Biol. 31*, 1137–1143.

Kravariti, L., Thomas, J., Sourmeli, S., Rodakis, G.C., Mauchamp, B., *et al.*, **2001**. The biolistic method as a tool for testing the differential activity of putative silkmoth chorion gene promoters. *Insect Biochem. Mol. Biol. 31*, 473–479.

Lampe, D.J., Akerley, B.J., Rubin, E.J., Mekalanos, J.J., Robertson, H.M., **1999**. Hyperactive transposase mutants of the *Himar1 mariner* transposon. *Proc. Natl Acad. Sci. USA 96*, 11428–11433.

Lampe, D.J., Churchill, M.E., Robertson, H.M., **1996**. A purified *mariner* transposase is sufficient to mediate transposition *in vitro*. *EMBO J. 15*, 5470–5479.

Lampe, D.J., Grant, T.E., Robertson, H.M., **1998**. Factors affecting transposition of the *Himar1 mariner* transposon *in vitro*. *Genetics 149*, 179–187.

Lampe, D.J., Walden, K.K.O., Sherwood, J.M., Robertson, H.M., **2000**. Genetic engineering of insects with *mariner* transposons. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 237–248.

Lee, H., Simon, J.A., Lis, J.T., **1988**. Structure and expression of ubiquitin genes of *Drosophila melanogaster. Mol. Cell. Biol. 8*, 4727–4735.

Leopold, R.A., Hughes, K.J., DeVault, J.D., **1996**. Using electroporation and a slot cuvette to deliver plasmid DNA to insect embryos. *Genet. Anal. 12*, 197–200.

Lerat, E., Brunet, F., Bazin, C., Capy, P., **1999**. Is the evolution of transposable elements modular? *Genetica 107*, 15–25.

Li, X., Heinrich, J.C., Scott, M.J., **2001a**. *piggyBac*-mediated transposition in *Drosophila melanogaster*: an evaluation of the use of constitutive promoters to control transposase gene expression. *Insect Mol. Biol. 10*, 447–456.

Li, X., Lobo, N., Bauser, C.A., Fraser, M.J., Jr., **2001b**. The minimum internal and external sequence requirements for transposition of the eukaryotic transformation vector *piggyBac*. *Mol. Genet. Genomics 266*, 190–198.

Lidholm, D.-A., Gudmundsson, G.H., Boman, H.G., **1991**. A highly repetitive, *mariner*-like element in the genome of *Hyalophora cecropia*. *J. Biol. Chem. 266*, 11518–11521.

Lidholm, D.-A., Lohe, A.R., Hartl, D.L., **1993**. The transposable element *mariner* mediates germline transformation in *Drosophila melanogaster. Genetics 134*, 859–868.

Lis, J.T., Simon, J.A., Sutton, C.A., **1983**. New heat shock puffs and β-galactosidase activity resulting from transformation of *Drosophila* with an *hsp70–LacZ* hybrid gene. *Cell 35*, 403–410.

Lobo, N.F., Hua-Van, A., Li, X., Nolen, B.M., Fraser, M.J., Jr., **2002**. Germ line transformation of the yellow fever mosquito, *Aedes aegypti*, mediated by transpositional insertion of a *piggyBac* vector. *Insect Mol. Biol. 11*, 133–913.

Lobo, N., Li, X., Hua-Van, A., Fraser, M.J., Jr., **2001**. Mobility of the *piggyBac* transposon in embryos of the vectors of Dengue fever (*Aedes albopictus*) and La Crosse encephalitis (*Ae. triseriatus*). *Mol. Genet. Genomics 265*, 66–71.

Lohe, A.R., Hartl, D.L., **1996a**. Germline transformation of *Drosophila virilis* with the transposable element *mariner. Genetics 143*, 365–374.

Lohe, A.R., Hartl, D.L., **1996b**. Autoregulation of *mariner* transposase activity by overproduction and dominant-negative complementation. *Mol. Biol. Evol. 13*, 549–555.

Lohe, A.R., Hartl, D.L., **2002**. Efficient mobilization of *mariner in vivo* requires multiple internal sequences. *Genetics 160*, 519–526.

Lohe, A.R., Lidholm, D.-A., Hartl, D.L., **1995**. Genotypic effects, maternal effects and grandmaternal effects of immobilized derivatives of the transposable element *mariner. Genetics 140*, 183–192.

Lorenzen, M.D., Berghammer, A.J., Brown, S.J., Denell, R.E., Klingler, M., *et al.*, **2003**. *piggyBac*-mediated germline transformation in the beetle *Tribolium castaneum*. *Insect Mol. Biol. 12*, 433–440.

Lorenzen, M.D., Brown, S.J., Denell, R.E., Beeman, R.W., **2002**. Cloning and characterization of the *Tribolium castaneum* eye-color genes encoding tryptophan

oxygenase and kynurenine 3-monooxygenase. *Genetics 160*, 225–234.

Loukeris, T.G., Arca, B., Livadras, I., Dialektaki, G., Savakis, C., **1995a**. Introduction of the transposable element *Minos* into the germ line of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 92*, 9485–9489.

Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S., Savakis, C., **1995b**. Gene transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science 270*, 2002–2005.

Lozovskaya, E.R., Nurminsky, D.I., Hartl, D.L., Sullivan, D.T., **1996**. Germline transformation of *Drosophila virilis* mediated by the transposable element *hobo*. *Genetics 142*, 173–177.

Lozovsky, E.R., Nurminsky, D., Wimmer, E.A., Hartl, D.L., **2002**. Unexpected stability of *mariner* transgenes in *Drosophila*. *Genetics 160*, 527–535.

Mamoun, C.B., Guzman, I.Y., Beverly, S.M., Goldberg, D.E., **2000**. Transposition of the *Drosophila* element *mariner* within the human malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol. 110*, 405–407.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., *et al.*, **1999**. Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nature Biotechnol. 17*, 969–973.

Matz, M.V., Lukyanov, K.A., Lukyanov, S.A., **2002**. Family of the green fluorescent protein: journey to the end of the rainbow. *BioEssays 24*, 953–959.

McGrane, V., Carlson, J.O., Miller, B.R., Beaty, B.J., **1988**. Microinjection of DNA into *Aedes triseriatus* ova and detection of integration. *Am. J. Trop. Med. Hyg. 39*, 502–510.

Medhora, M.M., MacPeek, A.H., Hartl, D.L., **1988**. Excision of the *Drosophila* transposable element *mariner*: identification and characterization of the *Mos* factor. *EMBO J. 7*, 2185–2189.

Miahle, E., Miller, L.H., **1994**. Biolistic techniques for transfection of mosquito embryos (*Anopheles gambiae*). *Biotechniques 16*, 924–931.

Michel, K., Atkinson, P.W., **2003**. Nuclear localization of the *Hermes* transposase depends on basic amino acid residues at the N-terminus of the protein. *J. Cell. Biochem. 89*, 778–790.

Michel, K.A., O'Brochta,, D.A., Atkinson, P.W., **2002**. Does the proposed DSE motif form the active center in the *Hermes* transposase? *Gene 298*, 141–146.

Michel, K., O'Brochta, D.A., Atkinson, P.W., **2003**. The C-terminus of the *Hermes* transposase contains a protein multimerization domain. *Insect Biochem. Mol. Biol. 33*, 959–970.

Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S., *et al.*, **2001**. *Hermes*-mediated germline transformation of the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol. Biol. 10*, 155–162.

Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P., *et al.*, **1987**. Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. *Science 237*, 779–781.

Morris, A.C., **1997**. Microinjection of mosquito embryos. In: Crampton, J.M., Beard, C.B., Louis, C. (Eds.), Molecular Biology of Insect Disease Vectors: A Methods Manual. Chapman and Hall, London, pp. 423–429.

Morris, A.C., Eggleston, P., Crampton, J.M., **1989**. Genetic transformation of the mosquito *Aedes aegypti* by micro-injection of DNA. *Med. Vet. Entomol. 3*, 1–7.

Mullins, M.C., Rio, D.C., Rubin, G.M., **1989**. *Cis*-acting DNA sequence requirements for *P*-element transposition. *Cell 3*, 729–738.

Nawa, S., Sakaguchi, B., Yamada, M.A., Tsujita, M., **1971**. Hereditary change in *Bombyx* after treatment with DNA. *Genetics 67*, 221–234.

Nawa, S., Yamada, S., **1968**. Hereditary change in *Ephestia* after treatment with DNA. *Genetics 58*, 573–584.

Nolan, T., Bower, T.M., Brown, A.E., Crisanti, A., Catteruccia, F., **2002**. *piggyBac*-mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. *J. Biol. Chem. 277*, 8759–8762.

O'Brochta, D.A., Atkinson, P.W., Lehane, M.J., **2000**. Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector. *Insect Mol. Biol. 9*, 531–538.

O'Brochta, D.A., Gomez, S.P., Handler, A.M., **1991**. *P* element excision in *Drosophila melanogaster* and related drosophilids. *Mol. Gen. Genet. 225*, 387–394.

O'Brochta, D.A., Handler, A.M., **1988**. Mobility of *P* elements in drosophilids and nondrosophilids. *Proc. Natl Acad. Sci. USA 85*, 6052–6056.

O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., **1994**. Interplasmid transposition of *Drosophila hobo* elements in non-drosophilid insects. *Mol. Gen. Genet. 244*, 9–14.

O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., **1996**. *Hermes*, a functional non-drosophilid insect gene vector. *Genetics 142*, 907–914.

O'Hare, K., Rubin, G.M., **1983**. Structures of *P* transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. *Cell 34*, 25–35.

Papadimitriou, E., Kritikou, D., Mavroidis, M., Zacharopoulou, A., Mintzas, A.C., **1998**. The heat shock 70 gene family in the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol. Biol. 7*, 279–290.

Peloquin, J.J., Thibault, S.T., Schouest, L.P., Jr., Miller, T.A., **1997**. Electromechanical microinjection of pink bollworm *Pectinophora gossypiella* embryos increases survival. *Biotechniques 22*, 496–499.

Peloquin, J.J., Thibault, S.T., Staten, R., Miller, T.A., **2000**. Germ-line transformation of pink bollworm (Lepidoptera: Gelechiidae) mediated by the *piggyBac* transposable element. *Insect Mol. Biol. 9*, 323–333.

Perera, O.P., Harrell, R.A., Handler, A.M., **2002**. Germline transformation of the South American malaria vector, *Anopheles albimanus*, with a *piggyBac/EGFP* transposon vector is routine and highly efficient. *Insect Mol. Biol. 11*, 291–297.

Phillips, J.P., Xin, J.H., Kirby, K., Milne, C.P., Krell, P., *et al.*, **1990**. Transfer and expression of an

organophosphate insecticide degrading gene from *Pseudomonas* in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 87*, 8155–8159.

Pinkerton, A.C., Michel, K., O'Brochta, D.A., Atkinson, P.W., **2000**. Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. *Insect Mol. Biol. 9*, 1–10.

Pinkerton, A.C., Whyard, S., Mende, H.M., Coates, C.J., O'Brochta, D.A., *et al.*, **1999**. The Queensland fruit fly, *Bactrocera tryoni*, contains multiple members of the *hAT* family of transposable elements. *Insect Mol. Biol. 8*, 423–434.

Pirrotta, V., **1988**. Vectors for *P*-mediated transformation in *Drosophila*. In: Rodriguez, R.L., Denhardt, D.T. (Eds.), Vectors: A Survey of Molecular Cloning Vectors and their Uses. Butterworth, Oxford, pp. 437–456.

Pirrotta, V., Steller, H., Bozzetti, M.P., **1985**. Multiple upstream regulatory elements control the expression of the *Drosophila white* gene. *EMBO J. 4*, 3501–3508.

Plautz, J.D., Day, R.N., Dailey, G.M., Welsh, S.B., Hall, J.C., *et al.*, **1996**. Green fluorescent protein and its derivatives as versatile markers for gene expression in living *Drosophila melanogaster*, plant and mammalian cells. *Gene 173*, 83–87.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J., **1992**. Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene 111*, 229–233.

Presnail, J.K., Hoy, M.A., **1994**. Transmission of injected DNA sequences to multiple eggs of *Metaseiulus occidentalis* and *Amblyseius finlandicus* (Acari: Phytoseiidae) following maternal microinjection. *Exp. Appl. Acarol. 18*, 319–330.

Reznikoff, W.S., **2000**. *Tn5* transposition. In: Craig, N.L., Craige, R., Gellert, M., Lambowitz, A.M. (Eds.), Mobile DNA II. American Society for Microbiology, Washington, DC, pp. 403–422.

Reznikoff, W.S., Bhasin, A., Davies, D.R., Goryshin, I.Y., Mahnke, L.A., *et al.*, **1999**. *Tn5*: a molecular window on transposition. *Biochem. Biophys. Res. Commun. 266*, 729–734.

Rio, D.C., Laski, F.A., Rubin, G.M., **1986**. Identification and immunochemical analysis of biologically active *Drosophila P* element transposase. *Cell 44*, 21–32.

Rio, D.C., Rubin, G.M., **1988**. Identification and purification of a *Drosophila* protein that binds to the terminal 31-base-pair inverted repeats of the transposable element *P*. *Proc. Natl Acad. Sci. USA 85*, 8929–8933.

Robertson, H.M., **1993**. The *mariner* transposable element is widespread in insects. *Nature 362*, 241–245.

Robertson, H.M., **2000**. Evolution of DNA transposons in eukaryotes. In: Craig, N.L., Craige, R., Gellert, M., Lambowitz, A.M. (Eds.), Mobile DNA II. American Society for Microbiology, Washington, DC, pp. 1093–1110.

Robertson, H.M., Lampe, D.J., **1995a**. Distribution of transposable elements in arthropods. *Annu. Rev. Entomol. 40*, 333–357.

Robertson, H.M., Lampe, D.J., **1995b**. Recent horizontal transfer of a *mariner* element between Diptera and Neuroptera. *Mol. Biol. Evol. 12*, 850–862.

Robertson, H.M., MacLeod, E.G., **1993**. Five major subfamilies of *mariner* transposable elements in insects, including the Mediterranean fruit fly, and related arthropods. *Insect Mol. Biol. 2*, 125–139.

Robertson, H.M., Zumpano, K.L., **1997**. Molecular evolution of an ancient *mariner* transposon, *Hsmar1*, in the human genome. *Gene 205*, 203–217.

Rong, Y.S., Golic, K.G., **2000**. Gene targeting by homologous recombination in *Drosophila*. *Science 288*, 2013–2018.

Rowan, K., Orsetti, J., Atkinson, P.W., O'Brochta, D.A., **2004**. *Tn5* as an insect gene vector. *Insect Biochem. Molec. Biol. 34*, 695–705.

Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., *et al.*, **1999**. *In vivo* transposition of *mariner*-based elements in enteric bacteria and mycobacteria. *Proc. Natl Acad. Sci. USA 96*, 1645–1650.

Rubin, G.M., Kidwell, M.G., Bingham, P.M., **1982**. The molecular basis of *P-M* hybrid dysgenesis: the nature of induced mutations. *Cell 29*, 987–994.

Rubin, G.M., Spradling, A.C., **1982**. Genetic transformation of *Drosophila* with transposable element vectors. *Science 218*, 348–353.

Sarkar, A., Coates, C.J., Whyard, S., Willhoeft, U., Atkinson, P.W., *et al.*, **1997a**. The *Hermes* element from *Musca domestica* can transpose in four families of cyclorrhaphan flies. *Genetica 99*, 15–29.

Sarkar, A., Collins, F.C., **2000**. Eye color genes for selection of transgenicinsects. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 79–91.

Sarkar, A., Yardley, K., Atkinson, P.W., James, A.A., O'Brochta, D.A., **1997b**. Transposition of the *Hermes* element in embryos of the vector mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol. 27*, 359–363.

Saville, K.J., Warren, W.D., Atkinson, P.W., O'Brochta, D.A., **1999**. Integration specificity of the *hobo* element of *Drosophila melanogaster* is dependent on sequences flanking the target site. *Genetica 105*, 133–147.

Scavarda, N.J., Hartl, D.L., **1984**. Interspecific DNA transformation in *Drosophila*. *Proc. Natl Acad. Sci. USA 81*, 7515–7519.

Scholnick, S.B., Morgan, B.A., Hirsh, J., **1983**. The cloned Dopa decarboxylase gene is developmentally regulated when reintegrated into the *Drosophila* genome. *Cell 34*, 37–45.

Senecoff, J.F., Bruckner, R.C., Cox, M.M., **1985**. The FLP recombinase of the yeast 2-micron plasmid: characterization of its recombination site. *Proc. Natl Acad. Sci. USA 82*, 7270–7274.

Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D.S., Desplan, C., **1997**. Direct regulation of rhodopsin 1 by *Pax-6/eyeless* in *Drosophila*: evidence for a conserved function in photoreceptors. *Genes Devel. 11*, 1122–1131.

Sherman, A., Dawson, A., Mather, C., Gilhooley, H., Li, Y., *et al.*, **1998**. Transposition of the *Drosophila* element *mariner* into the chicken germ line. *Nature Biotechnol. 16*, 1050–1053.

Siegal, M.L., Hartl, D.L., **1996**. Transgene coplacement and high efficiency site-specific recombination with the *Cre/loxP* system in *Drosophila*. *Genetics 144*, 715–726.

Simmons, M.J., Haley, K.J., Grimes, C.D., Raymond, J.D., Niemi, J.B., **2002**. A *hobo* transgene that encodes the *P*-element transposase in *Drosophila melanogaster*: autoregulation and cytotype control of transposase activity. *Genetics 161*, 195–204.

Spradling, A.C., **1986**. *P* element-mediated transformation. In: Roberts, D.B. (Ed.), *Drosophila*: A Practical Approach. Oxford, IRL Press, pp. 174–197.

Steller, H., Pirrotta, V., **1985**. A transposable *P* vector that confers selectable G418 resistance to *Drosophila* larvae. *EMBO J. 4*, 167–171.

Steller, H., Pirrotta, V., **1986**. *P* transposons controlled by the heat shock promoter. *Mol. Cell Biol. 6*, 1640–1649.

Sumitani, M., Yamamoto, D.S., Oishi, K., Lee, J.M., Hatakeyama, M., **2003**. Germline transformation of the sawfly, *Athalia rosae* (Hymenoptera: Symphyta), mediated by a *piggyBac*-derived vector. *Insect Biochem. Mol. Biol. 33*, 449–458.

Sundararajan, P., Atkinson, P.W., O'Brochta, D.A., **1999**. Transposable element interactions in insects: crossmobilization of *hobo* and *Hermes*. *Insect Mol. Biol. 8*, 359–368.

Sundraresan, V., Freeling, M., **1987**. An extrachromosomal form of the *Mu* transposon of maize. *Proc. Natl Acad. Sci. USA 84*, 4924–4928.

Taillebourg, E., Dura, J.M., **1999**. A novel mechanism for *P* element homing in *Drosophila*. *Proc. Natl Acad. Sci. USA 96*, 6856–6861.

Tamura, T., Thibert, T., Royer, C., Kanda, T., Eappen, A., *et al.*, **2000**. A *piggyBac* element-derived vector efficiently promotes germ-line transformation in the silkworm *Bombyx mori* L. *Nature Biotechnol. 18*, 81–84.

Thibault, S.T., Luu, H.T., Vann, N., Miller, T.A., **1999**. Precise excision and transposition of *piggyBac* in pink bollworm embryos. *Insect Mol. Biol. 8*, 119–123.

Thomas, J.L., Bardou, J., L'hoste, S., Mauchamp, B., Chavancy, G., **2001**. A helium burst biolistic device adapted to penetrate fragile insect tissues. *J. Insect Sci. 1*, 9.

Thomas, J.L., Da Rocha, M., Besse, A., Mauchamp, B., Chavancy, G., **2002**. 3xP3–EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards. *Insect Biochem. Mol. Biol. 32*, 247–253.

Tosi, L.R.O., Beverly, S.M., **2000**. *Cis* and *trans* factors affecting *Mos1 mariner* evolution and transposition *in vitro*, and it potential for functional genomics. *Nucl. Acids Res. 28*, 784–790.

Uhlirova, M., Asahina, M., Riddiford, L.M., Jindra, M., **2002**. Heat-inducible transgenic expression in the silkmoth *Bombyx mori*. *Devel. Genes Evol. 212*, 145–151.

Walker, V.K., **1990**. Gene transfer in insects. *Adv. Cell Culture 7*, 87–124.

Wang, H.H., Fraser, M.J., **1993**. TTAA serves as the target site for TFP3 lepidopteran transposon insertions in both nuclear polyhedrosis virus and *Trichoplusia ni* genomes. *Insect Mol. Biol 1*, 1–7.

Wang, H.H., Fraser, M.J., Cary, L.C., **1989**. Transposon mutagenesis of baculoviruses: analysis of TFP3 lepidopteran insertions at the FP locus of nuclear polyhedrosis viruses. *Gene 81*, 97–108.

Wang, W., Swevers, L., Iatrou, K., **2000**. *Mariner* (*Mos1*) transposase and genomic integration of foreign gene sequences in *Bombyx mori* cells. *Insect Mol. Biol. 9*, 145–155.

Warren, W.D., Atkinson, P.W., O'Brochta, D.A., **1994**. The *Hermes* transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the *hobo*, *Ac*, and *Tam*3 (*hAT*) element family. *Genet. Res. 64*, 87–97.

White, L.D., Coates, C.J., Atkinson, P.W., O'Brochta, D.A., **1996**. An eye color gene for the detection of transgenic non-drosophilid insects. *Insect Biochem. Mol. Biol. 26*, 641–644.

Wilson, C., Pearson, R.K., Bellen, H.J., O'Kane, C.J., Grossniklaus, U., *et al.*, **1989**. *P*-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Devel. 3*, 1301–1313.

Wilson, R., Orsetti, J., Klocko, A.D., Aluvihare, C., Peckham, E., *et al.*, **2003**. Post-integration behavior of a *Mos1* gene vector in *Aedes aegypti*. *Insect Biochem. Mol. Biol. 33*, 853–863.

Yamao, M., Katayama, N., Nakazawa, H., Yamakawa, M., Hayashi, Y., *et al.*, **1999**. Gene targeting in the silkworm by use of a baculovirus. *Genes Devel. 13*, 511–516.

Zhang, J.K., Pritchett, M.S., Lampe, D.J., Robertson, H.M., Metcalf, W.W., **2000**. *In vivo* transposon mutagenesis of the methanogenic archeon *Methanosarcina acetivorans C2A* using a modified version of the insect *mariner*-family transposable element *Himar1*. *Proc. Natl Acad. Sci. USA 97*, 9665–9670.

Zhang, H., Shinmyo, Y., Hirose, A., Mito, T., Inoue, Y., *et al.*, **2002**. Extrachromosomal transposition of the transposable element *Minos* in embryos of the cricket *Gryllus bimaculatus*. *Devel. Growth Differ. 44*, 409–417.

Zhang, L., Sankar, U., Lampe, D.J., Robertson, H.M., Graham, F.L., **1998**. The *Himar1 mariner* transposase cloned in a recombinant adenovirus vector is functional in mammalian cells. *Nucl. Acids Res. 226*, 3687–3693.

Zwiebel, L.J., Saccone, G., Zacharapoulou, A., Besansky, N.J., Favia, G., *et al.*, **1995**. The *white* gene of *Ceratitis capitata*: a phenotypic marker for germline transformation. *Science 270*, 2005–2008.

4.14 Insect Cell Culture and Recombinant Protein Expression Systems

P J Farrell, The University of Calgary, Canada
L Swevers and K Iatrou, Institute of Biology, National Centre for Scientific Research ''Demokritos'', Athens, Greece

4.14.1. Introduction

In the era of genomics, proteomics, and biotechnology, protein expression systems play key roles. For example, characterization of a gene now requires overexpression of the encoded protein by heterologous expression systems to realize its functional and structural characterization. Furthermore, production of proteins for therapeutic purposes is dependent on the use of host organisms that are genetically engineered for protein production at high levels.

Several host organisms have been used in recent years for recombinant protein expression purposes. These include bacteria, yeast, mammals, and insects, as well as cell lines derived from various mammalian and insect species. Each of these expression systems has unique advantages and deficiencies and, as a result, is more suitable than the others for specific applications while not as good for others.

Bacterial expression systems, for example, can direct very high levels of protein expression but the recombinant proteins generally lack the posttranslational modifications that take place in eukaryotes. Thus, for eukaryotic proteins that require appropriate modifications for functionality, bacterial expression systems are considered inappropriate. Bacteria also do not have eukaryotic-type secretion systems and normally secreted heterologous proteins usually accumulate in the bacterial cytoplasm in the form of insoluble aggregates, known as "inclusion bodies," which make the purification of functional proteins extremely difficult.

Yeast-based expression systems can normally yield high levels of recombinant proteins. However, the expression levels for particular classes of proteins such as secreted or membrane-anchored proteins are, in general, much more limited than those for intracellular proteins. Moreover, the patterns of posttranslational modifications including glycosylation are more limited in yeast than in higher eukaryotes and this limitation often affects biological activity.

Transformed mammalian cells are considered to represent excellent means for expression of membrane-anchored and secreted proteins. However,

mammalian cells need to be maintained in expensive media supplemented with CO_2 under carefully controlled conditions. Most importantly, mammalian cell lines are also potential sources of harmful pathogens, which can limit their utility for production of proteins of pharmaceutical value. Finally, mammalian systems are labor intensive because it requires several months for the generation of clones overexpressing stably recombinant proteins.

Insect cell-based expression systems, which can be used for *in vitro* production of virtually all types of heterologous proteins, accumulate most of the advantages of the other expression systems while lacking most of their disadvantages. Insect cell-derived expression systems are currently divided into two types, (1) the baculovirus expression system, which employs insect cell lines as hosts for baculovirus expression vectors and recombinant protein production in batch mode, and (2) transformed insect cell lines that overexpress continuously proteins from nonlytic plasmid-based expression vectors.

Following the development of the Baculovirus Expression System (BES) in 1983, insect cells have been employed extensively for the production of a wide variety of recombinant proteins using various versions of baculovirus-based expression vectors, and excellent results have been obtained for various research and industrial applications. As a result, the baculovirus/insect cell expression system is currently considered as the industry workhorse for recombinant protein production. As explained below, however, an important limitation of this system relates to the production of secreted proteins and proteins that require extensive posttranslational modifications. Furthermore, the baculovirus expression system is a transient production system because host cells are lysed and killed during each infection cycle.

More recently, a series of baculovirus-free expression vector systems have also emerged, which direct high-level recombinant protein production in insect cell lines, either simply transfected or stably transformed with relevant expression plasmid DNAs, and circumvent the disadvantages of the baculovirus expression system. These insect cell-based expression systems can be divided into two subgroups, one employing dipteran insect cell lines and another that utilizes lepidopteran insect cell lines. For the latter, the incorporation of genetic elements obtained from baculoviruses in the context of plasmid vectors has allowed the generation of extremely powerful expression vectors that direct the generation of large quantities of recombinant proteins. In general, continuous insect cell-based expression systems show outstanding features for the production of recombinant proteins, which, among others, include:

1. A fast process from cDNA cloning to production of recombinant protein – stable cell lines expressing recombinant protein are developed within 1–2 months.
2. High yields of secreted recombinant proteins that may surpass those achieved by the baculovirus system and mammalian cell systems.
3. Utilization of cell lines derived from a multitude of insect species.
4. Easy purification of secreted proteins from serum-free media.
5. Full capacity of posttranslational modifications – the cells maintain their integrity throughout the production process.
6. Continuous protein production – the transgene is stably integrated into the genome of the cells and protein production occurs continuously because no lysis occurs.

Irrespective of whether it is associated with the use of baculovirus or plasmid expression vectors, the use of insect cells can overcome the shortcomings of other established protein expression workhorses. Among others, the advantages of insect cells over other expression systems include the capacity to produce higher levels of soluble recombinant proteins that are folded correctly and extensively modified posttranslationally, the fact that both baculoviruses and insect cell lines are nonpathogenic to humans, and that insect cells can be grown in medium free of protein or other, potentially pathogenic, animal products.

This article is intended as a practical review of the developments in both baculovirus and plasmid-based protein expression systems employing insect cells. New applications of insect cells are also discussed including surface display and high-throughput screening for the identification of bioactive compounds. For related information on this topic, particularly in relation to applications related to baculoviruses and expression systems derived from them, the reader is also referred to **Chapters 6.8** and **6.9**.

4.14.2. Baculovirus Expression Systems

4.14.2.1. Brief History of the Baculovirus Expression System

Insect cells have certainly gained most of their popularity as hosts for production of foreign proteins using recombinant baculoviruses as expression vectors. For this application, the Open Reading Frames (ORFs) of heterologous genes substitute for ORFs of nonessential genes of a baculovirus

or heterologous ORFs are placed under the control of either viral promoters or nonviral promoters inserted into "neutral" sites of the baculovirus genome. Following infection of cultured insect cells (or larvae) with the recombinant baculovirus, the heterologous gene is expressed. By taking advantage of the several powerful promoters of nonessential genes, the levels and quality of the recombinant protein obtained from insect cells infected with the relevant recombinant baculoviruses are frequently superior to those obtained from other expression systems.

The original description of a recombinant baculovirus for foreign protein expression appeared in a series of articles from Max Summer's laboratory at Texas A&M University in 1983. Their model baculovirus system was the *Autographa californica* nucleopolyhedrosis virus (AcNPV), a member of the nuclear polyhedrosis virus (NPV) subfamily of baculoviruses that have the unique characteristic of producing large proteinaceous occlusion bodies (OBs) during the very late stages of infection. OBs constitute over 25% of the total protein mass within an infected cell (Smith *et al*., 1983a) and serve to protect the mature virions embedded within them from the damaging effects of the natural environment. OBs are mostly composed of a single protein, polyhedrin, whose gene was initially cloned from AcNPV (Smith *et al*., 1983b). Deletions were introduced in the cloned polyhedrin gene in plasmids, and several mutant AcNPVs with an OB negative (OB^-) phenotype were generated by homologous recombination following cotransfection of insect cells with wild-type AcNPV DNA and the plasmid deletions (Smith *et al*., 1983c). Although lacking the ability to synthesize the complete polyhedrin protein, OB^- AcNPVs were still able to infect cultured cells and produce progeny virus. Furthermore, the polyhedrin promoter was shown to remain as active in OB^- AcNPVs and to produce various forms of truncated polyhedrin protein at levels similar to those of the full length polyhedrin protein expressed by the wild-type AcNPV.

Having established that the highly expressed polyhedrin protein was not essential for the survival and propagation of AcNPV, the researchers at Texas A&M constructed various transfer vectors for inserting foreign genes into different positions of the polyhedrin gene region of the AcNPV genome by homologous recombination. Using human beta interferon (IFN-β) as a secreted reporter protein, OB^- recombinant viruses were obtained that expressed either chimeric polyhedrin-interferon proteins or authentic IFN-β under the control of the polyhedrin promoter. The recombinant IFN-β was secreted (with concomitant removal of its signal peptide), glycosylated and biologically active, and was expressed at levels up to 10 μg per ml or 100- to 500-fold higher than what was possible at that time using transformed mouse or bacterial expression systems, respectively (Smith *et al*., 1983b).

The successful expression of IFN-β marked the start of the baculovirus/insect cell protein expression system. Other reports of high-level recombinant protein expression followed soon afterwards, which included the production of a fusion between polyhedrin and *Escherichia coli* β-galactosidase (Pennock *et al*., 1984), human c-Myc (Myamoto *et al*., 1985), and human interleukin-2 (Smith *et al*., 1985).

4.14.2.2. Baculovirus Life Cycle

The baculovirus life cycle has been described in detail elsewhere (O'Reilly *et al*., 1992; Vialard *et al*., 1995; see **Chapter 6.8**).

Members of the *Baculoviridae* family are characterized by a rod-shaped enveloped virion and a circular, double-stranded and covalently closed DNA genome of 60–160 kbp (Mathews, 1982). They infect mainly insects, are not infectious to vertebrates, and are classified under two subfamilies: NPVs and granulosis viruses (GVs), which are members of the subfamily of *Eubaculovirinae* which comprises OB-forming viruses, while under the family of *Nudilibaculovirinae* are classified nonoccluded baculoviruses. Most baculoviruses have a very narrow host range, with the majority of the *Eubaculovirinae* infecting lepidopteran insects. NPVs are also the main constituents of expression vectors, with those species receiving the most attention being AcNPV, first isolated from larvae of the alfalfa looper *A. californica*, and the nuclear polyhedrosis virus of the silkworm *Bombyx mori* (BmNPV).

After an insect dies from an NPV infection, viral OBs, which serve to protect the virions embedded within them from the damaging effects in the natural environment such as UV radiation, are released from the carcass. When these OBs are ingested by an uninfected feeding insect, they are dissolved in the midgut (polyhedrin is solubilized due to the alkaline pH of the gut lumen), and the occluded virions are freed. Following fusion of the virions with midgut epithelial cells, the nucleocapsids migrate to the cell nuclei, where the genome is uncoated and transcription begins. A temporally regulated gene expression cascade ensues, that directs replication and formation of new virions (**Figure 1**).

Two forms of viruses are produced at different phases of the infection cycle: initially nucleocapsids are produced in the nucleus and enveloped upon

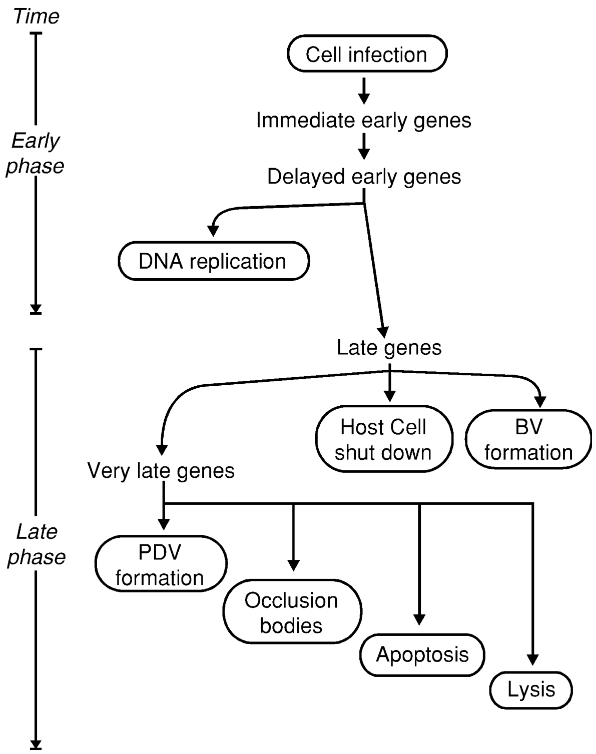


Figure 1 A simplified view of the viral gene expression cascade following baculovirus infection of an insect cell. The cascade is divided into two temporal halves, the first half terminating upon completion of DNA replication of the baculovirus genome, and the second one leading to the production of both budded virions (BVs) and polyhedra-derived virions (PDVs). The infection process ends with the lysis of the host cell.

budding from the cell's plasma membrane. The envelopes of these budded virions (BVs) are also distinguished by the presence of gp64 (also known as gp67), a baculovirus glycoprotein essential for BV entry into the cells by endocytosis (Whitford *et al.*, 1989; Monsma *et al.*, 1996). BVs are responsible for the systemic spread of the infection (secondary infection of neighboring cells or other tissues via the tracheal system of the infected insect). Later in the infection cycle, a second type of virion is generated. These are enclosed, either individually or in association with others, in an envelope of nuclear origin, prior to their incorporation into OBs. This second form, termed polyhedra-derived virions (PDVs), are distinguished from BVs by the association of several viral proteins, including gp41 (Whitford and Faulkner, 1992), p25 (Russell and Rohrmann, 1993), and p74, which is essential for PDV infectivity (Kuzio *et al.*, 1989), with the PDV envelope.

The baculovirus infection of insect cells derived from larval tissues and cultured *in vitro* is similar to that occurring in nature: susceptible cultured cells must be derived from susceptible insect species. OBs, appearing as obvious crystal structures under a simple light microscope, form in the nuclei and are released from lysed cells. Due to the nonalkaline nature of the insect cell culture medium (~pH 6.2), OBs remain insoluble and the PDVs are trapped within them. Thus, the infection is only spread to other cultured cells by BVs. PDVs extracted from OBs can also be used for the primary infection of cultured cells, albeit with significantly lower efficiency than BVs (Volkman and Summers, 1977).

The transcription of the 150 or so baculovirus genes following host cell infection is temporally regulated in four phases (**Figure 1**): immediate early and early genes are transcribed prior to viral DNA replication; late and very late genes are transcribed after replication. Immediate early gene promoters are activated by host factors immediately following uncoating, while delayed early, late, and very late gene promoters require the availability of viral transcription factors, synthesized during the previous phases, for transcriptional activation in the gene expression cascade. Approximately 20 activators of transcription have been identified in AcNPV including IE-1 (Guarino and Summers, 1986), IE-2 (or IE-N; Carson *et al.*, 1988), several late expression factors (LEFs; Lu and Miller, 1995; Rapp *et al.*, 1998) and one very late factor, VLF-1, that is necessary for transcription from the polyhedrin promoter (Yang and Miller, 1999) (for additional details, see **Chapter 6.8**).

4.14.2.3. Early Vectors and Techniques for Generating Recombinant Baculoviruses

Due to the large size of the baculovirus genome, it is difficult to routinely manipulate the baculovirus DNA for ligation of foreign genes directly into its genome. Until recently, the most common approach was to first clone the foreign gene into a plasmid "transfer vector," which is easy to manipulate using regular molecular biology techniques. The purpose of the transfer vector is to target an insertion site in the baculovirus genome for homologous recombination (via double-crossover). Thus, a generic transfer vector contains (1) a promoter, (2) a multiple cloning site for insertion of a heterologous ORF, (3) a poly(A) addition site, and (4) a few kilobase pairs of DNA sequence matching the destination locus in the baculovirus genome flanking elements 1–3. Usually, the destination locus is the polyhedrin region, because the polyhedrin gene promoter is very powerful and the polyhedrin gene itself is nonessential for the virus life cycle *in vitro*. Furthermore, the OB^- phenotype of recombinant viruses facilitates their selection. Most transfer vectors are designed

to simply swap, by homologous recombination, the polyhedrin ORF in the baculovirus genome with a foreign gene, while conserving the polyhedrin promoter and transcription termination [(poly(A) addition site)] regions. Various permutations of this basic approach have also been used (see below).

After the insertion of a foreign gene into the transfer vector is accomplished, the recombinant transfer vector is cotransfected with wild-type baculovirus DNA into insect cells. Within the nucleus, a relatively low frequency genetic exchange, by homologous recombination, occurs between the sequences of the transfer plasmid and the wild-type baculovirus DNA, and a small fraction of recombinant virus is generated. As an alternative to a transfer vector, PCR products generated with primers containing only 50 nucleotides of flanking DNA have also been used successfully, although the frequency of homologous recombination was significantly lower (Gritsun *et al*., 1997). The recombinant viruses can be discriminated visually and isolated from wild-type ones in virus plaque purification assays either based on their OB^{-} phenotype (for the cases of poyhedrin ORF disruption) or by virtue of other visual or molecular phenotypes expressed by them (in the cases where the polyhedrin gene is not disrupted). Another widely used technique for isolating a recombinant virus employs end-point dilution cloning and identity confirmation by PCR, dot-blot hybridization, immunoblotting or activity assays. Once isolated, the recombinant virus is amplified by one or more infection cycles in insect cells. At this point the virus stock is ready for recombinant protein expression. The whole process of preparation of the recombinant transfer vector, cotransfection, purification, amplification, and verification of protein expression usually takes 4 to 6 weeks to complete.

4.14.2.3.1. Polyhedrin promoter As has been already mentioned above, the polyhedrin promoter is by far the most frequently exploited promoter for recombinant protein expression using baculovirus vectors. Initially, transfer vector pAc373 (Smith *et al*., 1985) was used to target the AcNPV polyhedrin gene locus for foreign gene expression. This vector contained a deletion of approximately 182 bp between nucleotides −9 and +175 relative to the polyhedrin ATG translation start site. However, the early studies on the use of this specific and other related transfer vectors revealed that recombinant protein expression was less efficient when the transfer vectors encompassed disruptions in (1) the immediate vicinity of the normal polyhedrin translation start ATG codon, both in the 5′-UTR (Matsuura *et al*., 1987; Possee and Howard, 1987) and the polyhedrin N-terminal region (Luckow and Summers, 1988, 1989); (2) the 15–20 nt upstream of the polyhedrin mRNA transcription initiation site (Possee and Howard, 1987; Rankin *et al*., 1988); and, especially (3) the polyhedrin TAAG transcription initiation site itself (Possee and Howard, 1987; Rankin *et al*., 1988). In addition, it was quickly discovered that the polyhedrin sequences downstream of the poly(A) addition site cannot be deleted extensively due to the presence of an ORF necessary for AcNPV replication (Possee *et al*., 1991). To maximize foreign gene expression from the polyhedrin promoter, improved transfer vectors, such as pAcYM1 (Page, 1989) or pVL941 (Luckow and Summers, 1989), were designed subsequently, which targeted the polyhedrin locus while preserving, through point mutations (ATG to ATC or ATT), the overall context of the region surrounding the normal polyhedrin translation start site.

4.14.2.3.2. *p10* promoter The *p10* gene promoter, another powerful very late phase promoter, was also harnessed as an alternative to the polyhedrin promoter (Vlak *et al*., 1988). P10 is a 10 kDa protein of ambiguous function, probably involved in lysis (van Oers *et al*., 1993) or occlusion body formation (Williams *et al*., 1989), that is not essential for the production of either budded or extracellular virus (Vlak *et al*., 1988), at least *in vitro*. Due to the lack of a selectable phenotype from a recombinant virus targeting the *p10* locus, the β-galactosidase reporter protein was used to identify recombinant viruses. In a report related to comparative aspects of protein production using baculovirus-based vectors, the expression of β-galactosidase and a secreted reporter protein, juvenile hormone esterase (JHE), from recombinant AcNPVs using the *p10* gene promoter were found to be higher and 8 h earlier than that obtained with the polyhedrin promoter (Bonning *et al*., 1994), while work from another group suggested comparable levels of expression (DiFalco *et al*., 1997).

4.14.2.3.3. Basic protein promoter The basic protein is a late gene product associated with the viral DNA within the nucleocapsid. The harnessing of this promoter allows the expression of foreign genes at earlier times than those using the very late phase promoters of the polyhedrin and *p10* genes. As the presence of the basic protein gene was suspected to be essential for viral replication, its deletion was not expected to yield viable recombinant virus. For this reason, its promoter was duplicated and inserted into the polyhedrin gene locus (Hill-Perkins and

Possee, 1990). The promoter duplication was not reported to have an effect on virus stability. Maximal rates of expression of β-galactosidase from recombinant AcNPV using this promoter occurred 3 to 6 h earlier than from the polyhedrin gene promoter but the expression levels were reported to be lower than those obtained using the polyhedrin promoter. Later studies have found, however, that the yields of both β-galactosidase and JHE from recombinant AcNPVs employing the basic protein promoter were higher than those obtained using the polyhedrin promoter (Bonning *et al.*, 1994).

4.14.2.3.4. Constitutive cellular and early viral promoters For the expression of recombinant proteins requiring extensive posttranslational modifications and secretion, the use of late and very late promoters is suboptimal due to the compromising of the normal cellular processes during the late stages of baculovirus infection (Jarvis and Summers, 1989). Moreover, for biopesticide applications involving the expression of insecticidal proteins by recombinant baculoviruses *in vivo* (see **Chapter 6.9**), it is desirable to be in a position to express insect-toxic proteins at an early time in the baculovirus infection cycle.

One alternative to the use of late promoters was the employment of a constitutively active cellular promoter. A recombinant BmNPV was generated that expressed chloramphenicol acetyl-transferase (CAT) reporter protein under control of the *B. mori* cytoplasmic actin promoter (Johnson *et al.*, 1992). The CAT protein could be detected in the infected silkworm cells grown in culture as quickly as 5 h postinfection, compared to 20 h using the polyhedrin promoter, and the overall CAT yield was only threefold lower using the actin promoter relative to that obtained with the polyhedrin promoter. Furthermore, the actin promoter was also shown to function in cultured cells derived from other lepidopteran species such as *Spodoptera frugiperda* and *Choristoneura fumiferana* and could be potentially incorporated into other baculovirus species such as AcNPV.

Similarly, Morris and Miller (1992) generated several recombinant AcNPVs expressing CAT under control of promoter elements obtained from the following genes: *Drosophila melanogaster* hsp70, AcNPV IE-N, AcNPV IE-1, AcNPV IE-0, and AcNPV ETL. While CAT activity could be detected rapidly following infection in most cases, the levels of CAT produced using these promoters were one to two orders of magnitude lower compared to a conventional recombinant AcNPV that employed the polyhedrin promoter to drive foreign CAT expression. For secreted proteins, however, similar or higher overall levels of recombinant protein were obtained from recombinant AcNPVs employing the IE-1 promoter as the driver of foreign gene expression, as compared to a conventional baculovirus expression vector (Jarvis *et al.*, 1996).

4.14.2.3.5. Multiple promoters There are two strategies to accommodate applications, where cells are required to synthesize more than one protein, e.g., for production of antibodies or virus-like particles. One method is to infect cultures with more than one recombinant virus, each expressing a unique protein. This requires a careful determination of the stoichiometric balance of each virus required for achieving consistent ratios of each recombinant protein. The second approach employs multiple transfer vectors for generating a single AcNPV expressing simultaneously several different proteins. Duplication of the polyhedrin gene within one recombinant AcNPV was shown to be feasible (Emery and Bishop, 1987), and this approach was used to produce Bluetongue virus core-like particles (French and Roy, 1990) and monoclonal antibodies (zu Putlitz *et al.*, 1990). Transfer vectors have been also developed for the generation of a single recombinant AcNPV capable of producing up to five different recombinant proteins, by duplication of both the polyhedrin and p10 gene promoters, without causing genetic instability of the viral genome, at least over a course of six passages (Belyaev *et al.*, 1995).

4.14.2.4. Improvements in Baculovirus Expression Vectors

The generation of recombinant baculoviruses using the original protocols is often technically difficult, time consuming, and labor intensive. First, the frequency of a successful homologous recombination event between the transfer vector and wild-type baculovirus DNA is typically in the range of 0.2–1% (O'Reilly *et al.*, 1992), therefore one or more rounds of purification by plaque assay or dilution cloning are necessary for the isolation of a pure recombinant virus. Second, at least one further round of amplification is required in order to generate a suitable viral titer before the virus can be used in a small scale test for expression. In total, this usually takes at least 3–4 weeks and up to 6 weeks (O'Reilly *et al.*, 1992; Luckow *et al.*, 1993). However, over the last decade, several companies have marketed commercially available kits that have capitalized on ingenious molecular biology designs that circumvent some of the bottleneck problems

associated with the traditional methods of preparing recombinant baculoviruses and accelerate their identification and purification.

4.14.2.4.1. Linearized baculovirus DNA Linearization of AcNPV at the destination locus can increase the recovery of recombinant baculoviruses when the former is cotransfected with an appropriate transfer vector (Kitts *et al.*, 1990). AcRP23. LacZ AcNPV DNA (Becton Dickinson/Pharmingen; Possee and Howard, 1987) contains a *lacZ* gene in place of the polyhedrin gene, in addition to a unique *Bsu*361 site that has been introduced downstream of the polyhedrin promoter. Cotransfection of insect cells with *Bsu*361-linearized AcRP23.LacZ and a transfer vector targeting the polyhedrin locus results in approximately 30% of the progeny viruses being recombinant via homologous recombination (Kitts *et al.*, 1990). This reduces significantly the number of progeny viruses that have to be screened in order to achieve purification of recombinant ones. Furthermore, the isolation of recombinants in plaque assays or limiting dilution assays is facilitated by the fact that the recombinants do not produce LacZ and appear colorless, as opposed to the parental blue ones, in the presence of X-gal. Linearized AcUW1. LacZ DNA (Becton Dickinson/Pharmingen; Weyer *et al.*, 1990) is a modified AcNPV DNA similar to AcRP23.LacZ but designed to be used with transfer vectors targeting the *p10* gene promoter.

BaculoGold™ DNA (Becton Dickinson/Pharmingen) and BacPAK™ (Clontech) are modified AcNPV DNAs with three *Bsu*36I sites inserted in the vicinity of the polyhedrin locus (Kitts and Possee, 1993). Digestion of the DNAs with *Bsu*36I yields linear DNAs encompassing a lethal deletion in the essential ORF 1629 (Possee and Howard, 1987) that maps downstream of the polyhedrin locus, and this precludes the generation of a viable virus upon self-ligation of the restricted parental viral genome. When provided with a transfer vector spanning the deleted region, homologous recombination in the transfected insect cells rescues the lethal deletion. Over 99% of viruses produced by the transfected cells are recombinants and only one round of purification is necessary. These DNAs also contain a *lacZ* gene at the polyhedrin locus, which is substituted for by the gene of interest in a successful recombination event. Thus, the isolation of recombinant baculoviruses by plaque or limiting dilution assays is facilitated by the lack of expression of LacZ and the presence of colorless infected cells upon addition of X-gal. BaculoGold™ Bright (Becton Dickinson/Pharmingen) recombinant baculoviruses express the green fluorescence protein (GFP), that can be visualized 24 h postinfection (p.i.), under the control of the *p10* gene promoter. This can facilitate the purification of recombinant virus by fluorescence activated cell sorting (FACS) sorting of single GPF positive cells. The titration process is also simplified by FACS analysis or by a serial dilution and visualization using fluorescent microscopy.

BacVector® (Novagen) and Bac-N-Blue™ (Invitrogen) are modified linearized AcNPV DNAs similar to BaculoGold™ and BacPAK™. They also contain a lethal deletion in ORF1629 that is rescued by a transfer vector. The relevant transfer vectors target the polyhedrin locus and contain reporter genes encoding either β-glucuronidase (pBac, Novagen) or β-galactosidase (pBlueBac, Invitrogen) under the control of the AcNPV ETL promoter. This allows for visual identification of a successful homologous recombination event by the scoring of blue (recombinant) plaques in a background of colorless ones following the addition of X-gluc or X-gal, respectively. Finally, BacVector-3000 is a modified linearized AcNPV DNA (Novagen) containing deletions of several nonessential genes that, apparently, compete with target protein production, in addition to deletions of the genes encoding two baculovirus degrading enzymes, cathepsin and chitinase, which are claimed to destabilize recombinant proteins.

4.14.2.4.2. Shuttle vectors Certainly one of the most ingenious and fastest methods to generate recombinant AcNPV is the Bac-to-Bac® expression system (Luckow *et al.*, 1993), licensed to Invitrogen (formerly Life Technologies) by Monsanto. In this system, the recombination event between the AcNPV and transfer vector occurs in *E. coli* DH10B by site-specific transposase-mediated insertion, as opposed to homologous recombination in insect cells. The DH10Bac™ strain harbors a modified AcNPV genome, known as bacmid, that is essentially a large (136 kbp) shuttle plasmid containing, in addition to the baculovirus genome sequences, a kanamycin resistance marker, a low copy number mini-F replicon for retention and replication in *E. coli*, and the target site *att*Tn7 for the Tn7 bacterial transposon in the polyhedrin locus. A helper plasmid providing Tn7 transposase is also maintained in the DH10Bac™ strain by tetracyclcine resistance. When DH10Bac™ cells are transformed with a third plasmid containing the gene of interest flanked by Tn7 elements, transposition of the gene of interest occurs to the Tn7 target site in the polyhedrin locus of the bacmid. The successful transposition disrupts the expression of a

LacZ reporter gene in the bacmid, so that generation of a recombinant bacmid results in white *E. coli* colonies in blue background colonies arising from unaltered parent cells. The recombinant bacmid DNA is isolated from minipreps of white colonies and then used to transfect insect cells and generate viral stocks for small-scale recombinant protein expression. The whole process is reported to take 7–10 days (Luckow *et al.*, 1993).

Patel *et al.* (1992) also described a rapid method for generating recombinant baculoviruses by homologous recombination between a modified AcNPV genome propagated in yeast and a baculovirus transfer vector containing yeast sequences. With this yeast-baculovirus shuttle system, it is reported that recombinant viral stocks can be obtained within 10–12 days. This system is not available from a commercial supplier.

4.14.2.4.3. Direct cloning Several publications have also appeared describing direct cloning of the gene of interest into polyhedrin locus of the AcNPV genome (Ernst *et al.*, 1994; Lu and Miller, 1996). Modified AcNPV's were generated that could be linearized with restriction enzymes in the polyhedrin locus. Ligation with the foreign gene fragment *in vitro* and transfection of the ligation mix into insect cells resulted in the percentage of recombinant viruses approaching 100%. Invitrogen Corporation has recently developed Baculodirect™, a direct cloning strategy for generating recombinant AcNPV using the well-characterized lambda-phage site-specific recombination system (Gateway™) as opposed to a homologous recombination. In addition, the recombination event transferring the desired gene to the polyhedrin locus also includes the HSV thymidine kinase (TK) selection marker that is driven by the IE-1 promoter. Once the *in vitro* recombinase reaction is complete, the reaction contents are transfected into insect cells and selective pressure of ganciclovir is applied so that only those viruses harboring the TK resistance marker can replicate. Within 7 days, purified viral stocks are available for expression studies.

4.14.2.5. Cell Lines Used as Hosts for Baculovirus Expression Vectors

With the popularity of the BES and the fact that the vast majority of baculovirus expression vectors are based on AcNPV, it is not surprising that recent developments in insect cell culture have focused on cell lines derived from species that can support both the replication of AcNPV and the high level expression of foreign proteins under control of the AcNPV polyhedrin, p10 and basic protein gene promoters. AcNPV has a relatively broad host range among lepidopteran species and cell lines established from them, including the fall armyworm *S. frugiperda*, the cabbage looper *Trichoplusia ni*, the tobacco budworm *Heliothis virescens* and the gypsy moth *Lymantria dispar*. However, those cell lines that are the most widely used to date as hosts for protein expression include IPLB-Sf21AE (Vaughn *et al.*, 1977) established from *S. frugiperda* pupal ovaries, its subclone Sf9 (Summers and Smith, 1987) and BTI-Tn-5B1-4 or High Five™ cells established from *T. ni* embryos (Granados *et al.*, 1994). These cell lines are well characterized and have a history of good growth and recombinant protein expression in serum-free culture medium and in large-scale suspension cultures. In contrast to AcNPV, BmNPV has a narrow host range; Bm5 (Grace, 1967) and BMN-4 cells (Maeda, 1989) established from *B. mori* ovarian tissue cells are used as host cells for BmNPV infections. Although both Bm5 and BMN-4 lines can be propagated in serum-free media, Bm5 cells have superior growth characteristics in suspension culture (Keith *et al.*, 1999) and have been reported to produce recombinant protein in bioreactors (Zhang *et al.*, 1993).

4.14.2.6. Classes of Recombinant Proteins Expressed by Baculovirus Infected Insect Cells

Insect cells are capable of most types of posttranslational modifications that occur in mammalian cells. They include signal peptide cleavage, N- and O-linked glycosylation (although they may not be capable of a significant level of complex glycosylation), phosphorylation, acylation, palmitylation, myristoylation, prenylation, amidation, and carboxymethylation (see Luckow, 1991 for an extensive compilation to that date). Heterologous proteins are also correctly targeted to their subcellular destination including plasma membranes, cytosol, lysosomes, mitochondria, nuclei, or nucleoli. Hence, different classes of recombinant proteins from a variety of organisms have been expressed successfully using the baculovirus expression system. These include cytoplasmic proteins, structural proteins, lysozomal enzymes, membrane receptors, ion exchangers, transport proteins, and nuclear factors (Table 1).

Although it is unwise to make gross generalizations, some classes of proteins are certainly expressed at much higher levels than others, and each individual protein within a class will often have its own unique troublesome characteristics that a researcher will have to deal with: some proteins may form insoluble aggregates, others are more vulnerable to proteolysis (especially following

lysis when degradative enzymes are released), secretion may be inefficient, expression levels may be low and those proteins requiring a physiologically intact cell membrane may have to be harvested prematurely.

4.14.2.6.1. Intracellular proteins Clearly the most successful application of the baculovirus expression system is for expression of intracellular recombinant proteins. Expression levels over 100–200 mg l^{-1} are not uncommon (**Table 1**) and proteins may be released by the cells into the culture medium following virus-induced lysis to simplify purification. Insoluble aggregates of recombinant intracellular protein within the cell have occasionally been reported (Alnemri and Litwack, 1993; Rankl *et al*., 1994; Baldock *et al*., 2000). In rare cases, the fusion of an insect-derived signal peptide to the N-terminus of a normally intracellular targeted protein has been successful for secreting the intracellular protein (Mroczkowski *et al*., 1994; Laukkanen *et al*., 1996).

4.14.2.6.2. Secreted proteins Secreted proteins are usually expressed at much lower levels compared to intracellular proteins, usually in the order of 10 mg l^{-1} (**Table 1**). This is thought to be due to a breakdown in the secretory pathway in the late stages of infection, when foreign gene expression from late and very late promoters is maximal (Jarvis and Summers, 1989). Three approaches have been tried to improve the efficiency of secretion. In the first approach, the substitution of the native signal peptide of a heterologous secreted protein with an insect-specific signal peptide, such as those of the honeybee mellitin (Tessier *et al*., 1991; Jarvis *et al*., 1993), *Drosophila* cecropin B (Jarvis *et al*., 1993), AcNPV 64K (Jarvis *et al*., 1993), AcNPV gp67 (Golden *et al*., 1998; Murphy *et al*., 1993), AcNPV chitinase (Chen *et al*., 2000), and AcNPV egt (Murphy *et al*., 1993), was expected to make secretion more efficient. In some cases expression was improved dramatically using insect-specific signal peptides (Murphy *et al*., 1993; Chen *et al*., 2000), while in others the modifications proved ineffective (Jarvis *et al*., 1993; Golden *et al*., 1998). In the second approach, the use of an earlier promoter (but weaker than late and very late promoters) such as IE-1 to drive the expression of a heterologous secreted protein, before the secretory pathway was compromized, resulted in only a slight improvement in the expression level (Jarvis *et al*., 1996). The third approach was to coexpress a molecular chaperone protein such as immunoglobulin heavy chain binding protein (BiP), although no improvement in secretion of immunoglobulin was observed (Hsu *et al*., 1994). If glycosylation is not important for a particular secreted protein, one strategy to improve expression is to remove the signal sequence altogether. This will prevent its entry into the secretory pathway and disguise the protein as an intracellular protein. Unpublished results from Dr Bruce Hammock's laboratory (personal communication) have shown an improvement in the expression of active *H. virescens* JHE from 4 to 75 mg l^{-1} using this strategy (**Figure 2**).

4.14.2.6.3. Membrane proteins The reputation of the baculovirus expression system for lower expression levels of proteins destined for the secretory pathway compared to other proteins (Jarvis and Guarino, 1995) also applies to membrane proteins. This said, expression levels of membrane proteins are often higher than those achieved with other expression systems; the expression of membrane proteins in bacteria, for example, is largely unsuccessful. Certainly many laboratories have used the BES to express and purify a variety of membrane proteins. Some examples include ion exchangers (Li *et al*., 1992; Dale *et al*., 1996; Egger *et al*., 1999), G protein-coupled receptors (GPCRs) (Strosberg and Guillaume, 2000; reviewed by Bouvier *et al*., 1998; Carpentier *et al*., 2001), transport proteins (Smith *et al*., 1992; Tate, 1998; Gao *et al*., 1999; Miyasaka *et al*., 2001), adhesion molecules (Stoltenberg *et al*., 1993; Nagarajan and Selvaraj, 1999; Phan *et al*., 2001), and other membrane proteins (Xiao *et al*., 2000; Soares *et al*., 2001; Sun *et al*., 2002). Purified levels obtained are generally in the range of 0.1–10 mg l^{-1} (Chinni *et al*., 1998; Carpentier *et al*., 2001; Miras *et al*., 2001; Soares *et al*., 2001). There are numerous reports of either inactive, immature, misfolded membrane proteins or incompletely glycosylated forms of baculovirus-expressed membrane proteins (Pajot-Augy *et al*., 1995; Vasudevan *et al*., 1995; Loisel *et al*., 1997; Massotte *et al*., 1997; Tate, 1999; Sun *et al*., 2002). Improvements in the yields of active membrane proteins have been obtained by the coexpression of molecular chaperones including calreticulin, BiP, cannexin, and nina A (Tate, 1999; Miyasaka *et al*., 2001; Lenhard and Reilander, 1997).

For those studying membrane protein signaling events following ligand binding, there can be some difficulties using the BES. Obviously, there is the fact that an inherent property of the baculovirus is that it will ultimately kill the host cell. Thus, only a short window of opportunity exists for the study of receptor signaling events in live cells, between the moment of expression from a late- or very late-phase promoter and cell death. Some investigators have

Table 1 Expression levels of some secreted and intracellular proteins expressed using the baculovirus expression system. The expression levels reported are in mg per liter of *unprocessed* cell culture

Protein	*Baculovirus*	*Promoter*	*Level* ($mg\,l^{-1}$)	*Culture conditions*	*Reference*
Secreted					
Hu tPA	AcNPV/Sf9	Polyhedrin	2.5	n.r.	Steiner *et al.* (1988)
Hu SEAP	AcNPV/High Five	Polyhedrin	13	n.r.	Davis *et al.* (1992)
Rat PCE	AcNPV/Sf9	Polyhedrin	25	Shake flask	DiPersio *et al.* (1992)
Hu IL-8	AcNPV/Sf9	Polyhedrin	0.5–2	T-flask	Kang *et al.* (1992)
Hu LTA_4H	AcNPV/Sf9	Polyhedrin	100	10 l bioreactor	Gierse *et al.* (1993)
Hu C9	AcNPV/Sf21	Polyhedrin	0.7	Shake flask	Tomlinson *et al.* (1993)
HIV gp120	AcNPV/Sf9	Polyhedrin	10–15	5 l reactor	Murphy *et al.* (1993)
Hu procolipase	AcNPV/High Five	Polyhedrin	15	Spinner flask	Lowe (1994)
Hu PSA	AcNPV/Sf9	Polyhedrin	2–4	30 l bioreactor	Kurkela *et al.* (1995)
In JHE	AcNPV/Sf21	Polyhedrin	12	6-well plate	Bonning and Hammock (1995)
In JHE	AcNPV/Sf21	P10	13	6-well plate	Bonning and Hammock (1995)
In JHE	AcNPV/Sf21	Basic protein	16	6-well plate	Bonning and Hammock (1995)
In JHE	AcNPV/High Five	Polyhedrin	32	Shake flask	Bonning and Hammock (1995)
In JHE	AcNPV/Sf21	Polyhedrin	22	Spinner flask	Bonning and Hammock (1995)
Hu IL-5	AcNPV/Sf9	Polyhedrin	5–15	3 l reactor	Brown *et al.* (1995)
Ms OSF-2	AcNPV/Sf9	Polyhedrin	40	15 cm dish	Sugiura *et al.* (1995)
Hu HCII	AcNPV/High Five	Polyhedrin	3	T-flask	Ciaccia *et al.* (1995)
GalNAc-transferase	AcNPV/Sf9	Polyhedrin	2–3	Shake flask	Homa *et al.* (1995)
Hu IL-5	AcNPV/Sf9	Polyhedrin	5–15	n.r.	Brown *et al.* (1995)
Hu LIF	AcNPV/Sf21	Polyhedrin	12	Roller bottle	Geisse *et al.* (1996)
Hu ApoA-I	AcNPV/Sf21	Polyhedrin	40–50	1 l reactor	Sorci-Thomas *et al.* (1996)
Hu prorennin	AcNPV/Sf9	Polyhedrin	40–50	23 l reactor	Mathews *et al.* (1996)
Ms FAK	AcNPV/Sf9	Polyhedrin	19	1 l suspension	Withers *et al.* (1996)
In transferrin	AcNPV/Sf9	Polyhedrin	10	Roller bottles	Winzerling *et al.* (1996)
Hu prorennin	AcNPV/Sf9	Polyhedrin	0.5	Spinner flasks	Mathews *et al.* (1996)
Ms GHBP	AcNPV/Sf21	Polyhedrin	17	Shake flasks	Thordarson *et al.* (1996)
Chk avidin	AcNPV/Sf9	Polyhedrin	12.2	Shake flask	Airenne *et al.* (1997)
Hu MMP9	AcNPV/High Five	Polyhedrin	>300	8 l bioreactors	George *et al.* (1997)
Hu α_1m	AcNPV/High Five	Polyhedrin	50–150	n.r.	Wester *et al.* (1997)
Mn CatK	AcNPV/Sf21	Polyhedrin	7–10	20 ml cultures	McQueney *et al.* (1998)
Hu STC	AcNPV/Sf9	Polyhedrin	125	60 l bioreactor	Zhang *et al.* (1998)
Hu GL	AcNPV/High Five	Polyhedrin	5–12	4 l bioreactor	Canaan *et al.* (1998)
Hu Apo A-I mutants	AcNPV/Sf21	Polyhedrin	1–8	Spinner flask	Sviridov *et al.* (1999)
Hu OPG-Fc	AcNPV/High Five	Polyhedrin	15	Shake flask	Willard *et al.* (2000)
Hu prolactin	AcNPV/High Five	Polyhedrin	53–60	T-flask	Das *et al.* (2000)
Hu prolactin	AcNPV/Sf9	Polyhedrin	172	1 l bioreactor	Pereira *et al.* (2001)
In proPAP	AcNPV/Sf21	Polyhedrin	0.37	T-flask	Wang *et al.* (2001)
Hu MMP-9	AcNPV/Sf9	Polyhedrin	0.8	Spinner flask	Sadatmansoori *et al.* (2001)
Hu amylase	AcNPV/Sf9	Polyhedrin	6–10	Roller bottle	Ragunath *et al.* (2002)
In proPAP-2	AcNPV/Sf21	Polyhedrin	5.9	suspension	Ji *et al.* (2003)
Hu IL-3	AcNPV/High Five	Polyhedrin	1.5–3.0	2.5 l bioreactor	Ding *et al.* (2003)
Intracellular					
CAT	AcNPV/Sf21	Polyhedrin	>100	T-flask	Luckow and Summers (1988)
VP6	AcNPV/Sf9	Polyhedrin	350	4 l bioreactor	Caron *et al.* (1990)
β-gal	AcNPV/High Five	Polyhedrin	>500	Shake flask	Wickham *et al.* (1992)
VP4	AcNPV/Sf9	Polyhedrin	50	Spinner flasks	Juarbe-Osorio (1993)
CAT	BmNPV/Bm5	Polyhedrin	250	1.5 l bioreactor	Zhang *et al.* (1993)
Hu ACL	AcNPV/Sf9	Polyhedrin	200	Spinner flasks	Lord *et al.* (1997)
NF6B1 and RelA	AcNPV/Sf9	Polyhedrin	>10	Spinner flasks	Coleman *et al.* (1997)
Hu aminoacylase I	AcNPV/Sf21	Polyhedrin	100–200	n.r.	Pittelkow *et al.* (1998)
Hu M/NEI	AcNPV/Sf9	Polyhedrin	20	15 l bioreactor	Cooley *et al.* (1998)
Hu Syk	AcNPV/Sf21	Polyhedrin	30–40	n.r.	Baldock *et al.* (2000)
EBNA1	AcNPV/Sf9	Polyhedrin	30	Spinner flask	Meij *et al.* (2000)
Hu PARP	AcNPV/Sf9	Polyhedrin	8–80	1 l bioreactor	Pereira *et al.* (2001)
KSHV Pol	AcNPV/Sf9	Polyhedrin	5–15	Spinner flasks	Dorjsuren *et al.* (2003)

Most reports are obtained from a survey of the Protein Expression and Purification journal (n.r., not reported; Hu, human; Ms, mouse; In, insect; Chk, chicken; Mn, monkey; tPA, tissue plasminogen activator; SEAP, placental secreted alkaline phosphatase; PCE, pancreatic cholesterol esterase; IL-8, interleukin-8; LTA_4H, leukotriene A_4 hydrolase; C9, complement protein 9; HIV gp120,

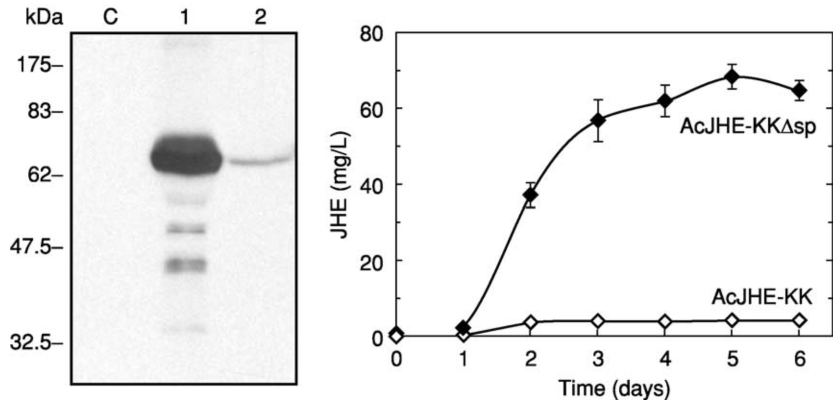


Figure 2 One technique to improve the expression level of secreted proteins from baculovirus infected insect cells involves the removal of the signal peptide encoded by the gene and the creation of an artificial ATG at the start of the mature polypeptide coding region in the expression vector. This modification prevents the nascent polypeptide from entering the secretory pathway. In this example, the yield of biologically active juvenile hormone esterase (JHE) present in the culture supernatant of recombinant AcNPV-infected Sf21 cells is enhanced approximately 20-fold when the JHE signal peptide is removed (AcJHE-KKΔsp) compared to the full length gene AcJHE-KK (Bonning *et al.*, 1997). Left panel: Western blot analysis of 5 μl culture media to confirm the relative JHE expression levels in static cultures. Lane C, control culture medium; lane 1, day 6 cultutre medium form AcJHE-KKΔsp infected Sf21 cells; lane 2, day 6 culture medium from AcJHE-KK infected Sf21 cells. Right panel: Batch production of JHE by AcJHE-KK or AcJHE-KKΔsp-infected Sf21 cells in serum-containing medium in 6-well plates over a 6-day period. Note that JHE produced by AcJHE-KKΔsp was not directed through the secretory pathway.

used membrane preparations from infected cells rather than the cells themselves to carry out their studies (Clawges *et al.*, 1997). In addition, coupling of heterologous receptors with endogenous insect cell host proteins, such as G proteins, has generally produced low responses upon ligand binding (see below). This problem has been circumvented by coexpressing a GPCR with specific G protein subunits in order to assess agonist binding (Butkerait *et al.*, 1995; Barr *et al.*, 1997; Bouvier *et al.*, 1998).

4.14.2.6.4. Compartmentalized proteins The BES has also been employed to synthesize and correctly process proteins targeted to subcellular compartments. A variety of mitochondrial proteins have been expressed using the BES, both membrane associated (Yet *et al.*, 1995; Bader *et al.*, 1998; Huang *et al.*, 2000) and located within the mitochondrial matrix (Wang and Kaguni, 1999; Holcomb *et al.*, 2000), although the latter reported incomplete processing and inefficient targeting. Lysosomal enzymes have also been successfully expressed (Tschantz *et al.*, 1999; Bromme and McGrath, 1996; Steed *et al.*, 1998), as has a nucleolar protein (Ren *et al.*, 1996) and a nuclear membrane protein (Bailer *et al.*, 1995).

4.14.2.7. Other Applications of the Baculovirus Expression System

4.14.2.7.1. Virus-like particles The baculovirus expression system has proven to be very effective for the production of heterologous virus-like and core-like particles (VLPs and CLPs) destined for use in immunization or structural studies. Some examples include the poliovirus VLP (Urakawa *et al.*, 1989), bluetongue virus CLP (French and Roy, 1990), Norwalk virus (Jiang *et al.*, 1992), the papillomavirus (Cann *et al.*, 1995), and herpes simplex virus (Newcomb *et al.*, 1999). VLPs and CLPs can be architecturally complex and comprising several structural proteins in different molar

human immunodeficiency virus glycoprotein 120; PSA, prostrate specific antigen; JHE, *Heliothis virescens* juvenile hormone esterase; IL-5, interleukin-5; OSF-2, murine osteoblast specific factor 2; HCII, heparin cofactor II; GalNAc-transferase, bovine UDP-GalNAc polypeptide *N*-acetyl galactosaminyl transferase; LIF, leukemia inhibitory factor; Apo A1, apolipoprotein A-I; FAK, focal adhesion kinase; GHBP, growth hormone binding protein; MMP9 matrix metalloproteinase-9; α_1m–α_1, microglobulin; CatK, cathepsin K; STC, stanniocalcin; GL, gastric lipase; OPG-Fc, osteoprotegerin-immunoglobulin Fc fusion; proPAP, *Manduca sexta* prophenoloxidase-activating proteinase precursor; proPAP-2, *M. sexta* prophenoloxidase-activating proteinase-2 precursor; IL-3, interleukin-3; CAT, bacterial chloramphenicol acetyl-transferase; VP6, rotavirus VP6; β-gal, beta galactosidase; VP4, rotavirus outer capsid protein; ACL, ATP citrate lyase; NFκB1/RelA, subunits of the NF-κB transcriptional activator of immunoglobulin κ light chain; M/NEI, monocyte/neutrophil elastase inhibitor; Syk, Syk protein; EBNAI, Epstein-Barr virus nuclear antigen 1; PARP, poly(ADP-ribose) polymerase; KSHV Pol, Kaposi's sarcoma-associated herpesvirus DNA polymerase).

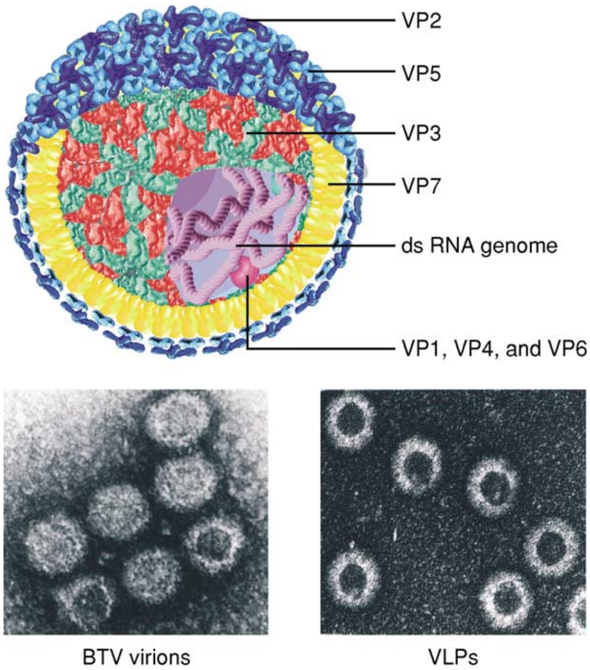


Figure 3 A comparison of electron micrographs of native blue-tongue virus (BTV) virions with recombinant BTV virus-like particles (VLPs) produced and self-assembled in baculovirus-infected insect cells. For optimal synthesis of the BTV VLPs, a quadruple gene expression vector was used to co-express BTV VP2, VP3, VP5, and VP7 proteins (Roy *et al.*, 1997). At the top of the figure, a model of the BTV virion is shown. Images kindly supplied by Dr Polly Roy (London School of Hygiene and Tropical Medicine, UK).

proportions. It was observed that the coexpression of the various VLP structural proteins in baculovirus-infected insect cells results in the self-assembly of the VLP (Urakawa *et al.*, 1989). Initially, the various structural proteins were produced by coinfection with individual recombinant baculoviruses. However, variation in the distribution of individual recombinant baculoviruses to each cell would result in differences in the quality of the VLP. Coexpression of proteins of the VLP or CLP using a single baculovirus with multiple promoters circumvents this problem (**Figure 3**; French and Roy, 1990; Belyaev *et al.*, 1995). The ratio of each protein expressed can be controlled through the use of baculovirus promoters of different strength (Roy *et al.*, 1997).

4.14.2.7.2. Surface display and antibody production The baculovirus virion has been developed as an eukaryotic alternative to the bacteriophage for the surface display of foreign proteins from an expression library and the selection of specific binding proteins (review: Grabherr *et al.*, 2001). By duplicating the essential major envelope glycoprotein gene encoding gp64 in the baculovirus genome, it was shown that foreign proteins (X) could be presented on the mature N-terminus of the duplicated X-gp64 (fusion) protein, in the envelope of BVs (Boublik *et al.*, 1995). A demonstration of the feasibility of using baculovirus display for the screening of a library has been described (Ernst *et al.*, 1998). Immunization of mice with purified virions displaying X-gp64 fusion proteins has also been useful for obtaining polyclonal and monoclonal antibodies against the antigen X (Lindley *et al.*, 2000). This procedure eliminates one bottleneck in the conventional antibody production process of having to generate purified protein X for immunization. Due to an immunostimulatory effect often associated with viral immunogens (Minev *et al.*, 1999), this method may be more effective than the conventional process for raising antibodies against certain antigens (Lindley *et al.*, 2000).

4.14.2.7.3. Expression of recombinant proteins in insect larvae Insect larvae have occasionally been employed, instead of cultured insect cells, as hosts for recombinant baculoviruses for the expression of recombinant proteins (Maeda *et al.*, 1985). Production in larvae can be inexpensive, high yielding, and easily scalable compared to cell culture. Yields around 1 mg per silkworm larva infected with recombinant BmNPV have been obtained for human interleukin-3 (Miyajima *et al.*, 1987), hepatitis B surface antigen (Higashihashi *et al.*, 1991), human CD66 antigens (Yamanaka *et al.*, 1996), grass carp growth hormone (Ho *et al.*, 1998), hepatitis E virus capsid protein (Sehgal *et al.*, 2003), and human fibroblast growth factor (Wu *et al.*, 2001). A bovine cardiac sodium–calcium exchanger has also been successfully expressed in *T. ni* larvae (Hale *et al.*, 1999). However, the recovery of the expressed protein may be more problematic from larvae than from tissue culture because of increased proteolysis in the insect hemolymph and contamination of preparations by abundant hemolymph proteins.

4.14.2.7.4. Baculovirus transducing and transforming vectors Because of their ability to enter efficiently host and nonhost insect cells alike, baculovirus vectors can be designed with the goal of overexpressing regulatory factors and studying their role during development. Because baculoviruses are normally lethal to the host tissues, expression studies are by necessity limited to a brief period of experimentation extended between the initial infection and the activation of the host cell lysis process (Iatrou and Meidinger, 1989). For lepidopteran insect systems, this problem can be

circumvented by the construction and use of mutant viruses that are unable to proceed through the late phases of the infection cycle. By contrast, infection of tissues of nonhost insects that are refractory to productive infection can result in more extended periods of expression and experimental observation (Oppenheimer *et al*., 1999).

Two approaches have been utilized for the generation of transgenic lepidopteran insects using baculoviruses. One exploits the fact that AcNPV can infect nonhost lepidopteran insect larvae without killing the insect (Mori *et al*., 1995; Oppenheimer *et al*., 1999). Due to this property, the AcNPV can be used as a vehicle to efficiently deliver a transgene to germline cells, which is then targeted to chromosome sites for homologous recombination and generation of transgenic progeny (Yamao *et al*., 1999). A second approach uses a recombinant baculovirus with a deleted *lef-8* gene (Iatrou *et al*., 2000). The *lef-8* gene encodes a subunit of the viral RNA polymerase (Passarelli *et al*., 1994) that is necessary for mRNA transcription from the promoters of late and very late-phase baculovirus genes. Loss-of-function mutations in this gene prevent the progression from the replication phase to the virulent phase of the baculovirus infection cycle (Shikata *et al*., 1998). Accordingly, deletions of the *lef-8* gene convert the baculovirus to a harmless, self-replicating extra-chromosomal entity (baculovirus artificial chromosome – BVAC), while host cells infected by it behave as normal ones (P.J. Farrell and K. Iatrou, unpublished results). Passage of the *lef-8* deficient baculovirus through a rescuing cell line that constitutively expresses the Lef-8 protein enables the production of BVAC inoculum that maintains full infectivity for and replicating capacity in the host cells (**Figure 4**).

The BVACs could potentially be used for prolonged protein expression by taking advantage of the high initial infection efficiency of cells by the baculovirus, the replication of the BVACs in them, and the subsequent mitotic transmission of the transgene-containing BVAC to the daughter cells. Furthermore, the prevention of both virus-induced damage to the secretory pathway and lysis would make BVACs more suitable for the expression of secreted and membrane proteins under the control of early viral or cellular promoters than conventional baculovirus expression vectors. Efforts are also being made to generate transgenic silkworms through the infection of germline cells with BVACs derived from BmNPV.

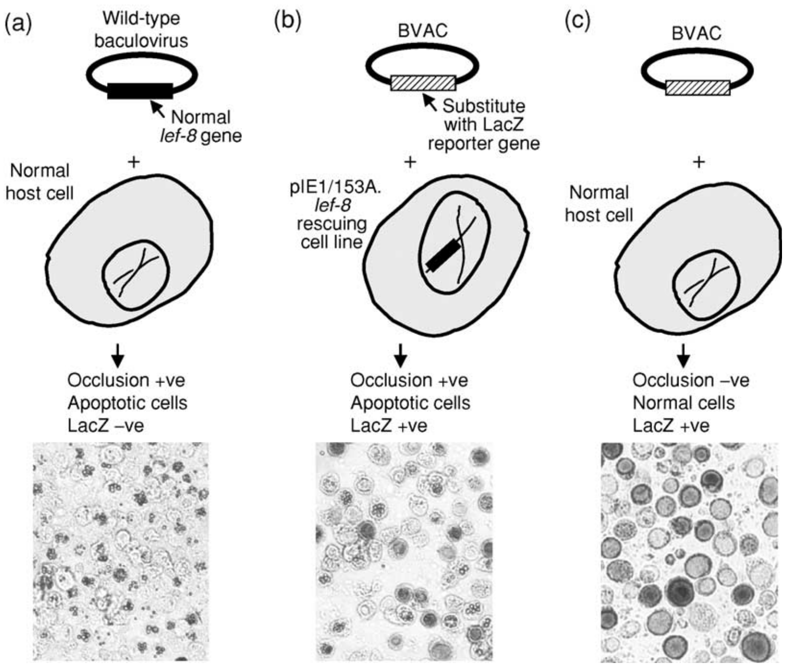


Figure 4 The potential for a nonlytic baculovirus is demonstrated. (a) Infection of normal insect cells by a wild-type baculovirus. The infected cells produce OBs (small, refractive objects) and eventually lyse. (b) A baculovirus artificial chromosome (BVAC) is created when the *lef-8* gene is eliminated and replaced with a reporter gene expression β-galactosidase (dark cells). When the *lef-8* gene product is supplied by a transformed cell line, the BVAC can complete the infection cycle that includes the formation of budded virions and occlusion bodies and cell lysis, in addition to the production of β-galactosidase. (c) The absence of the *lef-8* gene product, in normal permissive cells infected by a BVAC, prevents completion of the baculovirus infection cycle. OBs are absent, the cells appear and replicate normally, and also express β-galactosidase.

4.14.2.7.4. Transduction of mammalian cells Baculovirus virions were demonstrated to have the ability to enter certain cell lines derived from vertebrate species without evidence of viral gene expression (Volkman and Goldsmith, 1983; Carbonell and Miller, 1987). By incorporating mammalian expression cassettes into recombinant AcNPVs, several reports have appeared demonstrating that baculoviruses can serve as an efficient mode of gene transfer into a variety of primary and transformed mammalian cell lines, including those difficult to transfect using traditional methods, and express reporter proteins under the control of mammalian promoters (Hoffmann *et al*., 1995; Boyce and Bucher, 1996; Shoji *et al*., 1997; Yap *et al*., 1997; Condreay *et al*., 1999). Chromosomal integration and stable gene expression have also been demonstrated to be feasible by inclusion of a selectable marker (Condreay *et al*., 1999; Merrihew *et al*., 2001) or by integration signals from adeno-associated virus (AAV) into the baculovirus genome (Palombo *et al*., 1998). The advantages of using the baculovirus in this application include the high transfection efficiency (often greater than 90%; Condreay *et al*., 1999), low toxicity of the baculovirus to mammalian cells, and capacity of the baculovirus to carry large inserts. Recently, hybrids of the baculovirus and AAV were used to coinfect mammalian cells and produce high titer recombinant AAV for gene therapy applications (Sollerbrant *et al*., 2001).

4.14.3. Insect Cells as Hosts for Plasmid-Based Expression of Recombinant Proteins

4.14.3.1. Introduction

Several baculovirus-free, plasmid-based approaches to recombinant protein expression in insect cells have been developed. In this technique, a plasmid expression cassette harboring a gene of interest is introduced into the host insect cell line using a variety of transfection techniques. The recombinant protein will be transiently expressed for a few days after transfection whereupon the cells or their media containing the recombinant protein can be harvested. Stable cell lines expressing the recombinant protein of interest, either continuously or by induction, can be generated by applying an antibiotic resistance-selection scheme over a period of several weeks. The antibiotic resistance gene can either be present on the same expression cassette as the gene of interest or supplied by cotransfection with a separate plasmid. Antibiotic selection schemes conferring resistance to G418 (Jarvis *et al*., 1990), hygromycin B (Johansen *et al*., 1989), methotrexate (Shotkoski and Fallon, 1993), puromycin (McLachlin and Miller, 1997), and zeocin (Pfeifer *et al*., 1997) have all been used extensively with insect cell lines.

The production of recombinant proteins in transfected or stably transformed insect cells provides considerable advantages over both the baculovirus expression system and transformed mammalian cells for the case of applications involving secreted and membrane-anchored proteins: insect cell lines are safe to humans, introns are spliced correctly and efficiently from expressed genomic DNAs and lysis does not occur, therefore allowing continuous, as opposed to batch-type, expression of the proteins and limiting proteolysis and facilitating purification of the secreted proteins. Furthermore, insect cells can perform most essential posttranslational modifications as efficiently as mammalian ones, and membrane proteins can be expressed in a stable physiological environment. Finally, most insect cell lines can grow in serum-free media to high densities, and many insect cell lines, particularly of lepidopteran origin, are already well characterized in large-scale suspension culture because of their role as hosts in the baculovirus expression system.

Two major categories of plasmid-based expression systems have been developed utilizing promoters and cell lines derived from two different insect orders, Diptera and Lepidoptera. These are presented in more detail below.

4.14.3.2. Expression Systems Based on Dipteran Cell Lines

Dipteran expression vectors mostly utilize the strong constitutive actin 5C promoter of *D. melanogaster* (Angelichio *et al*., 1991) or the inducible metallothionein promoter of *Drosophila* (Johansen *et al*., 1989; Kovach *et al*., 1992; Millar *et al*., 1995; Hegedus *et al*., 1998; Zhang *et al*., 2001) to drive foreign protein expression, in conjunction to a hygromycin B antibiotic selection scheme to generate a polyclonal population of Schneider 2 cell lines (S2 cells; Schneider, 1972), which takes approximately 3 weeks to accomplish (Johansen *et al*., 1989). The latter system has been developed by SmithKline-Beecham Pharmaceuticals and made commercially available through Invitrogen Corporation as the Drosophila Expression System (DES®). *Drosophila* S2 cells can grow in serum-free medium in batch suspension culture to cell densities up to 15×10^6 cells ml^{-1}, although S2 cells are substantially smaller than most lepidopteran cell lines including Sf21, High Five™, and Bm5

Table 2 Expression levels of some secreted proteins from stably transformed insect cells

Protein	*Cells*	*Level (mg l^{-1})*	*Culture conditions*	*Reference*
D. melanogaster metallothionein promoter				
Hu IL-5	S2	22	n.r.	Johansen *et al.* (1995)
Modified HIV gp120	S2	2	n.r.	Culp *et al.* (1991)
Modified HIV gp120	S2	5–35	n.r.	Ivey-Hoyle *et al.* (1991)
Hu IgG$_1$	S2	1	n.r.	Kirkpatrick *et al.* (1995)
Hu SPC1	S2	3	n.r.	Denault *et al.* (2000)
Hu EPO	S2	2	Spinner flasks	Shin and Cha (2003)
AcNPV ie-1 promoter				
Hu tPA	Sf9	1.0	Static	Jarvis *et al.* (1990)
OpMNPV ie-2 promoter				
Ms IgG$_1$	Sf9	0.5–1	Spinner flasks	Li *et al.* (2001)
Modified Hu p97	Sf9	10	Spinner flasks	Hegedus *et al.* (1999)
Modified Hu Factor X	Sf9	18	Shake flasks	Pfeifer *et al.* (2001)
Hu plasminogen	S2	10–15	Spinner flasks	Nilsen and Castellino (1999)
B. mori cytoplasmic actin promoter, pIE1/153A vector				
Hu tPA	Bm5	135–160	Static-spinner flask	Farrell *et al.* (1999)
In JHE	Bm5	130–190	Static-spinner flask	Farrell *et al.* (1998)
Hu GM-CSF	High Five	27–46	Spinner flask-static	Keith *et al.* (1999)

n.r., not reported; Hu, human; Ms, mouse; In, insect; IL-5, Interleukin-5; HIV gp120, human immunodeficiency virus glycoprotein 120; IgG$_1$, immunoglobulin G$_1$; SPC1, subtilisin-like proprotein convertase 1; EPO, erythropoietin; tPA, tissue plasminogen activator; p97, melanotransferrin; JHE, *Heliothis virescens* juvenile hormone esterase; GM-CSF, granulocyte macrophage colony stimulating factor.

cells. Enzymes, membrane receptors, ion channels, viral antigens, and monoclonal antibodies have been successfully produced using these systems (see Table 2).

4.14.3.3. Expression Systems Based on Lepidopteran Cell Lines

Lepidopteran expression vectors utilize a variety of promoters derived from baculoviruses and lepidopteran cells, including the AcNPV *ie-1* promoter, the OpNPV *ie-2* promoter, and the *B. mori actin* gene promoter; these promoters function mainly in lepidopteran cell lines such as Sf21, Sf9, High Five™, or Bm5 cells.

4.14.3.3.1. Constitutive expression

4.14.3.3.1.1. Baculovirus immediate early promoters A lepidopteran insect cell-based expression system was initially developed by Dr Don Jarvis at Texas A&M University (Jarvis *et al.*, 1990). To generate stably transformed cell lines, Sf9 cells were cotransfected with a neomycin resistance plasmid, an expression vector employing the AcNPV immediate early gene promoter (*ie-1*) and a region containing mRNA polyadenylation signals (Guarino and Summers, 1987), followed by selection and isolation of G418 resistant clones over a period of 4 weeks following transfection. From the transformed insect cell clones, the expression levels of β-galactosidase (an intracellular protein) were approximately 100-fold lower than those obtained from a baculovirus expression vector employing the polyhedrin promoter, while the level of t-PA (a secreted protein) obtained was only two-fold lower. Despite the low values, these experiments demonstrated the potential of using stable lepidopteran cell lines for producing those recombinant proteins directed through the secretory pathway as an alternative to a baculovirus. The *ie-1* gene promoter was also shown to function in dipteran cell lines (Vanden Broeck *et al.*, 1995). Improvements in the expression vector were made by incorporating the AcNPV homologous repeat 5 (HR5) transcriptional enhancer element (Guarino *et al.*, 1986) upstream of the *ie-1* promoter (Jarvis *et al.*, 1996). HR5 was shown to act in *cis* to significantly stimulate the expression of a reporter protein from early baculovirus promoters such as *ie-1* and *p35* in transient expression assays (Rodems and Friesen, 1993; Pullen and Friesen, 1995). A series of vectors utilizing the HR5-enhanced AcNPV *ie-1* promoter are available from Novagen for stable or transient protein expression. A similar set of expression vectors has been developed based on the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) *ie-2* promoter (Theilmann and Stewart, 1992; Pfeifer *et al.*, 1997; Hegedus *et al.*, 1998), which can function in both lepidopteran and dipteran insect cell lines. These vectors are available from Invitrogen as the Insect Select™ vector set.

4.14.3.3.1.2.* Bombyx mori *cytoplasmic actin promoter A powerful lepidopteran expression cassette, pIE1/153A, was recently developed in the laboratory of Dr Kostas Iatrou at the University of Calgary. The cassette utilizes the silk moth (*B. mori*) cytoplasmic actin gene promoter (Mounier and Prudhomme, 1986; Johnson *et al.*, 1992), one of the stronger constitutive cellular promoters that is also active in a variety of transfected lepidopteran cell lines. Transcription from this cellular promoter was surprisingly found to be stimulated by the immediate early gene product (*ie-1*) of BmNPV, IE-1, a transcription factor capable of stimulating the *in vitro* rate of transcription from the actin promoter in *trans* by up to 100-fold (Lu *et al.*, 1996). Stimulation of the actin promoter by up to two orders of magnitude was also obtained by linking in *cis* the homologous repeat 3 (HR3) region of BmNPV in various orientations relative to the actin promoter (Lu *et al.*, 1997). Linkage of the *ie-1* gene and the HR3 enhancer element with the actin gene promoter in the pIE1/153A expression cassette resulted in a stimulation of foreign gene expression directed by the actin promoter by approximately 5000-fold in transient expression assays for two reporter proteins (Lu *et al.*, 1997). Stable cell lines have been generated by cotransfecting the cells with the expression cassette and a second plasmid conferring resistance to hygromycin B, puromycin, or G418. Reported expression levels of secreted proteins from stable cell lines transformed with this system have exceeded, by far, those obtained by the baculovirus expression system (Farrell *et al.*, 1998; Farrell *et al.*, 1999). Furthermore the expression plasmid functions in a wide variety of lepidopteran cell lines including those derived from *B. mori*, *T. ni*, *Plodia interpuntella*, *L. dispar*, *Mamestra brassicae*, *C. fumiferana*, and *S. frugiperda* (Keith *et al.*, 1999). The expression cassette is also suited to scaled-up transient expression protocols that can yield tens of milligrams of recombinant protein per liter in 5 days posttransfection (Farrel and Iatrou, 2004) (**Figure 5**), thus avoiding the time consuming and labour intensive process of stable antibiotic selection and the cloning of high expressors. Derivatives of the pIE1/153A expression cassette are available from Dr Iatrou.

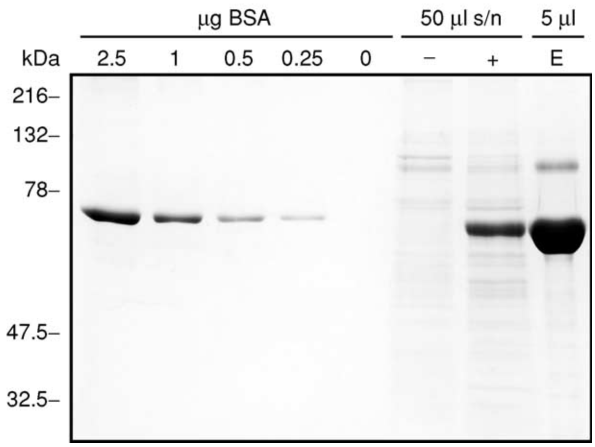


Figure 5 Transient expression and affinity purification of histidine-tagged human secreted alkaline phosphatase (SEAP; kindly provided by Eric Carpentier and Amine Kamen, Biotechnology Research Institute, Montreal, Canada) from a lipofectin-mediated transfection of High Five^{TM} cells in suspension culture using the pIE1/153A expression cassette. By comparison to a series of bovine serum albumin mass standards on the Coomassie blue-stained gel, approximately 50 µg ml^{-1} of SEAP was present in the culture supernatant 5 days after transfection and production in protein-free medium ESF-921 (lane marked "+" compared to control marked "−"). Two milligram of SEAP was recovered from one elution fraction (E) following Ni-ion affinity purification from 50 ml of culture supernatant.

4.14.3.4. Insect Cell-Based Expression Using Inducible Promoter Systems

In contrast to constitutive promoters, the use of inducible promoters has the advantage that it allows the production of proteins that are toxic or growth-inhibitory to the cells. In such cases, cells are first grown in batch mode (rather than continuously) to high densities. When sufficiently high densities are reached, the inducer is added to the cell culture to induce high-level protein expression. Of importance to the choice of an inducible expression system is the induction ratio of gene expression. In the absence of inducer, promoter activity should be minimal as to prevent accumulation of toxic proteins during the growth phase of the cells. Addition of the inducer should, subsequently, result in the accumulation of high amounts of protein.

4.14.3.4.1. Heat shock promoter The *Drosophila hsp70* promoter (Thummel and Pirrotta, 1992) has been widely used to achieve inducible gene expression in transgenic flies. The promoter is heat-inducible and functions during all stages of development and in all tissues (Steller and Pirrotta, 1984; Spradling, 1986). It has a low basal expression coupled with high inducibility (>100-fold at 42 °C) (Huynh and Zieler, 1999). The promoter is not only functional in *Drosophila* cells but also in mosquito and lepidopteran cell lines (Zhao and Eggleston, 1999; Helgen and Fallon, 1990; Lan and Riddiford, 1997).

While the *hsp70* promoter has been used to mediate expression of membrane proteins (Shotkoski *et al.*, 1996) and to drive expression of "helper" antibiotic resistance genes for the establishment of

permanently transformed cell lines (Lycett and Crampton, 1993; Vanden Broeck *et al.*, 1995) and transgenic mosquitoes (Miller *et al.*, 1987), its application for the inducible expression of proteins at high levels for functional or structural characterization is considered limited. Long-term exposure to high temperature, which is considered necessary to reach the highest expression levels, is generally detrimental to the cell cultures. Also the translational induction to heat shock is reported to be much lower than the transcriptional induction (Cherbas *et al.*, 1994), presumably because the heat shock interferes with posttranscriptional processing and translation.

Heat shock (*hsp70*) promoter-based reporter and expression constructs were also incorporated in baculovirus vectors to monitor infection of tissues and cells and to achieve protein production. In the context of the baculovirus genome in infected cells, however, gene expression from the *hsp70* promoter was constitutive rather than heat-inducible (Lee *et al.*, 2000; Moto *et al.*, 2003).

4.14.3.4.2. Metallothionein promoter Transcription of mammalian and *Drosophila* metallothionein genes is activated by heavy metal load via activation of the metal-responsive factor 1 (MTF-1) (Zhang *et al.*, 2001). Inducible expression of genes via the metallothionein promoter is achieved in *Drosophila* S2 cells and other dipteran cell lines (Johansen *et al.*, 1989; Kovach *et al.*, 1992; Millar *et al.*, 1995; Hegedus *et al.*, 1998). Protein expression via the metallothionein promoter is induced by the addition of cadmium or copper ions (10–1000 μM) and induction levels of several 100-fold are achieved (Bunch *et al.*, 1988; Otto *et al.*, 1987; Zhang *et al.*, 2001). The metallothionein promoter-based inducible system is considered suitable for the inducible expression of many different classes of proteins in dipteran cell lines.

A disadvantage of the metal ion-inducible expression system is that at the high metal ion concentrations required for achieving the highest expression levels, toxic effects are also observed on the cells, such as cytoplasmic vacuolization and granule formation as well as an associated reduction in their growth rate (Bunch *et al.*, 1988). Thus, optimal protein production requires determination of the right balance between degree of induction of gene expression and general cellular toxicity.

While the metal ion-mediated induction works efficiently in *Drosophila* and mammalian cell lines (Zhang *et al.*, 2001), in lepidopteran cell lines such as Sf9, Ld652Y, and Bm5, the metallothionein promoter was either not or only marginally functional (Hegedus *et al.*, 1998; V. Douris, L. Swevers and K. Iatrou, unpublished observations).

4.14.3.4.3. Ecdysone-inducible promoters Many insect tissue culture cell lines are sensitive to the insect molting hormone 20-hydroxyecdysone (20E) and respond to it by morphological changes and a decline in growth rates (Sorbrier *et al.*, 1989; Sohi *et al.*, 1995; Smagghe *et al.*, 2003). At the molecular level, 20E exerts its actions by binding to the ecdysone nuclear receptor (EcR) that exists as a heterodimer with the nuclear receptor Ultraspiracle (USP) (Thomas *et al.*, 1993; Yao *et al.*, 1993; Swevers *et al.*, 1996) and the hormone-bound complex directly activates transcription at ecdysone-response elements (EREs) in promoters of target genes (Antoniewski *et al.*, 1996; Vogtli *et al.*, 1998; Wang *et al.*, 1998; see **Chapter 3.5**). Early-response genes, which are directly regulated by the EcR/USP complex, are induced in insect cell lines after challenge with 20E (Sohi *et al.*, 1995; Chen *et al.*, 2002; Swevers *et al.*, 2003b), indicating that all transduction elements for gene activation by 20E are present.

Basal reporter cassettes that contain multiple repeats of an ERE derived from the *Drosophila hsp27* promoter are 1000- to 2000-fold induced by μM quantities of 20E in *Drosophila* S2 cells as well as *Bombyx*-derived (lepidopteran) Bm5 cells (Koelle *et al.*, 1991; Swevers *et al.*, 2003b). The induced expression levels are comparable to those obtained by strong constitutive promoters (Swevers *et al.*, 2003b) and silk moth (lepidopteran) transformed cell lines incorporating an ecdysteroid-inducible expression system have been described for production of recombinant proteins (Tomita *et al.*, 2001) as well as high-throughput screening for potential 20E agonists and antagonists (Swevers *et al.*, 2003b).

An advantage of the ecdysteroid-inducible expression system is that, in the absence of ligand, the EcR/USP heterodimer functions as a repressor of gene transcription (Tsai *et al.*, 1999) and the basal expression levels in the absence of the inducer are very low. Because 20E is a natural hormone, toxicity effects on the cell cultures are virtually nonexistent. Nevertheless, it remains to be investigated in detail whether the physiological effects of 20E on the function of the cells could potentially interfere with their capacity for protein production. It is expected that this will not be the case: using the baculovirus/lepidopteran cell line expression system, it was reported that the addition of ecdysteroids actually increased recombinant protein production (Sarvari *et al.*, 1990).

4.14.3.5. Classes of Recombinant Proteins Expressed by Transformed Insect Cells

4.14.3.5.1. Secreted proteins A variety of heterologous secreted proteins have been expressed from stable insect cell lines at similar or higher expression levels than can be obtained using baculovirus expression vectors (Table 2). Certainly the cellular or early-phase baculovirus promoters employed are not as transcriptionally powerful as late- and very late phase promoters such as those of the polyhedrin, p10 or basic protein genes. However, the fact that the cell's secretory pathway is not being damaged in the absence of viral infection, contributes to the improvement in the expression level and quality of the overexpressed protein compared to expression using recombinant baculovirus. The substitution of the native mammalian signal peptide coding sequence in a heterologous mammalian gene with a sequence encoding an insect-specific signal peptide was found to have no effect on the resulting expression level in transfected insect cells (Farrell *et al.*, 2000).

4.14.3.5.2. Membrane proteins As with secreted proteins, the absence of viral infection may provide a superior cellular environment for the production of membrane proteins which also traverse the secretory pathway. Several functional membrane proteins have been successfully expressed in stable insect cell lines including ion exchangers (Szerenscei *et al.*, 2000), transmitter gated ion channels (Joyce *et al.*, 1993; Millar *et al.*, 1994; Smith *et al.*, 1995; Atkinson *et al.*, 1996; Buckingham *et al.*, 1996), and GPCRs (Kleymann *et al.*, 1993; Swevers *et al.*, 2003a; see **Chapter 5.5**). As found with the baculovirus expression system (Bouvier *et al.*, 1998), coupling of heterologous GPCRs with endogenous insect G proteins may be inefficient following ligand binding, even in transformed insect cells (Figure 6). However, a strategy that has proven useful for the functional expression of heterologous GPCRs is the coexpression of mammalian G proteins in stably transformed insect cells (L. Swevers, K. Iatrou, E. Morou, N. Balatsos, and Z. Georgoussi, unpublished data; see also further below).

4.14.3.5.3. Intracellular proteins The use of transformed insect cells for the expression of intracellular proteins is of limited potential compared to the baculovirus expression system. There are a few reports on the concentrations of intracellular proteins obtained, however the promoters employed in stable gene expression from insect cells are considerably weaker than those utilized in baculovirus expression vectors (Jarvis *et al.*, 1990; Percival *et al.*, 1997; Pfeifer *et al.*, 1997). Furthermore, the purification of heterologous intracellular proteins, expressed at relatively low levels inside insect cells, from endogenous cellular protein mixtures is expected to be difficult. Secretion of the intracellular protein would facilitate the purification process. However, the mere fusion of an insect-specific signal peptide to the N-terminus of an intracellular protein failed to provide the means necessary to secrete intracellular proteins (Farrell *et al.*, 2000). It was realized that other biological signals in addition to a signal peptide are required for efficient secretion from insect and mammalian cells into an extracellular environment. These could be supplied by fusing the complete coding sequence of a secreted protein to that of an intracellular protein (Figure 7; Farrell *et al.*, 2000). The chimeric proteins produced are soluble and can be easily purified from

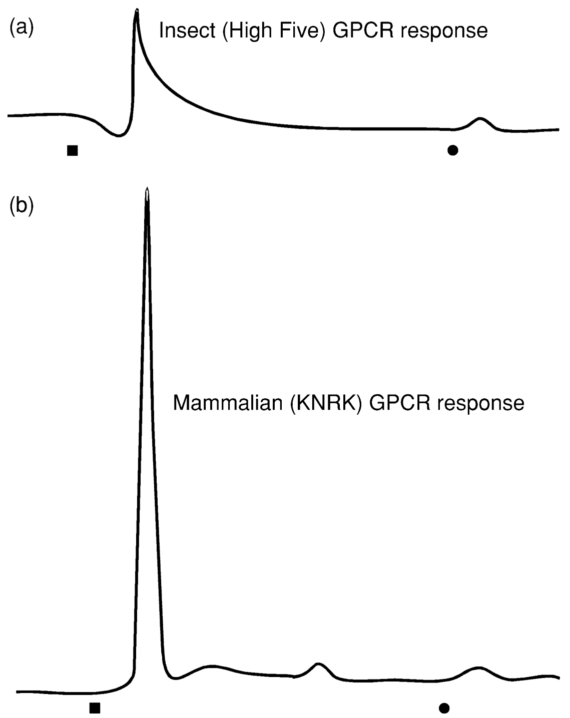


Figure 6 Fluorescent spectrophotometer outputs of calcium signaling assays to compare the G protein-coupled receptor (GPCR) response of a mammalian GPCR, the rat protease activated receptor 2 (rPAR2; Hollenberg *et al.*, 1996), expressed in stably transformed insect (panel a, High Five^{TM}) and mammalian (panel b, KNRK) cell lines. The amplitude reflects elevations in intracellular calcium with time, following exposure to the rPAR-2 peptide agonist, SLIGRL, at the time points denoted by the filled square and circle. Despite the fact that the receptor density was higher in the insect cell line (data not shown), the results imply that this mammalian GPCR does not couple well with the endogenous insect G proteins. Courtesy of Dr Morley Hollenberg, Mamoud Saifeddine and Bajhat Al-Ani (University of Calgary, Canada).

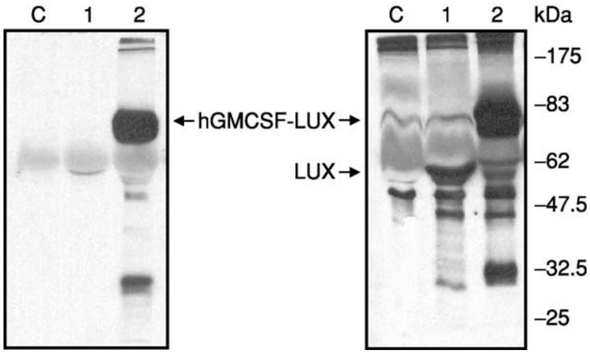


Figure 7 Secretion of luciferase from lepidopteran insect cells transfected with the pIE1/153A expression plasmid that encompasses a luciferase secretion module. Normally, luciferase (LUX) is expressed intracellularly, however, by fusing it with a secreted protein, in this case the human granulocyte macrophage colony stimulating factor (hGMCSF), the normally intracellular protein can be dragged into the supernatant as a chimera. High Five cell culture supernatants are shown in the SDS-PAGE/Western blots probed with (left) an anti-hGMCSF antibody and (right) an anti-LUX antibody. The lane marked ''C'' contains protein from a control transfection using the pIE1/153A expression vector without inserted transgene. Lanes 1 contain supernatants from cells transfected with vector pIE1/153.lux directing intracellular expression of luciferase, and Lanes 2 contain supernatants from cells transfected with vector pIE1/153A.hgmcsf-lux directing the secretion of the overexpressd hGMCSF-LUX fusion protein. Although some luciferase does leak from the cells naturally, a large proportion is secreted when expressed as a hGMCSF-LUX fusion protein.

the cell culture supernatant under nondenaturing conditions.

4.14.4. Insect Cell-Based High-Throughput Screening Systems

In cell-based detection systems, cell lines are engineered such that they respond to a biological stimulus by the generation of an easily detectable, fluorescent or luminescent, signal. Accordingly, cell-based detection systems integrate two relevant genetic elements, (1) an expression element that directs expression of the target molecule (e.g., a receptor) thus allowing the cells to respond specifically to bioactive compounds (e.g., ligands) that interact specifically with the target and initiate a downstream cell signaling response, and (2) a reporter element that allows the cells to generate an easily measurable response (fluorescent or luminescent signal) following activation of the expressed receptor by the bioactive compound.

Insect cell-based high throughput screening systems have been developed for two major types of receptors, nuclear hormone receptors (see **Chapters 3.4–3.6**) and membrane-anchored GPCRs (see **Chapter 5.5**). Because nuclear hormone receptors activate directly transcription at specific target sites in the DNA (Gronemeyer and Laudet, 1995), detection of nuclear receptor activation occurs by induction of expression of appropriate fluorescent (GFP or its variants) or luminescent (luciferase) reporter genes. On the other hand, GPCRs, which are subdivided into several classes according to the type of signal transduction pathway that they generate at the plasma membrane (cAMP up- or downregulation, release of Ca^{2+}, activation/inhibition of ion channels) (Hamm, 2001; Marinissen and Gudkind, 2001) require different reporter assays that are designed according to the specificity of each receptor class. Since the release of Ca^{2+} can be easily measured by fluorescent (e.g., employing the Ca^{2+}-sensitive fluorescent dyes fura-2 or fluo-3) or bioluminescent (coelenterazine/aequorin-based) methods (Grynkiewicz *et al.*, 1985; Knight *et al.*, 1991), activation of GPCRs that naturally function by Ca^{2+} release can be monitored by the detection of Ca^{2+} accumulation. For other GPCRs, the possibility exists to coexpress them with the mammalian $G\alpha_{16}$ protein, which can couple GPCRs of other transduction classes to the Ca^{2+} pathway (Kostenis, 2001).

4.14.4.1. Nuclear Receptors: The Ecdysone Receptor

Of the approximately 20 nuclear hormone receptors identified in the insect (*Drosophila* or *Anopheles*) genome (Adams *et al.*, 2000; Zdobnov *et al.*, 2002; see **Chapters 3.5** and **3.6**), only one has a clearly identified ligand. This is EcR, the receptor for the insect moulting hormone 20E (Escriva *et al.*, 1997). Because many insect cell lines express functional EcR receptors (Sohi *et al.*, 1995; Chen *et al.*, 2002; Swevers *et al.*, 2003a), they have the potential to be developed as screening systems for the 20E hormone (see **Chapter 3.4**).

A microplate-based bioassay, for the detection of 20E mimetic (agonistic and antagonistic) activities, was described that is based on changes in growth or morphology of the *Drosophila* B_{II} tumorous blood cell line (Clement *et al.*, 1993). This cell line is used to screen natural products from plants and fungi as well as collections of synthetic ecdysteroid derivatives for moulting and antimoulting hormone activity (Dinan *et al.*, 1997; Harmatha and Dinan, 1997; Dinan *et al.*, 2001; Harmatha *et al.*, 2002).

Another, more recently developed high throughput screening system for 20E agonists and antagonists, is based on silk moth-derived Bm5 cells that have been engineered to incorporate an ecdysteroid-responsive GFP reporter cassette in their genome

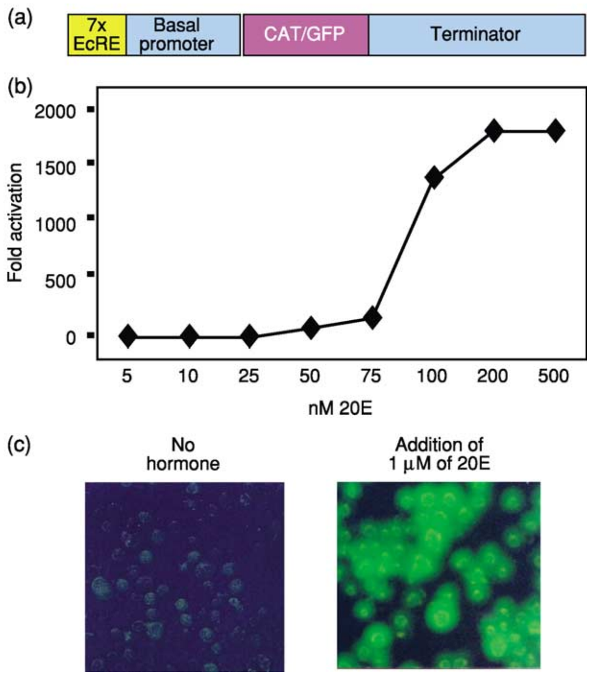


Figure 8 Establishment of a high-throughput screening system for ecdysone agonists and antagonists based on transformed silk moth-derived Bm5 cells. (a) Schematic presentation of the ecdysone-responsive reporter constructs. EcRE, ecdysone-responsive element; CAT, chloramphenicol acetyl transferase; GFP, green fluorescent protein. (b) Dose-response curve of the ecdysone-responsive CAT reporter construct in transient expression experiments. Shown is the fold inducibility in function at different hormone concentrations. (c) Induction of green fluorescence by 20E in a clonal transformed cell line that contains integrated copies of the ecdysone-responsive GFP reporter construct in their chromosomes.

(Swevers *et al.*, 2003b; **Figure 8**). The half-maximal response of activation of the reporter cassette is elicited by concentrations of 20E between 50 and 100 nM, which are comparable to the concentrations required for induction of morphological or growth inhibitory responses in other cell lines (Cherbas *et al.*, 1980; Dinan *et al.*, 1997). An important advantage of the ecdysteroid-responsive fluorescent cell lines is that the amount of fluorescence emitted by the cells can be easily detected and quantified from cells seeded in multiwell plates by a fluorescence plate reader, thus rendering the system amenable to high-throughput analysis. The ecdysteroid-inducible fluorescent Bm5 cell lines were used successfully to screen a collection of plant extracts as well as a chemical library of potential nonsteroidal agonists (dibenzoyl hydrazine derivatives) for detection of 20E agonist and antagonist activities (Swevers *et al.*, 2003b). The dibenzoyl hydrazine compounds selected by the high-throughput assay were also effective in larval toxicity tests, thus validating the usefulness of the fluorescent screening system for rapid selection of 20E mimics as potential new insecticides.

4.14.4.2. G Protein-Coupled Receptors

The majority of signal transduction by hormones or neurotransmitters is mediated by GPCRs (Vanden Broeck, 2001). In the two insects whose complete genome sequence is known, *D. melanogaster* and *Anopheles gambiae*, GPCRs comprise the largest number of genes, accounting for 1–2% of the total gene number (Hill *et al.*, 2002). Similarly, in the human genome more than 600 GPCR genes have been identified. Human GPCRs are considered important targets for drug development, and it is estimated that up to 40–50% of the available drugs are modulators of GPCR function (Kostenis, 2001).

Using the AcNPV-based baculovirus expression system, several types of mammalian GPCRs have been successfully expressed in *S. frugiperda* Sf9 and *T. ni* Hi5 cell lines (Vasudevan *et al.*, 1992; Ng *et al.*, 1995, 1997; Zhang *et al.*, 1995; Wehmeyer and Schulz, 1997; Ohtaki *et al.*, 1998). While the AcNPV-based expression system is not amenable to high throughput screening development because the cells are lysed at the end of the infection cycle, these studies nevertheless demonstrated that the receptors that were overexpressed were functional and could couple efficiently to downstream signaling elements such as ion channels and the adenylate cyclase system. Thus, using plasmid-based expression systems that do not result in cell lysis (Farrell *et al.*, 1998), transformed cell lines can be obtained that express constitutively functional GPCRs for screening of ligand mimetics.

Drosophila Schneider 2 cells have been used by several research groups to achieve permanent and functional expression of insect as well as mammalian GPCRs (Joyce *et al.*, 1993; Kleymann *et al.*, 1993; Millar *et al.*, 1995; Tota *et al.*, 1995; Vanden Broeck *et al.*, 1995; Buckingham *et al.*, 1996; Torfs *et al.*, 2000; Perret *et al.*, 2003). In the case of a tachykinin receptor from the stable fly *Stomoxys calcitrans*, activation resulted in the detection of Ca^{2+} release by fluorescent and luminescent methods, thus making the transformed cell line an effective system for the screening of tachykinin-like ligands (Torfs *et al.*, 2002).

In mammalian cell-based screening systems, the "promiscuous" Gα proteins, $G\alpha_{15}$ and $G\alpha_{16}$, have been used to couple the activation of any GPCR to the Ca^{2+} signaling pathway (Stables *et al.*, 1997; Kostenis, 2001). The presence of the $G\alpha_{16}$ protein results in the activation of phospholipase Cβ

(PLCβ) resulting in the production of the secondary messengers diacyl glycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). While DAG activates other downstream pathways through activation of protein kinase C, the action of IP_3 results in the release of Ca^{2+} from intracellular stores (Stables *et al*., 1997), which can be subsequently detected easily by fluorescent or bioluminescent methods (Grynkiewicz *et al*., 1985; Knight *et al*., 1991). Cell lines overexpressing $G\alpha_{16}$ protein can, therefore, be used to screen for ligands of GPCRs that normally couple to other second messenger or to ion channels.

This strategy has been employed recently in the case of transformed silk moth Bm5 cells that overexpress the murine δ opioid receptor: coexpression of the human $G\alpha_{16}$ protein in these cells has resulted in the stimulation of the coupling of the mouse receptor to the PLCβ/IP_3 pathway, thus making the transformed cell line a very effective screening system for ligands of the δ opioid receptor (L. Swevers, K. Iatrou, E. Morou, N. Balatsos and Z. Georgoussi, unpublished data).

4.14.5. Glycosylation of Recombinant Proteins in Insect Cells

Considerable attention has been paid to the glycosylation patterns of heterologous recombinant proteins produced in insect cells, particularly N-linked glycosylation. Several excellent and comprehensive reviews in this area have been published by Jarvis *et al*. (1998), Altmann *et al*. (1999), Marchal *et al*. (2001), and Jarvis (2003). In these reviews, it is agreed that oligomannose (five to nine mannose residues; Altmann *et al*., 1999) and fucosylated trimannose type carbohydrate (Jarvis *et al*., 1998) moieties dominate the N-linked glycosylation pattern of heterologous secreted and membrane proteins expressed in insect cells. The lack of complex glycosylation, such as the presence of terminal sialic acid found on many mammalian glycoproteins, excludes the use of insect cells to express mammalian glycoproteins for some applications. One solution to this limitation is to engineer baculoviruses (Jarvis *et al*., 1996; Wagner *et al*., 1996) or insect cell lines (Hollister *et al*., 1998) to express mammalian glycotransferases necessary for complex glycosylation of an over-expressed protein. The coexpression of two mammalian glycotransferases, β-1,4-galactosyltransferase and α-2,6-sialyltransferase, enabled an insect cell for the first time to produce an artificially sialylated baculovirus glycoprotein gp64 (Seo *et al*., 2001). This research will almost certainly extend the utility of insect cells to the production of "mammalized" complex glycoproteins.

4.14.6. Conclusions and Future Perspectives

Baculoviruses and insect cell lines have acquired a significant biotechnological niche for the production of proteins of general scientific and pharmaceutical value. While baculovirus vectors provide a superior system for high level expression of intracellular proteins, their use for the production of proteins that require an intact cellular environment is considered to be suboptimal. In contrast, the recently developed expression systems that employ baculovirus-derived genetic elements and direct continuous high level expression in transformed insect cells, not only circumvent the major drawbacks of the baculovirus expression systems for secreted and membrane-anchored proteins but can also be employed as high-throughput screening tools for the identification of bioactive substances with defined biological specificities.

For the baculovirus system, great technical improvements have occurred, mostly with regard to the efficiency and ease of generating recombinant viruses, with the best case being the generation of recombinant viruses through direct cloning into bacterially isolated bacmid chromosomal DNA. Because of the high degree of sophistication already achieved, relatively little improvement in this system is expected in the coming years. By contrast, the use of plasmid-based expression systems for insect cells is relatively recent and important improvements and new applications are expected to arise in the future.

Plasmid-based expression systems are not, in general, considered optimal for the production of intracellular proteins, because their isolation requires cell lysis and purification from a multitude of contaminating cellular proteins. Improvements in the production process are expected to include the redirection of normally intracellular recombinant proteins toward the extracellular medium, from which they can be purified with relative ease, particularly if the medium is serum-free. To test this strategy, secretion modules derived from secreted proteins, such as JHE and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been fused in-frame with the N-termini of intracellular proteins, such as the CAT enzyme and the BmCF1 nuclear receptor (Farrell *et al*., 2000). Efficient secretion to the extracellular medium has been reported and, because the engineered proteins also contained a histidine tag and an enteropeptidase cleavage site, single-step purification

could be achieved by metal-ion affinity binding coupled with proteolytic cleavage (Farrell *et al.*, 2000). Future modifications in this method may include the use of alternative affinity tags and proteolytic sites as well as improved versions of the secretion modules.

More recently, *Drosophila* S2 cell lines have been used as expression hosts for the purification of multiprotein complexes that are dedicated to the execution of particular cellular functions (Forler *et al.*, 2003). Key factors involved in particular cellular processes (e.g., splicing of mRNA precursor molecules, RNA quality control, nuclear export) were expressed as double-tagged proteins, while the expression of endogenous untagged proteins was suppressed by RNA interference. Tagged proteins, together with assembled interacting factors, were purified by the "tandem-affinity purification" strategy and component proteins of purified complexes were identified by Western blot and mass spectrometry (Forler *et al.*, 2003). The technique will be valuable for the characterization of multiprotein functional complexes operating in metazoan species, in general, and in insect ones, in particular.

Transformation of insect cell lines with expression constructs still represents a cumbersome process requiring selection of resistant cell clones after addition of antibiotics. It is expected, however, that alternatives to the method of selecting cells containing genomic integrants of plasmid vectors by antibiotic selection, including the use of vectors capable of directing autonomous integration into the host genome, will be developed in the immediate future. In fact, recent reports suggest that the use of a plasmid encompassing the genomic sequences of the *Junonia coenia* densovirus (JcDNV) directs autonomous genomic integration in insect cells grown in culture, as well as *in vivo*, without affecting the normal physiology of the host cells in any detectable way (Bossin *et al.*, 2003). Furthermore, it has also been reported that this system offers the additional advantage of integrated copy number manipulation through directed deletion of viral genome sequences from the expression plasmid (Bossin *et al.*, 2003). Although the production capabilities of this system have yet to be tested with specific protein models, it is anticipated that the incorporation in it of promoter systems that are in current use as drivers of recombinant protein expression in insect cells, will expand further the current capabilities and advantages of the nonlytic systems utilizing insect cells as hosts for continuous high level production of recombinant proteins over lytic ones. The major advantage of the use of the densovirus-based transformation system is the ease of selection of transformed cells by FACS analysis through the detection of red fluorescence in the cell nuclei (achieved by the nuclear expression of DsRed/viral capsid protein fusions directed by the use of the viral P9 promoter; Bossin *et al.*, 2003). Finally, the detection of efficient densovirus amplification and densovirus-derived transgene expression in *Drosophila* larval and adult tissues (Royer *et al.*, 2001) indicates that the densovirus-based transformation system is not limited to lepidopteran cell lines but can be applied to dipteran cell lines as well.

In mammalian systems, many nuclear receptors and GPCRs have been characterized at the molecular level using transformed cell lines, and this has led to the discovery of lead compounds (and drugs) that interfere specifically at very low doses with the function of the receptors. Equivalent data on insect receptors, however, are currently lacking to a great extent. Although the identification of the natural ligands for orphan insect (*Drosophila*) GPCRs is now occurring at a good pace, the identification of the relevant ligands was made possible through the use of heterologous expression systems such as transformed mammalian cell lines or *Xenopus* oocytes (Birgül *et al.*, 1999; Meeusen *et al.*, 2002; Staubli *et al.*, 2002; Johnson *et al.*, 2003). In this regard, we expect that insect cell lines would provide better host expression systems for insect GPCRs and could be employed for a more effective screening for compounds that interfere with their functions. The same applies for the identification of ligands for the approximately 20 orphan nuclear receptors that exist in the insect genome (Adams *et al.*, 2000; Zdobnov *et al.*, 2002). The transcriptional properties of several of these receptors have been characterized (e.g., the HNF-4 receptor (Kapitskaya *et al.*, 1998), the FTZ-F1 receptor (Suzuki *et al.*, 2001), and the HR3 and E75 receptors (Swevers *et al.*, 2002)) and it should be relatively straightforward to design cell-based reporter systems for screening of ligands, particularly small molecules capable of blocking their functions. In this regard, it is worth noting that the *Drosophila* DHR38/USP receptor complex was recently found to be activated by various ecdysteroids (Baker *et al.*, 2003), illustrating the as yet unrealized potential of orphan nuclear receptors as targets for interference by small molecule ligands. Thus, like their mammalian counterparts, insect orphan receptors can be considered as targets for interference by small molecules, which can be developed into environmentally friendly insecticides that function as "endocrine disruptors," and insect cell-based expression systems are anticipated to play an instrumental role in the discovery effort.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Airenne, K.J., Oker-Blom, C., Marjomaki, V.S., Bayer, E.A., Wilchek, M., *et al.*, **1997**. Production of biologically active recombinant avidin in baculovirus-infected insect cells. *Protein Expr. Purif. 9*, 100–108.

Alnemri, E.S., Litwack, G., **1993**. The steroid binding domain influences intracellular solubility of the baculovirus overexpressed glucocorticoid and mineralocorticoid receptors. *Biochemistry 32*, 5387–5393.

Altmann, F., Staudacher, E., Wilson, I.B.H., Marz, L., **1999**. Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconjugate J. 16*, 109–123.

Angelichio, M.L., Beck, J.A., Johansen, H., Ivey-Hoyle, M., **1991**. Comparison of several promoters and polyadenylation signals for use in heterologous gene expression in cultured Drosophila cells. *Nucleic Acids Res. 19*, 5037–5043.

Antoniewski, C., Mugat, B., Delbac, F., Lepesant, J.-A., **1996**. Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster. Mol. Cell. Biol. 16*, 2977–2986.

Atkinson, A.E., Henderson, J., Hawes, C.R., King, L.A., **1996**. Efficient membrane targeting of the chick nicotinic acetylcholine receptor a-subunit in insect cell lines. *Cytotechnology 19*, 37–42.

Bader, B., Knecht, W., Fries, M., Loffler, M., **1998**. Expression, purification and characterization of histidine-tagged rat and human flavoenzyme dihydroorotate dehydrogenase. *Protein Expr. Purif. 13*, 414–422.

Bailer, S.M., Berlin, W.K., Starr, C.M., Hanover, J.A., **1995**. Characterization of nuclear pore protein P62 produced using a baculovirus. *Protein Expr. Purif. 6*, 546–554.

Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., *et al.*, **2003**. The *Drosophila* orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell 113*, 731–742.

Baldock, D., Graham, B., Akhlaq, M., Graff, P., Jones, C.E., *et al.*, **2000**. Purification and characterization of human syk produced using a baculovirus expression system. *Protein Expr. Purif. 18*, 86–94.

Barr, A.J., Brass, L.F., Manning, D.R., **1997**. Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. *J. Biol. Chem. 272*, 2223–2229.

Belyaev, A.X., Hails, R.S., Roy, P., **1995**. High-level expression of five foreign genes by a single recombinant baculovirus. *Gene 156*, 229–233.

Birgül, N., Weise, C., Kreienkamp, H.-J., Richter, D., **1999**. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J. 18*, 5892–5900.

Bonning, B.C., Hammock, B.D., **1995**. Use of juvenile hormone esterase as a novel reporter enzyme in the baculovirus expression system. *J. Vir. Meth. 51*, 103–114.

Bonning, B.C., Roelvink, P.W., Vlak, J.M., Possee, R.D., Hammock, B.D., **1994**. Superior expression of juvenile hormone esterase and β-galactosidase from the basic protein promoter of *Autographa califonica* nuclear polyhedrosis virus compared to the p10 protein and polyhedrin promoters. *J. Gen. Virol. 75*, 1551–1556.

Bonning, B.C., Ward, V.K., van Meer, M.M.M., Booth, T.F., Hammock, B.D., **1997**. Disruption of lysosomal targeting is associated with insecticidal potency of juvenile hormone esterase. *Proc. Natl Acad. Sci. USA 94*, 6007–6012.

Bossin, H., Fournier, P., Royer, C., Barry, P., Cérutti, P., *et al.*, **2003**. *Junonia coenia* densovirus-based vectors for stable transgene expression in Sf9 cells: influence of the densovirus sequences on genomic integration. *J. Virol. 77*, 11060–11071.

Boublik, Y., Di Bonito, P., Jones, I.M., **1995**. Eukaryotic virus display, engineering the major surface glycoprotein of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. *Bio/Technol. 13*, 1079–1084.

Bouvier, M., Menard, L., Dennis, M., Marullo, S., **1998**. Expression and recovery of functional G-protein-coupled receptors using baculovirus expression systems. *Curr. Opin. Biotechnol. 9*, 522–527.

Boyce, F.M., Bucher, N.L., **1996**. Baculovirus-mediated gene transfer into mammalian cells. *Proc. Natl Acad. Sci. USA 93*, 2348–2352.

Bromme, D., McGrath, M.E., **1996**. High level expression and crystallization of recombinant human cathespin S. *Protein Sci. 5*, 789–791.

Brown, P.M., Scheid, M.P., O'Neill, G.P., Tagari, P.C., Nocholson, D.W., **1995**. A single-step purification of biologically active recombinant human interleukin-5 from a baculovirus expression system. *Protein Expr. Purif. 6*, 63–71.

Buckingham, S.D., Matsuda, K., Hosie, A.M., Baylis, H.A., Squire, M.D., *et al.*, **1996**. Wild-type and insecticide-resistant homo-oligomeric GABA receptors of *Drosophila melanogaster* stably expressed in a *Drosophila* cell line. *Neuropharmacology 35*, 1393–1401.

Bunch, T.A., Grinblat, Y., Goldstein, L.S.B., **1988**. Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nucl. Acids Res. 16*, 1043–1061.

Butkerait, P., Zheng, Y., Hallak, H., Graham, T.E., Miller, H.A., *et al.*, **1995**. Expression of the human 5-hydroxytryptamine1A receptor in Sf9 cells. Reconstitution of a coupled phenotype by co-expression of mammalian G protein subunits. *J. Biol. Chem. 270*, 18691–18699.

Cann, P.L., Touze, A., Enogat, N., Leboulleux, D., Mougin, C., *et al.*, **1995**. Detection of antibodies against human papillomavirus (HPV) type 16 virions by enzyme-linked immunosorbent assay using recombinant HPV 16 L1

capsids produced by recombinant baculovirus. *J. Clin. Microbiol. 33*, 1380–1382.

Canaan, S., Dupuis, L., Riviere, M., Faessel, K., Romette, J.-L., *et al.*, **1998**. Purification and interfacial behavior of recombinant human gastric lipase produced from insect cells in a bioreactor. *Protein Expr. Purif. 14*, 23–30.

Carbonell, L.F., Miller, L.K., **1987**. Baculovirus interaction with nontarget organisms: a virusborne reporter gene is not expressed in two mammalian cell lines. *Appl. Environm. Microbiol. 53*, 1412–1417.

Caron, A.W., Archambault, J., Massie, B., **1990**. High-level protein production in bioreactors using the baculovirus-insect cell expression system. *Biotechnol. Bioeng. 36*, 1133–1140.

Carpentier, E., Lebesgue, D., Kamen, A.A., Hogue, M., Bouvier, M., *et al.*, **2001**. Increased production of active human$_{\ni 2}$-andrenergic/$G_{\forall S}$ fusion receptor in Sf-9 cells using nutrient limiting conditions. *Protein Expr. Purif. 23*, 66–74.

Carson, D.D., Summers, M.D., Guarino, L.A., **1988**. Functional mapping of an AcNPV immediate early gene which augments the expression of the IE-1 *trans*-activated 39k gene. *Virology 162*, 444–451.

Chen, Y., Jin, M., Goodrich, L., Smith, G., Coppola, G., *et al.*, **2000**. Purification and characterisation of human α-galactosidase A expression in insect cells using a baculovirus vector. *Protein Expr. Purif. 20*, 228–236.

Chen, J.H., Turner, P.C., Rees, H.H., **2002**. Molecular cloning and induction of nuclear receptors from insect cell lines. *Insect Biochem. Mol. Biol. 32*, 657–667.

Cherbas, L., Moss, R., Cherbas, P., **1994**. Transformation techniques for *Drosophila* cell lines. *Methods Cell Biol. 44*, 161–179.

Cherbas, L., Yonge, C.D., Cherbas, P., Williams, C.M., **1980**. The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. *Wilhelm Roux's Archives 189*, 1–15.

Chinni, C., Bottomley, S.P., Duffy, E.J., Hemmings, B.A., Stone, S.R., **1998**. Expression and purification of the human thrombin receptor. *Protein Expr. Purif. 13*, 9–15.

Ciaccia, A.V., Cunningham, E.L., Church, F.C., **1995**. Characterization of recombinant heparin cofactor II expressed in insect cells. *Protein Expr. Purif. 6*, 806–812.

Clawges, H.M., Depree, K.M., Parker, E.M., Graber, S.G., **1997**. Human 5-HT1 receptor subtypes exhibit distinct G protein coupling behaviors in membranes from Sf9 cells. *Biochemistry 36*, 12930–12938.

Clement, C.Y., Bradbrook, D.A., Lafont, R., Dinan, L., **1993**. Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem. Mol. Biol. 23*, 187–193.

Coleman, T.A., Parmelee, D., Rao Thotakura, N., Nguyen, N., Burgin, M., *et al.*, **1997**. Production and purification of novel secreted human proteins. *Gene 190*, 163–171.

Cooley, J., Mathieu, B., Remold-O'Donnell, E., Mandle, R.J., **1998**. Production of recombinant human monocyte/neutrophil elastase inhibitor (rM/NEI). *Protein Expr. Purif. 14*, 38–44.

Condreay, J.P., Witherspoon, S.M., Clay, W.C., Kost, T.A., **1999**. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc. Natl Acad. Sci. USA 96*, 127–132.

Culp, J.S., Johansen, H., Hellmig, B., Beck, J., Matthews, T.J., *et al.*, **1991**. Regulated expression allows high level production and secretion of HIV-1 gp120 envelope glycoprotein in *Drosophila* Schneider cells. *Biotechnology 9*, 173–177.

Dale, W.E., Textor, J.A., Mercer, R.W., Simchowitz, L., **1996**. Expression and characterization of the human erythrocyte anion exchanger in a baculovirus/Sf-9 system. *Protein Expr. Purif. 7*, 1–11.

Das, T., Johns, P.W., Goffin, V., Kelly, P., Kelder, B., *et al.*, **2000**. High-level expression of biologically active human prolactin from recombinant baculovirus in insect cells. *Protein Expr. Purif. 20*, 265–273.

Davis, T.R., Munkenbeck Trotter, K., Granados, R.R., Wood, H.A., **1992**. Baculovirus expression of alkaline phosphatase as a reporter gene for evaluation of production glycosyltaion and secretion. *Biotechnology 10*, 1148–1150.

Denault, J.-B., Lazure, C., Day, R., Leduc, R., **2000**. Comparative characterization of two forms of recombinant Human SPC1 secreted from Schneider 2 cells. *Protein Expr. Purif. 19*, 113–124.

DiFalco, M.R., Bakopanos, E., Patricelli, M., Chan, G., Congote, L.F., **1997**. The influence of various insect cell lines p10 and polyhedrin promoters in the production of secreted insulin-like growth factor-interleukin-3 chimeras in the baculovirus expression system. *J. Biotechnol. 56*, 49–56.

Dinan, L., Bourne, P.C., Meng, Y., Sarker, S.D., Tolentino, R.B., *et al.*, **2001**. Assessment of natural products in the Drosophila melanogaster B(II) cell bioassay for ecdysteroid agonist and antagonist activities. *Cell. Mol. Life Sci. 58*, 321–342.

Dinan, L., Whiting, P., Girault, J.-P., Lafont, R., Dhadialla, T.S., *et al.*, **1997**. Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. *Biochem. J. 327*, 643–650.

Ding, H., Griesel, C., Nimtz, M., Conradt, H.S., Weich, H.A., *et al.*, **2003**. Molecular cloning, expression, purification, and characterization of soluble full-length, human interleukin-3 with a baculovirus-insect cell expression system. *Protein Expr. Purif. 31*, 34–41.

DiPersio, L.P., Kissel, J.A., Hui, D.Y., **1992**. Purification of pancreatic choilesterol esterase expressed in recombinant baculovirus-infected Sf9 cells. *Protein Expr. Purif. 3*, 114–120.

Dorjsuren, D., Badralmaa, Y., Mikovits, J., Li, A., Fisher, R., *et al.*, **2003**. Expression and purification of recombinant Kaposi's sarcoma-associated herpesvirus

DNA polymerase using a Baculovirus vector system. *Protein Expr. Purif. 29*, 42–50.

Egger, M., Ruknudin, A., Niggli, E., Lederer, W.J., Schulze, D.H., **1999**. Ni^{2+} transport by the human Na^+/Ca^{2+} exchanger expressed in Sf9 cell. *Am. J. Physiol. 276*, 1184–1192.

Emery, V.C., Bishop, D.H.L., **1987**. The development of multiple expression vectors for high level synthesis of eukaryotic proteins expression of LCMV-N and AcNPV polyhedrin protein by a recombinant baculovirus. *Protein Engineer. 1*, 359–366.

Ernst, W., Grabherr, R., Wegner, D., Borth, N., Grassauer, A., *et al.*, **1998**. Baculovirus surface display construction and screening of a eukaryotic epitope library. *Nucleic Acids Res. 26*, 1718–1723.

Ernst, W.J., Grabherr, R.M., Katinger, H.W.D., **1994**. Direct cloning into the *Autographa californica* nuclear polyhedrosis virus for generation of recombinant baculoviruses. *Nucleic Acids Res. 22*, 2855–2856.

Escriva, H., Safi, R., Hänni, C., Langlois, M.-C., Saumitou-Laprade, P., *et al.*, **1997**. Ligand binding was acquired during evolution of nuclear receptors. *Proc. Natl Acad. Sci. USA 94*, 6803–6808.

Farrell, P.J., Behie, L.A., Iatrou, K., **2000**. Secretion of intracellular proteins from animal cells using a novel secretion module. *Proteins–Struct. Funct. Genetics 41*, 144–150.

Farrell, P.J., Behie, L.A., Iatrou, K., **1999**. Transformed insect cells new sources of human tissue plasminogen activator. *Biotechnol. Bioeng. 64*, 426–433.

Farrell, P.J., Iatrou, K., **2004**. Transfected insect cells in suspension culture rapidly yield moderate quantities of recombinant proteins in protein-free culture medium. *Protein Expr. Purif. 36*, 177–185.

Farrell, P.J., Lu, M., Prevost, J., Brown, C., Behie, L.A., *et al.*, **1998**. High level of expression recombinant glycoproteins from transformed Lepidopteran insect cells using a novel expression vector. *Biotechnol. Bioeng. 60*, 656–663.

Forler, D., Köcher, T., Rode, M., Gentzel, M., Izaurralde, E., *et al.*, **2003**. An efficient protein complex purification method for functional proteomics in higher eukaryotes. *Nature Biotechnol. 21*, 89–92.

French, T.J., Roy, P., **1990**. Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. *J. Virol. 64*, 1530–1536.

Gao, X., McLean, H., Caveney, S., Donly, C., **1999**. Molecular cloning and functional characterization of a GABA transporter from the CNS of the cabbage looper *Trichoplusia ni*. *Insect Biochem. Mol. Biol. 29*, 609–623.

Geisse, S., Gram, H., Kleuser, B., Kocher, H.P., **1996**. Eukaryotic expression systems a comparison. *Protein Expr. Purif. 8*, 271–282.

George, H.J., Marchand, P., Murphy, K., Wiswall, B.H., Dowling, R., *et al.*, **1997**. Recombinant human 92-kDa type IV collagenase/gelatinase from baculovirus-infected insect cells expression purification and characterization. *Protein Expr. Purif. 10*, 154–161.

Gierse, J.K., Luckow, V.A., Askonas, L.J., Duffin, K.L., Aykent, S., *et al.*, **1993**. High-level expression and purification of human leukotriene A_4 hydrolase from insect cells infected with a baculovirus vector. *Protein Expr. Purif. 4*, 358–366.

Golden, A., Austen, D.A., van Schravendijk, M.R., Sullivan, B.J., Kawasaki, A.S., *et al.*, **1998**. Effect of promoters and signal sequences on the production of secreted HIV-1 gp120 protein in the baculovirus system. *Protein Expr. Purif. 14*, 8–12.

Grabherr, R., Ernst, W., Oker-Blom, C., Jones, I., **2001**. Development in the use of baculoviruses for the surface display of complex eukaryotic proteins. *Trends Biotechnol. 19*, 231–236.

Grace, T.D.C., **1967**. Establishment of a line of cells from the silkworm *Bombyx mori*. *Nature 216*, 613.

Granados, R.R., Li, G., Derkensen, A.C.G., McKenna, K.A., **1994**. A new insect cell line from *Trichoplusia ni* (BTI-Tn-5B1-4) susceptible to *Trichloplusia ni* single enveloped nuclear polyhedrosis virus. *J. Inverteb. Pathol. 64*, 260–266.

Gritsun, T.S., Mikhailov, M.V., Roy, P., Gould, E.A., **1997**. A new rapid and simple procedure for direct cloning of PCR products into baculoviruses. *Nucleic Acids Res. 25*, 1864–1865.

Gronemeyer, H., Laudet, V., **1995**. Nuclear receptors. *Protein Profile 2*, 1173–1308.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., **1985**. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem. 260*, 3440–3450.

Guarino, L.A., Gonzalez, M.A., Summers, M.D., **1986**. Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 60*, 224–229.

Guarino, L.A., Summers, M.D., **1986**. Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. *J. Virol. 57*, 563–571.

Guarino, L.A., Summers, M.D., **1987**. Nucleotide sequence and temporal expression of a baculovirus regulatory gene. *J. Virol. 61*, 2091–2099.

Hale, C.C., Zimmerschied, J.A., Bliler, S., Price, E.M., **1999**. Large-scale expression of recombinant cardiac sodium-calcium exchange in insect larvae. *Protein Expr. Purif. 15*, 121–126.

Hamm, H., **2001**. How activated receptors couple to G proteins. *Proc. Natl Acad. Sci. USA 98*, 4819–4821.

Harmatha, J., Dinan, L., **1997**. Biological activity of natural and synthetic ecdysteroids in the BII bioassay. *Arch. Insect Biochem. Physiol. 35*, 219–225.

Harmatha, J., Dinan, L., Lafont, R., **2002**. Biological activities of a specific ecdysteroid dimer and of selected monomeric structural analogues in the B(II) bioassay. *Insect Biochem. Mol. Biol. 32*, 181–185.

Hegedus, D.D., Pfeifer, T.A., Hendry, J., Theilmann, D.A., Grigliatti, T.A., **1998**. A series of broad host range shuttle vectors for constitutive and inducible expression

of heterologous proteins in insect cells. *Gene 207*, 241–249.

Hegedus, D.D., Pfeifer, T.A., Theilmann, D.A., Kennard, M.L., Gabathuler, R., *et al.*, **1999**. Differences in the expression and localization of human melanotransferrin in lepidopteran and dipteran insect cell lines. *Protein Expr. Purif. 15*, 296–307.

Helgen, J.C., Fallon, A.M., **1990**. Polybrene-mediated transfection of cultured lepidopteran cells: induction of a *Drosophila* heat shock promoter. *In Vitro Cell Dev. Biol. 26*, 731–736.

Higashihashi, N., Arai, Y., Enjo, T., Horiuchi, T., Saeki, Y., *et al.*, **1991**. High-level expression and characterization of hepatitis B virus surface antigen in silkworm using a baculovirus vector. *J. Vir. Meth. 35*, 159–167.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, **2002**. G protein-coupled receptors in *Anopheles gambiae*. *Science 298*, 176–178.

Hill-Perkins, M.S., Possee, R.D., **1990**. A baculovirus expression vector derived from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol. 71*, 971–976.

Ho, W.K., Meng, Z.Q., Lin, H.R., Poon, C.T., Leung, Y.K., *et al.*, **1998**. Expression of grass carp growth hormone by baculovirus in silkworm larvae. *Biochim. Biophys. Acta 1381*, 331–339.

Hoffmann, C.V., Sandig, G., Jennings, G.T., Rudolph, M., Schlag, P., *et al.*, **1995**. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc. Natl Acad. Sci. USA 92*, 10088–10103.

Holcomb, T., Taylor, L., Trohkimoinen, J., Curthoys, N.P., **2000**. Isolation characterization and expression of a human brain mitochondrial glutaminase cDNA. *Molec. Brain Res. 76*, 56–63.

Hollenberg, M.D., Saifeddine, M., Al-Ani, B., **1996**. Proteinase-activated receptor-2 in rat aorta structural requirements for agonist activity of receptor-activating peptides. *Molec. Pharmacol. 49*, 229–233.

Hollister, J.R., Shaper, J.H., Jarvis, D.L., **1998**. Stable expression of mammalian beta 1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. *Glycobiology 8*, 473–480.

Homa, F.L., Baker, C.A., Thomsen, D.R, Elhammer, A.P., **1995**. Conversion of bovine UDP-GalNAcpolypeptide *N*-acetylgalactosaminyltransferase to a soluble secreted enzyme and expression in Sf9 cells. *Protein Expr. Purif. 6*, 141–148.

Hsu, T.-A., Eiden, J.J., Bourgarel, P., Meo, T., Betenbaugh, M.J., **1994**. Effects of co-expressing chaperone BiP on functional antibody production in the baculovirus system. *Protein Expr. Purif. 5*, 595–603.

Huang, Y.-W., Lu, M.-L., Qi, H., Lin, S.-X., **2000**. Membrane-bound human 3-hydroxysteroid dehydrogenase overexpression with his-tag using a baculovirus system and single step purification. *Protein Expr. Purif. 18*, 169–174.

Huynh, C.Q., Zieler, H., **1999**. Construction of modular and versatile plasmid vectors for the high-level expression of single or multiple genes in insects and insect cell lines. *J. Mol. Biol. 288*, 13–20.

Iatrou, K., Farrell, P., Hashimoto, Y., **2000**. Baculovirus Artificial Chromosomes and Methods of Use. U.S. Patent Number 6,090,584.

Iatrou, K., Meidinger, R.G., **1989**. Bombyx mori nuclear polyhedrosis virus-based vectors for expressing passenger genes in silk moth cells under viral or cellular promoter control. *Gene 75*, 59–71.

Ivey-Hoyle, M., Culp, J.S., Chaikin, M.A., Hellmig, B.D., Mathews, T.J., *et al.*, **1991**. Envelope glycoproteins from biologically diverse isolates of immunodeficiency viruses have widely different affinities for CD4. *Proc. Natl Acad. Sci. USA 88*, 512–516.

Jarvis, D.L., **2003**. Developing baculovirus – insect cell expression systems for humanized recombinant glycoprotein production. *Virology 310*, 1–7.

Jarvis, D.L., Fleming, J.-A.G.W., Kovacs, G.R., Summers, M.D., Guarino, L.A., **1990**. Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Biotechnology 8*, 950–955.

Jarvis, D.L., Guarino, L.A., **1995**. Continuous foreign gene expression in stably transformed insect cells. In: "Methods in Molecular Biology Baculovirus Expression Protocols," vol. 39. Humana, Clifton, NJ, pp. 187–202.

Jarvis, D.L., Kawar, Z.S., Hollister, J.L., **1998**. Engineering N-glycosylation in the baculovirus-insect cell system. *Curr. Opin. Biotechnol. 9*, 528–533.

Jarvis, D.L., Summers, M.D., **1989**. Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell. Biol. 9*, 214–223.

Jarvis, D.L., Summers, M.D., Garcia, A., Jr., Bohlmeyer, D.A., **1993**. Influence of different signal peptides and prosequences on expression and secretion of human tissue plasminogen activator in the baculovirus system. *J. Biol. Chem. 268*, 16754–16762.

Jarvis, D.L., Weinkauf, C., Guario, L.A., **1996**. Immediate-early baculovirus vectors for foreign gene expression in transformed or infected insect cells. *Protein Expr. Purif. 8*, 191–203.

Ji C., Wang Y., Ross J., Jiang, H., **2003**. Expression and *in vitro* activation of *Manduca sexta* prophenoloxidase-activating proteinase-2 precursor (proPAP-2) from baculovirus-infected insect cells. *Protein Expr. Purif. 29*, 235–243.

Jiang, X., Wang, M., Graham, D.Y., Estes, M.K., **1992**. Expression self-assembly and antigenicity of the Norwalk virus capsid protein. *J. Virol. 66*, 6527–6532.

Johansen, H., van der Straten, A., Sweet, R., Otto, E., Maroni, G., *et al.*, **1989**. Regulated expression of high copy number allows production of a growth inhibitory oncogene product in Drosophila Schneider cells. *Genes Devel. 3*, 882–889.

Johansen, K., Appelbaum, E., Doyle, M., Hensley, P., Zhao, B., *et al.*, **1995**. Binding interactions of human interleukin 5 with its receptor alpha subunit. Large scale production structural and functional studies of

Drosophila-expressed recombinant proteins. *J. Biol. Chem. 270*, 9459–9471.
Johnson, E.C., Garczynski, S.F., Park, D., Crim, J.W., Nässel, D.R., *et al.*, **2003**. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 100*, 6198–6203.
Johnson, R., Meidinger, R.G., Iatrou, K., **1992**. A cellular promoter-based expression cassette for generating recombinant baculoviruses directing rapid expression of passenger genes in infected insects. *Virology 190*, 815–823.
Joyce, K.A., Atkinson, A.E., Bermudez, I., Beadle, D.J., King, L.A., **1993**. Synthesis of functional GABA-A receptors in stable insect cell lines. *FEBS Lett. 335*, 61–64.
Juarbe-Osorio, L.G., Gorziglia, M., Betenbaugh, M.J., **1993**. Recovery and characterization of a rotavirus outer capsid protein expressed in a recombinant insect cell system. *Protein Expr. Purif. 4*, 232–239.
Kapitskaya, M.Z., Dittmer, N.T., Deitsch, K.W., Cho, W.-L., Taylor, D.G., *et al.*, **1998**. Three isoforms of a hepatocyte nuclear factor-4 transcription factor with tissue- and stage-specific expression in the adult mosquito. *J. Biol. Chem. 273*, 29801–29810.
Kang, X.-Q., Wiggins, J., Mallick, S., Grant, S.R., **1992**. Production purification and characterization of human recombinant IL-8 from the eukaryotic expression system baculovirus. *Protein Expr. Purif. 3*, 313–321.
Keith, M.B.A., Farrell, P.J., Iatrou, K., Behie, L.A., **1999**. Screening of transformed insect cell lines for recombinant protein production. *Biotechnol. Prog. 15*, 1046–1052.
Kirkpatrick, R.B., Ganguly, S., Angelichio, M., Griego, S., Shatzmann, A., *et al.*, **1995**. Heavy chain dimers as well as complete antibodies are efficiently formed and secreted from *Drosophila* via a BiP-mediated pathway. *J. Biol. Chem. 270*, 19800–19805.
Kitts, P.A., Ayres, M.D., Possee, R.D., **1990**. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res. 18*, 5667–5672.
Kitts, P.A., Possee, R.D., **1993**. A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques 14*, 810–817.
Kleymann, G., Boege, F., Hahn, M., Hampe, W., Vasudevan, S., *et al.*, **1993**. Human β_2-adrenergic receptor produced in stably transformed insect cells is functionally coupled via endogenous GTP-binding protein to adenylyl cyclase. *Eur. J. Biochem. 213*, 797–804.
Knight, M.R., Campbell, A.K., Smith, S.M., Trewavas, A.J., **1991**. Recombinant aequorin as a probe for cytosolic free Ca^{2+} in *Escherichia coli. FEBS Lett. 282*, 405–408.
Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.
Kostenis, E., **2001**. Is $G\alpha_{16}$ the optimal tool for fishing ligands of orphan G-protein-coupled receptors? *Trends Pharmacol. Sci. 22*, 560–564.
Kovach, M.J., Carlson, J.O., Beaty, B.J., **1992**. A *Drosophila* metallothionein promoter is inducible in mosquito cells. *Insect Mol. Biol. 1*, 37–43.
Kurkela, R., Herrala, A., Henttu, P., Nal, H., Vihk, P., **1995**. Expression of active secreted human prostrate-specific antigen by recombinant baculovirus-infected insect cells on a pilot-scale. *Biotechnology 13*, 1230–1234.
Kuzio, J., Jaques, R., Faulkner, P., **1989**. Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology 173*, 759–763.
Lan, Q., Riddiford, L.M., **1997**. DNA transfection in the ecdysteroid-responsive GV1 cell line from the tobacco hornworm, *Manduca sexta. In Vitro Cell Dev. Anim. 33*, 615–621.
Laukkanen, M.-L., Oker-Blom, C., Keinanen, K., **1996**. Secretion of green fluorescence protein from recombinant baculovirus-infected cells. *Biochem. Biophys. Res. Commun. 266*, 755–781.
Lee, D.F., Chen, C.-C., Hsu, T.-A., Juang, J.-L., **2000**. A baculovirus superinfection system: efficient vehicle for gene transfer into *Drosophila* S2 cells. *J. Virol. 74*, 11873–11880.
Lenhard, T., Reilander, H., **1997**. Engineering the folding pathway of insect cells generation of a stably transformed insect cell line showing improved folding of a recombinant membraneprotein. *Biochem. Biophys. Res. Commun. 238*, 823–830.
Li, E., Brown, S.L., Dolman, C.S., Brown, G.B., Nemerow, G.R., **2001**. Production of functional antibodies generated in a nonlytic insect cell expression system. *Protein Expr. Purif. 21*, 121–128.
Li, Z., Smith, C.D., Smolley, J.R., Bridge, J.H.B., Frank, K.S., *et al.*, **1992**. Expression of the cardiac Na^+–Ca^{2+} exchanger in insect cells using a baculovirus vector. *J. Biol. Chem. 267*, 7828–7833.
Lindley, K.M., Su, J.L., Hodges, P.K., Wisely, G.B., Bledsoe, R.K., *et al.*, **2000**. Production of monoclonal antibodies using recombinant baculovirus displaying gp64-fusion proteins. *J. Immunol. Methods 234*, 123–135.
Loisel, T.P., Ansanay, H., St-Onge, S., Gay, B., Boulanger, P., *et al.*, **1997**. Recovery of homogeneous and functional β_2-andrenergic receptors from extracellular baculovirus particles. *Nature Biotechnol. 15*, 1300–1304.
Lord, K.A., Wang, X.-M., Simmons, S.J., Bruckner, R.C., Loscig, J., *et al.*, **1997**. Variant cDNA sequences of human ATPcitrate lyase cloning expression and purification from baculovirus-infected insect cells. *Protein Expr. Purif. 9*, 133–141.
Lowe, M.E., **1994**. Human pancreatic procolipase expressed in insect cells purification and characterization. *Protein Expr. Purif. 5*, 583–586.
Lu, M., Farrell, P.J., Johnson, R., Iatrou, K., **1997**. A baculovirus (BmNPV) repeat element functions as a powerful constitutive enhancer in transfected insect cells. *J. Biol. Chem. 272*, 30724–30728.

Lu, M., Johnson, R.R., Iatrou, K., **1996**. *Trans*-activation of a cell housekeeping gene promoter by the IE1 gene product of baculoviruses. *Virology 218*, 103–113.

Lu, A., Miller, L.K., **1995**. The roles of eighteen baculovirus late gene expression factor genes in transcription and DNA replication. *J. Virol. 69*, 975–982.

Lu, A., Miller, L.K., **1996**. Generation of recombinant baculoviruses by direct cloning. *Biotechniques 21*, 63–68.

Luckow, V.A., **1991**. Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: Prokop, A., Bajpai, R.K., Ho, S.C. (Eds.), Recombinant DNA Technology and Applications. McGraw Hill, New York, pp. 97–125.

Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., **1993**. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherischia coli. *J. Virol. 67*, 4566–4579.

Luckow, V.A., Summers, M.D., **1988**. Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus. *Virology 167*, 56–71.

Luckow, V.A., Summers, M.D., **1989**. High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology 170*, 31–39.

Lycett, G.J., Crampton, J.M., **1993**. Stable transformation of mosquito cell lines using a hsp70:neo fusion gene. *Gene 136*, 129–136.

Maeda, S., **1989**. *Bombyx mori* nuclear polyhedrosis virus and their use for expression of foreign genes in insect cells. In: Mitshuashi, J. (Ed.), Invertebrate Cell Systems and Applications, vol. 1. CRC, Boca Raton, London, pp. 167–181.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., *et al.*, **1995**. Production of human α-interferon in silkworm using a baculovirus vector. *Nature 315*, 592–594.

Marchal, I., Jarvis, D.L., Cacan, R., Verbert, A., **2001**. Glycoproteins from insect cells sialylated or not? *Biol. Chem. 382*, 151–159.

Marinissen, M.J., Gudkind, S.J., **2001**. G protein-coupled receptors and signalling networks: emerging paradigms. *Trends Pharmacol. Sci. 22*, 368–376.

Massotte, D., Baroche, L., Simonin, F., Yu, L., Kieffer, B., *et al.*, **1997**. Characterization of δ, κ and μ human opoid receptors overexpressed in baculovirus-infected insect cells. *J. Biol. Chem. 272*, 19987–19992.

Matsuura, Y., Possee, R.D., Overton, H.A., Bishop, D.H.L., **1987**. Baculovirus expression vectors the requirements for high level expression of proteins including glycoproteins. *J. Gen. Virol. 68*, 1233–1250.

Mathews, S., Dobeli, H., Pruschy, M., Bosser, R., D'Arcy, A., *et al.*, **1996**. Recombinant human rennin produced in different expression systems biochemical properties and 3D structure. *Protein Expr. Purif. 7*, 81–92.

Matthews, R.E.F., **1982**. Classification and nomenclature of viruses. *Intervirology 17*, 1–199.

McLachlin, J.R., Miller, L.K., **1997**. Stable transformation of insect cells to coexpress a rapidly selectable marker gene and an inhibitor of apoptosis. *In vitro Cell. Devel. Virol. Animal 33*, 575–579.

McQueney, M.S., Feild, J., Hanning, C.R., Brun, K., Ramachandran, K., *et al.*, **1998**. Cynomolgus monkey (*Macaca fascicularis*) cathespin K cloning expression purification and activation. *Protein Expr. Purif. 14*, 387–394.

Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., *et al.*, **2002**. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 15363–15368.

Meij, P., Vervoort, M.B., de Gooijer, K., Bloemena, E., Meijer, C.J., *et al.*, **2000**. Bioreactor-scale production and one-step purification of Epstein-Barr nuclear antigen 1 expressed in baculovirus-infected insect cells. *Protein Expr. Purif. 20*, 324–333.

Merrihew, R.V., Clay, W.C., Condreay, J.P., Witherspoon, S.M., Dallas, W.S., *et al.*, **2001**. Chromosomal integration of transduced recombinant baculovirus DNA in mammalian cells. *J. Virol. 75*, 903–909.

Millar, N.S., Baylis, H.A., Reaper, C., Bunting, R., Mason, W.T., *et al.*, **1995**. Functional expression of a cloned *Drosophila* muscarinic acetylcholine receptor in a stable *Drosophila* cell line. *J. Exp. Biol. 198*, 1843–1850.

Millar, N.S., Buckingham, S.D., Sattelle, D.B., **1994**. Stable expression of a functional homo-oligomeric *Drosophila* GABA receptor in a *Drosophila* cell line. *Proc. Roy. Soc. Lond. 258*, 307–314.

Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Coon, H.G., **1987**. Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. *Science 237*, 779–781.

Minev, B.R., Chaves, F.L., Mitchell, M.S., **1999**. Cancer vaccines novel approaches and a new promise. *Pharmacol. Ther. 81*, 121–139.

Miras, R., Cuillel, M., Catty, P., Guillain, F., Mintz, E., **2001**. Purification of heterologous sarcoplasmic reticulum Ca^{2+}-ATPase Serca1a allowing phosphoenzyme and Ca^{2+}-affinity measurements. *Protein Expr. Purif. 22*, 299–306.

Miyajima, A., Schreurs, J., Otsu, K., Kondo, A., Arai, K., *et al.*, **1987**. Use of the silkworm *Bombyx mori* and an insect baculovirus vector for high-level expression and secretion of biologically active mouse interleukin-3. *Gene 58*, 273–281.

Miyasaka, T., Kaminogawa, S., Shimizu, M., Hisatsune, T., Reinach, P.S., *et al.*, **2001**. Characterization of human taurine transporter expressed in insect cells using a recombinant baculovirus. *Protein Expr. Purif. 23*, 389–397.

Monsma, S.A., Oomens, A.G., Blissard, G.W., **1996**. The gp64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of an infection. *J. Virol. 70*, 4607–4616.

Mori, H., Yamao, M., Nakazawa, H., Sugahara, Y., Shirai, N., *et al.*, **1995**. Transovarian transmission

of a foreign gene in the silkworm *Bombyx mori* by *Autographa californica* nuclear polyhedrosis virus. *Biotechnology 13*, 1005–1007.

Morris, T.D., Miller, L.K., **1992**. Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines. *J. Virol. 66*, 7397–7405.

Moto, K., Kojima, H., Kurihara, M., Iwami, M., Matsumoto, S., **2003**. Cell-specific expression of enhanced green fluorescence protein under the control of neuropeptide gene promoters in the brain of the silkworm, *Bombyx mori* nucleopolyhedrosis-derived vectors. *Insect Biochem. Molec. Biol. 33*, 7–12.

Mounier, N., Prudhomme, J.C., **1986**. Isolation of actin genes in *Bombyx mori*. The coding sequence of a cytoplasmic actin gene expressed in the silk gland is interrupted by a single intron in an unusual position. *Biochemie 68*, 1053–1061.

Mroczkowski, B.S., Huvar, A., Lernhardt, W., Misono, K., Nielson, K., *et al.*, **1994**. Secretion of thermostable DNA polymerase using a novel baculovirus vector. *J. Biol. Chem. 269*, 13522–13528.

Murphy, C.A., McIntire, J.R., Davis, D.R., Hodgdon, H., Seals, J.R., *et al.*, **1993**. Enhanced expression and large-scale purification of recombinant HIV-1 gp120 in insect cells using the baculovirus egt and p67 signal peptides. *Protein Expr. Purif. 4*, 349–357.

Myamoto, C., Smith, G.E., Farrell-Towt, J., Chizzonite, R., Summers, M.D., *et al.*, **1985**. Production of human c-*myc* protein in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol. 5*, 2860–2865.

Nagarajan, S., Selvaraj, P., **1999**. Expression and characterization of glycolipid-anchored B7 (CD80) from baculovirus-infected insect cells protein transfer onto tumor cells. *Protein Expr. Purif. 17*, 273–281.

Newcomb, W.W., Homa, F.L., Thomsen, D.R., Trus, B.L., Cheng, N., *et al.*, **1999**. Assembly of the herpes simplex virus procapsid from purified components and identification of small complexes containing the major capsid and scaffolding proteins. *J. Virol. 73*, 4239–4250.

Ng, G.Y.K., Trogadis, J., Stevens, J., Bouvier, M., O'Dowd, B.F., *et al.*, **1995**. Agonist-induced desensitization of dopamine D1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D1 receptor internalization. *Proc. Natl Acad. Sci. USA 92*, 10157–10161.

Ng, G.Y.K., Varghese, G., Chung, H.T., Trogadis, J., Seeman, P., *et al.*, **1997**. Resistance of the dopamine $D2_L$ receptor to desensitization accompanies the upregulation of receptors on to the surface of Sf9 cells. *Endocrinology 138*, 4199–4206.

Nilsen, S.L., Castellino, F.J., **1999**. Expression of human plasminogen in *Drosophila* Schneider S2 cells. *Protein Expr. Purif. 16*, 136–143.

Ohtaki, T., Ogi, K., Masuda, Y., Mitsuoka, K., Fujiyoshi, Y., *et al.*, **1998**. Expression, purification, and reconstitution of receptor for pituitary adenylate cyclase-activating polypeptide. *J. Biol. Chem. 273*, 15464–15473.

Oppenheimer, D.I., MacNicol, A.M., Patel, N.H., **1999**. Functional conservation of the *wingless-engrailed* interaction as shown by a widely applicable baculovirus misexpression system. *Curr. Biol. 9*, 1288–1296.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., **1992**. Baculovirus Expression Vectors A Laboratory Manual. W.H. Freeman and Co, New York.

Otto, E., Allen, J.M., Young, J.E., Palmiter, R.D., Maroni, G., **1987**. A DNA segment controlling metal-regulated expression of the *Drosophila melanogaster* metallothionein gene. *Mol. Cell. Biol. 7*, 1710–1715.

Page, M.J., **1989**. p36C an improved baculovirus expression vector for producing high levels of mature recombinant proteins. *Nucleic Acids Res. 17*, 454.

Pajot-Augy, E., Couture, L., Bozon, V., Remy, J.J., Biache, G., *et al.*, **1995**. High-level expression of recombinant porcine LH receptor in baculovirus-infected insect cells or caterpillars. *J. Mol. Endocrinol. 14*, 1–66.

Palombo, F., Monciotti, A., Recchia, A., Cortese, R., Ciliberto, G., *et al.*, **1998**. Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. *J. Virol. 72*, 5025–5034.

Passarelli, A.L., Todd, J.W., Miller, L.K., **1994**. A baculovirus gene involved in late gene expression predicts a large polypeptide with a conserved motif of RNA polymerases. *J. Virol. 68*, 4673–4678.

Patel, G., Nasmyth, K., Jones, N., **1992**. A new method for the isolation of recombinant baculovirus. *Nucleic Acids Res. 20*, 97–104.

Pennock, G.D., Shoemaker, C., Miller, L.K., **1984**. Strong and regulated expression of *Escherichia coli* β-galactosidase in insect cells with a baculovirus vector. *Mol. Cell. Biol. 4*, 399–406.

Percival, M.D., Bastien, L., Griffin, P.R., Kargman, S., Ouellet, M., *et al.*, **1997**. Investigation of human cyclooxygenase-2 glycosylation heterogeneity and protein expression in insect and mammalian cell expression systems. *Protein Expr. Purif. 9*, 388–397.

Pereira, C.A., Pouliquen, Y., Rodas, V., Massotte, D., Mortensen, C., *et al.*, **2001**. Optimized insect cell culture for the production of recombinant heterologous proteins and baculovirus particles. *Biotechniques 6*, 1263–1268.

Perret, B.G., Wagner, R., Lecat, S., Brillet, K., Rabut, G., *et al.*, **2003**. Expression of EGFP-amino-tagged human mu opioid receptor in *Drosophila* Schneider 2 cells: a potential expression system for large-scale production of G-protein coupled receptors. *Protein Expr. Purif. 31*, 123–132.

Pfeifer, T.A., Guarna, M.M., Kwan, E.M., Lesnicki, G., Theilmann, D.A., *et al.*, **2001**. Expression analysis of a modified FactorX in stable insect cell lines. *Protein Expr. Purif. 23*, 233–241.

Pfeifer, T.A., Hegedus, D.D., Grigliatti, T.A., Theilmann, D.A., **1997**. Baculovirus immediate-early promoter-mediated expression of the Zeocin resistance gene for use as a dominant selectable marker in dipteran and lepidopteran insect cell lines. *Gene 188*, 183–190.

Phan, D., Han, E., Birrell, G., Bonnal, S., Duggan, L., *et al.*, **2001**. Purification and characterization of human

cell–cell adhesion molecule 1 (C-CAM1) expressed in insect cells. *Protein Expr. Purif. 21*, 343–351.

Pittelkow, S., Lindner, H., Rohm, K.-L., **1998**. Human and porcine aminoacylase I overproduced in a baculovirus expression vector system: evidence for structural and functional identity with enzymes isolated from kidney. *Protein Expr. Purif. 12*, 269–276.

Possee, R.D., Howard, S.C., **1987**. Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Res. 15*, 10233–10248.

Possee, R.D., Sun, T.P., Howard, S.C., Ayres, M.D., Hill-Perkins, M., *et al*., **1991**. Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis 9.4 kbp EcoRI-I and -R (polyhedrin gene) region. *Virology 185*, 229–241.

Pullen, S.S., Friesen, P.D., **1995**. Early transcription of the *ie-1* transregulator gene of *Autographa californica* nuclear polyhedrosis virus is regulated by DNA sequences within its 5′ noncoding leader region. *J. Virol. 69*, 156–165.

Ragunath, C., Sundar, K., Ramasubbu, N., **2002**. Expression characterization and biochemical properties of recombinant human salivary amylase. *Protein Expr. Purif. 24*, 202–211.

Rankin, C., Ooi, B.G., Miller, L.K., **1988**. Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression. *Gene 70*, 39–49.

Rankl, N.B., Rice, J.W., Gurganus, T.M., Barbee, J.L., Burns, D.J., **1994**. The production of an active protein kinase C-delta in insect cells is greatly enhanced by the use of the basic protein promoter. *Protein Expr. Purif. 5*, 346–356.

Rapp, J.C., Wilson, J.A., Miller, L.K., **1998**. Nineteen baculovirus open reading frames including LEF-12, support late gene expression. *J. Virol. 72*, 10197–10206.

Ren, Y., Busch, R., Durban, E., Taylor, C., Gustafson, W.C., *et al*., **1996**. Overexpression of human nucleolar proteins in insect cells: characterization of nucleolar protein p120. *Protein Expr. Purif. 7*, 212–219.

Rodems, S.M., Friesen, P.D., **1993**. The *hr5* transcriptional enhancer stimulates early expression from the *Autographica californica* nuclear polyhedrosis virus genome but is not required for virus replication. *J. Virol. 67*, 5776–5785.

Roy, P., Mikhailov, M., Bishop, D.H.L., **1997**. Baculovirus multigene expression vectors and their use for understanding the assembly process of architecturally complex virus particles. *Gene 190*, 119–127.

Royer, C., Bossin, H., Romane, C., Bergoin, M., Couble, P., **2001**. High amplification of a densovirus-derived vector in larval and adult tissues of *Drosophila*. *Insect Mol. Biol. 10*, 275–280.

Russell, R.L., Rohrmann, G.F., **1993**. Nucleotide sequence of the ubiquitin-39K gene region from the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *J. Gen. Virol. 74*, 1191–1195.

Sadatmansoori, S., MacDougall, J., Khademi, S., Cooke, L.S., Guarino, L., *et al*., **2001**. Construction expression and characterization of a baculovirally expressed catalytic domain of human matrix metalloproteinase-9. *Protein Expr. Purif. 23*, 447–452.

Sarvari, M., Csikos, G., Sass, M., Gal, P., Schumaker, V.N., *et al*., **1990**. Ecdysteroids increase the yield of recombinant protein produced in baculovirus insect cell expression system. *Biochem. Biophys. Res. Commun. 30*, 1154–1161.

Schneider, I., **1972**. Cell lines derived fromlate embryonic stages of *Drosophila melanogaster. J. Embryol. Exp. Morphol. 27*, 353–356.

Sehgal, D., Malik, P.S., Jameel, S., **2003**. Purification and diagnostic utility of a recombinant hepatitis E virus capsid protein expressed in insect larvae. *Protein Expr. Purif. 27*, 27–34.

Seo, N.-S., Hollister, J.R., Jarvis, D.L., **2001**. Mammalian glycotransferases expression allows sialoglycoprotein production by baculovirus-infected insect cells. *Protein Expr. Purif. 22*, 234–241.

Shikata, M., Sano, Y., Hashimoto, Y., Matsumoto, T., **1998**. Isolation and characterization of a temperature-sensitive mutant of *Bombyx mori* nucleopolyhedrovirus for a putative RNA polymerase gene. *J. Gen. Virol. 79*, 2071–2078.

Shin, H.S., Cha, H.J., **2003**. Statistical optimization for immobilized metal affinity purification of secreted human erythropoietin from Drosophila S2 cells. *Protein Expr. Purif. 28*, 331–339.

Shoji, I., Aizaki, H., Tani, H., Ishii, K., Chiba, T., *et al*., **1997**. Efficient gene transfer into various mammalian cells including non-hepatic cells by baculovirus vectors. *J. Gen. Virol. 78*, 2657–2664.

Shotkoski, F.A., Fallon, A.M., **1993**. The musquito dihydrofolate reductase gene functions as a dominant selectable marker in transfected cells. *Insect Biochem. Mol. Biol. 23*, 883–893.

Shotkoski, F., Zhang, H.-G., Jackson, M.B., ffrench-Constant, R.H., **1996**. Stable expression of insect GABA receptors in insect cell lines: promoters for efficient expression of *Drosophila* and mosquito *Rdl* GABA receptors in stably transformed mosquito cell lines. *FEBS Lett. 380*, 257–262.

Smagghe, G., Braeckman, B.P., Huys, N., Tirry, L., Raes, H., **2003**. Cultured mosquito cells *Aedes albopictus* C6/36 (Dip., Culicidae) responsive to 20-hydroxyecdysone and non-steroidal ecdysone agonist. *J. Appl. Ent. 127*, 1–7.

Smith, C.D., Hirayama, B.A., Wright, E.M., **1992**. Baculovirus-mediated expression of the Na^+/glucose cotransporter in Sf9 cells. *Biochim. Biophys. Acta 1104*, 151–159.

Smith, G.E., Fraser, M.J., Summers, M.D., **1983c**. Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome deletion mutations within the polyhedrin gene. *J. Virol. 46*, 584–593.

Smith, G.E., Ju, G., Ericson, B.L., Moschera, J., Lahm, H.-W., *et al*., **1985**. Modification and secretion of

human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Natl Acad. Sci. USA 82*, 8404–8408.

Smith, G.E., Summers, M.D., Fraser, M.J., **1983b**. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol. 3*, 2156–2165.

Smith, G.E., Vlak, J.M., Summers, M.D., **1983a**. Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *J. Virol. 45*, 215–225.

Smith, L.A., Amar, M., Harvey, R.J., Darlison, M.G., Earley, F.G.P., *et al.*, **1995**. The production of a stably transformed insect cell line expressing an invertebrate GABAA receptor β-subunit. *J. Recep. Signal Transduct. Res. 15*, 33–42.

Soares, M., Hanisch, F.-G., Finn, O.J., Ciborowski, P., **2001**. Recombinant human tumor antigen MUC1 expressed in insect cells structure and immunogenicity. *Protein Expr. Purif.* 22, 92–100.

Sohi, S.S., Palli, S.R., Cook, B.J., Retnakaran, A., **1995**. Forest insect cell lines responsive to 20-hydroxyecdysone and two nonsteroidal ecdysone agonists, RH-5849 and RH-5992. *J. Insect Physiol. 41*, 457–464.

Sollerbrant, K., Elmen, J., Wahlestedt, C., Acker, J., Leblois-Prehaud, H., *et al.*, **2001**. A novel method using baculovirus-mediated gene transfer for production of recombinant adeno-associated virus vectors. *J. Gen. Virol. 82*, 2051–2060.

Sorbrier, M.L., Chapel, S., Couderc, J.L., Micard, D., Lecher, P., *et al.*, **1989**. 20-OH-ecdysone regulates 60 C beta tubulin gene expression in Kc cells and during *Drosophila* development. *Exp. Cell Res. 184*, 241–249.

Sorci-Thomas, M.G., Parks, J.S., Kearns, M.W., Pate, G.N., Zhang, C., *et al.*, **1996**. High level secretion of wild-type and mutant forms of human proapoA-I using baculovirus-mediated Sf-9 cell expression. *J. Lipid Res. 37*, 673–683.

Spradling, A.C., **1986**. P element-mediated transformation. In: Roberts, D.B. (Ed.), *Drosophila*: A Practical Approach. IRL Press, Oxford, pp. 175–197.

Stables, J., Green, A., Marshall, F., Fraser, N., Knight, E., *et al.*, **1997**. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Anal. Biochem. 252*, 115–126.

Staubli, F., Jørgensen, T.J.D., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99*, 3446–3451.

Steed, P.M., Lasala, D., Liebman, J., Wigg, A., Clark, K., *et al.*, **1998**. Characterization of recombinant human cathespin B expressed at high levels in baculovirus. *Protein Science* 7, 2033–2037.

Steiner, H., Pohl, G., Gunne, H., Hellers, M., Elhammer, A, *et al.*, **1988**. Human tissue-type plasminogen activator synthesized by using a baculovirus vector in insect cells compared with human plasminogen activator produced in mouse cells. *Gene 73*, 449–457.

Steller, H., Pirrotta, V., **1984**. Regulated expression of genes injected into early *Drosophila* embryos. *EMBO J. 3*, 165–173.

Stoltenberg, J.K., Straney, R.A., Tritch, R.J., Mackin, W.M., George, H.J., **1993**. Expression and functional characterization of recombinant human vascular cell adhesion molecule-1 (VCAM1) synthesized by baculovirus-infected insect cells. *Protein Expr. Purif. 4*, 585–593.

Strosberg, D., Guillaume, J.-L., **2000**. Expression of β-andrenergic receptors in recombinant baculovirus-infected insect cells. *Methods Mol. Biol. 126*, 207–214.

Sugiura, T., Takamatsu, H., Kudo, A., Amann, E., **1995**. Expression and characterization of murine osteoblast-specific factor 2 (OSF-2) in a baculovirus expression system. *Protein Expr. Purif.* 6, 305–311.

Summers, M.D., Smith, G.G., **1987**. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station Bulletin 155.

Sun, S., Albright, C.F., Fish, B.H., George, H.J., Selling, B.H., *et al.*, **2002**. Expression purification and kinetic characterization of full-length human fibroblast activation protein. *Protein Expr. Purif. 24*, 274–281.

Suzuki, T., Kawasaki, H., Yu, R.T., Ueda, H., Umesono, K., **2001**. Segmentation gene product Fushi tarazu is an LXXLL motif-dependent coactivator for orphan receptor FTZ-F1. *Proc. Natl Acad. Sci. USA 98*, 12403–12408.

Sviridov, D., Luong, A., Pyle, L., Fidge, N., **1999**. Effectivity of expression of mature forms of mutant human apolipoprotein A-I. *Protein Expr. Purif. 17*, 231–238.

Swevers, L., Cherbas, L., Cherbas, P., Iatrou, K., **1996**. *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Molec. Biol. 26*, 217–221.

Swevers, L., Farrell, P.J., Kravariti, L., Xenou-Kokoletsi, M., Sdralia, N., *et al.*, **2003a**. Transformed insect cells as high throughput screening tools for the discovery of new bioactive compounds. *Comm. Agric. Appl. Biol. Sci. 68/2(b)*, 333–341.

Swevers, L., Ito, K., Iatrou, K., **2002**. The BmE75 nuclear receptors function as dominant repressors of the nuclear receptor BmHR3A. *J. Biol. Chem. 277*, 41637–41644.

Swevers, L., Kravariti, L., Ciolfi, S., Xenou-Kokoletsi, M., Ragoussis, N., *et al.*, **2003b**. A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. *FASEB J.* Express Article 10.1096/fj.03-0627fje.

Szerenscei, R.T., Tucker, J.E., Cooper, C.C., Winkfein, R.J., Farrell, P.J., *et al.*, **2000**. Minimal domain requirement for cation transport by the potassium-dependant Na–Ca+K exchanger: comparison with a NCKX homologue from *Caenorhabditis elegans*. *J. Biol. Chem. 275*, 669–676.

Tate, C.G., **1998**. Baculovirus-mediated expression of neurotransmitter transporters. *Methods Enzymol. 296*, 443–455.

Tate, C.G., **1999**. Molecular chaperones stimulate the functional expression of the cocaine-sensitive serotonin transporter. *J. Biol. Chem. 274*, 17551–17558.

Tessier, D.C., Thomas, D.Y., Khouri, H.E., Laliberte, F., Vernet, T., **1991**. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. *Gene 98*, 177–183.

Theilmann, D.A., Stewart, S., **1992**. Tandemly repeated sequence at the 3′ end of the IE-2 gene of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus is an enhancer element. *Virology 187*, 97–106.

Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., **1993**. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature 362*, 471–475.

Thordarson, G., Wu, K., Talamantes, F., **1996**. Purification and characterization or recombinant mouse growth hormone binding protein produced in the baculovirus expression system. *Protein Expr. Purif. 7*, 74–80.

Thummel, C.S., Pirrotta, V., **1992**. New pCaSpeR P-element vectors. *Drosopila Inf. Serv. 71*, 150.

Tomita, S., Kawai, Y., Woo, S.D., Kamimura, M., Iwabuichi, K., *et al.*, **2001**. Ecdysone-inducible foreign gene expression in stably-transformed lepidopteran insect cells. *In Vitro Cell Dev. Biol. Animal 37*, 564–571.

Tomlinson, S., Ueda, E., Maruniak, J.E., Garcia-Canedo, A., Bjes, E.S., *et al.*, **1993**. The expression of hemolytically active human complement protein C9 in mammalian insect and yeast cells. *Protein Expr. Purif. 4*, 141–148.

Torfs, H., Poels, J., Detheux, M., Dupriez, V., Van Loy, T., *et al.*, **2002**. Recombinant aequorin as a reporter for receptor-mediated changes of intracellular Ca^{2+}-levels in *Drosophila* S2 cells. Invert. *Neurosci. 4*, 119–124.

Torfs, H., Shariatmadari, R., Guerrero, F., Parmentier, M., Poels, J., *et al.*, **2000**. Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila Schneider* 2 cell line. *J. Neurochem. 74*, 2182–2189.

Tota, M.R., Sirotina, A., Strader, C.D., Graziano, M.P., **1995**. Interaction of [fluorescein-Trp^{25}]glucagons with the human glucagons receptor expressed in *Drosophila* Schneider 2 cells. *J. Biol. Chem. 270*, 26466–26472.

Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M., Evans, R.M., **1999**. SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Mol Cell. 4*, 175–186.

Tschantz, W.R., Zhang, L., Casey, P.J., **1999**. Cloning, expression, and cellular localization of a human prenylcysteine lyase. *J. Biol. Chem. 274*, 35802–35808.

Urakawa, T., Ferguson, M., Minor, P.D., Cooper, J., Sullivan, M., *et al.*, **1989**. Synthesis of immunogenic but non-infectious poliovirus particles in insect cells by a baculovirus expression vector. *J. Gen. Virol. 70*, 1453–1463.

Vanden Broeck, J., **2001**. Insect G protein-coupled receptors and signal transduction. *Arch. Insect Biochem. Physiol. 48*, 1–12.

Vanden Broeck, J., Vulsteke, V., Huybrechts, R., De Loof, A., **1995**. Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. *J. Neurochem. 64*, 2387–2395.

van Oers, M.M., Flipsen, J.T., Reusken, C.B., Sliwinsky, E.L., Goldbach, R.W., *et al.*, **1993**. Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol. 74*, 563–574.

Vasudevan, S., Hulme, E.C., Bach, M., Haase, W., Pavia, J., *et al.*, **1995**. Characterization of the rat m3 muscarinic acetylcholine receptor produced in insect cells infected with recombinant baculovirus. *Eur. J. Biochem. 227*, 466–475.

Vasudevan, S., Premkumar, L., Stowe, S., Gage, P.W., Reilander, H., *et al.*, **1992**. Muscarinic acetylcholine receptor produced in recombinant baculovirus infected Sf9 insect cells couples with endogenous G-proteins to activate ion channels. *FEBS Lett. 311*, 7–11.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., McCawley, P., **1977**. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In Vitro 13*, 213–217.

Vialard, J.E., Arif, B.M., Richardson, C.D., **1995**. Introduction to the molecular biology of baculoviruses. *Methods Mol. Biol. 39*, 1–24.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, N., Klinge-Roode, E.C., *et al.*, **1988**. Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expression of a p10-B-galactosidase fusion gene. *J. Gen. Virol. 69*, 765–776.

Vogtli, M., Elke, C., Imhof, M.O., Lezzi, M., **1998**. High level transactivation by the ecdysone receptor complex at the core recognition motif. *Nucleic Acids Res. 26*, 2407–2414.

Volkman, L.E., Goldsmith, P.A., **1983**. In-vitro survey of *Autographa-californica* nuclear polyhedrosis virus interaction with nontarget vertebrate host cells. *Appl. Environ. Microbiol. 45*, 1085–1093.

Volkman, L.E., Summers, M.D., **1977**. Autographa californica nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. *J Invertebr Pathol. 30*, 102–103.

Wagner, R., Liedtke, S., Kretzschmar, E., Geyer, H., Geyer, R., *et al.*, **1996**. Elongation of the N-glycans of fowl plague virus hemagglutinin expressed in *Spodoptera frugiperda* (Sf9) cells by coexpression of human beta 1,2-N-acetylglucosaminyltransferase I. *Glycobiology 6*, 165–175.

Wang, Y., Jiang, H., Kanost, M.R., **2001**. Expression and purification of *Manduca sexta* prophenoloxidase-activating proteinase precursor (proPAP) from baculovirus-infected insect cells. 2001. *Protein Expr. Purif. 22*, 328–337.

Wang, S.F., Miura, K., Miksicek, R.J., Segraves, W.A., Raikhel, A.S., **1998**. DNA binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex. *J. Biol. Chem. 273*, 27531–27540.

Wang, Y., Kaguni, L.S., **1999**. Baculovirus expression reconstitutes *Drosophila* mitochondrial DNA polymerase. *J. Biol. Chem. 274*, 28972–28977.

Wehmeyer, A., Schulz, R., **1997**. Overexpression of δ-opioid receptors in recombinant baculovirus-infected *Trichoplusia ni* "High 5" insect cells. *J. Neurochem. 68*, 1361–1371.

Wester, L., Johansson, M.U., Alerstrom, B., **1997**. Physiochemical and biochemical characterization of human α_1-microglobulin expressed in baculovirus-infected insect cells. *Protein Expr. Purif. 11*, 95–103.

Weyer, U., Knight, S., Possee, R.D., **1990**. Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectros. *J. Gen. Virol. 71*, 1525–1534.

Whitford, M., Faulkner, P., **1992**. Nucleotide sequence and transcriptional analysis of a gene encoding p41, a structural glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 66*, 4763–4768.

Whitford, M., Stewart, S., Kuzio, J., Faulkner, P., **1989**. Identification and sequence analysis of a gene encoding gp67 and abundant envelope glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 63*, 1393–1399.

Wickham, T.J., Davis, T., Granados, R.R., Shuler, M., Wood, H.A., **1992**. Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. *Biotechnol. Progr. 8*, 391–396.

Willard, D., Chen, W.-J., Barrett, G., Blackburn, K., Bynum, J., *et al.*, **2000**. Expression purification and characterization of the human receptor activator of NF-κB ligand (RANKL) extracellular domain. *Protein Expr. Purif. 20*, 48–57.

Williams, G.V., Rohel, D.Z., Kuzio, J., Faulkner, P., **1989**. A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *J. Gen. Virol. 70*, 187–202.

Winzerling, J.J., Pham, D.Q.-D., Kunz, S., Samaraweera, P., Law, J.H., *et al.*, **1996**. Purification of recombinant insect transferrin from large volumes of cell culture medium using high capacity Ni^{2+}-dipicolylamine gel. *Protein Expr. Purif. 7*, 137–142.

Withers, B.E., Keller, P.R., Fry, D.W., **1996**. Expression, purification and characterization of focal adhesion kinase using a baculovirus system. *Protein Expr. Purif. 7*, 12–18.

Wu, X., Kamei, K., Sato, H., Sato, S.I., Takano, R., *et al.*, **2001**. High-level expression of human acidic fibroblast growth factor and basic fibroblast growth factor in silkworm (*Bombyx mori* L.) using recombinant baculovirus. *Protein Expr. Purif. 21*, 192–200.

Xiao, Z., Jiang, X., Beckett, M.L., Wright, G.L., Jr., **2000**. Generation of a baculovirus recombinant prostrate-specific membrane antigen and its use in the development of a novel protein biochip quantitative immunoassay. *Protein Expr. Purif. 19*, 12–21.

Yamanaka, T., Kuroki, M., Kinugasa, T., Matsuo, Y., Matsuoka, Y., **1996**. Preparation and characterization of two human carcinoembryonic antigen family proteins of neutrophils CD66b and c in silkworm larvae. *Protein Expr. Purif. 7*, 438–446.

Yamao, M., Katayama, N., Nakazawa, H., Yamakawa, M., Hayashi, Y., *et al.*, **1999**. Gene targeting in the silkworm by use of a baculovirus. *Genes Develop. 13*, 511–516.

Yang, S., Miller, L.K., **1999**. Activation of baculovirus very late promoters by interaction with very late factor 1. *J. Virol. 73*, 3404–3409.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., *et al.*, **1993**. Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature 366*, 476–479.

Yap, C.C., Ishii, K., Aoki, Y., Aizaki, H., Tani, H., *et al.*, **1997**. A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. *Virology 231*, 192–200.

Yet, F.-S., Moon, Y.K., Sul, H.S., **1995**. Purification and reconstitution of murine mitochondrial glycerol-3-phosphate acyltransferase. Functional expression in baculovirus-infected insect cells. *Biochemistry 34*, 7303–7310.

Zdobnov, E.M., von Mering, C., Letunic, I., Torrents, D., Suyama, M., *et al.*, **2002**. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster. Science 298*, 149–159.

Zhang, B., Egli, D., Georgiev, O., Schaffner, W., **2001**. The *Drosophila* homolog of mammalian zinc finger factor MTF-1 activates transcription in response to heavy metals. *Mol. Cell. Biol. 21*, 4505–4514.

Zhang, J., Alfonso, P., Thotakura, N.R., Su, J., Buergin, M., *et al.*, **1998**. Expression purification and bioassay of human stanniocalcin from baculovirus-infected insect cells and recombinant CHO cells. *Protein Expr. Purif. 12*, 390–398.

Zhang, J., Kalogerakis, N., Behie, L.A., Iatrou, K., **1993**. A two-stage bioreactor system for the production of recombinant proteins using a genetically engineered baculovirus/insect cell system. *Biotechnol. Bioeng. 42*, 357–366.

Zhang, R., Cai, H., Fatima, N., Buczko, E., Dufau, M.L., **1995**. Functional glycosylation sites of the rat luteinizing hormone receptor required for ligand binding. *J. Biol. Chem. 270*, 21722–21728.

Zhao, Y.-G., Eggleston, P., **1999**. Comparative analysis of promoters for transient gene expression in cultured mosquito cells. *Insect Mol. Biol. 8*, 31–38.

zu Putlitz, J., Kubasek, W.L., Duchene, M., Marget, M., von Specht, B.U., *et al.*, **1990**. Antibody production in baculovirus-infected insect cells. *Biotechnology. 8*, 651–654.

5.1 Sodium Channels

D M Soderlund, Cornell University,
Geneva, NY, USA

5.1.1. Introduction

5.1.1.1. Function and Structure of Voltage-Sensitive Sodium Channels

Voltage-sensitive sodium channels mediate the transient increase in sodium ion permeability that underlies the rising phase of the electrical action potential in most types of excitable cells (Hille, 2001). In vertebrates, sodium channels are found in neurons and cardiac and skeletal muscle cells as well as glial and neuroendocrine cells (Goldin, 2002). In contrast, voltage-sensitive sodium channels in insects appear to be limited in distribution to neurons (Littleton and Ganetzky, 2000).

The principal structural element of voltage-sensitive sodium channels is a large (~260 kDa) α subunit that forms the ion pore and confers the functional and pharmacological properties of the channel (Catterall, 2000; Yu and Catterall, 2003). Sodium channel α subunits are pseudotetrameric proteins that contain four internally homologous domains, each of which contains six hydrophobic transmembrane helices and additional hydrophobic segments that contribute to the formation of the ion pore (**Figure 1**). The four internally homologous domains form a radially symmetrical assembly with the ion pore at the center (Guy and Seetharamulu, 1986; Yellen, 1998; Lipkind and Fozzard, 2000).

Voltage-sensitive sodium channel α subunits are part of a larger family of cation channels that also includes cyclic nucleotide-gated channels and voltage-sensitive potassium and calcium channels (Strong *et al.*, 1993). Whereas voltage-sensitive sodium and calcium channels share the pseudotetrameric four-domain structure shown in **Figure 1**, voltage-sensitive potassium channels and cyclic nucleotide-gated channels are composed of four separate subunits, each of which corresponds to a single domain of sodium and calcium channels. The phylogenetic distribution of voltage-sensitive cation channels and amino acid sequence comparisons among and between channel classes suggest that the earliest channels were tetramers of four separate subunits, like voltage-sensitive potassium channels (Strong *et al.*, 1993; Goldin, 2002). These data also suggest that four-domain calcium channels arose from such channels via two rounds of intragenic duplication. The four-domain voltage-sensitive sodium channels, which appear later in evolution than calcium channels, are likely to have arisen from duplication and divergence of voltage-sensitive calcium channels.

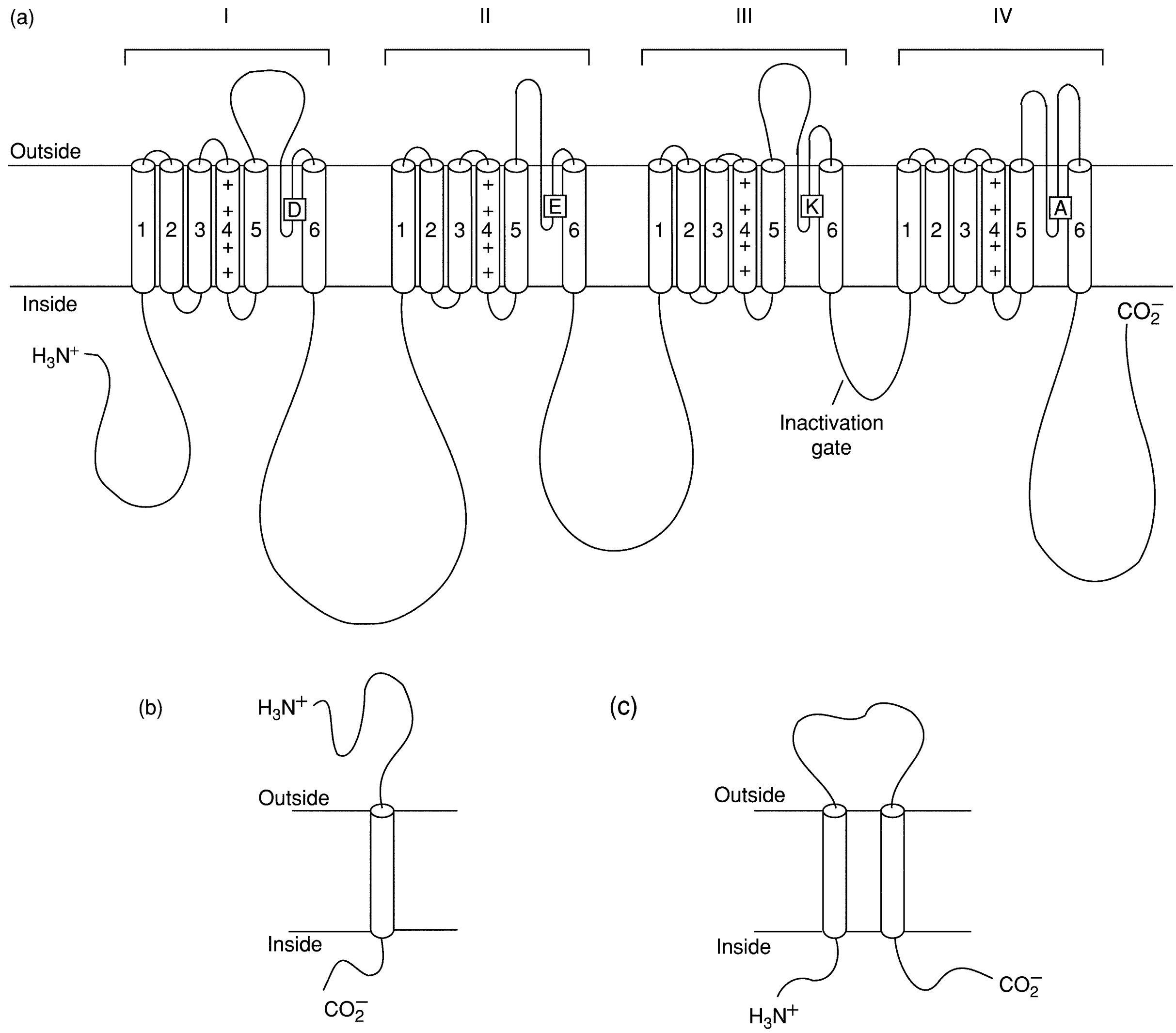


Figure 1 Diagrammatic representations of the structure of vertebrate and insect sodium channel subunits. (a) Sodium channel α subunit, showing the four internally homologous domains (I–IV), each containing six hydrophobic transmembrane helices. Also illustrated are other structural elements relevant to sodium channel function: the multiple positively charged residues (+) in the four S4 helices that constitute the voltage sensor, the four amino acids constituting the selectivity filter (the ''DEKA motif'') located in the pore-forming regions of the four internally homologous domains, and the inactivation gate peptide between the third and fourth domains. (b) Vertebrate sodium channel auxiliary β subunit. (c) Insect sodium channel auxiliary tipE subunit.

Further sodium channel gene duplication and divergence in the course of mammalian evolution has resulted in at least 10 sodium channel α subunit genes that differ in amino acid sequence, developmental and anatomical distribution, and biophysical and pharmacological properties (Goldin *et al.*, 2000; Goldin, 2002). In mammalian brain and skeletal muscle, sodium channel α subunits are associated with one or more smaller auxiliary β subunits that modulate the expression and functional properties of sodium channel α subunits (Catterall, 2000; Goldin, 2001).

5.1.1.2. Sodium Channels as Targets for Neurotoxicants

Voltage-sensitive sodium channels are the site of action of a wide structural variety of naturally occurring neurotoxins that contribute to the chemical ecology of predation and defense (Cestèle and Catterall, 2000; Blumenthal and Seibert, 2003; Wang and Wang, 2003). These sites, together with binding sites for synthetic neurotoxicants and drugs, identify at least 10 distinct binding domains associated with the voltage-sensitive sodium channel.

Five principal neurotoxin recognition sites, designated as sites 1–5 (Catterall, 1988, 1992), have been identified in both functional assays and in radioligand binding experiments (see **Table 1**). These sites are thought to represent physically non-overlapping domains of the sodium channel protein that interact allosterically as the channel protein changes conformation in response to the binding of one or more neurotoxins. Site 1 binds the water-soluble toxins tetrodotoxin (TTX) and saxitoxin (STX), which interact at or near the extracellular opening of the ion pore and block ion transport through the channel. Site 2 binds a structurally heterogeneous group of lipophilic toxins that alter both the opening (activation) and closing (inactivation) of sodium channels. Site 2 neurotoxins shift the voltage dependency of sodium channel activation toward more negative membrane potentials, thereby increasing the probability that channels will open at normal membrane resting potentials. These toxins also slow or completely block sodium channel inactivation, thereby prolonging the open state of the channel. Site 3 binds a group of polypeptide toxins, called α-toxins, isolated from scorpion venoms or sea anemone nematocysts. These toxins selectively prolong sodium channel inactivation without affecting the rate or voltage dependency of channel opening and allosterically enhance the action of compounds acting at site 2. Site 4 binds a second group of polypeptide scorpion toxins, called β-toxins, which selectively enhance sodium channel activation and do not interact allosterically with site 2 compounds. Site 5 binds the brevetoxins and ciguatoxin, lipophilic compounds isolated from marine dinoflagellates, which shift the voltage dependency of sodium channel activation and prolong inactivation in a manner similar to site 2 neurotoxins. However, these compounds bind at a site distinct from site 2 and interact allosterically with compounds acting at site 2.

Table 1 Identified and inferred binding domains on the voltage-sensitive sodium channel

Site[a]	*Active neurotoxins*[b]	*Physiological effect*
Binding domains identified with specific radioligands		
1	Tetrodotoxin	Inhibit ion transport
	Saxitoxin	
	μ-Conotoxin	
2	Veratridine	Persistent activation
	Batrachotoxin	
	Aconitine	
	Grayanotoxins	
	Pumiliotoxin-B	
	N-alkylamides	
3	α Scorpion toxins	Prolong inactivation
	Sea anemone toxins	
4	β Scorpion toxins	Enhance activation
5	Brevetoxins	Persistent activation
	Ciguatoxin	
6	δ-Conotoxins	Prolong inactivation
	Conus striatus toxin	
7	DDT and pyrethroids	Prolong inactivation
Binding domains inferred but not characterized with radioligands		
8	μO-conotoxins	Inhibit ion transport
9	Gonioporatoxin	Prolong inactivation
10	Local anesthetics	Inhibit ion transport
	Anticonvulsants	
	Antiarrhythmics	
	Pyrazolines	

[a]Sites 1–5 after Catterall (1988); sites 6 and 7 after Zlotkin (1999); sites 8–10 are assigned arbitrarily to distinguish them from sites 1–7.
[b]Insecticides are capitalized.

Subsequent research has identified two additional binding domains that are labeled with specific radioligands and shown to be distinct from sites 1 to 5. Site 6 (**Table 1**) binds δ-conotoxins and *Conus striatus* toxin (Gonoi *et al.*, 1987; Fainzilber *et al.*, 1994). Site 7 (**Table 1**) (also designated site 6 in some classifications) binds dichlorodiphenyltrichloroethane (DDT) and pyrethroids (Bloomquist and Soderlund, 1988; Lombet *et al.*, 1988; Trainer *et al.*, 1997). At least three other binding domains (arbitrarily designated as sites 8–10 in **Table 1**) have been postulated to account for the actions on sodium channels of μO-conotoxins (Terlau *et al.*, 1996), *Goniopora* coral toxin (Gonoi *et al.*, 1986), and local anesthetics, class I antiarrhythmics, and class I anticonvulsants (Catterall, 1987).

Sodium channels are widely recognized as the principal target site for diphenylethane (e.g., DDT) and pyrethroid insecticides (Sattelle and Yamamoto, 1988; Bloomquist, 1993; Soderlund, 1995; Narahashi, 1996). In addition, other toxicant-binding domains on the sodium channel are important target sites for the continued discovery and development of novel insect control agents. Two additional classes of synthetic insecticides alter sodium channel function by binding to sites that are distinct from the pyrethroid recognition site. Synthetic *N*-alkylamide insecticides, which are analogs of insecticidal natural products, produce excitatory effects by binding to site 2 (Ottea *et al.*, 1989, 1990), whereas pyrazolines (also called dihydropyrazoles) and structurally related insecticides suppress normal nerve activity by the voltage-dependent blockade of sodium channels in a manner similar to anticonvulsants (Salgado, 1990, 1992; Deecher *et al.*, 1991;

Deecher and Soderlund, 1991). The recent development of indoxacarb, the first commercial insecticide derived from pyrazoline-type compounds (Harder *et al*., 1996; Wing *et al*., 1998), underscores the continued relevance of the sodium channel as a target for insecticide development. Sodium channels are also the target of insect-selective polypeptide toxins from scorpion venoms (Zlotkin *et al*., 1995) which have been genetically engineered into insect baculoviruses; two potential biopesticide products based on this technology have proceeded to the stage of large-scale field tests (Cory, 1999).

5.1.1.3. Progress in Insect Sodium Channel Research Since 1985

The report in 1984 of the first DNA sequence for a voltage-sensitive ion channel, the voltage-sensitive sodium channel of the eel (*Electrophorus electricus*) electric organ (Noda *et al*., 1984), marked the beginning of a period of explosive growth in the application of molecular techniques to the study of ion channels that continues to the present. Since that time, studies of mammalian ion channels have identified the existence of gene families for both the α and β subunits of the voltage-sensitive sodium channel and have elucidated the structural domains of sodium channel α subunits that confer many of the biophysical and pharmacological properties of channel isoforms (Catterall, 2000; Goldin, 2001). Biomedical applications resulting from this research include the identification of sodium channel gene mutations that cause heritable diseases and a renewed interest in voltage-sensitive sodium channels as targets for therapeutic agents (Cannon, 2000; Clare *et al*., 2000; Baker and Wood, 2001; Goldin, 2001).

The corresponding expansion of information on voltage-sensitive sodium channels in insects during this period has occurred in two stages. The first of these involved the integration of molecular biology with classical genetic approaches in *Drosophila melanogaster* to further illuminate the nature of gene mutations that were implicated genetically as determinants of sodium channel function and to determine the molecular structures of the genes defined by these mutations. The second phase, in which the molecular and functional characterization of sodium channels was extended to include numerous other insect taxa, was driven principally by research to define the role of sodium channel gene mutations in insecticide resistance. This chapter reviews the literature on insect voltage-sensitive sodium channels since 1985 from both of these perspectives.

5.1.2. Sodium Channel Genes in Insects

5.1.2.1. Sodium Channel Genes in *Drosophila melanogaster*

5.1.2.1.1. *dsc1* The first putative insect sodium channel α subunit gene to be identified, *dsc1*, was isolated from *D. melanogaster* DNA libraries by low-stringency hybridization to a probe from the *E. electricus* sodium channel (Okamoto *et al*., 1987; Salkoff *et al*., 1987; Ramaswami and Tanouye, 1989). Sequence analysis of partial genomic DNA and cDNA clones identified sequences encoding the four internally homologous domains (Salkoff *et al*., 1987), but sequences of the 5′ and 3′ termini and the segments lying between homology domains I and II and homology domains II and III, all of which are regions of low sequence conservation in vertebrate genes, were not defined until the recent report of a full-length *dsc1* coding sequence (Kulkarni *et al*., 2002). The *dsc1* locus was initially mapped to cytogenetic region 60D-E on chromosome 2R (Okamoto *et al*., 1987; Salkoff *et al*., 1987; Ramaswami and Tanouye, 1989) and subsequently localized to cytogenetic region 60E5 by genome sequencing (Adams *et al*., 2000). Expression of the *dsc1* gene is under both anatomical and developmental regulation: expression in embryos and larvae is restricted to very few cells, some of which may be nonneuronal, whereas *dsc1*-derived transcripts are found at much higher levels in pupae and adults, where they are widely expressed in the central nervous system and retina (Tseng-Crank *et al*., 1991; Hong and Ganetzky, 1994). A recent genome-wide screen identified *dsc1* transcripts as targets for RNA editing at a site in the "inactivation gate" sequence (**Figure 1**) between homology domains III and IV (Hoopengardner *et al*., 2003).

Alignment of the dsc1 protein sequence with those of vertebrate sodium channels suggests that dsc1 may exhibit atypical ion selectivity. Two conserved amino acid residues, a lysine in the pore-forming region of homology domain III and an alanine in the corresponding location in homology domain IV, are crucial for the sodium ion selectivity typical of voltage-sensitive sodium channels. When either of these is mutated to a glutamate residue the resulting channel becomes permeable to calcium and other monovalent and divalent cations that normally do not permeate sodium channels (Heinemann *et al*., 1992). The dsc1 protein sequence contains a glutamate residue rather than the conserved lysine residue in the pore-forming region of homology domain III and therefore may form a channel that is permeable to calcium as well as sodium. This observation is intriguing in light of recent studies identifying a

novel sodium current in dorsal unpaired median neurons of the cockroach *Periplaneta americana* that is biophysically distinct from the more typical sodium channels present in the same neurons and is permeable to calcium as well as sodium (Grolleau and Lapied, 2000; Defaix and Lapied, 2001).

5.1.2.1.2. *para* The *para*^{ts} (*paralytic–temperature-sensitive*) locus at cytogenetic location 14D on the X chromosome of *D. melanogaster* was first identified as the site of a temperature-sensitive paralytic mutation (Suzuki *et al.*, 1971), which was subsequently found to cause temperature-sensitive impairment of action potential conduction in nerves (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980). Sequencing of genomic DNA and cDNA clones derived from the *para* locus showed that *para* encodes a protein having all of the structural hallmarks of voltage-sensitive sodium channel α subunits and exhibiting approximately 50% overall amino acid sequence identity to vertebrate sodium channel α subunits (Loughney *et al.*, 1989). The *para* gene product is quite divergent from the *dsc1* gene product in that the four conserved internal homology domains of the two *D. melanogaster* genes are only as similar to each other (~50% amino acid sequence identity) as they are to the corresponding regions of vertebrate sodium channel proteins.

Sequence analyses of partial cDNAs derived from the *para* loci of *D. melanogaster* and the related species *D. virilis* identified alternative mRNA splicing at eight sites involving seven optional exons (designated *a, b, e, f, h, i,* and *j*) and one pair of mutually exclusive exons (designated *c/d*), resulting in 256 possible unique structural variants from the *para* gene (Thackeray and Ganetzky, 1994, 1995; O'Dowd *et al.*, 1995; Warmke *et al.*, 1997). The locations of alternatively spliced exons in relation to other structural landmarks in the para protein are illustrated in **Figure 2**. Alternative splicing at

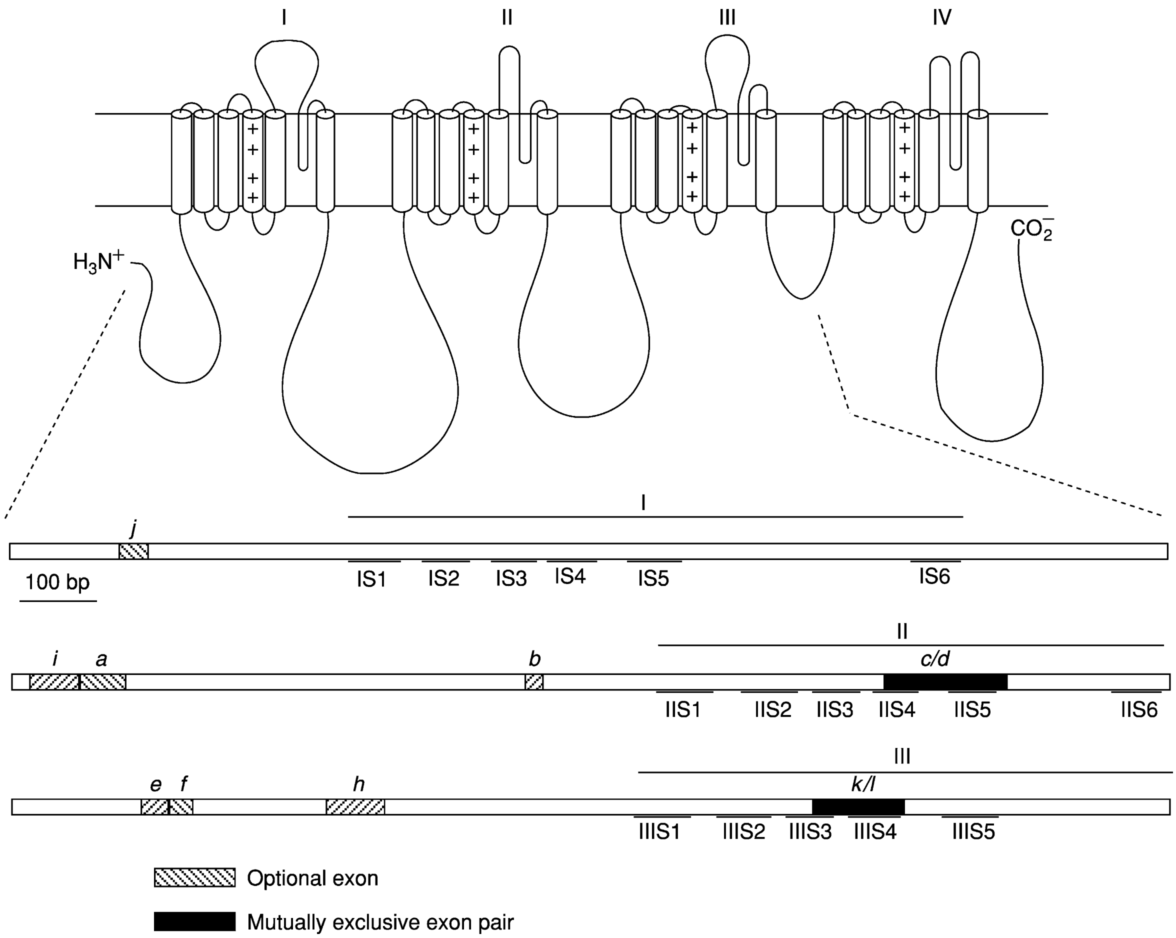


Figure 2 Diagram showing the approximate size and location of optional and mutually exclusive exons of in the *para* and *Vssc1* sodium channel genes in relation to other sodium channel structural landmarks. (Modified with permission from Lee, S.H, Ingles, P.J., Knipple, D.C., Soderlund, D.M., **2002**. Developmental regulation of alternative exon usage in the housefly *Vssc1* sodium channel gene. *Invert. Neurosci. 4,* 125–133.)

these sites produces a heterogeneous family of sodium channel α subunit transcripts. Additional transcript heterogeneity in these species is conferred by posttranscriptional RNA editing at three sites (Hanrahan *et al.*, 2000). Transcripts from the *para* locus are expressed strongly in the nervous system throughout development, but analysis of transcript pools from different developmental stages and anatomical regions revealed significant developmental and anatomical regulation of both alternative splicing and RNA editing (Tseng-Crank *et al.*, 1991; Hong and Ganetzky, 1994; Thackeray and Ganetzky, 1994, 1995; Hanrahan *et al.*, 2000).

5.1.2.1.3. *tipE* The *tipE* (*temperature-induced paralysis, locus E*) gene, at cytogenetic region 64B2, is the site of a temperature-sensitive paralytic mutation having a phenotype similar to that of *para* mutants (Kulkarni and Padhye, 1982; Jackson *et al.*, 1986; Feng *et al.*, 1995b). Sequence analysis of cDNAs derived from the *tipE* locus identified an open reading frame encoding a 452-amino acid protein that is clearly not a member of the voltage-sensitive ion channel family and exhibits no significant sequence similarity to any other proteins in sequence databases (Feng *et al.*, 1995a). The tipE protein contains two hydrophobic segments having properties consistent with the formation of transmembrane helices that flank a loop that contains five potential *N*-glycosylation sites. Transcripts from the original *tipE* mutant strain contain a premature stop codon between the two hydrophobic domains and therefore express a truncated tipE protein. Expression of the *tipE* gene in *D. melanogaster* is restricted to the nervous system and exhibits strong developmental regulation, so that transcript levels are very low in embryonic and larval stages but increase markedly in the late pupal stage. Genetic and functional criteria (see Section 5.1.3.1) suggest that the tipE protein may be a sodium channel auxiliary subunit, functionally analogous but structurally unrelated to vertebrate sodium channel β subunits.

5.1.2.1.4. Genetic and functional characterization of *D. melanogaster* mutants Gene dosage and interaction studies with $para^{ts}$ mutants provide evidence for a crucial physiological role for the para protein. Homozygous $para^{ts}$ mutants that exhibit temperature-sensitive paralysis in a wild-type background are unconditionally lethal in the presence of either nap^{ts} (*no action potential, temperature-sensitive*) or *tipE*, both of which also impair nerve function at nonpermissive temperatures (Ganetzky, 1984, 1986). Similarly, decreased dosage of $para^{+}$ is unconditionally lethal in a nap^{ts} background, but increased dosage of $para^{+}$ causes leg-shaking behavior, a sign of neuronal excitation, and suppresses the temperature sensitivity of nap^{ts} (Stern *et al.*, 1990).

The *smellblind* locus was originally identified as the site of mutations causing olfactory defects and was mapped close to *para* on the X chromosome (Lilly and Carlson, 1989). Two viable *smellblind* alleles exhibit temperature-dependent lethality to embryos at high temperatures and to adults at low temperatures, whereas four other alleles are recessive lethals (Lilly and Carlson, 1989; Lilly *et al.*, 1994b). None of the six *smellblind* alleles complements the lethality of two unconditionally lethal *para* alleles, and two of the recessive lethal alleles of *smellblind* contain rearrangements within the primary *para* transcript (Lilly *et al.*, 1994a). Taken together, these results show that *smellblind* is a novel class of *para* mutation. Interestingly, *smellblind* alleles do not exhibit the rapid, temperature-sensitive paralysis of $para^{ts}$ mutants, and the $para^{ts}$ mutants examined so far do not exhibit olfactory defects (Lilly *et al.*, 1994a).

Electrophysiological and pharmacological studies provide additional evidence for the functional importance of the *para* gene product. The temperature-sensitive failure of nerve conduction associated with $para^{ts}$ alleles is presynaptic to the cervical giant fibers, which remain electrically excitable at nonpermissive temperatures (Nelson and Wyman, 1989; Elkins and Ganetzky, 1990). Cultured neurons from embryos homozygous for different $para^{ts}$ alleles exhibit an allele-dependent reduction in sodium current density; in the case of $para^{ST76}$ the expression of sodium currents was reduced by 98% (O'Dowd *et al.*, 1989). One of these alleles, $para^{ts2}$, also caused a depolarizing shift in the voltage dependence of activation of sodium channels in cultured embryonic neurons. Cultured larval neurons from $para^{ts}$ individuals exhibit temperature-dependent resistance to veratridine, which binds to site 2 on the sodium channel and causes persistent channel activation (Suzuki and Wu, 1984). Also, several $para^{ts}$ strains are resistant to DDT and pyrethroid insecticides (Pittendrigh *et al.*, 1997).

The *tipE* mutation causes a temperature-dependent paralytic phenotype similar to that caused by $para^{ts}$ alleles (Jackson *et al.*, 1986). Three additional mutant *tipE* alleles, generated by γ-ray mutagenesis and characterized as deletions or translocations disrupting the *tipE* locus, confer the temperature-sensitive phenotype when heterozygous with the original ethylmethanesulfonate-induced *tipE* allele (Feng *et al.*, 1995b). This finding suggests that the original *tipE* mutation, identified

as a premature stop codon yielding a truncated gene product (Feng *et al.*, 1995a), is a loss-of-function mutation. In addition to conferring the conditional paralytic phenotype, the *tipE* mutation causes temperature-sensitive impairment of conduction in adult, but not larval, nerves (Ganetzky, 1986; Elkins and Ganetzky, 1990) and reductions of approximately 50% in the sodium current density of cultured embryonic neurons and in density of [^{3}H]saxitoxin binding sites in adult head preparations (Jackson *et al.*, 1986). Studies using cultured embryonic neurons show that $tipE^+$ is required for the sustained repetitive firing of sodium-dependent action potentials (Hodges *et al.*, 2002). Sodium currents in neurons from animals carrying the *tipE* mutation recover more slowly, and the minimum interpulse interval needed to produce a second full-amplitude action potential is longer, than those in neurons from wild-type animals. Expression of the wild-type $tipE^+$ transgene rescues all of the functional deficits conferred by the *tipE* mutation in these assays.

Interactions between *tipE* and $para^{ts}$ alleles provide further insight into the functional role of the tipE protein. As noted above, *tipE* interacts with various $para^{ts}$ alleles to cause unconditional lethality at temperatures that are permissive for single mutants (Ganetzky, 1986). It is interesting that some alleles of *para* exhibit partial viability when combined with *tipE*, whereas other allelic combinations are unconditionally lethal. These observations have been interpreted as evidence that the tipE and para proteins may interact physically (Ganetzky, 1986).

In contrast to the large body of information available about the functional significance of *para* and *tipE*, there is very little information on the role of *dsc1*. Embryonic neurons homozygous for a deficiency that includes the *dsc1* locus exhibit normal sensitivity to veratridine (Sakai *et al.*, 1989), thereby indicating the presence of functional sodium channels in these cells. Moreover, these neurons express voltage-sensitive sodium currents that are indistinguishable from those in wild-type cells (Germeraad *et al.*, 1992). These studies provide further evidence that *para* rather than *dsc1* encodes the sodium channels found in embryonic neurons.

Until recently, there were no mutations associated with the *dsc1* locus that might illuminate the function of the dsc1 protein. However, a series of *smell-impaired* (*smi*) mutants, created by single *P* element insertion mutagenesis and isolated on the basis of the loss of an olfactory avoidance behavioral phenotype, included one mutation (*smi60E*) mapping close to the *dsc1* locus (Anholt *et al.*, 1996). Subsequent studies showed that this mutant strain contains a *P* element transposon within the second intron of the *dsc1* gene (Kulkarni *et al.*, 2002). The reduction of olfactory avoidance behavior in this strain is correlated with a reduction in the abundance of *dsc1* transcripts, and excision of the *P* element restores wild-type behavior and *dsc1* transcript levels. These results provide the first clear indication of a physiologically defined role for the dsc1 protein.

5.1.2.2. Sodium Channel Genes in Other Insect Species

5.1.2.2.1. Orthologs of *para* The identification of the *para* gene product as a physiologically important voltage-sensitive sodium channel in *D. melanogaster* (Loughney *et al.*, 1989) and the concurrent development of the polymerase chain reaction (PCR) as a means of gene identification (Saiki *et al.*, 1988) provided the conceptual and technical framework for the isolation of *para*-orthologous DNA sequences from other arthropod species (Doyle and Knipple, 1991; Knipple *et al.*, 1991). The likely importance of voltage-sensitive sodium channels as sites of mutations conferring resistance to DDT and to pyrethroid insecticides provided further impetus for this effort (Soderlund and Knipple, 2003). As a result, full-length or partial genomic DNA or cDNA sequences now are known for *para*-orthologous sodium channel genes from at least 15 additional arthropod species.

Two concurrent and independent efforts led to the characterization of the full-length coding sequence of *Vssc1* (also called *Msc*), the *para* ortholog of the housefly (*Musca domestica*) (Ingles *et al.*, 1996; Williamson *et al.*, 1996). The inferred sequence of the Vssc1 protein is 90% identical to that of the most similar splice variant of the *para* gene product (Loughney *et al.*, 1989; Thackeray and Ganetzky, 1994). The initial sequence analyses of *Vssc1* cDNA clones obtained from adult fly heads did not identify alternatively spliced transcripts (Ingles *et al.*, 1996; Williamson *et al.*, 1996). A subsequent study investigated the issue of alternative splicing at the *Vssc1* locus in greater detail by examining the heterogeneity of multiple cDNA clones derived from *Vssc1* transcripts in three different developmental stages and two different adult body regions (Lee *et al.*, 2002). This investigation identified multiple alternative exons in the *Vssc1* gene, including a new pair of mutually exclusive exons (designated *k* and *l*) (**Figure 2**) encompassing part of transmembrane domain IIS3 and all of transmembrane domain IIS4. This splice site was not previously identified in the analysis of *para* transcripts in *Drosophila* species, which contain a segment homologous to exon *l* (Thackeray and Ganetzky, 1994, 1995; O'Dowd

et al., 1995; Warmke *et al.*, 1997), but a search of the *para* genomic sequence (Adams *et al.*, 2000) identified a segment upstream of the exon *l*-like sequence with substantial sequence similarity to exon *k* of *Vssc1* (Lee *et al.*, 2002). The amino acid sequences of exons *k* and *l* are highly divergent, differing at 16 of 41 amino acid residues, but retain conserved features of the voltage sensor structure in domain IIIS4. Other novel aspects of alternative splicing of *Vssc1* transcripts in *M. domestica* included the apparent constitutive expression of optional exons *h* and *i* and the apparent inactivation of exon *c* by a stop codon, so that all transcripts encoding full-length channel sequences contained only exon *d* at this site. Alternative splicing at all of the sites identified either in *Vssc1* or in the *para* genes of *D. melanogaster* or *D. virilis* could theoretically generate up to 512 structurally unique sodium channel splice variants. However, only a small subset of these variants (five or fewer) comprised more than 90% of the transcripts in each *M. domestica* cDNA pool examined.

The *para* ortholog of *Blattella germanica* (designated $para^{CSMA}$) encodes an inferred protein sequence that is 76–78% identical to the two dipteran sequences (Dong, 1997; Tan *et al.*, 2002a). Analysis of multiple $para^{CSMA}$ sequences identified novel aspects of alternative exon usage at some of the sites of alternative splicing found in *D. melanogaster* and *M. domestica*. For example, $para^{CSMA}$ transcripts contain two examples of segments corresponding to optional exon *j*, suggesting that the *B. germanica* gene may incorporate multiple variants of this exon. Similarly, the *B. germanica* transcripts contain three different exons (designated G1, G2, and G3) at the exon *k*/*l* splice site in homology domain III (Tan *et al.*, 2002a). Exons G1 and G2 correspond to exons *l* and *k* of *para* and *Vssc1*, whereas exon G3 exhibits no obvious sequence similarity to either exon G1 or G2 and contains an in-frame stop codon. Exon G3 corresponds exactly to an alternative exon in vertebrate $Na_v1.6$ sodium channels that contains a premature stop codon at the same position (Plummer *et al.*, 1997).

The structure of *hscp*, the ortholog of *para* in *Heliothis virescens*, was determined by sequence analyses of genomic DNA and PCR-amplified cDNA segments (Park *et al.*, 1999). The reported *hscp* coding sequence, which is incomplete at the 3′ end, gives a predicted protein sequence that is 80% identical to the corresponding coding region of *para*. Fifteen of the 19 introns identified in the *hscp* gene were conserved in location with respect to the corresponding introns in *para*, and alternatively spliced transcripts containing optional exon *j* and mutually exclusive exons *c* and *d* were detected. This study also identified several transcripts lacking sequences from the linker between homology domains I and II that contained premature stop codons. Unlike the truncation of the *M. domestica* and *B. germanica* transcripts by premature stop codons located in alternatively spliced exons, these *H. virescens* transcripts appear to be the result of splicing errors that resulted in missing exons and frameshifts.

The ortholog of *para* in the head louse, *Pediculus capitis*, encodes a voltage-sensitive sodium channel α subunit protein that is 73% identical to *para* and the *B. germanica* and *M. domestica* orthologs (Lee *et al.*, 2003). Full-length sequences from two strains of head louse and one strain of body louse (*P. humanus*) were 99.6% identical at the nucleotide level, providing further evidence that the head louse and body louse are conspecific. Analysis of multiple transcripts provided evidence for alternative splicing of a segment homologous to exon *j* of *para*. All of the transcripts contained segments corresponding to *para* optional exons *a*, *b*, and *h* and mutually exclusive exons *d* and *l* but did not contain segments corresponding to optional exons *i*, *e*, and *f*.

The ortholog of *para* in the varroa mite, *Varroa destructor* (designated *VmNa*) encodes a voltage-sensitive sodium channel α subunit that is only 51% identical to the *para* gene product of *D. melanogaster* (Wang *et al.*, 2003). Sequence analysis of multiple *VmNa* transcripts identified several novel aspects of alternative exon usage in this gene. Some *VmNa* transcripts contained an optional exon (designated exon 3) corresponding to the pair of mutually exclusive introns *k* and *l* in *Drosophila* species and *M. domestica* (**Figure 2**). Some *VmNa* transcripts also contained option exons at three sites not previously identified as sites of alternative splicing: exon 1, a segment of homology domain II containing part of the IIS2 and all of the IIS3 transmembrane helices; exon 2, a short inserted sequence in the domains II–III intracellular linker; and a retained intron in the C terminus containing an alternative stop codon. Channels lacking exon 1 or 3 would also lack critical determinants of channel organization and function and therefore may be inactive, whereas the alternative splicing of exon 2 and the alternative C terminus could be involved in the regulation of channel expression or functional properties.

Partial sequences of varying length also exist for the *para* orthologs from 10 additional arthropod species: *Anopheles gambiae* (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000); *Bemisia tabaci* (Morin *et al.*, 2002); *Boophilus microplus* (He *et al.*, 1999); *Culex pipiens* (Martinez-Torres *et al.*, 1999a); *Frankliniella occidentalis* (Forcioli *et al.*, 2002); *Helicoverpa armigera* (Head *et al.*, 1998); *Hematobia*

irritans (Guerrero *et al.*, 1997); *Leptinotarsa decemlineata* (Lee *et al.*, 1999b); *Myzus persicae* (Martinez-Torres *et al.*, 1999b); and *Plutella xylostella* (Schuler *et al.*, 1998).

5.1.2.2.2. Orthologs of *dsc1* The search for the *para* ortholog of *B. germanica* also yielded a partial cDNA of the ortholog of *dsc1* in this species (Dong, 1997). Subsequent work described the complete coding sequence of this gene (designated *BSC1*) and identified three regions of alternative exon usage (Liu *et al.*, 2001). Reverse transcription (RT)-PCR analyses documented the expression of *BSC1* transcripts not only in the nerve cord but also in muscle, gut, fat body, and ovary, a much broader pattern of expression than was found by *in situ* hybridization of *dsc1*-derived probes to *D. melanogaster* tissues (Hong and Ganetzky, 1994). RT-PCR assays also showed that the expression of *BSC1* splice variants was both tissue-specific and developmentally regulated (Liu *et al.*, 2001). So far, the only other ortholog of *dsc1* described in the literature is a partial genomic DNA sequence from *H. virescens* (Park *et al.*, 1999).

5.1.2.2.3. Orthologs of *tipE* The *tipE* gene is of interest because of its clear involvement in modifying sodium channel expression and function and the suggestion that it may encode a novel type of sodium channel auxiliary subunit. The ortholog of *tipE* in *Musca domestica*, designated *Vsscβ*, encodes a predicted protein that exhibits 72% overall amino acid sequence identity to the tipE protein and 97% identity within the hydrophobic regions identified as probable transmembrane domains (Lee *et al.*, 2000a). These results suggest that *tipE* and *Vsscβ* are substantially more divergent in sequence than are the two sodium channel α subunit genes from these species.

5.1.3. Functional Expression of Cloned Insect Sodium Channels

5.1.3.1. Expression of Functional Sodium Channels in *Xenopus* Oocytes

Unfertilized oocytes of the African clawed frog, *Xenopus laevis*, are a powerful tool for confirming the functional roles of neurotransmitter receptors and ion channels and for correlating channel structure with functional and pharmacological properties (Lester, 1988). Oocytes injected with cRNA (synthetic mRNA prepared from cloned cDNA) express sodium channels in the cell membrane that can be detected by conventional electrophysiological assays, such as two-electrode voltage clamp analysis of the currents carried by the expressed proteins in response to changes in membrane potential or the application of native or exogenous ligands (Goldin, 1992; Stühmer, 1992). This system, coupled with site-directed mutagenesis of cloned cDNAs, has been widely employed in structure–function studies of vertebrate sodium channels (Catterall, 2000; Goldin, 2001; Yu and Catterall, 2003).

Initial efforts to express functional sodium channel from synthetic *para* cRNA injected into oocytes produced voltage-gated sodium currents of very low amplitude, but coexpression of *para* and *tipE* cRNAs stimulated sodium current expression approximately 30-fold (Feng *et al.*, 1995a). Subsequent studies (Warmke *et al.*, 1997) showed that injection of large amounts of *para* cRNA was required to obtain more robust currents in the absence of *tipE* cRNA. These authors also showed that para/tipE channels inactivated more rapidly than those expressed from *para* alone. Thus, coexpression of *para* and *tipE* reconstitutes the functional properties of the native *D. melanogaster* voltage-sensitive sodium channel and provides evidence that the tipE protein may function as a sodium channel auxiliary subunit.

Efforts to express functional sodium channels in oocytes from *Vssc1* cRNA produced results similar to those with *para*, requiring large amounts of injected cRNA to yield low levels of sodium current expression, but coexpression with *tipE* cRNA resulted in the robust expression of voltage-gated sodium currents (Smith *et al.*, 1997; Lee *et al.*, 2000a). These Vssc1/tipE channels, like para/tipE channels, inactivated more rapidly than channels expressed from either *para* or *Vssc1* cRNA alone. Coexpression of Vssc1 with its conspecific auxiliary subunit, Vsscβ, was more effective than coexpression with tipE in enhancing the level of sodium current expression and accelerating the inactivation kinetics of Vssc1 sodium channels (Lee *et al.*, 2000a). Coexpression with *tipE* cRNA was also found to be necessary for the expression of functional sodium channels from $para^{CSMA}$ cRNA in oocytes (Tan *et al.*, 2002b). However, this study did not document the expression of functional channels in oocytes from $para^{CSMA}$ cRNA alone.

5.1.3.2. Pharmacological Properties of Expressed Insect Sodium Channels

Insect sodium channels expressed in oocytes retain sensitivity to insecticides and other natural and synthetic toxicants that alter sodium channel function. Insect sodium channels expressed in oocytes also are sensitive to blockade by nanomolar concentrations of tetrodotoxin (Warmke *et al.*, 1997; Smith *et al.*,

1998; Tan *et al*., 2002a). These channels are also susceptible to modification by the alkaloid batrachotoxin (Lee and Soderlund, 2001) and polypeptide toxins from sea anemone (Warmke *et al*., 1997) and scorpion (Shichor *et al*., 2002) venoms.

The effects of pyrethroids on expressed insect sodium channels have been extensively investigated in the context of the functional characterization of sodium channel mutations associated with pyrethroid resistance (see Section 5.1.4.5). When assayed under voltage clamp conditions, type I pyrethroids (i.e., cismethrin and permethrin) appear to bind predominantly to resting or inactivated channels, shifting the voltage dependence of activation to more negative potentials and causing a slowly activating sodium current. These compounds also produce characteristic sodium tail currents following a depolarizing pulse that decay with first-order time constants (Smith *et al*., 1997, 1998; Warmke *et al*., 1997; Zhao *et al*., 2000). In contrast to these results, type II pyrethroids (i.e., [1*R*,*cis*,α*S*]-cypermethrin and deltamethrin) exhibit profound use-dependent modification of sodium currents, which implies that these compounds bind preferentially to activated sodium channel states (Smith *et al*., 1998; Vais *et al*., 2000; Tan *et al*., 2002b). The tail currents caused by these compounds are more persistent than those caused by cismethrin or permethrin. In the case of deltamethrin, tail current decay is biphasic, rather than monophasic (Vais *et al*., 2000; Tan *et al*., 2002b); this finding has been interpreted as evidence for two binding sites on insect sodium channels with different affinities for this ligand (Vais *et al*., 2000).

5.1.3.3. Functional Characterization of Sodium Channel Splice Variants

The conservation of alternative exon structure and the developmental and anatomical regulation of alternative exon usage in the *para* sodium channel gene of *D. melanogaster* and its orthologs in other insect species imply that alternative splicing may generate a family of sodium channel proteins with differing functional properties, as has been found for other ion channels and receptors (Harris-Warwick, 2000). Most of the optional exons identified in *para* orthologs to date (see **Figure 2**) are located in intracellular domains of the sodium channel protein. These exons may therefore be involved in the regulation of sodium channel expression or function as the result of interactions with protein kinases or G proteins (Cukierman, 1996; Cantrell and Catterall, 2001). This interpretation is consistent with the existence in exons *a* and *i* of consensus protein kinase A phosphorylation sites (O'Dowd *et al*., 1995; Ingles *et al*., 1996). Similarly, exons 2 and 4 of the *VmNa* sodium channel are located in intracellular domains, and exon 2 contains contains a consensus protein kinase C phosphorylation site (Wang *et al*., 2003). The sole exception so far to the intracellular location of optional exons is exon 1 of the *VmNa* sodium channel sequence, which is located in the transmembrane region of domain II (Wang *et al*., 2003).

So far, there is little direct evidence bearing on the functional significance of the alternative splicing of optional exons. In embryonic *D. melanogaster* neurons, functional sodium channels were detected only in those cells having *para* transcripts containing exon *a* (O'Dowd *et al*., 1995). This study also documented enhanced sodium current expression in cells expressing channels that contain both exons *a* and *i*. Whereas these findings imply a critical role for exon *a* alone and the combination of exons *a* and *i* together in sodium channel regulation, the direct comparison in functional expression assays using *X. laevis* oocytes of variants of *para* that differ only by the presence or absence of exon *a* did not find any effects of exon *a* on sodium current expression or properties in this system (Warmke *et al*., 1997).

In contrast to the optional exons, the mutually exclusive exons in *para* orthologs occur within the transmembrane regions of homology domains II and III (**Figure 2**). There is no information on the functional role of the alternative splicing of exons *c* and *d*. In *D. melanogaster*, these exons differ by only two of 55 amino acid residues (Loughney *et al*., 1989). In *M. domestica*, all functional channels apparently contain only exon *d* because exon *c* contains an in-frame stop codon (Lee *et al*., 2002). These observations suggest that alternative splicing at the *c*/*d* site may play a role in posttranscriptional regulation rather than in the generation of functionally distinct channel variants.

The most significant functional effects of alternative exon usage have been documented for splice variants at the exon *k*/*l* site. Unlike exons *c* and *d*, exons *k* and *l* (corresponding to exons G2 and G1 in *B. germanica*) differ substantially in amino acid sequence (Lee *et al*., 2002; Tan *et al*., 2002a). Expression of *para*^{CSMA} variants containing either exon G1 or G2 in oocytes documents differences in the voltage dependence of both activation and inactivation of these channels (Tan *et al*., 2002a). Unexpectedly, this study also found substantial differences between these variants in their sensitivity to the pyrethroid insecticide deltamethrin. These results provide the first experimental evidence for functional differences between splice variants of insect sodium channels. Alternative splicing at the *k*/*l* site in

B. germanica and *V. destructor* also appears to be involved in the expression of inactive channel variants, in that exon G3 in the *para*^{CSMA} sequence encodes a truncated channel (Tan *et al.*, 2002a) and the absence of exon 3 in the *VmNa* sequence encodes a channel lacking one of the four voltage sensor regions (Wang *et al.*, 2003).

5.1.4. Sodium Channels and Knockdown Resistance to Pyrethroids

5.1.4.1. Knockdown Resistance

The knockdown resistance (*kdr*) trait, which confers resistance to the rapid knockdown action and lethal effects of DDT and pyrethrins, was first documented in houseflies in 1951 (Busvine, 1951) and isolated genetically in 1954 (Milani, 1954). The *kdr* trait confers resistance to both the rapid paralytic and lethal actions of all known pyrethroids, as well as the pyrethrins and DDT, but does not diminish the efficacy of other insecticide classes (Oppenoorth, 1985). Electrophysiological assays employing a variety of nerve preparations from larval and adult *kdr* insects (Bloomquist, 1988) provide direct evidence for reduced neuronal sensitivity as the basis for the *kdr* trait. A second resistance trait in the housefly (designated *super-kdr*) that confers much greater resistance to DDT and pyrethroids than that found in *kdr* strains has also been isolated genetically and mapped to chromosome 3 (Sawicki, 1978; Farnham *et al.*, 1987). The *kdr* and *super-kdr* traits are widely presumed to represent allelic variants at a single resistance locus on the basis of their similar spectra of resistance and their common localization to chromosome 3 in the housefly.

Resistance mechanisms similar to *kdr* have been inferred in a number of agricultural pests and disease vectors on the basis of cross-resistance patterns and the absence of synergism by compounds known to inhibit the esterase and monooxygenase activities involved in pyrethroid metabolism (Soderlund and Bloomquist, 1990; Bloomquist, 1993; Soderlund, 1997; Soderlund and Knipple, 1999). Confirming electrophysiological evidence for reduced neuronal sensitivity to pyrethroids also exists for at least six species: *H. virescens*, *Spodoptera littoralis*, *Culex quinquefasciatus*, *A. stephensi*, *B. germanica*, and *P. xylostella* (Bloomquist, 1988, 1993; Schuler *et al.*, 1998).

Pyrethroids are known to exert their insecticidal effects by altering the function of voltage-sensitive sodium channels in nerve membranes (see Section 5.1.1.2). Therefore, studies of *kdr*-like resistance have focused on mechanisms that might affect the regulation, pharmacology, or function of the sodium channel.

5.1.4.2. Altered Sodium Channel Regulation as a Mechanism of Knockdown Resistance

The possible role of reduced insecticide receptor density in knockdown resistance was initially implicated on the basis of resistance-associated reductions in the density of binding sites for [3H]STX (Rossignol, 1988), a radioligand that specifically labels site 1 of the sodium channel (see **Table 1**). However, further investigation of [3H]STX binding in susceptible, *kdr*, and *super-kdr* housefly strains (Grubs *et al.*, 1988; Sattelle *et al.*, 1988; Pauron *et al.*, 1989) revealed that a reduction in sodium channel density is not an obligatory component of the *kdr* or *super-kdr* phenotypes of the housefly. Moreover, comparisons of *Vssc1* transcript and protein levels in susceptible and *kdr* housefly strains did not document any differences between strains (Castella *et al.*, 1997).

Although these results rule out sodium channel downregulation as the mechanism underlying the *kdr* and *super-kdr* traits in the housefly, reduced sodium channel density could, in principle, produce a *kdr*-like phenotype. The relationship between sodium channel density and *kdr*-like resistance has been evaluated directly using the *napts* strain of *D. melanogaster*, in which the density of sodium channels (measured as binding sites for [3H]STX in head membrane preparations) is approximately half that of wild-type flies (Jackson *et al.*, 1984). Flies homozygous for *nap*^{ts} exhibit modest (~threefold) resistance to the lethal effects of DDT and pyrethroids that is also evident in delayed onset of paralysis and reduced physiological sensitivity of the adult central nervous system (Kasbekar and Hall, 1988; Bloomquist *et al.*, 1989). These results suggest that if such a mechanism were present in other insects that exhibit knockdown resistance, the magnitude of resistance observed would require a profound and readily detectable reduction in sodium channel density (Grubs *et al.*, 1988). Because reductions in sodium channel density more severe than that observed in the *nap*^{ts} strain of *D. melanogaster* would be anticipated to compromise viability, it is unlikely that reduced target density can account for *kdr*-like traits that confer significant levels of resistance.

5.1.4.3. Genetic Linkage between Knockdown Resistance and Sodium Channel Genes

Two studies employed restriction fragment length polymorphisms (RFLPs) in the *Vssc1* gene coupled with discriminating dose bioassays with DDT to demonstrate tight genetic linkage (within ~1 map

unit) of the *kdr* and *super-kdr* resistance trait and the *Vssc1* gene of *M. domestica* (Williamson *et al.*, 1993; Knipple *et al.*, 1994). In addition to providing strong genetic evidence for mutations at a sodium channel structural gene as the cause of knockdown resistance in the housefly, these two studies also provided the first experimental evidence for the widely presumed allelism of the *kdr* and *super-kdr* traits in this species.

Conceptually similar approaches were employed to investigate linkage between knockdown resistance traits and *para*-orthologous sodium channel sequences in other species. In the case of *B. germanica*, knockdown resistance was tightly linked (within ~0.2 map units) to an RFLP located with the *para*-orthologous sodium channel gene (Dong and Scott, 1994). The use of RFLP markers to assess the linkage between knockdown resistance and sodium channel gene sequences in *H. virescens* (Taylor *et al.*, 1993) was complicated by the use of a strain with multiple resistance mechanisms. Nevertheless, results of these assays suggested that one component of resistance was linked to an RFLP in the *para*-orthologous sodium channel gene of this species. In *L. decemlineata* (Lee *et al.*, 1999b) and *B. tabaci* (Morin *et al.*, 2002), sequencing of DNA from individual insects of susceptible and resistant phenotypes has identified sequence variants within sodium channel coding regions that are genetically linked with resistant phenotypes. Finally, some mutant alleles of *para* that exhibit the temperature-sensitive paralytic phenotype also exhibit resistance to pyrethroids at permissive temperatures (Hall and Kasbekar, 1989; Pittendrigh *et al.*, 1997).

5.1.4.4. Identification of Resistance-Associated Mutations

The selective alteration of sodium channel pharmacology in knockdown resistant insects and the genetic linkage of knockdown resistance traits and sodium channel gene sequences provided a strong impetus for the identification and functional characterization resistance-associated mutations in insect sodium channel genes. Results of these efforts are the subject of two recent comprehensive reviews (Soderlund and Knipple, 1999, 2003).

Comparison of partial and complete sequences from 15 housefly strains representing multiple examples of susceptible, *kdr*, and *super-kdr* phenotypes consistently identified two point mutations that were associated with resistant phenotypes (Figure 3): mutation of leucine to phenylalanine at

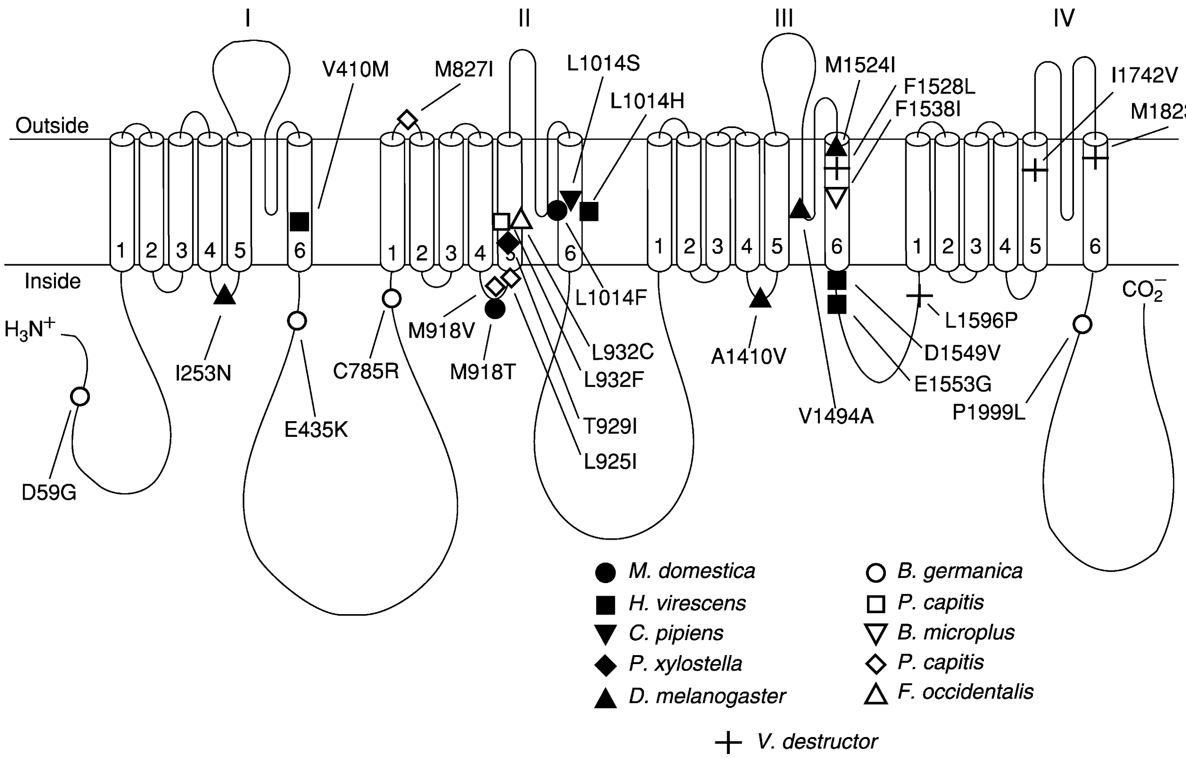


Figure 3 Locations and identities of sodium channel point mutations associated with knockdown resistance to pyrethroids. Symbols indicate the species in which each mutation was first identified (see also **Table 2**). (Modified with permission from Soderlund, D.M., Knipple, D.C. **2003**. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem. Mol. Biol. 33*, 563–577.)

amino acid residue 1014 (designated L1014F) in all *kdr* and *super-kdr* strains, and the additional mutation of methionine to threonine at residue 918 (designated M918T) only in *super-kdr* strains (Ingles *et al*., 1996; Miyazaki *et al*., 1996; Williamson *et al*., 1996). Mutations in *para*-orthologous sodium channel gene sequences corresponding to the L1014F mutation in the housefly have been also identified to date in eight additional pest species (**Table 2**): *B. germanica* (Miyazaki *et al*., 1996; Dong, 1997), *F. occidentalis* (Forcioli *et al*., 2002), *H. irritans* (Guerrero *et al*., 1997), *A. gambiae* (Martinez-Torres *et al*., 1998), *P. xylostella* (Schuler *et al*., 1998), *L. decemlineata* (Lee *et al*., 1999b), *M. persicae* (Martinez-Torres *et al*., 1999b), and *C. pipiens* (Martinez-Torres *et al*., 1999a). (For clarity and consistency and to facilitate comparisons between species, including those for which full-length sodium channel sequences are not available, all resistance-associated mutations in this report are numbered according to their positions in the amino acid sequence of the most abundant splice variant of the housefly Vssc1 sodium channel α subunit (GenBank Accession no. U38813).) In contrast to the many examples of mutations in other species corresponding to the L1014F mutation in the housefly, a mutation corresponding to the second-site M918T mutation that is associated with the *super-kdr* trait of the housefly has been found to date only in highly resistant populations of *H. irritans* (Guerrero *et al*., 1997).

The search for sodium channel gene mutations associated with knockdown resistance has also identified numerous novel mutations (**Figure 3**; **Table 2**). Studies with pyrethroid-resistant *H. virescens* populations identified a second mutation at sequence position 1014, L1014H (Park and Taylor, 1997), as well as three new mutations: V410M (Park *et al*., 1997), D1549V, and E1553G (Head *et al*., 1998). The latter two mutations were found together in sodium channel sequences from pyrethroid-resistant strains of both *H. virescens* and *H. armigera* (Head *et al*., 1998). Studies with knockdown-resistant populations of *C. pipiens* and *A. gambiae* identified a third variant at position 1014, L1014S (Martinez-Torres *et al*., 1999a;

Table 2 Sodium channel amino acid sequence polymorphisms associated with knockdown resistance in arthropod species

Species	*Mutations identified*[a]	*Reference*
Anopheles gambiae	L1014F	Martinez-Torres *et al.* (1998)
	L1014S	Ranson *et al.* (2000)
Bemisia tabaci	M918V; L925I	Morin *et al.* (2002)
Blattella germanica	L1014F[b]	Miyazaki *et al.* (1996), Dong (1997)
	L1014F+E435K+C785R[b]	Liu *et al.* (2000)
	L1014F+D59G+E435K+C785R+P1999L[b]	Liu *et al.* (2000)
Boophilus microplus	F1538I	He *et al.* (1999)
Culex pipiens	L1014F; L1014S	Martinez-Torres *et al.* (1999)
Drosophila melanogaster	I253N; A1410V; A1494V; M1524I	Pittendrigh *et al.* (1997)
Frankliniella occidentalis	L1014F+T929C	Forcioli *et al.* (2002)
Helicoverpa armigera	D1549V+E1533G	Head *et al.* (1998)
Heliothis virescens	L1014H	Park and Taylor (1997)
	V410M	Park *et al.* (1997)
	D1549V+E1533G	Head *et al.* (1998)
Hematobia irritans	L1014F+M918T	Guerrero *et al.* (1997)
Leptinotarsa decemlineata	L1014F	Lee *et al.* (1999b)
Musca domestica	L1014F; L1014F+M918T	Ingles *et al.* (1996), Miyazaki *et al.* (1996), Williamson *et al.* (1996)
Myzus persicae	L1014F	Martinez-Torres *et al.* (1999b)
Pediculus capitis	M827I+T929I+L932F[c]	Lee *et al.* (2000b), (2003)
Plutella xylostella	L1014F+T929I	Schuler *et al.* (1998)
Varroa destructor	L1596P+M1823I	Wang *et al.* (2002)
	F1528L+L1596P+I1742V+M1823I	

[a]Positions numbered according to the amino acid sequence of the most abundant splice variant of the housefly Vssc1 sodium channel protein (Ingles *et al*., 1996; Williamson *et al*., 1996).
[b]These mutations correspond to the D58G, E434K, C764R, L993F, and P1880L mutations in the full-length *Blattella germanica para*[CSMA] sodium channel (Dong, 1997; Liu *et al*., 2000).
[c]These mutations correspond to the M815I, T917I, and L920F mutations in the full-length *Pediculus capitis* sodium channel (Lee *et al*., 2003).
Modified from Soderlund, D.M., Knipple, D.C. **2003**. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem. Mol. Biol. 33*, 563–577.

Ranson *et al*., 2000). A novel mutation at Met918 (M918V) was recently identified in some pyrethroid-resistant *B. tabaci* populations (Morin *et al*., 2002). Characterization of sodium channel sequences from pyrethroid-resistant *P. xylostella* identified a novel putative second-site mutation (T929I) associated with the more commonly observed L1014F mutation in strains with high pyrethroid resistance (Schuler *et al*., 1998). The T929I mutation is also observed in combination with two other novel mutations, M827I and L932F, in pyrethroid-resistant *P. capitis* (Lee *et al*., 2000b, 2003). A second mutation at position 929 (T929C) was found in combination with the L1014F mutation in highly resistant populations of *F. occidentalis* (Forcioli *et al*., 2002). Recent studies with *B. tabaci* have also identified another novel mutation in this region of the sodium channel protein (L925I) that is tighly linked to pyrethroid resistance (Morin *et al*., 2002). Four novel putative second-site mutations (D59G, E435K, C785R, and P1999L) are found together with the L1014F mutation in one or more strains of *B. germanica* that exhibit high levels of pyrethroid resistance (Liu *et al*., 2000). Finally, a screen for pyrethroid resistance in strains of *D. melanogaster* having temperature-sensitive paralytic phenotypes that map to the *para* sodium channel identified four novel resistance-associated mutations: I253N, A1410V, A1494V, and M1524I (Pittendrigh *et al*., 1997).

The identification of mutations associated with pyrethroid resistance in *para*-orthologous sodium channel gene sequences also extends to noninsect arthropod species. Sodium channel sequences from populations of *B. microplus* that exhibit very high levels of pyrethroid resistance contain the F1538I mutation (He *et al*., 1999). Also, the *para*-orthologous sodium channel sequences obtained from populations of the mite *V. destructor* that are resistant to the pyrethroid fluvalinate contain four novel mutations (F1528L, P1596L, I1742V, and V1823I) (Wang *et al*., 2002), which include the first resistance-associated mutations identified in homology domain IV (**Figure 3**).

Among the 26 unique sodium channel amino acid sequence polymorphisms associated so far with pyrethroid resistance, those occurring at five sites have been found as single mutations in resistant populations: Val410 (V410M in *H. virescens*), Met918 (M918V in *Bemisia tabaci*); Leu925 (L925I in *B. tabaci*), Leu1014 (L1014F in several species, L1014H in *H. virescens*, and L1014S in *C. pipiens* and *A. gambiae*); and Phe1538 (F1538I in *B. microplus*). Mutations at six sites (M918T in *M. domestica* and *H. irritans*; T929I in *P. xylostella* and T929C in *F. occidentalis*; D59G, E435K, C785R, and P1999L in *B. germanica*) have been found in combination with the L1014F mutation in highly resistant strains and therefore have been hypothesized to function as second-site mutations that produce additive or synergistic enhancement the resistance caused by the L1014F mutation. The functional status of the remaining resistance-associated mutations is more ambiguous.

5.1.4.5. Functional Analysis of Resistance-Associated Mutations

The *X. laevis* oocyte expression system has been employed to characterize the effects of several resistance-associated mutations on the pyrethroid sensitivity and functional properties of expressed sodium channels. Most of these efforts have focussed on the mutations identified in sodium channels of *kdr* and *super-kdr* houseflies, but a more limited group of studies have also examined additional putative primary resistance mutations and certain putative secondary mutations. All of these studies involve the insertion of candidate mutations into wild-type sodium channel cDNAs, expression of wild-type and specifically mutated channels in oocytes, and direct comparison of the pharmacological properties of the expressed channels.

5.1.4.5.1. Functional analysis of the kdr and super-kdr mutations The effects of the L1014F mutation, the sodium channel gene mutation most commonly associated with knockdown resistance, on pyrethroid sensitivity have been examined in several sodium channel sequence contexts and with different pyrethroids as probes. Housefly Vssc1 sodium channels containing the L1014F substitution, coexpressed in oocytes with the *D. melanogaster* tipE protein, were approximately tenfold less sensitive to modification by cismethrin, a type I pyrethroid, than wild-type Vssc1/tipE channels (Smith *et al*., 1997). The L1014F mutation also accelerated the rate of decay of cismethrin-induced sodium tail currents. Similar experiments with wild type and specifically mutated *D. melanogaster* para sodium channels coexpressed with the tipE protein and deltamethrin as the test pyrethroid confirmed and extended these findings (Vais *et al*., 2000). Comparison of the actions of deltamethrin on wild-type para/tipE channels and channels containing the L1014F mutation reduced the sensitivity of expressed channels to deltamethrin approximately 17-fold and accelerated the rate of deltamethrin-induced tail current decay. More detailed analysis showed that mutated channels exhibited lower affinity for deltamethrin as well as a reduced availability of open

channel states due to enhanced closed-state inactivation. The effects of the L1014F mutation on deltamethrin sensitivity were also examined using *B. germanica* para^{CSMA} sodium channels coexpressed with tipE (Tan *et al.*, 2002b). In this study, the L1014F mutation conferred approximately sixfold resistance to deltamethrin but, unlike the results obtained with Vssc1/tipE and para/tipE channels, the reduction in pyrethroid sensitivity of mutated para^{CSMA}/tipE channels was not accompanied by an acceleration of tail current decay.

The discovery that the rat $Na_v1.8$ (also called SNS or PN3) sodium channel isoform was highly sensitive to both type I and type II pyrethroids (Smith and Soderlund, 2001) provided the opportunity to examine the impact of the L1014F mutation in a sodium channel sequence environment that is otherwise substantially divergent (i.e., only ~40% identical at the amino acid sequence level) from para-orthologous channels of insects. The L1014F mutation, introduced at the cognate conserved leucine residue of the rat $Na_v1.8$ channel, reduced the sensitivity of expressed channels to cismethrin more than tenfold and increased the rate of decay of the cismethrin-induced tail current. These effects were qualitatively identical to the effects of the L1014F mutation on the cismethrin sensitivity of Vssc1/tipE channels (Smith *et al.*, 1997).

The effects of the M918T mutation in the presence of the L1014F mutation, the combination of mutations associated with the *super-kdr* resistance trait of the housefly, have also been examined in both insect and mammalian sodium channel sequence contexts with both type I and type II pyrethroids as probes. Incorporation of the M918T and L1014F mutations into the Vssc1 protein gave rise to Vssc1/tipE channels that were completely insensitive to both cismethrin and [1*R*,*cis*,α*S*]-cypermethrin at the highest concentrations that could be achieved given the low aqueous solubility of these compounds (Lee *et al.*, 1999c). Similarly, rat $Na_v1.8$ sodium channels containing the M918T/L1014F double mutation were completely insensitive to modification by the highest attainable concentration of cismethrin (Soderlund and Lee, 2001). In the para/tipE sequence context the M918T/L1014F double mutation decreased the sensitivity of expressed channels to deltamethrin approximately 100-fold and also produced tail currents with monophasic, rather than biphasic, decay kinetics (Vais *et al.*, 2000). The latter effect was interpreted as evidence that the double mutation reduced the number of deltamethrin binding sites per channel from two to one.

The effects of the M918T single mutation on pyrethroid sensitivity has also been evaluated in multiple sequence contexts. Insertion of the M918T mutation into para/tipE channels gave channels that were twofold more resistant to deltamethrin than the doubly mutated (M918T/L1014F) channels; the M918T channels also exhibited monophasic tail current decay kinetics (Vais *et al.*, 2001). Insertion of the M918T mutation in Vssc1/tipE sodium channels significantly impaired sodium current expression in oocytes and produced channels that were not detectably modified by cismethrin (Lee *et al.*, 1999c). In rat $Na_v1.8$ channels, the M918T mutation caused a degree of resistance to cismethrin that was equivalent to that caused by the single L1014F mutation (Soderlund and Lee, 2001). These results suggest that the M918T mutation does not enhance resistance caused by the L1014F mutation in an additive or synergistic manner but rather provides a high level of resistance that supercedes the effect of the L1014F mutation alone.

In light of the profound reduction in pyrethroid sensitivity caused by the M918T single mutation in these assays, it is surprising that it has not been identified as a single mutation in pyrethroid-resistant insect populations. A likely explanation for this situation has emerged from studies of alternative exon usage in *para* and its orthologs (see Sections 5.1.2.1.2 and 5.1.2.2.1). In the *para* sequence of *D. melanogaster*, the Met918 residue occurs in mutually exclusive exons *c* and *d*, each of which is incorporated into a subpopulation of sodium channel sequence variants (Thackeray and Ganetzky, 1994). Exons homologous to exons *c* and *d* of *D. melanogaster* are also found among transcripts from the *para*-orthologous sodium channel gene of *H. virescens* (Park *et al.*, 1999) and *C. pipiens* (Martinez-Torres *et al.*, 1999a). In these situations, two independent point mutations (one each in exons *c* and *d*) would be required to insure that all channel variants contained the M918T substitution. In contrast, exon *c* in the housefly *Vssc1* gene contains a stop codon, so that all full-length *Vssc1* transcripts are derived solely from exon *d* (Lee *et al.*, 2002). Thus, a single point mutation in exon *d* of the housefly is sufficient to insure that all functional sodium channel splice variants in this species contain the M918T substitution. This interpretation suggests that the M918T mutation and other mutations at this site (such as the M918V mutation in *B. tabaci*) can only be selected in species in which alternative splicing at the exon *c*/*d* locus produces functional channels from only one of these alternative exons. Although consistent with available data on alternative splicing at the *Vssc1* locus in the housefly, this analysis does not explain the absence of the single M918T mutation in resistant strains of

this species. It therefore remains possible that a functional deficit associated with the M918T mutation is somehow complemented or rescued by the presence of the L1014F mutation (Lee *et al*., 1999c).

5.1.4.5.2. Functional analysis of other putative primary resistance mutations The effects of the V410M mutation, a single mutation associated with knockdown resistance in some *H. virescens* populations, has been examined in para/tipE, Vssc1/tipE, and para^{CSMA}/tipE sodium channels expressed in oocytes. Insertion of the V410M mutation into para/tipE channels resulted in a >10-fold reduction in permethrin sensitivity coupled with a ~45-fold acceleration in the rate of sodium tail current decay (Zhao *et al*., 2000). Similar results were obtained in Vssc1/tipE channels containing the V410M mutation, which decreased channel sensitivity to cismethrin by ~20-fold and accelerated the rate of tail current decay ~10-fold (Lee and Soderlund, 2001). In para^{CSMA}/tipE channels, the V410M mutation caused ~17-fold resistance to deltamethrin (Liu *et al*., 2002). In contrast to the effects of this mutation on the kinetics of channel modification by permethrin and cismethrin, resistance to deltamethrin in para^{CSMA}/tipE channels containing the V410M mutation was not accompanied by a significant acceleration in tail current decay kinetics. Although Met410 occurs in a region of the sodium channel known to be involved in the binding and action of batrachotoxin, the V410M mutation did not affect the potency of batrachotoxin as a modifier of Vssc1/tipE sodium channels in oocytes (Lee and Soderlund, 2001).

The V410M mutation is the only resistance mutation that has been examined both in the oocyte expression system and in its native cellular context. Assays with cultured adult central neurons from *H. virescens* homozygous for the V410M mutation documented a ~21-fold reduction in permethrin sensitivity of sodium channels in the resistant strain (Lee *et al*., 1999a). Although these authors did not report the time constants for the decay of permethrin-induced sodium tail current, inspection of their data suggests that reduced permethrin sensitivity in cultured neurons was apparently not correlated with a significant acceleration of tail current decay as was observed in some of the studies of the V410M mutation in oocytes (Zhao *et al*., 2000; Lee and Soderlund, 2001). In cultured neurons, channels containing the V410M mutation were approximately 2.6-fold more sensitive to the polypeptide scorpion toxin LqhαIT (Lee *et al*., 1999a).

The characterization of mutations at Leu1014 was expanded to include the L1014H mutation, another single mutation found in some knockdown-resistant *H. virescens* populations (Zhao *et al*., 2000). Incorporation of the L1014H mutation into para/tipE sodium channels conferred >tenfold resistance to permethrin, a type I pyrethroid. Permethrin-induced sodium tail currents recorded from channels containing the L1014H mutation decayed 57-fold more rapidly than those carried by wild-type para/tipE channels.

The effects of the F1538I mutation, identified in pyrethroid-resistant populations of *B. microplus*, have been assessed only in rat $Na_v1.4$ sodium channels that were also mutated to enhance baseline pyrethroid sensitivity (Wang *et al*., 2001). Deltamethrin caused use-dependent modification of these rat $Na_v1.4$ sodium channels. However, introduction of the F1538I mutation into these channels caused a loss of use-dependent modification by deltamethrin as well as a reduction in sensitivity to this compound.

5.1.4.5.3. Functional analysis of putative secondary mutations The effects of the T929I mutation, a putative second-site resistance mutation in *P. xylostella* and *P. capitis*, has been examined in para/tipE sodium channels expressed in oocytes (Vais *et al*., 2001). As a single mutation, the T929I substitution reduced the sensitivity of para/tipE channels to deltamethrin approximately tenfold. Deltamethrin-induced tail currents carried by channels containing the T929I mutation decayed rapidly with first-order kinetics. Channels containing both the T929I and L1014F mutations, the combination found in highly resistant populations of *Plutella xylostella* were more than 10 000-fold resistant to deltamethrin when compared to wild-type channels.

The effects of the E435K and C785R mutations, putative second-site mutations identified in several highly resistant *B. germanica* populations, on pyrethroid sensitivity have been examined in para^{CSMA}/tipE channels singly, together, and in combination with two primary resistance mutations. Channels containing the C785R mutation alone were identical to wild-type para^{CSMA}/tipE channels in their sensitivity to deltamethrin, but channels containing E435K single mutation and the E435K/C785R double mutation were more sensitive to deltamethrin than wild-type channels (Tan *et al*., 2002b). When either the E435K or C785R mutations were combined with the L1014F resistance mutation, the resulting double mutants were approximately 20-fold less sensitive to deltamethrin than channels containing the single L1014F mutation and approximately 100-fold less sensitive than wild-type para^{CSMA}/tipE channels. Channels

containing all three resistance mutations, a situation found in highly resistant *B. germanica* populations, were more than 500-fold less sensitive to deltamethrin than wild-type channels.

In a companion study, the E435K and C785R mutations were also evaluated as modifiers of deltamethrin resistance conferred by the V410M mutation (Liu *et al.*, 2002). Insertion of either the E435K or C785R mutation into $para^{CSMA}$/tipE channels containing the V410M mutation did not significantly affect the level of resistance conferred by the V410M mutation, but channels containing all three resistance mutations were approximately sixfold less sensitive to deltamethrin than channels containing only the V410M mutation and 100-fold less sensitive than wild-type $para^{CSMA}$/tipE channels.

5.1.5. Conclusions

5.1.5.1. Unique Features of Insect Sodium Channels

Although sodium channel function and structure is strongly conserved between evolutionarily divergent animal species, the molecular characterization of insect sodium channels has identified several distinctive features. First, in contrast to the family of sodium channel α subunit genes in mammalian species, insects appear to have only a single gene (*para* in *D. melanogaster* and its orthologs) that encodes voltage-gated, sodium-selective channels that are involved in action potential generation. However, insects are capable of generating a remarkable diversity of sodium channel variants from this locus by alternative exon usage and RNA editing. The number of sodium channel variants generated by these posttranscriptional modifications found in any given species appears to be only a fraction of the theoretical maximum, and the patterns of exon usage do not appear to be strongly conserved between species. So far there is scant information on the functional significance of alternative exon usage.

Insects also lack the sodium channel β subunit family of vertebrates and instead appear to use a novel family of glycoproteins, encoded by the *tipE* gene of *D. melanogaster* and its orthologs, as sodium channel auxiliary subunits. Genetic and cytochemical localization studies with *D. melanogaster* imply that the tipE protein is restricted in expression to the adult nervous system, suggesting that it may function as a modulator of sodium channel function and expression rather than as an obligatory auxiliary subunit. So far only two examples of *tipE*-like genes have been described, but their broader existence in insects is inferred by the heterologous coassembly of the *D. melanogaster* tipE protein with the *para*-orthologous sodium channel gene of *B. germanica*. It will be of interest to determine the breadth of distribution of *tipE*-like genes in the Insecta and in other animal taxa.

Finally, insect genomes also contain a second sodium channel α subunit-like gene, *dsc1* of *D. melanogaster* and its orthologs, that is equally divergent from the *para*-like sodium channel genes of insects and the vertebrate sodium channel α subunit gene family. The dsc1 protein is clearly not the sodium channel principally responsible for nerve action potential generation or the primary target for sodium channel-directed insecticides, and it may lack the stringent selectivity for sodium ions that is a hallmark of typical voltage-sensitive sodium channels. The functional significance of dsc1 and homologous proteins has remained obscure, but recent studies point to an as-yet undefined role in chemosensory pathways.

5.1.5.2. Sodium Channels and Pyrethroid Resistance

Molecular and genetic studies over the past decade have provided convincing evidence that point mutations in insect voltage-sensitive sodium channel genes are the primary cause of knockdown resistance to pyrethroids. Genetic linkage experiments and targeted DNA sequencing studies have consistently identified resistance-associated mutations in sodium channel genes that are orthologous to the *para* gene of *D. melanogaster.* Heterologous expression assays have documented the ability of many of these mutations, introduced into wild-type insect sodium channels either alone or in the combinations found in highly resistant populations, to reduce the sensitivity of expressed channels to pyrethroids exemplifying the type I and type II structural classes. Finally, the magnitude of resistance conferred by these mutations in heterologous expression assays *in vitro* has consistently been found to be in good agreement with the magnitude of resistance observed in resistant insect populations carrying the same mutations. These results not only identify the molecular mechanism of the *kdr* trait but also provide important confirmation that sodium channels encoded by the *Vssc1* gene are the principal target site for the toxic actions of DDT analogs and pyrethroids. Although the actions of DDT and pyrethroids on sodium channels have been characterized in great depth and detail over the past three decades, numerous other sites of action for some or all of these insecticides have also been proposed (Soderlund and Bloomquist, 1989; Bloomquist, 1993). The toxicological impact of the knockdown

resistance mutations in *para*-orthologous sodium channels implies that actions of DDT and pyrethroids at this target alone are sufficient to account for the toxic effects of these compounds in whole insects.

The search for sodium channel gene mutations in knockdown-resistant strains of various arthropod species has revealed the existence of resistance-associated polymorphisms at an unanticipated diversity of sites. Although not all of these polymorphisms have been shown to cause pyrethroid resistance, the multiplicity of sodium channel sequence polymorphisms associated with knockdown resistance contrasts with the situation for cyclodiene resistance in insects, which involves a mutation at a single amino acid residue in the subunit of the γ-aminobutyric acid receptor–chloride ionophore encoded by the *Rdl* gene in all cases that have been examined (ffrench-Constant, 1994). The diversity of sequence polymorphisms that are potentially involved in knockdown resistance poses a significant challenge for the use of this information in pyrethroid resistance monitoring and management.

The surprisingly large number of amino acid residues implicated as sites of resistance-causing mutations also poses challenges for the use of these mutations to map the pyrethroid binding site on the sodium channel. Not all of these residues are likely to interact physically with the pyrethroid molecule; mutations at some sites may indirectly alter the architecture of the pyrethroid site by altering or restricting the conformational flexibility of the sodium channel protein. As shown in **Figure 3**, most putative resistance mutations are located in the S5 and S6 helices and closely associated regions. Current models of sodium channel structure (Guy and Seetharamulu, 1986; Yellen, 1998; Lipkind and Fozzard, 2000) place the S5 and S6 domains in close proximity to each other adjacent to the inner pore of the channel. In this context, the clustering of a large number of resistance-associated mutations in the S5–S6 region of homology domain II implies an important role for this region of the sodium channel in determining, either directly or indirectly, the binding of pyrethroids. Recently, the crystal structure of a simple bacterial potassium channel was employed to generate a three-dimensional model of the S5–pore–S6 regions of the rat $Na_v1.4$ voltage-sensitive sodium channel (Lipkind and Fozzard, 2000). The use of such a model to identify the spatial relationships among the resistance-associated mutations in insect sodium channels may clarify the functional role of these residues in pyrethroid binding and action.

5.1.5.3. Future Exploitation of the Sodium Channel as an Insecticide Target

Despite the long and widespread use of DDT and then pyrethroids in the control of pests and disease vectors and the existence of knockdown resistance traits in populations of many pest and vector species, the pyrethroids remain an important and effective class of insecticides. Moreover, three key factors contribute to the continued value of the sodium channel as a target for future insecticides. First, toxicologically relevant sodium channels in insects are the products of a single gene and therefore exhibit conserved pharmacology in all neuronal tissues and insect life stages. Second, the value of sodium channel disruption as a mode of insecticidal action is amply demonstrated by the efficacy of sodium channel-directed natural toxins and insecticides. Third, toxin binding sites on the sodium channel other than the pyrethroid site (see **Table 1**) appear to be unaffected by mutations that confer pyrethroid resistance. In this context, each sodium channel binding domain can be envisioned as a separate potential target for insecticide discovery and development.

These factors in favor of the continued exploitation of sodium channels for insect control are counterbalanced by the evolutionary conservation of sodium channel structure, function, and pharmacology across animal taxa. Intrinsic selectivity of agents for insect sodium channels is uncommon, and the development of novel insecticides directed toward this target is complicated by the potential for toxicity to nontarget species. The example of pyrethroids is instructive in this regard: the notable safety of pyrethroids for humans is based principally on differential metabolism rather than differential target sensitivity, and the use of pyrethroids has been limited in some contexts by undesirable toxicity to aquatic vertebrate and invertebrate species. The development of indoxacarb, which exploits differential metabolic bioactivation as a mechanism of selective toxicity, illustrates the potential for the discovery of novel sodium channel-directed insecticides that exhibit acceptable safety and selectivity.

References

Adams, M., Celniker, S., Holt, R., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Anholt, R.R.H., Lyman, R.F., Mackay, T.F.C., **1996**. Effects of single *P*-element insertions on olfactory behavior in *Drosophila melanogaster. Genetics 143*, 293–301.

Baker, M.D., Wood, J.N., **2001**. Involvement of Na^+ channels in pain pathways. *Trends Pharmacol. Sci. 22*, 27–31.

Bloomquist, J.R., **1988**. Neurophysiological assays for the characterization and monitoring of pyrethroid resistance. In: Lunt, G.G. (Ed.), Neurotox '88: The Molecular Basis of Drug and Pesticide Action. Elsevier, Amsterdam, pp. 543–551.

Bloomquist, J.R., **1993**. Neuroreceptor mechanisms in pyrethroid mode of action and resistance. In: Roe, M., Kuhr, R.J. (Eds.), Reviews in Pesticide Toxicology. Toxicology Communications, Raleigh, NC, pp. 181–226.

Bloomquist, J.R., Soderlund, D.M., **1988**. Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. *Mol. Pharmacol. 33*, 543–550.

Bloomquist, J.R., Soderlund, D.M., Knipple, D.C., **1989**. Knockdown resistance to dichlorodiphenyltrichloroethane and pyrethroid insecticides in the *nap^ts^* mutant of *Drosophila melanogaster* is correlated with reduced neuronal sensitivity. *Arch. Insect Biochem. Physiol. 10*, 293–302.

Blumenthal, K.M., Seibert, A.L., **2003**. Voltage-gated sodium channel toxins: poisons, probes, and future promise. *Cell Biochem. Biophys. 38*, 215–237.

Busvine, J.R., **1951**. Mechanism of resistance to insecticide in houseflies. *Nature 168*, 193–195.

Cannon, S.C., **2000**. Spectrum of sodium channel disturbances in the nondystrophic myotonias and periodic paralyses. *Kidney Int. 57*, 772–779.

Cantrell, A.R., Catterall, W.A., **2001**. Neuromodulation of Na^+ channels: an unexpected form of cellular plasticity. *Nature Rev. 2*, 397–407.

Castella, C., Castells-Brooke, N., Bergé, J.-B., Pauron, D., **1997**. Expression and distribution of voltage-sensitive sodium channels in pyrethroid-susceptible and pyrethroid-resistant *Musca domestica*. *Invert. Neurosci. 3*, 41–47.

Catterall, W.A., **1987**. Common modes of drug action on sodium channels: local anesthetics, antiarrhythmics and anticonvulsants. *Trends Pharmacol. Sci. 8*, 57–65.

Catterall, W.A., **1988**. Structure and function of voltage-sensitive ion channels. *Science 242*, 50–61.

Catterall, W.A., **1992**. Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev. 72*, S15–S48.

Catterall, W.A., **2000**. From ionic currents to molecular mechanisms: structure and function of voltage-gated sodium channels. *Neuron 26*, 13–25.

Cestèle, S., Catterall, W.A., **2000**. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie 82*, 883–892.

Clare, J.J., Tate, S.N., Nobbs, M., Romanos, M.A., **2000**. Voltage-gated sodium channels as therapeutic targets. *Drug Discov. Today 11*, 506–520.

Cory, J.S., **1999**. Use and risk assessment of genetically modified baculoviruses. In: Beadle, D.J. (Ed.), Progress in Neuropharmacology and Neurotoxicology of Pesticides and Drugs. Royal Society of Chemistry, Cambridge, pp. 117–123.

Cukierman, S., **1996**. Regulation of voltage-dependent sodium channels. *J. Membrane Biol. 151*, 203–214.

Deecher, D.C., Payne, G.T., Soderlund, D.M., **1991**. Inhibition of [^{3}H]batrachotoxinin A 20-α-benzoate binding to mouse brain sodium channels by the dihydropyrazole insecticide RH 3421. *Pestic. Biochem. Physiol. 41*, 265–273.

Deecher, D.C., Soderlund, D.M., **1991**. RH 3421, an insecticidal dihydropyrazole, inhibits sodium channel-dependent sodium uptake into mouse brain preparations. *Pestic. Biochem. Physiol. 39*, 130–137.

Defaix, A., Lapied, B., **2001**. Characterization of a novel low-voltage-activated, tetrodotoxin-sensitive sodium channel permeable to calcium in insect pacemaker neurosecretory cells. *J. Physiol. 533P*, 54P–55P.

Dong, K., **1997**. A single amino acid change in the *para* sodium channel protein is associated with knockdown-resistance (*kdr*) to pyrethroid insecticides in the German cockroach. *Insect Biochem. Mol. Biol. 27*, 93–100.

Dong, K., Scott, J.G., **1994**. Linkage of *kdr*-type resistance and the *para*-homologous sodium channel gene in German cockroaches (*Blattella germanica*). *Insect Biochem. Mol. Biol. 24*, 647–654.

Doyle, K.E., Knipple, D.C., **1991**. PCR-based phylogenetic walking: isolation of *para*-homologous sodium channel gene sequences from seven insect species and an arachnid. *Insect Biochem. 21*, 689–696.

Elkins, T., Ganetzky, B., **1990**. Conduction in the giant nerve fiber pathway in temperature-sensitive paralytic mutants of *Drosophila*. *J. Neurogenet. 6*, 207–219.

Fainzilber, M., Kofman, O., Zlotkin, E., Gordon, D., **1994**. A new neurotoxin receptor site on sodium channels is identified by a conotoxin that affects sodium channel inactivation in molluscs and acts as an antagonist in rat brain. *J. Biol. Chem. 269*, 2574–2580.

Farnham, A.W., Murray, A.W.A., Sawicki, R.M., Denholm, I., White, J.C., **1987**. Characterization of the structure–activity relationship of *kdr* and two variants of *super-kdr* to pyrethroids in the housefly (*Musca domestica* L.). *Pestic. Sci. 19*, 209–220.

Feng, G., Deak, P., Chopra, M., Hall, L.M., **1995a**. Cloning and functional analysis of TipE, a novel membrane protein that enhances *Drosophila para* sodium channel function. *Cell 82*, 1001–1011.

Feng, G., Dèak, P., Kasbekar, D.P., Gil, D.W., Hall, L.M., **1995b**. Cytogenetic and molecular localization of *tipE*: a gene affecting sodium channels in *Drosophila melanogaster*. *Genetics 139*, 1679–1688.

ffrench-Constant, R.H., **1994**. The molecular and population genetics of cyclodiene insecticide resistance. *Insect Biochem. Mol. Biol. 24*, 335–345.

Forcioli, D., Frey, D., Frey, J.E., **2002**. High nucleotide diversity in the *para*-like voltage-sensitive sodium channel gene sequence in the western flower thrips (Thysanoptera: Thripidae). *J. Econ. Entomol. 95*, 838–848.

Ganetzky, B., **1984**. Genetic studies of membrane excitability in *Drosophila*: lethal interaction between two

temperature-sensitive paralytic mutations. *Genetics 108*, 897–911.

Ganetzky, B., **1986**. Neurogenetic analysis of *Drosophila* mutations affecting sodium channels: synergistic effects on viability and nerve conduction in double mutants involving *tip-E*. *J. Neurogenet. 3*, 19–31.

Germeraad, S.E., O'Dowd, D.K., Aldrich, R.W., **1992**. Functional assay of a putative *Drosophila* sodium channel gene in homozygous deficiency neurons. *J. Neurogenet. 8*, 1–16.

Goldin, A.L., **1992**. Maintenance of *Xenopus laevis* and oocyte injection. *Meth. Enzymol. 207*, 266–297.

Goldin, A.L., **2001**. Resurgence of sodium channel research. *Annu. Rev. Physiol. 63*, 871–894.

Goldin, A.L., **2002**. Evolution of voltage-gated Na^+ channels. *J. Exp. Biol. 205*, 575–584.

Goldin, A.L., Barchi, R.L., Caldwell, J.H., Hofmann, F., Howe, J.R., *et al.*, **2000**. Nomenclature of voltage-gated sodium channels. *Neuron 28*, 365–368.

Gonoi, T., Ashida, K., Feller, D., Schmidt, J., Fujiwara, M., *et al.*, **1986**. Mechanism of action of a polypeptide neurotoxin from the coral *Goniopora* in mouse neuroblastoma cells. *Mol. Pharmacol. 29*, 347–354.

Gonoi, T., Ohizumi, Y., Kobayashi, J., Nakamura, H., Catterall, W.A., **1987**. Action of a polypeptide toxin from the marine snail *Conus striatus* on voltage-sensitive sodium channels. *Mol. Pharmacol. 32*, 691–698.

Grolleau, F., Lapied, B., **2000**. Dorsal unpaired median neurones in the insect central nervous system: towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity. *J. Exp. Biol. 203*, 1633–1648.

Grubs, R.E., Adams, P.M., Soderlund, D.M., (**1988**). Binding of [^{3}H]saxitoxin to head membrane preparations from susceptible and knockdown-resistant houseflies. *Pestic. Biochem. Physiol. 32*, 217–223.

Guerrero, F.D., Jamroz, R.C., Kammlah, D., Kunz, S.E., **1997**. Toxicological and molecular characterization of pyrethroid-resistant horn flies, *Hematobia irritans*: identification of *kdr* and *super-kdr* point mutations. *Insect Biochem. Mol. Biol. 27*, 745–755.

Guy, H.R., Seetharamulu, P., **1986**. Molecular model of the action potential sodium channel. *Proc. Natl Acad. Sci. USA 83*, 508–512.

Hall, L.M., Kasbekar, D.P., **1989**. *Drosophila* sodium channel mutations affect pyrethroid sensitivity. In: Narahashi, T., Chambers, J.E. (Eds.), Insecticide Action: From Molecule to Organism. Plenum, New York, pp. 99–114.

Hanrahan, C.J., Palladino, M.J., Ganetzky, B., Reenan, R.A., **2000**. RNA editing of the *Drosophila para* Na^+ channel transcript: evolutionary conservation and developmental regulation. *Genetics 155*, 1149–1160.

Harder, H.H., Riley, S.L., McCann, S.F., Irving, S.N., **1996**. DPX-MP062: a novel broad-spectrum, environmentally soft, insect control compound. *Brighton Crop Protection Conference: Pests and Diseases,* 449–454.

Harris-Warwick, R.M., **2000**. Ion channels and receptors: molecular targets for behavioral evolution. *J. Comp. Physiol. A 186*, 605–616.

He, H., Chen, A.C., Davey, R.B., Ivie, G.W., George, J.E., **1999**. Identification of a point mutation in the *para*-type sodium channel gene from a pyrethroid-resistant cattle tick. *Biochem. Biophys. Res. Commun. 261*, 558–561.

Head, D.J., McCaffery, A.R., Callaghan, A., **1998**. Novel mutations in the *para*-homologous sodium channel gene associated with phenotypic expression of nerve insensitivity resistance to pyrethroids in heliothine lepidoptera. *Insect Mol. Biol. 7*, 191–196.

Heinemann, S.H., Terlau, H., Stühmer, W., Imoto, K., Numa, S., **1992**. Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature 356*, 441–443.

Hille, B., **2001**. Ion Channels of Excitable Membranes. Sinauer, Sunderland, MA.

Hodges, D.D., Lee, D., Preston, C.F., Boswell, K., Hall, L.M., *et al.*, **2002**. *tipE* regulates Na^+-dependent repetitive firing in *Drosophila* neurons. *Mol. Cell. Neurosci. 19*, 402–416.

Hong, C.-S., Ganetzky, B., **1994**. Spatial and temporal expression patterns of two sodium channel genes in *Drosophila*. *J. Neurosci. 14*, 5160–5169.

Hoopengardner, B., Bhalla, T., Staber, C., Reenan, R., **2003**. Nervous system targets of RNA editing identified by comparative genomics. *Science 301*, 832–836.

Ingles, P.J., Adams, P.M., Knipple, D.C., Soderlund, D.M., **1996**. Characterization of voltage-sensitive sodium channel gene coding sequences from insecticide-susceptible and knockdown-resistant housefly strains. *Insect Biochem. Mol. Biol. 26*, 319–326.

Jackson, F.R., Wilson, S.D., Hall, L.M., **1986**. The *tip-E* mutation of *Drosophila* decreases saxitoxin binding and interacts with other mutations affecting nerve membrane excitability. *J. Neurogenet. 3*, 1–17.

Jackson, F.R., Wilson, S.D., Strichartz, G.R., Hall, L.M., **1984**. Two types of mutants affecting voltage-sensitive sodium channels in *Drosophila melanogaster*. *Nature 308*, 189–191.

Kasbekar, D.P., Hall, L.M., **1988**. A *Drosophila* mutation that reduces sodium channel number confers resistance to pyrethroid insecticides. *Pestic. Biochem. Physiol. 32*, 135–145.

Knipple, D.C., Doyle, K.E., Marsella-Herrick, P.A., Soderlund, D.M., **1994**. Tight genetic linkage between the *kdr* insecticide resistance trait and a voltage-sensitive sodium channel gene in the housefly. *Proc. Natl Acad. Sci. USA 91*, 2483–2487.

Knipple, D.C., Payne, L.L., Soderlund, D.M., **1991**. PCR-generated conspecific sodium channel gene probe for the housefly. *Arch. Insect Biochem. Physiol. 16*, 45–53.

Kulkarni, N.H., Yamamoto, A.H., Robinson, K.O., Mackay, T.F.C., Anholt, R.R.H., **2002**. The DSC1 channel, encoded by the *smi60E* locus, contributes to

odor-guided behavior in *Drosophila melanogaster. Genetics 161*, 1507–1516.

Kulkarni, S.J., Padhye, A., **1982**. Temperature-sensitive paralytic mutations on the second and third chromosomes of *Drosophila melanogaster. Genet. Res. 40*, 191–199.

Lee, D., Park, Y., Brown, T.M., Adams, M.E., **1999a**. Altered properties of neuronal sodium channels associated with genetic resistance to pyrethroids. *Mol. Pharmacol. 55*, 584–593.

Lee, S.H., Dunn, J.B., Clark, J.M., Soderlund, D.M., **1999b**. Molecular analysis of *kdr*-like resistance in a permethrin-resistant strain of Colorado potato beetle. *Pestic. Biochem. Physiol. 63*, 63–75.

Lee, S.H., Gao, J.-R., Yoon, K.S., Mumcuoglu, K.Y., Taplin, D., *et al.*, **2003**. Sodium channel mutations associated with knockdown resistance in the human head louse, *Pediculus capitis* (De Geer). *Pestic. Biochem. Physiol. 75*, 79–91.

Lee, S.H., Ingles, P.J., Knipple, D.C., Soderlund, D.M., **2002**. Developmental regulation of alternative exon usage in the housefly *Vssc1* sodium channel gene. *Invert. Neurosci. 4*, 125–133.

Lee, S.H., Smith, T.J., Ingles, P.J., Soderlund, D.M., **2000a**. Cloning and functional characterization of a putative sodium channel auxiliary subunit gene from the housefly (*Musca domestica*). *Insect Biochem. Mol. Biol. 30*, 479–487.

Lee, S.H., Smith, T.J., Knipple, D.C., Soderlund, D.M., **1999c**. Mutations in the housefly *Vssc1* sodium channel gene associated with *super-kdr* resistance abolish the pyrethroid sensitivity of Vssc1/tipE sodium channels expressed in *Xenopus* oocytes. *Insect Biochem. Mol. Biol. 29*, 185–194.

Lee, S.H., Soderlund, D.M., **2001**. The V410M mutation associated with pyrethroid resistance in *Heliothis virescens* reduces the pyrethroid sensitivity of housefly sodium channels expressed in *Xenopus* oocytes. *Insect Biochem. Mol. Biol. 31*, 19–29.

Lee, S.H., Yoon, K.-S., Williamson, M.S., Goodson, S.J., Takano-Lee, M., *et al.*, **2000b**. Molecular analysis of *kdr*-like resistance in permethrin-resistant strains of head lice, *Pediculus capitis*. *Pestic. Biochem. Physiol. 66*, 130–143.

Lester, H.A., **1988**. Heterologous expression of excitability proteins: route to more specific drugs? *Science 241*, 1057–1063.

Lilly, M., Carlson, J., **1989**. *smellblind*: a gene required for *Drosophila* olfaction. *Genetics 124*, 293–302.

Lilly, M., Kreber, R., Ganetzky, B., Carlson, J.R., **1994a**. Evidence that the *Drosophila* olfactory mutant *smellblind* defines a novel class of sodium channel mutation. *Genetics 136*, 1087–1096.

Lilly, M., Riesgo-Escovar, J., Carlson, J., **1994b**. Developmental analysis of *smellblind* mutants: evidence for the role of sodium channels in *Drosophila* development. *Devel. Biol. 162*, 1–8.

Lipkind, G.M., Fozzard, H.A., **2000**. KcsA crystal structure as framework for a molecular model of the Na^+ channel pore. *Biochemistry 39*, 8161–8170.

Littleton, J.T., Ganetzky, B., **2000**. Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron 26*, 35–43.

Liu, Z., Chung, I., Dong, K., **2001**. Alternative splicing of the *BSC1* gene generates tissue-specific isoforms in the German cockroach. *Insect Biochem. Mol. Biol. 31*, 703–713.

Liu, Z., Tan, J., Valles, S.M., Dong, K., **2002**. Synergistic interaction between two cockroach sodium channel mutation and a tobacco budworm sodium channel mutation in reducing channel sensitivity to a pyrethroid insecticide. *Insect Biochem. Mol. Biol. 32*, 397–404.

Liu, Z., Valles, S.M., Dong, K., **2000**. Novel point mutations in the German cockroach *para* sodium channel gene are associated with knockdown resistance (*kdr*) to pyrethroid insecticides. *Insect Biochem. Mol. Biol. 30*, 991–997.

Lombet, A., Mourre, C., Lazdunski, M., **1988**. Interactions of insecticides of the pyrethroid family with specific binding sites on the voltage-dependent sodium channel from mammalian brain. *Brain Res. 459*, 44–53.

Loughney, K., Kreber, R., Ganetzky, B., **1989**. Molecular analysis of the *para* locus, a sodium channel gene in *Drosophila*. *Cell 58*, 1143–1154.

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Bergé, J.B., *et al.*, **1998**. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s. Insect Mol. Biol. 7*, 179–184.

Martinez-Torres, D., Chevillon, C., Brun-Barale, A., Bergé, J.B., Pasteur, N., *et al.*, **1999a**. Voltage-dependent Na^+ channels in pyrethroid-resistant *Culex pipiens* L mosquitoes. *Pestic. Sci. 55*, 1012–1020.

Martinez-Torres, D., Foster, S.P., Field, L.M., Devonshire, A.L., Williamson, M.S., **1999b**. A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Mol. Biol. 8*, 339–346.

Milani, R., **1954**. Comportamento mendeliano della resistenza alla azione abbattante del DDT: correlazione tran abbattimento e mortalita in *Musca domestica* L. *Riv. Parassitol. 15*, 513–542.

Miyazaki, M., Ohyama, K., Dunlap, D.Y., Matsumura, F., **1996**. Cloning and sequencing of the *para*-type sodium channel gene from susceptible and *kdr*-resistant German cockroaches (*Blattella germanica*) and housefly (*Musca domestica*). *Mol. Gen. Genet. 252*, 61–68.

Morin, S., Williamson, M.S., Goodson, S.J., Brown, J.K., Tabashnik, B.E., *et al.*, **2002**. Mutations in the *Bemisia tabaci para* sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. *Insect Biochem. Mol. Biol. 32*, 1781–1791.

Narahashi, T., **1996**. Neuronal ion channels as the target sites of insecticides. *Pharmacol. Toxicol. 78*, 1–14.

Nelson, J.C., Wyman, R.J., **1989**. Examination of paralysis in *Drosophila* temperature-sensitive paralytic mutations affecting sodium channels: a proposed mechanism of paralysis. *J. Neurobiol. 21*, 453–469.

Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., *et al.*, **1984**. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature 312*, 121–127.

O'Dowd, D.K., Gee, J.R., Smith, M.A., **1995**. Sodium current density correlates with expression of specific alternatively spliced sodium channel mRNAs in single neurons. *J. Neurosci. 15*, 4005–4012.

O'Dowd, D.K., Germeraad, S.E., Aldrich, R.W., **1989**. Alteration in the expression and gating of *Drosophila* sodium channels by mutations in the *para* gene. *Neuron 2*, 1301–1311.

Okamoto, H., Sakai, K., Goto, S., Takasu-Ishakawa, E., Hotta, Y., **1987**. Isolation of *Drosophila* genomic clones homologous to the eel sodium channel gene. *Proc. Japan Acad. Ser. B 63*, 284–288.

Oppenoorth, F.J., **1985**. Biochemistry and genetics of insecticide resistance. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 12. Pergamon, Oxford, pp. 731–773.

Ottea, J.A., Payne, G.T., Soderlund, D.M., **1989**. Activation of sodium channels and inhibition of [^{3}H]batrachotoxinin A-20-α-benzoate binding by an *N*-alkylamide neurotoxin. *Mol. Pharmacol. 36*, 280–284.

Ottea, J.A., Payne, G.T., Soderlund, D.M., **1990**. Action of insecticidal *N*-alkylamides at site 2 of the voltage-dependent sodium channel. *J. Agric. Food Chem. 38*, 1724–1728.

Park, Y., Taylor, M.F.J., **1997**. A novel mutation L1029H in sodium channel gene *hscp* associated with pyrehtroid resistance for *Heliothis virescens* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol. 27*, 9–13.

Park, Y., Taylor, M.F.J., Feyereisen, R., **1997**. A valine421 to methionine mutation in IS6 of the *hscp* voltage-gated sodium channel associated with pyrethroid resistance in *Heliothis virescens* F. *Biochem. Biophys. Res. Commun. 239*, 688–691.

Park, Y., Taylor, M.F.J., Feyereisen, R., **1999**. Voltage-gated sodium channel genes *hscp* and *hDSC1* of *Heliothis virescens* F. genomic organization. *Insect Mol. Biol. 8*, 161–170.

Pauron, D., Barhanin, J., Amichot, M., Pralavorio, M., Berge, J.-B., *et al.*, **1989**. Pyrethroid receptor in the insect Na^+ channel: alteration of its properties in pyrethroid-resistant flies. *Biochemistry 28*, 1673–1677.

Pittendrigh, B., Reenan, R., ffrench-Constant, R.H., Ganetzky, B., **1997**. Point mutations in the *Drosophila* sodium channel gene *para* associated with resistance to DDT and pyrethroid insecticides. *Mol. Gen. Genet. 256*, 602–610.

Plummer, N.W., McBurney, M.W., Meisler, M.H., **1997**. Alternative splicing of the sodium channel *SCN8A* predicts a truncated protein in fetal brain and non-neuronal cells. *J. Biol. Chem. 272*, 24008–24015.

Ramaswami, M., Tanouye, M.A., **1989**. Two sodium-channel genes in *Drosophila*: implications for channel-diversity. *Proc. Natl Acad. Sci. USA 86*, 2079–2082.

Ranson, H., Jensen, B., Vulule, J.M., Wang, X., Hemingway, J., *et al.*, **2000**. Identification of a point mutation in the votage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Mol. Biol. 9*, 491–497.

Rossignol, D.P., **1988**. Reduction in number of nerve membrane sodium channels in pyrethroid resistant houseflies. *Pestic. Biochem. Physiol. 32*, 146–152.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., *et al.*, **1988**. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science 239*, 487–491.

Sakai, K., Okamoto, H., Hotta, Y., **1989**. Pharmacological characterization of sodium channels in the primary culture of individual *Drosophila* embryos: neurons of a mutant deficient in a putative sodium channel gene. *Cell Diff. Devel. 26*, 107–118.

Salgado, V.L., **1990**. Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. *Pestic. Sci. 28*, 389–411.

Salgado, V.L., **1992**. Slow voltage-dependent block of sodium channels in crayfish nerve by dihydropyrazole insecticides. *Mol. Pharmacol. 41*, 120–126.

Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, N., *et al.*, **1987**. Genomic organization and deduced amino acid sequence of a putative sodium channel gene in *Drosophila*. *Science 237*, 744–749.

Sattelle, D.B., Leech, C.A., Lummis, S.C.R., Harrison, B.J., Robinson, H.P.C., *et al.*, **1988**. Ion channel properties of insects susceptible and resistant to insecticides. In: Lunt, G.G. (Ed.), Neurotox '88: The Molecular Basis of Drug and Pesticide Action. Elsevier, Amsterdam, pp. 563–582.

Sattelle, D.B., Yamamoto, D., **1988**. Molecular targets of pyrethroid insecticides. *Adv. Insect Physiol. 20*, 147–213.

Sawicki, R.M., **1978**. Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. *Nature 275*, 443–444.

Schuler, T.H., Martinez-Torres, D., Thompson, A.J., Denholm, I., Devonshire, A.L., *et al.*, **1998**. Toxicological, electrophysiological, and molecular characterisation of knockdown resistance to pyrethroid insecticides in the diamondback moth, *Plutella xylostella* (L.). *Pestic. Biochem. Physiol. 59*, 169–192.

Shichor, I., Zlotkin, E., Ilan, N., Chikashvili, D., Stühmer, W., *et al.*, **2002**. Domain 2 of *Drosophila para* voltage-gated sodium channel confers insect properties to a rat brain channel. *J. Neurosci. 22*, 4364–4371.

Siddiqi, O., Benzer, S., **1976**. Neurophysiological defects in temperature-sensitive paralytic mutants of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 73*, 3253–3257.

Smith, T.J., Ingles, P.J., Soderlund, D.M., **1998**. Actions of the pyrethroid insecticides cismethrin and cypermethrin on housefly *Vssc1* sodium channels expressed in *Xenopus* oocytes. *Arch. Insect Biochem. Physiol. 38*, 126–136.

Smith, T.J., Lee, S.H., Ingles, P.J., Knipple, D.C., Soderlund, D.M., **1997**. The L1014F point mutation in the housefly *Vssc1* sodium channel confers knockdown resistance to pyrethroids. *Insect Biochem. Mol. Biol. 27*, 807–812.

Smith, T.J., Soderlund, D.M., **2001**. Potent actions of the pyrethroid insecticides cismethrin and cypermethrin on rat tetrodotoxin-resistant peripheral nerve (SNS/PN3) sodium channels expressed in *Xenopus* oocytes. *Pestic. Biochem. Physiol. 70*, 52–61.

Soderlund, D.M., **1995**. Mode of action of pyrethrins and pyrethroids. In: Casida, J.E., Quistad, G.B. (Eds.), Pyrethrum Flowers: Production, Chemistry, Toxicology, and Uses. Oxford University Press, New York, pp. 217–233.

Soderlund, D.M., **1997**. Molecular mechanisms of insecticide resistance. In: Sjut, V. (Ed.), Molecular Mechanisms of Resistance to Agrochemicals. Springer, Berlin, pp. 21–56.

Soderlund, D.M., Bloomquist, J.R., **1989**. Neurotoxic actions of pyrethroid insecticides. *Annu. Rev. Entomol. 34*, 77–96.

Soderlund, D.M., Bloomquist, J.R., **1990**. Molecular mechanisms of insecticide resistance. In: Roush, R.T., Tabashnik, B.E. (Eds.), Pesticide Resistance in Arthropods. Chapman and Hall, New York, pp. 58–96.

Soderlund, D.M., Knipple, D.C., **1999**. Knockdown resistance to DDT and pyrethroids in the housefly (Diptera: Muscidae): from genetic trait to molecular mechanism. *Ann. Entomol. Soc. America 92*, 909–915.

Soderlund, D.M., Knipple, D.C., **2003**. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem. Mol. Biol. 33*, 563–577.

Soderlund, D.M., Lee, S.H., **2001**. Point mutations in homology domain II modify the sensitivity of rat $Na_v1.8$ sodium channels to the pyrethroid cismethrin. *Neurotoxicology 22*, 755–765.

Stern, M., Kreber, R., Ganetzky, B., **1990**. Dosage effects of a *Drosophila* sodium channel gene on behavior and axonal excitability. *Genetics 124*, 133–143.

Strong, M., Chandy, K.G., Gutman, G.A., **1993**. Molecular origin of voltage-sensitive ion channel genes: on the origins of electrical excitability. *Mol. Biol. Evol. 10*, 221–242.

Stühmer, W., **1992**. Electrophysiological recording from *Xenopus* oocytes. *Methods Enzymol. 207*, 319–339.

Suzuki, D.T., Grigliatti, T., Williamson, R., **1971**. Temperature-sensitive mutations in *Drosophila melanogaster*, VII. A mutation (*para^{ts}*) causing reversible adult paralysis. *Proc. Natl Acad. Sci. USA 68*, 890–893.

Suzuki, N., Wu, C.-F., **1984**. Altered sensitivity to sodium channel-specific neurotoxins in cultured neurons from temperature-sensitive paralytic mutants of *Drosophila*. *J. Neurogenet. 1*, 225–238.

Tan, J., Liu, Z., Nomura, Y., Goldin, A.L., Dong, K., **2002a**. Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *J. Neurosci. 22*, 5300–5309.

Tan, J., Liu, Z., Tsai, T.-D., Valles, S.M., Goldin, A.L., *et al.*, **2002b**. Novel sodium channel gene mutations in *Blattella germanica* reduce the sensitivity of expressed channels to deltamethrin. *Insect Biochem. Mol. Biol. 32*, 445–454.

Taylor, M.F.J., Heckel, D.G., Brown, T.M., Kreitman, M.E., Black, B., **1993**. Linkage of pyrethroid insecticide resistance to a sodium channel locus in the tobacco budworm. *Insect Biochem. Mol. Biol. 23*, 763–775.

Terlau, H., Stocker, M., Shon, K.-J., McIntosh, J.M., Olivera, B.M., **1996**. μO-conotoxin MrVIA inhibits mammalian sodium channels, but not through site I. *J. Neurophysiol. 76*, 1423–1429.

Thackeray, J.R., Ganetzky, B., **1994**. Developmentally regulated alternative splicing generates a complex array of *Drosophila para* sodium channel isoforms. *J. Neurosci. 14*, 2569–2578.

Thackeray, J.R., Ganetzky, B., **1995**. Conserved alternative splicing patterns and splicing signals in the *Drosophila* sodium channel gene *para*. *Genetics 141*, 203–214.

Trainer, V.L., McPhee, J.C., Boutelet-Bochan, H., Baker, C., Scheuer, T., *et al.*, **1997**. High affinity binding of pyrethroids to the α subunit of brain sodium channels. *Mol. Pharmacol. 51*, 651–657.

Tseng-Crank, J., Pollock, J.A., Hayashi, I., Tanouye, M.A., **1991**. Expression of ion channel genes in *Drosophila*. *J. Neurogenet. 7*, 229–239.

Vais, H., Williamson, M.S., Devonshire, A.L., Usherwood, P.N.R., **2001**. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest. Mgt. Sci. 57*, 877–888.

Vais, J., Williamson, M.S., Goodson, S.J., Devonshire, A.L., Warmke, J.W., *et al.*, **2000**. Activation of *Drosophila* sodium channels promotes modification by deltamethrin: reductions in affinity caused by knock-down resistance mutations. *J. Gen. Physiol. 115*, 305–318.

Wang, R., Huang, Z.Y., Dong, K., **2003**. Molecular characterization of an arachnic sodium channel gene from the varroa mite (*Varroa destructor*). *Insect Biochem. Mol. Biol. 33*, 733–739.

Wang, R., Liu, Z., Dong, K., Elzen, P.J., Pettis, J., *et al.*, **2002**. Association of novel mutations in a sodium channel gene with fluvalinate resistance in the mite, *Varroa destructor*. *J. Apicult. Res. 40*, 17–25.

Wang, S.-Y., Barile, M., Wang, G.K., **2001**. A phenylalanine residue at segment D3-S6 in $Na_v1.4$ voltage-gated Na^+ channels is critical for pyrethroid action. *Mol. Pharmacol. 60*, 620–628.

Wang, S.-Y., Wang, G.K., **2003**. Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. *Cell. Signal. 15*, 151–159.

Warmke, J.W., Reenan, R.A.G., Wang, P., Qian, S., Arena, J.P., *et al.*, **1997**. Functional expression of *Drosophila para* sodium channels: modulation by the membrane

protein *tipE* and toxin pharmacology. *J. Gen. Physiol. 110*, 119–133.

Williamson, M.S., Denholm, I., Bell, C.A., Devonshire, A.L., **1993**. Knockdown resistance (*kdr*) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*). *Mol. Gen. Genet. 240*, 17–22.

Williamson, M.S., Martinez-Torres, D., Hick, C.A., Devonshire, A.L., **1996**. Identification of mutations in the housefly *para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. *Mol. Gen. Genet. 252*, 51–60.

Wing, K.D., Schnee, M.E., Sacher, M., Connair, M., **1998**. A novel oxadiazine insecticide is bioactivated in lepidopteran larvae. *Arch. Insect Biochem. Physiol. 37*, 91–103.

Wu, C.-F., Ganetzky, B., **1980**. Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of *Drosophila melanogaster. Nature 286*, 814–816.

Yellen, G., **1998**. The moving parts of voltage-gated ion channels. *Q. Rev. Biophys. 31*, 239–295.

Yu, F.H., Catterall, W.A., **2003**. Overview of the voltage-gated sodium channel family. *Genome Biol. 4*, 207.1–207.7.

Zhao, Y., Park, Y., Adams, M.E., **2000**. Functional and evolutionary consequences of pyrethroid resistance mutations in S6 transmembrane segments of a voltage-gated sodium channel. *Biochem. Biophys. Res. Commun. 278*, 516–521.

Zlotkin, E., **1999**. The insect voltage-gated sodium channel as target of insecticides. *Annu. Rev. Entomol. 44*, 429–455.

Zlotkin, E., Moskowitz, H., Hermann, R., Pelhate, M., Gordon, D., **1995**. Insect sodium channel as the target for insect-selective neurotoxins from scorpion venom. In: Clark, J.M. (Ed.), Molecular Action of Insecticides on Ion Channels. American Chemical Society, Washington, DC, pp. 57–85.

5.2 The Insecticidal Macrocyclic Lactones

D Rugg, Fort Dodge Animal Health, Princeton, NJ, USA
S D Buckingham and D B Sattelle, University of Oxford, Oxford, UK
R K Jansson, Centocor, Malvern, PA, USA

5.2.1. Discovery

The naturally occurring avermectins and milbemycins are fermentation products of actinomycetes in the genus *Streptomyces*. They are 16-membered, macrocyclic lactones, which have structural similarities to antibacterial macrolides and antifungal polyenes but lack their antifungal or antibacterial activities and do not inhibit protein or chitin synthesis (Fisher and Mrozik, 1989). Avermectins are produced by the soil microorganism, *Streptomyces avermitilis* MA-4680 (NRRL 8165), which was first isolated at the Merck Research Laboratories in 1976 from a soil sample collected near a golf course in Japan by researchers at the Kitasato Institute (Campbell *et al*., 1984). Milbemycins were first described from a culture of *S. hygroscopicus* and are structurally similar to the avermectins but lack the disaccharide substituent at C13 (Takiguchi *et al*., 1980). Mishima *et al*. (1975) first reported the acaricidal activity of milbemycins. The anthelmintic activity of these milbemycins was later elucidated following testing of individual members of the milbemycin family (Mrozik *et al*., 1983). Milbemycins were also isolated from cultures of *S. cyanogriseus* in 1984 by workers at American Cyanamid and from *S. thermoarchaensis* by Glaxo researchers (Rock *et al*., 2002).

Four homologous pairs of closely related compounds are produced through the fermentation of *S. avermitilis* MA-4680 (NRRL 8165), each pair comprising a major and minor component, usually produced in the approximate ratio of 8 : 2 (Lasota and Dybas, 1991). A mixture of one of these pairs, avermectin B_1 (>80% B_{1a} and <20% B_{1b}) commonly called abamectin, was found to be active against nematodes (Burg *et al*., 1979; Egerton *et al*., 1979), insects (Ostlind *et al*., 1979; James *et al*., 1980; Wright, 1984), and mites (Putter *et al*., 1981).

Ivermectin, the semisynthetic 22,23-dihydro derivative of avermectin B_1, was the first commercially produced avermectin, introduced in 1981 for use against a range of nematode and arthropod parasites (Jackson, 1989). Like abamectin, ivermectin is a mixture of two homologs known as

ivermectin B_{1a} (>80%) and B_{1b} (<20%) (Campbell *et al.*, 1983). The natural avermectin abamectin was introduced as an antiparasitic drug and an agricultural insecticide and miticide in 1985 (Campbell, 1989). The natural milbemycins milbenock and milbemectin have been used as an agricultural miticide (Tsukamoto *et al.*, 1995) and in canine heartworm therapy (Jung *et al.*, 2003), respectively. An oxime derivative of milbemycin has been used widely for filarid and nematode control in dogs. Moxidectin, a synthetically modified milbemycin derived from the fermentation product nemadectin (Carter *et al.*, 1988), is used for insect and helminth control in animal health applications. In 1993, doramectin was introduced, also for use in animal health. This compound was derived from an induced mutation of *S. avermitilis* (Goudie *et al.*, 1993) and is structurally closer to abamectin than to ivermectin (Shoop *et al.*, 1995). A subsequent semisynthetic derivative, selamectin (the monosaccharide oxime), is used to control insect, acarid and helminth parasites of cats and dogs (Bishop *et al.*, 2000).

A targeted analoging program around abamectin resulted in a five-step semisynthetic avermectin, emamectin, in 1984 (Cvetovich *et al.*, 1994). MK-243 (the hydrochloride salt of emamectin) was discovered after screening several hundred avermectin derivatives in an *in vivo* screen using tobacco budworm, *Heliothis virescens* and southern armyworm, *Spodoptera eridania* (Dybas and Babu, 1988; Dybas *et al.*, 1989; Mrozik *et al.*, 1989; Mrozik, 1994). This compound was subsequently selected for further development for control of lepidopterans in crop protection. Synthesized aglycon derivatives of ivermectin have been reported to be potent flea insecticides (Cvetovich *et al.*, 1997), but have not been developed commercially.

Numerous papers have been published on the avermectins and milbemycins (Kornis, 1995). Several authors have reviewed the discovery, structure–activity relationships, environmental fate, spectrum of activity, and applications for their anthelminthic, insecticidal, and acaricidal activity (Campbell *et al.*, 1984; Fisher and Mrozik, 1984, 1989, 1992; Davies and Green, 1986; Strong and Brown, 1987; Dybas, 1989; Lasota and Dybas, 1991; Mrozik, 1994; Kornis, 1995; Shoop *et al.*, 1995; Fisher, 1997; Jansson and Dybas, 1998; Vercruysse and Rew, 2002a). In this chapter the chemistry, mode of action, biological activity, and applications of macrocyclic lactones, with special emphasis on their use as insecticides are reviewed.

5.2.2. Chemistry

5.2.2.1. Chemical Structure

The chemical structure of avermectin B_{1a} is shown in **Figure 1**. The milbemycins share a similar basic 16-carbon lactone but lack the disaccharide moiety of the avermectins (e.g., moxidectin) (**Figure 2**). These compounds have strong ultraviolet absorption at 245 nm, a feature which is of value in analytical detection. Avermectins and milbemycins are highly lipophilic, being soluble in most organic solvents but almost insoluble in water (Fisher and Mrozik, 1989). Milbemycins tend to be more lipophilic than avermectins (Hennessy and Alvinerie, 2002). Ultraviolet light rapidly decomposes

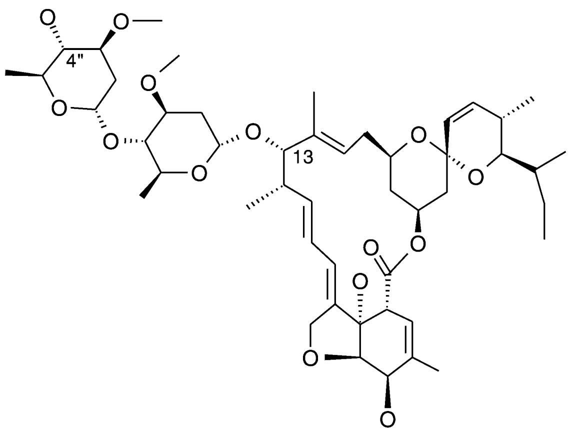


Figure 1 Chemical structure of avermectin B_{1a} (major component of abamectin).

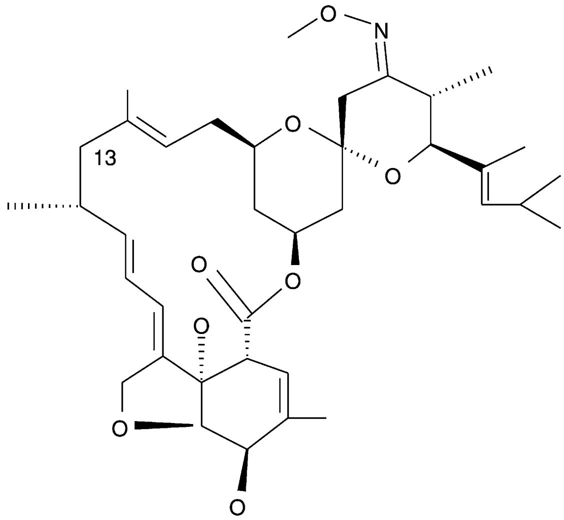


Figure 2 Chemical structure of moxidectin.

avermectins, leaving a large number of less toxic products (Halley *et al.*, 1989a) that lack any ultraviolet absorption (Mrozik *et al.*, 1988) as a residue. The half-life of ivermectin, exposed to sunlight as a thin film on glass, is about 3 h (Halley *et al.*, 1989b).

Ivermectin B_{1a} differs from abamectin in the loss of the 22,23 double bond, so that 22 and 23 are dihydrocarbons. The B_{1b} components differ from their respective B_{1a} structures by the replacement of a CH_2CH_3 group with a CH_3 (Fisher and Mrozik, 1989). Ivermectin is also commonly referred to as 22,23-dihydroavermectin B_1. Emamectin, an epimethyl amino substitution at the 4″ position of the sugar (**Figure 3**) was derived from abamectin using a five-step synthesis (Cvetovich *et al.*, 1994).

Doramectin was generated via a biosynthetic program. Mutated strains of *S. avermitilis* were used to generate novel avermectins (Goudie *et al.*, 1993). Doramectin (**Figure 4**) was selected for development as an endectocide (a compound that controls both internal and external parasites) from a number of compounds that differed from avermectins in

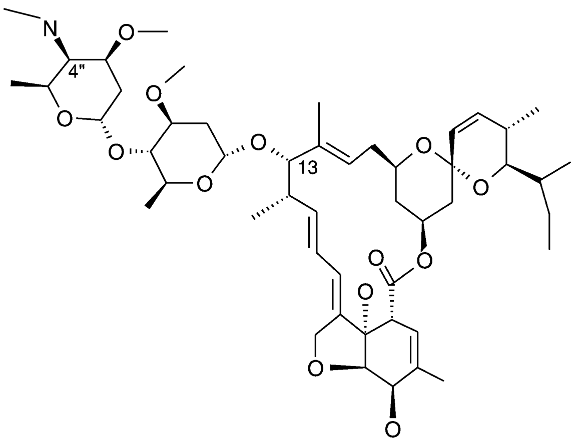


Figure 3 Chemical structure of emamectin.

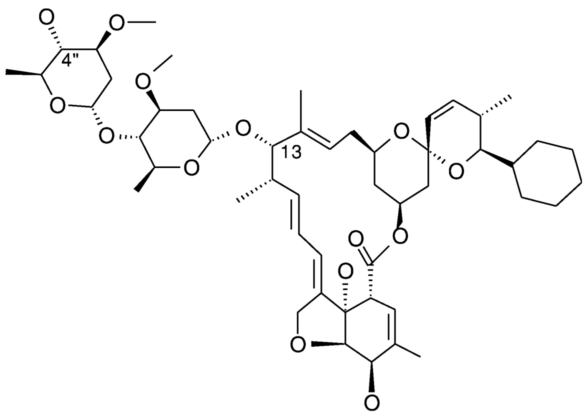


Figure 4 Chemical structure of doramectin.

substituents at the C25 position. Selamectin was chosen from a targeted chemistry research program aimed at identifying an avermectin that retained anthelminthic activity but with enhanced insecticidal activity, especially against fleas (Banks *et al.*, 2000).

Targeted synthesis programs with milbemycins have also led to commercial products. Moxidectin (**Figure 2**) was synthesized from the natural milbemycin Fα (nemadectin) via a multistep process (Carter *et al.*, 1988).

5.2.2.2. Structure–Activity Relationships

Structure–activity relationships of the avermectins and milbemycins are complicated due to the broad spectrum of activity against arthropods and helminths. However, reviews of the relationship between the activity of these compounds and chemical structure have shown some consistent patterns (Shoop *et al.*, 1995; Fisher, 1997). The major structural difference between avermectins and milbemycins is the lack of the disaccharide moiety at position C13 of the macrolide ring. Within avermectins, removal of the distal sugar results in a slight decrease in activity against nematodes. Removal of both sugars results in an avermectin aglycone with a hydroxy group at the C13 position with little useful activity against arthropods or helminths (Chabala *et al.*, 1980). When the oxygen is removed from the C13 position, the resultant deoxyavermectins recover potency and are structurally equivalent to milbemycins (Mrozik *et al.*, 1983; Shoop *et al.*, 1995). Generally, avermectins and milbemycins with lipophilic substituents at C13 are highly active, while polar substituents result in a loss of activity (Mrozik *et al.*, 1989). Similar structure–activity patterns have been seen with insects and mites.

For insecticidal activity, the most dramatic development was the synthesis of 4″-epi-amino avermectins (Fisher, 1997). In these compounds, the replacement of the hydroxy group on the distal sugar with an epimethylamino group resulted in a marked increase in potency against a variety of lepidopterans and reduced activity against mites.

5.2.3. Mode of Action

5.2.3.1. Biochemical and Molecular Action

Avermectins are potent endectocides. Ivermectin, the first and most commercially important member of this class, is the world's most successful animal health drug of all time (Raymond and Sattelle, 2002). While the molecular mode of action of avermectins initially proved difficult to define (Jackson,

1989), more recent evidence indicates that they act primarily on glutamate-gated chloride channels of helminths and insects, with additional effects (especially at high concentrations) on γ-aminobutyric acid (GABA) receptors.

Early evidence for GABA receptors as a site of action came from studies performed mostly on insects (Sattelle, 1990). Dihydroavermectin B_{1a}-sensitive GABA-binding sites were identified in the cockroach *Periplaneta americana* using [^{3}H]GABA binding (Lummis and Sattelle, 1985). Furthermore, avermectins were shown to act as a partial agonist at a GABA-binding site in honeybees (*Apis mellifera*) defined by [^{3}H]muscimol binding (Abalis and Eldefrawi, 1986). Deng and Casida (1992) examined the interactions of radiolabeled avermectin with nerve membranes from housefly (*Musca domestica*) heads and hypothesized that avermectins may either have multiple binding sites at each chloride channel, or may bind to GABA-gated channels at low concentrations and at other chloride channels at higher concentrations.

Evidence from vertebrate studies also pointed to GABA receptors as a site of action. Administered to rats, ivermectin acts as a GABA-ergic anxiolytic drug (Spinosa *et al*., 2002). Another study compared the potency of 25 avermectin analogs using a mouse seizure model with their direct action on recombinant $GABA_A$ receptors (Dawson *et al*., 2000). Interestingly, while their binding affinities to brain membrane and to recombinant $GABA_A$ receptors stably expressed in cell lines were found to be closely similar, suggesting $GABA_A$ receptors as the target, the rank order of affinity of these analogs at the [^{3}H]ivermectin binding site differed from that of their anticonvulsant activities, suggesting that their activities differed between subtypes of $GABA_A$ receptor. When the binding of the analogs to cell lines expressing known combinations of $GABA_A$ receptor subunits were measured, only modest differences in affinity (up to fivefold) were observed, compared to differences in anticonvulsant activity in the seizure model of up to 40-fold. Furthermore, their efficacy in potentiating GABA-evoked currents in *Xenopus laevis* oocytes expressing $GABA_A$ receptors correlated well with their anticonvulsant activity. The conclusion drawn, as reported earlier (Arena *et al*., 1993), was that the GABA receptor β subunit is a key determinant of avermectin potency. The study also suggested the presence of two distinct avermectin binding sites. Arena *et al*. (1993) had observed that there are twice as many avermectin binding sites as benzodiazepine sites correlating well with there being two β subunits in many $GABA_A$ receptors (Sigel and Buhr, 1997).

Certain evidence also suggested that avermectins act at sites other than $GABA_A$ receptors. For example, [^{3}H]22,23-dihydroavermectin-B_1 binding sites that were GABA-insensitive were identified in membrane preparations from *Caenorhabditis elegans* (Schaeffer and Haines, 1989). Tanaka and Matsumura (1985) found that avermectin stimulated chloride ion uptake in cockroach (*P. americana*) leg muscle; they suggested that this was not due to activity at the GABA binding site but rather a direct action on the chloride ion channel. Tanaka (1987) determined that abamectin and milbemycin act directly on a neuronal chloride channel of *P. americana*, and that this action could not be accounted for in terms of action on the GABA, picrotoxinin, or benzodiazepine sites on GABA-gated chloride channels. Nicholson *et al*. (1988) also deployed *P. americana* in studies of acetylcholine release from a central nervous system synaptosome preparation. The authors concluded that avermectins acted on a non-GABA-mediated chloride channel, with the resultant depolarization of the nerve terminal leading to increased acetylcholine release. Avermectin B_{1a} and moxidectin inhibited binding of 4-*n*-propyl-4′-ethnylbicycloorthobenzoate (EBOB) a noncompetitive blocker of the GABA-gated chloride channel in *Drosophila melanogaster* and *D. simulans* (Cole *et al*., 1995). Their efficacy in displacing EBOB was unaffected by the A302S mutation in the cloned *D. melanogaster* GABA receptor, RDL, that reduces sensitivity to convulsants, suggesting that either the main site of action of avermectin B_{1a} is not RDL, or that avermectin binding does not affect the RDL convulsant site. The dieldrin-resistant Maryland strain of *D. melanogaster*, which has the RDL A302S mutation, is susceptible to avermectin MK-243, abamectin, and abamectin 8,9-oxide (Bloomquist, 1994), again suggesting that these avermectins do not act at the convulsant site of the GABA receptor encoded by the *rdl* gene.

Evidence that glutamate-gated chloride channels are the primary site of the anthelminthic activity of avermectins came from a number of studies on nematodes. These studies showed that the interaction of avermectins with glutamate-gated chloride channels is distinct from any effect upon GABA-sensitive channels (Schaeffer and Haines, 1989; Arena *et al*., 1992, 1995). They also showed that the effects of ivermectin on its interaction with glutamate were concentration dependent. At low concentrations, ivermectin potentiated the response of the channel to glutamate, whereas at high concentrations, ivermectin opened the chloride channel directly (Arena *et al*., 1992). Thus, in nematodes, avermectins are believed to potentiate or directly open the glutamate-gated chloride channel (Arena *et al*., 1995).

Studies on cloned, expressed glutamate-gated chloride channels from nematodes and insects provide strong evidence that avermectins act upon these receptors. Cully *et al.* (1994) cloned two complementary DNAs encoding avermectin-sensitive glutamate-gated chloride channel subunits from the nematode *C. elegans*. These two genes (*GluClα1* and *GluClβ*) are newly discovered members of the cys-loop family of the ligand-gated ion channel superfamily. The α1 subunit gene is alternatively spliced to produce two subunits (Laughton *et al.*, 1997b). Cully *et al.* (1994) demonstrated that the potency of various avermectin compounds *in vivo* was correlated with their ability to potentiate glutamate-gated currents recorded when *GluClα1* and *GluClβ* are coexpressed in *Xenopus* oocytes, suggesting that these two subunits are components of the target of avermectins in nematodes. The α subunit possesses a binding site for glutamate but is not coupled to the opening of the channel (Etter *et al.*, 1996). The *GluClα1* gene is expressed in the pharyngeal muscle cells pm4 and pm5, and in some neurons (Dent *et al.*, 1997) and *GluClβ* is also expressed in pm4 (Laughton *et al.*, 1997a). When a transposon element is used to introduce a deletion in *GluClα1*, to eliminate its function, sensitivity of the animals to ivermectin remained but mRNA extracted from them and expressed in *Xenopus* oocytes resulted in the expression of glutamate receptors with reduced sensitivity to both ivermectin phosphate and to glutamate (Vassilatis *et al.*, 1997). Furthermore, in the mutant, binding of [^{3}H]ivermectin showed reduced binding affinity, indicating a loss of ivermectin binding sites. The same study also reported the cloning and functional expression of another glutamate-gated chloride channel, *GluClα2*, which when expressed in *Xenopus* oocytes responds reversibly to glutamate and irreversibly to avermectin.

Arguably, the strongest evidence that glutamate-gated chloride channels are targets for avermectins derives from genetics. Simultaneous mutation of genes encoding glutamate-gated chloride channel α-type subunits (*GluClα1*, *GluClα2*, and *GluClα3*) confers high-level resistance to ivermectin. Interestingly, only low-level (or no) resistance is conferred by mutation of any two of these three glutamate receptor genes (Dent *et al.*, 2000). It may prove to be a sound strategy to search for drug targets encoded by a multigene family because the development of drug resistance could be slower if several target genes have to be mutated simultaneously to confer resistance.

Similar results have been obtained using glutamate-gated chloride channels from other nematodes. Of the four glutamate-gated chloride channel subunits known in *Haemonchus contortus* (Delany *et al.*, 1998; Jagannathan *et al.*, 1999), two bind [^{3}H]ivermectin with high affinity when expressed in monkey kidney, COS-7, cells, which is not displaced by GABA, picrotoxin, or fipronil (convulsants that block GABA-gated chloride channels) or glutamate; and for the one channel examined, there was no difference in sequence between that from a wild-type and that from an ivermectin-resistant strain (Cheeseman *et al.*, 2001). The recently described tissue distribution of the four *H. contortus* avermectin-sensitive glutamate receptor subunits showed that these subunits are differentially expressed in the motor nervous system, as well as in other neurons (Portillo *et al.*, 2003).

Horoszok *et al.* (2001) reported the cloning of GLC-3, a glutamate-gated chloride channel from *C. elegans* that forms homooligomeric receptors when expressed in *Xenopus* oocytes. Ivermectin potently and irreversibly activated this receptor (EC_{50} the concentration that evokes a half-maximal response – 0.4 μM). The availability of a new homomeric avermectin-sensitive receptor from a helminth offers exciting possibilities for structure–function research into the site of action of these compounds.

Rohrer *et al.* (1995) identified the binding sites of ivermectin in two arthropods, *D. melanogaster* and *Schistocerca americana*. One subunit, DrosGluClα, was recently cloned from *D. melanogaster*. This subunit is sensitive to both glutamate and avermectins (Cully *et al.*, 1996). The same authors also found transcripts related to DrosGluClα in other insects, including cat flea, *Ctenocephalides felis*, fall armyworm, *Spodoptera frugiperda*, and cotton bollworm, *Helicoverpa zea*, but not in the two-spotted spider mite, *Tetranychus urticae*. It remains to be determined whether or not additional GluCl subunits are present in insects.

An exciting recent discovery may explain, in part, why avermectins appear to act at both GABA and glutamate receptors. Immunoprecipitation studies using antibodies to a *D. melanogaster* glutamate-gated chloride channel (*Dm*GluCl) showed that *Dm*GluClα antibodies precipitated all of the ivermectin and nodulisporic acid receptors solubilized by detergent from *Drosophila* head membranes, and that *Dm*RDL antibodies also immunoprecipitated all solubilized nodulisporic acid receptors and approximately 70% of the ivermectin receptors (Ludmerer *et al.*, 2002). The data led the authors to suggest that both *Dm*GluClα and RDL are components of nodulisporic acid and ivermectin receptors.

It has been suggested that avermectins may bind to a common site on all ligand-gated chloride

channels similar to the benzodiazepine site on mammalian GABA-receptors (Huang and Casida, 1997), and act in a tissue-specific manner either as positive or negative allosteric modulators. This would help to explain some of the additional effects that avermectins exert on chloride channels, such as their inhibition of strychnine binding to the mammalian glycine receptor (Graham *et al*., 1982); activation of multiple-gated chloride channels (glutamate, GABA, and acetylcholine) in crayfish (Zufall *et al*., 1989); and both potentiation and blocking of GABA-gated chloride channels in arthropods (Duce and Scott, 1985; Bermudez *et al*., 1991).

There is evidence for actions of avermectins at sites other than glutamate- or GABA-gated chloride channels. Avermectin C and avermectin A_1 increased calcium-dependent chloride currents of *Chara corallina* cells at low concentrations but blocked them at high concentrations (Driniaev *et al*., 2001). Avermectins A_2, B_1 (abamectin), B_2 and 22,23-dihydroavermectin B_1 (ivermectin) in the concentration range studied did not affect the chloride currents of *C. corallina* cells. There is also evidence for nicotinic acetylcholine receptor actions of avermectins. Ivermectin potentiates responses to acetylcholine mediated by heterologously expressed α7 nicotinic receptor subunits (Krause *et al*., 1998) with high potency (30 μM ivermectin potentiated responses to 30 μM acetylcholine (ACh) nearly threefold) through a presumed allosteric mechanism. However, responses to ACh mediated by ACR-16 (formerly known as Ce21), an α7-like homomer-forming nicotinic acetylcholine receptor subunit from *C. elegans* (Ballivet *et al*., 1996), with 47% identity with chicken α7 at the amino acid level, were not enhanced by the same concentration of ivermectin, but rather were slightly attenuated (Raymond *et al*., 2000).

In summary, avermectins affect invertebrates either by directly activating receptors for the neurotransmitters glutamate and GABA or by potentiating their actions, resulting in an influx of chloride ions into nerve cells and muscle. It appears that the anthelminthic properties of the avermectins are due predominantly to potentiation and/or direct opening of glutamate-gated chloride channels, whereas in insects, it is likely that avermectins bind to multiple sites, including both glutamate- and GABA-gated chloride channels as well as other insect chloride channels. However, not all insect chloride channels are sensitive to avermectins: one of the two chloride channels spontaneously active in cultured *D. melanogaster* larval neurons – the large conductance (35pS) "slow" channel – is insensitive to dihydroavermectin B_{1a} (Yamamoto and Suzuki, 1988). Nevertheless, there is now a wealth of evidence that the chloride ion flux resulting from the opening by avermectins of chloride channels in invertebrate nerve and muscle, particularly those gated by glutamate and GABA, results in disruption of activity and loss of function in these excitable cells and accounts for the potent endectocidal actions of these molecules.

5.2.3.2. Physiological Activity

5.2.3.2.1. Insects While avermectins are effective through both topical and oral routes in lepidopteran larvae, toxicity is generally greater through ingestion than by residual contact (Wright *et al*., 1985; Anderson *et al*., 1986; Bull, 1986; Corbitt *et al*., 1989). McKellar and Benchaoui (1996) surmised that the oral route made the major contribution to uptake of macrocyclic lactones in ectopararasitic arthropods. The higher *in vivo* activity of these compounds against sucking lice than against biting lice, and the potent effects on blood-feeding mites (Benz *et al*., 1989), supports this hypothesis. Similarly, abamectin, although having relatively poor contact activity, is potent when administered orally against cockroaches (Rust, 1986), lice (Rugg and Thompson, 1993), ants (Glancey *et al*., 1982; Baker *et al*., 1985), termites (Su *et al*., 1987), and vespid wasps (Chang, 1988).

In comparison with other neurotoxic agents, avermectins are considered to be relatively slow acting (Wright, 1986; Strong and Brown, 1987; Abro *et al*., 1988). Affected organisms usually die slowly, often over a period of days, and there is no quick knockdown effect like that achieved with the use of some pyrethroids and organophoshates (Jackson, 1989). Death usually follows paralysis and immobility. Agee (1985) described the symptoms of abamectin poisoning in *H. zea* larvae; the insects showed a gradual loss of locomotor coordination followed by lethargy and complete loss of locomotor activity. This slow action could possibly be due to either progressive paralysis of the organism's feeding, resulting in slow accumulation of the toxin, or starvation (Jackson, 1989). However, it is apparent that there is a direct neurotoxic effect, as adults of *H. zea* died more rapidly from abamectin poisoning than from starvation (Agee, 1985) and poisoned German cockroaches (*Blattella germanica*) ate less than controls, but the starved controls did not die (Cochran, 1985). The blood-sucking bug *Rhodnius prolixus* can survive for months after a blood meal, but dies within 9 days of ingestion of ivermectin (De Azambuja *et al*., 1985).

5.2.3.2.2. Acarids and helminths Symptoms of poisoning in acarids and helminths are similar to those of insects. The affected organisms cease feeding, suffer paralysis, and subsequently die (McKellar and Benchaoui, 1996). For most species, the oral route is considered the major means of uptake, although for some gastrointestinal and filarial nematodes, transcuticular absorption may be as significant as ingestion (Court *et al*., 1988).

5.2.3.2.3. Vertebrates Of the vertebrates, only fish are uniformly susceptible to macrocyclic lactone toxicity (Wislocki *et al*., 1989). Mammals are relatively insensitive to these compounds (Jackson, 1989). The signs of macrocyclic lactone poisoning in mammals are consistent with central nervous system toxicity. Symptoms include salivation, ataxia, muscle fasciculation, lingual paralysis, recumbency, apparent blindness, tremors, and depression. Lethal doses produce ataxia, convulsions, and coma (Campbell and Benz, 1984; Lankas and Gordon, 1989). These signs suggest poisoning of the central nervous system and are consistent with stimulation of GABA-gated chloride channels which are likely to be the active sites of macrocyclic lactones in mammals (Pong *et al*., 1980; Pong and Wang, 1982; Drexler and Sieghart, 1984a, 1984b, 1984c).

5.2.3.3. Sublethal Toxicity

Sublethal effects of macrocyclic lactones on insects include inhibition of feeding, developmental abnormalities, and disruption of reproduction (Strong and Brown, 1987). Cessation of feeding appears to be a symptom of toxicity as overt repellency is rarely observed with avermectins (Schuster and Everett, 1983; Cochran, 1985; Orton *et al*., 1992; Allingham *et al*., 1994). In most instances, reduction in the prolonged feeding activity of insects is due to the onset of poisoning (Strong and Brown, 1987).

Sublethal effects on development are varied. Cockroach nymphs that had been fed sublethal doses of abamectin were able to complete development (Cochran, 1985), but juvenile *R. prolixus* failed to molt (De Azambuja *et al*., 1985). Sublethal doses of abamectin fed to neonate tufted apple moth, *Platynota idaeusalis*, increased the developmental time from neonate to adult eclosion and greatly affected fecundity (Biddinger and Hull, 1999). In other lepidopterans, sublethal doses of abamectin may interfere with pupation and metamorphosis without marked disruption of larval development (Reed *et al*., 1985; Bull, 1986) and similar effects have been observed for ivermectin in dipterans (Meyer *et al*., 1980; Spradbery *et al*., 1985; Strong, 1986). Rugg (1995) found that abamectin, ivermectin, and moxidectin had some residual postfeeding effects resulting in death in the pupal stage of a dung-feeding fly, *Tricharea brevicornis*. Sublethal residues of avermectins have been shown to markedly delay the development of dung beetle larvae (Lumaret *et al*., 1993; Kruger and Scholtz, 1997) and produce morphological developmental abnormalities in dung-feeding flies (Clarke and Ridsdill-Smith, 1990; Strong and James, 1993) and dung beetles (Sommer *et al*., 1993).

Sublethal doses of these insecticides also produce various effects on reproduction. Female *R. prolixus* suffered a permanent reduction in egg production following ingestion of ivermectin (De Azambuga *et al*., 1985). In some Coleoptera, exposure to abamectin resulted in fewer eggs laid and lowered levels of hatching (Wright, 1984; Reed and Reed, 1986). Following abamectin ingestion, female *B. germanica* had fewer oocytes and produced fewer oothecae (Cochran, 1985). Also, female serpentine leafminer (*Liriomyza trifolii*) exposed to abamectin-treated leaves laid fewer eggs, possibly due to malfunction of the ovipositor after contact to abamectin (Schuster and Everett, 1983). They noted that ingestion of abamectin might have occurred and this could have an effect on toxicity to *Liriomyza*. Adult Australian blowfly *Lucilia cuprina* females fed sheep dung containing ivermectin residues show reduced fecundity, survival, and delayed reproductive development (Cook, 1991, 1993; Mahon and Wardhaugh, 1991). Orton *et al.* (1992) investigated the activity of avermectins as oviposition suppressants in *L. cuprina*. While the compounds tested were effective in suppressing oviposition, this was related to general toxic effects and was only apparent at levels that produced significant mortality. Cook (1993) found that sublethal doses of ivermectin affected mating behavior in male *L. cuprina*. The number of mating attempts was reduced and the duration of mating was longer in treated males than in nontreated males. When tsetse flies (*Glossina morsitans*) were fed on blood containing ivermectin, they aborted eggs and larvae. However, these flies produced normal larvae after refeeding on normal blood (Langley and Roe, 1984). Lepidopteran larvae treated with sublethal doses of abamectin that survived to adults displayed reduced mating, egg production, and hatching (Reed *et al*., 1985; Robertson *et al*., 1985; Wright, 1986). Sublethal doses of abamectin have been shown to irreversibly sterilize queens of the ant *Solenopsis invicta* (Glancey *et al*., 1982).

5.2.3.4. Metabolism

Oxidative metabolism is believed to be the major route for detoxification and excretion of

macrocyclic lactones in insects. Three main oxidative metabolites have been identified in mammals (Chiu and Lu, 1989; Zeng *et al.*, 1996). These metabolites are more polar than the parent compounds and greater hydrophilicity can enhance excretion of xenobiotics in insects. Studies with resistant and susceptible Colorado potato beetle (*Leptinotarsa decemlineata*) have implicated oxidative metabolism as a major mechanism in resistance to avermectins (Argentine, 1991; Argentine *et al.*, 1992; Yoon *et al.*, 2002).

5.2.4. Biological Activity

5.2.4.1. Spectrum and Potency

5.2.4.1.1. Insects The macrocyclic lactones are among the most potent insecticidal, acaricidal, and anthelminthic agents known. Avermectins have been shown to be toxic to most insects although potency and speed of action can vary widely (Strong and Brown, 1987). These compounds are used commercially to control pests in the orders Isoptera, Blattodea, Phthiraptera, Thysanoptera, Coleoptera, Siphonaptera, Diptera, Lepidoptera, and Hymenoptera. Abamectin, the compound most widely used for insect control, has been shown to be a potent stomach toxin in cockroaches (Cochran, 1985; Rust, 1986; Koehler *et al.*, 1991), fire ants (Glancey *et al.*, 1982), Argentine ants (Baker *et al.*, 1985), vespid wasps (Chang, 1988), termites (Su *et al.*, 1987), various blood-feeding Diptera (Miller *et al.*, 1986; Allingham *et al.*, 1994), and sheep-biting lice, *Bovicola ovis* (Rugg and Thompson, 1993). Among agricultural pests, the compound is highly toxic to tobacco hornworm (*Manduca sexta*), diamondback moth (*Plutella xylostella*), tomato pinworm (*Keiferia lycopersicella*), tobacco budworm (*H. virescens*), Colorado potato beetle (*L. decemlineata*), and the serpentine leafminer (*L. trifolii*) (lethal concentration (LC_{90}) values range between 0.009 and 0.19 $\mu g\,ml^{-1}$). However, it is less potent against certain Homoptera and most Lepidoptera (LC_{90} values range between 1 and >25 $\mu g\,ml^{-1}$) (Dybas, 1989; Lasota and Dybas, 1991; Jansson and Dybas, 1998). Although abamectin is toxic to certain aphids (e.g., LC_{90} values against black bean aphid, *Aphis fabae* and cotton aphid, *Aphis gossypii* range from 0.4 to 1.5 $\mu g\,ml^{-1}$) (Putter *et al.*, 1981; Dybas and Green, 1984), it was not as effective at controlling aphids in translaminar assays (Wright *et al.*, 1985). The reduced efficacy at controlling aphids is probably due to its prime activity as a stomach poison and its reduced concentrations in phloem tissue, where aphids actively feed. Poor residual control of aphids in the field has been confirmed in several studies (Dybas, 1989 and references therein).

Emamectin benzoate is a recently introduced avermectin that is highly potent to a broad spectrum of lepidopterous pests (Jansson and Dybas, 1998). LC_{90} values for emamectin benzoate against a variety of lepidopterous pests range between 0.002 and 0.89 $\mu g\,ml^{-1}$ (Dybas, 1989; Cox *et al.*, 1995b; Jansson and Dybas, 1998). The hydrochloride salt of emamectin was up to 1500-fold more potent against armyworm species, e.g., beet armyworm (*Spodoptera exigua*), than abamectin (Trumble *et al.*, 1987; Dybas *et al.*, 1989; Mrozik *et al.*, 1989). Emamectin hydrochloride was also 1720-, 884-, and 268-fold more potent to *S. eridania* than methomyl, thiodicarb, and fenvalerate, respectively, and 105- and 43-fold more toxic to cotton bollworm (*H. zea*) and tobacco budworm (*H. virescens*) larvae than abamectin (Dybas and Babu, 1988). Recent studies showed that emamectin benzoate was 875- to 2975-fold and 250- to 1300-fold more potent than tebufenozide to *H. virescens* and *S. exigua,* respectively. Emamectin benzoate was also 12.5- to 20-fold and 250- to 500-fold more potent than λ-cyhalothrin and 175- to 400-fold and 2033- to 8600-fold more potent than fenvalerate to these two Lepidoptera, respectively (Jansson *et al.*, 1998). Emamectin benzoate was 1.2- to 4.8-orders of magnitude more potent to lepidopterous pests of cole crops (e.g., *S. exigua, P. xylostella*, and cabbage looper, *Trichoplusia ni*), than other new insecticides, including chlorfenapyr, fipronil, and tebufenozide (Jansson and Dybas, 1998; Argentine *et al.*, 2002b).

Emamectin benzoate is markedly less toxic to most nonlepidopteran arthropods. Emamectin is 8- to 15-fold less toxic to the serpentine leafminer, *L. trifolii* and the two-spotted spider mite, *T. urticae,* respectively, than abamectin (Dybas *et al.,* 1989; Cox *et al.*, 1995a). Emamectin benzoate and abamectin are comparable in their potency against Mexican bean beetle, *Epilachna varivestis*, and Colorado potato beetle, *L. decemlineata* (Dybas, 1989). Emamectin benzoate is markedly less toxic to black bean aphid, *A. fabae*, than abamectin (Jansson and Dybas, 1998).

The macrocyclic lactones are widely used in animal health applications. The use of these compounds for antiparasitic therapy has been recently reviewed (Raymond and Sattelle, 2002; Vercruysse and Rew, 2002a). James *et al.* (1980) demonstrated the initial insecticidal activity of crude extracts and partially purified avermectins against Australian sheep blowfly, *L. cuprina*. Hughes and Levot (1990) examined the potencies of abamectin, ivermectin, and 4-deoxy-4-epi(methyl amino) avermectin B_1 in *in vitro*

assays with *L. cuprina*. All three avermectins were equally potent against newly hatched larvae, and an order of magnitude more potent than diazinon when it was first introduced for blowfly control. Similarly, ivermectin is about two orders of magnitude more potent than deltamethrin against pyrethroid-susceptible larvae of *L. cuprina* (Rugg *et al.*, 1995a). Ostlind *et al.* (1997) determined that ivermectin and emamectin were more potent than a number of common insecticides in a bioassay using larvae of *Lucilia sericata*. However, these workers found that abamectin was about 100-fold less toxic than ivermectin.

Avermectins have similar potency against most myiasis producing dipterans. Abamectin or ivermectin concentrations of 5–20 ng ml^{-1} in rearing medium were toxic to early stage larvae of *Cochliomia macellaria* (Chamberlain, 1982) and *Chrysomya bezziana* (Spradbery *et al.*, 1985). Similarly, avermectins are highly potent against parasitic larvae in the families Cuterebridae (Ostlind *et al.*, 1979; Roncalli, 1984), Gasterophilidae (Klei and Torbert, 1980; Craig and Kunde, 1981) and Oestridae (Preston, 1982; Yazwinski *et al.*, 1983).

Avermectins are also potent against hematophagous dipterans. Miller *et al.* (1986) demonstrated that the therapeutic anthelminthic (subcutaneous) dose of ivermectin would be effective against the blood-feeding horn fly, *Haematobia irritans*, for more than 2 weeks. Standfast *et al.* (1984) found that this same dose in cattle produced 99% mortality in engorged ceratopogonid midges 48 h after feeding for up to 10 days after treatment. *In vitro* studies have demonstrated that the subcutaneous dose of ivermectin in cattle kills 98% of buffalo flies fed for 7 days on blood taken 1 day after treatment (Kerlin and East, 1992). *In vitro* blood-feeding assays demonstrate that ivermectin is highly toxic to horn fly (88 h LC_{50} was 3–7 ng ml^{-1}) (Miller *et al.*, 1986) and buffalo fly (*H. irritans exigua*) (48 h LC_{50} was 30–60 ng ml^{-1}) (Allingham *et al.*, 1994). Adult tsetse fly (Langley and Roe, 1984), stable fly (*Stomoxys calcitrans*) (Miller *et al.*, 1986), and mosquitoes (Pampiglione *et al.*, 1985) tend to be less susceptible and generally survive feeding on animals treated at therapeutic dose rates.

Fleas are generally considered to be relatively insensitive to avermectins or milbemycins systemically administered at, or slightly above, doses that provide ectoparasite control in production animals (Strong and Brown, 1987; Zakson-Aiken *et al.*, 2001). However, Zakson-Aiken *et al.* (2001) examined a milbemycin and 19 avermectin aglycones, monosaccharides, and disaccharides for toxicity to fleas in an artificial membrane feeding system and found that 19 of these compounds produced between 52% and 97% mortality in fleas fed a dose of 20 μg ml^{-1}. The potencies of these compounds, which included abamectin, selamectin, ivermectin, and milbemycin D, were remarkably similar, yet the most potent compound was inactive when tested at 1 mg kg^{-1} subcutaneously in the dog (Zakson-Aiken *et al.*, 2001). By contrast, selamectin provides effective flea control for up to 4 weeks after topical application at 6 mg kg^{-1} on dogs (Banks *et al.*, 2000; Bishop *et al.*, 2000).

Sucking lice on cattle are effectively controlled by therapeutic doses of avermectins, but biting lice are rarely affected by oral or injectable doses (Benz *et al.*, 1989). However, topical administration to cattle does give effective control of biting lice (Benz *et al.*, 1989). *In vitro* studies with sheep biting lice indicate that efficacy is not due to direct contact activity, but rather to the differential distribution following application resulting in the availability of a toxic oral dose. Rugg and Thompson (1993) examined the toxicity of abamectin, ivermectin, and cypermethrin to the sheep-biting, louse *Damalinia ovis*. These workers found that avermectins were an order of magnitude less potent than cypermethrin when assessed in a contact bioassay. However, in an assay incorporating ingestion and contact action both macrocyclic lactones were an order of magnitude more potent than cypermethrin.

Macrocyclic lactones are also highly toxic to many dung-feeding insects, but toxicity may vary widely between life stages. Dung-feeding Diptera and Coleoptera are highly susceptible; however, adult dung beetles are relatively insensitive. Rugg (1995) found that adult *Onthophagus gazella* were four orders of magnitude less susceptible to ivermectin or moxidectin than larvae in *in vitro* bioassays. This relative insensitivity in adult dung beetles with no significant effects on adult mortality seen in mature beetles fed dung from cattle treated with ivermectin has been reported previously (Ridsdill-Smith, 1988; Wardhaugh and Rodriguez-Mendez, 1988; Fincher, 1992). However, newly emerged and immature adult beetles may be affected negatively by ivermectin residues (Wardhaugh and Rodriguez-Mendez, 1988; Houlding *et al.*, 1991). Adult dung beetles are sexually immature at emergence and have an initial period of intense feeding. It is during this period that they would be most susceptible to residues in feces. Nevertheless, the relative insensitivity of adult dung beetles is attributable to ingestion being the main route of toxicity, to the feeding behavior of the beetles, and the characteristics of the compounds. Adult dung beetles ingest only the liquid and colloidal constituents of dung,

as their mouthparts are unsuitable for solids (Bornemissza, 1970). Avermectins and similar compounds bind tightly to soil and organics, and are virtually insoluble in water (Halley *et al.*, 1989b) and could be expected to partition differentially to the solid components of dung. Thus, adults would be expected to ingest relatively low concentrations of these compounds when feeding on the liquid portion of dung.

Milbemycins generally tend to have lower toxicities to insects than the avermectins, but similar or higher potencies against some nematodes and acarids (McKellar and Benchaoui, 1996). *In vitro* bioassays have shown that moxidectin is 50- to 100-fold less toxic than ivermectin and abamectin to the sheep blowfly, *L. cuprina*, and about sixfold less potent than these two avermectins against sheep-biting lice (Rugg, 1995). This difference is clearly demonstrated in the effects of these compounds on dung feeding insects. An *in vitro* comparison of moxidectin and abamectin (Doherty *et al.*, 1994) found that abamectin was about 50-fold more toxic to larvae of a dung beetle (*O. gazella*) and buffalo fly than moxidectin when added to cattle feces. Rugg (1995) observed a similar result with both ivermectin and abamectin, which were at least an order of magnitude more potent than moxidectin to adults and larvae of a dung beetle and larvae of a dung-feeding fly (*T. brevicornis*). While ivermectin residues are generally considered to be toxic to some dung beetle larvae and fly larvae for up to 4 weeks after the treatment of cattle, there are differing reports for moxidectin. Strong and Wall (1994) found that moxidectin residues had no toxic effects on the larvae of beetles and cyclorrhaphous flies in cattle dung, and similarly, Fincher and Wang (1992) concluded that dung from moxidectin treated animals did not affect two species of dung beetles. However, Webb *et al.* (1991) reported that feces from cattle treated with a low, controlled-release dose of moxidectin were toxic to larvae of the face fly, *Musca autumnalis*, and concluded that the therapeutic injectable dose of moxidectin would be an effective control of *M. autumnalis* larvae in dung. Miller *et al.* (1994) reported significant mortality in horn fly larvae in dung for up to 28 days after oral or injectable treatment of cattle with moxidectin. Rank ordering of the negative effects of various avermectins and moxidectin on insect development in the dung of treated cattle was doramectin $>$ivermectin $\geq$ eprinomectin $\gg$ moxidectin (Floate *et al.*, 2001, 2002).

While moxidectin is generally less potent against insects than the avermectins in *in vitro* evaluations, it is commonly used at the same or similar therapeutic dose rates in production animals. Interestingly, the spectrum of insects controlled by these products is remarkably similar (Shoop *et al.*, 1995; McKellar and Benchaoui, 1996). The only macrocyclic lactones that have been specifically developed for primary insect control on animals are avermectins: ivermectin for blowfly and lice control on sheep (Eagleson *et al.*, 1993a, 1993b; Rugg *et al.*, 1993, 1995a, 1995b; Thompson *et al.*, 1994), and selamectin for flea control on cats and dogs (Benchaoui *et al.*, 2000; McTier *et al.*, 2000a, 2000b).

5.2.4.1.2. Acarids, copepods, and helminths Macrocyclic lactones are highly potent acaricides. Abamectin and natural milbemycins are widely used as agricultural miticides. Abamectin is highly effective against a broad spectrum of phytophagous mites and is one of the most potent acaricides with LC_{90} values ranging between 0.02 and 0.24 $\mu g\,ml^{-1}$ for mites in the families Tetranychidae, Eriophyidae, and Tarsonemidae (Lasota and Dybas, 1991). Eriophyid mites, such as the citrus rust mite, *Phyllocoptruta oleivora*, and various tetranychid mites, such as *T. urticae*, are among the most susceptible (Dybas, 1989). Other phytophagous mites, such as citrus red mite, *Panonychus citri* (LC_{90} = 0.24 $\mu g\,ml^{-1}$) are slightly more tolerant (Dybas, 1989). Similarly, ticks and parasitic mites are variably susceptible to the macrocyclic lactones. Much of the variation in potency against ticks relates to the different life cycle of ticks, the relatively slow action of these compounds against ticks, and the interactions with bioavailability of the compound in the host. These compounds tend to be much more effective against single host ticks; and are often considered relatively poor compounds against multihost ticks (McKellar and Benchaoui, 1996). In multihost ticks, adults are the infestive stage and are the least sensitive to macrocyclic lactones. Parasitic mites show similar variation: the deep burrowing sarcoptic mange mites that feed directly on blood and body fluids are highly sensitive, while chorioptic mange mites that live and feed on the superficial dermal surface are relatively insensitive (Benz *et al.*, 1989).

Macrocyclic lactones are also toxic to a number of parasitic copepods and ivermectin, emamectin benzoate, and doramectin have been used for control of parasitic sea lice in farmed salmon (Burka *et al.*, 1997; Davies and Rodger, 2000; Roth, 2000).

The macrocyclic lactones are highly potent nematicides, but have no useful activity against trematodes or cestodes. These compounds are especially toxic to the microfilarial stages of many filaroid nematodes (McKellar and Benchaoui, 1996). This extreme potency against microfilaria is best

demonstrated in the use of these compounds for heartworm control in dogs. Oral doses of 3–12 $\mu g\,kg^{-1}$ of moxidectin or ivermectin once a month provide effective protection against heartworm infection in dogs (Guerrero *et al.*, 2002). The oral use rate of these compounds for general nematode control in ruminants is about 200 $\mu g\,kg^{-1}$ (McKellar and Benchaoui, 1996).

5.2.4.2. Bioavailability

5.2.4.2.1. Crop protection uses Macrocyclic lactones are very susceptible to rapid photodegradation. The half-life of abamectin as a thin film under artificial light or simulated sunlight was 4–6 h and the rate of degradation of abamectin on petri dishes and on leaves was markedly greater in light than in dark environments (MacConnell *et al.*, 1989). The half-life of emamectin benzoate has been estimated to be 0.66 days on celery (Feely *et al.*, 1992) and is expected to be even shorter on cole crops. Photoirradiation of avermectins leads to the production a large number of decomposition products (Mrozik *et al.*, 1988), and a number of these photodegradates have also been identified for abamectin (Crouch *et al.*, 1991) and emamectin benzoate (Feely *et al.*, 1992). Many of these photodegradates show impressive insecticidal activity against larval lepidopterans (Argentine *et al.*, 2002a).

Despite the rapid degradation of avermectin insecticides in sunlight, significant amounts of these compounds are taken up rapidly via translaminar movement into sprayed foliage (Wright *et al.*, 1985; Dybas, 1989 and references therein). When abamectin was applied to plants held in the dark, the prolonged stability resulted in greater penetrability into leaves and more effective mite control (MacConnell *et al.*, 1989). Wright *et al.* (1985) demonstrated that this translaminar movement of abamectin produced good control of mites, but aphids were not controlled. They surmised that abamectin residues were probably abundant in the parenchyma tissues of leaves where mites feed, whereas abamectin probably did not distribute into the phloem tissue where aphids feed. The excellent residual efficacy of abamectin against several dipteran and lepidopteran leafminers is probably also due to translaminar movement and the presence of abamectin reservoirs in parenchyma tissue (Jansson and Dybas, 1998). This lack of true systemic activity (distribution into the phloem) in plants following spray application is demonstrated by the lack of residual efficacy against aphids. Spray applications of abamectin that had good contact efficacy provided little if any residual control of the aphids *Myzus persicae* (Johnson, 1985) and *A. fabae* (Green and Dybas, 1984). Similarly, the lack of downward transport of abamectin sprayed on foliage results in poor control of root knot nematodes (Stretton *et al.*, 1987; Cayrol *et al.*, 1993). By contrast, root dip, soil application or stem injections of avermectins have shown the potential to provide effective control of these parasites (Sasser *et al.*, 1982; Jansson and Rabatin, 1997, 1998).

5.2.4.2.2. Animal health uses In animal health, the high potency and lipophilic nature of the macrocyclic lactones allows extensive systemic delivery through a variety of routes. These compounds are used in oral, injectable, and topical formulations. The route of delivery, however, influences the bioavailability and distribution of the compounds in the host animal and thus the efficacy against different target parasites. Similarly, bioavailability can vary dramatically between species and be markedly affected by feed quantity (Ali and Hennessy, 1996) or composition (Taylor *et al.*, 1992) when orally administered. Efficacy against parasites may be dependent on transport in the plasma and distribution to vascular or nonvascular sites that constitute the parasite habitat. The pharmacokinetics of the macrocyclic lactones were reviewed by Hennessy and Alvinerie (2002).

Following most means of administration, macrocyclic lactones are eliminated fairly rapidly from the treated animals; elimination half-lives in various species range from about 3 to 10 days (see Hennessy and Alvinerie, 2002). Regardless of the means of administration or the species treated, the main route of elimination of the compounds is via feces with the compounds passing into the gastrointestinal tract in the bile (Hennessy and Alvinerie, 2002). A number of controlled release devices (Shoop and Soll, 2002) or sustained release formulations (Rock *et al.*, 2002; Shoop and Soll, 2002) using macrocyclic lactones have been used to provide long-term protection against parasites.

The most dramatic differences between application methods are seen with certain insect ectoparasites. While a number of blood-sucking parasites, such as sucking lice of cattle, are controlled by therapeutic doses of macrocyclic lactones administered orally or by injection, chewing lice are generally not susceptible. Topical applications (generally at about a 2.5-fold higher dose rate) are usually highly effective against chewing lice (Strong and Brown, 1987; Vercruysse and Rew, 2002b). Similar results have also been observed in lice on sheep (Coop *et al.*, 2002). While the efficacy and spectrum of these topical formulations against internal parasites is similar to the oral or injectable treatments, this

greater efficacy against chewing lice is thought to be a result of a high concentration of the active ingredient on the surface of the skin where these parasites feed (Titchener *et al*., 1994). Similarly, most of the macrocyclic lactone pour-on formulations for cattle have demonstrated a persistent efficacy against the blood-feeding horn fly and buffalo fly that is not seen with oral or injectable formulations (Vercruysse and Rew, 2002b).

This apparent greater efficacy of pour-on formulations against external parasites is presumably the result of deposition of the active compound on, and in, the skin. The high lipophilicity of the macrocyclic lactones is likely to result in the formation of depots of active ingredient in skin lipids and oil and fat secretions. This characteristic has been exploited in topical formulations of ivermectin that provide long-term residual control of blowfly and lice on sheep (Eagleson *et al*., 1993a, 1993b; Thompson *et al*., 1994), and selamectin which controls fleas on dogs and cats for at least 1 month (Benchaoui *et al*., 2000). Even greatly exaggerated oral or injectable doses of macrocyclic lactones are considered unlikely to provide residual control of fleas (Zakson-Aiken *et al*., 2001). In sheep, topically applied ivermectin is thought to bind to skin lipids and secretions and may be passively distributed around the sheep's body in this medium following application to a discrete site (Rugg and Thompson, 1997). Selamectin is thought to selectively partition into sebaceous glands of dogs and cats from where it is slowly released to the skin over an extended period (Hennessy and Alvinerie, 2002).

5.2.4.3. Safety and Selectivity

5.2.4.3.1. Crop protection uses Abamectin is generally considered to be nonphytotoxic under normal use even in sensitive ornamental plants (Wislocki *et al*., 1989), although mild foliar spotting has been noted in some ferns, daisies, and carnations (Dybas, 1989). Phytotoxicity was not affected by the addition of spray oils (Green *et al*., 1985), although, the addition of spray oils to abamectin has produced epidermal damage in pears that is not seen with the use of abamectin alone (Hilton *et al*., 1992).

Integrated pest management (IPM) involves the use of all available means (chemical, biological, cultural, physical, etc.) to achieve effective control of pests or pest damage. Central to the design of IPM programs are selective insecticides that reduce pest abundance and yet are innocuous to beneficial predators or parasites by allowing their survival and range expansion. The suitability of pesticides for use in IPM programs is based on the differential toxicity of a compound to pest and beneficial arthropod populations. Differential toxicity can occur through pharmacokinetic or metabolic differences between pests and beneficial organisms (physiological selectivity) or by a compound's unique qualities that result in toxic exposure of phytophagous pests with concomitant reduced exposure of beneficial organisms (ecological selectivity). The macrocyclic lactone insecticides possess both of these qualities, thereby making them highly selective and compatible with IPM.

The ecological selectivity of the avermectins and milbemycins results from a number of qualities of these compounds following application in the field. As noted earlier, avermectin insecticides are readily taken up by plant foliage. Foliar uptake via translaminar movement results in a reservoir of the toxicant inside the leaf (MacConnell *et al*., 1989). Any remaining compound on the outside of foliage is rapidly degraded by sunlight, thereby resulting in minimal residues of the compound on the plant surface soon after application. These qualities of the avermectin insecticides that result in the selective availability of the compounds to phytophagous pests and reduced exposure to nonphytophagous organisms are the main reasons for their compatibility with beneficial arthropods and IPM programs.

A number of researchers have shown that applications of abamectin did not disrupt the *Liriomyza* leafminer–parasitoid complex on a variety of vegetable crops (see Dybas, 1989). Trumble and Alvarado Rodriguez (1993) demonstrated that because of the reduced toxicity of abamectin to natural enemies, it was an important component of a multiple-pest IPM program on fresh market tomato that was based on intensive sampling, parasitoid releases, mating disruption, microbial pesticides, and abamectin. Abamectin has also been shown to be less detrimental to certain life stages of two leafminer parasitoids, *Diglyphus intermedius* and *Neochrysocharis punctiventris*, compared with other commonly used insecticides (Schuster, 1994).

The differential toxicity of abamectin to a variety of pest and predatory mite species is also well documented (see Lasota and Dybas, 1991). Hoy and Cave (1985) showed that although abamectin was toxic to the predator *Metaseiulus occidentalis*, it was more toxic to the two-spotted spider mite, *T. urticae*. Similar results have been found with other predatory mite species (Grafton-Cardwell and Hoy, 1983; Zhang and Sanderson, 1990). Hoy and Cave (1985) also showed that 2–4 day field-aged residues of abamectin were safe for *M. occidentalis*. Several other studies demonstrated that abamectin was less toxic to naturally occurring

and introduced natural enemies of pest mites, and for this reason, it selectively kills target pests and conserves their natural enemies (Dybas, 1989; Lasota and Dybas, 1991).

Zchorifein *et al.* (1994) found that abamectin was compatible with *Encarsia formosa*, an important component of IPM programs for greenhouse whitefly, *Trialeurodes vaporariorum*. No mortality was observed when adult parasitoids were exposed to 24 h residues of abamectin. They found that a management program based on the combined treatment of abamectin with releases of *E. formosa* maintained lower densities of the whitefly on poinsettia throughout the season and required fewer applications of abamectin compared with a management program based on applications of abamectin alone. Similarly, Brunner *et al.* (2001) found that while abamectin was highly toxic when applied topically to two parasitoids of leafrollers, 1-day-old residues were not toxic.

Like abamectin, emamectin benzoate is less toxic to beneficial arthropods such as honeybees, parasitoids, and predators. Kok *et al.* (1996) reported that emamectin hydrochloride had minimal adverse effects on two hymenopterous parasitoids (*Pteromalus puparum* and *Cotesia orobenae*) of Lepidoptera on broccoli. Contact activity of emamectin benzoate residues on alfalfa against two species of bees declined rapidly and was negligible 24 h after treatment (Chukwudebe *et al.*, 1997). They determined that bee mortality was directly related to transient dislodgeable residues and thus these beneficial insects could be expected to survive and colonize treated crops within relatively short intervals after applications of emamectin benzoate. Sechser *et al.* (2003) found that emamectin benzoate was relatively safe to a number of insect predators of sucking pests on cotton.

The selectivity of abamectin has been widely investigated in mites. Trumble and Morse (1993) showed that abamectin was compatible with *Phytoseiulus persimilis* in field-grown strawberry. They found that the highest net economic return was achieved when several releases of the predatory mite *P. persimilis* were integrated with suitably timed applications of abamectin. In a laboratory leaf-disc bioassay, adult females of the predatory mite *Amblyseius womersleyi* dipped in abamectin solution showed low mortality (16.6%), while all *T. urticae* females died within 24 h after dipping (Park *et al.*, 1996). In apple, abamectin was highly compatible with the phytoseiid mites *M. occidentalis* and *Amblyseius fallacis*. Abamectin was effective at reducing and maintaining numbers of European red mites (*Panonychus ulmi*) at or below the economic threshold for at least 3 weeks after application. Although predatory mite numbers declined initially, they quickly resurged resulting in favorable predator to prey ratios that were comparable or superior to those found in nontreated plots, and aided in mite suppression for the remainder of the growing season (Jansson and Dybas, 1998).

In glasshouse roses, Sanderson and Zhang (1995) found that full canopy applications of abamectin at 3–5 day spray intervals effectively controlled *T. urticae* populations, but were detrimental to the predatory mite *P. persimilis*. However, by applying abamectin to the upper canopy of plants only, excellent mite control in the marketable portion of the plant was achieved and predatory mite populations in the lower canopy were conserved. This demonstrated the potential for integrating abamectin with natural predators for control of a target pest, even under intense abamectin pressure.

5.2.4.3.2. Animal health uses The macrocyclic lactones are generally considered to be relatively safe in mammals. Glutamate-gated chloride channels have not been reported in mammals. Clinical signs are associated with neurotoxicity of the central nervous system as this is the site of GABA-gated chloride channels in mammals. The poor distribution of these compounds through the blood–brain barrier and/or their rapid removal by the transmembrane protein, P-glycoprotein, are thought be responsible for their lack of toxicity to mammals (Shoop and Soll, 2002; Abu-Qare *et al.*, 2003). Increased susceptibility to avermectins has been observed in Murray Grey cattle (Seaman *et al.*, 1987) and in some collie-breed dogs (Paul *et al.*, 1987). It is thought that a deficiency of P-glycoprotein in these animals results in increased accumulation of avermectins in the central nervous system and the resultant neurotoxicity (Shoop and Soll, 2002; Roulet *et al.*, 2003).

In animal health, nontarget effects of macrocyclic lactones are generally minimized due to their main use as systemic parasiticides. The compounds are applied discretely to the target host and not released into the environment. The major nontarget effects in animal health uses are on the insect fauna of dung of treated animals. Regardless of the route of administration, the major means of elimination of these compounds from animals is via the feces and the major component in feces is usually the parent compound (Campbell *et al.*, 1983; Chiu and Lu, 1989). Feed-through compounds for the control of pestiferous Diptera in dung have been in use, especially in cattle, for many years, yet little attention has been paid to the effects on nontarget dung

fauna (Strong, 1992). Similarly, most of the compounds used as external parasiticides for cattle are toxic to dung insects, but organophosphorus and pyrethroid compounds are more toxic to dung-inhabiting insects than avermectins (Bianchin *et al.*, 1992). However, there has been a great deal of attention given to possible environmental consequences of systemic parasiticides, especially avermectins and related compounds, with entire journal volumes (e.g., *Veterinary Parasitology* 48, 1993) and dedicated regulatory or scientific workshops (National Registration Authority, 1998; Alexander and Wardhaugh, 2001) devoted to this subject.

This rise in interest is probably related to the widespread use of these compounds. Their broad spectrum, encompassing both nematodes and arthropods, has resulted in their extensive use, and especially where gastrointestinal worms are the prime target, most arthropods could be considered nontarget organisms. Certainly, in countries where the introduction of dung beetles to control dung build-up and dung breeding pests have occurred, any compounds with adverse effects on these beneficial agents will gain some attention. Also, the differing toxicities of related compounds marketed by different companies may be perceived as providing a commercial advantage. Hence, authors have reported the effects of ivermectin in controlling dung-breeding pestiferous Diptera (e.g., Meyer *et al.*, 1980; Miller *et al.*, 1981; Marley *et al.*, 1993), or its toxicity to dung beetles (e.g., Ridsdill-Smith, 1988; Sommer and Overgaard Nielsen, 1992; Sommer *et al.*, 1992, 1993), and that dung degradation may be retarded (e.g., Wall and Strong, 1987; Sommer *et al.*, 1992) or not affected (e.g., Jacobs *et al.*, 1988; McKeand *et al.*, 1988; Wratten *et al.*, 1993). Other reports have appeared directly comparing the effects of avermectins and moxidectin on dung fauna (Fincher and Wang, 1992; Doherty *et al.*, 1994; Floate *et al.*, 2001, 2002).

There is little doubt that avermectins and related compounds have the potential to affect dung-inhabiting insects. Moxidectin has little if any impact on dung insects, but also is less efficacious against pestiferous fly larvae. The selection of these compounds for certain animal health uses should be evaluated on the basis of their efficacy against pest target and on concomitant environmental concerns. Thus, for example, ivermectin use in a controlled-release device for sheep provides added protection against breech strike (Rugg *et al.*, 1998a), and residues in dung likely reduce populations of the adult blowfly population (Cook, 1991, 1993), while exerting little impact on the rate of degradation of sheep dung where insects play a relatively minor role (Cook, 1993; King, 1993). Where there are concerns over possible adverse effects of treatment for gastrointestinal worm infestation on cattle dung fauna, moxidectin would be the compound of choice (Strong and Wall, 1994). If control of arthropod ectoparasites or their dung-feeding stages is of importance (Marley *et al.*, 1993), an avermectin may be preferred because of its greater toxicity to insects. Even so, a study examining the effects of commercial ivermectin use in cattle in South Africa (Scholtz and Krüger, 1995) found that dung insect populations recovered rapidly following an initial depression. They concluded that the long-term effects on dung fauna might not be as severe as previous studies have claimed. However, simulation of the impact of eprinomectin on dung beetle populations using computer modeling suggests that, in the absence of immigration, a single treatment of eprinomectin could reduce beetle activity by 25–35% in the next generation (Wardhaugh *et al.*, 2001). In studies directly comparing the effects of macrocyclic lactones in cattle dung, avermectins result in lethal and sublethal effects on dung fauna in feces excreted for a number of weeks after treatment, while moxidectin residues are relatively innocuous (Fincher and Wang, 1992; Doherty *et al.*, 1994; Floate *et al.*, 2001, 2002; Wardhaugh *et al.*, 2001).

5.2.5. Uses

5.2.5.1. Crop Protection

Abamectin is registered worldwide for control of mites and certain insect pests on a variety of ornamental and horticultural crops and on cotton. Abamectin is effective for controlling agromyzid leafminers, *Liriomyza* spp., Colorado potato beetle, *L. decemlineata*, diamond back moth, *P. xylostella*, tomato pinworm, *K. lycopersicella*, citrus leafminer, *Phyllocnistis citrella*, and pear psylla, *Cacopsylla pyricola*. Abamectin has also been incorporated into bait to control red imported fire ants and cockroaches. These and other urban pest applications were reviewed by Lasota and Dybas (1991).

Recent studies have shown that avermectins have potential as a component in Africanized honeybee abatement programs (Danka *et al.*, 1994). Another novel use is the injection of abamectin into trees which controls certain phytophagous arthropods, such as elm leaf beetle (*Pyrrhalta luteola*), for up to 83 days after application (Harrell and Pierce, 1994). The main commercial agricultural use of macrocyclic lactones is as acaricides. These compounds have demonstrated mite control and residual activity superior to most acaricides on a variety of ornamental crops, food crops, and cotton (Putter

et al., 1981; Dybas and Green, 1984; Dybas, 1989). Macrocyclic lactones are also highly effective for the control of plant parasitic nematodes following injection application (Jansson and Rabbatin, 1997, 1998; Takai *et al.*, 2003) or soil incorporation (Blackburn *et al.*, 1996). Emamectin benzoate, milbemectin, and nemadectin are used commercially for the control of pine wilt nematode in Japan.

Emamectin benzoate is used for broad-spectrum control of lepidopteran pests on certain vegetable crops. This compound is very effective against numerous lepidopteran pests of a variety of crops. It is expected that the compound will be used to control lepidopterous pests on a wide variety of vegetable, tree, and row crops and also for control of thrips on eggplant and tea in Japan (Jansson and Rabatin, 1997).

5.2.5.2. Animal and Human Health

The macrocyclic lactones have had a dramatic impact on animal health. Their potency and broad spectrum has resulted in this chemistry dominating the parasiticide market. Macrocyclic lactones have been commercialized for the control of nematodes and arthropod parasites for most common food-producing and companion animals and are widely used "off-label" for parasite control in many other species. Avermectins (e.g., emamectin) are also used for control of copepod parasites of farmed salmon (Davies and Rodger, 2000). In human health, ivermectin has been used since 1987 in a compassionate program in Africa and Central and South America to control *Onchocerca volvulus* which is the causative agent of river blindness in man (Shoop and Soll, 2002). The program was expanded recently to include the reduction of spread of elephantiasis caused by lymphatic filarid nematodes. Recently, moxidectin has been demonstrated to be safe and well tolerated in humans (Cotreau *et al.*, 2003). Moxidectin is more efficacious than ivermectin in *Onchocerca* models and is currently undergoing clinical trials prior to inclusion in filarid control programs (Molyneux *et al.*, 2003). Ivermectin has been approved for treatment of intestinal strongiloidosis scabies in humans, and is also useful for the treatment of louse infestations (Elgart and Meinking, 2003). These authors consider that there may be a number of additional uses for the macrocyclic lactones as antiparasitic medications for parasites of skin in humans.

5.2.6. Resistance

5.2.6.1. Insects

Resistance to the avermectins in insects has been reviewed by Clark *et al.* (1995). In general, a variety of biochemical and pharmacokinetic mechanisms may contribute to avermectin resistance in arthropods, although biochemical mechanisms tend to be more important among most arthropod systems studied to date. However, there are many conflicting reports on the importance of different resistance mechanisms. Similarly, cross resistance between avermectin insecticides/acaricides and other classes of chemistry is not well understood, and there are probably equal numbers of reports demonstrating cross-resistance as there are those refuting it.

Cross-resistance between abamectin and pyrethroids has been reported for field strains of houseflies (Scott, 1989; Geden *et al.*, 1990). Abro *et al.* (1988) proposed that there was a low level of cross resistance to abamectin in a field strain of diamondback moth that was highly resistant to DDT, malathion, and cypermethrin when assessed in a topical assay. However, the same population was equally susceptible to abamectin as the baseline colony in an ingestion bioassay. Roush and Wright (1986) found no evidence of cross-resistance to avermectins in houseflies resistant to diazinon, dieldrin, DDT, or permethrin, and Parella (1983) reported no cross-resistance to avermectins in a pyrethroid-resistant agromyzid fly. Campanhola and Plapp (1989) determined that there was no cross-resistance to abamectin in pyrethroid-resistant tobacco budworm, where both target site resistance and metabolic resistance to pyrethroids were present. Similarly, Cochran (1990) concluded that pyrethroid-resistant field populations of German cockroach were not cross-resistant to abamectin. Argentine and Clark (1990) examined a susceptible laboratory strain and a multiple-resistant field strain of Colorado potato beetle, and found no difference in their dose responses to abamectin. No cross-resistance to abamectin was observed in a laboratory-selected pyrethroid-resistant strain of citrus thrip, although cross-resistance was detected to DDT and some organophosphates and carbamates (Immaraju and Morse, 1990). Rugg and Thompson (1993) reported that pyrethroid-resistant sheep biting lice (*B. ovis*) were not cross-resistant to ivermectin.

Beeman and Stuart (1990) examined a number of pesticides against a field-collected strain of red flour beetle (*Tribolium castaneum*) resistant to lindane that had been further selected with dieldrin in the laboratory. These workers found cross-resistance to other cyclodienes, but not to abamectin nor to organophosphates. Resistance to cyclodienes, accounted for by channel mutants in the Rdl GABA-gated chloride channel subunit (review: ffrench-Constant, 1994), usually extends to all insecticides that block chloride ion channels, and this resistance is not

conferred to avermectins as these activate chloride channels and therefore act at a separate site (Bloomquist, 1993). Rohrer *et al.* (1995) showed that fipronil, which interacts with GABA-gated chloride channels (as an antagonist) did not affect ivermectin binding either in *Drosophila* head membranes or in locust neuronal membranes.

Ismail and Wright (1991) examined the toxicities of a number of insect growth regulators, and abamectin, to a susceptible strain of diamondback moth and to strains selected for resistance to the growth regulators chlorfluazuron and teflubenzuron. They found that cross-resistance to other growth regulators was variable and did not detect cross-resistance to abamectin. Recently, Zhao *et al.* (2002) showed that the resistance to spinosad in a field population of diamondback moth was not cross-resistant to emamectin benzoate.

Scott (1989) proposed that cross-resistance to abamectin in pyrethroid-resistant houseflies was polygenic and due to decreased cuticular penetration and enhanced metabolism mediated by mixed-function oxidases. Subsequently, Scott *et al.* (1991) reported laboratory selection of high-level abamectin resistance in these field-collected houseflies; after seven selections resistance ratios of >60 000-fold (determined by topical application) and 36-fold (determined by residual exposure) were reached. In their study the synergists piperonyl butoxide, diethyl maleate, and *S*,*S*,*S*-tributylphosphorotrithioate did not affect the toxicity of abamectin, indicating that resistance was not due to enhanced metabolism mediated by mixed function oxidases, glutathione transferases, or hydrolases. Konno and Scott (1991) found that resistance in this selected strain resulted from decreased cuticular penetration (>1700-fold) and altered abamectin binding (35-fold). This strain was also cross-resistant to two abamectin analogs and resistance was highly recessive.

Laboratory selection of abamectin against two strains of Colorado potato beetle resulted in resistance levels of 21-fold for a mutagen-induced resistance and 38-fold by direct selection. Both resistance factors were incompletely dominant and polyfactorial (Argentine and Clark, 1990). Oxidative metabolism and possibly carboxylesterase activity were responsible, in part, for resistance to abamectin (Argentine, 1991; Argentine *et al*., 1992). Lamine (1994) found that abamectin-resistant larvae of *L. decemlineata* were seven- to tenfold less susceptible to emamectin benzoate than the baseline colony in a topical bioassay. No cross-resistance between abamectin and emamectin benzoate was evident in adult beetles. Recent studies showed that *L. decemlineata* populations which were 15- to 23-fold less sensitive to abamectin in a contact, topical bioassay (Clark *et al*., 1995) were classified as susceptible to abamectin in a diet-based ingestion assay (Dively and Jansson, 1996). Recently, oxidative metabolism was confirmed as a principal resistance mechanism in laboratory-selected, abamectin-resistant Colorado potato beetle (Yoon *et al*., 2002; Gouamene-Lamine *et al*., 2003).

Hughes and Levot (1990) reported that a laboratory-selected strain of *L. cuprina* resistant to organophosphates and carbamates exhibited a low level cross-resistance (two- to threefold) to abamectin and ivermectin when compared to susceptible flies. However, emamectin benzoate was equally toxic to both strains. This strain also exhibited a low level of cross-resistance to pyrethroids (Sales *et al*., 1989). A laboratory-selected pyrethroid-resistant strain of *L. cuprina* that showed cross-resistance between an organophosphate and a carbamate insecticide (Sales *et al*., 1989) was subsequently selected with deltamethrin, diflubenzuron, and butacarb resulting in levels of resistance in excess of 1000-fold to all three compounds (Kotze and Sales, 1994). These workers also found that metabolic inhibitors significantly synergized the three selected strains, indicating the involvement of both monooxygenases (mixed-function oxidases) and esterases in these resistances. Rugg *et al.* (1995a) showed that the same pyrethroid- and carbamate-resistant laboratory strain of *L. cuprina* was not cross-resistant to ivermectin. Organophosphate-resistant field strains of *L. cuprina* have also been shown to have no cross-resistance to avermectins (Hughes and Levot, 1990; Rugg *et al*., 1998b).

Rugg *et al.* (1998b) selected larvae of *L. cuprina* with ivermectin for over 60 generations and achieved an eightfold increase in tolerance compared to the parental strain. This low-level resistance reverted rapidly in the absence of selection pressure, and although enzymatic metabolism was implicated, no specific resistance mechanisms were determined. Kotze (1995) found that the induction of monooxygenase and glutathione transferase enzymes with phenobarbital in insecticide-susceptible *L. cuprina* larvae resulted in increased tolerance to butacarb, diazinon, and diflubenzuron. In similar experiments, Rugg *et al.* (1998a) found no increase in tolerance to ivermectin in induced flies that had reduced susceptibility to diazinon.

Resistant field populations of *P. xylostella* from Malaysia were not cross-resistant to *Bacillus thuringiensis* (Iqbal *et al*., 1996). Lasota *et al.* (1996) showed that there was no cross-resistance between abamectin, emamectin benzoate, permethrin, and

methomyl in *P. xylostella* using an ingestion bioassay. Following laboratory selection of a field population of *P. xylostella* from China, testing of F_1 progeny from reciprocal crosses between abamectin-resistant and abamectin-susceptible strains indicated that resistance was autosomal and incompletely recessive and backcross studies suggested that the resistance was probably controlled by more than one gene (Liang *et al.*, 2003). Further, there was little cross-resistance between abamectin and four pyrethroids (deltamethrin, β-cypermethrin, fenvalerate, and bifenthrin) and no cross-resistance between abamectin and the acylureas chlorfluazuron or flufenoxuron.

Morse and Brawner (1986) reported LC_{50} values ranging from 7 to 21 mg l^{-1} in strains of citrus thrips that had not been exposed to abamectin. Immaraju and Morse (1990) retested one of these strains that had been maintained in the laboratory and also selected for resistance to fluvalinate, and determined LC_{50} values of 0.9 and 0.4 mg ml^{-1}, respectively. Responses to abamectin in nonexposed populations varied by over 50-fold suggesting that thrips may have a large inherent variation in susceptibility to abamectin. Immaraju *et al.* (1992) reported the occurrence of two abamectin-resistant populations of western flower thrips in Californian glasshouses. Resistance ratios (LC_{90}) of 18- and 798-fold were attributed to intensive use of abamectin during the previous year (five or ten applications, respectively).

In summary, resistance to avermectins has been demonstrated in a number of insects. In all instances, although not clearly determined, resistance is believed to be polygenic, relatively slow to develop, and able to revert rapidly in the absence of selection pressure. Metabolic mechanisms seem to be the most important route of resistance in the arthropod systems studied to date, and in all cases, a number of biochemical and sometimes physiological mechanisms are implicated. Cross-resistance between avermectins and other insecticide classes is similarly ill-defined; however, it is apparent that there is little significant cross-resistance to avermectins except possibly in one strain of housefly (Scott, 1989). The variations in responses of field strains resistant to other insecticide classes to avermectins may in some cases be due to enhanced metabolism resulting from previous exposure to other insecticides, although there is evidence to both support and refute this supposition. A number of species have also demonstrated inherent population differences in response to avermectins without the influence of any predisposing factors. A wide variation among field-collected strains has been seen in several insects which does not constitute cross-resistance, nor does it indicate that this variation predisposes insects to an increased likelihood for resistance development to avermectins.

Cross-resistance between the avermectins and milbemycins (specifically abamectin, ivermectin, doramectin, and moxidectin) has been demonstrated in a number of nematode species. Because abamectin is the only avermectin compound used widely in agriculture, there have been a few studies conducted to examine cross-resistance within the class. Sensitivity to emamectin benzoate in select abamectin-resistant strains has been assessed in several insects (e.g., *L. trifolii, L. decemlineata*, *P. xylostella*) and only minimal, if any, cross-resistance has been detected (Jansson and Dybas, 1998; Jansson and Rugg, unpublished data). The reasons for the lack of cross-resistance to these closely related compounds are unclear. It is possible that the changes in the derivatization of emamectin benzoate may result in the loss or protection of the chemical bonds that are subject to enzymatic cleavage in abamectin resistance. Additionally, because macrocyclic lactones have multiple sites of action and may affect a number of different receptors to activate or potentiate chloride ion influx, subtle differences in chemistry between the different avermectins and milbemycins may alter the relative activities of compounds at the different sites. This could affect the patterns and levels of cross-resistance within the class and also be a factor in the subtle differences seen in the spectra and potencies of these compounds.

5.2.6.2. Acarids

Laboratory selection of up to 15 generations of two species of tetranychid mites with abamectin resulted in no significant increase in resistance compared with a nonselected baseline colony (Hoy and Conley, 1987). Conversely, 20 rounds of selection against a predatory phytoseiid mite resulted in a gradual and modest shift in susceptibility to abamectin (Hoy and Ouyang, 1989). A recent survey of field populations of two-spotted spider mite in California (Campos *et al.*, 1995) found that mites varied widely in their susceptibility to abamectin, with up to a 658-fold difference in susceptibility in a residual exposure assay. Levels of resistance were correlated with the amount and time of abamectin usage, and laboratory selection of a single population over 38 generations resulted in more than a 100-fold increase in resistance. Despite the higher level of resistance in a residual assay, selected mites were still considered susceptible to abamectin in a direct contact bioassay. Further, despite the wide variation in responses among field populations of mites, no field failures of the product were reported.

In *T. urticae*, resistance to abamectin has been attributed to increased excretion and decreased absorption (Clark *et al*., 1995), monooxygenases (Campos *et al*., 1996), esterases (Campos *et al*., 1996, 1997; Jansson *et al*., unpublished data), glutathione-*S*-transferases (Clark *et al*., 1995), and decreased penetration (Clark *et al*., 1995). Levels of resistance in two spotted spider mite populations collected from the field were shown to drop markedly within 2–6 weeks after collection when maintained in the absence of selection pressure (Campos *et al*., 1995; Jansson *et al*., unpublished data). Bergh *et al*. (1999) reported wide variation in susceptibility to abamectin in citrus rust mite (*P. oleivora*) field populations with no clear relationship between susceptibility and product efficacy. While some populations from commercial groves with a history of exposure to abamectin were less susceptible to abamectin, mortality was only slightly lower than that of a pristine population. Also bioassay results indicated that the responses of these field populations did not change whether they were assessed before or after abamectin spray applications.

5.2.6.3. Helminths

Ivermectin resistance was initially detected in nematodes of sheep and goats, and was generally a result of intensive use over a number of years (Shoop, 1993). Ivermectin-resistant nematodes have been shown to have cross-resistance to other avermectins and milbemycins (Shoop *et al*., 1993). However, moxidectin was significantly more toxic to many parasitic nematodes (Shoop, 1993; Shoop *et al*., 1993; Kieran, 1994) and this difference in potency has resulted in moxidectin providing economic control of ivermectin-resistant parasites in field use situations. Conder *et al*. (1993) showed that the modes of action of ivermectin and moxidectin were qualitatively similar by examining changes in membrane conductance in crab muscle fibers. These workers also demonstrated cross-resistance to moxidectin in an ivermectin-resistant strain of *H. contortus* and confirmed that moxidectin was about fourfold more potent than ivermectin against this parasite in a jird model. Recently, Ranjan *et al*. (2002) reported the results of concurrent selection of *H. contortus* for 22 generations with ivermectin and moxidectin. These workers found that worms that had developed resistance through selection with either of these macrocyclic lactones were cross-resistant to the other compound. However, the rate of development of resistance differed between the two compounds and occurred more slowly with moxidectin, which was also markedly more potent than ivermectin. See Pritchard (2002) for the most recent, detailed review of the status of resistance to macrocyclic lactones in nematodes. Briefly, resistance is still a major concern for parasites of sheep and has also been detected in some cattle parasites. Resistance has been found to be associated with enhanced P-glycoprotein expression resulting in increased efflux from the target site, but there are thought to be a number of possible resistance mechanisms including various amino acid-gated anion subunit genes, genes associated with glutamate receptors and transporters, and genes involved in amphid structure and function. The multiplicity of possible sites for macrocyclic lactone binding and activity and their variable sensitivity to different compounds may be responsible for the differential potencies seen within and between avermectins and milbemycins and suggests that products of several genes may be involved in the mechanism of action of macrocyclic lactones. Similarly, these subtle differences in the ways in which ivermectin and moxidectin act at the molecular level could result in the differences seen between these compounds in potency, rates of development of resistance, and levels of cross-resistance.

5.2.7. Summary

The macrocyclic lactones are potent insecticides, nematicides, and acaricides. The class consists of two closely related groups of compounds, the avermectins and milbemycins, which are natural fermentation products or synthetic derivatives of natural products derived from actinomycetes. The broad spectrum of activity against a variety of insects, acarids, and nematodes has led to the widespread use of these compounds in animal and human health, crop protection, and urban pest control. Ivermectin has been the most successful antiparasitic drug introduced in animal health and abamectin has been the premier agricultural miticide in recent years.

Macrocyclic lactones affect invertebrates by either directly activating receptors for the neurotransmitters glutamate and GABA, or by potentiating their actions, resulting in an influx of chloride ions into nerve cells and muscle. It is currently believed that the anthelminthic properties of the avermectins are due predominantly to potentiation and/or direct opening of glutamate-gated chloride channels, whereas in insects, it is likely that avermectins bind to multiple sites, including glutamate and GABA-gated chloride channels as well as other insect chloride channels (Sattelle, 1990;

Bloomquist, 1993). The chloride ion flux resulting from the opening by these compounds of chloride channels in invertebrate nerve and muscle, particularly those gated by glutamate and GABA, results in disruption of activity and loss of function in these excitable cells and accounts for the potent actions of these molecules (review: Raymond and Sattelle, 2002). Toxicity is generally greater through ingestion than by residual contact and compared with other neurotoxic agents, macrocyclic lactones are considered to be relatively slow acting. Intoxicated organisms usually die slowly, often over a period of days, and there is no quick knockdown effect. Death usually follows paralysis and immobility. The comparatively poor contact activity of these compounds plus their short environmental persistence generally results in good compatibility with beneficial and nontarget organisms. These compounds have low relative toxicities to vertebrates and are not phytotoxic.

Resistance to avermectins has been demonstrated in a number of arthropods. In most instances, although not clearly determined, resistance is apparently polygenic, develops relatively slowly, and tends to revert rapidly in the absence of selection pressure. Metabolic resistance mechanisms seem to be the most important in the arthropod systems studied to date, and in all these cases a number of biochemical and physiological mechanisms are implicated. Cross-resistance between macrocyclic lactones and other insecticide classes is similarly ill-defined; however, it is apparent that there is little significant cross-resistance. The variations in responses of field strains resistant to other insecticide classes to avermectins may in some cases be due to enhanced metabolism resulting from previous exposure to other insecticides. A number of species have demonstrated wide inherent variation in response to avermectins without the influence of any predisposing factors. This natural variation in susceptibility does not constitute cross-resistance, nor is there evidence to indicate that this variation results in predisposition to develop increased resistance to avermectins.

Despite the widespread, intensive use of abamectin against a number of pests with a high propensity to develop resistance, development of resistance has been relatively slow and rare. The rapid reversion of resistance in the absence of selection pressure and the lack of any confirmed cases of cross-resistance make them amenable for use in resistance management programs. However, in order to prolong the longevity of these compounds in the market place, it will be necessary to minimize selection pressure in practice, especially in high use situations, through proper rotation, good pest management practices, and proper resistance management strategies.

References

Abalis, I.M., Eldefrawi, A.T., **1986**. [^{3}H]muscimol binding to a putative GABA receptor in honey bee brain and its interaction with avermectin B_{1a}. *Pestic. Biochem. Physiol. 25*, 279–287.

Abro, G.H., Dybas, R.A., Green, A.St.J., Wright, D.J., **1988**. Toxicity of avermectin B1 against a susceptible laboratory strain and an insecticide-resistant strain of *Plutella xylostella* (Lepidoptera: Plutellidae). *J. Econ. Entomol. 81*, 1575–1580.

Abu-Qare, A.W., Elmasry, E., Abou-Donia, M.B., **2003**. A role for P-glycoprotein in environmental toxicology. *J. Toxicol. Environ. Health 6*, 279–288.

Agee, H.R., **1985**. Neurobiology of the bollworm moth: effects of abamectin on neuronal activity and on sensory systems. *J. Agric. Entomol. 2*, 325–336.

Alexander, M., Wardhaugh, K., **2001**. Workshop on the effects of parasiticides on dung beetles. *CSIRO Entomol. Tech. Rep. 89*, 1–37.

Ali, D.N., Hennessy, D.R., **1996**. The effect of level of feed intake on the pharmacokinetic distribution, excretion and biotransformation of ivermectin in sheep. *J. Vet. Pharmacol. Therap. 19*, 89–94.

Allingham, P.G., Kemp, D.H., Thompson, D.R., Rugg, D., **1994**. Effect of ivermectin on three populations and a laboratory strain of *Haematobia irritans exigua* (Diptera: Muscidae). *J. Econ. Entomol. 87*, 573–576.

Anderson, T.E., Babu, J.R., Dybas, R.A., Mehta, H., **1986**. Avermectin B_1: ingestion and contact toxicity against *Spodoptera eridania* and *H. virescens* (Lepidoptera: Noctuidae). *J. Econ. Entomol. 79*, 197–201.

Arena, J.P., Liu, K.K., Paress, P.S., Frazier, E.G., Cully, D.F., *et al.*, **1995**. The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. *J. Parasitol. 81*, 286–294.

Arena, J.P., Liu, K.K., Paress, P.S., Schaeffer, J.M., Cully, D.F., **1992**. Expression of a glutamate-activated chloride current in *Xenopus* oocytes injected with *Caenorhabditis elegans* RNA: evidence for modulation by avermectin. *Brain Res. Mol. Brain Res. 15*, 339–348.

Arena, J.P., Whiting, P.J., Liu, K.K., McGurk, J.F., Paress, P.S., *et al.*, **1993**. Avermectins potentiate GABA-sensitive currents in *Xenopus* oocytes expressing cloned $GABA_A$ receptors. *Biophys. J. 64*, 325.

Argentine, J., **1991**. Two abamectin-resistant strains of Colorado potato beetle. *Resist. Pest Mgt 3*, 30–31.

Argentine, J.A., Clark, J.M., **1990**. Selection for abamectin resistance in Colorado potato beetle (Coleoptera: Chrysomelidae). *Pestic. Sci. 28*, 17–24.

Argentine, J.A., Clark, J.M., Lin, H., **1992**. Genetics and biochemical mechanisms of abamectin resistance in two isogenic strains of Colorado potato beetle. *Pestic. Biochem. Physiol. 44*, 191–207.

Argentine, J.A., Jansson, R.K., Starner, V.R., Halliday, W.R., **2002a**. Toxicities of emamectin benzoate homologues and photodegradates to Lepidoptera. *J. Econ. Entomol. 95*, 1185–1189.

Argentine, J.A., Jansson, R.K., Halliday, W.R., Rugg, D., Jany, C.S., **2002b**. Potency, spectrum and residual activity of four new insecticides under glasshouse conditions. *Fla. Entomol. 85*, 552–562.

Baker, T.C., van Vorhis Key, S.E., Gaston, L.K., **1985**. Bait-preference tests for the Argentine ant (Hymenoptera: Formicidae). *J. Econ. Entomol. 78*, 1083–1088.

Ballivet, M., Alliod, C., Bertrand, S., Bertrand, D., **1996**. Nicotinic acetylcholine receptors in the nematode *Caenorhabditis elegans*. *J. Mol. Biol. 258*, 261–269.

Banks, B.J., Bishop, B.F., Evans, N.A., Gibson, S.P., Goudie, A.C., *et al.*, **2000**. Avermectins and flea control: structure activity relationships and the selection of selamectin for development as an endectocide for companion animals. *Bioorg. Med. Chem. 8*, 2017–2025.

Beeman, R.W., Stuart, J.J., **1990**. A gene for lindane + cyclodiene resistance in the red flour beetle (Coleoptera: Tenebrionidae). *J. Econ. Entomol. 83*, 1745–1751.

Benchaoui, H.A., Clemence, R.G., Clements, P.J.M., Jones, R.I., Watson, P., *et al.*, **2000**. Efficacy and safety of selamectin against fleas on dogs and cats presented as veterinary patients in Europe. *Vet. Parasitol. 91*, 223–232.

Benz, G.W., Roncalli, R.A., Gross, S.J., **1989**. Use of ivermectin in cattle, sheep, goats and swine. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 215–229.

Bergh, J.C., Rugg, D., Jansson, R.K., McCoy, C.W., Robertson, J.L., **1999**. Monitoring the susceptibility of citrus rust mite (Acari: Eriophyidae) populations to abamectin. *J. Econ. Entomol. 92*, 781–787.

Bermudez, I., Hawkins, C.A., Taylor, A.M., Beadle, D.J., **1991**. Actions of insecticides on the insect GABA receptor complex. *J. Recept. Res. 11*, 221–232.

Bianchin, I., Honer, M.R., Gomes, A., Koller, W.W., **1992**. Effect of some antitick compounds/insecticides on *Onthophagus gazella*. *EMBRAPA Comunicado Technico 45*, 1–7.

Biddinger, D.J., Hull, L.A., **1999**. Sublethal effects of selected insecticides on growth and reproduction of a laboratory susceptible strain of tufted apple bud moth (Lepidoptera: Tortricidae). *J. Econ. Entomol. 92*, 314–324.

Bishop, B.F., Bruce, C.I., Evans, N.A., Goudie, A.C., Gration, K.A.F., *et al.*, **2000**. Selamectin: a novel broad-spectrum endectocide for dogs and cats. *Vet. Parasitol. 91*, 163–176.

Blackburn, K., Alm, S.R., Yeh, T.S., **1996**. Avermectin B_1, isazofos, and fenamiphos for control of *Hopolaimus galeatus* and *Tylenchorhynchus dubius* infesting *Poa annua*. *J. Nematol. 28*(Suppl.), 687–694.

Bloomquist, J.R., **1993**. Toxicology, mode of action and target site-mediated resistance to insecticides acting on chloride channels. *Comp. Biochem. Physiol. 106*, 301–314.

Bloomquist, J.R., **1994**. Cyclodiene resistance at the insect GABA receptor/chloride channel complex confers broad cross resistance to convulsants and experimental phenylpyrazole insecticides. *Arch. Insect Biochem. Physiol. 26*, 69–79.

Bornemissza, G.F., **1970**. Insectary studies on the control of dung breeding flies by the activity of the dung beetle, *Onthophagus gazella* F. (Coleoptera: Scarabaeinae). *J. Austral. Entomol. Soc. 9*, 31–41.

Brunner, J.F., Dunley, J.E., Doerr, M.D., Beers, E.H., **2001**. Effect of pesticides on *Colpoclypeus florus* (Hymenoptera: Eulophidae) and *Trichogramma platneri* (Hymenoptera: Trichogrammatidae), parasitoids of leafrollers in Washington. *J. Econ. Entomol. 94*, 1075–1084.

Bull, D.L., **1986**. Toxicity and pharmacodynamics of avermectin in the tobacco budworm, corn earworm, and fall armyworm (Noctuidae: Lepidoptera). *J. Agric. Food Biochem. 34*, 74–78.

Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, S.A., *et al.*, **1979**. Avermectins, new family of potent antihelminthic agents: producing organism and fermentation. *Antimicrob. Agents Chemother. 15*, 361–367.

Burka, J.F., Hammell, K.L., Horsberg, T.E., Johnsons, G.R., Rainnie, D.J., *et al.*, **1997**. Drugs in salmonid aquaculture: a review. *J. Vet. Pharmacol. Therap. 20*, 333–349.

Campanhola, C., Plapp, F.W., Jr., **1989**. Toxicity and synergism of insecticides against susceptible and pyrethroid-resistant neonate larvae and adults of the tobacco budworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 82*, 1527–1533.

Campbell, W.C. (Ed.) **1989**. Ivermectin and Abamectin. Springer-Verlag, New York.

Campbell, W.C., Benz, G.W., **1984**. Ivermectin: a review of efficacy and safety. *J. Vet. Pharmacol. Therap. 7*, 1–16.

Campbell, W.C., Burg, R.W., Fisher, M.H., Dybas, R.A., **1984**. The discovery of ivermectin and other avermectins. In: Magee, P.S., Kohn, G.K., Menn, J.J. (Eds.), Pesticide Synthesis through Rational Approaches. American Chemical Society, Washington, DC, pp. 5–20.

Campbell, W.C., Fisher, M.H., Stapley, E.O., Albers-Schonberg, T., Jacob, T.A., **1983**. Ivermectin: a potent new antiparasitic agent. *Science 221*, 923–928.

Campos, F.C., Dybas, R.A., Krupa, D.A., **1995**. Susceptibility of twospotted spider mite (Acari: Tetranychidae) populations in California to abamectin. *J. Econ. Entomol. 88*, 225–231.

Campos, F.C., Krupa, D.A., Dybas, R.A., **1996**. Susceptibility of populations of twospotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae) from Florida, Holland, and the Canary Islands to abamectin and characterization of abamectin resistance. *J. Econ. Entomol. 89*, 594–601.

Campos, F.C., Krupa, D.A., Jansson, R.K., **1997**. The evaluation of a petri plate assay for the assessment of abamectin susceptibility in *Tetranychus urticae* Koch (Acari: Tetranychidae). *J. Econ. Entomol. 90*, 742–746.

Carter, G.T., Nietsche, J.A., Hertz, M.R., **1988**. LL-F28249 antibiotic complex: a new family of antiparasitic macrocyclic lactones: isolation, characterization and structures of LL-F28249α,β,γ,λ. *J. Antibiotics 41*, 519–529.

Cayrol, J.C., Dijan, C., Frankowski, J.P., **1993**. Efficacy of abamectin B_1 for control of *Meloidogyne arenaria*. *Fund. Appl. Nematol. 16*, 239–246.

Chabala, J.C., Mrozik, H., Tolman, R.L., Eskola, P., Lusi, A., *et al.*, **1980**. Ivermectin, a new broad-spectrum antiparasitic agent. *J. Med. Chem. 23*, 1134–1136.

Chamberlain, W.F., **1982**. Evaluation of toxicants against the secondary screwworm. *Insectic. Acaric. Tests 7*, 255.

Chang, V., **1988**. Toxic baiting of the western yellowjacket (Hymenoptera: Vespidae) in Hawaii. *J. Econ. Entomol. 81*, 228–235.

Cheeseman, C.L., Delany, N.S., Woods, D.J., Wolstenholme, A.J., **2001**. High-affinity ivermectin binding to recombinant subunits of the *Haemonchus contortus* glutamate-gated chloride channel. *Mol. Biochem. Parasitol. 114*, 161–168.

Chiu, S.-H.L., Lu, A.Y.H., **1989**. Metabolism and tissue residues. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 131–143.

Chukwudebe, A.C., Cox, D.L., Palmer, S.J., Morneweck, L.A., Payne, L.D., *et al.*, **1997**. Toxicity of emamectin benzoate foliar dislodgeable residues to two beneficial insects. *J. Agric. Food Chem. 45*, 3689–3693.

Clark, J.M., Scott, J.G., Campos, F., Bloomquist, J.R., **1995**. Resistance to avermectins: extent, mechanisms, and management implications. *Annu. Rev. Entomol. 40*, 1–30.

Clarke, G.M., Ridsdill-Smith, T.J., **1990**. The effect of avermectin B_1 on developmental stability in the bush fly, *Musca vetustissima*, as measured by fluctuating asymmetry. *Entomol. Exp. Appl. 54*, 265–269.

Cochran, D.G., **1985**. Mortality and reproductive effects of avermectin B_1 fed to German cockroaches. *Entomol. Exp. Appl. 37*, 83–88.

Cochran, D.G., **1990**. Efficacy of abamectin fed to German cockroaches (Dictyoptera: Blattellidae) resistant to pyrethroids. *J. Econ. Entomol. 83*, 1243–1245.

Cole, L.M., Roush, R.T., Casida, J.E., **1995**. *Drosophila* GABA-gated chloride channel: modified [^{3}H]EBOB binding site associated with Ala→Ser or Gly mutants of Rdl subunit. *Life Sci. 56*, 757–765.

Conder, G.A., Thompson, D.P., Johnson, S.S., **1993**. Demonstration of co-resistance of *Haemonchus contortus* to ivermectin and moxidectin. *Vet. Rec. 132*, 651–652.

Cook, D.F., **1991**. Ovarian development in females of the Australian sheep blowfly *Lucilia cuprina* (Diptera: Calliphoridae) fed on sheep faeces and the effects of ivermectin residues. *Bull. Entomol. Res. 81*, 249–256.

Cook, D.F., **1993**. Effect of avermectin residues in sheep dung on mating of the Australian sheep blowfly *Lucilia cuprina*. *Vet. Parasitol. 48*, 205–214.

Coop, R.L., Barger, I.A., Jackson, F., **2002**. The use of macrocyclic lactones to control parasites of sheep and goats. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 303–321.

Corbitt, T.S., Green, A.S.J., Wright, D.J., **1989**. Relative potency of avermectin B_1 against larval stages of *Spodoptera littoralis* and *Heliothis virescens*. *Crop Protect. 8*, 127–132.

Cotreau, M.M., Warren, S., Ryan, J.L., Fleckenstein, L., Vanapalli, S.R., *et al.*, **2003**. The antiparasitic moxidectin: safety, tolerability, and pharmacokinetics in humans. *J. Clin. Pharmacol. 43*, 1108–1115.

Court, J.P., Murgatroyd, R.C., Livingstone, D., Rohr, E., **1988**. Physiochemical characteristics of non-electrolytes and their uptake by *Brugia pahangi* and *Dipetalonema viteae*. *Mol. Biochem. Parasitol. 38*, 183–227.

Cox, D.L., Remick, D., Lasota, J.A., Dybas, R.A., **1995a**. Toxicity of avermectins to *Liriomyza trifolii* (Diptera: Agromyzidae) larvae and adults. *J. Econ. Entomol. 88*, 1415–1419.

Cox, D.L., Knight, A.L., Biddinger, D.G., Lasota, J.A., Pikounis, B., *et al.*, **1995b**. Toxicity and field efficacy of avermectins against codling moth (Lepidoptera: Tortricidae) on apples. *J. Econ. Entomol. 88*, 708–715.

Craig, T.M., Kunde, J.M., **1981**. Controlled evaluation of ivermectin in Shetland ponies. *Am. J. Vet. Res. 42*, 1422–1429.

Crouch, L.S., Feely, W.F., Arison, B.H., Vanden Heuvel, W.J., Colwell, L.F., *et al.*, **1991**. Photodegradation of avermectin B_{1a} thin films on glass. *J. Agric. Food Chem. 39*, 1310–1319.

Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H., *et al.*, **1994**. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature 371*, 707–711.

Cully, D.F., Paress, P.S., Liu, K.K., Schaeffer, J.M., Arena, J.P., **1996**. Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. *J. Biol. Chem. 271*, 20187–20191.

Cvetovich, R.J., Kelly, D.H., DiMichele, L.M., Shuman, R.F., Grabowski, E.J.J., **1994**. Synthesis of 4″-epi-amino-4″-deoxyavermectins B_1. *J. Org. Chem. 59*, 7704–7708.

Cvetovich, R.J., Senanayake, C.H., Amato, J.S., DiMichelle, L.M., Bill, T.J., *et al.*, **1997**. Practical synthesis of 13-O-[(2-methoxyethoxy) methyl]-22,23-dihydroavermectin B_1 aglycon [dimedectin isopropanol, MK-324] and 13-epi-O-(2-methoxymethyl)-22,23-dihydroavermectin B_1 aglycon [L-694,554], flea active ivermectin analogues. *J. Org. Chem. 62*, 3989–3993.

Danka, R.G., Loper, G.M., Villa, J.D., Williams, J.L., Sugden, E.A., *et al.*, **1994**. Abating feral Africanized honey bees (*Apis mellifera* L.) to enhance mating control of European queens. *Apidologie 25*, 520–529.

Davies, G.H., Green, R.H., **1986**. Avermectins and milbemycins. *Nat. Prod. Rep. England 3*, 87–121.

Davies, I.M., Rodger, G.K., **2000**. A review of the use of ivermectin as a treatment for sea lice [*Lepeophtheirus salmonis* (Kroyer) and *Caligus elongatus* (Nordmann)] infestation in farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture Res. 31*, 869–883.

Dawson, G.R., Wafford, K.A., Smith, A., Marshall, G.R., Bayley, P.J., *et al.*, **2000**. Anticonvulsant and adverse effects of avermectin analogs in mice are mediated through the gamma-aminobutyric acid(A) receptor. *J. Pharmacol. Exp. Ther. 295*, 1051–1060.

De Azambuja, P., Gomes, J.E.P.L., Lopes, F., Garcia, E.S., **1985**. Efficacy of ivermectin against the bloodsucking insect, *Rhodnius prolixus* (Hemiptera, Triatominae). *Mem. Inst. Oswaldo Cruz. 80*, 439–442.

Delany, N.S., Laughton, D.L., Wolstenholme, A.J., **1998**. Cloning and localisation of an avermectin receptor-related subunit from *Haemonchus contortus*. *Mol. Biochem. Parasitol. 97*, 177–187.

Deng, Y., Casida, J.E., **1992**. House fly head GABA-gated chloride channel: toxicologically relevant binding site for avermectins coupled to a site for ethynylbicycloorthobenzoate. *Pestic. Biochem. Physiol. 43*, 116–122.

Dent, J.A., Davis, M.W., Avery, L., **1997**. Avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. *EMBO J. 16*, 5867–5879.

Dent, J.A., Smith, M.M., Vassilatis, D.K., Avery, L., **2000**. The genetics of ivermectin resistance in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA 97*, 2674–2697.

Dively, G.P., Jansson, R.K., **1996**. Baseline monitoring of susceptibility to abamectin in the Colorado potato beetle (*Leptinotarsa decemlineata* (Say)). *Pestic. Resist. Newslett. 8*, 8–12.

Doherty, W.M., Stewart, N.P., Cobb, R.M., Kieran, P.J., **1994**. *In vitro* comparison of the larvicidal activity of moxidectin and abamectin against *Onthophagus gazella* (F.) (Coleoptera: Scarabaeidae) and *Haematobia irritans exigua* De Meijere (Diptera: Muscidae). *J. Austral. Entomol. Soc. 33*, 71–74.

Drexler, G., Sieghart, W., **1984a**. Properties of high affinity binding site for tritium-labelled avermectin B_{1a}. *Eur. J. Pharmacol. 99*, 269–277.

Drexler, G., Sieghart, W., **1984b**. Evidence for association of a high affinity avermectin binding site with benzodiazepine receptor. *Eur. J. Pharmacol. 101*, 201–207.

Drexler, G., Sieghart, W., **1984c**. Sulphur-35-labelled *tert*-butyl-bicyclophosphorothionate and avermectin bind to different sites associated with the gamma-aminobutyric acid-benzodiazepine receptor complex. *Neurosci. Lett. 50*, 273–277.

Driniaev, V.A., Mosin, V.A., Kruglyak, E.B., Sterlina, T.S., Kataev, A.A., *et al.*, **2001**. Effect of avermectins on Ca^{2+}-dependent Cl^- currents in plasmalemma of *Chara corallina* cells. *J. Membr. Biol. 182*, 71–79.

Duce, I.R., Scott, R.H., **1985**. Actions of dihydroavermectin B_{1a} on insect muscle. *Br. J. Pharmacol. 85*, 395–401.

Dybas, R.A., **1989**. Abamectin use in crop protection. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 287–310.

Dybas, R.A., Babu, J.R., **1988**. 4″-Eoxy-4″-methylamino-4″-epiavermectin B_1 hydrochloride (MK-243): a novel avermectin insecticide for crop protection. In: Proc. Br. Crop Protect. Conf., Pests and Diseases, vol. 1. pp. 57–64.

Dybas, R.A., Green, A.S.J., **1984**. Avermectins: their chemistry and pesticidal activity. *Proc. Br. Crop Protect. Conf., Pests and Diseases 9B-3*, 947–954.

Dybas, R.A., Hilton, N.J., Babu, J.R., Preiser, F.A., Dolce, G.J., **1989**. Novel second-generation avermectin insecticides and miticides for crop protection. In: Demain, A.L., Somkuti, G.A., Hunter-Cevera, J.C., Rossmoore, H.W. (Eds.), Novel Microbial Products for Medicine and Agriculture. Society for Industrial Microbiology, Fairfax, VA, pp. 203–212.

Eagleson, J.S., Thompson, D.R., Scott, P.G., Cramer, L.G., Barrick, R.A., **1993a**. Field trials to confirm the efficacy of ivermectin jetting fluid for control of blowfly strike in sheep. *Vet. Parasitol. 51*, 107–112.

Eagleson, J.S., Thompson, D.R., Scott, P.G., Cramer, L.G., **1993b**. The efficacy of ivermectin jetting fluid for control of the sheep biting louse (*Damalinia ovis*). *Austral. J. Exp. Agric. 33*, 843–845.

Egerton, J.R., Ostlind, D.A., Blair, L.S., Eary, C.H., Suhayda, D., *et al.*, **1979**. Avermectins, new family of potent antihelminthic agents: efficacy of the B_{1a} component. *Antimicrob. Agents Chemother. 15*, 372–378.

Elgart, G.W., Meinking, T.L., **2003**. Ivermectin. *Dermatol. Clin. 21*, 277.

Etter, A., Cully, D.F., Schaeffer, J.M., Liu, K.K., Arena, J.P., **1996**. An amino acid substitution in the pore region of a glutamate gated chloride channel enables the coupling of ligand binding to channel gating. *J. Biol. Chem. 271*, 16035–16039.

Feely, W.F., Crouch, L.S., Arison, B.H., Vanden Heuvel, W.J.A., Colwell, L.F., *et al.*, **1992**. Photodegradation of 4″-(epimethylamino)-4″-deoxyavermectin B_{1a} thin films on glass. *J. Agric. Food Chem. 40*, 691–696.

ffrench-Constant, R.H., **1994**. The molecular and population genetics of cyclodiene insecticide resistance. *Insect Biochem. Mol. Biol. 24*, 335–345.

Fincher, G.T., **1992**. Injectable ivermectin for cattle: effects on some dung-inhabiting insects. *Environ. Entomol. 21*, 871–876.

Fincher, G.T., Wang, G.T., **1992**. Injectable moxidectin for cattle: effects in two species of dung burying beetles. *Southwest. Entomol. 17*, 303–306.

Fisher, M.H., **1997**. Structure–activity relationships of the avermectins and milbemycins. In: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miamoto, J., Thompson, D.G. (Eds.), Phytochemicals for Pest Control. American Chemical Society, Washington, DC, pp. 220–238.

Fisher, M.H., Mrozik, H., **1984**. The avermectin family of macrolide-like antibiotics. In: Omura, S. (Ed.), Macrolide Antibiotics. Academic Press, New York, pp. 553–606.

Fisher, M.H., Mrozik, H., **1989**. Chemistry. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 1–23.

Fisher, M.H., Mrozik, H., **1992**. The chemistry and pharmacology of avermectins. *Annu. Rev. Pharmacol. Toxicol. 32*, 537–553.

Floate, K.D., Spooner, R.W., Colwell, D.D., **2001**. Larvicidal activity of endectocides against pest flies in the dung of treated cattle. *Med. Vet. Entomol. 16*, 1–4.

Floate, K.D., Colwell, D.D., Fox, A.S., **2002**. Reductions of non-pest insects in dung of cattle treated with endectocides: a comparison of four products. *Bull. Entomol. Res. 92*, 471–481.

Geden, C.J., Steinkraus, D.C., Long, S.J., Rutz, D.A., Shoop, W.L., **1990**. Susceptibility of insecticide-susceptible and wild house flies (Diptera: Muscidae) to abamectin on whitewashed and unpainted wood. *J. Econ. Entomol. 83*, 1935–1939.

Glancey, B.M., Lofgren, C.S., Williams, D.F., **1982**. Avermectin B_{1a}: effects on the ovaries of red imported fire ant queens (Hymenoptera: Formicide). *J. Med. Entomol. 19*, 743–747.

Gouamene-Lamine, C.N., Yoon, K.S., Clark, J.M., **2003**. Differential susceptibility to abamectin and two bioactive avermectin analogs in abamectin-resistant and -susceptible strains of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). *Pestic. Biochem. Physiol. 76*, 15–23.

Goudie, A.C., Evans, N.A., Gration, K.A.F., Bishop, B.F., Gibson, S.P., *et al.*, **1993**. Doramectin: a potent novel endectocide. *Vet. Parasitol. 49*, 5–15.

Grafton-Cardwell, E.E., Hoy, M.A., **1983**. Comparative toxicity of avermectin B1 to the predator *Metaseiulus occidentalis* (Nesbitt) (Acarina: Phytoseiidae) and the spider mites *Tetranychus urticae* Koch and *Panonychus ulmi* (Koch) (Acari: Tetranychidae). *J. Econ. Entomol. 76*, 1216–1220.

Graham, D., Pfeiffer, F., Betz, H., **1982**. Avermectin B_{1a} inhibits the binding of strychnine to the glycine receptor of rat spinal cord. *Neurosci. Lett. 29*, 173–176.

Green, A.St.J., Dybas, R.A., **1984**. The control of *Liriomyza* leafminers and other insect species with avermectin B_1. *Proc. Brit. Crop Protect. Conf., Pests and Diseases 3*, 1135–1141.

Green, A.St. J., Heijne, B., Schreurs, J., Dybas, R.A., **1985**. Serpentine leafminer (*Liriomyza trifolii*) (Burgess) control with abamectin (MK-936) in Dutch ornamentals, a review of the processes involved in the evolution of the use directions, and a summary of the results of phytotoxicity evaluations. *Med. Fac. Landbouww. Rijksuniv. Gent 50/2b*, 623–632.

Guerrero, J., McCall, J.W., Genchi, C., **2002**. The use of macrocyclic lactones in the control and prevention of heartworm and other parasites in dogs and cats. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 353–369.

Halley, B.A., Jacob, T.A., Lu, A.Y.H., **1989a**. The environmental impact of the use of ivermectin: environmental effects and fate. *Chemosphere 18*, 1543–1563.

Halley, B.A., Nessel, R.J., Lu, A.Y.H., **1989b**. Environmental aspects of ivermectin usage. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 162–172.

Harrell, M.O., Pierce, P.A., **1994**. Effects of trunk-injected abamectin on the elm leaf beetle. *J. Arboric. 20*, 1–3.

Hennessy, D.R., Alvinerie, M.R., **2002**. Pharmacokinetics of the macrocyclic lactones: conventional wisdom and new paradigms. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 97–123.

Hilton, R.J., Riedl, H., Westigard, P.H., **1992**. Phytotoxicity response of pear to application of abamectin–oil combinations. *Hortscience 27*, 1280–1282.

Horoszok, L., Raymond, V., Sattelle, D.B., Wolstenholme, A.J., **2001**. GLC-3: a novel fipronil and BIDN-sensitive, but picrotoxinin-insensitive, L-glutamate-gated chloride channel subunit from *Caenorhabditis elegans*. *Br. J. Pharmacol. 132*, 1247–1254.

Houlding, B., Ridsdill-Smith, T.J., Bailey, W.J., **1991**. Injectable ivermectin causes a delay in Scarabaeinae dung beetle egg-laying in cattle dung. *Austral. Vet. J. 68*, 185–186.

Hoy, M.A., Cave, F.E., **1985**. Laboratory evaluation of avermectin as a selective acaricide for use with *Metaseiulus occidentalis* (Nesbitt) (Acarina: Phytoseiidae). *Exp. Appl. Acarol. 1*, 139–152.

Hoy, M.A., Conley, J., **1987**. Selection for abamectin resistance in *Tetranychus urticae* and *T. pacificus* (Acari: Tetranychidae). *J. Econ. Entomol. 80*, 221–225.

Hoy, M.A., Ouyang, Y.-L., **1989**. Selection of the western predatory mite, *Metaseiulus occidentalis* (Acari: Phytoseiidae), for resistance to abamectin. *J. Econ. Entomol. 82*, 35–40.

Huang, J., Casida, J.E., **1997**. Avermectin B_{1a} binds to high- and low-affinity sites with dual effects on the gamma-aminobutyric acid-gated chloride channel of cultured cerebellar granule neurons. *J. Pharmacol. Exp. Therap. 281*, 261–266.

Hughes, P.B., Levot, G.W., **1990**. Toxicity of three avermectins to insecticide susceptible and resistant larvae of *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *J. Austral. Entomol. Soc. 29*, 109–111.

Immaraju, J.A., Morse, J.G., **1990**. Selection for pyrethroid resistance, reversion, and cross-resistance with citrus thrips (Thysanoptera: Thripidae). *J. Econ. Entomol. 83*, 698–704.

Immaraju, J.A., Paine, T.D., Bethke, J.A., Robb, K.L., Newman, J.P., **1992**. Western flower thrips (Thysanoptera: Thripidae) resistance to insecticides in coastal California greenhouses. *J. Econ. Entomol. 85*, 9–14.

Iqbal, M., Verkerk, R.H.J., Furlong, M.J., Ong, P.C., Rahman, S.A., *et al.*, **1996**. Evidence for resistance to *Bacillus thuringiensis* (*Bt*) subsp. *kurstaki* HD-1, *Bt* subsp. *aizawai* and abamectin in field populations of

Plutella xylostella from Malaysia. *Pestic. Sci. 48*, 89–97.

Ismail, F., Wright, D.J., **1991**. Cross-resistance between acylurea insect growth regulators in a strain of *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) from Malaysia. *Pestic. Sci. 33*, 359–370.

Jackson, H.C., **1989**. Ivermectin as a systemic insecticide. *Parasitol. Today 5*, 146–156.

Jacobs, D.E., Pilkington, J.G., Fisher, M.A., Fox, M.T., **1988**. Ivermectin therapy and degradation of cattle faeces. *Vet. Rec. 123*, 400.

Jagannathan, S., Laughton, D.L., Critten, C.L., Skinner, T.M., Horoszok, L., *et al.*, **1999**. Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans* gbr-2 (avr-14) gene. *Mol. Biochem. Parasitol. 103*, 129–140.

James, P.S., Picton, J., Riek, R.F., **1980**. Insecticidal activity of the avermectins. *Vet. Rec. 106*, 59.

Jansson, R.K., Dybas, R.A., **1998**. Avermectins: biochemical mode of action, biological activity, and agricultural importance. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer, New York, pp. 152–170.

Jansson, R.K., Rabatin, S., **1997**. Curative and residual efficacy of injection applications of avermectins for control of plant parasitic nematodes on banana. *J. Nematol. 29* (Suppl.), 695–702.

Jansson, R.K., Rabatin, S., **1998**. Potential of foliar, dip, and injection applications of avermectins for control of plant parasitic nematodes. *J. Nematol. 30*, 65–75.

Jansson, R.K., Halliday, W.R., Argentine, J.A., **1998**. Comparison of miniature and high volume bioassays for screening insecticides. *J. Econ. Entomol. 90*, 1500–1507.

Jansson, R.K., Brown, R., Cartwright, B., Cox, D., Dunbar, D.M., *et al.*, **1996**. Emamectin benzoate: a novel avermectin derivative for control of lepidopterous pests. In: Proc. 3rd Intern. Workshop Mgt Diamondback Moth and Other Crucifer Pests, Kuala Lumpur, Malaysia, pp. 171–177.

Johnson, A.W., **1985**. Abamectin for tobacco insect control. *Tobacco Sci. 29*, 135–138.

Jung, M., Saito, A., Buescher, G., Maurer, M., Graf, J.-F., **2003**. Chemistry, pharmacology and safety: milbemycin oxime. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallinford, pp. 51–74.

Kerlin, R.L., East, I.J., **1992**. The survival and fecundity of buffalo flies after treatment of cattle with three anthelminthics. *Austral. Vet. J. 69*, 283–285.

Kieran, P.J., **1994**. Moxidectin against ivermectin-resistant nematodes-a global view. *Austral. Vet. J. 71*, 18–20.

King, K.L., **1993**. Methods for assessing the impact of avermectins on the decomposer community of sheep pastures. *Vet. Parasitol. 48*, 87–97.

Klei, T.R., Torbert, B.J., **1980**. Efficacy of ivermectin (22,23-dihydroavermectin B_1) against gastro-intestinal parasites in ponies. *Am. J. Vet. Res. 41*, 1747–1750.

Koehler, P.G., Atkinson, T.H., Patterson, R.S., **1991**. Toxicity of abamectin to Cockroaches (Dictyoptera: Blattellidae, Blattidae). *J. Econ. Entomol. 84*, 1758–1762.

Kok, L.T., Lasota, J.A., McAvoy, T.J., Dybas, R.A., **1996**. Residual foliar toxicity of 4″-epimethylamino-4″-deoxyavermectin B1 hydrochloride (MK-243) and selected commercial insecticides to adult hymenopterous parasites, *Pteromalus puparum* (Hymenoptera: Pteromalidae) and *Cotesia orobenae* (Hymenoptera: Braconidae). *J. Econ. Entomol. 89*, 63–67.

Konno, Y., Scott, J.G., **1991**. Biochemistry and genetics of abamectin resistance in the house fly. *Pestic. Biochem. Physiol. 41*, 21–28.

Kornis, G.I., **1995**. Avermectins and milbemycins. In: Godfrey, C.R.A. (Ed.), Agrochemicals from Natural Products. Dekker, New York, pp. 215–255.

Kotze, A.C., **1995**. Induced insecticide tolerance in *Lucilia cuprina* (Diptera: Calliphoridae) larvae following dietary phenobarbital treatment. *J. Austral. Entomol. Soc. 34*, 205–209.

Kotze, A.C., Sales, N., **1994**. Cross-resistance spectra and effects of synergists in insecticide-resistant strains of *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Bull. Entomol. Res. 84*, 355–360.

Krause, R.M., Buisson, B., Bertrand, S., Corringer, P.-J., Galzi, J.-L., *et al.*, **1998**. Ivermectin: a positive allosteric effector of the α7 neuronal nicotinic acetylcholine receptor. *Molec. Pharmacol. 53*, 283–294.

Kruger, K., Scholtz, C.H., **1997**. Lethal and sublethal effects of ivermectin on the dung-breeding beetles *Euoniticellus intermedius* (Reiche) and *Onitis alexis* Klug (Coleoptera, Scarabaeidae). *Agric. Ecosys. Environ. 61*, 123–131.

Lamine, C.N.G., **1994**. Biochemical factors of resistance and management of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). MS thesis, University of Massachusetts, Amherst.

Langley, P.A., Roe, J.M., **1984**. Ivermectin as a possible control agent for tsetse fly, *Glossina morsitans*. *Entomol. Exp. Appl. 36*, 137–143.

Lankas, G.R., Gordon, L.R., **1989**. Toxicology. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 89–112.

Lasota, J.A., Dybas, R.A., **1991**. Avermectins, a novel class of compounds: implications for use in arthropod pest control. *Annu. Rev. Entomol. 36*, 91–117.

Lasota, J.A., Shelton, A.M., Bolognese, J.A., Dybas, R.A., **1996**. Baseline toxicity of avermectins to diamondback moth (Lepidoptera: Plutellidae) populations: implications for susceptibility monitoring. *J. Econ. Entomol. 89*, 33–38.

Laughton, D.L., Lunt, G.G., Wolstenholme, A.J., **1997a**. Reporter gene constructs suggest the *Caenorhabditis elegans* avermectin receptor β-subunit is expressed solely in the pharynx. *J. Exp. Biol. 200*, 1509–1514.

Laughton, D.L., Lunt, G.G., Wolstenholme, A.J., **1997b**. Alternative splicing of a *Caenorhabditis elegans* gene produces two novel inhibitory amino acid receptor

subunits with identical ligand binding domains but different ion channels. *Gene 201*, 119–125.

Liang, P., Gao, X.W., Zheng, B.Z., **2003**. Genetic basis of resistance and studies on cross-resistance in a population of diamondback moth, *Plutella xylostella* (Lepidoptera : Plutellidae). *Pest Mgt Sci. 59*, 1232–1236.

Ludmerer, S.W., Warren, V.A., Williams, B.S., Zheng, Y., Hunt, D.C., *et al.*, **2002**. Ivermectin and nodulisporic acid receptors in *Drosophila melanogaster* contain both gamma-aminobutyric acid-gated Rdl and glutamate-gated GluCl alpha chloride channel subunits. *Biochemistry 41*, 6548–6560.

Lumaret, J.P., Galante, E., Lumberas, C., Mena, J., Bertrand, M., *et al.*, **1993**. Field effects of ivermectin residues on dung beetles. *J. Appl. Ecol. 30*, 428–436.

Lummis, S.C.R., Sattelle, D.B., **1985**. Binding sites for 4-aminobutyric acid and benzodiazepines in the central nervous system of insects. *Pestic. Sci. 16*, 695–697.

MacConnell, J.G., Demchak, R.J., Preiser, F.A., Dybas, R.A., **1989**. Relative stability, toxicity, and penetrability of abamectin and its 8,9-oxide. *J. Agric. Food. Chem. 37*, 1498–1501.

Mahon, R.J., Wardhaugh, K.G., **1991**. Impact of dung from ivermectin-treated sheep on oogenesis and survival of adult *Lucilia cuprina*. *Austral. Vet. J. 68*, 173–177.

Marley, S.E., Hall, R.D., Corwin, R.M., **1993**. Ivermectin cattle pour-on: duration of a single late spring treatment against horn flies, *Haematobia irritans* (L.) (Diptera: Muscidae) in Missouri, USA. *Vet. Parasitol. 51*, 167–172.

McKeand, J., Bairden, K., Ibarra-Silva, A.M., **1988**. The degradation of bovine pats containing ivermectin. *Vet. Rec. 122*, 587–588.

McKellar, Q.A., Benchaoui, H.A., **1996**. Avermectins and milbemycins. *J. Vet. Pharmacol. Therap. 19*, 331–351.

McTier, T.L., Jernigan, A.D., Rowan, T.G., Holbert, M.S., Smothers, C.D., *et al.*, **2000a**. Dose selection of selamectin for efficacy against adult fleas (*Ctenocephalides felis felis*) on dogs and cats. *Vet. Parasitol. 91*, 177–185.

McTier, T.L., Jones, R.L., Holbert, M.S., Murphy, M.G., Watson, P., *et al.*, **2000b**. Efficacy of selamectin against adult flea infestations (*Ctenocephalides felis felis* and *Ctenocephalides canis*) on dogs and cats. *Vet. Parasitol. 91*, 187–199.

Meyer, J.A., Simco, J.S., Lancaster, J.L., **1980**. Control of face fly larval development with ivermectin, MK-933. *Southwest. Entomol. 5*, 207–209.

Miller, J.A., Kunz, S.E., Oehler, D.D., Miller, R.W., **1981**. Larvicidal activity of Merck MK-933, an avermectin, against the horn fly, stable fly, face fly and house fly. *J. Econ. Entomol. 74*, 608–611.

Miller, J.A., Oehler, D.D., Scholl, P. J., **1994**. Moxidectin: pharmacokinetics and activity against horn flies (Diptera: Muscidae) and trichostrongyle nematode egg production. *Vet. Parasitol. 53*, 133–143.

Miller, J.A., Oehler, D.D., Siebernaler, A.J., Kunz, S.E., **1986**. Effect of ivermectin on survival and fecundity of horn flies and stable flies (Diptera: Muscidae). *J. Econ. Entomol. 79*, 1564–1569.

Mishima, H., Kurabayashi, M., Tamura, C., Sato, S., Kuwano, H., *et al.*, **1975**. Structures of milbemycin beta1, beta2, and beta3. *Tetrahedron Lett. 16*, 711–714.

Molyneux, D.H., Bradley, M., Hoerauf, A., Kyelem, D., Taylor, M.J., **2003**. Mass drug treatment for lymphatic filariasis and onchocerciasis. *Trends Parasitol 19*, 516–522.

Morse, J.G., Brawner, O.L., **1986**. Toxicity of pesticides to *Scirtothrips citri* (Thysanoptera: Thripidae) and implications to resistance management. *J. Econ. Entomol. 79*, 565–570.

Mrozik, H., **1994**. Advances in research and development of avermectins. In: Hedin, P.A., Menn, J.J., Hollingsworth, R.M. (Eds.), Natural and Engineered Pest Management Agents. American Chemical Society, Washington, DC, pp. 54–73.

Mrozik, H., Chabala, J.C., Eskola, P., Matzuk, A., Waksmunski, F., *et al.*, **1983**. Synthesis of milbemycins from avermectins. *Tetrahedron Lett. 24*, 5333–5336.

Mrozik, H., Eskola, P., Linn, B.O., Lusi, A., Shih, T.L., *et al.*, **1989**. Discovery of novel avermectins with unprecedented insecticidal activity. *Experentia 45*, 315–316.

Mrozik, H., Eskola, P., Reynolds, G.F., Arison, B.H., Smith, G.M., *et al.*, **1988**. Photoisomers of avermectins. *J. Organ. Chem. 53*, 1820–1823.

National Registration Authority, **1998**. *NRA special review of macrocyclic lactones. NRA Special Review Series 98.3* 1–79.

Nicholson, R., Robinson, P.S., Palmer, P.J., Casida, J.E., **1988**. Ivermectin-stimulated release of neurotransmitter in the insect central nervous system: modulation by external chloride and inhibition by a novel trioxabicyclooctane and two polychlorocycloalkane insecticides. In: Neurotoxicology '88, Abstr. 96.

Orton, C.J., Watts, J.E., Rugg, D., **1992**. Comparative effectiveness of avermectins and deltamethrin in suppressing oviposition in *Lucilia cuprina* (Diptera: Calliphoridae). *J. Econ. Entomol. 85*, 28–32.

Ostlind, D.A., Cifelli, S., Lang, R., **1979**. Insecticidal activity of the antiparasitic avermectins. *Vet. Rec. 105*, 168.

Ostlind, D.A., Felchetto, T., Misura, A., Ondeyka, J., Smith, S., *et al.*, **1997**. Discovery of a novel indole diterpene insecticide using first instars of *Lucilia sericata*. *Med. Vet. Entomol. 11*, 407–408.

Pampiglione, S., Majori, G., Petrangeli, G., Romi, R., **1985**. Avermectins, MK-933 and MK936, for mosquito control. *Trans. Roy. Soc. Trop. Med. Hyg. 79*, 797–799.

Parella, M.P., **1983**. Evaluation of selected insecticides for control of permethrin-resistant *Liriomyza trifolii* (Diptera: Agromyzidae) on chrysanthemums. *J. Econ. Entomol. 76*, 1460–1464.

Park, C.G., Yoo, J.K., Lee, J.O., **1996**. Toxicity of some pesticides to two-spotted spider mite (Acari: Tetranychidae) and its predator *Amblyseius womersleyi* (Acari: Phytoseiidae). *Korean J. Appl. Entomol. 35*, 232–237.

Paul, A.J., Tranquilli, W.J., Seward, R.L., Todd, K.S., DiPietro, J.A., **1987**. Clinical observations in collies given ivermectin orally. *Am. J. Vet. Res. 48*, 684–685.

Pong, S.-S., Wang, C.C., **1982**. Avermectin B_{1a} modulation of gamma-aminobutyric acid receptors in rat brain membranes. *J. Neurochem. 38*, 375–379.

Pong, S.-S., Wang, C.C., Fritz, L.C., **1980**. Studies on the mechanism of action of avermectin B_{1a}: stimulation of release of gamma-aminobutyric acid from brain synaptosomes. *J. Neurochem. 34*, 351–358.

Portillo, V., Jagannathan, S., Wolstenholme, A.J., **2003**. Distribution of glutamate-gated chloride channel subunits in the parasitic nematode *Haemonchus contortus*. *J. Comp. Neurol. 462*, 213–222.

Preston, J.M., **1982**. The avermectins: new molecules for use in warble fly control. In: Boulard, C., Thornberry, H. (Eds.), Warble Fly Control in Europe. A.A. Balkema, Rotterdam, pp. 17–20.

Pritchard, R.K., **2002**. Resistance against macrocyclic lactones. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 163–182.

Putter, I., MacConnell, J.G., Preiser, F.A., Haidri, A.A., Ristich, S.S., *et al.*, **1981**. Avermectins: novel insecticides, acaracides and nematicides from a soil microorganism. *Experentia 37*, 963–964.

Ranjan, S., Wang, G.T., Hirschlein, C., Simkins, K.L., **2002**. Selection for resistance to macrocyclic lactones by *Haemonchus contortus* in sheep. *Vet. Parasitol. 103*, 109–117.

Raymond, V., Mongan, N.P., Sattelle, D.B., **2000**. Anthelmintic actions of homomer-forming nicotinic acetylcholine receptor subunits: chicken α7 and ACR-16 from the nematode *Caenorhabditis elegans*. *Neuroscience 101*, 785–791.

Raymond, V., Sattelle, D.B., **2002**. Novel animal-health drug targets for ligand-gated chloride channels. *Nature Rev. Drug Disc. 1*, 427–436.

Reed, D.K., Reed, G.L., **1986**. Activity of avermectin B_1 against the striped cucumber beetle (*Acalymma vittatum*) (Coleoptera: Chrysomelidae). *J. Econ. Entomol. 79*, 943–947.

Reed, D.K., Tromley, N.J., Reed, G.L., **1985**. Activity of avermectin B_1 against codling moth (Lepidoptera: Olethreutidae). *J. Econ. Entomol. 78*, 1067–1071.

Ridsdill-Smith, T.J., **1988**. Survival and reproduction of *Musca vetustissima* Walker (Diptera: Muscidae) and a scarabaeinae dung beetle in dung of cattle treated with avermectin B1. *J. Austral. Entomol. Soc. 27*, 175–178.

Robertson, J.L., Richmond, C.E., Preisler, H.K., **1985**. Lethal and sublethal effects of avermectin B_1 on the western spruce budworm (Lepidoptera: Tortricidae). *J. Econ. Entomol. 78*, 1129–1132.

Rock, D.W., DeLay, R.L., Gliddon, M.J., **2002**. Chemistry, pharmacology and safety: moxidectin. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 75–96.

Rohrer, S.P., Birzin, E.T., Costa, S.D., Arena, J.P., Hayes, E.C., *et al.*, **1995**. Identification of neuron-specific ivermectin binding sites in *Drosophila melanogaster* and *Schistocerca americana*. *Insect Biochem. Mol. Biol. 25*, 11–17.

Roncalli, R.A., **1984**. Efficacy of ivermectin against *Oestrus ovis* in sheep. *Vet. Med. Small Anim. Clinic. 79*, 1095–1097.

Roth, M., **2000**. The availability and use of chemotherapeutic sea lice control products. *Contrib. Zool. 69*, http://dpc.uba.uva.nl/ctz/vol69/nr01/a12.

Roulet, A., Puel, O., Gesta, S., Lepage, J.F., Drag, M., *et al.*, **2003**. MDR1-deficient genotype in collie dogs hypersensitive to the P-glycoprotein substrate ivermectin. *Eur. J. Pharmacol. 460*, 85–91.

Roush, R.T., Wright, J.E., **1986**. Abamectin toxicity to houseflies (Diptera: Muscidae) resistant to synthetic organic insecticides. *J. Econ. Entomol. 79*, 562–564.

Rugg, D., **1995**. Toxicity and tolerance to ivermectin in some insects associated with livestock and the interaction of ivermectin with the skin and fleece of sheep. PhD thesis, University of Sydney.

Rugg, D., Thompson, D.R., **1993**. A laboratory assay for assessing the susceptibility of *Damalinia ovis* (Schrank) (Phthiraptera: Trichodectidae) to avermectins. *J. Austral. Entomol. Soc. 32*, 1–3.

Rugg, D., Thompson, D.R., **1997**. The interactions of ivermectin with the fleece and skin of sheep. In: Proc. 4th Int. Congr. Sheep Vet., Armidale, NSW, pp. 393–397.

Rugg, D., Kotze, A.C., Thompson, D.R., Rose, H.A., **1998b**. Susceptibility of laboratory-selected and field strains of *Lucilia cuprina* (Diptera: Calliphoridae) to ivermectin. *J. Econ. Entomol. 91*, 601–607.

Rugg, D., Sales, N., Kotze, A.C., Levot, G.W., **1995a**. Susceptibility to ivermectin of pyrethroid-resistant *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *J. Austral. Entomol. Soc. 34*, 69–70.

Rugg, D., Thompson, D.R., Boyle, R., Eagleson, J.S., **1995b**. Field efficacy of an ivermectin jetting fluid for control of the sheep body louse *Bovicola* (*Damalinia*) *ovis* in New Zealand. *N. Z. Vet. J. 43*, 48–49.

Rugg, D., Thompson, D.R., Scott, P.G., Cramer, L.G., Barrick, R.A., **1993**. Efficacy of ivermectin jetting fluid against strike by some primary and secondary blowflies of sheep. *Austral. Vet. J. 70*, 180–182.

Rugg, D., Thompson, D.R., Gogolewski, R.P., Allerton, G.R., Barrick, R.A., *et al.*, **1998a**. Efficacy of ivermectin in a controlled-release capsule for the control of breech strike in sheep. *Austral. Vet. J. 76*, 350–354.

Rust, M.K., **1986**. Managing household pests. In: Bennett, G.W., Owens, J.M. (Eds.), Advances in Urban Pest Management. Van Nostrand Reinhold, New York, pp. 335–386.

Sales, N., Levot, G.W., Hughes, P.B., **1989**. Monitoring and selection of resistance to pyrethroids in the Australian sheep blowfly, *Lucilia cuprina*. *Med. Vet. Entomol. 3*, 287–291.

Sanderson, J.P., Zhang, Z.Q., **1995**. Dispersion, sampling, and potential for integrated control of two-spotted spidermite (Acari: Tetranychidae) on greenhouse roses. *J. Econ. Entomol. 88*, 343–351.
Sasser, J.N., Kirkpatrick, J.L., Dybas, R.A., **1982**. Efficacy of avermectins for root knot control in tobacco. *Plant Dis. 66*, 961–965.
Sattelle, D.B., **1990**. GABA receptors of insects. *Adv. Insect Physiol. 22*, 1–113.
Schaeffer, J.M., Haines, H.W., **1989**. Avermectin binding in *Caenorhabditis elegans*: A two-state model for the avermectin binding site. *Biochem. Pharmacol. 38*, 2329–2338.
Scholtz, C.H., Krüger, K., **1995**. Effects of ivermectin residues in cattle dung on dung insect communities under extensive farming conditions in South Africa. In: Stork, N.E., Harrington, R. (Eds.), Insects in a Changing Environment. Academic Press, New York, pp. 465–471.
Schuster, D.J., **1994**. Life-stage specific toxicity of insecticides to parasitoids of *Liriomyza trifolii* (Burgess) (Diptera, Agromyzidae). *Int. J. Pest Mgt 40*, 191–194.
Schuster, D.J., Everett, P.H., **1983**. Response of *Liriomyza trifolii* (Diptera: Agromyzidae) to insecticides on tomato. *J. Econ. Entomol. 76*, 1170–1174.
Scott, J.G., **1989**. Cross-resistance to the biological insecticide abamectin in pyrethroid resistant strains of house flies. *Pestic. Biochem. Physiol. 34*, 27–31.
Scott, J.G., Roush, R.T., Liu, N., **1991**. Selection of high-level abamectin resistance from field-collected house flies, *Musca domestica*. *Experentia 47*, 288–291.
Seaman, J.T., Eagleson, J.S., Corrigan, M.J., Webb, R.F., **1987**. Avermectin B_1 toxicity in a herd of Murray Grey cattle. *Austral. Vet. J. 64*, 271–278.
Sechser, B., Ayoub, S., Monuir, N., **2003**. Selectivity of emamectin benzoate to predators of sucking pests on cotton. *J. Plant Dis. Protect. 110*, 184–194.
Shoop, W.L., **1993**. Ivermectin resistance. *Parasitol. Today 9*, 154–159.
Shoop, W.L., Haines, H.W., Michael, B.F., Eary, C.H., **1993**. Mutual resistance to avermectins and milbemycins: oral activity of ivermectin and moxidectin against ivermectin-resistant and susceptible nematodes. *Vet. Rec. 133*, 445–447.
Shoop, W.L., Mrozik, H., Fisher, M.H., **1995**. Structure and activity of avermectins and milbemycins in animal health. *Vet. Parasitol. 59*, 139–156.
Shoop, W., Soll, M., **2002**. Ivermectin, abamectin and eprinomectin. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 1–29.
Sigel, E., Buhr, A., **1997**. The benzodiazepine binding site of $GABA_A$ receptors. *Trends Pharmacol. Sci. 18*, 425–429.
Sommer, C., Overgaard Nielsen, B., **1992**. Larvae of the dung beetle *Onthophagus gazella* Fabricus (Coleoptera: Scarabaeidae) exposed to lethal and sublethal ivermectin concentrations. *J. Appl. Entomol. 114*, 502–509.
Sommer, C., Gronvold, J., Holter, P., Nansen, P., **1993**. Effects of ivermectin on two afrotropical dung beetles, *Onthophagus gazella* and *Diastellopalpus quinquedens* (Coleoptera: Scarabaeidae). *Vet. Parasitol. 48*, 171–179.
Sommer, C., Steffanson, B., Overgaard Nielsen, B., Gronvold, J., Vagn Jensen, K.M., *et al.*, **1992**. Ivermectin excreted in cattle dung after subcutaneous injection or pour-on treatment: concentrations and impact on dung fauna. *Bull. Entomol. Res. 82*, 257–264.
Spinosa, H., de, S., Stilck, S.R., Bernardi, M.M., **2002**. Possible anxiolytic effects of ivermectin in rats. *Vet. Res. Commun. 26*, 309–321.
Spradbery, J.P., Tozer, R.S., Drewett, N., Lindsay, M.J., **1985**. The efficacy of ivermectin against larvae of the screw-worm fly (*Chrysomya bezziana*). *Austral. Vet. J. 62*, 311–314.
Standfast, H.A., Muller, M.J., Wilson, D.D., **1984**. Mortality of *Culicoides brevitarsis* (Diptera: Ceratopogonidae) fed on cattle treated with ivermectin. *J. Econ. Entomol. 77*, 419–421.
Stretton, A.O.W., Campbell, W.C., Babu, J.R., **1987**. Biological activity and mode of action of avermectins. In: Veech, A., Dickson, D.W. (Eds.), Vistas on Nematology. Society for Nematologists, Marceline, MO, pp. 136–146.
Strong, L., **1992**. The use and abuse of feed-through compounds in cattle treatments. *Bull. Entomol. Res. 82*, 1–4.
Strong, L., **1986**. Inhibition of pupariation and adult development in *Calliphora vomitoria* treated with ivermectin. *Entomol. Exp. Appl. 41*, 157–164.
Strong, L., Brown, T.A., **1987**. Avermectins in insect control and biology: a review. *Bull. Entomol. Res. 77*, 357–389.
Strong, L., James, S., **1993**. Some effects of ivermectin on the yellow dung fly, *Scatophaga stercoraria*. *Vet. Parasitol. 48*, 181–191.
Strong, L., Wall, R., **1994**. Effects of ivermectin and moxidectin on the insects of cattle dung. *Bull. Entomol. Res. 84*, 403–409.
Su, N.-Y., Tamashiro, M., Haverty, M.I., **1987**. Characterization of slow-acting insecticides for the remedial control of the Formosan termite (Isoptera: Rhinotermitidae). *J. Econ. Entomol. 80*, 1–4.
Takiguchi, Y., Mishna, H., Okuda, M., Tenao, M., **1980**. Milbemycins, a new family of macrolide antibiotics: fermentation, isolation and physico-chemical properties. *J. Antibiotics 33*, 1120–1127.
Takai, K., Suzuki, T., Kawazu, K., **2003**. Development and preventative effect against pine wilt disease of a novel liquid formulation of emamectin benzoate. *Pest Mgt Sci. 59*, 365–370.
Tanaka, K., **1987**. Mode of action of insecticidal compounds acting at an inhibitory synapse. *J. Pestic. Sci. 12*, 549–560.
Tanaka, K., Matsumura, F., **1985**. Action of avermectin B_{1a} on the leg muscles and the nervous system of the American cockroach. *Pestic. Biochem. Physiol. 29*, 124–135.
Taylor, S.M., Mallon, T.R., Blanchflower, W.J., Kennedy, D.G., Green, W.P., **1992**. Effects of diet on plasma concentrations of oral anthelminthics for cattle and sheep. *Vet. Rec. 130*, 264–268.

Thompson, D.R., Rugg, D., Scott, P.G., Cramer, L.G., Barrick, R.A., **1994**. Rainfall and breed effects on the efficacy of ivermectin jetting fluid for the prevention of fly strike and treatment of infestations of lice in long-woolled sheep. *Austral. Vet. J. 71*, 161–164.

Titchener, N.R., Parry, J.M., Grimshaw, W.T.R., **1994**. Efficacy of formulations of abamectin, ivermectin and moxidectin against sucking and biting lice of cattle. *Vet. Rec. 134*, 452–453.

Tsukamoto, T., Sato, K., Kinoto, T., Yanai, T., Nishida, A., *et al.*, **1995**. Syntheses of 8,9-epoxy- and 5-*O*-acyl-8,9-epoxymilbemycin A4 and their activity against *Tetranychus urticae*. *Biosci. Biotech. Biochem. 59*, 226–230.

Trumble, J.T., Alvarado Rodriguez, B., **1993**. Development and economic evaluation of an IPM program for fresh market tomato production in Mexico. *Agric. Ecosyst. Environ. 43*, 267–284.

Trumble, J.T., Morse, J.P., **1993**. Economics of integrating the predaceous mite *Phytoseiulus persimilis* (Acari: Phytoseiidae) with pesticides in strawberries. *J. Econ. Entomol. 86*, 879–885.

Trumble, J.T., Moar, W.J., Babu, J.R., Dybas, R.A., **1987**. Laboratory bioassays of the acute and antifeedant effects of avermectin B1 and a related analogue on *Spodoptera exigua* (Hubner). *J. Agric. Entomol. 4*, 21–28.

Vassilatis, D.K., Arena, J.P., Plasterk, R.H., Wilkinson, H.A., Schaeffer, J.M., *et al.*, **1997**. Genetic and biochemical evidence for a novel avermectin-sensitive chloride channel in *Caenorhabditis elegans*: isolation and characterization. *J. Biol. Chem. 272*, 33167–33174.

Vercruysse, J., Rew, R.S. (Eds.) **2002a**. Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford.

Vercruysse, J., Rew, R.S., **2002b**. General efficacy of the macrocyclic lactones to control parasites of cattle. In: Vercruysse, J., Rew, R.S. (Eds.), Macrocyclic Lactones in Antiparasitic Therapy. CAB International, Wallingford, pp. 185–222.

Wall, R., Strong, L., **1987**. Environmental consequences of treating cattle with the antiparasitic drug ivermectin. *Nature 327*, 418–421.

Wardhaugh, K.G., Rodriguez-Mendez, H., **1988**. The effects of the antiparasitic drug, ivermectin, on the development and survival of the dung breeding fly, *Orthelia cornicina* (F.) and the scarabaeine dung beetles, *Copris hispanus* L., *Bubas bubalus* (Oliver) and *Onitis belial* F. *J. Appl. Entomol. 106*, 381–389.

Wardhaugh, K.G., Longstaff, B.C., Morton, R., **2001**. A comparison of the development and survival of the dung beetle, *Onthophagus taurus* (Schreb.) when fed on the faeces of cattle treated with pour-on formulations of eprinomectin or moxidectin. *Vet. Parasitol. 99*, 155–168.

Webb, J.D., Burg, J.G., Knapp, F.W., **1991**. Moxidectin evaluation against *Solenoptes capillatus* (Anoplura: Linognathidae), *Bovicola bovis* (Mallophaga: Trichodectidae), and *Musca autumnalis* (Diptera: Muscidae) on cattle. *J. Econ. Entomol. 84*, 1266–1269.

Wislocki, P.G., Grosso, L.S., Dybas, R.A., **1989**. Environmental aspects of use in crop protection. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 182–214.

Wratten, S.D., Mead-Briggs, M., Gettinby, G., Ericsson, G., Baggot, D.G., **1993**. An evaluation of the potential effects of ivermectin on the decomposition of cattle dung pats. *Vet. Rec. 133*, 365–371.

Wright, J.E., **1984**. Biological activity of $AVMB_1$ against the boll weevil (Coleoptera: Curculionidae). *J. Econ. Entomol. 77*, 1029–1032.

Wright, D.E., **1986**. Biological activity and mode of action of avermectins. In: Ford, M.D., Lunt, G.G., Deay, R.C., Usherwood, P.N.R. (Eds.), Neuropharmacology and Pesticide Action. Ellis Horwood, Chichester, pp. 174–202.

Wright, J.E., Jenkins, J.N., Villavaso, E.J., **1985**. Evaluation of avermectin B_1 (MK-936) against *Heliothis* spp. in the laboratory and in field plots and against the boll weevil in field plots. *Southwest. Entomol. 7* (Suppl.), 11–16.

Yamamoto, D., Suzuki, N., **1988**. Patch-clamp analysis of single chloride channels in primary neurone cultures of *Drosophila*. In: Lunt, G.G. (Ed.), Neurotoxicology ’88. pp. 375–381.

Yazwinski, T.A., Greenway, T., Preston, B.L., Pote, L.M., Featherstone, H., *et al.*, **1983**. Antiparasitic activity of ivermectin in naturally parasitized sheep. *Am. J. Vet. Res. 44*, 2186–2187.

Yoon, K.S., Nelson, J.O., Clark, J.M., **2002**. Selective induction of abamectin metabolism by dexamethasone, 3-methylcholanthrene, and phenobarbital in Colorado potato Beetle, *Leptinotarsa decemlineata* (Say). *Pestic. Biochem. Physiol. 73*, 74–86.

Zakson-Aiken, M., Gregory, L.M., Meinke, P.T., Shoop, W.L., **2001**. Systemic activity of the avermectins against the cat flea (Siphonaptera: Pulicidae). *J. Med. Entomol. 38*, 576–580.

Zchorifein, E., Roush, R.T., Sanderson, J.P., **1994**. Potential for integration of biological and chemical control of greenhouse whitefly (Homoptera: Aleyrodidae) using *Encarsia formosa* (Hymenoptera: Aphelinidae) and abamectin. *Environ. Entomol. 23*, 1277–1282.

Zeng, Z., Andrew, N.W., Woda, J.M., Halley, B.A., Crouch, L.S., *et al.*, **1996**. Role of cytochrome P450 isoforms in the metabolism of abamectin and ivermectin in rats. *J. Agric. Food Chem. 44*, 3374–3378.

Zhang, Z., Sanderson, J.P., **1990**. Relative toxicity of abamectin to the predatory mite *Phytoseiulus persimilis* (Acari: Phytoseiidae) and the two-spotted spider mite (Acari: Tetranychidae). *J. Econ. Entomol. 83*, 1783–1790.

Zhao, J.Z., Li, Y.X., Collins, M.L., Gusukuma-Minuto, L., Mau, R.F.L., *et al.*, **2002**. Monitoring and characterization of diamondback moth (Lepidoptera : Plutellidae) resistance to spinosad. *J. Econ. Entomol. 95*, 430–436.

Zufall, F., Franke, C., Hatt, H., **1989**. The insecticide avermectin B_{1a} activates a chloride channel in crayfish muscle membrane. *J. Exp. Biol. 142*, 191–205.

5.3 Neonicotinoid Insecticides

P Jeschke and R Nauen, Bayer CropScience AG,
Monheim, Germany

5.3.1. Introduction

The discovery of neonicotinoids as important novel pesticides can be considered a milestone in insecticide research of the past three decades. Neonicotinoids represent the fastest-growing class of insecticides introduced to the market since the commercialization of pyrethroids (Nauen and Bretschneider, 2002). Like the naturally occurring nicotine, all neonicotinoids act on the insect central nervous system (CNS) as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Bai *et al.*, 1991; Liu and Casida, 1993a; Yamamoto, 1996; Chao *et al.*, 1997; Zhang *et al.*, 2000; Nauen *et al.*, 2001), but, with remarkable selectivity and efficacy against pest insects while being safe for mammals. As a result of this mode of action there is no cross-resistance to conventional insecticide classes, and therefore the neonicotinoids have begun replacing pyrethroids, chlorinated hydrocarbons, organophosphates, carbamates, and several other classes of compounds as insecticides to control insect pests on major crops (Denholm *et al.*, 2002). Today the class of neonicotinoids are part of a single mode of action group as defined by the Insecticide Resistance Action Committee (IRAC; an Expert Committee of Crop Life) for pest management purposes (Nauen *et al.*, 2001).

Neonicotinoids are potent broad-spectrum insecticides possessing contact, stomach, and systemic activity. They are especially active on hemipteran pest species, such as aphids, whiteflies, and planthoppers, but they are also commercialized to control coleopteran and lepidopteran pest species (Elbert *et al.*, 1991, 1998). Because of their physicochemical properties they are useful for a wide range of different application techniques, including foliar, seed treatment, soil drench, and stem application in

several crops. Due to the favorable mammalian safety characteristics (Matsuda *et al.*, 1998; Yamamoto *et al.*, 1998; Tomizawa *et al.*, 2000) neonicotinoids like imidacloprid are also important for the control of subterranean pests, and for veterinary use (Mencke and Jeschke, 2002).

5.3.2. Neonicotinoid History

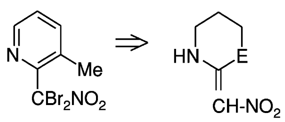
In the early 1970s, the former Shell Development Company's Biological Research Center in Modesto, California, invented a new class of nitromethylene heterocyclic compounds capable of acting on the nAChR. Starting with a random screening to discover lead structures from university sources, Shell detected the 2-(dibromo-nitromethyl)-3-methyl pyridine (SD-031588) (**Figure 1**) from a pool of chemicals from Prof. Henry Feuer of Purdue University (Feuer and Lawrence, 1969), which revealed an unexpected low-level insecticidal activity against housefly and pea aphid.

Further structural optimization of this insecticide lead structure led to the active six-membered tetrahydro-2-(nitromethylene)-2*H*-1,3-thiazine, nithiazine (SD-03565, SKI-71) (Soloway *et al.*, 1978, 1979; Schroeder and Flattum, 1984; Kollmeyer *et al.*, 1999). The molecular design by Shell appears rational and straightforward. The chemistry had been largely concentrated on the nitromethylene enamine skeleton (Kagabu, 2003a). Today, this early prototype can be considered as the first generation of the so-called neonicotinoid insecticides (**Figure 2**). Nithiazine showed higher activity than parathion against housefly adults (*Musca domestica*), and 1662 times higher activity against the target insect, the lepidopteran corn earworm larvae (*Helicoverpa zea*), combined with good systemic behavior in plants and low mammalian toxicity (Soloway *et al.*, 1978, 1979; Kollmeyer *et al.*, 1999; Tomizawa and Casida, 2003). However, due to the photochemically unstable 2-nitromethylene chromophore (**Figure 3**) in the field tests, nithiazine was never commercialized for broad agricultural use (Soloway *et al.*, 1978, 1979; Kagabu and Medej, 1995; Kagabu, 1997a; Kollmeyer *et al.*, 1999). Alternatively, photostabilization using the formyl moiety was not adequate for practical application (Kollmeyer *et al.*, 1999). Nevertheless, a knock-down fly product against *M. domestica* containing nithiazine as the active ingredient of a housefly trap device for poultry and animal husbandry has recently been commercialized (Kollmeyer *et al.*, 1999).

In the early 1980s synthesis work was initiated at Nihon Tokushu Noyaku Seizo K. K. (presently Bayer CropScience K. K.) on the basis of this first remarkable neonicotinoid lead structure and the unique insecticidal spectrum of activity (Kagabu *et al.*, 1992; Moriya *et al.*, 1992). Instead of the lepidopteran larva *H. zea*, the target insect for studying structure–activity relationships (SARs) and optimizing biological activity was the green rice leafhopper (*Nephotettix cincticeps*), because it is a major hemipteran pest of rice in Japan (Kagabu, 1997a). At the beginning of the project, a new pesticide screening method using rice seedlings was developed for continuous monitoring of the combined systemic and contact activities of compounds over 2 weeks against leafhoppers and planthoppers (Sone *et al.*, 1995).

The six-membered 2-(nitromethylene)-tetrahydro-1,3-thiazine ring was replaced with different N^1-substituted *N*-heterocyclic ring systems. Starting with 1-methyl-2-(nitromethylene)-imidazolidine, it was found that the activity depends on ring size (5 > 6 > 7-ring system). The N^1-benzylated 2-(nitromethylene)-imidazolidine 5-ring was the most active one. Introduction of substituted benzyl residues in the 1-position, like the *para*-chlorobenzyl moiety, and of nitrogen-containing *N*-hetarylmethyl

1970–1978 (Shell)

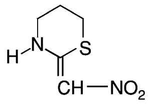
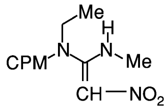
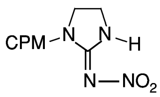
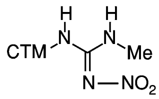
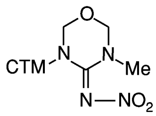
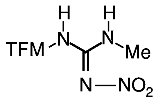
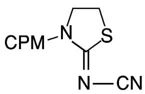
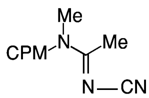


SD-031588

Compound		Toxicity index (TI)^a	
Shell no.	E	Pea aphid	Corn earworm
SD-031588		~5	0
SD-033420	CH_2	~3	155
SD-035347	NH	+	5.2
SD-035651	S	24	1662
Nithiazine (SKI-71)			

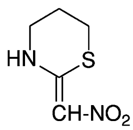
^{a}TI = (LC_{50} parathion/LC_{50} test compound) × 100.

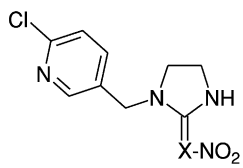
Figure 1 Development of nithiazine (SKI-71) by Shell. (Data from Kollmeyer, W.D., Flattum, R.F., Foster, J.P., Powel, J.E., Schroeder, M.E., *et al.*, **1999**. Discovery of the nitromethylene heterocycle insecticides. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 71–89 and Kagabu, S., **2003a**. Molecular design of neonicotinoids: past, present and future. In: Voss, G., Ramos, G. (Eds.), Chemistry of Crop Protection: Progress and Prospects in Science and Regulation. Wiley–VCH, New York, pp. 193–212.)

Pharmacophores *[—N—C(E)=X—Y]*	*Structure type* *Ring system* *(R^1—R^2, R^1—Z—R^2)*	*Noncyclic structure* *(R^1, R^2)*
Nitroenamines (E = S, N) (nitromethylenes) [—N—C(E)=CH—NO_2]	 Nithiazine	 Nitenpyram (1987)[a]
	 Imidacloprid (1985)[a]	 Clothianidin[b] (1989)[a]
Nitroguanidines (E = N) [—N—C(N)=N—NO_2]	 Thiamethoxam (1992)[a]	 (±)-Dinotefuran (1993)[a]
Cyanoamidines (E = S, Me) [—N—C(E)=N—CN]	 Thiacloprid (1986)[a]	 Acetamiprid (1988)[a]

[a]Year of the first patent application (priority date) covering the insecticide.

Figure 2 Chemical structures of the first generation neonicotinoid nithiazine and commercialized neonicotinoids displaying the different types of pharmacophors. CPM, 6-chloro-pyrid-3-ylmethyl; CTM, 2-chloro-1,3-thiazol-5-ylmethyl; TFM, (±)-6-tetrahydro-fur-3-ylmethyl. (Reproduced with permission from Jeschke, P., Schindler, M., Beck, M., **2002**. Neonicotinoid insecticides: retrospective consideration and prospects. *Proc. BCPC: Pests and Diseases 1*, 137–144.)



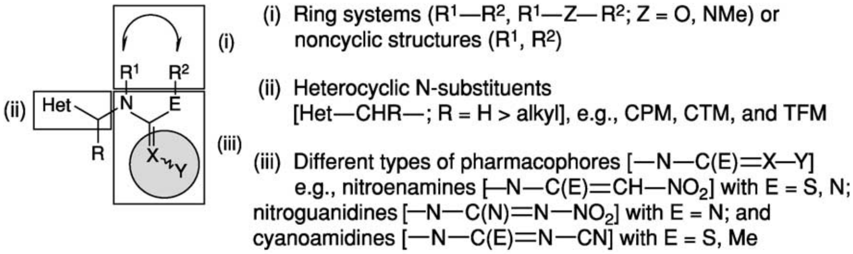


	Nithiazine (SKI-71)	*(NTN32692) X=—CH=*	*(NTN33893) Imidacloprid X=—N=*
λ_{max} (nm)	343	323	269
Water solubility (g l^{-1} at 20 °C)	200	3.4	0.57
LC_{90} (ppm)	40	0.32	0.32

Figure 3 Electronic absorption (λ_{max} in nm), water solubility (g l^{-1} at 20 °C), and insecticidal efficacy (ppm) of nithiazine (SKI-71), nitromethylene (NTN32692), and imidacloprid (NTN33893) against green rice leafhopper (*Nephotettix cincticeps*). LC_{90}, concentration at which >90% of insects are killed (Mencke and Jeschke, 2002).

residues, like the pyrid-3-ylmethyl moieties, enhanced the insecticidal activity by a factor of 5 and 25 when compared with the N^1-benzylated 2-(nitromethylene)-imidazolidine (Kagabu, 1997a). Yamamoto had previously recognized that the pyrid-3-ylmethyl residue was an essential moiety for insecticidal activity, by referring to the nAChR agonist model of Beers and Reich (1970), and thus synthesized a number of pyrid-3-yl-amines (Yamamoto and Tomizawa, 1993).

This chemical optimization procedure led to discovery of 1-(6-chloro-pyrid-3-ylmethyl)-2-nitro-methylene-imidazolidine (NTN32692), which had over 100-fold higher activity than nithiazine against *N. cincticeps* (using a strain resistant to organophosphorus compounds, methylcarbamates, and pyrethroides) (Kagabu *et al.*, 1992; Moriya *et al.*, 1992). However, the 2-nitromethylene chromophore of NTN32692 absorbs strongly (Figure 3) in sunlight (wavelength 290–400 nm), and rapidly decomposes



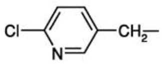
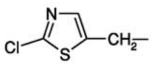
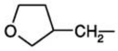
Structure of Het-CHR-	*Chemical name of this moiety*	*Abbreviation*
	6-Chloro-pyrid-3-ylmethyl-	CPM
	2-Chloro-1,3-thiazol-5-ylmethyl-	CTM
	(±)-6-Tetrahydro-fur-3-ylmethyl-	TFM

Figure 4 Structural segments for neonicotinoids.

in the field to noninsecticidal compounds. In order to avoid absorption of sunlight, a more stable functional group was necessary. After preparation of about 2000 compounds, imidacloprid (NTN33893) containing a 2-(*N*-nitroimino) group (see Section 5.3.3.1.1), the first member of the second-generation neonicotinoids, emerged from this project (Elbert *et al*., 1990, 1991; Bai *et al*., 1991; Nauen *et al*., 2001). The presence of an *N*-nitroimino group at the 2-position of the imidazolidine ring makes little difference to insecticidal activity compared with compounds that contain a nitomethylene group at this position, but the presence of $=N{-}NO_2$ significantly reduces affinity for the receptor (Liu *et al*., 1993b; Tomizawa and Yamamoto, 1993; Yamamoto *et al*., 1998). This suggests that the reduction in binding, resulting from the presence of the nitrogen atom at the 2-position, is compensated by the increase in hydrophobicity, which enhances transport to the target sites (Yamamoto *et al*., 1998; Matsuda *et al*., 2001). Compared with nithiazine, the biological efficacy of imidacloprid against the green rice leafhopper could be enhanced 125-fold. Furthermore, imidacloprid is about 10 000-fold more insecticidal than (*S*)-nicotine, a natural insecticide (Tomizawa and Yamamoto, 1993; Yamamoto *et al*., 1995). This breakthrough to the novel systemic insecticide imidacloprid was achieved by coupling a special heterocyclic group, the 6-chloro-pyrid-3-ylmethyl (CPM) residue (**Figure 4**), to the 2-(*N*-nitroimino)-imidazolidine building-block, new in this class of chemistry.

With the introduction of the insecticide imidacloprid to the market in 1991 Bayer AG began a successful era of the so-called CNI^{TM}s (chloronicotinyl insecticides *syn.* neonicotinoids), a milestone in insecticide research. Imidacloprid has, in the last decade, become the most successful, highly effective, and best selling insecticide worldwide used for crop protection and veterinary pest control.

In connection with these excellent results, a parallel change to other electron-withdrawing substituents like 2-*N*-cyanoimino, having essentially shorter maximum electronic absorptions than 290 nm, led to the discovery of thiacloprid (see Section 5.3.3.1.2), a second member of the CNI group. Attracted by Bayer's success with imidacloprid, several different companies such as Takeda (now Sumitomo Chemical Takeda Agro), Agro Kanesho, Nippon Soda, Mitsui Toatsu (now Mitsui Chemicals, Inc.), Ciba Geigy (now Syngenta), and others initiated intensive research and developed their own neonicotinoid insecticides. Research in these companies was facilitated because neonicotinoid chemistry showed a relatively broad spectrum of activity (Wollweber and Tietjen, 1999; Roslavtseva, 2000). Since the market introduction of imidacloprid, neonicotinoids have become the fastest-growing class of chemical insecticides. This tremendous success can be explained by their unique chemical and biological properties, such as broad-spectrum insecticidal activity, low application rates, excellent systemic characteristics such as uptake and translocation in plants, new mode of action, and favorable safety profile.

5.3.3. Chemical Structure of Neonicotinoids

In general, all these commercialized or developed compounds can be divided into ring systems

containing neonicotinoids, and neonicotinoids having noncyclic structures which differ in their molecular characteristics. Structural requirements for both ring systems containing neonicotinoids and neonicotinoids having noncyclic structures consist of different segments (Nauen *et al.*, 2001): the bridging fragment [R^1–R^2] (i) and for the noncyclic type compounds, the separate substituents [R^1, R^2] (i), the heterocyclic group [Het] (ii), the bridging chain [–CHR–] and the functional group [=X–Y] as part of the pharmacophore [–N–C(E)=X–Y] (iii) (**Figures 2** and **4**, **Table 1**).

The methylene group is normally used as the bridging chain (–CHR– with R = H). Other groups such as an ethylene or substituted methylene group decrease the biological activity. The pharmacophore (iii) can be represented by the group [–N–C(E) =X–Y], where =X–Y is an electron-withdrawing group and E is a NH, NR′, CH_2, O, or S moiety. It is well known that the pharmacophore type influences the insecticidal activity of the neonicotinoids. In general, the greatest insecticidal activity is observed for the compounds containing the nitroenamine (nitromethylene) [–N–C(E)=CH–NO_2; E = S, N], nitroguanidine [–N–C(E)=N–NO_2; E = N], or cyanoamidine [–N–C(E)=N–CN; E = S, CH_3] pharmacophore (Shiokawa *et al.*, 1995). Besides its influence on biological activity, the pharmacophore is also responsible for some specific properties such as photolytic stability, degradation in soil, metabolism in plants, and toxicity to different animals (Kagabu *et al.*, 1992; Moriya *et al.*, 1992; Minamida *et al.*, 1993; Tomizawa and Yamamoto, 1993; Shiokawa *et al.*, 1994; Tabuchi *et al.*, 1994).

The term "neonicotinoid" (Yamamoto, 1998, 1999) was originally proposed for imidacloprid and related insecticidal compounds with structural similarity to the insecticidal alkaloid (*S*)-nicotine, which has a similar mode of action (Tomizawa and Yamamoto, 1993; Tomizawa and Casida, 2003). In addition, a variety of terms have been used to subdivide this chemical class based on structural fragments (**Figure 4**): (a) chloronicotinyls (CNIs, heterocyclic group CPM) (Leicht, 1993), (b) thianicotinyls (heterocyclic group CTM) (Maienfisch *et al.*, 1999a), (c) furanicotinyls (heteroalicyclic group, TFM) (Wakita *et al.*, 2003), (d) nitroimines or nitroguanidines, (e) nitromethylenes, (f) cyanoimines (functional group as part of the pharmacophore), (g) first generation with moiety CPM, (h) second generation with moiety CTM or TFM (Maienfisch *et al.*, 1999a, 2001b), and third generation with moiety TFM (regarding the type of the heterocyclic group) (Wakita *et al.*, 2003).

5.3.3.1. Ring Systems Containing Commercial Neonicotinoids

Ring systems containing neonicotinoids can have as bridging fragment [R^1–R^2] (i) an alkylene group in the building-block, e.g., ethylene for imidacloprid and thiacloprid (five-membered ring systems) which can also be interrupted by heteroatoms like oxygen (six-membered ring systems) as shown in the case of thiamethoxam.

5.3.3.1.1. 1-[(6-Chloro-3-pyridinyl)-methyl]-*N*-nitro-2-imidazolidinimine (imidacloprid, NTN 33893) Imidacloprid was discovered in 1985 as a novel insecticide with a unique structure and with hitherto unrecognized insecticidal performance (Moriya *et al.*, 1992, 1993; Kagabu, 1997a, 2003a). The trade names for soil application/seed treatment are Admire™ and Gaucho® and the trade names for foliar application are Confidor® and Provado®, while for termite control it is Premise® (see Section 5.3.10).

The first laboratory synthesis was carried out by reduction of 6-chloronicotinoyl chloride to 2-chloro-5-hydroxymethyl-pyridine using an excess $NaBH_4$ in water, and conversion to the chloride by $SOCl_2$. Imidacloprid was obtained by the coupling reaction of 2-chloro-5-chloromethyl-pyridine (CCMP) (Diehr *et al.*, 1991; Wollweber and Tietjen, 1999) with the 5-ring building-block 2-nitro-imino-imidazolidine, in acetonitrile with potassium carbonate as base. This method was also successfully applied to the synthesis of [^{3}H]imidacloprid using $NaB[^3H]_4$ (Latli *et al.*, 1996).

Differences in photostability between the 2-nitromethylene group in compound NTN32692 (λ_{max} = 323 nm) and the 2-(*N*-nitroimino) group in imidacloprid (λ_{max} = 269 nm) (**Figure 3**) were examined by quantum chemical methods like AM1 and *ab initio* calculations (Kagabu, 1997a; Kagabu and Akagi, 1997). Crystallographic analysis of imidacloprid revealed a coplanar relationship of the imidazolidine 5-ring to the nitroimino group in position 2 (Born, 1991; Kagabu and Matsuno, 1997). Shorter ring C–N bond lengths and longer exocyclic C=N bond lengths were observed, suggesting the formation of a planar electron delocalized diene system, a so-called push–pull olefin (Kagabu and Matsuno, 1997). An intramolecular hydrogen bond between $^1NH \cdots O_2N{-}N{=}C^2$ was confirmed by characteristic resonances in the nuclear magnetic resonance (NMR) spectra (correlation spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY)). In addition, the infrared (IR) spectrum showed a highly chelated ^{1}NH absorption by intense bands at 3356 and 3310 cm^{-1} (Kagabu *et al.*, 1998).

Table 1 Chemical classification of nithiazine and commercialized neonicotinoids

Common name	*Scientific name* — *IUPAC name*	*Chemical Abstracts name*	*CAS registry no.*	*Development codes*	*Empirical formula*	*Molecular weight* (g mol^{-1})
Nithiazine	2-Nitromethylene-1,3-thiazinane	Tetrahydro-2-(nitromethylen)-2H-1,3-thiazine	[58842-20-9]	SD-035651[a] I*N*-A0159[b]	$C_5H_8N_2O_2S$	160.2
Imidacloprid	1-(6-Chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylidenamine	1-[(6-Chloro-3-pyridinyl)-methyl]-*N*-nitro-2-imidazolidinimine	[138261-41-3]	NTN 33893	$C_9H_{10}ClN_5O_2$	255.7
Thiacloprid	*N*-[3-(6-Chloro-pyridin-3-ylmethyl)-thiazolidin-2-ylidene]-cyanamide	Cyanamidine, [3-[(6-chloro-3-pyridinyl)methyl-2-thiazolidinylidene]	[111988-49-9]	YRC 2894	$C_{10}H_9ClN_4S$	252.8
Thiamethoxam	3-(2-Chloro-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitro)amine	3-[(2-Chloro-5-thiazolyl)methyl]tetrahydro-5-methyl-*N*-nitro-4H-1,3,5-oxadiazin-4-imine	[153719-23-4]	CGA293′343	$C_8H_{10}ClN_5O_3S$	291.7
Nitenpyram	(*E*)-*N*-(6-Chloro-3-pyridylmethyl)-*N*-ethyl-*N*′-methyl-2-nitrovinylidenediamine	*N*-[(6-Chloro-3-pyridinyl)methyl]-*N*-ethyl-*N*′-methyl-2-nitro-1,1-ethenediamine	[120738-89-8] [150824-47-8] (*E*-isomer)	TI-304	$C_{11}H_{15}ClN_4O_2$	270.7
Acetamiprid	(*E*)-N^1-[(6-Chloro-3-pyridyl)methyl]-N^2-cyano-N^1-methyl-acetamidine	(*E*)-*N*-[(6-chloro-3-pyridinyl)methyl]-*N*′-cyano-*N*-methyl-ethanimidamide	[135410-20-7]	NI-25	$C_{10}H_{11}ClN_4$	222.7
Clothianidin	(*E*)-1-(2-Chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine	[C(*E*)]-*N*-[(2-Chloro-5-thiazolyl)methyl]-*N*′-methyl-*N*″-nitroguanidine	[210880-92-5]	TI-435	$C_6H_8ClN_5O_2S$	249.7
(±)-Dinotefuran	(*RS*)-1-methyl-2-nitro-3-(tetrahydro-3-furylmethyl)guanidine	*N*-Methyl-*N*′-nitro-*N*″-[(tetrahydro-3-furanyl)methyl]guanidine	[165252-70-0]	MTI 446	$C_7H_{14}N_4O_3$	202.2

[a] Shell/DuPont.
[b] DuPont.

Studies were also done using comparative molecular field analysis (CoMFA), a technique for analysis of three-dimensional quantitative structure–activity relationships (3D QSAR) (Cramer *et al.*, 1988; Okazawa *et al.*, 1998). The model showed that the nitrogen atom of the imidacloprid CPM residue interacts with a hydrogen-donating site of nAChR, and the nitrogen atom at the 1-position of the imidazolidine 5-ring interacts with a negatively charged domain (Okazawa *et al.*, 2000). In comparison with (*S*)-nicotine the selective insecticidal mode of action of imidacloprid was well examined in detail by different researchers (Yamamoto *et al.*, 1995; Kagabu, 1997b; Matsuda *et al.*, 1999; Tomizawa, 2000). There studies focused on the chemical structure, state of ionization, and hydrophobicity of both molecules (**Figure 5**).

The principal target insect pests of imidacloprid in agricultural and veterinary medicinal use are sucking insects. Since imidacloprid provides a partial positively charged δ^+ nitrogen atom (not ionized, full or partial) instead of an ammonium head as does (*S*)-nicotine, imidacloprid has little restriction for translocation into the target area compared to (*S*)-nicotine (Graton *et al.*, 2003). The interatomic distances between the 2-(*N*-nitroimino)-imidazolidine nitrogen and the pyridyl nitrogen are 5.9 Å, which is a suitable value to bind to the electron-rich and hydrogen-donating recognition sites on nAChR (Yamamoto *et al.*, 1995; Matsuda *et al.*, 1999). The electron-donating atom for hydrogen bonding at 5.9 Å apart from the fully or partial positive charged δ^+ nitrogen is required for potent interaction with the anionic subsite of the insect nAChR (Yamamoto *et al.*, 1995; Tomizawa, 2000). In mammals, imidacloprid has weak effects on any nAChR, while nicotine affects mostly peripheral nACRs resulting in higher toxicity.

The deduced electron deficiency of the nitrogen atom of imidacloprid was proved explicitly by ^{15}N NMR spectroscopic measurements (Yamamoto *et al.*, 1995). Tomizawa *et al.* (2000) recently claimed that the *N*-nitro group of imidacloprid is much more important than the bridgehead nitrogen in electrostatic interaction with nAChRs' because a Mulliken charge of the bridgehead nitrogen, calculated by the MNDO method combined with the PM3 method (semi-empirical molecular orbital technique

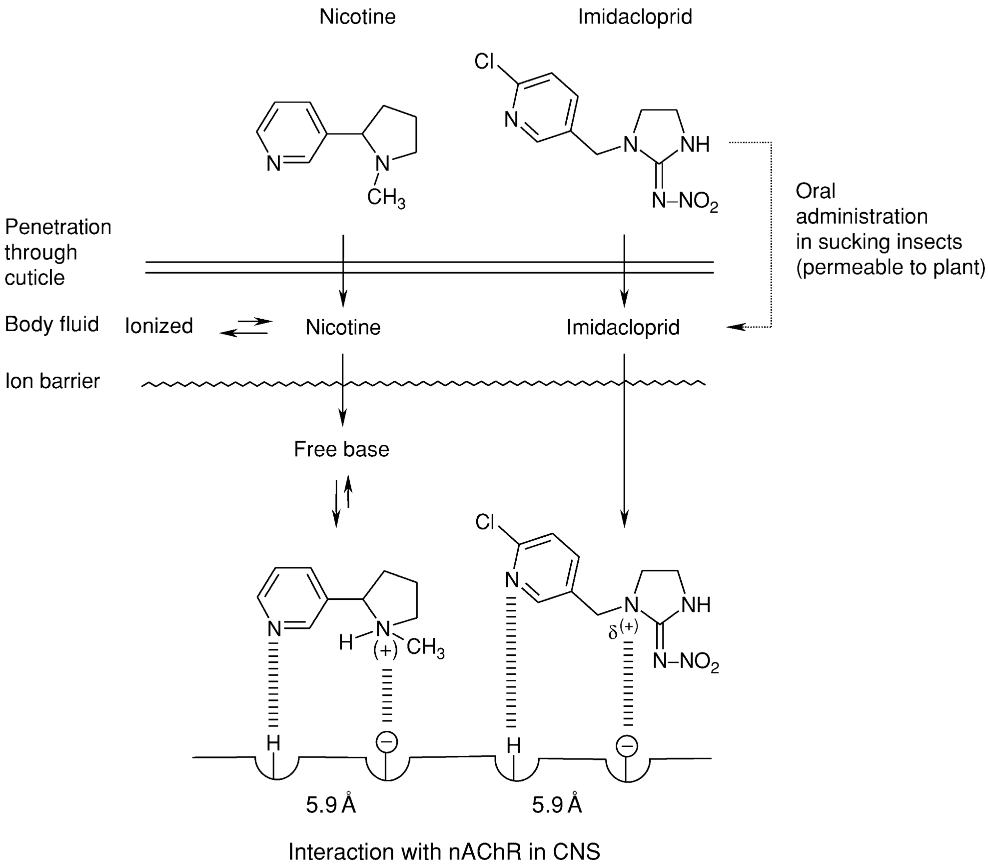


Figure 5 Insecticidal mode of action of (*S*)-nicotine and imidacloprid. (Adapted from Tomizawa, M., **2000**. Insect nicotinic acetylcholine receptors: mode of action of insecticide and functional architecture of the receptor. *Jap. J. Appl. Entomol. Zool. 44*, 1–15.)

for calculating electronic structure), was only marginally positive. However, Matsuda *et al*. (2001) suggested, that the electron-withdrawing power of the *N*-nitro group might be increased by a hydrogen bond involving the *N*-nitro group oxygen and hydrogen-bondable and positively charged amino acid residue, namely lysine or arginine, on the insect receptor, thereby strengthening its interaction with imidacloprid. Kagabu (1996) predicted the important contribution of this *N*-nitro group and its hydrogen-bondable property for insecticidal activity.

5.3.3.1.2. [3-(6-Chloro-3-pyridinyl)methyl-2-thiazolidinylidene]-cyanamidine (thiacloprid, YRC 2894) The second member of the CNI™ family from Bayer AG launched in 2000 was thiacloprid (Elbert *et al*., 2000; Yaguchi and Sato, 2001). Similar to imidacloprid, this neonicotinoid thiacloprid (YRC 2894, registered worldwide under the trade name Calypso™, and in Japan and Switzerland under the trade names Bariard™ and Alanto™), also contains the unique CPM moiety attached to the cyclic 2-(cyanoimino)-thiazolidine (CIT) building-block. Thiacloprid can be synthesized by a convergent one-step technical process starting from two key intermediates, CIT and CCMP (Diehr *et al*., 1991; Wollweber and Tietjen, 1999; Jeschke *et al*., 2001). Thiacloprid crystallizes in two different modifications depending on the solvent. The compound crystallizes from dichloromethane as form I (melting point 136 °C) and from isopropanol as form II (melting point 128.3 °C). From its physicochemical data, the technical ingredient is form I. The results of X-ray crystallographic analysis and an X-ray powder diffraction profile indicate that each form, I ($0.6 \times 0.3 \times 0.2\,mm^3$) and II ($0.5 \times 0.2 \times 0.1\,mm^3$), crystallizes with different cell constants in a monoclinic crystal system in the space group $P2_1/c$ (Jeschke *et al*., 2001). **Figure 6** shows the Ortep plot of the crystal structure of the form I. Interestingly, in the crystal lattice of form II, two symmetric independent molecules in the asymmetric unit cell are fixed (**Figure 6**).

With regard to the configuration of the pharmacophore [–N–C(S)=N–CN] moiety, thiacloprid exists only in the *Z*-configuration in both forms, I and II. In conjunction with the X-ray results, thiacloprid exists in solution exclusively in the stable *Z*-configuration. With respect to the C=N double bond, only one configuration is seen in all NMR spectra (^1H, ^{13}C, HMQC, HMBC, NMR spectroscopy), deduced from very sharp proton and carbon signals. In conjunction with X-ray results, there is no evidence for isomerization of the *Z*-isomer in DMSO-d_6 or $CDCl_3$ solution. Thiacloprid shows a single peak maximum at 242 nm in its ultraviolet (UV) spectrum, and it does not absorb natural sunlight. This explains thiacloprid's better photostability in comparison to other neonicotinoids (Jeschke *et al*., 2001).

Starting from forcefield methods (MMFF94s), the final energies, geometries, and properties were obtained at DFT (Becke and Perdew (BP) functional, triple-zeta valence plus (TZVP) polarization basis, conductor-like screening model – realistic solvent (COSMO-RS) for solvent effects; Perdew, 1986;

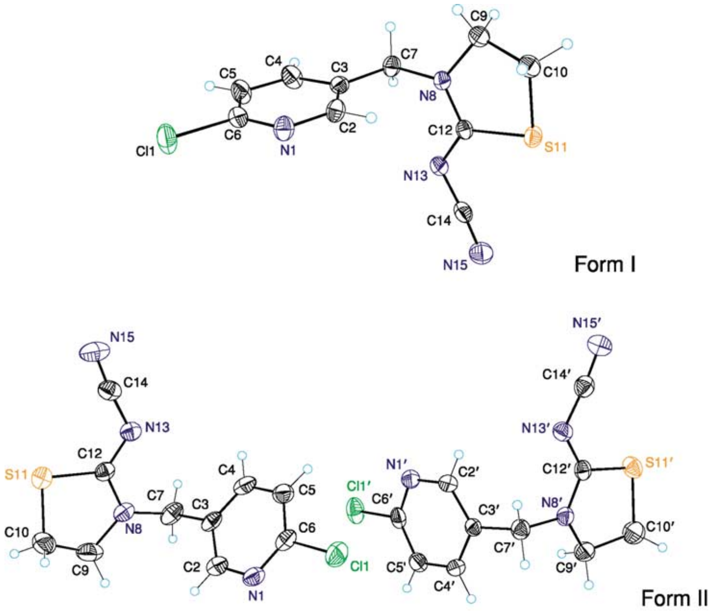


Figure 6 Ortep plot of thiacloprid form I and form II (Jeschke *et al*., 2001).

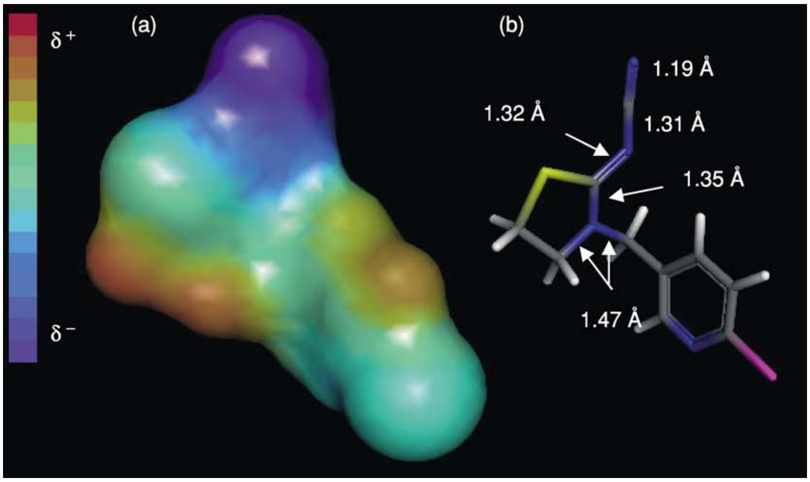


Figure 7 DFT (BR/TZVP) optimized geometry of thiacloprid (Jeschke *et al.*, 2001). (a) Atomic charges projected onto a Conolly surface; (b) some interatomic distances in Å.

Becke, 1988; Klamt, 1995) level of theory (Jeschke *et al.*, 2001) (**Figure 7**). It was found that, in the gas phase, *Z*-configured thiacloprid is about 4 kcal mol^{-1} lower in energy than the *E*-isomer. In water, this difference is increased by approximately 1 kcal mol^{-1}. The preference for the *Z*-configuration stems mainly from steric reasons. The nitrile moiety of the *Z*-isomer has virtually no close contacts to any other atom (sulfur to nitrile-carbon distance 3.1 Å). In the *E*-isomer, however, the largest possible distance, between the nitrile-carbon and the hydrogen atoms attached to the bridging carbon, is around 2.3 Å. Quantum chemical calculations show that the strong delocalization of the C=N double bond does not reduce the "double bond character" significantly (i.e., the torsional barrier for rotation around the C=N double bond is not decreased). The molecular dipole moment (derived from the electric field dependence of the DFT wave function) is calculated to be 10.2 D in water. This, together with the spatial relation of the pharmacophoric feature (–N–C(S)=N–CN), is very much in line with the pharmacophore model of imidacloprid.

5.3.3.1.3. 3-[(2-Chloro-5-thiazolyl)methyl]tetrahydro-5-methyl-*N*-nitro-4H-1,3,5-oxadiazin-4-imine (thiamethoxam, CGA 293′343) Ciba started a research program on neonicotinoids in 1985 (Maienfisch *et al.*, 2001a), and investigated variations of the imidacloprid nitroimino-heterocycle (Maienfisch *et al.*, 2001b). Thiamethoxam (Senn *et al.*, 1998; Maienfisch *et al.*, 1999a, 1999b) was discovered, and developed by Ciba Crop Protection (from 1996 Novartis Crop Protection, now Syngenta Crop Protection) in 1991. Thiamethoxam has been marketed since 1998 under the trademarks Actara® for foliar and soil treatment, and Cruiser® for seed treatment. Thiamethoxam can be synthesized starting from *N*-methyl-nitroguanidine by treatment with formaldehyde in the presence of formic acid (Göbel *et al.*, 1999). Finally alkylation with 2-chlorothiazol-5-ylmethylchloride in *N*,*N*-dimethyl-formamide and potassium carbonate as a base afforded the active ingredient in good yields (Maienfisch *et al.*, 1999a).

The SAR profile of this compound demonstrates the rather limited variability of the pharmacophore (–N–C(N)=N–NO_2). Insecticidal activity was only observed if the functional group is a strongly electron-withdrawing group and has a hydrogen accepting head, like *N*-nitroimino and *N*-cyanoimino. As pharmacophore *N*-substituent, a methyl group is clearly superior to hydrogen, an acyl substituent or C_2–C_4 alkyl group. The biological results have led to a general SAR profile for these novel compounds, which can be summarized as follows (Maienfisch *et al.*, 1999b, 2002):

1. All variations of the pharmacophore (N–C(N)=N–NO_2) resulted in a loss of activity.
2. Substitution of the CTM moiety by another N-containing heterocycle reduced the overall insecticidal activity, whereas CPM gave the best results.
3. A perhydro-1,3,5-oxadiazine ring system is clearly better than all other heterocyclic systems like perhydro-1,3,5-thiadiazines, hexahydro-1,3,5-triazines, or hexahydro-1,3-diazines.
4. Introduction of *N*-methyl at the 5-position led to strong increase of insecticidal activity (in contrast to the SAR of imidacloprid), whereas other substituents resulted in clear loss of activity.

5.3.3.2. Neonicotinoids Having Noncyclic Structures

From the new chemical class of ring system containing neonicotinoids noncyclic structures having the same mode of action can also be deduced. These noncyclic type neonicotinoids can have as separate substituents [R^1, R^2] (i), e.g., for R^1 hydrogen or alkyl like methyl (acetamiprid) and ethyl (nitenpyram) and in the case of E = NH for the substituent R^2 alkyl like methyl (clothianidin and dinotefuran), respectively.

5.3.3.2.1. *N*-[(6-chloro-3-pyridinyl)methyl]-*N*-ethyl-*N*′-methyl-2-nitro-1,1,-ethenediamine (nitenpyram, TI-304) Starting from the cyclic nithiazine (Soloway *et al*., 1978), the acyclic nitenpyram (Bestguard™) was discovered during optimization of substituents of an acyclic nitroethene (Minamida *et al*., 1993). The insecticidal activity of 1-(3-pyridylamino)-2-nitroethene compounds against the brown planthopper and the green leafhopper were studied. It was found that the 1-methylamino-1-(pyrid-3-ylmethylamino)-2-nitroethene was more active than the corresponding 1-ethyl, 1-methoxy, or 1-thiomethoxy derivatives. Introduction of sterically large amino groups like ethylamino, isopropylamino, hydrazino, *N*-pyrrolidino, or N-morpholino decreased the activity against the green rice leafhopper. Furthermore, the methylene bridge between the pyrid-3-yl and nitrogen was replaced with other linkages. However, all attempts at shortening and lengthening the linkage failed to increase the insecticidal activities against the brown planthopper and the green rice leafhopper (Akayama and Minamida, 1999). Heterocyclic aromatic substituents, different from pyrid-3-yl, were incorporated into the nitroethen structure. The replacement of pyrid-3-yl with 6-fluoro-pyrid-3-yl, 6-chloro-pyrid-3-yl, 6-bromo-pyrid-3-yl, and 2-chloro-1,3-thiazol-5-yl enhanced the activity against the brown planthopper.

5.3.3.2.2. (*E*)-*N*-[(6-chloro-3-pyridinyl)methyl]-*N*′-cyano-*N*-methyl-ethanimidamide (acetamiprid, NI-25) Acetamiprid (Takahashi *et al*., 1992; Matsuda and Takahashi, 1996) has an *N*-cyanoamidine structure, which contains in analogy to imidacloprid and thiacloprid the CPM moiety. This neonicotinoid was invented during a search for nitromethylene derivatives by Nippon Soda Co. Ltd. in 1989 and was registered in 1995 in Japan. The insecticide is marketed under the trade name Mospilan® for crop protection. The noncyclic acetamiprid was discovered by optimization studies of special 2-*N*-cyanoimino compounds with an imidazolidine 5-ring obtained from Nihon Bayer (Yamada *et al*., 1999). The noncyclic 2-nitromethylene and 2-*N*-nitroimine derivatives are less active than the corresponding ring structures, but numerous noncyclic derivatives show excellent activities against the armyworm and aphid as well as the cockroach. Regarding the substituent on the amino group, the *N*-methyl group exhibited the highest activity against the diamondback moth, while derivatives with hydrogen, *N*-methyl, and *N*-ethyl showed potent activity against the cotton aphid (Yamada *et al*., 1999).

5.3.3.2.3. [C(*E*)]-*N*-[(2-chloro-5-thiazolyl)methyl]-*N*′-methyl-*N*″-nitroguanidine (clothianidin, TI-435) As a result of continuous investigations of noncyclic neonicotinoids, researchers from Nihon Bayer Agrochem and Takeda in Japan found that these noncyclic neonicotinoids showed high activities against sucking insects. In the optimization process, Takeda researchers were able to demonstrate that compounds containing the nitroguanidine moiety, coupled with the thiazol-5-ylmethyl residue, have increased activity against some lepidopteran pests (Uneme *et al*., 1999). This is in accordance with the general pharmacophore [–N–C(E)=X–Y], where =X–Y is an electron-withdrawing group such as =N–NO_2, and E represents the NH–Me unit. After further optimization in this subclass, clothianidin (TI-435) emerged as the most promising derivative from this program (Ohkawara *et al*., 2002; Jeschke *et al*., 2003). Clothianidin has already been registered in Japan for foliar and soil applications under the trade names Dantotsu™ and Fullswing™ (Sumitomo Chemical Takeda Agro), and also in Korea, Taiwan, and other countries. Registrations have also been granted in North America and Europe for seed treatment under the brand name Poncho® (Bayer CropScience).

In the novel noncyclic structure of clothianidin (TI-435) (i.e., R = H; E = NHMe), the *N*-nitroguanidine pharmacophore [–N–C(N)=N–NO_2] is similar to that of imidacloprid, but the CPM group has been replaced by the CTM moiety (Ohkawara *et al*., 2002) (**Figure 2**). Clothianidin crystallizes under normal laboratory conditions using common solvents as needlelike crystals containing no additional solvent or water molecules. The best-quality crystals for X-ray structure analysis were obtained by slow evaporation of a methanol/H_2O (1 : 1) solution at room temperature. **Figure 8** shows a photograph using polarized light of the crystal needle with the dimensions $0.90 \times 0.30 \times 0.07\ mm^3$ used for X-ray structure analysis (Jeschke *et al*., 2003). Clothianidin (TI-435) was subjected to conformational sampling using forcefield methods (MMFF94s).

The calculated free energies indicate that at room temperature (RT), the preferred orientation of the *N*-nitroimino group is in the *trans* position; the *Z*-isomer with lowest energy is more than 2.6 kcal mol^{-1} above the optimal *E*-isomer. NMR experiments are in agreement that the *N*-nitroimino group strongly prefers one orientation only. From calculations as well as the X-ray structure, one can see that the three C–N bonds involving atom C5 have some double bond character. It is worth mentioning that the C5–N3 bond, which is the only formal C=N double bond within the *N*-nitroimino moiety, is slightly longer than the formal single bonds C5–N5 and C5–N2. This is reflected by the torsional angles found around the C–N bonds during conformational analysis; the respective values are all 180° or −180°. The *N*-methyl group can flip easily from the anti position into a syn position. The energies of the respective clothianidin (TI-435) conformers, relative to the optimal structure, are below 1.5 kcal mol^{-1}. All these findings are in line with the experimental ^{13}C NMR spectrum, which shows a relatively broad singlet at 138.8 ppm for the atom C3. This can already be understood qualitatively from the conformational arguments, like rotations of the C–N single bonds and modifications of the positioning of the CTM moiety (Jeschke *et al.*, 2003).

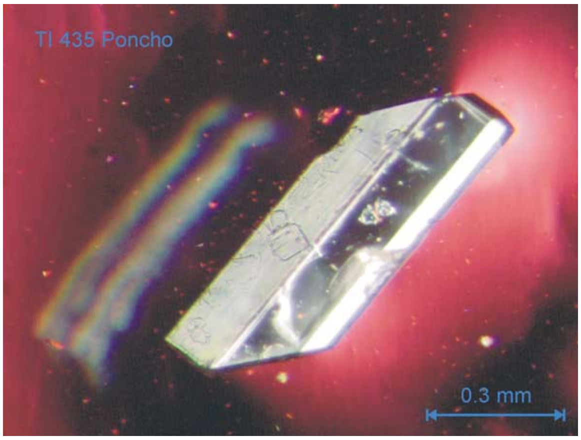


Figure 8 Digital stereo photomicrograph of a single crystal of clothianidin (TI-435) grown from methanol/water at room temperature (Jeschke *et al.*, 2003).

5.3.3.2.4. (±)-*N*-methyl-*N*′-nitro-*N*″-[(tetrahydro-3-furanyl)methyl]guanidine (dinotefuran, MTI-446) (±)-Dinotefuran (MTI-446) was discovered by Mitsui Chemicals Inc., and is highly effective as an agonist of nAChRs (Zhang *et al.*, 2000). It has a broad spectrum of activity against insect pests and has low mammalian toxicity (Kodaka *et al.*, 1998, 1999a, 1999b; Hirase, 2003). The trade names for the commercialized product are Starkle® and Albarin®. Similar to imidacloprid and clothianidin, it has a *N*-nitroguanidine pharmacophore [–N–C(N)=N–NO_2], but an alicyclic (±)-tetrahydro-3-furylmethyl (TFM) moiety instead of the halogenated heteroaromatic CPM or CTM moiety in the other neonicotinoids. The discovery of (±)-dinotefuran resulted from the idea of incorporating an *N*-nitroimino fragment into the structure of acetylcholine (Kodaka *et al.*, 1998; Wakita *et al.*, 2003). (±)-Dinotefuran bears a nonaromatic oxygen atom in the position corresponding to that of the aromatic nitrogen atom of the other neonicotinoids. The potencies of (±)-dinotefuran and a competitive nAChR antagonist (α-bungarotoxin, BGT) in inhibiting [^{3}H]epibatidine binding to *Periplaneta americana* nerve cord membranes were examined (Mori *et al.*, 2001). (±) and (+) Dinotefuran inhibited [^{3}H]epibatidine binding with IC_{50} (inhibitory concentration, where 50% of the tritiated ligand is displaced in membrane preparations from cockroach nerve) values of 890 and 856 nM, respectively. The (−)-enantiomer was about twofold less effective. In contrast the (+)-enantiomer was approximately 50-fold more insecticidal than the (−)-enantiomer of dinotefuran (Table 2).

The SAR between the tetrahydrofuran ring moiety and the insecticidal activity was investigated (Kiriyama and Nishimura, 2002), and it was found that:

Table 2 Potency of dinotefuran and its isomers in inhibiting [^{3}H]EPI and [^{3}H]α-BGT binding, and their *in vivo* activities

Compound	*[^{3}H]EPI binding, IC_{50} (nM)[a]*	*[^{3}H]α-BGT binding, IC_{50} (nM)[a]*	*Knockdown, KD_{50} (nmol g^{-1})*	*Insecticidal activity, LD_{50} (nmol g^{-1})[a]*
(±)-Dinotefuran	890 (626–1264)	36.1 (24.9–52.2)	0.351	0.173 (0.104–0.287)
(+)-Dinotefuran	856 (590–1241)	9.58 (6.79–13.52)	0.123	0.0545 (0.0396–0.0792)
(−)-Dinotefuran	1890 (1230–2890)	69.8 (47.1–103.4)	6.70	2.67 (1.55–4.69)

[a]95% confidence limits in parentheses.

BGT, bungarotoxin; EPI, epibatidine.

Reproduced with permission from Mori, K., Okumoto, T., Kawahara, N., Ozoe, Y., **2001**. Interaction of dinotefuran and its analogues with nicotinic acetylcholine receptors of cockroach nerve cords. *Pest Mgt Sci. 58*, 190–196.

1. The tetrahydro-fur-2-ylmethyl derivative (shift of oxygen position) reflected diminished activity.
2. The tetrahydro-fur-3-yl moiety is important; the cyclopentanylmethyl (deoxy derivative), the 3-methoxy-*N*-propyl (open-ring ether) and the *N*-methyl-pyrrolidine-3-ylmethyl derivatives showed very low or negligible activity.
3. Introduction of a methyl group at the 4- or 5-position of the tetrahydro-fur-3-yl ring gave intermediate activity (Wakita *et al*., 2003), but introduction of methyl at the 2- or 3-position of the THF ring system and in α-position of the side chain decreases activity. An ethyl group reduced greatly activity even if it was at the 4-position (Wakita *et al*., 2003).

The structural factors in the *N*-nitro guanidine moiety of (±)-dinotefuran were described as follows:

1. Deletion or extension to the alkyl group, like ethyl, of the terminal *N*-methyl group and addition of one more *N*-methyl group usually decrease the activity.
2. Carbocyclic cyclization to 5- and 6-ring systems maintains the activity.
3. Perhydro-1,3,5-oxadiazine 6-ring cyclization similar to that in thiamethoxam is inactive.
4. Modification of the *N*-nitroimino chromophore of dinotefuran to the nitromethylene, but not to the *N*-cyanoimino group maintains the insecticidal activity.

These results indicate that the modification of this moiety of (±)-dinotefuran results in drastic changes in potency and that the incorporation of structural fragments known from previous neonicotinoid insecticides does not necessarily lead to compounds retaining higher activity (Mori *et al*., 2001). Also in this structural type the distance of three methylene chains between the nitrogen on the imidazolidine ring and the hydrogen acceptor oxygen was important (Wakita *et al*., 2003).

5.3.3.3. Bioisosteric Segments of Neonicotinoids

Retrospective considerations regarding bioisosteric segments of the developed and commercial neonicotinoids gave insight into general structural requirements (segments i–iii, **Figure 4**) for all the different ring systems and noncyclic structures (Jeschke *et al*., 2002). Several common molecular features, when comparing compounds with ring systems, like imidazolidine (imidacloprid), and its isosteric alternatives, like thiazolidine (thiacloprid), perhydro-1,3,5-oxadiazine (thiamethoxam), or hexahydro-1,3,5-triazine (Agro Kanesho's AKD-1022) and the functional groups like *N*-nitroimino [=N–NO_2], *N*-cyanoimino [=N–CN], or nitromethylene [=CH–NO_2] of these insecticides, have been described (Tomizawa *et al*., 2000). After superposition of the most active derivatives, it was possible to state the molecular shape similarity of the second generation neonicotinoids. It was found that electrostatic similarity of the most active compounds correlates well with the binding affinity (Nakayama and Sukekawa, 1998; Sukekawa and Nakayama, 1999), and a similar correlation was obtained by CoMFA (Nakayama, 1998; Okazawa *et al*., 1998).

5.3.3.3.1. Ring systems versus noncyclic structures

In comparison to the corresponding ring systems, the noncyclic structures exhibit similar broad insecticidal activity by forming a so-called quasi-cyclic conformation when binding to the insect nAChR (Kagabu, 2003a). Thus, the three commercial noncyclic structures – nitenpyram, acetamiprid, and clothianidin – can be regarded as examples, if retrosynthetic considerations are carried out (Jeschke *et al*., 2002; Kagabu, 2003a). The noncyclic neonicotinoides are generally less lipophilic than the corresponding neonicotinoids with a ring structure (see Section 5.3.3.4).

Based on the CoMFA results, a binding model for imidacloprid was described. This model clarified that the nitrogen of the CPM moiety interacts with a hydrogen-donating site of the nAChR, and that the nitrogen atom at the 1-position of the imidazolidine ring interacts with the negatively charged domain (Nakayama, 1998; Okazawa *et al*., 1998). Furthermore, the binding activity of noncyclic structures (e.g., acetamiprid, nitenpyram, and related compounds) to the nAChR of houseflies was measured and the results were analyzed using CoMFA. Superposition of stable conformations of nitenpyram, acetamiprid, and imidacloprid showed that the preferred regions for negative electrostatic potentials near the oxygen atoms of the nitro group, as well as the sterically forbidden regions beyond the imidazolidine 3-nitrogen atom of imidacloprid, were important for binding (Okazawa *et al*., 2000). The area around the 6-chloro atom of the CPM moiety was described as a sterically permissible region. Apparently the steric interactions were more important for noncyclic neonicotinoids than for the cyclic derivative, imidacloprid. Finally, it was also demonstrated that the noncyclic structures bind to the nAChR recognition site in a manner similar to ring structures like imidacloprid, and that the electrostatic properties of the noncyclic amino and cyclic imidazolidine structures affected their binding affinity (**Figure 9**).

Figure 10 shows atom-based aligments of imidacloprid, clothianidin, dinotefuran and acetamiprid.

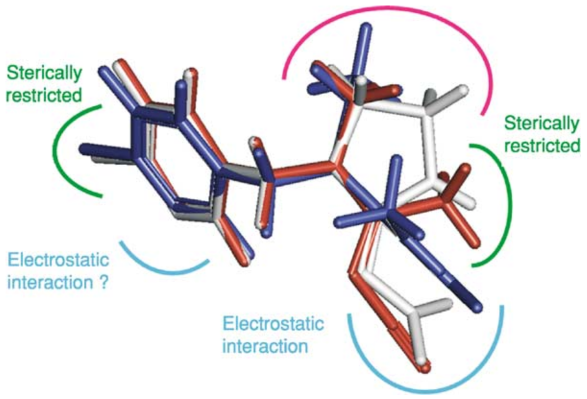


Figure 9 Stable conformations and predicted properties of binding site: imidacloprid (white), nitenpyram (blue), acetamiprid (red). (Adapted from Akazawa *et al.*, 2000.)

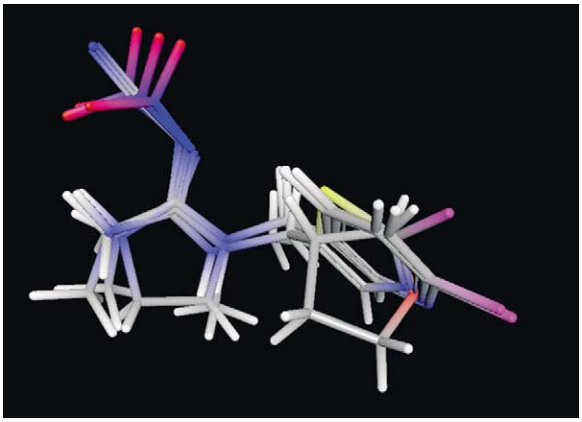


Figure 10 Alignment of DFT/BP/SVP/COSMO optimized geometries of imidacloprid, clothianidin, dinotefuran, and acetamiprid. The alignment was done by minimization of the mutual spatial distance of three pharmacophoric points, namely (i) the positively charged carbon atom connected to the =N–NO_2 and =N–CN moiety, respectively, (ii) the nitro/cyano groups themselves, and (iii) the nitrogens of the aromatic rings and the oxygen of the tetrahydrofuran ring.

The aligment shown does not necessarily reflect the active conformation. However, the following arguments hold true for each of these family of conformers. The ion pairs of the aromatic nitrogen atoms in imidacloprid, clothianidin, and acetamiprid point in the same direction, and that this is also true for one of the two ion pairs of oxygen in dinotefuran. The tetrahydrofuryl ring of dinotefuran is more or less perpendicular to the heteroaromatic ring systems of the other neonicotinoids.

Visual inspection of the nucleophilic Fukui functions (**Figure 11**) shows that the tetrahydrofuryl ring of dinotefuran differs dramatically from the other neonicotinoids under investigation. While the latter all show some large contributions at the aromatic moiety, the tetrahydrofuryl moiety seems to be much less attractive for nucleophilic attack.

5.3.3.3.2. Isosteric alternatives to the heterocyclic *N*-substituents The nitrogen-containing hetarylmethyl group as *N*-substituent (CPM, CTM) has a remarkably strong influence on the insecticidal activity. X-ray crystal structure analysis of imidacloprid and related neonicotinoids indicated that distances between the van der Waals surface of the CPM nitrogen and the atomic center of the pharmacophoric nitrogen are 5.45–6.06 Å (Tomizawa *et al.*, 2000). This range coincides with the distance between the ammonium nitrogen and carbonyl oxygen of acetylcholine, and between the nitrogen atoms of (*S*)-nicotine (Kagabu, 1997a). Alternatively, the CPM and CTM moieties were assumed to be able to participate in hydrogen bonding, like the pyridine ring of (*S*)-nicotine, and that this is important for the insecticidal activity. The CTM substituent generally confers higher potency in the clothianidin and *N*-desmethyl-thiamethoxam series than the CPM moiety in the imidacloprid, thiacloprid, acetamiprid, and nitenpyram series (Zhang *et al.*, 2000). Surprisingly, replacing both CPM and CTM by an oxygen-containing five-membered heterocycle resulted in a novel *N*-substituent TFM, that led to the development of the insecticide (±)-dinotefuran. It was found that the TFM structure can be taken as an isoster of the CPM and CTM moiety (Kagabu *et al.*, 2002). In an attempt to understand this, the hydrogen bonding regions of CPM, CTM, and TFM were projected onto their respective Connolly surfaces (Jeschke *et al.*, 2002) (**Figure 12**).

5.3.3.3.3. Bioisosteric pharmacophors The particularly high potency of the neonicotinoids bearing *N*-nitroimino, *N*-cyanoimino, or nitromethylene moieties, which have a negative electrostatic potential, implies a positive electrostatic potential for the corresponding insect nAChR recognition site (Nakayama and Sukekawa, 1998). Therefore, considerable attention has been given to the possible involvement of the pharmacophoric nitrogen in neonicotinoid action. In order to understand better the structural requirements, binding activity was analyzed using CoMFA (Akamatsu *et al.*, 1997). SAR analyses have also been performed for *in vitro* activities (Nishimura *et al.*, 1994). In particular, 3D QSAR procedures are helpful to predict the receptor–ligand interaction (Nakayama, 1998; Okazawa *et al.*, 1998,

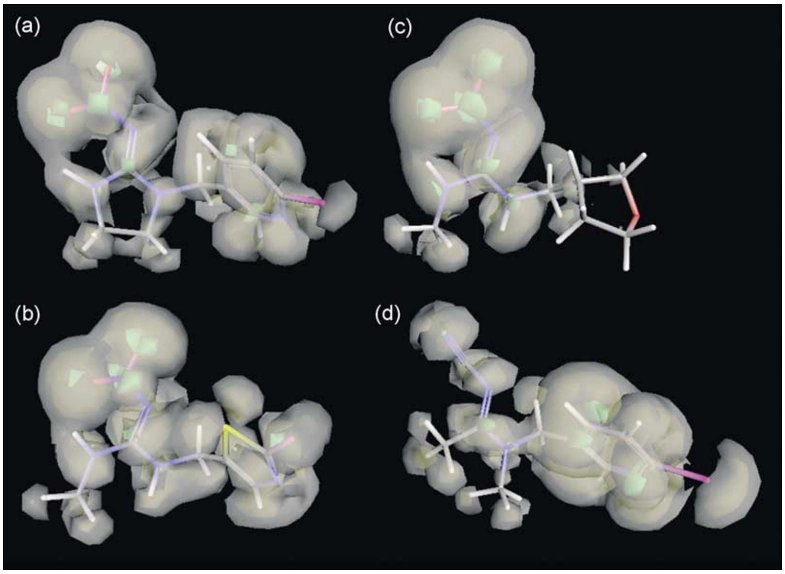


Figure 11 Isosurfaces for Fukui functions for nucleophilic attack of (a) imidacloprid, (b) clothianidin, (c) dinotefuran, and (d) acetamiprid. Three levels of isosurfaces are displayed: 0.005 (green, opaque), 0.001 (yellow, transparent), and 0.0005 (white, transparent).

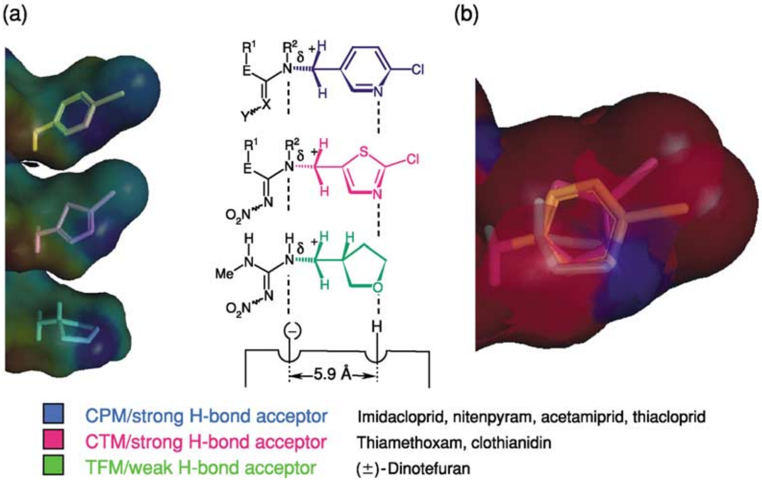


Figure 12 Isosteric alternatives to the heterocyclic *N*-substituents. Connolly surfaces of the CPM moiety from imidacloprid, nitenpyram, acetamiprid (blue), CTM moiety from thiamethoxam and clothianidin (magenta), and TFM from (±)-dinotefuran (green). Atomic charges have been driven via the Mulliken partitioning scheme DFT/BP/TZVP/COSMO Kohn–Sham orbitals. (a) H-bonding potentials is mapped onto the Connolly surface; (b) atomic charges are projected onto the Connolly surface. (Reproduced with permission from Jeschke, P., Schindler, M., Beck, M., **2002**. Neonicotinoid insecticides: retrospective consideration and prospects. *Proc. BCPC: Pests and Diseases 1*, 137–144.)

2000).

As described in an alternative binding model for imidacloprid, the interatomic distance of 5.9 Å between the oxygen of the nitro group (at the van der Waals surface) and the nitrogen in 1-position was also noted as adequate (Kagabu, 1997a). That means the oxygen of the nitro group and the cyano nitrogen are well suited as acceptors for hydrogen bonding with the nAChR, in place of ring nitrogen atoms in CPM and CTM or the ring oxygen in TFM. Thus the π-conjugated system composed of a *N*-nitroimino or *N*-cyanoimino group and the conjugated nitrogen in 1-position are considered essential moieties for the binding of neonicotinoids to the putative cationic subsite in insect nAChR (**Figure 13**). On the other hand, Maienfisch *et al.* (1997) reported that a nitroenamine isomer (N^3-free

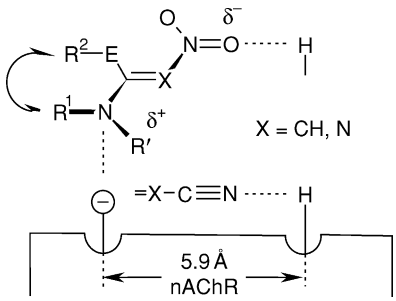


Figure 13 Binding to putative cationic subsite in insect nAChR (Kagabu, 1997a).

imidacloprid) posseses similar insecticidal activity to imidacloprid (Boëlle *et al.*, 1998).

5.3.3.4. Physicochemistry of Neonicotinoids

The physicochemical properties of the ring system and noncyclic neonicotinoids played an important role in their successful development. In addition, photostability is a significant factor in field performance of this class of insecticides (see Section 5.3.2). As described (Kagabu and Medej, 1995; Kagabu, 1997a; Kagabu and Akagi, 1997), the energy gap for the different functional groups [=X–Y], from the ground state to the single state, excited in the order [=N–CN] > [=N–NO_2] > [=CH–NO_2]. For practical application of neonicotinoids, such as for soil and seed treatment, as well as for foliar application, the uptake and translocation in plants is crucial for their insecticidal activity. Thereby, not only the bioisosteric segments of neonicotinoids (see Section 5.3.3.3.3) but also the whole molecular shape (**Figure 12**), including the resultant water solubility, has to be considered. Neonicotinoid insecticides are push–pull olefins made up of conjugated electron donating and accepting groups (Kagabu, 1997a). Such polar, nonvolatile molecules have a high water solubility (**Table 3**) and low P_{OW} values compared with other nonpolar insecticidal classes. The following characteristics were described from Kagabu (1997a):

1. Noncyclic neonicotinoids are less lipophilic than the corresponding ring system neonicotinoids.
2. Concerning the functional group [=X–Y] as part of the pharmacophore [–N–C(E)=X–Y] (iii), water solubility increases in the order of [=CH–NO_2] > [=N–CN] > [=N–NO_2].
3. Regarding E, the lipophilicity increased in the order of S > C > O > NH (Kagabu, 1996).

Somewhat more lipophilic neonicotinoids should be better for seed treatment application, because uptake by roots is more effective than in the case of more hydrophilic compounds (Briggs *et al.*, 1982) (**Figure 14**, **Table 3**). Due to the excellent systemic properties of neonicotinoids conferred by the moderate water solubility, these insecticides are effective against their main target pests, which are sucking insects, like aphids, leafhoppers, and whiteflies.

5.3.3.4.1. Physicochemical properties of commercialized neonicotinoids The physicochemical properties of a compound are related to its structural formula. However, it is not possible to predict the behavior of a substance, with sufficient certainity, only on the basis of its formula and physicochemical properties (Stupp and Fahl, 2003).

5.3.3.4.1.1. Imidacloprid Due to the special moieties like the CPM residue and the 2-(*N*-nitroimino)-imidazolidine 5-ring system, imidacloprid has very weak basic properties under environmental conditions (see Section 5.3.8.1.2). Water solubility and low partition coefficient in octanol–water are not influenced by pH values between 4 and 9, and at 20 °C (Krohn and Hellpointer, 2002) (**Table 3**). The low partition coefficient of imidacloprid indicates that it has no potential to accumulate in biological tissues and further enrichment in the food chain. The occurrence of imidacloprid in the air is determined by its low vapor pressure of 4×10^{-10} Pa which excludes volatilization from treated surfaces. The rapid uptake and translaminar transport of imidacloprid from the treated upper leaf side to the lower surface is excellent, as observed in cabbage leaves (Elbert *et al.*, 1991), and in rice and cucumber (Ishii *et al.*, 1994). However, imidacloprid has a considerable acropetal mobility in xylem of plants. In contrast, its penetration and translocation in cotton leaves was less pronounced, as qualitatively reflected by phosphor-imager autoradiography (Buchholz and Nauen, 2001). This xylem mobility makes imidacloprid especially useful for seed treatment and soil application, but it is equally effective for foliar application (Elbert *et al.*, 1991).

Due to its lack of any acidic hydrogen, the pK_a of imidacloprid is >14, and therefore its transport within the phloem is unlikely, as been shown in several studies (Stein-Dönecke *et al.*, 1992; Tröltzsch *et al.*, 1994). Systemic properties were examined using ^{14}C-labeled imidacloprid. The translocation of imidacloprid in winter wheat and its uptake and translocation from treated cotton seeds into the growing parts of the cotton plant is described by Elbert *et al.* (1998). Registered patterns of use of imidacloprid in agriculture now include traditional foliar spray application as well as soil drench application, drip irrigation, trunk (injection) application, stem or granular treatments and seed treatment.

Table 3 Physicochemical properties of commercialized neonicotinoids

	Ring systems			*Noncyclic structures*			
Physical and chemical properties	*Imidacloprid*	*Thiacloprid*	*Thiamethoxam*	*Nitenpyram*	*Acetamiprid*	*Clothianidin*	*(±)-Dinotefuran*
Color and physical state	Colorless crystals	Yellow/ crystalline powder	Slightly cream/ crystalline powder	Pale yellow crystals	Colorless crystals	Clear/coloress solid powder	
Melting point (°C)	144	136	139.1	83–84	98.9	176.8	94.5–101.5
Henry's law constant (Pa × m^3 mol^{-1}) (at 20 °C)	2×10^{-10}				$<5.3 \times 10^{-8}$ (calc)	2.9×10^{-11}	
Density (g ml^{-1}) (at 20 °C)	1.54	1.46		1.40 (at 26 °C)	1.330	1.61	1.33
Vapor pressure (Pa)							
(at 25 °C)			6.6×10^{-9}		$<1 \times 10^{-6}$	1.3×10^{-10}	
(at 20 °C)	4×10^{-10}			1.1×10^{-9}		3.8×10^{-11}	
Solubility in water (g l^{-1}) (at 20 °C)	0.61	0.185	4.1 (at 25 °C)	840 (pH 7.0)	4.20 (at 25 °C)	0.327	54.3 ± 1.3
Solubility in organic solvents (g l^{-1} at 25 °C)							
Dichloromethane	67 (at 20 °C)	160 (at 20 °C)	43.0	nd		1.32	
N-hexane	<0.1	nd	0.00018				
N-heptane	nd	<0.1 (at 20 °C)	nd	nd		<0.00104	
Methanol	10 (at 20 °C)	nd	10.2	670 (at 20 °C)		6.26	
Ethanol							
Xylene	nd	0.3 (at 20 °C)	nd	4.5 (at 20 °C)		0.0128	
Toluene	0.68		0.63				
1-Octanol	nd	1.4 (at 20 °C)	0.63	nd		0.938	
Acetone	50 (at 20 °C)	64 (at 20 °C)	42.5	290 (at 20 °C)		15.2	
Acetonitrile		52 (at 20 °C)	78.0	430 (at 20 °C)			
Ethyl acetate	nd	9.4 (at 20 °C)	5.74	33 (at 20 °C)		2.03	
Partition coefficient in octanol–water (at 25 °C)	3.72 (at 21 °C)						
log P_{ow}	0.57 (at 22 °C)	1.26 (at 20 °C)		−0.64	0.8	0.7	−0.644
log P_{ow} (pH 4.7) (at 20 °C)			−0.13 (at pH 6.8)		No significant variations with pH	0.9	
log P_{ow} (pH 9.0) (at 20 °C)						0.9	
Dissociation constant pK_a (at 20 °C)			No dissociation in range pH 2–12	3.1 and 11.5	0.7 (at 25 °C) Weak base	11.09	No dissociation in range pH 1.4–12.3
Flammability		Not highly				Not highly	
Surface tension (mN m^{-1}) (at 20 °C)		66				79.6	

nd, not determined.

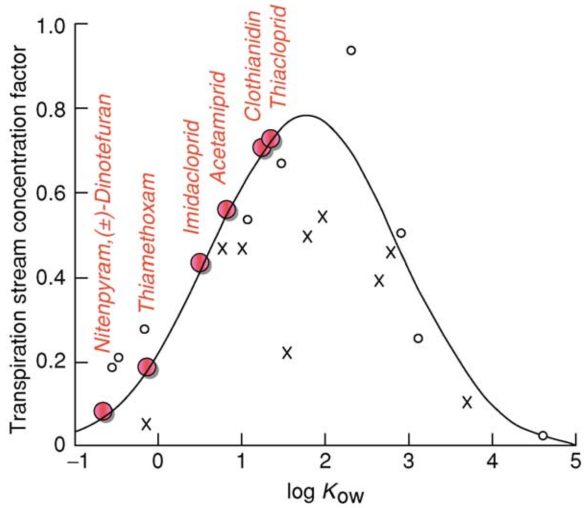


Figure 14 Systemicity of neonicotinoids. Relationship between the translocation of neonicotinoids to barley shoots following uptake by the roots (expressed as the transpiration stream concentration factor), and the octan-1-ol/water partition coefficients (as log K_{ow}). (Diagram and experimental data taken from Briggs, G., Bromilow, R.H., Evans, A.A., **1982**. Relationships between lipophilicity and root uptake and translocation of non-ionized chemicals by barley. *Pestic. Sci. 13*, 495–504). Because of the higher lipophilicity, thiacloprid and clothianidin show the best uptake by the roots from solution, whereas nitenpyram and (±)-dinotefuran are more xylem mobile than the other neonicotinoids.

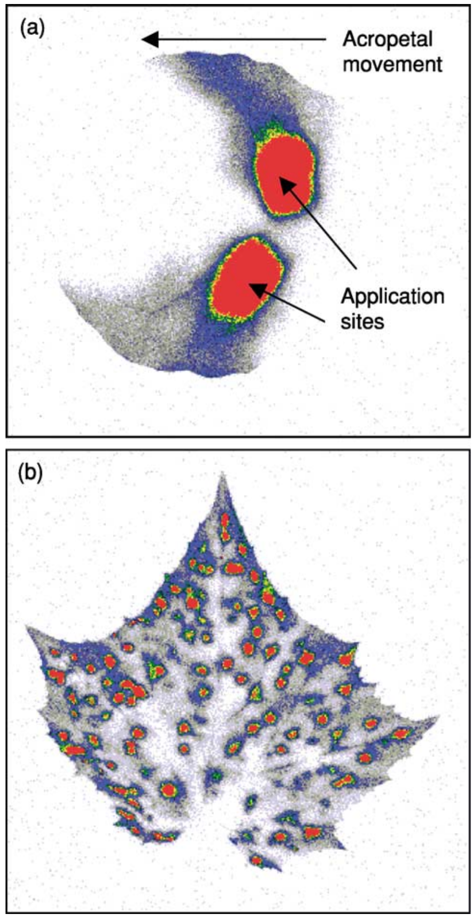


Figure 15 (a) Translocation of [^{14}C]-thiacloprid in cabbage plants 1 day after application of 2 × 5 µl droplets onto the first true leaf. Radioactivity on the surface of the cuticle (residue) was removed by cellulose-acetate stripping; (b) translocation pattern of thiacloprid sprayed to a cucumber leaf.

Because of imidacloprid's systemic properties it is evenly distributed in young, growing plants (Elbert *et al.*, 1991, 1998).

5.3.3.4.1.2. ***Thiacloprid*** It is stable towards hydrolysis even under conditions of heavy rain. Once applied to leaves, thiacloprid shows good rainfastness and photostability, and remains in or on leaves for a considerable time, thus allowing a continous penetration of the active ingredient into the leaf. Its half-life in water at pH 5, 7, and 9 is over 500 h. Photolysis in water (buffered at pH 7) shows a half-life of >100 days. On soil surfaces thiacloprid is also stable under sunlight irradiation (Jeschke *et al.*, 2001). The penetration and translocation behavior of thiacloprid in cabbage was comparable to those reported for imidacloprid (Buchholz and Nauen, 2002). The amounts of thiacloprid stripped off from cabbage application sites were 23% and 17% at 1 day and 7 days after treatment, respectively. Levels in the true leaf increased from 63% to 77% as measured 1 day and 7 days after application, respectively. These results demonstrate that thiacloprid is readily taken up by cabbage leaves, thus providing good systemic control of leaf-sucking pests. The visualization of the translocation pattern of [^{14}C]thiacloprid-equivalents by phosphor-imaging technology revealed xylem mobility, i.e., translocation of the active ingredient in the upwards direction, even 1 day after application to cabbage leaves (**Figure 15a**). This xylem mobility is further highlighted in **Figure 15b**, showing excellent distribution of thiacloprid in cucumber leaves after spray application.

5.3.3.4.1.3. ***Thiamethoxam*** It is a crystalline, odorless compound with a melting point of 139.1 °C. This neonicotinoid has a relatively high water solubility of 4.1 g l^{-1} at 25 °C and a low partition coefficient (log P_{OW} = −0.13 at pH 6.8). In the range of pH 2 to 12 no dissociation is observed (Maienfisch *et al.*, 2001a). These properties favor a rapid and efficient uptake in plants and xylem transport (Widmer *et al.*, 1999) (**Table 3**). Through this systemic activity, all plant parts situated acropetally from the application can be protected (Maienfisch *et al.*, 1999a, 2001a). Thia-

methoxam is hydrolytically very stable at pH 5 with a half-life greater than 1 year at room temperature and stable at pH 7 (estimated half-life at room temperature is 200–300 days) (Maienfisch *et al.*, 2001a). This neonicotinoid is more labile at pH 9, where the half-life is a few days. It is rapidly photolytically degraded with a half-life of about 1 h as a droplet deposit on Teflon. But no decomposition was observed after storage of the active ingredient (a.i.) or formulated product at 54 °C for 2 months; however, at temperatures above 150 °C, exothermic decomposition occurs (Maienfisch *et al.*, 1999a). Under laboratory soils, thiamethoxam degrades at moderate to slow rates. Under field conditions, degradation is generally faster, because of the higher microbial activity of field soils and exposure to light as important degradation pathways (Maienfisch *et al.*, 1999a).

5.3.3.4.1.4. Nitenpyram It is highly soluble in water (840 g l^{-1}), and shows good systemic action (Kashiwada, 1996) and no phytotoxicity, characteristics which enable the various application methods of the neonicotinoid (Akayama and Minamida, 1999). The partition coefficient is low (−0.64) (**Table 3**).

5.3.3.4.1.5. Acetamiprid It is stable in buffer solutions at pH 4, 5, and 7 and under sunlight. It is degraded slowly at pH 9 at 45 °C. Hydrolysis of acetamiprid in 0.5–1 N sodium hydroxide at 90–95 °C for 2 h gave quantitatively *N*-methyl-(6-chloro-pyrid-3-yl)-methylamine (Tokieda *et al.*, 1997). Because of its high solubility in water (**Table 3**), acetamiprid shows a high systemic and translaminar insecticidal activity (Yamada *et al.*, 1999) and is currently used only foliarly.

5.3.3.4.1.6. Clothianidin It has no acidic or alkaline properties at the relevant pH (Stupp and Fahl, 2003). Therefore, the pH of the aqueous system has no influence on its physicochemical properties. It is stable to hydrolysis in a pH range of 4 to 9, but photolysis contributes significantly to its degradation in the environment resulting in an elevated mineralization rate. The water solubility of clothianidin is relatively low (0.327 g l^{-1} at 20 °C) in comparison to the other neonicotinoids that have a nitroguanidyl moiety. This is also reflected by the octanol–water partition coefficient, indicating a favorable adsorption to soil (log P_{OW} = 0.7 at 25 °C) (**Table 3**). On basis of these physicochemical properties no bioaccumulation is expected nor any volatilization, and thus no significant amounts of the substance are to be expected in the atmosphere (Stupp and Fahl, 2003).

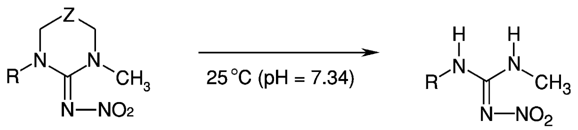
5.3.3.5. Proneonicotinoids

Pharmaceutical conceptions in prodrug design generally focus on its potential in overcoming pharmacokinetic problems and poor oral absorption (Testa and Mayer, 2001). Prodrug activation occurs enzymatically, nonenzymatically, or also sequentially (enzymatic step followed by nonenzymatic rearrangement) (Testa and Mayer, 1998). Depending on both the drug and its prodrug, the therapeutic gain may be modest, marked, or even significant (Balant and Doelker, 1995). The field of neonicotinoid chemistry reflects, e.g., with Mannich adducts, some significant examples for useful proneonicotinoids in crop protection (see Sections 5.3.3.5.1 and 5.3.3.5.3).

5.3.3.5.1. Ring cleavage to noncyclic neonicotinoids The six-membered perhydro-1,3,5-thiadiazine (Z = S), hexahydro-1,3,5-triazine (Z = NR), and perhydro-1,3,5-oxadiazine (Z = O) containing neonicotinoids can be prepared by a Mannich type cyclization reaction by treatment, for example, cyclization of the *N*-nitroguanidine moiety with two molar equivalents of formaldehyde (HCHO) in the presence of one or more equivalents of hydrogen sulfide (H_2S), an appropriate amine ($R{-}NH_2$) or water (H_2O) in a suitable solvent. It is known that these cyclic Mannich adducts can cleave into their noncyclic compounds depending on the reaction conditions (pH values). Efficient syntheses of the active ingredient clothianidin, which is readily performed by ring cleavage reaction of the *bis*-aminal structure ($N{-}CH_2{-}N$ bonds) within the 6-ring system of the N,N′-dialkylated 2-(*N*-nitroimino)-hexahydro-1,3,5-triazine, have been previously described (Maienfisch *et al.*, 2000; Jeschke *et al.*, 2003).

The hydrolysis (ring cleavage) of different 6-ring intermediates giving noncyclic neonicotinoids were studied in a physiological salt solution at 25 °C (pH 7.34). It was found that the perhydro-1,3,5-oxadiazines (Z = O) have noteably longer half-lives than the appropriate hexahydro-1,3,5-triazines as *bis*-aminals (Kagabu, 2003a) (**Figure 16**).

Agro Kanesho's hexahydro-1,3,5-triazine (Z = NMe) AKD-1022, a six-membered ring compound, is a proinsecticide of clothianidin (Bryant, 2001). Recently, it was demonstrated that thiamethoxam, having a perhydro-1,3,5-oxadiazine (Z = O) structure, is also an *in vivo*, easy-to-cleave neonicotinoid precursor for the highly active noncyclic neonicotinoid clothianidin (Nauen *et al.*, 2003). This suggests a prodrug principle in the mode of action of thiamethoxam, rather than differences in the binding



Substituent	*Half-life $t_{1/2}$ (in days) at 25 °C (pH = 7.34)*		
R	*Z = O*	*Z = S*	*Z = N—CH$_3$*
2-Chloro-1,3-thiazol-5-ylmethyl- (CTM)	132	12	5.3
6-Chloro-pyrid-3-ylmethyl- (CPM)	72	6	8

Figure 16 Half-life $t_{1/2}$ (days) of neonicotinoids in salt solution at 25 °C (pH 7.34). (Reproduced from Kagabu, S., **2003a**. Molecular design of neonicotinoids: past, present and future. In: Voss, G., Ramos, G. (Eds.), Chemistry of Crop Protection: Progress and Prospects in Science and Regulation. Wiley–VCH, New York, pp. 193–212.)

site (Kayser *et al.*, 2002). Clothianidin is one of the most prominent metabolites in the leaves of thiamethoxam-treated (soil drench) cotton plants. Thiamethoxam is not only rapidly metabolized in plants but also (within minutes) converted to clothianidin in noctuid larvae. The level of clothianidin detected in the hemolymph is quite high, i.e., physiologically relevant, and considering the electrophysiological results in an interaction with the nAChR in the CNS can be assumed to occur (Nauen *et al.*, 2003). Therefore clothianidin is likely to be responsible for the insecticidal potency of thiamethoxam, and not the parent compound itself, an important fact for resistance management strategies (see Section 5.3.9).

5.3.3.5.2. Mannich adducts as useful precursors The first patent applications covering a number of substituted 5-nitro-tetrahydro-1,3-pyrimidines, the so-called Mannich adducts of certain insecticidally active nitromethylene compounds, were published in 1988 (e.g., compound Bay T 9992, Bayer AG). Due to their potent toxicity to insects and arthropods, numerous different companies produced compounds of this type (Nauen *et al.*, 2001). These Mannich adducts can be prepared via an aminomethylation reaction by the treatment of the CH-acidic nitromethylenes [–N–C(E)=CH–NO_2; E = NHR^2], which react as nitroenamines (Rajappa, 1981), with one or more equivalents of an amine (R^3–NH_2), and at least two molar equivalents of the electrophile formaldehyde (HCHO) in a suitable solvent like alcohols, water, polar aprotic solvents such as tetrahydrofuran, and *N*,*N*-dimethyl-formamide, or solid paraformaldehyde (**Figure 17**; Mannich adduct formation, pathway A). In some cases, a small amount of a strong, nonoxidizing acid, such as hydrochloric acid, can be used as a catalyst. The Mannich adducts have a wide range of substituents R^3 which inhibit the

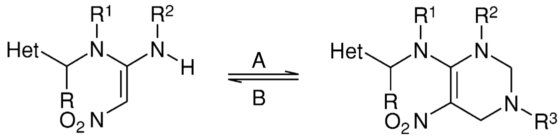


Figure 17 Formation and hydrolysis of Mannich adducts. (Reproduced from Nauen, R., Ebbinghaus-Kintscher, A., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Iishaaya, I., (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 77–105.)

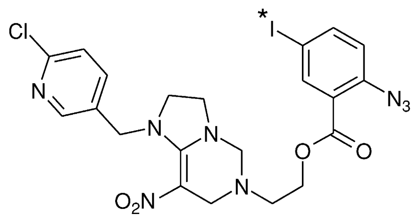
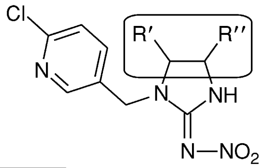


Figure 18 [^{125}I] azidoneonicotinoid (AN) photoaffinity radioligand, a useful probe to explore the structure and diversity of insect nAChRs. (Reproduced from Tomizawa, M., Casida, J.E., **2001**. Structure and diversity of nicotinic acetylcholine receptors. *Pest Mgt Sci.* *57*, 914–922.)

[^{3}H]imidacloprid binding site of *Drosophila* or *Musca* nAChR by 50% at 0.7–24.0 nM (Latli *et al.*, 1997). However, at different pH values, hydrolysis of the Mannich adducts can be observed (**Figure 17**; Mannich adduct cleavage, pathway B).

Application of more stable Mannich adducts in protein biochemistry led to the synthesis of the [^{125}I]azidoneonicotinoid (AN) probe by using 1-(6-chloro-pyrid-3-ylmethyl)-2-nitromethylene-imidazolidine (NTN32692) as the photoaffinity radioligand (Latli *et al.*, 1997; Maienfisch *et al.*, 2003) (**Figure 18**). This probe facilitated the identification of insecticide-binding site(s) in insect nAChRs.

In the first photoaffinity labeling of an insect neurotransmitter receptor, [^{125}I]AN exhibited high



—R′HC—CHR″—	—H_2C—CH_2—	**—HC=CH—**	—(HO)HC—CH_2—	—H_2C—CH(OH)—	—(HO)HC—CH(OH)—
pI_{50} value	9.3	**9.6**	8.5	7.6	5.5

Figure 19 Modification of the imidacloprid bridging chain and pI_{50} values for the nicotinic acetylcholine receptor (nAChR) from housefly head membranes. (Reproduced from Nauen, R., Ebbinghaus-Kintscher, A., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Iishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 77–105.)

affinity (8 nM), and successfully identified a 66 kDa polypeptide in *Drosophila* head membranes. Several cholinergic ligands and neonicotinoid insecticides, like imidacloprid and acetamiprid, strongly inhibited this photoaffinity labeling. Thus, the labeled polypeptide is pharmacologically consistent with the ligand- and insecticide-binding subunit of *Drosophila* nAChR (Tomizawa and Casida, 1997, 2001).

5.3.3.5.3. Active metabolites of neonicotinoids From the metabolic pathway (see Section 5.3.7.1) of imidacloprid it is known that hydroxylation of the imidazolidine ring leads in general to the mono- (R′ = —H; R″ = —OH) and the bishydroxylated (R′, R″ = —OH) derivatives, both of which have reduced affinity (**Figure 19**). Alternatively, the mono (R′ = —OH; R″ = —H) derivative reflects a higher level of efficacy (pI_{50} value 8.5) (Nauen *et al.*, 2001; Sarkar *et al.*, 2001). Interestingly, the olefin metabolite showed a higher pI_{50} value for nAChR from housefly head membranes than imidacloprid, and provides superior toxicity to some homopterans after oral ingestion (Nauen *et al.*, 1998b, 1999b). This result suggests that for the central ring system the exact rearrangement of the ring atoms, and not the electronic effect of the ring system, seems to be necessary for insecticidal activity. A similar phenomenon has been described for the conjugated pyridone derivatives (Kagabu, 1999).

5.3.4. Biological Activity and Agricultural Uses

The biological activity and agricultural uses of neonicotinoid insecticides are enormous' and these insecticides are continuing to see new uses (Elbert and Nauen, 2004). It is definitely beyond the scope of this chapter to provide a full overview of the agronomic and horticultural cropping systems that use neonicotinoid insecticides and readers interested in these aspects should refer to many articles and book chapters published during the past decade, e.g., Elbert *et al.* (1991, 1998), Yamamoto (1999), Kiriyama and Nishimura (2002), and Elbert and Nauen (2004). In order to provide a flavor of the agricultural uses of neonicotinoids, and the affected target pests, a few examples considering imidacloprid are given below. Imidacloprid is presently the most widely used neonicotinoid insecticide worldwide.

5.3.4.1. Efficacy on Target Pests

Due to their unique properties – high intrinsic acute and residual activity against sucking and some chewing insect species, high efficacy against aphids, whiteflies, leafhoppers and planthoppers, and the Colorado potato beetle, and excellent acropetal translocation – neonicotinoids can be used in a variety of crops (**Figure 20**). These uses include: aphids on vegetables, sugar beet, cotton, pome fruit, cereals, and tobacco; leafhoppers, planthoppers, and water weevil on rice; whiteflies on vegetables, cotton, and citrus; lepidopteran leafminer on pome fruit and citrus; and wireworms on sugar beet and corn (**Table 4**). Termites and turf pests such as white grubs can also be controlled by imidacloprid (Elbert *et al.*, 1990, 1991).

Neonicotinoids such as imidacloprid and thiamethoxam also control important vectors of virus diseases, thereby impairing the secondary spread of viruses in various crops. This control has been observed, e.g., for the persistent barley yellow dwarf virus (BYDV) transmitted by *Rhopalosiphum padi* and *Sitobion avenae* (Knaust and Poehling, 1992). Seed treatments proved highly effective in controlling barley yellow dwarfvirus vectors and the subsequent infection, in a series of field trials in southern England. Sugar beet seed, pelleted with imidacloprid, was well protected especially against infections with beet mild yellow virus transmitted by the peach potato aphid, *Myzus persicae* (Dewar and Read, 1990).

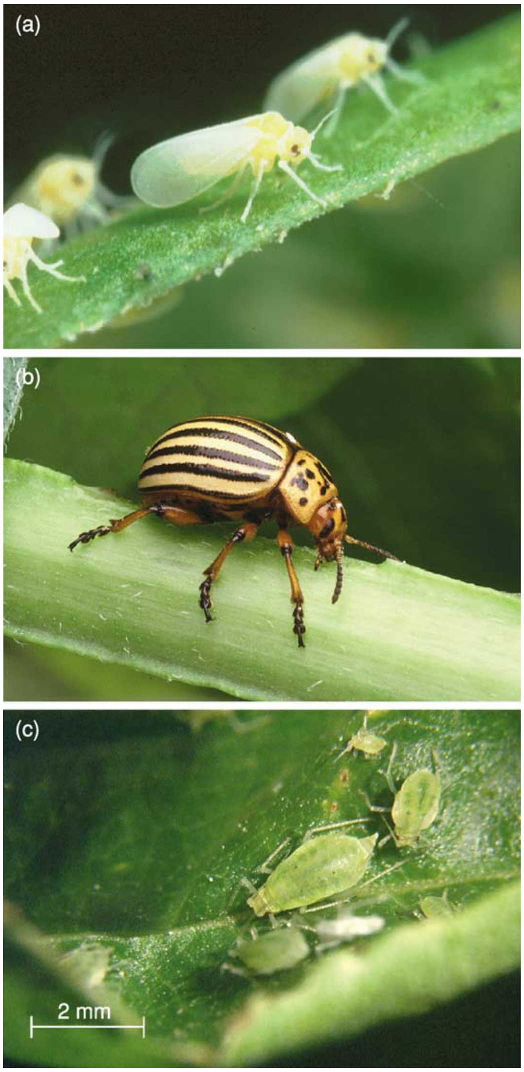


Figure 20 Important pest insects targeted by neonicotinoid insecticides. (a) Sweet-potato whitefly, *Bemisia tabaci*; (b) Colorado potato beetle, *Leptinotarsa decemlineata*; (c) green peach aphid, *Myzus persicae*.

Table 4 Insecticidal efficacy of imidacloprid after foliar application against a variety of pest insects under laboratory conditions

Pest species	*Developmental stage*	*LC_{95}, rounded (ppm)*
Homoptera		
Aphis fabae	Mixed	8
Aphis gossypii	Mixed	1.6
Aphis pomi	Mixed	8
Brevicoryne brassicae	Mixed	40
Myzus persicae	Mixed	1.6
Myzus persicae (tobacco)	Mixed	8
Phorodon humuli	Mixed	0.32
Laodelphax striatellus	Third instar	1.6
Nephotettix cincticeps	Third instar	0.32
Nilaparvata lugens	Third instar	1.6
Sogatella furcifera	Third instar	1.6
Pseudococcus comstocki	Larvae	1.6
Bemisia tabaci	Second instar	8
Trialeurodes vaporariorum	Adult	40
Hercinothrips femoralis	Mixed	1.6
Coleoptera		
Leptinotarsa decemlineata	Second instar	40
Lema oryzae	Adult	8
Lissorhoptrus oryzophilus	Adult	40
Phaedon cochleariae	Second instar	40
Lepidoptera		
Chilo suppressalis	First instar	8
Helicoverpa armigera	Second instar	200
Plutella xylostella	Second instar	200
Heliothis virescens	Egg	40
Spodoptera frugiperda	Second instar	200

5.3.4.2. Agricultural Uses

Due to their high systemicity, diverse ways of application are feasible and these methods have been introduced into practice. Soil treatments can be done by incorporation of granules, injection, application with irrigation water, spraying, and use of tablets. Plants or plant parts can be treated by seed dressing, pelleting, implantation, dipping, injection, and painting. These methods have led to a more economic and environmentally friendly use of these products, fitting well into various integrated pest management (IPM) programmes. In this section, imidacloprid has been chosen as an example of a neonicotinoid, and a few examples of its use in various crops is given. Such uses include treatments for rice in Japan, cotton in the mid-south of the USA, vegetables in Mexico, and cereals in France. In these crops the neonicotinoid insecticides targets mainly early-season pests.

5.3.4.2.1. Rice In Japan, nursery box application of insecticides is a standard procedure, and it is currently used in 50% of the rice-growing area. This type of treatment is directed against early-season pests like *Lissorhoptrus oryzophilus* (rice water weevil) and *Lema oryzae* (rice leaf beetle). In contrast to commercially available products like carbosulfan, benfuracarb, and propoxur, which control mainly rice water weevil and rice leaf beetle, imidacloprid covers the whole spectrum of early-season

pests including *N. cincticeps* (green rice leafhopper), *Laodelphax striatellus* (smaller brown planthopper), and also mid- and late-season hopper species, like *Sogatella furcifera* (white-backed planthopper) and *Nilaparvata lugens* (brown planthopper). Benefits of such nursery box applications include, reduced labor input, longer residual effect, and reduced side effects against nontarget organisms when compared to surface treatments.

Based on 5-year field trials, a use pattern for imidacloprid against the main rice pests has been established. When applied as a granule, 2 granular GR with 1 g a.i. per nursery box (= 200 g a.i. ha^{-1}) 0–3 days before transplanting, the product gives full protection for 50–90 days against different hoppers species. By controlling *L. striatellus,* a 91% reduction of the rice stripe virus was observed. An excellent residual effect was detected up to 50 days after transplanting against *L. oryzophilus*, and up to 60 days against *L. oryzae*.

Apart from the seedling box application, imidacloprid can be used as a granule for water surface application, as a wettable powder (WP) or as a dust formulation for foliar treatment, and also as a seed dressing to control the above mentioned pests on rice. Under field conditions of such use, no apparent effects from imidacloprid treatments were observed against populations of the spiders *Pardosa pseudoannulata* and *Tetragnatha vermiformis*.

Therefore, imidacloprid can be regarded very effective against rice insects and flexible to use, but it is most efficient in nursery box application. It outperforms other conventional insecticides due to its broader efficacy and lower dose rates. Virus diseases are suppressed through effective vector control, and low toxicity has been observed against important beneficials in rice.

5.3.4.2.2. Cotton The main cotton pests in the mid South of the USA are *Thrips tabaci, Lygus lineolaris* (tarnished plant bug), *Neurocolpus nubilis* (clouded plant bug), *Anthonomus grandis* (boll weevil), *Heliothis virescens* (tobacco budworm), and *Helicoverpa zea* (bollworm). Other pests such as *Pseudatomoscelis seriatus* (cotton fleahopper), *Aphis gossypii* (cotton aphid), *Spodoptera exigua* (beet armyworm) and *S. frugiperda* (fall armyworm) are of lesser importance in this region.

Thrips tabaci attacks the terminal bud and two to four true leaves. The plant is damaged by a reduced stand, retarding growth, killed buds, and delayed fruiting, with significant yield reduction. *Lygus lineolaris* damages by feeding on pinhead squares, which causes young squares to shed. Terminal bud injury leads to multiple branched plants ("crazy cotton"). Symptoms caused by *N. nubilis* are similar to those of *L. lineolaris*.

It is thus evident that damage caused by early-season pests, such as thrips and bugs, can be very important. Loss of first-position squares can have detrimental effects on the yield potential of the plant. If normal fruiting of cotton plants is prevented, this can cause a delay in maturity, which in turn necessitates additional late-season insecticidal applications to control bollworms, tobacco budworms, and boll weevils.

A 480 FS formulation (Gaucho®) is applied for seed dressing, against early infestations of thrips and aphids, at 250 g a.i. per 100 kg cotton seed (37 g a.i. ha^{-1}). Against bugs and later attack of aphids, three foliar split applications at low dose rates are recommended (Provado® 1.6 F, 3 × 15 g a.i. ha^{-1} (Table 4). These should be done as band sprays at intervals of 7–10 days. The first application starts 30 days after emergence of the five leaf stage, subsequent to the Gaucho® protection period. The *Heliothis–Helicoverpa* complex is controlled by conventional insecticides in mid-season, whereas boll weevil and armyworms are controlled by azinphos-methyl or cyfluthrin, respectively.

5.3.4.2.3. Vegetables Virus diseases have been the main factors affecting the tomato and chilli production in Mexico during the past 20 years, and these diseases have led to significant yield reductions in many regions. These crops are grown on approximately 160 000 ha with constant virus infestation in 30–35% of the fields, with persistent viruses being the most important in Mexican agriculture. In tomato crops, viruses can infest up to 100% of the crop. The damage depends on the type of virus, the population density of vectors, and the developmental stage of the crop. Protection by conventional insecticide treatments under plastic covered greenhouses, however, has not given satisfactory results (Elbert and Nauen, 2004).

5.3.4.2.4. Cereals The traditional method in controlling virus vectors in Europe is regular monitoring of aphid attack of young wheat and barley plants after emergence in autumn. If the threshold of 10% infested plants with at least one aphid per plant has been surpassed, usually a pyrethroid will be sprayed. Monitoring has to be continued, because the residual effect of the treatment may be insufficient for vector control. Depending on climatic conditions and the duration of the aphid attack, a second or a third application may be required. This rather time consuming and difficult procedure can be avoided by a seed treatment with imidacloprid.

Barley yellow dwarf virus vectors such as *R. padi* and *S. avenae* are controlled and the secondary spread of the disease is inhibited. In contrast to one or several sprays, seed dressing as the only treatment assures prolonged protection during the critical period, when virus transmission is of importance. Furthermore pyrethroid sprays with a broad spectrum of activity can be substituted by only one application of imidacloprid, which has virtually no effects on beneficials and other nontarget organisms. High yield varieties achieve their optimum yield potential only if they are sown early, which implies an enhanced risk of infestation with aphids and, consequently, with barley yellow dwarf virus. As Gaucho® controls aphids and suppresses the infection with this virus, early-sown cereals are especially well protected against insects and diseases. Therefore, a better tillering is achieved and 15–25% of the seed can be saved.

Both imidacloprid seed treatment and pyrethroid spray application have very good to excellent effects against the vector *R. padi*. The acute effect of the pyrethroid was better due to its quick knockdown activity. Imidacloprid alternatively acts more slowly, so, it takes more time to kill invading aphids, but its residual effect is much more pronounced. Thus imidacloprid use results in long-lasting control of aphids and, as a consequence, considerably better prevention of virus symptoms. A yield increase of 26% over untreated controls was achieved in comparison with, 10% achieved by pyrethroid treatment.

Field trials over 4 years in France confirmed that both wheat and barley are well protected against the above-mentioned pests and diseases. In an average of 76 trials in wheat and 66 trials in barley a yield increase of 3.1 dt ha^{-1} and 4.4 dt ha^{-1}, respectively; was obtained with the Gaucho® treatment when compared with untreated.

5.3.4.3. Foliar Application

Spray applications are especially used against pests attacking crops such as cereals, maize, rice, potatoes, vegetables, sugar beet, cotton, and deciduous trees. Table 4 shows the acute activity (estimated LC_{95} (lethal concentration at which 95% of insects are killed) in ppm a.i.) of imidacloprid against a variety of pests, following foliar application (dip and spray treatment) of host plants under laboratory and greenhouse conditions. Imidacloprid was very active to a wide range of aphids. The most susceptible was the damson hop aphid *Phorodon humuli* (LC_{95} 0.32 ppm), which is often highly resistant against conventional insecticides (Weichel and Nauen, 2003). Imidacloprid was highly effective against some of the most important rice pests, such as leafhoppers and planthoppers, rice leaf beetle *L. oryzae* and rice water weevil *L. oryzophilus*. Although imidacloprid is generally less effective against biting insects, its efficacy against the Colorado potato beetle *Leptinotarsa decemlineata* is relatively high (LC_{95} 40 ppm). The LC_{95} values for the second or third instar larvae of some of the most deleterious noctuid pest species of the order Lepidoptera, *Helicoverpa armigera, Spodoptera frugiperda*, and *Plutella xylostella*, were approximately 200 ppm, and higher than those for the species mentioned above (Elbert *et al*., 1991).

5.3.4.4. Soil Application and Seed Treatment

Typical soil insect pests such as *Agriotes* sp., *Diabrotica balteata*, or *Hylemyia antiqua* were controlled by incorporation of 2.5–5 ppm a.i. into the soil. Higher concentrations of imidacloprid are necessary to control *Reticulitermes flavipes* (7 ppm) and *Agrotis segetum* (20 ppm). However, imidacloprid activity is much more pronounced against early-season sucking pests, which attack the aerial parts of a wide range of crops. Soil concentrations as low as 0.15 ppm a.i. gave excellent control of *Myzus persicae* and *Aphis fabae* on cabbage in greenhouse experiments (Elbert *et al*., 1991). A good residual activity is essential for the protection of young plants.

5.3.5. Mode of Action

The biochemical mode of action of neonicotinoid insecticides has been studied and characterized extensively in the past 10 years. They act selectively on insect nAChRs, a family of ligand-gated ion channels located in the CNS of insects and responsible for rapid neurotransmission. The nAChR is a pentameric transmembrane complex, and each subunit consists of an extracellular domain containing the ligand binding site and four transmembrane domains (Nauen *et al*., 2001; Tomizawa and Casida, 2003). Neonicotinoid insecticides bind to the acetylcholine binding site located on the hydrophilic extracellular domain of α-subunits. Their ability to displace tritiated imidacloprid from its binding site correlates well with their insecticidal efficacy (Liu and Casida, 1993a; Liu *et al*., 1993b). [^{3}H]imidacloprid binds with nanomolar affinity to nAChR preparations from insect tissues, and next to the less specific α-bungarotoxin, it is the preferred compound in radioligand competition studies (Lind *et al*., 1998; Nauen *et al*., 2001). Furthermore, electrophysiological studies revealed that neonicotinoid insecticides act agonistically on nAChR, and this interaction is again very well correlated with

their potential to control target pest species. In contrast antagonistic compounds of mammalian nAChR were shown to be far less active as insecticides (Nauen *et al.*, 1999a). All neonicotinoid insecticides act with nanomolar affinity against housefly and other insect nAChRs, except thiamethoxam, which exhibits a comparatively low affinity for the [^{3}H]imidacloprid binding site. This low affinites was attributed to its proneonicotinoid nature (see Sections 5.3.3.5 and 5.3.3.5.1), as it was shown to be activated to clothianidin in insects and plants (Nauen *et al.*, 2003).

The mode of action of neonicotinoid insecticides has been described in several excellent reviews which should serve as a primary source for further informations (Kagabu, 1997a; Matsuda *et al.*, 2001; Nauen *et al.*, 2001; Tomizawa and Casida, 2003). These reviews describe our current knowledge of the structure and function of insect nAChR, characterized by receptor binding studies, phylogenetic considerations regarding receptor homologies between orthologs from different animal species, and electrophysiological investigations, as well as the description of a vast range of a structurally diverse receptor ligands, of course with an emphasis on neonicotinoid insecticides.

5.3.6. Interactions of Neonicotinoids with the Nicotinic Acetylcholine Receptor

5.3.6.1. Selectivity for Insect over Vertebrate nAChRs

Electrophysiological measurements reported in numerous studies have revealed that nAChRs are widely expressed in the insect nervous system on both postsynaptic and presynaptic nerve terminals, on the cell bodies of interneurons, motor neurons, and sensory neurons (Goodman and Spitzer, 1980; Harrow and Sattelle, 1983; Sattelle *et al.*, 1983; Breer, 1988; Restifo and White, 1990). Schröder and Flattum (1984) using extracellular electrophysiological recordings were the first to identify that the site of action of the nitromethylene nithiazine was on the cholinergic synapse. A number of subsequent electrophysiological and biochemical binding studies revealed that the primary target of the neonicotinoids were the nAChRs (Benson, 1989; Sattelle *et al.*, 1989; Bai *et al.*, 1991; Leech *et al.*, 1991; Cheung *et al.*, 1992; Tomizawa and Yamamoto, 1992, 1993; Zwart *et al.*, 1992; Liu and Casida, 1993a; Tomizawa *et al.*, 1996). Recent electrophysiological studies indicate that imidacloprid acts as an agonist on two distinct nAChR subtypes on cultured cockroach dorsal unpaired motoneuron (DUM) neurons (Buckingham *et al.*, 1997), an α-bungarotoxin (α-BGTx) sensitive nAChR with "mixed" nicotinic/muscarinic pharmacology and an α-BGTx insensitive nAChR. Such electrophysiological observations were supported by binding studies with [^{3}H]imidacloprid in membrane preparations from *M. persicae*. These ligand competition studies revealed the presence of high and low-affinity nAChR binding sites for imidacloprid in *M. persicae* (Lind *et al.*, 1998).

The identification of multiple putative nAChR subunits by molecular cloning is consistent with a substantial diversity of insect nAChRs (Gundelfinger, 1992). At present, at least five different subunits have been cloned from *Drosophila melanogaster* (Schulz *et al.*, 1998), from the locust *Locusta migratoria* (Hermsen *et al.*, 1998), and from the aphid *M. persicae* (Huang *et al.*, 1999). Despite the considerable number of subunits identified, only a few functional receptors were obtained after expression of different subunit combinations in *Xenopus* oocytes or cell lines. Initial work suggested that some subunits can form homooligomeric functional receptors when expressed in *Xenopus* oocytes. This was shown for Lα1 from the locust *Schistocerca gregaria* (Marshall *et al.*, 1990; Amar *et al.*, 1995), and for the Mpα1 and Mpα2 from *M. persicae* (Sgard *et al.*, 1998). However, the expression of these subunits was not very effective and generated only small inward currents (5–50 nA) following application of nicotine or acetylcholine. Alternatively, all three *Drosophila* α subunits (ALS, SAD, and Dα2) can form functional receptors in *Xenopus* oocytes when coexpressed with a chicken neuronal β2 subunit (Bertrand *et al.*, 1994; Matsuda *et al.*, 1998; Schulz *et al.*, 1998), suggesting that additional insect nAChR subunits remain to be cloned. Radioligand binding studies using several *M. persicae* α subunits coexpressed with a rat β2 subunit in the *Drosophila* S2 cell line also indicate pharmacological diversity in *M. persicae* (Huang *et al.*, 1999). In these binding studies it was shown that imidacloprid selective targets were formed by Mpα2 and Mpα3, but not Mpα1 subunits. These examples indicate that our understanding of the complexity of insect nAChR is still limited, and that electrophysiology will play an essential role in determining the significance of certain subunit combinations in the mode of action of neonicotinoid and other insecticidally active ligands.

5.3.6.2. Whole Cell Voltage Clamp of Native Neuron Preparations

The use of isolated neurons from insect CNS for electrophysiological studies is a suitable tool to

investigate the mode of action of new insecticidal compounds, which act on a range of neuronal target sites. Primary neuronal cell cultures from *H. virescens* larvae, one of the most important lepidopteran pest species, is one of these suitable tools. *H. virescens* neurons respond to the application of ACh, with a fast inward current of up to 5 nA at a holding potential of −70 mV. The current reversed at a holding potential close to 0 mV, indicating the activation of nonspecific cation channels, i.e., nAChRs.

Figure 21 illustrates the whole cell currents elicited by application of 1 μM nithiazine and the second generation neonicotinoids, acetamiprid, nitenpyram, and clothianidin. All of these compounds act as agonists on the nAChR, but the potency and agonistic efficacy of each of these compounds were quite different. Imidacloprid and clothianidin were the most potent compounds in this *Heliothis* preparation with an EC_{50} of 0.3 μM (Table 5). In the case of imidacloprid there was good agreement with electrophysiological measurements

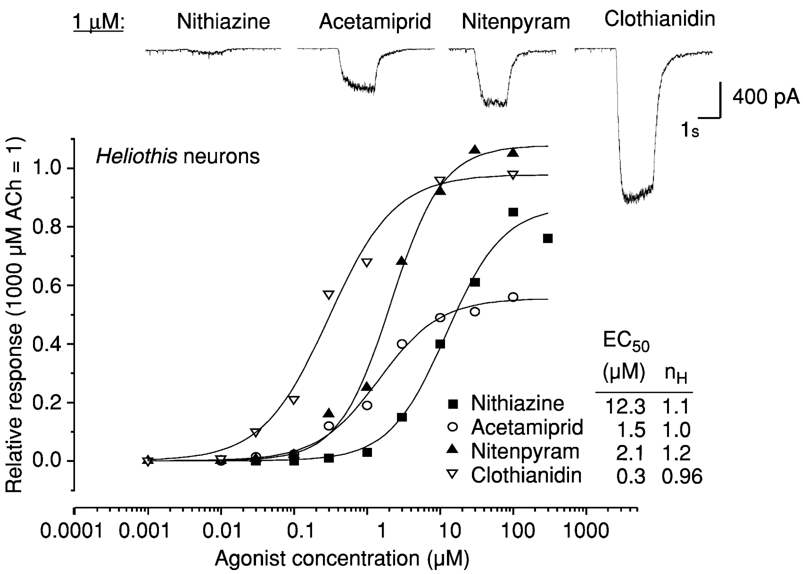


Figure 21 Whole cell current responses of a neuron isolated from the CNS of *Heliothis virescens* after application of different neonicotiniods. The dose-response curve was fitted by the Hill equation. All currents were first normalized to mean amplitudes elicited by 10 μM ACh before and after each test concentration was applied, and then normalized to the relative amplitude elicited by 1000 μM ACh. EC_{50} values given correspond to the half maximal activation of nAChR by each agonist. The Hill coefficient (n_H) of all tested compounds was close to 1. The upper inset shows the corresponding responses for the neonicotinoids at 1 μM (holding potential −70 mV). All currents were obtained from the very same neuron. (Reproduced with permission from Nauen, R., Ebbinghaus-Kintscher, A., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Iishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 77–105.)

Table 5 Comparison between electrophysiological and [^{3}H]imidacloprid displacement potencies for different neonicotinoids and epibatidine on insect nAChRs. Electrophysiological data (EC_{50} and relative (agonist) efficacy) were obtained from neuron cell bodies isolated from the CNS of *Heliothis virescens.* EC_{50} and relative efficacy values represent the mean of n separate experiments on different neurons. The inhibition of [^{3}H]imidacloprid binding to nAChR in housefly head membrane preparations by the compounds is expressed as pI_{50} value (pI_{50} values (= −log M) correspond to the concentration of cold ligand displacing 50% of bound [^{3}H]imidacloprid from housefly head membranes)

Compound	*n*	*EC_{50} (μM ± SD)*	*Relative efficacy (1 mM ACh = 1)*	*[^{3}H]imidacloprid pI_{50}*
Imidacloprid	4	0.31 ± 0.15	0.14 ± 0.02	9.3
Clothianidin	3	0.33 ± 0.03	0.99 ± 0.08	9.2
Acetamiprid	3	1.07 ± 0.37	0.56 ± 0.05	8.7
Nitenpyram	3	1.66 ± 0.38	0.98 ± 0.07	8.6
(±)-Epibatidine	3	1.69 ± 0.79	0.20 ± 0.05	6.2
Nithiazine	4	9.60 ± 3.20	0.79 ± 0.06	6.8

Reproduced with permission from Nauen, R., Ebbinghaus-Kintscher, A., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Iishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 77–105.

recorded from isolated cockroach neurons, where imidacloprid exhibited an EC_{50} of 0.36 μM (Orr *et al.*, 1997). Acetamiprid, nitenpyram, and the natural toxin epibatidine exhibited an EC_{50} of between 1 and 2 μM (**Table 5**). Similar values were also observed for the cockroach preparation, with an EC_{50} between 0.5 and 0.7 μM for epibatidine and acetamiprid (Orr *et al.*, 1997). Nithiazine had the lowest potency, with an EC_{50} of about 10 μM. The neonicotinoids, clothianidin, and nitenpyram were full agonists, whereas acetamiprid, epibatidine, and imidacloprid were partial agonists. The maximal response elicited by saturable concentrations of imidacloprid (100 μM) was only 15% of the maximal current obtained by 1000 μM ACh. In earlier work on isolated cockroach neurons (Orr *et al.*, 1997) and isolated locust neurons (Nauen *et al.*, 1999b), it was also found that imidacloprid acted as a partial agonist on insect nAChRs. The partial agonistic action of imidacloprid was also observed with chicken α4β2 nAChRs, and on a hybrid nAChR formed by the coexpression of a *Drosophila* α subunit (SAD) with the chicken β2 subunit in *Xenopus* oocytes (Matsuda *et al.*, 1998). Imidacloprid activates very small inward currents in clonal rat phaeochromocytoma (PC 12) cells, thus also indicating partial agonistic actions (Nagata *et al.*, 1996).

Furthermore, single-cell analysis revealed that imidacloprid activates predominatly a subconductance of approximately 10 pS, whereas acetylcholine activated mostly the high conductance state with 25 pS. Multiple conductance states were also observed in an insect nAChR reconstituted into planar lipid bilayers (Hancke and Breer, 1986) and on locust neurons (van den Breukel *et al.*, 1998).

5.3.6.3. Correlation between Electrophysiology and Radioligand Binding Studies

There is a good correlation between electrophysiological measurements, using isolated *Heliothis* neurons, and radioligand binding studies on housefly head membranes regarding the affinity of different ligands to nAChRs (**Figure 22**). This good correlation for the neonicotinoids may indicate that houseflies (binding data) and tobacco budworms (electrophysiology) have similar binding sites for imidacloprid and related compounds. Biochemical investigations using [^{3}H]imidacloprid as a radioligand in a number of different insect membrane preparations, e.g., from *Periplaneta americana*, *Lucilia sericata*, *D. melanogaster*, *Manduca sexta*, *H. virescens*, *Ctenocephalides felis*, *M. persicae*, and *N. cincticeps*, indicate that many (if not all) insects have high specific imidacloprid binding sites with nearly identical K_d values of ~1–10 nM (Lind *et al.*, 1998). However, it was also found that only the homopteran species seems to have an additional very high affinity binding site.

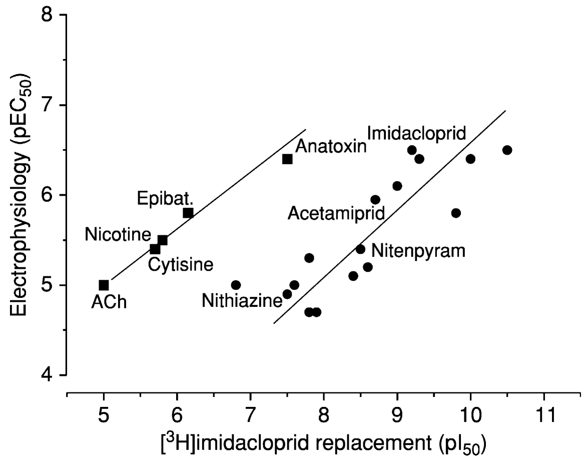


Figure 22 Comparison between electrophysiological and binding potencies of different neonicotinoids and nicotinoids. Electrophysiological data were obtained from neuron cell bodies isolated from the CNS of *Heliothis virescens*. pEC_{50} values (= −log *M*) correspond to the half maximal activation of nAChR by each agonist. Binding data: pI_{50} values (= −log *M*) correspond to the concentration of cold ligand displacing 50% of bound [^{3}H]imidacloprid from housefly head membranes. (Reproduced from Nauen, R., Ebbinghaus-Kintscher, A., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Iishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 77–105.)

In general, the pI_{50} values obtained by the displacement of specifically bound [^{3}H]imidacloprid from housefly head membranes were two to four orders of magnitude higher than the electrophysiologically determined pEC_{50} values obtained from isolated *Heliothis* neurons. Similar differences in biochemical binding and functional assay studies were also observed for different vertebrate nAChRs (review: Holladay *et al.*, 1997). A possible explanation for this phenomenon might be provided by considering the following: it is generally accepted that each nAChR can exist in multiple states, i.e., a resting state, an active (open) state, and one or more desensitized state(s), each of which has different affinities for ligands. The active state has a low affinity for ACh (K_d ranging from about 10 μM to 1000 μM), whereas the desensitized state(s) has a higher affinity (K_d ranging from about 10 nM to 1 μM) for nicotinic ligands (Lena and Changeux, 1993).

The kinetics of the transitions between these states have been resolved for *Torpedo* nAChR *in vitro*. The rate of isomerization between the resting and active state lies in the μs to ms timescale, and

within the desensitized state over a timeframe of seconds to minutes (Changeux, 1990, cited in Lena and Changeux, 1993). Because binding studies are conducted over a timescale of minutes to hours they may reflect interaction with the desensitized state(s), whereas electrophysiological studies measure the interaction of ligands with the active state. Considering this, it is surprising that there is a direct correlation between electrophysiological and biochemical binding studies for natural alkaloids such as (−)-nicotine, cytisine, (±)-epibatidine and anatoxin A (**Figure 22**). For these compounds the pI_{50} and pEC_{50} values were in the same range, with a good correlation. It is a general observation that natural alkaloids such as nicotine and epibatidine exhibit an agonistic potency in electrophysiological assays on isolated cockroach neurons (Bai *et al.*, 1991; Buckingham *et al.*, 1997; Orr *et al.*, 1997), *Heliothis* neurons (**Figure 22**), and locust neurons (Nauen *et al.*, 1999b) (see below). This potency is comparable to highly insecticidal neonicotinoids like imidacloprid. Matsuda *et al.* (1998), using a hybrid receptor formed by the coexpression of the *Drosophila* α subunit SAD with the chicken ß2 observed a comparable agonistic potency for both (−)-nicotine and imidacloprid. However, the agonistic potency of (+)-epibatidine was about two orders of magnitude greater. In contrast, all binding studies using [^{3}H]imidacloprid on housefly head membranes (Liu and Casida, 1993a, 1993b; Liu *et al.*, 1995; Wollweber and Tietjen, 1999), whitefly preparations (Chao *et al.*, 1997; Nauen *et al.*, 2002; Rauch and Nauen, 2003), and *Myzus* preparations (Nauen *et al.*, 1996, 1998a; Lind *et al.*, 1998), indicate that imidacloprid has a considerably higher potency in replacing specifically bound [^{3}H]imidacloprid than (−)-nicotine.

5.3.7. Pharmacokinetics and Metabolism

Neonicotinoids have moderate and different water solubility. They are nonionized and not readily hydrolyzed at physiological pH values (**Table 3**). The substances are biodegradable, therefore no accumulation was observed in mammals or through food chains (Roberts and Hutson, 1999). Neonicotinoid metabolism involves mostly detoxification reactions evident in three pathways (Tomizawa and Casida, 2003): (1) the brief period for poisoning at sublethal doses in both insects and mammals, (2) the synergistic effects of detoxification inhibitors on insecticidal action, and (3) the potency of metabolites relative to the parent compounds on nAChRs. The pharmacokinetics of neonicotinoids and imidacloprid have been investigated in *S. littoralis*, and the obtained profiles were consistent with high metabolism and an important role of nontarget tissues as sinks in this less responsive species (Greenwood *et al.*, 2002).

5.3.7.1. Metabolic Pathways of Commercial Neonicotinoids

5.3.7.1.1. Imidacloprid The metabolism of imidacloprid is strongly influenced by the method of application (Nauen *et al.*, 1998b). Depending on time and plant species, imidacloprid is degraded more or less completely, as comparative studies in many field crops have revealed (Araki *et al.*, 1994). The uptake and translocation of [^{14}C]imidacloprid after foliar application was studied in cotton. These studies showed that imidacloprid is taken up readily when mixed with the adjuvant Silwet L-77, then translocated acropetally and rapidly metabolized (Nauen *et al.*, 1999b). Experiments with seed-treated cotton plants revealed that only 5% of the applied imidacloprid was taken up by the young plant, and that 27 days after sowing, approximately 95% of the parent compound was metabolized (Tröltzsch *et al.*, 1994).

From results of soil metabolism studies it was found that imidacloprid is completely degradable and will not persist in soil. Under standard laboratory conditions the aerobic degradation of imidacloprid has a half-life (DT_{50}) of 156 days. The a.i. is degraded to carbon dioxide, and any residues are firmly bound to the soil matrix (Krohn and Hellpointer, 2002). Radioactively labeled imidacloprid shows that other degradation products, >10% of the applied radioactivity, do not occur. Sunlight and anaerobic conditions accelerated the degradation of imidacloprid. The degradation pathway shows that the neonicotinoid itself, and not the individual degradation products of the parent compound, is present in soil (Krohn and Hellpointer, 2002). Imidacloprid is not stabile in an aqueous environment, and in such an environment sunlight further accelerates the degradation.

The metabolism of imidacloprid was studied in tomatoes, potatoes, and maize (Roberts and Hutson, 1999) (**Figure 23**). In experiments using tomatoes, [^{14}C-*pyridinyl*]imidacloprid was applied to fruits. The main metabolites were the *N*-desnitro-imidacloprid and the 4- and 5-hydroxy derivatives (see Section 5.3.3.5.3). Small amounts of the cyclic urea, the *N*-nitroso compound were identified. A similar pattern of metabolites to the above mentioned was described for vines, together with a trace of the bicyclic triazinone. Traces of parent imidacloprid

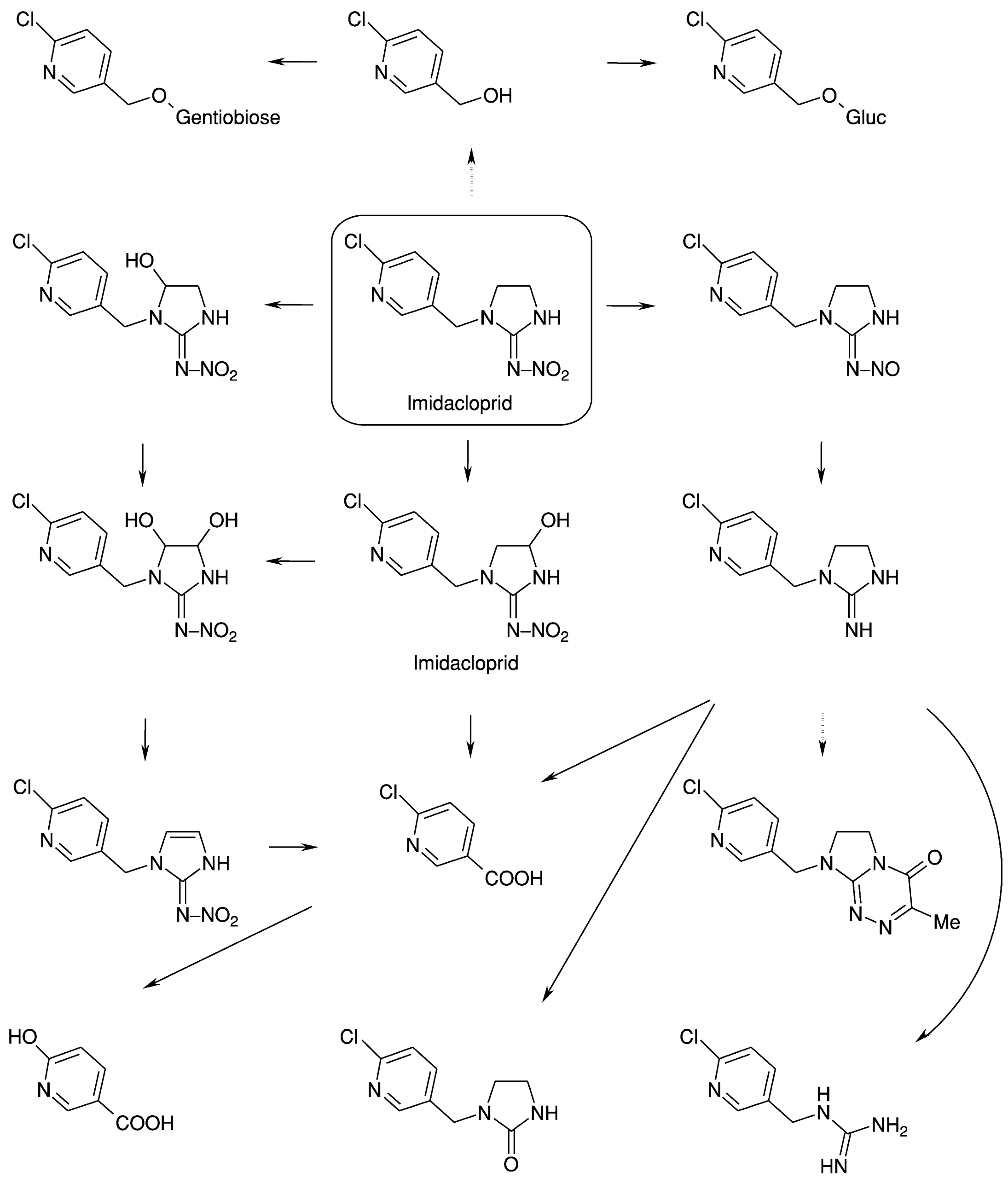


Figure 23 Proposed pathway of imidacloprid metabolism in plants. (Reproduced with permission from Roberts, T.R., Hutson, D.H. (Eds.), **1999**. Metabolic Pathways of Agrochemicals, Part 2, Insecticides and Fungicides. Cambridge University Press, Cambridge.)

and 6-chloro-nicotinic acid (6-CNA) were detected in the tubers. Koester (1992) studied the comparative metabolism of imidacloprid in suspension cultures of cells of several plant species including soybean and cotton, and found that the initial pathway of degradation proceeded mainly by monohydroxylation of the imidazolidine ring and dehydration to the olefin (see Section 5.3.3.5.3). Small amounts of 6-CNA and the 6-chloro-picolyl alcohol (6-CPA) and its glucopyranosid were formed. These identified metabolites are consistent with those found in whole plants.

5.3.7.1.2. Thiacloprid The metabolic pathway of the systemic thiacloprid, in quantitative and also qualitative terms, is similar in all crops (fruiting crops, cotton) investigated (Klein, 2001). The parent compound labeled in [methylene-^{14}C]-position is always the major component. The presence of high amounts of 6-CNA in cotton seed is due to

the accumulation of this acid in the seed as a phloem sink, after being secreted from the apoplasm into the phloem as a trap compartment for weak acids (Klein, 2001). It originated from leaf metabolism, where it was also detected (Klein, 2001). All main metabolites identified in plants were also detected in the rat and livestock metabolism study. By using two different labeling positions ([methylene-^{14}C] and [thiazolidine-^{14}C]), besides the unchanged parent compound, 25 metabolites were isolated and identified in the rat excreta. In goats and hens the residue levels in tissues and organs, as well as in milk and eggs, were low due to the fast elimination. There was no bioaccumulation of residues in any of the tissues. Generally, the metabolism of thiacloprid proceeded via the following pathways (Klein, 2001):

- hydroxylation of the parent compound at the 4-position of 1,3-thiazolidine ring and oxidative cleavage at the methylene bridge leading to 6-CPA and 6-CNA followed by conjugation of these two aglycones with sugars
- the corresponding 1,3-thiazolidine ring metabolites mainly comprised of the free 1,3-thiazolidine, the 4-hydroxylated 1,3-thiazolidine and its 4,5-dehydro (olefinic) derivative, as well as conjugates
- the *N*-nitrile moiety was hydrolyzed to the corresponding *N*-amide derivative, which was then *N*-hydroxylated
- the 1,3-thiazolidine ring system was cleaved in 1-position and the sulfur oxidized or methylated, forming the sulfonic acid or the *S*-methy-sulfoxyl and *S*-methyl-sulfonyl group, respectively.

5.3.7.1.3. Thiamethoxam In metabolism studies, the bulk of thiamethoxam (84–95%) was excreted in the urine with a small amount (2.5–6%) in the feces within 24 h, primarily as unchanged parent compound. This was also observed in rats, mice, ruminants, and poultry. Hydrolysis of the perhydro-1,3,5-oxadiazine ring system in thiamethoxam followed by *N*-demethylation is described as the major metabolic pathway; two other compounds are formed by loss of the *N*-nitro group from either metabolite. Reduction of the *N*-nitro group to a hydrazine, and subsequent conjugation with 2-oxo-propionic or acetic acids results in several major metabolites. Thiamethoxam metabolism was well investigated in plants like maize, rice, pears, and cucumbers. The active ingredient exhibits a systemic behavior, i.e., it is translocated via the roots into the whole plant. An accumulation was observed at the leaf borders, with very low residue levels. The same metabolites were found in cereals and fruits. An aqueous photolysis study with radiolabeled thiamethoxam indicated that the neonicotinoid degrades significantly under photolytic conditions (Schwartz *et al.*, 2000).

5.3.7.1.4. Nitenpyram The polar nitenpyram has a relatively short persistence in soil (DT_{50} 1–15 days), which probably offsets the relatively weak sorption that might otherwise lead to mobility through soils. In plants the metabolites 2-[*N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl]amino-2-methyliminoacetic acid (CPMA) and *N*-(6-chloro-3-pyridylmethyl)-*N*-ethyl-*N*-methylformamidine (CPMF) were formed. Presumably CPMF was produced in the field by decarboxylation of CPMA (Tsumura *et al.*, 1998). Animal metabolism was investigated by oral administration of nitenpyram labeled with ^{14}C to rats. As a result, 95–98% of the ^{14}C administered was excerted into urine within 2 days after treatment and no accumulation of ^{14}C in the internal organs was observed. The results reflect the high water solubility (840 g l^{-1}), and the low partition coefficient ($\log P_{OW} = -0.64$ at 25 °C) of nitenpyram may contribute to its low toxicity against mammals (Akayama and Minamida, 1999).

5.3.7.1.5. Acetamiprid Acetamiprid is a mobile, rapidly biodegradable compound in most soil. The primary degradation pathway is aerobic soil metabolism. Acetamiprid is relatively stable to hydrolysis at environmental temperatures, and photodegrades relatively slowly in water, whereas it is rapidly degraded in soils to form *N*-(6-chloro-pyrid-3-yl-methyl)-*N*-methyl-amine and 6-CNA. The DT_{50} values of acetamiprid in clay loam or light clay soils were in range of 1–2 days. In soil from the field or that used in container studies, acetamiprid degraded very rapidly (half-life 12 days). The main pathway was nitrile oxidation of acetamiprid into the N-amidino derivative, which cleaved, affording the *N*-(6-chloro-pyrid-3-ylmethyl)-*N*-methyl-amine and finally to 6-CNA. Mineralization to carbon dioxide followed.

Acetamiprid accounted for the majority of the residue in the crops, and metabolites occurred in negligible amounts. The main component found in plant metabolism studies was the parent acetamiprid. Small amounts of *N*-demethylacetamiprid were formed which cleaved to give 6-CNA and to a lesser extent oxidized to 6-CPA. The latter in turn either formed a conjugate with glucose or was also thought to form a *N*-(6-chloro-pyrid-3-ylmethyl)-*N*-methylamine as a minor metabolite (Tokieda *et al.*, 1998; Roberts and Hutson, 1999) (**Figure 24**). The

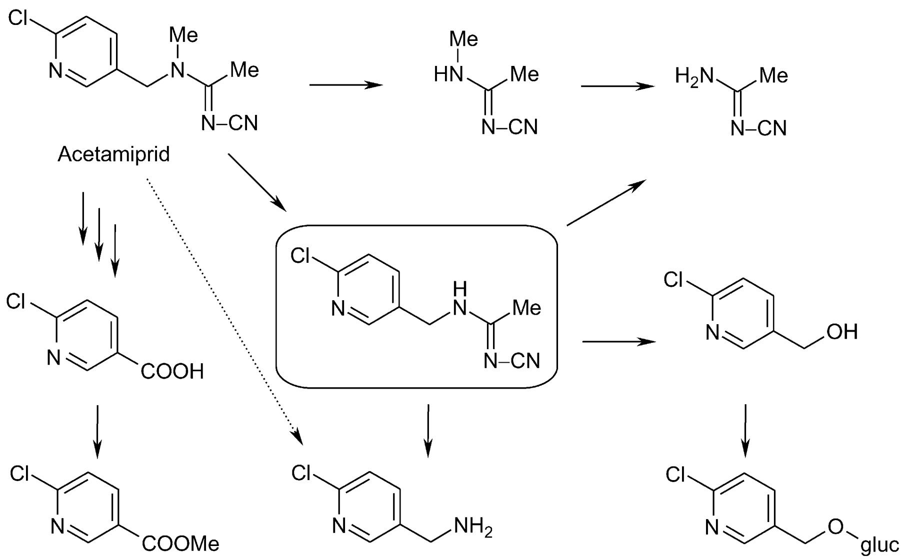


Figure 24 Proposed pathway of acetamiprid metabolism in plants. (Reproduced with permission from Roberts, T.R., Hutson, D.H. (Eds.), **1999**. Metabolic Pathways of Agrochemicals, Part 2, Insecticides and Fungicides. Cambridge University Press, Cambridge.)

metabolic fate of [2,6-^{14}C-*pyridine*]acetamiprid was investigated in eggplants and apples following application to foliage or fruit, and in cabbage plants after application to foliar and soil. Parent acetamiprid was slowly metabolized to similar products in all plants. Acetamiprid was absorbed by and penetrated the foliage well but it was poorly translocated (Roberts and Hutson, 1999).

5.3.7.1.6. Clothianidin Studies of the metabolic pathway of clothianidin in maize and sugar beet show a clear and consistent picture (Klein, 2003). The parent compound is always the major component of the residue in the edible parts of the crops. Up to seven metabolites were identified, but most of them did not exceed 10% of the total radioactive residues. Furthermore, all the main metabolites identified in plants were also detected in rat metabolism studies (Klein, 2003; Yokota *et al.*, 2003). Generally, the following reactions are involved in the metabolic pathway of clothianidin in maize and sugar beets after seed treatment application:

- *N*-demethylation to form the *N*-demethyl clothianidin and subsequent hydrolysis to produce the *N*-(2-chloro-1,3-thiazol-5-ylmethyl)urea;
- hydrolysis of the *N*′-nitroimino moiety to form *N*-(2-chloro-1,3-thiazol-5-yl)-*N*′-methylurea and subsequent *N*-demethylation to form *N*-(2-chloro-1,3-thiazol-5-yl)urea;
- denitrification (reduction) to form *N*-(2-chloro-1,3-thiazol-5-yl)-*N*-methylguanidine and C–N bond cleavage within the compound to form *N*-methylguanidine;
- C–N bond cleavage (loss of the 1,3-thiazol-5-ylmethyl moiety) to form *N*-methyl-*N*′-nitroguanidine;
- *N*-demethylation of *N*-methyl-*N*′-nitroguanidine to form *N*′-nitroguanidine;
- denitrification (reduction of *N*-methyl-*N*′-nitroguanidine to form *N*-methylguanidine);
- C–N bond cleavage of *N*-(2-chloro-1,3-thiazol-5-yl)-*N*′-nitroguanidine to form nitroguanidine; and
- C–N bond cleavage (loss of the *N*′-nitroimino moiety) and subsequent oxidation of the intermediate to form 2-chloro-1,3-thiazol-5-carboxylic acid.

Unchanged parent compound is the major constituent of the residue at harvest. The major pathway of metabolism involves *N*-demethylation of clothianidin to produce *N*-(2-chloro-1,3-thiazol-5-ylmethyl)-*N*′-nitroguanidine. In some crop parts *N*-methylguanidine made up some 20% of the residue at harvest (Klein, 2003).

5.3.8. Pharmacology and Toxicology

Because of the target site selectivity, neonicotinoids are more toxic to aphids, leafhoppers, and other sensitive insects than to mammals and aquatic life (Matsuda *et al.*, 1998; Yamamoto *et al.*, 1998; Tomizawa *et al.*, 2000; Tomizawa and Casida, 2003). Comparative binding studies on insects nAChRs indicate that their ability to displace tritiated imidacloprid from its binding site correlates well with their insecticidal efficacy (Kagabu, 1997a; Nauen *et al.*, 2001).

5.3.8.1. Safety Profile

The introduction of imidacloprid as the first CNI was a milestone in the general effort to enhance the environmental safety of crop protection agents (Krohn and Hellpointer, 2002) (**Tables 6** and 7).

5.3.8.1.1. Mammalian toxicity The safety factor of imidacloprid, that means the ratio of the lethal doses for mammals to insects, can be estimated as 7300 from the LD_{50} value of 0.062 mg kg^{-1} for *M. persicae* by topical application and an oral LD_{50} value for rats of 450 mg kg^{-1} (**Table 6**) (Elbert *et al*., 1990). Therefore imidacloprid ranks as an insecticide with high selective toxicity.

Thiacloprid possesses a low acute toxicity. Birds and mammals are at neither acute nor chronic risk when thiacloprid is applied at the recommended dose under practical conditions.

After oral dosing to rats, thiamethoxam is rapidly and completely absorbed, and up to 90% of the applied material is readily eliminated as parent compound through the urine. Thiamethoxam has a low acute toxicity when applied to rats either orally, dermally, or by inhalation (**Table 6**). The neonicotinoid is not irritant to skin and eyes, and has no sensitizing potential (Maienfisch *et al*., 1999a, 2001a). Acute and absorption subchronic neurotoxicity studies in rats showed that thiamethoxam is devoid of any neurotoxic potential. However, mechanistic studies showed thiamethoxam demonstrated a phenobarbital-like induction of enzymes in mouse liver. In repeat dose feeding studies in rodents and dogs, the liver and kidneys (rat only) were the main target organs. In life-time rodent feeding studies, mice showed an increased incidence of liver tumors, which was specific to this species since a chronic rat study showed thiamethoxam had no tumorigenic potential (Maienfisch *et al*., 2001a). A 12-month dog feeding study produced some alterations in blood chemistry, and a minimal change was seen in testes weight in two males at 1500 ppm, and tubular atrophy was observed at 750 and 1500 ppm. Dermal absorption studies in rats, and *in vitro* studies with rat and human skin, resulted in very low absorption of thiamethoxam. After an exposure time of 6 h the systemic absorption of thiamethoxam was between 0.4% and 2.7% within 6 h, and between 0.8% and 2.9% within 48 h. Oral reproductive toxicity studies showed no evidence of developmental impairment or teratogenic potential (Maienfisch *et al*., 1999a).

Nitenpyram has a minimal adverse effect against mammals (Akayama and Minamida, 1999) (**Table 6**).

Acetamiprid is of relatively low toxicity to mammals, and it has been classified as an "unlikely" human carcinogen. In mammals, acetamiprid caused generalized, nonspecific toxicity and did not appear to have specific target organ toxicity. Acetamiprid has relatively low acute and chronic toxicity in mammals, and there was no evidence of carcinogenicity, neurotoxicity, and/or endocrinic disruption. Aggregate risk estimates for acetamiprid for food and water do not raise concern for acute and chronic levels of exposure. The acute oral toxicity to male rat (LD_{50} 217 mg kg^{-1}) and mouse (LD_{50} 198 mg kg^{-1}), as well as female rat (LD_{50} 146 mg kg^{-1}) and mouse (LD_{50} 184 mg kg^{-1}) is noncritical. The acute dermal toxicity to male and female rats is low (>2000 mg kg^{-1}). Toxicological tests for acetamiprid, e.g., irritation to skin and eyes (rabbit), sensitization to skin (guinea pig), and mutagenicity, as evaluated by the Ames test, were all negative.

5.3.8.1.2. Environmental fate The fate of crop protection agents such as neonicotinoids in the environment includes its behavior in the soil, water, and air compartments (Stupp and Fahl, 2003). After application of a crop protection agent (as a formulation) to crops or in/on soil, a part of the active substance is not taken up by the plants remains, e.g., on the soil. Other parts of the agent can be transported to neighboring water bodies via drift, wind erosion, or runoff, or can enter the air by evaporation from the plant surface (Stupp and Fahl, 2003). Therefore, different physicochemical and biological processes are involved in the environmental behavior of an active substance, i.e., hydrolytic and photochemical degradation, microbial transformation and decomposition like metabolism, sorption to the soil, and translocation of the active substance and its degradation products.

Formulated imidacloprid is stable at room temperature in the dark under usual storage conditions; however, the active ingredient is labile in alkaline media (Kagabu and Medej, 1995, 2003b). Numerous field trials carried out in Europe and the USA on the degradation of imidacloprid demonstrate that the active ingredient does not accumulate in the soil (Krohn and Hellpointer, 2002). There is no likelihood of an accumulatiom occurring following repeated yearly applications. Long-term studies in the UK indicate that the maximum concentrations in the soil reaches a plateau, having a relatively low residual level ($\leq$0.030 mg kg^{-1}) (Krohn and Hellpointer, 2002). Imidacloprid is not stable in an aqueous environment, where sunlight further accelerates its degradation. Those characteristics influence the environmental behavior of imidacloprid on surface water and in water sediment such as in rivers and lakes, as well as in fog and rain. The

Table 6 Toxicological properties of commercialized neonicotinoids (selected relevant data)

		Ring systems			*Noncyclic structures*			
Study type	*Species*	*Imidacloprid*[a]	*Thiacloprid*[b]	*Thiamethoxam*[c]	*Nitenpyram*[d]	*Acetamiprid*[d]	*Clothianidin*[e]	*(±)-Dinotefuran*[d]
Acute oral (LD_{50} mg a.i. kg^{-1} bw)	Rat	~450 (m/f)	836 (m) 444 (f)	1563	1680 (m) 1575 (f)	217 (m) 146 (f)	>5000 (m/f)	2804 (m) 2000 (f)
Acute dermal (24 h) (LD_{50} mg a.i. kg^{-1} bw)	Rat	>5000 (m/f)	>2000 (m/f)	>2000	>2000 (m/f)		>2000 (m/f)	
Acute inhalation (4 h, aerosol) (LC_{50} mg a.i. m^{-3} air)	Rat	>5323 (dust)	>2535 (m) ~1223 (f)	3720	>5800 (m/f)	>0.29 (m/f)	>6141 (m/f)	
Skin irritation (4 h)	Rabbit	No irritation	No irritation	No irritation	No irritation	No irritation	No irritation	Acute percutaneous
Eye irritation	Rabbit	None	No irritation	No irritation	Very slight	No irritation	No irritation	Acute percutaneous
Skin sensitation	Guinea pig	No skin sensitation	No skin sensitation	No skin sensitation	No skin sensitation		No skin sensitation	No skin sensitation

[a]Elbert *et al.* (1990).
[b]Elbert *et al.* (2000).
[c]Maienfisch *et al.* (1999a).
[d]Tomlin (2000).
[e]Ohkawara *et al.* (2002).
m, males; f, females; a.i., active ingredient; bw, body weight.

Table 7 Environmental profile of commercialized neonicotinoids (selected relevant data)

	Ring systems			*Noncyclic structures*			
Test species (acute toxicity test)	*Imidacloprid*[a]	*Thiacloprid*[b]	*Thiamethoxam*[c]	*Nitenpyram*[d,g]	*Acetamiprid*[e]	*Clothianidin*[f]	*(±)-Dinotefuran*[e]
Birds							
Mallard duck (LC_{50} mg a.i. kg^{-1} diet)	>5000 (food)	>5000 (food)[g]	>5200 (food)	>5620 (food)		>5200 (food)	1000
Bobwhite quail (LD_{50} mg a.i. kg^{-1} bw)	152	2716	1552	2250 (capsule)	180	>2000	
Japanese quail (LD_{50} mg a.i. kg^{-1} bw)	31	49					>2000
Fish							
Rainbow trout (LC_{50} mg a.i. l^{-1})	211 (96 h)	29.6 (96 h)	>125 (96 h)	>10 (96 h)		>100 (96 h)	>40 (48 h)
Bluegill sunfish (LC_{50} mg a.i. l^{-1})		24.5 (96 h)	>114 (96 h)			>120 (96 h)	
Carp (LC_{50} mg a.i. l^{-1})	280 (96 h)			>1000 (96 h)	>100		>1000 (96 h)
Invertebrates							
Water flea, *Daphnia magna* (EC_{50} mg a.i. l^{-1})	85 (48 h)	>85.1 (48 h)	>100 (48 h)	>100 (48 h)[h]	>1000	>120 (48 h)	1000 (48 h)
Earthworm, *Eisenia foetida* (LC_{50} mg a.i. kg^{-1} dry soil)	10.7 (14 days)	105 (14 days)[g]	>1000 (14 days) (soil)	32.2 (14 days)	>1000	13.21 (14 days) (dry soil)	

[a] Pflüger and Schmuck (1991).
[b] Schmuck (2001).
[c] Maienfisch *et al.* (1999a).
[d] Akayama and Minamida (1999).
[e] Tomlin (2000).
[f] Ohkawara *et al.* (2002).
[g] Technical material.
[h] Unpublished data; Sumitomo Chemical Takeda Agro, 1999.

occurrence of imidacloprid in the air is determined by its low vapor pressure of 4×10^{-10} Pa, which excludes volatilization from treated surfaces (Krohn and Hellpointer, 2002).

Thiacloprid possesses a favorable environmental profile and will disappear from the environment after having been applied as a crop protection chemical. The effect of technical and formulated (SC 480) thiacloprid on respiratory (carbon turnover) and nitrogen mineralization rates in soil were examined in a series of 28-day studies on soil microflora (Schmuck, 2001). Thiacloprid had no adverse effect either on the microbial mineralization of nitrogen in different soil types like silty sand (0.6% org.C, pH_{KCl} 5.7) or loamy silt soil (2.3% org.C, pH_{KCl} 7.1) after addition of lucerne–grass–green meal ($5\,g\,kg^{-1}$) and at both the maximum and $10\times$ application rates (Schmuck, 2001). The half-lives in soil measured under field conditions of northern Europe ranged from 9 to 27 days, and in southern Europe from 10 to 16 days (Krohn, 2001). The major metabolites formed are only intermediates before complete mineralization to carbon dioxide occurs. Under practical field conditions thiacloprid is not toxic to earthworms (*Eisenia foetida*) or soil microorganisms (**Table 7**).

Furthermore, thiacloprid and its formulations pose a favorably low toxicity hazard to honeybees and bumblebees. Consequently, the compound can be applied to flowering crops at the recommended rates without posing a risk to bees (Calypso™ up to $200\,g\,a.i.\,ha^{-1}$). In addition, Calypso™ does not affect the pollination efficacy of pollinators (Elbert *et al.*, 2000). Thiacloprid must be classified as being only slightly mobile in soil, and hence it has low potential for leaching into groundwater. In the aquatic environment thiacloprid will also undergo rapid biotic degradation, with a half-life of 12–20 days (Krohn, 2001). Because of its low vapor pressure (3×10^{-10} Pa) and its water solubility of $0.185\,g\,l^{-1}$, the potential for its volatilization is negligible. Acute and chronic toxicity testing in fish and water-fleas (*Daphnia magna*) indicates a very low to negligible risk to these species following accidental contamination with thiacloprid by spray drift. Reproduction of water-fleas and fish was also not affected at environmentally relevant water concentrations of thiacloprid. Other aquatic invertebrates revealed a higher sensitivity to thiacloprid, especially insect larvae and amphipod species such as *Chironomus* and *Hyallela*. Insect larvae were also identified as the most sensitive species in a pond study. However, the rapid dissipation of thiacloprid in water bodies allows for a rapid recovery of affected aquatic insect populations. Algae, represented by *Scenedesmus subspicatus* and *Pseudokirchneriella subcapitata*, are not sensitive to thiacloprid. Toxicity exposure ratio values indicate a high margin of safety for algal species. Harmful concentrations of thiacloprid or its metabolites are most unlikely in aquatic ecosystems. There is no potential for bioconcentration in fish due to the log P_{OW} of 1.26 for thiacloprid. The metabolites occurring in groundwater are of no concern as noted by toxicological, ecotoxicological, and biological testing.

In laboratory soils, thiamethoxam degrades at moderately slow rates (Maienfisch *et al.*, 1999a). The compound is photolyzed rapidly in water. In natural aquatic systems, e.g., rice paddies, degradation also occurs in the absence of light by microbial degradation. Based on the low vapor pressure (**Table 3**) and the results of soil volatility studies, significant volatilization is not expected. Thiamethoxam has a low toxicity by ingestion to birds, and is practically nontoxic to fish, *Daphnia* and molluscs. In addition, the earthworm species *E. foetida* and green algae were also found to be insensitive (Maienfisch *et al.*, 2001a). Thiamethoxam can be classified as slightly to moderately harmful to most beneficial insects, but safe to predatory mites in the field (Maienfisch *et al.*, 1999a). Alternatively, thiamethoxam and all other nitroguanidines have to be considered toxic to bees. However, thiamethoxam showed no bioaccumulation potential, and is moderately mobile in soil and degrades at fast to moderate rates under field conditions (Maienfisch *et al.*, 1999a).

Nitenpyram rapidly decomposed in water, under the conditions of 12 000 lux irradiation intensity using a 500 W xenon lamp (half-life at pH 5, 18–21 min; in purified water, 16 min), and in soil under aerobic conditions (half-lives flooded <1 day; upland 1–3 days) (Akayama and Minamida, 1999).

Toxicity of acetamiprid to fish and *Daphnia* is very low, with LD_{50} values for carp and *Daphnia* of $>100\,mg\,l^{-1}$ and $>1000\,mg\,l^{-1}$, respectively (Yamada *et al.*, 1999). Acetamiprid shows less adverse effects on both natural and enemies, such as predaceous mites, and beneficial insects, as well as honeybee and bumblebee (Takahashi *et al.*, 1998).

Chlothianidin degraded moderately under field conditions and was found to be a neonicotinoid of medium mobility based on laboratory experiments (Stupp and Fahl, 2003). With regard to its behavior in aquatic environments, photolysis contributes significantly to degradation, and clothianidin finally mineralizes to carbondioxide. Its degradation in water/sediment systems under aerobic conditions is moderate. The main part of the applied active

substance is bound irreversibly to the sediment, thus avoiding subsequent contamination (Stupp and Fahl, 2003). In addition, in a water/sediment study degradation of clothianidin was observed to be significantly faster (factor 2–3) under anaerobic conditions than in aerobic conditions (Stupp and Fahl, 2003). Based on the physicochemical properties of clothianidin (see Section 5.3.3.4) no volatilization, and thus, no significant amounts of this compound can be expected in the atmosphere. Therefore clothianidin does not appear to represent a risk for the environment.

5.3.9. Resistance

Resistance in arthropod pest species comprises a change in the genetic composition of a population in response to selection by pesticides, such that control of the pest species in the field may be impaired at the recommended application rates. Selection for resistance in pest populations (including weeds, fungi, mites, and insects) due to frequent applications of agrochemicals has been one of the major problems in modern agriculture. Resistance to insecticides and acaricides appeared very early, and the extent and economic impact remain greater than for other agrochemicals. Between the beginning of the last century and the mid-1950s, the number of resistant arthropod species grew gradually with only a few resistant species described per decade. However, this rate increased markedly after the introduction of the organophosphates to more than 30 new resistant species every 2 years through the early 1980s. Ten years later, more than 500 arthropod species were known to be resistant to at least one insecticide or acaricide (Georghiou, 1983; Green *et al*., 1990). Among them are 46 hopper and aphid species, which show in some cases very high levels of resistance to conventional types of insecticide chemistry, i.e., organophosphates, and carbamate and pyrethroid insecticides. The invention and subsequent commercial development of neonicotinoid insecticides, such as imidacloprid, has provided agricultural producers with invaluable new tools for managing some of the world's most destructive crop pests, primarily those of the order Hemiptera (aphids, whiteflies, and planthoppers) and Coleoptera (beetles), including species with a long history of resistance to earlier-used products (**Figure 20**). However, the speed and scale with which imidacloprid, the commercial forerunner of neonicotinoids, was incorporated into control strategies around the world prompted widespread concern over the development of imidacloprid resistance (Cahill *et al*., 1996; Denholm *et al*., 2002).

To a large extent these pessimistic forecasts have not been borne out in practice. Imidacloprid has proved remarkably resilient to resistance, and cases of resistance that have been reported are still relatively manageable and/or geographically localized. The existence of strong resistance in some species has nonetheless demonstrated the potential of pests to adapt and resist field applications of neonicotinoids. The ongoing introduction of new molecules (e.g., acetamiprid, thiamethoxam, nitenpyram, thiacloprid, and clothianidin), unless carefully regulated and coordinated, seems bound to increase exposure to neonicotinoids, and to enhance conditions favoring resistant phenotypes.

5.3.9.1. Activity on Resistant Insect Species

The incidence and management of insect resistance to neonicotinoid insecticides was recently reviewed by Denholm *et al.* (2002). Resistance to neonicotinoid insecticides is still rare under field conditions and baseline susceptibility data have been provided in the past and more recently especially for imidacloprid in order to monitor for early signs of resistance in some of the most destructive pest insects (Cahill *et al*., 1996; Elbert *et al*., 1996; Elbert and Nauen, 1996; Foster *et al*., 2003; Nauen and Elbert, 2003; Rauch and Nauen, 2003; Weichel and Nauen, 2003). Baseline susceptibility data and derived diagnostic concentrations to monitor resistance are usually calculated from composite log-dose probit-mortality lines, including the combined curves of several strains of a certain pest collected from all over the world or at least different parts of the world where the compound is supposed to be used. A compilation for imidacloprid of such data is given for some of the most important pests, i.e., *Myzus persicae, Aphis gossypii, Phorodon humuli, Bemisia tabaci, Trialeurodes vaporariorum*, and *Leptinotarsa decemlineata* (**Table 8**).

There have been only low levels of tolerance detected in European and Japanese samples of *M. persicae* (Devine *et al*., 1996; Nauen *et al*., 1996), but it was not possible to link this tolerance to specific biochemical markers (Nauen *et al*., 1998a). In most cases the lower susceptibility to imidacloprid and other neonicotinoids was correlated with a decreased efficacy of nicotine (Devine *et al*., 1996; Nauen *et al*., 1996). Furthermore differences in hardiness were sometimes observed in field strains bioassayed directly upon receipt, which allow strains to survive longer in comparison to susceptible laboratory populations, when exposed to imidacloprid-treated leaves (Nauen and Elbert, 1997). However, such effects disappeared when the exposure time (especially in systemic bioassays) was extended

Table 8 Baseline susceptibility of some high risk pests to imidacloprid

Species	*Diagnostic dose*	*Bioassay system/assessment time*	*Reference*
Myzus persicae	15 ppm	Leaf dip (6-well plate)	Nauen and Elbert (2003)
Myzus persicae	2.25 ng per aphid	Topical	Foster *et al.* (2003)
Aphis gossypii	13 ppm	Leaf dip (6-well plate)	Nauen and Elbert (2003)
Phorodon humuli	13 ppm	Leaf dip (6-well plate)	Weichel and Nauen (2003)
Bemisia tabaci	16 ppm	Systemic bioassay	Cahill *et al.* (1996)
Bemisia tabaci	1 ppm	Leaf dip	Rauch and Nauen (2003)
Leptinotarsa decemlineata	8 ppm	Artificial diet (larvae)	Olson *et al.* (2000)
Leptinotarsa decemlineata	0.2 μg per beetle	Topical (adult)	Nauen (unpublished data)

from 48 h to 72 h, and also after maintaining such strains under laboratory conditions for some weeks (Nauen and Elbert, 1997). More recently Foster *et al.* (2003b) demonstrated that tolerance to imidacloprid in *M. persicae* from different regions in Europe also provided cross-tolerance to acetamiprid. The authors were able to show a clear correlation between ED_{50} values of acetamiprid and imidacloprid for strains with a different degree of tolerance. However, tolerance factors compared to a susceptible reference population never exceeded factors of 20, and field failures were not seen (Foster *et al.*, 2003b).

One species of major concern over the last decade is the tobacco or cotton whitefly, *B. tabaci*; this is a serious pest in many cropping systems worldwide and several biotypes of this species have been described (Perring, 2001). The most widespread biotype is the B-type, which is also known as *B. argentifolii* Bellows & Perring. The B-type whitefly is a common pest, particularly in cotton, vegetables, and ornamental crops, both by direct feeding and as a vector of numerous plant pathogenic viruses. In southern Europe, it coexists with another biotype, the Q-type, which was originally thought to be restricted to the Iberian peninsula, but which is now also known to occur in some other countries throughout the Mediterranean area, including Italy and Israel (Brown *et al.*, 2000; Palumbo *et al.*, 2001; Nauen *et al.*, 2002; Horowitz *et al.*, 2003). The biotypes B and Q can easily be distinguished by randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) or native polyacrylamide gel electrophoresis (PAGE) and subsequent visualization of their nonspecific esterase banding pattern (Guirao *et al.*, 1997; Nauen and Elbert, 2000).

As a consequence of extensive exposure to insecticides, *B. tabaci* has developed resistance to a wide range of chemical control agents (Cahill *et al.*, 1996). The need for a greater diversity of chemicals for whitefly control in resistance management programs has been met by the introduction of several insecticides with new modes of action, which are unaffected by mechanisms of resistance to organophosphates or pyrethroids. Since the introduction of imidacloprid, the neonicotinoids have been the fastest-growing class of insecticides. Imidacloprid exhibits an excellent contact and systemic activity and therefore has been largely responsible for the sustained management of *B. tabaci* in horticultural and agronomic production systems worldwide. Beside imidacloprid, there are other neonicotinoids with good efficacy against whiteflies, e.g., acetamiprid and thiamethoxam.

In Israel, monitoring of resistance in *B. tabaci* to imidacloprid and acetamiprid was initiated in 1996 in cotton and greenhouse ornamental crops. After 2 years of use in cotton, no apparent resistance to imidacloprid and acetamiprid was reported (Horowitz *et al.*, 1998). However, 3 years of acetamiprid use in greenhouses in Israel resulted in a 5–10-fold decrease in susceptibility of *B. tabaci* to acetamiprid (Horowitz *et al.*, 1999). In the past only a few cases of lowered neonicotinoid susceptibility in B-type *B. tabaci* have been described, among them strains from Egypt and Guatemala that were recently reported (El Kady and Devine, 2003; Byrne *et al.*, 2003). In Arizona, where imidacloprid has been used since 1993, monitoring of *B. tabaci* populations from cotton fields, melon fields, and greenhouse vegetables suggested reduced susceptibility to imidacloprid from 1995 to 1998, but subsequent monitoring showed that these populations had actually regained and sustained susceptibility to imidacloprid in 1999 and 2000 (Li *et al.*, 2000, 2001). Furthermore, imidacloprid use in Arizona and California remains high, but no signs of reduced control in the field have been reported yet (Palumbo *et al.*, 2001). B-type whiteflies have been shown to develop resistance to imidacloprid under selection pressure in the laboratory (Prabhaker *et al.*, 1997). There was a moderate increase of resistance of up to 17-fold in the first 15 generations, but 82-fold resistance after 27 generations. However, resistance was not stable and disappeared after a few generations without insecticide pressure. Resistance to

imidacloprid conferring a high level of cross-resistance to thiamethoxam and acetamiprid was first demonstrated, and best studied in Q-type *B. tabaci* from greenhouses in the Almeria region of southern Spain, but was also detected in single populations from Italy and recently Germany as well (Nauen and Elbert, 2000; Nauen *et al.*, 2002; Rauch and Nauen, 2003). Neonicotinoid resistance seem to remain stable in all field-collected Q-type strains maintained in the laboratory without further selection pressure (Nauen *et al.*, 2002). Neonicotinoid cross-resistance was also reported in B-type whiteflies from cotton in Arizona but at lower levels (Li *et al.*, 2000). More recently a high level of cross-resistance between neonicotinoids was also described in a B-type strain of *B. tabaci* from Israel, and resistance factors detected in a leaf-dip bioassay exceeded 1000-fold (Rauch and Nauen, 2003).

The Colorado potato beetle, *L. decemlineata* has a history of developing resistance to virtually all insecticides used for its control. The first neonicotinoid, i.e., imidacloprid was introduced for controlling Colorado potato beetles in North America in 1995. Concerns over resistance development were reinforced when extensive monitoring of populations from North America showed about a 30-fold variation in LC_{50} values from ingestion and contact bioassays against neonates (Olsen *et al.*, 2000). Much of this variation appeared unconnected with imidacloprid use, and was probably a consequence of cross-resistance from chemicals used earlier. Lowest levels of susceptibility occurred in populations from Long Island, New York, an area that has experienced the most severe resistance problems of all with *L. decemlineata*. Zhao *et al.* (2000) and Hollingworth *et al.* (2002) independently studied single strains collected from different areas in Long Island, both treated intensively with imidacloprid between 1995 and 1997. In the first study, grower's observations of reduced control were supported by resistance ratios for imidacloprid of 100-fold and 13-fold in adults and larvae, respectively. The second study reported 150-fold resistance from topical application bioassays against adults. In this case the strain was also tested with thiamethoxam, which had not been used for beetle control at the time of collection. Interestingly, resistance to thiamethoxam (about threefold) was far lower than to imidacloprid.

Other reports referring to resistance to neonicotinoid insecticides were on species of lesser importance, including species either from field-collected populations or artificially selected strains. Among these were the small brown planthopper, *Laodelphax striatellus* (Sone *et al.*, 1997), western flower thrips, *Franklienella occidentalis* (Zhao *et al.*, 1995), houseflies, *Musca domestica* and German cockroach, *Blattella germanica* (Wen and Scott, 1997), *Drosophila melanogaster* (Daborn *et al.*, 2001), *Lygus hesperus* (Dennehy and Russell, 1996), and brown planthoppers, *Nilaparvata lugens* (Zewen *et al.*, 2003).

5.3.9.2. Mechanisms of Resistance

Many pest insects and spider mites have developed resistance to a broad variety of chemical classes of insecticides and acaricides, respectively (Knowles, 1997; Soderlund, 1997). One of the three major classes of mechanisms of resistance to insecticides in insects is allelic variation in the expression of target proteins with modified insecticide binding sites, e.g., acetylcholinesterase insensitivity towards organophosphates and carbamates, voltage-gated sodium channel mutations responsible for knockdown resistance to pyrethroids, and a serine to alanine point mutation (*rdl* gene) in the γ-aminobutyric acid (GABA)-gated chloride channel ($GABA_A$-R) at the endosulfan/fipronil/dieldrin binding site (ffrench-Constant *et al.*, 1993; Williamson *et al.*, 1993, 1996; Mutero *et al.*, 1994; Feyereisen, 1995; Dong, 1997; Soderlund, 1997; Zhu and Clark, 1997; Bloomquist, 2001; Gunning and Moores, 2001; Siegfried and Scharf, 2001). The second – and often most important – class of resistance mechanisms in insect pest species is metabolic degradation involving detoxification enzymes such as microsomal cytochrome P-450 dependent monooxygenases, esterases, and glutathione *S*-transferases (Hodgson, 1983; Armstrong, 1991; Hemingway and Karunarantne, 1998; Bergé *et al.*, 1999; Devonshire *et al.*, 1999; Feyereisen, 1999; Hemingway, 2000; Field *et al.*, 2001; Scott, 2001; Siegfried and Scharf, 2001). The third, least important mechanism is an altered composition of cuticular waxes which affects penetration of toxicants. Reduced penetration of insecticides through the insect cuticle has often been described as a contributing factor, in combination with target site insensitivity or metabolic detoxification (or both), rather than functioning as a major mechanism on its own (Oppenoorth, 1985). Most of the mechanisms mentioned above affect in many cases the efficacy of more than one class of insecticides, i.e., constant selection pressure to one chemical class could to a greater or lesser extent confer cross-resistance to compounds from other chemical classes (Oppenoorth, 1985; Soderlund, 1997).

When the first neonicotinoid insecticide was introduced to the market in 1991, aphids were considered to be high risk pests with regard to their potential to develop resistance to this class of

chemicals. They have a high reproductive potential, and extremely short life cycle allowing for numerous generations in a growing season. Combined with frequent applications of insecticides that are usually required to maintain aphid populations below economic thresholds, resistance development is facilitated in these species, resulting in control failures. Such control failures have been reported for organophosphorus compounds for many decades, and more recently also for pyrethroids (Foster *et al*., 1998, 2000; Devonshire *et al*., 1999; Foster and Devonshire, 1999).

One of the major aphid pests is the green peach aphid, *M. persicae*. Resistance of *M. persicae* to insecticides is conferred by increased production of a carboxylesterase, named E4 or FE4, which provides cross-resistance to carbamates, organophosphorus, and pyrethroid insecticides (Devonshire and Moores, 1982; Devonshire, 1989). This esterase overproduction was shown to be due to gene amplification (Field *et al*., 1988, 2001). It was the sole resistance mechanism reported in *M. persicae* for more than 20 years, and only recently an insensitive (modified) acetylcholinesterase was described as a contributing factor in carbamate resistance in *M. persicae* (Moores *et al*., 1994a, 1994b; Nauen *et al*., 1996). The insensitive acetylcholinesterase in *M. persicae* confers strong resistance to pirimicarb, and a little less to triazamate (Moores *et al*., 1994a, 1994b; Buchholz and Nauen, 2001). Due to the improvement of molecular biological techniques, Martinez-Torres *et al.* (1998) recently showed that knockdown resistance to pyrethroids, caused by a point mutation in the voltage-gated sodium channel, is also present in *M. persicae*. In summary, resistance to all major classes of aphicides occurs in *M. persicae*; however, the only class of insecticides not yet affected by any of the mechanisms described above are the neonicotinoids, including its most prominent member imidacloprid (Elbert *et al*., 1996, 1998a; Nauen *et al*., 1998a; Horowitz and Denholm, 2001).

The most comprehensive studies on the biochemical mechanisms of resistance to neonicotinoid insecticides using an agriculturally relevant pest species were performed in whiteflies, *B. tabaci* (Nauen and Elbert, 2000; Nauen *et al*., 2002; Rauch and Nauen, 2003; Byrne *et al*., 2003). Biochemical examinations revealed that neonicotinoid resistance in Q-type *B. tabaci* collected in 1999 was not associated with a lower affinity of imidacloprid to nAChRs in whitefly membrane preparations (Nauen *et al*., 2002). This was confirmed more recently by testing strains ESP-00, GER-01, and ISR-02 obtained in the years 2000–2002 by Rauch and Nauen (2003). Although neonicotinoid resistance was very high in these strains (up to 1000-fold), the authors found just a 1.3-fold and 1.7-fold difference in binding affinity between strains, and concluded that target site resistance is not involved in neonicotinoid resistance in those strains investigated. Piperonyl butoxide, a monooxygenase inhibitor, is generally used as a synergist to suppress insecticide resistance conferred by microsomal monooxygenases. Experiments with whiteflies pre-exposed to piperonyl butoxide suggested a possible involvement of cytochrome P-450 dependent monooxygenases in neonicotinoid resistance (Nauen *et al*., 2002). Rauch and Nauen (2003) biochemically confirmed that whiteflies resistant to neonicotinoid insecticides showed a high microsomal 7-ethoxycoumarin O-deethylase activity, i.e., up to eightfold higher compared with neonicotinoid susceptible strains. Furthermore, this enhanced monooxygenase activity could be correlated with imidacloprid, thiamethoxam, and acetamiprid resistance. Significant differences between glutathione *S*-transferase and esterase levels were not found between neonicotinoid resistant and susceptible strains of *B. tabaci* (Rauch and Nauen, 2003). Several metabolic investigations in plants and vertebrates showed that imidacloprid and other neonicotinoids undergo oxidative degradation, which may lead to insecticidally toxic and nontoxic metabolites (Araki *et al*., 1994, Nauen *et al*., 1999b; Schulz-Jander and Casida, 2002). Metabolic studies in *B. tabaci in vivo* revealed that the main metabolite in neonicotinoid-resistant strains is 5-hydroxy-imidacloprid, whereas no metabolism could be detected in the susceptible strain (**Figure 23**) (see Section 5.3.7.1.1). One can therefore suggest that oxidative degradation is the main route of imidacloprid detoxification in neonicotinoid resistant Q-type whiteflies (Rauch and Nauen, 2003). Compared to imidacloprid, the 5-hydroxy metabolite showed a 13-fold lower binding affinity to whitefly nAChR. This result was in accordance with previous studies with head membrane preparations from the housefly (Nauen *et al*., 1998). The binding affinity expressed as the IC_{50} was highest with olefine (0.25 nM) > imidacloprid (0.79) > 5-hydroxy (5 nM) > 4-hydroxy (25 nM) > dihydroxy (630 nM) > guanidine and urea (>5000 nM). The biological efficacy in feeding bioassays with aphids correlated also with the relative affinities of the metabolites towards the housefly nAChR (Nauen *et al*., 1998b). The lower binding affinity of 5-hydroxy-imidacloprid compared to imidacloprid coincides with its lower efficacy against *B. tabaci* in the sachet test (17-fold). These data show that differences between binding to the

nAChR are comparable to the efficacy differences in adult bioassays (Rauch and Nauen, 2003).

Daborn *et al*. (2001) described *D. melanogaster* mutants exhibiting an eight- and sevenfold resistance to imidacloprid and DDT, respectively, which showed an overexpression of the P-450 gene *Cyp6g1*. However, it was not tested whether recombinant Cyp6g1 itself can metabolize imidacloprid; potentially it could metabolize a *trans*-acting factor which then upregulates presently uncharacterized P-450 genes. However, it was demonstrated that the recombinant cytochrome P-450 isozyme CYP3A4 from human liver can selectively metabolize the imidazolidine moiety of imidacloprid, resulting in 5-hydroxy-imidacloprid as the major metabolite (Schulz-Jander and Casida, 2002). It would therefore be fruitful to investigate if homologs of *Cyp6g1* and *Cyp3a4*, or similar genes, are present in the *B. tabaci* genome, and if overexpression of these genes is associated with neonicotinoid resistance.

Although considerable resistance to neonicotinoid insecticides was also reported for populations of the Colorado potato beetle from Long Island, NY, the underlying mechanisms associated have not been very well studied. Pharmacokinetic investigations on susceptible and resistant strains did not reveal any differences in the uptake and excretion of ^{14}C-labeled imidacloprid, nor were there any differences in metabolic conversion found (Hollingworth *et al*., 2002). The authors suggested a modification of the target site to be responsible for the observed resistance. But, preliminary investigations revealed no differences between resistant and susceptible strains in receptor binding studies using [^{3}H]imidacloprid (Nauen and Hollingworth, unpublished data).

Resistance development to imidacloprid would be disastrous in many regions, so there is a demand for an effective resistance management program, and interest in finding and developing new active ingredients for pest control.

5.3.9.3. Resistance Management

Monitoring and detection of insecticide resistance in order to recommend and implement effective resistance management strategies is currently one of the most important areas of applied entomology. This program is necessary to sustain the activity of as many active ingredients with different modes of action as possible by using alternate spray regimes, rotation, and sophisticated application techniques (Denholm and Rowland, 1992; McKenzie, 1996; Denholm *et al*., 1998, 1999; Horowitz and Denholm, 2001). Historically, the problem of insecticide resistance was tackled by continuously introducing new active ingredients for pest insect control. The number of insecticides available is high, but the biochemical mechanisms targeted by all these compounds is rather limited (Casida and Quistad, 1998; Nauen and Bretschneider, 2002). A pronounced decline in the introduction of new insecticides since the late 1970s revealed the limitations of a strategy relying on the continuous introduction of new active compounds (Soderlund, 1997).

This problem has been recognized by the regulatory authorities; and the European Plant Protection Organization (EPPO) recently published guidelines outlining the background work on resistance required for registration of a new active ingredient (Heimbach *et al*., 2000). These requirements, which are provided by agrochemical companies as an essential part of the registration dossier, include

- baseline susceptibility studies (testing several strains of a target species known to be prone to resistance development);
- monitoring (continuous studies on the development of resistance of target species by simple bioassays after the launch of a new compound); and
- possible resistance management strategies (how the new compound should be combined with others in order to expand its life time in the field).

5.3.10. Applications in Nonagricultural Fields

Because of the high insecticidal efficacy together with its nonvolatility and stability under storage conditions, imidacloprid (Premise®) has been successfully applied as a termiticide (Jacobs *et al*., 1997a; Dryden *et al*., 1999), and has also been used for control of turf pests such as white grubs (Elbert *et al*., 1991). Furthermore, imidacloprid is the active ingredient of the insecticide Merit®, and is commonly incorporated into fertilizers for early control of grubs in turf (Armbrust and Peeler, 2002). In addition, imidacloprid was the first neonicotinoid to be used in a gel bait formulation for cockroach control. The imidacloprid gel showed outstanding activity even after 27 months' under various conditions (Pospischil *et al*., 1999). As an endoparasiticide imidacloprid exerted activity against the gastrointestinal nematode *Haemonchus contortus* in sheep only at higher concentrations (Mencke and Jeschke, 2002). Synergistic mixtures containing imidacloprid are patented and these are useful against textile-damaging insects, such as

moth (*Tineola bisselliella, Tinea pellionella*) and beetles (*Attagenus, Anthrenus*) (Mencke and Jeschke, 2002). Salmon-parasitizing crabs are controlled by addition of 100 ppm imidacloprid to seawater (Mencke and Jeschke, 2002). Due to its toxicological properties – favorable mammalian safety characteristics (Yamamoto *et al*., 1995) (**Table 6**), the absence of eye/skin irritation and skin sensitization potential – imidacloprid has been developed for control of lice in humans (Mencke and Jeschke, 2002) and veterinary medicine (Werner *et al*., 1995). Imidacloprid (worldwide trademark: Advantage®) is the first neonicotinoid to have been developed for topical application in animals (Griffin *et al*., 1997) (see Section 5.3.10.1).

Nitenpyram (Capstar®), a fast-acting, orally administered flea treatment, is absorbed into blood of the host animal, and is thus readily available for uptake by feeding fleas (Rust *et al*., 2003). Therefore, administration of nitenpyram is effective in eliminating adult fleas for up to 48 h after treatment.

5.3.10.1. Imidacloprid as a Veterinary Medicinal Product

The cat flea (*Ctenocephalides felis*), the primary ectoparasite of companion animals worldwide, will feed on a wide variety of animals in addition to cats and dogs (Rust and Dryden, 1997), although it is not equally well adapted to all hosts (Williams, 1993). Fleas threaten the health of humans and animals due to bite reactions and transmission of diseases (Krämer and Mencke, 2001), and in addition are major nuisance pests. Therefore, flea control, is necessary.

In veterinary medicine, fleas are the primary cause for flea allergic dermatitis. This dermatitis results when the adult flea injects saliva into the host during blood feeding, which accelerates immunological response, leading to secondary infections of the skin.

The last aspect of veterinary importance is the role of fleas in disease, for example, transmission of the cestode *Dipylidium caninum*. The role of fleas in human disease transmission has been known since historic times. The Oriental rat flea (*Xenopsylla cheopis*) is the major transmitter of *Yersinia pestis*, the bacterium that causes the bubonic plague in humans. The cat flea is also capable of transmitting *Y. pestis*, and human plague cases and even deaths associated with infected cats and dogs have been occasionally reported (Rust *et al*., 1971).

Furthermore, a variety of bacteria and viruses have been reported to be transmitted by the dog flea (*C. canis*) as well as the cat flea (*C. felis*) (Krämer and Mencke, 2001). Moreover, cat owners have a high incidence of cat scratch fever, a zoonotic disease caused by the Gram-negative bacterium *Bartonella henselae*. Recent research showed that flea feces are the major means of disease transmission between cats, and from cats to humans (Malgorzata *et al*., 2000).

5.3.10.1.1. Insecticidal efficacy in veterinary medicine Recommendations for the treatment of fleas on companion animals, and the selection of an insecticide and its formulation, are generally based upon the species and age of the animal to be treated, the level of infestation, the rate of potential reinfestation, and the thoroughness of environmental treatment. However, the selection of an insecticide formulation by a pet owner is actually based on economics and the product's ease of use (Williams, 1993). Another factor in the choice of a flea treatment by pet owners is the safety and toxicology of the insecticide (Krämer and Mencke, 2001).

Imidacloprid, 10% spot-on, was designed to offer a dermal treatment, which means it is applied externally onto a small dorsal area (a spot) of the animal's skin. Criteria for the selection of an appropriate topical flea formulation are good solubility of the compound, good adhesion to the skin, good spreading properties, good local and systemic tolerance, stability, and compatibility with legal standards. The imidacloprid spot-on formulation, which meets all these requirements, contains 10 g a.i. in 100 ml nonaqueous solution. The efficacy of this formulation for flea control on cats and dogs has been reported (Krämer and Mencke, 2001). Imidacloprid applied at the target therapeutic dosage of 10.0 $mg\,kg^{-1}$ killed 99% of the fleas within 1 day of treatment, and continued to provide 99–100% control of further flea infestation for at least 4 weeks (Hopkins *et al*., 1996; Arther *et al*., 1997) (**Figure 25**).

Studies using flea-infested cats (Jacobs *et al*., 1997b) proved that imidacloprid possess considerable potency against adult fleas on cats, and retains a high level of activity for 4–5 weeks. Imidacloprid was effective for both immediate relief from an existing flea burden (the therapeutic effect), and for longer-term flea control (the prevention or prophylactic effect) (**Table 9**). Imidacloprid spreads and acts using animal skin as the main carrier. The compound was shown to be localized in the water-resistant lipid layer of the skin surface, produced by sebaceous glands, and spread over the body surface and onto the hair (Mehlhorn *et al*., 1999). Insecticide spread over the skin surface was also reported from a clinical study that observed fast onset of flea control, as early as 6 h posttreatment (Everett *et al*., 2000). Therefore if the superficial fatty layer of

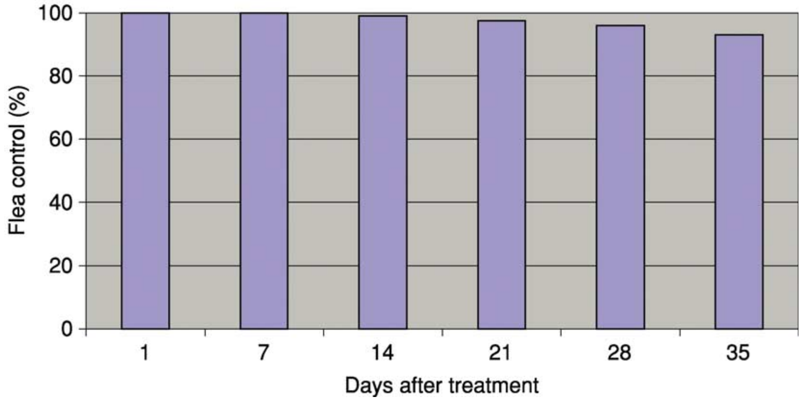


Figure 25 Flea control achieved with imidacloprid 10% spot-on as confirmed by a dose-confirmation study in flea infested dogs (Hopkins *et al.*, 1996).

Table 9 Geometric mean flea counts of two groups of cats, an untreated control and a group treated with imidacloprid 10% spot-on at a dosage of 10.0 mg kg^{-1} bw at day 0 of the study

Weeks after treatment	*After 1 day*			*After 2 days*		
	Control	*Treated*	*Reduction (%)*	*Control*	*Treated*	*Reduction (%)*
0	36.7	0.01	99.8	31.7	0.0	100.0
1	34.1	0.0	100.0			
2	31.0	0.2	99.3	27.3	0.0	100.0
3	32.1	1.0	97.0	28.9	0.1	99.7
4	36.2	5.0	86.1	27.8	0.9	96.7
5	33.8	9.5	71.9	26.8	3.1	88.3
6	28.1	20.1	28.4	24.8	10.6	57.3

Reproduced from Jacobs, D.E., Hutchinson, M.J., Krieger, K.J., **1997b**. Duration of activity of imidacloprid, a novel adulticide for flea control, against *Ctenocephalides felis* on cats. *Vet. Rec. 140*, 259–260.

the skin is removed by repeated swabbing with alcohol fleas consumed the same amount of blood in treated and untreated dogs. Thus imidacloprid localized in the lipid layer of the skin acts on adult fleas by contact. It was reported that imidacloprid is not taken up by the flea during blood feeding, but is absorbed via the flea's smooth, nonsclerotized intersegmental membranes that are responsible for the insect's mobility. Mehlhorn *et al.* (1999) concluded that this seems reasonable because of imidacloprid's lipophilicity renders it incapable of passing through the sclerotized cuticle. Moreover, initial damage was seen in the ganglia close to the ventral body side (i.e., in subesophageal and thoracic ganglia). Fleas affected by imidacloprid treatment showed characteristic pathohistological changes (Mehlhorn *et al.*, 1999). Muscle fibers and tissue around the subesophageal ganglion were damaged, with the mitochondria and axons showing intensive vacuolization (**Figure 26**). Imidacloprid's mode of action corresponded with the structural findings (Mehlhorn *et al.*, 1999), which showed overall destruction of the mitochondria, damage of the nerve cells, and disintegration of the insect muscles (**Figure 26**).

Imidacloprid's activity on ectoparasitic insects results from its presence within the lipid layer of the host body surface. Since this lipid layer is always present, imidacloprid remains available for a prolonged time (Hopkins *et al.*, 1996; Mehlhorn *et al.*, 2001a), and reduces the likelihood of its removal during water exposure (Mehlhorn *et al.*, 1999).

The spectrum of imidacloprid activity to ectoparasites is not confined to fleas. It has also proven to be highly effective against both sucking lice (*Linognathus setosus*), and biting or chewing lice (*Trichodectes canis*) (Hanssen *et al.*, 1999). Furthermore imidacloprid acted rapidly on all motile stages of sheep keds (Mehlhorn *et al.*, 2001b). Sheep keds of the species *Melophagus ovinus* are wingless parasitic insects belonging to the dipteran family Hippobiscidae. Besides skin infection, which results in the loss wool quality and meat production, sheep keds are also known to transmit diseases, such as trypanosomiasis. However, ticks did not prove to

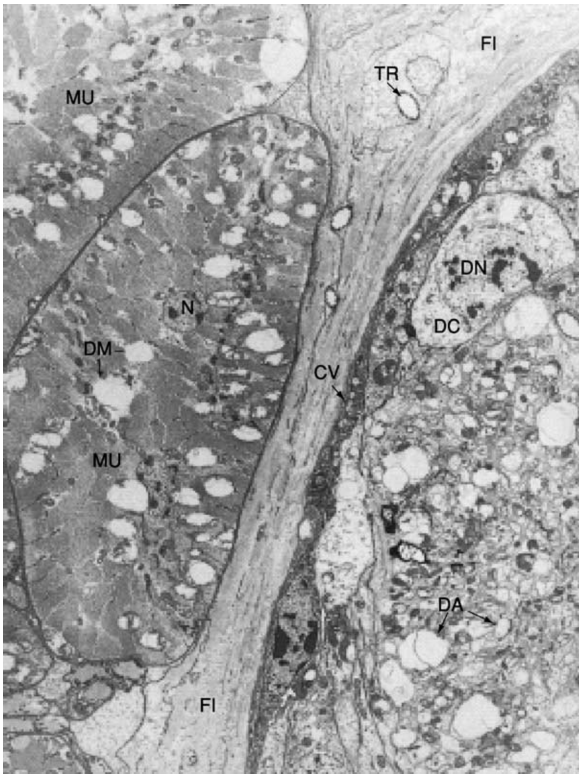


Figure 26 Transmission electron micrograph of a section through an adult cat flea exposed to imidacloprid for 1 h *in vitro*. Note the extensive damage at the level of muscle fibers and the subesophagal ganglion (vacuoles). CV, cellular cover of the ganglion; DA, degenerating axon; DC, degenerating nerve cell; DM, degenerating mitochondrion; FI, Fibrillar layer of connective tissue; Mu, Muscle fiber; TR, tracheole. Magnification ×25 000. (Reproduced with permission from Mehlhorn, H., Mencke, N., Hansen, O., **1999**. Effects of imidacloprid on adult and larval stages of the cat flea *Ctenocephalides felis* after *in vivo* and *in vitro* experiments: a light- and electronmicroscopy study. *Parasitol. Res. 85*, 625–637.)

be sensitive to complete control by imidacloprid (Young and Ryan, 1999).

5.3.10.1.2. Larvicidal activity Apart from the fast-acting adulticidal activity of imidacloprid on flea populations, its larvicidal effects have also been investigated. Reinfestation of animals, from earlier deposited eggs, larvae, and preemerged adult fleas, can be overcome by the use of effective larvicidal compounds or insecticides acting on both the adult and the immature stages of fleas. In early studies using imidacloprid, larvicidal activity was observed in the immediate surrounding of treated dogs (Hopkins *et al.*, 1997). Skin debris collected from treated dogs, when mixed into flea rearing media, showed high flea larva mortality. In repeated tests using the skin debris samples, collected at day 7 post-treatment, the larval mortality remained high at

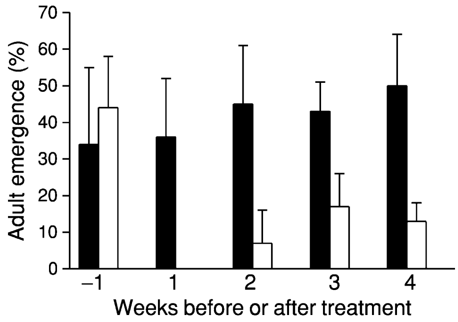


Figure 27 Emergence of adult fleas from flea eggs incubated on blankets used by untreated (control) or imidacloprid treated cats. (Reproduced from Jacobs, D.E., Hutchinson, M.J., Ewald-Hamm, D., **2000**. Inhibition of immature *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) development in the immediate environment of cats treated with imidacloprid. *J. Med. Entomol. 37*, 228–230.)

100% even after 51 days (Arther *et al.*, 1997). In *in vitro* studies, flea larvae survived for only 6 h when placed on clipped hair from imidacloprid treated dogs (Mehlhorn *et al.*, 1999). Similar results have also been reported for cats (Jacobs *et al.*, 2000). Adult flea emergence was reduced by 100%, 84%, 60%, and 74% in the first, second, third, and fourth week postimidacloprid treatment, in comparison to untreated controls (Figure 27). This persistent larvicidal activity is important, because in the absence of any larvicidal effect of an applied adulticide, reinfestation would occur from eggs deposited prior to treatment (Jacobs *et al.*, 2001). Furthermore, cats wander freely outdoors, and thus may visit sites shared with other flea-infested domestic or wild animals.

5.3.10.1.3. Flea allergy dermatitis (FAD) FAD is a disease in which a hypersensitive state is produced in a host in response to the injection of antigenic material from flea salivary glands (Carlotti and Jacobs, 2000). Synonyms for FAD include flea bite allergy and flea bite hypersensitivity. In cats, the disease is also known as feline miliary dermatitis and feline eczema. FAD is one of the most frequent causes of skin conditions in small animals, and a major clinical entity in dogs. FAD is the commonest nonroutine reason for pet owners to seek veterinary advice. Hypersensitivity to flea bites is not only of importance to domestic pets, but is also an important cause of the common skin disease in humans, termed papular urticaria. Detailed investigations carried out on patients exposed to flea-infested pets have shown that the incidence of such reactions is quite high.

Several field studies have been conducted, focusing on the efficacy of imidacloprid on cats and dogs with clinical signs of FAD (Krämer and Mencke, 2001). The efficacy of imidacloprid in flea removal,

and the resolution of FAD was tested in dogs and cats from single- and multiple-animal households (Genchi *et al.*, 2000). Flea infestation was examined and FAD dermatitis lesions were ranked according to severity of typical clinical signs. Flea numbers dropped significantly after treatment of animals from both single- and multiple-animal households. In dogs clinical signs of FAD prior to treatment, decreased from 38% to 16% by day 14, and 6% by day 28, thus verifying a rapid adulticidal and high residual activity that lasted at least 4 weeks. There was an effective control of parasites, with rapid improvement of allergy until almost complete remission up to 28 days following the first application. Recently studies on the effect of imidacloprid on cats with clinical signs of FAD confirmed field data published by Genchi *et al.* (2000). Clinical signs of FAD, especially alopecia and pruritus were resolved after monthly treatment using imidacloprid (Keil *et al.*, 2002) (**Figure 28**). Furthermore, controlling FAD is enhanced when blood feeding of fleas is reduced. This so-called "sublethal effect" or "antifeeding effect" was reported using very low concentrations of imidacloprid (Rust *et al.*, 2001, 2002).

5.3.10.1.4. Imidacloprid as combination partner in veterinary medicinal products The ability of acaricides to repel or kill ticks, before they attach to a host and feed, is important for the prevention of transmission of tick born pathogens (Young *et al.*, 2003). K9 Advantix™, an effective tick control agent (Spencer *et al.*, 2003, Mehlhorn *et al.*, 2003), is a spot-on product containing 8.8% (w/w) imidacloprid and 44% (w/w) permethrin. The mixture repels and kills four species of ticks, including *Ixodes scapularis*, for up to 4 weeks. It also repels and kills mosquitoes, and kills flea adults and larvae. Furthermore, a combination containing imidacloprid 10% (w/v) and permethrin 50% (w/v) in a spot-on formulation, has repellent and insecticidal efficacy, against the sand fly species (*Phlebotomus papatasi*) (Mencke *et al.*, 2003), ticks (*I. ricinus, Rhipicephalus sanguineus*), and flea (*C. felis felis*) (Epe *et al.*, 2003) on dogs.

Another combination product, (Advantage Heart™ (10% w/v imidacloprid plus 1% w/v moxidectin), a macrolide antihelmintic, has been developed as a spot-on for dermal application to kittens and cats (Arther *et al.*, 2003). It is intended for monthly application for control of flea infestations and intestinal nematodes, and for prevention of feline heartworm disease. It controls and treats not only established adult gastrointestinal parasites, but also developmental stages, including fourth instar larvae and immature adults of *Toxocara cati* in cats (Hellmann *et al.*, 2003; Reinemeyer and Charles, 2003). Furthermore, the spot-on combination is safe and highly efficacious against *T. canis* and Ancylostomatidae in naturally infested dogs (Hellmann *et al.*, 2003), as well as against *Sarcoptes scabiei* var. *canis* on dogs (Fourie *et al.*, 2003).

5.3.11. Concluding Remarks and Prospects

The discovery of neonicotinoids as a new class of nAChR ligands can be considered a milestone in insecticide research, and permits an understanding of the functional properties of insect nAChRs. Up to now the most current information regarding nAChRs originated from research with vertebrate receptors. The world market for insecticides is still dominated by compounds irreversibly inhibiting acetylcholinesterase, an important enzyme in the CNS of insects. The market share of these inhibitors,

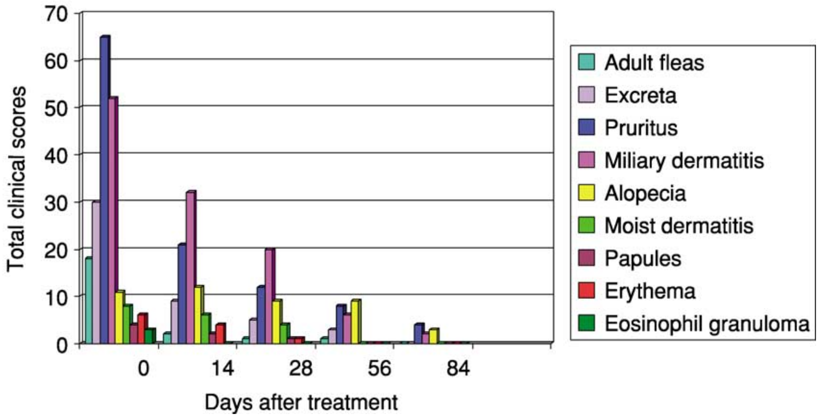


Figure 28 Resolution of signs of flea allergy dermatitis (FAD) in cats treated with imidacloprid over a 84-day study period (Keil *et al.*, 2002).

Table 10 Modes of action of the top selling 100 insecticides/acaricides and their world market share (excluding fumigants, endotoxins and those insecticides with unknown mode of action)

	Market share (%)		
Mode of action	*1987*	*1999*	*Change (%)*
Acetylcholinesterase	71	52	−20.0
Voltage-gated sodium channel	17	18	+1.4
Acetylcholine receptor	1.5	12	+10.0
GABA-gated chlorine channel	5.0	8.3	+3.3
Chitin biosynthesis	2.1	3.0	+0.9
NADH dehydrogenase	0	1.2	+1.2
Uncouplers	0	0.7	+0.7
Octopamine receptor	0.5	0.6	+0.1
Ecdysone receptor	0	0.4	+0.4

Reproduced with permission from Nauen, R., Bretschneider, T., **2002**. New modes of action of insecticides. *Pestic. Outlook 12*, 241–245. © The Royal Society of Chemistry.

and those insecticides acting on the voltage-gated sodium channel, account for approximately 70% of the world market (Nauen and Bretschneider, 2002) (**Table 10**).

Today, the neonicotinoids are the fastest-growing group of insecticides (estimated marked share in 2005: about 15%), with widespread use in most countries in many agronomic cropping systems, especially against sucking pests but also against ectoparasitic insects. The relatively low risk and target specificity of the products, combined with their suitability for a range of application methods, also will ensure their success as important insecticides in integrated pest management (IPM) strategies.

References

Akamatsu, M., Ozoe, Y., Ueno, T., Fujita, T., Mochida, K., *et al*., **1997**. Sites of action of noncompetitive GABA antagonists in housflies and rats: three-dimensional QSAR analysis. *Pestic. Sci. 49*, 319–332.

Akayama, A., Minamida, I., **1999**. Discovery of a new systemic insecticide, nitenpyram and its insecticidal properties. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 127–148.

Amar, M., Thomas, P., Wonnacott, S., Lunt, G.G., **1995**. A nicotinic acetylcholine receptor subunit from insect brain forms a non-desensitizing homo-oligomeric nicotine acetylcholine receptor when expressed in *Xenopus* oocytes. *Neurosci. Lett. 199*, 107–110.

Araki, Y., Bornatsch, W., Brauner, A., Clark, T., Dräger, G., *et al*., **1994**. Metabolism of imidacloprid in plants. In: Proc. IUPAC Congress, Washington 2B–157.

Armbrust, K.I., Peeler, H.B., **2002**. Effects of formulation on the run-off of imidacloprid from turf. *Pest Mgt Sci. 58*, 702–706.

Armstrong, W.N., **1991**. Glutathione *S*-transferases: reaction, metabolism, structure, and function. *Chem. Res. Toxicol. 4*, 131–142.

Arther, R.G., Bowman, D.D., McCall, J.W., Hansen, O., Young, D.R., **2003**. Feline Advantage Heart™ (imidacloprid and moxidectin) topical solution as monthly treatment for prevention of heartworm infection (*Dirofilaria immitis*) and control of fleas (*Ctenocephalides felis*) on cats. *Parasitol. Res. 90*, 137–139.

Arther, R.G., Cunningham, J., Dorn, H., Everett, R., Herr, L.G., *et al*., **1997**. Efficacy of imidacloprid for removal and control of fleas (*Ctenocephalides felis*) on dogs. *Am. J. Vet. Res. 58*, 848–850.

Bai, D., Lummis, S.C.R., Leicht, W., Breer, H., Satelle, D.B., **1991**. Actions of imidacloprid and related nitromethylen on cholinergic receptors of an identified insect motor neuron. *Pestic. Sci. 33*, 197–204.

Balant, L.P., Doelker, E., **1995**. Metabolic considerations in prodrug design. In: Wolff, M.E. (Ed.), Burger's Medicinal Chemistry and Drug Discovery, 5th edn., vol. 1. Wiley-Interscience, New York, pp. 949–982.

Becke, A.D., **1988**. Density-functional exchange-energy approximation with correct asymtotic behavior. *Phys. Rev. A 38*, 3098–3100.

Beers, W.H., Reich, E., **1970**. Structure and activity of acetylcholine. *Nature 228*, 917–922.

Benson, J.A., **1989**. Insect nicotinc acetylcholine receptors as targets for insecticides. In: McFarlane, N.R., Farnham, A.W. (Eds.), Progress and Prospects in Insect Control, BCPC Monograph, vol. 43. British Crop Protection Council, pp. 59–70.

Bergé, J.B., Feyereisen, R., Amichot, M., **1999**. Cytochrome P-450 monooxygenases and insecticide resistance in insects. In: Denholm, I., Pickett, J.A., Devonshire, A.L. (Eds.), Insecticide Resistance: From Mechanisms to Management. CAB International, Wallingford, pp. 25–31.

Bertrand, D., Ballivet, M., Gomez, M., Bertrand, S., Phannavong, B., *et al*., **1994**. Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate β2 subunit and *Drosophila* α subunits. *Eur. J. Neurosci. 6*, 869–875.

Bloomquist, J.R., **2001**. GABA and glutamate receptors as biochemical sites for insecticide action. In: Ishaaya, I. (Ed.), Biochemical Sites Important in Insecticide Action and Resistance. Springer, Berlin, pp. 17–41.

Boëlle, J., Schneider, R., Géradin, P., Loubinoux, B., Maienfisch, P., *et al*., **1998**. Synthesis and insecticidal evaluation of imidacloprid analogs. *Pestic. Sci. 54*, 304–307.

Born, L., **1991**. The molecular and crystal structure of imidacloprid (phase 2). *Pflanzenschutz-Nachrichten Bayer 44*, 137–144.

Breer, H., **1988**. Receptors for acetylcholine in the nervous system of insects. In: Lund, G.G. (Ed.), Neurotox 88: Molecular Basis of Drug and Pesticide Action. Excerpta Medica, Amsterdam, pp. 301–309.

Briggs, G., Bromilow, R.H., Evans, A.A., **1982**. Relationships between lipophilicity and root uptake and translocation of non-ionized chemicals by barley. *Pestic. Sci. 13*, 495–504.

Brown, J.K., Perring, T.M., Cooper, A.D., Bedford, I.D., Markham, P.G., **2000**. Genetic analysis of *Bemisia* (Hemiptera: Aleyrodidae) populations by isoelectric focusing electrophoresis. *Biochem. Genet. 38*, 13–25.

Bryant, R., **2001**. Chemistries for the newer groups of agrochemical active ingredients. *Agro-Food-Industry Hi-Tech 9/10*, 58–63.

Buchholz, A., Nauen, R., **2001**. Organophosphate and carbamate resistance in *Myzus persicae* Sulzer and *Phorodon humuli* Schrank (Homoptera: Aphididae): biological and biochemical considerations. *Mitteil. Deutschen Gesell. Allg. Angew. Entomol. 13*, 227–232.

Buchholz, A., Nauen, R., **2002**. Translocation and translaminar bioavailability of two neonicotinoid insecticides after foliar application to cabbage and cotton. *Pest Mgt Sci. 58*, 10–16.

Buckingham, S.D., Lapied, B., Le Corronc, H., Grolleau, F., Sattelle, D.B., **1997**. Imidacloprid actions on insect neuronal acetylcholine receptors. *J. Exp. Biol. 200*, 2685–2692.

Byrne, F.J., Castle, S., Prabhaker, N., Toscano, N.C., **2003**. Biochemical study of resistance to imidacloprid in B biotype *Bemisia tabaci* from Guatemala. *Pest Mgt Sci. 59*, 347–352.

Cahill, M., Gorman, K., Day, S., Denholm, I., Elbert, A., *et al.*, **1996**. Baseline determination and detection of resistance to imidacloprid in *Bemisia tabaci* (Homoptera: Aleyrodidae). *Bull. Entomol. Res. 86*, 343–349.

Carlotti, D.N., Jacobs, D.E., **2000**. Therapy, control and prevention of flea allergy dermatitis in dogs and cats. *Vet. Dermatol. 11*, 83–98.

Casida, J.E., Quistad, G.B., **1998**. Golden age of insecticide research: past, present, or future? *Annu. Rev. Entomol. 43*, 1–16.

Chao, S.L., Dennehy, T.J., Casida, J.E., **1997**. Whitefly (Hemiptera: Aleyrodidae) binding site for imidacloprid and related insecticides: a putative nicotinic acetylcholine receptor. *J. Econ. Entomol. 90*, 879–882.

Cheung, H., Clarke, B.S., Beadle, D.J., **1992**. A patch-clamp study of the action of a nitromethylene heterocycle insecticide on cockroach neurons growing *in vitro*. *Pestic. Sci. 34*, 187–193.

Cramer, R.D., Paterson, D.E., Bunce, J.D., **1988**. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. *J. Am. Chem. Soc. 110*, 5959–5967.

Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., ffrench-Constant, R., **2001**. DDT resistance in *Drosophila* correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genom. 266*, 556–563.

Denholm, I., Cahill, M., Dennehy, T.J., Horowitz, A.R., **1999**. Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly *Bemisia tabaci*. In: Denholm, I., Pickett, J.A., Devonshire, A.L. (Eds.), Insecticide Resistance: From Mechanisms to Management. CAB International, Wallingford, pp. 81–93.

Denholm, I., Devine, G., Foster, S., Gorman, K., Nauen, R., **2002**. Incidence and management of insecticide resistance to neonicotinoids. *Proc. Brighton Crop Protection Conference: Pests and Diseases 1*, 161–168.

Denholm, I., Horowitz, A.R., Cahill, M., Ishaaya, I., **1998**. Management of resistance to novel insecticides. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action, Mechanism and Application. Springer, New York, pp. 260–282.

Denholm, I., Rowland, M.W., **1992**. Tactics for managing pesticide resistance in arthropods: theory and practice. *Annu. Rev. Entomol. 37*, 91–112.

Dennehy, T.J., Russell, J.S., **1996**. Susceptibility of *Lygus* bug populations in Arizona to acephate (Orthene) and bifenthrin (Capture) with related contrasts of other insecticides. In: Proceedings Beltwide Cotton Conferences, Nashville, TN, USA, vol. 2. pp. 771–776.

Devine, G., Harling, Z., Scarr, A.W., Devonshire, A.L., **1996**. Lethal and sublethal effects of imidacloprid on nicotine-tolerant *Myzus nicotianae* and *Myzus persicae*. *Pestic. Sci. 48*, 57–62.

Devonshire, A.L., **1989**. Insecticide resistance in *Myzus persicae*: from field to gene and back again. *Pestic. Sci. 26*, 375–382.

Devonshire, A.L., Denholm, I., Foster, S., **1999**. Insecticide resistance in the peach potato aphid, *Myzus persicae*. In: Denholm, I., Ioannidis, P.M. (Eds.), Combating Insecticide Resistance. AgroTypos SA, Athens, pp. 79–85.

Devonshire, A.L., Moores, G.D., **1982**. A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach potato aphids (*Myzus persicae*). *Pestic. Biochem. Physiol. 18*, 235–246.

Dewar, A.M., Read, L.A., **1990**. Evaluation of an insecticidal seed treatment, imidacloprid, for controlling aphids on sugar beet. *Proc. Brighton Crop Protection Conference: Pests and Diseases 2*, 721–726.

Diehr, H.-J., Gallenkamp, B., Jelich, K., Lantzsch, R., **1991**. Synthesis and chemical-physical properties of the insecticide imidacloprid (NTN33893). *Pflanzenschutz-Nachrichten Bayer 44*, 107–112.

Dong, K., **1997**. A single amino acid change in the para sodium channel protein is associated with knockdown resistance (kdr) to pyrethroid insecticides in German cockroaches. *Insect Biochem. Mol. Biol. 27*, 93–100.

Dryden, M.W., Perez, H.R., Ulitchny, D.M., **1999**. Control of fleas on pets and in homes by use of imidacloprid or lufenuron and pyrethrin spray. *J. Am. Vet. Med. Assoc. 215*, 36–39.

El Kady, H., Devine, G.J., **2003**. Insecticide resistance in Egyptian populations of the cotton whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Pest Mgt Sci. 59*, 865–871.

Elbert, A., Becker, B., Hartwig, J., Erdelen, C., **1991**. Imidacloprid: a new systemic insecticide. *Pflanzenschutz-Nachrichten Bayer 44*, 113–136.

Elbert, A., Erdelen, C., Kühnhold, J., Nauen, R., Schmidt, H.W., *et al.*, **2000**. Thiacloprid, a novel neonicotinoid insecticide for foliar application. *Proc. Brighton Crop Protection Conference: Pests and Diseases 1*, 21–26.

Elbert, A., Nauen, R., **1996**. Bioassays for imidacloprid for a resistance monitoring against the whitefly *Bemisia tabaci*. *Proc. Brighton Crop Protection Conference: Pests and Diseases 2*, 731–738.

Elbert, A., Nauen, R., **2004**. New applications for neonicotinoid insecticides using imidacloprid as an example. In: Horowitz, A.R., Ishaaya, I. (Eds.), Novel Approaches to Insect Pest Management in Field and Protected Crops. Springer, New York, pp. 29–44.

Elbert, A., Nauen, R., Cahill, M., Devonshire, A.L., Scarr, A.W., *et al.*, **1996**. Resistance management with chloronicotinyl insecticides using imidacloprid as an example. *Pflanzenschutz-Nachrichten Bayer 49*, 5–53.

Elbert, A., Nauen, R., Leicht, W., **1998**. Imidacloprid, a novel chloronicotinyl insecticide, biological activity and agricultural importance. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action, Mechanism, and Application. Springer, New York, pp. 50–73.

Elbert, A., Overbeck, H., Iwaya, K., Tsuboi, S., **1990**. Imidacloprid, a novel systemic nitromethylene analog insecticide for crop protection. *Proc. Brighton Crop Protection Conference: Pests and Disease 1*, 21–28.

Epe, C., Coati, N., Stanneck, D., **2003**. Efficacy of the compound preparation imidacloprid 10% (w/v)/permethrin 50% (w/v) spot-on against ticks (*I. ricinus, R. sanguineus*) and fleas (*C. felis*) on dogs. *Parasitol. Res. 90*, 122–124.

Everett, R., Cunningham, J., Arther, R., Bledsoe, D.L., Mencke, N., **2000**. Comparative evaluation of the speed of flea kill of imidacloprid and selamectin in dogs. *Vet. Therap. 1*, 229–234.

Feuer, H., Lawrence, J.P., **1969**. The alkyl nitrate nitration of active methylene compounds. 6. A new synthesis of α-nitroalkyl heterocyclics. *J. Am. Chem. Soc. 91*, 1856–1857.

Feyereisen, R., **1995**. Molecular biology of insecticide resistance. *Toxicol. Lett. 82*, 83–90.

ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., Chalmers, A.E., **1993**. A point mutation in *Drosophila* GABA receptor confers insecticide resistance. *Nature 363*, 449–451.

Field, L.M., Blackmann, R.L., Devonshire, A.L., **2001**. Evolution of amplified esterase genes as a mode of insecticide resistance in aphids. In: Ishaaya, I. (Ed.), Biochemical Sites Important in Insecticide Action and Resistance. Springer, Berlin, pp. 209–219.

Field, L.M., Devonshire, A.L., Forde, B.G., **1988**. Molecular evidence that insecticide resistance in peach potato aphids, *Myzus persicae* (Sulz), results from amplification of an esterase gene. *Biochem. J. 251*, 309–315.

Foster, S.P., Denholm, I., Devonshire, A.L., **2000**. The ups and downs of insecticide resistance in peach potato aphids (*Myzus persicae*) in the UK. *Crop Protect. 19*, 873–879.

Foster, S.P., Denholm, I., Harling, Z.K., Moores, G.D., Devonshire, A.L., **1998**. Intensification of resistance in UK field populations of the peach potato aphid, *Myzus persicae* (Homoptera: Aphididae) in 1996. *Bull. Entomol. Res. 88*, 127–130.

Foster, S.P., Denholm, I., Thompson, R., **2003**. Variation in response to neonicotinoid insecticides in peach potato aphids, *Myzus persicae* (Hemiptera: Aphididae). *Pest Mgt Sci. 59*, 166–173.

Foster, S.P., Devonshire, A.L., **1999**. Field-simulator study of insecticide resistance conferred by esterase-, MACE-, and kdr-based mechanisms in the peach potato aphid, *Myzus persicae* (Sulzer). *Pestic. Sci. 55*, 810–814.

Fourie, L.J., Du Rand, C., Heine, J., **2003**. Evaluation of the efficacy of an imidacloprid 10%/moxidectin 2.5% spot-on against *Sarcoptes scabiei* var. *canis* on dogs. *Parasitol. Res. 90*, 135–136.

Genchi, C., Traldi, G., Bianciardi, P., **2000**. Efficacy of imidacloprid on dogs and cats with natural infestations of fleas, with special emphasis on flea hypersensitivity. *Vet. Therap. 2*, 71–80.

Georghiou, G.P., **1983**. Management of resistance in arthropods. In: Georghiou, G.P., Saito, T. (Eds.), Pest Resistance to Pesticides. Plenum, New York, pp. 769–792.

Göbel, T., Gsell, L., Huter, O.F., Maienfisch, P., Naef, R., *et al.*, **1999**. Synthetic approaches towards CGA 293′343: a novel broad-spectrum insecticide. *Pestic. Sci. 55*, 355–357.

Goodman, C.S., Spitzer, N.C., **1980**. Embryonic development of neurotransmitter receptors in grasshopers. In: Sattelle, D.B., Hall, L.M., Hidebrand, J.G. (Eds.), Receptors for Neurotransmitters, Hormones and Pheromones in Insects. Elsevier, Amsterdam, pp. 195–307.

Graton, J., Berthelot, M., Gal, J.-F., Laurence, C., Lebreton, J., *et al.*, **2003**. The nicotinic pharmacophore: thermodynamics of the hydrogen-bonding complexation of nicotin, nornicotine, and models. *J. Organ. Chem. 68*, 8208–8221.

Green, M.B., LeBaron, H.M., Moberg, W.K., **1990**. Managing Resistance to Agrochemicals: From Fundamental to Practical Strategies. American Chemical Society, Washington, DC.

Greenwood, R., Ford, M.G., Scarr, A., **2002**. Neonicotinoid pharmacokinetics. *Proc. Brighton Crop Protection Conference: Pest and Diseases 1*, 153–160.

Griffin, L., Krieger, K., Liege, P., **1997**. Imidacloprid: a new compound for control of fleas initiated dermatitis. *Suppl. Comp. Cont. Educ. Prac. Vet. 19*, 17–20.

Guirao, P., Beitia, F., Cenis, J.L., **1997**. Biotype determination of Spanish populations of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Bull. Entomol. Res. 87*, 587–593.

Gundelfinger, E.D., **1992**. How complex is the nicotinic receptor system of insects? *Trends Neurosci. 15*, 206–211.

Gunning, R.V., Moores, G.D., **2001**. Insensitive acetylcholinesterase as sites for resistance to organophosphates and carbamates in insects: insensitive acetylcholinesterase confers resistance in Lepidoptera.

In: Ishaaya, I. (Ed.), Biochemical Sites Important in Insecticide Action and Resistance. Springer, Berlin, pp. 221–238.

Hancke, W., Breer, H., **1986**. Channel properties of an insect neuronal acetylcholine receptor protein reconstituted in planar lipid bilayers. *Nature 321*, 171–175.

Hanssen, I., Mencke, N., Asskildt, H., Ewald-Hamm, D., Dorn, H., **1999**. Field study on the insecticidal efficacy of Advantage against natural infestans of dogs with lice. *Parasitol. Res. 85*, 347–348.

Harrow, I.D., Sattelle, D.B., **1983**. Acetylcholine receptors on the cell body membrane of giant interneurone 2 in the cockroach *Periplaneta americana*. *J. Exp. Biol. 105*, 339–350.

Heimbach, U., Kral, G., Niemann, P., **2000**. Implementation of resistance risk analysis of plant protection products in the German authorisation procedure. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 7B-2. pp. 771–776.

Hellmann, K., Knoppe, T., Radeloff, I., Heine, J., **2003**. The anthelmintic efficacy and the safety of a combination of imidacloprid and moxidectin spot-on in cats and dogs under field conditions in Europe. *Parasitol. Res. 90*, 142–143.

Hemingway, J., **2000**. The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochem. Mol. Biol. 30*, 1009–1015.

Hemingway, J., Karunarantne, S.H.P.P., **1998**. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med. Vet. Entomol. 12*, 1–12.

Hermsen, B., Stetzer, E., Thees, R., Heiermann, R., Schrattenholz, A., *et al.*, **1998**. Neuronal nicotinic receptors in the locust *Locusta migratoria*. *J. Biol. Chem. 273*, 18394–18404.

Hirase, K., **2003**. Dinotefuran: a novel neonicotenoid insecticide. *Agrochem. Japan 82*, 14–16.

Hodgson, E., **1983**. The significance of Cytochrome P-450 in insects. *Insect Biochem. 13*, 237–246.

Holladay, M.W., Dart, M.J., Lynch, J.K., **1997**. Neuronal nicotinic acetylcholine receptors as targets for drug discovery. *J. Med. Chem. 40*, 4169–4194.

Hollingworth, R.M., Mota-Sanchez, D., Whalon, M.E., Graphius, E., **2002**. Comparative pharmokinetics of imidacloprid in susceptible and resistant Colorado potato beetles. In: Proc. 10th IUPAC Int. Congr. Chem. Crop Protection, Abstracts 1, p. 312.

Hopkins, T.J., Kerwick, C., Gyr, P., Woodley, I., **1996**. Efficacy of imidacloprid to remove and prevent *Ctenocephalides felis* infestations on dogs and cats. *Austral. Vet. Practit. 26*, 150–153.

Hopkins, T.J., Woodley, I., Gyr, P., **1997**. Imidacloprid topical formulation: larvicidal effect against *Ctenocephalides felis* in the surrounding of treated dogs. *Suppl. Comp. Cont. Educ. Pract. Vet. 19*, 4–10.

Horowitz, A.R., Denholm, I., **2001**. Impact of insecticide resistance mechanisms on management strategies. In: Ishaaya, I. (Ed.), Biochemical Sites Important in Insecticide Action and Resistance. Springer, Berlin, pp. 323–338.

Horowitz, A.R., Denholm, I., Gorman, K., Cenis, J.L., Kontsedalov, S., *et al.*, **2003**. Biotype Q of *Bemisia tabaci* identified in Israel. *Phytoparasitica 31*, 94–98.

Horowitz, A.R., Denholm, I., Gorman, K., Ishaaya, I., **1999**. Insecticide resistance in whiteflies: current status and implication for management. In: Denholm, I., Ioannidis, P. (Eds.), Combating Insecticide Resistance. AgroTypos SA, Athens, pp. 8996–8998.

Horowitz, A.R., Weintraub, P.G., Ishaaya, I., **1998**. Status of pesticide resistance in arthropod pest in Israel. *Phytoparasitica 26*, 31–240.

Huang, Y., Williamson, M.S., Devonshire, A.L., Windass, J.D., Lansdell, S.J., *et al.*, **1999**. Molecular characterization and imidacloprid selectivity of nicotinic acetylcholine receptor subunits from the peach potato aphid *Myzus persicae*. *J. Neurochem. 73*, 380–389.

Ishii, Y., Kobori, I., Araki, Y., Kurogochi, S., Iwaya, K., *et al.*, **1994**. HPLC determination of the new insecticide imidacloprid and its behavior in rice and cucumber. *J. Agric. Food Chem. 42*, 2917–2921.

Jacobs, D.E., Hutchinson, M.J., Ewald-Hamm, D., **2000**. Inhibition of immature *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) development in the immediate environment of cats treated with imidacloprid. *J. Med. Entomol. 37*, 228–230.

Jacobs, D.E., Hutchinson, M.J., Fox, M.T., Krieger, K.J., **1997a**. Comparison of flea control strategies using imidacloprid or lufenuron in a controlled simulated home environment. *Am. J. Vet. Res. 58*, 1260–1262.

Jacobs, D.E., Hutchinson, M.J., Krieger, K.J., **1997b**. Duration of activity of imidacloprid, a novel adulticide for flea control, against *Ctenocephalides felis* on cats. *Vet. Rec. 140*, 259–260.

Jacobs, D.E., Hutchinson, M.J., Stannek, D., Mencke, N., **2001**. Accumulation and persistence of flea larvicidal activity in the immediate environment of cats treated with imidacloprid. *Med. Vet. Entomol. 15*, 342–345.

Jeschke, P., Moriya, K., Lantzsch, R., Seifert, H., Lindner, W., *et al.*, **2001**. Thiacloprid (Bay YRC2894): a new member of the chloronicotinyl insecticide (CNI) *Pflanzenschutz-Nachrichten Bayer 54*, 147–160.

Jeschke, P., Schindler, M., Beck, M., **2002**. Neonicotinoid insecticides: retrospective consideration and prospects. *Proc. Brighton Crop Protection Conference: Pests and Diseases 1*, 137–144.

Jeschke, P., Uneme, H., Benet-Buchholz, J., Stölting, J., Sirges, W., *et al.*, **2003**. Clothianidin (TI-435): the third-member of the chloronicotinyl insecticide (CNI^{TM}) family. *Pflanzenschutz-Nachrichten Bayer 56*, 5–24.

Kagabu, S., **1996**. Studies on the synthesis and insecticidal activity of neonicotinoid compounds. *J. Pestic. Sci. 21*, 237–239.

Kagabu, S., **1997a**. Chloronicotinyl insecticides: discovery, application, and future perspective. *Rev. Toxicol. 1*, 75–129.

Kagabu, S., **1997b**. Molecular structures and properties of imidacloprid and analogous compounds. *Phytoparasitica 25*, 347.

Kagabu, S., **1999**. Discovery of chloronicotinylinsecticides. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 91–106.

Kagabu, S., **2003a**. Molecular design of neonicotinoids: past, present and future. In: Voss, G., Ramos, G. (Eds.), Chemistry of Crop Protection: Progress and Prospects in Science and Regulation. Wiley–VCH, New York, pp. 193–212.

Kagabu, S., **2003b**. Insecticides: imidacloprid. In: Plimmer, J.R. (Ed.), Encyclopedia of Agrochemicals. Wiley-Interscience, New York, pp. 933–944.

Kagabu, S., Akagi, T., **1997**. Quantum chemical consideration of photostability of imidacloprid and related compounds. *J. Pest. Sci. 22*, 84–89.

Kagabu, S., Matsuda, K., Komai, K., **2002**. Preparation of dinotefuran related compounds and agonistic action on SADβ2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *J. Pestic. Sci. 27*, 374–377.

Kagabu, S., Matsuno, H., **1997**. Chloronicotinyl insecticides. 8. Crystal and molecular structures of imidacloprid and analogous compounds. *J. Agric. Food Chem. 45*, 276–281.

Kagabu, S., Medej, S., **1995**. Stability comparison of imidacloprid and related compounds under simulated sunlight, hydrolysis conditions, and to oxygen. *Biosci. Biotechnol. Biochem. 59*, 980–985.

Kagabu, S., Moriya, K., Shibuya, K., Hattori, Y., Tsuboi, S., *et al.*, **1992**. 1-(6-Halonicotinyl)-2-nitromethylene-imidazolidines as potential new insecticides. *Biosci. Biotechnol. Biochem. 56*, 362–363.

Kagabu, S., Yokoyama, K., Iwaya, K., Tanaka, M., **1998**. Imidacloprid and related compounds: structure and water solubility of *N*-alkyl derivatives of imidacloprid. *Biosci. Biotechnol. Biochem. 62*, 1216–1224.

Kashiwada, Y., **1996**. Bestguard (nitenpyram, TI-304) – a new systemic insecticide. *Agrochem. Japan 68*, 18–19.

Kayser, H., Lee, C., Wellmann, H., **2002**. Thiamethoxam and imidacloprid bind to different sites on nicotinic receptors: conserved pharmacology among aphids. In: Proc. 10th IUPAC Int. Congr. Chem. Crop Protection, Abstracts 1, p. 305.

Keil, K., Wellington, J., Ciszewski, D., **2002**. Efficacy of Advantage™ in controlling flea allergy dermatitis in cats. *Suppl. Comp. Cont. Educ. Pract. Vet. 24*, 6–9.

Kiriyama, K., Nishimura, K., **2002**. Structural effects of dinotefuran and analogues in insecticidal and neural activities. *Pest Mgt Sci. 58*, 669–676.

Klamt, A., **1995**. Conductor-like screening model for real solvents. A new approach to the quantitative calculation of solvation phenomena. *J. Phys. Chem. 99*, 2224–2235.

Klein, O., **2001**. Behaviour of thiacloprid (YRC 2894) in plants and animals. *Pflanzenschutz-Nachrichten Bayer 54*, 209–240.

Klein, O., **2003**. Behaviour of clothianidin (TI-435) in plants and animals. *Pflanzenschutz-Nachrichten Bayer 56*, 75–101.

Knaust, H.J., Poehling, H.M., **1992**. Studies of the action of imidacloprid on grain aphids and their efficiency to transmit BYD virus. *Pflanzenschutz-Nachrichten Bayer 45*, 381–408.

Knowles, C.O., **1997**. Mechanisms of resistance to acaricides. In: Sjut, V. (Ed.), Molecular Mechanisms of Resistance to Agrochemicals, vol. 13. Springer, Berlin, pp. 57–77.

Kodaka, K., Kinoshita, K., Wakita, T., Yamada, E., Kawahara, N., *et al.*, **1998**. MTI-446: a novel systemic insect control compound. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 1. pp. 21–26.

Kodaka, K., Kinoshita, K., Wakita, T., Yamada, E., Kawahara, N., *et al.*, **1999a**. Synthesis and activity of novel insect control compound MTI-446 and its derivatives. In: Proc. 24th Annu. Mtg Pestic. Sci. Soc. Japan Abstracts, p. 85.

Kodaka, K., Kinoshita, K., Wakita, T., Yamada, E., Kawahara, N., *et al.*, **1999b**. Synthesis and activity of novel insect control compound MTI-446 and its derivatives. In: Proc. 15th Ass. Pestic. Design Res. Abstracts, p. 27.

Koester, J., **1992**. Comparative metabolism [pyridinyl-14C-methyl]imidacloprid in plant cell suspension cultures. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 2. pp. 901–906.

Kollmeyer, W.D., Flattum, R.F., Foster, J.P., Powel, J.E., Schroeder, M.E., *et al.*, **1999**. Discovery of the nitromethylene heterocycle insecticides. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 71–89.

Krämer, F., Mencke, N., **2001**. Flea biology and control: the biology of the cat flea, control and prevention with imidacloprid in small animals. Springer, New York.

Krohn, J., **2001**. Behaviour of thiacloprid in the environment. *Pflanzenschutz-Nachrichten Bayer 54*, 281–290.

Krohn, J., Hellpointer, E., **2002**. Environmental fate of imidacloprid. *Pflanzenschutz-Nachrichten Bayer 55* (special edn.), 3–26.

Latli, B., Than, C., Morimoto, H., Williams, P.G., Casida, J.E., **1996**. [6-Chloro-3-pyridylmethyl-^{1}H]neonicotinoids as high affinity radioligands for the nicotinic acetylcholine receptor: preparation using NaB^1H_4, and LiB^1H_4. *J. Labeled Compd. Radiopharm. 38*, 971–978.

Latli, B., Tomizawa, M., Casida, J.E., **1997**. Synthesis of a novel [^{125}I]neonicotinoid photoaffinity probe for the *Drosophila* nicotinic acetylcholine receptor. *Bioconjugate Chem. 8*, 7–14.

Leech, C.A., Jewess, P., Marshall, J., Sattelle, D.B., **1991**. Nitromethylene actions on *in situ* and expressed insect nicotinic acetylcholine receptors. *FEBS Lett. 290*, 90–94.

Leicht, W., **1993**. Imidacloprid: a chloronicotinyl insecticide. *Pestic. Outlook 4*, 17–24.

Léna, C., Changeux, J.-P., **1993**. Allosteric modulations of the nicotinic acetylcholine receptor. *Trends Neurosci. 16*, 181–186.

Li, Y., Dennehy, T.J., Li, S., Wigert, M.E., Zarborac, M., *et al.*, **2001**. Sustaining Arizona's fragile success in whitefly resistance management. In: Proc. Beltwide Cotton Conferences, National Cotton Council, Memphis, TN, pp. 1108–1114.

Li, Y., Dennehy, T.J., Li, X., Wigert, M.E., **2000**. Susceptibility of Arizona whiteflies to chloronicotinyl insecticides and IGRs: new developments in the 1999 season. In: Proc. Beltwide Cotton Conferences, National Cotton Council, Memphis, TN, pp. 1325–1330.

Lind, R.J., Clough, M.S., Reynolds, S.E., Earley, F.G.P., **1998**. [^{3}H]Imidaclorid labels high- and low-affinity nicotinic acetylcholine receptor-like binding sites in the aphid *Myzus persicae* (Hemiptera: Aphididae). *Pestic. Biochem. Physiol. 62*, 3–14.

Liu, M.-Y., Casida, J.E., **1993a**. High affinity binding of [^{3}H]imidacloprid in the insect acetylcholine receptor. *Pestic. Biochem. Physiol. 1993*, 40–46.

Liu, M.-Y., Lanford, J., Casida, J.E., **1993b**. Relevance of [^{3}H]imidacloprid binding site in housefly head acetylcholine receptor to insecticidal activity of 2-nitromethylenen- and 2-nitroimino-imidazolidines. *Pestic. Biochem. Physiol. 46*, 200–206.

Liu, M.-Y., Latli, B., Casida, J.E., **1995**. Imidacloprid binding site in *Musca* nicotinic acetylcholine receptor: interactions with physostigmine and a varity of nicotinic agonists with chloropyridyl and chlorothiazolyl substituents. *Pestic. Biochem. Physiol. 52*, 170–181.

Maienfisch, P., Angst, M., Brandl, F., Fischer, W., Hofer, D., *et al.*, **2001a**. Chemistry and biology of thiamethoxam: a second generation neonicotinoid. *Pest Mgt Sci. 57*, 906–913.

Maienfisch, P., Brandl, F., Kobel, W., Rindlisbacher, A., Senn, R., **1999a**. CGA 293,343: a novel, broad-spectrum neonicotinoid insecticide. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 177–209.

Maienfisch, P., Gonda, J., Jacob, O., Kaufmann, L., Pitterna, T., *et al.*, **1997**. In: 214th Am. Chem. Soc. Natl Mtg Abstracts, Agro-018.

Maienfisch, P., Gsell, L., Rindlisbacher, A., **1999b**. Synthesis and insecticidal activity of CGA 293343: a novel broad-spectrum insecticide. *Pestic. Sci. 55*, 351–355.

Maienfisch, P., Haettenschwiler, J., Rindlisbacher, A., Decock, A., Wellmann, H., *et al.*, **2003**. Azido-neonicotinoids as candidate photoaffinity probes for insect nicotinic acetylcholine receptors. 1. *Chimia 57*, 710–714.

Maienfisch, P., Huerlimann, H., Haettenschwiler, J., **2000**. A novel method for the preparation of *N*,*N*′-disubstituted-*N*″-nitroguanidines, including a practical synthesis of the neonicotinoid insecticide clothianidin. *Tetrahedron Lett. 41*, 7187–7191.

Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., *et al.*, **2001b**. The discovery of thiamethoxam: a second generation neonicotinoid. *Pest Mgt Sci. 57*, 165–176.

Maienfisch, P., Rindlisbacher, A., Huerlimann, H., Haettenschwiler, J., Desai, A.K., *et al.*, **2002**. 4-Nitroimino-1,3,5-oxadiazines: a new type of neonicotinoid insecticides. *ACS Symp. Series 800*, 219–230.

Malgorzata, G., Mikolajczyk, M.S., O'Reilly, K.L., **2000**. Clinical disease in kittens inoculated with a pathogenic strain of *Bartonella henselae*. *Am. J. Vet. Res. 61*, 375–379.

Marshall, J., Buckingham, S.D., Shingai, R., Lunt, G.G., Goosey, M.W., *et al.*, **1990**. Sequence and functional expression of a single α subunit of an insect nicotinic acetylcholine receptor. *EMBO J. 9*, 4391–4398.

Martinez-Torres, D., Foster, S.P., Field, L.M., Devonshire, A.L., Williamson, M.S., **1998**. A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Mol. Biol. 8*, 1–8.

Matsuda, K., Buckingham, S.D., Freeman, J.C., Squire, M.D., Baylis, H.A., *et al.*, **1998**. Effects of the α subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. *Br. J. Pharmacol. 123*, 518–524.

Matsuda, K., Buckingham, S.D., Freeman, J.C., Squire, M.D., Baylis, H.A., *et al.*, **1999**. Role of the α subunit of nicotinic acetylcholine receptor in the selective action of imidacloprid. *Pestic. Sci. 55*, 211–213.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M., *et al.*, **2001**. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci. 22*, 573–578.

Matsuda, M., Takahashi, H., **1996**. Mospilan (acetamiprid, NI-25): a new systemic insecticide. *Agrochem. Japan 68*, 12–20.

McKenzie, J.A., **1996**. Ecological and Evolutionary Aspects of Insecticide Resistance. Academic Press, San Diego, CA.

Mehlhorn, H., D'Haese, J., Mencke, N., Hanssen, O., **2001b**. *In vivo* and *in vitro* effects of imidacloprid on sheep keds (*Melophagus ovinus*): a light and electron microscopic study. *Parasitol. Res. 87*, 331–336.

Mehlhorn, H., Hansen, O., Mencke, N., **2001a**. Comparative study on the effects of three insecticides (fipronil, imidacloprid, selamectin) on the developmental stages of the cat flea (*Ctenocephalides felis* Buche 1835): a light and electron microscopic analysis of *in vivo* and *in vitro* experiments. *Parasitol. Res. 87*, 198–207.

Mehlhorn, H., Mencke, N., Hansen, O., **1999**. Effects of imidacloprid on adult and larval stages of the cat flea *Ctenocephalides felis* after *in vivo* and *in vitro* experiments: a light- and electronmicroscopy study. *Parasitol. Res. 85*, 625–637.

Mehlhorn, H., Schmahl, G., Mencke, N., Bach, T., **2003**. The effect of an imidacloprid and permethrin combination against developmental stages of *Ixodes ricinus* ticks. *Parasitol. Res. 90*, 119–121.

Mencke, N., Jeschke, P., **2002**. Therapy and prevention of parasitic insects in veterinary medicine using imidacloprid. *Curr. Topics Med. Chem. 2*, 701–715.

Mencke, N., Volf, P., Volvofa, V., Stanneck, D., **2003**. Repellent efficacy of a combination containing imidacloprid and permethrin against sand flies (*Phlebotomus papatasi*) on dogs. *Parasitol. Res. 90*, 108–111.

Minamida, I., Iwanaga, K., Tabuchi, T., Aoki, I., Fusaka, T., *et al.*, **1993**. Synthesis and insecticidal activity of acylic nitroethene compounds containing a heteroarylmethylamino group. *J. Pestic. Sci. 18*, 41–48.

Moores, G.D., Devine, G.J., Devonshire, A.L., **1994a**. Insecticide-insensitive acetylcholinesterase can enhance esterase-based resistance in *Myzus persicae* and *Myzus nicotianae*. *Pestic. Biochem. Physiol. 49*, 114–120.

Moores, G.D., Devine, G.J., Devonshire, A.L., **1994b**. Insecticide resistance due to insensitive acetylcholinesterase in *Myzus persicae* and *Myzus nicotianae*. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 1. pp. 413–418.

Mori, K., Okumoto, T., Kawahara, N., Ozoe, Y., **2001**. Interaction of dinotefuran and its analogues with nicotinic acetylcholine receptors of cockroach nerve cords. *Pest Mgt Sci. 58*, 190–196.

Moriya, K., Shibuya, K., Hattori, Y., Tsuboi, S., Shiokawa, K., *et al.*, **1992**. 1-(6-Chloronicotinyl)-2-nitroimino-imidazolidines and related compounds as potential new insecticides. *Biosci. Biotechnol. Biochem. 56*, 364–365.

Moriya, K., Shibuya, K., Hattori, Y., Tsuboi, S., Shiokawa, K., *et al.*, **1993**. Structural modification of the 6-chloropyridyl moiety in the imidacloprid skeleton: introduction of a five-membered heteroaromatic ring and the resulting insecticidal activity. *Biosci. Biotech. Biochem. 57*, 127–128.

Mutero, A., Pralavorio, M., Bride, J.M., Fournier, D., **1994**. Resistance-associated point mutations in insecticide insensitive acetylcholinesterase. *Proc. Natl Acad. Sci. USA 91*, 5922–5926.

Nagata, K., Aistrup, G.L., Song, J.-H., Narahashi, T., **1996**. Subconductance-state currents generated by imidacloprid at the nicotinic acetylcholine receptor in PC 12 cells. *NeuroReport 7*, 1025–1028.

Nakayama, A., **1998**. Molecular similarity and structure–activity relationship of neonicotinoid insecticides. *J. Pestic. Sci. 23*, 336–343.

Nakayama, A., Sukekawa, M., **1998**. Quantitative correlation between molecular similarity and receptor-binding activity of neonicotinoid insecticides. *Pestic. Sci. 52*, 104–110.

Nauen, R., Bretschneider, T., **2002**. New modes of action of insecticides. *Pestic. Outlook 12*, 241–245.

Nauen, R., Ebbinghaus, U., Tietjen, K., **1999a**. Ligands of the nicotinic acetylcholine receptor as insecticides. *Pestic. Sci. 55*, 608–610.

Nauen, R., Ebbinghaus-Kintscher, A., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Iishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 77–105.

Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V.L., Kaussmann, M., **2003**. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pest. Biochem. Physiol. 76*, 55–69.

Nauen, R., Elbert, A., **1997**. Apparent tolerance of a field-collected strain of *Myzus nicotianae* to imidacloprid due to strong antifeeding response. *Pestic. Sci. 49*, 252–258.

Nauen, R., Elbert, A., **2000**. Resistance of *Bemisia* spp. (Homoptera: Aleyrodidae) to insecticides in southern spain with special reference to neonicotinoids. *Pest Mgt Sci. 56*, 60–64.

Nauen, R., Elbert, A., **2003**. European monitoring of resistance to common classes of insecticides in *Myzus persicae* and *Aphis gossypii* (Homoptera: Aphididae) with special reference to imidacloprid. *Bull. Entomol. Res. 93*, 47–54.

Nauen, R., Hungenberg, H., Tollo, B., Tietjen, K., Elbert, A., **1998a**. Antifeedant-effect, biological efficacy and high affinity binding of imidacloprid to acetylcholine receptors in tobacco associated *Myzus persicae* (Sulzer) and *Myzus nicotianae* Blackman (Homoptera: Aphididae). *Pestic. Sci. 53*, 133–140.

Nauen, R., Reckmann, U., Armborst, S., Stupp, H.P., Elbert, A., **1999b**. Whitefly-active metabolites of imidacloprid: biological efficacy and translocation in cotton plants. *Pestic. Sci. 55*, 265–271.

Nauen, R., Strobel, K., Tietjen, K., Otsu, Y., **1996**. Aphicidal activity of imidacloprid against a tobacco feeding strain of *Myzus persicae* (Homoptera: Aphididae) from Japan closely related to *Myzus nicotianae* and highly resistant to carbamates and organophosphates. *Bull. Entomol. Res. 86*, 165–171.

Nauen, R., Stumpf, N., Elbert, A., **2002**. Toxicological and mechanistic studies on neonicotinoid cross resistance in Q-type Bemisia tabaci (Hemiptera: Aleyrodidae). *Pest Mgt Sci. 58*, 868–875.

Nauen, R., Tietjen, K., Wagner, K., Elbert, A., **1998b**. Efficacy of plant metabolites of imidacloprid against *Myzus persicae* and *Aphis gossypii* (Homoptera: Aphididae). *Pestic. Sci. 52*, 53–57.

Nishimura, K., Kanda, Y., Okazawa, A., Ueno, T., **1994**. Relationship between insecticidal and neurophysiological activities of imidacloprid and related compounds. *Pestic. Biochem. Physiol. 50*, 51–59.

Ohkawara, Y., Akayama, A., Matsuda, K., Andersch, W., **2002**. Clothianidin: a novel broad-spectrum neonicotinoid insecticide. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 1. pp. 51–58.

Okazawa, A., Akamatsu, M., Nishiwaki, H., Nakagawa, Y., Miyagawa, H., *et al.*, **2000**. Three-dimensional quantitative structure–activity relationship analysis of acyclic and cyclic chloronicotinyl insecticides. *Pest Mgt Sci. 56*, 509–515.

Okazawa, A., Akamatsu, M., Ohoka, A., Nishiwaki, H., Cho, W.-J., Nakagawa, Y., *et al.*, **1998**. Prediction of the binding mode of imidacloprid and related compounds to housefly head acetylcholine receptors using three-dimensional QSAR analysis. *Pestic. Sci. 54*, 134–144.

Olsen, E.R., Dively, G.P., Nelson, J.O., **2000**. Baseline susceptibility to imidacloprid and cross-resistance

patterns in Colorado potato beetle (Coleoptera: Chrysomelidae) populations. *J. Econ. Entomol. 93*, 447–458.

Oppenoorth, F.J., **1985**. Biochemistry and genetics of insecticide resistance. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 12. Pergamon, Oxford, pp. 731–773.

Orr, N., Shaffner, J., Watson, G.B., **1997**. Pharmacological characterization of an epibatidine binding site in the nerve cord of *Periplaneta americana*. *Pestic. Biochem. Physiol. 58*, 183–192.

Palumbo, J.C., Horowitz, A.R., Prabhaker, N., **2001**. Insecticidal control and resistance management for *Bemisia tabaci*. *Crop Protect. 20*, 739–765.

Perdew, J.P., **1986**. Density functional approximation for the correlation energy of the inhomogeneous electron gas. *Phys. Rev. B 33*, 8822–8824.

Perring, T.M., **2001**. The *Bemisia tabaci* species complex. *Crop Protect. 20*, 725–737.

Pflüger, W., Schmuck, R., **1991**. Ecotoxicological profile of imidacloprid. *Pflanzenschutz-Nachrichten Bayer 44*, 145–158.

Pospischil, R., Schneider, U., Böcker, T., Junkersdorf, J., Nentwig, G., *et al.*, **1999**. Efficacy of imidacloprid for cockroach control in a gel bait formulation. *Pflanzenschutz-Nachrichten Bayer 52*, 386–400.

Prabhaker, N., Toscano, N.C., Castle, S.J., Henneberry, T.J., **1997**. Selection for imidacloprid resistance in silverleaf whiteflies from the Imperial Valley and development of a hydroponic bioassay for resistance monitoring. *Pestic. Sci. 51*, 419–428.

Rajappa, S., **1981**. Nitroenamines: preparation, structure, and synthetic potential. *Tetrahedron Lett. 37*, 1453–1480.

Rauch, N., Nauen, R., **2003**. Biochemical markers linked to neonicotinoid cross-resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Arch. Insect Biochem. Physiol. 54*, 165–176.

Reinemeyer, C.R., Charles, S., **2003**. Evaluation of the efficacy of a combination of imidacloprid and moxidectin against immature *Toxocara cati* in cats. *Parasitol. Res. 90*, 140–141.

Restifo, L.L., White, K., **1990**. Molecular and genetic approaches to neurotransmitter and neuromodulator systems in *Drosophila*. *Adv. Insect. Physiol. 22*, 115–219.

Roberts, T.R., Hutson, D.H. (Eds.), **1999**. Metabolic Pathways of Agrochemicals, Part 2, Insecticides and Fungicides. Cambridge University Press, Cambridge.

Roslavtseva, S.A., **2000**. Neonicotinoids as a new promising group of insecticides. *Agrokhimiya 1*, 49–52.

Rust, J.H., Jr., Cavanaugh, D.C., O'Shita, R., Marshall, J.D., Jr., **1971**. The role of domestic animals in the epidemiology of plague. 1. Experimental infection of dogs and cats. *J. Infect. Dis. 124*, 522–526.

Rust, M.K., Dryden, M.W., **1997**. The biology, ecology, and management of the cat flea. *Annu. Rev. Entomol. 42*, 451–473.

Rust, M.K., Hinkle, N.C., Waggoner, M., Mencke, N., Hansen, O., *et al.*, **2001**. The influence of imidacloprid on adult cat flea feeding. *Suppl. Comp. Cont. Educ. Vet. Pract. 23*, 18–21.

Rust, M.K., Waggoner, M., Hinkle, N.C., Mencke, N., Hansen, O., *et al.*, **2002**. Disruption of adult cat flea feeding by imidacloprid. *Suppl. Comp. Cont. Educ. Vet. Pract. 24*, 4–5.

Rust, M.K., Waggoner, M.M., Hinkle, N.C., Stansfield, D., Barnett, S., **2003**. Efficacy and longevity of nitenpyram against adult cat fleas (Siphonaptera: Pulicidae). *J. Med. Entomol. 40*, 678–681.

Sarkar, M.A., Roy, S., Kole, R.K., Chowdhury, A., **2001**. Persistence and metabolism of imidacloprid in different soils of West Bengal. *Pest Mgt Sci. 57*, 598–602.

Sattelle, D.B., Buckingham, S.D., Wafford, K.A., Sherby, S.M., Barkry, N.M., *et al.*, **1989**. Actions of the insecticide 2(nitromethylene)tetrahydro-1,3-thiazine on insect and vertebrate nicotinic acetylcholine recptors. *Proc. Roy. Soc. B 237*, 501–514.

Sattelle, D.B., Harrow, I.D., Hue, B., Pelhate, M., Gepner, J.I., *et al.*, **1983**. α-Bungarotoxin blocks excitatory synaptic transmission between cercal sensory neurons and giant interneurons. *J. Exp. Biol. 107*, 473–489.

Schmuck, R., **2001**. Ecotoxicological profile of the insecticide thiacloprid. *Pflanzenschutz-Nachrichten Bayer 54*, 161–184.

Schröder, M.E., Flattum, R.F., **1984**. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pestic. Biochem. Physiol. 22*, 148–160.

Schulz, R., Sawruk, E., Mülhardt, C., Bertrand, S., Baumann, A., *et al.*, **1998**. Dα3, a new functional α subunit of nicotinic acetylcholine receptors from *Drosophila*. *J. Neurochem. 71*, 853–862.

Schulz-Jander, D.A., Casida, J.E., **2002**. Imidacloprid insecticide metabolism: human cytochrome P-450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicol. Lett. 132*, 65–70.

Schwartz, B.J., Sparrow, F.K., Heard, N.E., Thede, B.M., **2000**. Simultaneous derivatization and trapping of volatile products from aqueous photolysis of thiamethoxam insecticide. *J. Agric. Food Chem. 48*, 4671–4675.

Scott, J.G., **2001**. Cytochrome P-450 monooxygenases and insecticide resistance: lessons from CYP6D1. In: Ishaaya, I. (Ed.), Biochemical Sites Important in Insecticide Action and Resistance. Springer, Berlin, pp. 255–267.

Senn, R., Hofer, D., Hoppe, T., Angst, M., Wyss, P., *et al.*, **1998**. CGA 293343: a novel broad-spectrum insecticide supporting sustainable agriculture worlwide. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 1. pp. 27–36.

Sgard, F., Fraser, S.P., Katkowska, M.J., Djamgoz, M.B.A., Dunbar, S.J., *et al.*, **1998**. Cloning and functional characterization of two novel nicotinic acetylcholine receptor α subunits from the insect pest *Myzus persicae*. *J. Neurochem. 71*, 903–912.

Shiokawa, K., Tsuboi, S., Iwaya, K., Moriya, K., **1994**. Development of a chloronicotinyl insecticide, imidacloprid. *J. Pestic. Sci. 19*, 209–217; 329–332.

Shiokawa, K., Tsuboi, S., Moriya, K., Kagabu, S., **1995**. Chloronicotinyl insecticides: development of imidacloprid. In: 8th Int. Congr. Pestic. Chemi.: Option 2000, pp. 49–59.

Siegfried, B.D., Scharf, M.E., **2001**. Mechanisms of organophosphate resistance in insects. In: Ishaaya, I. (Ed.), Biochemical Sites Important in Insecticide Action and Resistance. Springer, Berlin, pp. 269–291.

Soderlund, D.M., **1997**. Molecular mechanisms of insecticide resistance. In: Sjut, V. (Ed.), Molecular Mechanisms of Resistance to Agrochemicals. Springer, Berlin, pp. 21–56.

Soloway, S.B., Henry, A.C., Kollmeyer, W.D., Padgett, W.M., Powell, J.E., *et al.*, **1978**. Nitromethylene heterocycles as insecticides. In: Shankland, D.L., Hollingworth, R.M., Smyth, T., Jr. (Eds.), Pesticide and Venom Neurotoxicology. Plenum, New York, pp. 153–158.

Soloway, S.B., Henry, A.C., Kollmeyer, W.D., Padgett, W.M., Powell, J.E., *et al.*, **1979**. Nitromethylene insecticides. *Adv. Pestic. Sci. 2*, 206–217.

Sone, S., Hattori, Y., Tsuboi, S., Otsu, Y., **1997**. Difference in suceptibility to imidacloprid of the population of the small brown planthopper, *Laodelphax striatellus* Fallen, from various localities in Japan. *J. Pestic. Sci. 20*, 541–543.

Sone, S., Yamada, Y., Tsuboi, S., **1995**. New pesticide screening method for leaf hoppers and plant hoppers using rice seedlings. *Jap. J. Appl. Entomol. Zool. 39*, 171–173.

Spencer, J.A., Butler, J.M., Stafford, K.C., Pough, M.B., Levy, S.A., *et al.*, **2003**. Evaluation of permethrin and imidacloprid for prevention of *Borrelia burgdorferi* transmission from blacklegged ticks (*Ixodes scapularis*) to *Borrelia burgdorferi*-free dogs. *Parasitol. Res. 90*, 106–107.

Stein-Dönecke, U., Führ, F., Wieneke, J., Hartwig, J., Leicht, W., **1992**. Influence of soil moisture on the formulation of dressing zones and uptake of imidacloprid after seed treatment of winter wheat. *Pflanzenschutz-Nachrichten Bayer 45*, 327–368.

Stupp, H.-P., Fahl, U., **2003**. Environmental fate of clothianidin (TI-435; Poncho®). *Pflanzenschutz-Nachrichten Bayer 56*, 59–74.

Sukekawa, M., Nakayama, A., **1999**. Application of molecular similarity analysis in 3D-QSAR of neonicotinoid insecticides. *J. Pestic. Sci. 24*, 38–43.

Tabuchi, T., Fusaka, T., Iwanaga, K., **1994**. Studies on acyclic nitroethene compounds. 3. Synthesis and insecticidal activity of acyclic nitroethene compounds containing (6-substituted)-3-pyridylamino group. *J. Pestic. Sci. 19*, 119–125.

Takahashi, H., Mitsui, J., Takakusa, N., Matsuda, M., Yoneda, H., *et al.*, **1992**. NI-25, a new type of systemic and broad sprectrum insecticide. In: Proc. Brighton Crop Protection Conference: Pests and Diseases, vol. 1. pp. 89–96.

Takahashi, H., Takakusa, N., Suzuki, J., Kishimoto, T., **1998**. Development of a new insecticide, acetamiprid. In: Abstracts 23rd Ann. Mtg Pestic. Sci. Soc. Japan, p. 23.

Testa, B., Mayer, J.M., **1998**. Design of intramolecularly activated prodrugs. *Drug Metab. Rev. 30*, 787–807.

Testa, B., Mayer, J.M., **2001**. Concepts in prodrug design to overcome pharmacokinetic problems. In: Testa, B., Waterbeemd, H. van de, Folkers, G., Guy, R. (Eds.), Pharmacokinetic Optimization in Drug Re search: Biological, Physicochemical, and Computational Strategies. Verlag Helvetica Chimica Acta, Zurich, Switzerland, pp. 85–95.

Tokieda, M., Ozawa, M., Kobayashi, S., Gomyo, T., **1997**. Method for determination of total residues of the insecticide acetamiprid and its metabolites in crops by gas chromatography. *J. Pestic. Sci. 22*, 77–83.

Tokieda, M., Ozawa, M., Kobayashi, S., Gomyo, T., **1998**. In: Proc. 9th Int. Congr. Pestic. Chem., 2, Poster 7C-029.

Tomizawa, M., **2000**. Insect nicotinic acetylcholine receptors: mode of action of insecticide and functional architecture of the receptor. *Jap. J. Appl. Entomol. Zool. 44*, 1–15.

Tomizawa, M., Casida, J.E., **1997**. [^{125}I]azidoneonicotinoid photoaffinity labeling of insecticide-binding subunit of *Drosophila* nicotinic acetylcholine receptor. *Neurosci. Lett. 237*, 61–64.

Tomizawa, M., Casida, J.E., **2001**. Structure and diversity of nicotinic acetylcholine receptors. *Pest Mgt Sci. 57*, 914–922.

Tomizawa, M., Casida, J.E., **2003**. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol. 48*, 339–364.

Tomizawa, M., Latli, B., Casida, J.E., **1996**. Novel neonicotinoid–agarose affinity column for *Drosophila* and *Musca* nicotinic acetylcholine receptors. *J. Neurochem. 67*, 1669–1676.

Tomizawa, M., Latli, B., Casida, J.E., **1999**. Structure and function of insect nicotinic acetylcholine receptors studied with nicotinoid insecticide affinity probes. In: Yamamoto, I., Casida, J.E. (Eds.), Nicotinoid Insecticides and Nicotinic Acetylcholine Receptor. Springer, New York, pp. 271–292.

Tomizawa, M., Lee, D.L., Casida, J.E., **2000**. Neonicotinoid insecticides: molecular feature conferring selectivity for insect versus mammalian nicotinic receptors. *J. Agric. Food Chem. 48*, 6016–6024.

Tomizawa, M., Yamamoto, I., **1992**. Binding of nicotinoids and the related compounds to the insect nicotinic acetylcholine receptor. *J. Pestic. Sci. 17*, 231–236.

Tomizawa, M., Yamamoto, I., **1993**. Structure activity relationships of neonicotinoids and imidacloprid analogs. *J. Pestic. Sci. 18*, 91–98.

Tomlin, C.D.S., **2000**. The pesticide Manual, 12th edn. British Crop Protection Council.

Tröltzsch, C.M., Führ, F., Wieneke, J., Elbert, A., **1994**. Einfluss unterschiedlicher Bewässerungsverfahren auf die aufnahme von Imidacloprid durch Baumwolle

nach Saatgutbeizung. *Pflanzenschutz-Nachrichten Bayer 47*, 249–303.

Tsumura, Y., Nakamura, Y., Tonagai, Y., Kamimoto, Y., Tanaka, Y., *et al.*, **1998**. Determination of neonicotinoid pesticide nitenpyram and its metabolites in agricultural products. *Shokuhin Eiseigaku Zasshi 39*, 127–134.

Uneme, H., Iwanaga, K., Higuchi, N., Kando, Y., Okauchi, T., *et al.*, **1999**. Syntheses and insecticidal activity of nitroguanidine derivatives. *Pestic. Sci. 55*, 202–205.

van den Breukel, I., van Kleef, R.G.D.M., Zwart, R., Oortgiesen, M., **1998**. Physiostigmine and acetylcholine differentially activate nicotinic receptor subpopulations in *Locusta migratoria* neurons. *Brain Res. 789*, 263–273.

Wakita, T., Kinoshita, K., Yamada, E., Yasui, N., Kawahara, N., *et al.*, **2003**. The discovery of dinotefuran: a novel neonicotinoid. *Pest Mgt Sci. 59*, 1016–1022.

Weichel, L., Nauen, R., **2003**. Monitoring of insecticide resistance in damson hop aphids, *Phorodon humuli* Schrank (Hemiptera: Aphididae) from German hop gardens. *Pest Mgt Sci. 59*, 991–998.

Werner, G., Hopkins, T., Shmidl, J.A., Watanabe, M., Krieger, K., **1995**. Imidacloprid, a novel compound of the chloronicotinyl group with outstanding insecticidal activity in the on-animal treatment of pests. *Pharmacol. Res. 31* (Suppl.), 136.

Wen, Z.M., Scott, J.G., **1997**. Cross-resistance to imidacloprid in strains of German cockroach (*Blattella germanica*) and housefly (*Musca domestica*). *Pestic. Sci. 49*, 367–371.

Widmer, H., Steinemann, A., Maienfisch, P., **1999**. Chemical and physical properties of thiamethoxam (CGA 293343). In: Abstracts 218th Am. Chem. Sci. Natl Mtg, p. 134.

Williams, B., **1993**. Reproductive success of cat fleas, *Ctenocephalides felis*, on calves as unusual host. *Med. Vet. Entomol. 7*, 94–98.

Williamson, M.S., Denholm, I., Bell, C.A., Devonshire, A.L., **1993**. Knockdown resistance (kdr) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*). *Mol. Gen. Genet. 240*, 17–22.

Williamson, M.S., Martinez-Torres, D., Hick, C.A., Devonshire, A.L., **1996**. Identification of mutations in the housefly *para*-type sodium channel gene associated with knock-down resistance (kdr) to pyrethroid insecticides. *Mol. Gen. Genet. 252*, 51–60.

Wollweber, D., Tietjen, K., **1999**. Chloronicotinyl insecticides: a success of the new chemistry. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 109–125.

Yaguchi, Y., Sato, T., **2001**. Thiacloprid (Bariard), a novel neonicotinoid insecticide for foliar application. *Agrochem. Japan 79*, 14–16.

Yamada, T., Takahashi, H., Hatano, R., **1999**. A novel insecticide, acetamiprid. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and Nicotinic Acetylcholine Receptor. Springer, New York, pp. 149–175.

Yamamoto, I., **1996**. Neonicotinoids: mode of action and selectivity. *Agrochem. Japan 68*, 14–15.

Yamamoto, I., **1998**. Nicotine: old and new topics. *Rev. Toxicol. 2*, 61–69.

Yamamoto, I., **1999**. Nicotine to neonicotinoids: 162 to 1997. In: Yamamoto, I., Casida, J.E. (Eds.), Neonicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer, New York, pp. 3–27.

Yamamoto, I., Tomizawa, M., **1993**. New development of nicotinoid insecticides. In: Mitsui, T., Matsumura, F., Yamaguchi, I. (Eds.), Pesticide Development: Molecular Biological Approaches. Pesticide Sci. Soc. Japan, Tokyo, pp. 67–83.

Yamamoto, I., Tomizawa, M., Saito, T., Miyamoto, T., Walcott, E.C., *et al.*, **1998**. Structural factors contributing to insecticidal and selective actions of neonicotinoids. *Arch. Insect. Biochem. Physiol. 37*, 24–32.

Yamamoto, I., Yabuta, G., Tomizawa, M., Saito, T., Miyamoto, T., *et al.*, **1995**. Molecular mechanism for selective toxicity of nicotinoids and neonicotinoids. *J. Pestic. Sci. 20*, 33–40.

Yokota, T., Mikata, K., Nagasaki, H., Ohta, K., **2003**. Absorption, tissue distribution, excretion, and metabolism of clothianidin in rats. *J. Agric. Food Chem. 51*, 7066–7072.

Young, D.R., Arther, R.G., Davis, W.L., **2003**. Evaluation of K9 Advantix™ vs. Frontline Plus® topical treatments to repel brown dog ticks (*Rhipicephalus sanguineus*) on dogs. *Parasitol. Res. 90*, 116–118.

Young, D.R., Ryan, W.G., **1999**. Comparison of Frontline® Top Spot™, Preventic® collar alone or combined with Advantage® in control of flea and tick infestations in water immersed dogs. In: Proc. 5th Int. Symp. Ectoparas. Pets, Fort Collins, Colorado, April, p. 24.

Zewen, L., Zhaojun, H., Yinchang, W., Lingchun, Z., Hongwei, Z., *et al.*, **2003**. Selection for imidacloprid resistance in *Nilaparvata lugens*: cross-resistance patterns and possible mechanisms. *Pest Mgt Sci. 59*, 1355–1359.

Zhang, A., Kayser, H., Maienfisch, P., Casida, J.E., **2000**. Insect nicotinic acetylcholine receptor: conserved neonicotinoid specifity of [^{3}H]imidacloprid binding site. *J. Neurochem. 75*, 1294–1303.

Zhao, J.Y., Liu, W., Brown, J.M., Knowles, C.O., **1995**. Insecticide resistance in field and laboratory strains of Western flower thrips (Thysanoptera: Thripidae). *J. Econ. Entomol. 88*, 1164–1170.

Zhao, J.Z., Bishop, B.A., Graphius, E.J., **2000**. Inheritance and synergism of resistance to imidacloprid in the Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol. 93*, 1508–1514.

Zhu, K.Y., Clark, J.M., **1997**. Validation of a point mutation of acetylcholinesterase in Colorado potato beetle by polymerase chain reaction coupled to enzyme inhibition assay. *Pestic. Biochem. Physiol. 57*, 28–35.

Zwart, R., Oortgiesen, M., Vijverberg, H.P., **1992**. The nitromethylene heterocycle 1-(pyridin-3-yl-methyl)-2-nitromethylene-imidazolidine distinguishes mammalian from insect nicotinic receptor subtypes. *Eur. J. Pharmacol. 228*, 165–169.

5.4 GABA Receptors of Insects

S D Buckingham and D B Sattelle, University of Oxford, Oxford, UK

5.4.1. Introduction

Receptors for the neurotransmitter γ-aminobutyric acid (GABA) are abundant in the nervous systems of insects (Sattelle, 1990) where, as in vertebrates, they play a major role in inhibition. In 1985 when the first set of volumes of this series entitled *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* was published, little was known about these proteins, despite the already established use of insect preparations to address fundamental questions in neurobiology, and despite the fact that GABA receptors (GABARs) were already considered to be possible insecticide targets. Since then, our understanding of insect GABARs has been transformed.

Since 1985, studies on GABARs have benefited from the application of molecular biology (molecular cloning, functional expression, and determination of their gene structure), electrophysiology (single-channel and whole-cell patch clamp recordings), integrative physiology (demonstrating a key role for such receptors in particular insect behaviors), genome analysis (yielding a complete set of ligand gated anion channels for the fruit fly, *Drosophila melanogaster* and the mosquito, *Anopheles gambiae*, and genetics (providing a detailed understanding of mutations that underly insecticide resistance). Radioligand binding studies have generated a detailed description of the pharmacology of insect GABAR binding sites. Electrophysiological studies, some using identified neurons, have contributed important information on GABARs, both functional native and expressed recombinant receptors. Patch clamp methods, in particular, have provided key insights, enabling: (1) the analysis of single channel properties; and (2) description of the effects of chemicals on receptor kinetics. A number of studies have examined the distribution of GABARs and other molecular components of insect GABAergic synapses using antibodies to GABA, enzymes involved in GABA synthesis, and, more recently, antibodies to cloned GABAR subunits. Thus, arange of complementary experimental approaches have led to new discoveries on the roles of GABARs in sensory receptive field tuning, learning, and even a possible role in the insect circadian clock.

A major advance in the study of insect GABARs resulted from the cloning of three candidate *Drosophila* ionotropic GABAR subunits, one of which resistant to DieLdrin(RDL) readily forms a functional, recombinant, homomeric GABA gated chloride channel. This was followed by the discovery of RDL orthologs in several insect orders. Thus, for the first time, it was possible to study in detail the physiology and pharmacology of an insect GABAR of known molecular composition. Consequently, in the years since the publication of the first edition of *Comprehensive Insect Physiology, Biochemistry and Molecular Biology*, there has been considerable growth in our understanding of insect GABARs (Hosie *et al.*,

1997) and their vertebrate counterparts (Whiting, 2003).

Insect GABAR subtypes were not well established in 1985, although even then data were beginning to emerge, to question earlier notions that benzodiazepine binding sites were not present in insects (Nielsen *et al.*, 1978). Today, we know that insect GABARs rival the complexity of their vertebrate counterparts, and possess a number of sites for modulation by not only benzodiazepines, but also several other allosteric modulators. Again, in 1985, there was no evidence for the existence of an insect metabotropic receptor – today, three candidate insect metabotropic GABARs have been cloned and their characterization is now in progress.

This chapter concentrates on new information that has accumulated since the previous edition of this series. After a brief review of findings up to 1985, we discuss the more recent findings on the physiology and pharmacology of insect ionotropic GABARs, examining each major chemical binding site in turn. Evidence for distinct subtypes of native insect GABARs is also discussed. Attention is then drawn to findings derived from the cloning and functional expression of recombinant insect GABARs, and their contributions to our understanding of (1) native receptors and (2) insecticide modes of action. Next, we consider the roles of ionotropic GABARs in insect behavior. Finally, new findings on metabotropic GABARs are described.

By 1985, GABA was already known to be an inhibitory neurotransmitter at nerve–muscle junctions and in the central nervous system (CNS) of arthropods (Kuffler and Edwards, 1958; Usherwood and Grundfest, 1965; Otsuka *et al.*, 1966), but very little is still known of insect GABARs. Most information was based on electrophysiology performed on native nerve and muscle receptors. However, GABA had been shown to induce hyperpolarizing responses in (mostly unidentified) central neurons of several insect species, including postsynaptic membranes of interneurons (Callec and Boistel, 1971; Callec, 1974; Hue *et al.*, 1979) and unidentified cell bodies (Kerkut *et al.*, 1969; Pitman and Kerkut, 1970; Walker *et al.*, 1971; Roberts *et al.*, 1981) in the terminal abdominal ganglion of the cockroach, *Periplaneta americana*. Dissociated cells of locust thoracic ganglia (Giles and Usherwood, 1985), as well as identified dorsal unpaired median neurons (DUMETi) that innervate the *Extensor tibiae* muscle of the embryonic grasshopper *Schistocerca nitens* (Goodman and Spitzer, 1980), were also found to respond to GABA with hyperpolarizations. Peripheral GABARs located on somatic muscle fibers had been described in some considerable detail (Cull-Candy and Miledi, 1981; Cull-Candy, 1982). These early studies established that GABA mediates hyperpolarizing, largely inhibitory responses in insect neurons, but other than showing a sensitivity to picrotoxinin, little was known of the details of the pharmacology of these responses.

5.4.2. Ionotropic GABARs of Insects

5.4.2.1. Lessons from Vertebrate GABARs

Our understanding of both insect and vertebrate GABARs is based on binding studies, electrophysiology of *in situ* receptors and electrophysiology of expressed recombinant receptors, each approach offering its own particular advantages. Electrophysiological responses of neurons in insect or vertebrate nervous systems as well as cultured neurons provide a detailed account of the pharmacology, ion permeability, and kinetic properties of native GABARs. It is particularly convenient where identified neurons, or classes of neurons, are studied, as the analysis is restricted to a circumscribed (in some cases, perhaps homogeneous) class of receptors. Electrophysiological studies using heterologously expressed recombinant receptors offer the further advantage that the subunit composition of the receptors is known. Binding studies complement these functional approaches. However, when applied to native tissues the technique often pools data from multiple GABARs (as well as any other binding sites for the radioligand) from all cell types in the tissue under investigation. As functional and binding experiments yield different types of information, care must be exercised when comparing data obtained using these different techniques. For example, electrophysiological and binding studies typically address different states of the same receptor, which can also contribute to some of the apparent discrepancies.

Nineteen ionotropic GABAR subunit molecules have been cloned from vertebrates (Whiting, 2003). They are members of the dicysteine-loop (cys-loop) family of ionotropic neurotransmitter receptors that also includes the nicotinic acetylcholine receptors (nAChRs) (Karlin, 2002), serotonin type 3 (5-HT_3) receptors (5HT_3Rs) (Reeves and Lummis, 2002), and strychnine sensitive glycine receptors (GlyRs) (Betz *et al.*, 1999) of vertebrates as well as glutamate gated chloride channels (GluCls), a 5-HT gated chloride channel and a histamine gated chloride channel of invertebrates (review: Raymond and Sattelle, 2002). Each receptor molecule consists of five subunits arranged symmetrically around an integral, hydrophobic ion pore (**Figure 1**). Each polypeptide

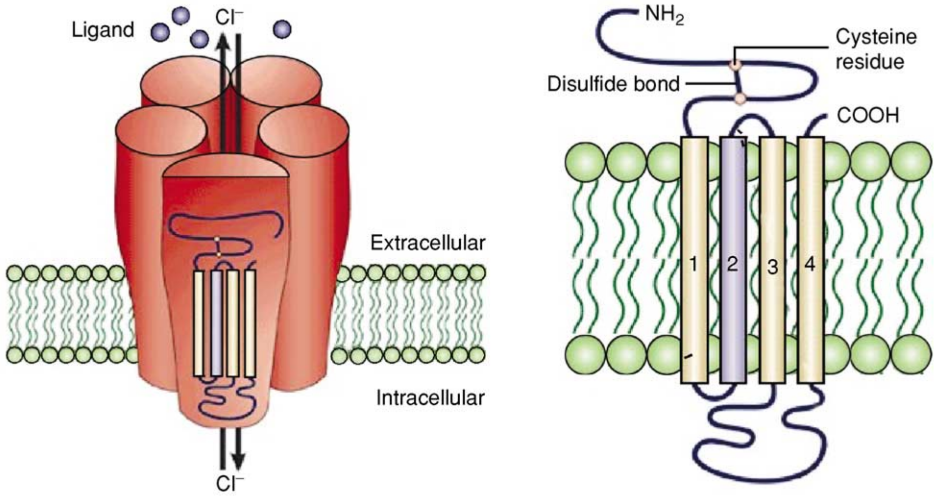


Figure 1 A schematic representation of the three-dimensional structure of ionotropic GABA receptors. Although more data are available for the structure of vertebrate ionotropic GABA receptors, it is probable that insect GABA receptors have a similar structure. (From Raymond, V., Sattelle, D.B., **2002**. Novel animal-health drug targets from ligand-gated chloride channels. *Nat. Rev. Drug Discov. 6*, 427–436.)

subunit possesses a long N-terminal extracellular domain, which contains residues that contribute to the neurotransmitter (GABA) binding site and four transmembrane regions (M1–M4) (**Figure 1**). The second transmembrane domain (M2) contains most of the residues that line the chloride channel (Karlin and Akabas, 1995; Smith and Olsen, 1995). Vertebrate ionotropic GABARs may be divided into two pharmacological categories: $GABA_A$ receptors, which are all antagonized by the alkaloid, bicuculline, and regulated by a wide range of allosteric modulators such as benzodiazepines, barbiturates, and pregnane steroids; and $GABA_C$ receptors, which are insensitive to bicuculline as well as the majority of modulators of $GABA_A$ receptors (Bormann, 2000; Zhang *et al.*, 2001). $GABA_C$ receptors are less liable to desensitize than $GABA_A$ receptors (Johnston, 1996). This physiological and pharmacological characterization is mirrored by corresponding structural differences between the two receptor subtypes, in that they are composed of distinct subunits. $GABA_A$ receptors are composed of subunits from several different classes (α_{1-6} β_{1-3} γ_{1-3}, δ, ε, π, and θ) (Hedblom and Kirkness, 1997; Thompson *et al.*, 1998; Neelands *et al.*, 1999; Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000; Whiting, 2003), whereas $GABA_C$ receptors are believed to be composed of the three known isoforms of the ρ subunit, ρ_{1-3} (Sieghart, 1995; Djamgoz, 1995; McKernan and Whiting, 1996). $GABA_A$ receptors are complex allosteric proteins and contain, in addition to the agonist binding site, distinct sites for the actions of barbiturates, steroids, loreclezole, furosemide, picrotoxin (PTX), zinc, lanthanum, volatile anaesthetics, benzodiazepines, and the anaesthetic, propofol. Although it should be noted that there is not perfect consensus on this division of vertebrate GABARs into A and C subtypes, this has been the view of the majority of workers to date and has received recent support (Bormann, 2000). This view will therefore be adopted for the purposes of this review.

Functional expression studies, in which various combinations of subunits are heterologously expressed in *Xenopus laevis* oocytes or cell lines, have shown that the pharmacology of such recombinant vertebrate receptors is strongly influenced by subunit composition. For example, the specific subtype of β subunit present is a critical determinant of agonist pharmacology, whereas the α and γ subunits are important in conferring sensitivity to benzodiazepines. When the α_1 subtype is present in the heterologously expressed receptor it exhibits a pharmacology that resembles benzodiazepine type 1 pharmacology defined on the basis of *in situ* native receptor studies. Also, the inclusion of either the α_2 or α_3 subunit confers to the receptor the classical benzodiazepine type 2 pharmacology (Pritchett *et al.*, 1989). The γ_2 subunit is essential for benzodiazepine activity (Pritchett *et al.*, 1989), and the specific subtype of γ subunit present in the receptor plays a major role in determining the receptor's responsiveness to allosteric modulators, such as benzodiazepines. Taken together, experiments using recombinant expression have suggested that native vertebrate $GABA_A$ receptors are composed of at least α, β, and γ subunits, a conclusion supported by several immunoprecipitation studies

(Mohler *et al.*, 1992; Khan *et al.*, 1994; Araujo *et al.*, 1996; Jechlinger *et al.*, 1998; Nusser *et al.*, 1999). About 35% of $GABA_A$ receptors in the brain contain $\alpha_1\beta_2\gamma_2$ subunits, 20% contain $\alpha_3\beta_3\gamma_2$, and 15% contain $\alpha_2\beta_3\gamma_2$ subunits. Other combinations are present, but they are less common (Whiting, 2003).

With fewer laboratories working in the field the growth in our knowledge of insect GABARs has been slower than that of their vertebrate counterparts. It is clear, however, that while all vertebrate and insect ionotropic GABARs are blocked by the plant derived toxin, picrotoxinin, a number of pharmacological differences distinguish insect GABARs from vertebrate $GABA_A$ and $GABA_C$ receptors. For example, insect ionotropic GABARs do not readily fit the vertebrate classification. There are two principal differences. First, the majority of insect GABARs, unlike $GABA_A$ receptors, are insensitive to bicuculline, although bicuculline sensitive insect GABARs do exist. Secondly, most insect GABARs are pharmacologically distinct from $GABA_C$ receptors in that they are subject to allosteric modulation by benzodiazepines and barbiturates (Sattelle, 1990). There are other minor differences in aspects of their detailed pharmacology, each of which will be addressed in the following sections.

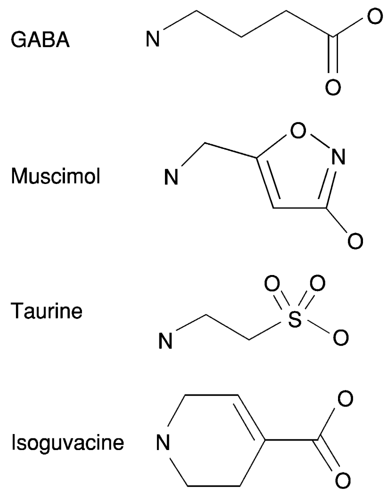


Figure 2 The molecular structures of some common agonists of vertebrate and insect ionotropic GABA receptors.

5.4.2.2. Pharmacology of *In Situ* Insect GABARs

5.4.2.2.1. Agonist site Early binding studies on cockroach (*P. americana*) nerve cord preparations using [^{3}H]GABA and [^{3}H]muscimol indicated that insect GABARs, unlike vertebrate $GABA_A$ receptors, were insensitive to 3-aminopropanesulfonic acid (3-APS). Also, muscimol is of the same, or higher, potency as GABA on insect GABARs (Lummis and Sattelle, 1985, 1986). The most potent displacers of [^{3}H]GABA binding to cockroach nerve cord preparations were found to be GABA itself and muscimol, with IC_{50}s of 130 and 710 nM respectively. In contrast, the other vertebrate agonists tested (thiomuscimol, 4,5,6,7-tetrahydroisoxazolo[5,4-c] pyridin-3-ol (THIP), 3-APS, and isoguvacine) had IC_{50}s greater than 30 mM (Lummis and Sattelle, 1986). The dissociation rate for GABA binding was estimated to be around 0.116 min^{-1} and the estimated association rate was approximately 316 $mM^{-1}\,min^{-1}$. The molecular structures of some GABAR agonists are illustrated in **Figure 2**.

Later, electrophysiological studies were performed on GABARs in an identified *Periplaneta* motor neuron (the fast coxal depressor, D_f) (Sattelle *et al.*, 1988), and on cloned *Drosophila* GABARs containing only RDL subunits heterologously expressed in oocytes or insect cell lines (Millar *et al.*, 1994; Hosie and Sattelle, 1996a, 1996b; Buckingham *et al.*, 1996). The only major difference between the [^{3}H]GABA displacement profile and the functional studies is that isoguvacine is a potent agonist on native, functional insect GABARs (Sattelle *et al.*, 1988), providing a further distinction from vertebrate GABARs. The reason for this apparent discrepancy between physiology and binding data is unclear, but may be attributable to the fact that the two methods probe different receptor states, and that the two approaches may probe different pools of receptor subtypes. The sensitivity of insect GABARs to ZAPA ((*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid) (Taylor *et al.*, 1993) also distinguishes them from vertebrate $GABA_A$ receptors, which are largely insensitive to this compound (Woodward *et al.*, 1993; Qian and Dowling, 1993).

Reports from several laboratories show a lack of action of established vertebrate $GABA_B$ agonists or antagonists on insect GABARs (Lees *et al.*, 1987; Sattelle *et al.*, 1988; Murphy and Wann, 1988; Bermudez *et al.*, 1991; Wolff and Wingate, 1998). However, this does not preclude the existence of $GABA_B$ receptors with unusual pharmacology. For example, the $GABA_B$R agonist baclofen and the $GABA_B$ antagonist saclofen were inactive on GABARs of D_f (Sattelle *et al.*, 1988). However, more recent experiments have identified $GABA_B$-like responses, which differ significantly in their pharmacology from vertebrate $GABA_B$ receptors in being insensitive to baclofen (Bai and Sattelle, 1995). The presence of these insect $GABA_B$ receptors was only detected when responses mediated through ionotropic GABARs were blocked by PTX.

Most insect ionotropic GABARs are relatively insensitive to the GABA analog CACA (*cis*-aminocrotonic acid), which is an agonist of vertebrate $GABA_C$, but not $GABA_A$, receptors. For example, visual interneurons in the fly, *Calliphora erythrocephala*, are CACA insensitive (Brotz and Borst, 1996). However, it should be noted that CACA activates GABARs that mediate inhibition by identified filiform hair receptors of an identified projection interneuron, a circuit that mediates wind elicited responses (Gauglitz and Pfluger, 2001).

Taurine (2-aminoethanesulfonic acid), an abundant amino acid in both vertebrates and insects, may act as a coagonist with GABA in certain insects. It is abundant in the nervous system of the locust, *Schistocerca gregaria* (Whitton *et al*., 1987), where its distribution changes following periods of intense muscular activity. When ionophoretically applied to isolated locust neurons in culture, taurine evokes membrane conductance increases very similar to those evoked by GABA (Whitton *et al*., 1994). Indeed, responses to both GABA and taurine are both blocked by PTX and enhanced by flunitrazepam, consistent with their acting through the same receptor. It was therefore suggested that taurine may act at the same site as GABA, serving to modulate the GABA response. In the migratory locust, *Locusta migratoria*, taurine is coexpressed with GABA in local and intersegmental inhibitory interneurons but not in efferent neurosecretory or paracrine cells, suggesting a role in preventing hyperexcitation during stressful conditions (Stevenson, 1999).

5.4.2.2.2. Competitive antagonists A consistent feature of most, but not all, insect GABARs is that they are insensitive to the plant derived alkaloid, bicuculline (**Figure 3**), a defining blocker of vertebrate $GABA_A$ receptors (Curtis *et al*., 1971; Watson and Girdlestone, 1995). Extrasynaptic GABARs on the motor neuron, D_f (Sattelle *et al*., 1988; Buckingham *et al*., 1994), and synaptic GABARs on the giant interneuron 2 of *P. americana* (Buckingham *et al*., 1994) are insensitive to bicuculline, as are many locust neuron ionotropic GABARs (Benson, 1988; Lees *et al*., 1987). Bicuculline does not block either the cloned *Drosophila* GABAR, RDL (Millar *et al*., 1994), or the cloned *Heliothis virescens* RDL-like GABAR (Wolff and Wingate, 1998). GABA mediated inhibitory postsynaptic potentials (IPSPs) recorded in an identified locust (*S. gregaria*) interneuron are also bicuculline insensitive (Watson and Burrows, 1987). Similarly, bicuculline insensitivity is a feature of GABARs that mediate inhibition by identified filiform hair receptors of an identified projection interneuron, a circuit that mediates wind elicited responses (Gauglitz and Pfluger, 2001). Responses to muscimol in motion sensitive visual interneurons of the fly, *C. erythrocephala*, are also insensitive to bicuculline (Brotz and Borst, 1996). Furthermore, insensitivity to bicuculline is also seen for certain spider GABARs (Panek *et al*., 2002).

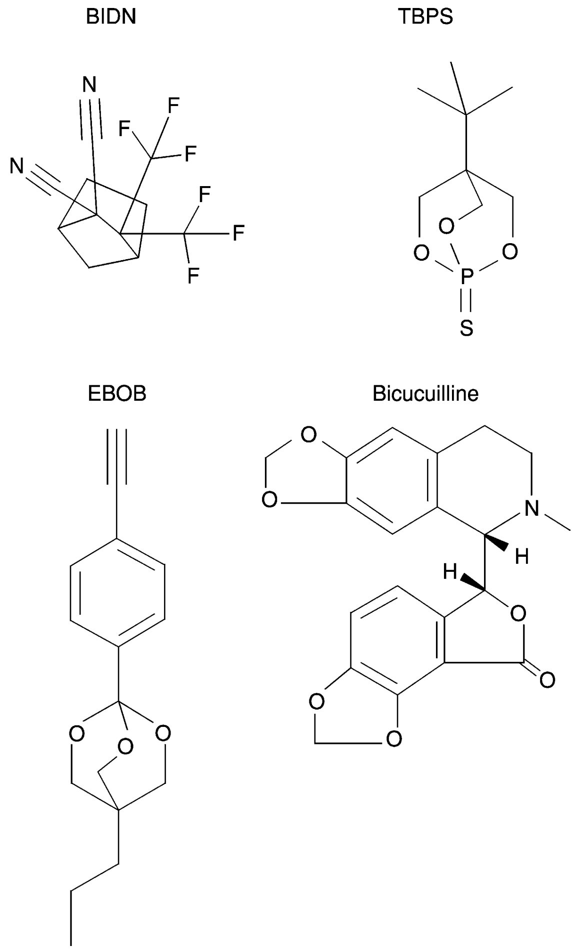


Figure 3 The molecular structures of some common convulsant antagonists of vertebrate and insect ionotropic GABA receptors.

However, there is a body of evidence that bicuculline does block ionotropic GABARs of some adult *Manduca sexta* abdominal ganglion neurons (Roberts *et al*., 1981; Waldrop *et al*., 1987; Waldrop 1994) as well as electrically or odor evoked IPSPs and GABA responses in projection neurons of antennal lobes of this species (Christensen *et al*., 1998). Recently, Sattelle *et al.* (2003) have also shown that bicuculline insensitive GABARs are present in the larval nervous system of *M. sexta*. Further, the observation that bicuculline is effective in recovering behaviors disrupted by the systemic administration of GABA transporter blockers (Leal and Neckameyer, 2002) suggests that, despite the lack of bicuculline action on RDL, bicuculline

may nonetheless block some *D. melanogaster* GABARs. In addition, bicuculline also blocks certain nicotinic acetylcholine receptors (nAChRs) of insects (Benson, 1988; Buckingham *et al*., 1994) and vertebrates (Rothlin *et al*., 1999).

One attempt to resolve the issue of bicuculline sensitivity tested the possibility that such divergent reports may reflect pharmacological differences between extrasynaptic and synaptic GABARs (Buckingham *et al*., 1994). The cockroach, *P. americana*, offers a convenient model for such an approach because of the number of indentified neurons with ready access to both their cell bodies and their synapses. Motor neuron D_f has a population of well-characterized GABARs on its cell body membrane, and giant interneuron 2 (GI 2) not only presents an accessible cell body, but also receives synaptic inputs from nerve X. IPSPs measured in GI 2 using the oil gap recording method in response to electrical stimulation of nerve X were blocked by both bicuculline (100 μM) and PTX (0.01 μM). However, bicuculline injected directly into the neuropil in the region of these synapses did not block IPSPs. The apparent block of synaptic GABARs by bicuculline was found to be due to inhibition of nAChRs upstream in the synaptic pathway. While the study failed to find differences in the pharmacology of synaptic and extrasynaptic GABARs, it did show that three separate populations of GABARs exist: synaptic receptors in the neuropilar branches of GI 2, extrasynaptic GABARs on the cell body of GI 2, and extrasynaptic GABARs on D_f, all of which are bicuculline insensitive. The finding that bicuculline acts on nAChRs does not, however, explain the block of IPSPs by bicuculline in *M. sexta* interneurons (Waldrop *et al*., 1987), since ACh responses in this preparation are insensitive to direct bicuculline application (Waldrop and Hildebrand, 1988).

Thus, there is some crossover between the pharmacophore for ligand binding sites of insect ionotropic GABARs and nAChRs. Whereas most insect GABARs are insensitive to bicuculline, many insect ACh receptors, in contrast, are blocked by bicuculline (Buckingham *et al*., 1994; Waldrop, 1994). At the same time, imidacloprid, an insecticide acting as a partial agonist of nAChRs, can also partially block GABARs of honeybee Kenyon cells, albeit at relatively high concentrations (Deglise *et al*., 2002).

In addition to their abundant expression in the CNS, and in contrast to vertebrates, insect GABARs are also expressed on muscle fibers. In many respects, these muscle insect GABARs resemble those of the nervous system in that they are sensitive to muscimol and to the GABA channel blocker, picrotoxinin (Scott and Duce, 1987; Murphy and Wann, 1988). However, until 1997, no detailed comparison had been reported for muscle and neuronal GABARs in the same animal for any insect species. One study, therefore, set out directly to compare GABARs on fibers of the coxal levator muscle (182c,d) of the cockroach, *P. americana* (Schnee *et al*., 1997), with the well-characterized GABARs on the motor neuron, D_f, of the same species (Sattelle *et al*., 1988). Both were sensitive to PTX and insensitive to bicuculline, but there were significant pharmacological differences between them. Unlike the neuronal receptors, the muscle GABARs were insensitive to isoguvacine. The EC_{50} of muscimol relative to GABA was also much lower for muscle than for D_f. The muscle GABARs were also sensitive to certain cyclodienes: 12-ketoendrin, endrin, and heptachlor epoxide all reduced GABA responses with IC_{50}s in the 1–10 nM range. Similarly, micromolar concentrations of the cyclodiene, heptachlor, produced a complete block of muscle receptors, whereas concentrations as high as 100 μM resulted in about 50% block of GABARs on cockroach motor neuron D_f (Lummis *et al*., 1990). Micromolar *tert*-butyl-bicyclo[2.2.2]phosbrothionate (TBPS) and tert-butyl-bicylo orthobenzoate (TBOB) were almost without effect on muscle receptors. Hence, in the cockroach there are some clear differences between neuronal and muscle GABARs. The observation that muscle GABARs are significantly more sensitive to cyclodienes than the GABARs of motor neuron D_f is interesting in view of the fact that mutants of RDL are resistant to cyclodienes, yet RDL is expressed exclusively in the nervous system. This discrepancy remains to be addressed.

5.4.2.2.3. Noncompetitive antagonists and convulsants Several convulsants (**Figure 3**) are noncompetitive antagonists of insect GABARs and this site is the target of many insecticides, including the cyclodienes (such as endrin, dieldrin), hexachlorocyclohexanes (such as lindane), and bicycloorthobenzoates (such as 1-(4-ethynylphenyl)4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane (EBOB)) (Matsumura and Ghiasuddin, 1983; Lummis and Sattelle, 1986; Wafford *et al*., 1989; Lummis *et al*., 1990; Deng *et al*., 1993; Anthony *et al*., 1994). The earliest evidence that cyclodienes act at the convulsant site came from binding studies using [^{3}H]dihydropicrotoxinin, which indicated that the cyclodiene and PTX binding sites were similar (Matsumura and Ghiasuddin, 1983). Further, houseflies resistant to dieldrin show cross-resistance to many polychlorocycloalkanes and phenylpyrazoles, and to some bicycloorthobenzoates, but not to avermectins

(Deng *et al.*, 1991; Deng and Casida 1992; Cole *et al.*, 1993; Casida, 1993).

Initial studies using labeled probes for the convulsant site were hampered by the limitations of [^{3}H]dihydropicrotoxinin as a probe. These were largely overcome as improved radioligands became available. Specific, saturable binding of [^{35}S]TBPS to cockroach nerve cord membranes yielded a K_d of around 18 nM and B_{max} of around 177 fmol mg^{-1} protein (Lummis and Sattelle, 1986). This binding was inhibited by GABA and dihydroavermectin B_{1a} at micromolar concentrations, suggesting the presence of an allosteric linkage between these sites in insect GABARs.

Radioligand binding and toxicity approaches used by John Casida's laboratory have been particularly fruitful not only in identifying the sites of action of many insecticidally relevant compounds, but also in providing a pharmacological dissection of the convulsant site. The [^{3}H]EBOB binding site of housefly heads was found to be identical to, or to overlap with, a wide array of structurally distinct insecticides (Deng *et al.*, 1991) including cyclodienes, bicycloorthobenzoates, and PTX, but not avermectins. This was pursued further in a comprehensive study of the convulsant site examining the displacement of labeled EBOB and TBPS by 29 different compounds (Deng *et al.*, 1993). Binding of [^{35}S]TBPS to housefly head membranes had a K_d of 145 nM and B_{max} of 2.4 pmol mg^{-1} protein, and both [^{35}S]TBPS and [^{3}H]EBOB were displaced by a number of polychlorocycloalkanes, bicycloorthobenzoates, and phenylpyrazoles. The binding parameters resembled those of vertebrate tissues with respect to pH dependence, anion specificity and kinetics of association, and dissociation. Comparing the modes of competition of each compound class (i.e., competitive versus noncompetitive) and their relevance to toxicity, this study concluded that four subsites can be defined pharmacologically: site A (the EBOB site) overlaps with site C (the phenylpyrazole site) and with site B (the TBPS site), but sites B and C do not overlap. The avermectin site (site D) is distinct from all of the other three sites, but is allosterically linked to sites A and C.

Similar studies added weight to the conclusion that insect GABARs differ significantly from their vertebrate counterparts at the noncompetitive binding sites. For instance, they are significantly less sensitive to the polychlorocycloalkane, cage convulsants (such as TBPS and TBOB), and PTX, compounds that by contrast are potent inhibitors of vertebrate GABARs (Rauh *et al.*, 1990). A single exception to this rule is furnished by the polychlorocyclohexane insecticide, heptachlor, and its epoxide metabolite, which more potently displaced labeled TBPS from *Musca domestica* membranes than from rat (Lummis *et al.*, 1990). However, when muscle GABARs of the cockroach, *P. americana*, were compared to those on an identified motor neuron of the same species (Schnee *et al.*, 1997), muscle GABARs were found to be more sensitive to lindane and to heptachlor and its epoxide metabolite.

One fertile approach to understanding the convulsant site has been to compare the efficacies of a systematically varying set of PTX analogs in either blocking the receptor or in displacing radiolabeled probes. Such studies on vertebrate tissues led to the proposal (Ozoe and Matsumura, 1986) that the key elements in determining picrotoxane activity are the presence of a bulky lipophilic group and at least two electronegative centers. Several studies have emphasized the importance of two groups in the PTX molecule (the hydroxyl and the isopropenyl groups) in conferring high lethality (Jarboe *et al.*, 1968), on displacing [^{3}H]-dihydropicrotoxin (Ticku *et al.*, 1978), and as well as affecting electrophysiological responses of *in situ* (Kudo *et al.*, 1984) and recombinant GABARs (Anthony *et al.*, 1993). These conclusions were drawn in particular from the observation that fluoropicrotoxinin, which substitutes a fluorine at the hydroxyl position, is almost equally active as the parent molecule, whereas PTX acetate is much reduced in potency, and that picrotin, which adds a hydroxyl group to the isopropenyl, is also comparatively ineffective. Such findings suggest that for vertebrates, the hydroxyl group may act as a hydrogen bond acceptor, that substitutions at the hydroxyl group are liable to steric hindrance, and that the isopropenyl group must be lipophilic or electron-rich (Anthony *et al.*, 1993) for maximal activity. To determine whether the same is true for insect GABARs, one study (Anthony *et al.*, 1994) examined the actions of a series of PTX analogs on the well-defined GABARs on the identified motor neuron, D_f, of the cockroach, *P. americana*. The rank order of potency was the same as for expressed chick optic lobe GABARs, suggesting that the convulsant sites of insect and vertebrate $GABA_A$ receptors share certain common key features. Similar findings were obtained for RDL, a cloned insect GABAR (see Section 5.4.2.4.3.2).

The reduced sensitivity of insect GABARs to TBPS slowed the development of our understanding of insect convulsant site probes. Recently, however, two compounds have been deployed with selectivity for insect over vertebrate GABARs, BIDN, and fipronil (**Figures 3 and 4**).

BIDN ([^{3}H]3,3-bis-trifluoromethyl-bicyclo[2,2,1] heptane-2,2-dicarbonitrile) was developed using a

combined biochemical and pharmacophore modeling approach (Rauh *et al*., 1997a, 1997b). A large library of compounds was systematically screened for their ability to displace [^{35}S]TBPS from rat brain membranes. Several bicyclic dinitriles were identified and further screened for their selectiviy for insect GABARs over rat GABARs. This approach identified the common structural features of known convulsant site ligands, which helped to determine the key pharmacophore features (**Figure 5**) This was done by manually aligning each of a series of polycyclic dinitriles over a representation of the PTX molecule using the Sybyl software package (SYBYL Molecular Modeling Software, Tripos Associates, Inc., St. Louis, MO). Key pharmacophore elements identified were the presence of a polarizable moiety separated by a fixed distance from two hydrogen bond-accepting (i.e., electronegative) elements (**Figure 5**), confirming and extending the model proposed earlier by Ozoe and Matsumura (1986). A number of new potential ligands were developed from this pharmacophore model. Of these, the most useful was BIDN (Rauh *et al*., 1997a, 1997b).

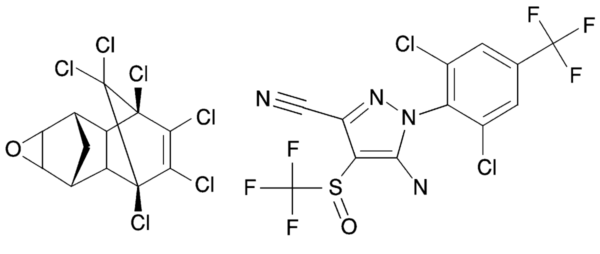


Figure 4 The molecular structures of two potent antagonists of insect ionotropic GABA receptors, dieldrin and fipronil, both of which have been used as insecticides.

Binding of [^{3}H]BIDN to southern corn rootworm (*Diabrotica undecimpunctata*) membranes was saturable and specific with high affinity (26 nM) and a Hill coefficient of unity suggesting that BIDN binds to a single class of receptor sites (Rauh *et al*., 1997b). Such high-affinity, specific binding was confirmed in the same study for five other insect species, including *P. americana* and *M. domestica*, whereas binding of BIDN to rat membranes was of lower affinity (around 250 nM). The same study examined the actions of BIDN using electrophysiological techniques, which showed that BIDN blocks GABARs of the identified cockroach motor neuron D_f as well as those present on the coxal levator muscles of the same species. BIDN also blocks GABA evoked depolarizations of thoracic neurons in the southern corn rootworm, (Rauh *et al*., 1997b). The estimated IC_{50} for BIDN action against central (on the cell body of motor neuron, D_f) and peripheral (on coxal levator muscle fibers) GABARs of *P. americana* was 0.9 μM and 2.5 μM, respectively. Although showing a degree of selectivity for insect over vertebrate receptors, the vertebrate toxicity of BIDN was a factor precluding its further development as a commercial pesticide.

Another insect GABAR ligand is the potent, widely used insecticide active on insect GABARs, the phenyl pyrazole, fipronil. Fipronil is a potent antagonist of cloned insect GABARs (Hosie *et al*., 1997; Grolleau and Sattelle, 2000) and has also been used as the basis for the development of a photo-affinity probe for the convulsant site (Sirisoma *et al*., 2001). There is abundant evidence that BIDN and fipronil act at the convulsant site. Fipronil competes for EBOB binding to housefly head membranes (Ratra and Casida, 2001). BIDN (Hosie *et al*., 1995b), picrotoxinin (Shirai *et al*., 1995), and fipronil (Hosie

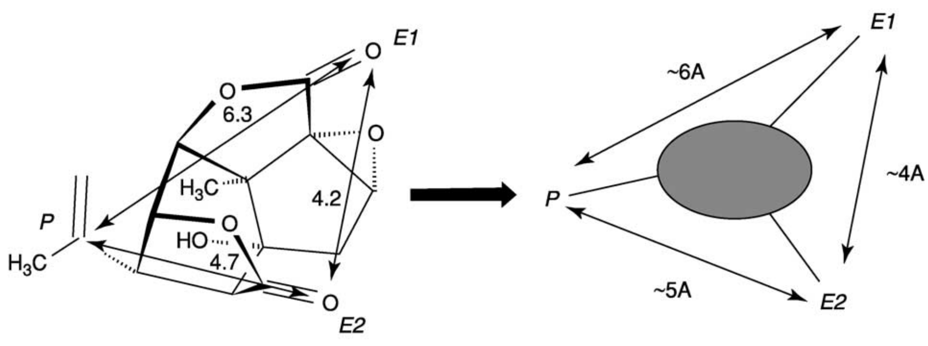


Figure 5 The ideal pharmacophore for the picrotoxin molecule. This was derived from a number of structure–function studies that have revealed the structural features that confer potency to picrotoxin analogs. This was used to predict that a series of bicyclic dinitriles would have potency at the picrotoxinin site. (From Rauh, J.J., Holyoke, C.W., Kleier, D.A., Presnail, J.K., Benner, E.A., *et al*., **1997b**. Polycyclic dinitriles: a novel class of potent GABAergic insecticides provides a new radioligand, [^{3}H]BIDN. *Invert. Neurosci. 3*, 261–268.)

et al., 1995b) all block RDL (see below) in a voltage independent, partly competitive and partly noncompetitive manner. However, although dieldrin displaces [^{3}H]BIDN binding competitively, PTX does not (Sattelle *et al.*, 1995), suggesting that the PTX and the dieldrin site are not identical. This confirms an earlier report (Deng *et al.*, 1993) that identified no less than four convulsant antagonist binding sites in the housefly GABAR that can be differentiated by [^{3}H]EBOB and [^{35}S]TBPS. It is therefore likely that BIDN, fipronil, EBOB, and PTX act at distinct but overlapping sites. Together, these compounds therefore provide a powerful tool-kit with which to further dissect the actions of insecticides on GABARs. Single channel properties of RDL expressed in a *Drosophila* cell line suggested that BIDN and fipronil both reduce the mean open time of GABAR chloride channels, but do so by acting at distinct sites (Grolleau and Sattelle, 2000) – see Section 5.4.2.4.3.2.

There are no doubt a number of naturally occurring ligands with GABAergic actions that await discovery. An interesting class of compounds acting at this site is the α and β thujones, the active ingredients of absinthe and present in certain herbal medicines. These compounds act at the convulsant site to block GABARs noncompetitively, and have insecticidal activity (Hold *et al.*, 2000, 2001). It will be of interest to study possible actions of other plant derived compounds on insect GABARs.

5.4.2.2.4. Allosteric modulators

5.4.2.2.4.1. Benzodiazepines In 1985, there was still some controversy as to whether insect nervous tissues possess receptors for benzodiazepines. One report suggested a lack of benzodiazepine receptors in invertebrates (Neilsen *et al.*, 1978) but this was followed by a number of reports (Abalis *et al.*, 1983; Lummis and Sattelle, 1986; Robinson *et al.*, 1985) of sensitivity of insect GABARs to benzodiazepines. All these reports indicated that the pharmacology of the insect benzodiazepine site differs from most of its vertebrate counterparts. Specific [^{3}H]flunitrazepam binding to cockroach (*P. americana*) nerve cord membranes yielded an estimated K_d of 383 nM and B_{max} of 5.5 pmol mg^{-1} protein (Lummis and Sattelle, 1986). The most potent displacer of this binding was Ro5-4864 ($IC_{50} = 1\,\mu M$), which was only slightly more potent than flunitrazepam ($IC_{50} = 1.6\,\mu M$). Clonezapam was much less effective ($IC_{50} = 25\,\mu M$). The pharmacological profile of the [^{3}H]flunitrazepam binding site in *P. americana* was Ro5-4864 ($IC_{50} = 0.6\,\mu M$) > diazepam ($IC_{50} = 1.0\,\mu M$) > flunitrazepam ($IC_{50} = 1.6\,\mu M$) ≫ clonazepam ($IC_{50} = 25\,\mu M$) ≫ Ro15-1788 ($IC_{50} = 100\,\mu M$). Allosteric linkage between the agonist and benzodiazepine sites was confirmed by the fact that 0.1 mM GABA enhanced flunitrazepam binding by some 150%. Furthermore, flunitrazepam enhanced responses to GABA in locust neuronal somata (Lees *et al.*, 1987; Whitton *et al.*, 1994) and in the motor neuron D_f of the cockroach at micromolar concentrations (Sattelle *et al.*, 1988). The mode of action of benzodiazepines seems to be, as in vertebrates, an increase in the frequency of opening of the GABA gated channel (Shimahara *et al.*, 1987).

The potent displacement of [^{3}H]flunitrazepam binding in cockroach by Ro5-4864 and its potentiation of GABA responses suggests that cockroach CNS benzodiazepine receptors resemble vertebrate peripheral benzodiazepine binding sites rather than central ones (Lummis and Sattelle, 1986). These peripheral binding sites in vertebrates are located principally in the kidney and liver, where they are associated with calcium channels. Nevertheless, the observation that flunitrazepam and GABA binding are allosterically linked, along with physiological evidence discussed below, confirm that the [^{3}H]flunitrazepam binding site in insect CNS is indeed part of a GABAR.

Flunitrazepam and diazepam have no effect upon GABARs of coxal levator muscle of cockroach, even at concentrations of up to 100 μM (Schnee *et al.*, 1997) or upon the cloned *H. virescens* GABAR (Wolff and Wingate, 1998). Flunitrazepam does, however, evoke responses to GABA in the cockroach D_f motor neuron (Sattelle *et al.*, 1988) and other neuronal preparations (review: Sattelle, 1990). That there are differing accounts of the actions of benzodiazepines upon insect GABARs is not surprising, given the great diversity in benzodiazepine pharmacology of vertebrate GABARs and the acute sensitivity of its pharmacology on subunit composition. A sensitivity to Ro5-4864 is probably the most significant distinguishing feature since the site of action of this compound in vertebrates is not on GABARs. Further, the lack of evidence for actions of benzodiazepines on insect muscle GABARs may represent an additional distinction between muscle and neuronal GABAR subtypes in insects, although more benzodiazepines would have to be tested to confirm this hypothesis.

5.4.2.2.4.2. Barbiturates Although there have been relatively few studies, there is evidence that insect GABARs possess a site for the action of barbiturates. Enhancement of responses to GABA recorded in locust neurons by barbiturates has been reported (Lees *et al.*, 1987), and phenobarbital enhances responses mediated by the heterologously expressed *H. virescens* GABAR (Wolff and Wingate, 1998). In contrast, sodium pentobarbital at 100 μM failed to modify

responses to GABA recorded in cockroach coxal levator muscle (Schnee *et al.*, 1997).

5.4.2.2.4.3. Other allosteric modulators In addition to the benzodiazepine and barbiturate allosteric modulator sites, there are reports of modulatory actions of other compounds. Pregnane steroids displace [^{35}S]TBPS binding from *M. domestica* membranes, but with low affinity, while the insect steroids ecdysone and 20-hydroxyecdysone are almost without effect (Rauh *et al.*, 1990). Other allosteric modulators of insect GABARs include thymol (Gonzalez-Coloma *et al.*, 2002). Priestley *et al.* (2003) have shown that thymol acts at a novel allosteric site. Propofol, widely used as an anaesthetic, enhances GABA responses mediated through RDL by up to about nine-fold with an EC_{50} of about 8 μM (Pistis *et al.*, 1996a). The action of propofol on vertebrate $GABA_A$ receptors is critically dependent upon the presence of a specific amino acid in the second transmembrane domain of the receptor (Pistis *et al.*, 1999).

5.4.2.3. Diversity: Existence of Subtypes

One of the most convincing demonstrations of the existence of multiple GABAR subtypes is provided where different tissues within the same animal possess receptors of distinct pharmacology. Bicuculline sensitive and insensitive GABARs are present in the nervous system of the moth, *M. sexta* (Waldrop, 1994; Waldrop *et al.*, 1987a,b; Sattelle *et al.*, 2003). In *P. americana*, muscle receptors differ from nervous system receptors in their insensitivity to isoguvacine and higher sensitivity to channel blockers, along with differences in their sensitivity to barbiturates and benzodiazepines, although the latter is less likely to be useful in defining the muscle subtype given the wide variation in benzodiazepine pharmacology among different GABARs. Taken together, however, the differences in pharmacology between insect muscle and neuronal receptors is sufficient to justify considering them as distinct receptor subtypes.

An even more convincing demonstration of the existence of GABAR subtypes is furnished where two types of GABARs can be demonstrated on the same cells. Here, work on identified cells is particularly compelling. DUM (dorsal unpaired median) neurons of the cockroach have been shown to possess at least two subtypes of ionotropic GABAR, one of which is regulated by intracellular calcium ions through a calmodulin pathway, while the other is sensitive to CACA but seemingly not regulated by intracellular calcium (Alix *et al.*, 2002).

Clearly, not only are insect GABARs to be distinguished pharmacologically from the major vertebrate subtypes, but there is also considerable evidence to suggest that insect GABARs are not of one pharmacologically uniform type. However, there is not enough data available yet to group all the known insect GABARs into a clearly delimited classification scheme. We would argue, however, that a classification into bicuculline sensitive versus insensitive and a subset of bicuculline insensitive muscle receptors is justified on the basis of currently available evidence.

5.4.2.4. Molecular Biology

5.4.2.4.1. Cloned receptors Ionotropic GABAR subunits have been cloned from eight species of insect. These fall into three distinct types: RDL, which has been found in a number of insect species, and lignad-gated chloride channel 3 (LCCH3) and GABAA and glycine receptor-like subunit of *Drosophila* (GRD), which are only known to date in *Drosophila*. All three are structurally similar to vertebrate $GABA_A$ receptor subtypes, and more generally to the cys-loop family of neurotransmitter receptors: they have four transmembrane domains of which the second possesses an amphipathic helix and a dicysteine motif in the extracellular domain (see Hosie *et al.*, 1997). GRD resembles both ionotropic GABA and glycine receptors of vertebrates (Harvey *et al.*, 1994). It has 33–44% identity to vertebrate GABAR subunits, but is distinguished by a large insertion between the dicysteine loop and the first transmembrane spanning region. Such a feature is not seen in any other ligand gated chloride channel. Its highest sequence identity is to vertebrate $GABA_A$ α subunits (40–44% identity), but it has almost the same degree of sequence identity to vertebrate glycine receptor α subunits (40–41% identity). GRD does not form receptors in *Xenopus* oocytes, but its presumed M2 region contains seven of the eight amino acid motif (TTVLTMTT) that is a signature of ligand gated chloride channels (Harvey *et al.*, 1994). Furthermore, *Xenopus* oocytes, injected with mRNA encoding the GRD subunit, do not respond to a wide range of amino acids and glutamate receptor agonists, neither does GRD form functioning receptors when coinjected with bovine $GABA_A$ α1 or β1 subunit or rat glycine β subunits (Harvey *et al.*, 1994). In light of the failure of GRD to support GABA responses, it is probably significant that GRD lacks any equivalent to the ligand binding domain II (TGSY) of $GABA_A$ receptors. There is, therefore, reason to suspect that GRD may not even be a GABAR subunit. LCCH3 has

high (approximately 47%) identity to vertebrate $GABA_A$ β subunits (Henderson *et al*., 1993, 1994).

5.4.2.4.2. RDL: a model insect ionotropic GABAR The *rdl* (resistance to dieldrin) gene was originally isolated from a naturally occurring dieldrin resistant strain of *D. melanogaster* (ffrench-Constant *et al*., 1991, 1993a). The *rdl* locus maps to position 66F of chromosome III. It has nine exons, alternative splicing of two of which gives rise to four gene products (**Figure 6**), of which the most extensively studied are RDLac and RDLdb. The *rdl* gene is not limited to *Drosophila*, nor indeed even to the Diptera. RDL-like GABAR subunits with 85–99% amino acid identity have also been cloned from *Myzus persicae* (Anthony *et al*., 1998), *H. virescens* (Wolff and Wingate, 1998), *Aedes aegypti* (Thompson *et al*., 1993a), *Drosophila simulans* (ffrench-Constant *et al*., 1993a), *M. domestica* (Thompson *et al*., 1993a), *Blatella germanica* (Thompson *et al*., 1993b; Kaku and Matsumura, 1994), *Tribolium castaneum* (Miyazaki *et al*., 1995), and *Hypothenemus hampei* (Andreev *et al*., 1994), indicating that it is widespread throughout insect orders. The RDL polypeptide has between 30% and 38% identity with vertebrate GABAR subunits (about the same identity among different vertebrate subunit types).

RDL GABAR subunits encoded by the *rdl*gene are distributed throughout the adult (Harrison *et al*., 1996) and embryonic (Aronstein and ffrench-Constant, 1995) nervous system of *Drosophila*, particularly in the optic lobes, ellipsoid body, fan-shaped body, ventrolateral cerebrum, and glomeruli of antennal lobes. RDL is not expressed in muscle. Furthermore, in *Drosophila*, RDL expression is largely confined to the neuropil, with no discernible expression on neuronal cell body membranes or muscle (Harrison *et al*., 1996), although cell bodies were clearly stained in *P. americana* (Sattelle *et al*.,

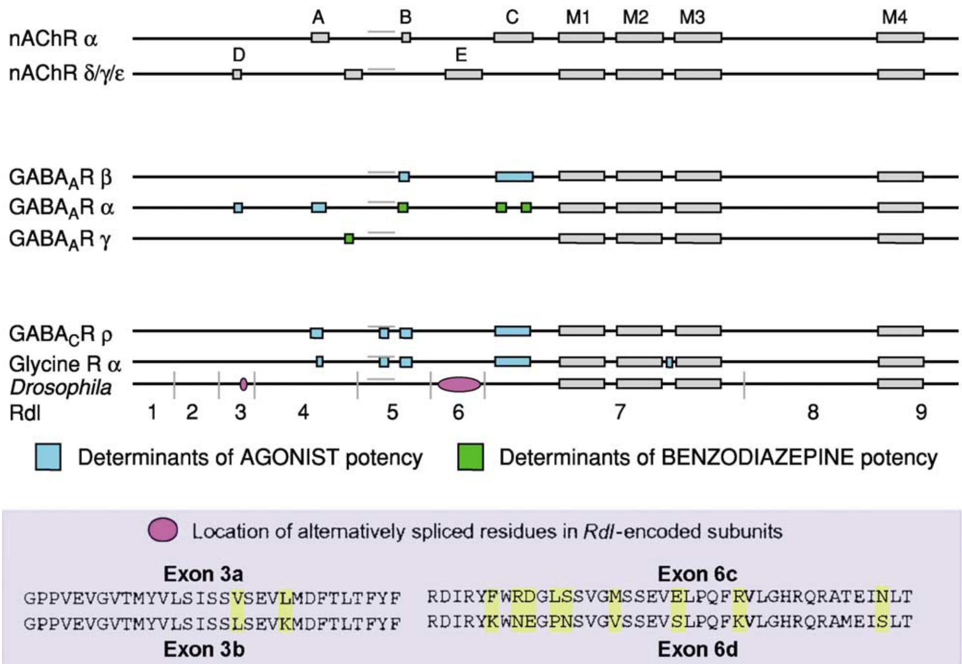


Figure 6 The locations of the determinants of agonist potency of cys-loop receptors are highly conserved. In this schematic alignment of cys-loop receptor subunits, the location of the cysteine-loop is indicated by the line above the subunit. The location of known determinants of agonist potency in nACh receptors, termed loops A–E, are marked yellow, while those of benzodiazepine potency are marked green. The exon boundaries of Rdl-encoded polypeptides are marked by vertical red lines. As a result of the alternative splicing of two exons, the *Drosophila* Rdl gene encodes four polypeptides, each of which exhibits characteristic features of $GABA_A$ receptor subunits. The alternatively spliced exons (3 and 6) encode regions of the extracellular N-terminal domain. There are two variant forms of each exon; those of exon 3 are termed ''a'' and ''b'' and differ by two residues, while the alternate forms of exon 6, termed ''c'' and ''d'', differ at 10 residues. Thus, depending on the splice variants present in a given polypeptide, the different Rdl encoded subunits may be referred to as RDL_{ac}, RDL_{bd}, etc. mRNAs encoding all four splice variants have been identified in embryonic *D. melanogaster*. The positions of these variant residues are marked in purple. The two alternate residues encoded by exon 3 lie close to a known determinant of the agonist potency of GABA receptors, while the 10 variant residues encoded by splice variants of exon 6 span a region that is poorly conserved in vertebrate GABA receptor subunits, but which corresponds to determinants of agonist potency in nAChRs. (Reproduced with permission from Hosie, A.M., Aronstein, K., Sattelle, D.B., ffrench-Constant, R.H., **1997**. Molecular biology of insect neuronal GABA receptors. *Trends Neurosci. 20*, 578–583.)

2000) and *Acheta domesticus* (Strambi *et al.*, 1998). RDL is very strongly expressed (Harrison *et al.*, 1996), where the pattern of staining is compartmentalized, suggesting that some, but not all, Kenyon cells (the principal interneurons of the mushroom bodies) receive GABAergic inputs (Brotz *et al.*, 1997). The significance of this pattern of expression is discussed in the following section. The distribution of RDL staining corresponds closely to staining for glutamic acid decarboxylase (GAD; the enzyme involved in the synthesis of GABA) (Buchner *et al.*, 1988), GABA, and synaptotagmin (DiAntonio *et al.*, 1993), suggesting that they contribute to the formation of synaptic receptors. Similar patterns of distribution in the mushroom bodies have been observed in the case of the cockroach *P. americana* (Sattelle *et al.*, 2000) and the cricket *A. domesticus* (Strambi *et al.*, 1998), where again RDL distribution closely matched GABA staining, although details of the pattern of staining differed somewhat across different species.

When expressed in *Xenopus* oocytes, RDL forms receptors (presumably homomeric, as no endogenous GABAR subunits have been reported in these cells) that respond to GABA with thresholds around 1–10 μM (ffrench-Constant *et al.*, 1993a, 1993b; Hosie *et al.*, 1995a, 1995b) as well as in baculovirus transfected sf9 cells (Lee *et al.*, 1994) and a *Drosophila* S2 cell line (Millar *et al.*, 1994). These currents reverse close to the equilibrium potential for chloride ions. Responses are dose-dependent and there is little evidence of rapid (<20 s) desensitization. The responses to GABA are very robust, often in the microampere range (ffrench-Constant *et al.*, 1993a, 1993b). The stable, inducible expression of RDL in the S2-RDL *Drosophila* cell line is particularly convenient: it allows long-term maintenance of a cell line expressing insect GABARs of known molecular composition, facilitating single channel kinetic analysis of ligand–receptor interactions. Both wild-type and mutant (RDL A302S) receptors have been expressed in these cell lines (Lee *et al.*, 1994; Millar *et al.*, 1994).

5.4.2.4.2.1. Comparison of RDL with* in situ *GABARs of insects Currently there is little information on *Drosophila* native GABARs with which to compare the pharmacology or physiology of recombinant RDL receptors. However, it has been shown that GABA responses of *Drosophila* motor neurons reverse around the resting potential and are blocked by PTX (Rohrbough and Broadie, 2002). Neurons cultured from embryonic *Drosophila* form synapses with one another, which include GABAergic synapses (Lee and O'Dowd, 1999). More recently, GABAergic synapses between embryonic *Drosophila* neurons have been shown to be PTX-sensitive, and this sensitivity is reduced in neurons derived from the dieldrin insensitive mutant strain (Lee *et al.*, 2003), suggesting that these synaptic connections involve RDL. Also, *Drosophila* CNS midline precursors in culture show responses to GABA (Schmidt *et al.*, 2000). Similarly, GABA gated currents of cultured *Drosophila* neurons are also blocked by PTX but are insensitive to bicuculline (Zhang *et al.*, 1994). Thus, what little is known of *Drosophila* GABARs *in situ* suggests that they resemble typical bicuculline insensitive insect GABARs.

5.4.2.4.3. Pharmacology of heterologously expressed RDL

5.4.2.4.3.1. The agonist site RDL of *D. melanogaster*, and its equivalent from the mosquito, *Aedes aegypti*, form functional homomers when expressed in *Xenopus* oocytes (ffrench-Constant *et al.*, 1993a, 1993b; Hosie *et al.*, 1996; Shotkoski *et al.*, 1994) as well as when stably expressed in a *Drosophila* cell line (Millar *et al.*, 1994). *Drosophila melanogaster* RDL expressed in *Xenopus* oocytes has a rank order of agonist potency of muscimol = GABA = TACA > CACA ≫ glycine (Hosie *et al.*, 1995a, 1995b). Similarly, the rank order of potency of agonists acting on RDL stably expressed in an insect cell line is GABA = TACA > CACA ≫ glycine = taurine (Buckingham *et al.*, 1996). The lack of activity of glycine is of interest, since the amino acid sequence of RDL more closely resembles that of glycine receptors than it does of many GABAR subunits (ffrench-Constant and Rocheleau, 1992). RDL is also insensitive to 3-APS yet is activated by isoguvacine (Hosie and Sattelle, 1996a). The high potency of muscimol (**Figure 2**), the sensitivity of RDL to CACA and isoguvacine, along with its insensitivity to 3-APS, are all features characteristic of insect nervous system GABARs, but not of insect muscle (see above). This supports the observation that RDL is widely expressed in the nervous system but not in muscle.

An interesting set of findings resulting from a chance observation of atypical agonist induced currents in RDL led to the discovery of the strong pH sensitivity of RDL. The GABA agonists THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) and ZAPA (*Z*-3-[(aminoiminomethyl)thio]prop-2-enoic acid) appeared to lead to a desensitizing response, followed by a pronounced "off" response upon agonist washout (Matsuda *et al.*, 1996). Further investigation showed this to be the result of acidification of the test saline by the agonists. When this was corrected, more typical responses were observed.

What is particularly significant about this, however, is that the apparent off response was due to a pH dependency of the efficacy of these two compounds–preacification of the saline greatly reduced the amplitude of the responses to these agonists. The actions of other agonists tested on RDL, including GABA, were pH independent. Other studies had shown that protonation of histidine residues underlies pH dependent actions of GABA on recombinant mammalian receptors (Krishek *et al*., 1996), as well as the pH dependency of the action of zinc ions (Wang *et al*., 1995). This points to further differences between the agonist binding sites of RDL and vertebrate $GABA_A$ receptors. From the pKa of GABA and THIP at the pH of the uncorrected test solutions (about 4.5), about 76% of the hydroxyls of GABA would be dissociated and about 50% of the hydroxyls of THIP would be dissociated. Thus, some of the differences in pH sensitivity of these drugs may be due to the importance of the dissociated state of the acidic group in ligand binding. Alternatively, these differences may also be attributable to pH dependent states of the histidine residues that form part of the receptor. There are seven histidine residues in the extracellar N-terminal of RDL, and future studies of the effects of point mutations involving each of these residues may help uncover new insights into the agonist actions on RDL.

Another key feature in which RDL resembles *in situ* insect GABARs is its insensitivity to bicuculline. This insensitivity to bicuculline is seen whether RDL is expressed transiently in *Xenopus* oocytes (Hosie and Sattelle, 1996a) or stably expressed in a *Drosophila* cell line (Millar *et al*., 1994). Bicuculline insensitivity is a feature also possessed by the *rdl* gene cloned from *H. virescens* (Wolff and Wingate, 1998). Since, as argued above, most insect GABARs are bicuculline insensitive, RDL thus resembles the majority of insect GABARs, including those expressed on cultured *Drosophila* neurons (Zhang *et al*., 1994).

In some respects, RDL is more similar to $GABA_C$ receptors and the recombinant receptors formed by expression of the vertebrate ρ subunit in their insensitivity to bicuculline and the ability to form functional homo-oligomers. However, it is clear that the agonist site of RDL is dissimilar to that of $GABA_C$ receptors in its preferred pharmacophore. At the sequence level, RDL bears no more closer resemblance to $GABA_C$ receptors than it does to $GABA_A$ receptors, and in fact more closely resembles glycine receptors (ffrench-Constant and Rocheleau, 1992). Thus, while RDL homomers are to be distinguished from $GABA_A$ receptors by their bicuculline insensitivity and the low potency of 3-aminosulfonic acid, they are also to be distinguished from $GABA_C$ receptors by the high efficacy of muscimol and isoguvacine.

5.4.2.4.3.2. The convulsant antagonist site The RDL channel expressed in *Xenopus* oocytes or the *Drosophila* S2 cell line is effectively blocked by 1–10 μM concentrations of PTX, TBPS, EBOB, dieldrin, BIDN, and fipronil (ffrench-Constant *et al*., 1993a, 1993b; Millar *et al*., 1994; Shirai *et al*., 1995; Buckingham *et al*., 1996). For structures of these molecules see **Figures 3–5**. The IC_{50} for PTX block of RDL expressed in *Drosophila* cells was estimated to be 2.5 μM, whereas 10 μM TBPS reduced GABA evoked currents by less than 50%. This higher efficacy of PTX compared to TBPS is also typical of *in situ* insect GABARs, whereas the reverse is true for vertebrate GABARs. Curiously, the RDL homolog from *H. virescens* is insensitive to dieldrin (Wolff and Wingate, 1998). The block of RDL mediated GABA responses by PTX has both competitive and noncompetitive features, in that it reduces the maximal response to GABA and shifts the EC_{50} of the GABA response (Shirai *et al*., 1995). A similar action of PTX was reported for a crustacean GABAR (Smart and Constanti, 1986). As is the case for insect GABARs *in situ*, PTX is a more potent blocker of RDL than TBPS (Buckingham *et al*., 1996), whereas the reverse is true for vertebrate $GABA_A$ receptors.

The convenience of the availability of a robust, functional homomeric GABAR was exploited by Shirai and coworkers (Shirai *et al*., 1995) to determine the key structural elements of the PTX molecule that determine its potency. Using the same approach as that on expressed vertebrate $GABA_A$ receptors (Anthony *et al*., 1993) and *in situ* insect receptors (Anthony *et al*., 1994), the study agreed with earlier ones in that picrotin and PTX acetate were found to be of less efficacy in blocking the GABA response, whereas fluoropicrotoxin was of similar potency to PTX. Thus, the PTX recognition site of RDL resembles that of *in situ* insect GABARs. The potential that RDL offers over *in situ* receptors, namely that the subunit composition is known, could be exploited further in elucidating the mechanisms of action of PTX and related compounds.

These structure–function studies did not uncover any significant difference between vertebrate and insect GABARs at the convulsant site, yet such differences presumably exist, as is inferred from the selectivity of certain insecticides known to act at the convulsant site. Such differences did, however, emerge in a subsequent study by Sattelle and colleagues (Hosie *et al*., 1996), which used a related series of PTX-like compounds, the picrodendrins, to probe the convulsant antagonist site. Picrodendrins

and related tutin analogs are terpenoid toxins present in extracts of a plant of the Euphorbiaceae, *Picrodendron baccatum*. These plants are known as "mata becerro," or calf-killer, in the Dominican Republic where they have been used to kill bed bugs and lice (Ozoe *et al*., 1994). The picrodendrins are structurally similar to PTX, and share its polycyclic lactone picrotoxane skeleton, acting at a similar site in the channel of vertebrate $GABA_A$ receptors (Ozoe *et al*., 1994). The series of terpenoids studied differed mainly in their substituents at the C9 position. In most cases, these substituents were a spiro-γ-butyrolactone ring, providing one of at least two electronegative groups believed to be necessary for picrotoxane activity (Ozoe and Matsumura, 1986). Alteration of these substituents produced differing effects on their potency on RDL when compared to their effectiveness in displacing $[^{35}S]$TBPS from rat membranes (Ozoe *et al*., 1994). First of all, most of the terpenoids were significantly less effective at blocking RDL than at displacing TBPS from rat membranes. More importantly, however, there were considerable differences in their rank order of potency on RDL compared to rat GABARs. Molecules that were most potent against RDL shared an olefin binding that brought the side chains closer to the plane of the spiro-γ-butyrolactone ring than the other compounds. This study succeeded in showing the importance of the position of the electronegative centers in the PTX-like family, which had not emerged from studies restricted to the C4 substituents and the isopropenyl moiety.

The bicyclic dinitrile BIDN, developed in the face of a need for a new, specific ligand for the insect convulsant site, is a potent blocker of recombinant RDL GABA receptors. At 1 μM, BIDN reversibly reduces the amplitude of RDL mediated GABA responses in *Xenopus* oocytes, with only a slight dependence on membrane potential (Hosie *et al*., 1995b). This action was dose dependent, with an IC_{50} that depended upon the concentration of GABA used. When tested against 20 μM GABA the IC_{50} for BIDN action was estimated to be 1.0 μM, whereas when tested against 100 μM GABA it was estimated at 20 μM. This is suggestive of allosteric interactions between the BIDN and GABA ligand binding sites. Like PTX, the action of BIDN displayed both competitive and noncompetitive elements, in that it reduced the maximum GABA induced currents and shifted the dose–response curve to the right, pointing to allosteric actions of BIDN. BIDN also reversibly blocks RDL mediated responses in the S2-RDL and S2-RDL(A302S) cell lines (Buckingham *et al*., 1996). In both the *Xenopus* oocyte and S2 cell line expression systems, it was noticed that GABA responses in the presence of BIDN lacked the desensitization that was so clearly present in control responses (Buckingham *et al*., 1996). One plausible interpretation of this might simply be that, like PTX, BIDN exerts its effects through an enhancement of desensitization, but favoring a different state than PTX. Evidence that BIDN and fipronil both operate by reducing open-state probability, but through different mechanisms, also comes from single channel studies (Grolleau and Sattelle, 2000).

RDL expressed in *Xenopus* oocytes and in a *Drosophila* cell line is sensitive to block by the phenylpyrazole insecticide, fipronil (Millar *et al*., 1994; Hosie *et al*., 1995a). In *Xenopus* oocytes, RDL mediated responses to 50 μM GABA are reduced by up to 80% by 100 μM fipronil. Recovery from fipronil block was very slow, and still incomplete after 10 min washout. This differs from the actions of both PTX and BIDN, which reverse completely and rapidly. Like the block by BIDN (Hosie *et al*., 1995b), fipronil block was voltage independent (Hosie *et al*., 1995a). A further similarity to the actions of both BIDN and PTX is that the action of fipronil consists of both competitive and noncompetitive elements, and is also dependent on the concentration of agonist. Thus, fipronil, BIDN, and PTX share many features (voltage independence, agonist dependence, and mixed mode of action) suggesting that they operate through similar mechanisms. However, it must be noted that whereas PTX and the cyclodienes are competitive inhibitors of $[^3H]$EBOB binding, fipronil is a noncompetitive displacer of $[^3H]$EBOB binding, at least in *M. domestica* (Deng *et al*., 1991, 1993). The possible significance of this in understanding the mechanisms of insecticide resistance is discussed below.

Differences in the action of fipronil and BIDN on RDL were examined in a single channel patch-clamp study (Grolleau and Sattelle, 2000). The ability of RDL to form functional homomers simplifies the interpretation of experiments on the effects of the A302S mutation on single channel properties of the channel. When applied to outside-out patches from S2-RDL cells, GABA (50 μM) evoked inward currents that were completely blocked by 100 μM PTX. As had been reported for outside-out patches of *Xenopus* oocytes expressing RDL, the current–voltage relationship showed slight inward rectification at positive potentials. The conductance of the single channel currents was around 36 pS, but interestingly a 71 pS conductance was observed when the application of GABA was longer than 80 ms. When either 1 μM fipronil or 1 μM BIDN was present in

the external saline, all the GABA gated channels were completely blocked. At lower concentrations, near their respective IC_{50} values, the duration of channel openings was shortened. Interestingly, the blocking action of BIDN resulted in the appearance of a novel channel conductance (17 pS). Examination of the effects of coapplication of these two convulsants (applying 300 nM BIDN with fipronil concentrations ranging from 100 to 1000 nM) revealed an additional BIDN induced dose dependent reduction in the maximum open probability. Thus, while both BIDN and fipronil shorten the duration of wild-type RDLac GABAR channel openings, they appear to do so by acting at distinct sites.

The findings reported to date on the actions of convulsants on RDL agree very closely with their actions on *in situ* insect GABARs. The agonist dependence and mixed competitive/noncompetitive action are consistent with a model of PTX action in which the PTX molecule binds preferentially to an agonist-bound conformational state of the receptor and hence increases the probability of a transition into a desensitized state. Such a conclusion was advanced to explain similar properties of PTX action at the crayfish neuromuscular junctional GABARs (Smart and Constanti, 1986) and the finding that the rate of development of a steady-state blockade of GABARs on rat sympathetic neurons was enhanced when GABA is applied in brief, rapidly repeated doses (Newland and Cull-Candy, 1992).

RDL has also been used to predict cross-resistance to insecticides acting at the convulsant site. Dieldrin resistant RDL mutant GABARs are not only comparatively insensitive to PTX, but are also insensitive to BIDN (Hosie *et al.*, 1995b). Similar results were obtained for fipronil (Hosie *et al.*, 1995a) although other workers reported different results (Wolff and Wingate, 1998). Furthermore, a novel tricyclic dinitrile, KN244, blocks RDL and the sensitivity of dieldrin resistant RDL to it is reduced 100-fold (Matsuda *et al.*, 1999).

*5.4.2.4.3.3. **Allosteric modulators*** Responses mediated by RDL are sensitive to potentiation by benzodiazepines, but their benzodiazepine pharmacology differs from *in situ* insect receptors. Insect GABA responses are potentiated by Ro 5-4864 and flunitrazepam. Responses to GABA mediated by RDL expressed in *Xenopus* oocytes (Hosie and Sattelle, 1996b) were potentiated by micromolar concentrations of Ro 5-4864. In contrast, the same receptor expressed in the *Drosophila* S2 cell line appeared to be insensitive to Ro 5-4864 (Millar *et al.*, 1994), although enhancement was obtained when the concentration of the benzodiazepine was increased to 100 μM (Buckingham *et al.*, 1996b). This illustrates that caution must be exercised in interpreting negative data until a wide range of ligands is tested. However, flunitrazepam, which is active at GABARs on cockroach motor neuron, D_f, at micromolar concentrations, was found to be ineffective on RDL in either expression system even at concentrations of 100 μM (Millar *et al.*, 1994; Hosie and Sattelle, 1996b). This represents a departure from most *in situ* insect receptors studied to date. Significantly, the identified motor neuron D_f of the cockroach stains positively to the RDL antibody (Sattelle, personal communication), suggesting that in this cell at least, RDL may coassemble with another subunit. Flunitrazepam is also reported to have no effect on a GABAR cloned from *H. virescens* (Wolff and Wingate, 1998). Nonetheless, the comparatively low sensitivity of RDL to benzodiazepines, coupled with its sensitivity to the "peripheral" benzodiazepine, Ro 5-4864, are both characteristic of insect receptors compared to that of vertebrates.

That RDL should differ from *in situ* receptors principally at the benzodiazepine site is not surprising, given the wide variation in benzodiazepine pharmacology of GABARs generally, and may indicate that RDL coassembles with another subunit subtype *in situ*. Since only two other GABARs have been cloned from *Drosophila* to date, these are the obvious candidates for the other subunit. However, it is unlikely that LCCH3 is the additional subunit, since, as described above, receptors formed by coexpression of RDL with LCCH3 are quite unlike insect receptors. In any case, the lack of overlap of expression of these two subunits at any stage of development makes it highly unlikely that they coassemble *in vivo*. The question of whether RDL forms a homomer *in situ* is discussed in a separate section below. Unfortunately, coexpression of RDL with GRD has yet to be described. While expression studies on the agonist pharmacology of three of the four RDL splice variants has been described in some detail (Chen *et al.*, 1994; Hosie and Sattelle, 1996a), benzodiazepine pharmacology of these variants, or combinations of them, has not yet been assayed.

Like *in situ* insect receptors, RDL is potentiated by barbiturates. In both the *Xenopus* oocyte and the *Drosophila* S2 cell line expression systems, RDL mediated GABA responses were enhanced by either sodium phenobarbitone or sodium pentobarbitone by up to 50% of control values (Hosie and Sattelle, 1996b; Buckingham *et al.*, 1996). Similar results were reported for the RDLbd splice variant, whose responses were enhanced up to fivefold (Belelli *et al.*,

1996a). Similarly, the RDL equivalent cloned from *H. virescens* is also subject to barbiturate (phenobarbital) enhancement (Wolff and Wingate, 1998). In contrast to vertebrate GABARs, which are also sensitive to barbiturates, elevation of the barbiturate to high (1 mM) concentrations did not evoke *Drosophila* RDL mediated currents in the absence of GABA (Belelli *et al*., 1996a; Buckingham *et al*., 1996). It is therefore likely that the two components of barbiturate action on vertebrate receptors – the enhancement of the GABA response and the direct opening of the channel in the absence of GABA – are mediated by two separate sites, of which only one may be present on insect GABARs.

RDL is comparatively insensitive to steroids. The steroid, 5α-pregnan-3α-ol-20-one, enhances human $\alpha_3\beta_1\gamma_{2L}$ receptors, but at high concentrations had only a minimal effect on RDLbd (Belelli *et al*., 1996) and RDLac (Millar *et al*., 1994; Hosie and Sattelle, 1996b). Insect GABARs *in situ* are also highly insensitive to steroids (Rauh *et al*., 1990).

The anticonvulsant, loreclezole, is a potentiator of vertebrate recombinant GABARs containing the β_2 or β_3 subunit (Wafford *et al*., 1995), but not of those containing a β_1 subunit (Wingrove *et al*., 1994). This is attributed to the presence of a serine in the β_2 and β_3 subunits, which is an asparagine at the equivalent position in the β_1. Curiously, although a mutation of this serine to a methionine abolishes loreclezole sensitivity (Stevenson *et al*., 1995), RDL (which has methionine at this position) is sensitive to loreclezole (Belelli *et al*., 1995; Hosie and Sattelle, 1996b).

RDL is also sensitive to a number of other allosteric modulators. Isoflurane enhances GABA responses mediated by expressed RDL and blocks at higher concentrations. This block is not seen for the cyclodiene resistant A302S mutant, suggesting that the block is due to an interaction at the PTX site (Edwards and Lees, 1997).

These data amply illustrate the usefulness of the cloned RDL receptor in advancing our understanding of insect GABARs. More than this, however, RDL, because of its ability to form functional homomers, has also been of use in structure/function studies on allosteric modulator sites of vertebrate GABARs. For instance, RDL is insensitive to the anesthetic etomidate, as are vertebrate β_1 subunits. Vertebrate β_2 and β_3 subunits, however, do confer sensitivity to etomidate and this is attributable to the presence of an asparagine residue at equivalent positions in a transmembrane-spanning domain, which is a methionine at the equivalent position in RDL. Mutating M314 to an asparagine in RDL rendered the expressed receptors sensitive to enhancement by etomidate (McGurk *et al*., 1998). As in vertebrate recombinant receptors, this sensitivity is stereospecific, whereas the native sensitivity to pregnane steroids or pentobarbitone remained unchanged. Conversely, the N289M mutation introduced into vertebrate β_2 abolished the etomidate sensitivity. Such complementarity of effects of mutation encourages confidence in interpreting experiments using an invertebrate receptor as a model of vertebrate receptors, especially in view of the comparative simplicity of interpreting data obtained using homomers.

5.4.2.4.3.4. Other (RDL) ligands RDL, like GABARs of the motor neuron, D_f, of the cockroach (Bai, 1994), is insensitive to zinc (Hosie and Sattelle, 1996b), which blocks many vertebrate $GABA_A$ receptors (Hosie *et al*., 2003), as well as ρ receptors.

5.4.2.4.4. Conclusions There can be little doubt that the availability of RDL has accelerated the pace of research into the molecular pharmacology of insect GABARs. The chief advantage that it confers, in addition to the convenience of performing experiments on oocytes, is that hitherto studies aimed at uncovering the mechanisms of drug–receptor interaction for insect GABARs have been hindered by the fact that in no case is the subunit composition of any *in situ* GABAR known. It is of great significance that a number of different laboratories have confirmed independently that whatever the expression system chosen (*Xenopus* oocytes, *Drosophila* S2 cells, *Spodoptera* Sf9 cells), the pharmacology of the expressed RDL receptor does not differ significantly. Indeed one paper (Buckingham *et al*., 1996) examined this issue directly. This encourages confidence that RDL is not coexpressing with GABAR subunits native to the respective expression systems, and that we can be reasonably certain that the subunit composition of the expressed RDL receptor is a homomeric pentamer. To appreciate the significance of such an advantage, we have only to look at the advances in understanding of nicotinic receptors that emerged from research using another homomer forming receptor, the vertebrate nicotinic α_7 receptor.

Since RDL homomers so closely mimic *in situ* GABARs, and since RDL antibody staining corresponds closely to GABA immunoreactivity, it is likely that RDL contributes to most of the GABARs in the insect brain. In view of the conclusion drawn above that there is pharmacological diversity of subtypes of GABARs in insects, the question of the source of this diversity remains to be addressed. The occurrence of alternative splicing and RNA

editing may add to functional diversity of the small *Drosophila* GABAR gene family. Evidence for this possibility is discussed in the following section.

5.4.2.4.5. Alternative splicing in GABA receptor subunits RDL is expressed in the form of four splice variants, arising from alternative splicing of two of its nine exons (exons 3 and 6). These four distinct polypeptides show characteristic structural features of a GABAR subunit. The regions in which the splice variants differ align closely with regions known to be determinants of agonist potency in vertebrate ionotropic receptor subunits. This is highly unusual. Although there are many examples of alternatively spliced GABAR subunit genes in vertebrates, the splicing usually occurs in their large intracellular loops. All four of these splice variants are expressed in embryonic *Drosophila* (ffrench-Constant and Rocheleau, 1992), yet their functional roles remain to be determined.

Since the pharmacological diversity observed among vertebrate $GABA_A$ receptors arises at least in part from the assorted assembly from different subunit isoforms, the alternate splicing of the *rdl* gene may serve a physiological role by increasing receptor diversity. Since RDL appears to be a component of the majority of insect GABARs, such a splicing mechanism may represent another route to subunit diversity in a small GABAR gene family. Evidence that alternative splicing confers pharmacological diversity comes from expression studies that have described pharmacological differences between these variants at the agonist site. For example, the three splice variants tested to date in functional expression studies (RDLac, RDLad, RDLbd) all differ in their EC_{50} values for GABA (Hosie and Sattelle, 1996a). The region in which these variants differ include loop F of the GABA binding site.

5.4.2.4.6. Does RDL form a homomer *in situ*? Despite our limited knowledge of the pharmacology of native GABARs of *Drosophila*, it is clear that RDL homomers mimic closely the pharmacology of insect GABARs. The general features that distinguish most insect GABARs from those of vertebrates – insensitivity to bicuculline, comparative insensitivity to benzodiazepines, sensitivity to the peripheral benzodiazepine Ro 5-4864, insensitivity to TBPS, and sensitivity to isoguvacine – are all seen in RDL homomers. The only challenge to this, the insensitivity of RDL to flunitrazepam, is perhaps not particularly serious in view of the diversity of benzodiazepine pharmacology amongst $GABA_A$ receptors studied to date and the acute dependence of benzodiazepine pharmacology upon subunit composition. Where this difference in benzodiazepine pharmacology gains significance, though, lies in the observation that a *Drosophila* anti-RDL antibody stains motor neuron D_f of the cockroach (Sattelle, personal observation), which is known to possess GABARs sensitive to micromolar concentrations of flunitrazepam (Sattelle *et al*., 1988). Since RDL, which is flunitrazepam insensitive, is expressed on D_f, which is flunitrazepam sensitive, this difference hints at the existence of another subunit subtype in these cells.

Single channel recordings also provide reason to suspect that RDL may not always exist as a homomer *in situ*. Single, GABA gated channels recorded from *Xenopus* oocytes injected with *rdl* cRNA were inwardly rectifying, with inward and outward currents of around 21 pS and 12 pS, respectively (Zhang *et al*., 1994). GABA gated single channels recorded from *Drosophila* larval neurons in culture, although also inwardly rectifying, had inward and outward conductances of around 28 and 29 pS, respectively (Zhang *et al*., 1994), a value significantly different from expressed RDL homomers. Mean open times of RDL homomers in *Xenopus* oocytes were significantly shorter than single GABA gated channels in cultured *Drosophila* neurons: around 62 ms compared to 118 ms, respectively. A simple explanation for these findings would be that RDL coexpresses with another subunit *in situ*.

However, single channels recorded from a *Drosophila* S2 cell line stably expressing RDL were estimated to be 36 pS (Grolleau and Sattelle, 2000), a value also significantly different from values for RDL recorded in oocytes. Both these studies used the same concentration of GABA (50 μM) and both used brief applications of GABA to outside-out patches. So it is likely that this difference is due to the different expression environments or perhaps the different ionic compositions of the recording media. Given that no other differences in pharmacology have been detected to date between the two expression systems, and that both studies identified a slight inward rectification, the latter is the more likely explanation. It is interesting to note that Grolleau and Sattelle (2000) reported the emergence of a larger conductance when the duration of the GABA pulse was prolonged to over 80 ms, suggesting that the conductance properties of this channel may be more complex than hitherto suspected.

Does RDL coexpress with the other two insect GABAR subunits known to date, LCCH3 or GRD? LCCH3 (Henderson *et al*., 1993) closely resembles vertebrate $GABA_A$ receptor β subunits. Neither LCCH3 nor GRD form functional homo-oligomeric receptors in *Xenopus* oocytes (Harvey

et al., 1994; Zhang *et al.*, 1994). When coexpressed with RDL in baculovirus transfected *Spodoptera frugiperda* Sf21 cells, however, LCCH3 forms a receptor that exhibits a biphasic response (Zhang *et al.*, 1994), which only becomes clear during prolonged applications of GABA (>1 s). The first component was a rapidly activating, rapidly desensitizing current with kinetics indistinguishable from those of RDL expressed alone. The second component, however, was of much slower kinetics of activation and desensitization, and was attributed to the RDL/LCCH3 combination. The coapplication of 500 μM PTX completely abolished the fast component and blocked about half of the slow current. In contrast, bicuculline (100 μM) completely abolished the slow component leaving the fast component unaffected. One simple explanation of these findings is that the fast component is attributable to RDL homomers that, in accord with other reports, is PTX sensitive and bicuculline insensitive, and that the slow response is mediated by RDL/LCCH3 multimers. This provides evidence that LCCH3 can indeed form a receptor when coexpressed with RDL, but the resulting receptor differs significantly from the pharmacology of all known insect GABARs with respect to picrotoxinin. Their sensitivity to bicuculline further distinguishes them from GABARs of cultured *Drosophila* neurons (Zhang *et al.*, 1994). Furthermore, expression of LCCH3 and RDL do not overlap in the adult–LCCH3 is expressed in cell bodies (Aronstein *et al.*, 1996) whereas RDL expression is restricted to neuropil and is not observed in *Drosophila* cell bodies (Aronstein *et al.*, 1995), although it is observed in cell bodies in other species (Harrison *et al.*, 1996). We therefore conclude that it is unlikely that RDL coexpresses with LCCH3 *in vivo*. It would be most interesting to establish whether perhaps LCCH3 contributes to the formation of bicuculline sensitive GABARs of the kind that are known to exist in *M. sexta* (Waldrop *et al.*, 1987).

One intriguing recent finding suggests that the additional subunit, from which we have excluded LCCH3 as a candidate, may not even be a GABAR subunit. Immunoprecipitation studies using antibodies to a *D. melanogaster* glutamate gated chloride channel (DmGluCl) showed that: (1) DmGluCl alpha antibodies precipitated all of the ivermectin and nodulisporic acid receptors solubilized by detergent from *Drosophila* head membranes; and (2) they also immunoprecipitated all solubilized nodulisporic receptors, but only approximately 70% of the ivermectin receptors (Ludmerer *et al.*, 2002). These data suggest that both DmGluCl α and RDL are components of nodulisporic acid and ivermectin receptors.

Finally, it is of interest to note the recent finding by O'Dowd and colleagues that RDL is involved at GABAergic synapses formed in cultures of embryonic *Drosophila* neurons (Lee *et al.*, 2003). Furthermore, the authors conclude that other subunit(s) are also involved.

5.4.2.5. Intracellular Modulation of GABARs

Ionotropic receptors are potentially susceptible to modulation by intracellular factors. This may alter their density, kinetics, or pharmacology, and may be an important component in long-term changes in synaptic function, such as may be involved in learning. One type of GABAR present on cockroach (*P. americana*) DUM neurons is regulated by changes in intracellular calcium through a calcium dependent protein kinase pathway (Alix *et al.*, 2002). Similarly, maintenance of GABA responses in isolated neurons from *P. americana* abdominal ganglia requires phosphorylation as well as, possibly, a nucleotide recognition site unrelated to PKA-dependent phosphorylation (Watson and Salgado, 2001). However, although RDL contains possible sites for intracellular phosphorylation (ffrench-Constant *et al.*, 1991), no rundown of RDL mediated responses is seen in *Drosophila* S2 cells in whole-cell patch clamp mode, even when no ATP is included in the pipette (Buckingham, personal observation). This does not, however, exclude the possibility that RDL is subject to modulation by intracellular signaling pathways. The latter is likely to provide a fertile area of study in the future, especially in view of the observations that suggest that RDL may be closely implicated in learning.

5.4.2.6. GABARs as Targets for Insecticides

Insect GABARs are the major targets for several chemically distinct classes of insecticidally active molecules: trioxabicyclooctanes, dithianes, silatranes, lindane, toxaphene, bi- and tricyclic dinitriles, chlorinated cyclodienes, and picrotoxinin (Deng *et al.*, 1991; Rauh *et al.*, 1997). These sites overlap, since [^{3}H]EBOB is displaced by all these compounds. The importance of the convulsant site for the insecticidal action is illustrated by the close linear correlation between the effectiveness of their displacing [^{3}H]EBOB binding from house fly head membranes and their LD_{50} (Deng *et al.*, 1991).

5.4.2.6.1. Picrotoxin Picrotoxin was used as an early insecticide in the preservation of lard (Bentley and Trimen, 1875). Picrotoxin contains picrotin and picrotoxinin. The radiolabeled form of picrotoxinin ([^{3}H]DHPTX) was used as an early probe of insect GABARs, but was later superseded by [^{35}S]TBPs.

5.4.2.6.2. Polychlorocyclohexanes Chemicals of this group, notably lindane, benzene hexachloride, and α-endosulfan, were used intensively as insecticides in the postwar years, until recent environmental concerns led to their disuse, with the exception of α-endosulfan. Binding (Lawrence and Casida, 1984) and electrophysiological (Wafford *et al.*, 1989) studies confirmed that they act at ionotropic GABARs.

5.4.2.6.3. Bicyclophosphorus esters and bicycloorthobenzoates The radioligand probe [^{35}S]TBPS overtook [^{3}H]DHPTX as the most useful probe of vertebrate GABA gated chloride channels. However, insect GABARs are comparatively insensitive to TBPS (Lummis and Sattelle, 1986) compared to vertebrate $GABA_A$ receptors, and the bicyclophosphorus esters have comparatively low insecticidal activity (Casida *et al.*, 1988). The next development arose from the discovery that the addition of a substitute benzene ring to the bicyclic ring greatly increased insecticidal potency (Palmer and Casida, 1985), as well as affinity for the convulsant site (Lummis and Sattelle, 1986). Selection for the optimal substituents gave rise to the bicycloorthobenzoates, of which the most frequently used are EBOB (1-(4′-ethyphenyl)-4-[2,3]propyl-2,6,7-trioxabicyclo [2.2.2]octane) (**Figure 3**) and TBOB. EBOB has proven particularly useful in characterizing the convulsant site of houseflies (Cole and Casida, 1992; Deng *et al.*, 1993) and *Drosophila* (Cole *et al.*, 1995).

5.4.2.6.4. Phenylpyrazoles The phenylpyrazole insecticide fipronil (**Figure 4**) is an effective pest control agent even at low doses (Tingle *et al.*, 2003). It has many characteristics that make it suitable: it degrades slowly with a half-life of 36 h to 7 months and it leaches out slowly into groundwater. Unfortunately, its toxicity to certain vertebrates may limit the use of fipronil insecticides.

Fipronil competes for the [^{3}H]EBOB binding site, along with α-endosulfan and lindane (Ratra and Casida, 2001), indicating that it acts at the convulsant site. RDL from *Drosophila* and *Heliothis* are both blocked micromolar concentrations of fipronil (Hosie *et al.*, 1995a; Wolff and Wingate, 1998), and fipronil reduces GABA gated currents in RDL by shortening the duration of openings (Grolleau and Sattelle, 2000). In addition to GABA gated chloride channels, fipronil also blocks glutamate gated chloride channels on DUM neurons of cockroach (Raymond *et al.*, 2000), but not histamine gated chloride channels of *Drosophila* eye (Zheng *et al.*, 2002). GLC-3, a homomer-forming recombinant glutamate gated chloride channel from *C. elegans*, is blocked by fipronil.

That fipronil and dieldrin both act at similar sites on insect GABARs allowed the prediction that cross-resistance between these insecticides will occur (Hosie *et al.*, 1995a). This was confirmed in a recent study that showed cross-resistance to dieldrin in a fipronil resistant mosquito line, and cross-resistance to fipronil in a dieldrin resistant mosquito line (Kolaczinski and Curtis, 2004).

5.4.2.6.5. Avermectins Avermectins (**Figure 7**) have been used as insecticides (e.g., abamectin) as well as endectocides (e.g., ivermectin) (review: Bloomquist, 1996 and (see **Chapter 5.2**). In addition to their actions on inhibitory glutamate receptors, avermectins also displace binding of EBOB to *Drosophila* membranes at 20 nM. This is also true of the dieldrin resistant mutant form of RDL (Cole *et al.*, 1995). Ivermectin binds to neuronal membranes of *D. melanogaster* and the locust, with nanomolar K_ds (Rohrer *et al.*, 1995). Although glutamate gated chloride channels are probably the principal site of action of avermectins, their insecticidal actions may also be due, at least in part, to actions on GABARs (Bloomquist, 1996). Their insecticidal actions can have a deleterious ecological effect where they are used as endectocides on cattle, as the presence of avermectins excreted in the dung is detrimental to dung dwelling insects (McCracken, 1993; Strong, 1993), slowing normal biodegradation.

5.4.2.7. Mechanisms of Insecticide Resistance

Resistance to insecticides is common in field populations of many insect species (Georghiou, 1986). Early binding studies provided evidence that resistance to cyclodienes is due largely to changes in the target site. The dissociation constant and saturation level of a cyclodiene resistant strain (Lpp) of the cockroach, *B. germanica*, differed from those of the wild-type (Matsumura *et al.*, 1987). These differences corresponded to an approximately tenfold reduction in affinity. A number of studies on geographically separate populations of *D. melanogaster* provided clear evidence of cross-resistance between cyclodiene insecticides and PTX (Kadous *et al.*, 1983; Matsumura and Ghiasuddin, 1983; Wafford *et al.*, 1989; Deng *et al.*, 1991; ffrench-Constant and Roush, 1991), again implicating changes in the convulsant site as a mechanism of resistance to the action of insecticides.

In the *rdl* (resistant to dieldrin) *Drosophila* strain, insecticide resistance arises from the substitution of a single amino acid (A302S), which contributes to

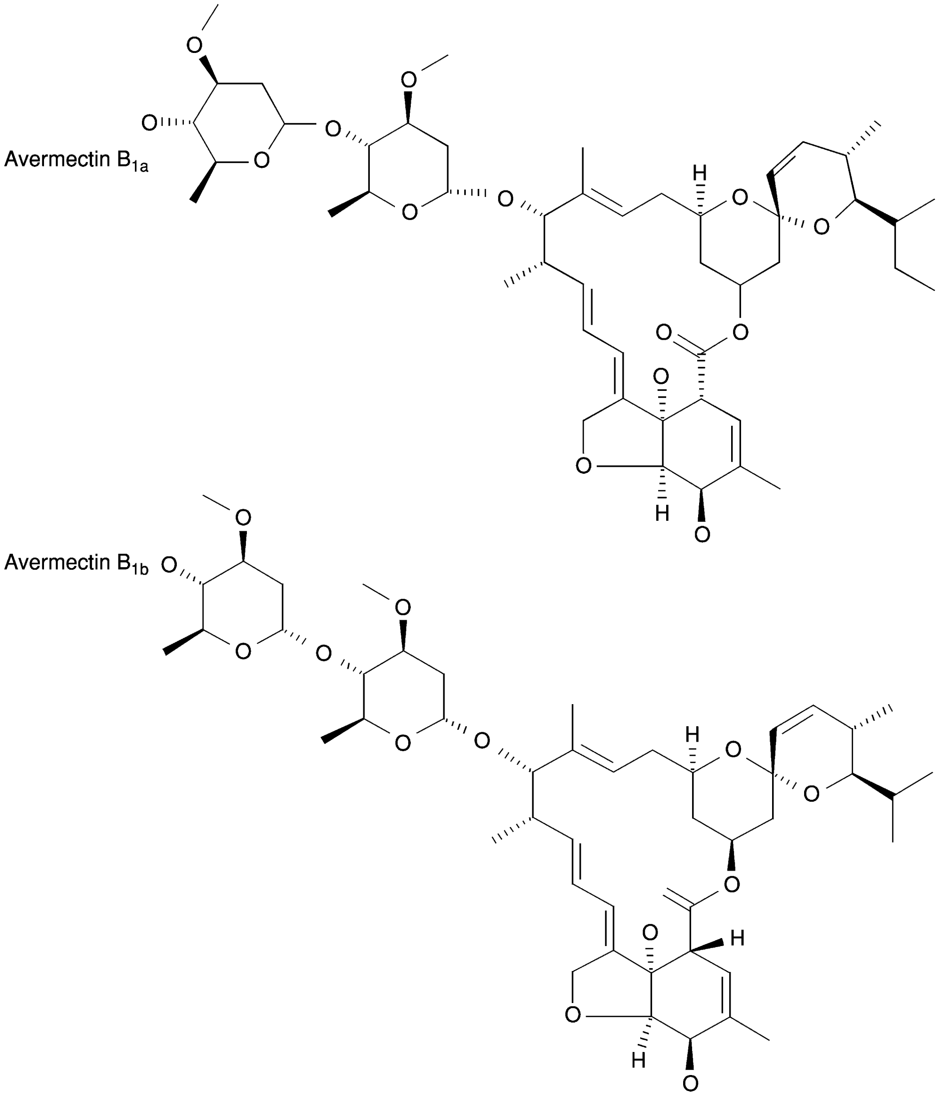


Figure 7 The molecular structures of avermectin B_{1a} and avermectin B_{1b}.

the M2 pore forming region of the GABAR channel. It is close to a site that determines charge selectivity in nAChRs (Imoto *et al.*, 1988; Leonard *et al.*, 1988). A similar mutation (A285S) confers dieldrin insensitivity to a cloned *H. virescens* RDL equivalent (Wolff and Wingate, 1998). Similarly, cyclodiene resistance in the aphid, *Nasonovia ribisnigri*, a main pest of salad crops, is also due to an A302S mutation (Rufingier *et al.*, 1999). In the peach aphid, *M. persicae*, cyclodiene resistance is due to a mutation of A302, but in this case three mutations 5 A302Gly, Ser (TCG), and Ser (AGT), can generate resistance (Anthony *et al.*, 1998). Study of wild *T. castaneum* suggests multiple origins of cyclodiene resistance (Andreev *et al.*, 1999), whereas the mutation probably has a single origin in the case of *D. melanogaster* (ffrench-Constant *et al.*, 2000). Indeed, the central importance of the RDL A302S mutation is reflected in the observation that a mutation of this amino acid is implicated in cyclodiene resistance in at least 60 strains of *D. melanogaster* and *D. simulans* collected worldwide (ffrench-Constant *et al.*, 1993a).

The A302S mutant receptor has a greatly reduced sensitivity to PTX. When expressed in *Xenopus* oocytes, wild-type RDL supports GABA responses that are completely blocked by 10 µM PTX, whereas RDL-A302S mediated responses are only about 50% blocked by the same concentration (ffrench-Constant *et al.*, 1993a, 1993b). The same study showed that sensitivity to dieldrin is also reduced. Dieldrin at 10 µM reduced the amplitude of wild-type responses by some 75%, whereas the mutant was almost completely insensitive to the

concentrations tested. Similar results are obtained regardless of the expression system. GABA responses mediated by RDL stably expressed in a *Drosophila* cell line (S2) were blocked by PTX with an IC_{50} of approximately 2 μM, whereas in the case of an S2 cell line stably expressing the A302S mutant, 100 μM PTX produced a maximum block of only about 30% (Buckingham *et al*., 1996). The A302S mutation is not known to have any effect on agonist pharmacology.

The A302S substitution confers resistance to a variety of naturally occurring (e.g., picrotoxinin, picrodendrin-O) and man-made (e.g., fipronil) insecticides, which act allosterically as noncompetitive antagonists of insect GABARs. The A302S substitution also renders RDL homomers and native receptors resistant to the antagonists TBPS, lindane, and BIDN (Belelli *et al*., 1995; Buckingham *et al*., 1996; Hosie *et al*., 1995b). However, these compounds interact noncompetitively in radioligand binding studies, suggesting that they have distinct, if overlapping, binding sites. The A285S mutant RDL of *H. virescens* is also resistant to fipronil and picrotoxinin to similar degrees as the *Drosophila* RDL mutant (Wolff and Wingate, 1998).

Studies on vertebrate $GABA_A$ receptors have suggested that PTX blocks the channel via an allosteric mechanism in which it binds preferentially to activated channels and stabilizes them in the agonist-bound, closed conformation (Smart and Constanti, 1986; Newland and Cull-Candy, 1992). It appears that one effect of the RDL A302S mutation is to stabilize the open conformation and reduce the rate of desensitization (Zhang *et al*., 1994). In addition, the A302S mutation lowers the binding affinity for PTX (Cole *et al*., 1995; Lee *et al*., 1994). Since the sites for all the convulsant antagonists overlap, we can assume that similar mechanisms apply to other cyclodienes. In this context, it is interesting to observe that although the substitution of a serine for an alanine does not introduce any change in net charge, the added hydroxyl group is more bulky and may restrict access of the PTX molecule by steric interference (ffrench-Constant *et al*., 1993a, 1993b). It has also been observed (Hosie *et al*., 1995a) that the A302S mutation both alters the rate of desensitization and reduces the potency of PTX as well as that of fipronil. PTX and dieldrin both displace EBOB binding competitively in *M. domestica*, whereas fipronil's displacement of EBOB is noncompetitive. It has been suggested (Zhang *et al*., 1994) that the A302S mutation must either alter both the fipronil and PTX binding sites, or perhaps instead, alters the allosteric linking mechanism by which convulsants reduce GABA responses, or both.

5.4.2.8. Molecular Basis of Selectivity of Insecticides for Insect GABARs

Many currently used insecticides have been developed for their selective toxicity to insects compared to vertebrates. Much of this is attributable to differences between the sites of action within the channel pore. For example, fipronil is more toxic to insects than to vertebrates because it has a higher affinity for insect GABARs (Hainzl *et al*., 1998). However, binding of EBOB, fipronil, lindane, and endosulfan to housefly head membranes is comparable to the binding to human recombinant β_3 GABARs, attributable to sequence similarities at key points on their receptors. Studies of recombinant human $GABA_A$Rs show subunit dependency of insecticide affinity (Ratra and Casida, 2001). Similarly, the potency of lindane, fipronil, EBOB, and α-endosulfan on human recombinant $GABA_A$ receptors is dependent on the specific type of α or γ subunit present (Ratra *et al*., 2001). A detailed study on the actions of picrodendrin and tutin antagonists on RDL expressed in oocytes (Hosie *et al*., 1996) suggested that insect and vertebrate GABARs differ in their ideal convulsant site pharmacophore. The same conclusion is confirmed by similar studies using systematic variations of a class of molecules. For instance, acyclic esters compete with EBOB and changes to these molecules result in increases in potency against housefly head membranes, whereas their potency against rat GABARs is unchanged or reduced (Hamano *et al*., 2000). A study using a series of 28 picrotoxane terpenoids concluded that rat GABARs require a spiro-γ as butyrolactone moiety at the 13 position and certain substituents at the 4-position, whereas these are not critical for interactions with *Musca* receptors. The electronegativity of the 16-carbon atom and the presence or absence of the 4- and 8-hydroxyl groups are important determinants of nor-diterpenes for *Musca* GABARs, again suggestive of differences in rat binding sites compared to *M. domestica* (Ozoe *et al*., 1998).

Differences in receptor sites are not the only mechanisms of insecticide selectivity. Fipronil is an interesting case, in that metabolic detoxification contributes positively to insect selectivity. Although selective toxicity of fipronil for insects is at least partly due to its higher affinity for the convulsant site, it is also due to differences in the rate at which it is broken down to its more active sulfone metabolite (Hainzl *et al*., 1998).

5.4.2.9. Single Channel Properties of Insect GABARs

It has proved difficult to obtain patch-clamp recordings of single GABA gated chloride channels of

insects, and this has been attributed to a heterogenous distribution of GABARs over the cell membrane (Pichon and Beadle, 1988). Noise analysis of cultured embryonic cockroach (*P. americana*) brain neurons gave an estimate of single channel conductance of 18.6 pS and mean open time of 11.8 ms (Shimahara *et al.*, 1987). Single channel recordings of GABA gated currents in cultured adult *P. americana* neurons indicated two open conductance states, with single channel conductances of around 17 and 11 pS and two mean open times of around 0.28 and 1.43 ms, as well as three closed states with mean durations of 0.25, 2.22, and 43.7 ms (Malecot and Sattelle, 1990). An RDL splice variant, RDL_{ac}, expressed in a *Drosophila* cell line has single channel conductance of 36 pS. Mean open time distribution was best fitted to two time constants, suggesting that there are two open states (Grolleau and Sattelle, 2000).

5.4.2.10. Distribution

GABARs are present throughout the insect brain (Sattelle, 1990), but this distribution is not uniform. GABAR localization at the tissue level reflects not only behavioral roles, such as in learning and receptive field sharpening, but possibly also its role in development. Fine details of distribution also provide clues to their possible functional roles. For example, GABARs are distributed over the terminals of primary auditory afferents in certain crickets, and are located near output synapses, suggesting that they play a role in presynaptic inhibition (Hardt and Watson, 1999).

A number of studies have indicated particularly dense GABAR expression in brain structures associated with learning, in accord with the observations described below, which point to a central role of GABAergic inhibitory pathways in the consolidation of memory. It has been mentioned above that antibodies to RDL, a *D. melanogaster* GABAR subunit, shows strong immunoreactivity in cockroach, *P. americana* (Harrison *et al.*, 1996) and cricket *A. domesticus* (Strambi *et al.*, 1998) mushroom bodies. Similar results were obtained in the cricket *Gryllus bimaculatus* (Schurmann *et al.*, 2000), and the fly *C. erythrocephala* (Brotz *et al.*, 1997). The three types of GABA immunoreactive neurons in the mushroom bodies of the cockroach (Yamazaki *et al.*, 1998) are described below. We can therefore conclude that high levels of expression of RDL-like GABARs is a common feature of insect mushroom bodies.

In addition to high level GABAR expression, there is evidence that GABAergic intrinsic neurons in the mushroom bodies form distinct compartments. In the fly *C. erythrocephala*, a RDL antibody stains concentrically around an unstained core in the pedunculus and α and β lobes of the mushroom body, whereas the γ-lobes showed a compartmentalized RDL staining. That only some of the neurons, presumably Kenyon cells, should be stained suggests that only some Kenyon cells have GABARs and are therefore non homogeneous (Brotz *et al.*, 1997). Similar compartmentalization is seen in the arborizations of GABAergic input neurons in *G. bimaculatus* and *D. melanogaster* (Schurmann, 2000). The functional significance of this compartmentalization is as yet unknown, but may be related to the finding that each Kenyon cell specifically connects corresponding layers of the calyces and lobes (Grunewald, 1999).

In addition to intrinsic neurons of the mushroom body, certain input and output neurons are also GABAergic (Nishino and Mizunami, 1998), although most of them are cholinergic, at least in *D. melanogaster* and *G. bimaculatus* (Schurmann, 2000). The calycal giants (the input neurons to the Kenyon cells of the mushroom bodies) are GABA immunoreactive (Nishino and Mizunami, 1998). In *D. melanogaster* and *G. bimaculatus*, GABA immunoreactive presynaptic fibers of extrinsic neurons intermingle with nonimmunoreactive fibers in the mushroom bodies (Schurmann, 2000).

There may be close association between GABA expression and expression of nitric oxide (NO) in the insect nervous system. The synthesis of NO is under the control of NADPH-diaphorase. In the locust, almost all GABAergic neurons in the brain were also found to be NADPH-diaphorase positive (Seidel and Bicker, 1997), suggesting that GABA and NO could be cotransmitters.

Distribution of GABAergic neurons is also likely to be determined by developmental constraints. GABA immunoreactive neurons are linearly determined in *M. sexta* (Witten and Truman, 1991). In this species, and probably in eight other insect orders, GABA immunoreactive neurons are restricted to six lineages (Witten and Truman, 1998). Similarly, the approximately 360 GABAergic neurons in the larval midbrain of *T. molitor* form ten distinct clusters. The number of GABAergic somata, but not the number of clusters, increases during development (Wegerhoff, 1999). As NADPH-diaphorase activity in grasshoppers is detected as early as embryonic stage 50%, and since all NADPH-diaphorase positive local interneurons of adult antennal lobe also express GABA immunoreactivity, this is the earliest reported appearance of GABA immunoreactivity in the embryonic antennal lobe (Seidel and Bicker, 2002).

Antibodies to GABA and GABAR associated proteins also stain other parts of the insect CNS that perform roles in sensory and motor processing. In the locust *S. gregaria*, GABA immunoreactivity and GAD immunoreactivity are both observed in about 100 bilateral pairs of tangential neurons that connect the lateral accessory lobes to distinct layers of the central body in the central complex, a structure that plays a role in motor control and visual orientation (Homberg *et al.*, 1999). The brain of the moth *M. sexta* contains some 20 000 GABAergic neurons, most of which are optic lobe neurons (Homberg *et al.*, 1987), although there is also abundant staining in the mushroom bodies (Homberg *et al.*, 1987) and the antennal lobes (Hoskins *et al.*, 1986). In locusts GABA has also been shown to be colocalized with locustatachykinin immunoreactive local interneurons, a distinct subset of antennal lobe neurons (Ignell, 2001). In *D. melanogaster*, the RDL antibody stains the calyces of mushroom bodies, glomeruli of antennal lobes, lower central body and corpora cardiaca, as well as medulla and lobula regions of the optic lobe: regions associated with olfactory, visual, and mechanosensory processing (Harrison *et al.*, 1996). In the same species, immunoreactivity to GABA is reported in the larval antennal lobe and the tritocerebral-subesophageal ganglion. Whereas choline acetyltransferase (ChAT) is expressed only in subsets of olfactory and gustatory afferents, GABA is expressed in most, if not all, local interneurons (Python and Stocker, 2002). Immunostaining for an insect GABA transporter in *M. sexta* closely matches that for GABA, with staining in parts of the optic and antennal lobes, mushroom body, lateral protocerebrum, and central complex (Umesh and Gill, 2002).

GABARs are also present on identified neurons of certain insects, notably the fast coxal depressor motor neuron (D_f) (Sattelle *et al.*, 1988), the GI2 escape neuron of the cockroach *P. americana* (Hue, 1991; Buckingham *et al.*, 1994), and fg1 (a neuron in the frontal ganglion of *M. sexta*), as well as unidentified dorsal unpaired median neurons of *P. americana* (Sattelle *et al.*, 2003).

5.4.2.11. GABARs and Behavior

By 1985 evidence had already accumulated pointing to the roles of GABARs in fast inhibitory responses on giant interneurons that process sensory input in the cockroach *P. americana*, and in auditory processing in the cricket. Recent work has expanded considerably on these findings, as well as added to the list of roles played by GABARs in generating insect behavior.

Demonstrations of the involvement of GABARs in behavior usually consist of descriptions of GABARs or GABAergic neurons in circuits or structures known to be involved in specific behaviors, or reports of effects of GABAergic drugs exerting specific actions on discrete aspects of behavior. Two areas that have received considerable attention over the past 10 years have been insect learning, where GABARs have been shown to be particularly abundant in the antennal lobe and mushroom bodies, and the tuning of sensory encoding, in which GABAergic inhibitory pathways have been shown to sharpen receptive fields or enhance sensory discrimination.

5.4.2.11.1. Insect learning There have been a number of convincing demonstrations that structures in the insect brain known to be involved in the acquisition, consolidation, or retrieval of memory are rich in ionotropic GABARs. RDL antibody stains structures in the brain of the cricket *A. domesticus* that are associated with learning (Strambi *et al.*, 1998). Particularly dense staining was observed in the mushroom bodies (especially the upper part of the peduncle and the two arms of the posterior calyx) and antennal lobe. Subsequent electrophysiological studies have further provided direct evidence that picrotoxinin sensitive GABARs are present on Kenyon cells of *A. domesticus* (Cayre *et al.*, 1999).

Although it is difficult to determine from immunohistochemical data the exact role that GABAergic neurons play in learning, there are indications that one such role is in providing inhibitory feedback. In the honeybee, *A. mellifera*, 50% of the approximately 110 GABA immunoreactive neurons in the mushroom body appear to be feedback neurons (Grunewald, 1999). Here they connect specific layers of the output regions (α and β lobes and pedunculi) with the input regions (the calyces). In the same species, GABA immunoreactive processes were found to synapse onto fine fibers (70%) and large, non-GABAergic boutons (probably from intrinsic and extrinsic mushroom body neurons, respectively) (Ganeshina and Menzel, 2001). These synapses are components of microcircuits, probably involving feed-forward and feedback loops. Similar microcircuits have been identified in the calyx neuropil of the *D. melanogaster* mushroom body (Yasuyama *et al.*, 2002). Here also, extrinsic GABA immunoreactive neurons appear to form a network of fine fibers codistributed with mainly Kenyon cell dendrites, onto which they make divergent synaptic inputs as well as onto boutons of projection neurons. Each microcircuit takes the form of a glomerulus consisting of a large cholinergic bouton at its core, surrounded by tiny, vesicle-free Kenyon cell

dendrites and input from GABA fibers. The pattern of staining suggests that Kenyon cells receive excitatory input from cholinergic cells along with postsynaptic inhibition from GABA cells.

GABAergic pathways appear also to provide substantial input to, and output from, the mushroom bodies. In the cockroach, *P. americana*, Yamazaki and colleagues (Yamazaki *et al.*, 1998) identified three types of GABA immunoreactive neurons in cockroach mushroom body. The first type consisted of a number of neurons (7–9) that ascended from the circumesophageal connective and projected into the calyces, suggesting that they were input neurons. A second type consisted of a larger number of cells (around 40), with dendrites ramifying in the junction between the pedunculus and the lobes, which are thought possibly to be inhibitory input neurons. Finally, a small number of large cells arborize around the alpha-lobe and project into large areas of calyces.

Mushroom bodies of the cockroach are also known to receive inputs from four giant interneurons, the calycal giants. These, too, are immunoreactive to GABA and input directly onto Kenyon cells. They are spontaneously rhythmically active, and this activity is inhibited by olfactory, visual, tactile, and air current stimuli (Nishino and Mizunami, 1998), and so may play a role in sensitizing Kenyon cells to olfactory stimuli through disinhibition.

Thus, inhibitory pathways are important in insect learning, but lack of functional data has so far precluded a detailed analysis of the different roles of GABARs. However, a number of exciting studies suggest that GABAergic circuits might contribute to the establishment of olfactory memory through the coordination of spike timing. For example, GABA affects the synchronous oscillation of intracellular calcium in Kenyon cells in *Drosophila*, an activity thought to be involved in memory consolidation (Rosay *et al.*, 2001). The possible role of GABARs in this activity is discussed in the following section.

5.4.2.11.2. Stimulus encoding and tuning

5.4.2.11.2.1. Odor representation in the antennal lobes Neurons in the antennal lobes of locusts and honeybees respond to certain odor stimuli (**Figure 8**) with synchronized, oscillatory activity (MacLeod and Laurent, 1996; review: Kauer, 1998). There is ample evidence that GABARs play a key role in the formation of this synchronization. For instance, the injection of GABA antagonists into the first olfactory relay neuropil of locust abolishes synchronization of odor specific neural assemblies in the antennal lobe (MacLeod and Laurent, 1996). Significantly, the temporal response patterns of individual neurons remain unaffected, even though such patterns include some hyperpolarization. Thus, this action of fast, ionotropic GABARs is specific to the formation of synchronization. Similar findings have been reported for the honeybee (review: Kauer, 1998).

The use of optical imaging techniques, such as calcium imaging, and the use of fluorescent, voltage sensitive dyes, allows the activity of large numbers of cells to be monitored simultaneously. In the honeybee, *A. mellifera*, spatiotemporal odor response patterns observed in olfactory glomeruli of the antennal lobes in response to odor stimuli can be mapped to identified glomeruli. By examining the effects of GABA or PTX injection in the antennal lobe, it can be shown that there exist two separate inhibitory networks that render such response patterns more confined, i.e., they enhance their spatial contrast (Sachse and Galizia, 2002). One of these networks is GABAergic and modulates overall antennal lobe activity, and the other is PTX insensitive and glomerulus specific. Confirmation that GABAergic connections underlie such synchronized oscillations is provided by computer simulations. For example, simulations show that GABA synapses forming inhibitory connections between local interneurons and projection interneurons of the antennal lobe allow the formation of dynamic assemblies of neurons, which oscillate together and synchronize transiently (Bazhenov *et al.*, 2001a). Thus, GABA-ergic synapses coordinate the activity of a large number of neurons to produce rhythmic, coordinated firing.

5.4.2.11.2.2. Fine-tuning of odor representation In many insect sensory systems, lateral inhibition serves the role of sharpening stimuli and enhancing the contrast of stimulus representation. In the antennal lobe, odor identity and duration of stimulation are represented both spatially, in the pattern of activity in antennal lobe glomeruli, and temporally, in the pattern of activity in groups of neurons. Spatial encoding is seen in response to electrical stimulation of the antennal nerve in the silkworm moth, *B. mori*, which is followed by postsynaptic depolarizations in the antennal lobe. These depolarizations can be monitored using optical recordings with voltage sensitive dyes (Ai *et al.*, 1998). GABA mediated IPSPs occur in the antennal lobe within 3 ms of these postsynaptic depolarizations, and these IPSPs, like the depolarizations, are not uniform over the antennal lobe, suggesting that the pattern of excitation is specific. GABAergic IPSPs also occur in the macroglomerular complex and some ordinary glomeruli in the antennal lobe. Recent evidence using computer simulations suggests that in the locust

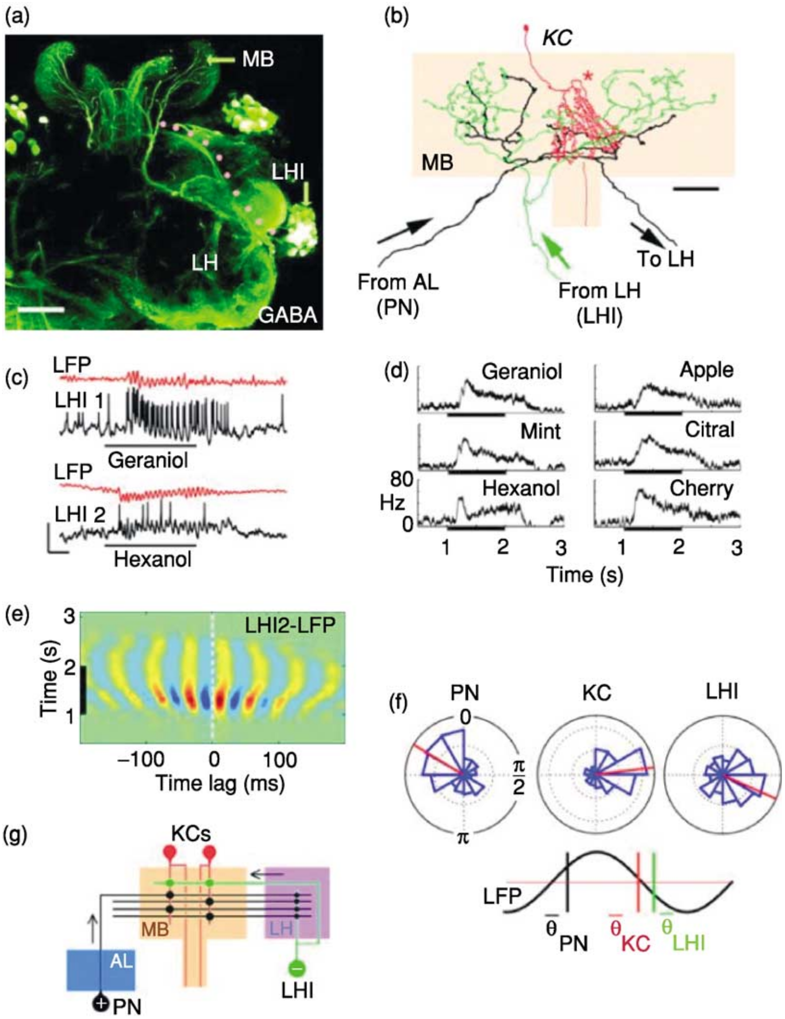


Figure 8 Feed-forward inhibition of KCs by LHIs. (a) Immunolabeling by antibody to GABA (37). Cluster of about 60 reactive somata (LHI) and tract of LHI axons running to the MB (stipples) are shown. The terminals of one of these axons in the MB are shown in (b). Scale bar = 100 μ. (b) PN axon (black) projects to the MB calyx (orange) and to the lateral horn (LH) (41). LHI axon (green) projects to the calyx (this study). PN and LHI axons terminate on KC dendrites (red). Neurons were stained by iontophoresis of cobalt hexamine (KC, PN) or neurobiotin (LHI) in separate preparations and were drawn with a camera lucida. Note varicosities in LHI and PN axon collaterals. Asterisk, KC axon. Scalebar = 50 μ. (c) Representative odor evoked responses of two LHIs and simultaneously recorded LFPs (5 to 40 Hz bandpass). Note membrane potential oscillations, locked to the LFP. Identity and delivery (1 s long) of stimulus indicated by black bar. LHI, 20 mV; LFP, 400 μV; 200 ms. (d) Instantaneous firing rate of LHI1 (in (C)) in response to various odors. Lower edge of profile shows mean instantaneous rate averaged across trials; profile thickness, SD. All LHIs responded to all odors tested, with response profiles that varied little across different odors. (e) Sliding cross-correlation between LFP and LHI2 traces (spikes clipped). Red, maxima; blue, minima. Strong locking is present throughout the response (odor delivery, vertical bar). Lower edge of correlation stripes just precedes stimulus onset due to width of the correlation window (200 ms). (f) Phase relationships between PN, KC, and, LHI action potentials, and LFP. (Upper) Polar plots. LFP cycle maxima defined as 0 rad, minima as π rad (PNs: 3 cell-odor pairs, 388 spikes; LHIs: 17 cell-odor pairs, 2632 spikes; KCs: 18 cells, 862 spikes). Mean phases are shown in red. Gridlines are scaled in intervals of 0.10 (probability per bin). (Lower) Schematic diagram showing LFP and mean firing phases. (g) Circuit diagram. (Reprinted with permission from Perez-Orive, J., Mazor, O., Turner, G. C., Cassenaer, S., Wilson, R.I., **2002**. Oscillations and sparsening of odor representations in the mushroom body. *Science 297*(5580), 359–365.)

these characteristic spatial patterns of activity seen in response to odor stimuli arise from competition among neurons with GABAergic outputs. GABAergic interconnections between local interneurons create competition between them that results in coordinated, phase-locked activity in separate groups of neurons. Thus, GABARs contribute to spatial and temporal patterns of activity that encode the stimulus (Bazhenov *et al.*, 2001b).

In addition to spatial representation of the odor stimulus, temporal synchronization plays a role in odor discrimination, as well as in memory. In

honeybees (Stopfer *et al*., 1997; Hosler *et al*., 2000), block of odor evoked oscillatory synchronization by PTX results in an impairment of discrimination of similar, but not that of dissimilar, odors. This points to a role of synchronization in sharpening odor discrimination.

The circuitry by which GABAergic neurons perform this is unknown. However, in the antennal lobe of the cockroach, *P. americana*, GABAergic neurons form connections that are suggestive of both feedforward and feedback inhibition (Distler *et al*., 1998). In cockroach antennal lobe glomeruli, a major processing center in olfaction, GABAergic local interneurons mediate inhibitory feedback connections onto both antennal receptor neurons and uniglomerular projection neurons. Each GABAergic neuron innervates a large number of antennal lobe glomeruli, thus potentially passing information to neighboring glomeruli (Distler and Boeckh, 1997, 1998). It is presumably these connections between local interneurons and excitatory projection neurons that create the coordinated, distributed oscillations in response to odor stimulation, which, in locusts at least, encode odor identity.

In addition to the identity of an odor stimulus, antennal lobe neurons also encode the duration of the odor. Hence, continuous application of an odor evokes a complex wave of depolarization and hyperpolarization, whereas a pulsatile stimulus evokes a simple train of spikes (Christensen *et al*., 1998). Here again, GABARs play a key role. GABA evoked IPSPs in *M. sexta* projection neurons of the antennal lobe resemble not only electrically evoked IPSPs, but also odor evoked IPSPs. For example, both GABA and odor evoked IPSPs are blocked by bicuculline. At the same time, administration of bicuculline also reversibly alters the temporal pattern of odor evoked activity in the projection neurons, providing circumstantial evidence that GABARs help shape the temporal pattern of activity (Christensen *et al*., 1998). This may also indicate a particular role for the bicuculline sensitive subtype of GABAR.

5.4.2.11.2.3. Involvement of GABA inputs in fine-tuning of sensory pathways may be a general phenomenon In a manner reminiscent of the sharpening of the spatial patterns of odor responses described above, GABAergic inputs appear to be responsible for fine tuning of sensory inputs in other sensory systems. An identified auditory neuron, AN1, of the bushcricket, *Ancistrura nigrovittata*, is finely tuned to the fundamental frequency of the male song. GABA input to this neuron greatly sharpens its selectivity for the fundamental frequency, severely attenuating subthreshold inputs in response to lower, and more so to higher, frequency sounds (Stumpner, 1998). This effect is highly specific in that other inputs through other neurotransmitters are responsible for side and frequency dependent inhibitions.

A similarly striking example is seen in the escape behavior of the cockroach, *P. americana*. Escape responses to wind stimuli are mediated by giant interneurons (Levi and Camhi, 2000), some of which respond selectively to wind from a particular direction, while others are omnidirectional (Buno *et al*., 1981; Westin *et al*., 1988; Okumah and Kondoh, 1996). Those that show a pronounced directional sensitivity respond to wind in a linear manner whereas those that are omnidirectional in their sensitivity respond nonlinearly. The synaptic input onto these cells results in a depolarization followed by a delayed hyperpolarization. This hyperpolarization was blocked by PTX , resulting in an altered response of an identified neuron (Neuron 101) from linear to nonlinear (Okumah and Kondoh, 1996). Hence, GABAergic synapses contribute to the transfer function of these neurons. Furthermore, Hill and Blagburn (1998) demonstrated that the receptive fields of GI6 and GI7 (which means the directional sensitivity) are sharpened by GABA mediated inputs.

GABARs on the terminals of afferent fibers probably also play a major role in the modulation of sensory input through presynaptic inhibition. In the locust, presynaptic inhibition of tegula afferents mediated through GABARs modulates the amplitude of postsynaptic potentials in the hind-wing motor neurons in this monosynaptic pathway in a phase dependent manner (Buschges and Wolf, 1999). GABARs also mediate inhibition by identified filiform hair receptors of an identified projection interneurone in a circuit that mediates wind elicited responses (Gauglitz and Pfluger, 2001). In addition, GABA ergic neurons modulate transmitter release at synapses between the fore-wing stretch receptor and wing depressor motor neurons of *L. migratoria* (Judge and Leitch, 1999). Consistent with this role is the observation that GABARs are distributed over the terminals of primary auditory afferents in certain crickets, and are located near output synapses, again suggesting that they play a role in presynaptic inhibition (Hardt and Watson, 1999).

Clearly, then, GABARs of insects probably play major roles in sharpening receptive fields of various sensory systems, as well as acting at "higher" sensory integration centers in aiding stimulus discrimination and the consolidation of memory.

5.4.2.11.3. Other behaviors involving GABARs While attention has already been drawn to the role for GABARs (presumably including the cloned *Drosophila* GABAR subunit, RDL) in memory

consolidation, the pattern of staining of cockroach brains with an RDL antibody also suggests a wider involvement with olfactory, visual, and mechanosensory processing (Sattelle *et al.*, 2000). In the locust, *S. gregaria*, GABA and GAD immunoreactivity is observed in about 100 bilateral pairs of tangential neurons that connect the lateral accessory lobes to distinct layers of the central body in the central complex, a structure that plays a role in motor control and visual orientation (Homberg *et al.*, 1999).

GABA is not only present in the insect nervous system, but also occurs in the periphery. GABARs have been demonstrated on skeletal muscle of cockroach (Schnee *et al.*, 1997), and those present on the flexor tibiae muscle of locust have received considerable attention (Cull-Candy and Miledi, 1981; Cull-Candy, 1982). GABA also plays a role in visceral functions. For instance, GABA decreases the heart rate of pupal *Drosophila*, although curiously it has no effect upon Hear rate of larval or adult flies (Zornik *et al.*, 1999). GABAergic neurons inhibit the prothoracic gland of *P. americana* up to the 17th day of the molt cycle, after which cessation of their input elicits competence of the prothoracic gland through disinhibition (Richter and Bohm, 1997).

Recent evidence also points to a role of GABAergic neurons in higher levels of behavior. GABA-immunoreactive neurons connect the noduli of accessory medulla to the medulla and to the lamina via processes in the distal tract of the cockroach, *Leucophaea maderae*. Interestingly, the accessory medulla of *D. melanogaster* and of *L. maderae* is thought to be the location of the circadian pacemaker. Injection of GABA into the vicinity of the accessory medulla resets the circadian motor activity of the cockroach in a stable, phase-dependent manner, suggesting that, along with Mas-allatotropin, GABA plays a role in circuits relaying photic information from circadian photoreceptors to the central pacemaker (Petri *et al.*, 2002).

Roles of GABARs in behavior can also be gauged indirectly through alterations in GABA transporter function. Disrupting GABA transporter function *in vivo*, thereby prolonging the effects of GABA at the synapse, in adult female *D. melanogaster* reduced locomotor activity, disrupted geotaxis, and induced convulsions with secondary loss of locomotor activity, without damaging feeding activity or female sexual receptivity (Leal and Neckameyer, 2002).

5.4.3. Metabotropic GABARs of Insects

Very little is known of insect $GABA_B$ (metabotropic) receptors, largely because baclofen is ineffective in many invertebrate preparations (Sattelle *et al.*, 1988; Benson, 1989). However, $GABA_B$-like responses were observed in giant interneurons of the terminal abdominal ganglion of the cockroach (Hue, 1991). Similarly, the motor neuron, D_f, of the same species was thought not to possess $GABA_B$ receptors, until it was shown that responses to $GABA_B$ receptor agonists could be observed when the ionotropic GABARs are blocked by PTX (Bai and Sattelle, 1995). These responses, which appeared to be mediated by a potassium conductance, differed in their pharmacology from vertebrate $GABA_B$ receptors in their insensitivity to baclofen (a property shared by $GABA_B$ responses in the cockroach GI2 and reduced sensitivity to the highly potent vertebrate $GABA_B$ agonist, SK&F97541. It was also shown recently that cultured Kenyon cells of *A. domesticus* possess $GABA_B$ receptors, in addition to PTX sensitive receptors (Cayre *et al.*, 1999). This was soon followed by the cloning, sequencing, and functional expression of $GABA_B$-like receptors from *D. melanogaster.*

5.4.3.1. A Cloned Insect Metabotropic ($GABA_B$) Receptor

Three second-messenger linked, $GABA_B$-like receptors have been cloned from *Drosophila* to date (Mezler *et al.*, 2001). Two of them have high sequence similarity to mammalian $GABA_BR1$ and $GABA_BR2$, whereas the third has no known mammalian counterpart. All three are expressed in the embryonic nervous system, and two are expressed in similar regions and may coexpress *in vivo* to form the functional receptor. R1 and R2 coexpressed in oocytes produce receptors with pharmacology dissimilar to vertebrates in that they are insensitive to baclofen. R3, however, did not produce functional receptors in any combination. These *Drosophila* $GABA_B$ receptors have similar intron positions and exon size to human $GABA_BRs$ (Martin *et al.*, 2001). More detailed physiological, pharmacological, and immunohistochemical experiments are needed in order to establish functional roles for these receptors.

5.4.4. Conclusions

With the cloning of ionotropic insect GABARs, along with detailed immunohistochemical studies and improved optical recording techniques, we have seen remarkable advances in our understanding of insect GABARs over the past 15 years. In addition, the recent cloning of metabotropic GABARs opens up a vast new area of research aimed at establishing the functional role of these receptors in the insect nervous system.

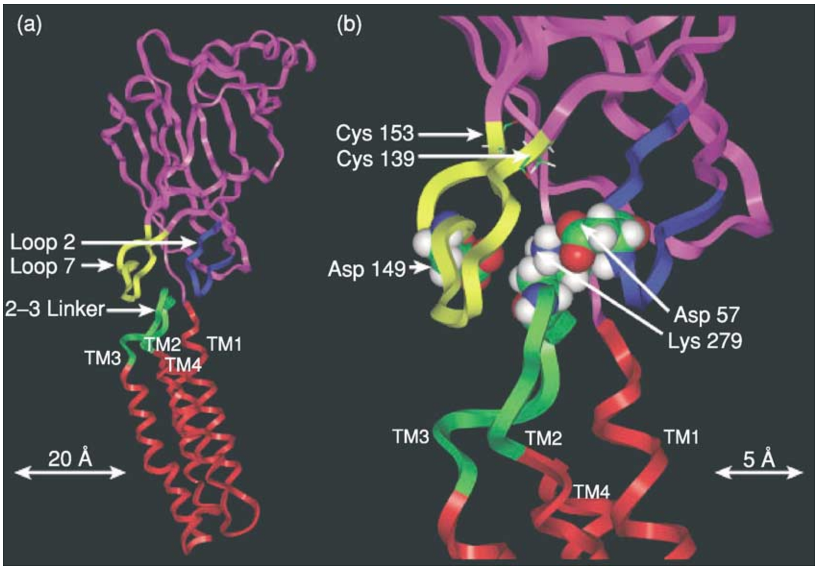


Figure 9 A molecular model of the $GABA_A$ receptor α1 subunit: (a) a model of a whole $GABA_A$ receptor α1 subunit viewed from the center of the ion channel; (b) detailed view of potential contacts between the extracellular and transmembrane domains. (From Kash, T.L., Jenkins, A., Kelley, J.C., Trudell, J.R., Harrison, N.L., **2003**. Coupling of agonist binding to channel gating in the GABAA receptor. *Nature 421*, 272–274.)

Our survey of the more recent findings in the area of insect GABARs leads us to suggest that these receptors are more complex than once was thought, that they form a class distinct from any vertebrate ionotropic GABAR, and that several distinct subtypes exist. Also, a single subunit, RDL, has both alternative splicing and RNA editing. It appears that GABARs play important roles, through inhibition, in stimulus discrimination, receptive field sharpening and memory formation.

Future investigations into this exciting class of neurotransmitter receptors will undoubtedly benefit from the developments in genomics and proteomics to facilitate the study of coexpressed genes and interacting gene products. Other areas that require further attention include the role played by GABARs in insect development, and the specific roles of GABAR subunit variants. The highly tractable development patterns of insects are likely to provide key insights into these strategic questions.

The recently developed technique of double-stranded RNA interference (RNAi) offers some exciting opportunities for future research into insect GABARs. For instance, this approach could be used to assess the roles of GRD and LCCH3, as well as probing further the role of RDL in synaptic transmission, insect learning, sensory tuning, and the circadian clock.

The recent crystal structure of the acetylcholine binding protein (Brjck *et al.*, 2001) now permits homology modeling of the N-terminal domain of a variety of cys-loop neurotransmitter receptors, including insect GABARs. In the future, it will be of interest to attempt molecular modeling of those parts of the RDL extracellular N-terminal domain (see Figure 9) that contribute to the agonist binding sites. This would permit elucidation of the mechanisms by which ligands dock to the four alternative splice variants of RDL to be compared, and introduces the possibility of undertaking parallel site-directed and *in silico* mutagenesis studies. Such a model may be of considerable use in the rational design of a future generation of insecticides incorporating both an improved level of safety and a reduced level of impact on nontarget species.

References

Abalis, I.M., Eldefrawi, F.E., Eldefrawi, A.T., **1983**. Biochemical identification of putative GABA/benzodiazepine receptors in house fly thorax. *Pestic. Biochem. Physiol. 20*, 39–48.

Ai, H., Okada, K., Hill, E.S., Kanzaki, R., **1998**. Spatiotemporal activities in the antennal lobe analyzed by an optical recording method in the male silkworm moth *Bombyx mori*. *Neurosci. Lett. 24*, 135–138.

Alix, P., Grolleau, F., Hue, B., **2002**. Ca^{2+}/calmodulin-dependent protein kinase regulates GABA-activated Cl^- current in cockroach dorsal unpaired median neurons. *J. Neurophysiol. 87*, 2972–2982.

Andreev, D., Rocheleau, J., Phillips, T.W., Beeman, R., ffrench-Constant, R.H., **1994**. A PCR diagnostic for cyclodiene insecticide resistance in the red flour beetle, *Tribolium castaneum*. *Pestic. Sci. 41*, 345–349.

Andreev, D., Kreitman, M., Phillips, T.W., Beeman, R.W., ffrench-Constant, R.H., **1999**. Multiple origins of

cyclodiene insecticide resistance in *Tribolium castaneum* (Coleoptera: Tenebrionidae). *J. Mol. Evol. 48*, 615–624.

Anthony, N.M., Holyoke, C.W., Sattelle, D.B., **1993**. Actions of picrotoxin analogues on a chick optic lobe $GABA_A$ receptor expressed in *Xenopus* oocytes. *Mol. Pharmacol. 3*, 63–67.

Anthony, N.M., Holyoke, C.W., Sattelle, D.B., **1994**. Blocking actions of picrotoxin analogues on insect (*Periplaneta americana*) GABARs. *Neurosci. Lett. 171*, 67–69.

Anthony, N., Unruh, T., Ganser, D., ffrench-Constant, R., **1998**. Duplication of the Rdl GABAR subunit gene in an insecticide-resistant aphid, *Myzus persicae*. *Mol. Gen. Genet. 260*, 165–175.

Araujo, F., Tan, S., Ruano, D., Schoemaker, H., Benavides, J., *et al.*, **1996**. Molecular and pharmacological characterization of native cortical gamma-aminobutyric $acid_A$ receptors containing both alpha1 and alpha3 subunits. *J. Biol. Chem. 271*, 27902–27911.

Aronstein, K., ffrench-Constant, R., **1995**. Immunocytochemistry of a novel GABAR subunit Rdl in *Drosophila melanogaster. Invert. Neurosci. 1*, 25–31.

Aronstein, K., Auld, V., ffrench-Constant, R., **1996**. Distribution of two GABAR-like subunits in the *Drosophila* CNS. *Invert. Neurosci. 2*, 115–120.

Bai, D., **1994**. Neurotransmitter Receptor Subtypes on Identifiable Insect Neurons. Ph.D. thesis. University of Cambridge.

Bai, D., Sattelle, D.B., **1995**. A $GABA_B$ receptor on an identified insect motor neurone. *J. Exp. Biol. 198*, 889–894.

Bazhenov, M., Stopfer, M., Rabinovich, M., Huerta, R., Abarbanel, H.D., *et al.*, **2001a**. Model of transient oscillatory synchronization in the locust antennal lobe. *Neuron 30*, 553–567.

Bazhenov, M., Stopfer, H., Rabinovich, M., Abarbanel, H.D., Sejnowski, T.J., *et al.*, **2001b**. Model of cellular and network mechanisms for odor-evoked temporal patterning in the locust antennal lobe. *Neuron 30*, 569–581.

Belelli, D., Hope, A.G., Callachan, H., Hill-Venning, C., Peters, L.A., *et al.*, **1995**. A mutation in the putative M2 domain of a *Drosophila* GABAR subunit differentially affects antagonist potency. *Br. J. Pharmacol. 116*, 442.

Belelli, D., Callachan, H., Hill-Venning, C., Peters, J.A., Lambert, J.J., **1996**. Interaction of positive allosteric modulators with human and *Drosophila* recombinant GABARs expressed in *Xenopus laevis* oocytes. *Br. J. Pharmacol. 118*, 563–576.

Benson, J.A., **1988**. Bicuculline blocks the response to acetylcholine and nicotine but not to muscarine or GABA in isolated insect neuronal somata. *Brain Res. 458*, 45–71.

Benson, J.A., **1989**. A novel GABAR in the heart of a primitive arthropod, *Limulus polyphemus*. *J. Exp. Biol. 147*, 421–438.

Bentley, R., Trimen, H., **1875**. *Anamirta paniculata*. In: Medicinal Plants, Being Descriptions With Original Figures of the Principal Plants Employed in Medicine and an Account of their Properties and Uses, Part 2, Item 14. J and A Churchill, London, p. 5.

Bermudez, I., Hawkins, C.A., Taylor, A.M., Beadle, D.J., **1991**. Actions of insecticides on the insect GABAR complex. *J. Recept. Res. 11*, 221–232.

Betz, H., Kuhse, J., Schmieden, V., Laube, B., Kirsch, J., *et al.*, **1999**. Structure and functions of inhibitory and excitatory glycine receptors. *Ann. New York Acad. Sci. 868*, 667–676.

Bloomquist, J.R., **1996**. Ion channels as targets for insecticides. *Annu. Rev. Entomol. 41*, 163–190.

Bonnert, T.P., McKernan, R.M., Farrar, S., le Bourdelles, B., Heavens, R.P., *et al.*, **1999**. Theta, a novel gamma-aminobutyric acid type A receptor subunit. *Proc. Natl Acad. Sci. USA 96*, 9891–9896.

Bormann, J., **2000**. The 'ABC' of GABARs. *Trends Pharmacol. Sci. 21*, 16–19.

Brejc, K., van Dijk, W.J., Klaassen, R.V., Schuurmans, M., van Der Oost, J., *et al.*, **2001**. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature 411*, 269–276.

Brotz, T.M., Borst, A., **1996**. Cholinergic and GABAergic receptors on fly tangential cells and their role in visual motion detection. *J. Neurophysiol. 76*, 1786–1799.

Brotz, T.M., Bochenek, B., Aronstein, K., ffrench-Constant, R.H., Borst, A., **1997**. Gamma-aminobutyric acid receptor distribution in the mushroom bodies of a fly (*Calliphora erythrocephala*): a functional subdivision of Kenyon cells? *J. Comp. Neurol. 23*, 42–48.

Buchner, E., Bader, R., Buchner, S., Cox, J., Emson, P.C., *et al.*, **1988**. Cell specific immunoprobes for the brain of normal and mutant *Drosophila melanogaster.* I. Wild-type visual system. *Cell Tissue Res. 253*, 357–370.

Buckingham, S.D., Hue, B., Sattelle, D.B., **1994**. Actions of bicuculline on cell body and neuropilar membranes of identified insect neurones. *J. Exp. Biol. 186*, 235–244.

Buckingham, S.D., Matsuda, K., Hosie, A.M., Baylis, H.A., Squire, M.D., *et al.*, **1996**. Wild-type and insecticide-resistant homo-oligomeric GABARs of *Drosophila melanogaster* stably expressed in a *Drosophila* cell line. *Neuropharmacology 35*, 1393–1401.

Buno, W. Jr, Crispino, L., Monti-Bloch, L., Mateos, A., **1981**. Dynamic analysis of cockroach giant interneuron activity evoked by forced displacement of cercal thread-hair sensilla. *J. Neurobiol. 12*, 561–578.

Buschges, A., Wolf, H., **1999**. Phase-dependent presynaptic modulation of mechanosensory signals in the locust flight system. *J. Neurophysiol. 81*, 959–962.

Callec, J.J., **1974**. Synaptic transmission in the central nervous system of insects. In: Treherne, J.E. (Ed.), Insect Neurobiology. Amsterdam, Elsevier/North Holland, pp. 119–185.

Callec, J.J., Boistel, J., **1971**. Role possible du GABA comme mediateur inhibiteur du systeme de fibres geantes chez la blatte (*Periplaneta americana*). *J. Physiol. Paris 63*, 119A.

Casida, J.E., **1993**. Insecticide action at the GABA-gated chloride channel: Recognition, progress, and prospects. *Arch. Insect Biochem. Physiol. 22*, 13–23.

Casida, J.E., Nicholson, R.A., Palmer, C.J., **1988**. Trioxabicyclooctanes as probes for the convulsant site of the GABA-gated chloride channel in mammals and arthropods. In: Lunt, G.G. (Ed.), Neurotox 88: Molecular Basis of Drug and Insecticide Action. Elsevier, New York, pp. 125–144.

Cayre, M., Buckingham, S.D., Yagodin, S., Sattelle, D.B., **1999**. Cultured insect mushroom body neurons express functional receptors for acetylcholine, GABA, glutamate, octopamine, and dopamine. *J. Neurophysiol. 81*, 1–14.

Chen, R., Belelli, D., Lambert, J.J., Peters, J.A., Reyes, A., *et al.*, **1994**. Cloning and functional expression of a *Drosophila* gamma-aminobutyric acid receptor. *Proc. Natl Acad. Sci. USA 91*, 6069–6073.

Christensen, T.A., Waldrop, B.R., Hildebrand, J.G., **1998**. Multitasking in the olfactory system: context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *J. Neurosci. 18*, 5999–6008.

Cole, L.M., Casida, J.E., **1992**. GABA-gated chloride-channel binding site for 4′-ethynyl-4-normal-[2,3-h-3(2)]propylbicycloorthobenzoate ([^{3}H]EBOB) in vertebrate brain and insect head. *Pestic. Biochem. Physiol. 44*, 1–8.

Cole, L.M., Nicholson, R.A., Casida, J.E., **1993**. Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pestic. Biochem. Physiol. 46*, 47–54.

Cole, L.M., Roush, R.T., Casida, J.E., **1995**. *Drosophila* GABA-gated chloride channel: Modified [^{3}H]EBOB binding site associated with Ala→ Ser or Gly mutants of Rdl subunit. *Life Sci. 56*, 757–767.

Cull-Candy, S.G., **1982**. Properties of postsynaptic channels activated by glutamate and GABA in locust muscle fibers. In: Evered, D., O'Connor, M., Whelan, J. (Eds.), Neuropharmacology of Insects. Ciba Foundation Symposium, Vol. 88. Pitman, London, pp. 70–82.

Cull-Candy, S.G., Miledi, R., **1981**. Junctional and extrajunctional membrane channels activated by GABA in locust muscle fibers. *Proc. R. Soc. Lond. B 211*, 527–535.

Curtis, D.R., Duggan, A.W., Felix, D., Johnston, G.A., **1971**. Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. *Brain Res. 32*(1), 69–96.

Deglise, P., Grunewald, B., Gauthier, M., **2002**. The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. *Neurosci. Lett. 324*, 86.

Deng, Y., Casida, J.E., **1992**. House fly head GABA-gated chloride channel: toxicologically relevant binding site for avermectins coupled to site for ethynylbicycloorthobenzoate. *Pestic. Biochem. Physiol. 43*, 116–122.

Deng, Y., Palmer, C.H., Casida, J.E., **1991**. House fly brain γ-aminobutyric acid-gated chloride channel: target for multiple classes of insecticides. *Pestic. Biochem. Physiol. 41*, 60–65.

Deng, Y., Palmer, C.J., Casida, J.E., **1993**. House fly head GABA-gated chloride channel: four putative insecticide binding sites differentiated by [^{3}H]EBOB and [^{35}S]TBPS. *Pestic. Biochem. Physiol. 47*, 98–112.

DiAntonio, A., Burgess, R.W., Chin, A.C., Deitcher, D.L., Scheller, R.H., *et al.*, **1993**. Identification and characterization of *Drosophila* genes for synaptic vesicle proteins. *J. Neurosci. 13*, 4924–4935.

Distler, P.G., Boeckh, J., **1997**. Synaptic connections between identified neuron types in the antennal lobe glomeruli of the cockroach, *Periplaneta americana*: II. Local multiglomerular interneurons. *J. Comp. Neurol. 383*, 529–540.

Distler, P.G., Boeckh, J., **1998**. An improved model of the synaptic organization of insect olfactory glomeruli. *Ann. New York Acad. Sci. 855*, 508–510.

Distler, P.G., Gruber, C., Boeckh, J., **1988**. Synaptic connections between GABA-immunoreactive neurons and uniglomerular projection neurons within the antennal lobe of the cockroach, *Periplaneta americana*. *Synapse 29*, 1–13.

Djamgoz, M.B.A., **1995**. Diversity of GABARs in the vertebrate outer retina. *Trends Neurosci. 18*, 118–120.

Edwards, M.D., Lees, G., **1997**. Modulation of a recombinant invertebrate gamma-aminobutyric acid receptor-chloride channel complex by isoflurane: effects of a point mutation in the M2 domain. *Br. J. Pharmacol. 122*, 726–732.

ffrench-Constant, R.H., Rocheleau, T.A., **1992**. *Drosophila* cyclodiene resistance gene shows conserved genomic organization with vertebrate γ-aminobutyric acidA receptors. *J. Neurochem. 59*, 1562–1565.

ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., Chalmers, A.E., **1993b**. A point mutation in a *Drosophila* GABAR confers insecticide resistance. *Nature 363*, 449–451.

ffrench-Constant, R.H., Steichen, J.C., Rocheleau, T.A., Aronstein, K., Roush, R.T., **1993a**. A single-amino acid substitution in a γ-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in *Drosophila* populations. *Proc. Natl Acad. Sci. USA 90*, 1957–1961.

ffrench-Constant, R.H., Roush, R.T., **1991**. Gene mapping and cross resistance in cyclodiene insecticide resistant *Drosophila melanogaster* (Mg). *Genet. Res. 57*, 17–21.

ffrench-Constant, R.H., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J., Roush, R.T., **1991**. Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: An invertebrate γ-aminobutyric acid subtype A receptor locus. *Proc. Natl Acad. Sci. USA 88*, 7209–7213.

ffrench-Constant, R.H., Anthony, N., Aronstein, K., Rocheleau, T., Stilwell, G., **2000**. Cyclodiene insecticide resistance: from molecular to population genetics. *Annu. Rev. Entomol. 45*, 449–466.

Ganeshina, O., Menzel, R., **2001**. GABA-immunoreactive neurons in the mushroom bodies of the honeybee: an electron microscopic study. *J. Comp. Neurol. 437*, 335–349.

Gauglitz, S., Pfluger, H.J., **2001**. Cholinergic transmission via central synapses in the locust nervous system. *J. Comp. Physiol. A 187*, 825–836.

Georghiou, G.P., **1986**. The magnitude of the resistance problem. In: Pesticide Resistance: Strategies and Tactics for Management. National Academy Press, Washington, DC, pp. 14–43.

Giles, D.P., Usherwood, P.N.R., **1985**. The effect of putative amino acid neurotransmitters on somata isolated from neurons of the locust central nervous system. *Comp. Biochem. Physiol. C 80*, 231–236.

Gonzalez-Coloma, A., Valencia, F., Martin, N., Hoffmann, J.J., Hutter, L., *et al.*, **2002**. Silphinene sesquiterpenes as model insect antifeedants. *J. Chem. Ecol. 28*, 117–129.

Goodman, C.S., Spitzer, N.C., **1980**. Embryonic development of neurotransmitter receptors in grasshoppers. In: Sattelle, D.B., Hall, L.M., Hildebrand, J.G. (Eds.), Receptors for Neurotransmitters, Hormones and Pheromones in Insects. Amsterdam, Elsevier/North-Holland, pp. 195–209.

Grolleau, F., Sattelle, D.B., **2000**. Single channel analysis of the blocking actions of BIDN and fipronil on a *Drosophila melanogaster* GABAR (RDL) stably expressed in a *Drosophila* cell line. *Br. J. Pharmacol. 130*, 1833–1842.

Grunewald, B., **1999**. Morphology of feedback neurons in the mushroom body of the honeybee, *Apis mellifera*. *J. Comp. Neurol. 404*, 114–126.

Hainzl, D., Cole, L.M., Casida, J.E., **1998**. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem. Res. Toxicol. 11*, 1529–1535.

Hamano, H., Nagata, K., Fukada, T.N., Shimotahira, H., Ju, X.L., *et al.*, **2000**. 5-[4-(3,3-Dimethylbutoxycarbonyl)phenyl]-4-pentynoic acid and its derivatives inhibit ionotropic gamma-aminobutyric acid receptors by binding to the 4′-ethynyl-4-n-propylbicycloorthobenzoate site. *Bioorg. Med. Chem. 8*, 665–674.

Hardt, M., Watson, A.H., **1999**. Distribution of input and output synapses on the central branches of bushcricket and cricket auditory afferent neurones: immunocytochemical evidence for GABA and glutamate in different populations of presynaptic boutons. *J. Comp. Neurol. 403*, 281–294.

Harrison, J.B., Chen, H.H., Sattelle, E., Barker, P.J., Huskisson, N.S., *et al.*, **1996**. Immunocytochemical mapping of a C-terminus anti-peptide antibody to the GABAR subunit, RDL in the nervous system in *Drosophila melanogaster. Cell Tissue Res. 284*, 269–278.

Harvey, R.J., Schmitt, B., Hermans-Borgmeyer, I., Gundelfinger, E.D., Betz, H., *et al.*, **1994**. Sequence of a *Drosophila* ligand-gated ion-channel polypeptide with an unusual amino-terminal extracellular domain. *J. Neurochem. 62*, 2480–2483.

Hedblom, E., Kirkness, E.F., **1997**. A novel class of $GABA_A$ receptor subunit in tissues of the reproductive system. *J. Biol. Chem. 272*, 15346–15350.

Henderson, J.E., Soderlund, D.M., Knipple, D.C., **1993**. Characterization of a putative γ-aminobutyric acid (GABA) receptor *β* subunit gene from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 193*, 474–482.

Henderson, J.E., Knipple, D.C., Soderlund, D.M., **1994**. PCR-based homology probing reveals a family of GABAR-like genes in *Drosophila melanogaster. Insect Biochem. Mol. Biol. 24*, 363–371.

Hill, E.S., Blagburn, J.M., **1998**. Indirect synaptic inputs from filiform hair sensory neurons contribute to the receptive fields of giant interneurons in the first-instar cockroach. *J. Comp. Physiol. A 183*, 467–476.

Hold, K.M., Sirisoma, N.S., Ikeda, T., Narahashi, T., Casida, J.E., **2000**. Alpha-thujone (the active component of absinthe): gamma-aminobutyric acid type A receptor modulation and metabolic detoxification. *Proc. Natl Acad. Sci. USA 97*, 4417–4418.

Hold, K.M., Sirisoma, N.S., Casida, J.E., **2001**. Detoxification of alpha- and beta-Thujones (the active ingredients of absinthe): site specificity and species differences in cytochrome P450 oxidation *in vitro* and *in vivo*. *Chem. Res. Toxicol. 14*, 589–595.

Homberg, U., Kingan, T.G., Hildebrand, J.G., **1987**. Immunocytochemistry of GABA in the brain and suboesophageal ganglion of *Manduca sexta*. *Cell Tissue Res. 248*, 1–24.

Homberg, U., Vitzthum, H., Muller, M., Binkle, U., **1999**. Immunocytochemistry of GABA in the central complex of the locust *Schistocerca gregaria*: identification of immunoreactive neurons and colocalization with neuropeptides. *J. Comp. Neurol. 409*, 495–507.

Hosie, A.M., Aronstein, K., Sattelle, D.B., ffrench-Constant, R.H., **1997**. Molecular biology of insect neuronal GABA receptors. *Trends Neurosci. 20*, 578–583.

Hosie, A.M., Baylis, H.A., Buckingham, S.D., Sattelle, D.B., **1995a**. Actions of the insecticide, fipronil, on dieldrin-sensitive and -resistant GABARs of *Drosophila melanogaster. Br. J. Pharmacol. 115*, 909–912.

Hosie, A.M., Dunne, E.L., Harvey, R.J., Smart, T.G., **2003**. Zinc-mediated inhibition of GABA(A) receptors: discrete binding sites underlie subtype specificity. *Nat. Neurosci. 6*, 362–369.

Hosie, A.M., Sattelle, D.B., **1996a**. Agonist pharmacology of two *Drosophil*a GABAR splice variants. *Br. J. Pharmacol. 119*, 1577–1585.

Hosie, A.M., Sattelle, D.B., **1996b**. Allosteric modulation of an expressed GABA-gated chloride channel of *Drosophila melanogaster. Br. J. Pharmacol. 117*, 1229–1237.

Hosie, A.M., Shirai, Y., Buckingham, S.D., Rauh, J.J., Roush, R.T., *et al.*, **1995b**. Blocking actions of BIDN, a bicyclic dinitrile convulsant compound, on wild-type and dieldrin-resistant GABAR homo-oliomers of *Drosophila melanogaster* expressed in *Xenopus* ootyes. *Brain Res. 693*, 257–260.

Hosie, A.M., Ozoe, Y., Koike, K., Ohmoto, T., Nikaido, T., *et al.*, **1996**. Actions of picrodendrin antagonists on

dieldrin-sensitive and - resistant *Drosophila* GABARs. *Br. J. Pharmacol. 119*, 1569–1576.

Hoskins, S.G., Homberg, U., Kingan, T.G., Christensen, T.A., Hildebrand, J.G., **1986**. Immunocytochemistry of GABA in the antennal lobes of the sphinx moth, *Manduca sexta*. *Cell Tissue Res. 244*, 243–252.

Hosler, J.S., Buxton, K.L., Smith, B.H., **2000**. Impairment of olfactory discrimination by blockade of GABA and nitric oxide activity in the honey bee antennal lobes. *Behav. Neurosci. 114*, 514–525.

Hue, B., **1991**. Functional assay for GABAR subtypes of a cockroach giant interneuron. *Arch. Insect Biochem. Physiol. 18*, 147–157.

Hue, B., Pelhate, M., Chanelet, J., **1979**. Pre- and postsynaptic effects of taurine and GABA in the cockroach central nervous system. *Can. J. Sci. Neurol. 6*, 243–250.

Ignell, R., **2001**. Monoamines and neuropeptides in antennal lobe interneurons of the desert locust, *Schistocerca gregaria*: an immunocytochemical study. *Cell Tissue Res. 306*, 143–156.

Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., *et al.*, **1988**. Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. *Nature 335*, 645–648.

Jarboe, C.H., Porter, L.A., Buckler, R.T., **1968**. Structural aspects of picrotoxinin action. *J. Med. Chem. 11*, 729–731.

Jechlinger, M., Pelz, R., Tretter, V., Klausberger, T., Sieghart, W., **1998**. Subunit composition and quantitative importance of hetero-oligomeric receptors: $GABA_A$ receptors containing alpha6 subunits. *J. Neurosci. 18*, 2449–2457.

Johnston, G.A., **1996**. GABAc receptors: relatively simple transmitter-gated ion channels? *Trends Pharmacol. Sci. 17*, 319–323.

Judge, S., Leitch, B., **1999**. Modulation of transmitter release from the locust forewing stretch receptor neuron by GABAergic interneurons activated via muscarinic receptors. *J. Neurobiol. 40*, 420–431.

Kadous, A.A., Ghiasuddin, S.M., Mantusura, F., Scott, J.G., Tanaka, K., **1983**. Difference in the picrotoxin receptor between the cyclodiene-resistant and susceptible strains of the German cockroach. *Pestic. Biochem. Physiol. 19*, 157–166.

Kaku, K., Matsumura, F., **1994**. Identification of the site of mutation within the M2 region of the GABAR of the cyclodiene-resistant German cockroach. *Comp. Biochem. Physiol. Biochem. Mol. Biol. 108*, 367–376.

Karlin, A., Akabas, M.H., **1995**. Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron 15*, 1231–1244.

Karlin, A., **2002**. Emerging structure of the nicotinic acetylcholine receptors. *Nat. Rev. Neurosci. 3*, 102–114.

Kash, T.L., Jenkins, A., Kelley, J.C., Trudell, J.R., Harrison, N.L., **2003**. Coupling of agonist binding to channel gating in the GABAA receptor. *Nature 421*, 272–274.

Kauer, J.S., **1998**. Olfactory processing, a time and place for everything. *Curr. Biol. 8*, R282–R283.

Kerkut, G.A., Walker, R.J., Pitman, R., **1969**. Ionophoretic application of ACh and GABA onto insect central neurones. *Comp. Biochem. Physiol. 31*, 611–633.

Khan, Z.U., Gutierrez, A., De Blas, A.L., **1994**. The subunit composition of a $GABA_A$/benzodiazepine receptor from rat cerebellum. *J. Neurochem. 63*, 371–374.

Kolaczinski, J.H., Curtis, C.F., **2004**. Chronic illness as a result of low-level exposure to synthetic pyrethroid insecticides, a review of the debate. *Food Chem. Toxicol. 42*, 697–706.

Krishek, B.J., Amato, A., Connolly, C.N., Moss, S.J., Smart, J.G., **1996**. Proton sensitivity of the $GABA_A$ receptor is associated with the receptor subunit composition. *J. Physiol 492*, 431–443.

Kudo, Y., Niwa, H., Tanaka, A., Yamada, K., **1984**. Actions of picrotoxinin and related compounds on the frog spinal cord: the role of a hydroxyl group at the 6-position in antagonizing the actions of amino acids and presynaptic inhibition. *Br. J. Pharmacol. 81*, 373–380.

Kuffler, E.W., Edwards, C., **1958**. Mechanisms of gamma aminobutyric acid (GABA) action and its relation to synaptic inhibition. *J. Neurophysiol. 32*, 589–610.

Lawrence, J.J., Casida, J.E., **1984**. Interactions of lindane, toxaphene and cyclodienes with brain-specific *t*-butylbicyclophosphorothionate receptor. *Life Sci. 35*, 171–178.

Leal, S.M., Neckameyer, W.S., **2002**. Pharmacological evidence for GABAergic regulation of specific behaviors in *Drosophila melanogaster. J. Neurobiol. 50*, 245–261.

Lee, H.J., Rocheleau, T., Zhang, H.G., Jackson, M.B., ffrench-Constant, R.H., **1994**. Expression of a *Drosophila* GABAR in a baculovirus insect cell system–functional expression of insecticide susceptible and resistant GABARs from the cyclodiene resistance gene Rdl. *FEBS Lett. 335*, 315–318.

Lee, D., O'Dowd, D.K., **1999**. Fast excitatory synaptic transmission mediated by nicotinic acetylcholine receptors in *Drosophila* neurons. *J. Neurosci. 19*, 5311–5321.

Lee, D., Su, H., O'Dowd, D., **2003**. GABA receptors containing Rdl subunits mediate fast synaptic transmission in *Drosophila* neurones. *J. Neurosci. 23*, 4625–4634.

Lees, G., Beadle, D.J., Neumann, R., Benson, J.A., **1987**. Responses to GABA by isolated insect neuronal somata-pharmacology and modulation by a benzodiazepine and a barbiturate. *Brain Res. 401*, 267–278.

Leonard, R.J., Labarca, G.G., Charnet, P., Davidson, N., Lester, H.A., **1988**. Evidence that the M2 membrane-spanning region lines the ion channel pore of the nicotinic receptor. *Science 242*, 1578–1581.

Levi, R., Camhi, J.M., **2000**. Population vector coding by the giant interneurons of the cockroach. *J. Neurosci. 20*, 3822–3829.

Ludmerer, S.W., Warren, V.A., Williams, B.S., Zheng, Y., Hunt, D.C., *et al.*, **2002**. Ivermectin and nodulisporic acid receptors in *Drosophila melanogaster* contain both gamma-aminobutyric acid-gated Rdl and glutamate-gated GluCl alpha chloride channel subunits. *Biochemistry 41*, 6548–6560.

Lummis, S.C.R., Sattelle, D.B., **1985**. Insect CNS GABA receptors. *Neurosci. Lett. 60*, 13–18.

Lummis, S.C.R., Sattelle, D.B., **1986**. Binding sites for [^{3}H]GABA, [^{3}H]flunitrazepam and [^{35}S]TBPS in insect CNS. *Neurochem. Int. 9*, 287–293.

Lummis, S.C.R., Buckingham, S.D., Rauh, J.J., Sattelle, D.B., **1990**. Blocking actions of heptachlor at an insect central nervous system GABAR. *Proc. R. Soc. B. 240*, 97–106.

MacLeod, K., Laurent, G., **1996**. Distinct mechanisms for synchronization and temporal patterning of odor-encoding. *Science 274*, 976–979.

Malecot, C.O., Sattelle, D.B., **1990**. Single-channel recordings from insect neuronal GABA-activated chloride channels. *J. Exp. Biol. 151*, 495–501.

Martin, S.C., Russek, S.J., Farb, D.H., **2001**. Human GABA(B)R genomic structure: evidence for splice variants in GABA(B)R1 but not GABA(B)R2. *Gene 278*, 63–79.

Matsuda, K., Hosie, A.M., Buckingham, S.D., Squire, M.D., Baylis, H.A., *et al*., **1996**. pH-dependent actions of THIP and ZAPA on an ionotropic *Drosophila melanogaster* GABAR. *Brain Res. 739*, 335–338.

Matsuda, K., Hosie, A.M., Holyoke, C.W., Rauh, J.J., Sattelle, D.B., **1999**. Cross-resistance with dieldrin of a novel tricyclic dinitrile GABAR antagonist. *Br. J. Pharmacol. 127*, 1305–1307.

Matsumura, F., Ghiasuddin, S.M., **1983**. Evidence for similarities between cyclodiene-type insecticides and picrotoxin in their action mechanisms. *J. Environ. Sci. Hlth B. 18*, 1–14.

Matsumura, F., Tanaka, K., Ozoe, Y., **1987**. GABA-related systems as targets for insecticides. In: Hollingworth, R.M., Green, M.B. (Eds.), Sites of Action of Neurotoxic Pesticides, ACS Symposium Series 356. American Chemical Society, Washington DC, pp. 44–70.

McCracken, D.I., **1993**. The potential for avermectins to affect wildlife. *Vet. Parasitol. 48*, 273–280.

McGurk, K.A., Pistis, M., Belelli, D., Hope, A.G., Lambert, J.J., **1998**. The effect of a transmembrane amino acid on etomidate sensitivity of an invertebrate GABAR. *Br. J. Pharmacol. 124*, 13–20.

McKernan, R., Whiting, P., **1996**. Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci. 19*, 139–143.

Mezler, M., Muller, T., Raming, K., **2001**. Cloning and functional expression of GABA(B) receptors from *Drosophila*. *Eur. J. Neurosci. 13*, 477–486.

Millar, N.S., Buckingham, S.D., Sattelle, D.B., **1994**. Stable expression of a functional homo-oligomeric *Drosophila* GABAR in a *Drosophila* cell line. *Proc. R. Soc. Lond. B. Biol. Sci. 258*, 307–314.

Miyazaki, M., Matsumura, F., Beeman, R.W., **1995**. DNA sequence and site of mutation of the GABAR of cyclodiene-resistant red flour beetle, *Tribolium castaneum*. *Comp. Biochem. Physiol. Biochem. Mol. Biol. 111*, 399–406.

Mohler, H., Benke, D., Mertens, S., Fritschy, J.M., **1992**. $GABA_A$-receptor subtypes differing in alpha-subunit composition display unique pharmacological properties. *Adv. Biochem. Psychopharmacol. 47*, 41–53.

Murphy, V.F., Wann, K.T., **1988**. The action of GABAR agonists and antagonists on muscle membrane conductance in *Schistocerca gregaria*. *Br. J. Pharmacol. 95*, 713–722.

Neelands, T.R., Fisher, J.L., Bianchi, M., Macdonald, R.L., **1999**. Spontaneous and gamma-aminobutyric acid (GABA)-activated GABA(A) receptor channels formed by epsilon subunit-containing isoforms. *Mol. Pharmacol. 55*, 168–178.

Newland, C.F., Cull-Candy, S.G., **1992**. On the mechanism of action of picrotoxin on GABAR channels in dissociated symphathetic neruones of the rat. *J. Physiol. 447*, 191–213.

Nielsen, M., Braestrup, C., Squires, R.F., **1978**. Evidence for a late evolutionary appearance of brain-specific benzodiazepine receptors: an investigation of 18 vertebrate and 5 invertebrate species. *Brain Res. 141*, 342–346.

Nishino, H., Mizunami, M., **1998**. Giant input neurons of the mushroom body: intracellular recording and staining in the cockroach. *Neurosci. Lett. 246*, 57–60.

Nusser, Z., Ahmad, Z., Tretter, V., Fuchs, K., Wisden, W., *et al*., **1999**. Alterations in the expression of $GABA_A$ receptor subunits in cerebellar granule cells after the disruption of the alpha6 subunit gene. *Eur. J. Neurosci. 11*, 1685–1697.

Okuma, J., Kondoh, Y., **1996**. Neural circuitry underlying linear representation of wind information in a nonspiking local interneuron of the cockroach. *J. Comp. Physiol. A 179*, 725–740.

Otsuka, M., Iversen, L.L., Hall, Z.W., Kravitz, E.A., **1966**. Release of gamma-aminobutyric acid from inhibitory nerves of lobster. *Proc. Natl Acad. Sci. USA 56*, 1110–1115.

Ozoe, Y., Akamatsu, M., Akamatsu, M., Higata, T., Ikeda, I., *et al*., **1998**. Picrodendrin and related terpenoid antagonists reveal structural differences between ionotropic GABARs of mammals and insects. *Bioorg. Med. Chem. 6*, 481–492.

Ozoe, Y., Hasegawa, H., Mochida, K., Koike, K., Suzuki, Y., *et al*., **1994**. Picrodendrins, a new group of picrotoxane terpenoids: structure-activity profile of action at the $GABA_A$ receptor-coupled picrotoxinin, binding site in rat brain. *Biosci. Biotech. Biochem. 58*, 1506–1507.

Ozoe, Y., Matsumura, F., **1986**. Structural requirements for bridged bicyclic compounds acting on picrotoxin receptor. *J. Agric. Food Chem. 34*, 126–134.

Palmer, C.H., Casida, J.E., **1985**. 1,4-Disubstituted 2,6,7-trioxabicyclo[2.2.2]octanes: a new class of insecticides. *J. Agric. Food. Chem. 33*, 976.

Panek, I., French, A.S., Seyfarth, E.A., Sekizawa, S., Torkkeli, P.H., **2002**. Peripheral GABAergic inhibition of spider mechanosensory afferents. *Eur. J. Neurosci. 16*, 96–104.

Perez-Orive, J., Mazor, O., Turner, G.C., Cassenaer, S., Wilson, R.I., *et al*., **2002**. Oscillations and sparsening of odor representations in the mushroom body. *Science 297*(5580), 359–365.

Petri, B., Homberg, U., Loesel, R., Stengl, M., **2002**. Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J. Exp. Biol. 205*, 1459–1469.

Pichon, Y., Beadle, D., **1988**. Acetylcholine, GABA and glutamate receptor channels in cultured insect neurones. In: Lunt, G.G. (Ed.), Neurotox '88: Molecular Basis of Drug and Pesticide Action. Elsevier Science Publishers, BV, Amsterdam, pp. 325–337.

Pistis, M., Belelli, D., McGurk, K., Peters, J.A., Lambert, J.J., **1999**. Complementary regulation of anaesthetic activation of human (α6-β3-γ2L) and *Drosophila* (RDL) GABA receptors by a single amino acid residue. *J. Physiol. 515*(Pt 1), 3–18.

Pitman, R.M, Kerkut, G.A., **1970**. Comparison of the actions of iontophoretically-applied acetylcholine and gamma aminobutyric acid with the EPSP and IPSP in cockroach central neurons. *Comp. Gen. Pharmac. 1*, 221–230.

Priestley, C.M., Williamson, E.M., Wafford, K.A., Sattelle, D.B., **2003**. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABA(A) receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster. Br. J. Pharmacol. 140*(8), 1363–1372.

Pritchett, D.B., Luddens, H., Seeburg, P.H., **1989**. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science 245*, 1389–1392.

Python, F., Stocker, R.F., **2002**. Immunoreactivity against choline acetyltransferase, gamma-aminobutyric acid, histamine, octopamine, and serotonin in the larval chemosensory system of *Drosophila melanogaster. J. Comp. Neurol. 390*, 455–469.

Qian, H., Dowling, J.E., **1993**. Novel GABA responses from rod-driven retinal horizontal cells. *Nature 361*, 162–164.

Ratra, G.S., Casida, J.E., **2001**. GABAR subunit composition relative to insecticide potency and selectivity. *Toxicol. Lett. 122*, 215–222.

Ratra, G.S., Kamita, S.G., Casida, J.E., **2001**. Role of human GABA(A) receptor beta3 subunit in insecticide toxicity. *Toxicol. Appl. Pharmacol. 172*, 233–240.

Rauh, J.J., Lummis, S.C.R., Sattelle, D.B., **1990**. Pharmacological and biochemical properties of insect GABARs. *Trends Pharmacol. Sci. 11*, 325–329.

Rauh, J.J., Benner, E., Schnee, M.E., Cordova, D., Holyoke, C.W., *et al.*, **1997a**. Effects of [^{3}H]-BIDN, a novel bicyclic dinitrile radioligand for GABA-gated chloride channels of insects and vertebrates. *Br. J. Pharmacol. 121*, 1496–1505.

Rauh, J.J., Holyoke, C.W., Kleier, D.A., Presnail, J.K., Benner, E.A., *et al.*, **1997b**. Polycyclic dinitriles: a novel class of potent GABAergic insecticides provides a new radioligand, [^{3}H]BIDN. *Invert. Neurosci. 3*, 261–268.

Raymond, V., Sattelle, D.B., Lapied, B., **2000**. Co-existence in DUM neurones of two GluCl channels that differ in their picrotoxin sensitivity. *Neuroreport 230*, 45–48.

Raymond, V., Sattelle, D.B., **2002**. Novel animal-health drug targets from ligand-gated chloride channels. *Nat. Rev. Drug Discov. 6*, 427–436.

Reeves, D.C., Lummis, S.C., **2002**. The molecular basis of the structure and function of the 5-HT_3 receptor: a model ligand-gated ion channel. *Mol. Membr. Biol. 19*, 11–26.

Richter, K., Bohm, G.A., **1997**. The molting gland of the cockroach *Periplaneta americana*: secretory activity and its regulation. *Gen. Pharmacol. 29*, 17–21.

Roberts, C.J., Krogsgaard-Larsen, P., Walker, R.J., **1981**. Studies of the action of GABA, muscimol and related compounds on *Periplaneta* and *Limulus* neurons. *Comp. Biochem. Physiol. 69C*, 7–11.

Robinson, T.N., MacAllan, D., Lunt, G.G., Battersby, M., **1985**. γ-aminobutyric acid receptor complex of insect CNS: characterization of a benzodiazepine binding site. *J. Neurochem. 47*, 1955–1962.

Rohrbough, J., Broadie, K., **2002**. Electrophysiological analysis of synaptic transmission in central neurons of *Drosophila* larvae. *J. Neurophysiol. 88*, 847–860.

Rohrer, S.P., Birzin, E.T., Costa, S.D., Arena, J.P., Hayes, E.C., *et al.*, **1995**. Identification of neuron-specific ivermectin binding sites in *Drosophila melanogaster* and *Schistocerca americana*. *Insect Biochem. Mol. Biol. 25*, 11–17.

Rosay, P., Armstrong, J.D., Wang, Z., Kaiser, K., **2001**. Synchronized neural activity in the *Drosophila* memory centers and its modulation by amnesiac. *Neuron 30*, 759–770.

Rothlin, C.V., Katz, E., Verbitsky, M., Elgoyhen, A.B., **1999**. The alpha9 nicotinic acetylcholine receptor shares pharmacological properties with type A gamma-aminobutyric acid, glycine, and type 3 serotonin receptors. *Mol. Pharmacol. 55*, 248–254.

Rufingier, C., Pasteur, N., Lagnel, J., Martin, C., Navajas, M., **1999**. Mechanisms of insecticide resistance in the aphid *Nasonovia ribisnigri* (Mosley) (Homoptera: Aphididae) from France. *Insect. Biochem. Mol. Biol. 29*, 385–391.

Sachse, S., Galizia, C.G., **2002**. Role of inhibition for temporal and spatial odor representation in olfactory output neurons, a calcium imaging study. *J. Neurophysiol. 87*, 1106–1117.

Sattelle, D.B., **1990**. GABA receptors of insects. *Adv. Insect Physiol. 22*, 1–113.

Sattelle, D.B., Bai, D., Chen, H.H., Skeer, J.M., Buckingham, S.D., **2003**. Bicuculline-insensitive GABA-gated Cl^- channels in the larval nervous system of the moth *Manduca sexta*. *Invert. Neurosci. 5*(1), 37–43.

Sattelle, D.B., Pinnock, R.D., Wafford, K.A., David, J.A., **1988**. GABARs on the cell-body membrane of an identified insect motor neurone. *Proc. R. Soc. Lond. B 232*, 443–456.

Sattelle, D.B., Harrison, J.B., Chen, H.H., Bai, D., Takeda, M., **2000**. Immunocytochemical localization

of putative gamma-aminobutyric acid receptor subunits in the head ganglia of *Periplaneta americana* using an anti-RDL C-terminal antibody. *Neurosci. Lett. 289*, 197–200.

Sattelle, D.B., Holyoke, C.W., Schnee, M.E., Cordova, D., Bennet, E.A., *et al.*, **1995**. A bicyclic dinitrile radioligand for studying GABA-gated chloride channels. *J. Physiol. 483*, 193P.

Schmidt, H., Luer, K., Hevers, W., Technau, G.M., **2000**. Ionic currents of *Drosophila* embryonic neurons derived from selectively cultured CNS midline precursors. *J. Neurobiol. 44*, 392–413.

Schnee, M., Rauh, J., Buckingham, S.D., Sattelle, D.B., **1997**. Pharmacology of skeletal muscle GABA-gated chloride channels in the cockroach *Periplaneta americana*. *J. Exp. Biol. 200*, 2947–2955.

Schurmann, F.W., **2000**. Acetylcholine, GABA, glutamate and NO as putative transmitters indicated by immunocytochemistry in the olfactory mushroom body system of the insect brain. *Acta. Biol. Hung. 51*, 355–362.

Schurmann, F.W., Ottersen, O.P., Honegger, H.W., **2000**. Glutamate-like immunoreactivity marks compartments of the mushroom bodies in the brain of the cricket. *J. Comp. Neurol. 418*, 227–239.

Scott, R.H., Duce, I.R., **1987**. Pharmacology of GABA receptors on skeletal muscle fibres of the locust (*Schistocerca gregaria*). *Comp. Biochem. Physiol. C 86*(2), 305–311.

Seidel, C., Bicker, G., **1997**. Colocalisation of taurine-with transmitter-immunoreactivities in the nervous system of the migratory locust. *Brain Res. 769*, 273–280.

Seidel, C., Bicker, G., **2002**. Developmental expression of nitric oxide/cyclic GMP signaling pathways in the brain of the embryonic grasshopper. *Brain. Res. Devel. Brain Res. 138*, 71–79.

Shimahara, T., Pichon, Y., Lees, G., Beadle, C.A., Beadle, D.J., **1987**. Gamma-aminobutyric acid receptors on cultured cockroach brain neurones. *J. Exp. Biol. 131*, 231–244.

Shirai, Y., Hosie, A.M., Buckingham, S.D., Holyoke, C.W.Jr, Baylis, H.A., *et al.*, **1995**. Actions of picrotoxin analogues on an expressed, homo-oliomeric GABAR of *Drosophila melanogaster. Neurosci. Lett. 188*, 1–4.

Sieghart, W., **1995**. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol. Rev. 47*, 181–234.

Sinkkonen, S.T., Hanna, M.C., Kirkness, E.F., Korpi, E.R., **2000**. GABA(A) receptor protein epsilon and theta subunits display unusual structural variation between species and are enriched in the rat locus ceruleus. *J. Neurosci. 20*, 3588–3595.

Sirisoma, N.S., Ratra, G.S., Tomizawa, M., Casida, J.E., **2001**. Fipronil-based photoaffinity probe for *Drosophila* and human beta 3 GABARs. *Bioorg. Med. Chem. Lett. 11*, 2979–2981.

Smart, T.G., Constanti, A., **1986**. Studies on the mechanism of action of picrotoxin and other convulsants at the crustacean muscle GABAR. *Proc. R. Soc. Lond. Ser. B 227*, 191–216.

Smith, G.B., Olsen, R.W., **1995**. Functional domains of $GABA_A$ receptors. *Trends Neurosci. 16*, 162–168.

Stevenson, P.A., **1999**. Colocalisation of taurine with transmitter-immunoreactivities in the nervous system of the migratory locust. *J. Comp. Neurol. 404*, 86–96.

Stevenson, A., Wingrove, P.B., Whiting, P.J., Wafford, K.A., **1995**. Beta-carboline gamma-aminobytyric acid (A) receptor inverse agonists modulate gamma-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. *Mol. Pharmacol. 49*, 965–969.

Stopfer, M., Bhagavan, S., Smith, B.H., Laurent, G., **1997**. Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature 390*, 70–74.

Strambi, C., Cayre, M., Sattelle, D.B., Augier, R., Charpin, P., *et al.*, **1998**. Immunocytochemical mapping of an RDL-like GABAR subunit and of GABA in brain structures related to learning and memory in the cricket *Acheta domesticus*. *Learn. Mem. 5*, 78–89.

Strong, L., **1993**. Overview: the impact of avermectins on pastureland ecology. *Vet. Parasitol. 48*, 3–17.

Stumpner, A., **1998**. Picrotoxin eliminates frequency selectivity of an auditory interneuron in a bushcricket. *J. Neurophysiol. 79*, 2408–2415.

Taylor, C.P., Vartanian, M.G., Yuen, P.W., Bigge, C., Suman-Chauhan, N., *et al.*, **1993**. Potent and stereospecific anticonvulsant activity of 3-isobutyl GABA relates to *in vitro* binding at a novel site labeled by tritiated gabapentin. *Epilepsy Res. 14*(1), 11–15.

Thompson, S.A., Bonnert, T.P., Whiting, P.J., Wafford, K.A., **1998**. Functional characteristics of recombinant human GABA(A) receptors containing the epsilon-subunit. *Toxicol. Lett. 100–101*, 233–238.

Thompson, M., Shotkoski, F., ffrench-Constant, R., **1993a**. Cloning and sequencing of the cyclodiene insecticide resistance gene from the yellow fever mosquito *Aedes aegypti*. Conservation of the gene and resistance associated mutation with *Drosophila*. *FEBS Lett. 325*, 187–190.

Thompson, M., Steichen, J.C., ffrench-Constant, R.H., **1993b**. Conservation of cyclodiene insecticide resistance-associated mutations in insects. *Insect Mol. Biol. 2*, 149–154.

Ticku, K., Ban, M., Olsen, R.W., **1978**. Binding of [^{3}H]a-dihydropicrotoxinin, a γ-aminobytyric acid synaptic antagonist to rat brain membranes. *Mol. Pharmacol. 14*, 391–402.

Tingle, C.C., Rother, J.A., Dewhurst, C.F., Lauer, S., King, W.J., **2003**. Fipronil: environmental fate, ecotoxicology, and human health concerns. *Rev. Environ. Contam. Toxicol. 176*, 1–66.

Umesh, A., Gill, S.S., **2002**. Immunocytochemical localization of a *Manduca sexta* gamma-aminobutyric acid transporter. *J. Comp. Neurol. 448*, 388–398.

Usherwood, P.N.R., Grundfest, H., **1965**. Peripheral inhibition in skeletal muscle of insects. *J. Neurophysiol. 28*, 497–518.

Wafford, K.A., Sattelle, D.B., Gant, D.B., Eldefrawi, A.T., Eldefrawi, M.E., **1989**. Noncompetitive inhibition of

GABARs in insect and vertebrate CNS by endrin and lindane. *Pest. Biochem. Physiol. 33*, 213.

Wafford, K.A., Bain, C.J., Quirk, K., McKernan, R.M., Wingrove, P.B., *et al.*, **1995**. A novel allosteric modulatory site on the $GABA_A$ receptor γ subunit. *Neuron 12*, 775–782.

Waldrop, B., **1994**. Physiological and pharmacological properties of responses of GABA and ACh by abdominal motor neurons in *Manduca sexta*. *J. Comp. Physiol. A 175*, 754–785.

Waldrop, B., Christensen, T.A., Hildebrand, J.G., **1987**. GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, *Manduca sexta*. *J. Comp. Physiol. A 161*, 23–32.

Waldrop, B., Hildebrand, J.G., **1988**. Physiology and pharmacology of acetylcholinergic responses of interneurones in the antennal lobes of the sphinx moth, *Manduca sexta*. *J. Comp. Physiol. A 164*, 433–441.

Walker, R.J., Crossman, A.R., Woodruff, G.N., Kerkut, G.A., **1971**. The effects of bicuculline on the gamma-aminobutyric acid (GABA) receptors of neurons of *Periplaneta americana* and *Helix aspersa*. *Brain Res. 33*, 75–82.

Wang, T.-L., Hackam, A., Guggino, W.B., Cutting, G.R., **1995**. A single histidine residue is essential for zinc inhibition of GABA rho receptors. *J. Neurosci. 15*, 7684–7691.

Watson, A.H.D., Burrows, M., **1987**. Immunocytochemical and pharmacological evidence for GABAergic spiking local interneurones in the locust. *J. Neurosci. 7*, 1741–1751.

Watson, S., Girdlestone, D., **1995**. TiPS on nomenclature. *Trends Pharmacol. Sci. 16*(1), 15–16.

Watson, G.B., Salgado, V.L., **2001**. Maintenance of GABAR function of small-diameter cockroach neurons by adenine nucleotides. *Insect Biochem. Mol. Biol. 31*, 207–212.

Wegerhoff, R., **1999**. GABA and serotonin immunoreactivity during postembryonic brain development in the beetle *Tenebrio molitor. Microsc. Res. Tech. 45*, 154–164.

Westin, J., Ritzmann, R.E., Goddard, D.J., **1988**. Wind-activated thoracic interneurons of the cockroach: I. Responses to controlled wind stimulation. *J. Neurobiol. 19*, 573–588.

Whiting, P.J., **2003**. GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery? *Drug Discov. Today 8*, 445–450.

Whitton, P.S., Strang, R.H.C, Nicholson, R.A., **1987**. The distribution of taurine in the tissues of some species of insects. *Insect Biochem. 17, 573–577.*

Whitton, P.S., Nicholson, R.A., Strang, R.H.C., **1994**. Electrophysiological responses of isolated locust (*Schistocerca gregaria*) somata to taurine and GABA application. *J. Insect Physiol. 40*, 195–199.

Wingrove, P.B., Wafford, K.A., Bain, C., Whiting, P.J., **1994**. The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the $\beta 2$ and $\beta 3$ subunit. *Proc. Natl Acad. Sci. USA 91*, 4569–4573.

Witten, J.L., Truman, J.W., **1991**. The regulation of transmitter expression in postembryonic lineages in the moth *Manduca sexta*. II. Role of cell lineage and birth order. *J. Neurosci. 11*, 1990–1997.

Witten, J.L., Truman, J.W., **1998**. Distribution of GABA-like immunoreactive neurons in insects suggests lineage homology. *J. Comp. Neurol. 398*, 515–528.

Wolff, M.A., Wingate, V.P., **1998**. Characterization and comparative pharmacological studies of a functional gamma-aminobutyric acid (GABA) receptor cloned from the tobacco budworm, *Heliothis virescens* (Noctuidae: Lepidoptera). *Invert. Neurosci. 3*, 305–315.

Woodward, R.M., Polenzani, L., Miledi, R., **1993**. Characterization of bicuculline/baclofen-insensitive (ρ-like) γ-aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of γ-aminobutyric $acid_A$ and γ-aminobutyric $acid_B$ receptor agonists and antagoinsts. *Mol. Pharmacol. 43*, 609–625.

Yamazaki, Y., Nishikawa, M., Mizunami, M., **1998**. Three classes of GABA-like immunoreactive neurons in the mushroom body of the cockroach. *Brain Res. 788*, 80–86.

Yasuyama, K., Meinertzhagen, I.A., Schurmann, F.W., **2002**. Synaptic organization of the mushroom body calyx in *Drosophila melanogaster. J. Comp. Neurol. 183*, 467–476.

Zhang, H.-G., ffrench-Constant, R.H., Jackson, M.B., **1994**. A unique amino acid of the *Drosophila* GABAR with influence on drug sensitivity by two mechanisms. *J. Physiol. 479*, 65–75.

Zhang, D., Pan, Z.H., Awobuluyi, M., Lipton, S.A., **2001**. Structure and function of GABA(C) receptors: a comparison of native versus recombinant receptors. *Trends Pharmacol. Sci. 22*, 121–132.

Zheng, Y., Hirschberg, B., Yuan, J., Wang, A.P., Hunt, D.C., *et al.*, **2002**. Identification of two novel *Drosophila melanogaster* histamine-gated chloride channel subunits expressed in the eye. *J. Biol. Chem. 277*, 2000–2005.

Zornik, E., Paisley, K., Nichols, R., **1999**. Neural transmitters and a peptide modulate *Drosophila* heart rate. *Peptides 20*, 45–51.

5.5 Insect G Protein-Coupled Receptors: Recent Discoveries and Implications

Y Park, Kansas State University, Manhattan, KS, USA
M E Adams, University of California, Riverside, CA, USA

5.5.1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins that sense and transduce extracellular signals into cellular responses. The binding of small ligands to the extracellular domains of these proteins elicits a cascade of intracellular responses through activation of heterotrimeric G proteins. In insects, many signaling molecules and their corresponding biological functions have been identified, but knowledge of the signal transduction subsequent to receptor binding has been limited by the relative paucity of information about GPCR structure and function.

It is clear that remarkably diverse physiological processes are mediated by GPCRs. Some of these include sensing of environmental signals such as light, odors, and taste. Others sense extracellular signaling molecules such as calcium, nucleotides, neurotransmitters, biogenic amines, and neuroendocrine peptides. Evolution of GPCRs for specific functions in a vast array of organisms is one interesting area of study, for which insects in many ways are ideal for comparative studies. In addition to their value for basic science, GPCRs mediate vital physiological events in insects that may provide promising targets for development of pest control measures as well as therapeutic targets for promotion of human health.

The advent of the postgenomics era is providing excellent opportunities to explore insect GPCRs and their biological functions. Genomic surveys of GPCRs in the fruit fly *Drosophila melanogaster* and in the malaria mosquito *Anopheles gambiae* have identified at least ~200 and 276 GPCRs, respectively (Brody and Cravchik, 2000; Hill *et al.*, 2002a). Sequence analysis reveals an appreciable degree of groupwise homology relationships between insect and vertebrate GPCRs, suggesting many GPCRs have common ancestry dating more than 600 million years ago (Benton and Ayala, 2003). Data mining combined with functional studies of insect GPCRs has begun to reveal specific ligand–receptor interactions.

Deorphaning insect GPCRs by finding cognate ligands in *Drosophila* is now being followed by expansion of physiological studies in other species of insects that are more amenable to physiological experiments. The use of genomics as a springboard for understanding physiological mechanisms is complemented by advances in molecular probes, genetics, and imaging technologies, leading to a new era of "molecular physiology." This review will cover

recent progress in the molecular and functional characterization of insect GPCRs.

5.5.2. Structure–Function Relationships for GPCRs

GPCRs are characterized by conserved core structures, consisting of an extracellular N-terminus, seven transmembrane (TM) α-helices each connected by alternating extracellular and intracellular loops (e1, e2, e3, i1, i2, and i3), and an intracellular C-terminus. The structure of the seven transmembrane protein bovine rhodopsin revealed by X-ray crystallography epitomizes the generalized bauplan predicted for GPCRs (Palczewski *et al.*, 2000; Teller *et al.*, 2001; review: Filipek *et al.*, 2003) (**Figure 1**). A number of important general structural features emerging from studies of vertebrate GPCRs are worth summarizing here as a prelude to discussions of insect GPCRs. Of course, caution is necessary in extrapolating knowledge from these works directly to other groups of phylogenetically diversified GPCRs.

The location of ligand binding sites varies considerably between different families of GPCRs. Indeed, GPCRs can be broadly categorized according to ligand size and location of the binding site. Such information is critical in the design of candidate drugs with either agonist or antagonist properties. For instance, GPCRs for small ligands such as retinoic acid, odorants, and biogenic amines appear to have a binding pocket surrounded by the TM bundle (Gotzes and Baumann, 1996; Vaidehi *et al.*, 2002). However, ligand binding to other groups of GPCRs involves extracellular epitopes of the protein, including the N-terminus and extracellular loops (Bockaert and Pin, 1999). In the case of the metabotropic glutamate and γ-aminobutyric acid B ($GABA_B$) receptors, the large N-terminus is composed of two lobes arranged for trapping ligands (Hermans and Challiss, 2001). In *Drosophila*, the N-terminal ectodomain of methuselah, an orphan GPCR associated with extended lifespan, consists of three β structure regions, which meet to form a shallow interdomain groove. This region characterized by an exposed tryptophan residue forms a putative ligand binding site by analogy with ectodomains of other GPCRs (West *et al.*, 2001).

The seven TM helices of GPCRs are arranged in an anticlockwise sequential manner when viewed from the extracellular aspect (**Figure 1**). Ligand binding induces changes in core structure, and in particular TM3 is thought to have a major role in receptor activation by altering the relative orientation of TM6 (Bockaert and Pin, 1999). Likewise, the conserved tripeptide signature of the TM3 cytoplasmic interface ([D,E]R[Y,W]) is considered to be important in receptor activation. The TM helices often are interrupted by distortions of the α-helix backbone, which may serve as hinge points

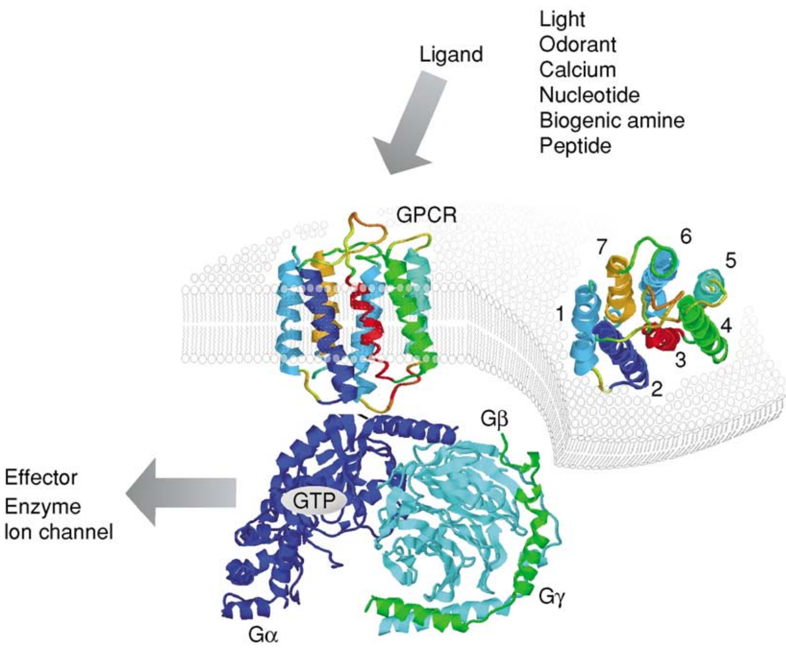


Figure 1 A diagram summarizing G protein-coupled receptor (GPCR) signaling. An array of seven transmembrane segments is shown according to the bovine rhodopsin structure. The trimeric G protein consisting of Gα, Gβ, and Gγ is coupled to the GPCR. Activation of the GPCR by ligands binding to the extracellular cell surface is amplified by intracellular effector proteins.

underlying conformational changes induced by ligand binding.

The cytoplasmic face of GPCRs interacts with heterotrimeric G-proteins, which dissociate upon receptor activation to trigger specific signal transduction pathways (**Figure 1**). Specificity for particular subtypes of heterotrimeric G-proteins is an important factor in determining downstream pathways. The *Drosophila* genome presents at least seven genes encoding Gα, six for Gβ, and two for Gγ subunits. The subunit Gα is in direct contact with the cytoplasmic interface of GPCRs (Bourne, 1997; Wess, 1998; Horn *et al*., 2000; Moller *et al*., 2001). Prediction of G protein subtype coupling specificity has been attempted in vertebrate GPCRs through identification of characteristic cytoplasm-facing sequence motifs (Bourne, 1997; Wess, 1998; Horn *et al*., 2000; Moller *et al*., 2001). This is a nontrivial exercise, considering reports showing GPCRs coupled with multiple G proteins as well as agonist-dependent changes in GPCR coupling with different G proteins (Robb *et al*., 1994; Reale *et al*., 1997; Cordeaux *et al*., 2001; Cordeaux and Hill, 2002).

Proper functioning of GPCRs often may depend on oligomerization with other proteins (Gomes *et al*., 2001; Angers *et al*., 2002). Such interactions may serve to localize receptors for effective coupling with downstream pathways and/or to influence desensitization, which regulates receptor levels on the cell surface and temporal parameters of the biological response (Pierce and Lefkowitz, 2001; Brady and Limbird, 2002; Kohout and Lefkowitz, 2003).

5.5.3. Evolution of Insect GPCRs

In a pioneering study, Hewes and Taghert (2001) performed a comprehensive phylogenetic analysis of *Drosophila* peptide GPCRs covering the entire array of annotated genes in the *Drosophila* genome. The analysis revealed 42 putative peptide GPCRs arranged within 15 monophyletic subgroups together with vertebrate peptide GPCRs. The relationships established in this study also appear to be valid generally for the *Anopheles* mosquito (Hill *et al*., 2002a). Evolutionary relationships between *Drosophila* and vertebrate peptide GPCRs have provided impetus for formulation of hypotheses to guide the process of deorphaning insect GPCRs. The success of this approach has been enhanced by many instances of previously established vertebrate ligand–receptor relationships. As this process advances, coevolutionary relationships between peptide GPCRs and their ligands have been brought into clearer focus.

Ligand–receptor coevolution has occurred as a consequence of reciprocal evolutionary influences between two interacting proteins – ligand and receptor – while the original definition of coevolution emphasized interactions between organisms (see Ridley, 1993). Assuming GPCRs share a common ancestral gene, diverse GPCRs must have arisen through multiple steps of gene duplication types of evolutionary processes (Chothia *et al*., 2003). In phylogenetic analyses, closely related GPCRs have cognate ligands also related each other, indicating that an ancestral ligand–receptor partnership has effectively rooted new sets of ligand–receptors with a novel function. The ligand–receptor partners appear to be maintained during evolution without punctuated changes of their relationships; thus coevolution may be a common phenomenon.

In the coevolution of ligand–receptor partners, it has been proposed that divergence of the receptor precedes the evolution of its cognate novel neuropeptide ligand (Darlison and Richter, 1999). Such reasoning is based on the frequent occurrence of multiple receptors for a single peptide. This hypothesis, however, may be questioned because many peptide encoding genes have multiple repeats of isopeptides and processing sites. A good example is the tetrapeptide FMRFamide, for which eight homologous repeats appear in the *Drosophila* propeptide gene (Schneider and Taghert, 1988, 1990). There also appears to be a significant degree of flexibility in the system, evidenced first by the presence of multiple receptors for a given ligand that are coupled to different G proteins (Darlison and Richter, 1999; Park *et al*., 2003) and second by differential selectivity of receptors to a number of isopeptides (Darlison and Richter, 1999). Further details regarding processes of ligand–receptor coevolution can be found in a number of recent publications (van Kesteren *et al*., 1996; Darlison and Richter, 1999; Goh and Cohen, 2002).

While discerning evolutionary relationships among GPCRs is challenging, establishing such relationships for signaling peptides is a much more daunting task. The main problem lies in the fact that only a small number of functionally important, conserved amino acids within peptide sequences defines their evolutionary relationships. A valuable search tool in bioinformatics, the position-specific iterated BLAST (PSI-BLASAT: Altschul *et al*., 1997), provides a powerful means of using small short, conserved sequence motifs for searching distantly related peptides. The method uses a score matrix generated from multiple alignments of the highest scoring hits in each round of the search. Conservation of consensus amino acids in the multiple

sequence alignment implies functionally and evolutionarily important amino acid residues common for a group of signaling peptides.

A good example of ligand–receptor coevolution was found for a group of neuropeptides having a C-terminal PRXamide motif (Pro-Arg-Xxx with C-terminal amide). This motif is common to an array of peptides occurring in both insects and vertebrates, including neuromedin U (NMU), cardioacceleratory peptide 2b (CAP2b), pyrokinins, and ecdysis triggering hormone (ETH). This group of peptides activates a cluster of GPCRs related to vertebrate receptors for NMU and thyrotropin releasing hormone (Park *et al.*, 2002) (**Figure 3**). While phylogenetic relationships among receptors are established almost exclusively on the basis of amino acid sequence, the patterns of pharmacological cross-reactivity for these GPCRs to cognate PRXamide peptides provide a new and "functional" criterion for inferring evolutionary relationships for ligand–receptor pairs.

Despite the frequent correspondence of insect and vertebrate ligand–receptor relationships, a number of *Drosophila* GPCRs are found to be relatively unique (Hewes and Taghert, 2001), such as *Drosophila* GPCRs having bootstrap values of less than 300 in 1000 pseudosamples. For example, closely related GPCRs within the group CG13229, CG13803, and CG8985 are found to have no significant relationship with other vertebrate GPCRs. Recently, two of these receptors CG13803 and CG8985 were found to be activated by Dromyosuppressin (Johnson *et al.*, 2003a; Egerod *et al.*, 2003a) having the C-terminal motif FLRLamide, a member of FMRFamide-related peptides (FaRPs) containing a C-terminal RFamide. Such unique GPCRs could arise either through rapidly evolving branches of GPCRs, or through recent loss of the related GPCRs in the vertebrates.

5.5.4. Insect GPCRs

5.5.4.1. Classification of GPCRs

Classification of the GPCR superfamily by families and groups has been made based on a combination of natural ligand categories and receptor sequence homology (Kolakowski, 1994; Horn *et al.*, 2000; Hewes and Taghert, 2001). For the following discussions, these classifications are used to cover comprehensive categories of GPCRs in all organisms. Accordingly, insect GPCRs are classified into the following families: family A, rhodopsin and various peptide receptors; family B, secretin-like peptide receptors and methuselah (mth); family C, metabotropic glutamate receptors, atypical GPCRs such as

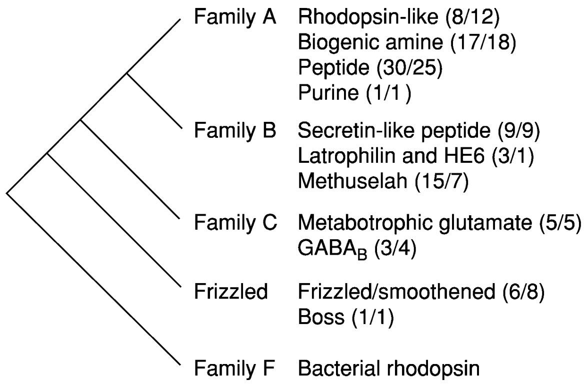


Figure 2 A conceptual phylogenetic relationship of the GPCR superfamily. The numbers of annotated genes for *Drosophila* (left) and *Anopheles* (right) are in parentheses. The conceptual tree is still not congruent and reconstructed based on Josefsson (1999).

frizzled/smoothened and bride of sevenless (boss), and odorant receptors (**Figure 2**).

Assuming all groups of GPCRs (or 7-TM proteins) are ancestrally related, a conceptual tree depicting evolutionary relationships among them can be drawn based on combined results from previous studies (**Figure 2**), while construction of a congruent tree is still controversial because of its high sequence divergency (Josefsson, 1999). The number of *Drosophila* genes encoding putative GPCRs as a fraction of the total in each group is shown in parenthesis for each category (**Figure 2**). In rooting the tree on family F, which includes bacterial rhodopsin, the group frizzled appears to be the most ancestral form. Family A with ~90 GPCRs is the largest group, and includes biogenic amines, peptides, rhodopsin, and ~30 orphan receptors (Hill *et al.*, 2002a). Given the excellent phylogenetic analysis for *Drosophila* peptide receptors in the previous study (Hewes and Taghert, 2001). We provide **Tables 1–3** summarizing the GPCRs that have been cloned so far. This table includes *Drosophila* data and GPCRs from other insects, but not the GPCRs of *A. gambiae* where experimental data is unavailable.

5.5.4.2. Family A: Biogenic Amine, Rhodopsin, and Neuropeptide Receptors

5.5.4.2.1. Biogenic amine receptors Most small signaling molecules used as transmitters, modulators, and hormones are common to vertebrates and invertebrates, including insects. The most prominent are acetylcholine, glutamate, GABA, and the biogenic amines serotonin, dopamine, and histamine (**Table 1**). Norepinephrine and epinephrine, abundant in the vertebrates, are absent or present only in very low amounts in insects (Maxwell *et al.*,

Table 1 Insect G protein-coupled receptors for biogenic amines

Name	*Synonym (GenBank accession number)*	*Species*	*Cellular response (expression system)*	*Expression pattern*	*Reference*
Serotonin receptor					
5-HT2	CG1056, 5HT-dro1, 5HT2dro, Dm5HT2 (X81835)	*Drosophila melanogaster*		Coexpression with pair-rule gene *ftz* in embryo	Colas *et al.* (1999, 1995)
5HT7	CG12073, 5HT-dro1, 5HT7dro, Dm5HTdro1, 5HT-R1 (M55533)	*Drosophila melanogaster*	Increased cAMP (Sf9, NIH 3T3)	Adult head, larval ventral ganglion	Witz *et al.* (1990), Saudou *et al.* (1992), Obosi *et al.* (1996)
5-HT1A	CG16720, 5HT-dro2A (Z11489)	*Drosophila melanogaster*	Inhibition cAMP (COS-7)	Ventral unpaired median neurons	Saudou *et al.* (1992)
5-HT1B	CG15113, 5HT-dro2B (Z11490)	*Drosophila melanogaster*	Inhibition cAMP (COS-7)	Larval ventral ganglion	Saudou *et al.* (1992), Obosi *et al.* (1996)
CG8007	(AE003679, AAN13390)	*Drosophila melanogaster*			Brody and Cravchik (2000)
Aedes 5-HT7	(AF296125)	*Aedes aegypti*		Malpighian tubules, tracheolar cells	Pietrantonio *et al.* (2001)
Bm5HT	B150 *Bombyx* (X95604)	*Bombyx mori*			von Nickisch-Rosenegk *et al.* (1996)
Hv5HT	K15 *Heliothis* (X95605)	*Heliothis virescens*			von Nickisch-Rosenegk *et al.* (1996)
Dopamine receptor					
DopR1	CG9652, dDA1, DmDop1 (X77234)	*Drosophila melanogaster*	Increased cAMP (HEK-293)	Somata of optic lobe	Gotzes *et al.* (1994), Gotzes and Baumann (1996)
DopR2	CG7569, CG18741, DopR99B, DAMB (U34383)	*Drosophila melanogaster*	Ca^{2+} mobilization (*Xenopus* oocyte), Increased cAMP (*Xenopus* oocyte, HEK-292, S2)	Mushroom body	Feng *et al.* (1996), Han *et al.* (1996), Reale *et al.* (1997)
DD2R	CG17004 (AY150862)	*Drosophila melanogaster*	Increased cAMP (CRE-luciferase assay in HEK-293)	Head and body	Hearn *et al.* (2002)
AmDop1	(CAA73841)	*Apis mellifera*	Increased cAMP (HEK-293)		Blenau *et al.* (1998)
AmBAR6	D2 (AAM19330)	*Apis mellifera*		Mushroom body	Ebert *et al.* (1998), Blenau and Baumann (2001)
Muscarinic acetylcholine receptor					
AchR-60C	CG4356, mAChR-1, DM1 (M23412)	*Drosophila melanogaster*	Increased IP3 metabolism (mouse Y1)	Mushroom body, optic lobe, antennal lobe	Shapiro *et al.* (1989), Blake *et al.* (1993), Hannan and Hall (1996)
CG7918	(AE003677, AAF54188)	*Drosophila melanogaster*			Brody and Cravchik (2000)
Octopamine/tyramine receptor					
OAMB	CG3856, CG15698, DmOCT1B (AF065443)	*Drosophila melanogaster*	Increased cAMP (S2), increased cAMP (CHO-K1), Ca^{2+} mobilization (CHO-K1)	Mushroom body	Arakawa *et al.* (1990), Robb *et al.* (1994), Han *et al.* (1998)
BmOcr	B96 *Bombyx* (X95607)	*Bombyx mori*			von Nickisch-Rosenegk *et al.* (1996)

Continued

Table 1 Continued

Name	*Synonym (GenBank accession number)*	*Species*	*Cellular response (expression system)*	*Expression pattern*	*Reference*
HvOcr	K50 *Heliothis* (X95606)	*Heliothis virescens*	Inhibition cAMP (LLC-PK1)	Antennae	von Nickisch-Rosenegk *et al.* (1996)
TyrR	CG7485, Tyr-dro, honoka (X54794, AB073914)	*Drosophila melanogaster*	Inhibition cAMP, Ca^{2+} mobilization (NIH 3T3)	Antennae, CNS	Saudou *et al.* (1990), Kutsukake *et al.* (2000)
Amtyr1	(AJ245824)	*Apis mellifera*	Inhibition cAMP, Ca^{2+} mobilization (HEK-293)	Mushroom body, CNS	Blenau *et al.* (2000)
TyrLoc	Tyr-Loc (X69520)	*Locusta migratoria*	Inhibition cAMP, Ca^{2+} mobilization (murine erythroleukemia cell (MEL-C88L, S2)		Vanden Broeck *et al.* (1995), Poels *et al.* (2001)
MabOcR	(AAK14402)	*Mamestra brassicae*			

1978; Evans, 1980). Prominent amines in insects and crustaceans are octopamine and tyramine (Evans, 1980). Octopamine is thought to play a modulatory role in arousal, paralleling that of noradrenaline in the sympathetic system of vertebrates (Evans, 1993; Blenau and Baumann, 2001). Octopamine and tyramine, while only present as "trace amines" in the vertebrates, may nevertheless play functional roles (Borowsky *et al.*, 2001). Among the biogenic amines known to be signaling molecules in insects, histamine is so far the only one for which GPCRs are unknown; electrophysiological data provide conclusive evidence for gating of ionotropic receptors by histamine. This contrasts with histamine receptors in the vertebrates, where both ionotropic and GPCR receptors are known (Roeder, 2003).

The evolutionary patterns of biogenic amine receptors among vertebrates and invertebrates are generally in accordance with monophyletic ancestries (Roeder, 2002), although recent gene duplication events have led to rapid expansions of certain vertebrate receptor groups. Furthermore, there are apparent shifts in ligand specificity in a number of receptor phylogenies, for which the ligands are structurally similar. This phenomenon is particularly apparent for *Bombyx* and *Heliothis* octopamine receptors, which occur in a monophyletic clade with tyramine receptors (Blenau *et al.*, 1998; Ebert *et al.*, 1998; Roeder, 2002).

Numerous functions of biogenic amines in insects are described at the behavioral and physiological levels, including modulation of sensory input and motor output, learning and memory, development, and elevated states of arousal (Blenau and Baumann, 2001; Roeder, 2002). Modulation of olfactory and visual stimuli is indicated by expression patterns of amines in sensory lobes, and by physiological experiments. For example, sensitization of pheromone receptor cells by octopamine and serotonin is indicated in *Bombyx mori* and *Manduca sexta* (Pophof, 2000; Dolzer *et al.*, 2001), and a possible role for serotonin in feedback from higher centers in the brain is indicated from morphological studies (Hill *et al.*, 2002b). Octopamine plays a major hormonal role in energy metabolism during locust flight by acting directly on the fat body and indirectly through release of adipokinetic hormone (Orchard *et al.*, 1993). Setting the day state in circadian cycling by serotonin is suggested for a cricket (*Gryllus bimaculatus*) (Saifullah and Tomioka, 2002), and dopamine receptors are thought to be targets of circadian modulation (Andretic and Hirsh, 2000). Egg diapause in *B. mori* appears to be controlled by dopaminergic pathways in the mother (Noguchi and Hayakawa, 2001).

In a growing number of cases, biogenic amine receptor-specific functions are reported. The *honoka* mutant in *Drosophila* carries a tyramine receptor defect and shows reduced responses to repellent odors. In addition, this mutant exhibited reduced tyramine modulation at the neuromuscular junction, while normal modulation by octopamine was observed (Kutsukake *et al.*, 2000). A role for the serotonin receptor subtype 5-HT2 in embryonic development was shown by a *Drosophila* mutant (Colas *et al.*, 1995, 1999). In these studies, serotonin signaling was found to be necessary for increased

Table 2 Insect G protein-coupled receptors for neuropeptides: family A group III and group V, and family B

Name	Synonym (GenBank accession no.)	Species	Active ligand[a]	Vertebrate homology group	Evidence/functional assay (expression system)[b]	Expression pattern	Reference
Family A group III							
CCKLR-17D3	CG6857 (AE003509, AAF48857)	*Drosophila melanogaster*		Cholecytokinin/ gastrin R	Homology to CCKLR-17D1		McBride *et al.* (2001), Kubiak *et al.* (2002)
CCKLR-17D1	CG6881, CG6894, CG32540, dsk-r1 (AE003510, AAF48879)	*Drosophila melanogaster*	Sulfakinin	Cholecytokinin/ gastrin R	Ca^{2+} mobilization (HEK-293, CHO-K1, SH-EP)		McBride *et al.* (2001), Kubiak *et al.* (2002)
NepYr	CG5811 (M81490)	*Drosophila melanogaster*		Neurokinin R	Inward current (*Xenopus* oocyte)		Li *et al.* (1992)
Leucokinin receptor	CG10626 (AE003566, NM139711, AY070926)	*Drosophila melanogaster*	Leucokinin	Neurokinin R	Ca^{2+} mobilization (S2)	Malpighian tubules, CNS	Radford *et al.* (2002)
Myokinin receptor	(AF228521)	*Boophilus microplus*		Neurokinin R	Homology cloning		Holmes *et al.* (2000)
Takr86C	CG6515, NKD (M77168)	*Drosophila melanogaster*	Tachykinin	Neurokinin R	Increased IP3 (mouse NIH-3T3), translocation of β-arrestin2	CNS	Monnier *et al.* (1992), Johnson *et al.* (2003b)
Takr99D	CG7887, DTKD (X62711)	*Drosophila melanogaster*	Tachykinin	Neurokinin R	Inward current (*Xenopus* oocyte), translocation of β-arrestin2	CNS	Li *et al.* (1991), Johnson *et al.* (2003b)
STKR	*Stomoxys* tachykinin receptor	*Stomoxys calcitrans*	Tachykinin-like	Neurokinin R	Ca^{2+} mobilization (S2)		Guerrero (1997), Torfs *et al.* (2001, 2002a)
LTKR	*Leucophaea* tachykinin receptor (AJ310390)	*Leucophaea maderae*		Neurokinin R	Homology cloning	Brain	Johard *et al.* (2001)
SNPF-R	CG7395, Drm-sNPF-R, NPFR76F, GPCR60 (NP_524176)	*Drosophila melanogaster*	Short neuropeptide F	Neurokinin R	Ca^{2+} mobilization (CHO-K1/Gα_{16}), inward current (*Xenopus* oocyte)		Mertens *et al.* (2002), Feng *et al.* (2003)
NPFR1	CG1147 (AF364400)	*Drosophila melanogaster*	Neuropeptide F	Neurokinin R	Ca^{2+} mobilization (CHO-K1/Gα_{16}), ^{125}I-NPF binding (CHO), translocation of β-arrestin2	CNS, midgut	Garczynski *et al.* (2002), Johnson *et al.* (2003b)

Continued

Table 2 Continued

Name	*Synonym (GenBank accession no.)*	*Species*	*Active ligand*[a]	*Vertebrate homology group*	*Evidence/functional assay (expression system)*[b]	*Expression pattern*	*Reference*
FMRFamide receptor	CG2114 (NM139501)	*Drosophila melanogaster*	FMRFamide	Neurokinin R	Ca^{2+} mobilization (CHO-K1/Gα_{16}), translocation of β-arrestin2	Trachea, gut, fat body, Malpighian tubules, ovary	Cazzamali and Grimmelikhuijzen (2002), Meeusen *et al.* (2002), Johnson *et al.* (2003b)
Allatostatin B receptor	CG14484, CG18192 (NP611241)	*Drosophila melanogaster*	Allatostatin B	Bombesin R	translocation of β-arrestin2		Johnson *et al.* (2003a)
PRXa receptor 1	CG9918 (AF522191)	*Drosophila melanogaster*	Cardioacceleratory peptide CAP2b	Neuromedin U R	Inward current (*Xenopus* oocyte)		Park *et al.* (2002)
PRXa receptor 2	CG8784 (AF522189)	*Drosophila melanogaster*	Hugin	Neuromedin U R	Inward current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1/Gα_{16})		Park *et al.* (2002), Rosenkilde *et al.* (2003)
PRXa receptor 3	CG8795 (AF522190)	*Drosophila melanogaster*	Hugin	Neuromedin U R	Inward current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1/Gα_{16})		Park *et al.* (2002), Rosenkilde *et al.* (2003)
HezPBAN R	AY319852	*Helicoverpa zea*	HezPBAN	Neuromedin U R	Ca^{2+} mobilization (Sf9)		Choi *et al.* (2003)
CAP2b receptor	CG14575 (AF522193)	*Drosophila melanogaster*	Cardioacceleratory peptide (CAP2b)	Neuromedin U R	Inward current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1/Gα_{16})		Iversen *et al.* (2002), Park *et al.* (2002)
ETH receptor a	CG5911a (AY220741)	*Drosophila melanogaster*	Ecdysis triggering hormone (ETH)	Neuromedin U R / thyrotropin releasing hormone R	Ca^{2+} mobilization (CHO-K1/Gα_{16})		Iversen *et al.* (2002), Park *et al.* (2003)
ETH receptor b	CG5911b (AY220742)	*Drosophila melanogaster*	Ecdysis triggering hormone (ETH)	Neuromedin U/ thyrotropin releasing hormone R	Ca^{2+} mobilization (CHO-K1/Gα_{16})		Iversen *et al.* (2002), Park *et al.* (2003)
Myosuppressin receptor 1	CG8985 (AF545042)	*Drosophila melanogaster*	Myosuppressin	Neuromedin U/ thyrotropin releasing hormone R	Ca^{2+} mobilization (CHO-K1/Gα_{16}), translocation of β-arrestin2, inhibition cAMP (HEK-292)		Egerod *et al.* (2003a), Johnson *et al.* (2003a)

Myosuppressin receptor 2	CG13803 (AF544244)	*Drosophila melanogaster*	Myosuppressin	Neuromedin U/ thyrotropin releasing hormone R	Ca^{2+} mobilization (CHO-K1/Gα_{16}), translocation of β-arrestin2, inhibition cAMP (HEK-292), GTPγS binding (HEK-292)		Egerod *et al.* (2003a), Johnson *et al.* (2003a)
Proctolin receptor	CG6986 (NM167020)	*Drosophila melanogaster*	Proctolin	Neuromedin U/ thyrotropin releasing hormone R	Ca^{2+} mobilization (HEK-293), translocation of β-arrestin2	Brain, medulla, hindgut, pericardial cell, heart	Egerod *et al.* (2003b), Johnson *et al.* (2003b)
Family A group V							
Corazonin receptor	CG10698 (AF522192, AF373862)	*Drosophila melanogaster*	Corazonin	Vasopressin R	Inward current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1/Gα_{16}), translocation of β-arrestin2		Cazzamali *et al.* (2002), Park *et al.* (2002), Johnson *et al.* (2003b)
Bombyx AKH receptor	BAKHR (AF403542)	*Bombyx mori*	Adipokinetic hormone (AKH)	Gonadotropin releasing hormone R	Ca^{2+} mobilization (CHO-K1/Gα_{16})		Staubli *et al.* (2002)
AKH receptor	CG11325, GRHR, DAKHR (AF522194, AAC61523)	*Drosophila melanogaster*	Adipokinetic hormone (AKH)	Gonadotropin releasing hormone R	Inward current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1/Gα_{16})		Park *et al.* (2002), Staubli *et al.* (2002)
CCAP receptor	CG6111 (AF522188)	*Drosophila melanogaster*	Crustacean cardioactive peptide (CCAP)	Vasopressin R	Inward current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1/Gα_{16})		Park *et al.* (2002), Staubli *et al.* (2002)
Drostar1	CG7285 (AAG54080, NM140783)	*Drosophila melanogaster*	Allatostatin C	Somatostatin R/ opioid R	GIRK current (*Xenopus* oocyte)	Corpora allata	Kreienkamp *et al.* (2002a), Johnson *et al.* (2003a)
Drostar 2	CG13702 (NM140782)	*Drosophila melanogaster*	Allatostatin C	Somatostatin R/ opioid R	GIRK current (*Xenopus* oocyte)	Optic lobe, corpora allata	Kreienkamp *et al.* (2002a), Johnson *et al.* (2003a)
DAR-1	AlstR, EG:121E7.2, CG2872 (AF163775, NM166982)	*Drosophila melanogaster*	Allatostatin A	Galanin	GIRK current (*Xenopus* oocyte), Ca^{2+} mobilization (CHO-K1), GTPγS binding (CHO-K1)		Birgul *et al.* (1999), Larsen *et al.* (2001)
DAR-2	AR-2, CG10001	*Drosophila melanogaster*	Allatostatin A	Galanin	Ca^{2+} mobilization (CHO-K1), GTPγS binding (CHO-K1)	Gut	Lenz *et al.* (2000, 2001), Larsen *et al.* (2001)

Continued

Table 2 Continued

Name	Synonym (GenBank accession no.)	Species	Active ligand[a]	Vertebrate homology group	Evidence/functional assay (expression system)[b]	Expression pattern	Reference
Pea-AlstR	*Periplaneta* AlstR (AF336364)	*Periplaneta americana*	Allatostatin A	Galanin	GIRK current (*Xenopus* oocyte)		Auerswald *et al.* (2001)
BAR	*Bombyx* AlstR (AH011256)	*Bombyx mori*	Allatostatin A	Galanin	Ca^{2+} mobilization (CHO-K1/$G\alpha_{16}$)	Gut	Secher *et al.* (2001)
LGR1	CG7665, Fsh (U47005)	*Drosophila melanogaster*	nd	FSH/LH/TRH	FSH receptor homology cloning		Hauser *et al.* (1997), Nishi *et al.* (2000)
LGR2	CG8930, Rickets (AF142343)	*Drosophila melanogaster*	Bursicon	FSH/LH/TRH	Mutant insensitive to bursicon-like factor	Embryo and pupae	Eriksen *et al.* (2000), Nishi *et al.* (2000), Baker and Truman (2002)
Family B							
Mas-DH-R	*Manduca* DH receptor (U03489)	*Manduca sexta*	Mas-diuretic hormone	Corticotropin releasing hormone R	[^{3}H]Mas-DH binding and cAMP (COS-7), [^{125}I], Mas-DH binding and cAMP (Sf-9)		Reagan (1994, 1995)
Acd-DH-R	*Acheta* DH receptor (U15959)	*Acheta domesticus*	Acheta-diuretic hormone	Corticotropin releasing hormone R	[^{3}H]Acd-DH binding and cAMP (COS-7),		Reale *et al.* (1997)
Bm-DH-R	*Bombyx* DH receptor	*Bombyx mori*		Corticotropin releasing hormone R	Homology cloning		Ha *et al.* (2000)

[a]The most active or putative ligand suggested in the respective reference(s).
[b]Methods used for cloning and assay, or other evidence provided.

Table 3 Family C: Metabotropic glutamate receptors and $GABA_B$ receptors

Name	*Synonym*	*Evidence*	*Expression*	*Reference*
mGluRA	CG11144, metabotropic glutamate receptor A, DmGluRA,	Inhibition of AC (HEK 293), GIRK current (*Xenopus* oocyte)	CNS	Raymond *et al.* (1999)
mGluRB	CG30361, CG18447, CG8692, metabotropic glutamate receptor B,	Homology		Brody and Cravchik (2000)
GABA-B-R1	CG15274, D-$GABA_B$R1	GIRK current (*Xenopus* oocyte)	CNS	Mezler *et al.* (2001)
GABA-B-R2	CG6706, D-$GABA_B$R2, GH07312	GIRK current (*Xenopus* oocyte)	CNS	Mezler *et al.* (2001)
GABA-B-R3	CG3022, $GABA_B$R3	Homology	CNS	Mezler *et al.* (2001)

cell adhesiveness required for cell–cell intercalation during gastrulation. Expression patterns of the serotonin 5-HT7-like receptor in *Aedes* Malpighian tubules and tracheolar cells are suggestive of possible functions in the control of diuresis and respiration (Pietrantonio *et al.*, 2001).

5.5.4.2.2. Rhodopsin Rhodopsins are a class of retinal-binding chromophores widespread in visual cells of animals. In each rhodopsin, the light-absorbing retinal binds to a pocket within the seven-transmembrane opsin protein. Incoming photons induce isomerization of retinal, which passes protons to its partner opsin, resulting in G protein activation. The eyes of insects are tuned to specific spectral ranges through diversity in genes encoding the opsin structure, whereby amino acid sequence variability occurs in the vicinity of the retinal binding pocket. The coupling of opsins to G-proteins leads to visual transduction through the opening or closing of cation channels in the visual cell. The evolution of genes encoding rhodopsins with different spectral properties have been described in various insect species (review: Briscoe and Chittka, 2001).

The insect compound eye is composed of cartridges called ommatidia, each organized as a set of eight photoreceptor retinula cells (R1–R8). In *Drosophila,* the compound eye expresses seven rhodopsin genes (*Rh1–Rh7*), which show unique expression patterns and spectral properties. A specialized portion of the plasma membrane in each retinula cell is convoluted via microvilli to form the rhodopsin-rich rhabdomere. The rhabdomere of each retinula cell faces the others within the cartridge to create the composite rhabdom, where light is absorbed. Rhodopsin Rh1, also called ninaE (neither inactivation nor after potential E), is expressed in retinula cells R1–R6 (O'Tousa *et al.*, 1985; Feiler *et al.*, 1988). Rh2 is expressed in ocelli (Cowman *et al.*, 1986), simple eyes located on the vertical side of the head, while Rh3 and Rh4 are expressed in R7 in nonoverlapping subsets of ommatidia (Fryxell and Meyerowitz, 1987). Rh5 and Rh6 are expressed in R8 in nonoverlapping subsets of ommatidia (Chou *et al.*, 1996, 1999). Rh7 (CG5638) has been identified in the genome sequence, but has not been associated with a physiological function as yet (Brody and Cravchik, 2000).

Insect rhodopsins are classified generally into three categories according to their responsiveness to ultraviolet, blue, and green colors (Townson *et al.*, 1998). In some cases, rhodopsins sensitive to red color have been identified in the Lepidoptera and Hymenoptera (Briscoe and Chittka, 2001), where adaptive evolution appears to have taken place through replacement of amino acids adjacent to the retinal binding site and thus shifting the spectral sensitivity (Briscoe, 2002). Recent expansions of the rhodopsin gene in different species of insects are observed in the phylogenetic relationships among rhodopsins in the orders Hymenoptera, Diptera, and Lepidoptera (Montell, 1999; Salcedo *et al.*, 1999; Briscoe, 2001; Briscoe and Chittka, 2001; Hill *et al.*, 2002a).

The phototransduction cascade in invertebrates begins with light-absorbing rhodopsin coupled to G_q. This activates phosphoinositide turnover through activation of phospholipase C β (PLCβ), liberation of inositol trisphosphate (IP3), and the opening of transient receptor potential (TRP) channels, leading to inward cation (Na^+ and Ca^{2+}) flux and depolarizing potentials in retinula cells (Alvarez *et al.*, 1996; Montell, 1999; Bahner *et al.*, 2000; Hardie and Raghu, 2001). Invertebrate phototransduction contrasts with that of vertebrates, where at low light intensities a large "dark current" flows through cyclic guanidine monophosphate (cGMP) nucleotide gated sodium channels to maintain a relatively depolarized state (approximately −40 mV). Under dark conditions, photoreceptor cells maintain high levels of cytosolic cGMP and hence a high number of open cyclic nucleotide gated sodium channels. Light absorbance by rhodopsin activates the G protein transducin (G_t), leading to elevation of phosphodiesterase, destruction of cGMP, and consequent closing of sodium channels (Shichida and Imai, 1998). Through this

mechanism, vertebrate photoreceptors hyperpolarize in response to light stimuli.

5.5.4.2.3. Neuropeptide receptors in family A Neuropeptides are ubiquitous signaling molecules acting mainly through GPCRs for activation of cellular responses. Numerous signaling peptides in insects have been characterized by their abilities to activate visceral activity, heartbeat, or glandular secretion (reviews: Gäde *et al*., 1997; Nassel, 2002). Studies of neuropeptide signaling have been accomplished mainly in large insects where biochemical studies and physiological assays are relatively easy. Subsequent findings of neuropeptides in various species show that amino acid sequences of neuropeptides are generally well conserved. With the opening of the postgenomic era, genomic sequences in *Drosophila* and *Anopheles* revealed more than 23 and 35 genes, respectively, encoding neuropeptides (Hewes and Taghert, 2001; Riehle *et al*., 2002). Peptidomics of the central nervous system (CNS) of *Drosophila* (Baggerman *et al*., 2002) identified 28 neuropeptides including eight novel peptides using capillary liquid chromatography in conjunction with tandem mass spectrometry.

Studies of neuropeptide receptors in vertebrates have identified a large number of GPCRs grouped as family A with rhodopsins and biogenic amine receptors (Kolakowski, 1994). Phylogenetic analysis of *Drosophila* and *Anopheles* genome sequences for putative GPCRs revealed ~40 GPCRs in family A in each species (Hewes and Taghert, 2001; Hill *et al*., 2002a). Analysis of phylogenetic relationships revealed that many insect neuropeptide receptors occur in monophyletic groups with vertebrate receptors, implying evolution from common ancestors in each group (Hewes and Taghert, 2001; Hill *et al*., 2002a). Family A is further classified into several subgroups, and insect peptide GPCRs currently are placed into group III or group V (Kolakowski, 1994; Hewes and Taghert, 2001).

Numerous insect GPCRs in family A group III and group V have been cloned and expressed, and functional assays have been key to identification of their ligands (**Table 2**). The earliest efforts to clone neuropeptide GPCRs started with cDNA library screenings that targeted *Drosophila* homologs of the vertebrate neuropeptide Y and tachykinin receptors (Li *et al*., 1991, 1992; Monnier *et al*., 1992). Following identification of these receptors in *Drosophila*, subsequent investigations located their homologs in the stable fly (*Stomoxys Calcitrans*) and cockroach (Leucophaea maderae) (Guerrero, 1997; Torfs *et al*., 2000, 2001, 2002a; Johard *et al*., 2001).

Recent approaches in functional genomics have contributed to the deorphaning of a large number of insect GPCRs. Functional analyses have been designed for screening sets of GPCRs and ligands belonging to a phylogenetic clade. This approach assumes ligand–receptor coevolution (see Section 5.5.6) and utilizes an established ligand–receptor pair as a baseline for formulation of testable hypotheses.

Such an approach was taken to identify likely receptors for peptide ligands having C-terminal PRXamide motifs (Park *et al*., 2002). The PRXa peptides in *Drosophila* and other insects fall into three classes: pyrokinins including the pheromone biosynthesis activating neuropeptide (PBAN) (–FXPRXa) (Raina *et al*., 1989; Matsumoto *et al*., 1990), cardioactive CAP2b-like peptides (–FPRXa) (Morris *et al*., 1982; Huesmann *et al*., 1995), and ETHs (–PRXa) (Zitnan *et al*., 1996; Adams and Zitnan, 1997; Park *et al*., 1999, 2002). In the vertebrates, PRXa motifs are found in the peptides NMU, vasopressin (AVP), and pancreatic polypeptide (PP), for which receptors already have been identified (Thibonnier *et al*., 1994; Fujii *et al*., 2000; Howard *et al*., 2000). Functional analyses demonstrated that *Drosophila* GPCRs related to the monophyletic NMU receptor group are activated by all three classes of *Drosophila* PRXa peptides (**Figure 3**). PRXa receptors are now being investigated in other species of insects, such as *Manduca*, where the physiological functions of the neuropeptides are better characterized (Kim and Adams, unpublished data), while genuine functions of the PRXamide receptors in *Drosophila* also are being investigated.

Drosophila GPCRs related to the vasopressin receptor failed to respond to PRXa peptides, but instead was activated by crustacean cardioactive peptide (CCAP), corazonin, and adipokinetic hormone (AKH), none of which falls into the PRXa peptide group (Park *et al*., 2002). *Drosophila* sulfakinin receptors also were identified through the logic of ligand–receptor coevolution upon finding homology in ligands between vertebrate cholecystokinin/gastrin and *Drosophila* sulfakinin (Kubiak *et al*., 2002). A *Drosophila* neuropeptide F receptor was identified, and found to belong to one of four *Drosophila* GPCRs in the neuropeptide Y receptor subgroup (Garczynski *et al*., 2002; Mertens *et al*., 2002).

Another productive approach for deorphaning GPCRs is reverse physiology (see Section 5.5.5). Receptors for allatostatin type A (DAR-1) (Birgul *et al*., 1999) and allatostatin type C (Drostar1, Drostar2) (Kreienkamp *et al*., 2002a) were identified through isolation from tissue extracts of native

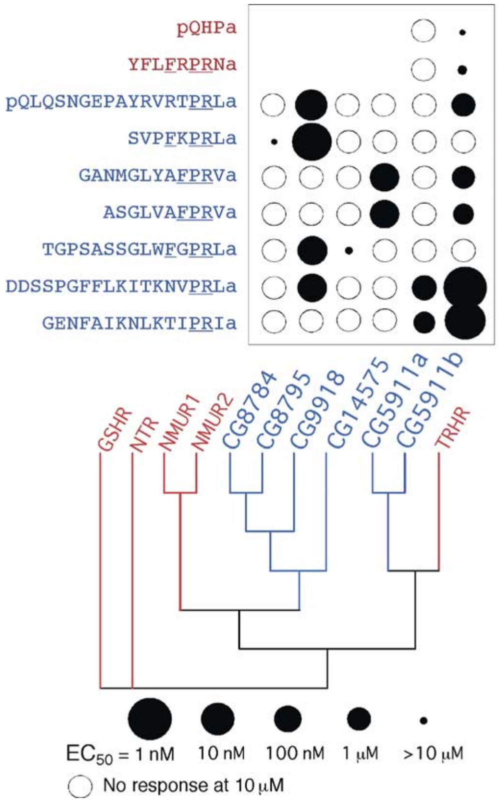


Figure 3 Ligand–receptor specificity for *Drosophila* PRXamide and related peptides. The amino acid sequence of each ligand is shown at the left, with the names of each peptide on the right. Circles indicate relative activity of ligands to each receptor. The larger the circle, the more potent is the peptide. Empty circles indicate ''no response'' at concentrations up to 10 μM, the highest concentration tested. The tree and names of neuropeptide receptors are: GSHR, GH secretagogue receptor; NTR, neurotensin receptor; NMUR1, neuromedin U receptor 1; NMUR2, neuromedin U receptor 2; TRHR, thyrotropin releasing hormone receptor; *Drosophila* PRXamide receptor GPCRs with CG numbers. Red letters are vertebrate peptides and GPCRs. Blue letters are *Drosophila* PRXamide peptides and GPCRs. (Data from Park, Y., Kim, Y.J., Adams, M.E., **2002**. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand–receptor coevolution. *Proc. Natl Acad. Sci. USA 99*, 11423–11428; and Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., **2003**. Two subtypes of ecdysis triggering hormone receptor in *Drosophila melanogaster*. *J. Biol. Chem. 278*, 17710–17715).

neuropeptide ligands that activate the receptor. Closely related genes in *Drosophila* and other insect species subsequently cloned and expressed, and showed their sensitivity to related allatostatin peptides (Lenz *et al.*, 2000, 2001; Auerswald *et al.*, 2001; Larsen *et al.*, 2001; Secher *et al.*, 2001). The reverse physiology approach also was successfully employed in identification of the *Drosophila* FMRFamide receptor (Meeusen *et al.*, 2002) and the leucokinin receptor in the snail *Lymnaea* (Cox *et al.*, 1997). Subsequent searches for leucokinin receptor orthologous genes in insects led to identification of novel leucokinin receptors in the cattle tick, *Boophilus microplus* (Holmes *et al.*, 2000, 2003) and in *Drosophila* (Radford *et al.*, 2002).

A gene homologous to the adenosine-like receptor has been found in both *Drosophila* and *Anopheles* genomes (Brody and Cravchik, 2000; Hill *et al.*, 2002a), but biological data are unavailable as yet. The findings in *Drosophila* will facilitate GPCR studies in other arthropod species where biological functions of those neuropeptides have been well characterized (see Section 5.5.5).

5.5.4.3. Family B: Secretin-Like Receptors

Insect GPCRs belonging to family B consist of secretin-like receptors (five genes in *Drosophila*) (**Table 3**), the *methuselah* group (∼11 genes), human epididymis-specific protein 6 (HE6; two genes), and latrophilin (one gene) (Brody and Cravchik, 2000; Hewes and Taghert, 2001; Hill *et al.*, 2002a). Two insect neuropeptides, diuretic hormone and amnesiac (Feany and Quinn, 1995; Zhong, 1995; Zhong and Pena, 1995), are similar to corticotropin releasing factor (CRF) and the pituitary adenylate cyclase activating peptide (PACAP), respectively, of vertebrate neuropeptides that are grouped with receptors in the family B. All five secretin-like receptors in the *Drosophila* family B (Brody and Cravchik, 2000; Hewes and Taghert, 2001) appear to have orthologs in the *Anopheles* genome (Hill *et al.*, 2002a).

The first receptor for an insect diuretic hormone (DH-R) was identified by screening a *Manduca* expression library with a tritiated ligand (Reagan, 1994). The sequence of the *Manduca* DH-R revealed that it is an ortholog of the vertebrate CRF receptor (Hewes and Taghert, 2001). Activation of the *Manduca* DH-R receptor expressed in COS-7 and Sf9 insect cells triggered cAMP elevation (Reagan, 1995). An orthologous gene from the house cricket, *Acheta domesticus*, was cloned and characterized in a functional expression system (Reagan, 1996). The orthologous *Drosophila* DH-R sequence reveals two closely related genes as candidates (CG32843 and CG4395), but no experimental evidence has appeared as yet.

The *methuselah* (*mth*) group of receptors, found by screening for *Drosophila* mutants with extended lifespan (Lin *et al.*, 1998), comprises a large group of GPCRs unique to insects. Phylogenetic analysis of the closely related species *A. gambiae* and *D. melanogaster* revealed recent expansions in the number of copies of the *mth* genes (Hill *et al.*, 2002a). Examination of *Drosophila mth* mutants suggested that

this gene product controls synaptic efficacy at the glutamatergic neuromuscular junction by increasing the rate of neurotransmitter exocytosis from the presynaptic terminal (Song *et al.*, 2002). The crystal structure of the *mth* ectodomain revealed potential ligand binding features in a shallow interdomain groove, although the ligand(s) have yet to be identified (West *et al.*, 2001).

Two genes encoding the GPCRs grouped with the human epididymis-specific protein 6 (HE6) were identified in *Drosophila*, but not in *Anopheles* (Brody and Cravchik, 2000; Hill *et al.*, 2002a). This receptor in vertebrates has an unusually long extracellular region characteristic of cell adhesion proteins, indicating potential roles in cell adhesion and signaling (Stacey *et al.*, 2000; Kierszenbaum, 2003). The genomes of *Drosophila* and *Anopheles* each have genes orthologous to vertebrate latrophilin. Vertebrate latrophilin is a putative receptor or binding protein for α-latrotoxin, a major component of black widow spider (genus *Lactrodectus*) venom. It appears to be involved in triggering calcium independent exocytosis, thus is also known as CIRL (calcium-independent receptor for α-latrotoxin) (Henkel and Sankaranarayanan, 1999). Latrophilin is thought to be a component of a large NMDA-receptor-associated signaling complex (Kreienkamp *et al.*, 2002b).

5.5.4.4. Family C: Metabotropic Glutamate Receptor and GABA$_B$ Receptor

Along with its well-known role in excitatory neuromuscular transmission, the neurotransmitter glutamate mediates both excitation and inhibition within the insect CNS (Parmentier *et al.*, 1998; Washio, 2002). Likewise, GABAergic inhibitory transmission occurs both in the peripheral and central nervous systems. In the CNS, these transmitters mediate both fast and slow transmission via ionotropic and metabotropic receptors, respectively. While our understanding of glutamate and GABA transmission in invertebrates is derived mostly from work on ionotropic receptors, metabotropic receptors are receiving increased attention because of new information gathered from genome sequences.

Analysis of the *Drosophila* genome revealed two genes encoding putative metabotropic glutamate receptors (mGluR) and three genes for metabotropic GABA$_B$ receptors, along with two other related genes not yet annotated (Brody and Cravchik, 2000). The *Anopheles* genome shows orthologous relationships with corresponding *Drosophila* genes in this group (Hill *et al.*, 2002a).

Drosophila mGluR receptors appear to function in the CNS by coupling to G$_i$, inhibiting activation of adenylyl cyclase (Parmentier *et al.*, 1996, 1998), although linkage to K^+ and Ca^{2+} channel modulation downstream could serve excitatory roles as well.

GABA is a major inhibitory neurotransmitter in the CNS of both vertebrates and invertebrates. Postsynaptic, ionotropic GABA$_A$ and GABA$_C$ receptors mediate fast inhibitory responses, while slow transmission occurs via metabotropic GABA$_B$ receptors. In vertebrates, GABA$_B$ receptors mediate reduction of transmitter release presynaptically (Slesinger *et al.*, 1997) and hyperpolarization postsynaptically (Isaacson and Vitten, 2003). Pharmacological studies of the GABA$_B$ receptor in the cockroach *Periplaneta americana* demonstrated hyperpolarizing, ionotropic responses of the fast coxal depressor motor neuron (Bai and Sattelle, 1995).

Heterodimerization of GABA$_B$R1 and R2 subunits is necessary for constitution of a functional receptor in vertebrates (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998). Furthermore, it is proposed that ligand binding involves only the GABA$_B$R1 subunit (Kniazeff *et al.*, 2002). Orthologous relationships of the subtypes GABA$_B$R1 and R2 are extended to *Drosophila* and the nematode *Caenorhabditis elegans* (Kniazeff *et al.*, 2002). As in the vertebrates, functional *Drosophila* GABA$_B$ receptors require heterodimerization of GABA$_B$R1 and R2 subunits (Mezler *et al.*, 2001). A third *Drosophila* subunit, GABA$_B$R3, appears to be an insect-specific subtype expressed in the CNS.

5.5.4.5. Other GPCRs: Odorant Receptor, Gustatory Receptor, Atypical 7TM Proteins

Analysis of genomic sequences in *Drosophila* and *Anopheles* indicates that the largest groups of GPCRs in insects are odorant and gustatory receptors (~70 genes for each) (Clyne *et al.*, 1999, 2000; Scott *et al.*, 2001; Hill *et al.*, 2002a). These receptors appear to have evolved quickly and comprise largely divergent subgroups having little if any conserved amino acid sequences in most cases. Evidence for functionality as odorant receptors has been provided by heterologous expression and bioassay (Stortkuhl and Kettler, 2001; Wetzel *et al.*, 2001). A gustatory receptor sensing trehalose was identified from a *Drosophila* mutant (Ishimoto *et al.*, 2000; Dahanukar *et al.*, 2001). Homology based cDNA library screening led to cloning of a number of putative odorant receptors in *Heliothis virescens*, and to demonstration of tissue-specific expression in antennae. This work thus has expanded olfactory receptor studies from *Drosophila* to other insect species (Krieger *et al.*, 2002).

Another group of putative receptors having seven transmembrane topology is classified separately as

"atypical 7TM proteins." This class includes frizzled (*fz*), smoothened, and bride of sevenless (bos), most of which were initially identified as *Drosophila* mutants. The function of *fz*, four homologous copies of which are found in *Drosophila* and *Anopheles*, appears to be in establishing morphogenic symmetry and cell polarity during development (Bhanot *et al.*, 1999; Strutt, 2001).

5.5.5. Intracellular Signaling Pathways Triggered by GPCRs

Activation of GPCRs by extracellular ligands at the cell surface leads to a rich diversity of intracellular signal transduction pathways, beginning with activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins), comprised of α, β, and γ subunits (**Figure 1**). The α subunit binds guanosine nucleotides, GDP in the resting state and GTP following activation of the GPCR with which the G-protein is associated. Upon GPCR activation, GTP binding to the α subunit of the G-protein leads to dissociation of the α and βγ subunits. The βγ dimer does not dissociate further under physiological conditions. Both α and βγ subunits can bind independently to target proteins to initiate cellular responses.

The type of intracellular signaling that ensues GPCR activation depends on the associated G protein, most importantly the Gα subunit. Coupling with $G\alpha_s$ activates adenylyl cyclase (AC), whereas coupling with $G\alpha_i$ and $G\alpha_o$ suppresses AC activity. Coupling with $G\alpha_q$ activates phospholipase C, initiating phosphoinositide turnover and intracellular Ca^{2+} mobilization. Prediction of coupling specificity between GPCR and G protein subtypes has been attempted using precedent data from vertebrate GPCRs (Bourne, 1997; Wess, 1998; Horn *et al.*, 2000; Moller *et al.*, 2001). To what extent such methods are predictive for insect GPCR signaling remains an open question.

Numerous instances of Gβγ signaling also have been documented in the context of $G\alpha_{i/o}$ signaling. The target proteins for Gβγ include AC, PLCβ, potassium channels, and phosphatidylinositol 3-kinase. Perhaps the best known of these is K channel modulation following parasympathetic activation of heart muscarinic acetylcholine receptors to slow heartbeat frequency (Kofuji *et al.*, 1995). This response results from dissociation of $G\alpha_i$ and Gβγ, with the latter acting directly as a ligand to open G protein coupled inwardly rectifying potassium channels (GIRK) and hyperpolarize myocardial cells (Reuveny *et al.*, 1994; Wickman *et al.*, 1994). This signaling pathway provides a particularly clear example of how direct G protein modulation of ion channels leads to an important physiological response.

Analysis of the *Drosophila* genome sequence reveals seven, six, and two genes that are homologous to vertebrate Gα, Gβ, and Gγ subunits, respectively (**Table 4**). The seven Gα homologous genes are classified as $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i/o}$, the latter including $G\alpha_t$, the well-known transducin involved in phototransduction (Simon *et al.*, 1991; Neves *et al.*, 2002) (**Figure 4**). Genes homologous to the Gβ subunit appear to have larger diversity than their vertebrate counterparts (**Figure 4**). Only two copies of the Gγ subunit are found in *Drosophila* (**Figure 4**). Alternatively spliced forms of these genes appear to provide additional flexibility to signaling systems (de Sousa *et al.*, 1989; Ray and Ganguly, 1994; Talluri *et al.*, 1995).

Immunolocalization of G protein α subunits in *Drosophila* demonstrated that expression of $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i/o}$ is highly restricted in the CNS (Wolfgang *et al.*, 1990). Expression in photoreceptor synaptic terminals and antennal lobes suggests functional roles in transmission of primary sensory information. Distinct expression patterns in embryonic stages implies involvement of G protein-mediated sensory transmission in embryonic development (Wolfgang *et al.*, 1991). Expression profiles of Gα subtypes in *M. sexta* also were examined by immunohistochemistry, where they were shown to be expressed in subsets of CNS cells (Copenhaver *et al.*, 1995).

A salient characteristic of GPCR-induced signaling is the opportunity for amplification along multiple steps of the pathway. For example, a single GPCR binding event leads to dissociation of α and βγ subunits, each of which can trigger downstream signaling pathways. In instances where the G protein subunit triggers second messengers through activation of enzymes such as AC or PLC, a second level of amplification occurs. Downstream activation of additional enzymes such as kinases or phosphatases provides a third level of amplification. Thus activation of a very small number of GPCRs can elicit highly significant biochemical responses in target cells. The diversity of downstream signal transduction pathways is summarized in several reviews, and generalized current concepts based vertebrate studies may well be applicable to insects (Simon *et al.*, 1991; Morris and Malbon, 1999; Neves *et al.*, 2002).

5.5.6. Assignment of GPCR Functions

GPCRs regulate a plethora of cellular functions by modulating the activities of diverse intracellular

Table 4 *Drosophila* trimeric G protein subunits Gα, Gβ, and Gγ

Group	*Name*	*Synonym*	*Expression pattern*	*Reference*
Gα subunit				
Gq	Gα49B	CG17759, dgq, DGqα, DGαq	Chemosensory cells, photoreceptor cells	Lee *et al.* (1990, 1994), Talluri *et al.* (1995), Bahner *et al.* (2000), Ratnaparkhi *et al.* (2002)
	CG17760			
Gi/o	Gαo47A	CG2204, DGαo, dgo, Goα	Brain, ovary, cardioblast, CNS	de Sousa *et al.* (1989), Schmidt *et al.* (1989), Wolfgang *et al.* (1990, 1991), Fremion *et al.* (1999)
	Gαi65A	CG10060, Gαi , Giα, DGα1,	CNS	Wolfgang *et al.* (1990, 1991)
Gs	Gα73B	CG12232, Gfα	Embryonic stage, midgut	Quan *et al.* (1993)
	Gαs60A	CG2835, Gαs, Gsα, Dgsα, dgs, Gs	Neuropil in CNS	Wolfgang *et al.* (1990, 1991, 2001)
	Concertina	CG17678, cta	Embryonic mesoderm	Parks and Wieschaus (1991)
Gβ subunit				
	CG2812			
	CG3004			
	EG:86E4.3	CG17766		
	Gβ13F	CG10545, dgβ, Gβ, G-protein β 13F	Eye	Yarfitz *et al.* (1988, 1991)
	Gβ5			
	Gβ76C	CG8770		
Gγ subunit				
	Gγ1	CG8261, D-Gγ1, dg1α	CNS	Ray and Ganguly (1994)
	Gγ30A	CG18511, CG3694, Gγe	Eye	Schulz *et al.* (1999)

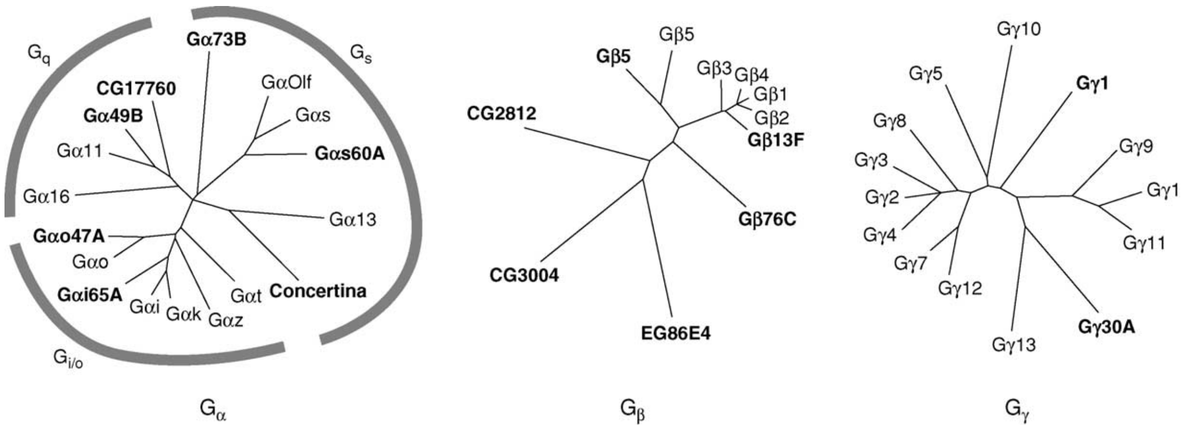


Figure 4 Phylogram for *Drosophila* G proteins with vertebrate G proteins. *Drosophila* G proteins are in bold letters.

target proteins, including ion channels, metabolic enzymes, and transporters. These actions lead to cellular responses important for embryonic, postembryonic, and reproductive development, homeostatic processes, responses to environmental stimuli, and induction of various context-driven behavioral states.

Strategies for functional identification of GPCRs have gone through several phases since the initial breakthroughs with β-adrenergic and muscarinic acetylcholine receptors (Dixon *et al.*, 1986; Kubo *et al.*, 1986; Yarden *et al.*, 1986). These first discoveries began with direct protein purification and partial amino acid sequencing of receptors with known ligands. This allowed for construction of oligonucleotide probes, cDNA library screening, and eventual cloning and sequencing of entire receptors. The next wave of GPCR identification occurred through expression cloning of, among others, neurokinin2 and serotonin receptors using early stage oocytes of the African clawed toad, *Xenopus laevis* (Masu *et al.*, 1987; Julius *et al.*,

1988). Taking advantage of sequence information from earlier work, homology cloning based on nucleotide sequence identity provided relatively easy and productive approaches to further receptor identification. Such homology searches led to the identification of the first insect GPCR, the muscarinic acetylcholine receptor (Shapiro *et al.*, 1989). Finally, availability of entire genome sequences from an increasing number of organisms has led to an explosive phase of *in silico* discoveries of putative GPCRs (Kim and Carlson, 2002), while homology search using cDNA library screening and degenerate polymerase chain reaction (PCR) is still highly effective for GPCR identification in species for which whole genome sequences are not yet available.

Whereas early efforts focused on receptors with known function, the recent era of genome discovery has generated numerous "orphan receptors" with unknown ligands and uncertain functions. For example, large numbers of putative GPCR in insects have been identified and analyzed from the genome sequences of *Drosophila* and *Anopheles* (Hewes and Taghert, 2001; Hill *et al.*, 2002a). Homology analysis of putative *Drosophila* GPCRs and particularly for neuropeptide receptors (Hewes and Taghert, 2001) has provided a foundation for GPCR deorphaning based on comparisons with mammalian genomes. This analysis, combined with improvements in heterologous expression systems and reporter constructs, has increased the rate of GPCR deorphaning during the past several years.

Deorphaning putative GPCRs through identification of endogenous ligands depends primarily on heterologous expression within a suitable bioassay system, and characterization of interactions between candidate ligands through biochemical or functional methods. The choice of expression and assay systems for a given GPCR gene entail practical considerations based on knowledge of previous studies. Since the assay primarily focuses on ligand–receptor interaction rather than endogenous downstream pathways, it often uses an exogenously introduced reporter system. The most frequently used expression systems have been the *Xenopus* oocyte expression system and various cell lines. The expression system needs to be chosen according to certain criteria: paucity of receptor endogenous to the expression system, which might cross-react with ligands under investigation, robust expression of the heterologous receptor, localization of the receptor on the cell membrane, appropriate posttranslational processing, and efficient coupling to downstream signaling pathways for activation of reporters. **Tables 1–3** summarize assay systems currently used for studies of insect GPCRs.

5.5.6.1. Functional GPCR Assays: Coupling with Reporters

Given the diversity of functions regulated by GPCRs, it is not surprising that they are targeted by a high percentage of pharmaceutical drugs currently on the market. For this reason, considerable efforts have been devoted to development of methods for high-throughput screening of novel GPCR agonists and antagonists. It seems likely that similar approaches may lead to future insectidical agents.

The most straightforward functional assays for GPCRs in oocytes or cell lines register Ca^{2+} mobilization as an increased fluorescence or luminescence. This cellular response is most commonly a result of $G\alpha_q$ activation and phosphoinositol metabolism, leading to IP3 mediated Ca^{2+} release from intracellular stores (Neves *et al.*, 2002). Commonly used Ca^{2+} sensors generate fluorescence or luminescence signals (see Section 5.5.5.3). Many orphan GPCRs are coupled to other $G\alpha$ subunits that do not mobilize calcium. Consequently, efforts have been made to utilize more promiscuous G proteins coupled to the PLCβ pathway. One very successful method has been the use of a $G\alpha_q$ variant known as $G\alpha_{16}$, which couples widely divergent GPCRs to the phosphoinositide turnover pathway via PLC, leading to Ca^{2+} mobilization (review: Stables *et al.*, 1997; Kostenis, 2001) (**Figures 5 and 6**). For example, expression of a *Drosophila* odorant receptor with exogenous $G\alpha_{16}$ in *Xenopus* oocytes was the system used to identify its cognate odorant ligand (Wetzel *et al.*, 2001). Other approaches have involved engineering of chimeric G proteins (e.g., $G\alpha_{16/z}$) which couple a diversity of GPCRs to PLCβ (Liu *et al.*, 2003).

For fluorescence assays, cells expressing GPCRs are pretreated with membrane permeant calcium indicators such as Fura2-AM, fast green AM, or fluo-3 AM (Meeusen *et al.*, 2002; Choi *et al.*, 2003; Johnson *et al.*, 2003a). For luminescence responses in cells expressing aequorin, pretreatment with the cofactor coelenterazine is necessary. Fluorescence or luminescence microplate readers having injection ports can be used for introduction of the cells into microplate wells that contain various concentrations of ligand. For high-throughput assay, simultaneous injection and reading of a plate for 96 or 384 wells also has been developed (Le Poul *et al.*, 2002).

Coupling to G_s normally leads to increases in AC activity. Experiments with the *Xenopus* oocyte expression system have demonstrated that $G\alpha_s$ activation can gate exogenous inwardly rectifying potassium channels GIRK1 and GIRK2 through the direct action of $G\beta\gamma$ (Kofuji *et al.*, 1995; Lim *et al.*,

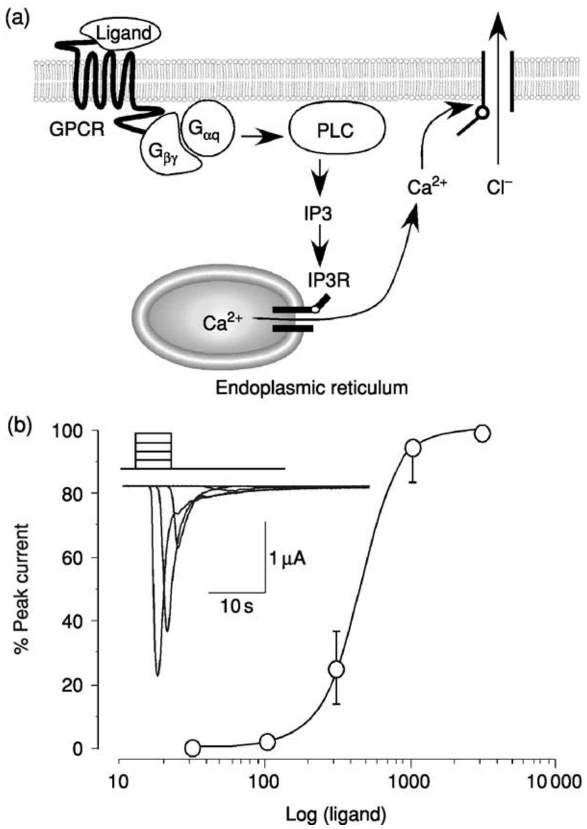


Figure 5 (a) A presumed signaling pathway in the *Xenopus* oocyte assay system for orphan GPCRs. GPCR expressed from injected cRNA transduce signal through phospholipase C (PLC), inositol triphosphate (IP3), and IP3 receptor triggering Ca^{2+} mobilization from intracellular Ca^{2+} storage. Ca^{+} activates chloride channels, resulting in inward currents. (b) Progressively higher concentrations of ligands generate larger currents. Mean values and corresponding standard deviations from multiple experiments are plotled as a function of ligand concentration.

1995; Peleg *et al.*, 2002). Expression of these exogenous GIRK subtypes in the proper stoichiometery can lead to robust inward K^+ currents, and hence provide a convenient assay for activation of $G\alpha_s$.

Other approaches for detecting $G\alpha_s$ activation have included measurements of increased cAMP levels (i.e., serotonin receptor 5HTR7) (Witz *et al.*, 1990; Saudou *et al.*, 1992; Obosi *et al.*, 1996). For $G\alpha_i$ activation, inhibition of AC activity can be detected through a reduction in forskolin-inducible cAMP increase (i.e., tyramine receptors) (Kutsukake *et al.*, 2000; Poels *et al.*, 2001).

5.5.6.2. Expression of GPCRs in *Xenopus* Oocytes

Advantages of using unfertilized *Xenopus* oocytes are many, not the least of which is the absence of endogenous GPCRs (Lee and Durieux, 1998). Injection of complementary RNA prepared with 5′ and 3′ untranslated regions of *Xenopus* β-globin gene results in sufficient expression in stage 5 or 6 oocytes for robust bioassays (Jespersen *et al.*, 2002). The oocyte contains sufficient machinery to link activation of exogenously introduced GPCRs to cellular responses.

The most routine use of oocytes is coupling of heterologously expressed GPCRs with endogenous G_q, leading to mobilization of intracellular Ca^{2+} through PLCβ activation and phosphoinositide turnover. This results in opening of Ca^{2+} dependent Cl^- channels on the oocyte plasma membrane and large inward currents that can be quantified by the two-electrode voltage clamp technique (**Figure 5**). This assay can be made considerably more flexible through the introduction of $G\alpha_{16}$, chimeric G proteins, or coupling to GIRK K^+ channels.

A precise perfusion system is required for generation of large sets of data using oocytes. Park *et al.* (2002) used a liquid chromatography pump and associated Rheodyne injection valve, supplying a continuous flow of buffer at a flow rate of $1.2\,\text{ml}\,\text{min}^{-1}$. Various concentrations of ligands were applied in standard 100 μl volumes of ligand through the injection valve, providing a 5 s exposure of ligand solution to the oocyte (Park *et al.*, 2002). This system allows precise application of a given concentration of ligand in a continuous flow of buffer, while caution must be exercised in the use of lipophilic ligands that may persist in the walls of the perfusion system even after extensive washes.

A challenge using *Xenopus* oocytes for GPCR assay is rapid desensitization following receptor activation. The degree of desensitization is highly variable and dependent on the specific GPCR expressed (Park *et al.*, 2002). In the case of *Drosophila* ETH receptors, the assay in oocytes was confounded by desensitization (Park, unpublished data). Even very low concentrations that did not elicit measurable currents were sufficient to cause desensitization. In such a case, alternative expression and assay systems using cell lines are preferable, where measurement of β-arrestin activity has proved to be a very effective method (see Section 5.5.6.4).

5.5.6.3. Assay of GPCR Activation in Cell Lines

Expression of GPCRs in cell lines currently is the method of choice both for deorphaning receptors and for large-scale, rapid-throughput drug screening for agonists and antagonists. Considerations in the choice of cell lines include high-level expression and proper processing of the exogenous GPCR gene, knowledge of the endogenous signaling systems operating, and the efficacy of the reporter system.

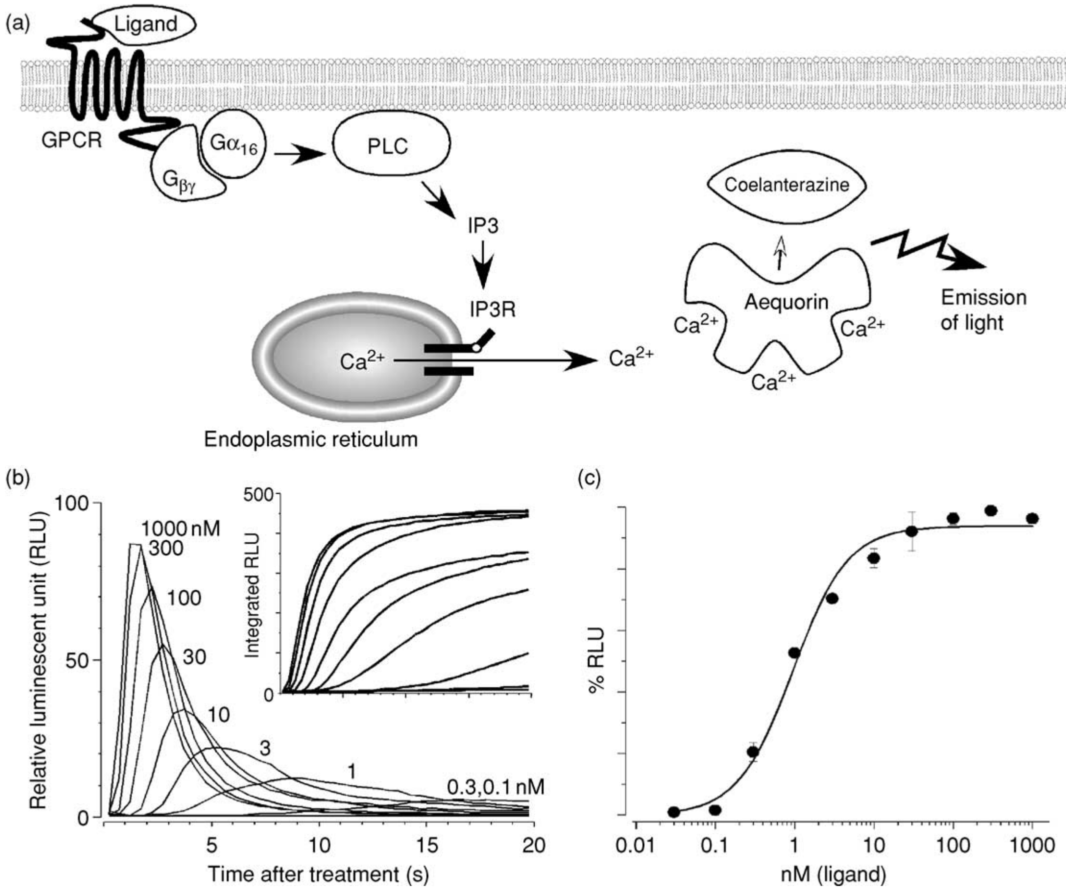


Figure 6 (a) A presumed signaling pathway operating in the aequorin assay for orphan GPCRs. The expressed heterologous GPCR transduces ligand binding through $G\alpha_{16}$, phospholipase C (PLC), inositol triphosphate (IP_3), and IP_3 receptor, resulting in Ca^{2+} mobilization from intracellular stores. Binding of Ca^{2+} to aequorin with the cofactor coelanterazine results in emission of light. (b) Progressively higher concentrations of ligand generate a more intense light response. (c) Integrated luminescence during a given time is used to generate the concentration–response curve.

Cell lines generally used for expression of insect GPCRs are Chinese hamster ovary (CHO-K1) cells, human embryonic kidney (HEK) cells, and Schneider's *Drosophila* (S2) cells (see **Tables 1 and 2**). All of these cells are known for their efficient coupling of GPCR activation to endogenous downstream signaling pathways. For instance, GPCRs expressed in CHO-K1 cells can be coupled to the PLC–calcium mobilization pathway, presumably via activation of G_q. A summary of downstream coupling mechanisms and signaling pathways for GPCRs in each cell line can be found at http://www.tumor-gene.org. Characteristics of *Drosophila* S2 cells are found in two recent publications (Van Poyer *et al.*, 2001; Torfs *et al.*, 2002b).

Both ligand binding assays and functional assays can be performed using cell line expression systems, depending on the need and situation. Affinity constants, receptor density, and kinetics of binding of heterologously expressed receptors can be investigated by using radioligands and compared with data obtained from native receptors (e.g., Johnson *et al.*, 2003b; Meeusen *et al.*, 2002). An advantage of the binding assay is that the biochemical properties of the expressed GPCR can be determined independent of coupling efficiency to downstream signaling and reporter activation.

5.5.6.4. Other GPCR Reporter Assays

While functional assays for GPCRs have been improved and refined through introduction of various reporter molecules into the expression system, rapid desensitization can severely attenuate responses. An effective strategy for dealing with this problem is to detect the interactions of β-arrestin with the GPCR. In this instance, desensitization of the GPCR can be monitored using bioluminescence resonance energy transfer (BRET). GPCR fused to *Renilla* luciferase is used for the cell line stably expressing arrestin fused to green fluorescent protein (GFP) (Barak *et al.*, 1997; Bertrand *et al.*, 2002). Activation of the GPCR leads to interaction between the

arrestin::GFP and GPCR::luciferase resulting in the BRET from the donor luciferase to the acceptor GFP. GPCR activation can be also measured by downstream gene regulation. Activation of GPCR coupled to AC and elevation of cAMP is monitored by expression of reporter gene controlled by cAMP responsive elements (CREs) (Chen *et al.*, 1995; Durocher *et al.*, 2000). It is thought that the CRE is activated though both $G\alpha_s$ and $G\alpha_q$ pathways (Durocher *et al.*, 2000). A simpler approach is to simply follow migration of a β-arrestin–GFP fusion protein to the cell membrane following GPCR activation (Barak *et al.*, 1997; Johnson *et al.*, 2003b).

The use of heterologous expression systems to obtain pharmacological profiles of GPCRs should be interpreted with appropriate caution, since it is often likely that critical transduction components unique to the natural physiological system will be absent. Thus the ligand affinities of certain GPCRs may be under the strong influence of native G protein subtypes with which they are associated (Kostenis, 2001). Even more complexity can be introduced in instances where relative stoichiometries of receptors to other cellular signaling components affect the assay system (Kenakin, 1997, 2003). Finally, the availability of specific G proteins appears to affect ligand binding affinity, as in the case of dopamine receptors (Reale *et al.*, 1997; Cordeaux and Hill, 2002; Gazi *et al.*, 2003).

5.5.6.5. Identifying Biological Functions of GPCRs

Understanding the biological function of a particular signaling pathway has been advanced through examination of ligand actions in large insects amenable to physiological experimentation. Important tools for endocrinological studies have been classical physiological experiments involving ligation, surgical extirpation and implantation of organs and tissues, parabiosis, and transplantations followed by bioassay-guided chemical isolation of the ligand on a particular system. Study of receptors mediating these processes provides further in-depth understanding of the mode of action of chemical signals at the molecular and cellular levels.

Biochemical and pharmacological data obtained through heterologous expression of a GPCR provides the first step in understanding molecular mechanisms of a signaling pathway and association of a putative authentic receptor with its ligand and physiological actions. Experimental evidence supporting an endogenous ligand–receptor interaction could include: highest affinity functional or biochemical interaction between the ligand and the receptor (usually at nanomolar to picomolar concentrations of ligand), spatial and temporal expression patterns of the GPCR that concurs with biological data, and mutant phenotypes that are in accordance with biological function of the signaling pathway.

Chemical isolation of unknown ligands for heterologously expressed orphan GPCRs is an increasingly useful method for functional analysis, especially with recently developed tools in proteomics. This approach is referred as reverse physiology (Birgul *et al.*, 1999) or reverse pharmacology (Civelli *et al.*, 2001) where the natural ligand for a receptor is sought. This approach contrasts with general approaches in which receptors are sought for a given ligand. Reverse physiology was successful in associating the peptide allatostatin A with its physiological receptor by isolating the peptide activating a *Drosophila* GPCR that is homologous to the somatostatin receptor of vertebrates (Birgul *et al.*, 1999). Three steps of peptide purification by cation-exchange column and reverse phase chromatography succeeded in isolation of a fraction active on the GPCR of interest. The final active fraction purified was analyzed by a matrix-assisted laser desorption ionization post source decay (MALDI-PSD) time-of flight spectrum for obtaining the sequence. A similar approach was successful for deorphaning *Drosophila* CG2114 as an FMRFamide receptor (Meeusen *et al.*, 2002).

Multidisciplinary approaches have been successful in a number of cases. Dow and his group studied signaling systems *Drosophila* Malpighian tubules mediating diuretic functions (review: Dow and Davies, 2003). Three neuroendocrine peptides and their signaling pathways implicated in the activation of fluid secretion were examined: cardioacceleratory peptide, diuretic hormone, and leucokinin. A combination of physiology, pharmacology, and *Drosophila* genetics elucidated downstream signaling pathways at the molecular and cellular levels. Specific GPCRs activated by leucokinin (CG10626) (Radford *et al.*, 2002) and cardioacceleratory peptide (CG14575) (Park *et al.*, 2002) were identified in functional assays. In the case of the leukokinin receptor, immunohistochemistry has been used to study its expression in stellate cells, a subtype of Malpighian tubule epithelial cell previously shown to respond to leucokinin by Ca^{2+} mobilization activating chloride current and fluid secretion (Rosay *et al.*, 1997; Radford *et al.*, 2002).

5.5.7. Conclusions

We find ourselves in an exciting era of exponential growth of knowledge regarding GPCR structure

and function. Knowledge of these receptors obtained in basic science could well to be applicable to the development of innovative pest control measures. Disruption of neuropeptide signaling systems through use of peptide hormone mimetics targeting the GPCRs also shows increasing promise (Nachman *et al.*, 2001, 2002a, 2002b). Chemical properties of pyrokinin analogs have been improved for increased cuticle penetrability and for resistance to peptidases (Nachman *et al.*, 2002a, 2000b). A novel method for delivering compounds to target GPCRs was attempted through use of a fusion protein combining lectin with allatostatin (Fitches *et al.*, 2002). By this means, allatostatin is delivered to insect hemolymph following oral ingestion and inhibits feeding and growth of the insect tomato moth (*Lacanobia oleracea*). With developing new biotechnology, GPCRs, particularly those mediating peptide signaling, may be excellent targets for pest control strategies providing an optimal range of specificity.

References

Adams, M.E., Zitnan, D., **1997**. Identification of ecdysis-triggering hormone in the silkworm *Bombyx mori*. *Biochem. Biophys. Res. Commun. 230*, 188–191.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., *et al.*, **1997**. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res. 25*, 3389–3402.

Alvarez, C.E., Robison, K., Gilbert, W., **1996**. Novel Gq alpha isoform is a candidate transducer of rhodopsin signaling in a *Drosophila* testes-autonomous pacemaker. *Proc. Natl Acad. Sci. USA 93*, 12278–12282.

Andretic, R., Hirsh, J., **2000**. Circadian modulation of dopamine receptor responsiveness in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 97*, 1873–1878.

Angers, S., Salahpour, A., Bouvier, M., **2002**. Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu. Rev. Pharmacol. Toxicol. 42*, 409–435.

Arakawa, S., Gocayne, J.D., McCombie, W.R., Urquhart, D.A., Hall, L.M., *et al.*, **1990**. Cloning, localization, and permanent expression of a *Drosophila* octopamine receptor. *Neuron 4*, 343–354.

Auerswald, L., Birgul, N., Gäde, G., Kreienkamp, H.J., Richter, D., **2001**. Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. *Biochem. Biophys. Res. Commun. 282*, 904–909.

Baggerman, G., Cerstiaens, A., De Loof, A., Schoofs, L., **2002**. Peptidomics of the larval *Drosophila melanogaster* central nervous system. *J. Biol. Chem. 277*, 40368–40374.

Bahner, M., Sander, P., Paulsen, R., Huber, A., **2000**. The visual G protein of fly photoreceptors interacts with the PDZ domain assembled INAD signaling complex via direct binding of activated Galpha(q) to phospholipase cbeta. *J. Biol. Chem. 275*, 2901–2904.

Bai, D., Sattelle, D., **1995**. A $GABA_B$ receptor on an identified insect motor neurone. *J. Exp. Biol. 198*, 889–894.

Baker, J.D., Truman, J.W., **2002**. Mutations in the *Drosophila* glycoprotein hormone receptor, rickets, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. *J. Exp. Biol. 205*, 2555–2565.

Barak, L.S., Ferguson, S.S., Zhang, J., Caron, M.G., **1997**. A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J. Biol. Chem. 272*, 27497–27500.

Benton, M.J., Ayala, F.J., **2003**. Dating the tree of life. *Science 300*, 1698–1700.

Bertrand, L., Parent, S., Caron, M., Legault, M., Joly, E., *et al.*, **2002**. The BRET2/arrestin assay in stable recombinant cells: a platform to screen for compounds that interact with G protein-coupled receptors (GPCRS). *J. Recept. Signal Transduct. Res. 22*, 533–541.

Bhanot, P., Fish, M., Jemison, J.A., Nusse, R., Nathans, J., *et al.*, **1999**. Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during *Drosophila* embryonic development. *Development 126*, 4175–4186.

Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., **1999**. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. *EMBO J. 18*, 5892–5900.

Blake, A.D., Anthony, N.M., Chen, H.H., Harrison, J.B., Nathanson, N.M., *et al.*, **1993**. *Drosophila* nervous system muscarinic acetylcholine receptor: transient functional expression and localization by immunocytochemistry. *Mol. Pharmacol. 44*, 716–724.

Blenau, W., Balfanz, S., Baumann, A., **2000**. Amtyr1: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. *J. Neurochem. 74*, 900–908.

Blenau, W., Baumann, A., **2001**. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol. 48*, 13–38.

Blenau, W., Erber, J., Baumann, A., **1998**. Characterization of a dopamine D1 receptor from *Apis mellifera*: cloning, functional expression, pharmacology, and mRNA localization in the brain. *J. Neurochem. 70*, 15–23.

Bockaert, J., Pin, J.P., **1999**. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J. 18*, 1723–1729.

Borowsky, B., Adham, N., Jones, K.A., Raddatz, R., Artymyshyn, R., *et al.*, **2001**. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc. Natl Acad. Sci. USA 98*, 8966–8971.

Bourne, H.R., **1997**. How receptors talk to trimeric G proteins. *Curr. Opin. Cell Biol. 9*, 134–142.

Brady, A.E., Limbird, L.E., **2002**. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. *Cell. Signal. 14*, 297–309.

Briscoe, A.D., **2001**. Functional diversification of lepidopteran opsins following gene duplication. *Mol. Biol. Evol. 18*, 2270–2279.

Briscoe, A.D., **2002**. Homology modeling suggests a functional role for parallel amino acid substitutions between bee and butterfly red- and green-sensitive opsins. *Mol. Biol. Evol. 19*, 983–986.

Briscoe, A.D., Chittka, L., **2001**. The evolution of color vision in insects. *Annu. Rev. Entomol. 46*, 471–510.

Brody, T., Cravchik, A., **2000**. *Drosophila melanogaster* G protein-coupled receptors. *J. Cell Biol. 150*, F83–F88.

Cazzamali, G., Grimmelikhuijzen, C.J., **2002**. Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 12073–12078.

Cazzamali, G., Saxild, N., Grimmelikhuijzen, C., **2002**. Molecular cloning and functional expression of a *Drosophila* corazonin receptor. *Biochem. Biophys. Res. Commun. 298*, 31–36.

Chen, W., Shields, T.S., Stork, P.J., Cone, R.D., **1995**. A colorimetric assay for measuring activation of Gs- and Gq-coupled signaling pathways. *Analyt. Biochem. 226*, 349–354.

Choi, M.Y., Fuerst, E.J., Rafaeli, A., Jurenka, R., **2003**. Identification of a G protein-coupled receptor for pheromone biosynthesis activating neuropeptide from pheromone glands of the moth *Helicoverpa zea*. *Proc. Natl Acad. Sci. USA 100*, 9721–9726.

Chothia, C., Gough, J., Vogel, C., Teichmann, S.A., **2003**. Evolution of the protein repertoire. *Science 300*, 1701–1703.

Chou, W.H., Hall, K.J., Wilson, D.B., Wideman, C.L., Townson, S.M., *et al.*, **1996**. Identification of a novel *Drosophila* opsin reveals specific patterning of the R7 and R8 photoreceptor cells. *Neuron 17*, 1101–1115.

Chou, W.H., Huber, A., Bentrop, J., Schulz, S., Schwab, K., *et al.*, **1999**. Patterning of the R7 and R8 photoreceptor cells of *Drosophila*: evidence for induced and default cell-fate specification. *Development 126*, 607–616.

Civelli, O., Nothacker, H.P., Saito, Y., Wang, Z., Lin, S.H., *et al.*, **2001**. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. *Trends Neurosci. 24*, 230–237.

Clyne, P.J., Warr, C.G., Carlson, J.R., **2000**. Candidate taste receptors in *Drosophila*. *Science 287*, 1830–1834.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., *et al.*, **1999**. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron 22*, 327–338.

Colas, J.F., Launay, J.M., Kellermann, O., Rosay, P., Maroteaux, L., **1995**. *Drosophila* 5-HT2 serotonin receptor: coexpression with fushi-tarazu during segmentation. *Proc. Natl Acad. Sci. USA 92*, 5441–5445.

Colas, J.F., Launay, J.M., Vonesch, J. L., Hickel, P., Maroteaux, L., **1999**. Serotonin synchronises convergent extension of ectoderm with morphogenetic gastrulation movements in *Drosophila*. *Mech. Devel. 87*, 77–91.

Copenhaver, P.F., Horgan, A.M., Nichols, D.C., Rasmussen, M.A., **1995**. Developmental expression of heterotrimeric G proteins in the nervous system of *Manduca sexta*. *J. Neurobiol. 26*, 461–484.

Cordeaux, Y., Hill, S.J., **2002**. Mechanisms of cross-talk between G-protein-coupled receptors. *Neurosignals 11*, 45–57.

Cordeaux, Y., Nickolls, S.A., Flood, L.A., Graber, S.G., Strange, P.G., **2001**. Agonist regulation of D(2) dopamine receptor/G protein interaction: evidence for agonist selection of G protein subtype. *J. Biol. Chem. 276*, 28667–28675.

Cowman, A.F., Zuker, C.S., Rubin, G.M., **1986**. An opsin gene expressed in only one photoreceptor cell type of the *Drosophila* eye. *Cell 44*, 705–710.

Cox, K.J., Tensen, C.P., Van der Schors, R.C., Li, K.W., van Heerikhuizen, H., *et al.*, **1997**. Cloning, characterization, and expression of a G-protein-coupled receptor from *Lymnaea stagnalis* and identification of a leucokinin-like peptide, PSFHSWSamide, as its endogenous ligand. *J. Neurosci. 17*, 1197–1205.

Dahanukar, A., Foster, K., van der Goes van Naters, W.M., Carlson, J.R., **2001**. A Gr receptor is required for response to the sugar trehalose in taste neurons of *Drosophila*. *Nat. Neurosci. 4*, 1182–1186.

Darlison, M.G., Richter, D., **1999**. Multiple genes for neuropeptides and their receptors: co-evolution and physiology. *Trends Neurosci. 22*, 81–88.

de Sousa, S.M., Hoveland, L.L., Yarfitz, S., Hurley, J.B., **1989**. The *Drosophila* Go alpha-like G protein gene produces multiple transcripts and is expressed in the nervous system and in ovaries. *J. Biol. Chem. 264*, 18544–18551.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., *et al.*, **1986**. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature 321*, 75–79.

Dolzer, J., Krannich, S., Fischer, K., Stengl, M., **2001**. Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol. 204*, 2781–2794.

Dow, J.A.T., Davies, S.A., **2003**. Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev. 83*, 687–729.

Durocher, Y., Perret, S., Thibaudeau, E., Gaumond, M.H., Kamen, A., *et al.*, **2000**. A reporter gene assay for high-throughput screening of G-protein-coupled receptors stably or transiently expressed in HEK293 EBNA cells grown in suspension culture. *Analyt. Biochem. 284*, 316–326.

Ebert, P.R., Rowland, J.E., Toma, D.P., **1998**. Isolation of seven unique biogenic amine receptor clones from the honey bee by library scanning. *Insect Mol. Biol. 7*, 151–162.

Egerod, K., Reynisson, E., Hauser, F., Cazzamali, G., Williamson, M., *et al.*, **2003a**. Molecular cloning and functional expression of the first two specific insect myosuppressin receptors. *Proc. Natl Acad. Sci. USA 100*, 9808–9813.

Egerod, K., Reynisson, E., Hauser, F., Williamson, M., Cazzamali, G., *et al.*, **2003b**. Molecular identification of the first insect proctolin receptor. *Biochem. Biophys. Res. Commun. 306*, 437–442.

Eriksen, K.K., Hauser, F., Schiott, M., Pedersen, K.M., Sondergaard, L., *et al.*, **2000**. Molecular cloning, genomic organization, developmental regulation, and a knock-out mutant of a novel leu-rich repeats-containing G protein-coupled receptor (DLGR-2) from *Drosophila melanogaster. Genome Res. 10*, 924–938.

Evans, P.D., **1980**. Biogenic amines in the insect nervous system. *Adv. Insect Physiol. 15*, 317–473.

Evans, P.D., **1993**. Molecular tudies on insect octopamine receptors. In: Pichon, Y. (Ed.), Comparative Molecular Neurobiology. Birkhauser, Basel, pp. 286–296.

Feany, M.B., Quinn, W.G., **1995**. A neuropeptide gene defined by the *Drosophila* memory mutant amnesiac. *Science 268*, 869–873.

Feiler, R., Harris, W.A., Kirschfeld, K., Wehrhahn, C., Zuker, C.S., **1988**. Targeted misexpression of a *Drosophila* opsin gene leads to altered visual function. *Nature 333*, 737–741.

Feng, G., Hannan, F., Reale, V., Hon, Y.Y., Kousky, C.T., *et al.*, **1996**. Cloning and functional characterization of a novel dopamine receptor from *Drosophila melanogaster. J. Neurosci. 16*, 3925–3933.

Feng, G., Reale, V., Chatwin, H., Kennedy, K., Venard, R., *et al.*, **2003**. Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster. Eur. J. Neurosci. 18*, 227–238.

Filipek, S., Teller, D.C., Palczewski, K., Stenkamp, R., **2003**. The crystallographic model of rhodopsin and its use in studies of other G protein-coupled receptors. *Annu. Rev. Biophys. Biomol. Struct. 32*, 375–397.

Fitches, E., Audsley, N., Gatehouse, J.A., Edwards, J.P., **2002**. Fusion proteins containing neuropeptides as novel insect contol agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect Biochem. Mol. Biol. 32*, 1653–1661.

Fremion, F., Astier, M., Zaffran, S., Guillen, A., Homburger, V., *et al.*, **1999**. The heterotrimeric protein Go is required for the formation of heart epithelium in *Drosophila*. *J. Cell Biol. 145*, 1063–1076.

Fryxell, K.J., Meyerowitz, E.M., **1987**. An opsin gene that is expressed only in the R7 photoreceptor cell of *Drosophila*. *EMBO J. 6*, 443–451.

Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Habata, Y., *et al.*, **2000**. Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. *J. Biol. Chem. 275*, 21068–21074.

Gäde, G., Hoffmann, K.H., Spring, J.H., **1997**. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev. 77*, 963–1032.

Garczynski, S.F., Brown, M.R., Shen, P., Murray, T.F., Crim, J.W., **2002**. Characterization of a functional neuropeptide F receptor from *Drosophila melanogaster. Peptides 23*, 773–780.

Gazi, L., Nickolls, S.A., Strange, P.G., **2003**. Functional coupling of the human dopamine D(2) receptor with Galpha(i1), Galpha(i2), Galpha(i3) and Galpha(o) G proteins: evidence for agonist regulation of G protein selectivity. *Br. J. Pharmacol. 138*, 775–786.

Goh, C.S., Cohen, F.E., **2002**. Co-evolutionary analysis reveals insights into protein–protein interactions. *J. Mol. Biol. 324*, 177–192.

Gomes, I., Jordan, B.A., Gupta, A., Rios, C., Trapaidze, N., *et al.*, **2001**. G protein coupled receptor dimerization: implications in modulating receptor function. *J. Mol. Med. 79*, 226–242.

Gotzes, F., Balfanz, S., Baumann, A., **1994**. Primary structure and functional characterization of a *Drosophila* dopamine receptor with high homology to human D1/5 receptors. *Recept. Channels 2*, 131–141.

Gotzes, F., Baumann, A., **1996**. Functional properties of *Drosophila* dopamine D1-receptors are not altered by the size of the N-terminus. *Biochem. Biophys. Res. Commun. 222*, 121–126.

Guerrero, F.D., **1997**. Transcriptional expression of a putative tachykinin-like peptide receptor gene from stable fly. *Peptides 18*, 1–5.

Ha, S.D., Kataoka, H., Suzuki, A., Kim, B.J., Kim, H.J., *et al.*, **2000**. Cloning and sequence analysis of cDNA for diuretic hormone receptor from *Bombyx mori*. *Mol. Cells 10*, 13–17.

Han, K.A., Millar, N.S., Davis, R.L., **1998**. A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci. 18*, 3650–3658.

Han, K.A., Millar, N.S., Grotewiel, M.S., Davis, R.L., **1996**. DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron 16*, 1127–1135.

Hannan, F., Hall, L.M., **1996**. Temporal and spatial expression patterns of two G-protein coupled receptors in *Drosophila melanogaster. Invertebr. Neurosci. 2*, 71–83.

Hardie, R.C., Raghu, P., **2001**. Visual transduction in *Drosophila*. *Nature 413*, 186–193.

Hauser, F., Nothacker, H.P., Grimmelikhuijzen, C.J., **1997**. Molecular cloning, genomic organization, and developmental regulation of a novel receptor from *Drosophila melanogaster* structurally related to members of the thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone/choriogonadotropin receptor family from mammals. *J. Biol. Chem. 272*, 1002–1010.

Hearn, M.G., Ren, Y., McBride, E.W., Reveillaud, I., Beinborn, M., *et al.*, **2002**. A *Drosophila* dopamine 2-like receptor: molecular characterization and identification of multiple alternatively spliced variants. *Proc. Natl Acad. Sci. USA 99*, 14554–14559.

Henkel, A.W., Sankaranarayanan, S., **1999**. Mechanisms of alpha-latrotoxin action. *Cell Tissue Res. 296*, 229–233.

Hermans, E., Challiss, R.A., **2001**. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. *Biochem. J. 359*, 465–484.

Hewes, R.S., Taghert, P.H., **2001**. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. *Genome Res. 11*, 1126–1142.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., *et al.*, **2002a**. G protein-coupled receptors in *Anopheles gambiae*. *Science 298*, 176–178.
Hill, E.S., Iwano, M., Gatellier, L., Kanzaki, R., **2002b**. Morphology and physiology of the serotonin-immunoreactive putative antennal lobe feedback neuron in the male silkmoth *Bombyx mori*. *Chem. Senses 27*, 475–483.
Holmes, S.P., Barhoumi, R., Nachman, R.J., Pietrantonio, P.V., **2003**. Functional analysis of a G protein-coupled receptor from the Southern cattle tick *Boophilus microplus* (Acari: Ixodidae) identifies it as the first arthropod myokinin receptor. *Insect Mol. Biol. 12*, 27–38.
Holmes, S.P., He, H., Chen, A.C., lvie, G.W., Pietrantonio, P.V., **2000**. Cloning and transcriptional expression of a leucokinin-like peptide receptor from the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). *Insect Mol. Biol. 9*, 457–465.
Horn, F., van der Wenden, E.M., Oliveira, L., IJzerman, A.P., Vriend, G., **2000**. Receptors coupling to G proteins: is there a signal behind the sequence? *Proteins 41*, 448–459.
Howard, A.D., Wang, R., Pong, S.-S., Mellin, T.N., Strack, A., *et al.*, **2000**. Identification of receptors for neuromedin U and its role in feeding. *Nature 406*, 70–74.
Huesmann, G.R., Cheung, C.C., Loi, P.K., Lee, T.D., Swiderek, K.M., *et al.*, **1995**. Amino acid sequence of CAP-2b, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*. *FEBS Lett. 371*, 311–314.
Isaacson, J.S., Vitten, H., **2003**. $GABA_B$ receptors inhibit dendrodendritic transmission in the rat olfactory bulb. *J. Neurosci. 23*, 2032–2039.
Ishimoto, H., Matsumoto, A., Tanimura, T., **2000**. Molecular identification of a taste receptor gene for trehalose in *Drosophila*. *Science 289*, 116–119.
Iversen, A., Cazzamali, G., Williamson, M., Hauser, F., Grimmelikhuijzen, C.J., **2002**. Molecular identification of the first insect ecdysis triggering hormone receptors. *Biochem. Biophys. Res. Commun. 299*, 924–931.
Jespersen, T., Grunnet, M., Angelo, K., Klaerke, D.A., Olesen, S.P., **2002**. Dual-function vector for protein expression in both mammalian cells and *Xenopus laevis* oocytes. *Biotechniques 32*, 536–538, 540.
Johard, H.A., Muren, J.E., Nichols, R., Larhammar, D.S., Nassel, D.R., **2001**. A putative tachykinin receptor in the cockroach brain: molecular cloning and analysis of expression by means of antisera to portions of the receptor protein. *Brain Res. 919*, 94–105.
Johnson, E.C., Bohn, L.M., Barak, L.S., Birse, R.T., Nassel, D.R., *et al.*, **2003a**. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. *J. Biol. Chem. 278*, 52172–52178.
Johnson, E.C., Garczynski, S.F., Park, D., Crim, J.W., Nassel, D.R., *et al.*, **2003b**. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 100*, 6198–6203.
Jones, K.A., Borowsky, B., Tamm, J.A., Craig, D.A., Durkin, M.M., *et al.*, **1998**. GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature 396*, 674–679.
Josefsson, L.G., **1999**. Evidence for kinship between diverse G-protein coupled receptors. *Gene 239*, 333–340.
Julius, D., MacDermott, A.B., Axel, R., Jessell, T.M., **1988**. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science 241*, 558–564.
Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., *et al.*, **1998**. GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature 396*, 683–687.
Kenakin, T., **1997**. Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol. Sci. 18*, 456–464.
Kenakin, T., **2003**. A guide to drug discovery: predicting therapeutic value in the lead optimization phase of drug discovery. *Nature Rev. Drug Discov. 2*, 429–438.
Kierszenbaum, A.L., **2003**. Epididymal G protein-coupled receptor (GPCR): two hats and a two-piece suit tailored at the GPS motif. *Mol. Reprod. Devel. 64*, 1–3.
Kim, J., Carlson, J.R., **2002**. Gene discovery by e-genetics: *Drosophila* odor and taste receptors. *J. Cell Sci. 115*, 1107–1112.
Kniazeff, J., Galvez, T., Labesse, G., Pin, J.P., **2002**. No ligand binding in the GB2 subunit of the GABA(B) receptor is required for activation and allosteric interaction between the subunits. *J. Neurosci. 22*, 7352–7361.
Kofuji, P., Davidson, N., Lester, H.A., **1995**. Evidence that neuronal G-protein-gated inwardly rectifying K+ channels are activated by G beta gamma subunits and function as heteromultimers. *Proc. Natl Acad. Sci. USA 92*, 6542–6546.
Kohout, T.A., Lefkowitz, R.J., **2003**. Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol. Pharmacol. 63*, 9–18.
Kolakowski, L.F., Jr., **1994**. GCRDb: a G-protein-coupled receptor database. *Recept. Channels 2*, 1–7.
Kostenis, E., **2001**. Is Galpha16 the optimal tool for fishing ligands of orphan G-protein-coupled receptors? *Trends Pharmacol. Sci. 22*, 560–564.
Kreienkamp, H.J., Larusson, H.J., Witte, I., Roeder, T., Birgul, N., *et al.*, **2002a**. Functional annotation of two orphan G-protein-coupled receptors, Drostar1 and -2, from *Drosophila melanogaster* and their ligands by reverse pharmacology. *J. Biol. Chem. 277*, 39937–39943.
Kreienkamp, H.J., Soltau, M., Richter, D., Bockers, T., **2002b**. Interaction of G-protein-coupled receptors with synaptic scaffolding proteins. *Biochem. Soc. Trans. 30*, 464–468.
Krieger, J., Raming, K., Dewer, Y.M., Bette, S., Conzelmann, S., *et al.*, **2002**. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci. 16*, 619–628.

Kubiak, T.M., Larsen, M.J., Burton, K. J., Bannow, C.A., Martin, R.A., *et al.*, **2002**. Cloning and functional expression of the first *Drosophila melanogaster* sulfakinin receptor DSK-R1. *Biochem. Biophys. Res. Commun. 291*, 313–320.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., *et al.*, **1986**. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature 323*, 411–416.

Kutsukake, M., Komatsu, A., Yamamoto, D., Ishiwa-Chigusa, S., **2000**. A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster. Gene 245*, 31–42.

Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., *et al.*, **2001**. Type A allatostatins from *Drosophila melanogaster* and *Diplotera punctata* activate two *Drosophila* allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. *Biochem. Biophys. Res. Commun. 286*, 895–901.

Le Poul, E., Hisada, S., Mizuguchi, Y., Dupriez, V.J., Burgeon, E., *et al.*, **2002**. Adaptation of aequorin functional assay to high throughput screening. *J. Biomol. Screen. 7*, 57–65.

Lee, A., Durieux, M.E., **1998**. The use of *Xenopus laevis* oocytes for the study of G protein-coupled receptors. In: Lynch, K.R. (Ed.), Identification and Expression of G Protein-Coupled Receptors. Wiley-Liss, New York, pp. 73–96.

Lee, Y.J., Dobbs, M.B., Verardi, M.L., Hyde, D.R., **1990**. *dgq*: a *Drosophila* gene encoding a visual system-specific G alpha molecule. *Neuron 5*, 889–898.

Lee, Y.J., Shah, S., Suzuki, E., Zars, T., O'Day, P.M., *et al.*, **1994**. The *Drosophila dgq* gene encodes a G alpha protein that mediates phototransduction. *Neuron 13*, 1143–1157.

Lenz, C., Williamson, M., Grimmelikhuijzen, C.J., **2000**. Molecular cloning and genomic organization of a second probable allatostatin receptor from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 273*, 571–577.

Lenz, C., Williamson, M., Hansen, G.N., Grimmelikhuijzen, C.J., **2001**. Identification of four *Drosophila* allatostatins as the cognate ligands for the *Drosophila* orphan receptor DAR-2. *Biochem. Biophys. Res. Commun. 286*, 1117–1122.

Li, X.J., Wolfgang, W., Wu, Y.N., North, R.A., Forte, M., **1991**. Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides. *EMBO J. 10*, 3221–3229.

Li, X.J., Wu, Y.N., North, R.A., Forte, M., **1992**. Cloning, functional expression, and developmental regulation of a neuropeptide Y receptor from *Drosophila melanogaster. J. Biol. Chem. 267*, 9–12.

Lim, N.F., Dascal, N., Labarca, C., Davidson, N., Lester, H.A., **1995**. A G protein-gated K channel is activated via beta 2-adrenergic receptors and G beta gamma subunits in *Xenopus* oocytes. *J. Gen. Physiol. 105*, 421–439.

Lin, Y.J., Seroude, L., Benzer, S., **1998**. Extended lifespan and stress resistance in the *Drosophila* mutant *methuselah*. *Science 282*, 943–946.

Liu, A.M., Ho, M.K., Wong, C.S., Chan, J.H., Pau, A.H., *et al.*, **2003**. Galpha(16/z) chimeras efficiently link a wide range of G protein-coupled receptors to calcium mobilization. *J. Biomol. Screen. 8*, 39–49.

Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., *et al.*, **1987**. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature 329*, 836–838.

Matsumoto, S., Kitamura, A., Nagasawa, H., Kataoka, H., Orikasa, C., *et al.*, **1990**. Functional diversity of a neurohormone produced by the subesophageal ganglion: molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide. *J. Insect Physiol. 36*, 427–432.

Maxwell, G.D., Tait, J.F., Hildebrand, J.G., **1978**. Regional synthesis of neurotransmitter candidates in the CNS of the moth *Manduca sexta*. *Comp. Biochem. Physiol. C 61*, 109–119.

McBride, E.W., Reveillaud, I., Ren, Y., Kopin, A.S., **2001**. Molecular cloning, genomic organization and expression of a *Drosophila* cholecystokinin-like receptor. *Annu. Drosophila Res. Conf. 42*, 432C.

Meeusen, T., Mertens, I., Clynen, E., Baggerman, G., Nichols, R., *et al.*, **2002**. Identification in *Drosophila melanogaster* of the invertebrate G protein-coupled FMRFamide receptor. *Proc. Natl Acad. Sci. USA 99*, 15363–15368.

Mertens, I., Meeusen, T., Huybrechts, R., De Loof, A., Schoofs, L., **2002**. Characterization of the short neuropeptide F receptor from *Drosophila melanogaster. Biochem. Biophys. Res. Commun. 297*, 1140–1148.

Mezler, M., Muller, T., Raming, K., **2001**. Cloning and functional expression of GABA(B) receptors from *Drosophila*. *Eur. J. Neurosci. 13*, 477–486.

Moller, S., Vilo, J., Croning, M.D., **2001**. Prediction of the coupling specificity of G protein coupled receptors to their G proteins. *Bioinformatics 17*, S174–S181.

Monnier, D., Colas, J.F., Rosay, P., Hen, R., Borrelli, E., *et al.*, **1992**. NKD, a developmentally regulated tachykinin receptor in *Drosophila*. *J. Biol. Chem. 267*, 1298–1302.

Montell, C., **1999**. Visual transduction in *Drosophila*. *Annu. Rev. Cell Devel. Biol. 15*, 231–268.

Morris, A.J., Malbon, C.C., **1999**. Physiological regulation of G protein-linked signaling. *Physiol. Rev. 79*, 1373–1430.

Morris, H.R., Panico, M., Karplus, A., Lloyd, P.E., Riniker, B., **1982**. Elucidation by FAB-MS of the structure of a new cardioactive peptide in *Aplysia*. *Nature 300*, 643–645.

Nachman, R.J., Strey, A., Isaac, E., Pryor, N., Lopez, J.D., *et al.*, **2002a**. Enhanced *in vivo* activity of peptidase-resistant analogs of the insect kinin neuropeptide family. *Peptides 23*, 735–745.

Nachman, R.J., Teal, P.E., Strey, A., **2002b**. Enhanced oral availability/pheromonotropic activity of

peptidase-resistant topical amphiphilic analogs of pyrokinin/PBAN insect neuropeptides. *Peptides 23*, 2035–2043.

Nachman, R.J., Teal, P.E., Ujvary, I., **2001**. Comparative topical pheromonotropic activity of insect pyrokinin/PBAN amphiphilic analogs incorporating different fatty and/or cholic acid components. *Peptides 22*, 279–285.

Nassel, D.R., **2002**. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol. 68*, 1–84.

Neves, S.R., Ram, P.T., Iyengar, R., **2002**. G protein pathways. *Science 296*, 1636–1639.

Nishi, S., Hsu, S.Y., Zell, K., Hsueh, A.J., **2000**. Characterization of two fly LGR (leucine-rich repeat-containing, G protein-coupled receptor) proteins homologous to vertebrate glycoprotein hormone receptors: constitutive activation of wild-type fly LGR1 but not LGR2 in transfected mammalian cells. *Endocrinology 141*, 4081–4090.

Noguchi, H., Hayakawa, Y., **2001**. Dopamine is a key factor for the induction of egg diapause of the silkworm, *Bombyx mori*. *Eur. J. Biochem. 268*, 774–780.

O'Tousa, J.E., Baehr, W., Martin, R.L., Hirsh, J., Pak, W.L., *et al.*, **1985**. The *Drosophila* ninaE gene encodes an opsin. *Cell 40*, 839–850.

Obosi, L.A., Schuette, D.G., Europe-Finner, G.N., Beadle, D.J., Hen, R., *et al.*, **1996**. Functional characterisation of the *Drosophila* 5-HTdro1 and 5-HTdro2B serotonin receptors in insect cells: activation of a G(alpha)s-like protein by 5-HTdro1 but lack of coupling to inhibitory G-proteins by 5-HTdro2B. *FEBS Lett. 381*, 233–236.

Orchard, I., Jan-Marino, R., ALange, A.B., **1993**. A multifunctional role for octopamine in locust flight. *Annu. Rev. Entomol. 38*, 227–249.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., *et al.*, **2000**. Crystal structure of rhodopsin: a G protein-coupled receptor. *Science 289*, 739–745.

Park, Y., Kim, Y.J., Adams, M.E., **2002**. Identification of G protein-coupled receptors for *Drosophila* PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand–receptor coevolution. *Proc. Natl Acad. Sci. USA 99*, 11423–11428.

Park, Y., Kim, Y.J., Dupriez, V., Adams, M.E., **2003**. Two subtypes of ecdysis triggering hormone receptor in *Drosophila melanogaster. J. Biol. Chem. 278*, 17710–17715.

Park, Y., Zitnan, D., Gill, S.S., Adams, M.E., **1999**. Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster. FEBS Lett. 463*, 133–138.

Parks, S., Wieschaus, E., **1991**. The *Drosophila* gastrulation gene concertina encodes a G alpha-like protein. *Cell 64*, 447–458.

Parmentier, M.L., Joly, C., Restituito, S., Bockaert, J., Grau, Y., *et al.*, **1998**. The G protein-coupling profile of metabotropic glutamate receptors, as determined with exogenous G proteins, is independent of their ligand recognition domain. *Mol. Pharmacol. 53*, 778–786.

Parmentier, M.L., Pin, J.P., Bockaert, J., Grau, Y., **1996**. Cloning and functional expression of a *Drosophila* metabotropic glutamate receptor expressed in the embryonic CNS. *J. Neurosci. 16*, 6687–6694.

Peleg, S., Varon, D., Ivanina, T., Dessauer, C.W., Dascal, N., **2002**. G(alpha)(i) controls the gating of the G protein-activated K(+) channel, GIRK. *Neuron 33*, 87–99.

Pierce, K.L., Lefkowitz, R.J., **2001**. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat. Rev. Neurosci. 2*, 727–733.

Pietrantonio, P.V., Jagge, C., McDowell, C., **2001**. Cloning and expression analysis of a 5HT7-like serotonin receptor cDNA from mosquito *Aedes aegypti* female excretory and respiratory systems. *Insect Mol. Biol. 10*, 357–369.

Poels, J., Suner, M.M., Needham, M., Torfs, H., De Rijck, J., *et al.*, **2001**. Functional expression of a locust tyramine receptor in murine erythroleukaemia cells. *Insect Mol. Biol. 10*, 541–548.

Pophof, B., **2000**. Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. *J. Comp. Physiol. A 186*, 307–313.

Quan, F., Wolfgang, W.J., Forte, M., **1993**. A *Drosophila* G-protein alpha subunit, Gf alpha, expressed in a spatially and temporally restricted pattern during *Drosophila* development. *Proc. Natl Acad. Sci. USA 90*, 4236–4240.

Radford, J.C., Davies, S.A., Dow, J.A., **2002**. Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem. 277*, 38810–38817.

Raina, A.K., Jaffe, H., Kempe, T.G., Keim, P., Blacher, R.W., *et al.*, **1989**. Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science 244*, 796–798.

Ratnaparkhi, A., Banerjee, S., Hasan, G., **2002**. Altered levels of Gq activity modulate axonal pathfinding in *Drosophila*. *J. Neurosci. 22*, 4499–4508.

Ray, K., Ganguly, R., **1994**. Organization and expression of the *Drosophila melanogaster* D-G gamma 1 gene encoding the G-protein gamma subunit. *Gene 148*, 315–319.

Raymond, V., Hamon, A., Grau, Y., Lapied, B., **1999**. DmGluRA, a *Drosophila* metabotropic glutamate receptor, activates G-protein inwardly rectifying potassium channels in *Xenopus* oocytes. *Neurosci. Lett. 269*, 1–4.

Reagan, J.D., **1994**. Expression cloning of an insect diuretic hormone receptor, a member of the calcitonin/secretin receptor family. *J. Biol. Chem. 269*, 9–12.

Reagan, J.D., **1995**. Functional expression of a diuretic hormone receptor in baculovirus-infected insect cells: evidence suggesting that the N-terminal region of diuretic hormone is associated with receptor activation. *Insect Biochem. Mol. Biol. 25*, 535–539.

Reagan, J.D., **1996**. Molecular cloning and function expression of a diuretic hormone receptor from the house cricket, *Acheta domesticus*. *Insect Biochem. Mol. Biol. 26*, 1–6.

Reale, V., Hannan, F., Hall, L.M., Evans, P.D., **1997**. Agonist-specific coupling of a cloned *Drosophila melanogaster* D1-like dopamine receptor to multiple second messenger pathways by synthetic agonists. *J. Neurosci. 17*, *6545–6553*.

Reuveny, E., Slesinger, P.A., Inglese, J., Morales, J.M., Iniguez-Lluhi, J.A., *et al.*, **1994**. Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits. *Nature 370*, 143–146.

Ridley, M., **1993**. Evolution. Blackwell Scientific Publications, Oxford.

Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., **2002**. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science 298*, 172–175.

Robb, S., Cheek, T.R., Hannan, F.L., Hall, L.M., Midgley, J.M., *et al.*, **1994**. Agonist-specific coupling of a cloned *Drosophila* octopamine/tyramine receptor to multiple second messenger systems. *EMBO J. 13*, 1325–1330.

Roeder, T., **2002**. Biochemistry and molecular biology of receptors for biogenic amines in locusts. *Microsc. Res. Tech. 56*, 237–247.

Roeder, T., **2003**. Metabotropic histamine receptors: nothing for invertebrates? *Eur. J. Pharmacol. 466*, 85–90.

Rosay, P., Davies, S.A., Yu, Y., Sozen, A., Kaiser, K., *et al.*, **1997**. Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci. 110*, 1683–1692.

Rosenkilde, C., Cazzamali, G., Williamson, M., Hauser, F., Sondergaard, L., *et al.*, **2003**. Molecular cloning, functional expression, and gene silencing of two *Drosophila* receptors for the *Drosophila* neuropeptide pyrokinin-2. *Biochem. Biophys. Res. Commun. 309*, 485–494.

Saifullah, A.S., Tomioka, K., **2002**. Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*. *J. Exp. Biol. 205*, 1305–1314.

Salcedo, E., Huber, A., Henrich, S., Chadwell, L.V., Chou, W.H., *et al.*, **1999**. Blue- and green-absorbing visual pigments of *Drosophila*: ectopic expression and physiological characterization of the R8 photoreceptor cell-specific Rh5 and Rh6 rhodopsins. *J. Neurosci. 19*, 10716–10726.

Saudou, F., Amlaiky, N., Plassat, J.L., Borrelli, E., Hen, R., **1990**. Cloning and characterization of a *Drosophila* tyramine receptor. *EMBO J. 9*, 3611–3617.

Saudou, F., Boschert, U., Amlaiky, N., Plassat, J.L., Hen, R., **1992**. A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. *EMBO J. 11*, 7–17.

Schmidt, C.J., Garen-Fazio, S., Chow, Y.K., Neer, E.J., **1989**. Neuronal expression of a newly identified *Drosophila melanogaster* G protein alpha 0 subunit. *Cell Reg. 1*, 125–134.

Schneider, L.E., Taghert, P.H., **1988**. Isolation and characterization of a *Drosophila* gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH2 (FMRFamide). *Proc. Natl Acad. Sci. USA 85*, 1993–1997.

Schneider, L.E., Taghert, P.H., **1990**. Organization and expression of the *Drosophila* Phe-Met-Arg-Phe-NH2 neuropeptide gene. *J. Biol. Chem. 265*, 6890–6895.

Schulz, S., Huber, A., Schwab, K., Paulsen, R., **1999**. A novel Ggamma isolated from *Drosophila* constitutes a visual G protein gamma subunit of the fly compound eye. *J. Biol. Chem. 274*, 37605–37610.

Scott, K., Brady, R., Jr., Cravchik, A., Morozov, P., Rzhetsky, A., *et al.*, **2001**. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell 104*, 661–673.

Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., *et al.*, **2001**. Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. *J. Biol. Chem. 276*, 47052–47060.

Shapiro, R.A., Wakimoto, B.T., Subers, E.M., Nathanson, N.M., **1989**. Characterization and functional expression in mammalian cells of genomic and cDNA clones encoding a *Drosophila* muscarinic acetylcholine receptor. *Proc. Natl Acad. Sci. USA 86*, 9039–9043.

Shichida, Y., Imai, H., **1998**. Visual pigment: G-protein-coupled receptor for light signals. *Cell. Mol. Life Sci. 54*, 1299–1315.

Simon, M.I., Strathmann, M.P., Gautam, N., **1991**. Diversity of G proteins in signal transduction. *Science 252*, 802–808.

Slesinger, P.A., Stoffel, M., Jan, Y.N., Jan, L.Y., **1997**. Defective gamma-aminobutyric acid type B receptor-activated inwardly rectifying K^+ currents in cerebellar granule cells isolated from weaver and Girk2 null mutant mice. *Proc. Natl Acad. Sci. USA 94*, 12210–12217.

Song, W., Ranjan, R., Dawson-Scully, K., Bronk, P., Marin, L., *et al.*, **2002**. Presynaptic regulation of neurotransmission in *Drosophila* by the g protein-coupled receptor methuselah. *Neuron 36*, 105–119.

Stables, J., Green, A., Marshall, F., Fraser, N., Knight, E., *et al.*, **1997**. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Analyt. Biochem. 252*, 115–126.

Stacey, M., Lin, H.H., Gordon, S., McKnight, A.J., **2000**. LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. *Trends Biochem. Sci. 25*, 284–289.

Staubli, F., Jorgensen, T.J., Cazzamali, G., Williamson, M., Lenz, C., *et al.*, **2002**. Molecular identification of the insect adipokinetic hormone receptors. *Proc. Natl Acad. Sci. USA 99*, 3446–3451.

Stortkuhl, K.F., Kettler, R., **2001**. Functional analysis of an olfactory receptor in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 98*, 9381–9385.

Strutt, D.I., **2001**. Asymmetric localization of frizzled and the establishment of cell polarity in the *Drosophila* wing. *Mol. Cell 7*, 367–375.

Talluri, S., Bhatt, A., Smith, D.P., **1995**. Identification of a *Drosophila* G protein alpha subunit (dGq alpha-3) expressed in chemosensory cells and central neurons. *Proc. Natl Acad. Sci. USA 92*, 11475–11479.

Teller, D.C., Okada, T., Behnke, C.A., Palczewski, K., Stenkamp, R.E., **2001**. Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry 40*, 7761–7772.

Thibonnier, M., Auzan, C., Madhun, Z., Wilkins, P., Berti-Mattera, L., *et al.*, **1994**. Molecular cloning, sequencing, and functional expression of a cDNA encoding the human V-1a vasopressin receptor. *J. Biol. Chem. 269*, 3304–3310.

Torfs, H., Detheux, M., Oonk, H.B., Akerman, K.E., Poels, J., *et al.*, **2002a**. Analysis of C-terminally substituted tachykinin-like peptide agonists by means of aequorin-based luminescent assays for human and insect neurokinin receptors. *Biochem. Pharmacol. 63*, 1675–1682.

Torfs, H., Oonk, H.B., Broeck, J.V., Poels, J., Van Poyer, W., *et al.*, **2001**. Pharmacological characterization of STKR, an insect G protein-coupled receptor for tachykinin-like peptides. *Arch. Insect Biochem. Physiol. 48*, 39–49.

Torfs, H., Poels, J., Detheux, M., Dupriez, V., Van Loy, T., *et al.*, **2002b**. Recombinant aequorin as a reporter for receptor-mediated changes of intracellular Ca2+-levels in *Drosophila* S2 cells. *Invertebr. Neurosci. 4*, 119–124.

Torfs, H., Shariatmadari, R., Guerrero, F., Parmentier, M., Poels, J., *et al.*, **2000**. Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila* Schneider 2 cell line. *J. Neurochem. 74*, 2182–2189.

Townson, S.M., Chang, B.S., Salcedo, E., Chadwell, L.V., Pierce, N.E., *et al.*, **1998**. Honeybee blue- and ultraviolet-sensitive opsins: cloning, heterologous expression in *Drosophila*, and physiological characterization. *J. Neurosci. 18*, 2412–2422.

Vaidehi, N., Floriano, W.B., Trabanino, R., Hall, S.E., Freddolino, P., *et al.*, **2002**. Prediction of structure and function of G protein-coupled receptors. *Proc. Natl Acad. Sci. USA 99*, 12622–12627.

van Kesteren, R.E., Tensen, C.P., Smit, A.B., van Minnen, J., Kolakowski, L.F., *et al.*, **1996**. Co-evolution of ligand–receptor pairs in the vasopressin/oxytocin superfamily of bioactive peptides. *J. Biol. Chem. 271*, 3619–3626.

Van Poyer, W., Torfs, H., Poels, J., Swinnen, E., De Loof, A., *et al.*, **2001**. Phenolamine-dependent adenylyl cyclase activation in *Drosophila* Schneider 2 cells. *Insect Biochem. Mol. Biol. 31*, 333–338.

Vanden Broeck, J., Vulsteke, V., Huybrechts, R., De Loof, A., **1995**. Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. *J. Neurochem. 64*, 2387–2395.

von Nickisch-Rosenegk, E., Krieger, J., Kubick, S., Laage, R., Strobel, J., *et al.*, **1996**. Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol. 26*, 817–827.

Washio, H., **2002**. Glutamate receptors on the somata of dorsal unpaired median neurons in cockroach, *Periplaneta americana*, thoracic ganglia. *Zool. Sci. 19*, 153–162.

Wess, J., **1998**. Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol. Therapeut. 80*, 231–264.

West, A.P., Jr., Llamas, L.L., Snow, P.M., Benzer, S., Bjorkman, P.J., **2001**. Crystal structure of the ectodomain of Methuselah, a *Drosophila* G protein-coupled receptor associated with extended lifespan. *Proc. Natl Acad. Sci. USA 98*, 3744–3749.

Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Stortkuhl, K.F., Hovemann, B., *et al.*, **2001**. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. *Proc. Natl Acad. Sci. USA 98*, 9377–9380.

White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., *et al.*, **1998**. Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature 396*, 679–682.

Wickman, K.D., Iniguez-Lluhl, J.A., Davenport, P.A., Taussig, R., Krapivinsky, G.B., *et al.*, **1994**. Recombinant G-protein beta gamma-subunits activate the muscarinic-gated atrial potassium channel. *Nature 368*, 255–257.

Witz, P., Amlaiky, N., Plassat, J.L., Maroteaux, L., Borrelli, E., *et al.*, **1990**. Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. *Proc. Natl Acad. Sci. USA 87*, 8940–8944.

Wolfgang, W.J., Hoskote, A., Roberts, I.J., Jackson, S., Forte, M., **2001**. Genetic analysis of the *Drosophila* Gs(alpha) gene. *Genetics 158*, 1189–1201.

Wolfgang, W.J., Quan, F., Goldsmith, P., Unson, C., Spiegel, A., *et al.*, **1990**. Immunolocalization of G protein alpha-subunits in the *Drosophila* CNS. *J. Neurosci. 10*, 1014–1024.

Wolfgang, W.J., Quan, F., Thambi, N., Forte, M., **1991**. Restricted spatial and temporal expression of G-protein alpha subunits during *Drosophila* embryogenesis. *Development 113*, 527–538.

Yarden, Y., Rodriguez, H., Wong, S.K., Brandt, D.R., May, D.C., *et al.*, **1986**. The avian beta-adrenergic receptor: primary structure and membrane topology. *Proc. Natl Acad. Sci. USA 83*, 6795–6799.

Yarfitz, S., Niemi, G.A., McConnell, J.L., Fitch, C.L., Hurley, J.B., **1991**. A G beta protein in the *Drosophila* compound eye is different from that in the brain. *Neuron 7*, 429–438.

Yarfitz, S., Provost, N.M., Hurley, J.B., **1988**. Cloning of a *Drosophila melanogaster* guanine nucleotide

regulatory protein beta-subunit gene and characterization of its expression during development. *Proc. Natl Acad. Sci. USA 85*, 7134–7138.

Zhong, Y., **1995**. Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in *Drosophila*. *Nature 375*, 588–592.

Zhong, Y., Pena, L.A., **1995**. A novel synaptic transmission mediated by a PACAP-like neuropeptide in *Drosophila*. *Neuron 14*, 527–536.

Zitnan, D., Kingan, T.G., Hermesman, J., Adams, M. E., **1996**. Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science 271*, 88–91.

5.6 Scorpion Venoms

E Zlotkin, The Hebrew University of Jerusalem, Israel

5.6.1. A Chemical Ecology Perspective on Scorpion Venoms

Scorpions comprise an order of predatory, essentially nocturnal, venomous arachnids, which occupy temperate, desert, and tropical habitats. More than 1259 species of scorpion in 16 living families and 155 genera have been described (Fet *et al.*, 2000). The Buthidae family, with 48 genera and over 500 species, is the largest and most widespread of the scorpion families occupying all six faunal regions (Sissom, 1990). The buthids provide all the species that are dangerous to public health and are the most studied venoms; they include the insect selective neurotoxins, which are the main topic of interest in this article. This section deals with aspects in scorpion's biology related to their venom apparatus. Buthids, and other scorpions, employ their venom on two occasions. First, and most importantly, when they hunt and seize their prey and, second, when they are threatened. In both cases, scorpion stinging is an automatic response triggered by the reception of certain signals stimuli (see below).

5.6.1.1. Venoms

Venom is defined as a mixture of substances that is produced in specialized glands in the body of a venomous animal and introduced by the aid of a stinging-piercing apparatus into the body of its prey or opponent in order to immobilize/paralyze it. The vast majority of venomous animals (such as snakes, many spiders, certain marine snails, and various cnidarians) are slow, even static, predators that feed on freshly killed prey, which are comprised of mobile and relatively vigorous animals. Such a drastic difference in the locomotory capacity of the venomous predator compared to its potential prey demands special adaptations for prey encounter. These include behavioral adaptations in the form of ambush hunting tactics, and the development of the venomous apparatus. The latter allows for quick prey immobilization at the earliest possible moment in any encounter, with relatively low venom doses (see below). The goal of fast action at low doses is achieved with the aid of two devices. The first is a physical mechanical device, the stinging instrument,

which enables direct venom delivery into the prey's circulatory system, or in close proximity to the nervous and neuromuscular target tissues that control and operate the animal's locomotory system. The second is a chemical device in the form of the neuroactive constituents of the venom (neurotoxins).

For the present discussion, a neurotoxin is defined as a substance that affects the function of excitable tissues, due to specific recognition and binding affinity to given sites in these tissues. The various neurotoxins obtained from animal venoms are commonly classified according to their effects and sites of action in the nervous systems. They are thus defined as: ion channel toxins, which modify ion conductance; presynaptic toxins, which affect neurotransmitter release; and postsynaptic toxins, which interfere with the binding and the resulting effect on neurotransmitters (Zlotkin, 1987, 1991). A common characteristic of the various biologically active venom constituents, including neurotoxins, is their protein-polypeptide nature, which has dual significance. First, polypeptides, through their high diversity of covalent structures and the resulting spatial arrangements, may reveal a highly diverse array of functional specificities, such as a site directed highly selective neurotoxicity (see below). Second, polypeptides are the most available structures for modifications through evolutionary adaptations. Scorpions, as venomous organisms, provide a perfect example of the above principles (see below).

5.6.1.2. Scorpions

Scorpions, and their derived venoms, have attracted the attention of investigators mainly because of their hazard to human life and health. In nature, however, encounters between humans and scorpions are coincidental. The stinging activity of scorpions in the field is mainly directed towards arthropods, their prey/food (**Figure 1** and see below).



Figure 1 The yellow Israeli scorpion (*Leiurus quinquestriatus hebraeus*) attacking a large locust (*Locusta migratoria*). (Reproduced from Zlotkin, E., **1987**. Pharmacology of suvival: insect selective neurotoxins derived from scorpion venom. *Endeavour 11* (4), 168–174.)

Detailed coverage of the scorpion's biology, including its systematics, biogeography, life histories, feeding behavior, and prey encounters has been presented in previous and recent literature. Thus, the reader is urged to consult the following textbooks: *Venomous Invertebrates* (Bücherl, 1971); *Arthropod Venoms* (Bettini, 1978); *The Biology of Scorpions* (Polis, 1990a); and the most recent advanced study, *Scorpion Biology and Research* (Brownell and Polis, 2001). Several aspects related to venom delivery and employment deserve particular attention:

The sting. The scorpion body is divided into two major components, the cephalothorax and abdomen (opisthosoma). The abdomen is subdivided into a broad anterior preabdomen (mesosoma) and a narrow tail-like postabdomen (metasoma) (**Figure 1**). The latter consists of five segments plus the telson or "sting." The telson is subdivided into a bulbous vesicle and the needle-like oculeus (Levy and Amitai, 1980; Hjelle, 1990). The vesicle of the telson contains a pair of symmetrically localized venom glands. Each gland is provided with medial and dorsal compressor muscles that press the gland against the cuticle along its exterior lateral and ventral surfaces (**Figure 2**). The glands are separated by a median vertical muscular septum. Each gland is provided with an exit duct, which communicates to the exterior via two apertures just before the tip of the oculeus (**Figure 2**; Hjelle, 1990).

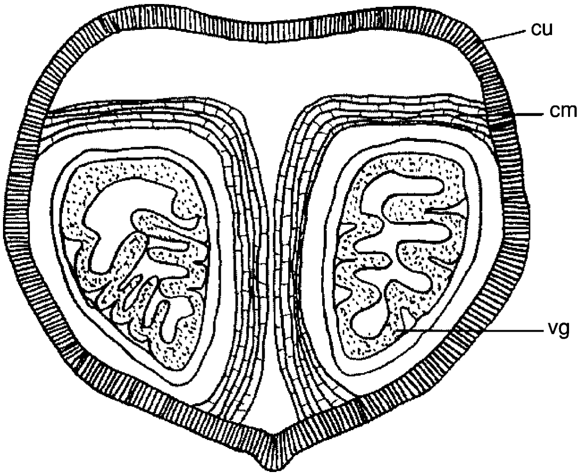


Figure 2 Diagrammatic representation of scorpion venom glands. cm, compressor muscles; cu, cuticle; vg, venom gland, showing secretory epithelial folding typical of the Buthidae. (Reproduced from Hjelle, J.T., **1990**. Anatomy and morphology. In: Polis, G. (Ed.), The Biology of Scorpions. Stanford University Press, pp. 9–63.)

Prey and predators. Scorpions are obligatory predators with a diverse diet composed mainly of insects and various arachnids, such as spiders, solifuges, and centipedes (McCormick and Polis, 1990). However, scorpions also eat crustaceans (isopods and gastropods) and occasionally vertebrates (small snakes, lizards, and rodents). Prey availability and abundance undergo seasonal changes (McCormick and Polis, 1990). Polis (1990) reported on 150 taxa of vertebrate and invertebrate organisms, which prey on scorpions, including birds, lizards, and insectivorous mammals. Certain Israeli fantoed geeko lizards, which prey on the extremely venomous yellow scorpion (*Leiurus quinquestriatus hebraeus*), were shown to possess a behavioral protection coupled with a physiological tolerance to the scorpion sting (Zlotkin *et al*., 2003). Thus, in addition to prey capture, the venom apparatus is also employed for deterrence of enemies. The existence of an aposematic coloration in animals, such as in scolopendrids and black widow spiders, may serve as a simple demonstration of its dual function. In nocturnal web or ambush (hidden) hunters, this aposematic coloration does not interfere with the predatory function of the venom apparatus.

Habitat plasticity. In contrast to the relative uniformity in their predatory feeding behavior, scorpions reveal a wide flexibility in their habitat diversity. Scorpions occupy diverse habitats including deserts, savanna, grasslands, temperate forests, tropical forests, rain forests, and intertidal zones, and snow covered mountains (Polis, 1990). Their habitat plasticity is exemplified by the distribution of *L. q. hebraeus* in Israel and the Sinai peninsula (Figure 3). These regions include heavily vegetated pockets of tropical climate (Tiberias lake and Jordan Valley), vegetated regions with a Mediterranean climate (Judean mountains), and extremely dry regions of Saharo-Sindic climate (central and southern Sinai). Similarly, this scorpion is found in various types of soils such as terra rossa, basalt, rendzina, loess, and stony desert (Levy and Amitai, 1980).

In desert habitats, scorpions appear to be one of the most successful and important predators in terms of density, diversity, biomass, and their role in community energetics and structure. The scorpions' success is attributed to their adaptability to extreme physical conditions (extreme cooling, immersion under water, high temperatures, extreme drought), their ability to conserve water, and an extremely low metabolic rate (the lowest for arthropods), which enable them to endure long periods of starvation (about a year) (McCormick and Polis, 1990; see Section 5.6.1.2.1). One of the strongest expressions of the ecological adaptability and success of scorpions is revealed by the chemical and pharmacological flexibility of their venoms.

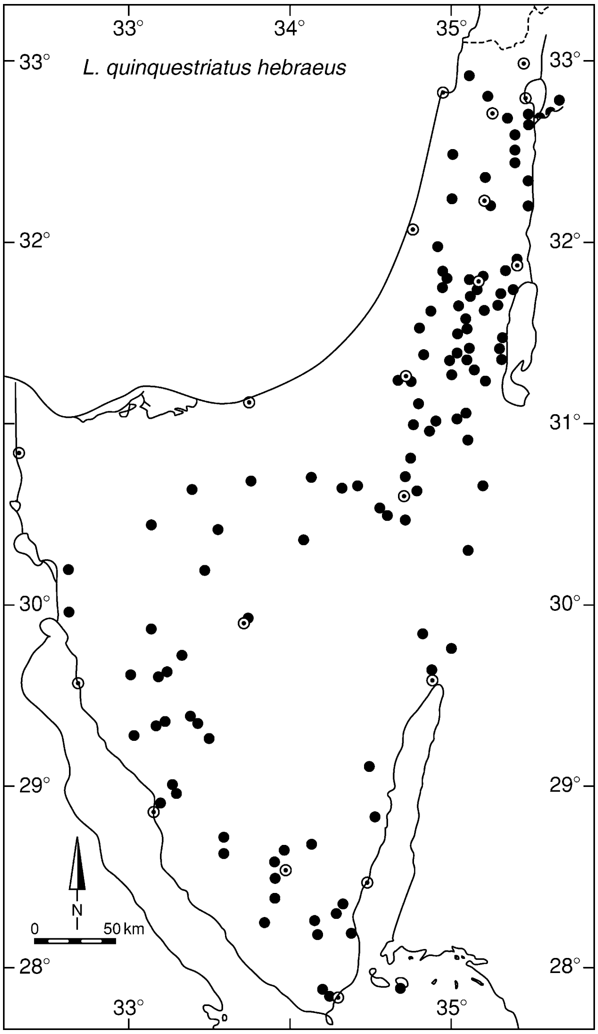


Figure 3 The distribution of the yellow Israeli scorpion (*Leiurus quinquestriatus hebraeus*, Buthidae) in Israel and Sinai. (Reproduced from Levy, G., Amitai, P., **1980**. Fauna Palestina: Scorpions. Magnes.)

5.6.1.2.1. Ambush hunters Foraging activity is reviewed by McCormick and Polis (1990). Briefly, foraging scorpions may either sit and wait or actively hunt prey. Active search for prey demands a comparatively greater expenditure of energy, and is, therefore, a less common foraging pattern. The majority are "sit and wait" ambush predators. For example, the sand scorpion (*Paruroctonus mesaensis* – see below) emerges from its burrow and remains motionless within a meter of the opening. Insects and other prey are detected (see below) as they pass close to the waiting scorpion. After

making contact with prey using its pedipalps a scorpion may or may not sting depending on the ratio of prey size to the predator's pedipalp size. Chelicerae initiate digestion by tearing the prey into smaller particles, which are collected in the preoral cavity.

McCormick and Polis (1990) stated that scorpions are able to consume large quantities of food at one time. Scorpions have an extremely low metabolic rate, much lower than that of insects. Various studies on insect metabolic rates (review: Keister and Buck, 1973) revealed an average value of 1000 mm O_2 $g^{-1}h^{-1}$ at 25 °C. Scorpions, however, have metabolic rates of 122.9 and 35.1 mm O_2 $g^{-1}h^{-1}$ for *Euscorpius* and *Nebo hierichonticus*, respectively (Dresco-Derouet, 1964a, 1964b). The above combination of consuming large quantities of food and low metabolic rates may explain the unusual capacity of the scorpion to survive starvation for 6–12 months or more (McCormick and Polis, 1990).

Most scorpions are opportunistic predators that accept most items of suitable size and physical-technical palatability. However, their preference for arthropods and insects is due to their availability. Therefore, small rodents and reptiles may occasionally be included in their menu. Detailed specification of scorpions' diet is described by McCormick and Polis (1990).

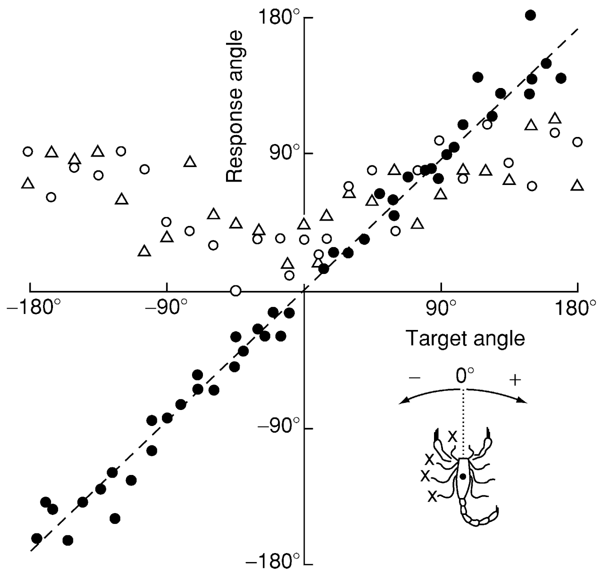


Figure 4 Directional orientation of *Paruroctonus mesaensis* to nearby disturbances of the sand. With eyes covered, *P. mesaensis* accurately turns to face the source of a substrate disturbance 8–10 cm away (filled circles). When the basitarsal slit sensilla are ablated unilaterally on all four legs, the angle of turning (open symbols) becomes biased in the direction of legs with sensors remaining intact. (+, –), Indicate right and left directions, respectively. (Reproduced from Brownell, P., Polis, G., **2001**. Scorpion Biology and Research. Oxford University Press, Oxford.)

5.6.1.2.2. Prey detection The precision in prey detection by scorpions as demonstrated by Brownell's extensive studies with the sand dune scorpion (*P. mesaensis*) is, presumably, achieved by many species (Brownell, 2001). Simple observations reveal that sand scorpions prey on insects and other small arthropods that crawl or fly towards the scorpion as it sits motionless (in ambush) outside the burrow. When a large insect such as cricket or burrowing cockroach moves within 20–30 cm of the hunting scorpion, it displays awareness of the prey by tensing its legs and opening its pedipalps. Prey movements trigger orientation responses of the scorpion, which rotates and moves forward to bring it 5–10 cm closer to the target (Brownell, 2001).

In summary, the sand scorpion is a nocturnal predator of small arthropods. Its prey detection is achieved not by vision or auditory stimulus, but rather by a mechanosensory organ. This latter organ is comprised of the ground-vibration sensitive' basitarsal slit sensilla and hairs of the tarsal leg segments, which perceive mechanical waves conducted through dry sand. The role of the basitarsal slit sensilla, in directional orientation of the sand scorpion, is exemplified in Figure 4. At close distances the *trichobotria* hairs on the pedipalps detect air currents induced by prey movements, and these guide the prey capturing appendages to the target. Finally, it may be noted that sensory behavioral prey detection is aimed at identifying the size and direction of the prey (Brownell, 2001). The hairs do not distinguish between phyletic categories of prey organisms. Such distinction, however, can be provided by the specific pharmacology of scorpion venom components.

5.6.1.3. Regulated Venom Secretion

5.6.1.3.1. Collection Scorpion venom is commonly collected by the aid of rapid, time-saving, "electrical milking," where venom is forcibly released by an electrical stimulation of the telson. A scorpion can also be induced to spontaneously release venom by mechanical stimulation of its telson (manual venom) (el Ayeb and Rochat, 1985; Martin *et al.*, 1987). A more convenient method to obtain a natural venom is by inducing a scorpion to sting a hydrophobic parafilm membrane, from which the deposited natural venom can be easily collected by a callibrated glass capillary (Zlotkin and Shulov, 1969; Zlotkin and Gordon, 1985).

5.6.1.3.2. Diversified venom composition Scorpion venom is substantially composed of components of unknown biological activity, such as the

nonsoluble acidic mucoproteins, nucleotides, and various low molecular weight salts and organic substances (Simard and Watt, 1990). Low levels of non-neurotoxic enzymatic activity have been detected occasionally, such as phosphatase, hyaluronidase (spreading factor), or phospholipase (Simard and Watt, 1990). However, the lethal and paralytic toxicities and pathogenicity of scorpion venom are derived from low molecular weight, single chain basic polypeptides, which specifically affect ion conductance in excitable tissues (see below).

When different samples of either pooled or individually collected venom derived from scorpions belonging to the same species were compared, chemically or immunologically, obvious differences in composition were revealed among geographically separated scorpions (el Ayeb and Rochat, 1985) as well as different methods of venom collection (el Ayeb and Rochat, 1985; Martin *et al*., 1987). Also, a careful analysis of scorpion venom derived from natural stings has revealed a curious variability among venom samples collected from successive stings of individual scorpions (Yahel-Niv and Zlotkin, 1979; Inceoglu *et al*., 2003). The above variability, however, displays an consistent pattern (see below).

5.6.1.3.3. Naturally secreted venom The employment of the device used for the collection of scorpion venom immediately after being released by natural stings (Zlotkin and Shulov, 1969) facilitated the analysis of successive venom drops deposited by continuous stinging from individual *L. q. hebraeus* scorpions. The authentic natural venom was examined for its appearance, protein content, electrophoretic mobility, and paralytic activity to blowfly larvae. It was shown that venom obtained by a series of successive stings changes its appearance in the following order: transparent, opalescent, and viscous secretions. As shown in Table 1, venom collected from individual scorpions demonstrates a wide variability in the number of venom-yielding stings, volume of secreted venom, protein content, and total and specific toxicities. The opalescent venom secretion exhibited the highest total and specific toxicities, in contrast to the transparent venom, which possessed the lowest toxicity. Electropherograms of opalescent and viscous venom secretions closely resemble each other, and both differ from the transparent venom, which had fewer protein bands and lower toxicity (Yahel-Niv and Zlotkin, 1979). A similar diversity in the composition of the successively secreted

Table 1 The diversity in the properties of *L. quinquestriatus hebraeus* venom obtained from individual scorpions by natural stings

Property	*Range*[a]	*Average ± SD*[a]
Venom yielding stings (number)		
Total venom	37–93	65 ± 16
Transparent venom	13–47	27 ± 12
Opalescent venom	6–24	13 ± 5
Viscous venom	8–38	24 ± 11
Volume (μl)		
Total venom	18.5–52	36 ± 11
Transparent venom	5.5–14	8.2 ± 2.4
Opalescent venom	6.0–16	10.8 ± 3.7
Viscous venom	6.5–29	16.5 ± 7.6
Protein content[b]		
Total venom (μg)	1 920–6 490	3 689 ± 1 387
Transparent venom concentration ($\mu g\,\mu l^{-1}$)	90–160	115 ± 33.4
Opalescent venom concentration ($\mu g\,\mu l^{-1}$)	70–180	129 ± 33.7
Viscous venom concentration ($\mu g\,\mu l^{-1}$)	40–200	81 ± 48.0
Specific toxicity (CPU mg^{-1})[c]		
Transparent venom	450–33 300	7 300 ± 14 000
Opalescent venom	930–100 000	27 000 ± 21 200
Viscous venom	1 430–50 000	14 300 ± 17 000
Total toxicity (CPU)[c]		
Transparent venom	500–4 330	1 200 ± 1 500
Opalescent venom	960–52 000	25 900 ± 23 000
Viscous venom	1 100–30 330	9 700 ± 17 400

[a] Ranges, averages, and standard deviations were based on 10 determinations each.
[b] Determined by the method of Lowry.
[c] CPU-contraction paralysis unit as determined on larvae of the blowfly *Sarcophaga falculata* according to Zlotkin *et al.* (1971a).
Reproduced with permission from Yahel-Niv, A., Zlotkin, E., **1979**. Comparative studies on venom obtained from individual scorpions by natural stings. *Toxicon 17* (5), 435–446.

venom has been recently demonstrated in the venom of the South African scorpion *Parabuthus transvaalicus* (Buthidae) (Inceoglu *et al*., 2003) with far-reaching pharmacological implications. As with the *Leiurus*, the *Parabuthus* scorpion, when stimulated to sting, first secretes a transparent venom (the prevenom) followed by an opaque (cloudy dense) secretion named venom. Similar to the transparent venom of *Leiurus*, the prevenom of *Parabuthus* comprises a relatively small fraction of the volume (22% and 5%, respectively) and of the total protein (25% and 10%, respectively). However, beyond the above similarity, the *Parabuthus* prevenom reveals a qualitative-fundamental difference. In contrast to the *Leiurus* transparent venom which possesses only about 3–4% of total toxicity with four times lower specific toxicity than the opaque venom (Table 1), the prevenom of *Parabuthus* possesses a higher specific toxicity. This toxicity includes higher insect paralysis (×3), mouse lethality (×1.2), and pain production (×3.5) when compared to the dense cloudy venom (Table 2). The potent toxicity of the prevenom is surprising in view of the data presented in Figure 5, showing that it is devoid of the 6–7 kDa long chain polypeptides, which include the potent insect and mammalian neurotoxic

Table 2 Comparative properties of prevenom and venom of *Parabuthus transvaalicus*

	Prevenom	*Venom*
Protein (mg ml^{-1}, $n = 8$)	7.2–14.3	53.4–85.2
Potassium salt (mM)	80.2 ± 4.5	5.4 ± 0.4
Volume secreted (μl)	1.2 ± 0.6	21 ± 5.2
PD_{50} insect μg protein/100 mg larvae	0.28	0.81
μl venom/100 mg larvae	0.028	0.016
LD_{99} mouse μg protein/20 g mouse	4.1	4.8
μl venom/20 g mouse	0.5	0.1
Pain response (foot licking for 10 min after injection)	32 ± 2.6	9.3 ± 2.0

Reproduced with permission from Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., *et al*., **2003**. One scorpion, two venoms: prevenom of *Parabuthus transvoalicus* acts as an alternative type of venom with distinct mechanism of action. *Proc. Natl Acad. Sci. USA 100* (3), 922–927.

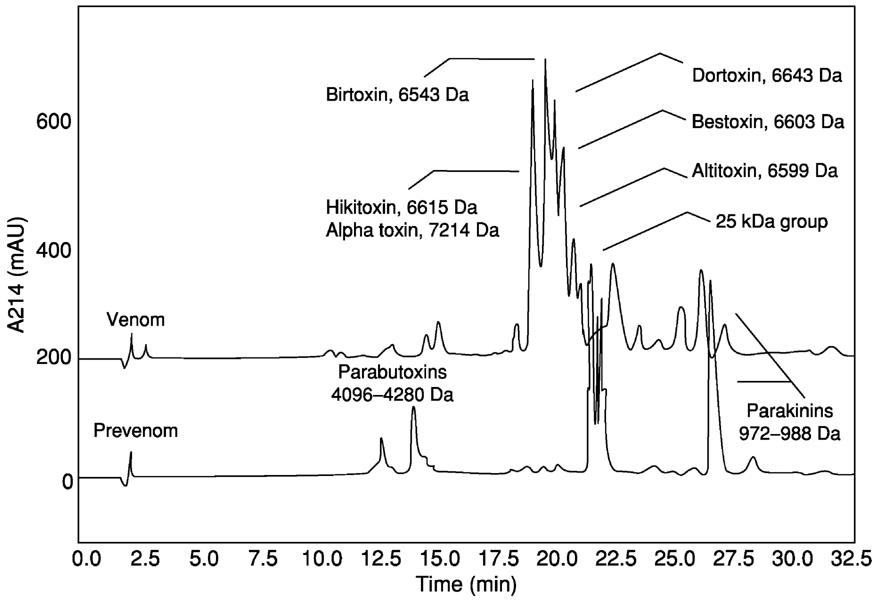


Figure 5 RP-HPLC profiles of prevenom and venom from *Parabuthus transvaalicus*. The venoms are ran on a C8 microbore analytical column and the absorbance at 214 nm is monitored and components are identified by online mass spectrometry (LC-MS). Both prevenom and venom contain the 0.9–1.2 kDa group, the 3–4 kDa polypeptide group, and the 25 kDa group. However, most of the protein in venom corresponds to the 6–7 kDa peptide group. Moreover, the three protein groups in prevenom are major species because of the lack of the 6–7 kDa group. (Reproduced from Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., *et al*., **2003**. One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proc. Natl Acad. Sci. USA 100* (3), 922–927.)

polypeptides that affect the voltage-gated sodium channels (see Sections 5.6.2 and 5.6.3). The pharmacologic uniqueness of the prevenom is attributed (Inceoglu *et al.*, 2003) to the presence of a high concentration (80 mM) of K^+ salt and certain peptides (**Figure 5**) that block the voltage-gated rectifying K^+ channels. The combined action of these two factors causes a strong local and prolonged depolarization that blocks neuronal conduction and induces pain. Therefore, it appears that the depolarizing effect of high extracellular K^+ provided by the prevenom, serves as a pharmacological substitute to the long chain sodium channel gating modifiers. Therefore, it is claimed that by virtue of its unique composition the prevenom economizes the metabolically expensive polypeptides, positively cooperates with the opaque venom, and fulfills an important defensive role as a pain producer (Inceoglu *et al.*, 2003).

It may be concluded that prevenom functions as a separate chemical and functional entity (one scorpion two venoms). This concept challenges the "pharmacological monopoly" of neurotoxic polypeptides and provides an efficient alternative solution to their insecticidal action (Inceoglu *et al.*, 2003). Thus, the transparent venom of *Leiurus*, due to its low and marginal toxicity, is not equivalent to the prevenom of *Parabuthus*. It may, however, fulfill a synergistic role with the opaque venom deserving experimental clarification. In contrast to the *Parabuthus* prevenom, the low toxicity of the first secreted transparent *Leiurus* venom contradicts the fundamental needs of the slow predator, which has to paralyze its mobile prey in the fastest possible manner. Alternatively, it provides a rational explanation for the phenomenon of multiple stinging of a mobile and vigorous prey, such as the migratory locust (Zlotkin, 1987). In other words, the process of stinging begins with a metabolically cheap venom and continues with a more potent ("expensive") venom when needed.

The phenomenon of the *Leiurus* transparent venom is very reminiscent of the observation of Bücherl (1955) who found that drops of electrically milked venom of *Tityus serrulatus* and *T. bahiensis* become successively more opalescent and viscous and more toxic to mice. Alternatively, the *Parabuthus* prevenom phenomenon is supported by an early observation (Miranda *et al.*, 1964), which indicated that the specific toxicity (by weight) of the "manual venom" (see above) was about four times that of the electrically obtained venom.

Finally, given the background of the above-mentioned variability in the successively secreted scorpion venom, it appears that scorpion venom production and secretion is a regulated timed process, enabling precise control of the prey's response and behavior. Such a goal may be achieved, for example, by the preprogramed secretion of insect selective neurotoxins (see Section 5.6.3.2.5).

5.6.1.4. Phyletic Specificity

5.6.1.4.1. An ecological point of view Organisms show a range of tolerance to various environmental factors. Ecologists use the prefixes "steno" and "eury" to refer to organisms with a narrow and wide, respectively, range of tolerance to a given factor. Thus, a stenothermic organism will tolerate only narrow variations in the ambient temperature. For example, the cold adapted antartic fish, *Trematomus bernacchi*, tolerates temperatures between −2 °C and +2 °C only. In other words, a steno type tolerance is exhibited by an organism that has acquired an adaptation to a strictly stable environment. The scorpions, as demonstrated by their geographical distribution, exhibit a *eury* type of tolerance to factors such as temperature, light, and humidity. However, they have strict requirements for prey organisms, which substantially consist of small arthropods and soft bodied insects. This steno type of feeding provides the necessary prerequisite for a chemical-pharmacological adaptation to the strict food supply.

5.6.1.4.2. Animal group specificity Animal group specificity refers to the phenomenon where animal venom and/or its derived toxins reveal toxicity to a given group of organisms, but are not effective against other organisms. For example, the venoms of marine nemertine worms (Rhynchoceola) and sea anemones (Cnidaria) are capable of affecting a wide range of animals, but their toxicity to crustacea is due, substantially, to a specific group of polypeptide neurotoxins (molecular weight (M_r) 3–6 kDa) that do not affect vertebrate and other invertebrate animals (Kem, 1976; Schweitz *et al.*, 1981). In cases where a venomous organism feeds on a given and limited group of animals, the phyletic specificity is manifested at the level of the whole venom. For example, the venomous marine Conidae snails are subdivided according to their feeding habits into fish, mollusk, and worm eaters. A series of early bioassays (Endean and Rudkin, 1963, 1965) revealed that the above feeding preference is associated with the specific toxicity of their venoms, which are aimed at the respective groups of prey animals. Moreover, this notion of animal group selectivity was demonstrated in the form of mollusk selective δ-conotoxins, which slow inactivation exclusively in the mollusk voltage-gated sodium channels (VGSCs) (Fainzilber

et al., 1994), or the insect selective ω-atracotoxins derived from Australian funnel web spiders that selectively block the insect voltage-gated calcium channels (Fletcher *et al.*, 1997; Wang *et al.*, 1999).

This phenomenon of animal group selective neurotoxins represents a chemical adaptation of the venom to the animal's specific prey or, more precisely, to certain peculiarities in the structure and function of the prey's nervous system. These animal group selective toxins indicate the occurrence of subtypes of voltage-gated channels specific to given groups of organisms (Zlotkin, 1991; see below).

5.6.1.4.3. Ion channel subtypes As a key structure of excitability (see Section 5.6.4.3.1) in biological systems, the VGSC is targeted by many toxicants (**Table 3**) derived from plant and animal systems (Catterall, 1980; Cestele and Catterall, 2000), as well as various synthetic pesticides (**Table 3**) (Soderlund and Knipple, 1995; Zlotkin, 1999). These toxicants interfere with all three fundamental functions of the VGSC, namely its selective ion transport (groups 1 and 9), voltage dependent initial increase in permeability (activation, groups 2, 4, 5), and the subsequent voltage dependent return to the resting sodium permeability (inactivation, groups 3, 6, 7, 8). The scorpion venom neurotoxins are represented in **Table 3** by the beta toxins (site 4 activation enhancers) and alpha toxins (site 3, which substantially inhibit inactivation) (Soderlund and Knipple, 1995; Gordon, 1997b; Section 5.6.2).

Sodium channel neurotoxins that are transport inhibitors or are gating (activation/inactivation) modifiers share two important features. First, they differentiate between the three distinct functional states (resting, open, inactivated) of the channel, and show an increased affinity to the specific conformation of a given state. Second, the various site groups of bound toxins interact with one another through an allosteric interaction or coupling (**Table 3**). This aspect of cooperativity may have far-reaching practical agronomical implications (Section 5.6.5.2).

Table 3 Neurotoxin and insecticide binding sites on the voltage-gated sodium channel

Site[a]	*Toxicant*[b]	*Physiological effect*	*Allosteric coupling*[c]
1	Tetrodotoxin (TTX) Saxitoxin (STX) μ-Conotoxin	Inhibit transport	+3, 5, −2
2	Barachotoxin (BTX) Veratridine Aconitine Grayanotoxin *N*-Alkylamides	Cause persistent activation	+3, −6
3	α-Scorpion toxins Sea anemone II toxin (ATXII)	Inhibit inactivation Enhances persistent activation	+2
4	β-Scorpion toxins	Shift voltage dependence of activation	
5	Brevetoxins Ciguatoxins	Shift voltage dependence of activation	+2, 4, −3
6	δ-Conotoxins (δ-TxVIA)	Inhibit inactivation	
7	DDT and analogs Pyrethroids	Inhibit inactivation[d] Shift voltage dependence and rate of activation	+2, 3, 5
8	*Goniopora* coral toxin *Conus striatus* toxin	Inhibit inactivation	
9	Local anesthetics Anticonvulsant Dihydropyrazoles	Inhibit transport	+2

[a]Sites 1–5 according to Catterall (1992), site 6 according to Fainzilber *et al.* (1994), site 7 according to Pauron *et al.* (1989) and Soderlund and Knipple (1995). Sites 8 and 9 were numbered arbitrarily in order to distinguish them from the others. Site 9 according to Soderlund and Knipple (1995) and Payne-Gregory *et al.* (1998). Substances are considered to belong to a given site if they compete with each other in binding assays and induce similar electrophysiological effects.
[b]Toxins and insecticides.
[c]Allosteric coupling refers to modulations induced by occupancy of a receptor site by the corresponding neurotoxin on the binding of neurotoxins at the indicated receptor site(s). Positive modulation (+) indicates enhancement of binding of the toxin at its indicated receptor site and/or stimulation of Na^+ influx. Negative modulation (−) refers to decrease in toxin binding at the indicated receptor site (Gordon, 1997a).
[d]See Soderlund and Knipple (1995).
Reproduced with permission from Zlotkin, E., **1999**. The insect voltage-gated sodium channel as target of insecticides. *Annu. Rev. Entomol. 44,* 429–455.

An additional feature of the pharmacological diversity of the VGSCs is revealed by the ability of different toxicants to identify minor differences in ion channel subtypes. For example, neurotoxic conotoxins, derived from the fish hunting *Conus geographus* snail, are able to distinguish among certain categories of voltage-gated calcium and sodium channels (Zlotkin, 1991). Similarly, as discussed in Section 5.6.3.3.2, the alpha scorpion toxins can be separated into several categories each representing a binding preference to a distinct subtype of the voltage-gated sodium channels. The first indication of channel subtypes that can be distinguished by neurotoxins was demonstrated by the animal group (phyletic) selectivity of scorpion venom neurotoxins (Zlotkin *et al.*, 2000). The insect selective neurotoxin AaIT (see below), derived from the venom of the North African scorpion *Androctonus australis* Hector, provided the first example of an animal group specific toxin (Zlotkin *et al.*, 1971c). As shown below, this finding has led to certain practical agronomical applications (see Section 5.6.5).

5.6.1.4.4. Factors specific to arthropods The symptoms of scorpion envenomation in arthropods mainly indicate a stimulatory effect on the skeletal musculature (Rathmayer *et al.*, 1978), which, in blowfly larvae, is expressed as an immediate and sustained contraction of body muscles accompanied by a complete paralysis (**Figure 6a**). The degree and duration of paralysis are dose dependent (Zlotkin *et al.*, 1971a). This paralysis of blowfly larvae was hence employed as a quick, specific, and sensitive bioassay for the evaluation of the potency of scorpion venoms, and for the detection and monitoring of AaIT toxin purification (see below).

There is an apparent resemblance between the effects of scorpion venoms on arthropods and on mammals, as observed by the similarity in action of scorpion venom on neuromuscular preparations derived from insects, crustaceans (Parnas and Russel, 1967; Parnas *et al.*, 1970), and mammals (Katz and Edwards, 1972; Brazil *et al.*, 1973; Zlotkin *et al.*, 1978). In all these preparations, the scorpion venom stimulates skeletal muscles due to its presynaptic excitation of exposed motor nerve endings, resulting in the release of the corresponding transmitter. This similarity in action could have led to the conclusion that the effect of scorpion venoms on mammals and arthropods was due to the same chemical factors in crude venoms.

However, using the blowfly larvae assay (**Figure 6**), Zlotkin *et al.* (1971a) showed that there was no correlation between larval paralysis and mice lethal potencies for 16 different Buthidae venoms, suggesting that the two toxic activities may result from different factors. These results were supported by the finding that the AaHI and AaHII toxins, which were strongly lethal to mammals and isolated according to their toxicity to mice (Rochat *et al.*, 1967; Miranda *et al.*, 1970), were inactive against blowfly larvae (Zlotkin *et al.*, 1971). Final proof of toxin diversity between those affecting mammals and those paralyzing insects was obtained by starch-gel-electrophoretic separation of the venom of *A. australis* and six other Buthidae scorpions. Three of these venoms contained more than one fraction that caused larval paralysis. These fractions were lethal to fly larvae, and were readily inactivated by trypsin, thus demonstrating their protein nature (Zlotkin *et al.*, 1972b).

These early findings led to the detection and isolation of AaIT (see below) and other insect selective neurotoxins. The existence of an additional group of toxins, the crustacean toxins, was initially demonstrated by the fact that, in contrast to the crude venom of *A. australis*, both the insect selective AaIT and the highly mammalian toxic AaHII, were unable to affect an isopod (terrestrial crustacean) or another scorpion (**Table 4**). These data suggested the presence of discrete neurotoxins (Zlotkin *et al.*, 1972a, 1975) specifically affecting those arthropods. Using a bioassay of isopod paralysis, coupled with gel filtration and ion exchange column chromatography, a protein specifically toxic to isopods (crustacean toxin, AaCT) was isolated and purified from the venom of *A. australis* (Zlotkin *et al.*, 1975). Several crustacean toxins have also been isolated from the venom of scorpionid scorpion *Scorpio maurus palmatus* (Lazarovici *et al.*, 1984) and certain South American scorpions (Possani *et al.*, 1999, 2000).

As shown in **Table 5**, the animal group specificity of various neurotoxins, in addition to the conventional toxicity assay with intact animals (**Table 4**), was also demonstrated on neuromuscular preparations from mammal, insect, crustacean, and arachnid. The crustacean toxin has the highest specific toxicity to the crustacean and arachnid neuromuscular junctions. In other words, from a chemical ecology point of view, the presence of animal group specific toxins (antimammals, anti-insects, anticrustaceans), in the venom of *A. australis*, represents a specific pharmacological adaptation to the four major classes of scorpion prey or enemy organisms, namely, the insects, arachnids, isopods, and vertebrates.

Finally, it is noteworthy that this concept of insect selectivity has been well accepted. In recent years, hundreds of publications have reported

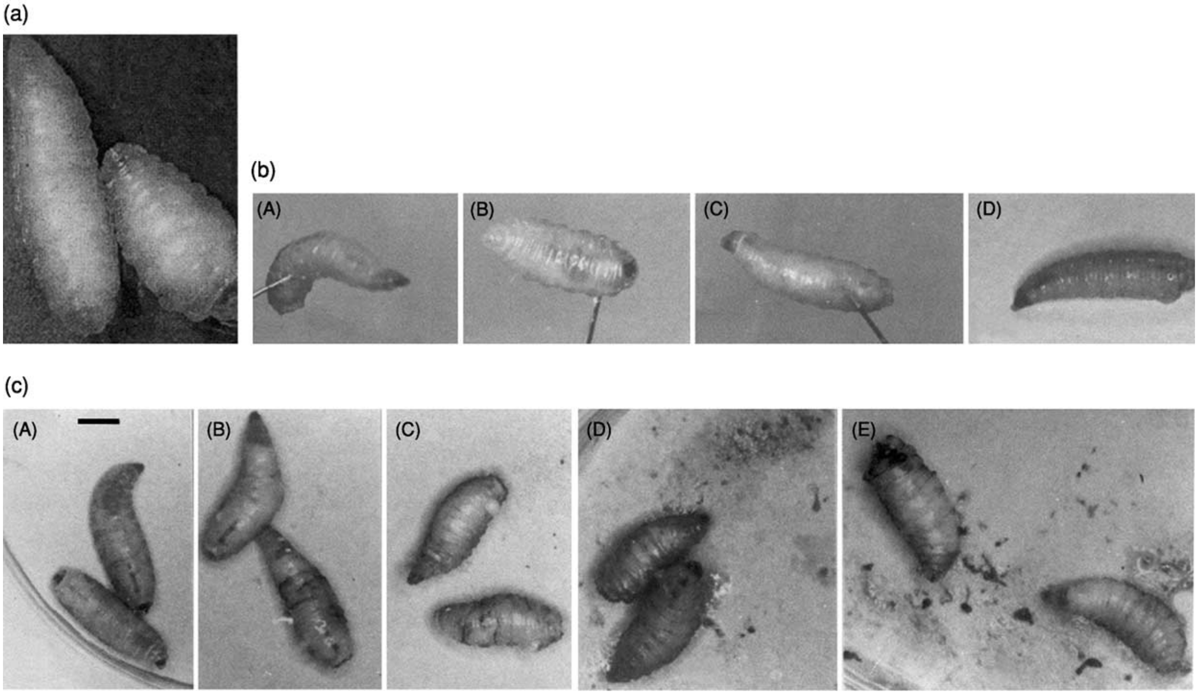


Figure 6 The response of *Sarcophaga falculata* blowfly larvae to the insect specific toxins derived from Buthidae scorpions, affecting voltage-gated sodium channels (VGSCs). The larvae (100–120 mg of body weight) are injected with 2–5 μl of saline solution containing the test substance into the ventral hind part of the body cavity. (a) Shows the fast contraction paralysis induced by the excitatory toxins. The contracted larva (right) was photographed about 5 s after injection compared with a saline injected larva (left). (b) Typical response of a larva injected with one paralytic unit (50 ng) of the depressant LqhIT2 toxin: (A) the larva mounted on the needle connected to a Hamilton syringe is wiggling; (B) 5–10 s after injection the larva reveals a transient fast contraction of 2–3 s duration, followed by the development of progressive flaccidity; (C) the larva 60 s after injection reveals the flaccid extended paralysis; and (D) 5 min after injection it is completely motionless with an extended body. (c) Demonstrates the delayed extended contraction paralysis induced by injection of 1 paralytic unit (30 ng) of an α type toxin (LqhαIT) to two larvae: (A) before injection (scale bar = 2.5 mm); (B) 1 min after injection – mobile and without detectable change in body form; (C) 5 min – obvious contraction and immobility; (D) 8 min – fully contracted and paralyzed. The contraction causes release of hemolymph from the injection site. The hemolymph darkens upon exposure to air; and (E) 20 min – still contracted but with partial recovery of mobility. The above responses were used as assays to monitor the isolation of the above toxins by column chromatography. (According to Zlotkin, E., Eitan, M., Bindokas, V.P., Adams, M.E., Moyer, M., *et al.*, **1991**. Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry 30* (19), 4814–4821; and Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., *et al.*, **1990**. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure, and mode of action. *Biochemistry 29* (25), 5941–5947.)

the characterization of additional insect selective scorpion toxins (Possani *et al.*, 2000).

5.6.2. The Vertebrate Affecting α and β Toxins

This article is motivated and directed by entomological–chemoecological considerations. However, like any other aspects of toxinology, early studies of scorpion venoms were pursued for clinical and public health purposes, using vertebrates as test animals and experimental systems. Thus, the present section provides a short overview on the “classical” chemistry and pharmacology of scorpion venom, to serve as a background for the entomological aspects that follow. The reader is invited to consult some early (Zlotkin *et al.*, 1978; Rochat *et al.*, 1979; Possani, 1984) and more recent (Simard and Watt, 1990; Martin-Eauclaire and Couraud, 1995; Loret and Hammock, 2001) review articles for more detailed information.

5.6.2.1. The Buthid Envenomation Syndrome

The clinical and public health aspects of scorpion stings can be summarized by the following statements:

1. Fewer than 25 species of scorpions, belonging to eight genera, are lethal to humans and all belong to the family Buthidae.

Table 4 The selective toxicity of AaIT and AaHII toxins

Test animal	A. australis *crude venom*	*AaHII*	*AaIT*
Flies, adults (*Sarcophaga*)	+	−[a]	+
Beetle, adults (*Tenebrio*)	+	−[a]	+
Beetle, larvae (*Tenebrio*)	+	−[a]	+
Cockroaches, adults (*Periplaneta, Blatella)*	+	−[a]	+
Locusts, adults, larvae (*Locusta)*	+	−[a]	+
Crickets, adults, larvae (*Gryllus*, *Acheta*)	+	−[a]	+
Arachnids (*Buthus*, scorpions)	+	−[a]	−[a]
Crustaceans (*Armadillidium*, Isopods)	+	−[a]	−[a]
Laboratory mice	+	+	−[a]

[a]The toxins were injected in doses that were at least 20 times higher than their equivalent active doses of the crude venom as estimated on the basis of their purification yields.

Reproduced with permission from Zlotkin, E., **1986**. The interaction of insect selective neurotoxins from scorpion venoms with insect neuronal membranes. In: Ford, M.G., Lunt, G.G., Reay, R.C., Usherwood, P.N.R. (Eds.), Neuropharmacology and Pesticide Action. Ellis Horwood, Chichester, pp. 352–383.

Table 5 The relative activity of different toxins derived from the venom of the scorpion *Androctonus australis* in several nerve–muscle preparations[a] and insect paralysis

Toxic material[b]	*Insect*[c]	*Crustacean*[d]	*Arachnid*[e]	*Mammal*[f]	*Insect paralysis*[g]
Crude venom	1	1	1	1	1
Insect toxin	115	No effect	No effect	No effect	25
Crustacean toxin	18	127	67	0.7	0.7
AaHI	7	27	11	1	5.7
AaHII	2	4	5	25	0.2

[a]Based on a comparison of the average minimal doses causing an evoked repetitive muscular response. Numbers express the activity relative to that of the crude venom.

[b]The different toxins were purified from the crude venom of the scorpion *Androctonus australis* Hector (Miranda *et al.*, 1970; Zlotkin *et al.*, 1971, 1975).

[c]Locust leg extensor tibiae preparation (Walther *et al.*, 1976).

[d]The crayfish walking leg dactylus opener preparation (Rathmayer *et al.*, 1978).

[e]The claw closer muscle preparation in the leg of a mygalomorph spider (Ruhland *et al.*, 1977).

[f]The guinea pig ileum smooth muscle preparation (Tintpulver *et al.*, 1976).

[g]Determined by injection into the body cavity of second and third instar *Locusta migratoria*.

Reproduced with permission from Ruhland, M.E., Zlotkin, E., Rathmayer, W., **1977**. The effect of toxins from the venom of the scorpion *Androctonus australis* on a spider nerve–muscle preparation. *Toxicon 15* (2), 157–160.

2. The public health hazard is declining due to recent progress in the availability of medical services. Scorpion stings, however, continue to account for hundreds of death worldwide each year. Morbidity and mortality are higher in children.
3. Scorpion neurotoxins induce local and systemic effects expressed in severe cardiovascular, respiratory, and muscular manifestations that precede death. The latter may occur within a few hours after envenomation.
4. Serotherapy, coupled to symptom directed drugs, can prevent lethality.
5. The toxicity of scorpion venom to humans is attributed to low molecular weight (4–8 kDa) basic polypeptides, which affect various ion channels in excitable membranes.
6. Envenomation in humans has three major symptoms: (a) muscular effects, in the form of contraction, twitchings, and fibrillations, are the most common abnormalities; (b) cardiovascular effects, such as electrocardiographic abnormalities, hypertension, and pulmonary edema, being the most dangerous; and (c) respiratory effects revealed by typical irregularities of respiratory movements (“Chayne Stokes” type), including respiratory paralysis, often occur.
7. All the above manifestations are a consequence of either an indirect effect, mediated by the nervous tissue innervating the target organs (heart, blood vessels, muscles), or a direct effect, mediated by the heart, skeletal, and smooth muscles related to the target organs.

8. The fact that a scorpion sting can kill or even affect a human being is astonishing, considering the small amount of venom injected. Yahel-Niv and Zlotkin (1979) evaluated that an average sting of *L. q. hebraeus* has a volume of 0.55 µl, which includes 57 µg of protein. Similar data were reported by Rochat *et al.* (1979) for the deadly North African scorpion, *A. australis* Hector, which injects only 100–200 µg (dry weight) of the whole venom, of which only 5% comprises active neurotoxins.
9. Both indirect and direct effects are produced by the same fundamental mechanisms, namely, an action on the VGSCs by the so-called α or β neurotoxins (see Section 5.6.2.2) present in the venom.

5.6.2.2. Toxins Affecting the Vertebrate VGSCs

Scorpion neurotoxins that affect the vertebrate VGSCs are subdivided into two categories, the α and β toxins. Both are long chain scorpion toxins (60–70 amino acids, four disulfide bridges) (Loret and Hammock, 2001), similar to the insect selective neurotoxins (see Sections 5.6.3 and 5.6.4).

5.6.2.2.1. Alpha toxins These were the first buthid scorpion toxins studied. They mimic and reproduce the effect and symptoms of the crude venoms to vertebrates (Miranda *et al.*, 1970). The α toxins are characteristic of "old world" Afro-Asian buthid scorpions. Some α toxins, however, were found in the venom of the South American *Tityus* and *Centruroides* scorpions (Barhanin *et al.*, 1982; Meves *et al.*, 1984). The scorpion α toxins are the most studied group in toxinology (see the above review articles), as they:

1. prolong action potentials due to the slowing of sodium channel inactivation (Adam *et al.*, 1966; Wang and Strichartz, 1983; Benoit and Dubois, 1987);
2. synergize interaction with lipophilic alkaloidal sodium channel activators (receptor site 2, **Table 3**), as revealed by sodium flux (Catterall and Nurenberg, 1973; Catterall, 1977) and binding studies (Catterall, 1976; Couraud *et al.*, 1978; Jover *et al.*, 1978);
3. reveal a voltage dependent binding affinity to excitable membranes (Catterall, 1976), and a mutual displacability with the sea anemone As TxII (Catterall and Beress, 1978; Couraud *et al.*, 1978);
4. bind to the α subunit of the VGSC as observed by photoaffinity labeling (Beneski and Catterall, 1980; Sharkey *et al.*, 1984; Jover *et al.*, 1988); and
5. occupy loop linking segments S3 and S4 of domain IV of the α subunit of the VGSC as their receptor binding site (Rogers *et al.*, 1996; Benzinger *et al.*, 1998).

5.6.2.2.2. Beta toxins The β toxins differ from the α toxins and have the following characteristics:

1. They are derived, substantially, from the American buthid scorpion belonging to the subfamilies of Centrurinae and Tityinae.
2. They induce similar (but not identical) symptoms to those of the α toxins when assayed on intact animals (Martin-Eauclaire and Couraud, 1995).
3. Unlike the α toxins, the β toxins (Cahalan, 1975) induce repetitive firing, due to the appearance of an abnormal Na^+ current upon repolarization (Meves *et al.*, 1982; Wang and Strichartz, 1982; Simard *et al.*, 1986).
4. The β toxins bind to receptor site 4 (**Table 3**) of the sodium channel (Jover *et al.*, 1980; Barhanin *et al.*, 1982) and generate a shift in the voltage dependence of activation to a hyperpolarizing value (Vijverberg *et al.*, 1984; Jonas *et al.*, 1986).
5. The β toxins do not compete with α toxins in binding assays (Jover *et al.*, 1980), although both groups interact with the same sodium channels.
6. Unlike the α toxins, the binding of the β toxins is independent of membrane potential not modified by the lipophilic alkaloids (receptor group 2, **Table 3**) or sea anemone toxins (Jover *et al.*, 1988).
7. The receptor binding site of the β toxins on the VGSC was identified with the aid of chimeric sodium channels composed of combinations between segments derived from the mammalian skeletal muscle-brain sodium channel isoforms (which show that β toxins induced a negative shift in the voltage dependence of activation) and cardiac sodium channels (which are resistant to the β toxin voltage dependent activation). This approach showed that β toxins bind to loops S1–S2 and S3–S4 in domain II of the α subunit. These data also confirmed the critical role domain II plays in β toxin binding to sodium channels (see Section 5.6.4.3), particularly the G845 site in II S3–S4 loop. This amino acid is replaced by N in the β toxin resistant cardiac sodium channel (Cestele *et al.*, 1998; Cestele and Catterall, 2000).

5.6.2.3. Short Toxins

In addition to the two categories (α, β) of long scorpion toxins, which affect the VGSC, scorpion

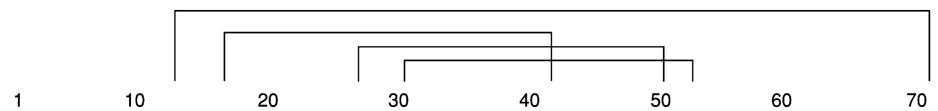
venoms were shown to possess a series of "short" neurotoxins (30–40 amino acids, 2–3 disulfide bridges), which affect the conductance of voltage-gated potassium, calcium, and chloride channels. These short toxins are beyond the scope of this article, which focuses on the insect selective long chain VGSC scorpion toxins. However, the reader is urged to consult the reviews of Martin-Euclaire and Couraud (1995) and Loret and Hammock (2001). It appears that these short toxins, which were assayed largely in vertebrate systems, may serve as an additional source of insect selective neurotoxins.

5.6.2.4. Toxin Structures

The various α and β type VGSC scorpion toxins, when classified according to their phyletic specificity, binding properties, and electrophysiological effects, were recently (Possani *et al.*, 1999) subdivided into not less than 10 functional groups. Because of the importance of public health considerations, this section, however, deals with the so-called, "classical" α and β toxins that affect mammals and are represented by functional groups 1 and 2 in the review article by Possani *et al.* (1999). **Figure 7** provides the primary, covalent, and secondary structures of these classical mammalian toxins.

Fontecilla-Camps *et al.* (1980, 1982, 1988) have developed models of three-dimensional structures of scorpion toxins based on crystallographic data, computer graphics, and energy minimization. These models suggest spatial arrangements for the α and β toxins are similar. In spite of the diversity in their primary structure (Martin-Eauclaire and Couraud, 1995; Loret and Hammock, 2001), all toxins reveal a conserved hydrophobic surface, which is thought to be involved in interaction with the excitable membranes and their respective binding sites (Fontecilla-Camps *et al.*, 1982; Fontecilla-Camps, 1989).

It has been demonstrated (see above cited recent review articles) that all scorpion toxins share a similar basic molecular scaffold composed of four disulfide bridges and a highly conserved secondary structure, which has three stands of antiparallel β sheet (β_1, β_2, β_3) and one stretch of α helix. Covalent bonding between the α helix and the β sheet is specified in Section 5.6.4.1.1. A closer observation of the primary structures reveals diversified variable segments in the amino acid sequences, which occupy several regions (indicated by the gaps in **Figure 7**) in the primary structure. It has been proposed that these variable loop segments (Meves *et al.*, 1986; Jablonsky *et al.*, 1995; Possani *et al.*, 1999), are supposed to confer diverse pharmacological specificities. For further details the reader is urged to consult Possani *et al.* (1999) and Gurevitz *et al.* (2002). **Figure 7** demonstrates the arrangement of the secondary structures along the amino acid sequence, while **Figure 8** provides an example of the spatial arrangement of typical α and β toxins that are toxic to mammals.




```
              [B1]                    [----α----]    [--β2--]     [--β3----]
                      1   2           3    4           5           6  7                  8
AaHII    VKDGYIVDDVN-CTYFCGR-----NAYCNEECTKL-KGES-GYCQWASPYGNACYCYKLPDHVRTKGP---GR--CH
AaHI     KRDGYIVYPNN-CVYHCVP-----P--CDGLCKKN-GGSS-GSCSFLVPSGLACWCKDLPDNVPIKDT--SRK--CT
LqqV     LKDGYIVDDKN-CTFFCGR-----NAYCHDECKKK-GGES-GYCQWASPYGNACWCYKLPDRVSIKE---GR--CN
BotIII   VKDGYIVDDRN-CTYFCGR-----NAYCHEECTKL-KGES-GYCQWASPYGNACYCYKVPDHVRTKGP---GR--CN
TsIV-5   KKDGYPVEYDN-CAYICWNY---DNAYCDKLCKDK-KADS-GYCYWVHIL---CYCYGLPDSEPTK----TNGK-CKS

              [B1]                       [---α---]        [-β2]        [-β3]
CssII     KEGYLVSKSTGCKYECLKL--GDNDYCLRECKQQYGKSSGGYCYAF-----ACWCTHLYEQAVVWPL--PNKT-CN
CsEI      KDGYLVEK-TGCKKTCYKL--GENDFCNRECHWKHIGGSYGYCYGF-----GCYCEGLPDSTQTWPL--PNKT-C
Cn2       KEGYLVDKNTGCKYECLKL--GDNDYCLRECHKQQYGKGAGGYCYAF-----ACWCTHLYEQAIVWPL--PNKR-CS
Cn3       KEGYLVELGTGCKYECFKL--GDNDYCLRECHKARYGKGAGGYCYAF-----GCWCTQLYEQAVVWPL--KNKT-CR
```

Figure 7 Primary, secondary, and covalent structures of certain old world α toxins active on mammals (upper) and classical mammal specific β toxins (lower). Sequences were aligned in reference to Cys residues. Gaps were introduced to maximize similarities. Cys residues are numbered in the order they occur in the sequence. The regions of secondary structure are indicated in brackets above the sequences as α helices (α) and β strands (β). Abbreviations correspond to the scorpions: *Aa*H, *Androctonus australis* Hector; *Bot, Buthus occitanus tunetanus*; *Cn, Centruroides noxius* Hoffmann; *CsE, Centruroides sculpturatus* Ewing; *Css, Centruroides suffusus suffusus*; *Lqq, Leiurus quintquestriatus quinquestriatus*; *Ts, Tityus serrulatus*. (Reproduced with permission from Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^+-channels. *Eur. J. Biochem. 264* (2), 287–300.)

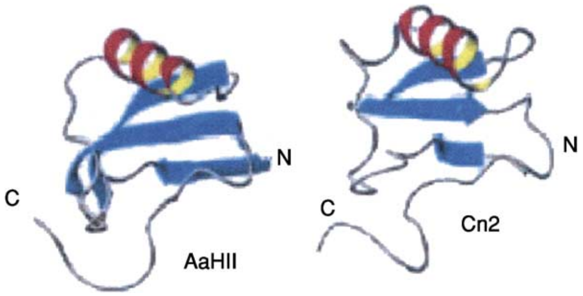


Figure 8 The spatial arrangement of α (AaHII) and β (Cn2) mammal toxic scorpion toxins derived from the venoms of the North African *Androctonus australis* and the Central American *Centruroides noxius* buthid scorpions, respectively. The two toxins reveal a similar orientation with the N and C-termini. (Reproduced with permission from Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^{+}-channels. *Eur. J. Biochem. 264* (2), 287–300.)

5.6.3. Insect Selective Neurotoxins: Pharmacology

5.6.3.1. The Pharmacological Ballet of Blowfly Larvae

5.6.3.1.1. The responsive larvae As indicated (see Section 5.6.2.4) scorpion toxins affecting the VGSCs, are presently subdivided into at least 10 functional groups. However, the three major groups of insect selective toxins, which are modifiers of the gating of the VGSCs, the excitatory depressant and α toxins, were detected on the basis of an intuitive observation of the behavioral response of blowfly larvae (Zlotkin *et al*., 2000).

Because blowfly larvae are continuously mobile with strictly segmented body musculature and have a soft and flexible integument, they can modify their mobility and body form in response to any disturbance in their neuromuscular system. As such, blowfly larvae provide a simple system to distinguish among the various insect selective toxins derived from scorpion venoms (**Figure 6**). Thus, excitatory toxins induce an immediate fast contraction paralysis (Zlotkin *et al*., 1971b, 1971c), while depressant toxins induce a preliminary transient contraction followed by a slowly progressing flaccid extension of the body (Zlotkin *et al*., 1991) (**Figure 6**). The alpha toxins show the so-called "delayed and extended contraction paralysis" (Eitan *et al*., 1990; **Figure 6**).

5.6.3.1.2. Isolation and purification The first scorpion venom derived insect selective neurotoxin was the excitatory toxin derived from the venom of *A. australis* Hector (AaIT). The isolation and purification of AaIT was monitored by the contraction paralysis assay of blowfly larvae (**Figure 6**). AaIT was isolated by using the column chromatography protocol developed by Miranda *et al*. (1970) for the purification of the first α toxins affecting mammals (see above). The protocol included water extraction, dialysis, recycling sephadex G50 gel filtration, and ion exchange equilibrium chromotography by anion (DEAE–Seph. A50) and cation (Amberlite, CG50) exchangers. However, presently, reverse phase columns are replacing the ion exchange chromatography (Eitan *et al*., 1990; Zlotkin *et al*., 1991). The final product, AaIT, contained 95% of the toxicity of the original venom, with a 267-fold increase in the specific toxicity. AaIT resembles other sodium channel scorpion neurotoxic polypeptides and comprises of a single chain polypeptide of 70 amino acids, cross-linked by four disulfide bridges. It is noteworthy that two additional AaIT isotoxins were isolated from the venom of *A. australis*: AaIT1, which differs by one amino acid (E25Q), and AaIT2, which differs by four amino acids (D2W, Y23N, E25Q, and P67T) (Loret *et al*., 1990a, 1990b). It was shown by modifications of Lys 28 or Lys 51 that these two amino acids are important for toxicity, and that their simultaneous modification led to a 40-fold reduction in insect toxicity. The primary structures of additional excitatory insect selective neurotoxins were derived from buthid scorpions such as *L. q. hebreaeus* (Froy *et al*., 1999), *L. q. quinquestriatus* (Kopeyan *et al*., 1990), *Buthus martensi* (Xiong *et al*., 1999), and *Buthotus judaicus* (BjxtrIT; Froy *et al*., 1999). With the exception of BjxtrIT toxin, these toxins had very similar amino acid sequences and identical location of the cysteine groups.

5.6.3.2. Complementary Duality of Excitatory and Depressant Toxins

5.6.3.2.1. Opposite as well as similar symptoms The blowfly larvae bioassay (**Figure 6a**) was used to monitor the purification of the excitatory insect selective toxins (Zlotkin *et al*., 1971a, 1985; Teitelbaum *et al*., 1979; Lester *et al*., 1982). The existence of depressant, flaccidity inducing insect toxins (such as BjIT2, LqqIT2, and LqhIT2) in Buthidae scorpion venoms was suggested by two pieces of evidence: (1) a progressive increase in the yield of the total contractive activity during the purification of the excitatory fly larvae-contractive toxins (Zlotkin *et al*., 1971a; Teitelbaum *et al*., 1979) suggested the removal of an antagonistic flaccidity inducing factor; and (2) the finding that venom of (*B. judaicus*), which is toxic to insects, did not induce in fly larvae the typical contraction paralysis but rather mixed, intermediate symptoms

that are between the two opposite forms of response presented in Figure 6a and b. Upon injection of 2 LD_{50} units of the crude venom into locusts, two phases of response were noticed. First, an immediate "knock-down" effect in the form of an excitatory paralysis (violent trembling of legs and contractions of thorax and abdominal muscles) for up to 30 min after injection. Second, after a partial recovery from this excitatory paralysis, there was a prolonged period of gradual progressive immobilization of the animal leading to death between 24 and 48 h following injection (Lester *et al.*, 1982). Subsequent systematic fractionation of the *B. judaicus* venom showed that the above duality in paralytic symptoms represents the presence of two categories of toxins that specifically affect insects, the excitatory and depressant toxins (Lester *et al.*, 1982). The depressant toxins, similar to the excitatory toxins, were shown to induce a transient short (1–2 s) phase of contractility that preceded the onset of flaccid paralysis (Figure 6b). As shown below, this similarity has additional manifestations.

5.6.3.2.2. Neuromuscular effects Simple considerations concerning symptomatology, doses, and speed of response indicated that the excitatory toxins, as well as the depressant toxins, substantially affected the nervous system.

The stimulatory action of the excitatory toxins on the skeletal musculature, as demonstrated in several insects (Zlotkin *et al.*, 1971a, 1971b; Walther *et al.*, 1976; Rathmayer *et al.*, 1978; Lester *et al.*, 1982), could have been attributed to either one or a combination of effects on the central nervous system (CNS), skeletal muscles, and/or peripheral motor neurons. Two separate lines of evidence, physiological and morphological, have clearly attributed the fast paralysis of the excitatory insect selective toxins to a presynaptic action, which is mediated by the terminal branches of motor nerves (Rathmayer *et al.*, 1978; Zlotkin, 1986; Fishman *et al.*, 1991). The latter conclusion is exemplified by Figure 9, which combines conventional electrophysiology and autoradiography to demonstrate that the peripheral terminal branches of the insect motor nerves are targeted by the excitatory AaIT toxin.

5.6.3.2.2.1. Depressant toxins LqhIT2 is a depressant toxin purified from the venom of the Israeli yellow scorpion. The effects of this toxin on prepupal housefly neuromuscular preparations mimic the effects on the intact animal (Figure 10), i.e., a brief period of repetitive bursts of junction potentials is followed by suppression of their amplitude and, finally, by a block of neuromuscular transmission.

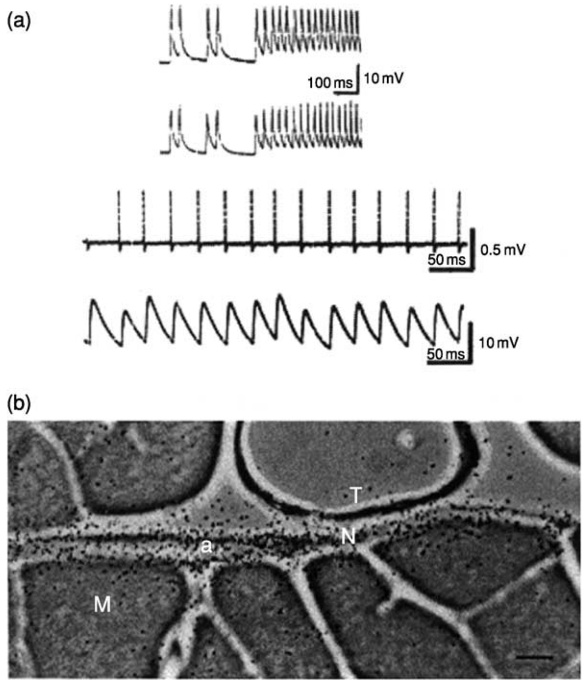


Figure 9 The peripheral terminal branches of the insect motor nerves are targeted by the AaIT. (a) Effect of AaIT on the neuromuscular preparation of the locust extensor tibiae muscle. Top: start of two trains of slow excitatory junction potential synchronously recorded from two distant locations on the muscle 50 min after application of 0.15 μg ml^{-1} of toxin. Bottom: spontaneous neuromuscular activity caused by toxin. Synchronous recording from the ''slow'' motor axon (upper trace) and a fiber in the main part of the extensor tibiae muscle (lower trace) 65 min after application of toxin. These data clearly indicate a presynaptic excitatory action on the motor nerve (Walther *et al.*, 1976). (b) Autoradiography of [^{125}I] AaIT in a terminal branch of motor nerve at its close proximity to the respective muscles in *P. americana* injected with a dose of the radioiodinated toxin, which induces fast paralysis. Note the obvious affinity of the toxin (photographic grains) to the nerves (N, nerve; a, axon) when compared to the surrounding muscles (M) and tracheae (T). (Reproduced with permission from Zlotkin, E., Fishman, Y., Elazar, M., **2000**. AaIT: from neurotoxin to insecticide. *Biochimie 82* (9–10), 869–881.)

Loose patch clamp recordings indicate that this repetitive activity has a presynaptic origin in the motor nerve, and closely resembles the effect of the excitatory toxin AaIT. The final synaptic block is attributed to neuronal membrane depolarization, which results in an increase in spontaneous transmitter release. This effect is not induced by the excitatory toxin which causes repetitive firing of the motor nerve (Figure 10d) and contraction of the larvae. The dominant effect of the depressant toxin is a complete block of neuromuscular transmission resulting in the final flaccid paralysis. In previous studies, using the cockroach ventral nerve cord derived giant axon, the insect specific depressant toxins LqqIT2 and $BjIT_2$ were shown

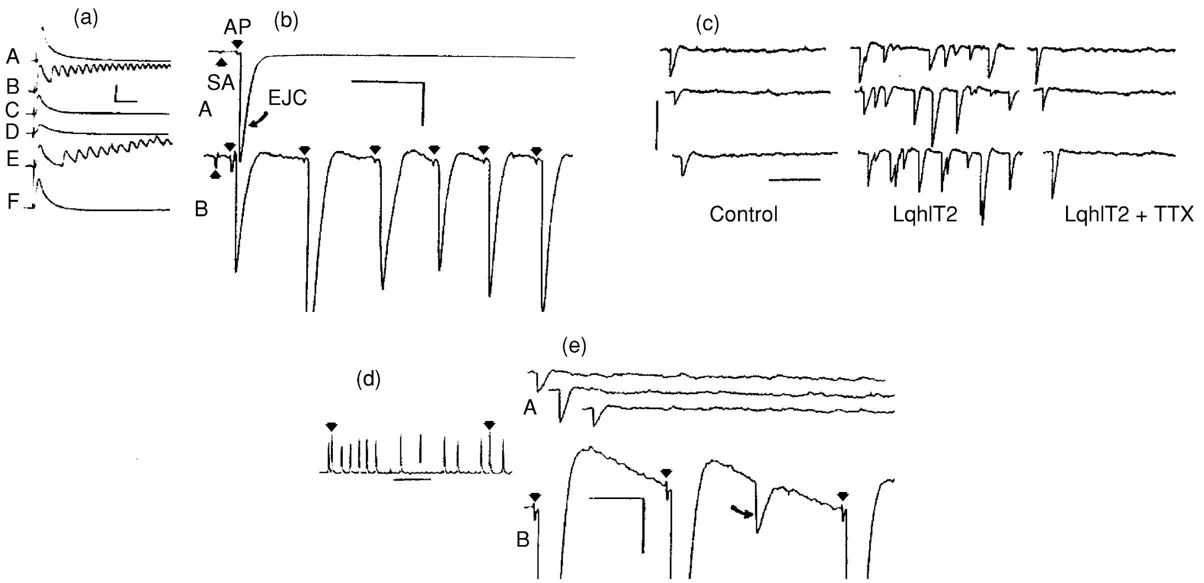


Figure 10 Neuromuscular effects of the depressant (LqhIT2) and excitatory (AaIT) insect toxins. (a) The depressant insect toxin LqhIT2 (40 nM) causes a brief phase of repetitive motor neuron activity followed by gradual suppression of excitatory junction potentials (EJPs) in prepupal *M. domestica*. (b) LqhIT2 induces repetitive presynaptic action potentials and excitatory junction currents (EJCs) in housefly muscle: (A) nerve stimulation (shown as a stimulus artifact, SA) generates a nerve terminal action potential (AP) resulting in an EJC of 2.7 nA amplitude; (b) following treatment with 30 nM LqhIT2, a single stimulus leads to repetitive action potentials (arrows) and EJCs. (c) LqhIT2 increases the spontaneous rate of neurotransmitter release, as measured by the frequency of miniature excitatory junction currents (MEJCs) recorded with a loose patch electrode. (d) AaIT induces repetitive activity in housefly motor neurons without suppressing EJPs or depolarizing the resting membrane potential of the muscle. Bath application of 125 nM toxin induces sporadic EJP activity. (e) Spontaneous junction current activity is shown before (A) and after (B) treatment with AaIT. No increase in spontaneous MEJC frequency was observed after treatment with AaIT. For details see text. For scorpion abbreviations see legend to **Figure 8**. (Reproduced with permission from Zlotkin, E., Eitan, M., Bindokas, V.P., Adams, M.E., Moyer, M., *et al.*, **1991**. Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry 30* (19), 4814–4821.)

to block evoked action potentials, due to a tetrodotoxin sensitive depolarization of the insect axonal membrane and suppression of the sodium current (Lester *et al.*, 1982; Zlotkin *et al.*, 1985). The data presented in Figure 10 suggest that in the prepupal housefly preparation the blocking action of LqhIT2 is a consequence of membrane depolarization. Owing to the difficulty in measuring toxin induced changes in the motor nerve terminal directly, the depolarization was indirectly indicated by an increase in the frequency of miniature end plate junction potentials (MEJCs) (Zlotkin *et al.*, 1991). Such end terminal depolarization could sharply limit invasion of action potentials into nerve terminals and, thereby, suppress excitatory junction potential evoked transmitter release. The ability of very low LqhIT2 concentrations (5 nM) to increase MEJC frequency suggests that depolarization may be a primary effect of the toxin on the motor system. The absence of an increase in MEJC activity following AaIT treatment (Figure 10e) suggests that this excitatory toxin causes minimal, if any, depolarization of motor axon terminals. Its predominant effect is to produce sustained repetitive activity in the motor nerve (Figure 10d and e), as previously shown in a locust neuromuscular preparation (Walther *et al.*, 1976).

Thus, the excitatory toxin has a single effect on the motor nerve, inducing sustained repetitive activity, while the depressant toxin induces a transient phase of repetitive activity, followed by motor nerve terminal depolarization that produces the ultimate block of neuromuscular transmission. The distinctive characteristics differentiating excitatory (AaIT) from depressant (LqhIT2) insect toxins recall those previously observed with type I and type II pyrethroid insecticides. Like the excitatory toxins, the type I pyrethroids produce a slowing of sodium channel inactivation and repetitive action potential activity. In contrast, the type II pyrethroids, like the depressant insect toxins, primarily depolarize the resting membrane potential (Narahashi, 1986).

5.6.3.2.3. Effects on sodium conductance The mechanism of the AaIT's induced (see above) axonal repetitive firing was clarified by studies on an

isolated giant axon from the CNS of the cockroach *Periplaneta americana* (Pelhate and Zlotkin, 1981, 1982) in current and voltage clamp assays, using the double oil gap single fiber technique (Pichon and Boistel, 1967). The AaIT induced repetitive firing was accompanied by a 2–7 mV depolarization of the axonal membrane. Voltage clamp experiments were performed in the presence of 2×10^{-6} M diaminopyridine, thus selectively blocking potassium current (Pelhate and Pichon, 1974). Under voltage clamp, AaIT slowed Na^+ inactivation most effectively at low negative values of applied voltage and increased the peak sodium current by about 20%. It was concluded (Pelhate and Zlotkin, 1981, 1982) that the repetitive activity induced by AaIT results from the voltage dependent modulation of sodium inactivation coupled with an increase in sodium permeability. AaIT did not affect potassium current. The excitatory toxins BjIT1 (Lester *et al.*, 1982) and LqIT1 (Zlotkin *et al.*, 1985), when assayed on the cockroach isolated axonal preparation, have yielded data closely resembling those of the AaIT. Their induced repetitive firing is attributable to an increase of sodium conductance and the slowing of its turning off. Recent studies have supported the above basic conclusion by revealing that the excitatory toxins shift the threshold of insect sodium channel activation to a more negative membrane potential and increase peak sodium currents (Gordon *et al.*, 2002). The recently purified excitatory insect toxin (BjxtrIT, Froy *et al.*, 1999), when assayed on the identical system of the cockroach giant axon (Pichon and Boistel, 1967), has mimicked the AaIT toxin. In current clamp experiments, BjxtrIT depolarized the resting membrane potential and generated spontaneous action potentials (**Figure 11a** and **b**). In voltage clamp experiments, a series of voltage steps from the holding potential of −60 mV up to +50 mV, in the absence (control) and presence of the BjxtrIT toxin, revealed that BjxtrIT opens insect sodium channels at very negative potential values. This element is shown in **Figure 11d** in the form of the curves on relative sodium conductance under control conditions and in the presence of toxin. As shown, BjxtrIT causes a shift in the sodium activation voltage curve to more negative values by 12–15 mV.

An essentially similar result, namely a shift in the voltage dependent activation to more negative potentials, was revealed in the action of the AaIT toxin in central neurons of a lepidopterous insect, the tobacco budworm, *Heliothis virescens* (Lee and Adams, 2000). Neurons were prepared from thoracic and abdominal ganglia of male moths or last instar larvae. Neuronal sodium currents were obtained by whole cell recordings. The shift in voltage dependent activations is demonstrated in **Figure 12c**, which reveals that inspection of I/V curves before and after toxin exposure showed a shift in voltage dependent activation at more negative potentials. The shift in voltage dependent activation caused by AaIT is concentration dependent (not shown) (Lee and Adams, 2000).

The progressively developing flaccidity of experimental insects caused by the depressant toxins BjIT2 and LqIT2 was explained through data obtained using the *in vitro* cockroach axonal preparation (Lester *et al.*, 1982; Zlotkin *et al.*, 1985). The first effect was a progressive, irreversible depolarizing action, as shown by a continuous decrease in the value of the resting membrane potential. The second effect was the progressive suppression of the sodium current. The combination of these two effects explains the progressive block of the evoked action potentials. The blocking effect of the depressant toxins on axonal excitability may account for the induction of flaccidity and progressive paralysis in the test animals.

A more recent study (Ben Khalifa *et al.*, 1997) has clarified the mechanism of the blocking effect, emphasizing the fact that depressant toxins are sodium channel openers. This fundamental conclusion was reached in two successive studies employing the BotIT2 and the recombinant LqhIT2 depressant toxins on the isolated giant axon and isolated dorsal unpaired median (DUM) neuron of the American cockroach, under current and voltage clamp conditions, using the double oil gap and the patch clamp techniques, respectively (Stankiewicz *et al.*, 1996; Ben Khalifa *et al.*, 1997). In current clamp conditions, these toxins reproduce the effects previously seen with BjIT2 and LqIT2; namely, membrane depolarization, transient repetitive activity, and the occurrence of occasional plateau potentials upon artificial repolarization. However, the voltage clamp experiments in these studies were performed under voltage pulses of long duration (75–100 ms), and they reveal that the toxins target the sodium channel activation in a similar way to the β toxins affecting mammals (see above).

Accordingly, the clearest effect of the BotIT2 and LqhIT2 toxins is the development of a slow activating-deactivating sodium current, which follows or accompanies the previously seen decrease of the fast peak sodium current (**Figure 13**). It is noteworthy that a similar decrease of the peak sodium current is also induced by beta toxins (Wang and Strichartz, 1983), accompanied by the development of the slow sodium current (Meves *et al.*, 1982, 1984). Analysis of the above phenomena (Stankiewicz *et al.*, 1996;

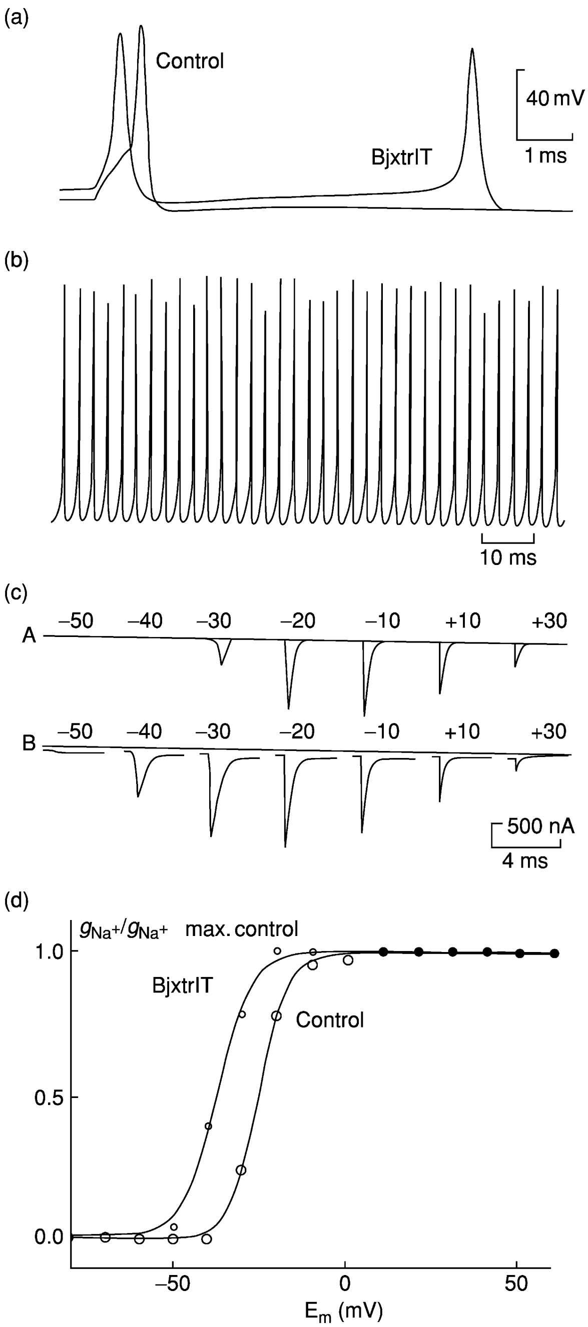


Figure 11 Effect of BjxtrIT on electrical activity and sodium currents of a cockroach isolated axon. (a) Two superimposed recordings of resting potentials and evoked action potentials under control conditions. A 0.5 ms current pulse of 10 nA evoked only one action potential of 95 mV in amplitude and 0.5 ms in duration (~20 mV above the resting potential). Six minutes after BjxtrIT application, the resting potential decreased by 6 mV; the action potential reached its threshold more rapidly and a second spontaneous action potential was measured 6.5 ms later, giving an instantaneous frequency of 154 Hz. (b) Spontaneous activity of the action potential after 7 and 10 min, respectively, after BjxtrIT application. Action potential frequencies higher than 200 Hz were obtained, and no ''plateau potentials'' could be measured. (c) Families of Na^+ currents recorded in the presence of 0.5 mM 3,4 diaminopyridine during 5 ms voltage pulses. (A): control; (B): after 6 min in the presence of Bj-strIT. Note that after BjxtrIT application, Na^+ currents developed at more negative potential values than in the control. The horizontal lines indicate the zero current level. (d) Voltage dependence of the sodium conductance (expressed as g_{Na^+}/g_{Na^+} max. control) calculated from current families as illustrated. Note the shift of the activation curve by 12.5 mV towards more negative potentials after addition of BjxtrIT. Bj, *Buthotus judaicus*. (Reproduced with permission from Froy, O., Zilberberg, N., Gordon, D., Turkov, M., Gilles, N., *et al.*, **1999**. The putative bioactive surface of insect-selective scorpion excitatory neurotoxins. *J. Biol. Chem. 274* (9), 5769–5776.)

Ben Khalifa *et al.*, 1997) has suggested that the balance between the fast transient and the slow sodium current components observed under BotIT2 and LqhIT2 would support the hypothesis of progressive toxin mediated transformation of fast-activating sodium channels into slow ones. The existence of two gating modes of sodium channels (fast and slow) has been reported in certain types of cells (Barres *et al.*, 1989; Kirsch and Brown, 1989),

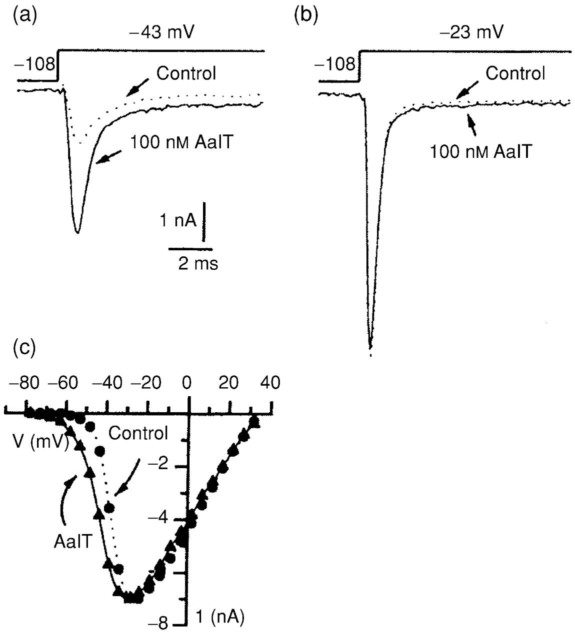


Figure 12 Voltage dependent action of AaIT (100 nM) on sodium channels in *H. virescens* neurons. (a) Peak sodium current evoked from a holding potential (V_T = −108 mV) to a test potential to −43 mV was more than doubled by AaIT, and steady-state sodium current also was increased. (b) Exposure to the toxin causes little change in peak or steady-state current at a test potential of −23 mV. (c) The current–voltage curve is shifted to more hyperpolarizing potentials in the voltage range −50 to −30 mV. (Reproduced with permission from Lee, D., Adams, M.E., **2000**. Sodium channels in central neurons of tobacco budworm, *Heliothis virescens*: basic properties and modification by scorpion toxins. *J. Insect Physiol. 46* (4), 499–508.)

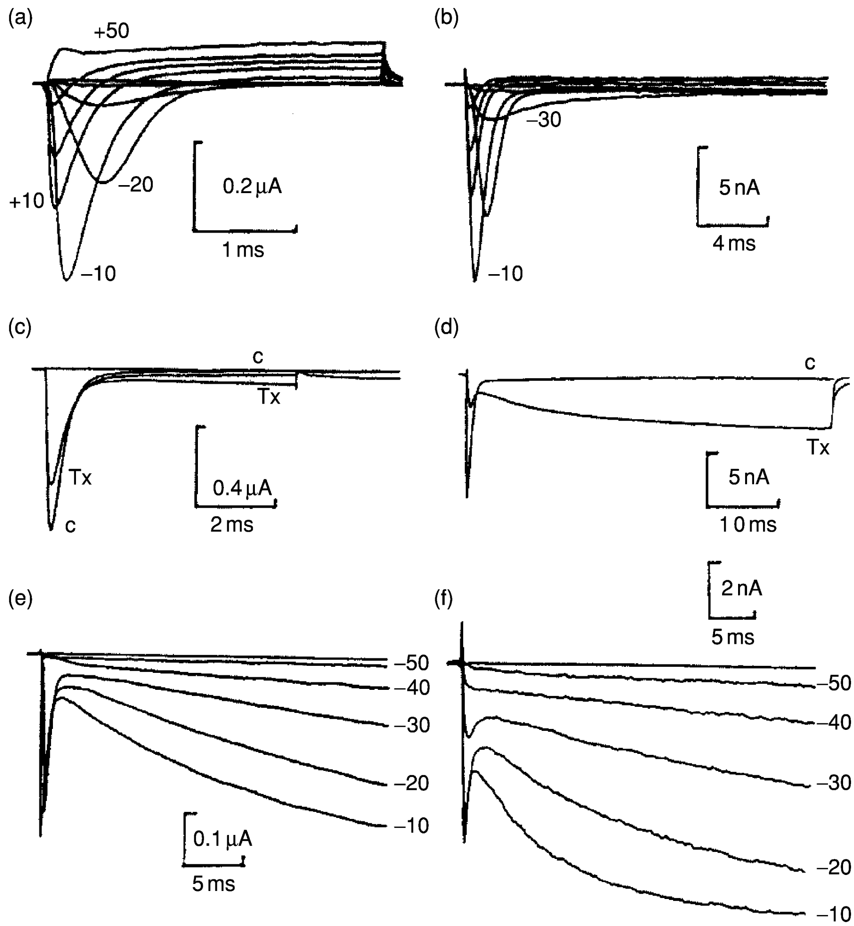


Figure 13 Transmembrane sodium currents recorded in giant axon (a, c, e) and in DUM neurone (b, d, f). (a, b) Control families of sodium currents recorded at different voltage pulses from a holding potential (HP) of −60 mV and −90 mV in axon and DUM neurone, respectively. (c, d) Superimposed sodium currents recorded before (c) and after application (Tx) of BotIT2 (1.5×10^{-6} M for axon and 3×10^{-6} M for DUM neurone). (e, f) Families of sodium currents after BotIT2. The pulse values are indicated near each trace. (Reproduced from Stankiewicz, M., Grolleau, F., Lapied, B., Borchani, L., el Ayeb, M., *et al.*, **1996**. BotIT2, a toxin paralytic to insects from the *Buthus occitanus tunetanus* venom modifying the activity of insect sodium channels. *J. Insect Physiol. 42* (4), 397–405.

where each channel switches between different structural conformations that underlie fast and slow gating. The toxins may interact with the above gating mechanism, transforming fast channels into slow ones. Furthermore, these slow currents may account for the global decrease in sodium peak conductance, which in turn accounts for the toxin induced flaccidity. In other words, the flaccidity is not due to a blockage of sodium channels, but rather to modified channels that open at more negative potentials and activate slowly. Thus, the depressant toxins are sodium channel openers.

5.6.3.2.4. Mutual displacability Differences in their primary structures, symptoms, and the effects on sodium conductance have raised the expectation that the excitatory and depressant toxins occupy separate receptor binding sites, similar to the α and β scorpion toxins affecting vertebrates. However, a series of competitive displacement binding assays, as shown in Figure 14, reveals that the radioiodinated AaIT toxin binding to locust synaptosomal membrane vesicles is displaced with a similar affinity by various excitatory and depressant insect selective toxins. Figure 15 reveals that the depressant LqhIT2 toxin possesses two noninteracting binding sites in locust neuronal membranes: a high-affinity ($K_{D1} = 0.9 \pm 0.6$ nM) and low-capacity ($B_{max} = 0.1 \pm 0.07$ pmol mg^{-1}) binding site, as well as a low-affinity ($K_{D2} = 185 \pm 13$ nM) and high-capacity ($B_{max} = 10.0 \pm 0.6$ pmol ng^{-1}) binding site. The data presented in Figure 16 reveal that the high-affinity site (responsible for the paralytic action) serves as a target for binding competition by the excitatory insect toxin AaIT. It is noteworthy that a scorpion β toxin (Ts VII), which was previously

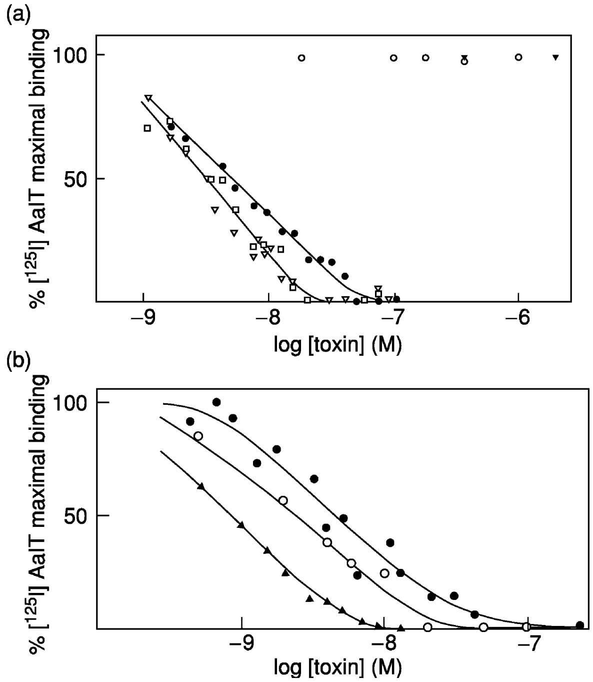


Figure 14 Displaceability of the [^{125}I] AaIT by the excitatory and depressant insect toxins. Ten microliters of the locust synaptosomal membrane vesicles (35 μg of protein) were added to 190 μl of the standard binding medium (0.15 M choline chloride, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1% BSA) containing 1.5 nM of [^{125}I] AaIT and increasing concentrations of each of the following unlabeled toxins: (a) AaIT (□); BjIT1 (●); BjIT2 (▽); AaMT2 (▼); *C. soffusus* β-mammal toxin 2 (○); (b) AaIT(○) LqIT1 (▲); LqIT2(●). The membranes were incubated for 30 min at 20–22 °C. For scorpion abbreviations see legend to **Figures 7, 10 and 11**. (Reproduced with permission from Gordon, D., Jover, E., Couraud, F., Zlotkin, E., **1984**. The binding of the insect selective neurotoxin (AaIT) from scorpion venom to locust synaptosomal membranes. *Biochim. Biophys. Acta 778*, 349–358; and Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M.F., *et al.*, **1985**. An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding Site. *Arch. Biochem. Biophys. 240* (2), 877–887.)

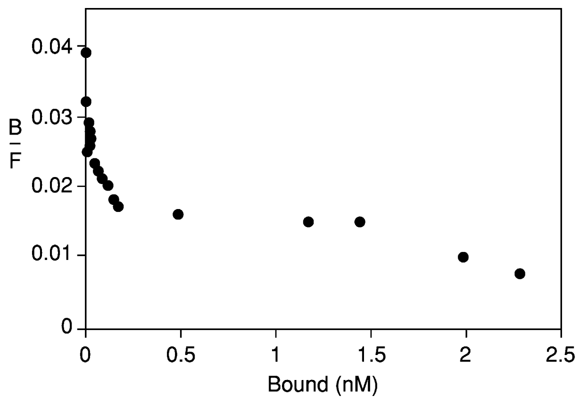


Figure 15 Scatchard analysis of a saturation curve of LqhIT2 binding to insect neuronal membranes. Locust neuronal membranes (155 μg of protein) were incubated with 72.5 pM [^{125}I]LqhIT2 and increasing concentrations of unlabeled LqhIT2 for 30 min at 22 °C in 0.4 ml of binding buffer, and the amount of specifically bound [^{125}I]LqhIT2 was determined following rapid filtration. Nonspecific binding (30–50% of total binding) was determined in the presence of 1 μM LqhIT2 and was subtracted from all data points. The concentration of LqhIT2 up to 5 μM did not reduce nonspecific binding. The specific radioactivity and the amount of bound toxin were recalculated for each toxin concentration, and the Scatchard plot was drawn using the computer program LIGAND. Scatchard plot analysis yielded the best fit ($P < 0.05$) using a two binding site model. The K_D values, obtained from three separate experiments, are $K_{D1} = 0.9 \pm 0.6$ nM, $B_{max1} = 0.1 \pm 0.07$ pmol per mg protein and $K_{D2} = 185 \pm 13$ nM, $B_{max2} = 10.0 \pm 0.6$ pmol per mg of protein. The total concentration of binding sites (R_T) used in the Hill plot was determined from Scatchard analysis. $N_H = 0.996$. For scorpion abbreviations see legend to **Figure 6**. (Reproduced with permission from Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W.A., *et al.*, **1992**. Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies. *Biochemistry 31* (33), 7622–7628.)

shown to compete with the AaIT on its binding sites in fly and cockroach CNS neuronal membranes (De Lima *et al.*, 1986), as well as with other β toxins in rat brain membranes (Barhanin *et al.*, 1982), was shown (Gordon *et al.*, 1992) to displace the [^{125}I] LqhIT2 from its high-affinity binding sites in the locust neuronal preparation as well. Thus, it may be concluded that the excitatory and depressant insect selective neurotoxins are able to mutually displace each other from a common receptor site. The latter is the high-affinity site of the LqhIT2 toxin, which is also shared by the β toxin TSVII (see below) (Gordon *et al.*, 1992). The above data suggested that the two different kinds of insect selective toxins either share a common binding site or possess separate, but mutually related, interacting binding sites on the insect neuronal sodium channel. The latter possibility, namely, separate binding sites, was suggested through the employment of site directed antibodies corresponding to conserved putative extracellular segments of the vertebrate sodium channels, coupled with binding assays of the radioiodinated AaIT and LqhIT2 toxins. These studies showed that the antibody mediated inhibition of [^{125}I] AaIT binding differs from that of LqhIT2 (Gordon *et al.*, 1992). Three out of the four antibodies that inhibited LqhIT2 binding only partially affected AaIT binding. These data suggest that the receptors to the depressant and excitatory insect toxins: (1) comprise an integral part of the insect sodium channel; (2) are formed by segments of external loops of the sodium channel; and (3) are in close proximity but are not identical in spite of the competitive interaction between these toxins (Gordon *et al.*, 1992).

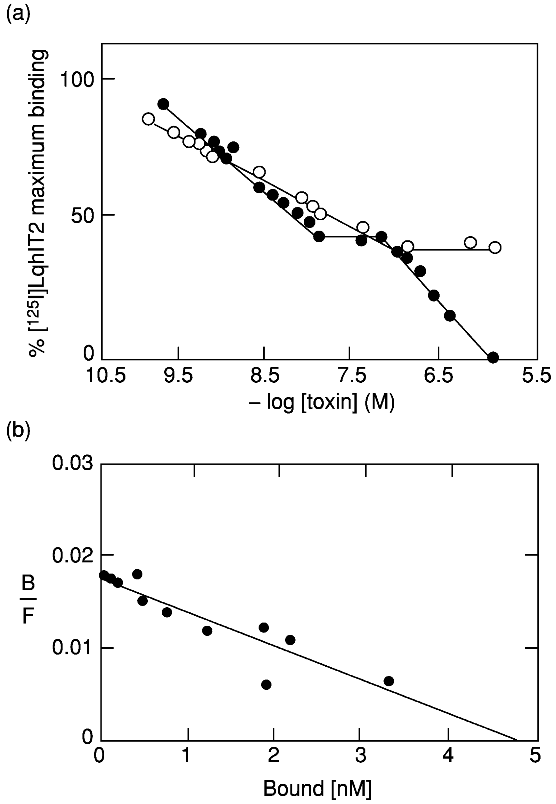


Figure 16 Displacement of [^{125}I]LqhIT2 binding by LqhIT2 and AaIT. Locust neuronal membranes were incubated for 30 min at 22 °C in the presence of 72.5 pM [^{125}I]LqhIT2 and increasing concentrations of LqhIT2. (●, 150 µg of membrane protein) or AaIT (○, 114 µg of membrane protein). Nonspecific binding of [^{125}I]LqhIT2, determined in the presence of 1 µM LqhIT2 was subtracted. The nonspecific binding was not affected by higher LqhIT2 concentrations, up to 5 µM. (a) Plots of [^{125}I]LqhIT2 bound as a function of log toxin concentration. (b) Scatchard analysis of a separate experiment in which 72.5 pM [^{125}I]LqhIT2 and 0.5 µM unlabeled AaIT (to saturate the high-affinity binding sites of LqhIT2) were incubated with 191 µg of locust membrane protein in the presence of increasing concentrations of unlabeled LqhIT2. The data were analyzed using the computer program LIGAND. The binding parameters of LqhIT2, representing the second low-affinity binding site, are $K_D = 194 \pm 36$ nM and $B_{max} = 13.8 \pm 6.9$ pmol per mg protein. (Reproduced with permission from Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W.A., *et al.*, **1992**. Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies. *Biochemistry 31* (33), 7622–7628.)

5.6.3.2.5. Complementary duality From a chemical ecology point of view the mutual displaceability among the excitatory and depressant toxins that occur in the same venom is puzzling. Such a paradox should have been eliminated by an evolutionary process. Its consistent occurrence in certain buthid venoms may suggest that it may fulfill an ecological role.

In Section 5.6.1.3, the change in composition of scorpion venom with the process of successive secretion is discussed. Lester *et al.* (1982) showed that a locust injected with *B. judaicus* crude venom displays a biphasic mode of paralysis, namely, a fast "knock-down," excitatory paralysis followed by a delayed progressive immobilization (similar to the effect of the depressant BjIT2 toxin) (see Section 5.6.3.2.1). Taking the above into account, it is tempting to assume that in successive venom secretions, the first released venom drops should contain predominantly excitatory toxin and that subsequent secretions should contain mainly the depressant toxin. The above possibility is supported by recent preliminary data based on blowfly larvae assays and RP-HPLC separation of buthid scorpion venom, indicating that the first stings contain mainly excitatory toxin while in subsequent stings, the depressant toxin predominates (Zlotkin, unpublished data).

An additional possibility is that the combined presence of the excitatory and depressant toxins fulfills a complementary or even cooperative role in the envenomated insect. Such an assumption is supported by the data presented in Figure 17 revealing a diversity in the mutual displaceability between the excitatory and depressant toxins in various insects. As shown in Figure 17, AaIT competes for all the high-affinity sites of LqhIT2 in cockroach and locust nervous system (Figures 15 and 16) and partially in the fly head neuronal membranes, but there is no displaceability between the two toxins in the lepidopterous larvae neuronal membranes. The insecticidal cooperativity towards noctuid larvae has been most recently revealed between an excitatory (LqhIT1) and a depressant (LqhIT2) toxin through the construct of a "double recombinant" *Autographa californica* nucleopolyhedrovirus (AcMNPV) (Regev *et al.*, 2003; see also Section 5.6.5.2). In other words the lack of competitive displaceability between the excitatory and depressant toxin (Figure 17; Moskowitz *et al.*, 1994) in the neuronal membranes of lepidopterous larvae enables the above insecticidal cooperativity.

5.6.3.2.6. The excitatory and depressant toxins are β toxins In the β toxin class, most toxins, which have been shown to be active on mammals, also show some activity against insects (Possani, 1984; De Lima *et al.*, 1986). The most selective group of toxins, which are toxic exclusively to insects, are the excitatory and depressant toxins. These toxins modify the activation process of the insect voltage-gated sodium channels (see Section 5.6.3.2.3). Therefore, these insect selective toxins represent the β toxin category among the Old World scorpion venoms (Stankiewicz *et al.*, 1996; Gordon, 1997a,1997b).

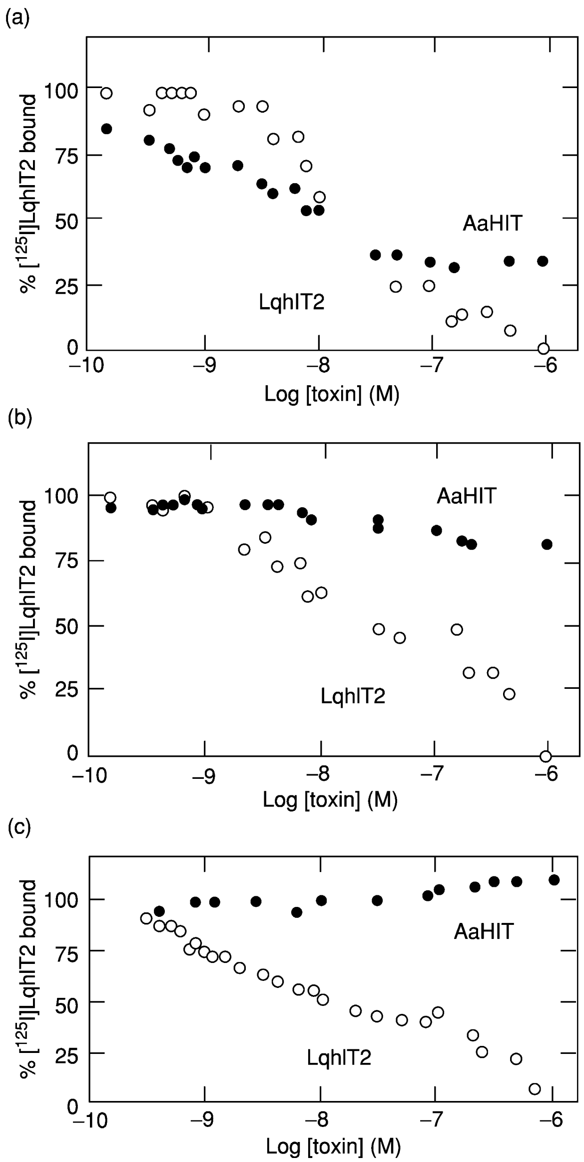


Figure 17 Competition for [^{125}I]LqhIT2 depressant toxin binding by the excitatory toxin AaHIT on various insect neuronal membranes. Neuronal membranes of (a) cockroach (*Periplaneta americana*), (b) blowfly head (*Sarcophaga falculata*), or (c) lepidopterous larvae (*Spodoptera littoralis*) were incubated for 1 h at room temperature in the presence of [^{125}I]LqhIT2 and increasing concentrations of AaHIT (●) or LqhIT2 (○). Nonspecific binding, measured in the presence of 1 μM LqhIT2, was subtracted. The depressant toxin reveals the two binding sites on every insect neuronal preparation while the excitatory AaHIT competes for all the high-affinity sites in cockroach (and locust), partially in fly and not at all in lepidopterous larvae neuronal membranes. For scorpion abbreviations see legend to **Figure 6**. (Reproduced with permission from Moskowitz, H., Herrmann, R., Zlotkin, E., Gordon, D., **1994**. Variability among insect sodium channels revealed by binding of selective neurotoxins. *Insect Biochem. Mol. Biol. 224*, 13–19.)

Two additional considerations support the similarity of the excitatory and depressant insect selective toxins to the β toxin category. First, their specific binding to the insect neuronal membranes is voltage independent (Gordon *et al.*, 1984; Moskowitz *et al.*, 1994) similar to that observed with mammalian sodium channels (Jover *et al.*, 1980; Lazdunski *et al.*, 1986). Second, the two categories of toxins were displaced by a typical β toxin (TS VII) from their binding sites in insect neuronal preparations. The latter was revealed in fly head synaptosomes using labeled excitatory AaIT toxin (De Lima *et al.*, 1986), and in locust synaptosomal vesicles by labeled depressant LqhIT2 toxin (Gordon *et al.*, 1992). Finally, the concluding remark of Stankiewicz *et al.* (1996) is noteworthy. They suggested that the excitatory and depressant toxins possess similar modes of action, and are distinguished essentially by their time course (kinetics). The depressant toxins drastically prolong the opening time of the sodium channel and, therefore, induce a depolarization that blocks neuronal conductance. The excitatory toxin induces a channel opening time of shorter duration, coupled to a voltage dependent slowing of inactivation, which favors the repetitive activity (Stankiewicz *et al.*, 1996).

5.6.3.3. Alpha Toxins Affecting Insects

5.6.3.3.1. LqhαIT toxin: the prototype This toxin was detected and isolated utilizing the blowfly bioassay monitoring a delayed and sustained contraction paralysis (**Figure 6c**; Eitan *et al.*, 1990). Like the excitatory and depressant insect selective neurotoxins, LqhαIT was highly toxic to insects, but it differed from these toxins in two important ways. First, LqhαIT lacked strict selectivity for insects. It was highly toxic to custaceans and had a measurable but low toxicity to mice. Second, it did not displace the excitatory toxin [^{125}I]-AaIT from its binding sites in the insect neuronal membrane. Such indicates the binding sites for LqhαIT are different from those shared by the excitatory and depressant toxins. However, in its primary structure (see **Figure 18**) and its effect on excitable tissues, LqhαIT strongly resembles the well characterized mammalian active α scorpion toxins. Its amino acid sequence was 55–75% identical with these α toxins. Such degree of similarity is comparable to that seen among the α toxins themselves. Voltage and current clamp studies showed that LqhαIT

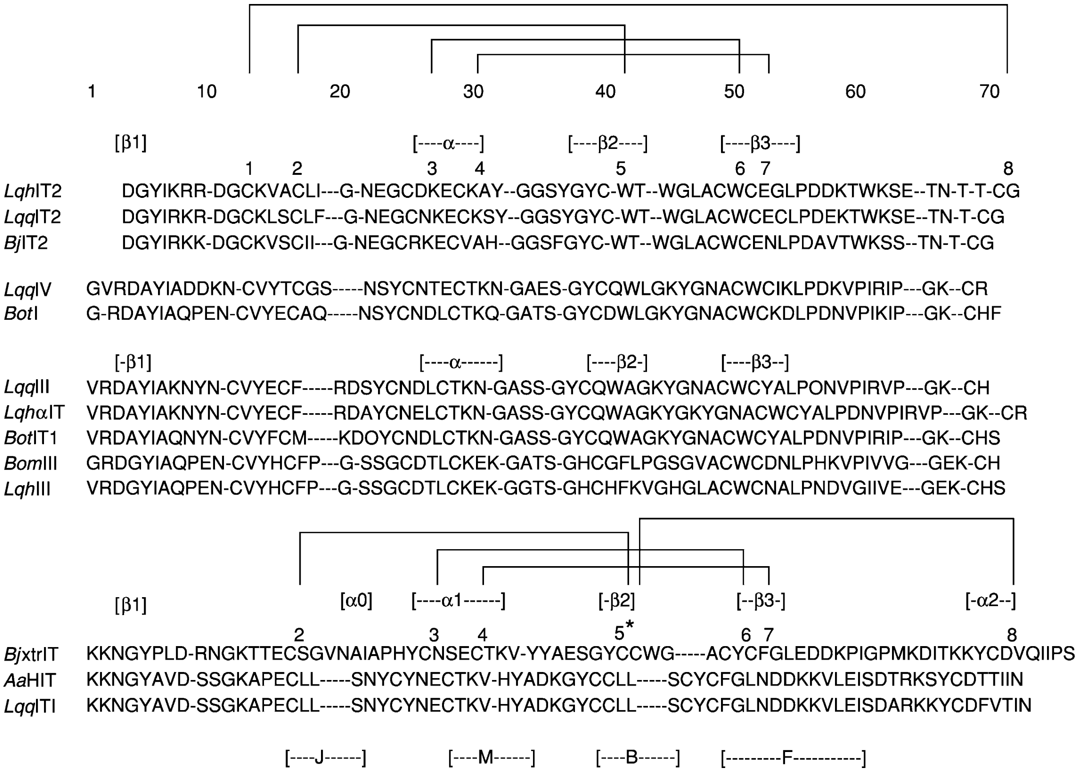


Figure 18 Primary covalent and secondary structures of representatives of the three major groups of the scorpion venom derived insect specific toxins affecting the VGSCs. Sequences were aligned taking as reference the Cys residues. Gaps were introduced in order to maximize similarities. Cys residues are numbered in the order of their appearance from N- to C-terminal. The regions of secondary structure revealed by three-dimensional studies are indicated in brackets above the sequences α (α helix), β (β strand). The asterisk on the right side of the fifth cysteine residue of the excitatory toxins indicates the position of the putative substitute of Cys 1 in the previous toxins. Bracketed J, M, B, and F regions at the bottom of the table indicate the position of the variable loops. Abbreviations correspond to the scorpions: *Aa*H, *Androctonus australis* Hector; *Bj*, *Buthotus judaicus*; *Bom*, *Buthus occitanus mordocei*; *Bot*, *Buthus occitanus tunetanus*; *Lqh*, *Leiurus quinquestriatus hebraeus*; *Lqq*, *Leiurus quintquestriatus quinquestriatus*. (Reproduced with permission from Possani, L.D., Becerril. B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^{+}-channels. *Eur. J. Biochem. 264* (2), 287–300.)

caused an extreme prolongation of the action potential in both cockroach giant axon (**Figure 19**) and rat skeletal muscle preparations, because of slowing inactivation of sodium currents. It has been concluded that LqhαIT is an α toxin, which acts on insect sodium channels (Eitan *et al*., 1990). However, due to its potential insecticidal value, a recent study that used neurons from thoracic and abdominal ganglia from the tobacco budworm, *H. virescens*, deserves attention (Lee and Adams, 2000). When treated with LqhαIT, in contrast to the excitatory AaIT, the toxin was shown to slow channel inactivation dramatically without modifying voltage dependent activation (**Figure 20**). Lee and Adams (2000) suggested that the differences in the poisoning symptoms of AaIT and LqhαIT can be explained by their different effects on sodium conductance. AaIT causes stimulus independent spontaneous action potentials in injected insects and *in vitro* neuromuscular preparations (see Section 5.6.3.2.2) leading to the typical symptoms of tetanic paralysis. In contrast, LqhαIT did not have any effect on the activation, but strongly inhibits the inactivation process. These considerations provide a reasonable explanation for the fact that LqhαIT causes repetitive activity only when motor axons are previously activated, leading to hyperactivity but not tetanic contractions (Lee, 1997).

5.6.3.3.2. The pharmacologic diversity of the α toxins As shown in Section 5.6.2.2, α scorpion toxins slow sodium current inactivation in various excitable preparations (Gordon and Gurevitz, 2003). The mammalian VGSCs are expressed by a gene family, which produce at least 12 known sodium channel subtypes that are distinguished by their location in various excitable tissues and their TTX sensitivity. Scorpion α toxins serve as a proper tool

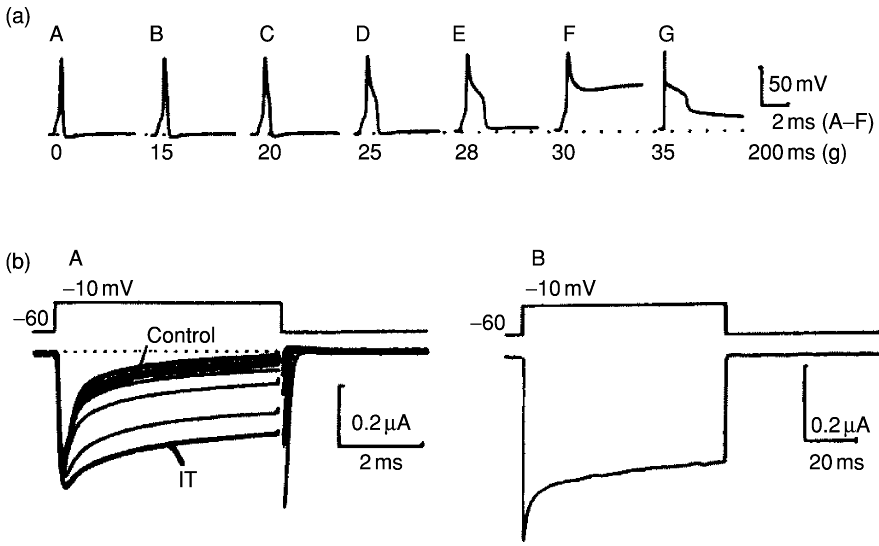


Figure 19 Effect of LqhαIT toxin on action potentials and sodium currents of isolated insect axon. (a) Effect on action potentials of the isolated cockroach axon. (A–G) Records of action potentials evoked by a short (0.5 ms) depolarizing current pulse of 10 nA. The number under the records indicates the time in minutes. Note the different time scale in grams. (b) Effect of LqhαIT toxin (10^{-6} M) on the sodium current of the isolated cockroach axon – a voltage-clamp experiment in the presence of 2×10^{-4} M 3,4-diaminopyridine. (A) Superimposed records of the sodium currents. In the control, the sodium current completely inactivates in less than 6 ms. In the presence of LqhαIT toxin (IT), the sodium inactivation is progressively slowed. The records were made every 20 s. (B) Record of the maintained sodium current. This record, which shows a very slow decrease in the sodium inward current during the voltage pulse, was taken 3 min after LqhαIT application. The slowest time constant was 250 ms instead of about 2.0 ms as observed in the absence of toxin. (Reproduced with permission from Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., *et al.*, **1990**. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure, and mode of action. *Biochemistry 29* (25), 5941–5947.)

for the identification of the various mammalian sodium channels (Gordon *et al.*, 2002). Thus, α toxins could have been subdivided according to their preference for mouse brain or rat synaptosomes, their abilities to affect rat brain sodium current or rat pyramidal neurons current, or even to distinguish between skeletal muscle, cardiac muscle, or motor nerve sodium channels (Gilles *et al.*, 1999, 2000; Gordon *et al.*, 2002). However, from a strictly entomological point of view, the scorpion α toxins are currently subdivided into three categories: (1) the "classical" α toxins, such as AaH2 and Lqh2, which are active in the mammalian brain; (2) α toxins that are very active in insects, namely LqhαIT and Lqq3 (Gordon *et al.*, 2002); and (3) α-like toxins, such as Lqh3, Bom 3, and Bom 4, which are unable to displace AaH2 (Gordon *et al.*, 2002). These toxins are active in both the mammalian and insect CNS.

All scorpion α toxins compete with the LqhαIT binding site on the insect VGSC receptor site 3 (**Table 3**). However, their binding affinity for insect and various mammalian sodium channels, as well as their voltage dependence and allosteric modulation by other channel toxicants, varies greatly (Gordon *et al.*, 1996, 2002; Gilles *et al.*, 2001, 2002).

5.6.4. Insect Selective Neurotoxins: the Molecular Level

5.6.4.1. Structure–Function Relationships

5.6.4.1.1. Primary, secondary, and tertiary structures **Figure 18** shows the primary covalent and secondary structures of the depressant, the excitatory, and the α insect specific toxins (Possani *et al.*, 1999). The depressant and the α insect specific toxins possess the typical structural arrangement revealed by the vertebrate affecting α and β toxins (Section 5.6.2.4; **Figures 7 and 8**), namely, a single chain of about 60–65 amino acids cross-linked by four disulfide bridges and a conserved secondary structure composed of an α helix adjacent to a triple-stranded antiparallel β sheet (see Section 5.6.2.4). The helix motif is linked to the β_3 strand by two of the four disulfide bonds (Cys 3 and 4 connected to Cys 6 and 7). A third, structurally conserved disulfide bond is the one between the Cys residues 2 and 5. These three conserved bonds occur in all scorpion toxins (including the excitatory insect specific toxins and the sodium channel blockers) (Possani *et al.*, 1999), and they play a role in the stabilization of the structurally conserved core of scorpion toxins. The fourth disulfide bridge in

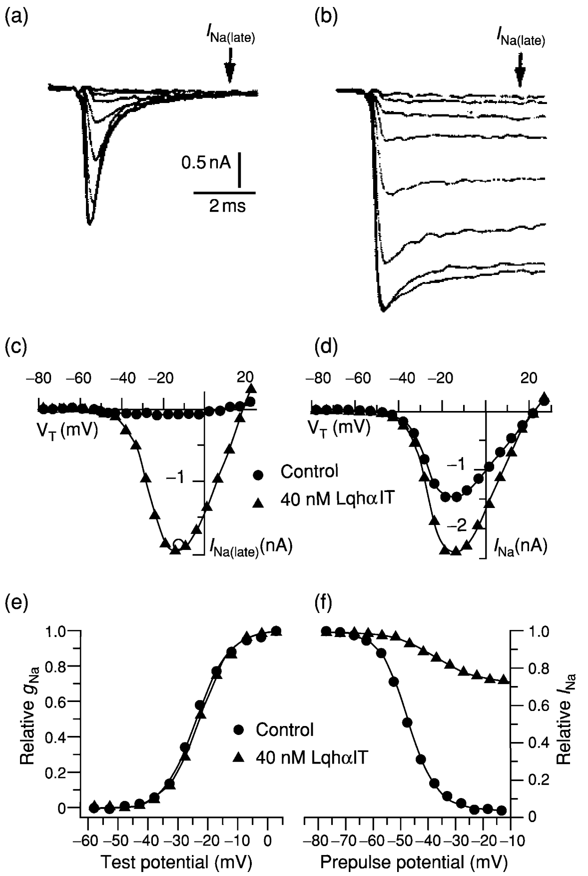


Figure 20 Effects of LqhαIT on sodium channels in cultured larval *H. virescens* neurons. (a) Superimposed sodium currents evoked by V_Ts from −48 to −13 mV (in 5 mV intervals) prior to the toxin exposure. (b) LqhαIT (40 nM) inhibited channel inactivation and increased peak sodium currents. (c) Late sodium currents $I_{Na(late)}$ taken at the time indicated by down arrows in (a) and (b) plotted as a function of test potential before (●) and after (▲) application of LqhαIT. $I_{Na(late)}$ was elevated in a voltage-dependent manner by LqhαIT. (d) *I–V* curves for peak sodium current before (●) and after (▲) application of LqhαIT. (e) Data from (d) are plotted as relative sodium conductance (g_{Na}), showing that the toxin did not modify voltage dependent activation of sodium channels. (f) Inhibition of steady-state inactivation by LqhαIT. Note that all sodium channels were inactivated by a −15 mV prepulse prior to toxin treatment, while ~70% of sodium channels failed to inactivate under the same conditions after application of the toxin. (Reproduced with permission from Lee, D., Adams, M.E., **2000**. Sodium channels in central neurons of the tobacco budworm, *Heliothis virescens*: basic properties and modification by scorpion toxins. *J. Insect Physiol. 46* (4), 499–508.)

the long-chain toxins is established between Cys residues adjacent to N- and C-terminals – Cys 1 and Cys 8 (Figure 18).

As shown in Figure 18, the fourth disulfide bond in the excitatory insect specific toxins is formed between the additional Cys residue in their β_2 strand (replacing Cys 1) and the C-terminal Cys 8 residue. However, this difference in the covalent bonding of the excitatory toxins does not affect the overall

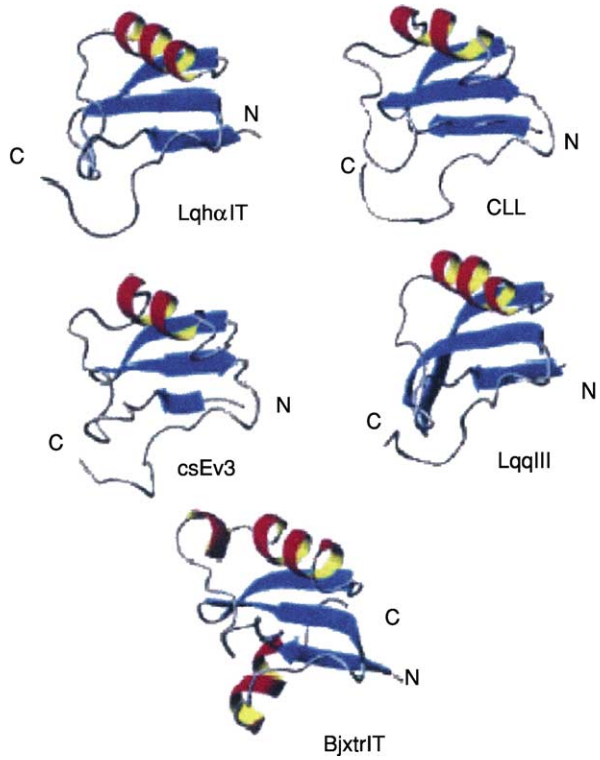


Figure 21 Spatial arrangement of some representatives of buthid scorpion toxins affecting arthropods. All toxins are similarly oriented with N- and C-terminals shown. LqhαIT is an insect specific α toxin. CLL is a crustacean toxin. CsEv3 is lethal to crickets. LqqIII is an α-like toxin that affects sodium inactivation in insects. BjxtrIT is an insect selective excitatory toxin; it is the only one that possesses two α helices. (Reproduced with permission from Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^+-channels. *Eur. J. Biochem. 264* (2), 287–300.)

folding and spatial arrangements of these molecules (Figure 21). The various conserved elements of secondary structures are connected by variable loops, which are solvent exposed turns (the bracketed J, M, B, and F regions at the bottom of Figure 18), and these correspond to the most structurally variable regions of the toxin considered to be responsible for their pharmacological specificity. A recent study by Froy *et al.* (1999) reports the isolation of a new insect selective toxin (BjxtrIT) from the venom of the Black Judean scorpion (*B. judaicus*) which, in spite of its higher molecular mass (76 amino acids, 8455 Da) and only 49% sequence identity, mimics the mode of action of AaIT. Although the spatial arrangement of BjxtrIT resembles the basic pattern of the scorpion neurotoxins it reveals two unique segments when compared to other excitatory toxins. First, it possesses five additional amino acid residues just before the α helix motif. In this extra region, the three-dimensional analysis revealed the presence of an α helix. The second peculiarity of this molecule

is the formation of an additional short α helix by residues 63–69 (**Figure 21**).

5.6.4.1.1.1. Functional groups of the VGSC active scorpion neurotoxins Use of a diverse set of experimental systems that allowed testing of various animals, neuronal preparations, and molecular approaches enabled a more detailed subdivision of the VGSC active scorpion neurotoxins (Gordon, 1997b; Gordon *et al.*, 1998, 2002). Possani *et al.* (1999) divided these toxins into 10 functional groups, according to their mode of action and animal group specificity: (1) classical mammal toxic α toxins with AaHII as prototype (Possani, 1984; Kirsch *et al.*, 1989; Gordon, 1997); (2) classical mammal toxic β toxins (Jover *et al.*, 1980; Couraud *et al.*, 1982; Zamudio *et al.*, 1992; Dehesa-Davila *et al.*, 1996); and (3) β toxins with dual, mammal/insect specificity, mediated by separate receptors. This latter group is represented by toxin Ts7 (Ts gamma) (De Lima *et al.*, 1986, 1989; De Lima and Martin-Eauclaire, 1995); (4) depressant insect selective neurotoxins (Zlotkin *et al.*, 1991); (5) α toxins affecting insects not very specifically, as represented by LqhαIT toxin (Eitan *et al.*, 1990) and LqqIII toxin (Kopeyan *et al.*, 1993); these toxins inhibit sodium inactivation and displace AaH2 (Possani *et al.*, 1999); (6) excitatory insect selective neurotoxins represented by the AaIT and BjxtrIT toxins (Zlotkin *et al.*, 1971c; Froy *et al.*, 1999); (7) New World toxins active against insects and crustaceans (Lebreton *et al.*, 1994; Selisko *et al.*, 1996; Garcia *et al.*, 1997); (8)–(10) it also includes three additional groups of various intermediate forms, weakly affecting insects and vertebrates (Possani *et al.*, 1999). The above subdivisions represent the pharmacologic flexibility of scorpion neurotoxins, but the rules governing the structure–function relationships still remain to be determined.

5.6.4.1.2. Bioactive surface and positive charges A key concept in understanding the biological role of a protein is that its function is derived from its three-dimensional structure. In monomeric proteins, such as the scorpion toxins, the tertiary structure is the highest level of organization. In spite of their functional diversity, scorpion toxins reveal a conserved spatial arrangement, which is formed by the disulfide covalent linkage between the α helix and the β sheet strands (see Section 5.6.4.1). The expected disagreement between the conserved structure (**Figures 18 and 21**) and their pharmacologic diversity can be demonstrated by two examples: the first reveals a large difference in action between two molecules that possess a strong spatial similarity, and the second reveals a pharmacological similarity between two molecules that differ in their spatial folding. For example, the toxin AaHSTR1, derived from the dangerous scorpion *A. australis*, is not toxic in spite of the fact that it exhibits a primary structure and a spatial arrangement similar to β-type mammal toxic scorpion toxins (Blanc *et al.*, 1997). The structure is composed of a short α helix (residues 26–33) connected by a tight turn to a triple stranded antiparallel β sheet (sequences 3–6, 38–41, and 44–48). The second contrary example is provided by comparison of the α scorpion toxins and sea anemone toxins, which compete on the receptor site 3 of the VGSC and inhibit sodium inactivation (**Table 3**). BgII, similar to other sea anemone toxins, is a short protein (4–5 kDa) cross-linked by three disulfide bridges (Kem and Dunn, 1988). As shown in **Figure 22** it is devoid of an α helix and its entire folding differs from that of the α AaH2 scorpion toxin. Superimposition of the three-dimensional structures of the above toxins (**Figure 22**) reveals four basic amino acid residues located in similar position and orientation. Such features may form two positively charged poles on the surfaces of both toxins. These aspects of the toxin surface and positive charge deserve further attention.

The above structure–function dilemma of scorpion toxins is better rationalized by the notion of an interplay between two structural motives; the scaffold provided by the covalently stabilized secondary structure serves as a supporter of a more variable entity the “bioactive surface.” Protein surface is of interest because it is the part of the protein that is accessible to another molecule and, hence, is where ligands bind. The accessible surfaces of molecules can be localized and mapped based on hydrophobicity and charge. It is generally accepted that the molecular basis for specific binding interactions lies in the uneven charge distribution over the surface of the protein (Stryer, 1995). It has been proposed that long chain scorpion toxins, which act on sodium channels, have on their surface a group of aromatic and hydrophobic residues. These residues are localized in a “conserved hydrophobic surface,” which include the toxins’ active domain (Fontecilla-Camps *et al.*, 1988; Landon *et al.*, 1997). Bearing this in mind, it is noteworthy that scorpion toxins are basic proteins in which the positive charge seems to be critical for binding. For example, Bot-XI is structurally homologous to AaH2, but is much less potent presumably due to the replacement of a surface lysine, position 58 in AaH2 (Sampieri *et al.*, 1987). Furthermore, chemical modifications of lysine residues in scorpion toxins strongly decrease their toxicity (Sampieri and Habersetzer-Rochat,

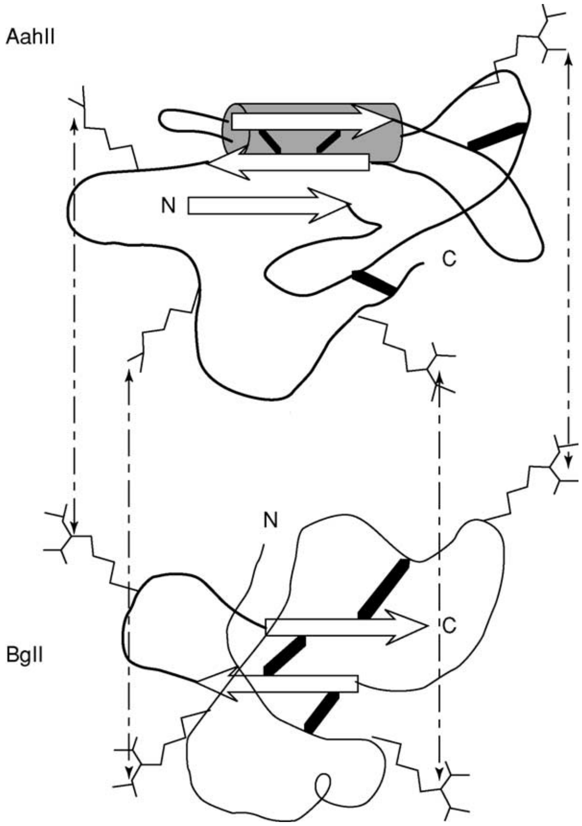


Figure 22 Comparison of structures for scorpion toxin AahII and a sea anemone toxin BgII. Although different in overall structure, the two toxins compete for the same binding site on the sodium channel. Superimposition of the two structures reveals four basic amino acid residues (dashed double-headed arrows) located in the same position within the two proteins (Arg 62, Lys 58, Lys 50, and Arg 18 in AahII and Arg 5, Arg 14, Arg 27, and Arg 36 in BgII (Loret *et al.*, 1994). (Reproduced from Loret, E., Hammock, B.D., **2001**. Structure and neurotoxicity of venoms. In: Bronwell, P., Polis, G. (Eds.), Scorpion Biology and Research. Oxford University Press, Oxford, p. 204.)

1978; Loret *et al.*, 1994). Similarly, chemical modification of conserved basic residues between the α helix and β sheet of AaIT (**Figure 18**) decreases its toxicity without markedly changing toxin binding affinity (Loret *et al.*, 1990a,1990b). A further discrepancy between toxicity and binding affinity was demonstrated by the ancestral scorpion toxin AaHIT4, which binds to the insect sodium channel with the same affinity as the AaIT, but has much lower toxicity (Loret *et al.*, 1991). The importance of the basic residues is exemplified in **Figure 22**.

5.6.4.2. Structural Features of the Insect Selective Toxins

5.6.4.2.1. The approach In the past, chemical modifications of specific amino acids served as the main approach to the study of toxicity–structure relationships (Darbon *et al.*, 1983; el Ayeb *et al.*, 1986; Kharrat *et al.*, 1989). However, using molecular biology techniques it is now possible to perform site directed mutagenesis of small peptides through the *Escherichia coli* expression system (Gurevitz and Zilberberg, 1994; Zilberberg *et al.*, 1996; Gurevitz *et al.*, 1998, 2002). Such studies are now feasible because genes encoding the most potent anti-insect α toxin, LqhαIT, and the excitatory BjxtrIT toxin have been isolated from cDNA libraries made from the telsons of *L. q. hebraeus* and *B. judaicus* (Zilberberg *et al.*, 1991; Zilberberg and Gurevitz, 1993; Froy *et al.*, 1999). The resulting expressed and renatured toxins have mimicked the native, chemically purified toxins in toxicity, binding, and electrophysiological assays (Zilberberg *et al.*, 1996; Turkov *et al.*, 1997; Froy *et al.*, 1999). The availability of a fully active recombinant toxin, coupled with site directed mutagenesis and structural analysis, facilitated elucidation of the toxins' bioactive surfaces.

5.6.4.2.2. The bioactive surface The issue of the functional diversity of the α toxin subtypes (see Section 5.6.3.3.2) was submitted to an intensive molecular treatment (Gurevitz *et al.*, 2002), including the most recent functional expression of chimeric toxin constructs (Gordon and Gurevitz, 2003). Briefly:

- The α toxins Lqh2 and LqhαIT, which reveal a very close similarity in their three dimensional structures (Gurevitz *et al.*, 2002) represent the classical mammal and insect active subtypes, respectively. LqhαIT binds to insect VGSCs with high affinity, but is almost devoid of any binding to rat brain sodium channels. Lqh2 binds with high affinity to rat brain VGSC, but with very low affinity to the insect sodium channels.
- Mutagenesis of LqhαIT (more than 40 point mutations) has revealed two sets of amino acids that constitute the bioactive surface, namely: F17, R18, W38, N44 and Y10, R58, V59, K62 (Gurevitz *et al.*, 2002) (**Figure 23**).
- The above information has suggested an active site exchange. That in the structural elements that carry the bioactive residues have been exchanged between the two toxins, resulting in chimeric constructs that acquired the functional selectivity of the "donor" toxin (Gordon and Gurevitz, 2003). These results emphasize similarity in the localization of bioactive surfaces on various α toxins, and that small differences on these surfaces dictate the pharmacological selectivity of these toxins (Gordon and Gurevitz, 2003).

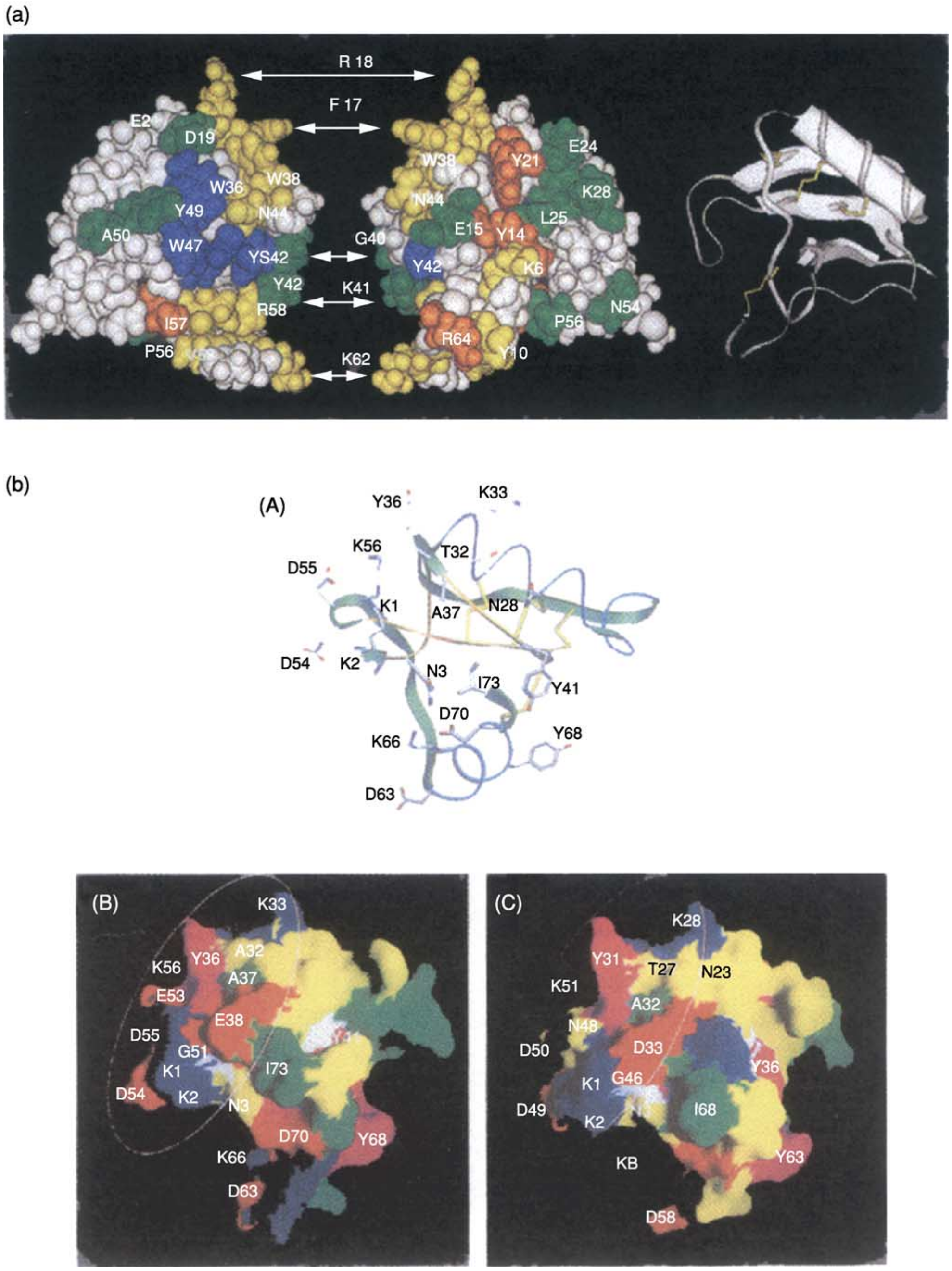


Figure 23 The putative bioactive surfaces of LqhαIT and BjxtrIT. The three-dimensional models are based on the solution and crystal structures, respectively. (a) LqhαIT with identical orientation of the carbon backbone (right) and the space-filling model (middle). Modified residues affecting bioactivity are in yellow and orange. Residues, which belong to the ''conserved hydrophobic surface'' are in blue. (b) Putative bioactive surface of scorpion excitatory toxins: (A) a ribbon diagram of the BjxtrIT structure (Oren *et al.*, 1998) with the side-chain residues indicated in panel B; (B, C) the putative bioactive surfaces of BjxtrIT and AaIT, respectively, highlighted by the ellipsoid (for details see Froy *et al.*, 1999). (Reproduced with permission from Gurevitz, M., Zilberberg, N., Froy, O., Turkov, M., Wilkunsky, R., **2002**. Diversification of toxin sites on a conserved protein scaffold – a scorpion recipe for survival. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 239–253.)

5.6.4.2.3. The structural uniqueness of the excitatory toxins: the C-terminal stretch The AaIT excitatory toxin has two structural characteristics namely the extension of the C-terminus and the formation of a different disulfide bridge (Darbon *et al.*, 1982). Both are confined to a region adjacent to the conserved hydrophobic surface thought to be implicated in the toxic activity (Fontecilla-Camps,

1989; Figure 18). A two-dimensional nuclear magnetic resonance study of the AaIT toxin (Darbon *et al.*, 1991) suggested that differences in their selectivity are due to the position of the C-terminal peptides with respect to the hydrophobic surface (see below).

The functional significance of the C-terminal region was emphasized by a recent study with the excitatory toxin BjxtrIT (Froy *et al.*, 1999). The toxin was submitted to an extensive mutagenesis of amino acids that are on the surface. Out of the 26 mutations introduced, substitutions of Asp 55, Asp 70, Ser 76, Asn 28, and Tyr 36 caused a fivefold reduction of toxicity, while substitution of the C-terminus hydrophobic residues Ile 73 and Ile 74 had a much larger effect of over 100-fold toxicity reduction (Froy *et al.*, 1999). Use of a similar approach with LqhαIT and the depressant LqhIT2 further emphasized the pharmacological role of the C-terminus. Mutations of LqhαIT revealed that substitution of Lys 8, Tyr 10, Phe 17, Arg 18, Arg 58, Val 59, and Lys 62, each reduced toxicity fivefold. The latter three belong to the C-terminal tail, and the other amino acids contribute to the outer surface. Similarly, in LqhIT2, the residues Asn 58 and Gly 61 of the C-terminal tail and Asp 8 Lys 11 and Lys 26 of its adjacent bioactive surface, when modified showed a fivefold decrease in toxicity.

In excitatory toxins, however, the C-terminus dictates the entire design-form of the active surface. Figure 24 demonstrates a superposition of the α backbones of four categories of scorpion toxins affecting VGSCs, and it exemplifies the 180 degree positional shift of the C-terminal tail of BjxtrIT and its fourth disulfide bridge, when compared to the other toxin categories. As shown in Figure 24, the shift of the C-terminal tail dictates (in reference to the scaffold) the orientation of the bioactive surface, which comprises the distal part of the C-terminal tail.

It has been suggested that the phyletic specificity of scorpion toxins is mediated by the "evolutionary wiggling of the C tail" (Gurevitz *et al.*, 2001), as performed by virtue of the change in the fourth disulfide bridge (Figure 24) in the excitatory insect selective toxins (Gurevitz *et al.*, 2002). Given this background of the hypothetical "evolutionary tail wiggling" it is noteworthy that a previous study of Loret *et al.* (1990b) suggested a "pharmacological" C tail wiggling. The significance of the C-terminal segment was indicated by a circular dichroism (CD) study with the dual active TSVII toxin. This β toxin was shown to affect insects through an AaIT receptor and affect mammals through the receptor of a potent mammal toxin β toxin (De Lima *et al.*, 1986). It has been suggested that when in contact



Figure 24 Superposition of the Cα backbone of scorpion toxins affecting sodium channels. Four toxins representing distinct pharmacological groups are delineated in ribbon: the α toxin, LqhαIT, the β toxin Cn2, and the excitatory toxin, BjxtrIT have blue, magenta, and red C-tails, respectively. Note: the last three residues could not be seen in the X-ray structure of BjxtrIT, but they cover, most likely, residue 38 in the center of the bioactive surface. In addition, a model of a depressant toxin, LqhIT2, is presented with a green C-tail (the model was constructed by alignment with Cn2 using the MODELLER and HOMOLOGY modules of Insight II, MSI, Cambridge, UK). The α helix and β strands are highlighted (barrel and arrows, respectively). The conserved three disulfide bonds are depicted in yellow lines, whereas the fourth disulfide bond is pinpointed in yellow balls and sticks. (Reproduced with permission from Gurevitz, M., Zilberberg, N., Froy, O., Turkov, M., Wilkunsky, R., **2002**. Diversification of toxin sites on a conserved protein scaffold – a scorpion recipe for survival. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 239–253.)

with the insect sodium channel, a supplementary α helix in the C-terminal region from residues 51 to 60 is formed (which is similar to the one described for the AaIT) (Loret *et al.*, 1990b; Figure 18), and this might endow the TSVII toxin with insect sodium channel binding properties. Conversely, the formation of a supplementary antiparallel β strand might give the TSVII its ability to bind to mammalian-type sodium channels, as with the β-type toxins (Loret *et al.*, 1990b). To summarize, the dual functions of the TSVII are attributed to a presumably adaptive modification of secondary structure and the conformational flexibility of the C-terminal region.

5.6.4.3. Interaction with the Insect VGSC

5.6.4.3.1. The voltage-gated sodium channels The pharmacology of VGSCs was reviewed previously by Catterall (1980, 1992, 1995), Gordon

(1997b), and Goldin (1999). For insecticidal aspects, the reader is referred to recent review articles by Bloomquist (1996), Zlotkin (1999), chapters in Clark (1995), Ishaaya (2001), and Section 5.6.5, as well as Section 5.6.1.4.3.. Briefly, VGSCs mediate an increase in sodium permeability during the initial rapidly rising phase of action potential in excitable tissues. They are plasma membrane proteins composed of a large pore-forming α subunit that is generally accompanied by one or two auxillary subunits (Feng *et al.*, 1995; Shichor *et al.*, 2002). The α subunit is composed of four repeated domains (D1–D4), each consisting of six transmembrane α helix segments (S1–S6) and a pore loop (between segments S5 and S6) (Catterall, 1995). Modifiers of VGSCs are used as drugs (Kallen *et al.*, 1993) and insecticides (Zlotkin, 1999) in spite of their limited and harmful lack of specificity to channel subtypes.

As shown in **Table 3**, voltage-gated sodium channels are the molecular targets for a broad range of neurotoxins that act at at least six distinct receptor sites on channel protein. These toxins can be subdivided into three categories: (1) toxins that physically block the pore and prevent sodium conductance comprised of hydrophilic compounds of low molecular mass and larger polypeptide toxins; (2) alkaloid toxins and related lipid soluble toxins that alter voltage dependent gating of sodium channels via an allosteric mechanism, through binding to intramembranous receptor sites; and (3) polypeptide toxins that alter channel gating by voltage sensor trapping through binding to extracellular receptor sites (Cestele and Catterall, 2000). The excitatory and depressant scorpion toxins belong to the latter category, but they are also able to exclusively identify insect VGSCs, and are able to distinguish between insect and their vertebrate-mammalian counterparts.

5.6.4.3.2. Domain II is targeted by the insect selective β toxins

5.6.4.3.2.1. The chimeric channel The aim of a study by Shichor *et al.* (2002) was to clarify the selective basis of AaIT toxin interaction with insect VGSCs by localizing the region that is targeted by the toxin. The approach taken was dictated by the following four considerations:

1. Insect VGSC is the exclusive target for excitatory as well as depressant toxins (see above and Zlotkin *et al.*, 2000).
2. Because of its mode of action AaIT belongs to the major β category of scorpion venom neurotoxins (see Sections 5.6.3 and 5.6.2.6).
3. The β toxins shift the voltage dependence of activation to more negative potentials (Section 5.6.3.2.6) by binding to receptor site 4 (Catterall, 1992). This site was shown to be in domain II (D2) (Marcotte *et al.*, 1997; Tsushima *et al.*, 1999) and includes the external loop S3–S4 of the vertebrate VGSC (Cestele *et al.*, 1998).
4. It has been assumed that like in mammalian channels, the β toxin site on insect channels also resides in D2 and is thereby targeted by AaIT.

With this background, a chimeric mammalian–insect channel composed of the rat brain sodium channel (rBIIA), in which D2 was replaced by the Para (paralytic temperature sensitive) *Drosophila* sodium channel D2, was constructed. As shown below, the chimeric channel was functional, despite a large phylogenetic distance between insects and mammals, and acquired the sensitivity to the anti-insect selective toxin AaIT.

The approach was comprised of construction of a chimeric cDNA (using restriction nucleases and PCR), generation of the respective cRNA, injection into *Xenopus* oocytes, and monitoring the sodium current by the two-electrode voltage clamp method (Shichor *et al.*, 2002).

5.6.4.3.2.2. The chimeric channel as susceptible to AaIT The main body of information is presented in **Figure 25** and its legend. First, in contrast to the insect Para channel, the mammalian rBIIA is not sensitive to AaIT (**Figure 25a**). Second, the chimeric channel is functional, as demonstrated by its capacity to produce voltage-gated sodium currents, which are sensitive to TTX and are regulated by the mammalian auxiliary subunit β1 (**Figure 25b**) but not by the insect TipE auxiliary subunit. The latter is consistent with the finding that the binding site for the β1 subunit was localized on domain D4 in the RBIIA channel (Qu *et al.*, 1999). Third, the chimera, similar to the *Drosophila* Para/TipE and in contrast to the RBIIA/β1 subunit, acquired sensitivity to the AaIT toxin (**Figure 25c**).

Analysis of the voltage dependence of activation of the chimeric channel revealed a statistically significant negative shift of 6 mV in V1/2; in the presence of AaIT (**Figure 25c**, middle and bottom). This effect resembles the negative shift in the voltage dependence of activation of sodium currents in insect neurons induced by AaIT (see Section 5.6.3.2.2 and Lee and Adams, 2000). The responsiveness of the chimeric channel to AaIT revealed an additional characteristic of the β toxins. Similar to the effect of the β toxin Css-IV on the rat brain rBIIA

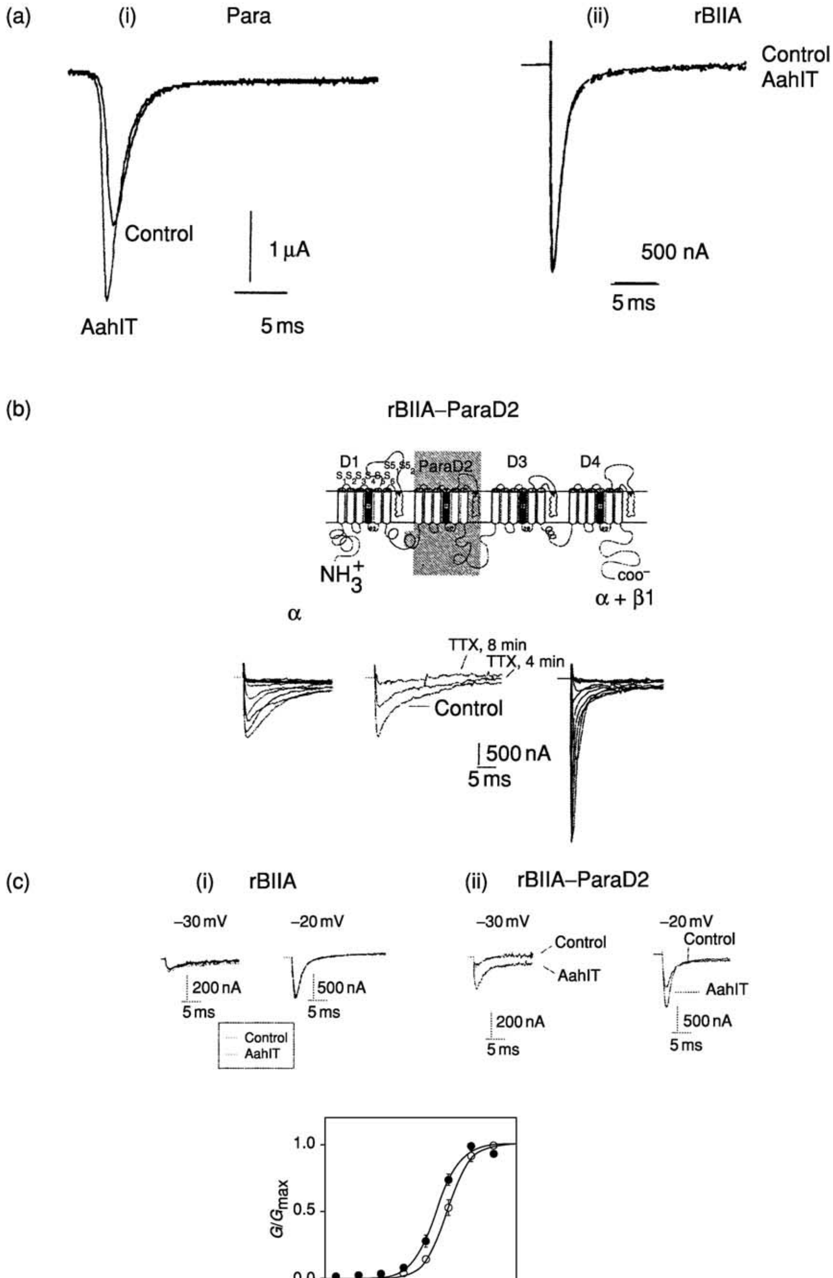


Figure 25 Domain II of *Drosophila* Para voltage-gated sodium channel confers sensitivity to AaIT toxin as revealed by sodium currents recorded from recombinant *Xenopus* oocytes in voltage clamp assays. (a) The rat brain channel (rBIIA) in contrast to the insect Para channel is not sensitive to the excitatory insect selective toxin (AaIT). (i) Para together with its auxiliary subunit TipE responds to 1 μM AaIT. (ii) rBIIA together with the auxiliary β1 subunit is resistant to 3 μM AaIT. (b) The expressed chimeric channel α subunit (α) is able to produce sodium currents (left) in a TTX sensitive (middle) and β1 (right) dependent fashion. (c) AaIT affects the rBIIA–Para D2 chimera but not the rBIIA channels. Top panel: currents elicited after step depolarization to −30 or −20 mV before and 10 min after application of 3 μM (i) or 1.4 μM (ii) AaIT. Concentrations up to 10 μM were checked to give the same results. Bottom panel: normalized conductance (G/G_{max}) voltage relationships. ○, control; ●, AaIT. Note the negative shift in the voltage dependence of activation of the sodium currents. (Reproduced with permission from Shichor, I., Zlotkin, E., Ilan, N., Chikashvili, D., Stuhmer, W., *et al.*, **2002**. Domain 2 of *Drosophila* para voltage-gated sodium channel confers insect properties to a rat brain channel. *J. Neurosci. 22* (11), 4364–4371.)

channel (Cestele *et al*., 1998), a depolarizing conditioning prepulse (to +50 mV) potentiated the effect of AaIT on the chimeric rBIIA-Para D2 currents. It may be concluded that the phyletic selectivity of AaIT is conferred by D2 of the insect VGSC (Shichor *et al*., 2002). Owing to the mutual displaceability between the excitatory and depressant toxins (see Section 5.6.3.2.4), and their assignment to the β toxins category, it appears that domain II also provides the binding region for the depressant toxins.

The above-demonstrated homology in the β toxin receptor sites between insect and mammalian sodium channels raises the question whether the external loop S3–S4 in D2 of the mammalian (RBIIA) channel, shown to comprise the receptor binding site for the β scorpion toxins (Cestele *et al*., 1998), also provides the binding site for the insect selective neurotoxins on the insect VGSC. The data presented in **Figure 26** show that the S3–S4–D2 loop is highly conserved in insect Para, rat brain, and skeletal muscle sodium channels. Furthermore, the Gly 845 residue in the RBIIA is essential for the binding of TsVII β toxin (Cestele *et al*., 1998), or its equivalent Asn residue in the cardiac channels. Both residues were shown to occur in the Para channels of fruit flies, domestic flies, and cockroaches. Therefore, it has been suggested (Gordon *et al*., 2002) that a separate or additional region in the insect Para D2 is most likely involved in the selective recognition by the excitatory (and depressant) insect selective scorpion neurotoxins of insect sodium channels.

```
             ----S3-----       *     -----S4----
rSkM1        L V E L G L A N V Q G L S V L R S F R
rBIIA        - M - - - - - - - E - - - - - - - - -
rBI          - - - - - - - - - E - - - - - - - - -
rH1          - M - - - - S R M G N - - - - - - - -
hH1          - M - - - - S R M S N - - - - - - - -
Drosophila   - L - - - - E G - - - - - - - - - - -
Musca        - L - - - - E G - - - - - - - - - - -
Cockroach    - L - - - - E G - - - - - - - - - - -
```

Figure 26 Comparison of S3–S4–D2 sequences of various mammalian and insect channels. Gly845 of rBIIA is highlighted by an asterisk (Cestele *et al*., 1998). Residues in bold are equivalents of gly845 in other channel subtypes: hSkM1 and rSkM1, skeletal muscle from human and rat; rBII and rBI, rat brain; hH1 and rH1, cardiac channels of human and rat (Goldin, 2001). *Drosophila, Musca*, and cockroach, Para channel from *D. melanogaster* (Loughney *et al*., 1989), *M. domestica* (Williamson *et al*., 1996), and *Blatella germanica* (Dong, 1997). (Reproduced with permission from Gordon, D., Gilles, N., Bertrand, D., Molgo, J., Nicholson, G., *et al*., **2002**. Scorpion toxins differentiating among neuronal sodium channel subtypes: nature's guide for design of selective drugs. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 215–238.)

However, in spite of the above structural homogeneity between the insect and mammalian VGSCs, it may be safely concluded that domain D2 determines the selectivity of the AaIT toxin to insects.

5.6.5. Applicative Agrotechnical Aspects

Recent progress in the fields of molecular biology and biotechnology has enabled the practical employment of pharmacologically useful polypeptides. The agronomical usefulness of insect selective neurotoxic polypeptides follows from (1) the growing public awareness to health hazards and environmental damage, and (2) the evolution of insect resistance, both of which derive from the massive use of classical industrial insecticides. The plant protective utility of the insect selective toxins is based on a combination of the following technical possibilities: their mimicry by nonpeptidic agents, their inclusion in recombinant insect infective microorganisms, the design of modified insect gut permeable polypeptides, their inclusion in transgenic insect protected plants, or their combined application with conventional insecticides. The section below addresses some of these aspects.

5.6.5.1. Baculoviral Bioinsecticides

5.6.5.1.1. Recombinant baculoviruses The technology of insecticidal recombinant baculoviruses has been the subject of several reviews (Maeda, 1989; Bonning and Hammock, 1992, 1996; O'Reilly *et al*., 1992; Loret and Hammock, 2001; see also Section 5.6.5). Given this broad background, focus is on key aspects specifically related to the scorpion venom insect selective neurotoxins. Owing to its proteinaceous nature and inaccessibility by topical or oral applications, the insect selective AaIT toxin cannot serve as a classical insecticide. Furthermore, it has a low toxicity to lepidopterous larvae (see below and Section 5.6.3.2.5), major pests of field crops. This problem is solved when the insecticidal action of AaIT is mediated by a recombinant baculovirus.

Baculoviruses are insect specific pathogens used as biological, environmentally clean insecticides. However, the baculoviruses act slowly enabling the insect pest larvae to feed continuously on the crop. This drawback is solved through the use of genetically engineered baculoviruses, which express genes encoding insecticidal neurotoxins (Bonning and Hammock, 1996). The foreign gene is subcloned into a transfer vector plasmid, which is cotransfected with parental baculovirus DNA into an appropriate insect cell line, so that the foreign gene

is transferred to the baculovirus by homologous recombination. The infectivity of baculoviruses is limited to a few closely related species within a single insect family. Thus, an important factor in the environmental safety of baculoviral insecticides chosen for their species selectivity is the fact that they complement natural predators instead of replacing them, as in the case of many chemical insecticides. Naturally occurring viruses are evolutionarily adapted for self-preservation and propagation. It follows that a part of the baculovirus replication strategy is to keep its host alive as long as possible to maximize progeny production. Genetic engineering is used to improve the efficacy of the viruses as pesticides by reducing the time it takes the virus to stop their insect hosts feeding (Bonning and Hammock, 1996).

5.6.5.1.2. The tolerance of lepidopterous larvae to AaIT The interaction of the fast-acting, neurotoxic and insect selective polypeptide AaIT with lepidopterous larvae tissues was studied through assays of toxicity, chromatography, binding, and autoradiography. The native and/or radioiodinated toxin was shown to induce a delayed, slow, progressive paralysis (within 24–28 h) of *Spodoptera* larvae at relatively high doses (paralytic unit: 2.4 μg/100 mg). The larvae of six species representing five families of Lepidoptera responded similarly to the toxin. The toxin is stable *in vitro* using insect hemolymph; however, 80% of its toxicity is lost in the insect's body within 24 h, accompanied by a progressive process of degradation and elimination by the excretory system. The toxin is devoid of accessibility to peripheral-terminal branches of *Spodoptera* motor nerves *in situ*, strongly contrasting with those of the toxin susceptible *Periplaneta* nerves (**Figure 9**).

It was concluded that the tolerance of lepidopterous larvae to AaIT can be substantially attributed to pharmacokinetic aspects of toxin accessibility barriers and degradation processes (Herrmann *et al.*, 1990). However, a subsequent study (Fishman *et al.*, 1997) revealed the occurrence of a pharmacodynamic aspect in the tolerance of lepidopterous larvae. This aspect was demonstrated in studies of the kinetics of binding dissociation of [^{125}I]AaIT from neuronal membranes of susceptible and tolerant insects. Stable binding is shown in susceptible insects such as cockroaches and locusts, which have a dissociation half time of approximately 9 and 5 min, respectively. This contrasts strongly with the fast half-times of dissociation of 7 s in *Spodoptera littoralis* (Noctuidae: Lepidoptera) larvae and 9 s for the terebrionid beetle *Trachyderma philistina* (Coleoptera), which are both relatively tolerant to AaIT. Such differences in binding kinetics may reflect a structural and functional diversity of sodium channels in different insect species that is responsible for their diverse susceptibilities to neurotoxic polypeptides.

To summarize, it has been shown that the relative tolerance of lepidopterous larvae to the AaIT toxin is based on a combination of two separate groups of factors. First, these are those that affect the accumulation of the critical dose at the site of action (the so-called pharmacokinetic factors), such as toxin impermeability of the terminal motor branches (**Figure 9**) and its degradation and elimination process. Second, these are factors that affect AaIT interaction with its receptor site pharmacodynamics as revealed by fast dissociation ($t1/2 =$ seconds) from lepidopterous neuronal membranes, strongly contrasting with susceptible insects ($t1/2 =$ minutes) (**Table 6**, **Figure 27**). As shown below, the above tolerance is overcome by the aid of an AaIT expressing recombinant baculovirus.

5.6.5.1.3. AaIT expressing recombinant baculoviruses In spite of the relative tolerance of lepidopterous larvae to the excitatory toxin, several laboratories have been designing AaIT based

Table 6 Paralytic potency and dissociation constants of AaIT in various insects and their respective neuronal membranes

Species	*PD$_{50}$*[a] *(μg 100 mg^{-1} body mass)*	*$10^{-3} \times k_{-1}$ (s^{-1})*	*t1/2*[a] *(s)*
Cockroach *Periplaneta americana*	0.024	1.33 ± 0.4	522 ± 130
Locust *Locusta migratoria*	0.3	2.57 ± 0.5	270 ± 55
Beetle *Adesmia* sp.	1.4	25.5 ± 7.5	30 ± 8.0
Moth larva *Spodoptera littoralis*	2.4	98.8 ± 20	7.2 ± 2.4
Beetle *Trachyderma philistina*	>10	76.3 ± 23	9.0 ± 2.8

[a]*t*1/2 is the time for half-dissociation calculated from the value of the dissociation constant according to $t1/2 = \ln(2/k_{-1})$ where k_{-1} is the dissociation constant (Benet, 1978).

Values are means ± SD ($N = 3$).

Reproduced with permission from Fishman, L., Hermann, R., Gordon, D., Zlotkin, E., **1997**. Insect tolerance to a neurotoxic polypeptide: pharmacokinetic and pharmacodynamic aspects. *J. Exp. Biol. 200* (7), 1115–1123.

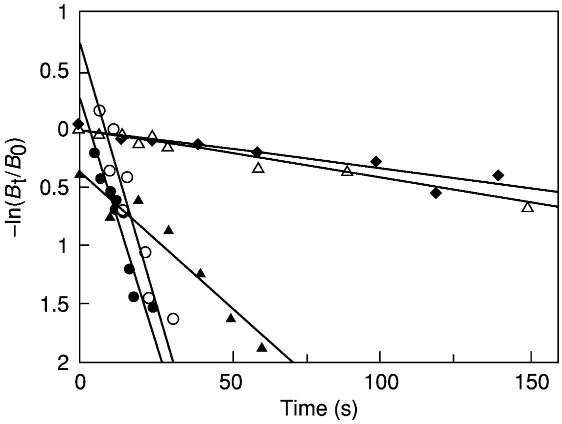


Figure 27 Time course of dissociation of [^{125}I] AaIT from five insect neuronal preparations by the dilution method. The figure shows the analysis of the dissociation curves by linearization. The values of the dissociation constants were determined directly from the slope and the half-time was calculated accordingly. ○, *Spodoptera littoralis* larvae; ▲, *Adesmia* sp.; ◆, *Periplaneta americana*; ●, *Trachyderma philistina*; △, *Locusta migratoria*. (Reproduced with permission from Fishman, L., Herrmann, R., Gordon, D., Zlotkin, E., **1997**. Insect tolerance to a neurotoxic polypeptide: pharmacokinetic and pharmacodynamic aspects. *J. Exp. Biol.* *200* (7), 1115–1123.)

recombinant baculoviruses. An obvious enhancement of the insecticidal capacity was achieved by providing the *A. californica* multiple nuclear polyhedrosis baculovirus (AcMNPV), which infects various important lepidopterous pest insects, with the gene encoding AaIT (McCutchen *et al.*, 1991; Stewart *et al.*, 1991). This approach, with the AcMNPV-AaIT virus, has even yielded the first successful field trial of a genetically improved baculovirus (Cory, 1994). Besides the above *A. californica* (Ac-AaIT) recombinant baculovirus, the AaIT gene was used to design additional recombinant baculoviruses such as *Helicoverpa zea* NPV (Hz-AaIT) (Treacy *et al.*, 2000), which has revealed an insecticidal superiority over Ac-AaIT baculovirus on heliothine larvae in greenhouse and field trials. The HzNPV was considered to be a better vectoring agent (versus AcNPV) for designing recombinant clones targeted at multi-species heliothine insects. Similarly, a recombinant *Trichoplusia ni* nuclear polyhedrosis virus (TnNPV-AaIT) was designed in China and significantly shortened the time of killing of agronomically important insects. Furthermore, the mosquitocidal capacity of AaIT was demonstrated by infecting mosquitos with a recombinant AaIT expressing Sindbis virus. The mosquitocidal action of AaIT make it a potential candidate for inclusion in molecular recombinant methods of mosquito control (Higg *et al.*, 1995). Besides the excitatory AaIT toxin, the α insect specific LqhαIT was also expressed by a recombinant baculovirus AcNPV-LqhαIT, which has demonstrated an enhanced insecticidal potency against *H. armigera* larvae. Similarly, the AcNPV-LqhIT2 recombinant baculovirus expressing the depressant insect selective toxin was shown to be superior to the one expressing the AaIT toxin (Gershburg *et al.*, 1998). As indicated above (see Section 5.6.3.2.5) the double recombinant virus simultaneously expressing the excitatory and depressant toxins has demonstrated an insecticidal superiority over the single recombinant Ac baculovirus (Regev *et al.*, 2003). Apart from the scorpion venom derived excitatory and depressant insect selective toxins, a mite toxin (Tomalski and Miller, 1991), a sea anemone toxin, and a spider toxin (Krapcho *et al.*, 1995; Prikhodko *et al.*, 1996) were also employed for the construction of recombinant baculoviruses.

5.6.5.1.4. Baculoviral potentiation In contrast to the low and slow toxicity of the native toxin in lepidopterous larvae (Herrmann *et al.*, 1990), when these larvae are infected with a AaIT expressing recombinant baculovirus (such as BmNPV-AaIT), they show a reduction of survival time and the amount of host plant they damage when compared with the wild-type virus (Cory, 1994). The expressed toxin found in the circulation of the infected insects is chemically identical to the native toxin (Stewart *et al.*, 1991), mimics its symptoms of paralysis (Maeda, 1989), and requires a much lower hemolymph titer to induce a similar paralysis than the injected native toxin. The latter phenomenon, the enhanced activity of the expressed toxin, is designated as toxin potentiation and is demonstrated in **Table 7**.

Preliminary observations revealed that infection with the wild-type virus does not significantly affect the insect's susceptibility to injected native toxin. It was thus assumed that the above potentiation is a consequence of a pharmacokinetic-targeting cooperativity between the recombinant baculovirus and its expressed AaIT toxin. This hypothesis was most recently examined by toxicity assays, immunocytochemical microscopy, and electrophysiology, using silkworm larvae infected with wild-type BmNPV and the recombinant BmNPV-AaIT viruses.

Recent data (Elazar *et al.*, 2001) suggest that toxin potentiation is a consequence of its continuous expression in the virus infected tracheal epithelia, which ramify in the insect body. This, therefore, provides a local supply of freshly produced toxin, which translocates to the insect CNS thereby providing the expressed toxin with critical target sites inaccessible to the native toxin. This conclusion is supported

Table 7 Baculoviral potentiation; the concentration of the native and recombinant AaIT in the hemolymph of paralyzed lepidopterous larvae

Insect[a]	*Native toxin concentration (ng μl^{-1})*[b]	*Recombinant toxin concentration (ng μl^{-1})*[c]	*Potentiation*
H. virescens (tobacco budworm)	70[d]	1.0[e]	70
B. mori (silkworm)	45[f]	1.3[f]	35

[a]Fourth instar larvae were employed.
[b]The concentration of the native toxin was obtained by dividing the value of the paralytic dose (PD_{50}) and the hemolymph volume of lepidopterous larvae (Herrmann *et al.*, 1990).
[c]The concentration of the recombinant toxin in the hemolymph of the larvae infected by the recombinant baculoviruses (AcMNPV-AaIT; BmNPV-AaIT) was monitored by the blowfly larvae contraction paralysis assay (Zlotkin *et al.*, 1971a) on samples of hemolymph collected from the paralyzed larvae.
[d]From Herrmann *et al.* (1995).
[e]From McCutchen *et al.* (1991).
[f]From Elazar *et al.* (2001).
Reproduced with permission from Elazar, M., Levi, R., Zlotkin, E., **2001.** Targeting of an expressed neurotoxin by its recombinant baculovirus. *J. Exp. Biol. 204* (15), 2637–2645.

by the data presented in **Figure 28**, which indicate that: (1) the recombinant AaIT toxin is expressed by a recombinant BmNPV AaIT virus in the tracheal epithelium of the tracheae, which are either enveloping or branching into the ventral nerve cord (VNC) (**Figure 28a**); (2) the tracheae surrounding the VNC are, in essence, an integral part of the protective sheath enveloping the insect's CNS; (3) as in **Figure 28b**, the high resolution immunogold method provides striking evidence for the presence of the recombinant AaIT deep inside the insect CNS; and (4) as in **Figure 28c**, the virus mediated presence of the recombinant toxin in the CNS has a functional significance. It reveals an obvious increase in the externally recorded spontaneous electrical activity in the VNCs derived from recombinant baculovirus (RBV) infected silkworm larvae when compared to wild-type baculovirus. The increase in the CNS derived electrical activity is correlated to the timing of the onset of paralysis in RBV infected larvae (data not shown).

The manner in which the virus localizes toxin delivery and toxin translocation is addressed by Engelhard *et al.* (1994), who examined the process of systemic host infection by the budded form of the baculovirus. The use of AcMNPV containing a *lacZ* reporter gene revealed that the major conduit for systemic viral infection, enabling its rapid spreading throughout the host insect, is through the tracheal system (Engelhard *et al.*, 1994), which is also accessible to external infection (Kirkpatrick *et al.*, 1994). The suitability of the tracheal system to guide the recombinant baculovirus and its expressed neurotoxin follows from the fact that the tracheal system is distributed among the various tissues, is intimately associated with individual cells, and is flexibly expanding and adapting itself to the growing organism by aid of the tracheoblasts (Engelhard *et al.*, 1994).

To summarize, toxin potentiation results from an interaction of the recombinant baculovirus with its expressed toxin, where the virus serves as a vector and provider of the toxin to the insect's nervous system via an existing and newly formed accessibility route, thereby producing CNS targeted neurotoxicity.

5.6.5.2. Cooperativity

5.6.5.2.1. The pharmacologic flexibility of the insect VGSC Presently, recombinant baculoviruses provide the most amenable system for the delivery of clonable insecticidal polypeptides for pest control purposes. A great deal of effort has been invested in the study of recombinant baculoviruses, and a number of cooperatively interacting combinations of substances is possible. Cooperative combinations can be delivered either as a cocktail of recombinant baculoviruses (O'Reilly *et al.*, 1992), each expressing separate insecticidal polypeptides, or the design of double recombinants, which express cooperative combinations of toxins in single virus preparations. A third possibility includes the combined delivery of recombinant baculoviruses with conventional organic insecticides.

The design of the above combinations is based on the so-called pharmacologic flexibility of the insect voltage-gated sodium channel (**Table 3**) as recently reviewed by Gordon (1997a), Gordon *et al.* (2002), and Zlotkin (1999), briefly:

The insect VGSC closely resembles its vertebrate counterpart in two major reaspects. First, in its structure and function and, second, in its pharmacological diversity and flexibility (**Table 3**). Despite their fundamental similarity, the most obvious pharmacologic distinction between the insect and mammal sodium channels is provided by the insect selective scorpion venom neurotoxins. However, the above toxins, when coupled to various binding (Moskowitz *et al.*, 1994) and toxicity (Herrmann *et al.*, 1995) assays have showed additional pharmacologic subdivisions among insect VGSCs. As presented in **Figure 17** the degree of displaceability between the excitatory and depressant toxins

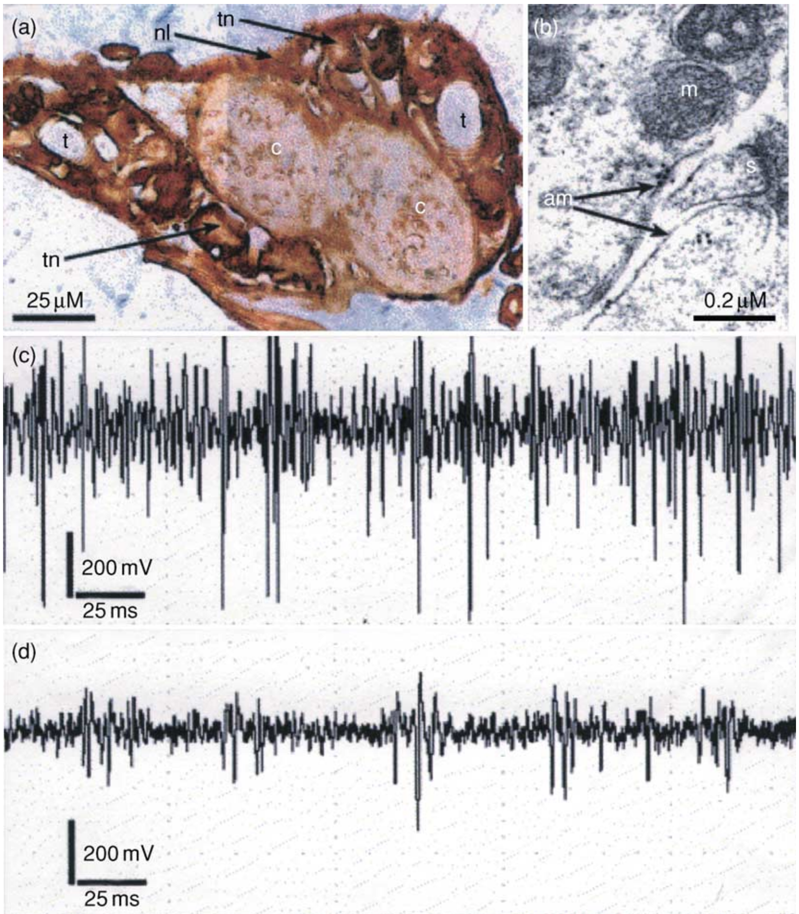


Figure 28 The expressed AaIT toxin is targeted by its recombinant baculovirus to the insect central nervous system (CNS). (a) Recombinant AaIT in the ventral nerve cord of silkworm larvae infected with BmNPV-AaIT. The expressed toxin is detected by anti-AaIT antibody and visualized by the horseradish peroxidase reaction (dark pigmentation, brown). The expressed AaIT is located in the cytoplasm of tracheal epithelial cells inside the neural lamella (nl) enveloping the two connectives (c) in close proximity to the abdominal ganglion. Note the hypertrophied tracheal epithelium with swollen nuclei (tn) and the cytoplasm loaded with the recombinant toxin. Scale bar = 25 μm. (b) Recombinant AaIT inside an abdominal ganglion of a silkworm larva infected with the above (a) recombinant baculovirus. The AaIT is detected by anti-AaIT antibody, visualized by gold-attached antibody and revealed by electron microscopy. AaIT is shown on axonal membranes (am, arrows) in the neuropil mass of the ganglion. Am, axonal membrane; c, connective; m, mitochondria; nl, neural lamella; s, synapse; t, trachea; tn, tracheal epithelium nuclei. Scale bar = 0.2 μm. (c, d) Spontaneous electrical activity recorded from VNC dissected from the 4th instar silkworm larvae 40–45 h following infection by the wild-type virus (WTBV) (d) and the recombinant, AaIT, expressing baculovirus (BmAaIT NPV) (c). Note the obvious difference in the intensity and frequency of the recorded responses from the infected ventral nerve cords. (Reproduced with permission from Elazar, M., Levi, R., Zlotkin, E., **2001**. Targeting of an expressed neurotoxin by its recombinant baculovirus. *J. Exp. Biol. 204* (15), 2637–2645.)

varies among various insect neuronal preparations (Moskowitz *et al*., 1994). Similarly, the positive cooperativity of insecticidal scorpion toxins varies among different test insects (Herrmann *et al*., 1995). Further, sodium channel subtyping was revealed by diverse toxins and neuronal preparations. For example, the binding of LqhαIT to locust neuronal membranes was cooperatively enhanced by veratridine due to increasing affinity and receptor site capacity (Gordon and Zlotkin, 1993). This is similar to the allosteric positive modulation that occurs between α toxins and veratridine (a plant lipophilic alkaloid; **Table 3**) in rat brain sodium channels. Alternatively, in contrast to negative modulation of α toxins in rat brain synaptosomes by brevetoxin (**Table 3**), brevetoxin was shown to increase the binding of LqhαIT to locusts' sodium channels (Cestele *et al*., 1995; Gordon, 1997a). Such differences indicate a structural/functional distinction between channel subtypes. The differential allosteric modulation may provide a new approach to increase selective activity of pesticides on target

organisms by the simultaneous application of allosterically interacting drugs (Gordon, 1997).

The allosteric modulation approach offers two forms of insecticidal interactions shown below: positive cooperativity and allosteric antagonism (or negative cross-resistance). In other words, the insect voltage-gated ion channel is targeted by two categories of insecticidal substances: industrial synthetic organic compounds and baculovirus mediated neurotoxic polypeptides. As shown below, they may be used in a cooperative fashion.

5.6.5.2.2. Positive cooperativity A recent examination of the insecticidal potency of various scorpion venom neurotoxins (Herrmann *et al.*, 1995) exemplifies this principle. It has been shown that a combination of two toxins increased the insecticidal activity five- to tenfold, with no apparent increase in mammalian toxicity. Synergistic combinations were obtained between toxins (such as AaIT and LqhαIT) that do not reveal competitive displaceability in binding assays and do not belong to the same site category. It is noteworthy that a similar cooperativity was demonstrated between the excitatory (AaIT) and depressant (LqIT2) toxins in their toxicity to lepidopterous larvae. These toxins do not displace each other in their binding to lepidopterous neuronal preparations (Moskowitz *et al.*, 1994), and they reveal an enhancement of recombinant baculovirus insecticidal efficacy against *H. virescens*, when simultaneously expressed by AcMNPV (see Section 5.6.3.2.4; Regev *et al.*, 2003). A second example of positive cooperativity is provided by a recent study (Gilles *et al.*, 2003), which reveals by the aid of binding studies (not toxicity assay on intact insects) that the binding affinity of LqhαIT toxin to locust CNS (but not to rat brain sodium channels) is allosterically increased by certain pyrethroids and brevetoxin-1 (**Figure 29**). Brevetoxin-1 also increases the binding of the excitatory AaIT (but not BjxtrIT) to insect sodium channels. This information is presented as an approach to tackle pyrethroid resistance by the aid of a combined use of synergistically acting insecticidal compounds (Gilles *et al.*, 2003, see also Section 5.6.5.2.1).

5.6.5.2.3. Allosteric antagonism (negative cross-resistance) An economically and environmentally more useful approach to resistance management is provided by the phenomenon defined as "negative cross-resistance," which is also designated as "allosteric antagonism." "Resistance" is officially defined by the World Health Organization as the "development of an ability in a strain of an organism to tolerate doses of toxicant, which would prove lethal

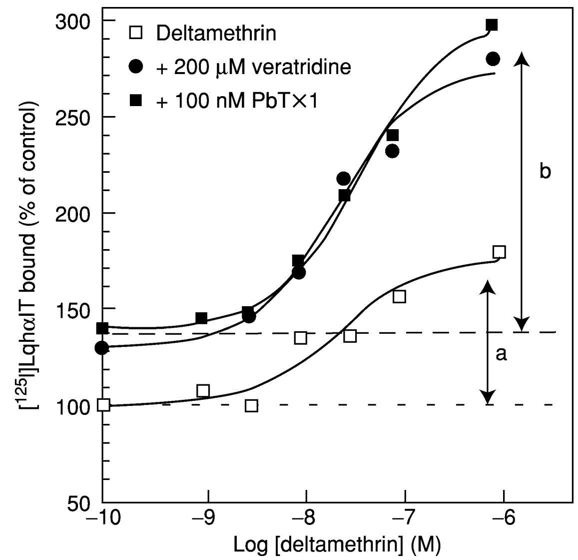


Figure 29 Effect of pyrethroids on LqhαIT binding to locust neuronal membranes. Synergic effect of deltamethrin on LqhαIT binding in the presence of saturating concentrations of deltamethrin alone (a) and together with 200 μM veratridine or 100 nM brevetoxin (PbTx-1) (b). Maximal specific binding of [^{125}I] LqhαIT alone (control 100%) and with veratridine or brevetoxin (without pyrethroid) are indicated by the lower and upper dashed lines, respectively. (Reproduced with permission from Gilles, N., Gurevitz, M., Gordon, D., **2003**. Allosteric interactions among pyrethroid, brevetoxin, and scorpion toxin receptors on insect sodium channels raise an attenative approach for insect control. *FEBS Lett. 540* (1–3), 81–85.)

to the majority of individuals in a normal (susceptible) population of the species" (Mullin and Jeffrey, 1992). Insecticide resistance, documented in almost all arthropod species in which it has been studied, is a major obstacle to the control of agriculturally and medically important pests (Hassall, 1990). Resistance can be acquired through the relatively rare behavioral or the more common physiological action. The physiological resistance may be subdivided into two main categories: the "metabolic" (modified permeability, excretion, detoxification-degradation) and the "altered target site" resistance. The knock-down resistance (KDR) phenomenon, which represents the most common form of resistance against DDT and pyrethroids, can be induced by various metabolic factors (Mullin and Jeffrey, 1992; Scott and Wheelock, 1992); but it is substantially attributed to an altered target site, namely, point mutations in the insect *para* type voltage-gated sodium channel (Taylor *et al.*, 1993; Williamson *et al.*, 1996; Dong, 1997; Pittendrigh *et al.*, 1997; Zlotkin *et al.*, 1999). As shown above, a similar pattern of resistance (namely, increased detoxification and modified target site) was revealed in the pre-existing tolerance of lepidopterous larvae and

certain tenebrionid beetles to the insect selective AaIT polypeptide (Herrmann *et al*., 1990; Fishman *et al*., 1997).

A reasonable possibility for insecticidal cooperativity is based on the assumption that if a certain population (or strain) of insects has developed through insecticidal-selective pressure, a modified voltage-gated ion channel, the presumed conformational change induced by the mutation can modify channel pharmacology. Such an assumption can be exemplified both by earlier and more recent findings that suggest the occurrence of an "allosteric antagonism" (negative cross-resistance) between the pyrethroid resistance phenomenon (knock-down resistance, KDR) and increased susceptibility to the insect sodium channel neurotoxicants. Such a phenomenon was previously observed in KDR houseflies and *N*-alkylamides (such as BTG 501–502). The latter were shown to be up to four times more effective against the super KDR strain (530) of houseflies (Elliot *et al*., 1986). It has also been shown (McCutchen *et al*., 1997) that neonate pyrethroid resistant (KDR) tobacco budworms exhibited a significantly reduced (by 33%) median lethal time (LT_{50}), when infected with a recombinant baculovirus expressing the AaIT toxin (AcAaIT) when compared with the wild-type budworms. Further, as shown in **Table 8**, KDR and super-KDR houseflies injected with the AaIT toxin revealed a 9- and 14-fold increase in their susceptibility to the AaIT toxin, respectively (Zlotkin *et al*., 1999). It is noteworthy that similar allosteric antagonism was shown by the acquisition of hypersensitivity to pyrethroids by TTX-resistant dorsal root ganglion sodium channels (Tatebayashi and Narahashi, 1994).

As discussed in Section 5.6.4.3, the AaIT toxin interacts with domain II of the insect VGSC at a receptor binding site that is different from those of the low molecular weight and lipophilic DDT and pyrethroids (Soderlund and Knipple, 1995). The latter presumably act at the intramembranous region of the channel, similarly to the lipid-soluble alkaloid gating modifiers, such as veratridine or batrachotoxin (Hille, 1992). With this background of binding site diversity, the data presented in **Table 8** demonstrate the pharmacological flexibility of sodium channels, which has implications for the management of insecticide resistance.

Briefly, the suitability of the insect sodium channel to serve as a target for future insecticides depends on: (1) its critical role in excitability (Hille, 1992); (2) its pharmacological diversity exhibited in the multiplicity of binding sites for various neurotoxicants, including four groups of industrial

Table 8 The paralytic effect (PD_{50}) of AaIT toxin to wild-type (WT) and pyrethroid-knockdown resistant (KDR) houseflies[a]

Housefly strain[b]	*PD_{50} (ng/insect ± SD)*[c]	*Susceptibility ratio*[d]
WT-"Cooper"	21.1 ± 1.35 (3)[e]	1.0
KDR[f]	2.3 ± 0.39 (3)	9.2
Super-KDR[g]	1.5 ± 0.25 (3)	14.0

[a]From Zlotkin *et al.* (1999). Represents the "knockdown" assay: determined on mobile (alert) flies 5 min following injection.
[b]Bred in IACR, Rothamsted, UK.
[c]PD_{50} – paralytic dose 50%.
[d]Susceptibility ratio: PD_{50} of WT/PD_{50} of strain.
[e]Numbers in parentheses indicate the number of independent assays performed.
[f]KDR-stock 579 homozygous for a point mutation (L1014F) in the hydrophobic S6 transmembrane segment of homology domain II of the Para sodium channel, which confers resistance of about one order of magnitude to pyrethroid insecticides (Williamson *et al*., 1996).
[g]Super-KDR-stock 530, possessing two point mutations in the *para* gene: the first is the above KDR (L1014F) mutation. The second point mutation (M918T) is located in the intracellular loop, connecting segments 4 and 5 in homology domain II (Pittendrigh *et al*., 1997). The super-KDR strain is about 500-fold resistant to deltamethrin (Williamson *et al*., 1996; Pittendrigh *et al*., 1997).
Reproduced with permission from Zlotkin, E., **1999**. The insect voltage-gated sodium channel as target of insecticides. *Annu. Rev. Entomol. 44*, 429–455.

insecticides (**Table 3**); (3) it being pharmacologically distinguishable from vertebrate sodium channels as revealed by heterologous expression of the *Drosophila* Para sodium channel (Warmke *et al*., 1997) and the insect selectivity of neurotoxins derived from arachnid venoms; and (4) its pharmacological flexibility, as exhibited by the "allostereic antagonism" (**Tables 3 and 8**).

"Allosteric antagonism" indicates that binding of one ligand to the sodium channel protein induces a conformational change that affects its functionality and responsiveness to other toxicants acting at a different site on the channel protein (Soderlund and Knipple, 1995; Gordon, 1997a; **Table 3**). A similar conformational change in the channel protein could be induced by point mutations of the kind that confer insecticide resistance. Such a change may affect the responsiveness of the channel to various toxicants either by antagonizing or enhancing their effects. The decreased, as well as increased, susceptibilities to AaIT by the different sodium channel mutated fly strains (Zlotkin *et al*., 1999) provide evidence for such effects.

Finally, the above phenomenon of the allosteric antagonism (or negative cross-resistance) should be viewed against the background of the increasing agroeconomical significance of pyrethroid resis-

tance (Elliot *et al.*, 1986), and the appearance of bioinsecticides based on recombinant baculovirus expressing neurotoxins (Cory, 1994). These developments emphasize the need to clarify the molecular basis underlying the allosteric coupling between toxicant binding sites on the ion channel, in order to assess its implications for insecticide resistance management.

5.6.5.3. Oral Toxicity

The clonability of insect selective neurotoxic polypeptides and their availability to genetic manipulations, raises the possibility of employing them in the design of transgenic insect protected plants.

In order to exert their neurotoxic effectivity, the plant expressed polypeptide toxin has to cross the insect's digestive system in an intact, pharmacologically functional form. Some of the data mentioned below indicate that such a possibility is feasible.

A series of previous studies showed that the midgut of the blowfly *Sarcophaga falculata* is permeable to polypeptides, such as the amphipathic cobra venom cardiotoxin (Mr 7 kDa) (Primor and Zlotkin, 1978, 1980; Primor *et al.*, 1980; Fishman *et al.*, 1984) and the excitatory AaIT toxin (Zlotkin *et al.*, 1992). By the aid of a radioiodinated toxin ([^{125}I]-AaIT) it has been shown that about 0.5–2.0% of the orally fed toxin crosses the gut in an active-toxic form within 30–80 min. The gut crossing occurs through a certain, morphologically defined segment of the midgut (Fishman *et al.*, 1984; Zlotkin *et al.*, 1992). Since proteins such as the serum proteins (Nogge, 1971), immunoglobulins (Schlein *et al.*, 1976; Nogge and Giannetti, 1980), and horseradish peroxidase (HRP; Fishman and Zlotkin, 1984) were shown to readily cross the gut of *Sarcophaga* flies, the pesticidal significance of the above information is doubtful (Zlotkin *et al.*, 1992).

However, two recent reports indicate the feasibility of using insect protected transgenic plants. The first study reported on insect resistant transgenic plants engineered with insecticidal genes of venom neurotoxins, namely, tobacco plants expressing the AaIT neurotoxin (Yao *et al.*, 1996). Insect bioassays showed that some transgenic plants had notable insect resistant activity. A similar result was obtained with *Agrobacterium tumefaciens* mediated transformation of rice with a spider insecticidal gene, conferring resistance to the leaf borer and the striped stem borer (Huang *et al.*, 2001). Insect bioassays demonstrated that the transgenic plants had an enhanced resistance to these pests. These encouraging results suggest that the digestive system of lepidopterous insect pests is permeable to insecticidal polypeptides. Such a conclusion is supported by a second recent report (Fishman *et al.*, 2003), which revealed that adult *Spodoptera* noctuid moths respond by paralysis within 10–30 min when given an orally delivered solution of crude scorpion venom. When the same venom is delivered by injection, a similar but faster paralysis occurs at a roughly 30 times lower dosage. The orally induced paralysis is noticeably enhanced by the addition of proteolytic blockers to the ingested toxic solutions. It appears that the moth's gut is permeable to polypeptides and the degree of the oral toxicity is determined by the proteolytic activity in the gut lumen. The above considerations can serve as guidelines for the study and design of gut permeable, insect selective neurotoxic polypeptides.

5.6.6. Concluding Remarks

This article focuses on the insect selective neurotoxic polypeptides derived from buthid scorpion venoms that affect voltage-gated sodium channels. The topic is treated from ecological, pharmacological, structural–molecular, and applicative points of view. A question that may be asked is what is the lesson provided by the insect selective toxins? Four possible answers are offered to this question:

1. From an ecological-evolutionary point of view one is impressed by the diversity in the composition of scorpion venom, strongly contrasting with the conservation of their morphology and anatomy (living fossils). It would appear that the selective pressure is mainly directed to and absorbed by the venom gland, which is an essential instrument for their survival (Zlotkin, 1983). The diversity and multiplicity of the phyletic toxins can be explained on the basis of an assumption that natural selection coevolved distinct types of ion channels in various groups of animals at the same time as the scorpions evolved specific peptide toxins that are aimed to affect the normal function of these ion channels for the purpose of prey capture or defense from predators (Possani *et al.*, 1999).
2. From a strictly neuropharmacological point of view, the insect selective toxins reveal the structural/functional uniqueness of the insect voltage-gated sodium channel, and serve as probes and pharmacological tools for the study of its chemistry and functional significance. In fact, these toxins provided the first clear indication for the occurrence of pharmacologically

distinguishable ion channel subtypes (Zlotkin *et al*., 2000).

3. As an exercise in the functional anatomy of neurotoxic polypeptides, the scorpion toxins provide a model for the design of useful polypeptides. They possess the appropriate balance between structural rigidity (provided by the peptidic backbone of the toxin molecule or its conserved "scaffold") and flexibility (provided by the "variable loops" and their aliphatic chains, such as arginine and lysine amino acids, which possess the respective functional guanidinium and amino groups). Thus, in scorpion toxins, the basic residues connected to the scaffold serve as multipoint interaction sites with the target molecules (Loret and Hammock, 2001). The above positively charged and hydrophobic residues form the bioactive surface.
4. The agronomical-applicative aspect serves as the main lesson of these insect selective toxins. It suggests a technological combination between the structural modifiability of the polypeptide insect selective neurotoxins and the pharmacological (allosteric) flexibility of the insect voltage-gated sodium channels. Such combination may enable the design of an insecticidal-resistance management strategy, based on synergistic or negative cross-resistance coupling among neurotoxins or among neurotoxins and classical organic insecticides. It also encourages the development of new modes for the delivery of the clonable and modifiable insect selective neurotoxins.

Acknowledgments

The topic of scorpion neurotoxins was recently presented and discussed in a series of review articles to which the reader is referred: Simard and Watt (1990), Martin-Eauclaire and Couraud (1995), Gordon *et al*. (1998), Zlotkin (1999), Possani *et al*. (2000), Zlotkin *et al*. (2000). The following recent text books provide an additional perfect source of updated information on the subcellular-molecular level: Bon (2000) and Menez (2002). However, in preparing the present review the following articles were especially useful by providing comprehensive and well-designed overviews on the neuropharmacology (Gordon *et al*., 2002), chemistry (Possani *et al*., 1999), structure–function (Gurevitz *et al*., 2002), and applicability (Loret and Hammock, 2001) of scorpion neurotoxins-affecting voltage-gated sodium channels.

Reported studies conducted by the author were supported by US-Israel BSF and BARD foundations as well as the Ciba-Geigy Company.

References

Adam, K.R., Schmidt, H., Stampfli, R., Weiss, C., **1966**. The effect of scorpion venom on single myelinated nerve fibres of the frog. *Br. J. Pharmacol. 26* (3), 666–677.

Barhanin, J., Giglio, J.R., Leopold, P., Schmid, A., Sampaio, S.V., *et al*., **1982**. *Tityus serrulatus* venom contains two classes of toxins. *Tityus* gamma toxin is a new tool with a very high affinity for studying the Na^+ channel. *J. Biol. Chem. 257* (21), 12553–12558.

Barres, B.A., Chun, L.L., Corey, D.P., **1989**. Glial and neuronal forms of the voltage-dependent sodium channel: characteristics and cell-type distribution. *Neuron 2*, 1375–1388.

Ben Khalifa, R., Stankiewicz, M., Pelhate, M., Serrano-Hernandez, S.E., Possani, L.D., *et al*., **1997**. Action of babycurus-toxin 1 from the east African scorpion Babycurus centrurimorphus on the isolated cockroach giant axon. *Toxicon 35* (7), 1069–1080.

Beneski, D.A., Catterall, W.A., **1980**. Covalent labeling of protein components of the sodium channel with a photoactivable derivative of scorpion toxin. *Proc. Natl Acad. Sci. USA 77* (1), 639–643.

Benet, L.Z., **1978**. Effect of route of administration and distribution on drug action. *J. Pharmacokinet. Biopharm. 6* (6), 559–585.

Benoit, E., Dubois, J.M., **1987**. Properties of maintained sodium current induced by a toxin from *Androctonus* scorpion in frog node of Ranvier. *J. Physiol. 383*, 93–114.

Benzinger, G.R., Kyle, J.W., Blumenthal, K.M., Hanck, D.A., **1998**. A specific interaction between the cardiac sodium channel and site-3 toxin Anthopleurin B. *J. Biol. Chem. 273*, 80–84.

Bettini, S., **1978**. Arthropod Venoms. Springer.

Blanc, E., Hassani, O., Meunier, S., Mansuelle, P., Sampieri, F., *et al*., **1997**. 1H-NMR-derived secondary structure and overall fold of a natural anatoxin from the scorpion *Androctonus australis* hector. *Eur. J. Biochem. 247* (3), 1118–1126.

Bloomquist, J.R., **1996**. Ion channels as targets for insecticides. *Annu. Rev. Entomol. 41*, 163–190.

Bon, C., **2000**. The Natural Toxins, vol. 82. Biochimie, Elsevier, Paris.

Bonning, B.C., Hammock, B.D., **1992**. Development and potential of genetically engineered viral insecticides. *Biotechnol. Genet. Eng. Rev. 10*, 455–489.

Bonning, B.C., Hammock, B.D., **1996**. Development of recombinant baculoviruses for insect control. *Annu. Rev. Entomol. 41*, 191–210.

Brazil, O.V., Excell, B.J., de Sa, S.S., **1973**. Proceedings: the importance of phospholipase A in the action of the crotoxin complex at the frog neuromuscular junction. *J. Physiol. 234* (2), 63P–64P.

Brownell, P., **2001**. Sensory ecology and oriented behaviours. In: Bronwell, P., Polis, G. (Eds.), Scorpion Biology and Research. Oxford University Press, Oxford, p. 159.

Brownell, P., Polis, G., **2001**. Scorpion Biology and Research. Oxford University Press, Oxford.

Bücherl, W., **1955**. Studien uber einige Brasilieniche Skorpione und ihre Trockengifte. *Arzneimittel-Forch. 5*, 68–71.

Bücherl, W., **1971**. Classification, biology and venom extraction of scorpions. In: Bücherl, W., Buckley, E. (Eds.), Venomous Animals and their Venoms, vol. 2. Academic Press, New York, pp. 317–347.

Cahalan, M.D., **1975**. Modification of sodium channel gating in frog myelinated nerve fibres by *Centruroides sculpturatus* scorpion venom. *J. Physiol. 244* (2), 511–534.

Catterall, R.D., **1992**. Cellular and molecular biology of voltage gated sodium channels. *Am. J. Physiol. 72*, 15–48.

Catterall, W.A., **1976**. Purification of a toxic protein from scorpion venom which activates the action potential Na^+ ionophore. *J. Biol. Chem. 251* (18), 5528–5536.

Catterall, W.A., **1977**. Membrane potential-dependent binding of scorpion toxin to the action potential Na^+ ionophore. Studies with a toxin derivative prepared by lactoperoxidase-catalyzed iodination. *J. Biol. Chem. 252* (23), 8660–8668.

Catterall, W.A., **1980**. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol. 20*, 15–43.

Catterall, W.A., **1995**. Structure and function of voltage-gated ion channels. *Annu. Rev. Biochem. 64*, 493–531.

Catterall, W.A., Beress, L., **1978**. Sea anemone toxin and scorpion toxin share a common receptor site associated with the action potential sodium ionophore. *J. Biol. Chem. 253* (20), 7393–7396.

Catterall, W.A., Nurenberg, M., **1973**. Sodium uptake associated with activation of action potential ionophores of cultured neuroblastoma and muscle cells. *Proc. Natl Acad. Sci. USA 70* (12), 3759–3763.

Cestele, S., Ben Khalifa, R.B., Pelhate, M., Rochat, H., Gordon, D., **1995**. Alpha-scorpion toxins binding on rat brain and insect sodium channels reveal divergent allosteric modulations by brevetoxin and veratridine. *J. Biol. Chem. 270* (25), 15153–15161.

Cestele, S., Catterall, W.A., **2000**. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie 82* (9–10), 883–892.

Cestele, S., Qu, Y., Rogers, J.C., Rochat, H., Scheuer, T., *et al.*, **1998**. Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3–S4 loop in domain II. *Neuron 21* (4), 919–931.

Clark, J.M., **1995**. Molecular Actions of Insecticides on Ion Channels. American Chemical Society, Washington, DC.

Cory, J.S., Hirst, M.L., Williams, T., Halls, R.S., Gouson, D., *et al.*, **1994**. Field trial of a genetically improved baculovirus insecticide. *Nature 371*, 138–140.

Couraud, F., Jover, E., Dubois, J.M., Rochat, H., **1982**. Two types of scorpion toxin receptor sites, one related to the activation, the other to the inactivation of the action potential sodium channel. *Toxicon 20*, 9–16.

Couraud, F., Rochat, H., Lissitzky, S., **1978**. Binding of scorpion and sea anemone neurotoxins to a common site related to the action potential Na^+ ionophore in neuroblastoma cells. *Biochem. Biophys. Res. Commun. 83* (4), 1525–1530.

Darbon, H., Jover, E., Couraud, F., Rochat, H., **1983**. Photoaffinity labeling of alpha- and beta-scorpion toxin receptors associated with rat brain sodium channel. *Biochem. Biophys. Res. Commun. 115* (2), 415–422.

Darbon, H., Weber, C., Braun, W., **1991**. Two-dimensional 1H nuclear magnetic resonance study of AaH IT, an anti-insect toxin from the scorpion *Androctonus australis* Hector. Sequential resonance assignments and folding of the polypeptide chain. *Biochemistry 30* (7), 1836–1845.

Darbon, H., Zlotkin, E., Kopeyan, C., van Rietschoten, J., Rochat, H., **1982**. Covalent structure of the insect toxin of the North African scorpion *Androctonus australis* Hector. *Int. J. Pept. Protein Res. 20* (4), 320–330.

De Lima, M.E., Martin, M.F., Diniz, C.R., Rochat, H., **1986**. *Tityus serrulatus* toxin VII bears pharmacological properties of both beta-toxin and insect toxin from scorpion venoms. *Biochem. Biophys. Res. Commun. 139* (1), 296–302.

De Lima, M.E., Martin-Euclaire, M.F., **1995**. The toxins purified from *Tityus serrulatus* (Lutz and Mello) venom. *J. Toxicol. Toxin Rev. 14*, 457–481.

De Lima, M.E., Martin-Euclaire, M.F., Hue, B., Loret, E., Diniz, C.R., *et al.*, **1989**. On the binding of two scorpion toxins to the central nervous system of the cockroach *Periplaneta americana*. *Insect Biochem. 151* (1), 187–192.

Dehesa-Davila, M., Ramirez, A.N., Zamudio, F., Gurrola-Briones, G., Lievano, A., *et al.*, **1996**. Structural and functional comparison of toxins from the venom of the scorpions *Centruroides infamatus, Centruroides limpidus limpidus and Centruroides noxius*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 113*, 331–339.

Dong, K., **1997**. A single amino acid change in the para sodium channel protein is associated with knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect Biochem. Mol. Biol. 27* (2), 93–100.

Dresco-Derouet, L., **1964a**. Le metabolisme respiratoire des scorpiones chez quelques especes a differente temperatures. *Bull. Mus. Natl d'Hist. Nat. (Paris) ser. 2 36*, 97–99.

Dresco-Derouet, L., **1964b**. Le metabolisme respiratoire des scorpions. II. Mesures de l'intensite respiratoire chez quelque especes a differentes temperatures. *Bull. Mus. Natl d'Hist. Nat. (Paris), ser. 2 36*, 533–557.

Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., *et al.*, **1990**. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure, and mode of action. *Biochemistry 29* (25), 5941–5947.

el Ayeb, M., Bahraoui, E.M., Granier, C., Rochat, H., **1986**. Use of antibodies specific to defined regions of scorpion alpha-toxin to study its interaction with its receptor site on the sodium channel. *Biochemistry 25* (21), 6671–6678.

el Ayeb, M., Rochat, H., **1985**. Polymorphism and quantitative variations of toxins in the venom of the scorpion *Androctonus australis* Hector. *Toxicon 23*, 755–760.

Elazar, M., Levi, R., Zlotkin, E., **2001**. Targeting of an expressed neurotoxin by its recombinant baculovirus. *J. Exp. Biol. 204* (15), 2637–2645.

Elliot, M., Farnham, A.W., Janes, N.F., Johnson, D.M., Pulman, D.A., *et al.*, **1986**. Insecticidal amides with selective potency against a resistant (super-kdr) strain of houseflies (*Musca domestica* L.). *Agric. Biol. Chem. 50*, 1347–1349.

Endean, R., Rudkin, C., **1963**. Studies of the venom of some Conidae. *Toxicon 1*, 49–64.

Endean, R., Rudkin, C., **1965**. Further studies of the venoms of Conidae. *Toxicon 2* (4), 225–249.

Engelhard, E.K., Kam-Morgan, L.N., Washburn, J.O., Volkman, L.E., **1994**. The insect tracheal system: a conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. *Proc. Natl Acad. Sci. USA 91* (18), 3224–3227.

Fainzilber, M., Kofman, O., Zlotkin, E., Gordon, D., **1994**. A new neurotoxin receptor site on sodium channels is identified by a conotoxin that affects sodium channel inactivation in molluscs and acts as an antagonist in rat brain. *J. Biol. Chem. 269* (4), 2574–2580.

Feng, G., Deak, P., Chopra, M., Hall, L.M., **1995**. Cloning and functional analysis of TipE, a novel membrane protein that enhances *Drosophila* para sodium channel function. *Cell 82* (6), 1001–1011.

Fet, V., Sisson, W.D., Lowe, G., Braunwalder, M.E., **2000**. Catalog of the Scorpions of the World (1758–1998). New York Entomological Society, New York.

Fishman, L., Herrmann, R., Gordon, D., Zlotkin, E., **1997**. Insect tolerance to a neurotoxic polypeptide: pharmacokinetic and pharmacodynamic aspects. *J. Exp. Biol. 200* (7), 1115–1123.

Fishman, L., Kagan, M.L., Zlotkin, E., **1991**. Accessibility of the insect nervous system to a neurotoxic polypeptide. *J. Exp. Zool. 257*, 10–23.

Fishman, L., Uliel, S., Zlotkin, E., **2003**. Neurotoxic peptide translocation through an insect gut. In: 14th World Congress on Animal, Plant and Microbial Toxins, September 9–15, Adelaide, Australia.

Fishman, L., Primor, N., Zlotkin, E., **1984**. The selective permeability of fleshfly midgut to an orally toxic cobra venom cardiotoxic. *J. Exp. Biol. 229*, 189–195.

Fishman, L., Zlotkin, E., **1984**. A diffusional route of transport of horseradish peroxidase through the midgut of a fleshfly. *J. Exp. Zool. 229*, 189–195.

Fletcher, J.I., Smith, R., O'Donoghue, S.I., Nilges, M., Connor, M., *et al.*, **1997**. The structure of a novel insecticidal neurotoxin, omega-atracotoxin-HV1, from the venom of an Australian funnel web spider. *Natl Struct. Biol. 4* (7), 559–566.

Fontecilla-Camps, J.C., **1989**. Three-dimensional model of the insect-directed scorpion toxin from *Androctonus australis* Hector and its implication for the evolution of scorpion toxins in general. *J. Mol. Evol. 29* (1), 63–67.

Fontecilla-Camps, J.C., Almassy, R.J., Suddath, F.L., Bugg, C.E., **1982**. The three-dimensional structure of scorpion neurotoxins. *Toxicon 20* (1), 1–7.

Fontecilla-Camps, J.C., Almassy, R.J., Suddath, F.L., Watt, D.D., Bugg, C.E., **1980**. Three-dimensional structure of a protein from scorpion venom: a new structural class of neurotoxins. *Proc. Natl Acad. Sci. USA 77* (11), 6496–6500.

Fontecilla-Camps, J.C., Habersetzer-Rochat, C., Rochat, H., **1988**. Orthorhombic crystals and three-dimensional structure of the potent toxin II from the scorpion *Androctonus australis* Hector. *Proc. Natl Acad. Sci. USA 85* (20), 7443–7447.

Froy, O., Zilberberg, N., Gordon, D., Turkov, M., Gilles, N., *et al.*, **1999**. The putative bioactive surface of insect-selective scorpion excitatory neurotoxins. *J. Biol. Chem. 274* (9), 5769–5776.

Garcia, C., Becerril, B., Selisko, B., Delepierre, M., Possani, L.D., **1997**. Isolation, characterization and comparison of a novel crustacean toxin with a mammalian toxin from the venom of the scorpion *Centruroides noxius* Hoffmann. *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 116* (3), 315–322.

Gershburg, E., Stockholm, D., Froy, O., Rashi, S., Gurevitz, M., *et al.*, **1998**. Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. *FEBS Lett. 422* (2), 132–136.

Gilles, N., Blanchet, C., Shichor, I., Zaninetti, M., Lotan, I., *et al.*, **1999**. A scorpion alpha-like toxin that is active on insects and mammals reveals an unexpected specificity and distribution of sodium channel subtypes in rat brain neurons. *J. Neurosci. 19* (20), 8730–8739.

Gilles, N., Chen, H., Wilson, H., Le Gall, F., Montoya, G., *et al.*, **2000**. Scorpion alpha and alpha-like toxins differentially interact with sodium channels in mammalian CNS and periphery. *Eur. J. Neurosci. 12* (8), 2823–2832.

Gilles, N., Gurevitz, M., Gordon, D., **2003**. Allosteric interactions among pyrethroid, brevetoxin, and scorpion toxin receptors on insect sodium channels raise an alternative approach for insect control. *FEBS Lett. 540* (1–3), 81–85.

Gilles, N., Harrison, G., Karbat, I., Gurevitz, M., Nicholson, G.M., *et al.*, **2002**. Variations in receptor site-3 on rat brain and insect sodium channels highlighted by binding of a funnel-web spider delta-atracotoxin. *Eur. J. Biochem. 269* (5), 1500–1510.

Gilles, N., Leipold, E., Chen, H., Heinemann, S.H., Gordon, D., **2001**. Effect of depolarization on binding kinetics of scorpion alpha-toxin highlights conformational changes of rat brain sodium channels. *Biochemistry 40* (48), 14576–14584.

Goldin, A.L., **1999**. Diversity of mammalian voltage-gated sodium channels. *Ann. N. Y. Acad. Sci. 868*, 38–50.

Goldin, A.L., **2001**. Nomenclature of voltage gated sodium channels. *Neuron 28*, 365–368.

Gordon, D., **1997a**. A new approach to insect-pest control – combination of neurotoxins interacting with

voltage sensitive sodium channels to increase selectivity and specificity. *Invert. Neurosci. 3* (2–3), 103–116.

Gordon, D., **1997b**. Sodium channels as a target for neurotoxins: mode of action and interaction of neurotoxins with receptor sites on sodium channels. In: Gutman, Y., Lazarovici, P. (Eds.), Toxins and Signal Transduction. Harwood, Amsterdam, pp. 119–149.

Gordon, D., Gilles, N., Bertrand, D., Molgo, J., Nicholson, G., *et al.*, **2002**. Scorpion toxins differentiating among neuronal sodium channel subtypes: nature's guide for design of selective drugs. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 215–238.

Gordon, D., Gurevitz, M., **2003**. The selectivity of scorpion alpha-toxins for sodium channel subtypes is determined by subtle variations at the interacting surface. *Toxicon 41* (2), 125–128.

Gordon, D., Jover, E., Couraud, F., Zlotkin, E., **1984**. The binding of the insect selective neurotoxin (AaIT) from scorpion venom to locust synaptosomal membranes. *Biochim. Biophys. Acta 778*, 349–358.

Gordon, D., Martin-Euclaire, M.F., Cestele, S., Kopeyan, C., Carlier, E., *et al.*, **1996**. Scorpion toxins affecting sodium current inactivation bind to distinct homologous receptor sites on rat brain and insect sodium channels. *J. Biol. Chem. 271* (14), 8034–8045.

Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W.A., *et al.*, **1992**. Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies. *Biochemistry 31* (33), 7622–7628.

Gordon, D., Savarin, P., Gurevitz, M., Zinn-Justin, S., **1998**. Functional anatomy of scorpion toxins affecting sodium channels. *J. Toxicol. Toxin Rev. 17*, 131–159.

Gordon, D., Zlotkin, E., **1993**. Binding of an alpha scorpion toxin to insect sodium channels is not dependent on membrane potential. *FEBS Lett. 315* (2), 125–128.

Gurevitz, M., Froy, O., Zilberberg, N., Turkov, M., Strugatsky, D., *et al.*, **1998**. Sodium channel modifiers from scorpion venom: structure-activity relationship, mode of action and application. *Toxicon 36* (11), 1671–1682.

Gurevitz, M., Gordon, D., Ben-Natan, S., Turkov, M., Froy, O., **2001**. Diversification of neurotoxins by C-tail 'wiggling': a scorpion recipe for survival. *FASEB J. 15* (7), 1201–1205.

Gurevitz, M., Zilberberg, N., **1994**. Advances in molecular genetics of scorpion neurotoxins. *J. Toxicol. Toxin Rev. 13*, 65–100.

Gurevitz, M., Zilberberg, N., Froy, O., Turkov, M., Wilkunsky, R., *et al.*, **2002**. Diversification of toxin sites on a conserved protein scaffold – a scorpion recipe for survival. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 239–253.

Hassall, K., **1990**. The Biochemistry and Uses of Pesticides. VCH, Weinheim.

Herrmann, R., Fishman, L., Zlotkin, E., **1990**. The tolerance of lepidopterous larvae to an insect selective neurotoxin. *Insect Biochem. 20*, 625–637.

Herrmann, R., Moskowitz, H., Zlotkin, E., Hammock, B.D., **1995**. Positive cooperativity among insecticidal scorpion neurotoxins. *Toxicon 33*, 1099–1102.

Higg, S., Olson, K.E., Klimowski, L., Powers, A.M., Carlson, J.O., *et al.*, **1995**. Mosquito sensitivity to a scorpion neurotoxin expressed using an infectious Sindbis virus vector. *Insect Mol. Biol. 4* (2), 97–103.

Hille, B., **1992**. Ionic Channels of Excitable Membranes. Sinauer, Sunderland.

Hjelle, J.T., **1990**. Anatomy and morphology. In: Polis, G. (Ed.), The Biology of Scorpions. Stanford University Press, pp. 9–63.

Huang, J.Q., Wel, Z.M., An, H.L., Zhu, Y.X., **2001**. *Agrobacterium tumefaciens*-mediated transformation of rice with the spider insecticidal gene conferring resistance to leaffolder and striped stem borer. *Cell Res. 11* (2), 149–155.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., *et al.*, **2003**. One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proc. Natl Acad. Sci. USA 100* (3), 922–927.

Ishaaya, I.E., **2001**. Biochemical Sites of Insctίcide Action and Resistance. Springer, Berlin-Heidelberg.

Jablonsky, M.J., Watt, D.D., Krishna, N.R., **1995**. Solution structure of an Old World-like neurotoxin from the venom of the New World scorpion *Centruroides sculpturatus* Ewing. *J. Mol. Biol. 248* (2), 449–458.

Jonas, P., Vogel, W., Arantes, E.C., Giglio, J.R., **1986**. Toxin gamma of the scorpion *Tityus serrulatus* modifies both activation and inactivation of sodium permeability of nerve membrane. *Pflugers Arch. 407* (1), 92–99.

Jover, E., Martin-Moutot, N., Couraud, F., Rochat, H., **1978**. Scorpion toxin: specific binding to rat synaptosomes. *Biochem. Biophys. Res. Commun. 85* (1), 377–382.

Jover, E., Martin-Moutot, N., Couraud, F., Rochat, H., **1980**. Binding of scorpion toxins to rat brain synaptosomal fraction. Effects of membrane potential, ions, and other neurotoxins. *Biochemistry 19* (3), 463–467.

Jover, E., Massacrier, A., Cau, P., Martin, M.F., Couraud, F., **1988**. The correlation between Na^+ channel subunits and scorpion toxin-binding sites. A study in rat brain synaptosomes and in brain neurons developing in vitro. *J. Biol. Chem. 263* (3), 1542–1548.

Kallen, R.G., Cohen, S.A., Barchi, R.L., **1993**. Structure, function and expression of voltage-dependent sodium channels. *Mol. Neurobiol. 7* (3–4), 383–428.

Katz, N.L., Edwards, C., **1972**. The effect of scorpion venom on the neuromuscular junction of the frog. *Toxicon 10* (2), 133–137.

Keister, M., Buck, J., **1973**. Respiration: some exogenous and endogenous effects on rate of respiration. In: Rockstein, M. (Ed.), The Physiology of Insecta, vol. 6. Academic Press, New York, pp. 469–509.

Kem, W.R., **1976**. Purification and characterization of a new family of polypeptide neurotoxins from the heteronemertine *Cerebratulus lacteus* (Leidy). *J. Biol. Chem. 251* (14), 4184–4192.

Kem, W.R., Dunn, B.M., **1988**. Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon 26* (11), 997–1008.

Kharrat, R., Darbon, H., Rochat, H., Granier, C., **1989**. Structure/activity relationships of scorpion alpha-toxins. Multiple residues contribute to the interaction with receptors. *Eur. J. Biochem. 181* (2), 381–390.

Kirkpatrick, B.A., Washburn, J.O., Engelhard, E.K., Volkman, L.E., **1994**. Primary infection of insect tracheae by *Autographa californica* M nuclear polyhedrosis virus. *Virology 203* (1), 184–186.

Kirsch, G.E., Brown, A.M., **1989**. Kinetic properties of single sodium channels in rat heart and rat brain. *J. Gen. Physiol. 93*, 85–99.

Kirsch, G.E., Skattebol, A., Possani, L.D., Brown, A.M., **1989**. Modification of Na channel gating by an alpha scorpion toxin from *Tityus serrulatus*. *J. Gen. Physiol. 93* (1), 67–83.

Kopeyan, C., Mansuelle, P., Martin-Euclaire, M.F., Rochat, H., Miranda, F., **1993**. Characterization of toxin III of the scorpion *Leiurus quinquestriatus quinquestriatus*: a new type of alpha-toxin highly toxic both to mammals and insects. *Nat. Toxins 1* (5), 308–312.

Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Bahraoui, E.M., *et al.*, **1990**. Primary structure of scorpion anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus*. *FEBS Lett. 261* (2), 423–426.

Krapcho, K.J., Kral, R.M., Jr., Vanwagenen, B.C., Eppler, K.G., Morgan, T.K., **1995**. Characterization and cloning of insecticidal peptides from the primitive weaving spider *Diguetia canities*. *Insect Biochem. Mol. Biol. 25* (9), 991–1000.

Landon, C., Sodano, P., Hetru, C., Hoffmann, J., Ptak, M., **1997**. Solution structure of drosomycin, the first inducible antifungal protein from insects. *Protein Sci. 6* (9), 1878–1884.

Lazarovici, P., Menashe, M., Zlotkin, E., **1984**. Toxicity to crustacea due to polypeptide-phospholipase interaction in the venom of a chactoid scorpion. *Arch. Biochem. Biophys. 229* (1), 270–286.

Lazdunski, M., Frelin, C., Barhanin, J., Lombet, A., Meiri, H., *et al.*, **1986**. Polypeptide toxins as tools to study voltage-sensitive Na^+ channels. *Ann. N. Y. Acad. Sci. 479*, 204–220.

Lebreton, F., Delepierre, M., Ramirez, A.N., Balderas, C., Possani, L.D., **1994**. Primary and NMR three-dimensional structure determination of a novel crustacean toxin from the venom of the scorpion *Centruroides limpidus limpidus* Karsch. *Biochemistry 33* (37), 11135–11149.

Lee, D., **1997**. Pharmacological and biophysical properties of insect neuronal sodium channels. Ph. D. thesis, University of California, Riverside.

Lee, D., Adams, M.E., **2000**. Sodium channels in central neurons of the tobacco budworm, *Heliothis virescens*: basic properties and modification by scorpion toxins. *J. Insect Physiol. 46* (4), 499–508.

Lester, D., Lazarovici, P., Pelhate, M., Zlotkin, E., **1982**. Purification, characterization and action of two insect toxins from the venom of the scorpion *Buthotus indiacus*. *Biochim. Biophys. Acta 688*, 229–236.

Levy, G., Amitai, P., **1980**. Fauna Palestina: Scorpions. Magnes.

Loret, E., Hammock, B.D., **2001**. Structure and neurotoxicity of venoms. In: Bronwell, P., Polis, G. (Eds.), Scorpion Biology and Research. Oxford University Press, Oxford, p. 204.

Loret, E.P., del Valle, R.M., Mansuelle, P., Sampieri, F., Rochat, H., **1994**. Positively charged amino acid residues located similarly in sea anemone and scorpion toxins. *J. Biol. Chem. 269* (24), 16785–16788.

Loret, E.P., Mansuelle, P., Rochat, H., Granier, C., **1990a**. Neurotoxins active on insects: amino acid sequences, chemical modifications, and secondary structure estimation by circular dichroism of toxins from the scorpion *Androctonus australis* Hector. *Biochemistry 29* (6), 1492–1501.

Loret, E.P., Martin-Eauclaire, M.F., Mansuelle, P., Sampieri, F., Granier, C., *et al.*, **1991**. An anti-insect toxin purified from the scorpion *Androctonus australis* Hector also acts on the alpha- and beta-sites of the mammalian sodium channel: sequence and circular dichroism study. *Biochemistry 30* (3), 633–640.

Loret, E.P., Sampieri, F., Roussel, A., Granier, C., Rochat, H., **1990b**. Conformational flexibility of a scorpion toxin active on mammals and insects: a circular dichroism study. *Proteins 8* (2), 164–172.

Loughney, K., Kreber, R., Ganetzky, B., **1989**. Molecular analysis of the para locus, a sodium channel gene in *Drosophila*. *Cell 58* (6), 1143–1154.

Maeda, S., **1989**. Expression of foreign genes in insects using baculovirus vectors. *Annu. Rev. Entomol. 34*, 351–372.

Marcotte, P., Chen, L.Q., Kallen, R.G., Chahine, M., **1997**. Effects of *Tityus serrulatus* scorpion toxin gamma on voltage-gated Na^+ channels. *Circ. Res. 80* (3), 363–369.

Martin, M.F., Rochat, H., Marcotte, P., Bougis, P.E., **1987**. Use of high performance liquid chromatography to demonstrate quantitative variation in components of venom from the scorpion *Androctonus australis* Hector. *Toxicon 25*, 569–573.

Martin-Eauclaire, M.F., Couraud, F., **1995**. Scorpion neurotoxins: effects and mechanisms. In: Chang, L.W., Dyer, R.S. (Eds.), Handbook of Neurotoxicology. Dekker, New York, pp. 683–716.

McCormick, S.J., Polis, G., **1990**. Prey, predators and parasites. In: Polis, G. (Ed.), The Biology of Scorpions. Stanford University Press, pp. 294–320.

McCutchen, B.F., Choudary, P.V., Crenshaw, R., Maddox, D., Kamita, S.G., *et al.*, **1991**. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Biotechnology N Y 9* (9), 848–852.

McCutchen, B.F., Hoover, K., Preisler, H.K., Betana, M.D., Herrmann, R., *et al.*, **1997**. Interactions of

recombinant and wild-type baculoviruses with classical insecticides and pyrethroid-resistant tobacco budworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 90* (5), 1170–1180.

Menez, A., **2002**. Prospectives in Molecular Toxinology. Wiley, Chichester.

Meves, H., Rubly, N., Watt, D.D., **1982**. Effect of toxins isolated from the venom of the scorpion *Centruroides sculpturatus* on the Na currents of the node of Ranvier. *Pflugers Arch. 393* (1), 56–62.

Meves, H., Rubly, N., Watt, D.D., **1984**. Voltage-dependent effect of a scorpion toxin on sodium current inactivation. *Pflugers Arch. 402* (1), 24–33.

Meves, H., Simard, J.M., Watt, D.D., **1986**. Interactions of scorpion toxins with the sodium channel. *Ann. N. Y. Acad. Sci. 479*, 113–132.

Miranda, F., Kupeyan, C., Rochat, H., Rochat, C., Lissitzky, S., **1970**. Purification of animal neurotoxins. Isolation and characterization of eleven neurotoxins from the venoms of the scorpions *Androctonus australis* hector, *Buthus occitanus tunetanus* and *Leiurus quinquestriatus quinquestriatus*. *Eur. J. Biochem. 16* (3), 514–523.

Miranda, F., Rochat, H., Lissitzky, S., **1964**. Sur les neurotoxins deux especes de scorpions nord-africains. I. Purifications de neurotoxins (scorpamines) d'*Androctonus australis* (L.) et de *Buthus occitanus* (Am.). *Toxicon 2*, 51–56.

Moskowitz, H., Herrmann, R., Zlotkin, E., Gordon, D., **1994**. Variability among insect sodium channels revealed by binding of selective neurotoxins. *Insect Biochem. Mol. Biol. 224*, 13–19.

Mullin, C.A., Jeffrey, C.S., **1992**. Molecular Mechanisms of Insecticide Resistance. American Chemical Society, Washington, DC.

Narahashi, T., **1986**. Nerve membrane ionic channels as the target of toxicants. *Arch. Toxicol. Suppl. 9*, 3–13.

Nogge, G., **1971**. Absorption of serum proteins by hemolymph in larvae of *Hypoderma bovis* (De Geer) (Diptera, Hypodermatidae). *Experientia 27* (5), 524–525.

Nogge, G., Giannetti, M., **1980**. Specific antibodies: a potential insecticide. *Science 209* (4460), 1028–1029.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., **1992**. Baculovirus Expression Vectors: A Laboratory Manual. Freeman, New York.

Parnas, I., Avgar, D., Shulov, A., **1970**. Physiological effects of venom of *Leiurus quinquestriatus* on neuromuscular systems of locust and crab. *Toxicon 8* (1), 67–79.

Parnas, I., Russel, F.E., **1967**. Effects of venom on nerve muscles and neuromuscular junction. In: Russel, F.E., Saunders, P.R. (Eds.), Animal Toxins. Pergamon, Oxford and New York, pp. 410–427.

Pauron, D., Barhanin, J., Amichot, M., Prelavorio, M., Berge, J.B., *et al.*, **1989**. Pyrethroid receptor in the insect Na^+ channel: alteration of its properties in pyrethroid-resistant flies. *Biochemistry 28*, 1673–1678.

Payne-Gregory, T., Deecher-Darlene, C., Soderlund, D.M., **1998**. Structure activity relationship for the action of dihydropyrazole insecticides on mouse brain sodium channels. *Pestic. Biochem. Physiol. 60*, 177–180.

Pelhate, M., Pichon, Y., **1974**. Selective inhibitors of potassium current in the giant axon of the cockroach. *J. Physiol. 242*, 90–91.

Pelhate, M., Zlotkin, E., **1981**. Voltage dependent slowing of the turn off of Na^+ current in the cockroach giant axon induced by the scorpion venom insect toxin. *J. Physiol. (Lond.) 319*, 30–31.

Pelhate, M., Zlotkin, E., **1982**. Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on isolated giant axons of the cockroach (*Periplaneta americana*). *J. Exp. Biol. 97*, 67–77.

Pichon, Y., Boistel, J., **1967**. Current–voltage relations in the isolated giant axon of the cockroach under voltage-clamp conditions. *J. Exp. Biol. 47* (2), 343–355.

Pittendrigh, B., Reenan, R., ffrench-Constant, R.H., Ganetzky, B., **1997**. Point mutations in the *Drosophila* sodium channel gene para associated with resistance to DDT and pyrethroid insecticides. *Mol. Gen. Genet. 256* (6), 602–610.

Polis, G.A., **1990a**. Ecology. In: Polis, G. (Ed.), The Biology of Scorpions. Stanford University Press, pp. 247–293.

Polis, G.A., **1990b**. The Biology of Scorpions. Stanford University Press.

Possani, L.D., **1984**. Structure of scorpion toxins. In: Tu, T. (Ed.), Insect Poisons, Allergens and Other Invertebrate Venoms, vol. 2. Dekker, New York, pp. 513–550.

Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^+-channels. *Eur. J. Biochem. 264* (2), 287–300.

Possani, L.D., Merino, E., Corona, M., Bolivar, F., Balthazar, B., **2000**. Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie 82*, 861–868.

Prikhodko, G.G., Robson, M., Warmke, J.W., Cohen, C.J., Smith, M.M., **1996**. Properties of three baculovirus-expressing genes that encode insect-selective toxins: μAga-IV, As.II and Sh.I. *Biol. Control 7*, 236–244.

Primor, N., Teitelbaum, Z., Zlotkin, E., **1980**. Penetrability of orally toxic protein from cobra venom through the gut of a blowfly. *Biochim. Biophys. Acta 627* (1), 71–81.

Primor, N., Zlotkin, E., **1978**. Oral toxicity of venoms and toxins to blowflies. In: Rosenberg, P. (Ed.), Toxins: Animal, Plant and Microbial. Pergamon, Oxford, pp. 539–547.

Primor, N., Zlotkin, E., **1980**. Penetrability of proteins through the digestive system of *Sarcophaga falculata* blowfly. *Biochim. Biophys. Acta 627* (1), 82–90.

Qu, Y., Rogers, J.C., Chen, S.F., McCormick, K.A., Scheuer, T., *et al.*, **1999**. Functional roles of the extracellular segments of the sodium channel alpha subunit in voltage-dependent gating and modulation by beta1 subunits. *J. Biol. Chem. 274* (46), 32647–32654.

Rathmayer, W., Ruhland, M., Tintpulver, M., Walther, C., Zlotkin, E., **1978**. The effect of toxins derived from

the venom of the scorpion *Androctonus australis* Hector on neuromuscular transmission. In: Rosenberg, P. (Ed.), Toxins: Animal, Plant and Microbial. Pergamon, Oxford, pp. 629–637.

Regev, A., Rivkin, H., Inceoglu, B., Gershburg, E., Hammock, B.D., *et al.*, **2003**. Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively. *FEBS Lett. 537* (1–3), 106–110.

Rochat, C., Rochat, H., Miranda, F., Lissitzky, S., **1967**. Purification and some properties of the neurotoxins of *Androctonus australis* Hector. *Biochemistry 6* (2), 578–585.

Rochat, H., Bernard, P., Couraud, F., **1979**. Scorpion toxins: chemistry and mode of action. *Adv. Cytopharmacol. 3*, 325–334.

Rogers, J.C., Qu, Y., Tanda, T.N., Scheuer, T., Catterall, W.A., **1996**. Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3–S4 extracellular loop in the domain IV of the sodium channel α subunit. *J. Biol. Chem. 271*, 15950–15962.

Ruhland, M., Zlotkin, E., Rathmayer, W., **1977**. The effect of toxins from the venom of the scorpion *Androctonus australis* on a spider nerve–muscle preparation. *Toxicon 15* (2), 157–160.

Sampieri, F., Habersetzer-Rochat, C., **1978**. Structure-function relationships in scorpion neurotoxins. Identification of the supperreactive lysine residue in toxin I of *Androctonus australis* Hector. *Biochim. Biophys. Acta 535* (1), 100–109.

Sampieri, F., Habersetzer-Rochat, C., Martin, M.F., Kopeyan, C., Rochat, H., **1987**. Amino acid sequence of toxin XI of the scorpion *Buthus occitanus tunetanus*. Evidence of a mutation having an important effect upon neurotoxic activity. *Int. J. Pept. Prot. Res. 29* (2), 231–237.

Schlein, Y., Spira, D.T., Jacobson, R.L., **1976**. The passage of serum immunoglobulins through the gut of *Sarcophaga faculata* Pand. *Ann. Trop. Med. Parasitol. 70*, 227–230.

Schweitz, H., Vincent, J.P., Barhanin, J., Frelin, C., Linden, G., *et al.*, **1981**. Purification and pharmacological properties of eight sea anemone toxins from *Anemonia sulcata*, *Anthopleura xanthogrammica*, *Stoichactis giganteus*, and *Actinodendron plumosum*. *Biochemistry 20* (18), 5245–5252.

Scott, J., Wheelock, G.D., **1992**. Characterization of the cytochrome p450 responsible for pyrethroid resistance in the housefly. In: Mullin, C.A., Jeffrey, C.S. (Eds.), Molecular Mechanisms of Insecticide Resistance. American Chemical Society, Washington, DC, pp. 16–30.

Selisko, B., Garcia, C., Becerril, B., Delepierre, M., Possani, L.D., **1996**. A new insect selective toxin from *Centruroides noxius* Hoffman: cDNA, primary structure, three dimensional model and electrostatic potentials in comparison to other toxin variants. *Eur. J. Biochem. 242*, 235–242.

Sharkey, R.G., Beneski, D.A., Catterall, W.A., **1984**. Differential labeling of the alpha and beta 1 subunits of the sodium channel by photoreactive derivatives of scorpion toxin. *Biochemistry 23* (25), 6078–6086.

Shichor, I., Zlotkin, E., Ilan, N., Chikashvili, D., Stuhmer, W., *et al.*, **2002**. Domain 2 of *Drosophila* para voltage-gated sodium channel confers insect properties to a rat brain channel. *J. Neurosci. 22* (11), 4364–4371.

Simard, J.M., Meves, H., Watt, D.D., **1986**. Effect of toxins VI and VII from the scorpion *Centruroides sculpturatus* on the sodium currents of the frog Node of Ranvier. *Pflugers Arch. 406*, 620–628.

Simard, J.M., Watt, D.D., **1990**. Venoms and toxins. In: Polis, G. (Ed.), The Biology of Scorpions. Stanford University Press, p. 414.

Sissom, W.D., **1990**. Systematics, biogeography and paleontology. In: Polis, G. (Ed.), The Biology of Scorpions. Stanford University Press, p. 64.

Soderlund, D.M., Knipple, D.C., **1995**. Action of insecticides on sodium channels: multiple target sites and site specific resistance. In: Clark, J.M. (Ed.), Molecular Action of Insecticides on Ion Channels. American Chemical Society, Washington, DC, pp. 97–108.

Stankiewicz, M., Grolleau, F., Lapied, B., Borchani, L., el Ayeb, M., *et al.*, **1996**. BotIT2, a toxin paralytic to insects from the *Buthus occitanus tunetanus* venom modifying the activity of insect sodium channels. *J. Insect Physiol. 42* (4), 397–405.

Stewart, L.M., Hirst, M., Lopez Ferber, M., Merryweather, A.T., Cayley, P.J., *et al.*, **1991**. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature 352* (6330), 85–88.

Stryer, L., **1995**. Biochemistry, 4th edn. Freeman.

Tatebayashi, H., Narahashi, T., **1994**. Differential mechanism of action of the pyrethroid tetramethrin on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J. Pharmacol. Exp. Ther. 270* (2), 595–603.

Taylor, M.F., Heckel, D.G., Brown, T.M., Kreitman, M.E., Black, B., **1993**. Linkage of pyrethroid insecticide resistance to a sodium channel locus in the tobacco budworm. *Insect Biochem. Mol. Biol. 23* (7), 763–775.

Teitelbaum, Z., Lazarovici, P., Zlotkin, E., **1979**. Selective binding of the scorpion venom insect toxin to insect nervous tissue. *Insect Biochem. 9*, 343–346.

Tintpulver, M., Zerachia, T., Zlotkin, E., **1976**. The action of toxins derived from scorpion venom on the ileal smooth muscle preparation. *Toxicon 14* (15), 371–377.

Tomalski, M.D., Miller, L.K., **1991**. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature 352* (6330), 82–85.

Treacy, M.F., Rensner, P.E., All, J.N., **2000**. Comparative insecticidal properties of two nucleopolyhedrovirus vectors encoding a similar toxin gene chimer. *J. Econ. Entomol. 93* (4), 1096–1104.

Tsushima, R.G., Borges, A., Backx, P.H., **1999**. Inactivated state dependence of sodium channel modulation by beta-scorpion toxin. *Pflugers Arch. 437* (5), 661–668.

Turkov, M., Rashi, S., Noam, Z., Gordon, D., Ben Khalifa, R., *et al.*, **1997**. *In vitro* folding and functional analysis of an anti-insect selective scorpion depressant neurotoxin produced in *Escherichia coli*. *Protein Expr. Purif. 10* (1), 123–131.

Vijverberg, H.P., Pauron, D., Lazdunski, M., **1984**. The effect of *Tityus serrulatus* scorpion toxin gamma on Na channels in neuroblastoma cells. *Pflugers Arch. 401* (3), 297–303.

Walther, C., Zlotkin, E., Rathmayer, W., **1976**. Action of different toxins from the scorpion *Androctonus australis* on the locust nerve-muscle preparation. *J. Insect Physiol. 22*, 1187–1194.

Wang, G.K., Strichartz, G., **1982**. Simultaneous modifications of sodium channel gating by two scorpion toxins. *Biophys. J. 40* (2), 175–179.

Wang, G.K., Strichartz, G.R., **1983**. Purification and physiological characterization of neurotoxins from venoms of the scorpions *Centruroides sculpturatus* and *Leiurus quinquestriatus*. *Mol. Pharmacol. 23* (2), 519–533.

Wang, X., Smith, R., Fletcher, J.I., Wilson, H., Wood, C.J., *et al.*, **1999**. Structure-function studies of omega-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. *Eur. J. Biochem. 264* (2), 488–494.

Warmke, J.W., Reenan, R.A., Wang, P., Qian, S., Arena, J.P., *et al.*, **1997**. Functional expression of *Drosophila* para sodium channels. Modulation by the membrane protein TipE and toxin pharmacology. *J. Gen. Physiol. 110* (2), 119–133.

Williamson, M.S., Martinez-Torres, D., Hick, C.A., Devonshire, A.L., **1996**. Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Mol. Gen. Genet.* **252** (1–2), 51–60.

Xiong, Y.M., Ling, M.H., Lan, Z.D., Wang, D.C., Chi, C.W., **1999**. The cDNA sequence of an excitatory insect selective neurotoxin from the scorpion *Buthus martensi* Karsch. *Toxicon 37* (2), 335–341.

Yahel-Niv, A., Zlotkin, E., **1979**. Comparative studies on venom obtained from individual scorpions by natural stings. *Toxicon 17* (5), 435–446.

Yao, B., Fan, Y., Zeng, Q., Zhao, R., **1996**. Insect-resistant tobacco plants expressing insect-specific neurotoxin AaIT. *Chin. J. Biotechnol. 12* (2), 67–72.

Zamudio, F., Saavedra, R., Martin, B.M., Gurrola-Briones, G., Herion, P., *et al.*, **1992**. Amino acid sequence and immunological characterization with monoclonal antibodies of two toxins from the venom of the scorpion *Centruroides noxius* Hoffmann. *Eur. J. Biochem. 204* (1), 281–292.

Zilberberg, N., Gordon, D., Pelhate, M., Adams, M.E., Norris, T.M., *et al.*, **1996**. Functional expression and genetic alteration of an alpha scorpion neurotoxin. *Biochemistry 35* (31), 10215–10222.

Zilberberg, N., Gurevitz, M., **1993**. Rapid isolation of full length cDNA clones by "inverse PCR": purification of a scorpion cDNA family encoding alpha-neurotoxins. *Anal. Biochem. 209* (1), 203–305.

Zilberberg, N., Zlotkin, E., Gurevitz, M., **1991**. The cDNA sequence of a depressant insect selective neurotoxin from the scorpion *Buthotus judaicus*. *Toxicon 29* (9), 1155–1158.

Zlotkin, E., **1983**. Insect selective toxins derived from scorpion venoms: an approach to insect neuropharmacology. *Insect Biochem. 13*, 219–236.

Zlotkin, E., **1986**. The interaction of insect selective neurotoxins from scorpion venoms with insect neuronal membranes. In: Ford, M.G., Lunt, G.G., Reay, R.C., Usherwood, P.N.R. (Eds.), Neuropharmacology and Pesticide Action. Ellis Horwood, Chichester, pp. 352–383.

Zlotkin, E., **1987**. Pharmacology of survival: insect selective neurotoxins derived from scorpion venom. *Endeavour 11* (4), 168–174.

Zlotkin, E., **1991**. Venom neurotoxins – models for selective insecticides. *Phytoparasitica 19*, 177–182.

Zlotkin, E., **1999**. The insect voltage-gated sodium channel as target of insecticides. *Annu. Rev. Entomol. 44*, 429–455.

Zlotkin, E., Devonshire, A. L., Warmke, J. W., **1999**. The pharmacological flexibility of the insect voltage gated sodium channel: toxicity of AaIT to knockdown resistant (kdr) flies. *Insect Biochem. Mol. Biol. 29*, 849–853.

Zlotkin, E., Eitan, M., Bindokas, V.P., Adams, M.E., Moyer, M., *et al.*, **1991**. Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry 30* (19), 4814–4821.

Zlotkin, E., Fishman, L., Shapiro, J.P., **1992**. Oral toxicity to flesh flies of a neurotoxic polypeptide. *Arch. Insect. Biochem. Physiol. 21* (1), 41–52.

Zlotkin, E., Fishman, Y., Elazar, M., **2000**. AaIT: from neurotoxin to insecticide. *Biochimie 82* (9–10), 869–881.

Zlotkin, E., Fraenkel, G., Miranda, F., Lissitzky, S., **1971a**. The effect of scorpion venom on blowfly larvae – a new method for the evaluation of scorpion venoms potency. *Toxicon 9* (1), 1–8.

Zlotkin, E., Gordon, D., **1985**. Detection, purification and receptor-binding assays on insect-selective neurotoxins derived from scorpion venom. In: Breer, H., Miller, T.A. (Eds.), Neurochemical Techniques in Insect Research. Springer, Berlin, pp. 243–295.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M.F., *et al.*, **1985**. An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys. 240* (2), 877–887.

Zlotkin, E., Martinez, H., Rochat, H., Miranda, F., **1975**. A protein toxic to crustacea from the venom of the scorpion *Androctonus australis*. *Insect Biochem. 5*, 243–250.

Zlotkin, E., Milman, T., Sion, G., Werner, Y.L., **2003**. Predatory behaviour of gekkonid lizards, *Ptydactylus* spp., towards the scorpion *Leiurus quinquestriatus*

heraeus and their tolerance of its venom. *J. Nat. Hist. 37*, 641–646.

Zlotkin, E., Miranda, F., Kopeyan, C., Lissitzky, S., **1971b**. A new toxic protein in the venom of the scorpion *Androctonus australis* Hector. *Toxicon 9* (1), 9–13.

Zlotkin, E., Miranda, F., Lissitzky, S., **1972a**. A factor toxic to crustacean in the venom of the scorpion *Androctonus australis* Hector. *Toxicon 10* (3), 211–216.

Zlotkin, E., Miranda, F., Lissitzky, S., **1972b**. Proteins in scorpion venoms toxic to mammals and insects. *Toxicon 10* (3), 207–209.

Zlotkin, E., Miranda, T., Rochat, H., **1978**. Chemistry and pharmacology of Buthidae scorpion venoms. In: Bettini, S. (Ed.), Arthropod Venoms, vol. 48. Springer, Berlin, Heidelberg, NewYork, pp. 317–369.

Zlotkin, E., Rochat, H., Kopeyan, C., Miranda, F., Lissitzky, S., **1971c**. Purification and properties of the insect toxin from the venom of the scorpion *Androctonus australis* Hector. *Biochimie 53* (10), 1073–1078.

Zlotkin, E., Shulov, A., **1969**. A simple device for collecting scorpion venom. *Toxicon 7*, 331–332.

5.7 Spider Toxins and their Potential for Insect Control

F Maggio, B L Sollod, H W Tedford, and G F King, University of Connecticut Health Center, Farmington, CT, USA

5.7.1. Introduction

Insects are the most diverse and successful animals on the planet, with the number of extant species estimated to be ~5 million (Novotny *et al*., 2002). However, the ability of insects to inhabit a wide variety of ecological niches has inevitably brought them into conflict with humans. Although only a small minority of insects are classified as pests, they nevertheless destroy 20–30% of the world's food supply (Oerke, 1994) and transmit a diverse array of human and animal pathogens.

In terms of agronomic importance, lepidopterans are the most pernicious insects, with ~40% of all chemical insecticides directed against heliothine species (Brooks and Hines, 1999). However, from a global human health perspective, mosquitoes are the most problematic arthropods, being responsible for the transmission of malaria, filariasis, and numerous arboviruses. Although malaria is unquestionably the most devastating insect-borne disease, causing an estimated 2 million deaths per year (Gubler, 1998), the increasing incidence of epidemics caused by mosquito-borne arboviruses (e.g., dengue, West Nile, Japanese encephalitis, yellow fever, and Rift Valley fever) is a mounting public health issue (Gubler, 2002). Other insects of significant public health importance include sandflies, tsetse flies, fleas, and triatomid bugs, which transmit the causative agents of leishmaniasis, trypanosomiasis (African sleeping sickness), plague, and Chagas disease, respectively (Gubler, 1998; Gratz, 1999).

5.7.2. Chemical Control of Insect Pests

Humans have been engaged in internecine chemical warfare with insects ever since Paris Green (copper acetoarsenite) was introduced as an insecticide by French grape growers in the 1870s (Winston, 1997; Dent, 2000). However, our chemical assault on insects dramatically intensified with the introduction of dichlorodiphenyltrichloroethane (DDT), the first synthetic organic pesticide, during World War II (Casida and Quistad, 1998). DDT was effective not only in the agricultural sector, but formed the basis of initially successful malaria eradication programs (Attaran *et al*., 2000). The subsequent introduction of organophosphate and carbamate insecticides in the 1960s (Casida and Quistad, 1998) encouraged the prevailing view that chemical insecticides would prove to be a universal remedy for the control of insect pests.

This optimistic outlook was unwarranted for several reasons. First, chemical control subjects the insect population to Darwinian selection and the development of resistance is as inevitable as the evolution of bacterial resistance to overprescribed antibiotics. Moreover, the fact that the vast majority of

insecticides act on one of just four nervous system targets – the voltage-gated sodium channel, the nicotinic acetylcholine receptor (nAChR), the γ-aminobutyric acid (GABA)-gated chloride channel, and acetylcholinesterase – promoted the development of cross-resistance to different families of insecticides (Brogdon and McAllister, 1998). As of 1992, more than 500 species of insects and mites, including 56 anopheline and 39 culicine mosquitoes, had developed resistance to one or more classes of insecticides (Feyereisen, 1995; Brogdon and McAllister, 1998). Second, most chemical insecticides are relatively broad-spectrum, and indiscriminate use can lead to catastrophic environmental, ecological, and human health-related problems. Because these chemicals kill nontarget arthropods, including natural enemies of the targeted pest, there are several instances where insecticide application has exacerbated rather than ameliorated the targeted insect-pest problem (Winston, 1997; Dent, 2000).

The publication in 1962 of Rachel Carson's landmark book *Silent Spring* (Carson, 1962) substantially raised public awareness of the potential adverse impacts of synthetic insecticides and ultimately led to a paradigm shift in the development and use of these chemicals. Deregistration of DDT and other organochlorines in the 1970s in the USA stimulated the agrochemical industry to develop a new generation of insecticides that have increased biological activity (leading to reduced application rates/amounts), enhanced specificity, reduced environmental persistence, and decreased mammalian toxicity (Dent, 2000). Nevertheless, several classes of widely used insecticides will soon be lost from the marketplace due to either resistance development or deregistration by regulatory authorities following retrospective review. Combined with more demanding registration requirements for new agrochemicals, this is likely to decrease the pool of effective chemical insecticides in the near future (Rose, 2001; Zaim and Guillet, 2002). Thus, there is a pressing need to isolate safe insecticidal compounds and to develop viable alternatives to chemical insecticides. In this chapter, the potential of natural insecticidal toxins derived from spider venoms to fuel developments in each of these areas will be reviewed.

5.7.3. Spiders and Their Venoms: A Brief Overview

Excluding insects, which are their primary prey, spiders are the most successful terrestrial invertebrates (Rash and Hodgson, 2002). There are about 38 000 described species (Platnick, 1997), with at least a similar number undescribed. The vast majority belong to the two major suborders Araneomorphae and Mygalomorphae. Araneomorphs (modern spiders) represent ~90% of the world's spiders. The primitive spiders, or mygalomorphs, use their silk in a rudimentary manner compared to modern web-weavers and they prey primarily on nonflying arthropods. This suborder includes most of the large spiders that have inspired popular myths and Hollywood movies, including the tarantulas (theraphosids) and trapdoors, which are both harmless to humans (Escoubas and Rash, 2004; Isbister and White, 2004), and the highly venomous Australian funnel-web spiders (Nicholson and Graudins, 2002; Isbister and White, 2004).

As for other venomous creatures, such as scorpions and cone snails, the venoms of most spiders are a heterogeneous mixture of inorganic salts, low molecular mass organic molecules (<1 kDa), small linear peptides (typically 2–4 kDa), disulfide-reticulated polypeptides (typically 3–8 kDa), and high molecular mass proteins including enzymes (>10 kDa) (Escoubas *et al.*, 2000; Gomez *et al.*, 2002; Rash and Hodgson, 2002; Kuhn-Nentwig *et al.*, 2004; Tedford *et al.*, 2004). These complex chemical cocktails have evolved for the primary purpose of killing or paralyzing arthropod prey, although some compounds may play an additional role in predigestion of the injected victim (Rash and Hodgson, 2002). In the following sections, a brief overview of the insecticidal peptide and polyamine components that have been isolated from spider venoms are provided.

5.7.4. Insecticidal Acylpolyamine Toxins from Spider Venom

Polyamine toxins are low molecular weight secondary metabolites found in the venom glands of spiders and wasps (Strømgaard *et al.*, 2001). They appear to be a common component of both araneomorph (Aramaki *et al.*, 1986; Grishin *et al.*, 1989; Quistad *et al.*, 1991) and mygalomorph (Fish-Fisher and Bohn, 1957; Gilbo and Coles, 1964; Skinner *et al.*, 1990) venoms, where their primary function is to induce rapid paralysis of envenomated prey. Although first identified in spider venoms more than 40 years ago, they were not structurally and functionally characterized until the mid-1980s (Strømgaard *et al.*, 2001; Rash and Hodgson, 2002; Mellor and Usherwood, 2004).

Acylpolyamine toxins typically contain an unbranched polyamine chain composed of two to four secondary amino groups separated from each

other by two to eight methylene groups (Strømgaard *et al*., 2001). One end of the chain usually terminates with a primary amino or guanidino group, while an aromatic moiety (such as a 2,4-dihydroxyphenylacetyl group or an indol-3-acetyl group) is typically attached to the other end via an amide bond (**Figure 1a**). Polyamine toxins are capable of low affinity, nonspecific interactions with a variety of proteins, and as such their pharmacology is complex and not amenable to description with a few generalizations. Although some polyamine toxins from wasp venom antagonize nAChRs, and argiotoxin-636 from the orb-weaving spider *Argiope lobata* inhibits voltage-gated Na^+ and K^+ currents in vertebrate neurons, these toxins are thought to have evolved primarily as antagonists of arthropod ionotropic glutamate receptors (Strømgaard *et al*., 2001; Mellor and Usherwood, 2004). While these toxins have proved to be valuable pharmacological tools, they have never been seriously exploited as insecticide leads, possibly due to undesirable characteristics such as poor phyletic specificity, undesirable pharmacokinetics, and difficulties with synthesis and purification (Strømgaard *et al*., 2001). However, none of these characteristics absolutely rules out a possible success for insecticide discovery strategies that

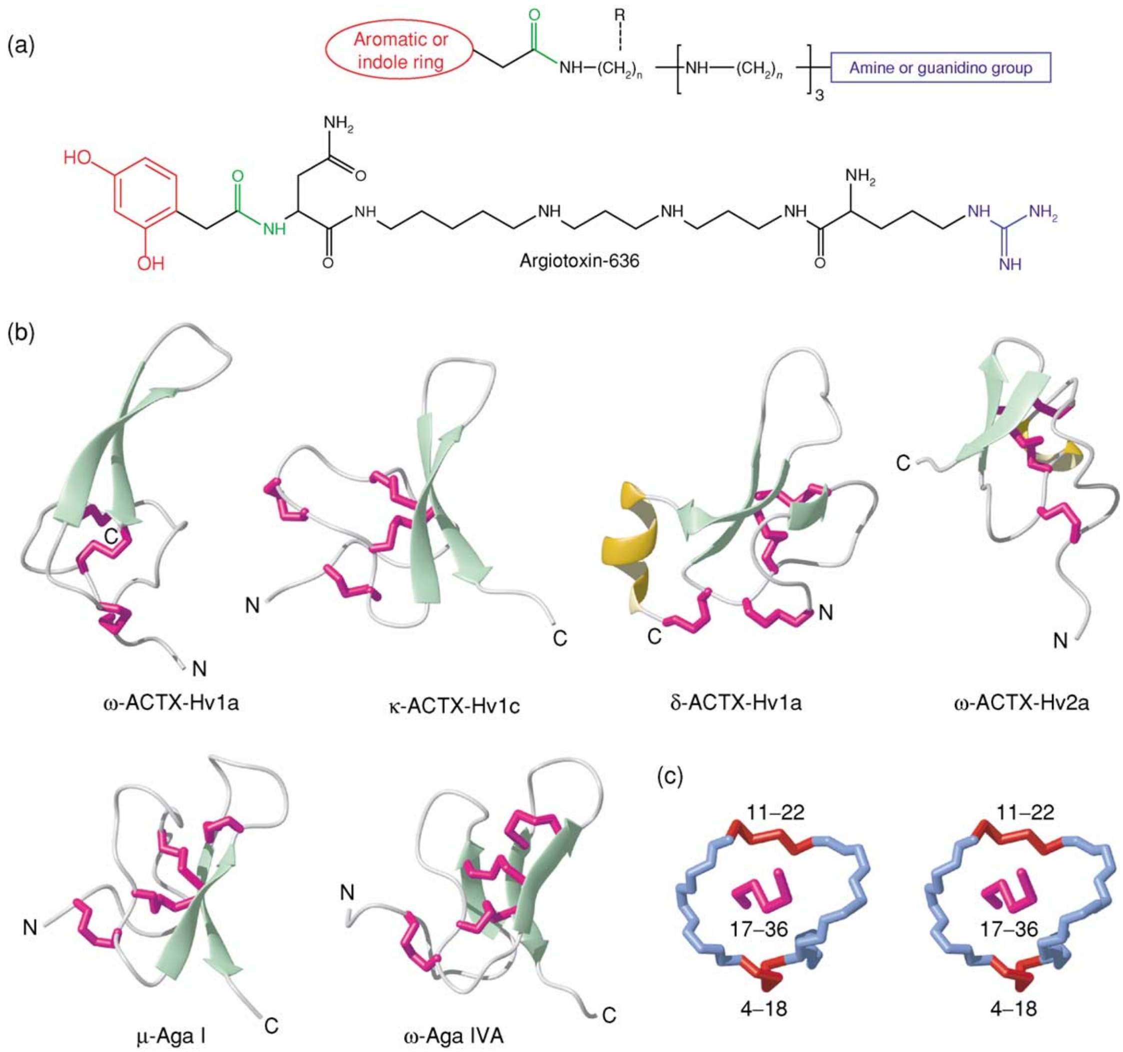


Figure 1 (a) The structure of argiotoxin-636 from the orb-weaving spider *Argiope lobata* below a generalized outline of the structure of spider venom acylpolyamines. (b) Schematic representations of the three-dimensional structure of the only six insecticidal spider toxins whose tertiary structure has been determined. The atracotoxins (ACTX) are from the Australian funnel-web spider *Hadroncyhe versuta* and the agatoxins (Aga) are from the unrelated American funnel-web spider *Agelenopsis aperta*. The β strands and α helices are shown in green and orange, respectively, and disulfide bridges are drawn as magenta tubes. The N- and C-termini of each toxin are labeled. Protein Data Bank accession codes are given in **Table 1**. (c) Stereo view of the cystine knot motif of ω-ACTX-Hv1a. The Cys17–Cys36 disulfide bond (magenta) is threaded through a closed loop formed by the Cys4–Cys18 and Cys11–Cys22 disulfide bridges (red) and the intervening sections of polypeptide backbone (blue).

exploit acylpolyamines either as rational design templates or as tracers in competitive ligand binding assays.

5.7.5. Insecticidal Peptide Toxins from Spider Venom

5.7.5.1. Spider Venom Polypeptides: An Underexploited Pharmacological Reservoir

Peptide toxins are expressed in the venom gland of spiders in a combinatorial fashion, and hence they tend to be the principal venom constituents (Escoubas *et al*., 2000; Escoubas and Rash, 2004; Kuhn-Nentwig *et al*., 2004; Tedford *et al*., 2004) (see Section 5.7.5.2). However, few spider venoms have been studied in sufficient detail to enable the extent and diversity of these peptidic components to be reliably estimated. A recent mass spectrometric analysis of 55 different tarantula venoms led to a conservative estimate of ~50 peptides per venom (Escoubas and Rash, 2004), and a similar level of peptide diversity has been reported in the venoms of the primitive hunting spider *Plectreurys tristis* (Quistad and Skinner, 1994), the Australian funnel-web spider *Hadronyche versuta* (Wang *et al*., 1999; Tedford *et al*., 2004), and the Central American wandering spider *Cupiennius salei* (Kuhn-Nentwig *et al*., 2004). If one assumes a total of ~80 000 spider species, then a conservative estimate of 20 pharmacologically distinct peptides per species leads to an estimated total of ~1.5 million spider venom polypeptides, which is a much larger pharmacological repertoire than the 50 000–100 000 peptides estimated to be present in the venoms of cone snails (Olivera and Cruz, 2001) and scorpions (Possani *et al*., 1999). It was recently estimated, based on available journal and patent literature, that <0.01% of the pharmacological diversity of spider venoms has been tapped thus far (Tedford *et al*., 2004).

Apart from studies of acylpolyamines, most early investigations of spider venoms focused on isolation of polypeptides with vertebrate neurotoxic activity. Some of these peptides, such as the ω-agatoxins (Olivera *et al*., 1994), have become invaluable tools in vertebrate pharmacology because of their selectivity for specific subtypes of vertebrate voltage-gated ion channels. Although genes encoding these peptides might be suitable for engineering recombinant insect viruses (see Section 5.7.6), their broad-spectrum toxicity makes them unsuitable as leads for chemical insecticides. However, the overwhelming majority of spiders are harmless to humans (Isbister and White, 2004), and therefore it is not surprising that appropriately designed venom screens have led to the discovery of insect-specific spider venom polypeptides (Skinner *et al*., 1992; Krapcho *et al*., 1995a, 1995b, 1997; Fletcher *et al*., 1997b; Johnson and Kral, 1997; Johnson *et al*., 1997, 1998; Atkinson *et al*., 1998; Balaji *et al*., 2000; Corzo *et al*., 2000; Wang *et al*., 2000, 2001; de Figueiredo *et al*., 2001; Lipkin *et al*., 2002; Zhang *et al*., 2003).

5.7.5.2. Expression and Evolution of Spider Venom Polypeptides: Rolling the Dice

Toxic polypeptides are found in the venoms of numerous animals, including scorpions, snakes, and cone snails, as well as the nematocysts of sea anemones. While these peptides are invariably expressed as longer precursors that are posttranslationally processed to yield the mature toxin, the nature of the precursor depends on the animal. In the aquatic cone snails and sea anemones, toxic polypeptides are initially translated as prepropeptides that are posttranslationally processed to yield the mature toxin (Olivera *et al*., 1999; Anderluh *et al*., 2000). In contrast, the precursors of most peptide toxins from snakes and scorpions lack a propeptide region (Goudet *et al*., 2002; Fujimi *et al*., 2003). Araneomorph and mygalomorph spiders generally conform to the prepropeptide paradigm (Wang *et al*., 2001; Gomez *et al*., 2002; Corzo *et al*., 2003). The precursor typically comprises an N-terminal signal sequence (16–25 residues) followed by a propeptide region of highly variable length (13–58 residues) which precedes a single downstream copy of the mature toxin sequence. For reasons that have yet to be determined, the propeptide region of most spider toxin precursors is rich in acidic residues (Santos *et al*., 1992; Diniz *et al*., 1993; Krapcho *et al*., 1995b; Wang *et al*., 2001; Liang, 2004; Ostrow *et al*., 2003).

Spider toxins are typically not produced as "one-offs." Rather, each toxin is generally expressed in the form of a small library of variants that can differ significantly or by as little as one amino acid in the mature toxin sequence (Skinner *et al*., 1989, 1992; Stapleton *et al*., 1990; Quistad and Skinner, 1994; Krapcho *et al*., 1995b; Sanguinetti *et al*., 1997; Johnson *et al*., 1998; Kalapothakis *et al*., 1998; Wang *et al*., 1999, 2000; Corzo *et al*., 2000, 2003; Lipkin *et al*., 2002; Kuhn-Nentwig *et al*., 2004; Liang, 2004). Recent comprehensive analyses of venom gland cDNA libraries from several species of Australian funnel-web spider indicate that a single species typically expresses three to ten variants of each peptide toxin (Sollod *et al*., 2003). Interspecies comparisons reveal that the mature toxin region has been poorly conserved during speciation whereas the signal sequences of the variants are nearly identical. This dichotomy

between a highly conserved signal sequence and highly variable mature toxin sequence suggests that spiders, like cone snails (Duda and Palumbi, 1999; Olivera *et al.*, 1999; Conticello *et al.*, 2001), have diversified their toxin pool by developing a mechanism for accelerated evolution (hypermutation) of the mature toxin region relative to the signal sequence. Remarkably, the cysteine residues, which are critical for determining the tertiary structure of the toxins, are strictly conserved within the otherwise hypervariable mature toxin region.

Thus, spiders are master combinatorial chemists; for hundreds of millions of years they have been synthesizing combinatorial peptide libraries using various cystine frameworks as a structural scaffold. Hypermutation of the mature toxin sequence has facilitated the evolution and optimization of toxins with new functionalities, while maintenance in the venom of a minilibrary of toxin variants presumably enables the spider to target slightly altered versions of the same receptor in different insects, or even differentially edited/spliced versions of the same receptor within a single insect.

5.7.5.3. Three-Dimensional Structure of Spider Venom Polypeptides: A Tale of Ropes and Knots

While it is difficult to make structural generalizations about spider venom polypeptides based on the small number of toxins that have been studied, some trends are apparent. The peptides that have been isolated thus far can be grouped into three categories:

1. Linear amphipathic peptides, typically of mass 2–4 kDa, with no disulfide bonds.
2. Medium-sized neurotoxic peptides, typically of mass 3–6 kDa and reticulated with three to five disulfide bonds.
3. Larger polypeptides (6–9 kDa) with four or more disulfide bonds.

There are few characterizations of the highly reticulated peptides with mass >6 kDa, and no three-dimensional structures have been determined for any member of this group. An extensive mass spectrometric analysis of 55 tarantula venoms (see figure 2b in Escoubas and Rash, 2004), as well as less detailed mass spectrometric analyses of Australian funnel-web spiders (Wang, 2000; Wilson, Wilson, 2001), suggests that the medium-sized neurotoxic peptides predominate in mygalomorph venoms. However, venom from the araneomorph *C. salei* appears to be markedly different: small amphipathic peptides appear to be the principal constituents of this venom, which also contains a higher proportion of high-mass peptides and less of the medium-sized neurotoxic peptides than mygalomorph venoms (Kuhn-Nentwig *et al.*, 2004).

The small linear peptides expressed in spider venoms have antimicrobial activity and in this respect they are similar to the cecropin-like peptides that are found in the hemolymph of numerous arthropods, where they form part of the innate immune system that protects against invasion by pathogenic organisms (Boman, 2003). However, the peptides found in the venoms of spiders and other invertebrates, including bees, wasps, ants, and scorpions, differ from the cecropins in that their antimicrobial activity appears to be a consequence of relatively nonspecific cytolytic activity against prokaryotic and eukaryotic cells. While these peptides appear to form amphipathic α helices in membrane-mimetic solvents or when bound to biological membranes, they exist in flexible "rope-like" conformations in the absence of lipid bilayers (Corzo *et al.*, 2001, 2002; Kuhn-Nentwig *et al.*, 2004). Some of these peptides are neurotoxic to insects, but their primary function appears to be to synergize the neurotoxic activity of the larger disulfide-reticulated peptides by optimizing their access to the insect nervous system (Corzo *et al.*, 2002; Kuhn-Nentwig, 2003; Kuhn-Nentwig *et al.*, 2004). The broad-spectrum activity of the linear cytolytic peptides makes them unsuitable as insecticide leads, although it has been suggested that genes encoding these toxins might synergize with neurotoxin transgenes in enhancing the efficacy of insect-specific viruses (Corzo *et al.*, 2002; Kuhn-Nentwig, 2003).

The majority of insect-specific toxins isolated from spider venom are medium-sized neurotoxic polypeptides (typically 30–50 amino acid residues) with three to five disulfide bonds (summarized in **Table 1**). Most of these toxins act presynaptically at insect neuromuscular junctions, either by enhancing or inhibiting neurotransmitter release, although several act at sites within the insect central nervous system (Johnson *et al.*, 1998; Bloomquist, 2003; Tedford *et al.*, 2004). Three-dimensional structures have been determined for six of these toxins (**Figure 1b**), and they all contain a structural motif known as the inhibitor cystine knot (ICK) (Pallaghy *et al.*, 1994; Norton and Pallaghy, 1998; Craik *et al.*, 2001). The cystine knot comprises a ring formed by two disulfides and the intervening sections of polypeptide backbone, with a third disulfide piercing the ring to create a pseudo-knot (see **Figure 1c**). Except for the special case of cyclic ICK peptides, cystine knots are not true topological knots since, at least theoretically, they can be untied by a nonbond-breaking geometrical transformation (Craik *et al.*, 2001). Nevertheless, the cystine knot

Table 1 Overview of neurotoxic insecticidal peptides isolated from spider venom

Spider	*Name of toxin*[a]	*Number of residues*	*Number of disulfide bonds*	*Molecular target*[b]	*Discovery reference*	*Insect selective?*	*Three-dimensional fold*[c]	*Structure reference*	*Insectophore mapped?*
Agelenopsis aperta	μ-Aga I[d]	36	4	VGSC	1	Yes/No	1EIT	2	No
	ω-Aga IA	66 + 3[e]	5	VGCC	3	No	No		No
	ω-Aga IIA	?[f]	?[f]	VGCC	3	No	No		No
	ω-Aga IVA	48	4	VGCC	4	No	1OAW	5	No
Aptostichus schlingeri	Aptotoxin I	74	4	Unknown	6	Unknown	No		No
	Aptotoxin III	38	4	Unknown	6	Unknown	No		No
	Aptotoxin VII	33	3	Unknown	6	Unknown	No		No
Calisoga sp.	Peptide A	39	4	Unknown	7	Yes	No		No
Coremiocnemis validus	Covalitoxin-II	31	3	Unknown	8	Yes	No		No
Cupiennius salei	CSTX-1	74	4	VGCC	9	No	No		No
Diguetia canities	DTX 9.2	56	4	VGSC	10, 11	Yes	No		No
Hadronyche versuta	ω-ACTX-Hv1a	37	3	VGCC	12	Yes	1AXH	12	Yes[g]
	ω-ACTX-Hv2a	45	3	VGCC	13	Yes	1G9P	13	No
	κ-ACTX-Hv1c	37	4	K^+ channel	14, 15	Yes	1DL0	14	Yes[h]
	δ-ACTX-Hv1a[i]	42	4	VGSC	16, 17	No	1VTX	18	No
Macrotheles gigas	Magi-2	40	3	VGSC	19	Yes	No		No
	Magi-3	46	5	VGSC	19	Yes	No		No
	Magi-5	29	3	VGSC	19	No	No		No
	Magi-6	36	4	Unknown	19	No	No		No
Oxyopes kitabensis	Oxytoxin 1	69	5	VGSC	20	Yes	No		No
Phoneutria nigriventer	Tx4(6-1)	48	5	VGSC	21, 22	Yes	No		No
Plectreurys tristis	Plectoxin II	44[j]	5	VGCC	23	Unknown	No		No
	Plectoxin V	46	5	Unknown	24	Unknown	No		No
	Plectoxin IX	46	4	Unknown	24	Unknown	No		No
	Plectoxin X	49	5	Unknown	24	Unknown	No		No
Segestria florentina	SFI1[k]	46	4	Unknown	25	Unknown	No		No
Selenocosmia huwena[l]	Huwentoxin-V	35	3	Unknown	26	Yes	No		No
Tegenaria agrestis	TaITX-1	50	3	Unknown CNS target	27	Yes	No		No

[a]If a venom contains several variants of a toxin, only the protoypic family member is listed.
[b]Abbreviations used: VGSC, voltage-gated sodium channel; VGCC, voltage-gated calcium channel; CNS, central nervous system.
[c]The Protein Data Bank accession number is given for toxins whose three-dimensional structure has been determined.
[d]*Agelenopsis aperta* contains several variants of this toxin (μ-Aga II to VI) (Skinner *et al.*, 1989). Similar toxins are found in the venom of the related American funnel-web spider *Hololena curta* (Stapleton *et al.*, 1990) and *Paracoeletes luctuosus* (Corzo *et al.*, 2000). Some of these toxins appear to be insect-selective, while others are neurotoxic to mice (Corzo *et al.*, 2000).

[e]This toxin is a heterodimer in which 66- and 3-residue peptides are linked by an intermolecular disulfide bond (Santos *et al.*, 1992).
[f]This 11 kDa toxin has never been fully sequenced (Adams *et al.*, 1990).
[g]Tedford *et al.* (2001, 2004).
[h]Maggio and King (2002).
[i]A similar toxin (Magi 4) is found in the venom of *Macrotheles gigas* (Corzo *et al.*, 2003).
[j]The C-terminal Thr residue in PLTX-II is amidated and *O*-palmitoylated (Branton *et al.*, 1993).
[k]A toxin with similar N-terminal sequence was isolated from *Segestria* sp. many years earlier and shown to be insect-selective, but the complete amino acid sequence was not reported (Johnson and Kral, 1997).
[l]Reclassified as *Ornithoctonus huwena* (Wang) in a recent taxonomic revision.
[1]Adams *et al.* (1989).
[2]Omecinsky *et al.* (1996).
[3]Adams *et al.* (1990).
[4]Mintz *et al.* (1992).
[5]Kim *et al.* (1995).
[6]Skinner *et al.* (1992).
[7]Johnson *et al.* (1997).
[8]Balaji *et al.* (2000).
[9]Kuhn-Nentwig *et al.* (2004).
[10]Krapcho *et al.* (1995b).
[11]Bloomquist *et al.* (1996).
[12]Fletcher *et al.* (1997b).
[13]Wang *et al.* (2001).
[14]Wang *et al.* (2000).
[15]Gunning, Maggio, King, and Nicholson (unpublished data).
[16]Brown *et al.* (1988).
[17]Nicholson *et al.* (1996).
[18]Fletcher *et al.* (1997a).
[19]Corzo *et al.* (2003).
[20]Corzo *et al.* (2002).
[21]Figueiredo *et al.* (1995).
[22]de Lima *et al.* (2002).
[23]Branton *et al.* (1993).
[24]Quistad and Skinner (1994).
[25]Lipkin *et al.* (2002).
[26]Zhang *et al.* (2003).
[27]Johnson *et al.* (1998).

provides peptidic neurotoxins with tremendous stability and resistance to proteases, thereby ensuring that the injected toxin is not degraded before reaching its nervous system target.

5.7.6. Toxin-Based Target Validation and Screening

Surprisingly, the molecular target of about 50% of the neurotoxins listed in **Table 1** has not been determined. However, it is striking that all spider venom-derived peptide neurotoxins with known molecular targets are directed against ion channels. Ion channels are well-validated insecticide targets (Bloomquist, 1996), with the vast majority of synthetic insecticides acting on voltage-gated sodium channels (the target of DDT, pyrethroids, oxadiazines, dihydropyrazoles, veratrum alkaloids, and *N*-alkylamides), nicotinic acetylcholine receptors (the target of neonicotinoids and spinosyns), or GABA/glutamate-gated chloride channels (the target of dieldrin, lindane, endosulfan, avermectins, and phenylpyrazoles). Of the major insecticide classes, only the organophosphate and carbamate insecticides (which target acetylcholinesterase) are not directed against this group of ligand- and voltage-gated ion channels.

Clearly, in order to minimize the potential for cross-resistance, it would be highly advantageous for new insecticides to act on targets outside of this established ion channel triumvirate. While the majority of well-characterized spider toxins appear to act on voltage-gated sodium channels (VGSCs), the overutilization of this target makes these toxins the least interesting in terms of developing high-throughput screens (HTSs) for small-molecule insecticide leads. It should be noted, however, that at least some of these toxins (e.g., δ-ACTX-Hv1a (Nicholson *et al*., 1996) and Magi 2, 3, and 5 (Corzo *et al*., 2003)) bind VGSCs at sites distinct from current-generation insecticides. Thus, the high penetrance in many insect populations of alleles conferring resistance to certain VGSC insecticides may not compromise newly developed chemical control agents that utilize different sodium channel binding sites (Bloomquist, 1996). In fact, insects with these resistance alleles might have increased susceptibility to such chemicals since insects with pyrethroid-resistance alleles are significantly more susceptible to both *N*-alkylamides and scorpion toxins that bind VGSCs at sites different from the pyrethroids (Zlotkin *et al*., 2000).

It is apparent from **Table 1** that many of the most potent spider venom toxins target neuronal voltage-gated calcium channels (VGCCs). This is not particularly surprising since insect VGCCs play a critical role in modulating cellular excitability and neurotransmitter release, and severe loss-of-function mutations in *Drosophila melanogaster* genes encoding the pore-forming α_1 subunits of both the L-type (*Dmca1D*) and P/Q/N-type (*Dmca1A*) VGCCs are embryonic lethal (Eberl *et al*., 1998; Smith *et al*., 1998; Kawasaki *et al*., 2002) (note that the designation of L- and P/Q/N-type channels is based solely on gene homologies with vertebrate VGCCs, not electrophysiological or pharmacological criteria). It is therefore remarkable that no commercial insecticides have ever been targeted against VGCCs. The lack of phyletic specificity of the ω-agatoxins possibly encouraged the misconception that insect VGCCs could not be selectively targeted. However, the highly insect-selective ω-atracotoxins (King *et al*., 2002; Tedford *et al*., 2004) demonstrate that such specificity is indeed possible. Hence, as suggested previously (Hall *et al*., 1994), insect VGCCs appear to be excellent HTS targets. The major impediment to implementing such HTSs will be development of heterologous expression systems for the relevant insect VGCCs. Thus far, there are no examples in the scientific or patent literature of heterologous expression of a functional arthropod VGCC.

An exciting recent development is the observation that the κ-atracotoxins (previously known as the Janus-faced atracotoxins) (Wang *et al*., 2000; King *et al*., 2002; Maggio and King, 2002) are selective for insect K^+ channels (Tedford *et al*., 2004). As far as we are aware, no synthetic chemical insecticides have ever been developed that target K^+ channels. Isolation of a lethal invertebrate-specific K^+ channel blocker was somewhat unexpected, since all loss-of-function K^+ channel alleles in *Drosophila* are viable (Wicher *et al*., 2001). Nevertheless, induction of a κ-ACTX-Hv1c transgene in *D. melanogaster* for just 10–15 min induces a lethal phenotype (Maggio, King, and Reenan, unpublished data), which strongly validates this ion channel as a viable insecticide target. In contrast with insect VGCCs, heterologous expression systems are available for numerous invertebrate K^+ channels (Wicher *et al*., 2001), and many HTS formats have been developed for K^+ channel targets (Kirsch *et al*., 2001; Ford *et al*., 2002).

An important consideration in implementing high-throughput insecticide screens with these ion channels is that one would like to mimic the phyletic specificity as well as the potency of the toxin used to validate the target. This contrasts with pharmaceutical development, where one can simply look for potent channel blockers (or activators) without regard for phyletic specificity. Ion channels are typically highly promiscuous with respect to available binding

sites, as exemplified by VGSCs which display at least nine topologically distinct binding sites for toxins and insecticides (Zlotkin *et al*., 2000). Thus, a screen designed to simply select for potent channel blockers has a high probability of yielding phyletically indiscriminate lead compounds that bind the channel at a location distinct from the binding site for the toxin used to validate the target. The probability of selecting lead compounds that are insect-selective is likely to be significantly increased if the primary screen is developed to identify molecules that compete with the toxin for its specific channel binding site; alternatively, a noncompetitive primary screen could be followed by a secondary screen that selects for such competitors.

5.7.7. Toxin-Based Bioinsecticides: A New Era of Biological Control

5.7.7.1. A Brief History of Conventional Biological Control Methods

Biological control methods were introduced well before the advent of synthetic insecticides, and in many instances were extremely successful. Indeed, the first use of biological control in North America was a spectacular success (Caltagirone and Doutt, 1989; Winston, 1997). In the 1880s, a massive outbreak of the accidentally introduced cottony-cushion scale (*Icerya purchasi*) threatened to decimate the infant Californian citrus industry. Over the winter of 1888–89, at a cost of only $1500, the US Department of Agriculture released the Australian ladybird beetle, *Rodolia cardinalis*, a voracious and specific predator of the cottony-cushion scale. Within a matter of months, the problematic scale was completely controlled within the introduced areas. Unfortunately, the introduction of broad-spectrum synthetic insecticides in the 1940s substantially diminished the success rate in subsequent predator/parasite release programs, largely because the introduced predators and parasites were just as susceptible to insecticides as the targeted pest insect. Ironically, cottony-cushion scale reappeared as a problem in California in the 1940s when citrus growers began experimenting with DDT and other insecticides that failed to discriminate between *Rodolia* and scale insects (Winston, 1997).

Another biological control method that has been used with variable success is the release of sterile insects. In this approach, male insects are sterilized, usually by irradiation, and released in large excess over the native male population. A textbook case was the total eradication of screwworm, a major pest of goats, from the Venezuelan island of Curaçao in the 1950s (Winston, 1997). However, Curaçao is small and well separated from the mainland and neighbouring islands, and very few sterile insect release (SIR) programs have worked when applied to less ecologically isolated areas. In part, this is because the cost of traditional SIR programs are astronomical compared with predator release programs due to the massive rearing facilities that need to be built and maintained to enable sterile males to be produced and released in requisite excess over the native population (Winston, 1997).

Other biological control methods include the release of insect pathogens (especially viruses, which are discussed in more detail below) (see Section 5.7.7.2 and **Chapters 6.8–6.10**), insect pheromones, and entomopathogenic fungi (reviews: Hall and Menn, 1999; Copping and Menn, 2000) (see **Chapter 6.11**).

5.7.7.2. Biological Control in the New Millennium

The genomics era has ushered in new methods of biological control. We now have the capacity to genetically manipulate insects and insect pathogens to have desired traits (Atkinson, 2002) (see **Chapter 5.12**). The potential benefits for insect release programs have been reviewed recently (Handler, 2002; Benedict and Robinson, 2003). The ability to engineer male sterility by genetic methods should increase success in future SIR programs by substantially improving the fitness level of released males. Moreover, genetic sexing techniques (Robinson, 2002) should enable facile elimination of females from the release population, which will enhance SIR by increasing the number of matings between sterile males and wild females.

An even more exciting development is the possibility of releasing insects carrying either a conditional lethal trait or female-killing allele (Handler, 2002). Both of these techniques, under optimal conditions, should be far superior to traditional SIR (Schliekelman and Gould, 2000a, 2000b). A conditional lethal trait that has been tested in *Drosophila*, but which has the advantage of being applicable to virtually all insect species that can be genetically manipulated, is temperature-sensitive alleles of genes encoding either diphtheria or ricin toxins. The insects are engineered to express only the A subunit of the toxin; in the absence of the B subunit that normally allows transmembrane movement, toxicity is cell-specific, thus preventing damage to predators. In principle, genes encoding insect-specific spider toxins could be used in a similar manner. It has been shown that the ω- and κ-atracotoxins can be expressed under heat shock control in *Drosophila*; insects are phenotypically normal at 18 °C but a heat shock to 37 °C

for just 10–15 min kills all flies (Tedford, Maggio, King, and Reenan, unpublished data). Since the toxins are not orally active, insects bearing these alleles would pose no threat to predatory organisms.

An alternative to SIR and parasite/predator release is incorporation of a transgene encoding a specific insecticidal component into the genome of plants or insect-specific pathogens. One recently introduced, and thus far highly successful, approach is the planting of transgenic crops that express insecticidal toxins, such as engineered corn and cotton crops that express δ-endotoxins from the soil bacterium *Bacillus thuringiensis* (Peferoen, 1997; Jenkins, 1999) (see **Chapter 6.6**). In addition to minimizing effects on nontarget organisms, *Bt* plants have generated increases in crop yields and significantly reduced insecticide use on many farms (Huang *et al*., 2002; Pray *et al*., 2002; Toenniessen *et al*., 2003). However, this technology has several potential drawbacks. First, constitutive expression of an insect toxin in transgenic plants is likely to expedite the development of resistance (Ferré and Van Rie, 2002; Morin *et al*., 2003), thus limiting the long-term benefit of the toxin transgene and compromising the effectiveness of *Bt* spores used widely by organic farmers. Second, some consumers are uncomfortable with, or philosophically opposed to, the introduction of nonplant proteins into their food (for a discussion of the emotive "Frankenfood" pejorative, see Dale (1999a, 1999b)). An additional concern associated with this technology is the possibility of transgene introgression into wild relatives of the engineered plant (Arnaud *et al*., 2003).

Bt endotoxins are uniquely suited to incorporation into transgenic plants as their activation is dependent upon the alkaline environment found in the gut of many insects, which ensures that these toxins are not active in humans and other vertebrates. In principle, the remarkable phyletic specificity of many spider-derived peptide neurotoxins makes them ideal for incorporation into transgenic plants. It was recently shown, for example, that rice transformed with a transgene encoding an insecticidal spider toxin was significantly less susceptible to infestation by the rice leaf-folder *Cnaphalocrocis medinalis* and striped stemborer *Chilo suppressalis* (Huang *et al*., 2001). However, most spider-derived polypeptide neurotoxins are not active when fed to insects, although it is possible that oral activity could be engineered by cofeeding with an agent that increases insect gut permeability, such as cytolytic peptides or even *Bt* endotoxins.

An alternative bioinsecticide strategy that is more specific, and which obviates the problem of introducing a foreign protein into the food supply, is the release of insect-specific viruses. Although there are many different types of insect viruses, all studies of this technology to date have focused on arthropod-specific baculoviruses, partly because of their proven inability to infect vertebrates (Gröner, 1986; Black *et al*., 1997; Kost and Condreay, 2002; Herniou *et al*., 2003). For most members of the nucleopolyhedrovirus (NPV) subgroup, infectivity is restricted to a few closely related lepidopterans, although some viruses only replicate efficiently in a single species (Black *et al*., 1997; Federici, 1999) (see **Chapter 6.8**). Thus, toxin exposure is limited to the targeted insect, while inculpable populations that are susceptible to chemical pesticides, including predators/parasites of the targeted insect, are unaffected.

Wild-type NPVs have become a key component of integrated pest management programs in several countries. They are used extensively in Brazil to protect soybean crops from the velvetbean caterpillar *Anticarsia gemmatalis* (Moscardi, 1999), and in China to safeguard cotton from the highly insecticide-resistant *Helicoverpa armigera* bollworm (Sun *et al*., 2002). In most situations, however, wild-type NPVs are not competitive with chemical pesticides because they typically take 5–10 days to kill their hosts, during which time the insect continues to feed and cause crop damage (Bonning and Hammock, 1996; Black *et al*., 1997). This shortcoming has been addressed by engineering recombinant baculoviruses that express heterologous insecticidal neurotoxins (Cory *et al*., 1994; Treacy, 1999; Inceoglu *et al*., 2001). Introduction of transgenes encoding peptidic neurotoxins from mites (Tomalski and Miller, 1991; Popham *et al*., 1997), scorpions (McCutchen *et al*., 1991; Stewart *et al*., 1991; Gershburg *et al*., 1998; Sun *et al*., 2002), spiders (Prikhod'ko *et al*., 1996, 1998; Hughes *et al*., 1997), and sea anemones (Prikhod'ko *et al*., 1996, 1998) have all been demonstrated to reduce the time interval between virus application and cessation of feeding or death (**Figure 2**). Importantly, introduction of a toxin transgene does not appear to alter the intrinsic infectivity of the virus or its natural host range (Black *et al*., 1997). (For more in depth discussion of naturally occurring and genetically modified baculoviruses see **Chapters 6.8** and **6.9**.)

Few attempts have been made to enhance the insecticidal efficacy of baculoviruses by incorporation of spider toxin transgenes. This is rather surprising since, as outlined in **Figure 2**, the most dramatic improvement obtained thus far in the insecticidal activity of *Autographa californica* NPV (AcNPV) resulted from incorporation of a transgene encoding μ-agatoxin IV from the American funnel-web spider

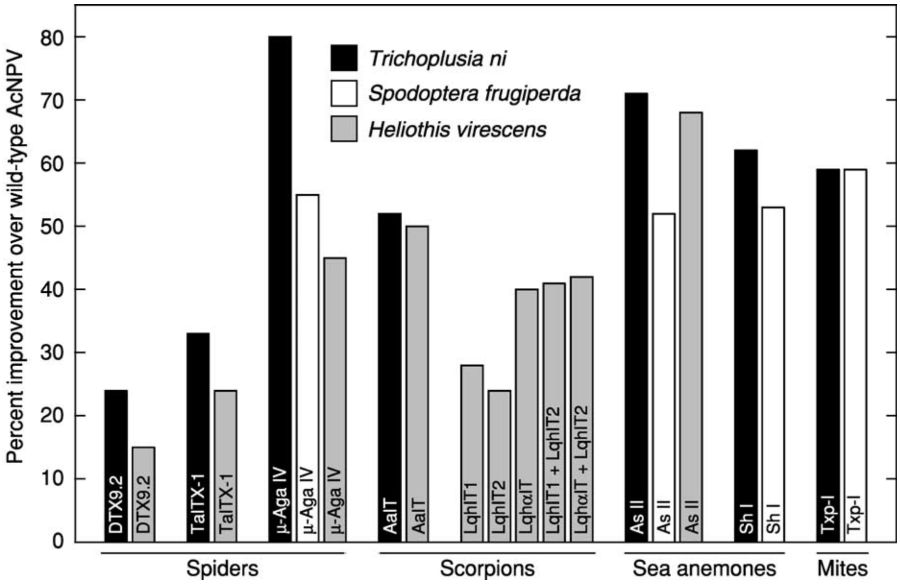


Figure 2 Histogram displaying the percent improvement (relative to wild-type virus) in the time required for 50% of a test population to be killed/paralyzed after feeding on *Autographa californica* nucleopolyhedrosis virus (AcNPV) engineered to contain transgenes encoding various arachnid or sea anemone toxins. It was difficult to make valid comparisons between studies because of differences in the manner of transgene insertion, choice of promoter driving the transgene, toxin signal sequence, and method of bioassay. Thus, this comparison is restricted to studies that used similar AcNPV constructs and which tested the virus on tobacco budworm (*Heliothis virescens*, light gray bars), fall armyworm (*Spodoptera frugiperd*a, white bars), or cabbage looper (*Trichoplusia ni*, black bars). In studies where a number of different constructs of the same transgene were tested, the construct that gave the best improvement over wild-type virus is reported here. Toxins are grouped taxonomically and their names are indicated on the histogram. (Data are from Lu, A., Seshagiri, S., Miller, L.K., **1996**. Signal sequence and promoter effects on the efficacy of toxin-expressing baculoviruses as biopesticides. *Biol. Control 7*, 320–332; Hughes, P.R., Wood, H.A., Breen, J.P., Simpson, S.F., Duggan, A.J., *et al.*, **1997**. Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. *J. Invertebr. Pathol. 69*, 112–118; Popham, H.J.R., Prikhod'ko, G.G., Felcetto, T.J., Ostlind, D.A., Warmke, J.W., *et al.*, **1998**. Effect of deltamethrin treatment on lepidopteran larvae infected with baculoviruses expressing insect-selective toxins μ-Aga-IV, As II, or Sh I. *Biol. Control 12*, 79–87; Prikhod'ko, G.G., Popham, H.J.R., Felcetto, T.J., Ostlind, D.A., Warren, V.A., *et al.*, **1998**. Effects of simultaneous expression of two sodium channel toxin genes on the properties of baculoviruses as biopesticides. *Biol. Control 12*, 66–78; and Regev, A., Rivkin, H., Inceoglu, B., Gershburg, E., Hammock, B.D., *et al.*, **2003**. Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively. *FEBS Lett. 537*, 106–110.)

Agelenopsis aperta. However, a complicating factor in comparing outcomes from different studies (apart from different bioassay strategies, as discussed in Treacy (1999)) is that the efficiency with which a particular toxin is expressed and folded in virus-infected insects is generally not determined. A highly potent insecticidal neurotoxin could be ineffective as a baculoviral transgene because the toxin fails to be properly processed and/or folded in virus-infected insects. This is a critical issue because all of the arachnid and sea anemone toxins used to enhance baculoviral efficacy contain three to seven disulfide bonds, and the number of possible disulfide isomers increases dramatically as the number of cysteine residues increases; for example, six cysteines can be paired in 15 different ways to form three disulfide bonds, whereas eight cysteines can be covalently linked in 105 different ways to form four disulfide bridges. Thus, if a toxin with four disulfide bonds folded in a completely stochastic manner, the yield of correctly folded toxin would be <1%. In this respect, it is worth noting that heterologous expression systems have not been developed for any of the spider toxin genes previously used to generate recombinant baculoviruses, and hence it is unknown whether significant amounts of properly folded, bioactive spider toxin are produced in virus-infected insects.

The ω- and κ-atracotoxins appear to be excellent prospects for engineering viral bioinsecticides. They are the only insect-specific spider toxins for which heterologous expression systems have been developed (Tedford *et al.*, 2001; Maggio and King, 2002), they fold efficiently even in the absence of cellular folding enzymes (Wang *et al.*, 2000, 2001; Tedford *et al.*, 2001), and bioactive toxin is expressed when transgenes encoding these toxins are introduced into *D. melanogaster* (Tedford, Maggio, King, and Reenan, unpublished data). Our preliminary studies indicate that AcNPV expressing either ω-ACTX-Hv1a or κ-ACTX-Hv1c transgenes kills *Heliothis*

virescens more quickly than wild-type virus; moreover, potency can be significantly improved by expressing both toxins simultaneously in the pest insect (Maggio, Mukherjee, and King, unpublished data). Coexpression of synergistic toxin combinations (Herrmann *et al.*, 1995; Prikhod'ko *et al.*, 1998) appears to be a promising avenue for future investigations since it was also recently noted that baculoviral efficacy could be improved by synergistic expression of excitatory and depressant scorpion neurotoxins (Regev *et al.*, 2003).

Viruses that target disease vectors have also been isolated, including NPVs specific for the mosquito genera *Aedes*, *Anopheles*, and *Culex* (Becnel *et al.*, 2001; Moser *et al.*, 2001). Until recently, none of these NPVs had been characterized at the molecular level. However, the genome sequence was recently determined for an exotic baculovirus specific for *Culex* mosquitoes (CuniNPV), which are key vectors of St. Louis encephalitis, Eastern equine encephalitis, and West Nile virus in the United States (Afonso *et al.*, 2001). This work opens up the possibility of incorporating spider toxin transgenes into CuniNPV and related mosquito viruses in order to improve their efficacy.

5.7.8. Spider Toxins as Templates for Mimetic Design

In general, polypeptides are not good drugs because of their poor pharmacokinetic properties (Latham, 1999). Linear peptides are typically not even good drug leads because their interconversion between a plethora of different solution conformations makes it difficult to elucidate meaningful structure–activity relationships (Kieber-Emmons *et al.*, 1997). In contrast, conformationally constrained peptides and peptidomimetic libraries have emerged as valuable tools in the drug discovery process. The cystine knot toxins found in spider venoms are more akin to these libraries with one exception: the spider toxins come preoptimized for the desired target. Cystine knot toxins essentially comprise a robust structural scaffold (the cystine framework) onto which four loops are tethered, each bounded by half-cystines (Tedford *et al.*, 2001). As outlined above (see Section 5.7.5.2), spiders have been performing combinatorial chemistry with this framework on an evolutionary timescale, randomly changing residues in the intercystine loops and selecting those peptides that are most effective in helping to kill or paralyze their insect prey.

Thus, in principle, the long evolutionary history of ICK spider toxins should ensure that they are better leads than those resulting from screens of peptide or peptidomimetic libraries. Why then have they not been utilized for rational insecticide design? The answer is obvious from a quick glance at **Table 1**. For rational design, one needs to know not just the three-dimensional structure of the toxin, but also the spatial arrangement of residues that are critical for interaction with the toxin target. Although dozens of peptide toxins have been isolated from spider venoms over the past 15 years, the target binding site, or "insectophore," has only been elucidated for ω-ACTX-Hv1a (Tedford *et al.*, 2001, 2004) and κ-ACTX-Hv1c (Maggio and King, 2002), and these insectophores were only mapped in the past 2 years.

Elucidation of the target binding sites for ω-ACTX-Hv1a (**Figure 3a**) and κ-ACTX-Hv1c (**Figure 3b**) provides an opportunity for rational design of small molecule mimetics based on the mapped insectophores, a process refered to as "insectophore cloning." Several recent examples have provided encouraging validation of this concept (review: Norton *et al.*, 2003). Particularly relevant are three recent studies that involved cloning the pharmacophores of peptide toxins that block vertebrate calcium or potassium channels (Menzler *et al.*, 2000; Baell *et al.*, 2001, 2002). In each case, an attempt was made to rationally design nonpeptidic analogs that topologically mimicked the functional moieties corresponding to three key amino acid hits from mutagenesis studies. In the most successful study, an attempt was made to clone the pharmacophore of the cone snail ICK peptide ω-conotoxin MVIIA, a specific blocker of vertebrate N-type VGCCs and a lead for the development of novel analgesics. An alkylphenyl backbone was decorated with chemical groups corresponding to the key functional residues Arg10, Leu11, and Tyr13 (**Figure 3c**). Even though other ω-conotoxin MVIIA residues are involved in the toxin–channel interaction, the rationally designed mimics nevertheless had IC_{50} values of ~3 μM against human N-type VGCCs (Menzler *et al.*, 2000).

Thus, it should be possible to clone topologically restricted spider toxin insectophores to produce nonpeptidic lead compounds with low micromolar potency against the desired insect targets. What remains to be determined is whether a rationally designed mimic can maintain the desired phyletic specificity of the parent toxin. An obvious caveat is that residues deemed not to be functionally important according to mutagenesis studies might nevertheless provide a steric impediment to binding to the vertebrate counterpart of the targeted insect channel. Failure to take this into account might lead to phyletically indiscriminate mimics.

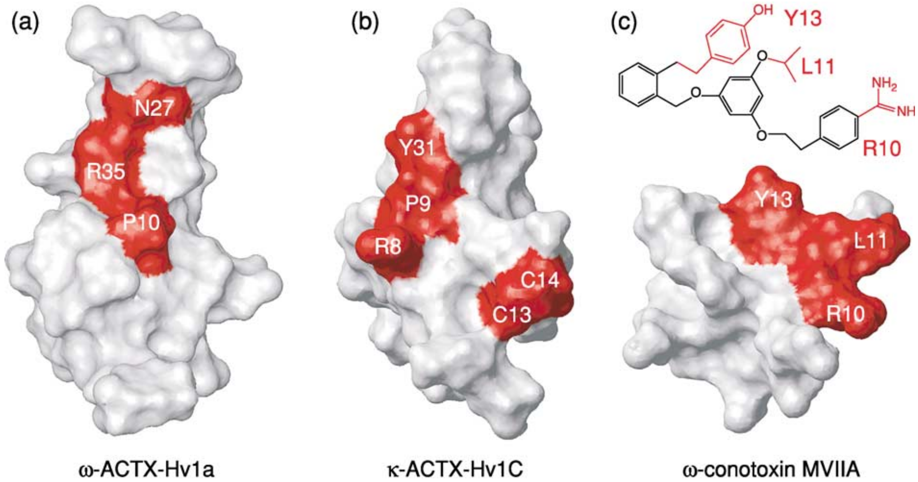


Figure 3 Molecular surface representation of (a) ω-ACTX-Hv1a (Protein Data Bank file 1AXH), (b) κ-ACTX-Hv1c (Protein Data Bank file 1DL0), and (c) ω-conotoxin MVIIA (Protein Data Bank file 1OMG) showing the channel binding surfaces (red) as mapped by mutagenesis studies (Nadasdi *et al.*, 1995; Tedford *et al.*, 2001, 2004; Maggio and King, 2002). Also shown in (c) is a rationally designed mimetic of the ω-conotoxin MVIIA pharmacophore, which blocks cloned human N-type VGCCs with $IC_{50} \sim 3\,\mu M$ (Menzler *et al.*, 2000).

5.7.9. Future Directions

Following a decade in which research on spider venoms was focused primarily on vertebrate-active compounds, there has been an acceleration of interest in recent years in the isolation and characterization of insect-specific toxins. So far, however, this immense reservoir of insecticidal compounds has been sparsely sampled, and it can be expected that many more insect-specific spider toxins will be isolated over the next few years. What remains to be seen is whether this basic research can be translated into viable insect control methods. In too few cases has there been a concerted effort to uncover the molecular target of these toxins, determine their three-dimensional structure, and elucidate detailed structure–function relationships. It is striking that the target binding site has been mapped in detail for only two spider toxins. Nevertheless, with appropriate focus on these aspects, it is likely that peptidic neurotoxins from spider venoms will facilitate discovery of new insecticide targets and provide molecular templates for rational design of novel chemical insecticides that act at these sites. Moreover, it should be possible to employ these spider toxins as bioinsecticides by incorporating genes encoding these toxins into insect-specific viruses, or by using such genes as conditional lethal alleles in transgenic insects.

Acknowledgments

We would like to acknowledge our many collaborators, especially Roger Drinkwater, Simon Gunning, Graham Nicholson, Robert Reenan, and David Wilson for allowing us to discuss unpublished collaborative results. Thanks to Susan Rowland and Scott Chouinard for critical appraisal of the manuscript, and to US National Science Foundation and the NIH National Institute of Allergy and Infectious Diseases for financial support.

References

Adams, M.E., Herold, E.E., Venema, V.J., **1989**. Two classes of channel-specific toxins from funnel web spider venom. *J. Comp. Physiol. A 164*, 333–342.

Adams, M.E., Bindokas, V.P., Hasegawa, L., Venema, V.J., **1990**. ω-Agatoxins: novel calcium channel antagonists of two subtypes from funnel web spider (*Agelenopsis aperta*) venom. *J. Biol. Chem. 265*, 861–867.

Afonso, C.L., Tulman, E.R., Lu, Z., Balinsky, C.A., Moser, B.A., *et al.*, **2001**. Genome sequence of a baculovirus pathogenic for *Culex nigripalpus*. *J. Virol. 75*, 11157–11165.

Anderluh, G., Podlesek, Z., Macek, P., **2000**. A common motif in proparts of cnidarian toxins and nematocyst collagens and its putative role. *Biochim. Biophys. Acta 1476*, 372–376.

Aramaki, Y., Yasuhara, T., Higashijima, T., Yoshioka, M., Miwa, A., *et al.*, **1986**. Chemical characterization of spider toxin, JSTX and NSTX. *Proc. Jpn. Acad. Ser. B 62*, 359–362.

Arnaud, J.F., Viard, F., Delescluse, M., Cuguen, J., **2003**. Evidence for gene flow via seed dispersal from crop to wild relatives in *Beta vulgaris* (Chenopodiaceae): consequences for the release of genetically modified crop species with weedy lineages. *Proc. Roy. Soc. B 270*, 1565–1571.

Atkinson, P.W., **2002**. Genetic engineering in insects of agricultural importance. *Insect Biochem. Mol. Biol. 32*, 1237–1242.

Atkinson, R.K., Howden, M.E.H., Tyler, M.I., Vonarx, E.J., **1998**. Insecticidal toxins derived from funnel web (*Atrax* or *Hadronyche*) spiders. US Patent No. 5 763 568.

Attaran, A., Roberts, D.R., Curtis, C.F., Kilama, W.L., **2000**. Balancing risks on the backs of the poor. *Nature Med. 6*, 729–731.

Baell, J.B., Forsyth, S.A., Gable, R.W., Norton, R.S., Mulder, R.J., **2001**. Design and Synthesis of Type-III Mimetics of ω-Conotoxin GVIA. *J. Comput. Aided Mol. Des. 15*, 1119–1136.

Baell, J.B., Harvey, A.J., Norton, R.S., **2002**. Design and synthesis of Type-III mimetics of ShK toxin. *J. Comput. Aided Mol. Des. 16*, 245–262.

Balaji, R.A., Sasaki, T., Gopalakrishnakone, P., Sato, K., Kini, R.M., *et al.*, **2000**. Purification, structure determination and synthesis of covalitoxin-II, a short insect-specific neurotoxic peptide from the venom of the *Coremiocnemis validus* (Singapore Tarantula). *FEBS Lett. 474*, 208–212.

Becnel, J.J., White, S.E., Moser, B.A., Fukuda, T., Rotstsein, M.J., *et al.*, **2001**. Epizootiology and transmission of a newly discovered baculovirus from the mosquitoes *Culex nigripalpus* and *C. quinquefasciatus*. *J. Gen. Virol. 82*, 275–282.

Benedict, M.Q., Robinson, A.S., **2003**. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. *Trends Parasitol. 19*, 349–355.

Black, B.C., Brennan, L.A., Dierks, P.M., Gard, I.E., **1997**. Commercialization of baculoviral insecticides. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 341–387.

Bloomquist, J.R., **1996**. Ion channels as targets for insecticides. *Annu. Rev. Entomol. 41*, 163–190.

Bloomquist, J.R., **2003**. Mode of action of atracotoxin at central and peripheral synapses of insects. *Invertebr. Neurosci. 5*, 45–50.

Bloomquist, J.R., Kinne, L.P., Deutsch, V., Simpson, S.F., **1996**. Mode of action of an insecticidal peptide toxin from the venom of a weaving spider (*Diguetia canities*). *Toxicon 34*, 1072–1075.

Boman, H.G., **2003**. Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med. 254*, 197–215.

Bonning, B.C., Hammock, B.D., **1996**. Development of recombinant baculoviruses for insect control. *Annu. Rev. Entomol. 41*, 191–210.

Branton, W.D., Rudnick, M.S., Zhou, Y., Eccleston, E.D., Fields, G.B., *et al.*, **1993**. Fatty acylated toxin structure. *Nature 365*, 496–497.

Brogdon, W.G., McAllister, J.C., **1998**. Insecticide resistance and vector control. *Emerg. Infect. Dis. 4*, 605–613.

Brooks, E., Hines, E., **1999**. Viral biopesticides for heliothine control: fact or fiction? *Today's Life Sci. January/February*, 38–44.

Brown, M.R., Sheumack, D.D., Tyler, M.I., Howden, M.E.H., **1988**. Amino acid sequence of versutoxin, a lethal neurotoxin from the venom of the funnel-web spider *Atrax versutus*. *Biochem. J. 250*, 401–405.

Caltagirone, L.E., Doutt, R.L., **1989**. The history of the *Vedalia* beetle importation to California and its impact on the development of biological control. *Annu. Rev. Entomol. 34*, 1–6.

Carson, R., **1962**. Silent Spring. Houghton Mifflin, Boston, MA.

Casida, J.E., Quistad, G.B., **1998**. Golden age of insecticide research: past, present, or future? *Annu. Rev. Entomol. 43*, 1–16.

Conticello, S.G., Gilad, Y., Avidan, N., Ben-Asher, E., Levy, Z., *et al.*, **2001**. Mechanisms for evolving hypervariability: the case of conopeptides. *Mol. Biol. Evol. 18*, 120–131.

Copping, L.G., Menn, J.J., **2000**. Biopesticides: a review of their action, applications and efficacy. *Pest Mgt. Sci. 56*, 651–676.

Cory, J.S., Hirst, M.L., Williams, T., Hails, R.S., Goulson, D., *et al.*, **1994**. Field trial of a genetically improved baculovirus insecticide. *Nature 370*, 138–140.

Corzo, G., Escoubas, P., Stankiewicz, M., Pelhate, M., Kristensen, C.P., *et al.*, **2000**. Isolation, synthesis and pharmacological characterization of δ-palutoxins IT, novel insecticidal toxins from the spider *Paracoelotes luctuosus* (Amaurobiidae). *Eur. J. Biochem. 267*, 5783–5795.

Corzo, G., Escoubas, P., Villegas, E., Barnham, K.J., He, W., *et al.*, **2001**. Characterization of unique amphipathic antimicrobial peptides from venom of the scorpion *Pandinus imperator. Biochem. J. 359*, 35–45.

Corzo, G., Gilles, N., Satake, H., Villegas, E., Dai, L., *et al.*, **2003**. Distinct primary structures of the major peptide toxins from the venom of the spider *Macrothele gigas* that bind to sites 3 and 4 in the sodium channel. *FEBS Lett. 547*, 43–50.

Corzo, G., Villegas, E., Gomez-Lagunas, F., Possani, L.D., Belokoneva, O.S., *et al.*, **2002**. Oxyopinins, large amphipathic peptides isolated from the venom of the wolf spider *Oxyopes kitabensis* with cytolytic properties and positive insecticidal cooperativity with spider neurotoxins. *J. Biol. Chem. 277*, 23627–23637.

Craik, D.J., Daly, N.L., Waine, C., **2001**. The cystine knot motif in toxins and implications for drug design. *Toxicon 39*, 43–60.

Dale, P.J., **1999a**. Public reactions and scientific responses to transgenic crops. *Curr. Opin. Biotechnol. 10*, 203–208.

Dale, P.J., **1999b**. Public concerns over transgenic crops. *Genome Res. 9*, 1159–1162.

de Figueiredo, S.G., de Lima, M.E., Cordeiro, M.N., Diniz, C.R., Patten, D., *et al.*, **2001**. Purification and amino acid sequence of a highly insecticidal toxin from the venom of the brazilian spider *Phoneutria nigriventer* which inhibits NMDA-evoked currents in rat hippocampal neurones. *Toxicon 39*, 309–317.

de Lima, M.E., Stankiewicz, M., Hamon, A., de Figueiredo, S.G., Cordeiro, M.N., *et al.*, **2002**. The toxin Tx4(6-1) from the spider *Phoneutria nigriventer* slows down Na^+

current inactivation in insect CNS via binding to receptor Site 3. *J. Insect Physiol. 48*, 53–61.

Dent, D., **2000**. Insect Pest Management. CAB International, Wallingford.

Diniz, M.R., Paine, M.J., Diniz, C.R., Theakston, R.D., Crampton, J.M., **1993**. Sequence of the cDNA coding for the lethal neurotoxin Tx1 from the Brazilian "armed" spider *Phoneutria nigriventer* predicts the synthesis and processing of a preprotoxin. *J. Biol. Chem. 268*, 15340–15342.

Duda, T.F., Jr., Palumbi, S.R., **1999**. Developmental shifts and species selection in gastropods. *Proc. Natl Acad. Sci. USA 96*, 10272–10277.

Eberl, D.F., Ren, D., Feng, G., Lorenz, L.J., Vactor, D.V., *et al.*, **1998**. Genetic and developmental characterization of *Dmca1D*, a calcium channel α_1 subunit gene in *Drosophila melanogaster. Genetics 148*, 1159–1169.

Escoubas, P., Diochot, S., Corzo, G., **2000**. Structure and pharmacology of spider venom neurotoxins. *Biochimie 82*, 893–907.

Escoubas, P., Rash, L., **2004**. Tarantulas: eight-legged pharmacists and combinatorial chemists. *Toxicon 43*, 555–574.

Federici, B.A., **1999**. Naturally occurring baculoviruses for insect pest control. In: Hall, F.R., Menn, J.J. (Eds.), Biopesticides: Use and Delivery. Humana Press, Totowa, NJ, pp. 301–320.

Ferré, J., Van Rie, J., **2002**. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol. 47*, 501–533.

Feyereisen, R., **1995**. Molecular biology of insecticide resistance. *Toxicol. Lett. 82–83*, 83–90.

Figueiredo, S.G., Garcia, M.E., Valentim, A.C., Cordeiro, M.N., Diniz, C.R., *et al.*, **1995**. Purification and amino acid sequence of the insecticidal neurotoxin Tx4(6-1) from the venom of the "armed" spider *Phoneutria nigriventer* (Keys). *Toxicon 33*, 83–93.

Fisher, F.G., Bohn, H., **1957**. Die Giftsekrete der Volgenspinnen. *Liebigs Ann. Chem. 603*, 232–250.

Fletcher, J.I., Chapman, B.E., Mackay, J.P., Howden, M.E.H., King, G.F., **1997a**. The structure of versutoxin (δ-atracotoxin-Hv1) provides insights into the binding of site 3 neurotoxins to the voltage-gated sodium channel. *Structure 5*, 1525–1535.

Fletcher, J.I., Smith, R., O'Donoghue, S.I., Nilges, M., Connor, M., *et al.*, **1997b**. The structure of a novel insecticidal neurotoxin, ω-atracotoxin-Hv1, from the venom of an Australian funnel web spider. *Nature Struct. Biol. 4*, 559–566.

Ford, J.W., Stevens, E.B., Treherne, J.M., Packer, J., Bushfield, M., **2002**. Potassium channels: gene family, therapeutic relevance, high-throughput screening technologies and drug discovery. *Progr. Drug Res. 58*, 133–168.

Fujimi, T.J., Nakajyo, T., Nishimura, E., Ogura, E., Tsuchiya, T., *et al.*, **2003**. Molecular evolution and diversification of snake toxin genes, revealed by analysis of intron sequences. *Gene 313*, 111–118.

Gershburg, E., Stockholm, D., Froy, O., Rashi, S., Gurevitz, M., *et al.*, **1998**. Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. *FEBS Lett. 422*, 132–136.

Gilbo, C.M., Coles, N.W., **1964**. An investigation of certain components of the venom of the female Sydney funnel web spider *Atrax robustus* Cambr. *Austr. J. Biol. Sci. 17*, 758–763.

Gomez, M.V., Kalapothakis, E., Guatimosim, C., Prado, M.A.M., **2002**. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. *Cell. Mol. Neurobiol. 22*, 579–588.

Goudet, C., Chi, C.W., Tytgat, J., **2002**. An overview of toxins and genes from the venom of the Asian scorpion *Buthus martensi* Karsch. *Toxicon 40*, 1239–1258.

Gratz, N.G., **1999**. Emerging and resurging vector-borne diseases. *Annu. Rev. Entomol. 44*, 51–75.

Grishin, E.V., Volkova, T.M., Arseniev, A.S., **1989**. Isolation and structure analysis of components from venom of the spider *Argiope lobata*. *Toxicon 27*, 541–549.

Gröner, A., **1986**. Specificity and safety of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses. CRC Press, Boca Raton, FL, pp. 177–202.

Gubler, D.J., **1998**. Resurgent vector-borne diseases as a global health problem. *Emerg. Infect. Dis. 4*, 442–450.

Gubler, D.J., **2002**. The global emergence/resurgence of arboviral diseases as public health problems. *Arch. Med. Res. 33*, 330–342.

Hall, F.R., Menn, J.J., **1999**. Biopesticides: Use and Delivery. Humana Press, Totowa, NJ.

Hall, L.M., Ren, D., Feng, G., Eberl, D.F., Dubald, M., *et al.*, **1994**. The calcium channel as a new potential target for insecticides. In: Clark, J.M. (Ed.), Molecular Action of Insecticides on Ion Channels. American Chemical Society, Washington, DC, pp. 162–172.

Handler, A.M., **2002**. Prospects for using genetic transformation for improved SIT and new biocontrol methods. *Genetica 116*, 137–149.

Herniou, E.A., Olszewski, J.A., Cory, J.S., O'Reilly, D.R., **2003**. The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol. 48*, 211–234.

Herrmann, R., Moskowitz, H., Zlotkin, E., Hammock, B.D., **1995**. Positive cooperativity among insecticidal scorpion neurotoxins. *Toxicon 33*, 1099–1102.

Huang, J., Rozelle, S., Pray, C., Wang, Q., **2002**. Plant biotechnology in China. *Science 295*, 674–676.

Huang, J.Q., Wel, Z.M., An, H.L., Zhu, Y.X., **2001**. *Agrobacterium tumefaciens*-mediated transformation of rice with the spider insecticidal gene conferring resistance to leaffolder and striped stem borer. *Cell Res. 11*, 149–155.

Hughes, P.R., Wood, H.A., Breen, J.P., Simpson, S.F., Duggan, A.J., *et al.*, **1997**. Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. *J. Invertebr. Pathol. 69*, 112–118.

Inceoglu, A.B., Kamita, S.G., Hinton, A.C., Huang, Q., Severson, T.F., *et al.*, **2001**. Recombinant baculoviruses for insect control. *Pest. Mgt. Sci. 57*, 981–987.

Isbister, G.K., White, J., **2004**. Clinical consequences of spider bite: recent advances in our understanding. *Toxicon 43*, 477–492.

Jenkins, J.N., **1999**. Transgenic plants expressing toxins from *Bacillus thuringiensis*. In: Hall, F.R., Menn, F.R. (Eds.), Biopesticides: Use and Delivery. Humana Press, Totowa, NJ, pp. 211–232.

Johnson, J.H., Bloomquist, J.R., Krapcho, K.J., Kral, R.M., Trovalto, R., *et al.*, **1998**. Novel insecticidal peptides from *Tegenaria agrestis* spider venom may have a direct effect on the insect central nervous system. *Arch. Insect Biochem. Physiol. 38*, 19–31.

Johnson, J.H., Kral, R.M., Jr., **1997**. Insecticidal peptides from *Segestria* sp. spider venom. US Patent No. 6 674 846.

Johnson, J.H., Kral, R.M., Jr., Krapcho, K. **1997**. Insecticidal peptides from spider venom. US Patent No. 5 688 764.

Kalapothakis, E., Penaforte, C.L., Beirão, P.S.L., Romano-Silva, M.A., Cruz, J.S., *et al.*, **1998**. Cloning of cDNAs encoding neurotoxic peptides from the spider *Phoneutria nigriventer. Toxicon 36*, 1843–1850.

Kawasaki, F., Collins, S.C., Ordway, R.W., **2002**. Synaptic calcium-channel function in *Drosophila*: analysis and transformation rescue of temperature-sensitive paralytic and lethal mutations of *cacophony. J. Neurosci. 22*, 5856–5864.

Kieber-Emmons, T., Murali, R., Greene, M.I., **1997**. Therapeutic peptides and peptidomimetics. *Curr. Opin. Biotechnol. 8*, 435–441.

Kim, J.I., Konishi, S., Iwai, H., Kohno, T., Gouda, H., *et al.*, **1995**. Three-dimensional solution structure of the calcium channel antagonist ω-agatoxin IVA: consensus molecular folding of calcium channel blockers. *J. Mol. Biol. 250*, 659–671.

King, G.F., Tedford, H.W., Maggio, F., **2002**. Structure and function of insecticidal neurotoxins from Australian funnel-web spiders. *J. Toxicol. Toxin Rev. 21*, 359–389.

Kirsch, D.R., Heinrich, J.N., Pausch, M.H., Silverman, S., Baumbach, W.M.L., *et al.*, **2001**. Molecular genetic screen design for agricultural and pharmaceutical product discovery. In: Seethala, R., Fernandes, P.B. (Eds.), Handbook of Drug Screening. Dekker, New York, pp. 153–188.

Kost, T.A., Condreay, J.P., **2002**. Recombinant baculoviruses as mammalian cell gene-delivery vectors. *Trends Biotechnol. 20*, 173–180.

Krapcho, K.J., Jackson, J.R.H., Johnson, J.H., DelMar, E.G., Kral, R.M., Jr., **1995a**. Insecticidally effective peptides. US Patent No. 5 441 934.

Krapcho, K.J., Jackson, J.R.H., VanWagenen, B.C., Kral, R.M., Jr., **1997**. Insecticidally effective peptides. US Patent No. 5 658 781.

Krapcho, K.J., Kral, R.M., Jr., VanWagenen, B.C., Eppler, K.G., Morgan, T.K., **1995b**. Characterization and cloning of insecticidal peptides from the primitive weaving spider *Diguetia canities*. *Insect Biochem. Mol. Biol. 25*, 991–1000.

Kuhn-Nentwig, L., **2003**. Antimicrobial and cytolytic peptides of venomous arthropods. *Cell. Mol. Life Sci. 60*, 2651–2668.

Kuhn-Nentwig, L., Schaller, J., Nentwig, W., **2004**. Biochemistry, toxicology and ecology of the venom of the spider *Cupiennius Salei* (Ctenidae). *Toxicon 43*, 543–553.

Latham, P.W., **1999**. Therapeutic peptides revisited. *Nature Biotechnol. 17*, 755–757.

Liang, S., **2004**. An overview of peptide toxins from the venom of the Chinese bird spider *Selenocosmia huwena* Wang [*Ornithoctonus huwena* (Wang)]. *Toxicon 43*, 575–585.

Lipkin, A., Kozlov, S., Nosyreva, E., Blake, A., Windass, J.D., *et al.*, **2002**. Novel insecticidal toxins from the venom of the spider *Segestria florentina*. *Toxicon 40*, 125–130.

Lu, A., Seshagiri, S., Miller, L.K., **1996**. Signal sequence and promoter effects on the efficacy of toxin-expressing baculoviruses as biopesticides. *Biol. Control 7*, 320–332.

Maggio, F., King, G.F., **2002**. Scanning mutagenesis of a Janus-faced atracotoxin reveals a bipartite surface patch that is essential for neurotoxic function. *J. Biol. Chem. 277*, 22806–22813.

McCutchen, B.F., Choudary, P.V., Crenshaw, R., Maddox, D., Kamita, S.G., *et al.*, **1991**. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Biotechnology 9*, 848–852.

Mellor, I.R., Usherwood, P.N.R., **2004**. Targeting ionotropic receptors with polyamine-containing toxins. *Toxicon 43*, 493–508.

Menzler, S., Bikker, J.A., Suman-Chauhan, N., Horwell, D.C., **2000**. Design and biological evaluation of non-peptide analogues of ω-conotoxin *MVIIA*. *Bioorg. Med. Chem. Lett. 10*, 345–347.

Mintz, I.M., Venema, V.J., Swiderek, K.M., Lee, T.D., Bean, B.P., *et al.*, **1992**. P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature 355*, 827–829.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., *et al.*, **2003**. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc. Natl Acad. Sci. USA 100*, 5004–5009.

Moscardi, F., **1999**. Assessment of the application of baculoviruses for control of Lepidoptera. *Annu. Rev. Entomol. 44*, 257–289.

Moser, B., Becnel, J., White, S., Afonso, C., Kutish, G., *et al.*, **2001**. Morphological and molecular evidence that *Culex nigripalpus* baculovirus is an unusual member of the family Baculoviridae. *J. Gen. Virol. 82*, 283–297.

Nadasdi, L., Yamashiro, D., Chung, D., Tarczy-Hornoch, K., Adriaenssens, P., *et al.*, **1995**. Structure–activity analysis of a *Conus* peptide blocker of N-type neuronal calcium channels. *Biochemistry 34*, 8076–8081.

Nicholson, G.M., Graudins, A., **2002**. Spiders of medical importance in the Asia–Pacific: atracotoxin, latrotoxin,

and related spider neurotoxins. *Clin. Exp. Pharmacol. Physiol. 29*, 785–794.

Nicholson, G.M., Little, M.J., Tyler, M., Narahashi, T., **1996**. Selective alteration of sodium channel gating by Australian funnel-web spider toxins. *Toxicon 34*, 1443–1453.

Norton, R.S., Baell, J.B., Angus, J.A., **2003**. Calcium channel blocking polypeptides: structure, function, and molecular mimicry. In: McDonough, S.I. (Ed.), Calcium Channel Pharmacology. Kluwer, Amsterdam, pp. 143–179.

Norton, R.S., Pallaghy, P.K., **1998**. The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon 36*, 1573–1583.

Novotny, V., Basset, Y., Miller, S.E., Weiblen, G.D., Bremer, B., *et al.*, **2002**. Low host specificity of herbivorous insects in a tropical forest. *Nature 416*, 841–844.

Oerke, E.-C., **1994**. Estimated crop losses due to pathogens, animal pests and weeds. In: Oerke, E.-C., Dehne, H.-W., Schönbeck, F., Weber, A. (Eds.), Crop Production and Crop Protection: Estimated Losses in Major Food and Cash Crops. Elsevier, Amsterdam, pp. 72–78.

Olivera, B.M., Cruz, L.J., **2001**. Conotoxins, in retrospect. *Toxicon 39*, 7–14.

Olivera, B.M., Miljanich, G.P., Ramachandran, J., Adams, M.E., **1994**. Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. *Annu. Rev. Biochem. 63*, 823–867.

Olivera, B.M., Walker, C., Cartier, G.E., Hooper, D., Santos, A.D., *et al.*, **1999**. Speciation of cone snails and interspecific hyperdivergence of their venom peptides: potential evolutionary significance of introns. *Ann. New York Acad. Sci. 870*, 223–237.

Omecinsky, D.O., Holub, K.E., Adams, M.E., Reily, M.D., **1996**. Three-dimensional structure analysis of μ-agatoxins: further evidence for common motifs among neurotoxins with diverse ion channel specificities. *Biochemistry 35*, 2836–2844.

Ostrow, K.L., Mammoser, A., Suchyna, T., Sachs, F., Oswald, R., *et al.*, **2003**. cDNA sequence and *in vitro* folding of GsMTx4, a specific peptide inhibitor of mechanosensitive channels. *Toxicon 42*, 263–274.

Pallaghy, P.K., Nielsen, K.J., Craik, D.J., Norton, R.S., **1994**. A common structural motif incorporating a cystine knot and a triple-stranded β-sheet in toxic and inhibitory polypeptides. *Protein Sci. 3*, 1833–1839.

Peferoen, M., **1997**. Progress and prospects for field use of *Bt* genes in crops. *Trends Biotechnol. 15*, 173–177.

Platnick, N.I., **1997**. Advances in Spider Taxonomy, 1992–1995: With Redescriptions 1940–1980. New York Entomological Society and The American Museum of Natural History, New York. (Updated online version available at http://research.amnh.org/entomology/spiders/catalog81-87/).

Popham, H.J.R., Li, Y., Miller, L.K., **1997**. Genetic improvement of a *Helicoverpa zea* nuclear polyhedrosis virus as a biopesticide. *Biol. Control 10*, 83–91.

Popham, H.J.R., Prikhod'ko, G.G., Felcetto, T.J., Ostlind, D.A., Warmke, J.W., *et al.*, **1998**. Effect of deltamethrin treatment on lepidopteran larvae infected with baculoviruses expressing insect-selective toxins μ-Aga-IV, As II, or Sh I. *Biol. Control 12*, 79–87.

Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^+ channels. *Eur. J. Biochem. 264*, 287–300.

Pray, C.E., Huang, J., Hu, R., Rozelle, S., **2002**. Five years of *Bt* cotton in china – the benefits continue. *Plant J. 31*, 423–430.

Prikhod'ko, G.G., Popham, H.J.R., Felcetto, T.J., Ostlind, D.A., Warren, V.A., *et al.*, **1998**. Effects of simultaneous expression of two sodium channel toxin genes on the properties of baculoviruses as biopesticides. *Biol. Control 12*, 66–78.

Prikhod'ko, G.G., Robson, M., Warmke, J.W., Cohen, C.J., Smith, M.M., *et al.*, **1996**. Properties of three baculovirus-expressing genes that encode insect-selective toxins: μ-Aga-IV, As II, and Sh I. *Biol. Control 7*, 236–244.

Quistad, G.B., Reuter, C.C., Skinner, W.S., Dennis, P.A., Suwanrumpha, S., *et al.*, **1991**. Paralytic and insecticidal toxins from the funnel web spider, *Hololena curta*. *Toxicon 29*, 329–336.

Quistad, G.B., Skinner, W.S., **1994**. Isolation and sequencing of insecticidal peptides from the primitive hunting spider, *Plectreurys tristis* (Simon). *J. Biol. Chem. 269*, 11098–11101.

Rash, L.D., Hodgson, W.C., **2002**. Pharmacology and biochemistry of spider venoms. *Toxicon 40*, 225–254.

Regev, A., Rivkin, H., Inceoglu, B., Gershburg, E., Hammock, B.D., *et al.*, **2003**. Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively. *FEBS Lett. 537*, 106–110.

Robinson, A.S., **2002**. Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes. *Genetica 116*, 5–13.

Rose, R.I., **2001**. Pesticides and public health: integrated methods of mosquito management. *Emerg. Infect. Dis. 7*, 17–23.

Sanguinetti, M.C., Johnson, J.H., Hammerland, L.G., Kelbaugh, P.R., Volkmann, R.A., *et al.*, **1997**. Heteropodatoxins: peptides isolated from spider venom that block $K_v4.2$ potassium channels. *Mol. Pharmacol. 51*, 491–498.

Santos, A.D., Imperial, J.S., Chaudhary, T., Beavis, R.C., Chait, B.T., *et al.*, **1992**. Heterodimeric structure of the spider toxin ω-Agatoxin IA revealed by precursor analysis and mass spectrometry. *J. Biol. Chem. 267*, 20701–20705.

Schliekelman, P., Gould, F., **2000a**. Pest control by the release of insects carrying a female-killing allele on multiple loci. *J. Econ. Entomol. 93*, 1566–1579.

Schliekelman, P., Gould, F., **2000b**. Pest control by the introduction of a conditional lethal trait on multiple loci: potential, limitations, and optimal strategies. *J. Econ. Entomol. 93*, 1543–1565.

Skinner, W.S., Adams, M.E., Quistad, G.B., Kataoka, H., Cesarin, B.J., *et al.*, **1989**. Purification and characterization of two classes of neurotoxins from the funnel

web spider, *Agelenopsis aperta*. *J. Biol. Chem. 264*, 2150–2155.

Skinner, W.S., Dennis, P.A., Lui, A., Carney, R.L., Quistad, G.B., **1990**. Chemical characterization of acylpolyamine toxins from venom of a trap-door spider and two tarantulas. *Toxicon 28*, 541–546.

Skinner, W.S., Dennis, P.A., Li, J.P., Quistad, G.B., **1992**. Identification of insecticidal peptides from venom of the trap-door spider, *Aptostichus schlingeri* (Ctenizidae). *Toxicon 30*, 1043–1050.

Smith, L.A., Peixoto, A.A., Kramer, E.M., Villella, A., Hall, J.C., **1998**. Courtship and visual defects of *cacophony* mutants reveal functional complexity of a calcium-channel α1 subunit in *Drosophila*. *Genetics 149*, 1407–1426.

Sollod, B.L., Wilson, D., Drinkwater, R., King, G.F., **2003**. Atracotoxin expression and evolution. In: Proc. 14th World Congr. Int. Soc. Toxinol. Adelaide, Australia, p. 23.

Stapleton, A., Blankenship, D.T., Ackermann, B.L., Chen, T.M., Gorder, G.W., *et al.*, **1990**. Curtatoxins: neurotoxic insecticidal polypeptides isolated from the funnel-web spider *Hololena curta*. *J. Biol. Chem. 265*, 2054–2059.

Stewart, L.M.D., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.A., *et al.*, **1991**. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature 352*, 85–88.

Strømgaard, K., Andersen, K., Krogsgaard-Larsen, P., Jaroszewski, J.W., **2001**. Recent advances in the medicinal chemistry of polyamine toxins. *Mini Rev. Med. Chem. 1*, 317–338.

Sun, X., Chen, X., Zhang, Z., Wang, H., Bianchi, F.J., *et al.*, **2002**. Bollworm responses to release of genetically modified *Helicoverpa armigera* nucleopolyhedroviruses in cotton. *J. Invertebr. Pathol. 81*, 63–69.

Tedford, H.W., Fletcher, J.I., King, G.F., **2001**. Functional significance of the β-hairpin in the insecticidal neurotoxin ω-atracotoxin-Hv1a. *J. Biol. Chem. 276*, 26568–26576.

Tedford, H.W., Sollod, B.L., Maggio, F., King, G.F., **2004**. Australian funnel-web spiders: master insecticide chemists. *Toxicon 43*, 601–618.

Toenniessen, G.H., O'Toole, J.C., DeVries, J., **2003**. Advances in plant biotechnology and its adoption in developing countries. *Curr. Opin. Plant Biol. 6*, 191–198.

Tomalski, M.D., Miller, L.K., **1991**. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature 352*, 82–85.

Treacy, M.F., **1999**. Recombinant baculoviruses. In: Hall, F.R., Menn, J.J. (Eds.), Biopesticides: Uses and Delivery. Humana Press, Totowa, NJ, pp. 321–340.

Wang, X.-H., **2000**. Discovery and characterization of insecticidal neurotoxins from Australian funnel-web spider venom. Ph.D. thesis, Department of Biochemistry, University of Sydney.

Wang, X.-H., Connor, M., Smith, R., Maciejewski, M.W., Howden, M.E.H., *et al.*, **2000**. Discovery and characterization of a family of insecticidal neurotoxins with a rare vicinal disulfide bond. *Nature Struct. Biol. 7*, 505–513.

Wang, X.-H., Connor, M., Wilson, D., Wilson, H.I., Nicholson, G.M., *et al.*, **2001**. Discovery and structure of a potent and highly specific blocker of insect calcium channels. *J. Biol. Chem. 276*, 40806–40812.

Wang, X.-H., Smith, R., Fletcher, J.I., Wilson, H., Wood, C.J., *et al.*, **1999**. Structure–function studies of ω-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. *Eur. J. Biochem. 264*, 488–494.

Wicher, D., Walther, C., Wicher, C., **2001**. Non-synaptic ion channels in insects: basic properties of currents and their modulation in neurons and skeletal muscles. *Progr. Neurobiol. 64*, 431–525.

Wilson, D.T.R., **2001**. The identification and characterization of Australian funnel-web spider venom. Ph.D. thesis, Institute for Molecular Biosciences, University of Queensland.

Winston, M.L., **1997**. Nature Wars: People vs. Pests. Harvard University Press, Cambridge, MA.

Zaim, M., Guillet, P., **2002**. Alternative insecticides: an urgent need. *Trends Parasitol. 18*, 161–163.

Zhang, P.F., Chen, P., Hu, W.J., Liang, S.P., **2003**. Huwentoxin-V, a novel insecticidal peptide toxin from the spider *Selenocosmia huwena*, and a natural mutant of the toxin indicates the key amino acid residues related to the biological activity. *Toxicon 42*, 15–20.

Zlotkin, E., Fishman, Y., Elazar, M., **2000**. AaIT: from neurotoxin to insecticide. *Biochimie 82*, 869–881.

5.8 Insecticidal Toxins from *Photorhabdus* and *Xenorhabdus*

R H ffrench-Constant, N Waterfield, and P Daborn, University of Bath, Bath, UK

5.8.1. Introduction

5.8.1.1. The Biology of *Photorhabdus* and *Xenorhabdus*

5.8.1.1.1. Bacteria, nematodes, and insects *Photorhabdus* and *Xenorhabdus* bacteria live in association with nematodes from the families Heterorhabditidae and Steinernematidae, respectively (Forst *et al*., 1997). Both genera bacteria are members of the Enterobacteriaceae, and are found as symbionts within the guts of the infective juvenile nematodes (ffrench-Constant *et al*., 2003). These infective juvenile nematodes seek out and invade insects, whereupon the bacteria are released from the mouth of the nematode directly into the insect blood system or hemocoel (Forst *et al*., 1997). The bacteria then multiply rapidly within the insect, apparently unaffected by the insect immune system (Silva *et al*., 2002). In the case of *Photorhabdus*, the bacteria first colonize the gut and grow rapidly in the hemocoel only, later colonizing all the other insect tissues (Silva *et al*., 2002). The nematodes also multiply within the insect cadaver, feeding off both the bacteria and the degraded insect tissues (Forst *et al*., 1997). Finally, a new generation of infective juvenile nematodes reacquires the bacteria and leaves the insect in search of new hosts.

During the course of this infection process the insect dies and the bacteria are inferred to secrete a range of toxins capable of killing the insect or attacking specific tissues (ffrench-Constant *et al*., 2003). For the purposes of this chapter, it is important to note that during this process the bacteria lie within the hemocoel of the insect or within the insect tissues. Therefore, any toxin action on the gut *in vivo* is likely to be from the hemocoel side of the gut. The existence of toxins acting from the lumen side of the gut is therefore, unexpected from the biology of the organism (Bowen *et al*., 1998). In this respect, this chapter is divided into sections on the orally active Toxin complexes (Tc's) and on the injectably active *M*akes *c*aterpillars *f*loppy (Mcf) toxins.

5.8.1.1.2. Bacterial nomenclature As this chapter involves the description of toxins from a wide range of *Photorhabdus* and *Xenorhabdus* strains, some description of current bacterial taxonomy is necessary to clarify subsequent discussion. The genus *Xenorhabdus* contains a number of species including *X. nematophilus*, *X. beddingii*, *X. bovienii*, and *X. poinarii* (Forst *et al*., 1997). The taxonomy of the genus *Photorhabdus*, however, is more complicated and has recently been revised. In this revision (Fischer-Le Saux *et al*., 1999), the genus was recently split into three species, *P. luminescens*, *P. temperata*, and *P. asymbiotica*. Two of these genera have also been subdivided into new subspecies. Thus, the *P. luminescens* group has three subspecies, *luminescens, akhurstii*, and *laumondii*, and the *P. temperata* group has one subspecies, *temperata*. The third species group, *P. asymbiotica*, which consists of isolates from human wounds recovered in the apparent absence of a nematode vector (Gerrard *et al*., 2003), has not yet been subdivided. Most of the genetic analyses have been performed on a limited number of strains: *P. luminescens* subsp. *akhurstii* strain

W14, *P. luminescens* subsp. *laumondii* strain TT01, and *P. temperata* strains K122 and NC1. For brevity these strains will be referred to as *Photorhabdus* strains W14, TT01, K122, or NC1, or simply W14, TT01, K122, and NC1, except where reference to their different specific designation is relevant to the discussion.

5.8.1.2. The Need for Alternatives to Bt

To date, the majority of transgenes deployed in insect resistant crops are *Cry* genes from *Bacillus thuringiensis* or "Bt." Despite the diversity of *Cry* genes cloned from *B. thuringiensis*, only a few Cry proteins are toxic towards specific pests. This observation has led to concerns about the development of resistance to specific Cry toxins and over the subsequent management of cross-resistance to other toxins occupying the same or similar binding sites (Ives, 1996; McGaughey *et al.*, 1998). These concerns are increasing as specific cases of laboratory-developed Bt resistance are documented in pest insects (McGaughey, 1985; Gahan *et al.*, 2001). *Bacillus thuringiensis* has also proved to be a useful source of other non-*Cry* genes such as the vegetative insecticidal proteins or "Vips" (Estruch *et al.*, 1996; Yu *et al.*, 1997); however, this organism clearly has a limited capacity to produce further toxins. The work described here on the isolation and characterization of insecticidal toxins from *Photorhabdus* and *Xenorhabdus* has been performed partly in the search for alternative novel insecticidal proteins for insect control.

5.8.2. The Toxin Complexes

5.8.2.1. Discovery of the Toxin Complexes

5.8.2.1.1. Purification and cloning of *Photorhabdus* toxins Despite the fact that *Photorhabdus* is released directly into the insect hemocoel by its nematode host, the culture supernatant of *Photorhabdus* strain W14 shows unexpected oral toxicity to the lepidopteran model *Manduca sexta* (Bowen and Ensign, 1998). A toxic high molecular weight-protein fraction was purified from the W14 supernatant by sequential ultrafiltration, dimethyl aminoethyl (DEAE) anion-exchange chromatography, and gel filtration (Bowen and Ensign, 1998). As a final purification step, high-performance liquid chromatography (HPLC) anion-exchange chromatography was used to separate four peaks or "Toxin complexes" A, B, C, and D (Bowen *et al.*, 1998). Purified Toxin complex A (Tca) has a median lethal dose of 875 ng cm^{-2} of diet against *M. sexta*, and is therefore as active as some Bt Cry proteins (Bowen *et al.*, 1998). The histopathology of orally ingested Tca shows the primary site of action to be the midgut, where cells of the midgut epithelium produce blebs as the epithelium itself disintegrates (Blackburn *et al.*, 1998). Interestingly, injection of purified Tca also results in destruction of the midgut with a similar histopathology, suggesting that Tca can act on the gut from either the lumen or the hemocoel (Blackburn *et al.*, 1998).

Each of the HPLC purified complexes migrates as a single or double species on a native gel but resolves into numerous different polypeptides on a denaturing sodium dodecylsulfate (SDS) gel (Bowen *et al.*, 1998). The *toxin complex* (*tc*) encoding genes were cloned by raising both monoclonal and polyclonal antisera against the purified complexes and using these to screen an expression library. Each of the four complexes Tca, Tcb, Tcc, and Tcd is encoded by four independent loci *tca, tcb, tcc*, and *tcd* (Bowen *et al.*, 1998). Each of these loci consists of an operon with successive open reading frames, for example, *tcaA, tcaB, tcaC*, and *tcaZ* (**Figure 1**). These open reading frames encode the different polypeptides that can be resolved from the native complexes as confirmed by N-terminal sequencing of the individual polypeptides resolved on an SDS gel (Bowen *et al.*, 1998). Genetic knockout of each of the *tca*, *tcb*, *tcc*, and *tcd* loci in turn showed that both *tca* and *tcd* contribute to oral toxicity to *M. sexta* and that removal of both these loci in the same strain renders the double mutant nontoxic (Bowen *et al.*, 1998).

Similar independent purification approaches to the supernatant of strain W14 identified two high molecular weight complexes, confusingly termed "toxin A" and "toxin B," with oral activity to the coleopteran *Diabrotica undecimpunctata howardi* (Guo *et al.*, 1999). These two toxins correspond to the species TcdA and TcbA, respectively, therefore confirming that both Tcd and Tcb also have activity against Coleoptera. The native molecular weights of TcdA and TcbA were estimated at 860 kDa, leading to the suggestion that they are tetramers of the 208 kDa species observed on an SDS gel (Guo *et al.*, 1999). These species can also be proteolytically cleaved by proteases found in the culture supernatant and an increase in insecticidal activity associated with the cleaved form of the toxin was reported (Guo *et al.*, 1999). However, the nature of the protease responsible and the relevance of the cleavage in the biological activity of the toxins remain obscure.

5.8.2.1.2. Cloning of *Xenorhabdus* toxins Supernatants of some *Xenorhabdus* strains also show oral

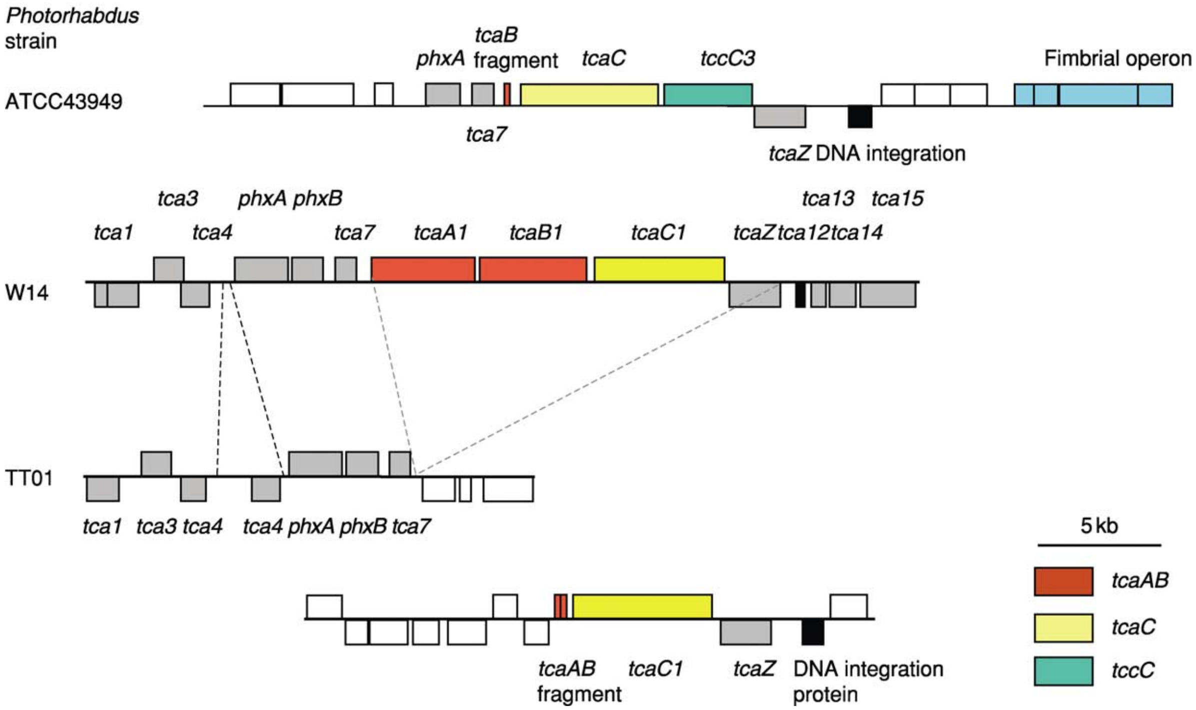


Figure 1 The *toxin complex a* (*tca*) locus from three different *Photorhabdus* strains highlighting the three different color-coded elements *tcaAB*, *tcaC*, and *tccC* (see key). Note that this locus is only complete in strain W14 (in which it confers oral toxicity) and that in TT01 the locus is deleted from the equivalent location in the genome and found as a partially complete copy elsewhere (see text for discussion).

toxicity to insects. Oral insecticidal activity against another lepidopteran, *Pieris brassicae*, was used to identify *tc* gene homologs from *X. nematophilus* PMF1296 (Morgan *et al*., 2001). In this study, a cosmid library was made in *Escherichia coli* and individual cosmids were screened for oral activity. Two overlapping cosmids with oral activity were recovered and one of these was completely sequenced. Transposon mutagenesis of the cosmids was then carried out to discover which open reading frames were involved in oral toxicity (Morgan *et al*., 2001). The genes encoding insecticidal activity were termed *Xenorhabdus protein toxins* (*xpt*) and insertions within the large gene *xptA1* were associated with a loss of oral activity (Morgan *et al*., 2001). The predicted amino acid sequences of the *xpt* genes show high similarity to the *tc* genes of *Photorhabdus* and their closest homologs have been indicated in a revised map of the open reading frames found in the cosmid (Figure 2). The orally toxic *Xenorhabdus* cosmid therefore contains two *tcdA* homologs transcribed in opposite directions (*xptA1* and *xpta2*) with *tcaC* (*xptC1*) and *tccC* (*xptB1*) homologs between them. Expression of one *tcdA*-like gene (*xptA1*) alone in *E. coli* was sufficient to reproduce oral activity in *E. coli* (Morgan *et al*., 2001). This data suggests that the two different genera of nematode symbionts use similar *toxin complex*-like genes.

5.8.2.1.3. Genomic organization of *Photorhabdus tc* genes Extended sequencing of the *tcd* locus in strain W14 revealed that clusters of *tcdA*-like genes were present in the *Photorhabdus* W14 genome (**Figure 3**). Further, these multiple *tcdA* and *tcdB*-like genes are inserted next to an AspV tRNA in a potential pathogenicity island (Waterfield *et al*., 2002). This island is a region of DNA inserted in putative "core" sequence, i.e., sequence similar to core sequences in *E. coli* and *Yersinia pestis*. The island contains multiple copies of *tccC*-like sequences, which may promote recombination, and also enteric repetitive intergenic consensus (ERIC) sequences (Waterfield *et al*., 2002). The region also contains numerous directly duplicated open reading frames and a fragment of a *tcdA*-like gene, suggesting that it may be prone to rearrangement. Finally, a comparison of pathogenicity islands or phage inserted at the Asp tRNA in other related bacteria shows similar genomic islands carrying other toxin genes and *rhs* elements in place of *tccC*-like elements (**Figure 4**). All these factors combine to support the concept that the *tcd*-island is a pathogenicity island. Moreover it is interesting to note that this island is adjacent to a

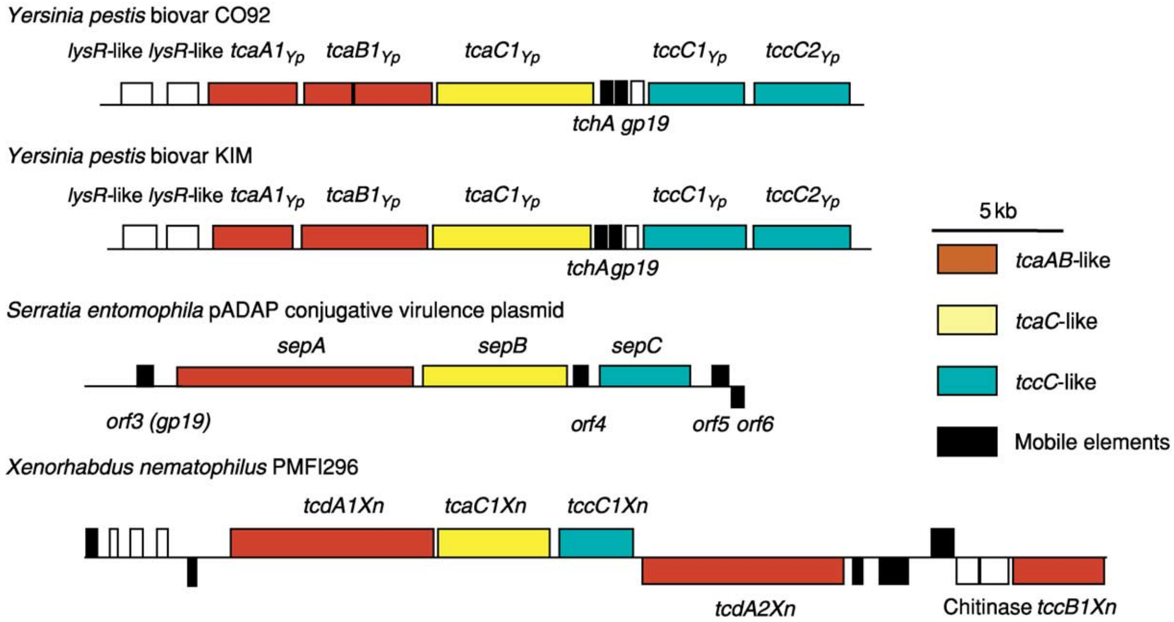


Figure 2 Comparison of *tc*-like loci in entomopathogenic bacteria. *Yersinia pestis* is vectored by the flea and carries a *tca*-like locus, complete in biovar KIM but with *tcaB1* disrupted in biovar CO92. *Serratia entomophila* is a free-living bacteria that infects grass grubs. In this species the *tc*-homologous *sepABC* is carried on a conjugative plasmid required for insect pathogenicity. In *Xenorhabdus nematophilus*, vectored by a nematode, *tc*-homologous genes lie on a cosmid clone recovered on the basis of oral toxicity to caterpillars. Note again that the color coding indicates homology with the three central *tcaAB*, *tcaC*, and *tccC*-like elements (see key).

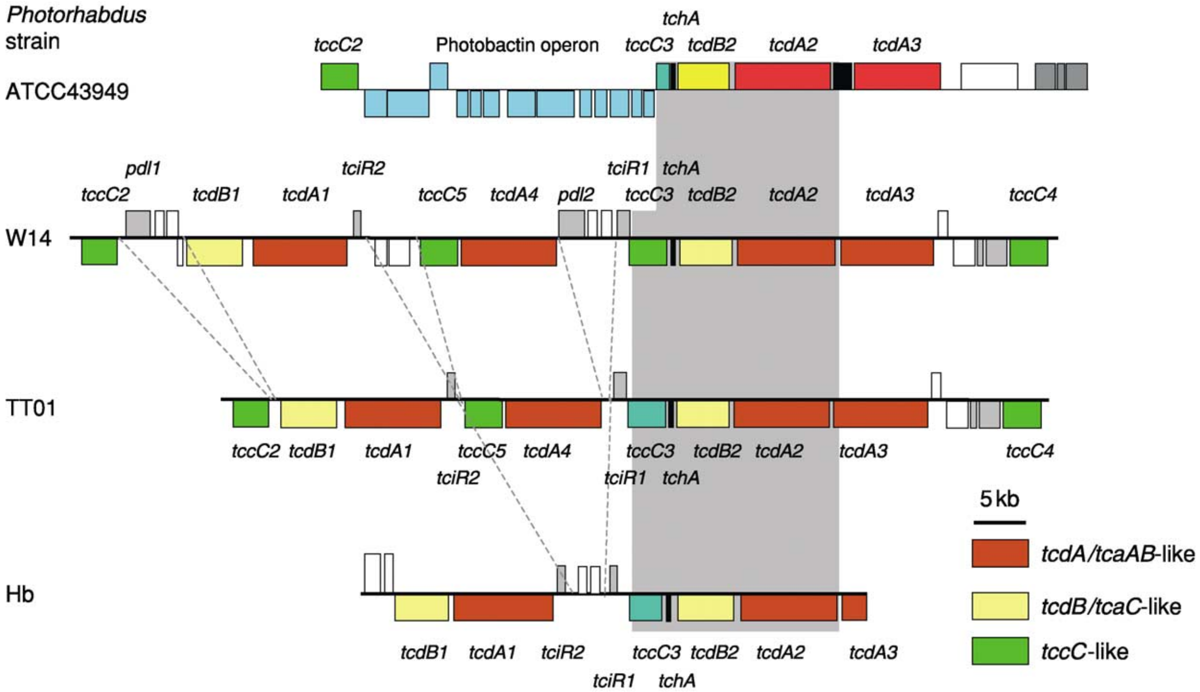


Figure 3 The *toxin complex d* (*tcd*) locus from four different *Photorhabdus* strains highlighting the three different color-coded *tcaAB/tcdA*, *tcaC/tcdB*, and *tccC*-like elements (see key). Comparison of the four loci shows a conserved core (boxed in gray) and then localized deletions within and between the different *tcd* homologs (see text). Note that the *tcd* locus from W14 can confer oral toxicity on recombinant *E. coli*.

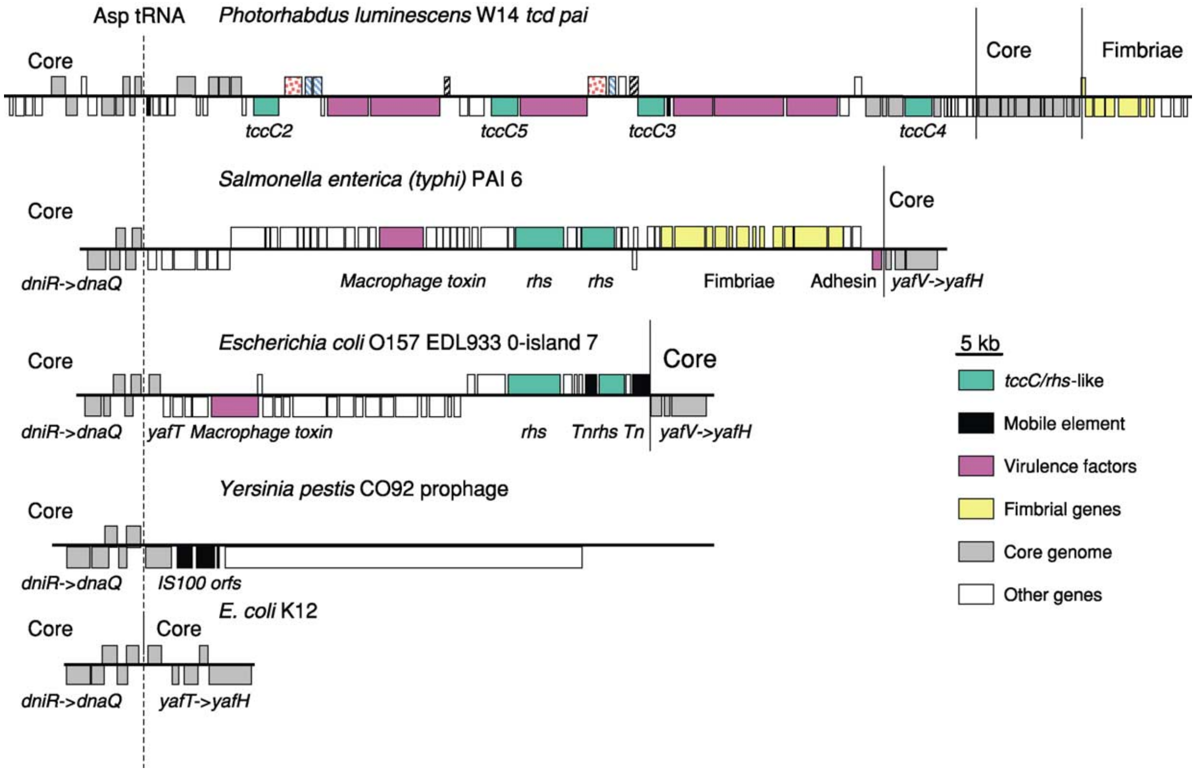


Figure 4 Comparison of *Photorhabdus* W14 *tcd* pathogenicity island with pathogenicity islands also inserted at the Asp tRNA in other related bacteria. The ''core'' sequence flanking the inserted island is shown. Note the common elements of the pathogenicity islands sharing insertion near a tRNA, toxin genes and mobile elements (see key).

gene *ngrA* implicated in nematode symbiosis (Ciche *et al*., 2001). Thus regions containing insect pathogenicity genes and nematode symbiosis factors may be linked in the W14 genome.

5.8.2.2. Homologs in Other Bacteria

Homologs of *tc* genes have now been found in other bacteria, both those pathogenic to insects and also bacteria with no obvious insect association (Waterfield *et al*., 2001b). Gene homologs are found in *Serratia entomophila*, a free-living bacterium causing "amber disease" in the New Zealand grass grub, *Costelytra zealandica* (Hurst *et al*., 2000). Larval disease symptoms after oral ingestion of *S. entomophila* include cessation of feeding, clearance of the gut, amber coloration, and eventual death. In *S. entomophila*, the disease determinants are encoded on a 115 kb plasmid designated pADAP, for amber disease-associated plasmid (Glare *et al*., 1993). Introduction of pADAP into *E. coli* is sufficient to confer oral toxicity on the whole bacteria (Hurst *et al*., 2000). The genes encoding the amber disease toxins were mapped to a 23 kb fragment of pADAP and then identified by insertional mutagenesis. The three disease-associated genes were termed *Serratia entomophila pathogenicity* (*sep*) genes (Hurst *et al*., 2000). These predicted products of these genes show high similarity to those of the *tc* genes and are again clearly homologs. Thus *sepA* is *tcd*-like, *sepB* is *tcaC*-like, and *sepC* is a *tccC*-like gene (**Figure 2**). The finding of *tc*-like genes in a bacterium with no known association with a nematode shows that this class of genes is not specific to nematode symbionts. Further, the presence of the *sep* genes on a plasmid supports the concept, derived from the *tcd* island of *Photorhabdus* W14 (Waterfield *et al*., 2002; ffrench-Constant *et al*., 2003), that the *tc* genes can be found on unstable or mobile stretches of DNA.

More recently, *tca*-like genes have also been discovered in both the sequenced genomes of *Y. pestis* (Parkhill *et al*., 2001; Deng *et al*., 2002), the causative agent of bubonic plague (Perry and Fetherston, 1997). Although highly pathogenic to man, *Y. pestis* is vectored by fleas and is therefore insect associated for part of its life cycle. Following uptake of an infected blood meal the midgut of the flea becomes "blocked," a process facilitating transmission of the infected blood to the next mammalian host (Hinnebusch *et al*., 1996). The hypothesis that the *tca* homolog in *Y. pestis* may be involved in midgut blockage was investigated by making a knockout

mutant of the gene and examining its effect on blood feeding in the flea. However, no direct effect of the *tca* mutant on midgut blockage was seen (ffrench-Constant, Perry, and Hinnebusch, unpublished data). The functional role of this gene in *Y. pestis* therefore remains unknown. The observation that one of the *tca* open reading frames is apparently disrupted in strain CO92 led Parkhill *et al.* (2001) to suggest the alternative hypothesis, that loss of *tca* function may actually be required for persistence of *Y. pestis* in the flea gut. Further, the presence of an intact *tca* homolog in the recent putative ancestor of *Y. pestis*, *Y. pseudotuberculosis* (S. Hinchcliffe, personal communication), supports the concept that *tca* function may have recently been lost.

Gene homologs can also be detected in the genomes of bacteria with no currently known associations with insects. In *Pseudomona syringae* pv tomato, there is a locus with *tcaC*, *tccB*, and *tccC* homologs (Figure 5). Although this bacterium has no known association with insects, as a plant pathogen it may have an undescribed insect vector. Finally, and most recently, *tc* homologs have been found in the genome of *Fibrobacter succinogenes*, a commensal of ruminants with no obvious insect association. This growing range of noninsect associated bacteria with *tc* gene homologs may suggest that this class of genes is widely found in Gram-negative bacteria and that insect toxicity has been acquired in only a limited subset of *tc* gene homologs. The inferred ancestral function of *tc* homologs, however, remains obscure.

5.8.2.3. Molecular Biology of the *toxin complex* Genes

5.8.2.3.1. *toxin complex* gene organization The previous survey of *tc* gene homologs found in a range of bacteria leaves us with a confusing array of gene names and homologies. However, several aspects of the genomic organization of these genes in different species begin to indicate which may be the minimal functional units of insect toxicity. First, a color-coded comparison of *tc* loci in different bacteria shows that the different loci are mainly variations on a simple theme of three basic gene types: (1) *tcdA*-like genes, (2) *tcaC*-like genes, and (3) *tccC*-like genes (Figures 1–3). Second, where isolated pieces of DNA can confer oral toxicity, such as the pADAP plasmid of *S. entomophila* (Hurst *et al.*, 2000) and the cosmids isolated from *X. nematophilus* (Morgan *et al.*, 2001), only these same three gene types are required for full toxicity. This effectively gives us a working hypothesis for attempts to recombinantly express *Photorhabdus* toxins, i.e., that combinations of these three types of genes are required for the expression of full oral toxicity.

5.8.2.3.2. Expression and structure of *Photorhabdus* toxins Following the demonstration that knockout of either *tca* or *tcd* in *Photorhabdus* W14 decreases oral toxicity and in the absence of orally toxic cosmids carrying these loci, it was required to define the minimal components necessary for full oral toxicity in *E. coli*. In these experiments plasmids were inserted into *E. coli* carrying either (1) *tcdA* alone, (2) *tcdA* and *tcdB*, or (3) *tcdA*, *tcdB*, and *tccC* together. Only the plasmid carrying all three genes confers oral toxicity when expressed in *E. coli* and toxicity is increased by induction of the culture with mitomycin, suggesting that Tc production may be related to stress induced by this phage-inducing agent. Electron microscopy of high molecular weight particle preparations of the recombinant protein showed the Tc complexes to be "ball

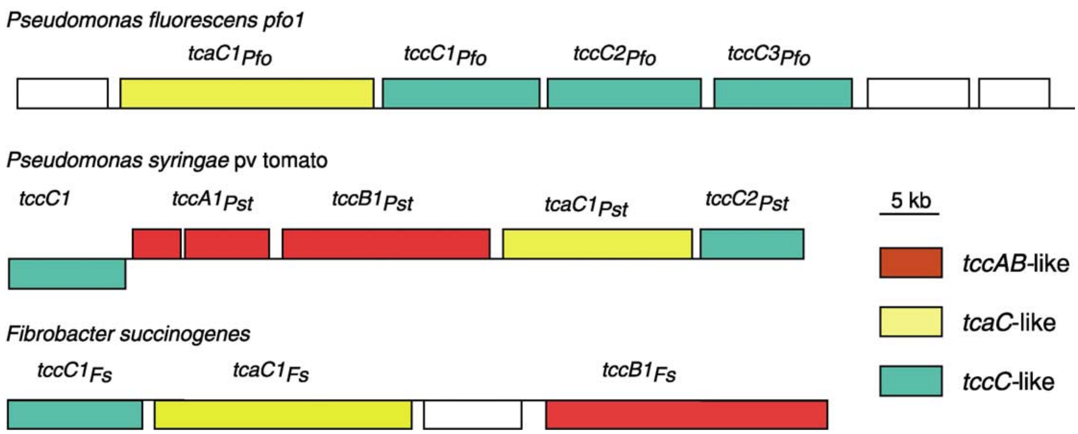


Figure 5 Comparison of *tc*-like loci in bacteria with no known insect association showing that *tc*-like genes can also be found in plant pathogens with no known insect vector like *Pseudomonas syringae* and even ruminal commensals like *Fibrobacter succinogenes* (see text for discussion).

and stick" shaped particles of 25 nm in length (Waterfield *et al.*, 2001a). Further, despite the fact that the presence of *tccC* is necessary for full oral toxicity, the particles themselves can be made by *tcdA* and *tcdB* alone. This suggests that TccC plays a critical role in the activation or export of the complex but does not change the physical structure of the *tcdAB*-derived structure.

Finally, despite the unexpected oral activity of the Tc toxins, how does *Photorhabdus* itself employ these toxins in a normal infection? Immunocytochemistry using an anti-TcaC antibody in insects infected with *Photorhabdus* W14 shows that Tca is produced during infection of the insect midgut (Silva *et al.*, 2002). During infection, the bacteria occupy specific grooves in the midgut, penetrating under the sheathing extracellular matrix to lie directly underneath the midgut epithelium. Within this sheltered niche the bacteria produce Tca, much of which appears to be associated with the bacterial outer membrane (Silva *et al.*, 2002). In this fashion the gut active toxin can be delivered in extreme proximity to the midgut epithelium itself.

5.8.3. The Makes Caterpillars Floppy Toxins

5.8.3.1. Discovery of Makes Caterpillars Floppy

5.8.3.1.1. Cloning of *mcf1* Although the Tc's account for the oral toxicity of some *Photorhabdus* supernatants, the nature of the toxins used by the bacteria growing within the hemocoel to actually kill the insect host remain unclear. To investigate potentially injectably active toxins individual cosmids were employed but this time injecting *E. coli* cultures carrying each cosmid individually into *M. sexta* caterpillars (Daborn *et al.*, 2002). By injecting 2×10^7 cells per culture, 300 clones were injected and a single cosmid that resulted in death of the insect was recovered. As well as killing the insect, the cosmid also induced a rapid loss of body turgor, termed the "floppy" phenotype (Daborn *et al.*, 2002). The cosmid was mutagenized with 126 single transposons to look for insertions showing the loss of either phenotype. The same insertions were also used as entry points to sequence the complete cosmid and identify open reading frames. Out of 126 insertions, 39 negated both phenotypes and all of these lay within a single 8.8 kb gene. This gene also caused both death and loss of body turgor when *E. coli* carrying this gene alone were injected into carterpillars. Therefore, this open reading frame was termed the *makes caterpillars floppy* gene or *mcf*. This open reading frame predicts a high molecular weight protein of 324 kDa. However, the predicted protein shows little homology to other proteins in the databases. Three regions of limited homology are present (**Figure 6**). First, the C-terminus shows similarity to a repeated sequence found in RTX-like toxins (Schaller *et al.*, 1999) potentially involved in toxin secretion. Second, part of the central domain is similar to the translocation domain from toxin B of *Clostridium difficile* (Hofmann *et al.*, 1997). Finally, a region in the N-terminus matches the consensus sequence for a Bcl-2 homology domain 3 (BH3) domain. Vertebrate proteins involved in apoptosis that contain only this domain

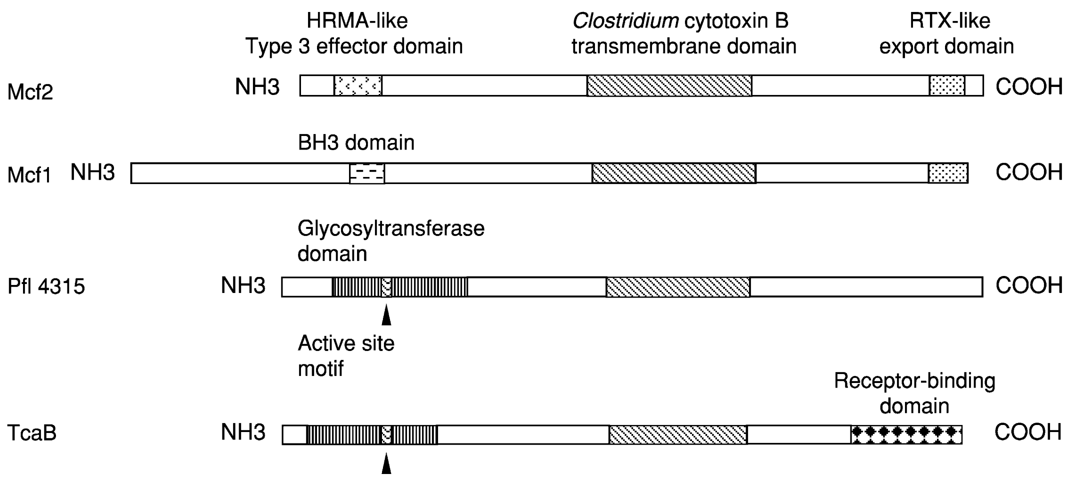


Figure 6 The Makes caterpillars floppy (Mcf) toxins 1 and 2 compared to toxins from *Pseudomonas fluorescens* (Pfl 4315) and *Clostridium* (confusingly termed TcaB but not a Tc toxin). Both Mcf1 and Mcf2 have domains in their N-termini putatively associated with apoptosis, namely a BH3 and HRMA-like domain, respectively. Two regions of similarity within this class of toxins are highlighted. First a transmembrane domain found in the *Clostridium* toxin and second an RTX-like export domain found in the C-terminus of both Mcf proteins.

are proapoptotic (Budd, 2001), leading to the working hypothesis that Mcf itself may also promote apoptosis. Interestingly, if proved, this would be the first BH3 domain found outside of eukaryotes.

The observation that *E. coli* expressing Mcf alone can survive in the presence of the insect immune system is unexpected. Normally, *E. coli* carrying a pUC18 plasmid are cleared from the insect, presumably via the action of both antibacterial peptides and the insect phagocytes or hemocytes. In contrast, *E. coli* carrying pUC18-*mcf* and a second green fluorescent protein (GFP) expressing plasmid persist longer in the hemolymph. Dissection of infected animals shows that the Mcf and GFP expressing bacteria can colonize tissues in the insect and that the hemocytes fail to encapsulate the bacteria in melanin. Therefore, it was speculated that this failure to encapsulate Mcf expressing bacteria was due to hemocytes, dying via Mcf induced apoptosis. To investigate whether Mcf can promote apoptosis in hemocytes, the hemocyte monolayers were exposed to recombinant Mcf. The results show that exposed hemocytes, disintegrate rapidly, within 6 h, by producing numerous characteristic blebs. The residual actin cytoskeleton also shows a characteristic punctate pattern of staining. This proapoptotic effect on hemocytes helps to explain how recombinant *E. coli* can persist in the insect but fails to explain how the insects lose body turgor and die.

To examine the cause of the floppy phenotype, the histopathology of insects infected with *E. coli* expressing Mcf was investigated. Given that both the Malpighian tubules and the midgut are responsible for osmoregulation, destruction of either of these organs could result in a rapid loss of body turgor. Sections of infected caterpillars showed that the Malpighian tubules were intact at the time of death but cells of the midgut epithelium were severely affected as early as 12 h post injection. Both goblet and columnar cells shed circular blebs, often including the nucleus, into the midgut lumen. Nuclei within the dying cells appear pycnotic and stain terminal deoxynucleotidyl transferase-mediated duTP end labeling (TUNEL) positive, suggesting that they are apoptotic. These observations are consistent with Mcf having a proapoptotic function and with its primary site of action being the insect midgut. However, some caution is necessary in interpreting these results as insects ingesting *B. thuringiensis* Cry of Vip toxins also show a similar histopathology of the midgut. More specific experiments to show that Mcf is proapoptotic are therefore needed.

Comparison of the genomic location of *mcf1*-like genes between *Photorhabdus* strains confirms two important points (Figure 7). First, *mcf1*-like genes appear to be present in all strains of *Photorhabdus*, as would be expected if Mcf1 is a dominant toxin. Second, like the *tc* toxins, the location of *mcf1* differs between genomes and is often associated with either a Phe tRNA or integrase genes. In fact in strain TT01, *mcf1* is adjacent to a Phe tRNA on one side and the downstream of the *tccC* locus, suggesting that in this case both a *tc* and *mcf* locus may be found together on the same pathogenicity

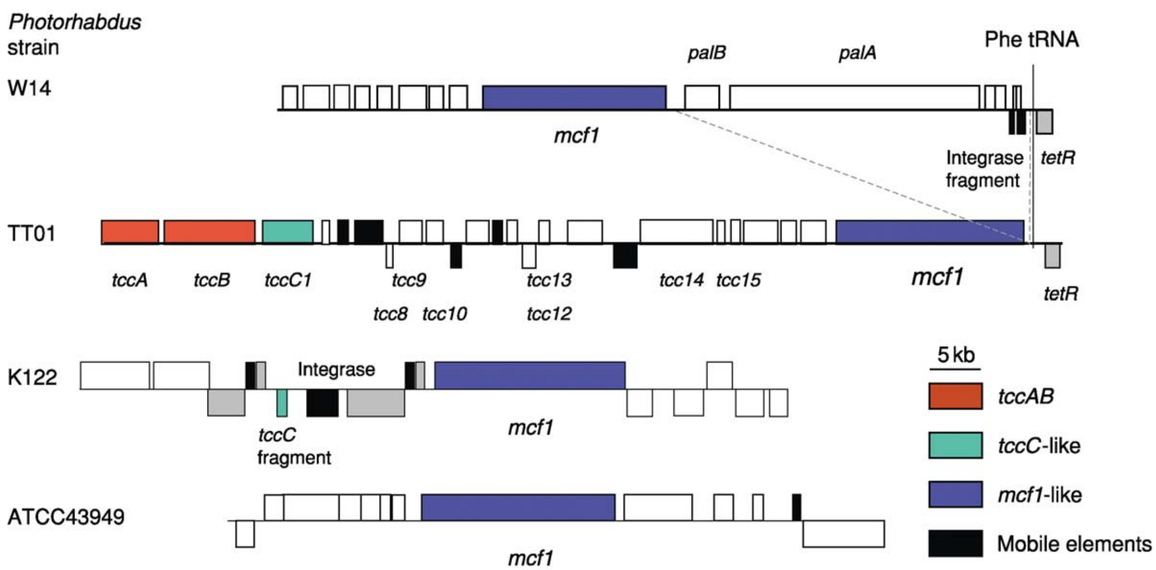


Figure 7 Comparison of *mcf1* containing loci from four different *Photorhabdus* strains. Note that *mcf1* lies alongside a Phe tRNA in both W14 and TT01; however, W14 also carries *palBA*. In the other two strains sequenced, K122 and ATCC43949, *mcf1* appears to occupy different genomic locations suggesting that the toxin is mobile within the *Photorhabdus* genome.

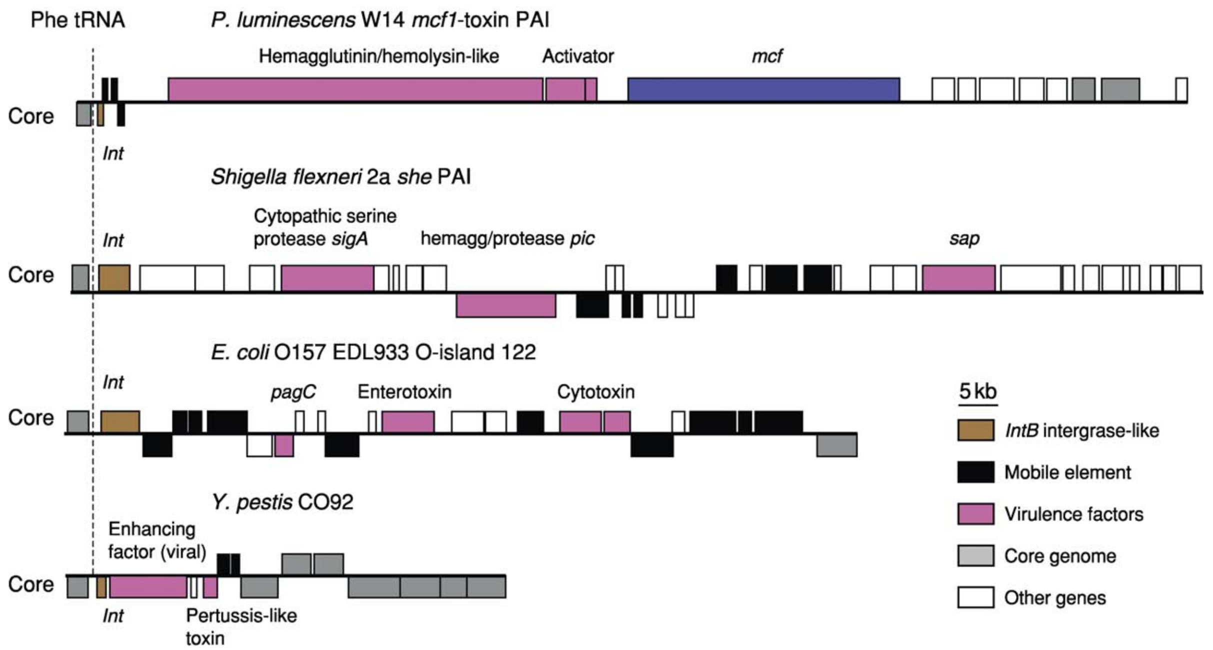


Figure 8 Comparison of *Photorhabdus* W14 *mcf1* pathogenicity island with pathogenicity islands also inserted at the Phe tRNA in related bacteria. The ''core'' sequence flanking the inserted island is shown. Note the common elements of the pathogenicity islands sharing insertion near a tRNA, toxin genes and mobile elements (see key). Note also the similarities in genomic organization with the *tcd* island in Figure 4.

island. Finally, comparison of the relative genomic location of the island containing *mcf1* in *Photorhabdus* strain W14 with other islands inserted at the Phe tRNA in related bacteria again show a similar pattern with other toxins being inserted alongside integrase genes or integrase gene fragments (Figure 8). This confirms that *Photorhabdus*, like related enteric bacteria, carries a range of toxin encoding pathogenicity islands inserted at tRNA genes.

5.8.3.1.2. Cloning of *mcf2* homologs Sample sequencing of the *P. luminescens* W14 genome revealed the presence of a second *mcf*-like open reading frame termed *mcf2*. The predicted Mcf2 protein is similar to Mcf1 (Figure 6) but is significantly shorter at the N-terminus. Further, rather than carrying BH3-like domain, part of the N-terminus shows similarity to HrmA from *Pseudomonas syringae* pv. *syringae* (Figure 6). The *hrmA* gene encodes a protein that has an avirulence (*avr*) phenotype in plants, travels a type III (Hrp) secretion system, and also elicits cell death when transiently expressed directly in tobacco cells. This suggests that HrmA is a type III delivered effector that can cause apoptosis in plant cells. The similarity of HrmA with part of Mcf2 suggests that Mcf2 may also be involved in inducing apoptosis but via a different mechanism than Mcf1. In this case, it is interesting to note how a type III effector protein such as HrmA, which is normally delivered directly into the cell, has become fused within the N-terminus of Mcf2 to be delivered within the high molecular weight protein via an undescribed mechanism. Comparisons of available sequence data from different *Photorhabdus* strains suggests that copies of *mcf1* are always present, suggesting it may be the dominant insect killing toxin (Figure 7). However, we remain uncertain about the distribution of *mcf2* and although this toxin is present in W14 and TT01, this gene is not present in currently available sequence from the *P. asymbiotica* isolate being sequenced at the Sanger Center. It is therefore not clear if *mcf2* is a required toxin present in all *Photorhabdus* strains like *mcf1* or if it is only present in a subset of strains.

5.8.4. Toxin Genomics

5.8.4.1. Microarray Analysis

The widespread distribution of the *tc* genes between different bacteria raises interesting questions as to their origins and transmission. To examine these questions in *Photorhabdus* strains the simple question should be answered: is oral toxicity monophyletic? In other words, do all *Photorhabdus* strains with orally toxic supernatants belong to the same

taxonomic group? To address this question, two parallel approaches were used. First, phylogenetic analysis of orally toxic and nonorally toxic strains using 16S DNA sequences was carried out. Second, hybridized genomic DNA from each strain to a microarray carrying 96 putative virulence factor genes from the orally toxic *Photorhabdus* strain W14 was done to investigate the minimal subset of *tc* genes required for oral toxicity.

The phylogenetic analysis showed that the six orally toxic strains examined fell into two distinct taxonomic groups. The first (IS5, EG2, IND, and W14 itself) lies within the previously recognized *P. luminescens* subsp. *akhurstii* subspecies. The second (Hb1 and Hm1) represents a different subgroup, *P. luminescens* subsp. *luminescens* (Figure 9). The microarray analysis also revealed groups of genes either conserved or variable between the strains examined (Figure 10). Conserved genes included genes such as *lux*, which produces light, a phenotype common to all *Photorhabdus* (Forst and Nealson, 1996). A series of putative virulence factors also appear conserved in most, or all, strains, including the toxin encoding gene *mcf1* (Daborn *et al.*, 2002), *rtxA1* and *rtxA2*-like genes, the operon encoding the *prtA* protease, and an *attachment and invasion locus* (*ail*) homolog (ffrench-Constant *et al.*, 2003). The type III secretion system (Waterfield *et al.*, 2002), often associated with virulence in other Gram-negative bacteria (Galan and Collmer, 1999), is also present in all strains. Other conserved genes include those encoding catalase, chitinase, ferrochetalese, and flagellae. Variable genes include bacteriocins, toxins/hemolysins, insertion elements, pili,

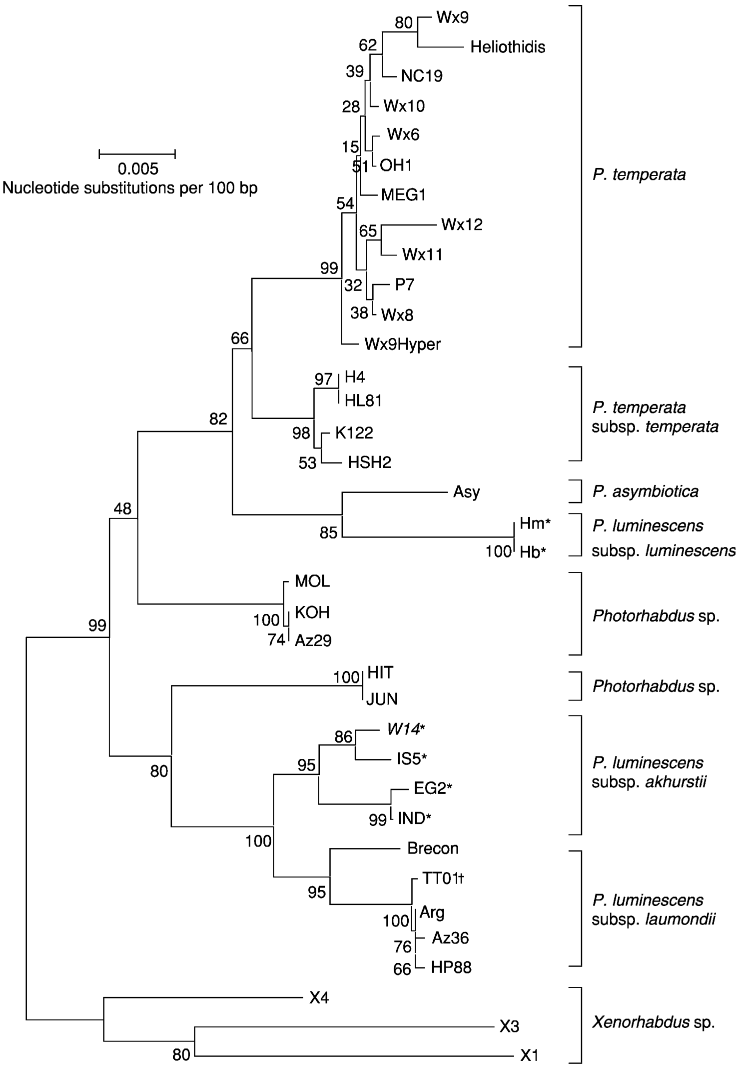


Figure 9 Phylogenetic analysis of orally and nonorally toxic strains used in the microarray analysis based on 16s DNA sequencing. Note that orally toxic strains (*) fall into two well-supported phylogenetic groups. Strain TT01 (†) has recently been completely sequenced (see text).

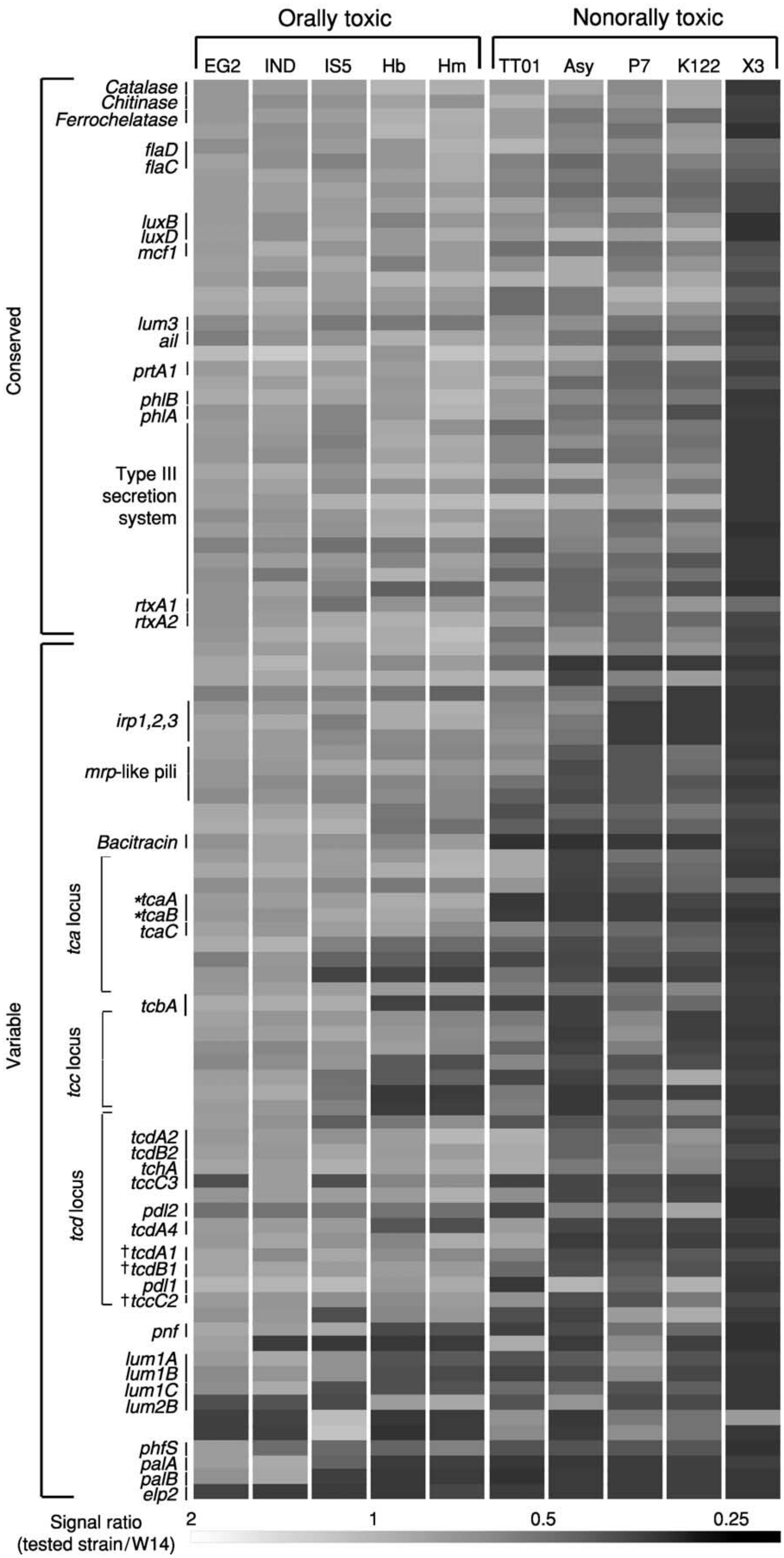
and genes involved in iron acquisition. Some toxin and hemolysin/hemagglutinin genes are also variable between strains. These include *pnf*, a *Photorhabdus necrotizing factor* that is found within a recently acquired region of the W14 genome (Waterfield *et al*., 2002) which is absent from TT01, and the *palA* and *palB* genes, encoding a hemolysin/hemagglutinin and its export activator (Waterfield *et al*., 2002). In W14, *palBA* is immediately downstream of the *mcf1* toxin gene and adjacent to a Phe tRNA (Waterfield *et al*., 2002) whereas in TT01 (**Figure 7**), *palBA* is deleted from the equivalent location, as predicted. In contrast, a second two-component hemolysin locus *phlAB* (Brillard *et al*., 2002), is predicted to be similar in all strains, and indeed potentially duplicated in strain Hm.

The *tc* genes correspond to variable genes, whose distribution is strikingly split between orally toxic and nontoxic strains (**Figure 11**). Orally toxic strains carry all three genes in the *tca* operon (*tcaA*, *tcaB*, and *tcaC*) whereas those lacking toxicity lack *tcaA* and *tcaB*. The genes of the *tca* operon are the only genes to show a perfect correlation with oral toxicity, and genes from *tcb, tcc*, and *tcd* are all variable across orally toxic strains. For the *tca* locus (**Figure 1**), a comparison of W14 and TT01 shows two important findings. First, the W14 *tcaABC* operon is absent from the equivalent location in TT01. Second, a *tca*-like locus is present elsewhere in TT01 but lacks most of *tcaA* and *tcaB*, which have been deleted, and retains only a *tcaC1*-like gene. These observations confirm that the presence of *tcaAB* is necessary for oral toxicity of the bacterial supernatant. Further, the fact that *tca*-like loci can be found at different locations in different genomes supports the concept that the *tca* locus is mobile, as suggested by the presence of either a transposase, in W14, or an integration protein, in TT01, adjacent to both *tca*-like loci (**Figure 1**). For the *tcb* locus, the suggestion that *tcbA* is lacking from TT01 is again confirmed by analysis of the genomic sequence, as *tcbA* is deleted from the equivalent location in TT01 and a transposase left in its place. Again comparing W14 and TT01, the array suggests that the *tcc* locus should be completely conserved, as supported by an examination of the genomic sequence. Finally for *tcd*, the genomic sequence (**Figure 3**) confirms that all the *tc* genes of the island are present in both W14 and TT01, including both *lysR*-like regulators. Further, the predicted loss of *tcdA4* in group 2 orally toxic strains is confirmed by an examination of this locus in the Hb strain. The array even predicts the loss of *pdl1* and *pdl2* from within the *tcd* island of TT01, again confirmed by the sequence (**Figure 3**). The localized deletion of *tcdA4*, and the apparent rearrangements mediated by *tccC*-like genes, again support the hypothesis that the *tcd* genes are encompassed in an unstable pathogenicity island. However, the consistent maintenance of four genetic elements within this island (*tcdA2, tcdB2*, a *gp13*-like holin, and the conserved region of *tccC3*), and the conserved organization of similar *tcd*-like genes in other bacteria (such as *sepA*, *sepB*, *orf4*, and *sepC* in *Serratia*), suggest that these genes form an invariant, but not orally toxic, core.

Previous analysis of strain W14 showed that either *tca* or *tcd* can independently contribute to oral toxicity. Thus plasmid clones of either *tca* or *tcd* from W14 confer recombinant oral toxicity when expressed in other nonorally toxic *Photorhabdus* strains, such as K122. This data is consistent with the current observation that the presence or absence of *tcaAB* is perfectly correlated with toxicity of the supernatant. However, all the *tc* genes within the *tcd* island are also present in some strains, such as TT01 (**Figure 3**), which lack orally toxic supernatants. To investigate the apparent lack of oral toxicity associated with *tcd* in TT01, the toxicity of the bacterial cells was examined independently of their supernatant and it was found that toxicity is associated with the cells rather than the supernatant (**Figure 12**). Confirmation that this novel cell-associated toxicity is *tcd* related now requires knockout or heterologous expression of this locus. In conclusion, even a very limited microarray can provide a powerful and predictive tool for correlating observed phenotypes with bacterial genotypes. Similar microarrays may therefore be useful in investigating other *Photorhabdus* phenotypes involved either in insect pathogenicity or symbiosis with their nematode hosts.

5.8.5. Conclusions

With the publication of the full genome sequence of strain TT01 and the ongoing sequencing of a *P. asymbiotica* strain at the Sanger Center, we are entering an era of comparative *Photorhabdus* genomics. As this review has indicated this now allows us to begin to determine where given toxin genes might have come from, and how they are passed between different strains. In the case of the *tc* genes, the appearance of orally toxic *tca* genes in divergent strains and the finding that these genes can occur at different locations in the genome support the hypothesis that they are mobile between strains. Microarray analysis of more toxin genes in a wider array of strains may also cast light on the origins of


Orally toxic
Nonorally toxic
EG2
IND
IS5
Hb
Hm
TT01
Asy
P7
K122
X3
Conserved
Catalase
Chitinase
Ferrochelatase
flaD
flaC
luxB
luxD
mcf1
lum3
ail
prtA1
phlB
phlA
Type III secretion system
rtxA1
rtxA2
Variable
irp1,2,3
mrp-like pili
Bacitracin
tca locus
*tcaA
*tcaB
tcaC
tcbA
tcc locus
tcd locus
tcdA2
tcdB2
tchA
tccC3
pdl2
tcdA4
†tcdA1
†tcdB1
pdl1
†tccC2
pnf
lum1A
lum1B
lum1C
lum2B
phfS
palA
palB
elp2
Signal ratio
(tested strain/W14)
2
1
0.5
0.25

other toxin genes such as *mcf1* and *mcf2*. Despite this recent explosion in sequence data, perhaps the most important biological questions remain largely unanswered. Thus, what is the biological role of the array of toxins that *Photorhabdus* encodes? Although it is easy to infer that these toxins may be necessary to kill the insect host, assessing the role of individual toxins via genetic knockout may be hampered by their functional redundancy. Thus, *tc* gene knockouts may show no oral activity but

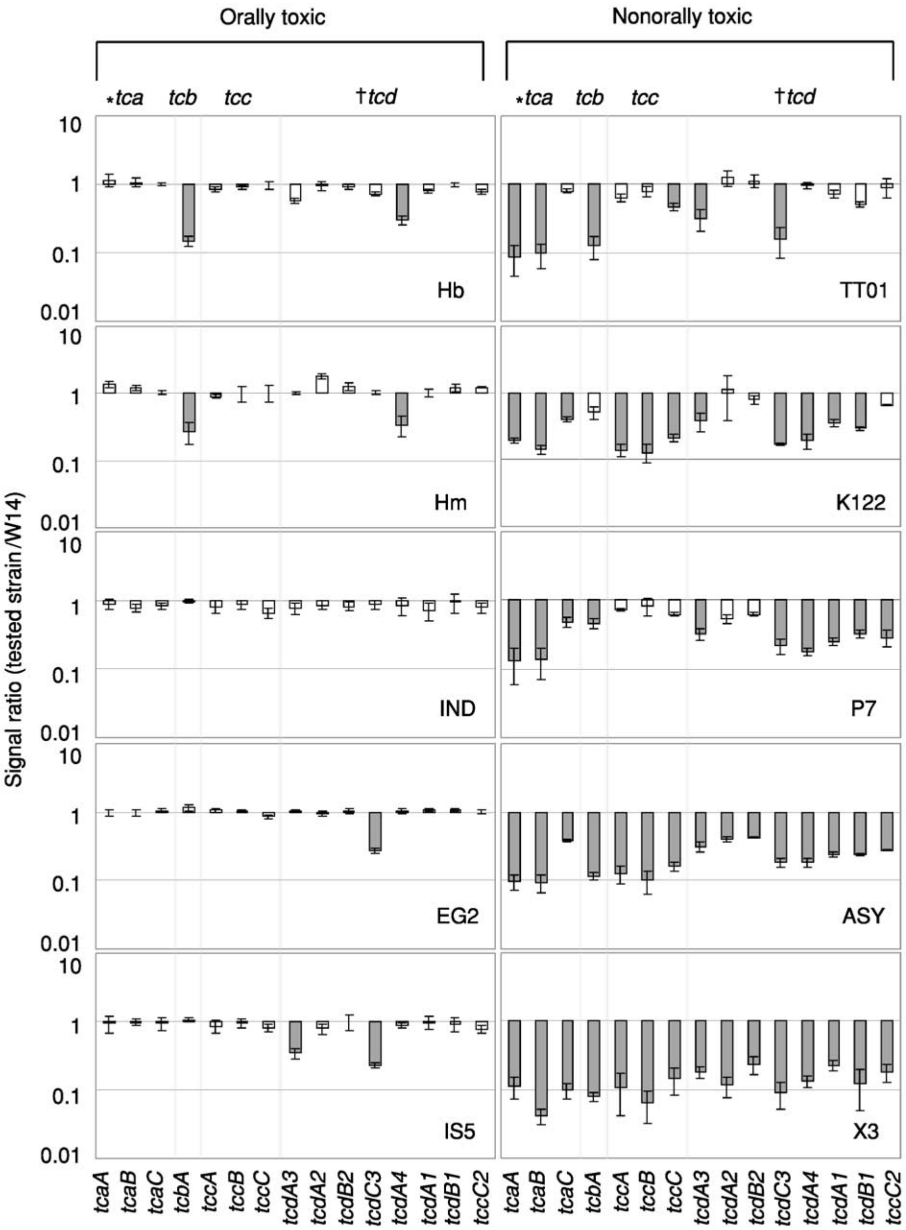


Figure 11 Detailed analysis of the array results for *tc* genes only. In this diagram ratios close to 1 predict the presence of a gene and ratios greater than 0.5 (shaded histograms) predict it to be absent. Note that only the presence of *tcaAB* (∗) is perfectly correlated with the presence of oral toxicity in the different strains (see text) and that *tcd* (†) is variable between strains.

Figure 10 Diagrammatic representation of microarray results where DNA from orally and nonorally toxic strains was hybridized to 96 W14 virulence factor genes. Genes have been divided into conserved (showing a consistent hybridization signal in all strains) and variable (those showing a variable signal between strains). Note for example that the type III secretion system appears present in all *Photorhabdus* strains, whereas members of the *tc* genes are variable (see text). The two loci implicated in oral toxicity are indicated: *tcaAB* (∗) and *tcdAB* (†).

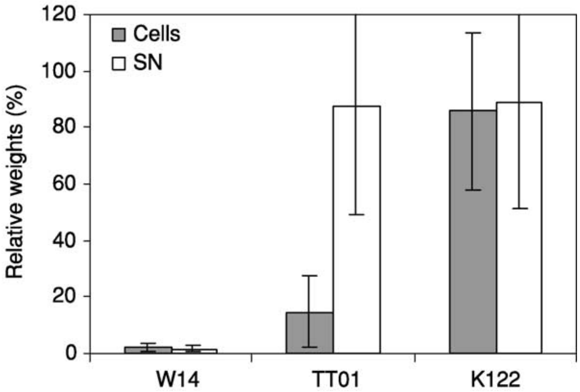


Figure 12 Comparison of supernatant (SN) versus cell-associated oral toxicity for three different *Photorhabdus* strains. Note that strain TT01 containing a complete copy of the *tcd* locus has no oral toxicity associated with the bacterial supernatant but does show oral toxicity associated with the bacterial cells (see text for discussion).

they can still kill the insect host. Further, mutants lacking *mcf1* take a longer time to kill insects but are still lethal. The current challenge is therefore to dissect out the individual roles of each toxin gene, even when they are members of large and rapidly growing gene families.

References

Blackburn, M., Golubeva, E., Bowen, D., ffrench-Constant, R.H., **1998**. A novel insecticidal toxin from *Photorhabdus luminescens*: histopathological effects of Toxin complex A (Tca) on the midgut of *Manduca sexta*. *Appl. Environ. Microbiol. 64*, 3036–3041.

Bowen, D., Rocheleau, T.A., Blackburn, M., Andreev, O., Golubeva, E., *et al.*, **1998**. Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science 280*, 2129–2132.

Bowen, D.J., Ensign, J.C., **1998**. Purification and characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. *Appl. Environ. Microbiol. 64*, 3029–3035.

Brillard, J., Duchaud, E., Boemare, N., Kunst, F., Givaudan, A., **2002**. The PhlA hemolysin from the entomopathogenic bacterium *Photorhabdus luminescens* belongs to the two-partner secretion family of hemolysins. *J. Bacteriol. 184*, 3871–3878.

Budd, R.C., **2001**. Activation-induced cell death. *Curr. Opin. Immunol. 13*, 356–362.

Ciche, T.A., Bintrim, S.B., Horswill, A.R., Ensign, J.C., **2001**. A phosphopantetheinyl transferase homolog is essential for *Photorhabdus luminescens* to support growth and reproduction of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *J. Bacteriol. 183*, 3117–3126.

Daborn, P.J., Waterfield, N., Silva, C.P., Au, C.P.Y., Sharma, S., *et al.*, **2002**. A single *Photorhabdus* gene *makes caterpillars floppy* (*mcf*) allows *Escherichia coli* to persist within and kill insects. *Proc. Natl Acad. Sci. USA 99*, 10742–10747.

Deng, W., Burland, V., Plunkett, G., 3rd, Boutin, A., Mayhew, G.F., *et al.*, **2002**. Genome sequence of *Yersinia pestis* KIM. *J. Bacteriol. 184*, 4601–4611.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., **1996**. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc. Natl Acad. Sci. USA 93*, 5389–5394.

ffrench-Constant, R., Waterfield, N., Daborn, P., Joyce, S., Bennett, H., *et al.*, **2003**. *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. *FEMS Microbiol. Rev. 26*, 433–456.

Fischer-Le Saux, M., Viallard, V., Brunel, B., Normand, P., Boemare, N.E., **1999**. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. *Int. J. Syst. Bacteriol. 49*, 1645–1656.

Forst, S., Dowds, B., Boemare, N., Stackebrandt, E., **1997**. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annu. Rev. Microbiol. 51*, 47–72.

Forst, S., Nealson, K., **1996**. Molecular biology of the symbiotic–pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. *Microbiol. Rev. 60*, 21–43.

Gahan, L.J., Gould, F., Heckel, D.G., **2001**. Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science 293*, 857–860.

Galan, J.E., Collmer, A., **1999**. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science 284*, 1322–1328.

Gerrard, J.G., McNevin, S., Alfredson, D., Forgan-Smith, R., Fraser, N., **2003**. *Photorhabdus* species: bioluminescent bacteria as emerging human pathogens? *Emerg. Infect. Dis. 9*, 251–254.

Glare, T.R., Corbett, G.E., Sadler, A.J., **1993**. Association of a large plasmid with amber disease of the New Zealand grass grub, *Costelytra zealandica*, caused by *Serratia entomophila* and *Serratia proteamaculans*. *J. Invertebr. Pathol. 62*, 165–170.

Guo, L., Fatig, R.O., 3rd, Orr, G.L., Schafer, B.W., Strickland, J.A., *et al.*, **1999**. *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins. *J. Biol. Chem. 274*, 9836–9842.

Hinnebusch, B.J., Perry, R.D., Schwan, T.G., **1996**. Role of the *Yersinia pestis hemin storage* (*hms*) locus in the transmission of plague by fleas. *Science 273*, 367–370.

Hofmann, F., Busch, C., Prepens, U., Just, I., Aktories, K., **1997**. Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. *J. Biol. Chem. 272*, 11074–11078.

Hurst, M.R., Glare, T.R., Jackson, T.A., Ronson, C.W., **2000**. Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. *J. Bacteriol. 182*, 5127–5138.

Ives, A.R., **1996**. Evolution of insect resistance to Bt-transformed plants. *Science 273*, 1412–1413.

McGaughey, W.H., **1985**. Insect resistance to biological insecticide *Bacillus thuringiensis*. *Science 229*, 193–195.

McGaughey, W.H., Gould, F., Gelernter, W., **1998**. Bt resistance management. *Nat. Biotechnol. 16*, 144–146.

Morgan, J.A., Sergeant, M., Ellis, D., Ousley, M., Jarrett, P., **2001**. Sequence analysis of insecticidal genes from *Xenorhabdus nematophilus* PMFI296. *Appl. Environ. Microbiol. 67*, 2062–2069.

Parkhill, J., Wren, B.W., Thomson, N.R., Titball, R.W., Holden, M.T., *et al.*, **2001**. Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature 413*, 523–527.

Perry, R.D., Fetherston, J.D., **1997**. *Yersinia pestis*: etiologic agent of plague. *Clin. Microbiol. Rev. 10*, 35–66.

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., *et al.*, **1999**. Characterization of apxIVA, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology 145*, 2105–2116.

Silva, C.P., Waterfield, N.R., Daborn, P.J., Dean, P., Chilver, T., *et al.*, **2002**. Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cell. Microbiol. 6*, 329–339.

Waterfield, N., Daborn, P.J., ffrench-Constant, R.H., **2002**. Genomic islands in *Photorhabdus*. *Trends Microbiol. 10*, 541–545.

Waterfield, N., Dowling, A., Sharma, S., Daborn, P.J., Potter, U., *et al.*, **2001a**. Oral toxicity of *Photorhabdus luminescens* W14 toxin complexes in *Escherichia coli*. *Appl. Environ. Microbiol. 67*, 5017–5024.

Waterfield, N.R., Bowen, D.J., Fetherston, J.D., Perry, R.D., ffrench-Constant, R.H., **2001b**. The toxin complex genes of *Photorhabdus*: a growing gene family. *Trends Microbiol. 9*, 185–191.

Yu, C.-G., Mullins, M.A., Warren, G.W., Koziel, M.G., Estruch, J.J., **1997**. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Appl. Environ. Microbiol. 63*, 532–536.

5.9 Amino Acid and Neurotransmitter Transporters

D Y Boudko, University of Florida,
St. Augustine, FL, USA
B C Donly, Agriculture and Agri-Food Canada,
London, ON, Canada
B R Stevens, University of Florida, Gainesville,
FL, USA
W R Harvey, University of Florida,
St. Augustine, FL, USA

5.9.1. Introduction

5.9.1.1. Role of Amino Acid Transporters in Insects

Insects use amino acids as substrates for protein synthesis, as essential cofactors for intermediary metabolism, as intracellular messengers between distinct metabolic pathways, and as intercellular messengers – neurotransmitters. Amino acids are precursors in a variety of catabolic and anabolic pathways in which amino acid transporters serve as rate-limiting components (Saier, 2000a); e.g., glutamate transporters regulate the synthesis of GABA (Mathews and Diamond, 2003). In addition some insects use amino acids rather than glucose as an energy source (Balboni, 1978; Gade and Auerswald, 2002) and as osmolytes (Patrick and Bradley, 2000).

5.9.1.2. Uniporters, Symporters, and Antiporters

The movement of amino acids across membranes is catalyzed by transporters, which are polypeptides arranged as monomers or multimers. The collective interaction of these transporters with specific substrates and electrochemical driving forces generated by primary ATP-driven pumps and secondary mineral ion transporters is termed a "transport system" (Christensen, 1964; Gerencser and Stevens, 1994). Amino acid transporters fall into three groups on the basis of their electrochemical profiles: uniporters, symporters, and antiporters. Uniporters facilitate diffusion of amino acids down their own electrochemical gradients. Thermodynamically, uniporters are equivalent to channels but differ kinetically in possessing specific amino acid binding sites and lower conductances than inorganic ion channels. In contrast, amino acid:ion symporters (cotransporters) use electrochemical motive forces of inorganic ions for the translocation of amino acids, stoichiometrically coupled to the ions, usually inorganic cations or anions. A cotransporter can move an amino acid up its electrochemical gradient while the cotransported ion moves down its electrochemical gradient (the amino acid undergoes secondary active transport). Under specific conditions the coupling stoichiometry between amino acid and ion may change, resulting in a so-called "molecular slip" (Nelson *et al.*, 2002). In contrast to symporters, antiporters move thermodynamically coupled solutes in opposite directions and utilize different electrochemical mechanisms.

5.9.1.3. Energization and Electrochemistry of Amino Acid Uptake

The energization and electrochemical mechanisms of amino acid transporters vary greatly, depending upon the available electrochemical conditions (summarized in **Figure 1**). For example, classically characterized reuptake of neurotransmitters by high-affinity neurotransmitter transporters (NTTs) utilizes a steady-state Na^+ electrochemical gradient across neuronal membranes. The Na^+ gradient is thought to be generated by a Na^+/K^+ ATPase and the amino acid uptake is inhibited by agents, such as ouabain or vanadate, that inhibit Na^+/K^+ ATPase activity (Clark and Amara, 1993). More recently, it has become apparent that a proton-translocating, vacuolar-type ATPase (H^+ V-ATPase) has a dominant role in energization of membranes interfacing low sodium ion domains (Wieczorek *et al.*, 1999a). For example, it energizes processes in intracellular membranes (endomembranes), e.g., vesicular and lysosomal amino acid carriers. It serves as an essential energization component in synaptic vesicles, where it contributes to the hyperpolarization of the vesicular membrane and therefore facilitates Na^+-coupled reuptake of neurotransmitters (Nelson and Lill, 1994). Amino acid uptake mediated by transporters is also energized by V-ATPases in plasma membranes of cells exposed to media with low Na^+ concentrations, as in the midgut lumen of caterpillars and mosquito larvae (Harvey *et al.*, 1998). Nanomolar concentrations of bafilomycin or concanamycin inhibit the V-ATPase selectively (Drose *et al.*, 2001), providing a valuable pharmacological tool for the identification of V-ATPase-dependent carrier mechanisms.

Whether an amino acid is anionic (−), zwitterionic (+/−), or cationic (+) depends on the local pH and on amino acid dissociation constants. Other conditions being constant, the lower the pH the more protonated is the amino acid and the more positive is its charge. Conversely, the higher the pH the less protonated and the more negative is the amino acid. This dependence of charge transition upon pH value is especially important in mosquito larvae and caterpillars where amino acid uptake occurs through membranes with large electrostatic and pH gradients. Although the electrochemical interaction between H^+ V-ATPases and amino acid transporters in insect midgut is poorly explored, a tentative experimental model may be suggested by analogy with inorganic ion transporters, which mediate lumen alkalinization of caterpillar and mosquito larval midgut. One such component recruited in alkalinization is H^+ V-ATPase-energized Cl^-/HCO_3^- exchange, which was found in larval midgut of *Aedes aegypti* (Boudko *et al.*, 2001a). Alkalinization of anterior midgut and related pH gradients along the midgut lumen appear to be chiefly, if not exclusively, generated by transepithelial exchange of

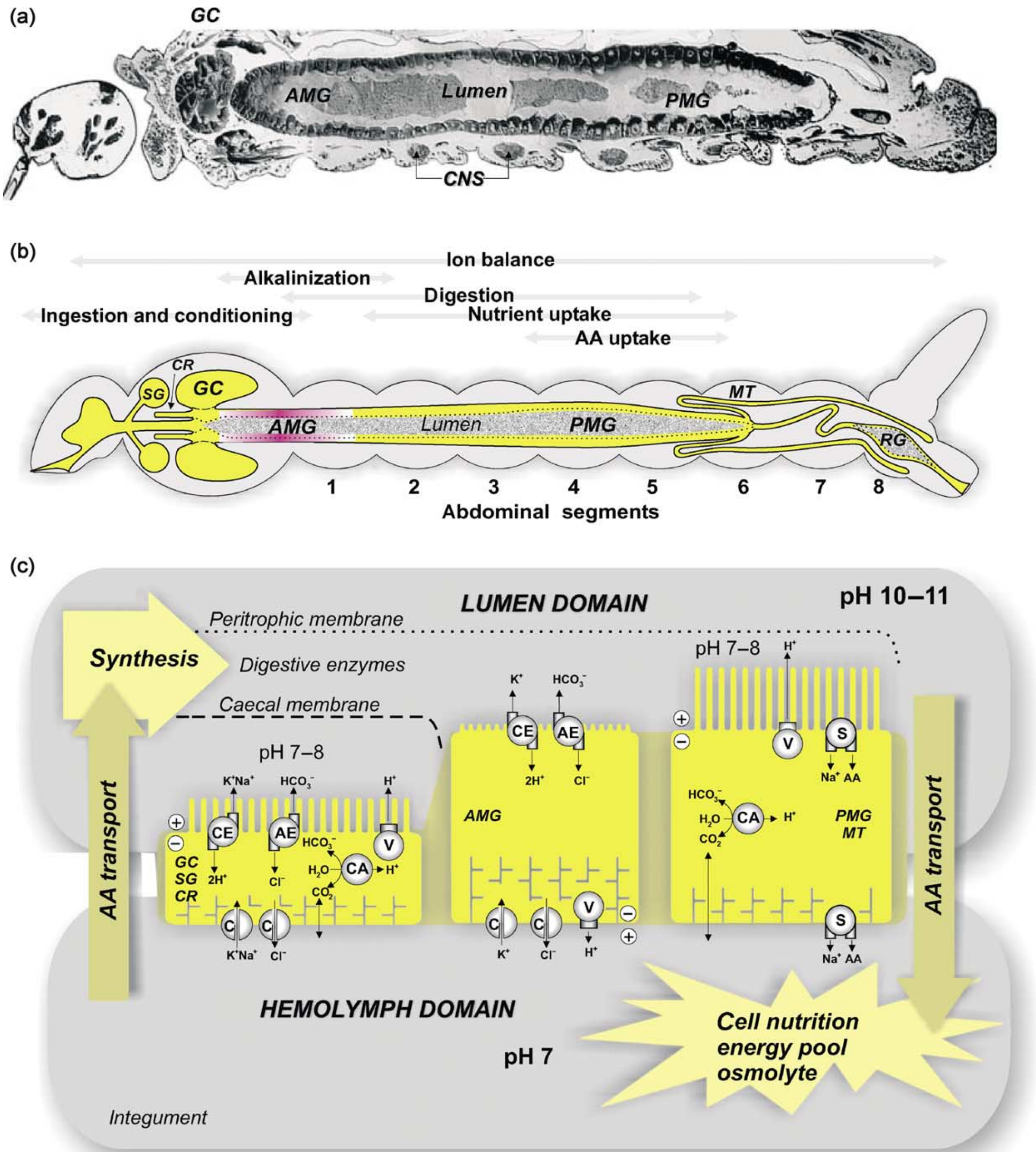


Figure 1 Energization and electrochemical mechanisms of amino acid uptake in insects (example from midgut of mosquito larvae). (a) Low magnification micrograph of longitudinal section showing general cytology of the larval midgut of 4th instar *Aedes aegypti*; (b) diagram illustrating distribution of specific physiological functions in alimentary canal of mosquito larvae; (c) simplified diagram showing putative electrochemical mechanisms that are involved in amino acid uptake in mosquito larvae. Abbreviations: AA, amino acids; AMG, anterior region of midgut; CR, cardia; CNS, central nervous system (ventral nerve cord ganglia); GC, gastric caeca; MT, Malpighian tubules; RG, rectal gland; PMG, posterior midgut; SG, salivary glands; S, symporter, V, H^+V-ATPase; C, channel; CE, cation exchanger; AE, anion exchanger; CA, carbonic anhydrase.

strong Cl^- and weak HCO_3^- anions that is energized by H^+ V-ATPases. So far no evidence for a significant role of Na^+/K^+ ATPases in energization of mosquito larval midgut cells has been obtained, but by analogy with known anionic pathways, a spatial exchange of strong inorganic cations and weak organic cations may mimic the role of Na^+/K^+ ATPases in generating the transmembrane Na^+ concentration gradients that are necessary for amino acid uptake. Alternatively, different motive

forces, e.g., for H^+, K^+, Cl^- as well as one for substrate itself can be used by nutrient amino acid transporters.

Since H^+ V-ATPase constantly moves protons from the cytosol to the exterior it renders the inside of its resident membrane negative to the outside, i.e., it strongly hyperpolarizes the resident membrane (Harvey, 1992). Due to electrical integrity of the insect midgut epithelia, both apical and basal membranes are hyperpolarized (Beyenbach, 2001). For example, even though the V-ATPase is resident on the apical plasma membranes of specific cells in posterior midgut of *Ae. aegypti* larvae (Zhuang *et al*., 1999), both membranes in such cells, as well as entire segments of posterior midgut epithelium, would be hyperpolarized relative to the lumen, implicating electrophoretic forces for charged amino acids and inorganic ions. Primary absorption of nutrient amino acids in posterior midgut of mosquito larvae (Clements, 1992) colocalizes with the residence and high activity of H^+ V-ATPases (Filippova *et al*., 1998; Zhuang *et al*., 1999; Boudko *et al*., 2001b). V-ATPases also colocalize with amino acid transporters in certain cells of the gastric caeca and cardia, where they appear to drive amino acid uptake from hemocoel to the cells (Boudko, unpublished data). These observations suggest that thermodynamic coupling between H^+ V-ATPases and amino acid transport mechanisms is widespread in insects. Significant correlations of H^+ V-ATPase expression and midgut invasion by malaria ookinetes has been reported in the Ross cells of adult *Anopheles gambiae* midgut (Cociancich *et al*., 1999). However, the role of V-ATPases in the infection mechanism remains to be clarified.

5.9.1.4. Structural Organization of Known Transporters

The physiological activity of most known amino acid transport systems is derived from transmembrane proteins that are monomers or form multimeric complexes within the plasma- and endomembranes. These proteins typically integrate 8–14 alpha-helical transmembrane spanning domains (TMDs) and a certain number of submembrane loops, which bear sites for interaction with the cytoskeleton and with other membrane and cytosolic proteins. Molecular identities of transport proteins have been explored mostly in mammals and, to some extent, in model insects (**Table 1**). In addition to monomeric transporters, there are several groups of unique transporters that act as obligatory heterodimers. For example, HAT heterodimers consist of a light chain, a catalytic subunit with 12–14 putative TMDs (SLC7), which is associated with a glycosylated globular heavy chain subunit that has only a single membrane spanning domain (SLC3) (see Section 5.9.7.2). Monomeric and heterodimeric transport systems appear in both nonpolarized cells and epithelial cells; however, they play different and most often complementary roles.

5.9.1.5. How Many Amino Acid Transporters Are Required?

Analysis of recently available genomes suggests that complex organisms require many transporters to regulate local amino acid concentrations in different tissues and cells. Due to their large size and ionic character, all amino acids require specific molecular carriers to pass lipid bilayers of cellular membranes. Mosquito larvae require 10 essential L-amino acids to reach the 2nd instar (Clements, 1992): three basic ones (arginine, lysine, and histidine) and seven neutral ones (valine, leucine, isoleucine, phenylalanine, tryptophan, threonine, and methionine). Thus, one would expect to find a subset of transporters for primary uptake of such amino acids through apical membranes in the posterior midgut as well as for their redistribution among specific body parts and tissues. Potentially, a single transporter gene may encode carriers for several amino acids with common structural features; however, several transporters would be necessary to reduce competition of amino acids for binding sites and to increase transport efficiency. A growing number of observations suggest that high-affinity uptake is required for a high degree of selectivity, implying that a large number of transporter phenotypes may exist. Nonessential amino acids, i.e., glycine, alanine, tyrosine, cysteine, serine, proline, aspartate, glutamate, and their derivatives, can be synthesized *de novo* within the insect organism. However, different tissues and cells have different requirements, often not matching nonessential amino acid anabolism. Hence, essential roles of transporters for nonessential amino acids have evolved and have become more prominent with increased tissue specialization and histological complexity of modern organisms. In addition to the population of plasma membrane amino acid transporters, a large number of specific transporters are necessary for translocation of amino acids between different intracellular domains. Moreover, insects undergo radical physiological and ecological transitions between larval and adult lifestyles. These transitions imply dramatic differences in amino acid requirements, which, together with certain requirements for genomic and phenotypic plasticity of amino acid transport mechanisms, may result in an increase of amino acid transporters being encoded. They may remain

Table 1 Classification of insect nervous system amino acid and neurotransmitter transporters

Transporter family	*TC number (Saier, 2000b)*	*Solute carrier orthology*	*Common names*	*Cloned insect genes*[a]	*Accession number*	*Substrates*
Vesicular monoamine transporter (VMOAT)	2.A.1.22	SLC18	H^+ dependent vesicular monoamine transporter/vesicular acetylcholine transporter family	drmVAChT	AAB86609	Monoamines, acetylcholine
Solute: sodium symporter (SSS)	2.A.21	SLC5	Na^+ dependent glucose transporter family	trnCHT	AY629593	Choline
Neurotransmitter:sodium symporter (NSS)	2.A.22	SLC6	Na^+/Cl^- dependent neurotransmitter transporter family	masGAT	S65673	GABA, monoamines, proline
				trnGAT	AAF70819	
				drmSERT	AAD10615	
				masSERT	AAN59781	
				drmDAT	AAF76882	
				trnDAT	AAN52844	
				trnOAT	AAL09578	
				masPROT	AF454914	
				drmINE	AAC47292	
				masINE	AAF21642	
				drmBLOT	CAB53640	
Dicarboxylate/amino acid:cation (Na^+ or H^+)	2.A.23	SLC1	Na^+/K^+ dependent excitatory amino acid transporter family	trnEAAT1	AAB84380	Glutamate, aspartate
				dipEAAT1	AAF71701	
				drmEAAT1	AAD09142	
				drmEAAT2	AAD47830	
				apmEAAT1	AAD34586	
				aeaEAAT	AAP76304	
Anion:cation symporter (ACS; VGLUT) family	2.A.1.14	SLC17	H^+ dependent vesicular glutamate transporter			Glutamate
Amino acid/auxin permease (AAAP) family	2.A.18	SLC32	H^+ dependent vesicular inhibitory amino acid transporters			GABA, glycine

[a]Additionally, some putative transporters identified by phylogenetic proximity with characterized neurotransmitter transporter are discussed in Section 5.9.5.

inactive in insect genomes until activated, usually by a hormone, at a particular developmental stage.

5.9.1.6. Neurotransmitter Transporters and Nutrient Amino Acid Transporters

Amino acid transporters can be divided into two major categories with respect to their physiological roles: neurotransmitter transporters (NTTs) and nutrient amino acid transporters (NATs). Many phylogenetically distinct groups of amino acid transporters include both categories.

NTT denotes a carrier of neurotransmitters, which may be amino acids or their derivatives. Classical examples are plasma membrane insect NTTs for the high-affinity reuptake of glutamate, aspartate, glycine, and γ-aminobutyric acid (GABA), as well as the monoamines, dopamine, octopamine, 5-hydroxytryptamine (5-HT or serotonin), and histamine. Plasma membrane NTTs transport neurotransmitters from synaptic clefts into glial cells and presynaptic neurons. Intracellular NTTs serve for accumulation of neurotransmitters into synaptic vesicles, implicating them as one of the most critical components of presynaptic maturation for chemical signaling.

NAT denotes a carrier of one or more of the 20 natural α-amino acids with no apparent involvement in signaling processes, although there is weak background for such discrimination. Classical examples of insect NATs are those that absorb amino acids from the midgut or Malpighian tubule lumen through the apical membrane of specialized epithelial cells. NATs in the basal membrane export amino acids from epithelial cells into the hemolymph, comprising electrochemically different transport systems. Different NAT types are expressed in the basal cell membranes of the mosquito larval cardia, gastric caeca, and salivary glands, where they take up specific amino acids from the hemolymph and supply substrates for the synthesis of peritrophic and other secretory peptides. NATs in membranes of internal organelles participate in intermediary metabolism by supplying amino acids for metabolic pathways. Although glutamate, aspartate, and glycine transporters can be classified as NTTs or NATs, depending upon their role in specific cell types, in this review they will be discussed along with GABA transporters in Section 5.9.3.

A great deal is known about NATs in prokaryotes, yeast, and humans (Palacin *et al.*, 1998; Wipf *et al.*, 2002), but what little is known about them in insects is restricted mainly to caterpillars, fruit flies, and mosquitoes (discussed in Section 5.9.8.5.2). Much more is known about neurotransmitter amino acid transporters of insects (discussed immediately below and in Section 5.9.3).

5.9.2. Physiology of Insect NTTs

5.9.2.1. Synaptic Neurotransmission

The nervous system is responsible for the acquisition, integration, and storage of information. Neurons are specialized cells that enable electrochemical communication with each other, sensory cells, and target cells. This intercellular communication occurs predominantly at synapses, through the exocytotic release of chemical transmitters from the presynaptic neuron (Pennetta *et al.*, 1999; Caveney and Donly, 2002; Watson and Schurmann, 2002). Released transmitters diffuse across the synaptic space and bind to receptors on the postsynaptic membrane, resulting in either depolarization or other effects on the target cell (**Figure 2**). Transmitters are stored in the presynaptic terminal in vesicles, which are stocked with these chemicals by the action of transporters located in the vesicular membranes. The necessary stores of transmitter are acquired both through the biosynthesis of new molecules and the reuptake of released ones. Recovery of released transmitter is accomplished by transporters located in the plasma membranes of the various cells impinging on the synaptic space, both neuronal and glial cells (**Figure 2**). Thus, transport of neurotransmitters is important for the functioning of the synapse at two levels: for the packaging of transmitters in presynaptic vesicles prior to their release by exocytosis and for their reuptake from the extracellular space into the cytoplasm (Krantz *et al.*, 1999).

5.9.2.2. Role of Plasma Membrane NTTs in Synaptic Transmission

The action of released neurotransmitters is terminated by three major mechanisms: diffusion away from the synapse, buffering by the receptor and transporter ligand binding sites, and removal via uptake by high-affinity transporters present on neighboring membranes of presynaptic, postsynaptic, and glial cells (**Figure 2**). Much of the progress in understanding transmitter clearance has been carried out on various glutamatergic synapses in the mammalian CNS. Initially, diffusion was believed to play the predominant role in determining the concentration profile of glutamate in the synaptic cleft. In this view, plasma membrane transporters only act externally to the cleft – to lower extracellular glutamate levels, to contain the transmitter to the

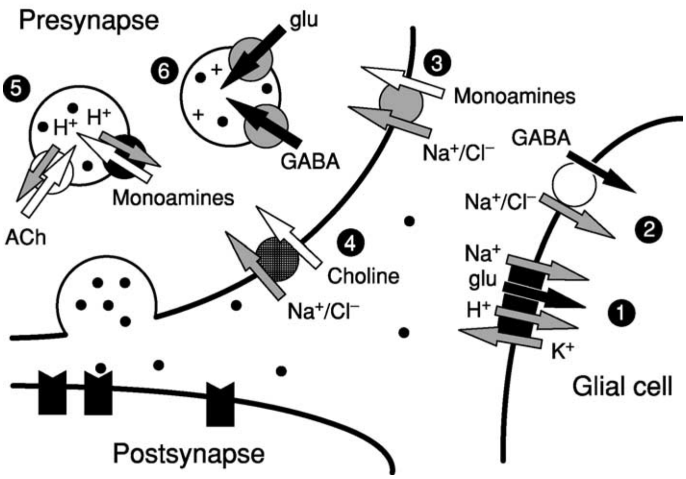


Figure 2 Neurotransmitters involved in insect synaptic signaling. Vesicles in the presynaptic neuron fuse with the presynaptic membrane and release neurotransmitters (•) that diffuse across the synaptic cleft and bind to receptors on the postsynaptic membrane. Unbound transmitters are taken up by various transport proteins in the plasma membranes of the presynaptic axon and adjacent glial cells: excitatory amino acid transporters (EAATs) that mediate Na^+ and K^+ dependent uptake of glutamate and aspartate into glutamatergic neurons or glial cells (1); and GABA and various monoamine transporters (SNFs) that mediate Na^+ and Cl^- dependent uptake of these ligands into glial cells (2) or back into neurons (3). A breakdown product of acetylcholine, choline, is transported into neurons by a different type of Na^+ and Cl^- dependent transporter that is related to glucose transporters (4). Neurotransmitters in neurons and glial cells are packaged into vesicles by transporters in the vesicular membrane, which are energized by proton/voltage gradients generated by the vacuolar H^+-ATPase. Transport of monoamines and acetylcholine into the vesicles is driven by a pH gradient (5), whereas transport of glutamate is driven by the electrical gradient (6). Vesicular GABA uptake is dependent on both gradients. Packaged neurotransmitters in neurons derived from this reuptake and from *de novo* synthesis are ready to be released when activated by an incoming voltage signal.

synapse, and to maintain a steep concentration gradient that promotes rapid diffusion (Krantz *et al*., 1999). Transporters were thought to play a limited role in the clearance of transmitter from the synapse. Moreover, because they cycle at very low speeds relative to receptor inactivation times, transporters were thought to affect only the slow components of glutamate removal (Otis *et al*., 1996). However, new data suggest that transporters play a dynamic role in the regulation of neurotransmission. For example, glutamate transporters may contribute to neurotransmitter inactivation on a rapid timescale. When present in high density, transporters may serve as binding sites without actually clearing the bound substrates (Seal and Amara, 1999). Also, Auger and Attwell (2000) found that glutamate removal by postsynaptic transporters at the climbing fiber synapse of rat Purkinje cells is very rapid, implying that translocation of glutamate may occur very early in the transport cycle. These observations suggest an active role for transporters in synaptic homeostasis, in which most of the presynaptically released transmitter is taken up rapidly and directly from the synapse. Other considerations affecting the relative roles of diffusion and uptake in regulating glutamate removal include the anatomy of individual synapses, with synapse diameter and the presence or absence of transporter-rich glia affecting the termination of postsynaptic excitation (Danbolt, 2001). Thus, the effects of diffusion and uptake on neurotransmitter concentration in the synaptic cleft depend upon the geometry of the cleft and adjacent spaces.

Analysis of amine transporter knockouts in mice also reveals a direct influence of transporters on the volume transmission of monoamines. Elimination of the relevant transporters revealed that by limiting neurotransmitter removal to diffusion alone, the extracellular transmitter concentration increased fivefold for dopamine, sixfold for serotonin, and twofold for norepinephrine (Gainetdinov and Caron, 2002; Torres *et al*., 2003). The persistence of dopamine in the extracellular space also increased 300-fold, relative to that in wild-type mice. Furthermore, intracellular transmitter concentrations were severely reduced, indicating reuptake is also crucial for the maintenance of the stores of these compounds. Therefore, the action of plasma membrane transporters is necessary for presynaptic homeostasis both for maintaining clearance kinetics and for providing adequate substrate levels.

Rapid removal of neurotransmitter from the synaptic cleft raises the prospect that transporter activity may influence the characteristics of postsynaptic

potentials, with broad implications for behavior and learning. However, this prospect has scarcely been explored.

5.9.2.3. Study of Insect NTTs

Much current knowledge on transporter structure and function has been derived from studies of vertebrate (particularly mammalian) examples. In general, transporters from insects are less well characterized than their vertebrate counterparts. However, several groups and even particular species of insect transporters are clearly orthologous to vertebrate transporters. Much of what is known of many of the specific categories of NTTs (from both a structural and functional perspective) have been inferred from discoveries in the mammalian CNS (reviews: Deken *et al*., 2002; Kanai *et al*., 2002; Torres *et al*., 2003). Commensurate with overall genome size, there appears to be less specialization in NTT complements in insects than in their mammalian equivalents. This relative simplicity may render NTTs promising targets for pest control as the insect nervous system possesses relatively less molecular redundancy to be overcome. The simplicity should also prove to make them tractable model systems for genetic and molecular dissection. Indeed, characterizing membrane transporters in the model organism *Drosophila melanogaster* has progressed rapidly and there has been significant progress in analyzing NTT transporters in Lepidoptera, as described below.

Insects may nevertheless provide a rich resource of NTT diversity, based on their extensive physiological variation and adaptation to diverse environments and lifestyles. In addition to the examples already identified by functional studies, the completion of the fruit fly and mosquito genomes has now allowed the entire complements of transporters from these two species to be categorized, as discussed below (Sections 5.9.5–5.9.9). Initial annotation of the *Drosophila* genome according to the gene ontology (GO) system resulted in the assignment of 665 transcripts within the "function" category as transporters (Adams *et al*., 2000). Comparison with the recently completed mosquito genome (Holt *et al*., 2002) has yielded further novel insights into insect transporters as well as a wealth of inferential insights based on comparison with the growing catalog of genomic databases (see Section 5.9.5).

5.9.2.4. Potential for Use of NTTs as Insecticide Targets

The importance of neurotransmitter reuptake at the synapse suggests that insect NTTs may serve as targets for insecticidal action. Many potent drugs that affect behavior by acting as "selective reuptake blockers" of plasma membrane NTTs have been identified in the mammalian brain (Torres *et al*., 2003). Gene knockouts (and antisense knockdowns) of various transporters in rodents have also demonstrated significant physiological consequences resulting from disruption of transporter function (Rothstein *et al*., 1996; Torres *et al*., 2003). In insects, the natural plant compound, cocaine, may exert behavioral and insecticidal effects through antagonism of the reuptake of amines in the nervous system, in particular that of octopamine (Nathanson *et al*., 1993). The role of GABAergic uptake activity in modulating behavior in *D. melanogaster* has been examined using a pharmacological approach (Leal and Neckameyer, 2002). Blocking GABA transporter function with reuptake inhibitors revealed specific effects on behavior including reduction of locomotor activity, geotactic abilities, and loss of the righting reflex. The specificity of the pharmacological effect to GABAergic activity was shown by the restoration of normal locomotor activity when inhibitors were applied in the presence of a GABA receptor antagonist. Thus, there is good reason to think that agents that block transporter activity may prove effective in perturbing pest behavior (by antifeedant or other insecticidal activities).

5.9.3. Molecular Biology of Insect NTTs

5.9.3.1. Classification of NTTs

The increasing availability of genomic information now allows a holistic approach to the analysis of the structure and function of specific transporters. By comparing complete genome complements of protein components in diverse organisms, it is possible to develop hierarchies based on structural conservation reflecting biological themes that have evolved in response to functional requirements. For membrane proteins that transport amino acids, a comparison of three sequenced eukaryotic genomes (*Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Homo sapiens*) distinguished five different transporter superfamilies based on phylogeny, substrate spectrum, transport mechanism, and cell specificity (Wipf *et al*., 2002). An even more comprehensive scheme has been devised by Saier (2000b) to classify all permeases based on both phylogeny and function. The resulting transporter classification (TC) system uses five criteria to group individual transporters within each family. Transporters in the nervous system belong to six separate

families in the TC system (**Table 1**). The primary distinction demarcating the various nervous system transporters is the site at which the transport system is active. Plasma membrane transporters transfer released neurotransmitter from the synaptic space back into the cytoplasm of neurons and glial cells using the inwardly directed Na^+ gradient across the plasma membrane (Masson *et al.*, 1999). Vesicular transporters located in neuronal synaptic vesicles further concentrate transmitters from the cytoplasm inside the vesicles using proton electrochemical gradients generated by the vacuolar type H^+-ATPase (Yelin and Schuldiner, 2002).

5.9.3.2. Plasma Membrane Neurotransmitter Transporters

It has long been recognized that nervous tissues are capable of taking up a variety of endogenous substrates by way of specific, high-affinity, Na^+-dependent plasma membrane transport. The energy for this uptake derives from coupling the inward transport of neurotransmitter to the flow of sodium ions, using the inwardly directed electrochemical Na^+ gradient generated by the Na^+/K^+-ATPase (see Section 5.9.1.3). The molecular identities of the membrane proteins responsible have become known over the last 10–15 years, revealing structural similarities to at least three families of transporters (**Table 1**; Krantz *et al.*, 1999). Members of the excitatory amino acid transporter (EAAT) family (for glutamate and aspartate) are distinguished by the counter-transport of potassium ions, and this group includes other members that transport neutral amino acids as substrate. Proteins in the sodium neurotransmitter symporter family (SNF) are characterized by 12 transmembrane domains and depend upon chloride for uptake activity. This family is made up of four subfamilies, encompassing a variety of substrates ranging from various amino acids to amines. Finally, the most recently characterized family is the glucose transporter family, which includes carriers for choline. All three of these families also include many other more distantly related prokaryotic transporters. A new classification scheme of insect SNF transporters is proposed in Section 5.9.8.5.

5.9.3.3. Excitatory Amino Acid Transporters (EAAT; SLC1)

5.9.3.3.1. Roles of glutamate and EAATs in insects L-Glutamate has been implicated in many important physiological processes in mammals including neuronal development, plasticity, and long-term potentiation, as well as in pathological conditions including epilepsy, cerebral ischemia, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and schizophrenia (Danbolt, 2001). L-Glutamate also is the most abundant amino acid in the insect CNS, acting as both an excitatory and an inhibitory neurotransmitter (Bicker *et al.*, 1988; Usherwood, 1994) via high-affinity binding/activation of postsynaptic receptors, which are classified as metabotropic and ionotropic subtypes (Monaghan *et al.*, 1989; Gasic and Hollmann, 1992). It also acts as the principal excitatory transmitter at neuromuscular junctions (Jan and Jan, 1976a, 1976b) binding to specific receptors on somatic muscles (Schuster *et al.*, 1991; Petersen *et al.*, 1997). Whereas, essential roles of glutamate in the insect CNS have been deduced from extensive immunohistochemical labeling of the ligand, its receptors, and transporters, its physiological roles, as well as their central mechanisms, remain poorly understood. Apparently, brain-specific glutamate in insects mimics processes known in the mammalian CNS, including afferent signaling and memory; e.g., it appears to mediate central mechanisms that are associated with long-term olfactory memory in the honeybee (Maleszka *et al.*, 2000), and to mediate *N*-methyl-D-aspartate (NMDA) type glutamate receptor-coupled regulatory pathways in the biosynthesis of juvenile hormone (Chiang *et al.*, 2002). Moreover, little is known about the physiology of glutamatergic transmission and glutamate/aspartate excitotoxicity in insects.

5.9.3.3.2. Insect EAATs Transporters of glutamate in the nervous system have recently been cloned and characterized from several insect species. The isolation of these genes was based on sequence similarities with vertebrate EAATs, using PCR with degenerate primers: sequence comparisons showed them to be members of this distinct family of transporters (dicarboxylate/amino acid:cation (Na^+ or H^+) symporter family by Saier, 2000b (TC 2.A.23); **Table 1**). This group corresponds to the mammalian solute ligand carrier group 1 (SLC1). Cloned insect EAATs comprise two from fruit flies (Seal *et al.*, 1998; Besson *et al.*, 1999), one from caterpillars (Donly *et al.*, 1997), one from cockroaches (Donly *et al.*, 2000), one from yellow fever mosquitoes (Umesh *et al.*, 2003), and one from honeybees (Kucharski *et al.*, 2000). They share up to 45% protein sequence identity and common secondary structures, which are characterized by six putative alpha-helical transmembrane domains and 2–4 beta-pleated domains resembling the beta-barrel structures of pore-forming ion channels (Seal *et al.*, 1998). Demonstrated substrates for these

insect EAATs include glutamate and also aspartate, which may sometimes act as a cotransmitter with glutamate. Biophysical studies with vertebrate transporters have shown that the transport of these substrates is electrogenic, involving the uptake of one amino acid$^-$ anion coupled to the cotransport of three Na^+ cations, one H^+ cation, and the countertransport of one K^+ cation (net inward flux of two positive charges per cycle). A novel property of vertebrate EAATs is the possession of a substrate-gated anion channel activity, as suggested by the presence of channel-like structures in the C-terminal portion of the protein. *Drosophila* EAAT1 protein expressed in frog oocytes induces a chloride flux that is consistent with this postulated channel-like activity (Seal *et al.*, 1998).

In situ hybridization detected the expression of both dmEAAT1 and dmEAAT2 in neuronal cells, however with distinct patterns for each transcript (Besson *et al.*, 1999). Similar extensive expression of EAAT has been detected in adult brain of the honeybee *Apis mellifera*, where it was associated with the mushroom bodies in which glial cells are rare (Kucharski *et al.*, 2000). In contrast, *in situ* hybridization, using *Trichoplusia ni* EAAT cDNA probes on whole mount caterpillar ganglia, identified a population of interfacial glial cells (Caveney and Donly, 2002). An immunohistochemical analysis on EAAT localization using antipeptide antibodies raised against the *T. ni* EAAT amino acid sequence produced selective staining in the ganglionic neuropil, as well as extensions of glial sheath cells wrapped around nerve processes that innervate skeletal muscles (Gardiner *et al.*, 2002). In contrast, an antipeptide antibody raised against the *A. aegypti* EAAT stained only the thoracic ganglia and not the muscles (Umesh *et al.*, 2003).

Comparisons between insect and vertebrate EAATs, based on structure, pharmacology, or expression, have failed to indicate clearly to which classes of vertebrate EAATs the insect transporters belong (Donly *et al.*, 1997; Seal *et al.*, 1998; Kucharski *et al.*, 2000). Most unexpected is the strong preference of fruit fly EAAT2 for aspartate as a substrate (Besson *et al.*, 2000). Sequence comparison among insect EAATs (**Figure 3**), reveals a cluster of putative aspartate transporters with similarity to the fruit fly EAAT2. Among the remaining glutamate transporters in the tree, there is no pattern relating to localization of expression, as there are examples of expression in both glial cells and neurons (drmEAAT1 is neuronal whereas trnEAAT is glial). Several putative EAATs (also compared in **Figure 3**) are derived from the recently completed mosquito (*A. gambiae*) genome and an early

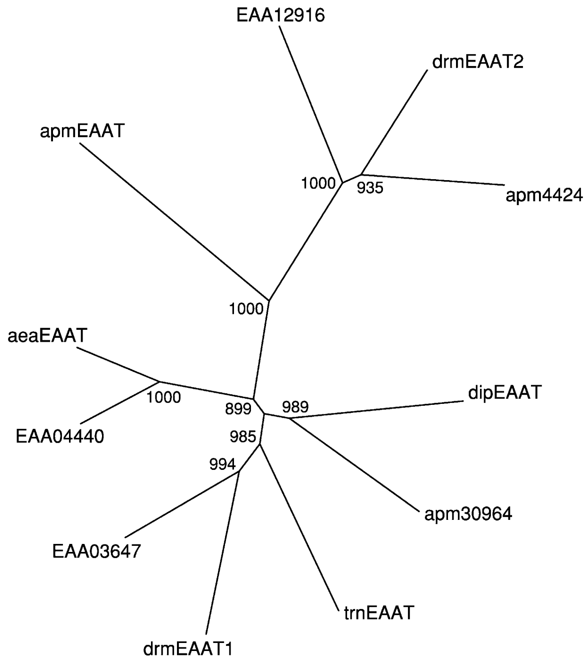


Figure 3 Dendrogram/bootstrap analysis of insect excitatory amino acid transporters. Sequence similarity was assessed using amino acid alignments in Clustal X 1.81 (Thompson *et al.*, 1997) and an unrooted tree was calculated by the ''neighbor joining'' method. Confidence values were derived by ''bootstrapping'' the dataset, using 1000 replicates and a generator seed value of 333 (Clustal X 1.81). The alignment was visualized using Treeview 1.6.6 (Page, 1996). The variable N- and C-terminal regions of the amino acid sequences were trimmed so that the region used for comparison extended from the first to the last predicted transmembrane domains. The accession numbers for the characterized transporters are found in **Table 1**. The three putative *Anopheles gambiae* EAAT sequences have accession numbers EAA12916, EAA04440, and EAA03647 as marked. The two putative honeybee EAAT sequences (in addition to the known apmEAAT) were deduced from contigs 30964 and 4424 from the initial assembly of the *A. mellifera* genome (Baylor Human Genome Sequencing Center).

assembly of the honeybee (*Ap. mellifera*) genome. Both genomes contain evidence for at least three potential EAATs. In contrast, the fruit fly genome contains only two EAATs. Based on this difference in EAAT complement between two related dipteran species, it seems possible that significant variability in EAAT complements may exist across the breadth of the Insecta.

5.9.3.4. Na^+/Cl^--Driven Neurotransmitter Symporters (SNF; SLC6)

The essential role of neurotransmitter uptake in synaptic transmission and integrative nerve function was established long before the cloning of the first transporter (Levi and Raiteri, 1974). The existence of a family of plasma membrane transporters that is responsible for Na^+ and Cl^--dependent transport in

the nervous system first became apparent when similarity was observed between two mammalian transporter cDNAs that were isolated based on protein purification and sequencing (Guastella *et al.*, 1990; Liu *et al.*, 1992a) and on expression cloning (Pacholczyk *et al.*, 1991). The similarity between these two transporters, one for GABA and the other for norepinephrine, was subsequently used to isolate a variety of other transporters in many organisms (including insects), which transport diverse substrates in the nervous system and other tissues. These transporters make up the Sodium Neurotransmitter Symporter family (SNF), which corresponds to the mammalian solute carrier group, SLC6 (TC 2.A.22; **Table 1**). All such transporters possess common features of structure and function, but nevertheless exhibit remarkable differences in their interaction with transmembrane electrochemical gradients for Na^+, K^+, and Cl^- as well as substrate selectivity, affinity, and carrier capacity. Protein sequence hydrophobicity and conformation profiles predict quite similar 12-TMD structures for these transporters, with cytosolic N- and C-terminals and an extended extracellular loop between TMDs 3 and 4 having a variable number of *N*-glycosylation sites (see Section 5.9.8.5).

Sequence comparison among vertebrate neurotransmitter transporters reveals five orthologous clusters among family members that can be defined according to substrate specificity as: (1) inhibitory neurotransmitter transporters for GABA and glycine; (2) aminergic neurotransmitter transporters for norepinephrine, serotonin, and dopamine; (3) the osmolyte transporters for betaine and taurine; (4) metabolic intermediate creatine transporters; and (5) amino acid transporters for proline and glycine (B^{0+} system). In addition, mammalian members of this family include several groups of "orphan" transporters for which no substrates and functions are yet known (Chen *et al.*, 2003; Lill and Nelson, 1998). Comparison of the amino acid sequences of insect examples also reveals orthologous clustering, however differences from the vertebrate results are evident (**Figures 4, 6,** and **10**). Phylogenetic analyses show very clear differences between two large clusters of insect SNF members (**Figures 6** and **10**), which can be defined as Neurotransmitter Transporters (NTTs) and nutrient amino acid transporters (NATs) based also on their functional characteristics, which have been determined for many cloned insect transporters (see discussion below and Section 5.9.8.5.2, respectively). Due to their clear functional and phylogenetic specificity, these clusters represent a first and most significant divergence within the insect SNF; these clusters can reasonably be defined as NTT and NAT subfamilies. In addition, most insect NTTs form orthologous pairings with mammalian complements mentioned above, as well as sharing substrate and physiological roles in neuronal signaling, each of which has been confirmed for at least one cloned and characterized insect transporter. Despite their phylogenetic proximity to B^{0+} transporters, insect NATs encompass no substantial correlations in ligand specificity to such mammalian transporters. In contrast, they represent a large and lineage-specific (paralogous) group of NATs, which apparently are absent in the mammals and some other lineages (see discussion in Section 5.9.8.5.2).

5.9.3.4.1. GABA transporters As in the vertebrate CNS, GABA serves as the primary inhibitory neurotransmitter in the insect nervous system (Callec, 1985). Immunocytochemical studies of GABAergic transmission sites using antibodies against GABA, glutamic acid decarboxylase (the essential enzyme for GABA synthesis), and various GABA receptor subunits have revealed an extensive pattern of GABA activity in the nervous systems of a variety of insect species. This pattern implies roles for GABA in diverse processes including visual and olfactory processing, as well as learning and memory (reviews: in Caveney and Donly, 2002; Umesh and Gill, 2002). The main mechanism by which GABA activity is terminated on the postsynaptic membrane is through reuptake into neurons and glial cells by high-affinity transporters (Callec, 1985). High-affinity insect GABA transporters (GATs) have been cloned and characterized from the hawkmoth, *Manduca sexta* (Mbungu *et al.*, 1995) and the cabbage looper, *T. ni* (Gao *et al.*, 1999). In each species, Northern analysis was used to localize expression of a single observed mRNA transcript to the nervous system. The hawkmoth masGAT protein has also been further localized by immunocytochemistry (Umesh and Gill, 2002). The results indicated that transporter immunoreactivity coincides closely with previously determined patterns of GABAergic activity, including staining of brain areas involved in processing visual and olfactory information.

The two characterized lepidopteran GATs show ionic stoichiometry and kinetics similar to those of the vertebrate GAT1 subtype (Mbungu *et al.*, 1995; Gao *et al.*, 1999). However, the pharmacological profiles of the lepidopteran transporters are distinct from any known vertebrate subtype, particularly in their insensitivity to several nipecotic acid derivatives, which are potent inhibitors of GAT1. Only one GAT has been found in each moth species; in

contrast, vertebrates exhibit diversity at two levels. First, the vertebrate GATs, which in the rat are enumerated as GATs 1–3, display unique cellular expression patterns and affinities for GABA. Second, various related transporters have been identified in vertebrates, showing affinities for other substrates including taurine, creatine, and betaine. The presence of multiple GATs has been reported in *Drosophila* (Neckameyer, 1998), which is in contrast to the apparently unified phenotype studied in two lepidopteran models (Mbungu *et al.*, 1995; Gao *et al.*, 1999). Analysis of the fruit fly and mosquito genomes shows that each contains only a single GAT locus (at least of the known Na^+- and Cl^--dependent type). Consequently, any diversity in GAT transcripts or proteins must result from alternative splicing of a single gene. In addition, no genes or transcripts that appear to encode taurine or creatine transporters have been identified in insects to date.

5.9.3.4.2. Monoamine transporters Monoamines that serve as neurotransmitters in insects include serotonin (5-HT), dopamine, octopamine, and histamine (Monastirioti, 1999). The neuroactive amines in insects differ from those of vertebrates in that octopamine appears to substitute functionally for norepinephrine in insects. Tyramine, the immediate precursor of octopamine, may also have a role in neurotransmission but its status has remained controversial. Recently, tyramine was shown to be the effective ligand of the fruit fly amine receptor (TyrR) in modifying larval neuromuscular junction potentials, producing new evidence for a role for tyramine as a neuromodulator (Nagaya *et al.*, 2002). Monoamines play diverse roles in insect behavior and physiology, acting in the nervous system as well as in many peripheral tissues. In the nervous system, serotonin, dopamine, and octopamine are slow-acting neurotransmitters that bind to G protein-coupled receptors and modulate various second messengers, such as cyclic nucleotides and Ca^{2+}. After being released, they are taken up by high-affinity transporters for reuse or inactivation by N-acetylation. Transporters for all three of these monoamines are present in insects, clustering according to their transport substrate in sequence comparisons of insect Na^+/ Cl^--dependent transporters (**Figures 4, 6,** and 10).

5.9.3.4.2.1. Serotonin transporter (SERT; 5HTT) Indolamine uptake activity in the insect nervous system was first demonstrated in nerve fibers of the locust brain (Klemm and Schneider, 1975), followed by the finding of high-affinity serotonin

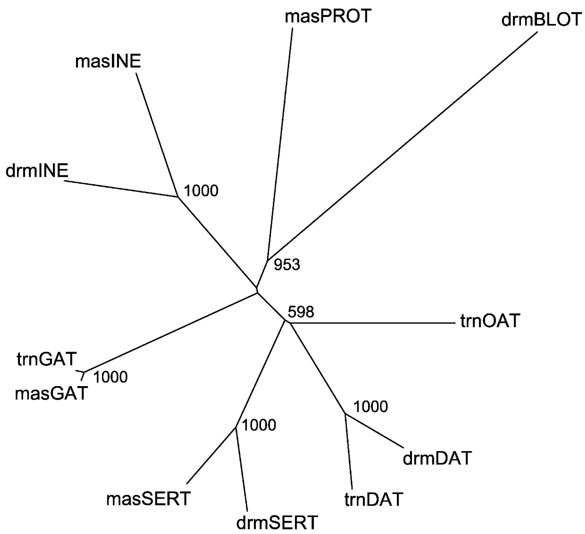


Figure 4 Dendrogram/bootstrap analysis of insect nervous system Na^+/Cl^- dependent neurotransmitter transporters. The sequences used were limited to those for which there is a characterized insect example published (accession numbers are listed in **Table 1**). The comparison was performed as in **Figure 3**, using amino acid sequences trimmed to the first and last predicted transmembrane domains.

transport activity in many other insect species. In insects serotonin is also involved in neuromuscular transmission (McDonald, 1975) and epithelial transport processes, such as fluid balance (Maddrell, 1962; O'Donnell and Maddrell, 1984; Brown and Nestler, 1985), as well as in modulation of acid/base balance and midgut alkalinization (D. Boudko, unpublished data). It also serves in the integration of complex behavior patterns in insects, including aggression and defense (Baier *et al.*, 2002; Kostowski *et al.*, 1975), feeding (Novak and Rowley, 1994) and mating arousal (Yellman *et al.*, 1997), circadian rhythms (Muszynska-Pytel and Cymboroski, 1978a; Muzynska-Pytel and Cymborowski, 1978b), and memory (Carew, 1996). The first insect serotonin transporter cDNA (dSERT) to be cloned and characterized was that from *D. melanogaster* (Corey *et al.*, 1994; Demchyshyn *et al.*, 1994). The fruit fly transporter has pharmacological properties that are distinct from those of mammalian SERTs, such as a much lower sensitivity to antidepressant antagonists. A SERT that was recently cloned and characterized from the hawkmoth, *M. sexta*, was less sensitive to cocaine binding than other SERTs (Sandhu *et al.*, 2002b). Structural modification of the cocaine-sensitive human SERT, by using chimeras that mimic fragments of the insect molecule, revealed important elements involved in the interaction with cocaine (Sandhu *et al.*, 2002b). This finding is relevant for human health and

important for understanding serotonin transporter biology; it also illustrates the value of arthropod models in uncovering structure– function relationships of complex transport proteins through comparative studies. Serotonin transporters are represented by a single gene in the *D. melanogaster* genome and apparently in the *An. gambiae* genome as well. Two conceptual translation entries seen in NCBI (EAA05837 and EAA03384) are identical by nucleotide sequence and have yet to be mapped, which together suggest that they may be fragments of a single gene copy (**Figure 6**).

5.9.3.4.2.2. Dopamine transporter (DAT) Dopamine is the most abundant catecholamine in the insect nervous system, with dopamine-containing neurons being widely distributed throughout a variety of species (Osborne, 1996). In contrast, only trace quantities of the vertebrate catecholamine transmitter, norepinephrine, can be detected in the insect nervous system. Dopaminergic neurons modulate both visceral and skeletal muscle activity, with dopamine affecting flight motor patterns in *M. sexta* and the escape circuit in cockroaches (Osborne, 1996). Dopamine release from peripheral nerve endings also stimulates salivary gland secretions in many species. The first insect dopamine transporter (DAT) was cloned and characterized from the fruit fly (Porzgen *et al.*, 2001). This transporter exhibited substrate selectivity similar to vertebrate DATs (dopamine and tyramine are preferred substrates), but showed a distinct pharmacological profile that is reminiscent of mammalian norepinephrine transporters (with high affinities for tricyclic antidepressants). A second DAT, isolated from the cabbage looper moth, has similar properties (Gallant *et al.*, 2003). These transporters cluster closely, along with another putative DAT sequence that is present in the mosquito genome (**Figures 4, 6, and 10**). It has been proposed that these invertebrate DATs represent conserved copies of a primordial catecholamine transporter that existed before the divergence of mammalian catecholamine carriers into subtypes responsible for dopamine and norepinephrine uptake (Porzgen *et al.*, 2001).

5.9.3.4.2.3. Octopamine transporter (OAT) Octopamine is the monohydroxylic analog of norepinephrine in insects. It is present in high concentrations in a range of insect tissues including the nervous system, where it may act as a neurotransmitter, neurohormone, and neuromodulator (Roeder, 1999). It modulates afferent and efferent signaling in locusts (Homberg, 2002; Leitch *et al.*, 2003). Na^+-dependent (and independent) octopamine uptake has been reported in various cockroach tissues (Sinakevitch *et al.*, 1994) and firefly lantern (Robertson and Carlson, 1976; Copeland and Robertson, 1982). In the honeybee, it mediates consolidation of olfactory memory (Farooqui *et al.*, 2003) and hygienic behavior (Spivak *et al.*, 2003) as well as complex social behavior, including labor establishment (Schulz *et al.*, 2002). Despite abundant neurotransmitter roles for octopamine, examination of the completed dipteran genomes reveals no alternative genes that may encode a Na^+/Cl^--dependent transporter for octopamine uptake. In these insects, it has been proposed that octopamine may be dispersed by an alternate mechanism, or perhaps taken up by a member of an unrelated transporter family (Porzgen *et al.*, 2001). Nevertheless, a novel transporter cDNA that was isolated and cloned from the lepidopteran *T. ni*, using RT-PCR with degenerate primers, showed remarkable sequence similarity to DAT transporters (Malutan *et al.*, 2002). However, this transporter is distinct from known DATs in that it has a high affinity for octopamine along with tyramine and dopamine; in addition it is expressed in a different subset of neuronal cells in the caterpillar nervous system than DAT. This octopamine transporter (OAT) showed relaxed ionic dependency relative to other insect monoamine transporters and was generally resistant to pharmacological inhibitors of monoamine transporters. Comparison of the OAT sequence with other Na^+/Cl^--dependent transporters shows that it clusters with mammalian norepinephrine transporters but not other insect transporters, including those for monoamines (**Figures 6 and 10**). OAT emerges almost equidistant from the serotonin and dopamine transporter branches in a high-resolution dendrogram (**Figure 4**). The extent to which this novel type of transporter is distributed among insect orders remains to be determined.

Finally, most of the histamine in the insect nervous system is in the retina and optic lamina, where it functions as an inhibitory neurotransmitter (Stuart, 1999). However, searches for a histamine transporter based on expected homology to other monoamine transporters have detected no putative candidates. This failure may indicate that the histamine transporter is a member of a novel or unrelated transporter family, as in the case of the delayed discovery of transporters for choline in the nervous system (see Section 5.9.3.5). Alternatively, functional screening may yet identify a histamine transporter.

5.9.3.4.3. Glycine and proline transporters In vertebrates, a distinct subfamily of Na^+/Cl^--dependent NTTs transports the amino acids glycine (GLYT)

and proline (PROT) (Deken *et al*., 2002). Multiple subtypes of transporters for the inhibitory amino acid glycine are expressed both in the CNS and in peripheral tissues. Although arthropods apparently lack receptors for glycine (Witte *et al*., 2002), sequence comparison with vertebrate GLYTs reveals potential Gly type transporters in both the *D. melanogaster* and *An. gambiae* genomes (CG5549 or NP_611836 and EAA07088, respectively). Vertebrates have only a single high-affinity proline transporter type (it is brain specific), which is thought to be associated with modulating activity at glutamatergic synapses (Deken *et al*., 2002). In insects, proline is an alternative energy source and major osmolyte, but its role in the nervous system remains unproven. Nevertheless, a Na^+/Cl^--dependent proline transporter was recently cloned from *M. Sexta*, where it is predominantly expressed in the nervous system (Sandhu *et al*., 2002a). This protein transported proline exclusively when heterologously expressed in *Xenopus* oocytes. Sequence comparison shows that the hawkmoth PROT is related to known vertebrate glycine and proline transporters (Sandhu *et al*., 2002a). The *D. melanogaster* and *An. gambiae* genomes also each contain a related sequence (CG7075 or NP_723303 and EAA12299, respectively) representing putative orthologs. Moreover, two gene products that apparently represent alternative splicoforms of a single gene in the 2L chromosome are present in *D. melanogaster* expressed sequence tags (ESTs for NP_723303 and NP_609135, **Figure 6**).

5.9.3.4.4. Orphan inebriated (ine) and bloated tubules (blot) transporters The identification of Na^+/Cl^--dependent transporter family members based on structural similarity has resulted in the discovery of many putative transporters for which the substrates are not known. Many of these so-called “orphan” transporters are larger than other Na^+/Cl^--dependent transporters, with extended fourth and sixth extracellular loops and a larger C-terminus. The expression patterns of these orphan transporters are often diverse, with extensive nervous system and peripheral distributions. Insect examples include the *Drosophila* blot (bloated tubules) and *ine* (inebriated) proteins, and the *M. sexta ine* protein (ms*ine*). The “inebriated” locus, relating to a characteristic “inebriated” behavior phenotype (Stern and Ganetzky, 1992) was initially cloned from *D. melanogaster* and its expression was mapped by *in situ* hybridization in the posterior hindgut, Malpighian tubules, anal plate, garland cells, and a subset of cells in the CNS of developing embryos (Soehnge *et al*., 1996). It has been proposed by analogy with other NTTs that the neuronal hyper-excitability characteristic of the *ine* phenotype is a result of failure in some neurotransmitter uptake due to *ine* mutations (Soehnge *et al*., 1996); however, the identity of any such ligands remains elusive. Additional transgenic characterization led to the hypothesis that a neuronal dm*ine* isoform mediates downregulation of sodium channels via a neurotransmitter uptake (Huang and Stern, 2002) whereas an epithelial dm*ine* isoform compensates osmotic load in the hindgut and Malpighian tubules via an osmolyte carrier (Huang *et al*., 2002). Substantial progress in characterization of *ine* has been made by the heterologous expression of an ine-orthologous transporter from *M. sexta* in *Xenopus* oocytes (Chiu *et al*., 2000). That study revealed that *ine* responds to hyper-osmotic stimuli via the inositol triphosphate-coupled release of intracellular Ca^{2+}, which subsequently activates Ca^{2+}-dependent K^+ channels. The phylogenetic analysis indicates a single *ine* gene in *An. gambiae* and two splicoforms of a single *ine* gene in *D. melanogaster* (CG15444, located on the L2 chromosome). The insect *ine* group, with undefined ligand and transient functions, represents an evolutionary root of the NTTs and apparently provides primordial genetic sources in the differentiation of GLYT and GAT and monoamine neurotransmitter transporters. Therefore, the reasons why substrates for these putative transporters have remained elusive may not only be technical, but biological as well. The primary function of some of these proteins may be to regulate transmembrane ion fluxes in different tissues including the CNS.

Another orphan gene, named by similarity with a mammalian orphan group, has been extensively analyzed in *Drosophila* (Johnson *et al*., 1999). This gene, named bloated tubules (*blot*), exhibits complex and dynamic expression patterns during embryogenesis and supports processes that are essential for cell division, differentiation, and epithelial morphogenesis. Insect *ine* and *blot* do not cluster in high-resolution phylogenetic analyses. Nevertheless, their broader phylogenetic proximity with other uncharacterized “orphan type” sequences, as well as the related nutrient-type transporters, msKAAT1 and ms CAATCH1, from *M. sexta* and aeAAT1 and agAAT8 from mosquito (**Figures 6 and 10**; Section 5.9.8.5.2) indicates that several discrete functional groups may eventually emerge from under the “orphan” umbrella. The analysis showed five putative orphan genes in *An. gambiae* and four genes encoding seven presently known isoforms in *D. melanogaster*, which are in phylogenetic proximity with *blot* and represent a transition between the SNF

(Na^+/Cl^--dependent transporter) and PAT (proton amino acid transporter) families (Figure 6).

5.9.3.5. Choline Transporters (CHTs)

Acetylcholine (ACh) is the principal excitatory neurotransmitter in the insect CNS, although it has not been shown to have any role in transmission at the insect neuromuscular junction (Callec, 1985). ACh released from neurons activates postsynaptic receptors of both the ionotropic and metabotropic type and is then eliminated exclusively by enzymatic and nonenzymatic degradation, rather than a reuptake mechanism, as with other transmitters. Extracellular cholinesterases, widely exploited as insecticide targets, catalyze hydrolysis of ACh to choline and acetate; then specific transport systems remove the choline from the synaptic cleft via neuronal high-affinity, Na^+-driven reuptake. The resulting neuronal choline uptake represents a rate-limiting initial step in the ACh synthesis pathway in insects (Breer and Knipper, 1990).

An insect choline transporter (CHT) was isolated first from locust synaptosomes, using monoclonal antibodies that specifically blocked choline uptake *in vivo* (Knipper *et al*., 1989). The transporter was shown to be sensitive to the choline uptake inhibitor hemicholinium-3 and was localized by immunohistochemistry in ganglionic neuropils in the locust CNS. Initial attempts to identify CHT cDNAs in mammals using homology-based PCR were unsuccessful until 2000 when a first draft of the *C. elegans* genomic project became available. Using this information Okuda and colleagues cloned a nematode CHT (cho-1) and almost immediately thereafter the high-affinity hemicholinium-3 sensitive rat CHT1 (Okuda and Haga, 2000). All currently known CHTs are characterized by a 13-transmembrane domain configuration and are members of the sodium ion-dependent glucose transporter family, which corresponds to mammalian SLC5, with a SLC5A7 membership tag assigned to CHTs. This group includes transporters for diverse substrates, including glucose and galactose (urea and water), I^- (ClO_4^-, SCN^-, NO_3^-, Br^-), biotin, lipoate and pantothenate, as well as choline. In addition, SLC5 members may act as glucose *myo*-inositol (glucose) Na^+ (H^+) channels. This family of transporters is present in species from cyanobacteria to humans. They are placed in the Solute:Sodium Symporter (SSS) family (Saier, 2000b; TC 2.A.21), which comprises one of the largest groups of solute carriers in mammals (SLC5; Table 2). A putative insect CHT recently cloned from the cabbage looper (NCBI; trnCHT: AY629593; see Table 1) shows

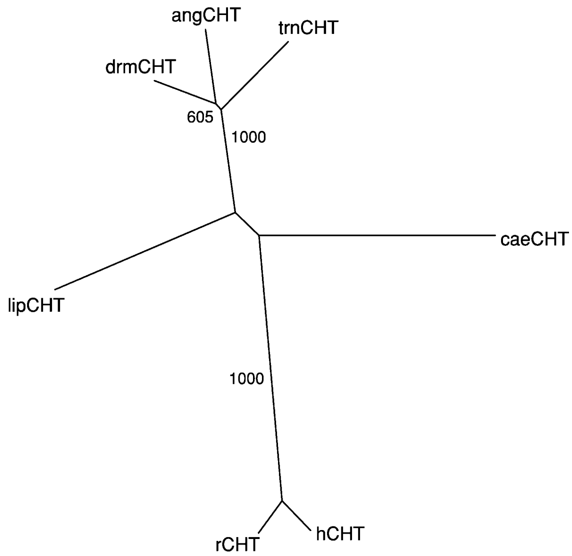


Figure 5 Dendrogram/bootstrap analysis of the amino acid sequences of insect choline transporters. Putative insect CHT sequences are included in the analysis, resulting in a discrete clade separate from the other invertebrate CHTs. The comparison was performed as in Figure 3, using amino acid sequences trimmed to the first and last predicted transmembrane domains. Accession numbers for the sequences are: human (hCHT; NP_068587), rat (rCHT; NP_445973), horseshoe crab (lipCHT; AAG41055), nematode (caeCHT; NP_502539), fruit fly (drmCHT; NP_650743), mosquito (angCHT; EAA07459), and cabbage looper (trnCHT; AY629593).

strong similarity to the known CHTs, and forms an orthologous cluster with putative CHT sequences from the fruit fly and mosquito genomes (Figure 5). The pharmacological effects of blocking choline uptake by this CHT may be different from blocking other neurotransmitter transporters since ACh synthesis should be suppressed without transmitter concentration building up in the synaptic space. It remains to be seen whether CHT may serve as a useful target for insecticide activity.

5.9.3.6. Vesicular Membrane Transporters

Neurotransmitters are taken up from the synaptic cleft by plasma membrane transporters and are sequestered in synaptic vesicles, thereby protecting the cell from potential toxicity and protecting the transmitters from intraneuronal metabolism (Masson *et al*., 1999). Low-affinity transporters, which are located in the vesicular membrane, transfer the transmitters into the vesicle lumen. Accumulation of transmitter in vesicles also facilitates uptake of transmitter into the cell by plasma membrane transporters, since it lowers the concentration gradient across the neuronal membrane. Vesicular transport can employ H^+ and ligand-specific electrochemical

motive forces that are induced by the activity of H^+ V-ATPases located on the vesicular membrane. Typically, such transporters exchange one or more protons inside the vesicle with a neurotransmitter molecule in the cytoplasm. There is less molecular information on these transporters than on their plasma membrane counterparts; however, it has become clear from sequence comparisons that the vesicular transporters characterized to date fall into three separate families; these are discussed immediately below.

5.9.3.6.1. Vesicular inhibitory amino acid transporters (vIAATs or vGATs) A vesicular GABA transporter was initially identified in *C. elegans* as a product of the *unc*-47 gene that mimics a GABA-defective behavior phenotype (McIntire *et al.*, 1993). This gene encodes a 10-TMD protein that colocalizes with synaptic vesicles of GABAergic neuronal terminals, where it mediates low-affinity vesicular accumulation of inhibitory neurotransmitters via an H^+/GABA(Gly) antiporter mechanism ($K_m^{GABA(Gly)} = 5(25)$ mM; McIntire *et al.*, 1997). Subsequently, when mammalian orthologs were cloned it was recognized that glycine may also be a physiological substrate, leading to the term vesicular inhibitory amino acid transporter (vIAAT, more commonly vGAT). These transporters are distantly related to other transporters that resemble SLC36 and SLC38 in mammals and bear some resemblance to a group of permeases that place them in the Amino Acid/Auxin Permease (AAAP) family of transporters (TC 2.A.18) in the TC classification scheme (Saier, 2000b; see also phylogenetic analysis in Section 5.9.8.1). Functional characterization of rodent orthologous transporters revealed that they contribute to GABA- and glycine-ergic signaling and glycine-dependent vesicular accumulation of GABA (Sagne *et al.*, 1997). However, no insect vIAAT has been characterized as yet.

5.9.3.6.2. Vesicular monoamine and acetylcholine transporters (vMATs and vAChTs) The first recognized and most thoroughly studied vesicular transporters make up the Vesicular Neurotransmitter Transporter (VNT) family (TC 2.A.1.22), a group classified within the Major Facilitator superfamily (Saier, 2000b). They include the vesicular transporters for monoamines (vMATs) and for acetylcholine (vAChT). These proteins have 12 predicted TMDs, which differ from the vIAATs (above) whose sequences predict only 10 (Masson *et al.*, 1999). Many vMATs and vAChT related transporters have been cloned from a variety of organisms, but the only insect vesicular transporter that has been studied to date is the *D. melanogaster* vAChT (Kitamoto *et al.*, 1998; Kitamoto *et al.*, 2000a; Yasuyama *et al.*, 2002). Additional data and analysis of phylogenetic proximity of dipteran vMATs to *H. sapiens* vMATs are included in Sections 5.9.5 and 5.9.9.1. Like the gene for nematode and vertebrate transporters, the gene for the fruit fly vAChT nests completely within the first intron of the choline acetyl transferase gene, encoding the enzyme that synthesizes ACh (Kitamoto *et al.*, 1998). Mutation analysis of the fly vAChT gene demonstrated that the function of the transporter is essential for viability, because mutants are recessive lethal (Kitamoto *et al.*, 2000b). VAChT is involved in cholinergic transmission since decreased activity in heterozygous mutants resulted in defects in the giant fiber pathway, a neural circuit known to contain cholinergic synapses. Functional studies of the fruit fly vAChT in an isolated cell system should yield information on its kinetics.

5.9.3.6.3. Vesicular glutamate transporters (vGLUTs) Vesicular transporters for glutamate were only recently recognized when a human brain-specific inorganic phosphate transporter (BNPI) was found to also transport glutamate (Bellocchio *et al.*, 2000). Three subtypes of mammalian vesicular glutamate transporter are now known (SLC17; see Section 5.9.9.4), but no insect examples have yet been studied. Based upon their structure, these transporters also belong to the Major Facilitator superfamily (see above), but within a different family, the Anion:Cation Symporter (ACS) family (TC 2.A1.14; **Table 1**).

5.9.4. Nutrient Amino Acid Transport in Insects

5.9.4.1. Early Studies of Insect Amino Acid Uptake

Clements (1992) reviewed the work on amino acid uptake in Malpighian tubules of living insects by Wigglesworth, Ramsay, Phillips, and others. Giordana *et al.* (1989), Castagna *et al.* (1997), and Wolfersberger (2000) reviewed the physiology and biochemistry of amino acid uptake in isolated midguts. In the early 1970s, Signe Nedergaard (1972) reported the transport of alpha-aminoisobutyric acid from lumen to blood-side of the isolated caterpillar midgut by a voltage-dependent process that requires oxygen and K^+ but is independent of active K^+ transport. Studying brush border membrane vesicles from caterpillar midgut, Hanozet, Giordana, and Sacchi (Hanozet *et al.*, 1980) showed

that phenylalanine is cotransported with K^+ by a voltage-dependent process. Since the K^+ pump is electrogenic (Harvey *et al.*, 1968) and oxygen dependent (Harvey *et al.*, 1967), it appeared that it uses energy from ATP hydrolysis to charge the apical plasma membrane (lumen positive to cell) and that the voltage drives a positively charged K^+ amino acid complex across the membrane, carrying both solutes into the cell. The oxygen dependence arises because the midgut has little anaerobic reserve, ATP levels dropping as oxygen pressure drops (Mandel *et al.*, 1980). After the K^+ pump-containing, goblet cell apical membrane was isolated (Cioffi and Wolfersberger, 1983; Harvey *et al.*, 1983), the K^+ pump was shown to consist of an H^+ translocating ATPase (Wieczorek *et al.*, 1989) that charges the membrane and is thought to drive secondary K^+/$2H^+$ exchange (Wieczorek *et al.*, 1991). The membrane charging by the V-ATPase has been confirmed many times (Wieczorek *et al.*, 1999b) but the nature of the secondary K^+/H^+ exchange remains unresolved. The working hypothesis for amino acid uptake is that the H^+ V-ATPase charges the apical membrane (lumen positive to cell) and that the voltage drives amino acid:K^+ cotransport from lumen to cell.

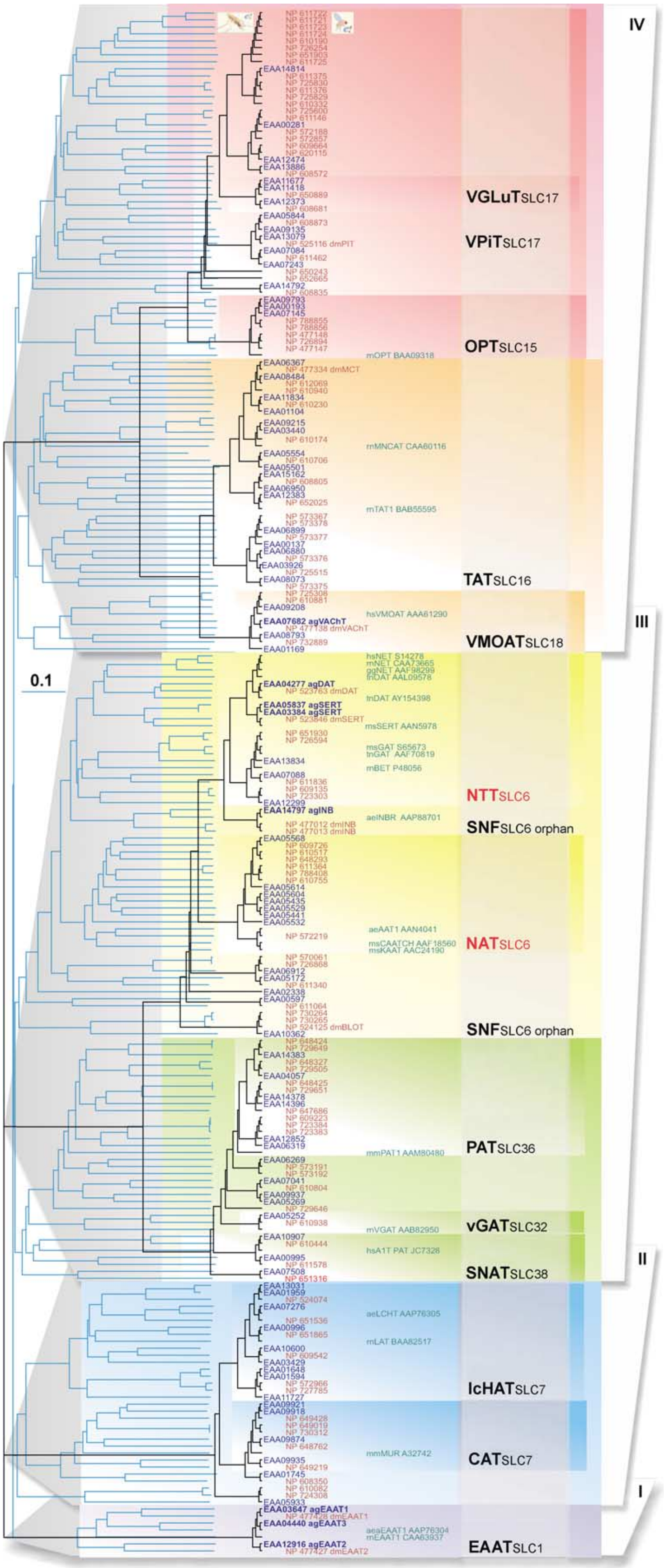
5.9.4.2. Amino Acid Uptake by Brush Border Membrane Vesicles

Several major amino acid transport systems have been characterized in brush border membrane vesicles (BBMV) from lepidopteran midgut. Uptake has been studied in *Philosamia cynthia* (Giordana and Parenti, 1994), *M. sexta* (Hennigan *et al.*, 1993a, 1993b; Reuveni and Dunn, 1994; Bader *et al.*, 1995; Liu and Harvey, 1996a, 1996b), *Bombyx mori* (Parenti *et al.*, 2000; Leonardi *et al.*, 2001a, 2001b) and *Pieris brassicae* (Reuveni and Dunn, 1994; Giordana *et al.*, 1989; Wolfersberger, 2000). Facilitative transport systems are widespread (Wolfersberger, 2000) along with separate K^+- or Na^+-dependent neutral and cationic amino acid transporters, which are driven mainly by the membrane potential (Giordana and Parenti, 1994; Castagna *et al.*, 1997). Some evidence suggests that leucine uptake is partially modulated by a hormonal and nutrient-related mechanism (Leonardi *et al.*, 2001b). Nutrient glutamate transport appears to be anomalous (Xie *et al.*, 1994). In summary, four principal transport systems were characterized: (1) a broad spectrum neutral amino acid:K^+ cotransport system; (2) an arginine K^+ cotransport system; (3) an acidic amino acid system with anomalous properties; and (4) a glycine or proline transporter. Extensive kinetics studies on BBMV showed that the transport systems had low affinity for amino acids (K_m around 1 mM) and K^+ (K_m around 30 mM). Presteady state kinetic studies on BBMV suggested a symmetric on/off mechanism (Parenti *et al.*, 1992). The kinetic studies also suggested that there might be more than one transporter for each amino acid and that several amino acids use the same transporter. Hence, the kinetic parameters from BBMV studies represent average values rather than values from single transporters.

Surprisingly, there are no available references on amino acid uptake by isolated brush border membrane vesicles from larval midgut of mosquitoes. There is a single report of uptake of radiolabeled amino acids in intact midgut of adult *Anopheles stephensi* (Schneider *et al.*, 1986). There is a published method for vesicle preparation from midgut preparations, but it was used only for *Bacillus sphaericus* binding studies (Nielsen-LeRoux and Charles, 1992). Therefore, most existing insect vesicle transport data are from caterpillars, taking advantage of their large size in preparation of large tissue samples. Nevertheless, dipteran larvae of *An. gambiae* and *D. melanogaster* serve in the postgenomic era as more convenient and comprehensive models for molecular, cellular, and integrative studies of transport biology, including explorations of amino acid transport metabolons. BBMV studies on caterpillars confirmed voltage-driven, amino acid transport.

5.9.5. Postgenomic Analysis of Insect AATs

In the postgenomic era, there is a rapid advance toward the identification of virtually all transport proteins in a growing number of genomic models. This advance facilitates the accumulation and systematization of molecular and physiological data, which makes it possible to deduce the function of particular transporters by their phylogenetic proximity, which in turn enables one to identify new species and groups of transporters with previously unknown functions. This review takes advantage of this new opportunity. A matrix of dipteran transporters, together with reference sequences of characterized amino acid transporters, was used to establish phylogenetic reciprocity within comprehensive genomic populations of insect amino acid transporters. The resulting phylogram (**Figure 6**) incorporates the best reciprocal matches from predicted gene products of *D. melanogaster* (Adams *et al.*, 2000) and *An. gambiae* (Holt *et al.*, 2002), along with characterized metazoan amino acid transporters, which are deposited in the National Center for Bioinformatics (NCBI) databases. The properties of insect amino acid transporters will be discussed in the framework of the classical divisions


IV
III
II
I
0.1
VGLuT SLC17
VPiT SLC17
OPT SLC15
TAT SLC16
VMOAT SLC18
NTT SLC6
SNF SLC6 orphan
NAT SLC6
SNF SLC6 orphan
PAT SLC36
vGAT SLC32
SNAT SLC38
lcHAT SLC7
CAT SLC7
EAAT SLC1

of neurotransmitter phenotypes and a novel division of nutrient amino acid phenotypes. Then this description is integrated into a new scheme that discloses phylogenetic relationships of such transporters in the order in which they are self-clustered in the low-resolution, unrooted phylogram (Figure 6), which was constructed by using ClustalX software for progressive multiple sequence alignment and comparison (Thompson *et al*., 1997). Since there are an indefinite number of phenotypic transitions and putative substrates within several phylogenetic groups, an entire group of amino acid transporters was included if at least one of its members, or its reciprocal sequence homolog, serves as a carrier of amino acids or related substrates. The final phylogenetic tree (Figure 6) is a product of multiple iterations of comprehensive subsets of sequences. It includes 85 gene products of *An. gambiae* (blue font color, proximal column) and 108 gene products of *D. melanogaster*, which are encoded by 88 genes, but some are represented by several established splicoforms (red font color, middle column). Included also are 25 transporters, which were cloned and characterized from other insect species (green font color, distal column). Several *An. gambiae* gene products, which are definitely orthologs of cloned and characterized transporters, are indicated in bold. Six families that differ substantially in their electrochemical transport mechanisms are represented by different background colors. In addition to the integrated low-resolution tree in Figure 6, analyses of phylogenetic proximity of mammalian and dipteran transporters for each particular family are included, which are shown here as ClustalX dendrograms, phylograms, and/or cladograms, depending upon the view that is best suited for representing peculiarities within a particular group. Due to the lack of clear consensus in transport protein classification, we will follow the most natural solute carrier nomenclature (SLC), which rests on a comprehensive subset of characterized *H. sapiens* and other mammalian transporters. Four large clusters that exhibit phylogenetic proximity between different families of insect amino acid transporters are introduced in Figure 6 as panels with a Roman integer.

Special emphasis is placed on the insect Nutrient Amino Acid Transporter (NAT) subfamily which is introduced here for the first time. It resides within the SNF, and apparently represents a group of lineage-specific, essential nutrient amino acid transporters, which appear to be good targets for insect population control (see Section 5.9.4).

Figure 6 Comprehensive low-resolution phylogram (blue tree) and cladogram (black tree) of dipteran amino acid transporters. The unrooted phylogenetic trees were constructed using Clustal X software (Thompson *et al*., 1997), which utilizes a neighbor-joining algorithm for tree reconstruction (Saitou and Nei, 1987), converted to Windows metafile by TreeView(Win32) (Page, 1996) and decorated with CorelDraw software. This phylogenetic analysis includes 193 dipteran sequences (Smith-Waterman all-against-all e-value cutoff $<10^{-5}$), which represent putative orthologs of 10 solute carrier families (SoLute Carrier numbers; SLC with Arabic integers) and 15 identified[1] phenotypes of amino acid transporters (large font on the transparent ribbon). Sequences are represented by nonredundant subsets of gene product accession numbers, which can be used to retrieve these sequences from NCBI protein databases (National Center for Biotechnology Information (***NCBI***)[2]). First row from the left (blue font) are 85 putative gene products identified in *An. gambiae*, each of which is represented by a single splicoform; second row (red font) 108 gene products found in *D. melanogaster*, which are encoded by 88 genes, but some are represented by several established splicoforms; third row (green font) represent 25 characterized transporters from various species that are used to reference tree clusters[3]. Common transporter phenotype abbreviations are used, e.g.: EAAT, excitatory amino acid transporter; CAT, cationic amino acid transporter; lcHAT, light chain subunit of heterodimeric amino acid transporter; SNAT, sodium neutral amino acid transporter; vGAT, vesicular GABA transporter; PAT, proton amino acid transporter; SNF, sodium neurotransmitter transporter family; NAT, nutrient amino acid transporter; NTT, neurotransmitter transporter; vMAT, vesicular monoamine transporter; TAT, T-system amino acid transporter; OPT, oligopeptide transporter; P_iT, inorganic phospate (P_i) transporter; vGluT, vesicular glutamate transporter. Sequences from different lineages are shown in different font color, e.g: *Ae. aegypti*, green; *An. gambiae*, blue; *D. melanogaster*, red; *H. sapiens*, black. Accession numbers from first draft of *An. gambiae* genome annotation (EAA numbers) are duplicated by accession numbers for recently reviewed sequences (XP_#). Tree reconstruction was done from the unaltered alignment of sequences as they were deposited in the NCBI database, so be aware that some *An. gambiae* sequences may have incorrect composition of open reading frame as well as prediction of 3′ and 5′ ends, which could produce tree distortion. Scale bar are evolutionary distances in mutations per site. [All families in **Figure 6** are shown in amplified form in appropriate sections of this chapter.]

[1]Not all transport phenotypes are shown in this phylogram; see other figures for details.

[2]Low dash, which links NP and numerical integers, are missing due to file conversion and must be inserted to avoid accession errors, e.g. NP 477427 represents NP_477427. Accession numbers starting with EAA integer are from first draft of *An. gambiae* genome annotation; since a few of them will disappear during future revisions of genomic databases they are duplicated by new accession numbers started with XP_ in the high resolution phylogram (Figures 8–10 and 13–17).

[3]Each referencing sequence is represented by a transporter phenotype name following by an accession number, e.g., msCAATCH AAF18560 is *M. sexta* cation amino acid transporter channel; other species abbreviations here and on other figures are: ae, *Ae. aegypti*; dm, *D. melanogaster*; gg, *Gallus gallus*; hs, *Homo sapiens*; rn, *Rattus norvegicus*; mm, Mus musculus; tn, *Trichoplusia ni*.

5.9.6. AAT Cluster I (SLC1)

This type of transporter, abundant in metazoan lineages and present in bacteria and Archaea, is absent in plants and fungi (**Table 2**). Mammalian SLC1 transporters are extensively characterized, comprising two ASCT (alanine, serine, and cysteine transporters) and five EAAT genes in *Homo sapiens*. Phylogenetically, EAAT is a progeny of the ASCT group which together comprise only one SLC1 (solute carrier type 1) family in mammals. It is devoted to small nutrient and excitatory (glutamate and aspartate) amino acid transporters. Mammalian SLC1 genes encode two classically characterized carrier systems (**Table 2**). System ASC (ASCT1 and 2) serves as a facultative sodium ion driven substrate provider of glutamine, alanine, serine, cysteine, and asparagine in tissues with a low capacity or absence of anabolic pathways for the production of such amino acids (Arriza *et al.*, 1993; Utsunomiya-Tate *et al.*, 1996; Furuya and Watanabe, 2003; Wolfgang *et al.*, 2003) and in proliferating tissues, including tumors (Kekuda *et al.*, 1996; Li *et al.*, 2003). Intriguingly, some transporters of this group have been identified as retroviral receptors (Rasko *et al.*, 1999); for example, SLC5A1 (ASCT2) mammalian transporters are utilized as a surface binding receptor and an intracellular gateway by a diverse group of retroviruses (Marin *et al.*, 2003).

System $X^{A,G}$ (EAAT) maintains local glutamate/aspartate concentrations, including those in central excitatory insect synapses (Donly *et al.*, 2000), glial tissue (Kanai and Hediger, 1992; Kanai *et al.*, 1993; Kirschner *et al.*, 1994; Suchak *et al.*, 2003), visual and olfactory afferents (Arriza *et al.*, 1997; Besson *et al.*, 1999; Kucharski *et al.*, 2000), macrophages (Rimaniol *et al.*, 2000), and osteocytes (Huggett *et al.*, 2002). Mammalian EAAT genes appear to have diverged through adaptation for particular physiological conditions (Arriza *et al.*, 1994; Amara and Fontana, 2002) and specifically may expand their functions beyond the tuning of excitatory amino acids uptake at membranes that surround a synaptic cleft. For example, EAATs may serve as facultative providers of cysteine for glutathione synthesis (Chen and Swanson, 2003) or act as leak channels and/or ligand-gated chloride channels (Fairman *et al.*, 1995; Arriza *et al.*, 1997).

Apparently, the ASCT group is absent from dipteran genomes, which also appear to have few EAAT genes. EAAT comprises two established genes in *D. melanogaster* and three genes in *An. gambiae*, which split into two clusters in the dipterans (**Figure 6**), but have no obvious orthologs among the five mammalian transporters. A brief description of cloned EAATs from several insects is given above in Section 5.9.3.3.

5.9.7. AAT Cluster II (SLC7)

Like Cluster I, Cluster II corresponds to one solute carrier family in mammals (SLC7) that integrates two large functionally and structurally different groups of transporters, the cationic amino acid transporter (CAT) subfamily and the heterodimeric amino acid transporter (HAT) subfamily, based on phylogenetic, structural, and physiological properties (Verrey *et al.*, 2003). SLC7-related transporter heterogeneity has been partially explored in insects and one representative HAT-homologous sequence (AeaLAT) has recently been cloned from *Ae. aegypti* (Jin *et al.*, 2003). Phylogenetic analysis of insect transporters revealed three specific clusters. Two large and distal clusters apparently comprise insect versions of CAT and HAT subfamilies, respectively. The third small cluster is parental to the CAT/HAT cluster (**Figures 6** and **7**), which by its phylogenetic loci represents transporters similar to mammalian orphan SLC7A4, with an as yet indefinite function (Wolf *et al.*, 2002). In mammals SLC7 genes encode a broad variety of transport systems including sodium independent b^+, y^+, y^+L (for cationic AA) as well as $b^{0,+}$ and a sodium-dependent system $B^{0,+}$; y^+L (for neutral AAs) (Closs, 2002; Verrey *et al.*, 2003). Since very few insect transporters in this phylogenetic group are characterized, a brief description of characterized mammalian transporters is included below, along with a description of the phylogenetic pattern of related dipteran amino acid transporters (**Figure 7**).

5.9.7.1. Cationic Amino Acid Transporters (CATs)

The cationic amino acid transporter (CAT, SLC7A1–4) subfamily comprises 14 TMD transporters that are typically located on the plasma membrane (Wolf *et al.*, 2002). Most of these transporters regulate local concentrations of cationic amino acids through (1) facilitated diffusion and/or (2) differential amino acid exchange between relatively saturated physiological domains in organisms, e.g., growing and/or actively metabolizing cells, as well as nutrient and secretory epithelia (Verrey *et al.*, 2003). The first CAT was identified as the receptor for murine ecotropic leukaemia viruses in the mouse, MuLVR or mmCAT1, which mimics a y^+-transport system (sodium ion-independent L-arginine, L-lysine, and L-ornithine uptake) phenotype in *Xenopus* oocytes (Albritton *et al.*, 1989; Kim

Table 2 Classification properties and taxonomic proximity of insect amino acid transporters (last five columns indicate taxonomic proximity by Blast LINK, NCBI; value is protein number, cutoff max 200; database 13.10.2003)

Type	*Mammalian SLC members (protein name, respectively)*	*Insect protein*	*Transport systems (some aliases)*	*Substrate selectivity (weak substrate)*	*TMDs (other prediction)*	*Carrier type*	*Apparent affinity*	*Stoichiometry ↓ absorption ↑ emission* (uncoupled)	*Representative H. sapiens*	*Orthology (proximity) An. gambiae*	*Archaea*	*Bacteria*	*Metazoa*	*Fungi*	*Plant*
Cluster I															
ASCT	SLC1A4,5 (ASCT1,2)	Absent	BO,ATBO,SA AT	A,S,C,(T,Q)	8(6,10)	S	L,M	$\downarrow Na^+Sb(Cl^-)$	NP_003029	Figure 6	7	61	132		
EAAT	SLC1A1,2 (EAAT3,2)	EAAT2 (dEAAT2)	X^-_{AG}	E,D($K_m^O << K_m^E$ in dEAAT2)	8(6,10)	E S	L,M H	$\downarrow Na^+Sb\uparrow Sb$ $\downarrow 3Na^+Sb^-H^+\uparrow K^+$	NP_004161	[EAA12916]	5	62	132		
	SLC1A3,6, 7(EAAT1,4,5)	Absent	X^-_{AG}	E,D	8(6,10)	S	H	$\downarrow 3Na^+Sb^-H^+\uparrow K^+$	NP_004163		7	58	135		
		EAAT1(dEAAT1)	X^-_{AG}	E,D	8(6,10)	S	H	$\downarrow 2\text{–}3Na^+Sb^-H^+\uparrow K^+$		EAA03647	7	64	129		
		EAAT3(AeaEAAT)	X^-_{AG}	E,D	8(6,10)	S	H	$\downarrow 2\text{–}3Na^+Sb^-H^+\uparrow K^+$	Figure 6	EAA04440	7	63	130		
Cluster II															
	Insect specific				14					EAA05933		13	39		6
	Insect specific				14					EAA01745		56	125		17
CAT	SLC7A1(CAT1)		y^+	R,K,H	14	U,E	L,M	$\downarrow Sb$; $\downarrow Sb\uparrow Sb^+$	NP_003036			112	59		21
	SLC7A2(CAT2)		y^+	R,K,H	14	U	L	$\downarrow Sb$	NP_003037	Figure 7		110	59		19
	SLC7A3(CAT3)		y^+	R,K	14	U	L	$\downarrow Sb$	NP_116192			112	62		20
	SLC7A4(CAT4)		Orphan		14				NP_004 164	EAA09935		113	52		25
					14					EAA09874		97	63		20
					14					EAA09921		106	65		19
					14					EAA09918		107	64		20
HAT	SLC7A6		y^+L, y^+LAT2	K,R,Q,H,M,L,A,C	12+AP[1]	E	M,H	$\downarrow Sb^+ \uparrow Sb$; $\downarrow Na^+Sb \uparrow Sb^{+0}$	NP_003974		5	59	123	7	6
	SLC7A7		y^+L, y^+LAT1	K,R,Q,H,M,L	12+AP[1]	E	M,H	$\downarrow Na^+Sb \uparrow Sb^+$	NP_003973		7	62	117	7	7
		AeaHAT			12+AP[1]					EAA07276	7	70	114	6	3
					12+AP[1]				Figure 7	EAA13031	11	64	109	7	6
					12+AP[1]					EAA00996	10	64	113	6	5
	SLC7A5		L,LAT1	H,M,L,I,V,F,Y,W,(Q)	12+AP[1]	E	↓H↑ L	$\downarrow Sb\uparrow Sb$	NP_003477	[EAA01959]	5	77	106	7	5
	SLC7A8		L, LAT2	A,S,C,T,N,Q,R,H, M,L,I,V,F,Y,W	12+AP[1]	E	M	$\downarrow Sb\uparrow Sb$	NP_036376		10	63	119	6	1
	SLC7A10		Asc1	G,A,S,C,T (D & L isoforms)	12+AP[1]	E		$\downarrow Sb\uparrow Sb$	NP_062823		7	69	118	5	
					12+AP[1]					EAA03429	7	69	109	6	
					12+AP[1]					EAA10600	6	73	103	10	
	SLC7A11		x_c^+(xCT)	E, C-(substrate for GSH)	12+AP[1]	E	H	$\downarrow C\uparrow E$	NP_055146		7	67	119	7	
	SLC7A9		rBAT, b^{0+}	K,R,A,S,C,T,N, Q,H,M,I,L,V,F,Y,W	12+AP[1]	E	M	$\downarrow Sb^{+0}\uparrow Sb$	NP_055085		6	60	124	7	3

Continued

Table 2 Continued

Type	Mammalian SLC members (protein name, respectively)	Insect protein	Transport systems (some aliases)	Substrate selectivity (weak substrate)	TMDs (other prediction)	Carrier type	Apparent affinity	Stoichiometry ↓ absorption ↑ emission (uncoupled)	Representative H. sapiens	Orthology (proximity) An. gambiae	Archaea	Bacteria	Metazoa	Fungi	Plant
Cluster III															
SNAT	SCL38A3(SNAT3)		N1, SN1	Q,N,H	11	SE	L	↓Na^{+}Sb↑H^{+}	NP_006832				104	18	51
	SCL38A5(SNAT5)		N2, SN2	Q,N,H,S,G	11	SE	L	↓Na^{+}Sb↑H^{+}	NP_277053				109	18	47
	SCL38A1(SNAT1)		A1, ATA1	Q,N,H,S,G,A,C,M	11	S	L	↓Na^{+}Sb	NP_109599	**Figure 8**			108	17	46
	SCL38A2(SNAT2)		A2, ATA2	Q,N,H,S,G,A,C,M,P	11	S	L	↓Na^{+}Sb	NP_061849				105	17	49
	SCL38A4(SNAT4)		A3, ATA3	N,S,G,A,C,M,P	11	S	L	↓Na^{+}Sb	NP_060488				100	17	50
	SCL38A6		Orphan		11				NP_722518	[EAA10907]			112	17	42
	SCL38A7		Orphan		11				NP_612637	EAA00995		1	111	12	42
	SCL38A8		Orphan		11				NP_775785	[EAA07508]		1	86	4	30
vGAT	SLC32		VGABAT(VIAAT)	GAVA, G	10	E	L	↓Sb↑H^{+}(ves)	NP_542119	EAA05252			89	9	87
PAT	SLC36A3,4		Orphan		11(9)				NP_861439			2	118	15	53
	SLC36A1		IMINO(LYAA T1)	P,G,A,GABA	11(9)	S	L	↑SbH^{+} (lys); ↓SbH^{+} (ep)	NP_510968	**Figure 8**			110	15	68
	SLC36 A2		Tramdorin 1	P,G,A	11(9)	S	M	↑SbH^{+} (lys); ↓SbH^{+} (ep)	NP_861441				134	12	34
SNF^{+}	Earlier insect specific	NAT, dBLOT	orphan (blot)		12					EAA10362	1	2	190		
NAT	Insect specific	Earlier NAT	orphan		12					EAA00597			185		
NAT	Insect specific	aeAAT1,KAAT1, CAATCH1	NAT1(BO^{+})	F,C,H,A,S,M,I,Y,T,G, N,P,L-DOPA	12	S	M	↓2Na^{+}Sb[Cl−]; ↓2K^{+}Sb[Cl−]		[EAA05435]			200		
NAT	Insect specific	agAAT6	NAT2(BO^{+})	W,Y,5-HT,F,A	12	S	M	↓2Na^{+}Sb[Cl−]		EAA05614			200		
NAT	Insect specific	agAAT8	NAT3(BO^{+})	Y,F,L-DOPA,W,5-HT	12	S	M	↓2Na^{+}Sb[Cl−]	**Figure 10**	EAA05568			200		
NAT	Insect specific		NAT4(BO^{+})		12	S				EAA05532			200		
NAT	Insect specific		NAT5(BO^{+})		12	S				EAA05604			200		
NAT	Insect specific		NAT6(BO^{+})		12	S				EAA05441			200		
NAT	Insect specific		NAT7(BO^{+})		12	S				EAA05529			200		
Cluster III															
NNT	Earlier insect specific	NNT, dINE, msINE	Orphan(ine)		12			K^{+}(Ca^{+}) channel		EAA14797			200		
NNT	SLC6A5,7,9		GlyT, ProT,	G,P	12	S	H	↓2–3Na^{+}Sb[Cl−]	See **Figure 10**	[EAA12299]			200		
NNT	SLC6A14		BO^{+}(ATBO^{+})	K,R,A,S,C,T,N,O,H,M, I,L,V,F,Y,W	12	S	M	↓2Na^{+}Sb[Cl−]	NP_009162	[EAA07088]			200		
NNT	SLS6A8,6,11,12,13		GABA/Bet, CT	GABA, betaine, creatine	12	S	H	↓2Na^{+}Sb[Cl−]	**Figure 10**				200		
NNT	SLC6A1		GABAT	GABA, neuronal high affinity	12	S	H	2–3Na^{+}GABA[Cl]	NP_003033	EAA13834			200		
NNT	SLC6A3,2	tnOAT, tnDAT, dDAT	DAT, NET	DA, OA, NE	12	S	H	↓2Na^{+}Sb↓K^{+} [Cl−]	**Figure 10**	EAA04277			200		
NNT	SLC6A4	msSERT, dSERT	SERT	5HT	12	S	H	↓2Na^{+}5HT↑K^{+} [Cl−]	NP_001036	EAA05837			200		

Cluster VI															
VMOAT	SLC18A1		MAT, MOAT	DA, 5HT, [HA, EP, NE, OA]	12(10)	E		$\downarrow Sb^{+} \downarrow H^{+}$	NP_003044	[EAA09208]	7	110	61	1	
	SLC18A2		MAT, MOAT	DA, 5HT, HA, EP, NE	12(10)	E		$\downarrow Sb^{+} \downarrow H^{+}$	NP_003045	**Figure 14**	7	95	61	3	
	SLC18A3		AChT	ACh [Oa]	12(10)	E		$\downarrow Sb^{+} \downarrow H^{+}$	NP_003046	EAA07682	3	122	46	1	2
	Insect specific				12(10)					EAA08793	6	127	61	1	
	Insect specific				12(10)					EAA01169	5	105	65		
MCT	SLC16A1,7,8, 3(MCT1,2,3,4)		MCT	Lactate, pyruvate, ketone bodies	12+AP[2]	SE	LM	$\downarrow H^{+}Sb$; $\downarrow Sb \uparrow Sb$	NP_003042	**Figure 15**	2	27	158	10	
	SLC16A4,5,6,11,12,13		Orphan		12				NP_004686		1	26	126	5	
	SLC9,14		Orphan		12				XP_166145	[EAA11834]	3	26	131	2	1
	Insect specific	Putative dmMCT			12				**Figure 15**	EAA06367			194		
	Insect specific				12					EAA08073		15	139	5	
	SLC16A2		T3,T4	d, l aromatic amino acids	12	U	ML	$\downarrow Sb$	NP_006508		1	4	149	9	16
	SLC16A10		TAT	d, l aromatic amino acids	12	U	ML	$\downarrow Sb$	NP_061063	[EAA12383]	2	23	161	11	
	Insect specific				12				**Figure 15**	EAA05501	3	70	122	2	
OPT	SLC15A1 (PEPT1)			Di-, tri-peptides	12	S	L	$\downarrow 2H^{+}Sb^{0}$; $\downarrow$1–2$H^{+}Sb^{+/-}$	NP_005064			11	67	6	61
	SLC15A2 (PEPT2)			di-, tri-peptides	12	S	H	$\downarrow 2H^{+}Sb^{0}$; $\downarrow$1–2$H^{+}Sb^{+/-}$	NP_066568	**Figure 16**		14	68	5	80
	SLC15A1,2 (PHT1,2)			histidine, di-, tri-peptides	12	S		$\downarrow 2H^{+}Sb^{0}$; c1-2$H^{+}Sb^{+/-}$	NP_057666			5	34	5	154
	Insect species specific				12					EAA00193		17	68	2	91
	Insect species specific				12					EAA07145		19	63	4	78
PIT(ANT)	SLC17A1 (NPT1)	Absent	P_iT	P_iT organic ions	12	S	M	$\downarrow Na^{+}Pi$,$\downarrow Sb$ (?)	NP_005065	**Figure 17**		14	170		12
	SLC17A2,3,4 (NPT3,4)	Absent	Orphan		9				NP_005826		1	35	152		13
	SLC17A5 (AST)	Absent	Sialin	Sialic acid	10(12,6)	S	M	$\downarrow H^{+}Sb^{-}$	NP_036566			32	152		12
VGluT	SLC176,7,8(VGluT2,1,3)		DNPi,BNPi	E	9^{SUSOI}	E	ML	$\downarrow E \uparrow H^{+}(Cl^{-})$ ves	NP_064705	EAA12373		42	145		12
VGluT				E	12^{SUSOI}			$\downarrow E \uparrow H^{+}(Cl^{-})$ ves		EAA11677		42	146		12
VGluT				E	12^{SUSOI}			$\downarrow E \uparrow H^{+}(Cl^{-})$ ves		EAA11418		42	146		12
	Insect specific				11^{SUSOI}					EAA14814		40	147		12
	Insect specific									EAA00281		38	149		12
	Insect specific									EAA09135					
	Insect specific								**Figure 17**	EAA13079					
	Insect specific									EAA07243					
	Insect specific									EAA07084					
	Insect specific									EAA13886					
	Insect specific									EAA12474					
	Insect specific				7^{SUSOI}					EAA14792	1	68	117		12

Notes, abbreviations, and special characters: + and −, positive and negative charges; AP[1], ancillary protein (4F2hc/CD98 or rBAT); AP[2], ancillary protein (CD147); U, uniporters (facilitated diffusion), E, exchanger (antiporters); S, symporters (cotransporter); C, channels (high throughput tunneling of inorganic ions); Sb, substrate; H, M, L, apparent substrate affinity high $<10^{-5}$, medium 10^{-3}–10^{-4}, and low $>10^{-3}$, respectively; lys, lysosomal membrane; ep, epithelial membrane; ves, vesicular membrane; na, not available.

Ligands: specific amino acid are shown according to a single capital letter nomenclature; HA, histamine; DA, dopamine; 5HT, serotonin; NE, norepinephrine (noradrenalin); EP, epinephrine (adrenaline); ACh, acetylcholine; Ch, choline; GABA, gamma amino butyric acid; SUSOI, prediction from available open reading frame sequences using SUSOI algorithm and remote server (http://sosui.proteome.Bio.tuat.ac.jp/sosui_subunit.html) Mitaku Group, Department of Biotechnology, Tokyo University of Agriculture and Technology.

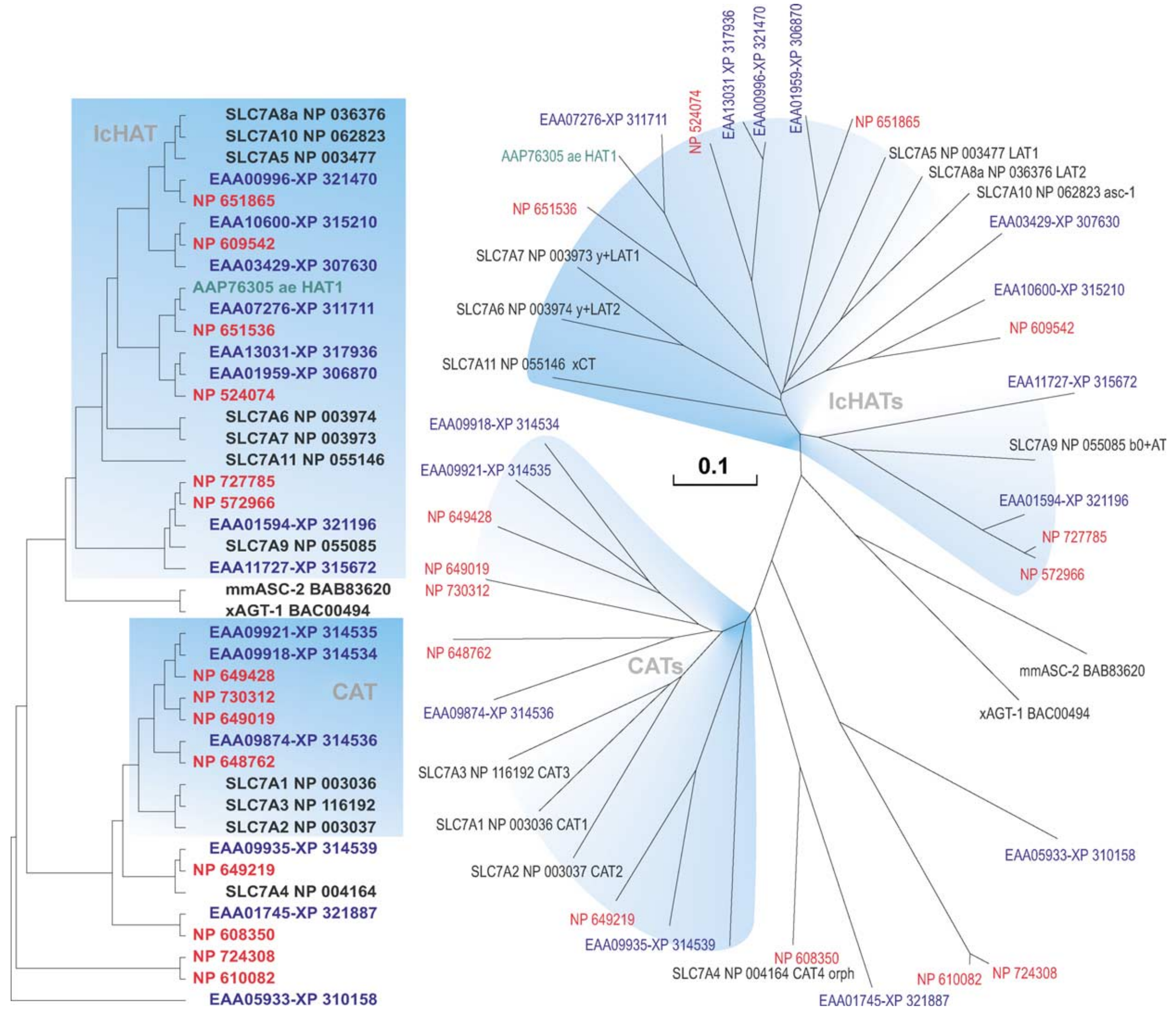


Figure 7 Phylogenetic proximity of dipteran and mammalian cationic amino acid transporter (CATs) and light chain subunits of heterodimeric amino acid transporter lcHATs (SLC7). Cladogram rooted with an EAA05933-XP_310158 *An. gambiae* sequence (left) and unrooted dendrogram (right) of putative dipteran CATs and lcHATs along with identified and partly characterized orthologs from *H. sapiens* genome.

et al., 1991). Now mammalian CAT subtypes 1–3 (SLC7A1–3, respectively) represent a large group of comparatively well-characterized Na^+-independent transporters. CATs in nonepithelial tissues predominantly function as y^+-system nutrient cationic amino acid providers. In epithelial tissues, CATs mediate basolateral transport which apparently is an essential component of transepithelial transport of amino acids. Thermodynamically, CAT types 2 and 3 mediate downhill diffusion (uniport) of selected cationic amino acids with extremely weak coupling or no coupling to other substrates. Since its substrates carry charge at physiological pH, CAT transport can be electrophoretic as well. In contrast, CAT type 1 is strongly trans-stimulated by intracellular substrates and may act as a thermodynamically coupled exchanger of specific intracellular and extracellular amino acids. Neutral amino acids can be recognized by most CATs with rather low apparent affinity (Closs, 2002). In mammals, CAT subtypes exhibit abundant but subtype-specific spatial expression and immunolabeling patterns and appear to be genetic factors of several metabolic and immune diseases, including cystinuria and lysinuric protein intolerance (Simell, 2001). In addition to their relatively universal function in absorption and emission of cationic amino acids including arginine, CATs serve as rate-limiting substrate providers for enzymatic production of nitric oxide (NO) in endothelial cells and macrophages. CATs undergo complex regulation at transcriptional (Fernandez *et al*., 2002) and posttranscriptional (Graf *et al*.,

2001) levels, which include modulation by substrates and nontransported ligands, as well as by cytoskeleton proteins (Zharikov *et al.*, 2001).

5.9.7.2. Heterodimeric Amino Acid Transporters (HATs)

The heterodimeric amino acid transporter (HAT, mammalian SLC7A5–11) subfamily comprises 12-TMD transport proteins (also referred to as light chain catalytic subunit, ~40 kDa) whose functional expression requires association with its ancillary counterpart peptide, a heavy chain (~80 kDa) subunit (Verrey *et al.*, 2003). The heavy chain subunits (mammalian SLC3) are currently represented by two proteins with apparent α-glucosidase homology: 4F2hc, the heavy chain of the lymphocyte activation antigen 4F2 (Hemler and Strominger, 1982) and rBAT, a type II membrane glycoprotein related to the $b^{0,+}$ amino acid transport phenotype (Bertran *et al.*, 1992; Tate *et al.*, 1992; Wells and Hediger, 1992). Heavy chain proteins have a single membrane spanning domain and an extracellular globular C-terminal domain in their secondary structure. Their interaction with light chain subunits is stabilized via 4F2hc-specific disulfide covalent binding (Chillaron *et al.*, 2001) or rBAT-specific noncovalent interaction (Pfeiffer *et al.*, 1998). The suggested role of the heavy chain SLC3 component is in coordinated delivery of the heterodimeric complex to the plasma membrane whereas the light chain SLC7 of HATs serves as a carrier mechanism (Palacin and Kanai, 2003; Verrey *et al.*, 2003).

In contrast to facilitators and nonobligatory exchangers like CATs, the obligatory exchangers, HATs, are associated with a great variety of transport systems that are diverse in terms of distribution and substrate selectivity, including L system for large neutral amino acids; asc system for small neutral amino acids, e.g., Ala, Ser, Cys; x_c^- system for anionic amino acids; and $y^+L/b^{0,+}$ system that combines carriers for cationic and neutral amino acids (Verrey *et al.*, 2003). Virtually all cloned HATs are obligatory exchangers of amino acids, except for the sodium ion driven y^+L transport of neutral amino acids by SLC7A6 or SLC7A7 and SLC3A3 HATs (Pfeiffer *et al.*, 1999; Broer *et al.*, 2000). The varied physiological roles of HATs have been extrapolated from their activity in heterologous expression systems, distribution, and earlier brush border vesicle analyses. For example, in nutritive epithelial tissues they serve as basolateral complements of apical B^0 and $b^{0,+}$ transporters, which are both essential for transepithelial absorption of amino acids (Fernandez *et al.*, 2003). They also may serve as tertiary active transport mechanisms for epithelial reabsorption of cysteine and dibasic amino acids (Chillaron *et al.*, 1996). In nonpolarized cells, heterodimeric transporters serve in several unique mechanisms in which nutrient neutral amino acid uptake is thermodynamically coupled to emission of metabolized or redundant intracellular neutral amino acids (Meier *et al.*, 2002). Notable examples of HAT function are neuronal and glial activity of a SLC7A11/xCT transporter, which is heterologously associated with a 4F2hc subunit (Sato *et al.*, 1999) and mediates 1 : 1 exchange of extracellular cysteine for intracellular glutamate. This transporter, therefore, represents a rate-limiting component in GSH (glutathione) synthesis pathways. GSH is a tripeptide of glycine, glutamate, and cysteine that is crucial for cells and tissues that tolerate endogenous free-radical stress, e.g., activated macrophages, hypothalamic neurons, a variety of glial cells and pancreas, as well as several cell culture lines (Bassi *et al.*, 2001; Bridges *et al.*, 2001; Sato *et al.*, 2002; Tomi *et al.*, 2003; Wang *et al.*, 2003).

5.9.7.2.1. SLC7 related genes in dipteran models Transporters structurally similar to SLC7 have been reported throughout all life kingdoms; however, these have quite specific patterns of taxonomic proximity for particular species (**Table 2**). The insect CAT cluster, defined by phylogenetic reciprocity to mammalian CATs, includes six genes in both dipteran models (**Figures 4** and **7**). However, insect CAT genes do not form exact orthological matches with mammalian CATs, except for the SLC7A4 (NP_004164) gene, which clusters with insect NP_649219 and EAA09935 sequences (**Figure 7**). In contrast, insect CATs exhibit better interspecific homology by forming four orthologous clusters when they are analyzed within frames of dipteran lineages (**Figure 6**). It is most likely that proximal insect CATs are functional equivalents of mammalian CATs 1–3, which evolved via lineage-specific gene duplications; however, exact functions remain elusive, pending cloning and functional characterization of such gene transcripts. In addition to the four CATs characterized in mammalian models, both *Anopheles* and *Drosophila* genomes include two more, apparently parental, genes that are at a lower phylogenetic level relative to CATs and HATs.

Insect HATs comprise nine putative genes in *An. gambiae* but only five genes in *D. melanogaster*, which together occupy five apparently orthologous clusters (**Figures 6** and **7**). Only one such group forms a cluster with mammalian SLC7A9 (NP_055085) transporters (**Figure 7**). Several *An. gambiae* genes are not mapped, so the question

about the exact number of HAT genes in this model remains to be clarified. One HAT gene cloned and characterized from *Ae. aegypti* (Jin *et al.*, 2003), AAP76305, appears to be orthologous to EAA07276 and NP_651536 genes in *An. gambiae* and *D. melanogaster*, respectively (see Section 5.9.7.2.3).

5.9.7.2.2. SLC3-related genes in dipteran models SLC3 proteins (heavy chain subunit of HAT; **Figure 8**) serve as obligatory cofactors of SLC7 transport proteins, but do not appear to be phylogenetically related to some membrane solute carrier family. In contrast, this subunit is homologous to the α-amylase or glycosyl hydrolase Family 13, all of which bear a unique catalytic $(\beta/\alpha)_8$-barrel domain with the active site being at the C-terminal end of the barrel beta-strands. These proteins share a functional signature in that they catalyze a variety of glycoside bond cleavage/formation reactions (MacGregor *et al.*, 2001). The extracellular domain of SLC3 members has up to 30% to 40% peptide identity with insect maltases and bacterial α-glucosidases (Palacin and Kanai, 2003), which in turn indicates a possible route of evolution common for α-amylase family proteins (Janecek, 1994). Nevertheless, rBAT (see below) lacks amylase-specific activity when heterologously expressed in *Xenopus* oocytes (Wells and Hediger, 1992); it appears to lack amylase-specific catalytic residues (Chillaron *et al.*, 2001) when compared to *Bacillus cereus* oligo 1,6-glucosidase (O1,6G), which is available in the Protein Data Bank (PDB) (Watanabe *et al.*, 1997).

At present, two members of mammalian SLC3 that form functional HATs are characterized (Palacin and Kanai, 2003): rBAT (SLC3A1) was initially identified by expression cloning in *Xenopus* oocytes (Bertran *et al.*, 1992; Tate *et al.*, 1992; Wells and Hediger, 1992); and 4F2hc (SLC3A2), was initially identified as a lymphocyte activation antigen (Hemler and Strominger, 1982). Both of these proteins appear to be widespread throughout the Metazoa. They also share remarkable homology with a number of putative genes from bacteria and fungi. However, the affiliation of these bacterial and fungal proteins with HAT forming proteins requires a detailed comparison of sequences and secondary

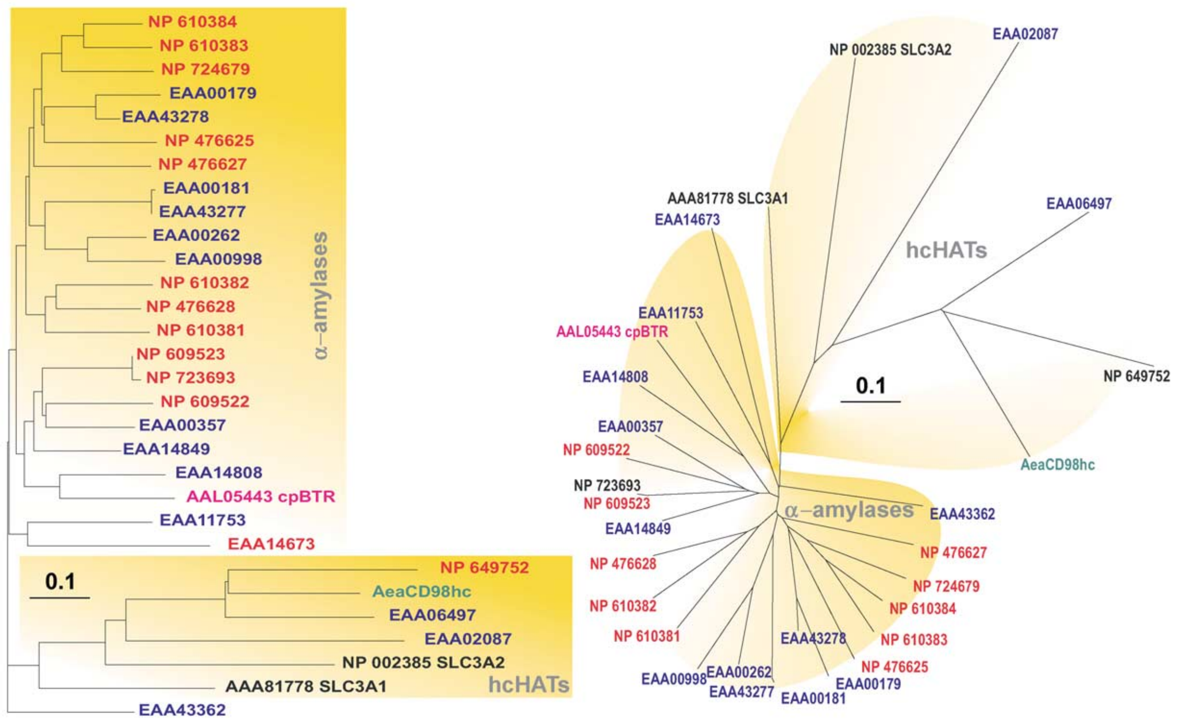


Figure 8 Phylogenetic proximity of mammalian HATs (hcHATs, SLC3) with dipteran orthologs of heavy chain subunits and with amylases. Unrooted phylogram (left) and dendrogram (right) of putative heavy chain subunit of HATs and amylases from dipteran genomes, along with known orthologs from *H. sapiens* genome (black font). Other font colors: green, heavy chain subunit of heterodimeric amino acid transporter from *Ae. aegypti*; blue, *An. gambiae* sequences; red, *D. melanogaster* sequences; pink, a binary toxin-binding alpha-glucosidase from *Culex pipiens*. Scale bar is evolutionary distances in mutations per site.

structures as well as functional assay, because of their apparent orthology with amylases. Intriguingly, SLC7 homologous proteins are present in plants and Archaea that lack SLC3 counterparts. This fact makes dialectic the obligatory requirement of SLC3 and CLC7 association at least in some lineages.

Two putative genes resembling the mammalian SLC3A2 heavy chain subunit are currently found in the *An. gambiae* genome (EAA06497, located on the X chromosome; and EAA02087, not yet mapped) whereas only one gene is present in *D. melanogaster* (NP_649753, located on the 3R chromosome). All other dipteran sequences with apparent SLC3 homology reside in an α-amylase specific gene cluster (**Figure 8**). Intriguingly, some products of these genes are recognized as toxin receptors, e.g., AAL05443, which encodes the receptor of the *B. sphaericus* binary toxin (BT) in *Culex pipiens* midgut (Darboux *et al.*, 2001). Another important finding from analysis of dipteran sequences is that heavy chain proteins apparently evolved not from α-amylases but from some common primordial ancestor (**Figure 6**).

5.9.7.2.3. Cloned insect HATs from *Ae. aegypti*; (AeaLAT + AeaCD98hc) Two complementary subunits (AeaLAT and AeaCD98hc, light chain and heavy chain subunits, respectively) of an insect HAT have been cloned recently from the mosquito *Ae. aegypti*. Like mammalian HATs, insect transporters require expression of complementary subunits to produce a functional L-amino acid transporter phenotype in *Xenopus* oocytes. Heterologously expressed mosquito AeaLAT+AeaCD98hc subunits mediate uptake of large neutral and basic amino acids, partly resembling y^+L system transporters. Small acidic and neutral amino acids appear to be poor substrates for this transporter (Jin *et al.*, 2003). Like mammalian HAT, these insect transporters can utilize a Na^+ gradient (upregulated ~44%) for the uptake of neutral amino acids (L-leucine; $K_m^{leu} = 67\,\mu M$), but not basic amino acids (L-lysine), which is virtually Na^+-independent at pH of 7.4 (Jin *et al.*, 2003). It has been suggested that the electrochemical gradient for amino acids at high extracellular concentrations appears to be the major factor that enables amino acid transport by AeaLAT+AeaCD98hc. AeaLAT showed high-level expression in the gastric caeca, Malpighian tubules, and hindgut of larvae. In caeca and hindgut, expression was in the apical cell membrane. However, in Malpighian tubules and midgut (which showed low-level expression), the transporter was detected in the basolateral membrane. This expression profile supports the conclusion that this AeaLAT + AeaCD98hc pair mediates a nutrient amino acid emission from posterior midgut epithelial cells to hemolymph (Jin *et al.*, 2003). A combination of similar basal y^+L HATs with apical $B^{0,+}$ transporters, such as msKAAT1, msCAATCH1, and (recently cloned from mosquito larvae midguts) aeAAT1 and agAAT8 may comprise a complete transepithelial transport metabolon for nutrient amino acid uptake. It has been stated that the apical location of AeaLAT in the gastric caeca implies its involvement in the absorption of amino acids from the lumen (Jin *et al.*, 2003). Similarly, its basal location in the midgut suggests that it may participate in the emission of amino acids or their derivatives to the hemolymph for circulation to the gastric caeca and cardia lumen where they are required for assembly of the peritrophic matrix.

5.9.8. AAT Cluster III (SLC6, SLC32, SLC36, SLC38)

5.9.8.1. SNATs and PATs (SLC38, SLC32, SLC36)

Sodium neutral amino acid transporters (SNATs), vesicular GABA Transporters (vGATs), and proton amino acid transporters (PATs), SLC38, SLC32, and SLC36, respectively, are grouped together because they represent phylogenetically related groups of transport proteins with a consecutive phylogenetic hierarchy as well as common motifs in their secondary structure, such as the presence of 10–12 TMDs. This clustering of insect transporters also corresponds to a previously identified, eukaryotic-specific, amino acid/auxin permease superfamily (Young *et al.*, 1999). Although the homology of these superfamilies of metazoan transporters is certain, to judge the exact orthology of particular insect transporters with known mammalian SNATs and PATs is problematic, because of extensive lineage-specific gene duplication/loss and lack of characterized insect transporters related to this superfamily.

5.9.8.2. Sodium Neutral Amino Acid Transporters, SNATs (SLC38)

This cluster represents the parental group in the SNAT + PAT superfamily, whose members fall into several consecutive phylogenetic levels in dipteran models (**Figures 6 and 9**). In mammals, characterized SNATs comprise System A and N transporters, each of which mediates a low-affinity transport ($0.3 < K_m^{substrate} < 2\,mM$) of varied subsets of small,

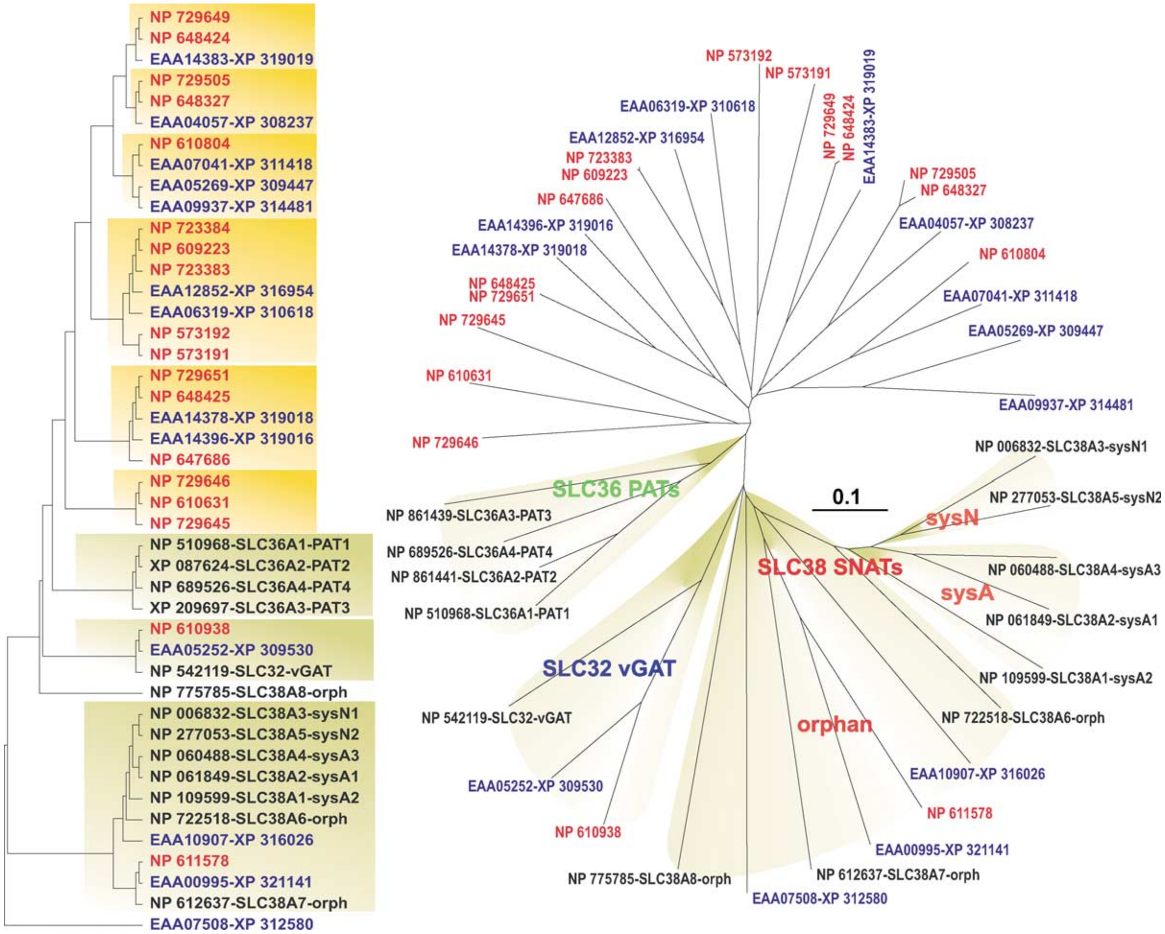


Figure 9 Phylogenetic transitions between dipteran and mammalian sodium neutral amino acid transporters (SNATs, SLC38), proton amino acid transporters (PATs, SLC36) and vesicular GABA transporter (vGAT, SLC32). Unrooted phylogram (left) and dendrogram (right). Font colors: blue, *An. gambiae* sequences; red, *D. melanogaster* sequences; black, *H. sapiens* sequences. Other abbreviations: sysA and sysN, systems A and system N transporters, respectively. Scale bar is evolutionary distances in mutations per site.

aliphatic amino acids (Mackenzie and Erickson, 2003). System A transporters appear to be broadly expressed in all mammalian tissues and to mediate Na^+-driven, electrophoretic, and pH sensitive neutral amino acid uptake through plasma membranes. In contrast, system N transporters have narrower substrate profiles and utilize proton motive forces, which may facilitate a reverse amino acid transport and consequently an active emission of catabolic amino acids (Chaudhry *et al*., 2001; Broer *et al*., 2002). Both systems are known to be tightly regulated via hormonal and intracellular signaling pathways and pH dependent allosteric modifications. SNATs are implicated in many physiological processes associated with reciprocal transcellular amino acid exchange, cellular nutrition, and metabolite secretion/detoxification pathways (Mackenzie and Erickson, 2003).

5.9.8.3. Vesicular GABA Transporters, vGATs (SLC32)

These transporters mediate vesicular accumulation of inhibitory neurotransmitters via an H^+/GABA(Gly) exchange (see Section 5.9.3.6.1). They presently comprise a single-member "family" in mammals designated SLC32 (Gasnier, 2003), which in insects represents a single level transition between A and N system transporters within the SLC38 family and proton amino acid transporters of the SLC36 family (**Figures 6** and **9**). A single vGAT gene encodes at least one splicoform in each dipteran model. The physiological role of vGATs is in vesicular accumulation of intracellular GABA, which is essential for inhibitory chemical signaling. Essential and universal throughout the Metazoa, inhibitory neurotransmission implicates a stabilizing

selection that would conserve vGAT sequences and would make it reasonable to identify insect vGAT genes by phylogenetic relevance with mammalian genes (**Figures 6 and 9**). Hence, the function of *An. gambiae* EAAT05252 and *D. melanogaster* NP_610938 in vesicular GABA uptake can be considered.

5.9.8.4. Proton Amino Acid Transporters, PAT (SLC36)

Proton amino acid transporters (PATs) comprise a group of electrophoretic H^+/amino acid (1 : 1) symporters that mediate cytosolic acquisition of small neutral amino acids from intracellular (lysosomal) and extracellular proteolysis. In mammals, the PAT group is represented by four genes that encode two well-characterized transporters, PAT1/LYAAT-1 and PAT2, and two orphan transporters (Boll *et al*., 2003a). The first orphan has been characterized as a rat brain-specific lysosomal amino acid transporter (rLYAAT-1) (Sagne *et al*., 2001) and also as a mouse intestinal epithelium-specific plasma membrane proton/amino acid transporter (mPAT1), which induces H^+ driven, low affinity ($K_m^{G,A,P} = 7$, 7.5, 2.8 mM) transport of Gly, Ala, Pro, and several structurally related derivatives in *Xenopus* oocytes (Boll *et al*., 2002). In contrast, PAT2 has a narrower substrate selectivity and higher affinity ($K_m^{G,A,P} = 0.59$, 0.26, 0.12 mM) (Boll *et al*., 2002). PATs are differentially and broadly expressed in mammalian tissues except for the orphan PAT3, which so far has been found only in adult mice testis (Boll *et al*., 2003b). Immunocytochemical studies show that rLYAAT-1 is associated with other cellular membranes, e.g., Golgi apparatus, ER, and occasionally with the plasma membrane (Agulhon *et al*., 2003).

Dipteran genomes comprise nine putative genes in *An. gambiae* and 11 genes, encoding 15 identified transcripts, in *D. melanogaster*. These transporters split into five orthologous groups within the dipteran framework; one additional paralogous group is present in *D. melanogaster* (**Figure 9**). However, the entire population of these dipteran transporters is paralogous to mammalian PATs.

The phylogeny of SNATs and PATs apparently reflects transitions of electrochemical properties observed among characterized transporters in these families. Such transitions were suggested earlier as an evolutionary trend from an H^+ driven ancestral carrier, e.g., in PATs and vGAT, toward aquisition of a Na^+ driven mechanism, e.g., in SNATs (Boll *et al*., 2003a; Chen *et al*., 2003). An inverse evolutionary trend, from a Na^+ dependent ancestral mechanism toward a H^+ driven mechanism, is apparent from the phylogenetic analysis. Although H^+ driven mechanisms are indeed older; the SNATs and PATs population is self-rooted and apparently shares a common ancestor with the Na^+ driven SNF (SLC6) transporters (**Figure 6**). This fact means that Na^+ driven mechanisms were already established before the SNAT and PAT families diverged. In addition, a Na^+ driven mechanism is encoded in the older A system of SLC38 transporters that reside on the plasma membrane (**Figures 6 and 9**). The ability to modify properties under low intracellular Na^+ demand (N system SLC38) and directly utilize proton motive forces (SLC36) presumably was acquired later during the establishment of phagocytosis and lysosomal digestion in earlier eukaryotes. SLC36 members residing in various endomembranes correspond with lysosomal traffic for which the apical intestinal membrane is just a single possible position. Paralogous clustering in insect and mammalian SLC36 is not surprising, since this group is under the pressure of diverse nutrient requirements in different tissues and cell types. In contrast, vGAT (SLC 32) serving in inhibitory neurotransmission evolved under a stabilizing pressure, which is reflected by its trans-specific orthology. Despite structural similarity, SLC32 and SLC36 exhibit striking differences in their kinetic mechanisms: SLC32 vGAT is an exchanger (antiporter) utilizing H^+ motive forces from synergetic activity modulated by Cl^- channels and V-ATPase for ligand accumulation (Gasnier, 2003), whereas SLC36 is a symporter utilizing both H^+ and ligand electrochemical motive forces for ligand secretion through lysosomal membranes and/or ligand uptake through plasma membranes (Boll *et al*., 2003a).

5.9.8.5. Sodium Neurotransmitter Symporter Family (SNF; SLC6)

The title of this amino acid transporter family, which is present in many protein databases, reflects a historical, rather than a biological, perspective. It is misleading in four ways. First, the root of this family is located among primitive primordial carriers that have broad substrate selectivity and apparently share a common ancestor with archaeal and bacterial transporters (**Table 1**). Second, members of at least five other, phylogenetically remote, families participate directly in neurotransmitter transport, namely, EAATs (SLC1), PATs (SLC36), vGAT (SLC32), vGluTs (SLC17), and vMAT (SLC16). Third, the SNF family includes a large number of nutrient amino acid transporters (NATs) and undefined transporter functions (blot, ine) along with the neurotransmitter transporters (NTTs) (See **Figures 6 and 10**; **Table 1**). Fourth, electrochemical

mechanisms encoded in these transporters can utilize transmembrane gradients for ions other than just Na^+ and/or Cl^-, e.g., K^+. Nevertheless, all SNF members share significant similarities in their sequences and secondary structures, which include 12 relatively conserved TMDs and a large extracellular loop between transmembrane domains 3 and 4, although prominent differences are present in this loop.

5.9.8.5.1. Neurotransmitter transporters, NTTs The following subsets of insect Na^+/Cl^- neurotransmitter transporters (SNF) are described above: GABA transporters (GAT; Section 5.9.3.4.1); dopamine transporters (DAT), octopamine transporters (OAT), and serotonin transporters (SERT or 5HTT; Section 5.9.3.4.2); glycine-proline transporters (Section 5.9.3.4.3); inebriated transporters (Ine; Section 5.9.3.4.4) (See also Sections 5.9.2 and 5.9.3).

5.9.8.5.2. Nutrient amino acid transporters (NATs) NATs represent a large and phylogenetically separate cluster of carrier proteins with specific physiological roles in insects and apparently in many other organisms. Here a new scheme of SNF classification that includes two subfamilies, NTTs and NATs (**Figure 10**) is introduceed. Phylogenetic analysis revealed six NAT genes in the *D. melanogaster* genome and seven genes in the *An. gambiae* genome (**Figures 6 and 10**). The properties of four currently cloned and characterized insect NATs, *M. sexta* KAAT1 and CAATCH1,

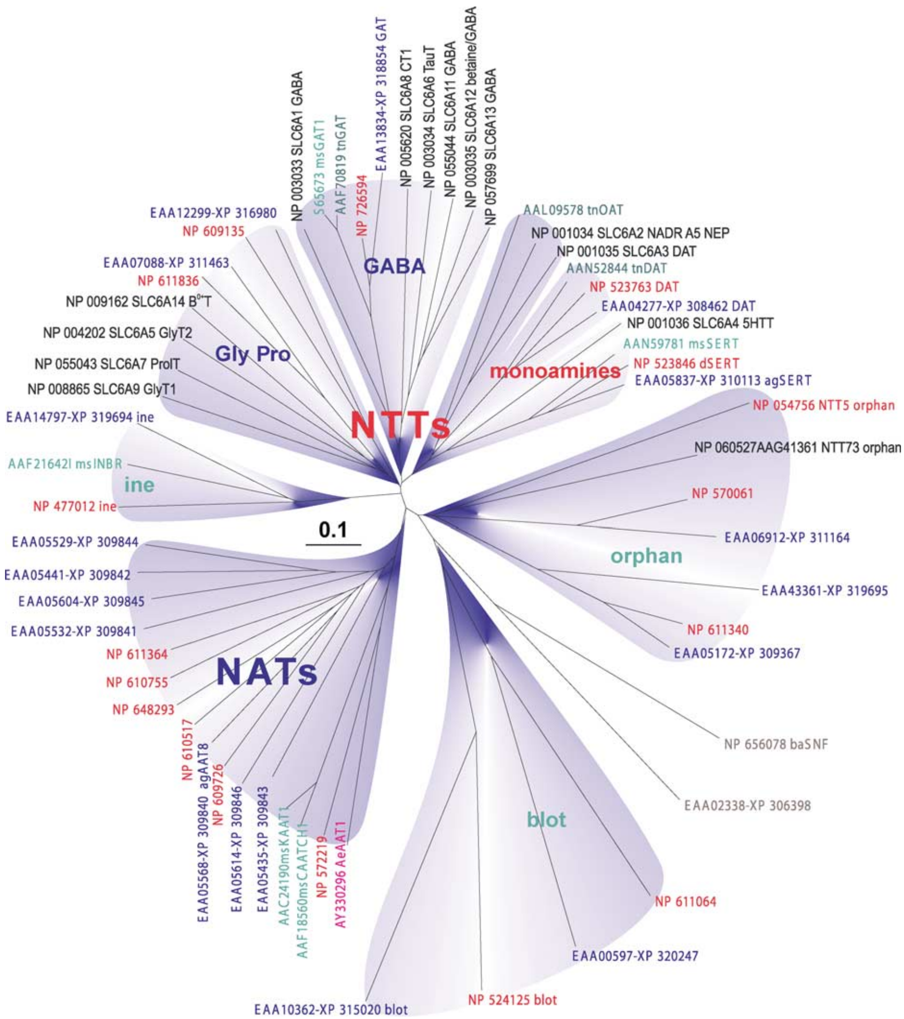
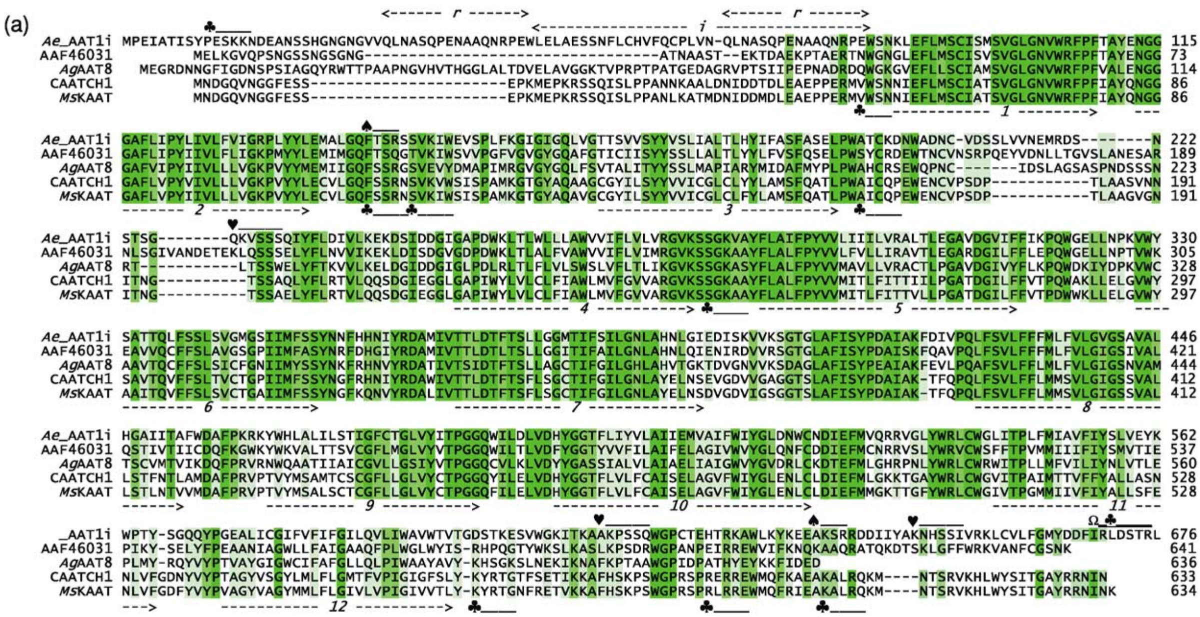


Figure 10 Unrooted neighbor-joining dendrogram of insect and mammalian neurotransmitter transporters (NTTs) and nutrient amino acid transporter (NATs) from the sodium neurotransmitter family (SNF, SLC6). Font colors: blue, *An. gambiae* sequences; red, *D. melanogaster* sequences; black, *H. sapiens* sequences; dark green, *T. ni*; light green, *M. sexta*; brown, bacterial SNF related transporter from *Bacillus anthracis* (NP_656078) and *An. gambiae* sequence, which apparently is symbiotic contamination (EAA02338). Scale bars are evolutionary distances in mutations per site.

Ae. aegypti AeAAT1 (Boudko *et al*., in press), and *An. gambiae* agAAT8, are reviewed here. Cloned insect NATs share common 12-TMD's secondary structure and high homology in protein sequences (**Figure 11**).

msKAAT1, a potassium and sodium ion coupled amino acid transporter, was the first NAT in the SNF family to be cloned. Efforts to clone it based on phylogenic proximity to the NTT cluster were unsuccessful and it was cloned by extensive heterologous expression-screening of RNA fractions from posterior midgut of *M. sexta* (Castagna *et al*., 1998). Because the msKAAT1 sequence is significantly similar to that of other SNF members, it is clear that the SNF category includes not only transporters for neurotransmitter uptake but also other transporters that mediate epithelial functions. *Xenopus* oocytes expressing KAAT1 induced uptake of neutral amino acids but not charged ones or GABA, resembling in this profile mammalian system B carriers (Castagna *et al*., 1998) as well as the earlier reported amino acid uptake in brush border vesicles from midgut epithelium of *M. sexta* (Hennigan *et al*., 1993a, 1993b). As expected from

(a)



(b)

	Ae_AAT1	AAF46031	AgAAT8	MsCAATCH1
AAF46031	54.9			
AgAAT8	46.9	47.2		
MsCAATCH1	48.2	48.3	44.6	
MsKAAT1	48.0	47.8	44.0	90.2

(c)

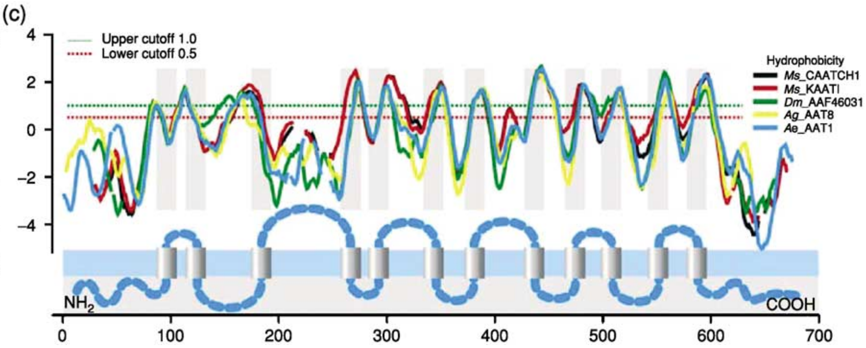


Figure 11 Molecular structure of insect NATs. (a) An open reading frame from the conceptual translation of *Ae. aegypti* Ae_AAT1i clone # 23 cDNA (AY330296) is aligned with the sequences of *M. sexta* KAAT1 (AAC241900) and CAATCH1 (AAF18560); *An. gambiae* AgAAT8 that was cloned in our laboratory (AAN40409) and a putatively homologous protein present in the *Drosophila* genome (AAF46031). (Sequence similarities are shown by the intensities of the green background.) Ae_AAT1 specific and universal homologous protein features are shown in upper and lower lines, respectively, as follows: ← # → similar transmembrane domains and their numbers; ← r →, internal sequence repeats; ← i →, insert that is absent in alternative Ae_AAT1 (AAN40410); ♣, protein kinase; C, phosphorylation sites ($n=10$); ♠, cGMP dependent protein kinase phosphorylation sites ($n=2$); ♥, myosin 1 heavy chain kinase phosphorylation sites ($n=3$); Ω, protein kinase C phosphorylation sites of all types ($n=1$). (b) Reciprocal amino acid sequence identity matrix. (c) Hydropathy plot and predicted transmembrane topology of all five transporters (TopPed II parameters were: GES-hydrophobicity scales; 1.0 upper and 0.5 lower cutoffs; 10 and 5, respectively; core and wage window sizes, 60; critical loop length, 2 for critical transmembrane spacer. Hydropathy values (vertical scale) and distribution of transmembrane spanning domain (insert at bottom) are aligned relative to amino acid position (horizontal scale).

the vesicle studies, msKAAT1 utilizes not only Na^+ but K^+ gradient as well. K_m^{K+} (1 mM Leu) = 32 ± 2.8 mM, which was five times higher than K_m^{Na+}. The high expression of msKAAT1 in absorptive columnar cells of the posterior midgut, which was confirmed by Northern blots and *in situ* hybridization, as well as the unusual mechanism that is adapted for K^+ rather than Na^+ motive forces for amino acid translocation, fits with the physiology of nutrition in caterpillar larvae, which have a low Na^+ and high K^+ plant diet. In addition, studies of pH dependency showed that msKAAT1 transport efficiency is maximal in the alkaline media that is physiologically normal for caterpillar midgut (Peres and Bossi, 2000).

Reciprocal ligand inhibition experiments show that msKAAT1 substrates require the presence of both the carboxylic group and the alpha-amino group, as well as the uncharged side chain (Vincenti *et al.*, 2000). The unique characteristics of msKAAT1 suggested that its inhibitors would represent a new type of lineage selective, and thus environmentally safe, insecticides and encouraged further analysis of its electrochemical, pharmacological, and structural properties. These studies led to several important findings, e.g., the ability of heterologous msKAAT1 to induce carrier coupled and uncoupled ion fluxes with specific ion selectivity profiles (Bossi *et al.*, 1999a, 1999b), the key role of the Y147 site in substrate selectivity and affinity (Liu *et al.*, 2003), the critical role of the E93 site in the functional folding of msKAAT1 (Sacchi *et al.*, 2003), and the irreversible pharmacological inhibition of msKAAT1 by modification of the R76 residue with an arginine-modifying reagent, phenylglyoxal (Castagna *et al.*, 2002).

msCAATCH1, a nutrient amino acid transporter that is also an ion channel, was cloned by a PCR based strategy using degenerate primers to conserved peptide motifs in the msSNF but avoiding msKAAT1-specific motives (Feldman *et al.*, 2000). In contrast to msKAAT1 and NTTs, the *Xenopus* oocyte expressed msCAATCH1 is Cl^- independent but combines a carrier associated current with a carrier independent leakage current, which can be blocked by methionine, alanine, leucine, histidine, glycine, and threonine. In addition, msCAATCH1 exhibits different substrate selectivity depending upon symported cations, e.g., preferring threonine in the presence of K^+ but preferring proline in the presence of Na^+ with apparent $K_m^{[Na+]Pro} = 330$ versus $K_m^{[Na+]Tre} = 35$ and $K_m^{[K+]Pro} = 1900$ versus $K_m^{[K+]Tre} = 235$ in μM, respectively. Simultaneous assay of isotope labeled amino acid uptake and voltage clamped currents revealed that transport and ligand gated channel mechanisms are thermodynamically uncoupled (Quick and Stevens, 2001). msCAATCH1 and msKAAT1 share 90.6% peptide sequence identity and could be alternative splicoforms of a single gene. In addition, they share the essential role of Y147 in the ligand affinity (Stevens *et al.*, 2002) along with the analogous role of Y140 in GAT1 (Bismuth *et al.*, 1997). Despite this striking structural similarity, the two msNATs encode transporters that are dramatically different in physiological and electrochemical properties. Hopefully, future studies of the relative expression and membrane location will clarify the physiological roles of these two transporters in the caterpillar midgut.

aeAAT1, the first NAT to be cloned from a mosquito larva, was cloned from a larval posterior midgut cDNA collection of the Yellow fever mosquito, *Ae. aegypti* (Boudko *et al.*, in press) using a PCR based similarity cloning approach (Feldman *et al.*, 2000) followed by rapid amplification of cDNA ends (RACE). Complete analysis revealed that aeAAT1 serves as a primary phenylalanine provider in mosquito larvae (Harvey *et al.*, 2003). Despite remarkable sequence similarity with msKAAT1 and msCAATCH1, the substrate and electrochemical profiles of aeAAT1 differ from the lepidopteran transporters. For example, the Cl^- dependency and ability to utilize both K^+ and Na^+ electrochemical gradients that are characteristic of msKAAT1 are absent in aeAAT1 (**Figure 12**). Additional screening of an entire-larval cDNA collection indicated the presence of two splicoforms, short (aeATT1) and long (aeAAT1i). Heterologous expression/characterization in *Xenopus* oocytes confirmed that long splicoform aeAAT1i is a low-affinity and high-throughput transporter with a specific preference for phenylalanine (substrate affinity profile F >> C > H > A ≈ S > M > I > Y > T ≈ G > N ≈ P > L-DOPA > GABA > L >> DA ≈ Octopamine ≈ 5HT; $K_m^{Phe} = 0.45$ mM; $K_m^{Na+} = 38$ mM; $\eta Na^+/\eta Phe \approx 2/1$; **Figure 12**). Whole mount *in situ* hybridization confirmed a specific spatial transcription of aeAAT1 in the posterior midgut, salivary glands, cardia, and specific basal cells of gastric caeca (**Figure 13**).

Although aeAAT1 shares some properties with msNATs and the well-known mammalian B^{0+} system, for which the molecular identity so far consists of but one cloned transporter (Sloan and Mager, 1999), differences in substrate affinity and electrochemical profile, which apparently are a result of intensive adaptive divergence, make uncertain the possibility of defining a clear orthology between NAT species. Two other insect transporters that mediate aromatic amino acid uptake are members

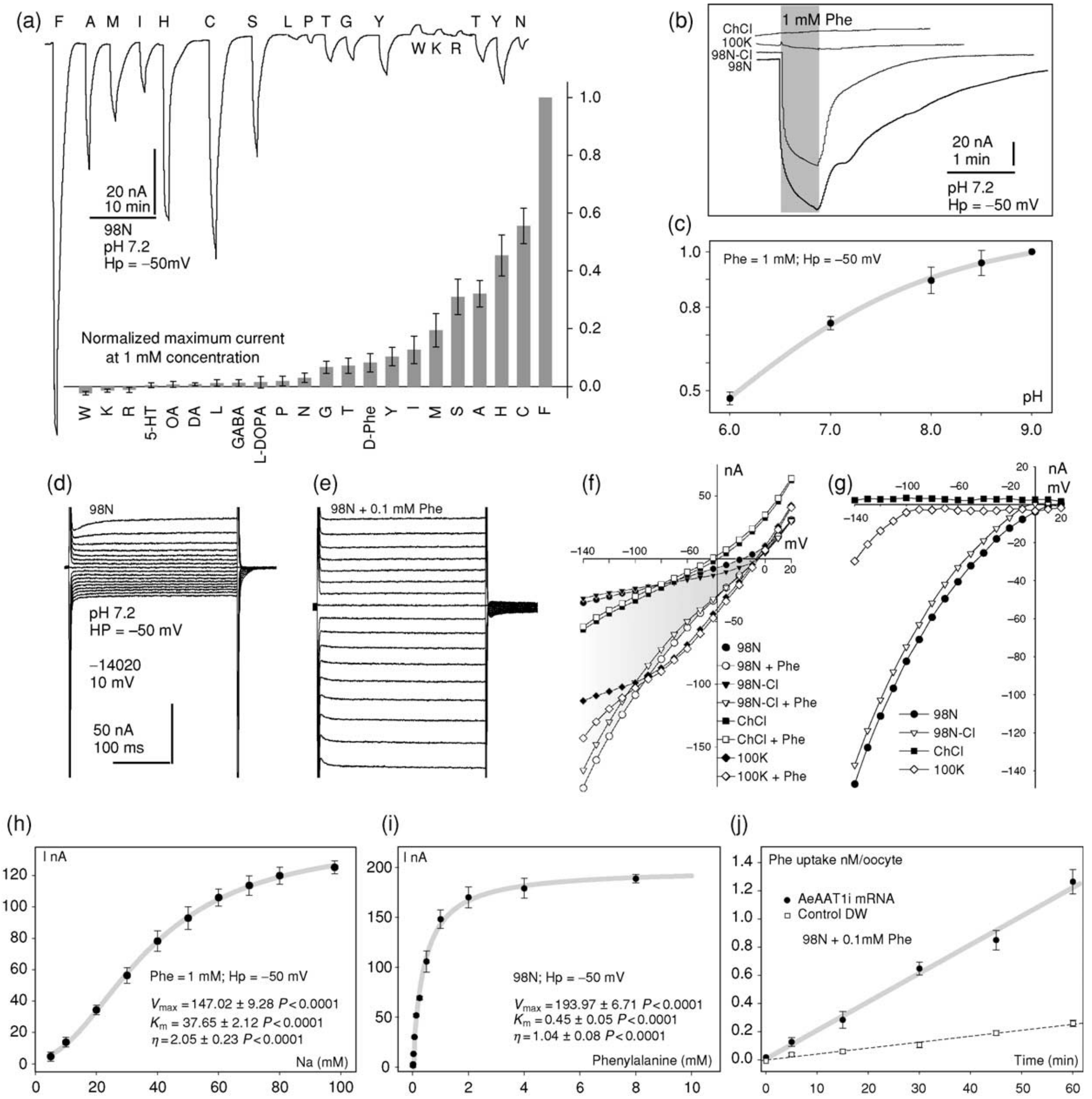


Figure 12 Electrochemical properties of Ae_AAT1i expressed in *Xenopus* oocytes. (a) Top: a trace representing amino acid induced currents in an oocyte injected with Ae_AAT1i mRNA (all amino acids were presented at 1 mM final concentration in 98 mM Na^+-containing saline at pH 7.2 for 30 s and washed until complete recovery of the background current). Bottom: amino acid and selected neurotransmitter sensitivity profiles in heterologously expressed Ae_ATT1i (bars are means of normalized responses ± SE; $n \geq 3$ for different oocytes). (b and c) Ion and pH dependency of phenylalanine induced currents. (d and e) Representative voltage-step induced currents in saline alone and after application of 0.1 mM phenylalanine. (f) I/V plots at different ionic conditions outside oocytes. Dots with similar shapes represent pairs of a control (no ligand) vs. 0.1 mM phenylalanine I/V plots. Gradual shading indicates a difference between I/V plots in control 98 saline and 98 saline with 0.1 mM phenylalanine. (g) Average I/V plot for the data from f. (h and i) Phenylalanine and sodium ion concentration dependency (means of current ± SE; $n = 3$ for each point); K_m, Michaelis-Menten constant and η, Hill coefficient values derived from these data, shown in graph; (j) Linear regression lines from phenylalanine uptake by RNA vs. water injected oocytes (concentration means ± SE, $n = 3$ or 2).

of the Na^+ independent TAT (Kim *et al.*, 2001) transport systems and heterodimeric LAT^+4F2hc group (Mastroberardino *et al.*, 1998; Rossier *et al.*, 1999), which are well characterized in mammals (SLC16 and $SLC7^+$SLC3 and SLC16 phylogenetic families, respectively). An increasing number of studies indicate that TAT and heterodimeric transport systems participate in the basolateral epithelial uptake/exchange of various organic solutes in growing cells (Verrey *et al.*, 2003). By contrast, aeAAT1

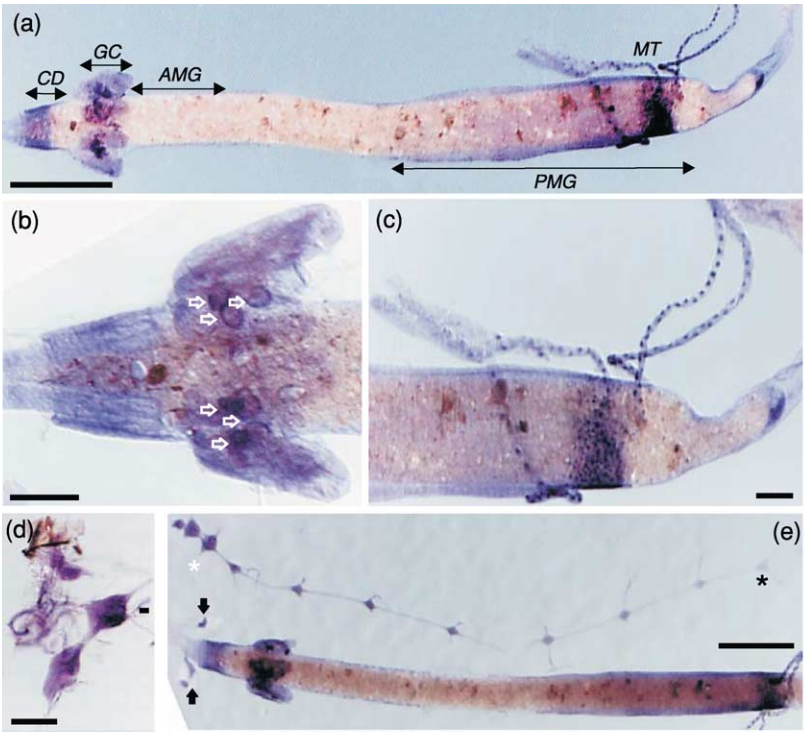


Figure 13 *In situ* hybridization of whole-mount early 4th instar *Ae. aegypti* larvae with Ae_ATT1i DIG mRNA probes. (a) Distribution of Ae_AAT1i (blue labeling). CD, cardia (esophageal invagination); GC, gastric caeca; AMG, anterior midgut; PMG, posterior midgut; MT, Malpighian tubules. (b and c) High magnification images of gastric caeca and posterior midgut, respectively. Large epithelial cells in the basal posterior part of GC are indicated by white outline arrows. (d) Image of cephalic ganglia isolated from the central nervous system (CNS). (e) Image from middle 4th instar larval preparation showing intensive and uniform Ae_AAT1 expression patterns in CD, GC, and PMG. Isolated ventral nerve cord showing different intensities of hybridization in anterior abdominal ganglia vs. posterior abdominal ganglia of the CNS (anterior and posterior ends of the cord, white and black asterisks, respectively). Black arrows point to salivary glands. Scale bar: (a, e) 1 mm; (b, c, d) 200 μm.

and other NATs appear to play a prominent role in the primary uptake of essential nutrient amino acids from the lumen to the epithelial cells through the apical brush border membranes in posterior midgut. aeAAT1 is also transcribed in the cardia and gastric caeca, where amino acid absorption is likely, because amino acids in these tissues are extensively depleted by the polymerization of the peritrophic matrix in the lumen of the cardia. These data support the hypothesis that aeAAT1 is a key transporter in phenylalanine-consuming pathways, such as the dopaminergic neurotransmission and ectoderm tanning pathways that are ubiquitous in the animal kingdom, as well as in insect-specific external cuticle sclerotization and polymerization of the peritrophic matrix.

agAAT8 (resembling predicted gene product EAAT05568) was cloned from larvae of *An. gambiae*, which serve as a genomic and tropical disease vector model. A novel, high throughput strategy was used for cloning and characterization of this transport protein (Boudko *et al.*, unpublished data). This strategy combines cyberanalysis to verify the correct 5′ and 3′ open reading frame (ORF) ends with PCR cloning from a high performance, tissue specific-cDNA collection (Matz, 2002) using long, ligation-ready, ORF-flanking primers and the efficient expression vector, pXOOM, which is designed for heterologous expression in *Xenopus* oocytes or mammalian cell lines (Jespersen *et al.*, 2002). In contrast to aeAAT1, agAAT8 has approximately equal apparent affinity for products of phenylalanine catabolism, including tyrosine and L-DOPA ($K_m^Y > 0.3$ mM). Although *in situ* hybridization revealed similar expression patterns for both mosquito transporters, some differences are prominent, e.g., the relative labeling intensity of aeAAT1 is significantly higher in the posterior midgut than in the gastric caeca, whereas labeling of agATT8 is approximately equal in the two regions. The transcript density of both transporters also varied depending upon developmental stage.

In summary, from the information currently available on insect NATs we conclude that this group comprises transporters that mediate the initial absorption of essential nutrient amino acids from the midgut lumen into the epithelial cells of the mosquito. Nutrient amino acid transporters do not possess the trans-specific orthology exhibited by neurotransmitter transporters (**Figures 6 and 10**), since they diverge through extensive gene duplication and losses during adaptation to particular environmental and nutrient niches. Most likely, similar paralogous groups of nutrient transporters will be defined in other insects and will be split into several, probably three, subclusters, each of which is adapted to transport an isomorphic subset of amino acids. In addition to encoding principal apical transporters, NATs may encode basolateral transporters through an alternative splicing of an anchoring motif. Owing to their crucial role in insect nutrition and phylogenetic specificity, NATs are considered to be excellent targets for the development of lineage-specific and environmentally safe mosquitocides.

5.9.8.5.3. Orphan transporters: transitions between SNF clusters These transporters are essential for physiology and development but their substrates and transport mechanisms are currently undefined. Reasons for failure to characterize orphan transporters could vary, e.g., inappropriate heterologous system, incorrect protein folding, or lack of function enabling interactions. Phylogenetic analysis indicates that orphan species represent functional and presumably evolutionary transitions between groups with defined functions (**Figure 6**). For example, orphan *ine* transporters represent a transition between neurotransmitter transporters and nutrient amino acid transporter subfamilies. SNF orphans cannot be defined as a subfamily since they do not form a true separate cluster but instead represent several consecutive levels in the phylogenetic tree. One orphan gene, named by similarity with a mammalian orphan group, has been extensively analyzed in *Drosophila* (Johnson *et al.*, 1999). This gene, named bloated tubules (*blot*), exhibits a complex and dynamic expression pattern during embryogenesis of supporting processes that are essential for cell division, differentiation, and epithelial morphogenesis. The analysis showed five putative orphan genes in *An. gambiae* and four genes encoding seven presently known isoforms in *D. melanogaster*, which represent transitions between SNF and PAT (proton amino acid transporters) superfamilies (**Figure 3**).

5.9.9. AAT Cluster IV (SLC15, SLC16, SLC17, SLC18)

Cluster IV represents one of the most diverse groups of transporters, many of which do not transport amino acids but are phylogenetically close to those which do so (**Figure 6**). It corresponds to distantly related families of mammalian transporters, including: vesicular acetylcholine and monoamine transporters (vAChT and vMATs, SLC18, see also Section 5.9.3.3.2). It is the best characterized group of vesicular transporters. It includes monocarboxylate cotransporters (MCTs, SLC16), which comprise one characterized T-type amino acid transporter (TATs, SLC16A10), H^+/oligopeptide transporters (OPTs, PEPTs, SLC15), and vesicular glutamate transporters (vGluTs). The latter represent a family that is united with transporters of type I phosphate and other organic and inorganic anions (SLC17). Since these transporters have not been characterized in insect lineages we include the most comprehensive subset of predicted genes from dipteran genomes in the phylogenetic analysis.

5.9.9.1. Vesicular Monoamine and Acetylcholine Transporters, vMATs and vAChT (SLC18)

The SLC18 family comprises specific transporters mediating high-affinity vesicular accumulation of biogenic amines that are essential for cholinergic and aminergic transmission in CNS and neuromuscular synapses throughout the Metazoa (Eiden *et al.*, 2003). These transporters apparently are not related to amino acid carriers but are included here because they are functionally similar to vesicular amino acid carriers from other phylogenetic groups (vGAT, SLC32 and vGluT, SLC17) and show distant relationships to monocarboxylate transporters (MCT, SLC16), with at least one member serving as an amino acid carrier. More direct phylogenetic links with SLC18 have been defined with toxin-extruding antiporters (TEXANs) (Yelin and Schuldiner, 1995), which are abundant in bacteria, fungi, and Archaea (**Table 2**) (Paulsen and Skurray, 1993). In bacteria these transporters appear to be essential components of interspecific antibiotic tolerance and drug resistance (Schuldiner *et al.*, 1994; Schuldiner *et al.*, 1995). All metazoan transporters that are involved in neuronal signaling are highly conserved structurally, and SLC18 members are no exception. They share remarkable similarities, in sequences and electrochemical signatures, with different lineages of the Metazoa (Rand *et al.*, 2000). A different, though highly conserved, SLC18 member's electrochemical

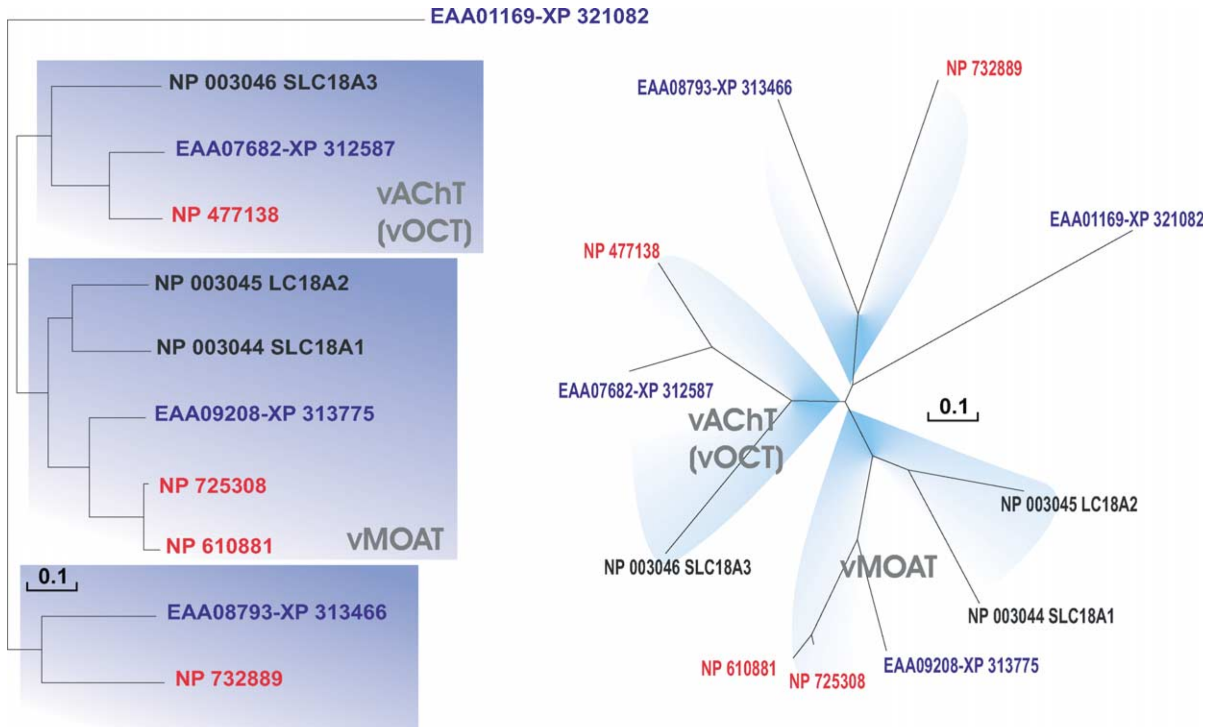


Figure 14 Phylogenetic proximity of dipteran and mammalian vesicular monoamine transporters (vMOATs; SLC18). Unrooted phylogram (left) and dendrogram (right). Font colors: blue, *An. gambiae* sequences; red, *D. melanogaster* sequences; black, *H. sapiens* sequences. Scale bars are evolutionary distances in mutations per site.

mechanism enables a high-affinity uptake of biogenic amines, which is thermodynamically coupled to H^+ V-ATPase generated proton motive forces. It exhibits a rigid stoichiometry of 1 ligand per $2H^+$ and is capable of vesicular accumulations to as high as 0.5 M concentrations of particular ligands, exceeding up to 10^3-fold and 10^5-fold transmembrane gradients for ACh and monoamines, respectively (Parsons, 2000).

Although many mammalian SLC18 members have been analyzed since the first rodent vMAT1 and vMAT2 (SLC18A1 and SLC18A2, respectively) was cloned (Erickson *et al.*, 1992; Liu *et al.*, 1992b), only one SLC18 related insect transporter has been cloned from *D. melanogaster* (NP_477131 or AAB86609; Kitamoto *et al.*, 2000a). A 12-TMD structure for this transporter has been predicted. However, since experimental proof is lacking, an alternative 10-TMD model, arising from a different prediction algorithm, can be considered as well (Rost, 1996; Eiden *et al.*, 2003). Based on EM and immunolabeling, dmvAChT colocalized with acetylcholine transferase in the brain of the adult fly where it appears to mediate specific components of synaptic wiring in glomerula (Yasuyama *et al.*, 2002). vMATs and vAChTs are encoded by four putative genes in the *An. gambiae* and three genes in the *D. melanogaster* genomes (Figures 6 and 14). vMATs and vAChTs form orthological clusters with mammalian SLC18 members. In contrast to the *H. sapiens* genome, only one gene encodes vMAT in dipteran genomes; however, two apparent splicoforms are encoded by dmvMAT (Figure 14). An additional dipteran specific group is absent in mammalian SLC18 (Figure 14).

5.9.9.2. Monocarboxylate Transporters and T System Amino Acid Transporters, MCTs and TATs (SLC16)

MCTs and TATs (SLC16) comprise a large family of H^+ coupled transporters and apparent uniporters (Bonen, 2000) that currently includes 14 members (Halestrap and Meredith, 2003) in *H. sapiens*, which, within the E value interval selected for these sequences, could be associated with 18 predicted gene products in NCBI and EMBL databases (Figure 15). Only five mammalian SLC16 members have been characterized (Halestrap and Meredith, 2003):

1. The first member was defined *in vivo* (Deuticke, 1982), subsequently cloned (Kim *et al.*, 1992; Garcia *et al.*, 1994), and designated MCT1 (SLC16A1); when heterologously expressed, it

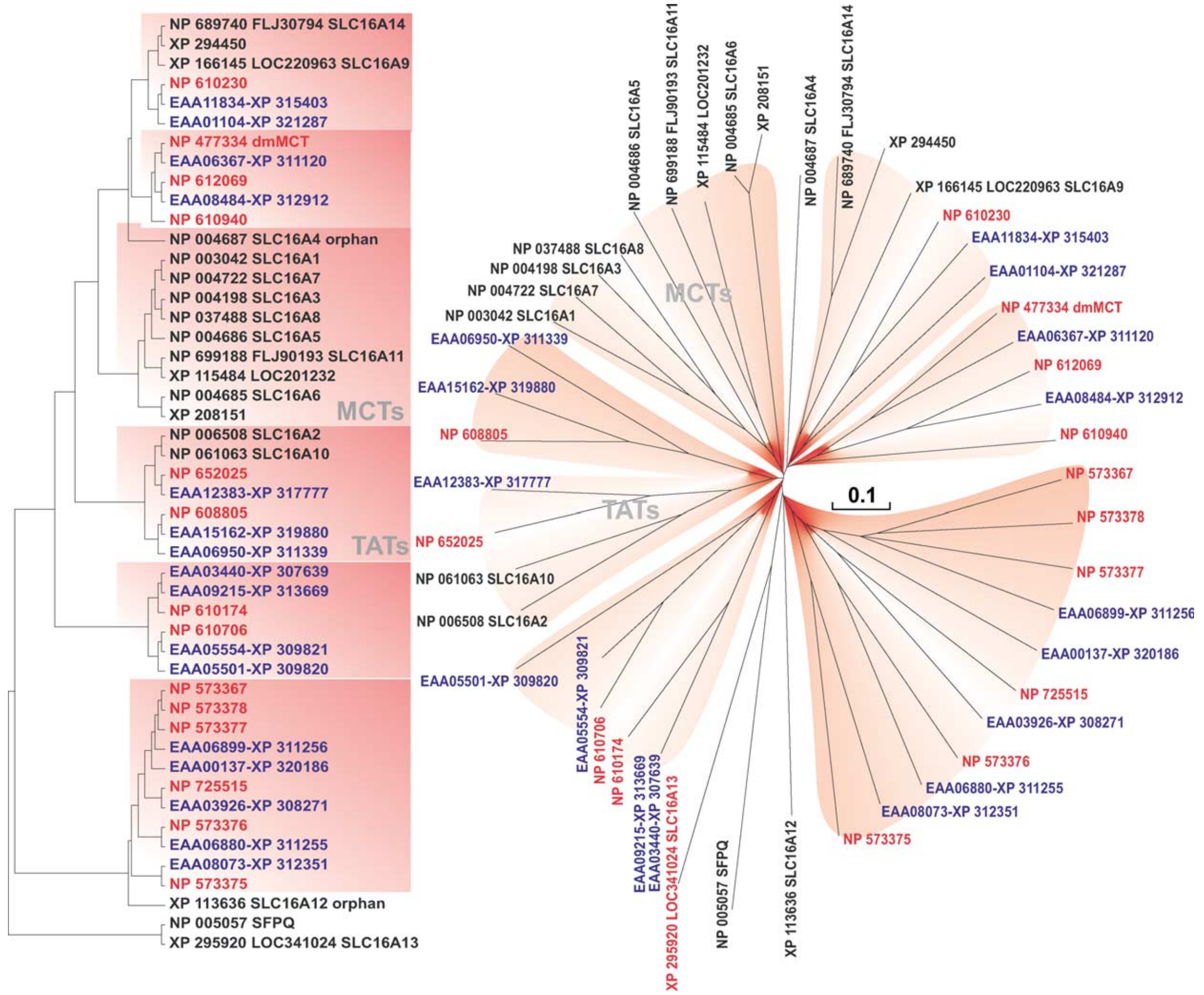


Figure 15 Phylogenetic distribution of dipteran and mammalian monocarboxilate and T-system amino acid transporters (MCTs and TATs; SLC16). Rooted cladogram (left) and unrooted dendrogram (right). Human orphan member of SLC16 (SLC16A13) and PTB associated splicing factor (NP_005057) are selected as outgroup. Font colors: blue, *An. gambiae* sequences; red, *D. melanogaster* sequences; black, *H. sapiens* sequences. Scale bars are evolutionary distances in mutations per site.

mediates a moderate affinity uptake of two to five carbon chain aliphatic monocarboxylates, e.g., $K_m^{pyruvate} = 0.7$ mM, $K_m^{propionate} = 1.5$ mM, and $K_m^{\text{L-lactate}} < 3$ mM (Broer *et al.*, 1998a; Manning Fox *et al.*, 2000).

2. Unlike MCT1, MCT2 (SLC16A2) expression intensity varies greatly in different mammalian models, e.g., its transcripts are weakly expressed in human (Price *et al.*, 1998) but are abundant in rodent (Garcia *et al.*, 1995; Jackson *et al.*, 1997) tissues where it induces a relatively high-affinity carrier; $K_m^{\text{L-lactate}} \approx 0.1$ mM and $K_m^{pyruvate} \approx 0.7$ mM (Lin *et al.*, 1998; Broer *et al.*, 1999).
3. MCT3 (SLC16A8), initially cloned from chick retinal pigment epithelium (Yoon *et al.*, 1997), mediates a low-affinity ($K_m^{\text{L-lactate}} > 6$ mM) basal transport (Yoon *et al.*, 1997; Philp *et al.*, 2001), which together with an apical MCT1 (Bergersen *et al.*, 1999) comprises a transepithelial pathway in the retinal pigment epithelium and choroid plexus.
4. MCT4 (SLC16A3) is abundant in various tissues (Price *et al.*, 1998; Wilson *et al.*, 1998; Pilegaard *et al.*, 1999; Dimmer *et al.*, 2000; Bergersen *et al.*, 2002; Meredith *et al.*, 2002) and has the weakest substrate affinity among MCTs, e.g., measured $K_m^{lactate}$ and $K_m^{pyruvate}$ values with different assay techniques are in the tenths millimolar range (Dimmer *et al.*, 2000; Manning Fox *et al.*, 2000).
5. T system transporter TAT1 (SLC16A10) mediates H^+ and Na^+ uncoupled, submillimolar affinity, active transport or facilitated diffusion of aromatic amino acids and some related metabolites (L-phenylalanine, L-tryptophan, L-tyrosine, and L-DOPA) across basolateral membranes of intestinal and renal epithelial cells as well as

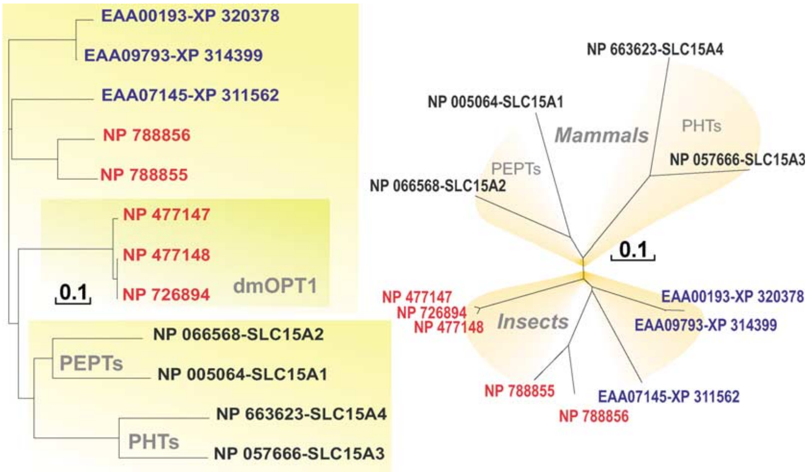


Figure 16 Paralogous distribution of dipteran and mammalian oligopeptide transporters (OPTs, SLC15). Unrooted phylogram (left) and dendrogram (right). Font colors correspond: blue, *An. gambiae* sequences; red, *D. melanogaster* sequences; black, *H. sapiens* sequences. Other abbreviations: dmOPTs, *D. melanogaster* oligopeptide transporters; PEPTs, *H. sapiens* peptide transporters; PHTs, *H. sapiens* peptide/histidine transporters. Scale bar is evolutionary distances in mutations per site.

plasma membranes of various nonpolarized cells, e.g., in placenta, liver, and muscle (Kim *et al*., 2001, 2002; Kim do *et al*., 2002). (SLC16A3 TATs should not be confused with sulfate transporter Tat1 (SLC23A11) (Toure *et al*., 2001; Vincourt *et al*., 2003) or bacteria/yeast specific TAT1 permeases (Bajmoczi *et al*., 1998)).

The tissue distributions of a few other members of SLC16 have been analyzed (Price *et al*., 1998) but no electrochemical and ligand data are yet available. A recent comprehensive review aids our SLC16 description by mentioning that heterologous expression of a TAT1 related SLC16A2 transporter in *Xenopus* oocytes revealed a thyroid hormone (T4 and T3) transporter phenotype, which has micromolar apparent substrate affinity and appears to be independent of sodium and proton motive forces (Halestrap and Meredith, 2003).

All characterized SLC16 members have a 12-TMD structure with a characteristic cytosolic loop between TMDs 6 and 7. At least some members require an auxiliary protein for trafficking and functional coordination in plasma membranes, e.g., MCT2 interacts with an unknown protein factor (Kirk *et al*., 2000) and MCT1 and MCT4 interact with CD147 (also known as OX-47, extracellular matrix metalloproteinase inducer (EMMPRIN), HT7, or basigin) (Kirk *et al*., 2000; Zhao *et al*., 2001; Halestrap and Meredith, 2003), which was confirmed using a fluorescence resonance energy transfer (FRET) technique (Wilson *et al*., 2002) and by reduction of transport by antisense silencing of a CD147 ortholog in *Xenopus* oocytes (Meredith and Halestrap, 2000).

Phylogenetic analysis discloses 15 putative genes in *An. gambiae* and 14 genes, corresponding with the number of identified transcripts, in *D. melanogaster* (Figures 6 and 15). One member of *D. melanogaster* MCTs was identified before annotation of the genome (CAB42050); however, it does not cluster with mammalian MCT1 (SLC16A1) but instead fits into an apparently orthologous phylogenetic cluster with four other putative members of dipteran MCTs (Figure 15). In fact, most MCTs form mammalian or dipteran lineage specific, paralogous clusters, except for orphans SLC16A9 and SLC16A14 and the characterized aromatic amino acid transporter TAT (SLC16A10) and putative thyroid hormone transporter (SLC16A2), which show proximity with dipteran transporters and would reasonably be expected to have similar physiological roles (Figure 16).

5.9.9.3. Oligopeptide Transporters, OPTs (SLC15)

Oligopeptide transporters (OPTs), also known as peptide transporters (PTRs) (Steiner *et al*., 1995) evolved as an efficient, energy-saving uptake mechanism complementing or replacing amino acid carriers in special cases, e.g., (1) they enable high throughput intracellular loading of nutrient amino acids in peptide form and (2) they remove specific and nonspecific oligopeptides, which may interfere with cellular signaling and cellular interactions from body fluids and subcellular media. In mammals,

OPTs mediate H^+ coupled uptake of small peptides or similar substrates into intestinal and renal epithelial cells, bile duct epithelium, glial and epithelia cells of the choroid plexus, lung, and mammary gland (Daniel and Kottra, 2003). OPTs mainly utilize transmembrane proton motive forces and appear to be reversible when heterologously expressed in HeLa cells or *Xenopus* oocytes (Fei *et al.*, 1994; Boll *et al.*, 1996) and *in vivo* (Tomita *et al.*, 1995).

The first proton-coupled peptide transporter PEPT1 (SLC15A1) was identified by expression-cloning from rabbit small intestine cDNA in the Hediger laboratory (Fei *et al.*, 1994). It mediates the uptake of small peptides, being independent of extracellular Na^+, K^+, and Cl^- gradients and, surprisingly, independent of the membrane potential as well. PEPT1 is transcribed in intestine, kidney, and liver with small amounts in brain. Simultaneously, PEPT1 was cloned by Murer and colleagues in attempts to define the molecular basis for proton-dependent peptide transport in BBMV from rabbit small intestinal epithelia (Boll *et al.*, 1994). This group also cloned an orthologous transporter PEPT2 (SLC15A2) from kidney cortex epithelia which, in contrast to low-affinity high-throughput PepT1, mediates high-affinity low-throughput transport of a virtually identical subset of substrates (Boll *et al.*, 1996). A large number of PepT1 and PEPT2 related OPTs have been identified by homology cloning in subsequent years (Liang *et al.*, 1995; Saito *et al.*, 1996; Rubio-Aliaga *et al.*, 2000; Chen *et al.*, 2002; Verri *et al.*, 2003), including one from insects, i.e., *D. melanogaster* OPT-1 (Roman *et al.*, 1998). More recently, two other OPTs were identified in mammals: a brain-specific peptide histidine transporter 1 (rat PHT1 a.k.a. human PTR4, SLC15A4; Yamashita *et al.*, 1997) and peptide histidine transporter 2 (rPHT2 a.k.a. hPTR3, SLC15A3) isolated from the rat lymphatic tissue (Sakata *et al.*, 2001). In contrast to the broad substrate affinity of PEPT1 and 2, PHT1 and 2 have more specific sets of substrates including histidine and some model di- and tripeptides. These transporters apparently play different, more specific physiological roles, since they are strongly expressed in specialized tissues such as brain for PTH1 (Yamashita *et al.*, 1997) and lymphatic system, lung, spleen, and thymus for PHT 2 (Sakata *et al.*, 2001). OPTs range from 450 to 700 amino acid residues, most of the eukaryotic members having ~650 amino acid residues and 12 predicted TMDs with cytoplasmic N- and C-termini (Daniel and Kottra, 2003).

5.9.9.3.1. *Drosophila* oligopeptide transporters, OPTs The only insect OPT characterized to date is from *D. melanogaster* (Roman *et al.*, 1998). The *opt* gene encodes a protein that mediates proton-dependent di- and tripeptide transport in intestinal and Malpighian tubular (renal) epithelia. Like mammalian OPTs, fruit fly OPT1 has 12 TMDs and a long (200 amino acid residue) fifth external loop. It is most similar to human renal PEPT2 and intestinal PEPT1 proteins, containing the highly conserved histidine residue, thought to be involved in peptide binding and transport, at position 88.

Up to 30% of total amino acids in the hemolymph of adult fruit flies are present in the form of di- or tripeptides, which serve in osmoregulation (Chen, 1966; Collett, 1976). Protein digestion in *Drosophila* occurs mainly in the midgut where resulting peptides and amino acids are rapidly taken up by the epithelium (Law *et al.*, 1977) and metabolized or transported to the hemolymph. Hemolymph amino acids are secreted into the Malpighian tubular lumen (equivalent to vertebrate glomerular filtration) but are reabsorbed in the hindgut. Compared to mammalian peptide transporters discussed above, little was known about the absorption of peptides in insects prior to the cloning of dmOPT1. The *opt1* gene encodes a high-affinity di- and tripeptide transporter that is proton dependent. It is transcribed in germinal and somatic tissues of both males and females where it is most highly expressed in nurse cells of the ovary. It is also expressed in epithelial cells of the midgut and rectum and is mildly expressed in neurons. Uptake was assayed in HeLa cells transfected with pCMVOPT1 or pCMV2 using lipofectamine. Tritiated L-alanylalanine uptake was measured in buffer with high NaCl and low KCl at pH 6.0. Evidence that the peptide transport is proton-coupled was a ten-fold decrease in uptake when extracellular pH was increased from 6 to 7 and a 20-fold decrease when it was increased from 6 to 8. Uptake was virtually abolished by FCCP (25 μM), which collapses the proton gradient. The K_m for OPT1-dependent alanylalanine transport at external pH = 6 was 48.8 μM (Roman *et al.*, 1998).

5.9.9.3.2. Phylogenetic characteristics of dipteran OPTs OPT (SLC15) related transporters are present in most life kingdoms, except for the Archaea (**Table 2**), being especially abundant in plants where they are in phylogenetic proximity to nitrate transporters. OPTs comprise three putative genes in the *An. gambiae* genome and three genes in the *D. melanogaster* genome with three different

splicoforms known for the single characterized example of a dmOPT1 gene (**Figure 16**). Within the framework of mammalian and dipteran lineages, OPTs form separate paralogous branches, indicating relatively recent gene duplication. Probably for the same reason, the proximity of different dipteran OPTs is difficult to deduce solely from the phylogenetic analysis.

5.9.9.4. Vesicular Glutamate and Phosphate Transporters, vGLUTs and P_iTs (SLC17)

In mammals, the SLC17 family comprises two physiologically distinct groups of characterized transporters: type I inorganic phosphate transporters (P_iTs) and vesicular glutamate transporters (vGLUTs). The P_iT family, which was initially restricted to transporters of inorganic phosphates across plasma membranes, has now been expanded to include transporters of various organic and inorganic anions across both plasma membranes and endomembranes. The vGluT family comprises extensively analyzed vesicular glutamate transporters, which are specialized to serve excitatory neurotransmission that enables endomembrane traffic and vesicular accumulation of excitatory amino acids. Since no SLC17-related insect transporter has yet been characterized, only a brief description of known SLC17 transporters was included from a recent comprehensive review (Reimer and Edwards, 2003).

5.9.9.4.1. Inorganic phosphate transporter, type I P_iTs (SLC17A1) The first member of mammalian SLC17A1 (NaP$_i$-1) was identified by expression cloning from a rabbit kidney cortex cDNA library, based on its ability to induce Na^+-dependent phosphate transport in *Xenopus* oocytes, with 750-fold higher apparent uptake than controls injected with total poly(A) RNA (Werner *et al.*, 1991). However, submillimolar substrate affinities of rabbit NaP$_i$-1 and the next-cloned ortholog from *H. sapiens* (Miyamoto *et al.*, 1995), as well as specific electrochemical properties of their heterologous expression in *Xenopus* oocytes, e.g., conductance for mineral and organic anions (Busch *et al.*, 1996a, 1996b; Broer *et al.*, 1998b) and low pH sensitivity (Busch *et al.*, 1996b), do not match characteristics of Na^+-dependent P_i uptake in brush border membrane vesicles (Amstutz *et al.*, 1985) and do not support a primary role for these transporters in high-affinity phosphate transport (Murer *et al.*, 2000; Reimer and Edwards, 2003). Indeed, two different P_iT types have been identified (Magagnin *et al.*, 1993; Kavanaugh *et al.*, 1994) that are unrelated to SLC17 Na^+/P_i^- cotransporters. One new type is a SLC34 family, type II P_iT, whose members mediate apical P_i uptake in various polarized epithelia (Murer *et al.*, 2003). Transporters of the other new type were originally described as retroviral receptors (Olah *et al.*, 1994) but are now recognized as type III P_iTs (SLC20), which appear to be basolateral components of P_i homeostasis (Collins *et al.*, 2003). A recent revision of P_iTs tends to support the earlier proposal that some members of the SLC17 family are facultative P_iTs, with major roles in the rapid acquisition of electrochemical and pH balance via anion secretion (Amstutz *et al.*, 1985). In fact, they may utilize a variety of electrochemical mechanisms ranging from facilitated diffusion to electrophoretic Na^+ coupled symport (Reimer and Edwards, 2003).

Intriguingly, despite a crucial role of P_i homeostasis in all organisms, and the presence of homologous proteins in other metazoa, as well as in bacteria, fungi, and plant lineages, there is no trace of SLC20 and SLC34 families in dipteran genomes and they appear to be absent in arthropods in general. Conversely, mammalian type I P_iTs do not include any dipteran sequences (**Figure 17**), which leaves uncertain the presence of SLC17 related P_iTs in dipteran genomes.

5.9.9.4.2. Sialin (SLC17A5) Extensive genetic analysis and BBMV studies revealed a physiological role for another SLC17 member, sialin (SLC17A5), which apparently mediates lysosomal H^+ coupled (1 : 1) emission of amino carbohydrates (sialic acid) from lysosomal catabolism of glycosylated membrane proteins, as well as serving endomembrane traffic of some monocarboxylic acids (Reimer and Edwards, 2003).

5.9.9.4.3. vGlutT (SLC17A6, 7, 8) Three members of mammalian SLC17 (SLC17A6, A7, and A8, a.k.a. vGluT1, 2 and 3) are recruited in excitatory neurotransmission, enabling vesicular storage of L-glutamate. Initially defined as plasma membrane, Na^+ coupled P_iTs, vGluT1 (Ni *et al.*, 1994), and vGluT2 (Aihara *et al.*, 2000) transport phenotypes have since been shown to be H^+ coupled carriers that mediate accumulation of L-glutamate into synaptic vesicles of neurons (Bellocchio *et al.*, 2000) and glutamatergic endocrine cells (Hayashi *et al.*, 2001; Takamori *et al.*, 2001). All characterized vGluTs have been morphologically associated with glutamatergic neurons of the postnatal brain and especially abundant at membranes of synaptic vesicles, although spatial differences in subtype distribution in the brain are prominent (Bellocchio *et al.*, 1998; Fremeau *et al.*, 2002; Varoqui *et al.*, 2002b). The neuronal specificity of vGluTs makes them

useful markers of excitatory neuronal components, e.g., recent demonstration of the existence of peripheral glutamatergic components in the gastroenteropancreatic system and testis (Hayashi *et al*., 2003). The expression of vGluTs occurs very early in embryogenesis (Gleason *et al*., 2003). High resolution immunoelectron microscopy also demonstrates a specific subcellular distribution of vGluT types, e.g., vGluT1 and vGluT2 are mainly associated with nerve terminals; in contrast, vGluT3 is present in vesicular structures of astrocytes and neuronal dendrites (Fremeau *et al*., 2002).

Apparently, all vGluTs share highly conserved, pmf driven, electrochemical mechanisms with nearly millimolar apparent K_m^{Glu}. Like P_iTs, vGluTs enable Cl^- and some other anionic conductances in BBMV and heterologous models (Bellocchio *et al*., 2000; Fremeau *et al*., 2001; Varoqui *et al*., 2002a). Vesicular glutamate accumulation has been shown to be inhibited by the broad specificity blocker of chloride conductance DIDS(4,4′-diisothiocyanatodihydrostilbene-2,2′disulfonic acid). Like all other endomembrane transporters, electrophoretic vGluTs are energized by electrogenic V-ATPase.

5.9.9.4.4. Phylogenetic characteristics of SLC17 transporters Before their molecular identities were established, P_iTs and vGluTs have been identified by comparative studies of vesicular glutamate uptake in BBMVs from neuronal tissues of several vertebrates and invertebrates (goldfish, frogs, turtles, pigeons, rats, fruit fly, and crayfish). Their

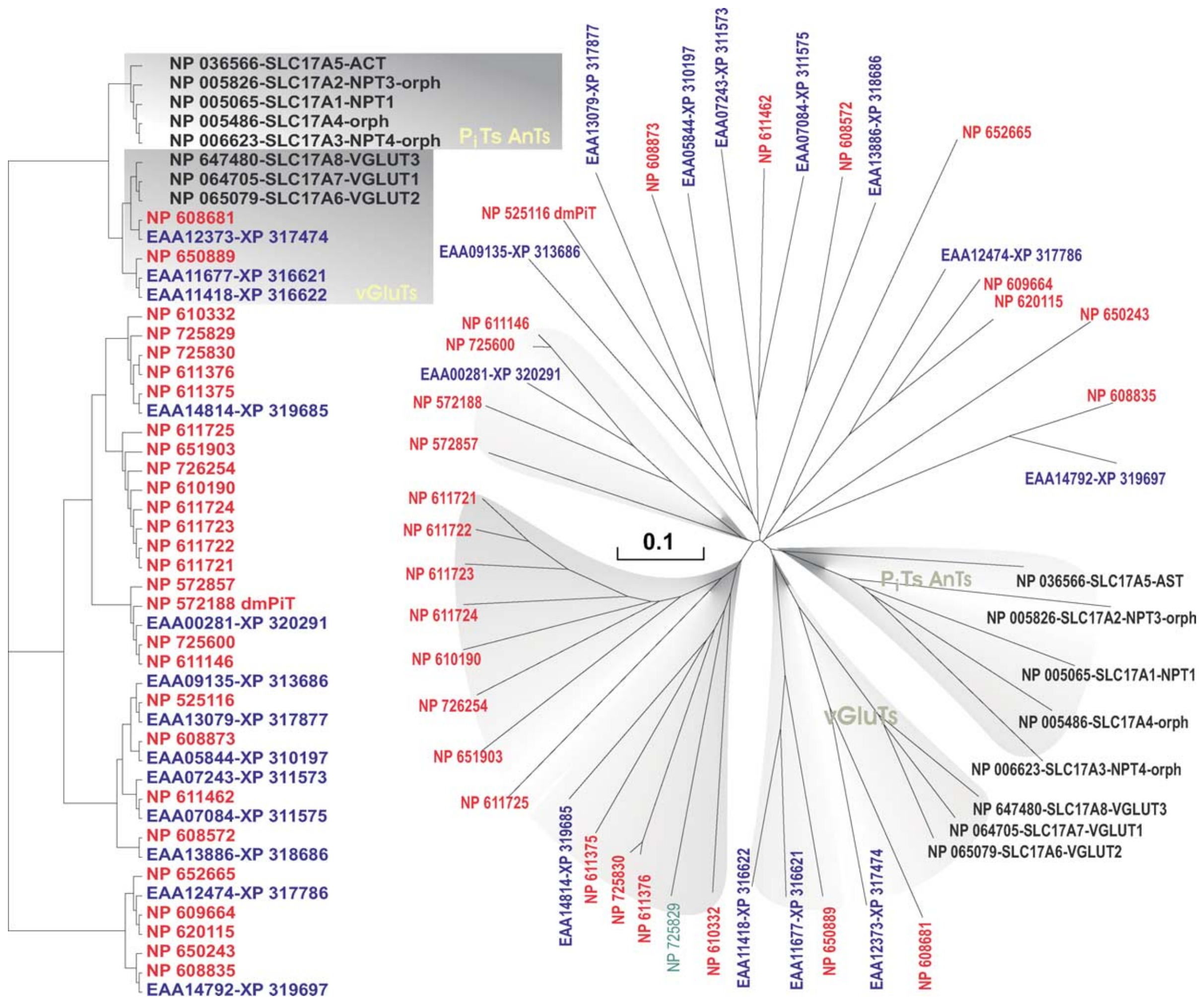


Figure 17 Phylogenetic proximity of putative organic and inorganic anion (P_iTs) and vesicular glutamate transporters (vGluTs) in dipteran and mammalian models (SLC17). Unrooted cladogram (left) and dendrogram (right). Other abbreviations: AST, anion/sugar transporter; NPT, sodium inorganic phosphate transporter[4]. Scale bars are evolutionary distances in mutations per site.

[4]NTP title reflects a historical drawback, since NTPs were initially cloned and characterized as inorganic phosphate transporter Type I. Later, the major role of this transporter phenotype in the transport of organic and inorganic anions was established.

properties, including millimolar Glu affinity, as well as V-ATPase energization and chloride stimulation, appear to be extremely conserved over 350–400 million years (Tabb and Ueda, 1991). Phylogenetic analysis indicates a single gene copy in *D. melanogaster* and *An. gambiae* genomes with close proximity to three, apparently paralogous, vGluT gene clusters in mammals (**Figure 17**). Two additional *An. gambiae* genes and one *D. melanogaster* gene are more distantly related to the vGluTs. No dipteran genes with proximity to P_iT transporters have been found to date. The earlier defined dmP_iT transporter gene (NP_572188) now appears to be phylogenetically separated from P_iT gene clusters; its function cannot be deduced from the phylogenetic pattern alone. Intriguingly, dipteran genomes include several new SLC17 gene clusters that are absent in mammals and have no matches with characterized genes in other lineages. Two of these clusters indicate remarkable gene duplications in *D. melanogaster* (**Figure 17**). In summary, relatively conserved VGluTs seem to have diverged through lineage-specific gene duplications that reflect increases in functional and morphological complexity of the CNS. Hence, a role for the genes that encode transporters related to the SLC17 family can be expected in insect vesicular glutamate homeostasis. In contrast, P_i absorption and homeostasis, which are essential for all organisms, appears to occur in insects through phylogenetically unrelated roots of transporters that are distinct from P_iTs of SLC20, SLC34, and even SLC17.

5.9.10. Summary and Perspectives

Practical benefits from characterizing neurotransmitter and nutrient amino acid transporters have extraordinary relevance to medicine and agriculture. The kinetic properties, abundance, variety, and uniqueness of NTTs and NATs make them prime targets to develop inexpensive insecticides and mosquitocides that are as potent as DDT and as safe as Bti (*Bacillus thuringiensis* subsp. *israelensis*). As providers of intracellular amino acids, plasma membrane transporters represent rate limiting components of crucial metabolic pathways. Low concentrations of high affinity or irreversibly binding inhibitors can block transport and abolish essential pathways, so such transport inhibitors may be potent insecticides. The abundance and variety of transport systems in insects provides a plethora of targets for potential amino acid transport inhibitors. This diversity may help to avoid the pitfalls of resistance that have doomed many previous insecticides such as DDT – before pests become resistant to one transport inhibitor another one can be developed. In particular, the unique nature of mosquito NATs provides the potential for agents to be developed that will inhibit them without affecting vertebrate or other insect NATs and NTTs. The most likely class of potential mosquitocides would be amino acid derivatives and peptides. An almost infinite variety of such compounds can be synthesized and screened making use of new tools from recent advances in insect transport physiology and molecular biology. Vincent Wigglesworth invented Insect Physiology to help control tropical diseases. Today, integrative physiology and functional genomics of *An. gambiae* and other disease vectors can build on this basic science to provide rational strategies for impacting insect borne diseases through vector modification to reduce disease transmissibility and insecticide application to reduce vector population size.

Acknowledgments

Supported in part by The Whitney Laboratory and NIH Research grant R01 AI-30464.

References

Adams, M.D., Celniker, S.E., Holt, R.A., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Agulhon, C., Rostaing, P., Ravassard, P., Sagne, C., Triller, A., *et al.*, **2003**. Lysosomal amino acid transporter LYAAT-1 in the rat central nervous system: an *in situ* hybridization and immunohistochemical study. *J. Comp. Neurol. 462*, 71–89.

Aihara, Y., Mashima, H., Onda, H., Hisano, S., Kasuya, H., *et al.*, **2000**. Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter. *J. Neurochem. 74*, 2622–2625.

Albritton, L.M., Tseng, L., Scadden, D., Cunningham, J.M., **1989**. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus-infection. *Cell 57*, 659–666.

Amara, S.G., Fontana, A.C., **2002**. Excitatory amino acid transporters: keeping up with glutamate. *Neurochem. Int. 41*, 313–318.

Amstutz, M., Mohrmann, M., Gmaj, P., Murer, H., **1985**. Effect of pH on phosphate transport in rat renal brush border membrane vesicles. *Am. J. Physiol. 248*, F705–F710.

Arriza, J.L., Eliasof, S., Kavanaugh, M.P., Amara, S.G., **1997**. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc. Natl Acad. Sci. USA 94*, 4155–4160.

Arriza, J.L., Fairman, W.A., Wadiche, J.I., Murdoch, G.H., Kavanaugh, M.P., *et al.*, **1994**. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci. 14*, 5559–5569.

Arriza, J.L., Kavanaugh, M.P., Fairman, W.A., Wu, Y.N., Murdoch, G.H., *et al.*, **1993**. Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. *J. Biol. Chem. 268*, 15329–15332.

Auger, C., Attwell, D., **2000**. Fast removal of synaptic glutamate by postsynaptic transporters. *Neuron 28*, 547–558.

Bader, A.L., Parthasarathy, R., Harvey, W.R., **1995**. A novel proline, glycine: K^+ symporter in midgut brush-border membrane vesicles from larval *Manduca sexta*. *J. Exp. Biol. 198*, 2599–2607.

Baier, A., Wittek, B., Brembs, B., **2002**. *Drosophila* as a new model organism for the neurobiology of aggression? *J. Exp. Biol. 205*, 1233–1240.

Bajmoczi, M., Sneve, M., Eide, D.J., Drewes, L.R., **1998**. TATI encodes a low-affinity histidine transporter in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun. 243*, 205–209.

Balboni, E., **1978**. A proline shuttle in insect flight muscle. *Biochem. Biophys. Res. Commun. 85*, 1090–1096.

Bassi, M.T., Gasol, E., Manzoni, M., Pineda, M., Riboni, M., *et al.*, **2001**. Identification and characterisation of human xCT that co-expresses, with 4F2 heavy chain, the amino acid transport activity system x(c) (-). *Pflugers Archiv. 442*, 286–296.

Bellocchio, E.E., Hu, H., Pohorille, A., Chan, J., Pickel, V.M., *et al.*, **1998**. The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. *J. Neurosci. 18*, 8648–8659.

Bellocchio, E.E., Reimer, R.J., Fremeau, R.T., Jr., Edwards, R.H., **2000**. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science 289*, 957–960.

Bergersen, L., Johannsson, E., Veruki, M.L., Nagelhus, E.A., Halestrap, A., *et al.*, **1999**. Cellular and subcellular expression of monocarboxylate transporters in the pigment epithelium and retina of the rat. *Neuroscience 90*, 319–331.

Bergersen, L., Rafiki, A., Ottersen, O.P., **2002**. Immunogold cytochemistry identifies specialized membrane domains for monocarboxylate transport in the central nervous system. *Neurochem. Res. 27*, 89–96.

Bertran, J., Werner, A., Moore, M.L., Stange, G., Markovich, D., *et al.*, **1992**. Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. *Proc. Natl Acad. Sci. USA 89*, 5601–5605.

Besson, M.T., Soustelle, L., Birman, S., **1999**. Identification and structural characterization of two genes encoding glutamate transporter homologues differently expressed in the nervous system of *Drosophila melanogaster. FEBS Lett. 443*, 97–104.

Besson, M.T., Soustelle, L., Birman, S., **2000**. Selective high-affinity transport of aspartate by a *Drosophila* homologue of the excitatory amino-acid transporters. *Curr. Biol. 10*, 207–210.

Beyenbach, K.W., **2001**. Energizing epithelial transport with the vacuolar H(+)-ATPase. *News Physiol. Sci. 16*, 145–151.

Bicker, G., Schafer, S., Ottersen, O.P., Storm-Mathisen, J., **1988**. Glutamate-like immunoreactivity in identified neuronal populations of insect nervous systems. *J. Neurosci. 8*, 2108–2122.

Bismuth, Y., Kavanaugh, M.P., Kanner, B.I., **1997**. Tyrosine 140 of the gamma-aminobutyric acid transporter GAT-1 plays a critical role in neurotransmitter recognition. *J. Biol. Chem. 272*, 16096–16102.

Boll, M., Daniel, H., Gasnier, B., **2003a**. The SLC36 family: proton-coupled transporters for the absorption of selected amino acids from extracellular and intracellular proteolysis. *Pflugers Arch.* 2003 May 14 (Epub ahead of print).

Boll, M., Foltz, M., Rubio-Aliaga, I., Daniel, H., **2003b**. A cluster of proton/amino acid transporter genes in the human and mouse genomes. *Genomics 82*, 47–56.

Boll, M., Foltz, M., Rubio-Aliaga, I., Kottra, G., Daniel, H., **2002**. Functional characterization of two novel mammalian electrogenic proton-dependent amino acid cotransporters. *J. Biol. Chem. 277*, 22966–22973.

Boll, M., Herget, M., Wagener, M., Weber, W.M., Markovich, D., *et al.*, **1996**. Expression cloning and functional characterization of the kidney cortex high-affinity proton-coupled peptide transporter. *Proc. Natl Acad. Sci. USA 93*, 284–289.

Boll, M., Markovich, D., Weber, W.M., Korte, H., Daniel, H., *et al.*, **1994**. Expression cloning of a cDNA from rabbit small-intestine related to proton-coupled transport of peptides, beta-lactam antibiotics and Ace-inhibitors. *Pflugers Arch. 429*, 146–149.

Bonen, A., **2000**. Lactate transporters (MCT proteins) in heart and skeletal muscles. *Med. Sci. Sports Exercise 32*, 778–789.

Bossi, E., Centinaio, E., Castagna, M., Giovannardi, S., Vincenti, S., *et al.*, **1999a**. Ion binding and permeation through the lepidopteran amino acid transporter KAAT1 expressed in *Xenopus* oocytes. *J. Physiol. – Lond. 515*, 729–742.

Bossi, E., Sacchi, V.F., Peres, A., **1999b**. Ionic selectivity of the coupled and uncoupled currents carried by the amino acid transporter KAAT1. *Pflugers Archiv. 438*, 788–796.

Boudko, D.Y., Kohn, A.B., Meleshkevitch, E.A., Dasher, M.K., Seron, T.J., *et al.*, Ancestry and progency of nutrient amino acid transporters. *Proc. Natl Acad. Sci. USA* (in press).

Boudko, D.Y., Moroz, L.L., Harvey, W.R., Linser, P.J., **2001a**. Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc. Natl Acad. Sci. USA 98*, 15354–15359.

Boudko, D.Y., Moroz, L.L., Linser, P.J., Trimarchi, J.R., Smith, P.J., *et al.*, **2001b**. *In situ* analysis of pH gradients in mosquito larvae using non-invasive, self-referencing, pH-sensitive microelectrodes. *J. Exp. Biol. 204*, 691–699.

Breer, H., Knipper, M., **1990**. Regulation of high affinity choline uptake. *J. Neurobiol. 21*, 269–275.

Bridges, C.C., Kekuda, R., Wang, H.P., Prasad, P.D., Mehta, P., *et al.*, **2001**. Structure, function, and regulation of human cystine/glutamate transporter in retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci. 42*, 47–54.

Broer, A., Albers, A., Setiawan, I., Edwards, R.H., Chaudhry, F.A., *et al.*, **2002**. Regulation of the glutamine transporter SN1 by extracellular pH and intracellular sodium ions. *J. Physiol. –Lond. 539*, 3–14.

Broer, A., Wagner, C.A., Lang, F., Broer, S., **2000**. The heterodimeric amino acid transporter 4F2hc/y+LAT2 mediates arginine efflux in exchange with glutamine. *Biochem. J. 349*, 787–795.

Broer, S., Broer, A., Schneider, H.P., Stegen, C., Halestrap, A.P., *et al.*, **1999**. Characterization of the high-affinity monocarboxylate transporter MCT2 in *Xenopus laevis* oocytes. *Biochem. J. 341*, 529–535.

Broer, S., Schneider, H.P., Broer, A., Rahman, B., Hamprecht, B., *et al.*, **1998a**. Characterization of the monocarboxylate transporter 1 expressed in *Xenopus laevis* oocytes by changes in cytosolic pH. *Biochem. J. 333*, 167–174.

Broer, S., Schuster, A., Wagner, C.A., Broer, A., Forster, I., *et al.*, **1998b**. Chloride conductance and Pi transport are separate functions induced by the expression of NaPi-1 in *Xenopus* oocytes. *J. Membr. Biol. 164*, 71–77.

Brown, C.S., Nestler, C., **1985**. Catecholamines and indolalkylamines. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 11. Pergamon Press, Oxford, pp. 436–497.

Busch, A.E., Biber, J., Murer, H., Lang, F., **1996a**. Electrophysiological insights of type I and II Na/Pi transporters. *Kidney Int. 49*, 986–987.

Busch, A.E., Schuster, A., Waldegger, S., Wagner, C.A., Zempel, G., *et al.*, **1996b**. Expression of a renal type I sodium/phosphate transporter (NaPi-1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions. *Proc. Natl Acad. Sci. USA 93*, 5347–5351.

Callec, J.J., **1985**. Synaptic transmission in the central nervous system. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 5. Pergamon Press, Oxford, pp. 139–179.

Carew, T.J., **1996**. Molecular enhancement of memory formation. *Neuron 16*, 5–8.

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V.F., Harvey, W.R., *et al.*, **1997**. Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis. *J. Exp. Biol. 200*, 269–286.

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V.F., Harvey, W.R., *et al.*, **1998**. Cloning and characterization of a potassium-coupled amino acid transporter. *Proc. Natl Acad. Sci. USA 95*, 5395–5400.

Castagna, M., Vincenti, S., Marciani, P., Sacchi, V.F., **2002**. Inhibition of the lepidopteran amino acid cotransporter KAAT1 by phenylglyoxal: role of arginine 76. *Insect Mol. Biol. 11*, 283–289.

Caveney, S., Donly, B.C., **2002**. Neurotransmitter transporters in the insect nervous system. *Adv. Insect Physiol. 29*, 55–149.

Chaudhry, F.A., Krizaj, D., Larsson, P., Reimer, R.J., Wreden, C., *et al.*, **2001**. Coupled and uncoupled proton movement by amino acid transport system N. *EMBO J. 20*, 7041–7051.

Chen, H., Pan, Y.X., Wong, E.A., Bloomquist, J.R., Webb, K.E., **2002**. Molecular cloning and functional expression of a chicken intestinal peptide transporter (cPepT1) in *Xenopus* oocytes and Chinese hamster ovary cells. *J. Nutr. 132*, 387–393.

Chen, N.H., Reith, M.E., Quick, M.W., **2003**. Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6. *Pflugers Arch*. 2003 Apr 29 (Epub ahead of print).

Chen, P.S., **1966**. Amino acid and protein metabolism in insect development. *Adv. Insect Physiol. 3*, 53–132.

Chen, Y., Swanson, R.A., **2003**. The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. *J. Neurochem. 84*, 1332–1339.

Chen, Z., Fei, Y.J., Anderson, C.M., Wake, K.A., Miyauchi, S., *et al.*, **2003**. Structure, function and immunolocalization of a proton-coupled amino acid transporter (hPAT1) in the human intestinal cell line Caco-2. *J. Physiol. 546*, 349–361.

Chiang, A.S., Lin, W.Y., Liu, H.P., Pszczolkowski, M.A., Fu, T.F., *et al.*, **2002**. Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA 99*, 37–42.

Chillaron, J., Estevez, R., Mora, C., Wagner, C.A., Suessbrich, H., *et al.*, **1996**. Obligatory amino acid exchange via systems bo,$^{+}$-like and y^{+}L-like. A tertiary active transport mechanism for renal reabsorption of cystine and dibasic amino acids. *J. Biol. Chem. 271*, 17761–17770.

Chillaron, J., Roca, R., Valencia, A., Zorzano, A., Palacin, M., **2001**. Heteromeric amino acid transporters: biochemistry, genetics, and physiology. *Am. J. Physiol. Renal Physiol. 281*, F995–F1018.

Chiu, C.-S., Ross, L.S., Cohen, B.N., Lester, H.A., Gill, S.S., **2000**. The transporter-like protein inebriated mediates hyperosmotic stimuli through intracellular signalling. *J. Exp. Biol. 203*, 3531–3546.

Christensen, H.N., **1964**. Transport system serving for mono-+ diamino acids. *Proc. Natl Acad. Sci. USA 51*, 337–344.

Cioffi, M., Wolfersberger, M.G., **1983**. Isolation of separate apical, lateral and basal plasma membrane from cells of an insect epithelium. A procedure based on tissue organization and ultrastructure. *Tissue Cell 15*, 781–803.

Clark, J.A., Amara, S.G., **1993**. Amino acid neurotransmitter transporters: structure, function, and molecular diversity. *Bioessays 15*, 323–332.

Clements, A.N., **1992**. The Biology of Mosquitoes. Chapman and Hall, London.

Closs, E.I., **2002**. Expression, regulation and function of carrier proteins for cationic amino acids. *Curr. Opin. Nephrol. Hypertens. 11*, 99–107.

Cociancich, S.O., Park, S.S., Fidock, D.A., Shahabuddin, M., **1999**. Vesicular ATPase-overexpressing cells determine the distribution of malaria parasite oocysts on the midguts of mosquitoes. *J. Biol. Chem. 274*, 12650–12655.

Collett, J.I., **1976**. Small peptides, a life-long store of amino acid in adult *Drosophila* and Calliphora. *J. Insect Physiol. 22*, 433–440.

Collins, J.F., Bai, L., Ghishan, F.K., **2003**. The SLC20 family of proteins: dual functions as sodium-phosphate cotransporters and viral receptors. *Pflugers Arch.* May 21 (Epub ahead of print).

Copeland, J., Robertson, H.A., **1982**. Octopamine as the transmitter at the firefly lantern – presence of an octopamine-sensitive and a dopamine-sensitive adenylatecyclase. *Comp. Biochem. Physiol. C–Pharmacol. Toxicol. Endocrinol. 72*, 125–127.

Corey, J.L., Quick, M.W., Davidson, N., Lester, H.A., Guastella, J., **1994**. A cocaine-sensitive *Drosophila* serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc. Natl Acad. Sci. USA 91*, 1188–1192.

Danbolt, N.C., **2001**. Glutamate uptake. *Prog. Neurobiol. 65*, 1–105.

Daniel, H., Kottra, G., **2003**. The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. *Pflugers Arch.* 2003 Aug 7 (Epub ahead of print).

Darboux, I., Nielsen-LeRoux, C., Charles, J.F., Pauron, D., **2001**. The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: molecular cloning and expression. *Insect Biochem. Mol. Biol. 31*, 981–990.

Deken, S.L., Fremeau, R.T.J., Quick, M.W., **2002**. Family of sodium-coupled transporters for GABA, glycine, proline, betaine, taurine, and creatine. In: Reith, M.E.A. (Ed.), Neurotransmitter Transporters: Structure, Function and Regulation. Humana Press Inc., Totowa, New Jersey, pp. 193–234.

Demchyshyn, L.L., Pristupa, Z.B., Sugamori, K.S., Barker, E.L., Blakely, R.D., *et al.*, **1994**. Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 91*, 5158–5162.

Deuticke, B., **1982**. Monocarboxylate transport in erythrocytes. *J. Membr. Biol. 70*, 89–103.

Dimmer, K.S., Friedrich, B., Lang, F., Deitmer, J.W., Broer, S., **2000**. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. *Biochem. J. 350*, 219–227.

Donly, B.C., Richman, A., Hawkins, E., McLean, H., Caveney, S., **1997**. Molecular cloning and functional expression of an insect high-affinity Na^+-dependent glutamate transporter. *Eur. J. Biochem. 248*, 535–542.

Donly, C., Jevnikar, J., McLean, H., Caveney, S., **2000**. Substrate-stereoselectivity of a high-affinity glutamate transporter cloned from the CNS of the cockroach *Diploptera punctata*. *Insect Biochem. Mol. Biol. 30*, 369–376.

Drose, S., Boddien, C., Gassel, M., Ingenhorst, G., Zeeck, A., *et al.*, **2001**. Semisynthetic derivatives of concanamycin A and C, as inhibitors of V- and P-type ATPases: structure-activity investigations and developments of photoaffinity probes. *Biochemistry 40*, 2816–2825.

Eiden, L.E., Schafer, M.K., Weihe, E., Schutz, B., **2003**. The vesicular amine transporter family (SLC18): amine/proton antiporters required for vesicular accumulation and regulated exocytotic secretion of monoamines and acetylcholine. *Pflugers Arch.* 2003 May 6 (Epub ahead of print).

Erickson, J.D., Eiden, L.E., Hoffman, B.J., **1992**. Expression cloning of a reserpine-sensitive vesicular monoamine transporter. *Proc. Natl Acad. Sci. USA 89*, 10993–10997.

Fairman, W.A., Vandenberg, R.J., Arriza, J.L., Kavanaugh, M.P., Amara, S.G., **1995**. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature 375*, 599–603.

Farooqui, T., Robinson, K., Vaessin, H., Smith, B.H., **2003**. Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J. Neurosci. 23*, 5370–5380.

Fei, Y.J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., *et al.*, **1994**. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature 368*, 563–566.

Feldman, D.H., Harvey, W.R., Stevens, B.R., **2000**. A novel electrogenic amino acid transporter is activated by K^+ or Na^+, is alkaline pH-dependent, and is Cl^--independent. *J. Biol. Chem. 275*, 24518–24526.

Fernandez, E., Torrents, D., Chillaron, J., Martin Del Rio, R., Zorzano, A., *et al.*, **2003**. Basolateral LAT-2 has a major role in the transepithelial flux of L-cystine in the renal proximal tubule cell line OK. *J. Am. Soc. Nephrol. 14*, 837–847.

Fernandez, J., Bode, B., Koromilas, A., Diehl, J.A., Krukovets, I., *et al.*, **2002**. Translation mediated by the internal ribosome entry Site of the cat-1 mRNA is regulated by glucose availability in a PERK kinase-dependent manner. *J. Biol. Chem. 277*, 11780–11787.

Filippova, M., Ross, L.S., Gill, S.S., **1998**. Cloning of the v-ATPase B subunit from *Culex Quinquefasciatus* and expression of the B and C subunits in mosquitoes. *Insect Mol. Biol. 7*, 223–232.

Fremeau, R.T., Jr., Burman, J., Qureshi, T., Tran, C.H., Proctor, J., *et al.*, **2002**. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc. Natl Acad. Sci. USA 99*, 14488–14493.

Fremeau, R.T., Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., *et al.*, **2001**. The expression of vesicular

glutamate transporters defines two classes of excitatory synapse. *Neuron 31*, 247–260.

Furuya, S., Watanabe, M., **2003**. Novel neuroglial and glioglial relationships mediated by L-serine metabolism. *Arch. Histol. Cytol. 66*, 109–121.

Gade, G., Auerswald, L., **2002**. Beetles' choice – proline for energy output: control by AKHs. *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 132*, 117–129.

Gainetdinov, R.R., Caron, M.G., **2002**. Monoamine transporters. In: Reith, M.E.A. (Ed.), Neurotransmitter Transporters: Structure, Function and Regulation. Humana Press Inc., Totowa, New Jersey, pp. 171–192.

Gallant, P., Malutan, T., McLean, H., Verellen, L., Caveney, S., *et al.*, **2003**. Functionally distinct dopamine and octopamine transporters in the CNS of the cabbage looper moth. *Eur. J. Biochem. 270*, 664–674.

Gao, X.J., McLean, H., Caveney, S., Donly, C., **1999**. Molecular cloning and functional characterization of a GABA transporter from the CNS of the cabbage looper, *Trichoplusia ni. Insect Biochem. Mol. Biol. 29*, 609–623.

Garcia, C.K., Brown, M.S., Pathak, R.K., Goldstein, J.L., **1995**. cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1. *J. Biol. Chem. 270*, 1843–1849.

Garcia, C.K., Li, X., Luna, J., Francke, U., **1994**. cDNA cloning of the human monocarboxylate transporter-1 and chromosomal localization of the Slc16a1 locus to 1p13.2-P12. *Genomics 23*, 500–503.

Gardiner, R.B., Ullensvang, K., Danbolt, N.C., Caveney, S., Donly, B.C., **2002**. Cellular distribution of a high-affinity glutamate transporter in the nervous system of the cabbage looper *Trichoplusia ni. J. Exp. Biol. 205*, 2605–2614.

Gasic, G.P., Hollmann, M., **1992**. Molecular neurobiology of glutamate receptors. *Annu. Rev. Physiol. 54*, 507–536.

Gasnier, B., **2003**. The SLC32 transporter, a key protein for the synaptic release of inhibitory amino acids. *Pflugers Arch.* 2003 May 16 (Epub ahead of print).

Gerencser, G.A., Stevens, B.R., **1994**. Thermodynamics of symport and antiport catalyzed by cloned or native transporters. *J. Exp. Biol. 196*, 59–75.

Giordana, B., Parenti, P., **1994**. Determinants for the activity of the neutral amino acid/K^+ symport in lepidopteran larval midgut. *J. Exp. Biol. 196*, 145–155.

Giordana, B., Sacchi, V.F., Parenti, P., Hanozet, G.M., **1989**. Amino acid transport systems in intestinal brush-border membranes from lepidopteran larvae. *Am. J. Physiol. 257*, R494–R500.

Gleason, K.K., Dondeti, V.R., Hsia, H.L., Cochran, E.R., Gumulak-Smith, J., *et al.*, **2003**. The vesicular glutamate transporter 1 (xVGlut1) is expressed in discrete regions of the developing *Xenopus laevis* nervous system. *Gene Expr. Patterns 3*, 503–507.

Graf, P., Forstermann, U., Closs, E. I., **2001**. The transport activity of the human cationic amino acid transporter hCAT-1 is downregulated by activation of protein kinase C. *Br. J. Pharmacol. 132*, 1193–1200.

Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., *et al.*, **1990**. Cloning and expression of a rat brain GABA transporter. *Science 249*, 1303–1306.

Halestrap, A.P., Meredith, D., **2003**. The SLC16 gene family – from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch.* Aug 28 (Epub ahead of print).

Hanozet, G.M., Giordana, B., Sacchi, V.F., **1980**. K^+-dependent phenylalanine uptake in membrane vesicles isolated from the midgut of *Philosamia cynthia* larvae. *Biochim. Biophys. Acta. 596*, 481–486.

Harvey, W.R., **1992**. Physiology of V-ATPases. *J. Exp. Biol. 172*, 1–17.

Harvey, W.R., Cioffi, M., Dow, J.A., Wolfersberger, M.G., **1983**. Potassium ion transport ATPase in insect epithelia. *J. Exp. Biol. 106*, 91–117.

Harvey, W.R., Haskell, J.A., Nedergaard, S., **1968**. Active transport by the Cecropia midgut. III. Midgut potential generated directly by active K-transport. *J. Exp Biol. 48*, 1–12.

Harvey, W.R., Haskell, J.A., Zerahn, K., **1967**. Active transport of potassium and oxygen consumption in the isolated midgut of *Hyalophora cecropia*. *J. Exp. Biol. 46*, 235–248.

Harvey, W.R., Maddrell, S.H.P., Telfer, W.H., Wieczorek, H., **1998**. H^+ V-ATPases energize animal plasma membranes for secretion and absorption of ions and fluids. *Am. Zool. 38*, 426–441.

Harvey, W.R., Pung, L., Meleshkevitch, E.A., Kohn, A., Boudko, D.Y., **2003**. Molecular and electrochemical integration of nutrient amino acid uptake in mosquito larvae. *Proc. 6th Int. Congr. Comp. Physiol. Biochem.* Mt. Buller, Australia.

Hayashi, M., Morimoto, R., Yamamoto, A., Moriyama, Y., **2003**. Expression and localization of vesicular glutamate transporters in pancreatic islets, upper gastrointestinal tract, and testis. *J. Histochem. Cytochem. 51*, 1375–1390.

Hayashi, M., Otsuka, M., Morimoto, R., Hirota, S., Yatsushiro, S., *et al.*, **2001**. Differentiation-associated Na^+-dependent inorganic phosphate cotransporter (DNPI) is a vesicular glutamate transporter in endocrine glutamatergic systems. *J. Biol. Chem. 276*, 43400–43406.

Hemler, M.E., Strominger, J.L., **1982**. Characterization of antigen recognized by the monoclonal antibody (4F2): different molecular forms on human T and B lymphoblastoid cell lines. *J. Immunol. 129*, 623–628.

Hennigan, B.B., Wolfersberger, M.G., Harvey, W.R., **1993a**. Neutral amino acid symport in larval *Manduca sexta* midgut brush-border membrane vesicles deduced from cation-dependent uptake of leucine, alanine, and phenylalanine. *Biochim. Biophys. Acta 1148*, 216–222.

Hennigan, B.B., Wolfersberger, M.G., Parthasarathy, R., Harvey, W.R., **1993b**. Cation-dependent leucine, alanine, and phenylalanine uptake at pH 10 in brush-border membrane vesicles from larval *Manduca sexta* midgut. *Biochim. Biophys. Acta 1148*, 209–215.

Holt, R.A., Subramanian, G.M., Halpern, A., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Homberg, U., **2002**. Neurotransmitters and neuropeptides in the brain of the locust. *Microsc. Res. Tech. 56*, 189–209.

Huang, X., Huang, Y., Chinnappan, R., Bocchini, C., Gustin, M.C., *et al.*, **2002**. The *Drosophila* inebriated-encoded neurotransmitter/osmolyte transporter: dual roles in the control of neuronal excitability and the osmotic stress response. *Genetics 160*, 561–569.

Huang, Y., Stern, M., **2002**. *In vivo* properties of the *Drosophila* inebriated-encoded neurotransmitter transporter. *J. Neurosci. 22*, 1698–1708.

Huggett, J.F., Mustafa, A., O'Neal, L., Mason, D.J., **2002**. The glutamate transporter GLAST-1 (EAAT-1) is expressed in the plasma membrane of osteocytes and is responsive to extracellular glutamate concentration. *Biochem. Soc. Trans. 30*, 890–893.

Jackson, V.N., Price, N.T., Carpenter, L., Halestrap, A.P., **1997**. Cloning of the monocarboxylate transporter isoform MCT2 from rat testis provides evidence that expression in tissues is species-specific and may involve post-transcriptional regulation. *Biochem. J. 324*, 447–453.

Jan, L.Y., Jan, Y.N., **1976a**. L-glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. *J. Physiol. 262*, 215–236.

Jan, L.Y., Jan, Y.N., **1976b**. Properties of the larval neuromuscular junction in *Drosophila melanogaster. J. Physiol. 262*, 189–214.

Janecek, S., **1994**. Sequence similarities and evolutionary relationships of microbial, plant and animal alpha-amylases. *Eur. J. Biochem. 224*, 519–524.

Jespersen, T., Grunnet, M., Angelo, K., Klaerke, D.A., Olesen, S.P., **2002**. Dual-function vector for protein expression in both mammalian cells and *Xenopus laevis* oocytes. *Biotechniques 32*, 536–538, 540.

Jin, X., Aimanova, K., Ross, L.S., Gill, S.S., **2003**. Identification, functional characterization and expression of a LAT type amino acid transporter from the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol. 33*, 815–827.

Johnson, K., Knust, E., Skaer, H., **1999**. bloated tubules (blot) encodes a *Drosophila* member of the neurotransmitter transporter family required for organisation of the apical cytocortex. *Devel. Biol. 212*, 440–454.

Kanai, Y., Hediger, M.A., **1992**. Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature 360*, 467–471.

Kanai, Y., Smith, C.P., Hediger, M.A., **1993**. The elusive transporters with a high affinity for glutamate. *Trends Neurosci. 16*, 365–370.

Kanai, Y., Trotti, D., Berger, U.V., **2002**. The high-affinity glutamate and neutral amino-acid transporter family. In: Reith, M.E.A. (Ed.), Neurotransmitter Transporters: Structure, Function and Regulation. Humana Press Inc., Totowa, New Jersey, pp. 255–312.

Kavanaugh, M.P., Miller, D.G., Zhang, W., Law, W., Kozak, S.L., *et al.*, **1994**. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. *Proc. Natl Acad. Sci. USA 91*, 7071–7075.

Kekuda, R., Prasad, P.D., Fei, Y.J., Torres-Zamorano, V., Sinha, S., *et al.*, **1996**. Cloning of the sodium-dependent, broad-scope, neutral amino acid transporter Bo from a human placental choriocarcinoma cell line. *J. Biol. Chem. 271*, 18657–18661.

Kim do, K., Kanai, Y., Matsuo, H., Kim, J.Y., Chairoungdua, A., *et al.*, **2002**. The human T-type amino acid transporter-1: characterization, gene organization, and chromosomal location. *Genomics 79*, 95–103.

Kim, C.M., Goldstein, J.L., Brown, M.S., **1992**. Cdna Cloning of Mev, a mutant protein that facilitates cellular uptake of mevalonate, and identification of the point mutation responsible for its gain of function. *J. Biol. Chem. 267*, 23113–23121.

Kim, D.K., Kanai, Y., Chairoungdua, A., Enomoto, A., Inatomi, J., *et al.*, **2002**. Identification and characterization of a novel epithelial aromatic amino acid transporter TAT1. *Jap. J. Pharmacol. 88*, 223P.

Kim, D.K., Kanai, Y., Chairoungdua, A., Matsuo, H., Cha, S.H., *et al.*, **2001**. Expression cloning of a Na^+-independent aromatic amino acid transporter with structural similarity to H+/monocarboxylate transporters. *J. Biol. Chem. 276*, 17221–17228.

Kim, J.W., Closs, E.I., Albritton, L.M., Cunningham, J.M., **1991**. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature 352*, 725–728.

Kirk, P., Wilson, M.C., Heddle, C., Brown, M.H., Barclay, A.N., *et al.*, **2000**. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J. 19*, 3896–3904.

Kirschner, M.A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Amara, S.G., **1994**. Mouse excitatory amino acid transporter EAAT2: isolation, characterization, and proximity to neuroexcitability loci on mouse chromosome 2. *Genomics 24*, 218–224.

Kitamoto, T., Wang, W., Salvaterra, P.M., **1998**. Structure and organization of the *Drosophila* cholinergic locus. *J. Biol. Chem. 273*, 2706–2713.

Kitamoto, T., Xie, X., Wu, C. F., Salvaterra, P. M., **2000a**. Isolation and characterization of mutants for the vesicular acetylcholine transporter gene in *Drosophila melanogaster. J. Neurobiol. 42*, 161–171.

Kitamoto, T., Xie, X., Wu, C.-F., Salvaterra, P.M., **2000b**. Isolation and characterization of mutants for the vesicular acetylcholine transporter gene in *Drosophila melanogaster. J. Neurobiol. 42*, 161–171.

Klemm, N., Schneider, L., **1975**. Selective uptake of indolamine into nervous fibers in brain of desert locust, Schistocerca-Gregaria Forskal (Insecta) – fluorescence and electron-microscopic investigation. *Comp. Biochem. Physiol. CPharmacol. Toxicol. Endocrinol. 50*, 177–182.

Knipper, M., Strotmann, J., Mädler, U., Kahle, C., Breer, H., **1989**. Monoclonal antibodies against the high affinity choline transport system. *Neurochem. Int. 14*, 217–222.

Kostowski, W., Tarchalska-Krynska, B., Markowska, L., **1975**. Aggressive behavior and brain serotonin and catecholamines in ants (*Formica rufa*). *Pharmacol. Biochem. Behav. 3*, 717–719.

Krantz, D.E., Chaudhry, F.A., Edwards, R.H., **1999**. Neurotransmitter transporters. In: Bellen, H. (Ed.), Neurotransmitter Release. Oxford University Press, Oxford, pp. 145–207.

Kucharski, R., Ball, E.E., Hayward, D.C., Maleszka, R., **2000**. Molecular cloning and expression analysis of a cDNA encoding a glutamate transporter in the honeybee brain. *Gene 242*, 399–405.

Law, J.H., Dunn, P.E., Kramer, K.J., **1977**. Insect proteases and peptidases. *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 389–425.

Leal, S.M., Neckameyer, W.S., **2002**. Pharmacological evidence for GABAergic regulation of specific behaviors in *Drosophila melanogaster. J. Neurobiol. 50*, 245–261.

Leitch, B., Judge, S., Pitman, R.M., **2003**. Octopaminergic modulation of synaptic transmission between an identified sensory afferent and flight motorneuron in the locust. *J. Comp. Neurol. 462*, 55–70.

Leonardi, M.G., Casartelli, M., Fiandra, L., Parenti, P., Giordana, B., **2001a**. Role of specific activators of intestinal amino acid transport in *Bombyx mori* larval growth and nutrition. *Arch. Insect Biochem. Physiol. 48*, 190–198.

Leonardi, M.G., Fiandra, L., Casartelli, M., Cappellozza, S., Giordana, B., **2001b**. Modulation of leucine absorption in the larval midgut of *Bombyx mori* (Lepidoptera, Bombycidae). *Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129*, 665–672.

Levi, G., Raiteri, M., **1974**. Exchange of neurotransmitter amino-acid at nerve-endings can simulate high affinity uptake. *Nature 250*, 735–737.

Li, R., Younes, M., Frolov, A., Wheeler, T.M., Scardino, P., *et al.*, **2003**. Expression of neutral amino acid transporter ASCT2 in human prostate. *Anticancer Res. 23*, 3413–3418.

Liang, R., Fei, Y.J., Prasad, P.D., Ramamoorthy, S., Han, H., *et al.*, **1995**. Human intestinal H^+/peptide cotransporter. Cloning, functional expression, and chromosomal localization. *J. Biol. Chem. 270*, 6456–6463.

Lill, H., Nelson, N., **1998**. Homologies and family relationships among Na^+/Cl^- neurotransmitter transporters. *Meth. Enzymol. 296*, 425–436.

Lin, R.Y., Vera, J.C., Chaganti, R.S., Golde, D.W., **1998**. Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. *J. Biol. Chem. 273*, 28959–28965.

Liu, Q.R., Mandiyan, S., Nelson, H., Nelson, N., **1992a**. A family of genes encoding neurotransmitter transporters. *Proc. Natl Acad. Sci. USA 89*, 6639–6643.

Liu, Y., Peter, D., Roghani, A., Schuldiner, S., Prive, G.G., *et al.*, **1992b**. A cDNA that suppresses MPP^+ toxicity encodes a vesicular amine transporter. *Cell 70*, 539–551.

Liu, Z., Harvey, W.R., **1996a**. Cationic lysine uptake by System R^+ and zwitterionic lysine uptake by System B in brush border membrane vesicles from larval *Manduca sexta* midgut. *Biochim. Biophys. Acta 1282*, 32–38.

Liu, Z., Harvey, W.R., **1996b**. Arginine uptake through a novel cationic amino acid:K^+ symporter, System R^+, in brush border membrane vesicles from larval *Manduca sexta* midgut. *Biochim. Biophys. Acta 1282*, 25–31.

Liu, Z., Stevens, B.R., Feldman, D.H., Hediger, M.A., Harvey, W.R., **2003**. K^+ amino acid transporter KAAT1 mutant Y147F has increased transport activity and altered substrate selectivity. *J. Exp. Biol. 206*, 245–254.

MacGregor, E.A., Janecek, S., Svensson, B., **2001**. Relationship of sequence and structure to specificity in the alpha-amylase family of enzymes. *Biochim. Biophys. Acta 1546*, 1–20.

Mackenzie, B., Erickson, J.D., **2003**. Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch.* 2003 Jul 4 (Epub ahead of print).

Maddrell, S.H., **1962**. A diuretic hormone in *Rhodnius Prolixus Stal. Nature 194*, 605–606.

Magagnin, S., Werner, A., Markovich, D., Sorribas, V., Stange, G., *et al.*, **1993**. Expression cloning of human and rat renal cortex Na/Pi cotransport. *Proc. Natl Acad. Sci. USA 90, 5979–5983.*

Maleszka, R., Helliwell, P., Kucharski, R., **2000**. Pharmacological interference with glutamate re-uptake impairs long-term memory in the honeybee, *Apis mellifera. Behav. Brain Res. 115*, 49–53.

Malutan, T., McLean, H., Caveney, S., Donly, C., **2002**. A high-affinity octopamine transporter cloned from the central nervous system of cabbage looper *Trichoplusia ni. Insect Biochem. Mol. Biol. 32*, 343–357.

Mandel, L.J., Moffett, D.F., Riddle, T.G., Grafton, M.M., **1980**. Coupling between oxidative metabolism and active transport in the midgut of tobacco hornworm. *Am. J. Physiol. 238*, C1–C9.

Manning Fox, J.E., Meredith, D., Halestrap, A.P., **2000**. Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. *J. Physiol. 529*, 285–293.

Marin, M., Lavillette, D., Kelly, S.M., Kabat, D., **2003**. N-linked glycosylation and sequence changes in a critical negative control region of the ASCT1 and ASCT2 neutral amino acid transporters determine their retroviral receptor functions. *J. Virol. 77*, 2936–2945.

Masson, J., Sagne, C., Hamon, M., El Mestikawy, S., **1999**. Neurotransmitter transporters in the central nervous system. *Pharmacol. Rev. 51*, 439–464.

Mastroberardino, L., Spindler, B., Pfeiffer, R., Skelly, P.J., Loffing, J., *et al.*, **1998**. Amino-acid transport by heterodimers of 4F2hc/CD98 and members of a permease family. *Nature 395*, 288–291.

Mathews, G.C., Diamond, J.S., **2003**. Neuronal glutamate uptake contributes to GABA synthesis and inhibitory synaptic strength. *J. Neurosci. 23*, 2040–2048.

Matz, M.V., **2002**. Amplification of representative cDNA samples from microscopic amounts of invertebrate tissue to search for new genes. *Methods Mol. Biol. 183*, 3–18.

Mbungu, D., Ross, L.S., Gill, S.S., **1995**. Cloning, functional expression, and pharmacology of a GABA transporter from *Manduca sexta*. *Arch. Biochem. Biophys. 318*, 489–497.

McDonald, T.J., **1975**. Neuromuscular pharmacology of insects. *Ann. Rev. Entomol. 20*, 151–166.

McIntire, S.L., Jorgensen, E., Horvitz, H.R., **1993**. Genes required for GABA function in *Caenorhabditis elegans*. *Nature 364*, 334–337.

McIntire, S.L., Reimer, R.J., Schuske, K., Edwards, R.H., Jorgensen, E.M., **1997**. Identification and characterization of the vesicular GABA transporter. *Nature 389*, 870–876.

Meier, C., Ristic, Z., Klauser, S., Verrey, F., **2002**. Activation of system L heterodimeric amino acid exchangers by intracellular substrates. *EMBO J. 21*, 580–589.

Meredith, D., Bell, P., McClure, B., Wilkins, R., **2002**. Functional and molecular characterisation of lactic acid transport in bovine articular chondrocytes. *Cell. Physiol. Biochem. 12*, 227–234.

Meredith, D., Halestrap, A.P., **2000**. OX47 (basigin) may act as a chaperone for expression of the monocarboxylate transporter MCT1 at the plasma membrane of *Xenopus laevis* oocytes. *J. Physiol. Lond. 526*, 23P–24P.

Miyamoto, K., Tatsumi, S., Sonoda, T., Yamamoto, H., Minami, H., *et al.*, **1995**. Cloning and functional expression of a Na(+)-dependent phosphate co-transporter from human kidney: cDNA cloning and functional expression. *Biochem. J. 305*, 81–85.

Monaghan, D.T., Bridges, R.J., Cotman, C.W., **1989**. The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol. 29*, 365–402.

Monastirioti, M., **1999**. Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc. Res. Tech. 45*, 106–121.

Murer, H., Forster, I., Biber, J., **2003**. The sodium phosphate cotransporter family SLC34. *Pflugers Arch. 16*, 16.

Murer, H., Hernando, N., Forster, I., Biber, J., **2000**. Proximal tubular phosphate reabsorption: molecular mechanisms. *Physiol. Rev. 80*, 1373–1409.

Muszynska-Pytel, M., Cymboroski, B., **1978a**. The role of serotonin in regulation of the circadian rhythm of locomotor activity in the cricket (*Acheta domesticus* L.) II. Distribution of serotonin and variations in different brain structure. *Comp. Biochem. Physiol. C 59*, 17–20.

Muzynska-Pytel, M., Cymborowski, B., **1978b**. The role of serotonin in regulation of the circadian rhythms of locomotor activity in the cricket (*Acheta domesticus* L.) I. Circadian variations in serotonin concentration in the brain and hemolymph. *Comp. Biochem. Physiol. C 59*, 13–15.

Nagaya, Y., Kutsukake, M., Chigusa, S.I., Komatsu, A., **2002**. A trace amine, tyramine, functions as a neuromodulator in *Drosophila melanogaster*. *Neurosci. Lett. 329*, 324–328.

Nathanson, J.A., Hunnicutt, E.J., Kantham, L., Scavone, C., **1993**. Cocaine as a naturally occurring insecticide. *Proc. Natl Acad. Sci. USA 90*, 9645–9648.

Neckameyer, W.S., **1998**. Dopamine and mushroom bodies in *Drosophila*: experience-dependent and-independent aspects of sexual behavior. *Learn. Mem. 5*, 157–165.

Nedergaard, S., **1972**. Active transport of alpha-aminoisobutyric acid by the isolated midgut of *Hyalophora cecropia*. *J. Exp. Biol. 56*, 167–172.

Nelson, N., Lill, H., **1994**. Porters and neurotransmitter transporters. *J. Exp. Biol. 196*, 213–228.

Nelson, N., Sacher, A., Nelson, H., **2002**. The significance of molecular slips in transport systems. *Nat. Rev. Mol. Cell. Biol. 3*, 876–881.

Ni, B., Rosteck, P.R., Jr., Nadi, N.S., Paul, S.M., **1994**. Cloning and expression of a cDNA encoding a brain-specific Na(+)-dependent inorganic phosphate cotransporter. *Proc. Natl Acad. Sci. USA 91*, 5607–5611.

Nielsen-LeRoux, C., Charles, J.-F., **1992**. Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. *Eur. J. Biochem. 210*, 585–590.

Novak, M.G., Rowley, W.A., **1994**. Serotonin depletion affects blood-feeding but not host-seeking ability in *Aedes triseriatus* (Diptera: Culicidae). *J. Med. Entomol. 31*, 600–606.

O'Donnell, M.J., Maddrell, S.H., **1984**. Secretion by the Malpighian tubules of *Rhodnius prolixus stal*: electrical events. *J. Exp. Biol. 110*, 275–290.

Okuda, T., Haga, T., **2000**. Functional characterization of the human high-affinity choline transporter. *FEBS Lett. 484*, 92–97.

Olah, Z., Lehel, C., Anderson, W.B., Eiden, M.V., Wilson, C.A., **1994**. The cellular receptor for gibbon ape leukemia virus is a novel high affinity sodium-dependent phosphate transporter. *J. Biol. Chem. 269*, 25426–25431.

Osborne, R.H., **1996**. Insect neurotransmission: neurotransmitters and their receptors. *Pharmacol. Ther. 69*, 117–142.

Otis, T.S., Wu, Y.C., Trussell, L.O., **1996**. Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. *J. Neurosci. 16*, 1634–1644.

Pacholczyk, T., Blakely, R.D., Amara, S.D., **1991**. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature 350*, 350–354.

Page, R.D., **1996**. TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci. 12*, 357–358.

Palacin, M., Estevez, R., Bertran, J., Zorzano, A., **1998**. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev. 78*, 969–1054.

Palacin, M., Kanai, Y., **2003**. The ancillary proteins of HATs: SLC3 family of amino acid transporters. *Pflugers Arch*. 2003 Jun 11 (Epub ahead of print).

Parenti, P., Forcella, M., Pugliese, A., Casartelli, M., Giordana, B., *et al*., **2000**. Substrate specificity of the brush border K^+-leucine symport of *Bombyx mori* larval midgut. *Insect Biochem. Mol. Biol. 30*, 243–252.

Parenti, P., Villa, M., Hanozet, G.M., **1992**. Kinetics of leucine transport in brush border membrane vesicles from lepidopteran larvae midgut. *J. Biol. Chem. 267*, 15391–15397.

Parsons, S.M., **2000**. Transport mechanisms in acetylcholine and monoamine storage. *FASEB J. 14*, 2423–2434.

Patrick, M.L., Bradley, T.J., **2000**. The physiology of salinity tolerance in larvae of two species of *Culex* mosquitoes: the role of compatible solutes. *J. Exp. Biol. 203*, 821–830.

Paulsen, I.T., Skurray, R.A., **1993**. Topology, structure and evolution of 2 families of proteins involved in antibiotic and antiseptic resistance in Eukaryotes and Prokaryotes – an analysis. *Gene 124*, 1–11.

Pennetta, G., Wu, M., Bellen, H.J., **1999**. Dissecting the molecular mechanisms of neurotransmitter release in Drosophila. In: Bellen, H. (Ed.), Neurotransmitter Release. Oxford University Press, Oxford, pp. 304–351.

Peres, A., Bossi, E., **2000**. Effects of pH on the uncoupled, coupled and pre-steady-state currents at the amino acid transporter KAAT1 expressed in *Xenopus* oocytes. *J. Physiol. Lond. 525*, 83–89.

Petersen, S.A., Fetter, R.D., Noordermeer, J.N., Goodman, C.S., DiAntonio, A., **1997**. Genetic analysis of glutamate receptors in *Drosophila* reveals a retrograde signal regulating presynaptic transmitter release. *Neuron 19*, 1237–1248.

Pfeiffer, R., Rossier, G., Spindler, B., Meier, C., Kuhn, L., *et al*., **1999**. Amino acid transport of y+L-type by heterodimers of 4F2hc/CD98 and members of the glycoprotein-associated amino acid transporter family. *EMBO J. 18*, 49–57.

Pfeiffer, R., Spindler, B., Loffing, J., Skelly, P.J., Shoemaker, C.B., *et al*., **1998**. Functional heterodimeric amino acid transporters lacking cysteine residues involved in disulfide bond. *FEBS Lett. 439*, 157–162.

Philp, N.J., Yoon, H.Y., Lombardi, L., **2001**. Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. *Am. J. Physiol. –Cell Physiol. 280*, C1319–C1326.

Pilegaard, H., Terzis, G., Halestrap, A., Juel, C., **1999**. Distribution of the lactate/H^+ transporter isoforms MCT1 and MCT4 in human skeletal muscle. *Am. J. Physiol. 276*, E843–E848.

Porzgen, P., Park, S.K., Hirsh, J., Sonders, M.S., Amara, S.G., **2001**. The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: a primordial carrier for catecholamines. *Mol. Pharmacol. 59*, 83–95.

Price, N.T., Jackson, V.N., Halestrap, A.P., **1998**. Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. *Biochem. J. 329*, 321–328.

Quick, M., Stevens, B.R., **2001**. Amino acid transporter CAATCH1 is also an amino acid-gated cation channel. *J. Biol. Chem. 276*, 33413–33418.

Rand, J.B., Duerr, J.S., Frisby, D.L., **2000**. Neurogenetics of vesicular transporters in *C. elegans*. *FASEB J. 14*, 2414–2422.

Rasko, J.E.J., Battini, J.-L., Gottschalk, R.J., Mazo, I., Miller, A.D., **1999**. The RD114/simian type D retrovirus receptor is a neutral amino acid transporter. *Proc. Natl Acad. Sci. USA 96*, 2129–2134.

Reimer, R.J., Edwards, R.H., **2003**. Organic anion transport is the primary function of the SLC17/type I phosphate transporter family. *Pflugers Arch*. 2003 Jun 17 (Epub ahead of print).

Reuveni, M., Dunn, P.E., **1994**. Proline transport into brush border membrane vesicles from the midgut of *Manduca sexta* larvae. *Comp. Biochem. Physiol. Comp. Physiol. 107*, 685–691.

Rimaniol, A.C., Haik, S., Martin, M., Le Grand, R., Boussin, F.D., *et al*., **2000**. Na^+-dependent high-affinity glutamate transport in macrophages. *J. Immunol. 164*, 5430–5438.

Robertson, H.A., Carlson, A.D., **1976**. Octopamine – presence in firefly lantern suggests a transmitter role. *J. Exp. Zool. 195*, 159–164.

Roeder, T., **1999**. Octopamine in invertebrates. *Progr. Neurobiol. 59*, 533–561.

Roman, G., Meller, V., Wu, K.H., Davis, R. L., **1998**. The *opt1* gene of *Drosophila melanogaster* encodes a proton-dependent dipeptide transporter. *Am. J. Physiol. Cell Physiol. 44*, C857–C869.

Rossier, G., Meier, C., Bauch, C., Summa, V., Sordat, B., *et al*., **1999**. LAT2, a new basolateral 4F2hc/CD98-associated amino acid transporter of kidney and intestine. *J. Biol. Chem. 274*, 34948–34954.

Rost, B., **1996**. PHD: predicting one-dimensional protein structure by profile-based neural networks. *Methods Enzymol. 266*, 525–539.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., *et al*., **1996**. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron 16*, 675–686.

Rubio-Aliaga, I., Boll, M., Daniel, H., **2000**. Cloning and characterization of the gene encoding the mouse peptide transporter PEPT2. *Biochem. Biophys. Res. Commun. 276*, 734–741.

Sacchi, V.F., Castagna, M., Mari, S.A., Perego, C., Bossi, E., *et al*., **2003**. Glutamate 59 is critical for transport function of the amino acid cotransporter KAAT1. *Am. J. Physiol. Cell Physiol. 285*, C623–C632.

Sagne, C., Agulhon, C., Ravassard, P., Darmon, M., Hamon, M., *et al*., **2001**. Identification and characterization of a lysosomal transporter for small neutral amino acids. *Proc. Natl Acad. Sci. USA 98*, 7206–7211.

Sagne, C., El Mestikawy, S., Isambert, M.F., Hamon, M., Henry, J.P., *et al*., **1997**. Cloning of a functional

vesicular GABA and glycine transporter by screening of genome databases. *FEBS Lett. 417*, 177–183.

Saier, M.H., **2000a**. Vectorial metabolism and the evolution of transport systems. *J. Bacteriol. 182*, 5029–5035.

Saier, M.H., Jr., **2000b**. A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol. Mol. Biol. Rev. 64*, 354–411.

Saito, H., Terada, T., Okuda, M., Sasaki, S., Inui, K., **1996**. Molecular cloning and tissue distribution of rat peptide transporter PEPT2. *Biochim. Biophys. Acta-Biomembr. 1280*, 173–177.

Saitou, N., Nei, M., **1987**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol. 4*, 406–425.

Sakata, K., Yamashita, T., Maeda, M., Moriyama, Y., Shimada, S., *et al.*, **2001**. Cloning of a lymphatic peptide/histidine transporter. *Biochem. J. 356*, 53–60.

Sandhu, S.K., Ross, L.S., Gill, S.S., **2002a**. Molecular cloning and functional expression of a proline transporter from *Manduca sexta*. *Insect Biochem. Mol. Biol. 32*, 1391–1400.

Sandhu, S.K., Ross, L.S., Gill, S.S., **2002b**. A cocaine insensitive chimeric insect serotonin transporter reveals domains critical for cocaine interaction. *Eur. J. Biochem. 269*, 3934–3944.

Sato, H., Tamba, M., Ishii, T., Bannai, S., **1999**. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem. 274*, 11455–11458.

Sato, H., Tamba, M., Okuno, S., Sato, K., Keino-Masu, K., *et al.*, **2002**. Distribution of cystine/glutamate exchange transporter, system x(c) (-), in the mouse brain. *J. Neurosci. 22*, 8028–8033.

Schneider, M., Rudin, W., Hecker, H., **1986**. Absorption and transport of radioactive-tracers in the midgut of the malaria mosquito, *Anopheles-Stephensi*. *J. Ultrastruct. Mol. Struct. Res. 97*, 50–63.

Schuldiner, S., Shirvan, A., Linial, M., **1995**. Vesicular neurotransmitter transporters: from bacteria to humans. *Physiol. Rev. 75*, 369–392.

Schuldiner, S., Shirvan, A., Stern-Bach, Y., Steiner-Mordoch, S., Yelin, R., *et al.*, **1994**. From bacterial antibiotic resistance to neurotransmitter uptake. *A common theme of cell survival. Ann. N. Y. Acad. Sci. 733*, 174–184.

Schulz, D.J., Barron, A.B., Robinson, G.E., **2002**. A role for octopamine in honey bee division of labor. *Brain Behav. Evol. 60*, 350–359.

Schuster, C.M., Ultsch, A., Schloss, P., Cox, J.A., Schmitt, B., *et al.*, **1991**. Molecular cloning of an invertebrate glutamate receptor subunit expression in *Drosophila* muscle. *Science 254*, 112–114.

Seal, R.P., Amara, S., **1999**. Excitatory amino acid transporters: a family in flux. *Annu. Rev. Pharmacol. Toxicol. 39*, 431–456.

Seal, R.P., Daniels, G.M., Wolfgang, W.J., Forte, M., Amara, S., **1998**. Identification and characterization of a cDNA encoding a neuronal glutamate transporter from *Drosophila melanogaster*. *Recept. Channels* 6, 51–64.

Simell, O., **2001**. Lysinuric protein intolerance and other cationic amino acidurias. In: Scriver, C.R., Beaudet, S.W. (Eds.), Metabolic and Molecular Bases of Inherited Diseases. McGraw-Hill, New York, pp. 4933–4956.

Sinakevitch, I.G., Geffard, M., Pelhate, M., Lapied, B., **1994**. Octopamine-like immunoreactivity in the dorsal unpaired median (Dum) neurons innervating the accessory-gland of the male cockroach *Periplaneta-Americana*. *Cell Tiss. Res. 276*, 15–21.

Sloan, J.L., Mager, S., **1999**. Cloning and functional expression of a human Na^+ and Cl^--dependent neutral and cationic amino acid transporter $B0^+$. *J. Biol. Chem. 274*, 23740–23745.

Soehnge, H., Huang, X., Becker, M., Whitley, P., Conover, D., *et al.*, **1996**. A neurotransmitter transporter encoded by the *Drosophila* inebriated gene. *Proc. Natl Acad. Sci. USA 93*, 13262–13267.

Spivak, M., Masterman, R., Ross, R., Mesce, K.A., **2003**. Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. *J. Neurobiol. 55*, 341–354.

Steiner, H.Y., Naider, F., Becker, J.M., **1995**. The PTR family: a new group of peptide transporters. *Mol. Microbiol. 16*, 825–834.

Stern, M., Ganetzky, B., **1992**. Identification and characterization of inebriated, a gene affecting neuronal excitability in *Drosophila*. *J. Neurogenet. 8*, 157–172.

Stevens, B.R., Feldman, D.H., Liu, Z., Harvey, W.R., **2002**. Conserved tyrosine-147 plays a critical role in the ligand-gated current of the epithelial cation/amino acid transporter/channel CAATCH1. *J. Exp. Biol. 205*, 2545–2553.

Stuart, A.E., **1999**. From fruit flies to barnacles, histamine is the neurotransmitter of arthropod photoreceptors. *Neuron 22*, 431–433.

Suchak, S.K., Baloyianni, N.V., Perkinton, M.S., Williams, R.J., Meldrum, B.S., *et al.*, **2003**. The 'glial' glutamate transporter, EAAT2 (Glt-1) accounts for high affinity glutamate uptake into adult rodent nerve endings. *J. Neurochem. 84*, 522–532.

Tabb, J.S., Ueda, T., **1991**. Phylogenetic studies on the synaptic vesicle glutamate transport system. *J. Neurosci. 11*, 1822–1828.

Takamori, S., Rhee, J.S., Rosenmund, C., Jahn, R., **2001**. Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). *J. Neurosci. 21*, RC182.

Tate, S.S., Yan, N., Udenfriend, S., **1992**. Expression cloning of a Na(+)-independent neutral amino acid transporter from rat kidney. *Proc. Natl Acad. Sci. USA 89*, 1–5.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., **1997**. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res. 25*, 4876–4882.

Tomi, M., Funaki, T., Abukawa, H., Katayama, K., Kondo, T., *et al.*, **2003**. Expression and regulation of

L-cystine transporter, system x(c) (-), in the newly developed rat retinal Muller cell line (TR-MUL). *Glia 43*, 208–217.

Tomita, Y., Takano, M., Yasuhara, M., Hori, R., Inui, K.I., **1995**. Transport of oral cephalosporins by the H^+/dipeptide cotransporter and distribution of the transport activity in isolated rabbit intestinal epithelial cells. *J. Pharmacol. Exp. Ther. 272*, 63–69.

Torres, G.E., Gainetdinov, R.R., Caron, M.G., **2003**. Plasma membrane monoamine transporters: structure, regulation and function. *Nat. Rev. Neurosci. 4*, 13–25.

Toure, A., Morin, L., Pineau, C., Becq, F., Dorseuil, O., *et al.*, **2001**. Tat1, a novel sulfate transporter specifically expressed in human male germ cells and potentially linked to RhoGTPase signaling. *J. Biol. Chem. 276*, 20309–20315.

Umesh, A., Cohen, B.N., Ross, L.S., Gill, S.S., **2003**. Functional characterization of a glutamate/aspartate transporter from the mosquito *Aedes aegypti*. *J. Exp. Biol. 206*, 2241–2255.

Umesh, A., Gill, S.S., **2002**. Immunocytochemical localization of a *Manduca sexta* gamma-aminobutyric acid transporter. *J. Comp. Neurol. 448*, 388–398.

Usherwood, P.N.R., **1994**. Insect glutamate receptors. *Adv. Insect. Physiol. 24*, 309–341.

Utsunomiya-Tate, N., Endou, H., Kanai, Y., **1996**. Cloning and functional characterization of a system ASC-like Na^+-dependent neutral amino acid transporter. *J. Biol. Chem. 271*, 14883–14890.

Varoqui, H., Schafer, M.K.H., Zhu, H.M., Weihe, E., Erickson, J.D., **2002a**. Identification of the differentiation-associated Na^+/P-I transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J. Neurosci. 22*, 142–155.

Varoqui, H., Schafer, M.K., Zhu, H., Weihe, E., Erickson, J.D., **2002b**. Identification of the differentiation-associated Na^+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J. Neurosci. 22*, 142–155.

Verrey, F., Closs, E.I., Wagner, C.A., Palacin, M., Endou, H., Kanai, Y., **2003**. CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch*. 2003. Jun 11 (Epub ahead of print).

Verri, T., Kottra, G., Romano, A., Tiso, N., Peric, M., *et al.*, **2003**. Molecular and functional characterisation of the zebrafish (*Danio rerio*) PEPT1-type peptide transporter. *FEBS Lett. 549*, 115–122.

Vincenti, S., Castagna, M., Peres, A., Sacchi, V.F., **2000**. Substrate selectivity and pH dependence of KAAT1 expressed in *Xenopus laevis* oocytes. *J. Membr. Biol. 174*, 213–224.

Vincourt, J.B., Jullien, D., Amalric, F., Girard, J.P., **2003**. Molecular and functional characterization of SLC26A11, a sodium-independent sulfate transporter from high endothelial venules. *FASEB J. 17*, 890–892.

Wang, H.Y., Tamba, M., Kimata, M., Sakamoto, K., Bannai, S., *et al.*, **2003**. Expression of the activity of cystine/glutamate exchange transporter, system x(c) (-), by xCT and rBAT. *Biochem. Biophys. Res. Commun. 305*, 611–618.

Watanabe, K., Hata, Y., Kizaki, H., Katsube, Y., Suzuki, Y., **1997**. The refined crystal structure of *Bacillus cereus* oligo-1,6-glucosidase at 2.0 angstrom resolution: structural characterization of proline-substitution sites for protein thermostabilization. *J. Mol. Biol. 269*, 142–153.

Watson, A.H.D., Schurmann, F.-W., **2002**. Synaptic structure, distribution, and circuitry in the central nervous system of the locust and related insects. *Microsc. Res. Tech. 56*, 210–226.

Wells, R.G., Hediger, M.A., **1992**. Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. *Proc. Natl Acad. Sci. USA 89*, 5596–5600.

Werner, A., Moore, M.L., Mantei, N., Biber, J., Semenza, G., *et al.*, **1991**. Cloning and expression of cDNA for a Na/Pi cotransport system of kidney cortex. *Proc. Natl Acad. Sci. USA 88*, 9608–9612.

Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J., Harvey, W.R., **1999a**. Animal plasma membrane energization by proton-motive V-ATPases. *Bioessays 21*, 637–648.

Wieczorek, H., Gruber, G., Harvey, W.R., Huss, M., Merzendorfer, H., **1999b**. The plasma membrane H^+-V-ATPase from tobacco hornworm midgut. *J. Bioenerg. Biomembr. 31*, 67–74.

Wieczorek, H., Putzenlechner, M., Zeiske, W., Klein, U., **1991**. A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. Biol. Chem. 266*, 15340–15347.

Wieczorek, H., Weerth, S., Schindlbeck, M., Klein, U., **1989**. A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem. 264*, 11143–11148.

Wilson, M.C., Jackson, V.N., Heddle, C., Price, N.T., Pilegaard, H., *et al.*, **1998**. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. *J. Biol. Chem. 273*, 15920–15926.

Wilson, M.C., Meredith, D., Halestrap, A.P., **2002**. Fluorescence resonance energy transfer studies on the interaction between the lactate transporter MCT1 and CD147 provide information on the topology and stoichiometry of the complex in situ. *J. Biol. Chem. 277*, 3666–3672.

Wipf, D., Ludewig, U., Tegeder, M., Rentsch, D., Koch, W., *et al.*, **2002**. Conservation of amino acid transporters in fungi, plants and animals. *Trends Biochem. Sci. 27*, 139–147.

Witte, I., Kreienkamp, H.-J., Gewecke, M., Roeder, T., **2002**. Putative histamine-gated chloride channel subunits of the insect visual system and thoracic ganglion. *J. Neurochem. 83*, 504–514.

Wolf, S., Janzen, A., Vekony, N., Martine, U., Strand, D., *et al.*, **2002**. Expression of solute carrier 7A4 (SLC7A4) in the plasma membrane is not sufficient to mediate

amino acid transport activity. *Biochem. J. 364*, 767–775.
Wolfersberger, M.G., **2000**. Amino acid transport in insects. *Annu. Rev. Entomol. 45*, 111–120.
Wolfgang, C.L., Lin, C., Meng, Q., Karinch, A.M., Vary, T.C., *et al.*, **2003**. Epidermal growth factor activation of intestinal glutamine transport is mediated by mitogen-activated protein kinases. *J. Gastrointest. Surg. 7*, 149–156.
Xie, T., Parthasarathy, R., Wolfersberger, M.G., Harvey, W.R., **1994**. Anomalous glutamate/alkali cation symport in larval *Manduca sexta* midgut. *J. Exp. Biol. 194*, 181–194.
Yamashita, T., Shimada, S., Guo, W., Sato, K., Kohmura, E., *et al.*, **1997**. Cloning and functional expression of a brain peptide/histidine transporter. *J. Biol. Chem. 272*, 10205–10211.
Yasuyama, K., Meinertzhagen, I.A., Schurmann, F.W., **2002**. Synaptic organization of the mushroom body calyx in *Drosophila melanogaster. J. Comp. Neurol. 445*, 211–226.
Yelin, R., Schuldiner, S., **1995**. The pharmacological profile of the vesicular monoamine transporter resembles that of multidrug transporters. *FEBS Lett. 377*, 201–207.
Yelin, R., Schuldiner, S., **2002**. Vesicular neurotransmitter transporters. In: Reith, M.E.A. (Ed.), Neurotransmitter Transporters: Structure, Function and Regulation. Humana Press Inc., Totowa, New Jersey, pp. 313–355.
Yellman, C., Tao, H., He, B., Hirsh, J., **1997**. Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated *Drosophila*. *Proc. Natl Acad. Sci. USA 94*, 4131–4136.
Yoon, H.Y., Fanelli, A., Grollman, E.F., Philp, N.J., **1997**. Identification of a unique monocarboxylate transporter (MCT3) in retinal pigment epithelium. *Biochem. Biophys. Res. Commun. 234*, 90–94.
Young, G.B., Jack, D.L., Smith, D.W., Saier, M.H., Jr., **1999**. The amino acid/auxin:proton symport permease family. *Biochim. Biophys. Acta 1415*, 306–322.
Zhao, C., Wilson, M.C., Schuit, F., Halestrap, A.P., Rutter, G.A., **2001**. Expression and distribution of lactate/monocarboxylate transporter isoforms in pancreatic islets and the exocrine pancreas. *Diabetes 50*, 361–366.
Zharikov, S.I., Sigova, A.A., Chen, S., Bubb, M.R., Block, E.R., **2001**. Cytoskeletal regulation of the L-arginine/NO pathway in pulmonary artery endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol. 280*, L465–L473.
Zhuang, Z., Linser, P.J., Harvey, W.R., **1999**. Antibody to H(+) V-ATPase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti*). *J. Exp. Biol. 202*, 2449–2460.

Relevant Websites

http://sosui.proteome.bio.tuat.ac.jp – Proteome Bio Tuat.
http://www.ncbi.nlm.nih.gov – NCBI.

5.10 Biochemical Genetics and Genomics of Insect Esterases

J G Oakeshott, CSIRO Entomology, Canberra, Australia
C Claudianos, Australian National University, Canberra, Australia
P M Campbell, CSIRO Entomology, Canberra, Australia
R D Newcomb, HortResearch, Auckland, New Zealand
R J Russell, CSIRO Entomology, Canberra, Australia

5.10.1. Introduction

5.10.1.1. Historical Perspective

Esterases have been a major part of insect biochemical research over the last 50 years. As an important component of insects' xenobiotic defence system, they have long been a focus for studies of chemical insecticide metabolism and resistance. Additionally, acetylcholinesterase (AChE) was a target for the early organophosphate (OP) and carbamate compounds. Other esterases have been implicated in the metabolism of specific hormones and pheromones and, consequently, in various aspects of insect development and behavior. Many esterases are easily scored by isozyme electrophoresis, with multiple isozymes and their high levels of genetic variation making them tractable, informative tools for a variety of biochemical and ecological genetic studies. For all these reasons esterase biochemistry featured prominently in the first edition of *Comprehensive Insect Biochemistry, Physiology and Pharmacology* in 1985.

At that time not a single insect esterase gene had been cloned but in the decade that followed several were isolated, particularly from *Drosophila*. The insect esterase genes cloned in this period all proved to derive from the one gene family, the carboxyl/cholinesterases (or just carboxylesterases in

the Pfam nomenclature – see Pfam ID 00135). Members of this family were also cloned from various prokaryotes and vertebrates, albeit some of the noninsect esterase genes cloned in the period clearly belonged to different gene families. Alternatively, genes for several noncatalytic proteins involved in various signaling processes in insects and other eukaryotes were found to be members of the carboxyl/cholinesterase gene family.

Critically too, the tertiary structures of AChE and some sequence-related microbial lipases were resolved in this period and shown to lie within the one structurally defined superfamily of proteins. This defining structure is called the α/β hydrolase fold. Catalytically active members of this superfamily all use the same reaction mechanism, involving a catalytic triad of residues headed by a serine or sometimes cysteine residue as a nucleophil. While hydrolases (including some esterases) in some other superfamilies have the same reaction mechanism, they achieve this with otherwise unrelated structures, apparently as an outcome of convergent evolution.

The last decade has seen a few cases of esterase-based insecticide resistance resolved at a molecular level. All these cases still involve the carboxyl/cholinesterase gene family. All involve OP resistance. They fall into essentially three groups, first involving mutant AChE molecules that are less sensitive to inhibition by the insecticide, second involving many-fold amplifications of genes encoding carboxylesterases with limited ability to hydrolyze the insecticide but substantial scope to sequester it, and the third involving structural mutations in certain carboxylesterases that improve their kinetics for insecticide hydrolysis. Remarkably, some of the same mutations are found in widely different species, which might suggest very limited options for evolving resistance. Given the empirical data on the structure of AChE, and modeled structures of the carboxylesterases based on AChE and the lipases, it is becoming possible to understand the effects of the resistance mutations in precise structural terms.

Of course the other qualitative advance during the last decade has been the application of modern genomic and proteomic technologies to the study of insect esterases. This has greatly facilitated the isolation of esterase genes and improved our understanding of their functions and sequence relationships. It appears that insect species may each have about 30–60 different members of the carboxyl/cholinesterase gene family, about three-quarters of them catalytically active, and that these comprise a majority but not all of the esterase isozymes that can be visualized after native polyacrylamide gel electrophoresis (PAGE). The insect members of the family fall into a small number of clades many of whose origins approach or, in a few cases, exceed the age of the class Insecta. Within some of these clades, however, there has been remarkably rapid evolution such that only a few orthologous genes can be identified except among quite closely related species.

This chapter reviews the biochemistry and genetics of insect esterases, focusing mainly on developments since the first edition of this series. It is written from a genomics perspective and the bulk of it is organized around the major clades of insect esterases as revealed by the comparative genomic analysis. First, however, some of the historical functional definitions and classifications applied to insect esterases are briefly revised.

5.10.1.2. Functional Definitions and Classifications

The esterase reaction, defined as the hydrolysis of an ester to its component alcohol and acid, encompasses hydrolysis of a diverse range of carboxylic, thio-, phospho-, and other ester substrates. It sits within a broader set of hydrolase reactions that also include glycosylases, proteases, amidases, and many others (Webb, 1992). Even more generally it can be considered a particular case, involving water, of the acylation of a nucleophil. Use of other nucleophils in otherwise similar reactions generates, for example, various dehalogenase activities (Ollis *et al.*, 1992).

Most of the discussion in this review concerns enzymes hydrolyzing esters of carboxylic acids (E.C. 3.1.1). This is a valid and useful functional grouping insomuch as reaction energetics are significantly different for the hydrolysis of esters like thio- and phospho-esters, where there is a strong negative charge in the immediate environment of the ester bond (Aldridge and Reiner, 1972). Nevertheless, there is still great diversity even among the carboxylic acid ester hydrolases.

One major distinction separates the lipases from others in the group (Phythian, 1998; Arpigny and Jaeger, 1999; Fojan *et al.*, 2000). Lipases have maximal activity for esters like triglycerides with simple alcohol groups and complex acid moieties, which are essentially insoluble in water. Most of the others have substrates with simple acid moieties but potentially complex alcohol moieties, which are at least partly soluble in water. It is this latter, nonlipase, group, which is the particular focus of this review.

Various classifications based on inhibitor sensitivities have been applied to subdivide the nonlipase group of carboxylic ester hydrolases. One early classification in use even before isozyme studies recognized A-, B-, and C-type esterases on the basis of

their interaction with OPs. While A-type esterases could hydrolyze these molecules, the B-type enzymes were essentially irreversibly inhibited by them, and the C-type enzymes neither hydrolyzed nor were inhibited by them (Aldridge and Reiner, 1972). Most, but not all, insect esterases studied to date would be classified as B-type esterases on this scheme.

A subsequent elaboration of the Aldridge and Reiner (1972) scheme has been used to classify eukaryotic esterase isozymes detectable after native PAGE and staining with various artificial ester substrates. It relies on three classes of inhibitors, the OPs, sulfhydral reagents, and the carbamate eserine sulfate, from which can be discerned four classes of enzymes (Holmes and Masters, 1967; Coates *et al.*, 1975; Healy *et al.*, 1991). These are:

- acetylesterases, which are unaffected by any of these inhibitors and generally prefer substrates with acetyl acid groups and aromatic alcohol groups;
- arylesterases, which are only inhibited by the sulfhydral reagents and generally prefer substrates with aromatic alcohol groups;
- carboxylesterases, which are only inhibited by the OPs and prefer aliphatic esters, generally of longer acids than acetate; and
- cholinesterases, which are inhibited by OPs and eserine sulfate, and prefer substrates with charged alcohol moieties like choline esters over other aromatic or aliphatic esters.

A further classification that had been applied extensively to esterase isozymes, especially in *Drosophila* and mosquitoes, groups the enzymes according to their preferential hydrolysis of the isomeric artificial substrates, α- and β-naphthyl acetate (see Sections 5.10.4.3 and 5.10.5.1 below). This distinction is made because hydrolysis of the two isomers leads to differently colored bands when the reaction is coupled with several of the common diazo staining dyes. The classification has little value as a predictor of enzyme function and indeed allelic differences between the two preferences have been described for some isozymes. The α- and β-based nomenclatures are retained herein for some widely studied enzymes and enzyme clusters, noting, however, that in themselves they represent no broader biological distinctions.

A survey of 22 soluble *Drosophila melanogaster* esterase isozymes identified 10 carboxylesterases, six cholinesterases, and three acetylesterases, with no aryl esterases, and three isozymes not clearly classifiable on this scheme (Healy *et al.*, 1991). Surveys of the major esterase isozymes of various mosquitoes and hemipterans yield broadly similar results, albeit consistently recovering minority numbers of aryl as well as acetylesterases (VedBrat and Whitt, 1975; Casabé and Zerba, 1981; Narang and Seawright, 1982).

As noted earlier, all invertebrate carboxyl and cholinesterases that have been sequenced to date lie in the same gene family, which is therefore called the carboxyl/cholinesterase gene family (Oakeshott *et al.*, 1993, 1995, 1999; Claudianos *et al.*, 2001). No sequence information has yet been reported for invertebrate acetyl- or arylesterases, although data for a few from prokaryotes and vertebrates put them in unrelated families (Campbell *et al.*, 2003). There are also a small number of functionally defined carboxylesterases from vertebrates that do not sit in the carboxyl/cholinesterase family (Zschunke *et al.*, 1991).

Several different classes of mechanism have been described for hydrolase reactions, ranging across general acid/base, electrostatic, and covalent catalyses (Voet and Voet, 1990). They may be one or two step reactions. Some use metal ions in various capacities. However, the mechanism used by the carboxyl/cholinesterases entails a two-step reaction which is independent of metal ions and has an acyl-enzyme intermediate (**Figure 1**). The serine of the active site is made more nucleophilic than usual by interaction with a histidine residue, which itself is oriented correctly by an interaction with an acidic residue. These three residues constitute the so-called catalytic triad. Their functions are analogous to those of the well-known catalytic triad of serine proteases where the same mechanism occurs. Since the structures of the two types of protein are otherwise unrelated, their shared catalytic mechanism is assumed to reflect convergent evolution.

In the first step of this reaction mechanism the oxygen of the serine's side chain makes a nucleophilic attack on the carbonyl carbon atom of the substrate, displacing the alcohol product and forming a covalent linkage with the acyl moiety of the substrate. In the second step, the oxygen of a water molecule makes a similar attack on the carbonyl carbon, hydrolyzing the covalent acyl-enzyme link to yield free enzyme and the acid product of the reaction. It has generally been assumed that the water molecule is activated by the histidine and acid residues of the catalytic triad as described for the first, acylation step (**Figure 1**), but Thomas *et al.* (1999) and Behra *et al.* (2002) have proposed that a conserved "additional serine" might sometimes provide this function instead. Both steps require a tetrahedral transition state in which charge is moved to the carbonyl oxygen. As with serine proteases, the active site of carboxylesterases has certain

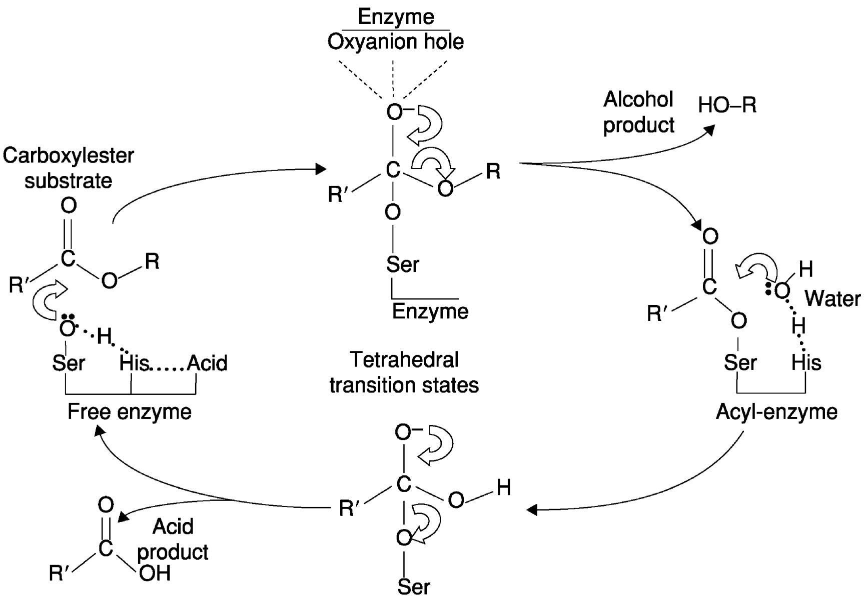


Figure 1 The carboxylesterase reaction mechanism. Carboxylesterases hydrolyze their substrates in two steps. First, the oxygen of a serine residue in the active site makes a nucleophilic attack on the carbonyl carbon of the substrate, displacing the alcohol product and forming a relatively stable acyl-enzyme. In the second step water makes a similar nucleophilic attack, this time displacing the serine residue to release the acid product of the reaction and regenerate the free enzyme. The serine of the active site is made more nucleophilic than usual by interaction with a histidine residue which is oriented correctly by an interaction with an acidic residue. During both nucleophilic substitution reactions there is a tetrahedral transition state the energy of which is reduced by an ''oxyanion hole'' in which the charge displaced onto the oxygen is accommodated by hydrogen bonding to the main-chain nitrogens of three amino acid residues around the active site. This mechanism is analogous to the mechanism of the serine proteases that also perform hydrolysis with a Ser–His–acid triad and main-chain nitrogens forming an oxyanion hole. The mechanism of serine proteases is frequently presented in detail in biochemistry textbooks with the similarity of carboxylesterases and serine proteases cited as an example of convergent evolution (e.g., Voet and Voet (1990), pp. 373–382).

conserved small residues, the main chain nitrogens of which are proposed to form an "oxyanion hole" to stabilize the transition states in both steps of the reaction by hydrogen bonding.

Hydrolysis of esters of carboxylic acids can be relatively undemanding energetically and examples of this reaction, including from the carboxyl/cholinesterases, have been described which approach diffusion limited kinetics (Taylor and Radic, 1994; Zera *et al.*, 2002; Devonshire *et al.*, 2003; Kamita *et al.*, 2003). Also, however, reactions of this type can often be relatively easily reversible, particularly in nonaqueous environments. The functional significance of the resultant esterification reactions has been well established for several bacterial "esterases," but the reverse reactions of eukaryotic esterases have received relatively little attention (Phythian, 1998).

5.10.2. Comparative Genomics of Insect Esterases

5.10.2.1. Overview

Consistent with their functional diversity, the sequences of the carboxyl/cholinesterases are also widely divergent. Even within the insect members there can be as little as about 20% amino acid identity between distant members of the family (Oakeshott *et al.*, 1999). In this and subsequent sections, it is shown how some of the sequence differences contribute to differences in the biochemistry of the proteins and their physiological functions.

Figure 2 presents a phylogeny of insect carboxyl/cholinesterase sequences. It includes all those identified in the fully sequenced *D. melanogaster* and *Anopheles gambiae* (African malaria mosquito) genomes (35 and 51, respectively), as well as 35 others for which there is both substantial sequence and some functional information from other insects. It therefore does not include the many apparently full length but functionally anonymous expressed sequence tag (EST) carboxyl/cholinesterase sequences in various insect EST databases. It also excludes several orthologs of *D. melanogaster* and *A. gambiae* sequences in other *Drosophila* and *Anopheles* species since these would simply appear in terminal bifurcations of the tree and provide little further information in terms of functional evolution. However, to give a broader context, all the lineages

of *Caenorhabditis elegans* (nematode) carboxyl/cholinesterases as identified by Oakeshott *et al.* (1999) that sit within the phylogeny (albeit for ease of tree building some nematode-specific radiations are simply represented by single sequences) are also included. Bacterial and vertebrate carboxyl/cholinesterases are not discussed, although some of their relationships to the major insect clades are known (Oakeshott *et al.*, 1999). No lipase sequences have yet been identified within the carboxyl/cholinesterase family in insects; some may sit as yet anonymously within the sequences included. However, the limited information available for insects, and the precedents in bacteria and vertebrates, suggest that most insect lipases will sit outside the clades shown in **Figure 2**, albeit many are in other families in the α/β hydrolase fold superfamily (Pistillo *et al.*, 1998; Arpigny and Jaeger, 1999; Nardini and Dijkstra, 1999; Adams *et al.*, 2000; Arrese *et al.*, 2001).

One obvious feature of the phylogeny is the high degree of separation of the insect clades from the *C. elegans* genes. There are only three clear overlaps, in the AChE, gliotactin, and neuroligin radiations. By cross-referencing the trees constructed by Oakeshott *et al.* (1999), it is also told that only these clades would likely be shared with known vertebrate carboxyl/cholinesterases. All the other clades in the phylogeny are thus insect-specific. While further sequencing may reveal representatives from other eukaryotes in some of these clades, it nevertheless seems likely that most of the insect carboxyl/cholinesterase lineages have evolved since the insects diverged from both the nematodes and the vertebrates.

The 35 non-*D. melanogaster*, non-*A. gambiae*, insect proteins in the phylogeny include 11 from other dipterans, eight from various lepidopterans, and two each from the Hymenoptera and Coleoptera, all in the Holometabola, and, for the Hemimetabola, 12 from various hemipterans. Although there are no nondipteran proteins in a few of the major clades at this point it is expected that most of these will also prove to have members from several orders, and indeed from both the Holo- and Hemimetabola, as more sequences become available. Notable here are the branch orders among the major clades; even some of the most derived clades contain members from different orders. Thus most of the major clades are older than the split between the Holo- and Hemimetabola.

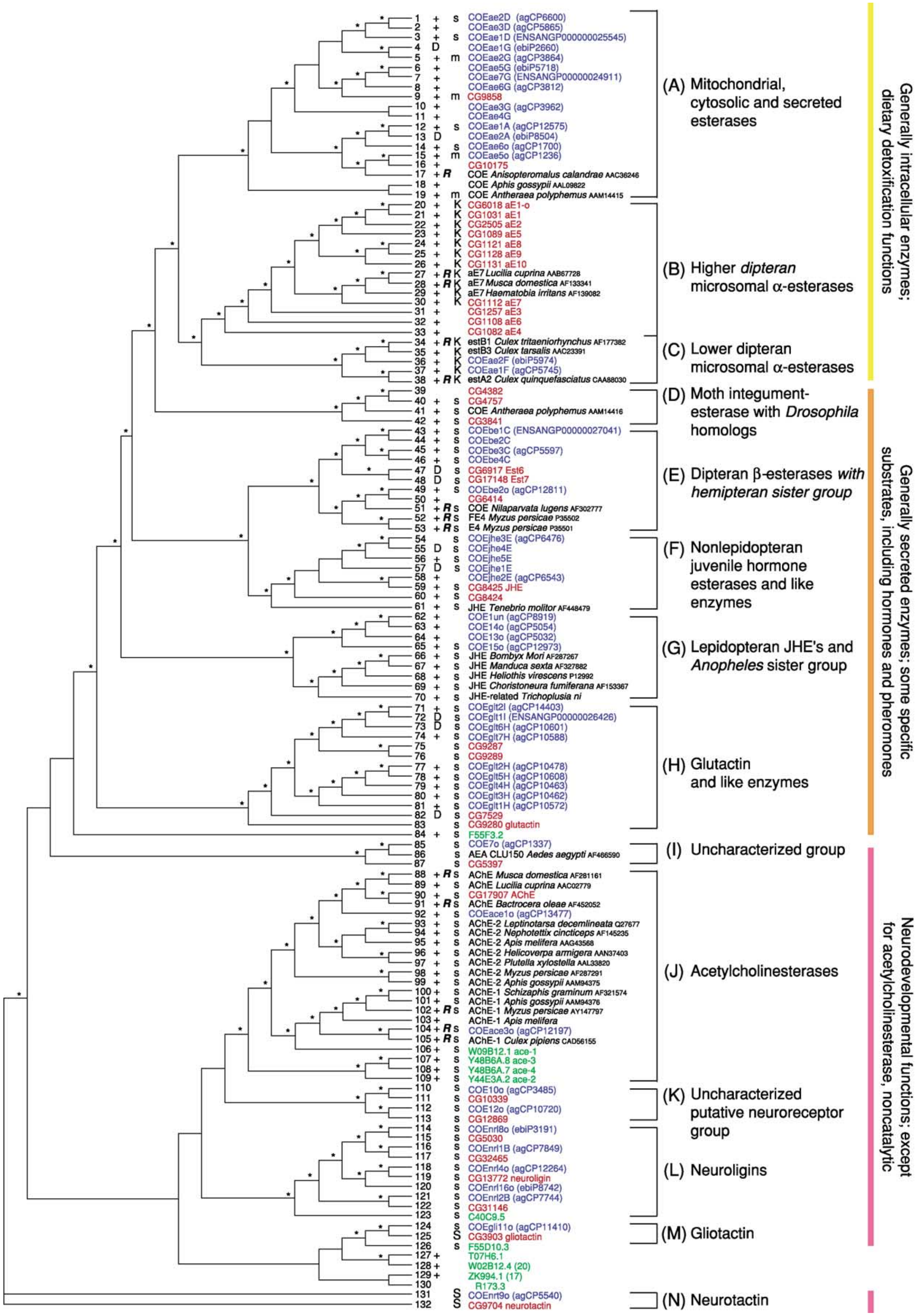
For the purposes of this review the radiations in the phylogeny containing insect sequences have been partitioned into 14 major clades, denoted A to N in **Figure 2**. In the rest of this section some major sequence and structural distinctions among these clades are outlined and below (see Sections 5.10.3, 5.10.4, and 5.10.5) are reviewed what is known of the functions of proteins within each of them.

One of the major discriminants among the 14 clades is catalytic capability, as inferred from the presence of a consensus catalytic triad (see also **Figure 4** below). Significantly, there are several noncatalytic clades, including the neuroligin, gliotactin, and neurotactin lineages (clades L, M, and N), as well as two clades of uncharacterized proteins (I and K), on this criterion. As discussed later, all functional evidence from characterized members of these clades confirms their catalytic incompetence. These clades were generated at relatively early branch points in the phylogeny and, in the case of the neuroligins and gliotactins, are also known to have vertebrate orthologs (see Section 5.10.3.2). Whilst the evidence from prokaryote members of the family suggests that catalytic competence is the ancestral state (Oakeshott *et al.*, 1999), it is nevertheless clear that a few noncatalytic functions developed early in the evolution of higher eukaryotes and have been retained in diverse lineages since. Loss of catalytic activity in these clades is also associated with the acquisition of additional domains as either N- or C-terminal extensions (depending on the clade) to the single carboxyl/cholinesterase domain (**Figure 3**).

The only major clade which contains significant proportions of both presumptively catalytic and noncatalytic members is the glutactin lineage (clade H) (see Section 5.10.4.1). Functional data confirm a noncatalytic role for glutactin and, like most of the other noncatalytic proteins above, it also has extra C-terminal transmembrane and cytoplasmic domains (**Figure 3**). However, it has apparently arisen relatively recently in a lineage which is predominantly composed of catalytically active proteins and, consistent with this, it lacks vertebrate orthologs.

Interestingly, while catalytic competence is generally a good taxonomic character for the insect carboxyl/cholinesterases, the identity of the catalytic triad is not. All those with an identifiable triad use serine as a nucleophil and histidine as the base. However, the acid residue may be aspartate or glutamate, and there are several lineages within the tree where both can be found.

Cellular/subcellular localization is another taxonomically informative character, at least for much of the phylogeny. The presence of N-terminal signal peptides and/or, in many cases, direct functional information indicate that all the major noncatalytic clades contain secreted proteins, albeit those characterized are known to be tethered to the cell


1 + s COEae2D (agCP6600)
2 + COEae3D (agCP5865)
3 + s COEae1D (ENSANGP000000025545)
4 D COEae1G (ebiP2660)
5 + m COEae2G (agCP3864)
6 + COEae5G (ebiP5718)
7 + COEae7G (ENSANGP00000024911)
8 + COEae6G (agCP3812)
9 + m CG9858
10 + COEae3G (agCP3962)
11 + COEae4G
12 + s COEae1A (agCP12575)
13 D COEae2A (ebiP8504)
14 + s COEae6o (agCP1700)
15 + m COEae5o (agCP1236)
16 + CG10175
17 + R COE Anisopteromalus calandrae AAC36246
18 + COE Aphis gossypii AAL09822
19 + m COE Antheraea polyphemus AAM14415
20 + K CG6018 aE1-o
21 + K CG1031 aE1
22 + K CG2505 aE2
23 + K CG1089 aE5
24 + K CG1121 aE8
25 + K CG1128 aE9
26 + K CG1131 aE10
27 + R K aE7 Lucilia cuprina AAB67728
28 + R K aE7 Musca domestica AF133341
29 + K aE7 Haematobia irritans AF139082
30 + K CG1112 aE7
31 + CG1257 aE3
32 + CG1108 aE6
33 + CG1082 aE4
34 + R K estB1 Culex tritaeniorhynchus AF177382
35 + K estB3 Culex tarsalis AAC23391
36 + K COEae2F (ebiP5974)
37 + K COEae1F (agCP5745)
38 + R K estA2 Culex quinquefasciatus CAA88030
39 CG4382
40 + s CG4757
41 + s COE Antheraea polyphemus AAM14416
42 + s CG3841
43 + s COEbe1C (ENSANGP00000027041)
44 + s COEbe2C
45 + s COEbe3C (agCP5597)
46 + s COEbe4C
47 D s CG6917 Est6
48 D s CG17148 Est7
49 + s COEbe2o (agCP12811)
50 + CG6414
51 + R s COE Nilaparvata lugens AF302777
52 + R s FE4 Myzus persicae P35502
53 + R s E4 Myzus persicae P35501
54 s COEjhe3E (agCP6476)
55 D s COEjhe4E
56 + s COEjhe5E
57 D s COEjhe1E
58 + COEjhe2E (agCP6543)
59 + s CG8425 JHE
60 + s CG8424
61 + s JHE Tenebrio molitor AF448479
62 + COE1un (agCP8919)
63 + COE14o (agCP5054)
64 + COE13o (agCP5032)
65 + s COE15o (agCP12973)
66 + s JHE Bombyx Mori AF287267
67 + s JHE Manduca sexta AF327882
68 + s JHE Heliothis virescens P12992
69 + s JHE Choristoneura fumiferana AF153367
70 + s JHE-related Trichoplusia ni
71 + s COEglt2I (agCP14403)
72 D s COEglt1I (ENSANGP00000026426)
73 D s COEglt6H (agCP10601)
74 + s COEglt7H (agCP10588)
75 s CG9287
76 s CG9289
77 + s COEglt2H (agCP10478)
78 + s COEglt5H (agCP10608)
79 + s COEglt4H (agCP10463)
80 + s COEglt3H (agCP10462)
81 + s COEglt1H (agCP10572)
82 D s CG7529
83 s CG9280 glutactin
84 + s F55F3.2
85 s COE7o (agCP1337)
86 s AEA CLU150 Aedes aegypti AF466590
87 s CG5397
88 + R s AChE Musca domestica AF281161
89 + s AChE Lucilia cuprina AAC02779
90 + s CG17907 AChE
91 + R s AChE Bactrocera oleae AF452052
92 + s COEace1o (agCP13477)
93 + s AChE-2 Leptinotarsa decemlineata Q27677
94 + s AChE-2 Nephotettix cincticeps AF145235
95 + s AChE-2 Apis melifera AAG43568
96 + s AChE-2 Helicoverpa armigera AAN37403
97 + s AChE-2 Plutella xylostella AAL33820
98 + s AChE-2 Myzus persicae AF287291
99 + s AChE-2 Aphis gossypii AAM94375
100 + s AChE-1 Schizaphis graminum AF321574
101 + s AChE-1 Aphis gossypii AAM94376
102 + R s AChE-1 Myzus persicae AY147797
103 + AChE-1 Apis melifera
104 + R s COEace3o (agCP12197)
105 + R s AChE-1 Culex pipiens CAD56155
106 + s W09B12.1 ace-1
107 + s Y48B6A.8 ace-3
108 + s Y48B6A.7 ace-4
109 + s Y44E3A.2 ace-2
110 s COE10o (agCP3485)
111 s CG10339
112 s COE12o (agCP10720)
113 s CG12869
114 s COEnrl8o (ebiP3191)
115 s CG5030
116 s COEnrl1B (agCP7849)
117 s CG32465
118 s COEnrl4o (agCP12264)
119 s CG13772 neuroligin
120 s COEnrl16o (ebiP8742)
121 s COEnrl2B (agCP7744)
122 s CG31146
123 s C40C9.5
124 s COEgli11o (agCP11410)
125 S CG3903 gliotactin
126 s F55D10.3
127 + T07H6.1
128 + W02B12.4 (20)
129 + ZK994.1 (17)
130 R173.3
131 S COEnrt9o (agCP5540)
132 S CG9704 neurotactin
(A) Mitochondrial, cytosolic and secreted esterases
(B) Higher dipteran microsomal α-esterases
(C) Lower dipteran microsomal α-esterases
(D) Moth integument-esterase with Drosophila homologs
(E) Dipteran β-esterases with hemipteran sister group
(F) Nonlepidopteran juvenile hormone esterases and like enzymes
(G) Lepidopteran JHE's and Anopheles sister group
(H) Glutactin and like enzymes
(I) Uncharacterized group
(J) Acetylcholinesterases
(K) Uncharacterized putative neuroreceptor group
(L) Neuroligins
(M) Gliotactin
(N) Neurotactin
Generally intracellular enzymes; dietary detoxification functions
Generally secreted enzymes; some specific substrates, including hormones and pheromones
Neurodevelopmental functions; except for acetylcholinesterase, noncatalytic

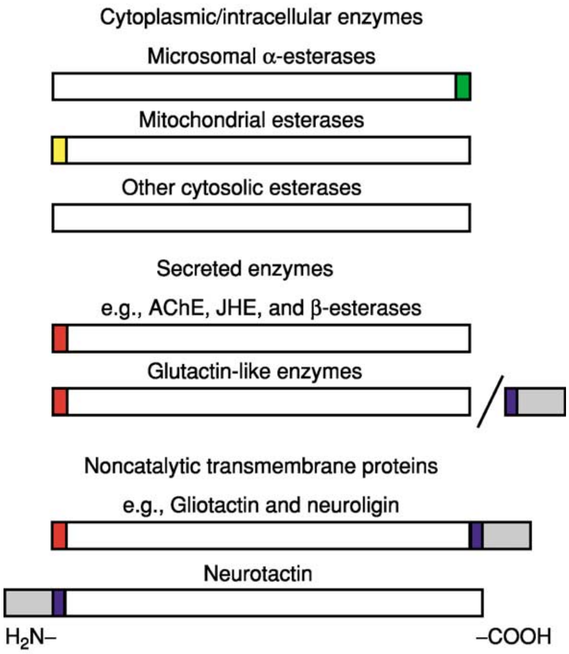


Figure 3 Schematic model (not to scale) of the consensus domain structure of insect carboxyl/cholinesterases in the various major clades indicated in **Figure 2**, showing N-terminal secretion (red) and mitochondrial targeting (yellow) and C-terminal endoplasmic retrieval (KDEL, green) signals for transmembrane proteins, the transmembrane (blue) and cytoplasmic (gray) domains. Slash symbol signifies that not all members of the glutactin clade have transmembrane domains (see Section 5.10.4.1). See also **Figure 2** regarding the secretion and endoplasmic reticulum retrieval signals.

membrane by motifs in their N- or C-terminal extensions (Figure 3) (see Sections 5.10.3.2 and 5.10.4.1). There are also some radiations of mainly catalytically competent enzymes that are secreted (D–H, J), but with the exception of glutactin and the AChE clade (J) these are not known to be membrane-associated. Members of the other major clades (A–C) are essentially all catalytically active enzymes and mostly intracellular, albeit they fall into two subgroups on the basis of subcellular localization. Members of one subgroup (B, C) can be classified as at least largely microsomal on the basis of functional data and/or the presence of C-terminal KDEL targeting motifs (Pelham, 1996; Yamamoto *et al.*, 2003). Members of the other subgroup (A) lack the latter motifs but cover a range of other localizations. A few contain conventional amino terminal secretion signals and some contain mitochondrial targeting signals (Bannai *et al.*, 2002). The rest are presumptively cytosolic, although some use of as yet unidentified nonconventional targeting signals, or extracellular translocation following membrane insertion (Emanuelsson *et al.*, 2000; Chou, 2001; Kriek *et al.*, 2003; Lai, 2003), cannot be discounted.

At the highest level, the phylogeny of insect carboxyl/cholinesterases can thus be partitioned quite cleanly into three groups on the basis of catalytic competence and cellular/subcellular localization. The oldest group (I–N) contains clades of secreted proteins that are generally membrane associated and, except for AChE, catalytically incompetent. Members of the next group (D–H) are also almost all secreted but, except for glutactin, not known to

Figure 2 Unrooted distance neighbor-joining tree showing a phylogeny of invertebrate carboxyl/cholinesterases. Sequences from the *Drosophila melanogaster* and *Anopheles gambiae* genomes (red and blue, respectively) are as annotated in Ranson *et al.* (2002). Sequences for various carboxyl/cholinesterases from other insects (black) were generally taken from the National Center for Biotechnology Information (NCBI) database; see text for inclusion criteria. The two exceptions were sequences 70 and 103, from Jones *et al.* (1994) and http://www.hgsc.bcm.tmc.edu. Sequences from the *Caenorhabditis elegans* genome (green) are as in Oakeshott *et al.* (1999) except that single representative sequences only were used for two nematode-specific lineages (see text and below). Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) with the BLOSUM 45 scoring matrix, with gap opening and gap extension penalties of 10.0 and 0.1, respectively, followed by some minor manual corrections to conform to known structural features of carboxyl/cholinesterases. The tree was constructed with PAUP* (Swofford, 2000) using standard distances and mean character differences. Nodes with at least 50% resampling frequency in 1000 bootstrap replications are indicated with an asteriks. To the right of the tree, in order, are: sequence numbers (1–132) as used elsewhere in this paper; catalytic competence as assessed by presence of a consensus catalytic Ser–His–Glu (+) or Ser–His–Asp (D) triad; evidence of involvement in chemical insecticide resistance (R, see text), and presence of a secretion signal (s) as assessed by the iPSORT algorithm of Bannai *et al.* (2002) and, if not secreted, of a mitochondrial (m) or KDEL endoplasmic reticulum targeting signal (K) as defined by Pelham (1996) and Yamamoto *et al.* (2003). (The upper-case S for sequences 125, 131, and 132 signifies that they lack a conventional N-terminal secretion signal according to iPSORT but empirical data establish that they are presented on the extracellular side of cell membranes (see Section 5.10.3.2).) Then follow sequence names and NCBI, Celera Protein and ENSEMBL database accession numbers and, if not *D. melanogaster*, *A. gambiae*, or *C. elegans*, species names. Note that *C. elegans* sequences 128 and 129 are single representative sequences for radiations of 20 and 17 proteins, respectively. Finally, major clades of sequences recognized in the text are named (and for cross-referencing to **Figure 6** below, alphabetically identified) and three putative functional ''superclades'' are also indicated. Note that some amino terminal sequence is missing for 18 of the proteins (4, 6, 7,13, 50, 58, 62–64, 78, 80, 103, 112, 114, 116, 120, 121, 126) and some C-terminal data are missing for 17 (6, 13, 54, 58, 60, 62–65, 77–80, 95, 103, 112, 118). Note also that the recently revised nomenclature of Weill *et al.* (2000, 2002, 2003) and Russell *et al.* (2004) for the AChE sequences have been followed (see also Section 5.10.3.1).

be membrane-tethered and they are almost all catalytically competent. The third and most derived group (A–C) contains catalytically competent enzymes covering a wide range of cellular/subcellular localizations, one subgroup generally being microsomal and the remainder a mix of presumptively secreted, mitochondrial, and cytosolic enzymes.

As discussed below (see Sections 5.10.3, 5.10.4, and 5.10.5), the available functional data suggest that these three groups are also qualitatively distinct in terms of their physiological roles. Characterized members of the first group, containing the secreted, membrane-associated, noncatalytic proteins and the AChE clade, are almost all involved in neurodevelopmental processes. At least four clades in the second group, mainly composed of secreted catalytic proteins, include various hormone and pheromone esterases. Many members of the third group, comprising mainly intracellular catalytically active proteins, appear to have broad substrate specificities and more general dietary and/or detoxification functions. The mutant enzymes conferring insecticide resistance occur in all three groups: AChE enzymes resistant to OP and carbamate inhibition in the first group and pesticide detoxifying enzymes in the third and, at least for some sap-sucking insects, also in the second. First, however, the phylogeny is examined in more detail, with particular regard to some major features of tertiary structure.

5.10.2.2. The Structural Context

Figure 4 diagrams the tertiary structure of the carboxyl/cholinesterase domain of the mature AChE monomer from *D. melanogaster* (DmAChE) (Harel *et al.*, 2000) (see Section 5.10.3.1). This structure is used as a model in the following sections because it is the only insect member of the carboxyl/cholinesterase protein family whose structure has been reported to this point. There are published structures for AChEs from a few different vertebrates, for the closely related vertebrate butyrylcholinesterase (BuChE) and another vertebrate carboxylesterase, and from a few sequence-related lipases from lower eukaryotes (Schrag and Cygler, 1993; Bourne and Shindyalov, 1998; Nicolet *et al.*, 2003). The structure of the carboxyl/cholinesterase domain of DmAChE is very similar to these others in its overall organization, notwithstanding over 60% amino acid sequence divergence from them. The core of the structure is the α/β hydrolase fold, comprising a major β-sheet of 11 strands arranged in a characteristic order, plus 14 α-helices in loops situated between the strands. Also in the loops are the components of the active site and catalytic gorge

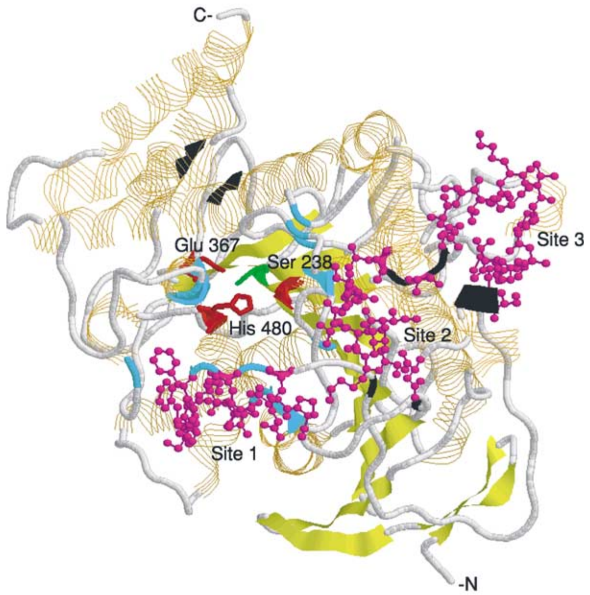


Figure 4 Three-dimensional cartoon structure of the carboxyl/cholinesterase domain of DmAChE (Harel *et al.*, 2000; PDB 1QO9; http://www.rcsb.org/pdb) generated by the RasMol program (http://www.openrasmol.org) looking down the catalytic gorge into the active site. Specifically indicated are residues contributing to the β-sheet (yellow; however, many are obscured behind the gorge and active site), several α-helices in the loops between β-strands (brown), six cysteines forming three disulfide bridges (black), the catalytic triad (Ser238, Glu367, His480, red) plus the candidate catalytic tetrad residue of Thomas *et al.* (1999) and Behra *et al.* (2002) (Ser264, green), 12 aromatic guidance residues lining the active site gorge (blue) and three peripheral substrate binding sites (purple). See also **Figure 6** for the relationship of these features to the primary sequence of the protein, and **Figure 5** for a schematic of the interior of the active site that cannot readily be shown in this figure.

and much of the external decoration for the structure. The structure is stabilized by three disulfide and four salt bridges.

There is quite detailed understanding of structure–function relationships in the regions of the AChE protein directly involved in catalysis. Many residues in these regions have been mutated *in vivo* in one or more of several cloned *ace* genes and the catalytic consequences for the protein determined (Doctor *et al.*, 1998; Ordentlich *et al.*, 1998; Villatte *et al.*, 2000a, 2000b; Boublik *et al.*, 2002; Pezzementi *et al.*, 2003 and references therein). **Figure 5** shows how the active site is built around the catalytic triad, with the nucleophilic serine protruding into the site on a conserved GXSXG pentapeptide termed the nucleophilic elbow. The rest of the site is largely composed of three subsites, which have variously been termed the leaving group pocket or P1 subsite (or in the particular case of AChE, anionic

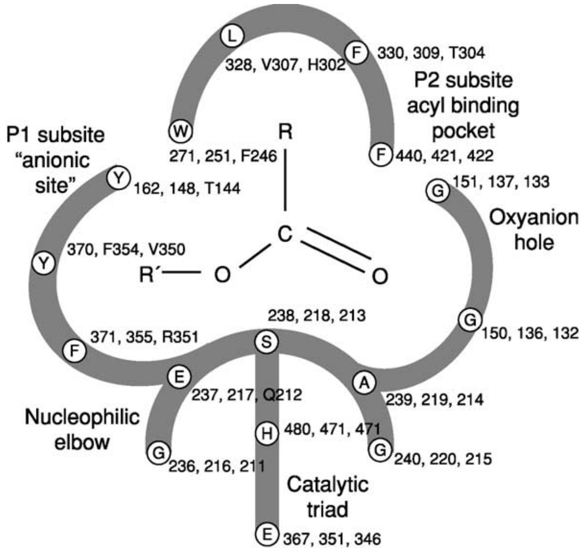


Figure 5 Schematic representation of the carboxyl/cholinesterase active site showing conserved amino acid residues. Each residue is shown with its single-letter code followed by its position in the sequences of DmAChE, LcαE7 (E3), and DmJHE, respectively. A letter is included where the residue in E3 or JHE differs from AChE. The residues of the catalytic triad, oxyanion hole, and nucleophilic elbow show strong conservation across active carboxyl/cholinesterases, while those constituting the subsites for the leaving and acyl groups of the substrate are more variable. These two subsites are described in greatest detail for AChE where they have often been called the P1 (or anionic) and P2 subsites, respectively. There is strong conservation of these sites across vertebrate and invertebrate AChEs (Harel *et al.*, 2000; Pezzementi *et al.*, 2003), but two of the indicated residues differ from AChE in E3 and seven of the eight differ in JHE.

site), the acyl pocket or P2 subsite, and oxyanion hole. These accommodate the alcohol, acid, and oxyanion moities of the substrate, respectively. Entry to the active site is via the catalytic gorge, which is over 20 Å long but only a few Å wide and is lined with several aromatic residues. Around the lip of the gorge are three "peripheral binding sites" which, when bound by substrate, trigger allosteric changes in the catalytic gorge that facilitate movement of substrate down the gorge. The end result is a particularly efficient enzyme with kinetics approaching diffusion limits (Taylor and Radic, 1994; Estrada-Mondaca and Fournier, 1998; Massoulie *et al.*, 1999).

Only two of the sequences in our phylogeny lack the additional serine (Ser264) which Thomas *et al.* (1999) and Behra *et al.* (2002) considered might, in combination with the established catalytic triad residues, form a "catalytic tetrad." One of these (33) is catalytically competent and the other (113) is not. Given its retention in so many catalytically inactive proteins, and its apparent absence in at least one catalytically competent enzyme, it seems likely to us that Ser264 has some highly conserved structural function in the active site, but not one directly involved in catalysis.

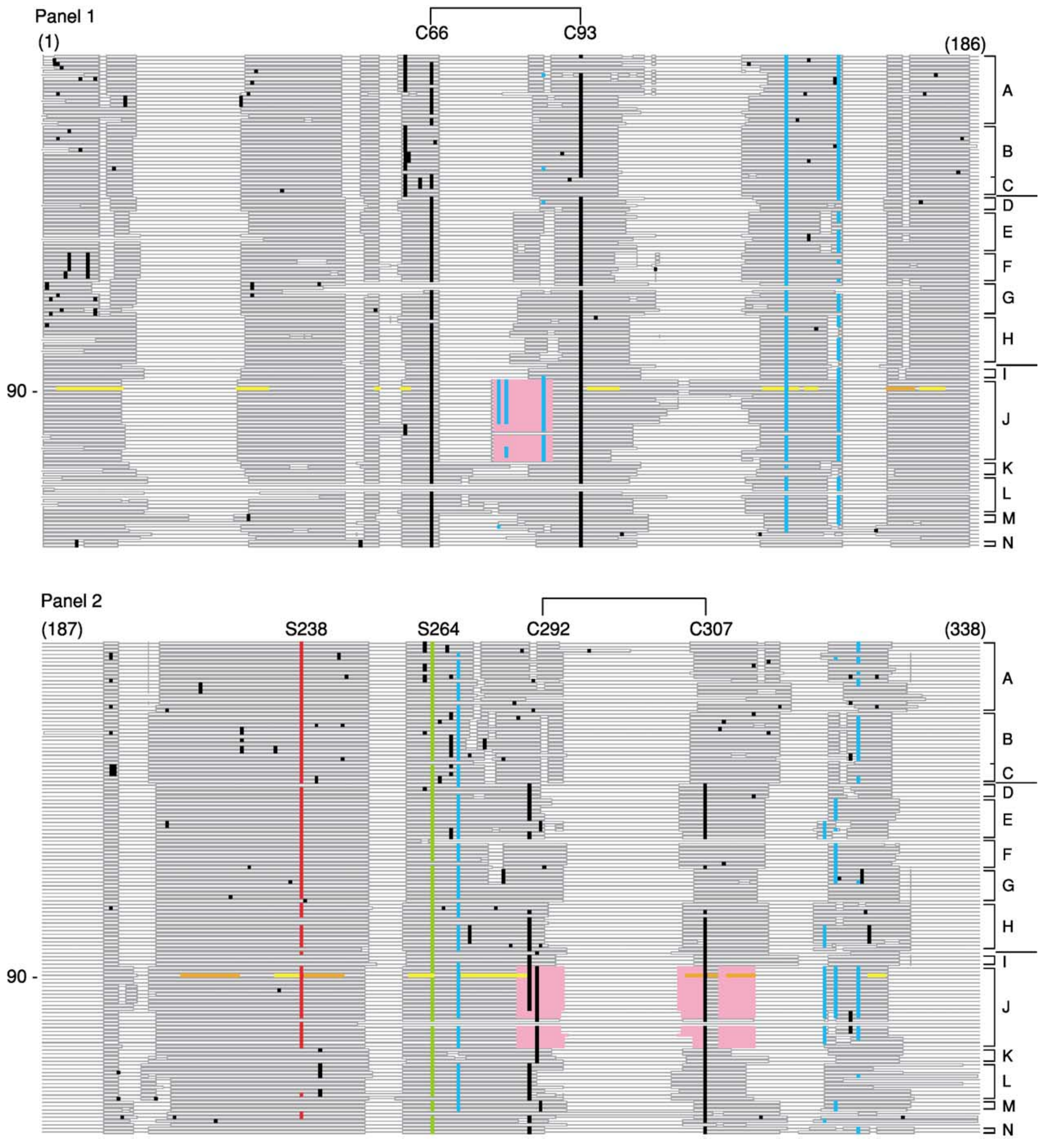
Figure 6 shows a qualitative alignment of the sequences in the phylogeny annotated with some of the major structural and/or functional motifs outlined above (a full alignment is available from the authors on request). In general terms, the alignment is consistent with the conservation of the overall arrangement of β-sheets and α-helices specifying the α/β hydrolase fold structure. Nevertheless there are also numerous clade-specific insertions/deletions, particularly in regions corresponding to loops towards the C-terminus of AChE. Many of these loops provide the external decoration to the core α/β hydrolase fold structure. Perhaps unsurprisingly some of the most distinctive arrangements occur in the noncatalytic clades. Importantly, however, the other catalytic clades also differ from AChE in aspects including two of the disulfide bridges, two of the peripheral substrate binding sites, and many of the aromatic gorge residues.

It is difficult to assess the significance of these differences in the absence of empirical structural information for any of the catalytic clades except AChE. However, they would seem to imply some major differences in the molecular mechanisms underpinning interactions with substrates. Modeling of a lepidopteran juvenile hormone esterase (JHE) indeed suggests at least two novel structural features of its active site that are instrumental to its particular catalytic capabilities (Thomas *et al.*, 1999) (see Section 5.10.4.2). There are also intriguing subclade-specific differences, for example, in the aromatic gorge residues of the hemipteran AChE-1 subclade, which may well have implications for catalysis, and the interactions with OP and carbamate insecticides (see Section 5.10.3.1). And, as discussed, the biochemistries of some of the insecticide resistance mutations imply major, allele-specific differences in aspects of structure affecting catalytic processes in certain catalytic carboxylesterases (see Sections 5.10.5.2, 5.10.6.1–5.10.6.3). It may be that a major reason for the proliferation of carboxyl/cholinesterases in higher eukaryotes is the functional diversity that can be achieved by relatively minor sequence changes in their catalytic machinery. Empirical structural data for some of the non-AChE catalytic clades would clearly now add greatly to our understanding of the functional diversity that has evolved among insect carboxyl/cholinesterases.

5.10.3. Acetylcholinesterase and Noncatalytic Neurodevelopmental Clades

The six major clades (I–N) containing insect sequences diverging earliest in the phylogeny (Figure 2) involve AChE and five clades of noncatalytic secreted proteins. The AChE is discussed first (see Section 5.10.3.1) because of the wealth of structure–function data available for it, followed by five noncatalytic clades (see Section 5.10.3.2).

Before doing so, however, it is worth noting some interesting parallels with the convergently evolved serine protease family of proteins in respect of the evolution of noncatalytic clades. Although they belong to a different structurally defined superfamily of proteins, the serine proteases generally use the same catalytic mechanism, built around the same catalytic triad and oxyanion hole, as the carboxyl/cholinesterases. (Indeed a few vertebrate esterases have been found in the serine protease family; see for example, Zschunke *et al.* (1991).) And, intriguingly, there are



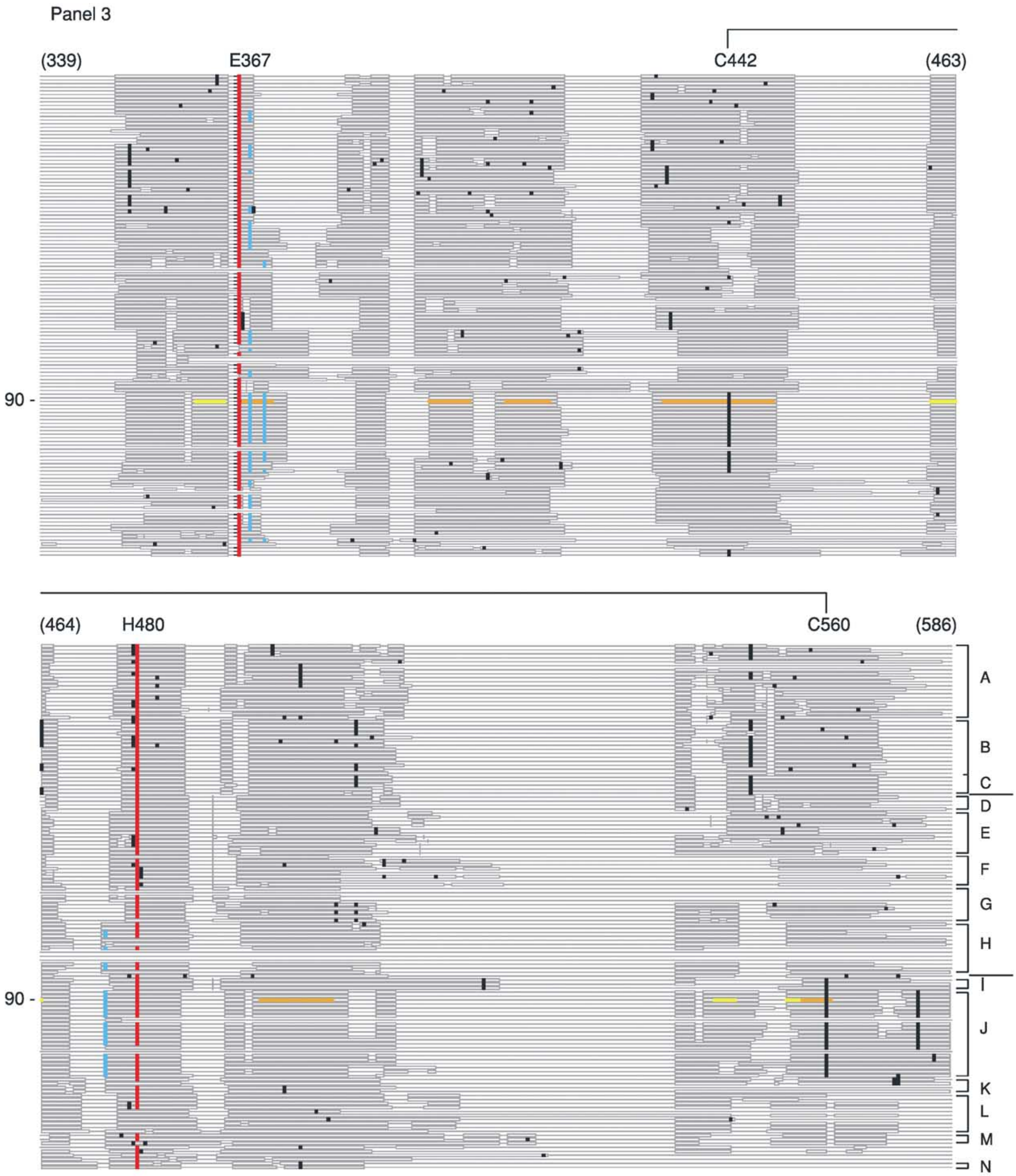


Figure 6 Block diagram generated by the GenDoc program (http://www.psc.edu) showing a qualitative alignment of the 132 carboxyl/cholinesterase amino acid sequences used to generate the phylogeny in **Figure 2**. Each sequence is simply represented as a series of blocks or lines to show the presence or absence of alignable residues. Sequences are ordered as in **Figure 2**, and their classification into the major clades recognized in **Figure 2** is shown on the right-hand side of each panel. The four panels cover sequences alignable to residues 1–186, 187–338, 339–463, and 464–585, respectively, in DmAChE (sequence 90 in the figure), which is used as a reference sequence because its tertiary structure is known (**Figure 4**). The β-strands and α-helices in DmAChE are shown in yellow and brown, respectively. Cysteines in all sequences are shown in black; those at residues 66 and 93, 292 and 307, and 442 and 560 in DmAChE are known to form three disulfide bonds. Aromatic residues corresponding to the aromatic gorge residues of DmAChE are shown in blue, conserved catalytic triad residues (Ser238, His480, and Glu367 in DmAChE) are shown in red, and the putative catalytic tetrad residue (Ser264 in DmAChE) of Thomas *et al.* (1999) and Behra *et al.* (2002) (which is present in all sequences covering the region except 33 and 113) in green. The known or presumptive peripheral substrate binding sites of all the AChE sequences are shown in pink. See **Figure 2** for the list of proteins lacking some amino or carboxy terminal sequence.

several lineages in the serine protease family, including in both vertebrates and invertebrates, that lack a consensus catalytic triad and are known to be catalytically incompetent. As with the carboxyl/cholinesterases, the ancestral state for the serine proteases, as seen in prokaryotes, is catalytic competence. However, early in the evolution of eukaryotes there was a proliferation of serine protease lineages with defective triads that took on ligand receptor roles in physiological processes ranging from neurodevelopmental regulation through to cellular immunity (Gabay and Almeida, 1993; Murugasu-Oei *et al*., 1995; Dimopoulos *et al*., 1997; Huang *et al*., 2000; Kwon *et al*., 2000; Kang *et al*., 2002). Many of these themes recur among the clades of noncatalytic carboxyl/cholinesterases.

5.10.3.1. Acetylcholinesterase

5.10.3.1.1. General biochemistry and genetics Although it also has other important roles, the major function of AChE is hydrolysis of the neurotransmitter acetylcholine after it has bound at cholinergic synapses. Hydrolysis is required to reset the synapses so that they can receive the next molecules of the neurotransmitter. Acetylcholine is an essential neurotransmitter in all higher eukaryotes but its role in insects is distinct from that in both vertebrates and nematodes. Accordingly insects also differ in their complement of *ace* genes and AChE proteins.

In vertebrates acetylcholine is an important neurotransmitter in both the central and peripheral nervous system and at neuromuscular junctions. Vertebrates only have one *ace* gene but, depending on the species, it can have three or four alternately spliced 3′ exons. This alternate splicing generates a range of variously oligomerized and soluble versus membrane-associated forms. The membrane-bound forms may be anchored with glycosyl phosphatidyl inositol (GPI) or complexed with collagen or the Type 1 transmembrane protein PRiMA (Massoulie *et al*., 1999; Perrier *et al*., 2002; Scholl and Scheiffele, 2003). The various forms are differentially expressed in the various cholinergic tissues above and in the blood and there are also some differences in their ontogenetic specificities. Vertebrates also have the closely related BuChE gene/enzyme system but this system is not directly involved in acetylcholine hydrolysis and it has no ortholog in nematodes or insects (Nicolet *et al*., 2003 and references therein).

Nematodes use acetylcholine as a neurotransmitter in both the central nervous system (CNS) and muscle tissue. *Caenorhabditis elegans* has four *ace* genes, but the products of two of these contribute the great majority of the organism's AChE activity (Massoulie *et al*., 1999, Combes *et al*., 2000, 2003). These two genes each encode a single transcript and class of product, one expressed as GPI-anchored dimers in neural tissue, and the other as a complex with collagen in muscle tissue and a small proportion of neurons. The form and function of the other two *ace* gene products are not well understood.

Insects use acetylcholine as a neurotransmitter in their CNS but their muscles largely use glutamate for this function. Accordingly their AChE is predominantly expressed as GPI-anchored dimers, with no equivalent of the collagen-complexed forms found in the muscles of vertebrates and nematodes (Massoulie *et al*., 1999). *Drosophila* and other higher Diptera (and possibly also a few lower Diptera) (Bourguet *et al*., 1998) only appear to have one *ace* gene but all other insects studied to date have two (Weill *et al*., 2002). Significantly, an acarine, the cattle tick *Boophilus microplus*, also has two (Baxter and Barker, 2002). In those arthropods with two *ace*/AChE gene/enzyme systems there appears to be just one system, *ace-1*/AChE-1 on the nomenclature of Weill *et al*. (2000, 2002, 2003) and Russell *et al*. (2004), which is responsible for neural acetylcholine hydrolysis, and it is also this one in which mutations conferring target site resistance to OP and carbamate insecticides may be found. The function of the other, *ace-2*/AChE-2, system is unknown; it is catalytically active but it is also speculated that it could have various cell–cell communication/adhesive properties, such as those described for *Drosophila* and vertebrate *ace*/AChE (Grisaru *et al*., 1999; Behra *et al*., 2002; Scholl and Scheiffele, 2003; Russell *et al*., 2004).

The currently available invertebrate AChE sequences form a strongly structured clade with four clearly discernable subclades in the phylogeny in **Figure 2**. One subclade, comprising solely the two inabundant *C. elegans* AChEs and its muscle/collagen-complexed AChE, is an outgroup to all the others. The next most distinct subclade includes the *C. elegans* AChE responsible for neural acetylcholine hydrolysis, plus the AChE-1 sequences of those insects and the tick which have two *ace* genes. The other two subclades are sister groups, one comprising the AChE-2s of the species with two *ace* genes, and the other comprising the AChEs of the higher Diptera. It follows that the single-*ace* genotype found in the higher Diptera has evolved by loss of the *ace-1* gene in an ancestor with a twin-*ace* genotype. And the intriguing corollary is that the originally noncholinergic AChE-2 in this ancestor has then reverted to a cholinergic role to cover for the loss of the AChE-1 (Weill *et al*., 2002; Russell *et al*., 2004).

The mature GPI-linked dimers that are the predominant form of AChE in *D. melanogaster* are produced through a complex set of posttranslational modifications of a primary 70 kDa translation product (Mutero and Fournier, 1992; Estrada-Mondaca and Fournier, 1998). This primary product comprises a 15 kDa N-terminal domain (in addition to a secretion signal), a ~55 kDa central carboxyl/cholinesterase domain, and a short C-terminal hydrophobic peptide. Dimerization occurs through an intermolecular disulfide linkage between homologous cysteine residues towards the C-terminal of the carboxyl/cholinesterase domain (Cys577) (**Figure 6**). The 15 and 55 kDa domains are proteolytically cleaved, albeit they remain noncovalently associated. Then the C-terminal hydrophobic peptide is replaced with the GPI anchor to the cell membrane. Several glycans are also added to the 15 and 55 kDa domains during processing.

At least four catalytically efficient AChE isoforms are clearly resolvable on native PAGE of *D. melanogaster* extracts (Fournier *et al.*, 1988, 1992; Zador, 1989; Healy *et al.*, 1991). The predominant one is the mature GPI-linked AChE dimer above (often also called the amphiphilic dimer). The others include amphiphilic monomers and hydrophilic dimers and monomers. The amphiphilic forms are largely localized to the CNS but the soluble, hydrophilic forms are not. It is unclear to what extent the alternate forms result from incomplete posttranslational processing as above, or are the result of the programmed deconstruction of the amphiphilic dimers. However, at least some contribution from both processes has been suggested. The amphiphilic dimers clearly have the major role in the cleavage of acetylcholine at synapses but it is also suggested that the other forms have other, as yet only poorly understood functions (Grisaru *et al.*, 1999). Notwithstanding their extra *ace* gene, insects in other orders still produce an essentially similar array of AChE isoforms (Toutant, 1989; Bourguet *et al.*, 1998).

Data for several species suggest that insect AChEs are generally, like their vertebrate counterparts, highly efficient kinetically (Gnagey *et al.*, 1987; Devonshire *et al.*, 1998; Chen *et al.*, 2001 and references therein). Specificity constants, k_{cat}/K_m, are of the order of $10^8\ M^{-1}\,s^{-1}$ for vertebrate AChEs with acetylcholine or (as more commonly measured) acetylthiocholine (Gibney *et al.*, 1990; Taylor and Radic, 1994). These values are the outcome of particularly high turnover rates (k_{cat} values approaching $10^4\ s^{-1}$) but only modest substrate affinities (K_m values approaching 50 μM). The latter are rationalized in terms of the likely high local concentrations of acetylcholine in the microenvironment of the synapse. Values for AChEs from the higher dipterans *D. melanogaster* and the Australian sheep blowfly *Lucilia cuprina* are three- to sixfold lower, due to slightly lower turnover values. Values for different isoforms of DmAChE are very similar to one another (Mutero and Fournier, 1992; Estrada-Mondaca and Fournier, 1998). There is one surprisingly low estimate of k_{cat} of around $2 \times 10^4\ min^{-1}$ for the Colorado potato beetle *Leptinotarsa decemlineata* (Zhu and Clark, 1995a) but the significance of this is unknown.

Two features of AChE biology in vertebrates that are generally associated with noncholinergic functions are also seen in insects, although evidence for the functional effects is still sparse for the insects (Darboux *et al.*, 1996; Scholl and Scheiffele, 2003). These features are the existence of nonneural soluble isoforms and the cell adhesive property. There are numerous studies showing effects of vertebrate AChE on the development of neuromuscular junctions and neurite outgrowth and many of these appear to depend on cell adhesive rather than catalytic properties of the enzyme (Sternfeld *et al.*, 1998; Grisaru *et al.*, 1999; Sharma *et al.*, 2001; Behra *et al.*, 2002, Scholl and Scheiffele, 2003). Soluble AChE is also overproduced in response to various stressors (Grisaru *et al.*, 1999; Lev-Lehman *et al.*, 2000). It is not known what parallels exist in insects but given the proliferation of closely related noncatalytic clades with clear functional effects they would seem well worth further investigation.

5.10.3.1.2. Target site insecticide resistance The activated or oxon forms of OP insecticides (see below) and carbamate insecticides act as essentially irreversible inhibitors, or suicide substrates, for AChE. This has proven true for all the well-characterized AChEs, although the insect AChEs seem particularly susceptible (Villatte *et al.*, 1998; Chen *et al.*, 2001; Boublik *et al.*, 2002). The inhibition occurs because the insecticides can serve as good substrates for the first step but poor substrates for the second step in the two-step reaction mechanism (**Figure 1**) (see Section 5.10.1.2). In other words they readily form a phosphorylated or carbamylated enzyme intermediate instead of the acyl-enzyme intermediate formed with carboxyl/choline ester substrates, but the phosphoryl- and carbamyl-enzyme intermediates are far more stable than the acyl-enzyme. The carbonyl carbon in the acyl enzyme intermediate is susceptible to nucleophilic attack by the oxygen of a water molecule, this oxygen being activated and oriented by residues in the active site. The slow to negligible hydrolysis of the

phosphoryl- and carbamyl-enzymes is presumably due to the inappropriate geometry of the phosphate group or an interfering effect of the nitrogen atom adjacent to the carbonyl carbon, respectively, that prevents hydrolysis by the enzyme's usual mechanism.

Inhibition as above for AChE probably occurs to varying degrees for all catalytically competent carboxyl/cholinesterases with OPs. To a lesser degree it may also occur for some of these other enzymes with some of the carbamates. Whatever their effects on the other carboxyl/cholinesterases, however, it is the consequences of the pesticides' inhibition of AChE on the functioning of cholinergic nerves that are assumed to underlie much of their insecticidal effect. Insects or acarines exposed to the insecticides can survive until a surprisingly high proportion of their AChE molecules are inhibited (Smissaert *et al*., 1975; Hoffmann *et al*., 1992; Devonshire *et al*., 2003), but above this threshold signal transduction down cholinergic nerves fails and paralysis occurs.

Well over 20 cases of target site resistance to OPs and/or carbamates have now been described in various insects and acarines (reviews: Fournier and Mutero, 1994; Bourguet *et al*., 1998; Devonshire *et al*., 1998; Russell *et al*., 2004). The cases cover most of the major orders of insect pests although hemipteran and dipteran cases predominate and there are relatively few lepidopteran examples (**Table 1**). There is a little evidence that upregulation of the amount of AChE can augment tolerance to the insecticides (Fournier *et al*., 1992; Charpentier *et al*., 1998) but high-level resistance seems only to be achieved by amino acid substitutions affecting the catalytic machinery of the enzyme that make it less sensitive to inhibition by the insecticides.

Russell *et al*. (2004) have discerned two major patterns of target site resistance to OPs and/or carbamates among many of the cases that have been most thoroughly characterized so far (**Table 1**). Each pattern is reported from several orders, and Pattern Two also from acarines, and there are examples of both from the genus *Culex*. Pattern One resistance is characterized at a bioassay level by much greater resistance to carbamates than to OPs and, at a biochemical level, by a much greater reduction in the sensitivity of AChE to the carbamates than to the OPs. The target site resistance reported in the mosquito *Culex pipiens*, the cotton aphid *Aphis gossypii*, and the peach potato aphid *Myzus persicae* are well-characterized examples of Pattern One resistance. Pattern Two resistance is characterized by more equivalent levels of resistance and/or reductions in AChE sensitivity to the two classes of insecticide. Some well-characterized examples of Pattern Two resistance involve the mosquito *Culex tritaeniorhynchus*, *D. melanogaster*, the housefly *Musca domestica*, the olive fruit fly *Bactrocera oleae*, and the silverleaf whitefly *Bemisia tabaci*. Importantly, the two types of pattern as defined by the bioassay and biochemical criteria also correspond to different sets of mutation in the catalytic machinery of AChE.

Molecular data on the resistance mutations are now available for 10 species in **Table 1**. A total of 18 mutations have been confidently associated with resistance in eight of the species, with four polymorphisms tentatively associated with resistance in the beetle *L. decemlineata* and the two-spotted spider mite *Tetranychus urticae*. Except for the *L. decemlineata* data, all cases involve the cholinergic form of AChE. As many as five mutations have been reported in each of *D. melanogaster* and *M. domestica*. However, the 22 differences involve just 11 DmAChE-equivalent sites. Mutations at five of the sites occur more than once in the data, sometimes involving the same substitutions in different species. Ten of the 11 sites lie in or near the active site/gorge.

At least on the current data, the options for Pattern One resistance mutations seem more constrained than those for Pattern Two resistance. A G151S mutation (on DmAChE numbering, which is used throughout this section) in the oxyanion hole has been associated with one clear case of Pattern One resistance, in *C. pipiens*, as well as two other anopheline cases almost certainly also of this pattern, in *Anopheles gambiae* and *A. albimanus* (Weill *et al*., 2002, 2003, 2004). It is unclear as yet how this relatively conservative mutation in the three lower dipterans reduces the sensitivity of the active site to inhibition by carbamates in particular. However, it is intriguing that a different mutation, to an aspartate, at the equivalent oxyanion hole residue of certain *L. cuprina* and *M. domestica* carboxylesterases actually confers metabolic resistance to OPs by allowing those enzymes to complete the second step of the reaction with OP substrates, albeit at the expense of their carboxylesterase activity (see Section 5.10.5.2).

However, Pattern One resistance in the hemipteran *M. persicae* is associated not with G151S but with a S371F change (Nabeshima *et al*., 2003). S371 is also close to the catalytic residues at the base of the gorge and has been implicated in substrate guidance and binding. While this is considered to be primarily with the alcohol portion of a carboxyl ester, its location (not obvious from the two-dimensional cartoon in **Figure 5**) is on the boundary between the anionic and acyl binding sites where it can also affect binding of the alkoxy group of OPs (Ordentlich *et al*., 1993, 1996; Millard *et al*.,

Table 1 Summary of cases of resistance to OPs and/or carbamates resulting from an insensitive AChE in various insect species; cases are included on the basis of bioassay and/or biochemical data implicating an insensitive AChE and, where possible, classified according to the two patterns of AChE insensitivity identified by Russell *et al.* (2004)[a]

Species	*Resistance profile*	*Resistance mutations*[b]	*Reference*
Pattern One: Carbamate resistance > OP resistance			
Thysanoptera			
Scirtothrips citri (citrus thrip)	AChE insensitivity is greater for propoxur than paraoxon in a resistant strain		Ferrari *et al.* (1993)
Hemiptera			
Aphis gossypii (cotton aphid)	Carbamate resistance (pirimicarb) is much greater than OP resistance (demeton-*S*-methyl, methamidiphos, monocrotophos, omethoate, pirimiphos-methyl)		Moores *et al.* (1996), Menozzi, cited in Weill *et al.* (2002), Li and Han (2002)
Myzus persicae (peach potato aphid)	Marked AChE insensitivity to pirimicarb (>100×) but no insensitivity to a range of other carbamates (e.g., methomyl, carbofuran, aldicarb, ethiofencarb) or OPs (demeton-*S*-methyl, dichlorvos, paraoxon)	S371F (gorge guidance residue near triad His/acyl pocket)	Moores *et al.* (1994), Williamson *et al.*, cited in Villatte and Bachmann (2002); Nabeshima *et al.* (2003)
Nephotettix cincticeps of Iwata and Hama (green rice leafhopper)	43-fold resistance to propoxur, 43-fold to carbaryl, 6-fold to malathion, 18-fold to malaoxon		Iwata and Hama (1972)
Coleoptera			
Leptinotarsa decemlineata of Wierenga and Hollingworth (Colorado potato beetle)	>550-fold resistance to carbofuran and 9-fold resistance to azinphosmethyl		Wierenga and Hollingworth (1993)
Lepidoptera			
Helicoverpa armigera (cotton bollworm)	35-fold resistance to thiodicarb, 75-fold to methomyl and no cross-resistance to OPs		Gunning *et al.* (1996a)
Heliothis virescens (tobacco budworm)	k_i values for AChE inhibition are 10- to 100-fold less for propoxur than for several OPs		Brown and Bryson (1992)
Lower Diptera			
Anopheles albimanus of various authors	k_i values are 100-fold less for propoxur than paraoxon		Ayad and Georghiou (1975)
	k_i values are ~10-fold less for propoxur than for malaoxon		Hemingway *et al.* (1985)
	Resistance levels are >1000-fold for propoxur, 73-fold for carbaryl and 1.2–38-fold for OPs		Hemingway and Georghiou (1983)
Culex pipiens complex (house mosquito)	k_i values for three strains are 1000-fold less for propoxur than malaoxon, paraoxon, and aldicarb; resistance factors for one strain are 1600-fold for propoxur, 18-fold for chlorpyriphos, 140-fold for malaoxon and 5-fold for temephos	G151S (oxyanion hole) in all strains	Weill *et al.* (2002, 2003, 2004)
Pattern Two: Carbamate resistance ~ OP resistance			
Hemiptera			
Bemisia tabaci (silverleaf whitefly)	Similar k_i values for OPs and carbamates		Byrne and Devonshire (1993, 1997)
Coleoptera			
Diabrotica virgifera (western corn rootworm)	Slight increase in AChE insensitivity to paraoxon in a strain resistant to a range of OPs and carbaryl		Miota *et al.* (1998)
Lepidoptera			
Spodoptera frugiperda (fall armyworm)	Similar k_i values for OPs and carbamates		Yu (1991)

Continued

Table 1 Continued

Species	*Resistance profile*	*Resistance mutations*[b]	*Reference*
Lower Diptera			
Culex tritaeniorhynchus	AChE from a resistant strain has 870-fold decrease in OP sensitivity, and to a lesser extent to carbamates	F371W (gorge guidance residue near triad His/acyl pocket)	Nabeshima *et al.* (2004)
Higher Diptera			
Bactrocera oleae (olive fruit fly)	k_i data indicate insensitivity to omethoate and paraoxon, but not to propoxur	G436S (near triad Glu) I161V (gorge guidance residue near anionic site)	Vontas *et al.* (2001, 2002)
Drosophila melanogaster (vinegar fly)	Similar levels of resistance to OPs (paraoxon, malaoxon) and carbamates (carbaryl, propoxur)	F77S (anionic site) I161V/T (gorge guidance residue near anionic site) G265A (near triad Ser) F330Y (acyl pocket)	Mutero *et al.* (1994)
Musca domestica of Walsh *et al.* (housefly)	Similar k_i values for OPs and carbamates	V182L (little effect) G265A/V (near triad Ser) F330Y (acyl pocket) G368A (near triad Glu)	Walsh *et al.* (2001)
Acarina			
Amblyseius potentillae (predatory mite)	Similar k_i values for paraoxon and propoxur		Anber and Overmeer (1988)
Tetranychus kanzawai (Kanzawa spider mite)	OP resistant strains with low cross-resistance to carbamates		Kuwahara (1982)
Unknown			
Thysanoptera			
Frankliniella occidentalis (western flower thrip)	Ten-fold decrease in AChE sensitivity to diazoxon inhibition in a resistant strain		Zhao *et al.* (1994)
Homoptera			
Cacopsylla pyri (European pear sucker)	Resistance to monocrotophos and 70-fold decrease in k_i for monocrotophos		Berrada *et al.* (1994)
Hemiptera			
Lygus hesperus (plant bug)	34-fold decrease in AChE sensitivity to paraoxon inhibition in a resistant strain		Zhu and Brindley (1990a)
Nephotettix cincticeps of Tomita *et al.*	AChE inhibition by propoxur		Tomita *et al.* (2000)
Coleoptera			
Leptinotarsa decemlineata	Resistance to azinphosmethyl	S276G (peripheral binding site/active site)[c]	Zhu and Clark (1995a, 1995b), Zhu *et al.* (1996)
Lower Diptera			
Anopheles albimanus of Weill *et al.*	Resistance to carbamates and OPs based on AChE insensitivity but details not reported	G151S (oxyanion hole)	Weill *et al.* (2004)
Anopheles gambiae (African malaria mosquito)	Resistance to carbosulfan	G151S (oxyanion hole)	N'guessan *et al.* (2003); Weill *et al.* (2003, 2004)
Higher Diptera			
Musca domestica of Kozaki *et al.*	>40-fold decrease in AChE sensitivity to fenitroxon in resistant individuals	G265A (near triad Ser) F330Y (acyl pocket)	Kozaki *et al.* (2001)
Acarina			
Boophilus microplus (southern cattle tick)	Resistance to OPs		Baxter and Barker (2002)
Tetranychus urticae (two-spotted spider mite)	Multi-resistant strain has AChE that is more sensitive to OPs and carbamates (not less)	S/G151S (oxyanion hole) F371F/C (gorge guidance residue near triad His/ acyl pocket) D/E160E (vicinity of gorge)	Anazawa *et al.* (2003)

1999). The clear inference from the *M. persicae* data is that it can also have profound effects on the binding of certain carbamates, at least in this hemipteran version of AChE-1.

In fact it is suspected that this *M. persicae* case may represent a subtype of Pattern One resistance that is specific for dimethyl carbamates like pirimicarb and the dimethyl carbamoyltriazole triazamate, and notably not including resistance to monomethyl carbamates such as propoxur that have been used to diagnose many of the mosquito and some of the other cases of Pattern One resistance above. Interestingly, the S371F change brings the *M. persicae* sequence into line with the status of this residue as a strongly conserved phenylalanine in most carboxyl/cholinesterases. Whatever benefit the serine might otherwise confer on susceptible aphids, it seems that it allows an accommodation of the slightly bulkier dimethyl carbamates at the base of the gorge that is hindered by the larger phenylalanine more usually found at this position in carboxyl/cholinesterases.

Another hemipteran, *A. gossypii*, also shows high resistance to pirimicarb, without cross-resistance to monomethyl carbamates, so it will be interesting to see if the same mutation occurs in its AChE-1. If so, it might suggest that the potency of the dimethyl carbamates as aphicides could at least in part be due to the unusual status of AChE-1 residue 371 in (susceptible) aphids. Interestingly, two other mutations to different residues which give very different resistance profiles have also been reported at residue 371 outside the Hemiptera and are discussed below.

A total of 12 mutations have been reported in three higher dipteran cases of known or presumptive Pattern Two resistance, involving *D. melanogaster*, *M. domestica*, and *B. oleae* (Mutero *et al.*, 1994; Kozaki *et al.*, 2001; Vontas *et al.*, 2001, 2002; Walsh *et al.*, 2001). These occur in just seven DmAChE-equivalent sites, with three of these sites (161, 265, 330) each used in two or three of the mutations. One of the 12 mutations, V182L, is located at some distance from the catalytic residues and apparently makes little contribution to resistance. However, the other 11 mutations all involve six sites at the base of the gorge, and most are substitutions to larger residues, which modeling suggests could act by constricting the space at the base of the gorge in such a way as to reduce the insecticides' access to the catalytic residues. Why they should effect Pattern Two as opposed to Pattern One resistance is as yet unclear.

Elegant kinetic analyses of AChE variants with different combinations of most of the 11 mutations indicate that most of them individually contribute towards the insensitivity phenotype. This is significant because various alleles in natural populations only contain a subset of the substitutions. Introduction of some of the same changes into the AChEs of *Aedes aegypti* and *L. cuprina* has a similar effect on the inhibition kinetics of baculovirus-expressed enzyme (Vaughan *et al.*, 1997b; ffrench-Constant *et al.*, 1998; Chen *et al.*, 2001); this supports the view that these or very similar mutations could underlie other cases of Pattern Two resistance.

Pattern Two resistance in *C. tritaeniorhynchus* involves a single change, F371W, not seen elsewhere as yet in this pattern (Nabeshima *et al.*, 2004). However, it does involve the same site at which other mutations have been associated with Pattern One resistance in *M. persicae* (above) and another very different resistance in the mite *T. urticae* (below). It fits the trend of changes to bulkier side chains evident in many of the mutations in both patterns. The fact that quite different insensitivity and resistance profiles can be obtained by the various substitutions at this site seen in different species may be an indication that the precise location of this residue around the boundary of the acyl and anionic sites differs among the AChEs of these species.

Russell *et al.* (2004) could not assign the case of target site resistance to azinphosmethyl in the beetle *L. decemlineata* to a pattern in the absence of toxicological or biochemical data on carbamate

[a]Cases were assigned to Pattern One or Two where bioassay and/or biochemical data were available for both OPs and carbamates. Pattern One resistance involves greater (target site) resistance factors and/or greater reductions in AChE sensitivity to carbamates than OPs. Pattern Two resistance involves more similar, or greater (target site) resistance or reductions in AChE sensitivity for the OPs compared to the carbamates. Cases for which an insensitive AChE has been reported, but for which the resistance profiles for both OPs and carbamates have not been reported, are classified as ''unknown.''

[b]All identified mutations except the S276G reported in *L. decemlineata* by Zhu *et al.* (1996) are in the cholinergic AChE, i.e., AChE in the higher Diptera and AChE-1 elsewhere (on the nomenclature of Weill *et al.* (2000), 2002, 2003 and Russell *et al.* (2004)). Residues are numbered according to the nomenclature for DmAChE (Harel *et al.*, 2000) with the residues in susceptible strains given before the numbers and those in resistant strains after them. A dash indicates that the mutation has yet to be identified. Bracketed comments on locations are taken from the authors or extrapolated from Harel *et al.* (2000).

[c]Weill *et al.* (2002) suggest that the association with resistance is not significant.

sensitivity. More importantly, Weill *et al.* (2003) have also expressed doubts about the significance of the resistance phenotype. This is important because molecular data (Zhu *et al.*, 1996) implicate an S276G change in the presumptively noncholinergic AChE-2 (Weill *et al.* (2003) and Russell *et al.* (2004) nomenclature) in this resistance. Neither this residue, nor this form of AChE have previously been implicated in resistance but modeling also puts the change in the vicinity of the active site, albeit not as close to the catalytic residues as most of those above. Zhu *et al.* (1996) suggest that the substitution could affect interactions with the pesticide either at the peripheral binding site or within the gorge. Clearly, further work is required to elucidate the biochemistry and molecular genetics of AChE-based resistance in this species.

The case of *T. urticae* is remarkable in that it apparently involves change(s) to AChE-1 that make it more, not less sensitive to inhibition by OPs and carbamates. No fixed differences have yet been reported in AChE-1 between susceptible and resistant strains. However, Anazawa *et al.* (2003) found that residue 151 in the oxyanion hole was polymorphic for Ser and Gly in susceptible strains but only Ser in resistant strains, residue 371 at the base of the gorge was polymorphic for Phe and Cys in resistant strains but only Phe in susceptibles, and residue 160, also near the active site, was polymorphic for Asp and Glu in susceptibles and only Glu in resistance. Residue 160 has not been implicated in resistance previously but both the other two have been. In both cases the precedent data (G151S, S371F, and F371W) associate changes to bulkier residues at these sites with reduced AChE sensitivity and target site resistance. In *T. urticae*, the F371F/C polymorphism associates a bulkier residue with lower AChE sensitivity but target site susceptibility, while the reverse is true for S/G151S. Whilst clearly difficult to interpret in detail, at face value the *T. urticae* results would seem to imply a very different AChE (in)sensitivity mechanism which nevertheless involves changes to some of the same residues implicated in the other AChE-based target site resistances so far resolved at a molecular level.

Some important insights into AChE-based target site resistance have also been gained from detailed analyses of the inhibition kinetics of various synthetic mutants of DmAChE. A series of studies (Villatte *et al.*, 1998, 2000a, 2000b; Boublik *et al.*, 2002) have examined a large number of substitutions at some of the sites recurrently implicated in natural resistance, as well as selected changes at over 20 other sites in the active site/gorge. DmAChE is already more sensitive than most characterized noninsect AChEs to inhibition by OPs and carbamates (as indeed are other well-characterized insect AChEs) (Chen *et al.*, 2001) but a small number of mutations were found to make it even more sensitive. Most changes, however, decreased sensitivity, and most also reduced acetylcholine hydrolytic activity, although many still allowed levels of acetylcholine hydrolysis predicted to be viable *in vivo*. Some changes had generally greater effect on the interactions with OPs than with carbamates, others had more impact on carbamate interactions, and some had large effects on both. There was also significant, mutation-specific variation both among OPs and among carbamates in the size of the effects. Some mutations masked the effects of others. Several mutations at sites like I161 and F371 where natural resistance has been reported were found to have large effects, but several sites closer to the lip of the gorge and the peripheral binding sites were also found to cause significant sensitivity changes. Overall the results suggest that there may be several more options for Patterns One or Two resistance, or indeed other classes of target site resistance mutations, than have been documented thus far, with the “choice” among options depending both on the particular pesticides involved and on genetic constraints like pre-existing codon usage and variability at the key sites.

It will also not be surprising if there are phylogenetic differences in the mutations underlying the various insensitivity phenotypes. There are already indications of this in the data to date. For example, the three lower dipteran cases of known or presumptive Pattern One resistance all involve a G151S change, whereas, in *M. persicae* the causative change appears to be S371F. Likewise, none of the several changes seen in the three higher dipteran cases of Pattern Two is the same as the one found underlying this pattern in *C. tritaeniorhynchus*. It is suspected that such differences reflect phylogenetic differences in the details of the geometry and composition of the gorge; significant structural differences are indeed suggested by the alignment in **Figure 6**, the hemipteran AChE-1 subclade being but one example. The differences between the higher Diptera and other lineages reflecting their use of nonorthologous AChEs for cholinergic functions are also expected.

Both Pattern One and Pattern Two resistances seem to compromise the kinetics of acetylcholine hydrolysis to some degree (Fournier and Mutero, 1994; Devonshire *et al.*, 1998), which can lead to a fitness cost for resistant genotypes in the absence of the insecticide (Roush and McKenzie, 1987). The costs have been most thoroughly explored in two cases of Pattern One resistance.

The insensitive AChE in *M. persicae* affects fitness in two ways (Foster *et al*., 2003). Aphids with the modified AChE reproduce more slowly and are more responsive to alarm pheromone. These effects have only recently been discerned as they are usually superimposed on fitness costs due to other resistance factors in clonal lines of this species (see Section 5.10.4.3.2). How they are mediated in terms of the physiology of AChE is as yet unclear.

A severe fitness cost has been described for the Pattern One resistance in *C. pipiens*; estimates by various means have put the fitness cost as high as about 60% (Chevillon *et al*., 1997; Lenormand *et al*., 1999). As a consequence there has been clinal variation in resistant allele frequencies away from geographic areas with heavy use of the insecticides towards those with much lighter use. There has also been regular seasonal variation in the frequency of the resistant allele in localities only seasonally exposed to the insecticide. Most recently a duplication of the *ace-1* locus has arisen and swept to high frequency in *C. pipiens* that combines both the susceptible wild-type and the resistant allele (Lenormand *et al*., 1998a). This combination apparently provides both sufficient resistant AChE-1 protein to ensure survival in the presence of the insecticide, and sufficient wild-type AChE-1 protein to restore near-wild-type neurotransmission in the absence of the insecticide.

5.10.3.2. Noncatalytic Clades

The five clades of noncatalytic proteins considered here comprise the neurotactins (clade N in **Figure 2**), which constitute an outgroup to all the others, and then the gliotactins (M), putative neuroligins (L), and two functionally anonymous radiations (I, K) which are all more closely related to the AChEs (J). expectThe greater phylogenetic separation of the neurotactins is also borne out by other aspects of their biology. For example, the neurotactins have an N-terminal extension which specifies transmembrane and intracellular domains, whereas all the others appear to specify these domains in C-terminal extensions (Grisaru *et al*., 1999). Nevertheless, as shown, there are some striking biochemical similarities between the neurotactins, gliotactins and AChE, and these are specified by their shared carboxyl/cholinesterase domains.

Drosophila melanogaster and *A. gambiae* each have a single neurotactin. In *D. melanogaster* it is only reported as a transmembrane glycoprotein in neuronal and epithelial tissue of embryos and larvae (Fremion *et al*., 2000). It does not have a consensus N-terminal secretion signal, presumably relying on a nonconventional signal or another mechanism, such as translocation following membrane insertion (see Section 5.10.2.2). Its ligand is a secreted protein, Amalgam, which is a member of the immunoglobulin superfamily (Liebl *et al*., 2003). However, there is as yet no evidence for a role in immunity in *Drosophila*. Instead, the Amalgam–neurotactin complex, aggregated with other as yet only partly ascertained proteins, acts as a heterophilic cell adhesion system in axon guidance processes during development (Hortsch *et al*., 1990; Speicher *et al*., 1998). Notably, the adhesive properties of neurotactin in *in vitro* systems can be mirrored by chimeric neurotactin proteins in which its carboxyl/cholinesterase domain is replaced with homologous sequences from the AChEs of *D. melanogaster* or the eel *Torpedo marmorata*, or *D. melanogaster* glutactin (Darboux *et al*., 1996) (see Section 5.10.4.1). *Inter alia*, this bears out the evidence for the cell adhesive capabilities of the AChEs (see Section 5.10.3.1.2).

Significantly, both the Amalgam-binding and cell adhesive functions of *D. melanogaster* neurotactin have been mapped to a segment of about 140 residues corresponding to the N-terminal region of the carboxyl/cholinesterase domain of AChE (in fact most of panel 1 in **Figure 6**) (Darboux *et al*., 1996; Fremion *et al*., 2000). Botti *et al*. (1998) have also shown strong conservation of surface charge around the lip of the gorge among AChE, neurotactin, and some of the other noncatalytics. All this suggests that N-terminal regions in the noncatalytics corresponding to some of the peripheral substrate binding machinery and parts of the gorge in AChE are responsible for their ligand binding functions.

There is no known vertebrate ortholog of insect neurotactin (although unfortunately there is an unrelated vertebrate peptide which is also called neurotactin) (Jung *et al*., 2000). There is another inactive carboxyl/cholinesterase in vertebrates, thyroglobulin, which is quite similar to insect neurotactin in its carboxyl/cholinesterase domain and which is also like insect neurotactin, and unlike the other noncatalytics has a membrane-spanning domain as an N-terminal extension (Grisaru *et al*., 1999; Scholl and Scheifele, 2003). However, thyroglobulin apparently functions in thyroid hormone delivery which clearly has no close parallel with insect neurotactin.

Although there are no functional data on the putative insect neuroligins, as a group they show clear sequence orthology with the well-studied vertebrate neuroligins and they have therefore been tentatively identified as such (Ranson *et al*., 2002). There are four neuroligin genes in the vertebrates studied to date, and these all express an array

of alternately spliced transcripts, generating further diversity at the protein level (Bolliger *et al*., 2001). Even greater diversity is seen in their ligands, the neurexins, which exist in hundreds of forms (Tabuchi and Sudhof, 2002). Neurexin diversity is again generated from about three genes, with alternate promoters and downstream splice variants generating the diversity. The well-studied neuroligin-1 group are postsynaptic membrane proteins that trigger synapse formation around axon contacts (Song *et al*., 1999; Clarris *et al*., 2002). Their extracellular carboxyl/cholinesterase domains attract and aggregate a subset of neurexins and certain other proteins, and their cytoplasmic domains then respond by assembling part of the exocytotic apparatus for the synapse (Dean *et al*., 2003). It is assumed that the diversity of neuroligins and neurexins gives specificity in the formation and organization of the synapses (Ichtchenko *et al*., 1996). There is some evidence for direct functional interactions between certain neuroligin-1 proteins and AChE in promoting neurite development (Grifman *et al*., 1998; Grisaru *et al*., 1999). At least in part this involves a level of functional overlap but how this is mediated in terms of ligands and specific sequence/structural motifs is unknown.

The insect neuroligin clade currently contains four pairs of *D. melanogaster* and *A. gambiae* orthologs, plus one additional *A. gambiae* sequence. EST and bioinformatic analyses also suggest at least some use of alternate splicing to generate additional diversity of gene products (Claudianos, unpublished data). Interestingly *D. melanogaster* only has one neurexin gene although, again, it apparently encodes at least a small number of different neurexin protein isoforms (Tabuchi and Sudhof, 2002; Schulte *et al*., 2003). Nevertheless it seems unlikely that the insect neuroligin/neurexin system involves the same scale of protein diversity as seen in their vertebrate orthologs, so there has probably been significant divergence in their function(s).

There are just a single *D. melanogaster* sequence and its *A. gambiae* ortholog in the insect gliotactin clade at this point. The *D. melanogaster* gliotactin is a transiently expressed transmembrane protein in the peripheral nervous system of the developing embryo, where it is an essential component of a paracellular structure called the septate junction (Genova and Fehon, 2003). The *A. gambiae* sequence has a conventional N-terminal secretion signal but the *D. melanogaster* sequence does not; like neurotactin above, it may use a nonconsensus secretion signal or a membrane insertion–translocation mechanism for extracellular presentation. Gliotactin acts in part as a transmembrane anchor for the septate junction structure, which also contains a small number of other proteins, notably including neurexin. There is no direct evidence that the neurexin acts as a ligand for the gliotactin although it might not be surprising, given the vertebrate data above indicating that neurexins are ligands for the related neuroligin receptors. There is evidence that the insect gliotactin and the septate junction function in part in axon guidance during development (Schulte *et al*., 2003). However, the major role of the complex appears to be maintenance of the transepithelial nerve–hemolymph permeability barrier. In mutant embryos with defective gliotactin the integrity of this barrier is broken down, the peripheral motor axons are exposed to high concentrations of potassium ions from the hemolymph, action potentials therefore do not propagate, and substantive paralysis results (Auld *et al*., 1995).

Consistent with the colocalization with neurexin in the septate junction, there are suggestions in the literature that *D. melanogaster* gliotactin is equivalent to the neuroligin-3 subset of vertebrate neuroligins, which are also found in ensheathing glia (Gilbert *et al*., 2001). However, this correspondence does not reflect genuine orthology at a sequence level (Claudianos, unpublished data) suggesting either a functional shift or functional overlap between the two receptor radiations.

One of the two functionally anonymous noncatalytic clades (K in **Figure 2**), comprising just two pairs of *D. melanogaster* and *A. gambiae* orthologs, is phylogenetically closely related to the neuroligins and conceivably could represent additional members of this neurexin binding functional group. Like the neuroligins, their sequences show C-terminal extensions beyond their carboxyl/cholinesterase domains with clear consensus transmembrane domains and downstream, presumptively cytoplasmic, domains. The other functionally anonymous noncatalytic clade (I in **Figure 2**) includes a single orthologous sequence from each of *D. melanogaster*, *A. gambiae*, and *A. aegypti*. It is less closely related to the neuroligins, or AChEs. Although it does have a C-terminal extension it does not contain any good match with consensus membrane-spanning motifs. Functional analysis of both these clades using all the tools of modern *Drosophila* genetics would seem to be highly worthwhile from here.

5.10.4. Secreted Catalytic Clades

There are five major clades of secreted, generally catalytically active carboxyl/cholinesterases (**Figure 2**). One contains the CNS adhesive protein glutactin and its relatives, most apparently catalytically active

(clade H) (see Section 5.10.4.1). Two others contain JHEs and similar enzymes albeit, interestingly, these two clades are not simply sister groups (clades F, G) (see Section 5.10.4.2). The fourth contains the well-studied dipteran β-esterases and their hemipteran relatives implicated in insecticide resistance (clade E) (see Section 5.10.4.3). Little is known about the fifth clade but it does include one enzyme putatively identified as a pheromone esterase (clade D) (see Section 5.10.4.4).

5.10.4.1. Glutactin and Related Proteins

This clade currently only contains *D. melanogaster* and *A. gambiae* sequences, four for the former and nine for the latter, which are arranged in two chromosomal clusters in each species. The only member of the clade for which there is currently functional information is *D. melanogaster* glutactin, which is an outgroup to all the others. This protein is catalytically inactive, as are two of the other *D. melanogaster* sequences. Conversely, the duplicate of *D. melanogaster* glutactin (82 in **Figure 2**) and all the *A. gambiae* sequences have consensus catalytic triads, and so are potentially catalytically competent. Glutactin is also one of only two proteins in this clade known to have additional carboxy terminal sequence specifying transmembrane and cytoplasmic domains, the other being another of the catalytically incompetent *D. melanogaster* proteins (76).

Little is yet known about the function of *D. melanogaster* glutactin (Olson *et al.*, 1990; Parker *et al.*, 1995; Darboux *et al.*, 1996; Grisaru *et al.*, 1999) and, as noted above, there are no known vertebrate orthologs to offer clues as to its function. The *D. melanogaster* protein is strongly anionic and heavily glycosylated. It is found in many basement membrane sites and is therefore inferred to be a component of the extracellular matrix. It is particularly abundant in the envelope of the CNS, so it fits the recurring theme of a nervous system cell surface adhesive protein seen above for AChE and the other noncatalytic members of the family. Nothing is known about possible ligands.

5.10.4.2. Juvenile Hormone Esterases

Juvenile hormone esterase (JHE) is one of the few insect esterases other than AChE to have a clearly defined physiological substrate. However, known insect JHEs fall into two distinct clades in the phylogeny in **Figure 2**. Known nonlepidopteran JHEs are found in a sister clade to the β-esterases (see Section 5.10.4.3), while known lepidopteran JHEs sit in a more distantly related radiation.

Juvenile hormones (JHs) are a family of sesquiterpenoid esters of methanol found throughout the Insecta (**Figure 7**). The ester moiety is stabilized by conjugation with a 2,3 double bond and it may be this feature of the substrate that requires a specific enzyme (Hammock, 1985). Hydrolysis of the conjugated methyl ester by JHE is traditionally regarded as inactivating the hormone, although mounting evidence suggests that the resultant JH acid also has a hormonal role in some circumstances, with or without re-esterification by a methyltransferase (Sparagana *et al.*, 1985; Ismail *et al.*, 1998, 2000). The most common form of JH is JHIII, which is found in all insect orders that have been investigated (Gilbert *et al.*, 2000). Other forms of JH have the same length of acid chain and generally also the 10(R),11 epoxide group. They differ principally in having various combinations of ethyl rather than methyl substituents on the acid chain (JH0, JHI, JHII), or by an additional 6,7 epoxide group (JHIII bisepoxide, JHB3). Some insects, notably the Lepidoptera and higher Diptera, may use mixtures of JHs, and the JH function may extend to other closely related compounds such as methyl farnesoate (JHIII without the epoxide) (Gilbert *et al.*, 2000).

JH has key roles in the regulation of insect development, metamorphosis, and reproduction (reviews: Gupta, 1990; Riddiford, 1996; Gilbert *et al.*, 2000; Truman and Riddiford, 2002). Certain developmental events require low JH titers and these often coincide with sharp peaks of JHE activity, suggesting that the role of JHE is to substantively clear the insect of JH (de Kort and Granger, 1981, 1996; Hammock, 1985; Roe and Venkatesh, 1990; Gilbert *et al.*, 2000). Some precisely timed experiments have shown peaks of JHE slightly after the JH titer had dropped, suggesting that the role of JHE could be to scavenge for traces of JH remaining after it has mostly

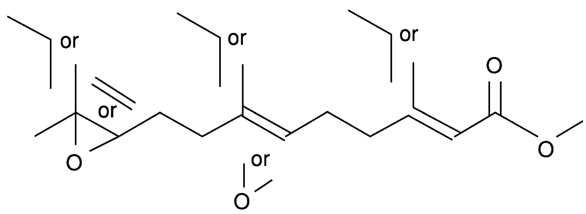


Figure 7 Juvenile hormone (JH) structures. The most common juvenile hormone (JHIII) is shown centrally. Other forms of JH have very similar structures. For example, JHIII bisepoxide has a second epoxide ring replacing the 6,7 double bond of JHIII while methylfarnesoate differs from JHIII by having a double bond in place of the 10,11 epoxide. These three forms of JH may act as a hormone blend in the higher Diptera (Yin *et al.*, 1995). Further forms of JH found particularly in Lepidoptera differ from JHIII by having ethyl side chains replacing one or more of the methyl side chains of JHIII (on C11 for JHII, on C11 and C7 for JHI, and on C11, C7, and C4 for JH0).

been removed by other means, for example, by JH epoxide hydrolases (Baker *et al.*, 1987).

Genetic variation in JHE activity can lead to differences in JH titer, which in turn can have profound effects on development. For example, such variation acts as a genetic switch between short-winged, sedentary and long-winged, migratory forms of some crickets (Zera and Huang, 1999; Zera and Harshman, 2001). Further evidence for a morphogenic role of JHE comes from the disruption of morphogenesis by application of more-or-less specific inhibitors of JHE or interfering RNAs that down-regulate JHE titers (Hajos *et al.*, 1999). The use of the interfering RNAs in final instars of the tobacco budworm *Heliothis virescens* led to aberrant morphogenesis, with the body organization remaining larvalike while the cuticle became pupalike.

The defining characteristics of a JH-specific esterase are a very high affinity for the hormone (typically a 20–200 nM K_m) combined with moderate turnover (k_{cat} of 0.6–4.3 s^{-1}), yielding specificity constants ($6–50 \times 10^6\ M^{-1}\ s^{-1}$) approaching the diffusion limits (Zera *et al.*, 2002; Kamita *et al.*, 2003). This should be sufficient to provide efficient hydrolysis of realistic *in vivo* concentrations of the hormone (Hammock, 1985). At the same time it makes an interesting contrast with AChE (see Section 5.10.3.1), which has an even higher specificity constant for its substrate but achieves this through a very high turnover and only modest K_m.

Most *Jhe* genes have been isolated by the classical reverse-genetics route in which protein purification and amino acid sequencing have then allowed the design of suitable polymerase chain reaction (PCR) primers or probes. Of particular importance has been a trifluoromethylketone affinity technique used for the purification of various leptidopteran JHEs and a coleopteran JHE (Hinton and Hammock, 2003). However, this method has generally proven difficult when applied to nonlepidopteran JHEs, due to either poor binding to the ligand or poor stability during extensive dialysis steps (Campbell *et al.*, 1998b; Zera *et al.*, 2002), although Campbell *et al.* (2001) achieved good recovery of *D. melanogaster* JHE activity when a carrier protein was added. JHE from the cricket *Gryllus assimilis* is the only example of purification from a hemimetabolous insect and its N-terminal sequence has been used to isolate part of the cognate gene (Zera *et al.*, 2002).

The identification of the *Jhe* gene of *D. melanogaster* became possible when the genome of that species was completely sequenced. This was done by matching a peptide mass fingerprint from the purified protein with a predicted gene sequence in the genome sequence (Campbell *et al.*, 2001). It was then possible to identify an orthologous gene from *A. gambiae* once the genome of that species became available (**Figure 2**) (Ranson *et al.*, 2002), although it remains to be demonstrated directly that the latter encodes a functional JHE.

The availability of complete sequences of insect genomes allows us to address an outstanding question from the classical studies on JHE. Do insects have more than one *Jhe*/JHE gene/enzyme system? Multiple JHE activities with different isoelectric points or other physical differences are sometimes found in one species (Khlebodarova *et al.*, 1996; Zera *et al.*, 2002 and references therein). It is generally not clear whether the different JHEs are the products of different genes, differently processed products of the same gene, or allelic variants. Also, it is possible for an esterase to show some JHE activity *in vitro* without evidence for an *in vivo* role in JH metabolism (Campbell *et al.*, 1998b). No study has clearly shown more than one *Jhe* gene in any particular species, although Hinton and Hammock (2003) had evidence for two products from one *Jhe* gene. Nevertheless it is plausible that an insect might use different *Jhe*/JHE systems in different locations (say in tissues and in circulation) or with specificity for different forms of JH. The latter is particularly plausible in the Diptera and Lepidoptera where endogenous forms of JH might act as mixtures of components, which differ in potency in assays, binding affinity to carriers, or relative abundance at different developmental stages (Yin *et al.*, 1995; Gilbert *et al.*, 2000).

The complete genome of *A. gambiae* shows a clade of five esterase sequences that are monophyletic with a pair of esterase sequences in *D. melanogaster*, one of which is the identified JHE (**Figure 2**). The closest relative of these seven dipteran sequences is the identified JHE of a coleopteran, the yellow mealworm *Tenebrio molitor* (Hinton and Hammock, 2003). Thus it seems probable that these eight sequences descend from an ancestral JHE. However, it is also quite possible that some of them no longer function in JH metabolism. Only the *A. gambiae* sequence most closely related to *D. melanogaster* JHE shows the GQSAG version of the GXSXG nucleophilic elbow motif associated with verified JHEs (see below). The second *D. melanogaster* sequence is clearly a duplication of the identified JHE and is adjacent on the chromosome but it also lacks the GQSAG motif. Perhaps one gene in each dipteran has retained the ancestral JHE function, while one duplicated gene in *D. melanogaster* and four in *A. gambiae* have evolved to other functions.

The lepidopteran JHEs form a distinct clade from the dipteran and coleopteran JHEs yet they share the

GQSAG motif. Falling between these two clades are the β-esterases (see Section 5.10.4.3). However, the β-esterase sequences lack the GQSAG motif, there is no physiological evidence suggesting that they might function as JHEs, and one, EST6, has been purified and shown to lack JHE activity *in vitro* (Myers *et al.*, 1993). This suggests that perhaps a single ancestral JHE whose origin predates the separation of several insect orders has subsequently given rise to a non-JHE radiation. Alternatively, the JHE function could have evolved twice in distinct esterase radiations subsequent to the separation of these orders. Interestingly, the lepidopteran JHE clade also includes four *A. gambiae* genes but no *D. melanogaster* genes. This again raises the possibility of multiple JHEs, although none of the four *A. gambiae* genes contains the GQSAG motif that is present in all the lepidopteran JHEs. It also suggests a loss of these genes in the higher Diptera.

Further evidence suggesting multiple JHEs or JHEs diverging to other roles is an unusual protein decribed as "JHE-related" from the cabbage looper *Trichoplusia ni* (Kadono-Okuda *et al.*, 2000). Although this sequence (70 in **Figure 2**) falls within the lepidopteran JHE clade, *T. ni* has another JHE that more closely resembles other lepidopteran JHEs. The JHE-related enzyme hydrolyzes JHIII but its affinity is fourfold less than the more typical JHE from *T. ni* hemolymph. The enzyme shows much lower sensitivity to certain inhibitors and a different expression pattern from classical lepidopteran JHEs. Kadono-Okuda *et al.* (2000) suggest that its function might not be to hydrolyze JH but instead to break down some other, as yet unknown, JH-like compound.

Even more atypical are two putative JHE sequences isolated from *L. decemlineata* (Vermunt *et al.*, 1998). These show only about 17% identity with the JHEs described above and lack the GXSXG nucleophilic elbow motif around the catalytic serine that is characteristic of all known carboxylesterases. Alternatively, these sequences show 29% identity with a bacterial protein that shows oral toxicity against several mosquito species and one lepidopteran (Duchaud *et al.*, 2003). These results could imply an entirely unrelated group of JHE enzymes. Importantly, however, there is as yet no direct demonstration that these genes express JHE activity.

While presence of the above-mentioned GQSAG motif is strongly correlated with JHE sequences, it is not diagnostic for JHEs. The consensus nucleophilic elbow sequence around the catalytic serine residue of all carboxyl/cholinesterases is GXSXG, the first X being most often Glu and the second most often Ala. Gln in the first X position is rare overall but present in all the confidently identified JHEs (Campbell *et al.*, 2001; Hinton and Hammock, 2003). Thomas *et al.* (1999) modeled the structure of a lepidopteran JHE on the known tertiary structures of an AChE and a lipase, and proposed that Gln rather than Glu at this position indirectly shortens the hydrogen bond between the His and Glu residues of the catalytic triad. This might partially account for the very low K_m values of JHEs for their substrates. Aside from the known JHEs and the probable *A. gambiae* JHE, a Gln at this position in an active carboxyl/cholinesterase only occurs twice in the *A. gambiae* genome (14 and 15 in **Figure 2**) and once in the *D. melanogaster* genome (82). The above-mentioned *T. ni* JHE-related enzyme has GQSCG, with the Gln in common with the other JHEs but the Cys residue otherwise unique in the phylogeny.

A second structural feature associated with JHEs is a predicted amphipathic helix with basic residues along one face, first identified in the lepidopteran JHEs (Thomas *et al.*, 1999) and also predicted for *D. melanogaster* JHE (Lys184, Arg191, Arg195) (Campbell *et al.*, 2001). The basic residues form a positively charged patch on the opposite surface of the protein from the active site gorge. This patch might function in the recognition of JHE by specific cells that take up and then degrade the enzyme, thereby contributing to the regulation of its titer in hemolymph (Thomas *et al.*, 1999). This feature is strongly associated with both the lepidopteran and nonlepidopteran JHE clades but seldom seen in otherwise apparently catalytically competent enzymes elsewhere in the phylogeny. Outside the JHE clades there is a trend for substitutions (numbered as per DmJHE) of the second (Arg191) and third (Arg195) basic residues in the face of the amphipathic helix with Gln or the acidic Glu or Asp residues, while the first (Lys184) is generally conserved among clades A to D but not elsewhere.

Beyond the two sequence motifs mentioned above, JHEs must have specific residues that interact with JH molecules to confer their very low K_m values and high specificities. An homology-based model of the tertiary structure of the lepidopteran *H. virescens* JHE could only dock JH in the active site with its orientation reversed relative to acetylcholine in AChE, such that the small alcohol moiety of JH was accommodated by the region homologous to the smaller "acyl binding pocket" while the longer acyl group was accommodated by the larger "anionic site" (Thomas *et al.*, 1999). Activity series with artificial substrates and inhibitors are generally consistent with this reversed-orientation hypothesis (Kamita *et al.*, 2003). Studies with JH analogs show that almost any departure from the JH structure

reduces binding (Campbell *et al.*, 1998b). Consistent with that observation, the *H. virescens* JHE model has JH making contacts with eight further residues (given below in HvJHE numbering) in addition to the residues of the catalytic triad (Thomas *et al.*, 1999). Two (Gly123, Gly124) are the generally conserved oxyanion hole residues. A further two (Pro295 and Phe127) occur often in the alignment. One (Ile449) is unusual but consistent with the generally small residues found adjacent to the His448 of the catalytic triad. The remaining three putative contact residues (Val75, Tyr77, and Phe338) show good conservation among the lepidopteran JHEs but no further and in particular they are not conserved among the nonlepidopteran JHEs. Instead they fall in regions of difficult alignment and indels in the overall esterase alignment (**Figure 6**), as one might expect if these are the regions that confer specificity for diverse substrates.

Much of the above speculation on JHE structure/function may soon be resolved. Kamita *et al.* (2003) announced the crystallization of JHE from the tobacco hornworm *Manduca sexta* with a JH analog, 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP), and expect a high resolution structure to follow soon.

Jhe has attracted significant interest over several years as a potential transgene for insertion into recombinant insect baculoviruses (Hammock *et al.*, 1990) (see **Chapter 6.9**). The objective has been to make the viruses commercially viable prospects as biological insecticides. A range of possible anti-JH effects might follow from an excess of virus-expressed JHE, which could be more effective than the JH-like effects of the chemical "juvenoid" insecticides. In larvae JH is a feeding stimulant so it was hoped that excess JHE would cause larvae to cease feeding while leaving the animals alive to allow further replication of the virus. Alternatively, an anti-JH effect at the time of a larval moult might have caused a precocious and fatal pupal molt.

In the first experiments with baculoviruses expressing wild-type HvJHE, first instar lepidopteran larvae were killed but older larvae were found to rapidly degrade the excess JHE (Hammock *et al.*, 1993; Bonning and Hammock, 1996). It appeared that the levels of circulating JHE were tightly regulated by specific uptake and degradation in pericardial cells. More recent work has therefore attempted to increase the efficacy of this JHE with mutations intended to interfere with its targeting for degradation, thereby allowing a greater increase of JHE activity in the insect (van Meer *et al.*, 2000; Shanmugavelu *et al.*, 2000 and references therein). When Lys29 and Lys524 in this JHE (not those in the amphipathic helix above) were replaced with arginine residues there was no reduction in the clearance of JHE from the hemolymph. However, less JHE was found in the lysosomes of pericardial cells, indicating that intracellular processing of the JHE taken up by the pericardial cells had been effected as intended. There was also less binding to a putative JHE binding protein and this correlated with improved insecticidal efficiency and reduced feeding damage and 17% of larvae showed contractile paralysis.

Curiously, more success was obtained with another mutation, which was actually expected to serve as a negative control. This mutation inactivated the enzyme by substituting a glycine for the catalytic serine residue. Viruses expressing this inactive enzyme killed the larvae more rapidly than did viruses expressing wild-type JHE and they also reduced larval feeding significantly. Some larvae (27%) died at the larval molt with extreme blackening of the cuticle. The remainder showed similar symptoms to larvae infected with the wild-type virus except that some also showed contractile paralysis. These pathologies differ from those seen with the first set of mutants, suggesting different mechanisms of action, but the mutations were not synergistic when combined in the same molecule (van Meer *et al.*, 2000). Enzyme-inactivating mutations of the other two residues of the catalytic triad resulted in viruses that were no more effective than the wild-type. There remains no satisfactory explanation for these results; at face value they could imply a non-catalytic function for the JHE used in these experiments. At the least they serve as dramatic reminders that the functions of JHEs and JHE-like enzymes are still very incompletely understood.

5.10.4.3. β-Esterases

This intensively studied clade (E) is the sister group to the nonlepidopteran JHEs. It comprises two subclades, each with *D. melanogaster* and *A. gambiae* representatives. One contains the so-called β-esterase cluster of *Drosophila*, and the other contains the hemipteran esterases implicated in metabolic resistance to OPs and some other insecticides.

5.10.4.3.1. The *Drosophila* β-esterase cluster The β-esterase cluster was so named by Korochkin *et al.* (1987) because it includes the major esterase isozymes apparent in PAGE analysis of whole organism homogenates of most *Drosophila* species using β-naphthyl acetate as a substrate. As outlined below (see Section 5.10.5.2), those authors also named another, α-esterase cluster, on the basis that it included the major esterase isozymes evident after staining PAGE gels using the isomeric α-naphthyl acetate as

substrate. In fact there are esterases that are polymorphic for α- and β-naphthyl acetate preferring forms (Zouros and van Delden, 1982), so the substrate preference does not necessarily indicate substantial sequence divergence. Nevertheless the names for the two clusters are now widely used and, with the caveat that there is imperfect correspondence with the substrate preference, the usage is retained here.

The β-esterase cluster has been characterized at a molecular and/or biochemical genetic level in many *Drosophila* species, mainly located in four species groups, covering both the subgenera *Sophophora* and *Drosophila* (reviews: Oakeshott *et al.*, 1990, 1993, 1995, 1999). In most species studied it contains two to four members arranged in a tightly linked cluster, with one member being an abundant hemolymph enzyme, another located in late larval integument and, in some cases, one being heavily expressed in male reproductive tissue (**Figure 8**). However, the roles of the different β-esterases in carrying out these functions vary greatly both among and within the species groups.

In the *melanogaster* species group in the subgenus *Sophophora*, the cluster comprises just two very tightly linked, tandemly arranged genes (Oakeshott *et al.*, 1995). In the ancestral state for this species group, as seen in *D. yakuba* and *D. erecta*, the upstream gene, *esterase6* (*Est6*), encodes a homodimer expressed mainly in the hemolymph of most life stages, while the product of the downstream gene, *esterase7* (*Est7*), also a homodimer, is essentially only seen in late larval integument (Dumancic *et al.*, 1997; Oakeshott *et al.*, 2001) (although EST analyses also reveal a brief pulse of expression in early embryos) (http://flygenome.yale.edu/Lifecycle/; see also Arbeitman *et al.*, 2002). However in the derived state, as seen in *D. melanogaster*, *D. simulans*, and *D. mauritiana*, all in the *melanogaster* subgroup of species, EST6 has become a monomer and acquired a major pulse of expression in the anterior sperm ejaculatory duct of the adult male (albeit still retaining strong hemolymph activity) (Oakeshott *et al.*, 2001). Furthermore, *D. melanogaster* at least shows a high frequency of null alleles of the *Est7* gene and it is postulated that it is becoming a functionless pseudogene in this species (Balakirev *et al.*, 2003).

Although little is known about the functional role of the ancestral hemolymph activity of EST6, there is abundant evidence that the acquired ejaculatory duct expression of EST6 in the melanogaster subgroup is important in reproductive biology (Meikle *et al.*, 1990; Richmond *et al.*, 1990). In these species the enzyme is transferred from the male to the female in the semen during mating. Most of it is not then retained in her reproductive tissue but rapidly translocated to her hemolymph, where traces of it survive for several days. It has also been suggested that the evolutionary shift of the enzyme to monomeric structure in the *melanogaster* subgroup has been to facilitate transport of this enzyme across the vaginal wall (Oakeshott *et al.*, 1995). How the male-donated enzyme in the female hemolymph impacts on the role of the female-produced EST6

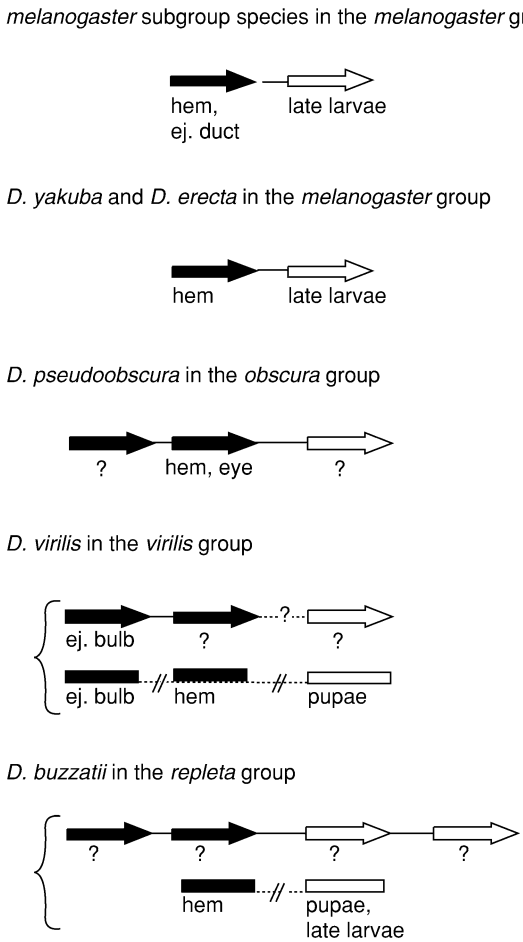


Figure 8 Organization of the β-esterase clusters in various *Drosophila* species as inferred from molecular and classical genetics (arrows and blocks respectively). *Est6* and its putative orthologs are shown in black, *Est7* and its putative orthologs are unshaded. For the molecular data, the arrows indicate directions of transcription. Major expression phenotypes are indicated below the arrows. Question marks indicate unknown linkage and orientation (between the arrows) or unknown expression (below the arrows). ····//··· indicates close linkage but precise relationship unknown. Hem, hemolymph; ej. duct, ejaculatory duct; ej. bulb, ejaculatory bulb. (Adapted from Oakeshott, J.G., Boyce, T.M., Russell, R.J., Healy, M.J., **1995**. Molecular insights into the evolution of an enzyme: esterase 6 in *Drosophila*. *Trends Ecol. Evol.* *10*, 103–110; and Oakeshott, J.G., Claudianos, C., Russell, R.J., Robin, G.C., **1999**. Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *BioEssays* *21*, 1031–1042.)

in her hemolymph is a mystery. However, elegant experiments reviewed in Richmond *et al.* (1990) comparing females mated to males bearing wild-type versus laboratory null alleles of *Est6* have shown clear consequences of the transfer of active enzyme for the female's subsequent reproductive behavior. In general terms there is a stimulation of egg laying behavior and an inhibition of receptivity to remating. There are separable short (<24 h) and long (5–7 days) term effects on receptivity. Interestingly some similar effects have also been described for several small peptides found in the semen but there is no evidence as to whether or how the EST6 and small peptide effects are related (Chapman *et al.*, 2003; Liu and Kubli, 2003). EST6 shows activity against a broad range of substrates *in vitro*, plus some limited peptidase/amidase activity, but its *in vivo* substrate(s) is (are) unknown (Richmond *et al.*, 1990).

The localization of EST7 activity to the integument of late larvae/prepupae suggests a fairly specific function during this major metamorphic transition. Alternatively, the high frequency of null alleles in *D. melanogaster* suggests that the function is dispensable, without significant effects on fitness in this species at least.

Detailed comparative genomic analyses have given us some strong clues as to the genetic basis of the acquired reproductive tract expression of EST6 in the *melanogaster* subgroup of species. It appears that the first step was the insertion of a complex macrotransposon into the distal portion of the ancestral *Est6* promoter (Oakeshott *et al.*, 2001; C.W. Coppin, B.C. Morrish, and J.G. Oakeshott, unpublished data). This insertion created a rudimentary promoter element directing ejaculatory duct expression. There was then a burst of nucleotide change in and around this element to hone its function, plus another pulse of change in the *Est6* coding region to adapt the protein to its new function. About 10% of the residues in the protein changed in a period of only 2–6 million years, and many of the changes were clustered in regions that would correspond to the peripheral substrate binding sites of AChE and an inferred ancestral dimerization site.

There is also evidence for functional effects of polymorphic variation in the *Est6* promoter and coding region within *D. melanogaster*. Most populations of the species segregate for two haplotypic forms of the promoter, the two forms differing at 13 sites spread over a ~400 bp region encompassing the introduced ejaculatory duct motifs (Odgers *et al.*, 1995). One of these two forms directs two- to three-fold more expression of the gene in the ejaculatory duct than the other (Odgers *et al.*, 2002). In the laboratory at least, such differences in the males are associated with quantitative differences in the egg laying profiles and remating behaviors of their mates (Saad *et al.*, 1994). There is no direct evidence as to whether these effects carry over to field populations but there is strong evidence from statistical tests of frequency distributions that these haplotype differences are not selectively equivalent in the field (Balakirev *et al.*, 2002; Odgers *et al.*, 2002). Most field populations of *D. melanogaster* are also polymorphic for two amino acid differences in the region of the EST6 protein corresponding to the peripheral substrate binding sites of AChE (Cooke and Oakeshott, 1989). There are kinetic differences between the corresponding allozymes for artificial substrates (White *et al.*, 1988), although the biological significance of these is unclear, either in the laboratory or in the field. Again, however, there is clear evidence that the allozymes are not selectively equivalent in the field. In this case there are large-scale latitudinal clines in their respective frequencies that recur across continents and take complementary forms in the northern and southern hemispheres (Anderson and Oakeshott, 1984).

The other lineage of the subgenus *Sophophora* in which the β-esterase cluster has been well characterized is the *obscura* species group, specifically *D. pseudoobscura* (Brady *et al.*, 1990; Brady and Richmond, 1990, 1992). In this species the cluster comprises three genes (**Figure 8**). The 3′ gene, termed *Est5C*, is orthologous to the 3′ gene (*Est7*) in the *melanogaster* cluster, while the two upstream genes, *Est5A* and *Est5B* (the latter sometimes just termed *Est5*), are duplicates of their common ancestor with *Est6* in the *melanogaster* cluster. The EST5 protein shows the high hemolymph activity seen for EST6 in the *melanogaster* group, albeit EST5 also shows high activity of unknown physiological significance in the eye. Nothing is known of the physiological roles of EST5A and EST5C, although their sequences show all the motifs expected for catalytic activity. Interestingly, some allozymes of EST5 differ in monomer/dimer status, suggesting that the ancestral dimeric status can be abolished by one or a very few amino acid differences (Arnason and Chambers, 1987).

Albeit less thoroughly characterized, it is clear that the β-esterase cluster in the subgenus *Drosophila* has diverged substantially from those outlined above for the two sophophoran species groups (**Figure 8**). Classical genetic analyses show a cluster of three genes encoding catalytically active β-esterases in *D. virilis*, in the *virilis* species group (Korochkin *et al.*, 1987, 1990). One of these, termed EST2, is

abundant in the hemolymph while another, ESTS, is abundant in the male reproductive tract. Unlike EST6, ESTS is produced in the ejaculatory bulb, not the duct, and, although transferred to the female, it is not transferred to hemolymph, but retained in the vagina. There are in fact reports of seminal fluid esterases that are retained in the vagina in various insects (Mikhailov and Torrado, 2000). Some of these have also been implicated in egg laying and remating behavior, with the mechanism suggested to involve the digestion of waxy matrices, which otherwise retain yet-to-be-used sperm in their sperm storage organs (Korochkin *et al*., 1976). This mechanism has been proposed to apply to *D. virilis*, in which mated females have a waxy plug at their entrance of their sperm storage organ whereas, notably, mated *D. melanogaster* females do not (Korochkin *et al*., 1976; Oakeshott *et al*., 1990; Healy *et al*., 1991).

Molecular work has also revealed a cluster of three β-esterase genes in *D. virilis* (Enikolopov *et al*., 1989; Korochkin *et al*., 1990, 1995; Sergeev *et al*., 1993, 1995) which map cytologically to the region where the classical genetics locates the three β-esterase isozyme loci. One of the cloned genes is heavily expressed in the ejaculatory bulb and was, therefore, originally nominated as the *EstS* gene. However, the sequence of the encoded protein shows a nonconservative substitution of the histidine in the catalytic triad which would almost certainly inactivate it. While it is tempting to put this down to a sequencing error, in fact (below) a parallel situation in the β-esterase cluster of *D. buzzatii*, in the *repleta* species group is seen. The alternative is to suggest that ESTS and perhaps also the other two β-esterase isozymes are encoded by as yet uncloned gene(s) at nearby chromosomal location(s). The corollary of this is that both an active and a catalytically inactive β-esterase are highly expressed in the ejaculatory bulb. Its abundant expression might suggest a function for the inactive protein, and as seen in the earlier sections there are many catalytically inactive carboxyl/cholinesterases, which have important ligand-binding functions. The *D. virilis* β-esterases clearly warrant further genetic and biochemical analyses.

Classical genetic analysis of *D. mojavensis* in the *repleta* species group shows a cluster of two β-esterase isozymes, one expressed in hemolymph and the other in late larvae (Zouros *et al*., 1982; Zouros and van Delden, 1982) and, although not mapped genetically, there is also evidence from several *repleta* species for a third β-esterase, which is highly expressed in male reproductive tissue (Kambysellis *et al*., 1968; Oakeshott *et al*., 1990). Molecular analysis of *D. buzzatii* in this species group has identified a cluster of three carboxyl/cholinesterase genes, which sequence similarities suggest should be its β-esterase cluster (East *et al*., 1990; Gomez and Hasson, 2003). However, the sequences of two of these show a glycine in place of the catalytic serine, which would clearly render them catalytically inactive. Given that *D. buzzatii* does express active hemolymph and late larval β-esterases (East *et al*., 1990), it appears that there may be two clusters of β-esterase or like genes in this species. And as with *D. virilis*, one of them appears to encode noncatalytic products.

The *A. gambiae* genome has a four-gene cluster which is clearly the sister group for the *Drosophila* β-esterase cluster (**Figure 2**). All four genes appear to encode catalytically active enzymes but there appears to be no information on their expression profiles. Several esterase isozymes are expressed in hemolymph and male reproductive tract in this species and other anophelines, but their relationship to the sequences in question is uncertain (Narang and Seawright, 1982; Vernick *et al*., 1988).

5.10.4.3.2. Amplified hemipteran β-esterases Also in part of the β-esterase clade (E) are a group of secreted esterases (which would actually be classified as α-esterases on the basis of their α/β-naphthyl acetate preferences) whose overexpression provides a major mechanism of resistance to OPs and carbamates in several hemipterans.

In *M. persicae* metabolic resistance to these insecticides is associated with a marked increase in total carboxylesterase activity (Needham and Sawicki, 1971). Four phenotypic classes have been identified; susceptible, moderately resistant (R1), highly resistant (R2), and extremely resistant (R3). Variously elevated levels of esterase activity are found in all three resistant classes, due to overexpression of either of two esterase isozymes, E4 and FE4 (Devonshire and Sawicki, 1979; Field *et al*., 1988, 1993). In some resistant lines the levels of the esterase protein reach 10 pM enzyme per aphid, or approximately 1% of the total soluble protein (Devonshire, 1989). The two enzymes differ slightly in molecular weight (E4 65 kDa, FE4 66 kDa) and they have slightly different catalytic activities. The genes encoding these enzymes are closely related (98% amino acid identity), genetically linked (19 kb apart), and likely to be recently derived by gene duplication (Field and Devonshire, 1998). Flanking sequences also differ between the two genes. The FE4 gene has a 1.7 kb deletion of promoter region compared with E4, resulting in a shorter 5′ untranslated region (94 bp). It is also likely that there are two further esterases

in the region, creating a cluster of at least four esterase genes even in susceptible *M. persicae* (Field and Devonshire, 1998).

Multiple mechanisms control the overexpression of the E4 and FE4 genes in resistant lines but gene amplification is the major one (Field *et al.*, 1999) (**Table 2**). Either gene can be amplified up to 80 times within a highly resistant R3 strain. Differences in resistance and esterase activity among the R1, R2, and R3 phenotypes largely correlate with the number of copies of the esterase genes. Normally only one of E4 or FE4 is amplified within a single line. The unit of amplification is large, approximately 24 kb for E4 and 20 kb for FE4, with each amplicon containing just one copy of the respective esterase gene and no other open reading frames. Amplicons are generally arranged in a head-to-tail configuration. In many resistant lines the E4 amplifications are also associated with a particular chromosomal translocation, with the amplicons generally all located at a single site on one of the translocation chromosomes. In one resistant clone, however, sites of E4 amplification have been found on three separate chromosomes. The FE4 gene is not associated with any translocations but the amplicons are distributed across the genome at from three to eight sites (Blackman *et al.*, 1995, 1999).

Restriction fragment length polymorphism (RFLP) patterns and 5′ sequence for E4 and FE4 genes from several resistant clones suggest that there was a single original amplification event for each of E4 and FE4 (Field *et al.*, 1994). Several subsequent mutations then generated the three major classes of resistance phenotype, the variation in the chromosomal sites for some of the E4 amplicons, and also some minor variation in gene copy number within the major phenotypic classes (Blackman *et al.*, 1995).

The levels of expression of E4 are also controlled by DNA methylation, with decreased methylation in some lines carrying amplifications resulting in reduced gene expression and reversion to a more susceptible phenotype (ffrench-Constant

Table 2 Esterase amplicons associated with insecticide resistance in *Myzus persicae* and *Culex pipiens*

Amplicon	*Genetics and biochemistry*	*References*
Myzus persicae		
E4	Secreted esterase with high affinity for OPs and carbamates, very slow turnover for OPs, and effectively none for carbamates. Up to 80-fold amplifications associated with a translocation (can be several sites) confers up to 500-fold resistance to OPs and 15-fold to carbamates. Quantitative variation in resistance correlates with amplification level. Reversion to susceptibility due to decreased flanking sequence methylation	Sawicki *et al.* (1978), Devonshire and Sawicki (1979), Devonshire and Moores (1982), Devonshire *et al.* (1986), ffrench-Constant and Devonshire (1988), Field *et al.* (1988, 1989, 1997), Blackman *et al.* (1995), Field and Devonshire (1998), Field (2000), Field and Blackman (2003)
FE4	As for E4 except for: slightly high turnover of OPs, amplification not associated with translocation, and no reversion due to altered methylation	As for E4
Culex pipiens complex		
Estα1	Low frequency in France, ≤70-fold amplification	Mouchès *et al.* (1987), Raymond *et al.* (1989), Chevillon *et al.* (1997), Lenormand *et al.* (1998b)
Estβ1²	Widespread in the Americas and China, ≤250-fold amplification, at least three sequence variants	Georghiou *et al.* (1980), Beyssat-Arnaouty *et al.* (1989), Mouchès *et al.* (1990), Cuany *et al.* (1993), Qiao and Raymond (1995), Vaughan *et al.* (1995)
Estβ6	Rare variant	Xu *et al.* (1994)
Estβ7	Rare variant	Xu *et al.* (1994)
Estβ8	Rare variant	Vaughan *et al.* (1995)
Estα2¹Estβ2¹	Common worldwide, partially replaced *Estβ1* in North America, but now declining in frequency in some European populations, ≤60-fold amplification	Raymond *et al.* (1987, 1991), Wirth *et al.* (1990), Rivet *et al.* (1993), Karunaratne *et al.* (1995), Qiao and Raymond, (1995), Guillemaud *et al.* (1996, 1997) Chevillon *et al.* (1997), Lenormand *et al.* (1998b), Hemingway *et al.* (2000), Weill *et al.* (2000), Pasteur *et al.* (2001)
Estα3Estβ1	South/Central American amplicon, two *Estβ1* sequence variants	De Silva *et al.* (1997)
Estα4Estβ4	Becoming more common in France, ≤50-fold amplification	Poirié *et al.* (1992), Chevillon *et al.* (1997), Lenormand *et al.* (1998b)
Estα5Estβ5	Becoming more common in Cyprus, ≤250-fold amplification	Poirié *et al.* (1992), Wirth and Georghiou (1996)

and Devonshire, 1988; Field *et al.*, 1989; Hick *et al.*, 1996; Field, 2000). No examples of such reversion have been described for the FE4 amplification suggesting that the phenomenon may be locus-specific. Methylation of the E4 gene in revertants is confined to CpG doublets within the coding region and is absent from CpG-rich regions in the 5′ and 3′ flanking DNA (Field, 2000). DNA methylation within a highly expressed gene is itself unusual in eukaryotes, methylation typically being associated with pretranscriptional gene silencing.

While the E4 and FE4 esterases are highly sensitive to inhibition by the insecticides they show poor hydrolytic activity against them. Thus the bimolecular rate constant (k_i) for the phosphorylation of E4 by paraoxon is about 133 μM^{-1} min^{-1} (Devonshire, 1977). However, its maximum rate of hydrolysis (k_3) for dimethyl OPs is about 3 h^{-1}, for diethyl OPs it is less than 1 h^{-1}, and it is even slower for carbamates (Devonshire and Moores, 1982). FE4 hydrolyzes both dimethyl and diethyl OPs approximately 1.5 times faster than E4 (Devonshire, 1989). Because their kinetics are so poor, the enzymes are thought to protect the organism from OPs and carbamates by first sequestering the insecticide and then, at least for dimethyl OPs, very slowly degrading it (Devonshire and Moores, 1989).

Very similar elevated esterase phenotypes are also associated with OP resistance in populations of a tobacco-feeding form (Abdel-Aal *et al.*, 1990, 1992; Wolff *et al.*, 1994) originally classified as a distinct species, *Myzus nicotianae* (Blackman, 1987). It is now clear that the elevated E4/FE4 esterases and encoding amplicons in this form are identical to those of *M. persicae*, and that the amplicons were acquired by introgression through sexual hybridization rather than by independent evolution (Field *et al.*, 1994). Given this, and studies of RFLP and randomly amplified polymorphic DNA (RAPD) patterns, it is now considered that *M. persicae* and *M. nicotianae* are conspecific (Clements *et al.*, 2000).

A similar case of amplification-based resistance has also been described in the brown rice planthopper *Nilaparvata lugens*. In this species two esterase isozymes stain more intensely in OP and carbamate resistant strains (Chen and Sun, 1994; Karunaratne *et al.*, 1999; Small and Hemingway, 2000a). The two isozymes are differently glycosylated forms of the same protein (Small and Hemingway, 2000a). The encoding gene is amplified three to seven times in strains resistant to OPs and carbamates, and there is an eight- to tenfold increase in measured esterase activity (Small and Hemingway, 2000b; Vontas *et al.*, 2000). Detailed kinetic analyses have not been reported for this system, but it is assumed that sequestration is a major part of the resistance mechanism.

The situation is more complicated in the greenbug *Schizaphis graminum*, where there are two types of esterase-based metabolic resistance to OPs, each associated with elevated staining intensities of esterase isozymes (Siegfried and Ono, 1993a, 1993b; Shufran *et al.*, 1996; Siegfried *et al.*, 1997; Ono *et al.*, 1999). One well-characterized type involves overexpression of a single esterase, whose gene shares intron positions and shows strong sequence similarity to E4/FE4 from *M. persicae* (about 73% identity at the amino acid level across the partial sequence available). The encoded enzyme cross-reacts strongly with antibody raised against *M. persicae* E4. Four- to eightfold amplification of this esterase gene confers broad spectrum OP resistance, while reversion to susceptibility occurs despite retention of the amplified genes because of changes in methylation. As in *M. persicae*, the amplified genes in resistant strains are methylated at CCGG sites, but revertants do not show methylation in upstream sequences. And also as with *M. persicae*, the mechanism of resistance seems primarily to involve sequestration rather than hydrolysis of the pesticide.

The second resistance mechanism in *S. graminum* is associated with elevated staining intensities of several esterase isozymes, although one, not the same as that in the first type above, clearly predominates. Patterns of antibody cross-reactivity suggest that none of the more intensely staining esterases in this so-called type 2 resistance are as closely related to *M. persicae* E4 as the overexpressed esterase in Type 1 resistance. The major type 2 esterase from *S. graminum* has been partially purified and shows slightly different electrophoretic mobility in type 2 strains, while Western blots using a type 2 esterase antibody suggest similar levels of expression across susceptible type 1 and type 2 strains (Siegfried and Zera, 1994; Siegfried *et al.*, 1997). These data suggest that Type 2 resistance in *S. graminum* is, unusually among the Hemiptera, not simply a result of overexpression and gene amplification. The extent to which it involves sequestration versus hydrolysis is therefore also an open question.

One feature of the hemipteran esterases like E4/FE4 and their relatives in clade E which may predispose them to a role in insecticide resistance is that they all appear to be secreted enzymes. It may be particularly useful for a sucking insect to secrete the enzyme into the gut, or perhaps preferentially into the salivary secretions, which are pumped into the plant as they feed. Salivary secretions are characteristically replete with digestive hydrolases, and this

may indeed be the ancestral function of the esterases recruited to a role in insecticide resistance.

Measurable fitness costs have been associated with the amplified esterases in at least some of the cases above (Dawson *et al.*, 1983; Foster *et al.*, 2003). These costs have been confounded in the case of *M. persicae* by the simultaneous presence in many clones of other resistance mechanisms with differing impacts on fitness (see Section 5.10.3.1.2). However the contributions from the various resistances are now being dissected, and the presence of the amplified esterase genes has been shown to slow the intrinsic rate of population increase (Foster *et al.*, 2003). With the possible exception of type 2 resistance in *S. graminum*, there are no known structural differences between the amplified esterases and their susceptible ancestors, so they would be expected still to perform the ancestral function. Nor does the metabolic cost associated with the production of such large amounts of an enzyme fully account for the fitness costs because, in *M. persicae* at least, revertants still appear to be less fit than susceptible aphids (Foster *et al.*, 1996).

5.10.4.4. Semiochemical Esterases

Clade D currently comprises one moth esterase and three *D. melanogaster* homologs. All appear catalytically active and three have good consensus secretion signals. While nothing is known of the functions of the *D. melanogaster* enzymes the moth esterase has been implicated in pheromone signaling. A clade A enzyme from the same species has also been tentatively implicated in this role but this section is used to review the topic of esterase involvement in pheromone and other semiochemically triggered signaling processes.

Sensitive and specific sensing of volatile chemical cues is crucial to the ability of insects to interpret their environment and signal to one another. Efficient degradation of the cues is an integral part of the signal reception and tranduction process and several specific odor degrading enzymes (ODEs) have now been identified in insects. Many of these ODEs are esterases because many of the semiochemical signals are esters (see **Chapter 3.15**). So far 231 esters, many of them C10–C16 acetates, have been identified as components of moth sex pheromone blends (http://www-pherolist.slu.se), while some of the 169 esters that have been identified in floral scents may be involved in the attraction of pollinators (Knudsen *et al.*, 1993). Little is known about esterases for other semiochemicals but there is quite detailed knowledge of some esterases involved in the reception of sex pheromones.

Sex pheromones are commonly found and mostly studied in Lepidoptera but they probably occur quite widely across insects (see **Chapters 3.14** and **3.15**). They are produced mainly by females to attract and court conspecific males. At least in the lepidopteran model systems studied, the males receive the signal in specialized sensillar hairs on their antennae. Inside these hollow sensillar hairs is an aqueous lymph where the external blend of volatiles is thought to be recreated for a subset of the molecules that can be bound by locally abundant pheromone binding proteins. The latter act to solubilize and transport the chemical signal to the dendrites of specific neurons that extend into the lymph. ODEs in the lymph compete to remove the signal, thereby maintaining the system's ability to respond to new stimuli. Receptors on the dendritic membrane, probably members of the G protein coupled receptor superfamily, specifically recognize the signal and trigger a signaling cascade that results in a neuronal impulse being sent to the antennal lobe and then the brain.

As well as the sensillar lymph, ODEs are also required at at least one and probably two other points in the process. One which is relatively well characterized involves cleaning pheromone off the external surface of the insect; most pheromone molecules are strongly hydrophobic and therefore prone to adhere to waxy surfaces. The males need to remove this material from their integument so that they do not become their own source of signal, while the females may need to remove it so that they can better control their signal (Vogt and Riddiford, 1981). The other point in the process where an esterase may be required is in the removal of bound pheromone off the receptor inside the olfactory neuron cells, if the signal remains bound to the receptor as it gets reset through vesicle cycling inside the cell (see **Chapter 3.15**).

The saturnid moth *Antheraea polyphemus* is one of the preferred models for studying the biochemistry of sex pheromone signal transduction, in large part because its large branched antennae and long sex pheromone-sensitive sensilla (sensillar tricoidea) are relatively amenable to study. Its sex pheromone is a 9 : 1 blend of the ester (*E*,*Z*)-6,11-hexadecadienyl acetate and the corresponding aldehyde (*E*,*Z*)-6, 11-hexadecadienal (Kochansky *et al.*, 1975). Four esterase isozymes extracted from *A. polyphemus* may have functions in the processing of the ester component of its pheromone blend: one from its scales, two from its antennal integument, and one from the lymph inside its sensillar tricoidea (Vogt and Riddiford, 1981, 1986; Klein, 1987).

An isozyme able to degrade radiolabeled (*E*,*Z*)-6, 11-hexadecadienyl acetate has been found in the body scales of both sexes (Vogt and Riddiford, 1986). The enzyme appears to be a carboxylesterase, as is it inhibited by typical OP inhibitors (Vogt and Riddiford, 1986). It requires solubilization by detergents suggesting some membrane association. It migrates very slowly on native PAGE and has a presumptively dimeric molecular mass of 100 kDa (Klein, 1987). Closely related species using different pheromones were found to have scale esterases that did not have activity against (*E*,*Z*)-6,11-hexadecadienyl acetate (Vogt and Riddiford, 1986).

Two soluble, OP-inhibitable esterase isozymes have also been associated with the antennal integument of both male and female *A. polyphemus* (Klein, 1987). One, of molecular mass 65 kDa, resolves as two forms under IEF (pI 5.85 and 6.0). The other, of 90 kDa, resolves as a single band under isoelectric focussing (IEF) (pI 5.0). The latter may be a homodimer as the molecular weight was estimated under native conditions. Neither of these is found in sensillar lymph but one, probably the 65 kDa enzyme, is also found in various other cuticular tissues, including wing, head and abdominal cuticle, and trachea, albeit not the brain, ventral nerve cord, fat body, or hemolymph (Vogt and Riddiford, 1981); hence the name integument esterase. As with the membrane-associated scale esterase above, this soluble integument esterase can hydrolyze the pheromone ester to the corresponding alcohol (Vogt and Riddiford, 1981).

An esterase isozyme has been identified in the sensillar lymph of male but not female *A. polyphemus*. This sensillar enzyme has a molecular mass typical of the subunit sizes of other carboxyl/cholinesterases (55 kDa) but an unusually low pI (3.0), and is not inhibited by several of the usual OP and other carboxyl/cholinesterase inhibitors (Vogt *et al.*, 1985; Klein, 1987). The pheromone analog 1,1,1-trifluoro-2-tetradecanone is a potent inhibitor (IC_{50} 5 nM) and kinetic analysis shows good affinity for the pheromone ($K_m = \sim 2 \times 10^{-6}$ M). Inhibition studies of the enzyme's ability to hydrolyze artificial substrates like naphthyl acetate with a range of pheromone-like compounds suggest that it has a high level of specificity for the native pheromone, including for the sites of unsaturation in its acyl chain backbone (Vogt *et al.*, 1985). Although it is perhaps four orders of magnitude less abundant than the pheromone binding protein, Vogt *et al.* (1985) have estimated that its V_{max} should be sufficient to clear half of the pheromone inside the sensillar lymph in 15 ms.

No esterases or other ODEs have yet been reported from inside pheromone-receptive neuronal cells but they may well occur there. It is known that many neurotransmitter-receiving G protein coupled receptors are internalized into the cell once activated by ligand binding (McClintock and Sammeta, 2003). The sensitivity of the neuron to further stimulation is then controlled by the balance between recycling to the cell surface and degradation of the receptor within the endosomes. If pheromone receptors are endocytosed in this fashion, then the ligand may well also become internalized and require hydrolysis within the neuron (see **Chapter 3.15**).

Ishida and Leal (2002) have used a degerate PCR approach to clone an esterase gene from *A. polyphemus* cDNA that they suggest encodes the 65 kDa integument esterase (clade D, sequence 41 in **Figure 2**). This assignment is based on the similar predicted size (61.7 kDa) and broadly similar pI (7.49) of protein 41 to the 65 kDa integument isozyme, the presence of a predicted secretion signal in protein 41, and the expression of the 41 gene in cuticular tissues, including male legs, as well as in male and female antennae. Esterase genes similar to 41 are also present in other moth antennal EST collections. One from female antennal cDNA of *M. sexta* is 70% identical to 41 over 120 amino acids (GenBank Accession no. BE015493), while another from male antennae of the light brown apple moth *Epiphyas postvittana* is 56% identical over 234 amino acids (R.D. Newcomb, unpublished data). Although *M. sexta* uses a sex pheromone composed of aldehydes, mainly (*E*,*E*,*Z*)-10,12,14-hexadecatrienal (Tumlinson *et al.*, 1989), *E. postvittana* uses a blend of two acetate esters (Bellas *et al.*, 1983).

Ishida and Leal (2002) also cloned another esterase gene that is expressed in the antennae of *A. polyphemus* using their degenerate PCR approach. This gene (19 in **Figure 2**) sits not in clade D as above, but in clade A. The authors suggest that it may encode the sensillar esterase, since it is expressed in male but not female antennae. It is also predicted to have a secretion signal (which would be necessary for it to be found in the sensillar lymph) under the software used by Ishida and Leal (2002), albeit not by the packages that are used (**Figure 2**). Other possible anomalies in respect of this assignment are the slightly larger size of the predicted protein (60 versus 55 kDa) and its substantially higher predicted pI (6.6 versus 3.0). The link between 19 and the sensillar esterase therefore remains to be confirmed.

It is nevertheless noteworthy that 19, like 41, shows significant similarity to sequences in three

antennal EST collections. In our phylogenetic analysis, 19 is most similar to an esterase from the cotton aphid *Aphis gossypii* (18) of no known function. However, esterase ESTs similar to 19 occur in male antennal cDNA libraries of *M. sexta* (GenBank Accession no. BF047018, 44% identical over 221 amino acids; GenBank Accession no. BF046933, 44% identical over 199 amino acids) and *E. postvittana* (42% identical over 321 amino acids) (R.D. Newcomb, unpublished data). Another esterase EST (GenBank Accession no. BP183087) very similar to 19 (70% identical over 148 amino acids) is also expressed in the pheromone gland of female silkmoth *Bombyx mori*. This moth uses an alcohol pheromone, bombycol ((*E*,*Z*)-10,12-hexadecadien-1-ol) (Butenandt *et al*., 1959). Perhaps an ester is made in the gland but is hydrolyzed to the alcohol for use as the sex pheromone.

The three *D. melanogaster* esterases (39, 40, and 42) in clade D have yet to be characterized functionally and no mutants are available. However, sites of expression can at least be identified from EST data. No ESTs have been detected for 39 at the time of writing but ESTs corresponding to 42 have been recovered in adult testis, and to 40 in adult head and salivary gland cDNA libraries. Microarray life cycle data are also available for 40 (http://flygenome.yale.edu/Lifecycle/) showing a peak of expression in prepupae and adults. It remains to be determined whether these esterase genes play any role in hormone hydrolysis in *Drosophila*.

We are unaware of any reports of esterases for plant semiochemicals among insects as yet. However, the large number of volatile plant esters which elicit specific responses in insects suggest that they probably exist. Three examples serve to illustrate the diversity of plant ester cues and insect responses to them. First, the noctuid moth *Autographa gamma* is attracted to a number of flowering plants that have in common the floral scent compound benzyl benzoate. Wind tunnel and electrophysiological experiments indicate that the response is specific to this ester (Plepys *et al*., 2002a, 2002b). Second, the volatile ester methyl salicylate is produced by many plants when they are attacked by herbivores (Shulaev *et al*., 1997) and females of many parasitoids use this compound as a cue to locate a host (Poecke *et al*., 2001). Finally, the ester ethyl (2*E*,4*Z*)-2,4-decadienoate produced by the Bartlett pear *Pyrus communis* is a powerful attractant for neonate larvae of the codling moth *Cydia pomonella* and is used by them to locate the fruit (Knight and Light, 2001). All these plant esters detected by insects will require rapid degradation by the insect to allow new incoming signal to be detected.

5.10.5. Intracellular Catalytic Clades

There are three major clades of mainly intracellular carboxyl/cholinesterases, all members so far identified apparently being catalytically active (clades A–C in **Figure 2**). Currently all but two of the sequences in these clades come from the Diptera. One clade is exclusively populated at the moment by an extensive radiation within the higher Diptera. Conversely, its sister clade, whilst not so extensively radiated, currently contains only lower dipteran sequences. These two clades, the lower and higher dipteran microsomal α-esterases (see Sections 5.10.5.1 and 5.10.5.2), are relatively intensively studied because of the involvement of certain members in metabolic resistance to various chemical insecticides. Less can be said as yet about the third major clade in this group, which appears to contain several cytosolic and a few secreted and mitochondrial enzymes. These enzymes are mainly from the lower Diptera, plus two from *D. melanogaster* and one each from a wasp, a moth, and an aphid. Interestingly the wasp esterase is also involved in insecticide resistance (see Section 5.10.5.3).

5.10.5.1. Lower Dipteran Microsomal α-Esterases

Both the anopheline and culicine mosquitoes studied to date contain a two-member cluster of genes encoding soluble/microsomal esterases. This two-member cluster in the mosquitoes constitutes clade C (**Figure 2**). In the culicines, where they are best studied, the two esterase genes are commonly designated *estα* and *estβ* because the corresponding enzymes, respectively, prefer α- and β-naphthyl acetate as substrates (Hemingway *et al*., 2000). However, they also appear as *est3* and *est2*, respectively, in some literature (see, e.g., Pasteur and Georghiou, 1981; Pasteur *et al*., 1981a). The *estα* and *estβ* genes share several intron positions and the amino acid sequences of the encoded Estα and Estβ proteins are about 50% identical (Vaughan *et al*., 1995, 1997a; Ranson *et al*., 2002). This compares with the 98% identity between the products of the duplicated aphid β-esterases implicated in OP and carbamate resistance (see Section 5.10.4.3.2), making duplication of the *estα* and *estβ* genes a much older event. Consistent with this, there is little immunological cross-reactivity between the two enzymes in *Culex pipiens* (Mouchès *et al*., 1987; Karunaratne *et al*., 1994).

The two genes are arranged head to head, with only about 600 bp between their coding regions in *A. gambiae* and about 1.7 kb between them in the wild-type (OP susceptible) *C. pipiens* (Vaughan *et al*., 1997a; Ranson *et al*., 2002). Although divergently

transcribed, in *C. pipiens* at least it appears that the two genes may share some promoter elements (Hawkes and Hemingway, 2002). Expression levels are not particularly high in susceptible *C. pipiens* and the two enzymes can be difficult to detect in some electrophoretic assays, despite their relatively high activities for the naphthyl ester substrates (Karunaratne *et al*., 1995). The *C. pipiens* enzymes separate mainly in the soluble fraction during purification but the presence of C-terminal KDEL motifs suggests at least a weak microsomal association (Fournier *et al*., 1987; Merryweather *et al*., 1990) (see Section 5.10.2.1). Estα and Estβ are highly polymorphic for isozyme variants in *C. pipiens*, with as much as 5% amino acid sequence differences reported among Estβ variants (Raymond *et al*., 1991; Hemingway and Karunaratne, 1998). Estα behaves as a homodimer while Estβ behaves as a monomer (Fournier *et al*., 1987). Monomer molecular weights of around 60 and 67 kDa for Estα and Estβ, respectively, are typical for carboxyl/cholinesterases (Fournier *et al*., 1987).

Mutations of the *estα* and *estβ* genes have been associated with broad spectrum OP resistance in *C. pipiens* populations from diverse localities around the world. These populations cover both *C. pipiens pipiens* and *C. pipiens quinquefasciatus*, and indeed many of the same alleles are found in both subspecies (e.g., Beyssat-Arnaouty *et al*., 1989; Hemingway and Karunaratne, 1998) (**Table 2**). In all cases characterized, resistance has been associated with overproduction of either or both of the Estα and Estβ proteins, and in almost all cases this in turn has been associated with amplification of either or both of the *estα* and *estβ* genes. One case of resistance apparently not due to amplification involves overproduction of the Estα1 variant by some other, as yet uncharacterized, mechanism (Rooker *et al*., 1996).

At least nine different *estα* and/or *estβ* amplicons have now been described in *C. pipiens*, at least one involving just *estα*, four just *estβ*, and four involving both (Pasteur *et al*., 1981b; Guillemaud *et al*., 1998; Hemingway and Karunaratne, 1998; Hemingway *et al*., 2000) (**Table 2**). These numbers are likely to be underestimates because several of the studies have only used isozyme mobilities to discriminate phenotypes and it is clear that these methods will miss many variants (Poirié *et al*., 1992; Vaughan *et al*., 1995; Karunaratne *et al*., 1995). While many populations are polymorphic for several amplicons, two are widespread, *estβ1²* and *estα2¹/estβ2¹*, with the latter particularly common and apparently replacing several amplicons, including the former, in many populations around the world over time (Raymond *et al*., 1987; Qiao and Raymond, 1995). Intriguingly now in France and Cyprus the *estα4/estβ4* and *estα5/estβ5* amplicons, respectively, are in turn superceding the *estα2¹/estβ2¹* amplicon (Poirié *et al*., 1992; Lenormand *et al*., 1998b). The amplifications characterized to date map to broadly the same region of the chromosome, but they are adjacent insertions rather than allelic substitutions of the wild-type genes (Nance *et al*., 1990; Wirth *et al*., 1990; Karunaratne *et al*., 1995; Hemingway *et al*., 2000).

Quantitative PCR and dot blot methods have also revealed substantial quantitative variation in amplicon copy number among strains with the same type of amplicon (Mouchès *et al*., 1985; Pasteur and Georghiou, 1989; Callaghan *et al*., 1998; Weill *et al*., 2000). This variation in turn underlies variation in levels of esterase activity and OP resistance (Villani *et al*., 1983; El-Khatib and Georghiou, 1985). Copy numbers as high as 250 have been reported, associated with activity increases of 500-fold and resistance factors of 800-fold (Mouchès *et al*., 1990; Poirié *et al*., 1992). Copy number is stable over many generations in homozygous laboratory lines in the absence of selection (Raymond *et al*., 1993). However, copy number has been readily increased in the laboratory by further OP selection (Wirth *et al*., 1990). It is suggested that unequal crossing-over generates at least some of the variation in copy number (Weill *et al*., 2000; Berticat *et al*., 2001). Differences in DNA methylation as seen in the amplified aphid esterases (see Section 5.10.4.3.2) have not been reported for *C. pipiens* *estα* or *estβ*.

The resistance mechanism mediated by the *M. persicae* E4/FE4 enzymes involves predominantly sequestration but with some role for very slow hydrolysis for dimethyl OPs (Devonshire, 1977; Devonshire and Moores, 1982) (see Section 5.10.4.3.2). However, the mechanism mediated by the amplified *C. pipiens* esterases appears to depend even more strongly on sequestration. Thus the overexpressed Estα2 and Estβ2 isozymes characterized to date are all highly reactive with OPs, but have negligible turnover rates for them (Ketterman *et al*., 1992, 1993; Karunaratne *et al*., 1993, 1995). For example, estimates of bimolecular rate constants for the phosphorylation of Estα2¹ or Estβ2¹ with various OPs range from 0.02 to 155 $\mu M^{-1} min^{-1}$ (18 and 17, respectively, for paraoxon, cf. 133 $\mu M^{-1} min^{-1}$ for *M. persicae* E4) (Devonshire, 1977) (see Section 5.10.4.3.2). The corresponding turnover rates (k_3) for paraoxon are only about 0.06 h^{-1} compared to 0.6 h^{-1} for *M. persicae* E4 (Devonshire, 1977). Coupled with their abundance – as much as 0.4% of the

total soluble protein of the organism (Karunaratne *et al.*, 1993) (cf. up to 1% for the *M. persicae* enzymes) (Devonshire, 1989) (see Section 5.10.4.3.2) – the overproduced enzymes can clearly provide the organism with an effective sink for insecticide.

There are several reports of significant differences in OP kinetics among the minority of the amplified Estα and Estβ isozymes that have been investigated in detail (Karunaratne *et al.*, 1993, 1995; Ketterman *et al.*, 1993; but see also Hemingway *et al.*, 2000). These include suggestions that the Estβ isozymes involved in amplification and resistance may have greater reactivity with OPs than Estβ isozymes that are not implicated in amplification and resistance. Further, thoroughgoing comparisons of the OP kinetics across amplified and nonamplified Estα and Estβ isozymes utilizing compounds used in the field might not only explain why particular isozymes are involved in resistance, but also elucidate the allele succession phenomena that are occurring among amplicons in the field.

Unlike the amplified aphid esterases, the amplified *C. pipiens* enzymes are only slightly reactive against carbamates biochemically (Cuany *et al.*, 1993; Karunaratne *et al.*, 1993) and do not confer carbamate resistance (Georghiou *et al.*, 1980; Priester *et al.*, 1981).

Two of the *C. pipiens* amplicons have been characterized intensively at a molecular level, one just involving the *estβ1*^{2} allele and the other involving both *estα2*^{1} and *estβ2*^{1}. In the first case, typified by the TEMR strain, the amplicon is some 30 kb in length, containing a highly conserved internal 25 kb core region (Mouchès *et al.*, 1990; Vaughan *et al.*, 1995; Rooker *et al.*, 1996). The amplicons are arranged in a tandem array adjacent to the wild-type locus on chromosome 2; they create a noticeably longer chromosome cytologically (Nance *et al.*, 1990; Wirth *et al.*, 1990). This *estβ1*^{2} amplicon was one of the first detected after the introduction of OPs, and has since declined in frequency in many localities where two-gene amplicons like *estα2*^{1}/*estβ2*^{1}, *estα4*/*estβ4*, and *estα5*/*estβ5* are proliferating (Raymond *et al.*, 1991; Poirié *et al.*, 1992; Rivet *et al.*, 1993; Lenormand *et al.*, 1998b).

The 25 kb *estα2*^{1}/*estβ2*^{1} amplicon as typified by the PelRR strain contains both esterase genes in a head-to-head configuration as per the wild-type unamplified genes (Villani *et al.*, 1983; Raymond *et al.*, 1987; Hemingway *et al.*, 1990). However, in PelRR the two genes are separated by 2.7 kb, whereas in susceptible strains like PelSS they are only separated by 1.7 kb, due to two insertions in the intergenic region in PelRR. There are also various single nucleotide substitutional differences between the intergenic regions of the two strains (Vaughan *et al.*, 1997a).

Although amplification of the two esterase genes in the PelRR strain results in a 1 : 1 ratio in gene number there is about threefold more Estβ2^{1} protein in PelRR flies than Estα2^{1} (Karunaratne, 1994, cited in Hawkes and Hemingway, 2002). Moreover, the two proteins differ in their tissue specificity (Pasteur *et al.*, 2001). Both are highly expressed in the alimentary canal, Malpighian tubules, and the oenocytes in the body cavity of larvae and adults. These are all tissues where a variety of detoxification functions are known to be carried out. However, Estβ2^{1} is also expressed in the CNS, where its proximity to the target site, AChE, may confer an additional level of protection. Presumably these differences can be accounted for by differences in promoter function between *estα2*^{1} and *estβ1*^{1}. They may be encoded by the intergenic insertions noted above. Gel shift assays have indeed indicated altered binding of at least one transcription factor in Pel RR (Hawkes and Hemingway, 2002).

Another explanation suggested for the success of the *estα2*^{1}/*estβ2*^{1} amplicon involves the presence of an additional gene on the amplicon. A functional aldehyde oxidase gene (*ao1*) is also amplified in this strain (Hemingway *et al.*, 2000), resulting in mosquitoes which also have elevated aldehyde oxidase activity. It is suggested that this might be advantageous for the detoxification of pesticides or other xenobiotics containing aromatic groups. The *estβ1*^{2} and *estα3*/*estβ1* amplicons were found not to contain intact functional copies of *ao1*.

Perhaps unsurprisingly, given the production of such large amounts of esterase protein, several of the amplification alleles are clearly associated with some fitness costs in the absence of insecticide. It appears that some resistant strains are not able to overwinter as well as susceptible strains (Gazave *et al.*, 2001), and that resistant males are not as successful in competing for mates as susceptible males (Berticat *et al.*, 2002). Amplicons with such fitness costs include *estα2*^{1}/*estβ1*^{1} but significantly, at least in terms of overwintering ability in French populations, apparently not *estα4*/*estβ4* (Chevillon *et al.*, 1997; Lenormand *et al.*, 1998b).

Intriguingly it seems that, along with insecticide resistance, the overexpressed *C. pipiens* esterases may also be associated with reduced levels of filarial parasites (McCarroll *et al.*, 2000). It will be of interest to see if any causal basis can be established for this correlation in the future. There were also earlier reports associating a particular esterase isozyme in *A. gambiae* with reduced transmission of the malarial parasite plasmodium (Collins *et al.*, 1986;

Vernick *et al*., 1988; Vernick and Collins, 1989) but more detailed investigations subsequently argued against any causal connection with the esterase (Crews-Oyen *et al*., 1993).

Although not yet fully characterized, amplifications of both *estα* and *estβ* genes have also been associated with broad spectrum OP resistance in *C. tritaeniorhynchus* and *C. tarsalis* (**Table 3**, below).

There are clearly fundamental similarities between the OP resistance mechanisms based on these amplified culicine α-esterases and the amplified hemipteran β-esterases reviewed above (see Section 5.10.4.3.2). In both cases high levels of resistance against a broad spectrum of OPs are principally achieved by a pesticide sequestration mechanism that uses massive gene amplification to dramatically overexpress a carboxylesterase isozyme with very high affinity for OPs. The versatility of this basic mechanism is borne out by some of the genetic and biochemical differences between the systems, for example, the relatively low levels of sequence similarity and the secreted versus intracellular locations of the enzymes. However in the following section we see how in the higher Diptera α-esterases with high affinity for OPs have also been recruited to a fundamentally different OP resistance mechanism. In this case a structural change in the enzyme converts it to an OP hydrolase, whilst qualitatively reducing its carboxylesterase activity in conventionally stained isozyme gels.

5.10.5.2. Higher Dipteran Microsomal α-Esterases

The *D. melanogaster* α-esterase radiation comprises 10 physically clustered genes plus one other located at a remote chromosomal location (Robin *et al*., 1996, 2000a, 2000b; Russell *et al*., 1996) (**Figure 9**). The 10 clustered α-esterase genes lie in a 60 kb segment that also contains four unrelated open reading frames and a closely related α-esterase pseudogene (*αE4a*, lying within an intron of *αE6*). All but one of the esterases in the cluster are oriented in the same direction. This α-esterase cluster is one of the largest clusters of sequence-related enzyme coding genes in the *D. melanogaster* genome (Adams *et al*., 2000).

At the nucleotide level, all members of the higher dipteran α-esterase radiation show a distinctive pattern of introns (Oakeshott *et al*., 1999). At the protein level, they show 37–66% amino acid identity, plus a distinctive pattern of small indels, a characteristic motif in a region corresponding to a peripheral binding site in AChE (plus some characteristic residues corresponding to the lining of the gorge in AChE) and the lack of an identifiable signal peptide, but almost all possess a carboxy terminal endoplasmic reticulum retention signal (Oakeshott *et al*., 1999) (**Figure 6**); hence their assignment as microsomal α-esterases.

All the esterase genes in the α-cluster are expressed to some degree. Diverse and noncomplementary roles are suggested by considerable differences between the genes in developmental patterns, overall expression levels, and tissue and sex specificities (Campbell *et al*., 2003). For example, three show varying degrees of male specificity, with one predominantly expressed in heads (*DmαE8*), another abundant in testes (*DmαE3*), and the third restricted to the germ line (*DmαE6*). Others are expressed broadly across feeding stages and this would be consistent with a role for at least some in the digestion of dietary or xenobiotic esters. A role in detoxification of xenobiotics is also implied by the role of several orthologs of one (*αE7*) in metabolic resistance to OPs (see below).

Figure 10 shows how some of the major events generating the *D. melanogaster* α-esterase radiation can be discerned by combining the evidence on the branch orders for their divergences in **Figure 2** with the information on their physical locations in **Figure 9**. Briefly, five major phases can be discerned. The first gave rise to four of the central genes in the extant cluster by a series of single gene duplications from the progenitor gene. The original progenitor then duplicated to generate one progenitor for the remainder of the genes on the left side of the cluster, and another progenitor for the remainder of those on its right side. Then successive single gene duplications of these latter two progenitors, respectively, generated the rest of the cluster, with one late-stage event also duplicating the left-terminal gene to generate the one remotely located member of the radiation. In general terms the radiation was thus generated from the inside outwards by a sequence of single gene duplications, mostly to adjacent locations and mostly in the same orientation.

This scenario can then be embellished by reference to available data on the composition of the α-esterase cluster in some other species, including fairly complete data for *D. pseudoobscura* and *D. buzzatii* and partial data for the *M. domestica* (**Figure 10**) (Oakeshott *et al*., 1999; Robin *et al*., 2000a; http://www.hgsc.bcm.tmc.edu). It is seen that most members of the cluster as now seen in *D. melanogaster* had arisen before the split of the drosophilids from the Muscidae (about 80 million years ago). However, it is also seen that the left end in particular remains a focus for ongoing change within the cluster, including both recurrent gene duplication and occasional gene loss. *Drosophila pseudoobscura*

Table 3 Summary of cases of esterases implicated in metabolic resistance to OPs (excluding malathion-specific resistance; see **Table 4**) and carbamates

Species	*Biochemistry*	*Reference*
Dictyoptera		
Blattella germanica (German cockroach)	Greater intensities of ≥3 OP- and carbamate-inhibitable esterase isozymes in OP and carbamate resistant strains	Prabhakaran and Kamble (1995), Scharf *et al.* (1997)
Thysanoptera		
Frankliniella occidentalis	Esterase activity in an OP resistant strain is lower and less sensitive to OP inhibition	Zhao *et al.* (1994)
Scirtothrips citri	Greater esterase activity in OP resistant strains	Ferrari *et al.* (1993)
Hemiptera		
Acyrthosiphon pisum (pea aphid)	Greater esterase activity in OP selected strains	Marullo *et al.* (1988)
Aphis gossypii	Greater esterase activity and ≥3 different or more intense esterase isozymes in OP and carbamate resistant strains	Furk *et al.* (1980), O'Brien *et al.* (1992)
Bemisia tabaci	Greater esterase activity in an OP-selected strain. However, the B-biotype $E_{0.14}$ esterase isozyme involved in SP resistance is not involved (see **Table 5**)	Bloch and Wool (1994), Byrne *et al.* (2000)
Brevicoryne brassicae (cabbage aphid)	Greater esterase activity in carbamate selected strains	Marullo *et al.* (1988)
Lygus hesperus	Greater OP-inhibitable esterase activity in strains whose OP resistance is also suppressed by carboxylesterase inhibitors	Zhu and Brindley (1990a, 1990b, 1992)
Myzus persicae	See **Table 2** and Section 5.10.4.3.2	
Myzus nicotianae[a] (tobacco aphid)	Greater esterase activity and more intense esterase isozymes in OP and carbamate resistant strains due to introgression of E4 and FE4 amplicons from *M. persicae* (see Section 5.10.4.3.2)	Abdel-Aal *et al.* (1990, 1992), Field *et al.* (1994), Wolff *et al.* (1994)
Nasonovia ribisnigri (currant-lettuce aphid)	Intense esterase isozyme in some carbamate resistant strains	Barber *et al.* (1999)
Nephotettix cincticeps	Greater esterase activity and ∼4 more intense esterase isozymes in OP resistant strains	Hasui and Ozaki (1984), Motoyama *et al.* (1984), Chiang and Sun (1996)
Nilaparvata lugens (brown rice planthopper)	Greater esterase activity and two more intense isozymes (generated by different glycosylation) due to a three- to sevenfold amplification of an esterase gene in OP and carbamate resistant strains (see Section 5.10.4.3.2)	Karunaratne *et al.* (1999), Small and Hemingway (2000a, 2000b), Vontas *et al.* (2000)
Phorodon humuli (damson-hop aphid)	Greater esterase activity and greater intensity of ≥4 esterase isozymes in OP and carbamate resistant strains	Lewis and Madge (1984), Wachendorff and Zoebelein (1988)
Schizaphis graminum (greenbug)	Increased esterase activity and (mainly) two more intense isozyme zones in strains whose OP resistance is partially suppressed by carboxylesterase inhibitors. Elevation in one zone (type I) is due to four- to eightfold amplification of an esterase gene with strong similarity to *M. persicae* E4/FE4. Reversion to susceptibility due to reduced methylation as with E4. Elevation in the other zone (type 2) apparently due to structural differences in an isozyme produced in similar amount in susceptible and resistant strains (see Section 5.10.4.3.2)	Siegfried and Ono (1993a, 1993b), Siegfried and Zera (1994), Shufran *et al.* (1996), Siegfried *et al.* (1997), Ono *et al.* (1999)
Coleoptera		
Diabrotica virgifera	Greater intensity of at least one major esterase isozyme zone and greater carbamate and OP hydrolytic activity *in vivo* in strains whose OP and carbamate resistance is suppressed by carboxylesterase inhibitors	Miota *et al.* (1998), Scharf *et al.* (1999a, 1999b), Zhou *et al.* (2002)
Leptinotarsa decemlineata	Novel OP-inhibitable esterase isozyme in an OP resistant strain	Anspaugh *et al.* (1995)
Oryzaephilus surinamensis (saw-toothed grain beetle)	Greater esterase activity in a chlorpyrifos-methyl (CPM) and fenitrothion (FT) resistant strain is due to greater amounts of an esterase isozyme zone with greater sensitivity to OP inhibition (but no OP hydrolytic activity). A strain with high CPM but low FT resistance produces an alternate isozyme zone still highly sensitive to CPM inhibition but less sensitive to FT inhibition	Collins *et al.* (1992), Conyers *et al.* (1998), Rossiter *et al.* (2001)

Continued

Table 3 Continued

Species	*Biochemistry*	*Reference*
Lepidoptera		
Cydia pomonella (codling moth)	Reduced esterase activities in some OP resistant strains	Bush *et al.* (1993)
Epiphyas postvittana (light brown apple moth)	Greater esterase activity due to greater intensities of many esterase isozymes in OP resistant strains	Armstrong and Suckling (1988)
Heliothis virescens	Novel metal-activated esterase isozyme insensitive to OP inhibition but with OP hydrolytic activity isolated from an OP (methyl paraoxon) resistant strain	Konno *et al.* (1990), Kasai *et al.* (1992)
	Greater esterase activity due to three more intense esterase isozymes in a carbamate selected strain whose resistance is partially suppressed by carboxylesterase inhibitors and which also shows elevated cross-resistance to OPs	Goh *et al.* (1995), Zhao *et al.* (1996)
	Greater esterase activity due to greater intensities of several esterase isozymes, one in place of an alternate esterase isozyme, in OP (profenofos) resistant strains	Harold and Ottea (1997, 2000)
Platynota idaeusalis (tufted apple bud moth)	Greater esterase activity due to greater intensities of one major and several minor esterase isozymes and greater OP hydrolytic activity *in vivo* in strains whose OP resistance is suppressed by carboxylesterase inhibitors	Karoly *et al.* (1996)
Diptera		
Aedes aegypti (yellow fever mosquito)	Greater OP-inhibitable esterase activity in strains whose OP resistance is suppressed by carboxylesterase inhibitors	Mazzarri and Georghiou (1995)
Anopheles albimanus	Greater esterase activity and a more intense zone of esterase isozymes in a strain whose OP resistance is suppressed by carboxylesterase inhibitors	Brogdon and Barber (1990)
Culex pipiens complex	See **Table 2** and Section 5.10.5.1	
Culex tarsalis	Greater esterase activity and more intense isozyme zone due to ~30-fold amplification of orthologs of *Estα*/*Estβ* in *C. pipiens* in an OP resistant strain (see Section 5.10.5.1)	Mouchès *et al.* (1987), Beyssat-Arnaouty *et al.* (1989), Raymond *et al.* (1989)
Culex tritaenorhynchus	Greater esterase activity and more intense isozyme due to amplification of an orthologs of *C. pipiens Estα*/*Estβ* in an OP resistant strain (see Section 5.10.5.1)	Karunaratne *et al.* (1998), Karunaratne and Hemingway (2000, 2001)
Haematobia irritans (buffalo fly)	Greater esterase activity and greater intensity of an esterase isozyme is associated with increased expression of the LcαE7 ortholog in some OP resistant strains (see Section 5.10.5.2)	Guerrero *et al.* (2000)
Lucilia cuprina (Australian sheep blowfly)	Reduced general esterase activity and E3 isozyme staining intensity and significant OP hydrolase activity associated with Asp137 (and Ser/Leu251) variants of LcαE7 in strains with OP resistance partially suppressible by carboxylesterase inhibitors (see Section 5.10.5.2)	Hughes and Devonshire (1982), Hughes and Raftos (1985), Russell *et al.* (1990), McKenzie *et al.* (1992), Newcomb *et al.* (1997, 2004), Campbell *et al.* (1998a, 1998c), Devonshire *et al.* (2003)
Lucilia sericata (green bottle fly)	Asp137 (and Ser/Leu251) variants of the ortholog of LcαE7 in populations known to segregate for general OP resistance (see Section 5.10.5.2)	R.D. Newcomb, C.G. Young, and J.R. Stevens, unpublished data
Musca domestica	Reduced general esterase activity and Ali isozyme staining intensity associated with Asp137 (and Ser/Leu251) variants of MdαE7 in OP resistant strains (see Section 5.10.5.2)	Oppenoorth and van Asperen (1960), Campbell *et al.* (1997), Claudianos *et al.* (1999, 2001), Scott and Zhang (2003)
Simulium damnosum and *Simulium sanctipauli* (black flies)	Greater esterase activity and greater *in vivo* OP hydrolytic activity in strains whose OP resistance is suppressed by carboxylesterase inhibitors	Hemingway *et al.* (1989)
Acarina		
Amblyseius pontentillae	Less intense esterase in an OP resistant strain; the isozyme is highly reactive with OPs but has insufficient OP hydrolytic activity to be a major contributor to resistance	Anber and Oppenoorth (1989), Oppenoorth (1990)
Boophilus microplus	Greater intensities of several esterase isozymes in OP resistant strains	Rosario-Cruz *et al.* (1997)
Tetranychus kanzawai	Greater esterase activity in strains whose OP resistance is suppressed by carboxylesterase inhibitors	Kuwahara *et al.* (1982)

[a]Note that *M. nicotianae* is now recognized as conspecific with *M. persicae* (see Section 5.10.4.3.2).

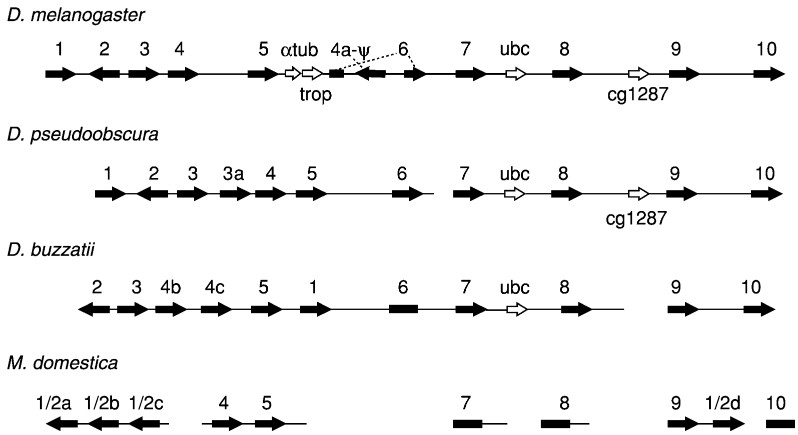


Figure 9 Organization of the α-esterase clusters as characterized to date from *D. melanogaster, D. pseudoobscura, D. buzzatii*, and *M. domestica* (Oakeshott *et al.*, 1999; Adams *et al.*, 2000; Robin *et al.*, 2000a; http://www.hgsc.bcm.tmc.edu). The whole of the cluster has been recovered from *D. melanogaster* and most of it at least from *D. pseudoobscura* and *D. buzzatii*, but it is only partly characterized in *M. domestica*. Nonesterase genes are unshaded (ubc, ubiquitin conjugating enzyme; trop, putative tropomyosin; αtub, α-tubulin; CG1287, functionally anonymous open reading frame). The contig in *D. melanogaster* is about 60 kb. Line breaks indicate uncharacterized gaps in *D. pseudoobscura, D. buzzatii*, and *M. domestica*. The orientations of some genes are unknown and accordingly shown as blocks rather than arrows. There is ambiguity as to the orthologs of the *Drosophila αE1* and *αE2* genes in *M. domestica* so the four candidates are shown as *αE1/2a–d*. Note that orthologs for six of the genes (*αE1/2*, *αE5*, *αE7*, *αE8*, *αE9*, *αE10*) have also been recovered from *Lucilia cuprina* but there is no information on their contig arrangement (Newcomb *et al.*, 1996).

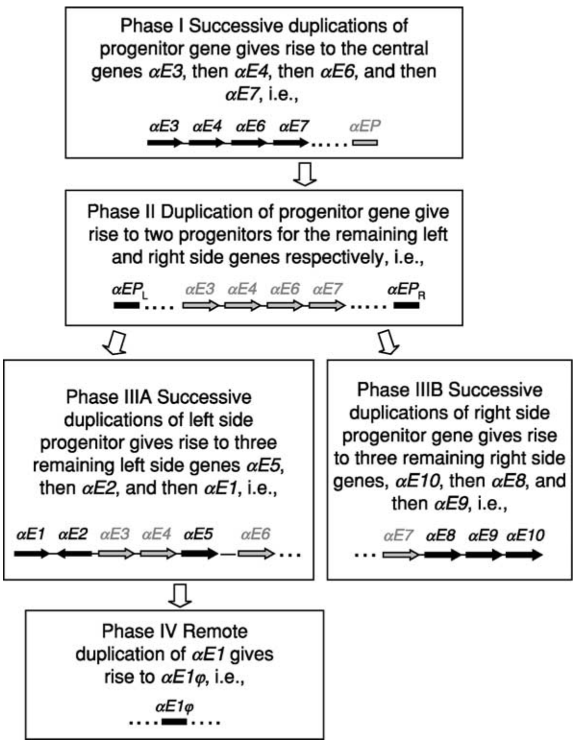


Figure 10 Schematic diagram showing a parsimonious reconstruction of the major phases in the evolution of the extant α-esterase cluster in *D. melanogaster*. Genes added in each phase are shown in bold, with pre-existing genes in gray. Genes of unknown orientation are shown as blocks rather than arrows. Distances are not to scale. *αEP* = progenitor α-esterase gene; αEP_L and αEP_R = progenitors for genes on the left and right side of the cluster, respectively.

lacks the *αE4a* pseudogene duplication and two of the nonesterase genes in the cluster, but has a tandem duplication of *αE3*. *Drosophila buzzatii* shows evidence of a different duplication of *αE4* and has its *αE1* in a different, central location within the cluster. Alternatively, *M. domestica* has two additional copies of *αE1/αE2*, including one at the other end of the cluster. It is not clear why the left end genes in particular should be a focus for change, but it may be relevant that they show relatively similar and broad expression profiles and lower levels of amino acid sequence conservation than the other genes.

Homologs of several of the α-esterases have also been identified in another higher dipteran, the calliphorid *L. cuprina* (Figure 9) (Oakeshott *et al.*, 1999) and, as with *M. domestica*, presumptive orthologs have been identified for several genes at the right end of the drosophilid cluster. The conservation of these orthologs across the Drosophilidae, Muscidae, and Calliphoridae clearly suggests the retention of important physiological functions, although as noted above there is as yet only indirect evidence as to the identities of these functions. The most highly conserved of the presumptive orthologs is αE7 (about 64% amino acid identities across the two lineages). This is also the one that has been implicated in metabolic resistance to OPs in a few muscid or calliphorid species involving what is often termed the mutant ali-esterase mechanism (Oppenoorth and van Asperen, 1960).

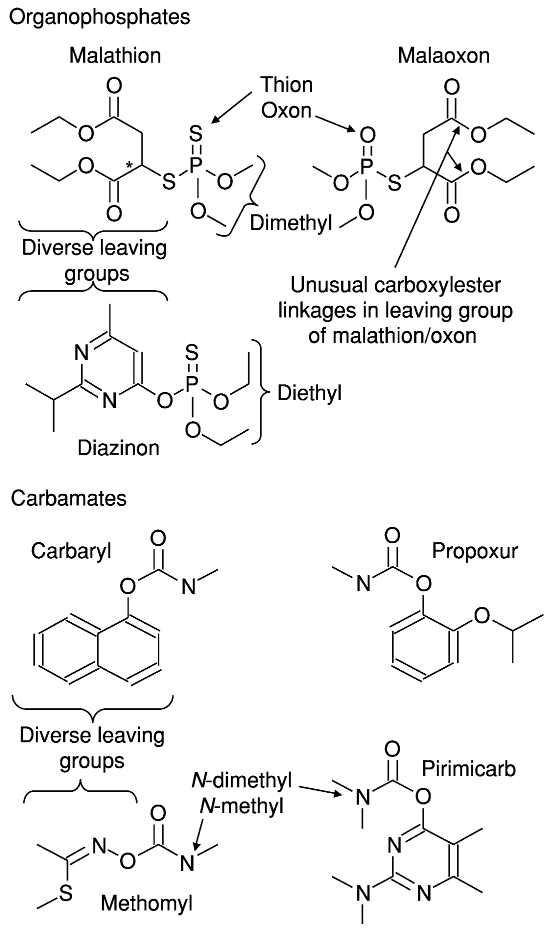


Figure 11 Structures of organophosphate (OP) and carbamate insecticides. OP insecticides all contain a central phosphorus atom. Most are produced in the thion form in which the phosphorus is linked by a double bond to a sulfur atom (phosphorothionates, exemplified here by diazinon, or phosphorothiolothionates when the leaving group is linked via another sulfur, exemplified here by malathion). Thion OPs are not the active forms of the insecticides but are converted *in vivo* by p450 oxidases to active oxon forms (phosphates, or phosphorothiolates when the leaving group is linked via a sulfur). Some OP insecticides (e.g., chlorfenvinphos) are produced in the oxon form and, therefore, do not require *in vivo* activation. The leaving groups are diverse electron-withdrawing groups that are displaced when oxon OPs react to phosphorylate the catalytic serine residue of a carboxyl/cholinesterase (**Figure 1**). They may be aromatic or aliphatic, may contain heteroatoms, and may be linked to the central phosphorus through an oxygen (most common), a sulfur or other atoms. The remaining two moieties attached to the central phosphorus are typically two methoxy groups (dimethyl) or two ethoxy groups (diethyl) although some other variations exist among commercial OPs. Malathion/oxon is unusual in that its leaving group contains two carboxylester linkages (and hydrolysis of either inactivates the OP). The optical center of malathion mentioned in the text is indicated with a asterisk. Carbamate insecticides resemble ordinary carboxylesters except that a nitrogen atom is adjacent to the carbonyl carbon of the carboxylester linkage. As with the OPs, the leaving groups are diverse, with varied sizes, aromatic or aliphatic groups, heteroatoms, etc. Where the leaving group is linked through a nitrogen atom rather than the more common carbon the carbamate is an oxime exemplified here by methomyl. The acyl group of most carbamate insecticides is *N*-methyl although there are variations, such as the *N*-dimethyl pirimicarb.

OP insecticides all contain a central phosphotriester moiety, which is crucial to their mode of action (**Figure 11**). It enables them to inhibit carboxyl/cholinesterases by acting as suicide substrates (see Section 5.10.3.1.2). Thus they rapidly phosphorylate the catalytic serine residue in an analogous manner to the first, acylation step in carboxylester hydrolysis. However, the second step (dephosphorylation for OPs rather than deacylation) proceeds at very slow to negligible rates, due to stereochemical differences between OP and carboxylester substrates. Carbamates (**Figure 11**) also inhibit by a similar mechanism. Commercial OP insecticides are mostly produced in an inactive "thion" form and must be activated to "oxons" by P450 enzymes in the insect before they inhibit as described above. Two of the three ester linkages around the central phosphorus in most commercial OPs involve methyl (dimethyl OPs) or ethyl groups (diethyl OPs), while the third is an electron-withdrawing "leaving group" (**Figure 11**). Hydrolysis of this third linkage detoxifies the insecticide. A few OP insecticides, most notably malathion, also contain carboxylester moieties within the leaving group, the hydrolysis of which also inactivates the insecticide. This leads to a special case of OP resistance, often termed malathion-specific resistance, which is discussed in detail below (see Section 5.10.6.2).

Three shared features of LcαE7 and MdαE7 have been suggested as predisposing them for a role in metabolic resistance to OPs (Campbell *et al.*, 1997). First, the wild-type enzymes are highly susceptible to inhibition by the activated, oxon forms of many OPs; indeed both interact more rapidly with many OPs than does the presumed target, AChE (Newcomb *et al.*, 1997; Chen *et al.*, 2001). For example, the k_i of LcαE7 with paraoxon is 6.3×10^7 $M^{-1}\,min^{-1}$ compared with 5.1×10^6 $M^{-1}\,min^{-1}$ for *L. cuprina* AChE (Newcomb *et al.*, 1997; Chen *et al.*, 2001). Second, the enzymes are expressed quite abundantly across the feeding life stages, larvae and adults, when the animals are likely to come in contact with insecticides (Parker *et al.*, 1991, 1996). Finally, they also show good kinetics (specificity constants $\sim 10^6\,M^{-1}\,s^{-1}$) towards the carboxylester groups of malathion (malathion carboxylesterase activity, MCE), leading to the involvement of certain alleles in malathion-specific resistance.

Resistance to a broad spectrum of OPs in both *M. domestica* and *L. cuprina* has been conferred by amino acid substitutions at either of two positions in the active site of αE7. In both species a Gly137Asp substitution confers one class of broad-spectrum resistance while Trp251Ser/Leu substitutions confer a different class of broad-spectrum resistance, coupled with malathion-specific resistance (Campbell *et al.*, 1998a, 1998c). None of the substitutions confers cross-resistance to carbamates. In both species it appears that each resistance substitution initially occurred at least twice in different allelic backgrounds although only one allele encoding each type of resistance is now relatively common in each species (Claudianos *et al.*, 1999, 2001; Smyth *et al.*, 2000; Scott and Zhang, 2003; Newcomb *et al.*, 2004).

Alleles showing the Gly137Asp substitution now dominate contemporary populations of both *L. cuprina* and *M. domestica* (Newcomb *et al.*, 1997, 2004; Claudianos *et al.*, 1999; Smyth *et al.*, 2000; Scott and Zhang, 2003). The resulting mutant enzyme has improved ability to hydrolyze OPs, with a preference for diethyl (55-fold increase in hydrolytic ability) over dimethyl OPs (33-fold increase) (Devonshire *et al.*, 2003). While these increases represent very low turnover numbers (about three turnovers per hour per molecule of enzyme), they are sufficient to account for modest levels of resistance to dimethyl OPs (4–12-fold) and about twofold greater resistance to diethyl OPs (7–20-fold resistance in adult *L. cuprina*) (Campbell *et al.*, 1998a, 1998c; Devonshire *et al.*, 2003). Interestingly, the turnover numbers of Asp137-αE7 with OPs are very comparable to those that the *M. persicae* E4/FE4 enzymes achieve for the particular case of malathion, and larger than those for the *M. persicae* enzymes with dimethyl OPs. In both cases Devonshire and Moores (1989) have argued that hydrolysis plays a significant supplementary role to sequestration in the resistance mechanism (see Section 5.10.4.3.2).

Asp137 is a nonconservative substitution of the Gly137 whose main chain nitrogen atom contributes to the oxyanion hole within the active site (**Figure 5**). The increased ability of the Asp137 enzyme to hydrolyze OPs may be due to the ability of this residue to activate a water molecule in a suitable orientation for nucleophilic attack and dephosphorylation of the phosphoryl-enzyme intermediate (Newcomb *et al.*, 1997), in an analogous manner to the second, deacylation step of carboxylester hydrolysis (**Figure 1**) (see Section 5.10.1.2). Thus the OP is turned over as a normal substrate rather than blocking the active site as a suicide substrate. A similar mechanism is proposed to explain the increased OP hydrolytic activity caused by a substitution to a histidine at a similar position in the oxyanion hole of human BuChE (Lockridge *et al.*, 1997). Forms of αE7 with Asp137 show drastically reduced carboxylesterase activities, possibly due to compromised functioning of the oxyanion hole for carboxylester hydrolysis (Newcomb *et al.*, 1997; Claudianos *et al.*, 1999). This then accounts for the association of OP resistance with apparent null phenotypes for the corresponding isozymes (E3 and Ali for *L. cuprina* and *M. domestica*, respectively) (Hughes and Raftos, 1985; Campbell *et al.*, 1997; Claudianos *et al.*, 1999) and reduced whole body homogenate activities for artificial substrates like naphthyl acetate or methyl butyrate (termed ali-esterase activity by Oppenoorth and van Asperen (1960)). It is this coincident gain of OP hydrolytic activity and loss of “ali-esterase” activity that defines the mutant ali-esterase mechanism.

Less abundant in *L. cuprina* and *M. domestica* populations are OP resistance alleles of α*E7* in which Trp251 is substituted by Leu or Ser (Claudianos *et al.*, 1999; Smyth *et al.*, 2000). Flies with these active site substitutions also show reduced ali-esterase activities as this mutation interferes with some, although not all of the susceptible enzyme’s carboxylesterase activities (Campbell *et al.*, 1998a). Like the Asp137 mutants, this enzyme has improved ability to hydrolyze OPs, except that in this case dimethyl OPs are preferred over diethyl OPs (34- and tenfold increases, respectively, over wild-type in OP turnover for the Leu251 enzyme from *L. cuprina*) (Devonshire *et al.*, 2003). This preference is reflected in two- to fivefold greater resistance to dimethyl OPs (5–27-fold resistance in adult *L. cuprina*) than their diethyl analogs (two to sixfold) (Campbell *et al.*, 1998c). Exceptional resistance levels are seen, however, for malathion (>130-fold) (see below). The effect of the Leu/Ser251 mutations is proposed to depend on replacement of a bulky aromatic group in the active site by smaller residues, which might provide less steric hindrance to the inversion about the tetrahedral phosphorus that must occur during hydrolysis of the serine–phosphorus bond (Campbell *et al.*, 1998a).

The exceptional resistance to malathion shown by *M. domestica* and *L. cuprina* with Leu or Ser251 variants of αE7 is due to the combination of MCE and OP hydrolase activities in these particular forms of the enzyme. Hydrolysis of the carboxylester groups of malathion accounts for the main products of detoxification of malathion in resistant flies, yet *in vitro* MCE activity seems poorly correlated with resistance (Smyth *et al.*, 2000). Flies with the Asp137 variant of αE7 show very low *in vitro* MCE activity (Asp137-αE7 loses nearly all carboxylesterase

activity) but do not differ in their susceptibility to malathion from flies showing general OP susceptibility and far more *in vitro* MCE activity (having Gly137/Trp251-αE7). Alternatively, flies with Leu/Ser251-αE7 show modest increases of *in vitro* MCE activity over OP susceptible flies yet show very high level resistance to malathion. Here one needs to remember that malathion is converted *in vivo* to malaoxon, a potent dimethyl OP esterase inhibitor. Campbell *et al.* (1998c) showed that the OP susceptible (Gly137/Trp251) form of αE7 rapidly loses its MCE activity in the presence of malaoxon, modeling the rapid loss of detoxification capacity that would occur in an OP susceptible fly exposed to malathion. However, the Leu251 form of the enzyme was able to sustain its MCE activity in the presence of malaoxon. Devonshire *et al.* (2003) and Heidari *et al.* (2004a) demonstrated that the Leu251 and Ser251 enzymes' MCE kinetics are only slightly improved over those of the OP susceptible form but their abilities to hydrolyze dimethyl OPs are greatly enhanced as described above. Thus the Leu251 and Ser251 enzymes are able to reactivate due to their OP hydrolase activity if their catalytic serines become phosphorylated by malaoxon and, therefore, they can continue to contribute their MCE activity to the task of detoxification.

Consistent with the specificity of the OP hydrolase activity, malathion resistant *M. domestica* and *L. cuprina* show little resistance to higher (nondimethyl OP) analogs of malathion, even though these molecules are identical around the carboxylester groups that are hydrolyzed by MCE (Campbell *et al.*, 1997, 1998c and references therein). Detoxification of malathion analogs by carboxylesterase activity only occurs for those analogs where the enzyme's minor OP hydrolase activity allows reactivation of the oxon OP-inhibited enzyme.

Although confirmatory bioassay data are not yet available for all the sequenced isolates, it appears that parallel mutations at the 137 and 251 sites of the orthologous αE7 enzyme may also confer the two types of resistance on another blowfly, *Lucilia sericata*. Thus this species is known to show the two types of resistance. Recent allelic sequencing work has revealed one (presumptive diethyl OP resistant) Asp137 haplotype from New Zealand and three unrelated (presumptive dimethyl OP/malathion-specific resistant) Ser251 or Leu251 haplotypes from Europe, North America, and Africa, in addition to seven unique (presumptive OP susceptible) Gly137/Trp251 *LsαE7* haplotypes (R.D. Newcomb, C.G. Young, and J.R. Stevens, unpublished data).

In light of the recurrence of αE7-based OP resistance, Heidari *et al.* (2004a) explored the catalytic consequences of various rational substitutions around the LcαE7 enzyme's active site. Substitutions at residue 137 generally reduced carboxylesterase activities, but none was as effective as the naturally occurring Asp for enhancing OP turnover. Substitutions of Trp251 with smaller residues generally produced enzymes with qualitatively similar properties to the naturally occurring Leu or Ser substitutions. This is consistent with the idea that the substitutions remove a steric constraint on OP hydrolysis. Notably, two of the synthetic mutants, bearing W251T and W251A, had quantitatively better dimethyl OP hydrolytic activity than the naturally occurring mutants. The fact that these mutants have not been found in the field may be because they would both require a double mutation in the wild-type (susceptible) codon. The synthetic W251G mutation was interesting for a different reason. Compared with all the other substitutions at this site, Gly251 led to lesser improvements in OP turnover but 20-fold better MCE kinetics. It is noteworthy then that a glycine substitution at the homologous position occurs in an overexpressed esterase from a parasitoid wasp, *Anisopteromalus calandrae*, which shows high malathion-specific resistance (Zhu *et al.*, 1999a, 1999b) (see Section 5.10.5.3). Substitutions of various other residues around the active site were mostly detrimental to OP hydrolysis and MCE activities, while the natural mutations combined in one molecule (Asp137 + Leu251) caused no improvement over either mutation alone.

As noted earlier, the function of αE7 prior to selection with OPs is unknown. However, it is noteworthy that αE7-mediated resistance in *L. cuprina* was initially associated with a fitness cost and measurable fluctuating (bilateral) asymmetry in the absence of the insecticide (McKenzie *et al.*, 1982; Davies *et al.*, 1996). Resistance due to αE7 in fact did not rise to high frequency until the 1970s when a *modifier* gene arose at another locus which redressed the cost and asymmetry problems (Davies *et al.*, 1996). The cost in OP resistant flies was presumably due to compromised activity towards the enzyme's native substrate and, consistent with this, the Asp137 enzyme shows more comprehensive loss of carboxylesterase activities and was associated with more severe asymmetry than the Leu/Ser251 enzymes. The biochemical function of the *modifier* gene product is unknown, although it is now known not to be the ortholog of the developmental gene *Notch* in *D. melanogaster* (P.C. Batterham, personal communication). Clearly identification of the *modifier* gene function would yield important insights into the function of αE7 beyond detoxification of OPs.

Another set of mutations has apparently also occurred in *L. cuprina* since the spread of the original α*E7* resistance alleles (Smyth *et al*., 2000; Newcomb *et al*., 2004). These are duplications of the chromosomal region containing α*E7* which result in two copies of this gene and others from the α-esterase cluster being carried on one chromosome. So far three independent duplications have been observed at low frequencies. Importantly, these duplications result in dissimilar resistance alleles on the same chromosome, Asp137 alleles linked with Leu251 alleles. As a result strains homozygous for these duplications show both high levels of malathion resistance and slightly higher resistance levels to diazinon (a diethyl OP) than do nonduplicate Asp137 strains (Campbell *et al*., 1998c).

An αE7 ortholog has also recently been implicated in OP resistance in the horn fly *Haemotobia irritans*, also in the Muscidae. In this case, however, resistance appears to have been achieved through overexpression of αE7 without amino acid changes (Guerrero, 2000). AChE is presumably protected because the OPs preferentially react with the very abundant HiαE7 in a fashion analogous to that of the overexpressed hemipteran esterases discussed earlier (see Section 5.10.4.3.2).

OP resistance has never been linked with αE7 in *D. melanogaster* and a possible reason is that the enzyme in this species tends to show lower rather than higher affinities for OPs when compared with its endogenous AChE (Parker *et al*., 1991, 1996; Chen *et al*., 2001). It is therefore suggested that little benefit would arise from mutations that might amplify the gene or enhance its turnover of OPs.

5.10.5.3. Nonmicrosomal Esterases

Although a relatively derived clade compared to others in **Figure 2**, this clade (A) contains sequences from both holo- and hemimetabolan orders, and it shows greater diversity in respect of cellular/subcellular localization than do any other clades. It contains a substantial proportion of the presumptively cytosolic enzymes in the phylogeny, plus the only presumptively mitochondrial enzymes and also three with good consensus secretion signals.

Little is yet known of the functions of clade A enzymes. We previously suggested one *D. melanogaster* member (9) may correspond to the developmental gene *cricklet* (Claudianos *et al*., 2001), but it is now clear that it does not (http://flybase.bio.indiana.edu/). The only specific function now established for a clade A enzyme is the high level of malathion-specific resistance attributed to a mutant form of a cytosolic esterase (17) in the parasitoid *A. calandrae* (Baker and Weaver, 1993; Baker *et al*., 1998a, 1998b; Zhu *et al*., 1999a, 1999b) (see Section 5.10.5.2).

Resistant strains of *A. calandrae* have a Trp to Gly substitution at a position equivalent to the Trp251Leu/Ser substitutions conferring malathion-specific resistance in *L. cuprina* and *M. domestica*. The effect is to improve the MCE kinetics of the enzyme. Heidari *et al*. (2004a) have since made a Trp251Gly change in the *L. cuprina* enzyme *in vitro* and expressed it in a baculovirus system, finding that it confers significantly greater benefits in respect of MCE activity than do the Trp251Leu/Ser mutations in this enzyme. The *A. calandrae* mutant thus provides strong support from a case of malathion-specific resistance in the field supporting the contention of Heidari *et al*. (2004a) that substitution of the Trp with any of several small residues might confer malathion-specific resistance.

Baker *et al*. (1998a) also found that the mutant *A. calandrae* enzyme is relatively unstable, at least *in vitro*. This is reminiscent of similar evidence for the Trp251Leu mutant in the blowfly enzyme (Smyth *et al*., unpublished data). Interestingly, there is also 30-fold upregulation of the parasitoid enzyme in resistant strains (Zhu *et al*., 1999b). This might compensate for any instability of the enzyme *in vivo*, or simply augment the resistance due to the structural mutation. There is at least some evidence for upregulation of the blowfly mutant as well (Whyard and Walker, 1994).

5.10.6. Other Esterases Implicated in Insecticide Resistance

As seen in the earlier sections that molecular/genomic approaches have yielded enormous insights into the mechanisms underlying certain cases of esterase-based insecticide resistance. However, these clearly cover just a small proportion of the evergrowing list of esterase-based resistance. Now attention can be turned to the great majority of these for which there is as yet no molecular information. Their biochemistry and (classical) genetics are summarized in an attempt to discern recurring similarities and differences compared to those now elucidated at a molecular level.

5.10.6.1. Resistance against Organophosphates and Carbamates

Table 3 lists over 20 cases involving insect species from six orders and three acarines in which changes in esterase activity have been associated with nonmalathion specific OP resistance and/or carbamate resistance. About one-third of the cases involve

hemipterans. Most involve OP resistance alone or OP plus carbamate resistance; only a few involve carbamate resistance alone. The most common finding is an association of resistance with increased esterase activity as measured spectrophotometrically or electrophoretically with artificial substrates like naphthyl acetate.

Only three cases of a decrease are found, all involving OP resistance, outside the higher dipteran cases (see Section 5.10.5.2). These involve the codling moth *Cydia pomonella*, the western flower thrip *Frankliniella occidentalis*, and the predacious mite *Amblyseius potentillae*. However, only the *A. potentillae* case has been characterized in any detail biochemically and in this case the esterase seems unlikely to be the major cause of resistance (Oppenoorth, 1990). This suggests that other instances of the mutant aliesterase mechanism may occur outside the higher Diptera, but are relatively uncommon.

The fact that the great majority of cases involve increases in esterase activity might suggest that sequestration as exemplified in the *Myzus* and *Culex* cases is the predominant mechanism for esterase-mediated metabolic resistance to OPs and carbamates. The data for several of the cases are indeed at least consistent with these paradigms but it is equally clear that others are not. Some that may involve sequestration are not readily interpreted in terms of the sort of gene amplification events seen in the *Myzus* and *Culex* cases. A few are most easily explained in terms of a hydrolytic mechanism, albeit not the mutant ali-esterase mechanism.

Clearly one key requirement of a sequestration mechanism is that the esterase involved has high binding affinity for the insecticide. This is demonstrated directly in terms of the inhibition of the esterase activity by OPs *in vitro* in a few of the cases tabulated (the cockcroach *Blattella germanica*, the beetles *Leptinotarsa decemlineata* and *Oryzaephilus surinamensis*, and the mosquito *A. aegypti*). There are also several others where it can reasonably be inferred because the resistance is suppressed *in vivo* in bioassays including OP reagents like *S,S,S*-tributylphosphorotrithioate (DEF). Most of these cases cover OP resistance but they do include a case of carbamate resistance, involving *H. virescens*. Such *in vitro* or *in vivo* inhibition is generally taken as evidence for involvement of a member of the carboxyl/cholinesterases, although it is worth noting that an esterase from the serine protease family would probably yield similar results.

Sequestration as seen in the earlier aphid and culicine examples relies principally on mutations enabling massive overexpression of the relevant enzymes. In a few of the cases in **Table 3**, most notably the green rice leafhopper *Nephotettix cincticeps*, saw-toothed grain beetle *O. surinamensis*, and *B. microplus*, there is direct evidence for increased enzyme amounts. In a couple of others, it is indirectly inferred from the lack of difference in K_m or k_i estimates with various substrates or inhibitors between the relevant esterases from susceptible and resistant strains. In most cases, however, there is no evidence on this point and in one case, involving carbamate resistance in *H. virescens*, it actually appears not to be the case (see below).

In a significant proportion of the cases potentially involving sequestration which have been investigated electrophoretically, the greater level of spectrophotometric esterase activity in resistant lines appears to be largely due to the greater intensity of a single esterase isozyme. Cases like this include the currant lettuce aphid *Nasonovia ribisnigri*, the beetles *L. decemlineata* and *O. surinamensis*, and the tufted apple bud moth *Platynota idaeusalis*. However, there are in fact more cases where multiple isozymes show more intense staining in resistant strains. Some of the latter are probably just post-translationally modified variants of single gene products, while others involving just a small number of isozymes could reflect coamplification and overexpression of a small cluster of esterase-encoding genes. However, there are some cases, like *E. postvittana, H. virescens*, and *B. microplus*, where four to ten very different esterase isozymes are more intense in some resistant strains (Armstrong and Suckling, 1988; Rosario-Cruz *et al*., 1997; Harold and Ottea, 2000). It is not obvious what the genetic basis for this resistance could be, although there have been suggestions that *trans*-acting regulatory "supergenes" (Sabourault *et al*., 2001) could have such an effect.

For a few of the species in **Table 3** one or more of the elevated esterases associated with resistance has been purified and characterized to some degree biochemically. For the cases involving *B. germanica* (Prabhakaran and Kamble, 1995), *N. cincticeps* (Chiang and Sun, 1996), *L. decemlineata* (Anspaugh *et al*., 1995), and *Anopheles albimanus* (Brogdon and Barber, 1990), soluble esterases with molecular masses (~50–60 kDa), pH optima, pI values, and inhibitor sensitivities typical of carboxylesterases have been described. Limited comparisons between the enzymes from susceptible and resistant strains have failed to find any differences in properties suggestive of catalytic or other structural differences, and tentative conclusions about sequestration mechanisms based on increased enzyme amounts have therefore been drawn. However, the following cases, involving *O. surinamensis*, the western corn

rootworm *Diabrotica virgifera*, and *H. virescens*, all involve novel features suggestive of variously different mechanisms.

Two elevated esterase phenotypes are associated with OP resistance in *O. surinamensis*. One (HH) gives high level resistance to both chlorpyrifos-methyl and fenitrothion (both dimethyl thion OPs) (**Figure 11**), while the other (MH) gives high resistance to chlorpyrifos-methyl, but only moderate protection against fenitrothion. Isoelectric focusing resolves intense zones containing three and two isozymes, respectively, in HH and MH strains that are not apparent in susceptible individuals. None of these intense bands have the same mobility in HH and MH strains but all five have the same N-terminal sequence. It is therefore thought that the variation within each of the two phenotypes is posttranslational in origin, while the differences between phenotypes could be genetic, possibly due to allelic substitutions downstream of the sequenced N-terminal region.

The five isozymes all behave as conventional carboxylesterases: about 71 kDa in molecular mass, readily inhibitable by OPs, and without detectable OP hydrolase activity. Significantly, however, there are large quantitative differences in their sensitivities to OP inhibition. The isozymes in both HH and MH are more susceptible to chlorpyrifos-methyl and fenitrothion inhibition than extracts from susceptible strains. However, the HH isozymes are highly sensitive to inhibition by both OPs, whereas the MH isozymes are significantly less sensitive to fenitrothion. Given the consistency with the resistance data, the parsimonious interpretation is that there are structural differences between the HH and MH isozymes affecting the avidity of their binding to fenitrothion and hence their ability to sequester it. These structural differences may also be responsible for the greater naphthyl acetate staining intensities of the isozmyes compared to susceptible forms or, alternatively, the intensity differences could be due to a different mutation(s), involving gene amplification or upregulation of expression.

Parathion resistance in some populations of *D. virgifera* is associated with greatly increased intensities of (principally) two esterase isozymes (Scharf *et al*., 1999a, 1999b; Zhou *et al*., 2002). Little is known about the biochemistries of these isozymes, but the strong correlation in their intensities across individuals suggests that they are either posttranslational variants of a single gene product or that they are coamplified or coregulated. The fact that the resistance is partially suppressible by DEF suggests that they are carboxylesterases. The paradox is that there is also very good evidence that resistance is associated with *in vivo* hydrolysis of OPs (Miota *et al*., 1998), whereas the mutant carboxylesterases conferring resistance through OP hydrolysis as per the mutant ali-esterase mechanism are associated with a loss, not a gain in carboxylesterase activity. Either the isozyme intensities and OP hydrolysis both reflect a single as yet undescribed mechanism, or they reflect two distinct mechanisms, one involving sequestration by the isozymes and the other involving hydrolysis by other, apparently electrophoretically cryptic enzymes. Significantly there are three other species where OP resistance is also both substantively suppressed by DEF and associated with OP hydrolytic activity *in vivo* (*P. idaeusalis* and two simulid species) (Hemingway *et al*., 1989; Karoly *et al*., 1996); all these cases might reflect a novel carboxylesterase mechanism involving enhanced hydrolysis for both OPs and the conventional staining substrates. Alternatively, there are at least two instances (*H. virescens* and the hemipteran *Triatoma infestans*; see below) where esteratic OP hydrolysis is clearly not related to carboxylesterases.

The biochemistries of two independent cases of OP resistance and one of carbamate resistance have been studied in *H. virescens*. In the case of the carbamate resistance three esterase isozymes resolvable by isoelectric focusing were found to be elevated in intensity in a thiodicarb-selected strain (Goh *et al*., 1995; Zhao *et al*., 1996). Two of these were unreactive against antibodies raised against either the amplified *M. persicae* esterase or the *M. domestica* αE7 esterase, but the third reacted to both these antibodies and amino terminal sequencing confirmed its similarity with these and other carboxylesterases. Similarity was greatest against the *Drosophila* and hemipteran β-esterases, which might suggest it belongs in clade E. Notably, immunoblotting did not show a clear difference in the amount of this isozyme between susceptible and resistant strains, suggesting that structural rather than expression differences could underlie its elevated esterase activity in the resistant strain. Unfortunately little information was obtained about the biochemistries of the other two isozymes showing elevated staining intensities in this strain. At this point neither sequestration nor hydrolysis can be discounted as the mechanism underlying this resistance.

Profenofos resistance in *H. virescens* has also been associated with increased staining intensities of multiple naphthyl acetate staining esterase isozymes (Harold and Ottea, 1997, 2000). Several different isozymes are involved and some are not always elevated in intensity. However, the intensity of one

isozyme is always elevated in resistant individuals from both the field and laboratory populations analyzed, and the intensity of this band is negatively correlated with that of another, suggesting that the two may be allelic alternatives. This would argue for a structural difference between the two forms. It is not clear whether any of the isozymes implicated in OP resistance in this work correspond with those associated with carbamate resistance above; Harold and Ottea (2000) used native PAGE whereas the latter were resolved by isoelectric focusing. However it is notable that the carbamate selected line did also show substantial DEF-suppressible cross-resistance to OPs (Zhao *et al.*, 1996), which clearly supports the possibility of some overlap.

The other case of OP resistance in *H. virescens* does not involve carboxylesterases. It involves a parathion resistant line in which an arylesterase isozyme has been associated with OP hydrolysis both *in vitro* and *in vivo* (Konno *et al.*, 1990; Kasai *et al.*, 1992). The 120 kDa enzyme is concentrated in the soluble fraction, is activated by some divalent metal ions (Co^{2+} and Mn^{2+} but not Ca^{2+}) and has a pH optimum between 8 and 9. It has relatively low affinity for methyl paraoxon (K_m over 700 μM) but apparently good turnover rate (V_{max} ~0.01 μM min^{-1} mg^{-1} semipure protein), which contrasts directly with the mutant ali-esterases of *L. cuprina* and *M. domestica* (see Section 5.10.5.2). It is apparently inactive against parathion, but shows activity for paraoxon and a range of other oxon OPs. Some aspects of its biochemistry seem reminiscent of mammalian paraoxonases, but the latter are much smaller molecules (~45 kDa) that are preferentially activated by Ca^{2+} (Primo-Parmo *et al.*, 1996; La Du *et al.*, 1999; Claudianos *et al.*, 2001). Its activity was found to be many-fold higher in the resistant strain, compared to the susceptible control strain, but there was no evidence as to whether this reflected a difference in amount or activity of the molecule.

Further insights into OP hydrolytic activities not associated with carboxylesterases come from earlier work on OP hydrolysis by a strain of the kissing bug *Triatoma infestans* (Casabé and Zerba, 1981; De Malkenson *et al.*, 1984). The resistance status of this strain is not clear. However, analysis of the hydrolytic activity of native gel slices for various radiolabeled OPs found three distinct zones of activity from extracts of this strain. None corresponded with slices containing carboxylesterase isozymes although, as expected, some of these sequestered significant amounts of the OPs. One zone containing an aryl esterase isozyme could hydrolyze both oxon and thion OPs (**Figure 11**), another containing an acetylesterase isozyme could hydrolyze a thion OP, and a third containing no detectable esterase isozyme could also hydrolyze the thion OP. The aryl esterase has some parallels with the *H. virescens* case elucidated by Konno *et al.* (1990) and Kasai *et al.* (1992), although the activity against the thion OP is a notable difference. More generally these data clearly indicate the potential for several esterase-based hydrolytic resistance mechanisms that do not involve carboxylesterases, or even detectable esterase isozymes.

5.10.6.2. The Special Case of Malathion Carboxylesterase

Malathion is unusual among OP insecticides in having two carboxyl ester linkages in addition to the phosphoester linkage common to all OPs (**Figure 11**). As well as the hydrolysis of the latter as with other OPs, malathion can also be detoxified by hydrolysis of either or both of its (α- and β-) carboxylester linkages (Matsumura and Hogendijk, 1964; Eto, 1974; Welling *et al.*, 1974; Raftos, 1986). We outlined above (see Sections 5.10.5.2 and 5.10.5.3) three specific cases, involving dipteran microsomal α-carboxylesterases and a cytosolic wasp carboxylesterase, where substitutions to smaller amino acids at a particular residue in their presumptive acyl pocket created an MCE capable of effective *in vivo* detoxification and high levels of malathion-specific resistance. In the case of the wasp, the amount of its mutant cytosolic MCE was also elevated, further boosting resistance. In this section the biochemistry of several other cases of resistance-conferring MCEs, which have not yet been elucidated at a molecular level is summarized.

However, it is first worth noting some particular features of the chemistry of the malathion molecule. One is that it has an asymmetric carbon in its diethyl succinate group (**Figure 11**) and commercial malathion insecticides are racemic mixes of the two stereo isomers generated (Talley *et al.*, 1998). Almost all the biochemistry of MCEs to date is likewise based on unresolved racemates. Also, malathion differs from most other commonly researched carboxylester substrates, like acetylcholine or naphthyl acetate, etc., which have simple acyl groups and more complex alcohol moieties. Malathion has simple alcohol moieties (both ethyl) and bulkier acyl groups (ethyl succinate linked to the organophosphorus moiety). In this it is reminiscent of juvenile hormone (**Figure 7**) (see Section 5.10.4.3). It also resembles the pyrethroids (see **Figure 12**, Section 5.10.6.3) in having the bulky acyl groups, albeit the latter have bulky alcohol moieties as well. This may explain occasional cases of esterase-mediated

malathion and pyrethroid cross-resistance outlined below.

Reports of so-called malathion-specific resistance range across several insect orders, although about half involve the Diptera (**Table 4**). High levels of resistance, 50- to thousands-fold, are commonplace. With a few significant caveats, the resistance also shows a high level of specificity for malathion. One caveat is that in the few cases where it has been tested it also covers a small number of other OP insecticides like phenthoate, which also have carboxylester bonds in their acyl groups (Beeman and Schmidt, 1982; Scott and Georghiou, 1986; Raftos, 1986). Another is that while most relevant studies clearly show a lack of high levels of cross-resistance to noncarboxylester OPs, they could not preclude the possibility of low level resistance to such compounds, as seen occurs in the intensively characterized *L. cuprina* and *M. domestica* cases (see Section 5.10.5.2). Finally, there is some evidence for a degree of cross-resistance to pyrethroids, at least in the hemipterans *N. lugens* and *N. cincticeps* (Motoyama *et al*., 1984; Chen and Sun, 1994; Karunaratne *et al*., 1999).

Because the phenotype is generally so clear-cut, malathion-specific resistance has proven relatively amenable to classical genetic analysis. Essentially single-locus control has been reported for the higher dipterans *M. domestica*, *L. cuprina*, and *Chrysomya putoria* (Townsend and Busvine, 1969; Shono, 1983; Raftos and Hughes, 1986), the mosquitoes *A. stephensi, A. culicifacies, A. arabiensis, A. subpictus* (Hemingway, 1983, 1985; Malcolm and Boddington, 1989; Karunaratne and Hemingway, 2001) and *C. tarsalis* (Matsumura and Brown, 1963), the Indian meal moth *Plodia interpunctella* (Beeman and Schmidt, 1982), and the beetles *Tribolium cataneum* and *Cryptolestes ferrugineus* (White and Bell, 1988; Spencer *et al*., 1998), amongst others. However, there can be two or more resistance alleles at the locus, including both multiple malathion-specific alleles (Picollo de Villar *et al*., 1983), or also including one or more broad-spectrum OP resistance alleles (Campbell *et al*., 1998c; Karunaratne *et al*., 1999; Smyth *et al*., 2000; Vontas *et al*., 2000; and see below).

It has generally been found that malathion-specific resistance can be supressed by the use of the OP triphenyl phosphate (TPP) as a synergist (**Table 4**). This is often regarded as diagnostic of malathion-specific resistance and strongly implicates a carboxylesterase-based mechanism. Thin layer chromatography (TLC) indeed shows that malathion-specific resistance is generally associated with esteratic degradation at both the α- and β-carboxylester bonds of malathion (**Figure 11**), although the α-monoacid is generally the majority product and the diacid is generally less abundant than either monoacid (Matsumura and Voss, 1964; Kao *et al*., 1985; Raftos, 1986; Malcolm and Boddington, 1989, and references therein). Quantitation of MCE activity as a V_{max} of whole body homogenates (as determined from an endpoint radiometric partition assay) also generally shows MCE activity to be elevated in malathion-specific resistant strains. Elevations of 50-fold or greater are commonly recorded, although notably they are often significantly less than the corresponding resistance factors (e.g., Beeman and Schmidt, 1982; Ziegler *et al*., 1987; Smyth *et al*., 1996; Spencer *et al*., 1998). Characteristically the elevated MCE activities are potently inhibited by OPs, particularly TPP (e.g., Plapp and Eddy, 1961; Dyte and Rowlands, 1968; Townsend and Busvine, 1969; Hemingway, 1985; Spencer *et al*., 1998).

However, there is a very variable relationship between elevated MCE activity and general carboxylesterase activity in malathion-specific resistance. As well as the well-characterized *L. cuprina* and *M. domestica* cases (see Section 5.10.5.2), those involving the blowfly *C. putoria,* the moth *P. interpunctella*, and the mite *Tetranychus urticae* are also associated with a reduced level of "general" esterase activity as assessed using methyl butyrate or various naphthyl esters as substrates. However, there are cases where such general esterase activities are essentially unchanged (e.g., in some of the anophelines) or substantially enhanced (e.g., *C. tarsalis* and some planthoppers and leafhoppers) in resistant strains. A similar range of results is found across the various isozyme studies. Elevated MCE activity is associated with reduced staining intensities of particular isozymes in *M. domestica* and *L. cuprina*, but with elevated staining intensities of particular isozymes in *N. cincticeps* and the brown planthopper *Laodelphax striatellus*. In *C. tarsalis* the majority of MCE is associated with a zymogram region lacking any detectable general esterase isozyme at all. The same is true of the bug *T. infestans*, although malathion resistance status was not reported in this case (Wood *et al*., 1985).

Importantly it is clear that even susceptible strains often have significant levels of MCE activity. Notwithstanding large quantitative differences, at least some MCE is found to be widely distributed across tissues, life stages and subcellular fractions in both susceptible and resistant strains (Townsend and Busvine, 1969; Beeman and Schmidt, 1982; Motoyama *et al*., 1984; Wood *et al*., 1985) (**Table 4**). Studies of *N. lugens* and *N. cincticeps* found

Table 4 Summary of cases of esterases implicated in malathion-specific metabolic resistance

Species	*Biochemistry*	*Reference*
Hemiptera		
Laodelphax striatellus (brown planthopper)	Some immunologically related esterase isozymes with *in vitro* malathion carboxylesterase (MCE) activity and identical amino terminal sequences are elevated in amounts in malathion-resistant strains	Miyata and Saito (1976), Sakata and Miyata (1994)
Nephotettix cincticeps	Four isozymes with high esterase activity, three of which had malathion hydrolytic activity in three strains showing moderate levels of resistance to malathion albeit also several other OPs and some SPs	Miyata and Saito (1976), Motoyama *et al.* (1984), Chiang and Sun (1996)
Nilaparvata lugens	An isozyme with high MCE activity (and some SP hydrolytic activity) is more intense in strains with low level resistance to malathion (and some SPs) but not most OPs	Chang and Whalon (1987), Chen and Sun (1994)
Coleoptera		
Cryptolestes ferrugineus (rust red grain beetle)	Slightly lower general esterase activity but increased MCE activity *in vivo* and *in vitro* in a strain showing a high level of triphenyl phosphate (TPP)-suppressible malathion resistance	Spencer *et al.* (1998)
Tribolium castaneum (red flour beetle)	TPP-suppressible malathion-specific resistance	White and Bell (1988)
Lepidoptera		
Plodia interpunctella (Indian meal moth)	General esterase activity reduced but MCE activity elevated 33-fold in a strain showing high TPP-suppressible resistance to malathion and cross-resistance to phenthoate	Beeman and Schmidt (1982)
Hymenoptera		
Anisopteromalus calandrae	General esterase activity unchanged but (cytosolic) MCE activity 10–30-fold higher in a malathion resistant strain; W to G mutation at the same site as the W251L MCE mutation in *L. cuprina* and *M. domestica* contributes to malathion resistance, as does upregulation of expression	Baker *et al.* (1998a, 1998b), Zhu *et al.* (1999a, 1999b)
Diptera		
Anopheles arabiensis	General esterase activity unchanged and no elevated esterase isozymes but kinetically efficient MCE in strains with high levels of TPP-suppressible malathion specific resistance	Hemingway (1985)
Anopheles culicifacies	General esterase activity essentially unchanged and no elevated esterase isozyme but high levels of MCE activity and *in vivo* malathion metabolism in strains with high levels of TPP-suppressible malathion specific resistance	Herath *et al.* (1987), Malcolm and Boddington (1989), Karunaratne and Hemingway (2001)
Anopheles stephensi	General esterase activity essentially unchanged and no elevated esterase isozymes but three isozymes have MCE activity in strains resistant to malathion and phenthoate	Hemingway (1982, 1983), Scott and Georghiou (1986)
Anopheles subpictus	High levels of MCE activity and monoacid and diacid metabolites produced by mass homogenates of resistant field strain	Karunaratne and Hemingway (2001)
Chrysomya putoria (green blowfly)	General esterase activity is reduced in TPP-suppressible malathion-specific resistance; the alkyloxy methyl group of malathion is important for high level resistance	Townsend and Busvine (1969)
Culex tarsalis	General esterase activity enhanced in a strain with 150-fold resistance specifically to malathion that is synergised by TPP; MCE activity is associated with a zymogram region lacking esterase activity	Matsumura and Brown (1963), Ziegler *et al.* (1987), Whyard *et al.* (1994)
Lucilia cuprina	Reduced levels of general esterase activity, slightly higher levels of MCE activity and significant OP hydrolase activity associated with Ser/Leu251 variants of the LcαE7 (E3) isozyme in strains with high levels of TPP-suppressible resistance to malathion and phenthoate (see Section 5.10.5.2)	Hughes *et al.* (1984), Raftos (1986), Raftos and Hughes (1986), Smyth *et al.* (1994, 1996, 2000), Whyard and Walker (1994), Campbell *et al.* (1998a, 1998c), Newcomb *et al.* (2004)
Lucilia sericata	Ser/Leu251 variants found in LcαE7 ortholog in populations known to segregate for malathion specific resistance (see Section 5.10.5.2)	R.D. Newcomb, C.G. Young, and J.R. Stevens, unpublished data

Continued

Table 4 Continued

Species	*Biochemistry*	*Reference*
Musca domestica	Reduced levels of general esterase activity, slightly higher levels of MCE activity and Ser/Leu251 variants of the MdαE7 (Ali) isozyme in strains with high levels of TPP-suppressible resistance to malathion (see Section 5.10.5.2)	Plapp and Eddy (1961), Picollo de Villar *et al.* (1983), Shono (1983), Kao *et al.* (1984, 1985), Claudianos *et al.* (1999, 2001)
Acarina		
Tetranychus urticae	Reduced levels of general esterase activity, increased MCE activity and increased phosphatase activity in a strain with 60-fold malathion resistance and 10-fold parathion resistance	Matsumura and Voss (1964)

measurable levels of MCE activity associated with a majority of the esterase isozymes in both susceptible and resistant strains (Chang and Whalon, 1987; Chen and Sun, 1994; Chiang and Sun, 1996). Together with the cases where MCE is associated with a nonstaining area of general esterase zymograms, this suggests that a range of MCEs is at least theoretically available from which mutants with improved activities might be selected to confer resistance.

As described above (see Section 5.10.5.2) one MCE mutation found in orthologous microsomal carboxylesterases from clade B (**Figure 2**) in both the higher dipterans *L. cuprina* and *M. domestica*. This active site mutation improves MCE kinetics very slightly (although with a specificity constant around $10^6\,M^{-1}\,s^{-1}$ it is already high) but also confers significant OP hydrolase activity, so the MCE enzyme escapes from otherwise essentially irreversible inhibition if it binds at the phospho end of malaoxon (the activated form of malathion) rather than the carboxylester end of malaoxon or malathion. The mutant enzyme also has reduced activity for several of the conventional artificial substrates used to measure general esterase activity. There is molecular evidence that a similar MCE mutation confers malathion specific resistance on the hymenopteran *A. calandrae*, albeit in this case it involves a soluble cytosolic esterase from clade A in **Figure 2**, and also appears to involve some upregulation in the amount of the esterase (see Section 5.10.5.3). Precise parallels in the biochemistry and toxicology of the phenotype have also led to suggestions that the malathion-specific resistance in another higher dipteran *C. putoria* and in the moth *P. interpunctella* would be due to the same mutation in a microsomal clade B esterase (Campbell *et al.*, 1997, 1998c; Smyth *et al.*, 2000). Some of the better-characterized other cases of resistance to see if they also have biochemistries matching these cases are now considered.

Several cases of malathion-specific resistance in anopheline mosquitoes have been characterized to some degree biochemically, and none has proven associated with elevated staining of any esterase isozymes although all show the characteristic TPP-suppressible, MCE-based mechanism (Hemingway, 1985; Herath *et al.*, 1987; Malcolm and Boddington, 1989; Hemingway *et al.*, 1998; Karunaratne and Hemingway, 2001). Kinetic evidence in several cases indicates the presence of a highly efficient MCE enzyme in resistant strains that is apparently absent in susceptible strains (Hemingway, 1985; Hemingway *et al.*, 1998). *Anopheles stephensi* in Pakistan express at least three esterase isozymes with MCE activity but none of them is overexpressed in resistant strains and indeed they are all inabundant in both susceptible and resistant strains (Jayawardena and Hemingway, cited in Hemingway *et al.*, 1998). All these data imply that a qualitative rather than quantitative change in an MCE confers malathion-specific resistance among these anophelines. The *A. gambiae* genome does not contain any sequences in clade B (although culicines do), but the biochemistry available for the anopheline MCEs does not yet suggest which clade they do belong to.

In the culicine mosquito *C. tarsalis*, malathion-specific resistant lines express in the mitochondria of their gut cells a kinetically efficient MCE, which is not detectable in susceptible strains (Ziegler *et al.*, 1987; Whyard *et al.*, 1994). There is also a cytosolic MCE in a broad range of tissues, but this is less efficient kinetically and equally expressed in both susceptible and resistant strains. It is thus suspected that the effect of the resistance mutation is to substantially elevate the expression of a previously inabundant enzyme that is otherwise appropriate in its kinetics and expression profile for *in vivo* detoxification of malathion. The resistance-associated enzyme is not an ortholog of the clade B esterase amplified in broad-spectrum OP resistance in other culicines (Tittiger and Walker, 1997). The only clade in **Figure 2** containing sequences with mitochondrial targeting signals is clade A, although not all clade A enzymes have such signals. There are many precedents for mitochondrially located enzymes

performing detoxification functions in other organisms, including for pesticides like malathion (Bachurin *et al.*, 2003 and references therein).

An unusual variant of the secreted clade E esterase associated with broad-spectrum OP resistance in the planthopper *N. lugens* (see Section 5.10.4.3.2) has been described from Japan and the Philippines. This esterase provides at least a low level of resistance to malathion and certain synthetic pyrethroids but not to most OPs (Chang and Whalon, 1987; Chen and Sun, 1994) (see Section 5.10.6.3). While the variant associated with the broad-spectrum OP resistance has been shown to involve a three- to sevenfold amplification of the encoding gene, nothing has yet been reported of the molecular nature of the malathion/pyrethroid resistant variant (Karunaratne *et al.*, 1999; Small and Hemingway, 2000a, 2000b; Vontas *et al.*, 2000). While as many as eight different esterase isozymes have MCE activity in *N. lugens*, it is the E1 isozyme, which is more intense in the malathion/pyrethroid resistant strain, that appears to have the best MCE activity.

At least four esterase isozymes are strongly overexpressed in field strains of the leafhopper *N. cincticeps* showing varying levels of resistance to many OPs, including moderate resistance to malathion, and also some resistance to pyrethroids (Miyata and Saito, 1976; Motoyama *et al.*, 1984). The net effect is over 40-fold higher carboxylesterase activity in the resistant strains. At least three of the isozymes have some MCE and pyrethroid hydrolytic activities. It has been proposed these isozymes provide resistance to malathion and pyrethroids by hydrolytic detoxification, and to the other OPs by sequestration (Motoyama *et al.*, 1984) (see Section 5.10.6.1). Whilst not discounting this possibility, the latest data suggest that the contribution to malathion resistance due to the MCE activities of these enzymes may be relatively minor (Chiang and Sun, 1996). Even without this *N. cincticeps* case, it is apparent that the structural mutations described in *L. cuprina* and *M. domestica*, and inferred in *C. putoria* and *P. interpunctella*, are just one mechanism conferring MCE based malathion-specific resistance. Although not yet resolved at the molecular level there are clearly other mechanisms, involving different mutations, nonorthologous enzymes, and possibly different clades, which could confer MCE based resistance.

5.10.6.3. Resistance against Synthetic Pyrethroids

Most commercial synthetic pyrethroid (SP) insecticides are carboxylesters, with relatively large and highly hydrophobic acid and alcohol groups (Casida *et al.*, 1983) (**Figure 12**). The early, type 1, SPs are smaller than the more modern type 2 compounds, which differ in having an α-cyano substituent on their alcohol group and, in many cases, dibromo instead of dichloro substituents on their acyl groups. Most SPs also show high levels of racemic complexity, with as many as three chiral centers, one around the carbon to which the cyano moiety attaches in the alcohol group and two across the cyclopropane ring in the acyl group. The type 2 SPs generally have greater potency, in part because their greater bulk makes them less susceptible to hydrolytic detoxification by carboxylesterases (Elliott, 1977; Soderlund and Casida, 1977a, 1977b; Casida *et al.*, 1983). The various isomers also differ in potency against many insects, albeit not always in the same way across species. For example, it is generally but not always true that the *cis* isomers across the cyclopropane ring are more potent insecticides than their *trans* alternatives and, again, this is at least partly because they appear to be less susceptible to hydrolysis by carboxylesterases (see, e.g., Casida *et al.*, 1983; Ruigt, 1985; Chang and Whalon, 1986; Byrne *et al.*, 2000).

Table 5 summarizes about a dozen cases where elevated esterase levels have been associated with metabolic resistance to SPs. The cases tabulated range across several orders of insects and two acarines, although lepidopterans account for about half the cases. None of the cases tabulated has yet been fully elucidated at a molecular genetic or even biochemical level. The problems in studying SP resistance at a biochemical level stem from several refractory properties of the compounds themselves, including their isomeric complexity, difficulties in separating some isomers, and their very limited (low μM) aqueous solubilities. These difficulties have been compounded until recently by the unavailability of sensitive and convenient fluorometric or colorimetric assays, so kinetic analyses had to be carried out with expensive radiometric reagents in tedious partition assays. Fortunately Wheelock *et al.* (2003) have recently developed fluorometric analogs of some SPs, which should now greatly expedite progress in resolving the biochemistry of SP resistance.

At a phenotypic level, esterase based metabolic resistance to SPs is often reported in association with P450-based resistance. The contribution that the esterase mechanism makes to the overall metabolic resistance can be readily demonstated experimentally by the use of respective inhibitors for the two enzyme systems, and the reliance of the P450 mechanism on NADP as a cofactor; however, it is difficult to quantify the relative contributions of the two mechanisms (Liu *et al.*, 1984; Ottea *et al.*, 1995;

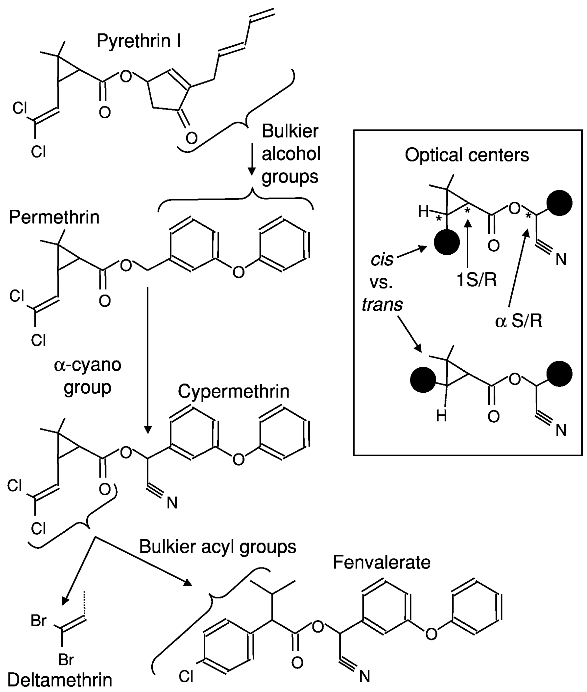


Figure 12 Pyrethroid structures. Pyrethrin I is shown first as an example of a natural pyrethroid. Synthetic pyrethroids (SPs) have shown increasing resistance to hydrolysis by esterases as the alcohol groups were made bulkier (e.g., permethrin), an α-cyano group was added (e.g., cypermethrin) and the acyl group was also made bulkier (e.g., fenvalerate), often by the replacement of the chlorine atoms with bromines (e.g., deltamethrin). Type 2 SPs contain the α-cyano moiety and often also the dibromo moiety. Pyrethroids generally have at least two and often three optical centers, one around the carbon to which the cyano moiety attaches and two across the cyclopropane ring, resulting in four or eight possible stereoisomers. Naming conventions describe stereoisomers as α R or S with respect to the carbon to which the cyano group attaches and 1R or S for the optical center on the cylcopropane ring closer to the carboxylester linkage. However, the alternatives at the other optical center on the cyclopropane ring are not described directly. Instead the combined effect of the two optical centers either side of the cyclopropane ring is then described as *cis* or *trans* for whether the bulkier moieties either side of the cyclopropane ring are placed closer together or further from each other.

Shan *et al.*, 1997; Shan and Ottea, 1998). Cross-resistance of the esterase component beyond SPs is unusual, although it has been reported for malathion in a small number of cases, e.g., *N. cincticeps* and *N. lugens* (Table 5), plus possibly also see *C. pipiens* (Bisset *et al.*, 1997). In these cases resistance may be mediated by a shared carboxylesterase-based mechanism. There is one report, involving *A. albimanus*, of cross-resistance to other OPs. The mechanism here is unknown but may involve amplification of an esterase that binds and sequesters both classes of compound (Brogdon and Barber, 1990). In a few cases multiple SPs have been tested; cross-resistance among SPs seems the rule, although (consistent with their heterogeneity in size and shape) resistance factors can vary substantially (Gunning *et al.*, 1999).

At a biochemical level, the usual finding is of elevated levels of esterase activity in *in vitro* assays of whole body extracts with compounds like naphthyl acetate as substrates. The increase at this level is generally not large, around two- to tenfold, although increases exceeding 50-fold have been reported, for example, in the cotton bollworm *Heliothis armigera*. However, the staining intensity of individual isozymes may be elevated over 100-fold in resistant strains, or in some cases they may be apparent in resistant strains but undetectable in susceptible strains. In some cases, like *Bemisia tabaci* and *B. microplus*, it essentially involves just a single band. More commonly it involves several bands; cases of this include *H. armigera*, the native budworm *Helicoverpa punctigera*, *H. virescens* and the tick *A. fallacis*. In *A. albimanus* the elevated intensity was resolved as a single band by native PAGE but separate bands by isoelectric focusing. There are no cases of SP resistance to our knowledge associated with reductions in staining intensity of esterase isozymes or the replacement of one isozyme band with another.

It is generally assumed that increased staining intensities of esterase isozymes associated with resistance are due to increases in the amounts of

Table 5 Summary of cases of esterases implicated in metabolic resistance to synthetic pyrethroids

Species	*Biochemistry*	*Reference*
Hemiptera		
Bemisia tabaci	Increased molar amount of the esterase isozyme $E_{0.14}$ is strongly associated with a marked increase in *in vivo* permethrin hydrolysis	Costa and Brown (1991), Byrne and Devonshire (1996), Byrne *et al.* (2000)
Nephotettix cincticeps	Increased amounts of four esterase isozymes with some SP (and malathion) hydrolytic activity tentatively associated with SP (and malathion) resistance; one isozyme with activity essentially only for fenvalerate. Activity greatest for *cis*-permethrin, then cypermethrin, then *trans*-permethrin (and then malathion)	Motoyama *et al.* (1984), Chiang and Sun (1996)
Nilaparvata lugens	Increased amount of the E1 isozyme in an SP and malathion resistant strain has more activity for *trans*-permethrin (and malathion) than *cis*-permethrin and cypermethrin. Several other isozymes also hydrolyse *cis*- and *trans*-permethrin (*trans* better than *cis)* and malathion	Chang and Whalon (1987), Chen and Sun (1994)
Coleoptera		
Leptinotarsa decemlineata	Increased *trans*-permethrin hydrolytic activity and general esterase activity in whole body homogenates of resistant individuals	Argentine *et al.* (1995)
Lepidoptera		
Helicoverpa armigera	Increased *in vivo* SP hydrolysis, general esterase activity and more intense staining of several esterase isozymes in strains whose resistance to multiple SPs is partially suppressible by nontoxic doses of OPs; increased staining intensity due to increased amount of enzyme	Gunning *et al.* (1996b, 1998, 1999), Campbell (2001)
Helicoverpa punctigera (native budworm)	Increased *in vivo* SP (fenvalerate) hydrolysis and more intense staining of several esterase isozymes in strains whose resistance is suppressible by DEF and propenofos	Gunning *et al.* (1997)
Helicoverpa zea (corn earworm)	Increased general esterase activity in resistant strains (not inducible by diet)	Muehleisen *et al.* (1989)
Heliothis virescens	Increased *in vivo* SP hydrolytic activity and general esterase activity and more intense staining of several esterase isozymes in resistant strains; increased staining intensity due to increased amount of enzyme; *trans*-permethrin hydrolyzed more rapidly than *cis*-permethrin	Dowd *et al.* (1987), Goh *et al.* (1995), Ottea *et al.* (1995), Shan and Ottea (1998)
Plutella xylostella (diamondback moth)	Esterase activity towards permethrin found in resistant strain	Liu *et al.* (1984)
Spodoptera exigua (beet armyworm)	Increased *in vivo* deltamethrin hydrolytic activity and general esterase activity in resistance strains	Delorme *et al.* (1988)
Spodoptera littoralis (Egyptian cotton leafworm)	Increased esterase activity associated with TPP-suppressible SP resistance	Riskallah (1983)
Diptera		
Anopheles albimanus	Increased intensity of an esterase isozyme zone in strains and individuals showing *S,S,S*-tributylphosphorotrithioate (DEF)-suppressible resistance to fenitrothion and deltamethrin	Brogdon and Barber (1990)
Acarina		
Amblyseius fallacis (predatory mite)	Many esterase isozymes in susceptible insects have some SP hydrolytic activity (preferentially *trans*- over *cis*-permethrin) but certain esterase isozymes from resistant strain have higher rates of SP hydrolysis	Chang and Whalon (1986)
Boophilus microplus	Increased *in vivo* SP hydrolytic activity and at least one unique esterase isozyme detected in strains whose SP resistance is at least partially suppressed by TPP	de Jersey *et al.* (1985), Jamroz *et al.* (2000)

the enzymes, and in some cases (*B. tabaci*, *N. cincticeps*, *N. lugens*, *H. armigera*, and *H. virescens*) immunochemical, tritium-labeling or other techniques have been used to validate this assumption. In *H. armigera* and the corn earworm *H. zea* it has been found that the elevated activity phenotype is stably expressed across a range of dietary conditions and is not the result of increased inducibility of the enzymes by the pyrethroids.

Although there may also be some protective effect due to the esterases binding and sequestering the pesticide (Goh *et al*., 1995; Gunning *et al*., 1999), there is good evidence from many studies indicating metabolism of SP by the esterases. In particular, increases in SP hydrolytic activity in whole body homogenates of resistant individuals have been recurrently reported. Examples of this include *B. tabaci*, *L. decemlineata, H. armigera*, *H. punctigera*, *H. virescens*, the beet armyworm *Spodoptera exigua*, *M. domestica*, and *B. microplus*. The generalities of greater hydrolytic activity for smaller compounds and *trans* isomers hold up in these extracts, but exceptions to both these generalities are also documented (Ishaaya and Casida, 1981) (**Table 5**).

As with malathion above, it appears that a substantial proportion of the esterase isozymes even in susceptible insects have some SP hydrolytic activity, at least *in vitro*. This has been mostly clearly demonstrated by Chang and Whalon (1986, 1987) who found that a substantial proportion of the esterase isozymes which they could resolve in *N. lugens* and *A. fallacis* had some SP hydrolytic activity; some of these were elevated in resistant strains but others were not. Evidence from other species is also consistent with these results (Abdel-Aal and Soderlund, 1980; Ishaaya and Casida, 1980; Yu, 1991) (**Table 5**).

Esterase isozymes with some SP hydrolytic activity have been purified and characterized to varying degrees from several species. Except for *B. tabaci*, evidence to implicate the particular enzymes studied in SP resistance is generally lacking (Byrne *et al*., 2000). It is clear that isozymes with SP hydrolytic activity can be found from many tissues and developmental stages, and from both soluble and micrososmal fractions (Abdel-Aal and Soderlund, 1980; Ishaaya and Casida, 1980; Motoyama *et al*., 1984). Evidence on inhibition by OPs, molecular mass, isoelectric points, pH optima, and immunological cross-reactivity are all consistent with the isozymes being in the carboxyl/cholinesterase family, without being sufficiently definitive to identify particular clades within the family (Abdel-Aal and Soderlund, 1980; Ishaaya and Casida, 1980; Motoyama *et al*., 1984; de Jersey *et al*., 1985; Chen and Sun, 1994; Goh *et al*., 1995; Chiang and Sun, 1996; Byrne *et al*., 2000). K_m estimates with SPs are generally in the low–mid μM range, with k_{cat} data generally lacking (Jao and Casida, 1974; Abdel-Aal and Soderlund, 1980; Yu, 1991). Interestingly, Abdel-Aal and Soderlund (1980) found quantitative differences in tissue distributions and inhibitor sensitivities between *cis* and *trans* permethrin preferring activities and therefore suggested that esterase-mediated metabolic resistance to SPs would accrue through the combined effect of several different esterases of differing substrate preferences.

The most detailed kinetic analyses of SP hydrolysis by insect esterases have recently been carried out by Heidari *et al*. (unpublished data) for *in vitro* expressed forms of the *L. cuprina* αE7/E3 enzyme and various natural and synthetic mutants of it. LcαE7 is not involved in SP resistance but the wild-type enzyme shows high SP hydrolytic activity, including preferences for type 1 compounds, dichloro substituents, and *cis*/*trans* isomers across the cycopropane ring, with relatively little stereospecificity with respect to the α-cyano group. However, a few variants of the enzyme with substitutions in its presumptive acyl pocket were variously found to have reversed the *cis*/*trans* and *R*/*S* stereospecificities, and at least quantitatively altered preferences for the other structural features. These results support the earlier studies above indicating that most esterase isozymes are individually quite specific for the variable structural features of SP insecticides, and they further show that good hydrolytic activities against a variety of SP structures can be achieved across different (even very closely related) esterases. They also caution against the assumption that the changes in esterase biochemistry conferring SP resistance will always affect isozyme amounts; clearly there is scope for point mutations affecting enzyme structures to improve the suite of SP hydrolytic activities of the organism.

Two predictions can be made about the genetics of esterase-based SP resistance from the biochemical findings to date: they might differ considerably between species and sometimes can be polygenic. Whilst accepting the possibility of some structural changes in relevant enzymes, most of the data to date suggest that the changes will generally affect isozyme amounts. Gene amplification has been suggested as one possible mechanism for this (Gunning *et al*., 1999) although it would probably need to involve multiple amplifications, or amplifications of multiple (closely linked) genes, to account for some of the cases of resistance involving increased staining of multiple isozymes. Regulatory changes are an alternative explanation, and certainly there is abundant evidence for regulatory variation affecting the amounts of esterase isozymes (Game and Oakeshott, 1990; Holloway *et al*., 1997; Odgers *et al*., 2002). Notably the best evidence for the coincident upregulation of multiple esterases appears to involve inducible systems (Salama *et al*., 1992; Callaghan *et al*., 1998; Campbell, 2001), which does not appear to apply to the esterases involved in SP resistance. It is therefore suspected that polygenic inheritance will sometimes be the case.

5.10.7. Concluding Remarks

The carboxyl/cholinesterase gene family has radiated extensively in the higher eukaryotes. Small numbers are found in a minority of prokaryotes and only a few occur in lower eukaryote genomes, but 30 or more have been found in each of the higher eukaryotic genomes annotated. In insects, it appears that this expansion corresponds at least in part with their recruitment to functions in neurodevelopmental processes and the transduction of hormonal and other signals, albeit the precise physiological functions of most remain largely, if not completely, unknown. The radiation in physiological function is paralleled at a biochemical level. Where known, the kinetics of individual enzymes for their particular physiological substrates can be highly efficient. However, the substrates for different members of the family differ quite widely in structure and, remarkably, a significant proportion of the family has moved into noncatalytic functions.

While a significant proportion of the major catalytic and noncatalytic clades appear to have origins as old as the Insecta, it is also clear from cases like glutactin, the noncatalytic β-esterases, and the evolution of insecticide resistance, that new radiations moving the family into new functions are still arising. In this respect it will be interesting to see the constitution of the family in the silkmoth *Bombyx mori* and honeybee *Apis mellifera,* whose genome sequences should be released before this article appears in print. Both species are clearly much more distantly related than *D. melanogaster* and *A. gambiae*, and an enormous diversity of esterase isozymes have been reported from several Lepidoptera – as many as 60 isozymes compared to just 20–30 in *D. melanogaster* (Campbell, 2001; Campbell *et al*., 2003).

Notwithstanding the expansion of the carboxyl/cholinesterase family in higher eukaryotes, it is also clear that other protein families contribute significantly to the complement of esteratic activities of higher organisms. Vertebrate and prokaryote precedents suggest that insect aryl and acetyl esterases will derive from other families (Oakeshott *et al*., 1993). There are also other insect esterase activities like the ortholog of vertebrate neuropathy target esterase (Borrás *et al*., 2003) that clearly have other, nonserine dependent reaction mechanisms. And there are several vertebrate precedents for other enzyme families with serine based reaction mechanisms like the serine proteases that have generated good esteratic activities (Zschunke *et al*., 1991). The carboxyl/cholinesterases probably contribute the majority of insect carboxylic ester hydrolase activities, but the contributions of other protein families are as yet very poorly understood.

We will not understand why the carboxyl/cholinesterase family has been such a versatile platform for the evolution of new functions until we know a great deal more about structure, function, and structure–function relationships within the family. Fortunately, use of the modern tools of functional genomics and metabolomics could greatly improve our knowledge of their physiological and biochemical functions in the near term. The structural information, however, will be more difficult to expand. AChE is the only insect member of the family whose structure is currently known, albeit with a structure for a lepidopteran JHE also imminent. Even the modeled structure of JHE has suggested some qualitative differences from AChE directly concerned with catalysis, like the reverse orientation of the acyl and leaving group pockets. There is also particular priority to solve structures in some of the clades with noncatalytic functions and those from which the various metabolic resistances arise. In both cases ligand-bound structures will be of particular interest.

Perhaps the most spectacular demonstration of the evolutionary adaptability of the carboxyl/cholinesterases is their likely involvement in well over 50 cases of resistance to chemical insecticides over the last 50 years. Many involve the older OP and carbamate compounds where mutations of major biochemical effect are required to achieve either target site or metabolic resistance. Several also now involve the SPs although none of these has as yet been resolved at a molecular level. Esterase based SP resistance is also a notable feat of molecular evolution given the bulk and chiral complexity of SP insecticides. Nor are the more recent insecticides immune from esterase based resistance mechanisms – there are already reports of esterase involvement in resistance to various insect growth regulators (Ishaaya and Degheele, 1988; El Saidy *et al*., 1989; Ishaaya and Klein, 1990) where at least one can rationalize a hydrolytic basis for detoxification. Intriguingly there are also reports of possible esterase involvement in metabolic resistance to imidacloprid (Wang *et al*., 2002) and even the proteinaceous crystal toxins of *Bacillus thuringiensis* (Dang and Gunning, 2002), where a mechanism is at this time much less readily rationalized biochemically.

This chapter has taken a genomics perspective to the cases of esterase mediated resistance that have been resolved at a molecular level and then analyzed the biochemistries of about 70 cases of such resistances that have not yet been resolved at a molecular level. These analyses generally support the contention that the options for evolving resistance are

limited. However, the biochemistry clearly indicates the existence of several more that have yet to be resolved at a molecular level in any species. Involvement of multiple clades of carboxyl/cholinesterases has already been observed, and it is clear that a few cases involve other protein families.

It has recently been suggested that there may be two broad classes of AChE insensitivity mutations conferring target site resistance to OPs and carbamates, one skewed towards OP resistance and the other to carbamate resistance. It also appears that there may be at least two subclasses within the carbamate resistant class, with one specific for dimethyl carbamates and possibly restricted to hemipterans. The validity of these classifications will only be tested by molecular work on several more independent cases of target site resistance. It also appears from biochemical analyses of synthetic mutants that there could be many more substitutional options available for conferring resistance than have been reported from natural populations to date. Understanding which are real options, and how they relate to the different patterns of insensitivity phenotype, will also require AChE structural data for additional taxa. Also important to our understanding of the evolution of AChE and its role in resistance will be much greater knowledge at a biochemical and molecular level of both the noncatalytic functions of AChE and of the partitioning of the catalytic and noncatalytic functions among the two AChE proteins found in most insects.

It appears that the gene amplification/sequestration mechanism, and possibly also the mutant aliesterase mechanism, found in the cases of metabolic resistance to OPs resolved at a molecular level so far will indeed recur in some other cases. However, the biochemistry clearly points to some significant variations on these themes, as well as some completely different mechanisms. The involvement of the metal activated aryl esterases in general OP resistance in *H. virescens* obviously constitutes at least one completely different mechanism. Whilst it seems that insects do not have homologs of the vertebrate paraoxonase subfamily of aryl esterases (Sorenson *et al*., 1995), they appear to have homologs of at least some prokaryote OP hydrolytic enzymes (Hou *et al*., 1996; Claudianos *et al*., 2001; Horne *et al*., 2002a, 2002b). Insect homologs of one of these, the metal-activated OPH (Pfam 02126), have been identified (Hou *et al*., 1996; Claudianos *et al*., 2001). *Spodoptera frugiperda* larvae infected with recombinant baculoviruses expressing high levels of a bacterial OPH show nearly 300-fold greater resistance to paraoxon than uninfected controls (Dumas *et al*., 1990) while *D. melanogaster* genetically transformed with this gene show over 20-fold greater paraoxon resistance than untransformed controls (Phillips *et al*., 1990; Benedict *et al*., 1994).

There are also diverse esterase biochemistries underlying malathion-specifc resistance, with the molecular biology only as yet resolved for the Leu/Ser251 versions of the mutant αE7/aliesterase mechanism.

Regulatory changes are clearly suggested by the increased staining intensities and abundances of some isozymes seen for general OP, malathion-specific and pyrethroid resistance. Some of these could be relatively straightforward changes to the promoters of the relevant esterase genes, as seems to be the case for at least one *C. pipiens* amplicon. However, one paradox is the finding of greater intensities for multiple isozymes in several cases of resistance. Some of these appear beyond the scope of explanations based on posttranslational enzyme modification or adjacent gene coamplifications. Alternative explanations involving *trans*-acting regulatory factors remain to be investigated.

With the notable exception of the carbamate–OP cross-resistance due to E4/FE4 sequestration in *M. persicae*, none of the esterase based metabolic resistances to carbamates and SPs have as yet been elucidated at a molecular level. A sequestration mechanism similar to the E4/FE4 model may underlie some of the many other cases of OP–carbamate cross-resistance reported. Some variant of it might also account for the occasional case of OP–SP cross-resistance. However hydrolysis could well underlie more of the cases not involving cross-resistance to OPs, which are now numerous for the SPs at least. It is clear that many insect esterases have significant hydrolytic activity against the carboxylester bonds in SPs *in vitro*. Little is known about hydrolysis of carbamates in insects but bacterial enzymes are known which have good hydrolytic activities against either the amide or carboxylester bonds in these compounds (Pohlenz *et al*., 1992). The nature of the chemistry involved in hydrolysis of the carbamates and SPs is so different, both from that for OPs and from each other, that we would not expect hydrolytic mechanisms to confer cross-resistance among the three classes of compound.

One final perspective on resistance is that what is known at the moment is a snapshot of the "first generation" of insect responses to the insecticides. There is little insight as to what preceded resistance or what will follow the first generation response. What were the predisposing features of the relevant esterase systems? Did the mutations pre-date the use of the pesticides or has the breakdown of

control waited on the occurrence of new mutations? Can insects develop "second generation" responses that substantively enhance their resistance or cross-resistance or avoid the costs of resistance? Has the repeated use of ester pesticides so depleted some insects' store of genetic variability in detoxifying enzymes that they will struggle to respond to new compounds? One is just beginning to see the power of modern genomic and other biotechnologies to reconstruct the past and predict the future. Recently genotyping of preserved specimens by PCR has in fact suggested that some resistance mutations did predate insecticide use (Newcomb *et al.*, 2004). Likewise the tools of *in vitro* evolution, already used to predict resistance futures in the field of antibiotic resistant microorganisms, might also be useful in plotting possible futures for some key pesticide resistance enzymes.

Acknowledgments

The authors thank Thom Boyce, Bronwyn, Campbell, Chris Coppin, Erica Crone, Alan Devonshire, Peter East, Carol Hartley, Irene Horne, Kerrie-Anne Smyth, Tara Sutherland, and David Tattersall for sharing their insights into various topics covered in this review. They are also indebted to the tireless work of Narelle Dryden in compiling the literature. Undoubtedly, some significant relevant papers have been missed here, for which JGO takes full responsibility; the authors apologise to those authors whose work has been missed or inadvertently misrepresented.

References

Abdel-Aal, Y.A.I., Lampert, E.P., Roe, R.M., Semtner, P.J., **1992**. Diagnostic esterases and insecticide resistance in the tobacco aphid, *Myzus nicotianae* Blackman (Homoptera: Aphididae). *Pestic. Biochem. Physiol. 43*, 123–133.

Abdel-Aal, Y.A.I., Soderlund, D.M., **1980**. Pyrethroid hydrolysing esterases in southern armyworm larvae: tissue distribution, kinetic properties and selective inhibtion. *Pestic. Biochem. Physiol. 14*, 282–289.

Abdel-Aal, Y.A.I., Wolff, M.A., Roe, R.M., Lampert, E.P., **1990**. Aphid carboxylesterases: biochemical aspects and importance in the diagnosis of insecticide resistance. *Pestic. Biochem. Physiol. 38*, 255–266.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., *et al.*, **2000**. The genome sequence of *Drosophila melanogaster. Science 287*, 2185–2195.

Aldridge, W.N., Reiner, E., **1972**. Enzyme Inhibitors as Substrates: Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids. North-Holland Publishing, Amsterdam.

Anazawa, Y., Tomita, T., Aiki, Y., Kozaki, T., Kono, Y., **2003**. Sequence of a cDNA encoding acetylcholinesterase from susceptible and resistant two-spotted spider mite, *Tetranychus urticae*. *Insect Biochem. Mol. Biol. 33*, 509–514.

Anber, H.A.I., Oppenoorth, F.J., **1989**. A mutant esterase degrading organophosphates in a resistant strain of the predacious mite *Amblyseius potentillae* (Garman). *Pestic. Biochem. Physiol. 33*, 283–297.

Anber, H.A.I., Overmeer, W.P.J., **1988**. Resistance to organophosphates and carbamates in the predacious mite *Amblyseius potentillae* (Garman) due to insensitive acetylcholinesterase. *Pestic. Biochem. Physiol. 31*, 91–98.

Anderson, P.R., Oakeshott, J.G., **1984**. Parallel geographic patterns of allozyme variation in two sibling *Drosophila* species. *Nature 308*, 729–731.

Anspaugh, D.D., Kennedy, G.G., Roe, R.M., **1995**. Purification and characterization of a resistance-associated esterase from the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Pestic. Biochem. Physiol. 53*, 84–96.

Arbeitman, M.N., Furlong, E.E.M., Imam, F., Johnson, E., Null, B.H., *et al.*, **2002**. Gene expression during the life cycle of *Drosophila melanogaster. Science 297*, 2270–2275.

Argentine, J.A., Lee, S.H., Sos, M.A., Barry, S.R., Clark, J.M., **1995**. Permethrin resistance in a near isogenic stain of Colorado potato beetle. *Pestic. Biochem. Physiol. 53*, 97–115.

Armstrong, K.F., Suckling, D.M., **1988**. Investigations into the biochemical basis of azinphosmethyl resistance in the light brown apple moth, *Epiphyas postvittana* (Lepidoptera, Tortricidae). *Pestic. Biochem. Physiol. 32*, 62–73.

Arnason, E., Chambers, G.K., **1987**. Macromolecular interaction and the electrophoretic mobility of esterase-5 from *Drosophila pseudoobscura*. *Biochem. Genet. 25*, 287–307.

Arpigny, J.L., Jaeger, K.E., **1999**. Bacterial lipolytic enzymes: classification and properties. *Biochem. J. 343*, 177–183.

Arrese, E.L., Canavoso, L.E., Jouni, Z.E., Pennington, J.E., Tsuchida, K., *et al.*, **2001**. Lipid storage and mobilization in insects: current status and future directions. *Insect Biochem. Mol. Biol. 31*, 7–17.

Auld, V.J., Fetter, R.D., Broadie, K., Goodman, C.S., **1995**. Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood–nerve barrier in *Drosophila*. *Cell 81*, 757–767.

Ayad, H., Georghiou, G.P., **1975**. Resistance to organophosphates and carbamates in *Anopheles albimanus* based on reduced sensitivity of acetylcholinesterase. *J. Econ. Entomol. 68*, 295–297.

Bachurin, S.O., Shevtsova, E.P., Kireeva, E.G., Oxenkrug, G.F., Sablin, S.O., **2003**. Mitochondria as a target for neurotoxins and neuroprotective agents. *Ann. NY Acad. Sci. 993*, 334–344.

Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. *In vivo* fluctuation of JH, JH acid, and ecdysteroid

titer, and JH esterase activity, during development of 5th stadium *Manduca sexta*. *Insect Biochem. 17*, 989–996.

Baker, J.E., Fabrick, J.A., Zhu, K.Y., **1998a**. Characterization of esterases in malathion-resistant and susceptible strains of the pteromalid parasitoid *Anisopteromalus calandrae*. *Insect Biochem. Mol. Biol. 28*, 1039–1050.

Baker, J.E., Perez-Mendoza, J., Beeman, R.W., Throne, J.E., **1998b**. Fitness of a malathion-resistant strain of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *J. Econ. Entomol. 91*, 50–55.

Baker, J.E., Weaver, D.K., **1993**. Resistance in field strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera, Pteromalidae) and its host, *Sitophilus oryzae* (Coleoptera, Curculionidae), to malathion, chlorpyrifos-methyl, and pirimiphos-methyl. *Biol. Control 3*, 233–242.

Balakirev, E.S., Balakirev, E.I., Ayala, F.J., **2002**. Molecular evolution of the *Est-6* gene in *Drosophila melanogaster*: contrasting patterns of DNA variability in adjacent functional regions. *Gene 288*, 167–177.

Balakirev, E.S., Chechetkin, V.R., Lobzin, V.V., Ayala, F.J., **2003**. DNA polymorphism in the β-esterase gene cluster of *Drosophila melanogaster. Genetics 164*, 533–544.

Bannai, H., Tamada, Y., Maruyama, O., Nakai, K., Miyano, S., **2002**. Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics 18*, 298–305.

Barber, M.D., Moores, G.D., Tatchell, G.M., Vice, W.E., Denholm, I., **1999**. Insecticide resistance in the currant-lettuce aphid, *Nasonovia ribisnigri* (Hemiptera: Aphididae) in the UK. *Bull. Entomol. Res. 89*, 17–23.

Baxter, G.D., Barker, S.C., **2002**. Analysis of the sequence and expression of a second putative acetylcholinesterase cDNA from organophosphate-susceptible and organophosphate-resistant cattle ticks. *Insect Biochem. Mol. Biol. 32*, 815–820.

Beeman, R.W., Schmidt, B.A., **1982**. Biochemical and genetic aspects of malathion-specific resistance in the Indian meal moth (Lepidoptera: Pyralidae). *J. Econ. Entomol. 75*, 945–949.

Behra, M., Cousin, X., Bertrand, C., Vonesch, J.L., Biellmann, D., *et al.*, **2002**. Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. *Nature Neurosci. 5*, 111–118.

Bellas, T.E., Bartell, R.J., Hill, A., **1983**. Identification of two components of the sex pheromone of the moth, *Epiphyas postvittana* (Lepidoptera, Tortricidae). *J. Chem. Ecol. 9*, 503–512.

Benedict, M.Q., Scott, J.A., Cockburn, A.F., **1994**. High-level expression of the bacterial *opd* gene in *Drosophila melanogaster*: improved inducible insecticide resistance. *Insect Mol. Biol. 3*, 247–252.

Berrada, S., Fournier, D., Cuany, A., Nguyen, T.X., **1994**. Identification of resistance mechanisms in a selected laboratory strain of *Cacopsylla pyri* (Homoptera, Psyllidae): altered acetylcholinesterase and detoxifying oxidases. *Pestic. Biochem. Physiol. 48*, 41–47.

Berticat, C., Boquien, G., Raymond, M., Chevillon, C., **2002**. Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. *Genet. Res. 79*, 41–47.

Berticat, C., Marquine, M., Raymond, M., Chevillon, C., **2001**. Recombination between two amplified esterase alleles in *Culex pipiens*. *J. Hered. 92*, 349–351.

Beyssat-Arnaouty, V., Mouches, C., Georghiou, G.P., Pasteur, N., **1989**. Detection of organophosphate detoxifying esterases by dot-blot immunoassay in *Culex* mosquitoes. *J. Am. Mosquito Control Assoc. 5*, 196–200.

Bisset, J., Rodriguez, M., Soca, A., Pasteur, N., Raymond, M., **1997**. Cross-resistance to pyrethroid and organophosphorus insecticides in the southern house mosquito (Diptera: Culicidae) from Cuba. *J. Med. Entomol. 34*, 244–246.

Blackman, R.L., **1987**. Morphological discrimination of a tobacco-feeding form of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and a key to New World *Myzus* (Nectarosiphon) species. *Bull. Entomol. Res. 77*, 713–730.

Blackman, R.L., Spence, J.M., Field, L.M., Devonshire, A.L., **1995**. Chromosomal location of the amplified esterase genes conferring resistance to insecticides in *Myzus persicae* (Homoptera, Aphididae). *Heredity 75*, 297–302.

Blackman, R.L., Spence, J.M., Field, L.M., Devonshire, A.L., **1999**. Variation in the chromosomal distribution of amplified esterase (FE4) genes in Greek field populations of *Myzus persicae* (Sulzer). *Heredity 82*, 180–186.

Bloch, G., Wool, D., **1994**. Methidathion resistance in the sweet potato whitefly (Aleyrodidae, Homoptera) in Israel: selection, heritability, and correlated changes of esterase activity. *J. Econ. Entomol. 87*, 1147–1156.

Bolliger, M.F., Frei, K., Winterhalter, K.H., Gloor, S.M., **2001**. Identification of a novel neuroligin in humans which binds to PsD-95 and has a widespread expression. *Biochem. J. 356*, 581–588.

Bonning, B.C., Hammock, B.D., **1996**. Development of recombinant baculoviruses for insect control. *Annu. Rev. Entomol. 41*, 191–210.

Borrás, T., Morozova, T.V., Heinsohn, S.L., Lyman, R.F., Mackay, T.F.C., *et al.*, **2003**. Transcription profiling in *Drosophila* eyes that overexpress the human glaucoma-associated trabecular meshwork-inducible glucocorticoid response protein/myocilin (TIGR/MYOC). *Genetics 163*, 637–645.

Botti, S., Felder, C., Sussman, J., Silman, I., **1998**. Electrotactins: a class of adhesion proteins with conserved electrostatic and structural motifs. *Protein Eng. 11*, 415–420.

Boublik, Y., Saint-Aguet, P., Lougarre, A., Arnaud, M., Villatte, F., *et al.*, **2002**. Acetylcholinesterase engineering for detection of insecticide residues. *Protein Eng. 15*, 43–50.

Bourguet, D., Fonseca, D., Vourch, G., Dubois, M.P., Chandre, F., *et al.*, **1998**. The acetylcholinesterase gene *Ace*: a diagnostic marker for the pipiens and

quinquefasciatus forms of the *Culex pipiens* complex. *J. Am. Mosquito Control Assoc. 14*, 390–396.

Bourne, P.E., Shindyalov, I.N., **1998**. A database of pairwise aligned 3-D structures for the acetylcholinesterases, lipases and other homologous proteins. In: Doctor, B.P., Gentry, M.K., Taylor, P., Quinn, D.M., Rotundo, R.L. (Eds.), Structure and Function of Cholinesterases and Related Proteins. Plenum, New York, pp. 457–462.

Brady, J.P., Richmond, R.C., **1990**. Molecular analysis of evolutionary changes in the expression of *Drosophila* esterases. *Proc. Natl Acad. Sci. USA 87*, 8217–8221.

Brady, J.P., Richmond, R.C., **1992**. An evolutionary model for the duplication and divergence of esterase genes in *Drosophila*. *J. Mol. Evol. 34*, 506–521.

Brady, J.P., Richmond, R.C., Oakeshott, J.G., **1990**. Cloning of the esterase-5 locus from *Drosophila pseudoobscura* and comparison with its homologue in *D. melanogaster*. *Mol. Biol. Evol. 7*, 525–546.

Brogdon, W.G., Barber, A.M., **1990**. Fenitrothion-deltamethrin cross-resistance conferred by esterases in Guatemalan *Anopheles albimanus*. *Pestic. Biochem. Physiol. 37*, 130–139.

Brown, T.M., Bryson, P.K., **1992**. Selective inhibitors of methyl parathion-resistant acetylcholinesterase from *Heliothis virescens*. *Pestic. Biochem. Physiol. 44*, 155–164.

Bush, M.R., Abdel-Aal, Y.A.I., Rock, G.C., **1993**. Parathion resistance and esterase activity in codling moth (Lepidoptera: Tortricidae) from North Carolina. *J. Econ. Entomol. 86*, 660–666.

Butenandt, A., Groschel, U., Karlson, P., Zillig, W., **1959**. N-acetyl tyramine, its isolation from *Bombyx* cocoons and its chemical and biological properties. *Arch. Biochem. Biophys. 83*, 76–83.

Byrne, F.J., Devonshire, A.L., **1993**. Insensitive acetylcholinesterase and esterase polymorphism in susceptible and resistant populations of the tobacco whitefly *Bemisia tabaci* (Genn.). *Pestic. Biochem. Physiol. 45*, 34–42.

Byrne, F.J., Devonshire, A.L., **1996**. Biochemical evidence of haplodiploidy in the whitefly *Bemisia tabaci*. *Biochem. Genet. 34*, 93–107.

Byrne, F.J., Devonshire, A.L., **1997**. Kinetics of insensitive acetylcholinesterases in organophosphate-resistant tobacco whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae). *Pestic. Biochem. Physiol. 58*, 119–124.

Byrne, F.J., Gorman, K.J., Cahill, M., Denholm, I., Devonshire, A.L., **2000**. The role of B-type esterases in conferring insecticide resistance in the tobacco whitefly, *Bemisia tabaci* (Genn.). *Pest Mgt Sci. 56*, 867–874.

Callaghan, A., Parker, P.J.A.N., Holloway, G.J., **1998**. The use of variance in enzyme activity as an indicator of long-term exposure to toxicant-stressed environments in *Culex pipiens* mosquitoes. *Funct. Ecol. 12*, 436–441.

Campbell, B.E., **2001**. The role of esterases in pyrethroid resistance in Australian populations of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Ph.D. thesis, Australian National University, Canberra.

Campbell, P.M., Harcourt, R.L., Crone, E.J., Claudianos, C., Hammock, B.D., *et al.*, **2001**. Identification of a juvenile hormone esterase gene by matching its peptide mass fingerprint with a sequence from the *Drosophila* genome project. *Insect Biochem. Mol. Biol. 31*, 513–520.

Campbell, P.M., Newcomb, R.D., Russell, R.J., Oakeshott, J.G., **1998a**. Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 28*, 139–150.

Campbell, P.M., Oakeshott, J.G., Healy, M.J., **1998b**. Purification and kinetic characterisation of juvenile hormone esterase from *Drosophila melanogaster*. *Insect Biochem. Mol. Biol. 28*, 501–515.

Campbell, P.M., Robin, G.C.D., Court, L.N., Dorrian, S.J., Russell, R.J., *et al.*, **2003**. Developmental expression and gene/enzyme identifications in the alpha-esterase gene cluster of *Drosophila melanogaster*. *Insect Mol. Biol. 12*, 459–471.

Campbell, P.M., Trott, J.F., Claudianos, C., Smyth, K.A., Russell, R.J., *et al.*, **1997**. Biochemistry of esterases associated with organophosphate resistance in *Lucilia cuprina* with comparisons to putative orthologues in other Diptera. *Biochem. Genet. 35*, 17–40.

Campbell, P.M., Yen, J.L., Masoumi, A., Russell, R.J., Batterham, P., *et al.*, **1998c**. Cross-resistance patterns among *Lucilia cuprina* (Diptera: Calliphoridae) resistant to organophosphorus insecticides. *J. Econ. Entomol. 91*, 367–375.

Casabé, N., Zerba, E.N., **1981**. Esterases of *Triatoma infestans* and its relationship with the metabolism of organophosphorus insecticides. *Comp. Biochem. Physiol. C 68*, 255–258.

Casida, J.E., Gammon, D.W., Glickman, A.H., Lawrence, L.J., **1983**. Mechanisms of selective action of pyrethroid insecticides. *Annu. Rev. Pharmacol. 23*, 413–438.

Chang, C.K., Whalon, M.E., **1986**. Hydrolysis of permethrin by pyrethroid esterases from resistant and susceptible strains of *Amblyseius fallacis*. *Pestic. Biochem. Physiol. 25*, 446–452.

Chang, C.K., Whalon, M.E., **1987**. Substrate specificities and multiple forms of esterases in the brown planthopper. *Pestic. Biochem. Physiol. 27*, 30–35.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., *et al.*, **2003**. The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. *Proc. Natl Acad. Sci. USA 100*, 9923–9928.

Charpentier, A., Villatte, F., Fournier, D., **1998**. Acetylcholinesterase increase in *Drosophila* as a mechanism of resistance to insecticide. In: Doctor, B.P., Gentry, M.K., Taylor, P., Quinn, D.M., Rotundo, R.L. (Eds.), Structure and Function of Cholinesterases and Related Proteins. Plenum, New York, pp. 503–507.

Chen, W.L., Sun, C.-N., **1994**. Purification and characterization of carboxylesterases of a rice brown planthopper,

Nilaparvata lugens Stäl. *Insect Biochem. Mol. Biol. 24*, 347–355.

Chen, Z.Z., Newcomb, R., Forbes, E., McKenzie, J., Batterham, P., **2001**. The acetylcholinesterase gene and organophosphorus resistance in the Australian sheep blowfly, *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 31*, 805–816.

Chevillon, C., Bourguet, D., Rousset, F., Pasteur, N., Raymond, M., **1997**. Pleiotropy of adaptive changes in populations: comparisons among insecticide resistance genes in *Culex pipiens*. *Genet. Res. 70*, 195–203.

Chiang, S.W., Sun, C.N., **1996**. Purification and characterization of carboxylesterases of a rice green leafhopper *Nephotettix cincticeps* Uhler. *Pestic. Biochem. Physiol. 54*, 181–189.

Chou, K.C., **2001**. Using subsite coupling to predict signal peptides. *Protein Eng. 14*, 75–79.

Clarris, H.J., McKeown, S., Key, B., **2002**. Expression of neurexin ligands, the neuroligins and the neurexophilins, in the developing and adult rodent olfactory bulb. *Int. J. Devel. Biol. 46*, 649–652.

Claudianos, C., Crone, E., Coppin, C., Russell, R., Oakeshott, J., **2001**. A genomics perspective on mutant aliesterases and metabolic resistance to organophosphates. In: Marshall Clark, J., Yamaguchi, I. (Eds.), Agrochemical Resistance: Extent, Mechanism and Detection. American Chemical Society, Washington, DC, pp. 90–101.

Claudianos, C., Russell, R.J., Oakeshott, J.G., **1999**. The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect Biochem. Mol. Biol. 29*, 675–686.

Clements, K.M., Sorenson, C.E., Wiegmann, B.M., Neese, P.A., Roe, R.M., **2000**. Genetic, biochemical, and behavioral uniformity among populations of *Myzus nicotianae* and *Myzus persicae*. *Entomol. Exp. Applic. 95*, 269–281.

Coates, P.M., Mestriner, M.A., Hopkinson, D.A., **1975**. A preliminary genetic interpretation of the esterase isozymes of human tissues. *Ann. Hum. Genet. 39*, 1–20.

Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., *et al.*, **1986**. Genetic selection of a plasmodium-refractory strain of the malaria vector *Anopheles gambiae*. *Science 234*, 607–610.

Collins, P.J., Rose, H.A., Wegecsanyi, M., **1992**. Enzyme activity in strains of the sawtoothed grain beetle (Coleoptera, Cucujidae) differentially resistant to fenitrothion, malathion, and chlorpyrifos-methyl. *J. Econ. Entomol. 85*, 1571–1575.

Combes, D., Fedon, Y., Grauso, M., Toutant, J.P., Arpagaus, M., **2000**. Four genes encode acetylcholinesterases in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*: cDNA sequences, genomic structures, mutations and *in vivo* expression. *J. Mol. Biol. 300*, 727–742.

Combes, D., Fedon, Y., Toutant, J.P., Arpagaus, M., **2003**. Multiple *ace* genes encoding acetylcholinesterases of *Caenorhabditis elegans* have distinct tissue expression. *Eur. J. Neurosci. 18*, 497–512.

Conyers, C.M., Macnicoll, A.D., Price, N.R., **1998**. Purification and characterisation of an esterase involved in resistance to organophosphorus insecticides in the saw-toothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). *Insect Biochem. Mol. Biol. 28*, 435–448.

Cooke, P.H., Oakeshott, J.G., **1989**. Amino acid polymorphisms for esterase-6 in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 86*, 1426–1430.

Costa, H.S., Brown, J.K., **1991**. Variation in biological characteristics and esterase pattern among populations of *Bemisia tabaci* and association of one population with silverleaf symptom induction. *Entomol. Exp. Applic. 61*, 211–219.

Crews-Oyen, A.E., Kumar, V., Collins, F.H., **1993**. Association of two esterase genes, a chromosomal inversion, and susceptibility to *Plasmodium cynomolgi* in the African malaria vector *Anopheles gambiae*. *Am. J. Trop. Med. Hyg. 49*, 341–347.

Cuany, A., Handani, J., Bergé, J., Fournier, D., Raymond, M., *et al.*, **1993**. Action of esterase B1 on chlorpyrifos in organophosphate-resistant *Culex* mosquitoes. *Pestic. Biochem. Physiol. 45*, 1–6.

Dang, H T., Gunning, R., **2002**. Resistance to *Bacillus thuringiensis* Delta-endotoxin CrylAc in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae). In: Akhurst, R.J., Beard, C.E., Hughes, P. (Eds.), Biotechnology of *Bacillus thuringiensis* and its Environmental Impact. CSIRO, Canberra, pp. 100–103.

Darboux, I., Barthalay, Y., Piovant, M., Hipeaujacquotte, R., **1996**. The structure–function relationships in *Drosophila* neurotactin show that cholinesterasic domains may have adhesive properties. *EMBO J. 15*, 4835–4843.

Davies, A.G., Game, A.Y., Chen, Z., Williams, T.J., Goodall, S., *et al.*, **1996**. Scalloped wings is the *Lucilia cuprina* Notch homologue and a candidate for the modifier of fitness and asymmetry of diazinon resistance. *Genetics 143*, 1321–1337.

Dawson, G.W., Griffiths, D.C., Pickett, J.A., Woodcock, C.M., **1983**. Decreased response to alarm pheromones by insecticide-resistant aphids. *Naturwissenschaften 70*, 254–255.

de Jersey, J., Nolan, J., Davey, P.A., Riddles, P.W., **1985**. Separation and characterization of the pyrethroid-hydrolyzing esterases of the cattle tick, *Boophilus microplus*. *Pestic. Biochem. Physiol. 23*, 349–357.

de Kort, C.A.D., Granger, N.A., **1981**. Regulation of the juvenile hormone titer. *Annu. Rev. Entomol. 26*, 1–28.

de Kort, C.A.D., Granger, N.A., **1996**. Regulation of JH titers: the relevance of degradative enzymes and binding proteins. *Arch. Insect Biochem. Physiol. 33*, 1–26.

De Malkenson, N.C., Wood, E.J., Zerba, E.N., **1984**. Isolation and characterization of an esterase of *Triatoma infestans* with a critical role in the degradation of organophosphorus esters. *Insect Biochem. 14*, 481–486.

De Silva, D., Hemingway, J., Ranson, H., Vaughan, A., **1997**. Resistance to insecticides in insect vectors of disease: Estα3, a novel amplified esterase associated with amplified Estβ1 from insecticide resistant strains of the mosquito *Culex quinquesfasciatus*. *Exp. Parasitol. 87*, 253–259.

Dean, C., Scholl, F.G., Choih, J., Demaria, S., Berger, J., *et al.*, **2003**. Neurexin mediates the assembly of presynaptic terminals. *Nat. Neurosci. 6*, 708–716.

Delorme, R., Fournier, D., Chaufaux, J., Cuany, A., Bride, J.M., *et al.*, **1988**. Esterase metabolism and reduced penetration are causes of resistance to deltamethrin in *Spodoptera exigua* HUB (Noctuidae: Lepidoptera). *Pestic. Biochem. Physiol. 32*, 240–246.

Devonshire, A.L., **1977**. The properties of a carboxylesterase from the peach-potato aphid, *Myzus persicae* (Sulz.), and its role in conferring insecticide resistance. *Biochem. J. 167*, 675–683.

Devonshire, A.L., **1989**. The role of electrophoresis in the biochemical detection of insecticide resistance. In: Loxdale, H.D., Hollander, J.D. (Eds.), Electrophoretic Studies on Agricultural Pests. Clarendon Press, Oxford, pp. 363–377.

Devonshire, A.L., Field, L.M., Foster, S.P., Moores, G.D., Williamson, M.S., *et al.*, **1998**. The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Phil. Trans. Roy. Soc. London B 353*, 1677–1684.

Devonshire, A.L., Heidari, R., Bell, K.L., Campbell, P.M., Campbell, B.E., *et al.*, **2003**. Kinetic efficiency of mutant carboxylesterases implicated in organophosphate insecticide resistance. *Pestic. Biochem. Physiol. 76*, 1–13.

Devonshire, A.L., Moores, G.D., **1982**. A carboxylesterase with broad substrate specificity causes organophosphorous, carbamate and pyrethroid resistance in peach potato aphids (*Myzus persicae*). *Pestic. Biochem. Physiol. 18*, 235–246.

Devonshire, A.L., Moores, G.D., **1989**. Detoxication of insecticides by esterases from *Myzus persicae*: is hydrolysis important? In: Reiner, E., Aldridge, W.N., Hoskin, F.C.G. (Eds.), Esterases Hydrolysing Organophosphorus Compounds. Ellis Horwood, Chichester, pp. 180–192.

Devonshire, A.L., Sawicki, R.M., **1979**. Insecticide resistant *Myzus persicae* as an example of evolution by gene duplication. *Nature 280*, 140–141.

Devonshire, A.L., Searle, L.M., Moores, G.D., **1986**. Quantitative and qualitative variation in the messenger-RNA for carboxylesterases in insecticide-susceptible and resistant *Myzus persicae* (Sulz). *Insect Biochem. 16*, 659–665.

Dimopoulos, G., Richman, A., Muller, H.M., Kafatos, F.C., **1997**. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. Natl Acad. Sci. USA. 94*, 11508–11513.

Doctor, B.P., Taylor, P., Quinn, D.M., Rotundo, R.L., Gentry, M.K. (Eds.), **1998**. Structure and Function of Cholinesterases and Related Proteins. Plenum, New York.

Dowd, P.F., Gagne, C.C., Sparks, T.C., **1987**. Enhanced pyrethroid hydrolysis in pyrethroid-resistant larvae of the tobacco budworm, *Heliothis virescens* (F). *Pestic. Biochem. Physiol. 28*, 9–16.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., *et al.*, **2003**. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat. Biotechnol. 21*, 1307–1313.

Dumancic, M.M., Oakeshott, J.G., Russell, R.J., Healy, M.J., **1997**. Characterization of the *EstP* protein in *Drosophila melanogaster* and its conservation in Drosophilids. *Biochem. Genet. 35*, 251–271.

Dumas, D.P., Wild, J.R., Raushel, F.M., **1990**. Expression of *Pseudomonas* phosphotriesterase activity in the fall armyworm confers resistance to insecticides. *Experientia 46*, 729–731.

Dyte, C.E., Rowlands, D.G., **1968**. The metabolism and synergism of malathion in resistant and susceptible strains of *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae). *J. Stored Prod. Res. 4*, 157–173.

East, P., Graham, A., Whitington, G., **1990**. Molecular isolation and preliminary characterisation of a duplicated esterase locus in *Drosophila buzzatii*. In: Barker, J.S.F., Starmer, W.T., MacIntyre, R.J. (Eds.), Ecological and Evolutionary Genetics of *Drosophila*. Plenum, New York, pp. 389–406.

El-Khatib, Z.I., Georghiou, G.P., **1985**. Geographic variation of resistance to organophosphates, propoxur and DDT in the southern house mosquito, *Culex quinquefasciatus*, in California. *J. Am. Mosquito Control Assoc. 1*, 279–283.

El Saidy, M.F., Auda, M., Degheele, D., **1989**. Detoxification mechanisms of diflubenzuron and teflubenzuron in the larvae of *Spodoptera littoralis* (Boisd). *Pestic. Biochem. Physiol. 35*, 211–222.

Elliott, M., **1977**. Synthetic pyrethroids. In: Elliott, M. (Ed.), Synthetic Pyrethroids. American Chemical Society, Washington, DC, pp. 1–28.

Emanuelsson, O., Nielsen, H., Brunak, S., Von Heijne, G., **2000**. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol. 300*, 1005–1016.

Enikolopov, G.N., Khechumyan, R.K., Kuzin, B.A., Korochkin, L.I., Georgiev, G.P., **1989**. Molecular organisation of a family of genes specifying *Drosophila virilis* esterases: tissue specific expression of the gene for *Drosophila virilis* esterase S. *Soviet Genet. 25*, 265–273.

Estrada-Mondaca, S., Fournier, D., **1998**. Stabilization of recombinant *Drosophila* acetylcholinesterase. *Protein Express. Purific. 12*, 166–172.

Eto, M., **1974**. Organophosphorus Pesticides: Organic and Biological Chemistry. CRC Press, Cleveland, OH.

Ferrari, J.A., Morse, J.G., Georghiou, G.P., Sun, Y.Q., **1993**. Elevated esterase-activity and acetylcholinesterase insensitivity in citrus thrips (Thysanoptera,

Thripidae) populations from the San Joaquin Valley of California. *J. Econ. Entomol. 86*, 1645–1650.

ffrench-Constant, R.H., Devonshire, A.L., **1988**. Monitoring frequencies of insecticide resistance in *Myzus persicae* (Sulzer) (Hemiptera, Aphididae) in England during 1985–86 by immunoassay. *Bull. Entomol. Res. 78*, 163–171.

ffrench-Constant, R.H., Pittendrigh, B., Vaughan, A., Anthony, N., **1998**. Why are there so few resistance-associated mutations in insecticide target genes? *Phil. Trans. Roy. Soc. London B 353*, 1685–1693.

Field, L.M., **2000**. Methylation and expression of amplified esterase genes in the aphid *Myzus persicae* (Sulzer). *Biochem. J. 349*, 863–868.

Field, L.M., Anderson, A.P., Denholm, I., Foster, S.P., Harling, Z.K., *et al.*, **1997**. Use of biochemical and DNA diagnostics for characterising multiple mechanisms of insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). *Pestic. Sci. 51*, 283–289.

Field, L.M., Blackman, R.L., **2003**. Insecticide resistance in the aphid *Myzus persicae* (Sulzer): chromosome location and epigenetic effects on esterase gene expression in clonal lineages. *Biol. J. Linnean Soc. 79*, 107–113.

Field, L.M., Blackman, R.L., Tyler-Smith, C., Devonshire, A.L., **1999**. Relationship between amount of esterase and gene copy number in insecticide-resistant *Myzus persicae* (Sulzer). *Biochem. J. 339*, 737–742.

Field, L.M., Devonshire, A.L., **1998**. Evidence that the E4 and FE4 esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* (Sulzer) are part of a gene family. *Biochem. J. 330*, 169–173.

Field, L.M., Devonshire, A.L., ffrench-Constant, R.H., Forde, B.G., **1989**. Changes in DNA methylation are associated with loss of insecticide resistance in the peach-potato aphid *Myzus persicae* (Sulz.). *FEBS Lett. 243*, 323–327.

Field, L.M., Devonshire, A.L., Forde, B.G., **1988**. Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem. J. 251*, 309–312.

Field, L.M., Javed, N., Stribley, M.F., Devonshire, A.L., **1994**. The peach potato aphid *Myzus persicae* and the tobacco aphid *Myzus nicotianae* have the same esterase-based mechanisms of insecticide resistance. *Insect Mol. Biol. 3*, 143–148.

Field, L.M., Williamson, M.S., Moores, G.D., Devonshire, A.L., **1993**. Cloning and analysis of the esterase genes conferring insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). *Biochem. J. 294*, 569–574.

Fojan, P., Jonson, P.H., Petersen, M.T.N., Petersen, S.B., **2000**. What distinguishes an esterase from a lipase: a novel structural approach. *Biochimie 82*, 1033–1041.

Foster, S.P., Harrington, R., Devonshire, A.L., Denholm, I., Devine, G.J.K.M.G., *et al.*, **1996**. Comparative survival of insecticide-susceptible and resistant peach-potato aphids *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) in low temperature field trials. *Bull. Entomol. Res. 86*, 17–27.

Foster, S.P., Kift, N.B., Baverstock, J., Sime, S., Reynolds, K., *et al.*, **2003**. Association of MACE-based insecticide resistance in *Myzus persicae* with reproductive rate, response to alarm pheromone and vulnerability to attack by *Aphidius colemani*. *Pest Mgt Sci. 59*, 1169–1178.

Fournier, D., Bergé, J.-B., Cardoso de Almeida, M.-L., Bordier, C., **1988**. Acetylcholinesterases from *Musca domestica* and *Drosophila melanogaster* brain are linked to membranes by a glycophospholipid anchor sensitive to an endogenous phospholipase. *J. Neurochem. 50*, 1158–1163.

Fournier, D., Bride, J.-M., Hoffmann, F., Karch, F., **1992**. Acetylcholinesterase: two types of modifications confer resistance to insecticide. *J. Biol. Chem. 267*, 14270–14274.

Fournier, D., Bride, J.-M., Mouchès, C., Raymond, M., Magnin, M., *et al.*, **1987**. Biochemical characterization of the esterases A1 and B1 associated with organophosphate resistance in the *Culex pipiens* L. complex. *Pestic. Biochem. Physiol. 27*, 211–217.

Fournier, D., Mutero, A., **1994**. Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comp. Biochem. Physiol. C 108*, 19–31.

Fremion, F., Darboux, I., Diano, M., Hipeau-Jacquotte, R., Seeger, M.A., *et al.*, **2000**. Amalgam is a ligand for the transmembrane receptor neurotactin and is required for neurotactin-mediated cell adhesion and axon fasciculation in *Drosophila*. *EMBO J. 19*, 4463–4472.

Furk, C., Powell, D.F., Heyd, S., **1980**. Primicarb resistance in the melon and cotton aphid, *Aphis gossypii* Glover. *Plant Pathol. 29*, 191.

Gabay, J.E., Almeida, R.P., **1993**. Antibiotic peptides and serine protease homologs in human polymorphonuclear leukocytes: defensins and azurocidin. *Curr. Opin. Immunol. 5*, 97–102.

Game, A.Y., Oakeshott, J.G., **1990**. Associations between restriction site polymorphism and enzyme activity variation for esterase 6 in *Drosophila melanogaster. Genetics 126*, 1021–1031.

Gazave, L., Chevillon, C., Lenormand, T., Marquine, M., Raymond, M., **2001**. Dissecting the cost of insecticide resistance genes during the overwintering period of the mosquito *Culex pipiens*. *Heredity 87*, 441–448.

Genova, J.L., Fehon, R.G., **2003**. Neuroglian, gliotactin, and the Na^+/K^+ ATPase are essential for septate junction function in *Drosophila*. *J. Cell Biol. 161*, 979–989.

Georghiou, G.P., Pasteur, N., Hawley, M.K., **1980**. Linkage relationships between organophosphate resistance and a highly active esterase-B in *Culex pipiens quinquefaciatus* Say from California. *J. Econ. Entomol. 73*, 301–305.

Gibney, G., Camp, S., Dionne, M., Macpheequigley, K., Taylor, P., **1990**. Mutagenesis of essential functional residues in acetylcholinesterase. *Proc. Natl Acad. Sci. USA 87*, 7546–7550.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on

future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Gilbert, M., Smith, J., Roskams, A.J., Auld, V.J., **2001**. Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. *Glia 34*, 151–164.

Gnagey, A.L., Forte, M., Rosenberry, T.L., **1987**. Isolation and characterization of acetylcholinesterase from *Drosophila*. *J. Biol. Chem. 262*, 13290–13298.

Goh, D.K.S., Anspaugh, D.D., Motoyama, N., Rock, G.C., Roe, R.M., **1995**. Isolation and characterization of an insecticide-resistance-associated esterase in the tobacco budworm *Heliothis virescens* (F). *Pestic. Biochem. Physiol. 51*, 192–204.

Gomez, G.A., Hasson, E., **2003**. Transpecific polymorphisms in an inversion linked esterase locus in *Drosophila buzzatii*. *J. Mol. Evol. 20*, 410–423.

Grifman, M., Galyam, N., Seidman, S., Soreq, H., **1998**. Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. *Proc. Natl Acad. Sci. USA 95*, 13935–13940.

Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H., **1999**. Structural roles of acetylcholinesterase variants in biology and pathology. *Eur. J. Biochem. 264*, 672–686.

Guerrero, F.D., **2000**. Cloning of a horn fly cDNA, *HiαE7*, encoding an esterase whose transcript concentration is elevated in diazinon-resistant flies. *Insect Biochem. Mol. Biol. 30*, 1107–1115.

Guillemaud, T., Lenormand, T., Bourguet, D., Chevillon, C., Pasteur, N., *et al*., **1998**. Evolution of resistance in *Culex pipiens*: allele replacement and changing environment. *Evolution 52*, 443–453.

Guillemaud, T., Makate, N., Raymond, M., Hirst, B., Callaghan, A., **1997**. Esterase gene amplification in *Culex pipiens*. *Insect Mol. Biol. 6*, 319–327.

Guillemaud, T., Rooker, S., Pasteur, N., Raymond, M., **1996**. Testing the unique amplification event and the worldwide migration hypothesis of insecticide resistance genes with sequence data. *Heredity 77*, 535–543.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1996a**. Insensitive acetylcholinesterase and resistance to thiodicarb in Australian *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). *Pestic. Biochem. Physiol. 55*, 21–28.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1996b**. Esterases and esfenvalerate resistance in Australian *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pestic. Biochem. Physiol. 54*, 12–23.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1997**. Esterases and fenvalerate resistance in a field population of *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in Australia. *Pestic. Biochem. Physiol. 58*, 155–162.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1998**. Inhibition of pyrethroid resistance related esterases by piperonyl butoxide in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Aphis gossypii* (Hemiptera: Aphididae). In: Jones, D.G. (Ed.), Piperonyl Butoxide: The Insecticide Synergist. Academic Press, San Diego, CA.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1999**. Esterase inhibitors synergise the toxicity of pyrethroids in Australian *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pestic. Biochem. Physiol. 63*, 50–62.

Gupta, A.P. (Ed.), **1990**. Morphogenetic Hormones of Arthropods: Recent Advances in Comparative Arthropod Morphology, Physiology, and Development. Part 1: Discoveries, Syntheses, Metabolism, Evolution, Modes of Action, and Techniques. Rutgers University Press, New Brunswick, NJ.

Hajos, J.P., Vermunt, A.M.W., Zuidema, D., Kuclsar, P., Varjas, L., *et al*., **1999**. Dissecting insect development: baculovirus-mediated gene silencing in insects. *Insect Mol. Biol. 8*, 539–544.

Hammock, B.D., **1985**. Regulation of juvenile hormone titre: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 431–472.

Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N., Maeda, S., **1990**. Expression and effects of the juvenile-hormone esterase in a baculovirus vector. *Nature 344*, 458–461.

Hammock, B.D., McCutchen, B.F., Beetham, J., Choudary, P.V., Fowler, E., *et al*., **1993**. Development of recombinant viral insecticides by expression of an insect-specific toxin and insect-specific enzyme in nuclear polyhedrosis viruses. *Arch. Insect Biochem. Physiol. 22*, 315–344.

Harel, M., Kryger, G., Rosenberry, T.L., Mallender, W.D., Lewis, T., *et al*., **2000**. Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. *Protein Sci. 9*, 1063–1072.

Harold, J.A., Ottea, J.A., **1997**. Toxicological significance of enzyme activities in profenofos-resistant tobacco budworms, *Heliothis virescens* (F.). *Pestic. Biochem. Physiol. 58*, 23–33.

Harold, J.A., Ottea, J.A., **2000**. Characterization of esterases associated with profenofos resistance in the tobacco budworm, *Heliothis virescens* (F.). *Arch. Insect Biochem. Physiol. 45*, 47–59.

Hasui, H., Ozaki, K., **1984**. Electrophoretic esterase patterns in the brown planthopper, *Nilaparvata lugens* Stäl (Hemiptera: Delphacidae) which developed resistance to insecticides. *Appl. Entomol. Zool. 19*, 52–58.

Hawkes, N.J., Hemingway, J., **2002**. Analysis of the promoters for the beta-esterase genes associated with insecticide resistance in the mosquito *Culex quinquefasciatus*. *Biochim. Biophys. Acta 1574*, 51–62.

Healy, M.J., Dumancic, M.M., Oakeshott, J.G., **1991**. Biochemical and physiological studies of soluble esterases from *Drosophila melanogaster*. *Biochem. Genet. 29*, 365–388.

Heidari, R., Devonshire, A.L., Campbell, B.E., Bell, K.L., Dorrian, S.J., *et al*., **2004a**. Hydrolysis of organophosphorus insecticides by *in vitro* modified carboxylesterase

E3 from *Lucilia cuprina*. *Insect Biochem. Mol. Biol. 34*, 353–363.

Hemingway, J., **1982**. The biochemical nature of malathion resistance in *Anopheles stephensi* from Pakistan. *Pestic. Biochem. Physiol. 17*, 149–155.

Hemingway, J., **1983**. The genetics of malathion resistance in *Anopheles stephensi* from Pakistan. *Trans. Roy. Soc. Trop. Med. Hyg. 77*, 106–108.

Hemingway, J., **1985**. Malathion carboxylesterase enzymes in *Anopheles arabiensis* from Sudan. *Pestic. Biochem. Physiol. 23*, 309–313.

Hemingway, J., Callaghan, A., Amin, A.M., **1990**. Mechanisms of organophosphate and carbamate resistance in *Culex quinquefasciatus* from Saudi Arabia. *Med. Vet. Entomol. 4*, 275–282.

Hemingway, J., Callaghan, A., Kurtak, D.C., **1989**. Temephos resistance in *Simulium damnosum* Theobald (Diptera, Simuliidae): a comparative study between larvae and adults of the forest and savanna strains of this species complex. *Bull. Entomol. Res. 79*, 659–669.

Hemingway, J., Coleman, M., Paton, M., McCarroll, L., Vaughan, A., *et al.*, **2000**. Aldehyde oxidase is coamplified with the world's most common *Culex* mosquito insecticide resistance-associated esterases. *Insect Mol. Biol. 9*, 93–99.

Hemingway, J., Georghiou, G., **1983**. Studies on the acetylcholinesterase of *Anopheles albimanus* resistant and susceptible to organophosphate and carbamate insecticides. *Pestic. Biochem. Physiol. 19*, 167–171.

Hemingway, J., Hawkes, N., Prapanthadara, L., Jayawardenal, K.G., Ranson, H., **1998**. The role of gene splicing, gene amplification and regulation in mosquito insecticide resistance. *Phil. Trans. Roy. Soc. London B 353*, 1695–1699.

Hemingway, J., Karunaratne, S.H., **1998**. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med. Vet. Entomol. 12*, 1–12.

Hemingway, J., Malcolm, C.A., Kissoon, K.E., Boddington, R.G., Curtis, C.F., *et al.*, **1985**. The biochemistry of insecticide resistance in *Anopheles sacharovi*: comparative studies with a range of insecticide susceptible and resistant *Anopheles* and *Culex* species. *Pestic. Biochem. Physiol. 24*, 68–76.

Herath, P.R.J., Hemingway, J., Weerasinghe, I.S., Jayawardena, K.G.I., **1987**. The detection and characterization of malathion resistance in field populations of *Anopheles culicifacies* B in Sri Lanka. *Pestic. Biochem. Physiol. 29*, 157–162.

Hick, C.A., Field, L.M., Devonshire, A.L., **1996**. Changes in the methylation of amplified esterase DNA during loss and reselection of insecticide resistance in peach-potato aphids, *Myzus persica*e. *Insect Biochem. Mol. Biol. 26*, 41–47.

Hinton, A.C., Hammock, B.D., **2003**. Juvenile hormone esterase (JHE) from *Tenebrio molitor*: full-length cDNA sequence, *in vitro* expression, and characterization of the recombinant protein. *Insect Biochem. Mol. Biol. 33*, 477–487.

Hoffmann, F., Fournier, D., Spierer, P., **1992**. Minigene rescues acetylcholinesterase lethal mutations in *Drosophila melanogaster. J. Mol. Biol. 223*, 17–22.

Holloway, G.J., Crocker, H.J., Callaghan, A., **1997**. The effects of novel and stressful environments on trait distribution. *Funct. Ecol. 11*, 579–584.

Holmes, R.S., Masters, C.J., **1967**. The developmental multiplicity and isoenzyme status of cavian esterases. *Biochim. Biophys. Acta 132*, 379–399.

Horne, I., Sutherland, T.D., Harcourt, R.L., Russell, R.J., Oakeshott, J.G., **2002a**. Identification of an *opd* (organophosphate degradation) gene in an agrobacterium isolate. *Appl. Envir. Microbiol. 68*, 3371–3376.

Horne, I., Sutherland, T.D., Oakeshott, J.G., Russell, R.J., **2002b**. Cloning and expression of the phosphotriesterase gene *hocA* from *Pseudomonas monteilii* C11. *Microbiology 148*, 2687–2695.

Hortsch, M., Patel, N.H., Bieber, A.J., Traquina, Z.R., Goodman, C.S., **1990**. *Drosophila* neurotactin, a surface glycoprotein with homology to serine esterases, is dynamically expressed during embryogenesis. *Development 110*, 1327–1340.

Hou, X., Maser, R.L., Magenheimer, B.S., Calvet, J.P., **1996**. A mouse kidney- and liver-expressed cDNA having homology with a prokaryotic parathion hydrolase (phosphotriesterase)-encoding gene: abnormal expression in injured and polycystic kidneys. *Gene 168*, 157–163.

Huang, Q., Deveraux, Q.L., Maeda, S., Salvesen, G.S., Stennicke, H.R., *et al.*, **2000**. Evolutionary conservation of apoptosis mechanisms: lepidopteran and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalian caspase-9. *Proc. Natl Acad. Sci. USA 97*, 1427–1432.

Hughes, P.B., Devonshire, A.L., **1982**. The biochemical basis of resistance to organophosphorus insecticides in the sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol. 18*, 289–297.

Hughes, P.B., Green, P.E., Reichmann, K.G., **1984**. Specific resistance to malathion in laboratory and field populations of the Australian sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae). *J. Econ. Entomol. 77*, 1400–1404.

Hughes, P.B., Raftos, D.A., **1985**. Genetics of an esterase associated with resistance to organophosphorus insecticides in the sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Bull. Entomol. Res. 75*, 535–544.

Ichtchenko, K., Nguyen, T., Sudhof, T.C., **1996**. Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J. Biol. Chem. 271*, 2676–2682.

Ishaaya, I., Casida, J.E., **1980**. Properties and toxicological significance of esterases hydrolyzing permethrin and cypermethrin in *Trichoplusia ni* larval gut and integument. *Pestic. Biochem. Physiol. 14*, 178–184.

Ishaaya, I., Casida, J.E., **1981**. Pyrethroid esterase(s) may contribute to natural pyrethroid tolerance of larvae of the common green lacewing. *Envir. Entomol. 10*, 681–684.

Ishaaya, I., Degheele, D., **1988**. Properties and toxicological significance of diflubenzuron hydrolase activity in *Spodoptera littoralis* larvae. *Pestic. Biochem. Physiol. 32*, 180–187.

Ishaaya, I., Klein, M., **1990**. Response of susceptible laboratory and resistant field strains of *Spodoptera littoralis* (Lepidoptera, Noctuidae) to teflubenzuron. *J. Econ. Entomol. 83*, 59–62.

Ishida, Y., Leal, W.S., **2002**. Cloning of putative odorant-degrading enzyme and integumental esterase cDNAs from the wild silkmoth, *Antheraea polyphemus*. *Insect Biochem. Mol. Biol. 32*, 1775–1780.

Ismail, S.M., Goin, C., Muthumani, K., Kim, M., Dahm, K.H., *et al.*, **2000**. Juvenile hormone acid and ecdysteroid together induce competence for metamorphosis of the Verson's gland in *Manduca sexta*. *J. Insect Physiol. 46*, 59–68.

Ismail, S.M., Satyanarayana, K., Bradfield, J.Y., Dahm, K.H., Bhaskaran, G., **1998**. Juvenile hormone acid: evidence for a hormonal function in induction of vitellogenin in larvae of *Manduca sexta*. *Arch. Insect Biochem. Physiol. 37*, 305–314.

Iwata, T., Hama, H., **1972**. Insensitivity of cholinesterase in *Nephotettix cincticeps* resistant to carbamate and organophosphorus insecticides. *J. Econ. Entomol. 65*, 643–644.

Jamroz, R.C., Guerrero, F.D., Pruett, J.H., Oehler, D.D., Miller, R.J., **2000**. Molecular and biochemical survey of acaricide resistance mechanisms in larvae from Mexican strains of the southern cattle tick, *Boophilus microplus*. *J. Insect Physiol. 46*, 685–695.

Jao, L.T., Casida, J.E., **1974**. Insect pyrethroid-hydrolyzing esterases. *Pestic. Biochem. Physiol. 4*, 465–472.

Jones, G., Venkataraman, V., Ridley, B., O'Mahony, P., Turner, H., **1994**. Structure, expression and gene sequence of a juvenile hormone esterase-related protein from metamorphosing larvae of *Trichoplusia ni*. *Biochem. J. 302*, 827–835.

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., *et al.*, **2000**. Analysis of fractalkine receptor CC(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol. 20*, 4106–4114.

Kadono-Okuda, K., Ridley, B., Jones, D., Jones, G., **2000**. Distinctive structural and kinetic properties of an unusual juvenile hormone-hydrolyzing esterase. *Biochem. Biophys. Res. Commun. 272*, 12–17.

Kambysellis, M.P., Johnson, F.M., Richardson, R.H., **1968**. Isozyme variability in species of the genus *Drosophila*. Distribution of the esterases in the body tissues of *Drosophila aldrichi* and *Drosophila mulleri* adults. *Biochem. Genet. 1*, 249–265.

Kamita, S.G., Hinton, A.C., Wheelock, C.E., Wogulis, M.D., Wilson, D.K., *et al.*, **2003**. Juvenile hormone (JH) esterase: why are you so JH specific? *Insect Biochem. Mol. Biol. 33*, 1261–1273.

Kang, D., Lundstrom, A., Liu, G., Steiner, H., **2002**. An azurocidin-like protein is induced in *Trichoplusia ni* larval gut cells after bacterial challenge. *Devel. Comp. Immunol. 26*, 495–503.

Kao, L.R., Motoyama, N., Dauterman, W.C., **1984**. Studies on hydrolases in various house fly strains and their role in malathion resistance. *Pestic. Biochem. Physiol. 22*, 86–92.

Kao, L.R., Motoyama, N., Dauterman, W.C., **1985**. The purification and characterisation of esterases from insecticide-resistant and susceptible house flies. *Pestic. Biochem. Physiol. 23*, 228–239.

Karoly, E.D., Rose, R.L., Thompson, D.M., Hodgson, E., Rock, G.C., *et al.*, **1996**. Monooxygenase, esterase, and gutathione transferase activity associated with azinphosmethyl resistance in the tufted apple bud moth, *Platynota idaeusalis*. *Pestic. Biochem. Physiol. 55*, 109–121.

Karunaratne, S.H.P.P., **1994**. Characterisation of multiple variants of carboxylesterases which are involved in insecticide resistance in the mosquito. *Culex quinquefasciatus*. Ph.D. thesis, University of London.

Karunaratne, S.H.P.P., Hemingway, J., **2000**. Insecticide resistance spectra and resistance mechanisms in populations of Japanese encephalitis vector mosquitoes, *Culex tritaeniorhynchus* and *Cx. gelidus* in Sri Lanka. *Med. Vet. Entomol. 14*, 430–436.

Karunaratne, S.H.P.P., Jayawardena, K.G., Hemingway, J., Ketterman, A.J., **1993**. Characterization of a beta-type esterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*. *Biochem. J. 294*, 575–579.

Karunaratne, S.H.P.P., Jayawardena, K.G., Hemingway, J., Ketterman, A.J., **1994**. Immunological cross-reactivity of a mosquito carboxylesterase-A2 antibody to other mosquito and vertebrate esterases and cholinesterase. *Biochem. Soc. Trans. 22*, 127S.

Karunaratne, S.H.P.P., Hemingway, J., **2001**. Malathion resistance and prevalence of the malathion carboxylesterase mechanism in populations of mosquito vectors of disease in Sri Lanka. *Bull. WHO 79*, 1060–1064.

Karunaratne, S.H.P.P., Hemingway, J., Jayawardena, K.G.I., Dassanayaka, V., Vaughan, A., **1995**. Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide-resistant and susceptible *Culex quinquefasciatus* mosquitoes. *J. Biol. Chem. 270*, 31124–31128.

Karunaratne, S.H.P.P., Small, G.J., Hemingway, J., **1999**. Characterization of the elevated esterase-associated insecticide resistance mechanism in *Nilaparvata lugens* (Stäl) and other planthopper species. *Int. J. Pest Mgt 45*, 225–230.

Karunaratne, S.H.P.P., Vaughan, A., Paton, M.G., Hemingway, J., **1998**. Amplification of a serine esterase gene is involved in insecticide resistance in Sri Lankan *Culex tritaeniorhynchus*. *Insect Mol. Biol. 7*, 307–315.

Kasai, Y., Konno, T., Dauterman, W.C., **1992**. Role of phosphorotriester hydrolases in the detoxication of organophosphorus insecticides. In: Chambers, J.E., Levi, P.E. (Eds.), Organophosphates: Chemistry, Fate

and Effects. Academic Press, San Diego, CA, pp. 169–182.

Ketterman, A.J., Jayawardena, K.G., Hemingway, J., **1992**. Purification and characterization of a carboxylesterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*. *Biochem. J. 287*, 355–360.

Ketterman, A.J., Karunaratne, S.H.P.P., Jayawardena, K.G.I., Hemingway, J., **1993**. Qualitative differences between populations of *Culex quinquefasciatus* in both the esterases A-2 and B-2 which are involved in insecticide resistance. *Pestic. Biochem. Physiol. 47*, 142–148.

Khlebodarova, T.M., Gruntenko, N.E., Grenback, L.G., Sukhanova, M.Z., Mazurov, M.M., *et al.*, **1996**. A comparative analysis of juvenile hormone metabolizing enzymes in two species of *Drosophila* during development. *Insect Biochem. Mol. Biol. 26*, 829–835.

Klein, U., **1987**. Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem. 17*, 1193–1204.

Knight, A.L., Light, D.M., **2001**. Attractants from Bartlett pear for codling moth, *Cydia pomonella* (L.), larvae. *Naturwissenschaften 88*, 339–342.

Knudsen, J.T., Tollsten, L., Bergstrom, L.G., **1993**. Floral scents: a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry 33*, 253–280.

Kochansky, J., Tette, J., Taschenberg, E.F., Cardé, R.T., Kaissling, K.-E., *et al.*, **1975**. Sex pheromone of the moth, *Antheraea polyphemus*. *J. Insect Physiol. 21*, 1977–1983.

Konno, T., Kasai, Y., Rose, R.L., Hodgson, E., Dauterman, W.C., **1990**. Purification and characterization of a phosphorotriester hydrolase from methyl parathion-resistant *Heliothis virescens*. *Pestic. Biochem. Physiol. 36*, 1–13.

Korochkin, L.I., Belyaeva, E.S., Matveeva, N.M., Kuzin, B.A., Serov, O.L., **1976**. Genetics of esterases in *Drosophila* Slow-migrating S-esterase in *Drosophila* of the *virilis* group. *Biochem. Genet. 14*, 161–182.

Korochkin, L.I., Ludwig, M., Tamarima, N., Uspenskyi, I., Yenikolopov, G., *et al.*, **1990**. Molecular genetic mechanisms of tissue-specific esterase isozymes and protein expression in *Drosophila*. In: Markert, C., Scandalios, J. (Eds.), Isozymes: Structure, Function, and Use in Biology and Medicine. Wiley-Liss, New York, pp. 399–440.

Korochkin, L.I., Ludwig, M.Z., Poliakova, E.V., Philinova, M.R., **1987**. Some molecular genetic aspects of cellular differentiation in *Drosophila*. *Soviet Sci. Rev. 1*, 411–466.

Korochkin, L.I., Panin, V.M., Pavlova, G.V., Kopantseva, M.R., Shostak, N.G., *et al.*, **1995**. A relatively small 5′ regulatory region of esterase-S gene of *Drosophila virilis* determines the specific expression as revealed in transgenic experiments. *Biochem. Biophys. Res. Commun. 213*, 302–310.

Kozaki, T., Shono, T., Tomita, T., Kono, Y., **2001**. Fenitroxon insensitive acetylcholinesterases of the housefly, *Musca domestica* associated with point mutations. *Insect Biochem. Mol. Biol. 31*, 991–997.

Kriek, N., Tilley, L., Horrocks, P., Pinches, R., Elford, B.C., *et al.*, **2003**. Characterization of the pathway for transport of the cytoadherence-mediating protein, PfEMP1, to the host cell surface in malaria parasite-infected erythrocytes. *Mol. Microbiol. 50*, 1215–1227.

Kuwahara, M., **1982**. Insensitivity of the acetylcholinesterase from the organophosphate-resistant Kanzawa spider mite, *Tetranychus kanzawai* Kishida (Acarina: Tetranychidae), to organophosphorus and carbamate insecticides. *Appl. Entomol. Zool. 17*, 486–493.

Kuwahara, M., Miyata, T., Saito, T., Eto, M., **1982**. Activity and substrate specificity of the esterase associated with organophosphorus insecticide resistance in the Kanzawa spider mite, *Tetranychus kanzawai* Kishida. *Appl. Entomol. Zool. 17*, 82–91.

Kwon, T.H., Kim, M.S., Choi, H.W., Joo, C.H., Cho, M.Y., *et al.*, **2000**. A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem. 267*, 6188–6196.

La Du, B.N., Aviram, M., Billecke, S., Navab, M., Primo-Parmo, S., *et al.*, **1999**. On the physiological role(s) of the paraoxonases. *Chemico-Biol. Interact. 120*, 379–388.

Lai, E.C., **2003**. Lipid rafts make for slippery platforms. *J. Cell Biol. 162*, 365–370.

Lenormand, T., Bourguet, D., Guillemaud, T., Raymond, M., **1999**. Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. *Nature 400*, 861–864.

Lenormand, T., Guillemaud, T., Bourguet, D., Raymond, M., **1998a**. Appearance and sweep of a gene duplication: adaptive response and potential for new functions in the mosquito *Culex pipiens*. *Evolution 52*, 1705–1712.

Lenormand, T., Guillemaud, T., Bourguet, D., Raymond, M., **1998b**. Evaluating gene flow using selected markers: a case study. *Genetics 149*, 1383–1392.

Lev-Lehman, E., Evron, T., Broide, R.S., Meshorer, E., Ariel, I., *et al.*, **2000**. Synaptogenesis and myopathy under acetylcholinesterase overexpression. *J. Mol. Neurosci. 14*, 93–105.

Lewis, G.A., Madge, D.S., **1984**. Esterase activity and associated insecticide resistance in the damson-hop aphid, *Phorodon humuli* (Schrank) (Hemiptera: Aphididae). *Bull. Entomol. Res. 74*, 227–238.

Li, F., Han, Z.J., **2002**. Two different genes encoding acetylcholinesterase existing in cotton aphid (*Aphis gossypii*). *Genome 45*, 1134–1141.

Liebl, E.C., Rowe, R.G., Forsthoefel, D.J., Stammler, A.L., Schmidt, E.R., *et al.*, **2003**. Interactions between the secreted protein Amalgam, its transmembrane receptor Neurotactin and the Abelson tyrosine kinase affect axon pathfinding. *Development 130*, 3217–3226.

Liu, H.F., Kubli, E., **2003**. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 100*, 9929–9933.

Liu, M.Y., Chen, J.S., Sun, C.N., **1984**. Synergism of pyrethroids by several compounds in larvae of the diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol. 77*, 851–856.

Lockridge, O., Blong, R.M., Masson, P., Froment, M.T., Millard, C.B., *et al.*, **1997**. A single amino acid substitution, Gly117His, confers phosphotriesterase (organophosphorus acid anhydride hydrolase) activity on human butyrylcholinesterase. *Biochemistry 36*, 786–795.

Malcolm, C.A., Boddington, R.G., **1989**. Malathion resistance conferred by a carboxylesterase in *Anopheles culicifacies* Giles (Species-B) (Diptera, Culicidae). *Bull. Entomol. Res. 79*, 193–199.

Marullo, R., Lovei, G.L., Tallarico, A., Tremblay, E., **1988**. Quick detection of resistant phenotypes with high esterase activity in two species of aphids (Homoptera, Aphididae). *J. Appl. Entomol. 106*, 212–214.

Massoulie, J., Anselmet, A., Bon, S., Krejci, E., Legay, C., *et al.*, **1999**. The polymorphism of acetylcholinesterase: post-translational processing, quaternary associations and localization. *Chemico-Biol. Interact. 120*, 29–42.

Matsumura, F., Brown, A.W.A., **1963**. Studies on carboxylesterase in malathion-resistant *Culex tarsalis*. *J. Econ. Entomol. 56*, 381–388.

Matsumura, F., Hogendijk, C.J., **1964**. The enzymatic degradation of malathion in organophosphae resistant and susceptible strains of *Musca domestica*. *Entomol. Exp. Applic. 7*, 179–193.

Matsumura, F., Voss, G., **1964**. Mechanism of malathion and parathion resistance in the two spotted spider mite, *Tetranychus urticae*. *J. Econ. Entomol. 57*, 911–917.

Mazzarri, M.B., Georghiou, G.P., **1995**. Characterization of resistance to organophosphate, carbamate, and pyrethroid insecticides in field populations of *Aedes aegypti* from Venezuela. *J. Am. Mosquito Control Assoc. 11*, 315–322.

McCarroll, L., Paton, M.G., Karunaratne, S.H., Jayasuryia, H.T., Kalpage, K.S., *et al.*, **2000**. Insecticides and mosquito-borne disease. *Nature 407*, 961–962.

McClintock, T.S., Sammeta, N., **2003**. Trafficking prerogatives of olfactory receptors. *Neuroreport 14*, 1547–1552.

McKenzie, J.A., Parker, A.G., Yen, J.L., **1992**. Polygenic and single gene responses to selection for resistance to diazinon in *Lucilia cuprina*. *Genetics 130*, 613–620.

McKenzie, J.A., Whitten, M.J., Adena, M.A., **1982**. The effect of genetic background on the fitness of diazinon resistance genotypes of the Australian sheep blowfly, *L. cuprina*. *Heredity 49*, 1–9.

Meikle, D.B., Sheehan, K.B., Phillis, D.M., Richmond, R.C., **1990**. Localisation and longevity of seminal-fluid esterase-6 in mated female *Drosophila melanogaster*. *J. Insect Physiol. 36*, 93–101.

Merryweather, A.T., Crampton, J.M., Townson, H., **1990**. Purification and properties of an esterase from organophosphate-resistant strain of the mosquito *Culex quinquefasciatus*. *Biochem. J. 266*, 83–90.

Mikhailov, A.T., Torrado, M., **2000**. Carboxylesterases moonlight in the male reproductive tract: a functional shift pivotal for male fertility. *Frontiers Biosci. 5*, E53–E62.

Millard, C.B., Kryger, G., Ordentlich, A., Greenblatt, H.M., Harel, M., *et al.*, **1999**. Crystal structures of aged phosphonylated acetylcholinesterase: nerve agent reaction products at the atomic level. *Biochemistry 38*, 7032–7039.

Miota, F., Scharf, M.E., Ono, M., Marcon, P., Meinke, L.J., *et al.*, **1998**. Mechanisms of methyl and ethyl parathion resistance in the western corn rootworm (Coleoptera : Chrysomelidae). *Pestic. Biochem. Physiol. 61*, 39–52.

Miyata, T., Saito, T., **1976**. Mechanism of malathion resistance in the green rice leafhopper, *Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae). *J. Pestic. Sci. 1*, 23–29.

Moores, G.D., Devine, G.J., Devonshire, A.L., **1994**. Insecticide-insensitive acetylcholinesterase can enhance esterase-based resistance in *Myzus persicae* and *Myzus nicotianae*. *Pestic. Biochem. Physiol. 49*, 114–120.

Moores, G.D., Gao, X., Denholm, I., Devonshire, A.L., **1996**. Characterisation of insensitive acetylcholinesterase in insecticide-resistant cotton aphids, *Aphis gossypii* Glover (Homoptera: Aphididae). *Pestic. Biochem. Physiol. 56*, 102–110.

Motoyama, N., Kao, L., Lin, P., Dauterman, W., **1984**. Dual role of esterases in insecticide resistance in the green rice leafhopper. *Pestic. Biochem. Physiol. 21*, 139–147.

Mouchès, C., Fournier, D., Raymond, M., Magnin, M., Bergé, J.-B., *et al.*, **1985**. Specific amplified DNA sequences associated with organophosphate insecticide resistance in mosquitoes of the *Culex pipiens* complex, with a note on similar amplification in the housefly, *Musca domestica* L. *C. R. Acad. Sci. Paris 301*, 695–700.

Mouchès, C., Magnin, M., Bergé, J.B., de Silvestri, M., Beyssat, V., *et al.*, **1987**. Overproduction of detoxifying esterases in organophosphate-resistant *Culex* mosquitoes and their presence in other insects. *Proc. Natl Acad. Sci. USA 84*, 2113–2116.

Mouchès, C., Pauplin, Y., Agarwal, M., Lemieux, L., Herzog, M., *et al.*, **1990**. Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. *Proc. Natl Acad. Sci. USA 87*, 2574–2578.

Muehleisen, D.P., Benedict, J.H., Plapp, F.W., Carino, F.A., **1989**. Effects of cotton allelochemicals on toxicity of insecticides and induction of detoxifying enzymes in bollworm (Lepidoptera, Noctuidae). *J. Econ. Entomol. 82*, 1554–1558.

Murugasu-Oei, B., Rodrigues, V., Yang, X.H., Chia, W., **1995**. Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the *Drosophila* embryo. *Genes Devel. 9*, 139–154.

Mutero, A., Fournier, D., **1992**. Post-translational modifications of *Drosophila* acetylcholinesterase. *J. Biol. Chem. 267*, 1695–1700.

Mutero, A., Pralavorio, M., Bride, J.M., Fournier, D., **1994**. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl Acad. Sci. USA 91*, 5922–5926.

Myers, M.A., Healy, M.J., Oakeshott, J.G., **1993**. Effects of the residue adjacent to the reactive serine on the substrate interactions of *Drosophila* esterase 6. *Biochem. Genet. 31*, 259–278.

N'guessan, R., Darriet, F., Guillet, P., Carnevale, P., Traore-Lamizana, M., *et al.*, **2003**. Resistance to carbosulfan in *Anopheles gambiae* from Ivory Coast, based on reduced sensitivity of acetylcholinesterase. *Med. Vet. Entomol. 17*, 19–25.

Nabeshima, T., Kozaki, T., Tomita, T., Kono, Y., **2003**. An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*. *Biochem. Biophys. Res. Commun. 307*, 15–22.

Nabeshima, T., Mori, A., Kozaki, T., Iwata, Y., Hidoh, O., *et al.*, **2004**. An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*. *Biochem. Biophys. Res. Commun. 313*, 794–801.

Nance, E., Heyse, D., Britton-Davidian, J., Pasteur, N., **1990**. Chromosomal organization of the amplified esterase B1 gene in organophosphate-resistant *Culex pipiens quinquefasciatus* Say (Diptera, Culicidae). *Genome 33*, 148–152.

Narang, S., Seawright, J. A., **1982**. Linkage relationships and genetic mapping in *Culex* and *Anopheles*. In: Steiner, W.W.M., Tabachnick, W.J., Rai, K.S., Narang, S. (Eds.), Recent Developments in the Genetics of Insect Disease Vectors. Stipes, Champaign, IL, pp. 231–280.

Nardini, M., Dijkstra, B.W., **1999**. Alpha/beta hydrolase fold enzymes: the family keeps growing. *Curr. Opin. Struct. Biol. 9*, 732–737.

Needham, P.H., Sawicki, R.M., **1971**. Diagnosis of resistance to organophosphorus insecticides in *Myzus persicae*. *Nature 230*, 125–126.

Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J., *et al.*, **1997**. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc. Natl Acad. Sci. USA 94*, 7464–7468.

Newcomb, R.D., East, P.D., Russell, R.J., Oakeshott, J.G., **1996**. Isolation of alpha cluster esterase genes associated with organophosphate resistance in *Lucilia cuprina*. *Insect Mol. Biol. 5*, 211–216.

Newcomb, R.D., Gleeson, D.M., Yong, C.G., Russell, R.J., Oakeshott, J.G., **2004**. Multiple mutations and gene duplications conferring organophosphorus insecticide resistance have been selected at the *Rop-1* locus of the sheep blowfly, *Lucilia cuprina*. *J. Mol. Evol.* (in press).

Nicolet, Y., Lockridge, O., Masson, P., Fontecilla-Camps, J.C., Nachon, F., **2003**. Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. *J. Biol. Chem. 278*, 41141–41147.

O'Brien, P.J., Abdel-Aal, Y.A., Ottea, J.A., Graves, J.B., **1992**. Relationship of insecticide resistance to carboxylesterases in *Aphis gossypii* (Homoptera, Aphididae) from midsouth cotton. *J. Econ. Entomol. 85*, 651–657.

Oakeshott, J.G., Boyce, T.M., Russell, R.J., Healy, M.J., **1995**. Molecular insights into the evolution of an enzyme: esterase 6 in *Drosophila*. *Trends Ecol. Evol. 10*, 103–110.

Oakeshott, J.G., Claudianos, C., Russell, R.J., Robin, G.C., **1999**. Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *BioEssays 21*, 1031–1042.

Oakeshott, J.G., Healy, M.J., Game, A.Y., **1990**. Regulatory evolution of the β-carboxyl esterases in *Drosophila*. In: Barker, J.S.F., Starmer, W.T., McIntyre, R.J. (Eds.), Ecological and Evolutionary Genetics of *Drosophila*. Plenum, New York, pp. 359–387.

Oakeshott, J.G., van Papenrecht, E.A., Boyce, T.M., Russell, R.J., Healy, M.J., **1993**. Evolutionary genetics of *Drosophila* esterases. *Genetica 90*, 239–268.

Oakeshott, J.G., van Papenrecht, E.A., Claudianos, C., Morrish, B.C., Coppin, C., *et al.*, **2001**. An episode of accelerated amino acid change in *Drosophila* esterase-6 associated with a change in physiological function. *Genetica 110*, 231–244.

Odgers, W.A., Aquadro, C.F., Coppin, C.W., Healy, M.J., Oakeshott, J.G., **2002**. Nucleotide polymorphism in the *Est6* promoter, which is widespread in derived populations of *Drosophila melanogaster*, changes the level of esterase 6 expressed in the male ejaculatory duct. *Genetics 162*, 785–797.

Odgers, W.A., Healy, M.J., Oakeshott, J.G., **1995**. Nucleotide polymorphism in the 5′-promoter region of esterase-6 in *Drosophila melanogaster* and its relationship to enzyme activity variation. *Genetics 141*, 215–222.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., *et al.*, **1992**. The α/β hydrolase fold. *Protein Eng. 5*, 197–211.

Olson, P.F., Fessler, L.I., Nelson, R.E., Sterne, R.E., Campbell, A.G., *et al.*, **1990**. Glutactin, a novel *Drosophila* basement membrane-related gylcoprotein with sequence similarity to serine esterases. *EMBO J. 9*, 1219–1227.

Ono, M., Swanson, J.J., Field, L.M., Devonshire, A.L., Siegfried, B.D., **1999**. Amplification and methylation of an esterase gene associated with insecticide-resistance in greenbugs, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae). *Insect Biochem. Mol. Biol. 29*, 1065–1073.

Oppenoorth, F.J., **1990**. Reevaluation of kinetic data for a paraoxon-hydrolyzing enzyme in a resistant strain of the predacious mite *Amblyseius potentillae*. *Pestic. Biochem. Physiol. 38*, 99–100.

Oppenoorth, F.J., van Asperen, K., **1960**. Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance. *Science 132*, 298–299.

Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segal, Y., *et al.*, **1996**. The architecture of human acetylcholinesterase active centre probed by interactions with selected organophosphate inhibitors. *J. Biol. Chem. 271*, 11953–11962.

Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segal, Y., *et al.*, **1998**. Functional characteristics of the oxyanion hole in human acetylcholinesterase. *J. Biol. Chem. 273*, 19509–19517.

Ordentlich, A., Barak, D., Kronman, C., Flashner, Y., Leitner, M., *et al.*, **1993**. Dissection of the human acetylcholinesterase active centre determinants of substrate specificity. *J. Biol. Chem. 268*, 17083–17095.

Ottea, J.A., Ibrahim, S.A., Younis, A.M., Young, R.J., Leonard, B.R., *et al.*, **1995**. Biochemical and physiological mechanisms of pyrethroid resistance in *Heliothis virescens* (F). *Pestic. Biochem. Physiol. 51*, 117–128.

Parker, A.G., Campbell, P.M., Spackman, M.E., Russell, R.J., Oakeshott, J.G., **1996**. Comparison of an esterase associated with organophosphate resistance in *Lucilia cuprina* with an orthologue not associated with resistance in *Drosophila melanogaster. Pestic. Biochem. Physiol. 55*, 85–99.

Parker, A.G., Russell, R.J., Delves, A.C., Oakeshott, J.G., **1991**. Biochemistry and physiology of esterases in organophosphate-susceptible and organophosphate-resistant strains of the Australian sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol. 41*, 305–318.

Parker, C.G., Fessler, L.I., Nelson, R.E., Fessler, J.H., **1995**. Drosophila UDP-glucose:glycoprotein glucosyltransferase: sequence and characterization of an enzyme that distinguishes between denatured and native proteins. *EMBO J. 14*, 1294–1303.

Pasteur, N., Georghiou, G.P., **1981**. Filter paper test for rapid determination of phenotypes with high esterase activity in organophosphate resistant mosquitoes. *Mosquito News 41*, 181–183.

Pasteur, N., Georghiou, G.P., **1989**. Improved filter paper test for detecting and quantifying increased esterase activity in organophosphate-resistant mosquitoes (Diptera: Culicidae). *J. Econ. Entomol. 82*, 347–353.

Pasteur, N., Iseki, A., Georghiou, G.P., **1981a**. Genetic and biochemical studies of the highly active esterases A′ and B associated with organophosphate resistance in mosquitoes of the *Culex pipiens* complex. *Biochem. Genet. 19*, 909–919.

Pasteur, N., Marquine, M., Hoang, T.H., Nam, V.S., Failloux, A.B., **2001**. Overproduced esterases in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) from Vietnam. *J. Med. Entomol. 38*, 740–745.

Pasteur, N., Sinegre, G., Gabinaud, A., **1981b**. Est-2 and Est-3 polymorphisms in *Culex pipiens* L. from Southern France in relation to organophosphate resistance. *Biochem. Genet. 19*, 499–508.

Pelham, H.R., **1996**. The dynamic organisation of the secretory pathway. *Cell Struct. Funct. 21*, 413–419.

Perrier, A.L., Massoulie, J., Krejci, E., **2002**. PRiMA: the membrane anchor of acetylcholinesterase in the brain. *Neuron 33*, 275–285.

Pezzementi, L., Johnson, K., Tsigelny, I., Cotney, J., Manning, E., *et al.*, **2003**. Amino acids defining the acyl pocket of an invertebrate cholinesterase. *Comp. Biochem. Physiol. B 136*, 813–832.

Phillips, J.P., Xin, J.H., Kirby, K., Milne, C.P., Krell, P., *et al.*, **1990**. Transfer and expression of an organophosphate insecticide-degrading gene from *Pseudomonas* in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 87*, 8155–8159.

Phythian, S.J., **1998**. Esterases. In: Kelly, D.R. (Ed.), Biotechnology, Vol. 8a: Biotransformations 1, 2nd edn. Wiley-VCH, Weinheim, pp. 194–241.

Picollo de Villar, M.I., Van Der Pas, L.J.T., Smissaert, H.R., Oppenoorth, F.J., **1983**. An unusual type of malathion-carboxylesterase in a Japanese strain of housefly. *Pestic. Biochem. Physiol. 19*, 60–65.

Pistillo, D., Manzi, A., Tino, A., Boyl, P.P., Graziani, F., *et al.*, **1998**. The *Drosophila melanogaster* lipase homologs: a gene family with tissue and developmental specific expression. *J. Mol. Biol. 276*, 877–885.

Plapp, F.W., Jr., Eddy, G.W., **1961**. Synergism of malathion against resistant insects. *Science 334*, 2043–2044.

Plepys, D., Ibarra, F., Francke, W., Lofstedt, C., **2002a**. Odour-mediated nectar foraging in the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae): behavioural and electrophysiological responses to floral volatiles. *Oikos 99*, 75–82.

Plepys, D., Ibarra, F., Lofstedt, C., **2002b**. Volatiles from flowers of *Platanthera bifolia* (Orchidaceae) attractive to the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae). *Oikos 99*, 69–74.

Poecke, R.M.P., van Posthumus, M.A., Dicke, M., **2001**. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitiod *Cotesia rubecula*: chemical, behavioural, and gene-expression analysis. *J. Chem. Ecol. 27*, 1911–1928.

Pohlenz, H.-D., Boidol, W., Schuttke, I., Streber, W.R., **1992**. Purification and properties of an *Arthobacter oxydans* P52 carbamate hydrolase specific for the herbicide phenmedipham and nucleotide sequence of the corresponding gene. *J. Bacteriol. 174*, 6600–6607.

Poirié, M., Raymond, M., Pasteur, N., **1992**. Identification of two distinct amplifications of the esterase-B locus in *Culex pipiens* (L.) mosquitos from Mediterranean countries. *Biochem. Genet. 30*, 13–26.

Prabhakaran, S.K., Kamble, S.T., **1995**. Purification and characterization of an esterase isozyme from insecticide resistant and susceptible strains of German cockroach, *Blattella germanica* (L.). *Insect Biochem. Mol. Biol. 25*, 519–524.

Priester, T.M., Georghiou, G.P., Hawley, M.K., Pasternak, M.E., **1981**. Toxicity of pyrethroids to organophosphate-, carbamate-, and DDT-resistant mosquitoes. *Mosquito News 41*, 143–150.

Primo-Parmo, S.L., Sorenson, R.C., Teiber, J., Ladu, B.N., **1996**. The human serum paraoxonase arylesterase gene (Pon1) is one member of a multigene family. *Genomics 33*, 498–507.

Qiao, C.L., Raymond, M., **1995**. The same esterase B1 haplotype is amplified in insecticide-resistant mosquitoes of the *Culex pipiens* complex from the Americas and China. *Heredity 74*, 339–345.

Raftos, D.A., **1986**. The biochemical basis of malathion resistance in the sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol. 26*, 302–309.

Raftos, D.A., Hughes, P.B., **1986**. Genetic basis of a specific resistance to malathion in the Australian sheep blowfly, *Lucilia cuprina* (Diptera, Calliphoridae). *J. Econ. Entomol. 79*, 553–557.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., *et al.*, **2002**. Evolution of supergene families associated with insecticide resistance. *Science 298*, 179–181.

Raymond, M., Beyssat-Arnaouty, V., Sivasubramanian, N., Mouchès, C., Georghiou, G.P., *et al.*, **1989**. Amplification of various esterase Bs responsible for organophosphate resistance in *Culex* mosquitoes. *Biochem. Genet. 27*, 417–423.

Raymond, M., Callaghan, A., Fort, P., Pasteur, N., **1991**. Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature 350*, 151–153.

Raymond, M., Pasteur, N., Georghiou, G.P., Mellon, R.B.W.M.C., Hawley, M.K., **1987**. Detoxification esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol. 24*, 24–27.

Raymond, M., Poulin, E., Boiroux, V., Dupont, E., Pasteur, N., **1993**. Stability of insecticide resistance due to amplification of esterase genes in *Culex pipiens*. *Heredity 70*, 301–307.

Richmond, R.C., Nielsen, K.M., Brady, J.P., Snella, E.M., **1990**. Physiology, biochemistry and molecular biology of the *Est-6* locus in *Drosophila melanogaster.* In: Barker, J.S.F., Starmer, W.T., MacIntyre, R.J. (Eds.), Ecological and Evolutionary Genetics of *Drosophila*. Plenum, New York, pp. 273–293.

Riddiford, L.M., **1996**. Molecular aspects of juvenile hormone action in insect metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G. (Eds.), Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, San Diego, CA, pp. 223–251.

Riskallah, M.R., **1983**. Esterases and resistance to synthetic pyrethroids in the Egyptian cotton leafworm. *Pestic. Biochem. Physiol. 19*, 184–189.

Rivet, Y., Marquine, M., Raymond, M., **1993**. French mosquito populations invaded by A2-B2 esterases causing insecticide resistance. *Biol. J. Linnean Soc. 49*, 249–255.

Robin, C., Russell, R.J., Medveczky, K.M., Oakeshott, J.G., **1996**. Duplication and divergence of the genes of the alpha-esterase cluster of *Drosophila melanogaster. J. Mol. Evol. 43*, 241–252.

Robin, G.C., Claudianos, C., Russell, R.J., Oakeshott, J.G., **2000a**. Reconstructing the diversification of alpha-esterases: comparing the gene clusters of *Drosophila buzzatii* and *D. melanogaster. J. Mol. Evol. 51*, 149–160.

Robin, G.C., Russell, R.J., Cutler, D.J., Oakeshott, J.G., **2000b**. The evolution of an alpha-esterase pseudogene inactivated in the *Drosophila melanogaster* lineage. *Mol. Biol. Evol. 17*, 563–575.

Roe, R.M., Venkatesh, K., **1990**. Metabolism of juvenile hormones: degradation and titre regulation. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods: Recent Advances in Comparative Arthropod Morphology, Physiology, and Development, Part 1. Rutgers University Press, New Brunswick, NJ, pp. 125–180.

Rooker, S., Guillemaud, T., Berge, J., Pasteur, N., Raymond, M., **1996**. Coamplification of esterase A and B genes as a single unit in *Culex pipiens* mosquitoes. *Heredity 77*, 555–561.

Rosario-Cruz, R., Miranda-Miranda, E., Garcia-Vasquez, Z., Ortiz-Estrada, M., **1997**. Detection of esterase activity in susceptible and organophosphate resistant strains of the cattle tick *Boophilus microplus* (Acari: Ixodidae). *Bull. Entomol. Res. 87*, 197–202.

Rossiter, L.C., Conyers, C.M., MacNicoll, A.D., Rose, H.A., **2001**. Two qualitatively different B-esterases from two organophosphate-resistant strains of *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) and their roles in fenitrothion and chlorpyrifos-methyl resistance. *Pestic. Biochem. Physiol. 69*, 118–130.

Roush, R.T., McKenzie, J.A., **1987**. Ecological genetics of insecticide and acaricide resistance. *Annu. Rev. Entomol. 32*, 361–380.

Ruigt, G.É.S.F., **1985**. Pyrethroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 12. Pergamon, Oxford, pp. 183–262.

Russell, R.J., Claudianos, C., Campbell, P.M., Horne, I.M., Sutherland, T.D., *et al.*, **2004**. Two major classes of target site insensitivity mutations confer resistance to organophosphate and carbamate insecticides. *Pestic. Biochem. Physiol. 79*, 84–93.

Russell, R.J., Dumancic, M.M., Foster, G.G., Weller, G.L., Healy, M.J., *et al.*, **1990**. Insecticide resistance as a model system for studying molecular evolution. In: Barker, J.S.F., Starmer, W.T., MacIntyre, R.J. (Eds.), Ecological and Evolutionary Genetics of *Drosophila*. Plenum, New York, pp. 293–314.

Russell, R.J., Robin, G.C., Kostakos, P., Newcomb, R.D., Boyce, T.M., *et al.*, **1996**. Molecular cloning of an alpha-esterase gene cluster on chromosome 3R of *Drosophila melanogaster. Insect Biochem. Mol. Biol. 26*, 235–247.

Saad, M., Game, A.Y., Healy, M.J., Oakeshott, J.G., **1994**. Associations of esterase-6 allozyme and activity variation with reproductive fitness in *Drosophila melanogaster. Genetica 94*, 43–56.

Sabourault, C., Guzov, V.M., Koener, J.F., Claudianos, C., Plapp, F.W., *et al.*, **2001**. Overproduction of a P450 that

metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase *(Md Alpha E7)* gene in resistant houseflies. *Insect Mol. Biol. 10*, 609–618.

Sakata, K., Miyata, T., **1994**. Biochemical characterization of carboxylesterase in the small brown planthopper *Laodelphax striatellus* (Fallén). *Pestic. Biochem. Physiol. 50*, 247–256.

Salama, M.S., Schouest, L.P., Miller, T.A., **1992**. Effect of diet on the esterase patterns in the hemolymph of the corn-earworm and the tobacco budworm (Lepidoptera, Noctuidae). *J. Econ. Entomol. 85*, 1079–1087.

Sawicki, R.M., Devonshire, A.D., Rice, A.D., Moores, G.D., Petzing, S.M., *et al.*, **1978**. The dectection and distribution of organophosphorus and carbamate insecticide-resistant *Myzus persicae* (Sulz.) in Britain in 1976. *Pestic. Sci. 9*, 189–201.

Scharf, M.E., Hemingway, J., Small, G.J., Bennett, G.W., **1997**. Examination of esterases from insecticide resistant and susceptible strains of the German cockroach, *Blattella germanica* (L.). *Insect Biochem. Mol. Biol. 27*, 489–497.

Scharf, M.E., Meinke, L.J., Siegfried, B.D., Wright, R.J., Chandler, L.D., **1999a**. Carbaryl susceptibility, diagnostic concentration determination, and synergism for US populations of western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol. 92*, 33–39.

Scharf, M.E., Meinke, L.J., Wright, R.J., Chandler, L.D., Siegfried, B.D., **1999b**. Metabolism of carbaryl by insecticide-resistant and-susceptible western corn rootworm populations (Coleoptera: Chrysomelidae). *Pestic. Biochem. Physiol. 63*, 85–96.

Scholl, F.G., Scheiffele, P., **2003**. Making connections: cholinesterase-domain proteins in the CNS. *Trends Neurosci. 26*, 618–624.

Schrag, J.D., Cygler, M., **1993**. 1.8 Å refined structure of the lipase from *Geotrichum candidum*. *J. Mol. Biol. 230*, 575–591.

Schulte, J., Tepass, U., Auld, V.J., **2003**. Gliotactin, a novel marker of tricellular junctions, is necessary for septate junction development in *Drosophila*. *J. Cell Biol. 161*, 991–1000.

Scott, J.G., Georghiou, G.P., **1986**. Malathion-specific resistance in *Anopheles stephensi* from Pakistan. *J. Am. Mosq. Control Assoc. 2*, 29–32.

Scott, J.G., Zhang, L., **2003**. The house fly aliesterase gene (MdalphaE7) is not associated with insecticide resistance or P450 expression in three strains of house fly. *Insect Biochem. Mol. Biol. 33*, 139–144.

Sergeev, P.V., Panin, V.M., Pavlova, G.V., Kopantseva, M.R., Shostak, N.G., *et al.*, **1995**. The expression of esterase-S gene of *Drosophila virilis* in *Drosophila melanogaster. FEBS Lett. 360*, 194–196.

Sergeev, P.V., Yenikolopov, G.N., Peunova, N.I., Kuzin, B.A., Khechumian, R.A., *et al.*, **1993**. Regulation of tissue-specific expression of the esterase S gene in *Drosophila virilis*. *Nucl. Acids Res. 21*, 3545–3551.

Shan, G., Ottea, J.A., **1998**. Contributions of monooxygenases and esterases to pyrethroid resistance in the tobacco budworm, *Heliothis virescens*. *Proc. Natl Cotton Counc. America 2*, 1148–1151.

Shan, G.M., Hammer, R.P., Ottea, J.A., **1997**. Biological activity of pyrethroid analogs in pyrethroid-susceptible and-resistant tobacco budworms, *Heliothis virescens* (F.). *J. Agric. Food Chem. 45*, 4466–4473.

Shanmugavelu, M., Baytan, A.R., Chesnut, J.D., Bonning, B.C., **2000**. A novel protein that binds juvenile hormone esterase in fat body tissue and pericardial cells of the tobacco hornworm *Manduca sexta* L. *J. Biol. Chem. 275*, 1802–1806.

Sharma, K.V., Koenigsberger, C., Brimijoin, S., Bigbee, J.W., **2001**. Direct evidence for an adhesive function in the noncholinergic role of acetylcholinesterase in neurite outgrowth. *J. Neurosci. Res. 63*, 165–175.

Shono, T., **1983**. Linkage group analysis of carboxylesterase in a malathion resistant strain of the housefly, *Musca domestica* L. (Diptera: Muscidae). *Appl. Entomol. Zool. 18*, 407–415.

Shufran, R.A., Wilde, G.E., Sloderbeck, P.E., **1996**. Description of three isozyme polymorphisms associated with insecticide resistance in greenbug (Homoptera: Aphididae) populations. *J. Econ. Entomol. 89*, 46–50.

Shulaev, V., Silverman, P., Raskin, I., **1997**. Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature 385*, 718–721.

Siegfried, B.D., Ono, M., **1993a**. Mechanisms of parathion resistance in the greenbug, *Schizaphis graminum* (Rondani). *Pestic. Biochem. Physiol. 45*, 24–33.

Siegfried, B.D., Ono, M., **1993b**. Parathion toxicokinetics in resistant and susceptible strains of the greenbug (Homoptera, Aphididae). *J. Econ. Entomol. 86*, 1317–1323.

Siegfried, B.D., Swanson, J.J., Devonshire, A.L., **1997**. Immunological detection of greenbug (*Schizaphis graminum*) esterases associated with resistance to organophosphate insecticides. *Pestic. Biochem. Physiol. 57*, 165–170.

Siegfried, B.D., Zera, A.J., **1994**. Partial purification and characterization of a greenbug (Homoptera, Aphididae) esterase associated with resistance to parathion. *Pestic. Biochem. Physiol. 49*, 132–137.

Small, G.J., Hemingway, J., **2000a**. Differential glycosylation produces heterogeneity in elevated esterases associated with insecticide resistance in the brown planthopper *Nilaparvata lugens* Stäl. *Insect Biochem. Mol. Biol. 30*, 443–453.

Small, G.J., Hemingway, J., **2000b**. Molecular characterization of the amplified carboxylesterase gene associated with organophosphorus insecticide resistance in the brown planthopper, *Nilaparvata lugens*. *Insect Mol. Biol. 9*, 647–653.

Smissaert, H.R., Abd El Hamid, F.M., Overmeer, W.P.J., **1975**. The minimum acetylcholinesterase (AChE) fraction compatible with life derived by aid of a simple model explaining the degree of dominance of resistance to inhibitors in AChE “mutants” *Biochem. Pharmacol. 24*, 1043–1047.

Smyth, K.A., Boyce, T.M., Russell, R.J., Oakeshott, J.G., **2000**. MCE activities and malathion resistances in field populations of the Australian sheep blowfly (*Lucilia cuprina*). *Heredity 84*, 63–72.

Smyth, K.A., Russell, R.J., Oakeshott, J.G., **1994**. A cluster of at least three esterase genes in *Lucilia cuprina* includes malathion carboxylesterase and two other esterases implicated in resistance to organophosphates. *Biochem. Genet. 32*, 437–453.

Smyth, K.A., Walker, V.K., Russell, R.J., Oakeshott, J.G., **1996**. Biochemical and physiological differences in the malathion carboxylesterase activities of malathion-susceptible and resistant lines of the sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol. 54*, 48–55.

Soderlund, D. M., Casida, J. E., **1977a**. Substrate specificity of mouse-liver microsomal enzymes in pyrethroid metabolism. In: Elliott, M. (Ed.), Synthetic Pyrethroids. American Chemical Society, Washington, DC, pp. 162–172.

Soderlund, D. M., Casida, J. E., **1977b**. Stereospecificity of pyrethroid metabolism in mammals. In: Elliott, M. (Ed.), Synthetic Pyrethroids. American Chemical Society, Washington, DC, pp. 173–185.

Song, J.Y., Ichtchenko, K., Sudhof, T.C., Brose, N., **1999**. Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc. Natl Acad. Sci. USA 96*, 1100–1105.

Sorenson, R.C., Primo-Parmo, S.L., Camper, S.A., La Du, B.N., **1995**. The genetic mapping and gene structure of mouse paraoxonase/arylesterase. *Genomics 30*, 431–438.

Sparagana, S.P., Bhaskaran, G., Barrera, P., **1985**. Juvenile homone acid methyltransferase activity in imaginal discs of *Manduca sexta* prepupae. *Arch. Insect Biochem. Physiol. 2*, 191–202.

Speicher, S., Garcia-Alonso, L., Carmena, A., Martin-Bermudo, M.D., De La Escalera, S., *et al.*, **1998**. Neurotactin functions in concert with other identified CAMs in growth cone guidance in *Drosophila*. *Neuron 20*, 221–233.

Spencer, A.G., Price, N.R., Callaghan, A., **1998**. Malathion-specific resistance in a strain of the rust red grain beetle *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *Bull. Entomol. Res. 88*, 199–206.

Sternfeld, M., Ming, G.L., Song, H.J., Sela, K., Timberg, R., *et al.*, **1998**. Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. *J. Neurosci. 18*, 1240–1249.

Swofford, D.L., **2000**. PAUP: Phylogenetic Analysis Using Parsimony, v4. Sinauer Associates, Sunderlund, MA.

Tabuchi, K., Sudhof, T.C., **2002**. Structure and evolution of neurexin genes: insight into the mechanism of alternative splicing. *Genomics 79*, 849–859.

Talley, T.T., Jianmongkol, S., Richardson, R., Radic, Z., Thompson, C.M., **1998**. Isomalathion stereoisomers: insecticide impurities or new probe of cholinesterase? In: Doctor, B.P., Gentry, M.K., Taylor, P., Quinn, D.M., Rotundo, R.L. (Eds.), Structure and Function of Cholinesterases and Related Proteins. Plenum, New York, pp. 531–538.

Taylor, J.L., Radic, Z., **1994**. The cholinesterases: from genes to proteins. *Annu. Rev. Pharmacol. Toxicol. 34*, 281–320.

Thomas, B.A., Church, W.B., Lane, T.R., Hammock, B.D., **1999**. Homology model of juvenile hormone esterase from the crop pest, *Heliothis virescens*. *Proteins: Struct. Funct. Genet. 34*, 184–196.

Thompson, J.D., Higgins, D.G., Gibson, T.J., **1994**. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res. 22*, 4673–4680.

Tittiger, C., Walker, V.K., **1997**. Isolation and characterization of an unamplified esterase B3 gene from malathion-resistant *Culex tarsalis*. *Biochem. Genet. 35*, 119–138.

Tomita, T., Hidoh, O., Kono, Y., **2000**. Absence of protein polymorphism attributable to insecticide-insensitivity of acetylcholinesterase in the green rice leafhopper, *Nephotettix cincticeps*. *Insect Biochem. Mol. Biol. 30*, 325–333.

Toutant, J.P., **1989**. Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. *Progr. Neurobiol. 32*, 423–446.

Townsend, M.G., Busvine, J.R., **1969**. The mechanism of malathion resistance in the blowfly *Chrysomyia putoria*. *Entomol. Exp. Applic. 12*, 243–267.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Tumlinson, J.H., Brennan, M.M., Doolittle, R.E., Mitchell, E.R., Brabham, A., *et al.*, **1989**. Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind-tunnel. *Arch. Insect Biochem. Physiol. 10*, 255–271.

van Meer, M.M.M., Bonning, B.C., Ward, V.K., Vlak, J.M., Hammock, B.D., **2000**. Recombinant, catalytically inactive juvenile hormone esterase enhances efficacy of baculovirus insecticides. *Biol. Control 19*, 191–199.

Vaughan, A., Hawkes, N., Hemingway, J., **1997a**. Co-amplification explains linkage disequilibrium of two mosquito esterase genes in insecticide-resistant *Culex quinquefasciatus*. *Biochem. J. 325*, 359–365.

Vaughan, A., Rocheleau, T., ffrench-Constant, R., **1997b**. Site-directed mutagenesis of an acetylcholinesterase gene from the yellow fever mosquito *Aedes aegypti* confers insecticide insensitivity. *Exp. Parasitol. 87*, 237–244.

Vaughan, A., Rodriguez, M., Hemingway, J., **1995**. The independent gene amplification of electrophoretically indistinguishable Beta-esterases from the insecticide-resistant mosquito *Culex quinquefasciatus*. *Biochem. J. 305*, 651–658.

VedBrat, S.S., Whitt, G.S., **1975**. Isozyme ontogeny during development of the mosquito, *Anopheles albimanus*. In: Markert, C.L. (Ed.), Isozymes, vol. 3. Academic Press, New York, pp. 131–143.

Vermunt, A.M.W., Koopmanschap, A.B., Vlak, J.M., De Kort, C.A.D., **1998**. Evidence for two juvenile hormone esterase-related genes in the Colorado potato beetle. *Insect Mol. Biol. 7*, 327–336.

Vernick, K.D., Collins, F.H., **1989**. Association of a plasmodium-refractory phenotype with an esterase locus in *Anopheles gambiae*. *Am. J. Trop. Med. Hyg. 40*, 593–597.

Vernick, K.D., Collins, F.H., Seeley, D.C., Gwadz, R.W., Miller, L.H., **1988**. The genetics and expression of an esterase locus in *Anopheles gambiae*. *Biochem. Genet. 26*, 367–379.

Villani, F., White, G.B., Curtis, C.F., Miles, S.J., **1983**. Inheritance and activity of some esterases associated with organophosphate resistance in mosquitoes of the complex of *Culex pipiens* L. (Diptera: Culicidae). *Bull. Entomol. Res. 23*, 154–170.

Villatte, F., Bachmann, T.T., **2002**. How many genes encode cholinesterase in arthropods? *Pestic. Biochem. Physiol. 73*, 122–129.

Villatte, F., Marcel, V., Estrada-Mondaca, S., Fournier, D., **1998**. Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. *Biosensors Bioelectron. 13*, 157–164.

Villatte, F., Ziliani, P., Estrada-Mondaca, S., Menozzi, P., Fournier, D., **2000a**. Is acetyl/butyrylcholine specificity a marker for insecticide resistance mutations in insect acetylcholinesterase? *Pest Mgt Sci. 56*, 1023–1028.

Villatte, F., Ziliani, P., Marcel, V., Menozzi, P., Fournier, D., **2000b**. A high number of mutations in insect acetylcholinesterase may provide insecticide resistance. *Pestic. Biochem. Physiol. 67*, 95–102.

Voet, D., Voet, J.G., **1990**. Biochemistry. Wiley, New York.

Vogt, R.G., Riddiford, L.M., **1981**. Pheromone binding and inactivation by moth antennae. *Nature 293*, 161–163.

Vogt, R.G., Riddiford, L.M., **1986**. Scale esterase: a pheromone-degrading enzyme from scales of silk moth *Antheraea polyphemus*. *J. Chem. Ecol. 12*, 469–482.

Vogt, R.G., Riddiford, L.M., Prestwich, G.D., **1985**. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. Natl Acad. Sci. USA 82*, 8827–8831.

Vontas, J.G., Cosmidis, N., Loukas, M., Tsakas, S., Hejazi, M.J., *et al.*, **2001**. Altered acetylcholinesterase confers organophosphate resistance in the olive fruit fly *Bactrocera oleae*. *Pestic. Biochem. Physiol. 71*, 124–132.

Vontas, J.G., Hejazi, M.J., Hawkes, N.J., Cosmidis, N., Loukas, M., *et al.*, **2002**. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. *Insect Mol. Biol. 11*, 329–336.

Vontas, J.G., Small, G.J., Hemingway, J., **2000**. Comparison of esterase gene amplification, gene expression and esterase activity in insecticide susceptible and resistant strains of the brown planthopper, *Nilaparvata lugens* (Stål). *Insect Mol. Biol. 9*, 655–660.

Wachendorff, U., Zoebelein, G., **1988**. Diagnosis of insecticide resistance in *Phorodon humuli* (Homoptera, Aphididae). *Entomol. General. 13*, 145–155.

Walsh, S.B., Dolden, T.A., Moores, G.D., Kristensen, M., Lewis, T., *et al.*, **2001**. Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance. *Biochem. J. 359*, 175–181.

Wang, K.Y., Liu, T.X., Yu, C.H., Jiang, X.Y., Yi, M.Q., **2002**. Resistance of *Aphis gossypii* (Homoptera: Aphididae) to fenvalerate and imidacloprid and activities of detoxification enzymes on cotton and cucumber. *J. Econ. Entomol. 95*, 407–413.

Webb, E.C., **1992**. Enzyme Nomenclature 1992: Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes. International Union of Biochemistry and Molecular Biology. Academic Press, San Diego, CA.

Weill, M., Berticat, C., Raymond, N., Chevillon, C., **2000**. Quantitative polymerase chain reaction to estimate the number of amplified esterase genes in insecticide-resistant mosquitoes. *Anal. Biochem. 285*, 267–270.

Weill, M., Fort, P., Berthomieu, A., Dubois, M.P., Pasteur, N., *et al.*, **2002**. A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the *ace* gene in *Drosophila*. *Proc. Roy. Soc. London B 269*, 2007–2016.

Weill, M., Lutfalla, G., Mogensen, K., Chandre, F., Berthomieu, A., *et al.*, **2003**. Insecticide resistance in mosquito vectors. *Nature 423*, 136–137.

Weill, M., Malcolm, C., Chandre, F., Mogensen, K., Berthomieu, A., *et al.*, **2004**. The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol. Biol. 13*, 1–7.

Welling, W., De Vries, A.W., Voerman, S., **1974**. Oxidative cleavage of a carboxyester bond as a mechanism of resistance to malaoxon in houseflies. *Pestic. Biochem. Physiol. 4*, 31–43.

Wheelock, C.E., Wheelock, A.M., Zhang, R., Stok, J.E., Morisseau, C., *et al.*, **2003**. Evaluation of alpha-cyanoesters as fluorescent substrates for examining inter-individual variation in general and pyrethroid-selective esterases in human liver microsomes. *Anal. Biochem. 315*, 208–222.

White, M.M., Mane, S.D., Richmond, R.C., **1988**. Studies of esterase 6 in *Drosophila melanogster.* 18. Biochemical differences between the slow and fast allozymes. *Mol. Biol. Evol. 5*, 41–62.

White, N.D.G., Bell, R.J., **1988**. Inheritance of malathion resistance in a strain of *Tribolium castaneum* (Coleoptera, Tenebrionidae) and effects of resistance genotypes on fecundity and larval survival in malathion-treated wheat. *J. Econ. Entomol. 81*, 381–386.

Whyard, S., Russell, R.J., Walker, V.K., **1994**. Insecticide resistance and malathion carboxylesterase in the

sheep blowfly, *Lucilia cuprina*. *Biochem. Genet. 32*, 9–24.

Whyard, S., Walker, V.K., **1994**. Characterization of malathion carboxylesterase in the sheep blowfly *Lucilia cuprina*. *Pestic. Biochem. Physiol. 50*, 198–206.

Wierenga, J.M., Hollingworth, R.M., **1993**. Inhibition of altered acetylcholinesterases from insecticide-resistant Colorado potato beetles (Coleoptera: Chrysomelidae). *J. Econ. Entomol. 86*, 673–679.

Wirth, M.C., Georghiou, G.P., **1996**. Organophosphate resistance in *Culex pipiens* from Cyprus. *J. Am. Mosq. Control Assoc. 12*, 112–118.

Wirth, M.C., Marquine, M., Georghiou, G.P., Pasteur, N., **1990**. Esterases A2 and B2 in *Culex quinquefasciatus* (Diptera: Culicidae): role in organophosphate resistance and linkage studies. *J. Med. Entomol. 7*, 202–206.

Wolff, M.A., Abdel-Aal, Y.A.I., Goh, D.K.S., Lampert, E.P., Roe, R.M., **1994**. Organophosphate resistance in the tobacco aphid (Homoptera, Aphididae): purification and characterization of a resistance-associated esterase. *J. Econ. Entomol. 87*, 1157–1164.

Wood, E.J., de Villar, M.I.P., Zerba, E.N., **1985**. Role of microsomal carboxylesterase in reducing the action of malathion in eggs of *Triatoma infestans*. *Pestic. Biochem. Physiol. 23*, 24–32.

Xu, J., Qu, F., Weide, L., **1994**. Diversity of amplified esterase B genes responsible for organophosphate resistance in *Culex quinquefasciatus* from China. *J. Med. Colleges Peoples Liberation Army 9*, 20–23.

Yamamoto, K., Hamada, H., Shinkai, H., Kohno, Y., Koseki, H., *et al.*, **2003**. The KDEL receptor modulates the endoplasmic reticulum stress response through mitogen-activated protein kinase signaling cascades. *J. Biol. Chem. 278*, 34525–34532.

Yin, C.M., Zou, B.X., Jiang, M.G., Li, M.F., Qin, W.H., *et al.*, **1995**. Identification of juvenile-hormone-III bisepoxide (JHB(3)), juvenile-hormone-III and methyl farnesoate secreted by the corpus allatum of *Phormia regina* (Meigen) *in vitro* and function of JHB(3) either applied alone or as a part of a juvenoid blend. *J. Insect Physiol. 41*, 473–479.

Yu, S.J., **1991**. Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (Smith, J.E.). *Pestic. Biochem. Physiol. 39*, 84–91.

Zador, E., **1989**. Tissue specific expression of the acetylcholinesterase gene in *Drosophila melanogaster. Mol. Gen. Genet. 218*, 487–490.

Zera, A.J., Harshman, L.G., **2001**. The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst. 32*, 95–126.

Zera, A.J., Huang, Y., **1999**. Evolutionary endocrinology of juvenile hormone esterase: functional relationship with wing polymorphism in the cricket, *Gryllus firmus*. *Evolution 53*, 837–847.

Zera, A.J., Sanger, T., Hanes, J., Harshman, L., **2002**. Purification and characterization of hemolymph juvenile hormone esterase from the cricket, *Gryllus assimilis*. *Arch. Insect Biochem. Physiol. 49*, 41–55.

Zhao, G., Rose, R.L., Hodgson, E., Roe, R.M., **1996**. Biochemical mechanisms and diagnostic microassays for pyrethroid, carbamate, and organophosphate insecticide resistance/cross-resistance in the tobacco budworm, *Heliothis virescens*. *Pestic. Biochem. Physiol. 56*, 183–195.

Zhao, G.Y., Liu, W., Knowles, C.O., **1994**. Mechanisms associated with diazinon resistance in western flower thrips. *Pestic. Biochem. Physiol. 49*, 13–23.

Zhou, Z., Scharf, M.E., Parimi, S., Meinke, L.J., Wright, R.J., *et al.*, **2002**. Diagnostic assays based on esterase-mediated resistance mechanisms in western corn rootworms (Coleoptera: Chrysomelidae). *J. Econ. Entomol. 95*, 1261–1266.

Zhu, K.Y., Brindley, W.A., **1990a**. Acetylcholinesterase and its reduced sensitivity to inhibition by paraoxon in organophosphate-resistant *Lygus hesperus* Knight (Hemiptera, Miridae). *Pestic. Biochem. Physiol. 36*, 22–28.

Zhu, K.Y., Brindley, W.A., **1990b**. Properties of esterases from *Lygus hesperus* Knight (Hemiptera: Miridae) and the roles of the esterases in insecticide resistance. *J. Econ. Entomol. 83*, 725–732.

Zhu, K.Y., Brindley, W.A., **1992**. Enzymological and inhibitory properties of acetylcholinesterase purified from *Lygus hesperus* Knight (Hemiptera, Miridae). *Insect Biochem. Mol. Biol. 22*, 245–251.

Zhu, K.Y., Clark, J.M., **1995a**. Comparisons of kinetic properties of acetylcholinesterase purified from azinphosmethyl-susceptible and azinphosmethyl-resistant strains of Colorado potato beetle. *Pestic. Biochem. Physiol. 51*, 57–67.

Zhu, K.Y., Clark, J.M., **1995b**. Cloning and sequencing of a cDNA encoding acetylcholinesterase in Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Insect Biochem. Mol. Biol. 25*, 1129–1138.

Zhu, K.Y., Lee, S.H., Clark, J.M., **1996**. A point mutation of acetylcholinesterase associated with azinphosmethyl resistance and reduced fitness in Colorado potato beetle. *Pestic. Biochem. Physiol. 55*, 100–108.

Zhu, Y.C., Dowdy, A.K., Baker, J.E., **1999a**. Detection of single-base substitution in an esterase gene and its linkage to malathion resistance in the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Pestic. Sci. 55*, 398–404.

Zhu, Y.C., Dowdy, A.K., Baker, J.E., **1999b**. Differential mRNA expression levels and gene sequences of a putative carboxylesterase-like enzyme from two strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Insect Biochem. Mol. Biol. 29*, 417–425.

Ziegler, R., Whyard, S., Downe, A.E.R., Wyatt, G.R., Walker, V.K., **1987**. General esterase, malathion carboxylesterase, and malathion resistance in *Culex tarsalis*. *Pestic. Biochem. Physiol. 28*, 279–285.

Zouros, E., van Delden, W., **1982**. Substrate-preference polymorphism at an esterase locus of *Drosophila mojavensis*. *Genetics 100*, 307–314.

Zouros, E., van Delden, W., Odense, R., van Dijk, H., **1982**. An esterase duplication in *Drosophila*: differences

in expression of duplicate loci within and among species. *Biochem. Genet.* 20, 929–942.

Zschunke, F., Salmassi, A., Kreipe, H., Buck, F., Parwaresch, M.R., *et al.*, **1991**. cDNA cloning and characterization of human monocyte macrophage serine esterase-1. *Blood* 78, 506–512.

Relevant Websites

http://www.sanger.ac.uk – The Sanger Institute; includes Pfam nomenclature.

http://www.flygenome.yale.edu – full sequence of *Drosophila melanogaster* genome.

http://www.phenolist.slu.se – up-to-date listing of insect pheromones.

http://www.flybase.bio.indiana.edu – comparative data on insect genomics.

http://www.ncbi.nlm.nih.gov – National Center for Biotechnology Information.

http://www.ensembl.org – ENSEMBL database.

http://www.hgsc.bcm.tmc – Honeybee esterases sequences.

http://www.rcsb.org – Crystal structure of AChE.

http://www.openrasmol.org – Structural modeling software.

http://www.psc.edu – Multiple alignment software.

http://www.pherolist.slu.se – Components of sex pheromone blends.

http://www.hgsc.bcm.tmc.edu – *D. pseudoobscura* esterase sequences.

5.11 Glutathione Transferases

H Ranson and J Hemingway, Liverpool School of Tropical Medicine, Liverpool, UK

5.11.1. Introduction

Glutathione transferases (GSTs) are found ubiquitously in aerobic organisms. They were first discovered in animals in 1961 where they were postulated to play a role in the detoxification of drugs (Booth *et al*., 1961). Their central role in detoxification and drug resistance pathways in mammals has now been established but additional functions are continually being attributed to this complex enzyme family. For example, GSTs are important mediators in oxidative stress responses, are involved in the synthesis of prostaglandins, and facilitate intracellular transport of hydrophobic compounds. Mammalian GSTs are particularly well studied due to their role in cancer epidemiology and treatment. Several GSTs can metabolize environmentally derived carcinogens and polymorphisms in these genes are linked to cancer risk. In addition, the overexpression of GSTs in tumor cells can contribute to drug resistance (Landi, 2000).

The vast majority of GSTs are cytosolic dimeric proteins with typical molecular masses of around 50 kDa. A small subset of trimeric microsomal GSTs that are very distantly related to the cytosolic GSTs occur in mammals and insects. Most organisms contain multiple forms of GSTs with both specific and overlapping substrate preferences.

In 1985, GSTs had only been identified in a limited number of insect species including the grass grub, greater wax moth, housefly, and American cockroach (Mannervik, 1985). The importance of this enzyme family in many different types of insecticide resistance had been demonstrated and multiple forms were known to exist in individual insects. That GSTs have now been studied in over 30 different insect species is testimony to the importance of this enzyme family. This review describes the diversity of the GSTs family in insects and highlights some of the diverse functions of this complex enzyme family.

5.11.2. Methods Used to Study Insect GSTs

Although a wide range of biochemical techniques have been exhaustively applied to the isolation of

GSTs from numerous insect species, relatively few enzymes have been purified to homogeneity. Frequently, after several purification steps, multiple bands were still visible after sodium dodecyl sulfate (SDS) electrophoresis and, moreover, given our current knowledge of the extent of certain insect GST classes, a single band on an SDS gel may not always be indicative of a homogeneous enzyme preparation. The choice of purification techniques also led to a systematic bias in the classes of GSTs identified with some GST classes being more amenable to purification. The methods employed have now been shown to capture only a relatively small subset of the total GST population. Thus, it is no surprise that advances in gene cloning and recombinant protein expression techniques were seized upon by those studying the role of individual GST enzymes in insects. However, early molecular biology approaches to the isolation of insect GSTs were also biased towards the isolation of certain subsets of GSTs and it was not until the era of large-scale genome sequencing projects that the full extent of this enzyme family was appreciated (see Section 5.11.3). Some of the various approaches that have been used to isolate and characterize insect GSTs are outlined below.

5.11.2.1. Isolation of Insect GSTs

5.11.2.1.1. Biochemical purification Early purification approaches used conventional column chromatographic methods such as ion exchangers, gel filtration, and hydroxyapatite chromatography to isolate GSTs. These approaches were very time consuming and often ineffective, and purification of many individual isoenzymes was really only feasible after the introduction of affinity chromatography (Yu, 1996). Original affinity chromatography columns used the glutathione conjugate of sulfobromophthalein immobilized to an agarose matrix as a ligand (e.g., Clark and Dauterman, 1982; Clark and Shamaan, 1984). Problems sometimes arose when recovering the bound enzyme from this matrix and most purification methods now use glutathione coupled via the sulfur atom or *S*-hexyl glutathione coupled via the α-amino group of the glutamyl residue as ligands (e.g., Grant *et al.*, 1991; Prapanthadara *et al.*, 1993). Enzymes that bind to the columns are usually eluted using glutathione and the GST activity of different fractions determined with the "general substrate" for all GSTs, i.e., 1-chloro-2,4-dinitrobenzene (CDNB). Once eluted, the multiple enzymes are sometimes further separated by nondenaturing polyacrylamide gel electrophoresis (PAGE), isoelectrofocusing, or chromatofocusing.

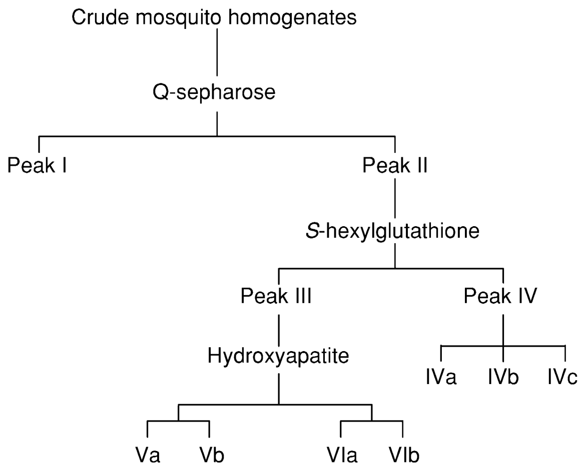


Figure 1 Partial purification of GSTs from the mosquito *A. gambiae* by column chromatography. Bound enzymes are shown on the right arm of the branch. (Adapted from Prapanthadara, L., Hemingway, J., Ketterman, A.J., **1995**. DDT-resistance in *Anopheles gambiae* (Diptera: culicidae) from Zanzibar, Tanzania, based on increased DDT-deyhdrochlorinase activity of glutathione *S*-transferases. *Bull. Ent. Res. 85*, 267–274.

There are two major limiting factors associated with the purification approaches outlined above. First, many GSTs do not bind to any of these affinity columns. It is generally reported that theta class GSTs are not retained by GSH-based affinity matrices (Meyer *et al.*, 1991) but, even within a particular GST class, individual enzymes can sometimes be divided into bound and unbound fractions (Sawicki *et al.*, 2003; Ortelli *et al.*, 2004). Second, CDNB is not a good substrate for all GSTs. Several GSTs have now been characterized that have low or undetectable activity with this substrate (e.g., Singh *et al.*, 2001; Sawicki *et al.*, 2003).

Nevertheless, despite these limitations, the application of biochemical purification techniques provided the first indications of the diversity, in terms of both physical properties and substrate range, of this enzyme family in insects. For example, using the purification scheme outlined in **Figure 1**, eight different fractions with CDNB conjugation activity, each containing multiple isozymes, were obtained from the mosquito *Anopheles gambiae* (Prapanthadara *et al.*, 1993).

A summary of the GSTs isolated biochemically from insects is given in **Table 1**. A review of the purification procedures used to isolate many of these enzymes is provided by Clark (1989).

5.11.2.1.2. Cloning of insect GSTs The approaches that have led to the successful cloning of insect GSTs can be broadly divided into five categories: (1) use

Table 1 Examples of glutathione transferases isolated from insects by biochemical or molecular cloning techniques

Insect order	*Species name*	*Common name*	*Number of enzymes isolated by biochemical techniques*[a]	*Number of genes cloned*	*Reference*
Coleoptera	*Costelytra zealandica*	New Zealand grass grub	3		Clark *et al.* (1985)
Dictyoptera	*Periplaneta Americana*	American cockroach	5	1	Arruda *et al.* (1995)
Diptera	*Aedes aegypti*	Yellow fever mosquito	2, 3	1	Grant *et al.* (1991), Grant and Hammock (1992)
	Anopheles gambiae	African malaria mosquito	7	28	Prapanthadara *et al.* (1993), Reiss and James (1993), Ranson *et al.* (1997, 1998, 2001), Ding *et al.* (2003)
	Culicoides variipennis sonorensis	Biting midge		1	Abdallah *et al.* (2000)
	Drosophila melanogaster	Fruit fly	1, 2	13	Toung *et al.* (1990, 1993), Sawicki *et al.* (2003), Beall *et al.* (1992)
	Lucilia cuprina	Sheep blowfly	1	1	Board *et al.* (1994)
	Musca domestica	Housefly	4, 2, 9	7	Clark *et al.* (1986), Chien *et al.* (1995), Wei *et al.* (2001)
Hemiptera	*Nilaparvata lugens*	Rice brown planthopper	1	1	Vontas *et al.* (2002)
	Triatoma infestans	Kissing bug	1		Wood *et al.* (1986)
Lepidoptera	*Choristoneura fumiferana*	Spruce budworm		1	Feng *et al.* (1999)
	Diatraea saccharalis	Sugar borer	2		Tiwari *et al.* (1991)
	Eoreuma loftni	Mexican rice borer	3		Tiwari *et al.* (1991)
	Galleria mellonella	Greater wax moth	1, 4		Clark *et al.* (1977), Baker *et al.* (1994)
	Helicoverpa zea	Corn earworm	3, 1		Chien and Dauterman (1991)
	Manduca sexta	Tobacco hornworm	2	3	Snyder *et al.* (1995), Rogers *et al.* (1999)
	Orthosia gothica	Hebrew character moth	4		Egaas *et al.* (1995)
	Plutella xylostella	Diamondback moth	1, 4, 3	1	Chiang and Sun (1993), Huang *et al.* (1998)
	Spodoptera frugiperda	Fall armyworm	4		Kirby and Ottea (1995)

[a]In species with more than one report of the isolation of GST enzymes, where it can be difficult to determine the relationship between the enzymes described in each study, multiple entries are given. Note also that the figures given do not distinguish between homodimers and heterodimers.

of GST-specific antibodies to screen cDNA expression libraries; (2) PCR using degenerate primers; (3) screening of cDNA or gDNA libraries with GST nucleotide probes; (4) searching nucleotide sequence databases; and (5) fortuitous discovery. Of these, the latter approach accounted for a surprising proportion of the early insect GST gene sequences determined and is still a valuable source of new sequences for species without large volumes of sequence data. Since many GST classes are encoded by closely related multigene families (and these are often clustered in the genome; see Section 5.11.5.1), the identification of the first gene within a family has often had a cascade effect with multiple additional members of the gene family being identified in quick succession. The difficult step is isolating the first member of a particular gene class. In *Drosophila melanogaster* and *Musca domestica*,

two of the first insect GSTs to be isolated were obtained by raising antibodies against GSTs purified by affinity chromatography from these species and using these to screen expression libraries (Toung *et al*., 1990; Fournier *et al*., 1992). A similar approach was used to isolate GSTs from the spruce budworm *Choristoneura fumiferana* (Feng *et al*., 1999) and the biting midge *Culicoides variipennis* (Abdallah *et al*., 2000), although in both of these cases, the antibodies were raised against unknown proteins rather than purified GSTs.

The degenerate PCR approach, using primers designed by homology to other known insect GSTs, was successfully employed to identify the first GST gene from the brown planthopper *Nilaparvata lugens* (Vontas *et al*., 2002) and the diamondback moth *Plutella xylostella* (Huang *et al*., 1998). *DmGST2* the first of a new class of GSTs to be identified in insects (now classified as the sigma class) was amplified as a secondary PCR product obtained during an unrelated project (Beall *et al*., 1992) and the orthologous gene from *A. gambiae* was also fortuitously isolated during a library screen using the coding region of a salivary gland-specific gene as a probe (Reiss and James, 1993). The recurrent isolation of GST clones using antibodies or DNA probes designed to bind to other protein or gene families may partly reflect the relatively high frequency with which some members of the GST family are expressed. For example, the accidentally amplified *DmGST2* described above has now been shown to account for about 2% of the total soluble protein in *Drosophila* (Singh *et al*., 2001).

Searching EST databases and partial or full genome sequence databases has led to the detection of fragments of GST-like sequence that have been used as a starting point for obtaining full-length gene sequences by PCR-based techniques (Snyder *et al*., 1995; Ranson *et al*., 2001; Sawicki *et al*., 2003).

Several insect GST genes have been expressed *in vitro* in order to characterize the recombinant proteins. The absence of posttranslational modification and their location in the cytosol make GST proteins amenable to expression in *Escherichia coli* (e.g., Ranson *et al*., 1997; Huang *et al*., 1998; Wei *et al*., 2001) although baculovirus expression systems have also been used (Feng *et al*., 1999). One of the potential limitations of using recombinant proteins to characterize GST activity is the absence of heterodimers. This problem has been elegantly addressed in plants by coexpressing two GST subunits and separating the recombinant proteins into homodimers and heterodimers by chromatofocusing or anion exchange chromatography. By constructing tandem expression systems, the transcription and translation of both subunits are coordinated helping to ensure that they are expressed at the same level (Dixon *et al*., 1999).

5.11.2.2. Characterization of Substrate Specificity

A number of model substrates are widely used to characterize the substrate specificity of GSTs, the most common of which is the conjugation of reduced glutathione (GSH) to CDNB. This assay is simple to conduct and the rate of conjugation can be monitored by measuring the change in optical density at 340 nm. The conjugation of GSH to 1,2-dichloro-4-nitrobenzene (DCNB) can also be measured under similar assay conditions although the activity of most insect GST enzymes is higher with CDNB than DCNB. Other model substrates used to characterize GST activity include *p*-nitrophenol acetate (*p*-NPA), *p*-nitrophenyl bromide (*p*-NPB), and ethacrynic acid. The selenium independent peroxidase activity of GSTs is usually detected using cumene hydroperoxide or *t*-butyl hydroperoxide (*t*-BHP).

A vast array of GST inhibitors have been described (Mannervik and Danielson, 1988) and the effects of these on GST enzymes can be useful in distinguishing between different enzymes. Those commonly used to study insect GSTs include bromosulfophthalein, cibacron blue, and glutathione derivatives (Vontas *et al*., 2002). Quinones can also have strong inhibitory effects on GSTs and this can be problematic in purification of insect GSTs (Grant *et al*., 1991).

These model substrates and inhibitors have been proved very valuable for characterizing and comparing the various GST enzymes, and many early approaches to the classification of insect GSTs relied heavily on data generated from such experiments (see below). More recent studies on insect GSTs have focused mainly on their role in the detoxification of xenobiotics or in protection against oxidative stress, and assays have now been developed to assess the role of individual enzymes in these physiological processes. For example, spectrophotometric, chromatographic, and radiolabeling techniques can detect products generated by the GST catalyzed metabolism of many insecticides and products of lipid peroxidation reactions (Clark *et al*., 1986; Prapanthadara *et al*., 1995; Singh *et al*., 2001).

5.11.3. Classification and Nomenclature

Mammalian GST nomenclature was originally based on substrate specificities. Five GST enzyme types

were recognized and referred to as glutathione-*S*-aryltransferase, glutathione-*S*-epoxidetransferase, glutathione-*S*-alkyltransferase, glutathione-*S*-aralkyltransferase, and glutathione-*S*-alkenetransferase. However, later studies on GSTs isolated from rat livers found that each enzyme recognized a broad and overlapping range of substrates and thus this nomenclature was abandoned in favor of the use of roman letters reflecting the order of elution from a CM-cellulose ion exchanger (Habig *et al.*, 1974). Subsequently, the realization that GSTs existed as both homo- and heterodimers led to a further change in nomenclature to reflect that subunit composition and individual subunits of GSTs were numbered in the order in which they were discovered and classified. These GSTs were divided into three distinct groups on the basis of their isoelectric points: the basic (α-ε), near neutral (μ), and acidic (π) groups (Mannervik, 1985). These groups were later designated as classes and assigned the Greek letters alpha, mu, and pi (Mannervik and Danielson, 1988).

In early studies on insect GSTs, where multiple enzymes were isolated from a species they were generally assigned numbers according to their order of elution from the various purification procedures employed or isoelectric points (Clark *et al.*, 1985; Prapanthadara *et al.*, 1993). Two immunologically distinct classes of GSTs were later recognized in houseflies and designated class I and class II (Fournier *et al.*, 1992). The first insect GST to be cloned was *Dm*GST1 from *D. melanogaster* (Toung *et al.*, 1990). The ortholog of this gene was later cloned from *M. domestica* and proposed to be the sole member of the class I GST gene family in this species (Fournier *et al.*, 1992). However, soon after, a cluster of eight GST genes (termed the *gstD* genes and including the original class I *DmGST1* gene) were identified in *D. melanogaster* (Toung *et al.*, 1993). Representatives of the class II family were also cloned from *D. melanogaster* and *A. gambiae* and in each species, the class II family was reported to be encoded by a single gene (Beall *et al.*, 1992; Reiss and James, 1993). As predicted by their immunological properties, these class II GSTs had very low levels of sequence identity to the class I genes and were proposed to have a different physiological function. This was supported by immunohistology studies on houseflies, which located the class I GSTs to the hemolymph and the class II GSTs predominately to the indirect flight muscles in the thorax (Franciosa and Berge, 1995).

The advent of large-scale EST and full genome sequencing data has led to a marked increase in the number of GST classes recognized in insects, plants, and mammals. A total of nine different classes of cytosolic GSTs have now been described (**Table 2**). In insects, it became clear that the existing classification of insect GSTs into class I and class II was inadequate and new guidelines were proposed for the naming of insect GSTs along the lines of the mammalian classification system (Chelvanayagam *et al.*, 2001).

Table 2 Classes of cytosolic glutathione transferases and their representation in insects

Class	*Lineage*	*Putative number in* A. gambiae	*Putative number in* D. melanogaster
Alpha	Mammals		
Beta	Bacteria		
Delta	Insects	15	11
Epsilon	Insects	8	14
Kappa	Mammals		
Lambda	Plants		
Mu	Mammals		
Omega	Mammals, insects, nematodes	1	5
Pi	Mammals		
Phi	Plant		
Tau	Plant		
Theta	Mammals, insects, plants	2	4
Sigma	Molluscs, helminths, nematodes, insects, mammals	1	1
Zeta	Plants, nematodes, mammals, insects	1	2

5.11.3.1. Nomenclature Guidelines for Insect GSTs

As a general rule, GST sequences that share over 40% amino acid sequence identity are assigned to the same class. A robust nomenclature requires a degree of flexibility in class assignments, however, and ideally other properties such as phylogenetic relationships, immunological properties, tertiary structure, and ability to form heterodimers should be employed in classification decisions. As in mammals, kinetic characteristics have proven a poor criterion for classification with several GSTs from different insect classes catalyzing similar reactions. At least six classes of insect GSTs have been identified in both *A. gambiae* and *D. melanogaster* (**Table 2**) (Ranson *et al.*, 2002). Despite the availability of full genome sequences for two insect species, the possibility that further insect GST classes

exist cannot be discounted. In particular, several of the assignments to the delta and epsilon classes are weakly supported by phylogenetic analysis and may be more correctly placed in a separate class (Ding *et al*., 2003).

Individual GST subunits are assigned names indicating the species they were isolated from and the GST class. They are also given a number that may either reflect the order of discovery or the genomic organization. Thus, *AgGSTd12* is the twelfth member of the *A. gambiae* delta class of GSTs to be identified. The proteins, represented by capital letters, are nonitalicized and contain information on the composition of the dimer. So the designation AgGSTD12-12 represents a homodimer of two AgGSTD12 subunits, whereas AgGSTD11-12 would be a heterodimer made up of a subunit of AgGSTD11 and a subunit of AgGSTD12. In practice, except where ambiguity may arise, the species prefix is usually omitted.

5.11.3.2. Classes of Insect GSTs

5.11.3.2.1. Delta class The delta class is one of the two largest classes of insect GSTs (**Table 2**). GSTs originally classified as belonging to insect class I have mostly been reclassified as delta GSTs. There are 15 delta GST genes in *A. gambiae* and 11 in *D. melanogaster* (Ranson *et al*., 2002). GSTs from this class are found uniquely in insects and have been cloned from many species including *Lucilia cuprina* (Board *et al*., 1994), *M. domestica* (Fournier *et al*., 1992), *N. lugens* (Vontas *et al*., 2002), and *C. variipennis* (Abdallah *et al*., 2000). Multiple recombinant delta GST homodimers have been produced *in vitro* and biochemically characterized. Most, but not all, have activity with CDNB and the majority bind to glutathione or *S*-hexylglutathione columns. Delta class GSTs have been implicated in resistance to all the major classes of insecticide (Wang *et al*., 1991; Tang and Tu, 1994; Vontas *et al*., 2002).

5.11.3.2.2. Epsilon class The epsilon class is also a large, insect-specific class. Members of this class have been cloned from *P. xylostella*, *M. domestica, Manduca sexta, A. gambiae*, and *D. melanogaster* (Snyder *et al*., 1995; Huang *et al*., 1998; Singh *et al*., 2000; Ranson *et al*., 2001; Wei *et al*., 2001). The majority of epsilon GSTs that have been characterized have low levels of activity with CDNB and are often not retained by glutathione affinity matrices. Both these factors may explain the relatively recent discovery of this class. As with the delta class, epsilon GSTs play an important role in the detoxification of insecticides (Huang *et al*., 1998; Wei *et al*., 2001; Ortelli *et al*., 2004).

5.11.3.2.3. Omega class The omega GST class was first identified in humans by bioinformatic techniques (Board *et al*., 2000). Members of the omega class have also been found in the mouse, rat, *Caenorhabditis elegans, Schistosoma mansoni, D. melanogaster*, and *A. gambiae* (Board *et al*., 2000; Ranson *et al*., 2002). In most species, the omega GSTs appear to be encoded by a single gene but five putative omega GST genes have been identified in *D. melanogaster* (Ranson *et al*., 2002). The human recombinant GSTO1-1 has low levels of activity with most typical GST substrates but high thiol transferase activity. Its physiological function is unclear but one suggestion is that it may play a housekeeping role in protecting against oxidative stress by removing *S*-thiol adducts from proteins (Board *et al*., 2000).

5.11.3.2.4. Sigma class The sigma class GSTs are related to the catalytically inactive cephalopod *S*-crystallin, a major component of eye lens (Ji *et al*., 1995). A structural role has also been suggested for the insect sigma GSTs (formally known as the insect class II GSTs), as in both *D. melanogaster* and *M. domestica* these proteins are found predominately in the indirect flight muscles in close association with troponin-H. Both of these enzymes also possess a proline/alanine rich N-terminal extension, which may aid attachment to the flight muscle. Recently, however, insect sigma GSTs (with or without the N-terminal extension) have been shown to be catalytically active (Singh *et al*., 2001). This class of proteins has low levels of activity with typical GST substrates but a high affinity for the lipid peroxidation product 4-hydroxynonenal (4-HNE). A means of reducing the levels of reactive oxygen species in highly metabolically active tissues such as flight muscles is essential and it is postulated that the high levels of GSTS1-1 found in this tissue may protect the flight muscle against by-products of oxidative stress (Singh *et al*., 2001). Sigma GSTs are found in all major eukaryotic taxonomic groups except plants. In addition to the housefly and *Drosophila* sigma GSTs described above, members of this class lacking the N-terminal extension have been cloned or purified from *M. sexta, Blattella germanica*, *A. gambiae*, and *C. fumiferana* (Reiss and James, 1993; Snyder *et al*., 1995; Arruda *et al*., 1997; Feng *et al*., 1999). In all cases to date, the sigma GSTs are encoded by a single gene, although in *A. gambiae*, two alternative transcripts from this gene have been detected (see Section 5.11.5.3.1).

5.11.3.2.5. Theta class Theta GST were first purified from mammalian livers. The first theta GSTs to be studied were unusual amongst the mammalian GSTs in that they had very low levels of activity with CDNB and were not retained by glutathione affinity matrices (Meyer *et al.*, 1991). Theta GSTs also occur in bacteria, plants, and insects and initially the theta GSTs were proposed to be the progenitor of all GST classes (Pemble and Taylor, 1992). However, as more GSTs were identified it became apparent that many GSTs, including the insect delta class, were inappropriately assigned to this class and proposals for the evolutionary pathway of GSTs were revised (see Section 5.11.8). Insects do possess several GST genes whose sequence identity and phylogeny warrant their inclusion in the theta class. These putative insect theta GSTs have not yet been biochemically characterized and their physiological role is unknown.

5.11.3.2.6. Zeta class The zeta class of GSTs was first identified by bioinformatic approaches. Zeta GSTs occur in many different species and their sequence is highly conserved, particularly at the N-terminus of the proteins where the SSCXWRVIAL motif is conserved in plants, insects, and mammals (Board *et al.*, 1997). The highly conserved structure of this protein suggests it plays an essential housekeeping role and in this regard, GSTZ1-1 catalyzes an important step in the tyrosine degradation pathway. Two putative zeta class GSTs have been identified in *D. melanogaster*, while a single zeta GST gene is found in *A. gambiae* (Ranson *et al.*, 2002).

5.11.3.2.7. Microsomal GSTs The microsomal GSTs have a different structure and genetic origin to the cytosolic GSTs but perform similar functions. The microsomal GST class is encoded by a single gene in *D. melanogaster*. Mutants that have this gene disrupted have reduced life spans but are still viable (Toba and Aigaki, 2000). Humans possess multiple microsomal GSTs some of which detoxify xenobiotic and lipid-derived products of reactive oxygen but other members of this class are involved in the synthesis of prostaglandins. Microsomal GSTs are sometimes referred to as membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Jakobsson *et al.*, 1999).

5.11.4. Protein Structure

There are two types of GST with very differing structures. The majority of insect GSTs are soluble dimeric proteins found in the cytosol. The second type, the microsomal class, are trimeric membrane associated proteins. At present, the three-dimensional structure has only been elucidated for four insect GSTs, three from the delta class and a single sigma class GST and much of our knowledge of the structure of insect GST proteins is extrapolated from knowledge of mammalian GSTs. Posttranslational modification of GSTs appears to be a very rare phenomenon and has not been reported in insects.

5.11.4.1. Cytosolic GSTs

Each soluble GST is a dimer of subunits ranging from 24 to 28 kDa in size. Each subunit has two binding sites, the G site and the H site. The highly conserved G site binds the tripeptide glutathione and is largely composed of amino acid residues found in the N-terminal of the protein. The H site or substrate binding site is more variable in structure and is largely formed from residues in the C-terminal (Mannervik, 1985). An alignment of amino acid sequences of representative insect GSTs from different classes is shown in **Figure 2**. The active site residue at the N-termini of the protein is shown in bold. This residue interacts with and activates the sulfhydryl group of glutathione to generate the catalytically active thiolate anion (Armstrong, 1991). This nucleophilic thiolate anion is then capable of attacking substrates bound to the H site. In most mammalian GSTs and in all sigma GSTs, the active site residue is a tyrosine (Wilce and Parker, 1994; Agianian *et al.*, 2003). In the delta, epsilon, theta, and zeta classes this role is performed by a serine residue while in the omega class, cysteine is the active site residue (review: Sheehan *et al.*, 2001). Generally, mutation of the active site residue results in reductions in GST activity of up to 90% (Rushmore and Pickett, 1993; Caccuri *et al.*, 1997). However, it is noteworthy that GSTD3-3 in *D. melanogaster* lacks the 15 N-terminal amino acids and yet is still catalytically active (Sawicki *et al.*, 2003). The determination of the three-dimensional structures of GST subunits have identified additional residues that interact with glutathione (Wilce *et al.*, 1995; Agianian *et al.*, 2003) (**Figure 2**) and perhaps an alternative residue substitutes for the active site tyrosine in GSTD3-3. The *cis*-proline at residue 53 is conserved in all GSTs and appears to be critical for the correct formation of the GSH binding site.

The H site, which binds the hydrophobic substrate, is more variable in structure and contributes to the substrate specificity of the enzyme. The H site of the *Drosophila* sigma GST, GSTS1-1, is polar and unusually open in structure (Agianian *et al.*, 2003) whereas the delta class GSTs from *A. gambiae* have



Figure 2 Alignment of representative amino acid sequence from each insect GST class: delta GST = *L. cuprina* (Acc. No. P42860], epsilon GST = *P. xylostella* (Acc. No. U66342], sigma GST = *D. melanogaster* (Acc. No. M95198], theta GST = *A. gambiae* (Acc. No. AF15526], zeta GST = *A. gambiae* (Acc. No. AF15522], omega GST = *A. gambiae* (Acc. No. AY255856]. The active site residues are shown in bold and the conserved proline necessary for maintaining the GST fold is marked with an asterisk. The extent of the N and C domains is indicated by an arrow below the alignment. The two GSTs in this alignment for which three-dimensional structures have been resolved (Wilce *et al.*, 1995; Agianian *et al.*, 2003) are annotated as follows. Residues putatively involved in the binding of glutathione are underlined, those involved in substrate binding are shown in italics. Beta sheets are highlighted in blue, alpha helices in pink.

a mixture of polar and hydrophobic pockets (Oakley *et al.*, 2001). Again, elucidation of three-dimensional structures has predicted a number of residues involved in substrate binding (Wilce *et al.*, 1995; Oakley *et al.*, 2001; Agianian *et al.*, 2003). The putative H site residues in the *D. melanogaster* sigma GST and the delta class GST1 from *L. cuprina* are shown in **Figure 2**.

Representative crystal structures of vertebrate GSTs are available for all the cytosolic GST classes found in insects. The general structure is the same for all classes with the major differences occurring at the active site and at the intersubunit interface. The unique structural features of the different GST classes were recently comprehensively reviewed (Sheehan *et al.*, 2001) and will only be covered briefly here.

Each monomer consists of two distinct domains joined by a variable linker region. The N-terminal domain consists of four beta sheets and three flanking alpha helices and adopts a conformation similar to the thioredoxin domain found in many proteins that bind GSH or cysteine (Sheehan *et al.*, 2001). A conserved proline in the *cis* conformation (marked with an asterisk in **Figure 2**) is found in all GST sequences and appears to be important in maintaining the protein in a catalytically competent structure (Wilce *et al.*, 1995). The C-terminal domain is larger and consists of a variable number of alpha helices. The omega GST class is unusual in possessing an N-terminal extension that forms a unique structural unit with the C-terminal domain in human omega GSTs (Board *et al.*, 2000). Aligning the putative insect omega GSTs with the mammalian GST sequence suggests this may be a general characteristic of the omega GST structure. A 45 amino acid residue N-terminal extension is present in some sigma class GSTs (**Figure 2**) but, unlike the

omega extension, this region of the protein is not essential for catalytic activity (Singh *et al.*, 2001; see Section 5.11.3.2.4).

The interface between the two subunits can be hydrophobic or hydrophilic and interactions between residues in both subunits are essential for dimer stability. Many GST classes have a V-shaped dimer interface with a "lock and key" motif linking the dimers. The "key," which can be aliphatic (zeta class and *D. melanogaster* sigma class) or aromatic (mammalian alpha, mu, and pi classes), from one subunit interacts with a hydrophobic pocket on the other subunit. The lock and key mechanism appears to be absent from the omega and theta classes of GSTs (Sheehan *et al.*, 2001).

Incompatibility in interfacial residues prevents heterodimers forming between two GST subunits from different classes. However, within a class, the formation of heterodimers can expand the range of functional proteins produced. The prevalence of GST heterodimers has not been widely studied in insects. However, an elegant series of experiments in plants, in which two GST subunits were consecutively expressed *in vitro*, found that even within a class not all GSTs will dimerize with each other to form heterodimers (Dixon *et al.*, 1999).

5.11.4.2. Microsomal GSTs

The microsomal GSTs are trimeric proteins with a molecular mass of approximately 50 kDa. The human MGST1 is a homotrimer found in the membranes of the endoplasmic reticulum and mitochondria. The three-dimensional structure of this protein has been resolved and is markedly different to the soluble GST classes. Each subunit consists of four transmembrane alpha helices. The homotrimer contains a single active site with the hydrophobic substrate binding site facing the cytosol (Schmidt-Krey *et al.*, 1999). The *D. melanogaster* microsomal GST class is encoded by a single gene and hence only homotrimeric proteins can be formed in this species (Toba and Aigaki, 2000). In contrast three distinct microsomal gene GSTs are transcribed in *A. gambiae* but their tertiary structure *in vivo* has not been resolved.

5.11.5. GST Gene Organization

The wide range of functions performed by GSTs is aided by the broad substrate specificities of individual enzymes and by the extensive nature of the GST supergene family in insects. Multiple gene duplications provide a degree of genetic security enabling individual GST genes to evolve to occupy new biochemical niches while still ensuring that essential functions are covered. The insect-specific GST classes in particular are characterized by paralogous clusters of genes, indicating that expansion has occurred by local gene duplications (see below). The permissiveness of GSTs to alterations in their primary structure has also facilitated the diversification of this enzyme family. Very dramatic changes in substrate specificity can be achieved by a small number of amino acid substitutions (Ortelli *et al.*, 2004). In addition, several genetic mechanisms have been described in insects that contribute further levels of heterogeneity to the GST supergene family. In this section, we discuss current knowledge of the genomic organization of the GST supergene family in insects and describe some specific examples of alternative splicing, gene conversion, and allelic variation.

5.11.5.1. Clustering of Paralogous Genes

The physical location of GST genes in the genomes of *A. gambiae* and *D. melanogaster* is shown in **Figure 3**. In both species, the genes are found on all three chromosomes but several large clusters exist. In fact, of the total 69 putative GST genes present in these two species only 17 are present as singletons. In both species, the majority of epsilon class GSTs are found sequentially on both chromosome strands of a single polytene division and there is evidence of recent internal duplications within these gene clusters (Ding *et al.*, 2003; Sawicki *et al.*, 2003). The 11 *D. melanogaster* delta GSTs are sequentially arranged on chromosome 3R, division 87B. The orthologous class in *A. gambiae* is larger and individual members are scattered over all chromosomes, but two closely linked clusters each consisting of six genes are found on chromosome 2R. These two paralogous *A. gambiae* delta class GST clusters may be a result of a segmental duplication (Ding *et al.*, 2003).

5.11.5.2. Intron Size and Position

The frequency of introns in the insect GST supergene family varies widely between species, particularly within the insect-specific delta and epsilon classes. In the *D. melanogaster* delta and epsilon GSTs and the *M. domestica* delta GSTs, the majority of the genes are uninterrupted by introns within the coding sequence (Zhou and Syvanen, 1997; Sawicki *et al.*, 2003) although some have introns in their 5′UTRs (Lougarre *et al.*, 1999). In contrast, the coding sequence of all but one of the *A. gambiae* GSTs is interrupted by at least one intron. The intron size in *A. gambiae* ranges from 64 to 13 937 bp (Ding *et al.*, 2003). A common intron is found in the N-terminal

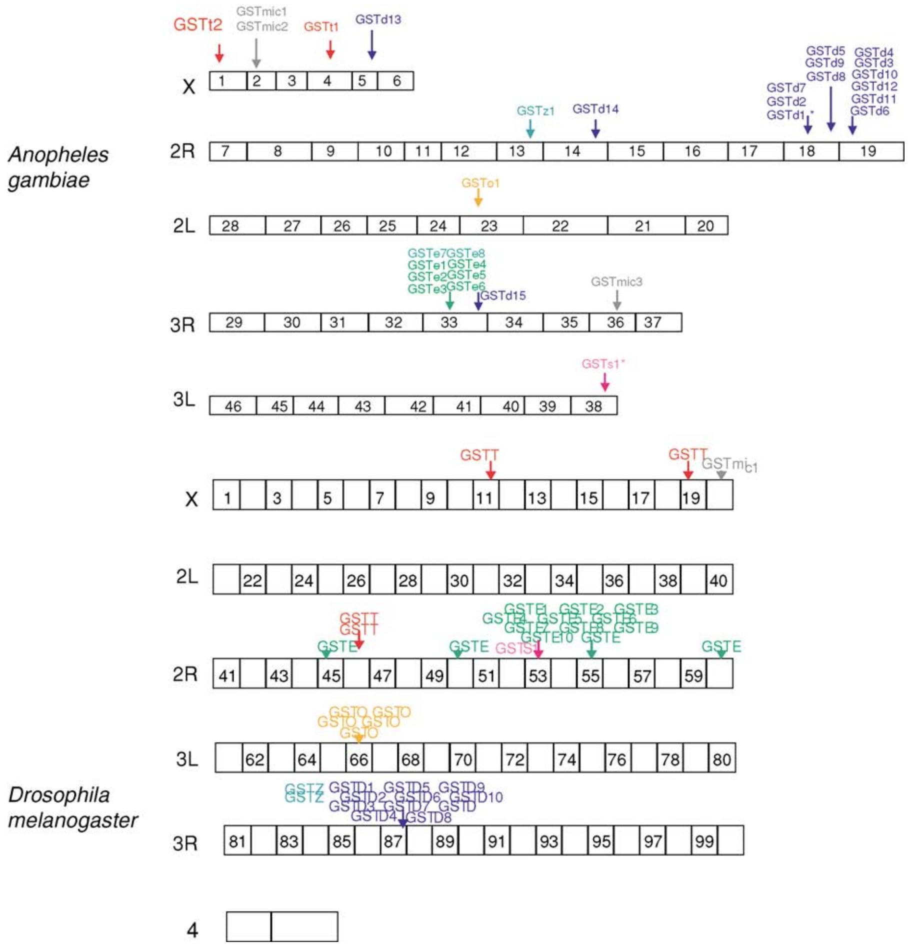


Figure 3 Chromosomal location of GST genes in two species of *Diptera*.

of 16 of the 28 cytosolic GST genes in this species (Ding *et al*., 2003).

5.11.5.3. Mechanisms for Generating Additional Heterogeneity

5.11.5.3.1. Alternative splicing of GSTs Alternative splicing of an RNA transcript can result in the production of different mRNAs, and thereby different proteins, from the same DNA segment. To date, alternative splicing of insect GST genes has only been reported in the *Anopheles* genus. A delta class GST *GSTd1* from *A. gambiae* (and its ortholog from *A. dirus*) is alternatively spliced to produce four distinct mature transcripts each encoding subunits with different biochemical properties (Ranson *et al*., 1998; Jirajaroenrat *et al*., 2001). In *A. gambiae*, the single sigma class GST gene is also alternatively spliced to produce two transcripts, both of which have been detected by RT-PCR (Ding *et al*., 2003). In both of these cases, the mature transcripts share a common 5′ exon (constitutive exon) but differ in their use of 3′ exons (Figure 4). The constitutive

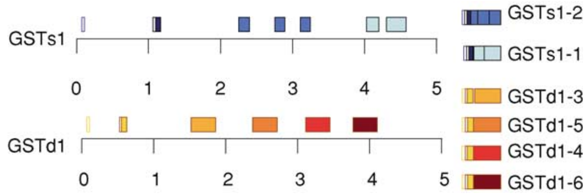


Figure 4 Alternative splicing of two *A. gambiae* GST genes. A schematic of the two genes is shown on the left and the mRNA transcripts on the right. The sigma class gene, *GSTs1*, produces two transcripts, *GSTs1-1* and *GSTs1-2*. The delta class *GSTd1* gene produces four distinct transcripts, *GSTd1-3* to *GSTd1-6*. Coding exons are shown by colored rectangles, 5′UTRs are shown by empty rectangles. The scale bar is in kilobases.

exon in each gene comprises the majority of the residues in the N-terminal domain whereas the variable exons encode the C-termini. As the carboxyl region of the GST subunit contains the majority of the residues involved in substrate binding (the H site), this form of alternative splicing is an efficient means of broadening the substrate recognition patterns of the GSTs with a minimal addition to the genome.

The factors governing choice of splice site in the *Anopheles* GSTs are unknown. For the delta GST at least, all four alternative transcripts are expressed throughout mosquito development and in both sexes. The expression pattern of this GST gene in different tissues has not yet been reported however, and it is possible that the choice of 3′ splice site is tissue dependent (Ranson *et al*., 1998).

5.11.5.3.2. GST fusion genes The delta class GSTs in *M. domestica* have an unusual genomic organization. Transcripts encoding five members of this class (*MdGST-1–MdGST-5)* have been detected in adult flies (Syvanen *et al*., 1994; Zhou and Syvanen, 1997). Screening a genomic library for clones containing these GST genes identified a complex array of loci encoding MdGST-3 and MdGST-4. At least three genomic loci encoding MdGST-3 and two encoding MdGST-4 were found, although none of these were identical to the sequence of the corresponding cDNA clones. Furthermore, several loci that appear to have resulted from the fusion of the 5′ end of one of these genes with the 3′ of another were identified in two different strains of *M. domestica* (Zhou and Syvanen, 1997). The open reading frame (ORF) is preserved in all of these fusion genes but, surprisingly, the breakpoints are not conserved and there are no introns at fusion sites, or elsewhere in the coding sequence. The genetic mechanism that produces these fusion genes is unclear and it is not known whether or not these fusion genes encode functional GSTs. Nevertheless, it is possible that a special recombination mechanism associated with the delta class GST family in *M. domestica* may contribute additional potential for functional diversity (Zhou and Syvanen, 1997).

5.11.5.3.3. Allelic variation Small numbers of amino acid substitutions can have dramatic effects on the substrate specificities of GST homodimers (Ketterman *et al*., 2001) and thus the maintenance of allelic variation in a population can provide further functional diversification. In *A. gambiae*, two alleles of the epsilon class GST *GSTe1*, differing at 12 amino acid residues, are found in laboratory and field populations. One of these encodes a protein with very high levels of peroxidase activity and it has been proposed that this may provide a fitness advantage in the presence of insecticide exposure, thus explaining its frequency in *A. gambiae* populations (Ortelli *et al*., 2003).

In addition to allelic variation, the formation of heterodimers can expand the range of functional proteins (see Section 5.11.4). The prevalence of GST heterodimers has not been widely studied in insects and it is often difficult to distinguish between genuine heterodimers and mixed populations of homodimers when characterizing GSTs isolated from insect tissues. An *in vitro* approach, similar to that adopted in plants (Dixon *et al*., 1999), may help reveal the importance of heterodimers in generating diversity in the insect GST family.

5.11.5.4. GST Pseudogenes

Pseudogenes can be produced by deleterious mutations, which result in the silencing of a gene. This type of pseudogene, known as an unprocessed pseudogene, normally occurs in a duplicate gene and frequently pseudogenes are found proximal to the genes from which they are derived. Unprocessed pseudogenes are rarely transcribed although some are transcribed and not translated or, occasionally, translated into nonfunctional proteins. Two possible unprocessed pseudogenes have been described in the GST delta class of *A. gambiae*. One of these is untranscribed and the second is transcribed but predicted to encode a nonfunctional protein (Ding *et al*., 2003). Both genes are located within the GST delta cluster on chromosome 2R. Originally, two of the *D. melanogaster* delta GSTs, *GSTD7* and *GSTD3*, were reported to be pseudogenes on the basis of abnormalities in their putative transcript lengths (Toung *et al*., 1993). However, transcripts for both of these have since been detected by real-time polymerase chain reaction (RT-PCR) and recombinant proteins derived from these cDNAs encode catalytically active proteins (Sawicki *et al*., 2003).

A different type of pseudogene, known as a processed pseudogene, is found in the GST supergene family in *D. melanogaster. Mgst1-psi* is a putative pseudogene of the single microsomal GST gene found in this species. This pseudogene contains a poly-A tail towards the 3′ end, has no introns, a premature stop codon, and a direct repeat at both ends. Presumably, it was derived from the reverse transcription and subsequent integration of *Mgst1* mRNA into the genome (Toba and Aigaki, 2000).

5.11.6. Functions of Insect GSTs

The primary function of GSTs is generally considered to be in the detoxification of both endogenous and xenobiotic compounds either by acting directly on these compounds or by catalyzing the secondary metabolism of a vast array of compounds that are oxidized by the cytochrome P450 family. Perhaps of equal importance is the role of GSTs in stress physiology. In addition, members of this enzyme family have been implicated in various biosynthetic

pathways and may be involved in the intracellular transport of ligands. In insects, the majority of studies have focused on their role in the detoxification of foreign compounds, in particular insecticides and plant allelochemicals, and in many cases the endogenous substrates of insect GSTs are unknown. The application of functional genomic approaches to this enzyme family will hopefully redress this balance and enable the housekeeping functions of these enzymes to be fully elucidated.

5.11.6.1. Endogenous Substrates

The little that is known about the endogenous substrates of insect GSTs is generally inferred from functions that have been attributed to mammalian enzymes. An exception to this is the antennae-specific GST identified in *M. sexta*, which is proposed to play a vital role in olfactory function both by detoxifying compounds that can interfere with sex pheromone detection and, more specifically, by conjugating GSH to aldehyde pheromones thereby terminating signals from sex-pheromone odorants (Rogers *et al*., 1999).

The wide range of substrates recognized by GSTs suggests they play a central role in cellular processes. One of these processes, GSH conjugation, is a key stage in the conversion of lipophilic compounds to water soluble metabolites that are more readily exported from the cell. GSTs are also important in the biosynthesis of prostaglandins (Meyer and Thomas, 1995). They can act as intracellular transporters of various nonsubstrate hydrophobic compounds in mammals and may be involved in similar processes in insects (Wilce and Parker, 1994). The zeta class of GST is one of the few enzymes with a clearly defined role in biosynthetic pathways in the cell. It catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate, an essential step in the catabolism of tyrosine (Blackburn *et al*., 1998).

5.11.6.2. Protection against Oxidative Stress

GSTs play a vital role in the inactivation of toxic products of oxygen metabolism. Reactive oxygen species (ROS), including hydrogen peroxide, superoxide anions, and hydroxyl radicals, are generated during aerobic respiration. These ROS trigger a cascade of reactions that can be highly damaging to the cells. The generation of ROS initiates the conversion of polyunsaturated fatty acids to lipid hydroperoxides. These in turn can give rise to highly reactive α,β-unsaturated aldehydes, e.g., 4-HNE. 4-HNE is toxic at high concentrations but at low concentrations it has important signaling functions affecting cell proliferation, apoptosis, and differentiation (Sawicki *et al*., 2003). GSTs have a protective effect at several different stages in this pathway. Peroxidase activity has been detected in insect GSTs from the delta, epsilon, and sigma classes using the model substrate cumene hydroperoxide (Vontas *et al*., 2001; Singh *et al*., 2001; Ortelli *et al*., 2004). Other delta and sigma insect GSTs can detoxify 4-HNE by conjugation with glutathione (Singh *et al*., 2001; Sawicki *et al*., 2003).

5.11.6.3. Detoxification of Xenobiotics

Insects, like all organisms, are continually being exposed to foreign chemical species. Some of these, such as insecticides, are in evolutionary terms relatively new threats, but other environmental pollutants, such as plant degradation products, have existed over a much longer timespan. GSTs can protect against these toxins by sequestration or, more commonly, by converting them to less toxic metabolites or conjugates that are tagged for export from the cell. Much attention has been focused on the role of insect GSTs in insecticide resistance because of the significant economic and public health problems associated with resistance. Insecticide resistance will be used to illustrate some of the different pathways of GST catalyzed detoxification.

5.11.6.3.1. Metabolism of insecticides Elevated GST activity has been implicated in resistance to at least four classes of insecticides. Higher enzyme activity is usually due to an increase in the amount of one or more GST enzymes, either as a result of gene amplification or, more commonly, through increases in transcriptional rate, rather than qualitative changes in individual enzymes (review: Hemingway and Ranson, 2000). An overview of GST involvement in insecticide resistance is shown in **Figure 5**.

5.11.6.3.1.1. Organophosphates This was the first insecticide class in which resistance due to increases in GST activity was detected. GSTs have been implicated in organophosphate resistance in many insect species (Hayes and Wolf, 1988). Recombinant GST enzymes from the diamondback moth and housefly have verified the role of these enzymes in the metabolism of organophosphate insecticides (Huang *et al*., 1998; Wei *et al*., 2001). Detoxification occurs via an *O*-dealkylation or *O*-dearylation reaction. In O-dealkylation, glutathione is conjugated with the alkyl portion of the insecticide, e.g., the demethylation of tetrachlorvinphos in resistant houseflies (Oppenoorth *et al*., 1979), whereas the reaction of glutathione with the leaving group, important in the detoxification of parathion and methyl parathion in

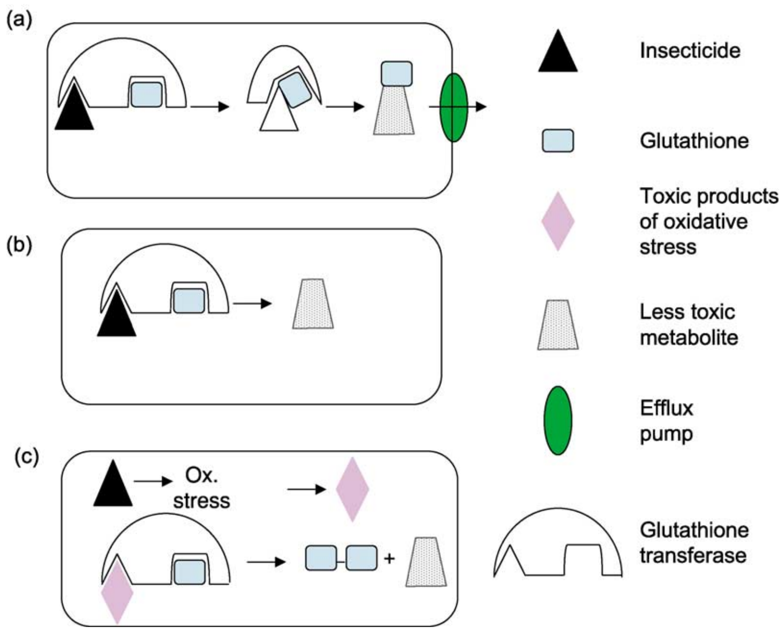


Figure 5 Overview of known GST involvement in insecticide resistance. (a) GSTs can detoxify insecticides via glutathione conjugation; the conjugates are then exported from the cell, e.g., demethylation of tetrachlorovinphos to demethyltetrachlorovinphos). (b) GSTs can detoxify the insecticide DDT via an elimination reaction. Glutathione acts as a proton source but no glutathione conjugate is produced (e.g., the dehydrochlorination of DDT to DDE). (c) GSTs with glutathione peroxidase activity can protect against insecticide induced oxidative stress.

the diamondback moth (Chiang and Sun, 1993), is an *O*-dearylation reaction.

GSTs can also catalyze the secondary metabolism of organophosphate insecticides. This insecticide class is usually applied in its noninsecticidal phosphorothionate form and activated to the insecticidal form by the action of cytochrome P450s in the insect. GSTs that can detoxify the active oxon analog have been described in mosquitoes and elevated activity of these enzymes is a cause of organophosphate resistance in *Anopheles subpictus* (Hemingway *et al*., 1991).

5.11.6.3.1.2. Organochlorines DDT dehydrochlorination is a major route of detoxification in insects. The enzyme catalyzing this reaction was not initially recognized as a member of the GST family partly due to the inability to detect even a transient GSH conjugate and also because of the lack of DDT dehydrochlorinase activity in some purified GSTs. Evidence that DDT dehydrochlorinase is a GST was finally provided in the housefly. Three enzymes with CDNB conjugating activity were isolated from a DDT resistant strain of housefly and two of these were also active with DDT as a substrate (Clark and Shamaan, 1984). The DDT dehydrochlorinase reaction proceeds via a base abstraction of hydrogen, catalyzed by the thiolate anion generated in the active site of the GST, leading to elimination of chlorine from DDT to generate DDE.

In addition to the housefly, an increased rate of DDT dehydrochlorination confers resistance to DDT in the mosquito *A. aegypti* (Grant *et al*., 1991) and *A. gambiae* (Prapanthadara *et al*., 1993).

5.11.6.3.2. Pyrethroids GSTs have not yet been shown to be involved in the direct metabolism of pyrethroid insecticides. Nevertheless, they play a very important role in conferring resistance to this insecticide class by detoxifying lipid peroxidation products induced by pyrethroids (Vontas *et al*., 2001). A delta class GST present in elevated quantities in a pyrethroid resistant strain of *N. lugens* was expressed *in vitro*. The recombinant protein had high peroxidase activity and a role for this enzyme in the prevention or repair of oxidative damage was proposed (Vontas *et al*., 2002). Elevated GSTs in other resistant insects may play a similar role. GSTs have also been suggested to protect against pyrethroid toxicity in insects by sequestering the insecticide (Kostaropoulos *et al*., 2001).

5.11.6.3.3. Plant chemicals Plants produce a wide variety of allelochemicals to protect themselves from pathogens and herbivores. The detoxification of these compounds presents a major challenge for

herbivorous insects and one consequence of this is that many insects feed on a very limited range of host plants. Many polyphagous species have highly evolved detoxification systems. Most studies on the metabolism of plant allelochemicals have focused on the role of cytochrome P450s (Fogleman *et al.*, 1998). However, GSTs can also metabolize many allelochemicals (review: Yu, 1996) and in some cases, the allelochemical acts as a regulator, inducing its own metabolism.

5.11.7. Regulation and Induction of GST Expression

In noninsect species, many GSTs are differentially regulated in response to various inducers or environmental signals or in a tissue developmental-specific manner. A similar complex pattern of expression is gradually being elucidated within the insect GST family. A detailed account of the effect of dietary compounds, insecticides, and laboratory inducers on GST expression is provided in two review articles (Clark, 1989; Yu, 1996). The main purpose of the following discussion is to outline some of the recent results from molecular studies on expression of individual GST genes and to highlight gaps in our knowledge.

5.11.7.1. Tissue and Life Stage Specificity of Expression

The majority of individual insect GSTs whose expression profiles have been determined are expressed constitutively in all life stages. Of course, the caveat here is that GSTs with a very restricted window of expression may have escaped detection in earlier studies. Nevertheless, in recent experiments looking at the global expression of the insect GSTs it was found that in *A. gambiae*, transcripts for all but one of the GST supergene family were detectable in 1-day-old adult mosquitoes by RT-PCR (Ding *et al.*, 2003). In these preliminary experiments no attempt was made to quantify the expression level in different developmental stages and it is apparent from several studies in other insects that the levels of individual enzymes can fluctuate widely during the life span of an insect. For example, using the less sensitive technique of Northern blotting, four delta class GSTs were undetectable in *D. melanogaster* adults (Toung *et al.*, 1993) and in the spruce budworm, *C. fumiferana,* a sigma GST was found at very low levels in feeding larvae but at high levels in diapausing larvae (Feng *et al.*, 1999).

Variations in the level of GST activity in different insect tissues have been reported in several species. In cases where the variation in activity is attributed to individual enzymes, such studies can provide valuable insights into the functions of different GSTs. Thus, the finding that sigma GSTs from housefly and *Drosophila* were predominately located in the indirect flight muscles in association with troponin H suggested that the role of this GST class was structural rather than catalytic (subsequently, however, these GSTs have been found to play a very important role in the protection against oxidative stress; Franciosa and Berge, 1995; Clayton *et al.*, 1998). Very high levels of GST activity have been reported in the fat body and midguts of insects. Both these tissues are important sites for the detoxification of xenobiotics and further studies characterizing the individual enzymes present in these tissues are needed.

5.11.7.2. Induction of GSTs

In a number of studies, insects have been exposed to various xenobiotics to determine the effect of these chemicals on GST expression levels (Ottea and Plapp, 1984; Yu, 1984). In the majority of cases, a model substrate, such as CDNB, is used to assay GST activity from crude insect homogenates and hence the results are general measurements of the activity of a large subset of GSTs and do not reveal much about fluctuations in the level of individual enzymes. A more complete picture of factors governing GST expression is available for the plant GST family. By studying the expression profile of 10 different *Arabidopsis* GSTs in response to a variety of inducers and stress treatments different signaling mechanisms were demonstrated to regulate the expression of individual GSTs in response to different stimuli (Wagner *et al.*, 2002). Although not resolved at the level of individual enzymes, a study in the fall armyworm, *Spodoptera frugiperda,* showed that different enzyme fractions were induced to varying extents by treatment with different inducers (Kirby and Ottea, 1995). Injection of larvae with 8-methoxypsoralen enhanced the activity of GSTs active against DCNB, but not those active against CDNB, whereas injection with pentamethylbenzene had the opposite effect. Coadministration of the inducers prevented some of these induction effects leading the authors to conclude that multiple mechanisms are involved in the regulation of these enzymes.

Other chemicals that induce GSTs include insecticides, fumigants, barbiturates, and plant allelochemicals (review: Yu, 1996). Several of these, e.g., paraquat and pyrethroid insecticides, are also inducers of oxidative stress. Repressors of insect GST activity have also been identified in the diet of

some insects. For example, GST activity in the tobacco budworm, *Heliothis virescens*, is decreased by a diet of wild tomato leaves (Clark, 1989).

5.11.7.3. Regulation of GST Expression

Most studies of the response of GSTs to environmental or artificial inducers suggest that regulation occurs at the transcriptional level. Several regulatory elements have been identified in the promoter regions of mammalian GSTs that may mediate their induction. These include xenobiotic response elements (XREs) and antioxidant or electrophile response elements (AREs/EpREs) (Rushmore and Pickett, 1993). Analysis of the promoters of the delta class GSTs from *D. melanogaster* identified several putative transcription factor binding sites including 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive elements (Toung *et al*., 1993). Other putative binding sites for transcription factors involved in developmental regulation were identified upstream of an epsilon GST in *A. dirus* (Udomsinprasert and Ketterman, 2002). In the absence of functional studies, the significance of these findings are unclear.

Genetic mechanisms responsible for the elevation in GST expression observed in many insecticide resistant strains have been the subject of many studies. In both the housefly and yellow fever mosquito (*Ae. aegypti*), modifications in *trans*-acting regulators have been implicated in increased GST expression. In *Ae. aegypti*, a mutation in a *trans*-acting repressor element is the proposed mechanism for the enhanced expression of a delta class GST in a DDT resistant strain (Grant and Hammock, 1992). A major regulatory gene on chromosome II of *M. domestica* has been proposed to regulate both GST and monooxygenase expression in insecticide resistant strains of houseflies (Plapp, 1984). Neither of these postulated regulators have been identified, although candidates have been proposed (Sabourault *et al*., 2001). Genetic mapping of the major genes controlling GST-based DDT resistance in *A. gambiae* also provided tentative evidence for a *trans*-acting regulator (Ranson *et al*., 2000) although in this species, mutations in promoter elements of the epsilon GST cluster are also associated with resistance (Ranson *et al*., 2001).

The significance of alternative splicing in regulating GST expression has not been fully investigated. Four alternative transcripts of a delta class gene and two of a sigma GST have been detected in *A. gambiae* (see Section 5.11.5.3.1) and it is possible that different inducers or stress treatments may affect the choice of splice site.

Regulation of GST enzyme levels may also occur by alterations in the stability of mRNA transcripts. Most studies on gene regulation rely on the detection of steady state mRNA levels and the role of differential stability of transcripts is often overlooked. An exception to this was a comprehensive study in *D. melanogaster* in which factors responsible for the observed difference in the protein levels of two delta GSTs, GSTD1 and GSTD21, were investigated (Tang and Tu, 1995). Although the transcription rate of *GSTD2* (previously known as *GSTD21*) was higher than that of the neighboring gene *GSTD1*, the steady state level of *GSTD1* mRNA was over 20-fold higher than that of *GSTD2*, suggesting that the *GSTD2* mRNA is unstable. Treatment with pentobarbital (PB) enhanced the stability of *GSTD2* mRNA, but also may have prevented its efficient translation, as no corresponding increase in the levels of GSTD2 protein was observed after PB treatment.

In general, posttranslational modification has not been widely reported in the GST family and there have been no confirmed reports in the insect GSTs. A glycosylated form of a GST from the human filarial parasite *Onchocerca volvulus* has been detected. This GST is unusual as it is located in the parasite cuticle at the host–parasite interface and hence is speculated to be involved in protecting the parasite from reactive oxygen produced during the host's immune response (Sommer *et al*., 2001). The detection of multiple forms of immunologically related GSTs during purification of housefly GSTs was initially interpreted as being suggestive of posttranslational modification (Fournier *et al*., 1992). However, the extent of the insect GST family, and the presence of multiple members within both the delta and epsilon classes, suggests that these multiple forms actually represented unique GST subunits.

5.11.8. Evolution of GSTs

GSTs are thought to have evolved from a thioredoxin-like ancestor to protect cells from oxygen toxicity (Hayes and McLellan, 1999). Multiple rounds of gene duplication and subsequent diversification have led to the range of GST proteins found in eukaryotes. The thioredoxin-like fold found in the N-terminal domain of GSTs occurs in many other glutathione binding proteins, including glutathione reductase, chloride intracellular channels (CLICs), and glutaredoxin (Bushweller *et al*., 1994; Xia *et al*., 2001; Cromer *et al*., 2002). The low levels of sequence identity between these proteins and GSTs led to the conclusion that the structural similarity was due to convergent rather than

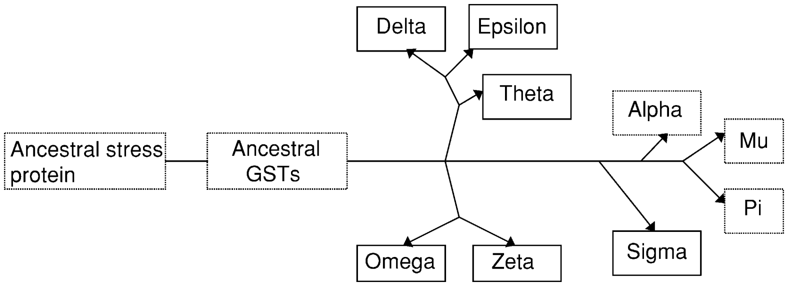


Figure 6 Suggested phylogenetic relationship between selected GST classes (those not present in insects are shown by dashed boxes). Schematic was modified from Sheehan *et al.* (2001) based on alignments of insect and mammalian GSTs in Ding *et al.* (2003).

divergent evolution (Wilce and Parker, 1994). However, the discovery of additional GST classes, in particular the omega class, warrants a reassessment of the relationship between these glutathione binding proteins and GSTs. For example, the CLIC1 sequence shows approximately 15% sequence identity with an omega class GST and structural similarity in both the N- and C-terminal domains suggesting that, despite the very different functions of these proteins, they have a common ancestor and belong in the same superfamily (Cromer *et al.*, 2002).

A schematic diagram showing the probable phylogenetic relationships between different GST classes is shown in **Figure 6**. The insect specific delta and epsilon GSTs have a common ancestor and are most closely related to the theta class to which they were originally assigned (Pemble and Taylor, 1992). The omega and zeta classes are also derived from a common ancestor and both of these classes are represented in a range of phylogenetic groups.

A comparative genomic analysis of the GSTs of the two Diptera, *A. gambiae* and *D. melanogaster*, found remarkably few clear orthologs between the two species (Ranson *et al.*, 2002). The orthologs that can be unambiguously identified were generally found within the noninsect-specific classes and these GSTs are presumably involved in essential steps in conserved physiological pathways, with subsequent constraints on their evolution. The two insect-specific classes, delta and epsilon, have independently radiated in *D. melanogaster* and *A. gambiae* suggesting that these GST classes play important roles in the adaptation of these insects to their specific biological niches rather than fulfilling general housekeeping functions. The majority of the delta and epsilon genes are tightly clustered in both species and the expanse of these GST classes is a result of local duplications of individual genes or blocks of genes (Holt *et al.*, 2002; Sawicki *et al.*, 2003).

5.11.9. Conclusions

Advances in genetics and biochemistry have unveiled a surprising complexity in the variety of the insect GST genes and enzymes in insects. The sequencing of insect genomes has been invaluable in unveiling the extent of the GST supergene family but clearly sequence analysis alone is not sufficient to uncover the *in vivo* function of individual enzymes. Furthermore, presently, full genome sequences are only publicly available for two Dipteran species. Extensive comparisons between the GST supergene family in these two species have enabled testable hypotheses about the broad roles of different GST classes to be formulated, but clearly the power of these comparative genomic approaches will be enhanced by expanding this analysis to other insect orders.

The role of GSTs in the detoxification of xenobiotics and, in particular insecticide detoxification, is rapidly being elucidated. However, much still remains to be learnt about the endogenous substrates of insect GSTs. Overexpression of individual GST genes via transient or stable transfection in insect cell lines or under expression via RNA interference or gene knockout studies will enable the role of GSTs in protecting cells against various external stimuli or chemical treatments to be studied. However, one of the biggest challenges for GST research is characterizing their roles in endogenous metabolic pathways. Deciphering the precise tissue and developmental expression profile of individual GST genes may provide some clues to their function and this type of data can now be more readily obtained for entire gene families via microarray-based experiments. Putative GST substrates are more readily assayed in an *in vitro* system, but frequently recombinant insect GSTs expressed in *E. coli* are sequestered in insoluble inclusion bodies. Overcoming this obstacle is a major priority for GST research.

References

Abdallah, M.A., Pollenz, R.S., Droog, F.N., Nunamaker, R.A., Tabachnick, W.J., *et al*., **2000**. Isolation and characterization of a cDNA clone coding for a glutathione *S*-transferase class delta enzyme from the biting midge *Culicoides variipennis sonorensis* Wirth and Jones. *Biochem. Genet. 38*, 377–390.

Agianian, B., Tucker, P.A., Schouten, A., Leonard, K., Bullard, B., *et al*., **2003**. Structure of a *Drosophila* sigma class glutathione *S*-transferase reveals a novel active site topography suited for lipid peroxidation products. *J. Mol. Biol. 326*, 151–165.

Armstrong, R.N., **1991**. Glutathione *S*-transferases: reaction mechanism, structure, and function. *Chem. Res. Toxicol. 4*, 131–140.

Arruda, L.K., Vailes, L.D., Benjamin, D.C., Chapman, M.D., **1995**. Molecular cloning of German cockroach (*Blattella germanica*) allergens. *Int. Arch. Allergy Immunol. 107*, 295–297.

Arruda, L.K., Vailes, L.D., Platts-Mills, T.A., Hayden, M.L., Chapman, M.D., **1997**. Induction of IgE antibody responses by glutathione *S*-transferase from the German cockroach (*Blattella germanica*). *J. Biol. Chem. 272*, 20907–20912.

Baker, W.L., Clark, A.G., Faulds, G., Nielsen, J.S., **1994**. Multiple glutatihone *S*-transferases in *Galleria mellonella*. *Insect Biochem. Molec. Biol. 24*, 301–307.

Beall, C., Fyrberg, C., Song, S., Fyrberg, E., **1992**. Isolation of a *Drosophila* gene encoding glutathione *S*-transferase. *Biochem. Genet. 30*, 515–527.

Blackburn, A.C., Woollatt, E., Sutherland, G.R., Board, P.G., **1998**. Characterization and chromosome location of the gene GSTZ1 encoding the human Zeta class glutathione transferase and maleylacetoacetate isomerase. *Cytogenet. Cell Genet. 83*, 109–114.

Board, P., Russell, R.J., Marano, R.J., Oakeshott, J.G., **1994**. Purification, molecular cloning and heterologous expression of a glutathione *S*-transferase from the Australian sheep blowfly (*Lucilia cuprina*). *Biochem. J. 299*, 425–430.

Board, P.G., Baker, R.T., Chelvanayagam, G., Jermiin, L.S., **1997**. Zeta, a novel class of glutathione transferases in a range of species from plants to humans. *Biochem. J. 328*, 929–935.

Board, P.G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L.S., *et al*., **2000**. Identification, characterization, and crystal structure of the Omega class glutathione transferases. *J. Biol. Chem. 275*, 24798–24806.

Booth, J., Boyland, E., Sims, P., **1961**. An enzyme from rat liver catalyzing conjugation with glutathione. *Biochem. J. 79*, 516–524.

Bushweller, J.H., Billeter, M., Holmgren, A., Wuthrich, K., **1994**. The nuclear magnetic resonance solution structure of the mixed disulfide between *Escherichia coli* glutaredoxin (C14S) and glutathione. *J. Mol. Biol. 235*, 1585–1597.

Caccuri, A.M., Antonini, G., Nicotra, M., Battistoni, A., Bello, M.L., *et al*., **1997**. Catalytic mechanism and role of hydroxyl residues in the active site of theta class glutathione *S*-transferases. Investigation of Ser-9 and Tyr-113 in a glutathione *S*-transferase from the Australian sheep blowfly *Lucilia cuprina*. *J. Biol. Chem. 272*, 29681–29686.

Chelvanayagam, G., Parker, M.W., Board, P.G., **2001**. Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. *Chem. Bio. Interact. 133*, 256–260.

Chiang, F., Sun, C., **1993**. Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorous insecticides. *Pest. Biochem. Physiol. 45*, 7–14.

Chien, C., Dauterman, W.C., **1991**. Studies on glutathione *S*-transferase in *Helicoverpa* (= *Heliothis*) *zea*. *Insect Biochem. 21*, 857–864.

Chien, C., Motoyama, N., Dauterman, W.C., **1995**. Separation of multiple forms of acidic glutathione *S*-transferase isozymes in a susceptible and a resistant strain of housefly, *Musca domestica* (L.). *Arch. Insect Biochem. Physiol. 28*, 397–406.

Clark, A.G., **1989**. The comparative enzymology of the glutathione *S*-transferases from non-vertebrate organisms. *Comp. Biochem. Physiol. B 92*, 419–446.

Clark, A.G., Dauterman, W.C., **1982**. The characterization by affinity chromatography of a glutathione *S*-transferase from different strains of housefly. *Pestic. Biochem. Physiol. 17*, 307–314.

Clark, A.G., Dick, G.K., Martindale, S.M., Smith, J.N., **1985**. Glutatione *S*-transferases from the New Zealand grass grub, *Costelytra zealandica*. *Insect Biochem. 15*, 35–44.

Clark, A.G., Letoa, M., Ting, W.S., **1977**. The purification by affinity chromatography of a glutathione *S*-transferase from larvae of *Galleria mellonella*. *Life Sci. 20*, 141–148.

Clark, A.G., Shamaan, N.A., **1984**. Evidence that DDT dehydrochlorinase from the housefly is a glutathione *S*-transferase. *Pestic. Biochem. Physiol. 22*, 249–261.

Clark, A.G., Shamaan, N.A., Sinclair, M.D., Dauterman, W.C., **1986**. Insecticide metabolism by multiple glutathione *S*-transferases in two strains of the housefly, *Musca domestica* (L.). *Pestic. Biochem. Physiol. 25*, 169–175.

Clayton, J.D., Cripps, R.M., Sparrow, J.C., Bullard, B., **1998**. Interaction of troponin-H and glutathione *S*-transferase-2 in the indirect flight muscles of *Drosophila melanogaster*. *J. Muscle Res. Cell Motil. 19*, 117–127.

Cromer, B.A., Morton, C.J., Board, P.G., Parker, M.W., **2002**. From glutathione transferase to pore in a CLIC. *Eur. Biophys. J. 31*, 356–364.

Ding, Y., Ortelli, F., Rossiter, L., Hemingway, J., Ranson, H., **2003**. The *Anopheles gambiae* glutathione transferase family: annotation, phylogeny and gene expression profiles. *BMC Genom. 13*, 35.

Dixon, D.P., Cole, D.J., Edwards, R., **1999**. Dimerisation of maize glutathione transferases in recombinant bacteria. *Plant Mol. Biol. 40*, 997–1008.

Egaas, E., Sandvik, M., Svendsen, N.O., Skaare, JU., **1995**. The separation and identification of glutathione *S*-transferase subunits from *Orthosia gothica*. *Insect Biochem. Mol. Biol. 25*, 783–788.

Feng, Q.L., Davey, K.G., Pang, A.S., Primavera, M., Ladd, T.R., *et al.*, **1999**. Glutathione *S*-transferase from the spruce budworm, *Choristoneura fumiferana*: identification, characterization, localization, cDNA cloning, and expression. *Insect Biochem. Mol. Biol. 29*, 779–793.

Fogleman, J.C., Danielson, P.B., Macintyre, R.J., **1998**. The molecular basis of adaptation in *Drosophila*. In: Hecht, M.K. (Ed.), Evolutionary Biology. Plenum, New York, p. 15.

Fournier, D., Bride, J.M., Poirie, M., Berge, J.B., Plapp, F.W., Jr., **1992**. Insect glutathione *S*-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *J. Biol. Chem. 267*, 1840–1845.

Franciosa, H., Berge, J.B., **1995**. Glutathione *S*-transferases in housefly (*Musca domestica*): location of GST-1 and GST-2 families. *Insect Biochem. Mol. Biol. 25*, 311–317.

Grant, D.F., Dietze, E.C., Hammock, B.D., **1991**. Glutathione *S*-transferase isozymes in *Aedes aegypti*: purification, characterization, and isozyme-specific regulation. *Insect Biochem. 21*, 421–433.

Grant, D.F., Hammock, B.D., **1992**. Genetic and molecular evidence for a trans-acting regulatory locus controlling glutathione *S*-transferase-2 expression in *Aedes aegypti*. *Mol. Gen. Genet. 234*, 169–176.

Habig, W.H., Pabst, M.J., Jakoby, W.B., **1974**. The glutathione *S*-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem. 249*, 7130–7139.

Hayes, J.D., McLellan, L.I., **1999**. Glutatione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res. 4*, 273–300.

Hayes, J.D., Wolf, C.R., **1988**. Role of glutathione transferase in drug resistance. In: Sies, H., Ketterer, B. (Eds.), Glutathione Conjugation. Academic Press, London, p. 315.

Hemingway, J., Miyamoto, J., Herath, P.R.J., **1991**. A possible novel link between organophosphorous and DDT insecticide resistance genes in *Anopheles:* supporting evidence from fenitrothion metabolism studies. *Pest. Biochem. Physiol. 39*, 49–56.

Hemingway, J., Ranson, H., **2000**. Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol. 45*, 371–391.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., *et al.*, **2002**. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Huang, H.S., Hu, N.T., Yao, Y.E., Wu, C.Y., Chiang, S.W., *et al.*, **1998**. Molecular cloning and heterologous expression of a glutathione *S*-transferase involved in insecticide resistance from the diamondback moth, *Plutella xylostella*. *Insect Biochem. Mol. Biol. 28*, 651–658.

Jakobsson, P.J., Thoren, S., Morgenstern, R., Samuelsson, B., **1999**. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc. Natl Acad. Sci. 96*, 7220–7225.

Ji, X., von Rosenvinge, E.C., Johnson, W.W., Tomarev, S.I., Piatigorsky, J., *et al.*, **1995**. Three-dimensional structure, catalytic properties, and evolution of a sigma class glutathione transferase from squid, a progenitor of the lens *S*-crystallins of cephalopods. *Biochemistry 34*, 5317–5328.

Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L., Ketterman, A.J., **2001**. Heterologous expression and characterization of alternatively spliced glutathione *S*-transferases from a single *Anopheles* gene. *Insect Biochem. Mol. Biol. 31*, 867–875.

Ketterman, A.J., Prommeenate, P., Boonchauy, C., Chanama, U., Leetachewa, S., *et al.*, **2001**. Single amino acid changes outside the active site significantly affect activity of glutathione *S*-transferases. *Insect Biochem. Mol. Biol. 31*, 65–74.

Kirby, M.L., Ottea, J.A., **1995**. Multiple mechanisms for enhancement of glutathione *S*-transferase activities in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol. 25*, 347–353.

Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E., Papadopoulou-Mourkidou, E., **2001**. Glutathione *S*-transferase in the defence against pyrethroids in insects. *Insect Biochem. Mol. Biol. 31*, 313–319.

Landi, S., **2000**. Mammalian class theta GST and differential susceptibility to carcinogens: a review. *Mutat. Res. 463*, 247–283.

Lougarre, A., Bride, J.M., Fournier, D., **1999**. Is the insect glutathione *S*-transferase I gene family intronless? *Insect Mol. Biol. 8*, 141–143.

Mannervik, B., **1985**. The isoenzymes of glutathione transferase. *Adv. Enzymol. Relat. Areas Mol. Biol. 57*, 357–417.

Mannervik, B., Danielson, U.H., **1988**. Glutathione transferases–structure and catalytic activity. *CRC Crit. Rev. Biochem. 23*, 283–337.

Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G.M., *et al.*, **1991**. Theta, a new class of glutathione transferases purified from rat and man. *Biochem. J. 274*, 409–414.

Meyer, D.J., Thomas, M., **1995**. Characterization of rat spleen prostaglandin H D-isomerase as a sigma-class GSH transferase. *Biochem. J. 311*, 739–742.

Oakley, A.J., Harnnoi, T., Udomsinprasert, R., Jirajaroenrat, K., Ketterman, A.J., *et al.*, **2001**. The crystal structures of glutathione *S*-transferases isozymes 1–3 and 1–4 from *Anopheles dirus* species B. *Protein Sci. 10*, 2176–2185.

Oppenoorth, F.J., Van der Pas, L.J.T., Houx, N.W.H., **1979**. Glutathione *S*-transferases and hydrolytic activity in a tetrachlorovinphos-resistant strain of housefly and their influence on resistance *Pestic. Biochem. Physiol. 11*, 176–188.

Ortelli, F., Rossiter, L.C., Vontas, J., Ranson, H., Hemingway, J., **2003**. Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide resistance locus, from the malaria vector. *Anopheles gambiae Biochem. J. 373*, 957–963.

Ottea, J.A., Plapp, F.W., **1984**. Glutathione *S*-transferases in the house fly: biochemical and genetic changes associated with induction and insecticide resistance. *Pest. Biochem. Physiol. 22*, 203–208.

Pemble, S.E., Taylor, J.B., **1992**. An evolutionary perspective on glutathione transferases inferred from class-theta glutathione transferase cDNA sequences. *Biochem. J. 287*, 957–963.

Plapp, F.W., **1984**. The genetic basis of insecticide resistance in the housefly: evidence that a single locus plays a major role in metabolic resistance to insecticides. *Pest. Biochem. Physiol. 22*, 194–201.

Prapanthadara, L., Hemingway, J., Ketterman, A.J., **1993**. Partial purification and characterization of glutathione *S*-transferases involved in DDT resistance from the mosquito *Anopheles gambiae*. *Pest. Biochem. Physiol. 47*, 119–133.

Prapanthadara, L., Hemingway, J., Ketterman, A.J., **1995**. DDT-resistance in *Anopheles gambiae* (Diptera: culicidae) from Zanzibar, Tanzania, based on increased DDT-deyhdrochlorinase activity of glutathione *S*-transferases. *Bull. Ent. Res. 85*, 267–274.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., *et al.*, **2002**. Evolution of supergene families associated with insecticide resistance. *Science 298*, 179–181.

Ranson, H., Collins, F., Hemingway, J., **1998**. The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione *S*-transferase family. *Proc. Natl Acad. Sci. USA 95*, 14284–14289.

Ranson, H., Jensen, B., Wang, X., Prapanthadara, L., Hemingway, J., *et al.*, **2000**. Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Mol. Biol. 9*, 499–507.

Ranson, H., Prapanthadara, L., Hemingway, J., **1997**. Cloning and characterization of two glutathione *S*-transferases from a DDT-resistant strain of *Anopheles gambiae*. *Biochem. J. 324*, 97–102.

Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X., *et al.*, **2001**. Identification of a novel class of insect glutathione *S*-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochem. J. 359*, 295–304.

Reiss, R.A., James, A.A., **1993**. A glutathione *S*-transferase gene of the vector mosquito, *Anopheles gambiae*. *Insect Mol. Biol. 2*, 25–32.

Rogers, M.E., Jani, M.K., Vogt, R.G., **1999**. An olfactory-specific glutathione-*S*-transferase in the sphinx moth *Manduca sexta*. *J. Exp. Biol. 202* (*Pt 12*), 1625–1637.

Rushmore, T.H., Pickett, C.B., **1993**. Glutathione *S*-transferases, structure, regulation, and therapeutic implications. *J. Biol. Chem. 268*, 11475–11478.

Sabourault, C., Guzov, V.M., Koener, J.F., Claudianos, C., Plapp, F.W., Jr., *et al.*, **2001**. Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (MdalphaE7) gene in resistant house flies. *Insect Mol. Biol. 10*, 609–618.

Sawicki, R., Singh, S.P., Mondal, A.K., Benes, H., Zimniak, P., **2003**. Cloning, expression and biochemical characterization of one Epsilon-class (GST-3) and ten Delta-class (GST-1) glutathione *S*-transferases from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class. *Biochem. J. 370*, 661–669.

Schmidt-Krey, I., Murata, K., Hirai, T., Mitsuoka, K., Cheng, Y., *et al.*, **1999**. The projection structure of the membrane protein microsomal glutathione transferase at 3 A resolution as determined from two-dimensional hexagonal crystals. *J. Mol. Biol. 288*, 243–253.

Sheehan, D., Meade, G., Foley, V.M., Dowd, C.A., **2001**. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J. 360*, 1–16.

Singh, S.P., Coronella, J.A., Benes, H., Cochrane, B.J., Zimniak, P., **2001**. Catalytic function of *Drosophila melanogaster* glutathione *S*-transferase DmGSTS1-1 (GST-2) in conjugation of lipid peroxidation end products. *Eur. J. Biochem. 268*, 2912–2923.

Singh, M., Silva, E., Schulze, S., Sinclair, D.A., Fitzpatrick, K.A., *et al.*, **2000**. Cloning and characterization of a new theta-class glutathione-*S*-transferase (GST) gene, gst-3, from *Drosophila melanogaster. Gene 247*, 167–173.

Snyder, M.J., Walding, J.K., Feyereisen, R., **1995**. Glutathione *S*-transferases from larval *Manduca sexta* midgut: sequence of two cDNAs and enzyme induction. *Insect Biochem. Mol. Biol. 25*, 455–465.

Sommer, A., Nimtz, M., Conradt, H.S., Brattig, N., Boettcher, K., *et al.*, **2001**. Structural analysis and antibody response to the extracellular glutathione *S*-transferases from *Onchocerca volvulus*. *Infect. Immun. 69*, 7718–7728.

Syvanen, M., Zhou, Z.H., Wang, J.Y., **1994**. Glutathione transferase gene family from the housefly *Musca domestica*. *Mol. Gen. Genet. 245*, 25–31.

Tang, A.H., Tu, C.P, **1994**. Biochemical characterization of *Drosophila* glutathione *S*-transferases D1 and D21. *J. Biol. Chem. 269*, 27876–27884.

Tang, A.H., Tu, C.P., **1995**. Pentobarbital-induced changes in *Drosophila* glutathione *S*-transferase D21 mRNA stability. *J. Biol. Chem. 270*, 13819–13825.

Tiwari, N.K., Singhai, S.S., Sharma, R., Meagher, R.L., Awasthi, Y.C., **1991**. Purification and characterization of glutathione transferases of *Eorenma loftini* and *Diatraea sacohara* (Lepidoptera: Pyralidae). *J. Econ. Entomol. 84*, 1424–1432.

Toba, G., Aigaki, T., **2000**. Disruption of the microsomal glutathione *S*-transferase-like gene reduces life span of *Drosophila melanogaster. Gene 253*, 179–187.

Toung, Y.P., Hsieh, T.S., Tu, C.P., **1990**. *Drosophila* glutathione *S*-transferase 1-1 shares a region of sequence homology with the maize glutathione *S*-transferase III. *Proc. Natl Acad. Sci. USA 87*, 31–35.

Toung, Y.P., Hsieh, T.S., Tu, C.P., **1993**. The glutathione *S*-transferase D genes. A divergently organized, intronless gene family in *Drosophila melanogaster. J. Biol. Chem. 268*, 9737–9746.

Udomsinprasert, R., Ketterman, A.J., **2002**. Expression and characterization of a novel class of glutathione *S*-transferase from *Anopheles dirus*. *Insect Biochem. Mol. Biol. 32*, 425–433.

Vontas, J.G., Small, G.J., Hemingway, J., **2001**. Glutathione *S*-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem. J. 357*, 65–72.

Vontas, J.G., Small, G.J., Nikou, D.C., Ranson, H., Hemingway, J., **2002**. Purification, molecular cloning and heterologous expression of a glutathione *S*-transferase involved in insecticide resistance from the rice brown planthopper, *Nilaparvata lugens*. *Biochem. J. 362*, 329–337.

Wagner, U., Edwards, R., Dixon, D.P., Mauch, F., **2002**. Probing the diversity of the Arabidopsis glutathione *S*-transferase gene family. *Plant Mol. Biol. 49*, 515–532.

Wang, J.Y., McCommas, S., Syvanen, M., **1991**. Molecular cloning of a glutathione *S*-transferase overproduced in an insecticide-resistant strain of the housefly (*Musca domestica*). *Mol. Gen. Genet. 227*, 260–266.

Wei, S.H., Clark, A.G., Syvanen, M., **2001**. Identification and cloning of a key insecticide-metabolizing glutathione *S*-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the housefly *Musca domestica*. *Insect Biochem. Mol. Biol. 31*, 1145–1153.

Wilce, M.C., Board, P.G., Feil, S.C., Parker, M.W., **1995**. Crystal structure of a theta-class glutathione transferase. *EMBO J. 14*, 2133–2143.

Wilce, M.C., Parker, M.W., **1994**. Structure and function of glutathione *S*-transferases. *Biochim. Biophys. Acta 1205*, 1–18.

Wood, E., Casabe, N., Melgar, F., Zebra, E., **1986**. Distribution and properties of glutathione *S*-transferases from *T. infestans*. *Comp. Biochem. Physiol. 84*, 607–617.

Xia, B., Vlamis-Gardikas, A., Holmgren, A., Wright, P.E., Dyson, H.J., **2001**. Solution structure of *Escherichia coli* glutaredoxin-2 shows similarity to mammalian glutathione-*S*-transferases. *J. Mol. Biol. 310*, 907–918.

Yu, S.J., **1984**. Interactions of allelochemicals with detoxication enzymes of insecticide-susceptible and resistant fall army-worms. *Pestic. Biochem. Physiol. 22*, 60–68.

Yu, S.J., **1996**. Insect glutathione *S*-transferases. *Zool. Stud. 35*, 9–19.

Zhou, Z.H., Syvanen, M., **1997**. A complex glutathione transferase gene family in the housefly *Musca domestica*. *Mol. Gen. Genet. 256*, 187–194.

5.12 Insect Transformation for Use in Control

P W Atkinson, University of California, Riverside, CA, USA
D A O'Brochta, University of Maryland Biotechnology Institute, College Park, MD, USA
A S Robinson, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria

5.12.1. Introduction

In the previous series of *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, the corresponding article by Whitten (1985) dealing, briefly, with insect transformation technology raised two points about the problems that the extension of this technology into the field would most likely face. The prescience of these forecasts is even more remarkable given that it was written shortly after the development of a genetic transformation technology for *Drosophila melanogaster* and so was authored some 10 years before the repeatable transposable element-mediated transformation of nondrosophilid pest insect species.

In writing this review we have chosen not to regurgitate information already presented in recent reviews on the subject (Robinson and Franz, 2000; Atkinson, 2002; Handler, 2002; Robinson, 2002; Wimmer, 2003; Robinson *et al*., 2004), and we will not discuss the issue of the development of a regulatory framework for the use of this technology. Two recent reports on the regulation of the release of transgenic arthropods have recently been published and the reader is referred to these for illumination as to the state of this issue (National Research Council, 2002; Pew Initiative on Food and Biotechnology, 2004). It would also be difficult to improve on Whitten's (1985) review, which comprehensively examined the history, conceptual basis, and application of genetic control in insects. Rather, under examination are two points raised by Whitten as a means to assess critically the current status of the application of genetically engineered insects to help solve problems in medicine and agriculture.

It must first be said that, some 20 years after the publication of Whitten's review, there is not one single example of the use of genetically engineered pest insects in the field. This is despite the first report of the transformation of an anopheline mosquito by Miller *et al*. (1987), and then, 8 years later, the first of several published reports ultimately describing five new transposable elements used to transform a range of nondrosophilid insect species, e.g., the Mediterranean fruit fly, *Ceratitis capitata*, and the yellow fever mosquito, *Aedes aegypti* (review: Robinson *et al*., 2004).

In his 1985 review, Whitten stated:

> Clearly it is unlikely that our technical ability to transform a species will prove lacking: rather, it is our uncertainty concerning what genetic modifications are desirable that will usually prove to be the major impediment.

The current state of the field supports this prediction. Repeatable genetic transformation technologies for nondrosophilid insects have now existed for 9 years. While the development of these did prove to be more problematic than expected and awaited the discovery of new transposable elements such as *Minos* (Franz *et al*., 1994), *Mos1* (Medhora *et al*., 1991), *piggyBac* (Cary *et al*., 1989), and *Hermes* (Warren *et al*., 1994), the application of these technologies to practical problems in medical, veterinary, and economic entomology has not occurred. Several reasons exist for this, the most important one being the difficulty in discovering genetic and biochemical systems that might prove to be effective genetic control strategies in the field. Even as recently as 2001, the most elegant examples of using genetic-based strategies to selectively eliminate females (e.g., for the augmentation of a sterile insect technology) occurred in *D. melanogaster* and with experimental population sizes several orders

of magnitude smaller than those required for the mass-rearing of insects in sterile insect technique (SIT) programs (Heinrich and Scott, 2000; Thomas *et al*., 2000). Both experiments utilized conventional *P* element-mediated genetic transformation of *Drosophila*. As discussed, research into the manipulation of genetic and biochemical systems has produced interesting advances to prevent the transmission of pathogens through mosquitoes and, using model systems, proof of principle tha transmission of pathogens can be diminished in genetically modified mosquitoes has been demonstrated (Ito *et al*., 2002; Moreira *et al*., 2002). While significant for the control of mosquitoes and the human diseases they vector, these experiments have little bearing on the development of corresponding technology in agriculturally important insect species, such as *C. capitata* and the many lepidopteran species that feed on crops. Strategies for introducing and testing genetic markers, such as the green fluorescent protein (GFP), into an SIT strain for the purpose of allowing easy identification of this strain from the field strain, have been discussed and such strains have been generated in the Caribbean fruit fly, *Anastrapha suspensa*, in which the green fluorescence has been found to be detectable up to 4 weeks postmortem (Handler, 2002). Strains having similar properties have also been developed in *C. capitata* (Franz, personal communication). The stability of this protein, although very important for its use as a marker, has raised concerns about the transfer of the protein to nontarget organisms through predation. Significant effort has been expended towards unraveling the genetic basis of sexual development in *C. capitata* with the goal being to use these genes and their promoters as the basis of new genetic sexing strategies. Important genes such as *double-sex* and *transformer* have been isolated and characterized, allowing comparisons to be made of their function with their structural homologs in *D. melanogaster* (Saccone *et al*., 2002). Sex-specific promoters from *C. capitata* have also been isolated (Christophides *et al*., 2000). To date, however, no genetically engineered strains of *C. capitata* designed for a genetic sexing strategy have been generated or tested. In lepidopterans, the only progress in pest species has been the genetic transformation of *Pectinophora glossypiella* using the *piggyBac* element and the consequent application for permits to examine the robustness of this genetically engineered strain in outdoor cage experiments (Peloquin *et al*., 2000).

Another factor contributing to the slow development of the technology is that genetic transformation of nondrosophilid insects is still not a routine robust technology. In this regard Ashburner *et al*. (1998) defined robustness:

> by the property that anyone skilled in the art can carry out the procedure, as is the case with transformation of *Drosophila*, a technique that has been performed in hundreds of laboratories world-wide.

Indeed, one of the reasons that *C. capitata* and *A. aegypti* are most frequently the subject of transformation experiments is the relative ease with which their embryos can be handled and injected. Combined with this is the very small number of laboratories in which nondrosophilid transformation is practiced. This is compounded by, in the case of tephritid fruit flies, quarantine laws that prevent many pest species from being reared in regions where they are classified as being exotic, thereby ruling out many research groups from developing genetically engineered strains of tephritids. Some 9 years after the successful demonstration of *Minos* transposable element-mediated transformation of *C. capitata* by Loukeris *et al*. (1995), the comparison with the rapid spread of *D. melanogaster* transformation protocols through the *Drosophila* community following the publication of this technique in 1982 by Rubin and Spradling (1982) could not be more stark. By 1991, *Drosophila* transformation was a routine technique that permeated *Drosophila* genetics. This has not been achieved as yet with mosquitoes, let alone many tephritids, lepidopterans, or coleopterans.

Another reason concerns the nature of the transposable elements used to transform nondrosophilid species. As mentioned above, the identification of new transposable elements such as *Mos1*, *Minos*, *Hermes*, *piggyBac*, and *Tn5* (Rowan *et al*., 2004), was a major step in achieving genetic transformation of these species. With the exception of *Tn5*, which has been developed as a transformation agent of bacteria, we know little about the genetic and biochemical behavior of these elements, particularly with respect to their behavior in nonhost insect species. Possible problems about deploying transposable elements, and the transgenes contained within them, was forecast by Whitten (1985):

> Thus the selfishness of DNA is clearly subject to rather strict rules of etiquette, presumably imposed by evolutionary constraints on its host. These constraints may force those workers who see practical benefits flowing from the availability of transposable element vectors for genomic modification of particular plants or animals, to tailor a system suited to the special genetic constraints of their particular candidate species.

Recently, the behavior of *Hermes, piggyBac*, and *Mos1* elements in *A. aegypti* was described by O'Brochta *et al.* (2003) while the immobility of *Mos1* in transgenic lines of *A. aegypti* was reported by Wilson *et al.* (2003). These studies reveal that, while these transposable elements can be used to genetically transform this mosquito species, they are then rendered relatively immobile in following generations. Furthermore, the mechanism of *Hermes* element transposition appears to differ between germline nuclei and somatic nuclei of *A. aegypti*. Both mechanisms are transposase dependent, yet cut-and-paste transposition occurs in somatic nuclei while a mechanism that results in the transposition of both the transposable element and flanking sequences occur in the germline. This difference in mechanisms of *Hermes* element transposition has not been seen in any of the higher dipteran species – *D. melanogaster* (O'Brochta *et al*., 1996; Guimond *et al*., 2003), *C. capitata* (Michel *et al*., 2001), and *Stomoxys calcitrans* (Lehane *et al*., 2000) – in which *Hermes* has been used as a gene vector. It is clear we have a very limited knowledge of how any of these transposable elements function either in their original host species, or in species into which they are introduced. This lack of knowledge, combined with the problem of identifying genetic systems they would carry into a population so as to be used in genetic control programs, has contributed to the inability to bring these transgenic-based control programs to fruition. In particular, those who propose transposable element-based strategies for spreading beneficial transgenes through field populations of insects need to take into account that the ability of a given element to transform a species does not necessarily mean that it can also subsequently spread through this same species. That the *P* element of *D. melanogaster* can do this in this species is no guarantee that other elements can also do the same. Kidwell and Ribeiro (1992) specify three critical parameters needed for a transposable element to spread through a population. These are:

- the basic reproductive rate of the insects possessing the transposable element;
- the infectivity of the element (which is a function of its transposition frequency, its ability to undergo replicative transposition, and its propensity to transpose to loci unlinked to the original donor site); and
- the size of the target insect population.

In a subsequent article (Ribeiro and Kidwell, 1994), they proposed seven questions that needed to be resolved if one is to believe that a transposable element can spread genes through an insect population. These are: (1) the instability of chimeric transposable elements containing transgenes, (2) stabilization of these transgenes following their fixation in the target population (if, indeed, this is possible), (3) the biochemical properties of the transposase, (4) the possibility of repression systems becoming active as the copy number of the transposable element increases in the genome, (5) position effects, (6) the promoters chosen to control expression of the transgene, and (7) the length of the chimeric transposable element. It must be emphasized that none of these determines how well a given transposable element will function as a gene vector in achieving transformation in the first place. It is also poignant that we know next to nothing about the answers to any of these questions for the four "new" transposable elements used to transform pest insect species.

5.12.2. The Status of Transgenic-Based Insect Control Programs

What then, is the current status of programs in which transgenic insects have been proposed to utilize transgenic insects? They can be categorized into three broad categories:

1. Those that are designed to impose a genetic load on the target population in order to reduce population size.
2. Those that are designed to only eliminate the pest phenotype of the insects in a receiving population without altering population size.
3. Those that are designed to increase the fitness of biological control agents or increase the production of products excreted by insects, such as honey or silk.

5.12.2.1. Load Imposition on the Target Population

The sterile insect technique is the classic example of controlling insects through the imposition of a genetic load. The method was developed during the 1940s and 1950s and is now a mature and widely accepted method for insect population suppression and, in some cases, eradication. The pest species is mass-reared and irradiated to induce dominant lethal mutations and rearrangements at high frequencies in the germlines of these insects. The mass-reared insects, preferably only males, are released into the receiving, field population where they mate with wild fertile insects. Fertile females mating with released sterile males are nonproductive with a net reproductive rate (R_o) of zero. In many species a small

proportion of the females mate more than once so the net effect on R_o is a function of sperm competitiveness in multiple-mated females. Successive releases in multiple generations continues to drive R_o down until either the population size is below its economic threshold (at which point it does not impact upon the economy of the relevant commodity) or until it is eradicated. The advantages of using this method of control have been discussed elsewhere (Hendrichs, 2000) but clearly rest on the species specificity of the method and its low environmental impact. Nonetheless implementing this "clean" control method faces some constraints including fitness costs associated with the development of specific strains and the effects of mass rearing, radiation, handling, and release on many aspects of field behavior (Robinson and Franz, 2000). Transgenic technology has the potential to positively impact existing SIT programs by improving aspects of insect production and monitoring and also has the potential to increase the use of SIT to include species that have otherwise been intractable to this technology.

Perhaps the biggest impact transgenic insect technology could have on any SIT program is to create strains of insects that permit the removal or elimination of females. The most significant effect that this would have on an SIT program is the reduction of the costs of mass-rearing and release because fewer insects need to be reared and released. In other programs, for example those that might involve vectors of human disease, removal of females prior to release may be essential since females would still be capable of biting and transmitting disease. Transgenic technology offers numerous options for creating strains that permit the selective removal of females prior to release. Conceptually the problem is one of expressing genes in one sex or the other that can be easily selected for or against. Using transgenic technology specificity of expression can be achieved by a variety of ways. First, expression can be controlled through the use of sex-specific promoters in combination with genes resulting in lethality. Sex-specific promoters from the vitellogenin and yolk protein genes have been characterized, and are examples of female-specific promoters that have been tested in transgenic insects with respect to possible applications in insect control programs (Heinrich and Scott, 2000; Kokoza *et al.*, 2000; Thomas *et al.*, 2000). These promoters are active in adult stages and would have limited application in SIT programs. They would not be useful for eliminating females at the larval stage and so any cost savings resulting from rearing both sexes would still be accrued. For eliminating females prior to mass-rearing, the challenge remains to identify promoters or other regulatory sequences that selectively eliminate females during, or immediately following, embryogenesis.

5.12.2.2. Challenges of Long-Term Gene Introduction into Natural Populations

Transgenic strategies relying on load imposition to reduce or eliminate a population involve the short-term introduction of "effector" genes (usually dominant or conditionally dominant lethals) into the gene pool of native populations. As described above for the SIT, approaches relying on inundative releases of nongenetically engineered mass-reared insects carrying dominant lethal mutations have been successful. More subtle approaches have been proposed whereby deleterious genes can be transmitted over the course of a few generations before the load effects are encountered (Schliekelman and Gould, 2000a, 2000b). The most ambitious plans involve the permanent and stable alteration of the genotypes of wild insects. These ideas present enormous and novel challenges to insect geneticists. Introducing new laboratory-produced genotypes into populations is relatively simple (e.g., inundative releases of radiation-sterilized flies), but maintaining those genotypes in the population and, in fact, having them increase in frequency is an unprecedented undertaking in applied insect genetics.

An allele can increase in frequency in populations for a variety of reasons. For example, if there is a fitness advantage associated with a genotype then over time this allele is expected to become more abundant. If selection pressures are sufficiently high then the forces of natural selection can result in relatively rapid changes in allele frequencies. The global spread of insecticide resistance in *Culex* mosquitoes is one of many such examples of selection driven increase in gene frequencies (Raymond, 1991). The fitness costs associated with transgenic mosquitoes are largely unknown and is an area of research in need of attention. A few reports on the ability of transgenics to compete with wild conspecifics or on fitness estimates based on life table analyses, consistently indicate that the process of transgenesis decreases the fitness of the host insect (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004). The sources of these fitness costs have not been precisely determined but are expected to be partitioned between costs associated with inbreeding during the process of creating a transgenic line of insects, transgene expression, and mutagenesis associated with transgene integration. It should be remembered, however, that genetic sexing strains generated through standard Mendelian genetics are less fit than

wild-type strains but are still successfully used in SIT programs. In *Drosophila* it has been estimated that transposing *P* elements results in a 1% reduction in viability per integration event, on average (Eanes *et al*., 1988). Thus, transgene integration is likely to result in fitness costs to the host organism. Expression of transgenes might also have effects on the fitness of the host organism. General costs associated with the metabolic load imposed by transgene expression are expected but have not been specifically measured (Billingsley, 2003). Individual transgenes, including marker genes, which have been generally considered to be "neutral," are likely to have costs associated with their expression. It is reasonable to assume, like others (Tiedje *et al*., 1989) that transgenic insects will be less fit relative to the nontransgenic host insect. Understanding the magnitude of fitness costs associated with transgenesis will permit insect geneticists to allow for any fitness costs in a given experiment. However, given that natural selection is unlikely to favor the increase in frequency of transgenes in populations, insect geneticists are still faced with the problem of driving a deleterious gene into populations quickly and effectively enough to alleviate the problem, economic or medical, caused by the target pest species.

Deleterious genes can be spread through populations by a number of mechanisms. For example, hitchhiking is a genetic phenomenon whereby genes closely linked to a particular gene under positive selection are also selected despite potentially negative fitness costs associated with them. Hitchhiking is a well-established phenomenon and insect geneticists could conceivably exploit it if a gene under strong positive selection could be identified and isolated. An alternative to hitchhiking is to link the deleterious transgene to a genetic element with transmission-skewing properties. Genetic segregation distorter systems and transposable elements have enhanced transmission potential under certain conditions. That is, organisms heterozygous for such elements give rise to a disproportionate number of gametes with the element or gene as compared to what would be expected based on simple Mendelian inheritance. A notable example of a transposable element with the ability to rapidly infiltrate populations of naive insects is the *P* element of *D. melanogaster.* Indeed, the natural history of *P* has been promoted to the status of a paradigm, and optimistic views by some vector biologists about the prospects of driving anti-*Plasmodium* genes into native populations of *Anopheles gambiae*, thereby eliminating malaria transmission, are based on exploiting the anticipated mobility properties of a *P*-like transposable element. It has been implicitly assumed that solution of the gene transformation problem in nondrosophilid insects through the development of transposable element based gene vectors would simultaneously solve the problem of gene drivers. This is an erroneous assumption.

Transposable elements differ in their abilities to spread through populations as illustrated by the differences observed between actively transposing *hobo* and *P* elements in *D. melanogaster.* The dynamics of movement and the final state achieved was element specific. A given element, such as the *P* element, may have drastically different drive potential in one species as compared to another. For example, while *P* elements effectively invade and spread in small laboratory cages of *D. melanogaster* they are relatively ineffective in *D. simulans* (Kimura and Kidwell, 1994).

There are a number of properties of a transposable element that are likely to influence its ability to spread through wild populations and these have already been listed based on previous studies (Kidwell and Ribeiro, 1992; Ribeiro and Kidwell, 1994). If we focus on the mechanism of transposition, then, clearly, activity of an element alone is not sufficient for an element to spread. Spread will require that transposition is associated with replication of the element to insure a net gain in allele frequency. Elements that transpose by a cut-and-paste mechanism are not expected to spread unless they are replicated in association with transposition. Class II transposable elements employ a number of mechanisms by which they can replicate. Element excision leaves a double-stranded break in the host DNA that is highly reactive and can initiate strand invasion and recombination using the sister chromatid as a template. Template-directed gap repair results in a copy of the moving element replacing the copy that has excised as a result of transposition. The frequency with which this type of repair takes place varies from element to element. The timing of transposition during the cell cycle is also a factor. Class II elements can also be replicated during the transposition process by timing their jumps to correspond to S phase of the cell cycle. If an element transposes after it has been replicated and targets an unreplicated region of a chromosome there will be a net gain of one element in the genome of one of the resulting mitotic products. The *Mos1* element of *D. mauritiana* appears to transpose during S phase in transgenic lines of *A. aegypti*; however, the stability of this element in these insects limits its use as a genetic drive mechanism in at least this species (O'Brochta *et al*., 2003). The proximity of the new

insertion sites relative to the donor site is also an important factor in determining the ability of the transposable element to spread. A highly active element that moves only, or even predominately, to tightly linked sites would not be a suitable agent to spread genes through an insect population, while an element with a reduced transposition rate that moved to unlinked loci either on the same or different chromosomes would be a viable spreading agent.

As mentioned above (see Section 5.12.1), at present little or no information exists as to the mobility properties of the four transposable elements used to genetically transform nondrosophilid insect species. Indeed, even for the relatively well-characterized *P* element of *D. melanogaster*, little is known about its mode of movement within and between chromosomes. This element does show a tendency to insert in or near the 5′ ends of genes, perhaps due to a relaxation of the DNA double helix during gene transcription. It has also been suggested that this element recognizes a structural feature at the insertion site rather than a strict canonical motif (Liao *et al*., 2000). This may well be true of other transposable elements and may well be an important factor in determining transposable element spread, but this remains an underexplored area of research.

5.12.2.3. Engineering of Beneficial Insects

Progress in this area has been limited to the stable introduction of genes, using the *piggyBac* transposable element, into the silkworm, *Bombyx mori* (Tamurua *et al*., 2000). Initially these experiments have been proof of principle experiments in which enchanced green fluorescent protein (EGFP) was used as a genetic marker to demonstrate that transformation could be achieved. Recently, Imamura *et al*. (2003) demonstrated that the GAL4/UAS system functions sufficiently in transgenic *B. mori* to enable tissue-specific expression of a reporter gene to occur. Transformation frequencies using the *piggyBac* transposable element as the gene vector were in the order of several percent. These experiments pave the way for gene identification using enhancer trapping in *Bombyx* which will further elevate the use of this species as a model system for other lepidopteran species. The extension of these techniques into practical benefits of silk production remains a challenge. While there have been reports of sperm-mediated transformation of the honeybee, *Apis mellifera* (Robinson *et al*., 2000), this technology has not yet been exploited by the honey industry and, since honey is a food, genetic engineering of its source may encounter public resistance. Similarly, the initial report of genetic transformation of the predatory mite *Metaseiulus occidentalis*, which is used as a biological control agent, has not been pursued in field applications (Presnail and Hoy, 1992).

5.12.3. Conclusion

Genetic transformation technologies have been successfully extended into selected nondrosophilid species using transposable elements. This significant and exciting success has, however, being confined to the laboratory where it has enabled novel genotypes to be constructed and tested. These technologies have yet to be extended to the field, despite many years elapsing since genetic transformation protocols were first established for key pest species such as *C. capitata* and *A. aegypti*. If the full potential and benefits of genetic modification of medically and agriculturally significant insect species is to be realized then there must be a stronger linkage between the formulation of ideas and the subsequent timely and safe testing of these in transgenic insect strains in the laboratory, and in the field. Key to this is improving the robustness of transgenic technology in these insect species. Alternatively, the wisdom of establishing a handful of insect transformation centers that would provide this service to the community needs to be explored. This may be particularly attractive for species, such as *A. gambiae*, that remain difficult to transform. Providing a central transformation center may encourage researchers to develop and test new concepts, confident that at least the transgenic insects containing the desired transgenes will be routinely produced in a timely manner. It is critical to demonstrate in the laboratory and then in field cage experiments clear and concrete examples of how transgenic insect technology is beneficial to the general public so that arguments about the benefits of these new approaches can be clearly made to this interested and undoubtedly concerned audience.

References

Ashburner, M., Hoy, M.A., Peloquin, J.J., **1998**. Prospects for the genetic transformation of arthropods. *Insect Mol. Biol.* *7*, 201–213.

Atkinson, P.W., **2002**. Genetic engineering in insects of agricultural importance. *Insect Biochem. Mol. Biol.* *32*, 1237–1242.

Billingsley, P.F., **2003**. Environmental constraints on the physiology of transgenic mosquitoes. In: Takken, W., Scott, T.W. (Eds.), Ecological Aspects for Application of

Genetically Modified Mosquitoes. Kluwer Academic Publishers, Dordrecht, pp. 149–161.

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E., *et al.*, **1989**. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology 172*, 156–169.

Catteruccia, F., Godfray, H.C., Crisanti, A., **2003**. Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. *Science 299*, 1225–1227.

Christophides, G.K., Livadaras, I., Savakis, C., Komitopoulou, K., **2000**. Two medfly promoters that have originated by recent gene duplication drive distinct sex, tissue and temporal expression patterns. *Genetics 156*, 173–182.

Eanes, W.F., Wesley, C., Hey, J., Houle, D., Ajioka, J.W., **1988**. The fitness consequences of *P* element insertion in *Drosophila melanogaster. Genet. Res. 52*, 17–26.

Franz, G., Loukeris, T.G., Dialektaki, G., Thompson, C.R., Savakis, C., **1994**. Mobile *Minos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. *Proc. Natl Acad. Sci. USA 91*, 4746–4750.

Guimond, N.D., Bideshi, D.K., Pinkerton, A.C., Atkinson, P.W., O'Brochta, D.A., **2003**. Patterns of *Hermes* element transposition in *Drosophila melanogaster. Mol. Gen. Genom. 268*, 779–790.

Handler, A.M., **2002**. Prospects for using genetic transformation for improved SIT and new biocontrol methods. *Genetica 116*, 137–149.

Heinrich, J.C., Scott, M.J., **2000**. A repressible female-specific lethal genetic system for making transgenic strains suitable for a sterile-release program. *Proc. Natl Acad. Sci. USA 97*, 8229–8232.

Hendrichs, J., **2000**. Use of the Sterile Insect Technique against key insect pests. *Sust. Devel. Int. 2*, 75–79.

Imamura, M., Nakai, J., Inoue, S., Quan, G.X., Kanda, T., *et al.*, **2003**. Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori. Genetics 165*, 1329–1340.

Irvin, N., Hoddle, M.S., O'Brochta, D.A., Carey, B., Atkinson, P.W., **2004**. Assessing fitness costs for transgenic *Aedes aegytpi* expressing the green fluorescent protein marker and transposase genes. *Proc. Natl Acad. Sci. USA 101*, 891–896.

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E.A., Jacobs-Lorena, M., **2002**. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature 417*, 387–388.

Kidwell, M.G., Ribeiro, J.M.C., **1992**. Can transposable elements be used to drive refractoriness genes into vector populations? *Parasitol. Today 8*, 325–329.

Kimura, K., Kidwell, M.G., **1994**. Differences in *P* element population dynamics between the sibling species *Drosophila melanogaster* and *Drosophila simulans*. *Genet. Res. 63*, 27–38.

Kokoza, V., Ahmed, A., Cho, W.L., Jasinskiene, N., James, A.A., *et al.*, **2000**. Engineering blood-meal activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc. Natl Acad. Sci. USA 97*, 9144–9149.

Lehane, M.J., Atkinson, P.W., O'Brochta, D.A., **2000**. *Hermes*-mediated genetic transformation of the stable fly, *Stomoxys calcitrans*. *Insect Mol. Biol. 9*, 531–538.

Liao, G.C., Rehm, E.J., Rubin, G.M., **2000**. Insertion site preferences of the *P* transposable element in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA 97*, 3347–3451.

Loukeris, T.G., Livadras, I., Arca, B., Zabalou, S., Savakis, C., **1995**. Gene transfer into the Medfly, *Ceratitis capitata*, using a *Drosophila hydei* transposable element. *Science 270*, 2002–2005.

Medhora, M., Maruyama, K., Hartl, D.L., **1991**. Molecular and functional analysis of the mariner mutator element *Mos1* in *Drosophila*. *Genetics 128*, 311–318.

Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S., *et al.*, **2001**. *Hermes*-mediated germline transformation of the Mediterranean fruit fly, *Ceratitis capitata*. *Insect Mol. Biol. 10*, 155–162.

Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P., *et al.*, **1987**. Stable integration and expression of a bacterial gene in the mosquito, *Anopheles gambiae*. *Science 237*, 779–781.

Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H., *et al.*, **2002**. Bee venom phosopholipase inhibits malaria parasite development in transgenic mosquitoes. *J. Biol. Chem. 277*, 40839–40843.

National Research Council, **2002**. Animal Biotechnology: Science-Based Concerns. The National Academies Press, Washington, DC.

O'Brochta, D.A., Sethuraman, N., Wilson, R., Hice, R.H., Pinkerton, A.C., *et al.*, **2003**. Gene vector and transposable element behavior in mosquitoes. *J. Exp. Biol. 206*, 3823–3834.

O'Brochta, D.A., Warren, W.D., Saville, K.J., Atkinson, P.W., **1996**. *Hermes*, a functional non-drosophilid gene vector from *Musca domestica*. *Genetics 142*, 907–914.

Peloquin, J.J., Thibault, S.T., Miller, T.A., **2000**. Genetic transformation of the pink bollworm *Pectinophora gossypiella* with the *piggyBac* element. *Insect Mol. Biol. 9*, 323–333.

Pew Initiative on Food and Biotechnology, **2004**. "Bugs in the System? Issues in the Science and Regulation of Genetically Modified Insects." Washington, DC, pp. 109.

Presnail, J.K., Hoy, M.A., **1992**. Stable genetic transformation of a beneficial arthropod, *Metaseiulus occidentalis* (Acari: Phytoseiidae), by a microinjection technique. *Proc. Natl Acad. Sci. USA 89*, 7732–7736.

Raymond, M., **1991**. Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature 350*, 151–153.

Ribeiro, J.M., Kidwell, M.G., **1994**. Transposable elements as population drive mechanisms: specification of critical parameter values. *J. Med. Entomol. 31*, 10–16.

Robinson, A.S., **2002**. Mutations and their use in insect control. *Mutat. Res. 511*, 113–132.

Robinson, A.S., Franz, G., Atkinson, P.W., **2004**. Insect transgenesis and its potential role in agriculture and human health. *Insect Biochem. Mol. Biol. 34*, 113–120.

Robinson, A.S., Franz, G., **2000**. The application of transgenic insect technology in the sterile insect technique. In: Handler, A.M., James, A.A. (Eds.), Insect Transgenesis: Methods and Applications. CRC Press, Boca Raton, FL, pp. 307–318.

Robinson, K.O., Ferguson, H.J., Cobey, S., Vassein, H., Smith, B.H., **2000**. Sperm-mediated transformation of the honey bee, *Apis mellifera*. *Insect Mol. Biol. 9*, 625–634.

Rowan, K.H., Orsetti, J., Atkinson, P.W., O'Brochta, D.A., **2004**. *Tn5* as an insect gene vector. *Insect Biochem. Mol. Biol. 34*, 695–705.

Rubin, G.M., Spradling, A.C., **1982**. Genetic transformation of *Drosophila* with transposable element vectors. *Science 218*, 348–353.

Saccone, G., Pane, A., Polito, L.C., **2002**. Sex determination in flies, fruitflies and butterflies. *Genetica 116*, 15–23.

Schliekelman, P., Gould, F., **2000a**. Pest control by the introduction of a conditonal lethal trait on multiple loci: potential, limitations, and optimal strategies. *J. Econ. Entomol. 93*, 1543–1565.

Schliekelman, P., Gould, F., **2000b**. Pest control by the release of insects carrying a female-killing allele on multiple loci. *J. Econ. Entomol. 93*, 1566–1579.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., *et al.*, **2000**. Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnol. 18*, 81–84.

Thomas, D.D., Donnelly, C.A., Wood, R.J., Alphey, L.S., **2000**. Insect population control using a dominant, repressible, lethal genetic system. *Science 287*, 2474–2476.

Tiedje, J.M., Colwell, R.K., Grossman, Y.L., Hodson, R.E., Lenski, R.E., *et al.*, **1989**. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology 70*, 298–315.

Warren, W.D., Atkinson, P.W., O'Brochta, D.A., **1994**. The *Hermes* transposable element from the housefly, *Musca domestica*, is a short inverted repeat-type element of the *hobo, Ac*, and *Tam3* (*hAT*) element family. *Genet. Res. 64*, 87–97.

Whitten, M.J., **1985**. The conceptual basis for genetic control. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 12. Pergamon, Oxford, pp. 465–528.

Wilson, R., Orsetti, J., Klocko, A.K., Aluvihare, C., Peckham, E., *et al.*, **2003**. Post-integration behavior of a *Mos1* mariner gene vector in *Aedes aegypti*. *Insect Biochem. Mol. Biol. 33*, 853–863.

Wimmer, E.A., **2003**. Innovations: applications of insect transgenesis. *Nature Rev. Genet. 4*, 225–232.

6.1 Pyrethroids

B P S Khambay and P J Jewess, Rothamsted Research, Harpenden, Hertfordshire, UK

6.1.1. Introduction

The introduction of synthetic pyrethroids in the 1970s signalled a new era for environmentally friendly, highly effective and selective insecticides. For example, deltamethrin when introduced was the most effective insecticide on the market, being over 100 times more active than DDT and with the benefit of not accumulating in the environment. More than 30 new pyrethroids, including several new structural types, have been commercialized in the past 20 years (Bryant, 1999; Tomlin, 2000). Despite the evolution of resistance to pyrethroids in many insect pests, pyrethroids continue to be an important class. Part of their success has been due to the implementation of resistance-management strategies and the exploitation of new applications.

Since the previous review in this series (Ruigt, 1985), research activity in the discovery of novel pyrethroids peaked in the early 1990s and has now largely ceased. Major advances have been made in understanding the mode of action, especially by the application of molecular biology techniques (see **Chapter 5.1**), and in the management of resistance in the field. This chapter will review the advances made in these areas with the emphasis on commercial compounds.

6.1.1.1. Pyrethrum Extract

Despite the introduction of a number of other natural insecticides, pyrethrum extract from the flowers of the pyrethrum daisy (*Tanacetum cinerariaefolium* Schulz –Bip.; syns *Chrysanthemum cinerariaefolium* Vis., *Pyrethrum cinerariaefolium* Trevir.) remains commercially the most important, with demand continually outstripping supply. Supplies from Kenya, the major producer, have been irregular for many years. A new pyrethrum industry has been established in Tasmania, Australia, which is currently the second biggest producer and is set to increase further. Their industry was founded on the basis of extensive research leading to the development of plant lines that flower simultaneously, thus allowing mechanical harvesting and thereby a significant saving in labor costs. In addition they produce a very high-quality product by further purification of the hexane extract with a new procedure based on partitioning with liquid carbon dioxide. Major applications of pyrethrum extract continue to center on control of flying insects (e.g., mosquitoes), pre-harvest clean-up, and human- and animal-health pests. With an increasing demand for organically grown produce, use of pyrethrum extract has also increased.

6.1.1.2. Synthetic Pyrethroids

The market share of synthetic pyrethroids remained for many years at around 20–25% of the global insecticide market but in the past few years has declined to around 17%. Cotton remains the biggest market for pyrethroid applications; however, due to resistance build-up, this market has steadily declined from nearly 50% to less than 40% of the total pyrethroid tonnage. With the exception of some Japanese and Chinese companies, most major agrochemical companies ceased research effort in the discovery of novel pyrethroids by the late 1990s.

Since 1985, major advances have been made in identifying novel pyrethroids that have substantially

increased the spectrum of activity to include control of mites, soil pests, rice pests (previously limited by the high fish toxicity of pyrethroids), and newer problem pests such as the cotton (also called the tobacco or sweet-potato) whitefly (*Bemisia tabaci*). In the past decade, commercial effort has been directed at introducing enriched isomer mixtures and the exploitation of niche markets including head-lice, termites, and bednets for the control of mosquitoes. Rather remarkably, sea-lice on salmonid fish are controlled by established pyrethroids (e.g., deltamethrin) which, despite their high fish toxicity, are being used at carefully selected doses. Another area of progress has been the development of novel formulations to extend the utility of established pyrethroids. For example, lambda-cyhalothrin formulated as micro-capsules allows slow release for public-health uses requiring long-term residual effects and when impregnated in plastic films controls termites with minimal operator exposure.

At the commercial level, global sales of pyrethroids in 2002 have been estimated at $1280M. Over 95% of the pyrethroid sales are accounted for by 12 active ingredients, of which deltamethrin continues to be the market leader with >16% share and sales exceeding $200M. Lamda-cyhalothrin is a close second with nearly 16% share; permethrin and cypermethrin account for 14% and 11% respectively, with all other pyrethroids contributing less than 7% each. Notably, although tefluthrin accounts for only 6% of the pyrethroid market, it is one of the most widely used soil insecticides in maize in the USA (Cheung and Sirur, 2002). The biggest concern in the sustained use of pyrethroids is the development of resistance. Though successful integrated pest-management strategies have been utilized, overall levels of resistance have continued to increase steadily. However, a greater understanding of the resistance mechanisms and the development of simple and high-throughput diagnostic procedures have aided the recognition of resistance mechanisms and hence facilitated the management of resistance.

6.1.2. Development of Commercial Pyrethroids

There are several reviews (Elliott, 1977, 1995, 1996; Davies, 1985; Henrick, 1995; Pap *et al*., 1996a; Matsuo and Miyamoto, 1997; Pachlatko, 1998; Bryant, 1999; Katsuda, 1999; Khambay, 2002; Soderlund *et al*., 2002) covering aspects of structure–activity relationships in pyrethroids. To appraise the advances made since 1985, it is useful to recollect the systematic approaches adopted earlier, in particular by Elliott, in the discovery of synthetic pyrethroids (see **Figure 1** for structures). The key to his success was the selection of pyrethrin I (**1**) over pyrethrin II (**2**) as the lead structure and the choice of test species (housefly *Musca domestica* and mustard beetle *Phaedon cochleariae*), which are model insects rather than agricultural pests. Pyrethrin I has superior insecticidal activity to pyrethrin II, though the latter gives better knockdown. This chapter will briefly review the progress in the development of pyrethroids using commercial pyrethroids as examples and for ease of readability these are not cited in chronological order.

Elliott's work culminated in the discovery of bioresmethrin (**3**) in 1967 followed by permethrin (**4**), cypermethrin (**5**), and deltamethrin (**6**) in 1977. These compounds have greater insecticidal activity than pyrethrin I, yet exhibit lower mammalian toxicity (**Table 1**) and initiated a major interest in pyrethroid research in agrochemical companies. These early synthetic compounds still account for a substantial share of the pyrethroid market despite the subsequent introduction of over 30 new pyrethroids. The majority of these compounds are closely related analogs. Replacement of the *E* (but not *Z*) vinyl chloride in cypermethrin with a CF_3 or a 4-chlorophenyl group retains activity and led to the discovery of cyhalothrin (**7**) and flumethrin (**8**), respectively. Both have good acaricidal activity and the latter is especially active against a range of tick species and is extensively used in animal health. Bromination of deltamethrin led to tralomethrin (**9**), a proinsecticide that breaks down to deltamethrin in the insect. Though most of the commercial pyrethroids are based on pyrethrin I, two have been developed using pyrethrin II as the lead structure. Kadethrin (**10**) emulates the high knockdown activity of pyrethrin II but acrinathrin (**11**) is more noted for its high activity against mites, ticks, lepidopteran, and sucking pests.

The other major influence in the development of pyrethroids came with the announcement of fenvalerate (**12**), a compound lacking the cyclopropane moiety. It was announced in 1976 by researchers at Sumitomo, around the same time as those disclosed by Elliott. Subsequently, many analogs were discovered, mainly with different substituents in the acyl moiety, especially at position 4. Examples include flucythrinate (**13**) and its derivative flubrocythrinate (**14**), also referred to as ZXI 8901) (Jin *et al*., 1996) with Br introduced at the 4″ position. Introduction of NH and optimization of substituents in the acyl moiety led to fluvalinate (**15**), which exhibits broad-spectrum insecticidal and miticidal

activity. In contrast to most other pyrethroids, it is essentially nontoxic to bees and is used to control mites in honeybee colonies and orchards.

Advances since 1985 in the development of new alcohol moieties have been limited. Recognition of the need for unsaturation in the side chain of the alcohol moiety of pyrethrin I had led to the development of the first commercial synthetic pyrethroid, allethrin (**16**). Substitution of the alkene with an alkyne group resulted in the more active analog prallethrin (**17**). Further systematic development resulted in the discovery of imiprothrin (**18**) and the more volatile, albeit only moderately insecticidal, empenthrin (**19**). The latter has low mammalian toxicity and is used in controlling insects that eat fabrics. Pyrethroids containing fluorinated benzyl alcohol moieties have long been recognized for their high vapor pressure but many exhibit high mammalian toxicity. An exception is transfluthrin (**20**) (acute $LD_{50} > 5000\,mg\,kg^{-1}$ to male rats), containing a tetrafluorobenzyl alcohol moiety, which has only recently been commercialised for domestic use. Although many pyrethroids exhibit high activity in contact assays against soil pests (e.g., the corn rootworm *Diabrotica balteata*), the majority are ineffective under field conditions because they do not possess the required physical properties, especially high volatility (Khambay *et al*., 1999). Tefluthrin (**21**), having a 4-methyltetrafluorobenzyl alcohol moiety, is sufficiently volatile and, despite its relatively high mammalian toxicity (acute LD_{50} $22\,mg\,kg^{-1}$ to male rats), is the only pyrethroid to have been commercialized as a soil insecticide. Two analogs of tefluthrin, metofluthrin (**22**) and profluthrin (**23**), have recently been developed for non-agricultural use by Sumitomo.

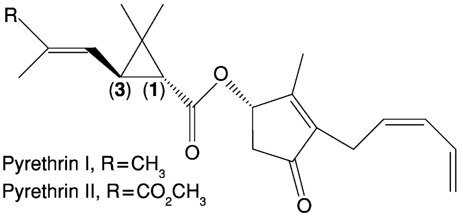
An interesting development has been the replacement of 3-phenoxybenzyl alcohol by 3-phenylbenzyl alcohol, which is sterically more restricted. Activity in this series is enhanced by the introduction of a CH_3 group at position 2 as in bifenthrin (**24**), which forces noncoplanarity of the phenyl rings presumably so matching the conformation of the target site. It has broad-spectrum activity and is particularly effective against some mite species. Although it is has one of the highest levels of mammalian and fish toxicity, it is commercially very successful.

The development of "hybrid" pyrethroids containing structural features both of DDT and of pyrethroids has been discussed previously (Ruigt, 1985). One compound, cycloprothrin (**25**), was eventually commercialized in 1987. Though it is much less insecticidal than deltamethrin, it has lower mammalian and fish toxicity and is therefore used to control pests in a range of applications including paddy rice, vegetables, tea, and cotton.

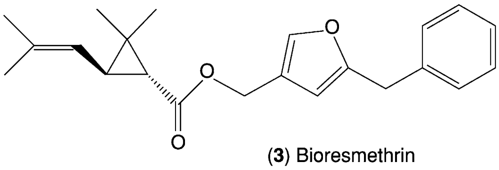
The most noteworthy advance in the past 20 years has been the discovery of the nonester pyrethroid etofenprox (**26**) in 1986. Further innovations led to silicon (e.g., silafluofen, (**27**)) and tin (e.g., (**28**)) analogs, the latter being nearly ten times more active than the corresponding carbon compounds. It had been a major objective of many researchers to replace the ester linkage in pyrethroids so as to achieve increased metabolic stability. A key feature of the nonester pyrethroids is their low toxicity to fish, and indeed etofenprox is used in rice paddy. However, it may be noted that even ester pyrethroids, despite having high intrinsic toxicity to fish, often manifest lower toxicity in the field than might be expected. This lower toxicity is observed because their concentration in water is limited by very low solubility, together with mitigation by strong sorption into organic matter such as pond sediments and river vegetation. However, the nonester pyrethroids have not yet made a significant commercial impact.

Of the many other variations, introduction of fluorine at the 4-position in the alcohol moiety has been the most significant. In general it leads to an appreciable enhancement of activity, albeit only to some species especially ticks (as in flumethrin (**8**)). Cyfluthrin (cypermethrin with a 4′-F) appears to have been introduced mainly on commercial considerations rather than on the grounds of efficacy. Furthermore, this substitution reduces metabolic attack at the 4′-position, thereby reducing the level of resistance in some insect species.

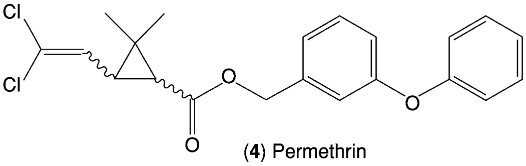
Commercial effort in the discovery of novel pyrethroids had all but ceased by the late 1990s, although efforts have continued to introduce single- or enriched-isomer mixtures (**Table 2**). Deltamethrin was the first pyrethroid to be commercialized as a single enantiomer by Rousell-Uclaf. This was followed by esfenvalerate (1*S*, α*S* component of fenvalerate) and acrinathrin. A single isomer of cyhalothrin, gamma-cyhalothrin (also called super-cyhalothrin), is currently under development. For cypermethrin, commercial production of a single isomer has not been possible; however, enriched isomer mixtures have been introduced to exploit specific properties and market opportunities. For example, the *trans* isomers of cypermethrin are known to be more readily metabolized in mammals than the *cis* isomers. Thus theta-cypermethrin, containing only the *trans* isomers, has a much higher safety margin than cypermethrin and, in addition to its use in agriculture, also has uses in veterinary and public health. Prallethrin also has potentially



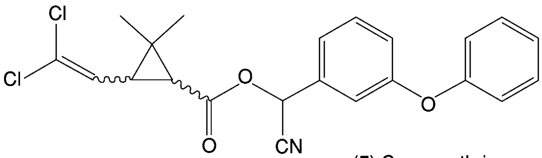
(**1**) Pyrethrin I, R=CH_3
(**2**) Pyrethrin II, R=CO_2CH_3



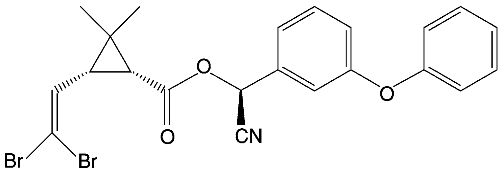
(**3**) Bioresmethrin



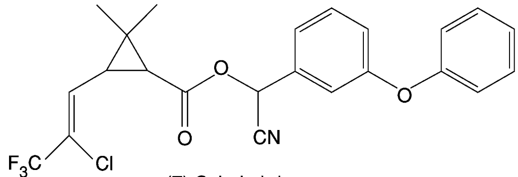
(**4**) Permethrin



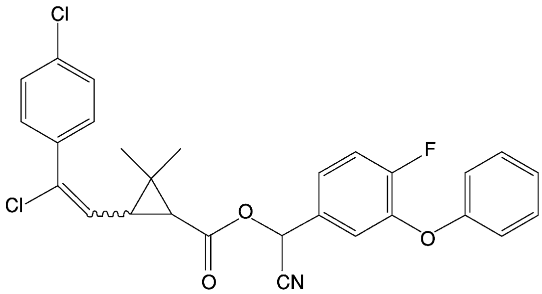
(**5**) Cypermethrin



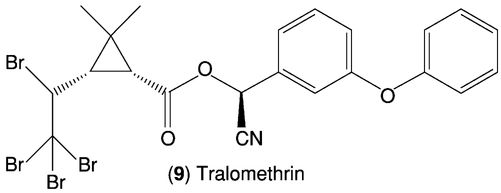
(**6**) Deltamethrin



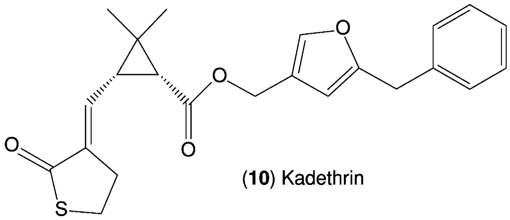
(**7**) Cyhalothrin



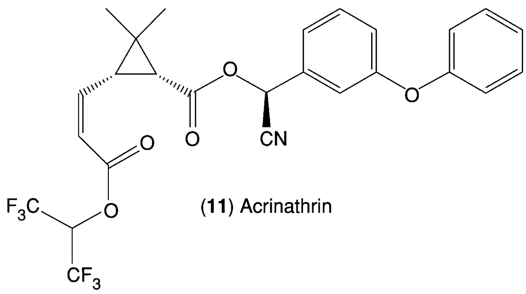
(**8**) Flumethrin



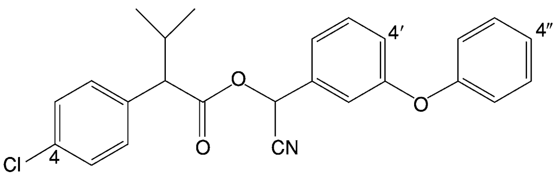
(**9**) Tralomethrin



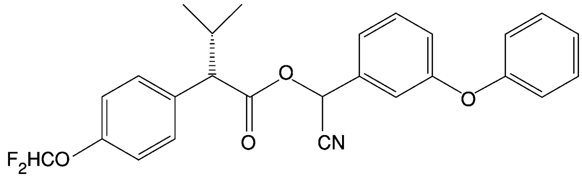
(**10**) Kadethrin



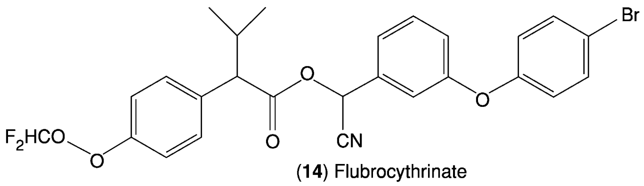
(**11**) Acrinathrin



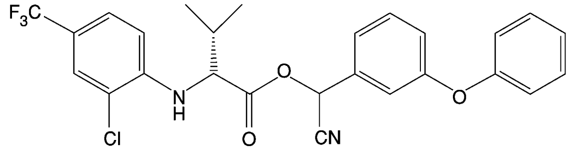
(**12**) Fenvalerate



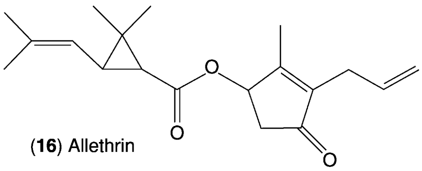
(**13**) Flucythrinate



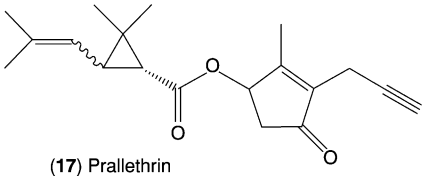
(**14**) Flubrocythrinate



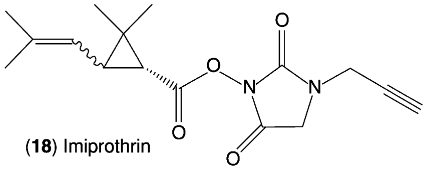
(**15**) Fluvalinate



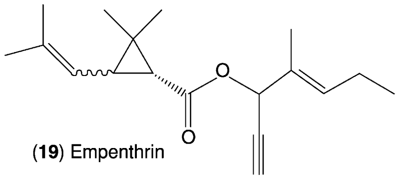
(**16**) Allethrin



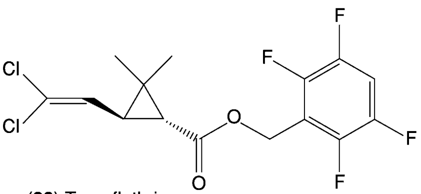
(**17**) Prallethrin



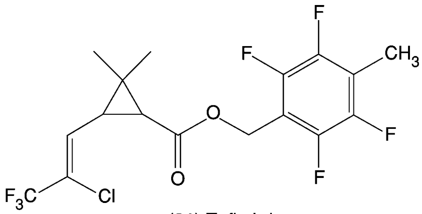
(**18**) Imiprothrin



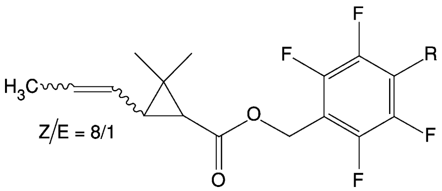
(**19**) Empenthrin



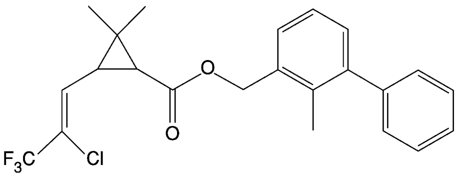
(**20**) Transfluthrin



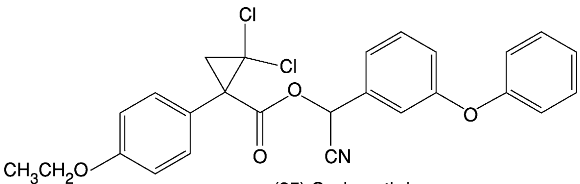
(**21**) Tefluthrin



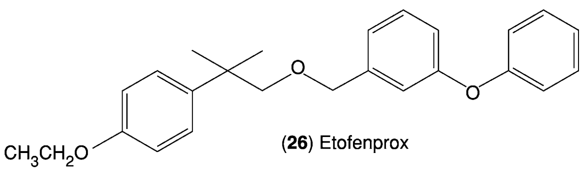
(**22**) Metofluthrin R=CH_2OCH_3
(**23**) Profluthrin R=CH_3



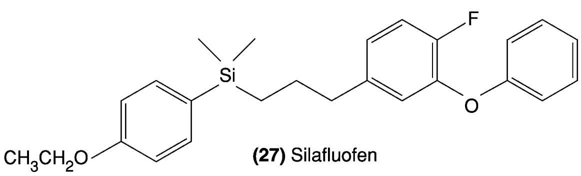
(**24**) Bifenthrin



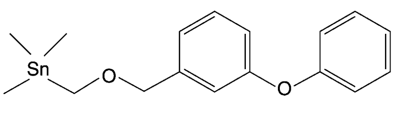
(**25**) Cycloprothrin



(**26**) Etofenprox



(27) Silafluofen



(28)

Figure 1 Commercial and novel pyrethroids (structures **1**–**28**).

Table 1 Insecticidal and mammalian toxicity of pyrethrin I and selected pyrethroids

	Representative median lethal dose ($mg\,kg^{-1}$)	
Pyrethroid	*Housefly, Musca domestica*	*Rat, oral*
Pyrethrin I (**1**)	30	420
Bioresmethrin (**3**)	6	800
Permethrin (**4**)	0.7	2000
Deltamethrin (**6**)	0.02	~100

Table 2 Isomer mixtures of pyrethroids

Pyrethroid	*Product*	*Number of isomers*	*Stereochemistry*
Cypermethrin		8	(1*R*)-*cis*, α*S*; (1*S*)-*cis*, α*R* (1*R*)-*cis*, α*R*; (1*S*)-*cis*, α*S* (1*R*)-*trans*, α*S*; (1*S*)-*trans*, α*R* (1*R*)-*trans*, α*R*; (1*S*)-*trans*, α*S*
	Alpha	2	(1*R*)-*cis*, α*S*; (1*S*)-*cis*, α*R*
	Beta	4	(1*R*)-*cis*, α*S*; (1*S*)-*cis*, α*R* (1*R*)-*trans*, α*S*; (1*S*)-*trans*, α*R*
	Theta	2	(1*R*)-*trans*, α*S*; (1*S*)-*trans*, α*R*
	Zeta	4	(1*R*)-*cis*, α*S*; (1*S*)-*cis*, α*S* (1*R*)-*trans*, α*S*; (1*S*)-*trans*, α*S*
Cyhalothrin		4	(1*R*)-*cis*, α*S*; (1*S*)-*cis*, α*R* (1*R*)-*trans*, α*S*; (1*S*)-*trans*, α*R*
	Lambda	2	(1*R*)-*cis*, α*S*; (1*S*)-*cis*, α*R*
	Gamma	1	(1*R*)-*cis*, α*S*
Fluvalinate		4	(2*R*), α*R*; (2*R*), α*S* (2*S*), α*S*; (2*S*), α*S*
	Tau	2	(2*R*), α*R*; (2*R*), α*S*

eight isomers but is sold as a mixture of two active *cis*/*trans* isomers, each with the 1*R* center at C1 and α*S* in the alcohol moiety.

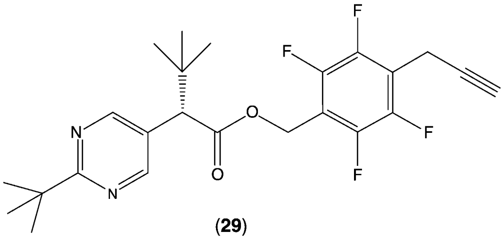
6.1.3. Structure–Activity Relationships in Pyrethroids

As described above, pyrethroids form a diverse class of insecticides highly effective against a broad spectrum of insect and acarine pests. The level of activity is determined by penetration, metabolism within the insect, and the requirements at the target site. As an illustration (see Figure 2 for structures), pyrethrin I has good activity against houseflies but poor activity against mites, whereas an experimental compound (**29**) has very high miticidal but poor insecticidal activity. Despite this diversity, it is possible to make some generalizations regarding structural requirements for high activity in pyrethroids. The seven-segment model (Figure 3) based on pyrethrin I, the lead structure originally adopted by Elliott (Elliott and Janes, 1978), serves as a good model to illustrate these general requirements and exceptions to them.

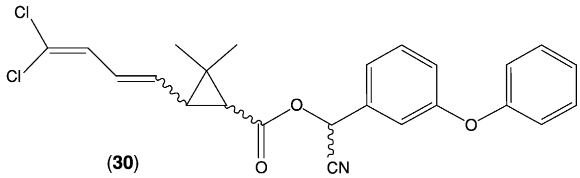
Segment A: Unsaturation generally leads to high activity. However, exceptions are known; for example fenpropathrin (**41**), containing the tetramethylcyclopropane acyl moiety (lacking in unsaturation), has high insecticidal and acaricidal activity. In general, large variations (steric and electronic) can be tolerated in this region. For example, (**30**) has an extended side-chain compared with cypermethrin and yet their activity is similar; flumethrin (**8**), possessing a bulky aromatic substituent, is highly active towards ticks.

Segment A + B: In ester pyrethroids, the gem-dimethyl group or its steric equivalent must be beta to the ester group. The required geometrical stereochemistry (*cis*/*trans*) across the cyclopropane ring (segment B) is influenced by the nature of the alcohol moiety, substituent at C3 and test species. In general, 1*R*-*trans*-chrysanthemates (as in bioresmethrin) are more active then the corresponding *cis* isomers. The vinyl dihalo substituents are generally more effective than the chrysanthemates; however, the relative activity of the *cis* and *trans* isomers varies considerably. For alcohols containing two rings (e.g., 3-phenoxybenzyl alcohol as in deltamethrin), the 1*R*-*cis* isomers are generally more active than the *trans* isomers, but with alcohol moieties that contain a single ring (e.g., pentafluorobenzyl as in fenfluthrin (**31**) and cyclopentenone as in allethrin) the converse is true, with the *trans* isomers being more active.

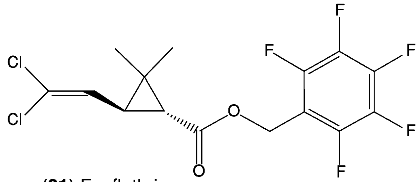
In pyrethrin II, the vinyl carbomethoxy group in the acid moiety has the *E* configuration. Its corresponding analogs, *cis* and *trans* esters of α-cyano-3-phenoxybenzyl alcohol, are equally active; the *Z* isomer of the nor homolog (e.g., (**32**)) of the *cis* (but not the *trans*) isomer was unexpectedly much more active. Acrinathrin incorporates these two features and, although only half as active as deltamethrin against houseflies, it is more active towards some species of mites, ticks, lepidopteran insects, and sucking pests. Furthermore, stereochemistry also influences species selectivity of pyrethroids, a process primarily determined by selective metabolism. For example, contrary to the above generalization for pyrethroids containing 3-phenoxybenzyl



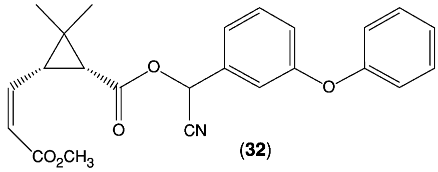
(**29**)



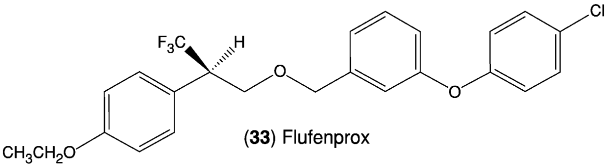
(**30**)



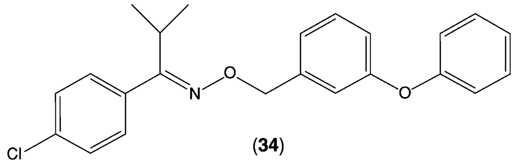
(**31**) Fenfluthrin



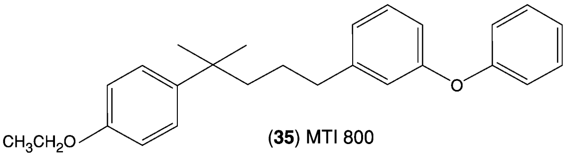
(**32**)



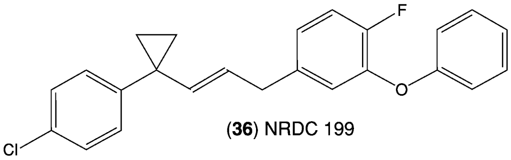
(**33**) Flufenprox



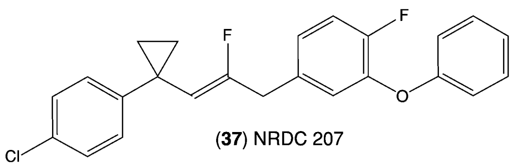
(**34**)



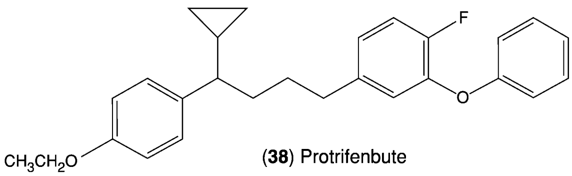
(**35**) MTI 800



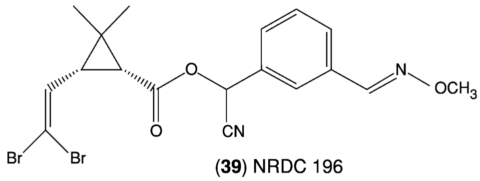
(**36**) NRDC 199



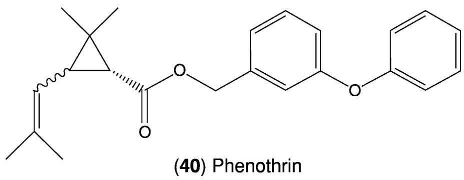
(**37**) NRDC 207



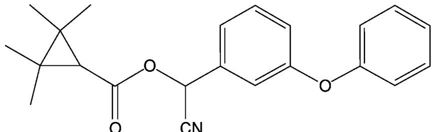
(**38**) Protrifenbute



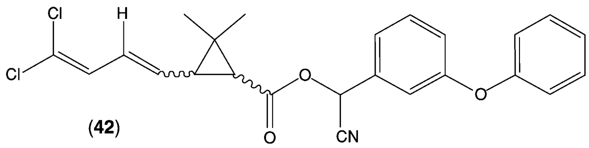
(**39**) NRDC 196



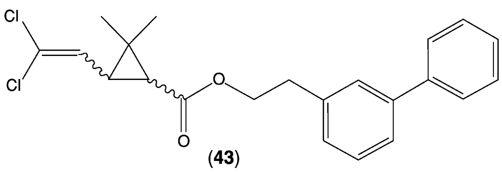
(**40**) Phenothrin



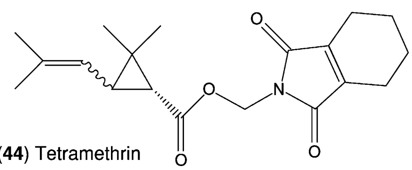
(**41**) Fenpropathrin



(**42**)



(**43**)



(**44**) Tetramethrin

Figure 2 Pyrethroids referred to in structure–activity relationships section (structures **29**–**44**).

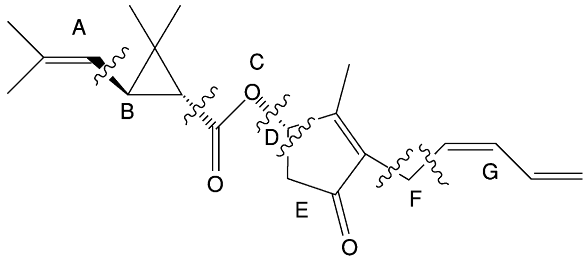


Figure 3 Schematic breakdown of the pyrethrin I molecule into seven segments.

alcohol, theta-cypermethrin (*trans* isomers) controls lepidopteran pests more effectively than α-cypermethrin (*cis* isomers), whereas the converse is true for dipterans.

When present, correct chirality at C1 of the acyl moiety (segment B) is crucial for activity. For both the esters of cyclopropane acids and for the fenvalerate series, activity requires the same spatial configuration at C1 (note that this is designated by *R* in the former and *S* in the latter by the nomenclature rules). Flufenprox (**33**), with an *R* configuration at C1, appears to contradict this generalization but apparently it is more similar to esfenvalerate than its corresponding *S* isomer.

Segment C: The ester linkage can be replaced by an isosteric group. However, changes are directly affected by the nature of segment B. For the cyclopropane esters, all isosteric replacements of the ester linkage substantially reduce activity. However, in the fenvalerate series, it can be replaced with retention of activity, for example (**34**) in which a ketoxime group replaces the ester linkage. The first such non-ester pyrethroid to be commercialized was etofenprox (**26**). Successful replacement of the ester group by an ether linkage was found to be largely dependent on the spatial arrangement of the gem-dimethyl group. In addition, the gem-dimethyl group could be replaced by cyclopropane and the ether link by other isosteric groups such as alkyl (MTI 800, (**35**)) and alkenyl (e.g., NRDC 199 (**36**) and NRDC 201 (**37**)). A subsequent important observation was that, in contrast to fenvalerate, replacement of the dimethyl with a cyclopropyl group in the nonester analogs (as in protrifenbute, (**38**)) resulted in a substantial (almost ×100) increase in activity.

Segments D and E: The carbon joined to the ester oxygen must be sp3 hybridized, with at least one proton attached to it and linked to an unsaturated center (e.g., an alkene). This can be a cyclic moiety (as in pyrethrin I) or substituted aromatic (as in permethrin). In the latter, introduction of an α-cyano group (e.g., deltamethrin) enhances activity only with benzyl alcohols with a 3-phenoxy or an equivalent cyclic substituent (e.g., 3-benzyl and 3-benzoyl). With other alcohols containing cyclic moieties (e.g., 5-benzyl-3-furylmethyl alcohol as in bioresmethrin and 3-phenyl as in bifenthrin), it reduces activity. With a single cyclic moiety (e.g., NRDC 196, (**39**)) containing an acyclic substituent, introduction of an α-cyano group has a negligible effect on activity. However, in the absence of a side-chain, introduction of a cyano group in such compounds leads to loss of activity (e.g., as with tefluthrin (**21**)). An ethynyl group is generally less effective, though in empenthrin it is more effective than a cyano group.

The chirality at the oxygenated carbon of the alcohol moiety (the α carbon) is also important for activity but, in contrast to the strict requirement at C1 of the acyl moiety, the required chirality appears to depend on the structure of the alcohol. For example, the orientation conferring high activity in the cyclopentanone (as in pyrethrin I) and acyclic (as in empenthrin) alcohol moieties is the opposite to that for the α-cyano-3-phenoxybenzyl alcohols (as in deltamethrin). Furthermore, the difference in the level of activity between the epimers at this center is much larger for the latter (see below). In addition, selectivity in pyrethroids between insect species can be due to selective metabolic processes. A good illustration is an elegant investigation by Pap *et al.* (1996b). They investigated the insecticidal activity of three structurally related series of pyrethroids (phenothrin (**40**), permethrin (**4**), and cypermethrin (**5**)) toward six insect species. The chirality requirement at C1 was as indicated above, and, as expected at the α-cyano center, the *S* isomer was more effective than *R* in all species; however, in the presence of metabolic inhibitors, the *R* isomer had comparable activity to the *S* towards mosquitoes but not the other species. Against houseflies, permethrin and phenothrin were less active than the corresponding α-cyano analogs. However, in the presence of the synergist piperonyl butoxide (PBO), the difference in the levels of activity was much lower. Both examples demonstrate that the α*R* isomer is more sensitive to metabolism and that in mosquitoes, but not the other species, unexpectedly binds to the sodium channel receptor in a comparable manner to the *S* isomer.

Segment F: The CH_2 group can be replaced by an oxygen or sulfur atom, or by an isosterically equivalent group such as carbonyl, with retention of variable levels of activity. In the case of benzyl alcohols, both cyclic and acyclic substituents can be attached either at position 3 or 4. However, cyclic substituents such as 3-phenoxybenzyl in the

4-position result in almost complete loss of activity, though a subsequent introduction of an α-cyano group enhances activity only for 3-substituted benzyl alcohols.

Segment G: Unsaturation in this region was initially considered essential for high activity. However, subsequent developments have indicated anomalies. Replacement of the diene in pyrethrin I with an alkene had led to the discovery of allethrin, the first synthetic pyrethroid to be commercialized. Although against houseflies it was as effective as pyrethrin I, it was significantly less effective against a range of other insect species. Indeed, this was the key observation made by Elliott that led to the development of bioresmethrin. However, since then many other highly effective alcohols have been discovered that contain unsaturated acyclic side-chains e.g., prallethrin and imiprothrin, both containing an alkynyl group. Two commercial compounds, transfluthrin (**20**) and empenthrin (**19**), both devoid of a side-chain, appear to contradict this generalization.

There are constraints in the overall shape, size, and the relative positioning of the various structural features within the pyrethroid molecule. Thus, despite the general belief, it is clear that the requirements for overall size are not stringent, though the alcohol moiety is limited to an overall size of no more than two benzene rings. The acid moiety can be varied substantially with retention of activity, for example flumethrin (**8**), fenpropathrin (**41**), and compound (**42**). In addition, although some of the regions of pyrethroid molecules can be varied independently of others, many require simultaneous modifications in adjacent regions (e.g., as in nonester pyrethroids). With regard to the relative positioning of the segments, compound (**43**), despite having the correct overall size, is nontoxic; this indicates that the relative positioning of the unsaturated center in the alcohol moiety to the ester linkage is crucial for activity. Thus, overall shape and size are both important for activity in pyrethroids.

Selectivity of pyrethroids is influenced by several factors, including rate of penetration, susceptibility to detoxification, and potency at the site of action. Finally, synergism in pyrethroids has been reported for a combination of active and inactive isomers, and between two structurally different pyrethroids (e.g., bioallethrin and bioresmethrin). Synergy is also known to arise with other classes of insecticides (e.g., organophosphates) that inhibit metabolic degradation (Naumann, 1990; Katsuda, 1999).

Quantitative structure–activity relationships (QSAR), using both *in vivo* and *in vitro* bioassay data with a range of pyrethroids, have been extensively investigated to gain a better understanding of structural/physical requirements for transport and binding of pyrethroids at the site of action (Ford *et al*., 1989; Ford and Livingstone, 1990; Hudson *et al*., 1992; Mathew *et al*., 1996; Chuman *et al*., 2000b). The most comprehensive QSAR studies with pyrethroids have been undertaken over the past 20 years by researchers at Kyoto University and collaborators (Nishimura *et al*., 1988, 1994; Matsuda *et al*., 1995; Nishimura and Okimoto, 1997, 2001; Akamatsu, 2002). For example, early studies (Nishimura *et al*., 1988 and references therein), using benzyl chrysanthemates and bioassays with the American cockroach *Periplaneta americana*, indicated that knockdown activity was strongly correlated with steric bulkiness (Van der Waals' volume) and both the hydrophobicity and position of substitution in the benzyl alcohol moiety. Thus, for *meta*-substituted derivatives, both hydrophobicity and steric factors were important, but for *para*-substituents activity varied parabolically with substituent volume. Both the rate of development of intoxication and the potency of the symptoms directly influenced knockdown activity and were dependent primarily on the pharmacokinetics of the transport process.

More recently, a direct relationship between changes in membrane potentials and physical properties of pyrethroids has been established (Matsuda *et al*., 1995). Using a diverse set of 39 ester pyrethroids, incorporating nine types of acyl and five types of alcohol moieties, they found a parabolic relationship between membrane potentials in crayfish (*Procambarus clarkii*) giant axons and the hydrophobicity of the compounds tested. They concluded that nonspecific penetration from outside the nerve membrane to the target site was the major factor influencing the rate of development of knockdown effects. The optimum lipophilicity for knockdown effect was calculated to be log K_{ow} 5.4 (where K_{ow} is the partition coefficient between 1-octanol/water). This was slightly higher than predicted values of about log K_{ow} 4.5 (Briggs *et al*., 1976; Kobayashi *et al*., 1988), indicating that other factors may also influence *in vivo* activity. Hence, less lipophilic pyrethroids can still exhibit high knockdown activity. Of particular note is imiprothrin, which gives high knockdown though having an exceptionally low log K_{ow} of 2.9. Interestingly, compounds of such low lipophilicity are expected to exhibit systemic movement in plants, though such activity has not been reported in pyrethroids. Given the complexity of structure–activity relationships in pyrethroids, it is not surprising that such QSAR studies have not led to the new commercial compounds.

It should be noted that commercial pyrethroids can exhibit significant phytotoxicity which can limit their application. For example, deltamethrin is too phytotoxic to be used on Sitka spruce (*Picea sitchensis*) transplants to control the large pine weevil (*Hylobius abietis*), and black pine beetles (*Hylastes* spp). Permethrin exhibits little phytotoxicity and cypermethrin is intermediate between the two (Straw *et al.*, 1996).

6.1.4. Mode of Action of Pyrethroids

6.1.4.1. Classification of Pyrethroids

Pyrethroids, which comprise a diverse range of structures, have historically been classified into two broad groups (Type I and Type II) on the basis of their biological responses (Table 3). Interpretation of most mode of action studies on insects has been predicated on this classification, though this is now considered to be an overly simplistic approach.

The two types of symptoms have been associated with the absence (Type I) or presence (Type II) of an α-cyano group. For example, permethrin exhibits Type I behavior; introduction of an α-cyano group gives cypermethrin which shows Type II behavior. This modification is accompanied by an increase in activity of an order of magnitude, and indeed all commercial Type II compounds have an α-cyano group in conjunction with a 3-phenoxybenzyl alcohol moiety. However, introduction of an α-cyano group does not always lead to an increase in activity. For example, in bioresmethrin it leads to a loss in activity and in NRDC 196 activity is only marginally increased. Furthermore, the level of activity is influenced by the choice of test species. Thus classification as Type I or II cannot be based solely on the presence/absence of an α-cyano group. It is most likely that Type I and Type II represent the extremes of a continuum, with many pyrethroids exhibiting intermediate properties.

Despite the uncertainty over such a classification, it has been suggested (Soderlund *et al.*, 2002; Vais *et al.*, 2003) that, on the basis of electrophysiological properties, there are two distinct binding sites for pyrethroids on the sodium channel. However, this would not explain why some pyrethroids (e.g., fluvalinate and bifenthrin, respectively with and without an α-cyano group) appear to manifest intermediate responses in insects, which behavior might indicate the presence of only a single binding site. The most likely explanation for the differential responses of Type I and Type II pyrethroids in *in vitro* assays lies in the occurrence of pharmacologically different voltage-gated sodium channels in insects (see below).

6.1.4.2. Site of Action

Pyrethroids are recognized for their rapid knockdown action on insects but are generally poor at killing them, a fact not fully appreciated for many years. Measurements of LD_{50} are usually made 24 and 48 h after treatment, at which times paralyzed insects are regarded as dead. However, insects often recover fully, even at higher doses (e.g., $5 \times LD_{50}$) (Bloomquist and Miller, 1985), over the following few days. In practice, such long-term recovery does not limit pyrethroid efficacy in the field, as death occurs by secondary processes including desiccation and predation.

Pyrethroids are nerve poisons that disrupt nerve conduction in both invertebrates and vertebrates

Table 3 Classification of pyrethroids

Response/action	*Type I*	*Type II*
Poisoning symptoms	• Rapid onset of symptoms even at sublethal levels • Hyperactivity often leading to knockdown • Low kill with high recovery • Inversely related to changes in temperature	• Slow onset of symptoms • Convulsion followed by paralysis • High kill with low recovery • Little effect of temperature change
Electrophysiological response in nerve tissue	• Repetitive discharges in axons	• Blockage of conduction at synapses
Action on sodium-channel function	• Monophasic and rapid decay of tail currents • Bind preferentially to closed channels	• Biphasic and very slow decay of tail currents • Bind preferentially to open channels
Level of resistance due to resistant houseflies with *super-kdr* mechanism	• Below 100-fold	• Over 200-fold

and their primary site of action is the voltage-gated sodium channels. However, there is controversy regarding the precise mode of action of pyrethroids and their binding site on the sodium channels (see **Chapter 5.1**).

Much of the work on the mode of action was reported during the 1980s but investigations on sodium channels have continued and a great deal of progress made. Many *in vitro* and *in vivo* investigations on mode of action and metabolism have been carried out on mammalian systems because of the interest in toxicology of these pesticides to mammals including humans and the expertise present in many laboratories for electrophysiology on mammalian neurons and muscles. Experiments have also used model systems such as squid and crustacean giant nerve fibers because of their large size and robustness. However, such studies may not be relevant to insects since there is considerable variation, not only in the metabolic capacities but also in the amino-acid sequences of the sodium channel proteins and thus their pharmacological properties. Another complication is that, in mammals, several genes express different sodium-channel isoforms, which exhibit differential responses to the two types of pyrethroids (Tatebayashi and Narahashi, 1994; Soderlund *et al*., 2002). Furthermore, each gene can express channels (splice variants) with varying pharmacological and biophysical properties (Tan *et al*., 2002). In insects, the voltage-gated sodium channels are encoded by only a single gene, which can also express splice-variants. Such channels, though having a common binding site, might nevertheless exhibit differences in their responses to pyrethroids. Such differences have been demonstrated in mammals but not yet in insects, though there is ample indirect evidence for this (see below).

These different factors give rise to the complex nature of pyrethroid action and therefore difficulty in defining in detail the mode of action. Early investigations into their mode of action were conducted on insect and mammalian nerve preparations but the interpretation of these measurements is somewhat controversial. Broad agreement has only been possible with measurements from sodium channels (see below) (Soderlund *et al*., 2002). Thus the summary presented below is a brief personal overview of the area.

6.1.4.2.1. Investigations based on nerve preparations Of the various symptoms induced by pyrethroids (hyperactivity, tremors, incoordination, convulsions followed by paralysis and death), only the initial toxicity symptoms can be linked to neurophysiological measurements and then only for Type I pyrethroids. They induce repetitive firing (short bursts of less than 5 s duration) in axonal nerve preparation in the sensory nerves accompanied by occasional large bursts of action potential in the ganglia (Nakagawa *et al*., 1982; Narahashi, 2002; Soderlund *et al*., 2002). Repetitive firing has been correlated to hyperactivity and uncoordinated movements leading to rapid knockdown in insects. This is also thought to stimulate the neurosecretory system causing an excessive release of diuretic hormones which eventually results in the disruption of the overall metabolic system in insects (Naumann, 1990 and references therein). The initial symptoms are manifested at very low concentrations ($0.1–0.001 \times LD_{50}$), it being estimated that less than 1% of the sodium channels need to be modified to induce repetitive firing (Song and Narahashi, 1996). In contrast, Type II pyrethroids, even at rather high concentrations, initially cause much less visible activity in insects, convulsions and rapid paralysis being the main symptoms. They cause slow depolarization of nerve membranes leading to block of nerve conduction. The concentration required ultimately to kill insects is much lower for Type II than for Type I compounds.

In general, Type I compounds have been viewed as more active than Type II in producing both visible symptoms and repetitive discharges in the peripheral nerves, though exceptions are known, especially when considering results from noninsect nerve preparations. For example, repetitive firing has been observed for both Type I and Type II pyrethroids in frog-nerve preparations (Vijverberg *et al*., 1986).

The first claimed correlation between a neurophysiological response and the poisoning action of pyrethroids related to the increased production of miniature excitatory postsynaptic potentials (MEPSPs) in response to depolarization of the nerve terminal caused by both Type I and Type II compounds (Salgado *et al*., 1983a, 1983b; Miller, 1998). This increase in MEPSPs results in depletion of neurotransmitter at the nerve-muscle synapses leading to nerve depolarization (paralysis) and also blockage of flight reflex responses. At low threshold concentrations, Type I pyrethroids induce repetitive firing and hyperactivity but not neurotransmitter release so that these processes are not linked to the toxic action of pyrethroids. These observations thus accounted for the apparent contradiction that though initial symptoms of Type I pyrethroids are positively correlated with temperature, the toxic action (release of neurotransmitter and conduction block) is negatively correlated with temperature. At high concentrations, Type I pyrethroids behave similarly to Type II pyrethroids and elicit toxic

symptoms in insects with neurotransmitter release and neuromuscular block. Thus, Type I pyrethroids affect the overall sensory nervous system but Type IIs affect only a specific part. This was elegantly demonstrated by Salgado *et al.* (1983a, 1983b), who showed that deltamethrin applied to the abdomen of houseflies took over an hour to reach the CNS, so that that the knockdown effect seen within 20 min could only be attributed to its action at the peripheral nervous system. Moreover, flies that had been knocked down by deltamethrin were able to fly when thrown in the air because communication along the CNS between the brain and the flight system had not yet been disrupted. In contrast, flies knocked down by Type I pyrethroids cannot fly when thrown in the air, indicating transmission block at the motor neurons. This effect can be reversed by increasing the temperature with Type I pyrethroids but not for Type II, in line with the observed *in vivo* toxicity symptoms.

6.1.4.2.2. Investigations based on sodium channels The most significant advances since 1985 in understanding pyrethroid action have been made by the application of molecular biological approaches to the functioning of voltage-gated channels (for a detailed review of this aspect, see **Chapter 5.1**). The ability to introduce specific mutations in the voltage-sensitive channels and to measure inter- and intra-cellular changes (by voltage- and patch-clamp experiments) has provided conclusive evidence that the primary target sites for pyrethroids are the voltage-gated sodium channels. In summary, pyrethroids interact with the voltage-gated sodium channels to produce tail currents, which can be used to quantify the potency of the insecticides, higher tail-current being associated with higher insecticidal activity (Nishimura and Okimoto, 2001). This action appears to account for symptoms of pyrethroid poisoning and actions on the peripheral and central neurons. The prolongation of these tail-currents is much longer and the effect is less reversible for Type II pyrethroids than Type I. The presumption that Type I and Type II pyrethroids represent the extremes of a continuum is supported by the observation that many pyrethroids exhibit an intermediate response. Thus, deltamethrin (Type II) and permethrin (Type I) exhibit Hill slopes (an indicator of the molar binding ratio) of $\sim$2 and $\sim$1, respectively but bifenthrin exhibits an intermediate slope of $\sim$1.4 (Khambay *et al.*, 2002). Finally, the link between single point mutations in sodium channels and resistance in insects has provided the most conclusive evidence that the voltage-gated sodium channels are the primary target for pyrethroids.

Other measurements on single sodium channels that confirm their being the primary target for pyrethroids include the negative temperature coefficient with toxicity being paralleled by a negative temperature dependence of channel depolarization. Furthermore, hyperactivity induced by Type I pyrethroids can be explained by the tail currents exceeding a certain threshold that then triggers repetitive firing and hyperactivity. Interestingly, it has recently (Motomura and Narahashi, 2001) been demonstrated that tetramethrin (**44**) (Type I) displaces fenvalerate (Type II) from mammalian sodium channels, implying that the former has a higher binding affinity. Also, tetramethrin displaces deltamethrin in single-channel studies. At first sight, this observation appears contradictory in that Type II pyrethroids are significantly more toxic than Type I pyrethroids in insects. However, the explanation may lie in the differences between sodium channels from insects and mammals.

6.1.4.3. Modeling Studies on Prediction of Putative Pharmacophores

Sodium channels are lipid-bound trans-membrane proteins and consequently it is difficult to obtain their crystal structure, especially with bound ligands. However, a recent publication (Jiang *et al.*, 2003) of the crystal structure of the voltage-gated potassium channel should aid progress. Indirect techniques involving binding of pyrethroids to the channel protein (e.g., with photo-reactive and radio-labelled pyrethroids) have been unsuccessful, primarily due to nonspecific binding to the channel and adjacent protein. Given these limitations, effort has been directed at modeling studies.

The "bioactive" conformation has been defined as that most probable at the binding site, and which is energetically stable and three-dimensionally similar to other compounds acting at the same site. Although ester pyrethroids such as deltamethrin have seven torsional axes, most conformational studies have focused on three axes (T2, T3, and T4 in **Figure 4**) around the ester linkage. Most studies have investigated common conformations of minimum energy and correlated them with toxicity/knockdown, overall electronic effects, geometric bulk, and partitioning coefficients (Naumann, 1990 and references therein; Chuman *et al.*, 2000a, 2000b). A "folded" form (**Figure 4**), also referred to as the "clamp" and "horseshoe", was identified as the active conformation.

As an example of the approaches used, Chuman *et al.* (2000a, 2000b) chose the nonester pyrethroid MTI 800 (representative of the most flexible series of pyrethroids) to identify the possible conformers. By comparison, they then deduced the likely

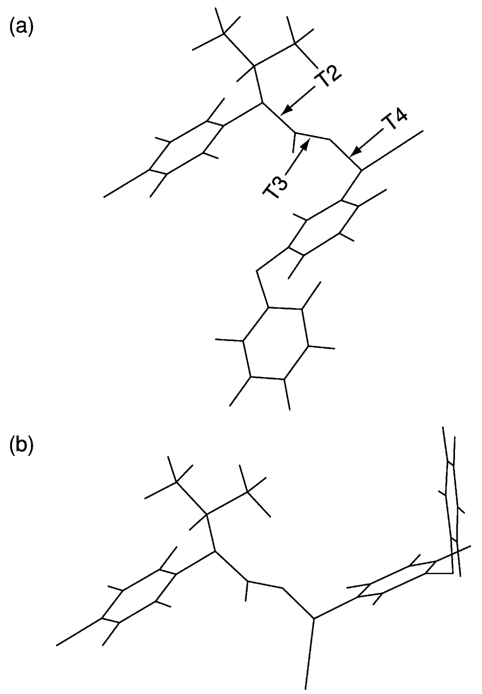


Figure 4 Folded and extended conformations of fenvalerate.

common conformations (within 10 kcal/mol of the ground energy) for the active isomers of pyrethrin I, deltamethrin, and fenvalerate (representatives of each of the three other main series of pyrethroids). To define the bioactive conformer, they also considered a further seven pyrethroid structures, both active and inactive. Similarity/dissimilarity searches using Cosine and Tanimoto coefficients together with superimposition considerations indicated the folded form as the common bioactive conformation for all four types of pyrethroids.

For deltamethrin (a cyclopropane ester), this bioactive folded conformation is close to minimum energy, and is similar to that both of the crystal structure and as observed in solution by NMR (under certain conditions). However, for fenvalerate (not containing a cyclopropane acid moiety), the extended form (**Figure 4**) has lower minimum energy than the bioactive folded conformation.

However, another recent study appears to contradict the above findings. Using new computational methodologies based on cluster analysis of molecular dynamics trajectories, Ford *et al.* (2002) proposed an "extended" conformation for the lethal action of both Type I and Type II pyrethroids, which would also account for the negative temperature coefficient. For knockdown activity (Type I compounds only), he proposed a different higher-energy conformation which is not accessible to Type II pyrethroids. Not being a minimum-energy conformation, this would be transient in nature, and would occur with a higher probability at elevated temperatures (i.e., positive temperature correlation with knockdown activity). Once again the key torsion angles, T2 and T4, lie around the ester bond, T3 being invariant throughout the dynamics simulations undertaken in this study.

6.1.5. Resistance to Pyrethroids

Under selection from repeated sprays of insecticides, individuals possessing biochemical mechanisms that can detoxify the insecticide more rapidly or are less sensitive to it are likely to be favoured. These resistant insects survive doses that would kill normally sensitive individuals. Genes encoding these mechanisms will then be passed on to the succeeding generations, resulting in pest populations that are not controlled effectively. This can lead to farmers increasing the rate or frequency of applications, imposing further selection pressure and ultimately leading to a situation whereby the pests become totally immune. Removal of selection pressure may result in the pest populations regaining some degree of sensitivity, particularly if there is a fitness cost to resistance such as longer development times or reduced over-wintering ability. Usually, however, the population never regains the degree of sensitivity of the naïve population, and often there appears to be little fitness cost so that high numbers of resistant pests remain in the population. Pesticide resistance has occurred with all insecticides; the existence of resistance mechanisms common to pyrethroids and other older insecticides meant that onset of resistance to the new compounds was quite rapid.

Many factors, in particular the type of crop and the history of insecticide use, can affect the selection and dominance of resistance mechanism(s) within an insect species. In addition, the relative importance of these mechanisms can change over time (Gunning *et al.*, 1991). Thus, management of resistance requires knowledge of the mechanism(s) present and ideally also their relative contribution. Though the nature of the resistance mechanisms can be identified relatively straightforwardly, there are, as yet, no established methods for predicting their relative importance in field strains of resistant insects. Such prediction requires a multidisciplinary approach based on both *in vivo* and *in vitro* assays. The concept of using novel selective inhibitors, designed using structure–activity investigations and ideally devoid of insecticidal activity, could prove particularly useful in determining the biochemical mechanism(s) responsible for resistance. The design of such inhibitors will need to take into consideration the variations in specificities of

metabolic enzymes amongst insect species. A greater input from chemists would be a key factor in such investigations.

There are three main mechanisms by which insects become less sensitive to pesticides: increased detoxification ("metabolic resistance"), reduced penetration, and target-site resistance. For many pests, the most important resistance mechanism for pyrethroids is considered to be site insensitivity, often referred to as knockdown resistance (*kdr*), caused by the inheritance of point mutations in the *para*-type sodium channel which is the target-site of the pyrethroids. The principal mechanisms of detoxification are esteratic cleavage of the ester bond and oxidative mechanisms catalysed by cytochrome P450. The other common mechanism of xenobiotic detoxification, dealkylation catalysed by glutathione-*S*-transferases (GSTs), is of lesser importance. Also, reduction in cuticular penetration and/or increased excretion has long been recognized as a resistance mechanism, though its contribution is likely to be as a modifier of metabolic or target-site resistance rather than as a major mechanism in its own right.

Advances in molecular techniques have led to the development of simple and efficient diagnostic kits for the detection of *kdr* resistance. Only a limited amount of work has been done to develop tests for resistance caused by metabolic enzyme(s), for which synergists are commonly used to detect their presence. The biggest problem currently is that the established inhibitors (e.g., piperonyl butoxide, PBO) are not specific and thus may give unreliable results. For example, PBO has been shown to inhibit both cytochrome P450 monooxygenases and esterases in Australian *H. armigera* (Gunning *et al*., 1998a).

When present, the site-insensitivity (*kdr* and especially *super-kdr*) mechanisms often predominate, although populations of pests frequently have multiple resistance mechanisms. The situation regarding the two principal metabolic mechanisms is much less clear and the relative importance of esterase or oxidative mechanisms may vary between species or even between populations of the same species.

Details of the resistance mechanisms and their management in the field are discussed in the following sections.

6.1.5.1. Resistance Mechanisms

6.1.5.1.1. Metabolic resistance

6.1.5.1.1.1. Metabolism of pyrethroids in insects

The principal metabolic pathways by which pyrethroids are degraded in insects were mostly evaluated prior to 1985 and are summarized in Roberts and Hutson (1999). Metabolism is conveniently divided into two phases: the initial biotransformation of a pesticide is referred to as Phase I metabolism, comprising mainly oxidative, reductive, and hydrolytic processes; Phase II metabolism is biotransformation, in which the pesticide or Phase I metabolite is conjugated with a naturally occurring compound such as a sugar, sugar acid, glutathione, or an amino acid. In general, the major Phase I degradative routes in insects and other animals (mostly mammals and birds) are similar. It is only in the details of their Phase II reactions whereby polar conjugates with sugars or amino acids are formed that there are major qualitative differences in the nature of the metabolites. As degradation studies on insects are not required for the registration of insecticides, such studies are usually only undertaken in order to understand specific questions concerning structure–activity relationships or to evaluate problems associated with resistance caused by enhanced metabolic breakdown. It is the latter reason that has seen the majority of insect metabolism studies performed.

Since 1985 there has been a vast increase in the knowledge of insect molecular genetics. The publication of the draft genome sequences for the fruit fly *Drosophila melanogaster* in 2000 and the mosquito *Anopheles gambiae* in 2002 has greatly increased the knowledge of the enzymes involved in metabolic degradation, particularly cytochromes P450. Consequently, recent research is beginning to make inroads into understanding which of the isozymes of cytochrome P450 are responsible for metabolizing different substructures of the pyrethroid molecule. The situation with respect to the nature of the carboxyesterases responsible for catalysing the hydrolysis of the pyrethroid ester bond is less clear and the carboxyesterases that catalyze the hydrolysis of pyrethroids in different species of insects may be different and nonhomologous.

Insects detoxify pyrethroids at varying rates and this degradative metabolism is important in understanding the detailed toxicology. Indeed, part of the relatively modest insecticidal activity of the natural pyrethrins is attributable to their rapid metabolic breakdown. Consequently, household sprays are usually formulated with synergists that inhibit the enzymes that catalyze this metabolic degradation and thereby enhance the insecticidal activity. Selection of insect strains possessing elevated levels of catabolizing enzymes is also an important mechanism in the development of decreased sensitivity (resistance) toward pyrethroids. There are two principal routes of detoxification of pyrethroids in insects: de-esterification catalysed by both esterases and cytochromes P450, and the hydroxylation of

aromatic rings or methyl groups by cytochromes P450, and these two mechanisms will be considered separately. The points of the chemical structure where a generalized 3-phenoxybenzyl pyrethroid molecule is detoxified are shown in Figure 5. The width of the arrow indicates the approximate extent of metabolic attack. It is in the detailed enzymology and molecular biology of the enzymes responsible for pyrethroid metabolism where there have been the most important advances.

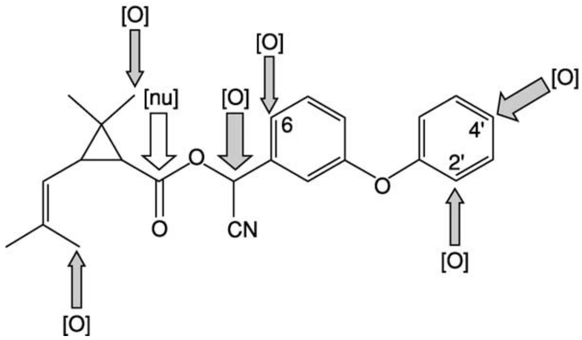
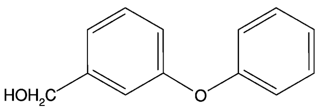
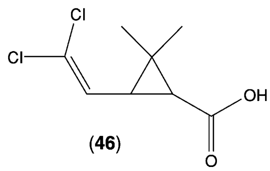


Figure 5 Points of metabolic attack in a generalized pyrethroid structure.

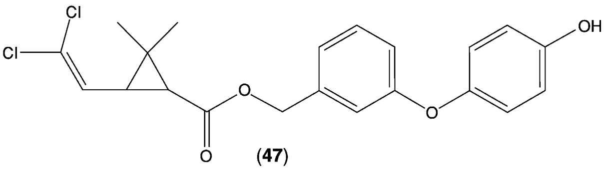
6.1.5.1.1.2. Metabolic pathways The most complete analysis of the metabolic pathways of pyrethroid degradation in insects has been by Shono (Shono *et al.*, 1978; reviewed by Soderlund *et al.*, 1983), who studied the metabolism of permethrin in American cockroaches (*Periplaneta americana*), houseflies (*Musca domestica*), and caterpillars (*Trichoplusia ni*). Forty-two metabolites (including conjugates) were identified, and the most important Phase 1 metabolites are shown in Figure 6. Metabolism occurred by ester cleavage to 3-phenoxybenzyl



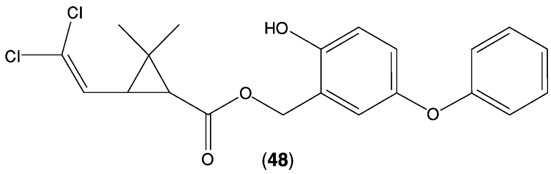
(**45**)



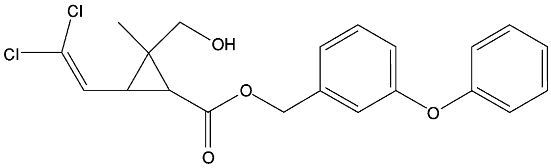
(**46**)



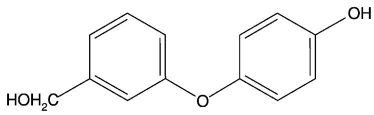
(**47**)



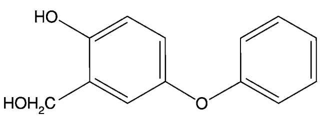
(**48**)



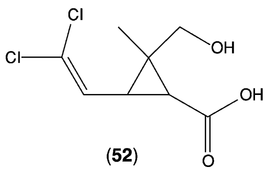
(**49**)



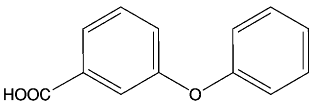
(**50**)



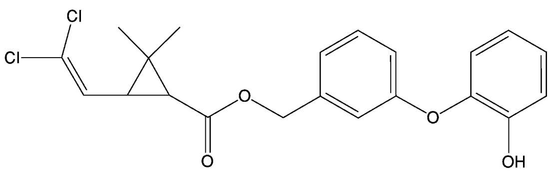
(**51**)



(**52**)



(**53**)



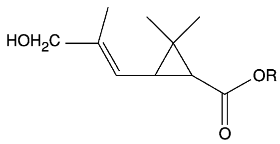
(**54**)

Figure 6 Metabolites from oxidative attack on permethrin (structures **45**–**54**).

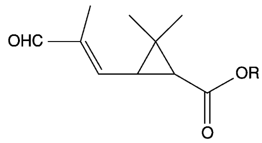
alcohol (3-PBA) (**45**) and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DCVA) (**46**). Hydroxylation also occurred at the 4′- and 6-positions of the phenoxybenzyl moiety and the *cis* or *trans* methyl groups of the DCVA moiety to give (**47**), (**48**), and (**49**) respectively. Hydroxylation of the *trans*-methyl group rather than the *cis*- was preferred. Ester cleavage of these hydroxylated metabolites gave the structures (**50**), (**51**), and (**52**) respectively. 3-PBA (**50**) was further oxidized to 3-phenoxybenzoic acid (**53**), as were the hydroxylated analogs of 3-PBA to their analogous benzoic acids.

A similar pattern of Phase I metabolites was observed with both *cis*- and *trans*-permethrin, although the relative proportion of certain of the conjugated structures was influenced by the stereochemistry. All metabolites found in the cockroach were also found in houseflies but the series of metabolites arising from the 6-hydroxylation of the 3-PBA moiety was only found in flies. Metabolites consisting of the whole hydroxylated molecule (e.g., (**47**), (**48**), and (**49**)) were exclusively found as their glucosides, whereas the ester cleavage products were found both free and as their glucoside and amino acid conjugates. All three insects conjugated DCVA with one or more of the amino acids glycine, glutamic acid, glutamine, and serine, in addition to forming glucose esters. This study did not identify the 2′-hydroxylated metabolite (**54**), a significant metabolite in mammalian studies. However, this metabolite and compounds derived from it were identified in a study of the metabolism of permethrin by the American bollworm *Helicoverpa zea*, the tobacco budworm *Heliothis virescens* (Lepidoptera, Noctuidae) (Bigley and Plapp, 1978) and the Colorado potato beetle, *Leptinotarsa decemlineata* (Soderlund *et al.*, 1987), in which it was the principal metabolite. Other later studies have confirmed this overall pattern in different insects, although some studies have shown more differences in the pattern of metabolites between *cis*- and *trans*-permethrin isomers.

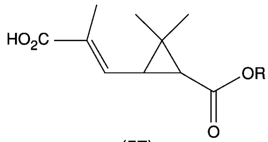
Holden (1979) showed that *trans*-permethrin was ester-cleaved at a higher rate than the *cis*-isomer in *P. americana*, an observation consistent with the findings that the *trans*-isomers of cyclopropane-containing pyrethroids are much better substrates for esterases than the *cis*-isomers (see below). This pattern of permethrin metabolism by insects can be taken as a template for the breakdown of other pyrethroids comprising esters of 2,2-dimethyl-3-(substituted)vinylcarboxylic acid with 3-PBA. Thus, the principal mechanisms of Phase I metabolism involve ester cleavage, both hydrolytic and oxidative, and aromatic substitution of one or



(**55**)



(**56**)



(**57**)

Figure 7 Metabolites from oxidative attack on the chrysanthemic acid moiety of allethrin (structures **55**–**57**).

other of the 3-PBA rings (the 4′-position is usually the major site) and the 2,2-dimethyl group of the acid moiety. Analogous reactions have been shown to take place with bifenthrin and deltamethrin in the bulb mite *Rhizoglyphus robini* to give the ester-cleaved products and the 4′-hydroxy metabolites (Ruzo *et al.*, 1988). Similarly, *trans*-cypermethrin gave *trans*-DCVA and both 2′- and 4′-hydroxy-cypermethrin in the cotton bollworm *Helicoverpa armigera* and in *H. virescens* (Lepidoptera) (Lee *et al.*, 1989). Fenvalerate, which lacks a cyclopropyl group, was metabolized via ester cleavage and 4′-hydroxylation in houseflies (Funaki *et al.*, 1994). In pyrethroids containing the chrysanthemic acid moiety (rather than a 2,2-dimethyl-3-dihalovinylcarboxylic acid), such as allethrin, phenothrin, and tetramethrin, the methyl groups of the isobutylene group are also subject to oxidative attack. Hydroxylation of these groups in allethrin to the alcohol (**55**) (**Figure 7**), followed by successive oxidation to the aldehyde (**56**) and carboxylic acid (**57**), occurred in houseflies (Yamamoto *et al.*, 1969). Tetramethrin was mainly metabolized via ester cleavage, with the production of the carboxyl derivative (**57**) of the *trans*-methyl group as a minor product (Yamamoto and Casida, 1968).

6.1.5.1.1.3. Ester hydrolysis All pyrethroids with the exception of the nonester compounds, such as MTI 800 which has a hydrocarbon linkage between the “acid” and “alcohol” moieties, are subject to esterase-catalyzed breakdown. Even etofenprox, which has an ether linkage in this position, is cleaved via oxidation of the benzylic carbon and hydrolysis of the resultant ester in the rat (Roberts and Hutson, 1999). The stereochemistry of the cyclopropanecarboxylic acid is important in determining the rate of esteratic detoxification, since the *trans*-isomers are very much better substrates for esterases than are the *cis*-isomers (Soderlund and Casida, 1977). Indeed, *cis*-pyrethroids possessing an α-cyano group (Type II

pyrethroids) and consequently a secondary ester are generally degraded via a microsomal P450 mechanism through oxidation of the α-carbon. Evidence from work with mammalian liver indicates that the esterases responsible for pyrethroid hydrolysis are also microsomal (Soderlund and Casida, 1977). This study also showed that the Type II pyrethroids cypermethrin and deltamethrin were the least susceptible to both esterase-catalysed hydrolysis and oxidation. Generally, the major route of Type II pyrethroid metabolism in mammals is via de-esterification catalysed by microsomal oxidases (e.g., Crawford and Hutson, 1977). Studies on insects have also concluded that ester cleavage is often mainly by an oxidative mechanism (Casida and Ruzo, 1980; Funaki *et al.*, 1994). The latter study concluded that the de-esterification of fenvalerate by pyrethroid-resistant houseflies was principally due to over-expression of cytochromes P450; only a small portion of the ester bond cleavage was caused by hydrolases.

Whether the ester cleavage is hydrolytic or oxidative is apparently largely dependent on the species. For example, *T. ni* (Ishaaya and Casida, 1980), *S. littoralis*, (Ishaaya *et al*., 1983), *S. eridana* (Abdelaal and Soderlund, 1980) (Lepidoptera), and *B. tabaci* (Jao and Casida, 1974; Ishaaya *et al*., 1987) (Hemiptera) all degraded *trans*-pyrethroids via a hydrolytic mechanism. Conversely, houseflies (Diptera) and the rust-red flour beetle *Tribolium castaneum* (Coleoptera) used an oxidative pathway (Ishaaya *et al*., 1987). A rather special case is that of the green lacewing *Chrysoperla carnea* agg. (Neuroptera), which is highly resistant to pyrethroids. This insect has been shown to contain high levels of a pyrethroid-hydrolyzing esterase that is able to catalyze the hydrolysis of *cis*-isomers, including those of Type II ester pyrethroids such as cypermethrin (Ishaaya and Casida, 1981; Ishaaya, 1993). How the over-production of esterases induces resistance to *cis*-pyrethroids such as deltamethrin, which are poor substrates for the enzyme(s) from other species, has been the cause of some conjecture. Devonshire and Moores (1989) presented evidence that *cis*-pyrethroids bind tightly to the active site and are thus sequestered by the large amounts of the resistance-associated esterases (E4 and FE4) produced by resistant peach-potato aphids *M. persicae*. Consequently, deltamethrin acts as a competitive inhibitor of esterase activity but is removed by binding to the protein rather than by hydrolysis. Selection pressure, whether artificially or from frequent commercial use of pyrethroids, frequently leads to multifactorial mechanisms of insecticide resistance, including esterases, cytochromes P450, target-site (*kdr* and *super-kdr*) mechanisms, and sometimes reduced penetration (e.g., Anspaugh *et al*., 1994; Pap and Toth, 1995; Ottea *et al*., 2000; Liu and Pridgeon, 2002). Such insect strains have extremely high resistance and usually show some degree of cross-resistance to all pyrethroids.

Most insect esterases that catalyze the hydrolysis of pyrethroid esters are soluble nonspecific B-type carboxylesterases. These have a wide substrate-specificity and model substrates, such as 1-naphthyl acetate, have been used to visualize electrophoretically distinct protein bands in *B. tabaci* (Byrne *et al*., 2000) and many other insect species. There is little information to date on the occurrence of microsomal esterases in insects analogous to those in mammals (e.g., Prabhakaran and Kamble, 1996). Most evidence for the involvement of esterases in pyrethroid breakdown is indirect and has used model substrates; however, direct evidence for pyrethroid hydrolysis has been obtained for esterases from several species, including the horn fly *Haematobia irritans* (Diptera) (Pruett *et al*., 2001). Concern has been raised of the validity of using model substrates to predict the breakdown of pyrethroids, and consequently some researchers have designed model substrates with pyrethroid-like structures. Thus, Shan and Hammock (2001) developed sensitive fluorogenic substrates based on DCVA coupled to the cyanohydrin of 6-methoxynaphthalene-2-carboxaldehyde. Hydrolysis of this ester and decomposition of the cyanohydrin regenerates this fluorescent aldehyde. An enzyme preparation that used this substrate from a cypermethrin-selected resistant strain of *H. virescens* (Lepidoptera) gave better selectivity (×5) than did 1-naphthyl acetate (×1.4). A similar approach, but using 1-naphthyl esters of the four stereoisomers of DCVA with detection of released 1-naphthol by diazo-coupling to Fast Blue RR, has been reported (Moores *et al*., 2002). In these experiments, only the (1*S*)-*trans*-isomer acted as a substrate for aphid esterases, in agreement with other experiments on the stereospecificity of insect pyrethroid esterases. Activity staining of electrophoretic gels using this novel substrate showed that the pyrethroid-hydrolyzing activity was distinct from the resistance-associated esterases visualized with 1-naphthyl acetate, indicating that the main mechanism for resistance was binding/sequestration rather than hydrolysis.

Work since 1985 has concentrated on understanding the nature of the esterases responsible for pyrethroid hydrolysis. *In vivo* studies have often yielded equivocal results and, using such tools as specific inhibitors of oxidative or hydrolytic metabolism, it has frequently been difficult to prove which mechanism is primarily responsible for pyrethroid catabolism. These problems have arisen in part

because there is no such thing as a specific inhibitor and some compounds thought to be specific inhibitors of cytochromes P450 (e.g., piperonyl butoxide) may also inhibit esterases (Gunning *et al.*, 1998b; Moores *et al.*, 2002).

Studies have included both *in vivo* work on whole insects or their tissues and *in vitro* studies with isolated enzymes. In resistant populations of many insect species, the mechanisms are most frequently due to both enhanced esterase and cytochrome P450 levels, so that dissection of the proportions of the different mechanisms is difficult. However, evidence that resistance ratios are reduced or abolished in the presence of reliably specific inhibitors such as organophosphates (Gunning *et al.*, 1999; Corbel *et al.*, 2003) can be taken as a good indication that enhanced esterase levels are responsible for metabolic resistance.

6.1.5.1.1.4. Cytochrome P450 monooxygenases These are a class of Phase I detoxification enzymes that catalyse various NADPH- and ATP-dependent oxidations, dealkylations, and dehydrogenations. Both microsomal and mitochondrial forms occur in insects. They are probably responsible for the most frequent type of metabolism-based insecticide resistance (Oppenoorth, 1985; Mullin and Scott, 1992; Scott and Wen, 2001). They are also a major mechanism for pyrethroid catabolism (Tomita and Scott, 1995). Their occurrence and importance in insect xenobiotic metabolism has been reviewed by Scott and Wen (2001). The super-family of cytochrome P450 genes has probably evolved by gene duplication and adaptive diversification, and comprises 86 functional genes in *D. melanogaster.* The large number of substrates metabolized by P450s is due both to the multiple isoforms and to the fact that each P450 may have several substrates (Rendic and DiCarlo, 1997). Because these enzymes may have overlapping substrate specificities, it is difficult to ascribe the function to individual P450 enzymes. In insects, although the importance of oxygenases in the metabolism of many substrates is known, the particular P450 isoforms involved have rarely been identified. For a general review on insect P450 enzymes and particularly their regulation, see **Chapter 4.1**.

Several P450 iso-enzymes have been isolated or expressed from insect sources. Regarding pyrethroid metabolism, the best-characterized P450 isoform is CYP6D1. This was originally purified from a strain of highly resistant (ca. ×5000) houseflies designated "Learn pyrethroid resistant" (LPR) selected by the continuous usage of permethrin to control flies in a New York State dairy. A reduced-penetration mechanism and *kdr* were also present in the strain. CYP6D1 has been purified (Wheelock and Scott, 1989) and sequenced via the use of degenerate primers derived from known protein sequences and PCR amplification (Tomita and Scott, 1995). Overproduction of this P450 isozyme was found to be the major mechanism of deltamethrin detoxification in microsomes derived from the LPR flies (Wheelock and Scott, 1992). The enzyme requires cytochrome b5 as a co-factor and is specific in its action, because only the 4′-hydroxy metabolite was produced from cypermethrin (Zhang and Scott, 1996). CYP6D1 was found to be the major and possibly the only P450 isoform responsible for pyrethroid metabolism in this strain of houseflies; consequently, the resistance ratios are very much less for pyrethroids such as fenfluthrin that do not have the 3-phenoxybenzyl group (Scott and Georghiou, 1986). The same mechanism was found to be responsible for PBO suppressible resistance to permethrin from a Georgia poultry farm in the USA (Kasai and Scott, 2000). In both these housefly strains, the mechanism was due to an increased (ca. ×10) transcription of the gene, leading to increased levels of CYP6D1 mRNA and higher levels of the enzyme. CYP6D1 is expressed in the insect nervous system and has been shown to protect the tissue from the effects of cypermethrin (Korytko and Scott, 1998). Clearly, from the metabolic specificity of CYP6D1, other isoforms of cytochromes P450 must also be implicated in pyrethroid metabolism, although which reactions are catalyzed by which isoform has yet to be determined.

It is characteristic of monooxygenases that they are inducible within an individual animal. The use of phenobarbitone to induce monooxygenase activity in rat liver is well known, and many other agents are capable of transiently up-regulating cytochromes P450. Phytophagous insects are exposed to many plant xenobiotics, for example monoterpenes which also induce P450 production. Such induction of P450s may incidentally induce an isoform also capable of metabolizing pyrethroids. For example, feeding larvae of *H. armigera* on mint (*Mentha piperita*) leaves induced a 4× resistance to pyrethroids compared with those fed on a semi-defined diet (Hoque, 1984; Terriere, 1984; Schuler, 1996; Scott *et al.*, 1998). CYP6B2 mRNA, a P450 isoform also implicated in pyrethroid resistance, is inducible by peppermint oil and specifically α-pinene in larvae of *H. armigera* (Ranasinghe *et al.*, 1997). This induction was rapid (ca. 4 h) and disappeared within a similar period of removing the stimulus. Clearly, the mechanism for the transient induction of P450s (Ramana, 1998) is different

from the situation with the LPR houseflies, in which CYPD1 is permanently up-regulated (Liu and Scott, 1998), although the precise mechanistic details still remain to be elucidated. In nonphytophagous arthropods for example the cattle tick *Boophilus microplus* which is not subjected to a barrage of allelochemicals in its diet, P450s were found to be of little importance in the induction of resistance to pyrethroids (Crampton *et al*., 1999), although the converse has been found for adults of the mosquito *Culex quinquefasciatus* (Kasai *et al*., 1998).

6.1.5.1.1.5. Model substrates When managing insecticide resistance, it is important that resistant alleles can be detected at low frequency in populations. Data from bioassays will only detect a quite high proportion of individuals with reduced sensitivity in the population. Consequently, it is useful to design biochemical or DNA (molecular) tests that can identify resistance in individual insects. Thus, model substrates commonly used to measure P450 levels *in vitro* need to be substrates of the relevant P450 isoforms that degrade the insecticide. Amongst such model substrates are the sensitive fluorogenic reagents 7-ethoxycoumarin, ethoxyresorufin, and methoxyresorufin, and the chromogenic substrate 4-nitroanisole. These compounds are dealkylated by monooxygenases to yield a fluorescent or colored product. Unfortunately, it has generally been found that these substrates are also isozyme specific, so that they may not be good indicators of P450-induced pyrethroid resistance. In the case of CYP6D1, methoxyresorufin was found to be a substrate, but ethoxyresorufin and 7-ethoxycoumarin were not (Wheelock and Scott, 1992). A similar variation in the activity of elevated oxygenases to 4-nitroanisole, benzo(a)pyrene, benzphetamine, and methoxyresorufin in the mid-gut of a multi-resistant (cypermethrin and thiodicarb) strain of *H. virescens* larvae was noted (Rose *et al*., 1995). In this strain, demethylation rates of both 4-nitroanisole and methoxyresorufin were useful as indicators of insecticide resistance; however, on such multi-resistant strains, several P450 isoforms are probably elevated in tandem making comparisons difficult. Consequently, the use of model substrates to estimate the levels of P450 monooxygenases in individual insects may only give equivocal information on levels of P450-derived metabolic resistance to pyrethroids.

6.1.5.1.1.6. Glutathione-*S*-transferases (GSTs) GSTs are important in the detoxification of organophosphorus insecticides and other electrophilic compounds, which are dealkylated and conjugated with glutathione (see **Chapter 5.11**). Pyrethroids are not electrophilic and would not be expected to be detoxified by this mechanism. However, there are several reports that have correlated enhanced levels of GSTs with pyrethroid resistance in a number of species, *S. littoralis* (Lagadic *et al*., 1993), *T. castaneum* (Reidy *et al*., 1990), and *Aedes aegypti* (Grant and Matsumura, 1989). Additionally, pyrethroids have been shown to induce production of GSTs in the honeybee *Apis mellifera* (Yu *et al*., 1984), fall armyworm *Spodoptera frugiperda* (Punzo, 1993), and German cockroach *Blatella germanica* (Hemingway *et al*., 1993). The role of the glutathione-*S*-transferase system as a mechanism of defence against pyrethroids is not fully understood, but it is thought that GST proteins sequester the pyrethroids (Kostaropoulos *et al*., 2001) or possibly protect the tissues from pyrethroid-induced lipid peroxidation (Vontas *et al*., 2001).

6.1.5.1.2. Cuticle penetration As a resistance mechanism, reduced penetration of the insect cuticle has been studied less and has generally been considered of subordinate importance to enhanced detoxification and target-site mutations. Where detected, it is usually found with other mechanisms, e.g., the "LPR strain" of houseflies referred to above. Reduced cuticular penetration of pyrethroids has been detected in resistant strains of a number of other species, including *H. armigera* (Gunning *et al*., 1991), *H. zea* (AbdElghafar and Knowles, 1996), *H. virescens* (Little *et al*., 1989), *S. exigua* (Delorme *et al*., 1988), the diamond-back moth *Plutella xylostella* (Noppun *et al*., 1989), *B. germanica* (Wu *et al*., 1998), and *B. microplus* (Schnitzerling *et al*., 1983). In all these cases, reduced cuticular penetration was found in addition to other resistance mechanisms, enhanced metabolism, and/or target-site resistance (*kdr*). Mechanisms involving reduced uptake appear not to give significant resistance to the lethal effects of insecticides but provide a more than additive effect when combined with other mechanisms. Thus, decreased penetration, although a minor factor on its own, can, when coupled with other mechanisms, increase resistance many-fold (Ahmad and McCaffery, 1999).

6.1.5.1.3. Target-site resistance Target-site resistance is the most important mechanism of pyrethroid resistance and its selection and spread have compromised the use of pyrethroid insecticides on many insect pests. It is a particularly important mechanism of pyrethroid resistance, as it confers a degree of loss of sensitivity to all members of the class. It is characterized by a reduction in the sensitivity of the insect nervous system and has been

termed "knockdown resistance" or *kdr* (Sawicki, 1985). The *kdr* trait was first identified in the early 1950s in houseflies (Busvine, 1951). It causes a loss of sensitivity to DDT and its analogs, and to pyrethrins and pyrethroids, which all owe their activity to interaction with the *para*-type voltage-gated sodium channel in nerve membranes. This loss of activity is characterized by a reduction in the binding of these insecticides to the sodium channel (Pauron *et al.*, 1989). An enhanced form of this resistance termed *super-kdr* has also been characterized in houseflies (Sawicki, 1978). Both the *kdr* and *super-kdr* traits were mapped to chromosome 3 and found to occupy the same allele, the *para*-type sodium channel. This has been confirmed by molecular cloning studies of these channels in *kdr* and *super-kdr* houseflies (Williamson *et al.*, 1996) and *kdr B. germanica* (Miyazaki *et al.*, 1996; Dong, 1997). The *super-kdr* resistance in houseflies is due to a methionine to threonine (M918T) point mutation in the gene encoding the *para* sodium channel. Both mutations were located with domain II of the ion channel. The L1014F mutation in IIS6 was found in both housefly and cockroach strains, and confers *kdr* resistance. To date, the M918T mutation has not been detected as a single substitution in any housefly strain, but only occurs in conjunction with L1014F. Mammals are intrinsically much less susceptible to pyrethroids and DDT. Significantly, mammalian neuronal sodium channels have an isoleucine rather than methionine in the position (874) that corresponds to the housefly *super-kdr* site (918). Site-directed mutation of this residue to methionine gives rise to a channel with >100x increased sensitivity to deltamethrin, suggesting that differential pyrethroid sensitivity between mammals and insects may be due in part to structural differences between the mammalian and insect sodium channels (Vais *et al.*, 2000). In some insect species, the existence of *kdr*-type target-site resistance has often been masked by efficient metabolic resistance mechanisms; an example is *M. persicae*, in which resistance-associated esterase is responsible for much of the reduced sensitivity towards organophosphates, carbamates, and pyrethroids. Any inference that target-site resistance might also be a factor has usually been based on the sensitivity of the insects in the presence of synergists such as PBO or DEF *S,S,S*,-tributyl phosphorotrithiolate, and the assumption that any residual decreased sensitivity is due to *kdr*-type target-site resistance.

Use of degenerate DNA primers for the *para*-type sodium channel and sequencing of the gene have resulted in the unequivocal identification of resistance-inducing mutations in the trans-membrane domain II (Martinez-Torres *et al.*, 1999b) of the channel in *M. persicae*, in which a leucine to phenylalanine (L1014F) mutation associated with *kdr* was identified. Insects containing this mutation could also be identified by the use of a discriminating dose of DDT, as DDT is unaffected by the enhanced-esterase mechanisms also present in most of the aphid clones. Indeed, of 58 aphid clones analysed for both *kdr*- and esterase-based mechanisms, only four contained an esterase (E4) mutation and not *kdr*. Estimates for resistance factors in aphids containing both mechanisms are 150–540-fold, in comparison to 3–4-fold (FE4 esterase alone) or 35-fold for *kdr* alone. Consequently, these dual-resistance mechanisms afford a level of decreased sensitivity whereby insects become totally immune to field dosages of pyrethroids. Similar *kdr* mutations have also been detected in cockroaches (Miyazaki *et al.*, 1996; Dong, 1997), *H. irritans* (Guerrero *et al.*, 1997), *P. xylostella* (Schuler *et al.*, 1998), and *An. gambiae* (Martinez-Torres *et al.*, 1998). In a pyrethroid-resistant strain of the tobacco budworm *H. virescens*, the same locus was mutated to histidine rather than phenylalanine (Park and Taylor, 1997)

As with metabolic resistance mechanisms, it is important to establish methods that can identify sodium-channel *kdr*-type mechanisms in single insects so that it is possible to adjust insect-control methods. The *kdr*-mutation of nerve insensitivity was originally identified by electrophysiology, and this method still remains a fundamental way of confirming nerve insensitivity. However, it is a specialized and rather cumbersome technique that is out of the question when attempting to test large numbers of an agricultural pest species. The DDT bioassay using a discriminating dose remains a useful method but may not completely discriminate between homozygous and heterozygous individuals. The most useful technique has been direct diagnosis of the mutation(s) based on PCR amplification and sequencing. The identification of the L1014F mutation in knockdown-resistant housefly strains has led to the development of several diagnostic assays for its occurrence in other species, including *H. irritans* (Guerrero *et al.*, 1997), the mosquitoes *An. gambiae* (Martinez-Torres *et al.*, 1998), and *Culex pipiens* (Martinez-Torres *et al.*, 1999a), as well as *M. persicae*. However, this technique will only identify known mutations and the test designed to detect L1014F in *An. gambiae* (Ranson *et al.*, 2000) did not detect the additional L1014S.

The molecular biology of knockdown resistance to pyrethroids has been reviewed (Soderlund and Knipple, 2003). Sequencing of the *para*-sodium

channel gene from several arthropod species has led to the discovery of a number of amino-acid polymorphisms in the protein. Of the 20 amino-acid polymorphisms each uniquely associated with pyrethroid resistance, those occurring at four sites have so far been found as single mutations in resistant populations. These are valine 410 (V410M in *H. virescens*; Park *et al*., 1997), methionine 918 (M918V in *B. tabaci*; Morin *et al*., 2002), leucine 1014 (L1014F in several species; L1014H in *H. virescens*; Park and Taylor, 1997; L1014S in *C. pipiens*; Martinez-Torres *et al*., 1999a), *An. gambiae* (Ranson *et al*., 2000), and phenylalanine 1538 (F538I, in *B. microplus*; He *et al*., 1999). All these sites have been located on trans-membrane regions of the channel and it is inferred that they are close to the binding site(s) of pyrethroids and DDT. Additionally, other polymorphisms have been found that only occur in the presence of L1014F and appear to act in a similar way to M918T in houseflies, causing enhanced resistance.

6.1.5.2. Resistance to Pyrethroids in the Field

The main cause of development of resistance in insects in the field is a persistent and high selection pressure as a consequence of repeated applications of a single class of insecticide (or another with the same mode of action). Therefore, despite effective control in the initial stages, a small number of survivors with innate resistance then rapidly multiply until control fails. In this regard, pyrethroids are no different from other insecticide classes. It is noteworthy that it was the development of resistance to pyrethroids that first prompted companies to take resistance seriously and to take joint action. Pyrethroids suffered an inherent disadvantage at the outset in that *kdr* also confers resistance to DDT, and prior use of DDT had already selected *kdr* alleles to significant levels in the same pests. Presently over 80 species have developed resistance to pyrethroids (Whalon *et al*., 2003).

Until the late 1970s, the major agrochemical companies had seen the development of resistance to established classes as commercially beneficial and motivation for the introduction of new classes. However, the increasing cost of discovery, together with the realization that pyrethroids could be rendered ineffective in the field within a much shorter period of time than other classes of insecticides, forced them to take collective action. In 1979 they set up the Pyrethroid Efficacy Group (PEG), which in 1984 become a sub-group of a larger international organization called the Insecticide Resistance Action Committee (IRAC). This group communicates its actions through a website (http://www.irac-online.org) and also sponsors the biannual Resistant Pest Management newsletter. Before the establishment of PEG, the investigation of resistance mechanisms and their consequences had remained the domain of academic research, and the development of resistance to pyrethroids was largely overlooked and even denied by some companies. The primary aim of PEG was to prolong the effectiveness of pyrethroids in the field. It encouraged and assisted in the investigation of all aspects of resistance to pyrethroids and particularly the development and implementation of insecticide-resistance management (IRM) strategies.

Persistent selection with a single class of insecticide will invariably lead to resistance, even if synergists are used. Therefore the overall aim is to minimize use of any single insecticide class so as to limit selection pressure and thereby conserve susceptibility in pest insects. This requires an in-depth knowledge of factors ranging from resistance mechanisms to genetic and ecological attributes of both pest and beneficial insects. Any strategy has also to integrate the judicious use of different insecticide types (namely those with different modes of action and synergy where possible) with other pest-management options (e.g., agronomic practices), together with regular monitoring of both the levels of resistance and the nature of the resistance mechanisms. Finally, the key requirement for the success of any strategy is the cooperation of the growers. By way of example, two IRM strategies are considered below which encompass these factors.

The first successful and the most publicized IRM strategy came from Australia. Synthetic pyrethroids were introduced in Australia in 1977 when there was already widespread resistance to virtually all established classes. However, within six years of introduction, resistance to these pyrethroids had also developed in commercially important insect species (Forrester *et al*., 1993). A resistance-management strategy for *H. armigera* was implemented in the 1983/4 season. A different approach was used for each class of insecticide, depending on the severity of the resistance risk and predicted selection pressure. It integrated the use of chemical and nonchemical control methods, especially biological and cultural. For chemical control, unrelated chemistries were used with a strong emphasis on pyrethroid/ovicide mixtures. In essence, a three-stage strategy was implemented. In Stage I (September to February), only endosulfan (an organochlorine) was permitted. Stage II (January to February) allowed a maximum of three pyrethroid sprays within a 42-day window (later reduced to 38 days), enough to control only one of the five *H. armigera*

generations present within a single growing season. In Stages I and II, ovicidal compounds (e.g., methomyl) could also be used. Organophosphates and carbamates were used in Stage III and also if required for additional sprays during Stages I and II.

The use of a "softer" insecticide (endosulfan) in the early season was deliberate to minimize disruption of beneficial parasitoids and predators and to avoid a potential upsurge of secondary pests such as mites, aphids, and whiteflies, which were not controlled by the pyrethroids available at the time. Examples of nonchemical countermeasures incorporated in the strategy to reduce selection pressure included the use of early-maturing crops, avoiding early-growing crops (e.g., maize) in adjacent fields, which may act as early-season nurseries for resistant *H. armigera*, and utilization of host plants in refugia to maintain a large pool of susceptible individuals, which would continually dilute the resistant population in the crop. Resistance was monitored on a weekly basis using a discriminating dose of fenvalerate with and without the synergist PBO. Later monitoring showed a rise in metabolic resistance attributed to MFOs and this resulted in the inclusion of PBO in the last of the maximum of three pyrethroid sprays. This strategy was successful for many years but the underlying trend of upward increase in the proportion of resistant insects continued and finally led to a complete reorganization of the strategy in the mid 1990s with a shift away from reliance on pyrethroids.

Initial resistance to pyrethroids was thought to be due to the presence of the knockdown site-insensivity resistance (*kdr*) mechanism probably as a direct result of cross-resistance to DDT. Over the years of the IRM strategy, metabolic resistance mechanisms appear to have taken over. However, there is still controversy over whether it is primarily due to elevated levels of esterases or mixed-function oxidases (MFOs). The main reason for the uncertainty concerns the role of synergists used in the studies. For example, PBO is now thought to inhibit both these types of enzymes. Furthermore, it has been suggested that such inhibitors may themselves have become resisted in the field over time (McCaffery, 1998), thus obscuring the mechanism of resistance.

The second IRM strategy involved the whitefly, *B. tabaci*, a representative sucking pest on a wide range of crops (Denholm *et al*., 1998). In 1995, overreliance on a limited range of pyrethroids in Arizona had led to a classic treadmill scenario, with farmers responding to rising levels of resistance by increasing the number of sprays (as many as 8–12 applications per season). In this pest, the haplodiploid breeding system encourages rapid selection and fixation of resistance genes. Males are produced uniparentally from unfertilized, haploid eggs, and females are produced biparentally from fertilized diploid eggs. In addition, for this (and other) highly polyphagous species, the interaction between pest ecology and resistance is complex and generally not well understood, making formulation of IRM strategies even more difficult. The strategy of Dennehy and Williams (1997) implemented in 1996 had several features in common with that for *H. armigera*. It too relies on the continuous availability of a pool of susceptible whiteflies in refugia (e.g., Brassica crops) throughout the year and alteration of agronomic practices (e.g., timing of planting) in crops to minimize whitefly numbers whilst still maintaining the level of natural enemies. The first of the three-stage IRM strategy involved use of a single spray each of pyriproxyfen and buprofezin (then newly available insect-growth regulators) with a 14- or a 21-day gap. The second stage allowed nonpyrethroid conventional insecticides and the third used pyrethroids synergized with acephate as late as possible in the season. A threshold of infestation was defined to initiate insecticide applications. Mixtures were limited to no more than two compounds and any one active ingredient was restricted to no more than two applications in one season. This strategy has been extremely successful in reducing the number of sprays required and regaining susceptibility both to synergized pyrethroids and key nonpyrethroid insecticides.

As for *H. armigera*, the need for monitoring, establishing threshold levels to trigger spray applications, and cooperation of the growers was key to the strategy. Long-term success of any IRM strategy depends on many factors because, even when an IRM strategy has been successful (McCaffery, 1998), effectiveness of synergized pyrethroids can be lost after just two applications in areas with a history of resistance to pyrethroids.

In conclusion, the key requirement for the development and sustainability of an IRM strategy is diagnosis of the resistance mechanism(s) in field populations. This is especially important when assessing the relative importance of individual mechanisms when several are present, which influences changes to the strategy with time. As alluded to earlier, there is still much uncertainty in the diagnosis of mechanisms. The use of established inhibitors or just *in vitro* bioassay data has been shown to be unreliable in this regard. For example in *M. persicae*, elevated esterase was considered for many years to be the main resistance mechanism.

It was only recently shown (Devonshire *et al.*, 1998) that this mechanism made only a minor contribution to the observed resistance to pyrethroids, with a *kdr* mechanism being the main contributor. This case further emphasized the need for not only accurately identifying the resistance mechanisms present but also their relative contribution. One way forward would be to design selective inhibitors on the basis of *in vivo* and *in vitro* structure–activity relationship studies from susceptible and resistant insects.

6.1.5.2.1. Chemistry-led options for circumventing resistance The established IRM strategies (considered above) successfully exploit a range of options including use of insecticides with different modes of action, agronomic practices, and biological control. However, there are two additional options involving insecticides that have hitherto not been fully exploited.

1. Negative cross-resistance is a phenomenon in which a resistant pest is rendered more susceptible to another class of pesticide. This phenomenon is rare in insects but for pyrethroids, two different classes, the *N*-alkylamides (Elliott *et al.*, 1986) and dihydropyrazoles (and related classes) (Khambay *et al.*, 2001), have been found to cause this effect. They both exhibit similar, albeit small (resistance factors of 0.4), levels of negative cross-resistance to houseflies with nerve insensivity (*super-kdr*) as the main resistance mechanism. The mechanisms involved are not properly understood. However this phenomenon is apparent only in the presence of the *super-kdr* mechanism, with *kdr* alone having little impact. BTG 502 (**Figure 8**; (58)) is representative of the *N*-alkylamides, a class with which this phenomenon was first identified. No compounds of this class have been commercialized to date, but see **Chapter 6.2**.

   In insects that have developed resistance to pyrethroids as a consequence of increased levels of metabolic enzymes (MFOs and esterases), negative cross-resistance has been demonstrated towards insecticides that require *in vivo* activation (i.e., pro-insecticides). For example, negative cross-resistance has been observed with indoxacarb in *H. armigera* with increased levels of esterases (Gunning, 2003). However, this compound has little effect in *M. persicae* with elevated levels of esterase (Khambay, unpublished data). This difference may be accounted for by the kinetics of the hydrolysis reaction. In susceptible *H. armigera*, hydrolysis is incomplete by the end of the bioassay time. The increased levels of esterases in resistant *H. armigera* generate more active compound over the same period, resulting in a higher level of activity. In *M. persicae*, the hydrolysis is complete during the test period even in susceptible insects and therefore no additional effects are observed if increased levels of esterases are present. Negative cross-resistance has also been observed for pyrethroid-resistant insects with increased levels of MFOs. Examples of such insecticides include the pyrazole chlofenapyr, the organophorothioates diazinon and chlorpyrifos, and the methylcarbamate propoxur.
2. Pyrethroids constitute a diverse class of chemical structures and levels of resistance to them also vary considerably. For example, in houseflies with the *super-kdr* resistance mechanism, the resistance factor for different pyrethroids can vary from less than 10 to over 500. Structure–activity relationships have been discerned and the levels of resistance factors (RFs, derived by dividing the LD50 of resistant insects by that of the corresponding susceptible insects) have been shown to be directly influenced by the nature of the alcohol group and the α substituent, but independent of the nature of the acid group (Farnham and Khambay, 1995a, 1995b; Beddie *et al.*, 1996). This is reflected in the description of

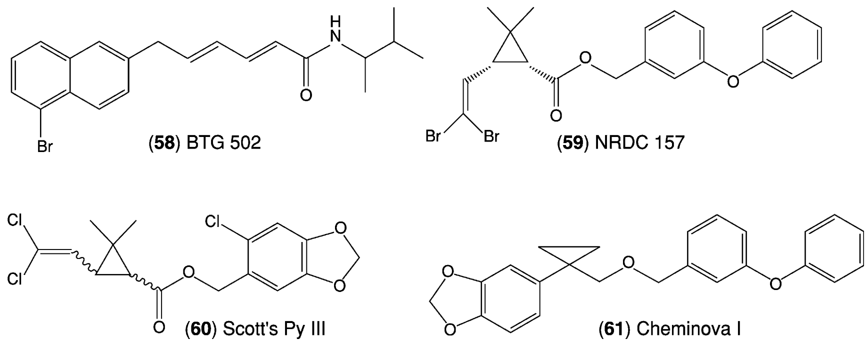


Figure 8 Compounds referred to in the resistance section (structures **58**–**61**).

Types I and II. Thus, highest RFs are shown by compounds with a 3-phenoxybenzyl (or equivalent) alcohol ester with an α-cyano substituent (e.g., deltamethrin) with an RF of 560. The RF of 91 for NRDC 157 (**59**) (deltamethrin analog lacking the α-cyano group) is significantly smaller, and indeed surprisingly the LD_{50} values against *super-kdr* flies are similar for the two compounds. This indicates that the additional target-site binding, and hence increased insecticidal activity, in susceptible flies gained by introducing an α-cyano group into NRDC 157 has been lost in *super-kdr* flies. The lowest RFs are observed for pyrethroids with alcohols containing a single ring and no α substituent, an example being tefluthrin with RF < 4.

In *H. armigera*, there has been some controversy regarding the resistance mechanism. Though it is accepted that the major mechanism is due to increased metabolism, it is unclear whether MFOs or esterases prevail. The highest RFs were shown by ester pyrethroids containing a phenoxybenzyl alcohol moiety in combination with an aromatic acid. The lowest RFs were with pyrethroids containing simple alcohol moieties as in bioallethrin. Resistance-breaking pyrethroids (RF < 1) were also identified e.g., those containing a methylenedioxyphenyl moiety as in Scott's Py III (**60**) and Cheminova 1 (**61**).

This approach clearly has potential to prolong the usefulness of pyrethroids as a class of insecticides. However, the agrochemical industries are unlikely to develop new pyrethroids purely for a role in "breaking" resistance to established compounds due to commercial considerations.

Despite the detection of resistance to pyrethroids soon after their introduction over 30 years ago, it has proved possible to manage it in many field situations. Therefore it is likely that pyrethroids will continue to be a useful tool for the foreseeable future.

Acknowledgments

We thank Dr Richard Bromilow for assistance in preparing this manuscript. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council, UK.

References

Abdelaal, Y.A.I., Soderlund, D.M., **1980**. Pyrethroid-hydrolyzing esterases in southern armyworm larvae – tissue distribution, kinetic properties, and selective inhibition. *Pestici. Biochem. Physiol. 14*, 282–289.

AbdElghafar, S.F., Knowles, C.O., **1996**. Pharmacokinetics of fenvalerate in laboratory and field strains of *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Econ. Entomol. 89*, 590–593.

Ahmad, M., McCaffery, A.R., **1999**. Penetration and metabolism of *trans*-cypermethrin in a susceptible and a pyrethroid-resistant strain of *Helicoverpa armigera*. *Pestici. Biochem. Physiol. 65*, 6–14.

Akamatsu, M., **2002**. Studies on three-dimensional quantitative structure–activity relationships in pesticides. *J. Pestici. Sci. 27*, 169–176.

Anspaugh, D.D., Rose, R.L., Koehler, P.G., Hodgson, E., Roe, R.M., **1994**. Multiple mechanisms of pyrethroid resistance in the German cockroach, *Blattella germanica* (L). *Pestici. Biochem. Physiol. 50*, 138–148.

Beddie, D.G., Farnham, A.W., Khambay, B.P.S., **1996**. The pyrethrins and related compounds. 41. Structure-activity relationships in non-ester pyrethroids against resistant strains of housefly (*Musca domestica* L). *Pestici. Sci. 48*, 175–178.

Bigley, W.S., Plapp, F.W., **1978**. Metabolism of cis-[C-14]-permethrin and trans-[C-14]-permethrin by tobacco budworm and bollworm. *J. Agric. Food Chem. 26*, 1128–1134.

Bloomquist, J.R., Miller, T.A., **1985**. Carbofuran triggers flight motor output in pyrethroid-blocked reflex pathways of the house-fly. *Pestici. Biochem. Physiol. 23*, 247–255.

Briggs, G.G., Elliott, M., Farnham, A.W., Janes, N., Needham, P.H., *et al.*, **1976**. Insecticidal activity of the pyrethrins and related compounds VIII. Relationship of polarity with activity in pyrethroids. *Pestici. Sci. 7*, 236–240.

Bryant, R., **1999**. Agrochemicals in perspective: analysis of the worldwide demand for agrochemical active ingredients. The Fine Chemicals Conference 99 [synopsis on the Internet] http://agranova.co.uk/agtalk99.asp Accessed 23 April 2004.

Busvine, J.R., **1951**. Mechanism of resistance to insecticide in houseflies. *Nature 168*, 193–195.

Byrne, F.J., Gorman, K.J., Cahill, M., Denholm, I., Devonshire, A.L., **2000**. The role of B-type esterases in conferring insecticide resistance in the tobacco whitefly, *Bemisia tabaci* (Genn). *Pest Manage. Sci. 56*, 867–874.

Casida, J.E., Ruzo, L.O., **1980**. Metabolic chemistry of pyrethroid insecticides. *Pestici. Sci. 11*, 257–269.

Cheung, K.W., Sirur, G.M., **2002**. Agrochemical Products Database, 2002. Cropnosis Limited, Edinburgh, UK.

Chuman, H., Goto, S., Karasawa, M., Sasaki, M., Nagashima, U., *et al.*, **2000a**. Three-dimensional structure–activity relationships of synthetic pyrethroids: 1. Similarity in bioactive conformations and their structure–activity pattern. *Quant. Struct.-Activ. Relationships 19*, 10–21.

Chuman, H., Goto, S., Karasawa, M., Sasaki, M., Nagashima, U., *et al.*, **2000b**. Three-dimensional structure–activity relationships of synthetic pyrethroids: 2. Three-dimensional and classical QSAR studies. *Quant. Struct.-Activ. Relationships 19*, 455–466.

Corbel, V., Chandre, F., Darriet, F., Lardeux, F., Hougard, J.M., **2003**. Synergism between permethrin and propoxur against *Culex quinquefasciatus* mosquito larvae. *Med. Vet. Entomol. 17*, 158–164.

Crampton, A.L., Green, P., Baxter, G.D., Barker, S.C., **1999**. Monooxygenases play only a minor role in resistance to synthetic pyrethroids in the cattle tick, *Boophilus microplus*. *Exp. Appl. Acarol. 23*, 897–905.

Crawford, M.J., Hutson, D.H., **1977**. Metabolism of pyrethroid insecticide (+/–)-alpha-cyano-3-phenoxybenzyl 2,2,3,3-tetramethylcyclopropanecarboxylate, WL 41706, in rat. *Pestici. Sci. 8*, 579–599.

Davies, J.H., **1985**. The pyrethroids: an historical introduction. In: Leahey, J.P. (Ed.), The Pyrethroid Insecticides. Taylor & Francis, London, pp. 1–41.

Delorme, R., Fournier, D., Chaufaux, J., Cuany, A., Bride, J.M., *et al.*, **1988**. Esterase metabolism and reduced penetration are causes of resistance to deltamethrin in *Spodoptera exigua* Hub (Noctuidae, Lepidoptera). *Pestici. Biochem. Physiol. 32*, 240–246.

Denholm, I., Cahill, M., Dennehy, T.J., Horowitz, A.R., **1998**. Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly *Bemisia tabaci*. *Phil. Trans. R. Soc. Lond. B Biol. Sci. 353*, 1757–1767.

Dennehy, T.J., Williams, L., **1997**. Management of resistance in *Bemisia* in Arizona cotton. *Pestici. Sci. 51*, 398–406.

Devonshire, A.L., Field, L.M., Foster, S.P., Moores, G.D., Williamson, M.S., *et al.*, **1998**. The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Phil. Trans. R. Soc. Lond. B Biol. Sci. 353*, 1677–1684.

Devonshire, A.L., Moores, G.D., **1989**. Detoxication of insecticides by esterases from *Myzus persicae* – is hydrolysis important? In: Reiner, E., Aldridge, W.N. (Eds.), Enzymes Hydrolysing Organophosphorus Compounds. Ellis Horwood, Chichester, UK, pp. 180–192.

Dong, K., **1997**. A single amino acid change in the para sodium channel protein is associated with knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect Biochem. Mol. Biol. 27*, 93–100.

Elliott, M., **1977**. Synthetic pyrethroids. In: Gould, R.F. (Ed.), Synthetic Pyrethroids. American Chemical Society, San Francisco, pp. 1–28.

Elliott, M., **1995**. Chemicals in insect control. In: Casida, J.E., Quinstad, G.B. (Eds.), Pyrethrum Flowers: Production, Chemistry, Toxicology, and Uses. Oxford University Press, New York, pp. 3–31.

Elliott, M., **1996**. Synthetic insecticides related to natural pyrethrins. In: Copping, L.G. (Ed.), Crop Protection Agents from Nature: Natural Products and Analogues. Royal Society of Chemistry, Cambridge, UK, pp. 254–300.

Elliott, M., Farnham, A.W., Janes, N.F., Johnson, D.M., Pulman, D.A., *et al.*, **1986**. Insecticidal amides with selective potency against a resistant (Super-Kdr) strain of houseflies (*Musca domestica* L). *Agric. Biol. Chem. 50*, 1347–1349.

Elliott, M., Janes, N., **1978**. Synthetic pyrethroids – a new class of insecticide. *Chem. Soc. Rev. 7*, 473–505.

Farnham, A.W., Khambay, B.P.S., **1995a**. The pyrethrins and related compounds. 39. Structure-activity relationships of pyrethroidal esters with cyclic side-chains in the alcohol component against resistant strains of housefly (*Musca domestica*). *Pestici. Sci. 44*, 269–275.

Farnham, A.W., Khambay, B.P.S., **1995b**. The pyrethrins and related-compounds. 40. Structure-activity relationships of pyrethroidal esters with acyclic side-chains in the alcohol component against resistant strains of housefly (*Musca domestica*). *Pestici. Sci. 44*, 277–281.

Feyereisen, R., **1999**. Insect P450 enzymes. *Ann. Rev. Entomol. 44*, 507–533.

Ford, M.G., Greenwood, R., Turner, C.H., Hudson, B., Livingstone, D.J., **1989**. The structure–activity relationships of pyrethroid insecticides. 1. A novel approach based upon the use of multivariate QSAR and computational chemistry. *Pestici. Sci. 27*, 305–326.

Ford, M.G., Hoare, N.E., Hudson, B.D., Nevell, T.G., Banting, L., **2002**. QSAR studies of the pyrethroid insecticides Part 3. A putative pharmacophore derived using methodology based on molecular dynamics and hierarchical cluster analysis. *J. Mol. Graphics Modelling 21*, 29–36.

Ford, M.G., Livingstone, D.J., **1990**. Multivariate techniques for parameter selection and data analysis exemplified by a study of pyrethroid neurotoxicity. *Quant. Struct.-Activ. Relationships 9*, 107–114.

Forrester, N.W., Cahill, M., Bird, L.J., Layland, J.K., **1993**. Management of pyrethroid and endosulfan resistance in *Helicoverpa armigera*. *Bull. Ent. Res* (Lepidoptera: Noctuidae) in Australia.

Funaki, E., Dauterman, W.C., Motoyama, N., **1994**. *In-vitro* and *in-vivo* metabolism of fenvalerate in pyrethroid-resistant houseflies. *J. Pest. Sci. 19*, 43–52.

Grant, D.F., Matsumura, F., **1989**. Glutathione S-transferase 1 and 2 in susceptible and insecticide resistant *Aedes aegypti*. *Pestici. Biochem. Physiol. 33*, 132–143.

Guerrero, F.D., Jamroz, R.C., Kammlah, D., Kunz, S.E., **1997**. Toxicological and molecular characterization of pyrethroid-resistant horn flies, *Haematobia irritans*: Identification of kdr and super-kdr point mutations. *Insect Biochem. Mol. Biol. 27*, 745–755.

Gunning, R.V., Devonshire, A.L., **2003**. Negative cross-resistance between indoxacarb and pyrethroids in the cotton bollworm, *Helicoverpa armigera*, in Australia: a tool for resistance management. The BCPC International Congress. Glasgow, British Crop Protection Council. pp. 789–794.

Gunning, R.V., Easton, C.S., Balfe, M.E., Ferris, I.G., **1991**. Pyrethroid resistance mechanisms in Australian *Helicoverpa armigera*. *Pestici. Sci. 33*, 473–490.

Gunning, R.V., Moores, G.D., Devonshire, A., **1999**. Esterase inhibitors synergise the toxicity of pyrethroids in Australian *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Pestici. Biochem. Physiol. 63*, 50–62.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1998a**. Inhibition of pyrethroid resistance related esterases by

piperonyl butoxide in Australian *Helicoverpa armigera* and *Aphis gossypii*. In: Jones, G. (Ed.), Piperonyl Butoxide: the Insecticide Synergist. Academic Press, London, pp. 215–226.

Gunning, R.V., Moores, G.D., Devonshire, A.L., **1998b**. Insensitive acetylcholinesterase and resistance to organophosphates in Australian *Helicoverpa armigera*. *Pestici. Biochem. Physiol. 62*, 147–151.

He, H.Q., Chen, A.C., Davey, R.B., Ivie, G.W., George, J.E., **1999**. Identification of a point mutation in the *para*-type sodium channel gene from a pyrethroid-resistant cattle tick. *Biochem. Biophys. Res. Commun. 261*, 558–561.

Hemingway, J., Dunbar, S.J., Monro, A.G., Small, G.J., **1993**. Pyrethroid resistance in German cockroaches (Dictyoptera, Blattelidae) – resistance levels and underlying mechanisms. *J. Econ. Entomol. 86*, 1631–1638.

Henrick, C.A., **1995**. Pyrethroids. In: Godfrey, C.R.A. (Ed.), Agrochemicals from Natural Products. Marcel Dekker, New York, pp. 63–145.

Holden, J.S., **1979**. Absorption and metabolism of permethrin and cypermethrin in the cockroach and the cotton leafworm larvae. *Pestici. Sci. 10*, 295–307.

Hoque, M.R., **1984**. Effect of different host plants and artificial diet on the tolerance levels of *Heliothis armigera* (Hubner) and *H. punctigera* (Wallengern) to various insecticides. MSc Thesis, University of Queensland, Australia.

Hudson, B.D., George, A.R., Ford, M.G., Livingstone, D.J., **1992**. Structure-activity relationships of pyrethroid insecticides. 2. The use of molecular dynamics for conformation searching and average parameter calculation. *J. Computer-Aided Mol. Design 6*, 191–201.

Ishaaya, I., **1993**. Insect detoxifying enzymes – their importance in pesticide synergism and resistance. *Arch. Insect Biochem. Physiol. 22*, 263–276.

Ishaaya, I., Ascher, K.R.S., Casida, J.E., **1983**. Pyrethroid synergism by esterase inhibition in *Spodoptera littoralis* (Boisduval) larvae. *Crop Protect. 2*, 335–343.

Ishaaya, I., Casida, J.E., **1980**. Properties and toxicological significance of esterases hydrolyzing permethrin and cypermethrin in *Trichoplusia ni* larval gut and integument. *Pestici. Biochem. Physiol. 14*, 178–184.

Ishaaya, I., Casida, J.E., **1981**. Pyrethroid esterase(s) may contribute to natural pyrethroid tolerance of larvae of the common green lacewing (Neuroptera, Chrysopidae). *Environ. Entomol. 10*, 681–684.

Ishaaya, I., Mendelson, Z., Ascher, K.R.S., Casida, J.E., **1987**. Cypermethrin synergism by pyrethroid esterase inhibitors in adults of the whitefly *Bemisia tabaci*. *Pestici. Biochem. Physiol. 28*, 155–162.

Jao, L.T., Casida, J.E., **1974**. Esterase inhibitors as synergists for (+)-trans-chrysanthemate insecticide chemicals. *Pestici. Biochem. Physiol. 4*, 456–464.

Jiang, Y.X., Lee, A., Chen, J.Y., Ruta, V., Cadene, M., *et al.*, **2003**. X-ray structure of a voltage-dependent K^+ channel. *Nature 423*, 33–41.

Jin, W.-G., Sun, G.-H., Xu, Z.-M., **1996**. A new broad spectrum and highly active pyrethroid-ZX 8901. Brighton Crop Protection Conference – Pests and Diseases, Brighton, British Crop Protection Council. pp. 455–460.

Kasai, S., Scott, J.G., **2000**. Overexpression of cytochrome P450CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. *Pestici. Biochem. Physiol. 68*, 34–41.

Kasai, S., Weerashinghe, I.S., Shono, T., **1998**. P450 monooxygenases are an important mechanism of permethrin resistance in *Culex quinquefasciatus* Say larvae. *Arch. Insect Biochem. Physiol. 37*, 47–56.

Katsuda, Y., **1999**. Development of and future prospects for pyrethroid chemistry. *Pestici. Sci. 55*, 775–782.

Khambay, B.P.S., **2002**. Pyrethroid insecticides. *Pestici. Outlook 13*, 49–54.

Khambay, B.P.S., Boyes, A., Williamson, M.S., Vias, H., Usherwood, P.N.R., **2002**. Defining Type I and Type II activity in insecticidal pyrethroids, 10th International Congress on the Chemistry of Crop Protection, Basel 4–9 August 2002, Abstract no. 3C. 49.

Khambay, B.P.S., Denholm, I., Carlson, G.R., Jacobson, R.M., Dhadialla, T.S., **2001**. Negative cross-resistance between dihydropyrazole insecticides and pyrethroids in houseflies, *Musca domestica*. *Pest Manage. Sci. 57*, 761–763.

Khambay, B.P.S., Farnham, A.W., Liu, M.G., **1999**. The pyrethrins and related compounds. Part XLII: Structure-activity relationships in fluoro-olefin non-ester pyrethroids. *Pestici. Sci. 55*, 703–710.

Kobayashi, T., Nishimura, K., Fujita, T., **1988**. Quantitative structure–activity studies of pyrethroids. 13. Physicochemical properties and the rate of development of the residual and tail sodium currents in crayfish giant-axon. *Pestici. Biochem. Physiol. 30*, 251–261.

Korytko, P.J., Scott, J.G., **1998**. CYP6D1 protects thoracic ganglia of houseflies from the neurotoxic insecticide cypermethrin. *Arch. Insect Biochem. Physiol. 37*, 57–63.

Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E., Papadopoulou-Mourkidou, E., **2001**. Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochem. Mol. Biol. 31*, 313–319.

Lagadic, L., Cuany, A., Berge, J.B., Echaubard, M., **1993**. Purification and partial characterization of glutathione-S-transferases from insecticide-resistant and lindane-induced susceptible *Spodoptera littoralis* (Boisd) larvae. *Insect Biochem. Mol. Biol. 23*, 467–474.

Lee, K.S., Walker, C.H., McCaffery, A., Ahmad, M., Little, E., **1989**. Metabolism of trans-cypermethrin by *Heliothis armigera* and *Heliothis virescens*. *Pestici. Biochem. Physiol. 34*, 49–57.

Little, E.J., McCaffery, A.R., Walker, C.H., Parker, T., **1989**. Evidence for an enhanced metabolism of cypermethrin by a monooxygenase in a pyrethroid-resistant strain of the tobacco budworm (*Heliothis virescens* F). *Pestici. Biochem. Physiol. 34*, 58–68.

Liu, N., Scott, J.G., **1998**. Increased transcription of CYP6D1 causes cytochrome P450 mediated insecticide

resistance in house fly. *Insect Biochem. Mol. Biol. 28*, 531–535.

Liu, N.N., Pridgeon, J.W., **2002**. Metabolic detoxication and the kdr mutation in pyrethroid resistant house flies, *Musca domestica* (L.). *Pestici. Biochem. Physiol. 73*, 157–163.

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Berge, J.B., *et al.*, **1998**. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* S.S. *Insect Mol. Biol. 7*, 179–184.

Martinez-Torres, D., Chevillon, C., Brun-Barale, A., Berge, J.B., Pasteur, N., *et al.*, **1999a**. Voltage-dependent Na^+ channels in pyrethroid-resistant *Culex pipiens* L mosquitoes. *Pestici. Sci. 55*, 1012–1020.

Martinez-Torres, D., Foster, S.P., Field, L.M., Devonshire, A.L., Williamson, M.S., **1999b**. A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Mol. Biol. 8*, 339–346.

Mathew, N., Subramanian, S., Kalyanasundaram, M., **1996**. Quantitative structure–activity relationship studies in pyrethroid esters derived from substituted 3-methyl-2-phenoxybutanoic acids against *Culex quinquefasciatus*. *Indian J. Chem. Sect. B Org. Chem. Med. Chem. 35*, 40–44.

Matsuda, K., Oimomi, N., Komai, K., Nishimura, K., **1995**. Quantitative structure–activity studies of pyrethroids. 32. Rates of change in membrane-potentials of crayfish and cockroach giant-axons induced by kadethric acid-esters and related-compounds. *Pestici. Biochem. Physiol. 52*, 201–211.

Matsuo, N., Miyamoto, J., **1997**. Development of synthetic pyrethroids with emphasis on stereochemical aspects. In: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J., Thompson, D.G. (Eds.), Phytochemicals for Pest Control. American Chemical Society, Washington, pp. 182–194.

McCaffery, A.R., **1998**. Resistance to insecticides in heliothine Lepidoptera: a global view. *Phil. Trans. R. Soc. Lond. B Biol. Sci. 353*, 1735–1750.

Miller, T., **1998**. Pyrethroid Insecticides. In: Chemistry & Toxicology of Insecticides. Web course at University of California, Riverside, Entomology 128 (Section 001). http://www.wcb.ucr.edu/wcb/schools/CNAS/entm/ tmiller/1/modules/pages28.html> 23 April 2004.

Miyazaki, M., Ohyama, K., Dunlap, D.Y., Matsumura, F., **1996**. Cloning and sequencing of the para-type sodium channel gene from susceptible and kdr-resistant German cockroaches (*Blattella germanica*) and house fly (*Musca domestica*). *Mol. Gen. Genet. 252*, 61–68.

Moores, G.D., Jewess, P.J., Boyes, A.L., Javed, N., Gunning, R.V., **2002**. Use of novel substrates to characterise esteratic cleavage of pyrethroids. The BCPC Conference: Pests and Diseases, Volumes 1 and 2. Proceedings of an international conference held at the Brighton Hilton Metropole Hotel, Brighton, UK, 18–21 November 2002. British Crop Protection Council, Farnham, UK, pp. 799–804.

Morin, S., Williamson, M.S., Goodson, S.J., Brown, J.K., Tabashnik, B.E., *et al.*, **2002**. Mutations in the *Bemisia tabaci* para sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. *Insect Biochem. Mol. Biol. 32*, 1781–1791.

Motomura, H., Narahashi, T., **2001**. Interaction of tetramethrin and deltamethrin at the single sodium channel in rat hippocampal neurons. *Neurotoxicology 22*, 329–339.

Mullin, C.J., Scott, J.G., **1992**. Biomolecular basis for insecticide resistance classification and comparisons. In: Mullin, C.J., Scott, J.G. (Eds.), Molecular Basis for Insecticide Resistance Diversity Among Insects. American Chemical Society, Washington, D.C., pp. 1–13.

Nakagawa, S., Okajima, N., Kitahaba, T., Nishimura, K., Fujita, T., *et al.*, **1982**. Quantitative structure–activity studies of substituted benzyl chrysanthemates. 1. Correlations between symptomatic and neurophysiological activities against American cockroaches. *Pestici. Biochem. Physiol. 17*, 243–258.

Narahashi, T., **2002**. Nerve membrane ion channels as the target site of insecticides. *Mini Rev. Med. Chem. 2*, 419–432.

Naumann, K., **1990**. Synthetic Pyrethroid Insecticides: Structures and Properties. Springer, Berlin.

Nishimura, K., Kato, S., Holan, G., Ueno, T., Fujita, T., **1994**. Quantitative structure–activity studies of pyrethroids. 31. Insecticidal activity of *meta*-substituted benzyl-esters of 1-(*para*-ethoxyphenyl)-2,2-dichlorocyclopropane and 2,2,3,3-tetrafluorocyclobutane-1-carboxylic acids. *Pestici. Biochem. Physiol. 50*, 60–71.

Nishimura, K., Kitahaba, T., Ikemoto, Y., Fujita, T., **1988**. Quantitative structure–activity studies of pyrethroids. 14. Physicochemical structural effects of tetramethrin and its related-compounds on knockdown activity against house-flies. *Pestici. Biochem. Physiol. 31*, 155–165.

Nishimura, K., Okimoto, H., **1997**. Quantitative structure–activity relationships of DDT-type compounds in a sodium tail-current in crayfish giant axons. *Pestici. Sci. 50*, 104–110.

Nishimura, K., Okimoto, H., **2001**. Effects of organosilicon pyrethroid-like insecticides on nerve preparations of American cockroaches and crayfish. *Pest Manage. Sci. 57*, 509–513.

Noppun, V., Saito, T., Miyata, T., **1989**. Cuticular penetration of s-fenvalerate in fenvalerate-resistant and susceptible strains of the diamondback moth, *Plutella xylostella* (L). *Pestici. Biochem. Physiol. 33*, 83–87.

Oppenoorth, F.J., **1985**. Biochemistry and genetics of insecticide resistance. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon, Oxford, UK, pp. 731–774.

Ottea, J.A., Ibrahim, S.A., Younis, A.M., Young, R.J., **2000**. Mechanisms of pyrethroid resistance in larvae and adults from a cypermethrin-selected strain of *Heliothis virescens* (F.). *Pestici. Biochem. Physiol. 66*, 20–32.

Pachlatko, J.P., **1998**. Natural Products in Crop Protection. 2nd International Electronic conference on Synthetic Organic Chemistry (ECSOC-2) <http://www.mdpi.org/ecsoc> September 1–30, 1998.

Pap, L., Bajomi, D., Szekely, I., **1996a**. The pyrethroids, an overview. *Internat. Pest Control 38*, 15–19.

Pap, L., Kelemen, M., Toth, A., Szekely, I., Bertok, B., **1996b**. The synthetic pyrethroid isomers II. Biological activity. *J. Environ. Sci. Health B Pestici. Food Contam. Agric. Wastes 31*, 527–543.

Pap, L., Toth, A., **1995**. Development and characteristics of resistance in the susceptible WHO/SRS house fly (*Musca domestica*) strain subjected to selection with beta-cypermethrin. *Pestici. Sci. 45*, 335–349.

Park, Y., Taylor, M.F.J., **1997**. A novel mutation L1029H in sodium channel gene hscp associated with pyrethroid resistance for *Heliothis virescens* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol. 27*, 9–13.

Park, Y., Taylor, M.F.J., Feyereisen, R., **1997**. A valine421 to methionine mutation in IS6 of the hscp voltage-gated sodium channel associated with pyrethroid resistance in *Heliothis virescens* F. *Biochem. Biophys. Res. Commun. 239*, 688–691.

Pauron, D., Barhanin, J., Amichot, M., Pralavorio, M., Berge, J.B., *et al.*, **1989**. Pyrethroid receptor in the insect Na^+ channel – alteration of its properties in pyrethroid-resistant flies. *Biochemistry 28*, 1673–1677.

Prabhakaran, S.K., Kamble, S.T., **1996**. Biochemical characterization and purification of esterases from three strains of German cockroach, *Blattella germanica* (Dictyoptera: Blattellidae). *Arch. Insect Biochem. Physiol. 31*, 73–86.

Pruett, J.H., Kammlah, D.M., Guerrero, F.D., **2001**. Variation in general esterase activity within a population of *Haematobia irritans* (Diptera: Muscidae). *J. Econ. Entomol. 94*, 714–718.

Punzo, F., **1993**. Detoxification enzymes and the effects of temperature on the toxicity of pyrethroids to the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 105*, 155–158.

Ramana, K.V., Kohli, K.K., **1998**. Gene regulation of cytochrome P450 – an overview. *Indian J. Exp. Biol. 36*, 437–446.

Ranasinghe, C., Headlam, M., Hobbs, A.A., **1997**. Induction of the mRNA for CYP6B2, a pyrethroid inducible cytochrome P450, in *Helicoverpa armigera* (Hubner) by dietary monoterpenes. *Arch. Insect Biochem. Physiol. 34*, 99–109.

Ranson, H., Jensen, B., Vulule, J.M., Wang, X., Hemingway, J., *et al.*, **2000**. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Mol. Biol. 9*, 491–497.

Reidy, G.F., Rose, H.A., Visetson, S., Murray, M., **1990**. Increased glutathione-S-transferase activity and glutathione content in an insecticide-resistant strain of *Tribolium castaneum* (Herbst). *Pestici. Biochem. Physiol. 36*, 269–276.

Rendic, S., DiCarlo, F.J., **1997**. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metabol. Rev. 29*, 413–580.

Roberts, T.R., Hutson, D.H. (Eds.), **1999**. Metabolic Pathways of Agrochemicals, Vol. 2. Royal Society of Chemistry, Cambridge, UK, pp. 579–725.

Rose, R.L., Barbhaiya, L., Roe, R.M., Rock, G.C., Hodgson, E., **1995**. Cytochrome P450–associated insecticide resistance and the development of biochemical diagnostic assays in *Heliothis virescens*. *Pestici. Biochem. Physiol. 51*, 178–191.

Ruigt, G.S.F., **1985**. Pyrethroids. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Pergamon Press, Oxford, UK, pp. 183–262.

Ruzo, L.O., Cohen, E., Capua, S., **1988**. Comparative metabolism of the pyrethroids bifenthrin and deltamethrin in the bulb mite *Rhizoglyphus robini*. *J. Agric. Food Chem. 36*, 1040–1043.

Salgado, V.L., Irving, S.N., Miller, T.A., **1983a**. Depolarization of motor-nerve terminals by pyrethroids in susceptible and kdr-resistant house-flies. *Pestici. Biochem. Physiol. 20*, 100–114.

Salgado, V.L., Irving, S.N., Miller, T.A., **1983b**. The importance of nerve-terminal depolarization in pyrethroid poisoning of insects. *Pestici. Biochem. Physiol. 20*, 169–182.

Sawicki, R.M., **1978**. Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. *Nature 275*, 443–444.

Sawicki, R.M., **1985**. The relevance of resistance in the study of the insecticidal activity of pyrethroids. *Abst. Pap. Am. Chem. Soc. 190*, 23–AGO.

Schnitzerling, H.J., Nolan, J., Hughes, S., **1983**. Toxicology and metabolism of some synthetic pyrethroids in larvae of susceptible and resistant strains of the cattle tick *Boophilus microplus* (Can). *Pestici. Sci. 14*, 64–72.

Schuler, M.A., **1996**. The role of cytochrome P450 monooxygenases in plant-insect interactions. *Plant Physiol. 112*, 1411–1419.

Schuler, T.H., Martinez-Torres, D., Thompson, A.J., Denholm, I., Devonshire, A.L., *et al.*, **1998**. Toxicological, electrophysiological, and molecular characterisation of knockdown resistance to pyrethroid insecticides in the diamondback moth, *Plutella xylostella* (L.). *Pestici. Biochem. Physiol. 59*, 169–182.

Scott, J.G., Georghiou, G.P., **1986**. Mechanisms responsible for high-levels of permethrin resistance in the housefly. *Pestici. Sci. 17*, 195–206.

Scott, J.G., Liu, N., Wen, Z.M., **1998**. Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 121*, 147–155.

Scott, J.G., Wen, Z.M., **2001**. Cytochromes P450 of insects: the tip of the iceberg. *Pest Manage. Sci. 57*, 958–967.

Shan, G.M., Hammock, B.D., **2001**. Development of sensitive esterase assays based on alpha-cyano-containing esters. *Analyt. Biochem. 299*, 54–62.

Shono, T., Unai, T., Casida, J.E., **1978**. Metabolism of permethrin isomers in American cockroach adults, house-fly adults, and cabbage-looper larvae. *Pestici. Biochem. Physiol. 9*, 96–106.

Soderlund, D.M., Casida, J.E., **1977**. Effects of pyrethroid structure on rates of hydrolysis and oxidation by mouse-liver microsomal-enzymes. *Pestici. Biochem. Physiol. 7*, 391–401.

Soderlund, D.M., Clark, J.M., Sheets, L.P., Mullin, L.S., Piccirillo, V.J., *et al.*, **2002**. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology 171*, 3–59.

Soderlund, D.M., Hessney, C.W., Jiang, M., **1987**. Metabolism of fenvalerate by resistant Colorado potato beetles. *J. Agric. Food Chem. 35*, 100–105.

Soderlund, D.M., Knipple, D.C., **2003**. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem. Mol. Biol. 33*, 563–577.

Soderlund, D.M., Sanborn, J.R., Lee, P.W., **1983**. Metabolism of pyrethrins and pyrethroids in insects. In: Hutson, D.H., Roberts, T.R. (Eds.), Progress in Pesticide Biochemistry and Toxicology. John Wiley, Chichester, UK.

Song, J.H., Narahashi, T., **1996**. Modulation of sodium channels of rat cerebellar Purkinje neurons by the pyrethroid tetramethrin. *J. Pharmacol. Exp. Ther. 277*, 445–453.

Straw, N.A., Fielding, N.J., Waters, A., **1996**. Phytotoxicity of insecticides used to control aphids on Sitka spruce, *Picea sitchensis* (Bong) Carr. *Crop Protect. 15*, 451–459.

Tan, J.G., Liu, Z.Q., Nomura, Y., Goldin, A.L., Dong, K., **2002**. Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *J. Neurosci. 22*, 5300–5309.

Tatebayashi, H., Narahashi, T., **1994**. Differential mechanism of action of the pyrethroid tetramethrin on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J. Pharmacol. Exp. Ther. 270*, 595–603.

Terriere, L.C., **1984**. Induction of detoxication enzymes in insects. *Annu. Rev. Entomol. 29*, 71–88.

Tomita, T., Scott, J.G., **1995**. cDNA and deduce protein-sequence of Cyp6d1 – the putative gene for a cytochrome-P450 responsible for pyrethroid resistance in the house fly. *Insect Biochem. Mol. Biol. 25*, 275–283.

Tomlin, C.D.S. (Ed.) **2000**. The Pesticide Manual: a World Compendium, 12th edn. British Crop Protection Council, Farnham, UK.

Vais, H., Atkinson, S., Eldursi, N., Devonshire, A.L., Williamson, M.S., *et al.*, **2000**. A single amino-acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides. *FEBS Letts 470*, 135–138.

Vais, H., Atkinson, S., Pluteanu, F., Goodson, S.J., Devonshire, A.L., *et al.*, **2003**. Mutations of the para sodium channel of *Drosophila melanogaster* identify putative binding sites for pyrethroids. *Mol. Pharmacol. 64*, 914–922.

Vijverberg, H.P.M., de Weille, J.R., Ruigt, G.S.F., **1986**. The effect of pyrethroid structure on the interaction with the sodium channel in the nerve membrane. In: Ford, M.G., Lunt, G.G., Reay, R.C., Usherwood, P.N.R. (Eds.), Neuropharmacology and Pesticide Action. Ellis Horwood, Chichester, UK.

Vontas, J.G., Small, G.J., Hemingway, J., **2001**. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem. J. 357*, 65–72.

Whalon, M., Mota-Sanchez, D., Hollingworth, R.M., Bills, P., Duynslager, L., **2003**. The Database of Arthropods Resistant to Pesticides [database on the Internet] Michigan State University <http://www.pesticideresistance.org/ DB/index.php> Accessed 23 April 2004.

Wheelock, G.D., Scott, J.G., **1989**. Simultaneous purification of a cytochrome P450 and cytochrome 65 housefly, *Musca domestica* L. *Insect Biochem. 19*, 481–488.

Wheelock, G.D., Scott, J.G., **1992**. The role of cytochrome-P450$_{ipr}$ in deltamethrin metabolism by pyrethroid-resistant and susceptible strains of house flies. *Pestici. Biochem. Physiol. 43*, 67–77.

Williamson, M.S., Martinez-Torres, D., Hick, C.A., Devonshire, A.L., **1996**. Identification of mutations in the housefly *para*-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Mol. Gen. Genet. 252*, 51–60.

Wu, D.X., Scharf, M.E., Neal, J.J., Suiter, D.R., Bennett, G.W., **1998**. Mechanisms of fenvalerate resistance in the German cockroach, *Blattella germanica* (L.). *Pestici. Biochem. Physiol. 61*, 53–62.

Yamamoto, I., Casida, J.E., **1968**. Syntheses of 14C-labeled pyrethrin I, allethrin, phthalthrin, and dimethrin on a submillimole scale. *Agric. Biol. Chem. 32*, 1382–1391.

Yamamoto, I., Kimmel, E.C., Casida, J.E., **1969**. Oxidative metabolism of pyrethroids in houseflies. *J. Agric. Food Chem. 17*, 1227–1236.

Yu, S.J., Robinson, F.A., Nation, J.L., **1984**. Detoxication capacity in the honey bee, *Apis mellifera* L. *Pestici. Biochem. Physiol. 22*, 360–368.

Zhang, M.L., Scott, J.G., **1996**. Cytochrome b(5) is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pestici. Biochem. Physiol. 55*, 150–156.

6.2 Indoxacarb and the Sodium Channel Blocker Insecticides: Chemistry, Physiology, and Biology in Insects

K D Wing, J T Andaloro, and S F McCann,
E. I. Du Pont de Nemours and Co., Stine–Haskell Research Laboratories, Newark, DE, USA
V L Salgado, Bayer CropScience AG, Monheim am Rhein, Germany

6.2.1. Introduction

There is an increasing need to discover novel chemical insecticides which act on unique biochemical target sites. This is necessary for insecticide resistance management, to maintain agricultural food and fiber production at reasonable economic levels, and also to retain the effectiveness of older and new classes of chemistry as useful tools.

The modes of action of the major insecticide classes are reviewed in Ishaaya (2001) and in this Encyclopedia. These insecticidal compounds are required not only for economic pest control, but also as critical probes to elucidate their respective biochemical target sites, allowing insights into their fundamental biological function.

While there is an increasing need for new agrochemicals, regulatory requirements for the registration of new insecticides continue to escalate, and society's requirements for safety to nontarget organisms and the environment and compatibility with ongoing agricultural practice, pose increasing challenges.

The voltage-gated sodium (Na^+) channel is a primary insecticide target for the synthetic and natural pyrethroids, DDT, *N*-alkylamides, and a host of natural product and peptide toxins (Zlotkin, 2001). At least ten independent target sites exist for these compounds, based on functional studies, and the compounds can exert allosteric effects on one another via these independent sites. In addition, it has been definitively shown that specific genetic mutations in the Na^+ channel in both laboratory and field insects can confer resistance to pyrethroids and DDT (see **Chapters 5.1** and **6.1**). The discovery of additional new Na^+ channel toxins acting at novel

sites thus creates the possibility of useful invertebrate control agents as well as the development of additional tools for understanding ion channel function.

The identification of the Na^+ channel blocker insecticides (SCBIs) represents such a discovery. These compounds all act at a unique site in insect voltage-gated Na^+ channels, which may correspond to the local anesthetic/anticonvulsant site. Our knowledge of the mode of action of indoxacarb derives largely from studies on the pyrazoline (also known as dihydropyrazole) forerunners of this family, carried out in the 1980s at the Rohm and Haas Company (Salgado, 1990, 1992). Pyrazolines were found to paralyze insects by blocking action potential initiation in nerve cells. The mechanism of this block was due to a voltage-dependent blocking action on voltage-gated Na^+ channels. This mechanism is also observed with many therapeutically useful local anesthetic, antiarrhythmic, and anticonvulsant drugs. The bioactivated form of indoxacarb, *N*-decarbomethoxylated DPX-MP062 (DCMP), also works in this manner. Indoxacarb's selective toxicity towards pest insects is due to its rapid bioactivation to the active metabolite DCMP, while higher animals primarily degrade indoxacarb to inactive metabolites via alternate routes.

This article will trace the chemical evolution of these compounds and their physiological activity in invertebrates, which eventually led to the commercial introduction of a member of this class, indoxacarb (Harder *et al*., 1996; Wing *et al*., 2000). The unique metabolic, insecticidal, and pest management control properties of indoxacarb will also be discussed.

6.2.2. Chemistry of the Na^+ Channel Blockers

6.2.2.1. Chemical Evolution and Structure–Activity of the Na^+ Channel Blocker Insecticides

The first insecticidal pyrazoline Na^+ channel blockers were reported in patents from Philips-Duphar in 1973 and are represented by PH 60–41 (Mulder and Wellinga, 1973) (**Figure 1**). These compounds were reported to have high levels of efficacy against coleopteran and lepidopteran pests. In 1985, Rohm and Haas reported pyrazolines such as RH-3421 with ester substituents on the pyrazoline 4-position (Jacobson, 1985). These compounds were later reported to have high insecticidal efficacy, low mammalian toxicity, and a rapid rate of dissipation in the environment (Jacobson, 1989). Subsequent work by DuPont on the SCBIs resulted in the discovery of several classes of related structures, all with similarly high levels of insecticidal activity (**Figure 2**). It was found that transposition of the N1 and C3 atoms in the pyrazoline core gave active compounds (compound B). Conformationally constrained pyrazolines, resulting from bridging the pyrazoline C4 postion with the C3 aryl substituent (forming indazoles and oxaindazoles) (compound C in **Figure 2**), were also highly active. Semicarbazones D are also insecticidal; these compounds are structurally similar to pyrazolines, but with the C5 carbon removed. Expanding the pyrazoline ring by one carbon gave highly active pyridazine compounds (compound E). Substituting oxygen or nitrogen for the C5 pyridazine atom gives oxadiazines and triazines, also with high insecticidal activity (compound F). Indoxacarb is, in fact, a representative of the oxadiazine subclass of SCBIs.

The insecticidal structure–activity relationships for the oxadiazines are summarized in **Figure 3**. For substituent R1, 4- or 5-Cl, Br, OCH_2CF_3, and CF_3 groups gave compounds with the highest activity. In the R2 position, 4-halo-phenyl and CO_2Me were the most active substituents. For groups at R3, 4-OCF_3, 4-CF_3, and 4-Br were best for activity. For the substituents on nitrogen (R4), CO_2CH_3 and $COCH_3$ were the most active, followed by H, Me, and Et.

6.2.2.2. Chemistry and Properties of Indoxacarb

Indoxacarb is synthesized as described in McCann *et al*. (2001, 2002) and Shapiro *et al*. (2002). Its

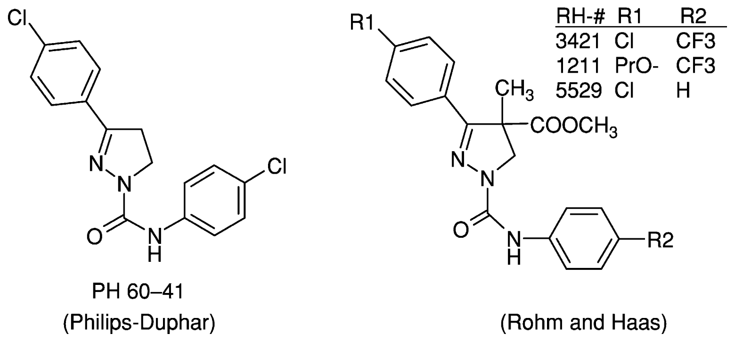


Figure 1 Structures of original pyrazoline Na^+ channel blockers.

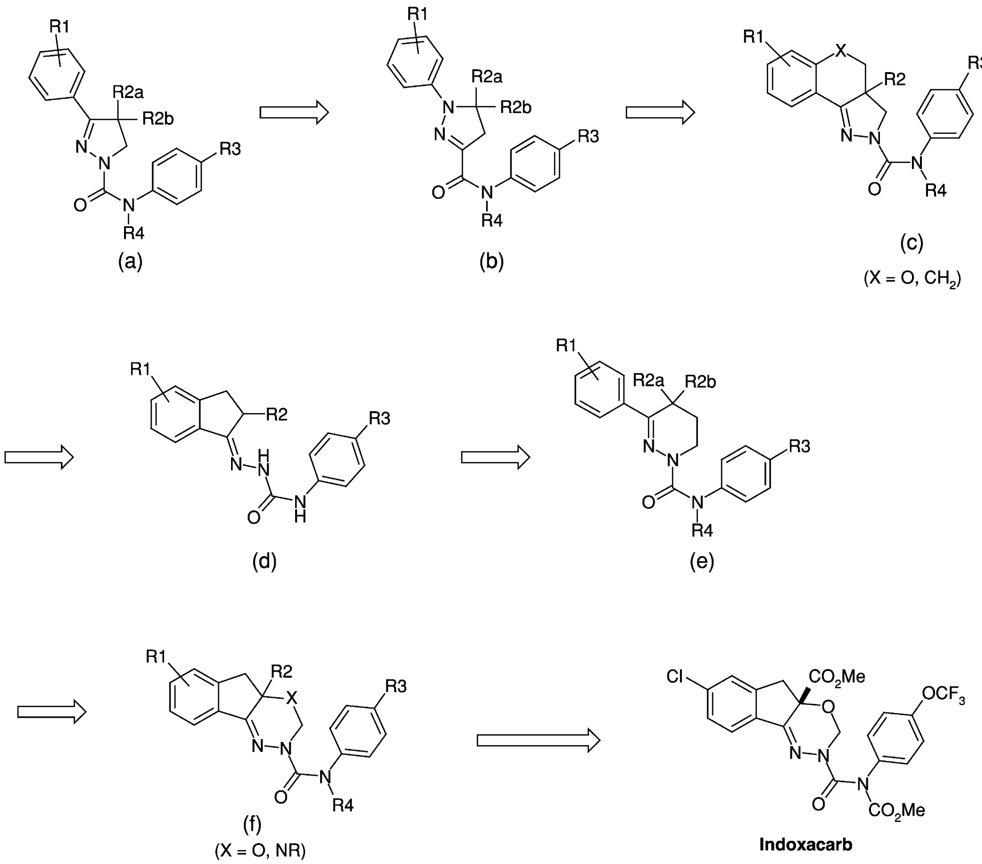


Figure 2 Structures of pyrazoline-like Na^+ channel blockers.

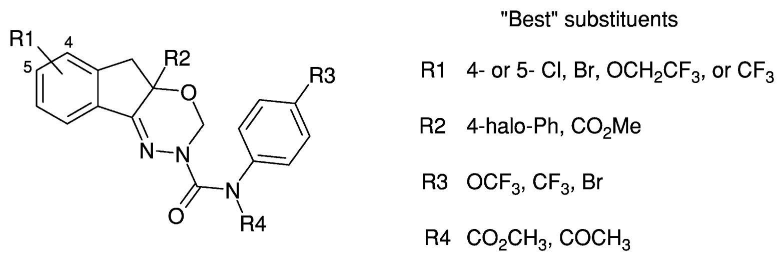


Figure 3 Structure–activity relationships for oxadiazine insecticides vs. *Spodoptera frugiperda*.

physical, toxicological, and environmental properties are shown in Table 1; the profile indicates a safe, environmentally benign compound with relatively low water solubility.

6.2.3. Metabolism and Bioavailability of Indoxacarb

6.2.3.1. Bioactivation of Indoxacarb

Indoxacarb requires metabolic activation by insects before it acts as a strong Na^+ channel blocker (Figure 4). An esterase or amidase-type of enzyme(s) cleaves the carbomethoxy group from the urea linkage, liberating the free urea DCMP, which then acts as the voltage-dependent Na^+ channel blocker. This was first demonstrated for the racemic compound DPX-JW062 (50 : 50 mixture of active *S* and inactive *R* enantiomers) being converted to the corresponding *N*-decarbomethoxylated DPX-JW062 (DCJW) (Wing *et al.*, 1998). The enzyme involved has been only partially characterized. It is clear that the enzyme is a hydrolase of some type, may be serine dependent. In Lepidoptera the enzyme is found in high concentrations in midgut cells (but not the gut contents) and in fat body, and also found in high concentrations in several subcellular fractions (nuclear, mitochondrial, microsomal, and cytosolic). The enzyme can be inhibited by several

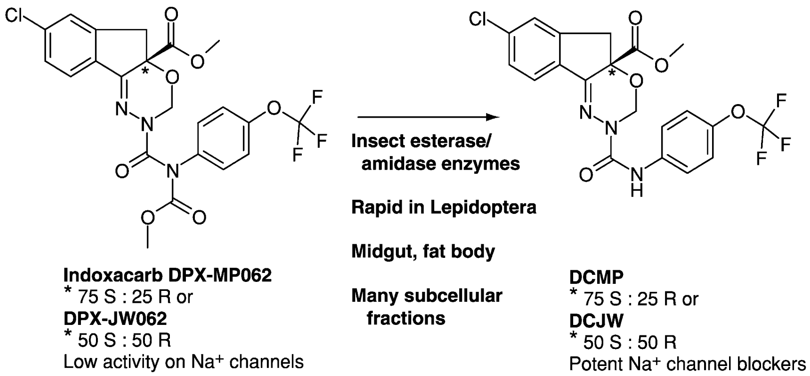


Figure 4 Bioactivation of oxadiazines to *N*-decarbomethoxylated Na^+ channel blocker metabolites in insects.

Table 1 Properties of indoxacarb (DPX-MP062)

Physical state (99% active enantiomer DPX-KN128)	Solid, powder
Melting point	88.1 °C
Solubility (for DPX-MP062, 75% DPX-KN128 + 25% R-enantiomer)	Water – 0.20 ppm *n*-Heptane – 1.72 mg ml^{-1} 1-Octanol – 14.5 mg ml^{-1} Methanol – 103 mg ml^{-1} Xylene – 117 mg ml^{-1} Acetonitrile – 139 mg ml^{-1} Ethyl acetate – 160 mg ml^{-1} Dichloromethane – >250 g kg^{-1} Acetone – >250 g kg^{-1} Dimethylformamide – >250 g kg^{-1}
Partition coefficient in octanol/water (KPX-KN128)	log K_{ow} = 4.65
Vapor pressure (DPX-KN128)	9.8×10^{-9} Pa at 20 °C 2.5×10^{-8} Pa at 25 °C
Acute toxicity of indoxacarb (DPX-MP062)	Oral LC_{50}: 1730 mg kg^{-1} (male rats); 268 mg kg^{-1} (female rats) Dermal LD_{50}: greater than 5000 mg kg^{-1} in rats Inhalation LC_{50}, 4 h: greater than 5.5 mg l^{-1} Dermal irritation: nonirritant Eye irritation: moderate eye irritant Ames test: negative
Environmental fate characteristics	''No major issues in the areas of soil persistence, mobility, and fish bioaccumulation for indoxacarb.''

Data from EPA, **2000**. Fact Sheet on Indoxacarb. Issued Oct. 30, 2000, Environmental Protection Agency, Washington, DC.

organophosphate esterase inhibitors but not the cytochrome p450 monooxygenase inhibitors piperonyl butoxide or 1-phenyl imidazole, nor the glutathione transferase inhibitor *N*-ethyl maleimide. Formal structural proof of the indoxacarb active metabolite was obtained by ^{14}C cochromatography and reversed phase high-performance liquid chromatography (HPLC)/electrospray mass spectrometry with authentic standards.

The bioactivation reaction is widespread throughout several orders of insects. Wing *et al.* (1998) first demonstrated that Lepidoptera representing *Trichoplusia, Spodoptera, Helicoverpa/Heliothis*, and *Manduca* species perform this reaction after oral administration of [^{14}C]DPX-JW062 within 3 h, by which time all larvae were observed to halt feeding, and to suffer a lack of righting, mild convulsions, and a distinct and irreversible pseudoparalysis. A more comprehensive study including Lepidoptera, Homoptera (*Nephotettix, Nilaparvata, Peregrinus, Myzus* species), and Hemiptera (*Lygus* species) indicate that onset of toxic symptoms in all of these insects is well correlated with formation of [^{14}C]DCJW from [^{14}C]DPX-JW062 or unlabeled indoxacarb to DCMP after oral administration (Wing *et al.*, 2000). However, it was noted that the bioactivation rate as well as speed of onset of symptoms was noticeably more rapid with Lepidoptera, and that the Lepidoptera quantitatively converted far more of the parent oxadiazine into the active metabolite than the other orders examined. In addition, there is no apparent stereoselectivity of activation for either the *S* (active Na^+ channel blocker) or *R* enantiomers of parent oxadiazine – both enantiomers appeared to be cleaved rapidly to the corresponding free ureas. However, it appears that all of the insecticidal activity resides in the *S*-enantiomer of DCJW or indoxacarb (Wing *et al.*, 1998) as has been observed for dihydropyrazoles (Hasan *et al.*, 1996). Bioactivation and subsequent cessation of feeding and pseudoparalysis in lepidopteran larvae was slower and required higher doses after topical, compared to oral administration.

While only these free ureas demonstrated strong Na^+ channel blocking activity, it was frequently

observed that both indoxacarb and its activated metabolite were similarly potent in intoxicating Lepidoptera (Wing *et al*., 1998; Tsurubuchi *et al*., 2001). This again argues for efficient bioactivation in this order of insects.

Indoxacarb bioactivation was also studied in the hemipteran cotton pest, the tarnished plantbug (*Lygus lineolaris*), compared to its hemipteran predator, big-eyed bug (*Geochoris punctipes*) (Tillman *et al*., 2001). These studies again demonstrate that the onset of neurotoxic symptoms in both bugs was well correlated with the appearance of the activated metabolite DCMP (albeit at slower rates than observed in Lepidoptera). The importance of oral uptake as a major route of bioactivation/intoxication, compared to tarsal absorption of residues or through dermal uptake, was clearly evident. Interestingly, laboratory studies showed both pest and predator to be similarly sensitive to indoxacarb, although *Geochoris* could tolerate higher levels of indoxacarb before showing signs of intoxication. However, in treated cotton fields, *Lygus* has been found to be more sensitive to indoxacarb treatment while *Geochoris* has been relatively refractory; further studies showed that this was largely due to infield behavior and the preference of *Geochoris* to feed on pest insects and their eggs as opposed to foliage, which significantly slowed oral indoxacarb uptake (Tillman *et al*., 2001).

In addition, all current evidence points to a SCBI structural requirement for an underivatized urea linkage to be present to exert strong, quasi-irreversible insect Na^+ channel blocking effects, as opposed to derivatized ureas.

6.2.3.2. Catabolism of Indoxacarb and Other Na+ Channel Blocker Insecticides

Relatively little is known about the metabolic breakdown of indoxacarb or other SCBIs in insects. On balance, it is clear that bioactivation is occurring much more rapidly than degradation after field application, when satisfactory insect control is observed. However, like any other novel insecticide, resistance management will be a key to maintaining the agricultural utility of this insecticide class.

Indeed, several field populations of North American oblique-banded leafroller (*Choristoneura rosaceana*) have shown a degree of insensitivity to indoxacarb, which is unique for Lepidoptera (Ahmad *et al*., 2002). Though this insect has never been exposed to indoxacarb for commercial control in the field, it has been treated with several other insecticide classes, including a number of organophosphates. Current studies indicate that resistant strains of this leafroller may be able to catabolize indoxacarb more rapidly than susceptible strains; the specific mechanisms and metabolites are currently being characterized (Hollingworth *et al*., unpublished data).

Interestingly, indoxacarb is apparently degraded primarily by higher animals via routes including cytochrome p450 mediated attack of the indanone and oxadiazine rings, while *N*-decarbomethoxylation is a relatively minor pathway (EPA, 2000; Scott, 2000). The rapid metabolic degradation is a critical factor responsible for the high nontarget animal safety of indoxacarb.

6.2.4. Physiology and Biochemistry of the Na+ Channel Blockers

6.2.4.1. Symptoms of SCBI Poisoning in Insects: Pseudoparalysis

Pyrazolines and indoxacarb produce identical acute neurotoxic symptoms in the American cockroach *Periplaneta americana*, progressing through initial incoordination (5–20 min after 1–10 μg injection), then tremors and prostration, and finally a distinctive pseudoparalysis, so named because the apparently paralyzed insects produce violent convulsions when disturbed. This pseudoparalysis was maintained for 3–4 days, after which the ability to move when disturbed waned. By 4–6 days, the insects could be considered dead. Lower doses caused a similar progression of symptoms, but the time before appearance of symptoms was delayed. This unusual pseudoparalysis was key to unraveling the complex mode of action of this family in insects.

Irreversible pseudoparalysis was also observed in lepidopterous larvae, with disturbance during the pseudoparalyzed state leading to squirming, and tremors of legs and mandibles evident upon microscopic examination. Higher doses of pyrazolines, especially in lepidopterous larvae, led within a few hours to flaccid paralysis (Salgado, 1990). Injection of 10 μg g^{-1} indoxacarb into fifth instar *Manduca sexta* larvae produced excitatory neurotoxic symptoms leading to convulsions and then relatively rapidly to flaccid paralysis (Wing *et al*., 1998). As discussed above, indoxacarb itself is converted *in vivo* to DCMP by esterase-like enzymes. The pseudoparalytic symptoms caused by indoxacarb have also been clearly observed in *Spodoptera frugiperda* larvae treated by injection of 20 μg g^{-1} indoxacarb or DCJW, and also in male *P. americana* injected with 1, 3, or 10 μg g^{-1} DCJW (Salgado, unpublished data).

Under field conditions, after ingesting or being directly sprayed with indoxacarb, insects will irreversibly stop feeding within a few minutes to 4 h; higher doses cause more rapid onset of symptoms.

Higher temperatures increase desiccation and speed of kill. Unlike the pyrethroids, indoxacarb exhibits a positive temperature coefficient. Uncoordinated insects may fall off the plant and desiccate, drown, or become subject to predation. Affected insects can stay alive for 4–96 h, depending on the dose of indoxacarb and susceptibility of the insect. Insects exposed to subparalytic doses eat much less than untreated larvae, develop more slowly, gain less weight, and pupate and emerge later than untreated insects.

6.2.4.2. Block of Spontaneous Activity in the Nervous System

RH-3421 and RH-1211, two highly active pyrazolines, were used as model compounds in an electrophysiological analysis of poisoned *P. americana*, together with RH-5529, which was useful for some experiments because of its ready reversibility (**Figure 1**). The upper panel in **Figure 5** compares extracellular recordings from three parts of the nervous system before and shortly after paralysis with 5 μg g^{-1} RH-3421. The connectives between the second and third thoracic ganglia are major pathways for interneurons within the central nervous system (CNS); the crural nerve is the major leg nerve, with both sensory and motor traffic; and the cercal nerve is exclusively sensory. In all cases, the complete absence of neural activity upon dihydropyrazole poisoning is striking. It has likewise been shown that activity in the CNS connectives of *M. sexta* larvae is completely blocked after paralysis with indoxacarb or DCJW via injection (Wing *et al*., 1998). The lower panel in **Figure 5** shows recordings from *S. frugiperda* larvae before and shortly after paralysis with both DCJW and indoxacarb. In the CNS connectives and also in the ventral nerve roots of the abdominal ganglia, which carry motor and sensory axons, there is no nerve activity in the paralyzed insects. The assessment of the state of the nervous system during poisoning is completed with **Figure 6**, which shows that even in a cockroach paralyzed for 24 h, tactile stimulation of the trochanter could elicit sensory spikes in the crural nerve that in turn initiate reflex motor activity in the same nerve. This is a clear demonstration that axonal conduction and synaptic transmission function more or less normally in paralyzed insects; the evident defect is that the nerves no longer generate action potentials spontaneously.

Normally, even in a quiescent insect, there is background or spontaneous action potential activity in the nervous system arising from pacemaker cells in the CNS and from tonic sensory receptors. Mechanoreceptors exist in many places on the cuticle to detect cuticular deformations or hair

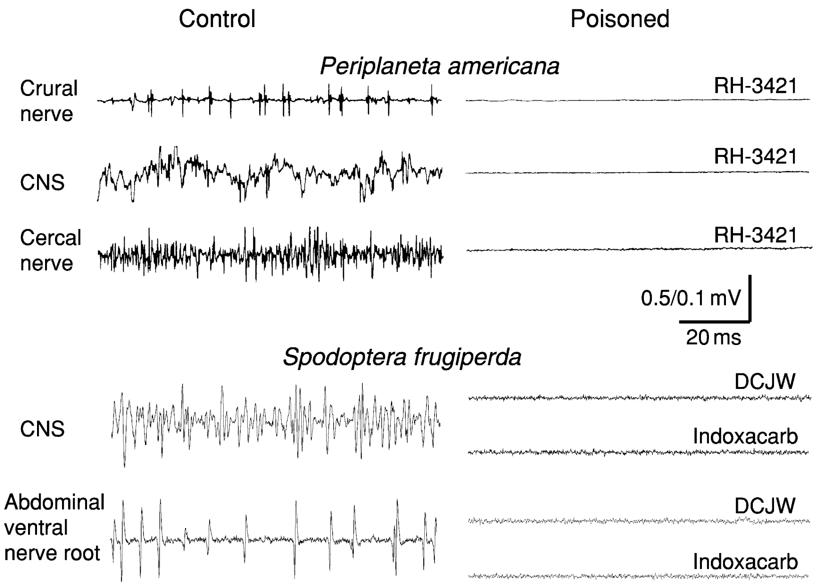


Figure 5 SCBI poisoning inhibits all spike activity in the nervous system. Representative extracellular recordings from the central nervous system (CNS) and peripheral nerves of paralyzed insects and untreated DMSO-injected controls. Adult male cockroaches were treated by injection of 5 μg g^{-1} RH-3421 and fifth instar *Spodoptera* larvae weighing 500 mg were treated with 5 μg g^{-1} DCJW or indoxacarb, 3–4 h before dissection of and recording from the paralyzed insects. (Cockroach data: reproduced with permission from Salgado, V.L., **1990**. Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. *Pestic. Sci. 28*, 389–411; © Society of Chemical Industry, permission is granted by John Wiley & Sons Ltd. on behalf of the SCI. *Spodoptera* data previously unpublished.)

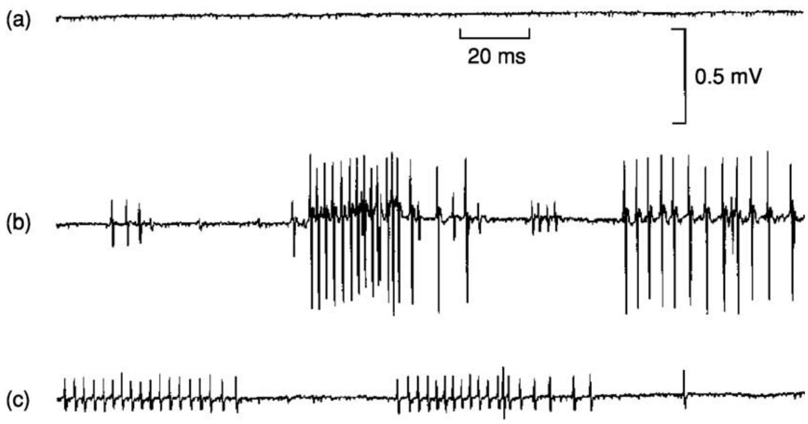


Figure 6 Sensory and motor nerve activity could still be elicited long after the onset of paralysis. These recordings, from nerve 5 (crural nerve) of a cockroach injected 24 h earlier with 5 $\mu g\,g^{-1}$ RH-3421, show that although there was no background activity (trace a), tactile stimulation of the ipsilateral trochanter elicited activity in several axons (trace b). Furthermore, cutting nerve 5 proximal to the recording site abolished the larger spikes (trace c), showing that the remaining smaller spikes were in primary sensory neurons while the larger ones in trace (b) were reflexly evoked motor spikes. (Reproduced with permission from Salgado, V.L., **1990**. Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. *Pestic. Sci. 28*, 389–411; © Society of Chemical Industry, permission is granted by John Wiley & Sons Ltd. on behalf of the SCI.)

deflections resulting from stresses due to the body's own movements or to external stimuli, and internally in the form of chordotonal organs or muscle stretch receptor organs to report on joint angles. Mechanoreceptors in general have both phasically and tonically responding sensory cells or units. The phasic units respond with a burst of activity at the start or end of a stimulus, but rapidly adapt in the presence of constant stimuli, because their function is to sensitively detect changes in stimulus level. The tonic units maintain a steady firing rate during stimulus presentation and give rise to slowly adapting sensations. In insect muscle stretch receptor organs, both phasic and tonic functions are subserved by a single neuron. A sudden elongation of this receptor induces firing initially at a high rate, which then declines to a constant rate that is proportional to static elongation, so that both velocity and position are encoded (Finlayson and Lowenstein, 1958).

The complete absence of neural activity in poisoned insects indicates that SCBIs block not only tonic sensory activity, but also pacemaker activity in the CNS. Both of these effects involve action potential generation in regions of neurons that are able to generate action potentials repetitively in response to constant stimuli. The ability of phasic receptors to respond long after paralysis at a high dose (Figure 6), suggests that phasic receptors are not as sensitive as the tonic ones, although they may also be affected at higher doses in Lepidoptera, when insects are completely paralyzed, as mentioned earlier. At this point in the electrophysiological analysis, the pseudoparalysis resulting from SCBI poisoning is apparently due to inhibition of spontaneous activity in tonic sensory receptors and pacemaker neurons. However, excitatory symptoms are also seen during the course of poisoning; a plausible hypothesis is that early in poisoning, before the compound reaches the CNS, sensory receptors in the periphery become blocked. In the absence of adequate sensory feedback to the CNS in response to attempted movements, there would be a tendency to overaccentuate these movements, resulting in the altered posture and gait that is observed. The quiet periods that predominate later in pseudoparalysis may be due to block of pacemaker activity in the CNS. An explanation of the tremors will be proposed below, after the cellular effects of the compounds that cause the observed block are considered. What is noteworthy, however, about the excitatory symptoms arising from a blocking action is the long time period over which apparent excitatory effects can be seen. Compounds with primary excitatory effects on neurons lead to continuous excitation, and within a few hours to complete paralysis due to, among other things, neuromuscular block, as seen for example with pyrethroids (Schouest *et al.*, 1986) or spinosad (Salgado, 1998). Insects poisoned with SCBIs, alternatively, do not suffer this pysiological exhaustion, and can therefore show strong tremors intermittently for several days after poisoning.

6.2.4.3. Block of Na^+ Channels in Sensory Neurons

A deeper understanding of the mode of action of the SCBIs required the investigation of their cellular effects on sensory neurons. Abdominal stretch receptors from cockroach adults and *M. sexta* larvae were shown to be blocked by various pyrazolines

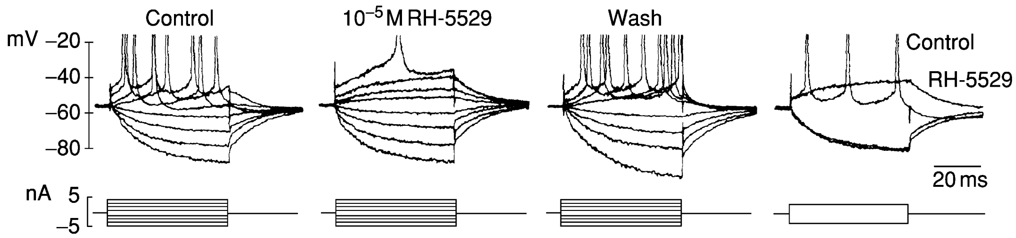


Figure 7 RH-5529 raised the threshold in current-clamped crayfish stretch receptors, without affecting passive membrane resistance. Records show voltage (upper) and current (lower) traces for current steps before, during, and after application of 10 µM RH-5529. Corresponding traces superimposed on the right show that the spike threshold was raised by RH-5529, with no change in resting potential or in the response to hyperpolarizing pulses. (Reproduced with permission from Salgado, V.L., **1990**. Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. *Pestic. Sci. 28*, 389–411; © Society of the Chemical Industry, permission is granted by John Wiley & Sons Ltd., on behalf of the SCI.)

(Salgado, 1990), but the slowly adapting stretch receptor (SASR) of the crayfish *Procambarus clarkii* (Wiersma *et al.*, 1953) was selected for studies of the cellular mechanism of action, because the greater size of its neuron enables intracellular recordings at the site of spike initiation.

Pyrazolines blocked the stretch receptor in crayfish with a potency similar to that in insects. In mechanoreceptors, membrane deformation resulting from elongation activates stretch-sensitive ion channels that induce a so-called generator current that depolarizes the membrane of the spike initiation zone to the threshold for action potential generation. In other words, the generator current resulting from mechanosensory transduction is encoded as a spike train in the spike initiation zone. Insertion of a microelectrode into the spike-initiation zone of an SASR neuron allowed the function of this transduction and spike-encoding process to be studied. Figure 7 shows a SASR neuron studied under current clamp, in which current pulses of varying amplitude were injected to either hyperpolarize or depolarize the membrane. Such currents bypass the sensory transduction process and allow direct assessment of the spike-encoding process. The voltage traces in response to the injected currents are shown in the upper row. For negative or hyperpolarizing pulses, represented as downward deflections of both current and voltage, RH-5529 did not affect the membrane response, indicating that it did not affect passive membrane properties (Figure 7). The singular effect of RH-5529 was to raise the threshold for spike generation in response to depolarizing pulses, making it more difficult for injected currents and, by inference, for generator currents, to elicit spikes. From this result, it was immediately clear that voltage-dependent Na^+ channels, whose activation determines the threshold and initiates the action potential, were blocked by the pyrazoline. This was also likely the mechanism of block in the spike initiation zones of CNS pacemaker neurons, where spikes are also generated by the activation of Na^+ channels in response to depolarization by summation of synaptic inputs and pacemaker currents.

6.2.4.4. Mechanism of Na^+ Channel Block

In order to better understand the action of SCBIs on Na^+ channels, further studies were carried out on crayfish giant axons treated with pyrazolines, with techniques that allowed the study of Na^+ channels under highly controlled conditions. A first hypothesis to explain the insensitivity of axonal Na^+ channels to pyrazolines was that the compounds block Na^+ channels in a voltage-dependent manner, and are therefore selective for channels in the spike initiation zone. Voltage-gated Na^+ channels are complex proteins, whose function is regulated by membrane potential through voltage-dependent conformational changes occurring on a timescale from less than a millisecond to several seconds (Pichon and Ashcroft, 1985). At the spike initiation zone, neurons operate near the threshold for action potential generation, in the range of −70 to −50 mV, where important conformational changes in Na^+ channels occur prior to opening. In contrast, axons have a resting potential near −90 mV, where Na^+ channels are predominantly in the resting state. However, when axons are depolarized to the range where Na^+ channels begin to undergo conformational changes, they become sensitive to pyrazolines (Salgado, 1990). Likewise, the extracellularly recorded compound action potential from motor nerves of *M. sexta* abdominal ganglia, which was likewise highly insensitive to DCJW, was rendered sensitive by depolarization of the nerves with a high K^+ saline (Wing *et al.*, 1998).

Figure 8 shows membrane currents evoked by test pulses to 0 mV before and after equilibration with 10 µM RH-3421 at various holding potentials. For each trace, after initial rapid downward and upward transients, the current became inward and peaked

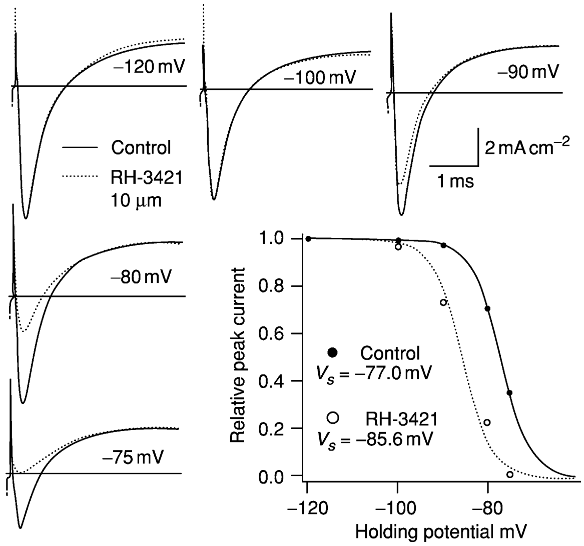


Figure 8 Dihydropyrazole block appears as a parallel shift of the steady state slow inactivation curve in the direction of hyperpolarization. Ionic current traces were scaled by a common factor so that the peak at −120 mV matched the peak before treatment with the dihydropyrazole. Peak I_{Na} was depressed most at depolarized potentials, whereas outward current, I_K, was not affected by the treatment. The graph shows plots of peak current normalized to the value at −120 mV. RH-3421 (10 μM) appears to shift the steady state inactivation relation to the left by 8.6 mV. (Reproduced with permission from Salgado, V.L., **1992**. Slow voltage-dependent block of Na^+ channels in crayfish nerve by dihydropyrazole insecticides. *Mol. Pharmacol. 41*, 120–126; © American Society for Pharmacology and Experimental Therapeutics.)

within 0.5 ms, then reversed and became outward. The inward (downward) peak is the Na^+ current and the outward (upward) steady state current is the K^+ current (Pichon and Ashcroft, 1985). Depolarization of the axon to potentials more positive than −90 mV in the control suppressed the Na^+ current by a process known as inactivation, which in this case had a midpoint potential of −77 mV. Na^+ channels have two partially independent inactivation processes, known as fast and slow inactivation. Fast inactivation occurs on a millisecond timescale and serves to terminate the action potential, while slow inactivation occurs on a much slower timescale, over hundreds of milliseconds, and performs a slow, modulatory function. Slow inactivation occurs at more negative potentials than fast inactivation, and is responsible for the inactivation seen in Figure 8. After equilibration of the axon with RH-3421, there was no effect on either Na^+ or K^+ current at −120 or at −100 mV (Figure 8). At more depolarized potentials, however, the Na^+ current was specifically depressed by RH-3421, whereas the K^+ current was unaffected. When peak Na^+ current is plotted against membrane potential, it appears that

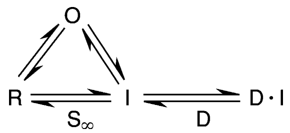


Figure 9 A model showing the resting (R), Open (O), and inactivated (I) states of the Na^+ channel, and specific interaction of the SCBIs (D) with the inactivated state.

RH-3421 has shifted the slow inactivation curve by 9 mV to the left.

The next step in the analysis is to consider the mechanism of this apparent shift of slow inactivation. Whereas fast inactivation occurs with a time course of hundreds of microseconds to a few milliseconds, and slow inactivation on the order of tens to hundreds of milliseconds, the changes in peak Na^+ current in the presence of SCBIs occur on a much slower timescale, on the order of 15 min. This slow readjustment of peak current in response to voltage change can therefore be attributed to a new process: the binding and unbinding of SCBIs to Na^+ channels.

The Na^+ channel undergoes transitions between many different conformations or states, which can be grouped into resting (R), open (O), and inactivated (I) states, each of which may have several substates. The transitions between these naturally occurring states are shown on the left in Figure 9. In this simplified model, $S_\infty = [R]/[R] + [I]$, is the steady-state slow inactivation parameter, which depends on membrane potential according to

$$S_\infty = \frac{1}{1 + \exp[(V - V_S)/k]} \qquad [1]$$

where V is membrane potential, V_S is the potential at which $S_\infty = 0.5$ (half of the channels are in the inactivated state), and k is a constant that includes the Boltzmann constant and describes the voltage sensitivity of the inactivation process (Hodgkin and Huxley, 1952).

This model ignores the fast-inactivated state, but interaction of the SCBIs with the channels is so slow, that it and the open state, O, can be ignored in the analysis. The observed enhancement of slow inactivation can be explained if we assume that the SCBI drug (D) binds selectively to an inactivated state, as also shown on the right-hand side in the model (Figure 9). Drug binding would then effectively remove channels from the I pool, which would be compensated for by mass action rearrangements among the other states, leading to net flux of more receptors from the R pool into the I and D • I pools. $K_I = [D] \times [I]/[D \bullet I]$ is defined as the equilibrium dissociation constant of the D • I complex.

In the presence of SCBI drug, two processes lead to the decrease in current upon depolarization: slow

inactivation and drug binding. Fortunately, slow inactivation can be removed from the measurements by hyperpolarizing to −120 mV before applying the depolarizing pulse to measure the peak current. Figure 10a shows the time course of block after stepping from −120 mV to various depolarized potentials, in an axon equilibrated with 1 μM RH-1211. The pulse protocol, shown in the inset in panel A (Figure 10), includes a 500 ms hyperpolarizing

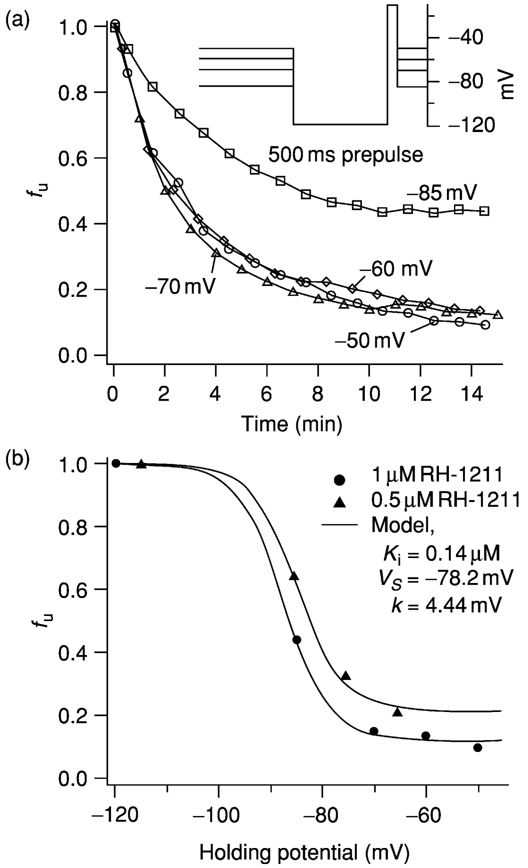


Figure 10 Block is asymptotic at strong depolarizations. The model predicts that block of Na^+ channels by SCBIs is voltage dependent only over the voltage range over which inactivation occurs. In order to observe block directly at strong depolarizations, where inactivation is complete, the axon was held at various potentials as in **Figure 8**, but a 500 ms hyperpolarizing prepulse to −120 mV was applied just before the 10 ms test pulse, during which I_{Na} was measured (see inset protocol). The prepulse was of sufficient amplitude and duration to remove inactivation completely, without interfering with block. (a) Time course of f_u, the fraction of channels unblocked, following steps from −120 mV to various other potentials, in an axon equilibrated with 1 μM RH-1211. Steady state block from this and another axon treated with 0.5 μM RH-1211 are plotted in (b). The solid curves in (b) were plotted according to eqn [2], with $K_i = 0.14$ μM, $V_h = 78.2$ mV, and $k = 4.44$ mV. (Reproduced with permission from Salgado, V.L., **1992**. Slow voltage-dependent block of Na^+ channels in crayfish nerve by dihydropyrazole insecticides. *Mol. Pharmacol. 41*, 120–126; © American Society for Pharmacology and Experimental Therapeutics.)

prepulse to −120 mV before each test pulse, which was long enough to completely remove slow inactivation, but not long enough to permit significant dissociation of D • I complexes, thus allowing direct assessment of block. The fraction of channels unblocked, f_u, is plotted against time, and shows that block, which is equivalent to the formation of D • I complexes, occurs with a time constant of 5–6 min at 1 μM. The voltage dependence occurs only over the range where slow inactivation occurs, saturating near −70 mV. This is seen clearly in Figure 10b, where f_u is plotted against holding potential. In terms of the model shown above, $f_u = ([R] + [I])/([R] + [D \bullet I] + [I])$. Substituting the above relations for K_I and S_∞ and eqn [1] into this expression, an equation for f_u as a function of [D] and V can be derived:

$$f_u = \frac{1}{1 + \frac{[D]}{K_I}\left(\frac{1}{1 + \exp[(V_S - V)/k]}\right)} \qquad [2]$$

The solid curves in Figure 10 were calculated from this equation, using a K_I of 140 nM, $V_S = -78.2$ mV and $k = 4.44$ mV, and fit the data quite well.

The quantitative correspondence of the blocking voltage dependence with model predictions supports the hypothesis that SCBIs bind selectively to the slow-inactivated state of the channel, in comparison with the resting state. However, because of the very slow binding reaction, in the equilibrium studies described to this point, only the resting and slow-inactivated states are populated significantly enough to participate in drug binding. In order to test whether SCBIs bind to the fast-inactivated state, the slow inactivation gate was removed by pretreatment of the internal face of the axon membrane with trypsin. After this pretreatment, block by the pyrazoline RH-1211 at −90 mV was comparable to that in intact axons, indicating that RH-1211 can block fast-inactivated channels as well. Treatment of the axon internally with the protein-modifying reagent *N*-bromoacetamide (NBA) is known to remove the fast inactivation gate. Pretreatment of axons with both trypsin and NBA therefore yields Na^+ channels that do not inactivate. At depolarized potentials, RH-1211 was just as potent at blocking these noninactivating channels as intact channels, showing that it can also block open channels (Salgado, 1992).

The metabolic bioactivation of DPX-JW062 and indoxacarb to *N*-decarbomethoxylated metabolites and the similarity of the mode of action of DCJW to pyrazolines was established by Wing *et al.* (1998), who showed that spontaneous activity and action

potential conduction in the CNS of *M. sexta* were blocked, consistent with block of Na^+ channels. These workers also established that the DCJW Na^+ channel block was voltage-dependent. Subsequently, studies in dorsal unpaired median (DUM) neurons isolated from the CNS of *P. americana* with the patch clamp technique have also shown the action of DCJW on Na^+ channels (Lapied *et al.*, 2001). Action potentials in these pacemaking neurons were blocked by 100 nM DCJW, and this action was shown to be due to block of voltage-dependent Na^+ channels. The block was very potent, with a 50% inhibitory concentration (IC_{50}) of 28 nM at −90 mV, in good agreement with the IC_{50} of racemic DCJW of 40 nM in blocking the compound action potential in *M. sexta* CNS determined by Wing *et al.* (1998). In addition, Wing *et al.* (1998) showed that *M. sexta* compound action potential block by DCJW was stereospecific for the *S*-enantiomer, which is also the only enantiomer toxic to insects.

Although the SCBIs enhance slow inactivation, it is important to realize that the slow inactivation of any particular channel is not affected. Instead, slow inactivation of the entire population of channels is enhanced because of the addition to the system of the highly stable SCBI-bound slow-inactivated state. In the presence of the SCBI, time for this very slow equilibration of the drug with the receptors must be allowed in order to measure slow inactivation properly. It is not enough to simply measure slow inactivation with pulses long enough to attain a steady state in the absence of SCBI. While pulses on the order of 1 s are long enough to attain steady-state slow inactivation in control axons, 15 min are required in the presence of insecticide (Salgado, 1992). Direct measurements of the effect of DCJW and indoxacarb on Na^+ channels have until now only been carried out with the whole cell voltage clamp method. With this technique, it is difficult to make measurements from a single cell for more than 30 min, so it is difficult to measure the effects of these slow-acting insectides under steady state conditions. The block on washing of DCJW requires at least 15 min to reach a steady state level and appears to be irreversible (Lapied *et al.*, 2001; Tsurubuchi and Kono, 2003; Zhao *et al.*, 2003). However, after equilibration of the cell with DCJW at a negative holding potential, the level of block increases in response to membrane depolarization on a faster time course, with steady state levels being attained within 2–3 min (Zhao *et al.*, 2003). Using 3 min conditioning pulses, Zhao *et al.* (2003) demonstrated that DCJW indeed enhanced slow inactivation of two different Na channel subtypes studied in isolated neurons; shifts of 12–13 mV in the direction of hyperpolarization were observed.

An additional effect on the *P. americana* DUM neurons observed by Lapied *et al.* (2001) was a strong hyperpolarization of the resting potential, associated with an increase in membrane resistance, indicating the block of a depolarizing conductance, thought to be the background Na^+ channels involved in the maintenance of the resting potential in these pacemaking neurons and characterized by Lapied *et al.* (1989, 1999). This finding is extremely interesting and should be investigated in more detail, because it demonstrates the existence of a second potential target site for SCBIs.

6.2.4.5. SCBIs Act at Site 10 on the Na^+ Channel

The voltage-dependent Na^+ channel blocking action of pyrazolines was immediately recognized as being similar to that of local anesthetics, class I anticonvulsants, and class I antiarrhythmics (Salgado, 1992), a structurally broad range of drugs (Clare *et al.*, 2000; Anger, 2001) all known to act at a common blocker site within the Na^+ channel pore. Consistent with the nomenclature of Soderlund (see **Chapter 5.1**), this will be called site 10. Like the SCBIs, drugs acting at site 10 all exhibit voltage dependence of block, deriving from selective binding to open and inactivated channel states.

Drugs acting at site 10 displace the binding of the radioligand [^{3}H]batrachotoxin (BTX)-B from the BTX binding site in the Na^+ channel (Postma and Catterall, 1984; Catterall, 1987). The interaction is clearly allosteric, because local anesthetics can block BTX-modified open channels without displacing the toxin (Wasserstrom *et al.*, 1993; Zamponi *et al.*, 1993), although they speed the dissociation rate of BTX from its binding site. Pyrazolines were also shown to potently displace specific binding of [^{3}H]BTX-B (Deecher *et al.*, 1991; Salgado, 1992). Payne *et al.* (1998) further examined the interaction between the pyrazoline RH-3421 and the local anesthetic dibucaine in BTX binding studies. Each of these compounds decreased the potency of the other as an inhibitor of BTX binding approximately as much as expected from the assumption that they share a common binding site. Furthermore, RH-3421 increased the dissociation rate of [^{3}H]BTX-B from its binding site, also expected from an action at site 10 (Deecher *et al.*, 1991).

Lapied *et al.* (2001) examined the interactions of DCJW with the local anesthetic lidocaine and the guanidinium blocker tetrodotoxin, which is known to block the pore from the external face, at a binding

site distinct from the local anesthetics (see **Chapter 5.1**). The IC_{50} for DCJW was not affected by the presence of an IC_{50} concentration of tetrodotoxin (TTX) in the external solution, consistent with independent action of the two compounds at distinct binding sites. In the presence of an IC_{50} concentration of lidocaine, however, the IC_{50} for DCJW was increased about 30-fold. A twofold shift in equilibrium binding would be expected from the hypothesis that both compounds act at the same site (Cheng and Prusoff, 1973), so the mechanism of the observed 30-fold shift is not fully understood. Nevertheless, available evidence is consistent with the action of the SCBIs at site 10.

Studies on permanently charged quaternary derivatives of local anesthetics were crucial in localizing site 10 within the Na^+ channel pore. Being permanently charged, these molecules cannot cross membranes and are therefore not clinically useful. However, when applied within the cell, they are able to enter the channel from the internal mouth, only when the channel is open, giving rise to a phenomenon known as use dependence – increase in the number of blocked channels with stimulation. Because the permanently charged quaternary molecule cannot fit through the ion-selective constriction in the channel known as the selectivity filter, it remains trapped within the channel after activation gate closure. Alternatively, more lipophilic compounds acting at site 10, when caught within the closed channel, can diffuse out laterally through lipophilic pathways in the channel wall. SCBIs behave like local anesthetics with very slow kinetics, and do not display use dependence (Zhao *et al.*, 2003), suggesting that the insecticides can only access the binding site from the lipophilic pathway and not through the internal mouth of the pore.

In addition to modulation of blocker access to the binding site by the activation gate, channel gating also modulates the equilibrium binding affinity of the channel for the molecules. Like the pyrazolines and DCMP/DCJW, there is little affinity of the blocker molecule for the resting state, but strong binding to the open and inactivated states. The bound molecule is thought to lie in a hydrophobic pocket that can be reached via either the aqueous or hydrophobic pathways. When bound, the molecule is thought to lie within the pore and block ion flow through it. Several excellent reviews on the interaction of blocking drugs with Na^+ channels have recently been published (Clare *et al.*, 2000; Anger *et al.*, 2001; Wang and Wang, 2003).

The fact that blockers acting at site 10 bind strongly to a number of states after channel opening but not to the resting state, indicates that the movements of the S6 segments (see Section 6.2.4.6) associated with channel activation lead to formation of the receptor for these compounds. The subsequent fast and slow inactivation steps are thought to involve processes at the internal membrane face that involve blocking of the pore by a so-called inactivation particle, presumably without further significant changes in the conformation of site 10 (see **Chapter 5.1**).

6.2.4.6. The Molecular Nature of Site 10 in Insects

The primary structure of the Na^+ channel α subunit consists of four homologous repeat domains (D1 to D4), each with six transmembrane segments S1 to S6. The six transmembrane segments of each domain are thought to be arranged into a quasicylindrical pseudosubunit, and all four of these pseudosubunit domains are arranged in pseudotetrametric fashion, forming the Na^+ channel pore in the space between them (see **Chapter 5.1**).

The existence of several mutations in the S6 segments from domains 1, 3, and 4 that affect potency of both tertiary and quaternary local anesthetics, the latter of which have access to the receptor only through aqueous pathways, indicates that the S6 segments line the pore of the Na channel (**Figures 11 and 12**). The voltage sensor for activation gating has been localized, also using site-directed mutagenesis, to the positively charged S4 segments, which are thought to exhibit voltage-dependent outward and/or rotary movements to open the activation gate, which is thought to be located at the cytoplasmic ends of the S6 segments. Although the voltage sensors are located on the S4 segments, their movement appears to be coupled to critical movements of the S6 segments, leading to opening of the activation gate at the cytoplasmic S6 ends, and modulation of three receptor sites known to be associated with the midsections of the S6 segments: the blocker site (site 10), the pyrethroid site (site 7), and the BTX site (site 2) (**Figure 13**). Several amino acid residues clustered around the middles of the S6 segments which, when mutated, affect binding of compounds to these sites, have been identified, and those associated with the binding of Na^+ channel blocking drugs have been the subject of much recent research because they potentially identify the faces of the S6 segments that face the pore (Yarov-Yarovoy *et al.*, 2002). These mutations are colored red in the sequence alignments in **Figure 11**. **Figure 12** shows a helical wheel representation of the four S6 segments arranged according to Kondratiev and Tomaselli (2003), who have only considered the residues

```
D1S6
0394 hmlffiviiflgsfylvnlilaivamsydelqk Musca domestica
0393 hmlffiviiflgsfylvnlilaivamsydelqk Blattella germanica
0405 hmlffiviiflgsfylvnlilaivamsydelqr Drosophila melanogaster
0401 hvlffvviiflgsfylvnlilaivamsydelqk Heliothis virescens
0403 hmlffiviiflgsfylvnlilaivamsydelqk Pediculus humanus humanus
0436 hmcffvviiflgsfylvnlilaivamsyddlqk Varroa destructor

D2S6
1001 cipfflatvvignlvvlnlflalllsnfgsssl Musca domestica
0980 cipfflatvvignlvvlnlflalllsnfgssnl Blattella germanica
1016 cipfflatvvignlvvlnlflalllsnfgsssl Drosophila melanogaster
0790 cipfflatvvignlvvlnlflalllsnfgsssl Heliothis virescens
1024 cipfflatvvignlvvlnlflalllsnfgsssl Pediculus humanus humanus
1078 cipfflatvvignlvvlnlflalllssfgasnl Varroa destructor

D3S6
1524 mylyfvffiifgsfftlnlfigviidnfneqkk Musca domestica
1505 mylyfvffiifgsfftlnlfigviidnfneqkk Blattella germanica
1536 mylyfvffiifgsfftlnlfigviidnfneqkk Drosophila melanogaster
1308 mylyfvffiifgsfftlnlfigviidnfneqkk Heliothis virescens
1546 mylyfvffiifgsfftlnlfigviidnfneqkk Pediculus humanus humanus
1698 mylyfvffiifgafftlnlfigviidnfneqkk Varroa destructor
xxxx mylyfvffiifgsfftlnlfigviidnfneqkk Boophilus microplus

D4S6
1824 gitfllsylvisflivinmyiavilenysqat Musca domestica
1805 giafllsylvisflivinmyiavilenysqat Blattella germanica
1836 gitfllsylvisflivinmyiavilenysqat Drosophila melanogaster
1846 gitfllsylvisflivinmyiavilenysqat Pediculus humanus humanus
1608 gityllsylvisflivinmyiavilenysqat Heliothis virescens
2000 avaylvsyliisflviinmyiavilenysqat Varroa destructor
xxxx avaylvsyliisflviinmyiavilenysqat Boophilus microplus
```

Figure 11 Sequence alignment of the pore-forming S6 transmembrane segments from various arthropod pest species, to show the location of residues known to be important for binding of local anesthetics (red), batrachotoxin (underscored), and pyrethroids (blue). Residues conforming with the consensus sequence are shown with a gray background.

known to affect blocker affinity, without alterations in the voltage dependence or kinetics of gating that would otherwise account for an increase in the IC_{50} for block. The S6 segments can be arranged so that all such residues, shown in red, face into the pore. A total of 10 residues affecting BTX action have also been identified scattered among the middle of all four S6 segments (Wang and Wang, 2003), but it is not yet clear if these are all associated with the binding site.

Residues in the S6 segments of domains 1, 2, and 3 that are known to affect pyrethroid sensitivity (see **Chapter 5.1**) are shown in blue in **Figures 11 and 12**. These are on the sides thought to face away from the pore. Mutations conferring pyrethroid resistance have also been identified in the S5 segments, indicating that the pyrethroid receptors may be located at the interfaces between S5 and S6 segments. The number of pyrethroid receptors per channel is not certain, but the locations of the resistance mutations in the S6 segments suggests that there could be as many as four receptors. Interestingly, quantitative studies with tetramethrin isomers on squid axon Na^+ channels indicated that there may be at least three pyrethroid receptors in each channel (Lund and Narahashi, 1982).

The extremely high conservation of the S6 segments among the arthropods compared in **Figure 11** is striking. The residues associated with the binding sites are all absolutely conserved. However, the P loops in the extracellular S5–S6 connecting segments dip deep into the external mouth of the barrel formed by the S6 segments to form the selectivity filter and the outer boundary of the inner chamber containing site 10 (Cronin *et al.*, 2003). The contributions of the P loops to the binding of blockers in site 10 have not yet been explored. **Figure 13** shows a diagram summarizing current knowledge of the locations of the S4, S5, and S6 segments and the three receptors associated with the S6 segments. TTX is also shown, binding to site 1 at the external mouth of the channel.

6.2.4.7. Biochemical Measurements of the Effects of SCBIs

While electrophysiology and in particular voltage-clamp measurements are essential for studying the detailed interactions of drugs with ion channels, certain receptor binding techniques are also useful, especially when quantitative potency data on many compounds is needed. Displacement of [^{3}H]BTX-B

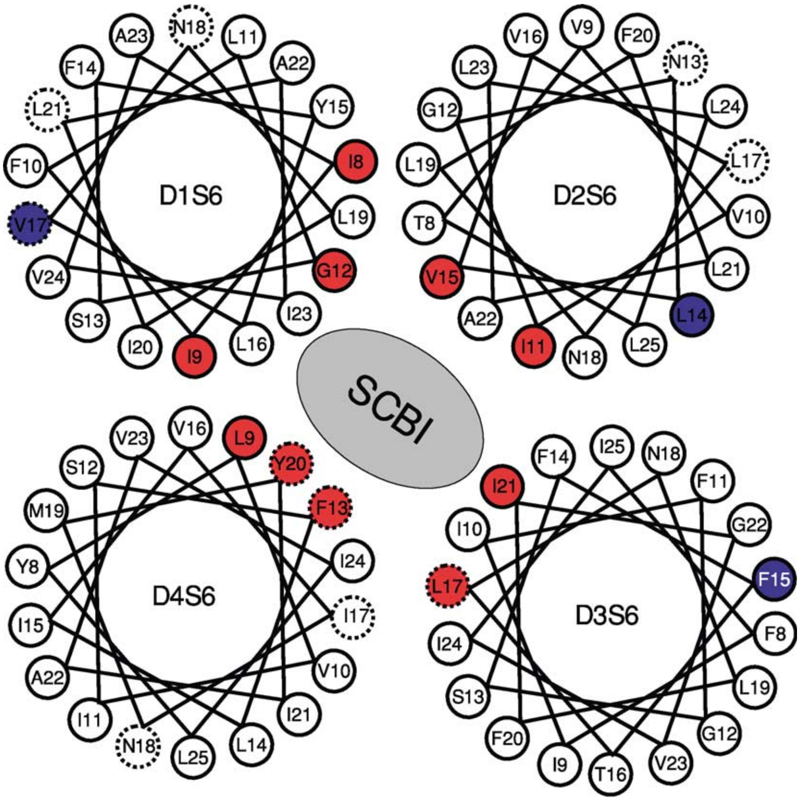


Figure 12 Helical wheel representation of residues 8–25 of the four S6 transmembrane segments arranged so that the residues known to be important for blocker binding (red) face the pore. Pyrethroid-binding residues are blue and BTX-binding residues are shown as broken circles. (Adapted from Kondratiev, A., Tomaselli, G.F., **2003**. Altered gating and local anesthetic block mediated by residues in the I-S6 and II-S6 transmembrane segments of voltage-dependent Na^+ channels. *Mol. Pharmacol. 64*, 741–752; and Wang, S.Y., Wang, G.K., **2003**. Voltage-gated Na^+ channels as primary targets of diverse lipid-soluble neurotoxins. *Cell Signal. 15*, 151–159.)

has been used effectively to quantify the potency of pyrazolines on Na^+ channels. The quantitative correspondence with electrophysiology and biological activity is good (Deecher *et al*., 1991; Salgado, 1992), and this is the method of choice for structure–activity relationship studies.

Block of the Na^+ flux through channels activated by the activator veratridine has also been used to measure effects of dihydropyrazoles. In this case, Na^+ flux can be assessed directly as flux of $^{22}Na^+$ into membrane vesicles (Deecher and Soderlund, 1991), or indirectly from the resulting depolarization as measured by voltage-sensitive dyes (Nicholson, 1992) or depolarization-triggered release of preloaded radiolabeled neurotransmitter (Nicholson and Merletti, 1990). With flux assays, however, veratridine is often used at a high concentration in order to achieve a strong signal, but since this activator acts at the BTX site, it inhibits the action of the pyrazolines (Deecher and Soderlund, 1991) and thereby interferes with accurate potency measurements.

6.2.4.8. Intrinsic Activity of Indoxacarb on Na^+ Channels

The initial mode of action studies with indoxacarb indicated that it was a proinsecticide, being metabolized to DCJW, which potently blocked the compound action potential in *M. sexta* nerve in a voltage-dependent manner (Wing *et al*., 1998). Indoxacarb itself also had this effect, but was much slower acting; while 1 μM DCJW blocked the compound action potential by 50% after 10 min, 40 min were required for indoxacarb to exert this same effect, and in that preparation one cannot be certain that the compound is not metabolized to DCJW within the ganglion. However, studies on patch-clamped neurons, which cannot metabolize the compound, confirmed that indoxacarb indeed has intrinsic activity on Na^+ channels. The effects of the two compounds were comparable, but while indoxacarb block was reversible with about 5 min washing or with hyperpolarization, block by DCJW was not reversible with either washing or hyperpolarization (Tsurubuchi and Kono, 2003; Zhao *et al*., 2003).

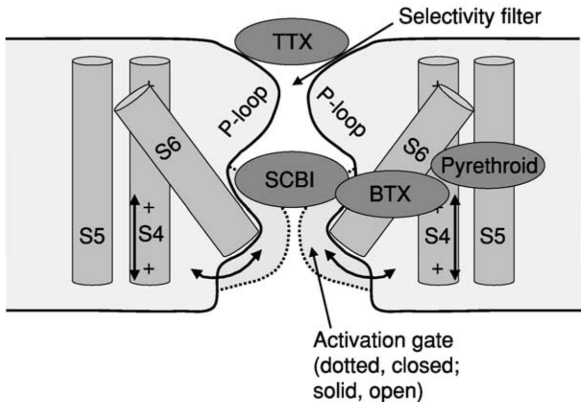


Figure 13 Diagrammatic representation of the location of the binding sites associated with the S6 segments of the Na^+ channel. The extracellular mouth of the channel is at the top of the figure. Two sets of the S4, S5, and S6 transmembrane helices are shown. Bidirectional arrows show the movements of the S4 and S6 segments associated with activation gating. The thick solid curves show the outline of the channel protein in the activated or open conformation, and the thick dotted line shows the activation gate at the cytoplasmic end of the S6 segments in the closed position. There is one tetrodotoxin (TTX) and one Na^+ channel blocker insecticide (SCBI) binding site per channel, but the number of batrachotoxin (BTX) and pyrethroid sites is not certain, although only one of each is shown. All three drug sites are in the middle of the S6 segments, and the pyrethroid site also includes residues in segment 5.

Table 2 Action of DCJW and indoxacarb on the abdominal stretch receptor organ of *Spodoptera frugiperda*

	Average percent block (five preparations, in exposure)	
Concentration[a] (M)	*DCJW*	*Indoxacarb*
3e-9	0	0
1e-8	40	0
3e-8	100	25
1e-7	100	60
3e-7	100	100

[a] In a saline containing, in mM, 28 NaCl, 16 KCl, 9 $CaCl_2$, 1.5 NaH_2PO_4, 1.5 mgCl_2, and 175 sucrose, pH 6.5, the stretch receptor fired continuously for several hours between 25 and 75 Hz.

The actions of indoxacarb and DCJW were also compared quantitatively on the abdominal stretch receptor organs of *S. frugiperda* (Table 2). In this preparation, the insecticides in solution are perfused rapidly over the body wall containing the stretch receptor, a small, thin organ directly exposed to the saline. Significant metabolism in this preparation is unlikely, but cannot be excluded. The organ fires continually at a rate of between 25 and 75 Hz for many hours, and is very sensitive to block by SCBIs. DCJW blocked activity consistently within 20–40 min at 3×10^{-8} M, but in five tests at 10^{-8} M the average percent block within 1 h was 40%. Indoxacarb was also intrinsically active, but 3- to 10-fold weaker than DCJW, blocking consistently at 3×10^{-7} M, but only 60% within 1 h at 1×10^{-7} M and 25% at 3×10^{-8} M.

In conclusion, it appears that indoxacarb is weaker (about three to ten times) than DCJW on Na^+ channels, and that the block is readily reversible. Furthermore, the much slower effect of indoxacarb on the compound action potentials in *M. sexta* ganglia in comparison with DCJW indicates that indoxacarb enters the ganglion much more slowly than does DCJW. Based on the appearance of insect symptoms concurrent with the appearance of high levels of metabolically formed DCJW in larval Lepidoptera (Wing *et al.*, 1998, 2000) and the greater potency and irreversibility of DCJW Na^+ channel block, it appears that the toxicologically significant compound after indoxacarb or DPX-JW062 administration is DCMP or DCJW, respectively.

6.2.4.9. Effects of SCBIs on Alternative Target Sites

Other possible target sites for SCBIs have been considered. The voltage-gated Ca^{2+} channel α subunits belong to the same protein superfamily as the voltage-gated Na^+ channels, and have a similar structure, composed of 24 transmembrane segments arranged into four domains of six segments each. Not unexpectedly, Ca^{2+} channels were also found to be blocked by pyrazolines, although at higher concentrations than Na^+ channels (Zhang and Nicholson, 1993; Zhang *et al.*, 1996). So far, there is no evidence that Ca^{2+} channel effects are significant in insect poisoning. In the initial work on the pyrazolines, it was observed in paralyzed cockroaches and *M. sexta* larvae that the heart continued to beat normally, and conduction across the cholinergic cercal–giant fiber synapses as well as γ-aminobutyric acid (GABA) and glutamate synapses on muscle, all Ca^{2+} channel-dependent processes, functioned normally (Salgado, 1990). Furthermore, DCJW had no effect on low-voltage activated and high-voltage activated calcium currents in isolated cockroach neurons (Lapied *et al.*, 2001).

A strong hyperpolarization of *M. sexta* muscle, on the order of 20 mV, was observed after poisoning by pyrazolines (Salgado, 1990). The effect was not reversible with prolonged washing and could not be reproduced by treatment with pyrazoline in solution, and therefore appears to be secondary and not related to the hyperpolarization of DUM neurons observed by Lapied *et al.* (2001). Muscles of *Periplaneta* or *Musca* were not hyperpolarized during pyrazoline poisoning (Salgado, 1990).

Table 3 Potency of indoxacarb to pest insects in the laboratory: larval ingestion and contact vs. adult contact

	Insecticidal potency of indoxacarb (LC_{50} values, ppm)		
		Direct spray contact	
Insects	*Ingestion*	*Larvae*	*Adults*[a]
Heliothis virescens	1.47	12.96	56
Helicoverpa zea	0.68	2.55	
Spodoptera frugiperda	0.61	5.25	
Spodoptera exigua	1.96	7.66	
Trichoplusia ni	0.28		45
Plutella xylostella	3.69		30
Empoasca fabae	1.11		
Cydia pomonella	7.68		
Lygus lineolaris	1.46		

[a] Toxicity measured with methylated seed oil (1%) or vegetable oil plus organosilicone surfactant (0.5%) at 7 days posttreatment.

6.2.5. Biological Potency of Indoxacarb

6.2.5.1. Spectrum and Potency of Indoxacarb in the Laboratory

Laboratory assays show that lepidopteran, hemipteran, and homopteran pests are inherently very sensitive to indoxacarb, and are significantly more affected when indoxacarb is ingested (Wing *et al.*, 2000). When treated plant leaves were fed to a variety of lepidopterous larvae, this resulted in 50% lethal concentration (LC_{50}) values ranging from 0.3 to 7.7 ppm when evaluated 3 days posttreatment (**Table 3**). These values are similar to LC_{50} values published by other researchers (Seal and McCord, 1996; Pluschkell *et al.*, 1998; Liu and Sparks, 1999; Liu *et al.*, 2002; Giraddi *et al.*, 2002; Smirle *et al.*, 2002; Ahmad *et al.*, 2003; Liu *et al.*, 2003), and indicates the high toxicity of this compound to Lepidoptera. Indoxacarb halts insect feeding within 4 h after ingestion. Insect mortality generally takes place within 2–4 days, and it is critical to evaluate both mortality after 72 h and feeding damage to assess the compound's true effectiveness. *Trichoplusia*, heliothines, and *Spodoptera* are the most sensitive among the lepidopterans while *Plutella* and *Cydia* are somewhat less sensitive. Variation in activity among species may be due to the rate of bioconversion of indoxacarb to the active metabolite DCJW or to the amount of product consumed. Insecticidal activity of indoxacarb against the sucking insects *Empoasca fabae* and *L. lineolaris* in the laboratory are similar to that of the lepidoptera (**Table 3**). Lepidopteran adults were also effected when they ingested a sugar water solution with indoxacarb.

The ingestion activity of indoxacarb to lepidopteran larvae is about three to nine times greater than its dermal contact activity (**Table 3**). For some insects however, such as *Lobesia botrana*, the potency of the two modes of entry is almost equivalent (Anonymous, 2000). Indoxacarb shows negligible activity when insects walk on a dried residue, and has no activity as a fumigant.

Indoxacarb was also significantly less active as a direct contact spray to moths compared to larvae; however, it is still effective. Directly spraying lepidopteran moths with indoxacarb plus methylated seed oil at 1% or a vegetable oil plus organosilicone surfactant at 0.5% resulted in LC_{50} values ranging from 30 to 56 ppm for lepidopteran adults at 7 days post treatment (**Table 3**). The ability of indoxacarb to provide moth control or suppression in the field will contribute to overall pest suppression.

Indoxacarb has inherent toxicity to select coleopteran (Parimi *et al.*, 2003), hemipteran (*Lygus*), homopteran (*Myzus*, *Empoasca, Nephotettix, Nilaparvata*), and dipteran (Chen *et al.*, 2003) pests after feeding on treated leaves or artificial diets (Wing *et al.*, 2000) (**Table 3**). Indoxacarb has low water solubility; however, leaf penetration and translaminar activity are responsible for controlling certain sucking pests and is enhanced by inclusion of oil-based formulations or tank-mixed adjuvants. Indeed, the compound is toxic in the field to plantbugs, fleahoppers, and leafhoppers; however, it is ineffective against species such as aphids because of poor systemicity and low phloem oral bioavailability. Overall, indoxacarb's utility as a field insecticide active against sucking insects is constrained by its very limited systemicity, compared to many neonicotinoids.

Indoxacarb exhibits ovilarvicidal activity on Lepidoptera such as *L. botrana*, *Eupoecilia ambiguella, Heliothis armigera*, and *Cydia pomonella* (Anonymous, 2000). Laboratory data indicates

that the probable mechanism of ovilarvicidal activity is due to adsorption of indoxacarb into the chorion and subsequent oral uptake as the neonate chews through the chorion to hatch. Indoxacarb directly sprayed onto the egg is more effective than placed in a dried residual deposit on which the eggs are laid.

6.2.5.2. Safety of Indoxacarb to Beneficial Insects

Beneficial insects walking over a dried residue of indoxacarb on a leaf are generally unaffected, as shown by Mead-Briggs *et al.* (1996) with the hoverfly *Episyrphus balteatus* and predatory mite *Typhlodromus pyri*. Indoxacarb is safe to the parasitoids *Eurytoma pini, Haltichella rhyacioniae, Bracon* sp., and *Macrocentrus ancylivorus* (Nowak *et al.*, 2001), *Cotesia marginiventris* and *Trichogramma pretiosum* (Ruberson and Tillman, 1999) and for the predator *G. punctipes* (Tillman *et al.*, 1998). Williams *et al.* (2003), Musser and Shelton (2003), Michaud and Grant (2003), Tillman *et al.* (2001), and Baur *et al.* (2003) also reported the safety of indoxacarb to beneficial insects. The insecticidal selectivity of indoxacarb against herbivorous pests is explained by Andaloro *et al.* (2000). There is little movement of product through the tarsi of insects thus rendering carnivorous predators and parasitic wasps largely insensitive to indoxacarb. However, any insect behavior that culminates in ingestion of the product, such as preening (ants), probing (nabids), or lapping exudates from the leaf (apple maggot flies) will impact insect survival. Indoxacarb controls fire ants, *Solenopsis* spp., on crops (Turnipseed and Sullivan, 2000) as a result of ants preening their antennae and legs, and subsequent ingestion of preened materials. Indoxacarb is formulated on a 5–7 μm silica particle and may readily adhere to the body of the insect through antennal drumming behavior and general locomotory activity.

6.2.5.3. Sublethal Effects of Indoxacarb

Liu *et al.* (2002) observed numerous effects of indoxacarb on the development of cabbage looper (*Trichoplusia ni*) larvae feeding on field-aged leaf residues 18–20 days old as well as on subsequent pupae and emerging adults. These effects were also documented on heliothine and *Spodoptera* species fed on diet with indoxacarb at 0.1 and 0.01 ppm (Andaloro, unpublished data). Larval development was greatly extended compared to the untreated check; survivors pupated 1–2 weeks later. Treated larvae were much smaller than untreated larvae. Some larvae could not molt or pupate properly, with exuvia remaining attached and sometimes severely restricting the body. Treated lepidopteran larvae and Colorado potato beetle (*Leptinotarsa decemlineata*) larvae sometimes are unable to dig into the soil and thus pupate on top of the soil. In the laboratory, lepidopteran adults emerging from treated larvae often have abnormal wings or are unable to fly. *Liriomyza* adults whose larvae were exposed to leaves treated with sublethal doses of indoxacarb emerged disoriented, stumbling, and unable to fly (Leibee, unpublished data). There are sufficient data indicating indoxacarb has significant growth regulating effects on the larvae, pupae, and adults of pest insect species observed which contribute to overall suppression of the field population.

6.2.5.4. Spectrum and Insecticidal Potency of Indoxacarb in the Field

Indoxacarb is active against a wide spectrum of insect pests from at least ten orders and well over 30 families (**Table 4**). Lepidopteran insects represent the majority of pests on the indoxacarb label but it is also used to control many species of plant bugs, leafhoppers, fleahoppers, weevils, beetles, flies, cockroaches, and ants. Ahmad *et al.* (2003), Brickle *et al.* (2001), and Andaloro *et al.* (2001) documented the activity of indoxacarb against lepidopteran pests on cotton, while Hammes *et al.* (1999), Tillman *et al.* (2001), and Teague *et al.* (2000) showed indoxacarb activity against hemipteran pests of cotton. The rates of indoxacarb range from approximately 40 to 125 g ha^{-1} active ingredient to control agricultural pests. These rate ranges are very consistent regardless of environmental conditions that can often cause degradation of performance of other registered products. Indoxacarb formulations are extremely stable to ultraviolet radiation, pH, and temperature extremes, and have a positive temperature correlation. Indoxacarb provides excellent rainfastness relative to most labeled products (Flexner *et al.*, 2000) providing growers with additional flexibility and control at labeled rates despite rainfall within hours after application.

Leaf penetration by indoxacarb not only results in activity against leafhoppers and plant bugs, but also pinworm, such as the tomato pinworm (*Keiferia lycopersicella*) and the tomato leafminer (*Tuta absoluta*), and provides suppression of lepidopteran leafminers such as the spotted tentiform leafminer (*Phyllonorycter blancardella*) and the citrus leafminer (*Phyllocnistis citrella*). Indoxacarb is also active against some dipteran leafminers such as *Liriomyza trifolii* on tomato and other vegetable crops.

The rate range for nonagricultural pests, such as structural, nuisance, or household pests ranges from 0.01% to 1.0% v/v concentration. Indoxacarb has shown excellent activity when prepared in a bait

Table 4 Spectrum of insects pests controlled by indoxacarb and range of indoxacarb rates for control[a]

Global field use rate range[b]	*Insect orders*	*Selected insect families*	*Selected representative genera*
40–125 g ha^{-1}	Lepidoptera	Arctiidae	*Estigmene*
		Crambidae	*Ostrinia, Diaphania, Hellula, Desmia, Herpetogramma, Loxostege, Pyrausta, Maruca, Neoleucinodes*
		Gelechiidae	*Phthorimaea, Keiferia, Anarsia, Tuta*
		Geometridae	*Operophtera*
		Gracillariidae	*Phyllonorycter, Phyllocnistis*
		Hesperiidae	*Lerodea*
		Lasiocampidae	*Malacosoma*
		Lymantriidae	*Lymantra*
		Noctuidae	*Heliothus/Helicoverpa, Spodoptera, Trichoplusia, Agrotis, Autographa, Alabama, Lithophane, Pseudoplusia, Mamestra, Chrysodeixis, Orthosia, Earias, Plusia, Diparopsis, Mythimma, Sylepta, Thaumetopea*
		Plutellidae	*Plutella*
		Pieridae	*Pieris, Colias*
		Pyralidae	*Hyphantria, Hellula, Maruca, Ostrinia*
		Sphingidae	*Manduca*
		Tortricidae	*Capua, Lobesia, Endopiza, Cydia, Grapholita, Epiphyas, Cnephasia, Argyrotaenia, Platynota, Eupoecillia, Sparganothis, Archips, Pandemis, Sasemia, Adoxophyes, Diparopsis*
		Zygaenidae	*Harrisinia*
40–125 g ha^{-1}	Coleoptera	Anobiidae	*Lasioderma*
		Chrysomellidae	*Diabrotica, Leptinotarsa, Chaetocnema*
		Curculionidae	*Hypera, Cryptophlabes, Anthonomus, Conotrachelus, Sitophilus, Phyllobius, Curculio, Diaprepes, Peritelus*
		Tenebrionidae	*Rhizopertha*
0.05–1%	Hymenoptera	Formicidae	*Linepithemia, Camponotus, Solenopsis, Monomorium, Lasius, Atta*
		Diprionidae	*Hoplocampa*
75–125 g ha^{-1}	Hemiptera	Miridae	*Lygus, Pseudatomoscelis, Creontiades, Tydia*
40–125 g ha^{-1}	Homoptera	Cicadellidae	*Empoasca, Typhlocyba, Erythroneura, Scaphoideus*
50–100 g ha^{-1}	Orthoptera	Acrididae	*Schistocerca, Locusta*
		Gryllotalpidae	*Scapteriscus*
0.1–1%	Blattaria	Blattellidae	*Blattella, Supella*
		Blattidae	*Periplaneta, Blatta*
50–125 g ha^{-1}	Diptera	Agromyzidae	*Liriomyza*
0.01–0.1%		Culicidae	*Anopheles*
0.01–0.1%		Muscidae	*Musca*
75–125 g ha^{-1}		Tephritidae	*Rhagoletis*
0.09–0.5%	Isoptera	Rhinotermitidae	*Reticulitermes*
0.1–1%	Thysanura	Lepismatidae	*Lepisma*

[a] The majority of these insects are on indoxacarb labels or development work is ongoing to include them on labels, or sufficient data exists indicating the activity of indoxacarb. The list of insect families and genera is not inclusive of the total insect control activity of indoxacarb.
[b] Indoxacarb rates are expressed in grams of active ingredient per hectare or percent of active ingredient published on global labels or used in laboratory and field experiments to achieve a high level of insect mortality.

against German cockroaches (*Blattella germanica*) (Appel, 2003) and fire ant species (Barr, 2003; Patterson, 2003). However, the suspension concentrate formulation is also effective against certain roach, ant, fly, silverfish, and termite species when the pest is sprayed directly or as a surface or soil residual spray.

6.2.5.5. Indoxacarb and Insecticide Resistance

Indoxacarb is highly active against insect pest species that are already resistant to other insecticide classes. This has been demonstrated in the housefly (*Musca domestica*) resistant to spinosad (Shono and Scott, 2003) and organophosphates (Sugiyama *et al.*, 2001); tobacco budworm (*Heliothis virescens*) resistant to spinosad (Young *et al.*, 2001); German cockroach resistant to pyrethroids (Appel, 2003; Dong, unpublished data); tortricid leafrollers resistant to organophosphates (Olszak and Pluciennik, 1998); cotton bollworm (*H. armigera*) resistant to cyclodienes, organophosphates, carbamates and pyrethroids (Ahmad *et al.*, 2003); diamondback moth (*Plutella xylostella*) resistant to several conventional chemistries and spinosad (Boyd, 2001);

beet armyworm (*Spodoptera exigua*) resistant to pyrethroids, organophosphates, and benzoylurea insect growth regulators (Eng, 1999); and *H. virescens* resistant to multiple conventional chemistries (Holloway *et al*., 1999).

It is likely that the insects mentioned above are resistant to conventional insecticides due to a variety of mechanisms, including increased metabolism, decreased penetration and target site insensitivity. Because indoxacarb is bioactivated via esterase/amidase enzymes, overproduction of esterases in insects resistant to organophosphates or pyrethroids could lead to faster liberation of the active toxin DCMP than in nonresistant insects. This suggests that resistant insects may in fact develop a negative cross-resistance to indoxacarb, as has been observed in laboratory strains of *H. armigera* (Gunning and Devonshire, 2003). However, it is important to note that we have no clear evidence thus far for negative cross-resistance to indoxacarb in the field, indicating the speed and ease with which susceptible Lepidoptera can bioactivate indoxacarb. The aggregate resistance data also indicate that insects with increased resistance to conventional chemistries also have highly sensitive Na^+ channel DCMP binding sites, as would be expected since there has been no previous exposure to commercial SCBIs in the field. Thus, indoxacarb is an excellent rotation partner for alternating modes of action in insect resistance management programs.

As has been mentioned, certain field strains of *C. rosaceana*, which have developed resistance to many conventional insecticides, are also poorly sensitive to indoxacarb (Ahmad *et al*., 2002). This may be due to an enhanced ability to detoxify indoxacarb; however, this insect is not on the indoxacarb US label and is an extremely unusual example of such poor susceptibility in Lepidoptera.

Insect pests representing eight species (*H. virescens, H. armigera, S. exigua, S. eridania, S. littoralis, P. xylostella,* and *T. absoluta*) in over 20 countries have been targeted for a sustained susceptibility monitoring program by DuPont Agricultural Products (Andaloro, unpublished data). These populations are evaluated by a feeding assay throughout each season in locations that have a history of insecticide resistance, and where a significant number of indoxacarb applications are made, so that early signs of resistance can be detected before widespread field failures occur. This proactive approach to indoxacarb resistance management is beneficial not only for preservation of this product in key markets, but also to maintain its usefulness as a rotation partner, thus promoting the longevity of other products with different modes of action, and thus ultimately benefiting the agricultural field in general.

6.2.6. Conclusions

In summary, indoxacarb is the first insecticide product that acts by blocking the voltage-dependent Na^+ channel at site 10. The mode of action of this novel SCBI class was determined by sequential observation of poisoned insect symptoms, electrophysiology (starting with intact neuronal preparations and progressing towards more targeted ion channel techniques), and confirmation with Na^+ flux and biochemical radioligand binding techniques. It is believed that this approach is the most direct and elegant to determine the mode of action of insect neurotoxins, as opposed to testing the toxin in broad panels of specific target site biochemical assays and trusting that positive responses are indicative of toxicologically significant modes of action. Thus far no qualitative difference has been observed in the mode of action of the subclasses of the SCBIs. Indoxacarb itself is able to bind to Na^+ channels, but the main toxicologically active principle is the insect bioactivation metabolite DCMP. SCBIs appear to bind to a novel site, which appears to correspond to site 10 within the channel pore, and have almost no effect on resting Na^+ channels, so that in axons that normally have a very negative resting potential, they exert no effect unless the cell is artificially depolarized. Alternatively, sensory neurons and certain pacemaking neurons in the CNS generate action potentials in a region of the cell with a relatively low resting potential, where currents flowing through the cell membrane depolarize it, giving rise to trains of action potentials at a frequency that is dependent on membrane potential. Because the Na^+ channels in these regions are continually shuttling through the inactivated state, and are even, because of the low resting potential, spending a significant amount of time in the inactivated state, they are highly susceptible to compounds such as the SCBIs, which bind to the inactivated state. Inactivation is voltage-dependent, and at any given potential there is a dynamic equilibrium between inactivated and resting channels. As inactivated channels become bound to the active compound, they are removed from this equilibrium, with the result that more channels become inactivated to take their place in the equilibrium. In a sense, SCBIs pull channels into the inactivated state. While local anesthetics can access their binding site via an aqueous pathway from the internal solution as well as via a lipid pathway, SCBIs may have access only via the lipid pathway.

At a physiological level, the very slow off-rate of the receptor-bound SCBI (as evidenced by extremely slow washout in electrophysiology) may be largely responsible for the irreversible feeding inhibition seen in poisoned insects. Since this occurs at very low doses of indoxacarb in lepidopteran larvae, it is likely that in pseudoparalyzed insects only a relatively small proportion of total Na^+ channels is poisoned. Thus once DCJW is pseudoirreversibly bound to the slow inactivated state, return of the Na^+ channel to the unpoisoned inactivated state is very slow, which helps explain the high potency of indoxacarb/DCMP in halting insect feeding.

Indoxacarb was designed as a proinsecticide to confer favorable environmental degradation and mammalian safety, while maintaining high levels of insecticidal activity. Proinsecticide design is a common strategy to enhance insecticide selectivity (Prestwich, 1990). The enzymatic cleavage in pest insects via *N*-decarbomethoxylation is a novel insecticide bioactivation. The divergent indoxacarb metabolic pathways (insects primarily to *N*-decarbomethoxylated sodium channel blockers, while mammals degrade the compound primarily via other routes leading to multiple inactive metabolites) ensures a high degree of nontarget organism safety, a critical feature for modern insecticides.

Also, since hydrolytic esterases are largely responsible for the bioactivation, it is likely that insects that have become resistant to pyrethroids and organophosphates via induction of detoxifying esterases may in fact liberate DCMP more rapidly than susceptible insects. Because of this feature, and DCMP's novel Na^+ channel target site, accumulated field evidence shows that indoxacarb is generally highly active against insects resistant to these other commercial insecticide classes, and is thus an excellent insect pest management tool. This includes the pyrethroids and DDT, which bind to a different site on the voltage-gated Na^+ channel.

These properties have contributed to indoxacarb's strong potency in the field against Lepidoptera and some other pest orders. The bioavailability characteristics of indoxacarb make it ideal for foliar applications, where a surface residue of a strong chewing insect compound is desired. Its limited leaf penetration allows control of some Homoptera, some Hemiptera and both lepidopteran and dipteran leafminers, and its broad spectrum also confers activity against many nonagricultural pests such as termites, cockroaches, and certain ants. However, as with any new insecticide class, proactive resistance management is key to ensuring the maintenance of this compound and its target site of action as effective pest management tools.

References

Ahmad, M., Hollingworth, R.M., Wise, J.C., **2002**. Broad-spectrum insecticide resistance in obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) from Michigan. *Pest Mgt Sci. 58*, 834–838.

Ahmad, M., Arif, M.I., Ahmad, Z., **2003**. Susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to new chemistries in Pakistan. *Crop Protect. 22*, 539–544.

Andaloro, J.T., Wing, K.D., Green, J.H., Lang, E.B., **2000**. Steward® dispersion and cotton leaf interactions: impact on cotton insect pests and safety to beneficial arthropods. *Proc. Beltwide Cotton Conf. 2*, 939–940.

Andaloro, J.T., Edmund, R.M., Castner, E.P., Williams, C.S., Sherrod, D.W., **2001**. Steward® SC field performance against heliothines: speed of action, symptmology, and behavior of treated larvae. *Proc. Beltwide Cotton Conf. 2*, 925–926.

Anger, T., Madge, D.J., Mulla, M., Riddall, D., **2001**. Medicinal chemistry of neuronal voltage-gated Na^+ channel blockers. *J. Med. Chem. 44*, 115–137.

Anonymous, **2000**. Indoxacarb Technical Bulletin for Europe, Middle East and Africa. DuPont de Nemours and Co., Nambsheim, France.

Appel, A.G., **2003**. Laboratory and field performance of an indoxacarb bait against German cockroaches (Dictyoptera: Blattellidae). *J. Econ. Entomol. 96*, 863–870.

Barr, C.L., **2003**. Fire ant mound and foraging suppression by indoxacarb bait. In: Proc. Red Imported Fire Ant Conf., Palm Springs, CA, pp. 50–51.

Baur, M.E., Ellis, J., Hutchinson, K., Boethel, D.J., **2003**. Contact toxicity of selective insecticides for non-target predaceous hemipterans in soybeans. *J. Entomol. Sci. 38*, 269–277.

Boyd, V.L., **2001**. A preemptive strike: adopt sound resistance management plan to reduce chances of problems like those in Hawaii. *The Grower 34*(4), 40.

Brickle, D.S., Turnipseed, S.G., Sullivan, M.J., **2001**. Efficacy of insecticides of different chemistries against *Helicoverpa zea* (Lepidoptera: Noctuidae) in transgenic *Bacillus thuringiensis* and conventional cotton. *J. Econ. Entomol. 94*, 86–92.

Catterall, W.A., **1987**. Common modes of drug action on Na^+ channels: local anesthetics, antiarrhythmics and anticonvulsants. *Trends Pharmacol. Sci. 8*, 57–65.

Chen, H., McPheron, B., Krawczyk, G., Hull, L., **2003**. Laboratory evaluation of surface residue toxicity of insecticides to apple maggot flies. *Int. Pest Control March/April 2003 45*, 92–93.

Cheng, Y.-C., Prusoff, W.H., **1973**. Relation between the inhibition constant (K_i) and the concentration of inhibitor which causes fifty percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol. 22*, 3099–3108.

Clare, J.J., Tate, S.N., Nobbs, M., Romanos, M.A., **2000**. Voltage-gated Na^+ channels as therapeutic targets. *Drug Discov. Today 5*, 506–520.

Cronin, N.B., Reilly, A., Duclohier, H., Wallace, B.A., **2003**. Binding of the anticonvulsant drug lamotrigine and the neurotoxin batrachotoxin to voltage-gated sodium channels induces conformational changes associated with block and steady-state activation. *J. Biol. Chem. 278*, 10675–10682.

Deecher, D.C., Soderlund, D.M., **1991**. RH 3421, an insecticidal dihydropyrazole, inhibits Na^+ channel-dependent Na^+ uptake in mouse brain preparations. *Pestic. Biochem. Physiol. 39*, 130–137.

Deecher, D.C., Payne, G.T., Soderlund, D.M., **1991**. Inhibition of [^{3}H]batrachotoxinin A 20-alpha-benzoate binding to mouse brain Na^+ channels by the dihydropyrazole insecticide RH 3421. *Pestic. Biochem. Physiol. 41*, 265–273.

Eng, O., **1999**. Indoxacarb: a potent compound for controlling the beet armyworm (*Spodoptera exigua*) on fruit vegetables in Malaysia. *Proc. 3rd Conf. Entomol. Soc. Malaysia* 101.

EPA, **2000**. Fact Sheet on Indoxacarb. Issued Oct. 30, 2000. Environmental Protection Agency, Washington, DC.

Finlayson, L.H., Lowenstein, O., **1958**. The structure and function of abdominal stretch receptors in insects. *Proc. Roy. Soc. London B 148*, 433–449.

Flexner, L., Green, J., Wing, K., Cameron, R., Saienii, J., *et al.*, **2000**. Steward® 150 SC rainfastness and residual control on cotton. *Proc. Beltwide Cotton Conf. 2*, 940.

Giraddi, R.S., Dasareddy, S.V., Lingappa, S.L., **2002**. Bioefficacy of new molecules of insecticides against gram pod-borer (*Helicoverpa armigera*) in pigeonpea (*Cajanus cajan*). *Indian J. Agric. Sci.* 72, 311–312.

Gunning, R.V., Devonshire, A.L., **2003**. Negative cross-resistance between indoxacarb and pyrethroids in the cotton bollworm, *Helicoverpa armigera*, in Australia: a tool for resistance management. *Proc. Brighton Crop Protect. Conf. 2*, 788.

Hammes, P., Sherrod, D.W., Hammes, G.G., **1999**. Cotton pest and beneficial insect management strategies with Steward insect control product in the Southeast US. *Proc. Beltwide Cotton Conf. 2*, 1224–1225.

Harder, H.H., Riley, S.L., McCann, S.F., Irving, S.N., **1996**. DPX-MP062: a novel broad-spectrum, environmentally soft insect control compound. *Proc. Brighton Crop Protects Conf. 1*, 48–50.

Hasan, R., Nishimura, K., Okada, M., Adamatsu, M., Inoue, M., *et al.*, **1996**. Stereochemical basis for the insecticidal activity of carbamoylated and acylated pyrazolines. *Pestic. Sci. 46*, 105–112.

Hodgkin, A.L., Huxley, A.F., **1952**. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. 117*, 500–544.

Holloway, J.W., Forrester, N.W., Leonard, B.R., **1999**. New insecticide chemistry for cotton IPM. *Proc. Beltwide Cotton Conf. 2*, 1086.

Ishaaya, I., **2001**. Biochemical processes related to insecticide action: an overview. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 1–16.

Jacobson, R.M., **1985**. N-aryl 3-aryl-4,5-dihydro-1H-pyrazole-2-pyrazolines. US Patent 5 798 311.

Jacobson, R.M., **1989**. A class of insecticidal dihydropyrazoles. In: Crombie, L.E. (Ed.), Recent Advances in the Chemistry of Insect Control. Royal Society of Chemistry, Cambridge, pp. 206–211.

Kondratiev, A., Tomaselli, G.F., **2003**. Altered gating and local anesthetic block mediated by residues in the I-S6 and II-S6 transmembrane segments of voltage-dependent Na^+ channels. *Mol. Pharmacol. 64*, 741–752.

Lapied, B., Malecot, C.O., Pelhate, M., **1989**. Ionic species involved in the electrical activity of single adult aminergic neurones isolated from the sixth abdominal ganglion of the cockroach *Periplaneta americana*. *J. Exp. Biol. 144*, 535–549.

Lapied, B., Stankiewicz, M., Grolleau, F., Rochat, H., Zlotkin, E., *et al.*, **1999**. Biophysical properties of scorpion alpha-toxin-sensitive background Na^+ channel contributing to the pacemaker activity in insect neurosecretory cells (DUM neurons). *Eur. J. Neurosci. 11*, 1449–1460.

Lapied, B., Grolleau, F., Sattelle, D.B., **2001**. Indoxacarb, an oxadiazine insecticide, blocks insect neuronal Na^+ channels. *Br. J. Pharmacol. 132*, 587–595.

Liu, T.-X., Sparks, A.N., **1999**. Efficacies of selected insecticides on cabbage looper and diamondback moth on cabbage in south Texas. *Subtrop. Plant Sci. 51*, 56–60.

Liu, T.-X., Sparks, A.N., Chen, W., Liang, G.-M., Brister, C., **2002**. Toxicity, persistence and efficacy of Indoxacarb on cabbage looper (Lepidoptera: Noctuidae). *J. Econ. Entomol. 95*, 360–367.

Liu, T.-X., Sparks, A.N., Chen, W., **2003**. Toxicity, persistence and efficacy of indoxacarb and two other insecticides on *Plutella xylostella* (Lepidoptera: Plutellidae) immatures in cabbage. *Int. J. Pest Mgt 49*, 235–241.

Lund, A.E., Narahashi, T., **1982**. Dose-dependent interaction of the pyrethroid isomer with sodium channels of squid axon membranes. *Neurotoxicology 3*, 11–24.

McCann, S.F., Annis, G.D., Shapiro, R., Piotrowski, D.W., Lahm, G.P., *et al.*, **2001**. The discovery of indoxacorb: oxadiazineses a new class of pyrazoline – type insecticides. *Pest Mgt Sci. 57*, 153–164.

McCann, S.F., Annis, G.D., Shapiro, R., Piotrowski, D.W., Lahm, G.P., *et al.*, **2002**. Synthesis and biological activity of oxadiazine and triazine insecticides: the discovery of Indoxacarb. In: Baker, D.R., Fenyes, J.G., Lahm, G.P., Selby, T.S., Stevenson, T.M. (Eds.), Synthesis and Chemistry of Agrochemicals, vol. 6. American Chemical Society, Washington, DC, pp. 166–177.

Mead-Briggs, M., Bakker, F.M., Grove, A.J., Primiani, M.M., **1996**. Evaluating the effects of multiple-application plant protection products on beneficial arthropods by means of extended laboratory tests: case study with predatory mites and hoverflies, and the insecticides

JW-062 and DPX-MP062. *Proc. Brighton Crop Protect. Conf. 1*, 307–313.
Michaud, J.P., Grant, A.K., **2003**. IPM-compatability of foliar insecticides for citrus: indices derived from toxicity to beneficial insects from four orders. *J. Insect Sci. 3*, 18–27.
Mulder, R., Wellinga, K., **1973**. Insecticidal 1-(phenylcarbamoyl)-2-pyrazolines. German Patent DE 2 304 584.
Musser, F.R., Shelton, A.M., **2003**. *Bt* sweet corn and selective insecticides: impacts on pests and predators. *J. Econ. Entomol. 96*, 71–80.
Nicholson, R.A., **1992**. Insecticidal dihydropyrazoles antagonize the depolarizing action of veratridine in mammalian synaptosomes as measured with a voltage-sensitive dye. *Pestic. Biochem. Physiol. 42*, 197–202.
Nicholson, R.A., Merletti, E.L., **1990**. The effect of dihydropyrazoles on release of [^{3}H]GABA from nerve terminals isolated from mammalian cerebral cortex. *Pestic. Biochem. Physiol. 37*, 30–40.
Nowak, J.T., McGravy, K.W., Fettig, C.J., Berisford, C.W., **2001**. Susceptibility of adult hymenopteran parasitoids of the Nantucket Pine Tip Moth (Lepidoptera: Tortricidae) to broad-spectrum and biorational insecticides in a laboratory study. *J. Econ. Entomol. 94*, 1122–1129.
Olszak, R.W., Pluciennik, Z., **1998**. Preliminary investigations on effectiveness of two modern insecticides in controlling codling moth, plum fruit moth and leafrollers. *Proc. Brighton Conf. Crop Protect. 2*, 57.
Parimi, S., Meinke, L.J., Nowatzki, T.M., Chandler, L.D., French, B.W., *et al.*, **2003**. Toxicity of insecticide–bait mixtures of insecticide resistant and susceptible western corn rootworms (Coleoptera: Chrysomelidae). *Crop Protect. 22*, 781–786.
Patterson, R.S., **2003**. Spring and fall applications of granular indoxacarb baits for fire ant control in Florida. In: Proc. Red Imported Fire Ant Conf., Palm Springs, CA, pp. 51–52.
Payne, G.T., Deecher, D.C., Soderlund, D.M., **1998**. Structure–activity relationships for the action of dihydropyrazole insecticides on mouse brain sodium channels. *Pestic. Biochem. Physiol. 60*, 177–185.
Pichon, Y., Ashcroft, F.M., **1985**. Nerve and muscle: electrical activity. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 5. Pergamon, Oxford, pp. 85–113.
Pluschkell, U., Horowitz, A.R., Weintraub, P.G., Ishaaya, I., **1998**. DPX-MP062: a potent compound for controlling the Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.). *Pestic. Sci. 54*, 85–90.
Postma, S.W., Catterall, W.A., **1984**. Inhibition of binding of [^{3}H]batrachotoxin A 20-a-benzoate to Na^+ channels by local anesthetics. *Mol. Pharmacol. 25*, 219–227.
Prestwich, G.D., **1990**. Proinsecticides: metabolically activated toxicants. In: Hodgson, E., Kuhr, R.J. (Eds.), Safer Insecticides – Development and Use. Marcel, New York, pp. 281–335.
Ruberson, J.R., Tillman, P.G., **1999**. Effect of selected insecticides on natural enemies in cotton: laboratory studies. *Proc. Beltwide Cotton Conf. 2*, 1210–1213.
Salgado, V.L., **1990**. Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. *Pestic. Sci. 28*, 389–411.
Salgado, V.L., **1992**. Slow voltage-dependent block of Na^+ channels in crayfish nerve by dihydropyrazole insecticides. *Mol. Pharmacol. 41*, 120–126.
Salgado, V.L., **1998**. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pestic. Biochem. Physiol. 60*, 91–102.
Schouest, L.P., Salgado, V.L., Miller, T.A., **1986**. Synaptic vesicles are depleted from motor nerve terminals of deltamethrin-treated house fly larvae, *Musca domestica. Pestic. Biochem. Physiol. 25*, 381–386.
Scott, M.T., **2000**. Comparative degradation and metabolism of indoxacarb, an oxadiazine insecticide. *Am. Chem. Soc. Mtg*, San Francisco, CA, Abstracts.
Seal, D.M., McCord, E., **1996**. Management of diamondback moths, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), in cole crops. *Nucl. Sci. Applic. 7*, 49–55.
Shapiro, R., Annis, G.D., Blaisdell, C.T., Dumas, D.J., Fuchs, J., *et al.*, **2002**. Toward the manufacture of indoxacarb. In: Baker, D.R., Fenyes, J.G., Lahm, G.P., Selby, T.P., Stevenson, T.M. (Eds.), Synthesis and Chemistry of Agrochemicals, vol. 6. American Chemical Society, Washington, DC, pp. 178–185.
Shono, T., Scott, J.G., **2003**. Spinosad resistance in the housefly, *Musca domestica*, is due to a recessive factor on autosome 1. *Pestic. Biochem. Physiol. 75*, 1–7.
Smirle, M.J., Lowery, D.T., Zurowski, C.L., **2002**. Resistance and cross-resistance to four insecticides in populations of oblique-banded leafroller (Lepidoptera: Tortricidae). *J. Econ. Entomol. 95*, 820–825.
Sugiyama, S., Tsurubuchi, Y., Karasawa, A., Nagata, K., Kono, Y., *et al.*, **2001**. Insecticidal activity and cuticular penetration of Indoxacarb and its *N*-decarbomethoxylated metabolite in organophosphorus insecticide-resistant and susceptible strains of the housefly *Musca domestica* (L.). *J. Pestic. Sci. 26*, 117–120.
Teague, T.G., Tugwell, N.P., Muthiah, S., Hornbeck, J.M., **2000**. New insecticides for control of tarnished plant bug: results from field and cage studies and laboratory bioassays. *Proc. Beltwide Cotton Conf. 2*, 1214–1217.
Tillman, P.G., Hammes, G.G., Sacher, M., Connair, M., Brady, E.A., *et al.*, **2001**. Toxicity of a formulation of the insecticide indoxacarb to the tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae), and the big-eyed bug, *Geochoris punctipes* (Hemiptera: Lygaeidae). *Pest Mgt Sci. 58*, 92–100.
Tillman, P.G., Mulrooney, J.E., Mitchell, W., **1998**. Susceptibility of selected beneficial insects to DPX-MP062. *Proc. Beltwide Cotton Conf. 2*, 1112–1114.
Turnipseed, S.G., Sullivan, M.J., **2000**. Activity of Steward® against plant bugs, bollworms, and predacious arthropods in cotton in South Carolina. *Proc. Beltwide Cotton Conf. 2*, 953–954.

Tsurubuchi, Y., Karasawa, A., Nagata, A., Shono, T., Kono, Y., **2001**. Insecticidal activity of oxadiazine insecticide indoxacarb and its *N*-decarbomethoxylated metabolite and their modulations of voltage-gated Na^+ channels. *Appl. Entomol. Zool. 36*, 381–385.

Tsurubuchi, Y., Kono, Y., **2003**. Modulation of sodium channels by the oxadiazine insecticide indoxacarb and its *N*-decarbomethoxylated metabolite in rat dorsal root ganglion neurons. *Pest Mgt Sci. 59*, 999–1006.

Wang, S.Y., Wang, G.K., **2003**. Voltage-gated Na^+ channels as primary targets of diverse lipid-soluble neurotoxins. *Cell Signal. 15*, 151–159.

Wasserstrom, J.A., Liberty, K., Kelly, J., Santucci, P., Myers, M., **1993**. Modification of cardiac Na^+ channels by batrachotoxin: effects on gating, kinetics, and local anesthetic binding. *Biophys. J. 65*, 386–395.

Wiersma, C.A.G., Furshpan, E., Florey, E., **1953**. Physiological and pharmacological observations on muscle organs of the crayfish, *Cambarus clarkii* Girard. *J. Exp. Biol 30*, 136–151.

Williams, L. III, Price, L.D., Manrique, V., **2003**. Toxicity of field-weathered insecticide residues to *Anaphes iole* (Hymenoptera: Mymaridae), an egg parasitoid of *Lygus lineolaris* (Heteroptera: Miridae), and implications for inundative biological control in cotton. *Biol. Control 26*, 217–223.

Wing, K.D., Schnee, M.E., Sacher, M., Connair, M., **1998**. A novel oxadiazine insecticide is bioactivated in lepidopteran larvae. *Arch. Insect Biochem. Physiol. 37*, 91–103.

Wing, KD, Sacher M., Kagaya, Y., Tsurubuchi, Y., Mulderig, L., *et al.*, **2000**. Bioactivation and mode of action of the oxadiazine indoxacarb in insects. *Crop Protect. 19*, 537–545.

Yarov-Yarovoy, V., McPhee, J.C., Idsvoog, D., Pate, C., Scheuer, T., *et al.*, **2002**. Role of amino acid residues in transmembrane segments IS6 and IIS6 of the Na^+ channel subunit in voltage-dependent gating and drug block. *J. Biol. Chem. 277*, 35393–35401.

Young, H.P., Bailey, W.D., Roe, R.M., **2001**. Mechanism of resistance and cross-resistance in a laboratory, Spinosad-selected strain of the tobacco budworm and resistance in laboratory-selected cotton bollworms. *Proc. Beltwide Cotton Conf. 2*, 1167–1170.

Zamponi, G.W., Doyle, D.D., French, R.J., **1993**. Fast lidocaine block of cardiac and skeletal muscle Na^+ channels: one site with two routes of access. *Biophys. J. 65*, 80–90.

Zhang, A., Nicholson, R.A., **1993**. The dihydropyrazole RH-5529 blocks voltage-sensitive calcium channels in mammalian synaptosomes. *Pestic. Biochem. Physiol. 45*, 242–247.

Zhang, A., Towner, P., Nicholson, R.A., **1996**. Dihydropyrazole insecticides: interference with depolarization-dependent phosphorylation of synapsin I and evoked release of L-glutamate in nerve-terminal preparations from mammalian brain. *Pestic. Biochem. Physiol. 54*, 24–30.

Zhao, X., Ikeda, T., Yeh, J.Z., Narahashi, T., **2003**. Voltage-dependent block of sodium channels in mammalian neurons by the oxadiazine insecticide indoxacarb and its metabolite DCJW. *Neurotoxicol. 24*, 83–96.

Zlotkin, E., **2001**. Insecticides affecting voltage-gated ion channels. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 43–76.

6.3 Insect Growth- and Development-Disrupting Insecticides

T S Dhadialla, Dow AgroSciences LLC,
Indianapolis, IN, USA
A Retnakaran, Canadian Forest Service,
Sault Ste. Marie, ON, Canada
G Smagghe, Ghent University, Ghent, Belgium

6.3.1. Introduction

In 1967 Carrol Williams proposed that the term "third generation pesticide" be applied to the potential use of the insect juvenile hormone (JH) as an insecticide, and suggested that it would not only be environmentally benign but that the pest insects would also be unable to develop resistance. However, it took several years before the first commercial juvenile hormone analog (JHA) made its debut (reviews: Retnakaran *et al*., 1985; Staal, 1975). Since then, several compounds that adversely interfere with the growth and development of insects have been synthesized, and have been collectively referred to as "insect growth regulators (IGRs)" (review: Staal, 1982). Concerns over eco-toxicology and mammalian safety have resulted in a paradigm shift from the development of neurotoxic, broad-spectrum insecticides towards softer, more environmentally friendly pest control agents such as IGRs. This search has led to the discovery of chemicals that: (1) interfere with physiological and biochemical systems that are unique to either insects in particular or arthropods in general; (2) have insect-specific toxicity based on either molecular target site or vulnerability to a developmental stage; and (3) are safe to the environment and nontarget species. At

the physiological level, opportunities to develop insecticides with such characteristics existed, *inter alia*, in the endocrine regulation of growth, development, reproduction, and metamorphosis. The rationale was that if the pest insect is treated with a chemical analog, which mimics the action of hormones like JHs and ecdysteroids, at an inappropriate stage the hormonal imbalance would force the insect to go through abnormal development leading to mortality. It was also thought that because such chemicals would in many instances work via the receptor(s) of these hormones, it was less likely for the affected population to develop target site resistance. Yet other target sites are the biosynthetic steps of cuticle formation, which insects share with other arthropods. Adversely interfering with this process would result in the inability of the intoxicated insect to molt and undergo further development. Unlike neurotoxic insecticides that are fast acting, IGRs are in general slow acting, which might result in more damage to the crop. However, some IGRs, such as ecdysone agonists, induce feeding inhibiton, significantly reducing the damage to below acceptable levels. The slow mode of action of some IGRs, such as chitin synthesis inhibitors, can be an advantage in controlling social insects, such as termites, where the material has to be carried to the brood and spread to other members.

It was in the early seventies that the first chitin synthesis inhibitor, a benzoylphenylurea, was discovered by scientists at Philips-Duphar BV, (now Crompton Corp., Weesp, The Netherlands), and marketed as Dimilin® by the Uniroyal Chemical Company (Crompton Corp. Middlebury, CT) in the USA. Since then several new analogs that interfere with one or more steps of cuticle synthesis have been synthesized and are being marketed for controlling various pests. These products are reviewed in Section 6.3.4, with emphasis on recent developments.

After the initial success with the synthesis of methoprene by Zoecon (Palo Alto, CA, USA) very few new JHAs with good control potential other than pyriproxyfen and fenoxycarb have been developed. These compounds have been particularly useful in targeting the eggs and embryonic development as well as larvae. The synthesis of pyriproxifen and fenoxycarb was a departure from the terpenoid structure of JHs and earlier JHAs, and is reviewed in Section 6.3.3. For extensive reviews on earlier JHAs the reader is referred to Retnakaran *et al.* (1985) and Staal *et al.* (1975).

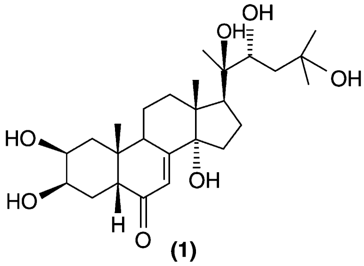
Early attempts in the 1970s to synthesize insecticides with molting hormone (20-hydroxyecdysone) activity failed because they were based on a cholesterol backbone, which resulted in chemical and metabolic instability of the steroid nucleus (Watkinson and Clarke, 1973). It took nearly two more decades before the first nonsteroidal ecdysone agonist, based on the bisacylhydrazine class of compounds, was synthesized (Hsu, 1991). Structure activity optimization of the first such compound over the subsequent few years led to the synthesis of four highly effective compounds that have since been commercialized (see Section 6.3.2.1). The reader is also referred to earlier reviews on the ecdysone agonist insecticides (Oberlander *et al.*, 1995; Dhadialla *et al.*, 1998).

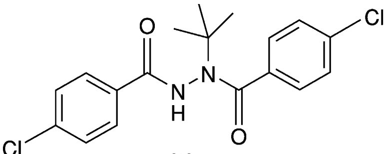
For more basic information, the reader is referred to the chapters in this series on insect hormones (ecdysteroids, JHs see **Chapter 3.4**) and cuticle synthesis (see **Chapter 4.2**) for further details on hormone physiology and chitin synthesis to augment the brief overviews presented in this chapter.

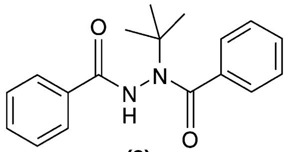
6.3.1.1. Physiological Role and Mode of Action of the Insect Molting Hormone

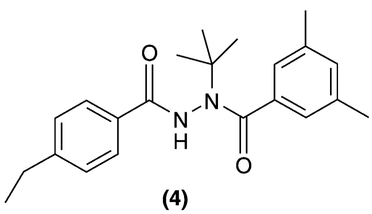
Amongst the animal kingdom, arthropods display a remarkable adaptability and diversity in inhabiting very different ecological niches. Between larval and adult stages of a given insect, an insect undergoes distinct developmental and morphological changes that help it to survive in different environments. For example, mosquito larvae are perfectly suited to surviving in an aqueous environment, whereas the adult mosquitos inhabit terrestrial and aerial environments. Larval or nymphal stages of most of the agricultural pests develop on host plant species, and the adult assumes an aerial space to look for food and a mate, returning to the host plant only to oviposit and start another life cycle.

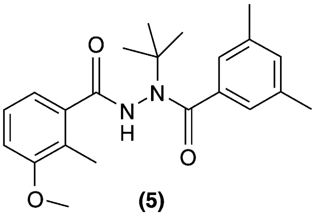
The growth and development from one stage to another is regulated by two main hormones, the steroidal insect molting hormone, 20-hydroxyecdysone (20E; **Figure 1, 1**) and the sesquiterpenoid JH, of which there are five types (**Figure 15**). Even as an insect embryo grows, it undergoes embryonic molts, where each molt is regulated by 20E. The molting process continues through the larval and pupal stages culminating in the adult stage. While molting to accommodate growth is regulated by 20E, the development from an egg to a larva to a pupa to an adult is regulated by the timing and titers of JH (**Figure 17**; also reviewed extensively by Riddiford, 1996). In the adult stage, both these hormones, being pleiotropic, change their roles to regulating reproductive processes (Wyatt and Davey, 1996).

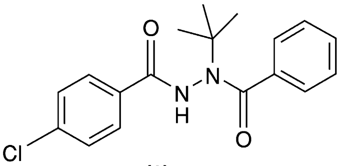

(1)


(2)


(3)


(4)


(5)


(6)

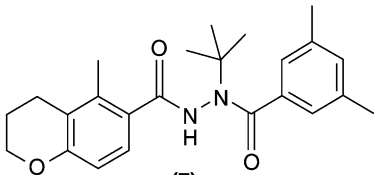

(7)

Figure 1 Chemical structures of 20-hydroxyecdysone (**1**), the first discovered symmetrically substituted dichloro-dibenzoylhydrazine (**2**), RH-5849 (**3**), tebufenozide (**4**), methoxyfenozide (**5**), halofenozide (**6**), and chromofenozide (**7**).

The molting process is initiated by an increase in the titer of 20E, and is completed following its decline and the release of eclosion hormone. In preparation for a molt and as the 20E titers increase, the larva stops feeding and apolysis of the epidermis from the old cuticle takes place leaving an ecdysial space that is filled with molting fluid containing inactive chitinolytic enzymes. During this time, the epidermal cells also reorganize in order that large quantities of protein can be synthesized for deposition of a new cuticle. This happens with up- and downregulation of a number of genes encoding a number of epidermal proteins and enzymes (Riddiford, 1994). The fall in the 20E titer triggers the activation of enzymes in the molting fluid for digestion of the procuticle underlying the old cuticle. This event is followed by the resorption of the molting fluid and preparation for ecdysis (Retnakaran *et al.*, 1997; Locke, 1998). Finally, when the 20E titer is cleared from the system, the eclosion hormone is released, which in turn results in the release of the ecdysis-triggering hormone, and all these events lead to the ecdysis of the larva leaving behind the remnants of the old cuticle (Truman *et al.*, 1983; Zitnanova *et al.*, 2001). With the completion of ecdysis, feeding resumes and endocuticular deposition continues during the intermolt period.

6.3.1.1.1. Molecular basis of 20E action Of the two hormones 20E and JH, the molecular basis of 20E action is much better understood. The pioneering work of Clever and Karlson (1960) and Ashburner (1973) on the action of 20E to induce/repress "puffs" in the polytene chromosomes of *Chironomus* and *Drosophila* led Ashburner and co-workers to propose a model for ecdysteroid action (Ashburner *et al.*, 1974). According to this model, 20E binds to an ecdysone receptor to differentially regulate several classes of "early" and "late" genes. While the "early" genes are activated by the 20E–receptor complex, the "late" genes are repressed. The protein products of the "early" genes such as E75 and CHR3 derepress the expression of the "late" genes such as DOPA decarboxylase

(DDC) and at the same time repress their own expression.

6.3.1.1.2. Ecdysone receptors The ecdysone receptor complex is a heterodimer of two proteins, ecdysone receptor (EcR) and ultraspiracle (USP), which is a homolog of the mammalian retinoic acid receptor (RXR) (Yao *et al*., 1992, 1995; Thomas *et al*., 1993). In several insects, both EcR and USP exist in several transcriptional and splice variants, presumably for use in a stage- and tissue-specific way (review: Riddiford *et al*., 2001). Both EcR and USP are members of the steroid receptor superfamily that have characteristic DNA and ligand binding domains. Ecdysteroids have been shown to bind to EcR only when EcR and USP exist as heterodimers (Yao *et al*., 1993), although additional transcriptional factors are required for ecdysteroid dependent gene regulation (Arbeitman and Hogness, 2000; Tran *et al*., 2000). Moreover, EcR can heterodimerize with RXR to form a functional ecdysteroid receptor complex in transfected cells (Yao *et al*., 1992; Tran *et al*., 2000). cDNAs encoding both EcR and USPs from a number of dipteran (Koelle *et al*., 1991; Imhof *et al*., 1993; Cho *et al*., 1995; Kapitskaya *et al*., 1996; Hannan and Hill, 1997, 2001; Veras *et al*., 1999), lepidopteran (Kothapalli *et al*., 1995; Swevers *et al*., 1995), coleopteran (Mouillet *et al*., 1997; Dhadialla and Tzertzinis, 1997), homopteran (Zhang *et al*., 2003; Dhadialla *et al*., unpublished data; Ronald Hill, personal communication), and orthopteran (Saleh *et al*., 1998; Hayward *et al*., 1999, 2003) insects, tick (Guo *et al*., 1997) and crab (Chung *et al*., 1998) have been cloned. Some of the EcRs and USPs have been characterized in ligand binding (Kothapalli *et al*., 1995; Kapitskaya *et al*., 1996; Dhadialla *et al*., 1998) and cell transfection assays (Kumar *et al*., 2002; Toya *et al*., 2002). In all cases, the DNA binding domains (DBDs) of EcRs show a very high degree of homology and identity. However, homology between the ligand binding domains (LBDs) of EcRs varies from 70% to 90%, although all EcRs studied so far bind 20E and other active ecdysteroids. The DBDs of USPs are also highly conserved. The USP LBDs, however, show very interesting evolutionary dichotomy: the LBDs from the locust, *Locusta migratoria*, the mealworm beetle, *Tenebrio molitor*, the hard tick, *Amblyoma americanum*, and the fiddler crab, *Uca puglitor*, show about 70% identity with their vertebrate homolog, but the same sequences from dipteran and lepidopteran USPs show only about 45% identity with those from other arthropods and vertebrates (Guo *et al*., 1997; Hayward *et al*., 1999; Riddiford *et al*., 2001). The functional significance of RXR-like LBDs in USPs of primitive arthropods is not well understood, because EcRs from the same insects still bind ecdysteroids (Guo *et al*., 1997; Chung *et al*., 1998; Hayward *et al*., 2003; Dhadialla, unpublished data for *Tenebrio molitor* EcR and USP (TmEcR/TmUSP)).

The crystal structures of USPs from both *Heliothis virescens* and *Drosophila melanogaster* have been elucidated by two groups (Billas *et al*., 2001; Clayton *et al*., 2001). The crystal structure of USP is similar to its mammalian homolog RXR, except that USP structures show a long helix-1 to helix-3 loop and an insert between helices 5 and 6. These variations seem to lock USP in an inactive conformation by displacing helix 12 from the agonist conformation. Both groups found that crystal structures of the two USPs had large hydrophobic cavities, which contained phospholipid ligands.

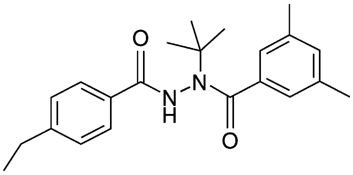
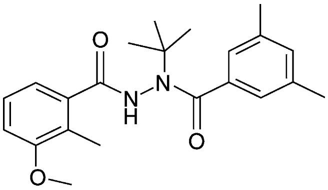
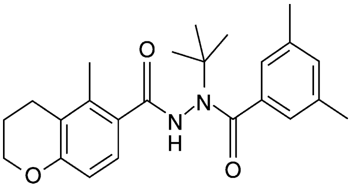
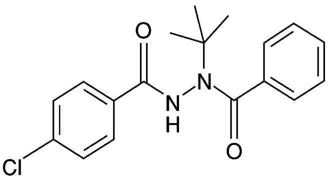
Finally, the crystal structures of *Heliothis viresens* EcR/USP (HvEcR/HuUSP) heterodimers liganded with an ecdysteroid or a nonsteroidal ecdysone agonist have been determined (Billas *et al*., 2003; see Section 6.3.2.2.2 for more details). The crystal structure of liganded EcR/USP from the silverleaf whitefly, *Bemesia tabaci*, has also been determined and awaits publication (Ronald Hill, personal communication).

6.3.2. Ecdysteroid Agonist Insecticides

6.3.2.1. Discovery of Ecdysone Agonist Insecticides and Commercial Products

Although attempts to discover insecticides with an insect molting hormone activity were made in the early 1970s (Watkinson and Clarke, 1973), it was not until a decade later that the first bisacylhydrazine ecdysone agonist ((**2**) in **Figure 1**) was serendipitously discovered by Hsu (1991) at Rohm and Haas Company, Springs House, PA, USA. Several years later, after several chemical iterations of this early lead, a simpler, unsubstituted, but slightly more potent analog, RH-5849 ((**3**) in **Figure 1**), was discovered (Aller and Ramsay, 1988). Further work on the structure and activity of RH-5849, which had commercial-level broad spectrum activity against several lepidopteran, coleopteran, and dipteran species (Wing, 1988; Wing and Aller, 1990), resulted in more potent and cost-effective bisacylhydrazines with a high degree of selective pest toxicity (review: Dhadialla *et al*., 1998). Of these, three bisacylhydrazine compounds, all substituted analogs of RH-5849, coded as RH-5992 (tebufenozide (**4**); **Figure 1**), RH-2485 (methoxyfenozide (**5**); **Figure 1**), and RH-0345 (halofenozide (**6**); **Figure 1**) have been commercialized (**Table 1**). Both tebufenozide and

Table 1 Bisacylhydrazine insecticides with ecdysone mode of action

Structure	*Common name*	*Coded as*	*Industry*	*Registered names*	*Pest spectrum*
	Tebufenozide	RH-5992	Rohm and Haas Co.,[a] Dow AgroSciences LLC[b]	MIMIC®, CONFIRM®, ROMDAN®	Lepidoptera
	Methoxyfenozide	RH-2485	Rohm and Haas Co.,[a] Dow AgroSciences LLC[b]	INTREPID®, RUNNER®, PRODIGY®, FALCON®	Lepidoptera
	Chromofenozide	ANS-118, CM-001	Nippon Kayaku, Saitame, Japan and Sankyo, Ibaraki, Japan	MATRIC®, KILLAT®	Lepidoptera
	Halofenozide	RH-0345	Rohm and Haas Co.,[a] Dow AgroSciences LLC[b]	MACH 2®	Lepidoptera, Coleoptera

[a]Discovered and commercialized by Rohm and Haas Company, Spring House, PA, USA.
[b]Now owned and commercialized by Dow AgroSciences, LLC., Indianapolis, IN, USA.

methoxyfenozide are selectively toxic to lepidopteran larvae (Hsu, 1991). However, methoxyfenozide is more potent than tebufenozide, and is toxic to a wider range of lepidopteran pests of cotton, corn, and other agronomic pests (Ishaaya *et al.*, 1995; Le *et al.*, 1996; Trisyono and Chippendale, 1997). Halofenozide has a broader and an overall insect control spectrum somewhat similar to that of RH-5849, but with significantly higher soil-systemic efficacy against scarabid beetle larvae, cutworms, and webworms (RohMid LLC, 1996). Another bisacylhydrazine, chromafenozide (7) coded as ANS-118; **Figure 1**) was discovered and developed jointly by Nippon Kayaku Co., Ltd., Saitama, Japan and Sankyo Co., Ltd. Ibaraki, Japan (Yanagi *et al.*, 2000; Toya *et al.*, 2002; **Table 1**). Chromafenozide, registered under the trade names, MATRIC® and KILLAT®, is commercialized for the control of lepidopteran larval pests of vegetables, fruits, vines, tea, rice, and ornamentals in Japan (Yanagi *et al.*, 2000; Reiji *et al.*, 2000).

6.3.2.1.1. Synthesis and structure–activity relationships (SAR) The synthesis of symmetrical and asymmetrical 1,2-dibenzoyl-1-*t*-butyl hydrazines can be achieved by the following two step reaction shown in **Figure 2**. In 1984, during the process of synthesizing compound number (**1** in **Figure 2**), which was to be used for the synthesis of another class of compounds, Hsu (Rohm and Haas Company) obtained an additional undesired product (**2** in **Figure 2**), 1,2-di (4-chlorobenzoyl)-1-*t*-butylhydrazine (A = 4-Cl). Testing of this undesired by-product revealed that it had ecdysteroid activity, and eventually led to the development of a bisacylhydrazine class of compounds. This serendipitous discovery can be attributed to the inquiring mind of Hsu, who decided to test this compound for biological activity even though it was a contaminant! By treating equivalent amounts of different benzoyl chlorides with **1** (benzoyl hydrazide), unsubstituted analogs (**3**) can be prepared as shown in **Figure 2**.

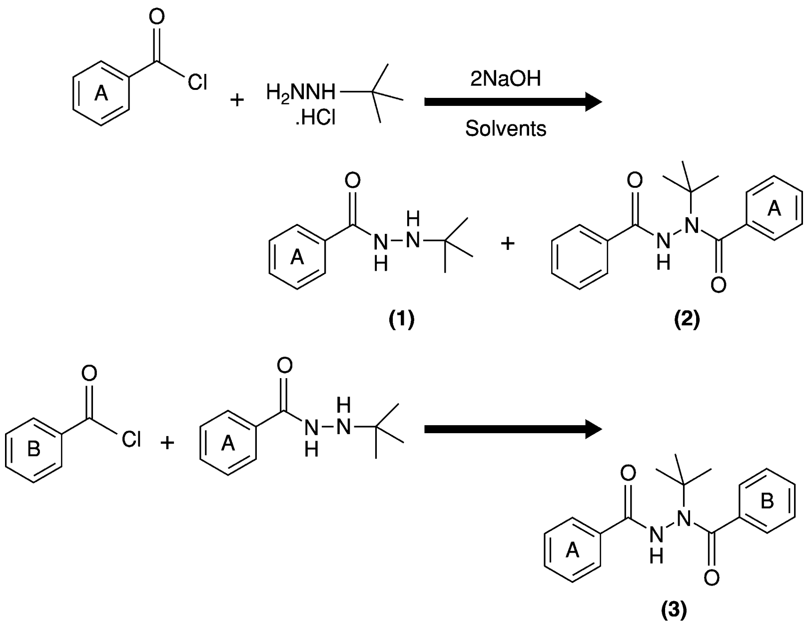
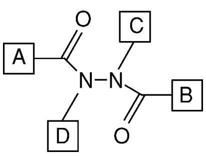


Figure 2 Synthetic routes for unsubstituted and substituted bisacylhydrazines. See text for details. (Reprinted with permission from Hsu, A.C.-T., Fujimoto, T.T., Dhadialla, T.S., **1997**. Structure-activity study and conformational analysis of RH-5992, the first commercialized nonsteroidal ecdysone agonist. In: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J., Thompson, D.G. (Eds.), Phytochemicals for Pest Control, ACS Symposium Series, vol. 658. American Chemical Society, pp. 206–219; © American Chemical Society.)

The discovery of the first ecdysone agonist (**1**) offered 18 possible sites for possible structural variation. Between 1985 and 1999, approximately 4000 structural bisacylhydrazine analogs were synthesized at the Rohm and Haas Company. This resulted in the selection of 22 candidate compounds for field testing; three of these were selected for commercial development.

Extensive SAR research was carried out around the bisacylhydrazine chemistry to discover additional more potent nonsteroidal agonists with different pest spectrum activity (Oikawa *et al*., 1994a, 1994b; Nakagawa *et al*., 1995a, 1995b, 1998, 2000, 2001a, 2001b; Hsu *et al*., 1997; Shimuzu *et al*., 1997; Smagghe *et al*., 1999b). Some general conclusions for the most active compounds can be drawn, in addition to those drawn by Hsu *et al*. (1997), as illustrated below:



A and B rings: phenyl, heterocyclic, or alkyl. The most active analogs are those that have different substituents on two phenyl rings (e.g., chromofenozide, halofenozide, tebufenozide, and methoxyfenozide). However, the phenyl ring furthest away from the *t*-butyl group (A region) can be replaced with alkyl chains without greatly affecting the biological activities (Shimuzu *et al*., 1997). Phenyl ring (A region) can have various 2, 3, 4, 5 methylene or ethylene dioxan substituents and maintain lepidopteran specific activity. Analogs derived from symmetrically substituted phenyl rings are generally less active than asymmetrically substituted compounds.

D region: In most cases, hydrogen is necessary for activity. When substituted with an unhydrolyzable group, the analogs tend not to be active.

C region: A bulky alkyl group like *t*-butyl is important for activity. The *t*-butyl group can be modified to a benzodioxan group without significantly decreasing the affinity of the compound.

Based on the X-ray crystal structures of RH-5849, a number of models have been developed that demonstrate superimposition of RH-5849 on the 20E structure (Chan *et al*., 1990; Mohammed-Ali *et al*., 1995; Cao *et al*., 2001; Kasuya *et al*., 2003). More recent X-ray crystallography studies of liganded (20E or diacylhydrazine) HvEcR/HvUSP show that the two compounds share common sites occupied by the side chain of 20E and the B ring of bisacylhydrazine (Billas *et al*., 2003).

6.3.2.2. Bisacylhydrazines as Tools of Discovery

The discovery of bisacylhydrazine has been a catalyst in stimulating research on the biological activity of ecdysteroids and nonsteroidal ecdysone agonists in whole insects, tissues, and in cells (reviews: Dhadialla *et al.*, 1998; Oberlander *et al.*, 1995). Because bisacylhydrazines have a greater metabolic stability than 20E *in vivo*, it was possible to confirm and further understand the effects of rising and declining titers of 20E during growth and development (Retnakaran *et al.*, 1995).

With the cloning and sequencing of cDNAs encoding EcRs from different insects it became clear that there were homology differences in the LBDs of the cloned EcRs but the functional significance of these differences was not apparent. In almost all insects, the effect of 20E is manifested by an interaction with EcR in the ecdysone receptor complex. Therefore, it is paradoxical that tebufenozide and methoxyfenozide, which also manifest insect toxicity via interaction with EcR, are predominantly toxic to lepidopteran insects and to a lesser degree to other insects. Through the use of these ecdysone agonists it became clear that while 20E can bind to different LBDs of EcRs from different orders of insects, the same LBDs bound bisacylhydrazines with unequal affinities, which reflected their insect specificity (reviewed by Dhadialla *et al.*, 1998). It also became clear that while a relatively low but similar affinity (K_d = 10–100 nM) of 20E to different EcRs was functional, only high-affinity (nM range) interactions of tebufenozide and methoxyfenozide with EcR were functionally productive (**Table 2**). On the other hand, the low affinity of halofenozide was most likely compensated for by its high metabolic stability in susceptible insects.

6.3.2.2.1. Molecular modeling Molecular modeling studies on both EcR LBDs (Wurtz *et al.*, 2000; Kasuya *et al.*, 2003) and bisacylhydrazines (Chan *et al.*, 1990; Mohammed-Ali *et al.*, 1995; Hsu *et al.*, 1997; Cao *et al.*, 2001) were conducted, perhaps partly prompted by the molecular modeling and X-ray crystal structure data of LBDs of steroid receptors from vertebrates and the search for new classes of nonsteroidal ecdysone agonists/antagonists. The results suggested that the tertiary protein structure of EcR LBDs is similar to that of vertebrate steroid receptor LBDs, and that helix 12 forms a "mouse trap" for a ligand in the binding cavity (**Figure 3**; see also description of crystal structures of EcR and USP below and **Chapter 3.5**). Results of molecular modeling and *in silico* docking experiments with 20E and RH-5849 have provided conflicting information about the orientation of 20E in the binding pocket. However, the recent description of the X-ray crystal structure of ligand bound HvEcR/HvUSP LBDs has provided definite answers to this (see below).

6.3.2.2.2. Mutational analysis In a very elegant study, Kumar *et al.* (2002) extended the *in silico* molecular modeling approach to construct a homology model of *Choristoneura fumiferana* EcR (CfEcR) LBD with 20E docked in to predict amino acid residues involved in interaction with 20E. The authors identified 17 amino acid residues as possibly being critical for 20E binding. In order to confirm this prediction, all 17 of these amino acids were mutated to alanine, except for A110 (where the 110 residue is numbered starting from helix one, which otherwise is A393 in the full-length CfEcR), which was mutated to proline. When the single point mutated LBDs were tested in ligand binding assays and transactivation assays (in insect and mammalian cells and in mice *in vivo*) the A110P mutant was ineffective in the two assays for a response to ecdysteroids (ponasterone A and 20E). While there was a 30% decrease in transactivation assays for the nonsteroidal bisacylhydrazines, their

Table 2 Relative binding affinities of ponasterone A and bisacylhydrazine insecticides to EcR/USPs in cellular extracts of dipteran, lepidopteran, and coleopteran cells and (biological activities)

	K_d *(nM)*		
Ligand	Drosophila *Kc cells*	*Plodia interpunctella*[a]	*Leptinotarsa decemlineata*[b]
Ponasterone A	0.7	3	3
Tebufenozide	192	3 (predominantly lepidoptera active)	218
Methoxyfenozide	124	0.5 (predominantly lepidoptera active)	ND
Halofenozide	493	129 (lepidoptera active)	2162 (coleoptera active)

[a]Imaginal wing disc cell line.
[b]Embryonic cell line.
ND, not determined.

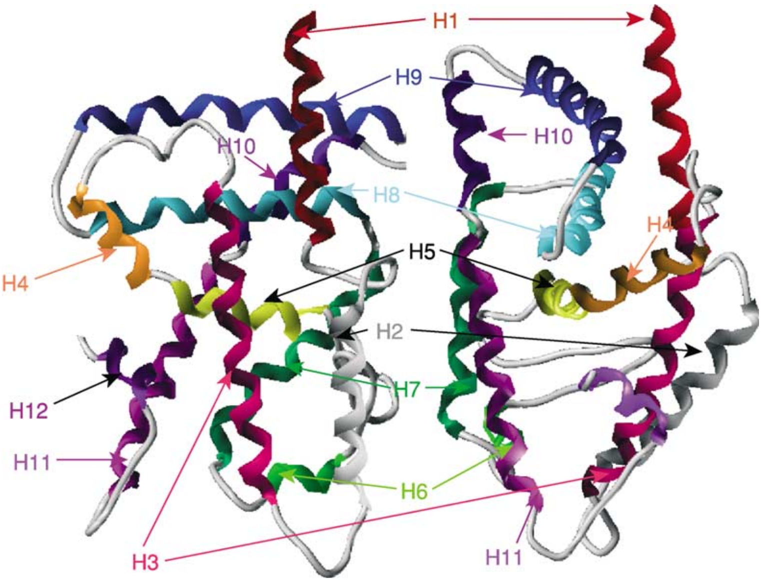


Figure 3 Different elevations of computer model(s) of the three-dimensional structure of the LBD of insect ecdysone receptors. The different helices are represented in different colors, and indicated by arrows and numbers. After a ligand has docked into the ligand binding pocket of EcR heterodimerizd with USP, helix 12 closes on the binding cavity like a ''mouse trap.''

ability to bind was unaffected. Changing A110 to leucine was as effective as the change to proline. Further work along these lines should make it possible not only to design new effective chemistries as ecdysone agonists/antagonists for pest control, but also to design EcR LBDs that result in productive binding with different chemistries, thus allowing their use in several gene switch applications.

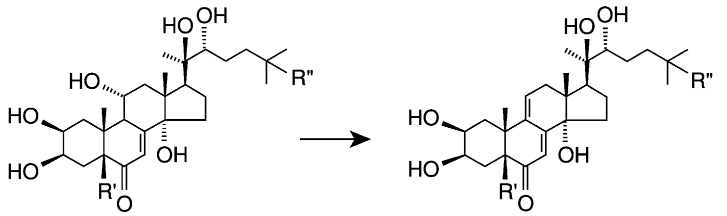
The necessity of EcR and USP forming a heterodimer for ligand interaction coupled with the difficulty in producing large enough quantities of EcR and USP LBDs required for X-ray diffraction studies have been overcome by Billas *et al.* (2003). These authors reported the crystal structures of the LBDs of the moth *H. virescens* EcR-USP heterodimer in complex with the ecdysteroid ponasterone A and with a nonsteroidal, lepidopteran specific agonist, BY106830 (a bisacylhydrazine). Comparison of the crystal structures liganded with ponasterone A and BY106830 revealed that the two ligands occupy very different but slightly overlapping spaces in the ligand binding pockets. The overlap of the two ligands was observed on the side chain of ponasterone A with the *t*-butyl group and the benzoyl ring closest to it in BY106830. The presence of the *t*-butyl group and its occupation in a hydrophobic groove of the HvEcR LB pocket confers lepidopteran specificity on it. Further examination of the residues in this hydrophobic cavity revealed that V384 in helix 5, which was conserved in lepidopteran insect EcR LBDs and is replaced by methionine in other insects, is essential for the lepidopteran specificity. The crystal structure observations supported the results of Kumar *et al.* (2002).

6.3.2.2.3. Ligand-dependent conformational changes Yao *et al.* (1995) used limited proteolysis protection to demonstrate that binding of muristerone A, a potent ecdysteroid, to *Drosophila melanogaster* EcR and USP (DmEcR/DmUSP) induces a conformation change in EcR that can be detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. In these experiments, incubation of muristerone A with DmEcR-^{35}S-methionine labeled/DmUSP proteins produced by transcription and translation of corresponding cDNAs in rabbit reticulocyte cell-free mixtures, afforded partial protection to EcR from trypsin as the protease. In an extension of this approach, Dhadialla *et al.* (unpublished data) used EcR-^{35}S-methionine labeled/USP proteins produced *in vitro* to demonstrate that muristerone A bound to DmEcR/DmUSP, *Aedes aegypti* EcR and USP (AeEcR/AeUSP), and CfEcR/CfUSP protected an EcR fragment of 36 kDa from limited proteolysis with trypsin, chymotrypsin, and proteinase K. On the other hand, the ability of tebufenozide (RH-5992) to induce a similar conformational change upon binding to EcR, which affords limited proteolytic protection as with muristerone A, correlated with its affinity to the various EcRs (**Table 3**). Interestingly, peptide fragments of a similar size (36 kDa)

Table 3 Size distribution of protease protected fragments of ^{35}S-labeled EcRs incubated in the presence of homologous USPs and an ecdysteroid, muristerone A, or a bisacylhydrazine, tebufenozide

	Protected fragment size (kDa)					
	DmEcR		*AaEcR*		*CfEcR*	
Protease	*Muristerone A*	*Tebufenozide*	*Muristerone A*	*Tebufenozide*	*Muristerone A*	*Tebufenozide*
Chymotrypsin	36		45	45	ND	ND
Trypsin	40		50	50	ND	ND
Proteinase K	36		36	36	36	36
K_d (nM)	336		28		0.5	

ND, not determined.
Cloned EcR and USP cDNAs from the three insects, *Drosophila melanogaster* (Dm), *Aedes aegypti* (Aa), and *Choristoneura fumiferana* (Cf) were expressed using *in vitro* transcription and translations of the rabbit reticulocyte system. However, EcRs were *in vitro* labeled with ^{35}S-methionine. Both EcR and USPs from the same species were incubated in the presence of 1 µm muristerone A or 1 µm tebufenozide and binding allowed to reach equilibrium. At that point the binding reactions were aliquated and subjected to increasing protease concentrations for 20 min at RT (limited proteolysis). The digests were analyzed by SDS-PAGE, and the labeled EcR bands visualized by fluorography. The K_d values are the same as those given in **Table 2**.



Ajugasterone C (R′ = H, R″ = H) ⟶ Dacryhainansterone (R′ = H, R″ = H)
Muristerone A (R′ = OH, R′ = H) ⟶ Kaladasterone (R′ = OH, R″ = H)
Turkesterone (R′ = H, R″ = OH) ⟶ 25-Hydroxydacryhainansterone (R′ = H, R″ = OH)

Figure 4 Schematic derivatization of ajugasterone C, muristerone A, and turkesterone to photo affinity ecdysteroid 7,9(11)-dien-6-ones, dacryhainansterone, kaladasterone, and 25-hydroxydacryhainansterone, respectively. (Adapted from Bourne, P.C., Whiting, P., Dhadialla, T.S., Hormann, R.E., Girault, J.-P., *et al.*, 2002. Ecdysteroid 7,9(11)-dien-6-ones as potential photoaffinity labels for ecdysteroid binding proteins. *J. Insect Sci.* 2, 1–11.

to the three EcRs with either muristerone A or tebufenozide (in the cases of AaEcR and CfEcR) bound were protected from proteolysis with proteinase K, suggesting similar ligand-induced conformational changes. Such a conformational change *in vivo* may be necessary for ligand-dependent transactivation of a gene.

With respect to the hypothesis of Billas *et al.* (2003) that in the EcR/USP heterodimer the EcR ligand binding pocket may conform to accommodate an active ligand, it is not clear if the changes seen in limited proteolysis experiments (above) are a result of just ligand-induced changes in the binding pocket or a result of ligand-induced changes in the whole of the EcR LBD.

6.3.2.2.4. Photoaffinity reagents for studying receptor–ligand interactions Before the X-ray crystal structures of the EcR/USP heterodimer were available, alternative approaches to understanding the interaction of ecdysteroid and nonsteroidal bisacylhydrazine ligands with residues in the EcR ligand binding pocket were explored. One such approach was to synthetically produce photoaffinity analogs of ecdysteroids (Bourne *et al.*, 2002) and bisacylhydrazines (Dhadialla *et al.*, unpublished data) as has been achieved for vertebrate steroids and their receptors (Katzenellenbogen and Katzenellenbogen, 1984). An ideal affinity labeling reagent should have: (1) a high affinity for the binding protein with low nonspecific binding to other proteins; (2) a photo-reactive functional group; and (3) a radiolabeled moiety for detection purposes.

Bourne *et al.* (2002) synthesized three ecdysteroid 7,9(11)-dien-7-ones (**Figure 4**; dacryhainansterone, 25-hydroxydacryhainansterone, and kaladasterone)

as photoaffinity ecdysteroids by dehydration of the corresponding 11α-hydroxy ecdysteroids (ajugasterone, turkesterone, and muristerone A, respectively). Of the three photoaffinity ecdysteroids, dacryhainansterone and kaladasterone showed the greatest potential for use in determining contact amino acid residues in EcR LBDs. All three dienone ecdysteroids retained their biological activity in the *D. melanogaster* B_{II} cell assay developed by Clement *et al.* (1993). However, upon irradiation with UV at 350 nM in the presence of bacterially expressed DmEcR/DmUSP and CfEcR/CfUSP, both dacryhainansterone and kaladasterone blocked subsequent specific binding of tritiated ponasterone A by >70%. These results clearly showed the potential of using radiolabeled dienones for further characterization of their interaction within EcR LBDs.

Photoaffinity analogs of bisacylhydrazines have also been produced and used to characterize their binding to EcRs from dipterans and lepidopterans (Dhadialla *et al.*, unpublished data). A tritiated benzophenone analog of methoxyfenozide (**Figure 5**), coded as RH-131039, displays the above-mentioned characteristic requirements of a photoaffinity reagent as well as the specificity to bind to lepidopteran EcR (CfEcR) with high affinity. Binding to dipteran EcR (DmEcR) could not be detected. Binding of either tritiated ponasterone A or tritiated RH-131039 to CfEcR/CfUSP could be competitively displaced using excess unlabeled RH-131039, tebufenozide, ponasterone A, or 20E. These results indicate that the ecdysteroids and the two nonsteroidal ecdysone agonists share a common binding site in the ligand binding pocket of CfEcR/CfUSP (**Figure 6**), an observation that has been confirmed by Billas *et al.* (2003) in their work on the crystal structure of liganded HvEcR/HvUSP. However, further experiments, which can now be done with the ecdysteroid and bisacylhydrazine photoaffinity compounds, need to be done to determine the ligand–receptor contact residues. Similar results were obtained for AaEcR/AaUSP, but not for DmEcR/DmUSP, indicating that even within an insect order, differences in the homology and folding of EcR LBDs is sufficient to discriminate between affinities of nonsteroidal ecdysone agonists. The bisacylhydrazine and ecdysteroid photoaffinity reagents described are additional useful tools that can be combined with data from chemical structure–activity relationships (SAR), analytical (liquid chromatography/mass spectrometery (LC/MS) and mass laser desorption ionization (MALDI)), and mutational analysis approaches to hypothesize and/or define the three-dimensional space of non-lepidopteran EcR LBDs. However, the availability of the liganded HvEcR/HvUSP LBD crystal structure provides the needed information for defining the three-dimensional ligand receptor interaction for homology modeling of such interactions with non-lepidopteran EcR LBDs, and for the design and discovery of new biologically potent ligands as insecticides for different insects orders.

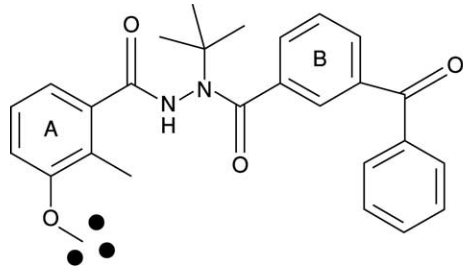


Figure 5 Structure of the tritiated photoaffinity benzophenone analog, RH-131039, of methoxyfenozide. The three dots on the methoxy substitution on the A-phenyl ring indicate tritiation of the methyl group. The benzophenone group on the B-phenyl ring can be activated with UV light for it to cross-link to the nearest amino acid residue.

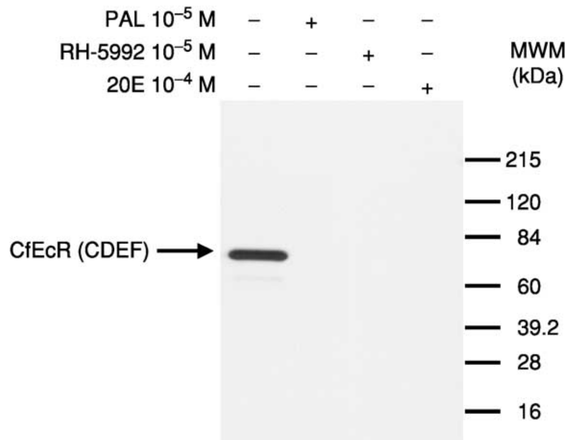


Figure 6 Fluorograph to show specific binding of tritiated RH-131039 (PAL; photo affinity ligand) to CDEF domain of *Choristoneura fumiferana* EcR (CfEcR) expressed by *in vitro* transcription and translation using rabbit reticulocyte system. CfEcR (CDEF domain) plus CfUSP was incubated with tritiated PAL in the presence of excess cold PAL, RH-5992, or 20E at the indicated concentrations under equilibrium binding conditions after which the reaction mixture was irradiated with $UV_{350\,nm}$ for 20 min at 4 °C. The reaction mixture was then mixed with SDS-PAGE sample buffer containing 1 mM DTT, heated for 5 min at 95 °C and then subjected to SDS-PAGE. The gel was dried and fluorographed to get the above image. The numbers on the right indicate relative migration of molecular weight markers (MWM).

6.3.2.3. Mode of Action of Bisacylhydrazines

In the earlier reviews on insecticides with ecdysteroid and JH activity (Oberlander *et al.*, 1995; Dhadialla *et al.*, 1998), much of the focus on mode of

action of ecdysone agonists was from data using RH-5849 and RH-5992. RH-5849, the unsubstituted bisacylhydrazine that was not commercialized, had a broader spectrum of insect toxicity than the four that have been commercialized. In this chapter, the focus will be on work published since 1996 on the commercialized bisacylhydrazine insecticides tebufenozide, methoxyfenozide, chromofenozide, and halofenozide. References will be made to earlier publications where relevant.

6.3.2.3.1. Bioassay A number of *in vitro* and *in vivo* assays have been used to study the effects and mode of action of the bisacylhydrazine insecticides (reviews: Oberlander *et al*., 1995; Dhadialla *et al*., 1998). Larvae of susceptible and nonsusceptible insects (described below), and a number of insect cell lines and dissected tissues have been used *in vitro* to understand the mode of action of bisacylhydrazines. Wing *et al*. (1988) were the first to use *D. melanogaster* embryonic Kc cells to demonstrate that like 20E, RH-5849 also induced aggregation and clumping of otherwise confluent cultures of Kc cells. Similar morphological effects of tebufenozide, methoxyfenozide, and halofenozide have also been demonstrated for cell lines derived from embryos or tissues of *Drosophila* (Clement *et al*., 1993), the mosquito, *Aedes albopictus* (Smagghe *et al*., 2003a), the midge, *Chironomus tentans* (Spindler-Barth *et al*., 1991), the forest tent caterpillar, *Malacosoma disstria*, the spruce budworm, *Choristoneura fumiferana* (Sohi *et al*., 1995), the Indian meal moth, *Plodia interpunctella* (Oberlander *et al*., 1995), the cotton boll weevil, *Anthonomus grandis* (Dhadialla and Tzertzinis, 1997), the European corn borer, *Ostrinia nubilalis*, the Southwestern corn borer, *Diatraea grandiosella*, and the cotton bollworm, *Helicoverpa zea* (Trisyono *et al*., 2000).

Some of these cell lines, imaginal wing discs, and larval claspers from susceptible insects have also been used to study the relative binding affinities, and biochemical and molecular effects of tebufenozide (Mikitani, 1996b), methoxyfenozide, or halofenozide (**Table 4**) and other ecdysone agonists (Nakagawa *et al*., 2002). Cytosolic and or nuclear extracts from 20E responsive cells and tissues containing functional ecdysone receptors, as well as bacterially expressed EcRs and USPs from different insects, have also been used to determine the relative binding affinities of bisacylhydrazines or to screen for new chemistries with similar mode of action in radiometric competitive receptor binding assays (**Table 5**).

Finally, in order to increase the throughput for screening a higher number of compounds, Tran *et al*. (2000) reconstituted ligand-dependent transactivation of *C. fumiferana* EcR in yeast. Reconstitution of the system required transforming yeast with four plasmids carrying cDNAs encoding CfEcR, CfUSP, or RXRγ, an activation factor, a glucocorticoid receptor interacting protein (GRIP), and a plasmid with four copies of an ecdysone response element (hsp27) in tandem fused to a reporter gene (**Figure 7**). The potency of a number of ecdysone agonists in transactivating the reporter gene in the reconstituted EcR ligand dependent assay correlated well with the known activities of the tested bisacylhydrazines in whole insect assays (**Figure 8**), indicating the utility of such assays for high throughput screening of new and novel chemistries with ecdysone mode of action. A similar yeast-based strain was also reconstituted using AaEcR/AaUSP (Tran *et al*., 2001).

6.3.2.3.2. Whole organism effects The commercialized bisacylhydrazine insecticides are toxic to susceptible insects mainly as a result of ingestion. Toxicity via topical application is expressed only when very high doses are applied. The effects of bisacylhydrazines in susceptible insects have been studied in a number of insects (Slama, 1995; Smagghe *et al*., 1996b, 1996c, 2002; Retnakaran *et al*., 1997; Dhadialla *et al*., 1998; Carton *et al*., 2000). In general, because bisacylhydrazines are more metabolically stable *in vivo* than ecdysteroids and because they are true ecdysone agonists at the receptor level, ingestion of bisacylhydrazines creates "hyperecdysonism" in susceptible insects,

Table 4 Relative affinities of 20E and bisacylhydrazines in imaginal wing evagination assays

Species	*20E*	*RH-5849*	*RH-5992*	*RH-2485*	*RH-0345*	*Reference*
Drosophilla melanogaster	0.011 μmol l^{-1}	1 μmol l^{-1}	1.1 μmol l^{-1}	ND	ND	Farkas and Slama (1999)
Chironomus tentans	278 nM	27 nM	7.3 nM	ND	230 nM	Smagghe *et al.* (2002)
Spodoptera littoralis	291 nM	44500 nM	403 nM	10.9 nM	472 nM	Smagghe *et al.* (2000a)
Galleria mellonella	321 nM	865 nM	8.9 nM	ND	ND	Smagghe *et al.* (1996a)
Leptinotarsa decemlineata	60.7 nM	461 nM	757 nM	ND	ND	Smagghe *et al.* (1996a)

ND, not determined.

Table 5 Relative binding affinities of bisacylhydrazine compounds to cellular extracts or *in vitro* expressed EcR and USP proteins from insects of different orders

	20E	*Pon A*	*RH-5849*	*RH-5992*	*RH-2485*	*RH-0345*	*Reference*
Drosophila melanogaster	145 nM	0.9 nM		336 nM			Dhadialla, (unpublished data), Cherbas *et al.* (1988)
Aedes aegypti	28 nM	2.8 nM		30 nM			Dhadialla, unpublished data; Kapitskaya *et al.* (1996)
Chironomus tentans		0.35 ± 0.28 and 6.5 ±2.4 nM					Grebe *et al.* (2000), Smagghe *et al.* (2001)
Spodoptera littoralis	158 nM			86.7 nM	24.3 nM		Smagghe *et al.* (2000a)
Plodia interpunctella	210 nM	3 nM		3 nM			Dhadialla, unpublished data
Galleria mellonella	106 nM		911 nM	22 nM			Smagghe *et al.* (1996a)
Spodoptera frugiperda (Sf9 cells)	166 nM	8.9 nM	363 nM	1.5 nM	3.5 nM		Nakagawa *et al.* (2000)
Anthonomus grandis	247 nM	6.1 nM		12 000 nM			Dhadialla and Tzertzinis (1997)
Leptinotarsa decemlineata	425 nM		740 nM	1316 nM			Smagghe *et al.* (1996a)
Tenebrio molitor		6 nM	>10 μM	>10 μM	>10 μM	>10 μM	Dhadialla, unpublished data
Locusta migratoria	1000 nM	1.18 nM	>10 μM	>10 μM	>10 μM	>10 μM	Hayward *et al.* (2003)
Bamesia argentfolli		8 nM	>10 μM	>10 μM	>10 μM	>10 μM	Dhadialla, unpublished data

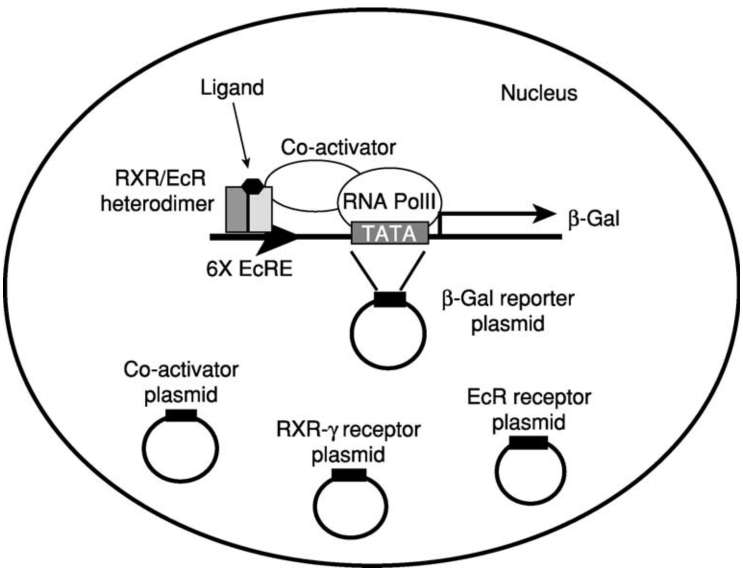


Figure 7 Schematic diagram showing a yeast cell transformed with plasmids carrying cDNAs encoding ecdysone receptor, RXR-γ, co-activator glucocorticoid receptor interaction protein (GRIP), and β-galactosidase (β-Gal) reporter gene fused to 6 heat shock protein 27 (hsp27) ecdysone response elements in a reconstituted and ecdysteroid (muristerone A) inducible high throughput screening system for discovery of new nonsteroidal ecdysone agonists.

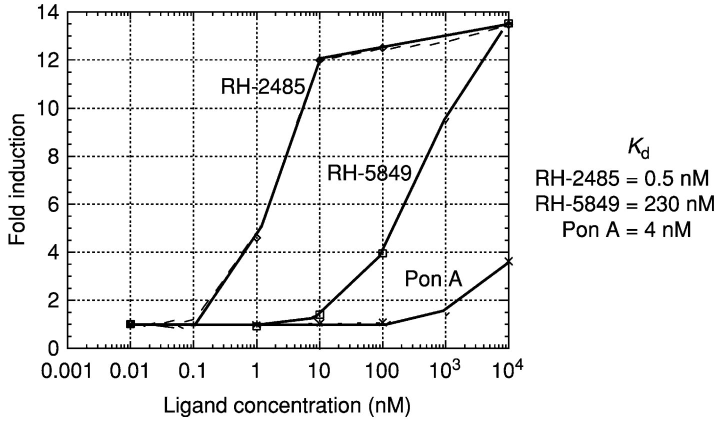


Figure 8 Dose-dependent induction of reporter (β-gal) gene in an ecdysone inducible, transformed yeast cell assay system, with RH-2485 (methoxyfenozide), RH-5849, and the phytoecdysteroid, ponasterone A. While the fold induction achieved with RH-2485 and RH-5849 correlate with their relative affinities (indicated on the right of the graph) for the ecdysone receptor (CfEcR) used in this study, the activation achieved by ponasterone does not correlate with its determined binding affinity to ecdysone receptor.

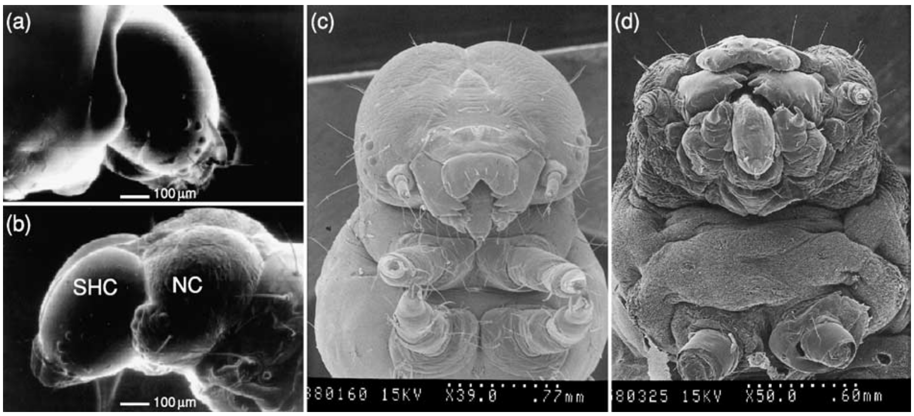


Figure 9 Scanning electron micrographs of control treated (a and c) and tebufenozide intoxicated (b and d) by ingestion (fava bean leaves dipped in a 10 ppm aqueous solution and 100 ng, respectively) second instar southern armyworm (a and b) and third instar spruce budworm (c and d) larvae 48 h after intoxication. In both control armyworm and budworm larvae (a and c), well formed sclerotized cuticle and mouthparts can be seen. In the intoxicated armyworm (b), the larvae is tricked into undergoing a precocious molt and the slipped head capsule (SHC) can be seen over the mouthparts. The new cuticle (NC) covering the head is soft, malformed, and unsclerotized. The soft unsclerotized mouthparts of the intoxicated spruce budworm (d) are shown after manually removing the slipped head capsule. In both cases, tebufenozide intoxicated larvae initiate a molt, slip head capsule, but are unable to complete a molt, and hence die of starvation, desiccation, and hemorrhage from the malformed unsclerotized new cuticle. (Adapted and composed from Dhadialla, T.S., Carlson, G.R., Le, D.P., **1998**. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol. 43*, 545–569; as well as other unpublished electron micrographs.)

thus inducing effects and symptomology of a molt event. One of the first effects of bisacylhydrazine ingestion by susceptible larvae is feeding inhibition within 3–14 h (Slama, 1995; Smagghe *et al*., 1996a; Retnakaran *et al*., 1997), which prevents further plant damage. During this time, synthesis of a new cuticle begins and apolysis of the new cuticle from the old one takes place. Subsequently, the intoxicated larvae become moribund, slip their head capsule (Figures 9 and 10), and, in extreme cases, the hind gut may be extruded (Figure 11). The new cuticle is not tanned or sclerotized. One resulting consequence is that the mouth parts under the slipped head capsule remain soft and mushy, preventing any crop damage even if the head capsule came off from mechanical or physical force. The larvae ultimately die as a result of their inability to complete a molt, starvation, and desiccation due to hemorrhage.

The reasons for the lethal precocious molt effects of bisacylhydrazines have been investigated at the ultrastructural level in *C. fumiferana* (Retnakaran *et al*., 1997), in beet armyworm, *Spodoptera exigua* (Smagghe *et al*., 1996c), in tomato looper, *Chrysodeixis chalcites* (Smagghe *et al*., 1997), in the Colorado potato beetle, *Leptinotarsa*



Figure 10 Control (left) and tebufenozide intoxicated by ingestion (right) larvae of the white tussock moth. While the control larva continues normal growth and development, the tebufenozide intoxicated larva undergoes a precocious lethal molt. In this case the intoxicated larva is in the slipped head capsule stage.



Figure 11 Photomicrograph showing the severe growth inhibitory effects of ingested tebufenozide in spruce budworm larvae. While both larvae show slipped head capsules, one larva (bottom) shows an additional effect, extrusion of the gut.

decemlineata (Smagghe *et al*.,1999c; Dhadialla and Antrium, unpublished observations), and in cultured abdominal sternites of the mealworm, *Tenebrio molitor* (Soltani *et al*., 2002). Some general conclusions can be drawn from these studies. Examination of the cuticle following intoxication with any of the three bisacylhydrazines revealed that the larvae synthesize a new cuticle that is malformed (Figure 12; Dhadialla and Antrim, unpublished data). Unlike during normal cuticle synthesis, the lamellate endocuticule deposition in bisacylhydrazine intoxicated larvae is disrupted and incomplete. The epidermal cells in intoxicated larvae have fewer microvilli, show hypertrophied Golgi complex and an increased number of vesicles compared to normal epidermal cells active in cuticle synthesis. The visual observations of precocious production of new cuticle have also been demonstrated by *in vivo* and *in vitro* experiments to demonstrate the inductive effects of tebufenozide and 20E on the amount of chitin in *S. exigua* larval cuticle and chitin synthesis in cultured claspers of *O. nubilalis*, respectively (Smagghe *et al*., 1997).

At the physiological level the state of "hyperecdysonism," coined by Williams (1967), manifested by bisacylhydrazines in intoxicated susceptible larvae is achieved by various mechanisms. Blackford and Dinan (1997) demonstrated that while larvae of the tomato moth, *Lacnobia oleracea*, detoxified ingested 20E as expected, it remained susceptible to ecdysteroid agonists RH-5849 and RH-5992. This suggested that the metabolic stability of the ecdysone agonists induced the "hyperecdysonism" state in the tomato moth larvae. In another study, RH-5849 was shown to repress steroidogenesis in the larvae of the blowfly, *Caliphora vicina*, as a result of its action on the ring gland (Jiang and Koolman, 1999). However, the production of ecdysteroids in abdominal sterintes of *T. molitor*, cultured *in vitro* in the presence of RH-0345, increased compared to that in control cultured abdominal sternites (Soltani *et al*., 2002). Ecdysteroid production, measured by an ecdysteroid enzyme immunoassay, by sternites cultured *in vitro* in the presence of 1–10 μM RH-0345 increased with increasing incubation times and concentrations of the bisacylhydrazine. Topical application of 10 μg RH-0345 to newly ecdysed pupae also caused a significant increase in hemolymph ecdysteroid amount as compared to control treated pupal hemolymph ecdysteroid levels. However, there was no effect in the timing of the normal ecdysteroid release in the hemolymph. Contrary to the *in vitro* and *in vivo* effects observed in the above *Tenebrio* study, Williams *et al*. (1997) observed that injection of RH-5849 into larvae of the tobacco hornworm, *Manduca sexta*, induced production of midgut cytosolic ecdysone oxidase and ecdysteroid phosphotransferase activities, which are involved in the inactivation of 20E. In addition, both 20E and RH-5849 caused induction of ecdysteroid 26-hydroxylase activity in the midgut mitochondria

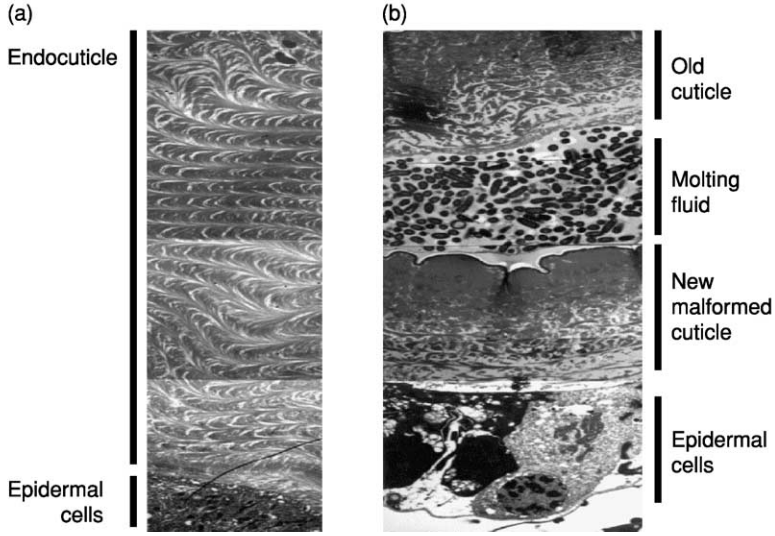


Figure 12 Transmission electron micrographs of the integument of control (a) and halofenozide (fava bean leaves dipped in a 100 ppm solution) ingested (b) second instar Colorado potato beetle larvae (same magnification). In the control integument, the epidermal cells appear normal (not clear in this figure) and the new cuticle is deposited in an ordered lamellate manner. In the integument of halofenozide intoxicated larva, the epidermal cells are highly vacuolated and the new cuticle is malformed, lacking ordered lamellate deposition. Although apolysis of the old cuticle has taken place, it is not shed, and electron dense granulated material (bacterial?) is seen in the ecdysial space. (Both micrographs taken at same magnification).

and microsomes. Subsequently, Williams *et al.* (2002) demonstrated that induction of ecdysteroid 26-hydroxylase by RH-5849, RH-5992 (tebufenozide), and RH-0345 (halofenozide) may be a universal action of bisacylhydrazines in susceptible lepidopteran larvae. These effects were not observed in nonsusceptible larvae of the waxmoth, *Galleria mellonella*, whose ecdysteroid receptor is capable of binding RH-5992 (Williams *et al.*, 2002). The binding in this case may not be sufficient for transactivation of genes that are involved in the molting process, and induced or repressed by 20E.

It is interesting to compare what happens at the molecular level in response to changing hormone levels in control insects compared to bisacylhydrazine intoxicated larvae as shown in **Figure 13**. Bisacylhydrazines, by virtue of their action via the ecdysone receptor, activate genes that are dependent upon increasing titers of 20E. However, those genes that are normally activated either by decreasing titers or absence of 20E are not expressed in the presence of ingested bisacylhydrazine in the hemolymph. During a normal molt cycle, the release of eclosion hormone to initiate the eclosion behavior in the molting larvae is dependent upon clearance of 20E titers in the hemolymph (Truman *et al.*, 1983). The presence of bisacylhydrazines in the hemolymph inhibits the release of eclosion hormone, thus resulting in an unsuccessful lethal molt.

The expression and repression of several genes during a molt cycle in lepidopteran (*M. sexta* and *C. fumiferana*) larvae treated with RH-5992 have been investigated (Retnakaran *et al.*, 1995, 2001; Palli *et al.*, 1995, 1996, 1999). In control larvae, at the time of the rise in titer of 20E, as well as those treated with RH-5992, early genes such as MHR3 (in *Manduca*), CHR3 (in *Choristoneura*), and E75, (in *Drosophila*) are expressed. As 20E titers decline, DDC, which requires a transient exposure to 20E followed by its clearance, is expressed. In RH-5992, intoxicated larvae genes like DDC are not expressed, which prevents tanning and hardening of the already malformed new cuticle. During the intermolt period, when 20E is absent, genes like those for the 14 kDa larval cuticle protein (LCP14) that are normally repressed in the presence of 20E, are expressed. Once again, the prolonged presence of RH-5992 in the hemolymph prevents the expression of LCP14, and perhaps other genes that would normally be depressed in the absence of 20E. As a consequence of all the changes induced or repressed by RH-5992, and other bisacylhydrazine insecticides, the molting process is completely derailed at the ultrastructural, physiological and molecular level leading to precocious lethal molt in susceptible larvae.

Farkas and Slama (1999) studied the effects of RH-5849 and RH-5992 on chromosomal puffing, imaginal disc proliferation, and pupariation in larvae of *D. melanogaster*. In this study, they demonstrated that the two bisacylhydrazine compounds acted like 20E in the induction of early

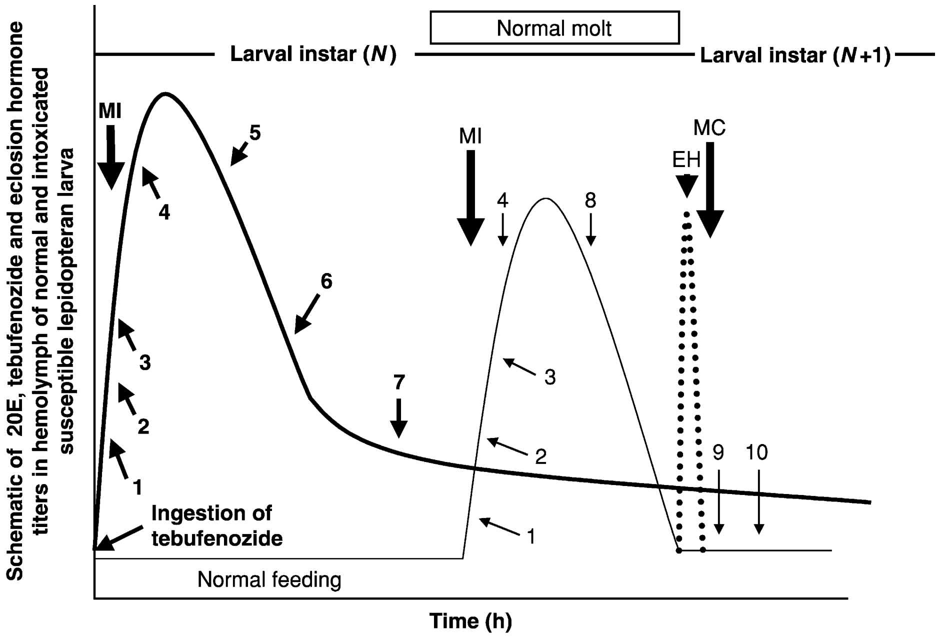


Figure 13 Schematic representation of titers 20E (thin line) and release of eclosion hormone (EH; dotted line), which triggers the ecdysis of the larva to complete a normal molt. The solid bold line represents relative titers of ingested tebufenozide in a susceptible lepidopteran larva (adapted from Dhadialla, unpublished data). Owing to the metabolic stability and amount of tebufenozide in the insect hemolymph, eclosion hormone, the release of which is normally dependent upon the complete decline of 20E, is not released and the intoxicated insect is not able to complete the molt, which leads to premature death. Both methoxyfenozide and halofenozide undergo similar metabolic fate, which is detrimental to intoxicated insect stage. The ecdysone agonists trigger a molt attempt anytime during the feeding stage of a susceptible larval instar. Events that take place during the molt and are dependent upon the increasing and decreasing titers of 20E are also shown. The numbers in bold and regular font represent different events triggered by tebufenozide and 20E, respectively. **1**, 1: Inhibition of feeding; **2**, 2: initiation of new cuticle synthesis; **3**, 3: apolysis of old cuticle from new cuticle resulting in an ecdysial space filled with molting fluid; **4**, 4: head capsule slippage; MI: molt initiated; MC: molt completed; **5**: derailment of the molting process; **6**: eclosion hormone is not released, and the larva stays trapped in its old cuticle and slipped head capsule covering the mouth parts (refer to Figure 9) causing it to starve; **7**: molt attempt is lethal and the ecdysone agonist intoxicated larvae dies of starvation, hemorrhage, and desiccation; 8: cuticle formation continues and molting fluid starts to be resorbed; 9: molt attempt is completed after release of EH and larva ecdyses into the next larval stage; 10: new cuticle hardens and the mouth parts are sclerotized so that the larva may continue its growth and development into the next stage.

chromosomal puffs (74EF and 75B), and regression of pre-existing puffs (25AC, 68C) in larval salivary glands. In the chromosomal puff assay, the ED_{50} for bisacylhydrazine compounds were, however, an order of magnitude less than for 20E. Results of additional experiments in this study using different assays, i.e., glycoprotein glue secretion, imaginal disc evagination, and rescue of phenotypic expression in ecdysone deficient mutants showed the potencies of the bisacylhydrazines were two orders of magnitude lower than for 20E. In spite of the quantitative differences in the assays used, the results confirmed the ecdysone-mimetic action of the two bisacylhydrazines.

In a study to understand the role of ecdysteroids in the induction and maintenance of the pharate first instar diapause larvae of the gypsy moth, *Lymantria dispar*, Lee and Denlinger (1997) demonstrated that a diapause specific 55 kDa gut protein could be induced in ligature isolated larval abdomens with injections of either 20E or RH-5992. They also demonstrated that the effect of KK-42, an imidazole derivative known to inhibit ecdysteroid biosynthesis, to prevent prediapausing pharate first instar larvae from entering diapause could be reversed by application of 20E or RH-5992. In this case, RH-5992 was two orders of magnitude more active than 20E.

Using 1,5-disubstituted imidazoles, Sonoda *et al.* (1995) demonstrated that the inductive effects of these compounds on precocious metamorphosis of *Bombyx mori* larvae could be reversed by tebufenozide.

6.3.2.4. Basis for Selective Toxicity of Bisacylhydrazine Insecticides

Unlike halofenozide, which is toxic to both coleopteran and lepidopteran larvae, both tebufenozide and methoxyfenozide are selectively toxic to lepidopteran larvae with a few exceptions of toxicity to

dipteran insects, like the midge, *C. tentans* (Smagghe *et al*., 2002) and the mosquito species (Darvas *et al*., 1998). This, at first, was a surprise, especially after the discovery that bisacylhydrazines are true ecdysone agonists, the activity of which, like that of 20E, is manifested via interaction with the ecdysone receptor complex. There are three basic reasons why an ingested insecticide may be differentially toxic to different insects: (1) metabolic differences between susceptible and nonsusceptible insects; (2) lack of transport to the target site in nonsusceptible insects; and (3) target site differences between susceptible and nonsusceptible insects.

Smagghe and Degheele (1994) found no differences in the pharmacokinetics and metabolism of ingested RH-5992 in two susceptible species, *Spodoptera exigua* and *S. exempta*, and a nonsusceptible insect, *Leptinotarsa decemlineata*. Similar results were obtained when the pharmacokinetics and metabolism of RH-5992 were investigated in larvae of susceptible *S. exempta* and the nonsusceptible Mexican bean beetle, *Epilachna verivesta* (Dhadialla and Thompson, unpublished results).

In competitive displacement EcR binding assays, it became apparent that one of the reasons for the differential selectivity of tebufenozide and methoxyfenozide might be due to differences in the LBDs of EcRs from different insects. While 20E binds to EcR complexes from insects from different orders with similar affinities, the affinity of tebufenozide and methoxyfenozide is very disparate for insects even within an insect order (**Table 2**). Hence, the very high affinity of tebufenozide and methoxyfenozide to lepidopteran EcR complexes correlates very well with their toxicity to larvae within this order, as well as those few amongst the Diptera. However, the same logic does not explain the toxicity of halofenozide and lack of toxicity of tebufenozide to coleopteran larvae, especially when the affinity of tebufenozide is higher than that of halofenozide for binding to EcRs from a coleopteran insect. Perhaps, in this case, the low binding affinity of halofenozide is compensated for by its greater metabolic stability (Smagghe and Degheele, 1994; Farinos *et al*., 1999).

To determine if there are additional reasons at the cellular level for the selective toxicity of tebufenozide, Sundaram *et al*. (1998) made use of four lepidopteran and dipteran cell lines all of which are responsive to 20E indicating the presence of functional EcRs. A lepidopteran cell line from *C. fumiferana* midgut cells (CF-203) and a dipteran cell line (DM-2) derived from *D. melanogaster* embryos responded to 20E in a similar dose-dependent induction of the transcriptional factor, hormone receptor 3 (HR3) CHR3 and DHR3 recspectively, which represents one of the early 20E inducible genes in the ecdysone signaling pathway. The lepidopteran CF-203 cells could produce CHR3 mRNA at 10^{-10} M RH-5992 concentration, as opposed to 10^{-6} M of this compound required for induction of very low amounts of DHR3 transcripts. In further experiments using ^{14}C-RH-5992, the authors were able to show that CF-203 cells accumulated and retained sixfold higher amounts of the radiolabeled compound than DM-2 cells over the same incubation period. Similar results were obtained with another lepidopteran cell line (MD-66) derived from *Malacosomma disstria*, and a dipteran cell line (Kc) from *D. melanogaster* embryos. This significantly higher retention of radiolabeled RH-5992 in lepidopteran cells compared to dipteran cells was in contrast to similar levels of retention of tritiated ponasterone A, a potent phytoecdysteroid. The extremely low amounts of RH-5992 in dipteran cells was due to an active efflux mechanism that was temperature-dependent and could be blocked with 10^{-5} M oubain, an inhibitor of Na^+, K^+-ATPase.

In developing a yeast based ecdysteroid responsive reporter gene assay, Tran *et al*. (2001) had to use a yeast strain that was deficient in the *pdr5* and *snq2* genes, both of which are involved in multiple drug resistance, to increase the sensitivity of the transformed strain to both steroidal and nonsteroidal ecdysone agonists. Subsequently, Retnakaran *et al*. (2001) demonstrated that while the wild-type yeast *Saccharomyces cervisiae* actively excluded tebufenozide, the *pdr5* deleted mutant strain retained significantly higher amounts of radiolabeled tebufenozide (Hu *et al*., 2001; Retnakaran *et al*., 2001). Retransformation of the mutant strain with the *pdr5* gene enabled the active exclusion of radiolabeled tebufenozide, thus once again suggesting a role for an ATP binding cassette transporter.

Some general conclusions can be drawn from the above studies regarding the selective insect toxicity of tebufenozide and methoxyfenozide. While the absence of significant differences in the pharmacokinetics and metabolism of these compounds in susceptible and nonsusceptible insects is not directly responsible for selective toxicity, it is indirectly, because the hormone levels of tebufenozide, after its ingestion by lepidopteran larvae, are about 2000-fold higher than the K_d equivalent for the lepidopteran EcR. The selective toxicity of tebufenozide and methoxyfenozide to lepidopteran larvae is probably due to the clearance of these agonists as shown in resistant cell lines where they are actively pumped out. It would be interesting to investigate if cells derived from *C. tentans* and mosquito tissues,

like lepidopteran cells, are devoid of an active mechanism to eliminate tebufenozide and methoxyfenozide, because ecdysone receptors from both these dipterans have high affinities for bisacylhydrazines (Kapitskaya *et al*., 1996; Smagghe *et al*., 2002) and are susceptible to tebufenozide (Smagghe *et al*., 2002).

6.3.2.5. Spectrum of Activity of Commercial Products

6.3.2.6.1. Chromofenozide (MATRIC®; KILLAT®; ANS-118; CM-001) Jointly developed by Nippon Kayaku Co. Ltd (Saitama, Japan), and Sankyo, Co. Ltd. (Ibaraki, Japan), chromofenozide is the latest amongst the nonsteroidal ecdysone agonist insecticides registered for control of lepidopteran pests on vegetables, fruits, vines, tea, rice, arboriculture, ornamentals, and other crops in Japan (Table 6). Chromofenozide has relatively safe mammalian, avian, and aquatic organism toxicology (Table 7). It also has no adverse effects on nontarget arthropods, including beneficials.

6.3.2.5.2. Halofenozide (MACH II; RH-0345) Unlike tebufenozide, methoxyfenozide, and chromofenozide, halofenozide is more soil systemic and has a broader pest spectrum of activity (Lepidoptera and Coleptera; Table 6). It was developed for control of beetle grub and lepidopteran larval pests of turf in lawns and on golf courses.

Halofenozide gave excellent control of the Japanese beetle (*Popillia japonica*) and the oriental

Table 6 Spectrum of pest activity of the four commercial ecdysone agonist insecticides

Tebufenozide and methoxyfenozide (Lepidoptera specific)	*Methoxyfenozide (Lepidoptera specific)*	*Chromofenozide (Lepidoptera specific)*	*Halofenozide (Lepidoptera and Coleoptera specific)*
Adoxophyes spp.	*Clysia ambiguella*	*Spodoptera littura*	*Agrotis ipsilon*
Anticarcia gemmatalis	*Grapholitha molesta*	*Spodoptera exigua*	*Spodoptera frugiperda*
Boarmia rhombodaria	*Heliothis* spp.	*Spodoptera littoralis*	Sod webworm *(Crambinae)*
Chilo suppressalis	*Helicoverpa zea*	*Plutella xylostella*	*Anomala orientalis*
Choristoneura spp.	*Ostrinia nubilalis*	*Cnaphalocrosis medinalis*	*Maladera canstanea*
Cnapholocrocis medinalis	*Phyllonorychter* spp.	*Ostrinia furnacalis*	*Ataenius spretulus*
Cydia spp.	*Tuta absoluta*	*Adoxophyes orana*	*Rhizotrogus majalis*
Diatraea spp.	*Keiferia lycopersicella*	*Heliothis virescens*	*Popillia japonica*
Diaphania spp.		*Lobesia botrana*	*Cotinis nitida*
Hellula spp., *Homona magnamima*		*Cydia pomonella*	*Cyclocephala* spp.
Lobesia botrana		*Chilo suppressalis*	*Phyllophaga* spp.
Lymantria dispar		*Anticarcia gemmatalis*	*Hyperodes near anthricinus*
Planotortrix spp.,			
Platynota idaeusalis			
Plusia spp., *Pseudoplusia includens*			
Rhicoplusia nu, Sparganothis spp.			
Spodoptera spp.			
Trichoplusia ni			
Epiphyas postvittana, Grapholitha prunivora			
Memestra brassicae, Operophthera brumata			

Table 7 Ecotoxicological profile of bisacylhydrazine insecticides

	Tebufenozide	*Methoxyfenozide*	*Chromofenozide*	*Halofenozide*
Avian: mallard duck, LC_{50} (8-day dietary)	>5000 mg kg^{-1}	>5620 mg kg^{-1}		>5000 mg kg^{-1}
Bobwhite quail, LC_{50} (8-day dietary)	>5000 mg kg^{-1}	>5620 mg kg^{-1}	>5000 mg kg^{-1} (Japanese quail, 14-day)	4522 mg kg^{-1}
Aquatic: bluegill sunfish, acute LC_{50} (96 h)	3.0 mg l^{-1}	>4.3 mg l^{-1}		>8.4 mg l^{-1}
Daphnia magna, acute EC_{50} (48 h)	3.8 mg l^{-1}	3.7 mg l^{-1}	>189 mg l^{-1} (3 h)	3.6 mg l^{-1}
Honeybee (oral and contact), acute LD_{50}	>234 μg bee^{-1}	>100 μg bee^{-1}	>100 μg bee^{-1} (contact) >133 μg bee^{-1} (oral)	>100 μg bee^{-1}
Earthworm, LC_{50} (14 days)	1000 mg kg^{-1}	>1213 mg kg^{-1}	>1000 mg kg^{-1}	980 mg kg^{-1}

beetle (*Exomala orientalis*) when applied at 1.1 kg active ingredient (a.i.) ha^{-1} to a mixed field population on turf plots (Cowles and Villani, 1996; Cowles *et al.*, 1999). In the same study, higher rates (1.7–2.2 kg a.i. ha^{-1}) were required to control the European chafer, *Rhizotrogus (Amphimallon) majalis (Razoumowsky)*, population. The Asiatic garden beetle, *Maladera castanaea* (Arrow), was not sensitive to any of the doses of halofenozide tested.

The recommended rates for the control of lepidopteran larvae (such as cutworms, sod webworms, armyworms, and fall armyworms) and beetle grubs (Japanese beetle, northern and southern masked chafer, June beetle, Black turfgrass ataenius beetle, green June beetle, annual bluegrass weevil larvae, bill bugs, aphodius beetles, European chafer, and oriental beetle) are 12 kg a.i. ha^{-1} and 18–24 kg a.i. ha^{-1}, respectively.

6.3.2.5.3. Tebufenozide (MIMIC; CONFIRM; ROMDAN; RH-5992) and methoxyfenozide (RUNNER; INTREPID; PRODIGY; RH-2485) Tebufenozide, at low use rates, shows remarkable insect selectivity for the control of lepidopteran larvae, including many members of the families Pyralidae, Pieridae, Tortricidae, and Noctuidae, which are pests of tree fruit, vegetables, row crops, and forests (**Table 6**; Le *et al.*, 1996; Dhadialla *et al.*, 1998; Carlson *et al.*, 2001). Laboratory studies have shown the following: tebufenozide administered through artificial diet or by leaves treated with tebufenozide is efficacious against *C. fumiferana* (Retnakaran *et al.*, 1999), corn earworm and fall armyworm (Chandler *et al.*, 1992), and beet armyworm (Chandler, 1994); both tebufenozide and methoxyfenozide are efficacious against laboratory reared and field populations of codling moth, *Cydia pomonella* (Pons *et al.*, 1999; Knight *et al.*, 2001), laboratory reared and multi-resistant cotton leafworm, *Spodoptera littoralis* (Smagghe *et al.*, 2000b), field populations of filbertworm, *Cydia latiferreana*, on hazelnut, and against *D. grandiosella* (Trisyono and Chippendale, 1998).

Tebufenozide has been used very successfully for the control of *C. fumiferana*, which is the most destructive insect defoliator of spruces, *Picea* sp., and balsam fir, *Abies balsamea* (L.) Mill, in North American forests (Sundaram *et al.*, 1996; Cadogan *et al.*, 1997, 1998, 2002). Cadogan *et al.* (1998) used a 240 g l^{-1} formulation of commercial tebufenozide (MIMIC® 240LV) mixed with water and a tracer dye (Rhodamine WT), which was sprayed onto the trees using a light-wing aircraft, to determine the foliar deposit of the insecticide and its efficacy against *C. fumiferana*. Single applications of 1 or 2 L ha^{-1} resulted in a 61.4–93.6% or 85.6–98.3% reduction, respectively, of the spruce budworm populations. Following double applications, the mean population reductions ranged from 93.6 to 98.3% compared to mean population reductions of 61% in untreated control fields. Defoliation in untreated control plots was about 92% and in plots with single or double spray applications, defoliation was 40–62% or 31–62%, respectively. These results indicated that a double spray application was most efficacious.

MIMIC 240LV has also provided effective control of Jackpine budworm, *Choristaneura pinus*, and *Lymantria dispar* in North American forests, as well as control of processionary caterpillar, *Thaumetopomea pityocampa*, and European pine shoot moth, *Rhyaciona buoliana*, in forests in Spain and Chile, respectively (Lidert and Dhadialla, 1996; Butler *et al.*, 1997).

Two of the major lepidopteran pests of tree-fruits are codling moth and leaf rollers, which have been controlled by frequent applications of azinphos-methyl and other organophosphate insecticides (Riedl *et al.*, 1999). With increasing requirements to use more selective insecticides without effecting biological control of other pests, much attention has been paid to the use of IGRs like chitin synthesis inhibitors, JHAs, and agonists of 20E. The effectiveness of tebufenozide against larvae of the codling moth, *C. pomonella*, has been tested both under laboratory and field conditions (Pons *et al.*, 1999; Knight, 2000). Pons *et al.* (1999) found that tebufenozide has ovicidal and larvicidal but not adulticidal activity against *C. pomonella*. Tebufenozide was about 73 times more effective when presented to neonate larvae in artificial diet as compared to treated apples; possibly because once the neonates have burrowed their way into the apples they are no longer exposed to the insecticides. However, in tebufenozide treated artificial diets, the susceptibility to the insecticide decreased with increasing age. Because of the feeding behavior of codling moth larvae, which generally do not feed and do not ingest large amounts of foliage or fruit as they burrow into the core of the fruit, the exposure to the insecticides is very limited. However, the reproductive and ovicidal effects of tebufenozide may be more important than its larvicidal effects (Knight, 2000). Knight (2000) found that in 30 field trials, tebufenozide exhibited both residual and topical ovicidal effects for codling moth egg masses laid on leaves. Similar results were obtained by Pons *et al.* (1999).

These studies indicated that the timing of tebufenozide sprays was important in not only preventing

codling moth injury to the apples, but also in controlling oblique banded leaf rollers for which both tebufenozide and methoxyfenozide have been shown to have lethal and sublethal effects (Riedl and Brunner, 1996; Sun *et al*., 2000). Knight (2000) proposed that delaying the first application of tebufenozide until after petal fall in apple for *Choristoneura rosaceana* (Harris) would correspond to an approximate codling moth timing of 30-degree days at which time this insect would be susceptible as well. This strategy, which targets the adults and eggs, was effective in the control of codling moth.

Both tebufenozide and methoxyfenozide are active primarily by ingestion, but also exhibit selective contact and ovicidal activity (Trisyono and Chippendale, 1997; Sun and Barrett, 1999; Sun *et al*., 2000).

6.3.2.6. Ecotoxicology and Mammalian Reduced Risk Profiles

Both tebufenozide and methoxyfenozide have been classified by the US Environmental Agency (EPA) as reduced risk pesticides because of their low acute and chronic mammalian toxicity, low avian toxicity, and their safety to most beneficial arthropods and compatibility with integrated pest management programs. Both tebufenozide and methoxyfenozide are toxic primarily to lepidopteran pests, but they are also toxic to a few dipteran (*C. tentans*, mosquitos) pests. Of the 150 insect species tested, both tebufenozide and methoxyfenozide were found to be safe to members of the insect orders Hymenoptera, Coleoptera, Diptera, Heteroptera, Hemiptera, Homoptera, and Neuroptera (Glenn Carlson, unpublished data). When used at rates that are 18- to 1500-fold greater than that producing 90% mortality in lepidopteran larvae, both tebufenozide and methoxyfenozide had little or no effect on a panel of non-lepidopteran pests (Coleoptera, Homoptera, mites, and nematodes; Trisyono *et al*., 2000; Carlson *et al*., 2001; Medina *et al*., 2001). Similarly, laboratory and/or field studies have shown that the two insecticides have little or no adverse effect on a wide range of non-lepidopteran beneficial insects, like honeybees and predatory insects/mites, when applied at rates used for control of lepidopteran larvae.

In 1998, methoxyfenozide became one of the only four pesticide products to be awarded the "Presidential Green Chemistry Award," which was established by the US Government during 1995 to recognize outstanding chemical processes and products that reduce negative impact on human health and the environment relative to the currently available technology.

The ecotoxicological and mammalian toxicity data for the four commercial ecdysone agonist insecticides are shown in **Tables 7 and 8**. These data clearly show the low mammalian, avian, and aquatic toxicity of these insecticides.

6.3.2.7. Resistance, Mechanism, and Resistance Potential

Tebufenozide is the first nonsteroidal ecdysone agonist with commercial application and has been used in Western Europe since the mid 1990s. In the USA, the first uses occurred concurrently in 1994 in Alabama and Mississippi under Section 18 exemptions (Walton *et al*., 1995; Dhadialla *et al*., 1998).

Resistance to tebufenozide was documented for the first time in the codling moth *C. pomonella* around Avignon in southern France (Sauphanor and Bouvier, 1995; Sauphanor *et al*., 1998b) and in the greenheaded leafroller, *Planotortrix octo*, in New Zealand (Wearing, 1998). A small survey with the beet armyworm, *S. exigua*, collected in different greenhouses in Western Europe (Spain, Belgium, The Netherlands) showed lack of resistance development to tebufenozide (Smagghe *et al*., 2003b). In this same period, Smagghe *et al*. (1999a) kept a laboratory strain of *S. exigua* under continuous pressure with sublethal concentrations of tebufenozide over 12 generations, and in the sixth generation a significantly lower toxicity (about five-fold) was observed. In addition, retention and

Table 8 Mammalian reduced risk profile of bisacylhydrazine insecticides

	Tebufenozide	*Methoxyfenozide*	*Chromofenozide*	*Halofenozide*
Acute oral LD_{50} (rat, mouse)	>5000 mg kg^{-1}	>5000 mg kg^{-1}	>5000 mg kg^{-1}	2850 mg kg^{-1}
Acute dermal LD_{50}	>5000 mg kg^{-1}	>2000 mg kg^{-1}	>2000 mg kg^{-1}	>2000 mg kg^{-1}
Eye irritation (rabbit)	Nonirritating	Nonirritating	slightly irritating	Moderately irritating, positive for contact
Dermal sensitization (guinea pig)	Nonsensitizer	Negative	Mildly sensitizing	Allergy
Ames assay	Negative	Negative	Negative	Negative
Acute inhalation	>4.3 mg l^{-1}	>4.3 mg l^{-1}		>2.7 mg l^{-1}
Reproduction (rat)	No effect	No effect	No effect	No effect

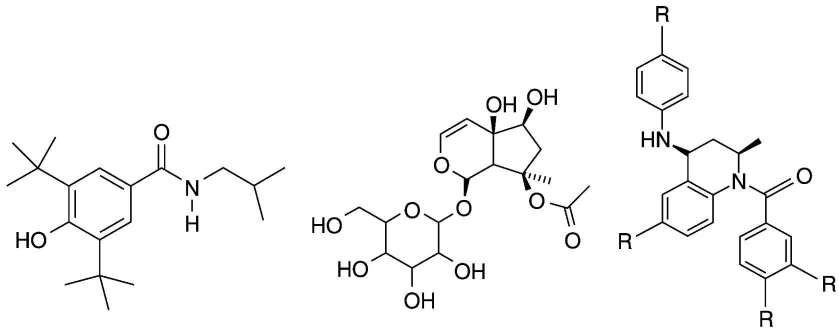
metabolic fate studies showed the importance of oxidative metabolism, leading to a rapid clearance from the body. Interestingly, a decrease in oviposition was noted with the lower toxicity indicating a possible fitness cost related to development of resistance (Smagghe and Degheele, 1997; Smagghe *et al.*, 1998). More recently, Moulton *et al.* (2002) reported resistance to tebufenozide in third instar larvae, which reached levels of 150-fold in a selected strain of *S. exigua* from Bangbuathong (Thailand) as compared with a laboratory reference strain. In this region of Thailand, many insecticides including organophosphates (OPs), pyrethroids, benzoylphenylurea (BPUs), and *Bacillus Thurigiensis* (Bt) formulations, and even new IGRs have been rendered ineffective due to ill-advised agricultural practices, most notably dilution of insecticide residues on leaves by overhead drench irrigation (Moulton *et al.*, 2002). This practice is likely to be responsible for the high incidence of insecticide multiresistance in this area and the highly accelerated rate of resistance development. When this Thailand strain was dosed with methoxyfenozide, 120-fold lower toxicity was observed. These selection assays with tebufenozide and methoxyfenozide showed a reduction in toxicity for both compounds, suggesting at least some commonality of resistance mechanism (Moulton *et al.*, 2002). A greenhouse raised strain of *S. exigua*, in southern Spain was also found to be resistant to tebufenozide and methoxyfenozide. Although the level of resistance was lower in this second strain, it was high enough to allow studies on the mechanism(s) of resistance. In general, a higher breakdown activity leads to lower levels of the parent toxophore. For tebufenozide and methoxyfenozide the major first phase route of detoxification was through oxidation (Smagghe *et al.*, 1998, 2003). In addition, piperonyl butoxide, a P450 inhibitor, significantly synergized the toxicity of tebufenozide and methoxyfenozide, whereas DEF, an esterase inhibitor, was ineffective (Smagghe *et al.*, 1998, 1999), indicating that a lower toxicity was more likely from an increase in oxidative activity, rather than in esterase activity. At the cellular level, Retnakaran *et al.* (2001) reported that tebufenozide accumulated selectively in lepidopteran Cf-203 (*C. fumiferana*) cells in contrast to dipteran Dm-2 cells (*D. melanogaster*), which actively excluded the compound (see Section 6.3.2.4 for more details). Perhaps such exclusion systems may also account for the fact that older instars of the white-marked tussock moth (*Orgyia leucostigma*) are resistant to tebufenozide (Retnakaran *et al.*, 2001). The characterization of all such possible resistance processes is essential to provide information that is helpful to prevent resistance from developing towards this valuable group of IGRs. But more information is needed with additional strains collected from the field, especially where growers have severe pest control problems, before a general interpretation can be formulated on resistance risks for this new type of IGRs in the field.

While the available data suggest oxidative metabolism as the primary reason for development of resistance to tebufenozide and methoxyfenozide, there is no evidence so far that suggests that target site modification could be involved as another route to resistance development in field or laboratory insect populations. However, at the cellular level, there is evidence that there could be alterations in the target site(s). Using insect cell lines, Cherbas and co-workers were the first to report that *in vitro* cultured Kc cells of *D. melanogaster* did not respond to 20E after continuous exposure (Cherbas, personal communication). Similarly, Spindler-Barth and Spindler (1998) reported that cells of another dipteran, *C. tentans*, maintained in the continuous presence of increasing concentrations of 20E or tebufenozide over a period of 2 years, developed resistance to both compounds. In these resistant subclones, all 20E regulated responses that are known to occur in sensitive cells were no longer detectable, suggesting that the hormone signalling pathway itself was interrupted (Spindler-Barth and Spindler, 1998; Grebe *et al.*, 2000). Further ligand binding experiments with extracts containing ecdysone receptors from susceptible and resistant cells indicated that ligand binding to EcR from resistant clones was significantly decreased. Moreover, an increase in 20E metabolism and a reduction in receptor concentration were noted in some clones. Similar effects have also been observed in another study using imaginal discs of *S. littoralis* selected for resistance to tebufenozide (Smagghe *et al.*, 2001).

While resistance to ecdysone agonist insecticides is inevitable, it can be prevented from occurring sooner with good resistance monitoring programs.

6.3.2.8. Oher Chemistries and Potential for New Ecdysone Agonist Insecticides

After the discovery of the first three bisacylhydrazine insecticides by scientists at Rohm and Haas Company, Spring House, PA, USA, a number of laboratories initiated such efforts to discover new and novel chemistries with ecdysone mode of action and different pest selectivity. Mikitani (1996) at Sumitomo Company, Japan, discovered a benzamide, 3,5,-di-*t*-butyl-4-hydroxy-N-isobutyl-benzamide



(1) **(2)** **(3)**

Figure 14 Chemical structures of new ecdysone agonists reported in literature: (**1**) 3,5-di-*t*-butyl-4-hydroxy-*N*-isobutyl-benzamide (DTBHIB) from Sumitomo; (**2**) 8-*O*-acetylhrapagide from Merck Research Laboratories, Westpoint, PA, USA and (**3**) tetrahydroquinoline from FMC Corporation, Princeton, NJ, USA. In (3) R = halide.

(DTBHIB; **Figure 14**) and Elbrecht *et al.* (1996) at Merck Research Laboratories, Westpoint, PA, USA, reported the isolation of an iridoid glycoside, 8-*O*-acetylharpagide (**Figure 14**), from *Ajuga reptans*. Both these compounds were reported to induce 20E-like morphological changes in *Drosophila* Kc cells, as well as competitively displace tritiated ponasterone A from *Drosophila* ecdysteroid receptors with potencies similar to that of RH-5849, the unsubstituted bisacylhydrazine. However, the insecticidal activity of these compounds was not described. Attempts to replicate the results of Mikitani (1996) using DTBHIB and analogs failed to demonstrate that these compounds were competitive inhibitors of tritiated ponasterone A binding to DmEcR/DmUSP produced by *in vitro* transcription and translation (Dhadialla, unpublished observations). On the other hand, Dinan *et al.* (2001) demonstrated that the results obtained by Elbrecht *et al.* (1996) were due to co-purification of ecdysteroids in their 8-O-acetylharpagide preparation. When used as a highly purified preparation, Dinan *et al.* (2001) found that 8-*O*-acetylharpagide was not active as an agonist or an antagonist in *D. melanogaster* BII cell bioassay, and neither did it compete with tritiated ponasterone A for binding to the lepidopteran ecdysteroid receptor complex from *C. fumiferana*.

Finally, scientists at FMC discovered a new tetrahydroquinoline (THQ) class of compounds (**Figure 14**) that competitively displaced tritiated ponasterone A from both dipteran (*D. melanogaster*) and lepidopteran (*H. virescens*) EcR and USP heterodimers (unpublished data). Interestingly, the most active analogs of this class of compounds bound the DmEcR/DmUSP with much higher affinity than the HvEcR/HvUSP. This is the reverse of what was observed with bisacylhydrazines. When tested for ligand binding to EcR/USP proteins from *L. migratoria, B. argentifoli, and T. molitor*, to which bisacylhydrazines show no measurable affinity, members of the THQ were found to bind with measurable affinity (μM range; Dhadialla and colleagues, unpublished results). Further work on this chemistry was continued at Rohm and Haas Company, Spring House, PA, USA, and its subsidiary, RheoGene LLC, Malvern, PA, USA, which resulted in the synthesis of a number of analogs that were active in transactivating reporter genes fused to different insect EcRs and heterodimeric partners (*L. migratoria* RXR, and human RXR) in mouse NIH3T3 cells (Michelotti *et al.*, 2003).

The discovery of THQs with ligand binding activities to various EcRs (Michelotti *et al.*, 2003), some of which do not interact with bisacylhydrazines, and the interpretation of X-ray crystal structure results of liganded HvEcR/HvUSP (Billas *et al.*, 2003) provides good evidence for the potential to discover new chemistries with ecdysone agonist activities. It should also be possible to design chemistries that specifically interact with EcR/USPs from a particular insect order.

6.3.2.9. Noninsecticide Applications of Nonsteroidal Ecdysone Agonists; Gene Switches in Animal and Plant Systems

A number of researchers started to explore the utilization of the ecdysone receptor as an inducible gene switch due to the knowledge that neither mammals nor plants have ecdysone receptors, and the discovery of bisacylhydrazine as true ecdysone agonists with reduced risk ecotoxicology and mammalian profiles. Gene switches are inducible gene regulation systems that can be used to control the expression of transgenes in cells, plants, or animals. There is a recognized need for tightly regulated eukaryotic molecular gene switch applications, such as for gene therapy, and in understanding the role played

by specific proteins in signaling pathways, cell differentiation, and development (Allgood and Eastman, 1997). Other applications include large-scale production of proteins in cells, cell-based high-throughput screening assays, and regulation of traits in both plants and animals. Unlike all other previous and current insecticides, commercial nonsteroidal ecdysone agonists are the first class of insect toxic chemistry that has led to such a high level of interest and utility outside the insecticide arena. The following is a brief overview of the use of bisacylhydrazine insecticides and EcRs as gene switches in mammalian and plant systems.

6.3.2.9.1. Gene switch in mammalian systems Christopherson *et al.* (1992) demonstrated that DmEcR could function as an ecdysteroid-dependent transcription factor in human 293 cells cotransfected with an RSV-based expression vector that encoded DmEcR and a reporter gene, which contained four copies of *Drosophila* Hsp27 linked to a MTV promoter-CAT construct. They demonstrated that the activity of DmEcR could not be activated by any of the mammalian steroid hormones tested. Even amongst the ecdysteroids, the phytoecdysteroid muristerone A was the best transactivator of the reporter gene, while 20E was not as effective. These authors further demonstrated that chimeric receptors containing the LBD of DmEcR and the DBD of glucocorticoid receptor could also function when cotransfected with plasmids containing glucocorticoid receptor response elements fused to a reporter gene. Subsequently, No *et al.* (1996) demonstrated that an EcR-based gene switch consisting of DmEcR, *Homo sapiens* RXR, and appropriate response elements fused to a reporter gene in mammalian cells or transgenic mice could be transactivated with micromolar levels of muristerone A. Muristerone was shown to maintain its activity when injected into mice and was not found to be toxic, teratogenic, or inactivated by serum binding proteins. Moreover, overexpression of modified DmEcR and RXR did not appear to to be toxic, at least in transfected cells. This study, like an earlier one (Yao *et al.*, 1995), demonstrated that HsRXR could substitute for its insect homolog and heterodimer partner for EcR, USP. However, in all these studies, the range of activating ligands was limited to ecdysteroids and, in particular, to muristerone A. In addition, RXR, which is present in almost all mammalian cells, was found not to be as good a partner for EcR as USP. Hence, very high endogenous levels were necessary for stimulation with muristerone A to occur.

With the availability of EcR and USPs from other insects, especially lepidopteran insects, and understanding the high affinity of nonsteroidal ecdysone agonists like tebufenozide and methoxyfenozide for lepidopteran EcRs/USPs, Suhr *et al.* (1998) demonstrated that the *B. mori* EcR (BmEcR), unlike DmEcR, could be transactivated to very high levels in mammalian cells with tebufenozide without adding an exogenous heterodimer partner like RXR. The endogenous levels of RXR were enough to provide the high transactivation levels. The work by Suhr *et al.* (1998) further demonstrated that while the D domain (hinge region) of EcR was necessary for high-affinity heterodimerization with USP, both D and E (LBD) domains were necessary for high-affinity interaction with RXR. By creating chimeric EcR using parts of E-domains from BmEcR and DmEcR, these investigators showed that a region in the middle of the E-domain (amino acids 402 to 508) in BmEcR constituted the region conferring high specificity for tebufenozide. Subsequent research by others has mainly focused on using EcRs and USPs from other insects as well as using different promoters for expression of the transfected genes (Kumar *et al.*, 2002; Dhadialla *et al.*, 2002; Palli and Kapitskaya, 2002; Palli *et al.*, 2002, 2003). Based on EcR homology modeling studies and site-directed mutagenesis of selected amino acid residues, Kumar *et al.* (2002) found that a CfEcR mutant, which had an A110P mutation in the LBD, was responsive only to nonsteroidal ecdysone agonists, including methoxyfenozide, but not to any of the ecdysteroids tested. This demonstrated that the affinity and functional specificity of an EcR for a ligand can be altered, thus offering the possibility of using multiplexed EcR-based gene switches that could regulate different traits with different ligands. Palli *et al.* (2003) developed a two-hybrid EcR based gene switch, which consisted of constructs containing DEF domains of CfEcR fused to the GAL4 DNA binding domain, and CfUSP or *Mus musculus* retinoid X receptor (MsRXR) EF domains fused to the VP16 activation domain. These constructs were tested in mammalian cells for their ability to drive a luciferase gene placed under the control of GAL4 response elements and synthetic TATAA promoter. This combination gave very low level basal activation of the reporter gene in the absence of the inducer. In the presence of the inducer, there was a rapid increase in the expression of the luciferase reporter gene, reaching levels as high as 8942-fold greater than basal level by 48 h. Withdrawal of the ligand resulted in 50% and 80% reduction of the reporter gene by 12 h and 24 h, respectively.

These studies clearly demonstrate the potential of utilizing EcR based gene switches that can be activated by ecdysteroids and nonsteroidal ecdysone agonists like tebufenozide, methoxyfenozide, and others. The fact that both tebufenozide and methoxyfenozide are registered as commercial insecticides, and have proven reduced risk mammalian and ecotoxicology profiles, makes them very attractive as inducers of the EcR based gene switches. The work of Kumar *et al.* (2002) clearly demonstrates the potential of mutating EcR to change its ligand specificity, thus opening additional possibilities of extending the use of the EcR gene switch in a multiplexed manner.

6.3.2.9.2. Gene switch for trait regulation in plants The reader is referred to very good recent reviews on this topic that not only describe the EcR-based chemically inducible gene regulation systems, but also other systems that have utility in plants (Jepson *et al.*, 1998; Zuo and Chua, 2000; Padidam, 2003). This section is restricted to descriptions of EcR-based gene switch systems.

In the mid-1990s, a number of agricultural companies initiated research to exploit the use of the EcR-based gene switch and nonsteroidal ecdysone agonists like tebufenozide as chemical inducers for regulation of traits (for example, fertility, flowering, etc.) in plants. Initial work was done using DmEcR and DmUSP as components of the gene switch (Goff *et al.*, 1996). In this case, the researchers used chimeric polypeptides (GAL4 DBD fused to LBD of DmEcR and VP16 activation domain fused to DmUSP) to activate the luciferase reporter gene fused to GAL4 response element in maize cells. In the presence of 10 μM tebufenozide, about 20- to 50-fold activation of luciferase expression was obtained. Subsequently, a number of researchers developed variants of EcR gene switches using different chimeric combinations of heterologous DBD, LBDs from different lepidopteran EcRs, and transactivation domains that could interact with appropriate response elements to transactivate a reporter gene in a ligand-dependent manner (Martinez *et al.*, 1999a; Unger *et al.*, 2002; Padidam *et al.*, 2003). For example, Jepson *et al.* (1996) and Martinez *et al.* (1999b) used chimeric *H. virescens* EcR (HvEcR) composed of glucocorticoid receptor transactivation and DBD fused to LBD of HvEcR and *GUS* reporter gene fused to glucocorticoid receptor response element for transfection of maize and tobacco protoplast. In both cases, weak transactivation of the *GUS* reporter gene was obtained with tebufenozide and muristerone A, though the response with the latter was much lower than with tebufenozide. However, 10 μM and higher concentrations of the two ligands were required to transactivate the reporter genes.

Padidam *et al.* (2003) used a chimeric *C. fumiferana* EcR (CfEcR) composed of a CfEcR LBD, GAL4 and LexA DBDs, and VP16 activation domains that could be activated with methoxyfenozide in a dose-dependent manner from a GAL4- or LexA-response element to express a reporter gene. These researchers used *Arabidopsis* and tobacco plants for transformation with the gene switch components and obtained several transgenic plants that had little or no basal level of expression in the absence of methoxyfenozide. In the presence of methoxyfenozide, reporter expression was several fold higher than in the absence of methoxyfenozide. The above studies provided ample evidence of the utility of EcR gene switch, especially those that utilize EcR from a lepidopteran species, and tebufenozide and methoxyfenozide as chemical inducers for trait regulation in plants.

Demonstration of the utility of EcR-based gene switch for trait regulation in maize was demonstrated by Unger *et al.* (2002). A mutation in maize (MS45), which results in male-sterile phenotype, could be reversed by complementation to gain fertility using methoxyfenozide-dependent chimeric receptor gene switch to express the wild-type MS45 protein in tapetum and anthers. These researchers used the EcR LBD from, *O. nubialis* to generate a chimeric receptor. The chimeric receptor was introduced into MS45 maize with the MS45 gene fused to the GAL4 response element, which in the absence of methoxyfenozide were male sterile. However, application of methoxyfenozide to plants containing either a constitutive promoter or anther specific promoter resulted in the restoration of fertility to MS45 plants grown in either the greenhouse or the field.

It is interesting to note that in all the above studies, except those by Goff *et al.* (1996), reporter transactivation response to tebufenozide, methoxyfenozide, or muristerone A via the EcR gene switch was obtained without the requirement of an exogenous heterodimeric partner (USP or RXR), suggesting that there may be other factor(s) in plants that can substitute for USP as a partner for EcR, or that EcR can function as a homodimer. However, as far as is known, there is no evidence of EcR binding an ecdysteroid or nonsteroid ligand in the absence of USP or RXR. Irrespective, these studies provide ample demonstration of the utility of EcR-based gene switch, which can be regulated with an ecdysteroid or a nonsteroidal ecdysone agonist. The use of nonsteroidal ecdysone agonists, like any of the commercialized products, is attractive because

their reduced risk for the environment and mammals, birds, aquatic animals, and beneficial arthropods has been found to be acceptable. The specificity and the high affinity with which the bisacylhydrazines bind ecdysteroid receptors also offers the opportunity to utilize different ligands and ecdysone receptors to regulate more than one trait in a plant or an animal system.

6.3.2.10. Conclusions and Future Prospects of Ecdysone Agonists

The discovery of bisacylhydrazine insecticides with a new mode of action, very high degree of selective insect toxicity and reduced risk to the environment, has spurred a lot of interest in this mode of action and chemistry. The biochemical and molecular information on insect EcRs from different orders of insects, crystal structures of liganded HvEcR/HvUSP, and of DmUSP and HvUSP, and the discovery of new non-bisacylhydrazine chemistries, offers potential for the discovery of other new and novel chemistries with ecdysone mode of action and those that would be selectively toxic to nonlepidopteran and coleopteran pests. Although the existing bisacylhydrazine insecticides are most efficacious when ingested, the use of this type of control method could be extended to act against other types of pests if ways could be found for these insecticides to be effective via topical or systemic applications. These bisacylhydrazine insecticides, because of their new mode of action, insect selectivity, and reduced risk ecotoxicology and mammalian profiles, are ideally suited for use in integrated insect management programs.

An altogether different benefit from the discovery of nonsteroidal ecdysone agonists as insecticides has been in the area of gene switch applications in plants and animals. In this particular application, it may be possible to alter the molecular structure of ecdysone receptor LBDs to accommodate new chemistries as gene switch ligands. This is an exciting active area of research that, if successfully applied, offers a number of opportunities in trait and gene regulation in both plant and animal systems. The search for new and novel chemistries that target ecdysone receptors will continue to be a fruitful area of research for several years.

6.3.3. Juvenile Hormone Analogs

Kopec (1922) extirpated the brain of a caterpillar and demonstrated that this prevented pupation; he attributed this phenomenon to a humoral factor produced by the brain. This discovery heralded the study of hormonal regulation of metamorphosis in insects. Wigglesworth (1936) later described the secretion of a hormone that prevents metamorphosis from a pair of glands, the corpora allata (CA) attached to the base of the brain; he called it the "status quo" or juvenile hormone (JH). A few years later, Fukuda (1994) described the prothoracic glands as the source of the molting hormone or ecdysone, which was later characterized as a steroid hormone (Butenandt and Karlson, 1954). At about the same time, the physiology of JH was elegantly worked out by Williams (1952). The chemical characterization of its structure, however, eluded researchers until 1967 when Röller and co-workers finally showed that it was a sesquiterpene, epoxy farnesoic acid methyl ester. At this time, Williams (1967) made the now famous statement, "third generation pesticides" in describing the use of JHs as environmentally safe control agents to which the insect will be unable to develop resistance, the first and second generation pesticides being the inorganic and the chlorinated hydrocarbons, respectively. The development of highly potent synthetic analogs of JH, which were several fold more active than the native hormone, gave credence to William's claim (Henrick *et al*., 1973).

This section describes the biological activity of JH and its analogs as well as their modes of action as understood today. Some of the major uses of JHAs for pest management are cataloged and finally prospects for future development are mentioned.

6.3.3.1. Juvenile Hormone Action

Juvenile hormone has a unique terpenoid structure and is the methyl ester of epoxy farnesoic acid (see also **Chapter 3.7**). This sesquiterpene exists in at least six different forms (**Figure 15**). JH III is the most common type and is present in most insects. Five different JHs, JH 0, I, II, III, and 4-methyl-JH I, have been described in lepidopterans. The bis-epoxide of JH, JH B_3, is found along with JH III in higher Diptera (Cusson and Palli, 2000). Various studies show that JH III is the main JH in most insects whereas JH I and II are the principal ones in Lepidoptera. Biosynthesis of JH proceeds through the mevalonate pathway with acetyl coenzyme A (acetyl CoA) serving as the building block. Propionyl CoA is used wherever ethyl side chains occur. Epoxidation and esterification are the terminal steps in the biosynthetic process (Schooley and Baker, 1985; Brindle *et al*., 1987).

JH secretion by the corpus allatum is regulated by two neurohormones, the allatotropins that stimulate secretion and the allatostatins that inhibit production. Severing the nerve connections or extirpating the neurosecretory cells results in the loss of

control (Tobe and Stay, 1985; Tobe *et al*., 1985; Lee *et al*., 2002). JH is cleared from the hemolymph by JH esterase, which selectively cleaves the methyl ester inactivating the hormone (Wroblewski *et al*., 1990; Feng *et al*., 1999). JH being highly lipophilic is made soluble by JH binding proteins (JHB) to facilitate transportation to the target sites as well as protect it from degradation from nonspecific esterases (Goodman, 1990). The receptor rich structure of the corpus allatum from the female cockroach, *Diploptera punctata*, is illustrated in **Figure 16** (Chiang *et al*., 2002).

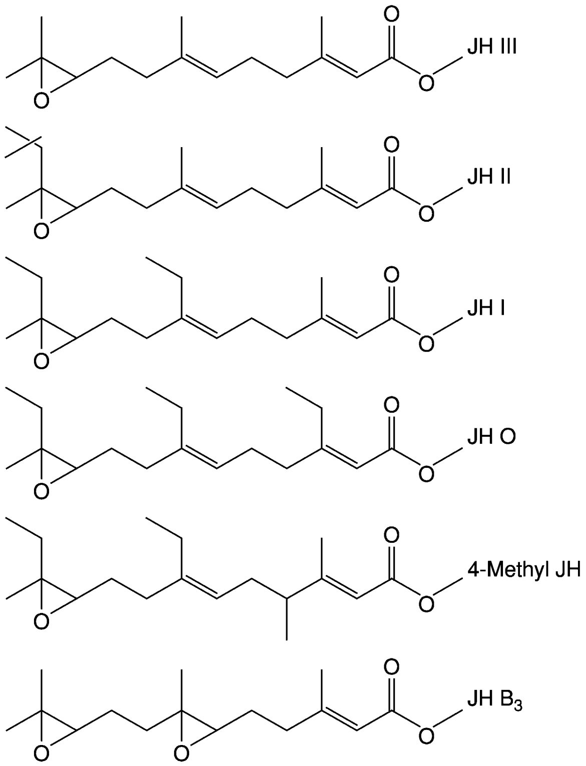


Figure 15 Chemical structure of naturally occurring JHs.

JH is perhaps one of the most pleiotropic hormones known and functions in various aspects of metamorphosis, reproduction, and behavior (Riddiford, 1994, 1996; Wyatt and Davey, 1996; Cusson and Palli, 2000; Palli and Retnakaran, 2000; Hiruma, 2003; Riddiford *et al*., 2003).

The major function of JH is the maintenance of the larval status or the so-called juvenilizing effect. During the last larval instar in lepidoptera there is an absence of JH during the commitment peak of 20E, which results in the reprogramming of metamorphosis towards pupation (**Figure 17**). In the absence of JH, this ecdysone peak induces the expression of the broad complex gene (*BrC* or *Broad*), which is a transcription factor that initiates the larval–pupal transformation through several microRNAs (miRNAs) (Zhou and Riddiford, 2002; Sempere *et al*., 2003). In adults, JH secretion resumes and is responsible for yolk protein (vitellogenin) synthesis and transport into the ovaries (Wyatt and Davey 1996). In addition, JH is also responsible for adult diapause where the ovaries do not develop due to the absence of JH and this effect

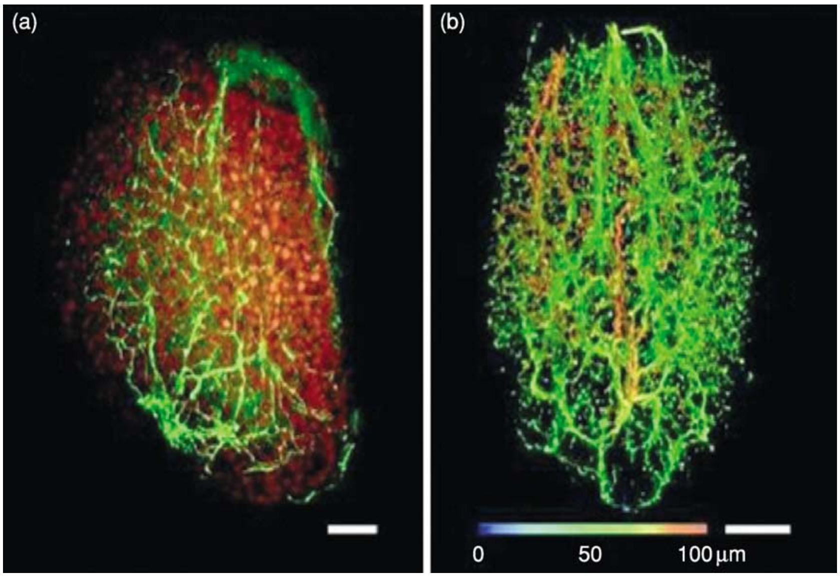


Figure 16 Corpus allatum of female cockroach, *Diploptera punctata*. (a) *N*-methyl-D-aspartate subtype of glutamate receptors (NMDAR) in the nerve fibers (green) involved in JH synthesis and nuclei (red) of parenchyma cells counterstained with propidium iodide. (b) Allatostatin-immunoreactive nerve fibers in the gland (depth code with different colors indicates distance of an object from the surface). Scale bar = 50 μm. (Reproduced with permission from Chiang, A.-S., *et al*., **2002**, Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA 99,* 37–42; © National Academy of Sciences, USA.)

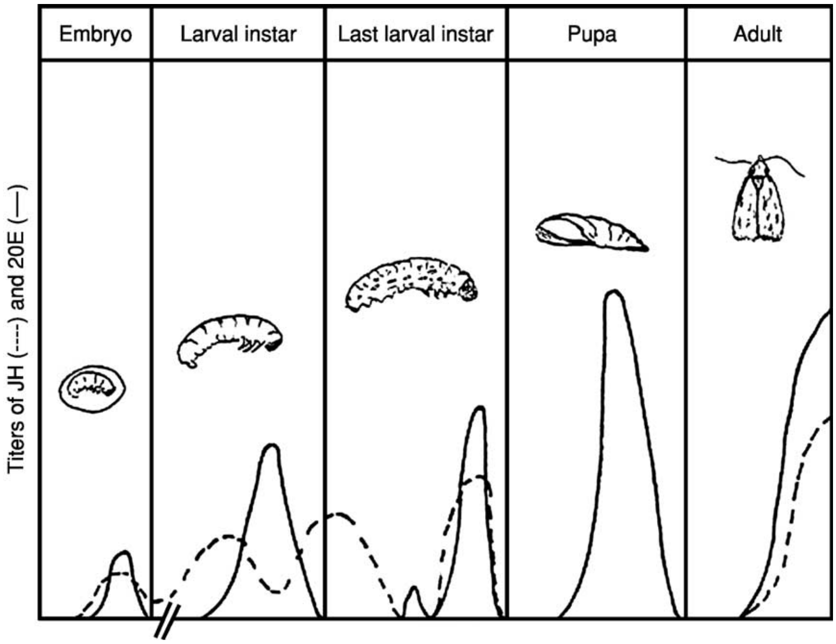


Figure 17 Juvenile hormone and 20-hydroxyecdysone (20E) titers in the various stages of a typical lepidopteran. MP, molt peak of 20E; CP, commitment peak of 20E; RD, reproduction peak of 20E.

can be reversed by applying JH. JH has also been shown to play a role in caste determination, pheromone production, polyphenism, migration, antifreeze protein production, female sexual behavior, male accessory gland secretion, etc. (Wyatt and Davey, 1996).

6.3.3.2. Putative Molecular Mode of Action of JH

The mode of action at the molecular level has not been completely understood at present. Three types of action have been hypothesized and varying degrees of support for each of these modes have been reported. First is the direct action where it directly induces the secretion of a product (Iyengar and Kunkel, 1995; Feng *et al*., 1999). Second is the indirect action where it modulates the action of 20E (Dubrovski *et al*., 2000; Zhou and Riddiford, 2002). In these two instances, the action is at the genomic level where JH has to bind to nuclear receptors to evoke its action. The third involves the role of JH in the transport of vitellogenin into the oocytes through a membrane receptor (Sevala and Davey, 1989). A search for a nuclear as well as a membrane receptor by many investigators over several years has not yielded any concrete results. A nuclear protein isolated from *M. sexta* epidermis using a human retinoic acid receptor as a probe turned out to be an ecdysone-induced transcription factor, a homolog of *Drosophila* hormone receptor 3 (DHR3). A 29 kDa epidermal nuclear protein was found to be a low-affinity JHB (Palli *et al*., 1994; Charles *et al*., 1996). A *D. melanogaster* mutant tolerant to the JHA, methoprene (Met) contained an 85 kDa protein that showed high affinity to JH III (Wilson and Fabian, 1986; Shemshedini *et al*., 1990). The gene was cloned and was identified as a member of the basic helix–loop–helix (bHLH) PAS family of transcriptional regulators (Ashok *et al*., 1998). Attempts to demonstrate the *Met* gene as the JH receptor have only added more confusion to the picture. The ultraspiracle (*USP*) gene, a homolog of the human retinoid-X receptor (RXR) and the heterodimeric partner of the EcR, was demonstrated to be involved in signal transduction of JH III in both yeast (Jones and Sharp, 1997) and Sf9 (Jones *et al*., 2001; Xu *et al*., 2002) cell based assays. In both cases, the cells were transformed with DNA sequences encoding DmUSP, in which JH III application resulted in transactivation. Isolated, recombinant DmUSP specifically bound JH III, and the authors report that *USP* homodimerizes and changes tertiary conformation, including the movement of the LBD α-helix 12 (Jones *et al*., 2001; Xu *et al*., 2002). Xu *et al*. (2002) also reported that ligand binding pocket point mutants of *USP* that did not bind JH III also acted as dominant negative suppressors of JH III activation of reporter promoter, and addition of wild-type *USP* rescued this activation. However, Hayward *et al*. (2003) were not able to demonstrate binding of tritiated JH III to USP from *L. migratoria*. In contrast to results obtained from Jones *et al*. (2001) and Xu *et al*. (2002), the crystal structure

of both HvUSP and DmUSP with a lipophilic ligand revealed the LBD α-helix 12 in an open, antagonist position (Billas *et al.*, 2001; Clayton *et al.*, 2001). While it is unclear whether *USP* is the true nuclear JH receptor, the evidence so far is at best equivocal.

Another approach to eluciding the molecular target(s) of JH action has been the use of JH responsive genes. Several have been identified, such as *jhp21* from *L. migratoria* (Zhang *et al.*, 1996). A response element in the promoter region of this gene binds to a protein, which is thought to be a transcription factor activated by JH (Zhou and Riddiford, 2002). Unfortunately, many of these JH responsive genes appear to be induced by JH at a slow rate, suggesting that they may not be the primary site of regulation. However, there are some genes, such as the JH esterase gene (*JHE*) from a *C. fumiferana* cell line, CF-203, that are induced by JH I within 1 h of administration (Feng *et al.*, 1999). This Cf *jhe* appears to be a primary JH responsive gene, which is induced by JH I and suppressed by 20E in a dose-dependent manner. Work in authors' laboratory has shown that a 30 bp minimal promoter region upstream of the Cf *jhe* transcription start site appears to bind to a nuclear protein isolated from the CF-203 cells (Retnakaran *et al.*, unpublished data). Whether or not it needs to be a high-affinity, low-abundance protein to qualify as a putative JH nuclear receptor is currently under investigation. It has been suggested that JH could act through a membrane receptor as well, as shown by the work of Davey on vitellogenin transport into the oocytes (Wyatt and Davey, 1996). JH acts on the follicle cells of the ovary and makes them contract, causing large spaces to appear between the cells; this process has been termed "patency." This intercellular space formation (patency) permits the vitellogenin from the hemolymph to enter the ovary and gain access to the surface of the oocyte and subsequently enter these cells. This phenomenon was originally described in *R. prolixus* but was later shown in many other species including locusts (Wyatt and Davey, 1996). This membrane-bound receptor appears to be coupled to a G protein and the signal is transduced through a protein kinase system activating an ATPase that permits, among other things, the transport of vitellogenin into the cells (**Figure 18**).

And so the search goes on to unambiguously identify the nuclear and membrane receptors of JH. Characterizing such a receptor would open the

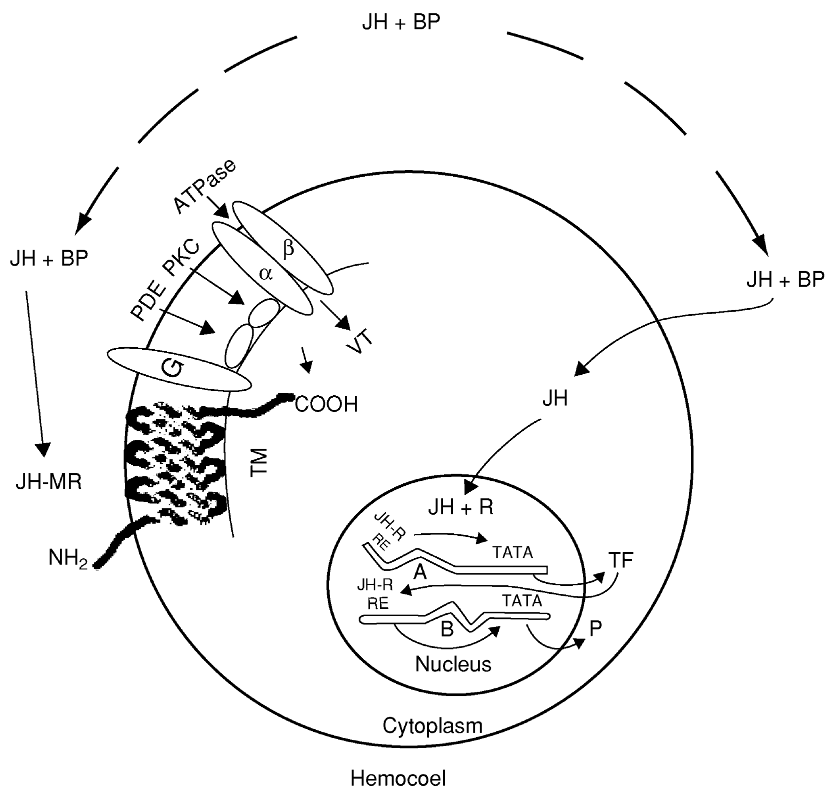


Figure 18 Putative dual receptor mechanism for JH action. A and B are two DNA transcripts; ATPase, Na^+K^+ ATPase with α and β subunits; BP, JH binding protein; G, G protein; JH, juvenile hormone; JH-BP, JH bound to binding protein; JH-MR, JH bound to membrane receptor; PDE, phosphodiesterase; PKC, protein kinase C; RE, hormone response element; TATA, transcription initiation site; TF, transcription factor; TM, transmembrane region. (Reproduced with permission from Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of JH. II. Roles of JH in adult insects. *Adv. Insect Physiol. 26,* 1–155.)

door for a more rational approach to developing target specific control agents.

6.3.3.3. Pest Management with JHAs

The idea that insects would be unable to develop resistance against JH if it was used as a control agent was one of the driving principles behind the impetus to develop this hormone as an insecticide (Williams, 1967). The difficulty and cost of synthesizing such a complex molecule with a labile epoxide moiety and susceptibility to degradation delayed the realization of this concept. However, it soon became apparent that several synthetic analogs of JH, many of them several fold more active than the native hormone, could be used as control agents. The discovery that some synthetic synergists of insecticides have intense JH activity (Bowers, 1968) was the harbinger for the development of various new analogs with diverse chemical structures. Consequently, the modes of action of these analogs may not necessarily be similar.

Over the years, numerous JHAs have been synthesized and their relative potencies, structure–activity relationships, and differential effects on various species have been studied (Slama *et al*., 1974; Romanuk, 1981). Many naturally occurring JHAs, also called juvenoids, have been isolated from plants such as the "paper factor" from the balsam fir tree (*Abies balsamea*), and juvocimenes from the sweet basil plant (*Ocimum basilicum*) (Bowers and Nishida, 1980). During coevolution, the plants probably developed these JHAs to defend themselves against insects. Some representative examples of these compounds are shown in **Figure 19**.

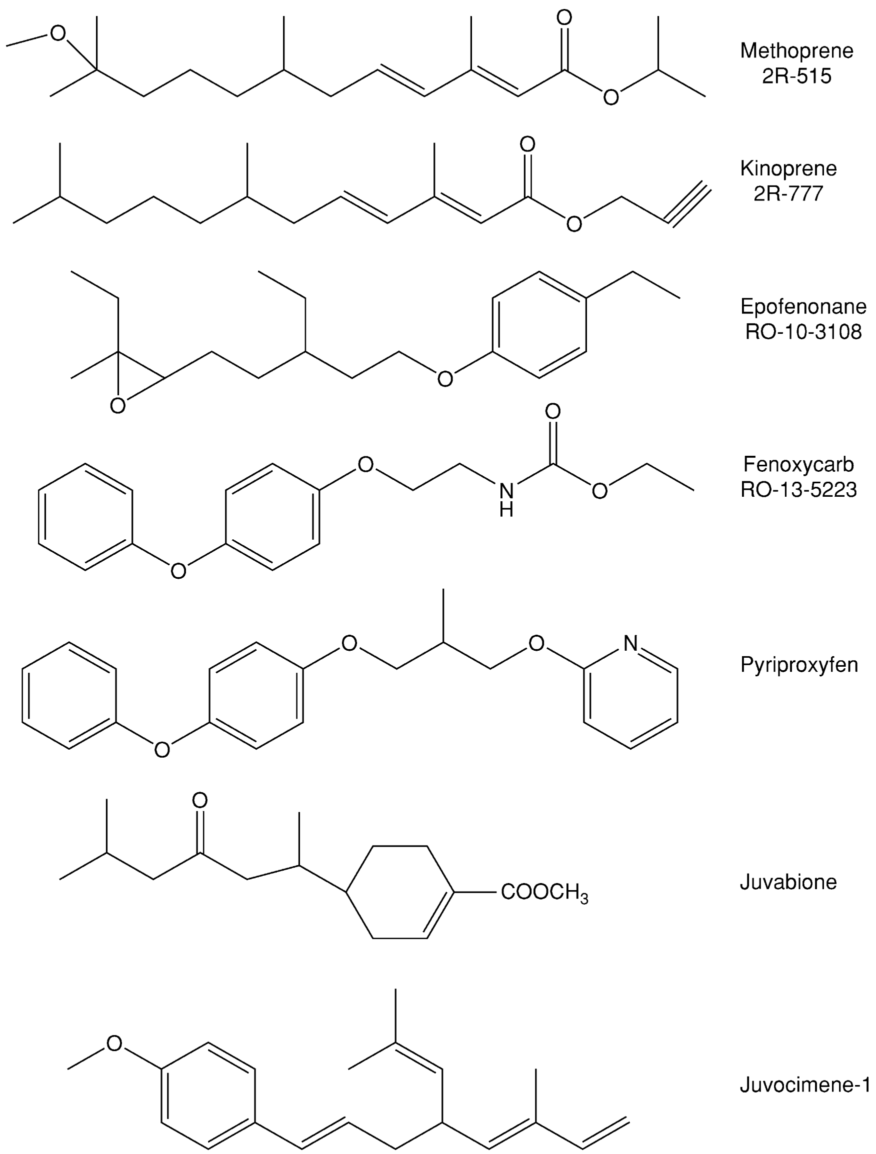


Figure 19 Some synthetic and naturally occurring JHAs.

JHAs can be broadly classified into two groups: the terpenoid JHAs such as methoprene and kinoprene and the phenoxy JHAs such as fenoxycarb and pyriproxyfen, with some such as epofenonane straddling the two (**Figure 19**).

6.3.3.3.1. Biological action of JHAs JHAs mimic the action of natural JH and, as such, can theoretically interfere with all the functions of JH. However, only a few such functions have been capitalized for control purposes (Retnakaran *et al.*, 1985). The organismal effects that have been exploited for control are relatively few, the most common being that seen on the last larval instar. Treatment of the larva in its last instar with JHA severely interferes with normal metamorphosis and results in various larval–pupal intermediates that do not survive (**Figure 20**). This effect, however, is not observed if the last instar larva is treated with the JHA methoprene during the first day or at the pharate stage (prepupal stage) of the stadium. If treated on day 1, the larva molts into a supernumerary larval instar and if it receives JHA at the pharate stage there is no effect (Retnakaran 1973a, 1973b; Retnakaran *et al.*, 1985). Morphogenetic control is ideally suited for controlling insects that are pests as adults, such as mosquitos and biting flies.

JHAs block embryonic development at blastokinesis and act as ovicides (Riddiford and Williams, 1967; Retnakaran, 1970). In some instances, when older eggs are treated, there is a delayed effect that is manifested at the last larval instar when it molts into a supernumerary instar (Riddiford, 1971). This type of ovicidal effect is useful especially in controlling fleas. In some cases where adults are treated, the material gets transferred to the eggs where they block embryonic development (Masner *et al.*, 1968). JHAs induce sterility in both sexes of adult tsetse flies (Langley *et al.*, 1990).

Adult or reproductive diapause is caused by the absence of JH secretion and this condition can be terminated by JHA treatment (De Wilde *et al.*, 1971; Retnakaran, 1974).

6.3.3.3.2. Properties and mode of action of selected JHAs

6.3.3.3.2.1. Methoprene (ALTOSID®, APEXSE®, DIANEX®, PHARORID®, PRECOR®, VIODAT®) Methoprene is perhaps one of the best known terpenoid JHAs developed for pest control. A mutant strain of *D. melanogaster* that was tolerant to methoprene, the so-called *Met* flies, was generated by Wilson and Fabian (1986). These flies were also found to be tolerant to JH III, JH B_3, and several JHAs but not to many classes of insecticides. It was therefore tempting to speculate that this would be an elegant source to discover the JH receptor. An 85 kDa protein isolated from the fat body of wild flies was found to bind with high affinity to JH III. The same 85 kDa protein from *Met* flies showed a sixfold lower affinity for JH III (Shemshedini *et al.*, 1990). When the *Met* gene was cloned, it became

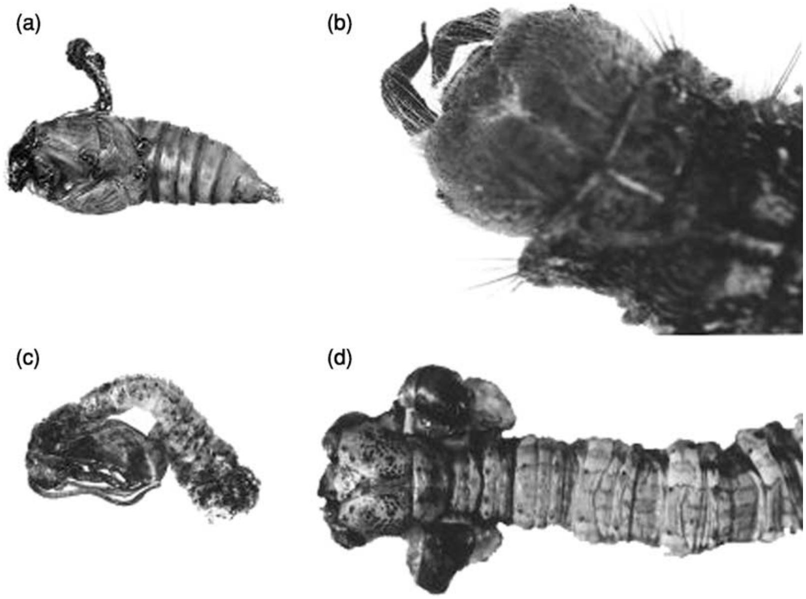


Figure 20 Morphogenetic effects of a JHA, methoprene, on the last larval instar of some lepidopteran insects. (a) A spruce budworm (*Choristoneura fumiferana*) pupa showing larval head and legs; (b) a forest tent caterpillar (*Malacosoma disstina*) showing aberrant mouthparts; (c) a pupa-like larva of the Eastern hemlock looper, *Lambdina fiscellaria*; (d) deformed Eastern hemlock looper larva with everted wing discs.

apparent that it was a bHLH-PAS and belonged to a family of transcriptional regulators, and this gene was not vital for the survival of the flies (Ashok *et al.*, 1998; Wilson and Ashok, 1998). This raises the possibility that JH activity could be exhibited by compounds that may interfere at any step during the synthesis, transportation, and target-site activity. Being extremely pleiotropic, the target-site activity could easily span a wide spectrum of functions. The methoprene-tolerant *Met* gene probably encodes a nonvital insecticide target protein of one type or another (Wilson and Ashok, 1998).

Methoprene is by far the most thoroughly studied JHA. Extensive EPA data collected over several years have shown that this JHA is relatively nontoxic to most nontarget organisms. Methoprene is rapidly broken down and excreted; its half-life in the soil is about 10 days. Mild toxicity to birds and some aquatic organisms has been observed. It has been used as a mosquito larvicide and for controlling many coleopterans, dipterans, homopterans, and siphonopterans (Harding, 1979).

6.3.3.3.2.2. Kinoprene (ENSTAR®) This is also an extremely mild JHA with little or no toxic effects. It breaks down in the environment and is relatively nonpersistent. It is relatively nonhazardous to bees and is nontoxic to birds, fish, and beneficial insects. It is decomposed by sunlight. It has been effectively used for controlling scales, mealy bugs, aphids, whiteflies, and fungal gnats. Its effects are morphological, ovicidal, and it acts as a sterilant (Harding, 1979).

6.3.3.3.2.3. Fenoxycarb (INSEGAR®, LOGIC®, TORUS®, PICTYL®, VARIKILL®) Fenoxycarb was the first phenoxy JHA found to be effective (Grenier and Grenier, 1993). It is unusual in having a carbamate moiety but does not show any carbamate-like toxicity. This JHA is considered slightly toxic to nontarget species, especially aquatic crustaceans, and carries the signal word "caution" on its label. It has been used as an effective control agent against fire ants (as bait), fleas, mosquitos, cockroaches, scale insects, and sucking insects. It is considered a general use insecticide. Unlike methoprene and kinoprene, fenoxycarb can be toxic to some of the beneficial insects such as neuropterans (Liu and Chen, 2001).

6.3.3.3.2.4. Pyriproxifen (KNACK®, SUMILARV®, ADMIRAL®) This JHA is a phenoxy analog similar to fenoxycarb. It is by far one of the most potent JHAs currently available. It is mildly toxic to some aquatic organisms but is nontoxic to bees. Its effects are similar to the other JHAs and causes morphogenetic effects as well as sterility. It has been used effectively in the form of bait for controlling the red imported fire ant (*Solenopsis invicta*) in California. It has been used for controlling aphids, scales, whiteflies, and the Pear psylla, and the tsetse fly (*Glossina morsitans*) in Zimbabwe. In the latter, treatment of both sexes results in pyriproxifen being transferred to the female uterus where the embryo is killed (Langley *et al.*, 1990).

6.3.3.4. Use of JHAs for Pest Management

JJHAs have been successfully used for controlling certain types of pests, especially where the pest is the adult stage. It has not been very effective against most lepidopteran agricultural pests because the larval stage is responsible for crop destruction. While some of the control measures tested since the previous edition of this series (Retnakaran *et al.*, 1985) are described below, **Table 9** summarizes the effects of the various JHA insecticides on agricultural and stored insect pests.

6.3.3.4.1. Control of public health and veterinary insects In most instances, the adult form is the active pest and, therefore, these species can be managed by JHA insecticides. Mosquitos, fleas, cockroaches, fire ants, and tsetse flies are some of the major pests that are vulnerable to JHAs (**Table 10**).

6.3.3.4.2. Anti-JH for pest management Compounds that prevent JH production, facilitate JH degradation, or destroy the corpus allatum all belong to this group. It is a catchall of various compounds that negate the activity of JH. Ideally, such compounds should be very effective pest control agents. Treatment of newly emerged larvae with such a compound would theoretically create miniature pupae, thus abbreviating the destructive part of the insect's life history. Many of the anti-JH compounds turned out to be highly toxic and did not pan out as good control agents. Once the JH receptor is characterized, it can be a target for control. Some of the effects that have been studied with anti-JH compounds are shown in **Table 11**.

6.3.3.5. Effects on Nontarget Invertebrates and Vertebrates

The effects of JHA insecticides have been studied and extensively reviewed (Grenier and Grenier, 1993; Miyamoto *et al.*, 1993; Palli *et al.*, 1995). The phenoxy JHAs, fenoxycarb, and pyriproxifen have been shown to be toxic to a number of dipteran, coleopteran, and hemipteran predators and parasitoids of scale insects (Mendel *et al.*, 1994).

Table 9 Control of agricultural and stored product insects with JHA insecticides

Insect species	*Host*	*Hormone analog*	*Activity*	*Reference*
Nephotettix cincticeps N. nigropictus N. virescens Recilia dorsalis (leafhoppers)	Rice	NC-170 4-chloro-5-(6-chloro-3-pyridylmethoxy)-2-(3,4-dichlorophenyl)-pyridazin-3(2H)-one	Good activity, 100 ppm suppressed population for more than 6 weeks	Miyake *et al.* (1991)
Adoxophyes orana Pandemis heparana Archips podana, Archips rosana (leafrollers)	Apple Orchards	Fenoxycarb and epofenonane	Adequate reduction, reinfestation never resulted in an increase to a harmful population level; good activity	de Reede *et al.* (1985)
Lycoriella mali (mushroom pest)	Compost	Methroprene	Resistance to this compound does not seem to be developing rapidly	Keil *et al.* (1988)
Heliothis virescens (tobacco budworm)	Cotton	Fenoxycarb	Good activity, opens new aspects for the control of lepidopterous pests with this compound	Masner *et al.* (1986)
Rhagoletis pomonella (apple maggot)	Apple	Pyriproxyfen	Good activity	Duan *et al.* (1995)
Ceratitis capitata (Mediterranean fruit fly)	Fruits in general	Methoprene	Good activity prevents adult eclosion	Saul *et al.* (1987)
Adoxophyes orana Pandemis heparana (leafrollers)	Apple orchards	Epofenonane and fenoxycarb	Good activity, the foliar residue remained active for at least 4 weeks and parasites seem to be less susceptible to it than the host itself	de Reede *et al.* (1984)
Manduca sexta (tobacco hornworm)	Tobacco plants	JHAs such as 1,3- and 1,4-bisNCS	Good activity, can disrupt insect growth and development	Ujváry *et al.* (1989)
Ceroplastes floridensis (Florida wax scale)	Guava plants	Fenoxycarb	Insects on treated plants deposited only a small amount of wax	Eisa *et al.* (1991)
Cacopsylla pyricola (Pear psylla)	Pear	Fenoxycarb	May still be a viable strategy for managing this	Krysan (1990)
Melanoplus sanaguinipes, M. differentialis (grasshopper)	Rangelands and crops	Fenoxycarb	Good activity; eliminates egg production and reduces oviposition	Capinera *et al.* (1991)
Spodoptera littura (tobacco cutworm)	Vegetable crops	Pyriproxyfen	Good activity, reduces the number of eggs	Hastakoshi (1992)
Christoneura rosaceana (leafroller)	Fruit trees	Fenoxycarb	Good activity, abnormal development is noted in all treatments	Aliniazee and Arshad (1998)
Cydia pomonella (codling moth)	Apple and pear	Phenoxyphenol pyridine and pyrazine carboxamides *N*-(4-phenoxyphenol) pyridinecarboxamides and *N*-(4-phenoxyphenyl) pyrazinecarboxamides	Good activity, prolongs development	Balcells *et al.* (2000)
Laodelphax striatellus (brown planthopper)	Rice	NC-170 4-chloro-5-(6-chloro-3-pyridylmethoxy)-2-(3,4-dichlorophenyl)-pyridazine-3(H)-one	Good activity, 100 ppm prevents larvae from entering diapause	Miyake *et al.* (1991)
Dacus dorsalis (oriental fruit fly)	Papayas	Methoprene	Good activity, higher doses showed no survival	Saul *et al.* (1987)
Trichoplusia ni (cabbage looper)	Cabbage	Methoprene	Good activity, amount of eggs is reduced	Campero and Haynes (1990)

Table 9 Continued

Insect species	*Host*	*Hormone analog*	*Activity*	*Reference*
Bemisia tabaci (sweet potato whitefly)	Cotton and vegetable crops	Pyriproxyfen	Good activity, strong translaminar effect and acts on all stages	Ishaaya and Horowitz (1992)
Bemisia tabaci (sweet potato whitefly)	Cotton and vegetable crops	insect growth regulators such as buprofezin and pyriproxyfen	activity not well seen	Palumbo *et al.* (2001)
Trialeurodes vaporariorum (greenhouse whitefly)	Greenhouse crops	Pyriproxyfen	Good activity, affects all stages	Ishaaya *et al.* (1994)
Reticulitermes santonensis Reticulitermes flaviceps Coptotermes formosanus (termites)	Wood	Carbamate derivative of 2-(4-hydroxybenzyl) cyclokexanone (W-328)	Good activity, 500 ppm concentration results in significant soldier differentiation	Hrdý *et al.* (2001)
Reticulitermes speratus (termites)		Ethyl(2-(*p*-phenoxy-phenoxy)ethyl)carbamate	Good activity, results in the collapse of the whole termite colony	Tsunoda *et al.* (1986)
Ips paraconfusus Lanier (California beetle)	Logs of ponderosa pine	Fenoxycarb	Good activity, acts as an effective chemosterilant	Chen and Border (1989)
Lipaphis erysimi (mustard aphid)	Leafy vegetables	Pyriproxyfen	Good activity, causes direct mortality, reduces longevity and inhibits progeny formation	Liu and Chen (2000)
Cacopsylla pyricola (Pear psylla)	Pear	Fenoxycarb	Good activity	Lyoussoufi *et al.* (1994)
Reticulitermes speratus (Japanese subterranean termite)	Wood in general	Ethyl (2-(*p*-phenoxy-phenoxy)ethyl)carbamate	Good activity, disturbance of caste differentiation results in collapse of the colony	Tsunoda *et al.* (1986)
Rhyzopertha dominica (lesser grain borer)	Wheat grain	2′,7′,-epoxy-3,7′-dimethyl-undec-2′-enyl6-thyl-3-pyridyl ether	Good activity, 4 ppm resulted in insect mortality	Mkhize (1991)
Tribolium castaneum (red flour beetle), *Rhyzopertha dominica* (lesser grain borer), *Sitophilus oryzae* (rice weevil)–red flour beetle	Wheat flour	Methoprene and pyriproxyfen	Good activity, 20 ppm reduced the population by 80–99%	Kostyukovsky *et al.* (2000)

However, the ectoparasite of the California red scale and Florida wax scale, *Aphytis holoxanthus*, was insensitive to pyriproxifen (Peleg, 1988). Fenoxycarb was also found to be toxic to *Colpoclypeus florus*, a parasitoid of *Adoxophyes orana* and *Pandemis heparana*. Pupation in the predatory coccinellid, *Chilocorus bipustulatus* L., was inhibited when larvae fed on the scale insect, *Chrysomphalus aonidum*, were dipped into a 0.025% solution of fenoxycarb (cited in Grenier and Grenier, 1993). Additional effects of JHA insecticides have been summarized in Table 12.

The toxicological profiles of JHA insecticides for mammalian (rat), aquatic (rainbow trout and Daphnia), predatory, and parasitoid insects have been summarized in Table 13. In general, JHA insecticides have low acute toxicity to fish, birds, and mammals. Both fenoxycarb and pyriproxifen have very low toxicity to adult bees. However, effects on brood development have been observed as a result of worker bees feeding pollen containing fenoxycarb residues to larvae (Wildboltz, 1988).

Last instars of certain aquatic insects are susceptible to JHA insecticides. Morphogenetic abnormalities have been observed in the heteropteran, *Notonecta unifaciata*, and in the dragon flies, *Anax junius* and *Pantala hymenaea*, with applications of fenoxycarb (Miura and Takahashi, 1987). Similarly, pyriproxifen also produced morphogenetic effects when applied to last instars of the

Table 10 Control of some insect pests of public health and veterinary importance with JHA insecticides

Insect species	*Host*	*Hormone analog*	*Activity*	*Reference*
Monomorium pharaonis (pharaoh ant)	Large office buildings, houses, apartment buildings, food establishments and hospitals	Pyriproxyfen	Good activity when used at concentrations of 0.25%, 0.5%, and 1%	Vail *et al.* (1995)
Culex quinquefasciatus Aedes aegypti (mosquitos)	Humans	VCRC/INS/A-23	Activity was good and the compound was found cost effective	Tyagi *et al.* (1985)
Chironomus fusciceps (midge)	Sulfur containing pods near residential areas	Pyriproxyfen	Good activity, at 0.00177 and 0.05369 ppm caused 50 to 90% emergence inhibition	Takagi *et al.* (1995)
Glossian morsitans, G. pallidipes (testse flies)	Cattle and humans	Pyriproxyfen	Good activity, it can replace pesticides	Hargrove and Langley (1993)
Blattella germanica (german cockroach)	Residences	Fenyxocarb and hydroprene	It can reduce the population	King and Bennett (1988)
Anopheles balabacensis (mosquitos)	Humans	Pyriproxyfen	Good activity, 0.005 ppb reduce the egg and sperm production and blood feeding and copulating activity	Iwanaga and Kanda (1988)
Ctenocephalides felis (cat flea)	Cat	Methoprene	Activity is 12.7 and 127 ng cm^{-2}, resulted in 15–5.2% adult emergence. Good control	Moser *et al.* (1992)
Solenopsis invicta (red imported fire ant)		Pyriproxyfen	Good activity, cause 80–85% reduction in colony size	Banks and Lofgren (1991)
Blattella orientalis (oriental cockroach)	Industrial, food manufacturing, and domestic premises	*S*-hydroprene	Good activity	Edwards and Short (1993)
Ctenocephalides felis (cat flea)	Cat	Methoprene	Good activity when cat fleas less than 24 h old were treated	Olsen (1985)
Culex pipiens pallens Culex tritaeniorhynchus	Humans	Pyriproxyfen	Activity remains unclear	Kamimura and Arakawa (1991)
Ctenocephalides felis and Ctenocephalides canis (dog and cat fleas)	Cat and dog	Methoprene and pyriproxifen	Good activity	Taylor (2001)
Culex pipiens (mosquitos)	Humans	4-alkoxyphenoxy- and 4-(alkylphenoxy) alkanaldoxime o-ethers	Good activity	Hayashi *et al.* (1989)
Culex pipiens	Humans	(Phenoxyphenoxy) and (benzylphenoxy) propyl ethers	Good activity	Niwa *et al.* (1989)
Alphitobius diaperinus (mealworm)	Poultry production	Methoprene and fenoxycarb	Good activity	Edwards and Abraham (1985)
Ctenocephalides felis (cat fleas)	Cats	CGA-2545′728	Good activity	Rasa *et al.* (2000)
Ctenocephalides felis (cat fleas)	Cats	Methoprene, pyriproxifen and fenoxycarb	Good activity	Miller *et al.* (1999)

Table 11 Insect control with some anti-JH compounds

Insect species	*Host*	*Hormone analog*	*Activity*	*Reference*
Manduca sexta (tobacco hornworm)	Tobacco, tomato	ETB (ethyl 4-[2-{*tert*-butyl carbonyloxy} butoxy]benzoate)	Some activity, 50 µg of ETB caused formation of larval–pupal intermediates after the fourth instar	Beckage and Riddiford (1983)
Bombyx mori (silkworm)	Mulberry	1-Citronellyl-5-phenylimidazole (KK-22)	Some activity, induction of precocious metamorphosis seems to correlate with prolongation of the larval developmental period in the third instar	Kuwano and Eto (1984)
Blattella germanica (German cockroach)	Household pest	Precocenes	Short-term activity seems to be effective and long-term activity shows that damage to CA is irreversible	Belles *et al.* (1985)
Bombyx mori (silkworm)	Mulberry	Ethoxy (KK-110) and 4-chlor-phenyl (KK-135)	Activity can be inhibited by stimultaneous application of methoprene	Kuwano *et al.* (1990)
Nilaparvata lugens (brown planthopper)	Rice	Precocene II	Activity with an increase in good ovarian growth	Ayoade *et al.* (1995)
Spodoptera littoralis (Egyptian cotton worm)	Cotton	Precocene I and II	Good activity but should be undertaken with care in order to minimize disruptive effects on parasitoids	Hagazi *et al.* (1998)
Hyphantria cunea (Fall webworm)	Hardwood trees	Fluoromevalonate (Fmev, ZR-3516)	Activity was shown as evoking varying degrees of ecdysial disturbance, which resulted in death of the insect	Farag and Varjas (1983)
Oxycarenus lavaterae (Lygaeid)	European forests	Precocenes	Activity shows a high degreee of uniformity	Belles and Baldellou (1983)
Bombyx mori (silkworm)	Mulberry	1-Benzyl-5-[(E)-2,6-dimethyl-1,5-heptadienyl] imidazole (KK-42)	Good activity, 100% precocious pupation	Kuwano *et al.* (1985)
Locusta migratoria migratorioides; Oncopeltus fasciatus; Schistocerca gergaria (locusts)	Various plants	Precocene II	Good activity, ovipositor protrusion, and death at higher concentrations	Degheele *et al.* (1986)
Nilaparvata lugens (Brown planthopper)	Rice	Precocene II (anti-allatin)	Good activity with high rate of mortality	Pradeep and Nair (1989)
Spodoptera mauritia (lawn army worm)	Agricultural crops	Fluoromevalonate (Fmev)	Good activity, induced various morphogenetic abnormalities, death before pupation occurred	Nair and Rajalekshmi (1989)
Drosophila melanogaster (fruit fly)	Various fruits	5-Ethoxy-6-(4-methoxyphenyl)methyl-1,3-benzodioxole J2581	Activity can be reversed by application of JH of methoprene	Song *et al.* (1990)
Diploptera punctata (cockroach)	Various types of diet	1,5-disubstituted imidaxoles	Good activity, powerful inhibitors of the last step of juvenile synthesis	Unnithan *et al.* (1995)
Manduca sexta (tobacco hornworm)	Tomato and tobaco	Farnesol dehydrogenase	Good activity, unique dehydrogenase that should be examined further	Sen and Garvin (1995)
Oncopeltus fasciatus (milkweed bug)	Milkwood	6,7-Dimethoxy-2,2-dimethyl 2*H*-chromene	Activity shows defiency of JH	Bowers and Unnithan (1995)

Continued

Table 11 Continued

Insect species	*Host*	*Hormone analog*	*Activity*	*Reference*
Heliothis virescens and *Trichoplusia ni* (cabbage looper)	Field crops and vegetables	Virus AcJHE-SG (transgenic virus with JH esterase gene)	Good activity, death occurred from contraction-paralysis and disruption of the normal sequence of events at the molt	Bonning *et al.* (1995)
Sogatella furcifera (whitebacked rice planthopper)	Rice	Precocene II	Activtiy showed inductio metathetely by itself occurred	Miyake and Mitsui (1995)
Zaprionus paravittiger (banana fruitfly)	Bananas	Precocene	Good activity, with 0.076, 0.1 ppm in 1 μL of acetone, adult longevity and fecundity were reduced	Rup Pushpinder and Baniwal (1985)
Musca domestica (housefly) *Diploptera punctata* (cockroach)	Various diets	2-(1-Imidazolyl)-2-methyl-1-phenyl-1-propanone and 2-methyl-1-phenyl-2-(1,2,4,-triazol-1-yl)-1-propanone	Activity was seen at 0.2 mM	Bélai *et al.* (1988)
Aphis craccivora (ground nut aphid)	Legumes	Precocene II	Good activity, with 2.0 μg/aphid, precocious metamorphosis was seen	Srivastava and Jaiswl (1989)
Pieris brassicae (cabbage butterfly) and *Leptimotarsa decemlineata* (Colorado potato beetle)	Vegetables and potato	2,2-Dimethylchromene derivatives	Good activity	Darvas *et al.* (1989)
Heliothis zea (corn earworm)	Corn	Precocene II	Good activity, growth and development were inhibited	Binder and Bowers (1991)

Table 12 Effects of JH analog insecticides on beneficial and aquatic species

Parasite/predator/aquatic	*Host/habitat*	*Hormone analog*	*Activity*	*Reference*
Chilocorus nigritus	Citrus red scale	Pyriproxyfen	Harmful to nontargeted species	Magaula and Samways (2000)
Daphnia pulex	Aquatic	Methoprene	Decrease in the incidence of all-male broods and an increase in the incidence of all-female broods	Peterson *et al.* (2000)
Aphanteles congregatus	Tobacco hornworm	Methoprene	Low dose would allow the insect to pupate normally	Beckage and Riddiford (1981)
Copidosoma floridanum	Cabbage looper	Methoprene	Slight activity, inhibited morphogenesis	Strand *et al.* (1991)
Encarsia pergandiella E.transvena, E.formosa	White fly	Pyriproxyfen	Relatively safe to parasitoids	Liu and Stansly (1997)
Moina macrocopa	Aquatic	Methoprene	Low activity on reproduction	Chu *et al.* (1997)
Ceroplastes destructor	Citrus orchards	Pyriproxyfen and fenoxycarb	Arrested the first and second instar	Wakgari and Giliomee (2001)
Chrysoperla rufilabris	Aphids	Fenoxycarb and pyriproxyfen	Ovicidal effect and delay in development	Liu and Chen (2001)
Rhithropanopeus harrishii (mud crab)	Aquatic	Methoprene	No activity was noticed	Celestial and McKenney (1993)

Table 13 Ecotoxicological profile of JHA insecticides (from various EPA reports)

Compound	*Mammals (rat) LD_{50}*	*Fish (rainbow trout) 96h LC_{50}*	*Crustacea (Daphnia) 48h LC_{50}*	*Honey bees LC_{50}*	*Predators*	*Parasitoids*
Methoprene[a]	$>34\,g\,kg^{-1}$	$>3.3.\,mg\,l^{-1}$	$>0.51\,mg\,l^{-1}$	$>1000\,mg\,bee^{-1}$	Minimal effects	Minimal effects
Kinoprene[b]	$>5\,g\,kg^{-1}$	$>20\,mg\,l^{-1}$	$>0.113\,mg\,l^{-1}$	Nontoxic	Minimal effects	Minimal effects
Fenoxycarb[c]	$>10\,g\,kg^{-1}$	$>1.6\,mg\,l^{-1}$	$>1.6\,mg\,l^{-1}$	>1000 ppm	Low toxicity	Low toxicity
Pyriproxifen[d]	$>5\,g\,kg^{-1}$	$>325\,mg\,l^{-1}$	$>400\,mg\,l^{-1}$	$>100\,mg\,bee^{-1}$	Low toxicity	Low toxicity

[a]ALTOSID®, APEXSE®, DIANEX®, PHARORID®, VIODAT®.
[b]ENSTAR®.
[c]INSEGAR®, LOGIC®, TORUS®, PICTYL®, VARIKILL®.
[d]KNACK®, SUMILARV®, ADMIRAL®.

dragonfly, *Orthetrum albistrum* and the midge, *Chironomus yoshimatsui* (Miyamoto *et al.*, 1993).

6.3.3.6. Conclusions and Future Research of JHAs

JHAs have not proven to be the wonderful control agents they were purported to be. However, they have advantages for controlling pests of public health. Many of them are environmentally attractive. The anti-JH compounds have, for the most part, remained at the experimental stage. Interfering with JH action will become an attractive option, once the JH receptor is characterized.

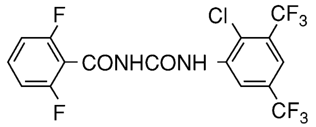
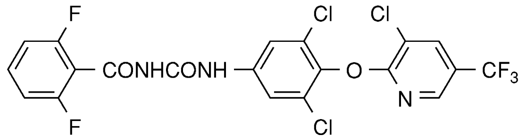
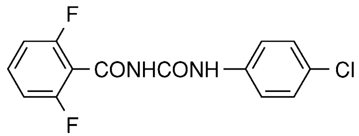
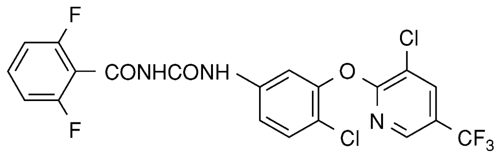
6.3.4. Insecticides with Chitin Synthesis Inhibitory Activity

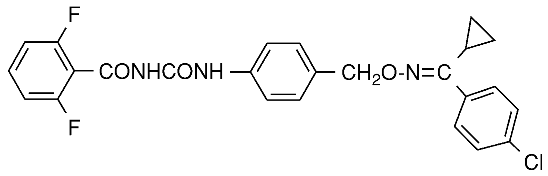
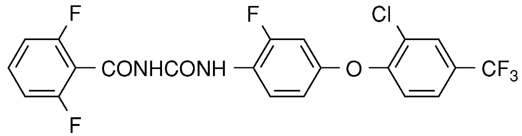
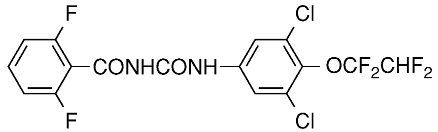
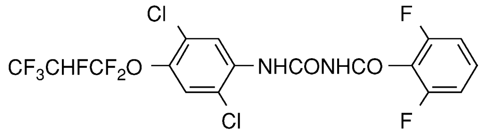
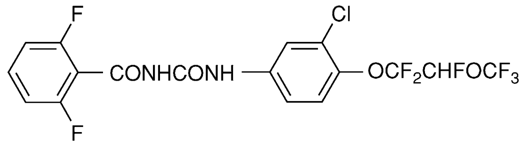
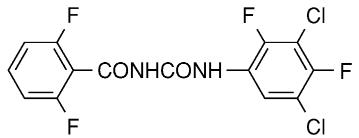
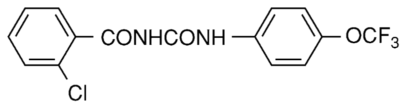
If the insect cuticle, which provides the exoskeletal structure, is disrupted during its formation, it is lethal to the insect. The cuticle needs to be waterproof for protection, soft and flexible to allow movement, extensible in between segments to allow for increase during feeding and growth, and also rigid to provide firm points of muscle attachment as well as for mandibles and claws. The cuticle is the product of a single layer of epidermal cells, and consists of different layers of which the procuticle contains chitin, a major component (30–60%) of insect cuticle. Chitin is a β-1,4-linked aminopolysaccharide homopolymer of *N*-acetylglucosamine (GlcNAc), and is by far one of the most abundant biological materials found on earth. This nitrogenous amino sugar polysaccharide is cross-linked to proteins via biphenyl linkages to form a protective matrix, which consists of a chitin microfibers–protein complex. Chitin biosynthesis, which is not fully understood, is a complex process consisting of a series of enzymatic steps beginning with a glucose unit, which is converted to GlcNAc that is linked with UTP, transported within the cell in combination with dolichol phosphate, and polymerized into chitin. The newly polymerized chitin is covalently linked to proteins to form chitin microfibrils in the cuticle. Polymerization to form chitin is catalyzed by the enzyme chitin synthase (CHS or chitin-UDP-glucosamine-transferase), which occurs in several forms (CHS 1, 2 and 3; EC 2.4.1.16). This enzyme has probably undergone sequential gene duplication and divergence during evolution, resulting in its expression in different forms in diverse species, one of which is the human hyaluronan synthase. It has a conserved amino acid sequence essential for chitin biosynthesis in yeasts, and it has evolved into two types: the fungal form, which occurs as an inactive zymogen requiring proteolysis for activation, and the arthropod form, which is membrane bound. For more details on chitin biosynthesis and cuticle formation, the reader should refer to **Chapter 4.2.** Over the past three decades, the chitin biosynthetic pathway has proven to be important for developing insect control agents that selectively inhibit any of the chitin synthetic steps in insects. Two types of insect regulatory chitin synthesis inhibitors (CSI) have been developed and used as commercial compounds for controlling agricultural pests: the benzoylphenyl ureas (BPUs), and buprofezin and cyromazine (Spindler *et al.*, 1990; Retnakaran and Oberlander, 1993; Londerhausen, 1996; Palli and Retnakaran, 1999).

6.3.4.1. Brief Review of Old Chitin Synthesis Inhibitors

The insecticidal activity of the first BPUs was discovered around 1970 by scientists at Philips-Duphar BV, (now Crompton Corp., Weesp, The Netherlands) and the first commercial compound of this series was diflubenzuron, Dimilin® (Van Daalen *et al.*, 1972; Grosscurt, 1978) marketed by Uniroyal Chemical (Table 14). Diflubenzuron and older CSIs have already been extensively reviewed by Retnakaran *et al.* (1985), Grosscurt *et al.* (1987), and Retnakaran and Wright (1987).

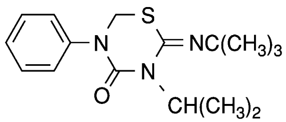
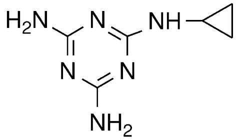
Table 14 Chemical structures of the chitin synthesis inhibitors (CSI), 11 benzoylphenyl ureas (BPUs), and buprofezin and cyromazine, their biological effects and their target pest species

Compound	*Chemical structure*	*Biological effects*	*Uses to control pest species*
Bistrifluron		Inhibition of larval molting leading to death	Whiteflies in tomato (*Trialeurodes vaporariorum* and *Bemisia tabaci*), and lepidopterous insects in vegetables, cabbage, persimmon, apples, and other fruits (e.g., *Spodoptera exigua*, *Plutella xylostella*, *Stathmopoda masinissa*, and *Phyllonorycter ringoniella*) at 75–400 g ha^{-1}
Chlorfluazuron		Acts as an antimolting agent, leading to death of the larvae and pupae	*Heliothis*, *Spodoptera*, *Bemisia tabaci*, and other chewing insects on cotton, and *Plutella*, *Thrips* and other chewing insects on vegetables. Also used on fruit, potatoes, ornamentals, and tea. Applied at 2.5 g hl^{-1}
Diflubenzuron		Nonsystemic insect growth regulator with contact and stomach action. Acts at time of insect molting, or at hatching of eggs	Wide range of leaf-eating insects in forestry, woody ornamentals, and fruit. Controls certain major pests in cotton, soybeans, citrus, tea, vegetables, and mushrooms. Also controls larvae of flies, mosquitos, grasshoppers, and migratory locusts. Used as an ectoparasiticide on sheep for control of lice, fleas, and blowfly larvae. Is suitable for inclusion in integrated control programs. Effective at 25–75 g a.i./ha against most leaf-feeding insects in forestry; in concentrations of 0.01–0.015% a.i. against codling moth, leaf miners, and other leaf-eating insects in top fruit; in concentrations of 0.0075–0.0125% a.i. against citrus rust mite in citrus; and at a dosage of 50–150 g a.i./ha against a number of pests in cotton (cotton boll weevil, armyworms, leafworms), soya beans (soya bean looper complex) and maize (armyworms). Also for control of larvae of mushroom flies in mushroom casing (1 g a.i. m^{-2}); mosquito larvae (25–100 g a.i. ha^{-1}); fly larvae in animal housings (0.5–1 g a.i. m^{-2} surface); and locusts and grasshoppers (60–67.5 g a.i. ha^{-1})
Fluazuron		Systemic acarine growth regulator with contact and stomach action inhibiting chitin formation	Ixodicide for strategic control of the cattle tick *Boophilus microplus* (including all known resistant strains) on beef cattle

Flucycloxuron		Nonsystemic acaricide and insecticide inhibiting the molting process in mites and insects. It is only active against eggs and larval stages. Adult mites and insects are not affected	Eggs and larval stages of Eriophyid and Tetranychid mite species on a variety of crops, including fruit crops, vegetables, and ornamentals. Also controls larvae of a number of insects. Because of its relative selectivity, it is well-suited for integrated control programs. Recommended concentration for control of mites on fruit crops is 0.01–0.015% a.i. On insects, good activity has been found against codling moth, leaf miners, and some leaf rollers in pome fruit, at the same concentration. In ornamentals grown under glass/plastic, lower concentrations can be used. In grapes, the recommended dosage is 125–150 g a.i. ha^{-1}
Flufenoxuron		Insect and acarid growth regulator with contact and stomach action. Treated larvae die at the next molt or during the ensuing instar. Treated adults lay nonviable eggs	Control of immature stages of many phytophagous mites (*Aculus*, *Brevipalpus*, *Panonychus*, *Phyllocoptruta*, *Tetranychus* spp.) and insect pests on pome fruit, vines, citrus fruit, tea, cotton, maize, soybeans, vegetables, and ornamentals
Hexaflumuron		Ingested, systemic insecticide	In agriculture for control of larvae of Lepidoptera, Coleoptera, Homoptera, and Diptera on top fruit, cotton, and potatoes. Major use now is in bait, for control of subterranean termites
Lufenuron		Insecticide/acaricide acting mostly by ingestion; larvae are unable to molt, and also cease feeding	Lepidoptera and Coleoptera larvae on cotton, maize, and vegetables; and citrus whitefly and rust mites on citrus fruit, at 10–50 g ha^{-1}. Also for the prevention and control of flea infestations on pets
Novaluron		Insecticide acts by ingestion and contact, affecting molting. Causes abnormal endocuticular deposition and abortive molting	Under development by Makhteshim Chemical Works for control of Lepidoptera, whitefly, and agromyzid leaf miners in top fruit, vegetables, cotton, and maize
Teflubenzuron		Antimolting agent, leading to death of the larvae and pupae	*Heliothis*, *Spodoptera*, *Bemisia tabaci* and other chewing insects on cotton; and *Plutella*, *Thrips* and other chewing insects on vegetables. Also used on fruit, potatoes, ornamentals, and tea. Applied at 2.5 g hl^{-1}.
Triflumuron		Ingested insecticide, acting by inhibition of molting	Lepidoptera, Psyllidae, Diptera, and Coleoptera on fruit, soybeans, vegetables, forest trees, and cotton. Also used against larvae of flies, fleas, and cockroaches in public and animal health

Continued

Table 14 Continued

Compound	*Chemical structure*	*Biological effects*	*Uses to control pest species*
Buprofezin		Probable chitin synthesis and prostaglandin inhibitor. Hormone disturbing effect, leading to suppression of ecdysis. Persistent insecticide and acaricide with contact and stomach action; not translocated in the plant. Inhibits molting of nymphs and larvae, leading to death. Also suppresses oviposition by adults; treated insects lay sterile eggs	Insecticide with persistent larvicidal action against Homoptera, some Coleoptera, and also Acarina. Effective against Cicadellidae, Deltocephalinae (leafhoppers), and Delphacidae (planthoppers) in rice, at 50–250 g ha^{-1}; Cicadellidae (lady beetle) in potatoes; Aleyrodidae (whitefly) in citrus, cotton, and vegetables, at 0.025–0.075 g ha^{-1}; Coccidae, Diaspididae (scale insects) and Pseudococcidae (mealybugs) in citrus and top fruit, at 25–50 g hl^{-1}; Tarsonemidae in vegetables, at 250–500 g ha^{-1}. Suitable for IPM programs
Cyromazine		Contact action, interfering with molting and pupation. When used on plants, action is systemic: applied to the leaves, it exhibits a strong translaminar effect; applied to the soil, it is taken up by the roots and translocated acropetally	Control of Diptera larvae in chicken manure by feeding to poultry or treating the breeding sites. Also used to control flies on animals. Used as a foliar spray to control leaf miners (*Liriomyza* spp.) in vegetables (e.g., celery, melons, tomatoes, lettuce), mushrooms, potatoes, and ornamentals, at 75–225 g ha^{-1}; also used at 190–450 g ha^{-1} in drench or drip irrigation

Data compiled from Palli, S.R., Retnakaran, A., **1999**. Molecular and biochemical aspects of chitin synthesis inhibition. In: Jollès, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser-Verlag, pp. 85–98; Tomlin, C.D.S., Ed., **2000**. The Pesticide Manual, 12th edn. British Crop Protection Council Publications; and Ishaaya, I., **2001**. Biochemical processes related to insecticide actions: an overview. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, pp. 1–16.

The discovery of diflubenzuron spawned the discovery and development of a whole array of new analogs by different agricultural companies (Table 14). These include: chlorfluazuron (AIM®, ATABRON®, HELIX®, JUPITER®; Ishihara Sangyo), flucycloxuron (ANDALIN®; Uniroyal Chemical), flufenoxuron (CASCADE®; BASF), hexaflumuron (CONSULT®, RECRUIT®, TRUENO®; Dow AgroSciences LLC), lufenuron (MATCH®; Syngenta AG), teflubenzuron (NOMOLT®, DART®, DIARACT®; BASF) and triflumuron (ALSYSTIN®, BAYCIDAL®; Bayer AG) (Zoebelein *et al.*, 1980; Haga *et al.*, 1982, 1992; Becher *et al.*, 1983; Sbragia *et al.*, 1983; Retnakaran *et al.*, 1985; Anderson *et al.*, 1986; Retnakaran and Wright, 1987; Grosscurt *et al.*, 1987; Sheets *et al.*, 2000; Tomlin, 2000).

6.3.4.2. New Chemistries and Products

In the last decade, some newer BPU analogs were discovered: novaluron (RIMON®), bistrifluron, fluazuron (ACATAK®), and noviflumuron (RECRUIT® III) by Makhteshim Chemicals, Dongbu Hannong Chemical Co., Syngenta AG, and Dow AgroSciences LLC, respectively (Bull *et al.*, 1996; Ishaaya *et al.*, 1996; Kim *et al.*, 2000; Tomlin, 2000; Karr *et al.*, 2004) (Table 14).

Two other IGRs, buprofezin (APPLAUD®) and cyromazine (NEOPREX®, TRIGARD®, VETRAZIN®), with chemistries different from BPUs, but which also interfere with molting and chitin biosynthesis were developed by Nihon Nohyaku and Ciba-Geigy AG (now Syngenta AG), respectively (Table 14; Hall and Foehse 1980; Williams and Berry, 1980; Kanno *et al.*, 1981; Reynolds and Blakey, 1989; Tomlin, 2000).

6.3.4.3. Mode of Action and SAR

Typically, the chemistry and symptoms of the CSIs are unique, and not similar to other insecticides. Studies with diflubenzuron, the most thoroughly investigated compound of the BPUs, revealed that it alters cuticle composition, especially inhibition of chitin, resulting in abnormal endocuticular deposition that affects cuticular elasticity and firmness, and causes abortive molting. The reduced chitin levels in the cuticle seem to result from inhibition of biochemical processes leading to chitin formation. To date, it is not clear whether inhibition of chitin synthetase is the primary biochemical site for the reduced level of chitin, since in some studies BPUs do not inhibit chitin synthetase in cell-free systems (Retnakaran *et al.*, 1985; Grosscurt and Jongsma, 1987; Retnakaran and Wright, 1987; Londerhausen, 1996; Oberlander and Silhacek, 1998; Palli and Retnakaran, 1999). In addition to chitin synthetase inhibition, other modes of action have been suggested for BPUs, such as: (1) inhibit the transport of UDP-GlcNAc across biomembranes; (2) block the binding of chitin to cuticular proteins resulting in inhibition of cuticle deposition and fibrillogenesis; (3) inhibit the formation of chitin due to an inhibition of the protease that activates chitin synthase, and activation of chitinases and phenoloxidases, which are both connected with chitin catabolism; (4) affect ecdysone metabolism, resulting in ecdysone accumulation that stimulates chitinase, which in turn digests nascent chitin; (5) block the conversion of glucose to fructose-6-phosphate; and (6) inhibit the DNA synthesis (Soltani *et al.*, 1984; Retnakaran *et al.*, 1985; Cohen, 1985; Retnakaran and Wright, 1987; Retnakaran and Oberlander, 1993; Mikólajczyk *et al.*, 1994; Zimowska *et al.*, 1994; Oberlander and Silhacek, 1998; Palli and Retnakaran, 1999; Oberlander and Smagghe, 2001). In addition, recent studies using imaginal discs and cell-free systems indicated that BPUs inhibit the 20-hydroxecdysone dependent GlcNAc incorporation into chitin, suggesting that BPUs affect ecdysone dependent biochemical sites, which lead to chitin inhibition (Mikolajczyl *et al.*, 1994; Zimowska *et al.*, 1994; Oberlander and Silhacek, 1998). BPUs act mainly by ingestion, and for the most part they are effective as larvicides, but in some species they also suppress oviposition. They act as ovicides, reducing the egg-laying rate or hindering the hatching process by inhibiting embryonic development. In most cases, the embryo was fully developed in the egg but the larva failed to hatch (Retnakaran and Wright, 1987; Ishaaya, 2001). BPUs were also found to reduce cyst and egg production in two free-living plant nematodes (Veech, 1978; Evans, 1985). In addition to being toxic by ingestion, some BPUs exhibit contact toxicity. Moreover, most BPUs, except hexaflumiron, have no systemic or translaminar activity (Retnakaran and Wright, 1987; Tomlin, 2000; Ishaaya, 2001).

The mode of action of buprofezin resembles that of the BPUs, although its structure is not analogous (Uchida *et al.*, 1985; De Cock and Degheele, 1998; Ishaaya, 2001). The compound strongly inhibits the incorporation of ^{3}H-glucose and GlcNAc into chitin. As a result of chitin deficiency, the procuticle of treated larval stages loses its elasticity and the insect is unable to molt. In addition, buprofezin may work as a prostaglandin inhibitor that may lead to suppression of ecdysis and slightly reduced DNA synthesis. It also exerts its effect on egg-hatch and on the larval stages in the rice planthopper, *Nilaparvata lugens*, and the whiteflies, *Bemesia tabaci and Trialeurodes vaporariorum* (De Cock *et al.*, 1995;

De Cock and Degheele, 1998; Ishaaya, 2001). It has no ovicidal activity but suppresses embryogenesis through adults (**Table 14**).

Although the exact mode of action of the IGR cyromazine, which is predominantly active against dipteran larvae, is not known, evidence has been presented to suggest that its target site for interference with sclerotization is different from that of BPUs (Biddington, 1985). In larvae intoxicated with cyromazine, the cuticle rapidly becomes less extensible and unable to expand compared with the cuticle of untreated larvae. The cuticle may be stiffer because of increased cross-linking between the various cuticle components, the nature of which remains unknown. As summarized in **Table 14**, cyromazine is an IGR with contact action interfering with molting and pupation. It has good systemic activity. When applied to the leaves, it exhibits a strong translaminar activity, and when applied to the soil it is taken up by the roots and translocated acropetally (Hall and Foehse, 1980; Awad and Mulla, 1984; Reynolds and Blakey, 1989; Viñuela and Budia, 1994; Tomlin, 2000).

With classical SAR (Hansch-Fujita), the effects of different substituents on the benzene rings in BPUs were analyzed for larvicidal activity against *Chilo suppressalis*, *S. littoralis*, and *B. mori* (Nakagawa *et al*., 1987, 1989a, 1989b). For the benzoyl moiety, the toxicity was higher with a higher total hydrophobicity, a higher electron withdrawal from the side chain, and a lower steric bulkiness of *ortho*-substituents. In addition, introduction of electron-withdrawing and hydrophobic substituents at the *para*-position of the phenyl (aniline) moiety enhanced the larvicidal activity, whereas bulkier groups were unfavorable. Stacking did not occur between the two aromatic moieties along the urea moiety (Sotomatsu *et al*., 1987). To ascertain the above results, the relative activity of BPUs was determined by measuring the incorporation of [^{14}C]-GlcNAc in larval integuments cultured *in vitro* (Nakagawa *et al*., 1989b). There was a colinear relationship between *in vitro* activities and *in vivo* larvicidal toxicities if the hydrophobic factor(s) participating in transport were considered separately. In addition, integuments of the three Lepidoptera were incubated in conditions with and without the synergists piperonylbutoxide (PB) and *S*,*S*,*S*-tributylphosphorotrithioate (DEF). In the Qualitative Structure–activity Relation (QSAR) equation measured without synergist, an electron-withdrawing effect was favorable to the activity, but an electron-donating group was favorable to the activity in the presence of PB. These results mean that electron-withdrawing groups are playing a role in suppressing the oxidative metabolism. DEF had no significant effect, suggesting that hydrolytic degradation of the phenyl moiety was not of significant consequence as compared to its oxidative degradation. In summary, the SAR results suggested that for BPUs the specific larvicidal spectrum is due to inherent differences in metabolism in addition to differences in the physiochemical substituent effects (Nakagawa *et al*., 1987, 1989a, 1989b; Sotomatsu *et al*., 1987).

6.3.4.4. Spectrum of Activity

Most CSI compounds are very potent against a variety of different pests with the highest activity towards lepidopterous insects and whiteflies. The main commercial applications are in field crops/agriculture, forestry, horticulture, and in the home as summarized in **Table 14** (Retnakaran and Wright, 1987; Tomlin, 2000; Ishaaya, 2001). Novaluron acts by ingestion and contact against lepidopterans (*S. littoralis*, *S. exigua*, *S. frugiperda*, *Tuta absoluta* and *Helicoverpa armigera)*, whitefly, *B. tabaci,* eggs and larvae, and different stages of the leafminer, *Liriomyza huidobrensis*. Bistrifluron is active against various lepidopteran pests and whiteflies an apple, Brassica leafy vegetables, tomato, persimmon, and other fruits (Kim *et al*., 2000). Hexaflumuron and the newer noviflumuron are now used in bait for control of subterranean termites (Sheets *et al*., 2000; Karr *et al*., 2004). As a specialty in the series of CSIs, fluazuron is the only ixodicide with a strong activity against cattle ticks (Kim *et al*., 2000) (**Table 14**).

Buprofezin has a persistent larvicidal action against sucking Homoptera, such as the greenhouse whitefly, *T. vaporariorum,* the sweet potato whitefly, *B. tabaci*, both of which are important pests of cotton and vegetables, the brown planthopper, *N. lugens* in rice, the citrus scale insects, *Aonidiella aurantii* and *Sassetia oleae,* and some Coleoptera and Acarina. In contrast, cyromazine is used for control of dipteran larvae in chicken manure. It is also used as a foliar spray to control leafminers (*Liriomyza* sp.) in vegetables and ornamentals, and to control flies on animals (Hall and Foehse, 1980; Williams *et al*., 1980; Kanno *et al*., 1981; Reynolds and Blakey, 1989; Tomlin, 2000) (**Table 14**).

6.3.4.5. Ecotoxicology and Mammalian Safety

Overall, CSIs have selective insect toxicities and as such are considered “soft insecticides.” For diflubenzuron, the harbinger of all BPUs, its environmental fate has been extensively investigated and was shown to be broken down by various microbial

Table 15 Environmental effects of some CSIs

Compound	*Mammals*	*Fish*	*Crustacae*	*Bees*	*Predators*	*Parasitoids*
Bistrifluron	Rat $LD_{50} > 5\,g\,kg^{-1}$	Carp LC_{50} (48 h) $> 0.5\,mg\,l^{-1}$		$LD_{50} > 100\,\mu g\,bee^{-1}$		
Chlorfluazuron	Rat $LD_{50} > 8.5\,g\,kg^{-1}$	Carp LC_{50} (48 h) $> 300\,mg\,l^{-1}$	*Daphnia* LC_{50} (48 h) $0.9\,\mu g\,l^{-1}$	$LD_{50} > 100\,\mu g\,bee^{-1}$	Highly toxic	No – very little effects
Diflubenzuron	Rat $LD_{50} > 4.6\,g\,kg^{-1}$	Zebra fish, rainbow trout LC_{50} (96 h) $>0.2\,mg\,l^{-1}$	*Daphnia* LC_{50} (48 h) $7.1\,\mu g\,l^{-1}$	Not hazardous $LD_{50} > 100\,\mu g\,bee^{-1}$	Safe – little effects	Safe-little adverse effects
Flucycloxuron	Rat $LD_{50} > 5\,g\,kg^{-1}$	Rainbow trout, sunfish LC_{50} (96 h) $>0.1\,mg\,l^{-1}$	*Daphnia* LC_{50} (48 h) $4.4\,\mu g\,l^{-1}$	$LD_{50} > 100\,\mu g\,bee^{-1}$	No – moderate effects	No – harmful effects
Flufenoxuron	Rat $LD_{50} > 3\,g\,kg^{-1}$	Rainbow trout LC_{50} (96 h) $> 100\,mg\,l^{-1}$			No adverse effects – harmful	No – little adverse effects
Hexaflumuron	Rat $LD_{50} > 5\,g\,kg^{-1}$	rainbow trout: not lethal, *Tilapia* $LC_{50} > 3\,\mu g\,l^{-1}$	*Daphnia* LC_{50} (96 h) $0.1\,\mu g\,l^{-1}$	$LD_{50} > 100\,\mu g\,bee^{-1}$	Very little effects	Harmless, but ectoparasites strongly affected
Lufenuron	Rat $LD_{50} > 2\,g\,kg^{-1}$	Carp, rainbow trout, sunfish LC_{50} (96 h) $> 30–70\,mg\,l^{-1}$		$LC_{50} > 38\,\mu g/bee^{-1}$ $LD_{50} > 8\,\mu g/bee^{-1}$	Safe – harmful	Moderately harmful
Novaluron	Rat $LD_{50} > 5\,g\,kg^{-1}$	Rainbow trout, sunfish LC_{50} (96 h) $> 1\,mg\,l^{-1}$	*Daphnia* LC_{50} (48 h) $58\,\mu g\,l^{-1}$	Nontoxic at recommended rates; $LC_{50} > 100\,\mu g\,bee^{-1}$ $LD_{50} > 1\,mg\,bee^{-1}$	Low toxicity – harmful	Little–harmful effects
Teflubenzuron	Rat $LD_{50} > 5\,g\,kg^{-1}$	Trout, carp LC_{50} (96 h) $>0.5\,mg\,l^{-1}$		$LD_{50} > 1\,mg\,bee^{-1}$	Harmless – highly toxic	No – very little effects
Triflumuron	Rat $LD_{50} > 5\,g\,kg^{-1}$	Rainbow trout LC_{50} (96 h) $>320\,mg\,l^{-1}$	*Daphnia* LC_{50} (48 h) $225\,\mu g\,l^{-1}$	Toxic	Harmless – moderately toxic	Moderately harmful
Buprofezin	Rat LD_{50} $2.4\,g\,kg^{-1}$	Carp, rainbow trout LC_{50} (48 h) $>2.7–1.4\,mg\,l^{-1}$	*Daphnia* LC_{50} (3 h) $50.6\,mg\,l^{-1}$	No effect at $2\,g\,l^{-1}$	Harmless – moderately toxic	Very little effects
Cyromazine	Rat LD_{50} $3.4\,mg\,kg^{-1}$	Carp, rainbow trout, sunfish LC_{50} (96 h) $>90\,mg\,l^{-1}$	*Daphnia* LC_{50} (48 h) $>9.1\,mg\,l^{-1}$	No effect up to $5\,\mu g$	Harmless – moderately toxic	Harmless – moderate effects

Data compiled from Darvas, B., Polgár, L.A., **1998**. Novel-type insecticides: specificity and effects on non-target organisms. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer, pp. 188–259; Tomlin, C.D.S. (Ed.), **2000**. The Pesticide Manual, 12th edn. British Crop Protection Council Publications; and Ishaaya, I., **2001**. Biochemical processes related to insecticide actions: an overview. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, pp. 1–16.

agents without accumulation in soil and water. The fear that spray run-off into streams may cause widespread mortality of nontarget species has not been realized. Moreover, its chitin synthesis inhibitory action is quite specific, and related biochemical processes, such as chitin synthesis in fungi and biosynthesis of hyaluronic acid and other mucopolysaccharides in chickens, mice, and rats, are not affected. A representative list of the salient environmental effects of CSIs against mammals (rat), vertebrates (fish), crustaceans, bees, predators, and parasitoids is given in **Table 15**. However, owing to selective toxicity towards arthropods, BPUs have, varying degrees of effects on crustaceans as well as beneficial insects, which requires their use with care. In this case, the IOBC/WPRS (International Organization for Biological and Integrated control of Noxious Animals and Plants, West Palaearctic Regional Section, 2004) sequential testing scheme has proven its value for testing the side effects on a species-by-species basis and under (semi-)field conditions (Hassan, 1992). However, since most of the BPU formulations need to be ingested to be effective, topical effects on parasites, predators, and pollinators are minimal. Retnakaran and Wright (1987), Perez-Farinos *et al*. (1998), and Medina *et al*. (2002) claimed that the selectivity of BPUs may result from a relatively low cuticular absorption in residual and direct contact assays. As summarized in **Table 15**, the majority of the CSIs are harmless or exert little adverse effect on bees, predators, or parasitoids, which renders these BPUs acceptable for inclusion in integrated pest management (IPM) programs (Elzen, 1989; Hassan *et al*., 1991, 1994; Vogt, 1994; Van de Veire *et al*., 1996; Darvas and Polgar, 1998; Sterk *et al*., 1999; Tomlin, 2000).

6.3.4.6. Resistance, Mechanism for Resistance and Resistance Potential

Pimprikar and Georghiou (1979) were the first to report very high levels of resistance (>1,000-fold) to diflubenzuron in a housefly population stressed with the compound. Since then, several research groups in different parts of the world have reported resistance to CSIs in Lepidoptera, Diptera, and whiteflies.

In Southeast Asia, selection of diamondback moth, *Plutella xylostella,* from Thailand during six generations with chlorfluazuron and teflubenzuron resulted in resistance levels of 109-fold and 315-fold, respectively (Ismail and Wright, 1991, 1992). While marked cross-resistance between chlorfluazuron and teflubenzuron was demonstrated, there was no evidence of cross-resistance to diflubenzuron, and little or no cross-resistance to flufenoxuron and hexaflumuron. Pretreatment with the synergists PB and DEF increased the toxicity of chlorfluazuron and teflubenzuron up to 34-fold and 28-fold, respectively, suggesting that microsomal monooxygenases and esterases are involved in resistance. In another study with *P. xylostella* from Malaysia, selection with diflubenzuron increased resistance to teflubenzuron, but had no effect on chlorfluazuron (Furlong and Wright, 1994). In this study, while use of PB and DEF suggested the involvement of microsomal monooxygenases and esterases in teflubenzuron resistance, glutathion-*S*-transferase (GSTs) had limited involvement.

As documented by Moffit *et al*. (1988) and Sauphanor *et al*. (1998), the codling moth *C. pomonella* shows very high levels of resistance to diflubenzuron in the USA and France. In southern France, failure in *C. pomonella* control was observed for several years, revealing a 370-fold resistance for diflubenzuron and cross-resistance with two other BPUs, teflubenzuron (7-fold) and triflumuron (102-fold), as well as to the ecdysone agonist, tebufenozide (26-fold) (Sauphanor and Bouvier, 1995). Interestingly, resistance to diflubenzuron was linked to cross-resistance to deltamethrin. In both cases, enhanced mixed-function oxidase and GST activities are involved in resistance, rather than target site modification. In addition, a fitness cost described in both resistant strains was mainly associated with metabolic resistance (Boivin *et al*., 2001). Finally, a lack of relationship between ovicidal and larvicidal resistance for diflubenzuron in *C. pomonella* may be due to different transport properties as well as differential enzymatic metabolism (Sauphanor *et al*., 1998).

In some strains of *S. littoralis*, resistance to diflubenzuron was also increased by a factor of about 300 (El-Guindy *et al*., 1983). El Saidy *et al*. (1989) reported that diflubenzuron and teflubenzuron were hydrolyzed rapidly by all tissues tested, and the gut wall was the most active tissue reaching 61% hydrolytic breakdown for diflubenzuron and 16% for teflubenzuron. Interestingly, profenofos and DEF could inhibit degradation of both BPUs tested under optimal conditions. Ishaaya and Casida (1980) had earlier reported that these organophosphorous compounds can inhibit insect esterases in larval gut integument. The strong synergism activity of DEF and profenofos indicated that the major route of detoxification in *S. littoralis* was through hydrolysis, while oxidative metabolism was found to be of minor importance for resistance.

In the Australian sheep blowfly, *Lucilia cuprina*, resistance to diflubenzuron was inherited in a codominant (S male × R female) or incompletely recessive (R male × S female) manner, and there was also some maternal influence on inheritance of

monooxygenase activities, suggesting that diflubenzuron resistance is polygenic and involves mechanisms additional to monooxygenases (Kotze and Sales, 2001).

McKenzie and Batterman (1998) reported the development of resistance to cyromazine in populations of *L. cuprina*, which, however, had remained susceptible for almost 20 years of exposure. This resistance in *L. cuprina* was controlled by a single gene in each variant and two resistance loci were identified: one (Cyr4) closely linked to the marker "reduced eyes" on chromosome IV, and the other, Cyr5, closely linked to the "stubby bristles" marker on chromosome V (Yen *et al*., 1996). Similarly, in *D. melanogaster*, cyromazine resistance was due to a mutation in a single, but different gene than the one identified in resistant *L. cuprina*. The resistance genes, designated *Rst(2)Cyr* and *Rst(3)Cyr*, were localized to puff positions 64 on chromosome II and 47 on chromosome III, respectively (Adcock *et al*., 1993).

With respect to resistance in *Musca domestica*, Keiding (1999) reviewed the period 1977 to 1994. In this period, only two IGRs, diflubenzuron and cyromazine, were widely used for fly control, either by direct contact application to breeding sites, manure, garbage, etc., or as an admixture in feed for poultry or pigs. Widespread use of diflubenzuron or cyromazine by direct treatment of manure has not led to resistance of practical importance except for resistance to diflubenzuron on a few farms in the Netherlands. However, there are reports showing that the use of either compound mixed with feed has resulted in the development of moderate to high resistance to these compounds. High resistance in adult flies to organophosphates and pyrethroids does not usually confer cross-resistance to the larvicidal effects of diflubenzuron or cyromazine, but cross-resistance between organophosphorous compounds and JHAs (methoprene) has been reported. In Denmark, Keiding *et al*. (1992) came to the same conclusions where housefly control with both compounds was carried out on farms for 1–9 years. On none of the farms was any general increase of tolerance to diflubenzuron found. In the same study, two strains were selected with diflubenzuron and cyromazine where moderate to high resistance developed if the selection pressure was strong, especially when used as feed-through applications on poultry farms where all feed contains diflubenzuron or cyromazine. If the treatment of fly breeding sources is less complete, resistance problems may not develop. More recently, Kristensen and Jespersen (2003) observed resistance in houseflies to diflubenzuron for the first time in Denmark, and some field populations with resistance to cyromazine. A fivefold cyromazine resistant strain was established and this was 3-, 5-, and 90-fold resistant to diflubenzuron, triflumuron, and methoprene, respectively. In reviewing the mechanism of resistance in houseflies to diflubenzuron and cyromazine, Ishaaya (1993) indicated that resistance was due to increased levels of detoxifying enzymes, decreased penetration, and enhanced excretion of the compounds. Cross-resistance between cyromazine and diflubenzuron was observed but the mechanism seems more target site related than metabolic.

For the control of whiteflies, novel compounds like buprofezin were introduced at the beginning of the 1990s (Horowitz *et al*., 1994; De Cock and Degheele, 1998). In Israel, a survey in cotton fields over 4 years (1989–1992) with two or three applications of buprofezin per season indicated a fivefold increase in tolerance in the *B. tabaci*, and the resistance ratio for resistance to pyriproxifen was >500-fold (Horowitz and Ishaaya, 1994). Based on the success of the program in Israel, the usefulness of buprofezin was once again demonstrated in a resistance management program in Arizona to control resistance to pyriproxifen in populations of the whitefly, *Bermisia argentifolii* in cotton (Dennehy and Williams, 1997). In another study in southern Spain where highly multiresistant strains of *B. tabaci* showed lower efficacy to buprofezin, pyriproxyfen resistance was not obvious (Elbert and Nauen, 2000). For the greenhouse whitefly, *T. vaporariorum*, which is a major pest problem in protected crops in Western Europe, a >300-fold resistance was scored in a strain collected in northern Belgium (De Cock *et al*., 1995). No cross-resistance was recorded for pyriproxyfen and diafenthiuron, indicating their potential in a resistance management program. In UK greenhouses, Gorman *et al*. (2002) also found very strong resistance to buprofezin in *T. vaporariorum*, and these strains were cross-resistant to teflubenzuron.

The chitin synthesis inhibition site has proven to be important for developing control agents that act against important groups of insect pests and many of these CSIs are environmentally safe. However, details of the exact mode of action of CSIs have not been elucidated so far. Although there were already some incidences of resistance to CSIs in the last two decades in different pest insects, these IGR compounds remain suitable for use as rotation partners in integrated and resistance management programs based on their new and se-

lective mode of action in contrast to broad-spectrum neurotoxins.

At present, it is believed that chitin synthesis has by no means been fully exploited as an attractive target and that opportunities exist to further discover new CSIs. A better understanding of the biosynthetic pathway for chitin synthesis and cuticle deposition, as well as precise mode of action of CSIs would allow for development of better and more efficient high throughput assays for discovery of new and novel CSIs.

6.3.5. Conclusions and Future Prospects of Insect Growth- and Development-Disrupting Insecticides

The three classes of insecticides reviewed in this chapter are much slower acting than those acting on neural target sites. The end user has, of course, been used to seeing insects die within a very short time following application of neuroactive insecticides. The discovery and availability of insecticides that inhibit growth and development of insects brought a paradigm shift from the faster acting neurotoxic insecticides. This change has necessitated educating the distributors and the users on the mode of action of these new insecticides. The bisacylhydrazine insecticides are generally faster acting than the JHA and CSI insecticides. Moreover, an attractive feature of the bisacylhydrazine insecticides is their ability to prevent crop damage by inhibition of feeding within 3–12 h after application.

Of the three classes of insecticides that disrupt growth and development in insects, the mode of action of nonsteroidal ecdysone agonist bisacylhydrazine insecticides is the best understood at the molecular level. This detailed understanding has been possible, both with the cloning and expression of cDNAs encoding EcR and USPs from several insects, and the availability of stable and easy to synthesize bisacylhydrazines. Unlike the JHAs and CSI, the molecular targets for bisacylhydrazine insecticides are not only known but the interaction of some of these insecticides with specific amino acid residues in the ligand binding pocket of the target ecdysone receptor are also known. Moreover, reasons for the selective insect toxicity of bisacylhydrazine insecticides are also well understood. Publication of the crystal structure of unliganded and liganded (with ecdysteroid and bisacylhydrazine) HvEcR/HvUSP, and the discovery of new nonsteroidal ecdysone agonist chemistries like tetrahydroquinolines, provides new tools, and suggests possibilities of discovering new ecdysone agonist insecticides. It should now be possible to use combinatorial chemistry approaches, around leads generated either via *in silico* screening or rational design (based on the three-dimensional structures and interactions of ligand in ligand binding pocket of EcR), to discover new and novel chemistries that target EcRs from specific insect orders. The discovery of additional new, novel, selective, and potent nonsteroidal ecdysone agonists will create opportunities to extend their applications in individually or simultaneously regulating different ecdysone receptor gene switches in plants and animals. Further, the discovery of non steroidal ecdysone agonist bisacylhydrazine insecticides has opened tremendous opportunities to explore both basic and applied biology.

The molecular basis of action of JHA and CSI insecticides is not well understood, although both classes of chemistries were discovered long before the bisacylhydrazine insecticides. The discovery of the JH receptor(s) has been elusive (see **Chapter 3.7**). To complicate matters further, it is not clear if the JHAs use the same molecular target/site as the natural JHs do (Dhadialla *et al*., 1998). Dhadialla *et al*. (1998) alluded to the possibility that the JH receptor may be a complex of two or more proteins, and that JH and JHAs could manifest their action by interacting either with different proteins in the complex or by interacting at different, but effective, sites. Consequently, the research to discover new JH agonist or antagonist chemistries has been slow. An antagonist of JH (different from a precocene type of mode of action) that acts either by inhibiting one of the JH biosynthesis steps or antagonizes the action of JH at the receptor level would be useful. With that, it may be possible to have agonists/antagonists of JH with insect selective toxicity, as has been possible for the ecdysone agonists.

Even though the precise mode of action of CSI insecticides is unknown, many analogs and variants of the original diflubenzuron, Dimilin®, have been synthesized and registered as insecticides. A better understanding of the biosynthetic pathway for chitin synthesis and cuticle deposition, and molecular characterization of the various enzymes involved, as well as the precise mode of action of CSIs, would allow for development of better and more efficient high-throughput assays for discovery of new and novel CSIs.

Finally, in spite of their slower speed of kill of insect pests than the faster acting neurotoxic insecticides, the three classes of insect growth and development disrupting insecticides are well suited for use in insect pest control. They are also suited for resistance management programs due to their novel and different modes of action. The bisacylhydrazine

insecticides are particularly attractive, due to their selective insect toxicity and high degree of mammalian and ecotoxicological reduced risk profiles.

Acknowledgments

The authors would like to thank Natalie Filion, CFS, Ontario, Canada, and Karan Dhadialla, University of Chicago, IL, USA, for sorting and typing the references in the required format. TSD also expresses his deep appreciation to Dow Agrociences LLC., Indianapolis, IN, USA (especially Drs. W. Kleshick and S. Evans) for support and encouragement in undertaking this writing. Part of the research was supported by grants from Gerome Canada and the Canadian Forest Service to AR and colleagues. GS also acknowledges the Fund for Scientific Research Flanders (FWO, Brussels, Belgium).

References

Adcock, G.J., Batterham, P., Kelly, L.E., McKenzie, J.A., **1993**. Cyromazine resistance in *Drosophila melanogaster* (Diptera, Drosophilidae) generated by ethyl methanesulfonate mutagenesis. *J. Econ. Entomol. 86*, 1001–1008.

Aliniazee, M.T., Arshad, M., **1998**. Susceptibility of immature stages of the obliqued banded leafroller, *Choristoneura rosaceana* (Lepidoptera: Tortricidae) to fenoxycarb. *J. Entomol. Soc. Br. Columbia 95*, 59–63.

Allgood, V.E., Eastman, E.M., **1997**. Chimeric receptors as gene switches. *Curr. Opin. Biotechnol. 8*, 474–479.

Aller, H.E., Ramsay, J.R., **1988**. RH-5849 – A novel insect growth regulator with a new mode of action. *Brighton Crop Prot. Conf. 2*, 511–518.

Anderson, M., Fisher, J.P., Robinson, J., Debray, P.H., **1986**. Flufenoxuron – an acylurea acaricide/insecticide with novel properties. *Brighton Crop Prot. Conf. 1*, 89–96.

Arbeitman, M.N., Hogness, D.S., **2000**. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell 101*, 67–77.

Asano, S., Kuwano, E., Eto, M., **1986**. Precocious metamorphosis induced by an anti-juvenile hormone compound applied to 3rd instar silkworm larvae, *Bombyx mori* L. (Leopidoptera: Bombycidae). *App. Entomol. Zool. 21*(2), 305–312.

Ashburner, M., **1973**. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melangaster*. I. Dependence upon ecdysone concentration. *Devel. Biol. 35*, 47–61.

Ashburner, M., Chiara, C., Meltzer, P., Richards, G., **1974**. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol. 38*, 655–662.

Ashok, M., Turner, C., Wilson, T.G., **1998**. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl Acad. Sci. USA 95*(6), 2761–2766.

Awad, T.I., Mulla, M.S., **1984**. Morphogenetic and histopathological effects induced by the insect growth regulator cyromazine in *Musca domestica* (Diptera, Muscidae). *J. Med. Entomol. 21*, 419–426.

Ayoade, O., Morooka, S., Tojo, S., **1995**. Induction of macroptery, precocious metamorphosis, and retarded ovarian growth by topical application of precocene II, with evidence for its non-systemic allaticidal effects in the brown planthopper, *Nilaparvata lugens*. *J. Insect Physiol. 42*(6), 529–540.

Balcells, M., Avilla, J., Profitos, J., Canela, R., **2000**. Synthesis of phenoxyphenyl pyridine and pyrazine carboxamides activity against *Cydia pomonella* (L.) eggs. *J. Agric. Food Chem. 48*, 83–87.

Banks, W.A., Lofgren, C.S., **1991**. Effectiveness of the insect growth regulator pyriproxyfen against the red imported fire ant *Hymenoptera formicidae*. *J. Entomol. Sci. 26*(3), 331–338.

Becher, H.M., Becker, P., Prokic-Immel, R., Wirtz, W., **1983**. CME, a new chitin synthesis inhibiting insecticide. *Brighton Crop Prot. Conf. 1*, 408–415.

Beckage, N.E., Riddiford, L.M., **1981**. Effects of methoprene and juvenile hormone on larval ecdysis, emergence, and metamorphosis of the endoparasitic wasp, *Apanteles Congregatus*. *J. Insect Physiol. 28*(4), 329–334.

Beckage, N.E., Riddiford, L.M., **1983**. Lepidopteran antijuvenile hormones: effects on development of *Apanteles congregatus* in *Manduca sexta*. *J. Insect Physiol. 29*(8), 633–637.

Bélai, I., Matolcsy, G., Farnsworth, D.E., Feyereisen, R., **1988**. Inhibition of insect cytochrome P-450 by some metyrapone analogues and compounds containing a cyclopropylamine moiety and their evaluation as inhibitors of juvenile hormone biosynthesis. *Pestic. Sci. 24*, 205–219.

Bellés, X., Baldellou, M.I., **1983**. Precocious metamorphosis induced by precocenes on *Oxycarenus lavaterae*. *Entomol. Exp. Appl. 34*(2), 129–133.

Bellés, X., Messegues, A., Piulachs, M.B., **1985**. Sterilization induced by precocenes on females of *Blatella gexmanica*(L): short and long term effects. *Zeitschrift fur Angewandte Entomologie 100*, 409–417.

Biddington, K.C., **1985**. Ultrastructural changes in the cuticle of the sheep blowfly, *Lucilia cuprina*, induced by certain insecticides and biological inhibitors. *Tissue Cell 17*, 131–140.

Billas, I.M.L., Moulinier, L., Rochel, N., Moras, D., **2001**. Crystal structure of the ligand-binding domain of the ultraspiracle protein USP, the ortholog of retinoid X receptors in insects. *J. Biol. Chem. 276*, 7465–7474.

Billas, I.M.L., Twema, T., Garnier, J.-M., Mitschler, A., Rochel, N., *et al.*, **2003**. Structural adaptability in the ligand-binding pocket of the ecdysone receptor. *Nature 426*, 91–96.

Binder, B.F., Bowers, W.S., **1991**. Behavioral changes and growth inhibition in last instar larvae of *Heliothis zea*

induced by oral and topical application of precocene II. *Entomol. Exp. Appl. 59*, 207–217.

Blackford, M., Dinan, L., **1997**. The tomato moth *Lacnobia oleracea* (Lepidoptera: Noctuidae) detoxifies ingested 20-hydroxyecdysone, but is susceptible to the ecdysteroid agonists RH-5849 and RH-5992. *Insect Biochem. Mol. Biol. 27*, 167–177.

Boivin, T., Chabert d'Hieres, C., Bouvier, J.C., Beslay, D., Sauphanor, B., **2001**. Pleiotropy of insecticide resistance in the codling moth, *Cydia pomonella*. *Entomol. Exp. Appl. 99*, 381–386.

Bonning, B.C., Hoover, K., Booth, T.F., Duffey, S., Hammock, B.D., **1995**. Development of a recombinant baculovirus expressing a modified juvenile hormone esterase with potential for insect control. *Arch. Insect Biochem. Physiol. 30*, 177–194.

Bourne, P.C., Whiting, P., Dhadialla, T.S., Hormann, R.E., Girault, J.-P., *et al.*, **2002**. Ecdysteroid 7,9(11)-dien-6-ones as potential photoaffinity labels for ecdysteroid binding proteins. *J. Insect Sci. 2*, 1–11 (available online at http://www. insectscience.org/2.11).

Bouvier, J.C., Boivin, T., Beslay, D., Sauphanor, B., **2002**. Age-dependent response to insecticides and enzymatic variation in susceptible and resistant codling moth larvae. *Arch. Insect Biochem. Physiol. 51*, 55–66.

Bowers, W.S., **1968**. Juvenile hormone: activity of natural and synthetic synergists. *Science 161*, 895–897.

Bowers, W.S., Nishida, R., **1980**. Juvocimences L potent juvenile hormone mimics from sweet basil. *Science 209*, 1030–1032.

Bowers, W.S., Unnithan, G.C., Fukushima, J., Toda, J., Sugiyama, T., **1995**. Synthesis and biological activity of furanyl anti-juvenile hormone compounds. *Pestic. Sci. 43*, 1–11.

Brindle, P.A., Baker, F.C., Tsai, L.W., Reuter, C.C., Schooley, D.A., **1987**. Sources of proprionate for the biogenesis of ethyl-branched insect juvenile hormones: role of isoleucine and valine. *Proc. Natl Acad. Sci. USA 84*, 7906–7910.

Bull, M.S., Swindale, S., Overend, D., Hess, E.A., **1996**. Suppression of *Boophilus microplus* populations with fluazuron – an acarine growth regulator. *Austral. Vet. J. 74*, 468–470.

Butenandt, A., Karlson, P., **1954**. Uber die isolierung eines metamorphose-hormons des insekten in kristallisierter from *Z. Naturforsch. Teil B 9*, 389–391.

Butler, L., Kondo, V., Blue, D., **1997**. Effects of tebufenozide (RH-5992) for gypsy-moth (Lepidoptera, Lymantriidae) suppression on nontarget canopy arthropods. *Env. Entomol. 26*(5), 1009–1015.

Cadogan, B.L., Retnakaran, A., Meating, J.H., **1997**. Efficacy of RH5992, a new insect growth regulator against spruce budworm (Lepidoptera: Tortricidae) in a boreal forest. *J. Econ. Entomol. 90*, 551–559.

Cadogan, B.L., Thompson, D., Retnakaran, A., Scharbach, R.D., Robinson, A., *et al.*, **1998**. Deposition of aerially applied tebufenozide (RH5992) on balsam fir (*Abies balsamea*) and its control of spruce budworm (*Choristoneura fumiferana* [Clem.]). *Pestic. Sci. 53*, 80–90.

Cadogan, B.L., Scharbach, R.D., Krause, R.E., Knowles, K.R., **2002**. Evaluation of tebufenozide carry-over and residual effects on spruce budworm (Lepidoptera: Tortricidae). *J. Econ. Entomol. 95*, 578–586.

Campero, D.M., Haynes, K.F., **1990**. Effects of methoprene on chemical communication, courtship, and oviposition in the cabbage looper (Lepidoptera: Noctuidae). *J. Econ. Entomol. 83*(6), 2263–2268.

Cao, S., Qian, X., Song, G., **2001**. *N′-tert*-butyl-*N′*-aroyl-*N*-(alkoxycarbonylmethyl)-*N*-aroylhydrazines, a novel nonsteroidal ecdysone agonist: synthesis, insecticidal activity, conformational, and crystal structure analysis. *Can. J. Chem. 79*, 272–278.

Capinera, J.L., Epsky, N.D., Turick, L.L., **1991**. Responses of *Malonoplus sanguinipes* and *M. differentialis* (Orthoptera: Acrididae) to fenoxycarb. *J. Econ. Entomol. 84*(4), 1163–1168.

Carlson, G.R., Dhadialla, T.S., Hunter, R., Jansson, R.K., Jany, C.S., *et al.*, **2001**. The chemical and biological properties of methoxyfenozide, a new insecticidal ecdysteroid agonist. *Pest Mgt. Sci. 57*, 115–119.

Carton, B., Heirman, A., Smagghe, G., Tirry, L., **2000**. Relationship between toxicity, kinetics and *in vitro* binding of nonsteroidal ecdysone agonists in the cotton leafworm and the Colorado potato beetle. *Med. Fac. Landbouww. Univ. Gent. 65*(2a), 311–322.

Celestial, D.M., McKenney, C.L.Jr., **1993**. The influence of an insect growth regulator on the larval development of the mud crab *Rhithropanopeus harrisii*. *Environ. Pollut. 85*(2), 169–173.

Chan, T.H., Ali, A., Britten, J.F., Thomas, A.W., Strunz, G.M., *et al.*, **1990**. The crystal structure of 1,2-dibenzoyl-1-*tert*-butylhydrazine, a non-steroidal ecdysone agonist, and its effects on spruce bud worm (*Choristoneura fumiferana*). *Can. J. Chem. 68*, 1178–1181.

Chandler, L.D., Pair, S.D., Harrison, W.E., **1992**. RH-5992, a new insect growth regulator active against corn earworm and fall armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 85*, 1099–1103.

Chandler, L.D., **1994**. Comparative effects of insect growth regulators on longevity and mortality of beet armyworm (Lepidoptera: Noctuidae) larvae. *J. Entomol. Sci. 29*, 357–366.

Charles, J.P., Wojtasek, H., Lentz, A.J., Thomas, B.A., Bonning, B.C., *et al.*, **1996**. Purification and reassessment of ligand binding by the recombinant, putative juvenile hormone receptor of the tobacco hornworm, *Manduca sexta*. *Arch. Insect. Biochem. Physiol. 31*, 371–393.

Cherbas, P., Cherbas, L., Lee, S.-S., Nakanishi, K., **1988**. [1251]iodo-ponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl Acad. Sci., USA 85*, 2096–2100.

Chen, N.M., Borden, J.H., **1989**. Adverse effect of fenoxycarb on reproduction by the california fivespined IPS,

IPS *Paraconfusus lanier* (Coleoptera: Scolytidae). *Can. Entomol. 121*, 1059–1068.

Chiang, A.-S., Lin, W-Y., Liu, H-P., Pszezalkowski, A., Fu, T-F., *et al.*, **2002**. Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl Acad. Sci. USA 99*, 37–42.

Cho, W.-L., Kapitskaya, M.Z., Raikhel, A.S., **1995**. Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. *Insect Biochem. Mol. Biol. 25*, 19–27.

Christopherson, K.S., Mark, M.R., Bajaj, V., Godowski, P.J., **1992**. Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl Acad. Sci. USA 89*, 6314–6318.

Chu, K.H., Wong, C.K., Chiu, K.C., **1997**. Effects of the insect growth regulator (S)-methoprene on survival and reproduction of the freshwater cladoceran, *Moina macrocopa*. *Environ. Pollut. 96*(2), 173–178.

Chung, A.C.-K., Durica D.S., Clifton, W., Roe, A., Hopkins, P.M., **1998**. Cloning of crustacean ecdysteroid receptor and retinoid-X-receptor gene homologs and elevation of retinoid-X-receptor mRNA by retinoic acid. *Mol. Cell. Endocrinol. 139*, 209–227.

Clayton, G.M., Peak-Chew, S.Y., Evans, R.M., Schwabe, J.W.R., **2001**. The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation. *Proc. Natl Acad. Sci. USA 98*, 1549–1554.

Clement, C.Y., Bradbrook, D.A., Lafont, R., Dinan, L., **1993**. Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem. Mol. Biol. 23*, 187–193.

Clever, U., Karlson, P., **1960**. Induktion von puff-veränderungen in den speicheldriisenchromosomen von *Chironomus tentans* durch ecdysone. *Exp. Cell. Res. 20*, 623–662.

Cohen, E., **1985**. Chitin synthetase activity and inhibition in different insect microsomal preparations. *Experientia 41*, 470–472.

Cowles, R.S., Villani, M.G., **1996**. Susceptibility of Japanese beetle, oriental beetle, and european chafer (Coleoptera: Scarabaedae) to halofenozide, an insect growth regulator. *J. Econ. Entomol. 89*(6), 1556–1565.

Cowles, R.S., Alm, S.R., Villani, M.G., **1999**. Selective toxicity of halofenozide to exotic white grubs (Coleoptera: Scarabaeidae). *J. Econ. Entomol. 92*(2), 427–434.

Cusson, M., Palli, S.R., **2000**. Can juvenile hormone research help rejuvenate integrated pest management? *Can. Entomol. 132*, 263–280.

Darvas, B., Pap, L., Kelemen, M., Laszlo, P., **1998**. Synergistic effects of verbutin with dibenzoylhydrazine-type ecdysteroid agonists on larvae of *Aedes aegypti* (Diptera: Culicidae). *J. Econ. Entomol. 91*, 1260–1264.

Darvas, B., Polgár, L.A., **1998**. Novel-type insecticides: specificity and effects on non-target organisms. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer, Berlin, pp. 188–259.

Darvas, B., Timár, T., Varjas, L., Kulcsár, P., Hosztafi, S., Bordás, B., **1989**. Synthesis of novel 2,2-dimethylchromene derivatives and their toxic activity on larvae of *Pieris brassicae* (Lep., Pieridae), and *Leptinotarsa decemlineata* (Col., Chrysomelidae)., *Acta Phytopathol. Entomol. Hungarica 24*(3–4), 455–472.

De Cock, A., Degheele, D., **1998**. Buprofezin: a novel chitin synthesis inhibitor affecting specifically planthoppers, whiteflies and scale insects. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer, pp. 74–91.

De Cock, A., Ishaaya, I., Van de Veire, M., Degheele, D., **1995**. Response of buprofezin-susceptible and -resistant strains of *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) to pyriproxyfen and diafenthiuron. *J. Econ. Entomol. 88*, 763–767.

De Reede, R.H., Alkema, P., Blommers, L.H.M., **1985**. The use of the insect growth regulator fenoxycarb and epofenonane against leafrollers in integrated pest management in apple orchards. *Entomol. Exp. Appl. 39*, 265–272.

De Reede, R.H., Groendijk, R.F., Wit, A.K.M., **1984**. Field tests with the insect growth regulators, epofenonane and fenoxycarb, in apple orchards against leafrollers and side-effects on some leafroller parasites. *Entomol. Exp. Appl. 35*, 275–281.

Dedos, S.G., Asahina, M., Fugo, H., **1993**. Effect of fenoxycarb application on the pupal–adult development of the silkworm, *Bombyx mori*. *J. Sericult. Sci. Japan 62*(4), 276–285.

Degheele, D., Fontier, H., Auda, M., De Loof, A., **1986**. Preliminary study on the mode of action of precocene II on *Musca domestica* L. *Lucilia caesar* L. *Med. Fac. Landbouww. Univ. Gent. 51*(1), 101–108.

Dennehy, T.J., Williams, L., **1997**. Management of resistance in *Bemisia* in Arizona cotton. *Pestic. Sci. 51*, 398–406.

DeWilde, J., De Kort, C.A.D., DeLoof, A., **1971**. The significance of juvenile hormone titers. *Mitt. Schweiz. Ent. Ges. 44*, 79–86.

Dhadialla, T.S., Carlson, G.R., Le, D.P., **1998**. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol. 43*, 545–569.

Dhadialla, T.S., Cress, D.E., Carlson, G.R., Hormann, R.E., Palli, S.R., *et al.*, **2002**. Ecdysone receptor, retinoid X receptor and ultraspiracle protein based dual switch inducible gene expression modulation system. *PCT Int. Appl. WO 2002029075*. pp. 79.

Dhadialla, T.S., Tzertzinis, G., **1997**. Characterization and partial cloning of ecdysteroid receptor from a cotton boll weevil embryonic cell line. *Arch. Insect Biochem. Physiol. 35*, 45–57.

Dinan, L., Whiting, P., Bourne, P., Coll, J., **2001**. 8-O-Acetylharpagide is not an ecdysteroid agonist. *Insect Biochem. Mol. Biol. 31*, 1077–1082.

Duan, J.J., Prokopy, R.J., Yin, C.-M., Bergwiler, C., Oouchi, H., **1995**. Effects of pyriproxyfen on ovarian

development and fecundity of *Rhagoletis pomonella* flies. *Entomol. Exp. Appl. 77*, 17–21.

Dubrovski, E.B., Dubrovskaya, V.A., Bilderback, A.L., Berger, E.M., **2000**. The isolation of two juvenile hormone-inducible genes in *Drosophila melanogaster. Devel. Biol. 224*(2), 486–495.

Edwards, J.P., Abraham, L., **1985**. Laboratory evaluation of two insect juvenile hormone analogues against *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae). *J. Stored Prod. Res. 21*(4), 189–194.

Edwards, J.P., Short, J.E., **1993**. Elimination of a population of the oriental cockroach (Dictyoptera: Blatidae) in a simulated domestic environment with the insect juvenile hormone analog (S)-hydropene. *J. Econ. Entomol. 86*(2), 436–443.

Eisa, A.A., El-Fatah, M.A., El-Nabawi, A., El-Dash, A.A., **1991**. Inhibitory effects of some insect growth regulators on developmental stages, fecundity and fertility of the florida wax scale, *Ceroplastes floridensis. Phytoparasitica 19*(1), 49–55.

El Saidy, M.F., Auda, M., Degheele, D., **1989**. Detoxification mechanism of diflubenzuron and teflubenzuron in the larvae of *Spodoptera littoralis* (Boisd.). *Pestic. Biochem. Physiol. 35*, 211–222.

Elbert, A., Nauen, R., **2000**. Resistance of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides in southern Spain with special reference to neonicotinoids. *Pest Manag. Sci. 56*, 60–64.

Elbrecht, A., Chen, Y., Jurgens, T., Hensens, O.D., Zink, D.L., *et al.*, **1996**. 8-*O*-acetyl-harpagide is a nonsteroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol. 26*, 519–523.

El-Guindy, M.A., El-Rafai, A.R.M., Abdel-Satter, M.M., **1983**. The pattern of cross-resistance to insecticides and juvenile hormone analogues in a diflubenzuron-resistant strain of the cotton leafworm, *Spodoptera littoralis. Pestic. Sci. 14*, 235–245.

Elzen, G.W., **1989**. Sublethal effects of pesticides on beneficial parasitoids. In: Jepson, P.C. (Ed.), Pesticides and Non-target Invertebrates. Intercept, pp. 29–159.

Evans, K., **1985**. An approach to control of *Globodera rostochiensis* using inhibitors of collagen and chitin synthesis. *Nematologica 30*, 247–250.

Farag, A.I., Varjas, L., **1983**. Precocious metamorphosis and moulting deficiencies induced by an anti-JH compound FMEV in the fall webworm, *Hyphantria cunea. Entomol. Exp. Appl. 34*, 65–70.

Farinos, G.P., Smagghe, G., Tirry, L., Castañera, P., **1999**. Action and pharmakokinetics of a novel insect growth regulator, halofenozide, in adult beetles of *Aubeonymus mariaefranciscae* and *Leptinotarsa decemlineata. Arch. Insect Biochem. Physiol. 41*, 201–213.

Farkas, R., Slama, K., **1999**. Effect of bisacylhydrazine ecdysteroid mimics (RH-5849 and RH-5992) on chromosomal puffing, imaginal disc proliferation and pupariation in larvae of *Drosophila melanogaster. Insect Biochem. Mol. Biol. 29*, 1015–1027.

Feng, Q.L., Ladd, T.R., Tomkins, B.L., Sundaram, M., Sohi, S.S., *et al.*, **1999**. Spruce budworm (*Christoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning. *Mol. Cell. Endocrinol. 148*, 95–108.

Fukuda, S., **1994**. The hormonal mechanism of larval moulting and metamorphosis in the silkworm. *J. Fac. Sci. Imp. Univ. Tokyo 4*, 477–532.

Furlong, M.J., Wright, D.J., **1994**. Examination of stability of resistance and cross-resistance patterns to acylurea insect growth regulators in field populations of the diamondback moth, *Plutella xylostella*, from Malaysia. *Pestic. Sci. 42*, 315–326.

Goff, S.A., Crossland, L.D., Privalle, L.S., **1996**. Control of gene expression in plants by receptor mediated transactivation in the presence of a chemical ligand. *PCT Int. Appl. WO 96/27673*. pp. 59.

Goodman, W.G., **1990**. A simplified method for synthesizing juvenile hormone–protein conjugates. *J. Lipid Res. 31*(2), 354–357.

Gordon, R., **1995**. Toxic effects of a commercial formulation of fenoxycarb against adult and egg stages of the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). *Can. Entomol. 127*, 1–5.

Gorman, K., Hewitt, F., Denholm, I., Devine, G.J., **2002**. New developments in insecticide resistance in the glasshouse whitefly (*Trialeurodes vaporariorum*) and the two-spotted spider mite (*Tetranychus urticae*) in the UK. *Pest Manag. Sci. 58*, 123–130.

Grebe, M., Rauch, P., Spindler-Barth, M., **2000**. Characterization of subclones of the epithelial cell line from *Chironomus tentans* resistant to the insecticide RH 5992, a non-steroidal moulting hormone agonist. *Insect Biochem. Mol. Biol. 30*, 591–600.

Grenier, S., Grenier, A.-M., **1993**. Fenoxycarb, a fairly new insect growth regulator: a review of its effects on insects. *Ann. Appl. Biol. 122*, 369–403.

Grosscurt, A.C., **1978**. Diflubenzuron: some aspects of its ovicidal and larvicidal mode of action and an evaluation of its practical possibilities. *Pestic. Sci. 9*, 373–386.

Grosscurt, A.C., ter Haar, M., Jongsma, B., Stoker, A., **1987**. PH 70–23: a new acaricide and insecticide interfering with chitin deposition. *Pestic. Sci. 22*, 51–59.

Guo, X., Harmon, M.A., Laudet, V., Manglesdorf, D.J., Palmer, M.J., **1997**. Isolation of a functional ecdysteroid receptor homologue from the ixodid tick *Amblyomma americanum* (L). *Insect Biochem. Mol. Biol. 27*, 945–962.

Haga, T., Toki, T., Koyanagi, T., Nishiyama, R., **1982**. Structure-activity relationships of a series of benzoylpyridyloxyphenyl-urea derivatives. *Int. Congr. Pestic. Chem. 2*, 7.

Haga, T., Toki, T., Tsujii, Y., Nishaymar, R., **1992**. Development of an insect growth regulator, chlorfluazuron. *J. Pestic. Sci. 17*, S103–S113.

Hagazi, E.M., El-Singaby, N.R., Khafagi, W.E., **1998**. Effects of precocenes (I and II) and juvenile hormone I on *Spodoptera littoralis* (Boisd.) (Lep., Noctuidae)

larvae parasitized by *Microplitis rufiventris Kok*. (Hym., Braconidae). *J. Appl. Entomol. 122*(8), 453–456.

Hall, R.D., Foehse, M.C., **1980**. Laboratory and field tests of CGA-72662 for control of the house fly and face fly in poultry, bovine, or swine manure. *J. Econ. Entomol. 73*, 564–569.

Hannan, G.N., Hill, R.J., **1997**. Cloning and characterization of LcEcR: a functional ecdysone receptor from the sheep blowfly, *Lucillia cupirina*. *Insect Biochem. Mol. Biol. 27*, 479–488.

Hannan, G.N., Hill, R.J., **2001**. *Lcusp*, an ultraspiracle gene from the sheep blowfly, *Lucilia cupirina*: cDNA cloning, developmental expression of RNA and confirmation of function. *Insect Biochem. Mol. Biol. 31*, 771–781.

Harding, W.C., **1979**. Pesticide profiles, part one: insecticides and miticides. *Univ. Maryland, Coop. Ext. Serv. Bull. 267*, 1–30.

Hargrove, J.W., Langley, P.A., **1993**. A field trial of pyriproxyfen-treated targets as an alternative method for controlling tsetse (Diptera: Glossinidae). *Bull. Entomol. Res. 83*, 361–368.

Hassan, S.A., **1992**. Guidelines for testing the effects of pesticides on beneficial organisms: description of test methods. *IOBC/WPRS Bull. 15*, 1–186.

Hassan, S.A., Bigler, F., Bogenschultz, H., Boller, E., Brun, J., *et al.*, **1991**. Results of the 5th joint pesticide testing program carried out by the IOBC/WPRS working group "Pesticides and beneficial organisms." *Entomophaga 36*, 55–67.

Hassan, S.A., Bigler, F., Bogenschultz, H., Boller, E., Brun, J., *et al.*, **1994**. Results of the 6th joint pesticide testing program of the IOBC/WPRS working group "Pesticides and beneficial organisms." *Entomophaga 39*, 107–119.

Hastakoshi, M., **1992**. An inhibitor mechanism for oviposition in the tobacco cutworm: *Spodoptera litura* by juvenile hormone analogue pyriproxyfen. *J. Insect Physiol. 38*(10), 793–801.

Hayashi, T., Iwamura, H., Nakagawa, Y., Fujita, T., **1989**. Development of (4-alkoxyphenoxy) alkanaldoxime O-ethers as potent insect juvenile hormone mimics and their structure-activity relationships. *J. Agric. Food Chem. 37*, 467–472.

Hayward, D.C., Bastiani, M.J., Truman, J.W.H., Riddiford, L.M., Ball, E.E., **1999**. The sequence of *Locusta* RXR, homologous to *Drosophila* ultraspiracle, and its evolutionary implications. *Devel. Genes Evol. 209*, 564–571.

Hayward, D.C., Dhadialla, T.S., Zhou, S., Kuiper, M.J., Ball, E.E., *et al.*, **2003**. Ligand specificity and developmental expression of RXR and ecdysone receptor in the migratory locust. *J. Insect Physiol. 49*, 1135–1144.

Henrick, C.A., Staal, G.B., Siddal, J.B., **1973**. Alkyl 3,7,1,1-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. *J. Agric. Food Chem. 21*, 354–359.

Hicks, B.J., Gordon, R., **1992**. Effects of the juvenile hormone analog fenoxycarb on various developmental stages of the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). *Can. Entomol. 124*, 117–123.

Hicks, B.J., Gordon, R., **1994**. Effect of the juvenile hormone analog fenoxycarb on post-embryonic development of the eastern spruce budworm, *Choristoneura fumiferana*, following treatment of the egg stage. *Entomol. Exp. Appl. 71*, 181–184.

Hill, T.A., Foster, R.E., **2000**. Effect of insecticides on the diamondback moth (Lepidoptera: Plutellidae) and its parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae). *J. Econ. Entomol. 93*(3), 763–768.

Hiruma, K., **2003**. Juvenile hormone action in insect development. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones. Elsevier Science, Amsterdam, pp. 528–535.

Horowitz, A.R., Ishaaya, I., **1994**. Managing resistance in insect growth-regulators in the sweet-potato whitefly (Homoptera, Aleyrodidae). *J. Econ. Entomol. 87*, 866–871.

Horowitz, A.R., Forer, G., Ishaaya, I., **1994**. Managing resistance in Bemisia tabaci in Israel with emphasis on cotton. *Pestic. Sci. 42*, 113–122.

Hrdý, I., Kuldová, J., Wimmer, Z., **2001**. A juvenile hormone analogue with potential for termite control: laboratory test with *Reticulitermes santonensis, Reticulitermes flaviceps* and *Coptotermes formosanus* (Isopt., Rhinotermitidae). *J. Appl. Entomol. 125*(7), 403–411.

Hsu, A.C.-T., Fujimoto, T.T., Dhadialla, T.S., **1997**. Structure-activity study and conformational analysis of RH-5992, the first commercialized nonsteroidal ecdysone agonist. In: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J., Thompson, D.G. (Eds.), Phytochemicals for Pest Control, ACS Symposium Series, vol. 658. American Chemical Society, pp. 206–219.

Hsu, AC.-T., **1991**. 1,2-Diacyl-1-alkyl-hydrazines; a novel class of insect growth regulators. In: Baker, D.R., Fenyes, J.G., Moberg, W.K. (Eds.), Synthesis and Chemistry of Agrochemicals, II. ACS Symposium Series, vol. 443. American Chemical Society, pp. 478–490.

Hu, W., Feng, Q., Palli, S.R., Krell, P.J., Arif, B.M., *et al.*, **2001**. The ABC transporter Pdr5p mediates the efflux of nonsteroidal ecdysone agonists in *Sacchromyces cerevisiae*. *Eur. J. Biochem. 268*, 3416–3422.

Imhof, M.O., Rusconi, S., Lezzi, M., **1993**. Cloning of a *Chironomus tentans* cDNA encoding a protein c(EcRH) homologous to the *Drosophila melanogaster* ecdysone receptor (dEcR). *Insect Biochem. Mol. Biol. 23*, 115–124.

Ishaaya, I., **1993**. Insect detoxifying enzymes – their importance in pesticide synergism and resistance. *Arch. Insect Biochem. Physiol. 22*, 263–276.

Ishaaya, I., **2001**. Biochemical processes related to insecticide actions: an overview. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, Berlin, pp. 1–16.

Ishaaya, I., Casida, J.E., **1980**. Properties and toxicological significance of esterases hydrolyzing permethrin and

cypermethrin in *Trichoplusia ni* larval gut integument. *Pestic. Biochem. Physiol. 14*, 178–184.

Ishaaya, I., De Cock, A., Degheele, D., **1994**. Pyriproxyfen, a potent suppressor of egg hatch and adult formation of the greenhouse whitefly (Homoptera: Aleyrodidae). *J. Econ. Entomol. 87*(5), 1185–1189.

Ishaaya, I., Horowitz, A.R., **1992**. Novel phenoxy juvenile hormone analog (pyriproxyfen) suppresses embryogenesis and adult emergence of sweetpotato whitefly (Homoptera: Aleyrodidae). *J. Econ. Entomol. 85*(6), 2113–2117.

Ishaaya, I., Yablonski, S., Horowitz, A.R., **1995**. Comparative toxicology of two ecdysteroid agonists, RH-2485 and RH-5992, on susceptible and pyrethroid resistant strains of the Egyptian cotton leafworm, *Spodoptera littoralis*. *Phytoparasitica 23*, 139–145.

Ishaaya, I., Yablonski, S., Mendelson, Z., Mansour, Y., Horowitz, A.R., **1996**. Novaluron (MCW-275), a novel benzoylphenyl urea, suppressing developing stages of lepidopteran, whitefly and leafminer pests. *Brighton Crop Prot. Conf. 2*, 1013–1020.

Ismail, F., Wright, D.J., **1991**. Cross-resistance between acylurea insect growth regulators in a strain of *Plutella xylostella* L. (Lepidoptera, Yponomeutidae). *Pestic. Sci. 33*, 359–370.

Ismail, F., Wright, D.J., **1992**. Synergism of teflubenzuron and chlorfluazuron in an acylurea-resistant field strain of *Plutella xylostella* L. (Lepidoptera, Yponomeutidae). *Pestic. Sci. 34*, 221–226.

Iwanaga, K., Kanda, T., **1988**. The effects of a juvenile hormone active oxime ether compound on the metamorphosis and reproduction of an anopheline vector, *Anopheles balabacensis* (Diptera: Culicidae). *Appl. Entomol. Zool. 23*(2), 186–193.

Iyengar, A.R., Kunkel, J.G., **1955**. Follicle cell calmodulin in *Blatella gexmanica*: transcript accumulation during vitellogenesis is regulated by juvenile hormone. *Devel. Biol. 170*, 314–320.

Jepson, I., Martinez, A., Greenland, A.J., **1996**. A gene switch comprising an ecdysone receptor or fusion product allows gene control by external chemical inducer and has agricultural and pharmaceutical applications. *PCT Int. Appl. WO 9637609*. pp. 121.

Jepson, I., Martinez, A., Sweetman, J.P., **1998**. Chemical inducible gene expression systems for plants. A review. *Pestic. Sci. 54*, 360–367.

Jiang, R.-J., Koolman, J., **1999**. Feedback inhibition of ecdysteroids: evidence for a short feedback loop repressing steroidogenesis. *Arch. Insect Biochem. Physiol. 41*, 54–59.

Jones, G., Sharp, P.A., **1997**. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl Acad. Sci. USA 94*, 13499–13503.

Jones, G., Wozniak, M., Chu, Y.-X., Dhar, S., Jones, D., **2001**. Juvenile hormone III-dependent conformational changes of the receptor ultraspiracle. *Insect Biochem. Mol. Biol. 32*, 33–49.

Kamimura, K., Arakawa, R., **1991**. Field evaluation of an insect growth regulator, pyriproxyfen, against *Culex pipiens* and *Culex tritaeniorhynchus*. *Jap. J. San. Zool. 42*(3), 249–254.

Kanno, H., Ikeda, K., Asai, T., Maekawa, S., **1981**. 2-Tert-butylimino-3-isopropyl-5-perhydro-1,3,5-thiodiazin 4-one (NNI 750), a new insecticide. *Brighton Crop Prot. Conf. 1*, 59–69.

Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., Raikhel, A.S., **1996**. The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell. Endocrinol. 121*, 119–132.

Karr, L.I., Sheets, J.J., King, J.E., Dripps, J.E., **2004**. Laboratory performance and pharmacokinetics of the benzoylphenylurea noviflumuron in eastern subterranean termites (Isoptera: Rhinotermitidae). *J. Econ. Entomol 97*, 593–600.

Kasuya, A., Sawada, Y., Tsukamoto, Y., Tanaka, K., Toya, T., *et al.*, **2003**. Binding mode of ecdysone agonists to the receptor: comparative modeling and docking studies. *J. Mol. Model. 9*, 58–65.

Katzenellenbogen, J.A., Katzellenbogen, B.S., **1984**. Affinity labeling of receptors for steroid and thyroid hormones. *Vit. Horm. 41*, 213–274.

Keiding, J., **1999**. Review of the global status and recent development of insecticide resistance in field populations of the housefly, *Musca domestica* (Diptera: Muscidae). *Bull. Entomol. Res. 89*, S9–S67.

Keiding, J., Elkhodary, A.S., Jespersen, J.B., **1992**. Resistance risk assessment of 2 insect development inhibitors, diflubenzuron and cyromazine, for control of the housefly *Musca domestica* L. 2. Effect of selection pressure in laboratory and field populations. *Pestic. Sci. 35*, 27–37.

Keil, C.B., Othman, M.H., **1988**. Effects of methoprene on *Lycoriella mali* (Diptera: Sciaridae). *J. Econ. Entomol. 81*(6), 1592–1597.

Kim, K.S., Chung, B.J., Kim, H.K., **2000**. DBI-3204: A new benzoylphenyl urea insecticide with a particular activity against whitefly. *Brighton Crop Prot. Conf. 1*, 41–46.

King, J.E., Bennett, G.W., **1988**. Mortality and developmental abnormalities induced by two juvenile hormone analogs on nymphal german cockroaches (Dictyoptera: Blattellidae). *J. Econ. Entomol. 81*(1), 225–227.

Knight, A.L., **2000**. Tebufenozide targeted against codling moth (Lepidoptera: Tortricidae) adults, eggs, and larvae. *J. Econ. Entomol. 93*(6), 1760–1767.

Knight, A.L., Dunley, J.E., Jansson, R.K., **2001**. Baseline monitoring of codling moth (Lepidoptera: Tortricidae) larval response to benzoylhydrazine insecticides. *J. Econ. Entomol. 94*, 264–270.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., *et al.*, **1991**. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell 67*, 59–77.

Kopec, S., **1922**. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull. 42*, 323–341.

Kostyukovsky, M., Chen, B., Atsmi, S., Shaaya, E., **2000**. Biological activity of two juvenoids and two ecdysteroids against three stored product insects. *Insect Biochem. Mol. Biol. 30*, 891–897.

Kothapalli, R., Palli, S.R., Ladd, T.R., Sohi, S.S., Cress, D., *et al.*, **1995**. Cloning and developmental expression of the ecdysone receptor gene from the spruce budworm, *Choristoneura fumiferana. Devel. Genet. 17*, 319–330.

Kotze, A.C., Sales, N., **2001**. Inheritance of diflubenzuron resistance and monooxygenase activities in a laboratory-selected strain of *Lucilia cuprina* (Diptera: Calliphoridae). *J. Econ. Entomol. 94*, 1243–1248.

Kristensen, M., Jespersen, J.B., **2003**. Larvicide resistance in *Musca domestica* (Diptera: Muscidae) populations in Denmark and establishment of resistant laboratory strains. *J. Econ. Entomol. 96*, 1300–1306.

Krysan, J.L., **1990**. Fenoxycarb and diapause: a possible method of control for pear psylla (Homoptera: Psyllidae). *J. Econ. Entomol. 83*(2), 293–299.

Kumar, M.B., Fujimoto, T., Potter, D.W., Deng, Q., Palli, S.R., **2002**. A single point mutation in ecdysone receptor leads to increased ligand specificity: implications for gene switch applications. *Proc. Natl Acad. Sci. USA 99*, 14710–14715.

Kuwano, E., Kikuchi, M., Eto, M., **1990**. Synthesis and insect growth regulatory activity of 1-neopentyl-5-substituted imidazoles. *J. Fac. Agric. Kyushu Univ. 35*(1–2), 35–41.

Kuwano, E., Takeya, R., Eto, M., **1985**. Synthesis and anti-juvenile hormone acitivty of 1-substituted-5-[(E)-2,6-dimethyl-1,5-heptadienyl]imidazoles. *Agric. Biol. Chem. 49*(2), 483–486.

Kuwano, E., Takeya, R., Eto, M., **1984**. Synthesis and anti-juvenile hormone activity of 1-citronellyl-5-substituted imidazoles. *Agric. Biol. Chem. 48*(12), 3115–3119.

Langley, P.A., Felton, T., Stafford, K., Oouchi, H., **1990**. Formulation of pyriproxyfen, a juvenile hormone mimic, for tsetse control. *Med. Vet. Entomol. 4*, 127–133.

Le, D.P., Thirugnanam, M., Lidert, Z., Carlson, G.R., Ryan, J.B., **1996**. RH-2485: A new selective insecticide for caterpillar control. *Proc. Brighton Crop Prot. Conf. 2*, 481–486.

Lee, K.-Y., Denlinger, D.L., **1997**. A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, *Lymantria dispar. J. Insect Physiol. 43*, 289–296.

Lee, K.-Y., Chamberlin, M.E., Horodyski, F.M., **2002**. Biological activity of *Manduca sexta* allalotropin-like peptides, predicted products of tissue-specific and developmentally regulated alternatively spliced mRNAs. *Peptides 23*, 1933–1941.

Legaspi, J.C., Legaspi, B.C., Jr., Saldana, R.R., **1999**. Laboratory and field evaluations of biorational insecticides against the Mexican rice borer (Lepidoptera: Pyralidae) and a parasitoid (Hymenoptera: Braconidae). *J. Econ. Entomol. 92*(4), 804–810.

Lidert, Z., Dhadialla, T.S., **1996**. MIMIC insecticide an ecdysone agonist for the global forestry market. In: Proceedings of the International Conference on Integrated Management of Forestry Lymantridae, Warsaw-Sekocin, pp. 59–68.

Liu, T.-X., Chen, T.-Y., **2000**. Effects of a juvenile hormone analog, pyriproxyfen, on the apterous form of *Lipaphis erysimi. Entomol. Exp. Appl. 98*, 295–301.

Liu, T.-X., Chen, T.-Y., **2001**. Effects of the insect growth regulator fenoxycarb on immature *Chrysoperla rufilabris* (Neuroptera: Chyrsopidae). *Fl. Entomol. 84*(4), 628–633.

Liu, T.-X., Stansly, P.A., **1997**. Effects of pyriproxyfen on three species of *Encarsia* (Hymenoptera: Aphelinidae), endoparasitoids of *Bemisia argentifolii* (Homoptera: Aleyrodidae). *Biol. Microbiol Cont. 90*(2), 404–411.

Locke, M., **1998**. Epidermis. In: Harrison, F.W., Locke, M. (Eds.), Microscopic Anatomy of Invertebrates, vol. 11A: Insecta. Wiley-Liss, New York, pp. 75–138.

Londerhausen, M., **1996**. Approaches to new parasiticides. *Pestic. Sci. 48*, 269–292.

Lopez, J.D., Jr., Latheef, M.A., Meola, R.W., **1999**. Effect of insect growth regulators on feeding response and reproduction of adult bollworm. *Proc. Beltwide Cotton Conf. 2*, 1214–1221.

Lyoussoufi, A., Gadenne, C., Rieus, R., d'Arcier, F.F., **1994**. Effects of an insect growth regulator, fenoxycarb, on the diapause of the pear psylla, *Cacopsylla pyri. Entomol. Exp. Appl. 72*(3), 239–244.

Magaula, C.N., Samways, M.J., **2000**. Effects of insect growth regulators on *Chilocorus nigritus* (Fabricius) (Coleoptera: Coccinellidae), a non-target natural enemy of citrus red scale, *Aonidiella aurantii* (Maskell) (Homoptera: Diaspididae), in southern Africa: evidence from laboratory and field trials. *African Entomol. 8*(1), 47–56.

Martinez, A., Sparks, C., Drayton, P., Thompson, J., Greenland, A., Jepson, I., **1999a**. Creation of ecdysone receptor chimeras in plants for controlled regulation of gene expression. *Mol. Gen. Genet. 261*, 546–552.

Martinez, A., Sparks, C., Hart, C.A., Thompson, J., Jepson, I., **1999b**. Ecdysone agonist inducible transcription in transgenic tobacco plants. *Plant J. 19*, 97–106.

Mascarenhas, V.J., Leonard, B.R., Burris, E., Graves, J.B., **1996**. Beet armyworm (Lepidoptera: Noctuidae) control on cotton in Louisiana. *Fl. Entomol. 79*, 336–343.

Masner, P., Angst, M., Dorn, S., **1986**. Fenoxycarb, an insect growth regulator with juvenile hormone activity: a candidate for *Heliothis virescens* (F.) control on cotton. *Pestic. Sci. 18*, 89–94.

Masner, P., Salama, K., Landa, V., **1968**. Natural and synthetic materials with insect hormone activity. IV. Specific female sterility effects produced by a juvenile hormone analogue. *J. Embryol. Exp. Morphol. 20*, 25–31.

McKenzie, J.A., Batterham, P., **1998**. Predicting insecticide resistance: mutagenesis, selection and response. *Phil. Trans. Roy. Soc. Lond. B Biol. Sci. 353*, 1729–1734.

Medina, P., Budia, F., Tirry, L., Smagghe, G., Viñuela, E., **2001**. Compatibility of spinosad, tebufenozide and azadirachtin with eggs and pupae of the predator *Chrysoperla carnea* (Stephens) under laboratory conditions. *Biocontrol Sci. Technol. 11*, 597–610.

Medina, P., Smagghe, G., Budia, F., del Estal, F., Tirry, L., *et al.*, **2002**. Significance of penetration, excretion, transovarial uptake to toxicity of three insect growth regulators in predatory lacewing adults. *Arch. Insect Biochem. Physiol. 51*, 91–101.

Mendel, Z., Blumberg, D., Ishaaya, I., **1994**. Effects of some insect growth regulators on natural enemies of scale insects (Hom.: Cocoidea). *Entomophaga 39*, 199–209.

Michelotti, E.L., Tice, C.M., Palli, S.R., Thompson, C.S., Dhadialla, T.S., **2003**. Tetrahydroquinolines for modulating the expression of exogenous genes via an ecdysone receptor complex. *PCT Int. Appl. WO 2003105849*. pp. 129.

Mikitani, K., **1996a**. A new nonsteroidal class of ligand for the ecdysteroid receptor 3,5-di-*tert*-butyl-4-hydroxy-N-isobutyl-benzamide. *Biochem. Biophys. Res. Commun. 227*, 427–432.

Mikitani, K., **1996b**. Ecdysteroid receptor binding activity and ecdysteroid agonist activity at the level of gene expression are correlated with the activity of dibenzoyl hydrazines in larvae of Bombyx mori. *J. Insect Physiol. 42*, 937–941.

Mikólajczyk, P., Oberlander, H., Silhacek, D.L., Ishaaya, I., Shaaya, E., **1994**. Chitin synthesis in *Spodoptera frugiperda* wing imaginal discs. I. Chlorfluazuron, diflubenzuron, and teflubenzuron inhibit incorporation but not uptake of [^{14}C]-*N*-acetyl-D-glucosamine. *Arch. Insect Biochem. Physiol. 25*, 245–258.

Miller, R.J., Broce, A.B., Dryden, M.W., Hopkins, T., **1999**. Susceptibility of insect growth regulators and cuticle deposition of the cat flea (Siphonaptera: Pulicidae) as a function of age. *J. Med. Entomol. 36*(6), 780–787.

Miura, T., Takahashi, R.M., **1987**. Impact of fenoxycarb, a carbamate insect growth regulator, on some aquatic invertebrates abundant in mosquito breeding habitats. *J. Am. Mosq. Control Assoc. 3*, 476–480.

Miyake, T., Mitsui, T., **1995**. Multiple physiological activity of an anti-juvenile hormone, precocene 2 on the whitebacked rice planthopper. *J. Pestic. Sci. 20*, 17–24.

Miyake, T., Haruyama, H., Mitsui, T., Sakurai, A., **1991**. Effects of a new juvenile hormone mimic, NC-170, on metamorphosis and diapause of the small brown planthopper, *Laodelphax striatellus*. *J. Pestic. Sci. 17*, 75–82.

Miyake, T., Haruyama, H., Ogura, T., Mitsui, T., Sakurai, A., **1990**. Effects of insect juvenile hormone active NC-170 on metamorphosis, oviposition and embryogenesis in leafhoppers. *J. Pest. Sci. 16*, 441–448.

Miyamoto, J., Hirano, M., Takimoto, Y., Hatakoshi, M., **1993**. Insect growth regulators for insect control, with emphasis on juvenile hormone analogs: present and future prospects. In: Duke, S.O., Menn, J.J., Plimmer, J.R. (Eds.), Pest Control with Enhanced Environmental Safety, ACS Symposium Series, vol. 524. American Chemical Society, Washington, DC, pp. 144–168.

Mkhize, J.N., **1991**. Activity of a juvenile hormone analog as a protectant against the lesser grain borer, *Rhizopertha dominica* (F.) (Coleoptera: Bostrichidae). *Insect Sci. Appl. 13*(2), 183–187.

Moffit, H.R., Westgrad, P.H., Mantey, K.D., Van de Baan, H.E., **1988**. Resistance to diflubenzuron in codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol. 81*, 1511–1515.

Mohammed-Ali, A.D., Chan, T.-H., Thomas, A.W., Strunz, G.M., Jewett, B., **1995**. Structure–activity relationship study of synthetic hydrazines as ecdysone agonists in the control of spruce budworm (*Choristoneura fumiferana*). *Can. J. Chem. 73*, 550.

Moser, B.A., Koehler, P.G., Patterson, R.S., **1992**. Effect of methoprene and diflubenzuron on larval development of the cat flea (Siphonaptera: Pulicidae). *J. Econ. Entomol. 85*(1), 112–116.

Mouillet, J.F., Delbecque, J.P., Quennedey, B., Delachambre, J., **1997**. Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. *Eur. J. Biochem. 248*, 856–863.

Moulton, J.K., Pepper, D.A., Jansson, R.K., Dennehy, T.J., **2002**. Pro-active management of beet armyworm (Lepidoptera: Noctuidae) resistance to tebufenozide and methoxyfenozide: baseline monitoring, risk assessment, and isolation of resistance. *J. Econ. Entomol. 95*, 414–424.

Muyle, H., Gordon, R., **1989**. Effects of selected juvenile hormone analogs on sixth instar larvae of the eastern spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae). *Can. Entomol. 121*, 1111–1116.

Nair, V.S.K., Rajalekshmi, E., **1989**. Effects of fluoromevalonate on penultimate and last instar larvae of *Spodoptera mauritia* Boisd. *Indian J. Expt. Biol. 27*(2), 170–173.

Nakagawa, Y., Akagi, T., Iwamura, H., Fujita, T., **1989a**. Quantitative structure-activity studies of benzoylphenylurea larvicides. VI. Comparison of substituent effects among activities against different insect species. *Pestic. Biochem. Physiol. 33*, 144–157.

Nakagawa, Y., Hattori, K., Minakuchi, C., Kugimiya, S., Ueno, T., **2000a**. Relationships between structure and molting hormonal activity of tebufenozide, methoxyfenozide, and their analogs in cultured integument system of *Chilo suppressalis* Walker. *Steroids 65*, 117–123.

Nakagawa, Y., Hattori, K., Shimizu, B., Akamatsu, M., Miyagawa, H., *et al.*, **1998**. Quantitative structure-activity studies of insect growth regulators XIV.

Three-dimensional quantitative structure-activity relationship of ecdysone agonists including dibenzoylhydrazine analogs. *Pestic. Sci. 53*, 267–277.

Nakagawa, Y., Minakuchi, C., Takahashi, K., Ueno, T., **2002**. Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Kc cell line. *Insect Biochem. Mol. Biol. 32*, 175–180.

Nakagawa, Y., Minakuchi, C., Ueno, T., **2000b**. Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Sf-9 cell line. *Steroids 65*, 537–542.

Nakagawa, Y., Matsutani, M., Kurihara, N., Nishimura, K., Fujita, T., **1989b**. Quantitative structure-activity studies of benzoylphenylurea larvicides. VIII. Inhibition of N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis* Walker. *Pestic. Biochem. Physiol. 43*, 141–151.

Nakagawa, Y., Shimuzu, B., Oikawa, N., *et al.*, **1995a**. Three-dimensional quantitative structure-activity analysis of steroidal and dibenzoylhydrazine-type ecdysone agonists. In: Hanch, C., Fujita, T. (Eds.), Classical and Three-Dimensional QSAR in Agrobiochemistry ACS Symposium, vol. 606. American Chemical Society, Washington, DC, pp. 288–301.

Nakagawa, Y., Smagghe, G., Kugimiya, S., *et al.*, **1999**. Quantitative structure-activity studies of insect growth regulators: XVI. Substituent effects of dibenzoylhydrazines on the insecticidal activity to Colorado potato beetle *Leptinotarsa decemlineata*. *Pestic. Sci. 55*, 909–918.

Nakagawa, Y., Smagghe, G., Tirry, L., Fujita, T., **2001a**. Quantitative structure–activity studies of insect growth regulators: XIX. Effects of substituents on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the beet armyworm *Spodoptera exigua*. *Pest Manag. Sci. 58*, 131–138.

Nakagawa, Y., Smagghe, G., Van Paemel, M., Tirry, L., Fujita, T., **2001b**. Quantitative structure-activity studies of insect growth regulators: XVIII. Effects of substituents on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the Colorado potato beetle *Leptinotarsa decemlineata*. *Pest Mgt. Sci. 57*, 858–865.

Nakagawa, Y., Sotomatsu, T., Irie, K., Kitahara, K., Iwamura, H., *et al.* , **1987**. Quantitative structure-activity studies of benzoylphenylurea larvicides. III. Effects of substituents at the benzoyl moiety. *Pestic. Biochem. Physiol. 27*, 143–155.

Nakagawa, Y., Soya, Y., Nakai, K., *et al.*, **1995b**. Quantitative structure-activity studies of insect growth regulators. XI. Stimulation and inhibition of N-acetylglucosamine incorporation in a cultured integument system by substituted *N-tert*-butyl-*N,N′*-dibenzoylhydrazines. *Pestic. Sci. 43*, 339–345.

Niwa, A., Iwamura, H., Nakagawa, Y., Fujita, T., **1989**. Development of (phenoxyphenoxy)- and (benzylphenoxy) propyl ethers as potent insect juvenile hormone mimetics. *J. Agric. Food Chem. 37*, 462–467.

No, D., Yao, T.-P., Evans, M.E., **1996**. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl Acad. Sci. USA 93*, 3346–3351.

Oberlander, H., Silhacek, D.L., Porcheron, P., **1995**. Non-steroidal ecdysteroid agonists: Tools for the study of hormonal action. *Arch. Insect Biochem. Physiol. 28*, 209–223.

Oberlander, H., Silhacek, D.L., **1998**. New perspectives on the mode of action of benzoylphenyl urea insecticides. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action: Mechanism and Application. Springer, pp. 92–105.

Oberlander, H., Smagghe, G., **2001**. Imaginal discs and tissue cultures as targets for insecticide action. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, Berlin, pp. 133–150.

Oikawa, N., Nakagawa, Y., Nishimura, K., Ueno, T., Fujita, T., **1994a**. Quantitative structure-activity analysis of larvicidal 1-(substituted benzoyl)-2-benzoyl-1-*tert*-butylhydrazines against *Chilo suppressalis*. *Pestic Sci. 41*, 139–148.

Oikawa, N., Nakagawa, Y., Nishimura, K., Ueno, T., Fujita, T., **1994b**. Quantitative structure-activity studies of insect growth regulators. X. Substituent effects on larvicidal activity of 1-*tert*-butyl-1-(2-chlorobenzoyl)-2-(substituted benzoyl)hydrazines against *Chilo suppressalis* and design synthesis of potent derivatives. *Pestic. Biochem. Physiol. 48*, 135–144.

Olsen, A., **1985**. Ovicidal effect on the cat flea, *Ctenocephalides felis* (Bouché), of treating fur of cats and dogs with methoprene. *Int. Pest Control.* January/February, 10–14.

Padidam, M., **2003**. Chemically regulated gene expression in plants. *Curr. Opin. Plant Biol. 6*, 169–177.

Padidam, M., Gore, M., Lu, D.L., Smirnova, O., **2003**. Chemical-inducible, ecdysone receptor-based gene expression system for plants. *Transgenic Res. 12*, 101–109.

Palli, S.R., Kapitskaya, M.Z., **2002**. Novel substitution variants of nuclear receptors and their use in a dual switch inducible system for regulation of gene expression. *PCT Int. Appl. WO 2002066615*. pp. 110.

Palli, S.R., Kapitskaya, M.Z., Kumar, M.B., Cress, D.E., **2003**. Improved ecdysone receptor-based inducible gene regulation system. *Eur. J. Biochem. 270*, 1308–1315.

Palli, S.R., Kumar, M.B., Cress, D.E., Fujimoto, T.T., **2002**. Substitution variants of nuclear receptors and their use in a dual switch inducible system for regulation of gene expression. *PCT Int. Appl. WO 2002066612*. pp. 148.

Palli, S.R., Ladd, T.R., Sohi, S.S., Cook, B.J., Retnakaran, A., **1996**. Cloning and developmental expression of *Choristoneura* hormone receptor 3, an ecdysone-inducible gene and a member of the steroid receptor superfamily. *Insect Biochem. Mol. Biol. 26*, 485–499.

Palli, S.R., Ladd, T.R., Tomkins, W., Primavera, M., Sundaram, M.S., *et al.*, **1999**. Biochemical and biological modes of action of ecdysone agonists on the spruce budworm. *Pestic. Sci. 55*, 656–657.

Palli, S.R., Primavera, M., Tomkins, W., Lambert, D., Retnakaran, A., **1995**. Age-specific effects of a non-steroidal ecdysteroid agonist, RH-5992, on the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Eur. J. Entomol. 92*, 325–332.

Palli, S.R., Retnakaran, A., **1999**. Molecular and biochemical aspects of chitin synthesis inhibition. In: Jollès, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, pp. 85–98.

Palli, S.R., Retnakaran, A., **2000**. Ecdysteroid and juvenile hormone receptors: properties and importance in developing novel insecticides. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, pp. 107–132.

Palli, S.R., Touchara, K., Charles, J., Bonning, B.C., Atkinson, J.K., *et al.*, **1994**. A nuclear juvenile hormone-bonding protein from larvae of *Manduca sexta*: A putative receptor for the metamorphic action of juvenile hormone. *Proc. Natl Acad. Sci. USA 91*(13), 6191–6195.

Palumbo, J.C., Horowitz, A.R., Prabhaker, N., **2001**. Insecticidal control and resistance management for *Bemisia tabaci*. *Crop Protect. 20*, 739–765.

Peleg, B.A., **1988**. Effect of a new phenoxy juvenile hormone analog on California red scale (Homoptera: Diaspididae), Florida wax scale (Homoptera: Coccidae) and the ectoparasite *Aphytis holoxanthus* DeBache (Hymenoptera: Aphelinidae). *J. Econ. Entomol. 81*, 88–92.

Perera, S.C., Ladd, T.R., Dhadialla, T.S., Krell, P.J., Sohi, S.S., *et al.*, **1999**. Studies on two ecdysone receptor isoforms of the spruce budworm, *Choristoneura fumiferana*. *Mol. Cell. Endocrinol. 152*, 73–84.

Perez-Farinos, G., Smagghe, G., Marco, V., Tirry, L., Castañera, P., **1998**. Effects of topical application of hexaflumuron on adult sugar beet weevil *Aubeonymus mariaefranciscae* on embryonic development: pharmacokinetics in adults and embryos. *Pestic. Biochem. Physiol. 61*, 169–182.

Peterson, J.K., Kashian, D.R., Dodson, S.I., **2000**. Methoprene and 20-OH-ecdysone affect male production in *Daphnia pulex*. *Environ. Toxicol. Chem. 20*(3), 582–588.

Pimprikar, G.D., Georghiou, G.P., **1979**. Mechanisms of resistance to diflubenzuron in the house fly, *Musca domestica* L. *Pestic. Biochem. Phsyiol. 12*, 10–22.

Pons, S., Riedl, H., Avilla, J., **1999**. Toxicity of the ecdysone agonist tebufenozide to codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol. 92*, 1344–1351.

Pradeep, A.R., Nair, V.S.K., **1989**. Morphogenetic effects of precocene II in the brown planthopper *Nilaparvata lugens* Stal. (Homoptera: Delphacidae). *Acta Entomol. Biochem. 86*(3), 172–178.

Rasa, Cordellia G., Meola, Roger W., Shenker, R., **2000**. Effects of a new insect growth regulator, CGA-255′728, on the different stages of the cat flea (Siphonaptera: Pulicidae). *Entomol. Soc. Am. 37*(1), 141–145.

Reiji, I., Shinya, N., Takashi, O., Yasushi, T., Keiji, T., *et al.*, **2000**. Ecdysone mimic insecticide, chromafenozide: field efficacy against the apple tortrix. *Annu. Rep. Sankyo Res. Lab. 52*, 59–62.

Retnakaran, A., **1970**. Blocking of embryonic development in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae), by some compounds with juvenile hormone activity. *Can. Entomol. 102*, 1592–1596.

Retnakaran, A., **1973a**. Hormonal induction of supernumerary instars in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Can. Entomol. 105*, 459–461.

Retnakaran, A., **1973b**. Ovicidal effect in the white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae), of a synthetic analogue of juvenile hormone. *Can. Entomol. 105*, 591–594.

Retnakaran, A., **1974**. Induction of sexual maturity in the white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae), by some analogue of juvenile hormone. *Can. Entomol. 106*, 831–834.

Retnakaran, A., Gelbic, I., Sundaram, M., Tomkins, W., Ladd, T., *et al.*, **2001**. Mode of action of the ecdysone agonist tebufenozide (RH-5992), and an exclusion mechanism to explain resistance to it. *Pest Mgt. Sci. 57*, 951–957.

Retnakaran, A., Granett, J., Ennis, T., **1985**. Insect growth regulators. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 12. Pergamon, Oxford, pp. 529–601.

Retnakaran, A., Hiruma, K., Palli, S.R., Riddiford, L.M., **1995**. Molecular analysis of the mode of action of RH-5992, a lepidopteran-specific, non-steroidal ecdysteroid agonist. *Insect Biochem. Mol. Biol. 25*, 109–117.

Retnakaran, A., Macdonald, A., Tomkins, W.L., Davis, C.N., Brownright, A.J., *et al.*, **1997**. Ultrastructural effects of a non-steroidal agonist, RH-5992, on the sixth instar larvae of spruce budworm, *Choristoneura fumiferana*. *J. Insect Physiol. 43*, 55–68.

Retnakaran, A., Oberlander, H., **1993**. Control of chitin synthesis in insects. In: Muzzarelli, R.A.A. (Ed.), Chitin Enzymology. European Chitin Society, pp. 89–99.

Retnakaran, A., Tomkins, W.L., Primavera, M.J., Palli, S.R., **1999**. Feeding behavior of the first-instar *Choristoneura fumiferana* and *Choristoneura pinus pinus* (Lepidoptera: Tortricidae). *Can. Entomol 131*, 79–84.

Retnakaran, A., Wright, J.E., **1987**. Control of insect pests with benzoylphenylureas. In: Wright, J.E., Retnakaran, A. (Eds.), Chitin and Benzoylphenyl Ureas. Dr. W. Junk Publishers, Dordrecht, Netherlands, pp. 205–282.

Reynolds, S.E., Blakey, J.K., **1989**. Cyromazine causes decreased cuticle extensibility in larvae of the tobacco hornworm, *Manduca sexta*. *Pestic. Biochem. Physiol. 35*, 251–258.

Riddiford, L.M., **1971**. Juvenile hormone and insect embryogenesis. *Mitt. Schweiz. Ent. Ges. 44*, 177–186.

Riddiford, L.M., **1994**. Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Adv. Insect Physiol. 24*, 213–274.

Riddiford, L.M., **1996**. Juvenile hormone: the status of its "status quo" action. *Arch. Insect Biochem. Physiol. 32*, 271–286.

Riddiford, L.M., Cherbas, P., Truman, J.W., **2001**. Ecdysone receptors and their biological functions. *Vit. Horm. 60*, 1–73.

Riddiford, L.M., Hiruma, K., Zhou, X., Nelson, C.A., **2003**. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster. Insect Biochem. Mol. Biol. 33*, 1327–1338.

Riddiford, L.M., Williams, C.M., **1967**. The effects of juvenile hormone analogues on the embryonic development of silkworms. *Proc. Natl Acad. Sci. USA 57*, 595–601.

Riedl, H., Blomefield, T.L., Gilomee, J.H., **1999**. A century of codling moth control in South Africa. II. Current and future status of codling moth management. *J. S. Afr. Soc. Hortic. Sci. 8*, 32–54.

Riedl, H., Brunner, J.F., **1996**. Insect growth regulators provide new pest control tools for Pacific Northwest orchards. In: Proc. 92nd Annu. Mtg. Washington State Hortic. Assoc., pp. 189–200.

RohMid, L.L.C., **1996**. RH-0345, Turf and Ornamental Insecticide. *Technical Infor. Bull.*, 9.

Roller, H., Dahm, K.H., Sweely, C.C., Trost, B.M., Ange, W., **1967**. The structure of the juvenile hormone. *Chem. Int. Ed. Engl. 6*, 179.

Romanuk, M., **1981**. Structure-activity relationships in selected groups of juvenoids. In: Sehnal, F., Zabra, A., Menn, J.J., Cymborowsti, B. (Eds.), Regulation of Insect Development and Behaviour, Part 1. Wroclaw Technical Univeristy Press, Wroclaw, Poland, pp. 247–260.

Rup, P.J., Baniwal, A., **1985**. Responses of *Zaprionus paravittiger* (Drosophilidae: Diptera) to anti-juvenile hormone, precocene. *Insect Sci. Appl. 6*(6), 671–675.

Saleh, D.S., Zhang, J., Wyatt, G.R., Walker, V.K., **1998**. Cloning and characterization of an ecdysone receptor cDNA from *Locusta migratoria*. *Mol. Cell. Endocrinol. 143*, 91–99.

Sariaslani, F.S., McGee, L.R., Ovenall, D.W., **1987**. Microbial transformation of Precocene II: Oxidative reactions by *Streptomyces griseus*. *Appl. Environ. Microbiol. 53*(8), 1780–1784.

Saul, S.H., Mau Ronald, F.L., Oi, D., **1995**. Laboratory trials of methoprene-impregnated waxes for disinfesting papayas and peaches of the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol. 78*, 652–655.

Saul, S.H., Mau Ronald, F.L., Kobayashi, R.M., Tsuda, D.M., *et al.*, **1987**. Laboratory trials of methoprene-impregnated waxes for preventing survival of adult oriental fruit flies (Diptera: Tephritidae) from infested papayas. *J. Econ. Entomol. 80*, 494–496.

Sauphanor, B., Bouvier, J.C., **1995**. Cross-resistance between benzoylureas and benzoylhydrazines in the codling moth, *Cydia pomonella* L. *Pestic. Sci. 45*, 369–375.

Sauphanor, B., Brosse, V., Monier, C., Bouvier, J.C., **1998a**. Differential ovicidal and larvicidal resistance to benzoylureas in the codling moth, *Cydia pomonella*. *Entomol Exp. Appl. 88*, 247–253.

Sauphanor, B., Bouvier, J.C., Brosse, V., **1998b**. Spectrum of insecticide resistance in *Cydia pomonella* (Lepidoptera: Tortricidae) in southeastern France. *J. Econ. Entomol. 91*, 1225–1231.

Saxena, R.C., Dixit, O.P., Sukumaran, P., **1992**. Laboratory assessment of indigenous plant extracts for anti-juvenile hormone activity in *Culex quinquefasciatus*. *Indian J. Med. Res. 95*, 204–206.

Sbragia, R., Bisabri-Ershadi, B., Rigterink, R.H., **1983**. XRD-473, a new acylurea insecticide effective against *Heliothis*. *Brighton Crop Prot. Conf. 1*, 417–424.

Schooley, D.A., Baker, F.C., **1985**. Juvenile hormone biosynthesis. In: Kerkut, G.A., Gilbert, K.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7. Pergamon, Oxford, pp. 363–389.

Sempere, L.F., Sokol, N.S., Dubrovsky, E.B., Berger, E.M., Ambros, V., **2003**. Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and Broad-Complex gene activity. *Devel. Biol. 259*, 9–18.

Sen, S.E., Garvin, G.M., **1995**. Substrate requirements for lepidopteran farnesol dehydrogenase. *J. Agric. Food Chem. 43*, 820–825.

Sevala, V.L., Davey, K.G., **1989**. Action of juvenile hormone on the follicle cells of *Rhodnius prolixus*: Evidence for a novel regulatory mechanism involving protein kinase c. *Experientia. 45*, 355–356.

Shaw, P.W., Walker, J.T.S., **1996**. Biological control of wooly apple aphid by *Aphelinus mali* in an integrated fruit production programme in Nelson. In: Proc. 49th N.Z. Plant Prot. Conf., pp. 59–63.

Sheets, J., Karr, L.L., Dripps, J.E., **2000**. Kinetics and uptake, clearance, transfer, and metabolism of hexaflumuron by eastern subterranean termites (Isoptera: Rhinotermitidae). *J. Econ. Entomol. 93*, 871–877.

Shemshedini, L., Wilson, T.G., **1990**. Resistance ot juvenile hormone and an insect growth regulator in *Drosophila* is associated with an altered cytosolic juvenile hormone-binding protein. *Proc. Natl Acad. Sci. USA 87*(6), 2072–2076.

Shimuzu, B., Nakagawa, Y., Hattori, K., Nishimura, K., Kurihara, N., *et al.*, **1997**. Molting hormone and larvicidal activities of aliphatic acyl analogs of dibenzoylhydrazine insecticides. *Steroids 62*, 638–642.

Singh, G., Sidhu, H.S., **1992**. Effect of juvenile hormone analogues on the morphogenesis of apteriform mustard sphid, *Lipaphis Erysimi* (Kalt.). *J. Insect Sci. 5*(1), 73–74.

Slama, K., **1995**. Hormonal status of RH-5849 and RH-5992 synthetic ecdysone agonists (ecdysoids) examined

on several standard bioassays for ecdysteroids. *Eur. J. Entomol. 92*, 317–323.

Slama, K., Romanuk, M., Sorm, F., **1974**. Insect Hormones and Bioanalogues. Springer, New York, NY, 477 pp.

Slama, K., Williams, C.M., **1996**. The juvenile hormone. V. The sensitivity of the bug, *Pyrrhocoris apterus*, to a hormonally active factor in american paper-pulp. *Biol. Bull. 130*, 235–246.

Smagghe, G., Braeckman, B.P., Huys, N., Raes, H., **2003a**. Cultured mosquito cells *Aedes albopictus* C6/36 (Dip., Culicidae) responsive to 20-hydroxyecdysone and non-steroidal ecdysone agonist. *J. Appl. Entomol. 127*, 167–173.

Smagghe, G., Carton, B., Decombel, L., Tirry, L., **2001**. Significance of absorption, oxidation, and binding to toxicity of four ecdysone agonists in multi-resistant cotton leafworm. *Arch. Insect Biochem. Physiol. 46*, 127–139.

Smagghe, G., Carton, B., Heirman, A., Tirry, L., **2000a**. Toxicity of four dibenzoylhydrazine correlates with evagination-induction in the cotton leafworm. *Pest. Biochem. Physiol. 68*, 49–58.

Smagghe, G., Carton, B., Wesemael, W., Ishaaya, I., Tirry, L., **1999a**. Ecdysone agonists – mechanism of action and application on *Spodoptera* species. *Pestic. Sci. 55*, 343–389.

Smagghe, G., Degheele, D., **1994**. The significance of pharmacokinetics and metabolism to the biological activity of RH-5992 (Tebufenozide) in *Spodoptera exempta, Spodoptera exigua*, and *Leptinotarsa decemlineata*. *Pestic. Biochem. Physiol. 49*, 224–234.

Smagghe, G., Degheele, D., **1997**. Comparative toxicity and tolerance for the ecdysteroid mimic tebufenozide in a laboratory and field strain of cotton leafworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 90*, 278–282.

Smagghe, G., Dhadialla, T.S., Derycke, S., Tirry, L., Degheele, D., **1998**. Action of the ecdysteroid agonist tebufenozide in susceptible and artificially selected beet armyworm. *Pestic. Sci. 54*, 27–34.

Smagghe, G., Dhadialla, T.S., Lezzi, M., **2002**. Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. *Insect Biochem. Mol. Biol. 32*, 187–192.

Smagghe, G., Eelen, H., Verschelde, E., Richter, K., Degheele, D., **1996a**. Differential effects of nonsteroidal ecdysteroid agonists in Coleoptera and Lepidoptera: Analysis of evagination and receptor binding in imaginal discs. *Insect Biochem. Mol. Biol. 26*, 687–695.

Smagghe, G., Gelman, D., Tirry, L., **1997**. *In vivo* and *in vitro* effects of tebufenozide and 20-hydroxyecdysone on chitin synthesis. *Arch. Insect Biochem. Physiol. 41*, 33–41.

Smagghe, G., Medina, P., Schuyesmans, S., Tirry, L., Viñuela, E., **2000b**. Insecticide resistant monitoring of tebufenozide for managing *Spodoptera exigua* (Hübner [1808]). *Bol. San. Veg. Plagas 26*, 475–481.

Smagghe, G., Nakagawa, Y., Carton, B., Mourad, A.K., Fujita, T., *et al.*, **1999b**. Comparative ecdysteroid action of ring-substituted dibenzoylhydrazines in *Spodoptera exigua*. *Arch. Insect Biochem. Physiol. 41*, 42–53.

Smagghe, G., Pineda, S., Carton, B., Del Estal, P., Budia, F., Viñuela, E., **2003b**. Toxicity and kinetics for methoxyfenozide in greenhouse-selected *Spodoptera exigua* (Lepidoptera: Noctuidae). *Pest Mgt. Sci. 59*, 1203–1209.

Smagghe, G., Salem, H., Tirry, L., Degheele, D., **1996b**. Action of a novel insect growth regulator tebufenozide against different developmental stages of four stored product insects. *Parasitica 52*, 61–69.

Smagghe, G., Viñuela, E., Budia, F., Degheele, D., **1996c**. *In vivo* and *in vitro* effects of the nonsteroidal ecdysteroid agonist tebufenozide on cuticle formation in *Spodoptera exigua*: An ultrastructural approach. *Arch. Insect Biochem. Physiol. 32*, 121–134.

Smagghe, G., Viñuela, E., Van Limbergen, H., Budia, F., Tirry, L., **1999c**. Nonsteroidal moulting hormone agonists: effects on protein synthesis and cuticle formation in Colorado potato beetle. *Entomol. Exp. Appl. 93*, 1–8.

Sohi, S.S., Palli, S.R., Retnakaran, A., **1995**. Forest insect cell lines responsive to 20-hydroxyecdysone and two nonsteroidal ecdysone agonists, RH-5849 and RH-5992. *J. Insect Physiol. 41*, 457–464.

Soltani, N., Aribi, N., Berghiche, H., Lakbar, S., Smagghe, G., **2002**. Activity of RH-0345 on ecdysteroid production and cuticle secretion in *Tenebrio molitor* pupae *in vivo* and *in vitro*. *Pestic. Biochem. Physiol. 72*, 83–90.

Soltani, N., Besson, M.T., Delachambre, J., **1984**. Effect of diflubenzuron on the pupal-adult development of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): growth and development, cuticle secretion, epidermal cell density and DNA synthesis. *Pestic. Biochem. Physiol. 21*, 256–264.

Song, Q., Ma, M., Ding, T., Ballarino, J., Wu, S.-J., **1990**. Effects of a benzodioxole, J2581 (5-ethoxy-6-(4-methoxyphenyl)methyl-1,3-benzodioxole), on vitellogenesis and ovarian development of *Drosophila melanogaster*. *Pestic. Biochem. 37*(1), 12–23.

Sonoda, M., Kuwano, E., Taniguchi, E., **1995**. Precocious metamorphosis induced by 1,5-disubstituted imidazoles is counteracted by tebufenozide (RH-5992), an ecdysteroid agonist. *Nippon Noyaku Gakkaishi 20*, 325–327.

Sotomatsu, T., Nakagawa, Y., Fujita, T., **1987**. Quantitative structure-activity studies of benzoylphenylurea larvicides. IV. Benzoyl ortho substituent effects and molecular conformation. *Pestic. Biochem. Physiol. 27*, 156–164.

Spindler, K.D., Spindler-Barth, M., Londershausen, M., **1990**. Chitin metabolism: a target for drugs against parasites. *Parasitol. Res. 76*, 283–288.

Spindler-Barth, M., Spindler, K.-D., **1998**. Ecdysteroid resistant subclones of the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). I. Selection and

characterization of resistant clones. *In Vitro Cell. Devel. Biol. Animal 34*, 116–122.

Spindler-Barth, M., Turberg, A., Spindler, K.-D., **1991**. On the action of RH 5849, a nonsteroidal ecdysteroid agonist, on a cell line from *Chironomus tentans*. *Arch. Insect Biochem. Physiol. 16*, 11–18.

Srivastava, U.S., Jaiswal, A.K., **1989**. Precocene II Induced effects in the aphid *Aphid craccivora* Koch. *Insect Sci. Appl. 10*(4), 471–475.

Sterk, G., Hassan, S.A., Baillod, M., Bakker, F., Bigler, F., *et al*., **1999**. Results of the seventh joint pesticide testing programme carried out by the IOBC/WPRS-Working Group Pesticides and Beneficial Organisms. *BioControl 44*, 99–117.

Staal, G.B., **1975**. Insect growth regulators with juvenile hormone activity. *Annu. Rev. Entomol. 20*, 417–460.

Staal, G.B., **1982**. Insect control with growth regulators interfering with the endocrine system. *Entomol. Exp. Appl. 31*, 15–23.

Stevens, P.S., Stevens, D., **1994**. An insect growth regulator for controlling leafrollers in kiwifruit. In: Proc. 47th N.Z. Plant Prot. Conf., pp. 310–313.

Strand, M.R., Goodman, W.G., Baehrecke, E.H., **1991**. The juvenile hormone titer of *Trichoplusia ni* and its potential role in embryogenesis of the polyembryonic wasp *Copidosoma floridanum*. *Insect Biochem. 21*(2), 205–214.

Suh, C.P.-C., Orr, D.B., Van Duyn, J.W., **2000**. Effect of insecticides on *Trichogramma exiguum* (Trichogrammatidae: Hymenoptera) preimaginal development and adult survival. *J. Econ. Entomol. 93*(3), 577–583.

Suhr, S.T., Gil, E.B., Senut, M.-C., Gage, F.H., **1998**. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl Acad. Sci. USA 95*, 7999–8004.

Sun, X., Barrett, B.A., **1999**. Fecundity and fertility changes in adult codling moth (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide. *J. Econ. Entomol. 92*, 1039–1044.

Sun, X., Barrett, B.A., Biddinger, D.J., **2000**. Fecundity and fertility changes in adult redbanded leafroller and obliquebanded leafroller (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide. *Entomol. Exp. Applic. 94*, 75–83.

Sundaram, K.M.S., Sundaram, A., Sloane, L., **1996**. Foliar persistence and residual activity of tebufenozide against spruce budworm larvae. *Pestic. Sci. 47*, 31–40.

Sundaram, M., Palli, S.R., Ishaaya, I., Krell, P.J., Retnakaran, A., **1998a**. Toxicity of ecdysone agonists correlates with the induction of CHR3 mRNA in the spruce budworm. *Pestic. Biochem. Physiol. 62*, 201–208.

Sundaram, M., Palli, S.R., Krell, P.J., Sohi, S.S., Dhadialla, T.S., *et al*., **1998b**. Basis for selective action of a synthetic molting hormone agonist, RH-5992 on lepidopteran insects. *Insect Biochem. Mol. Biol. 28*, 693–704.

Sundaram, M., Palli, S.R., Smagghe, G., Ishaaya, I., Feng, Q.L., *et al*., **2002**. Effect of RH-5992 on adult development in the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol. 32*, 225–231.

Swevers, L., Drevet, J.R., Lunke, M.D., Iatrou, K., **1995**. The silkmoth homolog of the *Drosophila* ecdysone receptor (B1 Isoform): cloning and analysis of expression during follicular cell differentiation. *Insect Biochem. Mol. Biol. 25*, 857–866.

Takagi, M., Tsuda, Y., Wada, Y., **1995**. Laboratory evaluation of a juvenile hormone mimic, pyriproxyfen, against *Chironomus fusciceps* (Diptera: Chironomidae). *J. Am. Mosquito Control Assoc. 11*(4), 474–475.

Tatar, M., Yin, C.-M., **2001**. Slow aging during insect reproductive diapause: Why butterflies, grasshoppers and flies are like worms. *Exp. Geront. 36*, 723–738.

Taylor, M.A., **2001**. Recent developments in ectoparasiticides. *Vet. J. 161*, 253–268.

Thomas, H.E., Stunnenberg, H.G., Stewart, A.F., **1993**. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature 362*, 471–475.

Tobe, S.S., Ruegg, R.P., Stay, B.A., Baker, F.A., Miller, C.A., *et al*., **1985**. Juvenile hormone titer and regulation in the cockroach *Diploptera punctata*. *Experientia 41*, 1028–1034.

Tobe, S.S., Stay, B., **1985**. Structure and regulation of the corpus allatum. *Adv. Insect Physiol. 18*, 305–432.

Tomlin, C.D.S. (Ed.) **2000**. The Pesticide Manual, 12th edn. British Crop Protection Council Publications.

Toya, T., Fukasawa, H., Masui, A., Endo, Y., **2002**. Potent and selective partial ecdysone agonist activity of chromafenozide in sfg cells. *Biochem. Biophys. Res. Comm. 292*, 1087–1091.

Tran, H.T., Askari, H.B., Shaaban, S., Price, L., Palli, S.R., *et al*., **2000**. Reconstruction of ligand-dependent transactivation of *Choristoneura fumiferana* ecdysone receptor in yeast. *J. Mol. Endocrinol. 15*, 1140–1153.

Tran, H.T., Shaaban, S., Askari, H.B., Walfish, P.G., Raikhel, A.S., *et al*., **2001**. Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J. Mol. Endocrinol. 27*, 191–209.

Trisyono, A., Chippendale, M., **1997**. Effect of the nonsteroidal ecdysone agonists, methoxyfenozide and tebufenozide, on the European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol. 90*(6), 1486–1492.

Trisyono, A., Chippendale, M., **1998**. Effect of the ecdysone agonists, RH-2485 and tebufenozide, on the Southwestern corn borer, *Diatraea grandiosella*. *Pestic. Sci. 53*, 177–185.

Trisyono, A., Goodman, C.L., Grasela, J.J., McIntosh, A.H., Chippendale, G.M., **2000**. Establishment and characterization of an *Ostrinia nubilalis* cell line, and its response to ecdysone agonists. *In Vitro Cell. Devel. Biol. Animal 36*, 400–404.

Truman, J.W., Rountree, D.B., Reiss, S.E., Schwartz, L.M., **1983**. Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta (L)*. *J. Insect Physiol. 29*, 895–900.

Tsunoda, K., Doki, H., Nishimoto, K., **1986**. Effect of developmental stages of workers and nymphs of *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae) on caste differentiation induced by JHA treatment. *Mat. Organismen Berlin 21*(1), 47–62.

Tyagi, B.K., Kalyanasundaram, M., Das, P.K., Somachary, N., **1985**. Evaluation of a new compound (VCRC/INS/A-23) with juvenile hormone activity against mosquito vectors. *Ind. J. Med. Res. 82*, 9–13.

Uchida, M., Asai, T., Sugimoto, T., **1985**. Inhibition of cuticle deposition and chitin biosynthesis by a new insect growth regulator buprofezin in *Nilaparvata lugens* Stål. *Agric. Biol. Chem. 49*, 1233–1234.

Ujváry, I., Matolcsy, G., Riddiford, L.M., Hiruma, K., Horwath, K.L., **1989**. Inhibition of spiracle and crochet formation and juvenile hormone activity of isothiocyanate derivatives in the tobacco hornworm, *Manduca sexta*. *Pestic. Biochem. Physiol. 35*, 259–274.

Unger, E., Cigan, A.M., Trimnell, M., Xu, R.-J., Kendall, T., *et al.*, **2002**. A chimeric ecdysone receptor facilitates methoxyfenozide-dependent restoration of male fertility in ms45 maize. *Transgenic Res. 11*, 455–465.

Unnithan, G.C., Andersen, J.F., Hisano, T., Kuwano, E., Feyereisen, R., **1995**. Inhibition of juvenile hormone biosynthesis and methyl fernesoate epoxidase activity by 1,5-disubstituted imidazoles in the cockroach, *Diploptera punctata*. *Pestic. Sci. 43*, 13–19.

Vail, K.M., Williams, D.F., **1995**. Pharaoh ant (Hymenoptera: Formicidae) colony development after consumption of pyriproxyfen baits. *J. Econ. Entomol. 88*(6), 1695–1702.

Van Daalen, J.J., Meltzer, J., Mulder, R., Wellinga, K., **1972**. A selective insecticide with a novel mode of action. *Naturwissenschaften 59*, 312–313.

Van de Veire, M., Smagghe, G., Degheele, D., **1996**. Laboratory test method to evaluate the effect of 31 pesticides on the predatory bug, *Orius laevigatus* (*Het.: Anthocoridae*). *Entomophaga 41*, 235–243.

Van Laecke, K., Degheele, D., **1991**. Detoxification of diflubenzuron and teflubenzuron in the larvae of the beet armyworm (*Spodoptera exigua*) (Lepidoptera: Noctuidae). *Pestic. Biochem. Physiol. 40*, 181–190.

Van Laecke, K., Smagghe, G., Degheele, D., **1995**. Detoxifying enzymes in greenhouse and laboratory strain of beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 88*, 777–781.

Veech, J.A., **1978**. The effect of diflubenzuron on the reproduction of free living nematodes. *Nematologica 24*, 312–320.

Veras, M., Mavroidis, M., Kokolakis, G., Gourzi, P., Zacharopoulou, A., *et al.*, **1999**. Cloning and characterization of CcEcR: An ecdysone receptor homolog from the Mediterranean fruit fly *Ceratitis capitata*. *Eur. J. Biochem. 265*, 798–808.

Viñuela, E., Budia, F., **1994**. Ultrastructure of *Ceratitis capitata* Wiedemann larval integument and changes induced by cyromazine. *Pestic. Biochem. Physiol. 48*, 191–201.

Vogt, H. (Ed.) **1994**. Pesticides and Beneficial Organisms, IOBC/WPRS Bulletin. vol. 17. IOBC/WPRS Publications.

Wakgari, W., Giliomee, J., **2001**. Effects of some conventional insecticides and insect growth regulators on different phenological stages of the white – scale, *Ceroplastes destructor* Newstead (Hemiptera: Coccidae), and its primary parasitoid, *Aprostocetus ceroplastae* (Girault) (Hymenoptera: Eulophidae). *Intern. J. Pest Manag. 47*(3), 179–184.

Walton, L., Long, J.W., Spivey, J.A., **1995**. Use of Confirm insecticide for control of beet armyworm in cotton under Section 18 in MS and AL. *Proc. Beltswide Cotton Conf. 2*, 46–47.

Watkinson, I.A., Clarke, B.S., **1973**. The insect moulting hormone system as a possible target site for insecticidal action. *PANS 14*, 488–506.

Wearing, C.H., **1998**. Cross-resistance between azinphosmethyl and tebufenozide in the greenheaded leafroller, *Planotortrix octo*. *Pestic. Sci. 54*, 203–211.

Wigglesworth, V.B., **1936**. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci. 79*, 91–121.

Wildboltz, T., **1988**. Integrated pest management in Swiss apple orchards: stability and risks. *Entomol. Exp. Appl. 49*, 71–74.

Williams, C.M., **1952**. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the *Cecropia* silkworm. *Biol. Bull. 103*, 120–138.

Williams, C.M., **1967**. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the *Cecropia* silkworm. *Biol. Bull. 121*, 572–585.

Williams, D.R., Chen, J.-H., Fisher, M.J., Rees, H.H., **1997**. Induction of enzymes involved in molting hormone (ecdysteroid) inactivation by ecdysteroids and an agonist, 1,2-dibenzoyl-1-*tert*-butylhydrazine (RH-5849). *J. Biol. Chem. 272*, 8427–8432.

Williams, D.R., Fisher, M.J., Smagghe, G., Rees, H.H., **2002**. Species specificity of changes in ecdysteroid metabolism in response to ecdysteroid agonists. *Pestic. Biochem. Physiol. 72*, 91–99.

Williams, R.E., Berry, J.G., **1980**. Evaluation of CGA 72662 as a topical spray and feed additive for controlling house fly breeding in chicken manure. *Poultry Sci. 59*, 2207–2212.

Wilson, T.G., Ashok, M., **1998**. Insecticide resistance resulting from an absence of target-site gene product. *Proc. Natl Acad. Sci. USA 95*(24), 14040–14044.

Wilson, T.G., Fabian, J., **1986**. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. *Devel. Biol. 118*(1), 190–201.

Wing, K.D., **1988**. RH 5849, a non-steroidal ecdysone agonist: effects on a *Drosophila* cell line. *Science 241*, 467–469.

Wing, K.D., Aller, H.E., **1990**. Ecdysteroid agonists as novel insect growth regulators. In: Cassida, J.E. (Ed.), Pesticides and Alternatives: Innovative Chemical and

Biological Approaches to Pest Control. Elsevier, pp. 251–257.

Wing, K.D., Slawecki, R., Carlson, G.R., **1988**. RH-5849, a nonsteroidal ecdysone agonist: effects on larval Lepidoptera. *Science 241*, 470–472.

Wroblewski, V.J., Harshman, L.G., Hanzlik, T.N., Hammock, B.D., **1990**. Regulation of juvenile hormone esterase gene expression in the tobacco budworm (*Heliothis virescens*). *Arch. Biochem. Biophys. 278*, 461–466.

Wurtz, *et al.*, **2000**. A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: A homology modeling and docking approach. *Protein Sci. 9*, 1073–1084.

Wyatt, G.R., Davey, K.G., **1996**. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.

Xu, Y., Fang, F., Chu, Y.-X., Jones, D., Jones, G., **2002**. Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor spiracle. *Eur. J. Biochem. 269*, 6026–6036.

Yanagi, M., Watanabe, T., Masui, A., Yokoi, S., Tsukamoto, Y., *et al.*, **2000**. ANS-118: A novel insecticide. *Proc. Brighton Crop Prot. Conf. 2*, 27–32.

Yanagi, M., **2000**. Development of a novel lepidopteran insect control agent, chromofenozide (MATRIC), and prospect of success. *Agrochem. Jpn. 76*, 16–18.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., *et al.*, **1995**. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature 366*, 476–479.

Yao, T.-P., Segraves, W.A., Oro, A.E., McKeown, M., Evans, R.M., **1992**. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell 71*, 63–72.

Yen, J.L., Batterham, P., Gelder, B., McKenzie, J.A., **1996**. Predicting resistance and managing susceptibility to cyromazine in the Australian sheep blowfly *Lucilia cuprina*. *Aust. J. Exp. Agri. 36*, 413–420.

Zhang, J., Cress, D.E., Palli, S.R., Dhadialla, T.S., **2003**. cDNAs encoding ecdysteroid receptors of whitefly and their use in screening for pesticides. *PCT Int. Appl. WO 2003027266*. pp. 85.

Zhang, J., Wyatt, G.R., **1996**. Cloning and upstream sequence of a juvenile hormone-regulated gene from the migatory locust. *Gene 175*(1–2), 193–197.

Zhou, X., Riddiford, L.M., **2002**. Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development 129*, 2259–2269.

Zimowska, G., Mikólajczyk, P., Silhacek, D.L., Oberlander, H., **1994**. Chitin synthesis in *Spodoptera frugiperda* wing discs. II. Selective action of chlorfluazuron on wheat germ agglutinin binding and cuticle ultrastructure. *Arch. Insect Biochem. Physiol. 27*, 89–108.

Zitnanova, I., Adams, M.E., Zitnan, D., **2001**. Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. *J. Exp. Biol. 204*, 3483–3495.

Zoebelein, G., Hammann, I., Sirrenberg, W., **1980**. BAY-SIR-8514, a new chitin synthesis inhibitor. *J. Appl. Entomol. 89*, 289–297.

Zuo, J., Chua, N.-H., **2000**. Chemical-inducible systems for regulated expression of plant genes. *Curr. Opin. Biotech. 11*, 146–151.

Relevant Website

http://www.iobc.wprs.org – International Organization for Biological and Integrated Control of Noxious Animals and Plants/West Palaearctic Regional Section **2004**.

6.4 Azadirachtin, a Natural Product in Insect Control

A J Mordue (Luntz), University of Aberdeen, Aberdeen, UK
E D Morgan, Keele University, Keele, UK
A J Nisbet, University of Melbourne, Werribee, Australia

6.4.1. Introduction

The dominance of synthetic pesticides, which began with the introduction of DDT in the 1940s, almost displaced completely the natural pesticides used before that time. Only pyrethrum and derris (the active compound is rotenone) survived in specialized uses. In the following half-century the problems of persistence and toxicity of the synthetics grew and regulation followed them. Slowly, one after the other was restricted in use, until we have reached the point where there are no longer available synthetic crop protectants for certain uses. At the same time fundamental research has uncovered entirely new ways of dealing with pests that range from use of pheromone traps through parasitoids to feeding deterrents. Unique among these new discoveries is the compound azadirachtin.

Azadirachtin is a colorless crystalline compound, found in the seeds of the neem tree, *Azadirachta indica* A. Juss. 1830. The compound is of great interest because it is a powerful feeding deterrent for a number of phytophagous insects, and has the power, at parts per million concentration, to inhibit growth and development of all insects ingesting it. This substance, together with a few compounds of closely related structure in the same seeds, provides the most powerful natural pesticide discovered since the era of synthetic pesticides. Its spectrum of activity includes many other arthropods and even annelids and nematodes. Yet it appears to have little or no toxicity for vertebrates. Its isolation, structure determination, synthesis, toxicity, mode of action, spectrum of activity, and practical application have all absorbed the interest of scientists of many disciplines

during the past four decades. Exploration of the properties and uses of azadirachtin continues, with a steady flow of about 45 papers per year appearing on various aspects of the compound.

A resurgence of interest in azadirachtin may be at hand. With the elucidation of its mode of action, which is drawing close, there will be the possibility of studying that site in detail with high-throughput screening methods to search for other molecules with greater field efficacy. Additionally, azadirachtin may prove to be an exciting tool for research scientists studying cellular processes and the cell cycle in invertebrate versus vertebrate cells.

6.4.1.1. The Neem Tree

The modern investigation of the properties of neem seeds began, as often happens, simultaneously in two directions, with the field work of Schmutterer in the Sudan, and laboratory work (unpublished) at the Anti-Locust Research Centre in London. In 1966, Morgan and Butterworth began a systematic search for the substance or substances in neem seeds responsible for the feeding deterrent effect on the desert locust *Schistocerca gregaria*, based on an antifeedant bioassay developed at the Anti-Locust Research Centre (Haskell and Mordue (Luntz), 1969). This work resulted in the isolation of the compound called azadirachtin (Butterworth and Morgan, 1968, 1971). It was soon shown to be effective systemically (Gill and Lewis, 1971), and to have growth-disrupting effects as well (Ruscoe, 1972). Neem products were slow to come to market while powerful synthetics dominated sales, but products are now available in several countries, including the USA, but elsewhere, the toxicity testing rules made for single-compound synthetics still make the licensing of neem products very difficult (Isman, 1997) (see Section 6.4.4).

Other compounds of related chemical structure and similar antifeedant properties have since been isolated from the seeds in smaller quantities. Therefore consideration must be given to the group when discussing the properties of azadirachtin. However, azadirachtin itself remains the most accessible and most abundant compound of the group.

The neem tree appears to be a native of Burma or the Indian subcontinent (Schmutterer, 2002). Indian sources say parts of the neem tree have been used since ancient times as protection against pests, citing the use of neem leaves for protection against bookworm and pests in stored rice, as well as in Ayurvedic medicine (Puri, 1999). The tree belongs to the family Meliaceae, which includes various species of mahogany, but the wood is not of high quality. There are only two other species recognized in the genus: *A. excelsa* (= *A. integrifolia*, locally known as marrango), a native of the Philippine Islands (Schmutterer and Doll, 1993), and the Thai neem tree, *A. siamensis*, very close in appearance to *A. indica*, and formerly thought to be a subspecies, but now awarded full species status. The latter forms hybrids with neem. All contain azadirachtin-like substances with similar properties (Kalinowski *et al*., 1997). Today neem is grown widely across the drier tropics from Australia to Africa, and South and Central America. It is easily grown, widely distributed, exists in large numbers (an estimated 14 million in India alone), so the seeds and their extracts are readily available.

6.4.2. Azadirachtin Research up to 1985

Research on azadirachtin from its discovery to 1985 is best summarized in three international symposia, two held in Germany, in 1980 (Schmutterer *et al*., 1981) and in 1983 (Schmutterer and Ascher, 1984), and one in Kenya in 1986 (Schmutterer and Ascher, 1987). In the 15–20 years following the isolation of azadirachtin, the major biological effects of neem seed extracts were established and shown to be due largely to the active ingredient azadirachtin. The antifeedant effects, insect growth regulatory effects, and sterility effects were established for a wide variety of phytophagous insects, and field trials were carried out to show the effectiveness of neem as a crop protection agent. Early promise of this, together with its large safety margin to vertebrates and potential effectiveness against other pests such as nematodes, led to an upsurge in neem research (Schmutterer and Ascher, 1984, 1987). By 1985 neem pesticides were promoted for use in developing countries by the Deutsche Gesellschaft für Technische Zusamenarbeit (GTZ) as a real alternative in biological and integrated pest control where sufficient raw material was available. In the Western world neem seed, neem oil, and several of their components were being tested in the USA as insect antifeedants and disruptants of normal growth and development. The first commercial neem seed formulation was patented in the USA (Larson, 1985) and granted registration by the Environment Protection Agency. Neem tree plantations for seed supplies were cultivated in the USA, Central and South America, and Australia.

By this time also the broad areas of its mode of action were established although the details were still not fully understood. Insect antifeedancy was shown to be particularly effective in lepidopteran pests compared with others such as the Coleoptera

and Hemiptera and was related to effects on the mouthpart chemoreceptors (Simmonds and Blaney, 1984). Studies on the insect growth regulatory (IGR) mode of action of azadirachtin in, for example, the blowfly *Calliphora vicinia*, the milkweed bug *Oncopeltus fasciatus*, the tobacco hornworm *Manduca sexta*, and the African migratory locust *Locusta migratoria* revealed that ecdysone biosynthesis and catabolism were affected by azadirachtin treatment to cause the major insect growth regulatory effects (Redfern *et al.*, 1982; Sieber and Rembold, 1983; Schlüter *et al.*, 1985; Dorn *et al.*, 1986; Bidmon *et al.*, 1987). In adult insects, such as *L. migratoria*, effects of azadirachtin on egg development were shown to be related to changes in both juvenile hormone (JH) and ecdysone titers (Rembold *et al.*, 1986). In addition to the effects of azadirachtin on JH and ecdysone and the interactions between both hormones, Schlüter (1987) was able to demonstrate in the Mexican bean beetle *Epilachna varivestis* that azadirachtin also affected cells in the process of proliferation and differentiation. Mitosis in the wing disc was blocked from metaphase onwards by azadirachtin injections.

By 1986 azadirachtin was established as an antifeedant, insect growth regulator, and sterilant with a novel mode of action. The basis for its mode of action was known to involve the neurosecretory–neuroendocrine axis and perhaps other sites including particular stages in cell division. Although the full structure had not yet been elucidated or synthetic routes explored, the use of neem extracts to produce insecticides with good market potential was thought to be feasible. Studies to overcome a number of problems preventing commercial use of neem relating to standardization and regulatory matters were still required, as were studies on its possible role in integrated pest management strategies.

6.4.3. Chemistry of Neem Products

Azadirachtin, formula $C_{35}H_{44}O_{16}$, m.p. 160 °C, $[\alpha]_D^{25}$-66°($CHCl_3$, $c = 0.5$) (**Figure 1**) belongs to the large group of plant triterpenoids, and to the narrower group of limonoids, which are frequently found in plants of the Meliaceae. Limonoids are triterpenoids that have their side chains shortened by four carbon atoms and the remaining four atoms cyclized as a furan ring (e.g., nimbin and salannin, and their desacetyl-derivatives; **Figure 1**). The molecule of azadirachtin is unusually highly oxygenated, and in a molecular model the surface is covered by functional

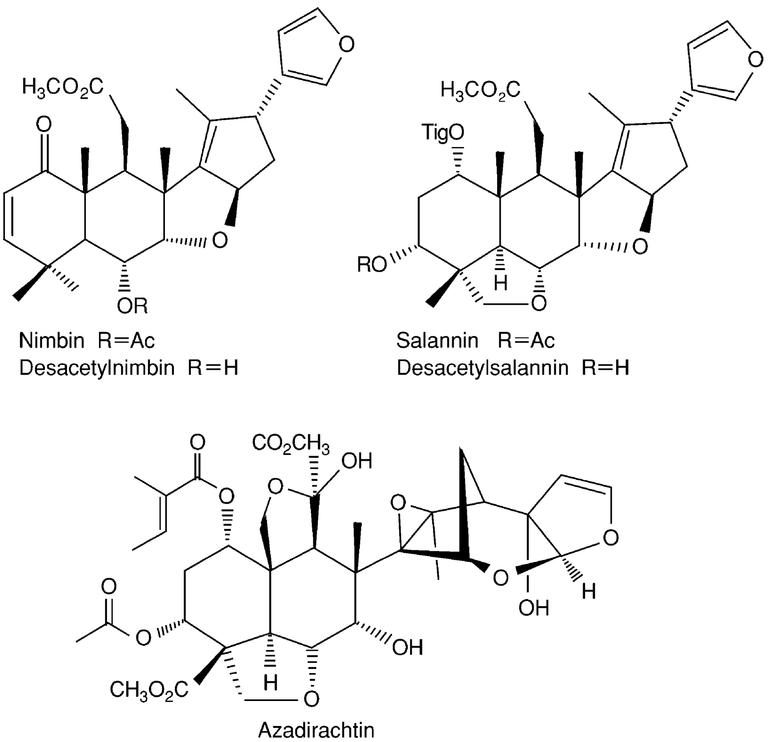


Figure 1 The structure of azadirachtin, together with the principal limonoids (nimbin and salannin, and their desacetyl-derivatives) from the seeds of neem (*Azadirachta indica*). Nimbin possesses almost no antifeedant effect and that of salannin is small compared to azadirachtin.

groups. The molecule contains an acetate, a tiglate ester, two methyl esters, a secondary and a tertiary alcohol, an epoxide, a vinyl ether, which is part of an acetal, and a hemiacetal. This overabundance of reactive groups makes its chemistry very difficult (Ley *et al*., 1993). Just how these contribute to its insecticidal properties is not yet understood. Another locust antifeedant called meliantriol of lower activity than azadirachtin was announced shortly before the isolation of azadirachtin (Lavie *et al*., 1967), but its isolation has never been repeated.

Structural studies on azadirachtin began immediately after its isolation, but because of its complexity and the sensitivity of some of the functional groups, progress was difficult. The structure was finally solved 18 years after isolation by a combination of modern nuclear magnetic resonance (NMR) techniques and X-ray crystallography (Bilton *et al*., 1987; Kraus *et al*., 1987; Turner *et al*., 1987). Synthesis of azadirachtin presents a great challenge to organic chemists. Great progress has been made towards it by the groups of Ley (Durand-Reville *et al*., 2002) and others (Nicolaou *et al*., 2002; Fukuzaki *et al*., 2002), but as yet it has not been completed.

6.4.3.1. Neem Limonoids

Largely because of the interest in azadirachtin, the triterpenoids of neem have been studied intensively. About 150 such compounds have now been described (Akhila and Rani, 1999), most of them found in very small quantities in various parts of the tree. Only about one-third of them have been tested for biological activity, and none has shown greater activity than the azadirachtin group.

Unfortunately many writers speak of "neem" as if that were a single commodity. The different parts of the neem tree all have different properties and contain different substances. The lack of more accurate description and the frequent lack of analytical data on what is contained in seed extracts have reduced the value of some of the published work on such extracts. The seeds are the only practical source of azadirachtin and its group of compounds. Any of this substance in other parts of the tree is in marginally small concentration.

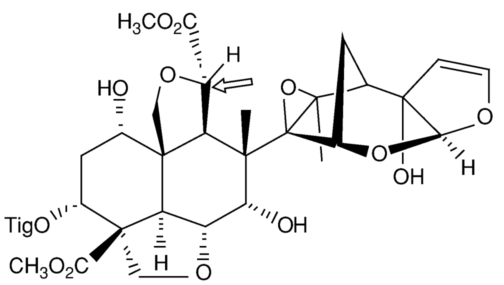
6.4.3.2. Isolation of Azadirachtin

The triterpenoid present in greatest quantity in the seeds is usually salannin (**Figure 1**), a simpler triterpenoid with only weak activity as a feeding deterrent. Azadirachtin, representing 0.1–1.0% (mean 0.6%) of the weight of the seed kernels is next in quantity. The other compounds of azadirachtin-like structure and biological properties occur in progressively smaller amounts. Chemically, they divide into three structural types (**Figure 2**): the azadirachtins, with a hemiacetal group at carbon atom number 11, the azadirachtols, without the hemiacetal, and the meliacarpins, in which the methoxycarbonyl ester at C29 is replaced by methyl. Not all compounds found fit easily into these types and new trace constituents continue to be found (e.g., Luo *et al*., 1999; Malathi *et al*., 2003). Rearrrangement products, known as azadirachtinins (**Figure 3**), of much lower biological activity, are found in the seeds and are also formed during isolation of the limonoids. The names azadirachtin A, azadirachtin B, etc., are sometimes used. These are incorrect names. For the correct chemical names for all these triterpenoids and limonoids, see Kraus (2002). Also present in lesser quantities are nimbin, 3-desacetylnimbin, and 6-desacetylsalannin (Johnson *et al*., 1996) (**Figure 1**), simpler limonoids of lesser activity and interest. Compounds of similar structure are found in the related *Melia* genus. For example, Persian lilac or chinaberry, *Melia azedarach*, contains meliacarpins (**Figure 2**), but the seeds are extremely hard and their extracts are toxic to mammals.

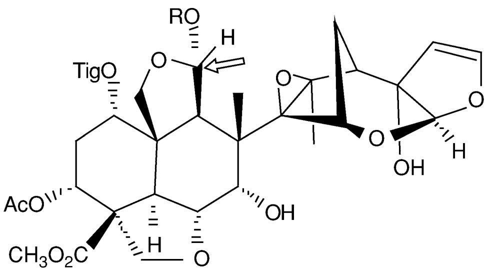
A number of isolation procedures have been described, but all require extraction with solvent from the ground seeds, followed by solvent partition to separate the more polar triterpenoids from the oil. After that, methods differ more, but all require some form of chromatography to separate the individual compounds, either gravity column chromatography (Johnson and Morgan, 1997a), flash columns (Jarvis *et al*., 1999), or preparative high-performance liquid chromatography (HPLC) (Lee and Klocke, 1987; Govindachari *et al*., 1990, 1996). Extraction by supercritical carbon dioxide has been examined, but is not as efficient as solvent extraction (Johnson and Morgan, 1997b; Ambrosino *et al*., 1999). There is a lot of current interest in microwave-assisted chemistry, and a microwave-assisted extraction of seeds has been described (Dai *et al*., 2001).

6.4.3.3. Analysis

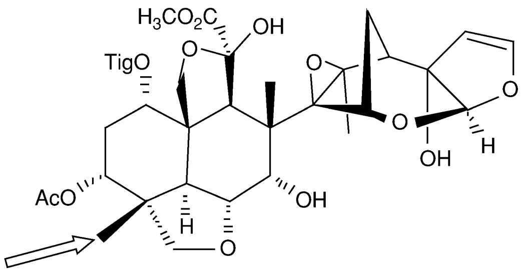
The number of triterpenoids in neem seeds and their similarity in physical properties makes analysis for the important pesticidal compounds both important and difficult. The standard method in use for some time is reverse-phase HPLC with ultraviolet (UV) absorption at short wavelength (214–218 nm) (e.g., Deota *et al*., 2000). Normal-phase supercritical fluid chromatography offers advantages where the equipment is available (Johnson and Morgan, 1997a). A colorimetric method for the whole group of triterpenoids, using vanillin–sulfuric acid has been



3-Tigloylazadirachtol

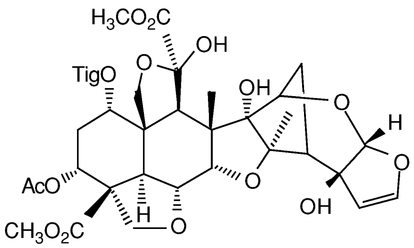


11-Demethoxycarbonylazadirachtin
(azadirachtin H) R=H
11-Acetyl-11-demethoxycarbonylazadirachtin
(marrangin) R=OAc

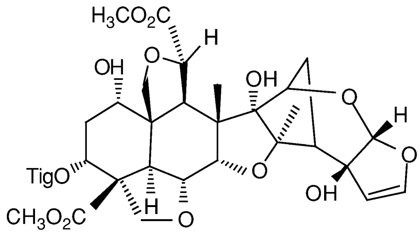


3-Acetyl-1-tigloyl-11-hydroxy-meliacarpin
(azadirachtin D)

Figure 2 Examples of minor products related to azadirachtin from neem seeds. The essential difference of structure that makes an azadirachtol or meliacarpin is indicated by an arrow. Marrangin is the chief insecticidal compound of the marrango tree (*Azadirachta excelsa*). The names azadirachtin A, azadirachtin B, etc., are sometimes used. These are incorrect names. For the correct chemical names for all teiterpernoids and limonoids, see Kraus (2002).



3-Acetyl-1-tigloylazadirachtinin
(11S)-epimer



3-Tigloylazadirachtolin

Figure 3 Rearrangement products from azadirachtin and 3-tigloylazadirachtol. These compounds, of much lower activity than the others illustrated, are formed by reaction between the epoxide ring and the hydroxyl group at C7.

reported, but this is rather nonspecific (Dai *et al*., 1999), and more specific enzyme-linked immunosorbent assays (ELISAs) have been developed that will give detection from 0.5 to 1000 ppb for azadirachtin and close relatives (Schutz *et al*., 1997, Hemalatha *et al*., 2001). Additionally, monoclonal antibodies to azadirachtin produced by phage display techniques are currently being assessed (Robertson, Mordue (Luntz), Mordue, and Porter, unpublished data). The most accurate and sensitive analytical method, but also the most costly in equipment, is liquid chromatography–mass spectrometry (Schaaf *et al*., 2000; Pozo *et al*., 2003).

6.4.3.4. Stability and Persistence

Azadirachtin is soluble in polar organic solvents (methanol, ethanol, acetone, chloroform, ethyl acetate) and slightly soluble in water ($1.29\,g\,l^{-1}$). Aqueous solutions are usually made by dissolving first in ethanol or acetone and carefully diluting with

water to avoid formation of the glassy mass when the solid is wetted with water, and which then dissolves very slowly. Solutions in organic solvents are stable almost indefinitely (Jarvis *et al*., 1998) but solutions in water at pH 7 or higher are unstable. Aqueous solutions are most stable from pH 4 to 6 (Jarvis *et al*., 1998).

While degradability is one great advantage in agricultural application of neem triterpenoids, the rate of disappearance is a little too high according to some authors. One report says azadirachtin is stable for less than 1 week under ambient conditions (Sundaram, 1996), another says its stability is less than 3 months (Brooks *et al*., 1996). Scott and Kaushik (2000) gave a half-life of 36–48 h in water exposed to sunlight. The DT_{50} (time for 50% of azadirachtin to disappear) in soil at 25 °C was 20 and 31.5 days in unautoclaved and autoclaved soil respectively (Stark and Walter, 1995). Probably the most comprehensive studies on the environmental behavior and stability of azadirachtin have been carried out by the Canadian Forest Service for its use on young trees (Sundaram, 1996). Its persistence on balsam fir and red oak foliage; its dissipation in forest nursery soils; absorption, leaching, and desorption from sandy loam forest soil; photostability on foliage; and rate of hydrolysis in natural waters have all been measured (Sundaram, 1996; Sundaram *et al*., 1997). They have also made efforts to formulate azadirachtin against hydrolysis and UV degradation, but no large-scale study of formulation for stability appears to have been made.

6.4.4. Neem Insecticides in Pest Control

6.4.4.1. Background

Today, after 40 years of discovery, research, and development of neem as a natural pesticide there are well-established products in the organic agriculture and niche markets in both North America and Western Europe. Azadirachtin, the main active ingredient of neem seeds, is an extremely effective antifeedant to many phytophagous insects, and an IGR and sterilant to all insects tested. Other constituents of neem seeds, including the oil, have been shown also to be effective control agents against plant nematodes and fungal pathogens.

The complexity of the azadirachtin molecule and our inability, as yet, to synthesize it has precluded its production as a synthetic pesticide, hence use of the natural product is the only choice. Neem as a botanical pesticide has many excellent attributes that include its broad-spectrum IGR effects, systemic action in some plants, minimal disruption of natural enemies and pollinators, rapid breakdown in the environment, and lack of toxicity to vertebrates. However, its initial promise has not been realized. Problems have arisen during the research and development phase and these relate to its mode of action, where (as with most IGRs) azadirachtin acts slowly and although feeding and crop damage ceases shortly after treatment, pest insects remain alive on treated crops, reducing acceptance by growers and consumers. Neem pesticides have limited persistence on plants and require multiple applications against certain pests when used as a stand-alone treatment. This, together with the cost of production of a consistent, high-quality product with 1% or more of azadirachtin by weight from annual supplies of seeds, results in the cost of neem pesticides to be some three times greater than synthetic pyrethroids (Isman, 2004). Regulatory issues have also featured large in the development of neem and have been a formidable barrier to the successful commercialization of neem-based pesticides. The Environmental Protection Agency of the USA chose to simplify the approval process of neem insecticides by recognizing azadirachtin as the sole active ingredient and deeming the remaining chemical components present in neem kernels to be “inert ingredients” (Isman, 2004). However, Europe and Japan, for example, have required identification and toxicological studies for all major constituents of neem, processes that have greatly delayed its introduction into these and other countries.

Despite these problems neem insecticides are established as valuable components in integrated crop management systems and accepted by organic producers. In California neem insecticides are used on over 60 food crops, particularly lettuce and tomato, and account for 0.25% of insecticide use on tomato (California EPA, 2001, in Isman, 2004). In Europe, Neemazal insecticides (Trifolio-M GmbH) have been cleared for use in Germany after the parent company completed toxicological studies on all compounds present in their neem products. Neem is expected to show continued sales growth in highly developed countries as new markets continue to be developed. Growth in organic food production and the gradual reduction, and ultimate elimination, of synthetic neurotoxic insecticides will both favor expansion of the market for neem. The introduction of neem products specifically formulated for particular crops and crop pests will also aid expansion, as would intensive education of growers.

In developing countries, simple inexpensive formulation of neem extracts must be encouraged as an

important source of pest control at the level of subsistence farming, as long as such unregulated products are not confused with legitimate regulated products of high technical standard.

6.4.4.2. Use in Integrated Pest Management

Azadirachtin exhibits good efficacy against key pests such as whiteflies, leaf miners, fungus gnats, thrips, aphids, and many leaf-eating caterpillars (Immaraju, 1998). Its use is recommended for insecticide resistance control, integrated pest control, and organic pest control programs. Research in community-level interactions with a view to the use of neem-based insecticides in IPM strategies are revealing subtle and complex effects that may or may not be useful. For example, the ability of azadirachtin to inhibit protein synthesis is demonstrated in the obliquebanded leafroller, *Choristoneura rosaceana* by an inhibition of midgut esterases (Smirle *et al*., 1996; Lowery and Smirle, 2000). This is seen also in resistant Colorado potato beetles (*Leptinotarsa decemlineata*) treated with both neem and *Bacillus thuringiensis* (Trisyono and Whalon, 1999) and may be a useful tool in the management of insect populations where insecticide resistance has developed as a result of elevated esterase activity.

Neem has been suggested for use in maintaining beneficial insect levels, as predators and parasitoids are not exposed directly to the insecticide on the plant and are not harmed by it, as shown in the brown citrus aphid and its parasitoid *Lysiphlebus testaceipes* (Tang *et al*., 2002) and the glasshouse whitefly *Trialeurodes vaporariorum* and its paraitoid *Encarsia formosa* (Simmonds *et al*., 2002). However, the indirect exposure of predators or parasitoids by feeding on or developing within prey exposed to azadirachtin may result in classical IGR effects on the beneficial if concentrations are high (Beckage *et al*., 1988; Osman and Bradley, 1993; Raguraman and Singh, 1998; Qi *et al*., 2001). Overall, however, if concentrations of neem insecticides are kept to the recommended levels no harm should ensue, for example, for the diamondback moth *Plutella xylostella* no adverse effects were apparent on the survival and foraging behavior of *Diadegma* parasitoids (Akol *et al*., 2002). There is evidence that larval honeybees have some resistance to azadirachtin in neem, and may also be safe from its effects by a lack of translocation back to the hive in nectar or pollen (Naumann and Isman, 1996).

6.4.4.3. Toxicity to Nontarget Organisms

Azadirachtin has displayed remarkable selectivity in toxicity studies, consistently showing no toxicity to vertebrates until very high concentrations are used. Investigations into mammalian toxicology of pure azadirachtin demonstrated a "no-observed-effect level" of 1500 mg kg^{-1} day^{-1} (the highest quantity tested) when azadirachtin was administered orally to rats for 90 days (Raizada *et al*., 2001). In addition to whole-animal toxicological data, a number of *in vitro* assays of the effects of azadirachtin on several mammalian cell lines have established that the compound is not effective against mammalian cells (measured as effects on proliferation, direct toxicity, and morphological alterations, e.g., micronucleation) until concentrations above 10 μM are used (Reed and Majumder, 1998; Akudugu *et al*., 2001; Salehzadeh *et al*., 2002; Robertson, 2004) (see Section 6.4.7.5).

Similarly with plants, phytotoxicity has been demonstrated particularly at the seedling stage, however, again not until high concentrations are used. Using tobacco seedlings Nisbet *et al*. (1996a) demonstrated toxic effects seen as short-term wilting and long-term deformation at 500 ppm pure azadirachtin. No effects on seedlings were seen at lower doses (1–200 ppm). Using turnip and barley seedlings no significant phytotoxicity was seen with 1–200 ppm azadirachtin (Stamatis, Allen, and Mordue (Luntz), unpublished data), although the dry weight of turnip seedlings 14 days post-treatment with 200 ppm azadirachtin was significantly lower than controls. Cell suspension cultures of *Agrostis stolonifera* when treated with azadirachtin at 150, 250, 500, and 1000 ppm during exponential growth showed significant loss in cell viability (measured as % viable cells in 10 μl cell suspension aliquots using fluorescein diacetate) within 2 h of treatment. The EC_{50} (concentration at which 50% of cells lost viability) at 2 h post-treatment was approximately 300 ppm (400 μM) (Stamatis, Zounos, Allen, and Mordue (Luntz), unpublished data).

6.4.4.4. Resistance

One of many advantages of the azadirachtin group of compounds in practical use is that development of resistance, which has become such a problem with single-compound synthetic pesticides, is delayed by use of the mixture. Resistance and desensitization can be avoided with the mixture of neem triterpenoids. Feng and Isman (1995) showed that the peach potato aphid *Myzus persicae* developed resistance to pure azadirachtin over 40 generations, but the same did not happen with neem seed extract. In another study with *Spodoptera litura* larvae on cabbage, the larvae became desensitized to pure azadirachtin in both choice and nonchoice tests, but did not desensitize to the seed extract, even

though the latter contained the same amount of azadirachtin (Bomford and Isman, 1996).

6.4.4.5. Systemic Action

The discovery by Gill and Lewis (1971) that azadirachtin was effective systemically was largely ignored for some years, but recently, that valuable aspect of its properties has again received attention. The uptake of azadirachtin from solution by cabbage leaves was demonstrated with the cabbage white butterfly *Pieris brassicae* (Arpaia and van Loon, 1993). Tobacco (*Nicotiana clevelandii*) seedlings also absorbed azadirachtin from solution through their roots and its subsequent translocation to leaves caused disturbances in the feeding behavior of the aphid *M. persicae*, resulting in a reduced ability of the aphids to acquire phloem-located viruses from infected seedlings (Nisbet *et al.*, 1993, 1996a). Sundaram *et al.* (1995) showed that neem extract watered around the roots of young aspen (*Populus tremuloides*) was taken up within 3 h and translocated to the stem and foliage within 3 days. Soil drenching of bean plants (*Phaseolus vulgaris*) with neem extract controlled the pea leaf miner *Lyriomyza huidobrensis* (Weintraub and Horowitz, 1997). More recently it has been shown that systemic dispersion of neem extract can be used to control *Ips pini* bark beetle in lodgepole pine (*Pinus contorta*) logs (Duthie-Holt *et al.*, 1999). No works appears to have been done to show whether all the azadirachtin-like compounds are translocated, or whether some more readily than others.

6.4.5. Antifeedant Effects of Azadirachtin

Azadirachtin has marked antifeedant activity against a large number of insect species, its effects being mediated through contact chemoreception (primary antifeedancy) and internal feedback mechanisms (secondary antifeedancy); the latter related to toxic effects on the gut, e.g., enzyme production, cell proliferation, and motility (Timmins and Reynolds, 1992; Nasiruddin and Mordue (Luntz), 1993a; Trumm and Dorn, 2000). Insects vary markedly in their behavioral sensitivity to azadirachtin. The desert locust *Schistocerca gregaria* and many species of Lepidoptera, are among the most sensitive, being deterred by as little as 0.007 ppm (in diets) whereas the Hemiptera and Coleoptera are much less sensitive with EC_{50} values of around 100 ppm or more (e.g., Isman, 1993; Mordue (Luntz) and Blackwell, 1993). Whereas *Schistocerca gregaria* prefers to starve to death rather than ingest azadirachtin, the primary antifeedant effect on aphids of azadirachtin applied systemically in plants occurs at levels far higher than those causing IGR and sterility effects (Lowery and Isman, 1993; Nisbet *et al.*, 1993, 1994; Mordue (Luntz) *et al.*, 1996). Hematophagous insects such as *Culex* mosquitoes are also less sensitive to the antifeedant effects of azadirachtin than to its IGR effects (Su and Mulla, 1998, 1999).

Whereas azadirachtin alone shows toxic IGR and sterilant actions against insects, several compounds in the plant biosynthetic pathway leading to azadirachtin have antifeedant effects against phytophagous insects (Aerts and Mordue (Luntz), 1997). The antifeedant role is an important phenomenon in the overall toxic effects of azadirachtin in those insects that are sensitive to it behaviorally and this phenomenon has been utilized in bioassays to explore the structure–activity relationships of the azadirachtin molecule. Both the decalin and the dihydrofuranacetal fragments of azadirachtin are important in eliciting antifeedant activity. Methylation of the hydroxy substitutions on the molecule and the addition of bulky groups to the dihydrofuran ring decrease antifeedant activity (Blaney *et al.*, 1990; Blaney and Simmonds, 1994; Govindachari *et al.*, 1995; Simmonds *et al.*, 1995).

The primary behavioral antifeedant response of insects to azadirachtin is mediated via neural input from the contact chemoreceptors. Inhibition of feeding behavior results from stimulation of deterrent receptors by azadirachtin often coupled with an inhibition of sugar receptors (Simmonds and Blaney, 1984). In *Spodoptera littoralis, Helicoverpa armigera*, and *H. assulta*, cells sensitive to sucrose and azadirachtin are mainly in the lateral sensillum styloconicum with azadirachtin evoking high impulse discharges (Simmonds *et al.*, 1995; Tang *et al.*, 2000). The firing of sucrose sensitive cells is reduced in the presence of azadirachtin and in some species the firing of the deterrent cells to azadirachtin is also reduced in the presence of sucrose (Simmonds and Blaney, 1996). This phenomenon, known as peripheral interaction, varies among insects and occurs in *S. littoralis* and *S. gregaria* but not in *L. migratoria* (Simmonds and Blaney, 1996). Investigations of the antifeedant mode of action by both electrophysiological recordings and behavioral analysis have shown that both polyphagous and oligophagous insects are behaviorally responsive to azadirachtin with the most responsive species being able to differentiate extremely small changes in the parent molecule (Blaney *et al.*, 1990, 1994; Nasiruddin and Mordue (Luntz), 1993b; Simmonds *et al.*, 1995). In Lepidoptera the antifeedant response is also correlated with increased neural activity of the chemoreceptors. A comparison of antifeedant

effects with physiological effects as measured by electrophysiological responses of taste receptors in lepidopteran larvae, antifeedant behavioral responses in locusts, and [^{3}H]dihydroazadirachtin-binding studies using insect cell lines and homogenates of locust testes is interesting. Results show that all of the effects are linked in terms of the potency of synthetic analogs of azadirachtin. This suggest a causal link between specific binding to membrane proteins and the ability of the azadirachtin molecule to exert biological effects (Mordue (Luntz) *et al.*, 1998).

6.4.6. Neuroendocrine Effects of Azadirachtin

6.4.6.1. Insect Growth Regulation

Over 400 species of insect to date have been shown to be susceptible to the neuroendocrine effects of azadirachtin, exhibiting the now classic symptoms of abnormal molts, larval–adult intermediates, mortality at ecdysis, and delayed molts, often resulting in greatly extended instar lengths. Many papers on a wide variety of insect taxa have allowed detailed descriptions of symptoms to be recorded that show consistency between all insects tested and an ED_{50} (effective dose for 50% of the population) of between 1 and 4 $\mu g\,g^{-1}$ bodyweight (Koul and Isman, 1991; Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz) *et al.*, 1995; Riba *et al.*, 2003). In *O. fasciatus, Epilachna varivestis*, and *L. migratoria* complete molt inhibition has been shown, by radioimmunoassay, to be due to a blockage of ecdysteroid synthesis and release, a delay in appearance of the last ecdysteroid peak, and a slow, abnormal decline in this peak (Redfern *et al.*, 1982; Sieber and Rembold, 1983; Schlüter *et al.*, 1985; Mordue (Luntz) *et al.*, 1986). In *Rhodnius prolixus* a single dose of azadirachtin reduced hemolymph ecdysteroid titers to levels too low for induction of ecdysis (Garcia *et al.*, 1990). This blockage of ecdysteroid production is not due to a direct action of azadirachtin on the prothoracic glands (Koolman *et al.*, 1988) but is caused by two specific actions of azadirachtin. These actions are in the cascade of events leading from brain activation and competency to molt to 20-hydroxyecdysone (20E) production in the hemolymph and its effects on target tissues. First, the release of the morphogenetic brain peptide prothoracicotropic hormone (PTTH) responsible for inducing synthesis and release of ecdysone from the prothoracic glands is blocked in azadirachtin-treated insects. *In vitro* cultures of brain gland complexes or prothoracic glands of *Calliphora vicinia, Bombyx mori, Heliothis virescens*, or *Manduca sexta* in the presence of azadirachtin and PTTH will produce and release normal levels of ecdysone (Bidmon *et al.*, 1987; Pener *et al.*, 1988; Barnby and Klocke, 1990). However, decapitation and ligation experiments in *B. mori, R. prolixus*, and *Peridroma saucia* have shown that release of PTTH is deficient in azadirachtin-treated insects (Koul *et al.*, 1987; Garcia *et al.*, 1990; Koul and Isman, 1991). This deficiency is due to effects on the corpus cardiacum, in terms of release of PTTH and indeed other peptide hormones related to molting such as eclosion hormone or bursicon (Sieber and Rembold, 1983; Mordue (Luntz) *et al.*, 1986; Bidmon *et al.*, 1987; Rembold *et al.*, 1989). Staining of neurosecretion with paraldehyde fuchsin revealed a build-up of material in the corpora cardiaca and neuropilar areas of the brain neurosecretory system in *L. migratoria* (Subrahmanyam *et al.*, 1989).

Experiments on *L. migratoria* with [^{3}H]dihydroazadirachtin revealed heavy labeling within the corpora cardiaca but not in the brain beyond the neurilemma barrier membrane (Subrahmanyam *et al.*, 1989). This suggests, that azadirachtin directly affects the corpora cardiaca by inhibiting the release of hormones, perhaps by operating via effects on neurotransmitters such as acetylcholine, γ-aminobutyric acid (GABA), serotonin, and octopamine (Bidmon *et al.*, 1987; Käuser *et al.*, 1987; Koolman *et al.*, 1988; Banerjee and Rembold, 1992). Also through that axis, azadirachtin has been shown to slow down the rate of synthesis and transport of PTTH by the brain neurosecretory cells, for example in *H. virescens* (Barnaby and Klocke, 1990). Additionally, studies on the ultrastructure of the ring complex of *Lucilia cuprina* after azadirachtin treatment revealed degenerative structural changes of the nuclei of all endocrine glands (prothoracic glands, corpus allatum, corpus cardiacum), suggesting that effects at the transcriptional–translational level were the key to the generalized disruption of neuroendocrine function (Meurant *et al.*, 1994).

Second, in addition to the effect of azadirachtin on PTTH synthesis, transport, and release, azadirachtin has direct effects on the production of ecdysone 20-monooxygenase from the midgut and fat body; the insect cytochrome P450-dependent hydroxylase responsible for the conversion of ecdysone to its more active metabolite, 20E. In *C. vicinia, Drosophila melanogaster, Aedes aegypti*, and *M. sexta*, 20-monooxygenase levels were affected within 1 h of azadirachtin exposure at levels varying between 10^{-4} and 10^{-5} M azadirachtin

(Bidmon *et al.*, 1987; Smith and Mitchell, 1988; Mitchell *et al.*, 1997). Such effects are also clearly demonstrated in *Tenebrio molitor* pupae where ecdysone hemolymph levels were shown to remain relatively constant after azadirachtin treatment (1 µg per insect), whereas there was a highly significant depletion of immunoreactive ecdysteroids (Marco *et al.*, 1990). Variations in successful recovery of development by treatment with exogenous 20E (Pener *et al.*, 1988; Barnby and Klocke, 1990; Marco *et al.*, 1990) support the fact that different target sites for azadirachtin exist and are manifested during growth and molting processes at different times.

In parallel with molting hormone deficiencies in azadirachtin-treated insects, changes in JH also play a significant role in the overall syndrome of azadirachtin poisoning at the molt. That JH hemolymph titers are reduced or delayed is demonstrated in a variety of ways. Azadirachtin inhibits JH-stimulated supernumerary molts in chilled *Galleria mellonella* larvae (Malczewska *et al.*, 1988) perhaps by a blockage of synthesis and release of JH. In the last instar larvae of *M. sexta*, azadirachtin causes supernumerary molts by delaying and extending the JH peak into the critical period for commitment to larval rather than pupal cuticle (Schlüter *et al.*, 1985; Beckage *et al.*, 1988). The importance of timing of hemolymph JH levels with ecdysteroid levels is demonstrated by the selective destruction of larval crochets or hooked setae on the prolegs in *M. sexta* (Reynolds and Wing, 1986; Beckage *et al.*, 1988). The epidermal cells producing such structures require the presence of both ecdysteroids and JH in order to make cuticle and in the absence of JH (e.g., on day 2 of the last larval instar) (Riddiford, 1981) the epidermis loses this ability. Other examples of an imbalance in the presence or absence of ecdysteroid and JH levels are seen in the occurrence of uneverted tanned pupal wing discs in *E. varivestis* (Schlüter, 1987), effects linked with high JH titers such as green hemolymph, brown and green cuticle, lack of black pigment, and supernumerary molts in locusts, *S. littoralis*, and *G. mellonella* (Schmutterer and Freres, 1990; Nicol and Schmutterer, 1991; von Friesewinkel and Schmutterer, 1991; Gelbic and Némec, 2001) and cuticular melanization resulting in black spots related to the absence of JH and low levels of ecdysteroids (Hori *et al.*, 1984; Koul *et al.*, 1987; Malczewska *et al.*, 1988).

It is of interest that azadirachtin potentiates the action of the ecdysteroid agonist RH-2485 in *S. littoralis* (Adel and Sehnal, 2000). There was no effect of azadirachtin on the hyperecdysonic action of high concentrations of diacylhydrazine RH-2485 to induce precocious molting (immediate and fatal molts) thus substantiating the view that azadirachtin does not bind to epidermal ecdysteroid receptors (Koolman *et al.*, 1988). However, azadirachtin was shown to potentiate supernumerary molts in early instar larvae of *S. littoralis* treated with low concentrations of RH-2485, perhaps explained by a slow decline in hemolymph JH levels. Azadirachtin also shows synergy with RH-2485 with regard to deaths at the molt and sterility (Adel and Sehnal, 2000).

6.4.6.2. Reproduction

Adverse effects of azadirachtin on ovarian and testes development, fecundity, and fertility are an important component of overall toxicity and insect control (Karnavar, 1987; Mordue (Luntz) and Blackwell, 1993; Mordue (Luntz), 2000). Sterility effects in females due to interference with vitellogenin synthesis and its uptake into oocytes has been demonstrated in many insects including *L. migratoria*, *O. fasciatus*, *S. exempta*, and *R. prolixus* (Rembold and Sieber, 1981; Dorn *et al.*, 1987; Feder *et al.*, 1988; Tanzubil and McCaffery, 1990). Reduced fecundity has been recorded in the fruit fly *Ceratitis capitata*, the leaf miner *Liriomyza trifolii*, peach potato aphid *Myzus persicae*, *S. exempta, S. littoralis*, and the southern green stink bug, *Nezara viridula* (Parkman and Pienkowski, 1990; Stark *et al.*, 1990; Tanzubil and McCaffrey, 1990; Nisbet *et al.*, 1994; Adel and Sehnal, 2000; Riba *et al.*, 2003); such fertility effects relate to resorption of yolk proteins in developing eggs (Rembold and Sieber, 1981) and lower viability of emerging larvae after treatment of the parental generation (Tanzubil and McCaffery, 1990). In males, fertility reduction is seen in a reduced potency in *O. fasciatus* (Dorn, 1986), a degeneration of spermatocytes in *Mamestra brassicae* (Shimizu, 1988) and blocked cell division in developing spermatocytes in *L. migratoria* leading to significantly smaller mature testes (Linton *et al.*, 1997).

The endocrine events causing reproductive malfunction are very similar to those seen with growth and molting and relate to disruptions in ecdysteroid production by the ovaries and in JH levels in the hemolymph. The latter are most probably caused by alterations to the brain neurosecretory peptides, the allatostatins and allatotropins that control synthesis and release of JH.

Whether production of JH esterase levels, controlling JH catabolism, is affected by azadirachtin is not yet known. In adult female *L. migratoria* ecdysteroid synthesis by the ovaries, together with oogenesis, is inhibited by 10 µg azadirachtin per insect (Rembold and Sieber, 1981), and JH titers

are decreased (Rembold *et al.*, 1986), these effects being associated with an accumulation of neurosecretory material in the corpora cardiaca and brain (Subrahmanyam *et al.*, 1989). In *R. prolixus* reduction in oocyte growth and egg production are caused also by reduced ecdysteroid levels in hemolymph and ovaries (Feder *et al.*, 1988; Garcia *et al.*, 1991). In the cotton stainer bug *Dysdercus koenigii* and the peach potato aphid *Myzus persicae* azadirachtin applied topically or in the diet inhibits and delays embryogenesis, resulting either in many of the embryos remaining at a late stage of development or in the viviparous aphid being born dead with undeveloped appendages (Koul, 1984; Nisbet *et al.*, 1994). It is thought that JH control of embryogenesis and the rate of development of embryos in aphids are important here.

In adult female earwigs, *Labidura riparia*, azadirachtin has been shown to have marked cytological effects on ovaries resulting in severely reduced ovarian development. Due to cyclical reproductive changes in *L. riparia* throughout adult life, relating to the production of eggs (vitellogenesis) and a nonvitellogenic period when the female cares for her eggs, there was the possibility of investigating the specific role of azadirachtin in vitellogenesis control. In this insect JH controls both vitellogenin production in the fat body and stimulates vitellogenin uptake by the ovaries. In addition, ovarian ecdysteroids play a role in vitellogenesis and, at the peak of production, oviposition. They also provide a feedback loop to the brain pars lateralis neurosecretory cells causing release of allatostatins, which block JH synthesis and release enabling successive follicle degenerations during the nonvitellogenic period. Azadirachtin treatment blocks vitellogenesis and corpus allatum activity as seen by ultrastructural studies and concomitantly increases allatostatin build-up in the pars lateralis as measured by antibodies to allatostatin 3 (*Blattella germanica* BLAST-3) (Sayah *et al.*, 1996, 1998). This phenomenon together with the fact that JH treatment rescues the effect of azadirachtin on vitellogenesis suggest that azadirachtin affects those peptides controlling corpus allatum activity in a similar manner to the azadirachtin effects on PTTH and hence ecdysone production during the molt.

Two major conclusions can be drawn regarding the mode of action of azadirachtin on neuroendocrine activity in insects during the molt and reproductive development. First, in both molting and sterility, the processes of synthesis, transport, and release of morphogenetic peptide hormones in the brain are at the center of the mode of action of azadirachtin. Second, in the molt (but not yet proved during reproductive development) the production of important enzymes for controlling hemolymph levels of active hormones (20-hydroxymonooxygenase levels that convert ecdysone to 20E) are inhibited by azadirachtin.

6.4.7. Studies on the Mode of Action of Azadirachtin

6.4.7.1. Studies using Insect Cell Lines

The analysis of direct cytological effects of azadirachtin in whole insects, or even in isolated insect tissues, is complicated by the multiplicity of physiological consequences resulting from the compound (see Section 6.4.6). This complexity has been addressed by analyzing the pharmacology of azadirachtin uptake and binding in cultured *S. frugiperda* Sf9 cells (derived from pupal ovarian tissue) and *D. melanogaster* Kc167 cells (derived from embryonic cells). Such cells rapidly accumulate [^{3}H]azadirachtin from culture medium and are enriched with binding sites compared with many tissue extracts derived from whole insects (Nisbet *et al.*, 1995, 1997; Mordue (Luntz) *et al.*, 1999; Mordue (Luntz), 2002; Robertson, 2004). The effects of azadirachtin on the proliferation and cell cycle events in these cells (Rembold and Annadurai, 1993; Salehzadeh *et al.*, 2002, 2003; Robertson, 2004), accompanied by the above binding studies have given detailed insights into the mode of action of the compound.

Initial studies examining the effects of azadirachtin on the proliferation of Sf9 cells used relatively high concentrations (1 μM) in the culture medium to produce profound effects on cell proliferation (Rembold and Annadurai, 1993; Mordue (Luntz) and Nisbet, 2000). Robertson (2004) has also shown significant reductions in the number of viable cells using the trypan blue exclusion assay within 48 h of treatment with 1 μM azadirachtin for both Sf9 and Kc167 cells. In addition to this effect on cell proliferation, azadirachtin evoked a characteristic cytotoxic effect on cultured cells. Reed and Majumdar (1998) used azadirachtin concentrations of about 0.1 μM in cell culture to demonstrate increases in cell volume, blebbings, blisters, and holes in cell membranes resulting in cytoplasmic extrusions. Since these studies much more refined work has been carried out to discover where in the cell azadirachtin is acting and at what concentration.

6.4.7.2. Effects on Cell Cycle Events

The effects of azadirachtin in blocking cell division have already been established in whole tissues such

as imaginal wing discs of *E. varivestis* (Schlüter, 1985, 1987) and in locust testes, where meiosis was blocked at prometaphase I (Linton *et al*., 1997). Salehzadeh *et al*. (2002) examined the effects of azadirachtin on the proliferation of Sf9 cells using dehydrogenase activity to estimate cell numbers and found an EC_{50} of 0.15 nM after 96 h incubation. Cell proliferation studies using [^{3}H]thymidine uptake during cell division revealed also an inhibition of mitosis in Sf9 cells 24 h post-treatment with an EC_{50} value of 1.5 nM azadirachtin (Robertson, 2004). This nuclear effect was confirmed by Comet assays that directly measure DNA damage. Significant damage was revealed in Sf9 cells within 6 h of treatment; with an EC_{50} value of 5 nM (Robertson, 2004). The reduction in proliferation was related to a time- and concentration-dependent antimitotic effect of azadirachtin on the cells, with concentrations between 10 nM and 5 μM causing the accumulation of mitotic figures, incomplete mitosis, cytokinesis, and an increased proportion of the cells in the G_2/M phase of the cell cycle (Salehzadeh *et al*., 2003). The "G_2" (GAP2) and the "M" (mitosis) stages occur after DNA synthesis and are the stages when the chromosomes separate and cytoplasmic division occurs. These events are dependent upon multiple cellular processes, including the synthesis and functioning of microtubules; a number of compounds (e.g., colchicine) interfere with these processes and inhibit mitosis. Azadirachtin and colchicine inhibit the cell cycle at similar points, an observation that encouraged Salehzadeh *et al*. (2003) to examine the ability of azadirachtin to prevent colchicine-flouroscein from binding to cells previously incubated for 4 h with 10 μM azadirachtin. A 45% reduction in fluorescence was recorded in azadirachtin-treated cells. This led to the conclusion that azadirachtin was binding either directly to tubulin or causing conformational changes that prevented colchicine binding (Salehzadeh *et al*., 2003). While [^{3}H]dihydroazadirachtin is rapidly incorporated by Sf9 cells and binds to the nuclei, it does so in a highly specific, high-affinity, saturable, and essentially irreversible manner (Nisbet *et al*., 1997). Based on these kinetic data, saturation of the azadirachtin-binding element of Sf9 cells requires much lower concentrations or shorter incubation times than 10 μM for 4 h. Thus, it seems unlikely that colchicine and azadirachtin share the same binding site in Sf9 cells; see Schlüter (1987) for a similar conclusion in wing bud development of *E. varivestis* and Billker *et al*. (2002) in microgametogenesis of *Plasmodium berghei*. An alternative explanation for the reduction in colchicine labeling in azadirachtin-treated cells is that the concentration of azadirachtin used, 10 μM, caused cytotoxic damage and reduced the availability of colchicine-binding sites.

6.4.7.3. Binding Studies

There is clearly substantial evidence to show that azadirachtin inhibits cell proliferation in Sf9 cells and that it does so by inhibiting mitotic events that rely on the correct synthesis and assembly of microtubules. [^{3}H]dihydroazadirachtin binds to the nuclei of Sf9 cells (Nisbet *et al*., 1997) and to fractions of homogenates of locust testes with very similar binding properties (Nisbet *et al*., 1995; Mordue (Luntz) *et al*., 1999). The binding sites on locust testes were determined by microautoradiography (Nisbet *et al*., 1996b) and shown to be associated with developing sperm tails, i.e., a region of intense microtubule synthesis. In addition azadirachtin interferes with spermatogenic meiosis in locust testes, arresting meiosis at metaphase I (Linton *et al*., 1997), an effect that could be due to disruption in microtubule synthesis and assembly. Treatment of homogenates of locus testes with nocodazole, which disrupts polymerized microtubules, or prolonged cold-stirring in buffers to promote microtubule depolymerization failed to reduce the binding of [^{3}H]dihydroazadirachtin (Nisbet *et al*., unpublished data).

In systems other than insect cell lines there is good evidence that azadirachtin interferes with microtubule function. In the malarial parasite *P. berghei* azadirachtin does not interfere with the assembly of microtubules during microgametogenesis but confocal laser microscopy and transmission electron microscopy have shown that the formation of mitotic spindles and axonemes was disrupted, i.e., the patterning of microtubules into more complex structures (Billker *et al*., 2002).

In the absence of a good insect cell model for analyzing protein secretion, mammalian cell lines have been used to attempt to understand a possible link between the effects of azadirachtin on protein synthesis, microtubule assembly, and secretory events. Incubation of mouse mammary acini with 0.5 mM azadirachtin reduced overall protein synthesis by about 75% but failed to prevent the secretion of [^{35}S]methionine-labeled proteins in pulse-chase experiments (Nisbet *et al*., unpublished data). The secretion of proteinaceous vesicles from murine mammary epithelial cells is dependent upon the structural integrity of a microtubular network and the existence of tubulins in their polymerized forms. In contrast to the lack of effect of azadirachtin on protein secretion, colchicine and nocodazole treatments of these cells disrupted microtubule assembly and

inhibited protein secretion (Patton, 1974; Rennison *et al.*, 1992). Salehzadeh *et al.* (2003) prepared tubulin from pig brains and repolymerized the protein *in vitro* in the presence of 0.1 mM azadirachtin, but this treatment only reduced polymerization by about 20%. In addition, tubulin and repolymerized microtubules prepared from sheep brains failed to specifically bind [^{3}H]dihydroazadirachtin *in vitro* (Nisbet *et al.*, unpublished data).

6.4.7.4. Effects on Protein Synthesis

Azadirachtin directly inhibits protein synthesis in a variety of tissues where cells are producing enzymes: midgut cells producing trypsin (Timmins and Reynolds, 1992), midgut and fat body cells producing 20-monooxygenases for ecdysone catabolism (Bidmon *et al.*, 1987; Smith and Mitchell, 1988), and midgut and fat body cells producing detoxification enzymes in insecticide-resistant insects (Lowery and Smirle, 2000). These effects in whole insects are seen also in insect cell lines where both cell division and protein synthesis are inhibited in a dose-dependent manner. Using *S. gregaria* injected with azadirachtin, Annadurai and Rembold (1993) were able to study polypeptide profiles of brain, corpora cardiaca, subesophageal ganglion, and hemolymph using high-resolution two-dimensional gel electrophoresis. Using Sf9 cells Robertson (2004) also carried out polypeptide separation with two-dimensional gels with spot analysis by hierarchical and K means clustering. Both groups concluded that differential effects on protein synthesis occurred although overall protein synthesis was reduced. Robertson (2004) showed that in Sf9 cells out of 381 protein spots 52.2% were decreased significantly in intensity, 28.9% were increased in size, and 18.9% were unchanged.

6.4.7.5. Studies Using Mammalian Cell Lines

Azadirachtin is not effective against mammalian cells (measured as effects on proliferation, direct toxicity, and morphological alterations, e.g., micronucleation) until concentrations above 10 μM are reached (Reed *et al.*, 1998; Akudugu *et al.*, 2001; Salehzadeh *et al.*, 2002; Robertson, 2004). On cultured rat dorsal root ganglion neurons, neuronal excitability was altered by modulation of potassium conductances, but only after azadirachtin treatment at 10 μM or higher (Scott *et al.*, 1999). In contrast, the EC_{50} for the effect of the compound on the viability of insect cells (Sf9) *in vitro* is several orders of magnitude lower than that for mammalian cell lines, e.g., 150 pM for Sf9 cells, >10 μM for mouse L929 cells (Salehzadeh *et al.*, 2002). Using mammalian cell lines to investigate azadirachtin mode of action it was shown that azadirachtin inhibited protein synthesis in mouse mammary cells at concentrations above 100 μM but had no effects on protein secretion from these cells (Nisbet *et al.*, unpublished data). Cytosolic concentrations of Ca^{2+} in mouse mammary cells were unaffected by the presence of 500 μM azadirachtin. It is therefore apparent that azadirachtin does not inhibit protein synthesis in mammalian cells as a secondary effect of instantaneous mobilization of calcium stores from the endoplasmic reticulum, as has been demonstrated for other protein synthesis inhibitors, e.g., vasopressin (Reilly *et al.*, 1998), arachidonate, carbachol, or 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxy-methyl) ester (BAPTA/AM) (Wong *et al.*, 1993). It is also clear from the lack of effect on resting calcium levels or protein secretion that azadirachtin does not induce reductions in protein synthesis through general toxicity or structural cellular damage and that the molecule may interfere with a specific cellular event involved in protein synthesis.

6.4.7.6. Resolving the Mode of Action of Azadirachtin

It would appear from current knowledge that azadirachtin may have more than one mode of action. First, it alters or prevents the formation of new assemblages of organelles or cytoskeleton resulting in the disruption of cell division, blocked transport and release of neurosecretory peptides, and inhibition of spermatozoa formation. Second, it inhibits protein synthesis in cells that are metabolically active and have been switched on to produce large amounts of protein such as digestive enzymes by midgut cells, cultured cells with high rates of proliferation, or cell production of mixed function oxidases for detoxification of xenobiotics. At the molecular level azadirachtin may act by altering or preventing transcription or translation of proteins expressed at particular stages of the cell cycle. Future work to elucidate this novel mode of action must now concentrate on the characterization and identification of binding sites using proteomic, microarray, and differential display techniques.

The mode of action of azadirachtin is unlikely to be resolved through the use of mammalian cell lines or proteins. The differences between homologous proteins in mammals and insects may in fact be the key to the specificity of azadirachtin. The use of modern protein purification and production methods should assist in resolving this question. The rapidly expanding range of proteomic tools available for synthesizing short peptides or recombinant polypeptides (e.g., phage display) could pro-

vide a high-throughput method of determining the amino acid residues and conformation required for azadirachtin binding, giving leads to the identity of the binding protein in insects.

Azadirachtin has profound effects on protein synthesis in insect cells and tissues and its antimitotic actions may result from the absence of proteins involved directly in microtubule assembly and function, or proteins involved in posttranslation modifications of these elements (e.g., protein kinases and phosphatases). Thorough stepwise analysis of the protein profile of cells treated with azadirachtin is possible by two-dimensional electrophoresis, such as in the studies initiated by Robertson (2004). This will provide valuable information on the time course of the effects of azadirachtin on the synthesis of individual proteins and even on the posttranslational modification of proteins. Modern molecular techniques to investigate the effects of azadirachtin on gene transcription will be invaluable to investigate this phenomenon. Differential gene expression in azadirachtin-treated cells at time points after treatment could be investigated using RNA arbitrarily primed-polymerase chain reaction (RAP-PCR) or cDNA subtraction to give an indication of which genes are upregulated or downregulated early in the process of the cytotoxic action of azadirachtin. With the advent of high-throughput expressed sequence tag (EST) sequencing and microarray analysis, EST databases could be generated from untreated insect cell lines, printed on microarrays, and hybridized with cDNA derived from treated and untreated cell lines over a wide range of time points after treatment. Ideally, these time-course analyses would be performed in azadirachtin-susceptible *Drosophila* cell lines (Robertson 2004; Mordue, Dhadialla, and Mordue (Luntz), unpublished data), to take advantage of the availability of the full annotated genome for this organism rather than attempting to determine homology with genes from Sf9 cells for example. Recent work has identified a putative Azadirachtin binding proteins with an apparent molecular mass of 591 kD. The protein was identified, by native polyacrylamide gel electrophoresis ligand overlay blots, in nuclear protein extracts from *Drosophila* KC167 cells using BSA-conjugated azadirachtin as a ligand (S.L. Robertson, T.S. Dhandialla, N. Weiting, S.V. Ley, W. Mordue, A.J. Mordue (Luntz), unpublished data).

References

Adel, M.M., Sehnal, F., **2000**. Azadirachtin potentiates the action of ecdysteroid agonist RH-2485 in *Spodoptera littoralis*. *J. Insect Physiol. 46*, 267–274.

Aerts, R.J., Mordue (Luntz), A.J., **1997**. Feeding deterrence and toxicity of neem triterpenoids. *J. Chem. Ecol. 23*, 2117–2132.

Akhila, A., Rani, K., **1999**. Chemistry of the neem tree (*Azadirachta indica* A. Juss.). *Prog. Chem Org. Nat. Prod. 78*, 47–149.

Akol, A.M., Sithanantham, S., Njagi, P.G.N., Varela, A., Mueke, J.M., **2002**. Relative safety of sprays of two neem insecticides to *Diadegma mollipla* (Holmgren), a parasitoid of the diamondback moth: effects on adult longevity and foraging behaviour. *Crop Protect. 21*, 853–859.

Akudugu, J., Gäde, G., Böhm, L., **2001**. Cytotoxicity of azadirachtin A in human glioblastoma cell lines. *Life Sci. 68*, 1153–1160.

Ambrosino, P., Fresa, R., Fogliano, V., Monti, S.M., Ritieni, A., **1999**. Extraction of azadirachtin A from neemseed kernels by supercritical fluid and its evalution by HPLC and LC/MS. *J. Agric. Food Chem. 47*, 5252–5256.

Annadurai, R.S., Rembold, H., **1993**. Azadirachtin A modulates the tissue specific 2D polypeptide patterns of the desert locust, *Schistocerca gregaria*. *Naturwissenschaften 80*, 127–130.

Arpaia, S., van Loon, J.J.A., **1993**. Effects of azadirachtin after systemic uptake into *Brassica oleracea* on larvae of *Pieris brassicae*. *Entomol. Exp. Applic. 66*, 39–45.

Banerjee, S., Rembold, H., **1992**. Azadirachtin-A interferes with control of serotonin pools in the neuroendocrine system of locusts. *Naturwissenschaften 79*, 81–84.

Barnby, M.A., Klocke, J.A., **1990**. Effects of azadirachtin on levels of ecdysteroids and prothoracicotropic hormone-like activity in *Heliothis virescens* (Fabr.) larvae. *J. Insect Physiol. 36*, 125–131.

Beckage, N.E., Metcalf, J.S., Nielsen, B.D., Nesbit, D.J., **1988**. Disruptive effects of azadirachtin on development of *Cotesia congregata* in host tobacco hornworm larvae. *Arch. Insect Biochem. Physiol. 9*, 47–65.

Bidmon, H.J., **1986**. Ultrastructural changes of the prothorax glands of untreated and with azadirachtin treated *Manduca sexta* larvae (Lepidoptera, Spingidae). *Entomol. Gen. 12*, 1–17.

Bidmon, H.J., Kaüser, G., Möbus, P., Koolman, J., **1987**. Effect of azadirachtin on blowfly larvae and pupae. In: Proceedings of the 3rd International Neem Conference, pp. 253–271.

Billker, O., Shaw, M.K., Jones, I.W., Ley, S.V., Mordue, A.J., *et al.*, **2002**. Azadirachtin disrupts formation of organised microtubule arrays during microgametogenesis of *Plasmodium berghei*. *J. Eukaryot. Microbiol. 49*, 489–497.

Bilton, J.N., Broughton, H.B., Jones, P.S., Ley, S.V., Lidert, Z., *et al.*, **1987**. An X-ray crystallographic, mass spectrometric and NMR study of the limonoid insect antifeedant azadirachtin and related derivatives. *Tetrahedron 43*, 2805–2815.

Blaney, W.M., Simmonds, M.S.J., Ley, S.V., Anderson, J.C., Smith, S.C., *et al*., **1994**. Effect of azadirachtin-derived decalin (perhydronaphthalene) and dihydrofuranacetal (furo[2,3-*b*]pyran) fragments on the feeding behavior of *Spodoptera littoralis* (perhydronaphthalene). *Pest. Sci. 40*, 169–173.

Blaney, W.M., Simmonds, M.S.J., Ley, S.V., Anderson, J.C., Toogood, P.L., **1990**. Antifeedant effects of azadirachtin and structurally related compounds on lepidopterous larvae. *Entomol. Exp. Applic. 55*, 149–160.

Bomford, M.K., Isman, M.B., **1996**. Desensitization of fifth instar *Spodoptera litura* to azadirachtin and neem. *Entomol. Exp. Applic. 81*, 307–313.

Brooks, M.W., Roy, S., Uden, P.C., Vittum, P., **1996**. The extraction and determination of azadirachtin from soil and insects by supercritical fluid extraction. *J. Testing Eval. 24*, 149–153.

Butterworth, J.H., Morgan, E.D., **1968**. Isolation of a substance that suppresses feeding in locusts. *J. Chem. Soc., Chem. Commun.* 23–24.

Butterworth, J.H., Morgan, E.D., **1971**. Investigation of the locust feeding inhibition of the seeds of the neem tree, *Azadirachta indica*. *J. Insect Physiol. 17*, 969–977.

Dai, J.M., Yaylayan, V.A., Raghavan, G.S.V., Pare, J.R., **1999**. Extraction and colorimetric determination of azadirachtin-related limonoids in neem seed kernels. *J. Agric. Food Chem. 47*, 3738–3742.

Dai, J.M., Yaylayan, V.A., Raghavan, G.S.V., Pare, J.R.J., Liu, Z., *et al*., **2001**. Influence of operating parameters on the use of microwave-assisted process (MAP) for the extraction of azadirachtin-related limonoids from neem (*Azadirachta indica*) under atmospheric pressure conditions. *J. Agric. Food Chem. 49*, 4584–4588.

Deota, P.T., Upadhay, P.R., Patel, K.B., Mehta, K.J., Kamath, B.V., *et al*., **2000**. Estimation and isolation of azadirachtin from neem *Azadirachta indica* A. Juss. seed kernels using high performance liquid chromatography. *J. Liqu. Chromatogr. 23*, 2225–2235.

Dorn, A., **1986**. Effects of azadirachtin on reproduction and egg development of the heteropteran *Oncopeltus fasciatus* Dallas. *J. Appl. Entomol. 102*, 313–319.

Dorn, A., Rademacher, J.M., Sehn, E., **1986**. Effects of azadirachtin on the moulting cycle, endocrine system and ovaries in last-instar larvae of the milkweed bug, *Oncopeltus fasciatus*. *J. Insect Physiol. 32*, 321–328.

Dorn, A., Rademacher, J.M., Sehn, E., **1987**. Effects of azadirachtin on reproductive organs and fertility in the large milkweed bug, *Oncopeltus fasciatus*. In: Proceedings of the 3rd International Neem Conference, pp. 273–288.

Durand-Reville, T., Gobbi, L.B., Gray, B.L., Ley, S.V., Scott, J.S., **2002**. Highly selective entry to the azadirachtin skeleton via a Claisen rearrangement/radical cyclization sequence. *Org. Lett. 4*, 3847–3850.

Duthie-Holt, M.A., Borden, J.H., Rankin, L.J., **1999**. Translocation and efficiency of a neem-based insecticide in lodgepole pine using *Ips pini* (Coleoptera: Scolytidae) as an indicator species. *J. Econ. Entomol. 92*, 180–186.

Feder, D., Valle, D., Rembold, H., Garcia, E.S., **1988**. Azadirachtin-induced sterilization in mature females of *Rhodnius prolixus*. *Z. Naturforsch. C 43*, 908–913.

Feng, R., Isman, M.B., **1995**. Selection for resistance to azadirachtin in the green peach aphid *Myzus persicae*. *Experientia 51*, 831–833.

Fukuzaki, T., Kobayashi, S., Hibi, T., Ikuma, Y., Ishihara, J., *et al*., **2002**. Studies aimed at the total synthesis of azadirachtin: a modeled connection of C-8 and C-14 in azadirachtin. *Org. Lett. 4*, 2877–2880.

Garcia, E.S., Gonzales, M.S., Azambuja, P., **1991**. Effects of azadirachtin in *Rhodnius prolixus*: data and hypotheses. *Mem. Inst. Oswaldo Cruz, Rio de Janiero 86*, 107–111.

Garcia, E.S., Luz, N., Azambuja, P., Rembold, H., **1990**. Azadirachtin depresses the release of prothoracicotropic hormone in *Rhodnius prolixus* larvae: evidence from head transplantations. *J. Insect Physiol. 36*, 679–682.

Gelbic, I., Némec, V., **2001**. Developmental changes caused by metyrapone and azadirachtin in *Spodoptera littoralis* (Boisd.) (Lep., Noctuidae) and *Galleria mellonella* (L.) (Lep., Pyralidae). *J. Appl. Entomol. 125*, 417–422.

Gill, J.S., Lewis, C.T., **1971**. Systemic action of an insect feeding deterrent. *Nature 232*, 402–403.

Govindachari, T.R., Gopalakrishnan, G., Suresh, G., **1996**. Isolation of various azadirachtins from neem oil by preparative high performance liquid chromatography. *J. Liqu. Chromatogr. 19*, 1729–1733.

Govindachari, T.R., Narasimhan, N.S., Suresh, G., Partho, P.D., Gopalakrishnan, G., *et al*., **1995**. Structure-related insect antifeedant and growth-regulating activities of some limonoids. *J. Chem. Ecol. 21*, 1585–1600.

Govindachari, T.R., Sandhya, G., Ganeshraj, S.P., **1990**. Simple method for the isolation of azadirachtin by preparative high-performance liquid chromatography. *J. Chromatogr. 513*, 389–391.

Haskell, P.T., Mordue (Luntz), A.J., **1969**. The role of mouthpart receptors in the feeding behaviour *Schistocerca gregaria*. *Entomol. Exp. Applic. 12*, 423–440.

Hemalatha, K., Venugopal, N.B.K., Rao, B.S., **2001**. Determination of azadirachtin in agricultural matrixes and commercial formulations by enzyme-linked immunosorbent assay. *J. AOAC Int. 84*, 1001–1010.

Hori, M., Hiruma, K., Riddiford, L. M., **1984**. Cuticular melanization in the tobacco hornworm larva. *Insect Biochem. 14*, 267–274.

Immaraju, J.A., **1998**. The commercial use of azadirachtin and its integration into viable pest control programmes. *Pest. Sci. 54*, 285–289.

Isman, M.B., **1993**. Growth-inhibitory and antifeedant effects of azadirachtin on six noctuids of regional economic importance. *Pest. Sci. 38*, 57–63.

Isman, M.B., **1997**. Neem and other botanical insecticides: barriers to commercialization. *Phytoparasitica 25*, 339–344.

Isman, M.B., **2004**. Factors limiting commercial success of neem insecticides in North America and Western Europe. In: Koul, O., Wahab, S. (Eds.), Neem Today

and in the New Millennium. Kluwer, London, pp. 33–41.

Jarvis, A.P., Johnson, S., Morgan, E.D., **1998**. Stability of the natural insecticide azadirachtin in aqueous and organic solvents. *Pest. Sci. 53*, 217–222.

Jarvis, A.P., Morgan, E.D., Edwards, C., **1999**. Rapid separation of triterpenoids from neem seed extracts. *Phytochem. Anal. 10*, 39–43.

Johnson, S., Morgan, E.D., **1997a**. Comparison of chromatographic systems for triterpenoids from neem (*Azadirachta indica*) seeds. *J. Chromatogr. A 761*, 53–63.

Johnson, S., Morgan, E.D., **1997b**. Supercritical fluid extraction of oil and triterpenoids from neem seeds. *Phytochem. Anal. 8*, 228–232.

Johnson, S., Morgan, E.D., Peiris, C.J., **1996**. Development of the major triterpenoids and oil in the fruit and seeds of neem (*Azadirachta indica*). *Ann. Bot. 78*, 383–388.

Kalinowski, H.O., Krack, C., Ermel, K., Chriathamjaree, C., **1997**. Isolation and characterization of 1-tigloyl-3-acetylazadirachtol from the seed kernels of the Thai neem *Azadirachta siamensis* Valeton. *Z. Naturforsch. B-J. Chem Sci. 52*, 1413–1417.

Karnavar, G.K., **1987**. Influence of azadirachtin on insect nutrition and reproduction. *Proc. Indian Acad. Sci. 96*, 341–347.

Kaüser, G., Brandtner, H.M., Bidmon, H.J., Koolman, J., **1987**. Ecdysone synthesis and release by the brain–ring gland complex of blowfly larvae. *J. Insect Physiol. 34*, 563–569.

Koolman, J., Bidmon, H.J., Lehmann, M., Kaüser, G., **1988**. On the mode of action of azadirachtin in blowfly larvae and pupae. In: Sehnal, F., Zabza, A., Denlinger, D.L. (Eds.), Endocrinological Fronteirs in Physiological Insect Ecology, vol. 1. Wrocław Technical University Press, Wrocław, pp. 55–67.

Koul, O., **1984**. Azadirachtin. 1. Interaction with the development of red cotton bugs. *Entomol. Exp. Applic. 36*, 85–88.

Koul, O., Amanai, K., Ohtaki, T., **1987**. Effect of azadirachtin on the endocrine events of *Bombyx mori*. *J. Insect Physiol. 33*, 103–108.

Koul, O., Isman, M.B., **1991**. Effects of azadirachtin on the dietary utilization and development of the variegated cutworm *Peridroma saucia*. *J. Insect Physiol. 37*, 591–598.

Kraus, W., **2002**. Azadirachtin and other triterpenoids. In: Schmutterer, H. (Ed.), The Neem Tree, 2nd edn. Neem Foundation, Mumbai, pp. 39–111.

Kraus, W., Bokel, M., Bruhn, A., Cramer, A., Klaiber, I., *et al.*, **1987**. Structure determination by nmr of azadirachtin and related compounds from *Azadirachta indica*. *Tetrahedron Lett. 26*, 6435–6438.

Larson, R.O., **1985**. Stable anti-pest neem seed extract. US Patent No. 4,556,562.

Lavie, D., Jain, M.K., Shpan-Gabrielith, S.R., **1967**. A locust phago-repellent from two *Melia* species. *J. Chem. Soc., Chem. Commun.* 910–911.

Lee, S.M., Klocke, J.A., **1987**. Combined Florisil, droplet counter-current and high performance liquid chromatography for preparative isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds. *J. Liqu. Chromatogr. 10*, 1151–1163.

Ley, S.V., Denholm, A.A., Wood, A., **1993**. The chemistry of azadirachtin. *Nat. Prod. Rep. 10*, 109–157.

Linton, Y.M., Nisbet, A.J., Mordue (Luntz), A.J., **1997**. The effects of azadirachtin on the testes of the desert locust, *Schistocerca gregaria*. *J. Insect Physiol. 43*, 1077–1084.

Lowery, D.T., Isman, M.B., **1993**. Antifeedant activity of extracts from neem, *Azadirachta indica*, to strawberry aphid, *Chaetosiphon fragaefolii*. *J. Chem. Ecol. 19*, 1761–1773.

Lowery, D.T., Smirle, M.J., **2000**. Toxicity of insecticides to obliquebanded leafroller, *Choristoneura rosaceana*, larvae and adults exposed previously to neem seed oil. *Entomol. Exp. Applic. 95*, 201–207.

Luo, X.-D., Ma, Y.-B., Wu, S.-H., Wu, D.-G., **1999**. Two novel azadirachtin derivatives from *Azadirachta indica*. *J. Nat. Prod. 62*, 1022–1024.

Malathi, R., Kabaleeswaran, V., Rajan, S.S., Gopalakrishnan, G., Govindachari, T.R., **2003**. Structure of azadirachtin-I, 11-β epimer. *J. Chem. Crystallogr. 33*, 229–232.

Malczewska, M., Gelman, D.B., Cymborowski, B., **1988**. Effect of azadirachtin on development, juvenile hormone and ecdysteroid titres in chilled *Galleria mellonella* larvae. *J. Insect Physiol. 34*, 725–732.

Marco, M-P., Pascual, N., Bellés, X., Camps, F., Messegeur, A., **1990**. Ecdysteroid depletion by azadirachtin in *Tenebrio molitor* pupae. *Pestic. Biochem. Physiol. 38*, 60–65.

Meurant, K., Sernia, C., Rembold, H., **1994**. The effects of azadirachtin-A on the morphology of the ring complex of *Lucilia cuprina* (Wied.) larvae (Diptera, Insecta). *Cell Tissue Res. 275*, 247–254.

Mitchell, M.J., Smith, S.L., Johnson, S., Morgan, E.D., **1997**. Effects of neem tree compounds azadirachtin, salannin, nimbin and 6-desacetylnimbin on ecdysone 20-monooxygenase activity. *Arch. Insect Biochem. Physiol. 35*, 199–209.

Mordue (Luntz), A.J., **2002**. The cellular actions of azadirachtin. In: Schmutterer, H. (Ed.), The Neem Tree *Azadirachta indica* A. Juss. and other Meliaceous Plants. VCH Verlagsgesellschaft, Weinheim, pp. 266–274.

Mordue (Luntz), A.J., Blackwell, A., **1993**. Azadirachtin: an update. *J. Insect Physiol. 39*, 903–924.

Mordue (Luntz), A.J., Evans, K.A., Charlet, M., **1986**. Azadirachtin, ecdysteroids and ecdysis in *Locusta migratoria*. *Comp. Biochem. Physiol. C 85*, 297–301.

Mordue (Luntz), A.J., Nisbet, A.J., **2000**. Azadirachtin from the neem tree (*Azadirachta indica*): its actions against insects. *Ann. Soc. Entomol. Brasil 29*, 615–632.

Mordue (Luntz), A.J., Nisbet, A.J., Jennens, L., Ley, S.V., Mordue, W., **1999**. Tritiated dihydroazadirachtin binding to *Schistocerca gregaria* testes and *Spodoptera* Sf9 cells suggests a similar mechanism of action for azadirachtin. In: Singh, R.P., Saxena, R.C. (Eds.), *Azadirachta*

indica A. Juss. IBH Publishing Company Pvt Ltd, New Delhi, pp. 247–258.

Mordue (Luntz), A.J., Nisbet, A.J., Nasiruddin, M., Walker, E., **1996**. Differential thresholds of azadirachtin for feeding deterrence and toxicity in locusts and an aphid. *Entomol. Exp. Applic. 80*, 69–72.

Mordue (Luntz), A.J., Simmonds, M.S.J., Ley, S.V., Blaney, W.M., Mordue, W., *et al.*, **1998**. Actions of azadirachtin, a plant allelochemical, against insects. *Pest. Sci. 54*, 277–284.

Mordue (Luntz), A.J., Zounos, A., Wickramananda, I.R., Allan, E.J., **1995**. Neem tissue culture and the production of insect antifeedant and growth regulatory compounds. *BCPC Symp. Proceed. 63*, 187–194.

Nasiruddin, M., Mordue (Luntz), A.J., **1993a**. The effect of azadirachtin on the midgut histology of the locusts, *Schistocerca gregaria* and *Locusta migratoria*. *Tissue Cell. 25*, 875–884.

Nasiruddin, M., Mordue (Luntz), A.J., **1993b**. The protection of barley seedlings from attack by *Schistocerca gregaria* using azadirachtin and related analogs. *Entomol. Exp. Applic. 70*, 247–252.

Naumann, K., Isman, M.B., **1996**. Toxicity of a neem (*Azadirachta indica* A. Juss) insecticide to larval honey bees. *Am. Bee J. 136*, 518–520.

Nicol, C.M.Y., Schmutterer, H., **1991**. Contact effects of seed oil from the Neem Tree, *Azadirachta indica* (A. Juss), on nymphs of the gregarious phase of the desert locust, *Schistocerca gregaria* (Forsk.). *J. Appl. Entomol. 111*, 197–205.

Nicolaou, K.C., Poecker, A.J., Follmann, M., Baati, R., **2002**. Model studies towards azadirachtin. 2. Construction of the crowded C8–C14 bond by transition metal chemistry. *Angew. Chem. Int. Edn. 41*, 2107–2110.

Nisbet, A.J., Mordue (Luntz), A.J., Grossman, R.B., Jennens, L., Ley, S.V., *et al.*, **1997**. Characterisation of azadirachtin binding to Sf9 nuclei *in vitro*. *Arch. Insect Biochem. Physiol. 34*, 461–473.

Nisbet, A.J., Mordue (Luntz), A.J., Mordue, W., **1995**. Detection of [22,23-^{3}H]dihydroazadirachtin binding sites on membranes from *Schistocerca gregaria* (Forskal) testes. *Insect Biochem. Mol. Biol. 25*, 551–558.

Nisbet, A.J., Mordue (Luntz), A.J., Mordue, W., Williams, L.M., Hannah, L., **1996b**. Autoradiographic localisation of [22,23-^{3}H]dihydroazadirachtin binding sites in desert locust testes. *Tissue Cell 28*, 725–729.

Nisbet, A.J., Woodford, J.A.T., Strang, R.H.C., **1994**. The effects of azadirachtin-treated diets on the feeding behaviour and fecundity of the peach potato aphid, *Myzus persicae*. *Entomol. Exp. Applic. 71*, 65–72.

Nisbet, A.J., Woodford, J.A.T., Strang, R.H.C., **1996a**. The effects of azadirachtin on the acquisition and inoculation of potato leafroll virus by *Myzus persicae*. *Crop Protect. 15*, 9–14.

Nisbet, A.J., Woodford, J.A.T., Strang, R.H.C., Connolly, J.D., **1993**. Systemic antifeedant effects of azadirachtin on the peach potato aphid *Myzus persicae*. *Entomol. Exp. Applic. 68*, 87–98.

Osman, M.Z., Bradley, J., **1993**. Effects of neem seed extracts on *Pholeastor (Apantales) glomeratus* L (Hym. Braconidae), a parasitoid of *Pieris brassicae* L. (Lep. Pieridae). *J. Appl. Entomol.–Z. Angew. Entomol. 115*, 259–265.

Parkman, P., Pienkowski, R.L., **1990**. Sublethal effects of neem seed extract on adults of *Liriomyza trifolii* (Diptera, Agromyzidae). *J. Econ. Entomol. 83*, 1246–1249.

Patton, S., **1974**. Reversible suppression of lactation by colchicine. *FEBS Lett. 48*, 85–87.

Pener, M.P., Rowntree, D.B., Bishoff, S.T., Gilbert, L.I., **1988**. Azadirachtin maintains prothoracic gland function but reduces ecdysteroid titres in *Manduca Sexta* pupae: *in vivo* and *in vitro* studies. In: Sehnal, F., Zabza, A., Denlinger, D.L. (Eds.), Endocrinological Frontiers in Physiological Insect Ecology. Wrocław Technical University Press, Wrocław, pp. 41–54.

Pozo, O.J., Marin, J.M., Sancho, J.V., Hernandez, F., **2003**. Determination of abamectin and azadirachtin residues in orange samples by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A. 992*, 133–140.

Puri, H.S., **1999**. Neem the Divine Tree. Harwood Academic, Amsterdam.

Qi, B.Y., Gordon, G., Gimme, W., **2001**. Effects of neem-fed prey on the predacious insects *Harmonia conformis* (Boisduval) (Coleoptera: Coccinellidae) and *Mallada signatus* (Schneider) (Neuroptera: Chrysopidae). *Biol. Control 22*, 185–190.

Raguraman, S., Singh, R.P., **1998**. Behavioral and physiological effects of neem (*Azadirachta indica*) seed kernel extract on larval parasitoid, *Bracon hebetor. J. Chem. Ecol. 24*, 1241–1250.

Raizada, R.B., Srivastava, M.K., Kaushal, R.A., Singh, R.P., **2001**. Azadirachtin, a neem biopesticide: subchronic toxicity assessment in rats. *Food Chem. Toxicol. 39*, 477–483.

Redfern, R.E., Kelly, T.J., Borkovec, A.B., Hayes, D.K., **1982**. Ecdysteroid titres and moulting aberrations in last stage *Oncopeltus* nymphs treated with insect growth regulators. *Pest. Biochem. Physiol. 18*, 351–356.

Reed, E., Majumdar, S.K., **1998**. Differential cytotoxic effects of azadirachtin on *Spodoptera frugiperda* and mouse cultured cells. *Entomol. Exp. Applic. 89*, 215–221.

Reilly, B.A., Brostrom, M.A., Brostrom, C.O., **1998**. Regulation of protein synthesis in ventricular myocytes by vasopressin: the role of sarcoplasmic/endoplasmic reticulum Ca^{2+} stores. *J. Biol. Chem. 273*, 3747–3755.

Rembold, H., Annadurai, R.S., **1993**. Azadirachtin inhibits proliferation of Sf9 cells in monolayer culture. *Z. Naturforsch. C 38*, 495–499.

Rembold, H., Sieber, K.P., **1981**. Inhibition of oogenesis and ovarian ecdysteroid synthesis by azadirachtin in *Locusta migratoria migratorioides* (R and F). *Z. Naturforsch. C 36*, 466–469.

Rembold, H., Schlagintweit, B., Ulrich, G.M., **1986**. Activation of juvenile hormone synthesis *in vitro* by a

corpus cardiacum factor from *Locusta migratoria*. *J. Insect Physiol. 32*, 91–94.

Rembold, H., Subrahmanyam, B., Müller, T., **1989**. Corpus cardiacum: a target for azadirachtin. *Experientia 45*, 361–363.

Rennison, M.E., Handel, S.E., Wilde, C.J., Burgoyne, R.D., **1992**. Investigation of the role of microtubules in protein secretion from lactating mouse mammary epithelial cells. *J. Cell Sci. 102*, 239–247.

Reynolds, S.E., Wing, K.D., **1986**. Interactions between azadirachtin and ecdysteroid-dependent systems in the tobacco hornworm, *Manduca sexta*. Abstracts 5th Int. Congr. Pest. Chem., IUPAC, Ottawa, Canada, 2 D/E-08.

Riba, M., Martí, J., Sans, A., **2003**. Influence of azadirachtin on development and reproduction of *Nezara viridula* L. (Het., Pentatomidae). *J. Appl. Entomol. 127*, 37–41.

Riddiford, L.M., **1981**. Hormonal control of epidermal cell development. *Am. Zool. 21*, 751–762.

Robertson, S.L., **2004**. Studies on the mode of action of azadirachtin from the neem tree *Azadirachta indica*, using insect cell lines. PhD thesis. University of Aberdeen, UK.

Ruscoe, C.N.E., **1972**. Growth disrupting effects of an insect antifeedant. *Nature New Biol. 236*, 159–160.

Salehzadeh, A., Akhkha, A., Cushley, W., Adams, R.L.P., Kusel, J.R., *et al.*, **2003**. The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells. *Insect Biochem. Mol. Biol. 33*, 681–689.

Salehzadeh, A., Jabbar, A., Jennens, L., Ley, S.V., Annadurai, R.S., *et al.*, **2002**. The effects of phytochemical pesticides on the growth of cultured invertebrate and vertebrate cells. *Pest Mgt Sci. 58*, 268–276.

Sayah, F., Fayet, C., Idaomar, M., Karlinsky, A., **1996**. Effect of azadirachtin on vitellogenesis of *Labidura riparia* (Insecta, Dermaptera). *Tissue and Cell 28*, 741–749.

Sayah, F., Idaomar, M., Soranzo, L., Karlinsky, A., **1998**. Endocrine and neuroendocrine effects of azadirachtin in adult females of the earwig *Labidura riparia*. *Tissue and Cell 30*, 86–94.

Schaaf, O., Jarvis, A.P., van der Esch, S.A., Giagnacovo, G., Oldham, N.J., **2000**. Rapid and sensitive analysis of azadirachtin and related triterpenoids from neem (*Azadirachta indica*) by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. A 886*, 89–97.

Schlüter, U., **1985**. Occurrence of weight-gain reduction and inhibition of metamorphosis and storage protein-formation in last larval instars of the mexican bean beetle, *Epilachna varivestis*, after injection of azadirachtin. *Entomol. Exp. Applic. 39*, 191–195.

Schlüter, U., **1987**. Effects of azadirachtin on developing tissues of various insect larvae. In: Proceedings of the 3rd International Neem Conference, pp. 331–348.

Schlüter, U., Bidmon, H.J., Grewe, S., **1985**. Azadirachtin affects growth and endocrine events in larvae of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 31*, 773–777.

Schmutterer, H. (Ed.) **2002**. The Neem Tree, 2nd edn. Neem Foundation, Mumbai.

Schmutterer, H., Ascher, K.R.S. (Eds.) **1984**. Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss) and Other Tropical Plants. German Agency for Technical Cooperation, Eschborn.

Schmutterer, H., Ascher, K.R.S. (Eds.) **1987**. Natural Pesticides from the Neem Tree and Other Tropical Plants. German Agency for Technical Cooperation, Eschborn.

Schmutterer, H., Ascher, K.R.S., Rembold, H. (Eds.) **1981**. Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss). German Agency for Technical Cooperation, Eschborn.

Schmutterer, H., Doll, M., **1993**. The marrango or Philippine neem tree, *Azadirachta excelsa* (= *A integrifolia)*: a new source of insecticides with growth-regulating properties. *Phytoparasitica 21*, 79–86.

Schmutterer, H., Freres, T., **1990**. Influence of neem-seed oil on metamorphosis, color and behavior of the desert locust, *Schistocerca gregaria* (Forsk), and of the African migratory locust, *Locusta migratoria migratorioides* (R and F). *Z. Pflanzenkrank. Pflanzenschutz. 97*, 431–438.

Schutz, S., Wengatz, I., Goodrow, M.H., Gee, S.J., Hummel, H.E., *et al.*, **1997**. Development of an enzyme-linked immunosorbent assay for azadirachtins. *J. Agric. Food Chem. 45*, 2363–2368.

Scott, I.M., Kaushik, N.K., **2000**. The toxicity of a neem insecticide to populations of culicidae and other aquatic invertebrates as assessed in *in situ* microcosms. *Arch. Environ. Contam. Toxicol. 39*, 329–336.

Shimizu, T., **1988**. Suppressive effects of azadirachtin on spermiogenesis of the diapausing cabbage armyworm, *Mamestra brassicae*, *in vitro*. *Entomol. Exp. Applic. 46*, 197–199.

Sieber, K.P., Rembold, H., **1983**. The effects of azadirachtin on the endocrine control of molting in *Locusta migratoria*. *J. Insect Physiol. 29*, 523–527.

Simmonds, M.S.J., Blaney, W.M., **1984**. Some effects of azadirachtin on lepidopterous larvae. In: Proceedings of the 2nd International Neem Conferences, pp. 163–180.

Simmonds, M.S.J., Blaney, W.M., **1996**. Azadirachtin: advances in understanding its activity as an antifeedant. *Entomol. Exp. Applic. 80*, 23–26.

Simmonds, M.S.J., Blaney, W.M., Ley, S.V., Anderson, J.C., Banteli, R., *et al.*, **1995**. Behavioral and neurophysiological responses of *Spodoptera littoralis* to azadirachtin and a range of synthetic analogs. *Entomol. Exp. Applic. 77*, 69–80.

Simmonds, M.S.J., Manlove, J.D., Blaney, W.M., Khambay, B.P.S., **2002**. Effect of selected botanical insecticides on the behavior and mortality of the glasshouse whitefly *Trialeurodes vaporariorum* and the parasitoid *Encarsia formosa*. *Entomol. Exp. Applic. 102*, 39–47.

Smirle, M.J., Lowery, D.T., Zurowski, C.L., **1996**. Influence of neem oil on detoxication enzyme activity in the obliquebanded leafroller, *Choristoneura rosaceana*. *Pest. Biochem. Phys. 56*, 220–230.

Smith, S.L., Mitchell, M.J., **1988**. Effects of azadirachtin on insect cytochrome-P450 dependent ecdysone 20-monooxygenase activity. *Biochem. Biophys. Res. Comm. 154*, *559–563*.

Stark, J.D., Vargas, R.I., Thalman, R.K., **1990**. Azadirachtin: effects on metamorphosis, longevity, and reproduction of three tephritid fruit-fly species (Diptera, Tephritidae). *J. Econ. Entomol. 83*, 2168–2174.

Stark, J.D., Walter, J.F., **1995**. Persistence of azadirachtin A and azadirachtin B in soil: effects of temperature and microbial activity. *J. Environ. Sci. Health B 30*, 685–698.

Su, T.Y., Mulla, M.S., **1998**. Antifeedancy of neem products containing azadirachtin against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae). *J. Vector Ecol. 23*, 114–122.

Su, T.Y., Mulla, M.S., **1999**. Effects of neem products containing azadirachtin on blood feeding, fecundity, and survivorship of *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae). *J. Vector Ecol. 24*, 202–215.

Subrahmanyam, B., Müller, T., Rembold, H., **1989**. Inhibition of turnover of neurosecretion by azadirachtin in *Locusta migratoria*. *J. Insect Physiol. 35*, 493–497.

Sundaram, K.M.S., **1996**. Azadirachtin biopesticide: a review of studies conducted on its analytical chemistry, environmental behaviour and biological effects. *J. Environ. Sci. Health B 31*, 913–948.

Sundaram, K.M.S., Campbell, R., Sloane, L., Studens, J., **1995**. Uptake, translocation, persistence and fate of azadirachtin in aspen plants (*Populus tremuloides* Michx) and its effect on pestiferous two-spotted spider-mite (*Tetranychus urticae* Koch). *Crop Protect. 14*, 415–421.

Sundaram, K.M.S., Sundaram, A., Curry, J., Sloane, L., **1997**. Formulation selection, and investigation of azadirachtin-A persistence in some terrestrial and aquatic components of a forest environment. *Pest. Sci. 51*, 74–90.

Tang, D.L., Wang, C.Z., Luo, L., Qin, J.D., **2000**. Comparative study on the responses of maxillary sensilla styloconica of cotton bollworm *Helicoverpa armigera* and oriental tobacco budworm *H. assulta* larvae to phytochemicals. *Sci. China Series C Life Sci. 43*, 606–612.

Tang, Y.Q., Weathersbee, A.A., Mayer, R.T., **2002**. Effect of neem seed extract on the brown citrus aphid (Homoptera: Aphididae) and its parasitoid *Lysiphlebus testaceipes* (Hymenoptera: Aphidiidae). *Environ. Entomol. 31*, 172–176.

Tanzubil, P.B., McCaffery, A.R., **1990**. Effects of azadirachtin and aqueous neem seed extracts on survival, growth and development of the African armyworm, *Spodoptera exempta*. *Crop Protect 9*, 383–386.

Timmins, W.A., Reynolds, S.E., **1992**. Azadirachtin inhibits secretion of trypsin in midgut of *Manduca sexta* caterpillars: reduced growth due to impaired protein digestion. *Entomol. Exp. Applic. 63*, 47–54.

Trisyono, A., Whalon, M.E., **1999**. Toxicity of neem applied alone and in combinations with *Bacillus thuringiensis* to Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol. 92*, 1281–1288.

Trumm, P., Dorn, A., **2000**. Effects of azadirachtin on the regulation of midgut peristalsis by the stomatogastric nervous system in *Locusta migratoria*. *Phytoparasitica 28*, 7–26.

Turner, C.J., Tempesta, M.S., Taylor, R.B., Zagorski, M.G., Termini, J.S., *et al.*, **1987**. An NMR spectroscopic study of azadirachtin and its trimethyl ether. *Tetrahedron 43*, 2789–2803.

von Friesewinkel, D.C., Schmutterer, H., **1991**. Kontaktwirkung von Niemol bei der afrikanischen Wanderheuschrecke *Locusta migratoria migratorioides* (R&F). *Z. Angew. Zool. 78*, 189–204.

Weintraub, P.G., Horowitz, A.R., **1997**. Systemic effects of neem insecticide on *Lyriomyza huidobrensis* larvae. *Phytoparasitica 25*, 283–289.

Wong, W.L., Brostrom, M.A., Kuznetsov, G., Gmitter-Yellen, D., Brostrom, C.O., **1993**. Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells. *Biochem. J. 289*, 71–79.

6.5 The Spinosyns: Chemistry, Biochemistry, Mode of Action, and Resistance

V L Salgado, Bayer CropScience AG, Monheim, Germany
T C Sparks, Dow AgroSciences, Indianapolis, IN, USA

6.5.1. Introduction

The spinosyns and associated spinosoids (semisynthetic analogs of the spinosyns) constitute a new and unique class of insect control agents. The spinosyns are fermentation-derived natural products that are chemically unique (Figure 1). In addition to their unique chemical structure, the spinosyns possess a novel mode of action coupled with an insecticidal efficacy on par with many pyrethroid insecticides, plus a very favorable toxicological profile (Bret *et al*., 1997; Sparks *et al*., 1999). Thus, the spinosyns exhibit many highly desirable attributes for an insect control agent.

The first commercial product, spinosad, was launched in 1997. An extensive discovery effort followed the initial isolation and identification of the first spinosyns. This effort led to the identification of an array of naturally produced spinosyns, as well as a variety of semi-synthetic analogs; the spinosoids.

In this chapter attempts are made to bring together aspects of the discovery of the spinosyns and spinosoids, their chemistry, biology, mode of action, structure–activity relationships, and resistance management. The reader is also referred to several reviews (Crouse *et al*., 1999; Sparks *et al*., 1999; Anzeveno and Green, 2002; Kirst *et al*., 2002b).

6.5.2. Discovery, Structure, and Biosynthesis of the Spinosyns

Among insecticidal insect control agents, natural products have in numerous instances become products or have been the model or inspiration for insecticidal products. At the very least, virtually all significant classes of insect control agents, including

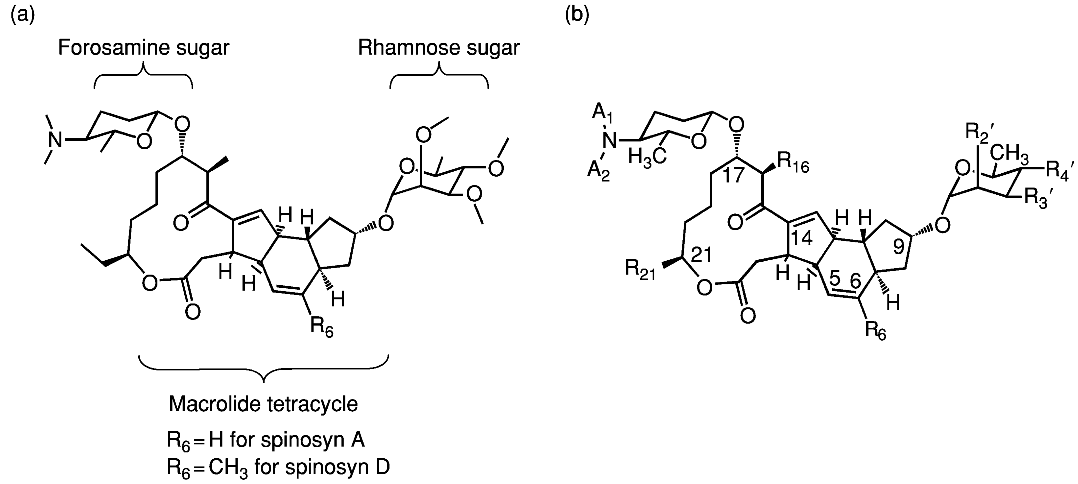


Figure 1 Structures of spinosad and spinosyns/spinosoids.

the organophosphates, carbamates, pyrethroids, neonicotinoids, and many others, can point to a model that exists among nature's chemical laboratory (Addor, 1995). As such, natural products have been and remain a very valuable source of inspiration for the discovery of new insect control agents, thereby providing a source of models, leads, and in some cases the insecticidal products.

There are two key elements to an effective natural products discovery program. The first is new, novel sources of natural products. The second is availability of new bioassays or approaches to screen or filter the natural products and their extracts. Many natural product discovery programs use the same source material coupled with new growth media, new extraction conditions, etc. Thus, new bioassays are central to the exploitation of these existing natural product libraries and hence, the discovery of new insecticidal natural products.

6.5.2.1. The Spinosyns

During the 1980s Lilly Research Laboratories had a program designed to look for rare and unique actinomycetes that were then fed into a whole series of biological screens. In late 1983 a new mosquito larvicide bioassay using early stadium *Aedes aegypti* larvae in 96-well microtiter plates was added to these biological screens. Because of the high throughput and small amount of material needed, a large number of broths could be screened at high sensitivity. An extract from a microorganism cultured from a soil sample collected in the Caribbean was found to be active in this assay (Thompson *et al.*, 1997). Refermentations of the sample (A83543) were active in subsequent mosquito bioassays, but it became of real interest when it showed contact and antifeedant activity against larvae of the southern armyworm (*Spodoptera eridania*) in a plant-based assay, coupled with little activity on spider mites (*Tetranychus urticae*), aphids (*Aphis gossypii*), or corn rootworm (*Diabrotica undecimpunctata*), thus demonstrating some inherent selectivity – a rare occurrence (Thompson *et al.*, 1997). Characterization of the microorganism showed it to be a new species of actinomycete from an unusual genus, *Saccharopolysopra spinosa* (Mertz and Yao, 1990).

From extracts of *S. spinosa*, a series of related A83543 factors, later named "spinosyns," were identified. The spinosyns were found to consist of two sugars, forosamine and 2′,3′,4′-tri-*O*-methylrhamnose, attached to a unique tetracyclic ring system containing a 12-member macrocycle (**Figure 1**). Among macrocyclic lactones, the spinosyns are unique (Kirst *et al.*, 1992; Shomi and Omura, 2002), possessing a structure that is truly novel among insect control agents. Other naturally occurring insecticidal macrocyclic lactones are known (e.g., avermectins, milbemycins), but these are based on entirely different 14-member macrocycles, with modes of action and activity spectra distinct from those of the spinosyns.

As observed with many natural products, the parent (wild type, WT) strain of *S. spinosa* produces a family of closely related spinosyns, which vary in methyl substitution patterns on the forosamine nitrogen, the 2′- and 3′-positions of the rhamnose, and at the C6, C16, and C21 positions of the tetracycle (Kirst *et al.*, 1992) (**Table 1**). The spinosyns produced from the parent strain included spinosyns A–H and J (I was not assigned) (**Table 1**).

Within this mixture, the two major components produced are spinosyn A (primary) and spinosyn D (secondary). An extract of the fermentation broth

Table 1 Structures of the spinosyns, pseudoaglycones, aglycones, and spinosoids

Number	*Compound*	*Strain*	*A1*[a]	*A2*	*R21*	*R16*	*R6*	*R2′*	*R3′*	*R4′*	*5,6*	*13,14*
Spinosyns												
1	A	WT	Me	Me	Et	Me	H	OMe	OMe	OMe	DB	DB
2	B	WT	H	[b]								
3	C	WT	H	H								
4	D	WT					Me					
5	E	WT			Me							
6	F	WT				H						
7	G	WT	[c]	[c]								
8	H	WT						OH				
9	J	WT							OH			
10	A C17-PSA	WT	na	na								
11	A C9-PSA	WT						na	na	na		
12	A aglycone	WT	na	na				na	na	na		
13	D C17-PSA	WT	na	na			Me					
14	D C9-PSA	WT					Me	na	na	na		
15	D aglycone	WT	na	na			Me	na	na	na		
7	H	H						OH				
16	Q	H					Me	OH				
17	R	H	H					OH				
18	S	H			Me			OH				
19	T	H						OH	OH			
8	J	J							OH			
20	L	J					Me		OH			
21	M	J	H						OH			
22	N	J	H				Me		OH			
23	K	K								OH		
24	O	K					Me			OH		
25	Y	K			Me					OH		
26	U	H + S						OH		OH		
27	V	H + S					Me	OH		OH		
28	P	J + S							OH	OH		
29	W	J + S					Me		OH	OH		
Novel spinosyns												
30	21-propyl A				nProp							
31	21-butenyl A	21B WT			Buten							
Spinosoids												
32	*N*-demethyl D	A	H				Me					
33	*N*-demethyl K	K	H							OH		
34	*N,N*-didemethyl K	K	H	H						OH		
35	2′-H A	H						H				
36	2′-H D	H					Me	H				
37	2′-*O*-ethyl A	H						OEt				
38	2′-*O*-vinyl	H						OVinyl				
39	2′-*O*-*n*-propyl A	H						OnProp				
40	2′-*O*-*n*-pentyl A	H						OnPent				
41	2′-*O*-benzyl A	H						OBenz				
42	2′-*O*-acetate A	H						OAcetate				
43	3′-H A	J							H			
44	3′-OH-*epi* A	J							OH-*epi*			
45	3′-*O*-methyl-*epi* A	J							OMe-*epi*			
46	3′-*O*-ethyl A	J							OEt			
47	3′*O*-vinyl A	J							OVinyl			
48	3′-*O*-*n*-propyl A	J							OnProp			
49	3′-*O*-isopropyl A	J							OIsoprop			
50	3′-*O*-isopropenyl A	J							OIsopropen			
51	3′-*O*-*n*-butyl A	J							OnBut			
52	3′-*O*-*n*-pentyl A	J							OnPent			
53	3′-*O*-allyl A	J							OAllyl			
54	3′-*O*-propargyl A	J							OProparg			
55	3′-*O*-CH_2CF_3 A	J							OCH_2CF_3			
56	3′-*O*-acetate A	J							OAcetate			
57	3′-*O*-methoxymethyl	J							OMeOMe			
58	3′-Methylene A	J							=Me			
59	4′-H A	K								H		
60	4′-*O*-ethyl A	K								OEt		
61	4′-*O*-acetate A	K								OAcetate		

Continued

Table 1 Continued

Number	*Compound*	*Strain*	*A1*[a]	*A2*	*R21*	*R16*	*R6*	*R2′*	*R3′*	*R4′*	*5,6*	*13,14*
62	2′,3′,4′-tri-*O*-ethyl A							OEt	OEt	OEt		
63	4″-*N*-ethyl B	C	Et	H								
64	4″-*N*-ethyl A	B	Et									
65	4″-*N*-*n*-propyl A	B	Prop									
66	4″-*N*-*n*-butyl A	B	Butyl									
67	4″-*N*,*N*-diethyl	C	Et	Et								
68	4″-OH	A	na	na								
69	5,6,13,14(α)-TH	A									SB	SB
70	5,6,13,14(β)-TH	A									SB	SB
71	13,14-α-DH	A										SB
72	13,14-β-DH	A										SB
73	7,11-Dehydro	A					Me					
74	5,6-α-epoxy	A									α-epoxy	
75	5,6-β-epoxy	A									β-epoxy	
76	5,6-DH	A									SB	
77	5,6-DH, 3′-*O*-ethyl	A							OEt		SB	
78	5,6-DH, 3′-*O*-*n*-propyl	A							OnProp		SB	
79	A C17-*O*-acetate	A	na	na								
80	A C17-*O*-DMAA	A	na	na								
81	A C17-*O*-DMAP	A	na	na								
82	A C17-*O*-DMAB	A	na	na								
83	A C17-*O*-NMPipz	A	na	na								
84	A C17-*O*-DMPipd	A	na	na								
85	A C9-*O*-L-lyxose[d]	A										
86	A C9-O-L-mannose[d]	A										

[a]Position on the spinosyn structure (see **Figure 1**).
[b]Dash indicates no change from substitution pattern for spinosyn A.
[c]The stereochemistry of 5″-methyl on the forosamine is axial.
[d]L-Lyxose is missing the 6-methyl of rhamnose; L-mannose is 6-hydroxy-rhamnose.
na, not applicable.; DB, double bond; DMAA, dimethylaminoacetate; DMAB, dimethylaminobutyrate; DMAP, dimethylaminoproprionate; DMPipd, dimethyl-*N*-piperidinyl acetate; NMPipz, *N*-methylpiperazinyl acetate; PSA, pseudoaglycone; SB, single bond; WT, wild type.

containing this naturally occurring mixture is known as spinosad, the active ingredient in a number of products (Tracer®, Success®, SpinTor®, Conserve®) marketed by Dow AgroSciences for insect control. Spinosad received registration from the US Environmental Protection Agency (EPA) for use in cotton insect control in February 1997. As discussed below (see Section 6.5.5.1.1.1), spinosyn A is the most active of the naturally occurring spinosyns from *S. spinosa*, followed closely by spinosyn D (Table 2). Thus, the most insecticidally active naturally occurring spinosyns produced by *S. spinosa* are also those that the microorganism naturally produces in the largest quantity.

Further research around the spinosyns required the availability of significant quantities of material. Because the wild-type parent strain only produced very small quantities of spinosyns, a strain improvement program was instituted to increase the titers of the spinosyns produced. During the process of strain improvement, a number of mutant strains were identified that had nonfunctional 2′- and/or 3′- and/or 4′-*O*-methyltransferases. Since these mutants were unable to methylate the hydroxyl groups on the 2′-, 3′-, or 4′-positions of the tri-*O*-methylrhamnose, a variety of new spinosyns were produced in addition to a few spinosyns seen only as minor factors in the wild-type strain (Sparks *et al.*, 1996, 1999).

The mutant strain possessing a nonfunctional 2′-*O*-methyltransferase produced the already existing spinosyn H (2′-*O*-demethyl analog of spinosyns A) as well as several new spinosyns: Q (2′-*O*-demethyl analog of spinosyn D), R (2′-*O*-demethyl analog of spinosyn B), S (2′-*O*-demethyl analog of spinosyn E), and T (2′-*O*-demethyl analog of spinosyn J). Likewise, a mutant with a nonfunctional 3′-*O*-methyltransferase produced spinosyn J (3′-*O*-demethyl analog of spinosyn A), a minor factor in the wild-type strain, along with the new spinosyns: M (3′-*O*-demethyl analog of spinosyn B), L (3′-*O*-demethyl analog of spinosyn D), and N (the 4′-*N*-demethyl analog of spinosyn L). The mutant possessing a nonfunctional 4′-*O*-methyltransferase produced three new spinosyns: K (4′-*O*-demethyl analog of spinosyn A), O (the 4′-*O*-demethyl analog of spinosyn D), and Y (the 4′-*O*-demethyl analog of spinosyn E) (Sparks *et al.*, 1996, 1999).

Table 2 Biological activity of spinosyns towards selected insect and mite species

Number	*Compound*	Heliothis virescens, *neonate (LC_{50}, ppm)*	Calliphora vicina, *larvae (LC_{50}, ppm)*	Stomoxys calcitrans, *adult (LC_{50}, ppm)*	Aphis gossypii *(LC_{50}, ppm)*	Macrosteles quadrilineatus *(LC_{50}, ppm)*	Tetranychus urticae *(LC_{50}, ppm)*
Spinosyns							
1	A	0.31	0.3–0.53	0.43	50 (42–88)	6.9	5.3
2	B	0.4	0.58	<0.5	11	5.1	0.9
3	C	0.8	0.63	0.45	33	14.5	8.4–29
4	D	0.8	0.44	0.55	50		3–19
5	E	4.6	1.95	0.16			100
6	F	4.5	2.50	0.80	>50		16
7	G	7.1					
8	H	5.7	1.1	0.31	>50		>50
9	J	>80	6.3	0.5	>50		~63
10	A C17-pseudoaglycone	>64		90			100
11	A C9-pseudoaglycone	>64					
12	A aglycone	>64					
13	D C17-pseudoaglycone	>64					100
14	D C9-pseudoaglycone	>64					
15	D aglycone	>64					
7	H	5.7	1.1	0.31	>50		95
16	Q	0.5	1.8	0.26	>50		14
17	R	14.5					
18	S	53				11.7	114
19	T	>64	>10	>10		>100	
8	J	>80	6.3	0.5	>50		~63
20	L	26	2.8	<5	>50	>100	48–67
21	M	22.6				>100	
22	N	40				61	>50
23	K	3.5	2.9	1.7	12	3.2	1.4
24	O	1.4		<5	11	6.9	0.8
25	Y	20					
26	U	22				8.2	>50
27	V	17				2.8	
28	P	>64	>10	6.1			
29	W	>64					
	Cypermethrin	0.18					
	Ethofenprox				0.3		
	Fenazaquin						1.0

Further variations in the methylation of the rhamnose sugar could also be obtained by the addition of sinefungin (Chen *et al.*, 1989), which in *S. spinosa* specifically blocked the 4′-O-methyltransferase during the fermentation process of the wild-type strain (Sparks *et al.*, 1996, 1999). When sinefungin was added to fermentations of the H (nonfunctional 2′-*O*-methyltranferase) and J (nonfunctional 3′-*O*-methyltranferase) mutant strains, several other new spinosyns (P, U, V, W) were produced that were missing methyl groups from the 4′-position in combination with missing methyl groups from either the 2′- or 3′-positions (Sparks *et al.*, 1996, 1999).

In general, the variations in all of the above-mentioned spinosyns center around (1) methylation of the forosamine amino group, (2) presence or absence of *O*-methyl groups on the rhamnose sugar, and (3) presence or absence of alkyl group(s) at the C6, C16, and C21 positions of the tetracyclic ring system. Other factors related to spinosyn A include the C17 pseudoaglycone (PSA), which lacks the forosamine sugar, the corresponding C9 PSA, missing the rhamnose moiety, and the aglycone, where both sugars are absent. The corresponding PSAs and aglycone were also observed for spinosyn D (**Table 1**).

Through the above process of isolating major and minor factors from the wild-type and mutant strains of *S. spinosoa*, more than 20 different spinosyns were identified (Sparks *et al.*, 1996, 1999) (**Table 1**).

6.5.2.2. The 21-Butenyl Spinosyns

Like Eli Lilly and Company, Dow AgroSciences has also had a long-standing natural products screening program. During the mid-1990s a high-throughput

screening (HTS) program was established that used a number of target insect pests including lepidopterous larvae, such as beet armyworm (*Spodoptera exigua*), in a 96-well format, as the primary natural products screening tool. This was accompanied by a number of secondary assays to further characterize extracts that were found to be active in the primary screen. One particular multiyear effort, to screen a very large number and variety of actinomycete broth extracts, relied on a novel bioassay as the secondary assay. From this screening effort an extract was found that was not only active in the primary diet-based screen, but that, on further testing, also exhibited interesting contact activity coupled with symptomology that was very reminiscent of the spinosyns. Further separation and isolation coupled to detailed physical characterization (nuclear magnetic resonance (NMR), liquid chromatography–mass spectroscopy (LC–MS), etc.) of this extract showed that the activity was indeed due to a spinosyn-like entity (Lewer *et al*., 2001, 2003). However, there were some structural differences relative to the then known spinosyns. The dominant, distinctive feature was the presence of a 2-butenyl group at the C21 position of the macrocyclic ring system in place of the heretofore typical ethyl-group of the spinosyns (Lewer *et al*., 2003) (**Figure 2**). The primary factor isolated from the mixture of 21-butenyl factors was the 21-butenyl homolog of spinosyn A (**Figures 1** and **2**, **Table 1**). To date more than 30 additional factors have been isolated, identified, and characterized (Lewer *et al*., 2003). These 21-butenyl spinosyns constitute a new family of spinosyns, for which research on the isolation, characterization, and chemistry is continuing (Crouse *et al*., 2002). Characterization of the microorganism that produces the 21-butenyl spinosyns showed it to be a new species of *Saccharopolyspora* closely related to, but distinct from, *S. spinosa*, which has been named *Saccharopolysopra pogona* (Lewer *et al*., 2003).

6.5.2.3. Spinosyn Biosynthesis

The spinosyns are 12-member macrocyclic lactones (macrolides). Based on incorporation studies using ^{13}C-labeled acetate, propionate, butyrate, and isobutyrate, the biosynthesis of the tetracycle of the spinosyns is consistent with a polyketide synthase-based (PKS) pathway (Kirst *et al*., 1992). As a macrolide, the spinosyns differ from the norm in that the spinosyn PKS pathway ultimately leads to the introduction of three intramolecular carbon–carbon bonds to form the spinosyn tetracycle (Waldron *et al*., 2001) (**Figure 3**). To this tetracycle a neutral sugar, rhamnose, is coupled at the C9 position, and then subsequently methylated via three O-methyltransferases. The final steps involve the addition of an amino sugar to the tetracycle at the C17 position. The amino sugar, forosamine, is also methylated prior to attachment to the spinosyn tetracycle – in this case at both positions of the nitrogen (Kirst *et al*., 1992; Waldron *et al*., 2000, 2001) (**Figure 3**).

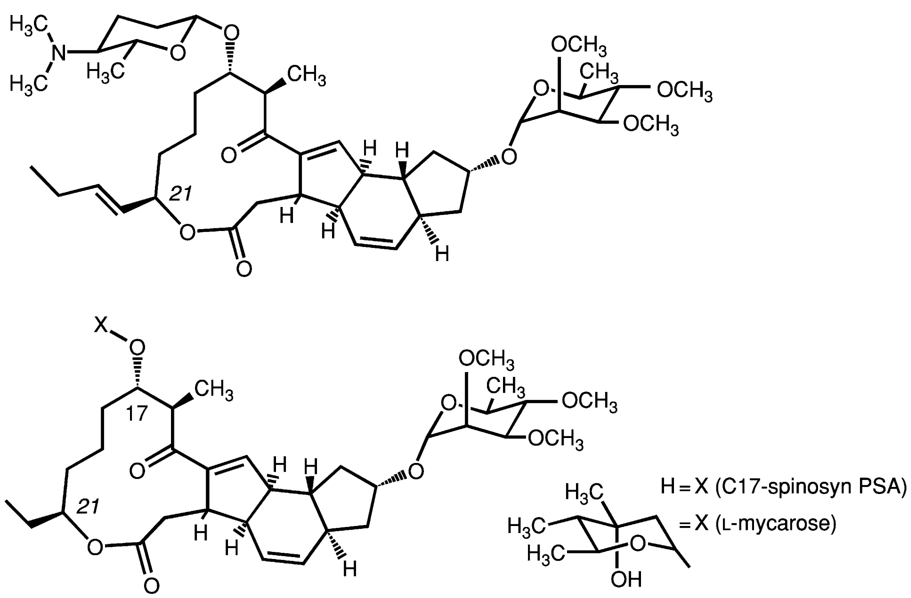


Figure 2 Structures for 21-butenyl spinosyn A (top), the spinosyn A C17-pseudoaglycone (PSA, bottom; X = H) and the C17 L-mycarose biotransformation product.

A large synthetic effort has gone into the modification of the forosamine and rhamnose moieties, as well as the core tetracycle (Crouse and Sparks, 1998; Crouse *et al*., 2001; Anzeveno and Green, 2002; Kirst *et al*., 2002b) (see Section 6.5.7). However, other approaches to macrolide modification are now possible. Sequencing the genes involved in spinosyn biosynthesis (Waldron *et al*., 2000, 2001; Madduri *et al*., 2001) has allowed the production of novel spinosyns through biotransformation and modification of the biosynthetic pathways. For example, a genetically engineered strain of *Saccharopolyspora erythraea* expressing the *spnP* glycosyltransferase gene was used to produce biotransformed spinosyns, wherein the β-D-forosamine moiety was replaced by α-L-mycarose at the C17 position (Gaisser *et al*., 2002). Alternatively, modifications to the basic tetracycle have been obtained via loading module swaps in the PKS, leading to a variety of novel substitutions at C21 and other positions (Burns *et al*., 2003; Martin, 2003). As one example, the ethyl group at C21 has been replaced with an *n*-propyl group (Martin, 2003), resulting in a unique spinosyn as active as spinosyn A (**Tables 1** and **3**).

6.5.2.4. Physical Properties of the Spinosyns

The spinosyns are high molecular weight (approximately 732 Da), relatively nonvolatile molecules (**Table 4**). As with the biological activity, small changes in structure can have far larger effects than might otherwise be expected. Although spinosyns A and D only differ by the presence of a methyl group at C6 (**Table 1**), this has a surprising effect on the physical properties, especially solvent solubilities. For example, spinosyn A is soluble in water at about 8.9 mg 100 ml^{-1}, while the water solubility of spinosyn D is only 0.05 mg 100 ml^{-1}. This pattern holds true for other solvents as well (**Table 4**).

6.5.3. Pharmacokinetics of Spinosad

6.5.3.1. Metabolism of the Spinosyns

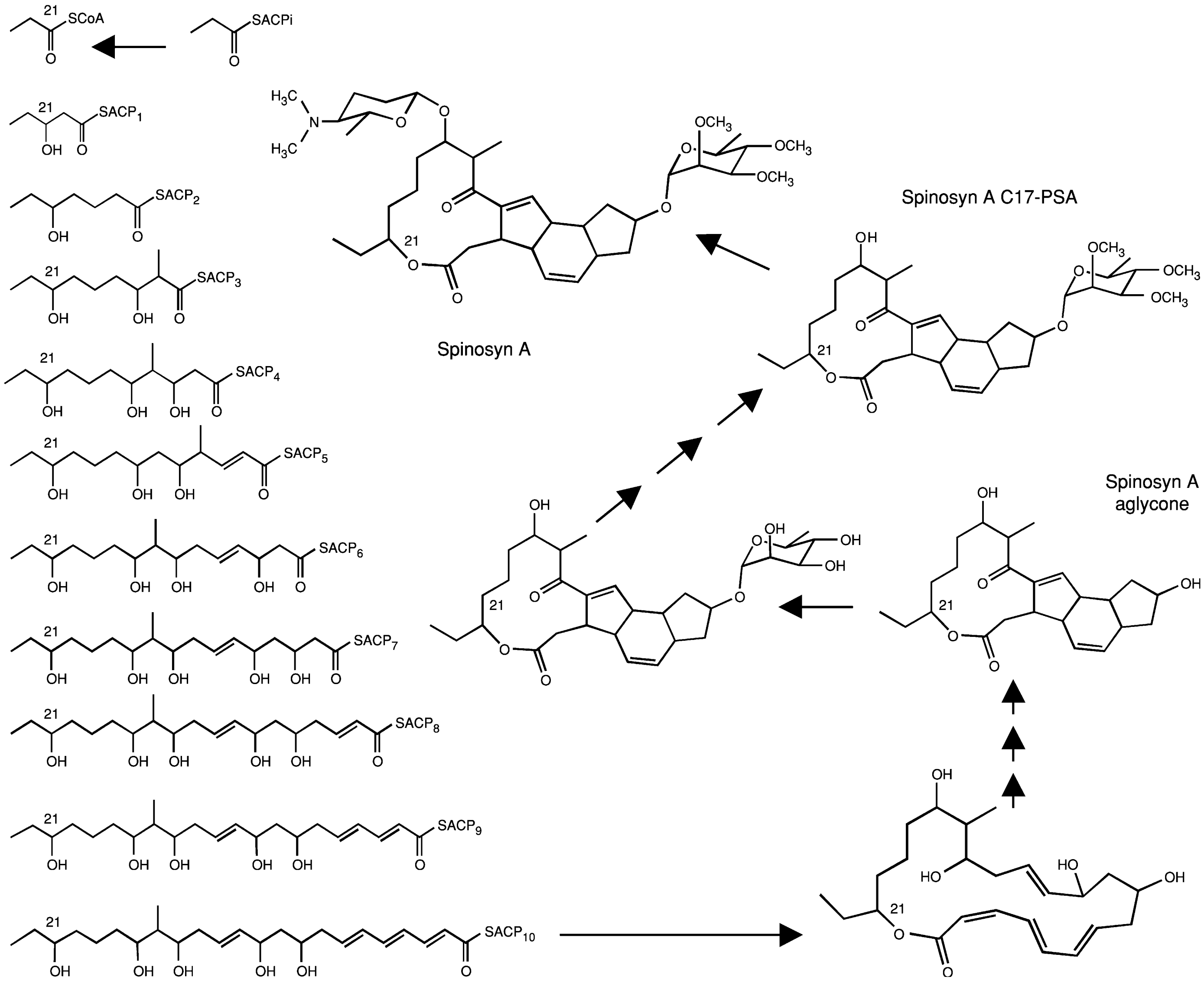
6.5.3.1.1. Mammalian and avian spinosyn metabolism Studies of ^{14}C-spinosyns A and D metabolism in rats identified fecal excretion as the major route of elimination for both spinosyns A and D. In the fecal material, between 52% (males) and 28% (females) of the administered dose of spinosyn A was excreted in the feces as parent or metabolites within 0–24 h post-dosing. Of this material only 6.4% (males) and 5.4% (females) was spinosyn A (Domoradzki *et al*., 1996). For spinosyn D, between 67.7% (males) and 73.3% (females) of the applied dose was present in the feces, of which 34.5% (males) and 35.2% (females) was the parent spinosyn D (Domoradzki *et al*., 1996). Thus, for both spinosyns A and D a substantial amount of metabolism had taken place in the first 24 h following administration. The primary pathways for the spinosyns in rats involved loss of a methyl group on the forosamine nitrogen (*N*-demethylation), and/or loss of one of the *O*-methyl groups on the rhamnose (*O*-demethylation) (**Figure 4**), both followed by conjugation. Conjugation of the parent was also noted as an important pathway in rats (Domoradzki *et al*., 1996).

In lactating goats, following 3 days of dosing with either spinosyn A or D, eight metabolites of spinosyn A and five metabolites of spinosyn D were detected. As with rats, *N*-demethylation was an important metabolic route. In addition, hydroxylation of the macrolide ring was noted as a primary metabolic pathway (Rainey *et al*., 1996) (**Figure 4**).

In studies with poultry, *N*-demethylation of the forosamine nitrogen and *O*-demethylation of the rhamnose moiety were also found to be the two primary metabolic pathways (Magnussen *et al*., 1996). In the latter case, the 2′/4′-*O*-demethyl metabolites (i.e., spinosyns H and K, respectively, for spinosyn A metabolism) predominated over the 3′-*O*-demethyl metabolites. Another pathway, involving removal of the forosamine sugar to form the C17 PSA, was identified, but appears to be secondary to the *N*- and *O*-demethylation (Magnussen *et al*., 1996) (**Figure 4**).

As demonstrated by these three studies, spinosyns A and D are readily metabolized in vertebrate and avian systems. Furthermore, *N*-demethylation of the forosamine nitrogen is a metabolic route that predominates in all three species. *O*-Demethylation of the rhamnose sugar is also important in rats and poultry. These particular pathways are both consistent with oxidative metabolism via monooxygenases and/or the action of glutathione transferases. In the rat studies, glutathione conjugates were noted for *O*-demethylated metabolites as well as for the parent spinosyns (A and D). The presence of hydroxylated macrolide metabolites in the goat provides direct support for involvement of monooxygenases in spinosyn metabolism.

6.5.3.1.2. Spinosyn metabolism in insects Spinosyns A and D appear to have a number of potential sites for metabolism, including *N*-demethylation of the forosamine, *O*-demethylation of the rhamnose, epoxidation of the 5,6 or 13,14 double bonds, opening of the macrocyclic lactone, etc. As shown above (see Section 6.5.3.1.1), some of these potential metabolites have been observed. However, compared to more conventional insect control agents,



Spinosyn A
Spinosyn A C17-PSA
Spinosyn A aglycone
SCoA
SACPi
SACP1
SACP2
SACP3
SACP4
SACP5
SACP6
SACP7
SACP8
SACP9
SACP10

the spinosyns are very large, unusual, complex molecules, and metabolic enzyme systems do exhibit distinct substrate specificities. Studies of spinosyn A metabolism in tobacco budworm (*H. virescens*) larvae show spinosyn A to penetrate slowly, and that the primary, and apparently only, component that was detected (within the limits of the experiment) was the parent, spinosyn A (Sparks *et al.*, 1997, 2001) (**Figure 5**). Thus, until 24 h post treatment there appears to be little or no metabolism of spinosyn A in pest insects such as larvae of *H. virescens*. In contrast, these same larvae readily metabolize the quinazoline acaricide, fenazaquin (Sparks *et al.*, 1997) (**Figure 5**). Subsequent studies with *H. virescens* larvae that were highly resistant (ca. 1000-fold) (Bailey *et al.*, 1999) to spinosad, also failed to show significant levels of metabolism (Young *et al.*, 2000). These observations are in clear distinction to those observed for mammals (see Section 6.5.3.1.1).

The apparent lack of spinosyn A metabolism by pest insects is also supported by synergist studies with the monooxygenase inhibitor, piperonyl butoxide (PBO). PBO is able to synergize the biological activity of the pyrethroid permethrin in house flies, but not that of spinosyn A (Sparks *et al.*, 1997). Likewise no PBO-based synergism was noted for spinosyn A or spinosad activity in studies involving *H. virescens* larvae (Sparks *et al.*, 1997) or in spinosad resistant larvae of the diamondback moth (*Plutella xylostella*) (Zhao *et al.*, 2002). Thus, within the limits of the available data, many pest insects appear to have a limited capacity to metabolize some spinosyns.

6.5.3.2. Spinosyn Penetration into Insects

The observed biological activity of any toxicant is a function of two primary parameters: (1) inherent activity at the target site, and (2) concentration of the toxicant at the target site as a function of time. Compared to more conventional insect control agents such as the organophosphates, carbamates, and pyrethroids, the spinosyns are large, complex, relatively high molecular weight molecules. Although the spinosyns, such as spinosyn A, exhibit good activity via topical or contact application, better activity is observed by oral application or by injection (Sparks *et al.*, 1995, 1997, 1998), suggesting that penetration through the insect cuticle may be comparatively slow. In contrast, insecticides such as cypermethrin are equitoxic to lepidopterous larvae by either topical application or injection (Sparks *et al.*, 1997), suggesting relatively rapid penetration through the cuticle of pest insect larvae. A time course of penetration of permethrin or spinosyn A into the hemolymph of larvae of the cabbage looper (*Trichoplusia ni*) (Sparks *et al.*, 1997) showed the rate of penetration for spinosyn A to be much slower than that of the pyrethroid permethrin. Likewise, studies of initial penetration rates (2–4 h) for a variety of insect control agents showed that spinosyn A possessed one of the slowest rates of penetration. Most organophosphate and pyrethroid insect control agents have initial rates of penetration that are several-fold higher; in some cases by more than an order of magnitude (**Table 5**). Interestingly, one compound that is reported to have a similarly slow rate of penetration is abamectin, another large macrolide (**Table 5**). Thus, in light of the very good biological activity possessed by many of the spinosyns, their comparatively slow rate of penetration may be well compensated for by a limited capacity of many pest insects to metabolize them.

6.5.3.3. Insecticidal Concentration in the Cockroach

A key parameter in understanding the mode of action of biologically active compounds is the aqueous concentration that needs to be in contact with the receptors in the body to cause the biological effect of interest. For pharmaceuticals, the therapeutic concentration is defined as the aqueous concentration of the substance in the patient's blood plasma needed to obtain a desired therapeutic effect. Likewise, the insecticidal concentration can be defined as the concentration of an insecticide in the aqueous phase of an insect's hemolymph needed to obtain a desired insecticidal effect. Hemolymph is a complex tissue, composed of cells suspended in a complex solution of salts and organic molecules, including high concentrations of proteins. Whole hemolymph concentration, as is usually measured for insecticides, is in itself not interesting for the mode of

Figure 3 Potential biosynthesis of spinosyn A. PSA, pseudoaglycone. (Adapted, in part, from information in Kirst, H.A., Michel, K.H., Mynderse, J.S., Chio, E.H., Yao, R.C., *et al.*, **1992**. Discovery, isolation, and structure elucidation of a family of structurally unique fermentation-derived tetracyclic macrolides. In: Baker, D.R., Fenyes, J.G., Steffens, J.J. (Eds.), Synthesis and Chemistry of Agrochemicals, vol. 3. American Chemical Society, Washington, DC, pp. 214–225; Waldron, C., Madduri, K., Crawford, K., Merlo, D.J., Treadway, P., *et al.*, **2000**. A cluster of genes for the biosynthesis of spinosyns, novel macrolide insect control agents produced by *Saccharopolyspora spinosa*. *Antonie van Leeuwenhoek 78*, 385–390; and Waldron, C., Matsushima, P., Rosteck, P.R., Jr., Broughton, M.C., Turner, J., *et al.*, **2001**. Cloning and analysis of the spinosad biosynthetic gene cluster of *Saccharopolyspora spinosa*. *Chem. Biol. 8*, 487–499.)

Table 3 Biological activity of novel spinosyns and spinosoids towards selected insect and mite species

Number	*Compound*	Heliothis virescens, *neonate* *(LC_{50}, ppm)*	Stomoxys calcitrans, *adult* *(LC_{50}, ppm)*	Aphis gossypii *(LC_{50}, ppm)*	Macrosteles quadrilineatus *(LC_{50}, ppm)*	Tetranychus urticae *(LC_{50}, ppm)*
Novel spinosyns						
30	21-Propyl A	0.16		27		
31	21-Butenyl A	0.29		5.2		1.4
Spinosoids						
32	*N*-demethyl D	5.6		>50		0.1
33	*N*-demethyl K	9.9			8.2	0.5
34	*N,N*-didemethyl K	7.4			11.1	3.4
35	2′-H A	0.23	0.24			134
36	2′-H D	0.23				25
37	2′-*O*-ethyl A	0.30				3.5
38	2′-*O*-vinyl	1.10				
39	2′-*O*-*n*-propyl A	0.30				
40	2′-*O*-*n*-pentyl A	2.00				
41	2′-*O*-benzyl A	1.80				
42	2′-*O*-acetate A	1.20	0.45		8.1	>100
43	3′-H A	0.36	0.47			5.2–>50
44	3′-OH-*epi* A	13.2		>50	>50	>50
45	3′-*O*-methyl-*epi* A	1.9		>50	>50	~50
46	3′-*O*-ethyl A	0.03		12.7		2.0
47	3′-*O*-vinyl A	<0.06-0.3		>50		14
48	3′-*O*-*n*-propyl A	0.05		9.3	~50	2.6
49	3′-*O*-isopropyl A	0.27		>50		3.6
50	3′-*O*-isopropenyl A	2.85		>50		>50
51	3′-*O*-*n*-butyl A	0.38		2.5		1.5
52	3′-*O*-*n*-pentyl A					2.5
53	3′-*O*-allyl A	0.06		>50		11
54	3′-*O*-propargyl A	0.05		>50		10
55	3′-*O*-CH_2CF_3 A	0.33		25		>50
56	3′-*O*-acetate A	33.0	0.41	>50	>100	22
57	3′-*O*-Me-*O*-Me	0.17		19		1.8
58	3′-Methylene	0.95		2.8	>50	8.9
59	4′-H A	4.1				
60	4′-*O*-ethyl A	0.24		>50		4.1
61	4′-*O*-acetate A	1.30	0.90		3.5	1.8
62	2′,3′,4′-tri-*O*-ethyl A	0.02		6.8		1.3
63	4″-*N*-ethyl B			>50		3.8
64	4″-*N*-ethyl A	0.29		15.2	~50	0.3
65	4″-*N*-*n*-propyl A	0.27		17.3	~50	0.35
66	4″-*N*-*n*-butyl A	14.1		5.3		0.4
67	4″-*N,N*-diethyl	>64		4.8		0.3
68	4″-OH	3.7	>5	~0.4		
69	5,6,13,14(α)-TH	35	0.41			
70	5,6,13,14(β)-TH	>64	45			32
71	13,14-α-Dihydro	4.7				18
72	13,14-β-Dihydro	>80	45			>50
73	7,11-Dehydro	0.2		>50		47.4
74	5,6-α-Epoxy	8	0.96			
75	5,6-β-Epoxy	0.63	0.96			0.50
76	5,6-DH	0.46	2.4	6.4		0.44
77	5,6-DH, 3′-*O*-ethyl	0.05		15		0.7
78	5,6-DH, 3′-*O*-*n*-propyl	0.04		1.7		0.4
79	A C17-*O*-acetate	>64	47			47
80	A C17-*O*-DMAA	>64	22			21
81	A C17-*O*-DMAP	16	9.5			
82	A C17-*O*-DMAB	16.6	8.6			
83	A C17-*O*-NMPipz	2.5	4.7		4	

Continued

Table 3 Continued

Number	*Compound*	Heliothis virescens, *neonate* *(LC_{50}, ppm)*	Stomoxys calcitrans, *adult* *(LC_{50}, ppm)*	Aphis gossypii *(LC_{50}, ppm)*	Macrosteles quadrilineatus *(LC_{50}, ppm)*	Tetranychus urticae *(LC_{50}, ppm)*
84	A C17-*O*-DMPipd	4.1	4.5		24.8	
85	A C9-*O*-L-lyxose[a]	1.01				1.3
86	A C9-*O*-L-mannose[a]	0.04				
	Cypermethrin	0.18				
	Ethofenprox				0.3	
	Fenazaquin					1.0

[a]L-Lyxose is missing the 6-methyl of rhamnose; L-mannose is 6-hydroxy-rhamnose.
DMAA, dimethylaminoacetate; DMAB, dimethylaminobutyrate; DMAP, dimethylaminoproprionate; DMPipd, dimethyl-*N*-piperidinyl acetate; NMPipz, *N*-methylpiperazinyl acetate.

Table 4 Physical properties of spinosyns A and D and 21-butenyl spinosyn A

Property	*Spinosyn A*	*Spinosyn D*	*21-Butenyl spinosyn A*
Appearance	Colorless solid	Colorless solid	Colorless solid
Empirical formula	$C_{41}H_{65}NO_{10}$	$C_{42}H_{67}NO_{10}$	$C_{43}H_{67}NO_{10}$
Molecular weight	731.96	745.98	757.99
Melting point	120 °C	170 °C	110 °C
Solubility (20 °C)			
Water (distilled)	89.4 ppm	0.495 ppm	
Water (pH 5)	290 ppm	28.7 ppm	184 ppm
Methanol	190 000 ppm	2500 ppm	
Hexane	4 480 ppm	743 ppm	
Acetone	168 000 ppm	10 100 ppm	
Vapor pressure (25 °C)	3×10^{-11} kPa	2×10^{-11} kPa	
Octanol/water			
Partition coefficient (pH 7.0, log P)	4.01	4.53	4.37

Adapted, in part, from Anonymous, **2001**. Spinosad Technical Bulletin. Dow AgroSciences, Indianapolis, IN; Crouse, G.D., Sparks, T.C., **1998**. Naturally derived materials as products and leads for insect control: the spinosyns. *Rev. Toxicol. 2*, 133–146; and Thompson, G.D., Dutton, R., Sparks, T.C., **2000**. Spinosad: a case study – an example from a natural products discovery programme. *Pest Mgt Sci. 56*, 696–702.

action because it can be much higher than the true aqueous concentration, due to partitioning into cell membranes and binding to hemolymph proteins. For most insecticides, it is not possible to measure hemolymph aqueous concentration directly, because of the difficulty of removing cells and proteins from whole hemolymph without also removing a portion of the hydrophobic insecticide from the aqueous phase. This problem can be circumvented by using an indirect method to estimate the insecticidal concentration, whereby the insecticide content of nerve cords isolated from poisoned, symptomatic insects is determined and compared with the content of nerve cords incubated with the insecticide in saline until a steady state has been reached.

In adult male American cockroaches (*Periplaneta americana*) poisoned with a threshold dose of spinosyn A, the nerve cord content was found to be 4.2 pmol. On the other hand, incubation of isolated nerve cords with 100 nM spinosyn A in saline gave a steady-state tissue content of 19.4 pmol. Assuming passive distribution of spinosyn A between the nerve cord and the aqueous medium *in vivo* and *in vitro* allows us to calculate by simple proportionality that a saline concentration of 21 nM would give the nerve cord content of 4.2 pmol, as observed at the threshold dose (Salgado *et al.*, 1998). Thus, it appears that 20 nM spinosyn A in the hemolymph should have an effect on the target receptors that is strong enough to cause symptoms (Table 6).

6.5.4. Mode of Action of Spinosyns

Because spinosad causes excitatory neurotoxic symptoms, a systematic investigation of the symptoms using neurophysiology was the most direct way to identify the target site. Although spinosad is

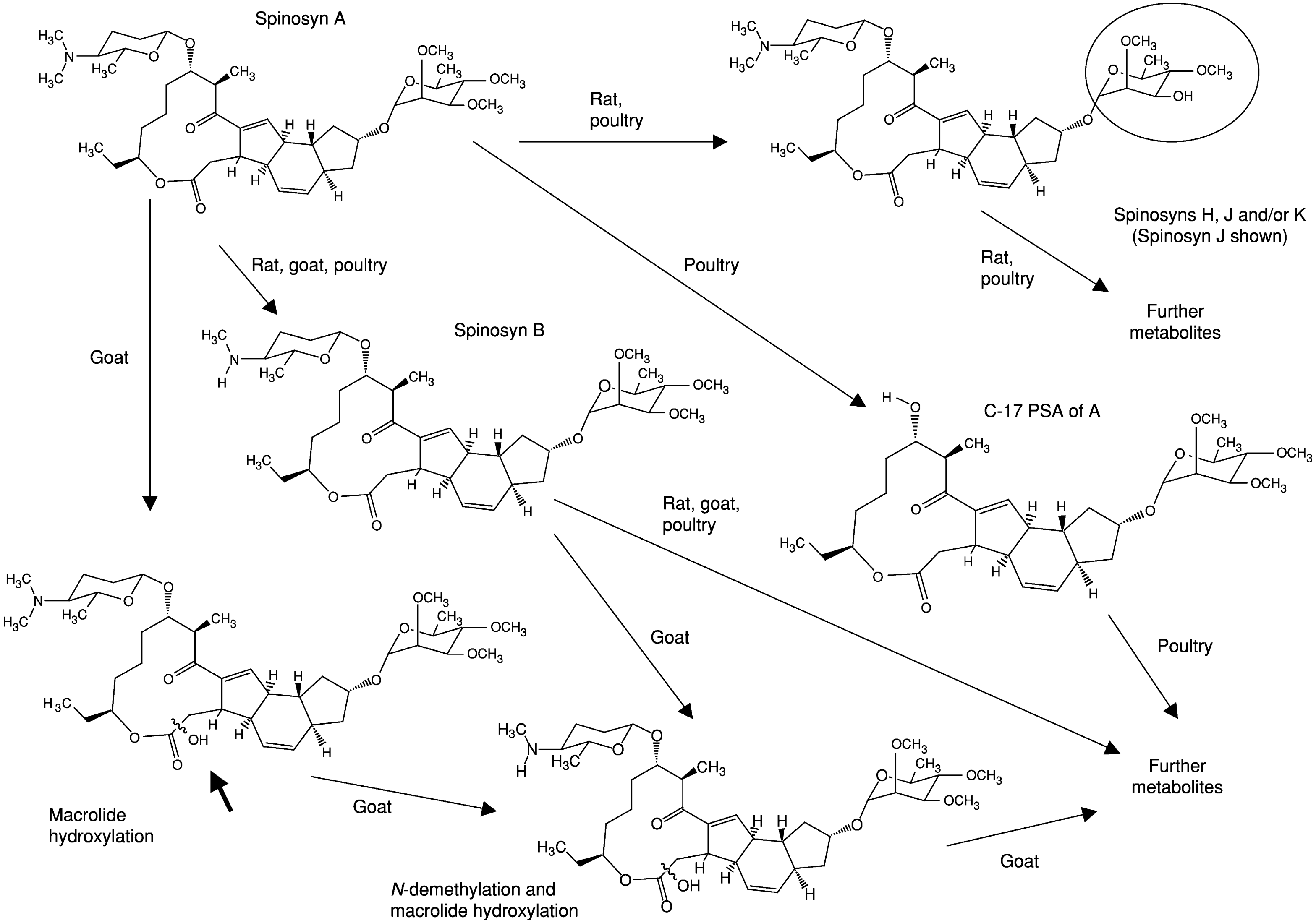


Figure 4 Spinosad metabolism in avian and mammalian systems. PSA, pseudoaglycone. (Adapted, in part, from information in Domoradzki, J.Y, Stewart, H.S., Mendrala, A.L., Gilbert, J.R., Markham, D.A., **1996**. Comparison of the metabolism and tissue distribution of ^{14}C-labeled spinosyn A and ^{14}C-labeled spinosyn D in Fischer 344 rats. Soc. Toxicol. Mtg. Anaheim, CA; Magnussen, J.D., Castetter, S.A., Rainey, D.P., **1996**. Characterization of spinosad related residues in poultry tissues and eggs following oral administration. In: 211th Natl. Mtg. American Chemical Society, New Orlean, LA, pp. AGRO 043; and Rainey, D.P., O'Neill, J.D., Castetter, S.A., **1996**. The tissue distribution and metabolism of spinosyn A and D in lactating goats. In: 211th Natl. Mtg. American Chemical Society, New Orlean, LA, pp. AGRO 045.)

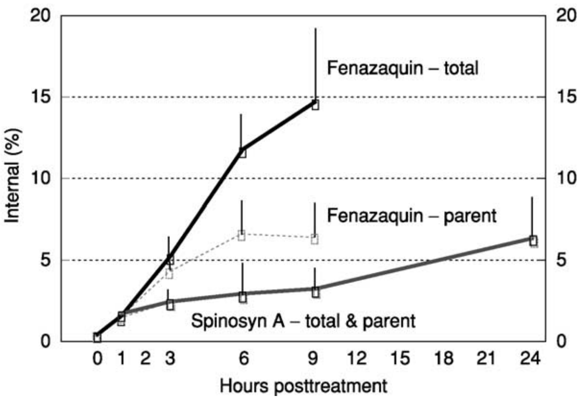


Figure 5 Metabolism of topically applied (1 μg per larva) fenazaquin and spinosyn A in last stadium *Heliothis virescens* larvae. Data points are means, bars give standard deviations. (Adapted from Sparks, T.C., Sheets, J.J., Skomp, J.R., Worden, T.V., Hertlein, M.B., *et al.*, **1997**. Penetration and metabolism of spinosyn A in lepidopterous larvae. In: Proc. 1997 Beltwide Cotton Conf., pp. 1259–1265 and previously unpublished data.)

targeted commercially at Lepidoptera and thrips, spinosyns and spinosoids are inherently broad spectrum, showing activity against insects in the orders Coleoptera, Diptera, Homoptera, Hymenoptera, Isoptera, Orthoptera, Lepidoptera, Siphonaptera, and Thysanoptera, as well as mites (Thompson *et al.*, 1995). American cockroaches were used as the test insects for mode of action studies because of their large size, well-described neuroanatomy and neurophysiology, and distinctive spinosad poisoning symptoms. Spinosyn A was used in all mode of action studies because it is the major component of spinosad, and is similar in biological activity to spinosyn D but much more soluble.

6.5.4.1. Poisoning Symptoms

Symptoms of spinosyn A poisoining in three insect species were described in detail by Salgado (1998) and can in all cases be divided into three phases.

Table 5 Initial rates of penetration and topical toxicities to *Heliothis virescens* larvae of various insecticides

Compound	*Time (h)*	*(%) Penetration*[a]	*Ratio to spinosyn A*	H. virescens *third instar larvae* *(LD_{50}, $\mu g\,g^{-1}$)*
Spinosyns				
Spinosyn A	3	2.3	1.0	1.12–2.4
Avermectins				
Abamectin	4	2.4	1.0	1.2
Pyrethroids and DDT				
DDT	4	10	4.3	52–152
λ-Cyhalothrin	3	8	3.5	0.929
Cypermethrin	3–4	35–36	15.2	0.25–1.61
Fenvalerate	3	3.3	1.4	0.870–1.89
Permethrin	2–3	9–18	3.9–7.8	1.33–2.79
Organophosphates				
Acephate	4	6	2.6	41.0–74.3
Chlorpyrifos	4	38.8	16.9	79.5
Chlorpyrifos-methyl	4	58	25.2	
Malathion	4	17	7.4	
Methyl parathion	4	28.9	12.6	8.3–20
Sulprofos	4	16	7.0	24
Carbamates				
Carbaryl	4	8	3.5	136–232
Cyclodienes				
Endrin	4	12	5.2	46.7
Formamidines				
Amitraz	4	12–15	5.2–6.5	
BTS-27271	4	9	3.9	
METI acaricide[b]				
Fenazaquin	3	5.3	2.3	~400

[a]Percent of material that was considered in the internal fraction.
[b]METI, mitochondrial electron transport inhibitor.
Adapted, in part, from Sparks, T.C., Sheets, J.J., Skomp, J.R., Worden, T.V., Hertlein, M.B., *et al.*, **1997**. Penetration and metabolism of spinosyn A in lepidopterous larvae. In: Proc. 1997 Beltwide Cotton Conf., pp. 1259–1265 and Crouse, G.D., Sparks, T.C., **1998**. Naturally derived materials as products and leads for insect control: the spinosyns. *Rev. Toxicol. 2*, 133–146.

Table 6 Dependence of various effects in *Periplaneta americana* on aqueous concentration of spinosyn A

Aqueous concentration (nM)	*Symptoms*[a]	*Increased CNS efferent activity*[a] *(%)*	*Activation of nicotinic acetylcholine receptor (nAChN)*[b] *(%)*	*Inhibition of small-neuron γ-aminobutynic acid receptor (GABA-R)*[c] *(%)*
1	None	0	4	>50
5	None	0	28	100
10	None	6	35	100
21	Difficulty righting	38	45	
30		54	52	
60	Prostration	68	65	
100		80	73	
1000		100	94	

[a]Symptoms and increased CNS afferent activity from Salgado *et al*. (1998).
[b]Activation of nAChN from Salgado and Saar (2004).
[c]Inhibition of small-neuron GABA-R from Watson (2001).

The earliest symptoms are due to prolonged involuntary muscle contractions that subtly alter the posture of the insect. This is evident in cockroaches and houseflies, where the first symptom is a lowering of the head and elevation of the tail, caused by involuntary straightening of the hindlegs. In the second phase of poisoning, the posture changes become so severe that the insects topple over and cannot right themselves, becoming prostrate. At this point, there are widespread fine tremors in all muscles, which sometimes can only be seen under a microscope. In hard-bodied insects, all appendages tremble constantly, and in soft-bodied insects such as caterpillars, the skin appears to crawl. In the last phase, the movements cease and the insects are paralyzed. The symptoms will be described in more detail for cockroaches, fruit flies, and tobacco budworm larvae.

The 24 h injection LD_{50} (median lethal dose) of spinosyn A was 0.74 (0.41–1.34) $\mu g\,g^{-1}$ for adult male *P. americana*. The first noticeable symptom was elevation of the body, due to depression of the coxae, extension of the legs, and flexion of the tarsae (**Figure 6b**). In this first poisoning phase, the cockroaches still walked around, and, if disturbed by touching, would resume a normal posture temporarily. These symptoms even persisted after the insect was decapitated (**Figure 6c**). As poisoning progressed to phase 2, the cockroaches became more uncoordinated and fell on their backs, as a result of asymmetric extension of the legs. The tarsae of the prostrate insects remained strongly flexed, and the insects showed tremors, which were not seen before prostration (**Figure 6d**). At the lowest effective dose, 0.625 $\mu g\,g^{-1}$, prostration occurred approximately 24 h after injection, whereas at 5 $\mu g\,g^{-1}$ it occurred within 4 h. The insects also showed other symptoms of excitation, such as wing beating and abdominal bloating resulting from their swallowing large amounts of air. The prostrate cockroaches eventually ceased trembling as they entered the third or quiescent poisoning phase, during which movement could still be elicited by a disturbance, but grew gradually weaker. The legs gradually relaxed as the insects became paralyzed, and no further movement could be elicited. No recovery was observed.

Adult male fruit flies, *D. melanogaster,* exposed to spinosyn A in sugar water, exhibited a characteristic set of sublethal symptoms. The 24 h LC_{50} (median lethal concentration) was 8.0 (5.7–12.1) ppm Initially, flies were able to remain upright and even walk around, but most had difficulty maintaining normal posture. When standing still, the tibiae slowly flexed and the tarsae extended, causing the legs to pull together and the body to rise. Approximately every 5 s, the fly compensated by spreading its legs into a normal stance, after which the process was repeated, with alternate slow pulling together, followed by rapid spreading, of the legs. While this effect occurred in nearly all flies at this concentration, some flies also held the wings abnormally. Normally, the wings are folded over the back, but in many poisoned flies they were either open straight out or folded down.

Heliothis virescens larvae prostrated by spinosyn A typically curled up due to abdominal flexion and lay on their sides, exhibiting widespread fine tremors. The true legs were extended and trembling, as were the mouthparts and even the surface of the soft cuticle. These excitatory effects were not obvious to the naked eye, but were easily seen with a stereomicroscope. Diuresis was also a common symptom of spinosyn A poisoning in *H. virescens*

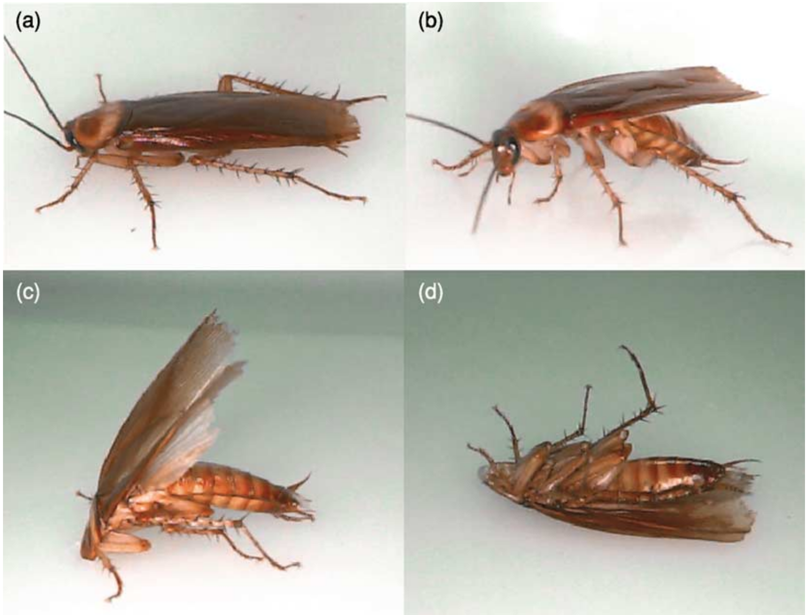


Figure 6 Typical symptoms shown by American cockroaches (*Periplaneta americana*) after injection with spinosyn A, in this case at 2 μg. (a) Control. (b) Depression of the legs elevates the body; note how all leg joints are extended. (c) The symptoms persist even after decapitation, and even worsen, as the whole body flexes. (d) Even after prostration, the tarsae remain strongly flexed, as seen in this decapitated insect. (Reprinted with permission from Salgado, V.L., **1998**. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pestic. Biochem. Physiol. 60*, 91–102; © Elsevier.)

larvae. The injection LD_{50} of spinosyn A against 50–70 mg *H. virescens* larvae was 0.23 (0.1–0.52) $\mu g\ g^{-1}$ (Salgado, 1998).

In summary, one of the first symptoms of spinosyn poisoning in all insects is leg extension, due to involuntary muscle contractions. Later in poisoning, all muscles appear to be affected.

6.5.4.2. Gross Electrophysiology

6.5.4.2.1. Spinosyns excite the central nervous system *in vivo* and *in vitro* Although many muscles are activated during spinosyn poisoning, tarsal flexion was an excellent model because it is one of the first symptoms to appear and persists throughout poisoning, even when the poisoned insect is decapitated and pinned on its back, allowing its physiological basis to be easily studied. This symptom is caused by contraction of the tarsal flexor muscle, located in the tibia (Snodgrass, 1952). The onset of spinosyn-A-induced tarsal flexion was accompanied by continual firing of the tarsal flexor muscle (**Figure 7a**). In control insects, the muscle was relaxed except for activity during sporadic movements (not shown). Cutting all contralateral nerve roots did not diminish flexion, and when all ipsilateral nerves except nerve 5, which innervates the tarsal flexor muscle, were cut, the tarsus still remained flexed, and the firing of the flexor muscle continued (**Figure 7b**). However, immediately upon severing nerve 5, the tarsus relaxed and the muscle recording became silent (not shown). Therefore, the smallest unit exhibiting the flexion was the leg and its ganglion attached through nerve 5, and it was concluded that spinosyn-induced tarsal flexion was caused by activation of the flexor motor neuron, driven by events occurring either within that neuron or elsewhere within that ganglion (Salgado, 1998).

Nerve recordings made from various nerves showed that central nervous system (CNS) and motor activity was generally increased in spinosyn A poisoned cockroaches, concomitant with appearance of excitatory symptoms and persisting even after paralysis. Alternatively, it could be demonstrated that the excitatory effects of spinosyn A were specifically on the CNS; there were no direct effects on axonal conduction, sensory receptor function, or neuromuscular function (Salgado, 1998).

The next step in analyzing the etiology of spinosyn-induced hyperactivity was to determine whether spinosyns could directly increase nerve activity when applied to isolated ganglia. **Figure 8** shows a ratemeter recording of the activity in a segmental nerve of an isolated larval housefly synganglion. In the control and before application of spinosyn A, the activity varied widely. Following treatment with 100 nM spinosyn A, the swings in activity became

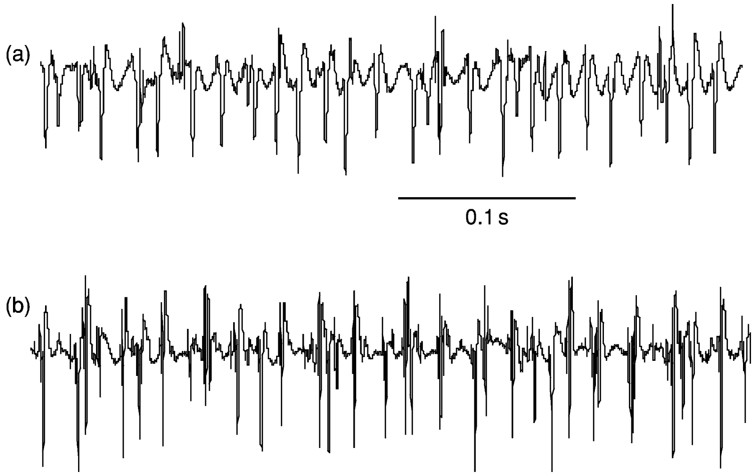


Figure 7 (a) Electromyogram showing continual firing of the metathoracic tarsal flexor muscle in a cockroach poisoned 4 h earlier by abdominal injection of 2.5 μg spinosyn A. (b) The activity persisted after all nerves to the metathoric ganglion, except ipsilateral nerve 5, were severed. (Reprinted with permission from Salgado, V.L., **1998**. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pestic. Biochem. Physiol. 60*, 91–102; © Elsevier.)

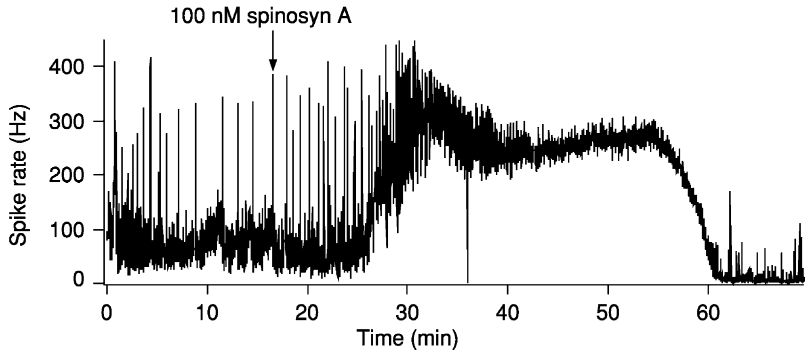


Figure 8 Spike rate recorded from a segmental nerve of an isolated housefly (*Musca domestica*) larval synganglion. Before spinosyn A, the activity varied mostly between 20 and 120 Hz, with occasional brief spikes to 400 Hz. After the addition of 100 nM spinosyn A, there was a delay of approximately 10 min, after which the activity increased greatly to a constant high level, with no dips below 200 Hz. After 25–30 min, the activity declined to below control levels. (Reprinted with permission from Salgado, V.L., **1998**. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pestic. Biochem. Physiol. 60*, 91–102; © Elsevier.)

much smaller and the activity stabilized at a high level, before eventually declining 20–30 min after the start of perfusion with spinosyn A. It was shown above that spinosyn A must attain an internal aqueous concentration near 20 nM in order to produce symptoms. This concentration applied in a saline bath was just sufficient to stimulate activity in isolated cockroach ganglia (**Table 6**). In other words, spinosyn A achieves a concentration in treated insects that is sufficient to cause the observed symptoms by direct actions on nerve ganglia. The ganglion from *Manduca sexta* is significantly more sensitive than the cockroach ganglion, giving a full response at 10 nM (Salgado *et al.,* 1998).

In summary, one of the first symptoms of spinosyn poisoning in all insects is leg extension, due to involuntary muscle contractions driven by activity in motor neurons, caused by a direct action of spinosyns within the ganglion. Later in poisoning, all muscles appear to be tonically activated. The elevated posture arises because, while opposing flexor and extensor muscles are both being activated, the stronger, weight-supporting, muscles overcome the weaker antagonists, so that each joint moves in the direction that elevates the body.

6.5.4.2.2. Mechanism of paralysis by spinosyns Spinosyn-poisoned insects eventually become paralyzed, but only after prolonged excitation, suggesting that paralysis results secondarily, from prolonged overexcitation of the nervous system. Interestingly, neural activity continues at high levels in paralyzed insects, indicating that paralysis is due to fatigue-related failure of the neuromuscular system. This

hypothesis was confirmed by showing that the maximal force exerted by the metathoracic femur in response to tetanic crural nerve stimulation faded more rapidly as poisoning progressed, and eventually disappeared concomitant with paralysis. The inhibitory neuromuscular synapses, which use γ-aminobutyric acid (GABA) as the neurotransmitter and are blocked after prostration with the GABA gated chloride channel blocker dieldrin, were shown to function normally in insects prostrated by spinosyn A (Salgado, 1998). In *in vitro* experiments on larval housefly muscle, spinosyn A at concentrations up to 1 μM had no effect on neuromuscular transmission, but at 5 μM and higher, the cell was slightly depolarized and the excitatory postsynaptic potential (EPSP) was enhanced. These concentrations are too high for these effects to be relevant during poisoning.

6.5.4.2.3. Spinosyns excite central neurons by inducing an inward, depolarizing, current Because spinosyns potently stimulate CNS activity (see Section 6.5.4.2.1), effects on neurons of the cockroach 6th abdominal ganglion were investigated *in situ* with intracellular microelectrodes. Dorsal unpaired median (DUM) neurons were chosen because they are spontaneously active and are the best-characterized neurons in the insect CNS, and known to contain many different receptors important for insecticide action, including nicotinic acetylcholine receptors (nAChRs) (Buckingham *et al.,* 1997), GABA (Le Corronc *et al.,* 2002) and inhibitory glutamate (Raymond *et al.,* 2000) receptors, and a variety of sodium, calcium, and potassium channels (Grolleau and Lapied, 2000). As reported previously (Dubreil *et al.,* 1994), DUM neurons were spontaneously activate and had random spontaneous unitary inhibitory postsynaptic potentials (IPSPs). Furthermore, stimulation of the connective evoked an IPSP in the DUM cell (Pitman and Kerkut, 1970; Dubreil *et al.,* 1994) (**Figure 9b**). At 200 nM, but not at 50 nM, spinosyn A depolarized DUM cells *in situ* (10 and 13 mV in two cells; compare the resting levels of the traces in **Figure 9b and c**) and increased the rate of spontaneous IPSPs considerably, but did not block the spontaneous or evoked IPSPs, even at concentrations as high as 1 μM ($n = 3$) (**Figure 9c**). It also increased the spontaneous firing rate, as can be seen by the two action potentials in this trace (the evoked IPSP abolished an action potential that was just starting). Picrotoxinin at 10 μM abolished the spontaneous and evoked IPSPs (**Figure 9d**), leading to further depolarization and excitation (note the increased action potential frequency). Thus, while spinosyn A did not appear to affect the inhibitory postsynaptic receptors in DUM neurons, or the ability to generate action potentials, it did have clear effects on the cells. While the increased spontaneous IPSP rates reflect changes occurring in presynaptic cells, the depolarization and increased firing rate appear to be due to a direct action of spinosyn A on the DUM cells themselves. It can be seen from the steady depolarization between action potentials (**Figure 9d**) that this firing is due to a continuous depolarizing stimulus flowing into the cell. This suggests that a spinosyn-induced inward current was responsible for the depolarization and increased firing rate. The depolarization was reversed by 10 μM methyllycaconitine (MLA), a selective nicotinic antagonist (**Figure 10**), indicating that it is mediated by nicotinic receptors. MLA also blocked the IPSP, consistent with the proposed multisynaptic pathway for this response, involving a nicotinic synapse (Dubreil *et al.,* 1994) (**Figure 10**).

6.5.4.2.4. Activation of nicotinic receptors by spinosyns generates a depolarizing inward current The intracellular recordings (**Figures 9 and 10**) indicated that spinosyn-A-induced an inward current in central neurons, probably mediated by nAChRs. This current was then directly investigated with voltage clamp experiments on isolated central neurons. The cells studied were a mixed population of neuronal somata, with diameters between 35 and 80 μm, isolated from the thoracic ganglia of the cockroach *P. americana* and studied with the single-electrode voltage clamp method (SEVC) as detailed by Salgado and Saar (2004). In ten cells tested, spinosyn A did indeed induce an inward current at concentrations between 50 nM and 1 μM. Furthermore, this current was completely blocked by 100 nM α-bungarotoxin (α-BGTX), a highly specific antagonist of nAChRs. Because the interaction of both α-BGTX and spinosyn A with the receptors was relatively slow, it was difficult to measure the dose response for block quantitatively. In the example in **Figure 11**, 3 nM α-BGTX blocked the spinosyn-A-induced current by approximately 75% before washout was begun, while 10 nM α-BGTX blocked the spinosyn-A-induced current by 85%, and 1000 nM blocked it completely. This sensitivity to α-BGTX is comparable to that of the nondesensitizing nAChR, which is blocked 78 ± 3% by 3 nM and 93 ± 1% by 10 nM α-BGTX (Salgado and Saar, 2004).

6.5.4.3. Nicotinic Receptors as the Spinosyn Target Site

6.5.4.3.1. Two subtypes of nicotinic receptors Two subtypes of α-BGTX-sensitive nicotinic receptors have been identified in insect neurons (van den

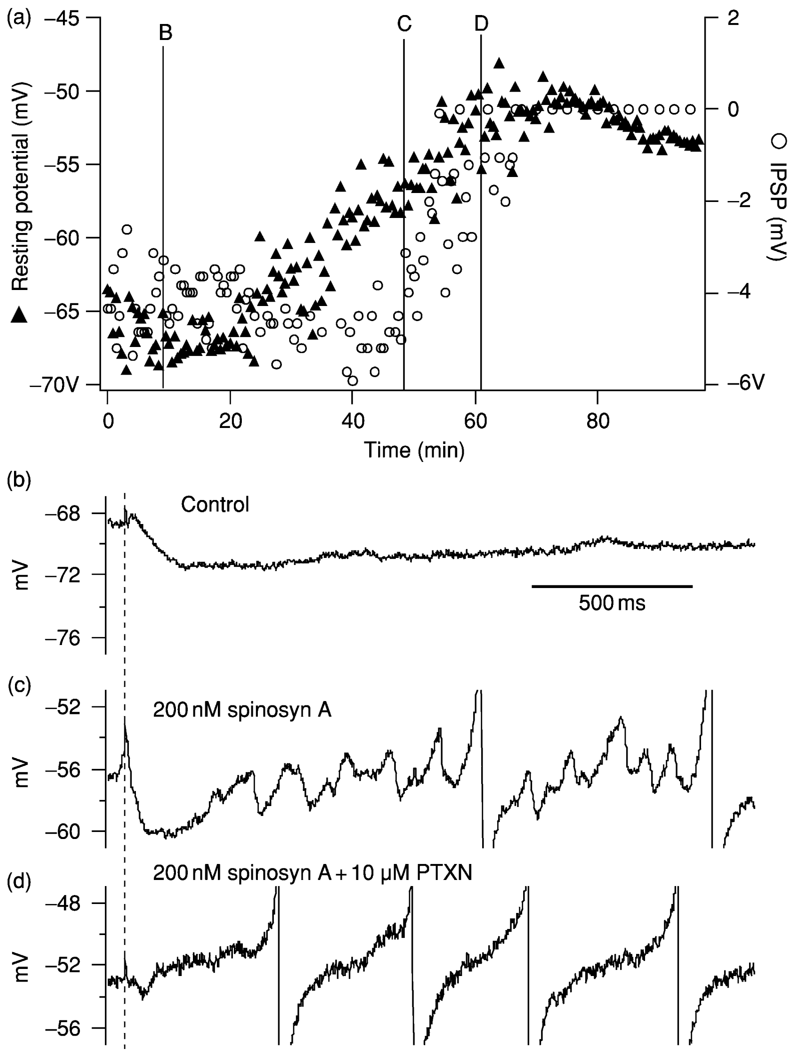


Figure 9 (a) Resting potential and inhibitory postsynaptic potential (IPSP) amplitude of a dorsal unpaired median (DUM) neuron in a cockroach terminal abdominal ganglion. (b–d) Sample recordings at the times indicated by the letters in panel A. The IPSP was evoked by connective stimulation at the vertical dashed line, 200 nM spinosyn A was added just after B, and 10 μM picrotoxinin (PTXN) was added just after point C. This example is representative of similar results obtained in three cells. The preparation for recording synaptic responses from DUM neurons is from Dubreil *et al.* (1994), with slight modifications. The terminal abdominal ganglion of adult male American cockroaches was removed with its associated peripheral nerves. The ganglion was desheathed, placed in an experimental chamber, and perfused with saline. Nerve XI and the anterior connectives were stimulated with suction electrodes, and compounds, dissolved in saline, were applied in the bath. Microelectrodes were filled with 3 M KOAc and had resistances between 110 and 150 MΩ (Salgado, unpublished data).

Beukel *et al.*, 1998; Nauen *et al.*, 2001). In American cockroaches, subtype nAChD receptors desensitize in the presence of agonists and are blocked by α-BGTX with a K_d of 21 nM, while subtype nAChN receptors are nondesensitizing and are blocked by α-BGTX with a K_d of 1.3 nM (Salgado and Saar, 2004). Both subtypes are blocked by MLA, with K_d values of 1 nM and 17 pM, respectively. Since nAChD receptors desensitize in the presence of agonists, they are antagonized during poisoning by nicotinic agonists such as the neonicotinoids (see **Chapter 5.3**), and this antagonism appears to cause the subacute symptoms of these compounds, such as feeding inhibition in aphids and inhibition of cleaning behavior in termites (Salgado and Saar, 2004). On the other hand, activation of nicotinic receptors by nicotinic agonists has long been known to cause excitatory symptoms (Schroeder and Flattum, 1984; Nauen *et al.*, 2001), which appear to be mediated by nAChN receptors.

6.5.4.3.2. Selective action of spinosyns on the nAChN subtype of nicotinic receptor Although almost all cockroach neurons studied contain both

nAChD and nAChN receptors, the two can be separated and studied independently using subtype-selective antagonists. nAChN receptors are extremely sensitive to MLA, with a K_d of 17 pM. Even though nAChD receptors are also very sensitive to MLA, their K_d of 1 nM permits selective block of only nAChN receptors with the inclusion of 100 pM MLA in the bath. Figure 12a shows the total ACh-evoked current in an isolated cockroach neuron and the pure nAChD current remaining after specific block of nAChN receptors by 100 pM MLA.

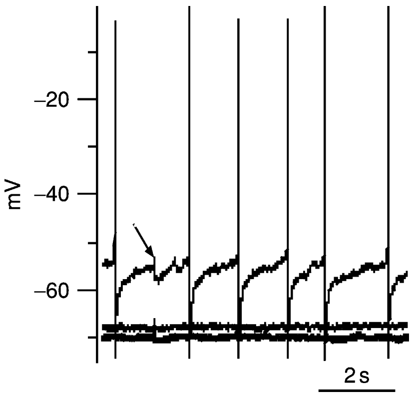


Figure 10 Reversal of spinosyn-A-induced depolarization by methyllycaconitine (MLA) in a DUM neuron. The lower trace is the control, with a resting potential near −70 mV. Spinosyn A at 1 μM depolarized the cell to −53 mV and induced firing. Addition of 10 μM MLA reversed the depolarization and stopped the firing (middle trace). Note that the small IPSP evoked by the stimulus (arrow) in the control became larger after the depolarization and was abolished by MLA. This example is representative of similar results obtained in two cells (Salgado, unpublished data).

On the other hand, selective block of nAChD receptors can be achieved by taking advantage of their property of desensitization. Desensitization is a property of the receptor itself, due to transition of activated receptors into a nonconducting state that binds agonists very tightly (Lena and Changeux, 1993). The desensitized state of the rat muscle nicotinic receptor, for example, has an affinity for agonists about 500-fold higher than that of the open state. The strong binding of agonists to the desensitized state can be used to specifically block the nAChD current (Figure 12b), where, in the presence of 100 nM imidacloprid (IMI), a potent nicotinic agonist (see **Chapter 5.3**), only the nAChN current remains functional, in a cell that has both receptor subtypes.

When nAChN receptors are blocked by the inclusion of 100 pM MLA in the bath, the effect of spinosyn A on nAChD receptors can be studied (Figure 12c). Spinosyn A blocked the nAChD receptors only very weakly, an average of only 23% at 10 μM (Salgado and Saar, 2004), and induced no inward current.

As mentioned above, the sensitivity of the spinosyn-A-induced nicotinic current to α-BGTX (IC_{50} (50% inhibitory concentration) of 1.8 nM measured by Salgado *et al.* (1997)) is very similar to that of nAChN receptors (IC_{50} of 1.2 nM measured by Salgado and Saar (2004), suggesting that spinosyn A activates nAChN receptors. Figure 12d shows the nAChN current, isolated with 100 nM IMI, in the presence of increasing concentrations of spinosyn A. As little as 1 nM spinosyn-A-induced a noticeable inward current and prolonged the action

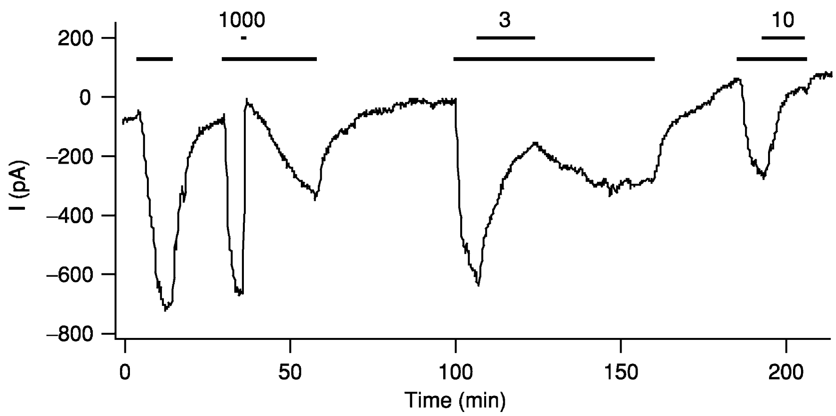


Figure 11 α-Bungarotoxin (BGTX) (thick bars, at concentrations indicated, in nM) block of the spinosyn A (thin bars, at 1 μM) induced inward current at −80 mV in an isolated voltage-clamped cockroach central neuron. The first application and washout of spinosyn A, between 0 and 35 min, reversibly induced a large inward (downward deflection) current. At the peak of the current evoked by the second application of spinosyn A, 1000 nM α-BGTX was applied, which rapidly blocked the current in the continued presence of spinosyn A; the inward current then slowly returned during washout of α-BGTX, but spinosyn A was also washed out, beginning at 60 min. The current evoked by the third application of spinosyn A, at 100 min, was blocked about 75% by 3 nM α-BGTX after about 15 min incubation, but the toxin was washed out before steady-state block was reached. The current evoked by the fourth spinosyn A application, though smaller, due to rundown, was blocked 85% by 10 nM α-BGTX (Salgado, unpublished data).

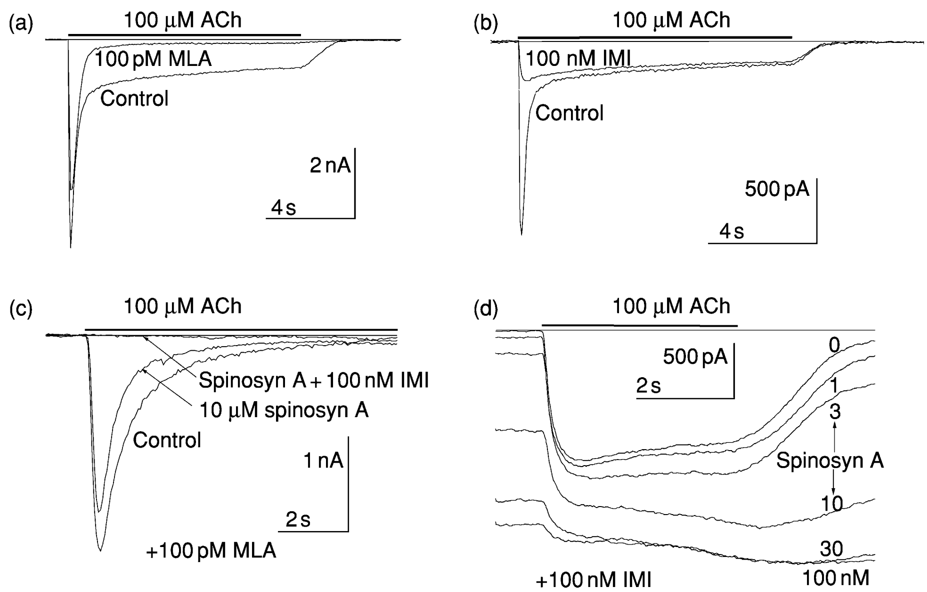


Figure 12 Pharmacological separation of cockroach nicotinic acetylcholine receptor (nAChR) subtypes and the action of spinosyn A on both subtypes. (a) Combined current in an isolated cockroach neuron and the pure nAChD current remaining after specific block of nAChN receptors by 100 pM MLA. (b) In the presence of 100 nM imidacloprid (IMI), only the nAChN current remains in a cell with both receptor subtypes. (c) When nAChN is blocked by the inclusion of 100 pM MLA in the bath, 10 μM spinosyn A slightly blocks the nAChD current. (d) nAChN current isolated with 100 nM IMI, alone and in the presence of increasing concentrations of spinosyn A (Salgado, unpublished data).

of ACh, and these effects increased dramatically with increasing concentrations of spinosyn A. Although the nAChN receptors do not desensitize, the control current drooped approximately 15% during the ACh pulse, possibly due to redistribution of ions near the membrane. At 3 nM spinosyn A, this droop was eliminated and at higher concentrations the current actually increased up to 10% during the ACh pulse, because of a synergistic interaction between spinosyn A and ACh on the nAChN receptor. This synergistic effect can also be seen from the fact that the current after the ACh pulses in the presence of spinosyn A decays much more slowly than in the control, an effect also noted by Salgado *et al.* (1997). The effect of spinosyn A on the nAChN receptors in **Figure 12d** was quantified by measuring the fraction of unmodified receptors remaining, taken as the amplitude of the rapid increase in current in the first second of ACh application as a fraction of the peak current in the control. For example, for the 10 nM spinosyn A treatment in **Figure 12d**, the result is 59% unmodified and for 100 nM it is 10%. The EC_{50} (concentration for 50% activation) for activation of nAChN receptors by spinosyn A, measured in the presence of 100 nM IMI to block nAChD receptors (**Figure 12d**) was 10 ± 1 nM ($n = 3$). Because of the synergistic interaction of spinosyns with competitive nicotinic agonists, the EC_{50} for activation of nAChN receptors was also measured in the absence of IMI, giving a value of 27 ± 4 nM ($n = 4$) (Salgado and Saar, 2004).

The synergistic interaction between spinosyn A and classical, competitive agonists acting at the ACh binding site shows that spinosyns are in fact allosteric agonists of nAChN. This is further supported by the fact that α-BGTX blocks the spinosyn-induced current noncompetitively and that spinosyn A does not displace binding of [^{3}H]α-BGTX (Salgado *et al.*, 1997). Under certain circumstances the synergism between spinosad and classical nicotinic agonists, such as neonicotinoids, is demonstrable in practice (Andersch *et al.*, 2003).

Note also in **Figure 12d** that the total current in the presence of 100 nM spinosyn A and 100 μM IMI is more than twice the total current evoked by ACh alone; spinosyn A is a more efficacious agonist than ACh and can therefore have very strong effects on cells that have a significant number of nAChN receptors. The sensitivity of any cell to the agonistic effects of spinosyns on nAChN receptors will be dependent on the number of those receptors and the input resistance of the cell, which together determine what proportion of the receptors need to be activated to significantly depolarize the cell and change its firing pattern. Symptoms arise in cockroaches at an internal aqueous concentration of 20 nM, at which 45% of nAChN receptors are

activated (Table 6). This represents the sensitivity of the most sensitive cells. This value appears relatively large, suggesting that cells have homeostatic mechanisms to compensate for a relatively large amount of inward current. In fact, DUM neurons first altered their firing pattern when the spinosyn A concentration reached 200 nM (Figure 9), at which 80% of nAChN receptors are activated. This may be related to the fact that DUM neurons fire spontaneously and have a relatively low input resistance.

6.5.4.3.3. Correlation of spinosyn biological activity with potency on nAChN receptors It has already been shown (Table 6) that symptoms appear when the internal aqueous concentration of spinosyn A reaches the range of 20 nM, which is also near the EC_{50} for activation of nAChN receptors, and is also in the range where significant nervous system stimulation is measurable. These results indicate that activation of nAChN receptors can account for the insecticidal activity of spinosyn A.

Potency in activating nAChN receptors was determined for 15 spinosyns and spinosoids, and the data are shown in Table 7 and plotted in Figure 13. There is a clear relation between activity on cockroach nicotinic receptors and insecticidal activity against first instar *H. virescens* larvae. These results provide further support for the importance of nAChR activation in spinosyn poisoning.

Table 7 Spinosyns and spinosoids tested for nicotinic activity. Each cell was calibrated with 100 nM spinosyn A and the potency of the test compound was determined by increasing the concentration gradually to bracket the magnitude of the response to 100 nM spinosyn A. The potency of the test compound relative to spinosyn A was determined by dividing 100 nM by the interpolated equi-effective concentration of the test compound. At least two determinations were made for each compound. The data are plotted in Figure 13

Number	*Spinosyn or spinosoid*	*Relative nicotinic potency*	*Relative* Heliothis *activity*
	4″-*N*-Me Quat	0.071	0.039
	13,14-Epoxide	0.112	0.18
74	5,6-β-Epoxide	0.225	0.32
8	Spinosyn H	0.26	0.088
73	5,6-α-Epoxide	0.45	0.089
72	5,6-H2	0.79	0.50
1	Spinosyn A	1.0	1.0
	5,6-H2, 4″-*N*-deMe	1.49	0.18
4	Spinosyn D	1.73	0.33
2	Spinosyn B	2.0	0.91
23	Spinosyn K	2.3	0.22
	3′-*O*-Et, 4″-*N*-deMe	3.3	0.83
77	5,6-H2, 3′-*O*-ethyl	5.9	3.8
62	2′,3′,4′-tri-*O*-ethyl A	8.2	12.5
46	3′-*O*-ethyl A	15	8.3

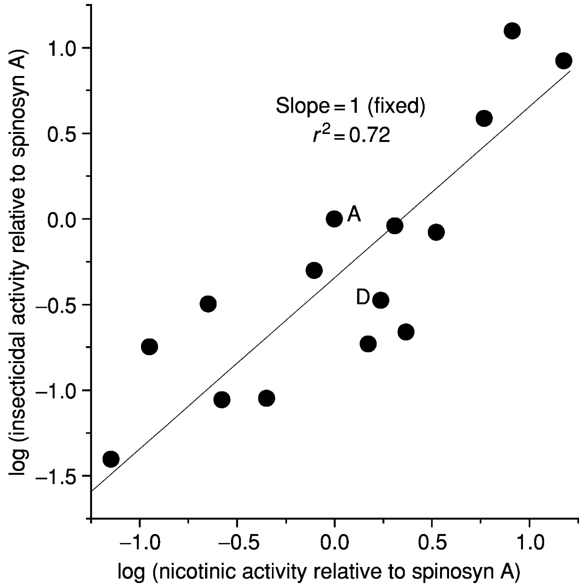


Figure 13 Relationship between relative insecticidal activity against first instar *Heliothis virescens* larvae in a drench assay and relative potency against nAChN receptors, for 15 spinosyns and spinosoids that were tested and had measurable activity in both assays. The regression line had a slope of 0.91, and $r^2 = 0.72$. The data and structural information for the spinosyns included in this figure are shown in Table 7. (Adapted from Salgado, V.L., Watson, G.B., Sheets, S.S., 1997. Studies on the mode of action of spinosad, the active ingredient in Tracer® insect control. Proc. 1996 Beltwide Cotton Production Conf., pp. 1082–1086.)

6.5.4.4. Effects of Spinosyns on GABA Receptors in Small Diameter Neurons of Cockroach

Watson (2001), using the whole-cell patch clamp method, found that in cockroach neurons with a diameter less than 20 μm, the GABA-activated chloride current was completely and irreversibly antagonized by as little as 5 nM spinosyn A (Figure 14), whereas GABA-activated chloride currents in cells larger than 25 μm were not affected. Over the same concentration range, a picrotoxinin-sensitive chloride current was activated by spinosyn A. The chloride current activated by spinosyn A is small in relation to the GABA response, an average of 124 pA in cells with GABA-activated chloride currents generally exceeding 2 nA, but because of the occurrence of GABA receptor antagonism and chloride current activation in the same concentration range, both of these effects might be due to a low efficacy partial agonist action of spinosyn A on a subtype of GABA receptor that appears to be found specifically in small neurons. This would presumably be an allosteric effect, due to binding of spinosyns at a modulatory site analogous to that for spinosyns on nicotinic receptors or avermectins

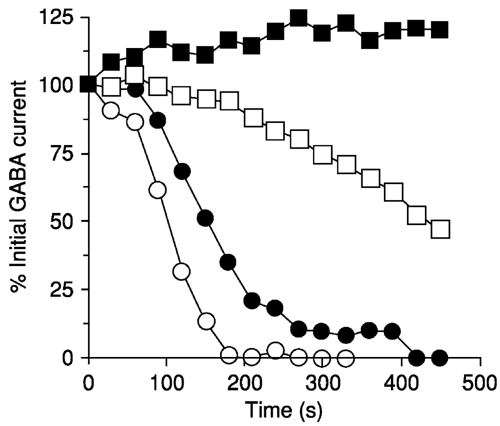


Figure 14 Representative data from individual neurons, showing loss of γ-aminobutyric acid (GABA) currents after the application of increasing concentrations of spinosyn A. With ATP in the recording electrode, responses to 20 mM GABA were fairly constant over time (filled squares). When spinosyn A was added to the perfusing medium at 1 nM (open squares), 5 nM (filled circles), and 100 nM (open circles), responses to GABA diminished over time. (Reprinted with permission from Watson, G.B., **2001**. Actions of insecticidal spinosyns on γ-aminobutyric acid responses from small-diameter cockroach neurons. *Pestic. Biochem. Physiol.* *71*, 20–28; © Elsevier.)

on glutamate and GABA gated chloride channels. Spinosyns did not antagonize the binding of [^{3}H]ivermectin to membranes prepared from several insect species, including cockroach (N. Orr, unpublished data). Although [^{3}H]ivermectin appears to bind to both GABA and GluCl receptors (Ludmerer *et al.*, 2002), it is not known how much of this binding is to the spinosyn-sensitive GABA receptor subtype present in small neurons. That component may be so small as to be undetectable in binding studies, but could nevertheless be very important physiologically.

The observed effect on GABA receptors in small neurons would be expected to have significant effects on the function of affected inhibitory synapses in poisoned insects, but it is not yet clear what the functions of these synapses are or what effect their disruption by spinosyns has on the insect. Since the activated current is relatively small, the overriding effect may be loss of inhibition caused by the loss of GABA receptor function. However, it cannot be excluded that the induced chloride current induces inhibition in some neurons. The excitatory symptoms first become significant in cockroaches at internal aqueous concentrations in the range of 20 nM, whereas block of the GABA receptors in small neurons is strong, if not complete, at 1 nM, and quite rapid and complete at 5 nM (**Figure 14**). While the activation of nicotinic receptors requires a somewhat higher spinosyn A concentration than the effects on GABA receptors in small neurons, its concentration dependence is such that it can account for the excitatory symptoms. Inhibition of GABA receptors in small neurons may act synergistically with nicotinic receptor activation to cause the excitatory symptoms.

In summary, spinosyns cause excitatory symptoms in insects by stimulating activity in the CNS. Activation of nondesensitizing nAChRs by an allosteric mechanism appears to be the primary mechanism by which spinosyns stimulate the neuronal activity, but it has also been shown that spinosyns potently antagonize GABA responses of small neurons, and this could also play a significant role in spinosyn poisoning. Both of these mechanisms are unique among insect control agents.

6.5.5. Biological Properties of the Spinosyns

6.5.5.1. Biological Activity and Spectrum of the Spinosyns

6.5.5.1.1. Pest insects The spinosyns are active against a broad range of insect pests, including many pest species among the Thysanoptera, Coleoptera, Isoptera, Hymenoptera, and Siphonaptera. However, for the spinosyns, the main focus for the activity is centered on the Lepidoptera and to a lesser degree the Diptera (Thompson *et al.*, 1995; Bret *et al.*, 1997; DeAmicis *et al.*, 1997; Sparks *et al.*, 1999; Kirst *et al.*, 2002a). The spinosyns are active against a wide variety of lepidopteran pest species including tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*), diamondback moth (*Plutella xylostella*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*S. exigua*), cabbage looper (*Trichoplusia ni*), American bollworm (*Heliothis armigera*), rice stemborer (*Chilo suppressalis*), and many others (Thompson *et al.*, 1995; Bret *et al.*, 1997). This broad spectrum of pest insect activity is coupled with a high level of potency. Spinosyn A, the most abundant factor produced by *S. spinosa*, is very active against pest lepidopterans, with LC_{50} values in the same range as some pyrethroid insecticides, such as permethrin, cypermethrin, or λ-cyhalothrin (**Table 8**).

6.5.5.1.1.1. Structure–activity relationships of spinosyns – Lepidoptera Among the spinosyns produced by *S. spinosa*, spinosyn A is the most active against pest lepidopterans such as *H. virescens* (**Table 2**). Interestingly, spinosyn A is also the most abundant factor produced in the wild-type strains of *S. spinosa*. Other spinosyns, such as B and C (the

4″-*N*-demethyl and 4″-*N*,*N*-didemethyl derivatives of spinosyn A, respectively) (Table 2), are nearly as active as spinosyn A against *H. virescens*, as is the second most abundant factor, the 6-methyl analog, spinosyn D. Loss of a methyl group at the 2′-, 3′-, or 4′-position of the rhamnose moiety reduces lepidopteran efficacy by about an order of magnitude, as measured by activity against *H. virescens* larvae (spinosyns H, J, K, respectively) (Table 2). A comparable loss in activity is also associated with loss of a methyl group at C16 or a reduction in alkyl size (ethyl to methyl) at C21 (spinosyns F and E, respectively) (Table 2). Interestingly, an increase in alkyl size at C21 (ethyl to *n*-propyl) results in a slight improvement in activity towards *H. virescens* larvae (Table 3), while a further alkyl extension at C21 (2-*n*-butenyl) is about as active as spinosyn A (C21 = ethyl). Among the spinosyns arising from mutant strains (2′, 3′, or 4′-demethyl rhamnose derivatives) (Table 2), virtually all were far less active than spinosyn A. The one notable exception to this is spinosyn Q (LC_{50} 0.5 ppm) which is close in activity to spinosyn A.

6.5.5.1.1.2. Structure–activity relationships of spinosyns – Diptera In general, the pattern of activity for the different spinosyns towards adult stable flies is similar to that observed for the Lepidoptera (Kirst *et al*., 2002a). For example, spinosyns A–D and Q are nearly equitoxic to the adult stable fly (*Stomoxys calcitrans*), while the C16 demethyl analog (spinosyn F) is less active, as is spinosyn K (Table 2). However, unlike with *H. virescens* larvae, spinosyns E, H, and J are as active as spinosyn A (Table 2). The difference for spinosyn J is especially interesting since it is at least 100-fold less active than spinosyn A against larvae of *H. virescens*. Thus, there may be some significant differences in the spinosyn target site in adult stable flies, in the metabolism/conjugation of spinosyn J, or both, compared to larvae of *H. virescens*. Larval blowfly (*Calliphora vicina*) activity of the tested spinosyns was even more like lepidopteran activity than *S. calcitrans* activity (Kirst *et al*., 2002a). As in *H. virescens* larvae, spinosyns A–D are as toxic as spinosyn A, while spinosyns E and F, and the 2′, 3′, or 4′-demethyl analogs (spinosyn H, J, and K, respectively) are less active than spinosyn A. However, unlike in larvae of *H. virescens*, spinosyn J still displayed a reasonable level of activity towards larvae of *C. vicina* (Table 2).

6.5.5.1.1.3. Structure–activity relationships of spinosyns – Homoptera Unlike members of the Lepidoptera and Diptera, homopterans such as aphids are not very susceptible to the spinosyns (DeAmicis *et al*., 1997; Sparks *et al*., 1999; Crouse *et al*., 2001). With the exception of spinosyns B, K, and O, the spinosyns of *S. spinosa* for which data are currently available are not very active against cotton aphid (*Aphis gossypii*) (Table 2). Spinosyn activity against the aster leafhopper (*Macrosteles quadrilineatus*) reflects a similar pattern in that spinosyn K is again the most active of the spinosyns tested, although comparatively, spinosyns A and B are also active. Interestingly, other 4′-*O*-demethyl spinosyns such as spinosyn O and the 2′,4′-di-*O*-demethyl spinosyns U and V are also quite active against the leafhoppers, relative to spinosyn A (Table 2).

6.5.5.1.1.4. Structure–activity relationships of spinosyns – Acarina The spinosyns are also active against tetranychid mite species such as the two-spotted spider mite (*Tetranychus urticae*) (Sparks *et al*., 1999; Crouse *et al*., 2001). As observed with some of the homopteran species, spinosyns K and O are among the most active of the natural spinosyns from *S. spinosa*, while spinosyns H and Q are comparatively weaker than spinosyn A (Table 3). Likewise, spinosyn B is also relatively active. As noted previously, there is only a weak correlation between activity towards neonate *H. virescens* larvae versus spinosyn activity towards *T. urticae* (Sparks *et al*., 1999). While improvements in mite activity are possible, the physical properties affecting residuality and translaminar characteristics are such that the spinosyns are typically not well suited for mite control compared to available commercial standards (Crouse *et al*., 2001).

6.5.5.1.2. Nontarget organisms

6.5.5.1.2.1. Beneficial insects The spinosyns, exemplified by spinosad, are highly efficacious against a variety of pest insect species, yet relatively weak against towards a variety of beneficial insect species, especially predatory insects. A detailed summary of studies involving the effects of spinosad on a wide variety of beneficial insects has recently been published (Williams *et al*., 2003). Spinosyns such as spinosad typically have little effect on predatory insects and mites (Williams *et al*., 2003). Where particular laboratory bioassays have shown some spinosad toxicity to predators (e.g., *Orius insidiosus*), corresponding greenhouse and field tests have generally shown spinosad to have little effect (Studebaker and Kring, 2003), principally due to the rapid environmental degradation of spinosad (Saunders and Bret, 1997; Crouse *et al*., 2001; Williams *et al*., 2003). Based on data in Williams *et al*. (2003), in more than 88% of the greenhouse/field assays

Table 8 Toxicity values for selected insect control agents

Compound	*Rat/mouse/rabbit (LD_{50}, mg kg^{-1})* Oral	Dermal[a]	*Avian; quail or mallard (LD_{50}, mg kg^{-1})*	*Fish*[b] *(LC_{50}, ppm)*	Daphnia *(LC_{50}, ppm)*	Heliothis virescens, *third instar, topical treatment (LD_{50}, $\mu g\ g^{-1}$)*	*VSR, oral/Hv*
DDT	87–500	1931	611	0.08t	0.0047	52–152	0.57–9.6
Cyclodienes							
Dieldrin	40–100	52–117	37	0.0012t	0.00024		
Endrin	3	12–19	14	0.00075t	0.0042	46.7	0.06
Endosulfan	18	74–130	805	0.0014t		73.3	0.25
Carbamates							
Carbaryl	307	>500 to >4000	>5000	1.95t	0.0056	136–232	1.32–2.25
Methomyl	17	>5000r	1100–3600	3.4t	0.0088	4.33–26.7	0.64–3.93
Aldicarb	0.9	2.5–5	594	0.6t	0.061	571	
Carbofuran	8	2550	190	0.38t			
Organophosphates							
Methyl parathion	9–42	63–72	90	3.7t	0.00014	11.6–65.0	0.14–3.62
EPN	7–65	22–262	349–437	0.21t	0.32	37	0.19–1.76
Chlorpyrifos	82–245	202–2000	940	0.0071t	0.0017	79.5	1.03–3.1
Acephate	866–945	>2000r		445t	>50	41.0–74.3	11.7–23
Methamidophos	13–30	110	8–11			85.7–150	0.09–0.35
Profenofos	400	472–1610		0.024t	0.0014	11	36.4
Pyrethroids							
Permethrin	2000 to >4000	>4000		0.0023t		1.33–2.79	717 to >3000
Fenvalerate	451	>5000	9932	0.0036t		0.40–1.89	239–1128
Cypermethrin	247	>2000	>10000	0.025t	0.0013	0.24–1.61	153–1029
λ-Cyhalothrin	56–79	632–696	>500–3950			0.93	60–85
Tralomethrin	1070–1250	>2000r				0.251	4263–4980
Insect growth regulators							
Pyriproxyfen	>5000	>2000	>2000	0.45–2.7	0.40		
Tebufenozide	>5000		>2150	3.0–5.7	3.8		
Methoxyfenozide	>5000	>2000	>5620	>4.3	3.7		
Neonicotinoids							
Imidacloprid	450	>5000	31–5000	211	32–85	350	na
Thiacloprid	444–836	>2000	2716q	30.5t	>85	12.6	na
Thiamethoxam	1563	>2000	576–1552	>114–125			
Acetamiprid	146–217	>2000		>100c	>100	~400	na
Dinetofuran	>2000	>2000	1000 to >2000	>40 to >1000			
Phenylpyrazoles							
Fipronil	100	>2000	31–2150	0.25–0.43	0.19		
Ethiprole	>2000	>2000					
Avermectins							
Abamectin	10.6–11.3		>2000	0.042	0.00032	1.16	9.1–9.7
Ememectin benzoate	70	>2000r	76–264	0.67		0.10	700
Pyrroles							
Chlorfenapyr	>626	>2000	10–34	7.4–11.6		4.5	>139
Oxadiazines							
Indoxacarb	>5000	>2000	>2250	0.5		0.93	>5376
Spinosyns							
Spinosad	3738 to >5000	>5000	>1333	6–30	93	1.28–2.25	1661 to >3906
Dihalopropenyl aryl ethers							
Pyridalyl	>5000	>5000	1133 to >5620	0.50t		0.82	>6097

[a]Dermal toxicity data, r = rabbit.
[b]Fish values are for carp unless otherwise indicated; t = trout.
VSR, vertebrate selectivity ratio: rat/mouse oral LD_{50}/topical LD_{50} for *H. virescens* larvae (see Section 6.5.5.1.2.2); na, not available.
Data adapted from Ware, G.W., **1982**. Pesticides: Theory and Application. W.H. Freeman, San Francisco, CA; Larson, L.L., Kenaga, E.E., Morgan, R.W., **1985**. Commercial and Experimental Organic Insecticides. Entomological Society of America, College Park,

involving predatory insects, the effects of spinosad on the predators fall into the harmless to slightly harmful categories, with the bulk (79%) in the harmless category.

Initial studies with spinosad suggested that there was some activity against hymenopterous parasitoids (*Encarsia formosa*) and honeybees (*Apis mellifera*), but that these pests were much less sensitive to spinosad compared to more conventional insect control agents such as the pyrethroids (e.g., cypermethrin) (Thompson *et al*., 1995, 2000). Subsequent studies, as recently reviewed by Williams *et al.* (2003), show hymenopterous parasitoids to have varying levels of susceptibility to spinosad, with many, unlike predaceous insects, tending to be relatively sensitive. While not all parasitoids are susceptible to spinosad (Elzen *et al*., 2000; Papa *et al*., 2002; Hill and Foster, 2003), studies with a wide variety of parasitoids show spinosad to be toxic to many of them, especially when applied directly to the parasite (Williams *et al*., 2003). However, unlike most of the lepidopteran and dipteran pest insect larvae targeted for control by spinosad, parasitoids are typically highly mobile. As such if they are able to escape the initial, direct contact with wet residues, these same parasitoids may not be highly impacted by the dried residues, especially after a few days (e.g., Tillman and Mulrooney, 2000). As noted above, spinosad is rapidly degraded in the environment (Sanders and Bret, 1997; Crouse *et al*., 2001). This rapid degradation contributes to observations in greenhouse and field studies that parasitoid populations, even those that are relatively sensitive to spinosad, can recover in a short period of time (1–2 weeks) (Williams *et al*., 2003).

Thus, as with all insect control agents, the use and efficacy of spinosad should be based on a variety of factors, including the presence and role of any beneficial insects. Compared to many of the organophosphate and pyrethroid insect control agents available for pest insect control, spinosad, as well as other recently introduced insect control agents (Sparks, 2004), have a reduced overall potential to adversely impact beneficial insects in the field.

6.5.5.1.2.2. Mammalian toxicology and ecotoxicology Among the critical attributes for any new insect control agent is the need to provide a high level of comparative safety to nontarget species and the environment.

The highly desirable trend with new insect control agents has been to increase efficacy towards target pest species while attempting to reduce the relative toxicity towards mammals and other nontarget species. Among the general populace, natural products are often viewed as inherently safer or "greener" than synthetic organic compounds, a view that ignores or is ignorant of the fact that many natural products are highly toxic to mammals and/or nontarget species (Thompson and Sparks, 2002). However, the spinosyns, in the form of spinosad, do indeed couple excellent levels of efficacy against pest insect species with a highly desirable environmental/toxicological profile.

Data for spinosad, as well as several new insect control agents, shows that toxicity to mammalian, avian, and aquatic species is relatively low when compared with many older and currently used insect control agents (**Table 8**). The relative selectivity of an insect control agent can be quantified (therapeutic index) by comparing the activity of a compound on a target insect pest with the activity towards a nontarget species. One such therapeutic index applied to insect versus mammalian activity is the vertebrate selectivity ratio (VSR) defined by Hollingworth (1976):

$$\mathrm{VSR} = \frac{\text{Acute rat oral LD}_{50}\text{ in mg kg}^{-1}}{\text{Insect LD}_{50}\text{ in }\mu\text{g g}^{-1}} \qquad [1]$$

The VSR for spinosad is among the most favorable in a set of now classic, current and newer insect control agents, when comparing *H. virescens* larvae topical toxicity and rat oral toxicity (Sparks *et al*., 1999; Thompson and Sparks, 2002) (**Table 8**). Thus, the superb mammalian toxicity profile observed for spinosad is further enhanced by the high level of efficacy towards the target insect pests, thereby contributing to spinosad's very favorable toxicology

MD, pp. 1–105; Sparks, T.C., Thompson, G.D., Kirst, H.A., Hertlein, M.B., Larson, L.L., *et al*., **1998**. Biological activity of the spinosyns, new fermentation derived insect control agents, on tobacco budworm (Lepidoptera: Noctuidae) larvae. *J. Econ. Entomol. 91*, 1277–1283; Hollingworth, R.M., **2001**. New insecticides: mode of action and selective toxicity. In: Baker, D.R., Umetsu, N.K., (Eds.), Agrochemical Discovery: Insect, Weed and Fungal Control. American Chemical Society, Washington, DC, pp. 238–255; Smith, P. (Ed.), **2001**. AgroProjects: PestProjects – 2001. PJB Publications, Richmond, UK; Thompson, G.D., Sparks, T.C., **2002**. Spinosad: a green natural product for insect control. In: Lankey, R.L., Anastas, P.T. (Eds.), Advancing Sustainability through Green Chemistry and Engineering. American Chemical Society, Washington, DC, pp. 61–73; and references therein; Sparks, T.C., **1996**. Toxicology of insecticides and acaricides. In: King, E.G., Phillips, J.R., Coleman, R.J. (Eds.), Cotton Insects and Mites: Characterization and Management. The Cotton Foundation, Memphis, TN, pp. 283–322; Sparks, T.C., **2004**. New insect control agents: modes of action and selectivity. In: Endersby, N.M., Ridland, P.M. (Eds.), The Management of Diamond back Moth and Other Crucifers Pests: Proc. 4th Intl. Workshop, Melbourne, Australia, 26–29 November 2001, pp. 37–44; extoxnet.orst.edu; and unpublished data.

profile. Additionally, tests conducted for EPA registration showed spinosad to neither leach nor persist in the environment. These factors all contributed to the EPA's registration of spinosad as a reduced risk pesticide in early 1997 (Thompson *et al*., 2000; Thompson and Sparks, 2002), and the bestowing of the Presidential Green Chemistry Award in 1999.

6.5.6. Resistance Mechanisms and Resistance Management

6.5.6.1. Cross-Resistance

Many of the primary insect pests in major markets have a history of developing resistance to insect control agents. Thus, efficacy against insecticide resistant pest insects and the potential for the development of resistance are increasingly important considerations in the development decision for any new insect control agent. Early in the development process, spinosad was examined for efficacy against a variety of insecticide resistant strains of pest lepidopterans, such as larvae of *H. virescens* and *P. xylostella* (Sparks *et al*., 1995). *Heliothis virescens* larvae collected from a number of locations in the mid-southern USA were subjected to discriminating dose assays for a variety of insecticides. Relative to the susceptible strains, larvae collected from almost all of these locations were poorly controlled by cypermethrin (pyrethroid) or profenophos (organophosphate). In contrast, spinosad provided very good to excellent control of all of the strains tested (Sparks *et al*., 1995), demonstrating its utility against insecticide-resistant strains possessing what was likely a mixture of resistance mechanisms (based on the diverse nature of the other insect control agents). Likewise, studies with laboratory strains of *P. xylostella* selected for high levels of resistance to pyrethroid, avermectin, or acylurea insecticides and known to possess enhanced glutathione transferase and/or monooxygenase activity (Chih-Ning Sun, personal communication), showed no appreciable levels of cross-resistance to spinosad (Sparks *et al*., 1995) (**Table 9**). One subsequent study with field-selected strains of *P. xylostella* resistant to the pyrethroid permethrin, has shown a low level of, but broad, cross-resistance to several insect control agents including spinosad and the avermectin emamectin benzoate (Shelton *et al*., 2000) (**Table 9**). However, virtually all other studies involving a variety of insecticide resistant species and strains have shown no cross-resistance to spinosad (**Table 9**). For example, a multiresistant strain of housefly (*Musca domestica*) that was highly resistant (1800-fold and 4200-fold) to the pyrethroids permethrin, and cypermethrin, respectively, exhibited no cross-resistance to spinosad (Liu and Yue, 2000) (**Table 9**). Spinosad was highly effective against a number of strains that were highly resistant to other insect control agents including abamectin (decreased penetration and altered target site), cyclodienes (*rdl*), pyrethroids (monooxygenases, knockdown resistance, and reduced penetration), and organophosphates (altered acetylcholinesterase) (Scott, 1998). Only with the multiresistant LPR strain of housefly was any cross-resistance noted, and that was only at a very low level (Scott, 1998). Taken in total, these data are most consistent with the presence of a unique mode of action for the spinosyns as well as what may be an apparent limited susceptibility of the spinosyn structure to insect metabolic systems.

6.5.6.2. Resistance Mechanisms

While almost all insecticide-resistant strains of pest insects have exhibited little or no cross-resistance to the spinosyns/spinosad, it is nearly axiomatic that if sufficient pressure is put on an insect population, resistance will be developed to any insect control agent, and the spinosyns/spinosad are certainly no exception. This has indeed been borne out in that there are now a few examples of spinosad resistance being developed in the laboratory and in the field.

Resistance to spinosad has been selected for in the laboratory. Bailey *et al.* (1999) used a topical selection protocol to generate a high level of spinosad resistance (toxicity ratio, TR = 1068-fold, topical assay) in larvae of *H. virescens* after 14 generations of selection using methods that favored the development of resistance (NC spinosad-R) (Young *et al*., 2001, 2003). Likewise, a spinosad resistant strain (NYSPINR) of *M. domestica* (TR = >150) was selected in the laboratory starting from a composite of field strains (Shono and Scott, 2003). From this multiresistant NYSPINR strain, a further spinosad resistant strain (*rspin*) was developed that was specifically resistant to only spinosad and also incorporated a number of recessive mutant markers (Shono and Scott, 2003). For spinosad resistant strains of both *H. virescens* (NC-spinosad-R) and housefly (*rspin*), cross-resistance to other insect control agents such as emamectin benzoate, indoxacarb, acetamiprid, abamectin, etc. was negligible (Young *et al*., 2001; Shono and Scott, 2003) (**Table 9**). Both studies also showed spinosad resistance to be linked to a single, non sex-linked, recessive gene (Shono and Scott, 2003; Wyss *et al*., 2003). Studies with the NYSPINR housefly strain also showed no synergism of spinosad activity with piperonyl butoxide, *S,S,S*-tributylphosphorothioate (DEF), or diethyl maleate (DEM), suggesting that metabolism was not likely to be the

basis for the observed resistance. In the case of the NC-spinosad-R *H. virescens* larvae, studies also showed that the resistance to spinosad did not appear to be associated with enhanced metabolism, decreased penetration, or excretion (Young *et al.*, 2000, 2001). At low concentrations, a decreased neural sensitivity was observed in the resistant strain (Young *et al.*, 2001). Thus, available data for both these spinosad resistant strains is most consistent with an altered target site resistance mechanism.

In addition to the laboratory selection studies, there are now a couple of studies indicating the presence of resistance to spinosad in field-collected insects. Surveys of beet armyworm (*Spodoptera exigua*) populations from the US and Thailand showed that there was a wide range in the biological response to spinosad when compared to a US Department of Agriculture (USDA) susceptible reference colony (Moulton *et al.*, 1999, 2000). Compared to the USDA larvae, toxicity ratios for an Arizona strain ranged from 14-fold to 20-fold, depending on assay method, while those of the Thailand strain ranged from 58-fold to 85-fold. Interestingly, the F_1 hybrids from the Thailand colony possessed toxicity ratios of 22-fold compared to the USDA strain, suggesting that the reduced susceptibility was incompletely dominant (Moulton *et al.*, 2000). Subsequent studies with the Thailand strain showed that susceptibility was returning after some time in laboratory culture (toxicity ratio declined to 15-fold) and that synergists (Piperonyl butoxide, DEF, and DEM) did not alter spinosad activity (Moulton *et al.*, 1999).

In mid-1998, spinosad was registered in Hawaii, for use on crucifers, for the control of *P. xylostella* larvae that had become resistant to all other then available insect control agents (Zhao *et al.*, 2002). Unfortunately, resistance management strategies incorporated into the spinosad label by the manufacturer did not foresee the particular cultural practices used in Hawaii. In many cases, the practices then in use resulted in nearly exclusive, almost continuous, year-round, weekly applications of spinosad to sequential crop plantings involving the same *P. xylostella* population, a situation that went on for more than 2 years. In 2000, field failures were observed with spinosad in Hawaii. Subsequent laboratory testing of the *P. xylostella* populations around the growing regions of the Hawaiian Islands demonstrated the presence of high levels of resistance to spinosad in several locations (Zhao *et al.*, 2002). Further laboratory selection, for nine generations, of one of the most resistant colonies (Pearl City, Oahu) with spinosad, increased resistance towards spinosad to an even larger degree (Zhao *et al.*, 2002). As noted above for the laboratory-selected spinosad-resistant strains of *H. virescens* and *M. domestica*, resistance to spinosad in this spinosad-selected strain of *P. xylostella* was also an autosomal, incompletely recessive trait (Zhao *et al.*, 2002), which has led to the rapid dissipation of resistance. Likewise, the synergists piperonyl butoxide and DEF did not alter the level of resistance to spinosad, and there was no cross-resistance to either emamectin benzoate or indoxacarb. While these data are of a limited nature, like those above, they are most consistent with an altered target site as the resistance mechanism.

6.5.6.3. Resistance Management

The development of any new insect control agent is a very expensive proposition. For a company to derive full value, a new molecule must remain in the marketplace for many years. Thus, the development of resistance has become an increasingly important consideration in the development of any new insect control agent. In light of spinosad's uniqueness, as well as the expense and effort that led to its development, insecticide resistance management (IRM) was a consideration at an early stage in the development of spinosad, (Thompson *et al.*, 2000). The goal of this effort was to increase the likelihood of spinosad's long-term efficacy and utility in the marketplace. One aspect of the IRM program was the incorporation of use patterns on the product label that reduced the likelihood of selecting back-to-back generations of insect pests. The IRM recommendation from the manufacturer included limiting use to three or fewer applications within a 30-day period, followed by another 30-day period during which spinosad was not to be used (Zhao *et al.*, 2002). For most lepidopterous pests, this spinosad-free period would allow at least one complete generation that had not been selected. A total of only six applications was allowed per crop (Zhao *et al.*, 2002). Another aspect of the spinosad IRM plan was implementation of a program to monitor the susceptibility of key insect pests.

As demonstrated repeatedly over the past 50 years, given time and very heavy selection pressure, resistance can develop to any insect control agent (Sparks *et al.*, 1993; Clark and Yamaguchi, 2002). Likewise, as demonstrated by the development of spinosad resistance in *P. xylostella* in Hawaii, not every use pattern or scenario can be foreseen. The practice of having multiple "crops" at different stages in adjacent fields effectively negated IRM recommendations for spinosad. However, the monitoring program that was in place was able to quickly provide needed susceptibility data for a number of sites in the Hawaiian Islands. While the initial data showed most sites to be free of problems, several

Table 9 Resistance and cross-resistance to spinosad

Species	*Strain*	*Select*	*Compound*	*Method*	*Toxicity ratio*	*Reference*
Spinosad resistant strains						
Heliothis virescens	NC spinosad-R	Lab G17	Spinosad	Larval dip	>1000	Young *et al.* (2001)
	NC spinosad-R	Lab G17	Permethrin	Larval dip	0.65	
	NC spinosad-R	Lab G17	Profenofos	Larval dip	1.70	
	NC spinosad-R	Lab G17	Emamectin benzoate	Larval dip	1.91	
	NC spinosad-R	Lab G17	Indoxacarb	Larval dip	1.45	
	NC spinosad-R	Lab G17	Acetamiprid	Larval dip	0.41	
Plutella xylostella	Pearl City	Field	Spinosad	Leaf dip	1080	Zhao *et al.* (2002)
	Pearl City	Field	Emamectin benzoate	Leaf dip	4	
	Pearl City	Field	Indoxacarb	Leaf dip	1.28	
Musca domestica	rspin	Laboratory	Spinosad	Topical	>150	Shono and Scott (2002)
	rspin	Laboratory	Indoxacarb	Topical	0.11	
	rspin	Laboratory	Abamectin	Topical	1.2	
	rspin	Laboratory	Fipronil	Topical	0.34	
	rspin	Laboratory	Cyfluthrin	Topical	0.42	
	rspin	Laboratory	Dimethoate	Topical	0.31	
	rspin	Laboratory	Chlorfenapyr	Topical	0.77	
	rspin	Laboratory	Methomyl	Topical	0.61	
Spodoptera exigua	Arizona	Field	Spinosad	Leaf dip	14–20	Moulton *et al.* (2000)
	Thailand	Field	Spinosad	Leaf dip	58–85	Moulton *et al.* (2000)
Spinosad cross-resistance						
Plutella xylostella	Fenvalerate-R	Laboratory	Cypermethrin	Topical	1520	Sun (1991), cited in
	Fenvalerate-R	Laboratory	Spinosad	Topical	3.2	Sparks *et al.* (1995)
	Tebufenozide-R		Cypermethrin	Topical	27	Sun (1991), cited in
	Tebufenozide-R		Spinosad	Topical	0.9	Sparks *et al.* (1995)
	Abamectin-R	Laboratory	Cypermethrin	Topical	64	Sun (1991), cited in
	Abamectin-R	Laboratory	Spinosad	Topical	2.4	Sparks *et al.* (1995)
Plutella xylostella	Ocean Cliff	Field	Permethrin	Leaf dip	126	Shelton *et al.* (2000)
	Ocean Cliff	Field	Spinosad	Leaf dip	14.5	
	Ocean Cliff	Field	Emamectin benzoate	Leaf dip	13.0	
	Ocean Cliff	Field	Chlorfenapyr	Leaf dip	1.0	
	Ocean Cliff	Field	Methomyl	Leaf dip	7.1	
	Oxnard	Field	Permethrin	Leaf dip	206	Shelton *et al.* (2000)
	Oxnard	Field	Spinosad	Leaf dip	12.8	
	Oxnard	Field	Emamectin benzoate	Leaf dip	6.0	
	Oxnard	Field	Chlorfenapyr	Leaf dip	1.7	
	Oxnard	Field	Methomyl	Leaf dip	6.5	
Plutella xylostella	His-hu 2001	Field	Abamectin	Leaf dip	2497	Kao and Cheng (2001)
	His-hu 2001	Field	Emamectin benzoate	Leaf dip	305	
	His-hu 2001	Field	Fipronil	Leaf dip	65	
	His-hu 2001	Field	Chlorfeapyr	Leaf dip	9	
	His-hu 2001	Field	Spinosad	Leaf dip	65	
	His-hu 2001	Field	Azadirachtin	Leaf dip	>1000	
	Lu-chu 2001	Field	Abamectin	Leaf dip	4988	Kao and Cheng (2001)
	Lu-chu 2001	Field	Emamectin benzoate	Leaf dip	153	
	Lu-chu 2001	Field	Fipronil	Leaf dip	104	
	Lu-chu 2001	Field	Chlorfeapyr	Leaf dip	10	
	Lu-chu 2001	Field	Spinosad	Leaf dip	59	

Continued

Table 9 Continued

Species	*Strain*	*Select*	*Compound*	*Method*	*Toxicity ratio*	*Reference*
	Lu-chu 2001	Field	Azadirachtin	Leaf dip	>1000	
Blattella germanica	Apyr-R	Field	Permethrin	Topical	97	Wei *et al.* (2001)
	Apyr-R	Field	Deltamethrin	Topical	480	
	Apyr-R	Field	Spinosad	Topical	1.3	
Musca domestica	AVER	Field	Abamectin	Topical	>1000	Scott (1998)
	AVER	Field	Spinosad	Topical	1.9	
	LPR (Multi-R)	Field–Lab.	Spinosad	Topical	4.3	
	OCR (cyclodiene)		Cyclodiene	Topical	high	
	OCR (cyclodiene)		Spinosad	Topical	0.4	
	Cornell-R	Field-Lab.	Spinosad	Topical	0.9	
	R12 (CYP6D1)	Laboratory	Spinosad	Topical	1.8	
	R3 (kdr and pen)	Laboratory	Spinosad	Topical	1.4	
Musca domestica	ALHF (multi R)	Field	Permethrin	Topical	1800	Liu and Yue (2000)
	ALHF (multi R)	Field	Cypermethrin	Topical	4200	
	ALHF (multi R)	Field	Spinosad	Topical	1.4	
Musca domestica	Several	Field	Permethrin	Glass	high	Scott *et al.* (2000)
	Several	Field	Cyfluthrin	Glass	high	
	Several	Field	Spinosad	Feeding	low	
	Several	Field	Fipronil	Glass	very low	
	Several	Field	Dimethoate	Glass	low	
	Several	Field	Methomyl	Feeding	low-high	
Choristoneura rosaceana	Berrien	Field	Azinphosmethyl	Diet	27	Ahmad *et al.* (2002)
	Berrien	Field	Chlorpyrifos	Diet	25	
	Berrien	Field	Spinosad	Diet	0.83	
	Berrien	Field	Cypermethrin	Diet	8	
	Berrien	Field	Methoxyfenozide	Diet	3.1	
	Berrien	Field	Indoxacarb	Diet	705	
Choristoneura rosaceana	Site 2	Field	Azinphosmethyl	Leaf disc	15.5	Smirle *et al.* (2003)
	Site 2	Field	Spinosad	Leaf disc	1.8	
Choristoneura rosaceana	Brown 97 (OP-R)	Field	Tebufenozide	Leaf dip	12.8	Waldstein and Reissig (2000)
	Brown-96 (OP-R)	Field	Spinosad	Leaf dip	2.0	

sites were also shown to exhibit significant levels of resistance to spinosad that increased with time and were associated with failures to control *P. xylostella* larvae in the field (Zhao *et al.*, 2002). As a response to these developments, a regional IRM program was implemented (Zhao *et al.*, 2002; Mau and Gusukuma-Minuto, 2004). Spinosad was voluntarily withdrawn from use where field failures had occurred and two newly registered products, emamectin benzoate and indoxacarb, were put into a rotation scheme (Mau and Gusukuma-Minuto, 2004). Following its withdrawal from use, susceptibility to spinosad increased rapidly such that for some of the locations (Maui and Hawaii) the thresholds for spinosad reintroduction were met within 6-8 months, setting the stage for spinosad's reintroduction in early 2002 (Mau and Gusukuma-Minuto, 2004). By the fall of 2002, resistance to spinosad had also fallen below the threshold in the remaining region (Oahu) such that it was reintroduced in early 2003 (Mau, personal communication). With its reintroduction, spinosad has become part of an IRM rotation scheme that also includes emamectin benzoate and indoxacarb (Mau, personal communication). Given the selection pressure that was placed on spinosad in several of the locations in Hawaii, it is not clear how long this rotation scheme will remain effective in the control of a pest that has developed resistance to virtually all insecticides previously used. Nevertheless, this experience with spinosad in Hawaii has demonstrated that IRM can indeed help extend, if not preserve, the life of an insect control agent.

6.5.7. Spinosyns and Spinosoid Structure–Activity Relationships

Exploration of the spinosyns included continued isolation and identification of new spinosyns (naturally occurring analogs of spinosyn A). This effort

ultimately led to the isolation more than 20 new spinosyns from *S. spinosa* (see Section 6.5.2.1), and an expanding number from *S. pogona* (Hahn *et al.*, 2002; Lewer *et al.*, 2003). At the same time, running in parallel with the spinosyn isolation program, was a long-standing effort aimed at the preparation of semisynthetic derivatives/analogs of the spinosyns, termed spinosoids. In light of the excellent activity against lepidopterans and the desire for greater utility, the spinosoid synthesis program had two goals. The first was to increase activity against lepidopterans, typically using *H. virescens* larvae as an indicator species. The second goal was, if possible, to expand the spectrum of this unique chemistry. To date, the total number of spinosyns and spinosoids resulting from this long-standing and continuing effort easily exceeds 1000 molecules, the vast majority being spinosoids.

Chemistry around the spinosyns has been focused on modifications to various functionalities in the spinosyn structure, which has been both limited by and facilitated by the novel chemical nature of these molecules. These modifications can loosely be grouped into:

- modifications of the tetracycle,
- modification/replacement of the forosamine sugar, and
- modification/replacement of the tri-*O*-methyl rhamnose sugar.

Due to limited quantities of starting material, initial modifications to the spinosyn structure were limited to relatively simple or straightforward modifications, most often associated with a reduced level of activity. Later modifications did ultimately succeed in devising spinosoids that were more active than the naturally occurring spinosyns (Sparks *et al.*, 2000a, 2001; Crouse *et al.*, 2001).

6.5.7.1. Modifications of the Tetracycle

The tetracycle of spinosad is a rather rigid structure, the shape of which is influenced, in part, by the conjugated 13,14 double bond. Hydration of the 13,14 double bond (compounds 71 and 72) alters the three-dimensional shape of the tetracycle, which is associated with a reduction in activity (Crouse and Sparks, 1998; Crouse *et al.*, 1999) (**Table 3**). The 13,14-α-dihydro (compound 71) results in less of a conformational change and hence less of a distortion than the corresponding 13,14-β-dihydro (compound 72), which is reflected in the better activity of the α-analog (**Table 3**). Not surprisingly, reduction of both the 5,6 and 13,14 double bonds (compounds 69 and 70) reduces activity against *H. virescens* larvae (Crouse *et al.*, 1999) and is neutral to negative for stable flies (Kirst *et al.*, 2002a) and mites (**Table 3**). In contrast, a reduction of only the 5,6 double bond (compound 70) has little effect on the three-dimensional shape or on activity towards *H. virescens* larvae, but reduces activity to stable flies, and is associated with an increase in activity against aphids and mites (Crouse *et al.*, 2001) (**Table 3**). Likewise, the 5,6-β-epoxy analog of spinosyn A is about as active against *H. virescens* larvae as spinosyn A, while the 5,6-α-epoxy analog (compound 74) is much less active (**Table 3**). Both 5,6-epoxides are slightly less effective against stable fly adults compared to spinosyn A, while the 5,6-β-epoxy analog is, just like the 5,6-dihydro derivative (compound 74), more active against mites (**Table 3**). Thus, for larvae of *H. virescens* and stable fly adults, alteration of the 5,6 double bond provides no improvement in activity, while some interesting increases in activity are noted for *T. urticae* (**Table 3**). In general, for *H. virescens* larvae, any modification to the internal structure of the tetracycle reduces activity (Crouse *et al.*, 1999), with the exception of the addition of extra unsaturation at the 7–11-position of spinosyn D (compound 73), which is about as active as spinosyn A (**Table 3**). Many other synthetic modifications have been made to the tetracycle (Crouse and Sparks, 1998; Crouse *et al.*, 1999) but none has provided a significant overall improvement in biological activity.

Where chemical synthesis has been unable to thus far succeed, mother nature, and the genetic manipulation thereof, have been able, in part, to fill the gap. Extension of the alkyl group at C21 to *n*-propyl (compound 30), produced through alteration of polyketidesyntase (PKS) modules (Burns *et al.*, 2003), provides a slight improvement in activity over spinosyn A against *H. virescens* larvae and aphids (**Table 3**). Further extension of the C21 position to 21-butenyl (compound 31), the primary factor produced by *S. pogona* (Lewer *et al.*, 2003), provides activity against *H. virescens* equivalent to spinosyn A. However, for *Aphis gossypii*, activity is further improved over C21-*n*-propyl (compound 30) (**Table 3**). Unfortunately, as with the other modifications that have improved intrinsic aphid and mite activity, the physical properties of the spinosyns thus far discovered generally preclude the effective plant mobility and residuality necessary for effective control of aphids or mites in the field (Crouse *et al.*, 2001).

6.5.7.2. Modification or Replacement of the Forosamine Sugar

As described above (see Section 6.5.7.1) for the spinosyns, removal of a methyl group from the forosamine nitrogen tends to be a fairly neutral

modification, resulting in a slight decrease (spinosyns A to B, spinosyns H to R, spinosyn L to N) or a modest increase in activity (spinosyn J to M). An increase in alkyl size for one of the positions on the forosamine nitrogen (i.e., ethyl and *n*-propyl; compounds 64 and 65) has little effect on *H. virescens* activity, while further increases in bulk greatly reduce activity (compounds 66 and 67). In contrast, *T. urticae* activity is only increased by these same modifications as is, to a lesser degree, activity against *A. gossypii* (Table 3). Replacement of the forosamine *N,N*-dimethylamine moiety with a hydroxyl (compound 68), reduces activity against *H. virescens* larvae while, apparently (response can be variable), increasing aphid activity (Table 3). Loss of the entire forosamine moiety results in major loss of activity (Sparks *et al.,* 1999) (Table 2) with only a small improvement noted by replacement with an acetate (C17-acetate, compound 79) group. Substitution of the forosamine with *N,N*-dimethylaminoacyl esters (compounds 80–82) only partially restores activity (Kirst *et al.,* 2002b) (Table 3). *N*-methylamino piperazinyl-acetate (compound 83) or *N,N*-dimethylamino-piperidinylacetate esters (compound 84) at C17 go further towards restoring activity against *H. virescens* and *Stomoxys calcitrans*, but still fall far short of the activity observed with forosamine (spinosyn, compound 1) (Kirst *et al.,* 2002b) (Table 3). A similar trend is observed with *S. calcitrans* (stable fly). Thus, for the lepidopterans, the above modifications to the forosamine do not lead to improvements in activity. In contrast, both aphid and mite activity were improved by replacement of the forosamine *N*-methyl groups with larger alkyl groups (compounds 64–67).

6.5.7.3. Modification or Replacement of the Tri-*O*-Methyl-Rhamnose Sugar

While many of the early synthetic modifications to the spinosyn structure were insecticidal, all of the early spinosoids were typified by a somewhat reduced or most often an almost total lack of *H. virescens* activity compared to spinosyn A. However, an exception was found in the form of the desmethoxy (2′-H or 3′-H) analogs of spinosyns H/Q and J, respectively (compounds 35, 36 and 43) (Table 3). These “desmethoxy” analogs were found to be as active against *H. virescens* larvae or slightly more so, than spinosyn A (Creemer *et al.,* 2000; Kirst *et al.,* 2002b) (Table 2). These modifications constituted the first clear indication that spinosoids at least matching the activity of the most active of the natural spinosyns (i.e., spinosyn A) were possible.

Concurrent with the synthetic efforts, several computer-aided modeling and design (CAMD) approaches were examined in an attempt to identify potential synthetic directions for improved analogs. Included among the computer modeling approaches initially used were comparative molecular field analysis (CoMFA) and Hansch-style quantitative structure–activity relationships (QSAR) (Crouse and Sparks, 1998). However, the large molecular size and complex structure rendered the available computing power insufficient to the task for these more conventional methodologies and generally made QSAR difficult. However, where conventional CAMD techniques were not successful, the application of artificial neural networks to spinosyn QSAR (Sparks *et al.,* 2000a) identified new directions that led to, as one example, new modified-rhamnose spinosoids. These latter compounds were in many cases far more active than spinosyn A against pest lepidopterans (Crouse and Sparks, 1998; Sparks *et al.,* 2000a, 2001; Anzeveno and Green, 2002).

As shown by the activity of the 2′, 3′, 4′-demethyl analogs from the mutant strains, *O*-demethylation generally leads to less active compounds compared to spinosyn A (Table 2). Loss of the rhamnose also leads to a large reduction in activity (Table 2). The first spinosoids possessing any kind of improvement in lepidopteran activity over spinosyn A were (as noted above) the des-methoxy (2′-H (compounds 35 and 36), or 3′-H (compound 43) analogs of spinosyns H/Q and J, respectively (Table 3). For the 2′-substituted spinosoids, the 2′-des-methoxy analog (compound 35) along with the 2′-*O*-ethyl (compound 37) and 2′-*O*-*n*-propyl (compound 39) represent the most active of the 2′-substitution-based analogs, which are as active or very slightly more so than spinosyn A. Likewise, modifications of the 4′-position of the rhamnose (e.g., compound 60) results in no real activity improvement over spinosyn A, with the exception of the 4′-des-methyoxy analog (compound 59), which generally remains equivalent or nearly so, to spinosyn K (4′-O-demethyl, compound 23) (Table 3). Thus, in general, modifications to the 2′- or 4′-positions do little to improve activity compared to spinosyn A.

Unlike the 2′- and 4′-positions (e.g., compounds 37 and 60), a genuine improvement in *H. virescens* activity was found to be associated with an increased size of the alkoxy groups on the rhamnose at the 3′-position (compounds 46–52) (Sparks *et al.,* 2000a). At the 3′-position, the activity optimum is between two to three carbons. Further increases in bulk or length resulted in a loss of activity (Sparks *et al.,* 2000a, 2001; Crouse *et al.,* 2001). The introduction of unsaturation into these short alkyl chains (e.g., compounds 47, 53, 54) was generally neutral in effect, while branching was detrimental (compounds

49 and 50) (Table 3). The 3′-O-vinyl analog (compound 47) could represent a very active configuration, but stability issues resulted in erratic results (Crouse *et al.*, 2001). Other modifications to the alkyl chains (halogenation, introduction of another oxygen, etc.) were generally not as effective as the simple alkyl chains (compounds 55 and 57) (Crouse *et al.*, 2001) (Table 3). While the optimum in 3′-O-alkyl chain length for lepidopterous larvae such as *H. virescens* (Crouse *et al.*, 2001), beet armyworm (*Spodoptera exigua*) and cabbage looper (*Trichoplusia ni*) (data not shown) is between two and three carbons (e.g., compounds 46, 48, 53 and 54), the optimum for aphids such as *A. gossypii* extends to four carbons (compound 51) (Crouse *et al.*, 2001) (Table 3). Unlike either aphids or lepidopterans, the longer chain 3′-O-alkyl groups (C2–C4) (compounds 46, 48, and 51, respectively) were essentially equivalent in activity for *T. urticae* (Table 3) and were only modestly more active than spinosyn A.

Interestingly, the 2′,3′,4′-tri-O-ethyl analog (compound 62) is essentially as potent against *H. virescens* larvae, *A. gossypii*, and *T. urticae* as the 3′-O-ethyl derivative (compound 46) (Table 3), further supporting the importance of the 3′-position of the rhamnose to insecticidal activity.

In addition to modifications to the tri-O-methylrhamnose moiety, the effects of rhamnose replacement with other sugar and nonsugar moieties were also investigated (Anzeveno and Green, 2002). These studies demonstrated that very little change around the rhamnose moiety is tolerated. Replacement sugars such as D-rhamnose, the β-anomeric rhamnose, furanose, and forosaminyl analogs (not shown) were much less active or inactive against *H. virescens* larvae and *T. urticae* (Anzeveno and Green, 2002). In contrast, removal of the methyl group at the 6′-position (L-lyxose analog, compound 85) had little effect on activity relative to spinosyn A, while addition of hydroxy to the 6′-methyl group (L-mannose, compound 86) provided a very significant improvement in activity over spinosyn A (Anzeveno and Green, 2002), nearly equaling that of the 3′-O-ethyl analog (compound 46). Replacement of the tri-O-methylrhamnose with nonsugar moieties such as simple alkyl groups or methoxy-substituted benzoyl groups (compounds not shown) all were found to be inactive (Anzeveno and Green, 2002).

6.5.7.4. Quantitative Structure–Activity Relationships and the Spinosyns

The above examples (see Sections 6.5.7.1–6.5.7.3) clearly demonstrate that very significant improvements in activity of the spinosyns are possible, both in terms of potency towards a particular pest or group of insect pests and greater spectrum. These improvements, especially those involving rhamnose ring modifications, were facilitated through the application of both classical and less conventional CAMD techniques early in the project. The QSAR analysis of the spinosyns conducted using artificial neural networks directly led to the identification and synthesis of far more potent spinosoids than had been seen up to that time (Sparks *et al.*, 2000a, 2001; Anzeveno and Green, 2002). The analysis identified several potential modifications to improve activity, including the lengthened alkyl groups on the rhamnose, and also pinpointed the 3′-position as the most important for activity (Sparks *et al.*, 2000a, 2001). While the neural net-based QSAR was able to identify useful targets for synthesis, understanding the physicochemical basis for the improved activity is not a question easily addressed with neural nets (Sparks *et al.*, 2001). Thus, more classical approaches have also been employed. Hansch-style multiple regression analysis (Kubinyi, 1993) of the spinosyns and spinosoids was able to establish some relationships between insecticidal activity against *H. virescens* larvae and several physicochemical parameters.

Among the spinosyns, the biological response of *H. virescens* larvae was best described by the three whole-molecule parameters, CLogP, Mopac dipole (dipole moment of the whole spinosyn molecule), and HOMO (highest occupied molecular orbital) (Sparks *et al.*, 2000b):

$$\begin{aligned}\text{Log } Hv\, \text{LC}_{50} &= -2.18\ \text{CLogP} + 0.61\ \text{Mopac dipole} \\ &\quad + 2.89\ \text{HOMO} + 33.74 \\ r^2 &= 0.824, \quad s = 0.372, \quad F = < 0.0001, \\ q^2 &= 0.724, \quad n = 18 \end{aligned} \qquad [2]$$

For a larger set of spinosyns and spinosoids, these same parameters (eqn [3]) were also able to explain much of the observed insecticidal activity towards *H. virescens* larvae (Sparks *et al.*, 2001). The addition of the squared term for CLogP (eqn [3]) also reflects the presence of an optimum of between two and three carbons at the rhamnose 3′-position, that is observed for *H. virescens* larval activity (Sparks *et al.*, 2001) (Table 3):

$$\begin{aligned}\text{Log } Hv\, \text{LC}_{50} &= -6.62\ \text{CLogP} + 0.67\ \text{CLogP}^2 \\ &\quad + 0.59\ \text{Mopac dipole} \\ &\quad + 3.09\ \text{HOMO} + 43.05 \\ r^2 &= 0.816, \quad s = 0.475, \quad F = < 0.0001, \\ q^2 &= 0.706, \quad n = 34 \end{aligned} \qquad [3]$$

For the spinosyns and spinosoids, HOMO appears to be directly influenced by substitution on the forosamine nitrogen (Sparks *et al.*, 2000b) suggesting that the primary drivers for activity are CLogP and Mopac dipole moment. In turn, Mopac dipole moment appears rather well correlated with the substitution pattern on the rhamnose moiety, and secondarily by substitution on the forosamine nitrogen (Sparks *et al.*, 2001). As implied by the above equations, the most active spinosyns and spinosoids tend to have smaller values for Mopac dipole moment and larger values (up to a point) for CLogP (Sparks *et al.*, 2001). Thus, these relationships provide a useful basis for understanding spinosyn and spinosoid activity not only against lepidopteran insect pests but also for other pest insects, including aphids and leafhoppers (Dintenfass *et al.*, 2001). CAMD and QSAR continue to play an important role in the investigation and exploitation of the spinosyn family of chemistry.

6.5.8. Conclusion

The natural spinosyns produced by select members of the genus *Saccharopolyspora*, and their semisynthetic derivatives, known as spinosoids, constitute a new and unique class of insect control agents. The spinosyns possess a novel mode of action, a very favorable toxicological profile coupled with high insecticidal efficacy against a broad range of pest insects, with commercial use focused on the Lepidoptera and, to a lesser degree, the Diptera. The commercial fermentation-derived product, spinosad, is a naturally occurring mixture of spinosyns A and D, which also happen to be the most insecticidally active of the natural spinosyns produced by *S. spinosa*. While possessing good contact activity, the spinosyns are most often more active orally, in part due to the slow cuticular penetration of the spinosyns compared to many insect control agents. Although spinosyns A and D are readily metabolized in vertebrate and avian systems, metabolism in insects is limited, compensating in part for slow penetration into insects. The spinosyns cause excitatory symptoms in insects by stimulating activity in the CNS. Activation of nondesensitizing nicotinic acetylcholine receptors by an allosteric mechanism appears to be the primary mechanism by which spinosyns stimulate neuronal activity, but antagonism of an as-yet-unidentified subtype of GABA receptor may also play a significant role in spinosyn poisoning. Through an extensive program of strain selection, genetic manipulation of the biosynthetic pathways and chemical synthesis, the effects of many modifications to the forosamine and rhamnose moieties as well as to the core tetracycle have been investigated. This experience, with guidance from classical modeling approaches as well as the application of QSAR methodologies utilizing artificial intelligence, has led to the synthesis of spinosoids with biological activity far exceeding that of spinosyn A. Because of its unique mode of action and limited susceptibility to metabolism in insects, pest insects resistant to other insecticides would typically not show cross-resistance to spinosad. However, as expected for any insect control agent put under heavy selection pressure, resistance to spinosad has been developed in the laboratory and has also recently appeared in the field. Available information suggests that resistance to spinosad is most likely target-site based, reinforcing the importance of the IRM recommendations developed by the manufacturer at the time of its launch that spinosad be used in rotation with products with other modes of action to ensure the long-term utility of this novel chemistry.

Acknowledgments

The authors would like to thank their many colleagues from Eli Lilly and Co., and Dow AgroSciences (DAS) who have contributed to our knowledge of the spinosyns. Special thanks are due to Mr. Jerry Watson (DAS), Dr Nailah Orr (DAS), Dr Gary Thompson (DAS), Dr Mark Hertlein (DAS), Dr Gary Crouse (DAS), Dr Paul Lewer (DAS), and Dr Ron Mau (University of Hawaii) for their many helpful discussions during the writing of this chapter.

References

Addor, R.W., **1995**. Insecticides. In: Godfrey, C.R.A. (Ed.), Agrochemicals from Natural Products. Dekker, New York, pp. 1–62.

Ahmad, M., Hollingworth, R.M., Wise, J.C., **2002**. Broad-spectrum insecticide resistance in obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) from Michigan. *Pest Mgt Sci. 58*, 834–838.

Anzeveno, P.B., Green, F.R., III, **2002**. Rhamnose replacement analogs of spinosyn A. In: Baker, D.R., Fenyes, J.G., Lahm, G.P., Selby, T.P., Stevenson, T.M. (Eds.), Synthesis and Chemistry of Agrochemicals, vol. VI. American Chemical Society, Washington, DC, pp. 262–276.

Anonymous, **2001**. Spinosad Technical Bulletin. Dow AgroSciences, Indianapolis, IN.

Andersch, W., Schnorbach, H.-J., Wollweber, D., **2003**. Synergistic insecticidal mixtures, Bayer AG. US Patent No. 6,686,387.

Bailey, W.D., Young, H.P., Roe, R.M., **1999**. Laboratory selection of a Tracer® resistant strain of tobacco

budworm and comparisons with field selected strains from the southeasetern US. Proc. 1999 Beltwide Cotton Production Conf., pp. 1221–1224.

Brett, B.L., Larson, L.L., Schoonover, J.R., Sparks, T.C., Thompson, G.D., **1997**. Biological properties of spinosad. *Down to Earth 52*, 6–13.

Buckingham, S.D., Lapied, B., Le Corronc, H., Satelle, D.B., **1997**. Imidacloprid actions on insect neuronal acetylcholine receptors. *J. Exp. Biol. 200*, 2685–2692.

Burns, L.S., Graupner, P.R., Lewer, P., Martin, C.J., Vousden, W.A., *et al.*, **2003**. Spinosyn Polyketide Synthase Fusion Products Synthesizing Novel Spinosyns and their Preparation and Use. Dow AgroSciences LLC, Indianapolis, IN.

Chen, T.S., Hensens, O.D., Schulman, M.D., **1989**. Biosynthesis. In: Campbell, W.C. (Ed.), Ivermectin and Abamectin. Springer, New York, pp. 55–72.

Clark, J.M., Yamaguchi, I., **2002**. Scope and status of pesticide resistance. In: Clark, J.M., Yamaguchi, I. (Eds.), Agrochemical Resistance: Extent, Mechanism and Detection. American Chemical Society, Washington, DC, pp. 1–22.

Creemer, L.C., Kirst, H.A., Paschal, J.W., Worden, T.V., **2000**. Synthesis and insecticidal activity of spinosyn analogs functionally altered at the 2′-, 3′- and 4′-positions of the rhamnose moiety. *J. Antibiotics 53*, 171–178.

Crouse, G.D., Sparks, T.C., **1998**. Naturally derived materials as products and leads for insect control: the spinosyns. *Rev. Toxicol. 2*, 133–146.

Crouse, G.D., Sparks, T.C., DeAmicis, C.V., Kirst, H.A., Martynow, J.G., *et al.*, **1999**. Chemistry and insecticidal activity of the spinosyns. In: Brooks, G.T., Roberts, T.R. (Eds.), Pesticide Chemistry and Bioscience: The Food–Environment Challenge. Royal Society of Chemistry, Cambridge, pp. 155–166.

Crouse, G.D., Sparks, T.C., Schoonover, J., Gifford, J., Dripps, J., *et al.*, **2001**. Recent advances in the chemistry of spinosyns. *Pest Mgt Sci. 57*, 177–185.

Crouse, G.D., Hahn, D.R., Graupner, P.R., Gilbert, J.R., Lewer, P., *et al.*, **2002**. Synthetic Macrolides of 21-Butenyl and Related Spinosyn Glycosides as Insecticides and Acaricides. PCT Int. Appl., Indianapolis, IN.

DeAmicis, C.V., Dripps, J.E., Hatton, C.J., Karr, L.L., **1997**. Physical and biological properties of the spinosyns: novel macrolide pest-control agents from fermentation. In: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J., Thompson, D.G. (Eds.), Phytochemicals for Pest Control. American Chemical Society, Washington, DC, pp. 144–154.

Dintenfass, L., DeAmicis, C.V., Dripps, J.D., Crouse, G.D., Kempe, M., *et al.*, **2001**. Structure–activity relationships of spinosyns and spinosoids. In: 221st Natl Mtg. American Chemical Society, San Diego, CA, Book of Abstracts, pp. AGRO 077.

Domoradzki, J.Y, Stewart, H.S., Mendrala, A.L., Gilbert, J.R., Markham, D.A., **1996**. Comparison of the metabolism and tissue distribution of ^{14}C-labeled spinosyn A and ^{14}C-labeled spinosyn D in Fischer 344 rats. Soc. Toxicol. Mtg. Anaheim, CA.

Dubreil, V., Sinakevitch, I.G., Hue, B., Geffard, M., **1994**. Neuritic GABAergic synapses in insect neurosecretory cells. *Neurosci. Res. 19*, 235–240.

Elzen, G.W., Maldonado, S.N., Rojas, M.G., **2000**. Lethal and sublethal effects of insecticides and an insect growth regulator on the boll weevil (Coleoptera: Curculionidae) ectoparasitoid *Catolaccus grandis* (Hymenoptera: Pteromalidae). *J. Econ. Entomol. 93*, 300–303.

Gaisser, S., Martin, C.J., Wilkinson, B., Sheridan, R.M., Lill, R.E., *et al.*, **2002**. Engineered biosynthesis of novel spinosyns bearing altered deoxyhexose substituents. *Chem. Commun. 2002*, 618–619.

Grolleau, F., Lapied, B., **2000**. Dorsal unpaired median neurons in the insect central nervous system: towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity. *J. Exp. Biol. 203*, 1633–1648.

Hahn, D.R., Balcer, J.L., Lewer, P., Gilbert, J.R., Graupner, P.R., **2002**. Pesticidal Spinosyn Derivatives Produced by Culturing Mutant *Saccharopolyspora* sp. Strains. PCT Int. Appl., Indianapolis, IN.

Hill, T.A., Foster, R.E., **2003**. Influence of selected insecticides on the population dynamics of diamondback moth (Lepidoptera: Plutellidae) and its parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae), in cabbage. *J. Entomol. Sci. 38*, 59–71.

Hollingworth, R.M., **1976**. The biochemical and physiological basis of selective toxicity. In: Wilkinson, C.F. (Ed.), Insecticide Biochemistry and Physiology. Plenum, New York, pp. 431–506.

Hollingworth, R.M., **2001**. New insecticides: mode of action and selective toxicity. In: Baker, D.R., Umetsu, N.K. (Eds.), Agrochemical Discovery: Insect, Weed and Fungal Control. American Chemical Society, Washington, DC, pp. 238–255.

Kao, C-.H., Cheng, E.Y., **2001**. Insecticide resistance in *Plutella xylostella* L. 11. Resistance to new introduced insecticides in Taiwan (1990–2001). *J. Agric. Res. China 50*, 80–89.

Kirst, H.A., Michel, K.H., Mynderse, J.S., Chio, E.H., Yao, R.C., *et al.*, **1992**. Discovery, isolation, and structure elucidation of a family of structurally unique fermentation-derived tetracyclic macrolides. In: Baker, D.R., Fenyes, J.G., Steffens, J.J. (Eds.), Synthesis and Chemistry of Agrochemicals, vol. 3. American Chemical Society, Washington, DC, pp. 214–225.

Kirst, H.A., Creemer, L.C., Naylor, S.A., Pugh, P.T., Snyder, D.E., *et al.*, **2002a**. Evaluation and development of spinosyns to control ectoparasites on cattle and sheep. *Curr. Topics Medic. Chem. 2*, 675–699.

Kirst, H.A., Creemer, L.C., Broughton, M.C., Huber, M.B.L., Turner, J.R., **2002b**. Chemical and microbiological modifications of spinosyns: exploring synergies between fermentation microbiology and organic chemistry. In: Baker, D.R., Fenyes, J.G., Lahm, G.P., Selby, T.P., Stevenson, T.M. (Eds.), Synthesis and Chemistry of Agrochemicals, vol. 6. American Chemical Society, Washington, DC, pp. 251–261.

Kubinyi, H., **1993**. QSAR: Hansch Analysis and Related Approaches. VCH Publishers, New York.

Larson, L.L., Kenaga, E.E., Morgan, R.W., **1985**. Commercial and Experimental Organic Insecticides. Entomological Society of America, College Park, MD, pp. 1–105.

Le Corronc, H., Alix, P., Hue, B., **2002**. Differential sensitivity of two insect GABA-gated chloride channels to dieldrin, fipronil and picrotoxinin. *J. Insect Physiol. 48*, 419–431.

Lena, C., Changeux, J.-P., **1993**. Allosteric modulations of the nicotinic acetylcholine receptor. *Trends Neurosci. 16*, 181–186.

Lewer, P., Hahn, D.R., Karr, L.L., Graupner, P.R., Gilbert, J.R., *et al.*, **2001**. Pesticidal Macrolides. PCT Int. Appl., Indianapolis, IN.

Lewer, P., Hahn, D.R., Karr, L.L., Graupner, P.R., Gilbert, J.R., *et al.*, **2003**. A new family of insecticidal spinosyns from a novel *Saccharopolyspora* strain. In: Proc. 44th Natl Mtg. Am. Soc. Pharmacognosy, Book of Abstracts, pp. 0:37.

Liu, N., Yue, X., **2000**. Insecticide resistance and cross-resistance in the house fly (Diptera: Muscidae). *J. Econ. Entomol. 93*, 1269–1275.

Ludmerer, S.W., Warren, V.A., Williams, B.S., Zheng, Y., Hunt, D.C., *et al.*, **2002**. Ivermectin and nodulisporic acid receptors in *Drosophila melanogaster* contain both γ-aminobutyric acid-gated *Rdl* and glutamate-gated GluCl α chloride channel subunits. *Biochemistry 41*, 6548–6560.

Magnussen, J.D., Castetter, S.A., Rainey, D.P., **1996**. Characterization of spinosad related residues in poultry tissues and eggs following oral administration. In: 211th Natl Mtg. American Chemical Society, New Orlean, LA, pp. AGRO 043.

Madduri, K., Waldron, C., Merlo, D.J., **2001**. Rhamnose biosynthesis pathway supplies precursors for primary and secondary metabolism in *Saccharopolyspora spinosa*. *J. Bacteriol. 183*, 5632–5638.

Martin, C., **2003**. Genetic engineering of *Saccharopolyspora spinosa* to generate a library of spinosyn analogues. In: 13th Int. Soc. Biol. Actinomycetes, Book of Abstracts.

Mau, R.F.L., Gusukuma-Minuto, L., **2004**. Diamondback moth, *Plutella xylostella* (L.), resistance management in Hawaii. In: Endersby, N.M., Ridland, P.M. (Eds.), The Management of Diamondback Moth and Other Crucifer Pests: Proc. 4th Intl workshop, Melbourne, Australia, 26–29 November 2001, pp. 307–311.

Mertz, F.P., Yao, R.C., **1990**. *Saccharopolyspora spinosa* sp. nor. isolated from soil collected in a sugar rum still. *Int. J. Syst. Bacteriol. 40*, 34–39.

Moultron, J.K., Pepper, D.A., Dennehy, T.J., **1999**. Studies of resistance of beet armyworm (*Spodoptera exigua*) to spinosad in field populations from the southern USA and southeast Asia. In: Proc. 1999 Beltwide Cotton Production Conf., pp. 884–889.

Moulton, J.K., Pepper, D.A., Dennehy, T.J., **2000**. Beet armyworm (*Spodoptera exigua*) resistance to spinosad. *Pest Mgt Sci. 56*, 842–848.

Nauen, R., Ebbinghaus-Kintscher, U., Elbert, A., Jeschke, P., Tietjen, K., **2001**. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In: Ishaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, Berlin, pp. 288–321.

Papa, G., de Almeida, F.J., Rotundo, M., **2002**. Effect of insecticides and methods of sampling on the population of beneficial arthropods, in cotton in Brazil. In: Proc. 2003 Beltwide Cotton Conf., pp. 1197–1201.

Pitman, R.M., Kerkut, G.A., **1970**. Comparison of the actions of iontophoretically applied acetylcholine and gamma aminobutyric acid with the EPSP and IPSP in cockroach central neurons. *Comp. Gen. Pharmacol. 1*, 221–230.

Rainey, D.P., O'Neill, J.D., Castetter, S.A., **1996**. The tissue distribution and metabolism of spinosyn A and D in lactating goats. In: 211th Natl Mtg. American Chemical Society, New Orlean, LA, pp. AGRO 045.

Raymond, V., Sattelle, D.B., Lapied, B., **2000**. Coexistence in DUM neurones of two GluCl channels that differ in their picrotoxin sensitivity. *Neuroreport 11*, 2695–2701.

Salgado, V.L., Watson, G.B., Sheets, S.S., **1997**. Studies on the mode of action of spinosad, the active ingredient in Tracer® insect control. Proc. 1996 Beltwide Cotton Production Conf., pp. 1082–1086.

Salgado, V.L., **1998**. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pestic. Biochem. Physiol. 60*, 91–102.

Salgado, V.L., Sheets, J.J., Watson, G.B., Schmidt, A.L., **1998**. Studies on the mode of action of spinosad: the internal effective concentration and the concentration dependence of neural excitation. *Pestic. Biochem. Physiol. 60*, 103–110.

Salgado, V.L., Saar, R., **2004**. Desensitizing and non-desensitizing subtypes of α-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. *J. Insect Physiol.* (in press).

Saunders, D.G., Bret, B.L., **1997**. Fate of spinosad in the environment. *Down to Earth 52*, 14–20.

Schroeder, M.E., Flattum, R.F., **1984**. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pestic. Biochem. Physiol. 22*, 148–160.

Scott, J.G., **1998**. Toxicity of spinosad to susceptible and resistant strains of house flies, *Musca domestica*. *Pestic. Sci. 54*, 131–133.

Scott, J.G., Alefantis, T.G., Kaufman, P.E., Rutz, D.A., **2000**. Insecticide resistance in house flies from caged-layer poultry facilities. *Pest Mgt Sci. 56*, 147–153.

Shelton, A.M., Sances, F.V., Hawley, J., Tang, J.D., Boune, M., *et al.*, **2000**. Assessment of insecticide resistance after the outbreak of diamondback moth (Lepidoptera: Plutellidae) in California in 1997. *J. Econ. Entomol. 93*, 931–936.

Shomi, K., Omura, S., **2002**. Discovery of new macrolides. In: Omura, S. (Ed.), Macrolide Antibiotics: Chemistry, Biology and Practice. Academic Press, New York, pp. 1–56.

Shono, T., Scott, J.G., **2003**. Spinosad resistance in the housefly, *Musca domestica*, is due to a recessive factor on autosome 1. *Pestic. Biochem. Physiol. 75*, 1–7.

Smirle, M.J., Lowery, D.T., Zurowski, C.L., **2003**. Susceptibility of leafrollers (Lepidoptera: Tortricidae) from organic and conventional orchards to azinphosmethyl, spinosad and *Bacillus thuringiensis*. *J. Econ. Entomol. 96*, 879–884.

Smith, P. (Ed.) **2001**. AgroProjects: PestProjects – 2001. PJB Publications, Richmond, UK.

Snodgrass, R.E., **1952**. A Textbook of Arthropod Anatomy. Comstock, Ithaca, NY.

Sparks, T.C., **1996**. Toxicology of insecticides and acaricides. In: King, E.G., Phillips, J.R., Coleman, R.J. (Eds.), Cotton Insects and Mites: Characterization and Management. The Cotton Foundation, Memphis, TN, pp. 283–322.

Sparks, T.C., **2004**. New insect control agents: modes of action and selectivity. In: Endersby, N.M., Ridland, P.M. (Eds.), The Management of Diamond back Moth and Other Crucifers Pests: Proc. 4th Intl. Workshop, Melbourne, Australia, 26–29 November 2001, pp. 37–44.

Sparks, T.C., Graves, J.B., Leonard, B.R., **1993**. Insecticide resistance and the tobacco budworm: past, present and future. *Rev. Pestic. Toxicol. 2*, 149–183.

Sparks, T.C., Thompson, G.D., Larson, L.L., Kirst, H.A., Jantz, O.K., *et al.*, **1995**. Biological characteristics of the spinosyns: a new class of naturally derived insect control agents. In: Proc. 1995 Beltwide Cotton Production Conf., pp. 903–907.

Sparks, T.C., Kirst, H.A., Mynderse, J.S., Thompson, G.D., Turner, J.R., *et al.*, **1996**. Chemistry and biology of the spinosyns: components of spinosad (Tracer®), the first entry into DowElanco's naturalyte class of insect control products. In: Proc. 1996 Beltwide Cotton Production Conf., pp. 692–696.

Sparks, T.C., Sheets, J.J., Skomp, J.R., Worden, T.V., Hertlein, M.B., *et al.*, **1997**. Penetration and metabolism of spinosyn A in lepidopterous larvae. In: Proc. 1997 Beltwide Cotton Conf., pp. 1259–1265.

Sparks, T.C., Thompson, G.D., Kirst, H.A., Hertlein, M.B., Larson, L.L., *et al.*, **1998**. Biological activity of the spinosyns, new fermentation derived insect control agents, on tobacco budworm (Lepidoptera: Noctuidae) larvae. *J. Econ. Entomol. 91*, 1277–1283.

Sparks, T.C., Thompson, G.D., Kirst, H.A., Hertlein, M.B., Mynderse, J.S., *et al.*, **1999**. Fermentation-derived insect control agents: the spinosyns. In: Hall, F.R., Menn, J.J. (Eds.), Biopesticides: Use and Delivery. Humana Press, Totowa, NJ, pp. 171–188.

Sparks, T.C., Anzeveno, P.B., Martynow, J.G., Gifford, J.M., Hertlein, M.B., *et al.*, **2000a**. The application of artificial neural networks to the identification of new spinosoids with improved biological activity toward larvae of *Heliothis virescens*. *Pestic. Biochem. Physiol. 67*, 187–197.

Sparks, T.C., Durst, G., Worden, T.V., **2000b**. Structure–activity relationships of the spinosyns. In: Proc. 2000 Beltwide Cotton Conf., pp. 1225–1229.

Sparks, T.C., Crouse, G.D., Durst, G., **2001**. Natural products as insecticides: the biology, biochemistry and quantitative structure–activity relationships of spinosyns and spinosoids. *Pest Mgt Sci. 57*, 896–905.

Studebaker, G.E., Kring, T.J., **2003**. Effects of insecticides on *Orius insidiosus* (Hemiptera: Anthocoridae) measured by field, greenhouse and Petri dish bioassays. *Florida Entomol. 86*, 178–185.

Thompson, G.D., Busacca, J.D., Jantz, O.K., Kirst, H.A., Larson, L.L., *et al.*, **1995**. Spinosyns: an overview of new natural insect management systems. In: Proc. 1995 Beltwide Cotton Conf., pp. 1039–1043.

Thompson, G.D., Michel, K.H., Yao, R.C., Mynderse, J.S., Mosburg, C.T., *et al.*, **1997**. The discovery of *Saccharopolyspora spinosa* and a new class of insect control products. *Down to Earth 52*, 1–5.

Thompson, G.D., Dutton, R., Sparks, T.C., **2000**. Spinosad: a case study – an example from a natural products discovery programme. *Pest Mgt Sci. 56*, 696–702.

Thompson, G.D., Sparks, T.C., **2002**. Spinosad: a green natural product for insect control. In: Lankey, R.L., Anastas, P.T. (Eds.), Advancing Sustainability through Green Chemistry and Engineering. American Chemical Society, Washington, DC, pp. 61–73.

Tillman, P.G., Mulrooney, J.E., **2000**. Effect of selected insecticides on the natural enemies *Coleomegilla maculata* and *Hippodamia convergens* (Coleoptera: Coccinellidae), *Geocoris punctipes* (Hemiptera: Lygaeidae), and *Bracon mellitor, Cardiochiles nigriceps*, and *Cotesia marginiventris* (Hymenoptera: Braconidae) in cotton. *J. Econ. Entomol. 93*, 1638–1643.

van den Beukel, I., van Kleef, R.G.D.M., Zwart, R., Oortgiesen, M., **1998**. Physostigmine and acetylcholine differentially activate nicotinic receptor subpopulatons in *Locusta migratoria* neurons. *Brain Res. 789*, 263–273.

Waldron, C., Madduri, K., Crawford, K., Merlo, D.J., Treadway, P., *et al.*, **2000**. A cluster of genes for the biosynthesis of spinosyns, novel macrolide insect control agents produced by *Saccharopolyspora spinosa*. *Antonie van Leeuwenhoek 78*, 385–390.

Waldron, C., Matsushima, P., Rosteck, P.R., Jr., Broughton, M.C., Turner, J., *et al.*, **2001**. Cloning and analysis of the spinosad biosynthetic gene cluster of *Saccharopolyspora spinosa*. *Chem. Biol. 8*, 487–499.

Waldstein, D.E., Reissig, W.H., **2000**. Synergism of tebufenozide in resistant and susceptible strains of obliquebanded leafroller (Lepidoptera: Tortricidae) and resistance to new insecticides. *J. Econ. Entomol. 93*, 1768–1772.

Ware, G.W., **1982**. Pesticides: Theory and Application. W.H. Freeman, San Francisco, CA.

Watson, G.B., **2001**. Actions of insecticidal spinosyns on γ-aminobutyric acid responses from small-diameter

cockroach neurons. *Pestic. Biochem. Physiol.* *71*, 20–28.

Wei, Y., Appel, A.G., Moar, W.J., Liu, N., **2001**. Pyrethroid resistance and cross-resistance in the German cockroach, *Blattella germainica* (L). *Pest Mgt Sci.* *57*, 1055–1059.

Williams, T., Valle, J., Viñuela, E., **2003**. Is the naturally derived insecticide spinosad compatible with insect natural enemies? *Biocont. Sci. Tech.* *13*, 459–475.

Wyss, C.F., Young, H.P., Shukla, J., Roe, R.M., **2003**. Biology and genetics of a laboratory strain of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), highly resistant to spinosad. *Crop Protect.* *22*, 307–314.

Young, H.P., Bailey, W.D., Wyss, C.F., Roe, R.M., Sheets, J.J., *et al.*, **2000**. Studies on the mechanism(s) of tobacco budworm resistance to spinosad (Tracer®). In: Proc. 2000 Beltwide Cotton Conf., pp. 1197–1201.

Young, H.P., Bailey, W.B., Roe, R.M., Iwasa, T., Sparks, T.C., *et al.*, **2001**. Mechanism of resistance and cross-resistance in a laboratory, spinosad-selected strain of the tobacco budworm and resistance in laboratory-selected cotton bollworms. In: Proc. 2001 Beltwide Cotton Conf., pp. 1167–1171.

Young, H.P., Bailey, W.D., Roe, R.M., **2003**. Spinosad selection of a laboratory strain of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), and characterization of resistance. *Crop Protect.* *22*, 265–273.

Zhao, J.-Z., Li, Y.-X., Collins, H.L., Gusukuma-Minuto, L., Mau, R.F.L., *et al.*, **2002**. Monitoring and characterization of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad. *J. Econ. Entomol.* *95*, 430–436.

Relevant Websites

http://www.extoxnet.orst.edu – Extension Toxicology Network, Oregon State University.

6.6 *Bacillus thuringiensis*: Mechanisms and Use

A Bravo and M Soberón, Instituto de Biotechnología, Cuernavaca Morelos, Mexico
S S Gill, University of California, Riverside, CA, USA

6.6.1. General Characteristics

Bacillus thuringiensis is a member of the *Bacillus cereus* group that also includes *B. cereus*, *B. anthracis*, and *B. mycoides* (Helgason *et al.*, 2000). The feature that distinguishes *B. thuringiensis* from the other members of the *B. cereus* group is its entomopathogenic properties. This bacterial species produces insecticidal proteins (δ-endotoxins) during sporulation phase as parasporal inclusions, which predominantly comprise one or more proteins, called Cry and Cyt toxins. These protein toxins are highly selective to their target insect, are innocuous to humans, vertebrates, and plants, and are completely biodegradable. Therefore, *B. thuringiensis* is a viable alternative for the control of insect pests in agriculture and disease vectors of importance in human public health.

Numerous *B. thuringiensis* strains have been isolated that show activity towards lepidopteran, dipteran, or coleopteran insects (Schnepf *et al.*, 1998). In recent years *B. thuringiensis* strains active against Hymenoptera, Homoptera, Orthoptera, and Mallophaga insect orders and to other noninsect organisms like nematodes, mites, and protozoa have been isolated (Crickmore *et al.*, 1998; Wei *et al.*, 2003). The entomopathogenic activity of *B. thuringiensis* is mainly due to Cry toxins. One feature that distinguishes these Cry proteins is their high selectivity for their target insect. More than 200 different *cry* genes have been isolated, and this constitutes an important arsenal for the control of a wide variety of insect pests. In this chapter we will summarize the present knowledge of the pathogenic properties of *B. thuringiensis*,

the mode of action of Cry toxins, and their application in agriculture and disease-vector control.

6.6.2. Virulence Factors and the PlcR Regulon

The *B. cereus* group is characterized by its pathogenic capabilities to a diverse group of organism, such as mammals in the case of *B. anthracis* and *B. cereus,* and insects in the case of *B. thuringiensis*. The pathological characteristics of the different members of the *B. cereus* group are mainly due to the presence of specific toxin genes that for the most part encoded by large, self-transmissible extrachromosomal plasmids (Schnepf *et al*., 1998). However, other chromosomal encoded virulence factors contribute to the pathogenic effects of different members of the *B. cereus* group (Dubois and Dean, 1995; Lereclus *et al*., 1996; Agaisse *et al*., 1999; Guttman and Ellar, 2000). In the case of *B. thuringiensis*, it has been known for some time that the spores synergize the effects of Cry proteins (Dubois and Dean, 1995). This synergistic effect was shown to be due to the production of several virulence factors, such as α-exotoxins, β-exotoxins, chitinases, phospholipases, and enterotoxins (Lereclus *et al*., 1996; Agaisse *et al*., 1999; Guttman and Ellar, 2000).

Mutation of genes encoding some of these virulence factors showed that they contribute to the pathogenicity of *B. thuringiensis* to insects (Lereclus *et al*., 1996; Fedhila *et al*., 2002). In the case of phosphatidylinositol specific phospholipase C (PI-PLC), disruption of the structural gene *plcA* diminished the synergistic effects of spores indicating that at least this virulence factor was necessary for an efficient virulence of the bacterium (Lereclus *et al*., 1996). This is also the case for the *inhA*2 gene that codes for a zinc-requiring metalloprotease (Fedhila *et al*., 2002). Characterization of the mechanism controlling *plcA* gene expression led to the discovery of a pleiotropic transcriptional regulator, PlcR. Mutations in the *plcR* gene abolished *plcA* gene expression and the virulence of the bacterium (Lereclus *et al*., 1996; Agaisse *et al*., 1999). A genetic screening of PlcR regulated genes identified several extra cellular virulence factors including a secreted RNase, an S-layer protein, phospholipase C, and enterotoxins, indicating that the PlcR regulon includes a diverse set of virulence factors (Agaisse *et al*., 1999). Also, the *inhA*2 gene was shown to be under the control of PlcR (Fedhila *et al*., 2003). A proteome analysis of secreted proteins from *B. cereus* strain ATCC 14579 showed that disruption of the *plcR* gene reduced the levels at least 56 exported proteins (Gohar *et al*., 2002). Alignment of the promoter regions of the PlcR regulated genes identified a 20-nucleotide palindromic sequence ("PlcR box") that is required for PlcR activation (Agaisse *et al*., 1999). Although, several PlcR regulated genes are also present in *B. cereus* and *B. anthracis* (Guttman and Ellar, 2000), only the *B. cereus* PlcR protein is functionally equivalent to that of *B. thuringiensis*, since the *B. anthracis plcR* gene is disrupted by a transposon-like sequence indicating that production of virulence factors in *B. anthracis* is blocked or may be different from that of *B. thuringiensis* and *B. cereus* (Agaisse *et al*., 1999). In fact, expression of a functional PlcR in *B. anthracis* leads to the activation of a large set of genes encoding enzymes and toxins that have protease, phospholipase, and hemolysis activities (Mignot *et al*., 2001).

PlcR regulated genes are expressed at the end of the vegetative growth phase during the stationary phase of growth (Lereclus *et al*., 1996). PlcR expression is negatively regulated by the sporulation key factor Spo0A, indicating that this gene is only temporally expressed in vegetative cells before the onset of sporulation (Lereclus *et al*., 1996). The PlcR protein senses cell density and is, therefore, part of a quorum sensing mechanism (Slamti and Lereclus, 2002). The *papR* gene, which encodes a 48 amino acid peptide and is located downstream of the *plcR* gene, is activated by PlcR and the gene product is secreted. Outside the cell, PapR is degraded by extra cellular proteases and the C-terminus end pentapeptide is internalized to the bacterium cytoplasm by the oligopeptide permease Opp (Slamti and Lereclus, 2002). Inside the cell, the pentapeptide binds PlcR activating the capacity of PlcR to bind to the PlcR box sequence. This binding then induces the activation of PlcR specific gene expression (Slamti and Lereclus, 2002). It was also shown that the PlcR activating peptide is strain specific for the activation of the PlcR regulon. Single amino acid changes on the activating PapR peptide were enough for strain-specific induction of the PlcR regulon (Slamti and Lereclus, 2002). These results suggest that quorum sensing of related *B. thuringiensis* strains is restricted by the sequence homology of the C-terminal pentapeptide of PapR. **Figure 1** shows the PlcR regulon network including the gene targets and the regulation of PlcR activation by PapR protein.

6.6.3. Insecticidal Toxins

6.6.3.1. Classification and Nomenclature

The major determinants of *B. thuringiensis* insecticidal properties are the δ-endotoxins (Schnepf *et al*., 1998). These endotoxins form two multigenic families, *cry* and *cyt*. Cry proteins are specifically toxic to orders of insects, Lepidoptera, Coleoptera,

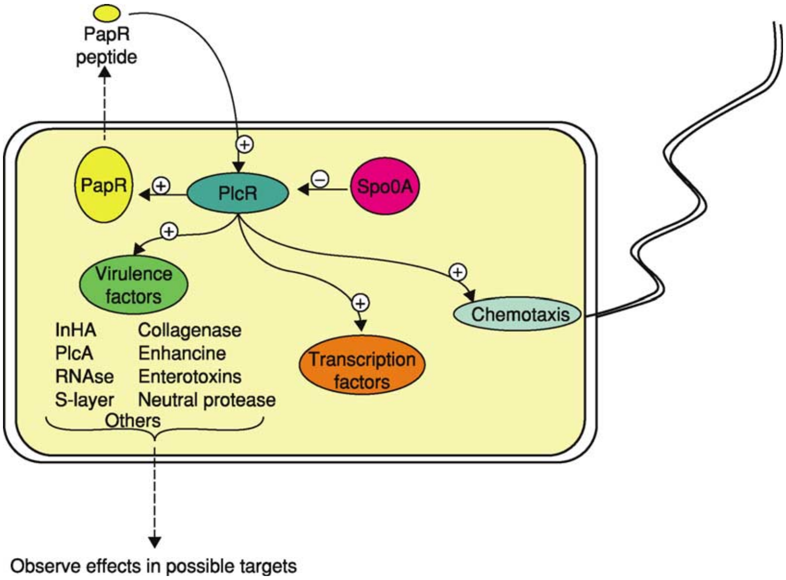


Figure 1 The PlcR regulon in *Bacillus thuringiensis*. Positive (+) and negative (−) regulation in the PlcR regulon are shown.

Hymenoptera, and Diptera. In contrast, Cyt toxins are mostly found in *B. thuringiensis* strains active against Diptera, although a few exceptions of coleopteran active strains containing Cyt proteins have been documented (Guerchicoff *et al*., 2001). Besides these crystals proteins, some *B. thuringiensis* and *B. cereus* strains produce a third group of insecticidal proteins called vegetative insecticidal proteins (Vip proteins) (Estruch *et al*., 1996). In contrast to crystal proteins, the Vip proteins are synthesized during the vegetative growth phase of the bacterium and secreted into the medium without forming crystal inclusions.

The Cry proteins comprise 40 subgroups with more than 200 members. The definition of Cry proteins is rather broad: a parasporal inclusion protein from *B. thuringiensis* that exhibits toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein (Crickmore *et al*., 1998). This definition includes the Cyt toxins although the mnemonic Cyt is given for Cry proteins that are structurally related to Cyt toxins (Crickmore *et al*., 1998, 2002). The nomenclature of Cry and Cyt proteins is based on primary sequence identity among the different protein sequences. Crystal proteins receive the mnemonic Cry or Cyt and four hierarchical ranks consisting of numbers, capital letters, lowercase letters, and numbers (e.g., Cry1Ab1). The ranks are given depending on the sequence identity shared with other Cry or Cyt proteins. A different number (first rank) is given to a protein if it shares less than 45% identity with all the others Cry proteins (Cry1, Cry2, etc.). The capital letter (second rank) is given if the protein shares less than 78% but more than 45% identity with a particular group of Cry proteins (e.g., Cry1A, Cry1B, etc.). The third rank, a lowercase letter, is given to distinguish proteins that share more than 78% but less than 95% identity with other Cry proteins (e.g., Cry1Aa, Cry1Ab, etc.). Finally a number is given to distinguish proteins that share more than 95% identity, but which are not identical and should be considered variants of the same protein (Crickmore *et al*., 1998, 2002). Although this system does not take into account the insect selectivity of Cry toxins, some subgroups of Cry toxins show toxicity against a particular genus of insects. Thus Cry1 proteins are active principally against lepidopteran insects, while the Cry3 proteins are toxic towards coleopterans. In the case of dipteran-active toxins, a surprisingly high number of different subgroups are active. For example, Cry proteins that share low sequence similarity (e.g., Cry1C, Cry2Aa, Cry4, Cry11, etc.) are active against mosquitoes.

Bacillus thuringiensis strains produce different proteins that are not related phylogenetically and all these subfamilies comprise the Cry family. Among these are: the three-domain Cry family; the larger group of Cry proteins (formed by 30 different Cry subgroups); the binary-like toxins (Cry35 and Cry36); and Mtx-like toxins (Cry15, Cry23, Cry33, Cry38, and Cry40), which are related to toxins produced by *B. sphaericus* (Crickmore *et al*., 1998, 2002). The Cry35 requires the Cry34 toxin to induce the lethal effects against *Diabrotica virgifera*

(Ellis *et al.*, 2002). The Cry34 is not related to other toxins but as mentioned above is the binary partner of Cry35. Similarly the coleopteran specific Cry23A toxin functions effectively only in the presence of the Cry37 protein (Donovan *et al.*, 2000). The sequence of Cry37 is not related to other proteins in the database but showed some similarities with Cry22 (Baum and Light Mettus, 2000) and Cry6, which are coleopteran specific (Thompson *et al.*, 2001) and also nematode specific toxins (Wei *et al.*, 2003).

The members of the three-domain family are globular molecules containing three distinct domains connected by single linkers. The alignment of their protein sequences revealed the presence of five conserved sequence blocks, although, some blocks are absent in some subgroups of the three-domain family. One particular feature of the members of this family is the presence of protoxins with two different lengths. One large group of protoxins is approximately twice as long as the majority of the toxins (130 or 70 kDa). The C-terminal extension found in the longer protoxins is dispensable for toxicity but is believed to play a role in the formation of the crystal inclusion bodies within the bacterium (de Maagd *et al.*, 2001).

Cyt toxins comprise two highly related gene families (*cyt1* and *cyt2*) (Crickmore *et al.*, 1998, 2002). Analysis of amino acid sequences shows that the different Cyt versions show a high degree of conservation in predicted α-helices and β-sheets (Guerchicoff *et al.*, 2001). Cyt toxins are also synthesized as protoxins and small portions of the N-terminus and C-terminus are removed to activate the toxin (Li *et al.*, 1996). Cyt proteins are almost exclusively found in dipteran active strains, although a few exceptions have been found as the presence of Cyt in coleopteran specific strains (Guerchicoff *et al.*, 2001). Cyt toxins synergize the toxic effect of some Cry proteins against different targets and also that of the *B. sphaericus* binary toxin (Wu *et al.*, 1994; Sayyed *et al.*, 2001; Wirth *et al.*, 2001). In the case of the three-domain Cry and Cyt proteins it is widely accepted that the primary action of these toxins is the formation of lytic pores in the midgut cells of susceptible insects. **Figure 2** shows the phylogenetic dendogram of Cry and Cyt toxins (Crickmore *et al.*, 2002).

Finally, Vip proteins comprise three families with seven members. The *vip1* and *vip2* genes are located in a single operon, and the proteins encoded by these genes constitute a binary toxin since both are necessary for toxicity against coleopteran larvae. The Vip1 and Vip2 proteins are 100 and 52 kDa in size, respectively (Warren, 1997). The mechanism of action of these proteins has not been clearly described. Nevertheless, the three-dimensional structure of Vip2 protein indicates that it may have ADP-ribosylating activity (Craig *et al.*, 1999). The Vip3 protein (88 kDa) is toxic to several lepidopteran larvae (Estruch *et al.*, 1996). A preliminary mode of action of Vip3 has been recently described. This protein is proteolytically activated by proteases present in the midgut juice extract or by trypsin to a 62 kDa resistant fragment, which binds two proteins of 80 and 100 kDa located in the apical membrane of the lepidopteran midgut cells. Vip3A also forms ionic pores that are voltage independent and cation selective characterized by long open times (Lee *et al.*, 2003).

6.6.3.2. Structure of Toxins

The crystal structures of the trypsin activated toxin Cry1Aa (lepidopteran specific), Cry3A and Cry3B (coleopteran specific), and Cry2Aa (dipteran–lepidopteran specific) protoxin have been solved (Li *et al.*, 1991; Grouchulski *et al.*, 1995; Galistki *et al.*, 2001; Morse *et al.*, 2001). Although the sequence similarity between these toxins is very low (20% and 17% identity of Cry2Aa with Cry3Aa and Cry1Aa, respectively) the overall structural topology of these proteins is very similar. **Figure 3** shows the three-dimensional structure of Cry1A, Cry3Aa toxins, and Cry2Aa protoxin (Morse *et al.*, 2001). The structure is composed of three structural domains. Domain I is a seven α-helix bundle in which a central helix α-5 is surrounded by six outer helices. This domain has been implicated in the membrane ion channel formation. The six α-helices are amphipathic and are long enough to span the 30-Å thick hydrophobic region of a membrane bilayer. Cry2Aa protoxin has an extra 49 amino acid region forming two extra α-helices in the N-terminal end that are cleaved out after proteolytic activation of the toxin (Morse *et al.*, 2001) (**Figure 3**). Domain II consist of three antiparallel β-sheets packed around a hydrophobic core in a "β-prism." This domain represents the most divergent part in structure among Cry toxin molecules (de Maagd *et al.*, 2001) and has been described as the selectivity-determining domain. Finally, domain III is a β-sandwich of two antiparallel β-sheets. Several lines of evidence indicate that domain III is implicated in insect selectivity and therefore involved in receptor binding. However, additional roles for this domain, such as in pore formation, have been suggested (Schwartz *et al.*, 1997c; de Maagd *et al.*, 2001).

Figure 4 shows the analysis of structural similarities of the three domains of Cry toxins with other proteins. This analysis revealed interesting features

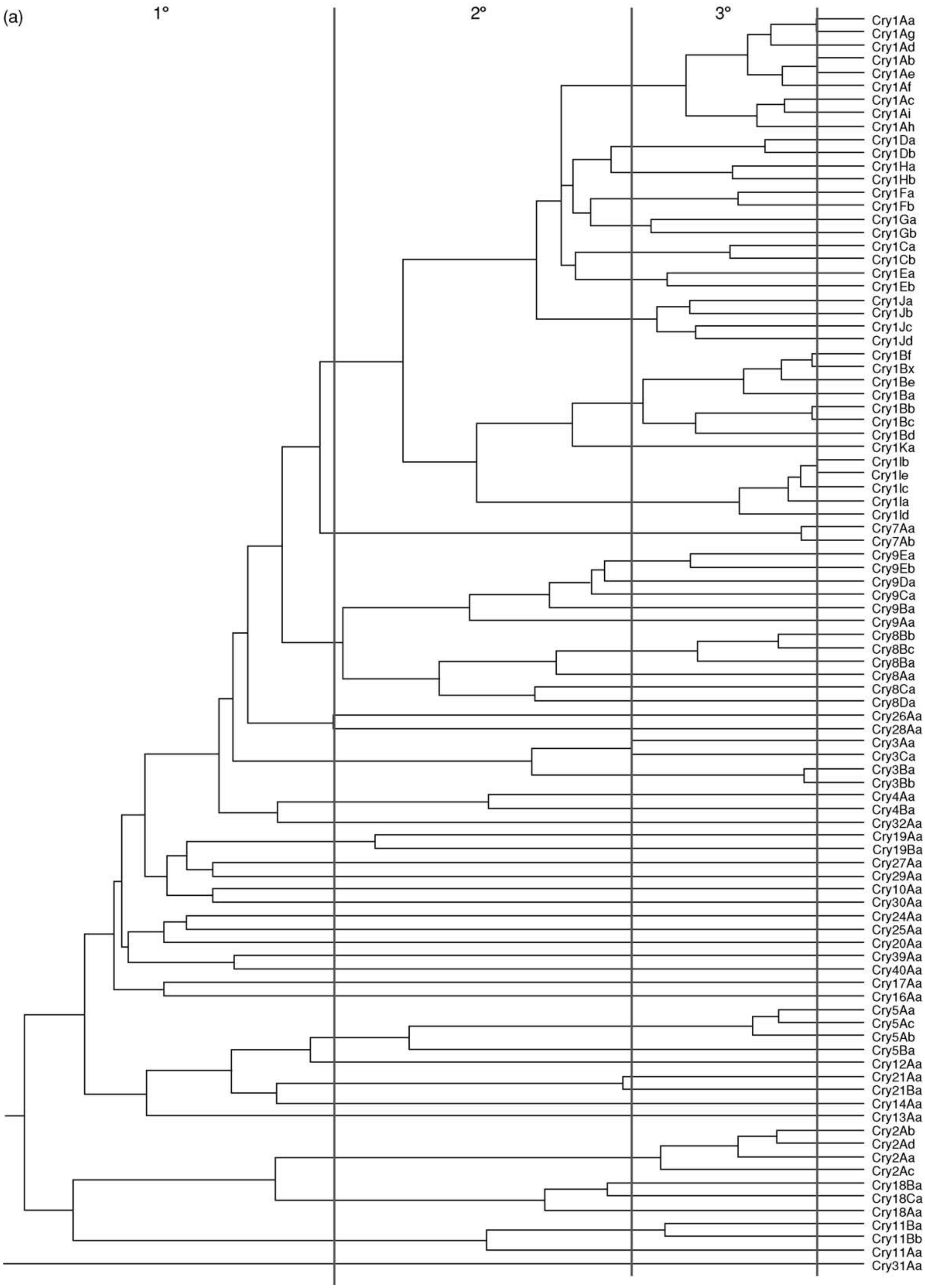


Figure 2 Continued

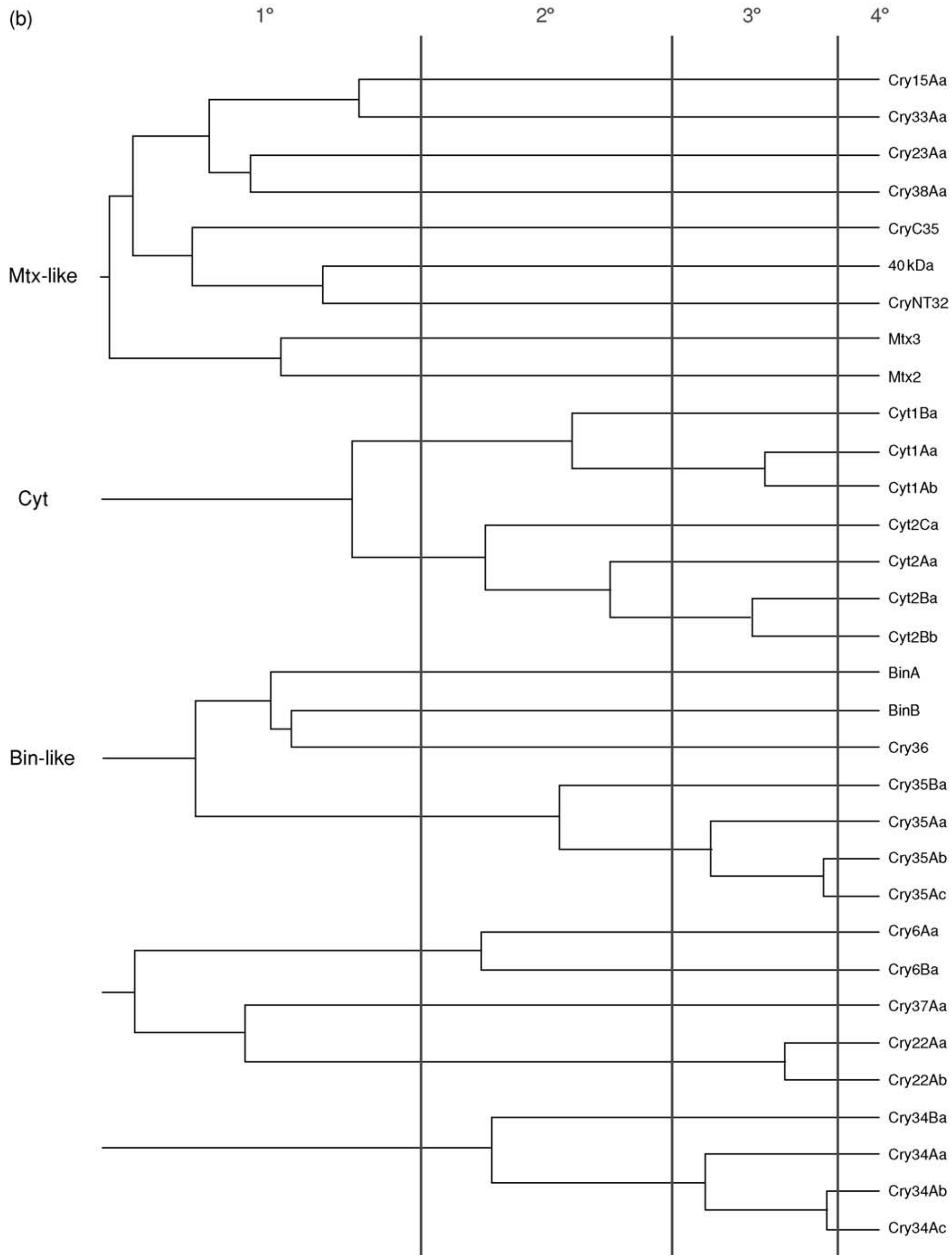


Figure 2 Dendograms of *cry* gene sequences. (a) The three domain sequences; (b) other *cry* gene sequences. Update and dendogram analysis can be found at http://www.biols.susx.ac.uk.

that could give hints on the possible role of these domains in the mode of action of Cry proteins. Domain I shares structural similarities with other pore forming toxins like colicin Ia and N (Protein Data Bank (PDB) codes: 1cii, 1a87), hemolysin E (1qoy), and diphtheria toxin (1ddt). This similarity suggests the role of this domain is in pore formation. In the case of domain II, structural similarities with several carbohydrate binding proteins like vitelline (1vmo), lectin jacalin (1jac), and lectin Mpa (1jot) supports the conclusion that this domain is involved in binding. Also for domain III, carbohydrate binding proteins with similar structures were identified as the cellulose binding domain of 1,4-β-glucanase CenC (1ulo), galactose oxidase (1gof), sialidase (1eut), β-glucuronidase (1bgh), the carbohydrate binding domain of xylanase U (1gmm), and β-galactosidase (1bgl). These results suggest

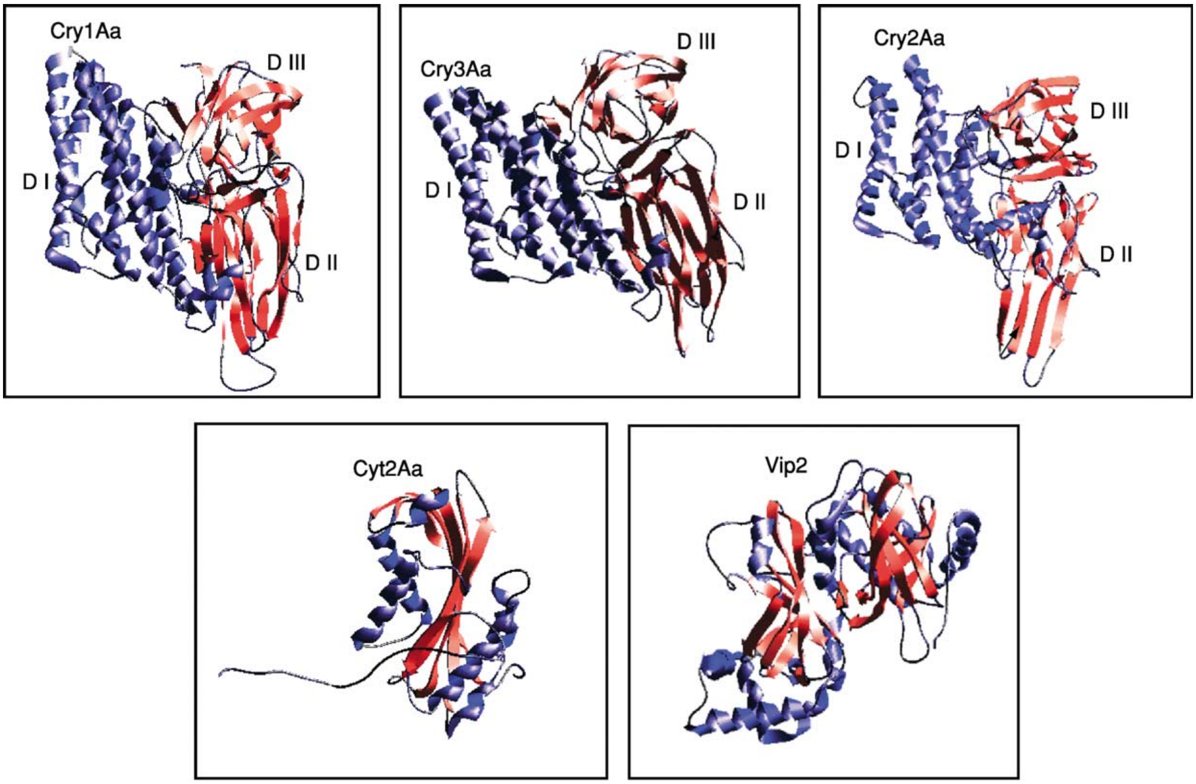


Figure 3 Three-dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis*. D I, II, III, domains I, II, and III.

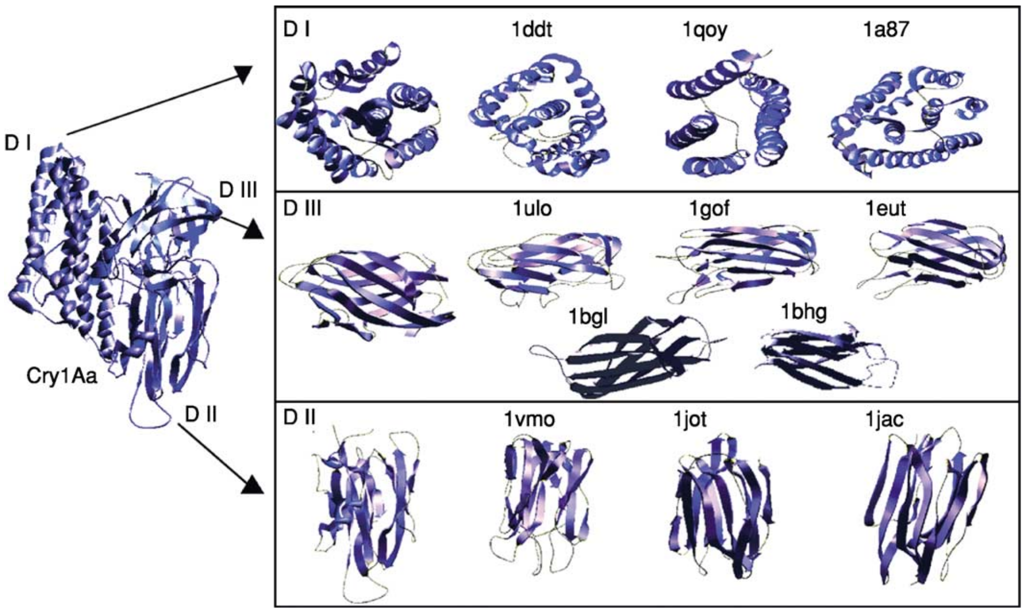


Figure 4 Proteins with structural similarity to the three domains of Cry toxins.

that carbohydrate moieties could have an important role in the mode of action of these three-domain Cry toxins, specifically in the recognition interaction of the toxin and its membrane receptors. As will be discussed later, the involvement of carbohydrate moieties in the mode of action of some Cry toxins has been demonstrated since different glycosylated proteins (aminopeptidase-N, a cadherin-like protein, and a glycoconjugate) have been identified as Cry toxin receptors in lepidopteran larvae.

Figure 3 also shows the ribbon representation of the three dimensional structure of Cyt2Aa toxin (Li *et al*., 1996). Cyt2Aa has single domain of α/β architecture comprising two outer layers of α-helical hairpins wrapped around a β-sheet. The Cyt2Aa protoxin is organized as a dimer, and proteolytic activation releases the active Cyt2Aa toxin monomer (Li *et al*., 1996; Gazit *et al*., 1997). Based on the length of the secondary structures it was proposed that the inner core of β-sheets (β-5, β-6, β-7, and β-3) could span the cell membrane (Gazit *et al*., 1997). However, analysis of membrane insertion capabilities of synthetic peptides corresponding to the different secondary structural elements suggested that α-helices A and C, rather than the β-sheets, could be the elements responsible for membrane interaction (Gazit *et al*., 1997).

Vip2A and Vip1A were isolated and characterized from a *B. cereus* strain (Warren, 1997). As mentioned above the structural genes *vip2* and *vip1* form an operon, and both Vip1 and Vip2 proteins constitute a binary toxin since both proteins are required for toxicity. Figure 3 shows the three-dimensional structure of Vip2 protein. Vip2 is a mixed α/β protein and is divided into two domains. The domains are structurally homologous although they share low amino acid identity. The overall fold of each domain resembles the catalytic domains of classical A-B toxins that have two components, a binding component (Vip1 presumably) and a catalytic ADP-ribosylating component (Vip2) (Warren, 1997). The cellular target of Vip2 is still unknown.

6.6.3.3. Evolution of Three-Domain Toxins

The increasing number of Cry toxins and the wide variety of target organisms that these proteins have puts forward the question of how evolution of this protein family created such a diverse arsenal of toxins. It has been proposed that toxins coevolved with their target insects (de Maagd *et al*., 2001). However, there are no studies that correlate the geographical distribution and Cry protein content of *B. thuringiensis* strains and the distribution of their target insect species in nature. In one particular study, a *B. thuringiensis* strain collection obtained from different climate regions of Mexico revealed that putative novel *cry* genes were more frequently found in *B. thuringiensis* strains isolated from tropical regions where the insect diversity is high (Bravo *et al*., 1998). Also the dipteran specific *cry11* and *cyt* genes were more frequently found in the rainy tropical regions than in the semiarid regions, which correlates with the distribution of dipteran insects (Bravo *et al*., 1998). A similar correlation between the frequencies of active strains with the geographical origin of the samples was presented by Bernhard *et al*. (1997) who reported that high number of *B. thuringiensis* strains active against *Heliothis virescens* in samples collected from North America where this species is a major agricultural pest. These results suggest that Cry toxins and insects may coevolve, although more comprehensive studies are needed to support this assumption.

Phylogenetic analysis of the complete Cry toxin or protoxin protein showed a broad correlation with toxicity (Bravo, 1997). However, analyses of the phylogenetic relationships of the isolated domains revealed interesting features regarding the creation of diversity in this family of proteins (Bravo, 1997; de Maagd *et al*., 2001). The analyses of phylogenetic relationships of isolated domain I and domain II sequences revealed that these domains coevolved. In the case of domain II, phylogenetic relationships showed a topology clearly related to the specificity of the toxin proteins (Bravo, 1997). This result is not surprising in spite of the fact that domain II is involved in receptor recognition and is therefore a determinant of insect selectivity (Bravo, 1997; de Maagd *et al*., 2001). Surprisingly, phylogenetic relationships of domain I sequences, that is involved in the pore formation of the toxin, showed a topology also clearly related to the selectivity of the toxin proteins (Bravo, 1997; de Maagd *et al*., 2001), suggesting that different types of domain I have been selected for acting in particular membrane conditions of their target insect (Bravo, 1997; de Maagd *et al*., 2001). The analysis of domain III sequences revealed a different topology due to the fact that several examples of domain III swapping among toxins occurred in nature (Bravo, 1997; de Maagd *et al*., 2001). Some toxins with dual specificity (coleopteran, lepidopteran) are clear examples of domain III swapping among coleopteran and lepidopteran specific toxins (Bravo, 1997; de Maagd *et al*., 2001). Cry1B toxins are good examples that illustrate domain III swapping in natural toxins. Five different Cry1B toxins have been described. These toxins share almost identical domain I and domain II sequences. However, Cry1B domain III sequences cluster with several Cry genes suggesting a very active process of domain III exchanges. The Cry1Ba domain III sequence is closely related to domain III sequences of Cry1Jb, Cry8Aa, and Cry9Da (Figure 5) but, in contrast, Cry1Bb and Cry1Bc share similar domain III sequences between them. In the case of Cry1Be, domain III clusters with domain III from Cry1Cb and Cry1Eb. Finally Cry1Bd domain III is similar to domain III of Cry1Ac toxin. Indeed Cry1B toxins

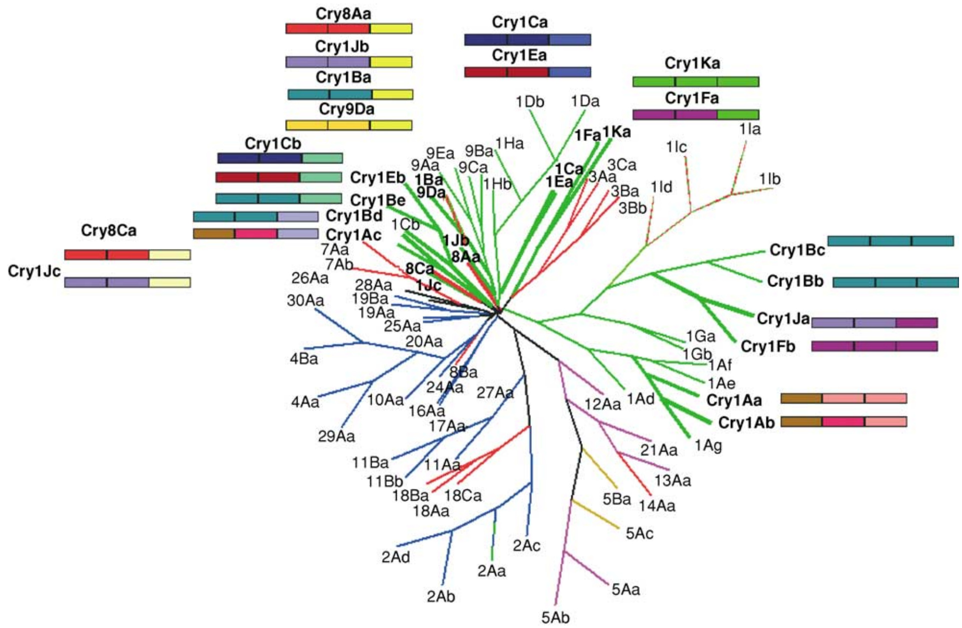


Figure 5 Phylogenetic relationships of Cry domain III sequences. Genes with similar domain III sequences are shown in the same color.

bioassays show that some of these toxins have different insect selectivities. For example, Cry1Ba is active against the coleopteran Colorado potato beetle, *Leptinotarsa decemlineata*, while Cry1Bd has higher activity against the lepidopteran diamondback moth, *Plutella xylostella,* than Cry1Ba and Cry1Bb toxins. A more comprehensive analysis of toxicity of Cry1B toxins could establish the relevance of domain III swapping in toxicity. However, this result suggests that domain III swapping could create novel specificities. Indeed, *in vitro* domain III swapping of certain Cry1 toxins results in changes in insect specificity as demonstrated for the hybrid protein containing domain I and II of Cry1Ab toxin and domain III of Cry1C which resulted in a toxin protein with increased toxicity towards *Spodoptera* sp. (Bosch *et al*., 1994; de Maagd *et al*., 2000, 2001). **Figure 5** shows the topology of the phylogenetic relationships of domain III sequences and the representation of Cry toxins in which domain III swapping occurred in nature during evolution of these proteins.

Phylogenetic analyses of the Cry toxin family shows that the great variability in the biocidal activity of this family has resulted from two fundamental evolutionary processes: (1) independent evolution of the three structural domains, and (2) domain III swapping among different toxins. These two processes have generated proteins with similar modes of action but with very different insect selectivities (Bravo, 1997; de Maagd *et al*., 2001).

6.6.4. Mode of Action of Three-Domain Cry Toxins

6.6.4.1. Intoxication Syndrome of Cry Toxins

As mentioned above (see Section 6.6.3.2), it is widely accepted that the primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming lytic pores in the apical microvilli membrane of the cells (Gill *et al*., 1992; Schnepf *et al*., 1998; Aronson and Shai, 2001; de Maagd *et al*., 2001). In order to exert its toxic effect, Cry protoxins in the crystalline inclusions are modified to membrane-inserted oligomers that cause ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding 60–70 kDa protease resistant proteins (Choma *et al*., 1990). Toxin activation involves the proteolytic removal of an N-terminal peptide (25–30 amino acids for Cry1 toxins, 58 for Cry3A, and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins (130 kDa protoxins). The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells of susceptible insects (Schnepf *et al*., 1998; de Maagd *et al*., 2001) before inserting into the membrane (Schnepf *et al*., 1998; Aronson and Shai, 2001). Toxin insertion leads to the formation of

lytic pores in microvilli apical membranes (Schnepf *et al*., 1998; Aronson and Shai, 2001). Cell lysis and disruption of the midgut epithelium releases the cell contents providing spores a rich medium that is suitable for spore germination leading to a severe septicemia and insect death (Schnepf *et al*., 1998; de Maagd *et al*., 2001).

6.6.4.2. Solubilization and Proteolytic Activation

Solubilization of long protoxins (130 kDa) depends on the highly alkaline pH that is present in guts of lepidopteran and dipteran insects, in contrast to coleopteran insect guts that have a neutral to slightly acidic pH (Dow, 1986). In a few cases, protoxin solubilization has been shown to be a determinant for insect toxicity. Cry1Ba is toxic to the coleopteran *L. decemlineata* only if the protoxin is previously solubilized *in vitro*, suggesting insolubility of the toxin at the neutral–acidic pH of coleopteran insects (Bradley *et al*., 1995). The C-terminal portion of protoxins contains many cysteine residues that form disulfide bonds in the crystal inclusions and, therefore, reducing the disulfide bonds is a necessary step for the solubilization of long Cry protoxins (Du *et al*., 1994). Differences in the midgut pH between lepidopteran and coleopteran midguts may be a reason for the bias in the utilization of arginine as basic amino acid over lysine in the lepidopteran specific toxins Cry1, Cry2, and Cry9 with exception of the Cry1I toxin, which is also active against coleopteran insects (Grochulski *et al*., 1995; de Maagd *et al*., 2001). The higher pK_a of arginine, compared with that of lysine, might be required for maintaining a positive charge even at the high pH of lepidopteran guts (up to pH 11) resulting in soluble toxins at alkaline pH.

Proteolytic processing of Cry toxins is a critical step involved not only on toxin activation but also on specificity (Haider and Ellar, 1989; Haider *et al*., 1989) and insect resistance (Oppert *et al*., 1997; Shao *et al*., 1998). Besides pH, lepidopteran and coleopteran insects differ in the type of proteases present in the insect gut; serine proteases are the main digestive proteases of Lepidoptera and Diptera, whereas cysteine and aspartic proteases are abundant in the midguts of Coleoptera (Terra and Ferreira, 1994). It has been reported that enhanced degradation of Cry toxins is associated with the loss of sensitivity of fifth instar *Spodoptera litoralis* larvae to Cry1C (Keller *et al*., 1996) and that serine protease inhibitors enhanced the insecticidal activity of some *B. thuringiensis* toxins up to 20-fold (MacIntosh *et al*., 1990). More recently, it was found that the low toxicity of Cry1Ab toxin to *S. frugiperda* could be explained in part by rapid degradation of the toxin on the insect midgut (Miranda *et al*., 2001). For several Cry proteins inactivation within the insect gut involves intramolecular processing of the toxin (Choma *et al*., 1990; Lambert *et al*., 1996; Audtho *et al*., 1999; Pang *et al*., 1999; Miranda *et al*., 2001). However, for several other Cry toxins, intramolecular processing is not always related to loss of toxicity and sometimes is required for proper activation of the toxin (Dai and Gill, 1993; Zalunin *et al*., 1998; Yamagiwa *et al*., 1999). Therefore, in some cases, differential proteolytic processing of Cry toxins in different insects could be a limiting step in the toxicity of Cry proteins (Miranda *et al*., 2001).

One interesting feature of Cry toxin activation is the processing of the N-terminal end of the toxins. The three-dimensional structure of Cry2Aa protoxin showed that two α-helices of the N-terminal region occlude a region of the toxin involved in the interaction with the receptor (Morse *et al*., 2001) (**Figure 3**). Several lines of evidences suggest that the processed N-terminal peptide of Cry protoxins might prevent binding to nontarget membranes (Martens *et al*., 1995; Kouskoura *et al*., 2001; Bravo *et al*., 2002b). *Escherichia coli* cells producing Cry1Ab or Cry1Ca toxins lacking the N-terminal peptide were severely affected in growth (Martens *et al*., 1995; Kouskoura *et al*., 2001). It was speculated that the first 28 amino acids prevented the Cry1A toxin from inserting into the membrane (Martens *et al*., 1995; Kouskoura *et al*., 2001). Recently, it was found that a Cry1Ac mutant that retains the N-terminus end after trypsin treatment binds nonspecifically to *Manduca sexta* membranes and was unable to form pores on *M. sexta* brush border membrane vesicles (Bravo *et al*., 2002b). Therefore, processing of the N-terminal end of Cry protoxins may unmask a hydrophobic patch of the toxin involved in toxin–receptor or toxin–membrane interaction (Morse *et al*., 2001; Bravo *et al*., 2002b).

6.6.4.3. Receptor Identification

The major determinant of Cry toxin selectivity is the interaction with specific receptors on the insect gut of susceptible insects (Jenkins and Dean, 2000). Therefore, receptor identification is fundamental for determining the molecular basis of Cry toxin action and also in insect resistance management that in many cases has been shown to correlate with defects in receptor binding (Ferré and Van Rie, 2002). A number of putative receptor molecules for the lepidopteran specific Cry1 toxins have been identified. In *M. sexta*, the Cry1Aa, Cry1Ab,

and Cry1Ac proteins bind to a 120 kDa aminopeptidase-N (APN) (Knight *et al*., 1994; Garczynski and Adang, 1995; Denolf *et al*., 1997) and to a 210 kDa cadherin-like protein ($Bt\text{-}R_1$) (Belfiore *et al*., 1994; Vadlamudi *et al*., 1995). Cadherins represent a large family of glycoproteins that are classically responsible for intercellular contacts. These proteins are transmembrane proteins with a cytoplasmic domain and an extracellular ectodomain with several cadherin repeats (12 in the case of $Bt\text{-}R_1$). The ectodomain contain calcium binding sites, integrin interaction sequences, and cadherin binding sequences. In *Bombyx mori*, Cry1Aa binds to a 175 kDa cadherin-like protein ($Bt\text{-}R_{175}$) (Nagamatsu *et al*., 1998, 1999) and to a 120 kDa APN (Yaoi *et al*., 1997). In *H. virescens* Cry1Ac binds to two proteins of 120 kDa and 170 kDa both identified as APN (Gill *et al*., 1995; Oltean *et al*., 1999). Also, a cadherin-like protein is involved in the mode of action of Cry1Ac toxin in *H. virescens* (Gahan *et al*., 2001). In *P. xylostella* and *Lymantria dispar* APNs were identified as Cry1Ac receptors (Valaitis *et al*., 1995; Lee *et al*., 1996; Denolf *et al*., 1997; Luo *et al*., 1997). In *L. dispar*, besides APN and cadherin-like receptors, a high molecular weight anionic protein ($Bt\text{-}R_{270}$) that binds Cry1A toxins with high affinity was identified (Valaitis *et al*., 2001). For Cry1C toxin an APN receptor molecule was identified in *Spodoptera litura* (Agrawal *et al*., 2002). Sequence analysis of various APN from lepidopteran insects suggests that APN's group into at least four classes (Oltean *et al*., 1999). *Plutella xylostella* and *B. mori* produce the four classes of APN and it is anticipated that several other lepidopteran insects may also produce the four isoforms (Nakanishi *et al*., 2002). Cry1A binding to the different *B. mori* APN isoforms revealed that this toxin only binds the 115 kDa isoform in ligand blot binding analysis (Nakanishi *et al*., 2002).

Surface plasmon resonance experiments showed that the binding affinity of Cry1A toxins to the *M. sexta* APN is in the range of 100 nM (Jenkins and Dean, 2000), while that of cadherin-like receptors ($Bt\text{-}R_1$) is in the range of 1 nM (Vadlamudi *et al*., 1995). This difference in the binding affinities between APN and $Bt\text{-}R_1$ suggest that binding to $Bt\text{-}R_1$ might be the first event on the interaction of Cry1A toxins with microvilli membranes and, therefore, the primary determinant of insect specificity.

Different experimental evidence supports the involvement of both Cry1A toxins receptors (APN and cadherin-like) in toxicity. Expression of the *M. sexta* and *B. mori* cadherin-like proteins, $Bt\text{-}R_1$ and $Bt\text{-}R_{175}$ respectively, on the surface of different cell lines render these cells sensitive to Cry1A toxins, although the toxicity levels were low (Nagamatsu *et al*., 1999; Dorsch *et al*., 2002; Tsuda *et al*., 2003). Cry1Aa toxin was shown to lyse isolated midgut epithelial cells; this toxic effect was inhibited if the cells were preincubated with anti-$Bt\text{-}R_{175}$ antisera in contrast with the treatment with anti-APN antisera, suggesting that cadherin-like protein $Bt\text{-}R_{175}$ is a functional receptor of Cry1Aa toxin (Hara *et al*., 2003). Also, a single-chain antibody (scFv73) that inhibits binding of Cry1A toxins to cadherin-like receptor, but not to APN, reduced the toxicity of Cry1Ab to *M. sexta* larvae (Gómez *et al*., 2001). Moreover, disruption of a cadherin gene by a retrotransposon-mediated insertion and its linkage to high resistance to Cry1Ac toxin in *H. virescens* YHD2 (a laboratory selected line) larvae supports a role for cadherin as a functional receptor (Gahan *et al*., 2001). Overall, these results suggest that binding to the cadherin-like receptor is an important step in the mode of action Cry1A toxins (**Figure 6**).

Regarding the APN receptor, several reports argue against the involvement of this protein in toxin activity. Mutants of Cry1Ac toxin affected on APN binding retained similar toxicity levels to *M. sexta* as the wild-type toxin (Burton *et al*., 1999). However, there are several reports describing point mutations of Cry toxins located in domain II that affected both APN binding and toxicity (Jenkins and Dean, 2000). Expression of APN in heterologous systems did not result in Cry1A sensitivity (Denolf *et al*., 1997) and, as mentioned above, an APN antibody did not protect *B. mori* midgut cells from Cry1Aa toxic effect in contrast to a cadherin antibody (Hara *et al*., 2003). However, two recent reports clearly demonstrate the importance of this molecule on the mode of action of Cry1 toxins. Inhibition of APN production in *S. litura* larvae by dsRNA interference showed that insects with low APN levels became resistant to Cry1C toxin (Rajagopal *et al*., 2002). Also, heterologous expression of *M. sexta* APN in midguts and mesodermal tissues of transgenic *Drosophila melanogaster* caused sensitivity to Cry1Ac toxin (Gill and Ellar, 2002). Additionally, previous reports demonstrated that incorporation of *M. sexta* APN into black lipid bilayers lowers the concentration of toxin needed for pore formation activity of Cry1Aa toxin (Schwartz *et al*., 1997b). Finally, all Cry1A APN receptors are anchored to the membrane by a glycosyl phosphatidylinositol (GPI) (Knight *et al*., 1994; Garczynski and Adang, 1995; Denolf *et al*., 1997; Oltean *et al*., 1999; Agrawal *et al*., 2002; Nakanishi *et al*., 2002). And treatment of *Trichoplusia ni* brush border membrane vesicles with PI-PLC, which cleaves GPI-anchored proteins from the membrane,

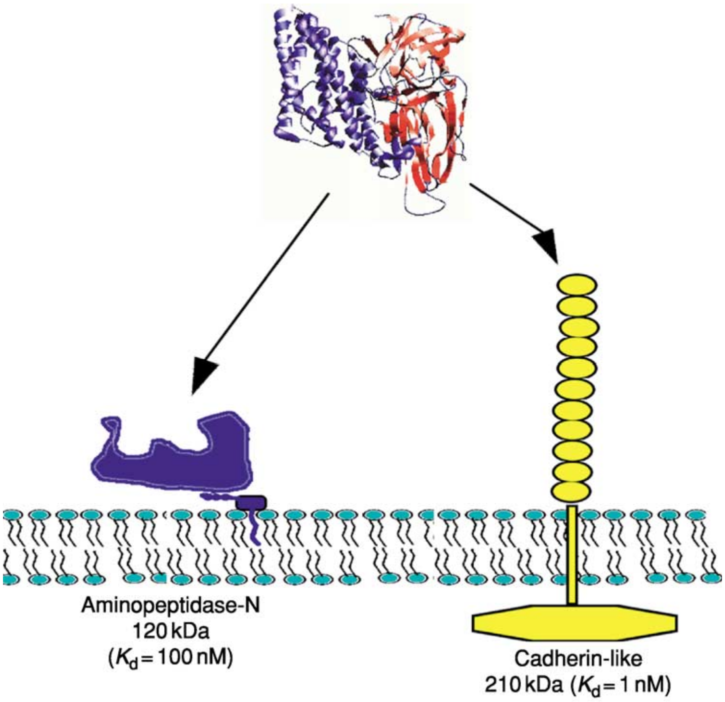


Figure 6 Receptor molecules of Cry1A proteins. K_d values are average affinity values of Cry1A toxins to aminopeptidase-N and cadherin receptors.

reduced Cry1Ac pore formation activity (Lorence *et al.*, 1997). These reports suggest that APN binding is also an important step in the mode of action of Cry1 toxins.

In the case of the dipteran specific Cry11A and Cry4B toxins, two binding proteins of 62 and 65 kDa were identified on brush border membrane vesicles from *Aedes aegypti* larvae (Buzdin *et al.*, 2002). The identity of these binding molecules and their role as receptors of Cry11A and Cry4B toxins still remains to be analyzed.

One interesting feature of the Cry1 receptor molecules identified so far is that all these proteins are glycosylated. As mentioned before, it is interesting to note that domain II and domain III of Cry toxins have structural homology to several protein domains that interact with carbohydrates. In the case of Cry1Ac, Cry5, and Cry14 toxins different experimental evidence suggest a role of carbohydrate recognition in the mode of action of these toxins. The domain III of Cry1Ac interacts with a sugar *N*-acetylgalactosamine on the aminopeptidase receptor (Masson *et al.*, 1995). In *Caenorhabditis elegans* resistance to the Cry5B and Cry14 toxins arises because of changes in the expression of enzymes involved in glycosylation (Griffits *et al.*, 2001). However, the structural similarities of domain II and III with carbohydrate binding proteins does not exclude the possibility that these domains may have protein–protein interactions with the receptor molecules. In fact some loop regions of domain II of Cry1Ab and the Bt-R_1 receptor do have protein–protein interaction (Gómez *et al.*, 2002a). The interaction between the toxin and its receptors can be complex involving multiple interactions with different toxin–receptor epitopes or carbohydrate molecules. Interestingly, the binding of Cry1Ac to two sites on the purified APN from *M. sexta* has been reported, and *N*-acetylgalactosamine could inhibit 90% of Cry1Ac binding, which does not inhibit the binding of Cry1Aa and Cry1Ab to the same APN receptor (Masson *et al.*, 1995). Only one of the Cry1Ac binding sites on APN is shared and recognized by Cry1Aa and Cry1Ab toxins (Masson *et al.*, 1995).

6.6.4.4. Toxin Binding Epitopes

The identification of epitopes involved in Cry toxin–receptor interaction will provide insights into the molecular basis of insect specificity and could help in the characterization of insect resistant populations in nature. Such studies would also aid in developing strategies to design toxins that could overcome receptor point mutations leading to Cry toxin resistance.

The toxin binding epitopes have been mapped for several Cry toxins. Domains II and III are the most variable regions of Cry toxins and had, therefore,

been subject to mutagenesis studies to determine their role in receptor recognition. Domain II was first recognized as an insect toxicity determinant domain based on hybrid toxin construction using *cry* genes with different selectivities (Ge *et al*., 1989). Site-directed mutagenesis studies of Cry1A toxins showed that some exposed loop regions, loop α-8, loop 2, and loop 3, of domain II are involved in receptor recognition (Rajamohan *et al*., 1996a, 1996b; Jenkins *et al*., 2000; Lee *et al*., 2000, 2001). Specifically, the loop 2 region of Cry1Ab toxin was shown to be important in the interaction with *M. sexta* brush border membrane vesicles. Characterization of mutants affected at Arg368-Arg369 in loop 2 indicated that these residues have an important role in the reversible binding of the toxin to brush border membrane vesicles, while Phe371 is involved in the irreversible binding of the toxin (Rajamohan *et al*., 1995, 1996b; Jenkins and Dean, 2000). Reversible binding is related to the initial interaction of the toxin to the receptor while irreversible binding is related to the insertion of the toxin into the membrane (Rajamohan *et al*., 1995). This result was interpreted as suggesting that Phe371 might be involved in membrane insertion (Rajamohan *et al*., 1995, 1996b). Also, evidence has been provided showing that Arg368-Arg369 in loop 2 of Cry1Ab are involved in APN binding since this mutant showed no binding to purified APN in surface plasmon resonance experiments (Jenkins and Dean, 2000; Lee *et al*., 2000). In this regard it is interesting to note that a truncated derivative of Cry1Ab toxin containing only domains II and III was still capable of receptor interaction but was affected in irreversible binding (Flores *et al*., 1997). Therefore, it is likely that mutations in Phe371 affect membrane insertion probably by interfering with conformational change, necessary for membrane insertion, after initial recognition of the receptor or as originally proposed this residue could be directly involved in membrane interaction (Rajamohan *et al*., 1995). Phylogenetic relationship studies of different Cry1 loop 2 sequences show that there is a correlation between the loop 2 amino acid sequences and cross-resistance with several Cry1 toxins in resistant insect populations, implying that this loop region is an important determinant of receptor recognition (Tabashnik *et al*., 1994; Jurat-Fuentes and Adang, 2001). Overall these results suggest that loop 2 of Cry1A toxins plays an important role in receptor recognition. Regarding loop α-8 and loop 3, mutations of some residues in these regions of Cry1A toxins affected reversible binding to brush border membrane vesicles or the purified APN receptor (Rajamohan *et al*., 1996a; Lee *et al*., 2001). The closely related toxins Cry1Ab and Cry1Aa have different loop 3 amino acid sequences even though they interact with the same receptor molecules (Rajamohan *et al*., 1996a). Analysis of Cry1Ab and Cry1Aa binding to the cadherin-like Bt-R_1 receptor in ligand blot experiments and competition with synthetic peptides corresponding to the toxin exposed loop regions, showed that, besides loop 2, loop 3 of Cry1Aa toxin was important for Bt-R_1 recognition (Gómez *et al*., 2002a). Although the Cry1A loop 1 region seems not to play an important role in receptor recognition this is certainly not the case for other Cry toxins. Mutagenesis of Cry3A loop 1 residues showed that this region, besides loop 3, is important for receptor interaction in coleopteran insects (Wu and Dean, 1996). Loop 1 residues of two Cry toxins with dual insecticidal activity (Cry1Ca and Cry2Aa, active against dipteran and lepidopteran insects) are important for toxicity against mosquitoes but not against lepidopterous insects (Widner and Whiteley, 1990; Smith and Ellar, 1994; Morse *et al*., 2001). Besides loop1 of Cry1C toxin, loops 2 and 3 are important for toxicity to dipteran and lepidopteran insects (Abdul-Rauf and Ellar, 1999). In the case of Cry2Aa toxin, an amino acid region involved in lepidopteran activity was not located in loops 1, 2, or 3 regions but was located to a different part of domain II in loops formed by β-5 and β-6, and by β-7 and β-8 (Morse *et al*., 2001). These results show that the loop regions of domain II are important determinants for receptor interaction, although other regions of this domain, in certain toxins, could also participate in receptor recognition.

As mentioned previously (see Section 6.6.3.3), domain III swapping indicated that this domain is involved in receptor recognition (Bosch *et al*., 1994; de Maagd *et al*., 2000), and it has been proposed that domain swap has been used as an evolutionary mechanism of these toxins (Bravo, 1997; de Maagd *et al*., 2001). The swapping of domain III between Cry1Ac and Cry1Ab toxins showed that the Cry1Ac domain III was involved in APN recognition (Lee *et al*., 1995; de Maagd *et al*., 1999a). The interaction of Cry1Ac domain III and APN was dependent on *N*-acetylgalactosamine (Gal-Nac) residues (Burton *et al*., 1999; Lee *et al*., 1999). Mutagenesis studies of Cry1Ac domain III identified Gln509-Asn510-Arg511 Asn506, and Tyr513 as the epitope for sugar recognition (Burton *et al*., 1999; Lee *et al*., 1999). The three-dimensional structure of Cry1Ac in the presence of the sugar confirmed that these residues are important for Gal-Nac recognition and that sugar binding has a conformational effect on the pore forming domain I (Li *et al*., 2001). In

the case of Cry1Ac toxin, a sequential binding mechanism to the APN receptor has been proposed (Jenkins *et al.*, 2000). The interaction of domain III to an *N*-acetylgalactosamine moiety in the receptor precedes the binding of loop regions of domain II (Jenkins *et al.*, 2000).

6.6.4.5. Receptor Binding Epitopes

In regard to the receptor binding epitopes, a region of 63 residues (Ile135–Pro198) involved in Cry1Aa binding was identified by analysis of truncated derivatives of *B. mori* APN. This site was specific for Cry1Aa toxin since it was not involved in Cry1Ac binding (Yaoi *et al.*, 1999; Nakanishi *et al.*, 2002). Nevertheless, this binding region is present in other APN molecules that do not bind Cry1Aa toxin when assayed by toxin overlay assays (Nakanishi *et al.*, 2002). This result can be explained if the epitope mapped was not accessible in native conditions. In fact it has been shown that denaturation of *M. sexta* APN exposes binding epitopes hidden under nondenaturating conditions (Daniel *et al.*, 2002). Therefore, the role of the mapped binding epitope in *B. mori* APN in toxicity remains to be analyzed.

Regarding cadherin-like receptors, a Cry1A binding epitope was mapped in Bt-R_1 and Bt-R_{175} receptor molecules by the analysis of truncated derivatives of these receptors in toxin overlay assays (Nagamatsu *et al.*, 1999; Dorsch *et al.*, 2002). In the case of Bt-R_1 and Bt-R_{175}, a toxin binding region of 70 amino acid residues was mapped in the cadherin repeat number 11 which is close to the membrane spanning region (Nagamatsu *et al.*, 1999; Dorsch *et al.*, 2002). The binding epitope was narrowed to 12 amino acids (^{1331}IPLPASILTVTV^{1342}) by using synthetic peptides as competitors. Binding of Cry1Ab toxin to the 70 residue toxin binding peptide was inhibited by synthetic peptides corresponding to loop α-8 and loop 2, suggesting that these loop regions are involved in the interaction with this receptor epitope (Gómez *et al.*, 2003). Using a library of single-chain antibodies displayed in M13 phage, a second Cry1A toxin binding region was mapped in the Bt-R_1 receptor (Gómez *et al.*, 2001). An scFv antibody (scFv73) that inhibited binding of Cry1A toxins to the cadherin-like receptor Bt-R_1, but not to APN, and reduced the toxicity of Cry1Ab to *M. sexta* larvae was identified (Gómez *et al.*, 2001). Sequence analysis of CDR3 region of the scFv73 molecule led to the identification of an eight amino acid epitope of *M. sexta* cadherin-like receptor, Bt-R_1 (^{869}HITDTNNK^{876}) involved in binding of Cry1A toxins. This amino acid region maps in the cadherin repeat 7 (Gómez *et al.*, 2001). Using synthetic peptides of the exposed loop regions of domain II of Cry1A toxins, loop 2 was identified as the cognate binding epitope of the *M. sexta* receptor Bt-R_1 ^{869}HITDTNNK^{876} site (Gómez *et al.*, 2002a). This finding highlights the importance of the ^{869}HITDTNNK^{876} binding epitope since extensive mutagenesis of loop 2 of Cry1A toxins has shown that this loop region is important for receptor interaction and toxicity (Rajamohan *et al*, 1995, 1996b; Jenkins and Dean, 2000; Jenkins *et al.*, 2000). Nevertheless, binding to cadherin repeat 7 was only observed in small truncated derivatives of Bt-R_1 (Gómez *et al.*, 2003) in contrast with larger truncated derivatives (Nagamatsu *et al.*, 1999; Dorsch *et al.*, 2002). Analysis of the dissociation constants of Cry1Ab binding to similar 70 amino acid peptides containing both toxin binding regions revealed that the toxin binds the epitope located in cadherin repeat 7 with sixfold higher affinity than cadherin repeat 11. Based on these results a sequential binding mechanism was proposed where binding of toxin to cadherin repeat 11 facilitates the binding of toxin loop 2 to the epitope in cadherin repeat 7 (Gómez *et al.*, 2003). Accumulating evidence indicate that proteins can interact with amino acid sequences displaying inverted hydropathic profiles (Blalock, 1995). The interactions of loop 2 with Bt-R_1 ^{865}NITI-HITDTNN^{875} region and of loops α-8 and 2 with ^{133}1IPLPASILTVTV^{1342} region were shown to be determined by hydropathic complementarity (Gómez *et al.*, 2002a, 2003).

As mentioned previously, it is generally accepted that the toxic effect of Cry proteins is exerted by the formation of a lytic pore. However, the fact that Cry1A toxins interact with protein molecules involved in cell–cell interactions (cadherin) within susceptible hosts could be relevant for the intoxication process as has been described for several other pathogens (Dorsch *et al.*, 2002). Targeting cell junction molecules seems to be representative of those bacteria that disrupt or evade epithelial barriers in their hosts. In this regard, it is remarkable that Cry1A toxins interact with at least two structural regions that are not close together in the primary sequence of Bt-R_1 (cadherin repeats 7 and 11). Although we cannot exclude the possibility that both sites could be located close together in the three-dimensional structure of Bt-R_1, we speculate that binding of Cry1A toxins could cause a conformational change in cadherin molecule that could interact with other cell-adhesion proteins, and consequently disrupt the epithelial cell layer (**Figure 7**).

As mentioned previously, mutagenesis studies have shown that besides domain II loop 2 and α-8,

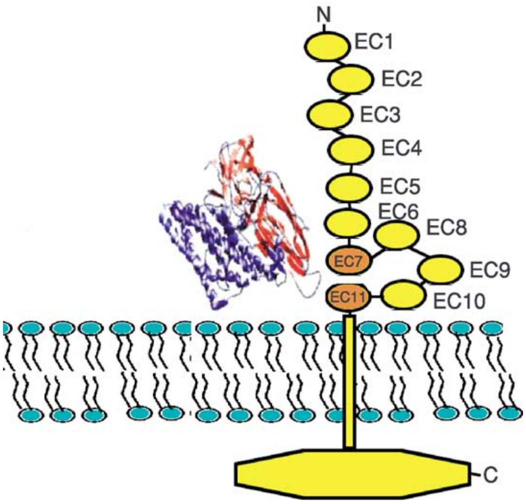


Figure 7 Structural regions on *Manduca sexta* cadherin receptor (Bt-R_1) involved in Cry1A binding. Ectodomains (EC) 7 and 11 contain Cry1A binding epitopes.

loop 3 of Cry1A toxins is important for receptor interaction and toxicity (Rajamohan *et al*., 1996a; Lee *et al*., 2001). The Bt-R_1 and the APN epitope involved in binding loop 3 regions still remains to be identified.

6.6.4.6. Cry Toxin–Receptor Binding Function in Toxicity

It is proposed that, following binding, at least part of the toxin inserts into the membrane resulting in pore formation (Schnepf *et al*., 1998). However, it is still largely unknown what is the role of receptor binding in promoting the insertion and oligomerization of the toxin. In the case of Cry1A toxins the differences of binding affinities between APN and Bt-R_1 (100 nM versus 1 nM K_d respectively) suggest that binding to Bt-R_1 is the first event in the interaction of Cry1A toxins with microvilli membranes.

In other noninsecticidal pore forming toxins, receptor binding facilitates a complete proteolytic activation of the toxin resulting in the formation of functional oligomers that are membrane insertion competent (Abrami and Van der Goot, 1999). In the case of Cry1A toxins, Gómez *et al*. (2002b, 2003) provided evidence that interaction of Cry toxin with its cadherin-like receptor is a necessary step for a complete proteolytic of the toxin. Incubation of Cry1Ab protoxin with the single-chain antibody scFv73 that mimics the cadherin-like receptor or with the toxin-binding peptides of Bt-R_1, and treatment with *M. sexta* midgut juice, resulted in toxin preparations with high pore formation activity *in vitro*. In contrast toxin preparations activated with same midgut juice proteases in the absence of the antibody showed very low pore formation activity (Gómez *et al*., 2002b, 2003). The high pore formation activity correlated with the formation of a 250 kDa oligomer composed of four Cry toxins that lacked the α-1 helix of domain I. The oligomer, in contrast to the 60 kDa monomer, has higher hydrophobicity as judged by 8-anilino-1-naphthalenesulfonate binding (Gómez *et al*., 2002b). The oligomer was membrane insertion competent in contrast with the monomer, as judged by measuring toxin membrane insertion using the intrinsic fluorescence of tryptophan residues (Raussel and Bravo, unpublished data). The 250 kDa oligomer could also be obtained by incubation of the Cry1Ab protoxin with brush border membrane vesicles isolated in the absence of protease inhibitors, presumably by the action of a membrane associated protease (Gómez *et al*., 2002b). Therefore toxin binding to cadherin facilitates the complete proteolytic activation of the toxin and the formation of a prepore structure that consists of four monomers. Characterization of the membrane insertion capabilities of the prepore, the kinetic properties of the ionic pore formed by this structure, and solving its three-dimensional structure could be important steps towards understanding the role of this insertion-intermediate structure in the mode of action of Cry toxins.

As mentioned previously APN receptors are also key molecules involved in the toxicity of Cry1 toxins. Cry1A APN receptors are anchored to the membrane by a GPI anchor. An indication of its possible role came from the characterization of membrane microdomains (lipid rafts) from microvilli membranes of *M. sexta* and *H. virescens* (Zhuang *et al*., 2002). Like their mammalian counterparts, *H. virescens* and *M. sexta* lipid rafts are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol anchored proteins (Zhuang *et al*., 2002). Lipid rafts have been implicated in membrane and protein sorting and in signal transduction (Simons *et al*., 2000). They have also been described as portals for different viruses, bacteria and toxins. The interaction of different bacterial toxins with their receptors located in lipid rafts is a crucial step in the oligomerization and insertion of toxins into the membrane (Cabiaux *et al*., 1997; Abrami and van der Goot, 1999; Rosenberg *et al*., 2000). Several Cry1A receptors, including the GPI anchored proteins, 120 and 170 kDa APNs from *H. virescens*, and the 120 kDa APN from *M. sexta* were preferentially partitioned into lipid rafts. After toxin exposure, Cry1A toxins were associated with lipid rafts and the integrity of these

microdomains was essential for *in vitro* Cry1Ab pore forming activity (Zhuang *et al*., 2002). Additionally, PI-PLC treatment of *T. ni* brush border membrane vesicles, resulting in cleavage of GPI-anchored proteins from membrane, reduced drastically Cry1Ac pore formation (Lorence *et al*., 1997). Therefore, the possible role of APN binding could be to drive Cry1A toxins, probably the prepore, to lipid rafts microdomains where the toxin inserts and forms pores. The participation of lipid rafts in the mode of action of Cry toxins could suggest a possible role of signal transduction events and/or the internalization of Cry toxins, since lipid rafts have an active role in these cellular processes. In the case of Cry5 toxin evidence was provided showing that toxin is internalized into the epithelial midgut cells after exposure of *C. elegans* nematodes to the toxin (Griffits *et al*., 2001).

Binding of Cry1A toxins to cadherin and APN receptors are key steps in the mode of action of these toxins. However, the participation of other uncharacterized molecules in the process of membrane insertion and pore formation of these toxins cannot be excluded.

6.6.4.7. Toxin Insertion

Following binding to their receptors, Cry toxins insert into membrane of midgut epithelial cells to form lytic pores. Insertion of Cry toxins into membranes requires a major conformational change in the toxin to expose a hydrophobic surface that can interact with the membrane bilayer. Domain I has been recognized as the pore forming domain based on mutagenesis studies (Wu and Aronson, 1992; Cooper *et al*., 1998), and on the similarity of the structure of this domain with other pore forming domains of bacterial toxins, such as colicin Ia, diphtheria translocation domain, and hemolysin (Aronson and Shai, 2001). The α-helices of domain I of Cry1 toxins are long enough to span the membrane and have an amphipathic character (Aronson and Shai, 2001). Helix α-5 is highly hydrophobic and conserved (conserved block 1) among all three-domain Cry toxin families (de Maagd *et al*., 2001). Cross-linking experiments done in Cry1Ac, that was genetically engineered to create disulfide bridges between some α-helices of domain I, showed that domain I swings away from the rest of the toxin exposing the α-7 helix for the initial interaction with the membrane, resulting in membrane insertion of the hairpin formed by helices α-4 and α-5 (Schwartz *et al*., 1997b). Analyses of the insertion capabilities of synthetic peptides corresponding to the seven α-helices of domain I from Cry3A showed that α-1 is the only helix that does not interact with the membrane, in contrast to the other helices (Gazit *et al*., 1998). Helices α-4 and α-5 were the only helices capable of adopting a transmembrane orientation (Aronson and Shai, 2001; Gazit *et al*., 1998). These results suggest that helices α-4 and α-5 insert into the membrane, while the rest of the helices remain on the membrane surface. These data agree with the proposed "umbrella model" of toxin insertion in which helices α-4 and α-5 insert into the membrane, leaving the rest of the α-helices in the interface of the membrane (Schwartz *et al*., 1997b). In view of the proposed role of the prepore structure in toxin insertion (Gómez *et al*., 2002b), it is tempting to speculate that in the three-dimensional structure of the prepore, four helices α-7 are exposed and form an hydrophobic surface that participates in the initial interaction of the tetramer with the membrane. Subsequently, the four hairpins formed by helices α-4 and α-5 insert into the membrane. The location of domains II and II in the membrane inserted state is unknown. However, with exception of helix α-1, membrane inserted Cry1Ac toxin resist proteinase K treatment suggesting that domains II and III might also, somehow, be inserted into the membrane (Aronson, 2000).

A different model of the inserted toxin, based on calorimetric determinations, proposed that the three-dimensional structure of the toxin does not change dramatically compared to the structure of the soluble monomer (Loseva *et al*., 2001). This model proposed that helices α-1 to α-3 are cleaved, and that the pore lumen is provided by residues of domain II and III, while the domain I hydrophobic surface (without helices α-1 to α-3) faces the lipid bilayer in a oligomeric structure (Loseva *et al*., 2001). In this regard it is interesting to note that mutagenesis of conserved arginine residues in Cry1Aa domain III affected the pore formation activity of Cry1Aa toxin, suggesting that domain III participates in channel activity (Schwartz *et al*., 1997b). Analysis of the pore formation activity of several Cry1 chimeric proteins established that the characteristics of the pore are influenced by domain II and domain III, because combination of domain III from Cry1Ab with domains I and II of Cry1C gave a protein that was more active than Cry1C in spodoptera frugiperda (Sf9) cells (Rang *et al*., 1999). Solving the structure of Cry toxins in the membrane inserted state will be important to determine the possible roles of domains II and III in pore formation.

6.6.4.8. Pore Formation

Based on the observation of large conductance states formed by several Cry toxins in synthetic planar lipid

bilayers (Lorence *et al.*, 1995; Peyronnet *et al.*, 2002) and the estimation of pore size at 10–20 Å (Von-Tersch *et al.*, 1994), it has been proposed that the pore could be formed by an oligomer of Cry toxins containing four to six toxin monomers. Moreover, intermolecular interaction between Cry1Ab toxin monomers is a necessary step for pore formation and toxicity (Soberón *et al.*, 2000). This conclusion was derived from studies that used two Cry1Ab mutant proteins that affected different steps in toxicity (binding and pore formation). Individually these mutant proteins had decreased toxicity to *M. sexta*; however, when assayed as a mixture of the two toxins, pore formation activity and toxicity against *M. sexta* larvae was recovered. These results show that monomers affected in different steps of their mode of action can form functional heterooligomers, and that oligomerization is a necessary step for toxicity (Soberón *et al.*, 2000). Recently, the structure of the pore formed by Cry1Aa toxin was analyzed by atomic force microscopy showing that the pore is a tetramer (Vie *et al.*, 2001). These data are in agreement with the proposition of a prepore structure composed of four monomers (Gómez *et al.*, 2002b). The regions of the toxin involved in oligomerization have not been determined; however, based on mutagenesis studies and analysis of toxin aggregation it has been suggested that some residues of helixα-5 may be implicated in this process (Aronson *et al.*, 1999; Vie *et al.*, 2001).

The pore activity of Cry toxins has been studied by a variety of electrophysiological techniques (Schwartz and Laprade, 2000), for example using synthetic membranes without receptor or in isolated brush border membrane vesicles containing natural receptors (Lorence *et al.*, 1995; Peyronnet *et al.*, 2001, 2002; Bravo *et al.*, 2002a). Also ion channels induced by various activated Cry1 toxins in its monomeric form – Cry1Aa (Grochulski *et al.*, 1995; Schwartz *et al.*, 1997a), Cry1Ac (Slatin *et al.*, 1990; Schwartz *et al.*, 1997a; Smedley *et al.*, 1997), and Cry1C (Schwartz *et al.*, 1993; Peyronnet *et al.*, 2002) – have been analyzed in black lipid bilayers. The channel formation of these toxins was extremely inefficient and in some studies was only achieved mechanically (Peyronnet *et al.*, 2001, 2002). The toxin concentrations needed to achieve channel formation in these conditions were two to three orders of magnitude higher than their *in vivo* insecticidal concentration. Conductance varied from 11 to 450 pS and multiple subconducting states are frequently observed showing unstable traces with current jumps of intermediate levels that are difficult to resolve. These high conductances are probably related to clusters of various numbers of identical size pores operating synchronously rather than pore oligomer structures of different sizes (Peyronnet *et al.*, 2002). Under non-symmetrical ionic conditions, the shift in the reversal potential (zero current voltage E_{rev}) towards the K^+ equilibrium potential (E_K) indicated that channels of Cry1 toxins are slightly cation selective. In fact, several reports indicated that Cry toxins form pores that are poorly selective to cationic ions including divalent cations (Lorence *et al.*, 1995; Kirouac *et al.*, 2002). As mentioned previously, the presence of receptor (APN) diminished the concentration, more than 100-fold, of Cry1Aa toxin required for pore formation activity in synthetic planar bilayers (Schwartz *et al.*, 1997a). Studies performed in lipid bilayers containing fused brush border membrane vesicles isolated from the target insect suggested that the channels formed by Cry1 toxins in the presence of their receptors have higher conductance than those formed in receptor free bilayers. The conductance of monomeric Cry1C induced channels ranged from 50 pS to 1.9 nS in bilayers containing brush border membrane vesicles from *S. frugiperda* (Lorence *et al.*, 1995). Similarly, the conductance of channels induced by the monomeric form of Cry1Aa toxin in bilayers containing membranes from *L. dispar* were about eightfold larger than the channels formed in the absence of receptor (Peyronnet *et al.*, 2001). However, the presence of multiple conductances is still observed and the instability of the currents induced in these studies suggested that even in the presence of receptors the insertion of monomers into the membrane does not involve a single conformation. In contrast, preliminary analysis of the currents induced by pure oligomer preparations in the absence of receptor showed highly stable conductance, suggesting a stable insertion of a single conformation of the toxin into the membrane (Muñoz-Garay and Bravo, unpublished data). Finally it is important to mention that, in contrast to other pore forming toxins, the pore formation activity of Cry1 proteins is not regulated by low pH, suggesting that Cry toxins are not internalized into acidic vesicles for insertion as other pore forming toxins (Tran *et al.*, 2001).

Overall, the mode of action of Cry toxins can be visualized as follows (**Figure 8**):

1. Solubilization of the crystal and activation of the protoxin by midgut proteases resulting in the monomer toxin production.
2. Binding of the monomer to the cadherin receptor located in the apical membrane of midgut cells, probably accompanied by a mild denaturation of the monomer that allows proteolytic cleavage of helix α-1. This cleavage might result in a conformational change and the formation of a

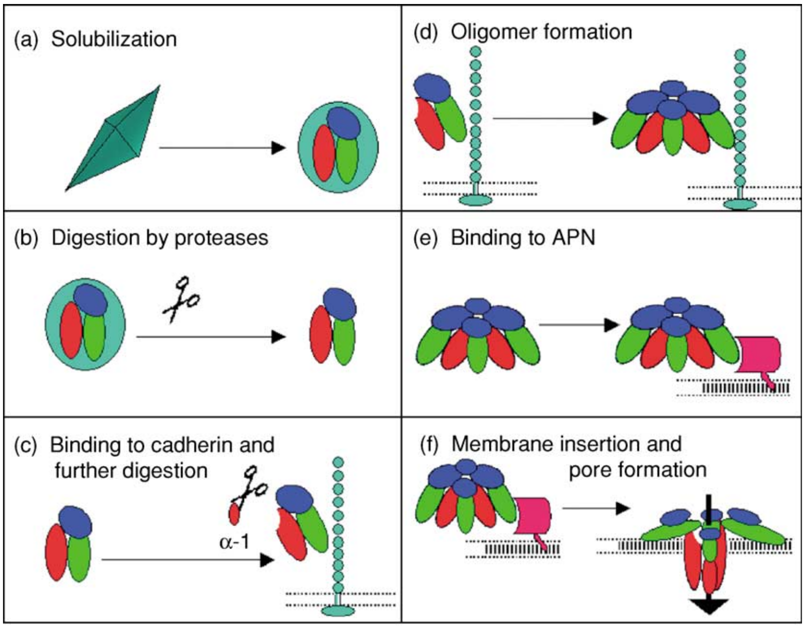


Figure 8 Mode of action of Cry toxins. (a) Crystals are solubilized and activated give rise to the monomeric toxin. (b) The toxin monomer binds the cadherin receptor, followed by proteolytic cleavage of helix α-1. (c) The tetramer is formed by intermonomeric contacts. (d) The toxin oligomer binds to the APN receptor. (e) The APN receptor and oligomeric Cry toxin localize to lipid rafts. (f) Following a conformational change the oligomer inserts into membrane forming a tetrameric pore.

molten globule state of the monomer exposing hydrophobic regions.

3. Formation of a tetramer by intermonomeric contacts.
4. Binding of the toxin oligomer to the APN receptor.
5. Mobilization of the APN receptor and Cry toxin to membrane microdomains.
6. A second conformational change of the oligomer, resulting in the insertion of the toxin into the membrane and formation of the membrane active tetrameric pore.

6.6.5. Synergism of Mosquitocidal Toxins

As previously mentioned (see Section 6.6.3.1) Cyt toxins synergizes the insecticidal effect of some Cry toxins. *Bacillus thuringiensis* subsp. *israelensis* is highly toxic to different mosquito species like *Aedes* spp., *Culex* spp., and blackfly, and also to *Anopheles* spp. but with lower toxicity (Margalith and Ben-Dov, 2000). This bacterium produces a crystal inclusion composed of at least four toxins: Cry4A, Cry4B, Cry11A, and Cyt1A (Huber and Luthy , 1981; Guerchicoff *et al.*, 1997). The toxicity of the crystal inclusion is greater, by far, than the toxicity of the isolated Cry and Cyt components. These Cry toxins are more toxic against mosquito larvae than Cyt1A with a difference in toxicity of at least 25-fold (Wirth *et al.*, 1997). However, the Cyt1A toxin synergizes the effect of the other Cry toxins. Toxicity of different combinations of Cry toxins with CytA1 was higher than the addition of expected toxicities of the isolated components (Crickmore *et al.*, 1995; Khasdan *et al.*, 2001). Also, the Cyt toxins synergize the toxicity of the binary toxins produced by *Bacillus sphaericus*. A ratio of 10 : 1 of *B. sphaericus* toxins to Cyt1A was 3600-fold more toxic to *A. aegypti* than *B. sphaericus* alone (Wirth *et al.*, 2001), and the presence of Cyt toxin can overcome the resistance of *Culex quinquefasciatus* to the binary toxins (Wirth *et al.*, 2001).

The mode of action of Cyt toxins includes the following steps: solubilization of the toxin under the alkaline pH and reducing conditions of the midgut, proteolytic processing of the protoxin, binding of the toxin to the epithelium surface of midgut cells, and pore formation leading to cell lysis (Li *et al.*, 1996, 2001). An important difference between Cyt and Cry toxins is the lack of a protein receptor for the Cyt toxin. Cyt toxins bind phospholipids and are capable of forming pores in cell lines of different origin (Gill *et al.*, 1987; Chow *et al.*, 1989; Li *et al.*, 1996, 2001). There are currently two proposed mechanisms of insertion and membrane perturbation by Cyt toxins. The first mechanism proposes that multimers of four Cyt toxin subunits form a β-barrel structured pore within the membrane, where the transmembrane region involves

the long β-sheets of the toxin (Promdonkoy and Ellar, 2003), whereas the second proposed mechanism suggests that the Cyt toxins exert their effect through a less specific detergent action (Butko *et al*., 1997; Butko, 2003).

Besides the synergistic effect of Cyt toxins, these proteins overcome or suppress resistance of mosquitoes to the Cry toxins (Wirth *et al*., 1997). *Culex quinquefasciatus* populations resistant to Cry4A, Cry4B, or Cry11A recovered sensitivity when assayed in the presence of Cyt toxin (Wirth *et al*., 1997). Furthermore, it has not been possible to select resistant mosquitoes against Cry toxins when selection is performed in the presence of Cyt toxin (Wirth *et al*., 1997). As mentioned previously (see Section 6.6.3.1), Cyt toxins are principally found in dipteran-active strains. However, a coleopteran-active strain containing a Cyt toxin has been described, although the possible synergistic effect of this Cyt toxin to other Cry proteins has not been analyzed (Guerchicoff *et al*., 2001). The molecular mechanism of the synergistic effect of Cyt toxins is still unknown. Cyt toxins could enhance any of the steps in the mode of action of Cry toxins, such as proteolytic activation, receptor binding, or toxin insertion. One plausible mechanism could be that Cyt toxins bind to microvilli membranes and then act as receptors of Cry toxins thus providing a binding site even in resistant insect populations where the Cry receptor molecules are affected. It would be interesting to investigate whether mosquitocidal Cry proteins are able of interacting with Cyt toxins and, if so, to map the binding epitopes. Engineering Cyt toxins for synergizing coleopteran- and lepidopteran-active toxins could be very useful in preventing the development of Cry resistant insect populations in nature.

6.6.6. Genomics

The genome size of different *B. thuringiensis* strains is in the range of 2.4–5.7 Mb (Carlson *et al*., 1994). Comparison of physical maps with that of *B. cereus* strains suggests that all *B. cereus* and *B. thuringiensis* have chromosomes with very similar organizations (Carlson *et al*., 1996). In May 2003 the complete genome sequence of type strains of the close *B. thuringiensis* relatives, *B. anthracis* (GenBank Accession no. AE016879) and *B. cereus* (GenBank Accession no. AE016877), were completed (Ivanova *et al*., 2003; Read *et al*., 2003). The *B. cereus* strain ATCC 14579 could be considered a *B. thuringiensis* strain based on its ribosomal 16S sequence, but does not produce crystals inclusions. The complete sequenced genome of *B. cereus* strain ATCC 1479 has 5.427 Mb (Ivanova *et al*., 2003). Analysis of putative coding regions and comparison to the sequence of the *B. anthracis* type strain showed that this group of bacteria lacks the capacity of metabolizing diverse types of carbohydrates that characterize typical soil bacteria like *Bacillus subtilis*. Carbohydrate catabolism in the *B. cereus* group is limited to glycogen, starch, chitin, and chitosan (Ivanova *et al*., 2003). In contrast, *B. cereus, B. anthracis*, and presumably *B. thuringiensis*, have a wide variety of peptide, amino acid transporters, and amino acid degradation pathways indicating that peptides and amino acids may be the most preferred nitrogen and carbon sources for these bacteria. These data led to the speculation that probably the habitat of the common ancestor of the *B. cereus* group was the insect intestine (Ivanova *et al*., 2003). The presence in the genome of several hydrolytic activities support this speculation, chitinolytic activities could participate in the degradation of chitin present in the peritrophic membrane, while the zinc metalloprotease could enhance degradation of the major component of the peritrophic membrane mucin. Also, several genes related to possible invasion, establishment, and propagation in the host were identified in both genome sequences (Ivanova *et al*., 2003; Read *et al*., 2003). Finally, the analysis of putative PlcR regulated genes gave a figure of 55 PlcR boxes controlling over 100 genes including four other transcriptional activators implying that the PlcR regulon is more complex than expected (Ivanova *et al*., 2003).

6.6.6.1. Sequence of Plasmid pBtoxis

Bacillus thuringiensis isolates usually harbor large plasmids, which are self-transmisible, containing δ-endotoxin genes that distinguish them from *B. cereus* strains (Schnepft *et al*., 1998). Therefore, the sequencing of plasmids containing *cry* genes could help to understand the evolution of *B. thuringiensis* and the spread of *cry* genes in nature. In 2002, the complete 128 kb sequence of a toxin encoded plasmid from *B. thuringiensis* subsp. *israelensis* (pBtoxis) was released (Berry *et al*., 2002). This plasmid contains 125 putative coding sequences with an average gene length of 725 bp (Berry *et al*., 2002). Sequence analysis showed that besides the known Cry toxins genes (*cry4Aa, cry4Ba, cry11A, cry10Aa, cyt2Ba*, and *cyt1Aa*) a putative novel *cry* gene was identified. Based on phylogenetic relationships with other Cyt toxins, the novel gene was named *cyt1Ca* (Berry *et al*., 2002). In contrast to the Cyt toxins, Cyt1Ca contains an additional domain located on the C-terminus (280 residues) which shares similarity

with domains present in other bacterial toxins that are involved on carbohydrate recognition (Berry *et al*., 2002). Therefore, it was proposed that Cyt1Ca could act by recognizing carbohydrate moieties in the cell surface (Berry *et al*., 2002). Based on sequence analysis, eight pseudogenes were identified. Among these, several gene fragments that shared significative sequence identity to described *cry* genes were identified, suggesting that the ancestors of plasmid pBtoxis contained other *cry* genes that were lost subsequently (Berry *et al*., 2002). Interestingly the *cry* gene fragments are located in nearby transposon related sequences indicating that gene transposition may have caused these genetic rearrangements and could be an important mechanism for toxin gene spread and evolution (Berry *et al*., 2002). Beside *cry* genes, the plasmid pBtoxis contains several putative genes coding for virulence factors (Berry *et al*., 2002). A second *plcA* gene, coding for Pl-PLC, was identified. However, this gene is probably not translated and no PlcR box was found in the promoter region of this gene. Interestingly, a PlcR box was identified in front of a peptide antibiotic production and export system, and this peptide antibiotic may be a novel virulence factor (Berry *et al*., 2002). Other genes related to sporulation and germination were identified, suggesting that plasmids could influence the sporulation and germination of the host strain (Berry *et al*., 2002). An interesting feature of the plasmid pBtoxis is that it shares similarity with a limited number of genes of plasmid pXO1, the virulence plasmid of *B. anthracis*; 29 of 125 open reading frames show amino acid sequence similarity to open reading frames present in pXO1 (Berry *et al*., 2002).

6.6.7. Mechanism of Insect Resistance

One of the major concerns regarding the use of *B. thuringiensis* is the generation of insect populations resistant to Cry toxins. Resistance to these toxins could be obtained by interfering with any of the steps involved in the mode of action of Cry toxins. The analysis of the molecular mechanism of insect resistance to *B. thuringiensis* toxins has been, for the most part, based on the study of insect resistant populations selected under laboratory conditions. These studies could provide important knowledge that allows for the rational design of procedures needed for resistance management in the field. However, resistance mechanisms of natural insect populations are likely not to compromise significantly the fitness of the insects to guarantee the fixation of resistant mutant alleles in the population. Therefore, the mutant alleles found in laboratory selected insect populations may not necessarily be found in nature although alleles found in nature are likely to be present in laboratory selected lines.

Selection of insects resistant to *B. thuringiensis* toxins in the laboratory has been performed for a variety of insect species. Lepidopteran insects resistant to *B. thuringiensis* toxins include *Plodia interpunctella, P. xylostella, H. virescens*, and *Spodoptera exigua* (Ferré and Van Rie, 2002), while coleopteran insect species include *L. decemlineata* and *Chrysomela scripta* (Ferré and Van Rie, 2002). As mentioned above (see Section 6.6.5), selection of Cry toxin resistant dipteran insects has been performed for *C. quinquefasciatus* (Wirth *et al*., 1997). Finally resistance to Cry toxins has also been achieved for the (noninsect) nematode *C. elegans* (Griffits *et al*., 2001). Until now only one insect species, *P. xylostella*, has developed resistance to Cry toxins in the field (Ferré and Van Rie, 2002). Only the resistance mechanisms in lepidopteran insects and in *C. elegans* nematode, have been characterized biochemically. Below we will summarize recent findings on the mechanisms involved in resistance to *B. thuringiensis* toxins.

6.6.7.1. Proteolytic Activation

As mentioned previously (see Section 6.6.4.2) Cry protoxins are activated by the action of midgut proteases. A line of *P. interpunctella* resistant to *B. thuringiensis* toxin had lower protease and protoxin activating activities. These differences were shown to be due to the lack of a major trypsin-like proteinase in the gut. Genetic analysis showed a cosegregation for the lack of the major protease and insect resistance to Cry1Ab toxin (Oppert *et al*., 1997). The consequence of reduced gut protease was a lower concentration of the activated toxin in the gut (Oppert *et al*., 1997). The resistant strain showed altered growth and morphology suggesting that this allele is unlikely to be selected in natural populations.

6.6.7.2. Receptor Binding

The most frequent mechanism of resistance to Cry toxins is altered receptor binding (Ferré and Van Rie, 2002). Different resistant populations from different lepidopteran species show an effect on toxin binding (Ferré and Van Rie, 2002). In a *P. interpunctella* strain selected for resistance to Cry1Ab, toxin binding affinity (K_d) was reduced 50-fold. Interestingly, this strain showed an increased susceptibility to Cry1C toxin which correlated with an increase number of binding sites to

this toxin (Ferré and Van Rie, 2002). Several receptor molecules for Cry1 toxins in *H. virescens* and *P. xylostella* have been hypothesized based on cross-resistance and susceptibility of different Cry1 resistant populations (Ferré and Van Rie, 2002). For *H. virescens*, three different receptors have been proposed: receptor A which binds Cry1Aa, Cry1Ab, and Cry1Ac toxins; receptor B which binds Cry1Ab and Cry1Ac toxins; and receptor C which recognizes Cry1Ac toxin. Based on the susceptibility of resistant insect populations, it was concluded that receptors B and C are unlikely to be involved in toxicity (Ferré and Van Rie, 2002). In the case of *P. xylostella*, four binding sites have been proposed (Ferré and Van Rie, 2002): receptor 1 is only recognized by Cry1Aa toxin; receptor 2 binds Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, and Cry1J; receptor 3 binds Cry1B; and receptor 4 binds Cry1C (Ferré and Van Rie, 2002). Cross-resistance of Cry1A toxins with Cry1F and Cry1J correlates with sequence similarity of loop regions in domain II, in particular loop 2, of these toxins (Tabashnik *et al.*, 1994; Jurat-Fuentes and Adang, 2001) (see Section 6.6.3.3). In the case of four *P. xylostella* strains that developed resistance in the field, reduced Cry1A binding correlated with resistance (Schnepf *et al.*, 1998; Ferré and Van Rie, 2002).

In the majority of the selected resistant insect lines described above there is no information regarding the characterization at the molecular level of mutations that leads to Cry resistance. In the case of the laboratory selected *H. virescens* Cry1Ac resistant line, YHD2, it was shown that a single mutation was responsible for 40–80% of Cry1Ac resistance levels. The mutation was mapped and shown to be linked to a retrotransposon insertion in the cadherin-like gene (Gahan *et al.*, 2001). As mentioned previously (see Section 6.6.4.5), this result shows that binding of Cry1A toxins to cadherin-like receptors is an important event in the mode of action of these toxins. Based on these results, it has been suggested that if mutations of cadherin genes are the primary basis of resistance in the field, the characterization of cadherin alleles could be helpful in monitoring field resistance (Morin *et al.*, 2003). The characterization of cadherin alleles in field derived and laboratory selected strains of the cotton pest pink bollworm (*Pectinophora gossypiella*) revealed three mutated cadherin alleles that were associated with resistance in this lepidopteran insect (Morin *et al.*, 2003). **Figure 9** shows the structural features of the four mutated alleles of cadherin genes associated with resistance in *H. virescens* and *P. gossypiella*.

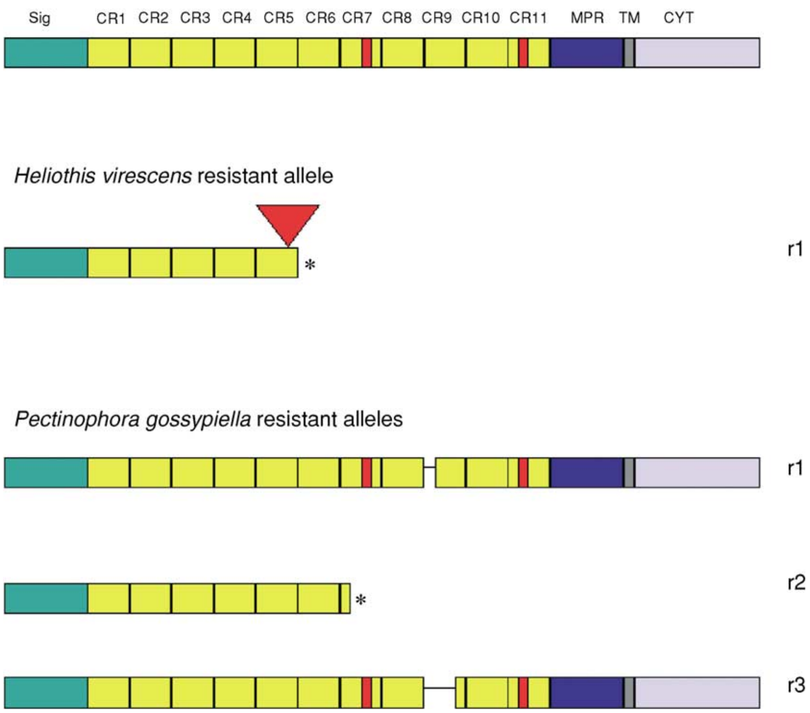


Figure 9 Gene structure of resistant cadherin alleles of *Heliothis virescens* (Gahan *et al.*, 2001) and *Pectinophora gossypiella* (Morin *et al.*, 2003). Sig corresponds to signal sequence for protein export; CR, cadherin repeat or repeated ectodomains; MPR, membrane proximal region; TM, transmembrane region; CYT, cytosolic domain; ∗, stop codon; red triangle, retrotransposon insertion; solid lines, deletions. Red sections on CR7 and CR11 are Cry1A binding epitopes mapped on *M. sexta* Bt-R_1 cadherin receptor.

6.6.7.3. Oligosaccharide Synthesis

Domain II and domain III of Cry toxins show structural similarity to carbohydrate binding domains present in other proteins, and in the case of Cry1Ac toxin, binding to the APN receptor involves the carbohydrate moiety *N*-acetylgalactosamine (de Maagd *et al*., 1999a; Burton *et al*., 1999) (see Section 6.6.3.3). Nevertheless, the most compelling evidence of the involvement of carbohydrate moieties on the mode of action of Cry toxins has come from the selection and characterization of nematode *C. elegans* Cry5B resistant lines (Griffits *et al*., 2001). Five independent *C. elegans* resistant strains were selected by feeding with *E. coli* cells expressing Cry5B toxin. Cloning and sequence of one gene responsible for the resistant phenotype (*bre-5*) showed that the mutated gene shares high sequence similarity to β-1,3-galactosyltransferase enzyme involved in carbohydrate synthesis (Griffits *et al*., 2001). The *bre-5* mutation confers cross-resistance to the Cry14A toxin which also has been shown to be toxic to insects (Griffits *et al*., 2001). The *bre-5* mutation had no effect on the fitness of the resistant nematodes (Griffits *et al*., 2001). These results suggest that carbohydrate moieties could play an important role in the mode of action (probably binding) of some Cry toxins.

In the case of the *H. virescens* YHD2 Cry1Ac resistant line, an altered pattern of glycosylation of two microvillar proteins of 63 and 68 kDa was shown to correlate with resistance (Jurat-Fuentes *et al*., 2002). As mentioned above (see Section 6.6.4.5), YHD2 strain contains several mutations responsible for its Cry1Ac resistant phenotype. Although the most important resistant allele is the disruption of the cadherin-like gene, altered glycosylation of microvilli membrane proteins increases its resistant phenotype (Jurat-Fuentes *et al*., 2002).

6.6.8. Applications of Cry Toxins

6.6.8.1. Forestry

One of the most successful applications of *B. thuringiensis* has been the control of lepidopteran defoliators that are pests of coniferous forests mainly in Canada and the United States. The strain HD-1, producing Cry1Aa, Cy1Ab, Cry1Ac, and Cry2Aa toxins, has been the choice for controlling these forests defoliators. Use of *B. thuringiensis* for the control spruce worm (*Choristoneura fumiferana*) started in the mid-1980s (van Frankenhuyzen, 2000). Afterwards *B. thuringiensis* has been used for the control of jackpine budworm (*C. pinus pinus*), Western spruce worm (*C. occidentalis*), Eastern hemlock looper (*Lambdina fiscellaria fiscellaria*) gypsy moth (*L. dispar*), and white-marked tussock moth (*Orgyia leucostigma*) (van Frankenhuyzen, 2000). In Canada, for the control of spruce worm, up to 6 million ha have been sprayed with *B. thuringiensis*, while up to 2.5 million ha have been sprayed for the control of other defoliators. In the case of United States up to 2.5 million ha are known to have been sprayed with *B. thuringiensis*. In these countries, 80–100% of the control of forests defoliators relies on the use of *B. thuringiensis*. In Europe, *B. thuringiensis* use for the control of defoliators greatly increased in the 1990s, reaching up to 1.8 million ha of forests treated by the end of the decade (van Frankenhuyzen, 2000). Successful application for the control of defoliators is highly dependent on proper timing, weather conditions, and high dosage of spray applications. These factors combine to determine the probability of larvae ingesting a lethal dose of toxin (van Frankenhuyzen, 2000). The use of *B. thuringiensis* in the control of defoliators has resulted in a significant reduction in the use of chemical insecticides for pest control in forests.

6.6.8.2. Control of Mosquitoes and Blackflies

As mentioned previously (see Section 6.6.5), *B. thuringiensis* subsp. *israelensis* is highly active against disease vector mosquitoes like *Aedes aegypti* (the vector of dengue fever), *Simulium damnosum* (the vector of onchocerciasis), and certain *Anopheles* species (vectors of malaria). The high insecticidal activity, the lack of resistance to this *B. thuringiensis* subspecies, the lack of toxicity to other organisms, and the appearance of insect resistant populations to chemical insecticides have resulted in the rapid use of *B. thuringiensis* subsp. *israelensis* as an alternative method for control of mosquitoes and blackfly populations.

In 1983, very soon after the discovery of *B. thuringiensis* subsp. *israelensis*, a control program for the eradication of onchocerciasis was launched in 11 countries in West Africa. This program was needed since the *S. damnosum* populations had developed resistance to organophosphates used for larval control (Guillet *et al*., 1990). At the present time, more than 80% of the region is protected by applications of this bacterial formulation and 20% with temephos (Guillet *et al*., 1990). As a consequence the disease has been controlled, protecting over 15 million children without the appearance of resistance this bacteria (Guillet *et al*., 1990).

In the Upper Rhine Valley in Germany, *B. thuringiensis* subsp. *israelensis* along with *B. sphaericus*

has been used to control *Aedes vexans* and *Culex pipiens pipiens* mosquitoes. Each year, approximately 300 km of river and 600 km^2 inundation areas are treated. Since 1981, more than 170 000 ha of mosquito breeding sites have been treated with different *B. thuringiensis* subsp. *israelensis* formulations. This program (KABS) has resulted in the reduction of mosquito populations by over 90% each year (Becker, 2000). These results are comparable to the use of *B. thuringiensis* subsp. *israelensis* for the control of *A. vexans* in the United States and in southern Switzerland (Becker, 2000). Again, no resistance to has been observed even after more than 20 years of the launch of the program (Becker, 2000).

High incidences of malaria in Hubei Province in China were reduced by more than 90% by the application of *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* into the Yangtze River. The vector mosquitoes *Anopheles sinensis* and *A. anthropophagus* populations were reduced 90% by weekly applications of fluid formulations of two *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* strains (Becker, 2000). In Brazil the outbreak of *A. aegypti* resistant populations to chemical insecticides in Rio de Janeiro and São Paulo led to the application of *B. thuringiensis* subsp. *israelensis* formulations for mosquito control (Regis *et al.*, 2000).

The success of vector control using *B. thuringiensis* subsp. *israelensis* will certainly spread the use of this *B. thuringiensis* subspecies around the world. However, the low activity of *B. thuringiensis* subsp. *israelensis* against certain vector mosquitoes, particular *Anopheles* spp., will require the isolation of strains with novel *cry* genes that are effective against different disease vectors.

6.6.8.3. Transgenic Crops

Bacillus thuringiensis is the leading biopesticide used in agriculture. However, only 2% of the insecticidal market consists of *B. thuringiensis* sprayable products; the rest consists mostly of chemical insecticides (Navon, 2000). Since the discovery of the HD-1 strain, formulations for lepidopteran pests control were released and products for the control of coleopteran insects are also available. However, the use of sprayable *B. thuringiensis* for insect control has been limited. Many reasons have contributed to this low acceptance of such commercial *B. thuringiensis* products. These include: the higher costs of commercial *B. thuringiensis* products; the lower efficacy of *B. thuringiensis* as compared to chemical insecticides; the limited persistence of *B. thuringiensis* products; the narrow insect spectrum of *B. thuringiensis;* and the fact that sucking and borer insects are poorly controlled (Navon, 2000).

However, the creation of transgenic crops that produce crystal *B. thuringiensis* proteins has been a major breakthrough, and substitution of chemical insecticides provides environmental friendly alternatives. In transgenic plants Cry toxin is produced continuously protecting the toxin from degradation and making it available to chewing and boring insects. Cry protein production has been improved by engineering *cry* genes with a codon usage compatible with that of plants, by removal of putative splicing signal sequences and by deletion of the C-terminal region of the protoxin (Schuler *et al.*, 1998). The first *B. thuringiensis* crop (Bt-potato) was commercialized in 1995. These transgenic plants expressed the Cry3A protein for protection against Colorado potato beetles resulting in 40% reduction in insecticidal use in 1997 (Schuler *et al.*, 1998). In 1996 Bt-cotton, producing the Cry1Ac toxin, was released to protect cotton from tobacco budworm, cotton bollworm, and pink bollworm. Also in 1996 Bt-maize, producing Cry1Ab toxin, was released for the control of European corn borer (*Ostrimia nubilalis*) larvae (Schuler *et al.*, 1998). By 2000, transgenic crops were grown on 44.3 million ha globally. Of this, 23% were insect resistant Bt-maize and 74% were herbicide resistant crops (James, 2001). Yield effects of insect resistant crops in the United States are less than 10% on average, but nevertheless use of insect resistant crops has considerably reduced the use of chemical pesticides (Qaim and Zilberman, 2003). However, the use of Bt-cotton in countries like China, Mexico, and India has showed that the use of this Bt-crop has a significant effect on yields and also in the use of chemical pesticides, since in underdeveloped countries yield loss is mainly due to technical and economical constraints that are overcome in part by the use of insect resistant crops (Qaim and Zilberman, 2003; Toenniessen *et al.*, 2003).

6.6.9. Public Concerns on the Use of *B. thuringiensis* Crops

Three major concerns have been raised on the use of Bt-crops: insect resistance, a direct effect on nontarget insects, and transgene flow to native landraces.

Widescale introduction of Bt-crops endangers the durability of *B. thuringiensis* as an insecticide in crops and in sprays since continuous exposure to Bt-plants will lead to selection for Cry toxin resistant insect populations. In response to this threat, resistance management strategies associated with the release of Bt-crops have been developed. The predominant approach involves the combination of

high production levels of the Cry protein combined with the establishment of refuge zones (de Maagd *et al*., 1999b; Conner *et al*., 2003). Refuges are areas of nontransgenic crops that are within close proximity of fields with Bt-plants. This procedure aims to maintain a population of susceptible insects that contain wild-type alleles. In most cases studied, resistance to Cry toxins is conferred by recessive mutations (de Maagd *et al*., 1999b; Ferré and Van Rie, 2002; Conner *et al*., 2003). Thus mating of homozygous individuals having susceptible alleles with heterozygous individuals having a resistant allele will give rise to susceptible heterozygote progeny, thereby delaying the development of resistance. Also, second generation transgenic crops are now being developed that produce two different Cry proteins that bind to different receptor molecules in the same insect. Thus, the possibilities of selecting mutants affected in receptor–toxin interaction are diminished exponentially (de Maagd *et al*., 1999b).

The massive use of *B. thuringiensis* sprays for forest defoliators raised concern on the effect of *B. thuringiensis* toxins on nontarget insects (Scriber *et al*., 2001). More recently, public concerns over the impact of Bt-crops on nontarget insects has increased based on results obtained on the toxicity of Bt-maize pollen to the Monarch butterfly (*Danaus plexippus*) (Losey *et al*., 1999). In this study, pollen, from a commercial variety of Bt-maize (N4640) expressing the *cry1Ab* gene, when spread onto milkweed (*Asclepias syriaca*) leaves was lethal to Monarch butterfly caterpillars in the laboratory (Losey *et al*., 1999). However, follow-up studies showed that low expression of *B. thuringiensis* toxin genes in pollen of most commercial transgenic hybrids, the lack of toxicity at the expected field rates, the absence of overlap between the pollen shed and larval activity, and the limited overlap in distribution of Bt-maize and milkweed, made field risks to the Monarch populations negligible (Gatehouse *et al*., 2002; Hellmich *et al*., 2001; Oberhauser *et al*., 2001; Pleasants *et al*., 2001; Sears *et al*., 2001; Stanley-Horn *et al*., 2001; Zangerl *et al*., 2001).

Concerns have also been raised about the effects of transgene introduction, or gene flow, on the genetic diversity of crop landraces and wild relatives in areas from which the crop originated. In 2001 the presence of transgenic DNA (from Bt-maize) constructs was reported in native maize landraces in Oaxaca, Mexico (Quist and Chapela, 2001). Oaxaca is considered part of the Mesoamerican center of origin and diversification of maize. Transgenic DNA was identified by polymerase chain reaction (PCR) based methodology, and the 35S promoter sequence was found in five of seven different native Criollo maize samples analyzed (Quist and Chapela, 2001). Moreover, inverse PCR reactions of genomic DNA of native landraces showed a high frequency of transgene insertion into a range of genomic contexts (Quist and Chapela, 2001). Nevertheless, based on criticisms on the interpretation of these data and possible false priming in the PCR reactions (Kaplinsky *et al*., 2002; Metz and Futterer, 2002), the journal *Nature* reconsidered its previous judgement on the acceptability of the results reported by Quist and Chapela, and concluded that the evidence provided was not sufficient to justify the publication (Editorial, 2002). However, more recent independent studies performed by the Mexican government (National Institute of Ecology (INE) and National Commission of Biodiversity (Conabio) claim to have evidence on the presence of transgenic DNA in native maize landraces, although publication of these results is still pending. In any case, gene flow is not restricted to transgenic plants, and naturally selected hybrids could also impact the populations and gene content of native landraces. Even if gene flow is confirmed, further studies on the possible impact of the gene flow from commercial hybrids (transgenic or nontransgenic) to native landraces should include long-term studies on the integrity of the transgenic constructs (or genes from nontransgenic hybrids) and the maintenance of these genes in the native landraces. These studies should provide a rational framework for taking decisions regarding the introduction and commercialization of transgenic crops in center of origin and diversification of crops. The final goal should be to assure the use of this environmental friendly technology without affecting the genetic diversity of natural landraces.

Acknowledgments

Research reported in this review was supported in part by DGAPA/UNAM IN216300, CONACyT 36505-N, and USDA 2002-35302-12539.

References

Abdul-Rauf, M., Ellar, D.J., **1999**. Mutations of loop2 and loop3 in domain II of *Bacillus thuringiensis* Cry1C delta-endotoxin affect insecticidal specificity and initial binding to *Spodoptera littoralis* and *Aedes aegypti* midgut membranes. *Curr. Microbiol. 39*, 94–98.

Abrami, L., van der Goot, F.G., **1999**. Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin. *J. Cell Biol. 147*, 175–184.

Agaisse, H., Gominet, M., Okstad, O.A., Kolsto, A.-B., Lereclus, D., **1999**. PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Mol. Microbiol. 32*, 1043–1053.

Agrawal, N., Malhotra, P., Bhatnagar, R.K., **2002**. Interaction of gene-cloned and insect cell-expressed aminopeptidase N of *Spodoptera litura* with insecticidal crystal protein Cry1C. *Appl. Environ. Microbiol. 68*, 4583–4592.

Aronson, A.I., Geng, C., Wu, I., **1999**. Aggregation of *Bacillus thuringiensis* Cry1A toxins upon binding to target insect larval midgut vesicles. *Appl. Environ. Microbiol. 65*, 2503–2507.

Aronson, A.I., **2000**. Incorporation of protease K in insect larval membrane vesicles does not result in the disruption of the integrity or function of the pore-forming *Bacillus thuringiensis* δ-endotoxin. *Appl. Environ. Microbiol. 66*, 4568–4570.

Aronson, A.I., Shai, Y., **2001**. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol. Lett. 195*, 1–8.

Audtho, M., Valaitis, A.P., Alzate, O., Dean, D.H., **1999**. Production of chymotrypsin-resistant *Bacillus thuringiensis* Cry2Aa1 δ-endotoxin by protein engineering. *Appl. Environ. Microbiol. 65*, 4601–4605.

Baum, J.A., Light Mettus, A.-M., **2000**. Polypeptide compositions toxic to Diabrotica insects, obtained from *Bacillus thuringiensis*; CryET70, and methods of use. Journal Patent PCT WO/00/26378-B.

Becker, N., **2000**. Bacterial control of vector-mosquitoes and black flies. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: From Laboratory to Field Application. Kluwer, London, pp. 383–398.

Belfiore, C.J., Vadlamudi, R.K., Osman, Y.A., Bulla, L.A. Jr., **1994**. A specific binding protein from *Tenebrio molitor* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *thuringiensis*. *Biochem. Biophys. Res. Commun. 200*, 359–364.

Bernhard, K., Jarret, P., Meadows, M., Butt, J., Ellis, D.J., *et al.*, **1997**. Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization and activity against insect pests. *J. Invertebr. Pathol. 70*, 59–68.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., *et al.*, **2002**. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israeliensis*. *Appl. Environ. Microbiol. 68*, 5082–5095.

Blalock, J.E., **1995**. Genetic origins of protein shape and interaction rules. *Nature Med. 1*, 876–878.

Bosch, D., Schipper, B., van der Kleij, H., de Maagd, R.A., Stiekema, J., **1994**. Recombinant *Bacillus thuringiensis* insecticidal proteins with new properties for resistance management. *Biotechnology 12*, 915–918.

Bradley, D., Harkey, M.A., Kim, M.-K., Biever, K.D., Bauer, L.S., **1995**. The insecticidal Cry1B crystal protein of *Bacillus thuringiensis* subsp. *thuringiensis* has dual specificity to coleopteran and lepidopteran larvae. *J. Invertebr. Pathol. 65*, 162–173.

Bravo, A., **1997**. Phylogenetic relationships of *Bacillus thuringiensis* δ-endotoxin family proteins and their functional domains. *J. Bacteriol. 179*, 2793–2801.

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., *et al.*, **1998**. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl. Environ. Microbiol. 164*, 4965–4972.

Bravo, A., Miranda, R., Gómez, I., Soberón, M., **2002a**. Pore formation activity of Cry1Ab toxin from *Bacillus thuringiensis* in an improved membrane vesicle preparation from *Manduca sexta* midgut cell microvilli. *Biochem. Biophys. Acta 1562*, 63–69.

Bravo, A., Sánchez, J., Kouskoura, T., Crickmore, N., **2002b**. N-terminal activation is an essential early step in the mechanism of action of the *B. thuringiensis* Cry1Ac insecticidal toxin. *J. Biol. Chem. 277*, 23985–23987.

Burton, S.L., Ellar, D.J., Li, J., Derbyshire, D.J., **1999**. N-Acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J. Mol. Biol. 287*, 1011–1022.

Butko, P., Huang, F., Pusztai-Carey, M., Surewicz, W.K., **1997**. Interaction of the delta-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis* with lipid membranes. *Biochemistry 36*, 12862–12868.

Butko, P., **2003**. Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. *Appl. Environ. Microbiol. 69*, 2415–2422.

Buzdin, A.A., Revina, L.P., Kostina, L.I., Zalunin, I.A., Chestukhina, G.G., **2002**. Interaction of 65- and 62-kDa proteins from the apical membranes of the *Aedes aegypti* larvae midgut epithelium with Cry4B and Cry11A endotoxins of *Bacillus thuringiensis*. *Biochemistry* (Moscow) *67*, 540–546.

Cabiaux, V., Wolff, C., Ruysschaert, J.M., **1997**. Interaction with a lipid membrane: a key step in bacterial toxins virulence. *Int. J. Biol. Macromol. 21*, 285–298.

Carlson, C.R., Caugant, D.A., Kolsto, A.B., **1994**. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl. Environ. Microbiol. 60*, 1719–1725.

Carlson, C.R., Johansen, T., Lecadet, M.-M., Kolsto, A.-B., **1996**. Genomic organization of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *berliner* 1715. *Microbiology 142*, 1625–1634.

Choma, C.T., Surewicz, W.K., Carey, P.R., Pozsgay, M., Raynor, T., *et al.*, **1990**. Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*: structural implications. *Eur. J. Biochem. 189*, 523–527.

Chow, E., Singh, G.J.P., Gill, S.S., **1989**. Binding and aggregation of the 25 kDa toxin of *Bacillus thuringiensis israelensis* to cell membranes: alteration by monoclonal antibodies and amino acid modifiers. *Appl. Environ. Microbiol. 55*, 2779–2788.

Conner, A.J., Glare, T.R., Nap, J.-P., **2003**. The release of genetically modified crops into the environment. 2. Overview of ecological risk assessment. *Plant J. 33*, 19–46.

Cooper, M.A., Carroll, J., Travis, E., Williams, D.H., Ellar, D.J., **1998**. *Bacillus thuringiensis* Cry1Ac toxin interaction with *Manduca sexta* aminopeptidase N in a

model membrane envionment. *Biochem. J. 333*, 677–683.

Craig, H.S., Putnam, C., Carozi, N., Tainer, J., **1999**. Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nature Struct. Biol. 6*, 932–936.

Crickmore, N., Bone, E.J., Wiliams, J.A., Ellar, D., **1995**. Contribution of individual components of the δ-endotoxins crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israeliensis*. *FEMS Microbiol. Lett. 131*, 249–254.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., *et al.*, **1998**. Revision of the nomenclture for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev. 62*, 807–813.

Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., *et al.*, **2002**. *Bacillus thuringiensis* toxin nomenclature. http:// www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.

Dai, S.M., Gill, S.S., **1993**. *In vitro* and *in vivo* proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. *Insect Biochem. Mol. Biol. 23*, 273–283.

Daniel, A., Sangadala, S., Dean, D.H., Adang, M.J., **2002**. Denaturation of either *Manduca sexta* aminopeptidase N or *Bacillus thuringiensis* Cry1A toxins exposes binding epitopes hidden under nondenaturating conditions. *Appl. Environmen. Microbiol. 68*, 2106–2112.

de Maagd, R.A., Bakker, P., Masson, L., Adang, M.J., Sangadala, S., *et al.*, **1999a**. Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. *Mol. Microbiol. 31*, 463–471.

de Maagd, R.A., Bosch, D., Stiekema, W., **1999b**. *Bacillus thuringiensis* toxin-mediated insect resistance in plants. *Trends Plant Sci. 4*, 9–13.

de Maagd, R.A., Weemen-Hendriks, M., Stiekema, W., Bosch, D., **2000**. Domain III substitution in *Bacillus thuringiensis* delta-endotoxin Cry1C domain III can function as a specific determinant for *Spodoptera exigua* in different, but not all, Cry1-Cry1C hybrids. *Appl. Environ. Microbiol. 66*, 1559–1563.

de Maagd, R.A., Bravo, A., Crickmore, N., **2001**. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet. 17*, 193–199.

Denolf, P., Hendrickx, K., VanDamme, J., Jansens, S., Peferoen, M., *et al.*, **1997**. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. *Eur. J. Biochem. 248*, 748–761.

Donovan, W.P., Donovan, J.C., Slaney, A.C., **2000**. *Bacillus thuringiensis* cryET33 and cryET34 compositions and uses therefore. US Patent No. 6 063 756.

Dorsch, J.A., Candas, M., Griko, N.B., Maaty, W.S.A., Midbo, E.G., *et al.*, **2002**. Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracelllar domain of Bt-R_1 in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol. 32*, 1025–1036.

Dow, J.A.T., **1986**. Insect midgut function. *Adv. Insect Physiol. 19*, 187–238.

Dubois, N.R., Dean, D.H., **1995**. Synergism between Cry1A insecticidal crystal proteins and spores of *Bacillus thuringiensis*, other bacterial spores and vegetative cells against *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae. *Environ. Entomol. 24*, 1741–1747.

Du, C., Martin, A.W., Nickerson, K.W., **1994**. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. *Appl. Envion. Microbiol. 60*, 3847–3853.

Editorial, **2002**. Biodiversity (communication arising): editorial note. *Nature 416*, 600.

Ellis, R.T., Stockhoff, B.A., Stamp, L., Schnepf, H.E., Schwab, G.E., *et al.*, **2002**. Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *Appl. Environ. Microbiol. 68*, 1137–1145.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., *et al.*, **1996**. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc. Natl Acad. Sci. USA 93*, 5389–5394.

Fedhila, S., Nel, P., Lereclus, D., **2002**. The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. *J. Bacteriol. 184*, 3296–3304.

Fedhila, S., Gohar, M., Slamti, L., Nel, P., Lereclus, D., **2003**. The *Bacillus thuringiensis* PlcR-regulated gene inhA2 is necessary, but not sufficient, for virulence. *J. Bacteriol. 185*, 2820–2825.

Ferré, J., Van Rie, J., **2002**. Biochemestry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol. 47*, 501–533.

Flores, H., Soberón, X., Sánchez, J., Bravo, A., **1997**. Isolated domain II and III from *Bacillus thuringiensis* Cry1Ab δ-endotoxin binds to lepidopteran midgut membranes. *FEBS Lett. 414*, 313–318.

Gahan, L.J., Gould, F., Heckel, D.G., **2001**. Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science 293*, 857–860.

Galitsky, N., Cody, V., Wojtczak, A., Debashis, G., Luft, J.R., *et al.*, **2001**. Structure of insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. *Acta Crystallogr. D57*, 1101–1109.

Garczynski, S.F., Adang, M.J., **1995**. *Bacillus thuringiensis* CryIA(c) δ-endotoxin binding aminopeptidase in the *Manduca sexta* midgut has a glycosyl-phosphatidylinositol anchor. *Insec. Biochem. Mol. Biol. 25*, 409–415.

Gatehouse, A.M.R., Ferry, N., Raemaekers, R.J.M., **2002**. The case of the monarch butterfly: a verdict is returned. *Trends Genet. 18*, 249–251.

Gazit, E., Burshtein, N., Ellar, D.J., Sawyer, T., Shai, Y., **1997**. *Bacillus thuringiensis* cytolytic toxin associates specifically with its synthetic helices A and C in the membrane state: implications for the assembly of

oligomeric transmembrane pores. *Biochemistry 36*, 15546–15556.

Gazit, E., La Rocca, P., Sansom, M.S.P., Shai, Y., **1998**. The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* δ-endotoxin are consistent with an umbrella-like structure of the pore. *Proc. Natl Acad. Sci. USA 95*, 12289–12294.

Ge, A.Z., Shivarova, N.I., Dean, D.H., **1989**. Location of the *Bombyx mori* specificity domain on a *Bacillus thuringiensis* delta-endotoxin. *Proc. Natl Acad. Sci. USA 79*, 6951–6955.

Gill, M., Ellar, D., **2002**. Transgenic *Drosophila* reveals a functional *in vivo* receptor for the *Bacillus thuringiensis* toxin Cry1Ac1. *Insect Mol. Biol. 11*, 619–625.

Gill, S.S., Cowles, E.A., Pietrantonio, P., **1992**. The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol. 37*, 615–636.

Gill, S.S., Singh, G.J.P., Hornung, J.M., **1987**. Cell membrane interaction of *Bacillus thuringiensis* subsp. *israelensis* cytolytic toxins. *Infect. Immun. 55*, 1300–1308.

Gill, S.S., Cowles, E.A., Francis, V., **1995**. Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem. 270*, 27277–27282.

Gohar, M., Okstad, O.A., Gilois, N., Sanchis, V., Kolsto, A.-B., *et al.*, **2002**. Two-dimensional electrophoresis analysis of the extrcellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. *Proteomics 2*, 784–791.

Gómez, I., Oltean, D.I., Sanchez, J., Gill, S.S., Bravo, A., *et al.*, **2001**. Mapping the epitope in cadherin-like receptors involved in *Bacillus thuringiensis* Cry1A toxin interaction using phage display. *J. Biol. Chem. 276*, 28906–28912.

Gómez, I., Miranda-Rios, J., Rudiño-Piñera, E., Oltean, D.I., Gill, S.S., *et al.*, **2002a**. Hydropathic complementarity determines interaction of epitope ^{869}HITDTNNK^{876} in *Manduca sexta* Bt-R_1 receptor with loop 2 of domain II of *Bacillus thuringiensis* Cry1A toxins. *J. Biol. Chem. 277*, 30137–30143.

Gómez, I., Sánchez, J., Miranda, R., Bravo, A., Soberón, M., **2002b**. Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Lett. 513*, 242–246.

Gómez, I., Dean, D.H., Bravo, A., Soberón, M., **2003**. Molecular basis for *Bacillus thuringiensis* Cry1Ab toxin specificity: two structural determinants in the *Manduca sexta* Bt-R_1 receptor interact with loops α-8 and 2 in domain II of Cy1Ab toxin. *Biochemistry 42*, 10482–10489.

Griffits, J.S., Whitacre, J.L., Stevens, D.E., Aroian, R.V., **2001**. *Bacillus thuringiensis* toxin resistance from loss of a putative carbohydrate-modifying enzyme. *Science 293*, 860–864.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.L., *et al.*, **1995**. *Bacillus thuringiensis* Cry1A(a) insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol. 254*, 447–464.

Guerchicoff, A., Ugalde, R.A., Rubinstein, C.P., **1997**. Identification and characterization of a previously undescribed *cyt* gene in *Bacillus thuringiensis* subsp. *israeliensis*. *Appl. Environ. Microbiol. 62*, 2716–2721.

Guerchicoff, A., Delécluse, A., Rubinstein, C.P., **2001**. The *Bacillus thuringiensis cyt* genes for hemolytic endotoxin constitute a gene family. *Appl. Envion. Microbiol. 67*, 1090–1096.

Guillet, P., Kurstak, D.C., Philippon, B., Meyer, R., **1990**. Use of *Bacillus thuringiensis israelensis* for onchocerciasis control in West Africa. In: de Barjac, H., Sutherland, D.J. (Eds.), Bacterial Control of Mosquitoes and Blackflies. Rutgers University Press, New Brunswick, NJ, pp. 187–201.

Guttmann, D.M., Ellar, D.J., **2000**. Phenotypic and genotypic comparisons of 23 strains from the *Bacillus cereus* complex for a selection of known and putative *B. thuringiensis* virulence factors. *FEMS Microbiol. Lett. 188*, 7–13.

Haider, M.Z., Ellar, D.J., **1989**. Functional mapping of an entomocidal δ-endotoxin: single amino acid changes produce by site directed mutagenesis influence toxicity and specificity of the protein. *J. Mol. Biol. 208*, 183–194.

Haider, M.Z., Smith, G.P., Ellar, D.J., **1989**. Delineation of the toxin coding fragments and an insect-specificity region of a dual toxicity *Bacillus thuringiensis* crystal protein gene. *FEMS Microbiol. Lett. 49*, 157–163.

Hara, H., Atsumi, S., Yaoi, K., Higurashi, S., Miura, N., *et al.*, **2003**. A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins to epithelial cells of *Bombyx mori* larvae. *FEBS Lett. 538*, 29–34.

Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., *et al.*, **2000**. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*: one species on the basis of genetic evidence. *Appl. Environ. Microbiol. 66*, 2627–2630.

Hellmich, R.L., Siegfried, B.D., Sears, M.K., Stanley-Horn, D.E., Daniels, M.J., *et al.*, **2001**. Monarch larvae sensitivity to *Bacillus thuringiensis*-purified proteins and pollen. *Proc. Natl Acad. Sci. USA 98*, 11925–11930.

Huber, H.E., Luthy, P., **1981**. *Bacillus thuringiensis* δ-endotoxin; composition and activation. In: Davidson, E.W. (Ed.), Pathogenesis of Microbiol Diseases. Allanheld Osmum, Totowa, NJ, pp. 209–234.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., *et al.*, **2003**. Genome sequence of *Bacillus cereus* and comparative analysis with *B. anthracis*. *Nature 423*, 87–91.

James, C., **2001**. Global review of commercialized transgenic crops: 2001. In: International Service for the Acquisition of AgricBiotech Applications Briefs No. 24. ISAAA, Ithaca, New York.

Jenkins, J.L., Dean, D.H., **2000**. Exploring the mechanism of action of insecticidal proteins by genetic engineering methods. In: Setlow, K. (Ed.), Genetic Engineering: Principles and Methods, vol. 22. Plenum, New York, pp. 33–54.

Jenkins, J.L., Lee, M.K., Valaitis, A.P., Curtiss, A., Dean, D.H., **2000**. Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. *J. Biol. Chem. 275*, 14423–14431.

Jurat-Fuentes, J.L., Adang, M.J., **2001**. Importance of Cry1 δ-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L). *Appl. Environ. Microbiol. 67*, 323–329.

Jurat-Fuentes, J.L., Gould, F.L., Adang, M.J., **2002**. Altered glycosylation of 63- and 68-kilodalton microvillar proteins in *Heliothis virescens* correlated with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins. *Appl. Environ. Microbiol. 68*, 5711–5717.

Kaplinsky, N., Braun, D., Lisch, D., Hay, A., Hake, S., *et al.*, **2002**. Biodiversity (communication arising): maize transgene results in Mexico are artifacts. *Nature 416*, 601.

Keller, M., Sneh, B., Strizhov, N., Prudovsky, E., Regev, A., *et al.*, **1996**. Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC. *Insect Biochem. Mol. Biol. 26*, 365–373.

Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S., Zaritsky, A., **2001**. Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. *Environ. Microbiol. 3*, 798–806.

Kirouac, M., Vachon, V., Noel, J.F., Girard, F., Schwartz, J.L., *et al.*, **2002**. Amino acid and divalent ion permeability of the pores formed by the *Bacillus thuringiensis* toxins Cry1Aa and Cry1Ac in insect midgut brush boreder membrane vesicles. *Biochim. Biophys. Acta 1561*, 171–179.

Knight, P., Crickmore, N., Ellar, D.J., **1994**. The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Mol. Microbiol. 11*, 429–436.

Kouskoura, T., Tickner, C., Crickmore, N., **2001**. Expression and cristalization of an N-terminally activated form of the *Bacillus thuringiensis* Cry1Ca toxin. *Curr. Microbiol. 43*, 371–373.

Lambert, B., Buysse, L., Decock, C., Jansens, S., Piens, C., *et al.*, **1996**. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. *Appl. Environ. Microbiol. 62*, 80–86.

Lee, M.K., Young, B.A., Dean, D.H., **1995**. Domain III exchanges of *Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. *Biochem. Biophys. Res. Comm. 216*, 306–312.

Lee, M.K., You, T.H., Young, B.A., Cotrill, J.A., Valaitis, A.P., *et al.*, **1996**. Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* CryIAc toxin. *Appl. Environ. Microbiol. 62*, 2845–2849.

Lee, M.K., You, T.H., Gould, F.L., Dean, D.H., **1999**. Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. *Appl. Environ. Microbiol. 65*, 4513–4520.

Lee, M.K., Rajamohan, F., Jenkins, J.L., Curtiss, A., Dean, D.H., **2000**. Role of two arginine residues in domain II, loop 2 of Cry1Ab and Cry1Ac *Bacillus thuringiensis* δ-endotoxin in toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopetidase N. *Mol. Microbiol. 38*, 289–298.

Lee, M.K., Jenkins, J.L., You, T.H., Curtiss, A., Son, J.J., *et al.*, **2001**. Mutations at the arginine residues in alpha 8 loop of *Bacillus thuringiensis* delta-endotoxin Cry1Ac affect toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N. *FEBS Lett. 497*, 108–112.

Lereclus, D., Agaisse, H., Gominet, S., Salamitou, S., Sanchis, V., **1996**. Identification of a gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. *J. Bacteriol. 178*, 2749–2756.

Li, J., Carroll, J., Ellar, D.J., **1991**. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature 353*, 815–821.

Li, J., Pandelakis, A.K., Ellar, D., **1996**. Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* ssp. *kyushuensis* and implications for membrane pore formation. *J. Mol. Biol. 257*, 129–152.

Li, J., Derbyshire, D.J., Promdonkoy, B., Ellar, D.J., **2001**. Structural implications for the transformation of the *Bacillus thuringiensis* δ-endotoxins from water-soluble to membrane-inserted forms. *Trans. Biochem. Soc. 29*, 571–577.

Lorence, A., Darszon, A., Díaz, C., Liévano, A., Quintero, R., *et al.*, **1995**. δ-Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers. *FEBS Lett. 360*, 217–222.

Lorence, A., Darszon, A., Bravo, A., **1997**. The pore formation activity of *Bacillus thuringiensis* Cry1Ac toxin on *Trichoplusia ni* membranes depends on the presence of aminopeptidase N. *FEBS Lett. 414*, 303–307.

Loseva, O.I., Toktopulo, E.I., Vasilev, V.D., Nikulin, A.D., Dobritsa, A.P., *et al.*, **2001**. Structure of Cry3A δ-endotoxin within phopholipid membranes. *Biochemistry 40*, 14143–14151.

Losey, J.E., Raylor, L.S., Carter, M.E., **1999**. Transgenic pollen harms monarch larvae. *Nature 399*, 214.

Luo, K., Tabashnik, B.E., Adang, M.J., **1997**. Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant diamondback moths (*Plutella xylostella*). *Appl. Environ. Microbiol. 63*, 1024–1027.

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., *et al.*, **1990**. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitor. *J. Agric. Food Chem. 38*, 1145–1152.

Margalith, Y., Ben-Dov, E., **2000**. Biological control by *Bacillus thuringiensis* subsp. *israeliensis*. In: Rechcigl, J.E., Rechcigl, N.A. (Eds.), Insect Pest Management: Techniques for Environmental Protection. CRC Press, Boca Raton, FL, pp. 243–301.

Martens, J.W.M., Visser, B., Vlak, J.M., Bosch, D., **1995**. Mapping and characterization of the entomocidal domain of the *Bacillus thuringiensis* CryIA(b) protoxin. *Mol. Gen. Genet. 247*, 482–487.

Masson, L., Lu, Y.-J., Mazza, A., Brosseau, R., Adang, M.J., **1995**. The Cry1A(c) receptor purified from *Manduca sexta* displays multiple specificities. *J. Biol. Chem. 270*, 20309–20315.

Metz, M., Futterer, J., **2002**. Biodiversity (communication arising): suspect evidence of transgenic contamination. *Nature 416*, 600–601.

Mignot, T., Mock, M., Robichon, D., Landier, A., Lereclus, D., *et al.*, **2001**. The incompatibility between the PlcR- and AtxA-controlled regulons may have selected a nonsense mutation in *Bacillus anthracis*. *Mol. Microbiol. 42*, 1189–1198.

Miranda, R., Zamudio, F., Bravo, A., **2001**. Processing of Cry1Ab δ-Endotoxin from *Bacillus thuringiensis* by midgut proteases: role in toxin activation and inactivation. *Insect Biochem. Mol. Biol. 31*, 1155–1163.

Morin, S., Biggs, R.W., Shriver, L., Ellers-Kirk, C., Higginson, D., *et al.*, **2003**. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc. Natl Acad. Sci. USA 100*, 5004–5009.

Morse, R.J., Yamamoto, T., Stroud, R.M., **2001**. Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure 9*, 409–417.

Nakanishi, K., Yaoi, K., Nagino, Y., Hara, H., Kitami, M., *et al.*, **2002**. Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella*: their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry1A toxin. *FEBS Lett. 519*, 215–220.

Nagamatsu, Y., Toda, S., Yagamuchi, F., Ogo, M., Kogure, M., *et al.*, **1998**. Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. *Biosci. Biotechnol. Biochem. 62*, 718–726.

Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A., Furukawa, Y., **1999**. The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. *FEBS Lett. 460*, 385–390.

Navon, A., **2000**. *Bacillus thuringiensis* application in agriculture. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: From Laboratory to Field Application. Kluwer, London, pp. 355–369.

Oberhauser, K.S., Prysby, M.D., Mattila, H.R., Stanley-Horn, D.E., Sears, M.K., *et al.*, **2001**. Temporal and spatial overlap between monarch larvae and corn pollen. *Proc. Natl Acad. Sci. USA 98*, 11913–11918.

Oltean, D.I., Pullikuth, A.K., Lee, H.-K., Gill, S.S., **1999**. Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. *Appl. Environ. Microbiol. 65*, 4760–4766.

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H., **1997**. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *J. Biol. Chem. 272*, 23473–23476.

Pang, A.S., Gringorten, J.L., Bai, C., **1999**. Activation and fragmentation of *Bacillus thuringiensis* δ-endotoxin by high concentrations of proteolytic enzymes. *Can. J. Microbiol. 45*, 816–825.

Peyronnet, O., Vachon, V., Schwartz, J.L., Laprade, R., **2001**. Ion channels induced in planar lipid bilayers by the *Bacillus thuringiensis* toxin Cry1Aa in the presence of gypsy moth (*Lymantria dispar*) brush border membranes. *J. Membr. Biol. 184*, 45–54.

Peyronnet, O., Nieman, B., Généreux, F., Vachon, V., Laprade, R., **2002**. Estimation of the radius of the pores formed by the *Bacillus thuringiensis* Cry1C δ-endotoxin in planar lipid bilayers. *Biochim. Biophys. Acta 1567*, 113–122.

Pleasants, J.M., Hellmich, R.L., Diveley, G.P., Sears, M.K., Stanley-Horn, D.E., *et al.*, **2001**. Corn pollen deposition on milkweeds in and near cornfields. *Proc. Natl Acad. Sci. USA 98*, 11919–11924.

Promdonkoy, B., Ellar, D.J., **2003**. Investigation of the pore forming mechanism of a cytolitic δ-endotoxin from *Bacillus thuringiensis*. *Biochem. J. 374*, 255–259.

Qaim, M., Zilberman, D., **2003**. Yield effects of genetically modified crops in developing countries. *Science 299*, 900–902.

Quist, D., Chapela, I.H., **2001**. Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. *Nature 414*, 541–543.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P., Bhatnagar, R.K., **2002**. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *J. Biol. Chem. 277*, 46849–46851.

Rajamohan, F., Alcantara, E., Lee, M.K., Chen, X.J., Curtiss, A., *et al.*, **1995**. Single amino acid changes in domain II of *Bacillus thuringiensis* Cry1Ab δ-endotoxin affect irreversible binding to *Manduca sexta* midgut membrane vesicles. *J. Bacteriol. 177*, 2276–2282.

Rajamohan, F., Hussain, S.-R.A., Cotrill, J.A., Gould, F., Dean, D.H., **1996a**. Mutations at domain II, loop 3, of *Bacillus thuringiensis* Cry1Aa and Cry1Ab δ-endotoxins suggest loop 3 is involved in initial binding to lepidopteran insects. *J. Biol. Chem. 271*, 25220–25226.

Rajamohan, F., Cotrill, J.A., Gould, F., Dean, D.H., **1996b**. Role of domain II, loop 2 residues of *Bacillus thuringiensis* Cry1Ab δ-endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. *J. Biol. Chem. 271*, 2390–2396.

Rang, C., Vachon, V., de Maagd, R.A., Villalon, M., Schwartz, J.-L., *et al.*, **1999**. Interaction between functional domains of *Bacillus thuringiensis* insecticidal proteins. *Appl. Environ. Microbiol. 65*, 2918–2925.

Read, T.D., Peterson, A.N., Tourasse, N., Baillie, L.W., Paulsen, I.T., *et al.*, **2003**. The genome sequence of

Bacillus anthracis Ames and comparison to closely related bacteria. *Nature 423*, 81–86.

Regis, L., da Silva, S.B., Melo-Santos, M.A.V., **2000**. The use of bacterial larvicides in mosquito and black fly control programmes in Brazil. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro 95*(Suppl. I), 207–210.

Rosenberger, C.M., Brumel, J.H., Finlay, B.B., **2000**. Microbial pathogenesis: lipid rafts as pathogen portals. *Current Biol. 10*, 823–825.

Sayyed, A.H., Crickmore, N., Wright, D.J., **2001**. Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* is toxic to the diamondback moth, *Plutella xylostella*, and synergizes the activity of Cry1Ac towards a resistant strain. *Appl. Environ. Microbiol. 67*, 5859–5861.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J.R., *et al*., **1998**. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev. 62*, 705–806.

Schuler, T.H., Poppy, G.M., Kerry, B.R., Denholm, I., **1998**. Insect-resistant transgenic plants. *Trends Biotechnol. 16*, 168–175.

Schwartz, J.L., Garneau, L., Savaria, D., Masson, L., Brousseau, R., *et al*., **1993**. Lepidopteran-specific crystal toxins from *Bacillus thuringiensis* form cation- and anion-selective channels in planar lipid bilayers. *J. Membr. Biol. 132*, 53–62.

Schwartz, J.L., Lu, Y.J., Sohnlein, P., Brousseau, R., Laprade, R., *et al*., **1997a**. Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. *FEBS Lett. 412*, 270–276.

Schwartz, J.L., Juteau, M., Grochulski, P., Cygler, M., Préfontaine, G., *et al*., **1997b**. Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. *FEBS Lett. 410*, 397–402.

Schwartz, J.L., Potvin, L., Chen, X.J., Brousseau, R., Laprade, R., *et al*., **1997c**. Single-site mutations in the conserved alternating arginine region affect ionic channels formed by CryIAa, a *Bacillus thuringiensis* toxin. *Appl. Environ. Microbiol. 63*, 3978–3984.

Schwartz, J.L., Laprade, R., **2000**. Membrane permeabilization by *Bacillus thuringiensis* toxins: protein insertion and pore formation. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: From Laboratory to Field Application. Kluwer, London, pp. 199–217.

Scriber, J.M., **2001**. *Bt* or not *Bt*: Is that the question? *Proc. Natl Acad. Sci. USA 98*, 12328–12330.

Sears, M.K., Hellmich, R.L., Stanley-Horn, D.E., Oberhauser, K.S., Pleasants, J.M., *et al*., **2001**. Impact of *Bt* corn pollen on monarch butterfly populations: a risk assessment. *Proc. Natl Acad. Sci. USA 98*, 11937–11942.

Shao, Z., Cui, Y., Liu, X., Yi, H., Ji, J., *et al*., **1998**. Processing of δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. *J. Invertebr. Pathol. 72*, 73–81.

Simons, K., Toomre, D., **2000**. Lipid rafts and signal transduction. *Nature Rev. Mol. Cell Biol. 1*, 31–39.

Slamti, L., Lereclus, D., **2002**. A cell–cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. *EMBO J. 17*, 4550–4559.

Slatin, S.L., Abrams, C.K., English, L.H., **1990**. Delta-endotoxins form cation-selective channels in planar lipid bilayers. *Biochem. Biophys. Res. Commun. 169*, 765–772.

Smedley, D.P., Armstrong, G., Ellar, D.J., **1997**. Channel activity caused by a *Bacillus thuringiensis* delta-endotoxin preparation depends on the method of activation. *Mol. Membr. Biol. 14*, 13–18.

Smith, D.J., Ellar, D.J., **1994**. Mutagenesis of two surface-exposed loops of the *Bacillus thuringiensis* CryIC δ-endotoxin affects insecticidal specificity. *Biochem. J. 302*, 611–616.

Soberón, M., Perez, R.V., Nuñez-Valdéz, M.E., Lorence, A., Gómez, I., *et al*., **2000**. Evidences for inter-molecular interaction as a necessary step for pore-formation activity and toxicity of *Bacillus thuringiensis* Cry1Ab toxin. *FEMS Microbiol. Lett. 191*, 221–225.

Stanley-Horn, D.E., Dively, G.P., Hellmich, R.L., Mattila, H.R., Sears, M.K., *et al*., **2001**. Assesing the impact of Cry1Ab-expressing corn pollen on monarch butterfly in field studies. *Proc. Natl Acad. Sci. USA 98*, 11931–11936.

Tabashnik, B.E., Finson, N., Johnson, M.W., Heckel, D.G., **1994**. Cross-resistance to *Bacillus thuringiensis* toxin Cry1F in the diamondback moth (*Plutella xylostella*). *Appl Environ. Microbiol. 60*, 4627–4629.

Terra, W., Ferreira, C., **1994**. Insect digestive enzymes: properties, compartamentalization and function. *Comp. Biochem. Physiol. B 109*, 1–62.

Toenniessen, G.H., O'Toole, J.C., DeVries, J., **2003**. Advances in plant biotechnology and its adoption in developing countries. *Curr. Opin. Plant Biol. 6*, 191–198.

Thompson, M., Knuth, M., Cardineau, G., **2001**. *Bacillus thuringiensis* toxins with improved activity. US Patent No. 6 303 364.

Tran, L.B., Vachon, V., Schwartz, J.L., Laprade, R., **2001**. Differential effects of pH on the pore-forming properties of *Bacillus thuringiensis* insecticidal crystal toxins. *Appl. Environ. Microbiol. 67*, 4488–4494.

Tsuda, Y., Nakatani, F., Hashimoto, K., Ikawa, S., Matsuura, C., *et al*., **2003**. Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm). *Biochem. J. 369*, 697–703.

Vadlamudi, R.K., Weber, E., Ji, I., Ji, T.H., Bulla, L.A., Jr., **1995**. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J. Biol. Chem. 270*, 5490–5494.

Valaitis, A.P., Lee, M.K., Rajamohan, F., Dean, D.H., **1995**. Brush border membrane Aminopeptidase-N in the midgut of the Gypsy Moth serves as the receptor for the CryIA(c) δ-endotoxin of *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol. 25*, 1143–1151.

Valaitis, A.P., Jenkins, J.L., Lee, M.K., Dean, D.H., Garner, K.J., **2001**. Isolation and partial characterization of Gypsy moth BTR-270 an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. *Arch. Insect Biochem. Physiol. 46*, 186–200.

van Frankenhuyzen, K., **2000**. Application of *Bacillus thuringiensis* in forestry. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: From Laboratory to Field Application. Kluwer, London, pp. 371–382.

Vie, V., Van Mau, N., Pomarde, P., Dance, C., Schwartz, J.L., *et al.*, **2001**. Lipid-induced pore formation of the *Bacillus thuringiensis* Cry1Aa insecticidal toxin. *J. Membr. Biol. 180*, 195–203.

Von-Tersch, M.A., Slatin, S.L., Kulesza, C.A., English, L.H., **1994**. Membrane-permeabilizing activities of *Bacillus thuringiensis* coleopteran-active toxin CryIIIB2 and CryIIIB2 domain I peptide. *Appl. Environ. Microbiol. 60*, 3711–3717.

Warren, G., **1997**. Vegetative insecticidal proteins: novel proteins for control of corn pests. In: Carozzi, N., Koziel, M. (Eds.), Advances in Insect Control: The Role of Transgenic Plants. Taylor and Francis, London, pp. 109–121.

Wei, J.-Z., Hale, K., Carta, L., Platzer, E., Wong, C., *et al.*, **2003**. *Bacillus thuringiensis* crystal proteins that target nematodes. *Proc. Natl Acad. Sci. USA 100*, 2760–2765.

Widner, W.R., Whiteley, H.R., **1990**. Location of the dipteran specific region in a lepidopteran–dipteran crystal protein from *Bacillus thuringiensis*. *J. Bacteriol. 172*, 2826–2832.

Wirth, M.C., Georghiou, G.P., Federeci, B.A., **1997**. CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proc. Natl Acad. Sci. USA 94*, 10536–10540.

Wirth, M.C., Delécuse, A., Walton, W.E., **2001**. CytAb1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and resistant *Culex quinquefasciatus* (Diptera: Culicidae). *Appl. Environ. Microbiol. 67*, 3280–3284.

Wu, D., Aronson, A.I., **1992**. Localized mutagenesis defines regions of the *Bacillus thuringiensis* δ-endotoxin involved in toxicity and specificity. *J. Biol. Chem. 267*, 2311–2317.

Wu, D., Johnson, J.J., Federeci, B.A., **1994**. Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. *Mol. Microbiol. 13*, 965–972.

Wu, S.-J., Dean, D.H., **1996**. Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CryIIIA δ-endotoxin. *J. Mol. Biol. 255*, 628–640.

Yamagiwa, M., Esaki, M., Otake, K., Inagaki, M., Komano, T., *et al.*, **1999**. Activation process of dipteran-specific insecticidal protein produced by *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol. 65*, 3464–3469.

Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., *et al.*, **1997**. Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. *Eur. J. Biochem. 246*, 652–657.

Yaoi, K., Nakanishi, K., Kadotani, T., Imamura, M., Koizumi, N., *et al.*, **1999**. *Bacillus thuringiensis* Cry1Aa toxin-binding region of *Bombyx mori* aminopeptidase N. *FEBS Lett. 463*, 221–224.

Zangerl, A.R., McKenna, D., Wraight, C.L., Carroll, M., Ficarello, P., *et al.*, **2001**. Effects of exposure to event 176 *Bacillus thuringiensis* corn pollen on monarch and black swallowtail caterpillars under field conditions. *Proc. Natl Acad. Sci. USA 98*, 11908–11912.

Zalunin, I.A., Revina, L.P., Kostina, L.I., Chestukhina, G.G., Stepanov, V.M., **1998**. Limited proteolysis of *Bacillus thuringiensis* CryIG and CryIVB δ-endotoxins leads to formation of active fragments that do not coincide with the structural domains. *J. Protein Chem. 17*, 463–471.

Zhuang, M., Oltean, D.I., Gómez, I., Pullikuth, A.K., Soberón, M., *et al.*, **2002**. *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. *J. Biol. Chem. 277*, 13863–13872.

6.7 Mosquitocidal *Bacillus sphaericus*: Toxins, Genetics, Mode of Action, Use, and Resistance Mechanisms

J-F Charles, Institut Pasteur, Paris, France
I Darboux and D Pauron, INRA, Sophia Antipolis, France
C Nielsen-Leroux, Institut Pasteur, Paris, France

6.7.1. Introduction

6.7.1.1. Generalities

Bacillus thuringiensis was the first bacterium described to have insecticidal activity. The discovery of a particular strain of *B. thuringiensis* serovar *israelensis* in the late seventies (Goldberg and Margalit, 1977; de Barjac, 1978) opened up the possibility of using microorganisms to control Diptera Nematocera vectors of disease agents, such as mosquitoes (Culicidae) and black-flies (Simuliidae).

The first strains of *B. sphaericus* to be active against mosquito larvae were isolated from moribund mosquito larvae (Kellen *et al*., 1965). The larvicidal activity of these isolates was very low, so they could not be used to control mosquitoes (**Table 1**). The identification of the SSII-1 strain in India (Singer, 1973) renewed interest in the use of this species as a biological control agent. However, it was only after the isolation of strain 1593 from dead mosquito larvae in Indonesia (Singer, 1974) that the potential of *B. sphaericus* as a real biological control agent for mosquito populations was taken seriously (Singer, 1977). This occurred because of the high level of mosquitocidal activity of this strain.

6.7.1.2. Comparison of the Properties of Mosquitocidal Strains of *Bacillus sphaericus*

B. sphaericus Meyer and Neide (Neide, 1904) is an aerobic bacterium that produces terminal spherical spores. It lacks a number of biochemical pathways, meaning that it is unable to metabolize sugars. This species is generally considered to be heterogeneous, based on the findings obtained through a variety of genetic and biochemical analyses. Given the large number of strains isolated, it became difficult to distinguish between toxic and atoxic strains, or between the toxic strains themselves.

The relationships between *B. sphaericus* strains have been examined by DNA homology (Krych *et al*., 1980). Five groups were initially identified but group II has since been further subdivided into IIA (over 79% identity, containing all toxic strains), and IIB. Two further systems were then developed: bacteriophage typing (Yousten *et al*., 1980; Yousten *et al*., 1984) and serotyping using flagellar antigens as for *B. thuringiensis* (de Barjac *et al*., 1985; de Barjac, 1990). Both techniques gave very similar classifications, with the mosquitocidal strains in group IIA. Currently, nine serotypes containing active strains have been identified (**Table 1**). Other

Table 1 Comparative properties of some mosquitocidal strains of *B. sphaericus*[a]

Strain	*Origin*	*Source*	*Flagellar serotype*	*Phage type*	*DNA group*	*RNA group*	*Fatty acid group*	*Mosquitocidal activity*[b]	*Crystal (Bin) genes*[c]	*Mtx genes*		
										1	*2*	*3*
Kellen K, Q	USA	*Culiseta incidens*	1a	1	IIA	?	AIII	Low	−	+	+	+
BS-197	India	?	1a	?	?	?	?	High	+	+	+	+
SSII-1	India	*Culex fatigans*	2a,2b	2	IIA	RIIA	AII	Moderate	−	+	+	+
IAB 881	Ghana	*Culex* sp. breeding site	3	−[d]	?	?	AV	High	1	+	?	?
LP1-G	Singapore	Mosquito breeding site	3	8	IIA	?	?	Moderate	4	±[e]	?	?
1593	Indonesia	*Culex fatigans*	5a,5b	3	IIA	RIIA	AIII	High	2	+	+	?
2362	Nigeria	*Simulium damnosum*	5a,5b	3	IIA	RIIA	AIII	High	2	+	+	+
1691	El Salvador	*Anopheles albimanus*	5a,5b	3	IIA	?	?	High	2	+	+	+
IAB 59	Ghana	*Anopheles gambiae*	6	3	?	?	?	High	1	+	+	+
31	Turkey	Mosquito breeding site	9a,9c	8	?	?	?	Low	−	+	+	+
2314-2	Thailand	?	9a,9b	−[d]	IIA	?	?	Low	−	+	+	+
2297	Sri Lanka	*Culex pipiens*	25	4	IIA	RIIA	AV	High	3	+	+	+
2173 (ISPC5)	India	*Culex fatigans*	26a,26b	−[d]	IIA	?	AIII	Moderate	−	−	−	−
IAB 872	Ghana	*Culex* sp. breeding site	48	3	?	?	?	High	1	+	+	+

[a]Data compiled from Krych *et al.* (1980), Yousten *et al.* (1980), de Barjac *et al.* (1985), Alexander and Priest (1990), Frachon *et al.* (1991), Guerineau *et al.* (1991), Thanabalu *et al.* (1991), Liu *et al.* (1996a,1996b), Thanabalu and Porter (1995, 1996), Priest *et al.* (1997), Humphreys and Berry (1998), and from data of the IEBC Collection (Pasteur Institute). +: present; −: absent; ?: no information available.

[b]Based on the lethal concentration 50% (LC_{50}) on *Culex pipiens* fourth instars.

[c]Based on the deduced amino-acid sequences, when known (see also **Table 2**).

[d]Strains not responding to any of the bacteriophages tested.

[e]− following the original publication (Humphreys and Berry, 1998); + following Z. Yuan, personal communication.

techniques such as numerical classification based on the taxonomy of phenotypic features (Alexander and Priest, 1990; Guerineau *et al.*, 1991), cellular fatty acid composition analysis (Frachon *et al.*, 1991), ribotyping (Aquino de Muro *et al.*, 1992; Miteva *et al.*, 1998), and random amplified polymorphic DNA analysis (Woodburn *et al.*, 1995) showed that most of the pathogenic strains are grouped in a few clusters. None of these methods can be used to predict the level of toxicity of a given strain. Strains can be divided into three groups according to their toxicity, but these groups do not correspond to the groups defined by any other classification method (**Table 1**). The genes encoding a number of toxins have been cloned (see below), and it has been suggested that hybridization experiments could be used to predict activity (Woodburn *et al.*, 1995). However, some strains that harbor toxin genes but display only weak mosquitocidal activity have been reported. Therefore, the only way to identify potentially valuable strains and to increase our knowledge of the real nature of the mosquito-larvicidal activity is to determine toxicity experimentally using mosquito larvae.

B. sphaericus strains are generally highly active against *Anopheles* and *Culex* larvae but are poorly, or are not toxic to *Aedes* larvae. However, susceptibility appears to depend on the species of mosquito considered, and thus can vary within a given genus (Berry *et al.*, 1993). Toxicity levels vary greatly between the larvicidal serotypes and even within the same serotype (Thiéry and de Barjac, 1989). Therefore, the relative potency of each strain is expressed as the specific titer on the different mosquito species and as the activity ratios derived from such titers (Thiéry and de Barjac, 1989).

Singer was the first to suggest that *B. sphaericus* produces toxins, rather than producing septicemia, like *B. thuringiensis* on Lepidoptera (Singer, 1973). A few years later, Davidson and Myers (Davidson and Myers, 1981) observed parasporal inclusions that they believed to participate in the toxic action of *B. sphaericus*. Indeed, all the most toxic strains produce such inclusions during sporulation (**Figure 1**). These inclusions have a crystalline ultrastructure and are released into the medium along with the spore after the completion of sporulation. The relationship between sporulation, crystal formation, and mosquitocidal activity, was first clearly established for strains belonging to serotype H5a,5b (Myers *et al.*, 1979; Broadwell and Baumann, 1986), H25 (Yousten and Davidson, 1982; de Barjac and Charles, 1983; Kalfon *et al.*, 1984), and H6 (de Barjac *et al.*, 1988). Later on, the partial purification of crystals toxic to mosquito larvae (Payne and Davidson, 1984) and studies showing mutants that failed to make crystals were not mosquitocidal (Charles *et al.*, 1988), confirmed the toxic nature and protein composition of the crystals. More recently, toxins different to the crystal toxins (Mtx toxins) were identified, thus renewing interest in *B. sphaericus* as a mosquitocidal agent (Thanabalu *et al.*, 1991; Thanabalu and Porter, 1995).

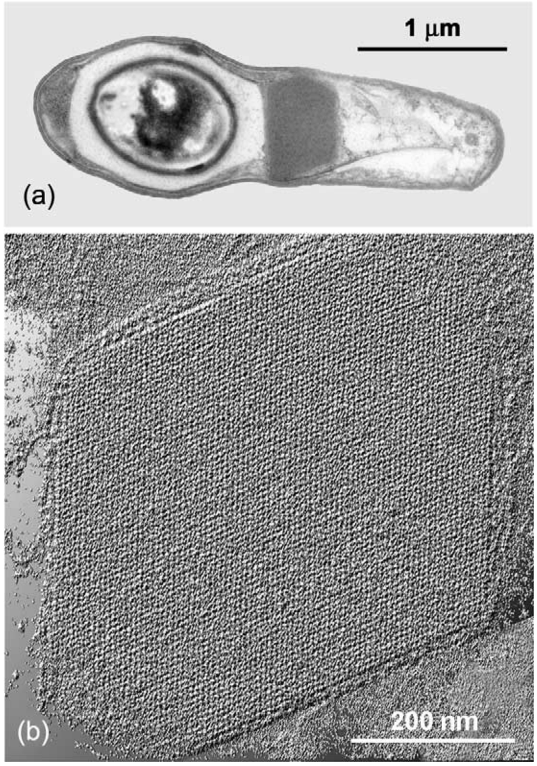


Figure 1 (a) *B. sphaericus* strain 2297 spore and crystal during the lysis of the sporangium, at the end of the sporulation process. (b) Detail of the crystal lattice.

There are three reviews summarizing *B. sphaericus* genetics, toxins and mode of action (Baumann *et al.*, 1991; Porter *et al.*, 1993; Charles *et al.*, 1996). The increasing field use of *B. sphaericus* has led to some cases of resistance. It is important to understand the nature and mode of action of *B. sphaericus* toxins and the mechanisms of resistance, if we are to restrict the development of resistance. These issues have been extensively investigated and an overview is given below.

6.7.2. Biochemistry and Genetics of *B. sphaericus* Toxins

At least two kinds of toxin (crystal toxins, or Bin toxins, and Mtx toxins) seem to account for the larvicidal activity of *B. sphaericus*. They differ both in their composition and time of synthesis. The crystal toxins are present in all highly active

strains (Table 1) and are produced during sporulation. The Mtx toxins are responsible for the activity of most of the less active strains, and seem to be synthesized only during the vegetative phase.

6.7.2.1. Crystal Toxins

The crystal toxin is composed of a 42-kDa protein and a 51-kDa protein, hereafter designated BinA and BinB, respectively (Baumann *et al*., 1987; Berry and Hindley, 1987; Hindley and Berry, 1987; Arapinis *et al*., 1988; Baumann *et al*., 1988; Hindley and Berry, 1988; Berry *et al*., 1989). These two proteins are synthesized in approximately equimolar amounts, assembled in crystal structures and visible at about stage III of sporulation (Yousten and Davidson, 1982; Kalfon *et al*., 1984; Baumann *et al*., 1985). The genes encoding both proteins have been cloned from several highly toxic strains. They encode proteins with predicted molecular masses of 51.4 and 41.9 kDa (Baumann *et al*., 1987; Berry and Hindley, 1987; Hindley and Berry, 1987; Arapinis *et al*., 1988; Baumann *et al*., 1988; Hindley and Berry, 1988; Berry *et al*., 1989). They appear to be organized in an operon, with a 174- to 176-bp intergenic region. A stem-loop structure (characteristic of transcription terminators) has been identified downstream from the *binA* gene (Figure 2, top; Hindley and Berry, 1987; Baumann *et al*., 1988). No sequences similar to *B. subtilis* sporulation promoters have been found upstream from the *binB* gene. However, both genes are expressed only during sporulation in *B. subtilis* (Baumann and Baumann, 1989). Moreover, *lacZ* fusion experiments have shown that transcription begins immediately before the end of exponential growth and continues into stationary phase in *B. sphaericus* (Ahmed *et al*., 1995). This probably allows sufficient amounts of the proteins for crystal formation to accumulate by stage III.

In mutants of *B. sphaericus* 2362 strain in which *spo0A* or *spoIIAC* sporulation genes have been disrupted by insertional mutations, toxicity on mosquito larvae is greatly reduced and no crystal

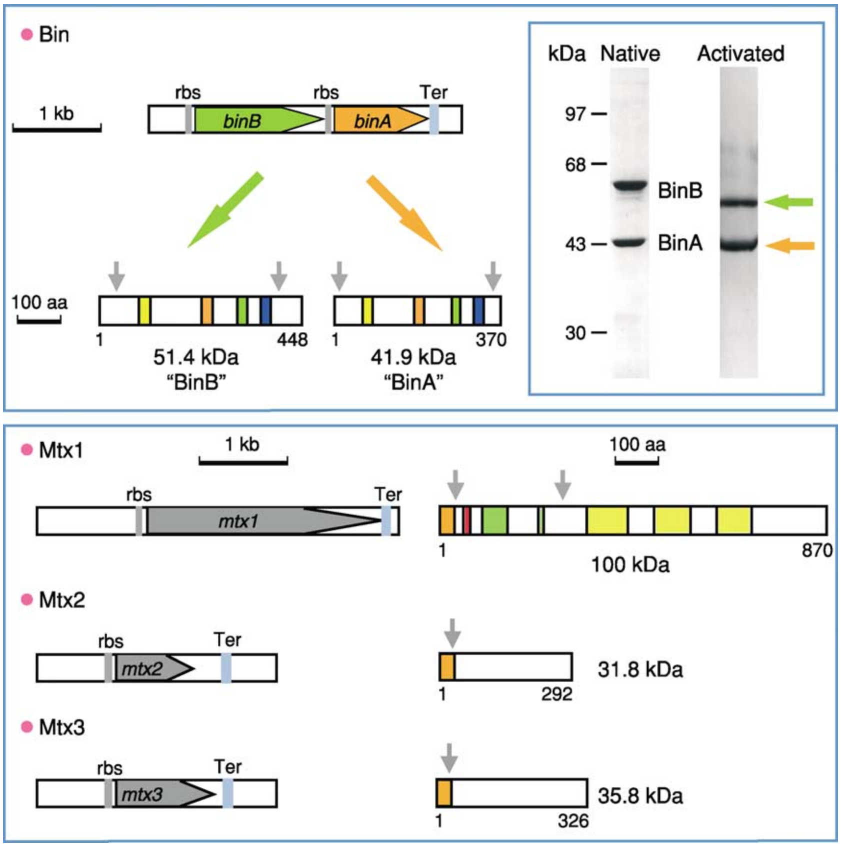


Figure 2 *B. sphaericus* toxin genes and schematic representation of corresponding polypeptides. Top, crystal (Bin) toxin: regions shared by BinB and BinA are represented by identical colors. Inset: SDS-PAGE (PolyAcrylamide Gel Electrophoresis) of native toxin and of toxin after activation by trypsin or by mosquito midgut juice. Bottom, Mtx toxins: the orange boxes indicate potential signal sequences; the colors in Mtx1 represent the potential transmembrane sequence (red), the two regions shared with ADP-ribosyltransferase toxins (green), and internal repeated sequences (yellow). Tryptic cleavage sites for each toxin are represented by gray arrows. Potential ribosome binding sites (rbs) and terminator sequences (Ter) are also indicated.

proteins are detected by SDS-PAGE or Western blotting (El-Bendary *et al.*, 2004). These results indicate that crystal protein synthesis is dependent on early and late mother cell sigma factors, as is the synthesis of most crystal proteins in *B. thuringiensis*.

In all the studied strains, toxin genes are chromosomal, although these strains contain plasmids (Aquino de Muro *et al.*, 1992). Both *binA* and *binB* have been identified in a wide variety of strains, and DNA hybridization has shown that all highly toxic strains tested contain very similar sequences (Aquino de Muro and Priest, 1994). The amino acid sequences of BinB and BinA are not similar to those of dipteran active toxins produced by *B. thuringiensis* serovar *israelensis* (*Bti*), or to any other toxins. However, BinB and BinA share four segments of sequence similarity (**Figure 2**, top; Baumann *et al.*, 1988), the significance of which remains unclear. Nevertheless, this indicates that BinB and BinA of *B. sphaericus* constitute a separate family of insecticidal toxins (Baumann *et al.*, 1988).

The toxin coding and flanking regions of strains 1593, 2362 and 2317.3 are identical over a span of 3479 nucleotides, whereas the sequences in strains IAB-59 and 2297 differ by 7 and 25 nucleotides, respectively (Berry *et al.*, 1989). These differences result in 5 and 3 amino acid differences in BinB, respectively, and in 1 and 5 amino acid differences in BinA, respectively. Partial or total sequencing of toxin genes led to the establishment of a classification system including four types of Bin toxin (types 1 to 4) (**Table 2**) (Priest *et al.*, 1997; Humphreys and Berry, 1998). The amino acid differences at positions 99, 135 (hydrophobic) and 267 (basic) of BinA are conservative. Type 1 and 2 BinA have a His at position 125, whereas types 3 and 4 contain an Asn. Type I BinA contains an acidic amino acid at position 104, whereas the other three types do not. Interestingly, BinA from type 4 (from strain LP1-G, only weakly active on mosquito larvae) has a serine (polar) at position 93 whereas types 1 to 3 contain a hydrophobic residue (Leu) (**Table 2**). Replacement of the serine at position 93 of BinA from LP1-G with a leucine restored the toxicity of the mutated type 4 toxin, whereas the replacement of the leucine with a serine at position 93 of the type 2 BinA from strain 1593 led to a significant loss in toxicity (Yuan *et al.*, 2001). This confirms that the amino acid at position 93 plays a key role in the toxicity of the BinA part of Bin toxins.

Amino acid residue substitutions at selected sites in the N- and C-terminal regions of type 2 BinA revealed the importance of other amino acids at the C-terminal end of the BinA peptide. For example, replacement of the Arg residue at position 312 by lysine or histidine (positively charged amino acids) completely destroys the biological activity of the binary toxin on *C. quinquefasciatus*, even though the binding of these mutants to mosquito brush border membranes is not altered (Elangovan *et al.*, 2000).

Table 2 Nomenclature and comparison of the four known Bin amino acid sequences from various *B. sphaericus* strains

		Type 1	*Type 2*	*Type 3*	*Type 4*
Polypeptide	*Amino acid position*	*IAB-59*[a], *IAB-872*[b] *IAB-881*[b], *9002*[b]	*1593*[a], *2362*[a], *2317.3*[a] *BSE18*[c], *C3-41*[d]	*2297*[a]	*LP1-G*[b]
BinA (41.9 kDa)		BinA1	BinA2	BinA3	BinA4
	93	Leu	Leu	Leu	Ser
	99	Val	Val	Phe	Val
	104	Glu	Ala	Ser	Ser
	125	His	His	Asn	Asn
	135	Tyr	Tyr	Phe	Phe
	267	Arg	Arg	Lys	Lys
BinB (51.4 kDa)		BinB1	BinB2	BinB3	BinB4
	69	Ala	Ser	Ser	Ser
	70	Lys	Asn	Asn	Asn
	110	Ile	Thr	Thr	Ile
	314	His	Leu	Tyr	His
	317	Leu	Phe	Leu	Leu
	389	Leu	Leu	Met	Met

[a]Berry *et al.* (1989).
[b]Humphreys and Berry (1998), Priest *et al.* (1997).
[c]Berry (personal communication).
[d]Yuan *et al.* (1998).

The replacement of alanine at some sites in the N- and C-terminal regions of both the BinA and BinB peptides results in the total loss of mosquitocidal activity. Surprisingly, toxicity is restored by mixing two nontoxic derivatives of the same peptide; *i.e.*, one mutated at the N-terminal end and the other mutated at the C-terminal end of either BinA or BinB (Shanmugavelu *et al.*, 1998). Thus, the altered binary toxins can functionally complement each other by forming oligomers.

The aggregation of both BinB and BinA has been analyzed by expressing crystal toxin components separately or together in homologous or heterologous *Bacillus* hosts. The proteins form amorphous inclusions when expressed independently in *B. subtilis* and in *B. sphaericus* or *B. thuringiensis* crystal-negative hosts (Charles *et al.*, 1993; Nicolas *et al.*, 1993). In contrast, crystals similar to those produced by natural *B. sphaericus* strains are found when the two genes are simultaneously expressed in either *B. sphaericus* or *B. thuringiensis* (Charles *et al.*, 1993; Nicolas *et al.*, 1993). No crystals can be detected in *B. subtilis* when both genes are present, unless they are fused, eliminating the intergenic region (Charles *et al.*, 1993). These results suggest that *B. sphaericus* and *B. thuringiensis*, but not *B. subtilis*, contain factors that help stabilize their protein and subsequent crystallization.

In vivo, BinA is slowly converted into a stable ~39-kDa protein, whereas BinB is rapidly converted into a stable ~43-kDa fragment (Baumann *et al.*, 1985; Broadwell and Baumann, 1987). *In vitro* deletion analysis was used to delineate the minimal active fragments of both proteins, which indeed correspond to the activated fragments (Broadwell *et al.*, 1990b; Clark and Baumann, 1990; Oei *et al.*, 1990; Sebo *et al.*, 1990). Thirty-two and 53 amino acids, at the N- and C-termini of BinB, respectively, can be eliminated without loss of toxicity (Clark and Baumann, 1990); deletions of 10 and 17 amino acids, at the N- and C-terminus of BinA, respectively, result in a protein similar to the 39-kDa activated fragment (**Figure 2**, top; Broadwell *et al.*, 1990b).

Sub-cloning experiments have shown that BinA alone is toxic for mosquito larvae (*C. pipiens*), although the activity is weaker than that of crystals containing both proteins (Nicolas *et al.*, 1993). In contrast, BinB alone is not toxic, although its presence enhances the larvicidal activity of BinA, suggesting synergy between the two polypeptides (de la Torre *et al.*, 1989; Broadwell *et al.*, 1990b; Oei *et al.*, 1990; Nicolas *et al.*, 1993). *In vitro* assays confirmed that the activated form of BinA alone is toxic for *C. quinquefasciatus* cells, whereas BinB appears to be inactive; however, no synergy between the components was observed *in vitro* (Baumann and Baumann, 1991). Although the simultaneous presence of both proteins appears necessary for full toxicity, the differing activities of the different *B. sphaericus* strains towards various mosquito species depends on the origin of BinA, as shown by analysis of *in vitro* mutated toxins (Berry *et al.*, 1993). Indeed, when amino acids were substituted in a region centered around position 100 of BinA, rendering BinA from strains IAB-59 and 2297 similar to that from strain 2362, the activity and specificity of these mutant toxins towards *Culex* and *Aedes* larvae was comparable, unlike the wild-type, indicating that this region is involved in specificity. Taken together, these observations suggest that BinA is the most important determinant of specificity and activity.

6.7.2.2. Mtx Toxins

Three types of Mtx toxin have been described to date: Mtx1, Mtx2 and Mtx3, with molecular weights of 97, 31.8 and 35.8 kDa, respectively (**Figure 2**, bottom). The genes encoding these proteins were initially cloned from the SSII-1 strain (Thanabalu *et al.*, 1991; Thanabalu and Porter, 1995; Liu *et al.*, 1996a), which has a moderate mosquitocidal activity (**Table 1**). In contrast to the crystal toxin genes, *mtx* genes are expressed during the vegetative growth phase, and sequences resembling vegetative promoters from *B. subtilis* have been found upstream from each gene (Thanabalu *et al.*, 1991; Thanabalu and Porter, 1995; Liu *et al.*, 1996a). *lacZ* fusion experiments confirmed these findings (Ahmed *et al.*, 1995). These proteins possess short N-terminal leader sequences characteristic of Gram-positive bacterial signal peptides (Thanabalu *et al.*, 1991; Thanabalu and Porter, 1995; Thanabalu and Porter, 1996). However, these toxins have been found associated with the cell membrane of *B. sphaericus*, indicating little or no cleavage of the signal sequence. The mature Mtx1 toxin can be further processed by gut proteases, leading to two fragments of 27 and 70 kDa, corresponding to the N- and C-terminal regions, respectively (Thanabalu *et al.*, 1992). The 70-kDa fragment possesses three repeated regions of about 90 amino acids (**Figure 2**, bottom), the function of which is unknown. The 27-kDa fragment contains a short putative transmembrane domain. These toxins do not display any similarity with each other or with crystal proteins or any other insecticidal proteins. In contrast, the 27-kDa fragment shares weak sequence similarities with the catalytic domains of various bacterial ADP-ribosyltransferases (**Figure 2**, bottom; Thanabalu

et al., 1991; Thanabalu *et al.*, 1992). Mtx2 and Mtx3 show similarities to two toxins active against mammalian cells; namely the epsilon-toxin from *Clostridium perfringens* and the cytotoxin from *Pseudomonas aeruginosa*, respectively (Thanabalu and Porter, 1995; Liu *et al.*, 1996a). The genes encoding Mtx toxins are found in numerous *B. sphaericus* strains (Liu *et al.*, 1993; Thanabalu and Porter, 1995; Liu *et al.*, 1996a; Thanabalu and Porter, 1996), including low, moderate and high toxicity strains (see **Table 1**).

6.7.3. Mode of Action of *B. sphaericus* Toxins

6.7.3.1. The Crystal Toxins

The mode of action of *B. sphaericus* crystal toxins has only been studied in mosquito larvae. However, there is one report of activity in adult *C. quinquefasciatus* after introduction by enema into the midgut, whereas no effect was recorded for *A. aegypti* adults (Stray *et al.*, 1988). Following ingestion of the spore/crystal complex by mosquito larvae, the protein crystal matrix is quickly dissolved in the lumen of the anterior stomach (Yousten and Davidson, 1982; Charles and Nicolas, 1986; Charles, 1987) by the combined action of midgut proteinases and of the high pH (Dadd, 1975; Charles and de Barjac, 1981). The toxin is released from *B. sphaericus* crystals in all species, even in the midgut lumen of nonsusceptible species such as *A. aegypti* (**Figure 3a**). Indeed, a number of studies have shown that the differences in susceptibility to *B. sphaericus* between mosquito species are not due to differences in the solubility and/or activation of the binary toxin (Baumann *et al.*, 1985; Davidson *et al.*, 1987; Nicolas *et al.*, 1990).

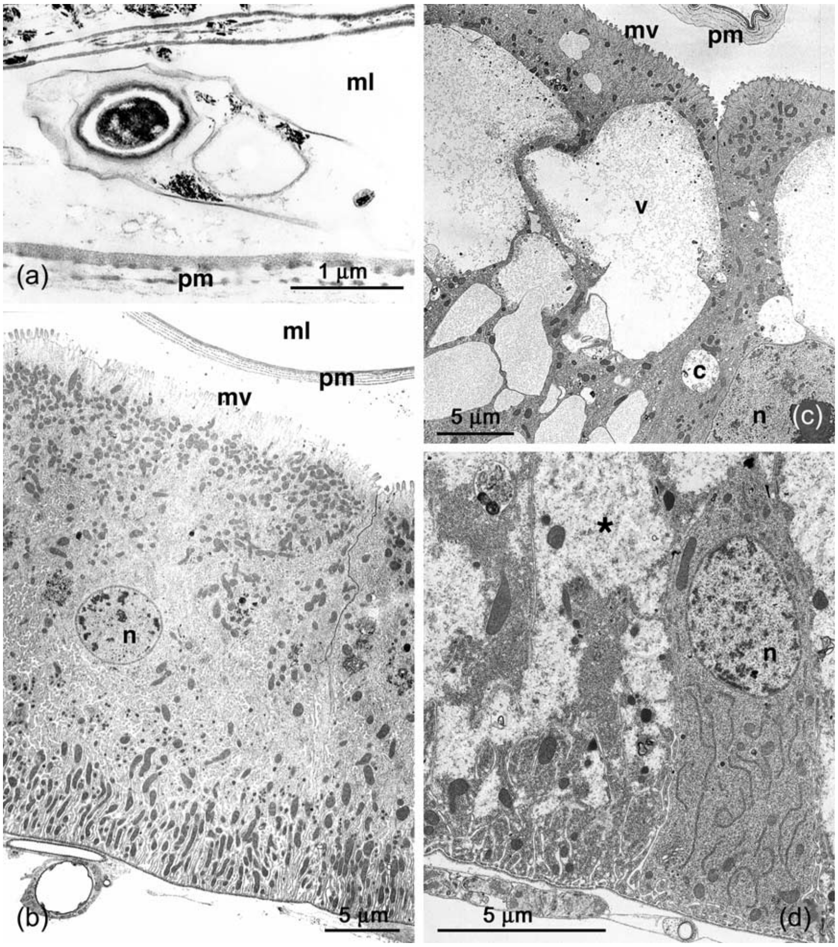


Figure 3 (a) Solubilization of the *B. sphaericus* 2297 crystal matrix within the midgut of an *Aedes aegypti* larva, under the combined action of proteinases and alkaline pH: only the envelopes initially surrounding the crystal are still visible. (b) Control midgut cell of an untreated *A. stephensi* larva. (c) Midgut cell of *C. pipiens* after feeding on *B. sphaericus*. (d) Midgut cell of *A. stephensi* after feeding on *B. sphaericus*. c, cytolysosome; ml, midgut lumen; mv, micovilli; n, nucleus; pm, peritrophic membrane; v, vacuoles; arrows indicate cytolysosomes; the star indicate areas of low electron density.

6.7.3.1.1. Cytopathology and physiological effects *Bti* toxins completely breakdown the larval midgut epithelium, whereas *B. sphaericus* does not. Nevertheless, midgut alterations start within 15 min of feeding on *B. sphaericus* spore/crystal complex (Davidson, 1981; Karch and Coz, 1983; Charles, 1987; Singh and Gill, 1988). There is no difference between midgut damage in *C. pipiens* after ingestion of spore/crystals from strains 1593 or 2297 (Davidson, 1981; Charles, 1987; Singh and Gill, 1988), even though these two strains belong to different Bin types. In contrast, symptoms of intoxication are not identical in different mosquito species. Large vacuoles (and/or cytolysosomes) appear in *C. pipiens* midgut cells, whereas large areas of low electron density appear in *Anopheles stephensi* midgut cells (compare **Figure 3c and d** with the control, **Figure 3b**). Mitochondrial swelling is a general feature described for *C. pipiens* and *A. stephensi*, as well as for *A. aegypti*, when intoxicated with a very high dose of spore/crystals (Charles, 1987). The midgut cells, especially cells of the posterior stomach and the gastric caecae, are the most severely damaged by the toxin, and Singh and Gill (Singh and Gill, 1988) also reported late damage to neural tissue and skeletal muscles.

Ultrastructural effects have also been observed in cultured cells of *C. quinquefasciatus* within few minutes of treatment with soluble and activated *B. sphaericus* toxin. These alterations consisted mainly in the swelling of mitochondria cristae and endoplasmic reticula, followed by the enlargement of vacuoles and the condensation of the mitochondrial matrix (Davidson *et al.*, 1987).

The physiological effects of *B. sphaericus* crystal toxin have been very poorly documented. The oxygen uptake of mitochondria isolated from *B. sphaericus*-treated *C. quinquefasciatus* larvae is inhibited, as is the activity of larval choline acetyl transferase (Narasu and Gopinathan, 1988). In addition, oxygen uptake by mitochondria isolated from rat liver is reduced by the *B. sphaericus* toxin (Narasu and Gopinathan, 1988).

6.7.3.1.2. Binding to a specific receptor in the brush border membrane As the differences in susceptibility between mosquito species do not seem to be due to the ability to solubilize and/or to activate the binary toxin, the variation in susceptibility is presumably due to cellular differences. Indeed, fluorescently-labeled toxin binds to the gastric caecae and the posterior stomach only in very susceptible *Culex* species. BinB does not bind to the gut of *Aedes aegypti*, whereas BinA weakly and nonspecifically binds in this species, and no regionalized binding occurs for *Anopheles* (Davidson, 1988; Davidson, 1989; Davidson and Yousten, 1990; Oei *et al.*, 1992). Furthermore, in *C. quinquefasciatus*, only BinB binds to the caecae and posterior stomach, and the binding of BinA appears to be conditioned by the binding of the BinB (**Figure 4b**), whereas BinA alone binds nonspecifically throughout the midgut (**Figure 4a**). This indicates that only one of the two components of the crystal toxin (putatively BinB) specifically binds to the midgut of susceptible larvae. The hypothesis that the N-terminal region of BinB is involved in regional binding of this protein in the larval midgut, and that the C-terminal region of BinB, as well as both the N- and C-terminal regions of BinA are involved in the interaction of the two components leading to the binding of BinA in the same regions as BinB, was supported by early *in vivo* binding studies using nontoxic deletion mutants of the crystal toxin (Oei *et al.*, 1992). The same authors also showed that the Bin toxin is only internalized when both components are present. However, further intracellular investigations are required to determine whether one or both components are internalized.

In vitro binding assays, using ^{125}I-labeled activated crystal toxin and brush border membrane fractions (BBMFs) isolated from susceptible or nonsusceptible mosquito larvae, confirmed this hypothesis. Indeed, direct binding experiments with *C. pipiens* BBMFs indicated that the toxin binds to a single class of specific receptor (**Figure 5**, left; Nielsen-LeRoux and Charles, 1992). No significant specific binding was detected with BBMFs from *A. aegypti* (**Figure 5**, right), which is consistent

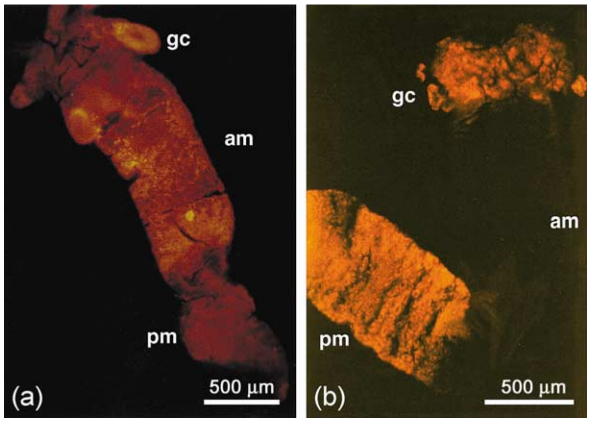


Figure 4 (a) Midgut of *C. quinquefasciatus* larvae only fed with fluorescently-labeled BinA. BinA weakly binds over the entire midgut. (b) Larvae fed with fluorescently-labeled BinA and unlabeled BinB; BinA binding is restricted to the gastric caecae and posterior stomach. am, anterior stomach; gc, gastric caeca; pm, posterior stomach. (Micrographs kindly provided by Dr C. Berry, Department of Biochemistry, Cardiff.)

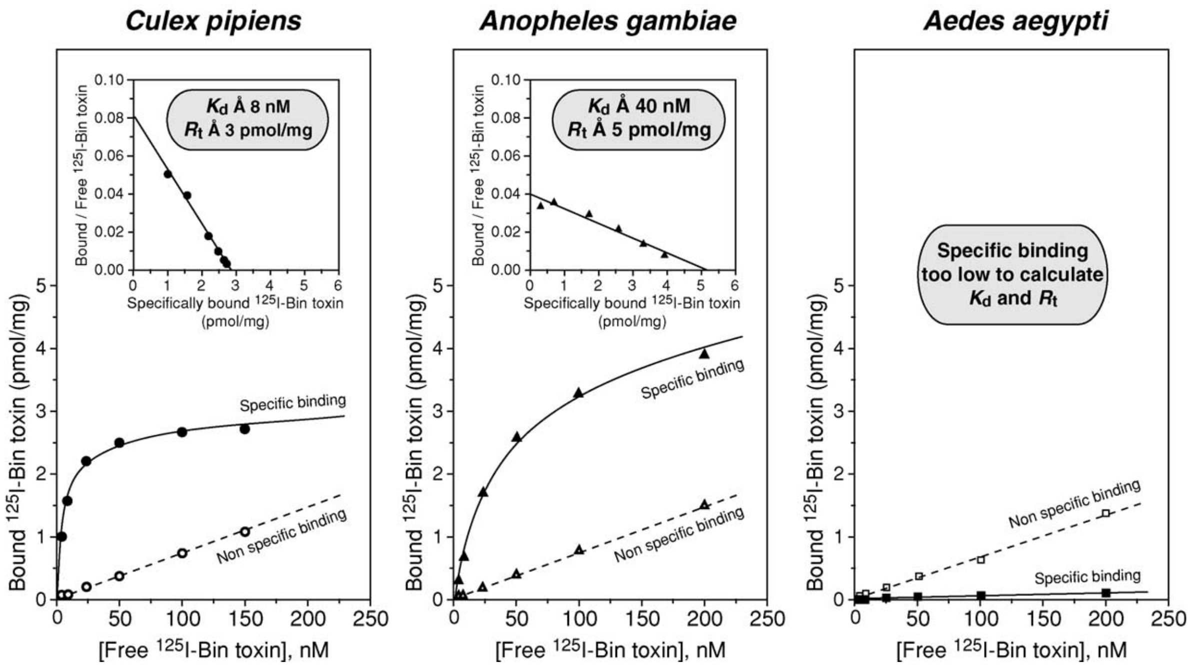


Figure 5 Binding of ^{125}I-labeled *B. sphaericus* Bin toxin to midgut brush border membranes of *C. pipiens*, *A. gambiae* and *A. aegypti*. Insets, specific binding in Scatchard plots. Values of dissociation constant (K_d) and receptor concentrations (R_t) are given when known.

with the lack of specific binding in fluorescence-labeling studies (Davidson, 1989; Davidson *et al.*, 1990). Similar results were obtained with other Culicidae species such as *Anopheles gambiae*, with minor differences in the values of the toxin/receptor binding characteristics: dissociation constants (K_d) were ~8 nM for *C. pipiens* and ~40 nM for *A. gambiae*, and receptor concentrations (R_t) were about 3 and 5 pmol mg^{-1} of BBMF protein respectively (**Figure 5**, center; Silva-Filha *et al.*, 1997). Both crystal toxin components bound to the membranes of all susceptible species, but the linearity of the Scatchard plots clearly indicated that only one of the components specifically bound to the receptor (**Figure 5**, insets).

Binding studies in which radiolabeled BinA or BinB were exposed separately to BBMFs confirmed that only BinB was able to bind specifically to *C. pipiens* midgut membranes, but that BinA alone was also able to bind midgut membranes from *A. gambiae* (Charles *et al.*, 1997).

6.7.3.1.3. Permeabilization of artificial lipid membranes Complementary studies using artificial lipid vesicles have been carried out to investigate further the respective roles of BinA and BinB in the toxic effect of the crystal toxin. This revealed that the whole Bin toxin and its individual components (BinA and BinB) are able to permeabilize receptor-free, large unilamellar phospholipid vesicles (LUVs) and planar lipid bilayers (PLBs) by forming pores (Schwartz *et al.*, 2001). Calcein-release experiments showed that LUV permeabilization is optimal at alkaline pH (which corresponds to the pH inside the mosquito larval midgut *in vivo*) and in the presence of acidic lipids. BinA is more efficient than BinB, and BinB facilitates the BinA effect. Stoichiometric (1:1) mixtures are more effective than the full Bin toxin, consistent with previous *in vivo* bioassay observations (Broadwell *et al.*, 1990a; Oei *et al.*, 1990; Baumann and Baumann, 1991; Nicolas *et al.*, 1993). In PLBs, BinA forms voltage-dependent channels of 100–200 pS with long open times and a high open probability. Larger channels (~400 pS) have also been observed. BinB, which inserts less easily, forms smaller channels (~100 pS) with shorter mean open times. The channels formed after the sequential addition of the two components, or when they are mixed in equal amounts (w/w), display BinA-like activity, supporting the hypothesis that BinA is principally responsible for pore formation in lipid membranes whereas BinB, the binding component of the toxin, promotes channel activity (Schwartz *et al.*, 2001).

6.7.3.1.4. Identification and cloning of the Bin toxin receptor Two important biochemical features have made it possible to purify the Bin toxin

receptor: (1) the availability of an iodinated derivative of the toxin that strongly binds to its receptor both in intact and solubilized membranes prepared from susceptible *Culex* mosquitoes (Nielsen-LeRoux and Charles, 1992); (2) the availability of midgut brush border membrane fractions (BBMFs) prepared from 4th instar larvae, constituting a rich source of receptors (Silva-Filha *et al.*, 1999). The Bin toxin receptor can be purified from BBMFs prepared from the midguts of larvae of a susceptible laboratory strain of *C. pipiens* (IP) by means of a classical protocol including solubilization with CHAPS plus anion exchange chromatography (Silva-Filha *et al.*, 1999). This receptor is a protein of approximately 60 kDa that is thought to be linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety, given the effect of phosphatidylinositol-specific phospholipase C (PI-PLC). This protein displays α-amylase-like activities, i.e., it has α-glucosidase (or maltase) and α-amylase *stricto sensu* activities. Moreover, partial microsequencing of the 60 kDa protein revealed significant similarities with identified insect maltases. Hence, this protein was named Cpm1 for *Culex pipiens* maltase 1 (Silva-Filha *et al.*, 1999).

The full-length *cpm1* cDNA was first cloned from the IP strain (Darboux *et al.*, 2001). The coding sequence is 1740 base pair-long and corresponds to a 580-amino acid protein (with a theoretical molecular weight of 66 kDa). It contains the tryptic peptides previously identified (Silva-Filha *et al.*, 1999) and peculiar structural features on both ends of the molecule. A hydrophobic signal peptide located at the N-terminal end of the protein targets the protein to the cell membrane. The C-terminal end contains a domain composed of three small amino acids (Ser^{558}-Ser^{559}-Ala^{560}) followed by hydrophilic residues and then a hydrophobic tail. These sequences fulfill the criteria for posttranslational maturation by linkage of a GPI moiety (Udenfriend and Kodukula, 1995; Eisenhaber *et al.*, 1998). Multiple alignments showed that this sequence shares a high level of similarity with maltase-like proteins from insects, especially over the first two-thirds of the molecule that are thought to bear the enzymatic activity responsible for hydrolyzing the terminal (α-1,4-linked glucose residues of various carbohydrates. Northern blotting experiments identified a single transcript of about 2 kb in the midgut of *Culex* larvae (Darboux *et al.*, 2001).

Cpm1 is not fully processed when expressed in *Escherichia coli*, but it is able to bind the Bin toxin specifically and has weak but significant α-glucosidase activity by hydrolyzing 4-nitrophenyl α-D-glucopyranoside. Much more conclusive data were obtained when *cpm1* cDNA was cloned in the insect Sf9 cell line. These cells are derived from pupal ovarian tissue originating from the Lepidoptera *Spodoptera frugiperda*. They have the enzymatic equipment required to maturate Cpm1 by adding the GPI moiety (Darboux *et al.*, 2002). Cpm1 can easily be detected at the surface of Sf9-IP cells, which express the receptor. Western blotting on the expression product of the *cpm1*/Sf9 construct, using a polyclonal antibody directed against the C-terminal end of the molecule (Darboux *et al.*, 2002), detects only one protein of about 66 kDa in the membrane fraction of transfected cells. Moreover, when transfected cells are treated with PI-PLC, no signal is observed in the membrane compartment whereas a strong signal is observed in the soluble fraction, indicating that the Cpm1 produced in Sf9 cells is indeed attached to the cell membranes by a GPI (Darboux *et al.*, 2002). The number of GPI-anchored proteins found in vertebrates and in invertebrates is increasing rapidly. They have numerous physiological roles (enzymatic activities, cell adhesion, transport and cell–cell signaling), but seem to be present in specialized micro-domains of the plasma membranes known as lipid rafts. Many studies have concentrated on the role of these rafts in cell trafficking and signal transduction (Simons and Toomre, 2000). When cells are transfected with a truncated *cpm1* sequence lacking its 3′ end (IP-Mut), no Cpm1 is found in the membrane fraction. Instead, Cpm1 is secreted and recovered in the culture medium. This secreted form exhibits strong α-glucosidase activity but little α-amylase activity compared to untransfected Sf9 cells (Darboux *et al.*, 2002). Binding experiments on *cpm1*/Sf9 cell membrane preparations showed that the native receptor is indeed expressed as the affinity of Bin for its site is very similar to that reported for *in vitro* experiments done on *Culex* BBMFs (about 10 nM). An anti-Cpm1 antibody was used to localize receptor expression sites in mosquito tissues. In the midguts of 4th instar larvae, a strong signal was detected both in the gastric caecum and in the posterior midgut, with a clear cutoff between the intermediate and posterior midguts (Darboux *et al.*, 2002). These data confirmed the localization of the Bin toxin binding site, which had been previously reported by use of fluorescently labeled derivatives (Davidson, 1988; Oei *et al.*, 1992).

6.7.3.2. The Mtx1 Toxin

Almost nothing is known about the mode of action of Mtx toxins other than Mtx1, because few studies have been carried out. The mosquitocidal activity of

Mtx1 was initially measured using fusion proteins synthesized in *E. coli*: Mtx1 is highly active against *C. quinquefasciatus* larvae (Thanabalu *et al.*, 1992), as the LC_{50} value is comparable to that of the crystal Bin proteins (15 ng/ml). Deletion analysis suggested that the 27 kDa fragment of Mtx1 is able to self ADP-ribosylate, whereas the 70 kDa fragment is assumed to play a role in the toxicity to cultured *C. quinquefasciatus* cells; however, both regions are necessary for toxicity to mosquito larvae (Thanabalu *et al.*, 1993).

Due to its cytotoxicity towards bacterial cells, the 27 kDa enzyme fragment cannot be produced alone, in the activated form, in *E. coli* expression systems. However, a nontoxic 32 kDa N-terminal truncated version of Mtx1 has been successfully expressed in *E. coli* and subsequently cleaved to an active 27 kDa enzyme fragment (Schirmer *et al.*, 2002b). *In vitro*, this 27 kDa fragment of Mtx1 is able to ADP-ribosylate numerous proteins in *E. coli* lysates, especially a 45 kDa protein identified as the *E. coli* elongation factor, Tu (EF-Tu). The inactivation of EF-Tu by Mtx1-mediated ADP-ribosylation and the resulting inhibition of bacterial protein synthesis probably play important roles in the cytotoxicity of the 27 kDa enzyme fragment of Mtx1 towards *E. coli* (Schirmer *et al.*, 2002b). Glu^{197} of the 27 kDa enzyme component was identified as the "catalytic" glutamate that is conserved in all ADP-ribosyltransferases (Schirmer *et al.*, 2002a). Transfection of mammalian HeLa cells with a vector encoding the 27 kDa fragment fused with a green fluorescent protein leads to cytotoxic effects characterized by cell rounding and formation of filopodia-like protrusions (Schirmer *et al.*, 2002a).

In vitro binding assays using isolated BBMFs from *C. pipiens* midguts indicate the presence of a membrane receptor to the 70-kDa fragment. The affinity of this receptor/toxin interaction is low (K_d ~1.4 μM), indicating that the 70-kDa component has a very low affinity for its binding site (**Figure 6a**; Charles and Berry, unpublished data). In addition, saturation of this binding site was not possible. Complementary competition experiments, using either the labeled P70 or the labeled Bin toxin as the labeled component, and unlabeled P70 or Bin toxin as the unlabeled competitors, showed that the Bin toxin can compete with the labeled P70 component, whereas the opposite is not true (**Figure 6b** and **c**). This suggests that the P70-Mtx1 component shares a common binding site with the Bin toxin, and that the Bin toxin can also bind to this binding site with a very low affinity.

After cleavage of Mtx1 by chymotrypsin-like proteases, the P70 proteolytic fragment of Mtx1 remains noncovalently bound to the N-terminal 27-kDa fragment (Schirmer *et al.*, 2002a). Thus, from a functional point of view, the binding of the P70 component of the toxin to a membrane receptor may play a key role in carrying the 27-kDa enzymatic fragment to its target site in mosquito midgut cells.

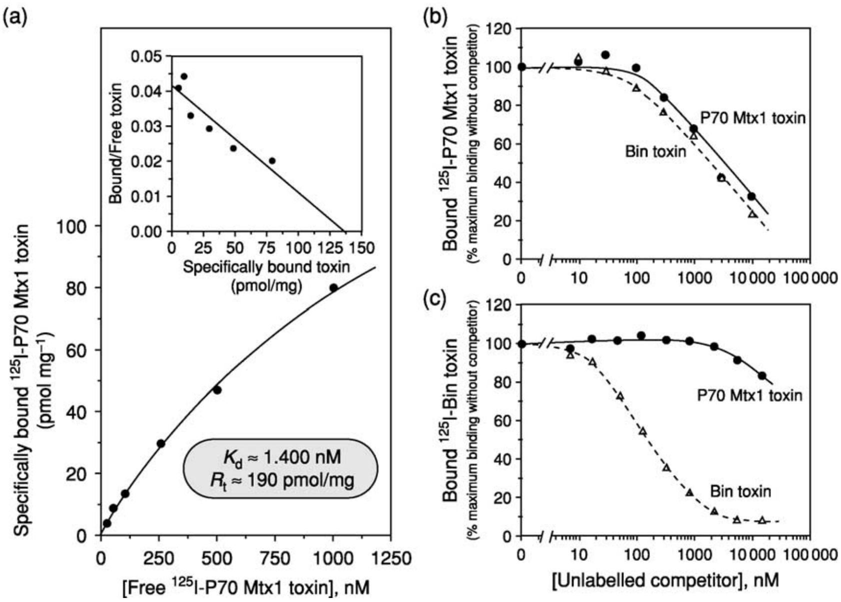


Figure 6 Direct binding of ^{125}I-labeled *B. sphaericus* P70-Mtx1 toxin to midgut brush border membranes of *C. pipiens* (a). Inset, specific binding in Scatchard plots. Competition experiments between ^{125}I-P70 and unlabeled P70 or Bin toxin (b), or between ^{125}I-Bin toxin and unlabeled P70 or Bin toxin (c).

6.7.4. Field Use of *B. sphaericus*

Due to the special properties of mosquitocidal *Bti* and *B. sphaericus* (e.g., environmental safety, high specificity of *B. sphaericus* and *Bti* toxins, relative ease of mass production, formulation and application, suitability for integrated control programs, and relatively low cost for development and registration), these bacilli were rapidly developed and used in many mosquito and/or black fly control programs. *Bti* has a broader application because unlike *B. sphaericus* it also has larvicidal activity against important vectors belonging to the *Aedes* and *Simulium* genera (see **Chapter 2.1**). *B. sphaericus* has been used to control *C. pipiens* and *C. quinquefasciatus* larvae since the late 1980s, and it is also used to control *Anopheles* spp. in some areas (**Table 3**; Karch *et al.*, 1992; Hougard *et al.*, 1993; Kumar *et al.*, 1994). Even though the larvicidal activity of *B. sphaericus* is mainly restricted to *Culex* and *Anopheles* species, it is stable, can recycle in organically polluted water (major breeding sites of *Culex* species), and is highly persistent (Lacey *et al.*, 1987; Correa and Yousten, 1995), giving it an advantage over *Bti*. Both *B. sphaericus* and *Bti* overcome the resistance of *Culex* and *Anopheles* to conventional insecticides.

In temperate regions, *B. sphaericus* is mainly used to reduce nuisance due to mosquito bites. It has been used on a large scale in Germany, especially in the Rhine Valley (Becker, 2000), and in France, where it was used to control *C. pipiens* (resistant to the organophosphate insecticide temephos) on the Mediterranean coast for more than 7 years before any problems of resistance arose (Sinègre *et al.*, 1993). However, the main interest (amount of product used and treated areas) is in tropical regions where *B. sphaericus* is used to control *Culex* and *Anopheles* populations, particularly to reduce the incidence of vector-born diseases like filariasis and malaria. In Goa (India), the use of *B. sphaericus* against *A. stephensi* larvae resulted in a notable reduction in the number of cases of malaria (Kumar *et al.*, 1994). This was also the case in China against *A. sinensis* (Becker, 2000; Yuan *et al.*, 2000). In the Democratic Republic of Congo, successful experimental field trials were run against *A. gambiae* in rice fields (Karch *et al.*, 1992). *C. quinquefasciatus* populations were reduced in the town of Maroua in Cameroon (Barbazan *et al.*, 1997). In the city of Recife, in Brazil, the experimental application of *B. sphaericus* successfully reduced the number of cases of filariasis by reducing the adult vector population of *C. quinquefasciatus* (Regis *et al.*, 1995).

Table 3 Examples of large-scale field use of *Bacillus sphaericus* against mosquito populations and the development of resistance

Target species	B. sphaericus *strain*	*Treatment frequency*	*Treatment period*	*Treated area*	*Locality/country*	*Resistance RR*[a]	*References*
A. stephensi	B-101	Weekly	9 months	2 km^2	Panaji city (India)	–	Kumar *et al.* (1996)
C. pipiens	2362	Every 21 days to 6 months	7 years	210 villages	Mediterranean Coast (France)	>20 000[b]	Sinègre *et al.* (1993, 1994)
C. quinquefasciatus	2362	Monthly/fortnightly	26 months	1.2 km^2	Recife city (Brazil)	10	Silva–Filha *et al.* (1995)
C. quinquefasciatus	1593M	Fortnightly	2 years	8 km^2	Kochi (India)	146	Rao *et al.* (1995)
C. quinquefasciatus	C3-41	3 times a month	8 years	8 km^2	Dongguan city (China)	22 000	Yuan *et al.* (2000)
C. quinquefasciatus	2362	Every 3 months	4 years	200 ha	Yaoundé (Cameroon)	–	Hougard *et al.* (1993)
C. quinquefasciatus	2362	Twice a year	2 years	2000 ha	Maroua (Cameroon)	–	Barbazan *et al.* (1997)

[a] The level of resistance is calculated as the ratio of the LC_{50} values of the resistant population to that of susceptible laboratory colonies.
[b] At isolated breeding sites.
–, resistance ratio value not reported.

Only a few of the highly larvicidal *B. sphaericus* strains are commercially sold: strain 2362 (VectoLex® and Spherimos®, Valent–Bioscience Corporation, USA) in the USA and Europe, strains 1593 (Biocide-S®) and 101-B (Sphericide®, Biotech International Ltd) in India, and strain C3-41 in China. These products are more or less adapted to various applications and larval breeding sites. As the success of a given strain is strongly dependent on the performance of the formulation, they have been improved over recent years, so that new products are now much more efficient (Skovmand and Bauduin, 1997; Fillinger *et al.*, 2003). There have been several attempts to produce these products locally in both Asia (Wuhan, China) and South America (Rio de Janeiro, Brazil), but only a few large international companies remain on the market.

6.7.5. Resistance To *B. sphaericus*

6.7.5.1. Introduction

Unlike *Bti*, towards which no cases of field resistance have appeared so far, several cases of larval resistance to *B. sphaericus* crystal toxin have been reported in *C. pipiens* and *C. quinquefasciatus* (**Table 3**). However, no resistance has yet been found in *B. sphaericus*-treated *Anopheles* populations. Indications for resistance to *B. sphaericus* were first reported in laboratory-selected populations (Georghiou *et al.*, 1992; Rodcharoen and Mulla, 1994), then by Pei *et al.* (2002), and a bit later from field-treated populations: from France (Sinègre *et al.*, 1994), India (Rao *et al.*, 1995), and Brazil (Silva-Filha *et al.*, 1995), and more recently from China (Yuan *et al.*, 2000) and Thailand (Mulla *et al.*, 2003) (**Table 4**).

The emergence of resistance was not that surprising given: (1) the apparent "one site" mode of action of the Bin toxin; (2) the ability of *B. sphaericus* to recycle and to persist in larval breeding zones, thus leading to increasing selection pressure (Lacey *et al.*, 1987; Correa and Yousten, 1995); and (3) the apparent high natural variation in susceptibility of *Culex* to *B. sphaericus* (Georghiou and Lagunes-Tejeda, 1991). However, resistance to *B. sphaericus* is also characterized by a large variation in resistance level, speed of appearance, and stability, indicating that factors such as selection pressure and the initial frequencies of resistance alleles in the mosquito population are very important.

We will summarize what is presently known about the genetics and mechanism of resistance to *B. sphaericus* with examples from both *C. pipiens* and *C. quinquefasciatus*. We will also discuss the mode of action and mechanism of resistance of the Bin toxin, as well as issues concerning the preservation of *B. sphaericus* as a mosquito control agent by searching for either natural or recombinant strains with low cross-resistance. Finally, we will discuss implications for application strategies.

Table 4 reports several examples of both laboratory-selected and initially field-selected resistance, from tropical and temperate areas, from places with high and low treatment frequencies and from various types of breeding sites. All populations reach very high levels of resistance (resistance ratio >10 000), either directly in the field or following further laboratory selection (Rao *et al.*, 1995; Nielsen-LeRoux *et al.*, 1997; Yuan *et al.*, 2000; Mulla *et al.*, 2003).

6.7.5.2. Genetics and Mechanisms of Resistance

Investigations into mosquito resistance to the Bin toxin under laboratory conditions were pioneered by Georghiou and co-workers. This group treated large numbers of early-4th instar larvae of a Californian population of susceptible *C. quinquefasciatus* (CpqS) with high concentrations of spore-crystal mixtures (Georghiou *et al.*, 1992; Wirth *et al.*, 2000b). Selection was very effective and resistance arose so rapidly in this colony (named CpqR or GEO) that the larvae were virtually insensitive to the *B. sphaericus* toxins by generation 12 (resistance ratio >100 000). Moreover, the F_1 offspring resulting from crosses between resistant and susceptible mosquitoes were almost totally susceptible, indicating that the resistance is recessive and borne by a single locus (Nielsen-LeRoux *et al.*, 1995; Wirth *et al.*, 2000b). Interestingly, a high level of resistance was also detected in first instar larvae, which suggests that the genetic factor of resistance is constitutive and not related to a particular developmental stage. Similar investigation with field-selected *B. sphaericus* resistant *C. pipiens* also showed monofactorial and recessive resistance, as F_1 progenies were as susceptible as the parental susceptible colony. In addition, dose-mortality Probit lines from back-cross progenies indicated that resistance of these colonies were likewise due to one major gene (Nielsen-LeRoux *et al.*, 1997; Oliveira *et al.*, 2003a). Until now, all studied cases of resistance were due to one major recessive gene that is sex linked in Mediterranean populations of *C. pipiens* (Nielsen-LeRoux *et al.*, 1997; Chevillon *et al.*, 2001; Nielsen-LeRoux *et al.*, 2002) but autosomally inherited in *C. quinquefasciatus* from Brazil and China (Oliveira *et al.*, 2003a) and possibly also in the GEO (CpqR) colony (Wirth *et al.*, 2000b). In general, no signs of a fitness cost have been reported in the laboratory (Oliveira *et al.*, 2003b), except for

Table 4 Characteristics of various *Culex pipiens* or *C. quinquefasciatus* colonies resistant to *B. sphaericus* strains

Country/year	Field (F) or Laboratory (L) selection	Colony	B. sphaericus *strain used for selection*	Level of resistance[a]	Genetics of resistance: Nb. genes	Genetics of resistance: Dom = D Res = R	Genetics of resistance: Linkage	Mechanisms of resistance[b] Binding characteristics: K_d (nM)	Mechanisms of resistance[b] Binding characteristics: R_t (pmol mg^{-1})	Reference
USA/1992	L	GEO	2362	>100 000	1	R	Autosomal	No binding	–	1, 2
USA/1994	L	JRMM-R	2362	37	?[c]	?	?	Binding[d]	–	3
Brazil/1995	F	Brazil-R	2362	10	?	?	?	11 ± 1	7 ± 1	4
India/1995	F/L	Kochi	1593M	146 (F)/>10 000 (L)	?	"D"[e]	?	No binding[e]	–	5
France/1994	F/L	SPHAE	2362	16 000 (F)/>50 000 (L)	1	R	Sex	3 ± 1	16 ± 1	6
Tunisia/1994	F/L	TUNIS	2362	>10 000 (L)	1	R	Sex	4 ± 1	3 ± 1	7
France/2000	F/L	BP	2362	>10 000 (F)/>50 000 (L)	1	R	Sex	No binding	–	7
China/2000	F/L	RLCq2(C3-41)	C3-41	22 000 (F)/>144 000 (L)	1	R	Autosomal	No binding	–	8
Brazil/2002	L	CqSF	2362	>162 000	1	R	Autosomal	No binding	–	8

[a]Level of resistance is calculated as the ratio of the LC_{50} values of the resistant colony to that of a susceptible colony.
[b]Binding experiments are done with (BBMF) brush border membrane fraction from homozygous laboratory selected resistant larvae.
[c]? No information available.
[d]Nielsen-LeRoux *et al.* (Unpublished data); not enough data to calculate K_d value.
[e]Poopathi and Nielsen-LeRoux (Unpublished data).
[1]Nielsen-LeRoux *et al.* (1995), [2]Wirth *et al.* (2000a), [3]Rodcharoen & Mulla (1994), [4]Silva-Filha *et al.* (1995), [5]Rao *et al.* (1995), [6]Nielsen-LeRoux *et al.* (1997),
[7]Nielsen-LeRoux *et al.* (2002), [8]Oliviera *et al.* (2003a).

one, the stable low level resistant JRMM-R (Rodcharoen and Mulla, 1994; Rodcharoen and Mulla, 1997). In the field, resistance appears to be unstable in the absence of selection pressure because, as soon after *B. sphaericus* treatment is interrupted, the exposed *C. quinquefasciatus* populations become susceptible to *B. sphaericus* again (Silva-Filha and Regis, 1997; Yuan *et al.*, 2000).

6.7.5.2.1. Molecular basis of laboratory-selected resistance As described above, the binding of BinB to the α-glucosidase (Cpm1) receptor at the apical membrane of midgut cells is essential for larvicidal activity. Thus, when resistance to *B. sphaericus* was first reported, the mechanisms of resistance were analyzed by comparing the kinetics of Bin receptor binding in the midguts of GEO and susceptible *C. quinquefasciatus* larvae (CpqS). As expected, the binding characteristics of ^{125}I-Bin to CpqS BBMFs were very similar to those previously reported for *C. pipiens* membranes (Nielsen-LeRoux and Charles, 1992). In contrast, Bin cannot specifically bind BBMFs from GEO mosquitoes (Nielsen-LeRoux *et al.*, 1995). In addition, when assayed on BBMFs prepared from F_1 (CpqS X GEO) larvae, ^{125}I-Bin bound to a single class of receptor, which is consistent with the suspected recessive nature of the resistance trait. Analysis of binding data with BBMFs from the backcross (BC) progeny showed that the binding sites are not saturated in the range of toxin concentrations used, and that the total amount of bound toxin is much lower than for the susceptible parental strain. LIGAND analysis showed that the experimental data obtained with the BC progeny fit a two-site model better than a one-site model. This possible existence of two classes of binding sites in the BC population is suggestive of genetic heterogeneity.

The next step was to determine whether this receptor was absent from the GEO mosquito midgut or whether it was present in a form that could no longer recognize the Bin toxin. In Northern blot experiments, total RNA extracted from GEO midguts was probed with a *cpm1* DNA fragment. The 2-kb transcript previously identified in IP mosquitoes was present in similar amounts in GEO mosquitoes (Darboux and Pauron, unpublished data). *In situ* hybridization experiments confirmed that *cpm1* transcripts are equally distributed both quantitatively and qualitatively in CpqS and GEO midguts. These transcripts were found in the regions that had been previously identified as Cpm1 reservoirs, i.e., cardia cells, the gastric caecae and the posterior midgut (Darboux *et al.*, 2002).

Nevertheless, the amount of Cpm1 protein differed completely in the two populations. Firstly, immunolocalization performed on the same type of cryosections as the *in situ* hybridizations failed to detect Cpm1 in any of the above-mentioned structures or anywhere else in the larval midgut. Secondly, Cpm1 was not detected in BBMFs from GEO mosquitoes by Western blotting (Darboux *et al.*, 2002). Taken together, these results suggest that the sequence encoding Cpm1 is altered in GEO. Analysis of the full cDNA sequence of $cpm1_{GEO}$ confirmed this hypothesis. The $cpm1_{GEO}$ sequence contains seven nonsilent mutations compared to $cpm1_{IP}$. Six of these mutations result in amino acid substitutions in the protein itself (Ala95Asp; Lys115Met; Glu178Thr; Asp230His; Asn265Asp; Leu486Met), and the seventh introduces a termination signal in the hydrophobic tail of the protein (Leu569Stop). To determine which of these mutations were involved in the resistance mechanism, Sf9 cells were transfected with various constructs corresponding to natural or chimeric forms of Cpm1. Western blotting, binding experiments and enzymatic assays were performed on the expression products of each construct. The $Cpm1_{GEO}$ form was unable to link to the plasma membranes of Sf9 cells (Figure 7), but accumulated in the extracellular medium, which explains why no signal could be detected both in BBMF Western blotting and by immunolocalization in midgut sections. Concomitantly, no binding activity was detected on membranes prepared from Sf9/$Cpm1_{GEO}$ cells, which confirms the previous results of *in vitro* binding of ^{125}I-Bin toxin to $BBMF_{GEO}$ (Darboux *et al.*, 2002).

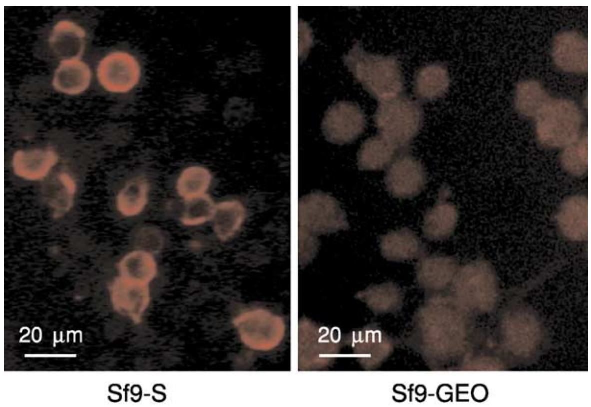


Figure 7 Ectopic expression of Cpm1. Sf9 cells were transfected with the $cpm1_{IP}$ sequence (Sf9-S) or the $cpm1_{GEO}$ one (Sf9-GEO). The anti-Cpm1 antibody gives a signal only on the plasma membrane of the Sf9-S cells (Castella and Pauron, unpublished data).

A chimeric form of Cpm1 was also constructed, where the GEO sequence was modified by replacing the early TAG codon by TTG (Stop569Leu) by site-directed mutagenesis in order to recover a "susceptible-like" hydrophobic tail. When transfected into Sf9 cells, this construct, named Sf9-GEOMut, was able to mature fully, and $Cpm1_{GEOMut}$ was detected in the membrane fraction of the cells to which it was linked by a GPI, as shown by its sensitivity to PI-PLC (Darboux *et al.*, 2002). Moreover, competition as well as saturation binding assays showed that ^{125}I-Bin binds membranes prepared from Sf9-GEOMut cells with the same affinity as those prepared from Sf9-IP cells. Hence, the six mutations present in the protein itself have no effect on the capacity of the receptor to recognize the toxin and are not involved in the resistance mechanism. They have no effect on the enzymatic activity of Cpm1, as shown by measurements done using the secreted form of $Cpm1_{GEO}$ (**Figure 8**).

$Cpm1_{GEO}$ hydrolyzes 4-nitrophenyl α-D-glucopyranoside as well as $Cpm1_{IPMut}$, which suggests that $Cpm1_{GEO}$ retains its physiological activity even when it is no longer bound to the membrane *in vivo*. If this is the case, this resistance mechanism may cause less pleiotropic effects than other mechanisms that result in the severe dysfunction in the physiology of the insect. In that respect, resistance to other *Bacillus* toxins may impair important functions such as cell–cell signaling in the case of the cadherin-like protein (Gahan *et al.*, 2001) or carbohydrate modifications in the case of the β-1,3-galactosyltransferase (Griffitts *et al.*, 2001). Resistance in GEO also contrasts with identified mechanisms of resistance to chemical insecticides by target modification, which is usually caused by a small number of point mutations in the receptor sequence that decrease its affinity for the pesticide (Pauron *et al.*, 1989; ffrench-Constant *et al.*, 1993; Mutero *et al.*, 1994).

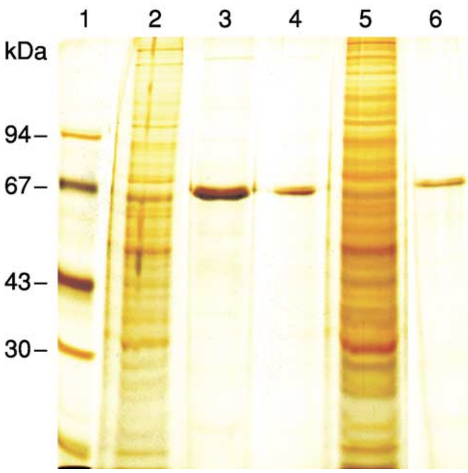


Figure 8 Silver staining of secreted forms of Cpm1. Lane 1, molecular weight markers. Lanes 2 and 5, crude extracellular medium of Sf9-IPMut and Sf9-GEO cells, respectively. Lanes 3 and 4, $Cpm1_{IPMut}$ purified by affinity chromatography. Lane 6 $Cpm1_{GEO}$ purified by affinity chromatography (Pauchet and Pauron, unpublished data).

A single point mutation in the $cpm1_{GEO}$ sequence results in Cpm1 molecules with a shortened hydrophobic tail (**Figure 9**). This hydrophobic stretch becomes too small to be recognized by the transamidase complex, thus preventing Cpm1 from being matured by the anchoring of a GPI moiety. Nevertheless, the unprocessed form of the receptor is translocated towards the apical membrane of the cell, where it is secreted. In the extracellular space, $Cpm1_{GEO}$ may still encounter and bind the Bin toxin but the complex formed can no longer promote the cytopathological effects of the toxin and the larva is not affected. This mechanism is the first example of a putative combination of two types of resistance mechanism to insecticides, i.e., target modification and sequestration of the toxic ligand.

6.7.5.2.2. Mechanisms of field-selected resistance

In vitro binding studies have also been conducted with other field-resistant mosquito colonies (see **Table 4**). In a number of cases, the mechanism of resistance was due to lack of measurable Bin toxin–receptor interaction. This was recently found for *C. quinquefasciatus* colonies from Brazil and China (Oliveira *et al.*, 2003a), from India (KOCHI colony, unpublished data), and for the *C. pipiens* BP colony from France (Nielsen-LeRoux *et al.*, 2002). Interestingly, the initial Bin toxin–receptor interactions of the highly *B. sphaericus*-resistant *C. pipiens* colonies SPHAE (Nielsen-LeRoux *et al.*, 1997) and TUNIS (Nielsen-LeRoux *et al.*, 2002), are similar to those of susceptible *C. pipiens* (*sensu lato*) colonies (reviewed in Nielsen-LeRoux *et al.*, 2002). Functional receptors have also been found in a low-level and stably resistant colony (Rodcharoen and Mulla, 1994; Nielsen-LeRoux, unpublished data). Another low-level resistant colony has been shown to have functional receptors, although the larvae collected in the field may not all have been homozygous resistant (Silva-Filha *et al.*, 1995).

There is evidence for more than one resistance mechanism to the Bin toxin in the *C. pipiens* complex worldwide and even within small areas like the West Mediterranean countries. Indeed, mutations in three West Mediterranean *C. pipiens* populations give rise to three different mutants: *sp-1*^{R} (SPHAE strain), *sp-2*^{R} (BP strain), and *sp-T*^{R} (TUNIS strain).

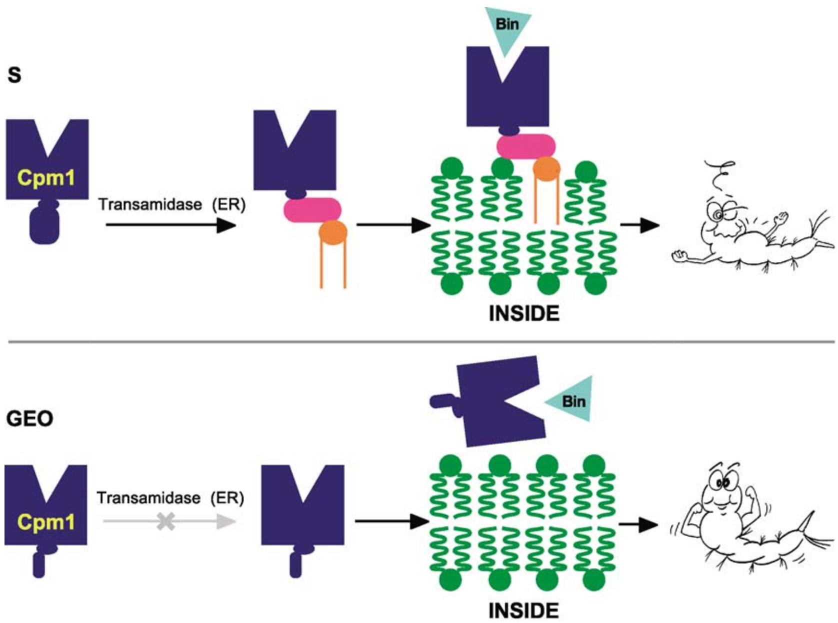


Figure 9 Schematic representation of the interaction between the Bin toxin and its receptor in a susceptible (S, top) and in a resistant (GEO, bottom) mosquito larva.

Mutations are all sex-linked and separately confer the same totally recessive resistance to *B. sphaericus*. However, binding experiments showed clear differences between the BP strain mutant and the other two mutants (Nielsen-LeRoux *et al.*, 2002). It is not clear whether mutations of TUNIS and SPHAE are identical or not, even though toxin receptor affinities are high in both cases and no gene recombination was observed when TUNIS and SPHAE were crossed (Chevillon *et al.*, 2001; Nielsen-LeRoux *et al.*, 2002). Moreover, the *cpm1* coding sequences of TUNIS and SPHAE are strictly identical (Darboux and Pauchet, unpublished data). *B. sphaericus* resistance in Californian *C. quinquefasciatus* populations may also be based on different mutations, as the laboratory-selected resistance of two Californian *C. quinquefasciatus* collected in the field was reported to be stable, one at a very high level (>100 000 fold, GEO strain) (Georghiou *et al.*, 1992; Wirth *et al.*, 2000b) and the other at a low level (35 fold, JRMM-R strain) (Rodcharoen and Mulla, 1994). A second study supports this hypothesis, because of the numerous *B. sphaericus*-resistant colonies tested for cross-resistance to other *B. sphaericus* strains, the only one that was resistant to all tested *B. sphaericus* strains was the JRMM-R colony (Nielsen-LeRoux *et al.*, 2001).

Cpm1 from the SPHAE strain appears to be totally functional, and its distribution in the larval midgut is indistinguishable from that in susceptible individuals (Castella and Pauron, unpublished data). In addition, the transcription pattern of $cpm1_{SPHAE}$ is identical to that of $cpm1_{IP}$ and the coding sequence of $cpm1_{SPHAE}$ displays no nonsilent mutations compared to the susceptible allele (Darboux and Pauchet, unpublished data). Hence, as no apparent differences in the activation process of the Bin toxin have been reported in SPHAE larvae (Nielsen-LeRoux *et al.*, 1997), the step involved in resistance in SPHAE presumably occurs after the Bin toxin interacts with its receptor. Determining whether this is dependent on the endocytosis of BinA, BinB, and/or the receptor, and whether they persist with $sp\text{-}1^{RR}$ larvae is likely to improve our understanding of how $sp\text{-}1^{R}$ interferes with the intoxication process.

When considering the mechanism underlying the resistance of the BP strain, it is worth noting the possibility of a tight physiological interaction between *sp-2* (BP) and *sp-1* (SPHAE). For example, the resistance of $sp\text{-}1^{RS}$ $sp\text{-}2^{RS}$ double heterozygotes is increased by over 100-fold, even though each mutation was totally recessive alone (Chevillon *et al.*, 2001). However, the BP colony may be different from SPHAE, as immunological characterization of Cpm1 in BP confirmed the binding data: no signal was ever detected either by Western blotting analysis of $BBMF_{BP}$ proteins or in cryosections of BP larva midgut (Castella and Pauron, unpublished data). In this strain, the resistance mechanism appears to be rather complex, as preliminary results

indicate that both the coding sequence and the pattern of transcription of *cpm1* are altered in BP (Darboux and Pauron, unpublished data).

6.7.6. Conclusions and Perspectives: Management of *B. sphaericus*-Resistance

The genes encoding BinA and BinB have been sequenced, and the regions important for the specificity and activity of these components identified. It is clear that both BinA and BinB are needed for full activity of the *B. sphaericus* crystal toxin: BinA alone is slightly toxic and might form pores in the cell membranes, and BinB appears to be responsible for the regionalized binding of the toxin to a specific midgut membrane receptor. Even if the nature of the Bin receptor, an α-glucosidase, is known in *Culex* spp., the possible intracellular actions of the Bin toxin(s) remain unclear. Indeed, immunolocalization investigations on the midguts of intoxicated *C. quinquefasciatus* larvae revealed that both BinA and BinB are present within cells a few hours after intoxication (Silva-Filha and Peixoto, 2003). These results show that the Bin toxin is internalized during the intoxication process and confirms previous results using fluorescently labeled Bin toxin (Davidson, 1988; Oei *et al.*, 1992). The importance of internalization of the Bin toxins into midgut cells is demonstrated by the fact that in the presence of the pore-forming or "detergent-like" Cyt1A toxin from *Bti*, the Bin toxin is internalized into *B. sphaericus*-resistant larval midgut cells (Federici *et al.*, personal communication). Further studies are required to elucidate the mode of action fully, as the final target molecules are unknown.

Further studies are also needed to determine the mode of action of the Bin toxin towards *Anopheles*, because there is some evidence suggesting that it differs from that towards *Culex*. As previously mentioned, no cases of resistance have yet been reported in *Anopheles* and *in vitro* binding experiments indicated that both BinA and BinB can bind to a receptor-binding site (Charles *et al.*, 1997).

The Mtx toxins have been studied less extensively than the Bin toxins, so further studies are needed, especially to determine how the enzymatic fragment from the Mtx1 toxin enters midgut cells to ADP-ribosylate target proteins.

As indicated above, there is a real risk that resistance to *B. sphaericus* will appear when it is used in large-scale *Culex* spp. control programs. Thus, it is important to investigate possible variations in toxic activity and specificity of different natural *B. sphaericus* strains, in order to evaluate the level of possible cross-resistance among different strains and the Bin toxins they produce. All *B. sphaericus*-resistant *Culex* spp. populations were selected using strains 2362, 1593 or C3-41 (Rodcharoen and Mulla, 1994; Nielsen-LeRoux *et al.*, 1995; Rao *et al.*, 1995; Yuan *et al.*, 2000), all of which belong to the same serotype and have identical Bin toxin genes (Bin2 type). However, there are small differences in the amino acid sequences of Bin toxins (**Table 2**), which may be important in the structure/function of the toxin/receptor complex, and therefore for larvicidal activity. Several groups have investigated the level of cross-resistance towards active *B. sphaericus* strains: 2297, IAB-881, IAB-872, LP1-G and IAB-59, in *Culex* spp. colonies resistant to strains 2362, 1593, or C3-41. The 2362-derived resistant JRMM-R colony is resistant to strains 2297 and 1593 (Rodcharoen and Mulla, 1994), and to IAB-59, IAB-881 and IAB-872 (Nielsen-LeRoux *et al.*, 2001). The GEO, SPHAE and the KOCHI colonies are resistant to all strains except IAB-59 (Poncet *et al.*, 1997; Poopathi *et al.*, 1999; Nielsen-LeRoux *et al.*, 2001). In addition, strain LP1-G is able to overcome resistance in *C. quinquefasciatus* larvae resistant to strain C3-41 from China (Yuan *et al.*, 2000).

Investigations on both strains IAB-59 and LP1-G suggested the presence of toxic factors other than the Bin and Mtx toxins. First, the crystals from LP1-G contain a natural nontoxic variant of the Bin toxin (Bin4 type) (Yuan *et al.*, 2001). Thus, although the whole strain is larvicidal, the toxic activity may come from other compounds produced by this strain. Second, crystals from the IAB-59 or IAB-872 Bin toxin (Bin1 type) are also nontoxic to the *B. sphaericus*-resistant *Culex* spp. colonies, which are also susceptible to the whole IAB-59 strain (Nielsen-LeRoux, unpublished data; Shi *et al.*, 2001; Pei *et al.*, 2002). In addition, the low level of cross-resistance to IAB-59 has recently been validated by selecting for resistance to this strain in laboratory conditions, in two *C. quinquefasciatus* populations collected in the field in China and Brazil (**Table 4**). The speed of appearance was much slower and the level of resistance much lower with the IAB-59 strain than with strains 2362 or C3-41. Finally, colonies that carry only a low level of resistance to IAB-59 are highly resistant to strains 2362 and C3-41, suggesting that resistance is directed against the Bin toxins regardless of whether it belongs to the Bin1 or Bin2 type. This is in agreement with *in vitro* toxin–receptor binding studies, which showed high competition among Bin toxins (Bin1, Bin2 and Bin4) and provided evidence for a common receptor-binding site (Nielsen-LeRoux *et al.*,

unpublished data). Further studies are required to evaluate whether strain IAB-59 is of commercial value as an alternative to *B. sphaericus* strains 1593, 2362, 101-B, and C3-41 (the only strains currently produced for use in the field). This depends on its potency and field performance. Additionally, several groups are currently trying to identify the genes encoding the additional toxic compounds from strains IAB-59 and LP1-G.

The fact that the Bin toxin behaves as a unique moiety during the binding step indicates that the probability of selecting resistance to *B. sphaericus* is much higher than for *Bti*, which contains at least four proteins that potentially act on different target molecules. Despite reports of laboratory and field resistance, the use of *B. sphaericus* for the control of mosquito larvae is still of interest. Nevertheless, it is important to collect information about baseline susceptibility before starting treatment. However, as resistance to *B. sphaericus* is recessive, it is only possible to detect resistance when it has already become homozygous and therefore difficult to handle. Thus, it may be possible to monitor the risk of resistance development in a given area by use of molecular nucleotide probes based on the mutation of resistance genes. However, resistance to *B. sphaericus* seems to be very complex and different probes would be needed to cover all possible mutations, only one of which has been identified so far (Darboux *et al.*, 2002).

Both from a practical and an ecological point of view, the best and most direct way to overcome and to manage *B. sphaericus* resistance is of course *Bti*, which is already commercialised. Fortunately, all *Culex* populations reported to display field resistance to *B. sphaericus* have been tested for susceptibility to *Bti*. No cross-resistance has been observed. There is even some evidence for increased susceptibility in some colonies (Rao *et al.*, 1995; Silva-Filha *et al.*, 1995; Yuan *et al.*, 2000). This was expected, because the multi-toxin complex of this bacterium does not share any receptor-binding sites with the *B. sphaericus* binary toxin (Nielsen-LeRoux and Charles, 1992). In several situations, it is recommended that *Bti* be used in rotation with *B. sphaericus* (Regis and Nielsen-LeRoux, 2000; Yuan *et al.*, 2000). Recent results obtained in Thailand have suggested that a mixture of *Bti* and *B. sphaericus* may help reduce the risk of appearance of *B. sphaericus* resistance while preserving the superior field performance of *B. sphaericus* compared to *Bti*, particularly in breeding sites rich in organic matter (Mulla *et al.*, 2003).

Alternatives to natural *Bti* and *B. sphaericus* strains include genetically modified organisms, as combining different toxins binding to different receptors in the same organism is also a good way of preventing resistance. In addition to the Bin toxins, other toxins from either *B. sphaericus* or other insecticidal microorganisms could be produced. For example, the expression of *Mtx1* genes in *B. sphaericus* during sporulation, i.e., under the control of a sporulation promoter, could allow the diversification of toxins. Similarly, *Bti* mosquitocidal toxin genes could be combined with *B. sphaericus* genes; some groups have already attempted this, for example by introducing the *Bti* genes encoding Cry4B, Cry4D or Cry11A into toxic *B. sphaericus* strains (Trisrisook *et al.*, 1990; Bar *et al.*, 1991; Poncet *et al.*, 1994; Poncet *et al.*, 1997). Conversely, *B. sphaericus* crystal toxin genes have been successfully introduced into toxic *Bti* (Bourgouin *et al.*, 1990). Although these studies did not demonstrate any increase in toxicity against *Anopheles* and *Culex*, such recombinants may delay the emergence of resistant insects.

Several attempts have been made to introduce or to combine toxin genes from *Bti*, *B. thuringiensis* serovar *jegathesan* (*Bt jeg*) and serovar *medellin* (*Bt med*), and to evaluate their activity towards both susceptible and *B. sphaericus*-resistant colonies. The introduction of Cry11A from *Bti* and Cry11Ba from *Bt jeg* partially restored the susceptibility of resistant colonies (Poncet *et al.*, 1997; Servant *et al.*, 1999). Nonspecific cytolytic toxins (Cyt1A and Cyt1B) also partially restored its toxicity towards two resistant colonies (Thiéry *et al.*, 1998; Wirth *et al.*, 2000a). A *Bti* strain harboring genes encoding the binary toxin as well as Cyt1 and Cry11B was more toxic than unmodified *Bti* IPS-82 (Park *et al.*, 2003). Several groups have also attempted to introduce and to overproduce entomopathogenic toxins into organisms living in the environment and serving as natural foods for mosquitoes, such as cyanobacteria (Liu *et al.*, 1996b), or to stably integrate the toxins encoding these genes into the chromosomes of other bacteria such as *Enterobacter amnigenus* (Tanapongpipat *et al.*, 2003). However, given our current knowledge of *B. sphaericus* resistance, the expression of Bin toxin as the only active compound should be avoided. No recombinant strains have yet proved to have a better field performance than the natural strains, and no products based on recombinant strains have yet been registered for use in mosquito control programs. Therefore there is a continuing need to identify new strains with novel modes of

action against the principal mosquito vectors of human diseases.

References

Ahmed, H.K., Mitchell, W.J., Priest, F.G., **1995**. Regulation of mosquitocidal toxin synthesis in *Bacillus sphaericus*. *Appl. Microbiol. Biotechnol. 43*, 310–314.

Alexander, B., Priest, F.G., **1990**. Numerical classification and identification of *Bacillus sphaericus* including some strains pathogenic for mosquito larvae. *J. Gen. Microbiol. 136*, 367–376.

Aquino de Muro, M., Priest, F.G., **1994**. A colony hybridization procedure for the identification of mosquitocidal strains of *Bacillus sphaericus* on isolation plates. *J. Invertebr. Pathol. 63*, 310–313.

Aquino de Muro, M., Mitchell, W.J., Priest, F.G., **1992**. Differentiation of mosquito-pathogenic strains of *Bacillus sphaericus* from non-toxic varieties by ribosomal RNA gene restriction patterns. *J. Gen. Microbiol. 138*, 1159–1166.

Arapinis, C., de la Torre, F., Szulmajster, J., **1988**. Nucleotide and deduced amino acid sequence of the *Bacillus sphaericus* 1593M gene encoding a 51.4 kD polypeptide which acts synergistically with the 42 kD protein for expression of the larvicidal toxin. *Nucleic Acids Res. 16*, 7731.

Bar, E., Lieman-Hurwitz, J., Rahamim, E., Keynan, A., Sandler, N., **1991**. Cloning and expression of *Bacillus thuringiensis israelensis* δ-endotoxin DNA in *B. sphaericus*. *J. Invertebr. Pathol. 57*, 149–158.

Barbazan, P., Baldet, T., Darriet, F., Escaffre, H., Haman, D.D., *et al.*, **1997**. Control of *Culex quinquefasciatus* (Diptera: Culicidae) with *Bacillus sphaericus* in Maroua, Cameroon. *J. Am. Mosq. Control Assoc. 13*, 263–269.

Baumann, L., Baumann, P., **1989**. Expression in *Bacillus subtilis* of the 51- and 42-kilodalton mosquitocidal toxin genes of *Bacillus sphaericus*. *Appl. Environ. Microbiol. 55*, 252–253.

Baumann, L., Baumann, P., **1991**. Effects of components of the *Bacillus sphaericus* toxin on mosquito larvae and mosquito-derived tissue culture-grown cells. *Curr. Microbiol. 23*, 51–57.

Baumann, P., Baumann, L., Bowditch, R.D., Broadwell, A.H., **1987**. Cloning of the gene of the larvicidal toxin of *Bacillus sphaericus* 2362: evidence for a family of related sequences. *J. Bacteriol. 169*, 4061–4067.

Baumann, L., Broadwell, A.H., Baumann, P., **1988**. Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. *J. Bacteriol. 170*, 2045–2050.

Baumann, P., Clark, M.A., Baumann, L., Broadwell, A.H., **1991**. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. *Microbiol. Rev. 55*, 425–436.

Baumann, P., Unterman, B.M., Baumann, L., Broadwell, A.H., Abbene, S.J., *et al.*, **1985**. Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for high-molecular-weight precursors. *J. Bacteriol. 163*, 738–747.

Becker, N., **2000**. Bacterial control of vector-mosquitoes and black flies. In: Charles, J.-F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: from Laboratory to Field Application. Kluwer Academic Publisher, Dordrecht, Boston, London, pp. 383–398.

Berry, C., Hindley, J., **1987**. *Bacillus sphaericus* strain 2362: identification and nucleotide sequence of the 41.9 kDa toxin gene. *Nucleic Acids Res. 15*, 5591.

Berry, C., Hindley, J., Ehrhardt, A.F., Grounds, T., de Souza, I., *et al.*, **1993**. Genetic determinants of host ranges of *Bacillus sphaericus* mosquito larvicidal toxins. *J. Bacteriol. 175*, 510–518.

Berry, C., Jackson-Yap, J., Oei, C., Hindley, J., **1989**. Nucleotide sequence of two toxin genes from *Bacillus sphaericus* IAB59: sequence comparisons between five highly toxinogenic strains. *Nucleic Acids Res. 17*, 7516.

Bourgouin, C., Delécluse, A., de la Torre, F., Szulmajster, J., **1990**. Transfer of the toxin protein genes of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* and their expression. *Appl. Environ. Microbiol. 56*, 340–344.

Broadwell, A.H., Baumann, P., **1986**. Sporulation-associated activation of *Bacillus sphaericus* larvicide. *Appl. Environ. Microbiol. 52*, 758–764.

Broadwell, A.H., Baumann, P., **1987**. Proteolysis in the gut of mosquito larvae results in further activation of the *Bacillus sphaericus* toxin. *Appl. Environ. Microbiol. 53*, 1333–1337.

Broadwell, A.H., Baumann, L., Baumann, P., **1990a**. Larvicidal properties of the 42 and 51 kilodalton *Bacillus sphaericus* proteins expressed in different bacterial hosts: evidence for a binary toxin. *Curr. Microbiol. 21*, 361–366.

Broadwell, A.H., Clark, M.A., Baumann, L., Baumann, P., **1990b**. Construction by site-directed mutagenesis of a 39-kilodalton mosquitocidal protein similar to the larva-processed toxin of *Bacillus sphaericus* 2362. *J. Bacteriol. 172*, 4032–4036.

Charles, J.-F., **1987**. Ultrastructural midgut events in Culicidae larvae fed with *Bacillus sphaericus* 2297 spore/crystal complex. *Ann. Inst. Pasteur/Microbiol. 138*, 471–484.

Charles, J.-F., de Barjac, H., **1981**. Variation du pH de l'intestin moyen d'*Aedes aegypti* en relation avec l'intoxication par les cristaux de *Bacillus thuringiensis* var. *israelensis* (sérotype H 14). *Bull. Soc. Path. Exot. 74*, 91–95.

Charles, J.-F., Nicolas, L., **1986**. Recycling of *Bacillus sphaericus* 2362 in mosquito larvae: a laboratory study. *Ann. Inst. Pasteur/Microbiol. 137B*, 101–111.

Charles, J.-F., Hamon, S., Baumann, P., **1993**. Inclusion bodies and crystals of *Bacillus sphaericus* mosquitocidal proteins expressed in various bacterial hosts. *Res. Microbiol. 144*, 411–416.

Charles, J.-F., Nielsen-LeRoux, C., Delécluse, A., **1996**. *Bacillus sphaericus* toxins: molecular biology and mode of action. *Annu. Rev. Entomol. 41*, 389–410.

Charles, J.-F., Kalfon, A., Bourgouin, C., de Barjac, H., **1988**. *Bacillus sphaericus* asporogenous mutants: ultrastructure, mosquito larvicidal activity and protein analysis. *Ann. Inst. Pasteur/Microbiol. 139*, 243–259.

Charles, J.-F., Silva-Filha, M.H., Nielsen-LeRoux, C., Humphreys, M., Berry, C., **1997**. Binding of the 51- and 42-kDa individual components from the *Bacillus sphaericus* crystal toxin on mosquito larval midgut membranes from *Culex* and *Anopheles* sp. *(Diptera: Culicidae). FEMS Microbiol. Lett. 156*, 153–159.

Chevillon, C., Bernard, C., Marquine, M., Pasteur, N., **2001**. Resistance to *Bacillus sphaericus* in *Culex pipiens* (Diptera: Culicidae): interaction between recessive mutants and evolution in southern France. *J. Med. Entomol. 38*, 657–664.

Clark, M.A., Baumann, P., **1990**. Modification of the *Bacillus sphaericus* 51- and 42-kilodalton mosquitocidal proteins: effects of internal deletions, duplications, and formation of hybrid proteins. *Appl. Environ. Microbiol. 57*, 267–271.

Correa, M., Yousten, A., **1995**. *Bacillus sphaericus* spore germination and recycling in mosquito larval cadavers. *J. Invertebr. Pathol. 66*, 76–81.

Dadd, R.H., **1975**. Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect Physiol. 21*, 1847–1853.

Darboux, I., Nielsen-LeRoux, C., Charles, J.-F., Pauron, D., **2001**. The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: molecular cloning and expression. *Insect Biochem. Mol. Biol. 31*, 981–990.

Darboux, I., Pauchet, Y., Castella, C., Silva-Filha, M.H., Nielsen-LeRoux, C., *et al.*, **2002**. Loss of the membrane anchor of the target receptor is a mechanism of bioinsecticide resistance. *Proc. Natl Acad. Sci. USA 99*, 5830–5835.

Davidson, E.W., **1981**. A review of the pathology of bacilli infecting mosquitoes, including an ultrastructural study of larvae fed *Bacillus sphaericus* 1593 spores. *Dev. Industr. Microbiol. 22*, 69–81.

Davidson, E.W., **1988**. Binding of the *Bacillus sphaericus* (Eubacteriales: Bacillaceae) toxin to midgut cells of mosquito (Diptera: Culicidae) larvae: relationship to host range. *J. Med. Entomol. 25*, 151–157.

Davidson, E.W., **1989**. Variation in binding of *Bacillus sphaericus* toxin and wheat germ agglutinin to larval midgut cells of six species of mosquitoes. *J. Invertebr. Pathol. 53*, 251–259.

Davidson, E.W., Myers, P., **1981**. Parasporal inclusions in *Bacillus sphaericus. FEMS Microbiol. Lett. 10*, 261–265.

Davidson, E.W., Bieger, A.L., Meyer, M., Shellabarge, R.C., **1987**. Enzymatic activation of the *Bacillus sphaericus* mosquito larvicidal toxin. *J. Invertebr. Pathol. 50*, 40–44.

Davidson, E.W., Oei, C., Meyer, M., Bieber, A.L., Hindley, J., *et al.*, **1990**. Interaction of the *Bacillus sphaericus* mosquito larvicidal proteins. *Can. J. Microbiol. 36*, 870–878.

Davidson, E.W., Yousten, A.A., **1990**. The mosquito larval toxin of *Bacillus sphaericus*. In: de Barjac, H., Sutherland, D.J. (Eds.), Bacterial Control of Mosquitoes and Blackflies. Rutgers University Press, New Brunswick, pp. 237–255.

de Barjac, H., **1978**. Une nouvelle variété de *Bacillus thuringiensis* très toxique pour les moustiques: *Bacillus thuringiensis* var. *israelensis,* sérotype 14. *C. R. Acad. Sci. Paris, sér. D 286*, 797–800.

de Barjac, H., **1990**. Classification of *Bacillus sphaericus* strains and comparative toxicity to mosquito larvae. In: de Barjac, H., Sutherland, D.J. (Eds.), Bacterial Control of Mosquitoes and Blackflies. Rutgers University Press, New Brunswick, pp. 228–236.

de Barjac, H., Charles, J.-F., **1983**. Une nouvelle toxine active sur les moustiques, présente dans des inclusions cristallines produites par *Bacillus sphaericus*. *C. R. Acad. Sci. Paris (série III) 296*, 905–910.

de Barjac, H., Larget-Thiéry, I., Cosmao Dumanoir, V., Ripouteau, H., **1985**. Serological classification of *Bacillus sphaericus* strains in relation with toxicity to mosquito larvae. *Appl. Microbiol. Biotechnol. 21*, 85–90.

de Barjac, H., Thiéry, I., Cosmao Dumanoir, V., Frachon, E., Laurent, P., *et al.*, **1988**. Another *Bacillus sphaericus* serotype harbouring strains very toxic to mosquito larvae: serotype H6. *Ann. Inst. Pasteur/Microbiol. 139*, 363–377.

de la Torre, F., Bennardo, T., Sebo, P., Szulmajster, J., **1989**. On the respective roles of the two proteins encoded by the *Bacillus sphaericus* 1593M toxin genes expressed in *Escherichia coli* and *Bacillus subtilis*. *Biochem. Biophys. Res. Comm. 164*, 1417–1422.

Eisenhaber, B., Bork, P., Eisenhaber, F., **1998**. Sequence properties of GPI-anchored proteins near the omega-site: constraints for the polypeptide binding site of the putative transamidase. *Protein Eng. 11*, 1155–1161.

El-Bendary, M., Priest, F., Charles, J.-F., Mitchell, W., **2004**. Crystal protein synthesis is dependent on early sporulation gene expression in *Bacillus sphaericus*. *Appl. Environ. Microbiol.* (in press).

Elangovan, G., Shanmugavelu, M., Rajamohan, F., Dean, D.H., Jayaraman, K., **2000**. Identification of the functional site in the mosquito larvicidal binary toxin of *Bacillus sphaericus* 1593M by site-directed mutagenesis. *Biochem. Biophys. Res. Commun. 276*, 1048–1055.

ffrench-Constant, R.H., Steichen, J.C., Ode, P.J., **1993**. Cyclodiene insecticide resistance in *Drosophila melanogaster* (Meigen) is associated with a temperature-sensitive phenotype. *Pest. Biochem. Physiol. 46*, 73–77.

Fillinger, U., Knols, B.G., Becker, N., **2003**. Efficacy and efficiency of new *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya. *Trop. Int. Health 8*, 37–47.

Frachon, E., Hamon, S., Nicolas, L., de Barjac, H., **1991**. Cellular fatty acid analysis as a potential tool for predicting mosquitocidal activity of *Bacillus sphaericus* strains. *Appl. Environ. Microbiol. 57*, 3394–3398.

Gahan, L.J., Gould, F., Heckel, D.G., **2001**. Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science 293*, 857–860.

Georghiou, G.P., Lagunes-Tejeda, A., **1991**. The occurrence of resistance to pesticides in arthropods. Food & Agriculture Organization of the United Nations, Rome.

Georghiou, G.P., Malik, J.I., Wirth, M., Sainato, K., **1992**. Characterization of resistance of *Culex quinquefasciatus* to the insecticidal toxins of *Bacillus sphaericus* (strain 2362). In: Cost, J., Chase, L. (Eds.), Mosquito Control Research, Annual Report. University of California Press, Berkeley, pp. 34–35.

Goldberg, L.J., Margalit, J., **1977**. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus, Aedes aegypti* and *Culex pipiens*. *Mosq. News. 37*, 355–358.

Griffitts, J.S., Whitacre, J.L., Stevens, D.E., Aroian, R.V., **2001**. Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. *Science 293*, 860–864.

Guerineau, M., Alexander, B., Priest, F.G., **1991**. Isolation and identification of *Bacillus sphaericus* strains pathogenic for mosquito larvae. *J. Invertebr. Pathol. 57*, 325–333.

Hindley, J., Berry, C., **1987**. Identification, cloning and sequence analysis of the *Bacillus sphaericus* 1593 41.9 kD larvicidal toxin gene. *Mol. Microbiol. 1*, 187–194.

Hindley, J., Berry, C., **1988**. *Bacillus sphaericus* strain 2297: nucleotidic sequence of a 41.9 kDa toxin gene. *Nucleic Acids Res. 16*, 4168.

Hougard, J.M., Mbentengam, R., Lochouarn, L., Escaffre, H., Darriet, F., *et al.*, **1993**. Control of *Culex quinquefasciatus* by *Bacillus sphaericus*: results of a pilot campaign in a large urban area in equatorial Africa. *Bull. WHO 71*, 367–375.

Humphreys, M.J., Berry, C., **1998**. Variants of the *Bacillus sphaericus* binary toxins: implications for differential toxicity of strains. *J. Invertebr. Pathol. 71*, 184–185.

Kalfon, A., Charles, J.-F., Bourgouin, C., de Barjac, H., **1984**. Sporulation of *Bacillus sphaericus* 2297: an electron microscope study of crystal-like inclusions biogenesis and toxicity to mosquito larvae. *J. Gen. Microbiol. 130*, 893–900.

Karch, S., Coz, J., **1983**. Histopathologie de *Culex pipiens* Linné (Diptera, Culicidae) soumis à l'activité larvicide de *Bacillus sphaericus* 1593-4. *Cah. ORSTOM, sér. Ent. méd. Parasitol. XXI*, 225–230.

Karch, S., Asidi, N., Manzambi, M., Salaun, J.J., **1992**. Efficacy of *Bacillus sphaericus* against the malaria vector *Anopheles gambiae* and other mosquitoes in swamps and rice fields in Zaire. *J. Am. Mosq. Control Assoc. 8*, 376–380.

Kellen, W., Clark, T., Lindergren, J., Ho, B., Rogoff, M., *et al.*, **1965**. *Bacillus sphaericus* Neide as a pathogen of mosquitoes. *J. Invertebr. Pathol. 7*, 442–448.

Krych, V.K., Johnson, J.L., Yousten, A.A., **1980**. Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. *Int. J. Syst. Bact. 30*, 476–482.

Kumar, A., Sharma, V., Sumodan, P., Thavaseluam, D., Kamat, R., **1994**. Malaria control utilising *Bacillus sphaericus* against *Anopheles stephensi* in Pinaji, Goa. *J. Am. Mosq. Control Assoc. 10*, 534–539.

Kumar, A., Sharma, V.P., Thavaselvam, D., Sumodan, P.K., Kamat, R.H., *et al.*, **1996**. Control of *Culex quinquefasciatus* with *Bacillus sphaericus* in Vasco City, Goa. *J. Am. Mosq. Control Assoc. 12*, 409–413.

Lacey, L.A., Day, J., Heitzman, C.M., **1987**. Long-term effects of *Bacillus sphaericus* on *Culex quinquefasciatus*. *J. Invertebr. Pathol. 49*, 116–123.

Liu, J.W., Hindley, J., Porter, A.G., Priest, F.G., **1993**. New high-toxicity mosquitocidal strains of *Bacillus sphaericus* lacking a 100-kilodalton-toxin gene. *Appl. Environ. Microbiol. 59*, 3470–3473.

Liu, J.W., Porter, A.G., Wee, B.Y., Thanabalu, T., **1996a**. New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-Kilodalton mosquitocidal toxins. *Appl. Environ. Microbiol. 62*, 2174–2176.

Liu, J.W., Yap, W.H., Thanabalu, T., Porter, A.G., **1996b**. Efficient synthesis of mosquitocidal toxins in *Asticcacaulis excentricus* demonstrates potential of Gram-negative bacteria in mosquito control. *Nature Biotechnol. 14*, 343–347.

Miteva, V., Gancheva, A., Mitev, V., Ljubenov, M., **1998**. Comparative genome analysis of *Bacillus sphaericus* by ribotyping, M13 hybridization, and M13 polymerase chain reaction fingerprinting. *Can. J. Microbiol. 44*, 175–180.

Mulla, M.S., Thavara, U., Tawatsin, A., Chomposri, J., Su, T., **2003**. Emergence of resistance and resistance management in field populations of tropical *Culex quinquefasciatus* to the microbial control agent *Bacillus sphaericus*. *J. Am. Mosq. Control Assoc. 19*, 39–46.

Mutero, A., Pralavorio, M., Bride, J.M., Fournier, D., **1994**. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl Acad. Sci. USA 91, 5922–5926.*

Myers, P., Yousten, A.A., Davidson, E.W., **1979**. Comparative studies of the mosquito-larval toxin of *Bacillus sphaericus* SSII-1 and 1593. *Can. J. Microbiol. 25*, 1227–1231.

Narasu, L.M., Gopinathan, K.P., **1988**. Effect of *Bacillus sphaericus* 1593 toxin on choline acetyl transferase and mitochondrial oxidative activities of the mosquito larvae. *Ind. J. Biochem. Biophys. 25*, 253–256.

Neide, E., **1904**. Botanische Beschneibung einiger sporenbildenden Bakterien. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 12*, 1–32.

Nicolas, L., Lecroisey, A., Charles, J.-F., **1990**. Role of the gut proteinases from mosquito larvae in the mechanism of action and the specificity of the *Bacillus sphaericus* toxin. *Can. J. Microbiol. 36*, 804–807.

Nicolas, L., Nielsen-LeRoux, C., Charles, J.-F., Delécluse, A., **1993**. Respective role of the 42- and 51-kDa component of the *Bacillus sphaericus* toxin overexpressed in *Bacillus thuringiensis*. *FEMS Lett. 106*, 275–280.

Nielsen-LeRoux, C., Charles, J.-F., **1992**. Binding of *Bacillus sphaericus* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. *Eur. J. Biochem. 210*, 585–590.

Nielsen-LeRoux, C., Charles, J.-F., Thiéry, I., Georghiou, G.P., **1995**. Resistance in a laboratory population of *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus* binary toxin is due to a change in the receptor on midgut brush-border membranes. *Eur. J. Biochem. 228*, 206–210.

Nielsen-LeRoux, C., Pasquier, F., Charles, J.-F., Sinègre, G., Gaven, B., *et al.*, **1997**. Resistance to *Bacillus sphaericus* involves different mechanisms in *Culex pipiens* mosquito larvae (Diptera: Culicidae). *J. Med. Entomol. 34*, 321–327.

Nielsen-LeRoux, C., Pasteur, N., Prêtre, J., Charles, J.-F., Ben Cheick, H., *et al.*, **2002**. High resistance to *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae): the complex situation of West-Mediterranean countries. *J. Med. Entomol. 39*, 729–735.

Nielsen-LeRoux, C., Rao, D.R., Rodcharoen-Murhy, J., Carron, A., Mani, T.R., *et al.*, **2001**. Various levels of cross-resistance to *Bacillus sphaericus* strains in *Culex pipiens* (Diptera: Culicidae) colonies resistant to *B. sphaericus* strain 2362. *Appl. Environ. Microbiol. 67*, 5049–5054.

Oei, C., Hindley, J., Berry, C., **1990**. An analysis of the genes encoding the 51.4- and 41.9-kDa toxins of *Bacillus sphaericus* 2297 by deletion mutagenesis: the construction of fusion proteins. *FEMS Microbiol. Lett. 72*, 265–274.

Oei, C., Hindley, J., Berry, C., **1992**. Binding of purified *Bacillus sphaericus* binary toxin and its deletion derivates to *Culex quinquefasciatus* gut: elucidation of functional binding domains. *J. Gen. Microbiol. 138*, 1515–1526.

Oliveira, C.M.O., Silva-Filha, M.H., Nielsen-LeRoux, C., Pei, G., Yuan, Z., *et al.*, **2003a**. Inheritance and mechanism of resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* from China and Brazil (Diptera: Culicidae). *J. Med. Entomol. 41*, 58–64.

Oliveira, C.M.O., Silva-Filha, M.H., Beltrán, J., Nielsen-LeRoux, C., Pei, C.G., *et al.*, **2003b**. Biological fitness of *Culex quinquefasciatus* (Diptera: Culicidae) larvae resistant to *Bacillus sphaericus*. *J. Am. Mosq. Control Assoc. 19*, 125–129.

Park, H.W., Bideshi, D.K., Federici, B.A., **2003**. Recombinant strain of *Bacillus thuringiensis* producing Cyt1A, Cry11B and the *Bacillus sphaericus* binary toxin. *Appl. Environ. Microbiol. 69*, 1331–1334.

Pauron, D., Barhanin, J., Amichot, M., Pralavorio, M., Bergé, J.-B., *et al.*, **1989**. Pyrethroid receptor in the insect Na^+ channel: alteration of its properties in pyrethroid-resistant flies. *Biochemistry 28*, 1673–1677.

Payne, J.M., Davidson, E.W., **1984**. Insecticidal activity of crystalline parasporal inclusions and other components of the *Bacillus sphaericus* 1593 spore complex. *J. Invertebr. Pathol. 43*, 383–388.

Pei, G.F., Oliveira, C.M.F., Yuan, Z.M., Nielsen-LeRoux, C., Silva-Filha, M.H., *et al.*, **2002**. A strain of *Bacillus sphaericus* causes slower development of resistance in *Culex quinquefasciatus*. *Appl. Environ. Microbiol. 68*, 3003–3009.

Poncet, S., Delécluse, A., Guido, A., Klier, A., Rapoport, G., **1994**. Transfer and expression of the *cryIVB* and *cryIVD* genes of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus* 2297. *FEMS Microbiol. Lett. 117*, 91–96.

Poncet, S., Bernard, C., Dervyn, E., Cayley, J., Klier, A., *et al.*, **1997**. Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the *cry11A* toxin gene from *Bacillus thuringiensis* subsp. israelensis. *Appl. Environ. Microbiol. 63*, 4413–4420.

Poopathi, S., Mani, T., Rao, R.D., Baskaran, G., Kabilan, L., **1999**. Cross-resistance to *Bacillus sphaericus* strains in *Culex quinquefasciatus* resistant to *B. sphaericus* 1593M. *Southeast Asian J. Trop. Med. Public Health 30*, 477–481.

Porter, A.G., Davidson, E.W., Liu, J.W., **1993**. Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. *Microbiol. Rev. 57*, 838–861.

Priest, F.G., Ebrup, L.V.Z., Carter, P.E., **1997**. Distribution and characterization of mosquitocidal toxin genes in some strains of *Bacillus sphaericus*. *Appl. Environ. Microbiol. 63*, 1195–1198.

Rao, D.R., Mani, T.R., Rajendran, R., Joseph, A.S.J., Gajanana, A., *et al.*, **1995**. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. *J. Am. Mosq. Control Assoc. 11*, 1–5.

Regis, L., Nielsen-LeRoux, C., **2000**. Management of resistance to bacterial vector control. In: Charles, J.-F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: from Laboratory to Field Application. Kluwer Academic Publisher, Dordrecht, Boston, London, pp. 419–433.

Regis, L., Silva-Filha, M.H.N.L., de Oliveira, C.M.F., Rios, E.M., da Silva, S.B., *et al.*, **1995**. Integrated control measures against *Culex quinquefasciatus*, the vector of filariasis in Recife. *Mem. Inst. Oswaldo Cruz. 90*, 115–119.

Rodcharoen, J., Mulla, M.S., **1994**. Resistance development in *Culex quinquefasciatus* (Diptera: Culicidae) to the microbial agent *Bacillus sphaericus*. *J. Econ. Entomol. 87*, 1133–1140.

Rodcharoen, J., Mulla, M.S., **1997**. Biological fitness of *Culex quinquefasciatus* (Diptera: Culicidae) susceptible and resistant to *Bacillus sphaericus*. *J. Med. Entomol. 34*, 5–10.

Schirmer, J., Wieden, H.-J., Rodnina Marina, V., Aktories, K., **2002a**. Inactivation of the elongation

factor Tu by mosquitocidal toxin-catalyzed mono-ADP-ribosylation. *Appl. Environ. Microbiol. 68*, 4894–4899.

Schirmer, J., Just, I., Aktories, K., Schirmer, J., Wieden, H.-J., *et al.*, **2002b**. The ADP-ribosylating mosquitocidal toxin from *Bacillus sphaericus*: proteolytic activation, enzyme activity, and cytotoxic effects. *J. Biol. Chem. 277*, 11941–11948.

Schwartz, J.-L., Potvin, L., Coux, F., Charles, J.-F., Berry, C., *et al.*, **2001**. Permeabilization of model lipid membranes by *Bacillus sphaericus* mosquitocidal binary toxin and its individual components. *J. Membr. Biol. 184*, 171–183.

Sebo, P., Bennardo, T., de la Torre, F., Szulmajster, J., **1990**. Delineation of the minimal portion of the *Bacillus sphaericus* 1593M toxin required for the expression of larvicidal activity. *Eur. J. Biochem. 194*, 161–165.

Servant, P., Rosso, M.L., Hamon, S., Poncet, S., Delécluse, A., *et al.*, **1999**. Production of Cry11A and Cry11Ba toxins in *Bacillus sphaericus* confers toxicity towards *Aedes aegypti* and resistant *Culex* populations. *Appl. Environ. Microbiol. 65*, 3021–3026.

Shanmugavelu, M., Rajamohan, F., Kathirvel, M., Elangovan, G., Dean, D.H., *et al.*, **1998**. Functional complementation of nontoxic mutant binary toxins of *Bacillus sphaericus* 1593M generated by site-directed mutagenesis. *Appl. Environ. Microbiol. 64*, 756–759.

Shi, Y., Yuan, Z., Cai, Q., Yu, J., Yan, J., *et al.*, **2001**. Cloning and expression of the binary toxin gene from *Bacillus sphaericus* IAB872 in a crystal-minus *Bacillus thuringiensis* subsp. *israelensis*. *Curr. Microbiol. 43*, 21–25.

Silva-Filha, M.H., Nielsen-LeRoux, C., Charles, J.-F., **1997**. Binding kinetics of *Bacillus sphaericus* binary toxin to midgut brush border membranes of *Anopheles* and *Culex* sp. *larvae. Eur. J. Biochem. 247*, 754–761.

Silva-Filha, M.H., Nielsen-LeRoux, C., Charles, J.F., **1999**. Identification of the receptor for *Bacillus sphaericus* crystal toxin in the brush border membrane of the mosquito *Culex pipiens* (Diptera: Culicidae). *Insect Biochem. Mol. Biol. 29*, 711–721.

Silva-Filha, M.H., Peixoto, C.A., **2003**. Immunocytochemical localization of the *Bacillus sphaericus* binary toxin components in *Culex quinquefasciatus* (Diptera: Culicidae) larvae midgut. *Pest. Biochem. Physiol. 77*, 138–146.

Silva-Filha, M.-H., Regis, L., **1997**. Reversal of a low-level resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* (Diptera: Culicidae) from an urban area of Recife, Brazil. *J. Econ. Entomol. 90*, 299–303.

Silva-Filha, M.-H., Regis, L., Nielsen-LeRoux, C., Charles, J.-F., **1995**. Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). *J. Econ. Entomol. 88*, 525–530.

Simons, K., Toomre, D., **2000**. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol. 1*, 31–39.

Sinègre, G., Babinot, M., Vigo, G., Jullien, J.-L., **1993**. *Bacillus sphaericus* et démoustication urbaine. In: Bilan de cinq années d'utilisation expérimentale de la spécialité Spherimos® dans le sud de la France. Document E.I.D.L.M. N°62. Entente Interdépartementale pour la Démoustication du Littoral Méditerranéen, Montpellier. 21pp.

Sinègre, G., Babinot, M., Quermel, J.-M., Gavon, B., **1994**. First field occurrence of *Culex pipiens* resistance to *Bacillus sphaericus* in southern France. Abstr. VIIIth Eur. Meet. Soc. Vector Ecol., 5–8 September, Barcelona, p. 17.

Singer, S., **1973**. Insecticidal activity of recent bacterial isolates and their toxins against mosquito larvae. *Nature 244*, 110–111.

Singer, S., **1974**. Entomogenous bacilli against mosquito larvae. *Dev. Industr. Microbiol. 15*, 187–194.

Singer, S., **1977**. Isolation and development of bacterial pathogens in vectors. In: "Biological regulation of vectors," DHEW Publication No. 77-1180. National Institutes of Health, Bethesda, MD, pp. 3–18.

Singh, G.J.P., Gill, S.S., **1988**. An electron microscope study of the toxic action of *Bacillus sphaericus* in *Culex quinquefasciatus* larvae. *J. Invertebr. Pathol. 52*, 237–247.

Skovmand, O., Bauduin, S., **1997**. Efficacy of granular formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West African countries. *J. Vector Ecol. 22*, 43–51.

Stray, J.E., Klowden, M.J., Hurlbert, R.E., **1988**. Toxicity of *Bacillus sphaericus* crystal toxin to adult mosquitoes. *Appl. Environ. Microbiol. 54*, 2320–2321.

Tanapongpipat, S., Nantapong, N., Cole, J., Panyim, S., **2003**. Stable integration and expression of mosquito-larvicidal genes from *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* into the chromosome of *Enterobacter amnigenus:* a potential breakthrough in mosquito biocontrol. *FEMS Microbiol. Lett. 221*, 343–248.

Thanabalu, T., Berry, C., Hindley, J., **1993**. Cytotoxicity and ADP-ribosylating activity of the mosquitocidal toxin from *Bacillus sphaericus* SSII-1: possible roles of the 27-kilodalton and 70-kilodalton peptides. *J. Bacteriol. 175*, 2314–2320.

Thanabalu, T., Hindley, J., Berry, C., **1992**. Proteolytic processing of the mosquitocidal toxin from *Bacillus sphaericus* SSII-1. *J. Bacteriol. 174*, 5051–5056.

Thanabalu, T., Hindley, J., Jackson-Yap, J., Berry, C., **1991**. Cloning, sequencing, and expression of a gene encoding a 100-kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-1. *J. Bacteriol. 173*, 2776–2785.

Thanabalu, T., Porter, A.G., **1995**. *Bacillus sphaericus* gene encoding a novel class of mosquitocidal toxin with homology to *Clostridium* and *Pseudomonas* toxins. *Gene 61*, 4031–4036.

Thanabalu, T., Porter, A.G., **1996**. A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. *Gene 170*, 85–89.

Thiéry, I., de Barjac, H., **1989**. Selection of the most potent *Bacillus sphaericus* strains, based on activity ratios determined on three mosquito species. *Appl. Microbiol. Biotechnol. 31*, 577–581.

Thiéry, I., Hamon, S., Delécluse, A., Orduz, S., **1998**. The introduction into *Bacillus sphaericus* of the *Bacillus thuringiensis* subsp. *medellin cyt1Ab1* gene results in higher susceptibility of resistant mosquito larva populations to *B. sphaericus*. Appl. Environ. Microbiol. *64*, 3910–3916.

Trisrisook, M., Pantuwatana, S., Bhumiratana, A., Panbangred, W., **1990**. Molecular cloning of the 130-kilodalton mosquitocidal δ-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. Appl. Environ. Microbiol. *56*, 1710–1716.

Udenfriend, S., Kodukula, K., **1995**. How glycosylphosphatidylinositol-anchored membrane proteins are made. *Annu. Rev. Biochem. 64*, 563–591.

Wirth, M.C., Walton, W.E., Federici, B.A., **2000a**. Cyt1A from *Bacillus thuringiensis* restores toxicity of *Bacillus sphaericus* against resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol. 37*, 401–407.

Wirth, M.C., Georghiou, G.P., Malik, J.I., Abro, G.H., **2000b**. Laboratory selection for resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from California, USA. *J. Med. Entomol. 37*, 534–540.

Woodburn, M.A., Yousten, A.A., Hilu, K.H., **1995**. Random amplified polymorphic DNA fingerprinting of mosquito-pathogenic and nonpathogenic strains of *Bacillus sphaericus*. *Int. J. Syst. Bacteriol. 45*, 212–217.

Yousten, A.A., de Barjac, H., Hedrick, J., Cosmao Dumanoir, V., Myers, P., **1980**. Comparison between bacteriophage typing and serotyping for the differentiation of *Bacillus sphaericus* strains. *Ann. Microbiol. (Inst. Pasteur) 131B*, 297–308.

Yousten, A.A., Davidson, E.W., **1982**. Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. *Appl. Environ. Microbiol. 44*, 1449–1455.

Yousten, A.A., Madhekar, N., Wallis, D.A., **1984**. Fermentation conditions affecting growth, sporulation, and mosquito larval toxin formation by *Bacillus sphaericus*. *Dev. Industr. Microbiol. 25*, 757–762.

Yuan, Z., Nielsen-Le Roux, C., Pasteur, N., Delécluse, A., Charles, J.-F., *et al*., **1998**. Detection of the binary toxin genes and proteins of several *Bacillus sphaericus* strains and their toxicity against susceptible and resistant *Culex pipiens*. *Acta Entomol. Sinica. 41*, 337–342.

Yuan, Z., Rang, C., Maroun, R.C., Juarez-Perez, V., Frutos, R., *et al*., **2001**. Identification and molecular structural prediction analysis of a toxicity determinant in the *Bacillus sphaericus* crystal larvicidal toxin. *Eur. J. Biochem. 268*, 2751–2760.

Yuan, Z., Zhang, Y., Cai, Q., Liu, E.Y., **2000**. High-level field resistance to *Bacillus sphaericus* C3-41 in *Culex quinquefasciatus* from southern China. *Biocontrol. Sci. Technol. 10*, 41–49.

6.8 Baculoviruses: Biology, Biochemistry, and Molecular Biology

B C Bonning, Iowa State University, Ames, IA, USA

6.8.1. Introduction

Initial interest in baculoviruses stemmed from their potential use in insect pest management (Moscardi, 1999). Modern baculovirology is driven by genetic enhancement of their insecticidal properties (van Beek and Hughes, 1998; Bonning *et al.*, 2002), their use for the study of fundamental biological processes (Clem, 2001; Manji and Friesen, 2001), for protein expression (Jarvis, 1997), and for gene therapy (Ghosh *et al.*, 2002; Kost and Condreay, 2002). Addressing their limitations for these purposes allows for increased understanding of baculovirus biology. As a result of the various applications of baculoviruses, they represent the best studied of the invertebrate viruses.

Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the type species of the *Nucleopolyhedrovirus* genus (Blissard *et al.*, 2000), has been particularly extensively studied both in terms of molecular biology and host range. Hence much of this review will focus on AcMNPV with particular emphasis on recent advances in baculovirus research. Relatively little molecular, genetic, and biochemical research has been conducted on granuloviruses (GV), in part because of the difficulty of establishing cell culture systems for GV propagation (Winstanley and Crook, 1993). Certain aspects of baculovirus biology such as baculovirus ecology (Cory *et al.*, 1997), epizootiology (Fuxa and Tanada, 1987), baculoviruses of nonlepidopteran hosts (Couch, 1991; Federici, 1997), and tritrophic interactions (Duffey *et al.*, 1995) are described elsewhere.

6.8.1.1. Taxonomy

Baculoviruses belong to the family Baculoviridae, a large family of bacilliform, occluded viruses that

infect arthropods, primarily the Lepidoptera (Adams and McClintock, 1991). Baculoviruses also have been isolated from members of the Diptera and Hymenoptera (Blissard *et al*., 2000). Members of the two genera within the Baculoviridae, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), are distinguished by occlusion body (OB) morphology and the cytopathology that they cause (Blissard *et al*., 2000). NPVs produce large polyhedra (typically 1–5 μm in diameter) with many virions or occlusion-derived virus (ODV), while GV produce smaller occlusion bodies or granules (150 nm in diameter by 400–600 nm in length) with a single virion (**Figure 1**). NPVs are designated as SNPV or MNPV according to whether the ODV contains single (S) or multiple (M) nucleocapsids (**Figure 1**). There are many more described MNPVs than SNPVs (Volkman, 1997). Because all of the MNPV characterized to date infect species within the order Lepidoptera, while SNPV have been isolated from species within the Lepidoptera, Hymenoptera, and Diptera, the progenitor NPV is believed to be an SNPV (Rohrmann, 1996). Despite morphological similarity to baculoviruses, at least one of the shrimp baculoviruses, the white spot syndrome virus, is unrelated at the amino acid level (Yang *et al*., 2001) and has been reclassified in the family Nimaviridae, genus *Whispovirus*.

The NPVs are divided into two groups on the basis of phylogenetic relatedness (Zanotto *et al*., 1993; Herniou *et al*., 2001). Group I NPVs have 17 genes that are absent from group II NPVs (Herniou *et al*., 2001). The two groups have distinct budded virus (BV) envelope fusion proteins, with GP64 in group I NPVs, and F (fusion) proteins in group II NPVs (Ijkel *et al*., 1999; Pearson *et al*., 2000). Homologs of GP64 are present in the thogotoviruses (Orthomyxoviridae) (Morse *et al*., 1992), while proteins related to F proteins are present in the genus *Errantivirus* (Metaviridae) (Malik *et al*., 2000; Rohrmann and Karplus, 2001; Pearson and Rohrmann, 2002).

GVs are classified according to tissue tropism: type 1 GVs such as *Trichoplusia ni* GV enter the host via the midgut epithelium but infect only the fat body. Because other important tissues are not affected, infection may be prolonged with lethargy only a day or two before death. For type 2 GV, tissue tropism is similar to that of NPVs affecting most tissues, while type 3 GVs are restricted to the midgut epithelium. *Harrisina brillians* GV is the only type 3 GV characterized to date and causes acute disease (Federici, 1997). The pathogenesis of NPV diseases has been studied in much greater detail compared to GV disease. A fundamental difference between the cytopathology of NPV and GV infections is that the nuclear membrane breaks down during replication of GV but not NPV, resulting in mixing of cytoplasm and nucleoplasm (Winstanley and Crook, 1993). GV-infected cells separate from each other and from the basement membrane, resulting in proliferation of regenerative cells adjacent to the basement membrane, which become infected as they differentiate.

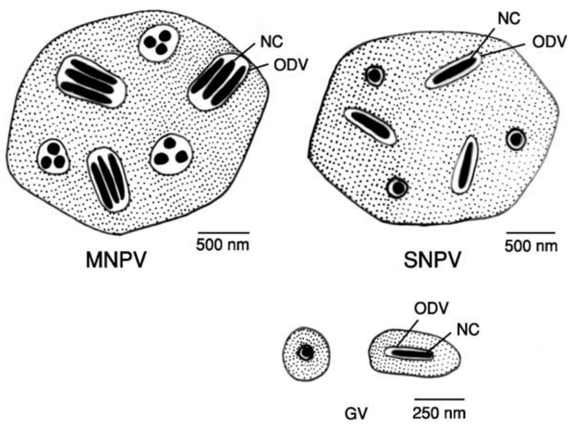


Figure 1 Schematic representation of occlusion bodies of multiple and single nucleopolyhedroviruses (MNPV and SNPV, respectively) and granuloviruses (GVs). The figure illustrates envelopment of multiple or single rod-shaped nucleocapsids (NC) within occlusion-derived virus (ODV) of the polyhedra of MNPV and SNPV, respectively. Each granule of the GV contains a virion with a single nucleocapsid. The relative sizes of the GV and NPV are indicated.

6.8.1.2. Nomenclature

Baculoviruses are named according to viral genus based on morphological characteristics, and the Latin name of the host from which they were first isolated. This system can be misleading because many baculoviruses infect more than one host species. AcMNPV for example, although first isolated from *A. californica*, infects at least 40 other species within the Lepidoptera (Gröner, 1986). Complexities also arise when the same virus is isolated from different host species (e.g., *Rachiplusia ou* MNPV and *Anagrapha falcifera* MNPV) (Hostetter and Puttler, 1991; Harrison and Bonning, 1999), and when different viruses are isolated from the same host species (e.g., *Mamestra configurata* MNPV A and B) (Li *et al*., 2002a, 2002b).

6.8.2. Structure

Baculoviruses exist as two phenotypes, BV and occlusion-derived virus (ODV), which have a common nucleocapsid structure and carry the same genetic information (Blissard, 1996; Rohrmann, 1992). ODV, contained within the occlusion bodies

(OB: polyhedra or granules), are responsible for transmission between host insects, while BV effect systemic transmission within the host, and virus propagation in cell culture.

6.8.2.1. Nucleocapsids

Nucleocapsids are tubular in shape with cap structures at each end (**Figure 2**). The genome is condensed to about 100-fold within the inner nucleoprotein core (Bud and Kelly, 1980). The supercoiled, circular genome of double-stranded DNA is complexed with a highly basic protein, P6.9 in NPV and VP12 in GV (Tweeten *et al.*, 1980; Wilson *et al.*, 1987). Binding of these arginine-rich molecules to the DNA produces a compact, insoluble DNA complex. The viral genomes appear to be prepackaged within the virogenic stroma (an electron-dense structure produced within the nucleus at the onset of viral DNA synthesis), before incorporation into the capsid shells (Fraser, 1986) (**Figure 3**). The end structures of nucleocapsids consist of a flat disk at the basal end and nipple structures at the apical end (Federici, 1986). Nucleocapsids attach to membranes at the apical end of the capsid. VP39 is the major component of the nucleocapsid in BV and OB of AcMNPV (Pearson *et al.*, 1988; Thiem and Miller, 1989). P80 and P24 are also associated with the capsid and PP78/83 is associated with the basal end complexed with EC27 and C42 (Braunagel *et al.*, 2001) (**Figure 2**). VP1054 and VP91 are associated with both BV and OB (Olszewski and Miller, 1997a; Russell and Rohrmann, 1997).

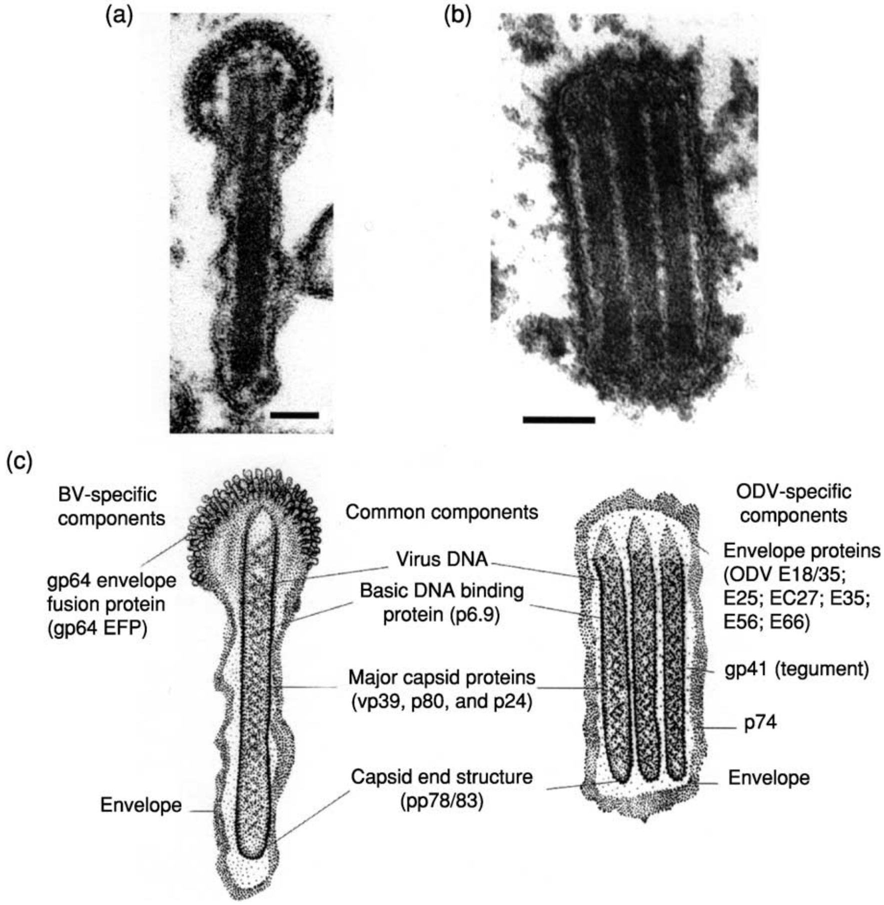


Figure 2 Structure of budded and occlusion-derived virus. (a) Transmission electron micrograph (TEM) of budded virus (BV) of *Lymantria dispar* MNPV showing glycoprotein spikes, or peplomers at the anterior end. Scale bar = 50 nm. (Reproduced with permission from Adams, J.R., Goodwin, R.H., Wilcox, T.A., **1977**. Electron microscopic investigations on invasion and replication of insect baculoviruses *in vivo* and *in vitro*. *Biol. Cellulaire 28*, 261–268.) (b) TEM of occlusion-derived virus (ODV) of *Agrotis ipsilon* MNPV (AgipMNPV) in a hemocyte of *Agrotis ipsilon*. Scale bar 100 nm. (c) Schematic representation of BV and ODV indicating virus-encoded structural components common to both or unique to each structure (after Funk *et al.*, 1997). References for structural proteins: P6.9 (Wilson *et al.*, 1987); VP39 (Thiem and Miller, 1989); P80 (Lu and Carstens, 1992); PP78/83 (Vialard and Richardson, 1993); ODV-E25 (Russell and Rohrmann, 1993); ODV-E66 (Hong *et al.*, 1994); ODV-E56 (Braunagel *et al.*, 1996a; Theilmann *et al.*, 1996); ODV-E18, -E35, and -E27 (Braunagel *et al.*, 1996b); GP41 (Whitford and Faulkner, 1992); p74 (Kuzio *et al.*, 1989); GP64 (group I NPVs: Whitford *et al.*, 1989) or F protein (group II NPVs and GVs: Pearson *et al.*, 2001).

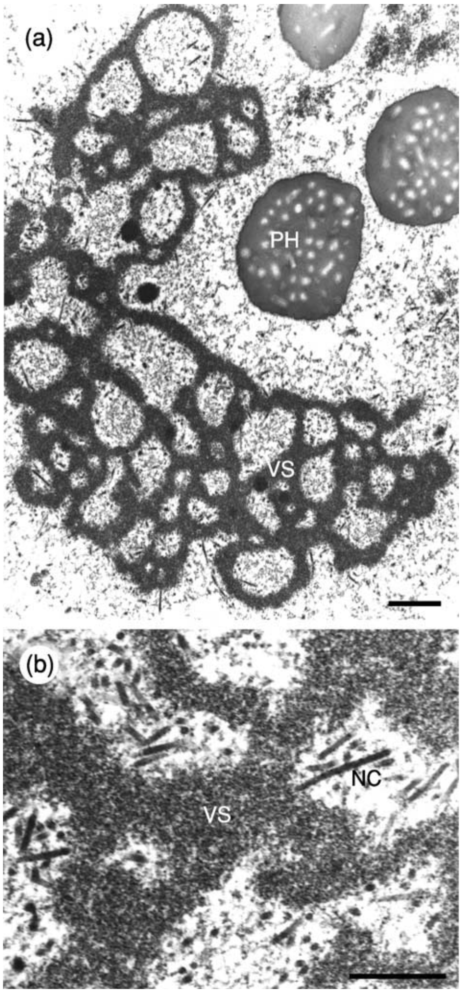


Figure 3 (a) Detail of the virogenic stroma (VS) and polyhedra (PH) within the nucleus of an AgipMNPV-infected hemocyte of *Agrotis ipsilon*. Scale bar = 1 µm. (b) Rod-shaped nucleocapsids (NC) produced within the virogenic stroma (VS). Scale bar = 500 nm.

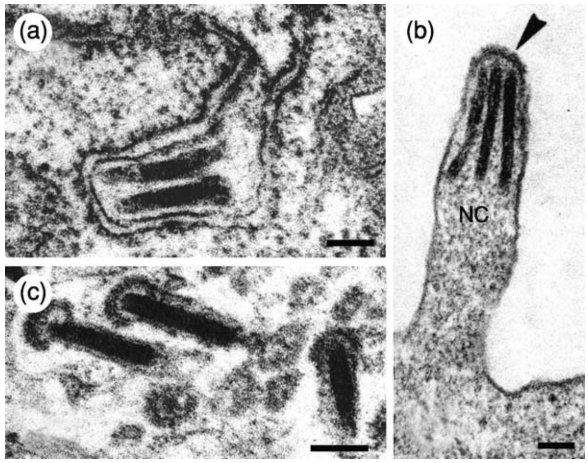


Figure 4 Production of budded virus. (a) Nucleocapsids become encased in a vesicle of nuclear membrane as they move out of the nucleus. Nucleocapsids are subsequently released into the cytoplasm as the vesicle breaks down. (*Autographa californica* MNPV in *Spodoptera exigua* fat body cell.) (b) Nucleocapsids move to and bud through regions of the plasma membrane that have been modified by virus-produced GP64 (arrow head). (*Helicoverpa zea* SNPV in *H. zea* cell line.) (c) Budded virus with distinctive peplomer structures at the anterior end. (*Trichoplusia ni* SNPV in *T. ni*.) Scale bars = 100 nm. (Reproduced with permission from Adams, J.R., Goodwin, R.H., Wilcox, T.A., **1977**. Electron microscopic investigations on invasion and replication of insect baculoviruses *in vivo* and *in vitro*. *Biol. Cellulaire 28*, 261–268.)

6.8.2.2. Budded Virus

Nucleocapsids initially move out of the nucleus for exit at the plasma membrane, and appear to move in vesicles derived from the nuclear membrane in association with filaments of actin. The presence of multivesicular aggregates containing many nucleocapsids suggests that fusion of vesicles may occur (Williams and Faulkner, 1997). The nuclear membrane-derived vesicle is lost in transit and unenveloped nucleocapsids align at the apical end with the plasma membrane, which is modified by the viral-encoded glycoprotein, GP64 (**Figures 4** and **5**). The GP64 envelope fusion protein is the major virus-encoded protein associated with the BV envelope. GP64 is expressed during the early and late phases (Blissard and Rohrmann, 1989; Whitford *et al*., 1989; Monsma and Blissard, 1995; Monsma *et al*., 1996) and accumulates at the plasma membrane (Volkman, 1986). On budding through the plasma membrane, nucleocapsids acquire a loose envelope with prominent spikes or peplomers consisting of GP64 at the apical end (Volkman and Goldsmith, 1984; Volkman, 1986). Ubiquitin is localized to the inner surface of the envelope (Guarino *et al*., 1995). GP64 is a membrane fusion protein that is activated at low pH and is involved in BV entry into the cell by endocytosis (Blissard and Wenz, 1992; Chernomordik *et al*., 1995; Monsma and Blissard, 1995). The BV envelope fuses with host endosomal membranes thereby releasing nucleocapsids into the cytoplasm. GP64 is essential for cell-to-cell movement of virus (Monsma *et al*., 1996). Baculovirus GP64 is related to the envelope glycoproteins of two orthomyxovirus-like arboviruses that are vectored by ticks. These proteins mediate membrane fusion and hemagglutination (Morse *et al*., 1992). GP64 is absent from ODV (Blissard and Rohrmann, 1989; Jarvis and Garcia, 1994) and also from the two variants of the single NPV of the S phenotype for which the genome has been completely sequenced (Chen *et al*., 2001, 2002).

GP64 is absent from group II NPVs, which have F proteins that serve a similar functional role. However, group I NPVs also encode F protein homologs that

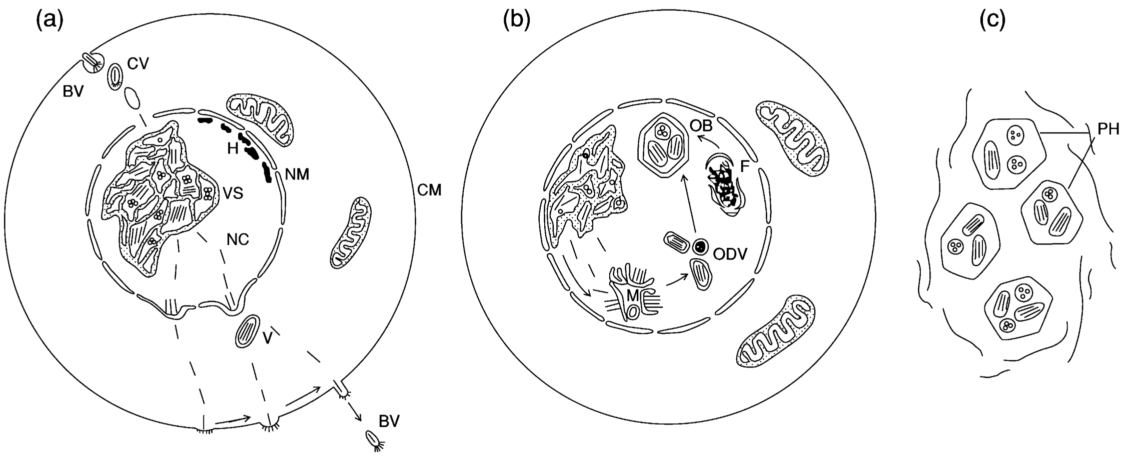


Figure 5 Production of budded virus and occlusion bodies during the late and very late phases of NPV infection, followed by cell lysis. (a) A budded virus (BV) binds to a putative receptor at the cell membrane (CM) and enters the cell by adsoprtive endocytosis within clathrin-coated vesicles (CV). The BV envelope fuses with host endosomal membranes thereby releasing nucleocapsids into the cytoplasm. Nucleocapsids uncoat at the nuclear membrane. Heterochromatin (H) becomes marginated and the virogenic stroma (VS) is produced at the onset of virus replication and the late phase of infection. Nucleocapsids (NC) produced within the virogenic stroma move out of the nucleus initially in vesicles (V) that appear to be derived form the nuclear membrane (NM). The vesicles dissociate and nucleocapsids bud through the GP64-modified regions of the plasma membrane to acquire a loose envelope with peplomers at the apical end. (b) A switch occurs at about 24 h after infection of the cell from BV to OB production, marking the onset of the very late phase of infection. Intranuclear vesicles dissociate to produce membranes (M) within the ring zone of the infected nucleus, which then envelope nucleocapsids to produce occlusion-derived virus (ODV). Fibrillar bodies (F) are produced that associate with polyhedral envelope precursor structures. ODV become embedded in the polyhedrin matrix, which is then surrounded by the polyhedral envelope. (c) Polyhedra (PH) are released on lysis of the infected cell.

are more divergent than those found in group II NPVs and GVs. The F protein homologs in group I NPVs appear to lack a fusion function (Pearson *et al.*, 2000; Lung *et al.*, 2003). There is overlap between the distribution of GP64 and the F protein homologs in the budded virus, but biochemical fractionation studies suggest that the F protein homologs are also associated with BV nucleocapsids. This localization suggests that the F protein homologs may play a role in the interaction between the budded virus envelope and the nucleocapsid potentially mediated by the cytoplasmic tail domain of the protein (Pearson *et al.*, 2001). However, the F protein homolog of AcMNPV is not essential for virus replication in cell culture or in insects. The AcMNPV F protein homolog has been shown to function as a viral pathogenicity factor that accelerates the mortality of infected hosts (Lung *et al.*, 2003).

The baculovirus F protein is related to the envelope protein of insect retroviruses in the genus *Errantivirus* (family Metaviridae). Evidence suggests that a *gypsy*-like noninfectious retrotransposon acquired a baculovirus F progenitor resulting in formation of a retrovirus (Malik *et al.*, 2000; Rohrmann and Karplus, 2001; Pearson and Rohrmann, 2002). A *Drosophila melanogaster* F homolog, which is not part of a retrovirus-like element, has also been identified, but it is unclear whether this is an insect gene, or a remnant of an integrated retrotransposon (Malik *et al.*, 2000; Rohrmann and Karplus, 2001). Hence the evolutionary events leading to the presence of F protein homologs in insects, baculoviruses, and insect retroviruses remain unclear. It is possible that the insect F homolog may represent the progenitor of both the baculovirus F protein and the envelope proteins of insect retroviruses.

Both viral and host-derived ubiquitin with an unusual phospholipid anchor have been found on the inner surface of the BV envelope (Guarino, 1990; Guarino *et al.*, 1995). Free ubiquitin is also present between the nucleocapsid and envelope and is almost as abundant as GP64, accounting for 2% of the weight of the virion. Ubiquitin is one of the most highly conserved of known proteins, but diverged considerably since acquisition by the baculoviruses. Baculovirus ubiquitin is the most divergent ubiquitin known with only 76% identity to animal ubiquitin (Guarino, 1990). A primary function of ubiquitin is to signal degradation of proteins by the 26S proteosome by covalent attachment of ubiquitin molecules to the protein. However, the viral protein supports protein degradation at only 40% the rate of the eukaryotic ubiquitin in *in vitro* assays, and multiubiquitination of some proteins was inhibited (Haas *et al.*, 1996). This suggests that virus ubiquitin may prevent degradation of

selected proteins during infection. Analysis of ubiquitin mutants of AcMNPV showed that ubiquitin may function in BV assembly although *ubi* is not an essential gene (Fraser *et al*., 1995; Reilly and Guarino, 1996). The baculovirus proteins IAP2, IE2, and PE38, which have RING finger domains, may function as ubiquitin ligase (E3) enzymes during baculovirus infection (Imai *et al*., 2003).

There are various other proteins that may be associated with BV including IE1 (Theilmann and Stewart, 1993) and actin (Lanier *et al*., 1996). The viral cathepsin V-Cath is associated with both the nucleocapsids and the envelopes of BV, and degrades actin (Lanier *et al*., 1996). This viral cathepsin L-like cysteine protease is required for liquefaction of the host insect during the final stages of virus infection. In the absence of this protease, tissues retain their integrity (Slack *et al*., 1995).

6.8.2.3. Occlusion-Derived Virus

At about 24 h post infection (p.i.), a switch occurs from BV to OB production, although the switch is not absolute (Faulkner, 1981) (**Figure 5**). The FP25K protein is a potential regulator of the switch from BV to OB production during baculovirus infection (Jarvis *et al*., 1992). During the very late phase, the nucleocapsids remain in the nucleus when sufficient FP25K protein is produced. The FP25K protein, which is expressed during the late phase, may be involved in the retention of the nucleocapsids within the nucleus (Harrison and Summers, 1995). FP25K is predominantly found in the cytoplasm of baculovirus-infected cells (Braunagel *et al*., 1999). Disruption of FP25K expression that occurs frequently on repeated passaging of virus in cell culture, leads to the few polyhedra (FP) phenotype with (1) enhanced BV production, (2) decreased polyhedra production, (3) increased synthesis of some BV structural proteins, (4) reduced levels of some ODV envelop proteins, (5) decreased polyhedrin protein synthesis, and (6) a block in the postmortem liquefaction of the host (Harrison and Summers, 1995; Harrison *et al*., 1996; Katsuma *et al*., 1999; Rosas-Acosta *et al*., 2001).

During the very late phase of infection, trilamellate membrane profiles appear within the ring zone of the nucleus and envelope nucleocapsids to form ODV (Stoltz *et al*., 1973; Fraser, 1986; Williams and Faulkner, 1997). These membranes may derive from invaginations of the inner nuclear membrane that produce microvesicles within the infected nucleus (Fraser, 1986; Hong *et al*., 1994) (**Figures 5** and **6**). Regulation of the ODV-specific components in the viral envelope may be important for the switch from BV to OB production (Braunagel and Summers, 1994; Braunagel *et al*., 1996b; Williams and Faulkner, 1997).

Nomenclature for some viral envelope proteins includes designation of protein source (BV or ODV), followed by location, E (envelope) or C (capsid), and the apparent molecular mass (Braunagel *et al*., 1996a). ODV-E66 (Hong *et al*., 1994, 1997) and ODV-E25 (Russell and Rohrmann, 1993) are both envelope proteins found in the virus-induced microvesicles in the cell nucleus. ODV-E56 is present in ODV envelopes, virus-induced intranuclear microvesicles, and the inner and outer nuclear membranes (Braunagel *et al*.,

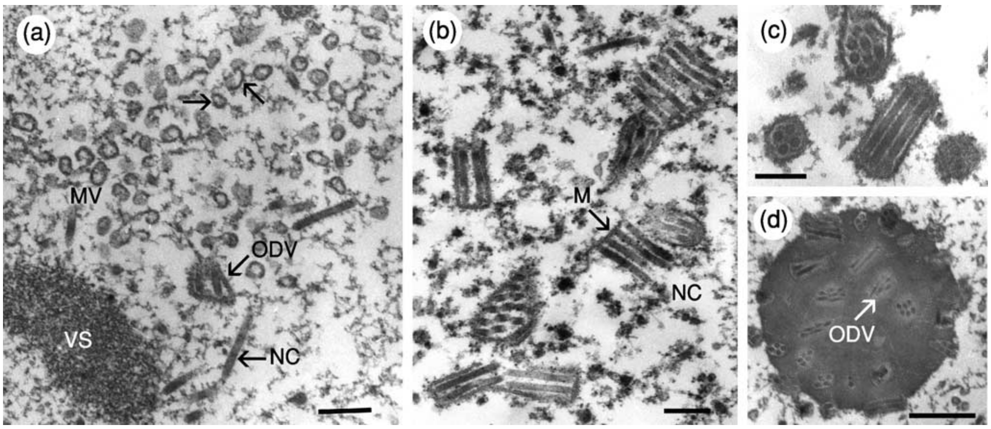


Figure 6 Production of occluded virus of AgipMNPV: intranuclear envelopment and occlusion of nucleocapsids. During the very late phase of infection, nucleocapsids (NC) are enveloped as they leave the virogenic stroma (VS). (a) Membrane profiles (arrows) appear to break down to produce microvesicles (MV), the occlusion-derived virus (ODV) envelope precursors in the ring zone of NPV-infected cells. (b) These microvesicles appear to produce trilamellar membrane profiles (M) distal to the nuclear membrane. Nucleocapsids (NC) align with these membranes, and (c) are enveloped to produce ODV. Transverse and longitudinal sections illustrate the bundling of multiple nucleocapsids within the ODV of MNPV. (d) The ODV are then occluded within the polyhedrin matrix. (a) and (d) fat body, (b) and (c) hemocyte of *Agrotis ipsilon*. Scale bar = 200 nm (a, b, and c), 500 nm (d).

1996a; Theilmann *et al.*, 1996). ODV-E35 shares the same N-terminus with ODV-E18 and may be translated from a transcript that includes both AcMNPV ORF143 (ODV-E18) and 144 (ODV-EC27) derived by ribosomal frameshifting at a step-loop structure (Braunagel *et al.*, 1996b). ODV-EC27 is present in ODV nucleocapsids and envelopes, and is also present in a modified form in BV (Braunagel *et al.*, 1996b). ODV-EC27 functions as a multifunctional viral cyclin with roles that may include arrest of the host cell cycle at G_2/M phase, or regulation of viral/cellular DNA replication (Belyavskyi *et al.*, 1998). P74 is associated with the ODV envelope and is essential for oral infectivity. It is hypothesized to function in attachment to or fusion with midgut epithelial cells (Kuzio *et al.*, 1989; Hill *et al.*, 1993; Faulkner *et al.*, 1997; Slack *et al.*, 2001). The *p74* gene lacks a conventional late promoter sequence, but is expressed at low levels during the late phase of infection.

The tegument protein GP41 is present between the envelope and the nucleocapsid of ODV (Whitford and Faulkner, 1992). GP41 is required for movement of the nucleocapsids out of the infected cell nucleus to produce BV (Olszewski and Miller, 1997b). Protein tyrosine phosphatase (PTP) has also been found associated with both BV and ODV (Li and Miller, 1995).

The lipid composition of the BV and ODV envelopes differs and ODV envelopes contain a greater density of protein (Braunagel and Summers, 1994). For AcMNPV BV derived from Sf9 cells, the major phospholipid constituent of the envelope is phosphatidylserine, comprising about 50% of the phospholipid content. For the ODV envelope, phosphatidylcholine and phosphatidylethanolamine are the predominant constituents. The BV envelope appears to be more fluid than that of ODV, which may be important for the different roles of these phenotypes in the baculovirus life cycle (Braunagel and Summers, 1994).

6.8.2.4. Occlusion Bodies

Large amounts of the occlusion matrix protein polyhedrin (or granulin) and a nonstructural protein P10 are produced during the very late phase of infection. These proteins accumulate in the cytoplasm (detectable at 10–12 and 14–16 h p.i.), and after about 20 h p.i. are also located in the nucleus. Polyhedrin becomes primarily intranuclear while P10 forms fibrillar bodies in both the cytoplasm and the nucleus (Vlak *et al.*, 1988; Williams *et al.*, 1989) (**Figure 7**). Large amounts of polyhedrin are required for production of OB. The ODV are embedded in the occlusion matrix of polyhedrin or granulin (Rohrmann, 1986, 1992; Funk *et al.*, 1997) (**Figure 6**). Polyhedrin is highly conserved (25–33 kDa) and related to the GV matrix protein granulin (with about 50% amino acid identity) that produces a paracrystalline lattice and accounts for 95% of the OB mass (Faulkner, 1981; Vlak and Rohrmann, 1985; Rohrmann, 1992). Point mutations in conserved domains of the polyhedrin gene have resulted in (1) a single cubic OB per cell with a paracrystalline matrix that occludes few virions (Brown *et al.*, 1980; Jarvis *et al.*, 1991), (2) small polyhedrin condensations that lack the lattice structure and do not occlude virions (Duncan *et al.*, 1983), and (3) large amorphous condensations of polyhedrin (Carstens *et al.*, 1992).

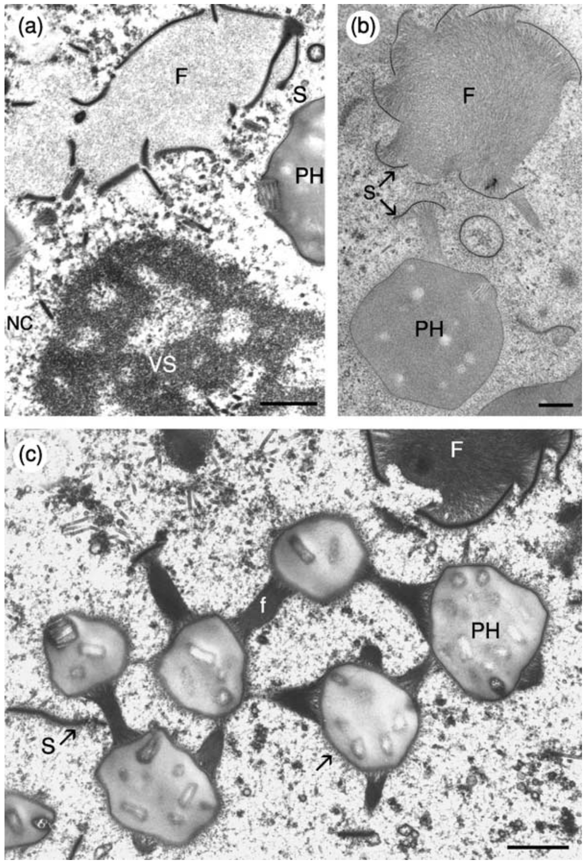


Figure 7 Maturation of the occlusion body. (a, b) Bilamellar fibrous sheets (S), which are calyx (polyhedral envelope) precursor structures, associate with the P10-rich fibrillar body (F). These sheets appear to form near to the nuclear membrane before incorporation into the fibrillar body. Nucleocapsids (NC), virogenic stroma (VS) and polyhedra (PH) are indicated. (c) The fibrillar bodies (F) and fibrillar structures (f) associated with the maturing polyhedra (PH) form a shell around the surface of the polyhedra prior to or during addition of the calyx (arrow). Addition of the calyx is the final step in OB maturation. (a), AgipMNPV, (b, c) *Rachiplusia ou* MNPV (RoMNPV). Scale bar = 500 nm.

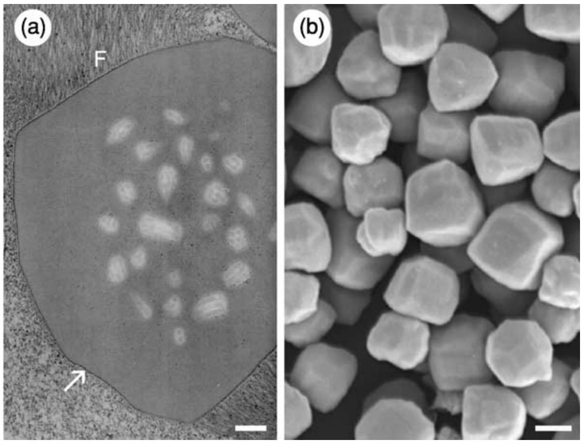


Figure 8 Occlusion bodies. (a) Mature polyhedron of RoMNPV showing polyhedral envelope or calyx (arrow) surrounding the polyhedron. The adjacent fibrillar body (F) is also indicated. Scale bar = 200 nm. (b) Scanning electron micrograph showing the polyhedral structure of occluded virus of RoMNPV derived from larvae of *Heliothis virescens*. Scale bar = 1 μm.

OB production is completed by addition of the polyhedron envelope or calyx (**Figure 8**), which consists primarily of carbohydrate and the phosphoprotein PP34, also known as the polyhedral envelope protein, PEP (Minion *et al.*, 1979; Gombart *et al.*, 1989). The precursor structures referred to as bilamellar fibrous sheets or electron-dense spacers form in the ring zone of the infected cell in association with P10 fibrillar bodies (Russell *et al.*, 1991) (**Figure 7**), although calyx formation is not dependent on the expression of P10 (van Lent *et al.*, 1990; Oers and Vlak, 1997). Thiol bonds link PP34 molecules to each other and to polyhedrin (Whitt and Manning, 1988). Mutants lacking the calyx show increased sensitivity to alkali and have pitted surfaces, suggesting possible loss of virions from the OB matrix (Zuidema *et al.*, 1989; Gross, 1994).

Occlusion bodies are resistant to desiccation and freezing (Jaques, 1985), and to putrefaction and various chemical treatments (Benz, 1986), but do not protect ODV well against the harmful effects of ultraviolet light (Ignoffo *et al.*, 1995, 1997). Baculoviruses lose most of their activity within 24 h as a result of damage to DNA when exposed to direct sunlight (Young and Yearian, 1974). On exposure to ultraviolet light, pyrimidine dimers are produced. Baculovirus expression of a pyrimidine dimer-specific glycosylase conferred some protection to BV but not to OB (Petrik *et al.*, 2003). It has been postulated that the baculovirus superoxide dismutase gene (*sod*) may function to protect OB from superoxide radicals generated on exposure to sunlight (Tomalski *et al.*, 1991; Ignoffo and Garcia, 1994).

6.8.3. Life Cycle

6.8.3.1. Infection

Baculoviruses infect the larval stages of insect hosts. Caterpillars typically ingest occlusion bodies (OB) (polyhedra or granules) on feeding, although NPVs can be transmitted via the egg. The polyhedrin or granulin matrix of the occlusion bodies dissolves rapidly in the alkaline conditions of the caterpillar midgut (pH 9.5–11.5), releasing the infectious ODV (**Figure 9**). Occlusion dissolution is also dependent on ionic strength (Kawanishi and Paschke, 1970) and is facilitated by midgut proteases (Granados and Williams, 1986). The ODV must then pass through the peritrophic membrane, an extracellular fibrous matrix of chitin, glycosaminoglycans, and protein that lines the midgut (Richards and Richards, 1977; Brandt *et al.*, 1978; Wang and Granados, 2001). GV and some NPV occlusions contain a viral enhancing factor, a metalloprotease called enhancin. Enhancin, which is released into the midgut on occlusion dissolution, facilitates penetration of the peritrophic membrane by degrading mucin (Derksen, 1988; Gallo *et al.*, 1991; Wang *et al.*, 1994; Gijzen *et al.*, 1995; Lepore *et al.*, 1996; Bischoff and Slavicek, 1997; Wang and Granados, 1997, 1998; Popham *et al.*, 2001). The chitin-binding agent calcofluor (M2R) inhibits formation of the peritrophic membrane (Wang and Granados, 2000) and increases the susceptibility of larvae to baculovirus infection. Calcofluor also the enhances the retention of infected midgut cells (Washburn *et al.*, 1998) and the relative contributions of these two effects to calcofluor-enhanced susceptibility of the host insect are unclear (Washburn *et al.*, 1998; Wang and Granados, 2001). It is also unclear how viruses that lack enhancin penetrate the peritrophic membrane. Possibilities include that virus infection occurs before the peritrophic membrane is fully formed, particularly in insects that produce the peritrophic membrane in response to feeding (Tellam, 1996), or through variation in peritrophic membrane structure in different species of Lepidoptera (Hopkins and Harper, 2001). Alternatively, there may be other virus proteins with peritrophic membrane-degrading activity.

Virions cross the peritrophic membrane and bind to the brush border microvilli of epithelial cells (**Figure 9**). ODV are highly infectious to midgut epithelial cells and less infectious to other tissues (Volkman and Summers, 1977). The primary target cells are the columnar cells of the midgut epithelium (Granados and Williams, 1986). ODV enter the cells by direct fusion of the viral and cellular membranes (Granados and Lawler, 1981), and the entry is likely

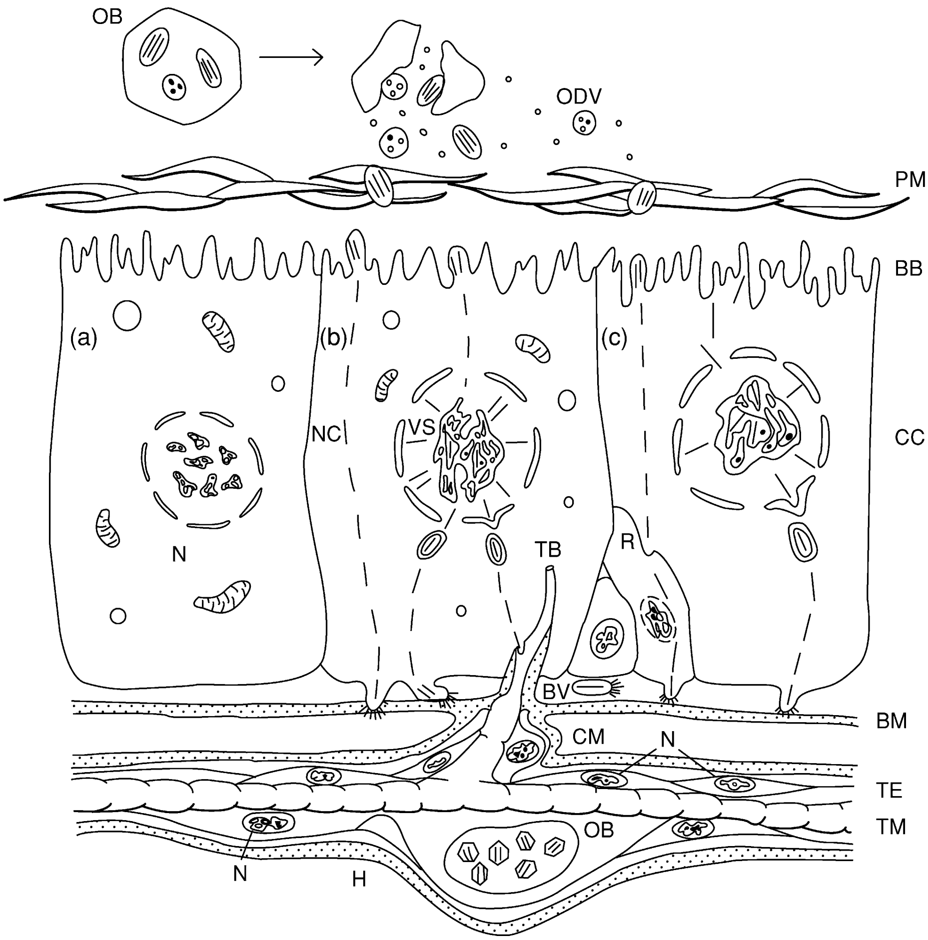


Figure 9 Infection of the midgut epithelium of the host insect by MNPV. Occlusion bodies (OB) dissolve in the midgut releasing ODV, which move through the peritrophic membrane (PM). (a) Uninfected columnar cell (CC) showing the normal distribution of heterochromatin in the nucleus (N); (b) columnar cell showing entry of virus at the brush boarder membrane (BB). Some nucleocapsids (NC) move to the nucleus, while other nucleocapsids undergo trancytosis. Infection of tracheoblasts (TB) closely associated with the midgut epithelium and subsequent infection of the tracheal epithelium (TE), as indicated by the presence of OB, is also shown. (c) Direct infection of regenerative cells (R), by transcytosis as for *Spodoptera exigua*, in addition to infection of columnar cell (CC). Budded virus acquires an envelope as it buds through the virus-modified plasma membrane. BV may enter the hemocoel (H) by budding through the basement membrane (BM) or via the tracheal epithelium (TE). Enlargement of the nucleus on infection of the cell is shown in (b) and (c). VS, virogenic stroma; CM, circular muscle; TM, tracheal matrix.

to involve a highly abundant or relatively non-specific receptor site (Horton and Burand, 1993). ODV entry is insensitive to inhibitors of adsorptive endocytosis.

Nucleocapsids move to the nucleus and uncoat, or may pass directly through the cell to infect other cells, or enter the hemocoel (Granados and Lawler, 1981; Keddie *et al.*, 1989) (**Figure 9**). The mechanism for movement of nucleocapsids within microvilli is unknown in the absence of microtubules (Volkman, 1997). BV bud from the basal side of the midgut epithelium and infectious virus can be detected in the hemolymph a few hours after infection (Granados and Lawler, 1981). When multiple nucleocapsids infect a single columnar epithelial cell, some nucleocapsids derived from ODV can be converted directly into BV without replication in the nucleus, taking advantage of GP64 that is expressed as an early gene on entry of other nucleocapsids into the nucleus (Washburn *et al.*, 2003a). Infection with the M or S phenotype of NPV results in infection of primary tissues with multiple or single nucleocapsids, respectively. Infection of secondary tissues is more rapid for the M phenotype supporting the hypothesis that ODV nucleocapsids can be repackaged as budded virus, while systemic infections of the S phenotype require virus replication in the primary target cells (Washburn *et al.*, 2003a). Direct acquisition of the envelope protein GP64 by ODV-derived nucleocapsids of the M phenotype allows

rapid movement of the virus through the midgut epithelial cells for further dissemination within the host insect (Washburn *et al*., 2003b). In the case of *Spodoptera exigua*, ODV-derived nucleocapsids of AcMNPV infect both the columnar cells and the underlying regenerative cells (Flipsen *et al*., 1995b) (**Figure 9**). Some lepidopteran larvae clear AcMNPV-infected cells from the midgut with almost full recovery of the tissue by 60 h p.i. (Keddie *et al*., 1989; Washburn *et al*., 2003b). The direct movement of virus through the midgut cells would provide an advantage to the virus in larvae that shed infected midgut epithelial cells and may have provided the driving force for evolution of the MNPV from the SNPV. The clearing of infected cells from the midgut may benefit MNPV by allowing the host insect to continue to feed (Volkman and Keddie, 1990).

Infection results in rapid formation of filamentous actin (F-actin) bundles, which may facilitate transport of nucleocapsids to the nucleus (Charlton and Volkman, 1993; Lanier and Volkman, 1998). Nucleocapids migrate to the nucleus and uncoat at the nuclear pore complex (GV) or just inside the nuclear membrane (Bilimoria, 1991). A capsid-associated protein kinase associated with NPV and GV virions may function in unpackaging of the viral genome by phosphorylation of the basic core protein P6.9 (Miller *et al*., 1983; Wilson and Consigli, 1985; Funk and Consigli, 1993). Transcription of immediate early genes rapidly follows with virus-specific RNA detectable within 30 min of infection (Chisholm and Henner, 1988). At this stage, F-actin aggregates form at the plasma membrane mediated by an early viral gene product, actin rearrangement-inducing factor-1 (ARIF-1) which colocalizes with the F-actin aggregates (Dreschers *et al*., 2001).

Numerous morphological changes occur in the cell and the nucleus during the course of infection. Within the cytoplasm, there are alterations to the cytoskeleton and endomembrane systems, and large fibrillar structures develop. Within the first hour after infection, thick microfilament cables form from nucleocapsid-induced rearrangement of cellular actin (Charlton and Volkman, 1991). These cables, which are associated with nucleocapsids prior to viral gene expression, are only present at 1–4 h p.i. The capsid proteins P39 and P78/83 appear to interact with actin and induce actin polymerization, thereby enabling movement of nucleocapsids to and/or into the nucleus (Lanier and Volkman, 1998). Within the first 6–8 h p.i., the nucleus swells, the cell becomes rounded, and the distribution of heterochromatin and morphology of the nucleolus change. Rounding of the cell appears to be the result of virus-mediated rearrangement of the microtubules and is the first cytopathic effect seen by light microscopy following infection (Volkman and Zaal, 1990) (**Figure 10**). Heterochromatin becomes marginated along the inner nuclear membrane. The transition from early to late gene expression marks the onset of viral replication, and at this time the virogenic stroma, or viral replication center, is produced in the nucleus (Knudson and Harrap, 1976; Young *et al*., 1993). The virogenic stroma is electron dense, contains intrastromal spaces, and has a convoluted periphery (**Figure 3**). Formation of the virogenic stroma is dependent on viral DNA synthesis and late gene expression (Carstens *et al*., 1994). During the late phase, F-actin appears in the virogenic stroma and in the surrounding ring zone next to the inner nuclear membrane (Charlton and Volkman, 1991) (**Figure 11**), associated with P78/83 and VP39 (Charlton and Volkman, 1991; Lanier and Volkman, 1998). Nuclear F-actin is required for production of nucleocapsids (Ohkawa and Volkman, 1999). Six early viral genes, including *ie-1*, have been implicated

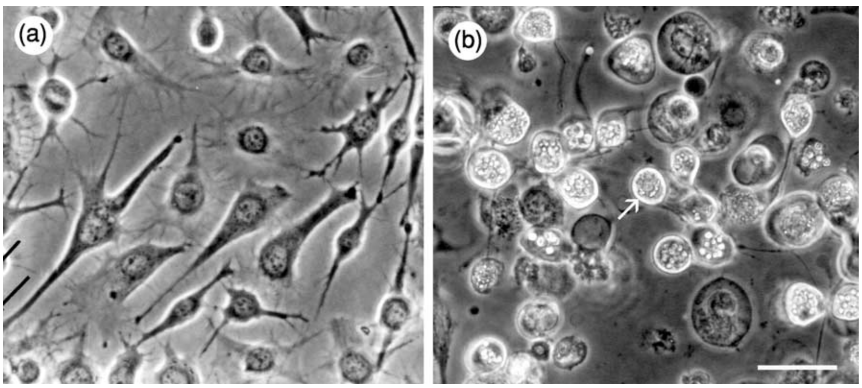


Figure 10 Cells of the *Manduca sexta* cell line GV1 illustrating (a) the normal fibroblast-like morphology with elongate extensions, and (b) rounding of cells on infection with AcMNPV. Refractile polyhedra are apparent within the distended nuclei by 3 days post infection (arrow). Rounding of the cells is the first response to baculovirus infection as seen by light microscopy. Photographs were taken under phase contrast. Scale bar = 50 μm.

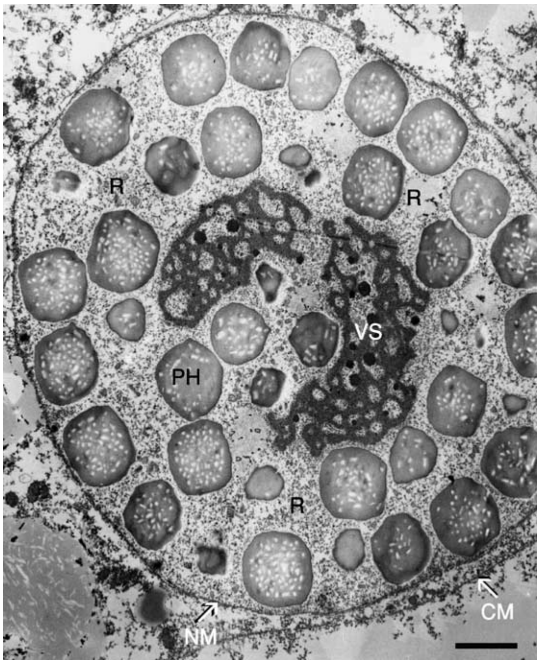


Figure 11 Hemocyte of *Agrotis ipsilon* infected with *Agrotis ipsilon* MNPV. The nucleus of the cell is shown with multiple polyhedra (PH) and the central, electron dense virogenic stroma (VS). The ring zone (R), nuclear membrane (NM), and cell membrane (CM) are indicated. Scale bar = 2 μm.

Table 1 Temporal classes of baculovirus gene expression; examples are provided to illustrate the progression of baculovirus gene expression from transregulatory genes, to those involved with DNA replication and production of budded and occluded virus in the late and very late stages of infection

Gene category	*Early gene*	*Late genes*	*Very late genes*
Transregulatory	*ie0, ie1, ie2*	*ie1*	
DNA replication	*dnapol*	*lef3*	
	p143		
Structural			
Nucleocapsid		*p6.9*	
		vp39	
		p80	
		p24	
		pp78/83	
		Tegument gp41	
BV	*gp64*	*gp64*	
		ubi	
ODV		*odv-e66, odv-e25*	
OB		*pep*	*polh*
Auxiliary	*egt*	*sod*	*p10*
	p35	*ubq*	
		cathepsin	
		ctl	
		pcna	
		ptp	
		protein kinase	

BV, budded virus; OB, occlusion bodies; ODV, occlusion-derived virus.

in recruitment of the monomeric G-actin to the nucleus (Ohkawa *et al.*, 2002). Progeny nucleocapsids appear within the virogenic stroma at about 10 h p.i. and synthesis of viral DNA continues for at least 12 h. As the virogenic stroma matures, the ring zone, a peristromal compartment of nucleoplasm appears (Figure 11). Intranuclear envelopment of virions and OB formation occurs in this compartment. Budded virus is produced during the late phase, and occluded virus is produced during the very late phase of virus infection (Table 1). The diameter of the nuclei of infected cells increases to five to ten times that of the nucleus of an uninfected cell (Federici, 1997). Baculovirus infection results in arrest of the cell cycle at the G_2/M phase, a phenomenon that may be mediated by viral proteins (Braunagel *et al.*, 1998).

Progeny nucleocapsids produced within the virogenic stroma initially bud through the plasma membrane modified by the virus-encoded protein GP64, to acquire a BV envelope (see Section 6.8.2.2). Later in infection nucleocapsids are enveloped and incorporated into the matrix of OB (see Section 6.8.2.3), while BV production is curtailed. Mature OB are released when the infected cell lyses. Large cytoplasmic vacuoles associate with both the nuclear and the outer plasma membranes and the nucleus disintegrates (van Oers *et al.*, 1993; Williams and Faulkner, 1997). The P10 protein, cellular microfilaments and virus-produced cathepsin and chitinase are required for OB release from the host insect (Williams *et al.*, 1989; Hawtin *et al.*, 1997; Thomas *et al.*, 2000).

Production of occlusion bodies in the midgut epithelial cells does not occur for most lepidopteran insects. The high levels of actin associated with the microvilli of the columnar epithelial cells may have an inhibitory effect on the production of occluded virus (Volkman *et al.*, 1996). The infected cells are sloughed off when the larva molts and the midgut epithelium is regenerated (Flipsen *et al.*, 1993).

6.8.3.2. Dissemination within the Host

With the exception of lepidopteran NPVs and an NPV of the cranefly *Tipula paludosa*, all other NPVs infect only the midgut epithelium (Federici, 1993). In contrast, lepidopteran NPVs transiently infect the midgut and then spread to virtually all other tissues. With the exception of the GV of *H. brillians*, which is restricted to the midgut, all other GVs transiently infect the midgut before invading other tissues. For lepidopteran NPVs, systemic spread may occur through direct penetration of the basement membrane into the hemocoel (Granados and Lawler,

1981; Flipsen *et al.*, 1995b; Federici, 1997) and via the tracheal system (Engelhard *et al.*, 1994) although there is debate over whether one route predominates the other (Federici, 1997). Following infection of the midgut epithelial cells, viruses infect the tracheolar cells that service the midgut (Washburn *et al.*, 2001). The tracheoles often penetrate the basement membrane to supply tissues with oxygen, and thereby provide a means for the virus to bypass the basement membrane barrier. However, because tracheolar cells also are covered by basement membrane, it is not clear how BV would then enter the hemocoel without crossing a basement membrane. Given that the pores in the basement membrane are too small to allow physical passage of BV (Reddy and Locke, 1990), penetration of the basement membrane by BV may be an enzymatic process or may be possible when the basement membrane is thin, in early instars and surrounding tracheoles that are suspended in the hemolymph (Federici, 1997; Volkman, 1997). Because the midgut epithelial cells contribute to production of the overlying basement membrane, it is also possible that infection of these cells disrupts maintenance of the basement membrane (Knebel-Mörsdorf *et al.*, 1996), or that enlargement of infected cells alters basement membrane structure thereby allowing passage of budded virus. Following infection of *Heliothis virescens* and *Helicoverpa zea* with AcMNPV, infection of tracheal cells occurred 6–10 h prior to the appearance of BV in the hemolymph, suggesting that BV entering the hemocoel may be derived from the tracheal cells (Trudeau *et al.*, 2001).

BV enter cells by adsorptive endocytosis (Volkman and Goldsmith, 1985; Blissard and Wenz, 1992) and are transported in clathrin-coated vesicles to the endosome where they fuse with the endosome membrane thereby releasing nucleocapsids into the cytoplasm (Blissard, 1996). Fusion is mediated by GP64, the major envelope glycoprotein as the endosomal pH decreases (Monsma and Blissard, 1995).

The infection spreads within a tissue by cell-to-cell transmission. The tissue tropism varies from mono-organotrophic (e.g., *Neodiprion sertifer* NPV and *Culex nigripalpus* NPV that infect only the midgut epithelium) to polyorganotrophic with infection of most tissues (Granados and Williams, 1986; Moser *et al.*, 2001). Once the tracheal matrix and hemocytes become infected in susceptible polyorganotrophic hosts, infection spreads rapidly to the epithelial and fat body tissues (Keddie *et al.*, 1989; Engelhard *et al.*, 1994; Flipsen *et al.*, 1995b). Infection then proceeds to nerve, muscle, pericardial cells, and reproductive and glandular tissues. Some tissues such as the Malpighian tubules and salivary glands are not well infected with only immediate early genes expressed suggesting that tissue tropism may be restricted by the availability of appropriate host factors to support virus replication (Knebel-Mörsdorf *et al.*, 1996).

The ability of the hemocytes of a host to support virus replication appears to be a key factor determining the susceptibility of some species to virus infection, through both virus amplification within the hemocoel, and the ability of the hemocytes to encapsulate sites of infection. In permissive hosts such as *H. virescens* and *T. ni*, the hemocytes play a primary role in amplification of virus in the hemocoel for initiation of new sites of infection. In some semipermissive hosts such as *H. zea*, although nucleocapsids of AcMNPV reach the nuclei of hemocytes, these cells do not support virus replication, and hence effectively remove virus from the hemocoel. The hemocytes of *H. zea* and *Manduca sexta* also appear to block virus dissemination by encapsulating infected tracheal elements possibly in response to ruptured basement membrane (Washburn *et al.*, 1996, 2000). Infection of hemocytes of the permissive host *H. virescens* compromises their ability to encapsulate (Trudeau *et al.*, 2001). There are contradictory reports on the role of hemocytes in virus dissemination within *Spodoptera frugiperda*, which is semipermissive to infection by AcMNPV. Clarke and Clem (2002) found lack of infection of hemocytes in this species, suggesting that the tracheal system may serve as the primary conduit for virus dissemination within this host. In contrast, Haas-Stapleton *et al.* (2003) found that hemocytes were infected by BV within the hemocoel. The opposing conclusions of these two studies may result from differential dispersal of virus from the sites of BV injection, between the first and second abdominal segment such that virus was injected to the rear of the abdomen (Clarke and Clem, 2002), versus through the planta of one of the prolegs (Haas-Stapleton *et al.*, 2003).

The response of the host insect to baculovirus infection varies dramatically according to the host–virus combination, which larval instar becomes infected, the virus dose acquired, and environmental conditions. As an example, about 4 days after infection of third instar *H. virescens* (a permissive host) with AcMNPV, host larvae become less responsive with reduced feeding activity (**Figure 12**). The cuticle becomes swollen and glossy in appearance as a result of hypertrophy of infected cells and tissues. In species that are lightly pigmented, the larvae take on a whitish coloration resulting from the polyhedra in the nuclei of fat body and epidermal cells. The baculovirus gene *ctl* encodes a small peptide with sequence similar to the conotoxins of predatory

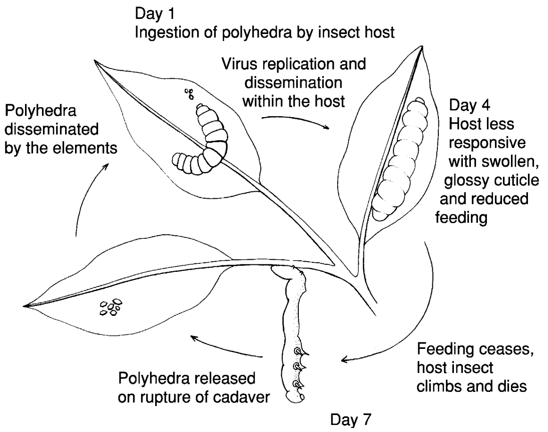


Figure 12 Effects of baculovirus infection on a susceptible host insect. The timing of the various stages varies according to the virus dose, larval instar, and environmental conditions. The timing of events would be typical for AcMNPV infection of third instar larvae of *Heliothis virescens*.

marine snails and aptotoxins of spiders (Eldridge *et al.*, 1992; Skinner *et al.*, 1992) (see **Chapter** 5.7). Conotoxins are calcium channel antagonists, while the aptotoxins are insecticidal causing rapid paralysis and death (Skinner *et al.*, 1992). While the function of baculovirus CTL remains to be determined, it may function to paralyze the insect during late stages of infection thereby promoting viral use of diminishing host resources (Eldridge *et al.*, 1992).

6.8.3.3. Dissemination from the Host

By 6–7 days after infection, feeding ceases. OB are released on lysis of infected cells. As the cells lyse, millions of polyhedra accumulate and the basement membrane degrades. Prior to death, many species exhibit negative geotropism and climb to the top of the vegetation, thereby facilitating broad dispersal of progeny virus. The cuticle becomes fragile and the dead insect liquefies. The baculovirus chitinase (*chiA*) and cathepsin (*v-cath*) genes, which are present in members of both NPV and GV, act in concert to facilitate release of occlusion bodies from the insect cadaver by degrading the cuticle and internal tissues (Hawtin *et al.*, 1995, 1997; Slack *et al.*, 1995; Kang *et al.*, 1998).

The chitinase of AcMNPV has both exo- and endo-chitinase activity against a range of substrates and is active from pH 3 to 10 (Hawtin *et al.*, 1995; Thomas *et al.*, 2000). It accumulates within the endoplasmic reticulum as a result of a C-terminal ER retention motif (KDEL). The chitinase is released as infected cells lyse (Thomas *et al.*, 1998). Removal of the ER retention motif and consequent secretion of the enzyme results in more rapid host liquefaction (Saville *et al.*, 2002). V-Cath is a cysteine protease that accumulates in the cell as an inactive proenzyme, proV-Cath (Bromme and Okamoto, 1995; Hom *et al.*, 2002). The viral cathepsin, which has a pH optimum of 5.0–5.5 is also active at pH 7 (Ohkawa *et al.*, 1994). ProV-Cath activation occurs on death of the infected cell possibly as a result of lysosomal protease release, and appears to contribute to death of the cell (Hom *et al.*, 2002). ChiA is required for the processing of V-Cath (Hom and Volkman, 2000).

A single dead insect may release as many as 10^{10} polyhedra (Entwistle and Evans, 1985) with the fat body, epidermis, and tracheal matrix producing the largest quantities of polyhedra (Aizawa, 1963). The amount of virus produced depends in part on the speed of kill of the host insect. In theory, the longer the insect survives, the more time the virus has for replication and production of progeny. For example, infection of the semipermissive host *Mamestra brassicae* with AcMNPV resulted in a higher virus yield than infection of the permissive host *T. ni* that succumbed more rapidly to infection (Hernandez-Crespo *et al.*, 2001). However, the yield of polyhedra may vary with species according to the physiological bases for the semipermissive character.

Liquefaction of the host cadaver increases transmission of the virus to conspecifics and facilitates dissemination in the environment. Polyhedra can survive outside the host in soil, in crevices of plants, or other refugia for years (Jaques, 1975), and are ubiquitous. Hence, in contrast to other viruses for which death of the host represents an evolutionary dead end, death of the baculovirus-infected host represents a successful replication strategy.

Predatory arthropods, scavengers, and parasitoids feeding on, or parasitizing baculovirus-infected lepidopteran larvae may become contaminated with virus and play an important role in dissemination of baculoviruses under field conditions (Ruberson *et al.*, 1991; Fuxa *et al.*, 1993; Chittihunsa and Sikorowski, 1995; Sait *et al.*, 1996; Vasconcelos *et al.*, 1996). Grazing mammals and birds also contribute to baculovirus dissemination (Entwistle and Evans, 1985; Fuxa, 1991).

The most common route for baculovirus transmission between the infected and healthy individuals is via horizontal transmission when a larva ingests occlusion bodies from the environment. Vertical transmission (from parent to offspring) is common for nucleopolyhedroviruses (Smits and Vlak, 1988; Kukan, 1999; Fuxa *et al.*, 2002). Artificial selection resulted in an increased rate of vertical transmission of *S. frugiperda* NPV suggesting a genetic basis for this characteristic (Fuxa and

Richter, 1991). Vertical transmission of baculoviruses may be important for species such as *S. frugiperda* that disperse or migrate such that successive generations are located at different geographic locations.

6.8.4. Virus Replication

Regulation of more than 100 baculovirus genes is complex and requires coordinated expression of temporally regulated early, late, and very late genes (Rohel and Faulkner, 1984). Each stage of virus replication is dependent on expression of genes in the preceding stage. Baculovirus gene expression appears to be regulated at the level of transcription, and includes a unique switch from transcription of early genes by host RNA polymerase II, to late and very late genes by a virus-encoded RNA polymerase (Fuchs *et al*., 1983; Guarino *et al*., 1998a, 1998b). *Trans*-acting protein factors (IE0, IE1, IE2, and PE38) interact with *cis*-acting DNA elements (upstream activation regions or homologous regions) in the virus genome to initiate early gene expression (Friesen, 1997).

6.8.4.1. Early Gene Expression

Early genes (transcribed before initiation of virus DNA replication) are distributed throughout the genome and transcription often results in overlapping RNAs that differ at the 5′ end. RNA transcripts are detectable within the first 15 min indicating that transcription likely begins on uncoating of the DNA in the nucleus. Early gene transcription peaks at 6–12 h p.i. in *S. frugiperda* cells. Downregulation of early gene transcription may result from expression of late genes or viral DNA replication. The expression of some early genes, such as *p35*, *gp64*, *39K*, and *ie1*, continues into the late phase as a result of the presence of both early and late promoter motifs. The early promoters, which are responsive to the host RNA polymerase II and host transcription factors, are readily distinguished from late promoters on the basis of nucleotide sequence. The early promoters consist of core transcription elements and *cis*-acting elements either in close proximity or within transcriptional enhancers. Most early promoters are TATA-containing (TATA+), initiator-containing (INR+), or composite (TATA+, INR+). The TATA element regulates the rate of transcription initiation and defines the start site for transcription. The INR motif (e.g., ATCA(G/T)T(C/T)) overlaps the RNA start site, contributes to basal promoter activity, and determines the start site in the absence of the TATA motif. The tetranucleotide CATG is the most conserved of the INR-like motifs of baculovirus early genes and is also prevalent in the promoters of arthropod genes (Cherbas and Cherbas, 1993). Both INR and TATA bind specific factors that recruit the TFIID transcription complex at the promoter. The presence of both motifs may enhance recruitment of the required factors to ensure adequate expression of early genes, and use of universal host transcription factors may facilitate gene expression in a broad range of tissue types. An upstream activation region (UAR), often present for early baculovirus promoters, potentiates transcription from the early promoter. The activity of the UAR may vary in a host-specific manner, which is consistent with the hypothesis that host transcription factors act via the UAR to potentiate basal promoter activity. Transient expression assays suggest that other early baculovirus genes including *dnapol* that do not have the conventional INR or TATA promoter motifs may be more dependent on virus-encoded transactivators (Ohresser *et al*., 1994). Downstream activating region (DAR) motifs contribute to basal promoter activity and are likely to stabilize protein interactions at the INR.

The homologous regions (*hrs*) are repetitive and highly conserved sequences that occur in multiple copies in the baculovirus genome. They are also unique to baculovirus genomes. *hrs* consist of repeated sequences encompassing direct repeats of imperfect palindromic sequences. These elements function as transcriptional enhancers of *cis*-linked early viral genes, and experimental data also indicate that they serve as origins of virus replication (Pearson *et al*., 1992; Rodems and Friesen, 1993). *hrs* may also contribute to late gene transcription (Lu and Miller, 1995b). Enhancement of transcription is most likely through enhancement of transcription initiation. The *hrs* vary in size according to the number of palindromic repeats and in the number of copies in different baculovirus genomes. Although host factors can mediate *hr*-stimulated transcription from viral promoters in the absence of viral proteins, *hr*-mediated enhancement of transcription is dramatically increased by IE1, which binds as a dimer directly or indirectly to the *hr* (Friesen, 1997). *hrs* also function as origins of DNA replication in transient replication assays (Ahrens *et al*., 1995b; Kool *et al*., 1995).

On initial infection when DNA template concentrations are low, baculoviruses express transregulatory proteins that stimulate early gene expression. The immediate early transregulator IE1 plays a key role in regulation of gene expression. IE1 activates expression of early genes (*p35*, *gp64*, 39K, *p143*, *dnapol*, *pe38*, *lef-1*, *lef-2*, *lef-3*, and *ie1*) and is

required for DNA replication and transcription of late genes. IE1 dramatically enhances expression of genes that are *cis*-linked to *hr* enhancers. IE1 down-regulates transcription of *pe38* and *ie2*. The *ie1* promoter along with associated upstream sequences is active in uninfected cells, and can be used to direct foreign gene expression in transformed lepidopteran cells (Jarvis *et al*., 1990). The steady state level of IE1 increases during infection although the function of this protein during virus replication remains to be determined.

IE0 is an immediate early transregulator identical to IE1 except for an additional 54 N-terminal residues. IE0 results from splicing of the upstream *ie0* exon and fusion to the *ie1* exon. RNA splicing is a rare event in baculovirus gene expression and probably only occurs during the early stages of infection (Kovacs *et al*., 1991). IE0 transactivates the *hr*-linked promoters of *ie-1* and *39K*. IE2 stimulates transcription of several promoters, but to a lesser extent than IE1. IE2 and PE38 contain motifs typical of transcription regulators, including a leucine zipper and a RING finger. PE38 stimulates expression from the *p143* helicase promoter.

6.8.4.2. DNA Replication

For *S. frugiperda* cells infected by AcMNPV, viral DNA replication occurs from 6 to 18 h p.i., and then declines. Viral DNA synthesis is dependent on production of early viral proteins. *Cis*-acting elements that may be involved in initiation of viral DNA synthesis have been identified by analysis of defective virus particles generated on repeated passage of AcMNPV in cell culture. These defective viruses lack large regions of the AcMNPV genome with four regions that become amplified. Two of these regions contain *hrs*, and both the *hr* and non-*hr* regions can support transient replication of plasmids (Kool *et al*., 1993b, 1994a). The efficiency of replication decreases with reduction of the number of palindromes present in the *hr* (Leisy *et al*., 1995). The presence of multiple *hrs* dispersed throughout the genome may provide redundancy in the event that an *hr* is deleted. Loss of a single *hr* element from *Bombyx mori* NPV or AcMNPV has no impact on virus replication (Majima *et al*., 1993; Rodems and Friesen, 1993). However, demonstration that *hrs* function *in vivo* as origins of replication is lacking. Non-*hr* origins of replication have been identified in AcMNPV and OpMNPV (Kool *et al*., 1993a; Pearson *et al*., 1993).

The isolation of conditional-lethal mutants that are defective in DNA replication has facilitated identification of virus genes that are involved in virus DNA replication (Lee and Miller, 1978; Brown *et al*., 1979; Gordon, 1984; Lu and Carstens, 1991; Ribeiro *et al*., 1994). Transient replication assays were used to identify regions of the baculovirus genome that are able to support replication of cotransfected origin-containing plasmid DNA (Kool *et al*., 1994a, 1994b; Ahrens *et al*., 1995a; Ahrens and Rohrmann, 1995a, 1995b; Lu and Miller, 1995b). Five genes were classified as essential (*p143*, *ie1*, *lef-1*, *lef-2*, and *lef-3*) and five as stimulatory (*dnapol*, *p35*, *ie-2*, *lef-7*, and *pe38*) for replication of AcMNPV DNA (Kool *et al*., 1994b; Lu and Miller, 1995b). The *p143* gene encodes a helicase, which acts as a host range determinant. Incorporation of a fragment of *B. mori* NPV (BmNPV) helicase into AcMNPV (with few amino acids differing between the AcMNPV and BmNPV sequences) allowed AcMNPV to infect *B. mori* cells and larvae (Maeda *et al*., 1993; Croizier *et al*., 1994; Argaud *et al*., 1998). *lef-1* is an early gene important for late and very late gene expression (Passarelli and Miller, 1993), and essential for DNA replication (Lu and Miller, 1995b). Conserved domains indicate that Lef-1 may be a baculovirus primase (Barrett *et al*., 1996). Lef-2 is required for late gene expression and DNA replication. Lef-2 interacts with Lef-1 suggesting that these two proteins may function in replication as a heterodimer (Evans *et al*., 1997). Lef-3 is a single-stranded DNA binding protein that is essential for DNA replication (Hang *et al*., 1995). Although Lu and Miller (1995b) classified *dnapol* as stimulatory in transient assays, other reports have shown that *dnapol* is essential for virus replication (Pearson *et al*., 1993; Kool *et al*., 1994a, 1994b; Ahrens and Rohrmann, 1995a). The host DNA polymerase, in conjunction with virus genes essential for replication, may be able to replicate viral DNA at a suboptimal level. P35 is an apoptotic suppressor of *S. frugiperda* cells infected with AcMNPV (Clem *et al*., 1991), and stimulates DNA replication by preventing apoptosis possibly induced by expression of late gene products or DNA replication (Clem and Miller, 1994). Under some circumstances IE1 has been reported to trigger apoptosis (Prikhod'ko and Miller, 1996). However, IE1 also induces expression of P35. The genes *ie-2* and *pe38* function as stimulators of DNA replication probably through activation of genes involved in replication. Both genes encode transactivators of early gene expression (Carson *et al*., 1988; Lu and Carstens, 1993). PE38 activates *p143*, the baculovirus helicase homolog, while IE-2 stimulates production of PE38 and IE1. The effects of IE-2 are cell line specific (Lu and Miller, 1995a). Lef-7 is a putative single-stranded DNA binding protein and, along with Hcf-1, is a cell line-specific regulator of

DNA replication (Lu and Miller, 1995a, 1996). In addition to the genes identified as being required for replication by transient replication assays, Lin and Blissard (2002b) found that *lef-11* is necessary for viral DNA replication in the context of virus infection.

Because alteration of only one residue in AcMNPV helicase enables this virus to replicate in the otherwise nonpermissive BmN cell line, it is likely that a host protein interacts with the viral helicase, and that this interaction has virus and cell line specificity. Hence, host factors may be involved in replication of baculovirus DNA in addition to the viral genes described here. The host cell topoisomerase would also be required to relieve torsional stress as the duplex DNA is unwound by P143.

Given that infection-dependent replication of origin-containing plasmids results in production of concatemers of plasmid DNA, baculovirus DNA may be amplified by a rolling circle mode of replication. The detection of multiple unit-length genome fragments from replicating viral DNA also supports this hypothesis (Leisy and Rohrmann, 1993; Oppenheimer and Volkman, 1997). The mechanism for production of unit length genomes *in vivo* however remains to be established. Alternatively, replication may involve the theta mechanism initially, rolling circle synthesis, and recombination at the late stage, as for herpesviruses (Mikhailov, 2003).

6.8.4.3. Late and Very Late Gene Expression

Expression of late and very late genes begins following initiation of viral DNA replication, and is dependent on viral DNA replication. The late phase genes, which are transcribed at about 6–24 h p.i., include those encoding the structural proteins of the nucleocapsid, such as the major capsid protein VP39 and the basic core protein P6.9. The very late genes, transcribed at 18–72 h p.i., encode polyhedrin or granulin. The P10 protein, which affects disintegration of the nucleus, is expressed during the very late phase (Roelvink *et al*., 1992). Production of the late and very late viral genes coincides with a dramatic decline in the host RNAs (6–18 h p.i.) (Ooi and Miller, 1988; Okano *et al*., 2001) and host protein synthesis (Carstens *et al*., 1979). The decline in host RNAs appears to result from one or more late viral genes and is concurrent with margination of host cell chromatin (Ooi and Miller, 1988). However, the amount of host TATA-binding protein (TBP), a transcription factor that localizes to sites of viral DNA replication, increases, possibly as a result of viral disruption of TBP turnover (Quadt *et al*., 2002). Transcription of mitochondrial encoded genes is also unaffected by baculovirus infection (Okano *et al*., 2001).

A unique four-subunit RNA polymerase is responsible for the switch from early to late and very late gene expression (Beniya *et al*., 1996). All four subunits are encoded by the viral genome, specifically by *lef-4*, *lef-8*, *lef-9*, and *p47* (Guarino *et al*., 1998a). Lef-4 is hypothesized to provide a capping function for RNA transcribed by the polymerase (Jin *et al*., 1998). Two separate enzyme activities can be distinguished that differ in their ability to transcribe the very late genes (Xu *et al*., 1995). This α-amanitin resistant polymerase requires the promoter element TAAG. Late promoters initiate from (A/G/T)TAAG, while the motif (T/A)ATAAGNA (T/A/C)T(T/A)T is conserved for promoters of *polyhedrin (polh)*, *granulin*, and *p10* genes (Rohrmann, 1986). The late and very late genes are distinguished by an additional promoter element, the burst element, in the untranslated leader of very late genes (Ooi *et al*., 1989).

Nineteen AcMNPV genes, referred to as late expression factor (LEF) genes, are required for optimal expression of late and very late proteins in transient expression assays (Lu and Miller, 1995b; Todd *et al*., 1995, 1996; Rapp *et al*., 1998; Li *et al*., 1999; Lin and Blissard, 2002a) (**Table 2**). A subset of these genes is required for the viral DNA replication (Kool *et al*., 1994a; Lu and Miller, 1995b; Lin *et al*., 2001; Guarino *et al*., 2002b; Lin and Blissard, 2002b) (**Table 2**). Among the LEF genes are *ie-1* and *ie-2*, which transactivate early gene expression and may

Table 2 Late expression factors required for optimal expression of late and very late viral proteins in transient expression assays

Function	*Late expression factors (LEFs)*
Transregulator	IE1
	IE2
DNA replication	DNAPOL (polymerase)
	LEF-1 (primase)
	LEF-2
	LEF-3
	P143 (helicase)
	P35 (antiapoptosis)
	LEF-7
	PE38
	LEF-11
Late gene transcription	LEF-4 (subunit of RNA polymerase)
	LEF-8 (subunit of RNA polymerase)
	LEF-9 (subunit of RNA polymerase)
	P47 (subunit of RNA polymerase)
	LEF-5 (initiation factor)
	LEF-6
	LEF-10
	LEF-12
	VLF-1

transactivate other *lefs* including the replication-associated *lefs* such as *dnapol*, *lef-3*, and *p143*. Many of the LEF genes are expressed during the early phase of gene expression. LEFs include the four genes *lef-4*, *lef-8*, *lef-9*, and *p47*, which encode subunits of the virus-encoded RNA polymerase (Lu and Miller, 1997; Gross and Shuman, 1998; Guarino *et al*., 1998a, 1998b). Lef-5 is an initiation factor and increases transcription from both late and very late promoters (Guarino *et al*., 2002a). Very late expression factor-1 (Vlf-1) appears to regulate transcription of very late genes (McLachlin and Miller, 1994). Vlf-1 has sequence similarity to integrase/resolvases and DNA-binding activity without integrase activity (McLachlin and Miller, 1994). It interacts with the burst element of very late gene promoters, thereby directing the viral-specific RNA polymerase to these sites (Yang and Miller, 1999). No other genes that regulate very late gene expression have been identified (Todd *et al*., 1996).

Expression of late and very late genes is regulated primarily at the level of transcription. Many late transcripts have mini-open reading frames, or are di- or polycistronic, which may provide mechanisms for translational regulation of downstream ORFs. There is also evidence of *cis*-acting transcriptional regulation of flanking genes, which may be common given that genes of different temporal classes are dispersed throughout the genome (Ooi and Miller, 1990; Gross and Rohrmann, 1993).

6.8.5. Effects on the Host

6.8.5.1. Virulence

Virulence is defined as the relative ability of a microorganism to overcome host defenses, or the degree of pathogenicity within a group or species (Poulin and Combes, 1999). Insect species are classified as permissive, semipermissive, or nonpermissive to virus infection, according to the degree of susceptibility (Bishop *et al*., 1995). Enhanced virulence against semipermissive species will result in host range expansion, and hence virulence and host range are intrinsically linked. The LD_{50} of a virus in a given host (the dose of virus required to kill 50% of the population) provides an indicator of the efficiency with which the baculovirus can lethally infect that host, and hence provides a measure of adaptation to the host. Various studies have been conducted to select for increased baculovirus virulence against a particular species under laboratory conditions (**Table 3**). The protocol typically involves repeated passaging of a baculovirus through the insect host. The mechanistic bases for the increased virulence in such cases remain to be determined however.

For fatal infection to occur, the baculovirus must overcome a number of barriers or defenses (that are distinct from mechanisms of resistance) (see Section 6.8.5.5) that may be presented by the host insect. The first potential barrier to baculovirus infection is the requirement for an alkaline environment in the midgut for dissolution of OB and release of ODV. The high alkalinity within the midgut of some Lepidoptera is hypothesized to protect larvae from toxic tannins in the diet (Berenbaum, 1980). Organisms without this characteristic would not be exposed to ODV, which are required for infection of the midgut epithelium. ODV must then negotiate the peritrophic membrane, a process that is poorly understood and likely varies with species according to temporal and structural properties of the peritrophic membrane (Tellam, 1996). Having negotiated the peritrophic membrane, various mechanisms may prevent virus replication within the cell, such as requirements for viral attachment, fusion and entry into the cell, apoptosis (Clem and Miller, 1993), shut down of protein synthesis (Du and Thiem, 1997), and incompatibility between the host cell and virus replication machinery (Croizier *et al*., 1994). Most viruses would not replicate in nonpermissive organisms because of restrictions at the molecular level. Studies on infection of cell lines with

Table 3 Examples of selection of baculoviruses for increased virulence by repeated passaging in the insect host

Virus	*Host insect*	*Number of passages*	*Increase in virulence (-fold)*	*Reference*
HzSNPV	*Helicoverpa zea*	5	1.8	Shapiro and Ignoffo (1970)
OpMNPV	*Trichoplusia ni*	7	10	Martignoni and Iwai (1986)
CfMNPV	*T. ni*	1		Stairs *et al.* (1981)
	Galleria mellonella			
OpMNPV	*Estigmene acrea*	6		Shapiro *et al.* (1982)
CfMNPV		9	25–250	
LdMNPV		9		
AcNPV	*T. ni*		12–15	Tompkins *et al.* (1981, 1988)
	Spodoptera exigua			
AcMNPV	*Plutella xylostella*		15	Kolodny Hirsch and Beek (1997)

BV show that most semi- and nonpermissive insect cells support expression from baculovirus early gene promoters, but not from late or very late promoters (Morris and Miller, 1992, 1993; Liu and Carstens, 1993). Expression from late promoters in some cases suggests that the block likely occurs during or after viral DNA replication. Interestingly, baculoviruses are able to enter mammalian cells and mammalian promoters are used to drive gene expression for gene therapy purposes (Van Loo *et al*., 2001; Ghosh *et al*., 2002; Kost and Condreay, 2002; Huser and Hofmann, 2003). Baculoviruses do not replicate in mammalian cell lines.

Even if the baculovirus is able to replicate in the midgut cells, Lepidopteran larvae are able to slough midgut cells that are infected by ODV (Briese, 1986b; Engelhard and Volkman, 1995; Washburn *et al*., 1995, 1998). Infected midgut epithelial cells are also shed during molting. Evasion of the shedding of infected cells is hypothesized to have led to evolution of the M phenotype of NPV, and the ability of ODV-derived nucleocapsids to cross the midgut epithelial cells and rapidly produce budded virus (Granados and Lawler, 1981; Washburn *et al*., 1999, 2003a) (see Section 6.8.3.1 and **Figure 9**).

Baculoviruses encounter developmental resistance, with infection of later instars requiring a higher viral inoculum. Larvae also become increasingly resistant to fatal baculovirus infection as they age within each instar (Evans, 1986; Teakle *et al*., 1986). In addition, baculovirus infection of some semipermissive species is curtailed by the inability of hemocytes to support virus replication, and by melanization of infected tissues (Trudeau *et al*., 2001).

The arms race between the host defenses and evolution of baculoviruses to counter those defenses contributes to the virulence of a baculovirus against a specific host. Genes encoding enhancin for degradation of the peritrophic membrane, ecdysteroid UDP-glucosyl transferase for inactivation of ecdysteroids, and suppressors of apoptosis provide particularly nice examples of baculovirus weapons in this arms race. Understanding of the barriers to baculovirus infection will facilitate genetic manipulation to enhance the virulence of baculovirus insecticides.

6.8.5.2. Host Range

NPVs have been isolated from more than 500 insect species in several orders (Lepidoptera, Hymenoptera, Diptera, Coleoptera, Thysanura, Trichoptera). GVs have only been identified from lepidopteran hosts and have been isolated from more than 100 species. The NPVs tend to infect only members of the genus, or in some cases the family of the host insect species from which the virus was originally isolated. Few detailed studies of baculovirus host range have been conducted, and there is a wide variation in bioassay methodology, which makes comparison between studies difficult. One potential shortcoming of laboratory-based host range studies is that the heterogeneity observed in baculoviruses isolated from the field (Lee and Miller, 1978; Smith and Summers, 1979) may be more representative for studies of host range than laboratory clones, and indeed may be important for virus survival (Weitzmann *et al*., 1992).

Lymantriid NPVs such as those from *Lymantria dispar* and *Orgyia antiqua* tend to have a narrow host range (Barber *et al*., 1993). Some viruses of noctuid species infect few species, while other viruses isolated from noctuids, such as *A. californica*, *A. falcifera*, and *M. brassicae* (Payne, 1986; Doyle *et al*., 1990; Hostetter and Puttler, 1991), infect 30–40 species from multiple families. AcMNPV infects 43 species of Lepidoptera within 11 families (Payne, 1986). The GVs and hymenopteran NPVs appear to have narrower host ranges than the lepidopteran NPVs.

Current research on baculovirus host range is driven in part by the requirement for assessment of baculovirus host range for registration of baculovirus insecticides. This task has become more important with the registration of recombinant baculovirus insecticides for assessment of potential risks from recombinant baculoviruses to nontarget organisms. Understanding the genetic bases for host range may allow manipulation of baculovirus host range characteristics for pest management purposes.

6.8.5.2.1. Host range genes There are eight baculovirus genes that are known to encode host range determinants (Miller and Lu, 1997) (**Table 4**), most of which have been identified by analysis of restriction of baculovirus replication to specific cell lines. Insertion or alteration of a single viral gene can influence host range. Alteration of a single residue in the *p143* gene, which encodes a DNA helicase homolog, was sufficient to enable AcMNPV to infect cell lines of *B. mori*, while alteration of two residues allowed AcMNPV to infect and kill larvae of *B. mori* (Croizier *et al*., 1994; Kamita and Maeda, 1997; Argaud *et al*., 1998).

Although TnGV and AcMNPV both replicate in *T. ni*, the DNA helicase of TnGV could not substitute for that of AcMNPV. This result suggests that specific protein–protein or protein–DNA interactions are necessary for a functional replication complex (Bideshi and Federici, 2000). Introduction of the host range factor 1 (*hrf-1*) gene of LdMNPV into AcMNPV enabled AcMNPV to infect *L. dispar* cell

Table 4 Nucleopolyhedrovirus genes implicated in host range or virulence

				Effect on replication of AcMNPV in:				
Gene	*Origin of gene*	*Function*	Manipulation	Bombyx mori	Trichoplusia ni	Spodoptera spp.	Lymantria dispar	*Reference*
p143	BmNPV	helicase	Hybrid gene	+ve				Croizier *et al.* (1994), Kamita and Maeda (1997), Argaud *et al.* (1998)
hrf-1	LdNPV	rescues tRNA	Insertion				+ve	Chen *et al.* (1998), Mazzacano *et al.* (1999)
hcf-1	AcMNPV	Unknown	Deletion		−ve			Lu and Miller (1996)
lef-7	AcMNPV	Unknown	Deletion			−ve		Chen and Thiem (1997)
p35	AcMNPV	Antiapoptosis	Deletion			−ve		Clem *et al.* (1991, 1994), Clem and Miller (1993), Chen and Thiem (1997), Zhang *et al.* (2002), Clarke and Clem (2003)
orf603	AcMNPV	Unknown	Deletion			−ve		Popham *et al.* (1998)
p94	AcMNPV	Unknown	Deletion					Clem *et al.* (1994)
ie0	AcMNPV	Transcriptional regulator	Deletion			+ve		Lu *et al.* (2003)

lines and larvae. The presence of *hrf*-1 in the AcMNPV genome resulted in a slight enhancement of AcMNPV virulence against *H. zea* (Chen *et al.*, 1998). Hrf-1 overcomes an apparent inhibition of tRNA synthesis in *L. dispar* cells infected with AcMNPV (Mazzacano *et al.*, 1999). Host cell-specific factor 1 (*hcf-1*) is required for replication of AcMNPV in *T. ni* cell lines but not for replication of AcMNPV in a *S. frugiperda* cell line (Lu and Miller, 1996). Hcf-1 was necessary for infection of *T. ni* larvae by injection, and disruption of *hcf-1* resulted in a 20–30% increase in time taken by the virus to kill the host insect following oral infection. The effect of *hcf-1* on virulence of AcMNPV in *T. ni* larvae would confer a significant advantage in the field. AcMNPV *lef-7* (late expression factor 7) is required for efficient infection of a *S. frugiperda* and a *S. exigua* cell line, but not a *T. ni* cell line (Chen and Thiem, 1997). AcMNPV ORF 603 is important for infection of *S. frugiperda* larvae but not for infection of *T. ni* larvae (Popham *et al.*, 1998). Disruption of expression of IE0, and concomitant increased expression of the apoptosis suppressor P35, enables AcMNPV to replicate in *S. littoralis* cells that undergo apoptosis on infection with wild-type AcMNPV (Lu *et al.*, 2003). The baculovirus antiapoptotic gene *p35* allows AcMNPV to replicate in a *S. frugiperda* cell line and in larvae, but is not required for replication in *T. ni* cells and larvae (Clem *et al.*, 1991, 1994; Clem and Miller, 1993). The presence in AcMNPV of at least one gene that specifically facilitates replication in *T. ni* larvae (*hcf-1*) and at least one gene that specifically facilitates replication in *S. frugiperda* larvae (*lef-7*) indicates that the ability to infect multiple species provides a selective advantage to the virus.

A DNA microarray of the AcMNPV genome has been used to identify six AcMNPV genes (*orf150*, *p10*, *pk2*, *lef-3*, *p35*, and *lef-6*) that are differentially regulated in cell lines derived from *S. frugiperda* (Sf9) and *T. ni* (High Five®) (Yamagishi *et al.*, 2003). Although the functional significance of these genes (with the exception of *p35*) in the two cell lines is unclear, these results are indicative of host cell-specific regulation of AcMNPV genes, suggesting differential viral gene expression for optimal replication in different cell types.

6.8.5.2.2. Antiapoptotic genes P35 is a 35 kDa suppressor of apoptosis that inhibits CED-3/ICE-like proteases produced in response to virus infection and involved in apoptosis of infected insect cells (Clem, 1997). Viruses lacking a functional *p35* gene induce apoptosis of the infected cell, with characteristic blebbing of the plasma membrane, nuclear condensation, and intranucleosomal cleavage of cellular DNA. Baculovirus-induced apoptosis is correlated with reduced infectivity, replication, and dissemination within the host insect (Zhang *et al.*, 2002; Clarke and Clem, 2003). P35 is expressed

during the early and late stages of infection to suppress apoptosis that occurs during the transition from the early to the late stage of infection. A reduction in OB production and lack of liquefaction of cadavers is observed in larvae of *T. ni* infected with *p35* mutants (Herschberger *et al.*, 1992; Clem *et al.*, 1994). P35 is the most broadly acting antiapoptotic protein known and is able to block apoptosis in a wide range of organisms. Hence it is of interest that P35 does not prevent apoptosis in cell lines from *S. littoralis* and *Choristoneura fumiferana* (Chejanovsky and Gershburg, 1995; Palli *et al.*, 1996).

A second family of baculovirus antiapoptotic genes, called *iap* (inhibitor of apoptosis) has been found in all baculoviruses except CuniNPV (Hughes, 2002). There are three *iap* genes in baculoviruses, but only EppoMNPV and OpMNPV have all three. AcMNPV has *iap1* and *iap2* in addition to *p35*, but the function of AcMNPV *iap* genes has not yet been demonstrated (Clem and Miller, 1994; Griffiths *et al.*, 1999). These Iap proteins may serve an auxiliary role in antiapoptotic activity (Viliplana and O'Reilly, 2003), act by blocking signal transduction pathways stimulated by various death stimuli (Clem, 1997), or may only function as inhibitors of apoptosis in certain hosts.

The protein P94 may induce apoptosis in tissues that are important for oral infection (Clem *et al.*, 1994). P35 is not required to inhibit apoptosis if P94 is not expressed (Kamita *et al.*, 1993; Clem, 1997). P94 is not required for infection of larvae of *S. frugiperda* or *T. ni*, but may be important for other species. If P94 induces apoptosis, there would be strong selection to acquire both of these genes. In line with this hypothesis, P35 in BmNPV is not required for infection of *B. mori* and most of the *p94* gene in this virus has been deleted (Kamita *et al.*, 1993; Clem, 1997).

6.8.5.3. Survival Time and Yield

The amount of time taken by the virus to kill the host varies widely, according to the host–virus combination, virus dose, larval instar, and environmental conditions, temperature in particular. In general, an increase in the dose of virus ingested results in a decrease in the survival time of the host insect (van Beek *et al.*, 1988). The survival time of 50% of the infected population (ST_{50}) and the LD_{50} both provide measures of pathogen virulence. The survival time of the host is also linked to the virus yield, with longer survival times allowing for additional rounds of virus replication, resources permitting. To promote increased survival time and virus yield, baculoviruses have acquired a gene that encodes ecdysteroid UDP-glucosyltransferase (*egt*).

Expression of the baculovirus *egt* gene has a complex effect on the host larva. By inactivating ecdysteroids, Egt allows the insect to live longer and grow larger thereby allowing increased production of virus. *egt* is expressed during the early phase of infection, and is secreted from infected cells rather than being retained in the endoplasmic reticulum as in the case of related mammalian proteins (O'Reilly, 1995). Egt catalyzes the conjugation of ecdysteroids with UDP-glucose or UDP-galactose and is specific for ecdysteroids (O'Reilly and Miller, 1989; O'Reilly *et al.*, 1992; Kelly *et al.*, 1995; Clark *et al.*, 1996). This sugar conjugation and inactivation of ecdysteroids suppresses host molting, prevents pupation, and prevents the conversion of permissive larvae into nonpermissive hosts. A critical level of Egt is required for the arrest of host development and hence the timing of arrest depends on the dose of virus received and also the timing of infection during the larval instar (O'Reilly, 1997). Suppression of molting causes infected larvae to continue to feed when they would otherwise cease feeding in preparation for the molt, and the yield of OB is increased (O'Reilly and Miller, 1991). Deletion of the *egt* gene from the AcMNPV genome results in a 20–30% reduction in the time taken by the virus to kill the host and an associated reduction in virus yield (O'Reilly and Miller, 1991). In *S. exigua*, Egt did not arrest development but Egt served to maintain the integrity of the Malpighian tubules of infected larvae (Flipsen *et al.*, 1995a). Hence, the role of Egt may not be dependent solely on inhibition of molting. Other evidence suggests that high titers of ecdysteroids are detrimental to virus replication and Egt may serve to mitigate these effects (O'Reilly *et al.*, 1995; O'Reilly, 1997).

6.8.5.4. Sublethal Effects and Latent Infections

The impact of baculoviruses on host populations extends beyond mortality of infected larvae, because of physiological effects that result from infection at sublethal doses of virus. Effects typical of sublethal infections include change in development time and sex ratio, and reduced fecundity and egg viability (Rothman and Myers, 1996a). The mechanisms of sublethal effects are poorly understood. Virus-induced alteration of hormonal and enzyme levels may affect host development or production of viable eggs or sperm (Subrahmanyam and Ramakrishnan, 1980; O'Reilly and Miller, 1989; Burand and Park, 1992). Latent viruses do not induce symptoms until activated by various stressors (Hughes *et al.*, 1993, 1997). Host insects that harbor latent viruses do not transmit virus horizontally, but can transmit virus vertically.

6.8.5.5. Resistance

Resistance is defined as physiological alteration in response to selection pressure from exposure to baculoviruses, resulting in decreased susceptibility of the host insect to the baculovirus. This is distinct from developmental resistance described above (see Section 6.8.5.1).

The development of resistance to baculoviruses is a key issue for use of baculoviruses in pest management but has received relatively little attention (Briese, 1986a, 1986b; Fuxa, 1993). Insects of the same species collected from different locations often vary in their susceptibility to a particular baculovirus, presumably because prior exposure in some localities has been selected for resistant populations (Fuxa, 1987). Resistance to and virulence of baculoviruses are likely to vary geographically according to frequency and levels of exposure. In Brazil, the resistance of the velvet bean caterpillar to *Anticarsia gemmatalis* MNPV increased according to the number of years of treatment at different sites (Abot *et al*., 1995). Several studies have shown that it is possible to select for resistant hosts under selection pressure from different baculoviruses, although not for all virus–host combinations (Kaomini and Roush, 1988). The cabbage looper, *T. ni*, has been shown to evolve up to 22-fold resistance to the virus TnSNPV (Milks and Myers, 2000), which was not associated with a detectable fitness cost and was stable in the absence of virus exposure (Milks *et al*., 2002). In contrast, resistance to other NPVs or a GV was associated with reduced pupal weight, developmental time, and/or egg production or hatch in *A. gemmatalis* (Fuxa and Richter, 1998), *S. frugiperda* (Fuxa *et al*., 1988), and *Plodia interpunctella* (Boots and Begon, 1993). *Spodoptera frugiperda* acquired 4.5-fold resistance to SfNPV following selection for seven generations (Fuxa *et al*., 1988); *P. interpunctella* acquired twofold resistance following exposure to *P. interpunctella* GV for 2 years (Boots and Begon, 1993); *A. gemmatalis* acquired fivefold resistance in eight generations in the laboratory, and >1000-fold resistance in 13 generations in the field in Brazil (Fuxa and Richter, 1998). As expected for resistance associated with fitness cost, resistance was rapidly lost on removal of virus selection from *S. frugiperda* and *A. gemmatalis* (Fuxa and Richter, 1989, 1998). There is also evidence that resistance to *S. frugiperda* NPV confers cross-resistance to other baculoviruses (Fuxa and Richter, 1990).

The mechanistic bases for resistance to baculoviruses have not been addressed. An additional complexity is that sublethal infection can cause the same effects as resistance such as altered development time and reduced fecundity. Assessment of field populations of the Western tent caterpillar *Malacosoma californicum pluviale*, for example, showed that effects were more consistent with sublethal infection than disease-induced resistance (Rothman and Myers, 1996b).

6.8.6. Baculovirus Genomics

6.8.6.1. General Properties of Baculovirus Genomes

At the time of writing, the genomes of 19 baculoviruses have been sequenced (**Table 5**). The sizes of the genomes range from approximately 80 to 180 kbp, due to the presence of unique genes, and gene duplications including the "baculovirus repeat ORFs" or *bro* genes (Gomi *et al*., 1999; Hayakawa *et al*., 1999; Kang *et al*., 2003). The genomes vary as a result of deletions, gene insertions, inversions, and duplications. Transposable elements have contributed to some of these modifications (Friesen, 1993; Jehle, 1996; Jehle *et al*., 1997). The organization of genes in NPV genomes is highly conserved, with rearrangements of genomic segments and presence or absence of auxiliary genes contributing to their diversity. The G+C content is highly variable, ranging from 28% to 59% of the genome.

In addition to the essential genes that are required for virus propagation (including genes required for virus gene expression, replication, virus assembly, and transmission), the large size of baculovirus genomes allows for inclusion of a second class of genes, the auxiliary genes (**Table 6**). These genes are not essential but confer some selective advantage to the virus, and may act at the cellular or organismal level (O'Reilly, 1997). These genes include *ubi*, *egt*, and *ctl*. Many baculovirus genes have yet to be characterized and the functions of many genes that have been studied remain unknown. Extensive analysis of 13 baculovirus genomes revealed a core of 30 common genes, including 10 of unknown function, and an additional 32 genes that are present in all of the lepidopteran baculoviruses (Herniou *et al*., 2003). In addition, 27 genes are specific to GVs and 14 to lepidopteran NPVs suggesting a role for these genes in the differences in the biology of these two groups. There are 17 genes that are specific to group I NPVs, including *gp64*.

There are numerous examples of baculovirus genes with homology to genes from eukaryotic or prokaryotic organisms (Possee and Rohrmann, 1997) (**Table 6**). The high frequency of homologous recombination during viral replication, combined with movement of genetic material by transposable

Table 5 Insect baculovirus genomes sequenced

Baculovirus	*Reference*
Nucleopolyhedroviruses (NPV)	
Adoxophyes honmai NPV	Nakai, Goto, Kang, Shikata, and Kunimi (unpublished data)
Autographa californica MNPV[a]	Ayres *et al.* (1994)
Bombyx mori NPV[a]	Gomi *et al.* (1999)
Choristoneura fumiferana MNPV	de Jong, Dominy, Lauzon, Arif, Carstens, and Krell (unpublished data)
Culex nigripalpus NPV	Afonso *et al.* (2001)
Epiphyas postvittana MNPV	Hyink *et al.* (2002)
Helicoverpa armigera SNPV[b]	Chen *et al.* (2001)
Helicoverpa zea SNPV[b]	Chen *et al.* (2002)
Lymantria dispar MNPV	Kuzio *et al.* (1999)
Mamestra configurata NPV A	Li *et al.* (2002b)
Mamestra configurata NPV B	Li *et al.* (2002a)
Orygia pseudotsugata MNPV	Ahrens *et al.* (1997)
Rachiplusia ou MNPV[a] (*Anagrapha falcifera* MNPV)	Harrison and Bonning (2003)
Spodoptera litura MNPV	Pang *et al.* (2001)
Spodoptera exigua MNPV	Ijkel *et al.* (1999)
Granuloviruses (GV)	
Cydia pomonella GV	Luque *et al.* (2001)
Phthorimaea operculella GV	Croizier, Taha, Croizier, and Lopez Ferber (unpublished data)
Plutella xylostella GV	Hashimoto *et al.* (2000)
Xestia c-nigrum GV	Hayakawa *et al.* (1999)

[a,b]Viruses with the same superscript letter are considered to be variants of the same virus (Blissard *et al.*, 2000).

elements (Miller and Miller, 1982; Fraser *et al.*, 1983; Friesen, 1993), provide possible mechanisms for genetic exchange between disparate organisms. Some of the genes such as *egt*, the ecdysteroid UDP-glucosyl transferase gene, and *sod*, the superoxide dismutase gene homolog, are most likely of insect origin. Indeed, a genome-wide survey revealed six genes likely to have been horizontally transferred into baculoviruses, namely *DNA ligase*, *rr1*, *gta*, *iap*, *chi*, and *egt*, of which *egt* and *iap* are derived from insects (Hughes, 2002; Hughes and Friedman, 2003). The AcMNPV chitinase gene *chiA* has the highest similarity to bacterial chitinase (Hawtin *et al.*, 1995). The higher G+C content of this gene is also more typical of the bacterial gene than other baculovirus genes. The F protein genes are also likely to have been acquired from insect hosts (Malik *et al.*, 2000; Rohrmann and Karplus, 2001).

The sequencing of multiple baculovirus genomes has allowed inference of baculovirus evolution and genetic diversity (Herniou *et al.*, 2003), horizontal gene transfer (Hughes and Friedman, 2003), identification of genes that have evolved in response to changing selection pressures (Harrison and Bonning, 2003, 2004), and identification of genes involved in virus–host insect interactions (Dall *et al.*, 2001).

6.8.6.2. Baculovirus Evolution

Comprehensive phylogenetic analyses of 30 genes common to 13 baculovirus genomes highlighted the division of baculoviruses into four groups: GVs, group I NPVs, group II NPVs, and the dipteran NPV CuniNPV (Herniou *et al.*, 2003). Phylogenetic analysis of *dnapol* from NPV and GV provides an example of this division (**Figure 13**). It is likely that the Baculoviridae will be reclassified to accommodate CuniNPV, which clearly does not belong with the lepidopteran NPVs.

Gene content mapping highlighted the fluid nature of baculovirus genomes including examples of repeated gene acquisitions and losses. Thirty-three genes were identified that have been acquired separately on two or even three occasions (Herniou *et al.*, 2003; Hughes and Friedman, 2003). Baculoviruses have sufficient flexibility with incorporation of additional genetic material to allow "sampling" for beneficial genes from the environment (Herniou *et al.*, 2001). Recombination between baculoviruses may occur when different baculoviruses infect the same cell. This is most likely to occur in the field between related viruses that share common hosts (Smith and Summers, 1980; Croizier and Ribeiro, 1992). Gene loss and acquisition may also result from the activity of transposable elements, several of which have been observed on culture of baculoviruses in cell lines. Sites favored by transposons include the *fp25K* gene resulting in several mutant viruses with the few polyhedra (FP) phenotype. *Trichoplusia ni* cell-derived transposable elements include IFP2 ("piggyBac") (Cary *et al.*, 1989), TFP3 ("tagalong") (Wang *et al.*, 1989), *hitchhiker* (Bauser *et al.*, 1996), and TED (Miller and Miller, 1982). For Sf cells, the elements IFP1.6 and IFP2.2 have been characterized (Beames and Summers, 1988, 1990), and a Tc1-like transposon has been identified in the genome of CpGV (Jehle *et al.*, 1995, 1997, 1998).

Shared protein phylogenetic profiles indicate the presence of viral proteins specific for infection of lymandtriid hosts. Only OpMNPV and LdMNPV that infect the lymantriids *L. dispar* and *Orygia pseudotsugata* encode *hrf1*, *ctl1*, and *ctl2*. Other baculoviruses encode one or other of the *ctl* genes,

Table 6 Baculovirus genes with homologs in other organisms

Gene	*Homolog*	*Other locations*
Virus propagation[a]		
iap	Inhibitor of apoptosis	Mammals, insects
fgf	Fibroblast growth factor	Mammals
gta	General transcription activator	Mammals
dnapol	DNA polymerase	Eukaryotes, prokaryotes, other virus families
vlf-1	Integrase/resolvase	Yeast, prokaryotes
pnk/pnl	Polynucleotide kinase and ligase	Phage, yeast, plants
alk-exo	Alkaline exonuclease	Eukaryotes, prokaryotes
gp64	Viral glycoprotein	Thogoto virus
gp37	Spindlin	Entomopoxviruses
dUTPase	dUTPase	Eukaryotes, prokaryotes, other virus families
rr 1, 2	Ribonucleotide reductase subunits	Eukaryotes, prokaryotes, other virus families
f	Envelope fusion protein	*Drosophila melanogaster*
Auxiliary genes[b]		
ptp	Protein tyrosine phosphatase	Eukaryotes, prokaryotes, poxviruses
ctl	Conotoxin	Cone snails, spiders
egt	Ecdysteroid UDP-glucosyl transferase	Eukaryotes, prokaryotes
pk1	Protein kinase	Eukaryotes, prokaryotes, other virus families
pk2	Protein kinase, eiF2-α-like	Eukaryotes
pcna	Proliferating cell nuclear antigen	Eukaryotes
sod	Superoxide dismutase	Eukaryotes, prokaryotes, poxviruses
ubi	Ubiquitin	Eukaryotes, prokaryotes
chiA	Chitinase	Eukaryotes, prokaryotes
cath	Cathepsin	Eukaryotes
p10	Filament-associated late protein	Entomopoxviruses

[a]Genes required for virus propagation including those encoding structural proteins, those required for virus gene expression, replication, production of progeny virus.
[b]Genes not required for virus replication but providing some selective advantage.
After Possee and Rohrmann (1997).

but not both, and *hrf1* is only found in OpMNPV and LdMNPV. The link between the presence of all three genes and infection of a particular lepidopteran family suggests that these proteins may function in specific interactions with this host family (Herniou *et al*., 2003).

6.8.6.3. Identification of Genes Involved in Virus–Insect Interactions

Dall *et al*. compared the genes of representative viruses from three different families that include viruses that infect insects, namely the Poxviridae, Baculoviridae, and Iridoviridae (Dall *et al*., 2001). Six groups of genes were identified that were present in all three families, present in viruses with insect hosts, and absent in members with vertebrate hosts. Four of the six groups of genes are found in baculoviruses, and include the fusolin/gp37 group, which encodes proteins that facilitate infection (Xu and Hukuhara, 1992; Mitsuhashi *et al*., 1998; Hukuhara *et al*., 1999). Analysis of the other three groups found in baculoviruses may provide leads for genetic improvement of baculovirus insecticides by modification of host range or virulence.

6.8.6.4. Identification of Genes that Have Undergone Adaptive Molecular Evolution

An alternative approach for identifying genes underlying host range or virulence is to compare the genomes of closely related baculoviruses that exhibit differential virulence to host species. The closely related viruses *Rachiplusia ou* MNPV (*A. falcifera* MNPV) (Harrison and Bonning, 1999) and AcMNPV have broad, overlapping host ranges but there are significant differences in the ability of these two viruses to infect at least six species. These species include the corn earworm *H. zea*, the European corn borer *Ostrinia nubilalis*, and the navel orangeworm *Amyelois transitella* (Lewis and Johnson, 1982; Hostetter and Puttler, 1991; Vail *et al*., 1993; Cardenas *et al*., 1997; Harrison and Bonning, 1999). On comparison of the genomes of these two viruses, it was found that the *hrs* of RoMNPV contain fewer palindromic repeats than in AcMNPV, homologs of five AcMNPV genes including *ctl* are missing from RoMNPV, and the composition of promoter motifs differed in 23 cases (Harrison and Bonning, 2003). On the basis that the primary selection pressure on a virus comes from its

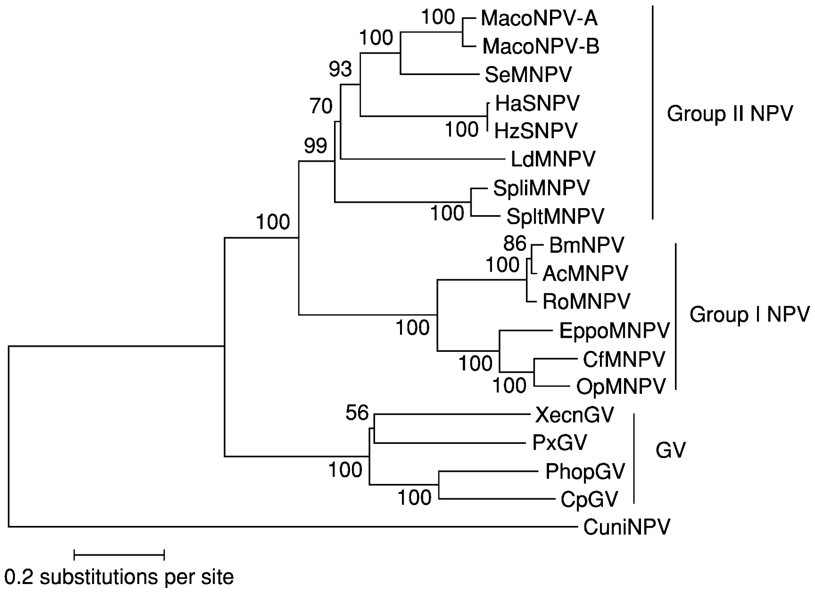


Figure 13 Phylogenetic analysis of predicted amino acid sequences of *dnapol* illustrating the separation of groups I and II NPVs from GVs and from the dipteran NPV derived from *Culex nigripalpus*. Phylogram produced by minimum evolution (ME) analysis of baculovirus DNA polymerase predicted amino acid sequences. Bootstrap values >50% (*n* = 1000 replicates) are shown at interior branches where they occur. The Group I and Group II NPV clades are indicated. AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus (Ayres *et al.*, 1994); BmNPV, *Bombyx mori* nucleopolyhedovirus (Gomi *et al.*, 1999); CfMNPV, *Choristoneura fumiferana* multiple nucleopolyhedrovirus (Liu and Carstens, 1995); CpGV, *Cydia pomonella* granulovirus (Luque *et al.*, 2001); CuniNPV, *Culex nigripalpus* nucleopolyhedrovirus (Afonso *et al.*, 2001); EppoMNPV, *Epiphyas postvittana* multiple nucleopolyhedrovirus (Hyink *et al.*, 2002); HaSNPV, *Helicoverpa armigera* single nucleopolyhedrovirus (Chen *et al.*, 2001); HzSNPV, *Helicoverpa zea* single nucleopolyhedrovirus (Chen *et al.*, 2002); LdMNPV, *Lymantria dispar* multiple nucleopolyhedrovirus (Kuzio *et al.*, 1999); MacoNPV-A, *Mamestra configurata* nucleopolyhedrovirus strain 90/2 (Li *et al.*, 2002b); MacoNPV-B, *Mamestra configurata* nucleopolyhedrovirus strain 96B (Li *et al.*, 2002a); OpMNPV, *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (Ahrens *et al.*, 1997); PxGV, *Plutella xylostella* granulovirus (Hashimoto *et al.*, 2000); PhopGV, *Phthorimaea operculella* granulovirus (accession number NC_004062); *Rachiplusia ou* multiple nucleopolyhedrovirus (Harrison and Bonning, 2003); SeMNPV, *Spodoptera exigua* multiple nucleopolyhedrovirus (Ijkel *et al.*, 1999); SpliMNPV, *Spodoptera littoralis* multiple nucleopolyhedrovirus (accession number AF215639); SpltMNPV, *Spodoptera litura* multiple nucleopolyhedrovirus (Pang *et al.*, 2001); XecnGV, *Xestia c-nigrum* granulovirus (Hayakawa *et al.*, 1999).

host, and that genes that have adaptively evolved may be involved in virulence, positive selection analysis was conducted on the genes of AcMNPV and RoMNPV (Yang *et al.*, 2000; Harrison and Bonning, 2003). Genes in which nonsynonymous (amino acid changing) substitutions are fixed at a higher rate than synonymous (silent) substitutions are identified as having undergone adaptive molecular evolution, or positive selection. This maximum likelihood analysis resulted in the identification of two genes, *arif-1* and *odv-e18*, predicted to have undergone positive selection. Odv-E18 is present in the envelope of occlusion-derived virus (Braunagel *et al.*, 1996b) and hence in an ideal location as a host range determinant for initial infection of midgut cells of the host insect. Arif-1 (actin-rearrangement-inducing factor) is involved with dissociation of the actin network of the host cell and the actin cables that form early during virus infection, and in production of actin aggregates at the plasma membrane (Dreschers *et al.*, 2001).

The strength of the positive selection analysis increases with increasing number of data sets. The positive selection analyses have been extended to include genes from multiple NPV genomes, and from this analysis nine genes predicted to have undergone positive selection have been identified (Harrison and Bonning, 2004). Alignments of 83 group I NPV genes were fitted to models of codon substitution that allow for varying selection intensity among codon sites. Genes predicted to have undergone positive selection include *ac38*, *lef-10*, *lef-12*, *odv-e18*, and *odv-e56*. NPV genes that have undergone positive selection may modulate the ability of different NPVs to replicate efficiently in cells (*lef-10*, *lef-12*) or establish primary infection of the midgut (*odv-e18*, *odv-e56*) of different host species.

6.8.7. Conclusion

Baculoviruses will continue to play an important role in the management of insect pests, as model

systems for studies of fundamental biological processes, as tools for recombinant protein expression, and as candidate vectors for gene therapy. The potential of mosquito baculoviruses such as CuniNPV for management of mosquito-borne disease will also be of particular interest. Sequencing of additional baculovirus genomes and comparative genomics will provide further insights into baculovirus biology and evolution. Data acquired from the comparative genomics studies such as identification of baculovirus genes that have undergone adaptive molecular evolution (Harrison and Bonning, 2003) and those potentially involved in interaction with the insect host (Dall *et al*., 2001) provide particularly useful leads for elucidation of how baculoviruses have evolved with their hosts. DNA microarrays of baculovirus genomes (Yamagishi *et al*., 2003), expressed sequence tag (EST) libraries (Okano *et al*., 2001), and RNA interference (Means *et al*., 2003) will be valuable tools for future study of baculovirus gene expression and virus–host cell interactions. Increased understanding of baculovirus biology will facilitate further optimization of baculovirus-related technologies.

Acknowledgments

Thanks to Drs Robert Harrison, Loy Volkman and Gary Blissard for critical reading of this chapter. Funded in part by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under Agreement Numbers 00-39210-9772 and 2003-35302-13558, as well as Hatch Act and State of Iowa funds.

References

Abot, A.R., Moscardi, F., Fuxa, J.R., Sosa-Gómez, D.R., Richter, A.R., **1995**. Susceptibility of populations of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) from Brazil and the United States to a nuclear polyhedrosis virus. *J. Entomol. Sci. 30*, 62–69.

Adams, J.R., McClintock, J.T., **1991**. Baculoviridae. Nuclear polyhedrosis viruses, part 1: Nuclear polyhedrosis viruses of insects. In: Adams, J.R., Bonami, J.R. (Eds.), Atlas of Invertebrate Viruses. CRC Press, Boca Raton, FL, pp. 87–204.

Adams, J.R., Goodwin, R.H., Wilcox, T.A., **1977**. Electron microscopic investigations on invasion and replication of insect baculoviruses *in vivo* and *in vitro*. *Biol. Cellulaire 28*, 261–268.

Afonso, C.L., Tulman, E.R., Lu, Z., Balinsky, C.A., Moser, B.A., *et al*., **2001**. Genome sequence of a baculovirus pathogenic for *Culex nigripalpus*. *J. Virol. 75*, 11157–11165.

Ahrens, C.H., Carlson, C., Rohrmann, G.F., **1995a**. Identification, sequence and transcriptional analysis of *lef-3*, a gene essential for *Orgyia pseudotsugata* baculovirus DNA replication. *Virology 210*, 372–382.

Ahrens, C.H., Pearson, M.N., Rohrmann, G.F., **1995b**. Identification and characterization of a second putative origin of DNA replication in a baculovirus of *Orgyia pseudotsugata*. *Virology 207*, 572–576.

Ahrens, C.H., Rohrmann, G.F., **1995a**. Identification of essential *trans*-acting regions required for DNA replication of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus: *lef-1* is an essential replication gene. *Virology 207*, 417–428.

Ahrens, C.H., Rohrmann, G.F., **1995b**. Replication of *Orgyia pseudotsugata* baculovirus DNA: *ie-1* and *lef-2* are essential and *ie-2*, *p34* and *Op-iap* are stimulatory genes. *Virology 212*, 650–662.

Ahrens, C.H., Russell, R.L.Q., Funk, C.J., Evans, J.T., Harwood, S.H., *et al*., **1997**. The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *Virology 229*, 381–399.

Aizawa, K., **1963**. The nature of infections caused by nuclear-polyhedrosis viruses. In: Steinhaus, E.A. (Ed.), Insect Pathology: An Advanced Treatise, vol. 1. Academic Press, New York, pp. 381–412.

Argaud, O., Croizier, L., López-Ferber, M., Croizier, G., **1998**. Two key mutations in the host-range specificity domain of the *p143* gene of *Autographa californica* nucleopolyhedrovirus are required to kill *Bombyx mori* larvae. *J. Gen. Virol. 79*, 931–935.

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D., **1994**. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology 202*, 586–605.

Barber, K.N., Kaupp, W.J., Holmes, S.B., **1993**. Specificity testing of the nuclear polyhedrosis virus of the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). *Can. Entomol. 125*, 1055–1066.

Barrett, J.W., Lauzon, H.A.M., Mercuri, P.S., Krell, P., Sohi, S.S., *et al*., **1996**. The putative LEF-1 proteins from two distinct *Choristoneura fumiferana* multiple nucleopolyhedroviruses share domain homology to eukaryotic primases. *Virus Genes 13*, 229–237.

Bauser, C.A., Elick, T.A., Fraser, M.J., **1996**. Characterization of *hitchhiker*, a transposon insertion frequently associated with baculovirus FP mutants derived upon passage in the TN-368 cell line. *Virology 216*, 235–237.

Beames, B., Summers, M.D., **1988**. Comparisons of host cell DNA insertions and altered transcription at the site of insertions in few polyhedra baculovirus mutants. *Virology 162*, 206–220.

Beames, B., Summers, M.D., **1990**. Sequence comparison of cellular and viral copies of host cell DNA insertions found in *Autographa californica* nuclear polyhedrosis virus. *Virology 174*, 354–363.

Belyavskyi, M., Braunagel, S.C., Summers, M.D., **1998**. The structural protein ODV-EC27 of *Autographa californica* nucleopolyhedrovirus is a multifunctional

viral cyclin. *Proc. Natl Acad. Sci. USA 95*, 11205–11210.

Beniya, H., Funk, C.J., Rohrmann, G.F., Weaver, R.F., **1996**. Purification of a virus-induced RNA polymerase from *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells that accurately initiates late and very late transcription *in vitro*. *Virology 216*, 12–19.

Benz, G.A., **1986**. Introduction: historical perspectives. In: Granados, R., Federici, B.A. (Eds.), The Biology of Baculoviruses. CRC Press, Boca Raton, FL, pp. 1–35.

Berenbaum, M., **1980**. Adaptive significance of midgut pH in larval Lepidoptera. *Am. Nat. 115*, 138–146.

Bideshi, D.K., Federici, B.A., **2000**. The *Trichoplusia ni* granulovirus helicase is unable to support replication of *Autographa californica* multicapsid nucleopolyhedrovirus in cells and larvae of *T ni*. *J. Gen. Virol. 81*, 1593–1599.

Bilimoria, S.L., **1991**. The biology of nuclear polyhedrosis viruses. In: Kurstak, E. (Ed.), Viruses of Invertebrates. Dekker, New York, pp. 1–72.

Bischoff, D.S., Slavicek, J.M., **1997**. Molecular analysis of an *enhancin* gene in *Lymantria dispar* nuclear polyhedrosis virus. *J. Virol. 71*, 8133–8140.

Bishop, D.H.L., Hirst, M.L., Possee, R.D., Cory, J.S., **1995**. Genetic engineering of microbes: virus insecticides – a case study. In: Darby, G.K., Hunter, P.A., Russell, A.D. (Eds.), 50 Years of Microbials. Cambridge University Press, Cambridge, pp. 249–277.

Blissard, G.W., **1996**. Baculovirus–insect cell interactions. *Cytotechnology 20*, 73–93.

Blissard, G.W., Black, B., Crook, N., Keddie, B.A., Possee, R., *et al.*, **2000**. Family Baculoviridae. In: Regenmortel, M.H.V.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy, 7th Report of the International Committee on the Taxonomy of Viruses. Springer, New York, pp. 195–202.

Blissard, G.W., Rohrmann, G.F., **1989**. Location, sequence, transcriptional mapping, and temporal expression of gp64 envelope glycoprotein gene of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology 170*, 537–555.

Blissard, G.W., Wenz, J.R., **1992**. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH dependent membrane fusion. *J. Virol. 66*, 6829–6835.

Bonning, B.C., Boughton, A.J., Jin, H., Harrison, R.L., **2002**. Genetic enhancement of baculovirus insecticides. In: Upadhyay, R.K. (Ed.), Advances in Microbial Control of Insect Pests. Kluwer Academic, London, pp. 109–125.

Boots, M., Begon, M., **1993**. Trade-offs with resistance to a granulosis virus in the Indian meal moth, examined by a laboratory evolution experiment. *Func. Ecol. 7*, 528–534.

Brandt, C.R., Adang, M.J., Spence, K.D., **1978**. The peritrophic membrane: ultrastructural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. *J. Invertebr. Pathol. 32*, 12–24.

Braunagel, S.C., Burks, J.K., Rosas-Acosta, G., Harrison, R.L., Ma, H., *et al.*, **1999**. Mutations within the *Autographa californica* nucleopolyhedrovirus FP25K gene decrease the accumulation of ODV-E26 and alter its intranuclear transport. *J. Virol. 73*, 8559–8570.

Braunagel, S.C., Elton, D.M., Ma, H., Summers, M.D., **1996a**. Identification and analysis of an *Autographa californica* nuclear polyhedrosis virus structural protein of the occlusion-derived virus envelope: ODV-E56. *Virology 217*, 97–110.

Braunagel, S.C., Guidry, P.A., Rosas-Acosta, G., Engelking, L., Summers, M.D., **2001**. Identification of BV/ODV-C42, an *Autographa californica* nucleopolyhedrovirus orf101-encoded structural protein detected in infected-cell complexes with ODV-EC27 and p78/83. *J. Virol. 75*, 12331–12338.

Braunagel, S.C., He, H., Ramamurthy, P., Summers, M.D., **1996b**. Transcription, translation, and cellular localization of three *Autographa californica* nuclear polyhedrosis virus structural proteins: ODV-E18, ODV-E35, and ODV-EC27. *Virology 222*, 100–114.

Braunagel, S.C., Parr, R., Belyavskyi, M., Summers, M.D., **1998**. *Autographa californica* nucleopolyhedrovirus infection results in Sf9 cell cycle arrest at G_2/M phase. *Virology 244*, 195–211.

Braunagel, S.C., Summers, M.D., **1994**. *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. *Virology 202*, 315–328.

Briese, D.T., **1986a**. Host resistance to microbial control agents. In: Franz, J.M. (Ed.), Biological Plant and Health Protection. Fischer Verlag, Stuttgart, pp. 233–256.

Briese, D.T., **1986b**. Insect resistance to baculoviruses. In: Federici, B.A., Granados, R. (Eds.), The Biology of Baculoviruses, vol. 2. CRC Press, Boca Raton, FL, pp. 237–263.

Bromme, D., Okamoto, K., **1995**. The baculovirus cysteine protease has a cathepsin B-like S2 subsite specificity. *Biol. Chem. Hoppe-Seyler 376*, 611–615.

Brown, M., Crawford, A.M., Faulkner, P.F., **1979**. Genetic analysis of a baculovirus *Autographa californica* nuclear polyhedrosis virus: isolation of temperature-sensitive mutants and assortment into complementation groups. *J. Virol. 31*, 190–198.

Brown, M., Faulkner, P., Cochran, M.A., Chung, K.L., **1980**. Characterization of two morphology mutants of *Autographa californica* nuclear polyhedrosis virus with large cuboidal inclusion bodies. *J. Gen. Virol. 50*, 309–316.

Bud, H.M., Kelly, D.C., **1980**. An electron microscope study of partially lysed baculovirus nucleocapsids: the intranucleocapsid packaging of viral DNA. *J. Ultrastruct. Res. 73*, 361–368.

Burand, J.P., Park, E.J., **1992**. Effect of a nuclear polyhedrosis virus infection on the development and

pupation of gypsy moth larvae. *J. Invertebr. Pathol. 60*, 171–175.

Cardenas, F.A., Vail, P.V., Hoffmann, D.F., Tebbets, J.S., Schreiber, F.E., **1997**. Infectivity of celery looper (Lepidoptera: Noctuidae) multiple nucleocapsid polyhedrosis virus to navel orangeworm (Lepidoptera: Pyralidae). *Environ. Entomol. 26*, 131–134.

Carson, D.D., Guarino, L.A., Summers, M.D., **1988**. Functional mapping of an AcNPV immediate early gene which augments expression of the IE-1 *trans*-activated 39k gene. *Virology 162*, 444–451.

Carstens, E.B., Chan, H., Yu, H., Williams, G.V., Casselman, R., **1994**. Genetic analysis of temperature-sensitive mutations in baculovirus late expression factors. *Virology 204*, 323–337.

Carstens, E.B., Tjia, S.T., Doerfler, W., **1979**. Infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus. I. Synthesis of intracellular proteins after virus infection. *Virology 99*, 386–398.

Carstens, E.B., Williams, G.V., Faulkner, P., Partington, S., **1992**. Analysis of polyhedra morphology mutants of *Autographa californica* nuclear polyhedrosis virus: molecular and ultrastructural features. *J. Gen. Virol. 71*, 3035–3040.

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E., *et al.*, **1989**. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viurses. *Virology 172*, 156–169.

Charlton, C.A., Volkman, L.E., **1991**. Sequential rearrangement and nuclear polymerization of actin in baculovirus-infected *Spodoptera frugiperda* cells. *J. Virol. 65*, 1219–1227.

Charlton, C.A., Volkman, L.E., **1993**. Penetration of *Autographa californica* nuclear polyhedrosis virus nucleocapsids into IPBL Sf21 cells induces actin cable formation. *Virology 197*, 245–254.

Chejanovsky, N., Gershburg, E., **1995**. The wild-type *Autographa californica* nuclear polyhedrosis virus induces apoptosis of *Spodoptera littoralis* cells. *Virology 209*, 519–525.

Chen, C.-J., Quentin, M.E., Brennan, L.A., Kukel, C., Thiem, S.M., **1998**. *Lymantria dispar* nucleopolyhedrovirus *hrf-1* expands the larval host range of *Autographa californica* nucleopolyhedrovirus. *J. Virol. 72*, 2526–2531.

Chen, C.-J., Thiem, S.M., **1997**. Differential infectivity of two *Autographa californica* nucleopolyhedrovirus mutants on three permissive cell lines is the result of *lef-7* deletion. *Virology 227*, 88–95.

Chen, X., Ijkel, W.F.J., Tarchini, R., Sun, X., Sandbrink, H., *et al.*, **2001**. The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. *J. Gen. Virol. 82*, 241–257.

Chen, X., Zhang, W.-J., Wong, J., Chun, G., Lu, A., *et al.*, **2002**. Comparative analysis of the complete genome sequences of *Helicoverpa zea* and *Helicoverpa armigera* single-nucleocapsid nucleopolyhedroviruses. *J. Gen. Virol. 83*, 673–684.

Cherbas, L., Cherbas, P., **1993**. The arthropod initiator: the capsite consensus plays an important role in transcription. *Insect Biochem. Mol. Biol. 23*, 81–90.

Chernomordik, L., Leikina, E., Cho, M.-S., Zimmerberg, J., **1995**. Control of baculovirus gp64-induced syncitium formation by membrane lipid composition. *J. Virol. 69*, 3049–3058.

Chisholm, G.E., Henner, D.J., **1988**. Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. *J. Virol. 62*, 3193–3200.

Chittihunsa, T., Sikorowski, P.P., **1995**. Effects of nonoccluded baculovirus (Baculoviridae) infection on *Microplitis croceipes* (Hymenoptera: Braconidae). *Biol. Control 24*, 1708–1712.

Clark, E.E., Tristem, M., Cory, J.S., O'Reilly, D.R., **1996**. Characterization of the ecdysteroid UDP-glucosyltransferase gene from *Mamestra brassicae* nucleopolyhedrosis virus. *J. Gen. Virol. 77*, 2865–2871.

Clarke, T.E., Clem, R.J., **2002**. Lack of involvement of haemocytes in the establishment and spread of infection in *Spodoptera frugiperda* larvae infected with the baculovirus *Autographa californica* M nucleopolyhedrovirus by intrahemocoelic injection. *J. Gen. Virol. 83*, 1565–1572.

Clarke, T.E., Clem, R.J., **2003**. *In vivo* induction of apoptosis correlating with reduced infectivity during baculovirus infection. *J. Virol. 77*, 2227–2232.

Clem, R.J., **1997**. Regulation of programmed cell death by baculoviruses. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 237–266.

Clem, R.J., **2001**. Baculoviruses and apoptosis: the good, the bad, and the ugly. *Cell Death Differ. 8*, 137–143.

Clem, R.J., Fechheimer, M., Miller, L.K., **1991**. Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science 254*, 1388–1389.

Clem, R.J., Miller, L.K., **1993**. Apoptosis reduces both the *in vitro* replication and the *in vivo* infectivity of a baculovirus. *J. Virol. 67*, 3730–3738.

Clem, R.J., Miller, L.K., **1994**. Control of programmed cell death by the baculovirus genes *p35* and *iap*. *Mol. Cell. Biol. 14*, 5212–5222.

Clem, R.J., Robson, M., Miller, L.K., **1994**. Influence of infection route on the infectivity of baculovirus mutants lacking the apoptosis-inhibitory gene *p35* and the adjacent gene *p94*. *J. Virol. 68*, 6759–6762.

Cory, J.S., Hails, R.S., Sait, S.M., **1997**. Baculovirus ecology. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 301–339.

Couch, J.A., **1991**. Nuclear polyhedrosis viruses of invertebrates other than insects. In: Adams, J.R., Bonami, J.R. (Eds.), Atlas of Invertebrate Viruses. CRC Press, Boca Raton, FL, pp. 205–226.

Croizier, G., Croizier, L., Argaud, O., Poudevigne, D., **1994**. Extension of A*utographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. *Proc. Natl Acad. Sci. USA 91*, 48–52.

Croizier, G., Ribeiro, H.C.T., **1992**. Recombination as a possible major cause of genetic heterogeneity in *Anticarsia gemmatalis* nuclear polyhedrosis virus populations. *Virus Res. 26*, 183–196.

Dall, D., Luque, T., O'Reilly, D., **2001**. Insect–virus relationships: sifting by informatics. *BioEssays 23*, 184–193.

Derksen, A.C.G., **1988**. Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology 167*, 242–250.

Doyle, C.J., Hirst, M.L., Cory, J.S., Entwistle, P.E., **1990**. Risk assessment studies: detailed host range testing of wild-type cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), nuclear polyhedrosis virus. *Appl. Environ. Microbiol. 56*, 2704–2710.

Dreschers, D., Roncarati, R., Knebel-Morsdorf, D., **2001**. Actin rearrangement-inducing factor of baculoviruses is tyrosine phosphorylated and colocalizes with F-actin at the plasma membrane. *J. Virol. 75*, 3771–3778.

Du, X., Thiem, S.M., **1997**. Responses of insect cells to baculovirus infection: protein synthesis shutdown and apoptosis. *J. Virol. 71*, 7866–7872.

Duffey, S.S., Hoover, K., Bonning, B.C., Hammock, B.D., **1995**. The impact of host plant on the efficacy of baculoviruses. *Rev. Pestic Toxicol. 3*, 137–275.

Duncan, R., Chung, K.L., Faulkner, P., **1983**. Analysis of a mutant of *Autographa californica* nuclear polyhedrosis virus with a defect in the morphogenesis of the occlusion body macromolecular lattice. *J. Gen. Virol. 64*, 1531–1542.

Eldridge, R., Li, Y., Miller, L.K., **1992**. Characterization of a baculovirus gene encoding a small conotoxin-like polypeptide. *J. Virol. 66*, 6563–6571.

Engelhard, E.K., Kam-Morgan, L.N.W., Washburn, J.O., Volkman, L.E., **1994**. The insect tracheal system: a conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. *Proc. Natl Acad. Sci. USA 91*, 3224–3227.

Engelhard, E.K., Volkman, L.E., **1995**. Developmental resistance in fourth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. *Virology 209*, 384–389.

Entwistle, P.F., Evans, H.F., **1985**. Viral control. In: Gilbert, L.I., Kerkut, G.A. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 12. Pergamon, Oxford, pp. 347–412.

Evans, H.F., **1986**. Ecology and epizootiology of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. 2. CRC Press, Boca Raton, FL, pp. 89–132.

Evans, J.T., Leisy, D.J., Rohrmann, G.F., **1997**. Characterization of the interaction between the baculovirus replication factors, LEF-1 and LEF-2. *J. Virol. 71*, 3114–3119.

Faulkner, P., **1981**. Baculovirus. In: Davidson, E.W. (Ed.), Pathogenesis of Invertebrate Microbial Diseases. Allanheld, Osmun, Totowa, NJ, pp. 3–33.

Faulkner, P., Kuzio, J., Williams, G.V., Wilson, J.A., **1997**. Analysis of p74, a PDV envelope protein of *Autographa californica* nucleopolyhedrovirus required for occlusion body infectivity *in vivo*. *J. Gen. Virol. 78*, 3091–3100.

Federici, B.A., **1986**. Ultrastructure of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. 1. CRC Press, Boca Raton, FL, pp. 61–88.

Federici, B.A., **1993**. Viral pathobiology in relation to insect control. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, vol. 2. Academic Press, New York, pp. 81–101.

Federici, B.A., **1997**. Baculovirus pathogenesis. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 33–60.

Flipsen, J.T.M., Lent, J.W.M.V., Goldbach, R.W., Vlak, J.M., **1993**. Expression of polyhedrin and p10 in the midgut of AcMNPV-infected *Spodoptera exigua* larvae: an immunoelectron microscopic investigation. *J. Invertebr. Pathol. 61*, 17–23.

Flipsen, J.T.M., Mans, R.M.W., Kleefsman, A.W., Knebel-Morsdorf, D., Vlak, J.M., **1995a**. Deletion of the baculovirus UDP-glucosyltransferase gene induces early degeneration of Malpighian tubules in infected insects. *J. Virol. 69*, 4529–4532.

Flipsen, J.T.M., Martens, J.W.M., Oers, M.M.V., Vlak, J.M., Lent, J.W.M.V., **1995b**. Passage of *Autographa californica* nuclear polyhedrosis virus through the midgut epithelium of *Spodoptera exigua* larvae. *Virology 208*, 328–335.

Fraser, M.J., **1986**. Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures. *J. Ultrastruct. Mol. Struct. Res. 95*, 189–195.

Fraser, M.J., Cary, L.C., Boonvisudhi, K., Wang, H.G.H., **1995**. Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. *Virology 211*, 397–407.

Fraser, M.J., Smith, G.E., Summers, M.D., **1983**. Acquisition of host cell DNA sequences by baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. *J. Virol. 47*, 287–300.

Friesen, P.D., **1993**. Invertebrate transposable elements in the baculovirus genome: characterization and significance. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects: Parasites, vol. 2. Academic Press, London, pp. 147–178.

Friesen, P.D., **1997**. Regulation of baculovirus early gene expression. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 141–170.

Fuchs, L.Y., Woods, M.S., Weaver, R.F., **1983**. Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *J. Virol. 43*, 641–646.

Funk, C.J., Braunagel, S.C., Rohrmann, G.F., **1997**. Baculovirus structure. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 7–32.

Funk, C.J., Consigli, R.A., **1993**. Phosphate cycling on the basic protein of *Plodia interpunctella* granulosis virus. *Virology 193*, 396–402.

Fuxa, J.R., **1987**. *Spodoptera frugiperda* susceptibility to nuclear polyhedrosis virus isolates with reference to insect migration. *Environ. Entomol. 16*, 218–223.

Fuxa, J.R., **1991**. Release and transport of entomopathogenic microorganisms. In: Levin, M.A., Strauss, H.S. (Eds.), Risk Assessment in Genetic Engineering. McGraw Hill, New York, pp. 83–113.

Fuxa, J.R., **1993**. Insect resistance to viruses. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, vol. 2. Academic Press, New York, pp. 197–209.

Fuxa, J.R., Mitchell, F.L., Richter, A.R., **1988**. Resistance of *Spodoptera frugiperda* (Lep.: Noctuidae) to a nuclear polyhedrosis virus in the field and laboratory. *Entomophaga 33*, 55–63.

Fuxa, J.R., Richter, A.R., **1989**. Reversion of resistance by *Spodoptera frugiperda* to nuclear polyhedrosis virus. *J. Invertebr. Pathol. 53*, 52–56.

Fuxa, J.R., Richter, A.R., **1990**. Response of nuclear polyhedrosis virus-resistant *Spodoptera frugiperda* larvae to other pathogens and to chemical insecticides. *J. Invertebr. Pathol. 55*, 272–277.

Fuxa, J.R., Richter, A.R., **1991**. Selection for an increased rate of vertical transmission of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Environ. Entomol. 20*, 603–609.

Fuxa, J.R., Richter, A.R., **1998**. Repeated reversion of resistance to nucleopolyhedrovirus by *Anticarsia gemmatalis*. *J. Invertebr. Pathol. 71*, 159–164.

Fuxa, J.R., Richter, A.R., Ameen, A.O., Hammock, B.D., **2002**. Vertical transmission of TnSNPV, TnCPV, AcMNPV, and possibly recombinant NPV in *Trichoplusia ni*. *J. Invertebr. Pathol. 79*, 44–50.

Fuxa, J.R., Richter, A.R., Strother, M.S., **1993**. Detection of *Anticarsia gemmatalis* nuclear polyhedrosis virus in predatory arthropods and parasitoids after viral release in Louisiana soybean. *J. Entomol. Sci. 28*, 51–60.

Fuxa, J.R., Tanada, Y., **1987**. Epizootiology of Insect Diseases. Wiley, New York.

Gallo, L.G., Corsaro, B.G., Hughes, P.R., Granados, R.R., **1991**. *In vivo* enhancement of baculovirus infection by the viral enhancing factor of a granulosis virus of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol. 58*, 203–210.

Ghosh, S., Parvez, M.K., Banerjee, K., Sarin, S.K., Hasnain, S.E., **2002**. Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. *Mol. Ther. 6*, 5–11.

Gijzen, M., Roelvink, P., Granados, R., **1995**. Characterization of viral enhancing activity from *Trichoplusia ni* granulosis virus. *J. Invertebr. Pathol. 65*, 289–294.

Gombart, A.F., Pearson, M.N., Rohrmann, G.F., Beaudreau, G.S., **1989**. A baculovirus PE-associated protein: genetic location, nucleotide sequence and immunocytochemical characterization. *Virology 169*, 182–193.

Gomi, S., Majima, K., Maeda, S., **1999**. Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *J. Gen. Virol. 80*, 1323–1337.

Gordon, J.D., **1984**. Phenotypic characterization and physical mapping of a temperature-sensitive mutant of *Autographa californica* nuclear polyhedrosis virus defective in DNA synthesis. *Virology 138*, 69–81.

Granados, R.R., Lawler, K.A., **1981**. *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology 108*, 297–308.

Granados, R.R., Williams, K.A., **1986**. *In vivo* infection and replication of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. 1. CRC Press, Boca Raton, FL, pp. 89–108.

Griffiths, C.M., Barnett, A.L., Ayres, M.D., Windass, J., King, L.A., *et al.*, **1999**. *In vitro* host range of *Autographa californica* nucleopolyhedrovirus recombinants lacking functional *p35*, *iap1* or *iap2*. *J. Gen. Virol. 80*, 1055–1066.

Gröner, A., **1986**. Specificity and safety of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. 1. CRC Press, Boca Raton, FL, pp. 177–202.

Gross, C.H., **1994**. The *Orgyia pseudotsugata* baculovirus p10 and polyhedrin envelope protein genes: analysis of their relative expression levels and role in polyhedron structure. *J. Gen. Virol. 75*, 1115–1123.

Gross, C.H., Rohrmann, G.F., **1993**. Analysis of the role of 5′ promoter elements and 3′ flanking sequences on the expression of a baculovirus polyhedron envelope protein gene. *Virology 92*, 273–281.

Gross, C.H., Shuman, S., **1998**. RNA 5′-triphosphatase, nucleoside triphosphatase, and guanylyltransferase activities of baculovirus LEF-4 protein. *J. Virol. 72*, 10020–10028.

Guarino, L.A., **1990**. Identification of a viral gene encoding a ubiquitin-like protein. *Proc. Natl Acad. Sci. USA 87*, 409–413.

Guarino, L.A., Dong, W., Jin, J., **2002a**. *In vitro* activity of the baculovirus late expression factor LEF-5. *J. Virol. 76*, 12663–12675.

Guarino, L.A., Jin, J., Dong, W., **1998a**. Guanylyltransferase activity of the LEF-4 subunit of baculovirus RNA polymerase. *J. Virol. 72*, 10003–10010.

Guarino, L.A., Mistretta, T., Dong, W., **2002b**. Baculovirus *lef-12* is not required for viral replication. *J. Virol. 76*, 12032–12043.

Guarino, L.A., Smith, G.E., Dong, W., **1995**. Ubiquitin is attached to membranes of baculovirus particles by a novel type of phopholipid anchor. *Cell 80*, 301–309.

Guarino, L.A., Xu, B., Jin, J., Dong, W., **1998b**. A virus-encoded RNA polymerase purified from baculovirus-infected cells. *J. Virol. 72*, 7985–7991.

Haas, A.L., Katzung, D.J., Reback, P.M., Guarino, L.A., **1996**. Functional characterization of the ubiquitin variant encoded by the baculovirus *Autographa californica*. *Biochemistry 35*, 5385–5394.

Haas-Stapleton, E., Washburn, J.O., Volkman, L.E., **2003**. Pathogenesis of *Autographa californica*

M nucleopolyhedrovirus in fifth instar *Spodoptera frugiperda*. *J. Gen. Virol. 84*, 2033–2040.

Hang, X., Dong, W., Guarino, L.A., **1995**. The *lef-3* gene of *Autographa californica* nuclear polyhedrosis virus encodes a single-stranded DNA-binding protein. *J. Virol. 69*, 3924–3928.

Harrison, R.L., Bonning, B.C., **1999**. The nucleopolyhedroviruses of *Rachiplusia ou* and *Anagrapha falcifera* are isolates of the same virus. *J. Gen. Virol. 80*, 2793–2798.

Harrison, R.L., Bonning, B.C., **2003**. Comparative analysis of the genomes of *Rachiplusia ou* and *Autographa californica* multiple nucleopolyhedrovirus. *J. Gen. Virol. 84*, 1827–1842.

Harrison, R.L., Bonning, B.C., **2004**. Application of maximum likelihood models to selection pressure analysis of group I nucleopolyhedrovirus genes. *J. Gen. Virol. 85*, 197–210.

Harrison, R.L., Jarvis, D.L., Summers, M.D., **1996**. The role of the AcMNPV *25K* gene "*FP25*," in baculovirus *polh* and *p10* expression. *Virology 226*, 34–46.

Harrison, R.L., Summers, M.D., **1995**. Mutations in the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus 25 kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. *J. Gen. Virol. 76*, 1451–1459.

Hashimoto, Y., Hayakawa, T., Ueno, Y., Fujita, T., Sano, Y., *et al.*, **2000**. Sequence analysis of the *Plutella xylostella* granulovirus genome. *Virology 275*, 358–372.

Hawtin, R.E., Arnold, K., Ayres, M.D., Zanotto, P.M., Howard, S.C., *et al.*, **1995**. Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology 212*, 673–685.

Hawtin, R.E., Zarkowska, T., Arnold, K., Thomas, C.J., Gooday, G.W., *et al.*, **1997**. Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. *Virology 238*, 243–253.

Hayakawa, T., Ko, R., Okano, K., Seong, S., Goto, C., *et al.*, **1999**. Sequence analysis of the *Xestia c-nigrum* granulovirus genome. *Virology 262*, 277–297.

Hernandez-Crespo, P., Sait, S.M., Hails, R.S., Cory, J.S., **2001**. Behavior of recombinant baculovirus in lepidopteran hosts with different susceptibilities. *Appl. Environ. Microbiol. 67*, 1140–1146.

Herniou, E.A., Luque, T., Chen, X., Vlak, J.M., Winstanley, D., *et al.*, **2001**. Use of whole genome sequence data to infer baculovirus phylogeny. *J. Virol. 75*, 8117–8126.

Herniou, E.A., Olszewski, J.A., Cory, J.S., O'Reilly, D.R., **2003**. The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol. 48*, 211–234.

Herschberger, P.A., Dickson, J.A., Friesen, P.D., **1992**. Site-specific mutagenesis of the 35 kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. *J. Virol. 66*, 5525–5533.

Hill, J.E., Kuzio, J., Wilson, J.A., Mackinnon, E.A., Faulkner, P., **1993**. Nucleotide sequence of the *p74* gene of a baculovirus pathogenic to the spruce budworm *Choristoneura fumiferana* multicapsid nuclear polyhedrosis virus. *Biochim. Biophys. Acta 1172*, 187–189.

Hom, L.G., Ohkawa, T., Trudeau, D., Volkman, L.E., **2002**. *Autographa californica* M nucleopolyhedrovirus ProV-CATH is activated during infected cell death. *Virology 296*, 212–218.

Hom, L.G., Volkman, L.E., **2000**. *Autographa californica* M nucleopolyhedrovirus chiA is required for processing of V-CATH. *Virology 277*, 178–183.

Hong, T., Braunagel, S.C., Summers, M.D., **1994**. Transcription, translation and cellular localization of PDV-E66: a structural protein of the PDV envelope of *Autographa californica* nuclear polyhedrosis virus. *Virology 204*, 210–222.

Hong, T., Summers, M.D., Braunagel, S.C., **1997**. N-terminal sequences from *Autographa californica* nuclear polyhedrosis virus envelope proteins ODV-E66 and ODV-E25 are sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles, and the envelope of occlusion derived virus. *Proc. Natl Acad. Sci. USA 94*, 4050–4055.

Hopkins, T.L., Harper, M.S., **2001**. Lepidopteran peritrophic membranes and effects of dietary wheat germ agglutinin on their formation and structure. *Arch. Insect Biochem. Physiol. 47*, 100–109.

Horton, H.M., Burand, J.P., **1993**. Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. *J. Virol. 67*, 1860–1868.

Hostetter, D.L., Puttler, B., **1991**. A new broad host spectrum nuclear polyhedrosis virus isolated from a celery looper, *Anagrapha falcifera* (Kirby) (Lepidoptera: Noctuidae). *Environ. Entomol. 20*, 1480–1488.

Hughes, A.L., **2002**. Evolution of inhibitors of apoptosis in baculoviruses and their insect hosts. *Infect. Genet. Evol. 2*, 3–10.

Hughes, A.L., Friedman, R., **2003**. Genome-wide survey for genes horizontally transferred from cellular organisms to baculoviruses. *Mol. Biol. Evol. 20*, 979–987.

Hughes, D.S., Possee, R.D., King, L.A., **1993**. Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. *Virology 194*, 608–615.

Hughes, D.S., Possee, R.D., King, L.A., **1997**. Evidence for the presence of a low-level, persistent baculovirus infection of *Mamestra brassicae* insects. *J. Gen. Virol. 78*, 1801–1805.

Hukuhara, T., Hayakawa, T., Wijonarko, A., **1999**. Increased baculovirus susceptibility of armyworm larvae feeding on transgenic rice plants expressing an entomopoxvirus gene. *Nature Biotechnol. 17*, 1122–1124.

Huser, A., Hofmann, C., **2003**. Baculovirus vectors: novel mammalian cell gene-delivery vehicles and their applications. *Am. J. Pharmacogenomics 3*, 53–63.

Hyink, O., Dellow, R.A., Olsen, M.J., Caradoc-Davies, K.M.B., Drake, K., *et al.*, **2002**. Whole genome analysis of the *Epiphyas postvittana* nucleopolyhedrovirus. *J. Gen. Virol. 83*, 957–971.

Ignoffo, C.M., Garcia, A., **1994**. Antioxidant and oxidant enzyme effects on the inactivation of inclusion bodies of the *Heliothis* baculovirus by simulated sunlight UV. *Environ. Entomol. 23*, 1025–1029.

Ignoffo, C.M., Garcia, C., Saathoff, S.G., **1997**. Sunlight stability and rain-fastness of formulations of *Baculovirus heliothis*. *Environ. Entomol. 26*, 1470–1474.

Ignoffo, G.M., Garcia, C., Zuidema, D., Vlak, J.M., **1995**. Relative *in vivo* activity and simulated sunlight-UV stability of inclusion bodies of a wild-type and an engineered polyhedra envelope-negative isolate of the nucleopolyhedrosis virus of *Autographa californica*. *J. Invertebr. Pathol. 66*, 212–213.

Ijkel, W.F.J., Strien, E.A. v., Heldens, J.G.M., Broer, R., Zuidema, D., *et al.*, **1999**. Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *J. Gen. Virol. 80*, 3289–3304.

Imai, N., Matsuda, N., Tanaka, K., Nakano, A., Matsumoto, S., *et al.*, **2003**. Ubiquitin ligase activities of *Bombyx mori* nucleopolyhedrovirus RING finger proteins. *J. Virol. 77*, 923–930.

Jaques, R.P., **1975**. Persistence, accumulation and denaturation of nuclear polyhedrosis and granulosis viruses. In: Summers, M.D., Engler, R., Falcon, L.A., Vail, P.V. (Eds.), Baculoviruses for Insect Pest Control: Safety Considerations. American Society of Microbiology, Washington DC, pp. 55–67.

Jaques, R.P., **1985**. Stability of insect viruses in the environment. In: Maramorosch, K., Sherman, K.E. (Eds.), Viral Insecticides for Biological Control. Academic Press, Orlando, FL, pp. 289–360.

Jarvis, D.L., **1997**. Baculovirus expression vectors. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 389–431.

Jarvis, D.L., Bohlmeyer, D.A., Garcia, A., **1991**. Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein. *Virology 185*, 795–810.

Jarvis, D.L., Bohlmeyer, D.A., Garcia, J.A., **1992**. Enhancement of polyhedrin nuclear localization during baculovirus infection. *J. Virol. 66*, 6903–6911.

Jarvis, D.L., Fleming, J.D.W., Kovacs, G.R., Summers, M.D., Guarino, L.A., **1990**. Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Bio/Technol. 8*, 950–955.

Jarvis, D.L., Garcia, A., **1994**. Biosynthesis and processing of the *Autographa californica* nuclear polyhedrosis virus gp64 protein. *Virology 205*, 300–313.

Jehle, J.A., **1996**. Transmission of insect transposons in baculovirus genomes: an unusual host–pathogen interaction. In: Tomiuk, J., Wohrmann, K., Sentker, A. (Eds.), Transgenic Organisms: Biological and Social Implications. Birlhauser Verlag, Basel, pp. 81–97.

Jehle, J.A., Fritsch, E.F., Nickel, A., Huber, J., Backhaus, H., **1995**. TC14.7: a novel lepidopteran transposon found in *Cydia pomonella* granulosis virus. *Virology 207*, 369–379.

Jehle, J.A., Linden, I.F. van der, Backhaus, H., Vlak, J.M., **1997**. Identification and sequence analysis of the integration site of transposon TCp3.2 in the genome of *Cydia pomonella* granulovirus. *Virus Res. 50*, 151–157.

Jehle, J.A., Nickel, A., Vlak, J.M., Backhaus, H., **1998**. Horizontal escape of the novel Tc10-like lepidopteran transposon TCp3.2 into *Cydia pomonella* granulovirus. *J. Mol. Evol. 46*, 215–224.

Jin, J., Dong, W., Guarino, L.A., **1998**. The LEF-4 subunit of baculovirus RNA polymerase has RNA 5′-triphosphatase and ATPase activities. *J. Virol. 72*, 10011–10019.

Kamita, S.G., Maeda, S., **1997**. Sequencing of the putative DNA helicase-encoding gene of the *Bombyx mori* nuclear polyhedrosis virus and fine-mapping of a region involved in host range expansion. *Gene 190*, 173–179.

Kamita, S.G., Majima, K., Maeda, S., **1993**. Identification and characterization of the *p35* gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis. *J. Virol. 67*, 455–463.

Kang, W., Tristem, M.N., Maeda, S., Crook, N.E., O'Reilly, D.R., **1998**. Identification and characterization of the *Cydia pomonella* granulovirus cathepsin and chitinase genes. *J. Gen. Virol. 79*, 2283–2292.

Kang, W.K., Imai, N., Iwanaga, M., Matsumoto, S., Zemskov, E.A., **2003**. Interaction of *Bombyx mori nucleopolyhedrovirus* BRO-A and host cell protein laminin. *Arch. Virol. 148*, 99–113.

Kaomini, M., Roush, R.T., **1988**. Absence of response to selection for resistance to nucleopolyhedrosis virus in *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). *J. Entomol. Sci. 23*, 379–382.

Katsuma, S., Noguchi, Y., Zhou, C.L.E., Kobayashi, M., Maeda, S., **1999**. Characterization of the 25K FP gene of the baculovirus *Bombyx mori* nucleopolyhedrovirus: implications for post-mortem host degradation. *J. Gen. Virol. 80*, 783–791.

Kawanishi, C.Y., Paschke, J.D., **1970**. The relationship of buffer pH and ionic strength on the yield of virions and nucleocapsids obtained by the dissolution of *Rachiplusia ou* nuclear polyhedra. *4th Int. Colloqu. Insect Pathol.* 127–146.

Keddie, B.A., Aponte, G.W., Volkman, L.E., **1989**. The pathway of infection of *Autographa californica* nuclear polyhedrosis virus in an insect host. *Science 243*, 1728–1730.

Kelly, T.J., Park, E.J., Masler, C.A., Burand, J.P., **1995**. Characterization of the glycosylated ecdysteroids in the hemolymph of baculovirus-infected gypsy moth larvae and cells in culture. *Eur. J. Entomol. 92*, 51–61.

Knebel-Mörsdorf, D., Flipsen, J.T.M., Roncarati, R., Jahnel, F., Kleefsman, A.W.F., *et al.*, **1996**. Baculovirus infection of *Spodoptera exigua* larvae: *lacZ* expression

driven by promoters of early genes *pe38* and *me53* in larval tissue. *J. Gen. Virol. 77*, 815–824.

Knudson, D.L., Harrap, K.A., **1976**. Replication of a nuclear polyhedrosis virus in a continuous cell culture of *Spodoptera frugiperda*: microscopy study of the sequence of events of the virus infection. *J. Virol. 17*, 254–268.

Kolodny Hirsch, D.M., van Beek, N.A.M., **1997**. Selection of a morphological variant of *Autographa californica* nuclear polyhedrosis virus with increased virulence following serial passage in *Plutella xylostella*. *J. Invertebr. Pathol. 69*, 205–211.

Kool, M., Ahrens, C., Goldbach, R.W., Rohrmann, G.F., Vlak, J.M., **1994a**. Identification of genes involved in DNA replication of the *Autographa californica* nuclear polyhedrosis virus. *Proc. Natl Acad. Sci. USA 91*, 11212–11216.

Kool, M., Ahrens, C.H., Vlak, J.M., Rohrmann, G.F., **1995**. Replication of baculovirus DNA. *J. Gen. Virol. 76*, 2103–2118.

Kool, M., Goldbach, R.W., Vlak, J.M., **1993a**. A putative non-hr origin of DNA replication of the *Hin*dIII-K fragment of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol. 75*, 3345–3352.

Kool, M., van den Berg, P.M., Tramper, J., Goldbach, R.W., Vlak, J.M., **1993b**. Location of two putative origins of DNA replication of *Autographa californica* nuclear polyhedrosis virus. *Virology 192*, 94–101.

Kool, M., Voeten, J.T.M., Vlak, J.M., **1994b**. Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis viral genome required for DNA replication. *Virology 198*, 680–689.

Kost, T.A., Condreay, J.P., **2002**. Recombinant baculoviruses as mammalian cell gene-delivery vectors. *Trends Biotechnol. 20*, 173–180.

Kovacs, G.R., Choi, J., Guarino, L.A., Summers, M.D., **1991**. Identification of spliced baculovirus RNAs expressed late in infection. *Virology 185*, 633–643.

Kukan, B., **1999**. Vertical transmission of nucleopolyhedrovirus in insects. *J. Invertebr. Pathol. 74*, 103–111.

Kuzio, J., Jaques, R., Faulkner, P., **1989**. Identification of *p74*, a gene essential for virulence of baculovirus occlusion bodies. *Virology 173*, 759–763.

Kuzio, J., Pearson, M.N., Harwood, S.H., Funk, C.J., Evans, J.T., *et al.*, **1999**. Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology 253*, 17–34.

Lanier, L.M., Slack, J.M., Volkman, L.E., **1996**. Actin binding and proteolysis by the baculovirus AcMNPV: the role of virion-associated V-CATH. *Virology 216*, 380–388.

Lanier, L.M., Volkman, L.E., **1998**. Actin binding and nucleation by *Autographa californica* M nucleopolyhedrovirus. *Virology 243*, 167–177.

Lee, H.H., Miller, L.K., **1978**. Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 27*, 754–767.

Leisy, D.J., Rasmussen, C., Kim, H.-T., Rohrmann, G.F., **1995**. The *Autographa californica* nuclear polyhedrosis virus homologous region 1a: identical sequences are essential for DNA replication activity and transcriptional enhancer function. *Virology 208*, 742–752.

Leisy, D.J., Rohrmann, G.F., **1993**. Characterization of the replication of plasmids containing hr sequences in baculovirus-infected *Spodoptera frugiperda* cells. *Virology 196*, 722–730.

Lepore, L.S., Roelvink, P.R., Granados, R.R., **1996**. Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. *J. Invertebr. Pathol. 68*, 131–140.

Lewis, L.C., Johnson, T.B., **1982**. Efficacy of two nuclear polyhedrosis viruses against *Ostrinia nubilalis* [Lep.: Pyralidae] in the laboratory and field. *Entomophaga 27*, 33–38.

Li, L., Donly, C., Li, Q., Willis, L.G., Keddie, B.A., *et al.*, **2002a**. Identification and genomic analysis of a second species of nucleopolyhedrovirus isolated from *Mamestra configurata*. *Virology 297*, 226–244.

Li, L., Harwood, S.H., Rohrmann, G.F., **1999**. Identification of additional genes that influence baculovirus late gene expression. *Virology 255*, 9–19.

Li, Q., Donly, C., Li, L., Willis, L.G., Theilmann, D.A., *et al.*, **2002b**. Sequence and organization of the *Mamestra configurata* nucleopolyhedrovirus genome. *Virology 294*, 106–121.

Li, Y., Miller, L.K., **1995**. Properties of a baculovirus mutant defective in the protein phosphatase gene. *J. Virol. 69*, 4533–4537.

Lin, G., Blissard, G.W., **2002a**. Analysis of an *Autographa californica* multicapsid nucleopolyhedrovirus *lef-6*-null virus: LEF-6 is not essential for viral replication but appears to accelerate late gene transcription. *J. Virol. 76*, 5503–5514.

Lin, G., Blissard, G.W., **2002b**. Analysis of an *Autographa californica* nucleopolyhedrovirus *lef-11* knockout: LEF-11 is essential for viral DNA replication. *J. Virol. 76*, 2770–2779.

Lin, G., Slack, J.M., Blissard, G.W., **2001**. Expression and localization of LEF-11 in *Autographa californica* nucleopolyhedrovirus infected Sf9 cells. *J. Gen. Virol. 82*, 2289–2294.

Liu, J.J., Carstens, E.B., **1993**. Infection of *Spodoptera frugiperda* and *Choristoneura fumiferana* cell lines with the baculovirus *Choristoneura fumiferana* nuclear polyhedrosis virus. *Can. J. Microbiol. 39*, 932–940.

Liu, J.J., Carstens, E.B., **1995**. Identification, localization, transcription, and sequence analysis of the *Choristoneura fumiferana* nuclear polyhedrosis virus DNA polymerase gene. *Virology 209*, 538–549.

Lu, A., Carstens, E.B., **1991**. Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. *Virology 181*, 336–347.

Lu, A., Carstens, E.B., **1992**. Nucleotide sequence and transcriptional analysis of the *p80* gene of *Autographa californica* nuclear polyhedrosis virus: a homologue of the *Orgyia pseudotsugata* nuclear polyhedrosis virus capsid-associated gene. *Virology 190*, 201–209.

Lu, A., Carstens, E.B., **1993**. Immediate early baculovirus genes transactivate the *p143* gene promoter of *Autographa californica* nuclear polyhedrosis virus. *Virology 195*, 710–718.

Lu, A., Miller, L.K., **1995a**. Differential requirements for baculovirus late expression factor genes in two cell lines. *J. Virol. 69*, 6265–6272.

Lu, A., Miller, L.K., **1995b**. The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. *J. Virol. 69*, 975–982.

Lu, A., Miller, L.K., **1996**. Species-specific effects of the *hcf-1* gene on baculovirus virulence. *J. Virol. 70*, 5123–5130.

Lu, A., Miller, L.K., **1997**. Regulation of baculovirus late and very late gene expression. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 217–235.

Lu, L., Du, Q., Chejanovsky, N., **2003**. Reduced expression of the immediate-early protein IE0 enables efficient replication of *Autographa californica* multiple nucleopolyhedrovirus in poorly permissive *Spodoptera littoralis* cells. *J. Virol. 77*, 535–545.

Lung, O.Y., Cruz-Alvarez, M., Blissard, G.W., **2003**. Ac23, an envelope fusion protein homolog in the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. *J. Virol. 77*, 328–339.

Luque, T., Finch, R., Crook, N., O'Reilly, D.R., Winstanley, D., **2001**. The complete sequence of the *Cydia pomonella* granulovirus genome. *J. Gen. Virol. 82*, 2531–2547.

Maeda, S., Kamita, S.G., Kondo, A., **1993**. Host range expansion of *Autographa californica* nuclear polyhedrosis virus (NPV) following recombination of a 0.6-kilobase-pair DNA fragment originating from *Bombyx mori* NPV. *J. Virol. 67*, 6234–6238.

Majima, K., Kobara, R., Maeda, S., **1993**. Divergence and evolution of homologous regions of *Bombyx mori* nuclear polyhedrosis virus. *J. Virol. 67*, 7513–7521.

Malik, H.S., Henikoff, S., Eickbush, T.H., **2000**. Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res. 10*, 1307–1318.

Manji, G.A., Friesen, P.D., **2001**. Apoptosis in motion: an apical, P35-insensitive caspase mediates programmed cell death in insect cells. *J. Biol. Chem. 276*, 16704–16710.

Martignoni, M.E., Iwai, P.J., **1986**. Propagation of multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* in larvae of *Trichoplusia ni. J. Invertebr. Pathol. 47*, 32–41.

Mazzacano, C.A., Du, X., Thiem, S.M., **1999**. Global protein synthesis shutdown in *Autographa californica* nucleopolyhedrovirus-infected Ld652Y cells is rescued by tRNA from uninfected cells. *Virology 260*, 222–231.

McLachlin, J.R., Miller, L.K., **1994**. Identification and characterization of *vlf-1*, a baculovirus gene involved in very late gene expression. *J. Virol. 68*, 7746–7756.

Means, J.C., Muro, I., Clem, R.J., **2003**. Silencing of the baculovirus Op-*iap3* gene by RNA interference reveals that it is required for prevention of apoptosis during *Orgyia pseudotsugata* M nucleopolyhedrovirus infection of Ld652Y cells. *J. Virol. 77*, 4481–4488.

Mikhailov, V.S., **2003**. Replication of the baculovirus genome. *Mol. Biol. 37*, 250–259.

Milks, M.L., Myers, J.H., **2000**. The development of larval resistance to a nucleopolyhedrovirus in not accompanied by an increased virulence in the virus. *Evol. Ecol. 14*, 645–664.

Milks, M.L., Myers, J.H., Leptich, M.K., **2002**. Costs and stability of cabbage looper resistance to a nucleopolyhedrovirus. *Evol. Ecol. 16*, 369–385.

Miller, D.W., Miller, L.K., **1982**. A virus mutant with an insertion of a *copia*-like transposable element. *Nature 299*, 562–564.

Miller, L.K., Adang, M.J., Browne, D., **1983**. Protein kinase activity associated with the extracellular and occluded forms of the baculovirus *Autographa californica* NPV. *J. Virol. 46*, 275–278.

Miller, L.K., Lu, A., **1997**. The molecular basis of baculovirus host range. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 217–235.

Minion, F.C., Coons, L.B., Broome, J.R., **1979**. Characterization of the PE of the nuclear polyhedrosis virus of *Heliothis virescens*. *J. Invertebr. Pathol. 34*, 303–307.

Mitsuhashi, W., Furuta, Y., Sato, M., **1998**. The spindles of an entomopoxvirus of Coleoptera (*Anomala cuprea*) strongly enhance the infectivity of a nucleopolyhedrovirus in Lepidoptera (*Bombyx mori*). *J. Invertebr. Pathol. 71*, 186–188.

Monsma, S.A., Blissard, G.W., **1995**. Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. *J. Virol. 69*, 2583–2595.

Monsma, S.A., Oomens, A.G.P., Blissard, G.W., **1996**. The gp64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J. Virol. 70*, 4607–4616.

Morris, T.D., Miller, L.K., **1992**. Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines. *J. Virol. 66*, 7397–7405.

Morris, T.D., Miller, L.K., **1993**. Characterization of productive and non-productive AcMNPV infection in selected insect cell lines. *Virology 197*, 339–348.

Morse, M.A., Marriott, A.C., Nuttall, P.A., **1992**. The glycoprotein of Thogoto virus (a tick-borne orthomyxo-like virus) is related to the baculovirus glycoprotein gp64. *Virology 186*, 640–646.

Moscardi, F., **1999**. Assessment of the application of baculoviruses for control of Lepidoptera. *Annu. Rev. Entomol. 44*, 257–289.

Moser, B.A., Becnel, J.J., White, S.E., Afonso, C., Kutish, G., *et al.*, **2001**. Morphological and molecular evidence that *Culex nigripalpus* baculovirus is an unusual member of the family Baculoviridae. *J. Gen. Virol. 82*, 283–297.

Oers, M.M.V., Vlak, J.M., **1997**. The baculovirus 10-kDa protein. *J. Invertebr. Pathol. 70*, 1–17.

Ohkawa, T., Majima, K., Maeda, S., **1994**. A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. *J. Virol. 68*, 6619–6625.

Ohkawa, T., Rowe, A.R., Volkman, L.E., **2002**. Identification of six *Autographa californica* multicapsid nucleopolyhedrovirus early genes that mediate nuclear localization of G-actin. *J. Virol. 76*, 12281–12289.

Ohkawa, T., Volkman, L.E., **1999**. Nuclear F-actin is required for AcMNPV nucleocapsid morphogenesis. *Virology 264*, 1–4.

Ohresser, M., Morin, N., Cerruti, M., Delsert, C., **1994**. Temporal regulation of a complex and unconventional promoter by viral products. *J. Virol. 68*, 2589–2597.

Okano, K., Shimada, T., Mita, K., Maeda, S., **2001**. Comparative expressed-sequence-tag analysis of differential gene expression profiles in BmNPV-infected BmN cells. *Virology 282*, 348–356.

Olszewski, J., Miller, L.K., **1997a**. Identification and characterization of a baculovirus structural protein, VP1054, required for nucleocapsid formation. *J. Virol. 71*, 5040–5050.

Olszewski, J., Miller, L.K., **1997b**. A role for baculovirus GP41 in budded virus production. *Virology 233*, 292–301.

Ooi, B.G., Miller, L.K., **1988**. Regulation of host RNA levels during baculovirus infection. *Virology 166*, 515–523.

Ooi, B.G., Miller, L.K., **1990**. Transcription of the baculovirus polyhedrin gene reduces the levels of an antisense transcript initiated downstream. *J. Virol. 64*, 3126–3129.

Ooi, B.G., Rankin, C., Miller, L.K., **1989**. Downstream sequence augments transcription from the essential initiation site of a baculovirus polyhedrin gene. *J. Mol. Biol. 210*, 721–736.

Oppenheimer, D.I., Volkman, L.E., **1997**. Evidence for rolling circle replication of *Autographa californica* M nucleopolyhedrovirus genomic DNA. *Arch. Virol. 142*, 2107–2113.

O'Reilly, D., **1995**. Baculovirus-encoded ecdysteroid UDP-glucosyltransferases. *Insect Biochem. Mol. Biol. 25*, 541–550.

O'Reilly, D., **1997**. Auxiliary genes. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 267–300.

O'Reilly, D.R., Brown, M.R., Miller, L.K., **1992**. Alteration of ecdysteroid metabolism due to baculovirus infection of the fall armyworm *Spodoptera frugiperda*: host ecdysteroids are conjugated with galactose. *Insect Biochem. Mol. Biol. 22*, 313–320.

O'Reilly, D.R., Kelly, T.J., Masler, E.P., Thyagaraja, B.S., Rohson, R.M., *et al.*, **1995**. Overexpression of *Bombyx mori* prothoracicotropic hormone using baculovirus vectors. *Insect Biochem. Mol. Biol. 25*, 475–485.

O'Reilly, D.R., Miller, L.K., **1989**. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. *Science 245*, 1110–1112.

O'Reilly, D.R., Miller, L.K., **1991**. Improvement of a baculovirus pesticide by deletion of the EGT gene. *Bio/Technol. 9*, 1086–1089.

Palli, S.R., Caputo, G.F., Sohi, S.S., Brownright, A.J., Ladd, T.R., *et al.*, **1996**. CfMNPV blocks AcMNPV-induced apoptosis in a continuous midgut cell line. *Virology 222*, 201–213.

Pang, Y., Yu, J., Wang, L., Hu, X., Bao, W., *et al.*, **2001**. Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. *Virology 287*, 391–404.

Passarelli, A.L., Miller, L.K., **1993**. Identification and characterization of *lef-1*, a baculovirus gene involved in late and very late gene expression. *J. Virol. 67*, 3481–3488.

Payne, C.C., **1986**. Insect pathogenic viruses as pest control agents. In: Franz, J.M. (Ed.), Biological Plant and Health Protection. Georg Fischer Verlag, Stuttgart, pp. 183–200.

Pearson, M.N., Bjornson, R.M., Ahrens, C.H., Rohrmann, G.F., **1993**. Identification and characterization of a putative origin of DNA replication in the genome of a baculovirus pathogenic for *Orgyia pseudotsugata*. *Virology 197*, 715–725.

Pearson, M.N., Bjornson, R.M., Pearson, G., Rohrmann, G.F., **1992**. The *Autographa californica* baculovirus genome: evidence for multiple replication origins. *Science 257*, 1382–1384.

Pearson, M.N., Groten, C., Rohrmann, G.F., **2000**. Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae. *J. Virol. 74*, 6126–6131.

Pearson, M.N., Rohrmann, G.F., **2002**. Transfer, incorporation, and substitution of envelope fusion proteins among members of the *Baculoviridae*, *Orthomyxoviridae*, and *Metaviridae* (insect retrovirus) families. *J. Virol. 76*, 5301–5304.

Pearson, M.N., Russell, R., Rohrmann, G.F., **2001**. Characterization of a baculovirus encoded protein that is associated with infected-cell membranes and budded virus. *Virology 291*, 22–31.

Pearson, M.N., Russell, R.L.Q., Rohrmann, G.F., Beaudreau, G.S., **1988**. P39, a major baculovirus structural protein: immunocytochemical characterization and genetic location. *Virology 167*, 407–413.

Petrik, D.T., Iseli, A., Montelone, B.A., Van Etten, J.L., Clem, R.J., **2003**. Improving baculovirus resistance to UV inactivation: increased virulence resulting from expression of a DNA repair enzyme. *J. Invertebr. Pathol. 82*, 50–56.

Popham, H.J.R., Bishop, D.H.L., Slavicek, J.M., **2001**. Both *Lymantria dispar* nucleopolyhedrovirus enhancin genes contribute to viral potency. *J. Virol. 75*, 8639–8648.

Popham, H.J.R., Pellock, B.J., Robson, M., Dierks, P.M., Miller, L.K., **1998**. Characterization of a variant of *Autographa californica* nuclear polyhedrosis virus with a nonfunctional ORF 603. *Biol. Control 12*, 223–230.

Possee, R.D., Rohrmann, G.F., **1997**. Baculovirus genome organization and evolution. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 109–140.

Poulin, R., Combes, C., **1999**. The concept of virulence: interpretations and implications. *Parasitol. Today 15*, 474–475.

Prikhod'ko, E.A., Miller, L.K., **1996**. Induction of apoptosis by baculovirus transactivator IE1. *J. Virol. 70*, 7116–7124.

Quadt, I., Mainz, D., Mans, R., Kremer, A., Knebel-Morsdorf, D., **2002**. Baculovirus infection raises the level of TATA-binding protein that colocalizes with viral replication sites. *J. Virol. 76*, 11123–11127.

Rapp, J.C., Wilson, J.A., Miller, L.K., **1998**. Nineteen baculovirus open reading frames, including *LEF-12*, support late gene expression. *J. Virol. 72*, 10197–10206.

Reddy, J.T., Locke, M., **1990**. The size limited penetration of gold particles through insect basal laminae. *J. Insect Physiol. 36*, 397–407.

Reilly, L.M., Guarino, L.A., **1996**. The viral ubiquitin gene of *Autographa californica* nuclear polyhedrosis virus is not essential for viral replication. *Virology 218*, 243–247.

Ribeiro, B.M., Hutchinson, K., Miller, L.K., **1994**. A mutant baculovirus with a temperature-sensitive IE-1 transregulatory protein. *J. Virol. 68*, 1075–1084.

Richards, A.G., Richards, P.A., **1977**. The peritrophic membrane of insects. *Annu. Rev. Entomol. 22*, 219–240.

Rodems, S.M., Friesen, P.D., **1993**. The hr5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. *J. Virol. 67*, 5776–5785.

Roelvink, P.W., Van Meer, M.M., De Kort, C.A.D., Possee, R.D., Hammock, B.D., *et al.*, **1992**. Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *J. Gen. Virol. 73*, 1481–1489.

Rohel, D.Z., Faulkner, P., **1984**. Time course analysis and mapping of *Autographa californica* nuclear polyhedrosis virus transcripts. *J. Virol. 50*, 739–747.

Rohrmann, G.F., **1986**. Polyhedrin structure. *J. Gen. Virol. 67*, 1499–1513.

Rohrmann, G.F., **1992**. Baculovirus structural proteins. *J. Gen. Virol. 73*, 749–761.

Rohrmann, G.F., **1996**. Evolution of occluded baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. 1. CRC Press, Boca Raton, FL, pp. 203–215.

Rohrmann, G.F., Karplus, P.A., **2001**. Relatedness of baculovirus and gypsy retrotransposon envelope proteins. *BMC Evol. Biol. 1*, 1–9.

Rosas-Acosta, G., Braunagel, S.C., Summers, M.D., **2001**. Effects of deletion and overexpression of the *Autographa californica* nuclear polyhedrosis virus FP25K gene on synthesis of two occlusion-derived virus envelope proteins and their transport into virus-induced intranuclear membranes. *J. Virol. 75*, 10829–10842.

Rothman, L.D., Myers, J.H., **1996a**. Debilitating effects of viral diseases on host Lepidoptera. *J. Invertebr. Pathol. 67*, 1–10.

Rothman, L.D., Myers, J.H., **1996b**. Is fecundity correlated with resistance to viral disease in the western tent caterpillar? *Ecol. Entomol. 21*, 396–398.

Ruberson, J.R., Young, S.Y., Kring, T.J., **1991**. Suitability of prey infected by nuclear polyhedrosis virus for development, survival, and reproduction of the predator *Nabis roseipennis* (Heteroptera: Nabidae). *Environ. Entomol. 20*, 1475–1479.

Russell, R.L.Q., Pearson, M.N., Rohrmann, G.F., **1991**. Immunoelectron microscopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major polyhedron-associated proteins. *J. Gen. Virol. 72*, 275–283.

Russell, R.L.Q., Rohrmann, G.F., **1993**. A 25 kilodalton protein is associated with the envelopes of occluded baculovirus virions. *Virology 195*, 532–540.

Russell, R.L.Q., Rohrmann, G.F., **1997**. Characterization of P91, a protein associated with virions of an *Orgyia pseudotsugata* baculovirus. *Virology 233*, 210–233.

Sait, S.M., Begon, M., Thompson, D.J., Harvey, J.A., **1996**. Parasitism of baculovirus-infected *Plodia interpunctella* by *Venturia canescens* and subsequent virus transmission. *Funct. Ecol. 10*, 586–591.

Saville, G.P., Thomas, C.J., Possee, R.D., King, L.A., **2002**. Partial redistribution of the *Autographa californica* nucleopolyhedrovirus chitinase in virus-infected cells accompanies mutation of the carboxy-terminal KDEL ER-retention motif. *J. Gen. Virol. 83*, 685–694.

Shapiro, M., Ignoffo, C.M., **1970**. Nucleopolyhedrosis of *Heliothis*: activity of isolates from *Heliothis zea*. *J. Invertebr. Pathol. 16*, 107–111.

Shapiro, M., Martignoni, M.E., Cunningham, J.C., Goodwin, R.H., **1982**. Potential use of the saltmarsh caterpillar as a production host for nucleoployhedrosis viruses. *J. Econ. Entomol. 75*, 69–71.

Skinner, W.S., Dennis, P.A., Li, J.P., Quistad, G.B., **1992**. Identification of insecticidal peptides from venom of the trap-door spider, *Aptostichus ichlingeri* (Ctenizidae). *Toxicon 30*, 1043–1050.

Slack, J.M., Dougherty, E.M., Lawrence, S.D., **2001**. A study of the *Autographa californica* multiple nucleopolyhedrovirus ODV envelope protein p74 using a GFP tag. *J. Gen. Virol. 82*, 2279–2287.

Slack, J.M., Kuzio, J., Faulkner, P., **1995**. Characterization of *v-cath*, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *J. Gen. Virol. 76*, 1091–1098.

Smith, G.E., Summers, M.D., **1979**. Restriction maps of five *Autographa californica* MNPV variants, *Trichoplusia ni* MNPV, and *Galleria mellonella* MNPV DNAs with endonucleases *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Xho*I, and *Eco*RI. *J. Virol. 30*, 828–838.

Smith, G.E., Summers, M.D., **1980**. Restriction map of *Rachiplusia ou* and *Rachiplusia ou–Autographa californica* baculovirus recombinants. *J. Gen. Virol. 33*, 311–319.

Smits, P.H., Vlak, J.M., **1988**. Biological activity of *Spodoptera exigua* nuclear polyhedrosis virus against *S. exigua* larvae. *J. Invertebr. Pathol. 51*, 107–114.

Stairs, G.R., Fraser, T., Fraser, M., **1981**. Changes in growth and virulence of a nuclear polyhedrosis virus

from *Choristoneura fumiferana* after passage in *Trichoplusia ni* and *Galleria mellonella*. *J. Invertebr. Pathol. 38*, 230–235.

Stoltz, D.B., Pavan, C., DaCunha, A., **1973**. Nuclear polyhedrosis virus: a possible example of *de novo* intranuclear membrane morphogenesis. *J. Gen. Virol. 19*, 145–150.

Subrahmanyam, B., Ramakrishnan, N., **1980**. The alteration of juvenile hormone titer in the haemolymph of *Spodoptera litura* (F.) due to baculovirus infection. *Experientia 36*, 471–472.

Teakle, R.E., Jensen, J.M., Giles, J.E., **1986**. Age-related susceptibility of *Heliothis punctiger* to a commercial formulation of nuclear polyhedrosis disease. *J. Invertebr. Pathol. 47*, 82–92.

Tellam, R.L., **1996**. The peritrophic membrane. In: Lehane, M.J., Billingsley, B.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, New York, pp. 86–114.

Theilmann, D.A., Chantler, J.K., Stewart, S., Flipsen, H.T.M., Vlak, J.M., *et al.*, **1996**. Characterization of a highly conserved baculovirus structural protein that is specific for occlusion-derived virions. *Virology 218*, 148–158.

Theilmann, D.A., Stewart, S., **1993**. Analysis of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus trans-activators IE-1 and IE-2 using monoclonal antibodies. *J. Gen. Virol. 74*, 1819–1826.

Thiem, S.M., Miller, L.K., **1989**. Identification, sequence, and transcriptional mapping of the major capsid protein gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 63*, 2008–2018.

Thomas, C.J., Brown, H.L., Hawes, C.R., Lee, B.Y., Min, M.K., *et al.*, **1998**. Localization of a baculovirus-induced chitinase in the insect cell endoplasmic reticulum. *J. Virol. 72*, 10207–10212.

Thomas, C.J., Gooday, G.W., King, L.A., Possee, R.D., **2000**. Mutagenesis of the active coding region of the *Autographa californica* nucleopolyhedrovirus *chiA* gene. *J. Gen. Virol. 81*, 1403–1411.

Todd, J.W., Passarelli, A.L., Lu, A., Miller, L.K., **1996**. Factors regulating baculovirus late and very late gene expression in transient-expression assays. *J. Virol. 70*, 2307–2317.

Todd, J.W., Passarelli, A.L., Miller, L.K., **1995**. Eighteen baculovirus genes, including *lef-11*, *p35*, 39K and *p47*, support late gene expression. *J. Virol. 69*, 968–974.

Tomalski, M.D., Eldridge, R., Miller, L.K., **1991**. A baculovirus homolog of a Cu/Zn superoxide dismutase gene. *Virology 184*, 149–161.

Tompkins, G.J., Dougherty, E.M., Adams, J.R., Diggs, D., **1988**. Changes in the virulence of nuclear polyhedrosis virus when propagated in altenative noctuid (Lepidoptera: Noctuidae) cell lines and hosts. *J. Econ. Entomol. 81*, 1027–1032.

Tompkins, G.J., Vaughn, J.L., Adams, J.R., Reichelderfer, C.F., **1981**. Effects of propagating *Autographa californica* nuclear polyhedrosis virus and its *Trichoplusia ni* variant in different hosts. *Environ. Entomol. 10*, 801–806.

Trudeau, D., Washburn, J.O., Volkman, L.E., **2001**. Central role of hemocytes in *Autographa californica* M nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*. *J. Gen. Virol. 75*, 996–1003.

Tweeten, K.A., Bulla, L.A., Consigli, R.A., **1980**. Characterization of an extremely basic protein derived from granosis virus nucleocapsids. *J. Virol. 33*, 866–876.

Vail, P.V., Hoffmann, D.F., Streett, D.A., Manning, J.S., Tebbets, J.S., **1993**. Infectivity of a nuclear polyhedrosis virus isolated from *Anagrapha falcifera* (Lepidoptera: Noctuidae) against production and postharvest pests and homologous cell lines. *Environ. Entomol. 22*, 1140–1145.

van Beek, N.A.M., Hughes, P.R., **1998**. The response time of insect larvae infected with recombinant baculoviruses. *J. Invertebr. Pathol. 72*, 338–347.

van Beek, N.A.M., Wood, H.A., Hughes, P.R., **1988**. Quantitative aspects of nuclear polyhedrosis virus infections in lepidopterous larvae: the dose-survival time relationship. *J. Invertebr. Pathol. 51*, 58–63.

van Lent, J.W.M., Goenen, J.T.M., Klinge-Roode, E.C., Rohrmann, G.F., Zuidema, D., *et al.*, **1990**. Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. *Arch. Virol. 111*, 103–114.

van Loo, N.-D., Fortunati, E., Ehlert, E., Rabelink, M., Grosveld, F., *et al.*, **2001**. Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids. *J. Virol. 75*, 961–970.

van Oers, M.M., Flipsen, J.T.M., Reusken, C.B.E.M., Sliwinsky, E.L., Goldbach, R.W., *et al.*, **1993**. Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol. 74*, 563–574.

Vasconcelos, S.D., Williams, T., Hails, R.S., Cory, J.S., **1996**. Prey selection and baculovirus dissemination by carabid predators of Lepidoptera. *Ecol. Entomol. 21*, 98–104.

Vialard, J.E., Richardson, C.D., **1993**. The 1629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. *J. Virol. 67*, 5859–5866.

Viliplana, L., O'Reilly, D., **2003**. Functional interaction between *Cydia pomonella* granulovirus IAP proteins. *Virus Res. 92*, 107–111.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klingeroode, E.C., *et al.*, **1988**. Functional studies on the *p10* gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-β-galactosidase fusion gene. *J. Gen. Virol. 69*, 765–776.

Vlak, J.M., Rohrmann, G.F., **1985**. The nature of polyhedrin. In: Maramorosch, K., Sherman, K.E. (Eds.), Viral Insecticides for Biological Control. Academic Press, New York, pp. 489–542.

Volkman, L.E., **1986**. The 64K envelope protein of budded *Autographa californica* nuclear polyhedrosis virus. *Curr. Topics Microbiol. Immunol. 131*, 103–118.

Volkman, L.E., **1997**. Nucleopolyhedrovirus interactions with their insect hosts. *Adv. Virus Res. 48*, 313–348.

Volkman, L.E., Goldsmith, P.A., **1984**. Budded *Autographa californica* NPV 64K protein: further biochemical analysis and effects of postimmunoprecipitation sample preparation conditions. *Virology 139*, 295–302.

Volkman, L.E., Goldsmith, P.A., **1985**. Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. *Virology 143*, 185–195.

Volkman, L.E., Keddie, B.A., **1990**. Nuclear polyhedrosis virus pathogenesis. *Semin. Virol. 1*, 249–256.

Volkman, L.E., Storm, K., Aivazachvili, V., Oppenheimer, D., **1996**. Overexpression of actin in AcMNPV-infected cells interferes with polyhedrin synthesis and polyhedra formation. *Virology 225*, 369–376.

Volkman, L.E., Summers, M.D., **1977**. *Autographa californica* nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali-liberated, and nonoccluded forms. *J. Invertebr. Pathol. 30*, 102–103.

Volkman, L.E., Zaal, K.J.M., **1990**. *Autographa californica* M nuclear polyhedrosis virus: microtubules, p10, and replication. *Virology 175*, 292–302.

Wang, H.H., Fraser, M.J., Cary, L.C., **1989**. Transposon mutagenesis of baculoviruses: analysis of TFP3 lepidopteran transposon insertions at the FP locus of nuclear polyhedrosis viruses. *Gene 81*, 97–108.

Wang, P., Granados, R.R., **1997**. An intestinal mucin is the target substrate for a baculovirus enhancin. *Proc. Natl Acad. Sci. USA 94*, 6977–6982.

Wang, P., Granados, R.R., **1998**. Observations on the presence of the peritrophic membrane in larval *Trichoplusia ni* and its role in limiting baculovirus infection. *J. Invertebr. Pathol. 72*, 57–62.

Wang, P., Granados, R.R., **2000**. Calcofluor disrupts the midgut defense system of insects. *Insect Biochem. Mol. Biol. 30*, 135–143.

Wang, P., Granados, R.R., **2001**. Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol. 47*, 110–118.

Wang, P., Hammer, D.A., Granados, R.R., **1994**. Interaction of *Trichoplusia ni* granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of four lepidopteran insects. *J. Gen. Virol. 75*, 1961–1967.

Washburn, J.O., Chan, E.Y., Volkman, L.E., Aumiller, J.J., Jarvis, D.L., **2003a**. Early synthesis of budded virus envelope fusion protein GP64 enhances *Autographa californica* multicapsid nucleopolyhedrovirus virulence in orally infected *Heliothis virescens*. *J. Virol. 77*, 280–290.

Washburn, J.O., Chen, E.Y., Volkman, L.E., Aumiller, J.J., Jarvis, D.L., **2001**. Comparative pathogenesis of *Helicoverpa zea* S nucleopolyhedrovirus in noctuid larvae. *J. Gen. Virol. 82*, 1777–1784.

Washburn, J.O., Haas-Stapleton, E.J., Tan, F.F., Beckage, N.E., Volkman, L.E., **2000**. Co-infection of *Manduca sexta* larvae with polydnavirus from *Cotesia congregata* increases susceptibility to fatal infection by *Autographa californica* M. *nucleopolyhedrovirus*. *J. Insect Physiol. 46*, 179–190.

Washburn, J.O., Kirkpatrick, B.A., Haas-Stapleton, E., Volkman, L.E., **1998**. Evidence that the stilbene-derived optical brightener M2R enhances *Autographa californica* M nucleopolyhedrovirus infection of *Trichoplusia ni* and *Heliothis virescens* by preventing sloughing of infected midgut epithelial cells. *Biol. Control 11*, 58–69.

Washburn, J.O., Kirkpatrick, B.A., Volkman, L.E., **1995**. Comparative pathogenesis of *Autographa californica* M nuclear polyhedrosis virus in larvae of *Trichoplusia ni* and *Heliothis virescens*. *Virology 209*, 561–568.

Washburn, J.O., Kirkpatrick, B.A., Volkman, L.E., **1996**. Insect protection against viruses. *Nature 383*, 767.

Washburn, J.O., Lyons, E.H., Haas-Stapleton, E.J., Volkman, L.E., **1999**. Multiple nucleocapsid packaging of *Autographa californica* nucleopolyhedrovirus accelerates the onset of systemic infection in *Trichoplusia ni*. *J. Virol. 73*, 411–416.

Washburn, J.O., Trudeau, D., Wong, J.F., Volkman, L.E., **2003b**. Early pathogenesis of *Autographa californica* multiple nucleopolyhedrovirus and *Helicoverpa zea* single nucleopolyhedrovirus in *Heliothis virescens*: a comparison of the 'M' and 'S' strategies for establishing fatal infection. *J. Gen. Virol. 84*, 343–351.

Weitzmann, M., Possee, R.D., King, L.A., **1992**. Characterization of two variants of *Panolis flammea* multiple nucleocapsid nuclear polyhedrosis virus. *J. Gen. Virol. 73*, 1881–1886.

Whitford, M., Faulkner, P., **1992**. Nucleotide sequence and transcriptional analysis of a gene encoding gp41, a structural glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 66*, 4763–4768.

Whitford, M., Stewart, S., Kuzio, J., Faulkner, P., **1989**. Identification and sequence analysis of a gene encoding gp67 an abundant glycoprotein of the baculovirus, *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 63*, 1393–1399.

Whitt, M.A., Manning, J.S., **1988**. A phosphorylated 34-kDa protein and a subpopulation of polyhedrin are thiol linked to the carbohydrate layer surrounding a baculovirus occlusion body. *Virology 164*, 33–42.

Williams, G.V., Faulkner, P., **1997**. Cytological changes and viral morphogenesis during baculovirus infection. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 61–107.

Williams, G.V., Rohel, D.Z., Kuzio, J., Faulkner, P., **1989**. A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *J. Gen. Virol. 70*, 187–202.

Wilson, M.E., Consigli, R.A., **1985**. Functions of a protein kinase activity associated with purified capsids of the granulosis virus infecting *Plodia interpunctella*. *Virology 143*, 526–535.

Wilson, M.E., Mainprize, T.H., Friesen, P.D., Miller, L.K., **1987**. Location, transcription and sequence of a baculovirus gene encoding a small arginine-rich polypeptide. *J. Virol. 61*, 661–666.

Winstanley, D., Crook, N., **1993**. Replication of *Cydia pomonella* granulosis virus in cell cultures. *J. Gen. Virol. 74*, 1599–1609.

Xu, B., Yoo, S., Guarino, L.A., **1995**. Differential transcription of baculovirus late and very late promoters: fractionation of nuclear extracts by phosphocellulose chromatography. *J. Virol. 69*, 2912–2917.

Xu, J., Hukuhara, T., **1992**. Enhanced infection of a nuclear polyhedrosis virus in larvae of the armyworm, *Pseudoletia separata*, by a factor in the spheroids of an entomopoxvirus. *J. Invertebr. Pathol. 60*, 259–264.

Yamagishi, J., Osobe, R., Takebuchi, T., Bando, H., **2003**. DNA microarrays of baculovirus genomes: differential expression of viral genes in two susceptible insect cell lines. *Arch. Virol. 148*, 587–597.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., *et al.*, **2001**. Complete genome sequence of the shrimp white spot bacilliform virus. *J. Virol. 75*, 11811–11820.

Yang, S., Miller, L.K., **1999**. Activation of very late promoters by interaction with very late factor 1. *J. Virol. 73*, 3404–3409.

Yang, Z., Nielsen, R., Goldman, N., Pedersen, A.-M.K., **2000**. Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics 155*, 431–449.

Young, J.H.C., MacKinnon, E.A., Faulkner, P., **1993**. The architecture of the virogenic stroma in isolated nuclei of *Spodoptera frugiperda* cells *in vitro* infected by *Autographa californica* nuclear polyhedrosis virus. *J. Struct. Biol. 110*, 141–153.

Young, S.Y., Yearian, W.C., **1974**. Persistence of *Heliothis* NPV on foliage of cotton, soybean, and tomato. *Environ. Entomol. 3*, 253–260.

Zanotto, P.M., Kessing, B.D., Maruniak, J.E., **1993**. Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. *J. Invertebr. Pathol. 62*, 147–164.

Zhang, P., Yang, K., Xiaojiang, D., Su, D., **2002**. Infection of wild-type *Autographa californica* multicapsid nucleopolyhedrovirus induces *in vivo* apoptosis of *Spodoptera litura* larvae. *J. Gen. Virol. 83*, 3003–3011.

Zuidema, D., Klinge-Roode, E.C., van Lent, J.W.M., Vlak, J.M., **1989**. Construction and analysis of an *Autographa californica* nuclear polyhedrosis virus mutant lacking the polyhedral envelope. *Virology 173*, 98–108.

6.9 Genetically Modified Baculoviruses for Pest Insect Control

S G Kamita, K-D Kang, and B D Hammock,
University of California, Davis, CA, USA
A B Inceoglu, Ankara University, Ankara, Turkey

6.9.1. Introduction

There are presently more than 20 known groups of insect pathogenic viruses, which are classified into 12 viral families (Tanada and Kaya, 1993; Blissard *et al.*, 2000). Among insect pathogenic viruses, members of the family Baculoviridae are the most commonly found and most widely studied. Baculoviruses are rod shaped, enveloped viruses with large, covalently closed, double-stranded DNA (dsDNA) genomes. Baculoviruses are made up of two genera: nucleopolyhedrovirus (NPV) and granulovirus (GV). The NPVs can be further divided into two groups (I and II) on the basis of the phylogenic relationships of 20 distinguishing genes (Herniou *et al.*, 2001). Baculoviruses produce two phenotypes, the budded virus (BV) and the occluded virus (OV), during their life cycles (Granados and Federici, 1986; Miller, 1997). BVs are predominantly produced during an early phase of infection and acquire their envelopes as they bud through the plasma membrane. BVs are responsible for the systemic or cell-to-cell spread of the virus within an infected insect. Continuous cell lines that support high-level production of BVs are available for NPVs, but not for GVs. The availability of these cell lines has been critical for the development of genetically modified NPVs. OVs are produced during a late phase of infection and are involved in the horizontal or larva-to-larva transmission of the virus. The OV of the NPV is known as a polyhedron (plural polyhedra) or polyhedral inclusion body, and that of the GV is known as a granule. Within the proteinaceous crystal matrix of the OV are embedded virions termed the occlusion derived virions (ODVs). Each ODV is formed by single or multiple nucleocapsids surrounded by an envelope. In the field, the polyhedron or granule structure protects the embedded ODV(s) from the environment. The morphology and biological properties of baculoviruses are reviewed in detail in **Chapter 6.8**.

Owing to their inherent insecticidal activities, natural baculoviruses have been used as safe and effective biopesticides for the protection of field and orchard crops, and forest in the Americas,

Europe, and Asia (Black *et al*., 1997; Hunter-Fujita *et al*., 1998; Vail *et al*., 1999; Moscardi, 1999; Copping and Menn, 2000; Lacey *et al*., 2001; see **Chapter 6.8**). Both NPVs and GVs have been successfully registered for use as microbial pesticides by commercial companies and government agencies. NPVs of the velvetbean caterpillar *Anticarsia gemmatalis* (AgMNPV) and *Helicoverpa armigera* (HaSNPV) are being used with particular success for the protection of soybean in Brazil (Moscardi, 1999) and cotton in China (Sun *et al*., 2002), respectively. Natural baculoviruses, however, are slower acting and more target specific (i.e., their host specificity is narrow) compared to synthetic chemical pesticides such as the pyrethroids. The general use of natural baculoviruses in developed countries has been limited except against forest pests primarily due to their slow speed of insect killing compared to chemical insecticides, and partially due to their relatively narrow host specificity, low field stability, and cost of production. As a natural control agent the "slow kill" characteristic allows the virus to replicate to tremendous numbers while allowing its host to feed for several days. Although an attribute in a natural control strategy, this trait is a severe limitation in modern agriculture. This trait and others such as narrow host specificity can and have been addressed by genetically modifying the baculovirus using recombinant DNA technology.

During the 1980s, the birth of genetically modified (GM) baculoviruses came along with exciting new research in the laboratories of Summers (Summers and Smith, 1987) and Miller (1988). They simultaneously exploited a combination of unique characteristics of NPVs to establish the baculovirus expression vector systems (BEVS) that are now in common use for basic research and commercial applications. These characteristics include: (1) the availability of the exceptionally strong polyhedrin gene (*polh*) promoter to drive foreign gene expression; (2) a selection system based upon the visualization of the nonessential (in cultured cells) gene product of the polyhedrin gene; (3) a dsDNA genome that can be easily modified; (4) a rod-shaped capsid that can extend to package additional DNA; and (5) a eukaryotic cell line that supports virus replication at high levels. *Autographa californica* multicapsid NPV (AcMNPV), originally isolated from the alfalfa looper *A. californica* (Vail *et al*., 1973, 1999), is the baculovirus type species. AcMNPV was used by the Summers and Miller laboratories as the parental baculovirus for BEVS. Another baculovirus, *Bombyx mori* NPV (BmNPV), isolated from the silk moth *B. mori*, was used by Maeda (1989a) as the parental baculovirus in an alternative BEVS that used larvae of *B. mori* for *in vivo* expression. The methodologies for the construction and use of recombinant AcMNPVs and BmNPVs for the expression of heterologous genes have been thoroughly described (Summers and Smith, 1987; O'Reilly *et al*., 1992; Richardson, 1995; Merrington *et al*., 1999). These methodologies, with slight modifications, have also been used for the construction of GM baculovirus pesticides.

The studies to date indicate that GM baculoviruses can easily become an integral part of pest insect control, especially in developing countries and for the control of insects that have become resistant to synthetic chemical pesticides (reviews: Hammock *et al*., 1993; McCutchen and Hammock, 1994; Miller, 1995; Bonning and Hammock, 1996; Wood, 1996; Harrison and Bonning, 2000a; Inceoglu *et al*., 2001a; Bonning *et al*., 2002). Several innovative and successful approaches have been taken to improve the speed of kill of a baculovirus by genetic modification. These approaches include: (1) insertion of a foreign gene into the baculovirus genome whose product alters the physiology of the target host insect or is toxic towards the target host; (2) deletion of an endogenous gene from the baculovirus genome; and (3) incorporation of active toxin into the OV. Combinations of these approaches have also been successful in terms of decreasing the time required to kill the host or more importantly the time required to stop host feeding. Safe and effective protection of the crop from feeding damage should be the goal of a GM baculovirus pesticide. Baculoviruses have been transformed from natural disease agents to efficient pesticides through the above-mentioned discoveries and innovative approaches. Here, numerous innovations that have been used to improve the efficacy of baculoviruses for crop protection are discussed. Additionally, studies that have addressed the safety of natural and GM baculoviruses, especially in terms of risk to humans, the environment, and nontarget beneficial insects are also covered. The individual sections of this chapter provide a detailed and comprehensive summary of the field, especially in terms of improving the insecticidal activity of GM baculoviruses. In Section 6.9.7, the reader will also find a discussion of the reasons why GM baculoviruses do not receive the interest that they deserve. The authors hope that after reading this review, the reader will be convinced that the currently available GM baculovirus pesticides are effective and safe, and should be used as biological pesticides.

6.9.2. Insertion of Hormone and Enzyme Genes

6.9.2.1. Hormones

Keeley and Hayes (1987) were two of the first to suggest the use of an insect neurohormone gene to increase the insecticidal activity of the baculovirus. They wrote:

> use of an insect baculovirus as an expression vector for neurohormone genes has several advantages as a pest control strategy. (1) Insect viruses are genera- or species-specific so that the virus has a limited host range and would not affect nontarget insects. (2) The natural hormone would be produced at continuous, high levels by the viral expression vector. (3) The combination of natural insect hormones with insect-specific viruses constitutes an ideal insect pest control agent from the environmental standpoint.

Menn and Bořkovec (1989) further suggested "a neuropeptide gene placed behind a strong non-essential viral promoter is capable of turning an infected cell into a neuropeptide factory within the insect..." Maeda (1989b) pioneered the field by being the first to put these concepts into practice by generating a recombinant BmNPV expressing a diuretic hormone gene that disrupted the normal physiology of larvae of the silkworm *B. mori*. Subsequently, at least four biologically active peptide hormones have been expressed using recombinant baculoviruses: eclosion hormone (Eldridge *et al.*, 1991), prothoracicotropic hormone (O'Reilly *et al.*, 1995), pheromone biosynthesis activating neuropeptide (Vakharia *et al.*, 1995; Ma *et al.*, 1998), and neuroparsin (Girardie *et al.*, 2001). Unfortunately, expression of a biologically active hormone by the recombinant baculovirus has only been modestly successful or unsuccessful (**Figure 1**) in terms of improving the speed of kill of the baculovirus. In hindsight, this lack of success is not completely unexpected since critical events in the insect's physiology and life cycle are often protected by sequestration (by physical means or through time) or by overlapping systems. As we learn more about the regulatory mechanisms and timing of the hormonal control systems of insects, we should be able to develop more refined approaches to the use of hormone genes to improve the baculovirus as a pesticide.

6.9.2.1.1. Diuretic hormone Diuretic and anti-diuretic hormones play critical roles in the excretion and retention of water by insects in response to changes in their environment (Holman *et al.*, 1990; Coast *et al.*, 2002; Gade, 2004). The tobacco hornworm *Manduca sexta* encodes a neuropeptide hormone consisting of 41 amino acid residues that stimulates diuresis (Kataoka *et al.*, 1989). Because of the relatively short length of this peptide hormone, Maeda (1989b) was able to generate a synthetic gene encoding the peptide and attempted to express this gene in fifth instar larvae of *B. mori* using BmNPV. The synthetic diuretic hormone (DH) gene was designed on the basis of the codon usage of the polyhedrin gene of BmNPV and the gene structure of an amidated peptide from the giant silk moth *Hyalophora cecropia*. The DH gene construct also included a signal sequence for secretion from a cuticle protein (CPII) of *Drosophila melanogaster* (Meigan) (Snyder *et al.*, 1982) and a glycine residue at the C-terminus for amidation. The recombinant BmNPV (BmDH5) carrying the synthetic DH gene killed fifth instar larvae about 1 day faster than the wild-type BmNPV (Maeda, 1989b). This corresponded to a roughly 20% improvement in speed of kill in comparison to the wild-type BmNPV. BmDH5 also caused a 30% reduction in hemolymph volume, which was hypothesized to be due to the excretion of water into the Malpighian tubules, and subsequently hindgut and outside (Maeda, 1989b). Biologically active DH, however, was not detected in the circulating hemolymph of these animals (D.A. Schooley, unpublished data). The 20% improvement in speed of kill is modest in comparison to more recent GM baculovirus constructs, and the bioassays were based on injection of BV rather than oral infection with polyhedra (the normal mode of baculovirus infection in the field). Nevertheless, these experiments generated a lot of excitement in the study of genetically modified baculoviruses as pesticides and established some of the groundwork for subsequent GM baculovirus pesticide constructs.

6.9.2.1.2. Eclosion hormone Eclosion hormones (EH) are neuropeptides that influence several aspects of pupal–adult ecdysis (i.e., eclosion) as well as larval–larval ecdyses (Holman *et al.*, 1990; Nijhout, 1994). The release of EH from the brain is controlled by a circadian clock within the brain and declining ecdysteroid titers. The overexpression of EH by a recombinant baculovirus has been hypothesized to induce the premature onset of eclosion behavior and molting. A cDNA encoding EH of *M. sexta* has been inserted into recombinant AcMNPVs and shown to express high levels of biologically active and secreted EH (Eldridge *et al.*, 1991, 1992b). Time-mortality bioassays in fifth or sixth instar larvae of *Spodoptera frugiperda* injected with vEHEGTD (a recombinant AcMNPV carrying the EH encoding cDNA at the ecdysteroid

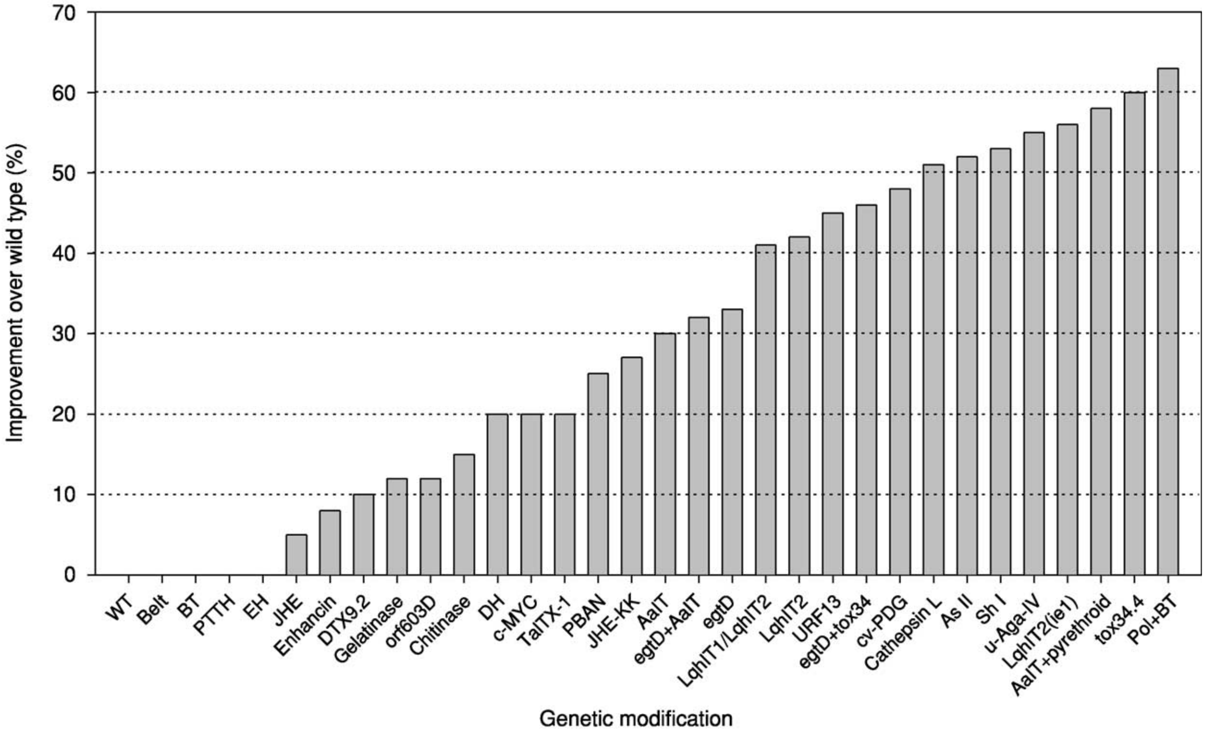


Figure 1 The speed of kill of the wild-type baculovirus can be dramatically improved by genetic modification. The genetic modification (insertion of a foreign gene or deletion of an endogenous gene) and percent improvement in speed of kill (or paralysis) relative to the wild-type virus or control virus is given. Because of differences in the parent virus, promoter, secretion signal, host strain and age, virus dose, and inoculation methods that were used, comparison between the different virus constructs is not possible. Abbreviations and reference(s): WT, wild type; Belt, insectotoxin-1 (Carbonell *et al.*, 1988); BT, *Bacillus thuringiensis* endotoxin (Merryweather *et al.*, 1990); PTTH, prothoracicotropic hormone (O'Reilly, D.R., **1995**. Baculovirus-encoded ecdysteroid UDP-glucosyltransferases. *Insect Biochem. Mol. Biol. 25*, 541–550); EH, eclosion hormone (Eldridge *et al.*, 1992b); JHE, juvenile hormone esterase (Hammock *et al.*, 1990a); enhancin, MacoNPV enhancin (Li *et al.*, 2003); DTX9.2, spider toxin (Hughes *et al.*, 1997); gelatinase, human gelatinase A (Harrison and Bonning, 2001); orf603D, deletion of AcMNPV *orf603* (Popham *et al.*, 1998a); chitinase (Gopalakrishnan *et al.*, 1995); DH, diuretic hormone (Maeda, 1989b); c-MYC, transcription factor (Lee *et al.*, 1997); TalTX-1, spider toxin (Hughes *et al.*, 1997); PBAN, pheromone biosynthesis activating neuropeptide (Ma *et al.*, 1998); JHE-KK, stabilized JHE (Bonning *et al.*, 1997b); AaIT, scorpion *Androctonus australis* insect toxin (McCutchen *et al.*, 1991; Stewart *et al.*, 1991); egtD + AaIT, insertion of *aait* at the *egt* gene locus (Chen *et al.*, 2000); egtD, deletion of ecdysteroid UDP-glucosyltransferase (*egt*) gene (O'Reilly and Miller, 1991); LqhIT1/LqhIT2, simultaneous expression of LqhIT1 and LqhIT2 (Regev *et al.*, 2003); LqhIT2, scorpion *Leiurus quinquestriatus* insect toxin 2 (Froy *et al.*, 2000); URF13, maize pore forming protein (Korth and Levings, 1993); egtD + tox34, insertion of *tox34* under the early *DA26* promoter at the *egt* gene locus (Popham *et al.*, 1997); cv-PDG, glycosylase (Petrik *et al.*, 2003); cathepsin L (Harrison and Bonning, 2001); As II, sea anemone toxin (Prikhod'ko *et al.*, 1996); Sh I, sea anemone toxin (Prikhod'ko *et al.*, 1996); μ-Aga-IV, spider toxin (Prikhod'ko *et al.*, 1996); LqhIT2(ie1), LqhIT2 under the early *ie1* promoter (Harrison and Bonning, 2000b); AaIT + pyrethroid, co-application of low doses of AcAaIT and pyrethroid (McCutchen *et al.*, 1997); tox34.4, mite toxin (Tomalski *et al.*, 1988; Burden *et al.*, 2000); Pol + BT, double expression of authentic polyhedrin and polyhedrin-BT-GFP fusion proteins (Chang *et al.*, 2003).

UDP-glucosyltransferase (*egt*) gene locus) showed that the median survival times (ST_{50}s) were reduced by approximately 29% (83 versus 117 h) and 17% (100 versus 120 h), respectively, in comparison to control larvae injected with wild-type AcMNPV. However, in comparison to control larvae injected with vEGTDEL (a control virus in which the *egt* gene was deleted), the ST_{50}s were not significantly different. Similar results were generated by time-mortality bioassays in neonate *S. frugiperda* that were orally inoculated with vEHEGTD, vEGTDEL, or AcMNPV. These findings indicated that deletion of the *egt* gene (rather than expression of EH) was responsible for the reduction in the ST_{50}. Eldridge *et al.* (1992b) thus concluded that EH expression does not provide enhanced biological control properties to AcMNPV. The role of the *egt* gene in improving speed of kill is discussed later in this chapter.

6.9.2.1.3. Prothoracicotropic hormone Prothoracicotropic hormones (PTTHs) or "brain hormones" are neurosecretory polypeptides that stimulate the

secretion of ecdysteroids from the prothoracic glands (Holman *et al*., 1990; Nijhout, 1994). The overexpression of PTTH by a recombinant baculovirus has been hypothesized to artificially elevate ecdysteroid levels resulting in the premature induction of molting, which in turn could disrupt insect feeding behavior (Menn and Borkovec, 1989; O'Reilly *et al*., 1995; Black *et al*., 1997). O'Reilly *et al.* (1995) have constructed a recombinant AcMNPV expressing the mature PTTH of *B. mori* (fused to a secretion signal sequence of sarcotoxin 1A of the flesh fly). This virus expressed PTTH in larvae of *S. frugiperda* resulting in the induction of higher than normal levels of hemolymph ecdysteroids. However, the expressed PTTH produced no observable effects on the development of *S. frugiperda* and, furthermore, inhibited the pathogenicity of the virus. In the same study, O'Reilly *et al.* (1995) expressed the mature PTTH-signal sequence construct using a mutant AcMNPV in which the AcMNPV encoded *egt* gene was knocked out. This virus apparently generated an even higher level of peak hemolymph ecdysteroid titer (1162 ± 476 versus $724 \pm 194\,\mathrm{pg}\,\mu\mathrm{l}^{-1}$) in comparison to a control virus that expressed PTTH but retained a normal *egt* gene. This higher ecdysteroid peak, however, was delayed by about 48 h (i.e., the peak occurred at 96 versus 48 h postinfection (p.i.)). These findings suggest that higher levels of hemolymph ecdysteroids are detrimental to the pathogenicity of the virus. In contrast, other studies (as will be discussed later) show that inactivation of the *egt* gene (putatively resulting in higher ecdysteroid levels) results in improved pathogenicity.

6.9.2.1.4. Pheromone biosynthesis activating neuropeptide Pheromone biosynthesis activating neuropeptide (PBAN) is a neurosecretory peptide (33–34 amino acid residues) of the brain that appears to be essential for pheromone biosynthesis and release in some lepidopterans (Holman *et al*., 1990; Nijhout, 1994). PBAN stimulates pheromone biosynthesis in conjunction with nervous stimuli during the dark phase of the photoperiod. Ma *et al.* (1998) expressed a PBAN of the corn earworm *Helicoverpa zea* (Hez-PBAN) fused to the bombyxin (an insect neurohormone) signal sequence for secretion using a recombinant AcMNPV (AcBX-PBAN). AcBX-PBAN expressed biologically active PBAN in cultured cells and larvae of *Trichoplusia ni*. In droplet feeding assays using preoccluded virus, neonate and third instar larvae of *T. ni* infected with AcBX-PBAN showed ST_{50}s that were reduced by 26% (58.3 versus 79.1 h) and 19% (70.2 versus 87.0 h), respectively, in comparison to larvae infected with a control virus. Although the mechanism for the improved speed of kill is unknown, Ma *et al.* (1998) did not observe any morphological differences between larvae that were infected with AcBX-PBAN or the control virus.

6.9.2.2. Juvenile Hormone Esterase

Wigglesworth (1935, 1936) was the first to identify a "juvenile factor" produced by the corpora allata that keeps larval insects in the juvenile state. Subsequently, Röller *et al.* (1967) and Meyer *et al.* (1970) showed the chemical structure of "juvenile hormone (JH)." Six JHs (JH-0, JH-I, JH-II, JH-III, 4-methyl JH-1, and JHB_3) have been identified to date, all of which are terpenoids derived from farnesenic acid (or its homologs) with an epoxide group at the 10, 11 position of one end and a conjugated methyl ester at the other end (Nijhout, 1994; Cusson and Palli, 2000). JH-III appears to be the most common of the JHs being found in all of the insect orders examined to date. In addition to their function as a "juvenile factor," JHs and/or their metabolites are involved in a diverse array of other functionalities including roles in development, metamorphosis, reproduction, diapause, migration, polyphenism, and metabolism (Riddiford, 1994; Gilbert *et al*., 2000; Truman and Riddiford, 2002). These diverse functionalities clearly indicate that the biosynthesis, transport, sequestration, and degradation of JH and/or its metabolites must be carefully regulated. Conversely, this careful regulation of JH titer opens a window of attack where a recombinant baculovirus expressing an appropriate protein(s) could disrupt the fine balance in JH titer and consequently the insect life cycle. Two pathways for the degradation of JH have been intensively studied in insects (Hammock, 1985; Roe and Venkatesh, 1990; de Kort and Granger, 1996). One involves a soluble esterase, JH esterase (JHE), that hydrolyzes the methyl ester moiety at one end of the JH molecule resulting in a carboxylic acid moiety (Kamita *et al*., 2003a). The other involves a microsomal epoxide hydrolase, JH epoxide hydrolase (JHEH) that hydrolyzes an epoxide moiety at the other end of the JH molecule to produce a diol.

Hammock *et al.* (1990a) first hypothesized that the natural insecticidal activity of the baculovirus AcMNPV could be improved by expression of a gene encoding JHE. The rationale behind this was that the recombinant AcMNPV would be ingested by early larval instars, and subsequently JHE would be produced at a point in development that is inappropriate for the insect. The observed result was that infected larvae reduced feeding and weight gain, and subsequently died slightly more quickly in

comparison to the wild-type AcMNPV (Hammock *et al.*, 1990a, 1990b; Eldridge *et al.*, 1992a). Two approaches to improve this technology have included: (1) improving the *in vivo* stability (i.e., reducing removal from the hemolymph and/or degradation) of the JHE enzyme by genetic modification of the JHE gene; and (2) increasing or altering the timing of gene expression by using alternative promoters to drive JHE expression. Recombinant baculoviruses expressing JHE have also been used as tools for hypothesis testing with regard to the biological activity of JHE within the insect host (van Meer *et al.*, 2000). JHE proteins from at least five different insect species including *Heliothis virescens* (Hammock *et al.*, 1990a; Bonning *et al.*, 1992), *Choristoneura fumiferana* (Feng *et al.*, 1999), *B. mori* (Hirai *et al.*, 2002), *M. sexta* (Hinton and Hammock, 2003a), and *T. molitor* (Hinton and Hammock, 2003b) have been expressed using recombinant AcMNPVs.

6.9.2.2.1. Increase in *in vivo* stability Studies have shown that recombinant JHE from *H. virescens*, when injected into larvae of *M. sexta*, is rapidly recognized and taken up into the pericardial cells from the hemolymph (Booth *et al.*, 1992; Ichinose *et al.*, 1992b, 1992a). This removal of JHE from the hemolymph occurs by a receptor mediated, endocytotic, saturable mechanism that does not involve passive filtration (Ichinose *et al.*, 1992a, 1992b; Bonning *et al.*, 1997a). The JHE is presumed to be degraded in lysosomes (Booth *et al.*, 1992). At least two putative JHE binding proteins may be involved in transport and/or degradation of JHE in the pericardial cells, including a putative heatshock cognate protein (HSP) (Bonning *et al.*, 1997a) and P29 (Shanmugavelu *et al.*, 2000, 2001). Receptor mediated endocytosis of JHE has been demonstrated in early and late larval instars of *M. sexta* (Ichinose *et al.*, 1992a). Although JHE is normally stable in hemolymph, the half-life of JHE injected into the hemolymph can be as little as 20 min under conditions where endogenous and exogenous proteins including bovine serum albumin, ovalbumin, and hemolymph JH binding protein have half-lives of days (Ichinose *et al.*, 1992a). Thus, the mechanism by which JHE is specifically recognized and removed by the pericardial cells could be a very important target for increasing the half-life of JHE in the hemolymph. There are three ways in which the uptake and degradation of JHE can be disrupted: (1) prevention of receptor mediated uptake by the pericardial cells; (2) disruption of transport to the lysosomes; and (3) prevention of lysosomal degradation.

Bonning *et al.* (1997b) identified two lysine residues that are likely to be on the surface of the JHE protein of *H. virescens* and potentially involved in uptake or degradation of JHE. These lysine residues are Lys-29 near the N-terminus, which is potentially involved in ubiquitin conjugation, and Lys-524, which is located within a putative lysosome targeting sequence. A mutated JHE protein (JHE-KK) in which both of these lysine residues were mutated to arginine residues showed decreased efficiency of lysosomal targeting (Bonning *et al.*, 1997b), and binds to the putative JHE binding protein (P29) with significantly less affinity than authentic JHE (Shanmugavelu *et al.*, 2000). However, JHE-KK as well as mutant JHEs in which only one of the lysine residues was mutated to an arginine residue showed similar catalytic activities and removal rates of JH from the hemolymph as the authentic JHE. A high-resolution crystal structure should assist with determining the basis of the interaction of JHE and the putative JHE binding protein P29 described by Shanmugavelu *et al.* (2000). These researchers hypothesize that P29 interacts with JHE of *H. virescens* and facilitates targeting of JHE to lysosomes within pericardial cells. Other putative JHE binding proteins have been identified in *M. sexta*, and their possible roles in the degradation of JHE remain to be elucidated (Shanmugavelu *et al.*, 2001). First instar larvae of *H. virescens* that were infected with a recombinant AcMNPV expressing JHE-KK (AcJHE-KK) under a very late viral promoter died approximately 22% faster (ST_{50} of 90 versus 116 h) than control larvae infected with a recombinant AcMNPV expressing the authentic JHE (AcJHE) (Bonning *et al.*, 1999). In similar experiments, first instar *T. ni* that were infected with AcJHE-KK died approximately 27% faster (ST_{50} of 83 versus 113 h) compared to control larvae infected with AcJHE. Kunimi *et al.* (1996) have analyzed the survival times of third to fifth instar *T. ni* that were infected with AcJHE-KK. In these older insects, they found that the ST_{50}s of AcJHE-KK infected third, fourth, and fifth larvae were reduced by roughly 4% (124.8 versus 130.2 h), 9% (126.6 versus 138.6 h), and 8% (135.0 versus 147.0 h), respectively, in comparison to control AcMNPV infected larvae. In similar bioassays of second to fifth instars of the soybean looper *Pseudoplusia includens*, AcJHE-KK infected larvae did not show a reduced survival time in comparison to AcMNPV infected control larvae (Kunimi *et al.*, 1997). The reasons for these differences in the survival times between species and instars are unknown, however, Bonning *et al.* (1999) speculated that host and/or age specific effects may be involved.

6.9.2.2.2. Gene silencing and RNA interference AcPR1 is a recombinant AcMNPV that produces biologically active JHE of *H. virescens* (Hanzlik *et al.*, 1989) under the very late baculoviral *p10* gene promoter (Roelvink *et al.*, 1992). AcPR2 is a recombinant AcMNPV in which the same JHE gene is placed under the *p10* promoter in the antisense direction. Transcription of this gene generates mRNAs that are antisense to the JHE mRNAs generated by AcPR1. These antisense JHE mRNAs are able to reduce the JHE activity produced by AcPR1 when AcPR1 and AcPR2 are coinfected into *Spodoptera frugiperda* (Roelvink *et al.*, 1992). By injection of AcPR2 into fifth instar *H. virescens*, Hajos *et al.* (1999) showed that AcPR2 downregulates JHE activity in more than 95% of the injected larvae. This effect was putatively due to direct RNA–RNA interaction between sense and antisense JHE RNAs. Although 95% of the infected larvae showed a reduction in JHE activity, only 25% of these larvae showed morphogenic alterations (Hajos *et al.*, 1999). These alterations were similar to those induced by the exogenous application of JH (Cymborowski and Zimowska, 1984) or JHE inhibitors (Hammock *et al.*, 1984) to the larvae. On the basis of the effectiveness of this baculovirus mediated gene silencing approach, Hajos *et al.* (1999) suggest that the identification of other host gene targets could greatly reduce lethal times or feeding damage of a GM baculovirus pesticide.

RNA interference (RNAi) is a sequence specific mechanism by which a targeted gene is silenced by the introduction of double-stranded RNA (dsRNA) that is homologous to the targeted gene (Hutvagner and Zamore, 2002; Denli and Hannon, 2003). Mechanistically, it is likely that the antisense RNA mediated gene silencing described by Hajos *et al.* (1999) involves the RNAi pathway. RNAi has been demonstrated in a diverse range of organisms including plants, fungi, arthropods, and mammals. One biological function of RNAi appears to be as a defense mechanism against RNA viruses in plants and other organisms. Recently, RNAi has been shown to be effective in lepidopteran cell lines (Means *et al.*, 2003; Valdes *et al.*, 2003) and larvae such as the giant silk moth, *H. cecropia* (Bettencourt *et al.*, 2002) and cabbage looper *T. ni* (Kramer and Bentley, 2003). Our laboratory has explored the use of RNAi to block the activity and effects of JHE in cultured *S. frugiperda* cells and larvae of *H. virescens*. In the larval experiments, a 0.5 kb dsRNA fragment that corresponds to the 5′-coding sequence of the JHE gene of *H. virescens* (Hanzlik *et al.*, 1989) was generated. Neonate *H. virescens* that ingested these dsRNAs within 6 h of hatching showed a statistically significant (two-sided Mann-Whitney test with an error type I of 99%) increase in weight (in comparison to control larvae) by the second and third days of the fifth larval instar. Additionally, injection of the same 0.5 kb dsRNAs into the larvae of *H. virescens* on the first day of the fifth instar resulted in a 16 h delay in the median time to pupation in comparison to water injected or untreated controls (**Figure 2**). Treatments were compared with Wilcoxon (Gehan) statistics on a 99% error type I. These comparisons indicated that RNAi against the JHE of *H. virescens* is effective at prolonging the juvenile state. The authors are currently in the process of constructing recombinant AcMNPVs carrying JHE gene derived sequences in a “head-to-head” manner such that the expressed RNAs will form a hairpin loop structure. Such a

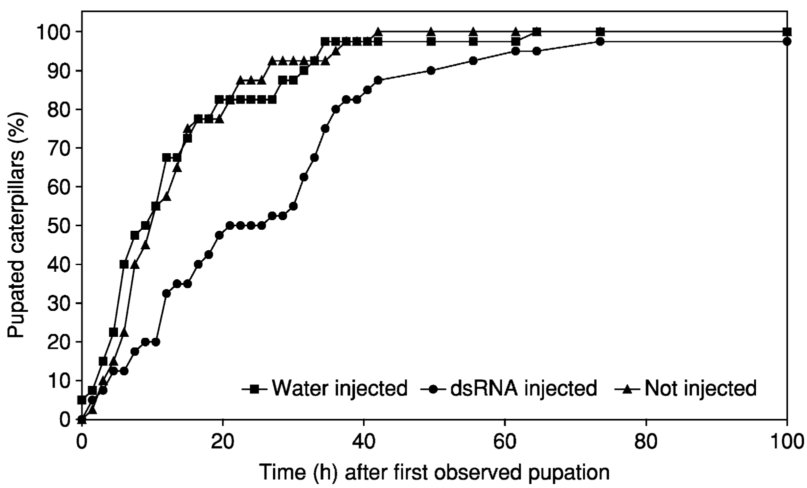


Figure 2 Percentage of larvae that pupate over time following treatment with double-stranded RNA (dsRNA), double-distilled water, or untreated. Pupation was scored on the basis of head capsule slippage at 1.5 h or longer intervals.

recombinant AcMNPV may be able to induce the RNAi response more efficiently. Of course, a recombinant virus that is effective at prolonging the juvenile state would be a disaster as a biological insecticide. However, these experiments are important in that they demonstrate the effectiveness of an RNAi approach in the regulation of insect neuroendocrinology and the results should be easily transferred to other significant physiological effectors.

The use of an authentic, insect-derived protein to combat the insect is conceptually elegant and potentially safer because one is only trying to express an endogenous protein at an inappropriate time or at higher levels in order to alter the physiology of the insect. Additionally, there is a perception that expressing the insect's own protein at an inappropriate time in development offers public relations advantages over the expression of toxins or proteases as will be discussed below. In practice, however, overexpression of an insect hormone or protein involved in hormone metabolism has not been dramatically effective. For example, the limited number of JHEs tested to date not enhance the speed of kill as well as scorpion toxins or proteases. However, with JHE there is a clear mechanism of action and an obvious path toward generating improved viruses by engineering the protein for greater stability in the insect. The recent homology model of Thomas *et al.* (1999) and crystal structure of the protein will greatly enhance these efforts.

6.9.2.3. Proteases

In order to better understand the rationale behind the use of protease encoding genes for improving speed of kill, a brief review of the midgut barriers faced by the baculovirus is given. The first barrier that the ODV encounters in the midgut is the fluid within the lumen. The high pH (8–11) and endogenous proteases found in the lumen are initially required to release ODVs from the polyhedra or granules. However, digestive enzymes or other factors within the lumen could also lead to the inactivation of the released ODV, if the ODVs do not quickly initiate infection. One such factor is a lipase, Bmlipase-1, isolated from the digestive juice of *B. mori* that shows antiviral activity against BmNPV (Ponnuvel *et al.*, 2003). The peritrophic membrane (PM; or peritrophic envelope) is the first physical barrier that the ODV faces (Richards and Richards, 1977; Brandt *et al.*, 1978; Tellam, 1996). The PM is composed of chitin fibrils in a protein–carbohydrate matrix. The specific composition and layout of the PM differs in different lepidopteran species and at different larval instars. The manner in which the ODVs pass this barrier is still not well understood. The second physical barrier that the virus faces is the midgut epithelium. The midgut epithelium is primarily composed of a single layer of columnar cells. The columnar cells show directionality (polarity) in which the luminal border is formed by microvilli (brush border membrane) and the hemocoelic border shows characteristic infolding with large numbers of mitochondria associated with the infolds. Regenerative and endocrine cells are also part of the midgut epithelium. Several pathways have been proposed for how the nucleocapsids pass through the midgut epithelium barrier following the fusion of ODVs with the microvillar membrane (Federici, 1997). One pathway involves entry of the nucleocapsid into the columnar cells, translocation through the cytoplasm, and budding through the hemocoelic border of the same cell without any viral replication. The other pathways involve the generation of progeny nucleocapsids within the columnar cells that can bud (1) through the hemocoelic border, (2) into the tracheal matrix via tracheoblasts, or (3) into regenerative cells. Once in the regenerative cells, the nucleocapsids may translocate through the cell without replication and through the hemocoelic border or virus replication may occur in these cells as well. Washburn *et al.* (2003) have proposed a "hybrid" pathway in which some nucleocapsids of MNPVs uncoat and start replication whereas others pass directly through the columnar cells. The third midgut associated barrier to systemic infection is the basement membrane (or basal lamina). The basement membrane (BM) is a fibrous matrix composed primarily of glycoproteins, type IV collagen, and laminin, which are secreted by the epithelial cells (Ryerse, 1998). The BM functions in several roles including structural support of the epithelium or surrounding tissues, filtration, and differentiation (Yurchenco and O'Rear, 1993).

6.9.2.3.1. Enhancins Enhancins are baculovirus encoded proteins that can enhance the oral infectivity of a heterologous or homologous baculovirus. Enhancin, also known as synergistic factor (SF) or virus enhancing factor (VEF), was first described by Tanada as a lipoprotein of 93–126 kDa found within the capsule of the GV of the armyworm *Pseudaletia unipuncta* (Yamamoto and Tanada, 1978a, 1978b) that enhances the infection of baculoviruses in lepidopteran larvae (Tanada, 1959). The enhancin of the GV of *T. ni* (TnGV) has been shown to possess metalloprotease activity (Lepore *et al.*, 1996; Wang and Granados, 1997). Tanada first suggested

that this factor is virus encoded (Tanada and Hukuhara, 1968) and Hashimoto *et al.* (1991) confirmed this by cloning and sequencing the *vef* gene from TnGV. Subsequently, enhancin gene homologs have been sequenced from the GVs of *H. armigera* and *P. unipuncta* (Roelvink *et al.*, 1995) and *Xestia c-nigrum* (four homologs; Hayakawa *et al.*, 1999). Enhancin gene homologs have also been identified in two NPVs, *Lymantria dispar* NPV (two homologs, E1 (Bischoff and Slavicek, 1997) and E2 (Popham *et al.*, 2001)) and *Mamestra configurata* NPV (Li *et al.*, 2003). Disruption of the E1 enhancin gene homolog of LdMNPV reduces viral potency from 1.4- to 4-fold in comparison to the wild-type LdMNPV but does not affect the killing speed of the virus (Bischoff and Slavicek, 1997). Enhancins appear to enhance virus infection by: (1) degrading high molecular weight proteins such as mucin (Wang and Granados, 1997), which are found in the PM (Derksen and Granados, 1988; Gallo *et al.*, 1991; Lepore *et al.*, 1996; Wang and Granados, 1997); and/or (2) enhancing fusion of the virus to the midgut epithelium cells (Ohba and Tanada, 1984; Kozuma and Hukuhara, 1994; Wang *et al.*, 1994).

Enhancin genes have been expressed by recombinant AcMNPVs in order to improve the ability of the virus to gain access to the midgut epithelium cells. A recombinant AcMNPV, AcMNPV-enMP2, carrying the enhancin gene of *M. configurata* NPV under its authentic promoter has been constructed (Popham *et al.*, 2001; Li *et al.*, 2003). In dose-mortality studies, AcMNPV-enMP2 shows a 4.4-fold lower median lethal dose ($LD_{50} = 2.8$ versus 12.4 polyhedra per larva) in second instar larvae of *T. ni* in comparison to the wild-type virus (Li *et al.*, 2003). In time-mortality studies, the ST_{50} of AcMNPV-enMP2 infected larvae was not significantly different from that of wild-type AcMNPV infected larvae when the larvae were inoculated at biologically equivalent doses (i.e., at an LD_{90} dose). However, when the larvae were inoculated with the same number (i.e., 32 polyhedra per larva) of AcMNPV-enMP2 or wild-type AcMNPV, the AcMNPV-enMP2 inoculated larvae showed an approximately 8% faster ST_{50} (130 versus 141 h). Li *et al.* (2003) suggest that this enhancement in speed of kill may be the result of increasing the number of initial infection foci in the host insect midgut rather than increasing the speed of the spread of the virus infection.

Hayakawa *et al.* (2000b) have generated a recombinant AcMNPV (AcEnh26) expressing the enhancin gene from *T. ni* granulovirus. In bioassays in which third instar larvae of *S. exigua* were fed a mixture of AcEnh26 infected Sf-9 cells and AcMNPV polyhedra, the LD_{50} of AcMNPV was 21-fold lower in comparison to feeding with a mixture of control virus infected Sf-9 cells and AcMNPV (2.69×10^3 versus 5.75×10^4 polyhedra per larva). In similar experiments, the LD_{50} of another baculovirus, *Spodoptera exigua* NPV (SeNPV), was ninefold lower (19.1 versus 162 polyhedra per larva) when coinfected with AcEnh26 infected Sf-9 cells. In an interesting twist to these experiments, Hayakawa *et al.* (2000b) engineered the enhancin gene from *T. ni* into tobacco plants and used a mixture of AcMNPV and lyophilized transgenic tobacco leaves for bioassay. They found a tenfold reduction in the LD_{50} of AcMNPV when the enhancin expressing tobacco was added to the bioassay. These findings suggest that expression of the enhancin gene in plants (Granados *et al.*, 2001; Cao *et al.*, 2002) or mixing enhancin expressing plant material in baculovirus pesticide formulations may be an effective method for improving the speed of kill of natural and recombinant baculoviruses.

6.9.2.3.2. Basement membrane degrading proteases As mentioned above, the basement membrane is the last midgut-associated barrier that the baculovirus virion must cross prior to the initiation of systemic infection. Harrison and Bonning (2001) have constructed recombinant AcMNPVs expressing three different proteases (rat stromelysin-1, human gelatinase A, and cathepsin L from the flesh fly *Sarcophaga peregrina*) that are known to digest BM proteins. Of these recombinant AcMNPVs, AcMLF9.ScathL expressing cathepsin L under the late baculovirus *p6.9* gene promoter generated a 51% faster speed of kill (ST_{50} of 48 versus 98 h) in comparison to wild-type AcMNPV in neonate larvae of *H. virescens*. Expression of gelatinase under the p6.9 promoter also improved speed of kill by approximately 12% (ST_{50} of 86.5 versus 98 h) in comparison to wild-type AcMNPV. On the basis of lethal concentration bioassays, there were no statistically significant differences in the virulence (i.e., LD_{50}) of these viruses in comparison to the wild-type AcMNPV. In bioassays to determine feeding damage, AcMLF9.ScathL infected larvae consumed approximately 27- and 5-fold less lettuce (29.27 and 5.21 cm^2, respectively) in comparison to mock or AcMNPV infected (1.1 cm^2) second instars of *H. virescens*, respectively. Although the mechanism of this improvement in speed of kill has yet to be determined, Harrison and Bonning (2001) speculated that digestion of BM proteins by the cathepsin L may: (1) hasten the spread of infection through the BM; (2) inappropriately

activate prophenoloxidase, a key enzyme involved in the formation of melanin during wound healing and the immune response in insects; and/or (3) cause BM damage that results in deregulation of ionic and molecular traffic between the hemocoel and the tissues. Harrison and Bonning (2001) also expressed cathepsin L under the baculovirus early *ie1* promoter using the recombinant baculovirus AcIE1TV3.ScathL. This virus, however, did not show any improvement in speed of kill. They speculated that expression under the early promoter did not produce sufficient cathepsin L to have an effect on the infected host. The effectiveness of protein expression using early, late, and/or constitutively expressed promoters will be further discussed later.

6.9.2.4. Other Enzymes and Factors

Chitin is an insoluble structural polysaccharide that is found in the exoskeleton and gut linings of insects (Cohen, 1987). Chitinases are enzymes that can degrade chitin into low molecular weight, soluble, and insoluble oligosaccharides (Kramer and Muthukrishnan, 1997). Genes encoding chitinases have been identified in several lepidopteran insects including *M. sexta* (Gopalakrishnan *et al.*, 1993), *B. mori* (Kim *et al.*, 1998; Daimon *et al.*, 2003), *Hyphantria cunea* (Kim *et al.*, 1998), *S. litura* (Shinoda *et al.*, 2001), and *C. fumiferana* (Zheng *et al.*, 2002). The genome of *B. mori* appears to encode multiple, potentially five different, chitinase genes (Daimon *et al.*, 2003). Chitinase and cysteine protease genes are also found in the genomes of NPVs and GVs (Ohkawa *et al.*, 1994; Hawtin *et al.*, 1995; Slack *et al.*, 1995; Kang *et al.*, 1998; Gong *et al.*, 2000). Baculovirus encoded chitinases and proteases (V-CATH) are thought to be involved in the degradation of the chitinous and proteinaceous components, respectively, of the host cadaver in order to induce liquefaction (O'Reilly, 1997; Hom and Volkman, 2000). Hom and Volkman (2000) have suggested another role of the baculovirus encoded chitinase, namely, proper folding of the nascent V-CATH polypeptides in the endoplasmic reticulum (ER). A recombinant AcMNPV, vAcMNPV· chi, has been generated that expresses the chitinase gene of *M. sexta* (Gopalakrishnan *et al.*, 1995). When fourth instar *S. frugiperda* were injected with vAcMNPV·chi, larval death occurred nearly 1 day earlier than those infected with the wild-type virus AcMNPV.

Carbohydrates are the major source of energy that powers insect metabolism and form the material basis of chitin in the cuticle of insects (Cohen, 1987). Thus, the availability of sugars is essential for normal growth and development. Trehalose, a disaccharide composed of two glucose molecules, is the major carbohydrate in the hemolymph of most insects (Friedman, 1978). Sato *et al.* (1997) have generated two recombinant AcMNPVs, vTREVL, and vERTVL, expressing a trehalase cDNA from the mealworm beetle *T. molitor* in the sense and antisense directions, respectively. In Sf-9 cell cultures and larvae of the cabbage armyworm *Mamestra brassicae* that were infected with vTREVL, biologically active trehalase was secreted into the supernatant and found in the hemolymph, respectively. The survival time of fifth instar larvae of *M. brassicae* that were infected with vTREVL was longer than control larvae infected with the wild-type AcMNPV. Interestingly, the vTREVL infected larvae showed considerably reduced melting in comparison to the AcMNPV infected larvae. The survival time of larvae infected with vERTVL was also slightly longer than that of control larvae infected with AcMNPV, but less than that of vTREVL.

URF13 is a mitochondrially encoded protein of maize that forms pores in the inner mitochondrial membrane (Korth *et al.*, 1991). Korth and Levings (1993) have generated recombinant, occlusion-negative AcMNPVs expressing authentic (BV13T) or mutated (BV13.3940) URF13 encoding genes under the *polh* promoter. When third to fourth instar larvae of *T. ni* were injected with BV13T or BV13.3940, 100% died by 60 h post injection. Control larvae that were injected with wild-type AcMNPV or a control β-galactosidase expressing AcMNPV lived up to 106 and 100 h p.i., respectively. The median lethal time (LT_{50}) of BV13T or BV13.3940 appeared reduced by about 45% (50 versus 94 h) in comparison to control virus infected larvae (estimate based on mortality graph). Although the mechanism of this toxicity was not determined, Korth and Levings (1993) showed that baculovirus expressed URF13 is localized in the membranes of cells of Sf-9 cultures and larvae of *T. ni*. They suggested that this membrane localization interferes with normal cellular functions. The URF13 induced cytotoxicity, however, appeared to be unrelated to pore formation, because the mutated URF13 expressed by BV13.3940 is unable to form membrane pores (Braun *et al.*, 1989), but the toxicity of BV13.3940 was the same as that of BV13T.

c-MYC is a transcription factor that is involved in various physiological processes including cell growth, proliferation, loss of differentiation, and

apoptosis in a variety of organisms (Pelengaris and Khan, 2003). Lee *et al.* (1997) have generated a recombinant AcMNPV carrying a 754 bp-long fragment of human *c-myc* exon II in the antisense direction under the *polh* promoter. When third or fourth instar *S. frugiperda* are injected with this virus at an average dose of 3500 plaque forming units (pfu) per mg of body weight, 75% of the larvae stop feeding by 3 days postinfection. This is roughly a twofold reduction in feeding in comparison to wild-type AcMNPV injected larvae, in which about 35% of the larvae stop feeding. This feeding cessation occurs roughly 2 days before death of the larva. Furthermore, the average survival time of the larvae is reduced by approximately 1 day. Lee *et al.* (1997) indicate two important advantages to this antisense strategy. First, this strategy does not require protein synthesis and also avoids any potential problems associated with posttranslational modifications or secretion. Second, since no foreign protein is produced the likelihood of insect resistance will be reduced.

Sunlight (i.e., UV radiation) is a major factor in the inactivation of baculoviruses in the field (Ignoffo and Garcia, 1992; Dougherty *et al.*, 1996; Black *et al.*, 1997; Ignoffo *et al.*, 1997). In an attempt to reduce UV inactivation, Petrik *et al.* (2003) have generated a recombinant AcMNPV that expresses an algal virus pyrimidine dimer specific glycosylase, cv-PDG (Furuta *et al.*, 1997), which is involved in the first steps of the repair of UV damaged DNA. The recombinant AcMNPV (vHSA50L) expresses the cv-PDG encoding the *A50L* gene under the *hsp70* promoter of *D. melanogaster*. Polyhedra of vHSA50L were not more resistant to UV inactivation than the wild-type AcMNPV, however, BV of vHSA50L were threefold more resistant to UV inactivation. In dose-mortality bioassays in neonate *T. ni*, the median lethal concentrations (LC_{50}s) of vHSA50L and AcMNPV were not significantly different. In neonates of *S. frugiperda*, however, the LC_{50} of vHSA50L was reduced by approximately 16-fold (7.1×10^5 versus 1.15×10^7 polyhedra per ml). Surprisingly, time-mortality bioassays (at an LC_{90} dose) in vHSA50L infected neonates of *S. frugiperda* indicated that the LT_{50} of vHSA50L was reduced by approximately 41% (73.6 versus 124.4 h). This reduction in LT_{50} was even greater (48%, 65.0 versus 124.4 h) for a construct (vHSA50-LORF) that carried *A50L* excluding the 5′-untrans--untranslated region under the *hsp70* promoter. In contrast to the neonate *S. frugiperda*, significant differences were not found in the LT_{50}s of neonate *T. ni* that were infected with vHSA50L or vHSA50LORF.

6.9.3. Insertion of Insect Selective Toxin Genes

Arthropods that feed on or parasitize other arthropods possess potent venoms that rapidly immobilize their prey. The venoms of arthropods such as scorpions, spiders, and parasitic wasps are composed of a mixture of salts, small molecules, proteins, and peptides (Zlotkin *et al.*, 1978, 1985; Zlotkin, 1991; Loret and Hammock, 1993; Gordon *et al.*, 1998; Possani *et al.*, 1999). Peptide toxins that are specifically active against invertebrates, vertebrates, or both invertebrates and vertebrates have been identified in these venoms. These peptide toxins generally target major ion channels such as the Na^+, K^+, Ca^{2+}, and Cl^- channels although there are several examples of peptides with unusual targets such as the intracellular calcium activated ryanodine channel (Fajloun *et al.*, 2000). Kinin-like peptides that interfere with physiological events have also been identified in venom (Inceoglu and Hammock, unpublished data). Arthropod venoms have been utilized as highly useful sources for the mining of highly potent and selective peptide toxins that selectively paralyze insects. Several of these peptides have been expressed by recombinant baculoviruses for the purpose of improving their speed of kill and insecticidal activity.

6.9.3.1. Scorpion Toxins

On average scorpion venom contains from 200 to 500 different peptides. It is conservatively assumed that about 10% of these peptides are insect selective toxins. Considering that there are roughly 1250 identified species of scorpion, this represents an enormous diversity (25 000 to 62 500) of potent and selective insecticidal peptides for pest control using baculovirus expression. Scorpion toxins are classified on the basis of size and pharmacological target site into two main groups: long and short chain neurotoxins (Zlotkin *et al.*, 1978; Loret and Hammock, 1993; see **Chapter 5.6**). Toxins with selective activity against insects are found in both the long and short chain groups. Long chain neurotoxins are polypeptides of 58–70 amino acid residues, cross-linked by four disulfide bonds, which mainly target voltage gated sodium channels and to some degree calcium channels (Zlotkin, 1991; Loret and Hammock, 1993; Gordon *et al.*, 1998). Short chain neurotoxins are peptides of 31–37 amino acid residues, cross-linked by three or four disulfide bonds, which mainly target potassium and chloride channels (Loret and Hammock, 1993). The long chain neurotoxins, the better studied of the two

main groups, are further classified into mammal or insect selective toxins, with the insect selective toxins being further classified on the basis of their mode of action into three subgroups: alpha insect toxins, excitatory toxins, and depressant toxins (Zlotkin *et al.*, 1995; Gordon *et al.*, 1998; Inceoglu *et al.*, 2001b). Each of these subgroups target different molecular sites and produce distinct effects upon the voltage gated sodium channel of the insect (Cestele and Catterall, 2000). In addition, when injected into larvae of the blowfly *S. falculata*, toxins in each of these subgroups display unique symptoms. Alpha insect toxins cause contractive paralysis that is delayed and sustained (Eitan *et al.*, 1990). Excitatory toxins, such as AaIT from the North African scorpion *Androctonus australis*, cause paralysis that is immediate and contractive (Zlotkin *et al.*, 1971, 1985; Zlotkin, 1991). In contrast, depressant toxins, such as LqhIT2 from the yellow Israeli scorpion *Leiurus quinquestriatus hebraeus*, cause transient (until 5 min post injection) contractive paralysis, followed by sustained, flaccid paralysis (Zlotkin *et al.*, 1985; Zlotkin, 1991).

Carbonell *et al.* (1988) were the first to attempt to express biologically active scorpion toxin, insectotoxin-1 of *Buthus eupeus* (*BeIt*), under control of the very late polyhedrin promoter using recombinant AcMNPV constructs (vBeIt-1, vBeIt-2, and vBeIt-3). The vBeIt-1 construct carried the 112 nucleotide-long *BeIt* gene 6 nucleotides downstream of the last nucleotide of the polyhedrin leader. The vBeIt-2 construct carried the *BeIt* gene fused to a 21 amino acid-long signal sequence for secretion of human β-interferon (Ohno and Taniguchi, 1981) 18 nucleotides downstream of the polyhedrin leader. The vBeIt-3 construct expressed *BeIt* as a fusion with the N-terminal 58 codons of the polyhedrin gene. All three constructs produced similar, high levels of *BeIt* specific transcripts from the polyhedrin promoter. The vBeIt-1 and vBeIt-2 constructs produced exceptionally low levels of the 4 kDa BeIt peptide, whereas the vBeIt-3 construct produced significant amounts of a 13–14 kDa polyhedrin-BeIt fusion peptide. Toxin specific biological activity, however, was not observed by bioassay in larvae of *T. ni*, *Galleria mellonella*, and *Sarcophaga*. Carbonell *et al.* (1988) speculated that this was the result of: (1) toxin instability; (2) a low sensitivity threshold of the toxin bioassay; and/or (3) inability of the polyhedrin-BeIt fusion to properly fold.

6.9.3.1.1. AaIT The insect-selective neurotoxin AaIT (*Androctonus australis* insect toxin 1), found in the venom of the scorpion *A. australis*, was the first scorpion toxin to be expressed by recombinant baculoviruses that showed biological activity (Maeda *et al.*, 1991; McCutchen *et al.*, 1991; Stewart *et al.*, 1991). AaIT is composed of a single polypeptide chain of 70 amino acid residues cross-linked by four disulfide bonds and is highly specific for the voltage gated sodium channel of insects (Zlotkin *et al.*, 2000; see **Chapter 5.6**). The toxin induces a neurological response similar to that evoked by the pyrethroid insecticides, but apparently acts at a different site within the sodium channel (see discussion below regarding toxin interactions with chemical pesticides). Maeda *et al.* (1991) constructed a recombinant BmNPV carrying a synthetic AaIT gene (Darbon *et al.*, 1982) that was linked to a signal sequence for secretion of bombyxin and driven by the very late polyhedrin (*polh*) gene promoter. The recombinant virus, BmAaIT, expressed biologically active AaIT that was secreted into the hemolymph of BmAaIT infected silkworm larvae. The BmAaIT infected larvae (second to fifth instar *B. mori*) displayed symptoms that were consistent with sodium channel binding by AaIT including body tremors, dorsal arching, feeding cessation, and paralysis beginning at 40 h p.i. Death occurred by 60 h p.i. This corresponded to an improvement in speed of kill of approximately 40% in comparison to control larvae infected with the wild-type BmNPV.

In related experiments, McCutchen *et al.* (1991) and Stewart *et al.* (1991) independently generated recombinant AcMNPVs expressing AaIT under the very late baculoviral *p10* promoter. The Stewart *et al.* construct, AcST-3, expressed AaIT as a fusion protein with the signal sequence of the baculoviral GP67 protein. The McCutchen *et al.* construct, AcAaIT, expressed AaIT as a fusion protein with the bombyxin signal sequence. Bioassays using orally inoculated, second instar larvae of *T. ni* indicated that the median lethal dose (LD_{50}) of AcST-3 was reduced by about 30% (44 versus 31 polyhedra per larva) in comparison to the wild-type AcMNPV. The median survival time (ST_{50}) of AcST-3 infected larvae (infected with approximately 17.4 polyhedra per larva) was reduced by about 25% (85.8 versus 113.1 h) in comparison to control larvae infected with AcMNPV. Third instar *T. ni* that are infected with a dose of AcST-3 that resulted in 100% mortality showed a 50% reduction in feeding damage in comparison to AcMNPV infected control larvae. The McCutchen *et al.* construct, AcAaIT, showed similar results by bioassay using second instar larvae of *H. virescens*. The LD_{50} of AcAaIT was reduced by about 39% (13.3 versus 21.9 polyhedra per larva) in comparison to AcMNPV. The ST_{50} of AcAaIT infected larvae (infected with 250 polyhedra per larva) was reduced by about 30% (88.0 versus

125 h) in comparison to control larvae infected with AcMNPV. By droplet feeding assays of neonate *H. virescens*, the ST_{50} of AcAaIT infected larvae was reduced by up to 46% (Inceoglu and Hammock, unpublished data). McCutchen *et al.* conducted additional experiments in which third instar larvae of *M. sexta* (an unnatural host of AcMNPV) were injected with AcAaIT (5 μl containing 1×10^6 pfu). They detected AaIT specific symptoms as early as 72 h post injection and found a 29% decrease (120 versus 168 h) in the speed of kill. Additional observations made during this study showed that larvae infected with AcAaIT typically were paralyzed and stopped feeding several hours prior to death. Furthermore, the yields of progeny viruses (polyhedra per cadaver) in AcAaIT infected larvae (third, fourth, or fifth instar *T. ni*) were only 20–32% of those of control larvae infected with AcMNPV (Kunimi *et al.*, 1996; Fuxa *et al.*, 1998). This suggested that the recombinant virus will be quickly outcompeted in the field by the wild-type virus (see below).

Hoover *et al.* (1995) have further characterized the paralytic effect of AaIT by bioassay using third instar *H. virescens* that were inoculated with either AcAaIT or AcMNPV. When the AcAaIT or AcMNPV infected larvae were placed on greenhouse cultivated cotton plants, it was found that the AcAaIT infected larvae fell off the plants approximately 5–11 h before death. This "knock-off" effect occurred before the induction of feeding cessation. As a consequence, the amount of leaf area consumed by the AcAaIT infected larvae was up to 62% and 72% less than that consumed by the AcMNPV and mock infected larvae, respectively (**Figure 3**). Knock-off effects have also been observed in field trials (on cotton) to assess the efficacy of recombinant AcMNPV (Cory *et al.*, 1994) or HaSNPV (Sun *et al.*, 2004) expressing AaIT. On the basis of this knock-off effect, Hoover *et al.* (1995) suggested that median survival time is not necessarily predictive of the reduction in the amount of feeding damage that results from the application of a recombinant baculovirus. Cory *et al.* (1994) and Hoover *et al.* (1995) also emphasized that the knock-off effect should reduce foliage contamination because unlike AcAaIT infected larvae that fall off the plant, wild-type virus infected larvae tend to die on the plant. Consequently, they suggested that the reduction in virus inoculum on the foliage should decrease the spread and recycling of the recombinant virus in comparison to the wild-type virus. A discussion of the fitness of a recombinant virus in comparison to the wild-type parent is given later in this chapter.

The AaIT gene has been expressed using other baculovirus vectors, for example, the NPVs of the mint looper *Rachiplusia ou* (RoMNPV; Harrison and Bonning, 2000b) and cotton bollworms *Helicoverpa zea* (HzNPV; Treacy *et al.*, 2000) and *H. armigera* (HaSNPV; Chen *et al.*, 2000; Sun

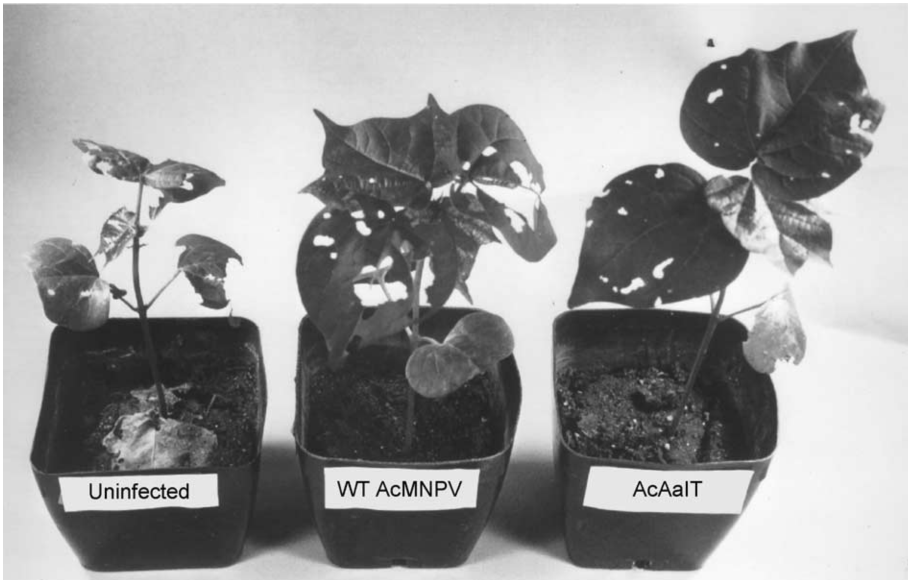


Figure 3 Damage to cotton plants by third instar larvae of *Heliothis virescens* that were inoculated with an LD_{99} dose of AcMNPV or AcAaIT or mock infected. On average, the leaf damage caused by AcAaIT infected larvae was reduced by 62% in comparison to AcMNPV infected larvae and 71.7% in comparison to mock infected larvae (Hoover *et al.*, 1995). (Reprinted with permission from Hoover, K., Schultz, C.M., Lane, S.S., Bonning, B.C., Duffey, S.S., *et al.*, **1995**. Reduction in damage to cotton plants by a recombinant baculovirus that knocks moribund larvae of *Heliothis virescens* off the plant. *Biol. Control* 5, 419–426; © Elsevier.)

et al., 2002, 2004). RoMNPV is also known as *Anagrapha falcifera* Kirby MNPV (Harrison and Bonning, 1999). Harrison and Bonning (2000b) have generated recombinant RoMNPVs (Ro6.9AaIT) expressing AaIT fused to the bombyxin signal sequence under the late *p6.9* promoter of AcMNPV. The *p6.9* promoter, like the very late promoters, requires the products of viral early genes and the initiation of viral DNA replication for its activation (Lu and Miller, 1997). However, it is activated earlier and drives higher levels of expression than the *p10* or *polh* promoters in tissue culture (Hill-Perkins and Possee, 1990; Bonning *et al.*, 1994). In dose-mortality bioassays (droplet feeding), there were no significant differences between the LC_{50}s of Ro6.9AaIT and the wild-type RoMNPV in neonate European corn borer *Ostrinia nubilalis* (8.1×10^6 and 1.0×10^7 polyhedra per ml, respectively) or *H. zea* (9.5×10^4 and 5.4×10^4 polyhedra per ml, respectively). The LC_{50}s, however, were significantly different in neonate *H. virescens* (1.67×10^5 and 8.4×10^4 polyhedra per ml, respectively). Survival-time bioassays (at an LC_{99} dose) of neonates of *O. nubilalis*, *H. zea*, and *H. virescens* infected with Ro6.9AaIT generated ST_{50}s that were reduced by approximately 34% (120 versus 181 h), 37% (81 versus 128 h), and 19% (72.5 versus 89.5 h), respectively, in comparison to control larvae infected with RoMNPV. Later in this chapter, recombinant HzNPV and HaSNPV constructs in which the AaIT gene was placed under a very late promoter or other alternative promoter (e.g., early, chimeric, or constitutive) and inserted at the *egt* gene locus of the virus are discussed.

6.9.3.1.2. Lqh and Lqq A series of highly potent insecticidal toxins have been identified and characterized from the venom of yellow Israeli scorpions, *L. quinquestriatus hebraeus* and *L. quinquestriatus quinquestriatus*. Both excitatory (e.g., LqqIT1, LqhIT1, and LqhIT5) and depressant (e.g., LqhIT2 and LqqIT2) insect selective toxins have been isolated from these scorpions (Zlotkin *et al.*, 1985, 1993; Kopeyan *et al.*, 1990; Zlotkin, 1991; Moskowitz *et al.*, 1998; see **Chapter 5.6**). Gershburg *et al.* (1998) have generated recombinant AcMNPVs expressing the excitatory LqhIT1 toxin under the very late *p10* (AcLIT1.p10) and early *p35* (AcLIT1.p35) gene promoters, and the depressant LqhIT2 toxin under the very late *polh* gene promoter (AcLIT2.pol). These constructs expressed LqhIT1 and LqhIT2 that were secreted to the outside of the cell under endogenous secretion signals. On the basis of time-mortality bioassays using neonate *H. armigera*, they found that the median effective times (ET_{50}s) for paralysis and/or death of AcLIT1.p10 and AcLIT2.pol were 66 and 59 h. This was an improvement of roughly 24% and 32%, respectively, in comparison to the wild-type AcMNPV (ET_{50} of 87 h). The ET_{50} was also reduced by about 16% (to 73 h) when LqhIT1 was expressed under the early promoter by AcLIT1.p35, although not as dramatically in comparison to AcLIT1.p10. Imai *et al.* (2000) have generated a recombinant BmNPV (BmLqhIT2) expressing LqhIT2 fused to a bombyxin signal sequence under the *polh* gene promoter. Fourth instar larvae of *B. mori* that were injected with BmLqhIT2 (2×10^5 pfu per larva) initially showed LqhIT2 specific symptoms at 48 h post injection and stopped feeding at roughly 82 h post injection. This was an improvement of roughly 35% in the ET_{50} in comparison to the wild-type BmNPV.

Harrison and Bonning (2000b) have generated recombinant AcMNPVs expressing LqhIT2 fused to a bombyxin signal sequence under the late *p6.9* (AcMLF9.LqhIT2) or very late *p10* (AcUW21.LqhIT2) gene promoters. Survival-time bioassays (at an LC_{99} dose) of neonate *H. virescens* infected with AcMLF9.LqhIT2 or AcUW21.LqhIT2 showed that the ST_{50}s (63.5 h) of larvae infected with these viruses were the same (Harrison and Bonning, 2000b). However, log-rank tests of the Kaplan-Meier survival curves of larvae infected by these viruses were significantly different ($P < 0.05$). In comparison to AcUW21.LqhIT2 infected neonate *H. virescens*, more of the AcMLF9.LqhIT2 infected neonates died at earlier times postinfection (Harrison and Bonning, personal communication). In comparison to control larvae infected with wild-type AcMNPV, AcMLF9.AaIT (recombinant AcMNPV expressing AaIT under the *p6.9* gene promoter), or AcAaIT, neonate *H. virescens* infected with AcMLF9.LqhIT2 or AcUW21.LqhIT2 showed reductions in median survival times of approximately 34% (63.5 versus 96.0 h), 10% (63.5 versus 71.0 h), and 10% (63.5 versus 70.5 h), respectively. Harrison and Bonning (2000b) have also generated recombinant RoMNPVs expressing LqhIT2 under the *p6.9* (Ro6.9LqhIT2) and *p10* (Ro10LqhIT2) promoters of AcMNPV. Ro10LqhIT2 produced visibly fewer polyhedra that potentially occluded fewer virions in Sf-9 cell cultures and thus were not used in bioassays. Survival-time bioassays (at an LC_{99} dose) in neonate larvae of the European corn borer *O. nubilalis* Hübner, *H. zea*, and *H. virescens* infected with Ro6.9LqhIT2 generated ST_{50}s that were approximately 41% (107 versus 181 h), 42% (74.5 versus 128 h), and 27% (65.5 versus 89.5 h) faster, respectively, than those generated in control neo-

nates infected with wild-type RoMNPV. The ST_{50}s of Ro6.9LqhIT2 infected neonate *H. zea* and *H. virescens* were also significantly lower than control neonates infected with a recombinant RoMNPV expressing AaIT under the *p6.9* promoter.

Chejanovsky *et al.* (1995) have generated a recombinant AcMNPV (AcLα22) that expresses the *L. quinquestriatus hebraeus* derived alpha toxin, LqhαIT (Eitan *et al.*, 1990). The LT_{50} of AcLα22 was roughly 35% faster (78 versus 120 h) than that of the wild-type AcMNPV in larvae of *H. armigera*. Since the LqhαIT toxin binds at a different site on the insect sodium channel from that of the excitatory toxins (Zlotkin *et al.*, 1978; Cestele and Catterall, 2000), Chejanovsky *et al.* (1995) suggested that a baculovirus expressing both alpha and excitatory toxins may yield a synergistic interaction between the toxins. However, they cautioned that LqhαIT toxin lacks absolute selectivity for insects (Eitan *et al.*, 1990); thus, a recombinant baculovirus expressing LqhαIT is not appropriate as a biological pesticide. The LqhαIT toxin, however, should be an effective tool to study the targeting of different types of toxins on the voltage gated sodium channel. The effectiveness of the use of multiple synergistic toxins is discussed below. Other examples of the expression of insect selective toxins from *L. quinquestriatus hebraeus* using alternative promoters, signal sequences for secretion, viral vectors, and/or insertion of the toxin gene at the *egt* gene locus are given later in this chapter.

6.9.3.2. Mite Toxins

The insect-predatory straw itch mite *Pyemotes tritici* encodes an insect paralytic neurotoxin TxP-I that induces rapid, muscle contracting paralysis in larvae of the greater wax moth *G. mellonella* (Tomalski *et al.*, 1988, 1989). TxP-I is encoded by the *tox34* gene as a precursor protein of 291 amino acid residues. The mature protein is secreted from the insect cell following cleavage of a 39 amino acid long signal sequence for secretion (Tomalski and Miller, 1991). The mode of action of TxP-I is unknown, however, it is highly toxic (even at a dose of $500\,\mu g\,kg^{-1}$) to lepidopteran larvae but not toxic to mice at a dose of $50\,mg\,kg^{-1}$. A recombinant, occlusion-negative AcMNPV (vEV-Tox34) expressing the *tox34* gene under a modified polyhedrin promoter (P_{LSXIV}; Ooi *et al.*, 1989) was shown to paralyze or kill fifth instar larvae of *T. ni* by 2 days post injection with 400 000 pfu of BV. In contrast, control larvae injected with BV of wild-type AcMNPV never showed symptoms of paralysis (Tomalski and Miller, 1991). Tomalski and Miller (1991, 1992) have constructed two other occlusion-negative AcMNPVs that express the *tox34* gene under early (vETL-Tox34) or hybrid late/very late (vCappolh-Tox34) gene promoters as well as an occlusion-positive AcMNPV (vSp-Tox34) expressing *tox34* under a different hybrid late/very late promoter. The activities of these constructs will be discussed later in this chapter. Lu *et al.* (1996) have constructed an occlusion-positive AcMNPV (vp6.9tox34) that utilizes the late *p6.9* promoter to drive expression of *tox34*. Use of this late promoter resulted in earlier, by at least 24 h, and higher level of TxP-I expression in comparison to TxP-I expression under a very late promoter. As discussed above, the *p6.9* gene promoter is not an early promoter, but is activated earlier and can drive higher levels of expression than very late promoters in tissue culture (Hill-Perkins and Possee, 1990; Bonning *et al.*, 1994). In time-mortality bioassays (at an LC_{95} dose), the median time to effect (ET_{50}) of vp6.9tox34 in neonate larvae of *S. frugiperda* and *T. ni* was reduced by approximately 56% (44.7 versus 101.3 h) and 58% (41.7 versus 99.0 h), respectively, in comparison to wild-type AcMNPV. In neonate *S. frugiperda* and *T. ni*, the earlier and higher level of TxP-I expression under the late promoter resulted in a 20–30% faster induction of effective paralysis or death in comparison to TxP-I expression under the very late gene promoter.

Burden *et al.* (2000) have constructed a slightly different TxP-I encoding gene (*tox34.4*) by RT-PCR of mRNAs purified from total RNA extracted from *P. tritici* using primers designed to amplify the *tox34* open reading of Tomalski and Miller (1991). A recombinant AcMNPV (AcTOX34.4) expressing TxP-I under the *p10* promoter has been generated. The LD_{50}s of AcTOX34.4 were not significantly different than those of the wild-type AcMNPV in dose-mortality bioassays of second (9.3 polyhedra per larva) and fourth (13.1 polyhedra per larva) instar larvae of *T. ni*. In time-mortality bioassays, second and fourth instar larvae of *T. ni* infected with AcTOX34.4 showed a 50–60% reduction (depending on virus dose and instar) in the mean time to death in comparison to control larvae infected with wild-type AcMNPV. There was also a dramatic reduction in the yield of progeny virus (number of polyhedra per μg of cadaver) at the time of death. Second and fourth instar larvae of *T. ni* that were infected with AcTOX34.4 produced roughly 85% and 95% lower yields of polyhedra per unit weight, respectively, in comparison to control larvae infected with AcMNPV. On the basis of pathogen–host model systems that describe how insect viruses may regulate host population density (as will be

discussed later in this chapter), Burden *et al.* (2000) suggested that the dramatic reduction in yield of AcTOX34.4 will cause it to be outcompeted by the wild-type AcMNPV.

6.9.3.3. Other Toxins

In addition to peptide toxins of scorpion and mite origin, insect-selective and highly potent toxins have been identified from other organisms including spiders, anemones, and bacteria. The spider *Agelenopsis aperta* and sea anemones *Anemonia sulcata* and *Stichadactyla helianthus* possess the insect selective toxins μ-Aga-IV, As II, and Sh I, respectively (Prikhod'ko *et al.*, 1996). Recombinant AcMNPVs carrying synthetic genes that express μ-Aga-IV (vMAg4p+), As II (vSAt2p+), or Sh I (vSSh1p+) under the very late P_{SynXIV} promoter have been constructed (Prikhod'ko *et al.*, 1996). The As II and Sh I toxins were each expressed as fusion peptides with a signal sequence derived from the flesh fly sarcotoxin IA, whereas μ-Aga-IV was expressed as a fusion peptide with the mellitin signal sequence of the honeybee, *Apis mellifera*. In neonate larvae of *T. ni*, the LC_{50} of vSSh1p+ was slightly lower than that of AcMNPV, but those of vMAg4p+ and vSAt2p+ were essentially identical. In contrast, in neonate larvae of *S. frugiperda* the LC_{50}s of all three viruses were increased by 3.75- to 5.25-fold in comparison to AcMNPV. In time-mortality bioassays, neonate larvae of *T. ni* and *S. frugiperda* infected (at an LC_{95} dose) with any of the recombinant viruses died more quickly than control larvae infected with AcMNPV. In neonate *T. ni* infected with vMAg4p+, vSAt2p+, and vSSh1p+, the ET_{50}s (median times to effectively paralyze or kill) were reduced by 17% (84.5 versus 102.0 h), 38% (62.8 versus 102.0 h), and 36% (65.3 versus 102.0 h), respectively. In neonate *S. frugiperda*, the ET_{50}s were reduced by 37% (65.7 versus 104.3 h), 31% (71.8 versus 104.3 h), and 35% (67.5 versus 104.3 h), respectively. Related constructs in which these toxins were expressed under a constitutive promoter or in tandem will be discussed below.

Hughes *et al.* (1997) have generated recombinant AcMNPVs expressing insect specific toxins DTX9.2 and TalTX-1 from the spiders *Diguetia canities* and *Tegenaria agrestis*, respectively. DTX9.2 was expressed by vAcDTX9.2 as a fusion protein with a signal sequence from the AJSP-1 gene of *T. ni* under the *polh* promoter. TalTX-1 (authentic signal sequence and mature protein) was expressed by vAcTalTX-1 under the *polh* promoter. In time-mortality bioassays, neonate larvae of *T. ni, S. exigua*, and *H. virescens* infected (at a less than LC_{50} dose) with vAcDTX9.2 generated ST_{50}s that were reduced by approximately 9% (62.6 versus 68.8 h), 9% (71.3 versus 78.5 h), and 10% (74.1 versus 81.9 h), respectively, in comparison to control larvae infected with a control virus (Ac-Bb1). Neonate *T. ni, S. exigua*, and *H. virescens* infected (at a less than LC_{50} dose) with vAcTalTX-1 generated ST_{50}s that were reduced by approximately 20% (55.5 versus 68.8 h), 18% (64.4 versus 78.5 h), and 19% (66.1 versus 81.9 h), respectively, in comparison to control larvae infected with Ac-Bb1. In all cases, larvae infected with the toxin expressing viruses stopped feeding prior to larvae infected with Ac-Bb1 or wild-type AcMNPV. Neonate *T. ni, S. exigua*, and *H. virescens* infected with vAcDTX9.2 showed FT_{50}s (median times to cessation of feeding) that were approximately 33, 28, and 42%, respectively, faster than control larvae infected with Ac-Bb1. Similarly, neonate *T. ni, S. exigua*, and *H. virescens* infected with vAcTalTX-1 showed FT_{50}s that were approximately 26, 17, and 37%, respectively, faster than control larvae. Interestingly, although the speed of kill of vAcTalTx-1 was faster, vAcDTX9.2 was able to stop feeding more quickly. Thus, Hughes *et al.* (1997) emphasized that enhanced speed of kill is not necessarily a reliable indicator of the enhanced speed with which feeding is stopped.

The genus *Bacillus* is composed of Gram-positive, endospore forming bacteria. During endospore formation, *Bacillus thuringiensis* (*Bt*) produces parasporal, proteinaceous, crystalline inclusion bodies that possess insecticidal properties. The biology and genetics of *Bt* and *Bt* toxins have been previously reviewed (Gill *et al.*, 1992; Schnepf *et al.*, 1998; Aronson and Shai, 2001). *Bt* encodes two major classes of lepidopteran-active toxins, cytolytic toxins and δ-endotoxins that are encoded by the *cyt* and *cry* genes, respectively. The δ-endotoxins of *Bt* are composed of large (>120 kDa) protoxin proteins that are solubilized in the alkaline conditions of the insect midgut and processed into the active toxin or toxins by proteases. The active toxins bind to specific receptors found on the insect's midgut epithelial cells and aggregate to form ion channels. Channel formation leads to disruption of the cell's transmembrane potential, which leads to osmotic cell lysis. Lethality is believed to be caused by (1) disruption of normal midgut function, which results in feeding cessation and starvation of young larvae, or (2) *Bt* septicemia.

Several studies have looked at the effectiveness of the expression of *Bt* toxin in terms of improving the insecticidal activity of the baculovirus. These studies have investigated the expression of full-length or truncated forms of *Bt* toxin genes

(e.g., *cryIAb*, *cryIAc*, etc.) that are placed under a very late promoter and expressed in AcMNPV (Martens *et al.*, 1990, 1995; Merryweather *et al.*, 1990; Ribeiro and Crook, 1993, 1998) or *Hyphantria cunea* NPV (Woo *et al.*, 1998). These studies showed that the *Bt* toxin is highly expressed by the baculovirus and subsequently processed into the biologically active form. However, *Bt* toxin expression did not improve the virulence of the virus (i.e., the LD_{50} of the recombinant virus is similar to or higher than the LD_{50} of the wild-type virus) or decrease the ST_{50} of larvae infected with the recombinant virus. These findings are not completely unexpected considering that the site of action of *Bt* toxins is the midgut epithelial cell, whereas the baculovirus expressed *Bt* protoxin is produced within the cytoplasm of cells within the insect body. Furthermore, the expressed protoxin (1) may not be digested to the active form because of the lack of appropriate proteases within the cytoplasm, (2) may be poorly secreted, or (3) the mature toxin may be cytotoxic to the cell (Martens *et al.*, 1995). In order to improve toxin secretion, several *Bt* toxin gene constructs have been generated in which a signal sequence of JHE of *H. virescens* was fused to the N-terminus of the toxin (Martens *et al.*, 1995). These toxins were translocated across the ER membrane, but they appeared to be retained within one of the ER or Golgi compartments. These findings suggest that the expression of the *Bt* toxin gene will have little or no effect in improving insecticidal activity once the virus crosses the midgut.

Chang *et al.* (2003) have taken a novel and apparently highly successful approach for the delivery of *Bt* toxin directly to the insect midgut epithelial cells using a recombinant baculovirus that occludes the toxin within its polyhedra. Their strategy was based upon the coexpression of (1) native polyhedrin and (2) a polyhedrin-foreign protein fusion from the same baculovirus (Je *et al.*, 2003). Chang *et al.* (2003) used this system to coexpress native polyhedrin and a polyhedrin-Cry1Ac-green fluorescent protein (GFP) fusion from the recombinant baculovirus ColorBtrus. ColorBtrus is a recombinant AcMNPV that produces apparently normal polyhedra that occlude *Bt* toxin and GFP. Although the Cry1Ac toxin was fused at both the N- and C-termini, this fusion protein could be digested by trypsin resulting in an immunoreactive protein that behaved identically to authentic Cry1Ac toxin. Chang *et al*. estimated that approximately 10 ng of Cry1Ac was contained in 1.5×10^6 ColorBtrus polyhedra. Furthermore, because of GFP incorporation, the ColorBtrus polyhedra fluoresced under UV light. This characteristic should allow the rapid monitoring and detection of ColorBtrus infected insects in the field (Chao *et al.*, 1996; Chang *et al.*, 2003). In dose-mortality bioassays using second or third instar larvae of the diamondback moth, *Plutella xylostella*, Chang *et al.* (2003) showed that the LD_{50} of ColorBtrus was reduced by roughly 100-fold in comparison to wild-type AcMNPV (28.3 versus 2798.3 polyhedra per larva, respectively). By time-mortality bioassays, the ST_{50} of larvae of *P. xylostella* infected with ColorBtrus was reduced by 63% (33.9 versus 92.8 h) in comparison to control larvae infected with wild-type AcMNPV when the larvae were inoculated at LD_{90} doses (i.e., 80 ColorBtrus or 10 240 AcMNPV polyhedra per larva). Chang *et al.* (2003) indicated that the ColorBtrus inoculated larvae displayed symptoms, such as feeding cessation, that were consistent with exposure to Cry1Ac. This suggested that feeding damage to the plant should cease very quickly after ingestion of ColorBtrus. Chang *et al.* (2003) further suggested that the "stacking" of multiple effectors (i.e., Cry1Ac toxicity and viral pathogenesis) by ColorBtrus should reduce the occurrence of resistance because the few individuals within the population that evolve resistance to the *Bt* toxin would also have to simultaneously evolve resistance to the baculovirus. Additionally, although the Cry1Ac toxin is highly toxic to a wide range of lepidopteran insects, it is not effective against *Spodoptera* species such as the beet armyworm *S. exigua* (Bai *et al.*, 1993). Since the beet armyworm is susceptible to AcMNPV, they suggested that this insect can be used for the efficient *in vivo* production of ColorBtrus or similar virus.

Besides the toxins mentioned here, new and interesting toxins are frequently being discovered. These novel insect specific toxins can potentially be expressed by baculoviruses. There is a great diversity of available toxins that can be expressed by GM baculoviruses either alone or in combination. We may have not yet reached the limits of improvement of the speed of kill of GM baculoviruses.

6.9.3.4. Improvement of Toxin Efficacy by Genetic Modifications

Thus far, the discussion on improving baculovirus pesticides has primarily focused on the expression of an insect selective toxin gene under late or very late baculoviral promoters. Here, the focus is on studies that have attempted to improve the efficacy of the toxin expressing constructs by (1) altering the timing and level of toxin expression, (2) improving folding and secretion efficiency of the toxin, and/or (3) expressing multiple toxins that target different

sites within the insect sodium channel. Related to this last point, studies on synergistic interactions between the expressed toxin and chemical pesticides are also discussed.

6.9.3.4.1. Alteration of the timing and level of expression In general, the first generation of recombinant baculovirus constructs used a single baculovirus derived very late (e.g., *polh* or *p10*) or late (e.g., *p6.9*) promoter to drive expression of the toxin gene (or other foreign gene). These promoters generally drive exceptionally high levels of protein expression (i.e., they are very strong promoters), but they require the products of viral early genes for activation. Thus, the very late/late promoters are not activated until a late stage of the baculovirus replication (Lu and Milller, 1997). At this late stage of infection, the translational machinery and processing pathways of the cell may be compromised (Jarvis *et al.*, 1990). Thus, a large amount of protein may be produced but not all of the protein will be functionally active due to poor folding or poor posttranslational modifications and the protein may also be retained within the cell due to poor secretion. Baculovirus early gene promoters, alternatively, are recognized by host transcription factors and are activated very soon after the viral DNA is uncoated in the nucleus. At this early stage of infection, the processing and secretory pathways of the cell should be in a less compromised state (in comparison to the late stage of infection). Thus, it is likely that a higher percentage of the expressed proteins will be properly folded, modified, and secreted. Earlier expression may be especially important for the proper folding of neurotoxins because they possess a relatively large number of disulfide bonds (e.g., the 70 amino acid-long AaIT peptide contains four disulfide bonds). Early gene promoters, however, generally drive very low levels of protein expression (i.e., they are weak promoters).

Several groups have attempted to circumvent the potential problems associated with protein expression under very late promoters using: (1) early gene promoters; (2) dual or chimeric promoters; and/or (3) constitutively expressed promoters to drive the expression of a foreign gene. Jarvis *et al.* (1996) have constructed a recombinant AcMNPV (*ie1*-AaIT) expressing the AaIT gene under the control of an immediate early gene (*ie1*) promoter of AcMNPV. In cultured Sf-9 cells infected with the *ie1*-AaIT construct, AaIT was detected as early as 4 h p.i. and continued to accumulate until at least 24 h p.i. At 24 h p.i., the amount of the expressed AaIT peaked and was about equal to that expressed by AcAaIT (AaIT expressed under the very late *p10* promoter). *In vitro* expression assays indicated that there was early and abundant expression of AaIT under the *ie1* promoter. These findings suggested that the *ie1*-AaIT construct should kill infected larvae more quickly than the AcAaIT construct. The results of the insect bioassays using neonate *H. virescens*, however, did not support this. Time-mortality bioassays showed that the *ie1*-AaIT virus killed neonate *H. virescens* only about 10% faster than the wild-type AcMNPV (LT_{50} of 87.0 versus 96.5 h). In comparison, AcAaIT killed the neonate *H. virescens* about 22% faster than AcMNPV (LT_{50} of 75.0 versus 96.5 h) in parallel experiments. Although Jarvis *et al.* (1996) were unclear as to the reason(s) for the slower speed of kill of *ie1*-AaIT in comparison to AcAaIT, they speculated that: (1) *ie1*-AaIT does not produce a threshold dose of AaIT that must be reached before kill rates can be enhanced; (2) duplication of the *ie1* promoter usurps limited cellular transcription factors needed by the endogenous *ie1* promoter for expression of the IE1 protein; (3) early expression of AaIT in midgut epithelial cells induces these cells to be shed thereby reducing the primary infection in these cells and/or dissemination of the virus into the hemocoel; and (4) the *ie1* promoter of AcMNPV has a narrower host range and/or tissue specificity in comparison to the virus as a whole. Interestingly, although *ie1*-AaIT infected larvae did not die as quickly as AcAaIT infected larvae, the relative growth rate of the *ie1*-AaIT infected larvae was about 13% lower (0.8 versus 0.92 mg per larva per day) than that of AcAaIT infected larvae. This suggested that even though they do not die as quickly, the *ie1*-AaIT infected larvae may consume less food.

van Beek *et al.* (2003) have compared the efficacy of recombinant AcMNPVs expressing either AaIT or LqhIT2 under various baculoviral early (*ie1* or *lef3*), early/late (*39K*), or very late (*p10*) gene promoters in larvae of *H. virescens, T. ni,* and *S. exigua*. The larvae were inoculated at both low (approximately LD_{50}) and high (approximately 100 times the LD_{50}) doses of each recombinant virus. By time-to-response bioassays using neonate and third instar *H. virescens* or *T. ni*, and second instar *S. exigua*, they found that the ET_{50} of the recombinant AcMNPV expressing AaIT under the early *hr5*/*ie1* promoter led in the majority of cases to a faster response, but a generalized pattern of response in terms of the effect of viral dose and instar was not found. The "*hr5*" designation indicates that the baculovirus *hr5* enhancer sequence was placed immediately upstream of the promoter sequence. By bioassays using third instar larvae of *H. virescens*, van Beek *et al.* found that there were no signifi-

cant differences in the ET_{50} of the recombinant AcMNPVs expressing LqhIT2 under an early (*hr5/ie1* or *hr5/lef3*) or early/late (*hr5/39K*) gene promoter. Furthermore, regardless of the larval instar or dose applied, when the early *hr5/ie1* promoter was used to drive the expression of LqhIT2 or AaIT, the ET_{50}s of the recombinant AcMNPV expressing LqhIT2 were consistently lower (by 8–32 h) in comparison to those of the recombinant AcMNPV expressing AaIT. This was putatively due to differences in the effectiveness of the two toxins.

As described above, Gershburg *et al.* (1998) have constructed recombinant AcMNPVs expressing the excitatory toxin LqhIT1 or depressant toxin LqhIT2 under either the early *p35* gene or very late *p10* gene promoters. They found that the recombinant AcMNPV expressing LqhIT1 under the *p35* promoter had a slightly slower ET_{50} (73 versus 66 h) in comparison to the recombinant AcMNPV expressing LqhIT1 under the *p10* promoter. However, they detected a clear paralytic effect when the *p35* promoter was used to express LqhIT1 even when they were unable to detect the LqhIT1 by immunochemical analysis. Thus, they suggested that baculovirus expression of toxin in cells that are adjacent to the target sites of the motor neural tissues overcomes the pharmacokinetic barriers that the toxin may face when, for example, purified toxin is injected to the insect. Consistent with this hypothesis, Elazar *et al.* (2001) have shown that the concentration of AaIT in the hemolymph of paralyzed *B. mori* larvae is approximately 50-fold lower when the paralyzing dose is delivered by a baculovirus (BmAaIT) rather than by the direct injection of purified AaIT. They hypothesized that baculovirus expression of AaIT provides a continuous, local supply of freshly produced toxin (via the tracheal system) to the neuronal receptors, thus providing access to critical target sites that are inaccessible to toxin that is injected. Therefore, a lower level of continuous expression under an early promoter may be sufficient to elicit a paralytic response.

As discussed above, Tomalski and Miller (1992) have constructed an occlusion-negative recombinant AcMNPV (vETL-Tox34) that expresses the *tox34* gene under the baculoviral early P_{ETL} promoter. In early fifth instar larvae of *T. ni*, this virus induced much slower paralysis (occurring after 48 h in 95% of larvae) in comparison to control larvae infected with recombinant AcMNPVs (vEV-Tox34, vCappolh-Tox34, or vSp-Tox34) expressing *tox34* under a very late or chimeric promoter (all larvae were paralyzed or dead by 48 h). Additionally, the average cumulative weight gain of vETL-Tox34 infected larvae at 24 h post injection was not significantly different to that of control larvae injected with AcMNPV or cell culture medium (mock infection). Lu *et al.* (1996) have also constructed a recombinant AcMNPV (vDA26tox34) expressing the *tox34* gene under the early *Da26* gene promoter. In comparison to vp6.9tox34 (*tox34* under a late promoter) or vSp-tox34 (*tox34* under a very late promoter) infected Sf-21 or TN-368 cells (derived from *S. frugiperda* and *T. ni*, respectively), TxP-I expression was detected at least 24 h earlier (but at dramatically lower levels) in Sf-21 and TN-368 cells infected with vDA26tox34. In time-mortality bioassays (at an LC_{95} dose), the ET_{50} of vDA26tox34 in neonate larvae of *S. frugiperda* and *T. ni* was reduced by approximately 39% (61.8 versus 101.3 h) and 28% (71.2 versus 99.0 h), respectively, in comparison to wild-type AcMNPV. However, this was 17.1 and 29.5 h slower, respectively, in comparison to neonate larvae of *S. frugiperda* and *T. ni* that were infected with vp6.9tox34 (ET_{50}s of 44.7 and 41.7 h, respectively). Even though the TxP-I is expressed significantly earlier under the early promoter, it is apparently not expressed in sufficient quantity, at least initially, to paralyze larvae of *S. frugiperda* or *T. ni*. Popham *et al.* (1997) have also expressed the *tox34* gene under the early *DA26* and late *p6.9* promoters (of AcMNPV) by recombinant HzSNPVs (HzEGTDA26tox34 and HzEGTp6.9tox34, respectively). They found that the ET_{50}s of HzEGTDA26tox34 and HzEGTp6.9tox34 in neonate larvae of *H. zea* were reduced by approximately 47% (35.4 versus 67.3 h) and 44% (38.0 versus 67.3 h), respectively, in comparison to a control virus. In contrast to the results of Lu *et al.* (1996), the early promoter was more effective in the case of HzSNPV and neonate *H. zea* suggesting that the effectiveness of a promoter will be virus and insect specific. With toxins of current potency, the use of early (and weak) promoters such as *ie1*, *lef3*, *p35, etl*, or *DA26* may or may not provide any benefit in terms of improved crop protection. However, if toxins that act at lower concentration (i.e., with much greater potency) are identified, the use of early promoters may offer a great advantage.

Tomalski and Miller (1992) have used a chimeric promoter ($P_{cap/polh}$) comprised of both the late capsid and very late polyhedrin promoter elements to drive the expression of the *tox34* gene in an occlusion-negative recombinant AcMNPV (vCappolh-Tox34). In Sf-21 cell cultures infected with vCappolh-Tox34, TxP-I is detected in the culture medium at 12 h p.i. at levels that are similar to those found in vEV-Tox34 (*tox34* under a very late promoter) infected Sf-21 cells at 24 h p.i. (i.e., high-

level expression occurred approximately 12 h earlier). When early fifth instar larvae of *T. ni* were injected with vCappolh-Tox34 approximately 50% of the insects were paralyzed by 24 h post injection. In comparison, only 10% or less of control virus (e.g., vEV-Tox34) infected larvae were paralyzed. Larvae infected with vCappolh-Tox34 also showed a significantly lower cumulative weight gain in comparison to control virus infected larvae. An occlusion-positive construct (vSp-Tox34) in which the *tox34* gene was placed under another hybrid late/very late promoter (P_{SynXIV}; Wang *et al.*, 1991) has also been constructed by Tomalski and Miller (1991, 1992). The induction of paralysis by vSp-Tox34 in fifth instar *T. ni* was slightly delayed, but all of the larvae were paralyzed or dead by 48 h post injection (as were vCappolh-Tox34 injected larvae; Tomalski and Miller, 1991, 1992). In time-mortality bioassays (at an LC_{95} dose), the ET_{50} of vSp-Tox34 in neonate larvae of *S. frugiperda* and *T. ni* was reduced by approximately 45% (55.4 versus 101.3 h) and 41% (58.5 versus 99.0 h), respectively, in comparison to control larvae infected with AcMNPV (Tomalski and Miller, 1992; Lu *et al.*, 1996). Furthermore, the yield (2.1×10^9 versus 3.5×10^9 polyhedra per larva) of vSP-Tox34 polyhedra from infected last instar larvae of *S. frugiperda* was reduced by approximately 40% in comparison to AcMNPV infected control larvae. Tomalski and Miller (1992) suggested that this reduction in yield will cripple the virus in terms of its ability to compete effectively with the wild-type virus in the environment.

Sun *et al.* (2004) have generated a recombinant HaSNPV (HaSNPV-AaIT) that expresses the AaIT gene under a chimeric promoter (ph-p69p) consisting of the late *p6.9* gene promoter inserted immediately downstream of the very late *polh* gene promoter in the same direction. The AaIT gene cassette was inserted at the *egt* gene locus of HaSNPV. Detailed dose-mortality bioassays indicated that the LD_{50}s of HaSNPV-AaIT was unchanged compared to wild-type (HaSNPV-WT) or *egt* gene-deleted (HaSNPV-EGTD) viruses in first to fifth instar larvae of the cotton bollworm, *H. armigera*. In time-mortality bioassays, first to fifth instar larvae of *H. armigera* infected with HaSNPV-AaIT showed reductions in the ST_{50}s of 10% (68.5 versus 76.5 h), of 20% (60.5 versus 75.5 h), 8% (71.0 versus 77.5 h), 4% (104.6 versus 109.0 h), and 10% (108.5 versus 121.0 h), respectively, in comparison to control larvae infected with HaSNPV-EGTD. In comparison to control (first to fifth instar) larvae infected with HaSNPV-WT, the corresponding reductions were 19, 28, 25, 21, and 18%, respectively. In third to fifth instar of *H. armigera* infected with HaSNPV-AaIT, the median times to feeding cessation (FT_{50}s) were reduced by 25% (51.5 versus 68.5 h), 22% (66.5 versus 85.0 h), and 12% (84.5 versus 96.5 h), respectively, in comparison to control larvae infected with HaSNPV-EGTD. In comparison to control larvae infected with HaSNPV-WT, the corresponding reductions in the FT_{50}s were 39%, 43%, and 30%, respectively. The role of the *egt* deletion in improving speed of kill is discussed further below. Sun *et al.* (2004) did not test constructs in which only the *polh* or *p6.9* gene promoter was used to drive expression of the AaIT gene, thus it was not possible to predict if expression under the dual promoter is more effective than expression under a single late or very late promoter.

The *hsp70* promoter of *D. melanogaster* (Snyder *et al.*, 1982) is constitutively active in insect cells (Vlak *et al.*, 1990; Zuidema *et al.*, 1990). Unlike very late or late gene promoters of the baculovirus, the *hsp70* promoter does not require the expression of baculovirus early gene products or the initiation of viral DNA synthesis for its activity. The *hsp70* promoter shows higher activity than early gene promoters, but it is not as active as baculoviral very late or late gene promoters (Morris and Miller, 1992, 1993). McNitt *et al.* (1995) have constructed occlusion-positive, recombinant AcMNPVs expressing AaIT (vHSP70AaIT) and TxP-I (vHSP70tox34) under the *hsp70* promoter. TxP-I was expressed at a significantly higher level under the *hsp70* promoter than under the early *DA26* promoter in both vHSP70tox34 infected Sf-21 and TN-368 cell cultures (McNitt *et al.*, 1995; Lu *et al.*, 1996). Additionally, TxP-I expression was detected as early as 6 and 12 h p.i., respectively, in the culture medium of vHSP70tox34 infected Sf-21 and TN-368 cells. Dose-mortality bioassays using neonate larvae of *S. frugiperda* or *T. ni* indicated that the LC_{50}s of vHSP70tox34 were unchanged from that of the AcMNPV. Time-mortality bioassays (at an LC_{95} dose) in neonate *S. frugiperda* and *T. ni* showed that the ET_{50}s of vHSP70tox34 were reduced by approximately 59% (41.8 versus 101.3 h) and 46% (53.8 versus 99.0 h), respectively, in comparison to control larvae infected with AcMNPV. In comparison to occlusion-positive, recombinant AcMNPVs expressing the *tox34* gene under very late (vSp-tox34) or late (vp6.9tox34) promoters, the ET_{50}s of vHSP70tox34 were faster than both vp6.9tox34 (44.7 h) and vSp-tox34 (55.4 h) in neonate *S. frugiperda*; and faster than vp6.9tox34 (41.7 h) and slower than vSp-tox34 (58.5 h) in neonate *T. ni*. This is surprising considering that the

overall level of TxP-I secretion under the *hsp70* promoter is substantially lower than that under the *p6.9* promoter (Lu *et al.*, 1996). On the basis of these findings Lu *et al.* (1996) suggested that expression from a relatively strong constitutive promoter such as *hsp70* is more effective, in some cases, than expression from a very strong late baculoviral promoter. Popham *et al.* (1997) have expressed the *tox34* gene under the *hsp70* promoter (HzEGT hsptox34) at the *egt* gene locus of a recombinant HzSNPV. Time-mortality bioassays (at an LC_{95} dose) in neonate *H. zea* showed that the ET_{50} of HzEGThsptox34 was reduced by 34% (44.1 versus 67.3 h) in comparison to control larvae infected with an *egt* deletion mutant of HzSNPV (HzEGT-del). In contrast to the results of Lu *et al.* (1996), however, expression of the *tox34* gene under the *hsp70* promoter was not as effective as expression under the early *Da26* (ET50 of 35.4 h) or late *p6.9* (ET_{50} of 38.0 h) promoters (both of these promoters were of AcMNPV origin).

The *hsp70* promoter has also been used to drive the expression of spider and sea anemone toxins. Prikhod'ko *et al.* (1998) have constructed recombinant AcMNPVs vhsMAg4p+, vhsSAt2p+, and vhsSSh1p+ that express the insect selective toxins μ-Aga-IV, As II, and Sh I, respectively, under the *hsp70* promoter. All of these constructs showed significantly reduced ET_{50}s (at an LD_{95} dose) in comparison to AcMNPV in neonate larvae of *S. frugiperda* and *T. ni*. In neonate *S. frugiperda* infected with vhsMAg4p+, vhsSAt2p+, or vhsSSh1p+, the ET_{50}s were reduced by approximately 55% (49.0 versus 110 h), 52% (52.5 versus 110 h), and 53% (51.9 versus 110 h), respectively. In neonate *T. ni*, the ET_{50}s were reduced by 42% (53.6 versus 99.7 h), 50% (49.0 versus 99.7 h), and 46% (52.9 versus 99.7 h), respectively. In both larval species, expression of μ-Aga-IV (*mag4* gene), As II (*sat2* gene), and Sh I (*ssh1* gene) under the *hsp70* promoter was more effective than expression under the very late P_{SynXIV} promoter (ET_{50}s of 60.0, 63.6, and 56.8 h, respectively, in *S. frugiperda*, and 84.4, 62.8, and 60.6 h, respectively, in *T. ni*).

Numerous studies (including several that are not included in our discussion above) have investigated the effectiveness of constitutive and baculoviral promoters (early, late, and very late) used either alone or in combination for the expression of the current generation of toxin genes. These studies, however, do not form a consensus opinion as to which promoter system is the most optimal in terms of improving insecticidal effectiveness of the toxin expressing baculovirus. The toxin gene, parental virus, and host-specific factors all appear to play important roles in determining the effectiveness of a particular promoter system. Given that an early or constitutive promoter does not provide a clear advantage (at least within the context of toxin genes of current potency), the use of a baculoviral late or very late promoter may provide a slight reduction in the risk that a toxin gene could be expressed in a nontarget insect. The argument here is that very late and late gene promoters are inactive in beneficial insects and other organisms (Heinz *et al.*, 1995; McNitt *et al.*, 1995) because the activation of these promoters requires (1) viral DNA replication and (2) late expression factors (LEF; Lu and Milller, 1997). In AcMNPV there are 18 *lef* genes that are required for optimal transactivation of expression from late and very late gene promoters. At least seven of the 18 *lef* genes appear to be involved in DNA replication and at least three *lef* gene products are part of a virus induced or virus encoded RNA polymerase. This RNA polymerase is required for transcription from late and very late genes. Thus, without viral DNA replication and the expression of essential *lef* genes, transcription from a late or very late gene promoter will not occur. In contrast, early and constitutive gene promoters can potentially be activated in nontarget insect cells (Morris and Miller, 1992, 1993; McNitt *et al.*, 1995). A discussion of the potential risks associated with gene transfer to nontarget insects and other organisms (e.g., mammals) is given below.

6.9.3.4.2. Better secretion and folding In order for a peptide or protein to be secreted to the outside of a eukaryotic cell, it must possess a signal sequence consisting of 15–30 hydrophobic amino acid residues at its N-terminal. Following the initiation of protein synthesis by ribosomes within the cytoplasm, the newly synthesized signal sequence is bound by a signal recognition particle (SRP). Subsequently, the SRP signal sequence–ribosome complex binds to a SRP receptor on the cytosolic surface of the rough ER membrane. The signal sequence peptide is then able to cross the ER membrane (and is generally cleaved off within the lumen of the ER) and the remainder of the protein is produced and secreted into the lumen of the ER. Once inside the ER lumen the peptide can undergo appropriate disulfide bond formation and folding. The mature protein then continues to the Golgi apparatus for subsequent release via secretory vesicles to the outside of the cell. In general, once inside the ER lumen, proteins are automatically transported through the Golgi apparatus and secreted (i.e., this is thought to be the default pathway) unless they possess a specific signal that directs them to another location.

Thus, the ability of the insect cell SRP to recognize the signal sequence on the baculovirus expressed protein is a key determinant for its eventual secretion to the outside of the cell. Signal sequences that originate from a variety of organisms (e.g., mammal, plant, yeast, insect, virus, etc.) are recognized by lepidopteran SRPs and can help direct baculovirus expressed proteins to the ER for subsequent processing and secretion from the cell (O'Reilly *et al.*, 1992). Signal sequences derived from proteins of insect origin, e.g., bombyxin from the silk moth *B. mori* (Adachi *et al.*, 1989), mellitin from the honeybee *A. mellifera* (Tessier *et al.*, 1991), and cuticle protein II (CPII) from *D. melanogaster* (Snyder *et al.*, 1982), have been shown to be functional when attached to a wide variety of proteins.

In order to improve processing, folding, and/or secretion of baculovirus expressed toxin, van Beek *et al.* (2003) have generated recombinant AcMNPVs expressing LqhIT2 (under the early *hr5/ie1* promoter) fused to various secretion signals of insect and noninsect origin. Signal sequences originating from bombyxin, CPII, adipokinetic hormone from *M. sexta* L. (Jaffe *et al.*, 1986), chymotrypsin of *Lucilia cuprina* (Weidemann) (Casu *et al.*, 1994), AcMNPV gp67 (Whitford *et al.*, 1989), and scorpion neurotoxins BjIT from *Hottentota judaicus* (Simon) (Zlotkin *et al.*, 1993), AaIT (Bougis *et al.*, 1989), and LqhIT2 (Zlotkin *et al.*, 1993) were tested. In time-mortality bioassays in third instar larvae of *H. virescens* (at a dose of polyhedra that was estimated to elicit a response in less than 50% of the larvae), they found that a recombinant AcMNPV expressing LqhIT2 fused to the bombyxin signal sequence elicited the fastest response time (ET_{50} of 62 h, estimate based on the graph). Recombinant AcMNPVs expressing LqhIT2 fused to the signal sequences of adipokinetic hormone (ET_{50} of 78 h), chymotrypsin (ET_{50} of 78 h), LqhIT2 (ET_{50} of 85 h), and gp67 (ET_{50} of 72 h) elicited intermediate response times. All of these viruses putatively expressed and secreted active toxin as evidenced by reduced survival times and the occurrence of paralysis. In contrast, recombinant AcMNPV expressing LqhIT2 fused to the signal sequences of CPII, BjIT, and AaIT appeared not to secrete active toxin because insects infected with these constructs exhibited symptomology that is typical of AcMNPV infection. In general, they found that the baculoviral gp67 or insect derived signal sequences (excluding CPII) worked better than the scorpion toxin derived signal sequences for the secretion of biologically active LqhIT2.

Lu *et al.* (1996) have constructed three recombinant AcMNPVs (vSp-BSigtox34, vSp-DCtox34, and vSp-tox21A/tox34) that express TxP-I (under the very late P_{SynXIV} promoter) fused to signal sequences originating from sarcotoxin IA of the flesh fly (Matsumoto *et al.*, 1986), CPII, and *tox21A* (a homolog of *tox34*) (Tomalski *et al.*, 1993), respectively. The levels of TxP-I secreted into the culture medium of Sf-21 cells infected with vSp-DCtox34 or vSp-tox21/tox34 were similar to control cells infected with vSp-tox34 (TxP-I fused to its own signal sequence). TxP-I secretion was undetectable on the basis of Western blotting in Sf-21 cell cultures infected with vSp-BSigtox34. By comparing the levels of TxP-I that were found within the cell (i.e., cell lysate) and in the culture medium (i.e., secreted), it appeared that the CPII secretion signal (vSp-DCtox34) was better than the *tox21A* signal sequence (vSp-tox21/tox34) in terms of being able to direct a higher percentage of the expressed TxP-I to the outside of the cell. TxP-I was only detected at very low levels within the cell lysates of Sf-21 cells infected with vSp-BSigtox34, thus it is difficult to determine whether the lack of TxP-I in the cell culture medium was the result of poor secretion or poor translation. In dose-mortality bioassays using neonate larvae of *T. ni*, the LC_{50}s of vSp-BSigtox34 and vSp-DCtox34 were similar to that of AcMNPV, whereas the LC_{50} of vSp-tox21A/tox34 was roughly double (5.3×10^4 versus 2.2×10^4 polyhedra per ml) that of AcMNPV. In time-mortality bioassays (at an LC_{95} dose) in neonate *T. ni*, the ET_{50}s of vSp-BSigtox34, vSp-DCtox34, and vSp-tox21A/tox34 were reduced by 26% (70.0 versus 94.6 h), 47% (49.9 versus 94.6 h), and 36% (60.8 versus 94.6 h), respectively, in comparison to control larvae infected with AcMNPV. However, in comparison to neonate *T. ni* infected with vSp-tox34 (a control virus expressing TxP-I fused to its authentic secretion signal, ET_{50} of 51.1 h), neonate *T. ni* infected with viruses expressing TxP-I fused to an alternative signal sequence did not show any improvements in ET_{50}. Additionally, although TxP-I secretion was undetected in Sf-21 cells infected with vSP-BSigtox34, neonate *T. ni* infected with this virus displayed paralysis indicating that the threshold level of toxin required for paralysis is low or that some cells within the larvae may more efficiently produce toxin. On the basis of these studies, Lu *et al.* (1996) suggested that the level of toxin secreted into the supernatant is generally diagnostic of how the virus will perform *in vivo*.

Chaperones found in the cytosol and ER (such as BiP, murine immunoglobulin heavy chain binding protein; Bole *et al.*, 1986) play key roles in protein folding, transport, and quality control in a wide

range of eukaryotic cells including insect cells (Ruddon and Bedows, 1997; Ailor and Betenbaugh, 1999; Trombetta and Parodi, 2003). The coexpression of BiP by a recombinant baculovirus has been shown to reduce aggregation and increase secretion of baculovirus expressed murine IgG in *T. ni* derived cells (Hsu *et al.*, 1994). Taniai *et al.* (2002) have investigated whether coexpression of BiP can improve the secretion of AcAaIT expressed AaIT (rAaIT) in Sf-21 cells. They found that coexpression of BiP increased the amount of soluble rAaIT, however, the amount of active rAaIT that was secreted into the culture medium was not improved. Protein disulfide isomerase (PDI) is another factor within the ER that is known to help protein folding and secretion in insect cells by catalyzing the oxidization, reduction, and isomerization of disulfide bonds *in vitro* (Hsu *et al.*, 1996; Ruddon and Bedows, 1997). Coexpression of a PDI expressing baculovirus with an AaIT expressing baculovirus was also ineffective in increasing the amount of active AaIT that was secreted into the culture medium (Taniai, Inceoglu, and Hammock, unpublished data).

6.9.3.4.3. Expression of multiple synergistic toxins At least six distinct receptor sites are found on the voltage gated sodium channels of mammals and insects that are the molecular targets of a broad range of neurotoxins. Two additional receptor sites are found on the insect sodium channel that are the molecular targets of insect selective excitatory and depressant scorpion toxins (Cestele and Catterall, 2000). On the basis of binding experiments using radiolabeled toxins, the depressant toxins bind to two noninteracting binding sites (one showing high affinity and the other low affinity) on the insect sodium channel (Gordon *et al.*, 1992; Zlotkin *et al.*, 1995; Cestele and Catterall, 2000). The excitatory toxins bind only to the high-affinity receptor site (Gordon *et al.*, 1992). Herrmann *et al.* (1995) have shown that when excitatory and depressant toxins are simultaneously coinjected into larvae of the blowfly *S. falculata* or *H. virescens*, the amount of toxin required to give the same paralytic response is reduced five- to tenfold in comparison to the amount required when only one of the toxins is injected. On the basis of this synergism, they suggested that speed of kill of recombinant baculovirus(es) could be further reduced by: (1) the coinfection of two or more recombinant baculoviruses that each express toxin genes with synergistic properties; or (2) genetic modification such that two or more synergistic toxin genes are simultaneously expressed.

Regev *et al.* (2003) have generated a recombinant AcMNPV (vAcLqIT1-IT2) that expresses both the excitatory LqhIT1 and the depressant LqhIT2 toxins under the very late *p10* and *polh* promoters, respectively. Time-response bioassays (at an LC_{95} dose) using neonate *H. virescens* showed that the ET_{50} of vAcLqIT1-IT2 was reduced by 41% (46.9 versus 79.1 h) in comparison to AcMNPV. In comparison, the ET_{50}s of recombinant AcMNPVs expressing only LqhIT1 (vAcLqIT1) or only LqhIT2 (vAcLqIT2) were 57.3 and 60.0 h, respectively. Thus, expression of both LqhIT1 and LqhIT2 by vAcLqIT1-IT2 resulted in a synergistic effect that reduced the ET_{50} by 10.4 h (18%) and 13.1 h (22%), respectively. A synergistic effect (15–19%) was also observed following coinfection of neonate *H. virescens* with a mixture of both vAcLqIT1 and vAcLqIT2 (ET_{50} of 48.8 h). Time-response bioassays (at an LC_{95} dose) using neonate *H. armigera* showed that the ET_{50} of vAcLqIT1-IT2 was reduced by 24% (69.1 versus 90.8 h) in comparison to AcMNPV. However, a synergistic effect was not elicited in comparison to neonate *H. armigera* infected with vAcLqIT1 or vAcLqIT2 alone. Since *H. armigera* is only a semi-permissive host of AcMNPV, Regev *et al.* (2003) hypothesized that inefficient oral infection may have diminished any synergistic effect. By intrahemocoelic injection (at an LD_{95} dose) of third instar larvae of *H. armigera*, they found that the ET_{50} of vAcLqIT1-IT2 (72 h) was reduced by 13 h (15%) and 70 h (49%) in comparison to larvae injected with only vAcLqIT1 or vAcLqIT2, respectively. In similar intrahemocoelic injection assays of third instar larvae of *S. littoralis* (nonpermissive by oral infection for AcMNPV), they found that the ET_{50} of vAcLqIT1-IT2 (54 h) was reduced by 43 h (44%) and 20 h (27%) in comparison to control larvae injected with only vAcLqIT1 or vAcLqIT2, respectively. Similar or decreased levels of synergism were found with a recombinant AcMNPV (vAcLqαIT-IT2) expressing both alpha and depressant scorpion toxins in orally and intrahemocoelically infected larvae of *H. virescens*, *H. armigera*, and *S. littoralis*.

Using thoracic neurons of the grasshopper *Schistocerca americana* Drury, Prikhod'ko *et al.* (1998) have shown that the spider and sea anemone toxins, μ-Aga-IV and As II, respectively, act at distinct sites on the insect sodium channel and synergistically promote channel opening. These toxins also showed synergism when injected into third instar blowfly *Lucilia sericata* and fourth instar fall armyworm *S. frugiperda*. Prikhod'ko *et al.* (1998) have generated recombinant AcMNPVs (vMAg4Sat2 and vhsMAg4SAt2) that coexpress both μ-Aga-IV and As II toxins under P_{SynXIV} or *hsp70* promoters, respectively. Time-response bioassay (at an LC_{95}

dose) using neonate *T. ni* or *S. frugiperda* showed that the ET_{50} of vMAg4Sat2 was reduced by 39% (60.6 versus 99.7 h) and 45% (56.8 versus 104.0 h), respectively, in comparison to AcMNPV. A synergistic effect, however, was not found in comparison to larvae infected with recombinant AcMNPVs expressing only μ-Aga-IV (vMAg4p+) or As II (vDASAt2). In similar bioassays, the ET_{50} of vhsMAg4SAt2 was reduced by 47% (52.3 versus 99.2 h) and 57% (48.3 versus 111.1 h), respectively, in comparison to AcMNPV. A synergistic effect, however, was again not found in comparison to larvae infected with vhsMAg4p+ or vDAhsSAt2. They hypothesized that synergism was not detected in the case of coexpression of these toxins under the very late P_{SynXIV} promoter (by vMAg4Sat2) because it takes time to activate this promoter during infection and, once activated, high enough levels of expression of either one of the toxins is sufficient to effectively debilitate the host insect. In the case of coexpression under the *hsp70* promoter by vhsMAg4SAt2, they hypothesized that lower ET_{50}s (i.e., a synergistic effect) may not be possible since it takes a minimum period of time (i.e., approximately 50 h) for the virus to initiate both primary and secondary infections within the host. Although synergism was not observed in terms of the ET_{50} (mean time at which 50% of the test larvae cease to respond to stimulus), synergism was observed in terms of the FT_{50} (mean time at which 50% of the test larvae cease feeding) of neonate *T. ni* infected with vhsMAg4SAt2 (at an LC_{95} dose by droplet feeding). The FT_{50} of vhsMAg4SAt2 infected neonates was lower than that of vMAg4p+, vDASAt2, and AcMNPV by approximately 25% (24.3 versus 32.3 h), 29% (24.3 versus 34.3 h), and 63% (24.3 versus 65.8 h). Prikhod'ko *et al.* (1998) suggested that 24 h may be the fastest time at which a recombinant baculovirus could stop feeding because of the time (approximately 12 h) that it takes to complete the primary infection cycle within the midgut. Furthermore, they suggested that an FT_{50} of 24 h is sufficiently rapid to make recombinant baculovirus pesticides competitive with chemical pesticides.

6.9.3.5. Toxin Interactions with Chemical Pesticides

Pyrethroids, carbamates, and organophosphates are common synthetic chemical pesticides that are used for protection against pest insects worldwide. Pyrethroids currently account for about 20% of the total market for insecticides (Khambay, 2002). Pyrethroids are the most commonly used chemical insecticides for the control of the tobacco budworm *H. virescens* and cotton bollworm *H. zea* in North America. Pyrethroids are divided into two groups, type I and type II, on the basis of the absence or presence, respectively, of a cyano (-CN) group in the alpha position of the carboxyl moiety. Both type I and type II pyrethroids target receptors in the sodium channel causing them to remain open and resulting in prolonged sodium influx that causes hyper-excitation of the nervous system of both insects and mammals. Insects, however, are more sensitive to pyrethroid action than mammals due to several factors including the relative concentration of pyrethroid metabolizing enzymes, binding kinetics of the pyrethroid to the receptor, and differential sensitivity of the insect and mammalian sodium channels to pyrethroid action (Soderlund *et al.*, 2002). Although the precise location of the pyrethroid receptor within the insect sodium channel is unknown, the binding site is apparently different from that of peptide toxins produced by spiders, sea anemones, and scorpions (Ghiasuddin and Soderlund, 1985; Herrmann *et al.*, 1995). The differential binding of pyrethroid and toxin molecules indicate that when they are both present within the sodium channel they can act independently, synergistically, or antagonistically.

McCutchen *et al.* (1997) have characterized the effect of low concentrations of six chemical pesticides (allethrin, cypermethrin, DDT, endosulfan, methomyl, and profenofos) on the efficacy of wild-type and recombinant baculoviruses. They found synergistic interactions when neonate *H. virescens* were exposed to AcAaIT and low concentrations (LC_{10}–LC_{20} at 24 h) of the type II pyrethroid (cypermethrin) and a carbamate (methomyl). The interactions of AcAaIT with allethrin, DDT, endosulfan, or profenofos were not synergistic but additive. Specifically, neonate *H. virescens* that were inoculated with 2000 polyhedra per larva (a greater than LC_{99} dose) of AcAaIT and immediately exposed to an LC_{10}–LC_{20} dose of cypermethrin or methomyl showed an LT_{50} of 30 h, a reduction of roughly 58% compared to control larvae infected with the virus alone. Neither synergistic nor antagonistic effects were observed in control experiments in which AcJHE.KK (a recombinant AcMNPV expressing a modified juvenile hormone esterase) was used in place of AcAaIT. Since the mode and site of action of JHE is putatively different from that of AaIT, this was not surprising. In contrast, the interactions of wild-type AcMNPV and the chemical pesticides (except for methomyl) were slightly antagonistic. McCutchen *et al.* (1997) have also examined the efficacy of AcAaIT in combination with a low dose of pyrethroid in a pyrethroid resistant strain (PEG strain) of *H. virescens*. Pyrethroid resistant neonate

H. virescens that were infected with AcAaIT (at a greater than LC_{99} dose) died approximately 11% faster (LT_{50} of 63 versus 71 h) than pyrethroid sensitive neonate *H. virescens* infected with AcAaIT. Furthermore, this decrease in the LT_{50} was not found in pyrethroid resistant neonates that were infected with wild-type AcMNPV (LT_{50}s of 94 and 92 h in the resistant and sensitive strains, respectively). McCutchen *et al.* (1997) thus suggested that the pyrethroid resistant larvae are more sensitive to the neurotoxin AaIT. They further suggested that the use of AcAaIT or other recombinant baculovirus might provide the means to deter the onset of insecticide resistance in the field and might be used to drive resistant populations towards susceptibility.

In related experiments, Popham *et al.* (1998b) tested the effectiveness of the coapplication of the type II pyrethroid deltamethrin and recombinant AcMNPVs expressing μ-Aga-IV, AS II, or Sh I (genes *mag4*, *sat2*, and *ssh1*, respectively) in neonate larvae of *T. ni* and *H. virescens*. The genes encoding these toxins were placed under the very late P_{SynXIV} (vMAg4p+, vSAt2p+, and sVVh1p+, respectively) or constitutively expressed *hsp70* (vhsMAg4p+, vhsSAt2p+, and vhsSSh1p+, respectively) promoters. By diet incorporation bioassays in neonate *T. ni* treated with a low dose (LC_{20}) of deltamethrin and a low dose (LC_{20}) of vMAg4p+, vSAt2p+, or vSSh1p+, they found additive effects in terms of larval mortality, but no synergistic effects. By droplet feeding bioassays in neonate *T. ni* treated with a low dose of deltamethrin and a high (LC_{75} or greater) dose of vMAg4p+, vSAt2p+, or vSSh1p+, they found that only the larvae exposed to both deltamethrin and vMAg4p+ showed a synergistic response in terms of FT_{50}, ET_{50}, and LT_{50}. In similar droplet feeding bioassays in neonate *T. ni* treated with a low dose of deltamethrin and a high dose of vhsMAg4p+, vhsSAt2p+, and vhsSSh1p+, they found no synergistic effects. Synergistic effects were observed in at least one time response parameter (FT_{50}, ET_{50}, or LT_{50}) in droplet feeding bioassays in neonate *H. virescens* treated with a low dose of deltamethrin and high dose of a toxin expressing AcMNPV. In all of the cases where a synergistic effect was observed, the coapplication of a high viral dose was also required. Thus, Popham *et al.* (1998b) suggested that (in contrast to the McCutchen *et al.* (1997) study discussed above) the coapplication of a pyrethroid and a recombinant baculovirus would confer little or no advantage in the field because low doses of the virus would most likely be applied. Furthermore, they suggested that the different outcomes of the McCutchen *et al.* (1997) study and their study might result from differences in the method of application of the pyrethroid.

A wide variety of venomous and nonvenomous animals, bacteria, and even plants possess peptide toxins that are highly potent and selective for lepidopteran insects and which have the potential to improve the insecticidal activity of recombinant baculoviruses. Rapid methods for the separation, purification, and identification of toxins from venom and other biological matrices have been developed (Nakagawa *et al*., 1997, 1998). These methods have been successfully used for the identification of a number of insecticidal peptide toxins, for example, AaIT5 from *A. australis* (Nakagawa *et al*., 1997) and ButaIT from the South Indian red scorpion *B. tamulus* (Wudayagiri *et al*., 2001). ButaIT is a short chain neurotoxin that targets the insect potassium channel, but is particularly interesting because it is highly selective towards the *Heliothine* subfamily but nontoxic against blowfly larvae and other insects. This high level of specificity may provide some unique advantages in comparison to other scorpion toxins. The concept of delivering individual toxins or combinations of toxins via baculoviruses remains a major route to practical application of insect selective peptide toxins. Whether used alone or in combination, the ever-increasing number of characterized insect selective toxins, and their compatibility and complementary nature to existing pest control strategies including chemical insecticides, provide us with extraordinary tools for "green" pest control.

6.9.4. Modification of the Baculovirus Genome

6.9.4.1. Deletion of the Ecdysteroid UDP-Glucosyltransferase Gene

Ecdysteroids are key molecules in the regulation of physiological events such as molting in insects. More than 60 different ecdysteroids have been isolated from insects and other arthropods, and about 200 phytoecdysteroids from plants (Nijhout, 1994; Henrich *et al*., 1999; Lafont, 2000; Dinan, 2001). Ecdysone was the first ecdysteroid to be isolated and is the best characterized. Following its production and secretion from the prothoracic glands, ecdysone is converted into 20-hydroxyecdysone in the hemolymph, epidermis, and fat body. 20-Hydroxyecdysone is the primary active form of the molting hormone in most insects. Ecdysteroids are often bound to specific carrier proteins that putatively help the ecdysteroid to penetrate cell membranes or increase the capacity of the hemo-

lymph for ecdysteroids. Like juvenile hormone, the titer of ecdysteroids in the hemolymph is determined by both their synthesis and their metabolism. During normal insect development, ecdysteroids are transported to the nucleus where they initiate a cellular response. The prevention of ecdysteroid transport results in the interruption of insect growth or abnormal development (or death).

Ecdysteroid UDP-glucosyltransferase is a baculovirus encoded enzyme that catalyzes the conjugation of sugar molecules to ecdysteroids (O'Reilly and Miller, 1989; O'Reilly, 1995). The conjugation of a hydrophilic sugar molecule to the ecdysteroid prevents it from crossing cellular membranes. Thus, conjugation effectively inactivates ecdysteroid function resulting in the inhibition of molting and pupation. O'Reilly and Miller (1989) were the first to identify a gene, *egt*, that encodes ecdysteroid UDP-glucosyltransferase in a baculovirus (AcMNPV). Homologs of the AcMNPV *egt* gene have been identified in approximately 90% (20 NPVs and 8 GVs, **Table 1**) of the baculovirus genomes that have been searched (Clarke *et al.*, 1996; Tumilasci *et al.*, 2003). Although the *egt* gene is commonly found in both NPVs and GVs, it is not essential for *in vitro* (in cultured insect cells) or *in vivo* (in larval hosts) replication of AcMNPV (O'Reilly and Miller, 1989, 1991). The *egt* gene has also been shown to be nonessential in the NPVs of *M. brassicae* (Clarke *et al.*, 1996), *L. dispar* (Slavicek *et al.*, 1999), *H. armigera* (Chen *et al.*, 2000), *H. zea* (Treacy *et al.*, 2000), *B. mori* (Kang *et al.*, 2000), and *A. gemmatalis* (Rodrigues *et al.*, 2001) (**Table 2**). Larvae of *S. frugiperda* or *T. ni* infected with vEGTDEL, an *egt* deletion mutant of AcMNPV, show earlier mortality and reduced feeding (by about 40%) in comparison to control larvae infected with wild-type AcMNPV (O'Reilly and Miller, 1991; Eldridge *et al.*, 1992a; Wilson *et al.*, 2000). Specifically, in time-mortality bioassays (at an LC_{90} or LC_{97} dose), the ST_{50} of vEGTDEL infected neonate *S. frugiperda* is reduced by approximately 22% (99.7 versus 127.2 h) in comparison to control larvae infected with AcMNPV. Furthermore, vEGTDEL infected larvae yield 23% fewer progeny viruses (polyhedra) in comparison to AcMNPV infected larvae. In second or fourth instar larvae of *T. ni*, vEGTDEL infection reduces the time to death by about 11% in comparison to AcMNPV infection. Virus yield is also lower in fourth instar larvae but not in the second instars. The *egt* gene thus provides a selective advantage to the virus in

Table 1 Baculoviruses carrying an ecdysteroid UDP-glucosyltransferase (*egt*) gene homolog

Virus	*Virus designation*	*Reference/accession No.*
Nucleopolyhedrovirus	*Autographa californica* MNPV (AcMNPV)	O'Reilly and Miller (1989)
	Anticarsia gemmatalis MNPV (AgMNPV)	Rodrigues *et al.* (2001)
	Amsacta albistriga NPV (AmalNPV)	AF204881
	Bombyx mori NPV (BmNPV)	Gomi *et al.* (1999)
	Buzura suppressaria NPV (BusuNPV)	Hu *et al.* (1997)
	Choristoneura fumiferana defective MNPV (CfDEFMNPV)	Barrett *et al.* (1995)
	Choristoneura fumiferana MNPV (CfMNPV)	Barrett *et al.* (1995)
	Ecotropis oblique NPV (EcobNPV)	AF107100
	Epiphyas postvittana MNPV (EppoMNPV)	Caradoc-Davies *et al.* (2001)
	Heliocoverpa armigera NPV (HearNPV)	Chen *et al.* (1997)
	Heliocoverpa zea NPV (HzNPV)	Popham *et al.* (1997)
	Lymantria dispar MNPV (LdMNPV)	Riegel *et al.* (1994)
	Mamestra configurata NPV (MacoNPV)	Li *et al.* (2002)
	Mamestra brassicae MNPV (MbMNPV)	Clarke *et al.* (1996)
	Orgyia pseudotsugata MNPV (OpMNPV)	Pearson *et al.* (1993)
	Rachiplusia ou MNPV (RoMNPV)	Harrison and Bonning (2003)
	Spodoptera exigua MNPV (SeMNPV)	Ijkel *et al.* (1999)
	Spodoptera frugiperda MNPV (SfMNPV)	Tumilasci *et al.* (2003)
	Spodoptera littoralis NPV (SpliNPV)	Kikhno *et al.* (2002)
	Spodoptera litura NPV (SpltNPV)	Pang *et al.* (2001)
Granulovirus	*Adoxophyes honmai* GV (AdhoGV)	Nakai *et al.* (2002)
	Adoxophyes orana GV (AdorGV)	Wormleaton and Winstanley (2001)
	Choristoneura fumiferana GV (CfGV)	AF058690
	Cydia pomonella GV (CpGV)	Luque *et al.* (2001)
	Epinotia aporema GV (EpapGV)	Manzan *et al.* (2002)
	Lacanobia oleracea GV (LoGV)	Smith and Goodale (1998)
	Phthorimaea operculella (PhopGV)	Taha *et al.* (2000)
	Plutella xylostella GV (PxGV)	Hashimoto *et al.* (2000)

Table 2 Genetic modifications at the *egt* gene locus

Parental virus	*Virus designation*	*Reference*
AcMNPV	VEGTZ	O'Reilly and Miller (1989)
	vEGTDEL	O'Reilly and Miller (1991), Treacy *et al.* (1997)
	vJHEEGTD	Eldridge *et al.* (1992a)
	vEGHEGTD	Eldridge *et al.* (1992b)
	VEGT⁻PTTHM	O'Reilly *et al.* (1995)
	AcMNPV-Δegt	Bianchi *et al.* (2000)
	vV8EGTdel, vp6.9*tox34*, vV8EE6.9*tox34*, vHSP70*tox34*, vV8EEHSP*tox34*	Popham *et al.* (1998a)
AgMNPV	VEGTDEL	Rodrigues *et al.* (2001)
	vAgEGTΔ-*lacZ*	Pinedo *et al.* (2003)
BmNPV	BmEGTZ	Kang *et al.* (2000)
HaSNPV (HearNPV)	HaLM2, HaCXW1, HaCXW2	Chen *et al.* (2000), Sun *et al.* (2002)
HzSNPV	HzEGTdel, HzEGTp6.9tox34, HzEGThsptox34, HzEGTDA26tox34	Popham *et al.* (1997)
LdMNPV	vEGT-	Slavicek *et al.* (1999)
MbMNPV	vEGTDEL	Clarke *et al.* (1996)

terms of the production of progeny. Conversely, this suggests that removal of the *egt* gene generates a virus that is less fit in comparison to the wild-type virus. In general, deletion of the *egt* genes of other NPVs has resulted in improvements in the speed of kill of 15–33% in comparison to the wild-type virus (e.g., Treacy *et al.*, 1997; Popham *et al.*, 1998a; Slavicek *et al.*, 1999; Treacy *et al.*, 2000; Chen *et al.*, 2000; Pinedo *et al.*, 2003). However, examples in which deletion of the *egt* gene does not reduce speed of kill are also found. Popham *et al.* (1997) found that deletion of the *egt* gene of HzSNPV had no effect in terms of reducing the ET_{50} (67.3 versus 65.4 h) in neonate larvae of *H. zea* in comparison to the wild-type virus. Bianchi *et al.* (2000) found that LT_{50} of AcMNPV-Δ*egt*, an *egt* gene deletion mutant of AcMNPV, was the same as that of wild-type AcMNPV in second and fourth instar larvae of *S. exigua*. Sun *et al.* (2004) found that the ST_{50} of neonate larvae of *H. armigera* infected with HaSNPV-EGTD (*egt* gene deletion mutant of HaSNPV) was not significantly improved over HaSNPV infected larvae, whereas second–fifth instar larvae were killed more rapidly. Thus, they suggested that *egt* gene deletion affects the speed of kill of the virus more strongly in later instars than in earlier instars.

Mechanistically, it is still unclear how deletion of the *egt* gene improves the speed of kill. In second instar larvae of *S. exigua*, Flipsen *et al.* (1995) found that an *egt* gene deletion mutant of AcMNPV caused earlier degradation of the Malpighian tubules in comparison to the wild-type AcMNPV. They speculated that this earlier degradation results in the faster speed of kill. Another possibility is that the general level of protein synthesis is elevated in larvae infected with the *egt* deletion virus (due to relatively higher levels of active ecdysteroids). Consistent with this hypothesis, Kang *et al.* (2000) found that virus encoded and virus induced proteins are expressed earlier in the infection cycle and at higher levels in larvae of *B. mori* that are infected with BmEGTZ, an *egt* gene deletion mutant of BmNPV, in comparison to the larvae infected with the wild-type BmNPV. Thus, mature progeny virions may be made and released more quickly in larvae infected with BmEGTZ than in larvae infected with BmNPV resulting in a faster systemic infection and death. Kang *et al.* (2000) have also shown that virus specific and certain host specific proteins are induced in a concentration dependent manner by the injection of purified ecdysteroids into BmEGTZ infected larvae (24 h prior to injection of ecdysteroids), but not in larvae infected with BmNPV. Furthermore, using a histochemical assay they showed that virus transmission is positively correlated with the amount of ecdysteroids that are injected into a larva (i.e., the higher the amount of ecdysteroids injected, the faster the transmission of the virus). In contrast, O'Reilly *et al.* (1995) found that the pathogenicity of a recombinant *egt*-gene-deleted AcMNPV that expresses biologically active PTTH (vEGT⁻PTTHM) is dramatically reduced (LC_{50} of 280×10^5 versus 3.8×10^5 polyhedra per ml) in comparison to vEGTDEL. This effect was not found in a PTTH expressing recombinant AcMNPV (vWTPTTHM) in which the *egt* gene was functional. In vEGT⁻PTTHM infected larvae of *S. frugiperda*, the titer of ecdysteroids spiked at around 96 h p.i. (1162 ± 476 pg μl^{-1}) and quickly dropped at 120 h p.i. (45 ± 12 pg μl^{-1}). In contrast, in vEGTDEL infected larvae, the titer of ecdysteroids increased at 72 h p.i. (519 ± 199 pg μl^{-1}) and remained high at 96 h p.i. (282 ± 55 pg μl^{-1}) and 120 h p.i. (334 ± 80 pg μl^{-1}). Although the peak ecdysteroid titer in vEGT⁻PTTHM infected larvae appeared to be significantly higher than that found in vEGTDEL infected larvae, this may not be the case because a higher percentage (54.5 ± 21.0% versus 32.3 ± 5.1%) of the ecdysteroids in the vEGT⁻PTTHM infected larvae were in the conju-

gated form in comparison to vEGTDEL infected larvae.

An elegant approach to further improve the *egt* gene deletion construct has been to insert an insect selective neurotoxin gene cassette into the *egt* gene locus, thus generating a toxin expressing, *egt* gene inactivated construct. For example, Chen *et al.* (2000) inserted an AaIT-GFP gene cassette (*aait* and green fluorescent protein (*gfp*) genes driven by the *polh* gene promoters of HaSNPV and AcMNPV, respectively) into the *egt* gene locus of HaSNPV (also known as HearNPV) in order to generate HaCXW2. In time-mortality bioassays, they found that the ST_{50} of second instar *H. armigera* infected with HaCXW2 was reduced by 32% (63.9 versus 94.4 h) and 8% (63.9 versus 69.5 h) in comparison to control larvae infected with wild-type HaSNPV or HaCXW1 (a recombinant HaSNPV carrying *gfp* at its *egt* gene locus). Sun *et al.* (2002, 2004) also showed that HaCXW1 and HaCXW2 were effective pest control agents under field conditions. In their field experiments, second instar *H. armigera* were allowed to feed on HaCXW1 or HaCXW2 treated plots (1.8×10^{10} polyhedra per 0.015 hectare plot containing 800 cotton plants) for 12 h, then the larvae were transferred to artificial diet and reared at ambient temperature. The ST_{50} values of the larvae infected with HaCXW1 or HaCXW2 in the field were reduced by 15% (101 versus 119 h) and 26% (88 versus 119 h), respectively, in comparison to control larvae that were field infected with HaSNPV. Treatment of cotton plants in the field with HaCXW1 or HaCXW2 reduced leaf consumption by approximately 50% and 63%, respectively. The results of additional field trials using HaSNPV-AaIT (a recombinant HaSNPV expressing AaIT under a chimeric ph-p69p promoter at the *egt* gene locus) are discussed below.

From these results it appears that deletion of the *egt* gene from the baculovirus genome is a useful strategy to improve its speed of kill and consequently its insecticidal activity, but by itself the reduction in speed of kill is too small to be of commercial value. Deletion of the *egt* gene, however, reduces the number of progeny viruses that are produced in comparison to the wild-type virus. Thus, the fitness of the *egt* gene deleted virus appears to be reduced in comparison to the wild-type virus. Conceptually, removal of an endogenous gene from its genome is safer than the insertion of a heterologous gene because a "new" gene will not be introduced into the environment. In practice, however, it is believed that there will be no significant difference in the risk of a recombinant baculovirus pesticide in which an endogenous gene is deleted from the genome or in which a heterologous gene is inserted into the genome. Safety issues and fitness of the virus are further discussed below.

6.9.4.2. Removal of Other Nonessential Genes

In a comparative study of seven completely sequenced lepidopteran baculovirus genomes, Hayakawa *et al.* (2000a) identified 67 putative genes that were common to all seven. When Herniou *et al.* (2001, 2003) added four additional lepidopteran NPVs and a GV (i.e., a total of 12 completely sequenced lepidopteran baculoviruses) 62 common genes were identified. Considering that the genomes of most baculoviruses encode well over 100 putative genes, these findings suggest that a large number of genes in the genome of lepidopteran baculoviruses may serve auxiliary or host specific functions or help to improve the fitness of the virus. This hypothesis is consistent with the finding that 61 out of the 136 putative genes of BmNPV appear not to be essential for viral replication in BmN cells, i.e., some level of viral replication was detected even when a gene was deleted by replacement with a *lac*Z gene cassette (Kamita *et al.*, 2003b; Gomi, Kamita, and Maeda, unpublished data). These findings suggest that there are a number of target genes that can be removed from the baculovirus genome in order to decrease fitness, alter host specificity, or simply to obtain a virus that may replicate faster or more efficiently.

Dai *et al.* (2000) have identified a mutant of *S. exigua* MNPV (SeXD1) that is lacking 10.6 kb of sequence (encoding 14 complete or partial genes including an *egt* gene homolog) that corresponds to the region between 13.7 and 21.6 map units in the wild-type SeMNPV genome. Dose-mortality bioassays in third instar larvae of *S. exigua* showed that the LD_{50}s of SeXD1 and SeMNPV (403 and 124 polyhedra per larva, respectively) were not significantly different. Time-mortality bioassays indicated that the ST_{50} of third instar *S. exigua* infected with SeXD1 was reduced by 25% (70.2 versus 93.1 h) in comparison to control larvae infected with SeMNPV. Dai *et al.* (2000) did not further examine which gene or genes in this 10.6 kb region are involved in decreasing the virulence of SeXD1 in comparison to SeMNPV. Considering that deletion of the *egt* gene from other baculoviruses has been shown to reduce the survival times of larvae infected with these viruses, the *egt* gene homolog of SeMNPV appears to be a prime candidate as the gene involved in reducing the virulence. However, several of the other genes that were deleted in this region are commonly found in other baculoviruses and may play roles in enhancing infection (e.g., *gp37*) or dissemination (e.g., v-*cath*, *chiA*, and

ptp-2) of the virus. Genes that help to enhance virulence or improve dissemination, but are nonessential, are potential targets for deletion in order to reduce the fitness of the virus.

As discussed above, the ST_{50} of larvae that are infected with an *egt* gene-deleted mutant baculovirus is often reduced in comparison to larvae that are infected with the wild-type virus. This reduction in survival time often results in the production of fewer progeny viruses (i.e., fewer polyhedra per larva) suggesting that the fitness of the *egt* gene deleted virus will also be reduced in comparison to the wild-type virus. Two other baculovirus genes that are involved in altering the virulence of the baculovirus are *orf603* (Popham *et al.*, 1998a) and *Ac23* (Lung *et al.*, 2003) both of AcMNPV origin. The LC_{50} of an AcMNPV variant (V8) in which the *orf603* gene is truncated is not significantly different from that of AcMNPV. The AcMNPV V8 variant (as well as other mutants in which the *orf603* gene was specifically inactivated) shows an ET_{50} that is reduced by approximately 12% (e.g., 88.5 versus 100.8 h) in comparison to a revertant virus (i.e., a virus in which the *orf603* mutation was repaired). In an attempt to further decrease the speed of kill of the V8 variant of AcMNPV, Popham *et al.* (1998a) inserted the *tox34* gene under the control of either the *p6.9* or *hsp70* promoter into the *egt* gene locus of V8 generating V8EEp6.*9tox34* and V8EEHSP*tox34*, respectively. In time-mortality bioassays in neonate larvae of *S. frugiperda* (at an LC_{95} dose), the ET_{50}s of V8EEp6.*9tox34* and V8EEHSP*tox34* were reduced by approximately 34% (58.2 versus 88.2 h) and 42% (51.2 versus 88.2 h), respectively, in comparison to control larvae infected with V8EGTdel, an *egt* gene deletion mutant of V8. However, in comparison to larvae infected with viruses in which the *orf603* gene was not deleted (but expressing the *tox34* gene under the *p6.9* or *hsp70* promoter at the *egt* gene locus), no differences were found in the ET_{50}s. They speculated that the relatively small decrease in speed of kill elicited by deletion of the *orf603* gene was masked by expression of the toxin gene.

A low-pH-activated fusion protein called GP64 is found in the envelopes of all group I NPVs, but lacking in group II NPVs. A different low-pH-activated envelope fusion protein called the F protein (Westenberg *et al.*, 2002) has been identified in group II NPVs. F proteins were first identified in LdMNPV (Pearson *et al.*, 2000) and SeMNPV (Ijkel *et al.*, 2000); however, F protein homologs are found in the genomes of all of the sequenced baculoviruses (i.e., both group I and group II NPVs and GVs) (Hayakawa *et al.*, 2000a; Herniou *et al.*, 2001). The F proteins of group II NPVs can functionally substitute for GP64 (Lung *et al.*, 2002), whereas the F proteins of group I NPVs appear not to function in membrane fusion (Pearson *et al.*, 2001). Lung *et al.* (2003) found that Ac23, the F protein homolog of AcMNPV, is not essential for AcMNPV replication in Sf-9 or High 5 cells or larvae of *T. ni*. The growth curve of *Ac23*null, an *Ac23* deletion mutant of AcMNPV, is indistinguishable to those of a repair virus of *Ac23*null (*Ac23*null-repair) or wild-type AcMNPV. *Ac23*null and *Ac23*null-repair also show indistinguishable lethal doses in larvae of *T ni*. However, in survival-time bioassays, the survival times of neonate or fourth instar *T. ni* that are infected with *Ac23*null are at least 28% (26 h) longer than those of control larvae infected with *Ac23*null-repair or AcMNPV (i.e., viruses that carry the *Ac23* gene). Interestingly, although not quantified, Sf-9 cells infected with *Ac23*null appear to produce significantly lower yields of polyhedra in comparison to Sf-9 cells infected with the control viruses (G.W. Blissard and O.Y. Lung, personal communication).

Wandering is a behavior that normally occurs only at the end of the larval stage in lepidopteran larvae (Nijhout, 1994; Goulson, 1997). At this stage, holometabolous insects stop feeding, void their gut, and look for a suitable location to pupate. Baculoviruses can artificially induce wandering in infected larvae to increase virus spread (Evans, 1986; Vasconcelos *et al.*, 1996; Goulson, 1997). It is recently found that baculovirus induced wandering requires a virus encoded protein tyrosine phosphatase (*ptp*) gene using a *ptp* gene deleted mutant of BmNPV (BmPTPD; S.G. Kamita, S. Maeda, and B.D. Hammock, unpublished data). Time-mortality bioassays (at an LC_{99} dose) showed that the ST_{50}s of BmPTPD or BmNPV infected neonate larvae of *B. mori* were not significantly different (88.1 and 81.7 h, respectively). BmPTPD infected neonates, however, did not display any symptoms of virus induced wandering as did the BmNPV infected neonates. Thus, the *ptp* gene is directly involved in increasing the dissemination of the virus. Removal of this gene may reduce the dispersal (and consequently fitness) of the virus in the field.

6.9.4.3. Alteration of Host Range

Although baculoviruses have been isolated from more than 400 insect species (Tanada and Kaya, 1993), most baculovirus isolates are permissive only in the original insect host from which they were isolated. Thus, the host range of individual baculoviruses is considered to be narrow. Among

baculoviruses, AcMNPV has a relatively wide host range being infectious against more than 30 insect species (Gröner, 1986) including a large number of pest insect species. The baculovirus must overcome a number of obstacles both at the organismal and cellular levels in order to replicate and eventually kill the insect host. At the organismal level, the polyhedra must first dissociate and release ODVs that can establish a primary infection. As discussed above, the dissociation of the polyhedra requires high pH and the presence of proteases, whereas the released ODVs must pass through at least three midgut barriers prior to establishing a systemic infection. At the cellular level, the BV must recognize and attach to a receptor on the cell surface, and enter the cell via endocytosis. Subsequently, the nucleocapsid must translocate to a nuclear pore, enter the nucleus, uncoat to release viral DNA, express early genes, initiate viral DNA synthesis, express late genes, and assemble and release mature virions. The baculovirus is able to express some early genes in a wide variety of insect cells (Miller and Lu, 1997; Thiem, 1997). Significantly, reduced levels of viral DNA synthesis and late gene promoter activity are also observed in some nonpermissive insect cells lines. Thus, the host range of a baculovirus may be limited, at least in terms of insect hosts, to its ability to: (1) express the entire complement of early genes; (2) synthesize genomic DNA at normal levels; (3) express late/very late genes at normal levels; and/or (4) assemble and release mature virions. Other key factors in the ability of a baculovirus to productively infect a host cell include its ability to inhibit: (1) the premature or global cessation of protein synthesis (Thiem, 1997) and (2) virus induced apoptosis (Miller *et al*., 1998; Clem, 2001).

The gypsy moth *L. dispar* and the *L. dispar* derived Ld652Y cell line are nonpermissive hosts of AcMNPV (Gröner, 1986; Du and Thiem, 1997a; Chen *et al*., 1998). AcMNPV infection of Ld652Y cells induces cytotoxicity and a premature cessation of host and viral protein synthesis (Du and Thiem, 1997b; Mazzacano *et al*., 1999). An *L. dispar* multicapsid NPV (LdMNPV) gene, host range factor 1 (*hrf-1*), has been identified that promotes AcMNPV replication in Ld652Y cells (Thiem *et al*., 1996; Du and Thiem, 1997a) and gypsy moth larvae (Chen *et al*., 1998). LdMNPV *hrf-1* does not function as an apoptotic suppressor (Du and Thiem, 1997b), however, *in vitro* translation assays indicate that the mechanism of translation arrest involves defective or depleted tRNA species (Mazzacano *et al*., 1999). In neonate larvae of *L. dispar*, the LC_{50} of a recombinant AcMNPV carrying the *hrf-1* gene of LdMNPV (vAcLdPD) is reduced by greater than 1800-fold (1.2×10^5 versus 2.2×10^8 polyhedra per ml) in comparison to AcMNPV. The LC_{50} of vAcLdPD, however, was still tenfold higher (1.2×10^5 versus 1.1×10^4 polyhedra per ml) than that of LdMNPV in neonate *L. dispar*. In second instar larvae of *H. zea*, the LC_{50} of vAcLdPD is 5.8-fold lower (5.49×10^5 versus 3.17×10^6 polyhedra per ml) than that of AcMNPV. However, the LC_{50} of AcLdPD was not significantly different to that of AcMNPV in second instar larvae of *P. xylostella* (AcMNPV resistant insect) and *S. exigua* (AcMNPV sensitive insect).

Two different types of antiapoptotic genes, *p35* and *iap*, have been identified in baculoviruses (Clem *et al*., 1996; Clem, 2001; Bortner and Cidlowski, 2002). The *p35* gene of AcMNPV was first shown by Clem *et al*. (1991) to function as an inhibitor of apoptosis in baculovirus infected Sf-21 cells. The P35 protein is an inhibitor of caspases (Bump *et al*., 1995; Xue and Horvitz, 1995) in a very wide variety of organisms (Clem, 2001; Bortner and Cidlowski, 2002). The first *iap* gene was identified by Crook *et al*. (1993) in *Cydia pomonella* GV. Subsequently, *iap* gene homologs have been identified in at least 10 other baculoviruses and a wide range of other organisms (Clem, 2001). The wild-type AcMNPV induces apoptosis in SL2 cells, a cell line derived from the Egyptian cotton worm *S. littoralis* (Chejanovsky and Gershburg, 1995; Du *et al*., 1999). The yield of AcMNPV BV in SL2 cells infected with AcMNPV is approximately 2700-fold lower than that of Sf-9 cells infected with AcMNPV. A recombinant AcMNPV (vHSP-P35) that overexpresses the *p35* gene of AcMNPV under the *hsp70* promoter does not induce apoptosis in SL2 cells; however, vHSP-P35 still produces low yields of BV (Gershburg *et al*., 1997). Lu *et al*. (2003) have found that AcMNPV is able to efficiently replicate in SL2 cells and larvae of *S. littoralis*, if it expresses higher levels of IE1 (an essential, multifunction protein that was first identified by its ability to transactivate baculovirus early genes) relative to IE0 (another protein that is capable of transactivating baculovirus early genes). The IE0 product is a longer form (by 54 N-terminal amino acids) of the IE1 product that is the result of transcriptional initiation from the *ie0* transcription initiation site and removal of the *ie0* intron (Friesen, 1997). Lu *et al*. used two approaches to increase the level of IE1 relative to IE0. First, they generated a mutant virus that expressed lower levels of IE0 by disrupting the *ie0* promoter by insertion of a 519 bp-long DNA fragment or a chloramphenicol acetyltransferase gene. Second, they generated

a recombinant AcMNPV (vHsp-1) that expressed a second copy of the *ie1* gene under the constitutively expressed *hsp70* promoter. Both of these approaches generated genetically modified AcMNPVs with host ranges that extended to *S. littoralis*.

At present, overexpression of AcMNPV *ie1* and insertion of LdMNPV *hrf-1* into the AcMNPV genome are the only ways in which the host range of AcMNPV can be extended to include pest insect species. Other AcMNPV genes that are involved in extending host range or dramatically increasing virulence include *p35* (Clem *et al.*, 1991; Hershberger *et al.*, 1992; Clem and Miller, 1993; Griffiths *et al.*, 1999), the DNA helicase gene *p143* (Maeda *et al.*, 1993; Croizier *et al.*, 1994), host cell specific factor 1 (*hcf-1*; Lu and Miller, 1995), late expression factor 7 (*lef-7*; Lu and Miller, 1995), and immediate early gene 2 (*ie2*; Prikhod'ko *et al.*, 1999). The roles of these genes in host range expansion and increase of virulence is discussed in the previous chapter.

6.9.5. Safety of GM Baculoviruses

The safety of a synthetic chemical or biologically based pesticide can never be assured with absolute confidence. Thus, when society considers whether or not a new pesticide is "safe," a better question to ask might be: What level of risk are we as a society willing to accept for a given benefit? In the case of chemical pesticides, the general public and a wide array of researchers (scientists, medical doctors, governmental regulators, statisticians, pathologists, ecologists, etc.) have analyzed the actual, potential, and perceived risks that they pose to human health and the environment. In general, all of these stakeholders agree that the benefits of chemical pesticides sufficiently outweigh any short- or long-term risks that they may pose. Society is thus willing (and should be willing) to accept some level of risk for a given benefit (e.g., increased yields of food, fiber, and feed from a given amount of natural resource). There are of course instances in which society has determined that the costs and risks associated with a particular pesticide (e.g., DDT) outweigh the benefits. In terms of the use of a GM baculovirus pesticide, this leads us to the following questions: (1) what are the risks associated with the field release of a GM baculovirus? (2) are these risks worth the benefits? and (3) are these risks any greater than the risks associated with the use of a chemical insecticide? A number of research studies and essays have addressed the first question. Miller (1995) and Hails (2001) suggest that the primary focus of assessing the risks of the field release of a GM baculovirus should include:

1. How the GM baculovirus affects nontarget species.
2. Whether the introduced gene provides a selective advantage to viral replication, survival, and/or host range (i.e., is fitness improved).
3. Should the introduced gene transfer to another organism, will this event provide a selective advantage to that organism.

6.9.5.1. Potential Effects of a GM Baculovirus on Nontarget Species

The potential of natural and recombinant baculoviruses to induce a deleterious effect upon vertebrates has been extensively studied (Burges, 1981; Black *et al.*, 1997). More than two dozen different baculoviruses have been tested in a wide range of vertebrates including 10 different mammalian species, birds, and fish. The baculoviruses in these tests were administered by various routes including inhalation, topical application, injection (intravenous, intracerebral, intramuscular, intradermal), and orally. Baculovirus induced deleterious effects were not observed in any of these tests. Direct and indirect tests on human subjects have also been conducted including oral administration of high (greater than 10^9 polyhedra) doses of polyhedra of HzSNPV over a period of 5 days (Heimpel and Buchanan, 1967). The blood of workers who were involved in the production of HzSNPV during a 26 month long period was analyzed for infectious baculovirus, baculoviral antigens, or baculoviral antibodies – none were detected (Black *et al.*, 1997). Furthermore, the ubiquitous baculovirus load from foods in our diet may be quite high (Heimpel *et al.*, 1973; Thomas *et al.*, 1974). For example, Heimpel *et al.* (1973) found that a square inch (approximately $6.5\,cm^2$) of cabbage taken from a store shelf may contain up to 2×10^6 polyhedra suggesting that consumption of a single serving of coleslaw ($16\,in^2$ of cabbage) exposes us to up to 3.2×10^7 polyhedra. These studies indicate that we live under constant exposure to baculoviruses with no apparent deleterious effects. The lack of deleterious effects is not completely unexpected since polyhedra are extremely insensitive to the neutral or acidic pH conditions of the vertebrate digestive system. Thus, any polyhedra that are normally ingested while eating coleslaw, for example, pass through the digestive tract and are either excreted intact or should any ODVs be released they are quickly inactivated first by the action of pepsin at low pH and subsequently by the pancreatic proteases at neutral pH (Black *et al.*, 1997; Miller and Lu, 1997).

Beginning in 1995, two studies have shown that baculoviruses can serve as highly efficient vehicles for the transfer of foreign genes into the nuclei of primary cultures of human, mouse, and rat hepatocytes and human hepatoma cell lines (Hofmann *et al*., 1995; Boyce and Bucher, 1996). Since then, baculoviruses have been shown to be able to transduce a number of mammalian cell types including human neural cells (Sarkis *et al*., 2000), human fibroblasts (Dwarakanath *et al*., 2001), human keratinocytes (Condreay *et al*., 1999), and several established mammalian cell lines (Boyce and Bucher, 1996; Shoji *et al*., 1997). Nonmammalian, vertebrate cell lines (e.g., fish cells) have also been transduced with a recombinant baculovirus (Leisy *et al*., 2003; Wagle and Jesuthasan, 2003). At first glance, these studies appear to raise concerns about the risks of baculoviruses that are used for pest control. However, upon careful consideration it is believed that they provide further evidence as to the safety of GM baculoviruses. First, these studies show that although the baculovirus can enter the mammalian cell, productive baculovirus infection does not occur even following inoculation at exceptionally high multiplicities of infection (MOI) of up to 1500 pfu per cell using a virus inoculum that was concentrated by ultracentrifugation (Hofmann *et al*., 1995; Barsoum *et al*., 1997; Shoji *et al*., 1997; Kost and Condreay, 2002; Hu *et al*., 2003). Furthermore, the inoculated cells did not show any visible cytopathic effects, grew normally, and showed normal plating efficiencies. Second, in all of these examples, the foreign gene is expressed only when placed under a promoter that is specific for mammalian cells (e.g., cytomegalovirus immediate early, Rous sarcoma virus long terminal repeat, simian virus 40, etc.). Foreign gene expression was never observed under a very late baculoviral promoter. Third, although transduction was successful in established cell lines and primary cultures, the direct application of baculoviruses for gene delivery to the liver *in vivo* is strongly reduced or inhibited because the baculovirus activates the complement (C) system (Sandig *et al*., 1996; Hofmann and Strauss, 1998). The C system represents a first line of host defense of the innate immune system for the elimination of foreign elements (Liszewski and Atkinson, 1993). Baculoviruses possess a number of characteristics including their inability to replicate in mammalian cells and induce any deleterious effects that make them ideal gene delivery vectors for human gene therapy and as a screening systems for proteins and other molecules of human health interest (Lotze and Kost, 2002).

All of the studies to date indicate that there are no known risks associated with human (or other vertebrate) exposure to baculoviruses. Thus, a second focus in the discussion of the safety of recombinant baculoviruses should focus on nontarget lepidopterans and other invertebrates such as predatory or beneficial insects. As discussed above and in the previous chapter, the normal host range of a baculovirus is generally limited to the lepidopteran insect species from which it was originally isolated (Gröner, 1986). This assessment of "host range," however, is potentially problematic because it is often based upon the ability of a baculovirus to elicit virus specific symptomology or death. Huang *et al*. (1997) have tested seven different recombinant NPVs (based on AcMNPV, BmNPV, LdMNPV, or OpMNPV) carrying a reporter gene encoding β-galactosidase, secreted alkaline phosphatase (SEAP), or luciferase under the control of an early (ETL) or very late (*polh*) promoters. These reporter viruses were tested in 23 different insect species from eight insect orders (Blattodea, Coleoptera, Diptera, Hemiptera, Homoptera, Lepidoptera, Neuroptera, and Orthoptera) and 17 families. The reporter viruses were initially injected into the test insects by hemocoelic injection. Insects that supported virus replication (i.e., on the basis of expression of the reporter protein) by injection were subsequently tested by *per os* (oral) inoculation with the preoccluded form of the virus. The use of these reporter viruses allowed the detection of both symptomless and pathogenic infections. By the injection experiments, β-galactosidase, SEAP, and luciferase activities were found only in the lepidopteran larvae. Consistent with previous reports, AcMNPV had the widest host range in the 10 lepidopteran hosts that were tested, whereas LdMNPV was the most host specific. Host range following *per os* inoculation was more limited in comparison to inoculation by injection. In fact two species, the monarch butterfly *Danaus plexippus* and gypsy moth *L. dispar*, that appeared to be susceptible (to AcMNPV, OpMNPV, and BmNPV) by injection were not susceptible by *per os* inoculation. Additionally, as pointed out by Cory and Myers (2003) the host range of a baculovirus in the field may be considerably narrower due to spatial or temporal differences that are not found in the laboratory.

Other studies have looked at whether natural enemies of lepidopterans such as parasitoids, scavengers, and predators (McNitt *et al*., 1995; McCutchen *et al*., 1996; Heinz *et al*., 1995; Li *et al*., 1999; Smith *et al*., 2000b; Boughton *et al*., 2003) are adversely affected when they prey upon larvae that are infected with recombinant or wild-

type viruses. The social wasp *Polistes metricus* is a beneficial insect that preys on lepidopteran larvae (Gould and Jeanne, 1984). McNitt *et al.* (1995) analyzed five developmental parameters (i.e., larval development time, pupal development time, larval mortality, mean nest size, and mean number of adults per nest) in order to assess colony health and individual vigor of *P. metricus* that fed on lepidopteran larvae infected with recombinant AcMNPVs expressing AaIT or TxP-I. These toxins were expressed under either the constitutive *hsp70* or very late *polh* promoter. There were no significant differences in any of the developmental parameters tested when the *P. metricus* fed on uninfected or virus infected (at 3 days before exposure to *P. metricus*) fourth or fifth instar *S. frugiperda*. In related experiments, McCutchen *et al.* (1996) allowed the parasitic wasp *Microplitis croceipes* to parasitize second instar *H. virescens* that were subsequently infected with AcAaIT, AcJHE.KK, or AcMNPV (at a greater than LC_{99} dose, 5×10^4 polyhedra per larva) at various times (0, 48, 72, 96, or 120 h) postparasitization. The survival of the parasitoid was less than 4% at 0 and 48 h postparasitization but increased gradually until 120 h postparasitization, when there was no significant difference in the virus or mock treated *H. virescens*. There were no significant differences in terms of time taken for emergence of the adult wasp, ability of the F_1 wasps to oviposit, or sex ratios of the F_1 wasps. However, there were significant differences in the time (205–216 versus 228 h) taken for emergence of the larval parasitoid from virus infected *H. virescens* in comparison to mock infected *H. virescens*. This was particularly evident in the AcAaIT infected larvae. McCutchen *et al.* (1996) suggested that the parasitoid larva responds to the poor condition of its host by prematurely emerging. Additionally, wasps that developed from AcAaIT or AcJHE.KK infected larvae were significantly smaller than wasps that developed from mock infected larvae.

The green lacewing, *Chrysoperla carnea* Stephens, insidious flower bug, *Orius insidiousus* (Say), red imported fire ant, *Solenopsis invicta* Buren, big-eyed bug, *Geocoris punctipes* (Say), convergent lady beetle, *Hippodamia convergens* Guerin-Meneville, and twelve-spotted lady beetle, *Coleomegilla maculata* DeGeer are generalist predators that attack larval lepidopterans such as *H. virescens*. Heinz *et al.* (1995) have looked at the development times of green lacewings and flower bugs that fed upon near-dead second instar larvae of *H. virescens* (three per day) that were infected with a greater than LC_{99} dose (5×10^4 polyhedra per larva) of AcAaIT or AcMNPV. They found no significant differences in the survival percentages of insects that fed upon AcAaIT-, AcMNPV-, or mock-infected *H. virescens*. There was a significant but short (19.1 versus 20.6 days) decrease in the larva-to-adult development time in green lacewings that fed upon AcAaIT or AcMNPV infected *H. virescens* in comparison to lacewings that fed upon mock infected *H. virescens*. One may speculate that the protein quality in the prey might be reduced due to overexpression and agglomeration of the toxin proteins resulting in these subtle differences. Li *et al.* (1999) have investigated the life history traits (rate of food consumption, travel speed, fecundity, and adult survival) of fire ants, big-eyed bugs, and convergent lady beetles fed on second instar *H. virescens* that were infected with one of seven viruses (AcMNPV, HzSNPV, HzSNPV expressing LqhIT2 under the *ie1* gene promoter, or AcMNPV expressing LqhIT2 or AaIT under the *ie1* or *p10* gene promoters) at 24 or 60 h prior to predation by the predator. No significant shifts in the life history characteristics were detected in predators that fed on any of the virus infected larvae in comparison to predators that fed on healthy larvae.

Boughton *et al.* (2003) have analyzed the adult survival, developmental time, and oviposition rates of green lacewings and twelve-spotted lady beetles fed upon first or second instar *H. virescens* that were infected with $100 \times LC_{50}$ doses of AcMLF9.ScathL (recombinant AcMNPV expressing a basement membrane degrading protease under the *p6.9* gene promoter) or AcMNPV. Unexpectedly, lacewings that fed on the AcMLF9.ScathL infected *H. virescens* showed significantly elevated survival rates in comparison to AcMNPV infected or uninfected *H. virescens*. Boughton *et al.* (2003) speculated that the protease expressed by AcMLF9.ScathL enhanced the efficiency of feeding of the lacewing. No significant differences were found in developmental time, time to onset of oviposition or mean daily egg production of lacewings that fed on virus infected or uninfected larvae. Most of the twelve-spotted lady beetles that fed exclusively on *H. virescens* (either uninfected or virus infected) died. There was no evidence that feeding on AcMLF9.ScathL infected larvae induced any adverse effects in comparison to AcMNPV infected larvae. Furthermore, neither the lacewing nor lady beetle exhibited any feeding preferences for AcMLF9.ScathL-, AcMNPV-, and mock-infected insects. On the basis of these findings, Boughton *et al.* (2003) concluded that there was no greater risk to insect predators by the use of AcMLF9.ScathL in comparison to the use of AcMNPV as a biological pesticide. Smith *et al.*

(2000a) have investigated the density and diversity of nontarget predators under field conditions following the application of recombinant baculoviruses (AcMNPV or HzSNPV expressing scorpion toxin LqhIT2 under the *ie1* gene promoter) on cotton. They found that predator densities and diversity were similar between recombinant and wild-type baculovirus treated plots. In contrast, the chemical pesticide (esfenvalerate) treated plots had consistently lower predator populations.

Taken together, these studies indicate that: (1) the amount of baculovirus expressed toxin or protease that accumulates in the larvae is not sufficient to induce any adverse effects on the predator; (2) the baculovirus is not infectious towards the predatory insects; and (3) the toxin or protease encoding gene is not expressed in the predatory insect. In some cases, there appear to be some costs associated with the beneficial insects that prey upon virus infected larvae. However, these costs are significantly lower in comparison to the costs associated with the application of synthetic chemical pesticides. The development of selective recombinant insecticides should augment any IPM program by reducing the impact on nontarget species, including beneficial insects. Consequently, the resurgence of primary pests and outbreaks of secondary pests should be minimized.

6.9.5.2. Fitness of GM Baculoviruses

Fitness is a term that describes the ability of an organism to produce progeny that survive to contribute to the following generation (Cory, 2000). In order to estimate the relative fitness of a recombinant baculovirus in comparison to the wild-type baculovirus, five key parameters should be assessed: speed of kill, yield, transmission, dispersal, and persistence. Speed of kill (or time to death), virus yield, and transmission rate can be easily determined by laboratory bioassays (as discussed above) and in some cases under field conditions. Although the relationship between speed of kill (generally quantified in terms of LT_{50}) and virus yield is complex, faster speed of kill generally results in dramatically lower virus yields. This correlation between improved speed of kill and reduced virus yield is found regardless of the parental virus that is genetically modified, and for modifications in which an effector gene is inserted or when an endogenous gene is deleted from the genome. For example, the speeds of kill of third, fourth, or fifth instar larvae of *T. ni* that are infected with AcAaIT or AcJHE.KK are reduced by approximately 30% and 8%, respectively, in comparison to control larvae infected with AcMNPV. These faster speeds of kill result in reductions of approximately 80% and 40% in the yields (polyhedra per milligram of cadaver) of AcAaIT and AcJHE.KK, respectively, in comparison to the yield of AcMNPV (Kunimi *et al.*, 1996). Dramatic reductions of up to 95% in virus yield (polyhedra per microgram of cadaver) are found in second and fourth instar larvae of AcTOX34.4 infected *T. ni* in comparison to control AcMNPV infected larvae (Burden *et al.*, 2000). The corresponding reductions in the mean times to death are 50–60%. A reduction in virus yield is also found by deletion of the *egt* gene of AgMNPV (Pinedo *et al.*, 2003). The mean lethal times of third instar larvae of *A. gemmatalis* infected with various doses of vAgEGTΔ-*lacZ* is reduced by 10–26% in comparison to control larvae infected with the same dose. The yield (polyhedra per gram of cadaver) of vAgEGTΔ-*lacZ* is reduced by approximately 50% in comparison to control larvae infected with the wild-type AgMNPV. Similar results are found in fifth instar larvae of *S. frugiperda* infected with vEGTDEL, which produce 23% fewer polyhedra per insect (the yield of virus per milligram of cadaver is not, however, significantly different) in comparison to control larvae infected with AcMNPV (O'Reilly and Miller, 1991). O'Reilly *et al.* (1991) and Ignoffo *et al.* (2000) speculated that this correlation between improved speed of kill and reduced virus yield results from the considerably reduced size of recombinant baculovirus infected larvae at the time of death.

Milks *et al.* (2001) have focused on intrahost competition between AcAaIT and AcMNPV or AcAaIT and TnSNPV in larvae of *T. ni* that were synchronously or asynchronously infected. They found no differences in the fitness of the genetically modified or wild-type viruses in terms of virus yield. The most important factors in these mixed infections were dose and timing. The virus that was inoculated at the highest dose or the virus that was first inoculated was the one that had the competitive advantage. These findings are not unreasonable when one considers that there are no significant differences in the replication rates of toxin gene carrying and wild-type baculoviruses in cell culture.

A key component of the transmission rate of a virus is its pathogenicity or potency (often quantified in terms of the LD_{50} or LC_{50}). In general, the pathogenicity of a baculovirus is not significantly changed following the insertion of a neurotoxin gene into its genome (McCutchen *et al.*, 1991; Tomalski and Miller, 1992; Prikhod'ko *et al.*, 1996; Harrison and Bonning, 2000b), although there are a few exceptions in which relatively small

(threefold or less) increases or decreases have been observed (Chen *et al.*, 2000; Harrison and Bonning, 2000b). Deletion of an endogenous baculovirus gene such as *egt* also results in no significant (O'Reilly and Miller, 1991; Slavicek *et al.*, 1999) or relatively small (Pinedo *et al.*, 2003) differences in pathogenicity. However, the pathogenicity of the virus can be dramatically increased (i.e., lower LD_{50} or LC_{50}) by 4- to 100-fold by genetic modifications that improve the ability of the virus to penetrate the midgut, i.e., by the expression of enhancins (Hayakawa *et al.*, 2000b; Popham *et al.*, 2001) or incorporation of *Bt* toxin into the polyhedra (Chang *et al.*, 2003). In these cases, it is believed that the enhancin or *Bt* toxin helps the virus to pass more easily through the midgut and establish a more rapid systemic infection. In contrast, however, there were no significant differences in the LC_{50} values of recombinant AcMNPVs expressing basement membrane degrading proteases (stromelysin-1, gelatinase A, of cathepsin L) under early or late promoters (Harrison and Bonning, 2001).

Another component of the transmission of a virus is the rate at which the virus and host come into contact. This component is obviously more difficult to quantify in the field. As described above, neurotoxin-induced paralysis will cause the insect to fall off the plant (Cory *et al.*, 1994; Hoover *et al.*, 1995; Sun *et al.*, 2004). This knock-off behavior results in lower levels of foliage contamination, thus the likelihood that a second host insect will come into contact with the recombinant virus (at least during feeding on the plant) will be reduced. In contrast, the wild-type virus infected larvae will most likely die on the plant, thereby increasing the potential for contact between the virus and host. Lee *et al.* (2001) have examined this behavioral effect in a greenhouse microcosm. They found that the wild-type AcMNPV out-competed recombinant viruses (AcAaIT or AcJHE.SG) for a niche in the greenhouse microcosm. AcMNPV and AcJHE epizootics lasted for 8 weeks after the initial release of the virus, whereas the AcAaIT epizootic ended by the fourth week after release. Additionally, AcMNPV polyhedra also increased to greater numbers in the soil in comparison to AcAaIT or AcJHE.SG after 8 weeks. A reduction in wandering behavior resulting from the inactivation of the *ptp* gene may also reduce foliage contamination as discussed above. In both of these examples, the transmission rate of the recombinant baculovirus should be reduced because the likelihood of a host insect and virus coming into contact is reduced.

Dispersal is another parameter that should be assessed in order to determine the fitness of a virus. Abiotic and biotic agents that are known to disperse baculoviruses include rainfall, air currents, predators, parasites, scavengers, and grazing mammals (Fuxa, 1991; Fuxa and Richter, 1994). Predatory and scavenging insects have been found to carry (within their digestive tracts) and disperse recombinant virus (in excrement) at rates of up to 125 cm per day over a period of up to 10 days (Lee and Fuxa, 2000). Thus, recombinant virus induced behavioral changes of the host larvae such as knock-off effects that reduce predation and scavenging will also reduce the dispersal of the virus. Additionally, the ability of the biotic agent itself to disperse the recombinant baculovirus may be reduced. For example, parasitic wasps that develop within larvae of *H. virescens* that are infected with recombinant baculoviruses are significantly smaller than wasps that develop within wild-type virus infected larvae (McCutchen *et al.*, 1996). Thus, the ability of the smaller wasps to travel, and subsequently disperse the recombinant virus, may be reduced because it has less energy reserves and reduced flight capability in comparison to wasps that developed on wild-type virus infected larvae.

Persistence is the final parameter that should be considered when determining the relative fitness of a genetically modified baculovirus. In general, inactivation by sunlight (ultraviolet radiation) is the primary route of inactivation of polyhedra in the environment (Ignoffo and Garcia, 1992; Black *et al.*, 1997; Ignoffo *et al.*, 1997). In contrast, soil and foliage are factors that can protect the virus from sunlight and subsequently increase its persistence (Peng *et al.*, 1999). Knock-off or other behavioral changes induced by the recombinant virus may increase their relative concentrations in the soil such that the recombinant's persistence is increased. In contrast, however, the transmission rate of this recombinant should be reduced because the potential for contact between the virus and host is reduced. The cuticle is another factor that can protect the virus from sunlight such that viral persistence is increased. Fuxa *et al.* (1998) found that the cadavers of *T. ni* that are killed by recombinant viruses (AcAaIT, AcJHE.KK, or AcJHE.SG) take much longer to disintegrate (4–7 days versus 1 day) in comparison to AcMNPV killed *T. ni*. Another report by Ignoffo and Garcia (1996) found, however, that there is no significant difference in the time to cell lysis following death by AcAaIT or AcMNPV (lysis took 1.7 versus 1.5 days, respectively). Clearly, viral fitness is dependent upon a large number of intricately integrated factors. Finally, it is also important to consider as suggested by Hammock (1992) that genetically modified baculoviruses are designed to

be biological insecticides and not biological control agents that will become permanently established. Thus, a recombinant baculovirus should by its very design show reduced fitness in comparison to the wild-type.

6.9.5.3. Movement of the Introduced Gene to Another Organism

The insecticidal efficacy of natural baculoviruses is dramatically improved by insertion of a foreign gene into the genome or inactivation (deletion) of an endogenous gene from the genome or a combination of both. The insertion strategy generally involves the insertion of an effector gene that encodes a protein that is detrimental to the target insect, alters its life cycle or stops it from feeding. The deletion strategy generally involves the inactivation of an endogenous gene (e.g., *egt* or *orf603*) by inserting another gene into its coding sequence. This other gene can be a marker gene such as *lacZ* or an effector gene as described above. In both cases, the genes are placed under a baculoviral or insect promoter. Several critical points should be kept in mind with these strategies. First, the genes are placed under promoters that are active only in insect cells (and in the case of late/very late baculoviral promoters, these promoters also require the products of baculoviral early genes for activity). Thus, should the effector gene and its promoter somehow jump to the genome of a noninsect cell, the gene will not be expressed. Second, the proteins encoded by the effector genes are chosen because they target some critical aspect of the pest insect life cycle or body. The proteins are not biologically active in the noninsects (although it is possible that they may induce an immunological response). Thus, if the effector gene somehow jumps to the genome of a noninsect cell, and if this gene is somehow expressed, detrimental effects will not result.

Genomic variants of baculoviruses are often found in individual field collected insects (Cherry and Summers, 1985; Maeda *et al*., 1990; Shapiro *et al*., 1991; Hodgson *et al*., 2001); this suggests that recombination and/or transposition events commonly occur between baculovirus genomes. Extensive homology between the donor and recipient DNA molecules and replication of both DNA molecules is required for high-frequency recombination to occur (Kamita *et al*., 2003b). Such conditions may occur when two heterologous viruses (that share some genomic homology) infect the same cell within the same insect. This scenario is the most likely one in which an effector gene of a GM baculovirus will jump to another organism (i.e., another insect virus). Should the effector gene jump to another virus under these conditions, the fitness of the new recombinant virus will be reduced in comparison to the original GM baculovirus and it too should be rapidly eliminated from the environment. In a second scenario in which the effector gene jumps from the GM baculovirus to the genome of the insect host, the effector gene could cause an adverse effect. However, these effects should be limited to a single individual because once this individual dies the effector gene will also "die." It is also possible that heterologous or random recombination events may lead to the movement of an effector gene to another organism. The same arguments that were made in regard to the homologous recombination based movement can be made in this case. However, the likelihood of heterologous recombination is much lower than the likelihood of homologous recombination.

6.9.6. Field Testing and Practical Considerations

Laboratory and greenhouse testing has generated a great deal of knowledge about the efficacy, safety, and environmental fate of GM baculovirus pesticides as described above. Mathematical models have also been generated to evaluate the effectiveness and ecological consequences of the release of GM baculoviruses (Dwyer and Elkinton, 1993; Dwyer *et al*., 1997; Dushoff and Dwyer, 2001). Field testing, however, over both the short-term (e.g., single growing season) and long-term (e.g., multiple seasons and years) is still necessary to confirm the findings of laboratory and greenhouse tests and the accuracy of mathematical models. The commercial potential of GM baculoviruses and practical considerations regarding their use can also be determined by field testing. Issues regarding the commercialization of GM baculovirus insecticides including marketing, *in vivo* and *in vitro* production, formulation, storage, and public acceptance are discussed in detail by Black *et al*. (1997).

Some of the earliest field trials of GM baculoviruses (occlusion-negative AcMNPVs carrying junk DNA or *lacZ* marker gene) were performed in England during the mid to late 1980s (Levidow, 1995; Black *et al*., 1997). In the USA, the first field trial (a 3-year study) of a GM baculovirus (a *polh* gene deleted AcMNPV that was co-occluded with wild-type AcMNPV) was begun in 1989 (Wood *et al*., 1994). These early field trials were performed primarily to analyze the environmental persistence of the virus. These trials showed that the persis-

tence of occlusion-negative constructs is exceptionally low. The first field trial to test the efficacy of an occlusion-positive, AaIT expressing AcMNPV (AcST-3) was performed in 1993 (Cory *et al.*, 1994). Cory *et al.* (1994) found that cabbage plants treated with AcST-3 showed 23–29% lower feeding damage (by third instar larvae of *T. ni*) in comparison to wild-type AcMNPV treated cabbage plants. This reduction in feeding damage (approximately 50% reduction) was not as large as that found in the laboratory trials (Stewart *et al.*, 1991). The reduction in feeding damage during the field trial was due to the earlier death of AcST-3 infected larvae in comparison to AcMNPV infected larvae. Cory *et al.* (1994) also found that the yield of AcST-3 was tenfold lower (9.9×10^7 versus 9.6×10^8 polyhedra per larva) in comparison to AcMNPV. Furthermore, they found that under the high (10^8 polyhedra per ml) dose treatments the majority (62%) of the larvae of AcST-3 treated cabbage were knocked off the plant whereas none were knocked off the AcMNPV treated cabbage. They speculated that both the behavioral (knock-off) and pathological (reduced yield per larva) differences between the recombinant and wild-type viruses would have important implications for risk assessment as discussed above.

The American Cyanamid Company performed the first field trials in the USA (one in Georgia and the other in Texas) to test the efficacy of an occlusion-positive, *egt* gene deleted, AaIT expressing AcMNPV in 1995 and 1996 (Black *et al.*, 1997). They found that this virus killed target insects faster than wild-type AcMNPV or an *egt* gene deleted AcMNPV, which resulted in increased control of the target insect. In 1997 and 1998, field trials were conducted by academic scientists and scientists at DuPont Agricultural Products (Smith *et al.*, 2000a) to assess (1) the efficacy of recombinant baculoviruses in protecting cotton, (2) the impact of recombinant virus introduction on predators, and (3) the ability of predators to disperse recombinant baculoviruses. Two occlusion-positive constructs (one based on AcMNPV and the other on HzSNPV) expressing LqhIT2 were tested. Depending upon the timing of the application, they found that the LqhIT2 expressing viruses were able to protect cotton from damage better than the wild-type virus (AcMNPV or HzSNPV) and as well as a synthetic chemical pesticide (esfenvalerate). Predator densities and diversity were similar between the recombinant and wild-type virus treated plots, whereas plots treated with the chemical pesticide had consistently smaller predator populations. Finally, they found that at 2–5 days after the initial application of virus, a very small percentage (0.2%) of predators that were caught and evaluated carried DNA from a recombinant virus. In 1998, Treacy *et al.* (2000) (American Cyanamid Company) conducted field trials in the USA (two in Georgia and one in North Carolina) to test the efficacy of two occlusion-positive constructs (AcMNPV and HzSNPV based) expressing AaIT in protecting cotton. They found that the recombinant HzSNPV (at 5 or 12×10^{11} polyhedra per hectare) protected cotton against heliothine complex larvae at slightly better levels than either the recombinant AcMNPV or *Bt* toxin (Dipel 2X, Abbott Laboratories, at 1121 g (WP) per hectare).

In 2000, Sun *et al.* (2004) conducted field trials in People's Republic of China (in Henan and Hubei provinces) to test the efficacy of HaSNPV-AaIT (an occlusion-positive recombinant HaSNPV expressing AaIT at the *egt* gene locus) in protecting cotton. Cotton plant plots treated with HaSNPV-AaIT showed significantly less damage to squares, flowers, and bolls in comparison to plots treated with the wild-type HaSNPV or HaSNPV-EGTD (HaSNPV with a deletion in its *egt* gene). In 2001 and 2002 (Hubei province), treatment of cotton plants during the entire growing seasons with HaSNPV-AaIT increased the yield of cotton lint by approximately 18% (1250 versus 1023 and 1800 versus 1474 kg per hectare in 2001 and 2002, respectively) in comparison to wild-type HaSNPV treated plots. The yield of cotton lint in plots treated with HaSNPV-AaIT was not significantly different from the yield (1083 and 1994 kg per hectare in 2001 and 2002, respectively) in plots treated with chemical pesticides (λ-Cyhalothrin EC, endosulfan, β-Cypermethrin on different dates).

Numerous field trials have been conducted over the past 10 years at geographically distant sites using several types of GM baculovirus constructs and under different cropping situations. These trials show that GM baculoviruses are effective and safe pesticides with efficacy that can be at levels similar to synthetic chemical pesticides or *Bt* toxin. The field trials also indicate that the recombinant baculovirus will not persist in the environment and has very little, if any, adverse activity against beneficial insects.

6.9.7. Concluding Remarks

The majority of people in both developed and developing countries are dependent upon others to produce or otherwise provide the minimum requirements of food, water, and/or shelter necessary for survival. A society must carefully manage and allocate its limited resources in order to provide these

minimum requirements for all of its members. Over the past five decades, the use of synthetic chemical pesticides has significantly increased the yields of food, fiber, and feed that producers are able to generate from a given amount of land. Although chemical pesticides have without a doubt improved the efficiency of agricultural output and reduced the incidence of disease by killing disease vectors, chemical pesticides are also a source of environmental pollution and both acute and chronic problems for human health. The inappropriate use of chemical pesticides has also helped to generate pesticide resistant insects. These problems are often intensified in developing countries because of poor governmental regulation and training in the appropriate use of these agents. Furthermore, the high costs of the newest generation of synthetic chemical pesticides are precluding their widespread use in many developing countries. These pressures in the agricultural industry are making biological control agents highly attractive as supplements or replacements for synthetic chemical pesticides in integrated pest management schemes.

While biological pesticides avoid chemical residue problems, many such agents lack sufficient potency and speed of action in the field (at least until recently) to be attractive alternatives. As discussed in this chapter, a fresh approach to biological insect control involves using the best genes of nature to enhance insecticidal properties of naturally occurring baculoviruses. Several laboratories, including the authors', have developed NPVs that have the knock-down speed of classical chemical insecticides on the noctuiid complex of insect pests while not harming nontarget species. The viruses are applied like classical insecticides, but they present no residue problems, and they are active on insects resistant to classical insecticides. Industry and academic scientists have overcome most of the limitations associated with natural baculovirus pesticides, especially in terms of effective crop protection, through the use of recombinant DNA techniques and other technologies. Furthermore, should baculoviruses appear to offer sufficient commercial potential for investment, many of the remaining limitations (e.g., those that are specific to a particular cropping situation) can be quickly addressed and overcome. The safety of baculoviruses has been thoroughly investigated and there is no evidence that natural or recombinant baculoviruses provide an increased threat to human or environmental health.

A large number of genetically modified baculovirus pesticides have been described in the scientific literature and, undoubtedly, many more have been tested in academic, governmental, and commercial laboratories. With the currently available genes and parental viruses, it is believed that the fastest speed of kill (median lethal time) that can be achieved is around 48 h because the virus will most likely have to undergo two replication cycles before the host insect is killed. Feeding cessation (median time to feeding cessation) will occur earlier, perhaps as early as 24–36 h postinfection. Feeding cessation at 24 h postinfection should be sufficient to make GM baculovirus pesticides competitive with synthetic chemical insecticides in terms of the protection of many types of crops. In terms of a “best performing” GM baculovirus pesticide, AcMNPV offers the widest host range in terms of infectivity against pest insect species. To this parental virus we would insert at the *egt* gene locus a gene encoding AaIT fused to a bombyxin signal sequence that is expressed under a chimeric late–very late promoter (e.g., P_{SynXIV}). We prefer the AalT encoding gene instead of the mite toxin encoding gene (although the mite toxin gene appears to be more insecticidal) because its mode of action is known and the action of AaIT is synergized with pyrethroid insecticides. LqhIT2 is a viable alternative to AaIT. Also, in comparison to the other insect selective toxins such as those from spiders and sea anemones, AaIT and LqhIT2 have undergone more testing. We prefer expression under a baculovirus late-very promoter because of the slightly improved selectivity that such a promoter has in comparison to baculovirus early or constitutive promoters. We prefer the bombyxin signal sequence because it has been shown to efficiently secrete a wide variety of proteins in a wide variety of insect cells. Deletion of the *egt* gene may reduce the fitness of the virus. Other parental viruses such as HzSNPV or RoMNPV are also viable options for pest species that are not highly susceptible to AcMNPV. Additionally, the coexpression of a protease or polyhedrin-*Bt* toxin fusion protein may help the virus to penetrate the midgut more quickly resulting in a faster systemic infection.

The safety and effectiveness of GM baculoviruses as pest insect controlling agents has been studied for more than 25 years. During this time, thousands of peer-reviewed studies on the basic biology, host range, ecology, efficacy, safety, and applications of natural and recombinant baculoviruses have been published. Baculoviruses are very commonly used in hundreds of academic and commercial laboratories as protein expression vectors, as gene transfer vehicles, for surface display, and as a model system to study large DNA viruses. Natural baculoviruses are used as effective pest insect control agents in several regions of the world. This chapter primarily reviews (1) major milestones in the development of

recombinant baculoviruses for pest insect control, (2) current strategies to generate recombinant baculoviruses with improved feeding prevention and speed of kill, and (3) potential risks associated with the use of recombinant baculoviruses for pest insect control. A tremendous knowledge base is now available regarding the efficacy and safety of genetically modified baculoviruses for pest insect control. Despite this, there are some that would argue that there are unseen and unpredictable risks associated with the release of any genetically modified organism into the environment. The authors agree with this, but would also like to argue that it is impossible to completely avoid all risk associated with the use of any pesticide. We believe that the benefits afforded by GM baculoviruses far outweigh any unseen or unpredicted risks that they pose, especially in comparison to the potential risks associated with the use of chemical pesticides or genetically modified plants. The current research indicates that GM baculovirus pesticides meet or exceed the high standard of expectation set by synthetic chemical insecticides. GM baculovirus pesticides are safe, effective, and ready for immediate use.

This chapter shows that the generation of exceptionally safe and efficient biological pesticides, which readily compete with chemical pesticides, has been achieved. The technology has been tested for many years and the results are not completely unsatisfactory, and indeed are favorable in many cases. However, GM baculoviruses still have limited use in the field. Therefore, it is concluded that these are other reasons why the implementation of an available, safe, and effective pest control technology is not moving at the pace it should have done. Several world-class pesticide manufacturing companies have shown interest in the technology throughout the years. They obtained and licensed critical patents and started their own research programs that positively synergized the efforts of others. On the one hand, this commercial interest has brought excitement and acceleration to the field, on the other hand, the cancellation of their biologicals programs after several years of research have taken a high toll on the commercialization of GM baculoviruses. Were the cancellations based on scientific knowledge or the ineffectiveness or safety of the viruses? Presented here are the studies that indicate otherwise. Was profitability an issue? GM baculoviruses now compete with chemical pesticides and examples of profitable industrial manufacturing schemes of non-GM baculoviruses exist. Was public perception an issue? Recombinant organisms have been safely used for many years now and chemical pesticides have more proven side effects than highly specific baculoviruses. Thus, we believe that the lack of implementation of GM baculoviruses is primarily due to a psychological effect.

Academic laboratories have already done their part by introducing GM baculovirus technology for the benefit of mankind and by presenting proof on its profitability. However, in the process, we may also have been shown that it is quite easy to take the technology and use it without regard to the intellectual property rights. This by itself should not be an excuse because legal ways to protect international patent rights exist. To negate the negative psychological effects of seemingly unsuccessful commercialization is, therefore, the greatest problem in this field of research as we see it. There is also more to do in terms of introducing the end-users to the merits of GM baculoviruses. One thing academic researchers might have done more efficiently is better outreach. This might have induced the agricultural cooperatives to look at this new alternative earlier on and might have helped them to start production on the farm or on a large scale. The intellectual property rights as they are currently held probably exclude this possibility. So why not publicize all relevant patents to encourage the technology rather than burying it? This is a possibility that all private and public patent holders should consider at this point. The Brazilian experience is exemplary and could be a model for other parts of the world. In that instance, even though studies were initiated by the government, cooperatives and private companies eventually showed interest in commercial production of non-GM baculoviruses.

Throughout the years, attempts have been made on many occasions to liaise with the leading scientists in the field in order to achieve a level of organization and to initiate private commercial interest. But the time has now come to put all the work done in perspective by either increasing the cooperation between everyone in the field to obtain a positive result or perhaps accepting the fact that this group of biopesticides will never reach the shelves. We believe that efforts should be directed towards more practical aspects of the field including increased outreach activities and free access to the technology. A discussion involving all interested parties of the problems associated with immediate commercialization will be critical. Countries where GM baculoviruses are actively used could of course provide insight into how to successfully commercialize GM baculoviruses. The studies to date on GM baculoviruses presented in this chapter clearly show that it would be a great waste to abandon this technology.

Acknowledgments

This work was funded in part by grants from the USDA (2003–35302-13499), NIEHS (P30 ES05707), and NIH (AI58267). We thank Dr. B.C. Bonning for critical review of the manuscript and N.M. Wolf for access to data prior to publication.

References

Adachi, T., Takiya, S., Suzuki, Y., Iwami, M., Kawakami, A., *et al.*, **1989**. cDNA structure and expression of bombyxin, an insulin-like brain secretory peptide, of the silkmoth, *Bombyxi mori*. *J. Biol. Chem. 264*, 7681–7685.

Ailor, E., Betenbaugh, M.J., **1999**. Modifying secretion and post-translational processing in insect cells. *Curr. Opin. Biotech. 10*, 142–145.

Aronson, A.I., Shai, Y., **2001**. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol. Lett. 195*, 1–8.

Bai, C., Degheele, D., Jansens, S., Lambert, B., **1993**. Activity of insecticidal crystal proteins and strains of *Bacillus thuringiensis* against *Spodoptera exempta* (Walker). *J. Invertebr. Pathol. 62*, 211–215.

Barrett, J.W., Krell, P.J., Arif, B.M., **1995**. Characterization, sequencing and phylogeny of the ecdysteroid UDP-glucosyltransferase gene from 2 distinct nuclear polyhedrosis viruses isolated from *Choristoneura fumferana*. *J. Gen. Virol. 76*, 2447–2456.

Barsoum, J., Brown, R., McKee, M., Boyce, F.M., **1997**. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. *Hum. Gene Ther. 8*, 2011–2018.

Bettencourt, R., Terenius, O., Faye, I., **2002**. Hemolin gene silencing by ds-RNA injected into *Cecropia* pupae is lethal to next generation embryos. *Insect Mol. Biol. 11*, 267–271.

Bianchi, F.J.J.A., Snoeijing, I., van der Werf, W., Mans, R.M.W., Smits, P.H., *et al.*, **2000**. Biological activity of SeMNPV, AcMNPV, and three AcMNPV deletion mutants against *Spodoptera exigua* larvae (Lepidoptera: Noctuidae). *J. Invertebr. Pathol. 75*, 28–35.

Bischoff, D.S., Slavicek, J.M., **1997**. Molecular analysis of an enhancin gene in the *Lymantria dispar* nuclear polyhedrosis virus. *J. Virol. 71*, 8133–8140.

Black, B.C., Brennan, L.A., Dierks, P.M., Gard, I.E., **1997**. Commercialization of baculoviral insecticides. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 341–387.

Blissard, G., Black, B., Crook, N., Keddie, B.A., Possee, R., *et al.*, **2000**. Family Baculoviridae. In: van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., McGeoch, D.J., Maniloff, J., Mayo, M.A., Pringle, C.R., Wickner, R.B. (Eds.), Virus Toxonomy: Classification and Nomenclature of Viruses. Academic Press, San Diego, pp. 195–202.

Bole, D.G., Hendershot, L.M., Kearney, J.F., **1986**. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol. 102*, 1558–1566.

Bonning, B.C., Booth, T.F., Hammock, B.D., **1997a**. Mechanistic studies of the degradation of juvenile hormone esterase in *Manduca sexta*. *Arch. Insect Biochem. Physiol. 34*, 275–286.

Bonning, B.C., Boughton, A.J., Jin, H., Harrison, R.L., **2002**. Genetic Enhancement of Baculovirus Insecticides. Kluwer Academic/Plenum Publishers, New York.

Bonning, B.C., Hammock, B.D., **1996**. Development of recombinant baculoviruses for insect control. *Annu. Rev. Entomol. 41*, 191–210.

Bonning, B.C., Hirst, M., Possee, R.D., Hammock, B.D., **1992**. Further development of a recombinant baculovirus insecticide expressing the enzyme juvenile hormone esterase from *Heliothis virescens*. *Insect Biochem. Mol. Biol. 22*, 453–458.

Bonning, B.C., Possee, R.D., Hammock, B.D., **1999**. Insecticidal efficacy of a recombinant baculovirus expressing JHE-KK, a modified juvenile hormone esterase. *J. Invertebr. Pathol. 73*, 234–236.

Bonning, B.C., Roelvink, P.W., Vlak, J.M., Possee, R.D., Hammock, B.D., **1994**. Superior expression of juvenile hormone esterase and β-galactosidase from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus compared to the p10 protein and polyhedrin promoters. *J. Gen. Virol. 75*, 1551–1556.

Bonning, B.C., Ward, V.K., VanMeer, M.M.M., Booth, T.F., Hammock, B.D., **1997b**. Disruption of lysosomal targeting is associated with insecticidal potency of juvenile hormone esterase. *Proc. Natl Acad. Sci. USA 94*, 6007–6012.

Booth, T.F., Bonning, B.C., Hammock, B.D., **1992**. Localization of juvenile hormone esterase during development in normal and in recombinant baculovirus-infected larvae of the moth *Trichoplusia ni*. *Tissue Cell 24*, 267–282.

Bortner, C.D., Cidlowski, J.A., **2002**. Cellular mechanisms for the repression of apoptosis. *Annu. Rev. Pharmacol. Toxicol. 42*, 259–281.

Boughton, A.J., Obrycki, J.J., Bonning, B.C., **2003**. Effects of a protease-expressing recombinant baculovirus on nontarget insect predators of *Heliothis virescens*. *Biol. Control 28*, 101–110.

Bougis, P.E., Rochat, H., Smith, L.A., **1989**. Precursors of *Androctonus australis* scorpion neurotoxins: structures of precursors, processing outcomes, and expression of a functional recombinant toxin II. *J. Biol. Chem. 264*, 19259–19265.

Boyce, F.M., Bucher, N.L.R., **1996**. Baculovirus-mediated gene transfer into mammalian cells. *Proc. Natl Acad. Sci. USA 93*, 2348–2352.

Brandt, C.R., Adang, M.J., Spence, K.D., **1978**. The peritrophic membrane: ultrastructural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. *J. Invertebr. Pathol. 32*, 12–24.

Braun, C.J., Siedow, J.N., Williams, M.E., Levings, C.S., **1989**. Mutations in the maize mitochondrial T-urf13 gene eliminate sensitivity to a fungal pathotoxin. *Proc. Natl Acad. Sci. USA 86*, 4435–4439.

Bump, N.J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., *et al*., **1995**. Inhibition of ice family proteases by baculovirus antiapoptotic protein P35. *Science 269*, 1885–1888.

Burden, J.P., Hails, R.S., Windass, J.D., Suner, M.M., Cory, J.S., **2000**. Infectivity, speed of kill, and productivity of a baculovirus expressing the itch mite toxin txp-1 in second and fourth instar larvae of *Trichoplusia ni*. *J. Invertebr. Pathol. 75*, 226–236.

Burges, H.D., **1981**. Safety, safety testing and quality control of microbial pesticides. In: Burges, H.D. (Ed.), Microbiol Control of Pests and Plant Diseases 1970–1980. Academic Press, New York, pp. 737–767.

Cao, J., Ibrahim, H., Garcia, J.J., Mason, H., Granados, R.R., *et al*., **2002**. Transgenic tobacco plants carrying a baculovirus enhancin gene slow the development and increase the mortality of *Trichoplusia ni* larvae. *Plant Cell Rep. 21*, 244–250.

Caradoc-Davies, K.M.B., Graves, S., O'Reilly, D.R., Evans, O.P., Ward, V.K., **2001**. Identification and *in vivo* characterization of the *Epiphyas postvittana* nucleopolyhedrovirus ecdysteroid UDP-glucosyltransferase. *Virus Genes 22*, 255–264.

Carbonell, L.F., Hodge, M.R., Tomalski, M.D., Miller, L.K., **1988**. Synthesis of a gene coding for an insect specific scorpion neurotoxin and attempts to express it using baculovirus vectors. *Gene 73*, 409–418.

Casu, R.E., Pearson, R.D., Jarmey, J.M., Cadogan, L.C., Riding, G.A., *et al*., **1994**. Excretory/secretory chymotrypsin from *Lucilia cuprina*: purification, enzymatic specificity and amino acid sequence deduced from mRNA. *Insect Mol. Biol. 3*, 201–211.

Cestele, S., Catterall, W.A., **2000**. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie 82*, 883–892.

Chang, J.H., Choi, J.Y., Jin, B.R., Roh, J.Y., Olszewski, J.A., *et al*., **2003**. An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin. *J. Invertebr. Pathol. 84*, 30–37.

Chao, Y.C., Chen, S.L., Li, C.F., **1996**. Pest control by fluorescence. *Nature 380*, 396–397.

Chejanovsky, N., Gershburg, E., **1995**. The wild-type *Autographa californica* nuclear polyhedrosis virus induces apoptosis of *Spodoptera littoralis* cells. *Virology 209*, 519–525.

Chejanovsky, N., Zilberberg, N., Rivkin, H., Zlotkin, E., Gurevitz, M., **1995**. Functional expression of an alpha anti-insect scorpion neurotoxin in insect cells and lepidopterous larvae. *FEBS Lett. 376*, 181–184.

Chen, C.J., Quentin, M.E., Brennan, L.A., Kukel, C., Thiem, S.M., **1998**. *Lymantria dispar* nucleopolyhedrovirus hrf-1 expands the larval host range of *Autographa californica* nucleopolyhedrovirus. *J. Virol. 72*, 2526–2531.

Chen, X.W., Hu, Z.H., Jehle, J.A., Zhang, Y.Q., Vlak, J.M., **1997**. Analysis of the ecdysteroid UDP-glucosyltransferase gene of *Heliothis armigera* single-nucleocapsid baculovirus. *Virus Genes 15*, 219–225.

Chen, X.W., Sun, X.L., Hu, Z.H., Li, M., O'Reilly, D.R., *et al*., **2000**. Genetic engineering of *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus as an improved pesticide. *J. Invertebr. Pathol. 76*, 140–146.

Cherry, C.L., Summers, M.D., **1985**. Genotypic variation among wild isolates of two nuclear polyhedrosis viruses isolated from *Spodoptera littoralis*. *J. Invertebr. Pathol. 46*, 289–295.

Clarke, E.E., Tristem, M., Cory, J.S., O'Reilly, D.R., **1996**. Characterization of the ecdysteroid UDP-glucosyltransferase gene from *Mamestra brassicae* nucleopolyhedrovirus. *J. Gen. Virol. 77*, 2865–2871.

Clem, R.J., **2001**. Baculoviruses and apoptosis: the good, the bad, and the ugly. *Cell Death Differ. 8*, 137–143.

Clem, R.J., Fechheimer, M., Miller, L.K., **1991**. Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science 254*, 1388–1390.

Clem, R.J., Hardwick, J.M., Miller, L.K., **1996**. Antiapoptotic genes of baculoviruses. *Cell Death Differ. 3*, 9–16.

Clem, R.J., Miller, L.K., **1993**. Apoptosis reduces both the *in vitro* replication and the *in vivo* infectivity of a baculovirus. *J. Virol. 67*, 3730–3738.

Coast, G.M., Orchard, I., Phillips, J.E., Schooley, D.A., **2002**. Insect diuretic and antidiuretic hormones. *Adv. Insect Physiol. 29*, 279–400.

Cohen, E., **1987**. Chitin biochemistry: synthesis and inhibition. *Annu. Rev. Entomol. 32*, 71–93.

Condreay, J.P., Witherspoon, S.M., Clay, W.C., Kost, T.A., **1999**. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc. Natl Acad. Sci. USA 96*, 127–132.

Copping, L.G., Menn, J.J., **2000**. Biopesticides: a review of their action, applications and efficacy. *Pest Mgt Sci. 56*, 651–676.

Cory, J.S., **2000**. Assessing the risks of releasing genetically modified virus insecticides: progress to date. *Crop Protect. 19*, 779–785.

Cory, J.S., Hirst, M.L., Williams, T., Halls, R.S., Goulson, D., *et al*., **1994**. Field trial of a genetically improved baculovirus insecticide. *Nature (Lond.) 370*, 138–140.

Cory, J.S., Myers, H., **2003**. The ecology and evolution of insect baculovirus. *Annu. Rev. Ecol. Evol. Syst. 34*, 239–272.

Croizier, G., Croizier, L., Argaud, O., Poudevigne, D., **1994**. Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. *Proc. Natl Acad. Sci. USA 91*, 48–52.

Crook, N.E., Clem, R.J., Miller, L.K., **1993**. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol. 67*, 2168–2174.

Cusson, M., Palli, S.R., **2000**. Can juvenile hormone research help rejuvenate integrated pest management? *Can. Entomol. 132*, 263–280.

Cymborowski, B., Zimowska, G., **1984**. Switchover in the sensitivity of the prothoracic glands to juvenile hormone in the cotton leafworm, *Spodoptera littoralis*. *J. Insect Physiol. 30*, 911–918.

Dai, X., Hajos, J.P., Joosten, N.N., vanOers, M.M., Ijkel, W.F.J., *et al.*, **2000**. Isolation of a *Spodoptera exigua* baculovirus recombinant with a 10.6 kbp genome deletion that retains biological activity. *J. Gen. Virol. 81*, 2545–2554.

Daimon, T., Hamada, K., Mita, K., Okano, K., Suzuki, M.G., *et al.*, **2003**. A *Bombyx mori* gene, BmChi-h, encodes a protein homologous to bacterial and baculovirus chitinases. *Insect Biochem. Mol. Biol. 33*, 749–759.

Darbon, H., Zlotkin, E., Kopeyan, C., Vanrietschoten, J., Rochat, H., **1982**. Covalent structure of the insect toxin of the North African scorpion *Androctonus australis* Hector. *Int. J. Pep. Prot. Res. 20*, 320–330.

de Kort, C.A.D., Granger, N.A., **1996**. Regulation of JH titers: the relevance of degradative enzymes and binding proteins. *Arch. Insect Biochem. Physiol. 33*, 1–26.

Denli, A.M., Hannon, G.J., **2003**. RNAi: an ever-growing puzzle. *Trends Biochem. Sci. 28*, 196–201.

Derksen, A.C.G., Granados, R.R., **1988**. Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology 167*, 242–250.

Dinan, L., **2001**. Phytoecdysteroids: biological aspects. *Phytochemistry 57*, 325–339.

Dougherty, E.M., Gurthrie, K.P., Shapiro, M., **1996**. Optical brighteners provide baculovirus activity enhancement and UV radiation protection. *Biol. Control 7*, 71–74.

Du, Q.S., Lehavi, D., Faktor, O., Qi, Y.P., Chejanovsky, N., **1999**. Isolation of an apoptosis suppressor gene of the *Spodoptera littoralis* nucleopolyhedrovirus. *J. Virol. 73*, 1278–1285.

Du, X.L., Thiem, S.M., **1997a**. Characterization of host range factor 1 (hrf-1) expression in *Lymantria dispar* M nucleopolyhedrovirus- and recombinant *Autographa californica* M nucleopolyhedrovirus-infected IPLB-Ld652Y cells. *Virology 227*, 420–430.

Du, X.L., Thiem, S.M., **1997b**. Responses of insect cells to baculovirus infection: protein synthesis shutdown and apoptosis. *J. Virol. 71*, 7866–7872.

Dushoff, J., Dwyer, G., **2001**. Evaluating the risks of engineered viruses: modeling pathogen competition. *Ecol. Appl. 11*, 1602–1609.

Dwarakanath, R.S., Clark, C.L., McElroy, A.K., Spector, D.H., **2001**. The use of recombinant baculoviruses for sustained expression of human cytomegalovirus immediate early proteins in fibroblasts. *Virology 284*, 297–307.

Dwyer, G., Elkinton, J.S., **1993**. Using simple models to predict virus epizootics in gypsy-moth populations. *J. Animal Ecol. 62*, 1–11.

Dwyer, G., Elkinton, J.S., Buonaccorsi, J.P., **1997**. Host heterogeneity in susceptibility and disease dynamics: tests of a mathematical model. *Am. Nat. 150*, 685–707.

Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., *et al.*, **1990**. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification, primary structure, and mode of action. *Biochemistry 29*, 5941–5947.

Elazar, M., Levi, R., Zlotkin, E., **2001**. Targeting of an expressed neurotoxin by its recombinant baculovirus. *J. Exp. Biol. 204*, 2637–2645.

Eldridge, R., Horodyski, F.M., Morton, D.B., O'Reilly, D.R., Truman, J.W., *et al.*, **1991**. Expression of an eclosion hormone gene in insect cells using baculovirus vectors. *Insect Biochem. 21*, 341–351.

Eldridge, R., O'Reilly, D.R., Hammock, B.D., Miller, L.K., **1992a**. Insecticidal properties of genetically engineered baculoviruses expressing an insect juvenile hormone esterase gene. *Appl. Environ. Microbiol. 58*, 1583–1591.

Eldridge, R., O'Reilly, D.R., Miller, L.K., **1992b**. Efficacy of a baculovirus pesticide expressing an eclosion hormone gene. *Biol. Control 2*, 104–110.

Evans, H.F., **1986**. Ecology and epizootiology of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. I. CRC Press, Boca Raton, pp. 89–132.

Fajloun, Z., Kharrat, R., Chen, L., Lecomte, C., Di Luccio, E., *et al.*, **2000**. Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca^{2+} release channel/ryanodine receptors. *FEBS Lett. 469*, 179–185.

Federici, B.A., **1997**. Baculovirus pathogenesis. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 33–59.

Feng, Q.L., Ladd, T.R., Tomkins, B.L., Sundaram, M., Sohi, S.S., *et al.*, **1999**. Spruce budworm (*Choristoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning. *Mol. Cell. Endocrinol. 148*, 95–108.

Flipsen, J.T.M., Mans, R.M.W., Kleefsman, A.W.F., Knebelmorsdorf, D., Vlak, J.M., **1995**. Deletion of the baculovirus ecdysteroid UDP-glucosyltransferase gene induces early degeneration of malpighian tubules in infected insects. *J. Virol. 69*, 4529–4532.

Friedman, S., **1978**. Treholose regulation, one aspect of metabolic homeostasis. *Annu. Rev. Entomol. 23*, 389–407.

Friesen, P.D., **1997**. Regulation of baculovirus early gene expression. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 141–170.

Froy, O., Zilberberg, N., Chejanovsky, N., Anglister, J., Loret, E., *et al.*, **2000**. Scorpion neurotoxins: structure/function relationships and application in agriculture. *Pest Mgt Sci. 56*, 472–474.

Furuta, M., Schrader, J.O., Schrader, H.S., Kokjohn, T.A., Nyaga, S., *et al.*, **1997**. Chlorella virus PBCV-1 encodes a homolog of the bacteriophage T4 UV damage repair gene *den*V. *Appl. Environ. Microbiol. 63*, 1551–1556.

Fuxa, J.A., Fuxa, J.R., Richter, A.R., **1998**. Host-insect survival time and disintegration in relation to population density and dispersion of recombinant and wild-type nucleopolyhedroviruses. *Biol. Control 12*, 143–150.

Fuxa, J.R., **1991**. Release and transport of entomopathogenic microorganisms. In: Levin, M., Strauss, H. (Eds.), Risk Assessment in Genetic Engineering. McGraw-Hill, New York, pp. 83–113.

Fuxa, J.R., Richter, A.R., **1994**. Distance and rate of spread of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) nuclear polyhedrosis virus released into soybean. *Environ. Entomol. 23*, 1308–1316.

Gade, G., **2004**. Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Annu. Rev. Entomol. 49*, 93–113.

Gallo, L.G., Corsaro, B.G., Hughes, P.R., Granados, R.R., **1991**. *In vivo* enhancement of baculovirus infection by the viral enhancing factor of a granulosis virus of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol. 58*, 203–210.

Gershburg, E., Rivkin, H., Chejanovsky, N., **1997**. Expression of the *Autographa californica* nuclear polyhedrosis virus apoptotic suppressor gene *p35* in nonpermissive *Spodoptera littoralis* cells. *J. Virol. 71*, 7593–7599.

Gershburg, E., Stockholm, D., Froy, O., Rashi, S., Gurevitz, M., *et al.*, **1998**. Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. *FEBS Lett. 422*, 132–136.

Ghiasuddin, S.M., Soderlund, D.M., **1985**. Pyrethroid insecticides – potent, stereospecific enhancers of mouse brain sodium channel activation. *Pestic. Biochem. Physiol. 24*, 200–206.

Gilbert, L.I., Granger, N.A., Roe, R.M., **2000**. The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol. 30*, 617–644.

Gill, S.S., Cowles, E.A., Pietrantonio, P.V., **1992**. The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol. 37*, 615–636.

Girardie, J., Chaabihi, H., Fournier, B., Lagueux, M., Girardie, A., **2001**. Expression of neuroparsin cDNA in insect cells using baculovirus vectors. *Arch. Insect Biochem. Physiol. 46*, 26–35.

Gomi, S., Majima, K., Maeda, S., **1999**. Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *J. Gen. Virol. 80*, 1323–1337.

Gong, C.L., Kobayashi, J., Jin, W., Wu, X.F., **2000**. Inactivation analysis of HcNPV cysteine protease gene and chitinase gene. *Acta Biochim. Biophys. Sinica 32*, 187–191.

Gopalakrishnan, B., Kramer, K.J., Muthukrishnan, S., **1993**. Properties of an insect chitinase produced in a baculovirus gene expression system. *Abstr. Papers Am. Chem. Soc. 205*, 79-Agro.

Gopalakrishnan, K., Muthukrishnan, S., Kramer, K.J., **1995**. Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem. Mol. Biol. 25*, 255–265.

Gordon, D., Moskowitz, H., Eitan, M., Warner, C., Catterall, W.A., *et al.*, **1992**. Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies. *Biochemistry 31*, 7622–7628.

Gordon, D., Savarin, P., Gurevitz, M., Zinn-Justin, S., **1998**. Functional anatomy of scorpion toxins affecting sodium channels. *J. Toxicol.-Toxin Rev. 17*, 131–159.

Gould, W.P., Jeanne, R.L., **1984**. *Polistes* wasps (Hymenoptera: Vespidae) as control agents for lepidopterous cabbage pests. *Environ. Entomol. 13*, 150–156.

Goulson, D., **1997**. *Wipfelkrankheit*: modification of host behaviour during baculoviral infection. *Oecologia 109*, 219–228.

Granados, R.R., Federici, B.A., **1986**. The Biology of Baculoviruses I and II. CRC Press, Boca Raton.

Granados, R.R., Fu, Y., Corsaro, B., Hughes, P.R., **2001**. Enhancement of *Bacillus thuringiensis* toxicity to lepidopterous species with the enhancin from *Trichoplusia ni* granulovirus. *Biol. Control 20*, 153–159.

Griffiths, C.M., Barnett, A.L., Ayres, M.D., Windass, J., King, L.A., *et al.*, **1999**. *In vitro* host range of *Autographa californica* nucleopolyhedrovirus recombinants lacking functional *p35*, *iap1* or *iap2*. *J. Gen. Virol. 80*, 1055–1066.

Gröner, A., **1986**. Specificity and safety of baculoviruses. In: Granados, R.R., Federici, B.A. (Eds.), The Biology of Baculoviruses, vol. 1. CRC Press, Boca Raton, pp. 178–196.

Hails, R., **2001**. Nature and genetically modified baculoviruses: environmentally friendly pest control or an ecological threat? *Outlook Agricult. 30*, 171–178.

Hajos, J.P., Vermunt, A.M.W., Zuidema, D., Kulcsar, P., Varjas, L., *et al.*, **1999**. Dissecting insect development: baculovirus-mediated gene silencing in insects. *Insect Mol. Biol. 8*, 539–544.

Hammock, B.D., **1985**. Regulation of juvenile hormone titer: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 7. Pergamon, New York, pp. 431–472.

Hammock, B.D., **1992**. Virus release evaluation. *Nature 355*, 119.

Hammock, B.D., Abdel-Aal, Y.A.I., Mullin, C.A., Hanzlik, T.N., Roe, R.M., **1984**. Substituted thiotrifluoropropanones as potent selective inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol. 22*, 209–223.

Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N., Maeda, S., **1990a**. Expression and effects of the juvenile hormone esterase in a baculovirus vector. *Nature 344*, 458–461.

Hammock, B.D., McCutchen, B.F., Beetham, J., Choudary, P.V., Fowler, E., *et al.*, **1993**. Development of recombinant viral insecticides by expression of an insect-specific toxin and insect-specific enzyme in

nuclear polyhedrosis viruses. *Arch. Insect Biochem. Physiol. 22*, 315–344.

Hammock, B.D., Wrobleski, V., Harshman, L., Hanzlik, T., Maeda, S., *et al.*, **1990b**. Cloning, expression and biological activity of the juvenile hormone esterase from *Heliothis virescens*. In: Hagedorn, H., Hildebrand, J., Kidwell, M., Law, J. (Eds.), Molecular Insect Science. Plenum, New York, pp. 49–56.

Hanzlik, T.N., Abdel-Aal, Y.A.I., Harshman, L.G., Hammock, B.D., **1989**. Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from *Heliothis virescens*: evidence for a catalytic mechanism of the serine carboxylesterases different from that of the serine proteases. *J. Biol. Chem. 264*, 12419–12425.

Harrison, R.L., Bonning, B.C., **1999**. The nucleopolyhedroviruses of *Rachiplusia ou* and *Anagrapha falcifera* are isolates of the same virus. *J. Gen. Virol. 80*, 2793–2798.

Harrison, R.L., Bonning, B.C., **2000a**. Genetic engineering of biocontrol agents of insects. In: Rechcigl, J.E., Rechcigl, N.A. (Eds.), Biological and Biotechnological Control of Insect Pests. Lewis Publishers, Boca Raton, FL, pp. 243–280.

Harrison, R.L., Bonning, B.C., **2000b**. Use of scorpion neurotoxins to improve the insecticidal activity of *Rachiplusia ou multicapsid nucleopolyhedrovirus*. *Biol. Control 17*, 191–201.

Harrison, R.L., Bonning, B.C., **2001**. Use of proteases to improve the insecticidal activity of baculoviruses. *Biol. Control 20*, 199–209.

Harrison, R.L., Bonning, B.C., **2003**. Comparative analysis of the genomes of *Rachiplusia ou* and *Autographa californica* multiple nucleopolyhedroviruses. *J. Gen. Virol. 84*, 1827–1842.

Hashimoto, Y., Corsaro, B.G., Granados, R.R., **1991**. Location and nucleotide sequence of the gene encoding the viral enhancing factor of the *Trichoplusia ni* granulosis virus. *J. Gen. Virol. 72*, 2645–2651.

Hashimoto, Y., Hayakawa, T., Ueno, Y., Fujita, T., Sano, Y., *et al.*, **2000**. Sequence analysis of the *Plutella xylostella* granulovirus genome. *Virology 275*, 358–372.

Hawtin, R.E., Arnold, K., Ayres, M.D., Zanotto, P.M.D., Howard, S.C., *et al.*, **1995**. Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology 212*, 673–685.

Hayakawa, T., Ko, R., Okano, K., Seong, S.-I., Goto, C., *et al.*, **1999**. Sequence analysis of the *Xestia c-nigrum* granulovirus genome. *Virology 262*, 277–297.

Hayakawa, T., Rohrmann, G.F., Hashimoto, Y., **2000a**. Patterns of genome organization and content in lepidopteran baculoviruses. *Virology 278*, 1–12.

Hayakawa, T., Shimojo, E., Mori, M., Kaido, M., Furusawa, I., *et al.*, **2000b**. Enhancement of baculovirus infection in *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae with *Autographa californica* nucleopolyhedrovirus or *Nicotiana tabacum* engineered with a granulovirus enhancin gene. *Appl. Entomol. Zool. 35*, 163–170.

Heimpel, A.M., Buchanan, L.K., **1967**. Human feeding tests using a nuclear polyhedrosis virus of *Heliothis zea*. *J. Invertebr. Pathol. 9*, 55–57.

Heimpel, A.M., Thomas, E.D., Adams, J.R., Smith, L.J., **1973**. The presence of nuclear polyhedrosis virus of *Trichoplusia ni* on cabbage from the market shelf. *Environ. Entomol. 2*, 72–75.

Heinz, K.M., McCutchen, B.F., Herrmann, R., Parrella, M.P., Hammock, B.D., **1995**. Direct effects of recombinant nuclear polyhedrosis viruses on selected non-target organisms. *J. Econ. Entomol. 88*, 259–264.

Henrich, V.C., Rybczynski, R., Gilbert, L.I., **1999**. Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. Vitamins and hormones. *Adv. Res. Appl. 55*, 73–125.

Herniou, E.A., Luque, T., Chen, X., Vlak, J.M., Winstanley, D., *et al.*, **2001**. Use of whole genome sequence data to infer baculovirus phylogeny. *J. Virol. 75*, 8117–8126.

Herniou, E.A., Olszewski, J.A., Cory, J.S., O'Reilly, D.R., **2003**. The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol. 48*, 211–234.

Herrmann, R., Moskowitz, H., Zlotkin, E., Hammock, B.D., **1995**. Positive cooperativity among insecticidal scorpion neurotoxins. *Toxicon 33*, 1099–1102.

Hershberger, P.A., Dickson, J.A., Friesen, P.D., **1992**. Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. *J. Virol. 66*, 5525–5533.

Hill-Perkins, M.S., Possee, R.D., **1990**. A baculovirus expression vector derived from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol. 71*, 971–976.

Hinton, A.C., Hammock, B.D., **2003a**. *In vitro* expression and biochemical characterization of juvenile hormone esterase from *Manduca sexta*. *Insect Biochem. Mol. Biol. 33*, 317–329.

Hinton, A.C., Hammock, B.D., **2003b**. Juvenile hormone esterase (JHE) from *Tenebrio molitor*: full-length cDNA sequence, *in vitro* expression, and characterization of the recombinant protein. *Insect Biochem. Mol. Biol. 33*, 477–487.

Hirai, M., Kamimura, M., Kikuchi, K., Yasukochi, Y., Kiuchi, M., *et al.*, **2002**. cDNA cloning and characterization of *Bombyx mori* juvenile hormone esterase: an inducible gene by the imidazole insect growth regulator KK-42. *Insect Biochem. Mol. Biol. 32*, 627–635.

Hodgson, D.J., Vanbergen, A.J., Watt, A.D., Hails, R.S., Cory, J.S., **2001**. Phenotypic variation between naturally co-existing genotypes of a lepidopteran baculovirus. *Evol. Ecol. Res. 3*, 687–701.

Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., *et al.*, **1995**. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc. Natl Acad. Sci. USA 92*, 10099–10103.

Hofmann, C., Strauss, M., **1998**. Baculovirus-mediated gene transfer in the presence of human serum or blood

facilitated by inhibition of the complement system. *Gene Ther. 5*, 531–536.
Holman, G.M., Nachman, R.J., Wright, M.S., **1990**. Insect neuropeptides. *Annu. Rev. Entomol. 35*, 201–217.
Hom, L.G., Volkman, L.E., **2000**. *Autographa californica* M nucleopolyhedrovirus *chiA* is required for processing of V-CATH. *Virology 277*, 178–183.
Hoover, K., Schultz, C.M., Lane, S.S., Bonning, B.C., Duffey, S.S., *et al.*, **1995**. Reduction in damage to cotton plants by a recombinant baculovirus that knocks moribund larvae of *Heliothis virescens* off the plant. *Biol. Control 5*, 419–426.
Hsu, T.-A., Eiden, J.J., Bourgarel, P., Meo, T., Betenbaugh, M.J., **1994**. Effects of co-expressing chaperone BiP on functional antibody production in the baculovirus system. *Prot. Exp. Purif. 5*, 595–603.
Hsu, T.-A., Watson, S., Eiden, J.J., Betenbaugh, M.J., **1996**. Rescue of immunoglobulins from insolubility is facilitated by PDI in the baculovirus expression system. *Prot. Exp. Purif. 7*, 281–288.
Hu, Y.C., Tsai, C.T., Chang, Y.J., Huang, J.H., **2003**. Enhancement and prolongation of baculovirus-mediated expression in mammalian cells: focuses on strategic infection and feeding. *Biotech. Prog. 19*, 373–379.
Hu, Z.H., Broer, R., Westerlaken, J., Martens, J.W.M., Jin, F., *et al.*, **1997**. Characterization of the ecdysteroid UDP-glucosyltransferase gene of a single nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria*. *Virus Res. 47*, 91–97.
Huang, X.P., Davis, T.R., Hughes, P., Wood, A., **1997**. Potential replication of recombinant baculoviruses in nontarget insect species: reporter gene products as indicators of infection. *J. Invertebr. Pathol. 69*, 234–245.
Hughes, P.R., Wood, H.A., Breen, J.P., Simpson, S.F., Duggan, A.J., *et al.*, **1997**. Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. *J. Invertebr. Pathol. 69*, 112–118.
Hunter-Fujita, F.R., Entwistle, P.F., Evans, H.F., Crook, N.E., **1998**. Insect Viruses and Pest Management. Wiley, Chichester.
Hutvagner, G., Zamore, P.D., **2002**. RNAi: nature abhors a double-strand. *Curr. Opin. Genet. Develop. 12*, 225–232.
Ichinose, R., Kamita, S.G., Maeda, S., Hammock, B.D., **1992a**. Pharmacokinetic studies of the recombinant juvenile hormone esterase in *Manduca sexta*. *Pestic. Biochem. Physiol. 42*, 13–23.
Ichinose, R., Nakamura, A., Yamoto, T., Booth, T.F., Maeda, S., *et al.*, **1992b**. Uptake of juvenile hormone esterase by pericardial cells of *Manduca sexta*. *Insect Biochem. Mol. Biol. 22*, 893–904.
Ignoffo, C.M., Garcia, C., **1992**. Combinations of environmental factors and simulated sunlight affecting activity of inclusion bodies of the *Heliothis* (Lepidoptera, Noctuidae) nucleopolyhedrosis virus. *Environ. Entomol. 21*, 210–213.
Ignoffo, C.M., Garcia, C., **1996**. Rate of larval lysis and yield and activity of inclusion bodies harvested from *Trichoplusia ni* larvae fed a wild or recombinant strain of the nuclear polyhedrosis virus of *Autographa californica*. *J. Invertebr. Pathol. 68*, 196–198.
Ignoffo, C.M., Garcia, C., Saathoff, S.G., **1997**. Sunlight stability and rain-fastness of formulations of baculovirus *Heliothis*. *Environ. Entomol. 26*, 1470–1474.
Ignoffo, C.M., Wong, J.F.H., McCutchen, W.F., Saathoff, S.G., Garcia, C., **2000**. Yields of occlusion bodies from *Heliothis virescens* (Lepidoptera: Noctuidae) and *Helicoverpa* (*Heliothis*) *zea* (Lepidoptera: Noctuidae) larvae fed wild or recombinant strains of baculoviruses. *Appl. Entomol. Zool. 35*, 389–392.
Ijkel, W.F.J., van Strien, E.A., Heldens, J.G.M., Broer, R., Zuidema, D., *et al.*, **1999**. Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *J. Gen. Virol. 80*, 3289–3304.
Ijkel, W.F.J., Westenberg, M., Goldbach, R.W., Blissard, G.W., Vlak, J.M., *et al.*, **2000**. A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. *Virology 275*, 30–41.
Imai, N., Ali, S.E.-S., El-Singabi, N.R., Iwanaga, M., Matsumoto, S., *et al.*, **2000**. Insecticidal effects of a recombinant baculovirus expressing scorpion toxin LqhIT2. *J. Seric. Sci. Jpn 69*, 197–205.
Inceoglu, A.B., Kamita, S.G., Hinton, A.C., Huang, Q.H., Severson, T.F., *et al.*, **2001a**. Recombinant baculoviruses for insect control. *Pest Mgt Sci. 57*, 981–987.
Inceoglu, B., Lango, J., Wu, J., Hawkins, P., Southern, J., *et al.*, **2001b**. Isolation and characterization of a novel type of neurotoxic peptide from the venom of the South African scorpion *Parabuthus transvaalicus* (Buthidae). *Eur. J. Biochem. 268*, 5407–5413.
Jaffe, H., Raina, A.K., Riley, C.T., Fraser, B.A., Holman, G.M., *et al.*, **1986**. Isolation and primary structure of a peptide from the corpora cardiaca of *Heliothis zea* with adipokinetic activity. *Biochem. Biophys. Res. Comm. 135*, 622–628.
Jarvis, D.L., Fleming, J.A., Kovacs, G.R., Summers, M.D., Guarino, L.A., **1990**. Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Bio/Technology 8*, 950–955.
Jarvis, D.L., Reilly, L.M., Hoover, K., Schultz, C.M., Hammock, B.D., *et al.*, **1996**. Construction and characterization of immediate early baculovirus pesticides. *Biol. Control 7*, 228–235.
Je, Y.H., Jin, B.R., Park, H.W., Roh, J.Y., Chang, J.H., *et al.*, **2003**. Baculovirus expression vectors that incorporate the foreign protein into viral occlusion bodies. *Biotechniques 34*, 81–87.
Kamita, S.G., Hinton, A.C., Wheelock, C.E., Wogulis, M.D., Wilson, D.K., *et al.*, **2003a**. Juvenile hormone (JH) esterase: Why are you so JH specific? *Insect Biochem. Mol. Biol. 33*, 1261–1273.
Kamita, S.G., Maeda, S., Hammock, B.D., **2003b**. High-frequency homologous recombination between

baculoviruses involves DNA replication. *J. Virol. 77*, 13053–13061.

Kang, K.-D., Lee, E.-J., Kamita, S.G., Maeda, S., Seong, S.-I., **2000**. Ecdysteroid stimulates virus transmission in larvae infected with *Bombyx mori* nucleopolyhedrovirus. *J. Biochem. Mol. Biol. 33*, 63–68.

Kang, W., Tristem, M., Maeda, S., Crook, N.E., O'Reilly, D.R., **1998**. Identification and characterization of the *Cydia pomonella* granulovirus cathepsin and chitinase genes. *J. Gen. Virol. 79*, 2283–2292.

Kataoka, H., Troetschler, R.G., Li, J.P., Kramer, S.J., Carney, R.L., *et al.*, **1989**. Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. Natl Acad. Sci. USA 86*, 2976–2980.

Keeley, L.L., Hayes, T.K., **1987**. Speculations on biotechnology applications for insect neuroendocrine research. *Insect Biochem. 17*, 639–651.

Khambay, B.P.S., **2002**. Pyrethroid insecticides. *Pestic. Outlook* 2, 49–54.

Kikhno, I., Gutierrez, S., Croizier, L., Croizier, G., Ferber, M.L., **2002**. Characterization of *pif*, a gene required for the *per os* infectivity of *Spodoptera littoralis* nucleopolyhedrovirus. *J. Gen. Virol. 83*, 3013–3022.

Kim, S.H., Park, B.S., Yun, F.Y., Je, Y.H., Woo, S.D., *et al.*, **1998**. Cloning and expression of a novel gene encoding a new antibacterial peptide from silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Comm. 246*, 388–392.

Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Barhraoui, E.M., *et al.*, **1990**. Primary structure of scorpion anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus*. *FEBS Lett. 261*, 424–426.

Korth, K.L., Kaspi, C.I., Siedow, J.N., Levings, C.S., **1991**. Urf13, a maize mitochondrial pore-forming protein, is oligomeric and has a mixed orientation in *Escherichia coli* plasma membranes. *Proc. Natl Acad. Sci. USA 88*, 10865–10869.

Korth, K.L., Levings, C.S., **1993**. Baculovirus expression of the maize mitochondrial protein Urf13 confers insecticidal activity in cell cultures and larvae. *Proc. Natl Acad. Sci. USA 90*, 3388–3392.

Kost, T.A., Condreay, J.P., **2002**. Recombinant baculoviruses as mammalian cell gene delivery vectors. *Trends Biotech. 20*, 173–180.

Kozuma, K., Hukuhara, T., **1994**. Fusion characteristics of a nuclear polyhedrosis virus in cultured cells: time course and effect of a synergistic factor and pH. *J. Invertebr. Pathol. 63*, 63–67.

Kramer, K.J., Muthukrishnan, S., **1997**. Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem. Mol. Biol. 27*, 887–900.

Kramer, S.F., Bentley, W.E., **2003**. RNA interference as a metabolic engineering tool: potential for *in vivo* control of protein expression in an insect larval model. *Metab. Eng. 5*, 183–190.

Kunimi, Y., Fuxa, J.R., Hammock, B.D., **1996**. Comparison of wild type and genetically engineered nuclear polyhedrosis viruses of *Autographa californica* for mortality, virus replication and polyhedra production in *Trichoplusia ni* larvae. *Entomol. Exp. Appl. 81*, 251–257.

Kunimi, Y., Fuxa, J.R., Richter, A.R., **1997**. Survival times and lethal doses for wild and recombinant *Autographa californica* nuclear polyhedrosis viruses in different instars of *Pseudoplusia includens*. *Biol. Control 9*, 129–135.

Lacey, L.A., Frutos, R., Kaya, H.K., Vail, P., **2001**. Insect pathogens as biological control agents: Do they have a future? *Biol. Control 21*, 230–248.

Lafont, R., **2000**. Understanding insect endocrine systems: molecular approaches. *Entomol. Exp. Appl. 97*, 123–136.

Lee, S.Y., Qu, X.Y., Chen, W.B., Poloumienko, A., MacAfee, N., *et al.*, **1997**. Insecticidal activity of a recombinant baculovirus containing an antisense c-myc fragment. *J. Gen. Virol. 78*, 273–281.

Lee, Y., Fuxa, J.R., **2000**. Transport of wild-type and recombinant nucleopolyhedroviruses by scavenging and predatory arthropods. *Microb. Ecol. 39*, 301–313.

Lee, Y., Fuxa, J.R., Inceoglu, A.B., Alaniz, S.A., Richter, A.R., *et al.*, **2001**. Competition between wild-type and recombinant nucleopolyhedroviruses in a greenhouse microcosm. *Biol. Control 20*, 84–93.

Leisy, D.J., Lewis, T.D., Leong, J.A.C., Rohrmann, G.F., **2003**. Transduction of cultured fish cells with recombinant baculoviruses. *J. Gen. Virol. 84*, 1173–1178.

Lepore, L.S., Roelvink, P.R., Granados, R.R., **1996**. Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. *J. Invertebr. Pathol. 68*, 131–140.

Levidow, L., **1995**. The Oxford baculovirus controversy: Safely testing safety? *Bioscience 45*, 545–551.

Li, J.B., Heinz, K.M., Flexner, J.L., McCutchen, B.F., **1999**. Effects of recombinant baculoviruses on three nontarget heliothine predators. *Biol. Control 15*, 293–302.

Li, Q.J., Donly, C., Li, L.L., Willis, L.G., Theilmann, D.A., *et al.*, **2002**. Sequence and organization of the *Mamestra configurata* nucleopolyhedrovirus genome. *Virology 294*, 106–121.

Li, Q.J., Li, L.L., Moore, K., Donly, C., Theilmann, D.A., *et al.*, **2003**. Characterization of *Mamestra configurata* nucleopolyhedrovirus enhancin and its functional analysis via expression in an *Autographa californica* M nucleopolyhedrovirus recombinant. *J. Gen. Virol. 84*, 123–132.

Liszewski, K., Atkinson, J.P., **1993**. The complement system. In: William, P.E. (Ed.), Fundamental Immunology. Raven Press, New York, pp. 917–939.

Loret, E.P., Hammock, B.D., **1993**. Structure and neurotoxicity of scorpion venom. In: Brownell, P.H., Polis, G. (Eds.), Scorpion Biology and Research. Oxford University Press, Oxford, pp. 204–233.

Lotze, M.T., Kost, T.A., **2002**. Viruses as gene delivery vectors: application to gene function, target validation, and assay development. *Cancer Gene Ther. 9*, 692–699.

Lu, A., Miller, L.K., **1995**. Differential requirements for baculovirus late expression factor genes in 2 cell lines. *J. Virol. 69*, 6265–6272.

Lu, A., Milller, L.K., **1997**. Regulation of baculovirus late and very late gene expression. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 193–216.

Lu, A., Seshagiri, S., Miller, L.K., **1996**. Signal sequence and promoter effects on the efficacy of toxin-expressing baculoviruses as biopesticides. *Biol. Control 7*, 320–332.

Lu, L.Q., Du, Q.S., Chejanovsky, N., **2003**. Reduced expression of the immediate-early protein IE0 enables efficient replication of *Autographa californica* multiple nucleopolyhedrovirus in poorly permissive *Spodoptera littoralis* cells. *J. Virol. 77*, 535–545.

Lung, O., Westenberg, M., Vlak, J.M., Zuidema, D., Blissard, G.W., **2002**. Pseudotyping *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. *J. Virol. 76*, 5729–5736.

Lung, O.Y., Cruz-Alvarez, M., Blissard, G.W., **2003**. Ac23, an envelope fusion protein homolog in the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. *J. Virol. 77*, 328–339.

Luque, T., Finch, R., Crook, N., O'Reilly, D.R., Winstanley, D., **2001**. The complete sequence of the *Cydia pomonella* granulovirus genome. *J. Gen. Virol. 82*, 2531–2547.

Ma, P.W.K., Davis, T.R., Wood, H.A., Knipple, D.C., Roelofs, W.L., **1998**. Baculovirus expression of an insect gene that encodes multiple neuropeptides. *Insect Biochem. Mol. Biol. 28*, 239–249.

Maeda, S., **1989a**. Expression of foreign genes in insects using baculovirus vectors. *Annu. Rev. Entomol. 34*, 351–372.

Maeda, S., **1989b**. Increased insecticidal effect by a recombinant baculovirus carrying a synthetic diuretic hormone gene. *Biochem. Biophys. Res. Comm. 165*, 1177–1183.

Maeda, S., Kamita, S.G., Kondo, A., **1993**. Host-range expansion of *Autographa californica* nuclear polyhedrosis virus (NPV) following recombination of a 0.6-kilobase pair DNA fragment originating from *Bombyx mori* NPV. *J. Virol. 67*, 6234–6238.

Maeda, S., Mukohara, Y., Kondo, A., **1990**. Characteristically distinct isolates of the nuclear polyhedrosis virus from *Spodoptera litura*. *J. Gen. Virol. 71*, 2631–2640.

Maeda, S., Volrath, S.L., Hanzlik, T.N., Harper, S.A., Majima, K., *et al.*, **1991**. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. *Virology 184*, 777–780.

Manzan, M.A., Lozano, M.E., Sciocco-Cap, A., Ghiringhelli, P.D., Romanowski, V., **2002**. Identification and characterization of the ecdysteroid UDP-glycosyltransferase gene of *Epinotia aporema* granulovirus. *Virus Genes 24*, 119–130.

Martens, J.W.M., Honee, G., Zuidema, D., Lent, J.W.M.V., Visser, B., *et al.*, **1990**. Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. *Appl. Environ. Microbiol. 56*, 2764–2770.

Martens, J.W.M., Knoester, M., Weijts, F., Groffen, S.J.A., Hu, Z.H., *et al.*, **1995**. Characterization of baculovirus insecticides expressing tailored *Bacillus thuringiensis* Cry1a(B) crystal proteins. *J. Invertebr. Pathol. 66*, 249–257.

Matsumoto, N., Okada, M., Takahashi, H., Ming, Q.X., Nakajima, Y., *et al.*, **1986**. Molecular cloning of a cDNA and assignment of the C-terminal of sarcotoxin IA, a potent antibacterial protein of *Sarcophaga peregrina*. *Biochem. J. 239*, 717–722.

Mazzacano, C.A., Du, X.L., Thiem, S.M., **1999**. Global protein synthesis shutdown in *Autographa californica* nucleopolyhedrovirus-infected Ld652Y cells is rescued by tRNA from uninfected cells. *Virology 260*, 222–231.

McCutchen, B.F., Choundary, P.V., Crenshaw, R., Maddox, D., Kamita, S.G., *et al.*, **1991**. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Bio/Technology 9*, 848–852.

McCutchen, B.F., Hammock, B.D., **1994**. Recombinant baculovirus expressing an insect-selective neurotoxin: characterization, strategies for improvement and risk assessment. In: Hedin, P.A., Menn, J.J., Hollingworth, R.M. (Eds.), Natural and Engineered Pest Management Agents. American Chemical Society, Washington, DC, pp. 348–367.

McCutchen, B.F., Herrmann, R., Heinz, K.M., Parrella, M.P., Hammock, B.D., **1996**. Effects of recombinant baculoviruses on a nontarget endoparasitoid of *Heliothis virescens*. *Biol. Control 6*, 45–50.

McCutchen, B.F., Hoover, K., Preisler, H.K., Betana, M.D., Herrmann, R., *et al.*, **1997**. Interactions of recombinant and wild-type baculoviruses with classical insecticides and pyrethroid-resistant tobacco budworm (Lepidoptera: Noctuidae). *J. Econ. Entomol. 90*, 1170–1180.

McNitt, L., Espelie, K.E., Miller, L.K., **1995**. Assessing the safety of toxin-producing baculovirus biopesticides to a nontarget predator, the social wasp *Polistes metricus* Say. *Biol. Control 5*, 267–278.

Means, J.C., Muro, I., Clem, R.J., **2003**. Silencing of the baculovirus Op-iap3 gene by RNA interference reveals that it is required for prevention of a apoptosis during *Orgyia pseudotsugata* M nucleopolyhedrovirus infection of Ld652Y cells. *J. Virol. 77*, 4481–4488.

Menn, J.J., Borkovec, A.B., **1989**. Insect neuropeptides – potential new insect control agents. *J. Agric. Food Chem. 37*, 271–278.

Merrington, C.L., King, L.A., Posse, R.D., **1999**. Baculovirus expression systems. In: Higgins, S.J., Hames, B.D. (Eds.), Protein Expression: A Practical Approach. Oxford University Press, Oxford, pp. 101–127.

Merryweather, A.T., Weyer, U., Harris, M.P.G., Hirst, M., Booth, T., *et al.*, **1990**. Construction of genetically engineered baculovirus insecticides containing the

Bacillus thuringiensis subsp. *kurstaki* HD-73 delta endotoxin. *J. Gen. Virol. 71*, 1535–1544.

Meyer, A., Hanzmannn, E., Schneiderman, H.A., Gilbert, L.I., Boyette, M., **1970**. The isolation and identification of the two juvenile hormones from the *Cecropia* silk moth. *Arch. Biochem. Biophys. 137*, 190–213.

Milks, M.L., Leptich, M.K., Theilmann, D.A., **2001**. Recombinant and wild-type nucleopolyhedroviruses are equally fit in mixed infections. *Environ. Entomol. 30*, 972–981.

Miller, L.K., **1988**. Baculoviruses as gene expression vectors. *Annu. Rev. Microbiol. 42*, 177–199.

Miller, L.K., **1995**. Genetically engineered insect virus pesticides: present and future. *J. Invertebr. Pathol. 65*, 211–216.

Miller, L.K., **1997**. The Baculoviruses. Plenum, New York.

Miller, L.K., Kaiser, W.J., Seshagiri, S., **1998**. Baculovirus regulation of apoptosis. *Semin. Virol. 8*, 445–452.

Miller, L.K., Lu, A., **1997**. The molecular basis of baculovirus host range. In: Miller, L.K. (Ed.), Plenum, New York, pp. 217–235.

Morris, T.D., Miller, L.K., **1992**. Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines. *J. Virol. 66*, 7397–7405.

Morris, T.D., Miller, L.K., **1993**. Characterization of productive and non-productive AcMNPV infection in selected insect cell lines. *Virology 197*, 339–348.

Moscardi, F., **1999**. Assessment of the application of baculoviruses for control of Lepidoptera. *Annu. Rev. Entomol. 44*, 257–289.

Moskowitz, H., Herrmann, R., Jones, A.D., Hammock, B.D., **1998**. A depressant insect-selective toxin analog from the venom of the scorpion *Leiurus quinquestriatus hebraeus*, purification and structure/function characterization. *Eur. J. Biochem. 254*, 44–49.

Nakagawa, Y., Lee, Y.M., Lehmberg, E., Herrmann, R., Herrmann, R., *et al.*, **1997**. Anti-insect toxin 5 (AaIT5) from *Androctonus australis*. *Eur. J. Biochem. 246*, 496–501.

Nakagawa, Y., Sadilek, M., Lehmberg, E., Herrmann, R., Herrmann, R., *et al.*, **1998**. Rapid purification and molecular modeling of AaIT peptides from venom of *Androctonus australis*. *Arch. Insect Biochem. Physiol. 38*, 53–55.

Nakai, M., Goto, C., Shiotsuki, T., Kunimi, Y., **2002**. Granulovirus prevents pupation and retards development of *Adoxophyes honmai* larvae. *Physiol. Entomol. 27*, 157–164.

Nijhout, H.F., **1994**. Insect Hormones. Princeton University Press, Princeton, NJ.

Ohba, M., Tanada, Y., **1984**. *In vitro* enhancement of nuclear polyhedrosis virus infection by the synergistic factor of a granulosis virus of the armyworm, *Pseudaletia unipuncta* (Lepidoptera, Noctuidae). *Ann. Virol. 135*, 167–176.

Ohkawa, T., Majima, K., Maeda, S., **1994**. A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. *J. Virol. 68*, 6619–6625.

Ohno, S., Taniguchi, T., **1981**. Structure of a chromosomal gene for human interferon β. *Proc. Natl Acad. Sci. USA 78*, 5305–5309.

Ooi, B.G., Rankin, C., Miller, L.K., **1989**. Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. *J. Mol. Biol. 210*, 721–736.

O'Reilly, D.R., **1995**. Baculovirus-encoded ecdysteroid UDP-glucosyltransferases. *Insect Biochem. Mol. Biol. 25*, 541–550.

O'Reilly, D.R., **1997**. Auxilary genes of baculoviruses. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum, New York, pp. 267–300.

O'Reilly, D.R., Kelly, T.J., Masler, E.P., Thyagaraja, B.S., Robson, R.M., *et al.*, **1995**. Overexpression of *Bombyx mori* prothoracicotropic hormone using baculovirus vectors. *Insect Biochem. Mol. Biol. 25*, 475–485.

O'Reilly, D.R., Miller, L.K., **1989**. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyltransferase. *Science 245*, 1110–1112.

O'Reilly, D.R., Miller, L.K., **1991**. Improvement of a baculovirus pesticide by deletion of the *egt* gene. *Bio/Technology 9*, 1086–1089.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., **1992**. Baculovirus Expression Vectors: A Laboratory Manual. W.H. Freeman, New York.

Pang, Y., Yu, J.X., Wang, L.H., Hu, X.H., Bao, W.D., *et al.*, **2001**. Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. *Virology 287*, 391–404.

Pearson, M.N., Bjornson, R.M., Ahrens, C., Rohrmann, G.F., **1993**. Identification and characterization of a putative origin of DNA-replication in the genome of a baculovirus pathogenic for *Orgyia pseudotsugata*. *Virology 197*, 715–725.

Pearson, M.N., Groten, C., Rohrmann, G.F., **2000**. Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae. *J. Virol. 74*, 6126–6131.

Pearson, M.N., Russell, R.L., Rohrmann, G.F., **2001**. Characterization of a baculovirus-encoded protein that is associated with infected-cell membranes and budded virions. *Virology 291*, 22–31.

Pelengaris, S., Khan, M., **2003**. The many faces of c-MYC. *Arch. Biochem. Biophys. 416*, 129–136.

Peng, F., Fuxa, J.R., Richter, A.R., Johnson, S.J., **1999**. Effects of heat-sensitive agents, soil type, moisture, and leaf surface on persistence of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. *Environ. Entomol. 28*, 330–338.

Petrik, D.T., Iseli, A., Montelone, B.A., Van Etten, J.L., Clem, R.J., **2003**. Improving baculovirus resistance to UV inactivation: increased virulence resulting from expression of a DNA repair enzyme. *J. Invertebr. Pathol. 82*, 50–56.

Pinedo, F.J.R., Moscardi, F., Luque, T., Olszewski, J.A., Ribeiro, B.M., **2003**. Inactivation of the ecdysteroid UDP-glucosyltransferase (*egt*) gene of *Anticarsia*

gemmatalis nucleopolyhedrovirus (AgMNPV) improves its virulence towards its insect host. *Biol. Control 27*, 336–344.

Ponnuvel, K.M., Nakazawa, H., Furukawa, S., Asaoka, A., Ishibashi, J., *et al.*, **2003**. A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. *J. Virol. 77*, 10725–10729.

Popham, H.J.R., Bischoff, D.S., Slavicek, J.M., **2001**. Both *Lymantria dispar* nucleopolyhedrovirus enhancin genes contribute to viral potency. *J. Virol. 75*, 8639–8648.

Popham, H.J.R., Li, Y.H., Miller, L.K., **1997**. Genetic improvement of *Helicoverpa zea* nuclear polyhedrosis virus as a biopesticide. *Biol. Control 10*, 83–91.

Popham, H.J.R., Pellock, B.J., Robson, M., Dierks, P.M., Miller, L.K., **1998a**. Characterization of a variant of *Autographa californica* nuclear polyhedrosis virus with a nonfunctional ORF 603. *Biol. Control 12*, 223–230.

Popham, H.J.R., Prikhod'ko, G.G., Felcetto, T.J., Ostlind, D.A., Warmke, J.W., *et al.*, **1998b**. Effect of deltamethrin treatment on lepidopteran larvae infected with baculoviruses expressing insect-selective toxins mu-Aga-IV, As II, or Sh 1. *Biol. Control 12*, 79–87.

Possani, L.D., Becerril, B., Delepierre, M., Tytgat, J., **1999**. Scorpion toxins specific for Na^+-channels. *Eur. J. Biochem. 264*, 287–300.

Prikhod'ko, E.A., Lu, A., Wilson, J.A., Miller, L.K., **1999**. *In vivo* and *in vitro* analysis of baculovirus *ie-2* mutants. *J. Virol. 73*, 2460–2468.

Prikhod'ko, G.G., Popham, H.J.R., Felcetto, T.J., Ostlind, D.A., Warren, V.A., *et al.*, **1998**. Effects of simultaneous expression of two sodium channel toxin genes on the properties of baculoviruses as biopesticides. *Biol. Control 12*, 66–78.

Prikhod'ko, G.G., Robson, M., Warmke, J.W., Cohen, C.J., Smith, M.M., *et al.*, **1996**. Properties of three baculoviruses expressing genes that encode insect-selective toxins: mu-Aga-IV, As II, and SH I. *Biol. Control 7*, 236–244.

Regev, A., Rivkin, H., Inceoglu, B., Gershburg, E., Hammock, B.D., *et al.*, **2003**. Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively. *FEBS Lett. 537*, 106–110.

Ribeiro, B.M., Crook, N.E., **1993**. Expression of full-length and truncated forms of crystal protein genes from *Bacillus thuringiensis* subsp. *kurstaki* in a baculovirus and pathogenicity of the recombinant viruses. *J. Invertebr. Pathol. 62*, 121–130.

Ribeiro, B.M., Crook, N.E., **1998**. Construction of occluded recombinant baculoviruses containing the full-length *cry1Ab* and *cry1Ac* genes from *Bacillus thuringiensis*. *Brazilian J. Med. Biol. Res. 31*, 763–769.

Richards, A.G., Richards, P.A., **1977**. The peritrophic membrane of insects. *Annu. Rev. Entomol. 22*, 219–240.

Richardson, C.D., **1995**. Baculovirus Expression Protocols. Humana Press, Totowa.

Riddiford, L.M., **1994**. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. In: Evans, P.D. (Ed.), Advances in Insect Physiology. Academic Press, San Diego, pp. 213–274.

Riegel, C.I., Lannerherrera, C., Slavicek, J.M., **1994**. Identification and characterization of the ecdysteroid UDP-glucosyltransferase gene of the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. *J. Gen. Virol. 75*, 829–838.

Rodrigues, J.C.M., De Souza, M.L., O'Reilly, D., Velloso, L.M., Pinedo, F.J.R., *et al.*, **2001**. Characterization of the ecdysteroid UDP-glucosyltransferase (*egt*) gene of *Anticarsia gemmatalis* nucleopolyhedrovirus. *Virus Genes 22*, 103–112.

Roe, R.M., Venkatesh, K., **1990**. Metabolism of juvenile hormones: degradation and titer regulation. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods, vol. I. Rutgers University Press, New Brunswick, pp. 126–179.

Roelvink, P.W., Corsaro, B.G., Granados, R.R., **1995**. Characterization of the *Helicoverpa armigera* and *Pseudaletia unipuncta* granulovirus enhancin genes. *J. Gen. Virol. 76*, 2693–2705.

Roelvink, P.W., VanMeer, M.M.M., deKort, C.A.D., Possee, R.D., Hammock, B.D., *et al.*, **1992**. Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *J. Gen. Virol. 73*, 1481–1489.

Roller, H., Dahm, K.H., Sweeley, C.C., Trost, B.M., **1967**. Die Struktur des Juvenilhormon. *Angew. Chem. 79*, 190–191.

Ruddon, R.W., Bedows, E., **1997**. Assisted protein folding. *J. Biol. Chem. 272*, 3125–3128.

Ryerse, J.S., **1998**. Basal laminae. In: Harrison, F.W., Locke, M. (Eds.), Insecta, vol. 11A. Wiley-Liss, New York, pp. 3–16.

Sandig, V., Hofmann, C., Steinert, S., Jennings, G., Schlag, P., *et al.*, **1996**. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. *Hum. Gene Ther. 7*, 1937–1945.

Sarkis, C., Serguera, C., Petres, S., Buchet, D., Ridet, J.L., *et al.*, **2000**. Efficient transduction of neural cells *in vitro* and *in vivo* by a baculovirus-derived vector. *Proc. Natl Acad. Sci. USA 97*, 14638–14643.

Sato, K., Komoto, M., Sato, T., Enei, H., Kobayashi, M.M., *et al.*, **1997**. Baculovirus-mediated expression of a gene for trehalase of the mealworm beetle, *Tenebrio molitor*, in insect cells, SF-9, and larvae of the cabbage armyworm, *Mamestra brassicae*. *Insect Biochem. Mol. Biol. 27*, 1007–1016.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., *et al.*, **1998**. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev. 62*, 775.

Shanmugavelu, M., Baytan, A.R., Chesnut, J.D., Bonning, B.C., **2000**. A novel protein that binds juvenile hormone esterase in fat body tissue and pericardial cells of the tobacco hornworm *Manduca sexta* L. *J. Biol. Chem. 275*, 1802–1806.

Shanmugavelu, M., Porubleva, L., Chitnis, P., Bonning, B.C., **2001**. Ligand blot analysis of juvenile hormone

esterase binding proteins in *Manduca sexta* L. *Insect Biochem. Mol. Biol. 31*, 51–56.

Shapiro, D.I., Fuxa, J.R., Braymer, H.D., Pashley, D.P., **1991**. DNA restriction polymorphism in wild isolates of *Spodoptera frugiperda* nuclear polyhedrosis virus. *J. Invert. Pathol. 58*, 96–105.

Shinoda, T., Kobayashi, J., Matsui, M., Chinzei, Y., **2001**. Cloning and functional expression of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using a recombinant baculovirus lacking the virus-encoded chitinase gene. *Insect Biochem. Mol. Biol. 31*, 521–532.

Shoji, I., Aizaki, H., Tani, H., Ishii, K., Chiba, T., *et al.*, **1997**. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. *J. Gen. Virol. 78*, 2657–2664.

Slack, J.M., Kuzio, J., Faulkner, P., **1995**. Characterization of V-Cath, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *J. Gen. Virol. 76*, 1091–1098.

Slavicek, J.M., Popham, H.J.R., Riegel, C.I., **1999**. Deletion of the *Lymantria dispar* multicapsid nucleopolyhedrovirus ecdysteroid UDP-glucosyl transferase gene enhances viral killing speed in the last instar of the gypsy moth. *Biol. Control 16*, 91–103.

Smith, C.R., Heinz, K.M., Sansone, C.G., Flexner, J.L., **2000a**. Impact of recombinant baculovirus applications on target heliothines and nontarget predators in cotton. *Biol. Control 19*, 201–214.

Smith, C.R., Heinz, K.M., Sansone, C.G., Flexner, J.L., **2000b**. Impact of recombinant baculovirus field applications on a nontarget heliothine parasitoid, *Microplitis croceipes* (Hymenoptera: Braconidae). *J. Econ. Entomol. 93*, 1109–1117.

Smith, I., Goodale, C., **1998**. Sequence and *in vivo* transcription of *Lacanobia oleracea* granulovirus *egt*. *J. Gen. Virol. 79*, 405–413.

Snyder, M., Hunkapiller, M., Yuen, D., Silvert, D., Fristrom, J., *et al.*, **1982**. Cuticle protein genes of *Drosophila* – structure, organization and evolution of 4 clustered genes. *Cell 29*, 1027–1040.

Soderlund, D.M., Clark, J.M., Sheets, L.P., Mullin, L.S., Piccirillo, V.J., *et al.*, **2002**. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology 171*, 3–59.

Stewart, L.M., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.J., *et al.*, **1991**. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature 352*, 85–88.

Summers, M.D., Smith, G.E., **1987**. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station Bulletin No. 1555.

Sun, X., Wang, H., Sun, X., Chen, X., Peng, C., *et al.*, **2004**. Biological activity and field efficacy of a genetically modified *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus expressing an insect-selective toxin from a chimeric promoter. *Biol. Control 29*, 124–137.

Sun, X.L., Chen, X.W., Zhang, Z.X., Wang, H.L., Bianchi, J.J.A., *et al.*, **2002**. Bollworm responses to release of genetically modified *Helicoverpa armigera* nucleopolyhedroviruses in cotton. *J. Invertebr. Pathol. 81*, 63–69.

Taha, A., Nour-el-Din, A., Croizier, L., Ferber, M.C., Croizier, G., **2000**. Comparative analysis of the granulin regions of the *Phthorimaea operculella* and *Spodoptera littoralis* granuloviruses. *Virus Genes 21*, 147–155.

Tanada, Y., **1959**. Synergism between two viruses of the armyworm *Pseudaletia unipuncta* (Haworth) (Lepidoptera, Noctuidae). *J. Insect Pathol. 6*, 378–380.

Tanada, Y., Hukuhara, T., **1968**. A nonsynergistic strain of a granulosis virus of the armyworm, *Pseudaletia unipuncta*. *J. Invertebr. Pathol. 12*, 263–268.

Tanada, Y., Kaya, H.K., **1993**. Insect Pathology. Academic Press, San Diego.

Taniai, K., Inceoglu, A.B., Hammock, B.D., **2002**. Expression efficiency of a scorpion neurotoxin, AaHIT, using baculovirus in insect cells. *Appl. Entomol. Zool. 37*, 225–232.

Tellam, R.L., **1996**. The peritrophic membrane. In: Lehane, M.J., Billingsley, B.F. (Eds.), Biology of the Insect Midgut. Chapman and Hall, New York, pp. 86–114.

Tessier, D.C., Thomas, D.Y., Khouri, H.E., Laliberte, F., Vernet, T., **1991**. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. *Gene 98* (2), 177–183.

Thiem, S.M., **1997**. Prospects for altering host range for baculovirus bioinsecticides. *Curr. Opin. Biotech. 8*, 317–322.

Thiem, S.M., Du, X.L., Quentin, M.E., Berner, M.M., **1996**. Identification of a baculovirus gene that promotes *Autographa californica* nuclear polyhedrosis virus replication in a nonpermissive insect cell line. *J. Virol. 70*, 2221–2229.

Thomas, B.A., Church, W.B., Lane, T.R., Hammock, B.D., **1999**. Homology model of juvenile hormone esterase from the crop pest, *Heliothis virescens*. *Proteins: Struct. Funct. Genet. 34*, 184–196.

Thomas, E.D., Heimpel, A.M., Adams, J.R., **1974**. Determination of the active nuclear polyhedrosis virus content of untreated cabbages. *Environ. Entomol. 3*, 908–910.

Tomalski, M.D., Bruce, W.A., Travis, J., Blum, M.S., **1988**. Preliminary characterization of toxins from the straw itch mite, *Pyemotes tritici*, which induce paralysis in the larvae of a moth. *Toxicon 26*, 127–132.

Tomalski, M.D., Hutchinson, K., Todd, J., Miller, L.K., **1993**. Identification and characterization of Tox21a – a mite cDNA encoding a paralytic neurotoxin related to Txp-I. *Toxicon 31*, 319–326.

Tomalski, M.D., Kutney, R., Bruce, W.A., Brown, M.R., Blum, M.S., *et al.*, **1989**. Purification and characterization of insect toxins derived from the mite, *Pyemotes tritici*. *Toxicon 27*, 1151–1167.

Tomalski, M.D., Miller, L.D., **1992**. Expression of a paralytic neurotoxin gene to improve insect baculoviruses as biopesticides. *Bio/Technology 10*, 545–549.

Tomalski, M.D., Miller, L.K., **1991**. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature 352*, 82–85.

Treacy, M.F., All, J.N., Ghidiu, G.M., **1997**. Effect of ecdysteroid UDP-glucosyltransferase gene deletion on efficacy of a baculovirus against *Heliothis virescens* and *Trichoplusia ni* (Lepidoptera: Noctuidae). *J. Econ. Entomol. 90*, 1207–1214.

Treacy, M.F., Rensner, P.E., All, J.N., **2000**. Comparative insecticidal properties of two nucleopolyhedrovirus vectors encoding a similar toxin gene chimer. *J. Econ. Entomol. 93*, 1096–1104.

Trombetta, E.S., Parodi, A.J., **2003**. Quality control and protein folding in the secretory pathway. *Annu. Rev. Cell Devel. Biol. 19*, 649–676.

Truman, J.W., Riddiford, L.M., **2002**. Endocrine insights into the evolution of metamorphosis in insects. *Annu. Rev. Entomol. 47*, 467–500.

Tumilasci, V.F., Leal, E., Marinho, P., Zanotto, A., Luque, T., *et al*., **2003**. Sequence analysis of a 5.1 kbp region of the *Spodoptera frugiperda* multicapsid nucleopolyhedrovirus genome that comprises a functional ecdysteroid UDP-glucosyltransferase (*egt*) gene. *Virus Genes 27*, 137–144.

Vail, P.V., Hostertter, D.L., Hoffmann, D.F., **1999**. Development of the multi-nucleocapsid nucleopolyhedroviruses (MNPVs) infectious to loopers (Lepidoptera: Noctuidae: Plusiinae) as microbial control agents. *Integr. Pest Mgt Rev. 4*, 231–257.

Vail, P.V., Jay, D.L., Hunter, D.K., **1973**. Infectivity of a nuclear polyhedrosis virus from the alfalfa looper, *Autographa californica*, after passage through alternate hosts. *J. Invertebr. Pathol. 21*, 16–20.

Vakharia, V.N., Raina, A.K., Kingan, T.G., Kempe, T.G., **1995**. Synthetic pheromone biosynthesis activating neuropeptide gene expressed in a baculovirus expression system. *Insect Biochem. Mol. Biol. 25*, 583–589.

Valdes, V.J., Sampieri, A., Sepulveda, J., Vaca, L., **2003**. Using double-stranded RNA to prevent *in vitro* and *in vivo* viral infections by recombinant baculovirus. *J. Biol. Chem. 278*, 19317–19324.

van Beek, N., Lu, A., Presnail, J., Davis, D., Greenamoyer, C., *et al*., **2003**. Effect of signal sequence and promoter on the speed of action of a genetically modified *Autographa californica* nucleopolyhedrovirus expressing the scorpion toxin LqhIT2. *Biol. Control 27*, 53–64.

van Meer, M.M.M., Bonning, B.C., Ward, V.K., Vlak, J.M., Hammock, B.D., **2000**. Recombinant, catalytically inactive juvenile hormone esterase enhances efficacy of baculovirus insecticides. *Biol. Control 19*, 191–199.

Vasconcelos, S.D., Cory, J.S., Wilson, K.R., Sait, S.M., Hails, R.S., **1996**. Modified behaviour in baculovirus-infected lepidopteran larvae and its impact on the spatial distribution of inoculum. *Biol. Control 7*, 299–306.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klingeroode, E.C., *et al*., **1990**. Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology 179*, 312–320.

Wagle, M., Jesuthasan, S., **2003**. Baculovirus-mediated gene expression in zebrafish. *Mar. Biotech. 5*, 58–63.

Wang, P., Granados, R.R., **1997**. An intestinal mucin is the target substrate for a baculovirus enhancin. *Proc. Natl Acad. Sci. USA 94*, 6977–6982.

Wang, P., Hammer, D.A., Granados, R.R., **1994**. Interaction of *Trichoplusia ni* granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of 4 lepidopteran insects. *J. Gen. Virol. 75*, 1961–1967.

Wang, X.Z., Ooi, B.G., Miller, L.K., **1991**. Baculovirus vectors for multiple gene expression and for occluded virus production. *Gene 100*, 131–137.

Washburn, J.O., Chan, E.Y., Volkman, L.E., Aumiller, J.J., Jarvis, D.L., **2003**. Early synthesis of budded virus envelope fusion protein GP64 enhances *Autographa californica* multicapsid nucleopolyhedrovirus virulence in orally infected *Heliothis virescens*. *J. Virol. 77*, 280–290.

Westenberg, M., Wang, H.L., Ijkel, W.F.J., Goldbach, R.W., Vlak, J.M., *et al*., **2002**. Furin is involved in baculovirus envelope fusion protein activation. *J. Virol. 76*, 178–184.

Whitford, M., Stewart, S., Kuzio, J., Faulkner, P., **1989**. Identification and sequence analysis of a gene encoding gp67, an abundant envelope glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol. 63*, 1393–1399.

Wigglesworth, V.B., **1935**. Functions of the *corpus allatum* of insects. *Nature 136*, 338.

Wigglesworth, V.B., **1936**. The function of the *corpus allatum* in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci. 79*, 91–121.

Wilson, K.R., O'Reilly, D.R., Hails, R.S., Cory, J.S., **2000**. Age-related effects of the *Autographa californica* multiple nucleopolyhedrovirus *egt* gene in the cabbage looper (*Trichoplusia ni*). *Biol. Control 19*, 57–63.

Woo, S.D., Kim, W.J., Kim, H.S., Jin, B.R., Lee, Y.H., *et al*., **1998**. The morphology of the polyhedra of a host range-expanded recombinant baculovirus and its parents. *Arch. Virol. 143*, 1209–1214.

Wood, H.A., **1996**. Genetically enhanced baculovirus insecticides. In: Gunasekaran, M., Weber, D.J. (Eds.), Molecular Biology of the Biological Control of Pests and Diseases of Plants. CRC Press, Boca Raton, pp. 91–104.

Wood, H.A., Hughes, P.R., Shelton, A., **1994**. Field studies of the coocclusion strategy with a genetically altered isolate of the *Autographa californica* nuclear polyhedrosis virus. *Environ. Entomol. 23*, 211–219.

Wormleaton, S.L., Winstanley, D., **2001**. Phylogenetic analysis of conserved genes within the ecdysteroid UDP-glucosyltransferase gene region of the slow-killing *Adoxophyes orana* granulovirus. *J. Gen. Virol. 82*, 2295–2305.

Wudayagiri, R., Inceoglu, B., Herrmann, R., Derbel, M., Choudary, P.V., *et al.*, **2001**. Isolation and characterization of a novel lepidopteran-selective toxin from the venom of South Indian red scorpion, *Mesobuthus tamulus*. *BMC Biochem. 2*, 16–23.

Xue, D., Horvitz, H.R., **1995**. Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature 377*, 248–251.

Yamamoto, T., Tanada, Y., **1978a**. Phospholipid, an enhancing component in synergistic factor of a granulosis virus of armyworm, *Pseudaletia unipuncta*. *J. Invertebr. Pathol. 31*, 48–56.

Yamamoto, T., Tanada, Y., **1978b**. Protein components of 2 strains of granulosis virus of armyworm, *Pseudaletia unipuncta* (Lepidoptera, Noctuidae). *J. Invertebr. Pathol. 32*, 158–170.

Yurchenco, P.D., O'Rear, J., **1993**. Supramolecular organization of basement membranes. In: Rohrback, D.H., Timpl, R. (Eds.), Molecular and Cellular Aspects of Basement Membranes. Academic Press, New York, pp. 19–47.

Zheng, Y., Zheng, S., Cheng, X., Ladd, T., Lingohr, E.J., *et al.*, **2002**. A molt-associated chitinase cDNA from the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol. 32*, 1813–1823.

Zlotkin, E., **1991**. Venom neurotoxins – models for selective insecticides. *Phytoparasitica 19*, 177–182.

Zlotkin, E., Fishman, Y., Elazar, M., **2000**. AaIT: from neurotoxin to insecticide. *Biochimie 82*, 869–881.

Zlotkin, E., Fraenkel, G., Miranda, F., Lissitzky, S., **1971**. The effect of scorpion venom on blow fly larvae; a new method for the evaluation of scorpion venom potency. *Toxicon 8*, 1–8.

Zlotkin, E., Gurevitz, M., Fowler, E., Adams, M.E., **1993**. Depressant insect selective neurotoxins from scorpion venom – chemistry, action, and gene cloning. *Arch. Insect Biochem. Physiol. 22*, 55–73.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M.F., *et al.*, **1985**. An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys. 240*, 877–887.

Zlotkin, E., Miranda, F., Rochat, H., **1978**. Venoms of buthinae. In: Bettini, S. (Ed.), Arthropod Venoms. Springer, New York, pp. 317–369.

Zlotkin, E., Moskowitz, H., Herrmann, R., Pelhate, M., Gordon, D., **1995**. Insect sodium channel as the target for insect-selective neurotoxins from scorpion venom. *ACS Symp. Ser. 591*, 56–85.

Zuidema, D., Schouten, A., Usmany, M., Maule, A.J., Belsham, G.J., *et al.*, **1990**. Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. *J. Gen. Virol. 71*, 2201–2210.

6.10 The Biology and Genomics of Polydnaviruses

B A Webb, University of Kentucky, Lexington, KY, USA
M R Strand, University of Georgia, Athens, GA, USA

6.10.1. Introduction to the Biological System

The Polydnaviridae are a unique group of viruses that are specifically associated with parasitoid wasps in the families Braconidae and Ichneumonidae (Webb *et al*., 2000). There are an estimated 40 000 species of parasitoids that carry polydnaviruses (PDVs) (Whitfield, 2000), and many of these species are of great economic importance as biological control agents of insect pests. The hosts of PDV-carrying parasitoids are almost exclusively larval stage Lepidoptera (moths and butterflies). Two genera of PDVs are currently recognized: the bracoviruses (BVs), which are associated with braconids, and the ichnoviruses (IVs), which are associated with ichneumonids (Webb *et al*., 2000). PDVs are the only viruses with segmented DNA genomes and all have a similar life cycle. PDVs persist as stably integrated proviruses in the genome of associated wasps and replicate in the ovaries of females. The life cycle and transmission of PDVs within the context of the parasitoid life cycle are shown schematically in Figure 1. When the female wasp lays an egg into a host, she injects a quantity of virus that infects hemocytes and other tissues. PDVs do not replicate in the wasp's host, but the virus confers protection from the host's immune system and alters other aspects of host physiology that allow the parasitoid to develop successfully. Thus, a true mutualism exists between PDVs and wasps, as viral transmission depends on parasitoid survival and parasitoid survival depends on infection of the host by the virus. The mutualistic association between PDVs and wasps has also profoundly affected the organization of PDV genomes such that they are unlike any other viruses. This chapter reviews the function of polydnaviruses in relation to our current understanding of polydnavirus genome structure and composition.

6.10.1.1. Fundamental Observations and the Discovery of Polydnaviruses

Like many other areas of biological research, the study of polydnaviruses began with a series of observations by early investigators. These observations then led to more detailed studies that continue to fascinate researchers today. To give the reader a historical perspective of the field, the discussion begins by recapitulating some of these early discoveries.

Entomologists have known for more than a century that the reproductive tracts of female wasps

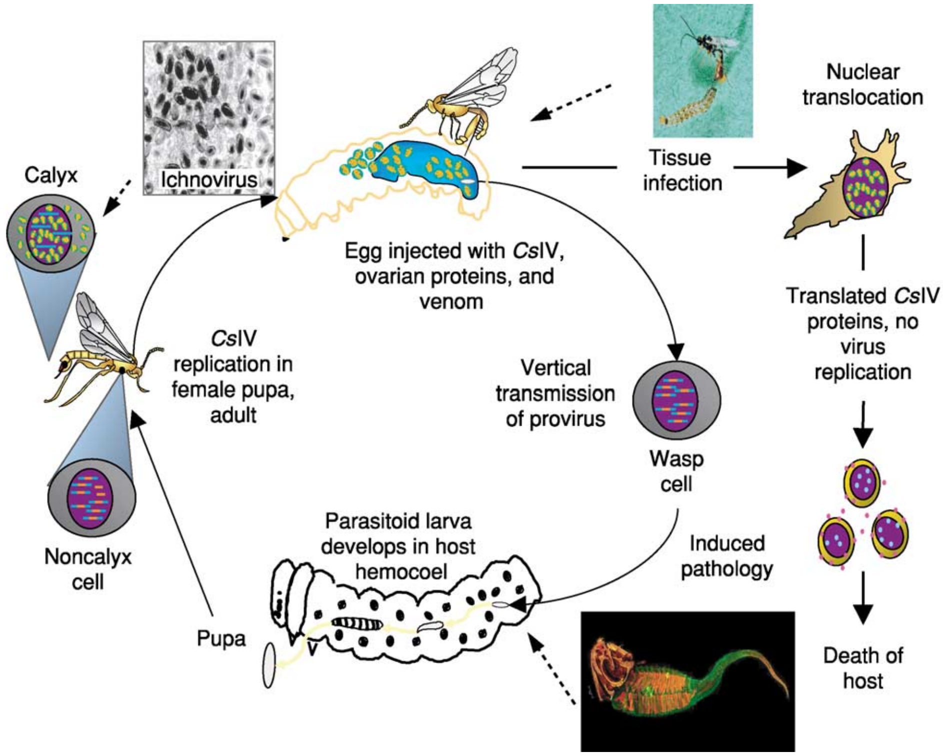


Figure 1 Parasitoid and polydnavirus life cycle. *Cs*IV, ichnovirus of *Campoletis sonorensis*.

could be easily isolated by anesthetizing the insect, grasping the ovipositor with forceps, and then gently pulling until the ovipositor separates from the wasp's body. Attached to the ovipositor are the common and lateral oviducts, ovaries, and accessory glands that together comprise the female's reproductive tract. Numerous investigators examined and described the reproductive morphology of parasitoids from many families (Clausen, 1940). In the late 1960s, however, researchers using a dissecting microscope and intense light noticed that the lateral oviducts of braconids and ichneumonids contained numerous eggs and a dense fluid with an iridescent blue color. Tearing of the oviduct resulted in extrusion of this highly viscous fluid that remained closely associated with the wasp eggs. Upon examination by electron microscopy, the fluid was found to consist of densely packed particles that structurally resembled viruses. The first species examined in this way was the campoplegine ichneumonid *Venturia canescens*. These observations led to the particles being called virus-like particles (VLPs) (Rotheram, 1967, 1973; Stoltz *et al*., 1976). Ironically, the VLPs of *V. canescens* are unusual in that they lack DNA (see Section 6.10.2). However, further characterization of VLPs from other species of ichneumonids led to the discovery that most contained double-stranded, circular DNAs (Vinson and Scott, 1975; Stoltz and Vinson, 1979a; Krell *et al*., 1982). Studies of braconids also identified DNA-containing virus like proteins (VLPs) that ultrastructurally resembled baculoviruses (Stoltz *et al*., 1976; Stoltz and Vinson, 1977). Based on the characteristics of these DNAs, morphology of the particles, and life cycle, these VLPs were recognized as a phylogenetically distinct family of viruses named the Polydnaviridae (Brown, 1986; Stoltz *et al*., 1995; Webb *et al*., 2000).

The virus-containing fluid of the oviduct was formally named "calyx fluid" upon learning that PDVs replicate specifically in the calyx cells that form the junction between the ovarioles and oviduct (Stoltz and Vinson, 1977, 1979). It was also recognized that the blue color of calyx fluid was due to diffraction of light through the densely packed viruses. To assess the functional importance of PDVs in parasitism, initial experiments focused on collecting calyx fluid, separating it from the wasp eggs, and then separately injecting wasp eggs and calyx fluid into host larvae. These studies demonstrated that wasp eggs rarely survive in the absence of calyx fluid, whereas hosts injected with calyx fluid exhibit many of the symptoms of hosts naturally parasitized by the wasp. Coinjection of calyx fluid and wasp eggs also resulted in successful development of the parasitoid that was indistinguishable from natural parasitism (Edson *et al*., 1981). This indicated that

PDVs alone are able to induce significant alterations in the physiology of the lepidopteran host. Coinjection of calyx fluid and wasp eggs resulted in successful development of the parasitoid that was indistinguishable from natural parasitism (Edson *et al*., 1981). Thus, PDVs induce changes in the physiology of the lepidopteran host that permit survival and development of wasp eggs and larvae. Viral transcripts from parasitized hosts were detected by investigators of several parasitoid–host systems (e.g., Blissard *et al*., 1987; Harwood *et al*., 1994; Hayakawa *et al*., 1994; Strand, 1994; Asgari *et al*., 1996; Galibert *et al*., 2003) but our understanding of virus function has benefited more recently from detailed analysis of PDV genomes (see Section 6.10.2). These data shed important light on the role PDVs have in the parasitized host (see Section 6.10.3), and in turn led to questions about the relationship between genome organization as related to biological activity (see Section 6.10.4). In particular, recent analyses strongly suggest that genome organization has been influenced by the restriction of viral replication to the parasitoid, which imposes important constraints on PDV function in parasitized hosts (Stoltz, 1993; Webb, 1998; Turnbull and Webb, 2002). The diversity of genes encoded by PDVs, and the physiological effects they have on parasitized hosts suggest that significant opportunities exist for using these viruses in insect control (see Section 6.10.5). Understanding the relationship between genome organization and physiological functions will also be essential to developing an understanding of the origins and evolution of PDVs (see Section 6.10.6). The genomic diversity and complexity of PDV genomes also raise important questions and opportunities regarding the direction and future of polydnavirus research (see Section 6.10.7).

In presenting this historical overview, an attempt has been made to convey the most important early observations of this field, while simultaneously outlining the chapter's organization. Research on polydnaviruses has now progressed well beyond these primary observations. But this background should be kept in mind, as we discuss PDV research in more technical detail because the significance and limitations of the work is best appreciated when related to the fundamental biology of these fascinating systems.

6.10.1.2. Parasitoid Biology and Phylogenetic Distribution of Polydnaviruses

Parasitoids are free-living insects as adults but are parasites of other arthropods during their immature stages. Hosts are usually located by the adult female, who lays her eggs in or on the host's body. The parasitoid larva then completes its development by feeding on host hemolymph and/or tissues. Parasitoids usually have limited host ranges and parasitize only a specific life stage (egg, larval, pupal, adult). Most parasitoids also complete their development by feeding on a single host, with hosts almost always being killed as a consequence of parasitization (Vinson and Iwantsch, 1980). Parasitoids in which a single offspring develops per host are referred to as solitary species, while parasitoids that produce two or more offspring per host are called gregarious. Parasitoids are also distinguished by whether larvae feed on the outside (ectoparasitoids) or inside (endoparasitoids) the hosts, and whether hosts cease to develop after parasitism (idiobionts), or remain mobile and continue to grow after parasitism (koinobionts). Idiobionts include ectoparasitoids that permanently paralyze their hosts and endoparasitoids that attack sessile host stages like eggs or pupae. Most koinobionts are endoparasitoids that parasitize insect larvae. Taxonomically, approximately 80% of all parasitoids belong to the order Hymenoptera (ants, bees, and wasps), 15% to the order Diptera (flies), and the remaining species are restricted to a few genera in other insect orders (Quicke, 1997).

The order Hymenoptera is divided into several lineages that include sawflies or woodwasps, and the monophyletic suborder Apocrita, which contains most species of parasitoids (Dowton and Austin, 1994; Whitfield, 1997). The Apocrita are usually divided into nine superfamilies. One of these superfamilies is the Ichneumonoidea, which itself is divided into the families Braconidae and Ichneumonidae (**Figure 2**). The Ichneumonoidea are all parasitoids and represents one of the largest recognized superfamilies in the animal kingdom with an estimated 100 000 species. As noted above, the family Polydnaviridae and the associated genera of bracoviruses and ichnoviruses are restricted exclusively to this superfamily. The phylogenetic relationships among braconids are sufficiently well characterized to conclude that the approximately 17 000 species of braconids that carry BVs comprise a monophyletic lineage, which has been associated with PDVs for at least 60 million years (Whitfield, 1997, 2000). This lineage consists of four subfamilies known to carry PDVs (Microgastrinae, Cardiochilinae, Miracinae, and Cheloninae) and several others that may carry PDVs but that have not been examined and confirmed to do so (Shaw and Huddleston, 1991; Dowton and Austin, 1994). The evolutionary transitions among subfamilies in the Ichneumonidae are less clear as no comprehensive phylogenetic analysis of the family has yet been published (Quicke *et al*.,

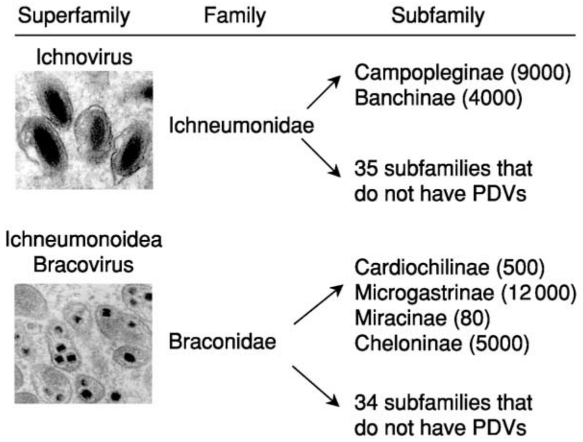


Figure 2 Phylogeny of PDV-containing parasitoids. Micrographs show ichnovirus and bracovirus virion structure (courtesy of D. Stoltz). Parenthetical numbers represent estimated number of species within each genus (estimates courtesy of M. Sharkey).

2000). However, nearly all species that carry IVs belong to the currently recognized subfamily Campopleginae, which contains approximately 9000 species. Previous studies suggest that IVs may also occur in at least some genera of Banchinae and Ctenopelmatinae (Stoltz and Whitfield, 1992). Recent analyses using both morphological and 28S rDNA data do not conclusively support the Campopleginae as monophyletic, nor do they resolve the relationship between campoplegines and banchines (Quicke *et al.*, 2000). Regardless of these uncertainties, the absence of PDVs among any of the basal extant lineages of the Ichneumonoidea strongly suggests that the association of BVs with braconids and IVs with ichneumonids arose independently. This conclusion is also corroborated by the distinct differences in morphology (see below) and nucleic acid sequences (see below) between IVs and BVs (Webb, 1998; Whitfield and Asgari, 2003).

All PDV-carrying ichneumonids are solitary endoparasitoids of lepidopteran larvae that completely consume the host and pupate within the remnant host cuticle. PDV-carrying braconids in the subfamilies Microgastrinae, Miracinae, and Cardiochilinae are either solitary or gregarious endoparasitoids. These species also parasitize larval stage Lepidoptera but unlike ichneumonids are primarily hemolymph feeders (Strand, 2000). Upon completing development, the larva chews its way out of the host to pupate. The moribund host usually retains a full complement of internal tissues but nonetheless dies shortly after wasp emergence (Beckage, 1993; Harvey and Strand, 2002). Braconids in the subfamily Cheloninae are solitary egg–larval parasitoids that oviposit into egg stage Lepidoptera, but their offspring complete development in the host's final larval stadium (Lawrence and Lanzrein, 1993).

The vast majority of PDV-carrying parasitoids have restricted host ranges, usually being able to parasitize only a few species of hosts. It is also common for the same host species to be parasitized by more than one species of PDV-carrying wasp. Hosts that are parasitized by a given wasp are referred to as being permissive, while species that do not support parasitoid development are nonpermissive or resistant. The permissive hosts for a given PDV-carrying species of parasitoid are almost always in the same family and often are in closely related genera.

Host insects respond to parasitoid attack behaviorally with defensive movements and evasive maneuvers. Even when parasitized, physiological responses to parasitization can still kill the parasitoid through immune responses, which can kill the parasitoid eggs or developing larvae (see Section 6.10.3.1.1). Indeed, whether or not the host immune system is able to recognize and kill the parasitoid often defines permissiveness. Thus, endoparasitoids must develop mechanisms to overcome host behavioral and physiological defenses to survive, while potential hosts have evolutionary needs to respond to those mechanisms. This life or death evolutionary competition occurs at every parasitization event, and therefore it is not surprising that the viruses, which mediate this process, are highly evolved and distinctive biological entities. There are also instances of parasitoid attack in which host larvae show behavioral and physiological symptoms of parasitization but the wasps fail to develop. Such host larvae are termed pseudoparasitized (Beckage and Gelman, 2004) and may represent up to 10% of the instances of "stinging" or apparent oviposition events. Similar phenotypic effects can be induced by injection of virus only, and it is likely that the natural instances of pseudoparasitism are due to oviposition of nonviable eggs or introduction of virus-containing calyx fluid into the hosts without an egg (Edson *et al.*, 1981; Cui *et al.*, 2000). It is possible that instances of pseudoparasitism described above result from successful immune responses to the parasitoid egg but failed immune responses to virus infection. In rarer instances, host larvae may show transient symptoms of parasitization such as a delays in growth but then recover to develop and pupate normally. This phenomenon is more commonly observed in semipermissive or nonpermissive hosts (Cui *et al.*, 2000). It is likely that these events represent failure of the polydnavirus to effectively suppress host immune responses, resulting in host responses that kill wasp eggs or larvae and/or suppress virus expression.

6.10.1.3. Polydnavirus Biology

Several reviews of PDV–parasitoid host associations have been written since their discovery. Some have emphasized the physiological effects of polydnaviruses on host insects (Stoltz and Vinson, 1979a; Beckage, 1993; Strand and Pech, 1995; Schmidt *et al.*, 2001). Others describe virion structure, genome organization, and transmission (Fleming and Krell, 1993; Stoltz, 1993; Webb, 1998; Turnbull and Webb, 2002; Kroemer and Webb, 2004), or provide comparative overviews of parasitoid–PDV associations (Stoltz and Vinson, 1979a; Stoltz and Whitfield, 1992). While literally tens of thousands of parasitoid species carry polydnaviruses, only a few of these species have been studied in any detail. The reader should keep this important point in mind and anticipate that new phenomena involving PDVs are likely to be discovered as additional species are examined. In this review, we consider polydnavirus systems are broadly considered, focusing both on traits that appear to be shared universally among species as well as on features that have been described in only one or two instances but that are nonetheless considered to be significant.

6.10.1.3.1. Polydnavirus life cycle PDV life cycles have been described as having "two arms" (Stoltz, 1993). Virus replication and vertical transmission occur only in the wasp, while viral genes disrupting physiology in the parasitized host function only in that "arm" of the life cycle (**Figure 1**). Both IVs and BVs replicate from proviral DNA in specialized cells of the wasp oviduct (i.e., the calyx cells). Virus replication is first detected in the late pupal stage with virus released from calyx cells by budding (in IVs) (Volkoff *et al.*, 1995) or cell lysis (in BVs) (Stoltz *et al.*, 1976) and accumulating to high concentrations in the oviduct lumen. When the wasp parasitizes its host a quantity of virus is delivered with the wasp egg. The virus enters lepidopteran cells where a host-specific subset of viral genes is expressed without detectable virus replication. Viral gene expression prevents the host immune system from killing the parasite egg thereby enabling wasp survival (Prevost *et al.*, 1990; Strand, 1994; Asgari *et al.*, 1996; Lavine and Beckage, 1996; Cui *et al.*, 2000). Virus transmission to subsequent generations is ensured by stable integration of proviral DNA in the wasp genome (Fleming and Summers, 1991; Gruber *et al.*, 1996; Savary *et al.*, 1999).

6.10.1.3.2. Polydnavirus morphology and physical characteristics Morphologically, all IVs have biconvex nucleocapsids, about 85 × 330 nm, surrounded by two unit membranes (**Figure 2**). BVs in contrast have cylindrical nucleocapsids of similar width (40 nm) but of highly variable length (25–100 nm) surrounded by a single unit membrane (Stoltz and Vinson, 1977) (**Figure 2**). Ichnovirus nucleocapsids are assembled in calyx cell nuclei and appear to acquire single envelope in the nucleus at the periphery of the virogenic stroma during virion assembly (Stoltz and Vinson, 1979a; Norton and Vinson, 1983; Yin *et al.*, 2003). IVs then appear to acquire transiently additional membranes upon exit from the nucleus and transit to the cellular membrane. However, these membranes are lost with the virion's second (external) membrane eventually acquired as viral particles bud from calyx cells. By contrast, BVs are singly enveloped and are released into the oviduct lumen by cell lysis (Stoltz *et al.*, 1976; Stoltz and Vinson, 1977; Wyler and Lanzrein, 2003). BVs may consist of a single nucleocapsid within a virion or multiple nucleocapsids may be embedded within a protein matrix in a manner similar to that of baculoviruses (Stoltz and Vinson, 1977, 1979a) (**Figure 2**). Ultrastructural studies indicated that BVs were released by cell lysis with continued production of virions requiring continued production of virus-producing cells from the calyx (Stoltz and Vinson 1979a). Although virions appeared to be released by cell lysis, analysis of calyx fluid from braconid wasps showed little evidence of cellular debris. This observation was clarified by studies of Wyler *et al.* (2003), who described a structure in the braconid calyx that serves to collect cellular debris, thereby preventing it from entering the calyx fluid, while allowing entry of virions.

Polydnavirus virions are complex with 4-25 proteins visualized by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Stoltz *et al.*, 1995; Webb *et al.*, 2000). Few of these proteins have been characterized. Genes encoding two proteins from the *Campoletis sonorensis* ichnovirus (*Cs*IV) have been cloned and analyzed. One of the two genes encodes a protein with a predicted molecular weight of 44 kDa and is not encoded within the viral genome (Deng *et al.*, 2000). The other encodes a 15 kDa protein that is virally encoded (Deng and Webb, 1999). Asgari *et al.* (2003) recently characterized two polydnavirus proteins as similar to the heat shock protein 70 and calreticulin, and hypothesized that these structural proteins may serve chaperonin-like functions. Other structural proteins have been isolated from the *V. canescens* VLPs (that lack nucleic acid) and associated with immune suppression of the host (Asgari *et al.*, 1998, 2002). One can speculate that these structural proteins on the VLPs are

related to PDVs but direct supportive evidence is lacking. Polydnavirus virions also have lipid and carbohydrate moieties but these are essentially uncharacterized (Stoltz *et al.*, 1995; Webb *et al.*, 2002).

6.10.1.3.3. General organization of polydnavirus genomes PDVs are unique among DNA viruses in having segmented double-stranded genomes (**Figure 3**). PDV segments are closed circular DNA molecules ranging in size from 2.0 to over 30 kb (Webb, 1998). BVs generally have fewer but larger segments than IVs (Kroemer and Webb, 2004). Conventionally, PDV segments are identified alphabetically from the smallest to the largest with comigrating DNA segments identified by a numerical suffix (e.g., O^1, O^2 etc.). DNA segment number is quite variable ranging from as few as 6 to over 50 (Stoltz *et al.* 1995; Tanaka *et al.*, unpublished data). Estimates of PDV genome sizes are complicated by comigrating segments and internal sequence homologies between segments (segment nesting) (**Figure 4**), but have been estimated to range from 150 to over 250 kb. A further complication in estimating genome size is that both BV and IV genomes have some segments that appear to be present in hypermolar ratios relative to other segments. The significance of this packaging strategy is considered below (see Section 6.10.4). It is also essential to emphasize that PDV genomes exist in two states: the circular episomal form packaged into

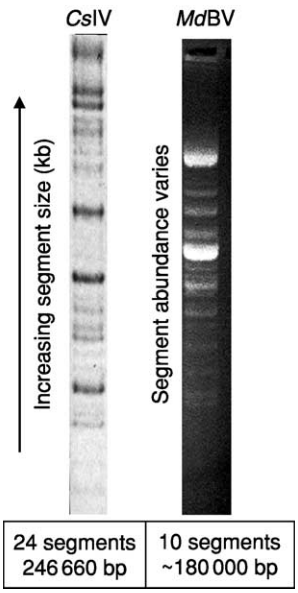


Figure 3 Polydnavirus genomes. Ichnovirus (*Cs*IV) and bracovirus (*Md*BV) genomes are shown after electrophoresis of undigested viral DNA on agarose gels and staining of DNA with ethidium bromide.

nucleocapsids during replication in calyx cells, and the linear proviral form integrated into the wasp genome that exists in all other cells of the wasp's body (Stoltz, 1993; Webb, 1998). Proviral segments seem to be dispersed within the wasp genome in IVs (Fleming and Summers, 1986) but appear to be organized in tandem array in BVs (Savary *et al.*, 1997; Pasquier-Barre *et al.*, 2002; Drezen *et al.*, 2003) (see Section 6.10.2).

Polymorphisms have been described from BV genomes and used to demonstrate Mendellian inheritance of variable segments (Stoltz *et al.*, 1986). Similar polymorphic loci were even present in individual laboratory wasps of a *Cotesia melanoscela* BV (*Cm*BV) laboratory colony having a highly similar genetic background (Stoltz and Xu, 1990). Stoltz and Xu (1990) suggested that inbreeding may lead to a greater number of polymorphisms due to continual interactions between homologous sequences, although relaxed selection pressures associated with the presence of related genes may also contribute.

Little is known about PDV genome packaging strategy. BV nucleocapsids are variable in length, and release of DNA from nucleocapsids by osmotic shock allowed release of circular DNA segments of variable length, which corresponded in size distribution to the nucleocapsid lengths (Gruber *et al.*, 1996). The interpretation of these data is that BV segments are singly encapsidated, and nucleocapsids and their lengths are determined by the size of the encapsidated segment (Gruber *et al.*, 1996; Strand *et al.*, unpublished data). There have been no experimental investigations of IV nucleocapsid packaging although the size of IV capsids is large enough to encapsidate an entire genome equivalent.

6.10.1.3.4. Timing and morphology of polydnavirus replication Replication of IVs is first detected in the late pupal stage of female wasps and continues during the adult stage. Replication is restricted to hypertrophied calyx cell nuclei and has no deleterious effect on the wasp (Stoltz and Vinson, 1979a; Norton and Vinson, 1983). Replication is closely linked with oviduct maturation and may be induced by increasing levels of the insect steroid hormone 20-hydroxyecdysone (20E) (Webb and Summers, 1992). The rate and cytology of replication differ between pupal and adult stages, with increased replication occurring following adult emergence (Volkoff *et al.*, 1995).

Replication of BV segments begins in the oviduct calyx cells during the pupal stage of female parasitoids with the amplification and excision of proviral segments (Albrecht *et al.*, 1994; Gruber *et al.*, 1996; Pasquier-Barre *et al.*, 2002). Wyler and Lanzrein

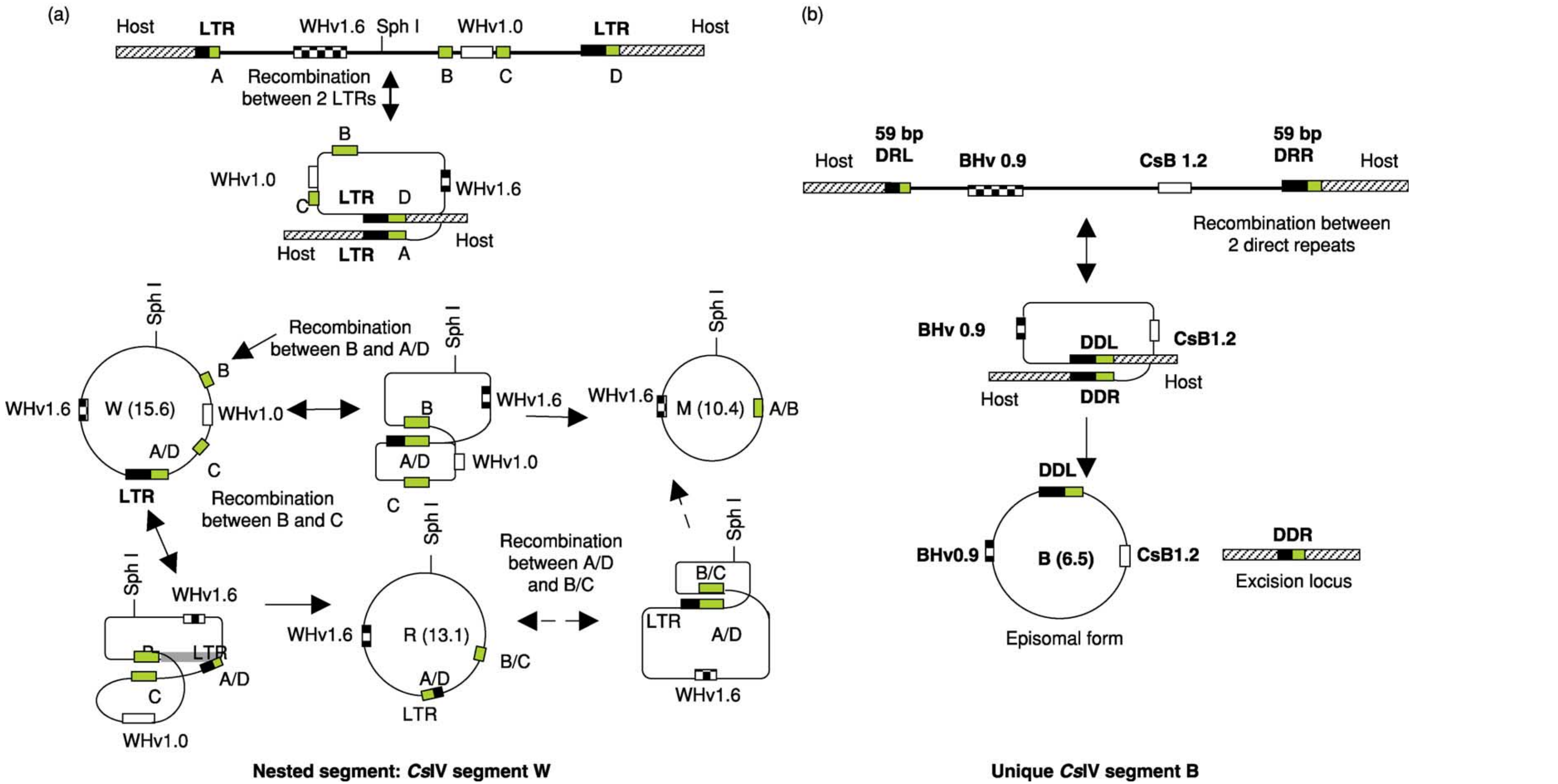


Figure 4 Segment types in ichnovirus genomes. Nested (a) and unique (b) segments are shown in their integrated state and episomal states. Linear schemata show proviral DNA while circular forms are encapsidated within virions. Junctional repeats thought to be important for excision of proviral segments and intramolecular recombination events in nested segments are indicated. DRL, direct repeat left; DRR, direct repeat right; LTR, long terminal repeat.

(2003) describe the morphogenic changes that occur in *Chelonus inanitus* as calyx cells differentiate and *Chelonus inanitus* BV (*Ci*BV) replication begins. As the calyx differentitates, elongated cells appear adjacent to the calyx region of the ovarioles. The calyx cells mature through several stages with cells lower in the calyx showing progressively larger nuclei, and increasing DNA content as measured by propidium iodide staining (Wyler and Lanzrein, 2003). Ribosomes and nuclear pores become abundant around the hypertrophied nuclei and as calyx cells differentiate, and are thought to be important for assembly and transport of mature virion proteins to the nucleus. As cells mature through intermediate stages, calyx cell nuclei become greatly enlarged and develop virogenic stroma with virion envelopes visible at the periphery of the stroma (Wyler and Lanzrein, 2003). Nuclei of mature calyx cells are filled with virions and the nuclear membrane breaks down just prior to cell lysis (Wyler and Lanzrein, 2003). An epithelial cell layer that separates the lysed calyx cells from the oviduct lumen appears to have a phagocytic role and capture debris from the lysed calyx cells. The epithelial layer also seems to be involved in transport of mature virions into the calyx fluid. Because BV calyx cells lyse, continued production of BV virions requires further differentiation of progenitor calyx cells and lysis of the cells after they mature.

6.10.1.3.5. Fate of polydnaviruses in parasitized hosts Most PDV-carrying parasitoids oviposit into the hemocoel of the host larva. Microscopic observations indicate that PDV infection of host tissues is rapid with viral nucleocapsids appearing in proximity of host cell nuclei as early as 2 h post oviposition (Stoltz and Vinson, 1979a). As IVs enter host cells the outer virion membrane appears to fuse to or dissolve the basement membrane, while the inner membrane of the virion fuses with the cellular membrane allowing the virion to enter the cell. The virion nucleocapsid then moves to the nucleus and releases viral DNA within the nucleus. BV virions appear to penetrate the basement membrane and then fuse with the cellular membrane. The BV nucleocapsid is translocated to nuclear pores, where the virion aligns with the pores and nucleocapsid contents, likely viral DNA, appear to be released into the nucleus through the nuclear pore. Although both IV and BV virions enter a variety of tissues, hemocytes are almost always the most heavily infected tissue in terms of the amount of detectable viral DNA (Stoltz and Vinson, 1979b; Strand *et al.*, 1992; Li and Webb, 1994; Strand, 1994; Yin *et al.*, 2003). Preferential infection of hemocytes could be due to the fact that wasps oviposit directly into the hemocoel, and hemocytes are the first cells virions encounter. The fact that hemocytes also lack a basement membrane may further facilitate infection. Chelonine braconids oviposit in host eggs making the process of infection of host tissues different from the scenario described above. These parasitoids often oviposit in host eggs before host tissues are formed, and even cells are often undifferentiated when virus is introduced. Wyder *et al.* (2003) showed that after oviposition *Ci*BV from *C. inanitus* initially remains close to but then enters cells of the host embryo 5–10 h post oviposition. Whether this delay in *Ci*BV infection occurs to allow differentiation of host cells is not known. However, most *Ci*BV DNA is still detected in hemocytes with lesser amounts being present in the fat body, nervous tissue, and the digestive system. The fact that *Ci*BV preferentially infects hemocytes may indicate that PDVs do have very specific tissue tropisms, suggesting that this tropism is also important for PDV pathogenesis in the chelonine wasps.

After infecting hemocytes and other host cells, BVs and IVs persist in episomal form until the host is consumed by the developing parasitoid. Viral transcripts are expressed in some host–parasite systems at near steady-state levels beginning 2–4 h after wasp oviposition and continuing until the wasp's progeny completes development (Theilmann and Summers, 1986; Strand *et al.*, 1992). In other systems, viral expression is transient (Asgari *et al.*, 1996) or in the case of chelonine braconids is delayed until late in the life cycle (Johner *et al.*, 1999) (see below). Unlike the situation in host larvae, some BV and IV DNA segments can persist for months or even years in established lepidopteran cell lines (Kim *et al.*, 1996; McKelvey *et al.*, 1996). Volkoff *et al.* (2001) detected expression of a viral gene (*K19*) over 3 years after infection of Sf9 cells by *Hd*IV from the ichneumonid *Hyposoter didymator*. More recently, Gunderson-Rindal and Lynn (2003) detected random insertion of some segments from *Glyptapanteles indiensis* BV (*Gi*BV) into chromosomal DNA of a *Lymantria dispar* cell line. Integration target sites shared a common motif (CATG), and upstream of this site were similarities to an insect retrotransposon that potentially suggest that viral DNA insertion involves a host-mediated transposition mechanism. These results also suggest the possibility that PDV genetic material could be horizontally transferred to lepidopteran hosts and exchanged between PDV genomes carried by different parasitoids that parasitize the same species of host.

6.10.2. Polydnavirus Genomics

As in other areas of biological research, genomic studies are significantly impacting our understanding of PDVs with the full implications of this work still to be realized. As a result, the polydnavirus genome website has been established and is updated periodically as new genome sequence information becomes available (http.//www.pdvdb-dev.ctegd.uga.edu). In organizing this section, first an overview of PDV genomes that applies to both IVs and BVs is provided. Then the unique organizational features of IV and BV genomes, and the major gene families they encode.

Polydnavirus genomes are unlike other viral genomes. Sequencing of encapsidated viral segments and genomes indicates that PDV genomes are largely noncoding (Cui and Webb, 1997; Wyder *et al.*, 2002). Generally, the genes encoded on these viral segments also have no homology with genes found on other viruses (Asgari *et al.*, 1997; Cui and Webb, 1997; Johner and Lanzrein, 1999; Hilgarth and Webb, 2002; Volkoff *et al.*, 2002; Falabella *et al.*, 2003) (see Section 6.10.6). Genes commonly encoded by DNA viruses such as viral DNA polymerases are absent and most of the genes that are found on PDV segments appear to be related to the pathogenic functions these viruses have in parasitized hosts. (Blissard *et al.*, 1989; Cui and Webb, 1997; Strand *et al.*, 1997) (see Section 6.10.3). Based on data generated in authors' laboratories and elsewhere, PDV genomes overall appear to have very low gene densities and many genes are spliced. PDV genomes also appear to have multiple copies of selected DNA segments and/or genes, and many genes have diverged into multiple variants that together form gene families.

6.10.2.1. Polydnavirus Genome Organization and Segment Types

As discussed above (see Section 6.10.1), PDV genomes are composed of multiple circular double-stranded DNA segments packaged in multiprotein capsids surrounded by single (BVs) or double (IVs) lipid envelopes (Webb, 1998; Turnbull and Webb, 2002). While both IV and BV genomes are composed of multiple circular dsDNAs, recent molecular analyses have discovered distinct differences in how their genomes are organized, how they replicate, and the genes they encode. Current knowledge of PDV genomics summarized below.

6.10.2.1.1. IV genomes IV genomes usually have more than 20 DNA segments ranging in size from 2 to 28 kb with genome sizes estimated to range from 75 kb to greater than 250 kb (Webb, 1998; Trudeau *et al.*, 2000). The genome of the *Cs*IV is composed of 22 nonredundant segments encoding approximately 248 kb of unique sequence (Turnbull and Webb, 2002; Lindstrom and Webb, unpublished data) (**Figure 4**). The *Campoletis chlorideae* IV genome, a closely related IV (*Cc*IV), has over 20 genomic segments and a genome estimated to be 130 kb (Yin *et al.*, 2003). Other IVs such as those associated with parasitoids of the genus *Hyposoter* are organized similarly to *Cs*IV (Stoltz *et al.*, 1981; Krell *et al.*, 1982; Volkoff *et al.*, 1995), although they tend to have smaller viral segments in greater numbers.

The sequence of the *Cs*IV genome has recently been completed. It is comprised of 22 nonredundant segments. The overall nonredundant *Cs*IV genome size (which excludes nested "daughter" segments) is 248 kb. There are at least 67 kb of partially redundant (daughter) segments making the genome size larger (~315 kb), if all the different genome segments are simply added. The *Cs*IV genome is approximately 60% A+T and greater than 60% noncoding. Portions of essentially every *Cs*IV segment have homology to other segments, primarily in coding regions. Every *Cs*IV segment encodes at least one gene, with the majority of the predicted genes transcribed in the same orientation within a segment. An exception is that the three genes on segment Q (12.1 kb) are encoded on opposite strands. Interestingly, segment Q is also the only segment known to encode members of more than one gene family (*rep* and *vinnexin*).

As discussed above (see Section 6.10.1), replication and assembly of IVs occurs only in the ovarian calyx cells of female parasitoids, and is temporally associated with changes in ecdysteroid titers that induce synthesis and melanization of the adult cuticle late in pupal development (Norton and Vinson, 1983; Whitfield, 1997). Limited data suggest that IV proviral segments are dispersed within chromosomal DNA of the parasitoid host (Fleming and Summers, 1991; Cui and Webb, 1997). DNA sequence across junction sites of integrated proviral IV segments shows that viral sequences are flanked by nucleic acid that is not encapsidated, suggesting that wasp genomic DNA separates IV segments (Fleming and Summers, 1991; Cui and Webb, 1997; Rattanadechakul and Webb, 2003). We do not currently know, however, whether or not IV segments are clustered on one or more chromosomes. The integration loci of *Cs*IV segments are surprisingly variable. For example, proviral DNA integration sites

may have short imperfect terminal repeats (e.g., 59 bp in segment B) or long perfect terminal repeats (e.g., 1186 bp in segment W) (Fleming and Summers, 1991; Cui and Webb, 1997). A single copy of the repeat is retained after recombination produces circular viral segments (Rattanadechakul and Webb, 2003).

Most IV segments excise from unique integration loci and form a stable segment. Segment B (**Figure 4**) is typical of such a "unique" segment. Segment B is 6516 bp in size and encodes two genes. *BHv*0.9 is a 900 bp mRNA expressed in parasitized insects. This gene is a member of the *Cs*IV *rep* gene family and contains a single copy of a 540 bp sequence also present on viral segments B, H, I, and O^1. The *BHv*0.9 gene does not have a signal peptide making the presumptive target of BHv0.9 the virus-infected cell. The segment B integration site is characterized by a 59 bp imperfect direct repeat with the excised segment having one copy of the repeat (Fleming, 1991; Rattanadechakul and Webb, 2003). Either the left or the right repeat can be present on an excised segment. Segments I, G^1, G2, G3, H, M^1, are also known to be orphan segments (Hilgarth and Webb, 2002). Unique segments may encode multiple genes from the same gene family with segment Z encoding nine *rep* genes, but outside of coding sequences the DNA sequence of these segments is "unique." There is no evidence of large regions of repetitive sequence within segments in the noncoding regions of these segments (Hilgarth, 2002). For example, the 6585 bp segment B has no repeats longer than 30 bp at 70% similarity. This and other repetitive sequences within the unique segments are almost exclusively found within expressed genes.

However, large repeats are obvious on many of the most abundant segments. Many of these abundant segments in IV genomes exhibit a phenomenon referred to as segment nesting. Nested segments also excise from unique integration loci but unlike unique segments, give rise to additional segments after excision via intramolecular recombination (**Figure 4**). *Campoletis sonorensis* and *H. fugitivus* IV segments W, V, and U (*Cs*IV), a U and L in *Hf* IV are known to be nested segments. Nested segments generate smaller "nested" segments from larger "parental" molecular through intramolecular recombination at intrasegmental repeats (Xu and Stoltz, 1993; Cui and Webb, 1997; Webb and Cui, 1998). Whether these repeats contribute functionally to segment nesting or whether the repeats result from the evolution of the phenomenon is not known. Segment nesting also is likely to occur in the *Hd*IV, as coding and noncoding sequences of segments C and G have been shown to cross hybridize strongly with larger genome segments (Y and Z, respectively) in *Hd* IV genomic Southern blots (Galibert *et al*., 2003). Segment nesting produces as many as five segments from a single proviral segment, with some viral genes preferentially retained on the progeny segments. Cui and Webb (1997) suggested that nesting provides a means for amplifying viral gene copy numbers. Webb and Cui (1998) manipulated virus infectious dose to show that viral gene copy number can directly affect viral protein titers in parasitized hosts. This may be particularly important for PDVs, as these viruses do not replicate after infection of lepidopteran cells (Strand *et al*., 1992) but expression of viral genes is essential for parasitoid (and virus) survival (see Section 6.10.4).

Segment nesting has been studied in the most detail in *Cs*IV segment W (15 812 bp) (Cui and Webb, 1997) (**Figure 4**). Segment W gives rise to segments R, M, and C^2 through intramolecular recombination events at a 350 bp recombination repeat, which is present in three copies on segment W. Segment W encodes four genes, of which two are expressed in parasitized insects (*WHv*1.0 and *WHv*1.6) and two are expressed in the wasp during virus replication (*WCs*1 and *WCs*2). *WHv*1.0 and *WHv*1.6 are members of the *cys-motif* gene family, while *WCs*1 and *WCs*2 genes are also related and comprise a viral gene family expressed in wasp ovarian cells during virus replication. The Segment W repeat is a 1186 bp direct repeat that is 100% identical and found at both termini of the integration locus. The excised segment contains a single copy of the recombination repeat. Segment W is itself composed of 2.5 copies of a 5 kb repeated region with significant internal sequence complexity, as indicated by an abundance of short direct and inverted repeats (Cui and Webb, 1997). Segment W appears to have evolved by sequential duplication of a 5 kb ancestral sequence that encoded a single *cys-motif* gene and a single *WCs* gene. There is also evidence that other nested segments result from repeated duplication with internal repeats, probably contributing to the phenomenon of segment nesting.

There may be a correlation between the integration repeat structure of viral segments and the efficiency with which they excise and/or replicate. Segment W has lengthy 100% identical terminal repeats and occurs as an abundant, nested segment in the *Cs*IV genome. By contrast, segment B has much shorter, imperfect terminal repeats and is a unique segment that is present in lower copy number (Webb, 1998; Rattanadechakul and Webb, 2003). Hypermolar segments are common in PDV genomes, suggesting the existence of an undescribed mechanism for controlling segment copy number (Webb, 1998; Webb and Cui, 1998; Turnbull and

Webb, 2002). Rattanadechakul and Webb (2003) suggest that gene families may be associated with particular segment types (nested versus unique; hypermolar versus nonhypermolar), and such organization may contribute to regulation of gene expression in parasitized hosts.

Savary *et al*. (1997) and Webb (1998) proposed that PDV replication may occur in a rolling-circle mechanism with excision of proviral DNA followed by amplification of circular episomes. A rolling-circle replication mechanism may be more efficient if genome segments are dispersed in the wasp genome. Data from Barre *et al*. (2002), Drezen *et al*. (2003), Marti *et al*. (2003) and Pasquier-Barre *et al*. (2002) support an alternative model for BVs. In this model, larger chromosome regions are amplified before excision of individual viral segments for encapsidation in virions. Individual viral segments do not replicate after excision from the amplified precursor (Pasquier-Barre *et al*., 2002; Drezen *et al*., 2003; Marti *et al*., 2003).

IV virions are uniform in size and shape, and have capsid dimensions that would support encapsidation of an entire genome complement (Krell *et al*., 1982). However, there are no data that directly address segment packaging in IVs. Experimentally, this is a difficult issue as virion shape is uniform and the virus does not replicate after infecting lepidopteran cells. More refined cytological and molecular studies are needed to better understand IV excision, amplification, and packaging.

6.10.2.1.2. BV genomes BV genomes are typically composed of fewer, larger segments than IV genomes, with size estimates ranging from 125–250 kb. Sequencing of the *Microplitis demolitor* BV (*Md*BV) genome was recently completed (Webb *et al*., unpublished data). *Md*BV consists of 15 segments that range in size from 4 kb (segment A) to 32 kb (segment O) (**Figure 3**). Total genome size is approximately 184 kb. Like *Cs*IV, *Md*BV segments exist in nonequimolar ratios with segment O being much more abundant than the others. The *Md*BV genome is also largely (>80%) noncoding and unlike *Cs*IV is AT-rich (65%). Some *Md*BV segments (like D–F, H, I) are unique, while others (O, M) consist of unique domains that are not shared by other segments, and repetitive domains that are shared among segments. No segment nesting *per se*, as occurs in IVs, has been found. Searches for open reading frames (ORFs) identify four gene families encoding predicted proteins of >100 amino acids on several *Md*BV segments. Some of these genes were known from prior studies while others were not. Several orphans without homology to known genes have also been identified. In addition, some segments, like D, have no ORFs for predicted proteins >15 amino acids. This suggests that these segments are either nonfunctional in parasitism or have functions other than gene expression. Sequence comparison also reveals no sequence homology between *Cs*IV and *Md*BV.

Analysis of *Glyptapanteles indiensis* BV (*Gi*BV) identifies 13 segments ranging from 11 kb to greater than 30 kb (Gundersen-Rindal and Dougherty, 2000). *Toxoneuron nigriceps* BV (*Tn*BV) (formerly *Cardichiles nigriceps*) has six major segments ranging from 6 kb to over 23 kb (Varricchio *et al*., 1999). The *Cotesia congregata* BV (*Cc*BV) has an unusually large number of segments for a BV, at least 20, which range from less than 10 kb to greater than 20 kb in size (de Buron and Beckage, 1992). Recent sequencing of the *Md*BV genome indicates that although genome segments appear larger and migrate slower than IV segments, most BV segments are similar in size to sequenced IV segments (Strand *et al*., unpublished data) (**Figure 3**). BV segments may appear relatively larger because of differences in higher-order structure as they appear to have relaxed circular topologies (Wyder *et al*., 2002), whereas IV segments are supercoiled (Krell *et al*., 1982). As relaxed circular topologies have slower mobilities on agarose gels, mobility of BV segments would be reduced relative to IV segments, and molecular weight standards thereby making BV segments appear relatively larger than IV segments.

In contrast to IV virions, BV virions vary in length with multiple nucleocapsids often observed within the singly enveloped virions (Stoltz and Vinson, 1977, 1979; Chen *et al*., 2003). There is evidence that individual capsids package single genome segments, with the length of the capsid corresponding to segment length (Albrecht *et al*., 1994). Experimental evidence also suggests that BV segments are clustered and occur in tandem arrays in the wasp chromosomal DNA (Savary *et al*., 1997; Belle *et al*., 2002; Wyder *et al*., 2002). Probes from three different viral segments (EP1, VP1, and VP2) in the *Cc*BV genome hybridized to the same location on subtelocentric chromosome 5 in adult wasp brain and gonad metaphase spreads, suggesting that *Cc*BV genome segments are clustered in a macrolocus near the telomeric end of this chromosome (Belle *et al*., 2002). Savary *et al*. (1997) showed that the proviral *Cc*BV EP1 segment is adjacent to proviral segment (A) in wasp genomic DNA providing further evidence that BV segments may be linked in tandem arrays. Wyder *et al*. (2002) have also shown that probes flanking regions of two known viral segments (*Ci*V12 and *Ci*V14) in the *C. inanitus* BV

(*Ci*BV) genome hybridize to other *Ci*BV segments in Southern blots of calyx DNA.

Like IV segments, BV segments exhibit hypermolarity (Wyder *et al*., 2002; Gunderson-Rindall and Lynn, 2003) and contain sequences that cross-hybridize to multiple segments. Probes made from three different regions of *Ci*V14 consistently hybridized to two viral genomic segments (one larger than expected) in Southern blots of *Ci*BV DNA suggesting that *Ci*V14 may be nested in a larger segment (Wyder *et al*., 2002).

As with IV segments, initiation of BV segment replication begins during the pupal stage of female parasitoids through amplification and excision of proviral segments (Albrecht *et al*., 1994; Gruber *et al*., 1996; Pasquier-Barre *et al*., 2002). Details of the morphogenic processes that occur during calyx cell differentiation and replication of *Ci*BV were discussed above (see Section 6.10.1). In terms of molecular events associated with replication, Marti *et al*. (2003) used quantitative polymerase chain reaction (PCR) to show that *C. inanitus* calyx cells become polyploid (estimated 30×) concurrent with nuclear hypertrophy and the onset of viral replication. With primers specific to one viral genome segment (*Ci*V12), the same study found that proviral sequences are selectively amplified in their integrated form following conversion of calyx cells to a polyploid state (Marti *et al*., 2003). From pupal stages 3b–6, the abundance of excised *Ci*V12 DNA is similar relative to the quantity of *Ci*V12 chromosomal excision locus, suggesting that viral DNA segments are not further amplified following conversion to individual circular episomes (Marti *et al*., 2003). A 15 bp imperfect direct repeat flanks the integrated form of *Ci*V12 and is involved in the excision of extrachromosomal segments from *C. inanitus* genomic sequences (Gruber *et al*., 1996). Extrachromosomal segments appear to form via juxtaposition and recombination of terminal repeats to generate a single copy of the repeat at the "empty" wasp genomic locus and in excised segments. Viral segments appear in females only after pupal stage 3 (Gruber *et al*., 1996), and coincident with maturation of the ovaries, oocytes, and ovarioles (Albrecht *et al*., 1994). Male wasps do not produce detectable levels of extrachromosomal *Ci*BV DNA in *C. inanitus* (Gruber *et al*., 1996).

In *C. congregata* quantitative PCR data indicate that virus replication begins by day 4 of the pupal stage. Pasquier-Barre *et al*. (2002) detected replication of the *Cc*BV EP1 segment within a large progenitor molecule containing both A and EP1 genome segments. Production of the progenitor molecule precedes recombination events that form individual A and EP1 circles, and individual circles are not amplified after formation (Pasquier-Barre *et al*., 2002).

The amplification of large progenitor molecules prior to episome formation, as proposed in Drezen *et al*. (2003), Marti *et al*. (2003), and Pasquier-Barre *et al*. (2002), may be an efficient mechanism of replication, particularly if viral genome segments are clustered in tandem arrays on a single chromosome and replicative cells are continuously produced from progenitor cells with virus released by cell lysis. For example, clustering of viral segments at one genomic locus could allow assembly of replication machinery at this locus. Replication of progenitor molecules prior to excision of individual circles allows for coamplification of segments but does raise questions as to how hypermolar segments are produced in BVs. There is evidence from the *Md*BV genome that at least some apparently hypermolar segments, as visualized on agarose gels, are actually due to multiple comigrating segments of approximately the same size (Deborde *et al*., unpublished data). Processes downstream of DNA replication could mediate control over segment hypermolarity. For example, segment copy number could depend on the efficiency with which individual episomes are excised from progenitor molecules, the efficiency with which they circularize, or the efficiency with which they are packaged into virions. Unlike circular episomes, linear progenitor molecules are not known to be encapsidated in BV virions (Pasquier-Barre *et al*., 2002).

6.10.2.2. IV Gene Families

PDV infection of host tissues coincides with severe physiological changes in the parasitized insect including alteration of host hormones, developmental arrest, and suppression of the cellular and humoral immune responses (Shelby and Webb, 1994; Balgopal *et al*., 1996; Webb, 1998; Shelby *et al*., 2000). Immunosuppression and developmental arrest require PDV gene expression (Fleming and Krell, 1993; Webb, 1998), and this viral gene expression occurs in the absence of viral DNA replication. An important characteristic of both IV and BV gene expression is that most viral genes are related to other viral genes that are expressed in the parasitized host. That is, they are members of gene families thought to be required for parasitism of the host and successful development of the parasitoid's offspring. Why PDV genes are needed in multiple copies remains to be explained.

PDV genes have been classified by their expression patterns, with class I genes expressed only in the parasitoid, class II genes expressed only in the

parasitized lepidopteran host, and class III genes expressed in both hosts (Theilmann and Summers, 1987, 1988; Stoltz, 1993). Some of class II genes encode members of gene families that alter host physiology, thereby enabling the survival of parasitoid progeny (Stoltz, 1993; Webb, 1998). Class I genes are likely involved in virus replication and packaging. Class III genes may play important roles in both the parasitoid and its lepidopteran host (Theilmann and Summers, 1987, 1988; Stoltz, 1993; Webb, 1998).

Few class I genes have been identified and characterized from IVs. Deng and Webb (1999) describe a 699 bp class I gene on *Cs*IV segment Y that encodes a 92 amino acid structural protein (p12) associated with *Cs*IV virions. Expression of mRNA from the *p12* gene coincides with the onset of viral replication in wasp ovaries, and the protein is detected only in extracts of ovarian tissues and on *Cs*IV virus particles. This pattern of *p12* expression suggests that it is a viral gene encoding a virion protein that is expressed only in the female parasitoid (Deng and Webb, 1999). By contrast, another *Cs*IV virion protein, p44, is encoded in the wasp genome but is not encapsidated (Deng and Webb, 2000). Genome sequence analyses indicate that many genes such as *p44* are involved in viral replication but are not encapsidated. By this definition they are not part of the viral genome, if one defines the viral genome on the basis of which genes are encapsidated. The only described class III genes are some members of the *rep* gene family from *Cs*IV (see Section 6.10.2.2.2). In contrast, many more class II genes from IVs have been studied. The most intensively studied IV in this regard is *Cs*IV. Below, the *Cs*IV class II genes that have been identified are first summarized. Then class II genes from other IVs are discussed.

6.10.2.2.1. *Cys-motif* genes The *cys-motif* genes are the most studied genes in the *Cs*IV genome These genes are recognized on the basis of a conserved, cysteine-rich motif. These motifs are composed of invariable cysteine residues flanked by hypervariable amino acids present in one or more copies in gene family members (Dib-Hajj *et al*., 1993; Turnbull and Webb, 2002). The *cys-motif* genes also have a highly conserved exon and intron structure, hydrophobic signal peptides, and N-terminal glycosylation sites in their predicted amino acid sequences (Blissard *et al*., 1987; Dib-Hajj *et al*., 1993; Cui and Webb, 1996). The nuclear magnetic resonance structure of the C-terminal cysteine-rich domain of one cys-motif protein (VHv1.1) indicates that the cysteine motifs of the proteins are held together by three disulfide bonds, forming a unique type of cystine knot (Einerwold *et al*., 2002). Residues lying within the cys-motif are highly variable, being less conserved than would be expected under conservative selection pressures (Dib-Hajj *et al*., 1993; Dupas *et al*., 2003).

*Cs*IV genome sequence analysis has identified ten known and putative *cys-motif* genes located on unique segments A, A′, F, and L, and on nested segments U, V, and W (Lindstrom *et al*., unpublished data). Functions and expression patterns of the V and W *cys-motif* genes (*VHv*1.1 and *VHv*1.4, and *WHv*1.0 and *WHv*1.6, respectively) have been studied in some detail (see Section 6.10.3). Blissard *et al*. (1987, 1989) suggested that the presence of multiple *cys-motif* gene variants may enable differential expression patterns and/or differential tissue targeting to provide optimal suppression of cellular immune responses during different stages of parasitoid development. Although there is little experimental data to support this possibility from study of the W and V *cys-motif* genes, investigations are under way to characterize expression patterns and functions of the newly discovered *cys-motif* genes.

6.10.2.2.2. The *rep* genes The *rep* genes were originally described based on a conserved 540 bp repeat and cross-hybridization of this repeat with several other *Cs*IV genome segments. (Theilmann and Summers, 1987, 1988; Fleming and Krell, 1993). Genome sequence analyses have shown that the *rep* gene family is the largest viral gene family identified to date, having 28 ORFs on ten unique *Cs*IV genome segments (Hilgarth and Webb, 2002; Volkoff *et al*., 2002). Although *rep* sequences are widespread within the *Cs*IV genome, most reside in predicted ORFs with *rep* related sequences detected in noncoding sequences showing evidence of rapid decay. The *rep* sequences encode predicted peptides that have an average amino acid identity of 43% (Hilgarth, 2002). The majority of the predicted *rep* genes encode a single copy of the repeat, although a number of predicted *rep* proteins possess two to five repeats arranged in tandem arrays (Theilmann and Summers, 1988; Fleming and Krell, 1993; Hilgarth and Webb, 2002).

Segments B, H, and O[1] have five *rep* genes that are expressed in parasitized *H. virescens* and/or *C. sonorensis* (Theilmann and Summers, 1987, 1988). Expression of *rep* genes can be detected in parasitized hosts at 2 h post parasitization (p.p.) Peak transcription is seen by 6 h p.p. with transcript levels essentially constant thereafter. The single segment B *rep* gene, *BHv*0.9, lacks introns and a signal peptide. It contains one 540 bp repeat element and is expressed in parasitized *H. virescens* hosts, but it is

not detected in *C. sonorensis* oviducts (Theilmann and Summers, 1988). *Rep* genes present on segments O^1 and H contain tandem arrays of *rep* sequences, and are expressed in the parasitized lepidopteran host and in female wasps.

More recently, *Cs*IV segment I (*I0.9*, *I1.1*, and *I1.2*) was sequenced and three additional members of the *rep* gene family were identified; the expression of these genes was detected in both *C. sonorensis* and parasitized *H. virescens* hosts (Hilgarth and Webb, 2002). Transcripts of *0.9 kb*, *1.1 kb*, and *1.2 kb* were detected by reverse transcriptase (RT)-PCR analyses, with expression levels of all three genes lower in *C. sonorensis* than in parasitized *H. virescens* tissues (Hilgarth and Webb, 2002). The *I0.9* and *I1.1* genes have a single copy of the 540 bp repeated sequence, whereas the *I1.2* encodes two repeats in a tandem array. Like other *rep* genes, segment I genes lack introns and signal peptide sequences. Anti-*rep* peptide antibodies detected *rep* protein products within cells although subcellular localization was not possible. Functions of the *rep*-encoded proteins remain largely uncharacterized (Hilgarth and Webb, 2002).

The *rep* gene family is also found in several other IV species. The *H. didymator* IV (*Hd*IV) has three *rep* genes expressed from segment E (*HdErep*1, *HdErep*2, and *HdErep*3), and the *Tranosema rostrale* IV (*Tr*IV) has one known and one putative *rep* gene expressed from segment F (*TrFrep*2 and *TrFrep*1) (Volkoff *et al*., 2002). Additional putative *rep* genes from *Hd*IV segment G and a polymorphic variant of *Hd*IV segment E have also been described (Volkoff *et al*., 2002; Galibert *et al*., 2003). Transcriptional analyses of the three *Hd*IV *rep* genes in lepidopteran cells shows that they encode transcripts of 790 nt, 1029 nt, and 936 nt, respectively (Volkoff *et al*., 2002). *HdErep*1 and *HdErep*2 transcripts are detected 4 h p.p./post infection (p.i.), with transcript levels maintained up to 8 days p.p. *HdErep*2 transcripts are detected at lower levels in *Hd*IV-infected cells and can be detected consistently only by RT-PCR (Volkoff *et al*., 2002). *TrFrep*1 was detected as a ~900 bp transcript showing peak expression by 1 day p.p. of sixth instar *Choristoneura fumiferana* larvae. Functions of the newly identified *Hd*IV and *Tr*IV *rep* genes also remain to be investigated (Volkoff *et al*., 2002).

6.10.2.2.3. The *vinnexin* genes The *Cs*IV viral innexin (*vinnexin*) gene family was identified by high homologies in protein sequence to invertebrate innexin gap junction proteins (Turnbull and Webb, 2002). The *Cs*IV *vinnexin* gene family has the fewest members containing only four genes (*Csvnx*-d1, *Csvnx*-q1, *Csvnx*-q2, and *Csvnx*-g1) on unique *Cs*IV segments D, G, and Q^2 (Turnbull and Webb, 2002). Three *C. sonorensis vinnexin* genes (*Csvnx*-d1, *Csvnx*-g1, and *Csvnx*-q2) are expressed in parasitized *H. virescens*, with *Csvnx*-d1 expression also detected in *C. sonorensis* tissues. Antibodies formed against a portion of the *Csvnx*-q2 protein localized to membranes of infected hemocytes (Turnbull and Webb, 2002). Innexin proteins are known to govern gap junction formation and cellular communication in invertebrate cells. Therefore, virally encoded *vinnexin* proteins may function to alter gap junction proteins in infected host cells by possibly modifying cell–cell communication during encapsulation responses in parasitized insects.

6.10.2.2.4. The *vankyrin* genes Annotation of the completed genome of *Cs*IV identified an additional novel gene family consisting of seven members on unique *Cs*IV segments P and I^2 (Kroemer and Webb, unpublished data) that contain ankyrin repeat domains. This viral ankyrin (*vankyrin*) gene family has weak but significant identities with the *Drosophila* dorsal/NF-κβ protein inhibitor (Iκβ) known as cactus. *Drosophila* cactus is a member of the Iκβ family of proteins, which are regulated inhibitors of the NF-κβ family of transcription factors (Ghosh *et al*., 1998). Gene targets of NF-κβ are highly diverse, but many are implicated in mammalian and insect immune responses, as well as in regulation of development (Engstrom *et al*., 1993; Kappler *et al*., 1993; Dushay *et al*., 1996; Ghosh *et al*., 1998; Manfruelli *et al*., 1999). Release of NF-κβ inhibition involves the direct degradation of Iκβ proteins bound to the transcription factors in the cytoplasm (Ghosh *et al*., 1998). Interestingly, the *Cs*IV-encoded *vankyrin* genes lack regulatory elements (nuclear export signals and destruction domains) associated with signal-induced and basal degradation of typical Iκβ proteins (Kroemer and Webb, unpublished data). Such a structure suggests a possible role for the *Cs*IV genes in irreversible binding to modify or inhibit NF-κβ signaling cascades within parasitized *H. virescens* hosts (see Section 6.10.3).

Recent sequence analyses of the *Hf*IV genome has also identified putative viral *ankyrin* genes. Homologs to the *Cs*IV *vankyrin* gene family are also likely to be present in the *Hd*IV genome, as probes of *Cs*IV *vankyrin* genes hybridize strongly to *Hd*IV DNA in Southern blot analyses (Volkoff *et al*., unpublished data). Conservation of this gene family suggests that it has an important function, and the putative association with ankyrin genes indicates that these genes may disrupt cellular signal transduction pathways.

6.10.2.2.5. Other IV genes The only other IV for which we have significant information on its encoded genes is *Hd*IV. In addition to having *rep*, *vinnexin*, and *vankyrin* genes, *Hd*IV also encodes a novel gene family composed of at least three genes (*M*24, *M*27, and *M*40) on genome segment M (Volkoff *et al*., 1999). *M* gene transcription can be detected from 4 h through the duration of parasitoid development. Members of the *M* gene family display similar protein structures with glycine- and proline-rich regions but are encoded by different *Hd*IV segments. Antibodies raised against M24 reacted with viral proteins and proteins in nonparasitized hosts, suggesting that some host peptides share epitopes with proteins encoded by the *M* genes. Volkoff *et al*. (1999) suggested the M proteins may exhibit host-related antigens as a mechanism for antigenic shielding of developing endoparasitoid larvae, although this hypothesis requires further experimental support.

Two other novel genes, *S6* and *P30*, were also recently described from *Hd*IV segments C and G, respectively. Both are expressed at high levels by 2 h p.i. in Sf9 cell cultures and within 1 day in parasitized *Spodoptera* larval tissues, primarily hemocytes (Galibert *et al*., 2003). The *S6* gene is predicted to encode a transmembrane protein, while the *P30* ORF encodes a predicted secreted and glycosylated protein with mucin-like motifs resembling those of the glc family in *Md*BV (see Section 6.10.2.3.1). The resemblence of the *Hd*IV P30 sequence to glc proteins of *Md*BV may further support the idea that common selection pressures may be driving convergent evolution of protein functions in the two PDV genera (Galibert *et al*., 2003). Characterization of the functions of P30 and S6 in the parasitized lepidopteran host may further support this hypothesis (Galibert *et al*., 2003) (see Section 6.10.3). Although most PDV genes are members of gene families, PDV genomes do encode some solitary genes that are essentially uncharacterized at this point. The *Cs*IV genome encodes at least seven solitary genes (not members of a gene family) that are recognized by gene finder programs. Similarly, the *Hd*IV genome encodes solitary genes including the *K19* gene from viral segment K that is persistently expressed in long-term *Hd*IV-infected cell lines (Volkoff *et al*., 2001). *K19* is a spliced gene containing two introns and encoding a cDNA with a short ORF and a long 3′ untranslated region composed of 13 imperfect sequence repeats. Functions of the *K19* gene and other solitary PDV genes remain to be investigated.

There are tens of thousands of IV- and BV-containing wasp species. Genes have been sequenced from only a few of these PDVs (~10); however, even these limited analyses demonstrate that viral genes vary significantly between species. Viral genomes may be specialized to meet requirements or constraints of specific host–parasitoid systems, and this specialization is evident in the viral genes and gene family compositions. The *T. rostrale* IV (*Tr*IV) seems well suited to test this possibility based on studies of its physiological effects that are discussed below (see Section 6.10.3). It would also be of interest to investigate the IVs from the Banchine group as phylogenetic analyses suggest independent origins for these viruses (see Section 6.10.1). One should expect to find functionally significant variation among the IVs as additional IV genomes are investigated and sequenced.

6.10.2.3. BV Genes

While BVs appear to be phylogenetically related (Whitfield, 2000; Whitfield and Asgari, 2003) (see Sections 6.10.1 and 6.10.6) there is only limited evidence of gene conservation among BVs from parasitoids in different genera characterized to date. This lack of conservation may reflect the fact that the polydnavirus systems, which have been characterized, largely reflect the three major divergent lineages of wasps within the BV containing groups. The lack of conservation also could reflect the rapidity of gene and genome evolution in polydnaviruses. Because there is relatively little evidence of gene family conservation among the BVs that have been studied to date, each species is discussed separately.

6.10.2.3.1. The *Microplitis demolitor* BV Genome analysis in combination with prior studies identified four families of class II genes. The *Md*BV *egf* gene family has three members (*egf*1.0, *egf*1.5, and *egf*0.4) encoded on viral genome segment O (Strand *et al*., 1997; Trudeau *et al*., 2000). The *egf* genes encode spliced transcripts with 5′ cysteine-rich regions that carry similarity to epidermal growth factor (EGF)-like cysteine-rich motifs (Strand *et al*., 1997; Trudeau *et al*., 2000). *Md*BV *egf* gene transcripts are detected in hemocytes of parasitized larvae, with highest levels of transcription observed between 12 h and 24 h p.p. followed by a rapid decline in expression (Strand *et al*., 1997). Peak expression of the *Md*BV *egf* genes coincides with physiological changes observed in host hemocytes; however, the exact function of the *egf* genes remains unclear (Strand *et al*., 1997). Secreted proteins containing EGF-like motifs (immunoevasive proteins (IEPs)) were described on the surfaces of eggs and PDV particles from the braconid endoparasite *Cotesia kariyai*, where they appear to promote immunoevasion (Tanaka *et al*., 2003). These data suggest that

*Md*BV *egf* genes may be derived from ancestral wasp genes having a role similar to that of the *C. kariyai* IEP proteins. The second class II gene is *glc*1.8 that was first described by Trudeau *et al.* (2000). Expression patterns of this gene are similar to that of *egf* genes in parasitized *Pseudoplusia includens*. The *glc*1.8 gene encodes a protein with hydrophobic N- and C-terminal domains flanking a heavily glycosylated core (Trudeau *et al.*, 2000). The glc1.8 protein, which localizes to the cellular surface of hemocytes, is important for disruption of host cellular immune response (Beck and Strand, 2003) (see Section 6.10.3). Annotation of the *Md*BV genome identified two additional gene families encoding predicted proteins with significant homologies to protein tyrosine phosphatases (PTPs) (see below) and Iκβ-like genes. PTPs are tyrosine dephosphorylating enzymes that occur in receptor-like and cytoplasmic forms often controlling intracellular signaling cascades (Stoker and Dutta, 1998; van Huijsduijen *et al.*, 2002). Receptor-like forms contribute to regulation of cell–cell adhesion, neuronal growth, and axon guidance, while cytoplasmic forms exhibit regulatory control over cell commitment and survival. Sequence analysis suggests all of the *Md*BV *PTP* genes are cytoplasmic. The discovery of Iκβ-like genes in the *Md*BV genome provide weak evidence that *Cs*IV and *Md*BV *vankyrin* genes are possibly derived from ancestral wasp sequences. Future experiments will likely be targeted to identify expression patterns and functions of *Md*BV-encoded *vankyrin* genes. Such studies will aid in understanding PDV evolution, and the forces driving convergent and/or divergent evolution among PDV genera.

6.10.2.3.2. *Cotesia* species BVs The *C. congregata* BV (*Cc*BV) genome encodes a family of three abundantly expressed genes: *EP*1, *EP*2, and *EP*3 (early protein 1, 2, and 3). The *EP* genes encode secreted, glycosylated proteins that are expressed within 30 min of *Manduca sexta* parasitization and accumulate to comprise over 10% of total hemolymph proteins by 24 h p.p. (Beckage *et al.*, 1987; Harwood *et al.*, 1994). The ability of EPs to be expressed in the parasitized host insect is closely correlated with the host range of the associated wasp, *C. congregata*. In permissive hosts EPs are rapidly and abundantly expressed, whereas nonpermissive hosts have greatly reduced expression of these proteins (Harwood *et al.*, 1998; Beckage and Tan, 2002). The physiological function(s) of EPs in the context of parasitized hosts is unclear.

Two genes (*Cr*V1 and *Cr*V2) from the *Cotesia rubecula* BV (*Cr*BV) are transiently expressed in parasitized hosts, *Pieris rapae* (Asgari *et al.*, 1997). *Cr*V1 (1.4 kb) and *Cr*V2 (1.1 kb) transcripts are only detected from 4–8 h p.p., with peak expression at 6 h p.p. (Asgari *et al.*, 1996). Both transcripts can be detected in fat body, with *Cr*V1 transcripts also being expressed in hemocytes of parasitized hosts. The highest levels of expression coincide with changes to hemocyte adhesive properties observed in parasitized insects (Asgari *et al.*, 1996). Analysis of the *Cr*V1 promoter with a reporter gene construct suggests that expression patterns may be mediated posttranscriptionally (Asgari and Schmidt, 2001). The CrV1 protein is glycosylated and contains a signal sequence allowing for secretion (Asgari *et al.*, 1996, 1997). CrV1 accumulates in the hemolymph, where a coiled-coil domain is important to binding and uptake of CrV1 by hemocytes (Asgari and Schmidt, 1997).

The *Cc*BV genome encodes a 1.4 kb transcript that is a homolog of *Cr*V1 (Le *et al.*, 2003). This transcript can be detected by 4 h p.p. in fat body and hemocytes and is seen until wasp emergence from the parasitized host. Interestingly, expression of the gene appears to require ovarian factors other than the virus itself as transcription of the *Cr*V1 homolog cannot be detected following injection of virus alone into *M. sexta* larvae (Le *et al.*, 2003). Expression of the *Cr*V1 homolog corresponds closely with the time of parasitoid emergence, and proteins may function in an analogous manner to CrV1 inhibiting hemocytic encapsulation in parasitized hosts (Asgari *et al.*, 1997; Le *et al.*, 2003). Using genomic sequence data, Whitfield (2002) has predicted that *Cr*V1 homologs exists in several additional *Cotesia* species.

Recently a novel lectin gene family shared by three *Cotesia* BVs has been described (Glatz *et al.*, 2003; Teramoto and Tanaka, 2003). Invertebrate lectins are often important recognition molecules in immune response, and function by binding carbohydrate moieties on the surfaces of foreign bodies (e.g., LPS in bacteria) during immune responses targeting them for hemocytic destruction (Glatz *et al.*, 2003; Teramoto and Tanaka, 2003). *Cr*BV encodes a gene (*Cr*V3) related to invertebrate C-type lectins that is expressed within 6 h p.p. in *P. rapae* larvae. CrV3 proteins are synthesized in parasitized fat body and hemocytes are heavily glycosylated, and are secreted into the hemolymph where they form oligomeric complexes (Glatz *et al.*, 2003). Two lectin genes (*Cky*811 and *Crf*111) were also recently described from the *C. kariyai* and *C. ruficrus* BV genomes (Teramoto and Tanaka, 2003). As with *Cr*V3, *Cky*811 and *Crf*111 transcripts can be detected within 6 h p.p. in hemocytes (also fat body for *Cky*811), and they encode proteins with C-type lectin motifs and signal peptides.

6.10.2.3.3. The *Chelonus inanitus* BV The BV associated with the egg–larval parasitoid *C. inanitus* (*Ci*BV) causes developmental arrest and disruption of the endocrine system in the last larval instars of parasitized *Spodoptera littoralis* hosts (Johner and Lanzrein, 2002) (see Section 6.10.3). Unlike gene expression patterns observed in larval parasitoids where viral transcripts are abundant shortly after parasitization, *Ci*BV gene expression appears to be delayed and synchronized with host larval development. *Ci*BV transcripts are detected in parasitized eggs but increase dramatically as host development proceeds. Transcripts are most abundant in the last larval instars of parasitized *S. littoralis* (Johner and Lanzrein, 2002). RT-PCR analyses of three *Ci*BV ORFs detected expression of the *Ci*BV genes in parasitized *S. littoralis* hosts and in pupae of female *C. inanitus*. One viral transcript was also expressed in the pupae of male *C. inanitus*, possibly providing evidence for transcriptional activity of proviral DNA (Johner and Lanzrein, 2002).

Recent analysis of four sequenced *Ci*BV segments (segments 12, 14, 14.5, and 16.8) did not detect ORFs related to previously described PDV genes (Wyder *et al.*, 2002). Seven predicted genes are encoded on the four sequenced *Ci*BV segments with preliminary evidence indicating that at least six of these genes are expressed. The known *Ci*BV genes contain multiple introns, with the two genes from segment 12 (*12g*1, *12g*2) having similarity to two genes of segment 14 (*14g*1, *14g*2). These data indicate that the two segments may represent an ancestrally duplicated sequence (Wyder *et al.*, 2002).

6.10.2.3.4. The *Glyptapanteles indiensis* BV As discussed above with *Md*BV, Chen *et al.* (2003b) also recently characterized a gene encoding a putative protein tyrosine phosphatase (*PTP*) gene in *Gi*BV. Using quantitative PCR, peak *Gi*BV *PTP* gene expression was detected in all tissues of parasitized *L. dispar* hosts within 2 h of parasitization followed by a decline apparent through 8 days. Since several *PTP* genes were also recently identified in the *Md*BV genome (see Section 6.10.2.3.1), this finding suggests that *PTP* genes may be conserved among BV genera (Chen *et al.*, 2003a).

Two additional novel *Gi*BV genes were recently recovered from a cDNA library of 24 h p.p. *L. dispar* hosts (Chen *et al.*, 2003b). These novel genes encode cDNA sequences and ORFs that hybridized to multiple genome segments suggesting they are members of gene families. Further investigations are required to better understand these potentially novel gene families in *Gi*BV.

6.10.2.3.5. The *Toxoneuron (Cardiochiles) nigriceps* BV *Toxoneuron nigriceps* BV (*Tn*BV) specific transcripts ranging in size from 0.3 to 3 kb were detected between 12 h and 48 h p.p. by Northern blot analysis of total RNA and polyadenylated RNA from parasitized *H. virescens* larvae (Varricchio *et al.*, 1999). Although functions of the viral transcripts are uncharacterized, the nucleotide sequence of one *Tn*BV gene (*Tn*BV1) sheds some light on its potential protein structure and cellular localization. *Tn*BV1 encodes a 467 nt spliced mRNA transcript expressed in the prothoracic glands of parasitized *H. virescens* larvae (Varricchio *et al.*, 1999). The deduced protein sequence lacks a signal peptide for secretion but includes 5′ and 3′ untranslated regions, a potential *N*-glycosylation site, and four potential phosphorylation sites. Recently, Falabella *et al.* (2003) characterized a second *Tn*BV gene (*Tn*BV2) transcribed in prothoracic glands and hemocytes of *H. virescens* larvae beginning by 6 h and peaking between 24 and 48 h p.p. *Tn*BV2 encodes a spliced gene with a signal peptide, several *N*-glycosylation and protein kinase C phosphorylation sites, and a conserved aspartyl-protease domain of a retroviral type.

6.10.3. Polydnavirus Function in Parasitized Hosts

As previously discussed, BVs and IVs accumulate to high densities in the calyces of female wasps and are injected into the parasitoid's lepidopteran host along with eggs, ovarian proteins (Ops), and venom during oviposition (Stoltz, 1993; Webb, 1998; Turnbull and Webb, 2002). Following infection of various tissues, PDV gene expression induces physiological alterations in the parasitized host that facilitate survival of the parasitoid's progeny. These include suppression of the host's immune system, changes in host growth and development, and alterations in metabolism (Strand and Pech, 1995; Schmidt *et al.*, 2001; Turnbull and Webb, 2002; Beckage and Gelman, 2003). Below are considered three aspects of polydnavirus function in hosts: the pathological effects PDVs have on host immunity and development, the function of isolated genes and their encoded proteins, and the potential role polydnaviruses play in successful parasitism.

6.10.3.1. Polydnavirus–Host Immune Interactions

The primary function of both BVs and IVs appears to be in protection of the developing parasitoid from the host's immune system (Schmidt *et al.*, 2001). Comparison of data across species indicate that

in some cases PDV-mediated immunosuppression requires direct infection and expression of viral genes in hemocytes, while in others immunosuppression involves both direct infection of hemocytes and secreted viral gene products (Li and Webb, 1994; Luckhart and Webb, 1996; Shelby and Webb, 1997). In some instances, PDVs also appear to have immune-related activities that do not involve gene expression. Insight into the molecular mechanisms underlying these effects derived from both genomic and functional data suggest that: (1) BVs and IVs immunosuppress hosts through the activity of different virulence genes; and (2) the shared organizational features of PDV genomes are likely important for function.

6.10.3.1.1. The host immune response toward the parasitoid Before considering the immunosuppressive functions of PDVs, it is important to briefly summarize how the host immune system responds to parasitoids in the absence of viral infection. Insects lack an acquired immune system but have a well-developed innate response that is subdivided into humoral and cellular defenses (Lavine and Strand, 2002). Humoral defenses include the production of antimicrobial peptides (Meister *et al.*, 2000; Lowenberger, 2001), reactive intermediates of oxygen or nitrogen (Bogdan *et al.*, 2000; Vass and Nappi, 2001), and proteolytic pathways like the phenoloxidase (PO) cascade that regulates melanization (Muta and Iwanaga, 1996; Gillespie *et al.*, 1997). Cellular defense refers to hemocyte-mediated immune responses (Lavine and Strand, 2002). The primary immune response toward parasitoids is encapsulation, which involves attachment of multiple layers of hemocytes to the surface of the parasitoid egg or larva.

As previously noted, PDV-carrying wasps are parasitoids of Lepidoptera whose hemocytes are divided into five classes: granular cells, plasmatocytes, spherule cells, oenocytoids, and prohemocytes (Gardiner and Strand, 1999; Nardi *et al.*, 2003). Granular cells are the main phagocytic cells in Lepidoptera and, along with humoral opsonins, are also essential for recognition of many encapsulation targets (Pech and Strand, 1996; Lavine and Strand, 2001; Lavine and Strand, 2002). Several humoral and cellular molecules have been identified from insects as pattern recognition receptors of pathogenic prokaryotes, but no humoral or cellular receptors have been identified and conclusively shown to recognize parasitoids (Lavine and Strand, 2002). Regardless, upon binding to a parasitoid, granular cells release cytokines that induce plasmatocytes to bind to the target and form a capsule (Pech and Strand, 1996; but see Willott *et al.* (1994) for an exception). The most potent known activator of lepidopteran plasmatocytes is the cytokine plasmatocyte spreading peptide (Clark *et al.*, 1997). Several other candidate molecules including eicosanoids have also been implicated in hemocyte activation events (Baines and Downer, 1992; Miller *et al.*, 1994; Diehl-Jones *et al.*, 1996). In turn, adhesion of plasmatocytes to the parasite and one another is mediated by integrins and potentially other classes of adhesion receptors like cadherins, Ig superfamily members, complement-like proteins, and selectins (Gillespie *et al.*, 1997; Levashana *et al.*, 2001). Once encapsulated, parasites are potentially killed by anoxia, reactive intermediates of oxygen (ROI) or nitrogen (RNI), toxic quinones associated with the melanization pathway, or cytotoxic molecules (Luckhart *et al.*, 1998; Nappi *et al.*, 2000).

6.10.3.1.2. Ichnovirus-immune interactions Edson *et al.* (1981) were the first to describe a role for IVs in protecting parasitoids from the host's immune system (see Section 6.10.1). In their study, *H. virescens* readily encapsulated *C. sonorensis* eggs in the absence of *Cs*IV but did not encapsulate the parasitoid when *Cs*IV was present. Furthermore, this effect required expression of one or more viral genes, as inactivated virus could not confer protection. Subsequent studies indicated that *Cs*IV and other IVs induce several alterations in hemocytes including cytoskeletal rearrangements, a decrease in the ability of granular cells and plasmatocytes to spread on foreign surfaces, and changes in the number of hemocytes in circulation (Stoltz and Guzo, 1986; Davies *et al.*, 1987; Guzo and Stoltz, 1987; Doucet and Cusson, 1996; Luckhart and Webb, 1996; Yin *et al.*, 2003). In the *Cs*IV system, hemocytes from noninfected hosts exhibit a decreased ability to attach to foreign surfaces when cultured in plasma from virus-infected hosts, which suggested that secreted proteins of host or viral origin could be involved in disruption of capsule formation (Davies *et al.*, 1987). Hemocytes from *Cs*IV-infected hosts are also unable to encapsulate eggs or other foreign targets, like glass, indicating that the host encapsulation response is broadly suppressed (Davies *et al.*, 1987). In contrast, *T. rostrale* IV (*Tr*IV) infection blocked encapsulation of *T. rostrale* eggs but had no effect on encapsulation of other targets suggesting suppression of the host encapsulation response is restricted to the parasitoid (Doucet and Cusson, 1996) (summarized by Lavine and Strand, 2002).

Ichnovirus infection also affects several elements of the humoral immune response. For example, *Cs*IV-infected hosts exhibit reduced levels of

lysozyme expression and several IVs are reported to reduce PO activity (Stoltz and Cook, 1983; Doucet and Cusson, 1996; Shelby *et al.*, 1998, 2000). Two major biochemical pathways exist, both involving the conversion of the inactive zymogen prophenoloxidase to the active PO, following recognition of foreign objects through activation of a serine protease cascade (Sugumaran, 1996). Recently, serine protease inhibitor activity was isolated from the calyx fluid of *V. canescens* (VcVLPs) (Beck *et al.*, 2000), though direct action on the serine proteases of the PO cascade was not shown. It is possible the *V. canescens* serine protease inhibitors block conversion of prophenolxidase to its active form as a mechanism for inhibiting defensive melanization. The second activation cascade involves synthesis of melanin by dopachrome isomerase activity on l-DOPA (Li *et al.*, 1994; Sugumaran, 1996). *Cs*IV infection reduces melanization by inhibiting synthesis of enzymes in the melanization pathway at the posttranscriptional level. The resultant changes in enzyme levels alter substrate availability and composition such that melanization is blocked (Hilgarth, 1995; Shelby *et al.*, 2000). In nonpermissive hosts of *C. sonorensis* melanization reactions are only temporarily depressed (Cui *et al.*, 2000) with recovery of melanization occurring as hemolymph, ovarial, and/or *Cs*IV protein titers decline.

While viral expression appears important in the immunosuppressive activities of IVs, a few studies indicate that some IVs passively protect parasitoids from encapsulation by coating the surface of the parasitoid egg. Surface features of the virions either prevent the parasitoid from being recognized as foreign by the host's immune system or in some way inhibit host hemocytes from being able to strongly adhere. This activity also does not require expression of any viral genes. For example, Cusson *et al.* (1998) found that *Tr*IV virions are physically associated with the chorion of *T. rostrale* eggs in a manner reminiscent of the VLPs of *V. canescens* that also associate themselves with the surface of the parasitoid egg. *Venturia canescens* VLPs lack nucleic acid but have capsids that are morphologically similar to other IVs (Rotheram, 1967). Hosts parasitized by *V. canescens* remain capable of mounting an encapsulation response against numerous foreign targets but are unable to encapsulate *V. canescens* because of VLPs and a hemomucin that coat the surface of their eggs (Rotheram, 1967; Feddersen *et al.*, 1986; Theopold *et al.*, 1996). The observations of Cusson *et al.* (1998) with *Tr*IV suggest a similar function.

Studies with *Cs*IV and selected other IVs indicate that virions infect several host tissues, and that hemocytes and fat body are principal sites of transcription (Fleming and Summers, 1986; Beliveau *et al.*, 2000; Galibert *et al.*, 2003; Yin *et al.*, 2003). However, few IV genes have been experimentally implicated in mediating any of the immune alterations described above. The *Cs*IV cys-motif proteins are secreted and bind to both granular cells and plasmatocytes (VHv1.1) (Li and Webb, 1994) or granular cells alone (VHv1.4) (Cui *et al.*, 1997). VHv1.1 and VHv1.4 are expressed in all infected tissues but the proteins are secreted and accumulate in the hemolymph where they bind to hemocyte surface proteins (Cui *et al.*, 1997). Experimental studies also indicate that VHv1.1 protein reduces but does not entirely block encapsulation of *C. sonorensis* eggs in hosts infected with a VHv1.1-expressing recombinant baculovirus (Li and Webb, 1994). Other IV genes are indirectly implicated in immunosuppression because of homology to genes with known function in immunity or because they are highly expressed in hemocytes. As discussed above (see Section 6.10.2), the *vankyrin* genes encoded by *Cs*IV and *Hf*IV (see above) share homology with insect Iκβ proteins. Work carried out in the past several years with *Drosophila* identifies NF-κβ transcription factors like Dif and Dorsal as important elements that activate transcription of antimicrobial peptide genes and stimulate other immune functions such as hematopoiesis (Qui *et al.*, 1999; Meister *et al.*, 2000). In most cells, NF-κβ factors are present in an inactive form in the cytoplasm of cells, retained due to complex formation with Iκβ proteins, like Cactus, that mask NF-κβ nuclear localization signals. Activation of transcription occurs when a signal is received and causes release of NF-κβ from its inhibitory Iκβ, followed by translocation of the NF-κβ transcription factor to the nucleus to activate gene expression (Qui *et al.*, 1999; Meister *et al.*, 2000). Thus, the presence of Iκβ-like genes in the genome of *Cs*IV suggest the virus may suppress the host immune response by binding host NF-κβs. The *P*30 ORF from *Hd*IV is likewise predicted to encode a mucin-like protein similar to the *glc* gene family of *Md*BV which has been shown to block adhesion of immune cells (Trudeau *et al.*, 2000; Beck and Strand, 2003; Galibert *et al.*, 2003) (see below). Products of the *vinnexin* genes from *Cs*IV share high homology to innexin gap junction proteins, and they are also expressed at high levels on *Cs*IV-infected host hemocytes (Turnbull and Webb, unpublished data). Gap junctions have been observed in hemocytic capsules between hemocytes but their functional significance was unknown. Identification of the *vinnexin* genes in PDV genomes suggests that disruption of normal

gap junction function may be a mechanism through which IVs prevent cellular encapsulation.

6.10.3.1.3. BV–host immune interactions BV-mediated immune suppression also usually involves infection of host tissues by viable, transcriptionally active virus (Guzo and Stoltz, 1987; Strand and Noda, 1991; Lavine and Beckage, 1995; Asgari *et al.*, 1996). The most important immunosuppressive activity is disruption of the host's encapsulation response. Whether disruption of capsule formation involves direct infection of hemocytes by virus, secretion of viral gene products into hemolymph which then interact with hemocytes, or both appears to vary among species. In the *M. demolitor* system, *Md*BV infection of *Pseudoplusia includens* irreversibly suppresses both encapsulation and phagocytosis (Strand and Noda, 1991; Strand and Pech, 1995; Lavine and Strand, unpublished data). Granular cells and plasmatocytes lose the capacity to bind to any foreign surface within 4 h of infection, and granular cells shortly thereafter undergo a high level of apoptosis. These alterations only occur if transcriptionally active virus directly infects hemocytes. Alterations in the adhesive properties of host hemocytes have also been described for BVs from other *Microplitis* species (Tanaka, 1982; Kadash *et al.*, 2003).

The effects of BVs from parasitoids in the genus *Cotesia* in contrast are more variable. Infection of *P. rapae* by *Cr*BV from *Cotesia rubecula* results in cytoskeletal alterations in hemocytes (Asgari *et al.*, 1996, 1997). These changes also correlate with reduced binding of *Helix pomatia* lectin which potentially binds a mucin on granular cells. Since transport of some surface mucins is mediated by actin filaments (Oliver and Specian, 1990), these results suggest that CrBV may interfere with transport of mucins and other factors to the cell surface that are required for adhesion. Severe and permanent inhibition of hemocyte binding to foreign surfaces is also reported for *Cm*BV from *C. melanoscela* and *Ck*BV from *C. kariyai* (Guzo and Stoltz, 1987; Tanaka, 1987).

In contrast, inhibition of encapsulation is accompanied by formation of hemocyte aggregations in *M. sexta* infected by *Cc*BV from *C. congregata* (Lavine and Beckage, 1996). Subsequent clearance of these aggregations is also accompanied by recovery of immune responses (Lovallo and Cox-Foster, 1999). FAD-glucose dehydrogenase (GLD) is activated only in immune activated plasmatocytes, making it a useful immune response marker (Cox-Foster and Stehr, 1994). Lovallo and Cox-Foster (1999) report that GLD activity increases after injection of ultraviolet-inactivated *Cc*BV, indicating that viral transcription is not responsible for this event. Uninfected hemocytes overlaid with purified *Cc*BV were still able to adhere, but preferentially reacted with other hemocytes rather than an introduced egg. Also, there was a reduction in overall adherence to glass (i.e., a foreign object) and formation of clumps of cells adhering to each other. Therefore, the virions were suggested to function as decoys that induced immune reactions to virus or virus-infected cells at a higher frequency than to the wasp egg. Cells participating in such aggregations would be subject to GLD-induced effects which include induction of apoptosis and cytolysis. Thus, GLD induction may contribute to changes in hemocyte populations and abrogation of an appropriately targeted encapsulation response to wasp eggs. The virus not destroyed during this "autoimmune response" is transcribed, presumably leading to sustained inhibition of immune responses via other mechanisms. In *Cc*BV, these symptoms occur in the majority of hemocytes by 24 h p.p. but are no longer evident by 8 days p.p. (Lavine and Beckage, 1996). Unlike other BV-carrying braconids, wasps in the genus *Chelonus* are all egg–larval parasitoids. Little is known about the role of chelonine BVs in immune suppression, but studies with *Ci*BV indicate that viral infection of the host protects *Chelonus* larvae from encapsulation but does not suppress capsule formation of latex beads. The mechanism underlying this parasitoid-specific effect is unknown as *Ci*BV is not known to affect the function of host hemocytes or humoral defense responses (Stettler *et al.*, 1998).

In terms of humoral immune responses, infection by several BVs reduces PO activity in the hemolymph of infected hosts (Stoltz and Cook, 1983; Beckage *et al.*, 1990; Strand and Noda, 1991). Upon bacterial immune challenge, hemocytes and fat body from *P. includens* and *Trichoplusia ni* upregulate expression of several antimicrobial peptides including cecropin, gloverin, lebosin, attacin, and lysozyme (Kang *et al.*, 1996a, 1996b; Liu *et al.*, 2000; Lundstrum *et al.*, 2002; Lavine and Strand, unpublished data). Interestingly, *Md*BV infection blocks inducible expression of these factors in fat body but has no effect on their expression in hemocytes (Lavine and Strand, unpublished data). This suggests that while cellular defense responses are compromised by *Md*BV infection, humoral responses that protect the host against prokaryotic pathogens remain at least partially functional.

As previously discussed for IVs, surface features of braconid eggs and larvae are sometimes as important in defense against encapsulation as BV infection of the host. Eggs of *C. kariyai* are coated by *Ck*BV virions, as well as other proteins secreted from ovarial cells (Hayakawa and Yazaki, 1997). Recent

studies identified two genes from *C. kariyai* that encode immunoevasive ovarial proteins (IEP1 and IEP2) (Tanaka *et al*., 2002). These genes are not encoded within the *Ck*BV genome but IEP proteins are on the surface of both *C. kariyai* eggs and *Ck*BV virions. IEP proteins also confer protection to eggs from encapsulation and elimination of *Ck*BV by hemocytes (Hayakawa and Yazaki, 1997; Tanaka *et al*., 2002). Virions do not coat the eggs of *T.* (*Cardiochiles*) *nigriceps* or *C. rubecula*, but the eggs of both species are coated with ovarial proteins that also inhibit encapsulation (Davies and Vinson, 1986; Asgari *et al*., 1998). In the case of *C. rubecula*, antisera raised against *Cr*BV cross-reacts with one of these proteins, Crp32, and when this protein is removed eggs are encapsulated by both nonparasitized and *Cr*BV infected hosts (Asgari and Schmidt, 1994). However, application of recombinant Crp32 to eggs restores protection (Asgari *et al*., 1998). Antibodies to Crp32 cross-react with two native proteins in host hemolymph. This indicates that Crp32 shares antigenic features with at least some host proteins and may protect eggs by conferring surface properties that resemble "self" to the host (Asgari *et al*., 1998). At the opposite extreme, eggs from *C. congregata* are fully susceptible to encapsulation and dependent upon *Cc*BV infection for protection, yet larvae have certain surface features that fully protect them from encapsulation even if transplanted into a nonparasitized host (Lavine and Beckage, 1996).

As with IVs, relatively few experimental studies clearly implicate specific BV genes in immunosuppression of hosts. Trudeau *et al.* (2000) described an *Md*BV gene, *glc* 1.8, that encodes a heavily glycosylated mucin-like protein, which is expressed by infected hemocytes and localizes to the cell surface. Beck and Strand (2003) recently used RNA interference (RNAi) to silence *glc*1.8 expression in a hemocyte-like cell line. Like hemocytes themselves, RNAi silencing of *glc*1.8 effectively restored normal adhesion activity to *Md*BV-infected cells suggesting that this gene plays a key role in disrupting encapsulation. Asgari and coworkers (Asgari *et al*., 1996, 1997; Asgari and Schmidt, 2002) reported that *Cr*BV transiently expressed two viral genes (*Cr*V1 and *Cr*V2) in the host fat body and hemocytes. Recombinant CrV1 protein, which binds to hemocytes via a putative leucine zipper domain, is endocytosed, and after endocytosis causes alterations in F-actin distribution and *H. pomatia* lectin binding, similar to what occurs in parasitized hosts. These results indicate that CrV1 is secreted and that altered lectin binding by hemocytes occurs after uptake of this viral protein. However, it is unclear from these studies whether CrV1 actually blocks *P. rapae* hemocytes from binding to or encapsulating any foreign target.

Other BV genes are indirectly implicated in immunosuppression because they share homology with known genes involved in regulation of immune responses in insects or other organisms. These include the Iκβ-like genes identified from *Md*BV that are structurally similar to the *Cs*IV *vankyrin* genes described previously (Webb and Strand unpublished data). The *PTP* genes identified from *Md*BV and *Gi*BV (Chen *et al*., 2003b) are also of interest as candidate suppressors of host cellular immune responses. *PTP*s have diverse functions but are particularly interesting from the perspective of immunity, because bacterial pathogens like *Yersinia pestis* express PTPs that block phagocytosis by dephosphorylating essential proteins in macrophages (Cornelis, 2002). Interestingly, the PTP from *Y. pestis* (YopH) also induces apoptosis in macrophages, either directly or indirectly by blocking NF-κB-dependent synthesis of antiapoptotic factors (Black *et al*., 2000; Cornelis, 2002). This suggests a role for either the *PTP* or *vankyrin* genes in the high level of apoptosis in granular cells infected by *Md*BV and potentially other BVs (Lavine and Beckage, 1995). Recent studies also describe a third gene family from three *Cotesia* BVs that encodes C-type lectins (Glatz *et al*., 2003; Teramato and Tanaka, 2003). As previously discussed, C-type lectins have been previously been implicated in insects as humoral pattern recognition receptors of prokaryotes (Jomori and Natori, 1991; Koizumi *et al*., 1999). What function these BV-encoded lectins might play in immunosuppression is unclear.

6.10.3.1.4. The potential effects of PDVs in disruption of insect antiviral defenses Little is known about antiviral defenses in insects. However, a few recent studies circumstantially suggest IVs and BVs reduce the efficacy of lepidopteran antiviral immunity. For example, coinfection with *Cs*IV allows the baculovirus *Autographa californica* M nucleopolyhedrovirus (*Ac*MNPV) in the normally resistant host, *Helicoverpa zea* (Washburn *et al*., 1995). *Manduca sexta* is a semipermissive host for the *Ac*MNPV but is more permissive when coinfected with *Cc*BV (Washburn *et al*., 2000). In both instances, increased permissiveness to *Ac*MNPV appears to be due to alterations in hemocyte function caused by the PDV. *Helicoverpa zea* hemocytes normally aggregate around foci of *Ac*MNPV infection and induce melanization (Trudeau *et al*., 2001). However, as previously discussed, IV and BV infection disrupt hemocyte adhesion and abundance, both of which likely

depress antiviral responsiveness. Other ovarial proteins like the IEPs from *C. kariyai* have also been implicated in helping BVs evade hemocyte antiviral responses (Tanaka *et al.*, 2003). Suppression of antiviral responses is also suggested by studies demonstrating that viral latency is sometimes broken by the immunosuppressive activity of PDVs (Stoltz and Makkay, 2003).

6.10.3.2. Polydnavirus–Host Developmental Interactions

The second major function of PDVs in parasitism is in host development. Lawrence (1986) divided parasitoids into "conformers" and "regulators" on the basis of their developmental interactions with the host. Conformers often coordinate their own development with that of the host but do not alter host development itself. Regulators in contrast disrupt host endocrine or other developmental pathways in a manner that facilitates successful development of the parasitoid. PDV-carrying parasitoids are almost always regulators that have a diversity of effects on host endocrine and nutritional physiology.

6.10.3.2.1. IV–host developmental interactions All known IV-carrying parasitoids are solitary, which means that only a single parasitoid offspring develops per host (Strand, 2000). Almost without exception, hosts parasitized by IV-carrying wasps exhibit reductions in weight gain, delays in molting, and are unable to undergo metamorphosis. The "juvenilization" of the host and disruption of metamorphosis are associated with specific alterations in endocrine physiology. Juvenile hormone (JH) is the key endocrine factor involved in maintenance of the larval stage, while juvenile hormone esterase (JHE) is the primary enzyme responsible for JH breakdown. *Cs*IV infection significantly reduces JHE activity in the host *H. virescens*, and it also suppresses 20-hydroxyecdysone (20E) titers by inducing the degeneration of the host's prothoracic glands (Dover *et al.*, 1988a and b; Shelby and Webb, 1997). Prothoracic gland degeneration, however, only occurs in the host's final instar indicating that *Cs*IV mediated inhibition of molting specifically blocks metamorphosis. Injection of *T. rostrale* calyx fluid (*Tr*IV) into penultimate instar *C. fumiferana* reduces JHE activity but it does not elevate JH titers (Cusson *et al.*, 2000). *Tr*IV also does not cause these glands to degenerate but it nonetheless suppresses 20E titers (Cusson *et al.*, 2000).

Ichnovirus infected hosts also exhibit alterations in metabolism. These include changes in the abundance of hemolymph proteins, free amino acids, and carbohydrate levels (Thompson, 1986; Vinson, 1990). Alterations in protein abundance may result from inhibition of protein synthesis at a posttranscriptional step (Shelby and Webb, 1994). This effect also appears to be selective. For example, *Cs*IV infection greatly reduces the abundance of arylphorin, a major hemolymph storage protein, but does not affect other major hemolymph proteins like Lipophorin and Transferrin (Shelby and Webb, 1994, 1997). Triglycerides and glycogen deposits in fat body are also greatly reduced in hosts parasitized by *C. sonorensis* (Hilgarth, 1995), but trehalose titers in hemolymph actually increase (Vinson, 1990). Overall, these data suggest IV infection affects nutrient stores of the host and may selectively alter specific factors in a manner that favors the developing parasite larvae. Thus far, no IV genes have been experimentally implicated in any of the endocrine or metabolic alterations that occur in infected hosts.

6.10.3.2.2. BV–host developmental interactions The developmental interactions between BV-carrying braconids and their hosts are more variable than those of IV-carrying ichneumonids. Microgastrine braconids like *Cotesia*, *Microplitis, Glyptapanteles* spp., and cardiochilines like *T. nigriceps* are all larval endoparasitoids that are either solitary (one offspring per host) or gregarious (multiple offspring per host). Most are able to oviposit in multiple instars of the host but their offspring complete development either obligately in the host's final instar or earlier depending on the species (Strand, 2000; Harvey and Strand, 2002). Viral expression in hosts parasitized by microgastrine and cardiochiline braconids usually begins within a few hours of parasitism (Turnbull and Webb, 2002). Hosts parasitized by solitary species thereafter exhibit severe reductions in weight gain, delays in molting, and inhibition of metamorphosis similar to those described for IV-carrying ichneumonids. Hosts parasitized by gregarious species are also inhibited from pupating but usually exhibit less severe alterations in weight gain. In contrast, chelonine braconids (*Chelonus, Ascogaster*) are all solitary, egg–larval parasitoids that induce precocious metamorphosis in their hosts such that initiation of pupation begins an instar earlier than normal (Lawrence and Lanzrein, 1993; Lanzrein *et al.*, 2001). The parasitoid then completes its larval development and emerges to pupate from the prepupal stage of the host. Viral expression in hosts parasitized by chelonines remains very low for many days after parasitism, but then expression of certain viral genes increases sharply immediately prior to the initiation of precocious metamorphosis (Johner *et al.*, 1999; Johner and Lanzrein, 2002).

BV infection contributes to many of the developmental alterations described in hosts although other wasp-derived factors, like teratocytes, venom, and the parasitoid larva itself (see above), also have important roles (Beckage and Gelman, 2003). For example, injection of *Md*BV from *M. demolitor* causes hosts to develop into larval–pupal intermediates, but injection of *Md*BV plus teratocytes results in complete inhibition of metamorphosis (Strand and Wong, 1991; Dover *et al.*, 1995). This occurs in part because *Md*BV or *Md*BV plus teratocytes elevate host JH titers (Balgopal *et al.*, 1996). These alterations appear to be due to both suppression of JHE activity and increased synthesis of JH by the host's corpora allata. They also implicate both *Md*BV and teratocytes in causing these changes. Studies with several other microgastrine and cardiochiline braconids also report an interactive role between BVs and teratocytes in developmental arrest and juvenilization of hosts (Pennacchio *et al.*, 1994; Dong *et al.*, 1996). Teratocytes secrete several proteins (Dahlman and Vinson, 1993), including TSP14 from *Microplitis croceipes* that shares some similarities with the *cys-motif* gene family of *Cs*IV (Dahlman *et al.*, 2003). Bioassays indicate that TSP14 inhibits protein synthesis and thus may contribute to developmental arrest by interfering with translation of certain host proteins (Dahlman *et al.*, 2003) (see Section 6.10.5).

Cotesia congregata also inhibits host metamorphosis by elevating hemolymph JH titers and interfering with processes that prevent prothoracic gland production and release of ecdysteroids (Beckage and Riddiford, 1982; Gelman *et al.*, 1998). These effects appear to involve *Cc*BV-mediated reductions in host JHE activity as well as secretion of JH by *C. congregata* larvae (Cole *et al.*, 2002). Inhibition of host metamorphosis by *C. kariyai* involves blockage of prothoracicotropic hormone release, while *T. nigriceps* disrupts ecdysteroid synthesis by prothoracic glands (Pennacchio *et al.*, 1994, 1997). In the case of *T. nigriceps*, experiments indicate that *Tn*BV infection of these glands reduces phosphorylation of proteins of the prothoracicotropic hormone-cAMP-prothoracic gland (Pennacchio *et al.*, 1998a, 1998b). Another factor implicated in causing the developmental delays that occur in hosts parasitized by *Cotesia* sp. is the discovery of bioactive peptides like growth blocking peptide and plasmatocyte spreading peptide (Hayakawa, 1990; Clark *et al.*, 1997; Strand *et al.*, 1999). In addition to functioning as an important cytokine in plasmatocyte activation, elevated titers of these factors also cause delays in molting and the onset of metamorphosis (Strand *et al.*, 1999). Studies with *C. kariyai* indicate that *Ck*BV infection induces a significant increase in growth blocking peptide titer in *Pseudaletia separata* (Hayakawa, 1991; Ohnishi *et al.*, 1995). Lastly, studies with *C. congregata* and *C. kariyai* describe alterations in levels and storage of neurotransmitters in the host nervous system that potentially impact both development and behavior (Noguchi *et al.*, 1995; Zitnan *et al.*, 1995).

Fewer studies have been conducted with BV-carrying egg–larval parasitoids but experiments with *C. inanitus* clearly indicate that calyx fluid (*Ci*BV), synergized by venom, developmentally arrest *S. littoralis* larvae in the prepupal stage by suppressing ecdysteroid synthesis and/or release (Grossniklaus-Burgen *et al.*, 1998; Steiner *et al.*, 1999; Lanzrein *et al.*, 2001). Reed and Brown (1998) also report that *Chelonus curvimaculatus* and *Ascogaster quadridentata* secrete ecdysteroids that may be involved in synchronizing the timing of parasitoid emergence from the host.

A diversity of nutritional alterations in hosts occur after BV infection that include dramatic changes in protein, lipid, and carbohydrate metabolism (summarized by Thompson, 1993; Thompson and Dahlman, 1998). These effects may be directly caused by expression of viral gene products and other factors like teratocytes, or indirectly arise as a consequence of the changes that occur in host endocrine physiology. Regardless, metabolic redirection is hypothesized to serve the parasitoid's nutritional needs at the expense of host tissues. Nakamatsu *et al.* (2001), for example, conducted experiments showing that *Ck*BV and venom from *C. kariyai* alter growth and metabolic efficiency of *P. separata*. Several hemolymph proteins are also down regulated by *Cc*BV from *C. congregata* and *Ck*BV from *C. kariyai*, including the hemolymph storage protein Arylphorin (Beckage and Kanost, 1993). Associated with these metabolic changes are reductions in the size of the host fat body and gonads (Yagi and Tanaka, 1992; Reed and Brown, 1998), suggesting that metabolic alterations in the host enhance nutrient availability to the parasitoid larva at the expense of host tissues.

As with IVs, almost no BV genes have been clearly linked to causing any of these endocrine, metabolic, or developmental alterations in hosts. In the *T. nigriceps–H. virescens* system, *Tn*BV potentially encodes several genes with protein kinase inhibitor (PKI) activity (Varricchio *et al.*, 1999). At least one *Tn*BV transcript, *Tn*PDV1, localizes to prothoracic gland cells with irregular morphology (Varricchio *et al.*, 1999). The transcript has a single putative N-linked glycosylation site, four potential phosphorylation sites, and lacks a signal peptide. Although *Tn*PDV1

does not exhibit any similarities to PKI genes in the National Center for Biotechnology Information nr-database, indicating that it may not be the source of the PKI activity, it may, however, be the source of observed cytological abnormalities. Similarly, the numerous *PTP* genes described previously could also play a role in disrupting protein kinase associated signaling pathways. Unlike gene expression patterns observed in larval parasitoids where viral transcripts are abundant shortly after parasitization, *Ci*BV gene expression patterns in hosts parasitized by *C. inanitus* are delayed and synchronized with the onset of precocious metamorphosis (Johner *et al*., 1999). Recent analysis of four sequenced *Ci*BV segments (12, 14, 14.5, 16.8) identifies several genes expressed in parasitized hosts including *14g*1 and *14g*2, which are upregulated fivefold and 1000-fold, respectively, in *S. littoralis* at the onset of precocious metamorphosis (Johner and Lanzrein, 2002). These genes share no significant homology with known genes, but the timing of their activity suggests they play an important role in regulating host development.

6.10.4. Polydnavirus Genome Structure–Function Relationships

The essential function of both IVs and BVs in the context of their symbiotic associations with parasitic wasps is inhibition of host cellular immune responses that otherwise kill endoparasitic wasp eggs and larvae. Cursory comparison of their genomes (**Figure 3**) suggests that they may use similar approaches in that PDVs are the only highly segmented DNA viruses. However, sequence analyses of representative IV and BV genomes reveals no sequence similarity but common structural features, suggesting that these viruses may have related strategies for acquiring and delivering pathogenic genes to host insects. Among the similar features are: (1) the presence of gene families distributed between abundant and relatively rare segments; (2) the existence of large amounts of noncoding sequence and spliced genes; and (3) the near complete absence of viral genes that can be associated with virus replication.

We consider some features of PDV gene families important for effective targeting of viral genes to disrupt host immunological responses. These include structural features that facilitate regulating gene dosage (hypermolarity, gene families, the presence of introns). Other features appear more relevant to maintenance of an effective symbiosis between the wasp and the virus. In the absence of replication, segment nesting may provide a mechanism for increasing gene copy number and elevating levels of specific gene products. Similarly, the presence of introns in some of the most highly expressed PDV genes suggests that this may enhance gene expression or mRNA stability as it is known to do in other eukaryotic systems (Le *et al*., 2003). Evolution of multiple variants in turn may provide the means to invade multiple hosts or interact with related signaling pathways in different cells or tissues.

The PDV literature emphasizes the obligate mutualisms between PDVs and their wasp hosts as contributing to the unusual features of PDV genome organization, replication, and function (Stoltz, 1993; Webb, 1998; Turnbull and Webb, 2002). Polydnaviruses are the biological mediators of parasitoid survival in the parasitized host, and they must evolve within the constraints imposed by these two very different types of insect associations. PDVs must be reliably transmitted, a function defined by the genetic assocation with the wasp genome. PDVs must also effectively disrupt parasitized host physiological systems, a function defined by the virus interaction with the genome of the lepidopteran host (Webb, 1998; Webb and Cui, 1998). In regard to PDV interactions with the wasp and lepidopteran genomes several characteristics of polydnavirus genomes are notable.

Polydnavirus genomes are integrated in wasp genomes. PDVs do not encode genes for structural proteins, viral polymerases, or transcription factors common in other DNA viruses. To support virus replication these genes must reside in the wasp genome and such viral genes have been transferred to the nuclear genome in both BVs and IVs during PDV evolution suggests that there is some benefit to having these genes encoded by the nuclear genome. One rather obvious benefit would be to allow more virulence genes to be packed into virions for delivery to the host during parasitization. Of potentially more significance (evolutionarily) is that translocating a gene essential for virus replication from a viral to a nuclear genome makes the virus dependent on the nuclear genome for its replication. In an evolutionary context such an event would fix the genetic association between (polydna)virus and host by making the association obligatory for virus replication. Such a genetically dependent association exposes the viral genome to evolutionary processes that lead to reduction and specialization of dependent microbial genomes (Andersson and Kurland, 1998). This evolutionary process has been termed “reductive evolution” and results in loss of genes that are nonessential to the mutualism to the viral genome, as well as amplification and/or elaboration of those microbial genes that are essential to the mutualism (Andersson

and Kurland, 1998). It is notable that PDVs genes are not truly "lost" but transferred to the nuclear genome in much the same way that many genes that were once in mitochondria are now expressed from nuclear genomes. Although reductive evolution is widespread in dependent obligate mutualisms, PDVs have two "hosts" with essential function in both. The evolutionary impact of these biological symbioses is thus more complex.

6.10.4.1. Gene Acquisition

Polydnavirus mutualisms have clearly been considered in light of the symbiotic theory for the origin of eukaryotic organelles (symbiogenesis) (Federici and Bigot, 2003), and the concept of reductive evolution (Webb, 1998; Shelby and Webb, 1999; Turnbull and Webb, 2002). However, the way in which PDVs acquire virulence genes has received relatively less consideration. PDVs do encode identifiable genes, and without exception these genes appear to be of eukaryotic rather than viral or microbial origin. Some PDV genes are quite closely related to cellular genes and have similar functional activities (e.g., PTPs, innexins). Presumably such genes were acquired from the wasp hosts and are now deployed against the parasitized host by the PDV. Moreover, many genes of cellular origin are members of viral gene families, suggesting a recurring organizational theme in which viral genes are acquired from the host, and their functions elaborated and specialized within the viral gene families. Some of these families have identifiable homologies to insect genes involved in signal transduction systems (e.g., *PTP*, *egf*, and *vankyrin*) or surface-mediated recognition (e.g., *glc* and *vinnexin*). It is also noteworthy that even though there is a consistent organizational theme in which PDV genomes encode relatively few PDV gene families (about three to seven), the complement of gene families differs markedly among viral species both within and between the PDV genera. For example, the BV gene families differ considerably among the systems studied to date, but studies of BV phylogeny strongly support the conclusion that all BV-containing wasps share a common ancestor (Whitfield, 1997). Thus, PDV genome organization is retained but the identity of the genes expressed shows considerable variation.

Proviral integration is stable through many wasp generations (Fleming and Summers, 1991), though low levels of excision occur in all tissues (Stoltz *et al.*, 1986) and some viral segments are able to integrate into cellular DNA after infection (Kim *et al.*, 1996; McKelvey *et al.*, 1996; Volkoff *et al.*, 2001).

6.10.4.2. Gene Amplification

Polydnavirus genome segments vary in number and molarity (Stoltz *et al.*, 1981), which results in unequal viral gene copy number and protein titers within the parasitized host (Webb and Cui, 1998). Segments contain large amounts of noncoding sequence, which may be comprised of highly repetitive regions (Webb *et al.*, unpublished data). The variability of PDV gene families suggests that gene duplication and divergence are common in the evolution of these viruses (Webb, 1998). Fleming and Krell (1993) suggest that PDV gene families provide mechanisms for tissue-specific and temporal regulation of gene expression, and there is some experimental support for this hypothesis (Asgari and Schmidt, 2001).

However, site and temporal regulation does little to explain the pronounced differences in segment molarity in PDVs. Gene duplication, segment hypermolarity, and segment nesting likely have important roles in regulation of gene dosage by providing a means to control mRNA levels of important genes. This is particularly important in PDV systems where viral functions are essential but occur in the absence of virus replication in parasitized hosts (Fleming and Krell, 1993; Webb, 1998). Webb and Cui (1998) documented a direct correlation between viral gene copy number and viral protein titers in parasitized hosts, and Cui *et al.* (2000) showed a link between gene expression patterns, gene function, and host range of *C. sonorensis* in relation to the VHv1.4 cys-motif protein.

The amplification of large progenitor molecules prior to episome formation may be an efficient mechanism of replication, particularly if viral genome segments are clustered in tandem arrays on a single chromosome and replicative cells are continuously produced from progenitor cells with virions released by cell lysis. For example, clustering of viral segments at one genomic locus could allow assembly of replication machinery at this locus. Replication of progenitor molecules prior to excision of individual circles allows for coamplification of segments, but does raise questions as to how hypermolar segments are produced in BVs. There is evidence from the *Md*BV genome that at least some apparently hypermolar segments, as visualized on agarose gels, are actually due to multiple segments of approximately the same size comigrating on these gels (Deborde *et al.*, unpublished data). Processes downstream of DNA replication could mediate control over segment hypermolarity. For example, segment copy number could depend on the efficiency with which individual episomes are excised from

progenitor molecules, the efficiency with which they circularize, or the efficiency with which they are packaged into virions.

6.10.5. Polydnavirus Research and Insect Control

This review began with a description of the parasitoid biology that led to discovery of basic biological phenomena as related to polydnavirus and wasp mutualisms. The primary role of PDVs in their mutualism is to inhibit insect immune responses and thereby permit successful parasitization. Secondarily some PDVs impact other aspects of host physiology thereby causing a rapid and pronounced inhibition of host growth and development. Conceptually, the isolation of viral genes that render lepidopteran insects more susceptible to disease and inhibit growth of host larvae should be useful for development of novel control strategies. One can easily envision the direct use of viral genes expressed in transgenic plants or recombinant baculoviruses to disrupt the physiology of pest insects and, thereby, inhibit insect growth or render the pest more susceptible to disease. Alternatively, PDVs genes and their encoded proteins may be used to study the affected lepidopteran physiological systems. In such an approach one would seek to identify novel insect targets that would enable targeted development of new classes of insecticidal compounds that inhibit insect growth and/or immunological systems. To this point, no PDV genes have been used directly for insect control, but there are clear indications that researchers are interested in and exploring such PDV genes and their potential utility.

Given the significance of the effects that PDVs have on insect physiology one might question the lack of movement towards developing PDV-based insect control strategies. However, PDVs are nonreplicative and therefore not amenable to mutational analyses. It is only in recent years that genomic approaches have elucidated the full complement of viral genes, and this work is only now entering the literature. As a result, analyses of PDV gene function is still at an early stage and, for its stage in development, shows reasonable promise.

There are reasons to suspect that PDV genes and proteins may have limitations that could limit there use in insect control strategies. In particular, the reliance of PDVs on gene families and mechanisms for controlling gene dosage suggest that it may be necessary to understand the roles of gene families and gene dosage in PDV function, before rational approaches can be developed to exploit PDV pathogenicity. For example, it may be necessary to express multiple viral genes from a single gene family to reproduce the biological effects caused by polydnavirus infection. Also there is good reason to question whether or not PDV genes that are normally expressed after infecting host cells would function when expressed from plant systems and subsequently ingested.

There are approaches that can be envisioned to overcome some of the potential limitations to utilizing PDV genes. Researchers are already investigating the potential for PDV genes to enhance other insect viruses, notably baculoviruses (Soldevila *et al.*, 1997; Washburn *et al.*, 2001). Such strategies could clearly overcome limitations associated with delivering PDV proteins to target insects via ingestion. Despite these cautionary considerations experimental evidence is developing that suggests that genes derived from PDVs have considerable promise for insect control.

6.10.5.1. Genes Inhibiting Insect Growth

One of the most dramatic effects of parasitization or PDV infection can be the reduction in insect growth. It is not uncommon for virus-infected insects to exhibit weight reductions of 50–75% relative to control insects over a 2–3 day period. However, isolation of genes inhibiting insect growth is problematic experimentally, in that it requires development of *in vivo* assays capable of direct measurement of "growth" or weight gain. Shelby and Webb (1994, 1996) approached this problem by showing that synthesis of lepidopteran storage proteins, which are abundantly synthesized in rapidly growing larvae, are dramatically inhibited by PDV infection. Subsequently, this effect was used to study the biochemical activity and ultimately associate inhibition of growth with a *cys-motif* gene from teratocytes (Dahlman *et al.*, 2003; Maiti *et al.*, 2003). This investigation documented that a *cys-motif* gene isolated from the teratocytes of a braconid wasp, *M. croceipes*, inhibited insect growth when injected (Dahlman *et al.*, 2003), or when ingested after expression of the recombinant protein in transgenic plants (Maiti *et al.*, 2003). This teratocyte gene, *TSP14*, is structurally related to the *Cs*IV *cys-motif* genes, suggesting that these viral genes may have direct effects on insect growth. Current work in this area is directed to expression and analysis of PDV function in similar assays.

6.10.5.2. Genes Disrupting Insect Immunity

Although inhibition of host immune responses is likely the most potent physiological effect of PDVs, this effect may not seem promising for utility in insect control. However, the aforementioned

expression of PDV genes in baculoviruses (Washburn *et al.*, 2001) had the objective of enhancing baculovirus pathogenicity and/or host range by using PDV genes to suppress the host immune response to baculovirus infection. So the immunosuppressive activity of PDVs is also being investigated. It is at least conceptually possible that a PDV immunosuppressive activity could be used to persistently inhibit pest immune systems, so that a high enough percentage of insects succumb to disease to suppress insect populations below economic injury levels. A potential advantage of such a strategy would be that insect resistance may be slow to develop, because the proximate cause of insect mortality would vary depending upon the pathogen infection that actually caused death of the pest insect.

Campoletis sonorensis parasitism disrupts humoral immunity and increases susceptibility of *H. virescens* larvae to opportunistic bacterial pathogens (Shelby *et al.*, 1998, 2000; Shelby and Webb, 1999). By their identity, the IV and BV *ankyrin* genes and the BV *PTP* genes suggest involvement of PDVs in direct regulation of signal transduction cascades in parasitized hosts. Signal transduction cascades could be a means through which PDVs regulate either host immunity or development. PTPs are involved in control of intracellular signaling cascades (Stoker and Dutta, 1998), and the presence of PTP-related genes in *Md*BV and *Gi*BV (Chen *et al.*, 2003b) implies virus-mediated control of PTP-regulated signal transduction in parasitized insects. Similarly, the presence Iκβ-related ORFs in IVs and BVs may be significant as insect immunological and developmental pathways are under NF-κβ mediated regulation and, therefore, potentially affected by these inhibitors of NF-κβ signaling cascades (Dushay *et al.*, 1996). The Iκβ-related ankyrin genes in PDVs may function to suppress NF-κβ signaling cascades as a means to alter development and humoral immunity in parasitized insects. At a minumum, further investigation of the emerging *PTP* and *ankyrin* gene families seems likely to expand our understanding of how PDV gene expression mediates lepidopteran host compatibility at the molecular level.

6.10.5.3. Host Range

The PDV-containing parasitoids are among the most successful groups both in terms of species diversity and sheer abundance of animals. One factor that may contribute to the success of these animals is the presence of PDVs which may provide a mutable set of genes that function as host range determinants for the parasitoid. This possibility has not been investigated in depth.

Kadash *et al.* (2003) recently examined the relatedness between three different bracovirus genomes in the genus *Microplitis* (*M. demolitor*, *M. mediator*, and *M. croceipes*), and assessed the ability of each bracovirus to protect the developing egg of an alternate *Microplitis* parasitoid. Cross-hybridization studies showed that the three BVs shared a high degree of homology in DNA sequences, but the three bracoviruses varied in their abilities to protect a parasitoid–egg of a different species in given host environments (*H. virescens* and *P. includens*) (Kadash *et al.*, 2003). Results of this study indicated that different evolutionary pressures acting on closely related parasitoids may be involved in the rapid divergence of BVs in braconid genera to allow wasps to rapidly adapt to different hosts environments. Webb and Cui (2000) documented a direct correlation between viral gene copy number and viral protein titers in parasitized hosts, and Cui *et al.* (2000) showed a link between gene expression patterns, gene function, and host range of *C. sonorensis* in relation to the VHv1.4 cys-motif protein. Such studies suggest that PDV genes do serve as host range determinants and are, therefore, potentially useful for insect control strategies designed to modify host range.

6.10.6. Polydnavirus Origins and Evolution

A long-standing question in the study of polydnaviruses is their evolutionary origin (Stoltz, 1993). One school of thought is that PDVs arose from a viral ancestor(s) that was associated with ichneumonoids, their hosts, or both. Evidence cited in favor of this scenario includes the observation that PDVs morphologically resemble other recognized families of viruses that infect insects. For example, IVs morphologically resemble ascoviruses that are also known to be horizontally transmitted to lepidopteran hosts by parasitoids (Bigot *et al.*, 1997; Whitfield and Asgari, 2003). Weak homology between ascovirus and some IV capsid proteins is also reported (Federici and Bigot, 2003). BV virions resemble some baculoviruses and nudiviruses (Stoltz and Vinson, 1979a; Federici and Bigot, 2003). Other similarities between PDVs and known DNA viruses include: (1) virions contain nucleic acid; (2) virions are infectious; and (3) replication and packaging of PDVs in calyx cells produce what appears to be virogenic stroma. On the other hand, the encapsidated genome of PDVs lacks several genes required for replication in the lepidopteran host. As discussed above (see Section 6.10.2), PDV genomes also have features more commonly associated with eukaryotes than viruses, such as large amounts of noncoding sequence, introns, and

multigene families. This has led some investigators to suggest that PDVs are no longer viruses at all but wasp secretions or novel organelles for delivering wasp genes to hosts (Federici and Bigot, 2003).

We would suggest the differences between these alternatives are largely semantic and that the ancient association between PDVs and parasitoids may well prevent the origin of these viruses from ever being determined with certainty. To put into context the difficulty of ferreting the origin of PDVs, it is instructive to consider the evolution of viruses more generally. The origin of viruses as biological entities can never be known with certainty, but all virus families that exist today likely arose from elements derived from some combination of both free-living organisms ("virus" progenitor) and host cells (Holland and Domingo, 1998). It is also clear that viruses can emerge and evolve by a variety of mechanisms including mutation, recombination, and reassortment. Recombination can create new viruses by capturing genes or sets of genes from either host cells or other viruses. Mutations, insertions, deletions, and rearrangement of genomes have also been well-described for many viruses in diverse families.

Genome sequence comparisons within virus families have yielded supportable phylogenetic relationships but also some important notes of caution. For example, the Baculoviridae is probably the best studied family of insect viruses, yet phylogenies for this group based on individual gene sequences have consistently produced conflicting results (Herniou *et al.*, 2003). This inconsistency could reflect unequal rates of gene evolution or real differences in the phylogeny of individual genes due to recombination events, duplications, and gene losses (Herniou *et al.*, 2001). The importance of the latter becomes clear when one considers that among 13 fully sequenced baculoviruses, 282 genes were specific to one virus, 243 genes had at least one homolog, and only 30 genes were shared by all genomes (Herniou *et al.*, 2003). Analysis of these shared genes in combination with gene order produced a well-supported phylogenetic tree for the Baculoviridae that now appears to consist of four major groups (Herniou *et al.*, 2003). Nonetheless, the pitfalls of anything short of such a broad analysis are clear.

No family level phylogeny exists for viruses as it is clear that there is no single origin for viruses (Domingo *et al.*, 1999; van Regenmortel *et al.*, 2000). For instance, there is no obvious way one can relate viruses of the size and complexity of Poxviridae (linear dsDNA, 130–375 kb, 150–300 genes) which infect insects and many other animals with Geminiviridae (circular ssDNA, 2.7–5.4 kb, 3–7 genes) that also infect insects as well as plants. Even comparisons among, for example, dsDNA virus families have yielded vague phylogenetic results. The main message to come from attempts at such broader phylogenetic studies is that virus evolution appears to be modular with recombinational arrangement of interchangeable genetic components occurring in different virus groups (Holland and Domingo, 1998). Numerous examples exist for a given family of viruses sharing a core component with another family while having nothing else in common. Thus, certain animal viruses, like picornaviruses and alphaviruses, encode genes or modules related to plant viruses with which they do not share the same morphology, number of genome components, or even genome organization. Comparison of different "supergroups" of viruses also almost always produces different phylogenies depending on which gene or module one compares. In summary, discerning ancestry beyond close relatives (like baculoviruses) has thus far resulted in severe blurring of perceived relationships among viruses.

The implications for understanding the origins of PDVs are obvious given recent estimates indicating that PDV–ichneumonoid associations arose at least 74 million years ago (Whitfield, 2000). Any similarities between individual genes shared between PDVs and another virus family must be viewed cautiously. Furthermore, a lack of significant similarity between PDV genomes and known virus families does not mean that PDVs themselves are not derived from viruses or properly classified as viruses themselves. Genomic analysis of PDVs is still at an early stage and arguably comparative data remain too weak to make phylogenetically meaningful comparisons either within PDVs or with viruses from other families. Even so, our limited knowledge of PDV genomes already suggests strikingly poor conservation in the class II genes that are expressed in parasitized hosts among PDVs outside the same genus (BVs) or subfamily (IVs). More important, potentially, for phylogenetic comparisons will be the structural genes that encode proteins for PDV nucleocapsids, as one may assume that the conservation of virion structure may reflect conservation among genes encoding virion proteins. However, very few of these genes have been identified as many appear to be encoded by the genome of the wasp rather than the encapsidated virus. Thus it is concluded that sequence information is still too weak to make firm comparisons between PDVs and viruses in other families. Even with additional data such comparisons may still yield ambiguous results given experiences with several other viral taxa.

It is also worth noting that many viruses have features strikingly similar to plasmids, episomes, and other mobile DNA or RNA elements like transposons or retrotransposons. The only clear distinction between many such mobile elements and viruses is the maturation of the latter within capsids or envelopes that affect efficient transmission and target cell receptor specificity. This is well illustrated by viruses like bacteriophage Mu (which is both a virus and transposon) and retroviruses (which are retrotransposons containing a functional envelope gene). While some authors have suggested that true viruses should show evidence of an evolutionary history independent of that of its host (Strauss *et al*., 1996), unambiguously discerning such independence is becoming increasingly more difficult as our comparative knowledge of animal and plant genomes rises. An extreme example is provided by maize in which numerous retroelement families account for 50% or more of the genome, such that large regions of host and selfish parasitic DNA can no longer be distinguished at all (Voytas, 1996). Again, the message from these broader comparisons is that most viruses share genetic features with their hosts and other mobile elements. In our view the balance of traits PDVs possess, particularly the encapsidation of nucleic acid for infection of other cells, favors viewing these entities as viruses rather than a type of organelle. If we reject the importance of envelopment of nucleic acid for infection of other cells, one has to wonder what is left to distinguish viruses from other biological entities.

We are more optimistic about the use of comparative genomic data for understanding the evolution of IVs and BVs to one another. Comparative studies with other viruses suggest that virus lineages that remain closely associated with a given host coevolve (Doolittle *et al*., 1989; Domingo *et al*., 1999). For example, papillomavirus types rarely if ever crosses host species barriers which is reflected in their phylogeny, since papillomavirus isolates from primates, ungulates, rabbits, and birds are separated by large distances (Ong *et al*., 1993). Similarly, the intimate association between individual PDVs and wasp species suggest that virus phylogeny is likely to mirror wasp phylogeny. Limited comparisons testing cophylogeny between BVs and wasps in the genus *Cotesia* have found essentially perfect correspondence (Whitfield and Asgari, 2003). The caveat for such studies is that the high divergence in class II genes already found among, for example, BVs suggests these factors may be poor choices for constructing supportable phylogenies for braconid PDVs as a whole. Provided whole genomes are ultimately available, employing groups of shared genes and genome organization, as has been done with baculoviruses, is more likely to yield clearer results than trees constructed using single genes. In conclusion, discerning the evolutionary relationships within the BVs and IVs is a potentially achievable goal, but discerning higher order relationships between BVs and IVs or between PDVs and other animal viruses will be more difficult and may not be possible.

6.10.7. The Future for Polydnavirus Research

In this review our understanding of PDVs, their association with parasitoids, and their function in parasitism has been summarized. In looking to the future, certain avenues of research that should receive priority are being suggested. These studies break along two lines: (1) advancing our understanding of PDV evolutionary relationships; and (2) advancing our understanding of PDV gene function, and the relationship between function and genome organization. Furthering our understanding of evolutionary relationships will require first and foremost more comparative genomic data. As discussed above (see Sections 6.10.2 and 6.10.4), the representative genomes of a selected IVs from campoplegine ichneumonids and a selected microgastrine braconid BVs have been fully sequenced. A few other campoplegine and banchine IVs as well as BVs from cardiochiline and chelonine braconids are in progress. At minimum, similar data are needed from at least one species in other subfamilies of ichneumonoids hypothesized to carry PDVs. In addition, genomic data from multiple campoplegine and microgastrine braconids from a common genus are needed. Together, these data would allow us to determine the rate at which PDV genomes are evolving, the relationship within and between BVs and IVs, and whether a cophylogeny exists between parasitoids and the viruses they carry.

Since encapsidated PDV genomes lack many genes required for replication these viral genes must now reside in the nuclear genome of their wasp host. Identifying these structural genes will be critical to understanding how PDVs replicate and potentially their relationship to other viruses. Evidence for integration of *C. congregata* BV segments in tandem array within the wasp genome is strong and suggestive that an ancestral provirus accounts for the genomic clustering of BV segments (Belle *et al*., 2002). Comparable data on other BVs and IVs is also needed.

The second area of priority should relate to host biology and the function of PDVs in parasitism. From the perspective of host immunity, our

knowledge of how insects regulate inducible humoral responses like antimicrobial peptide synthesis greatly exceeds our knowledge of hemocyte-mediated defense responses. This deficiency is well-illustrated by the recently published genome for *Anopheles gambiae* (Holt *et al*., 2002) where many genes regulating humoral defense responses are identifiable by homology but few genes regulating cellular defense responses are (Christophides *et al*., 2002). Future advances in understanding how PDV virulence genes suppress the insect immune system will depend on enhancing our understanding of the signaling pathways and processes that regulate insect cellular immune responses. Our understanding at the molecular level of how biosynthesis and release of insect endocrine factors are regulated is similarly weak, and will need to be improved in order to understand how viral genes developmentally arrest host growth. Lastly, developing bioassay systems beyond the hosts that a given parasitoid normally attacks will likely be needed to fully understand the mode of action of PDV genes. Especially attractive are insect systems like *Dr. melanogaster, A. gambiae*, and the honeybee *Apis mellifera* for which complete genomes are available. Development of genomic lepidopteran models would also, obviously, be of tremendous value to the study of PDVs.

Acknowledgments

We gratefully acknowledge the support of USDA (NRI-9902684; NRI 2001-52100-11332) and NSF (MCB-0094403) grants. This is publication 04-08-040 of the University of Kentucky Agricultural Experiment Station.

Reference

Albrecht, U., Wyler, T., Pfister-Wilhelm, R., Gruber, A., Stettler, P., *et al*., **1994**. PDV of the parasitic wasp *Chelonus inanitus* (Braconidae): characterization, genome organization and time point of replication. *J. Gen. Virol. 75*, 3353–3363.

Andersson, S.G., Kurland, C.G., **1998**. Reductive evolution of resident genomes. *Trends Microbiol. 6*, 263–268.

Asgari, S., Hellers, M., Schmidt, O., **1996**. Host haemocyte inactivation by an insect parasitoid: transient expression of a polydnavirus gene. *J. Gen. Virol. 77*, 2653–2662.

Asgari, S., Reineke, A., Beck, M., Schmidt, O., **2002**. Isolation and characterization of a neprilysin-like protein from *Venturia canescens* virus-like particles. *Insect Mol. Biol. 11*, 477–485.

Asgari, S., Schmidt, O., **1994**. Passive protection of eggs from the parasitoid, *Cotesia rubecula* in the host *Pieris rapae*. *J. Insect Physiol. 40*, 789–795.

Asgari, S., Schmidt, O., **2001**. Promoter studies of a polydnavirus gene from *Cotesia rubecula* (Hym: Braconidae). *Arch. Virol. 146*, 1979–1989.

Asgari, S., Schmidt, O., **2002**. A coiled-coil region of an insect immune suppressor protein is involved in binding and uptake by hemocytes. *Insect Biochem. Mol. Biol. 32*, 497–504.

Asgari, S., Schmidt, O., Theopold, U., **1997**. A polydnavirus-encoded protein of an endoparasitoid wasp is an immune suppressor. *J. Gen. Virol. 78*, 3061–3070.

Asgari, S., Theopold, U., Wellby, C., Schmidt, O., **1998**. A protein with protective properties against the cellular defense reactions in insects. *Proc. Natl Acad. Sci. USA 95*, 3690–3695.

Asgari, S., Zhang, G., Schmidt, O., **2003**. Polydnavirus particle proteins with similarities to molecular chaperones, heat-shock protein 70 and calreticulin. *J. Gen. Virol. 84*, 1165–1171.

Baines, D., Downer, R.G.H., **1992**. 5-Hydroxytryptamine-sensitive adenylate cyclase affects phagocytosis in cockroach hemocytes. *Arch. Insect Biochem. Physiol. 21*, 303–316.

Balgopal, M.M., Dover, B.A., Goodman, W.G., Strand, M.R., **1996**. Parasitism by *Microplitis demolitor* induces alterations in juvenile hormone titers and juvenile hormone esterase activity of its host, *Pseudoplusia includens*. *J. Insect Physiol. 42*, 337–345.

Beck, M., Strand, M.R., **2003**. RNA interference silences *Microplitis demolitor* bracovirus genes and implicates glc1.8 in disruption of adhesion in infected host cells. *Virology 314*, 521–535.

Beck, M., Strand, M.R., **2004**. Glc1.8 from *Microplitis demolitor* bracovirus induces a loss of adhesion and phagocytosis by Insect High Five and S2 cells. *J. Virol.* (in press).

Beck, M., Theopold, U., Schmidt, O., **2000**. Evidence for serine protease inhibitor activity in the ovarian calyx fluid of the endoparasitoid *Venturia canescens*. *J. Insect Physiol. 46*, 1275–1283.

Beckage, N.E., **1993**. Games parasites play: the dynamic roles of peptides and proteins in the host–parasite interaction. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, Vol. 1. Academic Press, New York, pp. 25–58.

Beckage, N.E., Gelman, D.B., **2004**. Wasp parasitoid disruption of host development: implications for new biologically based strategies of pest control. *Annu. Rev. Entomol. 49*, 299–330.

Beckage, N.E., Kanost, M.R., **1993**. Effects of parasitism by the braconid wasp *Cotesia congregata* on host hemolymph proteins of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol. 23*, 643–653.

Beckage, N.E., Metcalf, J.S., Nesbit, D.J., Schleifer, K.W., Zetlan, S.R., *et al*., **1990**. Host hemolymph monophenoloxidase activity in parasitized *Manduca sexta* larvae and evidence for inhibition by wasp polydnavirus. *Insect Biochem. 20*, 285–294.

Beckage, N.E., Riddiford, L.M., **1982**. Effects of parasitism by *Apanteles congregatus* on the endocrine physiology of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* *47*, 308–322.

Beckage, N.E., Tan, F.F., **2002**. Development of the braconid wasp *Cotesia congregata* in a semi-permissive noctuid host, *Trichoplusia ni*. *J. Invertebr. Pathol.* *81*, 49–52.

Beckage, N.E., Templeton, T.J., Nielsen, B.D., Cook, D.I., Stoltz, D.B., **1987**. Parasitism-induced hemolymph polypeptides in *Manduca sexta* (L.) larvae parasitized by the braconid wasp *Cotesia congregata* (Say). *Insect Biochem.* *17*, 439–455.

Beliveau, C., Laforge, M., Cusson, M., Bellemare, G., **2000**. Expression of a *Tranosema rostrale* polydnavirus gene in the spruce budworm, *Choristoneura fumiferana*. *J. Gen. Virol.* *81*, 1871–1880.

Beliveau, C., Levasseur, A., Stoltz, D., Cusson, M., **2003**. Three related TrIV genes: comparative sequence analysis and expression in host larvae and Cf-124T cells. *J. Insect Physiol.* *49*, 501–511.

Belle, E., Beckage, N.E., Rousselet, J., Poirie, M., Lemeunier, F., *et al*., **2002**. Visualization of polydnavirus sequences in a parasitoid wasp chromosome. *J. Virol.* *76*, 5793–5796.

Belshaw, R., Fitton, M., Herniou, E., Gimeno, C., Quicke, D.L.J., **1998**. A phylogenetic reconstruction of the Ichneumonoidea (Hymenoptera) based on the D2 variable region of 28S ribosomal RNA. *Syst. Entomol.* *23*, 109–123.

Berg, O.G., Kurland, C.G., **2000**. Why mitochondrial genes are most often found in nuclei. *Mol. Biol. Evol.* *17*, 951–961.

Black, D.S., Marie-Cardine, A., Schraven, B., Bliska, J.B., **2000**. The *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. *Cell. Microbiol.* *2*, 401–414.

Blissard, G.W., Smith, O.P., Summers, M.D., **1987**. Two related viral genes are located on a single superhelical DNA segment of the multipartite *Campoletis sonorensis* virus genome. *Virology* *160*, 120–134.

Blissard, G.W., Theilmann, D.A., Summers, M.D., **1989**. Segment W of *Campoletis sonorensis* virus: expression, gene products, and organization. *Virology* *169*, 78–89.

Blissard, G.W., Vinson, S.B., Summers, M.D., **1986**. Identification, mapping and *in vitro* translation of *Campoletis sonorensis* virus mRNAs from parasitized *Heliothis virescens* larvae. *J. Virol.* *57*, 318–327.

Bigot, Y., Drezen, J.M., Sizaret, P.Y., Rabouille, A., Hamelin, M.H., *et al*., **1997**. The genome segments of *Dp*RV, a commensal reovirus of the wasp *Diadromus pulchellus* (Hymenoptera). *Virology* *210*, 109–119.

Bogdan, C., Rollinghoff, M., Diefenbach, A., **2000**. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr. Opin. Immunol.* *12*, 64–76.

Brown, F., **1986**. The classification and nomenclature of viruses: summary of results of meetings of the International Committee on Taxonomy of Viruses in Sendai. *Intervirology* *25*, 141–143.

Carton, Y., Nappi, A., **1997**. *Drosophila* cellular immunity against parasitoids. *Parasitol. Today* *13*, 218–227.

Chen, Y.P., Gundersen-Rindal, D.E., **2003a**. Morphological and genomic characterization of the polydnavirus associated with the parasitoid wasp *Glyptapanteles indiensis* (Hymenoptera: Braconidae). *J. Gen. Virol.* *84*, 2051–2060.

Chen, Y.P., Taylor, P.B., Shapiro, M., Gundersen-Rindal, D.E., **2003b**. Quantitative expression analysis of a *Glyptapanteles indiensis* polydnavirus protein tyrosine phosphatase gene in its natural lepidopteran host, *Lymantria dispar*. *Insect Mol. Biol.* *12*, 271–280.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., *et al*., **2002**. Immunity-related genes and gene families in *Anopheles gambiae*. *Science* *298*, 159–166.

Clark, K.D., Pech, L.L., Strand, M.R., **1997**. Isolation and identification of a plasmatocyte-spreading peptide from the hemolymph of the lepidopteran insect *Pseudoplusia includens*. *J. Biol. Chem.* *272*, 23440–23447.

Clark, K.D., Witherell, A., Strand, M.R., **1998**. Plasmatocyte spreading peptide is encoded by an mRNA differentially expressed in tissues of the moth *Pseudoplusia includens*. *Biochem. Biophys. Res. Commun.* *250*, 479–485.

Clausen, C.P., **1940**. Entomophagous Insects. McGraw-Hill, New York.

Cole, T.J., Beckage, N.E., Tan, F.F., Srinivasan, A., Ramaswamy, S.B., **2002**. Parasitoid–host endocrine relations: self-reliance or cooptation? *Insect Biochem. Mol. Biol.* *32*, 1673–1679.

Cooke, J., Nowak, M.A., Boerlijst, M., Maynard-Smith, J., **1997**. Evolutionary origins and maintenance of redundant gene expression during metazoan development. *Trends Genet.* *13*, 360–364.

Cornelis, G.R., **2002**. *Yersinia* type III secretion: send in the effectors. *J. Cell Biol.* *158*, 401–408.

Cox-Foster, D.L., Stehr, J.E., **1994**. Induction and localization of FAD-glucose dehydrogenase (GLD) during encapsulation of abiotic implants in *Manduca sexta* larvae. *J. Insect Physiol.* *40*, 235–249.

Cui, L., Soldevila, A.I., Webb, B.A., **1997**. Expression and hemocyte-targeting of a *Campoletis sonorensis* polydnavirus cysteine-rich gene in *Heliothis virescens* larvae. *Arch. Insect Biochem.* *36*, 251–271.

Cui, L., Soldevila, A.I., Webb, B.A., **2000**. Relationships between polydnavirus gene expression and host range of the parasitoid wasp *Campoletis sonorensis*. *J. Insect Physiol.* *46*, 1397–1407.

Cui, L., Webb, B.A., **1996**. Isolation and characterization of a member of the cysteine-rich gene family from *Campoletis sonorensis* polydnavirus. *J. Gen. Virol.* *77*, 797–809.

Cui, L., Webb, B.A., **1997**. Homologous sequences in the *Campolitis sonorensis* polydnavirus genome are

implicated in replication and nesting of the W segment family. *J. Virol. 71*, 8504–8513.

Cusson, M., Laforge, M., Miller, D., Cloutier, C., Stoltz, D., **2000**. Functional significance of parasitism-induced suppression of juvenile hormone esterase activity in developmentally delayed *Choristoneura fumiferana* larvae. *Gen. Comp. Endocr. 117*, 343–354.

Cusson, M., Lucarotti, C., Stoltz, D., Krell, P., Doucet, D., **1998**. A polydnavirus from the spruce budworm parasitoid *Tranosema rostrale* (Ichneumonidae). *J. Invertebr. Pathol. 72*, 50–56.

Dahlman, D.L., Scheppers, E.L., Scheppers, T., Rana, R.L., Diluna, F.A., *et al.*, **2003**. A gene from a parasitic wasp expressed in teratocytes inhibits host protein synthesis, growth and development. *Insect Mol. Biol. 12*, 527–534.

Dahlman, D.L., Vinson, S.B., **1993**. Teratocytes: developmental and biochemical characteristics. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, Vol. 1, Parasites. Academic Press, New York, pp. 145–165.

Davies, H.D., Vinson, S.B., **1986**. Passive evasion by eggs of braconid parasitoid *Cardiochiles nigriceps* of encapsulation *in vitro* by haemocytes of host *Heliothis virescens*: possible role for fibrous layer in immunity. *J. Insect Physiol. 32*, 1003–1010.

Davies, D.H., Vinson, S.B., **1988**. Interference with function of plasmatocytes of *Heliothis virescens in vivo* by calyx fluid of the parasitoid *Campoletis sonorensis*. *Cell Tissue Res. 251*, 467–475.

Davies, D.H., Strand, M.R., Vinson, S.B., **1987**. Changes in differential haemocyte count and *in vitro* behaviour of plasmatocytes from host *Heliothis virescens* caused by *Campoletis sonorensis* PDV. *J. Insect Physiol. 33*, 143–153.

De Buron, I., Beckage, N.E., **1992**. Characterization of a polydnavirus (PDV) and virus-like filamentous particle (VLFP) in the braconid wasp *Cotesia congregata* (Hymenoptera: Braconidae). *J. Invertebr. Pathol. 59*, 315–327.

Deng, L., Webb, B.A., **1999**. Cloning and expression of a gene encoding a *Campoletis sonorensis* polydnavirus structural protein. *Arch. Insect Biochem. 40*, 30–40.

Deng, L., Stoltz, D.B., Webb, B.A., **2000**. A gene encoding a polydnavirus structural polypeptide is not encapsidated. *Virology 269*, 440–450.

Dib-Hajj, S.D., Webb, B.A., Summers, M.D., **1993**. Structure and evolutionary implications of a "cysteine-rich" *Campoletis sonorensis* polydnavirus gene family. *Proc. Natl Acad. Sci. USA 90*, 3765–3769.

Diehl-Jones, W., Mandato, C.A., Whent, G., Downer, R.G.H., **1996**. Monoaminergic regulation of hemocyte activity. *J. Insect Physiol. 42*, 13–19.

Domingo, E., Webster, R., Holland, J., **1999**. Origin and Evolution of Viruses. Academic Press, San Diego, CA.

Doolittle, R.F., Feng, D.F., Johnson, M.S., McClure, M.A., **1989**. Origins and evolutionary relationships of retroviruses. *Q. Rev. Biol. 64*, 1–30.

Dong, K., Zhang, D., Dahlman, D.L., **1996**. Down-regulation of juvenile hormone esterase and arylphorin production in *Heliothis virescens* larvae parasitized by *Microplitis croceipes*. *Arch. Insect Biochem. Physiol. 32*, 237–248.

Doucet, D., Cusson, M., **1996**. Role of calyx fluid in alterations of immunity in *Choristoneura fumiferana* larvae parasitized by *Tranosema rostrale*. *Comp. Biochem. Physiol. 114*, 311–317.

Dover, B.A., Davies, D.H., Vinson, S.B., **1988a**. Degeneration of last instar *Heliothis virescens* prothoracic glands by *Campoletis sonorensis* PDV. *J. Invert. Pathol. 51*, 80–91.

Dover, B.A., Davies, D.H., Vinson, S.B., **1988b**. Dose-dependent influence of *Campoletis sonorensis* PDV on the development and ecdysteroid titers of last-instar *Heliothis virescens* larvae. *Arch. Insect Biochem. Physiol. 8*, 113–126.

Dover, B.A., Menon, A., Brown, R.C., Strand, M.R., **1995**. Suprression of juvenile hormone esterase in *Heliothis virescens* by *Microplitis demolitor*. *J. Insect Physiol. 41*, 846–855.

Dover, B.A., Tanaka, T., Vinson, S.B., **1995**. Stadium-specific degeneration of host prothoracic glands by *Campoletis sonorensis* calyx fluid and its association with host ecdysteroid titers. *J. Insect Physiol. 41*, 947–955.

Dowton, M., Austin, A.D., **1994**. Molecular phylogeny of the insect order Hymenoptera: apocritain relationships. *Proc. Natl Acad. Sci. USA 91*, 9911–9915.

Drezen, J.-M., Provost, B., Espagne, E., Cattolico, L., Dupuy, C., *et al.*, **2003**. Polydnavirus genome: integrated vs. free virus. *J. Insect Physiol. 49*, 407–417.

Dupas, S., Turnbull, M., Webb, B.A., **2003**. Diversifying selection in a parasitoid's symbiotic virus among genes involved in inhibiting host immunity. *Immunogenetics 55*, 351–361.

Dushay, M.S., Asling, B., Hultmark, D., **1996**. Origins of immunity: *Relish*, a compound Rel-like gene in the antibacterial defense of *Drosophila*. *Proc. Natl Acad. Sci. USA 93*, 10343–10347.

Edson, K.M., Vinson, S.B., Stoltz, D.B., Summers, M.D., **1981**. Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. *Science 211*, 582–583.

Einerwold, J., Jaseja, M., Hapner, K., Webb, B.A., Copie, V., **2001**. Solution structure of the carboxyl-terminal cysteine-rich domain of the VHv1.1 polydnaviral gene product: comparison with other cysteine knot structural folds. *Biochemistry 40*, 14404–14412.

Engstrom, Y., Kadalayil, L., Sun, S-C., Samakovlis, C., Hultmark, D., *et al.*, **1993**. κβ-like motifs regulate the induction of immune genes in *Drosophila*. *J. Mol. Biol. 232*, 327–333.

Falabella, P., Varricchio, P., Gigliotti, S., Tranfaglia, A., Pennacchio, F., *et al.*, **2003**. *Toxoneuron nigriceps* polydnavirus encodes a putative aspartyl protease highly expressed in parasitized host larvae. *Insect Mol. Biol. 12*, 9–17.

Feddersen, I., Sander, K., Schmidt, O., **1986**. Virus-like particles with host protein-like antigenic determinants

protect an insect parasitoid from encapsulation. *Experientia 42*, 1278–1281.

Federici, B.A., Bigot, Y., **2003**. Origin and evolution of polydnaviruses by symbiogenesis of insect DNA viruses in endoparasitic wasps. *J. Insect Physiol. 49*, 419–432.

Fleming, J.G.W., Krell, P.J., **1993**. Polydnavirus genome organization. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, vol. 1, Parasites. Academic Press, San Diego, CA, pp. 189–225.

Fleming, J.G.W., Summers, M.D., **1986**. *Campoletis sonorensis* endoparasitic wasps contain forms of *C. sonorensis* virus DNA suggestive of integrated and extrachromosomal polydnavirus DNAs. *J. Virol. 57*, 552–562.

Fleming, J.G., Summers, M.D., **1991**. PDV DNA is integrated in the DNA of its parasitoid wasp host. *Proc. Natl Acad. Sci. USA 88*, 9770–9774.

Galibert, L., Rocher, J., Ravallec, M., Duonor-Cerutti, M., Webb, B.A., *et al.*, **2003**. Two *Hyposoter didymator* ichnovirus genes expressed in the lepidopteran host encode secreted or membrane-associated serine and threonine rich proteins in segments that may be nested. *J. Insect Physiol. 49*, 441–451.

Gardiner, E.M.M., Strand, M.R., **1999**. Monoclonal antibodies bind distinct classes of hemocytes in the moth *Pseudoplusia includens*. *J. Insect Physiol. 45*, 113–126.

Gelman, D.B., Reed, D.A., Beckage, N.E., **1998**. Manipulation of fifth-instar host (*Manduca sexta*) ecdysteroid levels by the parasitoid wasp *Cotesia congregata*. *J. Insect Physiol. 44*, 833–843.

Ghosh, S., May, M.J., Kopp, E.B., **1998**. NF-κβ and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol. 16*, 225–260.

Gillespie, J.P., Kanost, M.R., Trenczek, T., **1997**. Biological mediators of insect immunity. *Annu. Rev. Entomol. 42*, 611–643.

Glatz, R., Schmidt, O., Asgari, S., **2003**. Characterization of a novel protein with homology to C-type lectins expressed by the *Cotesia rubecula* bracovirus in larvae of the lepidopteran host, *Pieris rapae*. *J. Biol. Chem. 278*, 19743–19750.

Grossniklaus-Burgin, C., Pfister-Wilhelm, R., Meyer, V., Treiblmayr, K., Lanzrein, B., **1998**. Physiological and endocrine changes associated with polydnavirus/venom in the parasitoid–host system *Chelonus inanitus–Spodoptera littoralis*. *J. Insect Physiol. 33*, 305–321.

Gruber, A., Stettler, P., Heiniger, P., Schumperli, D., Lanzrein, B., **1996**. Polydnavirus DNA of the braconid wasp *Chelonus inanitus* is integrated in the wasp's genome and excised only in later pupal and adult stages of the female. *J. Gen. Virol. 77*, 2873–2879.

Gundersen-Rindal, D., Dougherty, E.M., **2000**. Evidence for integration of *Glyptapanteles indiensis* polydnavirus DNA into the chromosome of *Lymantria dispar in vitro*. *Virus Res. 66*, 27–37.

Gundersen-Rindal, D.E., Lynn, D.E., **2003**. Polydnavirus integration in lepidopteran host cells *in vitro*. *J. Insect Physiol. 49*, 453–462.

Gundersen-Rindal, D., Lynn, D.E., Dougherty, E.M., **1999**. Transformation of lepidopteran and coleopteran insect cell lines by *Glyptapanteles indiensis* PDV DNA. *In Vitro Cell Devel. Biol. Anim. 35*, 111–114.

Guzo, D., Stoltz, D.B., **1987**. Observations on cellular immunity and parasitism in the tussock moth. *J. Insect Physiol. 33*, 19–31.

Harwood, S.H., Grosovsky, A.J., Cowles, E.A., Davis, J.W., Beckage, N.E., **1994**. An abundantly expressed hemolymph glycoprotein isolated from newly parasitized *Manduca sexta* larvae is a PDV gene product. *Virology 205*, 381–392.

Harwood, S.H., McElfresh, J.S., Nguyen, A., Conlan, C.A., Beckage, N.E., **1998**. Production of early expressed parasitism-specific proteins in alternate sphingid hosts of the braconid wasp *Cotesia congregata*. *J. Invertebr. Pathol. 71*, 271–279.

Hayakawa, Y., **1990**. Juvenile hormone esterase activity repressive factor in the plasma of parasitized insect larvae. *J. Biol. Chem. 265*, 10 813–10 816.

Hayakawa, Y., **1991**. Structure of a growth-blocking peptide present in parasitized insect hemolymph. *J. Biol. Chem. 266*, 7982–7984.

Hayakawa, Y., Yazaki, K., **1997**. Envelope proteins of parasitic wasp symbiont virus protect the wasp eggs from the cellular immune reactions by the host insect. *Eur. J. Biochem. 246*, 820–826.

Hayakawa, Y., Yazaki, K., Yamanaka, A., Tanaka, T., **1994**. Expression of PDV genes from the parasitoid wasp *Cotesia kariyai* in two noctuid hosts. *Insect Mol. Biol. 3*, 97–103.

Harvey, J.A., Strand, M.R., **2002**. The developmental strategies of endoparasitoid wasps vary with host wasps vary with host feeding ecology. *Ecology 83*, 2439–2451.

Herniou, E.A., Luque, T., Chen, X., Vlak, J.M., Winstanley, D., *et al.*, **2001**. Use of whole genome sequence data to infer baculovirus phylogeny. *J. Virol. 75*, 8117–8126.

Herniou, E.A., Olszewski, J.A., Cory, J.S., O'Reilly, D.R., **2003**. The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol. 48*, 211–234.

Hilgarth, R., **1995**. Physiological effects of *Campoletis sonorensis* calyx fluid on *Heliothis virescens*. M.S. thesis, Kansas State University, Manhattan, KS.

Hilgarth, R., **2002**. Identification and analysis of the repeat element gene family in the *Campoletis sonorensis* polydnavirus *rep*. Ph. D. dissertation. University of Kentucky, Lexington, KY.

Hilgarth, R.S., Webb, B.A., **2002**. Characterization of *Campoletis sonorensis* ichnovirus segment I genes as members of the repeat element gene family. *J. Gen. Virol. 83*, 2393–2402.

Holland, J., Domingo, E., **1998**. Origin and evolution of viruses. *Virus Genes 16*, 13–21.

Holt, R.A., Subramanian, G.M., *et al.*, **2002**. The genome of the malaria mosquito *Anopheles gambiae*. *Science 298*, 129–149.

Johner, A., Lanzrein, B., **2002**. Characterization of two genes of the polydnavirus of *Chelonus inanitus* and their stage-specific expression in the host *Spodoptera littoralis*. *J. Gen. Virol. 83*, 1075–1085.

Johner, A., Stettler, P., Gruber, A., Lanzrein, B., **1999**. Presence of polydnavirus transcripts in an egg–larval parasitoid and its lepidopterous host. *J. Gen. Virol. 80*, 1847–1854.

Jomori, T., Natori, S., **1991**. Molecular cloning a cDNA for lipopolysaccharide-binding protein from the hemolymph of the American cockroach, *Periplaneta americana*. *J. Biol. Chem. 266*, 13318–13323.

Kadash, K., Harvey, J.A., Strand, M.R., **2003**. Cross-protection experiments with parasitoids in the genus *Microplitis* (Hymenoptera: Braconidae) suggest a high level of specificity in their associated bracoviruses. *J. Insect Physiol. 49*, 473–482.

Kang, D., Liu, G., Gunne, H., Steiner, H., **1996a**. PCR differential display of immune gene expression in *Trichoplusia ni*. *Insect Biochem. Mol. Biol. 26*, 177–184.

Kang, D., Lundstrom, A., Steiner, H., **1996b**. *Trichoplusia ni* attacin A, a differentially displayed insect gene coding for an antibacterial protein. *Gene 174*, 245–249.

Kappler, C., Meister, M., Lagueux, M., Gateff, E., Hoffmann, J.A., *et al.*, **1993**. Insect immunity: two 17 bp repeats nesting a κβ-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*. *EMBO J. 12*, 1561–1568.

Kim, M.K., Sisson, G., Stoltz, D.B., **1996**. Ichnovirus infection of an established gypsy moth cell line. *J. Gen. Virol. 77*, 2321–2328.

Kinuthia, W., Li, D., Schmidt, O., Theopold, U., **1999**. Is the surface of endoparasitic wasp eggs and larvae covered by a limited coagulation reaction? *J. Insect Physiol. 45*, 501–506.

Koizumi, N., Imai, Y., Morozumi, A., Imamura, M., Kadotani, T., *et al.*, **1999**. Lipopolysaccharide-binding protein of *Bombyx mori* participates in a hemocyte-mediated defense reaction against gram-negative bacteria. *J. Insect Physiol. 45*, 853–859.

Krell, P.J., Summers, M.D., Vinson, S.D., **1982**. A virus with a multipartite superhelical DNA genome from the ichneumonid parasitoid *Campoletis sonorensis*. *J. Virol. 43*, 859–870.

Kroemer, J.A., Webb, B.A., **2004**. Polydnavirus genes and genomes: emerging gene families and new insights into polydnavirus replication. *Annu. Rev. Entomol. 49*, 431–456.

Lanzrein, B., Pfister-Wilhelm, R., von Niederhausern, F., **2001**. Effects of an egg–larval parasitoid and its polydnavirus on development and the endocrine system of the hosts. In: Edwards, J.P., Weaver, R. (Eds.), Endocrine Interactions of Insect Parasites and Pathogens. BIOS Scientific Publishers, Oxford, pp. 95–109.

Lavine, M.D., Beckage, N.B., **1995**. Polydnaviruses: potent mediators of host insect immune dysfunction. *Parasitol. Today 11*, 368–378.

Lavine, M.D., Beckage, N.E., **1996**. Temporal pattern of parasitism-induced immunosuppression in *Manduca sexta* larvae parasitized by *Cotesia congregata*. *J. Insect Physiol. 42*, 41–51.

Lavine, M.D., Strand, M.R., **2001**. Surface characteristics of foreign targets that elicit an encapsulation response by the moth *Pseudoplusia includens*. *J. Insect Physiol. 47*, 965–974.

Lavine, M.D., Strand, M.R., **2002**. Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol. 32*, 1295–1309.

Lavine, M.D., Strand, M.R., **2003**. Hemocytes from *Pseudoplusia includens* express multiple alpha and beta integrin subunits. *Insect Mol. Biol. 12*, 441–452.

Lawrence, P., **1986**. Host–parasite hormonal interactions: an overview. *J. Insect Physiol. 32*, 295–298.

Lawrence, P.O., Lanzrein, B., **1993**. Hormonal interactions between insect endoparasites and their host insects. In: Thompson, S.N., Federici, B.A., Beckage, N.E. (Eds.), Parasites and Pathogens of Insects, Vol. 1, Parasites. Academic Press, New York, pp. 59–86.

Le, N.T., Asgari, S., Amaya, K., Tan, F.F., Beckage, N.E., **2003**. Persistence and expression of *Cotesia congregata* polydnavirus in host larvae of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol. 49*, 533–543.

Le Hir, H., Nott, A., Moore, M.J., **2003**. How introns influence and enhance eukaryotic gene expression. *Trends Biochem. Sci. 28*, 215–220.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., *et al.*, **2001**. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito *Anopheles gambiae*. *Cell 104*, 709–718.

Li, X., Webb, B.A., **1994**. Apparent functional role for a cysteine-rich polydnavirus protein in suppression of the insect cellular immune response. *J. Virol. 68*, 7482–7489.

Liu, C.T., Hou, R.F., Chen, C.C., **1998**. Formation of basement membrane-like structure terminates the cellular encapsulation of microfilariae in the haemocoel of *Anopheles quadrimaculatus*. *Parasitology 116*, 511–518.

Liu, G., Kang, D., Steiner, H., **2000**. *Trichoplusia ni* lebocin, an inducible immune gene with a downstream insertion element. *Biochem. Biophys. Res. Comm. 269*, 803–807.

Lovallo, N., Cox-Foster, D.L., **1999**. Alteration in FAD-glucose dehydrogenase activity and hemocyte behavior contribute to initial disruption of *Manduca sexta* immune response to *Cotesia congregata* parasitoids. *J. Insect Physiol. 45*, 1037–1048.

Lowenberger, C., **2001**. Innate immune response of *Aedes aegypti*. *Insect Biochem. Mol. Biol. 31*, 219–229.

Luckhart, S., Vodovotz, Y., Cui, L., Rosenberg, R., **1998**. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc. Natl Acad. Sci. USA 95*, 5700–5705.

Luckhart, S., Webb, B.A., **1996**. Interaction of a wasp ovarian protein and polydnavirus in host immune suppression. *Devel. Comp. Immunol. 20*, 1–21.

Lundstrom, A., Liu, G., Kang, D., Berzins, K., Steiner, H., **2002**. *Trichoplusia ni* gloverin, and inducible immune gene encoding an antibacterial insect protein. *Insect Biochem. Mol. Biol. 32*, 795–801.

Manfruelli, P., Reichhart, J.-M., Steward, R., Hoffmann, J.A., Lemaitre, B., **1999**. A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. *EMBO J. 18*, 3380–3391.

Maiti, I.B., Dey, N., Dahlman, D.L., Webb, B.A., **2003**. Antibiosis-type resistance in transgenic plants expressing a teratocyte secretory peptide (TSP) gene from a hymenopteran endoparasite (*Microplitis croceipes*). *Plant Biotech. J. 1*, 209–219.

Marti, D., Grossniklaus-Burgin, C., Wyder, S., Wyler, T., Lanzrein, B., **2003**. Ovary development and polydnavirus morphogenesis in the parasitic wasp *Chelonus inanitus*. I. Ovary morphogenesis, amplification of viral DNA and ecdysteroid titres. *J. Gen. Virol. 84*, 1141–1150.

McKelvey, T.A., Lynn, D.E., Gundersen-Rindal, D., Guzo, D., Stoltz, D., *et al.*, **1996**. Transformation of gypsy moth (*Lymantria dispar*) cell lines by infection with *Glyptapanteles indiensis* polydnavirus. *Biochem. Biophys. Res. Commun. 225*, 764–770.

Meister, M., Hetru, C., Hoffmann, J.A., **2000**. The antimicrobial host defense of *Drosophila*. In: Du Pasquier, L., Litman, G.W. (Eds.), Origin and Evolution of the Vertebrate Immune System. Springer, Berlin, pp. 17–36.

Miller, J.S., Nguyen, T., Stanley-Samuelson, D.W., **1994**. Eicosanoids mediate insect nodulation responses to bacterial infections. *Proc. Natl Acad. Sci. USA 91* 12418–12422.

Miller, L.K., **1996**. Insect viruses. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fundamental Virology. Lippincott-Raven, Philadelphia, PA.

Muta, T., Iwanaga, S., **1996**. The role of hemolympyh coagulation in innate immunity. *Curr. Opin. Immunol. 8*, 41–47.

Nakamatsu, Y., Gyotoku, Y., Tanaka, T., **2001**. The endoparasitoid *Cotesia kariyai* (Ck) regulates the growth and metabolic efficiency of *Pseudaletia separata* larvae by venom and Ck polydnavirus. *J. Insect Physiol. 47*, 573–584.

Nappi, A.J., Vass, E., Frey, F., Carton, Y., **2000**. Nitric oxide involvement in *Drosophila* immunity. *Nitric Oxide 4*, 423–430.

Nardi, J. B., Pilas, B., Ujhelyi, E., Garsha, K., Kanost, M. R., **2003**. Hematopoietic organs of *Manduca sexta* and hemocyte lineages. *Devel. Genes Evol. 213*, 477–491.

Noguchi, H., Hayakawa, Y., Downer, R.G.H., **1995**. Elevation of dopamine levels in parasitized insect larvae. *Insect Biochem. Mol. Biol. 25*, 197–201.

Norton, W.N., Vinson, S.B., **1983**. Correlating the initiation of virus replication with a specific pupal developmental phase of an ichneumonid parasitoid. *Cell Tissue Res. 231*, 387–398.

Oliver, M.G., Specian, R.D., **1990**. Cytoskeleton of intestinal goblet cells: role of actin filaments in baseline secretion. *Am. J. Physiol. 259*, G991–997.

Ohnishi, M., Tokuda, M., Masaki, T., Fujimura, T., Tai, Y., *et al.*, **1995**. Involvement of annexin-I in glucose-induced insulin secretion in rat pancreatic islets. *Endocrinology 136*, 2421–2426.

Ong, C.-K., Chan, S.-Y., Campo, M.S., Fujinaga, K., Mavromara-Nazos, P., *et al.*, **1993**. Evolution of human paillomavirus type 18: an ancient phylogenetic root in Africa and intratype diversity reflect coevolution with human ethnic groups. *J. Virol. 67*, 6424–6431.

Pasquier-Barre, F., Dupuy, C., Huguet, E., Moneiro, F., Moreau, A., *et al.*, **2002**. Polydnavirus replication: the EP1 segment of the parasitoid wasp *Cotesia congregata* is amplified within a larger precursor molecule. *J. Gen. Virol. 83*, 2035–2045.

Pech, L.L., Strand, M.R., **1996**. Granular cells are required for encapsulation of foreign targets by insect haemocytes. *J. Cell Sci. 109*, 2053–2060.

Pennacchio, F., Falabella, P., Sordetti, R., Varricchio, P., Malva, C., *et al.*, **1998a**. Prothoracic gland inactivation in *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *J. Insect Physiol. 44*, 845–857.

Pennacchio, F., Falabella, P., Vinson, S.B., **1998b**. Regulation of *Heliothis virescens* prothoracic glands by *Cardiochiles nigriceps* polydnavirus. *Arch. Insect Biochem. Physiol. 38*, 1–10.

Pennacchio, F., Sordetti, R., Falabella, P., Vinson, S.B., **1997**. Biochemical and ultrastructural alterations in prothoracic glands of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) last instar larvae parasitized by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae). *Insect Biochem. Mol. Biol. 27*, 439–450.

Pennacchio, F., Vinson, S.B., Tremblay, E., Ostuni, A., **1994**. Alteration of ecdysone metabolism in *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae induced by *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) teratocytes. *Insect Biochem. Mol. Biol. 23*, 383–394.

Prevost, G., Davies, D.H., Vinson, S.B., **1990**. Evasion of encapsulation by parasitoid correlated with the extent of host hemocyte pathology. *Entomol. Exp. Appl. 55*, 1–10.

Qui, P., Pan, P.C., Govind, S., **1999**. A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development 125*, 1909–1920.

Quicke, D.L.J., **1997**. Parasitic Wasps. Chapman and Hall, Cambridge.

Quicke, D.L.J., Fitton, M.G., Notton, D.G., Broad, G.R., Dolphin, K., **2000**. Phylogeny of the subfamilies of Ichneumonidae (Hymenoptera): a simultaneous molecular and morphological analysis. In: Austin, A.D., Dowton, M. (Eds.), Hymenoptera: Evolution, Biodiversity and Biological Control. CSIRO, Collingwood, pp. 74–83.

Rana, R.L., Dahlman, D.L., Webb, B.A., **2002**. Expression and characterization of a novel teratocyte protein of the braconid, *Microplitis croceipes*. *Insect Biochem. Mol. Biol. 32*, 1507–1516.

Ratcliffe, N.A., **1993**. Cellular defense responses of insects: unresolved problems. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, Vol. 1, Parasites. Academic Press, San Diego, CA, pp. 267–304.

Rattanadechakul, W., Webb, B.A., **2003**. Characterization of *Campoletis sonorensis* ichnovirus unique segment B and excision locus structure. *J. Insect Physiol. 49*, 523–532.

Reed, D.A., Brown, J.J., **1998**. Host/parasitoid interactions: critical timing of parasitoid-derived products. *J. Insect Physiol. 44*, 721–732.

Rotheram, S.M., **1967**. Immune surface of eggs of a parasitic insect. *Nature 214*, 700.

Rotherman, S.M., **1973**. The surface of the egg of a parasitic insect. 2. The ultrastructure of the particulate coat on the egg of *Nemeritis*. *Proc. Roy. Soc. London B 183*, 195–204.

Savary, S., Beckage, N., Tan, F., Periquet, G., Drezen, J.-M., **1997**. Excision of the polydnavirus chromosomal integrated EP1 sequence of the parasitoid wasp *Cotesia congregata* (Braconidae, Microgastrinae) at potential recombinase binding sites. *J. Gen. Virol. 78*, 3125–3134.

Savary, S., Drezen, J.-M., Tan, F., Beckage, N.E., Periquet, G., **1999**. The excision of PDV sequences from the genome of the wasp *Cotesia congregata* (Braconidae, Microgastrinae) is developmentally regulated but not strictly restricted to the ovaries in the adult. *Insect Mol. Biol. 8*, 319–327.

Schmidt, O., Theopold, U., Strand, M.R., **2001**. Innate immunity and evasion by insect parasitoids. *BioEssays 23*, 344–351.

Shaw, M.R., Huddleston, T., **1991**. Classification and biology of the braconid wasps (Hymenoptera). *Handbks. Ident. Br. Insects 7*, 1–126.

Shelby, K.S., Cui, L., Webb, B.A., **1998**. Polydnavirus-mediated inhibition of lysozyme gene expression and the antibacterial response. *Insect Mol. Biol. 7*, 265–272.

Shelby, K.S., Adeyeye, O.A., Okot-Kotber, B.M., Webb, B.A., **2000**. Parasitism-linked block of host plasma melanization. *J. Invertebr. Pathol. 75*, 218–225.

Shelby, K.S., Webb, B.A., **1994**. Polydnavirus infection inhibits synthesis of an insect plasma protein, arylphorin. *J. Gen. Virol. 75*, 2285–2292.

Shelby, K.S., Webb, B.A., **1997**. Polydnavirus infection inhibits translation of specific growth-associated host proteins. *Insect Biochem. Mol. Biol. 27*, 263–270.

Shelby, K.S., Webb, B.A., **1999**. Polydnavirus-mediated suppression of insect immunity. *J. Insect Phys. 45*, 507–514.

Soldevila, A.I., Heuston, S., Webb, B.A., **1997**. Purification and analysis of a polydnavirus gene product expressed using a poly-histidine baculovirus vector. *Insect Biochem. Mol. Biol. 27*, 201–211.

Soller, M., Lanzrein, B., **1996**. PDV and venom of the egg–larval parasitoid *Chelonus inanitus* (Braconidae) induce developmental arrest in the prepupa of its host *Spodoptera littoralis* (Noctuidae). *J. Insect Physiol. 42*, 471–481.

Steiner, B., Pfister-Wilhelm, R., Grossniklaus-Buergin, C., Rembold, H., Treiblmayr, K., *et al.*, **1999**. Titres of juvenile hormone I, II and III in *Spodoptera littoralis* (Noctuidae) from the egg to the pupal moult and their modification by the egg–larval parasitoid *Chelonus inanitus* (Braconidae). *J. Insect Physiol. 45*, 401–413.

Stettler, P., Trenczek, T., Wyler, T., Pfister-Wilhelm, R., Lanzrein, B., **1998**. Overview of parasitism associated effects on host haemocytes in larval parasitoids and comparison with effects of the egg–larval parasitoid *Chelonus inanitus* on its host *Spodoptera littoralis*. *J. Insect Physiol. 44*, 817–831.

Stoker, A., Dutta, R., **1998**. Protein tyrosine phosphatases and neural development. *BioEssays 20*, 463–472.

Stoltz, D.B., **1993**. The PDV life cycle. In: Thompson, S.N., Federici, B.A., Beckage, N.E. (Eds.), Parasites and Pathogens of Insects, Vol. 1, Parasites. Academic Press, San Diego, CA, pp. 167–187.

Stoltz, D.B., Beckage, N.E., Blissard, G.W., Fleming, J.G.W., Krell, P.J., *et al.*, **1995**. Polydnaviridae. In: Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A., Summers, M.D. (Eds.), Virus Taxonomy. Springer, New York, pp. 143–147.

Stoltz, D.B., Cook, D.I., **1983**. Inhibition of host phenoloxidase activity by parasitoid Hymenoptera. *Experientia 39*, 1022–1024.

Stoltz, D., Guzo, D., **1986**. Apparent haemocytic transformations associated with parasitoid-induced inhibition of immunity in *Malacosoma disstria* larvae. *J. Insect Physiol. 32*, 377–388.

Stoltz, D.B., Guzo, D., Cook, D., **1986**. Studies on PDV transmission. *Virology 155*, 120–131.

Stoltz, D.B., Krell, P.J., Vinson, S.B., **1981**. Polydisperse viral DNA's in ichneumonid ovaries: a survey. *Can. J. Microbiol. 27*, 123–130.

Stoltz, D., Makkay, A., **2003**. Overt viral diseases induced from apparent latency following parasitization by the ichneumonid wasp, *Hyposoter exiguae*. *J. Insect Physiol. 49*, 483–490.

Stoltz, D.B., Vinson, S.B., **1977**. Baculovirus-like particles in the reproductive tracts of female parasitoid wasps. 2. The genus *Apanteles*. *Can. J. Microbiol. 23*, 28–37.

Stoltz, D.B., Vinson, S.B., **1979a**. Viruses and parasitism in insects. *Adv. Virus Res. 24*, 125–171.

Stoltz, D.B., Vinson, S.B., **1979b**. Penetration into caterpillar cells of virus-like particles injected during oviposition by parasitoid ichneumonid wasps. *Can. J. Microbiol. 25*, 207–216.

Stoltz, D.B., Whitfield, J.B., **1992**. Viruses and virus-like entities in the parasitic Hymenoptera. *J. Hymen. Res. 1*, 125–139.

Stoltz, D.B., Xu, D., **1990**. Polymorphism in PDV genomes. *Can. J. Microbiol. 36*, 538–543.

Stoltz, D.B., Vinson, S.B., MacKinnon, E.A., **1976**. Baculovirus-like particles in the reproductive tracts of female parasitoid wasps. *Can. J. Microbiol. 22*, 1013–1023.

Strand, M.R., **1994**. *Microplitis demolitor* polydnavirus infects and expresses in specific morphotypes of *Pseudoplusia includens* haemocytes. *J. Gen. Virol. 75*, 3007–3020.

Strand, M.R., **2000**. Life history variation and developmental contraints in parasitoids. In: Hochberg, M., Ives, A.R. (Eds.), Population Biology of Parasitoids. Princeton University Press, Princeton, NJ, pp. 139–162.

Strand, M.R., Clark, K.D., **1999**. Plasmatocyte spreading peptide induces spreading of plasmatocytes but represses spreading of granulocytes. *Arch. Insect Biochem. Physiol. 42*, 213–223.

Strand, M.R., Clark, K.C., Gardiner, E.M.M., **1999**. Plasmatocyte spreading peptide does not induce *Microplitis demolitor* polydnavirus-infected plasmatocytes to spread on foreign surfaces. *Arch. Insect Biochem. Physiol. 42*, 213–223.

Strand, M.R., Hayakawa, Y., Clark, K.D., **2000**. Plasmatocyte spreading peptide (PSP1) and growth blocking peptide (GBP) are multifunctional homologs. *J. Insect Physiol. 46*, 817–824.

Strand, M.R., McKenzie, D.I., Grassl, V., Dover, B.A., Aiken, J.M., **1992**. Persistence and expression of *Microplitis demolitor* PDV in *Pseudoplusia includens*. *J. Gen. Virol. 73*, 1627–1635.

Strand, M.R., Noda, T., **1991**. Alterations in the haemocytes of *Pseudoplusia includens* after parasitism by *Microplitis demolitor. J. Insect. Physiol. 37*, 839–850.

Strand, M.R., Pech, L.L., **1995**. Immunological basis for compatibility in parasitoid–host relationships. *Annu. Rev. Entomol. 40*, 31–56.

Strand, M.R., Witherell, R.A., Trudeau, D., **1997**. Two *Microplitis demolitor* polydnavirus mRNAs expressed in hemocytes of *Pseudoplusia includens* contain a common cysteine-rich domain. *J. Virol. 71*, 2146–2156.

Strand, M.R., Wong, E.A., **1991**. The growth and role of *Microplitis demolitor* teratocytes in parasitism of *Pseudoplusia includens*. *J. Insect Physiol. 37*, 503–515.

Strauss, E.G., Strauss, J.H., Levine, A.J., **1996**. Virus evolution. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fundamental Virology. Lippincott-Raven, Philadelphia, PA, pp. 141–159.

Sugumaran, M., **1996**. Role of insect cuticle in immunity. In: Soderhall, K., Iwanaga, S. (Eds.), New Directions in Invertebrate Immunology. SOS Publications, Fair Haven, NJ, pp. 355–374.

Tanaka, T., **1982**. Effect of *Microplitis* parasitization on the ultrastructural morphology of hemocytes of *Leucania* hosts. In: Akai, H. (Ed.), The Ultrastructure and Functioning of Insect Cells. Society for Insect Cells, Tokyo, Japan, pp. 169–172.

Tanaka, T., **1987**. Morphological changes in haemocytes of the host *Pseudaletia separata* parasitized by *Microplitis mediator* or *Apanteles kariyae*. *Devel. Comp. Immunol. 1*, 57–67.

Tanaka, K., Matsumoto, H., Hayakawa, Y., **2002**. Detailed characterization of polydnavirus immunoevasive proteins in an endoparasitoid wasp. *Eur. J. Biochem. 269*, 2557–2566.

Tanaka, K., Tsuzuki, S., Matsumoto, H., Hayakawa, Y., **2003**. Expression of *Cotesia kariyai* polydnavirus genes in lepidopteran hemocytes and Sf9 cells. *J. Insect Physiol. 49*, 433–440.

Teramato, T., Tanaka, T., **2003**. Similar polydnavirus genes of two parasitoids, *Cotesia kariyai* and *Cotesia ruficrus*, of the host *Pseudaletia separata*. *J. Insect Physiol. 49*, 463–471.

Theilmann, D.A., Summers, M.D., **1987**. Physical analysis of the *Campoletis sonorensis* virus multipartite genome and identification of a family of tandemly repeated elements. *J. Virol. 61*, 2589–2598.

Theilmann, D.A., Summers, M.D., **1988**. Identification and comparison of *Campoletis sonorensis* virus transcripts expressed from four genomic segments in the insect hosts *Campoletis sonorensis* and *Heliothis virescens*. *Virology 167*, 329–341.

Theopold, U., Samakovlis, C., Erdjumentbromage, H., Dillon, N., Axelsson, B., *et al.*, **1996**. *Helix pomatia* lectin, an inducer of *Drosophila* immune response, binds to hemomucin, a novel suface mucin. *J. Biol. Chem. 271*, 12708–12715.

Thompson, S.N., **1986**. Effect of the insect parasite *Hyposoter exiguae* (Viereck) on the carbohydrate metabolism of its host *Trichoplusia ni*. *J. Insect Physiol. 32*, 287–293.

Thompson, S.N., **1993**. Redirection of host metabolism and effects on parasites nutrition. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, Vol. 1, Parasites. Academic Press, San Diego, CA, pp. 125–144.

Thompson, S.N., Dahlman, D.L., **1998**. Aberrant nutritional regulation of carbohydrate synthesis by parasitized *Manduca sexta* L. *J. Insect Physiol. 44*, 745–753.

Trudeau, D., Strand, M.R., **1998**. A limited role in parasitism for *Microplitis demolitor* PDV. *J. Insect Physiol. 44*, 795–805.

Trudeau, D., Washburn, J.O., Volkman, L.E., **2001**. Central role of hemocytes in *Autographa californica* M nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*. *J. Virol. 75*, 996–1003.

Trudeau, D., Witherell, R.A., Strand, M.R., **2000**. Characterization of two novel *Microplitis demolitor* PDV mRNAs expressed in *Pseudoplusia includens* haemocytes. *J. Gen. Virol. 81*, 3049–3058.

Turnbull, M.W., Webb, B.A., **2002**. Perspectives on polydnavirus origin and evolution. *Adv. Virus Res. 58*, 203–254.

van Huijsduijnen, R.H., Bombrun, A., Swinnen, D., **2002**. Selecting protein tyrosine phosphatases as drug targets. *Drug Discov. Today 7*, 1013–1019.

van Regnemeortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., *et al.* (Eds.) **2000**. Virus Taxonomy: classification and Nomenclature of Viruses Academic Press, New York.

Varricchio, P., Falabella, P., Sordetti, R., Graziani, F., Malva, C., Pennacchio, F., **1999**. *Cardiochiles nigriceps*

polydnavirus: molecular characterization and gene expression in parasitized *Heliothis virescens* larvae. *Insect Biochem. Mol. Biol. 29*, 1087–1096.

Vass, E., Nappi, A.J., **2001**. Fruit fly immunity. *Bioscience 51*, 529–535.

Vinson, S.B., **1990**. Physiological interactions between the host genus *Heliothis* and its guild of parasitoids. *Arch. Insect Biochem. Physiol. 13*, 63–81.

Vinson, S.B., Iwantsch, G.F., **1980**. Host regulation by insect parasitoids. *Q. Rev. Biol. 55*, 143–165.

Vinson, S.B., Scott, J.R., **1975**. Particles containing DNA associated with the oocyte of an insect parasitoid. *J. Invertebr. Pathol. 25*, 375–378.

Volkoff, A.-N., Cerutti, P., Rocher, J., Ohresser, M.C.P., Devauchelle, G., *et al.*, **1999**. Related RNAs in lepidopteran cells after *in vitro* infection with *Hyposoter didymator* virus define a new polydnavirus gene family. *Virology 263*, 349–363.

Volkoff, A.-N., Ravallec, M., Bossy, J., Cerutti, P., Rocher, J., *et al.*, **1995**. The replication of *Hyposoter didymator* PDV: cytopathology of the calyx cells in the parasitoid. *Biol. Cell 83*, 1–13.

Volkoff, A.-N., Rocher, J., Cerutti, P., Ohresser, M.C.P., d'Aubenton-Carafa, Y., *et al.*, **2001**. Persistent expression of a newly characterized *Hyposoter didymator* polydnavirus gene in long-term infected lepidopteran cell lines. *J. Gen. Virol. 82*, 963–969.

Volkoff, A.-N., Rocher, J., Duonor-Cerutti, M., Webb, B.A., Hilgarth, R., *et al.*, **2002**. Evidence for a conserved polydnavirus gene family: homologs of the *Cs*IV repeat element genes from two additional ichnoviruses. *Virology 300*, 316–331.

Voytas, D.F., **1996**. Retroelements in genome organization. *Science 274*, 737–738.

Washburn, J.O., Haas-Stapleton, E.J., Tan, F.F., Beckage, N.E., Volkman, L.E., **2000**. Co-infection of *Manduca sexta* larvae with PDV from *Cotesia congregata* increases susceptibility to fatal infection by *Autographa californica* M nucleopolyhedrovirus. *J. Insect Physiol. 46*, 179–190.

Washburn, J.O., Kirkpatrick, B.A., Volkman, L.E., **1995**. Comparative pathogenesis of *Autographa californica* M nuclear polyhedrosis virus in larvae of *Trichoplusia ni* and *Heliothis virescens*. *Virology 209*, 561–568.

Webb, B.A., **1998**. Polydnavirus biology, genome structure, and evolution. In: Miller, L.K., Balls, L.A. (Eds.), The Insect Viruses. Plenum, New York, pp. 105–139.

Webb, B.A., Beckage, N.E., Hayakawa, Y., Krell, P.J., Lanzrein, B., *et al.*, **2000**. Polydnaviridae. In: van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy: The Classification and Nomenclature of Viruses. Academic Press, San Diego, CA, pp. 253–259.

Webb, B.A., Cui, L., **1998**. Relationships between polydnavirus genomes and viral gene expression. *J. Insect Physiol. 44*, 785–793.

Webb, B.A., Summers, M.D., **1992**. Stimulation of PDV replication by 20-hydroxyecdysone. *Experientia 48*, 1018–1022.

Whitfield, J.B., **1997**. Molecular and morphological data suggest a single origin of the PDVs among braconid wasps. *Naturwissenschaften 84*, 502–507.

Whitfield, J.B., **2000**. Phylogeny of microgastroid braconid wasps, and what it tells us about polydnavirus evolution. In: Austin, A.D., Dowton, M. (Eds.), The Hymenoptera: Evolution, Biodiversity and Biological Control. CSIRO Publishing, Melbourne, pp. 97–105.

Whitfield, J.B., **2002**. Estimating the age of the polydnavirus/braconid wasp symbiosis. *Proc. Natl Acad. Sci. USA 99*, 7508–7513.

Whitfield, J.B., Asgari, S., **2003**. Virus or not? Phylogenetics of polydnaviruses and their wasp carriers. *J. Insect Physiol. 49*, 397–405.

Willott, E., Trenczek, T., Thrower, L.W., Kanost, M.R., **1994**. Immunochemical identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte types in *Manduca sexta*. *Eur. J. Cell Biol. 65*, 417–423.

Wyder, S., Blank, F., Lanzrein, B., **2003**. Fate of polydnavirus DNA of the egg-larval parasitoid *Chelonus inanitus* in the host *Spodoptera littoralis*. *J. Insect Physiol. 49*, 491–500.

Wyder, S., Tschannen, A., Hochuli, A., Gruber, A., Saladin, V., *et al.*, **2002**. Characterization of *Chelonus inanitus* polydnavirus segments: sequences and analysis, excision site and demonstration of clustering. *J. Gen. Virol. 83*, 247–256.

Wyler, T., Lanzrein, B., **2003**. Ovary development and polydnavirus morphogenesis in the parasitic wasp *Chelonus inanitus*. 2. Ultrastructural analysis of calyx cell development, virion formation and release. *J. Gen. Virol. 84*, 1151–1163.

Xu, D., Stoltz, D., **1993**. Polydnavirus genome segment families in the ichneumonid parasitoid *Hyposoter fugitivus*. *J. Virol. 67*, 1340–1349.

Yagi, S., Tanaka, T., **1992**. Retardation of testis development in the armyworm, *Pseudaletia separata*, parasitized by the braconid wasp, *Cotesia kariyai*. *Invertebr. Rep. Devel. 22*, 151–157.

Yin, L., Zhang, C., Qin, J., Wang, C., **2003**. Polydnavirus of *Campoletis chlorideae*: characterization and temporal effect on host *Helicoverpa armigera* cellular immune response. *Arch. Insect Biochem. Physiol. 52*, 104–113.

Zitnan, D., Kingan, T.G., Beckage, N.E., **1995**. Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of *Manduca sexta*. *Insect Biochem. Mol. Biol. 25*, 669–678.

Relevant Website

http://www.pdvdb-dev.ctegd.uga.edu – University of Georgia's database of polydnavirus genomes.

6.11 Entomopathogenic Fungi and their Role in Regulation of Insect Populations

M S Goettel, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada
J Eilenberg, The Royal Veterinary and Agricultural University, Frederiksberg C, Denmark
T Glare, AgResearch, Canterbury, New Zealand

6.11.1. Introduction

Entomopathogenic fungi play an important role in the regulation of insect populations. There is a diverse array of fungal insect pathogenic species from within four different classes. Adaptations range from obligate pathogens of specific insect species to generalists capable of infecting many host species to species that are facultative pathogens. Fungal epizootics are common in some insect species, while others are rarely affected. The earliest studies with entomopathogenic fungi were carried out in the 1800s, and these concentrated on developing ways of managing diseases that were devastating the silkworm industry (Steinhaus, 1975). In fact, the germ theory was first demonstrated by Bassi (1835 as cited by Steinhaus, 1975) using silkworms and the muscardine fungus, which was later named *Beauveria bassiana* in his honor. Bassi's studies on the disease in silkworms enabled him to extrapolate his findings into the field of human diseases. The stimulus for the idea of using entomopathogens to manage pest insects came largely from the silkworm disease studies. However, early attempts in using fungi as microbial control agents of pest insects were soon overshadowed by the development of chemical insecticides. It was not until the late 1950s that attempts to use entomopathogenic fungi for insect pest management resurfaced. To date, there are many commercial products available worldwide, based on less than 10 fungal species

(Shah and Goettel, 1999; Copping, 2001), but the potential for the development of many more remains high.

In this article, an overview of entomopathogenic fungi and their current and potential role in the regulation of pest insect populations is provided, emphasizing as much as possible the developments over the last 20 years. Reviews dealing with entomopathogenic fungi and their development as microbial control agents include those of Ferron (1985), McCoy *et al.* (1988), Evans (1989), Ferron *et al.* (1991), Glare and Milner (1991), Roberts and Hajek (1992), Tanada and Kaya (1993), Hajek and St. Leger (1994), Boucias and Pendland (1998), Wraight and Carruthers (1999), and several chapters in Butt *et al.* (2001) and Upadhyay (2003). For methods and techniques used to study these pathogens, readers are referred to Butt and Goettel (2000), and the chapters in Lacey (1997) and Lacey and Kaya (2000).

6.11.2. Entomopathogenic Fungi

Insect pathogens have a long history of recognition despite the relatively recent understanding of microbial infections. Descriptions of entomopathogenic fungi can be found in drawings from several centuries ago. Japanese descriptions of silkworm infections by muscardine fungi (probably *Beauveria bassiana*) and *Cordyceps* spp. infections of a number of insects date from around the nineth century (Samson *et al.*, 1988). Perhaps because of their prominent and often brightly colored fruiting bodies, *Cordyceps* species feature in many of the earliest references and drawings of entomopathogenic fungi. Species of *Cordyceps* have also been of practical value in several countries. Maori, the native people of New Zealand, were known to use extracts of *Cordyceps robertsii* as a pigment for traditional face tattoos (moko), while the Chinese have used *Cordyceps* infected caterpillars as traditional medicines for many years. Other entomopathogenic fungi had been recorded by the late eighteenth and through the nineteenth century. Flies infected by entomophthoralean fungi, where the distinct halo formed by forcibly discharged primary conidia could clearly be seen with the naked eye, were, for example, observed by Goethe (1817–1822).

Classification of these interesting fungi began with *Cordyceps* and other obvious insect-infecting fungi. While Cooke (1892) listed four groups parasitic to insects (*Cordyceps*, Entomophthorales, Laboulbeniales, and opportunitistic fungi such as *Cladosporium* and *Penicillium*), interest in the diversity of these fungi did not really blossom until the 1970s. Currently, at least 90 genera and more than 700 species, representing all the major classifications of fungi, have been implicated in diseases of insects.

With the advent of molecular comparisons as a tool in classification, the distinction between protozoa and fungi has been revisited and revised. Fungal taxonomy is still in a state of flux. As presently delimited, the kingdom Fungi may constitute a monophyletic group that shares some characters with animals, such as chitinous structures, storage of glycogen, and mitochondrial UGA coding for tryptophan. The Chytridiomycetes possess the primitive character of a single, smooth, posteriorly inserted flagellum and are basal to the other fungi (Cavalier-Smith, 1987; Barr, 1992). It does not include the Oomycetes, which have been moved to the kingdom Chromista with some algae (Leipe *et al.*, 1994).

While some phylogenists have moved the classes between kingdoms, the entomopathogens remain within four major classes: the Oomycetes, Chytridiomycetes, Ascomycetes, and Zygomycetes. Some unusual insect pathogenic species are found in other fungal classes, but most can be found in the Ascomycetes and Zygomycetes. A distinction must be made between disease causing species that in many cases result in mortality of the insect and those that live on insects, usually without causing mortality. The latter group includes species of Laboulbeniomycetes, which can be obligate parasites on insects, but have little effect on the health of their hosts (Tanada and Kaya, 1993) and Basidiomycota such as *Septobasidum* and *Uredinella*.

One of the most confusing aspects of fungal taxonomy is the treatment of species only known by an asexual stage. Many such species have been classified in the Deuteromycota within the class Hyphomycetes, which is essentially a form-class. This has led to many species having both a name for the sexual (teleomorph) stage and the asexual (anamorph) stage with no link between the names. With the advent of molecular identification tools, it has been possible to link many anamorph and teleomorph stages, or to substantially predict the teleomorph genera for a species described only through an anamorph stage. Many common insect pathogenic species are currently classified in the Hyphomycetes, such as *B. bassiana* and *Metarhizium anisopliae*, but with modern technology, it can be demonstrated that their affinities are with the Ascomycete genus *Cordyceps*. Whether separate pleomorphic and anamorphic names are still required is a current topic of debate around the International Code of Botanical Nomenclature. As

molecular analysis has the ability to link anamorphic stages with appropriate teleomorphic genera, the use of a dual nomenclature system is probably superfluous, but may be hard to lose in the short term.

The main classes that contain entomopathogenic species are reviewed in the following sections. Examples of insects infected with entomopathogenic fungi are presented in **Figures 1–15**.

6.11.2.1. Phylum Oomycota

Oomycetes are characterized by cellulose containing coenocytic hyphae, biflagellate zoospores, and usually contain no chitin. Sexual reproduction can occur between gametangia (antheridia and oogonia) on the same or different hyphae. While a number of species are saprophytes and parasites of animals and plants, two genera contain species pathogenic to mosquito larvae. The best studied species is *Lagenidium giganteum* (Lagenidiales), a pathogen of mosquito larvae (Glare and Milner, 1991; Kerwin and Petersen, 1997). Other species of *Lagenidium* can cause infections in aquatic crustaceans, such as crabs (e.g., Hatai *et al*., 2000).

Leptolegnia spp. (Saprolegniales) have also been reported to be pathogenic to mosquitoes, chironomids, and several other Diptera. These species are thought to infect via a secondary zoospore formed after encystment of the primary zoospore derived from sporangia (Zattau and McInnis, 1987).

6.11.2.2. Phylum Chytridiomycota

Chrytridiomycetes are characterized by cell walls containing chitin and no cellulose. Posteriorly uniflagellate zoospores and gametes settle and grow into a thallus, which becomes either a resting spore or coenocytic hyphae. This group is considered basal to the fungal branch under SSU rRNA phylogenetic comparisons. The Blastocladiales genus *Coelomomyces* contains most of the common entomopathogenic Chytridiomycetes. There are over 70 entomopathogenic species described in the *Coelomomyces* (Lucarotti *et al*., 1985). These species have been described from Diptera (mainly mosquitoes) and Heteroptera. Several species have an obligate intermediate host, such as copepods. They are characterized by formation of both thick walled resistant sporangia and flagellate zoospores. Other entomopathogenic species are known from *Coelomycidium* (Blastocladiales) and *Myriophagus* (Chytridiales); the former is found on blackflies and mosquitoes, and the latter has been reported as a pathogen on dipterous pupae by Sparrow, 1939 and Karling, 1948 (in Samson *et al*., 1988).

6.11.2.3. Phylum Zygomycota

Traditionally, the Zygomycota are separated on the basis of often nonseptate, multinuclear hyphae, and production of zygospores by copulation between gametangia. However, molecular analyses have not found the Zygomycota to be monophyletic (Jensen



Figure 1 Larvae of the European wireworm, *Agriotus obscurus*, killed by *Metarhizium anisopliae*. The progression of the emergence of the fungus from the cadavers and subsequent development of fungal structures is seen from left to right, with the larva on the right completely covered by the green spores of the fungus. (Photo courtesy of T. Kabaluk.)

et al., 1998), and some groups, such as the Glomales, may be placed elsewhere eventually (e.g., Bruns *et al*., 1993). *Basidiobolus ranarum* and other *Basidiobolus* species, traditionally considered as Zygomycetes, have now been placed in the Chytridiomycetes, despite being nonflagellate (Nagahama *et al*., 1995).



Figure 2 Tick (*Ixodes ricinus*) killed by *Metarhizium anisopliae*. (Photo courtesy of C. Nielsen.)

Within the true Zygomycota, the class Trichomycetes contains species often associated with insects. While Sweeney (1981) described the species *Smittium morbosum* (Trichomycetes) as a pathogen of mosquitoes, most associations of the Trichomycetes are symbiotic or weakly parasitic rather than true pathogens (Beard and Adler, 2002; Cafaro, 2002). Some species of *Mucor* (Mucorales) are occasionally associated with insect mortality.

The majority of entomopathogenic species within Zygomycota are contained in one order, the Entomophthorales. More than 200 entomopathogenic Entomophthorales species have been recognized. They commonly cause spectacular epizootics and are characterized in all genera but one (*Massospora*), by the production of forcibly discharged primary conidia. Many species are capable of producing various types of secondary conidia from the primary conidia and, in some cases, infection is obligatorily through a secondary conidium. Many species are also capable of producing resting spores, long-lived zygospores or azygospores. These fungi are generally obligate pathogens in nature, and many species are presently difficult or impossible to culture on artificial media.

Until around the 1960s, most entomopathogenic Entomophthorales were contained within a single genus, *Entomophthora*, but several years of taxonomic revision have placed the species into several genera. The main genera containing entomopathogenic species are *Conidiobolus, Entomophaga, Entomophthora, Erynia, Furia*, *Massospora, Neozygites, Pandora, Strongwellsea*, and *Zoophthora*.



Figure 3 Adult cabbage fly (*Delia radicum*) killed by *Beauveria bassiana*. This fungus produces clusters of white conidia. (Photo courtesy of J. Eilenberg.)



Figure 4 Adult weevil killed by *Paecilomyces farinosus*. The fungus produces extensive external growth and spores. (Photo courtesy of J. Martin.)



Figure 5 Pupae of the lepidopteran species *Dasyneura pudibunda* killed by *Cordyceps militaris*. The ascospores are produced in the orange asci. (Photo courtesy of J. Eilenberg.)



Figure 6 Alate termite (*Macrotermes* sp.) killed by *Cordycepioideus* sp. The fungus has only been found on alate termites. (Photo courtesy of J. Eilenberg.)

6.11.2.4. Ascomycota (and Deuteromycota)

Fungi from Ascomycota have septate and haploid mycelia and the sexual spores, ascospores, are produced in an ascus on a fruiting body, the ascomata. Typically, eight ascospores are produced in each ascus. No motile zoospores are produced. There has been continuing taxonomic confusion over classification of the teleomorphic (sexual) stages and the anamorphic (asexual) conidial stage when they are not produced by a single occurrence. *Cordyceps*, a genus containing many (>300) entomopathogenic species is probably the best known Ascomycete because the sexual and sometimes asexual stages are produced on highly visible stroma. The genus *Ascosphaera*, which is heterothallic and sexually dimorphic, contains species responsible for chalkbrood disease in bees. Unusually for entomopathogenic fungi, *Ascosphaera* spores are ingested by bee larvae and germinate in the gut (Gilliam and Vandenberg, 1990).

Many strains within Ascomycetes have apparently lost the ability to form the sexual stages, the teleomorph forms, and were classified in the form-class Hyphomycetes. Entomopathogenic species are known from over 40 genera, but the most important entomopathogenic species occur in *Aschersonia, Aspergillus, Beauveria, Culicinomyces, Gibellula, Hirsutella, Hymenostilbe, Lecanicillium* (formerly *Verticillium*), *Metarhizium, Nomuraea, Paecilomyces, Sorosporella*, and *Tolypocladium*.



Figure 7 Small Diptera (Chironomidae) killed by *Erynia conica*. The dead insect is covered by conidiophores from which conidia are discharged. (Photo courtesy of J. Eilenberg.)



Figure 9 Adult cantharid beetle (*Rhagonycha fulva*) infected with *Entomophthora muscae*. Conidia have been discharged from the abdomen and clusters of conidia are seen on the wings. (Photo courtesy of J. Eilenberg.)



Figure 8 Adult dipteran (*Ptychoptera contaminata*) killed by *Entomophaga* (= *Eryniopsis*) *ptychopterae*. No fungus growth is visible before incubation in a moist chamber. (Photo courtesy of J. Eilenberg.)



Figure 10 Small muscoid fly (*Coenosia testaceae*) infected with *Strongwellsea* sp. This fungus causes an abdominal hole in the host, while still alive. Conidia are projected from the hole and the infectious fly can disperse the disease. (Photo courtesy of J. Eilenberg.)

Most of these genera have been linked to one or several ascomycete genera. Such linkages can be demonstrated either biologically (an insect infected with an anamorph dies and a teleomorph is produced) or by using molecular tools showing the genetic relationship between anamorphs and teleomorphs (Huang *et al*., 2002; Liu *et al*., 2002). With the advent of molecular identification, many more links between anamorph and teleomorph stages will be made and, as discussed above, the need for the form-class Hyphomycetes will be unnecessary.

6.11.2.5. Basidiomycota

There are very few Basidiomycetes that have been implicated in insect pathogenesis. Some publications list the Septobasidiales genera *Septobasidium* and *Uredinella* as pathogenic to insects, but most



Figure 11 Colony of cereal aphids (*Sitobion avenae*). One individual (swollen, whitish-orange) is infected by *Pandora neoaphidis* and the discharged primary conidia may result in infection of the rest of the colony. (Photo courtesy of T. Steenberg.)



Figure 12 Ant infected with *Pandora formicae*. Before death, the ant climbs to the top of the canopy, turns the head downwards, and grasps the plant with its legs. The conidiophores then emerge and conidia are discharged, showering the understory, where presumably more hosts are present. (Photo courtesy of J. Eilenberg.)



Figure 13 Adult *Scatophaga stercoraria* killed by a member of the *Entomophthora muscae* complex. White bands of conidiophores emerge from the abdomen, which then produce forcibly discharged conidia. (Photo courtesy of A. Bruun Jensen.)



Figure 14 Adult *Delia radicum* filled with bright orange resting spores of *Strongwellsea castrans*. (Photo courtesy of J. Eilenberg.)

are considered symbiotic to insects, such as scales (Samson *et al.*, 1988).

6.11.2.6. Approaches to Classification

Traditionally, fungal taxonomy has been based on morphological, developmental, and physiological characteristics, from which the current structure of species, genera, and classes has emerged. While these characteristics worked very well for many years, the complexity of fungal life has led to use of some characteristics in classification that are not the result of coevolution, but parallel evolution of a common function. This has led to the incorrect placement of some species and genera in the phylogenetic scheme. In addition, the use of indirect methods, which evaluate phenotypic traits, have not been useful at



Figure 15 Earwig (*Forficula auricolaria*) infected with *Zoophthora forficulae*. The insect is completely covered with the fungus and is firmly attached to the vegetation by sticky rhizoids produced by the fungus. (Photo courtesy of J. Eilenberg.)

the subspecies level. Identification of specific isolates is necessary in biocontrol, both as a regulatory requirement and to advance understanding of ecology and epizootiology (see Section 6.11.4).

There have been many different approaches to classifying insect pathogenic fungi. In some cases, fungi have been defined functionally or ecologically, rather than phylogenetically, especially when techniques to directly determine phylogeny did not exist. The entomogenous fungi provide an example where the entomogenous habitat was seen as a defining criterion in many cases, whereas morphology and physiology formed the basis of many of the earlier efforts with other fungi. Early descriptions of *M. anisopliae* refer to *Entomophthora anisopliae* (Zimmermann *et al*., 1995), recognizing the relationship between two common entomogenous groups over dissimilarities in morphology and physiology. The mode and morphology of conidiation has been a main character used for classification.

In recent times, classification systems have attempted to take a more phylogenetic approach to systematics (e.g., Humber, 1984), but a true phylogenetic classification had not emerged for most organisms until the advent of molecular biology. Other approaches are still being explored. Chemotaxonomy has been attempted with some entomopathogenic groups such as *Beauveria*, utilizing the commercially available carbohydrate utilization strips system of API (i.e., API50CH) (Todorova *et al*., 1994; Rath *et al*., 1995). Isoenzymes have been investigated for their ability to discriminate between fungal strains (e.g., May *et al*., 1979; St. Leger *et al*., 1992b), but their power in phylogenetic classification is limited.

Molecular techniques that target RNA and DNA have been applied at every level of the taxonomic hierarchy. Many techniques have been applied, some of which directly sequence genes, and some of which represent differences in DNA sequence, such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) approaches. Pulse field gel electrophoresis (PFGE) allows the separation of DNA fragments as large as chromosomes and can be used for whole genome restriction digest comparisons. Chromosome length polymorphism was used by Viaud *et al*. (1996) to study nine isolates of *B. bassiana*. Mitochondrial DNA has been used to estimate intraspecies variation in *Lecanicillium* (= *Verticillium*) *lecanii* and *M. anisopliae* isolates (Typas *et al*., 1998). There are a number of reviews on the use of molecular characterization for entomogenous fungi (e.g., Clarkson, 1992; Driver and Milner, 1998).

With the development of molecular biology as a mainstream tool in classification, it became possible to test assumptions formed by studies based on morphology and other characteristics reliant on phylogenetic relationships. In macroevolution, the seven kingdoms are based largely on sequencing of small subunit ribosomal genes. While classifying fungi solely on the sequence of any gene is not wise, a general consensus is being reached over the evolutionary position of the fungi in respect to other groups based on rRNA gene sequences (e.g., Berbee and Taylor, 2001). Molecular analysis has shown that a number of species and genera classified as fungi, or in the kingdom Eumycota, are actually grouped with the Chromista. Many species have been placed in the fungi simply on the basis of hyphal growth, but are now being reconsidered.

The genera of entomopathogenic fungi are being reconsidered on the basis of sequencing of conserved genes and introns. Recent efforts to clarify classification of entomopathogenic fungi demonstrate the utility of molecular characterization at several levels. Zare *et al*. (2000), Gams and Zare (2001), Zare and Gams (2001) have redefined the genus *Verticillium* using rDNA sequencing, placing all insect pathogens in the genus *Lecanicillium*. Similarly, sequencing of large subunit rRNA gene revealed *Paecilomyces* may not be monophyletic (Obornik *et al*., 2001). Sequencing of the small subunit rDNA has also been used to examine phylogenetic relationships among the order Entomophthorales (Jensen *et al*., 1998). These molecular studies supported the use of spore discharge characteristics as an identifying characteristic for Entomophthorales.

Molecular studies have also been useful in positioning individual species, such as *Basidiobolus ranarum*, previously placed in the Zygomycota, as a nonflagellate species of chytrid (Nagahama *et al.*, 1995). The use of sequence level analysis also suggested reassigning of species such as *Eryniopsis ptychopterae* to *Entomophaga* (Hajek *et al.*, 2003).

As noted above, the use of DNA and RNA comparisons has never been more useful than when considering the fungal species that never reproduce sexually, because the characters of sexual reproduction are the basis for much of the traditional alignment of family, genera, and species. While the higher classification of fungi is in a state of flux, the form-class Hyphomycetes is likely to disappear in the near future, with DNA analysis making the need for a group for which a sexual stage is not known redundant. Linking of anamorphs and teleomorphs is now relatively straightforward. For example, sequencing of the ITS-5.8s region of nuclear RNA has recently been used to confirm a teleomorph, *Cordyceps bassiana*, of *Beauveria bassiana* (Huang *et al.*, 2002).

Strain characterization is a useful attribute, particularly for biocontrol, and in some cases, molecular techniques have assisted the placement of an important strain into species. The taxonomic position of *M. anisopliae* strain IMI 330189 used in the LUBILOSA program for locust control was confusing, as it was originally classified as *M. flavoviride* based on morphology of the conidia and some other features. Driver *et al.* (2000) used molecular techniques to revise the genus *Metarhizium* and demonstrate subspecific relationships among *M. anisopliae* and *M. flavoviride* strains, based largely on the sequence of the ITS-5.8s regions of rRNA. Sequencing of the ITS-5.8s region of *M. anisopliae* strain IMI 330189 revealed it to be a *M. anisopliae* subspecies, which they named *M. anisopliae* var. *acridum*. However, species cannot be erected on sequence data alone, and several of the subspecies "clades" identified by Driver *et al.* (2000) can only be delimited by sequence data at present.

The main drawback with molecular approaches to classification is the lack of agreement on standard techniques. This is not generally a problem for use in strain identification, but the choice of method does influence the relationships among species and genera. The use of rRNA sequences has become widespread, but as the studies of Rehner (2003) show, reliance on single region sequences may be misleading. Rehner (2003) used a multigene sequencing approach to clarify the taxonomic position of *Beauveria* species strains. This approach is a step beyond what is currently known about the molecular taxonomy of most entomopathogenic fungi and is likely to be a common approach in the future.

The contribution of molecular techniques to the development of entomopathogenic fungi has been enormous. The techniques have been used to clarify evolutionary relationships (e.g., Jensen *et al.*, 1998; Driver *et al.*, 2000) as well as allow development of theories of evolution around these, often obligate, pathogens. However, there are a number of difficulties with some current studies, such as incorrect typification and lack of holotypes (Samson, 1995). Few of the species have yet been studied in detail.

6.11.3. Biology and Pathogenesis

Survival of entomopathogenic fungi requires a delicate balance of interaction between the fungus, host, and the environment. In general, the life cycle of entomopathogenic fungi involves an infective spore stage, which germinates on the cuticle of the host, forming a germ tube that penetrates the cuticle and invades the hemocoel of the insect host (Hajek and St. Leger, 1994). The fungus then multiplies within the insect and kills it; death is due to toxin production by the fungus or multiplication to inhabit the entire insect. Under favorable environmental conditions, the fungus grows out of the cadaver, and forms conidiophores or analogous structures and sporulates. Alternatively, many species form some type of resting stage capable of surviving periods of adverse conditions before forming or releasing a type of spore. Spores need new hosts, so the fungus needs a strategy for dissemination. There are many variations and even exceptions to this generalized life cycle. However, the important point is that the environment and host are crucial to the survival and reproduction of the fungus.

6.11.3.1. Encounter with Host

The role of the host is both passive, in that it is the main nourishment for the fungus to produce reproductive structures in most cases, and active, in that the fungus needs to contact the host under suitable conditions. The role of environmental variables for terrestrial fungi is hard to overstate. For some fungi, such as conidia producing Hyphomycetes, germination, penetration, and sporulation can all require high humidity. Temperature is usually limiting, with infection occurring within a certain range. Hyphomycetes generally have temperature optima between 20 and 30 °C, while Entomophthorales, require 15–25 °C, suggesting that Entomophthorales are the more temperate fungi. Individual isolates within a single species can vary in temperature

optima. Similar temperatures are usually required for germination, sporulation, and discharge.

Some species are more dependent on high humidity than others. *Lecanicillium lecanii*, a hyphomycete, can require up to 16 h of 100% relative humidity (RH) at the leaf level in order to cause high mortality rates in whiteflies (Milner and Lutton, 1986). In other cases, humidity requirements appear lower, such as *Entomophthora muscae* infecting Diptera (Mullens and Rodriguez, 1985). However, it is often difficult to measure the actual humidity that the fungus is exposed to, due to microclimatic variability. Leaf transpiration may create adequate humidity at the leaf surface, which is difficult to measure (Vidal *et al*., 2003), allowing infection to occur even at low ambient humidity conditions (Fargues *et al*., 2003). Dew period can also be more important than ongoing high humidity (e.g., Milner and Lutton, 1986). Entomophthorales are often very dependent on >95% RH for sporulation and conidial germination, despite the occasional exception such as *E. muscae* (see Section 6.11.3.4).

Soil presents a range of abiotic and biotic factors that influences the life cycle of fungi. Moisture can be more constant and higher in the soil environment than on plants, but spore movement is more restricted than in the epigeal environments. The level of organic matter and pH can be important in the eventual infections levels, as well as in survival of propagules. In this way, soil is more similar to aquatic environments. In soil, other microbes and small invertebrates (e.g., springtails; Broza *et al*., 2001) can consume fungi directly, compete saprophytically, or be directly or indirectly antagonistic to the entomopathogens. It is not surprising that fungi are more effective in sterilized than unsterilized soils in the laboratory (e.g., Inglis *et al*., 1998). Microbes such as bacteria resident within insect hosts can compete within infected cadavers and, if the fungus does not have mechanisms to exclude these microbes, the fungus will be unable to colonize the cadaver sufficiently to allow sporulation.

Other abiotic conditions that influence encounters between the host and pathogens are wind and sunlight. Wind can assist spore dispersal but will also decrease humidity and remove free water in some cases. Sunlight and UV can be detrimental to the persistence of infectivity of most fungi. For example, Inglis *et al*. (1997a) found that the poor efficacy of *B. bassiana* against rangeland grasshoppers in a Canadian field trial was a result of conditions of temperature and light exposure, as the fungus was more virulent in greenhouses and shaded cages than in the open rangeland. However, there is significant variation in susceptibility to UV among different species and strains within species (Fargues *et al*., 1996).

Aquatic species of fungi are not subject to the influence of humidity, as they are generally infective only in water. Periodic drying of habitats is a constant problem, but most aquatic entomopathogens have strategies to survive drying. Similar to terrestrial environments, temperature can be a limiting factor. Chemical factors, such as salinity or organic pollutants in the water are also important.

Meteorological data do not always correlate with the occurrence of epizootics, partly because microclimatic conditions are not the same as macroclimatic conditions. There are still many gaps in our knowledge of the interaction between the pathogen, host, and environment, as demonstrated by the lack of prediction of epizootic and disease occurrence in most fungi.

6.11.3.2. Specificity and Host Range

Specificity of entomopathogenic fungi varies widely between genera, within genera, and even among strains of a species. The so-called muscardine fungi, *B. bassiana* and *M. anisopliae*, have well-documented broad host ranges, which include hundreds of insect species from several classes. Veen (1968) listed 204 insects naturally infected by *M. anisopliae*. In the intervening years, many more hosts have been reported and include species of Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Homoptera. Coleoptera are particularly prevalent hosts for *M. anisopliae* with over 70 scarab species included in the list of Veen (1968). Broad host lists, such as for *M. anisopliae*, are often misleading, as individual strains of entomopathogenic fungi can vary in their specificity. In addition, definition and delimitation of species can be arbitrary in these often asexual fungi, as demonstrated by recent molecular analyses of *Metarhizium* (i.e., Driver *et al*., 2000) and *Beauveria* (Rehner, 2003; see Section 6.11.2.6). For biocontrol applications, the isolate is more important than the species as a unit, partly due to variation in virulence to hosts among strains. There are implications in host range for determining the nontarget safety of fungal based bioinsecticides (Goettel *et al*., 1990a; Vestergaard *et al*., 2003). Based on published host ranges of the species *B. bassiana* or *M. anisopliae* it would be very difficult to convince regulators of their environmental safety, but it is commonly recognized that strains of *B. bassiana* and *M. anisopliae* are more restricted in their hosts than the species. A single strain of any entomopathogenic fungus rarely attacks both beneficial and pest species.

Some entomopathogenic fungi have been recorded from as limited a host range as a single species of insect. In the Entomophthorales, several species are known from only one host, but it is difficult to know if this is a true reflection of host range or the result of limited study. Few species have been cultured and even fewer have been bioassayed against a number of insects to determine potential host range. *Zoophthora radicans* (Entomophthorales) has been described from over 80 insect species from Diptera, Coleoptera, Lepidoptera, and Homoptera, but there is clear indication that strains are restricted, generally, to some species in a single insect class (Milner and Mahon, 1985). The individual strains are generally more pathogenic to the insects more closely related to the original host, but there are many exceptions.

Other groups of entomopathogenic fungi are limited in host range. *Coelomomyces opifexi*, for example, is known from only two mosquito species and a copepod intermediator (Glare and Milner, 1991). The many species of *Cordyceps* represent both broad and narrow host range species (e.g., Kobayasi, 1941), but here, again, information is limited.

In many cases, entomopathogenic fungi will kill certain species only in special situations. *M. anisopliae* is rarely recorded naturally as a pathogen of mosquitoes, but in the laboratory, many strains are highly pathogenic to mosquito larvae (Daoust and Roberts, 1982), indicating that other factors than susceptibility are important in occurrence of disease in nature. In some cases, the fungus can only kill weakened or stressed hosts. Behavioral avoidance can also lead to nonsusceptibility in the field, when laboratory bioassays indicate susceptibility. For example, *Aspergillus flavus* can be isolated from many wasp (*Vespula* spp.) nests in New Zealand and is highly pathogenic in the laboratory to *Vespula* (Glare *et al*., 1996), but the hygienic behavior of wasps is such that infections in healthy nests are not seen. *Zoophthora phalloides* is a pathogen of aphids, but shows distinct preferences in the field for some species. Remaudiére *et al*. (1981) recorded 72% of *Myzus ascalonicus* infected with *Z. phalloides* on a single bush, but only 6% of *Myzus ornatus* on the same bush. Conversely, *Pandora neoaphidis* on the same bush infected 71% of *M. ornatus* and only 1% of *M. ascalonicus*. The difference in susceptibility is likely due in part to the mode of infection by the two fungal species, as much as to possible differences in resistance among the aphid species. *Zoophthora phalloides* infects via the sessile capilliconidia, making it more effective against mobile hosts, while *P. neoaphidis* uses forcibly discharged primary and secondary conidia, which are more effective when they land directly on a host. Unsurprisingly, *M. ascalonicus* is a more mobile aphid than *M. ornatus*.

These results highlight the difference between laboratory and field susceptibility. If results from laboratory assays are to be used to predict activity in the field, pertinent environmental and exposure parameters must be incorporated as much as possible in the bioassay design (Butt and Goettel, 2000). For instance, in the laboratory, most bioassays do not allow for avoidance of the pathogen through biological, ecological, or behavioral methods. In the field, an insect may never come in contact with sufficient inoculum to succumb to infection. An example is caterpillars and *E. maimaiga*, a pathogen of gypsy moth. Hajek *et al*. (1996) examined the field incidence of caterpillars infected with *E. maimaiga* where high gypsy moth infections were occurring. They found only two individual caterpillars (1 of 318 *Malacosoma disstria* and 1 of 96 *Catocala ilia*) of a total of 1511 larvae from 52 species belonging to seven lepidopteran families infected with *Entomophaga maimaiga*. In the laboratory, more species were found to be susceptible and high percentages of the few species found infected in the field were infected in the laboratory. Despite *E. maimaiga* being a pathogen of the Lymantriidae in the laboratory, species other than gypsy moth are unlikely to be infected in the field unless they spend periods of time near the leaf litter where the fungus is sporulating (Hajek *et al*., 2000). Similarly, differential infections of mosquito hosts can be linked to position in the water profile, as bottom feeding species are more likely to come in contact with settling inoculum than surface feeders (Sweeney, 1981).

In most entomopathogenic fungi, there is differential virulence towards life stages of insects that are susceptible. Unlike bacterial, viral, and protozoan pathogens, most fungi directly penetrate the cuticle and do not need to be ingested. Therefore, entomopathogenic fungi have the potential to be active against nonfeeding stages such as pupae. Aquatic species, such as *Lagenidium* and *Coelomomyces*, rarely attack adult stages, although they can persist in them (see Section 6.11.3.4). Hyphomycetes such as *Metarhizium* and *Beauveria* often infect both adult and larval stages. Keller and others have used the ability of *Beauveria brongniartii* to infect both larval and adult stages of the European cockchafer, *Melolontha melolontha*, to develop biopesticide strategies based on spraying adults in order to transfer inoculum to the larvae in the soil (Keller *et al*., 1989). Most fungi, even if they attack all

life stages, show variability in virulence between the stages. Even within the larval stage, there is usually variability in susceptibility. For example, mosquito larvae were more susceptible to *Culicinomyces clavisporus* at earlier instars than later ones (Panter and Russell, 1984), while the scarab *Costelytra zealandica* is more resistant to *M. anisopliae* at the second compared to the third instar (Glare, 1994), indicating there is no general rule regarding which developmental stage will be more susceptible.

The development of resistance to entomopathogenic fungi in insect populations has rarely been documented. When the pea aphid, *Acyrthosiphon pisum*, was introduced to Australia, it became resistant to the pathogen, *P. neoaphidis*. Soon after the development of resistance, the fungus developed strains that were fully pathogenic for pea aphid, indicating a rapid genetic capacity to overcome resistance (Milner, 1982, 1985).

Genotyping using various molecular methods is becoming increasingly common in the identification of entomopathogenic fungi (see Section 6.11.2.6). However, the methods used to date rarely demonstrate a link between genotype and host preference where sufficient strains have been examined to give a meaningful result. There are exceptions, such as *B. bassiana* pathogenic to *Ostrinia nubilalis* and *Sitona* weevils, where some homogeneity among isolates from these hosts was found (Viaud *et al.*, 1996; Maurer *et al.*, 1997). In most cases, the molecular methods do not target genetic regions involved in specificity, but are aimed at random, nonspecific regions, or regions involved in basic cellular processes, such as ribosomal and mitochondrial regions. This suggests host specificity evolves relatively rapidly. Targeting of genes involved directly in virulence related events may lead to better matching between molecular identification and host range.

6.11.3.2.1. Vertebrate safety Fungi in general are well known allergens that produce toxins and are capable of infecting vertebrates. Fungal epizootics may be responsible for drastic reductions in invertebrate populations, populations that may be an important food source for some vertebrates. Consequently, the actions of entomopathogenic fungi may compromise vertebrate health and well-being in several ways.

Fungi are an important source of human allergens. However, entomopathogenic fungi are not typical human allergens (Gumowski *et al.*, 1991), indicating that exposure to fungi generated from natural epizootics does not generally induce allergenic reactions. Allergy problems have been documented in workers frequently exposed to certain entomopathogenic fungi such as *B. bassiana* (Saik *et al.*, 1990) and crude extracts from *M. anisopliae* contain potent allergens (Ward *et al.*, 1998). However, there were no reports of human hypersensitivity during mass production of *B. bassiana* by the Mycotech Corporation involving more than 30 000 person-hours of exposure (Goettel and Jaronski, 1997). Screening for allergies in 145 Tate and Lyle (Reading, UK) employees exposed to *L. lecanii* during its manufacture and of 31 employees involved in its field testing in greenhouses, concluded that the fungus is not significantly allergenic, even to a worker population that was more highly allergic to other allergens than average (Eaton *et al.*, 1986). Since all fungi are potentially allergenic, it is unreasonable and impractical to expect a total absence of allergic potential in microbial insecticides based on fungal pathogens. However, certainly every effort should be made to avoid human exposure during manufacture and application.

For the most part, entomopathogenic fungi are not virulent towards vertebrates (Saik *et al.*, 1990; Siegel and Shadduck, 1990). However, there are some exceptions. Certain species of entomopathogens such as *Conidiobolus coronatus* or *A. flavus* are well-known pathogens of vertebrates and, because of this, they are generally not considered for development as commercial biopesticides. An exception may be *Paecilomyces lilacinus*. Although as a species, it is known to cause human and other vertebrate infections and is an important emerging nosocomial fungal pathogen (Goettel *et al.*, 2001), the strain P 251, isolated in the Philippines, has been successfully developed as a safe microbial nematicide by the Australian Technology Innovation Corporation (Copping, 2001). This is another example of how fungal pathogens must be judged at the strain level, and not at the species level.

Although there have been no reports of infections in vertebrates directly resulting from the use of fungal pathogens as microbial control agents, there are several documented cases of "chance" occurrences. For instance, several entomopathogenic Hyphomycetes have been recovered from captive cold-blooded vertebrates (Austwick, 1983; Gonzalez Cabo *et al.*, 1995), although it is generally believed that these animals have been severely stressed by chilling. Laboratory challenge assays demonstrated pathogenicity of *B. bassiana* to silverside fish embryos and fry (Genthner and Middaugh, 1992), and of *M. anisopliae* to grass shrimp embryos (Genthner *et al.*, 1997) and silverside fish embryos and fry (Genthner and Middaugh, 1995). There have been several cases of human infection by entomopathogenic fungi reported for species presently in use as microbial control agents, with one fatality in an

immunoincompetent child, but none of these have been implicated from the commercial use of specific strains (Goettel *et al.*, 2001; Vestergaard *et al.*, 2003). The most recently reported case concerns the deep tissue infection of an immunosuppressed female by a *Beauveria* sp. (Henke *et al.*, 2002). The isolate in question was unable to grow *in vitro* at 37 °C, which indicates that the inability to grow at this temperature *in vitro* is insufficient to rule out possible infection and growth in mammalian tissues. Interestingly, this isolate was more virulent against a test insect, the Colorado potato beetle, as compared to a well-known entomopathogenic strain of *B. bassiana*.

Entomopathogenic fungi produce an array of metabolites (see Section 6.11.3.3.1 below), many of which are toxic or carcinogenic to vertebrates (Strasser *et al.*, 2000; Vey *et al.*, 2001). However, any hazard to vertebrates would require exposure to significant levels of these metabolites. Current evidence suggests that, unless infected insects are actively pursued and ingested (e.g., insect cadavers infected with *B. bassiana* are widely consumed in China for medicinal purposes), the risk to vertebrates should be minimal. To date, no detrimental effects have been noted in vertebrates fed insect cadavers. For instance, ring-necked pheasant chicks given feed coated with *M. anisopliae* var. *acridum* (as *M. flavoviride*) spores or infected grasshoppers for 5 days showed no significant changes in weight, growth rate, behavior, or mortality rate as compared to controls fed noninoculated food or noninfected grasshoppers (Smits *et al.*, 1999). Strasser *et al.* (2000) speculate that entomopathogenic fungi should pose no obvious risk to humans because toxin quantities produced *in vivo* are usually far lower than those produced *in vitro* and, therefore, toxin levels should never rise to harmful levels in the environment.

Fungal epizootics may deplete an important food source for certain vertebrates. However, at the same time, depletion of a pest is the desired effect in the use of an entomopathogenic fungus in microbial control. The possible effects on vertebrates due to the depletion of a food resource as a result of fungal epizootics, either natural or induced, are poorly documented. However, for the most part, depletion of the target host is the desired effect and can be more or less regulated in an integrated pest management program where fungi are used in large amounts (also called inundation). However, special attention must be paid if exotic fungi are to be used in the classical sense of biocontrol (Goettel and Hajek, 2001).

Stringent regulatory requirements address vertebrate safety issues. Before any entomopathogenic fungus can be registered as a microbial control agent, it must first undergo stringent testing for potential harmful effects on vertebrates, including mammals (see Laird *et al.*, 1990; Siegel, 1997). In principle, no harmful agent should ever make it onto the market. However, with the increasing development of immunosuppressive therapy in medicine, the increase in iatrogenic factors and nosocomial diseases, and the advent of new infectious diseases such as AIDS, the list of opportunistic fungi causing deep mycoses is increasing (Chabasse, 1994), and this includes some entomopathogens. Entomopathogens must be carefully scrutinized, but at the same time, regulations must not become unjustly stringent so as to significantly deter registration of safe and useful products.

6.11.3.3. Mode of Action and Host Reactions

Entomopathogenic fungi kill the host by a variety of means, from starvation through multiplication in the host, to production of toxins. Given the pathogenic habit, it is hardly surprising that entomopathogenic fungi produce extracellular enzymes and toxins. Entomopathogenic fungi produce a variety of chitinases and proteases, which aid penetration of the host physical defenses.

The main barrier to infection of insects by entomopathogenic fungi is the cuticle. For the majority of fungi, the route of infection is directly through the cuticle, not after ingestion. The fungus, therefore, needs enzymatic and/or physical means to penetrate this thick, multilayered shell. The process of infection starts with the spore contacting the cuticle of a host. In many cases, the conidia are adhesive to the cuticle, or secrete adhesive mucus as the conidium swells during pregermination (Hajek and St. Leger, 1994). Capilliconidia, the infective sessile spores of some *Zoophthora*, have a drop of adhesive on the end of the spore to assist attachment to passing insects (Glare *et al.*, 1985). Zoospores of *Lagenidium* encyst on the host surface, ensuring attachment (Kerwin and Petersen, 1997).

The exact mechanisms used by each fungus for penetration of the host cuticle may differ, but there are general processes and structures involved. *Metarhizium anisopliae*, for example, develops a number of specific structures to assist the anchoring and penetration of germ tubes, which arise from conidia (St. Leger, 1993). Appressoria and infection pegs assist the penetration of germ tubes. Once the penetrative tube has passed through the layers of the cuticle and epidermis, the fungus can proliferate in the hemocoel. For some hyphomycete fungi, such as *M. anisopliae*, this is initially performed by blastospores, while for some Entomophthorales,

proliferation can be by wall-less protoplasts (e.g., Butt *et al*., 1981; Bidochka and Hajek, 1998).

Injection of entomopathogenic fungi directly into the hemolymph can demonstrate that the cuticle is a major barrier to infection. However, the insect also has cellular and humoral methods of defense against fungal invasion. For example, when Bidochka and Hajek (1998) injected *Entomophaga aulicae* into gypsy moth larvae, a nonpermissive host, there was no evidence of restricted fungal growth, fungus specific induction of plasma proteins, or hemoctye encapsulation, but higher levels of phenoloxidase and prophenoloxidase-activating trypsin activity were found up to 96 h postchallenge. Bidochka and Hajek (1998) suggested that surface components of protoplasts, such as glycoproteins, may be implicated in activation of zymogenic trypsins in the insect, which in turn activate the prophenoloxidase cascade as a nonpermissive response.

6.11.3.3.1. Toxin production Some entomopathogenic fungi produce toxins, some of which are insecticidal and may assist in pathogenesis. Others produce metabolites that are antimicrobial, either to restrict saprophytic competition or protect against antagonistic microorganisms. Toxic metabolites from entomopathogenic fungi have been reviewed extensively by Roberts (1981) and, more recently, by Vey *et al*. (2001). The safety aspects of fungal metabolites have become an important issue, with implications for risk assessments, and these were reviewed by Strasser *et al*. (2000; see also Section 6.11.3.2.1).

Among the fungal metabolites that assist pathogenicity are the destruxins of *Metarhizium* spp. First described in 1961 (Kodaira, 1961), these cyclodepsipeptides are toxic to a number of insects. Susceptibility varies considerably, with a 30-fold difference between silkworm larvae and *Galleria* (Roberts, 1981). While the basic structure of destruxins is based on five amino acids and α-hydroxy acid, a number of isomers and congeners have been described (Vey *et al*., 2001). Destruxins have also been described from *Aschersonia* sp. (Krassnoff and Gibson, 1996) and from some plant pathogenic fungi. As well as toxicity at high doses, some destruxins reduce growth and reproduction (e.g., Brousseau *et al*., 1996). There is possibly a relationship between the chemical structure of the various destruxins and activity, with some forms more active against certain insect species (Vey *et al*., 2001). Destruxins can also be repellent or act as an antifeedant (Robert and Riba, 1989; Amiri *et al*., 1999; Thomsen and Eilenberg, 2000). Production of destruxins by some species and strains has been linked to increased host range, suggesting a role in pathogenic determination (Amiri-Besheli *et al*., 2000). Synthetic analogs to destruxins have been tested against lepidopteran insects and also proved toxic, and thus are potentially useful as novel pesticides (Thomsen and Eilenberg, 2000).

Some strains of *Beauveria* spp. produce beauvericin, a depsipeptide metabolite, which has shown toxicity to a number of invertebrates (Hamill *et al*., 1969; Roberts, 1981). Beauvericin has also been isolated from *Paecilomyces fumosoroseus* mycelium and the plant pathogenic fungus *Fusarium* spp. Activity has been classified as moderate against insects (Vey *et al*., 2001) and is not effective against all insects (Champlin and Grula, 1979). *Beauveria bassiana* also produces beauverolides, isarolides, and bassianolides, all cyclotetradepsipeptides, the former of which has been shown to be toxic to cockroaches (Frappier *et al*., 1975; Suzuki *et al*., 1977). Bassianolides have been shown to have activity against silkworms (Kanaoka *et al*., 1978). A further two nonpeptide toxins, bassianin and tenellin, have been isolated from *Beauveria* spp. and shown to inhibit the erythrocyte membrane ATPases (Jeffs and Khachatourians, 1997), but little else is known.

Linear peptidic efrapeptins are produced by *Tolypocladium* species and have some insecticidal and miticidal effects (Matha *et al*., 1988; Krasnoff *et al*., 1991). The species of *Tolypocladium* produce efrapeptins in varying amounts (Bandani *et al*., 2000). Bandani and Butt (1999) reported antifeedant and growth inhibitory properties.

Hirsutellin A is produced by *Hirsutella thompsonii* and is not proteolytic, but is toxic to a range of insects including *Galleria mellonella*, the mosquito, *Aedes aegypti* (Mazet and Vey, 1995), and mites (Omoto and McCoy, 1998). It is produced during liquid fermentation, and the sequence and amino acid composition is unlike any other known protein (Mazet and Vey, 1995). Other fungal toxins known to have some insecticidal properties include *Aspergillus* spp. Several toxins have been isolated from cultures of *Aspergillus*, such as kojic acid (Beard and Walton, 1969) and some mosquitocidal toxins (Toscano and Reeves, 1973). The production of aflatoxins by some strains of *Aspergillus* restricts interest in the group as insect pathogens, as these compounds have carcinogenic and tumor producing properties as well as some insecticidal properties.

It has not been demonstrated whether all entomopathogenic fungi produce toxins in the disease process and, indeed, if toxins are required for virulence. Quesada-Moraga and Vey (2003) demonstrated that *B. bassiana* can be pathogenic to the

locust regardless of toxin production. Furthermore, *in vivo* passage through a host or repeated subculture on artificial medium had a variable effect on toxin production; virulence and toxicogenic activity of one isolate was dependent on the mycological media that the inoculum was produced on, whereas virulence and toxicogenic activity of another isolate was greatly increased after two passages through the host.

In some cases, toxins are suspected, but not conclusively demonstrated. Some of the lower fungi, such as *Coelomycidium, Coelomomyces*, and the Entomophthorales, may possess only weak toxins, if any at all. It is more likely that they overcome their hosts by utilizing the nutrients and invading vital tissues (Roberts, 1981). Culture filtrates from entomophthoralean fungi injected into greater wax moth larvae (*Galleria*) resulted in blackening similar to that found in fully infected larvae, suggesting that toxins were active, but none were identified (Roberts, 1981). A short-lived cell lytic factor is thought to be responsible for death in lepidopteran hosts infected with *E. aulicae* (Milne *et al*., 1994).

Antibiotics are produced by entomopathogenic fungi in order to exclude saprophytes and resident microbes that compete for nutrients in the cadavers. Oosporein, a red-colored dibenzoquinone, is produced by strains of *Beauveria* spp. and has antiviral and antibacterial properties. Oosporein was found to inhibit the herpes simplex virus-1 DNA polymerase (Terry *et al*., 1992) and is active against Gram-positive, but not Gram-negative, bacteria (references in Vey *et al*., 2001). Beauvericin has shown antibiotic activity against bacteria (Ovchinnikov *et al*., 1971), and destruxin E has antivirus activity against nucleopolyhedrovirus (Quiot *et al*., 1980). The antibiotic phomalactone has been described from *H. thompsonii* var. *synnematos* (Krasnoff and Gupta, 1994) and was inhibitory to the entomopathogenic fungi *Beauveria, Tolypocladium*, and *Metarhizium*. Members within the *Cordyceps* also produce a number of metabolites that may be weak toxins or antibiotics. *Cordyceps* infected caterpillars are used as a traditional medicine in parts of Asia, and this may be partly based on the production of cordycepin, a weak antibiotic, by *Cordyceps* Zabra *et al*. (1996) reported that metabolites from *Pandora neoaphidis* had antibacterial activity.

6.11.3.3.2. Behavioral responses There have been relatively few detailed studies on the behavioral responses by hosts as a result of infection by entomopathogenic fungi. In the early stages of infection, in many cases, there are no noticeable symptoms; however, several days prior to death, symptoms become evident and include reduced feeding, activity, and coordination. Other responses include increased feeding, behavioral fever, altered mating or oviposition preferences, and positive or negative photo- or geotropism. Most responses are seemingly adaptations that favor either the host or the pathogen, while others may be the result of depletion of the host's nutritional reserves and the process of dying.

Reduced feeding has been reported in several insects. Examples include grasshoppers and locusts infected with *M. anisopliae* var. *acridum* (Prior *et al*., 1992; Thomas *et al*., 1997; Arthurs and Thomas, 2000), gypsy moth larvae infected with *E. maimaiga* (Hajek, 1989), *Plutella xylostella* larvae infected with *Z. radicans* (Furlong *et al*., 1997), and larvae of the Colorado potato beetle infected with *B. bassiana* (Fargues *et al*., 1994). In contrast, no change in consumption of food has been reported in some insects, such as *Plathypena scabra* (Lepidoptera, Noctuidae) infected with *Nomuraea rileyi* (Thorvilson *et al*., 1985) and *Cerotoma arcuata* (Coleoptera; Chrysomelidae) infected with *B. bassiana* (Lord *et al*., 1987). Increased feeding has been reported in *Lygus hesperus* (Noma and Strickler, 2000) and Colorado potato beetles infected with *B. bassiana* (Fargues *et al*., 1994). With the Colorado potato beetle, however, phagostimulation occurred only within the first 24 h after inoculation. Thereafter, there was no significant effect on consumption until day 2, and after that, consumption decreased significantly, resulting in an overall reduction in food consumption.

Intuitively, one could surmise that increased feeding during early stages of infection might favor either the host or pathogen. The pathogen might be favored if such increased feeding increases resources provided to it, although spore production of *E. maimaiga* in starved postinoculated gypsy moth larvae equaled production on fed larvae, suggesting, at least for this system, that larval feeding during the period of pathogen incubation within the host is not necessary for fungal development (Hajek, 1989). However, it may favor the host if such feeding provides more resources to fight off the pathogen. Most probably, decreased feeding just prior to death is a result of an overall shutting down of metabolic functions as the host nears death.

Some insects respond to fungal infection by altering their thermoregulatory behavior and achieving a higher than normal body temperature by basking in the sun or orienting on warmer surfaces. This is called "behavioral fever" and has been shown to either significantly slow the progress of infection or at times even eliminate the disease. This

phemonenon has been observed in fungal infections of *E. muscae* in flies (Watson *et al.*, 1993; Kalsbeek *et al.*, 2001a) and of *B. bassiana* and *M. anisopliae* var. *acridum* in grasshoppers and locusts (Inglis *et al.*, 1996b, 1997a; Blanford *et al.*, 1998; Blanford and Thomas, 2001). Clearly, the higher temperatures achieved during thermoregulation and/or behavioral fever exceed the pathogen's thermal threshold and are consequently detrimental to its growth and proliferation (Carruthers *et al.*,1992; Inglis *et al.*, 1996a, 1997b; Ouedraogo *et al.*, 2003). However, the ability to thermoregulate is possible only when environmental conditions are favorable and, consequently, behavioral fever is often not possible or is interrupted. For instance, a higher death rate of caged *B. bassiana* inoculated grasshoppers occurred in shaded than in nonshaded areas (Inglis *et al.*, 1997a). Even though some insects such as acridids infected by *M. anisopliae* var. *acridum* cannot escape infection, behavioral fever acts to prolong survival; only infected desert locusts that were allowed to "fever" were capable of surviving to adulthood, mating, and producing viable offspring (Elliot *et al.*, 2002). In addition to slowing the growth of fungal propagules within the host, elevated temperatures may increase the host immunity by maintaining high hemocyte population levels (Ouedraogo *et al.*, 2003) and inducing hemolymphal proteins (Ouedraogo *et al.*, 2002).

Insects infected with an entomopathogenic fungus may also alter their behavior during mating and oviposition. Housefly males preferably seek out *E. muscae*-killed females in their attempts to copulate (Møller, 1993). These males may themselves become infected or they may transmit conidia to uninfected females during copulation (Watson *et al.*, 1993). This behavior favors the dissemination of the fungus. The response to and production of sex pheromones by *Z. radicans* infected male and female *P. xylostella* moths is inhibited, thereby disrupting mating (Reddy *et al.*, 1998). Female carrot flies infected with *E. schizophorae* (as *E. muscae)* were unable to recognize their host plants and oviposited their eggs haphazardly instead of their normal deposition near the food plants (Eilenberg, 1987). It was concluded that, even though infection did not alter fecundity or fertility, *E. muscae* infected flies do not contribute to the development of the carrot fly population, as their eggs have no chance of survival on a nonhost plant. It is unclear whether this behavior provides any benefit to either the host or the pathogen.

Fungal infections are often responsible for inducing aberrant behaviors at or near host death. The result of such behaviors often are of benefit to the pathogen. The most common of these is to cause the infected host to move to elevated positions just prior to death. This is most common in insects succumbing to entomophthoralean infections. For instance, just prior to death, grasshoppers infected with *Entomophaga grylli* climb to the top of the canopy where they die firmly grasping onto the plant substrate by their first two pairs of legs (Carruthers *et al.*, 1997). Flies infected with *E. muscae* exhibit highly stereotyped behavior, which is characterized by four events: last locomotory movement to an elevated position, the last extension of the proboscis to the substrate, the start of upward wing movement, and the end of upward wing movement (Krasnoff *et al.*, 1995). Consequently, the flies die in elevated positions, with the proboscis extended and attached to the substrate, the abdomen angled away from the substrate, and the wings raised above the thorax. Since conidia are forcibly discharged by entomophthoralean fungi, this behavior acts to help distribute the spores over a wider area, allowing the spores to shower on susceptible hosts below. **Figure 16** shows an example of disease



Figure 16 Field situation showing the transmission of *Entomophthora* sp. (a) An adult cadaver of *Rhagonycha fulva* (Coleoptera) killed by *Entomophthora* sp. hangs from a leaf, attached by its mandibles. (b) An uninfected individual is attracted and is likely to get inoculated by physical contact with the conidia present on the cadaver and the leaf. (Photo courtesy of J. Eilenberg.)

transmission between cantharid hosts due to the exposed position of the fungus killed specimen.

6.11.3.4. Spore Production on Host

Spores are the main source of reproduction in entomopathogenic fungi. Among the groups, a number of strategies have been adopted to maximize the chances of finding new hosts to infect, and many different types of spores and sporulation structures have evolved to adapt to different habitats. Host death is a necessary prerequisite for sporulation in most, but not all, entomopathogenic fungi. The entomophthoralean fungus *Strongwellsea castrans*, for example, sporulates from one or two abdominal holes in still living flies (Eilenberg *et al.*, 2000a). However, in the majority of cases, spores are produced after the host has died and the fungus has fully colonized the cadaver.

In the asexual Ascomycetes (largely still classified as Hyphomycetes), production of robust primary conidia in large quantities is the main strategy for reproduction. Conidial production is dependent upon the size of the cadaver and nutritional factors, as well as species and strain of the fungus. In general, infections of a preferred host will generate 10^7–10^9 conidia/cadaver for terrestrial hosts (e.g., Glare, 1987; Luz and Fargues, 1998). For the fungus *N. rileyi* infecting *Helicoverpa armigera* and *H. punctigera* caterpillars, there was an approximately five- to tenfold increase in the number of spores produced in cadavers between 2nd, 3rd, and 5–6th instars (Glare, 1987). Under similar environmental conditions, conidial numbers appear to be mainly dependent upon the surface area of the host (Kish and Allen, 1976).

Most species from the Entomophthorales can forcibly discharge the primary spores, ejecting them away from the cadaver, and hopefully towards new hosts. Furthermore, many species then produce secondary conidia from the primary conidia, the spores of which are also forcibly discharged, and in some cases, capilliconidia are produced, which are detached from the capilli. Capilliconidia are sessile spores formed on top of a long capillary tube and represent a different strategy for infecting hosts, where new hosts encounter the spore when moving about. In some species, capilliconidia are the primary infective spore, e.g., *Z. phalloides* (Glare *et al.*, 1985) and *Neozygites floridana* (Nemoto and Aoki, 1975). Capilliconidia of some species can survive for days at reduced humidities (Uziel and Kenneth, 1991). **Figure 17** demonstrates how conidia may germinate and produce conidia of higher orders in the species *Entomophaga ptychopterae*.

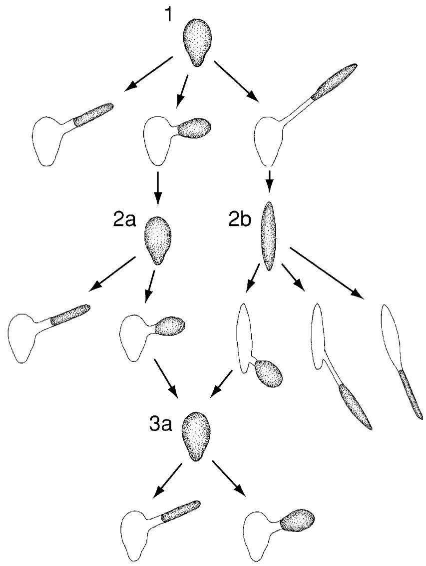


Figure 17 Conidia formation and germination in *Entomophaga* (formerly *Eryniopsis) ptychopterae*. A discharged primary conidium (1) can germinate with a germ tube or produce a secondary conidium of two different types: type 2a, which is similar in shape to the primary conidium or type 2b, which is narrow, ellipsoid, elongated, and produced on long conidiophores. Each type of secondary conidia can germinate with a germ tube or produce a tertiary conidium similar to the primary one (3a). This conidium can germinate with a germ tube or produce a quarternary conidium similar to the primary conidium. In addition, a type 2b secondary conidium can produce a tertiary conidium similar to a secondary conidium type 2b.

Production of primary and secondary spores by entomophthoralean fungi is generally very dependent on environmental conditions, as high humidity (even free water in some cases) is required for spore production (e.g., Steinkraus and Slaymaker, 1994). *Zoophthora phalloides* will only sporulate on aphid hosts at 100% RH and peak spore discharge takes place between 10 and 20°C (Glare *et al.*, 1986). This species produces up to 30 000 conidia per aphid cadaver. In the gypsy moth pathogen *E. maimaiga*, primary conidial production can be as high as 2.6×10^6 conidia per 5th instar cadaver (Shimazu and Soper, 1986). Not all entomophthoralean fungi depend on conditions of high humidity; for example, *Entomophthora muscae* can sporulate at just 50% RH (Kramer, 1980).

Inoculum density can influence eventual spore production on the cadavers. The aquatic *Culicinomyces clavisporus* produced less spores when the host was inoculated with higher doses of the pathogen (Cooper and Sweeney, 1986), and this is often the case for entomophthoralean fungi. This may be due to lack of complete colonization of the host by the fungus, or death due to the release of toxins rather than infection and multiplication.

Entomopathogenic fungi active in aquatic environments usually produce fewer spores than those infecting terrestrial insects. The mosquito pathogen *C. clavisporus* produces around 10^4 conidia/cadaver (Cooper and Sweeney, 1986). *Culicinomyces* produces nonmotile, true conidia, as do other Hyphomycetes. Other aquatic insect pathogenic fungi belong to fungal groups that can produce both motile and nonmotile spores. The oomycete *L. giganteum* produces both motile, biflagellate zoospores and sexually produced oospores (Kerwin and Petersen, 1997). The zoospores are released from both zoosporangial vesicles and oospores. Up to 20 000 sporangia can develop in a mosquito larval host, each releasing zoospores (Couch and Romney, 1973). Oospore production has been demonstrated to require sterols (Kerwin and Washino, 1983). These resistant and long-lived spores are produced within host cadavers. Another group of mosquito pathogens, *Coelomomyces* spp. (Chytridiomycetes) also has two infective stages. Resistant sporangia from mosquitoes release motile haploid zoospores, while the biflagellate diploid zygotes form within or after release from the obligate secondary host, usually a copepod (Lucarotti *et al*., 1985). The zygotes and zoospores are similar. Sporangia in infected mosquitoes can number from 100 to 15 000, releasing up to 5000 motile zoospores from each sporangium (Pillai and O'Loughlin, 1972).

Fluctuating conditions of temperature or humidity interrupt maximum spore production for most fungi. In addition, light and dark cycle influence the sporulation of some entomopathogens, but not others. At least nine entomophthoralean fungi cause host mortality in diurnal rhythms (Milner *et al*., 1984; Mullens, 1985; Tyrrell, 1987; Eilenberg, unpublished data), triggered by the onset of light (dawn) (Milner *et al*., 1984). This timing of host death seems important to allow the fungus to maximize the early morning dew period for sporulation. Conidial discharge of *Z. radicans* demonstrates circadian rhythms under alternating light and dark regimes, with more conidia discharged in the dark (Yamamoto and Aoki, 1983). *Erynia/Pandora neoaphidis* produced around 500 000 primary conidia from infected aphids under ideal conditions, but changing from high to lower humidities and back often stopped, or at least severely reduced, the number of conidia produced (Glare and Milner, 1991).

Resting stages represent a different strategy for entomopathogenic fungi. While primary spores are produced in large quantities by members of the Entomophthorales, they last for only a few days; however, resting spores can survive for months to even years. This allows them to survive periods of unfavorable conditions and cause infections the next season through production of germ conidia. Resting spores are produced within infected cadavers. Factors that influence the production of resting spores include developmental stage of the host, temperature, humidity, and inoculum density (e.g., Shimazu, 1979; Glare *et al*., 1989). With *Z. radicans*, which infects aphids, there is evidence of cytoplasmic based genetic elements involved in resting spore production, with more resting spores produced when aphids are inoculated with dual strains (Glare *et al*., 1989). The ability to produce resting spores varies with individual isolates. With *E. maimaiga* infecting gypsy moth, the primary determinant of resting spore production was larval instar, with infections of later instars producing the most resting spores (Hajek and Shimazu, 1996).

6.11.3.5. Transmission and Dispersal

Dispersal of infective propagules is a crucial component of survival, and entomopathogenic fungi use different strategies to maximize the chances of encountering new hosts. For Hyphomycetes and some other groups that produce copious numbers of spores, wind, rain, and invertebrates play a role in transmission. Generally, wind is a major aid for the distribution of conidia. The minimum airspeed required for dislodgement of *N. rileyi* was calculated to be 2.7 km h^{-1} (Garcia and Ignoffo, 1977).

Entomophthorales generally forcibly discharge the primary conidia, increasing the distribution of these short-lived conidia. Distribution can also be aided by the type of spore formed, with both the forcibly discharged and sessile capilliconidia produced by some Entomophthorales. The aphid pathogen *Neozygites fresenii* discharges about 3000 primary spores per cadaver, and Steinkraus *et al.* (1999) detected up to 90 000 primary conidia per cubic meter of air during the nighttime in a Louisiana cotton crop, indicating how successful forcible dispersal aided by air movement can be. However, for *N. fresenii*, most infections come from the capilliconidia, which are more resistant to the environment than primary conidia. With *Z. phalloides*, it has been demonstrated how aphids collect capilliconidia on their legs when walking across leaf surfaces, making this spore type ideal for infecting low-density mobile hosts (Glare *et al*., 1985).

Another mechanism to assist dispersal is through growth of hyphae out of a cadaver. The *Cordyceps* spp. and some of their anamorphs produce extensive stroma, which can grow more than 30 cm (e.g., Evans, 1982) and produce sexual and/or asexual spores at the end. Even common asexual species

such as *B. bassiana* sometimes grow several centimeters from the infected cadaver in soil. Movement of spores in the soil environment is much more limited than distribution in water or air. However, there is some movement of spores in soil, whether down the soil profile by water movement, or through distribution by invertebrates.

Insect behavior can assist dispersal. Some fungus mediated behavioral changes have been recorded, such as "summit disease" where infected grasshoppers climb to the top of plants to die (see Section 6.11.3.3.2). This may aid spore dispersal, but it has also been suggested that it is a mechanism by which the host reduces infection through increased exposure to UV. Movement of insects such as scavengers (e.g., collembolans; Dromph, 2001), natural enemies (e.g., coccinellid predators or parasitoids; Roy and Pell, 2000; see also Section 6.11.3.6), and pollinators (e.g., honeybees; Butt *et al.*, 1998) can act to disperse fungal inoculum. In some situations, insects may actually be attracted to diseased cadavers. For instance, adult *Rhagonycha fulva* have been observed to visit cohort cadavers (**Figure 16**). Use of insects to disseminate beneficial microbial control agents is further discussed by Vega *et al.* (2000).

Insect behavior can also lead to avoidance of infection. There is evidence that some insects actively avoid spores of entomopathogenic fungi. Scarab larvae were found to move away from soil containing *M. anisopliae* mycelia, but not from conidia (Villani *et al.*, 1999). The same species laid eggs preferentially in areas where mycelia were present, presumably because the mycelia respiration mimics plant root growth. Social insects are particularly adept at behavioral methods to avoid disease. For instance, termites have been shown to avoid conidia of *M. anisopliae* (Milner and Staples, 1996), and the common vespid wasps and honeybees use hygienic behavior to eject diseased individuals from the nest to reduce disease transmission (Gilliam *et al.*, 1983; Harcourt, unpublished data).

Some fungi use the host to aid dispersal directly. The fungus *S. castrans* sporulates from live hosts (**Figure 18**), thereby increasing distribution. Transmission in this species is also assisted by collembola, which can colonize the abdominal holes where the conidia are produced (Griffiths, 1985).

The production of resting spores is a major factor in transmission of many fungi, allowing the fungus to avoid unfavorable environmental conditions or lack of host. Resting spores do not synchronically germinate for most species of the Entomophthorales, increasing the temporal distribution of inoculum and the chances the fungus will eventually encounter new hosts. These spores can be long lived.

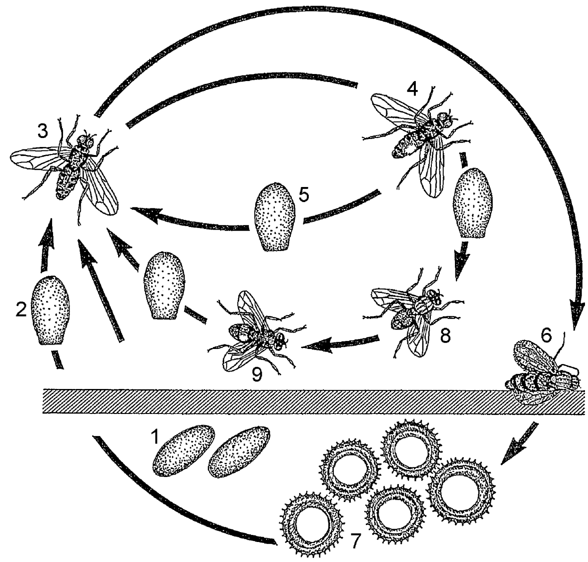


Figure 18 Life cycle of *Strongwelsea castrans*. (1) Pupae of *D. radicum* overwinter in the soil. (2) During spring, it is assumed that infective conidia of *S. castrans* are produced and discharged from resting spores in the soil. (3) Adult *D. radicum* emerge from pupae during spring and become infected by the conidia; one to two abdominal holes appear in the insect as a result of infection. (4) Conidia are forcibly discharged from the abdominal holes up to approximately 30 μm while the fly is still alive. (5) Discharged conidia, eventually after replicative conidiation, infect other adult *D. radicum*. Several successive infection cycles can take place during one season in the host population. (6) After midsummer, some *S. castrans* infected *D. radicum* develop resting spores instead of conidia. Following an incubation period, flies filled with resting spores die and drop to the soil surface. (7) Thick-walled resting spores survive in the soil layers during winter. (8) An alternative dipteran host gets infected. (9) A hole develops and primary conidia are discharged.

Persistence of *E. maimaiga* resting spores was found to be up to 6 years in forest soils (Weseloh and Andreadis, 1997), while the infection of the 17-year cicada by *Massospora* apparently depends upon resting spore germination over the full time frame (Soper *et al.*, 1976).

Transmission in many terrestrial fungi is dependent upon high humidity, with moisture required for sporulation and germination of conidia. It is not surprising that fungi have evolved to take advantage of the early morning dew through timing of host death. Timing death to occur just prior to dusk allows many entomophthoraleans to sporulate during the humidity of early morning. Despite the active discharge, conidia of Entomophthorales rarely reach above crop canopies and for *Pandora neoaphidis*, did not travel more than 1 m, even with wind assistance (Glare and Milner, 1991).

The ability to attack multiple life stages is important in disease transmission. Winged insects can be responsible for the spread of disease, especially

between populations and regions. Movement between sites for aquatic fungi, such as mosquito pathogens, is likely to be through adults, whether the adults are infected or just carrying spores. For instance, *Coelomomyces* infected adult female mosquitoes mate and take blood normally, but oviposit sporangia instead of eggs (Lucarotti, 1987). Small mammals and birds may also spread infective spores after ingesting diseased cadavers (Christensen *et al.*, 1977). Transmission between adults and larvae has been demonstrated for some Hyphomycetes, such as the beech-boring beetles of *Platypus* (Coleoptera) inoculated with *B. bassiana* (Glare *et al.*, 2002) and diamondback moth with *Z. radicans* (Pell *et al.*, 2001).

In aquatic environments, motile spores, such as the zoospores of *L. giganteum* and *Coelomomyces* spp., are able to move through the water in search of hosts. Even the resting stage, oospores and sporangia, produce zoospores, combining motility with long-term survival. Zoospores are the main dispersal stage for *L. giganteum* and are actively attracted to a chemotactic agent emitted by mosquito larvae (Domnas, 1981). *Coelomomyces* spp. zoospores may also be attracted to larvae in water (Kerwin, 1983; Federici and Lucarotti, 1986). Zoospores can travel up to 10 cm in still water (Jaronski, 1982) and many meters in natural systems, but cannot overcome physical barriers to move between discontinuous systems. This is not the only method entomopathogenic species employ to infect hosts in aquatic environments. The aquatic *Culicinomyces* infect hosts using sessile spores, which can persist in water for up to 8 days before settling (Sweeney, 1985).

Lastly, the intentional introduction of fungi as biocontrol agents can be a significant means of dispersal in some fungi, and is discussed in more detail in Section 6.11.6.

6.11.3.6. Interactions between Pathogens and Other Natural Enemies

Most insects have a variety of diverse natural enemies, which include predators, parasitoids, and numerous pathogens. Many of these compete for the same resource and, inevitably, with each other. Insects can become coinfected by two or more pathogens or by a combination of parasitoid and pathogen(s). Predators or parasitoids can prey upon pathogen infected hosts. Fungi may infect nontarget insects, including natural enemies, and natural enemies can also act to disperse fungal inoculum. The outcome of all of these interactions can be either nil, antagonistic, additive, or synergistic. For the most part, interactions between fungal entomopathogens and other natural enemies is positive (Roy and Pell, 2000).

The same insect population can be attacked by several species of fungi. Studies on aphids, like cereal aphids (*Diuraphis noxia, Rhopalosiphon padi, Sitobion avenae*, and others) and their fungal pathogens from Entomophthorales (*Conidiobolus obscurus*, *Entomophthora planchoniana, P. neoaphidis*, and others), have often documented this situation at the population level (Keller and Suter, 1980; Dedryver, 1981; Feng *et al.*, 1990). Each fungus species will contribute to the overall mortality in the aphid population, but may differ in their prevalence. For example, some studies documented that in cereal systems, *P. neoaphidis* was the most common species in 2 out of 4 years, while *E. planchoniana* was the most common in the other 2 years (Eilenberg *et al.*, unpublished data). In carrot flies, *Entomophthora schizophorae* is normally the only common fungal species, but one year in August, *Conidiobolus pseudapiculatus* was the most common species (Eilenberg, 1988; Eilenberg and Philipsen, 1988).

Different fungal species can affect different host stages in the same host population. For instance, the chrysomelid beetle, *Coleomera lanio*, is susceptible to *B. bassiana* and *M. anisopliae*, but the former infects larvae, pupae, and adults while the latter infects only pupae (Silveira *et al.*, 2002).

The same host specimen can be naturally infected by two fungi simultaneously, but it seems that this normally occurs at lower rates than would be expected from a statistical point of view. In laboratory studies, Inglis *et al.* (1999a) studied the effects of temperature on the competitive infection and colonization of grasshoppers coinoculated with *B. bassiana* and *M. anisopliae* var. *acridum*. They found that coapplication of the two fungi did not significantly affect the prevalence of mortality, but that in some instances, total fungal populations in the hemocoel of coinoculated grasshoppers were smaller than those in grasshoppers treated with each fungus alone, indicating a degree of interspecies antagonism. More *B. bassiana* than *M. anisopliae* was recovered from coinoculated grasshoppers incubated at a constant temperature of 25 °C, but as the amplitude of temperature differences increased, more *M. anisopliae* than *B. bassiana* was recovered, indicating that temperature influenced their competitiveness within the host cadaver. In contrast, using an avirulent strain of *B. bassiana* and a virulent strain of *M. anisopliae* var. *acridum*, and the desert locust as a host, Thomas *et al.* (2003) were able to demonstrate that even an avirulent pathogen can alter the virulence and reproduction of a second, highly virulent pathogen. Locusts preinoculated with the avirulent fungus prior to inoculation with

the virulent fungus exhibited a reduction in survival time and a significant decrease in sporulation of the virulent fungus compared to individuals inoculated with the virulent fungus alone.

Insect pathogenic fungi may also interact with other types of insect pathogens. Eilenberg *et al.* (2000a) documented that the fungus *S. castrans* and the microsporidium *Cystosporogenes deliaradicae* occurred commonly together in adult *Delia radicum*. In the same study, the bacterium *Bacillus thuringiensis* was also found sporulating in the abdomen in two fungus infected hosts, but serotyping and toxicity tests proved that the bacterial isolates belonged to serotypes that were avirulent to the host. As mentioned above, avirulent insect pathogens may play an important role in host–pathogen dynamics for the virulent insect pathogens. Another example of coinfection is found in the gypsy moth, *L. dispar*, which is naturally infected by the fungus *E. maimaiga* and the nuclear polyhedrosis virus LdNPV. Both pathogens are highly virulent and laboratory studies demonstrated that, depending on dosage and timing, there was sometimes an apparent synergistic interaction between the two pathogens, thereby significantly increasing overall mortality (Malakar *et al.*, 1999).

Insect pathogenic fungi can interact with parasitoids (Goettel *et al.*, 1990a). The two types of natural enemies may compete for the host's tissues and can, therefore, be regarded as antagonistic to one another. For instance, none of the parasitoids *Cotesia plutellae* and *Diadegma semiclausum* discriminated between healthy and *Zoophthora radicans* infected host *P. xylostella* larvae in oviposition experiments, although such infection always resulted in the death of the immature parasitoids (Furlong and Pell, 2000). The fungus also infects *D. semiclausum*, and can thus in several ways be regarded as potentially antagonistic in field situations. In contrast, some parasitoids are able to discriminate between healthy and infected hosts, avoiding oviposition in infected cadavers (Goettel *et al.*, 1990a). The parasitoid *Aphidius ervi* initiated fewer attacks on colonies of the aphid *Acyrtosiphon padi* that were subjected to the fungus *P. neoaphidis* on the previous day than on uninoculated colonies (Pope *et al.*, 2002). Several aspects in the interactions between the parasitoid *Aphelinus asychis*, the pathogen *P. farinosus*, and the host *D. noxia* demonstrate the potential of synergistic or additive interactions: the parasitoid discriminates fungal infected hosts thereby avoiding ovipositing in them; parasitoids emerging from fungal inoculated host populations are healthy; and there is a possibility that the fungus may be transmitted by the parasitoids (Mesquita and Lacey, 2001).

Insect predators can, of course, be infected by insect pathogenic fungi. Some insect pathogenic fungi occur in both predator and prey insects in specific ecosystems, but infection levels in the predators are usually low (Vestergaard *et al.*, 2003). It is still unknown whether the genotypes of the fungal isolates occurring in the different insect populations are identical and, furthermore, whether transmission of infection between predator and prey population is of significant importance. Predators can be antagonistic to fungal pathogens simply by eating fungus killed cadavers or parts thereof, thereby reducing subsequent fungal sporulation and spread. Roy *et al.* (1998) documented this effect in their studies of the predator *Coccinella septempunctata*, the fungus *P. neoaphidis*, and the prey and host aphid *Acyrthosiphon pisum*. The same predator could, however, vector the fungal disease to uninfected hosts and thereby enhance fungal dissemination and epizootic potential; this effect was found to be enhanced due to the increased activity of the pea aphid, *A. pisum*, to avoid the predators, at the same time resulting in a higher uptake of inoculum of *P. neoaphidis* and consequently higher infection levels (Roy *et al.*, 1998, 2001).

6.11.4. Evaluation of and Monitoring Fate

Evaluation of use of entomopathogenic fungi in biological control is an extremely important, yet complex, task. The most obvious evaluation criterion is control of the pest population. In situations where measurement of pest population changes is not difficult, the success or failure of biocontrol can be seemingly straightforward. However, even in such situations, the sublethal effects, such as reduced feeding, fecundity, or crop damage, should not be overlooked. In many situations, the actual number of pests is unimportant compared to the damage they cause or disease they vector. In these cases, evaluation depends more on reducing damage or disease than the pest populations themselves, the ultimate evaluation criterion being crop quality or yield.

With changes in regulations and concerns over safety of introduced agents, more emphasis is being placed on monitoring the fate of fungi (and other biological agents) after release. Monitoring the fate of entomopathogenic fungi in the environment can be important in ecological studies, for commercial evaluations, for measuring establishment, and for regulation and safety. In particular, there is concern

and interest in the release and monitoring of genetically modified organisms in the environment, including entomopathogenic fungi. Bidochka (2001) identified three main benefits from monitoring the fate of fungi: (1) identification of the introduced agent in a biological impact assessment, (2) economic cost benefit assessment, and (3) valuable information on fungal epidemiology.

Despite many years of research into entomopathogenic fungi, there remains an inadequate understanding of epizootiology, due in part to lack of specific methods for tracking individual isolates in the environment. The fate of released fungi, the genetic structure of fungal populations, and how to measure these components has not been well explored.

There are several possible ways in which to study persistence of artificially augmented fungal inoculum levels, including leaf and soil washes, leaf impressions, differential staining, bioassay of field collected substrates, and monitoring of sentinel insects (Goettel *et al.*, 2000). Comparisons can be made to untreated areas. When there are no longer differences in population levels between treated and untreated areas, it can be concluded that the augmented fungus has returned to background levels. The number of fungal propagules in the environment has often been used as a measure of the potential success of biocontrol applications. Propagule loadings have been measured by colony counts on semiselective media, where the fungus can be cultured, or through spore traps for fungi that discharge their spores into the air, such as the Entomophthorales. However, these methods do not distinguish between applied and background strains.

Methods that have been used to study the fate of entomopathogenic fungi at the specific strain level include phenotypic markers, vegetative compatibility, mating types and, more recently, a plethora of molecular approaches, which allow specific tracking of individual strains in most environments. When strains can be cultured, studies have used markers such as colony appearance or antibiotic resistance, but these techniques suffer from genetic instability and alterations due to environmental factors. Other nonmolecular based markers have been investigated, such as commercially available carbohydrate utilization or enzyme production strips system of API (i.e., API50CH; APIZyms), but with limited success (St. Leger *et al.*, 1986; Todorova *et al.*, 1994; Rath *et al.*, 1995). These systems can be used to distinguish groups, but are unlikely to distinguish individual isolates in most cases. Therefore their usefulness in tracking released strains is usually minimal.

Vegetative compatibility groups (VCGs) are subspecific groups within the same fungal species from which isolates can fuse hyphae to form heterokaryons and, thus, exchange genetic information (the parasexual cycle). Couteaudier and Viaud (1997) demonstrated that compatibility groups, within *Beauveria* spp. in Europe, could be used to delimit genetically incompatible groups and aid monitoring.

Many entomopathogenic fungi cannot be cultured and the lack of simple methods for isolation and specific strain characterization has made it difficult to conduct ecological studies on fungal persistence. The most important advances in monitoring the fate of entomopathogenic fungi in the environment have come from the application of molecular biology techniques. Precursors to DNA techniques were the use of allozymes to identify strains, and use of enzyme-linked immunosorbent assay (ELISA) for tracking in the environment. Application of allozyme analysis to strain detection in the environment has been attempted, although single isolate identification amongst strains from a single host has not proved possible. For example, the allozymes of *Beauveria* and *Metarhizium* spp. have a high degree of variability, indicative that the species had maintained a large effective population size for a long time (St. Leger *et al.*, 1992b). In some cases, isoenzymes have not been useful in separating applied and background strains (e.g., Trzebitzky and Lochelt, 1994) or showed little correlation with virulence (Reineke and Zebitz, 1996), but Lin and Lin (1998) found esterase patterns of *Beauveria* isolates correlated with geographical distribution and host. Allozymes have also been used with the Entomophthorales. Silvie *et al.* (1990) used allozymes to study the fate of *P. neoaphidis* released for greenhouse aphid control. Milner and Mahon (1985) used similar techniques to distinguish between an introduced Israeli strain of *Z. radicans* for aphid control and endemic strains pathogenic to flies and caterpillars. Hajek *et al.* (1990) used allozymes to confirm that the gypsy moth pathogen causing epizootics in North America was *E. maimaiga*. In further studies using allozymes and RFLPs, they demonstrated that *E. maimaiga* and *E. aulicae*, two very similar species, were attacking different caterpillars in the same area (Hajek *et al.*, 1991b). ELISA was also developed for *E. maimaiga* based on allozymes, but was cross-reactive for *E. aulicae* (Hajek *et al.*, 1991a).

Application of DNA techniques is making major advances in monitoring the fate of entomopathogenic fungi in the environment. There are numerous studies that have demonstrated the power of molecular biology in strain, species, and genera separation

(see Section 6.11.2.5). These same techniques are useful when applied to monitoring fungal isolates in the environment. For example, Enkerli *et al.* (2001) used 10 microsatellite markers to characterize *B. brongniartii* and could use these markers to demonstrate that strains applied in Switzerland in 1983 were still active over 8 years later. Specific identification of *B. brongniartii*, based on polymerase chain reaction (PCR) primers designed to group 1 intron insertions in the 28s rRNA gene, was used to identify and monitor an introduced strain for control of the scarab pest, *Hoplochelus marginalis*, in the Reunion Islands (Neuvéglise *et al.*, 1997). Other techniques, such as chromosomal length polymorphisms, RAPDs, and RFLP have all been used to assist monitoring strains in the environment. Molecular approaches to phylogeography of insect pathogens are also developing. Using a molecular genetics approach, Bidochka *et al.* (2001) found that genotypes of *M. anisopliae* in Canada were more influenced by habitat than host.

An extension of the use of molecular techniques for distinguishing strains important in biological control is the insertion of sequences (tags) or genes which can be used to specifically assist monitoring. For example, insertion of a green fluorescent protein gene was used to allow detection and tracking of a genetically modified *M. anisopliae* (Hu and St. Leger, 2002).

Media based detection methods, such as selective agars, can determine the number of viable propagules in the environment, but generally cannot allow separation between applied and background strains. Molecular approaches can allow separation of strains, but while these molecular approaches are extremely powerful, most techniques do not provide information on the activity (viability) of the propagules detected. Even though fungi can be detected, it is not possible to say whether the detected fungus is able to infect hosts. Use of *Galleria* or other sentinel insects, such as in the soil baiting method (Zimmermann, 1986), can demonstrate virulence, but gives no quantification of propagules. A combination of methods is required to monitor applied fungi in the environment, to provide information on the number of viable propagules of the applied fungus present over time.

6.11.5. Epizootiology and Its Role in Suppressing Pest Populations

Entomopathogenic fungi are well known for their ability to rapidly decimate insect outbreaks through spectacular epizootics, and it is principally this property that has spurred interest in the use of fungi for pest management. The term "epizootic" is used to describe a situation where the proportion of infected individuals is high or at least higher than the enzootic condition, i.e., the disease condition that is usually of low prevalence and constantly present in a host population (Tanada and Kaya, 1993). Prevalence is defined as the number or proportion of infected individuals at a given point in time (Fuxa and Tanada, 1987). Natural epizootics caused by fungi are an important factor in regulating pest insect populations, often alleviating the need for other interventions, such as application of chemical pesticides. Here, some examples of fungi with a role in suppressing pest populations in epigeal, soil, and aquatic environments are provided.

6.11.5.1. Epigeal Environment

Epizootics often occur within the epigeal environment. Infection may spread rapidly directly from infected insects to uninfected insects, from spores on leaves to insects feeding on the leaves, and also via spores present in the air.

High prevalence is often found among aphids (e.g., Feng *et al.*, 1991; Hollingsworth *et al.*, 1995; Hatting *et al.*, 2000). Hollingsworth *et al.* (1995) monitored the fungus *N. fresenii* in populations of *Aphis gossypii* in cotton in Arkansas, USA. Epizootics were common and prevalences could reach 90%. Such prevalences were detrimental to the aphid populations, which actually began to decline when the prevalence reached 15%. Also, aphids living in cold environments suffer from fungus infections. Nielsen *et al.* (2001) sampled *Elatobium abietinum* on sitka spruce in Iceland. Both *Entomophthora planchoniana* and *N. fresenii* were present in the aphids and were responsible for epizootics during the autumn, when daily mean temperatures were below 15 °C. However, the effect on the host population was not documented during this study.

Another group of insects often found infected with fungal pathogens are dipterans. Steinkraus *et al.* (1993) studied the prevalence of *Entomophthora muscae* in populations of *Musca domestica* and found prevalences of up to ~75% towards the end of the season. In this case, as in other cases, the epizootic builds up during the season. In studies of *M. domestica* and *Entomophthora schizophorae*, Six and Mullens (1996) documented that maximum daily temperatures higher than 26–28 °C correlated with low prevalences.

Entomopthoralean fungi from the *Entomophaga grylli* species complex are important pathogens of acridids worldwide (Carruthers *et al.*, 1997). They commonly cause epizootics, particularly following periods of high humidity or rain. Such epizootics

have frequently led to dramatic reductions in acridid populations, saving farmers and ranchers millions of dollars. The epizootics are very noticeable, as thousands of cadavers can be seen adhering to the tops of vegetation (see Section 6.11.3.3.2).

The host density-dependent development of epizootics was documented in the *L. dispar* populations infected with *E. maimaiga*. This fungus has shown remarkable dispersal over a period of several years (or maybe even decades) in north-eastern USA (Hajek, 1997, 1998, 1999). An interesting adaptation to the host is seen, since first instar larvae produce infective conidia of the fungus while larger larvae produce resting spores for winter survival. The effect on the host population is significant and the fungus is responsible for decimating large populations of this destructive, introduced pest.

Recent examples of epizootics in other insect groups from the epigeal environment include *N. parvispora* on thrips (*Frankliniella occidentalis*) in Spain (Montserrat *et al*., 1998), *Zoophthora phytonomi* on weevils in alfalfa in the USA (Morris *et al*., 1996), and *Entomophthora leyteensis* on whiteflies in the Philippines (Villacarlos *et al*., 2003). It appears that epizootics occur worldwide and in all major insect orders.

6.11.5.2. Soil Environment

Due to the cryptic nature of insects inhabiting the soil environment, natural epizootics most probably occur much more often than reported. It appears that the main source of inoculum of several important Hyphomycetes is the soil and epizootics have often been observed among soil living stages of insects (Steenberg *et al*., 1995; Fuhrer *et al*., 2001). Using insects as baits, a number of studies have demonstrated the presence of spores from the genera *Beauveria, Metarhizium, Paecilomyces*, and *Tolypocladium* in soil (Klingen *et al*., 2002; Keller *et al*., 2003). Among Ascomycetes, *Cordyceps* spp. have often been observed on insect cadavers on the soil surface. A study in Colombia documented, for example, that species of *Cordyceps* were common in populations of ants (Sanjuan *et al*., 2001). The effect of *Cordyceps* infections on insect populations is in need of further investigation.

The biological role of the soil ecosystem for the insect pathogenic fungi is, however, not clarified. The soil may simply act as a reservoir for a population of spores, which then infect host insects, or the soil ecosystems can be involved in a more complex interaction with insect pathogenic fungi. For instance, *B. brongniartii* was found in localities where the host, *Melolontha melolontha*, was absent for approximately 40 years (Keller *et al*., 2003). Either the fungus can survive by using other hosts or by saprophytic growth, or the spores can survive for many years. Hu and St. Leger (2002) suggest that the rhizosphere is an important interphase between insects, insect pathogenic fungi, and plants. Insects not susceptible to certain fungi may act to disperse fungal inoculum. Broza *et al*. (2001) and Dromph (2001) both documented that collembolans can be involved in transmission of insect pathogenic fungi without being infected themselves.

The presence of inoculum in the upper soil surface layers is important for the initiation of epizootics in the epigeal environment during spring. Nielsen *et al*. (2003) demonstrated that inoculum of epigeal, entomophthoralean pathogens (*C. obscurus*, *P. neoaphidis*) of cereal aphids (*Sitobion avenae* and *R. padi*) was present in the soil surface in early spring. Aphids became infected by these fungi when crawling on the soil surface. The epizootic would then develop in the epigeal environment.

6.11.5.3. Aquatic Environment

The aquatic environment is very different from terrestrial environments. Most insect pathogenic fungi infecting aquatic species have motile spores, such as zoospores, which can be actively attracted to the host (see Section 6.11.3.4). In aquatic environments, disease can be influenced by a range of abiotic and biotic factors, some of which are not experienced in other environments. For example, salinity, pH, organic and inorganic pollutants, and water movement can alter or stop infection. Salinity affects both *Coelomomyces* and *Lagenidium* infections. No *C. opifexi* infections were detected in salt water pools with over 20% salt, although the mosquito larval hosts could be detected in pools with twice that level of salt (Pillai, 1971). *Lagenidium giganteum* is even more susceptible to salinity with less than 0.05% salt levels in water required for high infection levels (Merriam and Axtell, 1982a). *Culicinomyces clavisporus* was more resistant to salt, infecting at 1.3% salt (Merriam and Axtell, 1982b). Temperature is also a major influence on disease and epizootiology, as with terrestrial fungi.

There has been substantial interest in some aquatic-active entomopathogenic fungi, mainly due to the pest status of mosquitoes and blackflies. However, many of these fungal species have complex life cycles unsuited to biocontrol, or have limited prevalence in most situations. *Coelomomyces* have an obligate intermediatory host (also referred to as an "alternate host"), often a copepod, between infecting mosquito larvae. This has both limited the occurrence of the pathogen, as well as its use in intentional introductions. Copepod abundance was

found to be the critical factor in epizootics caused by *C. punctatus* (Apperson *et al*., 1992). Pillai (1971) sampled over 400 pools in New Zealand and detected *C. opifexi* in only 21. When pools that had both mosquito and copepod hosts were intentionally inoculated, disease levels persisted for over 2 years, but disease incidence remained low (mean <16%). It seems that the pathogen remained in equilibrium with its hosts, even in a closed system of saltwater pools. There is evidence that *L. giganteum* persists better in high density than in low density populations, where the fungus may struggle to recycle.

Unlike the zoospore-infective species, the hyphomycete mosquito pathogen *C. clavisporus* is infective after ingestion. This reduces the need for a motile stage, as conidia present in the water are ingested, rather than needing to find and attach to the host. However, settling of conidia becomes a major factor in epizootiology. Applying high rates of conidia to ponds can kill up to 100% of mosquito larvae but there is no residual prevalence after 2 days (Sweeney, 1981). However, more recently, Seif and Shaarawi (2003) found that *C. clavisporus* was active for 5 days against mosquito larvae in brackish ponds in Egypt.

The ability to persist through times of habitat drying is a useful attribute for aquatic pathogens. *Lagenidium* and *Coelomomyces* both produce thick-walled resting stages (oospores and sporangia). *Lagenidium* can be unaffected by periodic drying (e.g., Fetter-Lasko and Washino, 1983).

Lagenidium giganteum and *C. clavisporus* have been the subject of development as biopesticides, which has resulted in epizootiological studies and inundative release. *Lagenidium giganteum* is now the basis of a commercially available mosquitocide. However, research on *C. clavisporus* peaked in the 1970–80s in Australia, and did not lead to a commercial product. Attempts at introducing *Coelomomyces* as a biocontrol agent in the classical sense into the Tokelau Islands provided inconclusive results (Laird, 1985).

6.11.6. Development as Inundative Microbial Control Agents

Inundation biological control is defined as

> The use of living organisms to control pests when control is achieved exclusively by the released organisms themselves
>
> Eilenberg *et al*. (2001)

It is attractive from the point of view that the effect on the target host is obtained with the released agent itself and is often regarded as *the* strategy in biological control with microorganisms. Terms like "biopesticide," "biological pesticide," or "mycopesticide" are frequently used to describe inundation biological control with fungi. These terms also reflect that this strategy is in many ways an "insecticidal approach" in that the inoculum has to be applied directly to the crop or host, often several times per season, which is attractive from a commercial point of view.

Inundation biological control is, however, not necessarily the most sustainable way to control pests, especially if the biological control agent is used simply as a replacement for chemical insecticides. Replacement of a chemical with a microbial control agent could result in the same type of problems associated with chemicals, namely development of populations resistant to the control agent, be it chemical or biological, as has occurred with the bacterial microbial agent *B. thuringiensis* (Tabashnik, 1994). Although no instances of the development of resistance to an entomopathogenic fungus have been documented to date, it must be realized that inundative use of entomopathogenic fungi has been limited and, therefore, the prerequisites for selection of a resistant population would, in most instances, not have been met as yet.

Many Hyphomycetes are well suited for the inundative strategy based on their relatively wider host ranges, ease of production, formulation and application, and a relatively long shelf life. Several species of fungi from Hyphomycetes have been developed commercially and are currently for sale worldwide (see Section 6.11.6.2 below).

Entomophthoralean fungi possess some characteristics that allow them to fit into the inundation biological control paradigm, while other characteristics suggest that these fungi would fail under these conditions (Pell *et al*., 2001). The generally high virulence of many species is a useful characteristic for this strategy, while the presence of replicative, infective but short-lived conidia hampers the development of "easy to use products." A further drawback is the fact that isolation *in vitro* is difficult and has been achieved only for very few species. Efficient mass production methods are mostly lacking as is knowledge on formulation.

In greenhouses, entomophthoralean fungi may have a better chance. The most thorough recent attempts were made by Shah *et al*. (2000). Experimental preparations based on alginate granules and mycelial mats of *P. neoaphidis* caused up to 36% infections in the potato aphid *Macrosiphon euphorbiae* after 14 days, although better results were obtained with *B. bassiana*.

In most temperate countries, greenhouses are the only place where inundative biological control of crops with entomophthoralean fungi could be recommended at present. Preparations of *P. neoaphidis* could be tested against aphids in greenhouses and may, due to some horizontal transmission effects afterwards, prove to have some advantages over other biological control agents used, including *Lecanicillium lecanii*. For some of the greenhouse pest–pathogen systems mentioned in Section 6.11.7, there could also be some prospects in inundation. Whatever the environment, a most promising release strategy could be a combination: an inundation with some immediate effect but including a significant inoculative effect.

6.11.6.1. Mass Production

The success of any commercial microbial control agent depends largely on the ability to produce adequate supplies of the agent to economically manage the target pest or pests. Entomopathogenic fungi produce different types of spores, depending on the growth environment. Aerial conidia are produced on the surface of cadavers, while blastospores, sometimes referred to as "hyphal bodies" or protoplasts, are produced in the host's hemocoel. Consequently, the fermentation medium and method will also dictate the type of spore that will be obtained. In submerged culture, primarily mycelia and blastospores are produced while aerial conidia are produced on the surface of liquid media or on solid substrates.

Efficient mass production methods are mostly lacking for entomophthoralean fungi as is knowledge on their formulation. Despite the development of methods for mass producing several species, to date, there are no commercialized products based on entomophthoralean fungi. Due to the short-lived nature of entomophthoralean conidia, attempts at mass production have targeted primarily mycelia or resting spores (Pell *et al*., 2001).

Many insect cadavers killed by fungi can be found in the environment in a dehydrated state; often, transfer of these cadavers to conditions of high humidity results in massive conidiogenesis and spore release (e.g., Tyrrell, 1988). This phenomenon has spurred the development of "dried mycelia" as a potential way of mass producing and using entomopathogenic fungi (McCabe and Soper, 1985). Mycelia are mass produced in deep fermentation, air dried in the presence of a sugar desiccant protectant, granulated, and stored dehydrated under low temperature. Upon rehydration, the fungus sporulates on the granule surface. This method is suitable both for entomophthoralean and hyphomycetous fungi. Despite some successful field demonstrations using dried mycelia against several insect pests (e.g., Pereira and Roberts, 1991; Krueger *et al*., 1992; Krueger and Roberts, 1997; Booth *et al*., 2000; Shah *et al*., 2000), this method has not yet been adopted commercially.

Some entomopathogenic fungi produce conidia or resting spores in submerged culture. Examples are resting spores of the entomophthoraleans, *Conidiobolus obscurus* (Latgé, 1980) and *E. maimaiga* (Kogan and Hajek, 2000), and conidia of the hyphomycetous fungi *Culicinomyces clavisporus* (Cooper and Sweeney, 1986) and *M. anisopliae* var. *acridum* (Jenkins and Prior, 1993). Submerged conidia of some species perform as well as aerially produced ones (e.g., *M. anisopliae* var. *acridum*; Jenkins and Thomas, 1996). For other species this is not so, e.g., submerged conidia of *C. clavisporus* performed worse than aerial conidia (Cooper and Sweeney, 1986). To date, there are no commercialized products based on conidia or resting spores produced in submerged culture.

Most Hyphomycetes produce blastospores in submerged culture, but for the most part, these are thin walled and short lived, and with few exceptions, are used primarily to produce starter batches for use in inoculation of aerial culture (see Section 6.11.6.2). In some cases, blastospores are more virulent than aerial conidia (e.g., Jackson *et al*., 1997; Lacey *et al*., 1999), and several commercial products consist of blastospores produced in submerged culture (e.g., *L. lecanii* and *P. fumosoroseus*; see below).

Solid state fermentation is used for production of aerial conidia of a number of commercially produced Hyphomycetes (see below). The solid substrates can be either nutritive, such as bran, cereals, or rice, or nonnutritive, such as vermiculite, clay, or sponge. Production of *B. bassiana* and *M. anisopliae* on rice is widely practiced in South America (Alves *et al*., 2003), Africa (Cherry *et al*., 1999), and China (Feng *et al*., 1994).

More details on the production of entomopathogenic fungi can be found in Bartlett and Jaronski (1988), Jenkins and Goettel (1997), Jenkins *et al*. (1998), and Wraight *et al*. (2001).

6.11.6.2. Commercialized Products

There are many commercial products available worldwide, most based on fungi from six to seven species from Hyphomycetes (Shah and Goettel, 1999; Copping, 2001; Wraight *et al*., 2001; Alves *et al*., 2003). Hyphomycetes are very well suited for commercialization due to their relatively wider host ranges, ease of production, shelf life, persistence, and ease of application.

6.11.6.2.1. *Beauveria bassiana* The most widely used species available commercially is *B. bassiana*. Products based on this species are available for use against a very wide variety of insect pests, from banana weevils (*Cosmopolites sordidus*) in Brazil (Alves *et al.*, 2003), pine caterpillars (*Dendrolimus* spp.) in China (Feng *et al.*, 1994), to the European corn borer, and greenhouse aphids in the Western world (Shah and Goettel, 1999; Copping, 2001). Formulations consist of aerially produced conidia produced as wettable powders or in emulsifiable oil.

The largest applications of *B. bassiana* have occurred in China, where an estimated 10 000 tons of conidia were produced annually for decades to control a wide range of forest and crop pests (Feng *et al.*, 1994). Most of these conidia were produced in state-owned, mechanized Chinese production facilities. However, with the recent transition from a state-planned and directed system to a market driven one, *B. bassiana* products have come to be considered as marginal and have not attracted the necessary attention from investors (Feng, 2003).

A solid culture process based on a packed bed of a proprietary starch granule has been developed to produce conidia that are marketed as Botanigard and Mycotrol in the USA and elsewhere (Bradley *et al.*, 1992). The products are used primarily to control aphids and whiteflies in commercial greenhouses. With host ranges that include over 700 species of arthropods (see Goettel *et al.*, 1990a), the market potential for more commercial products based on isolates of *B. bassiana* is large.

6.11.6.2.2. *Beauveria brongniartii* Products based on *B. brongniartii* are available against a wide variety of coleopteran, homopteran, lepidopteran, and dipteran pests in flowers, vegetables, oil palms, and other crops in Colombia (Alves *et al.*, 2003), against cerambicid beetles in Japan (Wraight *et al.*, 2001), and against the European cockchafer and other white grubs in Europe (Copping, 2001). Conidia are produced in solid-state fermentation either on barley kernels or clay granules. In Peru, the fungus has been widely used to control the Andean potato weevil (Alves *et al.*, 2003). The soil beneath potato storage facilities is treated with spores of the fungus prior to introduction of the potato tubers. Weevils become infected when the larvae drop to the soil to pupate.

6.11.6.2.3. *Lecanicillium lecanii* *Lecanicillium (= Verticillium) lecanii* is primarily marketed for control of greenhouse aphids, whiteflies, and thrips (Copping, 2001; Wraight *et al.*, 2001); however, products are also available in Colombia and Peru for control of lepidopteran, homopteran, and dipteran pests of flowers, vegetables, and other crops (Shah and Goettel, 1999; Alves *et al.*, 2003). Blastospores produced in submerged fermentation or conidia produced in solid-state fermentation are used in the various products. Previously, the requirement for very high humidity was a serious detriment in adopting this fungus for use in greenhouses, where high humidities also favor development of plant diseases. However, development of an improved formulation that decreases the humidity requirements of the fungus has alleviated much of this problem (Ravensberg, personal communication).

6.11.6.2.4. *Metarhizium anisopliae* (including var. *acridum*) Products based on *M. anisopliae* are available for a wide range of pests, including sugarcane spittle bugs (*Mahanarva* spp.) in Brazil (Alves *et al.*, 2003), red-headed cockchafer in Australia (Shah and Goettel, 1999), and termites in the USA (Copping, 2001). Products based on *M. anisopliae* var. *acridum* are available for control of grasshoppers and locusts in Africa (Lomer *et al.*, 2001) and Australia (Copping, 2001). Production systems vary, but most depend on culture in small bags or containers using rice as the substrate (Mendonca, 1992; Jenkins *et al.*, 1998). Such simple technologies may be more appropriate and economically feasible for smaller scale technologies, especially in developing countries, than larger, more sophisticated and capital intensive industrial scale production facilities (Swanson, 1997; Cherry *et al.*, 1999).

6.11.6.2.5. *Paecilomyces fumosoroseus* Formulations of *P. fumosoroseus* are marketed against whiteflies, thrips, spider mites, and aphids in Latin America, Europe, and North America (Copping, 2001; Alves *et al.*, 2003). Blastospores, produced in submerged culture, are dehydrated and formulated in a water dispersible bran granule or a water dispersible powder. The products are used primarily in greenhouse applications.

6.11.6.2.6. Others Numerous other products are currently available in many countries. Some examples include "*Entomophthora virulenta*" (= *Conidiobolus thromboides*) for control of whiteflies in Colombia (Alves *et al.*, 2003), *N. rileyi* for control of lepidopterans in Colombia (Shah and Goettel, 1999; Alves *et al.*, 2003), *P. lilacinus* for control of plant parasitic nematodes in Australia (Copping, 2001), and the Oomycete, *L. giganteum*, for control of mosquito larvae (Shah and Goettel, 1999; Copping, 2001).

Several products are based on microbial "cocktails." For instance, in Colombia, a product called

"Microbiol Completo" consists of a mixture of the fungi *B. bassiana*, *M. anisopliae*, *N. rileyi*, *P. fumosoroseus*, and the bacterium *Bacillus thuringiensis*. This mixture is marketed for use against a wide variety of pest lepidopterans, coleopterans, hemipterans, dipterans, and acari (Alves *et al*., 2003). Another cocktail, "Microbiol H.E.," contains a mixture of *B. bassiana*, *M. anisopliae*, *M. flavoviride*, *N. rileyi*, *P. fumosoroseus*, *P. lilacinus*, *V. lecanii*, and *Hirsutella thompsonii*, which includes activity against nematodes in addition to the pests listed under the Microbio Completo. Microbial cocktails not only increase host spectrum, but also may ensure activity under different environmental conditions. For instance, results from a field simulation study against grasshoppers suggested that *B. bassiana* itself would be more efficacious under cooler, cloudy conditions, although *M. anisopliae* var. *acridum* (as *flavoviride*) would be more efficacious under warmer, sunny conditions, while a cocktail of both fungi would be efficacious under both conditions (Inglis *et al*., 1997b).

6.11.6.3. Improvement of Efficacy

There are many factors that can affect the efficacy of biopesticides, such as slow speed of kill, moderate efficacy, poor storage characteristics, lack of field persistence after application, and expense (see Section 6.11.3; see also Inglis *et al*., 2001; Pell *et al*., 2001). Many of these factors can be overcome by strain selection, genetic improvement (St. Leger and Screen, 2001), formulation (Wraight *et al*., 2001), and application (Bateman and Chapple, 2001).

6.11.6.3.1. Strain selection Entomopathogenic fungal species are composed of often genetically heterogeneous collections of strains, which can have different properties on key characteristics, such as virulence to pest species. Natural variation between strains of any given species has long been used in the search for biocontrol fungi. The selection among strains for the more virulent, better environmental persistence, or some other important variable, is a constant theme in the literature. A common first step in the search for fungal control agents for any pest insect is to assay strains from a number of locations to select the most pathogenic (e.g., Vicentini *et al*., 2001; Shaw *et al*., 2002).

In addition to utilizing natural variation, there have been efforts to improve biocontrol fungi through selection. Improving virulence by passage through hosts has been attempted, sometimes with success. Ogarkov and Ogarkova (1999) reported an up to twofold increase in virulence of *B. bassiana* to *Trialeurodes vaporariorum* after passage through the host. *Lagenidium giganteum* increased virulence to two mosquito hosts by bi-weekly passage through another mosquito (Washino, 1981). Fargues and Robert (1983) restored lost virulence of *M. anisopliae* through serial passage through a scarab. These are all indicative of the potential for strain improvement through selection.

Improvement of other characteristics of entomopathogenic fungi have been investigated through selection. Increasing the endogenous reserves of conidia to enhance viability and desiccation tolerance was proposed by Hallsworth and Magan (1994). By growing cultures of *B. bassiana, M. anisopliae*, and *P. farinosus* under different conditions to obtain conidia with a modified polyol and trehalose content, conidia with increased intracellular levels of glycerol and erythritol were isolated. These conidia germinated more quickly and at lower water activity than unselected conidia (Hallsworth and Magan, 1995). Conidia with increased trehalose germinated more slowly but had better shelf life than unselected conidia.

More recently, there have been attempts to use biotechnological techniques, not just in direct genetic improvement of fungi, but to assist strain selection. As described in Sections 6.11.2.6 and 6.11.4, biochemical and molecular techniques have been used to identify specific strains of entomopathogenic fungi, allowing for more specific selection of strains following exposure to discriminatory conditions. For example, protoplast fusion has been used to combine characteristics of strains of *Beauveria* to improve the biocontrol potential. Fusion of a strain of *B. bassiana* from *Leptinotarsa decemlineata* with an insecticidal toxin-producing strain of *B. sulfurescens*, resulted in recovery of some di-auxotrophic mutants with enhanced activity against *L. decemlineata* and the caterpillar *O. nubilalis* (Couteaudier *et al*., 1996; Viaud *et al*., 1998). Molecular analysis demonstrated that the resulting mutants were mixtures of the two genotypes. Some of the resulting strains were classified as hypervirulent, as they killed the hosts more quickly than the parental strains. Virulence was stable after passage through the host, suggesting protoplast fusion may be an alternative method to direct genetic engineering for improving the biocontrol efficiency of entomopathogenic fungi (Viaud *et al*., 1998).

6.11.6.3.2. Genetic modification The use of genetic manipulation to overcome perceived limitations of entomopathogenic fungi for use as biocontrol agents is attractive. The research so far is limited when compared to other groups of organisms, but some interesting research has been done. The most

advanced research has been on the hyphomycete fungi *M. anisopliae* and *B. bassiana*.

Transformation systems have been developed for some entomopathogenic fungi. The first transformations of an entomopathogenic fungus were by Bernier *et al.* (1989) and Goettel *et al.* (1990b), who expressed a benomyl resistance gene in *M. anisopliae*. Electroporation and biolistics have also been used to transform *M. anisopliae* (St. Leger *et al.*, 1995). Polyethylene glycol (PEG) mediated transformation of protoplasts is another method for transformation of entomopathogenic fungi, as done with *P. fumosoroseus* and *P. lilacinus* (Inglis *et al.*, 1999b) using benomyl as the selective agent. Sandhu *et al.* (2001) developed a heterologous transformation system for *B. bassiana* and *M. anisopliae* based on the use of the *Aspergillus nidulan*s nitrate reductase gene (niaD), after EMS treatment of protoplasts and regeneration on chlorate medium.

Genetic modification of entomopathogenic fungi by direct manipulation has been led by St. Leger, and the area was recently reviewed by St. Leger and Screen (2001), covering both insect and plant pathogenic fungi. In the numerous publications, these researchers described the identification and cloning of protease genes, particularly the *pr1* cuticle degrading protease gene from *M. anisopliae* (St. Leger *et al.*, 1992a). Several other entomopathogenic Hyphomycetes (*A. flavus, B. bassiana, Paecilomyces farinosus, Tolypocladium niveum*, and *L. lecanii*) also have *pr1*-type enzymes (St. Leger *et al.*, 1991, 1992a). Efforts to produce more effective strains of fungi have included the overexpression of *pr1* by insertion of multiple copies, resulting in increased speed of kill of a host insect, although transformants had very poor sporulation ability (St. Leger, 2001). Modification of *pr1* gene expression in *M. anisopliae* resulted in melanization and cessation of feeding 25–30 h earlier than the wild-type disease in caterpillars (St. Leger *et al.*, 1996). The group has gone on to place the *pr1* gene under control of a constitutive promoter and included a green fluorescent protein encoding gene for tracking the fungi after field release (Hu and St. Leger, 2002). Genes involved in regulation of PR1 expression have also been identified from *M. anisopliae* (Screen *et al.*, 1997, 1998).

Chitinase, produced by many insect pathogenic fungi and implicated in pathogenesis, has also been the target of molecular studies. Several chitinase gene sequences from *Beauveria* and *Metarhizium* have been submitted to GenBank and described in publications (e.g., Bogo *et al.*, 1998). Overexpression of extracellular chitinase has also been demonstrated for *M. anisopliae* var. *acridum*, but did not alter virulence to the caterpillar, *M. sexta*, compared to the wild-type fungus (Screen *et al.*, 2001).

6.11.6.3.3. Formulation and application strategies There are a number of limitations that still affect the use of fungi. Formulation and application of fungal propagules has the potential to overcome some of these limitations. Jones and Burges (1998) defined four basic functions of formulation: (1) stabilization of the agent during production, distribution, and storage; (2) aiding the handling and application of the product to the target in the most appropriate manner and form; (3) protection of the agent from harmful environmental factors at the target site, thereby increasing persistence; and (4) enhancing efficacy of the agent at the target site by increasing activity, reproduction, contact, and interaction with the target pest.

Progress has been made in formulation science, and formulations for entomopathogens have been comprehensively reviewed by Burges (1998). Early formulations of entomopathogenic fungi often used just a weak detergent to get the hydrophobic spores into solution, without any efforts to use the formulations to improve efficacy. More recently, the use of oil as a formulation ingredient has been successfully used to overcome several problems. In the LUBILOSA program developing *M. anisopliae* var. *acridum* for control of locusts in Africa, the combination of oil formulations with ultra low volume (ULV) spraying has led to successful development of a biopesticide (Lomer *et al.*, 2001). Although it would seem impossible to use an entomopathogenic fungus requiring high humidity against locusts in the hot, dry conditions prevalent in much of their environment, studies have shown that ambient humidity may not be as critical as once thought (Fargues *et al.*, 1997; see also Section 6.11.3.1). Formulating *Metarhizium* conidia in nonevaporative diluents such as oils allowed the conidia to attach, spread (Inglis *et al.*, 1996a), and germinate on susceptible locusts, even at low ambient humidities (Bateman, 1997).

Formulation can also assist in extending shelf stability of fungal propagules generally desired by commercial producers. Spore survival after mass production varies greatly, depending on the species, but few entomopathogenic species of fungi can achieve the 1–2 years of shelf stability at formulation. Formulation in clay material is known to improve the shelf life of many conidial preparations (e.g., Shi, 1998). This appears to be due to the ability of clay to reduce the moisture content of the spores, as spores survive longer with less than 5% moisture (Wraight *et al.*, 2001). Moore *et al.* (1996) have

shown that survival of conidia of *M. anisopliae* was highest at low (<5%) relative humidity, and similar results have been found for other Hyphomycetes. Some oils can also maintain a shelf life of over 1 year for some Hyphomycetes, possibly due to maintenance of low spore moisture content.

Several groups have examined formulation of hyphal material from members of the Entomophthorales, which are more fragile than Hyphomycete conidia. McCabe and Soper (1985) patented a process of drying the mycelium of *Z. radicans* and coating it with sugar as a method for long-term storage and, more recently, Shah *et al.* (1999) demonstrated algination as a method for formulating *Pandora (Erynia) neoaphidis* mycelium. Formulations of dried mycelia of *M. anisopliae* and *B. bassiana* were developed by coating with a sugar solution before drying (Pereira and Roberts, 1990). Entomopathogenic fungi have also been formulated as granules (e.g., Reinecke *et al*., 1990) or, in some cases, applied without removal from the grains used as a production medium.

Persistence is a major issue with fungal biopesticides. One of the major factors affecting persistence is ultraviolet (UV) radiation, which rapidly degrades fungal spores. Use of various UV protectants in formulations has been tested, but with only limited success (Burges, 1998; Wraight *et al*., 2001). The most effective method to reduce UV degradation of spores has been to use application techniques that place the spores in the appropriate position to rapidly infect the host or to avoid UV, such as by applying subsurface. Subsurface application of entomopathogenic fungi has been used for *M. anisopliae* against scarabs (e.g., Logan *et al*., 2000) and other insect pests, but introducing large amounts of fungal inoculum into the soil and securing an even spread remains problematic. Methods that have been used include using seed drills for subsurface application of granules and hand application. The problems of spread of conidia after application to soil has led to the *Melolontha* researchers developing an area-wide approach based on augmentative applications of *B. brongniartii* for long-term suppression of pest populations (Hajek *et al*., 2001).

Compatibility between production, formulation, and application techniques is vital to successful use of microbial biopesticides. Application is a very specialized area of agricultural research, and has both its own technological and specific issues related to achieving adequate control, as well as coverage. Application technology can ensure infective propagules contact the target pest. Much of this technology is derived from our experience with chemical pesticides and may not be the most appropriate for use with fungal pathogens. Bateman and Chapple (2001) have recently reviewed spray application of mycopesticides in detail. Advances have been made with fungi, such as the use of ULV application technology. ULV has been used with the oil formulated *M. anisopliae* for locust control, as the droplets are resistant to evaporation (Lomer *et al*., 2001). Electrostatically charged ULV sprayers have been investigated for better coverage on leaf undersides (Sopp *et al*., 1989). Hydraulic spray systems have been used to apply water based formulations on crops, with air-blast and air-assist technologies primarily used for low-volume applications in fields and orchards. There are many variables to be considered in spray application, whether with ULV or with more conventional equipment. Droplet size is an important consideration, as there is a trade-off between small, more aerial droplets for better spread and large droplets, which contain more propagules and settle more rapidly. The size of the area to which fungal propagules are to be applied can alter the type of application equipment used. For large-scale applications, such as aerial applications, it is possible to use ULV applications of undiluted materials in some cases, whereas it can be difficult to get sufficient coverage on small areas using the undiluted sprays.

A number of novel application methods have been developed that do not rely on methods developed for chemical pesticide application technology. These methods, which rely on the behavior of the pest, may more closely mimic natural infection routes and have more chance of success in situations where conventional spray application is impossible, such as dispersed and highly mobile pests. “Lure and infect” uses an attractant to bring a susceptible insect to a trap containing a pathogen. After contamination in the trap, the insect leaves the trap and infects others, as demonstrated by the use of *Z. radicans* against diamondback moth. Furlong *et al.* (1995) showed that using pheromone lures to attract moths to traps containing sporulating *Z. radicans* can result in contamination and spread of the fungus through the target population. Autodissemination similarly uses trapping of adults in pathogen laced attractant traps then using those adults to disseminate the fungus into the population. This technique was successful in the Azores for introducing *M. anisopliae* to *Popillia japonica* populations (Klein and Lacey, 1999), but whether population suppression would occur depends on the control ability of the fungus. Pheromone impregnated pellets containing *B. bassiana* spores have also been tested against grain borers (Smith *et al*., 1999).

6.11.7. Development as Inoculative Microbial Control Agents

Inoculation biological control is defined as

> The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not that it will do so permanently.
>
> Eilenberg *et al.* (2001)

This is desirable because only a limited amount of inoculum (*in vivo* or *in vitro* acquired material) is released at each treatment. The inoculum is expected to proliferate and control the target pest over time, but additional releases would normally be needed in the future (e.g., next cropping season). Entomophthoralean fungi may fit very well in inoculation biological control because they have the ability to establish epizootics quickly in target pest populations, have a narrow host range, and are able to persist in the target insect population.

Inoculation biological control is being used in northeastern USA to initiate epizootics of *E. maimaiga* in populations of *L. dispar* (Hajek and Webb, 1999; Pell *et al.*, 2001). Resting spores are collected on sites with high populations of the target insect and high prevalence of the fungus, and then spread on soil in areas without the fungus, but with host infestation. Although the fungus is well established throughout the host's distribution area, the host continues to spread, and cadavers are distributed along the leading edge of this spread in an effort to halt the destruction caused by this introduced pest.

There are other possibilities for inoculative biological control using fungi, such as inoculation of a small amount of *B. brongniartii* to control soil dwelling beetles from Scarabaeidae (Eilenberg *et al.*, 2000b). Another possibility would be use of *E. muscae* and *S. castrans* against cabbage root flies (*D. radicum* and *D. floralis*). Moreover, indoor, inoculative releases to control *M. domestica* could be based on *in vivo* cultures of *E. muscae* or *E. schizophorae* (e.g., Kuramoto and Shimazu, 1997). Conidia are sufficiently persistent in the environment for this release (Kalsbeek *et al.*, 2001b). Indoor stables are favorable for studies on fungus dispersal in a confined environment.

Dissemination is essential for the success of inoculation biological control. Pell *et al.* (1993) and Furlong *et al.* (1995) demonstrated that autodissemination of *Z. radicans* was possible in field populations of the diamondback moth *P. xylostella* in Malaysia by using a pheromone trap to attract adult insects. After the adults left the trap, they dispersed the fungus to both larvae and adults, and larvae became infected. Further experiments of inoculative releases in England demonstrate that it is possible to induce epizootics in *P. xylostella* using *Z. radicans* (Pell and Wilding, 1994). Other examples are provided by Vega *et al.* (2000).

6.11.8. Use in Classical Biocontrol

Classical biological control is defined as

> The intentional introduction of an exotic biological control agent for permanent establishment and long-term pest control
>
> Eilenberg *et al.* (2001)

It is desirable from the point of view that limited inoculative releases of an organism will result in a long-lasting control of a pest (often an introduced pest species). Because the introduction of an exotic species has the potential of irreversible direct effects on nontarget species, or indirect effects through host depletion, it is important to gain as much information on the potential candidate's host range and epizootiology as possible prior to its introduction (Hajek and Butler, 2000; Hajek and Goettel, 2000; Goettel and Hajek, 2001; Goettel *et al.*, 2001).

There is great potential in the use of entomopathogenic fungi in classical biological control, especially against introduced pests. Fungi from Entomophthorales possess several characteristics favoring their use in classical biological control; they can establish epizootics quickly, they have mostly a narrow host range, and they persist in pest populations and in the environment. Unfortunately, in most cases, it is not known whether or not a given species is already present in a locality, and the tools needed for detailed studies of their spread after release (e.g., isolate specific characterization) are usually not fully developed (see Section 6.11.4).

As early as 1909, *E. maimaiga* infected gypsy moths, *L. dispar*, were collected in Japan and released in Boston, USA, in an attempt to control introduced *L. dispar*, but the fungus was not observed in local *L. dispar* populations for many years. It was, however, discovered in northeastern USA in 1989 and has now spread into many other areas within North America where *L. dispar* infestations were prevalent. It has not been ascertained if the fungus now commonly present is a result of the early release or if it was later accidentally imported (Hajek *et al.*, 1995; Pell *et al.*, 2001). A recent introduction of *E. maimaiga* into Bulgaria was successful (Pilarska *et al.*, 2000).

Another example of an establishment in a completely new environment was the release of an Israeli isolate of *Z. radicans* to control the clover

aphid *Therioaphis trifolii* in Australia. The experiments were limited in time and the area studied was small, yet it was shown that dispersal of the fungus up to at least 65 m took place within 2 months of release (Milner *et al.*, 1982). Another isolate of this same species was released in Illinois in 1984 to control the potato leafhopper, *Empoasca fabae* (McGuire *et al.*, 1987a). Epizootics of *Z. radicans* then occurred in *E. fabae* and contributed to regulation of the population, and there was evidence to suggest that one of the isolates might have become established (McGuire *et al.*, 1987b).

Neozygites floridana from South America has been considered for classical biological control of the cassava green mite (*Mononychellus tanajore*) in Africa (Elliot *et al.*, 2000). The data on dispersal in plots in Brazil indicated that the fungus would be capable of spread under African conditions and contribute to long-term control of the pest. However, it was concluded that *N. floridana* would not be capable of providing adequate control of the mite if used as the sole control agent. Releases of the Brazilian isolate were made in Benin in 1999 (Hountondji *et al.*, 2002). Postrelease monitoring revealed that the introduced isolate had become established, with infection prevalences of up to 36%, 48 weeks after initial introduction.

The use of fungi for classical biological control is at the present time a rather difficult issue. First, one must find a potential agent that does not already occur in the chosen environment. Then, laboratory studies are warranted to document that the proposed agent has potential for classical biological control. Finally, the authorities must be convinced that the agent for release and establishment poses no harm to the native fauna, and this is difficult, but not impossible, to accomplish (Hajek and Goettel, 2000; Goettel and Hajek, 2001). Today, the latter is difficult to achieve, due to the present attitude in society that more or less demands zero risk to biodiversity.

6.11.9. Role in Conservation Biocontrol

Conservation biological control (sometimes also called habitat manipulation) is defined as

> Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests
>
> Eilenberg *et al.* (2001)

It is probably the most attractive among all strategies for biological control, yet the least studied. No agents are released, but the farming systems and practice are modified in order to stimulate the naturally occurring predators, parasitoids, and insect pathogens, to regulate the pest insect populations below the damage threshold (Barbosa, 1998). Sufficient regulation by entomophthoralean fungi and other natural enemies may actually already exist for the vast number of crop herbivorous insect and mite species, which never do any damage because of low densities. The conservation strategy also fits well into modern sustainable agriculture, where actions to make direct use of the naturally occurring enemies of pests are taken into consideration, including increasing biodiversity and supporting a positive interaction between the crop, the flora, and fauna within the field and field margins, and landscape elements (Gurr and Wratten, 2000; Landis *et al.*, 2000). Fungi from the order Entomophthorales fit very well into this strategy (Pell *et al.*, 2001). They have the ability to establish natural epizootics depending on the host insect and a number of biotic and abiotic factors. A number of entomophthoralean species exist in each field, forest, private garden, greenhouse, stable and nature area. Certainly, this diversity of species (and isolates from each species) could be explored and exploited in conservation biological control.

Each system should be analyzed adequately. Studies on the epizootiology of *N. fresenii* in the cotton aphid, *Aphis gossypii*, in Arkansas have demonstrated that it is possible to monitor and to some extent predict the epizootic development of the fungus in the target insect population (Hollingsworth *et al.*, 1995; Steinkraus *et al.*, 1996; Pell *et al.*, 2001). The monitoring of the pest insect can therefore be supplemented with knowledge on the symptoms of fungal disease in the host, which would allow extension officers to modify their recommendations of intervention; e.g., if the pest population is high, but fungus infected, no pesticide treatment would be required as the pest population would soon succumb to the disease.

Habitat manipulation and modification of existing practices are often proposed as means to promote infections caused by entomopathogenic fungi (Pell *et al.*, 2001): more hedges, less spraying with chemicals (e.g., fungicides), and irrigation are all, at least theoretically, supportive to development of natural fungal epizootics among pest insects. It was recently shown that soil-occurring insect pathogenic fungi from Hyphomycetes are found in higher densities in organic grown fields compared to conventionally treated fields (Klingen *et al.*, 2002). To date, however, it has not been possible to document that a

specified practice will result in a sufficiently high infection level *every time* in field situations. Indoors it has been shown that intensive spraying with water supports the development of natural epizootics of *Erynia ithacensis* in fungus sciarid gnats (Huang *et al*., 1992), and such manipulation should be better studied and eventually adopted.

Among biotic factors influencing the success of entomopathogenic fungi are their interactions with other natural enemies. Such interactions may be positive, since predators and parasitoids may assist in the transmission of these fungi as passive vectors (see Sections 6.11.3.5 and 6.11.3.6). A better understanding of the interactions between entomopathogenic fungi and other natural enemies would allow us to use different biological control agents in different strategies. There is much potential for conservation biological control using entomopathogenic fungi, however, more studies are needed before we can take full advantage of this naturally occurring resource. Especially warranted are studies to clarify the importance of soil as a reservoir of inoculum and to determine whether crop rotation is supportive to infection of entomophthoralean fungi and other insect pathogenic fungi.

More information is needed to determine how landscape elements affect early initiation of epizootics. In both cases, the inclusion of studies of insect pathogens should be promoted strongly to complement the more detailed existing knowledge of interactions among predators, parasitoids, host and nonhost insects, cropping systems, and landscape elements. Despite the wish to make entomopathogenic fungi operational for conservation biological control, much work still remains. Compared with knowledge on predators and parasitoids, the knowledge on insect pathogens in natural systems is limited, and much observational and descriptive work is certainly still needed.

6.11.10. What Does the Future Hold?

Compared to other technologies, development of entomopathogenic fungi as inundative, inoculative, and classical microbial control agents is still in its infancy. Inundative application of mass produced spores has long been seen as an attractive alternative to the classical use of fungi for control. A number of fungal based biopesticides are now being produced. But, despite the number of "products" based on entomopathogenic fungi currently commercially produced for pest control throughout the world, the market share is still well below the potential suggested by their role in natural insect control. Although great strides have been made in improving methods of production, formulation, and application, a great many improvements are still possible. Production of species "cocktails," especially combining microbials from different classes or even with chemical or botanical antagonists is only just beginning. To date, all commercial products based on entomopathogenic fungi have been developed from wild-type field strains. In many cases, these products are developed with the insecticidal paradigm in mind, that is, strains with the widest host range are formulated to be applied by conventional equipment. These products are often difficult to market to clientele that are used to conventional chemical insecticides and expect tank mixing possibilities with either herbicides or fungicides (and this is often not compatible), a rapid kill (fungi are usually slower acting), and a broad spectrum of activity (even fungi with the broadest host spectrum have reduced spectra as compared to most chemicals in present use).

With only a few studies attempting genetic engineering on entomopathogenic fungi, it is hard to determine when or not modification of fungi will increase their efficacy in biocontrol. The current regulatory environment in many countries makes such research expensive, and public acceptability without demonstrated consumer (rather than farmer) gains may be some time off. Strain improvement through guided selection strategies is more likely to find acceptance in the short term.

In addition to development of entomopathogenic fungi for use in inundative applications, we see great potential in their use in conservation and classical biological control. Whatever strategies are contemplated, they should be considered with the intention not just of replacing chemical pesticides with entomopathogenic fungi, but contributing to the development of sustainable agriculture, horticulture, and forestry as well as the preservation of biodiversity and nature. With increasing public and political pressure to implement integrated pest management strategies, it is inevitable that use of entomopathogenic fungi will play an increasingly important role (Lacey and Goettel, 1995).

References

Alves, S.B., Pereira, R.M., Lopes, R.B., Tamai, M.A., **2003**. Use of entomopathogenic fungi in Latin America. In: Upadhyay, R.K. (Ed.), Advances in Microbial Control of Insect Pests. Kluwer, New York, pp. 193–211.

Amiri, B., Ibrahim, L., Butt, T.M., **1999**. Antifeedant properties of destruxins and their use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocon. Sci. Technol. 9*, 487–498.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M., Butt, T.M., **2000**. Inter- and intra-specific variation in destruxin production by the insect pathogenic *Metarhizium*, and its significance to pathogenesis. *Mycol. Res. 104*, 447–452.

Apperson, C.S., Federici, B.A., Tarver, F.R., Stewart, W., **1992**. Biotic and abiotic parameters associated with an epizootic of *Coelomomyces punctatus* in a larval population of the mosquito *Anopheles quadrimaculatus*. *J. Invertebr. Pathol. 60*, 219–228.

Arthurs, S., Thomas, M.B., **2000**. Effects of a mycoinsecticide on feeding and fecundity of the brown locust *Locustana pardalina*. *Biocont. Sci. Technol. 10*, 321–329.

Austwick, P.K.C., **1983**. Some mycoses of reptiles. *RMVN 18*, 779.

Barr, D.J.S., **1992**. Evolution and kingdoms of organisms from the perspective of a mycologist. *Mycologia 84*, 1–11.

Bandani, A.R., Butt, T.M., **1999**. Insecticidal, antifeedant and growth inhibitory activities of efrapeptins, metabolites of the entomogenous fungus *Tolypocladium*. *Biocon. Sci. Technol. 9*, 499–506.

Bandani, A.R., Khambay, B.P.S., Faull, J., Newton, R., Deadman, M., *et al.*, **2000**. Production of efrapeptins by *Tolypocladium* species and evaluation of their insecticidal and antimicrobial properties. *Mycol. Res. 104*, 537–544.

Barbosa, P. (Ed.), **1998**. Conservation Biological Control. Academic Press, San Diego.

Bartlett, M.C., Jaronski, S.T., **1988**. Mass production of entomogenous fungi for biological control of insects. In: Burge, R.N. (Ed.), Fungi in Biological Control Systems. Manchester University Press, Manchester, pp. 61–85.

Bateman, R., **1997**. The development of a mycoinsecticide for the control of locusts and grasshoppers. *Outlook Agric. 26*, 13–18.

Bateman, R., Chapple, A., **2001**. The spray application of mycopesticide formulations. In: Butt, T., Jackson, C., Magan, N. (Eds.), Fungal Biocontrol Agents. CABI Press, Wallingford, pp. 289–309.

Beard, C.E., Adler, P.H., **2002**. Seasonality of Trichomycetes in larval black flies from South Carolina, USA. *Mycologia 94*, 200–209.

Beard, R.L., Walton, G.S., **1969**. Kojic acid as an insecticidal mycotoxin. *J. Invertebr. Pathol. 14*, 53–59.

Berbee, M.L., Taylor, J.W., **2001**. Fungal molecular evolution: gene trees and geologic time. In: McLaughlin, D.J., McLaughlin, E.G., Lenke, P.A. (Eds.), The Mycota: a Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research, Systematics and Evolution, Part B. Springer GmbH, Berlin. pp. 229–245.

Bernier, L., Cooper, R.M., Charnley, A.K., Clarkson, J.M., **1989**. Transformation of the entomopathogenic fungus *Metarhizium anisopliae* to benomyl resistance. *FEMS Microbiol. Lett. 60*, 261–266.

Bidochka, M.J., **2001**. Monitoring the fate of biocontrol fungi. In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biocontrol Agents. CAB International, Wallingford, pp. 193–218.

Bidochka, M.J., Hajek, A.E., **1998**. A nonpermissive entomophthoralean fungal infection increases activation of insect prophenoloxidase. *J. Invertebr. Pathol. 72*, 231–238.

Bidochka, M.J., Kamp, A.M., Lavender, T.M., Dekoning, J., De Croos, J.N., **2001**. Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? *Appl. Environ. Microbiol. 67*, 1335–1342.

Blanford, S., Thomas, M.B., **2001**. Adult survival, maturation, and reproduction of the desert locust *Schistocerca gregaria* infected with the fungus *Metarhizium anisopliae* var *acridum*. *J. Invertebr. Pathol. 78*, 1–8.

Blanford, S., Thomas, M.B., Langewald, J., **1998**. Behavioural fever in the Senegalese grasshopper, *Oedaleus senegalensis*, and its implications for biological control using pathogens. *Ecol. Entomol. 23*, 9–14.

Bogo, M.R., Rota, C.A., Pinto, H., Ocampos, M., Correa, C.T., *et al.*, **1998**. A chitinase encoding gene (chit1 gene) from the entomopathogen *Metarhizium anisopliae*: isolation and characterization of genomic and full-length cDNA. *Curr. Microbiol. 37*, 221–225.

Booth, S.R., Tanigoshi, L., Dewes, I., **2000**. Potential of a dried mycelium formulation of an indigenous strain of *Metarhizium anisopliae* against subterranean pests of cranberry. *Biocont. Sci. Technol. 10*, 659–668.

Boucias, D.G., Pendland, J.C., **1998**. Principles of Insect Pathology. Kluwer Academic Publishers, Boston.

Bradley, C.A., Black, W.E., Kearns, R., Wood, P., **1992**. Role of production technology in mycoinsecticide development. In: Leatham, G.F. (Ed.), Frontiers in Industrial Mycology. Chapman and Hall, New York, pp. 160–179.

Brousseau, C., Charpentier, G., Bellonick, S., **1996**. Susceptibility of spruce budworm, *Choristoneura fumiferana* Clemens, to destruxins, cyclodepsipeptidic mycotoxins of *Metarhizium anisopliae*. *J. Invertebr. Pathol. 68*, 180–182.

Broza, M., Pereira, R.M., Stimac, J.L., **2001**. The nonsusceptibility of soil Collembolla to insect pathogens and their potential as scavengers of microbial pesticides. *Pedobiology 45*, 523–534.

Bruns, T.D., Vilgalys, R., Barns, S.M., Gonzalez, D., Hibbett, D.S., *et al.*, **1993**. Evolutionary relationships within the fungi: analysis of nuclear small subunit rRNA sequences. *Molec. Phylogen. Evol. 1*, 231–241.

Burges, H.D., **1998**. Formulation of mycoinsecticides. In: Burges, H.D. (Ed.), Formulation of Microbial Biopesticides. Kluwer, Dordrecht, pp. 131–185.

Butt, T.M., Goettel, M.S., **2000**. Bioassays of Entomogenous fungi. In: Navon, A., Ascher, K.R.S. (Eds.), Bioassays of Entomopathogenic Microbes and Nematodes. CAB International Press, Wallingford, pp. 141–195.

Butt, T.M., Beckett, A., Wilding, N., **1981**. Protoplasts in the *in vivo* life cycle of *Erynia neoaphidis*. *J. Gen. Microbiol. 127*, 417–421.

Butt, T.M., Jackson, C.W., Magan, N., **2001**. Fungi as Biocontrol Agents. CABI Publishing, Wallingford.

Butt, T.M., Carreck, N.L., Ibrahim, L., Williams, I.H., **1998**. Honey-bee-mediated infection of pollen beetles (*Meligethes aeneus* Fab.) by the insect-pathogenic fungus, *Metarhizium anisopliae*. *Biocont. Sci. Technol. 8*, 533–538.

Cafaro, M.J., **2002**. Species richness pattern in symbiotic gut fungi (Trichomycetes). *Fungal Divers. 9*, 47–56.

Carruthers, R.I., Larkin, T.S., Firstencel, H., Feng, Z., **1992**. Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology 73*, 190–204.

Carruthers, R.I., Ramos, M.E., Larkin, T.S., Hostetter, D.L., Soper, R.S., **1997**. The *Entomophaga grylli* (Fresenius) Batko species complex: its biology, ecology, and use for biological control of pest grasshoppers. *Mem. Entomol. Soc. Canad. 171*, 329–353.

Cavalier-Smith, T., **1987**. The origin of fungi and pseudofungi. In: Rayner, A.D.M., Brasier, C.M., Moore, D. (Eds.), Evolutionary Biology of the Fungi. Cambridge University Press, Cambridge, pp. 339–353.

Chabasse, D., **1994**. New opportunistic fungi appearing in medical pathology. A general review. *J. Mycol. Med. 4*, 9–28 (in French).

Champlin, F.R., Grula, E.A., **1979**. Noninvolvement of beauvericin in the entomopathogenicity of *Beauveria bassiana*. *Appl. Environ. Microbiol. 37*, 1122–1125.

Cherry, A.J., Jenkins, N.E., Heviefo, G., Bateman, R., Lomer, C.J., **1999**. Operational and economic analysis of a West African pilot-scale production plant for aerial conidia of *Metarhizium* spp. for use as a mycoinsecticide against locusts and grasshoppers. *Biocont. Sci. Technol. 9*, 35–51.

Christensen, J.B., Fetter-Lasko, J.L., Washino, R.K., Husbands, R.C., Kaffman, E.E., **1977**. A preliminary field study employing *Lagenidium giganteum*, a fungus, as a possible biological control agent against the pasture mosquito *Aedes nigromaculis*. *Proc. Calif. Vector Mosq. Control Assoc. 45*, 105.

Clarkson, J.M., **1992**. Molecular approaches to the study of entomopathogenic fungi. In: Lomer, C.J., Prior, C. (Eds.), Biological Control of Locusts and Grasshoppers. CAB International Press, Wallingford, pp. 191–199.

Cooke, M.C., **1892**. Handbook of Australian Fungi. Williams and Norgate, London.

Cooper, R.D., Sweeney, A.W., **1986**. Laboratory studies on the recycling potential of the mosquito pathogenic fungus *Culicinomyces clavisporus*. *J. Invertebr. Pathol. 48*, 152–158.

Copping, L.G. (Ed.), **2001**. The Biopesticide Manual, 2nd edn. British Crop Protection Council, Farnham.

Couch, J.N., Romney, S.V., **1973**. Sexual reproduction in *Lagenidium giganteum*. *Mycologia 65*, 250–252.

Couteaudier, Y., Viaud, M., **1997**. New insights into population structure of *Beauveria bassiana* with regard to vegetative compatibility groups and telomeric restriction fragment length polymorphisms. *FEMS Microbiol. Ecol. 22*, 175–182.

Couteaudier, Y., Viaud, M., Riba, G., **1996**. Genetic nature, stability, and improved virulence of hybrids from protoplast fusion in *Beauveria*. *Microbial Ecol. 32*, 1–10.

Daoust, R.A., Roberts, D.W., **1982**. Virulence of natural and insect passaged strains of *Metarhizium anisopliae* to mosquito larvae. *J. Invertebr. Pathol. 40*, 107–117.

Dedryver, C.A., **1981**. Biology of the cereal aphids in the west of France 2. Spatial and temporal distribution and field pathogenicity of 3 species of Entomophthoraceae. *Entomophaga 26*, 381–394.

Domnas, A.J., **1981**. Biochemistry of *Lagenidium giganteum* infection of mosquito larvae. In: Davidson, E.W. (Ed.), Pathogenesis of Invertebrate Microbial Diseases. Allanheld, Totowa, pp. 425–449.

Driver, F., Milner, R.J., **1998**. PCR applications to the taxonomy of entomopathogenic fungi. In: Bridge, P.D., Arora, D.K., Reddy, C.A., Elander, R.P. (Eds.), Applications of PCR in Mycology. CABI Press, Wallingford, pp. 153–186.

Driver, F., Milner, R.J., Trueman, J.W.H., **2000**. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycol. Res. 104*, 134–150.

Dromph, K., **2001**. Dispersal of entomopathogenic fungi by collembolans. *Soil Biol. Biochem. 33*, 2047–2051.

Eaton, K.K., Hennessy, T.J., Snodin, D.J., McNulty, D.W., **1986**. *Verticillium lecanii* allergological and toxicological studies on work exposed personnel. *Ann. Occup. Hyg. 30*, 209–217.

Eilenberg, J., **1987**. Abnormal egg-laying behaviour of female carrot flies (*Psila rosae*) induced by the fungus *Entomophthora muscae*. *Entomol. Expl. Appl. 43*, 61–65.

Eilenberg, J., **1988**. Occurrence of fungi from Entomophthorales in a population of carrot flies (*Psila rosae* F.). Results 1985 and 1986. *IOBC/WPRS Bull. 11*, 53–59.

Eilenberg, J., Philipsen, H., **1988**. The occurrence of Entomophthorales on the carrot fly (*Psila rosae* F.) in the field during two successive seasons. *Entomophaga 33*, 135–144.

Eilenberg, J., Hajek, A.E., Lomer, C., **2001**. Suggestions for unifying the terminology in biological control. *BioControl 46*, 387–400.

Eilenberg, J., Damgaard, P.H., Hansen, B.M., Pedersen, J.C., Bresciani, J., *et al.*, **2000a**. Natural co-prevalence of *Strongwellsea castrans*, *Cystosporogenes deliaradicae* and *Bacillus thuringiensis* in the host, *Delia radicum*. *J. Invertebr. Pathol. 75*, 69–75.

Eilenberg, J., Vestergaard, S., Harding, S., Nielsen, C., **2000b**. Potential for microbial control of Scarbaeidae and Curculionidae in Christmas tree and greenery production. In: Christensen, C.J. (Ed.), Improvements in Christmas Tree and Greenery Quality. Ministry of Environment, Danish Forest and Landscape Research Institute, Report No. 7, Copenhagen, pp. 22–23.

Elliot, S.L., Blanford, S., Thomas, M.B., **2002**. Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proc. R. Soc. Lond. 269*, 1599–1607.

Elliot, S.L., De Moraes, G.J., Delalibera, I., Da Silva, C.A.D., Tamai, M.A., *et al.*, **2000**. Potential of the

mite-pathogenic fungus *Neozygites floridana* (Entomophthorales: Neozygitaceae) for control of the cassava green mite *Mononychellus tanajore* (Acari: Tetranychidae). *Bull. Entomol. Res. 90*, 191–200.

Enkerli, J., Widmer, F., Gessler, C., Keller, S., **2001**. Strain-specific microsatellite markers in the entomopathogenic fungus *Beauveria brongniartii*. *Mycol. Res. 105*, 1079–1087.

Evans, H.C., **1982**. Entomogenous fungi in tropical forest ecosystems: an appraisal. *Ecol. Entomol. 6*, 47–60.

Evans, H.C., **1989**. Mycopathogens of insects of epigeal and aerial habitats. In: Wilding, N., Collins, N.M., Hammond, P.M., Webber, J.F. (Eds.), Insect–Fungus Interactions. Academic Press, London, pp. 205–238.

Fargues, J.F., Robert, P.H., **1983**. Effects of passage through scarabeid hosts on virulence and host specificity of two strains of the entomopathogenic Hyphomycete *Metarhizium anisopliae*. *Can. J. Microbiol. 29*, 576–583.

Fargues, J., Delmas, J.C., Lebrun, R.A., **1994**. Leaf consumption by larvae of the Colorado Potato Beetle (Coleoptera: Chrysomelidae) infected with the entomopathogen, *Beauveria bassiana*. *J. Econ. Entomol. 87*, 67–71.

Fargues, J., Ouedraogo, A., Goettel, M.S., Lomer, C.J., **1997**. Effects of temperature, humidity and inoculation method on susceptibility of *Schistocerca gregaria* to *Metarhizium flavoviride*. *Biocont. Sci. Technol. 7*, 345–356.

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A., Vidal, C., *et al.*, **1996**. Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia 135*, 171–181.

Fargues, J., Vidal, C., Smits, N., Rougier, M., Boulard, T., *et al.*, **2003**. Climatic factors on entomopathogenic Hyphomycetes infection of *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) in Mediterranean glasshouse tomato. *Biol. Control 28*, 320–331.

Federici, B.A., Lucarotti, C.J., **1986**. Structure and behaviour of the meiospore of *Coelomomyces dodgei* encystment in the copepod host *Acanthocyclops vernalis*. *J. Invertebr. Pathol. 48*, 259–268.

Feng, M.G., **2003**. Microbial control of insect pests with entomopathogenic fungi in China: a decade's progress in research and utilization. In: Upadhyay, R.K. (Ed.), Advances in Microbial Control of Insect Pests. Kluwer, New York, pp. 213–234.

Feng, M.G., Johnson, J.B., Halbert, S.E., **1991**. Natural control of cereal aphids (Homoptera, Aphididae) by entomopathogenic fungi (Zygomycetes, Entomophthorales) and parasitoids (Hymenoptera, Braconidae and Encyrtidae) on irrigated spring wheat in Southwestern Idaho. *Environ. Entomol. 20*, 1699–1710.

Feng, M.G., Johnson, J.B., Kish, L.P., **1990**. Survey of entomopathogenic fungi naturally infecting cereal aphids (Homoptera: Aphididae) of irrigated grain crops in Southwestern Idaho. *Environ. Entomol. 19*, 1534–1542.

Feng, M.G., Poprawski, T.J., Khachatourians, G.G., **1994**. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontr. Sci. Technol. 4*, 3–34.

Ferron, P., **1985**. Fungal control. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon, Oxford, pp. 313–346.

Ferron, P., Fargues, J., Riba, G., **1991**. Fungi as microbial insecticides against pests. In: Arora, D.K., Ajello, L., Mukerji, K.G. (Eds.), Handbook of Applied Mycology, Vol. 2: Humans, Animals, and Insects. Dekker, New York, pp. 665–706.

Fetter-Lasko, J.L., Washino, R.K., **1983**. In-situ studies on seasonality and recycling pattern in California USA of *Lagenidium giganteum* an aquatic fungal pathogen of mosquitoes. *Environ. Entomol. 12*, 635–640.

Frappier, F., Ferron, P., Pais, M., **1975**. Chimie des champignons entomopathogenes – le beauvellide, nouveau cyclodepsipeptide isole d'un *Beauveria tenella*. *Phytochemistry 14*, 2703–2705.

Fuhrer, E., Rosner, S., Schmied, A., Wegensteiner, R., **2001**. Studies on the significance of pathogenic fungi in the population dynamics of the lesser spruce sawfly, *Pritiphora abietina* Christ. (Tenthredinidae). *J. Appl. Entomol. 125*, 235–242.

Furlong, M.J., Pell, J.K., **2000**. Conflicts between a fungal entomopathogen, *Zoophthora radicans*, and two larval parasitoids of the diamond back moth. *J. Invertebr. Pathol. 76*, 85–94.

Furlong, M.J., Pell, J.K., Reddy, G.V.P., **1997**. Premortality effects of *Zoophthora radicans* infection in *Plutella xylostella*. *J. Invertebr. Pathol. 70*, 214–220.

Furlong, M.J., Pell, J.K., Choo, O.P., Rahman, S.A., **1995**. Field and laboratory evaluation of a sex pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bull. Entomol. Res. 85*, 331–337.

Fuxa, J.R., Tanada, Y., **1987**. Epizootiology of Insect Diseases. Wiley, New York.

Gams, W., Zare, R., **2001**. A revision of *Verticillium* Sect. *Prosteata*. III. Generic classification. *Nova Hedwigia 72*, 329–337.

Garcia, C., Ignoffo, C.M., **1977**. Dislodgement of conidia of *Nomuraea rileyi* from cadavers of cabbage looper, *Trichoplusia ni*. *J. Invertebr. Pathol. 30*, 114–116.

Genthner, F.J., Middaugh, D.P., **1992**. Effects of *Beauveria bassiana* on embryos of the Inland Silverside fish (*Menidia beryllina*). *Appl. Environ. Microbiol. 58*, 2840–2845.

Genthner, F.J., Middaugh, D.P., **1995**. Nontarget testing of an insect control fungus: effects of *Metarhizium anisopliae* on developing embryos of the inland silverside fish *Menida beryllina*. *Dis. Aquat. Org. 22*, 163–171.

Genthner, F.J., Foss, S.S., Gals, P.S., **1997**. Virulence of *Metarhizium anisopliae* to embryos of the grass shrimp *Palaemonetes pugio*. *J. Invertebr. Pathol. 69*, 157–164.

Gilliam, M., Vandenberg, J.D., **1990**. Fungi. In: Morse, R.A., Nowogrodzki, R. (Eds.), Honey Bee Pests, Predators, and Diseases. Cornell University Press, Ithaca, pp. 64–90.

Gilliam, M., Taber, S., Richardson, G.V., **1983**. Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie 14*, 29–39.

Glare, T.R., **1987**. Effect of host species and light conditions on production of conidia by *Nomuraea rileyi*. *J. Invertebr. Pathol. 50*, 67–69.

Glare, T.R., **1994**. Stage-dependant synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica*. *Biocontr. Sci. Technol. 4*, 321–329.

Glare, T.R., Milner, R.J., **1991**. Ecology of entomopathogenic fungi. In: Arora, D.K., Mukeriji, K.G., Pugh, J.G.F. (Eds.), Handbook on Applied Mycology, Vol. 2: Humans, Animals, and Insects. Dekker, New York, pp. 547–612.

Glare, T.R., Chilvers, G.A., Milner, R.J., **1985**. Capilliconidia as infective spores in *Zoophthora phalloides* (Entomophthorales). *Trans. Brit. Mycol. Soc. 85*, 463–470.

Glare, T.R., Harris, R.J., Donovan, B.J., **1996**. *Aspergillus flavus* as a pathogen of wasps, *Vespula* spp., in New Zealand. *N. Z. J. Zool. 23*, 339–344.

Glare, T.R., Milner, R.J., Chilvers, G.A., **1986**. The effect of environmental factors on the production, discharge and germination of primary conidia of *Zoophthora phalloides* Batko. *J. Invertebr. Pathol. 48*, 275–283.

Glare, T.R., Milner, R.J., Chilvers, G.A., **1989**. Factors affecting the production of resting spores by *Zoophthora radicans* in the spotted alfalfa aphid, *Therioaphis trifolii* f. *maculata*. *Canad. J. Bot. 67*, 848–855.

Glare, T.R., Placet, C., Nelson, T.L., Reay, S.D., **2002**. Potential of *Beauveria* and *Metarhizium* as control agents of pinhole borers (*Platypus* spp.). *N. Z. Plant Protect. J. 55*, 73–79.

Goethe, J.W. von, **1817–1822**. Zur morphologie. Erfahrung, betrachtung, folgerung durch lebens-ereignisse verbunden erster band. Edition Kuhn, D., (1954). Morphologisher Hefte, Neunter Band, Hermann Böhlaus Nachfolger, Weimar.

Goettel, M.S., Hajek, A.E., **2001**. Evaluation of nontarget effects of pathogens used for management of arthropods. In: Wajnberg, E., Scott, J.K., Quimby, P.C. (Eds.), Evaluating Indirect Ecological Effects of Biological Control. CABI Press, Wallingford, pp. 81–97.

Goettel, M.S., Jaronski, S.T., **1997**. Safety and registration of microbial agents for control of grasshoppers and locusts. *Mem. Entomol. Soc. Canad. 171*, 83–99.

Goettel, M.S., Inglis, G.D., Wraight, S.P., **2000**. Fungi. In: Lacey, L.A., Kaya, H.K. (Eds.), Field Manual of Techniques for the Application and Evaluation of Entomopathogens. Kluwer Academic Press, Dordrecht, pp. 255–282.

Goettel, M.S., Hajek, A.E., Siegel, J.P., Evans, H.C., **2001**. Safety of fungal biocontrol agents. In: Butt, T., Jackson, C., Magan, N. (Eds.), Fungal Biocontrol Agents – Progress, Problems and Potential. CABI Press, Wallingford, pp. 347–375.

Goettel, M.S., Poprawski, T.J., Vandenberg, J.D., Li, Z., Roberts, D.W., **1990a**. Safety to nontarget invertebrates of fungal biocontrol agents. In: Laird, M., Lacey, L.A., Davidson, E.W. (Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, pp. 209–231.

Goettel, M.S., St. Leger, R.J., Bhairi, S., Jung, M.K., Oakley, B.R., *et al.*, **1990b**. Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Curr. Genetics 17*, 129–132.

Gonzalez Cabo, J.F., Espejo Serrano, J., Barcena Asensio, M.C., **1995**. Mycotic pulmonary disease by *Beauveria bassiana* in a captive tortoise. *Mycoses 38*, 167–169.

Griffiths, G.C.D., **1985**. *Hypogastrura succinea* (Collembola Hypogastruridae) dispersed by adults of the cabbage maggot *Delia radicum* (Diptera Anthomyiidae) infected with the parasitic fungus *Strongwellsea castrans* (Zygomycetes Entomophthoraceae). *Canad. Entomol. 117*, 1063–1064.

Gumowski, P.I., Latgé, J.-P., Paris, S., **1991**. Fungal allergy. In: Arora, D.K., Mukeriji, K.G., Rugh, J.G.F. (Eds.), Handbook of Applied Mycology, Vol. 2: Humans, Animals, and Insects. Dekker, New York, pp. 163–204.

Gurr, G., Wratten, S., **2000**. Biological Control: Measures of Success. Kluwer, Dordrecht.

Hajek, A., **1989**. Food consumption by *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae infected with *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *Environ. Entomol. 18*, 723–727.

Hajek, A.E., **1997**. Fungal and viral epizootics in gypsy moth (Lepidoptera: Lymantriidae) populations in Central New York. *Biol. Cont. 10*, 58–68.

Hajek, A.E., **1998**. Pathology and epizootiology of *Entomophaga maimaiga* infections in forest Lepidoptera. *Microbiol. Molec. Biol. Rev. 63*, 814–835.

Hajek, A., **1999**. Pathology and epizootiology of *Entomophaga maimaiga* infections in forest Lepidoptera. *Microbiol. Molec. Biol. Rev. 63*, 814–835.

Hajek, A.E., Butler, L., **2000**. Predicting the host range of entomopathogenic fungi. In: Follett, P.A., Dunn, J.J. (Eds.), Nontarget Effects of Biological Control. Kluwer, Dordrecht, pp. 263–276.

Hajek, A.E., Goettel, M.S., **2000**. Guidelines for evaluating effects of entomopathogens on nontarget organisms. In: Lacey, L.A., Kaya, H.K. (Eds.), Field Manual of Techniques for the Application and Evaluation of Entomopathogens. Kluwer, Dordrecht, pp. 847–868.

Hajek, A.E., St. Leger, R.J., **1994**. Interactions between fungal pathogens and insect host. *Ann. Rev. Entomol. 39*, 293–322.

Hajek, A.E., Shimazu, M., **1996**. Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth *Lymantria dispar*. *Canad. J. Bot. 74*, 708–715.

Hajek, A.E., Webb, R.E., **1999**. Inoculative augmentation of the fungal entomopathogen *Entomophaga maimaiga*

as a homeowner tactic to control gypsy moth (Lepidoptera: Lymantriidae). *Biol. Cont. 14*, 11–18.

Hajek, A.E., Humber, R.A., Elkington, J.S., **1995**. Mysterious origin of *Entomophaga maimaiga* in North America. *Am. Entomol. 41*, 31–42.

Hajek, A.E., Wraight, S.P., Vandenberg, J.D., **2001**. Control of Arthropods Using Pathogenic Fungi. *Fungal Divers. Res. Ser. 6*, 309–347.

Hajek, A.E., Butler, L., Liebherr, J.K., Wheeler, M.M., **2000**. Risk of infection by the fungal pathogen *Entomophaga maimaiga* among Lepidoptera on the forest floor. *Environ. Entomol. 29*, 645–650.

Hajek, A.E., Butt, T.M., Strelow, L.I., Gray, S.M., **1991a**. Detection of *Entomophaga maimaiga* (Zygomycetes Entomophthorales) using ELISA. *J. Invertebr. Pathol. 58*, 1–9.

Hajek, A.E., Humber, R.A., Walsh, S.R.A., Silver, J.C., **1991b**. Sympatric occurrence of two *Entomophaga aulicae* (Zygomycetes Entomophthorales) complex species attacking forest Lepidoptera. *J. Invertebr. Pathol. 58*, 373–380.

Hajek, A.E., Jensen, A.B., Thomsen, L., Hodge, K., Eilenberg, J., **2003**. PCR-RFLP is used to investigate relations among species in the entomopathogenic genera *Eryniopsis* and *Entomophaga*. *Mycologia 95*, 262–268.

Hajek, A.E., Humber, R.A., Elkinton, J.S., May, B., Walsh, S.R.A., *et al.*, **1990**. Allozyme and restriction fragment length polymorphism analyses confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. *Proc. Natl Acad. Sci. USA 87*, 6979–6982.

Hajek, A.E., Butler, L., Walsh, S.R.A., Silver, J.C., Hain, F.P., *et al.*, **1996**. Host range of the gypsy moth (Lepidoptera: Lymantriidae) pathogen *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) in the field versus laboratory. *Environ. Entomol. 25*, 709–721.

Hallsworth, J.E., Magan, N., **1994**. Effects of KCl concentration on accumulation of acyclic sugar alcohols and trehalose in conidia of three entomopathogenic fungi. *Letters Appl. Microbiol. 18*, 8–11.

Hallsworth, J.E., Magan, N., **1995**. Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiol. Reading 141*, 1109–1115.

Hamill, R.L., Higgens, C.E., Boaz, H.E., Gorman, M., **1969**. The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. *Tetrahedron Lett. 49*, 4255–4258.

Hatai, K., Roza, D., Nakayama, T., **2000**. Identification of lower fungi isolated from larvae of mangrove crab, *Scylla serrata*, in Indonesia. *Mycoscience 41*, 565–572.

Hatting, J.L., Poprawski, T.J., Miller, R.M, **2000**. Prevalences of fungal pathogens and other natural enemies of cereal aphids (Homoptera: Aphididae) in wheat under dryland and irrigated conditions. *BioControl 45*, 179–199.

Henke, M.O., de Hoog, G.S., Gross, U., Zimmermann, G., Kraemer, D., *et al.*, **2002**. Human deep tissue infection with an entomopathogenic *Beauveria* species. *J. Clin. Microbiol. 40*, 2698–2702.

Hollingsworth, R.G., Steinkraus, D.C., McNew, R.W., **1995**. Sampling to predict fungal epizootics in cotton aphids. *Environ. Entomol. 24*, 1414–1421.

Hountondji, F.C.C., Lomer, C.J., Hanna, R., Cherry, A.J., Dara, S.K., **2002**. Field evaluation of Brazilian isolates of *Neozygites floridana* (Entomophthorales: Neozygitaceae) for the microbial control of cassava green mite in Benin, West Africa. *Biocontr. Sci. Technol. 12*, 361–370.

Hu, G., St. Leger, R., **2002**. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl. Environ. Microbiol. 68*, 6383–6387.

Huang, B., Li, C.R., Li, Z.G., Fan, M.Z., Li, Z.Z., **2002**. Molecular identification of the teleomorph of *Beauveria bassiana*. *Mycotaxon 81*, 229–236.

Huang, Y., Zhen, B., Li, Z., **1992**. Natural and induced epizootics of *Erynia ithacensis* in mushroom hothouse populations of yellow-legged fungus gnats. *J. Invertebr. Pathol. 60*, 254–258.

Humber, R.A., **1984**. Foundations for an evolutionary classification of the Entomophthorales (Zygomycetes). In: Wheeler, Q., Blackwell, M. (Eds.), Fungus–Insect Relationships: Perspectives in Ecology and Evolution. Columbia University Press, New York, pp. 166–183.

Inglis, G.D., Johnson, D.L., Goettel, M.S., **1996a**. Effect of bait substrate and formulation on infection of grasshopper nymphs by *Beauveria bassiana*. *Biocontr. Sci. Technol. 6*, 35–50.

Inglis, G.D., Johnson, D.L., Goettel, M.S., **1996b**. Effects of temperature and thermoregulation on mycosis by *Beauveria bassiana* in grasshoppers. *Biol. Contr. 7*, 131–139.

Inglis, G.D., Johnson, D.L., Goettel, M.S., **1997a**. Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) (Hyphomycetes: Sympodulosporae) of grasshoppers under field conditions. *Environ. Entomol. 26*, 400–409.

Inglis, G.D., Duke, G.M., Kawchuk, L.M., Goettel, M.S., **1999a**. Influence of oscillating temperatures on the competitive infection and colonization of the migratory grasshopper by *Beauveria bassiana* and *Metarhizium flavoviride*. *Biol. Control 14*, 111–120.

Inglis, G.D., Goettel, M.S., Butt, T.M., Strasser, H., **2001**. Use of hyphomycetous fungi for managing insect pests. In: Butt, T., Jackson, C., Magan, N. (Eds.), Fungal Biocontrol Agents. CABI Press, Wallingford, pp. 23–69.

Inglis, G.D., Johnson, D.L., Cheng, K.-J., Goettel, M.S., **1997b**. Use of pathogen combinations to overcome the constraints of temperature on entomopathogenic Hyphomycetes against grasshoppers. *Biol. Contr. 8*, 143–152.

Inglis, G.D., Johnson, D.L., Kawchuk, L.M., Goettel, M.S., **1998**. Effect of soil texture and soil sterilization on susceptibility of ovipositing grasshoppers to *Beauveria bassiana*. *J. Invertebr. Pathol. 71*, 73–81.

Inglis, P.W., Tigano, M.S., Valadares-Inglis, M.C., **1999b**. Transformation of the entomopathogenic fungi,

Paecilomyces fumosoroseus and *Paecilomyces lilacinus* (Deuteromycotina: Hyphomycetes) to benomyl resistance. *Gen. Mol. Biol. 22*, 119–123.

Jackson, M.A., McGuire, M.R., Lacey, L.A., Wraight, S.P., **1997**. Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Mycol. Res. 10*, 35–41.

Jaronski, S., **1982**. Oomycetes in mosquito control. In: Proc. 3rd Int. Colloq. Invertebr. Pathol., September 6–10, 1982. Brighton, England, pp. 420–424.

Jeffs, L.B., Khachatourians, G.G., **1997**. Toxic properties of *Beauveria* pigments on erythrocyte membranes. *Toxicon 35*, 1351–1356.

Jenkins, N.E., Goettel, M.S., **1997**. Methods for mass-production of microbial control agents of grasshoppers and locusts. *Mem. Entomol. Soc. Canad. 171*, 37–48.

Jenkins, N.E., Prior, C., **1993**. Growth and formation of true conidia by *M. flavoviride* in a simple liquid medium. *Mycol. Res. 97*, 1489–1494.

Jenkins, N.E., Thomas, M.B., **1996**. Effect of formulation and application method on the efficacy of aerial and submerged conidia of *M. flavoviride* for locust and grasshopper control. *Pesticide Sci. 46*, 299–306.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J., Lomer, C.J., **1998**. Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontr. News Infor. 19* (1), 21N–31N.

Jensen, A.B., Gargas, A., Eilenberg, J., Rosendahl, S., **1998**. Relationships of the insect-pathogenic order Entomophthorales (Zygomycota, Fungi) based on phylogenetic analyses of nuclear small subunit ribosomal DNA sequences (SSU rDNA). *Fungal Gen. Biol. 24*, 325–334.

Jones, K.A., Burges, H.D., **1998**. Technology of formulation and application. In: Burges, H.D. (Ed.), Formulation of Microbial Biopesticides. Kluwer, Dordrecht, pp. 7–30.

Kalsbeek, V., Mullens, B.A., Jespersen, J.B., **2001a**. Field studies of *Entomophthora* (Zygomycetes: Entomophthorales) – induced behaviour fever in *Musca domestica* (Diptera: Muscidae) in Denmark. *Biol. Contr. 21*, 264–271.

Kalsbeek, V., Pell, J.K., Steenberg, T., **2001b**. Sporulation by *Entomophthora schizophorae* (Zygomycetes: Entomophthorales) from housefly cadavers and the persistence of primary conidia at constant temperatures and relative humidities. *J. Invertebr. Pathol. 77*, 149–157.

Kanaoka, M., Isogai, A., Murakoshi, S., Ichione, M., Suzuki, A., *et al.*, **1978**. Bassianoilide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. *Agric. Biol. Chem. 42*, 629–635.

Keller, S., Suter, H., **1980**. Epizootiologische Untersuchungen über das *Entomophthora*-Auftre-ten bei feldbaulich wichtigen Blattlausarten. *Acta Oecologica 1*, 63–81.

Keller, S., Kessler, P., Schweizer, C., **2003**. Distribution of insect pathogenic fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metarhizium anisopliae*. *BioControl 48*, 307–319.

Keller, S., Keller, E., Schweizer, C., Auden, J.A.L., Smith, A., **1989**. Two large field trials to control the cockchafer *Melolontha melolontha* L. with the fungus *Beauveria brongniartii* (Sacc). Petch. In: BCPC Mono. No. 43 "Progress and Prospects in Insect Control." pp. 183–190.

Kerwin, J.L., **1983**. Biological aspects of the interactions between *Coelomomyces psoropborae* zygotes and the larvae of *Culiseta inornata*: host-mediated factors. *J. Invertebr. Pathol. 41*, 224–232.

Kerwin, J.L., Petersen, E.E., **1997**. Fungi: Oomycetes and Chytridiomycetes. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, pp. 251–268.

Kerwin, J.L., Washino, R.K., **1983**. Sterol induction of sexual reproduction in *Lagenidium giganteum*. *Exp. Mycol. 7*, 109–115.

Kish, L.P., Allen, G.E., **1976**. Conidial production of *Nomuraea rileyi* on *Pseudoplusia includens*. *Mycologia 68*, 436–439.

Klein, M.G., Lacey, L.A., **1999**. An attractant trap for autodissemination of entomopathogenic fungi into populations of the Japanese Beetle *Popillia japonica* (Coleoptera: Scarabaeidae). *Biocontr. Sci. Technol. 9*, 151–158.

Klingen, I., Eilenberg, J., Meadow, R., **2002**. Impact of farming systems, field margins and bait insect on the findings of insect pathogenic fungi in soil. *Agric. Ecosys. Environ. 91*, 191–198.

Kobayasi, Y., **1941**. The genus *Cordyceps* and its allies. *Sci. Rep. Tokyo Bunrika Daigaku Sect. B 5*, 53–260.

Kodaira, Y., **1961**. Biochemical studies on the muscardine fungi in the silkworms, *Bombyx mori*. *J. Fac. Text. Sci. Technol. Sinshu Uni. Sericult. 5*, 1–68.

Kogan, P.H., Hajek, A.E., **2000**. Formation of azygospores by the insect pathogenic fungus *Entomophthora maimaiga* in cell culture. *J. Invertebr. Pathol. 75*, 193–201.

Kramer, J.P., **1980**. The housefly mycosis caused by *Entomophthora muscae*: influences of relative humidity on infectivity and conidial germination. *J. N.Y. Ent. Soc. 88*, 236–240.

Krassnoff, S., Gibson, D.M., **1996**. New destruxins from the entomopathogenic fungus *Aschersonia* sp. *J. Nat. Prod. 59*, 485–489.

Krasnoff, S.B., Gupta, S., **1994**. Identification of the antibiotic phomalactone from the entomopathogenic fungus *Hirsutella thompsonii* var. *synnematosa*. *J. Chem. Ecol. 20*, 293–302.

Krasnoff, S.B., Watson, D.W., Gibson, D.M., Kwan, E.C., **1995**. Behaviorial effects of the entomopathogenic fungus, *Entomophthora muscae* on its host *Musca domestica*: postural changes in dying hosts and gated pattern of mortality. *J. Insect Physiol. 41*, 895–903.

Krasnoff, S.B., Gupta, S., St. Leger, R.J., Renwick, J.A., Roberts, D.W., **1991**. Antifungal and insecticidal properties of efrapeptins: metabolites of the fungus *Tolypocladium niveum*. *J. Invertebr. Pathol. 58*, 180–188.

Krueger, S.R., Roberts, D.W., **1997**. Soil treatment with entomopathogenic fungi for corn rootworm (*Diabrotica* spp.) larval control. *Biol. Contr. 9*, 67–74.

Krueger, S.R., Villani, M.G., Martins, A.S., Roberts, D.W., **1992**. Efficacy of soil applications of *Metarhizium anisopliae* (Metsch.) Sorokin conidia, and standard and lyophilized mycelial particles against scarab grubs. *J. Invertebr. Pathol. 59*, 54–60.

Kuramoto, H., Shimazu, M., **1997**. Control of house fly populations by *Entomophthora muscae* (Zygomycotina: Entomophthorales) in a poultry house. *Appl. Entomol. Zool. 32*, 325–331.

Lacey, L.A., **1997**. Manual of Techniques in Insect Pathology. Academic Press, San Diego.

Lacey, L.A., Goettel, M.S., **1995**. Current developments in microbial control of insect pests and prospects for the early 21st century. *Entomophaga 40*, 1–25.

Lacey, L.A., Kaya, H.K. (Eds.), **2000**. Field Manual of Techniques in Invertebrate Pathology. Kluwer, Dordrecht.

Lacey, L.A., Kirk, A.A., Millar, L., Mercadier, G., Vidal, C., **1999**. Ovicidal and larvicidal activity of conidia and blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay system allowing prolonged survival of control insects. *Biocontr. Sci. Technol. 9*, 9–18.

Laird, M., **1985**. Use of *Coelomomyces* in biological control: introduction of *Coelomomyces stegomyiae* into Nukunono, Tokelau Islands. In: Couch, J.N., Bland, C.E. (Eds.), The Genus Coelomomyces. Academic Press, New York, pp. 369–390.

Laird, M., Lacey, L.A., Davidson, E.W., **1990**. Safety of Microbial Insecticides. CRC Press, Boca Raton.

Landis, D.A., Wratten, S.D., Gurr, G.M., **2000**. Habitat management to conserve natural enemies of arthropod pests in agriculture. *Annu. Rev. Entomol. 45*, 175–201.

Latgé, J.P., **1980**. Sporulation de *Entomophthora obscura* Hall & Dunn en culture liquide. *Canad. J. Microbiol. 26*, 1038–1048.

Leipe, D.D., Wainright, P.O., Gunderson, J.H., Porter, D., Patterson, D.J., *et al.*, **1994**. The stramenopiles from a molecular perspective: 16S-like rRNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis*. *Phycologia. 33*, 369–377.

Lin, H., Lin, H.F., **1998**. Testing strain types of *Beauveria bassiana* by means of electrophoretic patterns of esterase isoenzyme. *Acta Phytophylacica Sinica 25*, 315–320.

Liu, Z.Y., Liang, Z.Q., Liu, A.Y., Yao, Y.J., Hyde, K.D., *et al.*, **2002**. Molecular evidence for teleomorph-anamorph connections in *Cordyceps* based on ITS-5.8S rDNA sequences. *Mycol. Res. 106*, 1100–1108.

Logan, D.P., Robertson, L.N., Milner, R.J., **2000**. Review of the development of *Metarhizium anisopliae* as a microbial insecticide, BioCane™, for the control of greyback canegrub *Dermolepida albohirtum* (Waterhouse) (Coleoptera: Scarabaeidae) in Queensland sugarcane. *Bulletin-OILB-SROP 23*, 131–137.

Lomer, C.J., Bateman, R.P., Johnson, D.L., Langewald, J., Thomas, M., **2001**. Biological Control of Locusts and Grasshoppers. *Annu. Rev. Entomol. 46*, 667–702.

Lord, J.C., Magalhaes, B.P., Roberts, D.W., **1987**. Effects of the fungus *Beauveria bassiana* (Bal.) Vuill behavior, oviposition, and susceptibility to secondary infections of adult *Ceroptoma arcuata* (Olivier, 1791) (Coleoptera: Chrysomelidae). *Ann. Soc. Entomol. Brasil 16*, 187–197.

Lucarotti, C.J., **1987**. *Coelomomyces stegomyiae* infection in adult *Aedes aegypti*. *Mycologia 79*, 362–369.

Lucarotti, C.J., Federici, B.A., Chapman, H.C., **1985**. Progress in the development of *Coelomomyces* fungi for use in integrated mosquito control programs. In: Laird, M., Miles, J.W. (Eds.), Integrated Mosquito Control Methodologies. Academic Press, New York, pp. 251–268.

Luz, C., Fargues, J., **1998**. Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. *J. Invertebr. Pathol. 72*, 97–103.

Malakar, R., Elkington, J.S., Hajek, A.E., Burand, J.P., **1999**. Within-host interactions of *Lymantria dispar* (Lepidoptera: Lymantridae) nucleopolyhedrosis virus and *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *J. Invertebr. Pathol. 73*, 91–100.

Matha, V., Weiser, J., Olejnicek, J., **1988**. The effect of tolypin in *Tolypocladium niveum* crude extract against mosquito and blackfly larvae in the laboratory. *Folia Parasitol. 35*, 381–383.

Maurer, P., Couteaudier, Y., Girard, P.A., Bridge, P.D., Riba, G., **1997**. Genetic diversity of *Beauveria bassiana* and relatedness to host insect range. *Mycol. Res. 101*, 159–164.

May, B., Roberts, D.W., Soper, R.S., **1979**. Intraspecific genetic variability in laboratory strains of *Entomophthora* as determined by enzyme electrophoresis. *Exp. Mycol. 3*, 289–297.

Mazet, I., Vey, A., **1995**. Hirsutellin A, a toxic protein produced in vitro by *Hirsutella thompsonii*. *Microbiol. Reading 141*, 1343–1348.

McCabe, D., Soper, R.S., **1985**. Preparation of an entomopathogenic fungal insect control agent. US Patent 4,530,834.

McCoy, C.W., Samson, R.A., Boucias, D.G., **1988**. Entomogenous fungi. In: Ignoffo, C., Mandava, N.B. (Eds.), CRC Handbook of Natural Pesticides, Vol. 5: Microbial Insecticides, Part A: Entomogenous Protozoa and Fungi. CRC Press, Boca Raton, pp. 151–236.

McGuire, M.R., Maddox, J.V., Ambrust, E.J., **1987a**. Effect of temperature on distribution and success of introduction of an *Empoasca fabae* (Homoptera: Cicadellidae) isolate of *Erynia radicans* (Zygomycetes: Entomophthoraceae). *J. Invertebr. Pathol. 50*, 291–301.

McGuire, M.R., Morris, M.J., Ambrust, E.J., Maddox, J.V., **1987b**. An epizootic caused by *Erynia radicans* (Zygomycetes: Entomophthoraceae) isolated from *Empoasca fabae* (Homoptera: Cicadellidae). *J. Invertebr. Pathol. 50*, 78–80.

Mendonca, A.F., **1992**. Mass production, application and formulation of *Metarhizium anisopliae* for control of

sugarcane froghopper, *Mahanarva posticata*, in Brazil. In: Lomer, C.J., Prior, C. (Eds.), Biological Control of Locusts and Grasshoppers. CABI Press, Wallingford, pp. 239–244.

Merriam, T.L., Axtell, R.C., **1982a**. Salinity tolerance of 2 isolates of *Lagenidium giganteum* (Oomycetes: Lagenidiales) a fungal pathogen of mosquito larvae. *J. Med. Entomol. 19*, 388–393.

Merriam, T.L., Axtell, R.C., **1982b**. Evaluation of the entomogenous fungi *Culicinomyces clavosporus* and *Lagenidium giganteum* for control of the salt marsh mosquito *Aedes taeniorhynchus*. *Mosq. News* 42, 594–602.

Mesquita, A.L.M., Lacey, L.A., **2001**. Interactions among the entomopathogenic fungus, *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes), the parasitoid *Aphelinus asychis* (Hymenoptera: Aphelinidae), and their aphid host. *Biol. Cont. 22*, 51–59.

Milne, R., Wright, T., Welton, M., Budau, C., Gringorten, L., *et al*., **1994**. Identification and partial purification of a cell-lytic factor from *Entomophaga aulicae*. *J. Invertebr. Pathol. 64*, 253–259.

Milner, R.J., **1982**. On the occurrence of pea aphids, *Acyrthosiphon pisum*, resistant to isolates of the fungal pathogen, *Erynia neoaphidis*. *Entomol. Exp. Appl. 32*, 23–27.

Milner, R.J., **1985**. Distribution in time and space of resistance to the pathogenic fungus, *Erynia neoaphidis* in the pea phid, *Acyrthosiphon pisum*. *Entomol. Exp. Appl. 37*, 235–240.

Milner, R.J., Lutton, G.G., **1986**. Dependence of *Verticillium lecanii* (Fungi: Hyphomycetes) on high humidities for infection and sporulation using *Myzus persicae* (Homoptera: Aphididae) as host. *Environ. Entomol. 15*, 380–382.

Milner, R.J., Mahon, R.J., **1985**. Strain variation in *Zoophthora radicans:* a pathogen on a variety of insect hosts in Australia. *J. Austral. Entomol. Soc. 24*, 195–198.

Milner, R.J., Staples, J.A., **1996**. Biological control of termites: results and experiences within a CSIRO project in Australia. *Biocontr. Sci. Technol. 6*, 3–9.

Milner, R.J., Holdom, D.G., Glare, T.R., **1984**. Diurnal patterns of mortality in aphids infected by entomophthoran fungi. *Entomol. Exper. Appl. 36*, 37–42.

Milner, R.J., Soper, R.S., Lutton, G.G., **1982**. Field release of an Israeli strain of the fungus *Zoophthora radicans* (Brefeld) Batko for biological control of *Therioaphis trifolii* (Monell) *f. maculata*. *J. Austral. Entomol. Soc. 21*, 113–118.

Møller, A.P., **1993**. A fungus infecting domestic flies manipulates sexual behaviour of its hosts. *Behav. Ecol. Sociobiol. 33*, 403–407.

Montserrat, M., CastaZé, C., Santamaria, S., **1998**. *Neozygites parvispora* (Zygomycota: Entomophthorales) causing an epizootic in *Frankliniella occidentalis* (Thysanoptera: Thripidae) in Spain. *J. Invertebr. Pathol. 71*, 165–168.

Moore, D., Douro-Kpindou, O.K., Jenkins, N.E., Lomer, C.J., **1996**. Effects of moisture content and temperature on storage of *Metarhizium flavoviride* conidia. *Biocontr. Sci. Technol.* 6, 51–61.

Morris, M.J., Roberts, S.J., Maddox, J.V., Armbrust, E.J., **1996**. Epizootiology of the fungal pathogen *Zoophthora phytonomi* (Zygomycetes: Entomophthorales) in field populations of alfalfa weevil (Coleoptera: Curculionidae) larvae in Illinois. *Great Lakes Entomol. 29*, 129–140.

Mullens, B.A., **1985**. Host age, sex, and pathogen exposure level as factors in the susceptibility of *Musca domestica* to *Entomophthora muscae*. *Entomol. Exp. Appl. 37*, 33–39.

Mullens, B.A., Rodriguez, J.L., **1985**. Dynamics of *Entomophthora muscae* (Entomophthorales: Entomopthoraceae) conidial discharge from *Musca domestica* (Diptera: Muscidae) cadavers. *Environ. Entomol. 14*, 317–322.

Nagahama, T., Sato, H., Shimazu, M., Sugiyama, J., **1995**. Phylogenetic divergence of the entomophthoralean fungi: evidence from nuclear 18S ribosomal RNA gene sequences. *Mycologia 87*, 203–209.

fNemoto, H., Aoki, J., **1975**. *Entomophthora floridana* (Entomophthorales: Entomophthoraceae) attacking the sugi spider mite, *Oligonychus honoensis* (Acarina: Tetranychidae) in Japan. *Appl. Entomol. Zool. 10*, 90–95.

Neuvéglise, C., Brygoo, Y., Riba, G., **1997**. 28s rDNA group-I introns: a powerful tool for identifying strains of *Beauveria brongniartii*. *Mol. Ecol. 6*, 373–381.

Nielsen, C., Eilenberg, J., Harding, S., Oddsdottir, E., Haldorsson, G., **2001**. Geographical distribution and host range of Entomophthorales infecting the green spruce aphid *Elatobium abietinum* Walker in Iceland. *J. Invertebr. Pathol. 78*, 72–80.

Nielsen, C., Hajek, A.E., Humber, R.A., Bresciani, J., Eilenberg, J., **2003**. Soil as an environment for winter survival of aphid-pathogenic Entomopththorales. *Biol. Contr. 28*, 92–100.

Noma, T., Strickler, K., **2000**. Effects of *Beauveria bassiana* on *Lygus hesperus* (Hemiptera: Miridae) feeding and oviposition. *Environ. Entomol. 29*, 394–402.

Obornik, M., Jirku, M., Dolezel, D., **2001**. Phylogeny of mitosporic entomopathogenic fungi: is the genus *Paecilomyces* polyphyletic? *Canad. J. Microbiol. 47*, 813–819.

Ogarkov, B.N., Ogarkova, G.R., **1999**. Influence of strain passages on virulence of entomopathogenic fungi from Moniliales. *Mikolog. Fitopatolog. 33*, 432–436.

Omoto, C., McCoy, C.W., **1998**. Toxicity of purified fungal toxin hirsutellin A to the citrus rust mite *Phyllocoprura oleivora*. *J. Invertebr. Pathol. 72*, 319–322.

Ouedraogo, R.M., Cusson, M., Goettel, M.S., Brodeur, J., **2003**. Inhibition of fungal growth in thermoregulating locusts, *Locusta migratoria*, infected by the fungus *Metarhizium anisopliae* var *acridum*. *J. Invertebr. Pathol. 82*, 103–109.

Ouedraogo, R.M., Kamp, A., Goettel, M.S., Brodeur, J., Bidochka, M.J., **2002**. Attenuation of fungal infection in thermoregulating *Locusta migratoria* is accompanied

by changes in hemolymph proteins. *J. Invertebr. Pathol. 81*, 19–24.

Ovchinnikov, Y.A., Ivanov, V.T., Mikhaleva, I.I., **1971**. The synthesis and some properties of beauvericin. *Tetrahed. Lett. 2*, 159–162.

Panter, C., Russell, R.C., **1984**. Rapid kill of mosquito larvae by high concentrations of *Culicinomyces clavisporus* conidia. *Mosq. News 44*, 242–244.

Pell, J.K., Wilding, N., **1994**. Preliminary caged field trials using the pathogen *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) against the diamond-back moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) in the U.K. *Biocontr. Sci. Technol. 4*, 71–75.

Pell, J.K., Macaulay, E.D.M., Wilding, N., **1993**. A pheromone trap for dispersal of the pathogen *Zoophthora radicans* Brefeld. (Zygomycetes: Entomophthorales) amongst populations of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Biocontr. Sci. Technol. 3*, 315–320.

Pell, J.K., Eilenberg, J., Hajek, A.E., Steinkraus, D.C., **2001**. Biology, ecology and pest management of Entomophthorales. In: Butt, T.M., Jackson, C.W., Magan, N. (Eds.), Fungi as Biocontrol Agents. CAB International Press, Wallingford, pp. 71–153.

Pereira, R.M., Roberts, D.W., **1990**. Dry mycelium preparations of entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. *J. Invertebr. Pathol. 56*, 39–46.

Pereira, R.M., Roberts, D.W., **1991**. Alginate and cornstarch mycelial formulations of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*. *J. Econ. Entomol. 84*, 1657–1661.

Pilarska, D., McManus, M., Hajek, A.E., Herard, F., Vega, F.E., *et al.*, **2000**. Introduction of the entomopathogenic fungus *Entomophaga maimaiga* Hum., Shim. & Sop. (Zygomycetes: Entomophthorales) to a *Lymantria dispar* (L.) (Lepidoptera: Lymantridae) population in Bulgaria. *J. Pest Sci. 73*, 125–126.

Pillai, J.S., **1971**. *Coelomomyces opifexi* (Coelomomycetaceae: Blastocladiales). Part 1. Its distribution and the ecology of infection pools in New Zealand. *Hydrobiologia 38*, 425–436.

Pillai, J.S., O'Loughlin, I.H., **1972**. *Coelomomyces opifexi* (Pillai and Smith) (Coeolomomycetaceae: Blastocladiales). 2. Experiments in sporangial germination. *Hydrobiologia 40*, 77–86.

Pope, T., Croxson, E., Pell, J.K., Godfray, H.C.J., Muller, C.B., **2002**. Apparent competition between two species of aphids via the fungal pathogen *Erynia neoaphidis* and its interaction with the aphid parasitoid *Aphidius ervi*. *Ecol. Entomol. 27*, 196–203.

Prior, C., Moore, D., Reed, M., LePatourel, G., Abraham, Y.J., **1992**. Reduction of feeding by the dessert locust, *Schistocerca gregaria*, after infection with *Metarhizium flavoviride*. *J. Invertebr. Pathol. 60*, 304–307.

Quesada-Moraga, E., Vey, A., **2003**. Intra-specific variation in virulence and *in vitro* production of macromolecular toxins active against locust among *Beauveria bassiana* strains and effects of *in vitro* and *in vivo* passage on these factors. *Biocontr. Sci. Technol. 13*, 323–340.

Quiot, J.-M., Vey, A., Vago, C., Pais, M., **1980**. Action antivirale d'une mycotoxine. Etude d'une toxine de l'hyphomycete *Metarhizium anisopliae* (Metsch.) Sorok. en culture cellulaire. *CR Acad. Sci. Ser. D (Paris) 291*, 763–766.

Rath, A.C., Carr, C.J., Graham, B.R., **1995**. Characterization of *Metarhizium anisopliae* strains by carbohydrate utilization (API50CH). *J. Invertebr. Pathol. 65*, 152–161.

Reddy, G.V.P., Furlong, M.J., Pell, J.K., Poppy, G.M., **1998**. *Zoophthora radicans* infection inhibits the response to and production of sex pheromone in the diamondback moth. *J. Invertebr. Pathol. 72*, 167–169.

Rehner, S.A., **2003**. A multigene phylogeny of *Beauveria*: new insights into species diversity, biogeography, host affiliation and life history. In: Proc. 36th Ann. Meet. Soc. Invertebr. Pathol., July 26–30, 2003. Vermont, USA, p. 102 (abstract only).

Reineke, A., Zebitz, C.P.W., **1996**. Protein and isoenzyme patterns among isolates of *Beauveria brongniartii* with different virulence to European cockchafer larvae (*Melolontha melolontha* L.). *J. Appl. Entomol. 120*, 307–315.

Reinecke, P., Andersch, W., Stenzel, K., Hartwig, J., **1990**. BIO 1020, a new microbial insecticide for use in horticultural crops. *Brighton Crop Prot. Conf.: Pests Disease 1*, 49–84.

Remaudiére, G., Latgé, J.-P., Michel, M.F., **1981**. Ecologie comparee des entomophthoracees pathogenes de pucerons en France littorale et continentale. *Entomophaga 26*, 157–178.

Robert, P.H., Riba, G., **1989**. Toxic and repulsive effect of spray *per os* and systemic application of destruxin E to aphids. *Mycopathologia 108*, 170–183.

Roberts, D.W., Hajek, A.E., **1992**. Entomopathogenic fungi as bioinsecticides. In: Leatham, G.F. (Ed.), Frontiers in Industrial Mycology. Chapman and Hall, New York, pp. 144–159.

Roy, H.E., Pell, J.K., **2000**. Interactions between entomopathogenic fungi and other natural enemies: implications for biological control. *Biocontr. Sci. Technol. 10*, 737–752.

Roy, H.E., Pell, J.K., Alderson, P.G., **2001**. Targeted dispersal of the aphid pathogenic fungus *Erynia neoaphidis* by the aphid predator *Coccinella septempunctata*. *Biocontr. Sci. Technol. 11*, 99–110.

Roy, H.E., Pell, J.K., Clark, S.J., Alderson, P.G., **1998**. Implications of predator foraging on aphid pathogen dynamics. *J. Invertebr. Pathol. 71*, 236–247.

Saik, J.E., Lacey, L.A., Lacey, C.M., **1990**. Safety of microbial insecticides to vertebrates-domestic animals and wildlife. In: Laird, M., Lacey, L.A., Davidson, E.W.

(Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, pp. 115–132.

Samson, R.A., **1995**. Constraints associated with taxonomy of biocontrol fungi. *Canad. J. Bot. 73* (Suppl. 1), S83–S88.

Samson, R.A., Evans, H.C., Latgé, J.-P., **1988**. Atlas of Entomopathogenic Fungi. Springer, Berlin.

Sandhu, S.S., Kinghorn, J.R., Rajak, R.C., Unkles, S.E., **2001**. Transformation system of *Beauveria bassiana* and *Metarhizium anisopliae* using nitrate reductase gene of *Aspergillus nidulans*. *Indian J. Exp. Biol. 39*, 650–653.

Sanjuan, T., Henao, L.G., Amat, G., **2001**. Spatial distribution of *Cordyceps* spp. (Ascomycotina: Clavicipitaceae) and its impact on the ants in forests of the Amazonia Colombian foothill. *Rev. Biol. Tropic. 49*, 945–955.

Screen, S.E., Hu, G., St. Leger, R.J., **2001**. Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. *J. Invertebr. Pathol. 78*, 260–266.

Screen, S., Bailey, A., Charnley, K., Cooper, R., Clarkson, J., **1997**. Carbon regulation of the cuticle-degrading enzyme PR1 from *Metarhizium anisopliae* may involve a trans-acting DNA-binding protein CRR1, a functional equivalent of the *Aspergillus nidulans* CREA protein. *Curr. Gen. 31*, 511–518.

Screen, S., Bailey, A., Charnley, K., Cooper, R., Clarkson, J., **1998**. Isolation of a nitrogen response regulator gene (nrr1) from *Metarhizium anisopliae*. *Gene 221*, 17–24.

Seif, A.I., Shaarawi, F.A., **2003**. Preliminary field trials with *Culicinomyces clavosporus* against some Egyptian mosquitoes in selected habitats. *J. Egyptian Soc. Parasitol. 33*, 291–304.

Shah, P.A., Goettel, M.S., **1999**. Directory of Microbial Control Products and Services. Society for Invertebrate Pathology, Gainesville.

Shah, P.A., Aebi, M., Tuor, U., **2000**. Infection of *Macrosiphum euphorbiae* with mycelial preparations of *Erynia neoaphidis* in a greenhouse trial. *Mycol. Res. 104*, 645–652.

Shaw, K.E., Davidson, G., Clark, S.J., Ball, B.V., Pell, J.K., *et al*., **2002**. Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*. *Biol. Control 24*, 266–276.

Shi, Z.M., **1998**. Technology for conidial preparation of *Beauveria bassiana*. In: Li, Y.W., Li, Z.Z., Liang, Z.Q., Wu, J.W., Wu, Z.K., Xu, Q.F. (Eds.), Study and Application of Entomogenous Fungi in China. Academic Periodical Press, Beijing, pp. 114–115.

Shimazu, M., **1979**. Resting spore formation of *Entomophthora sphaerosperma* Fresenius (Entomophthorales: Entomophthoraceae) in the brown plant hopper, *Nilaparvata lugens* (Stal) (Hemiptera: Delphacidae). *Appl. Entomol. Zool. 14*, 383–388.

Shimazu, M., Soper, R.S., **1986**. Pathogenicity and sporulation of *Entomophaga maimaiga* Humber, Shimazu, Soper and Hajek (Entomophthorales Entomophthoraceae) on larvae of the gypsy moth *Lymantria dispar* L. (Lepidoptera Lymantriidae). *Appl. Entomol. Zool. 21*, 589–596.

Siegel, J.P., **1997**. Testing the pathogenicity and infectivity of entomopathogens to mammals. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, pp. 325–336.

Siegel, J.P., Shadduck, J.A., **1990**. Safety of microbial insecticides to vertebrates – humans. In: Laird, M., Lacey, L.A., Davidson, E.W. (Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, pp. 101–114.

Silveira, R.D., dos Anjos, N., Zanuncio, J.C., **2002**. Natural enemies of *Coelomera lanio* (Coleoptera: *Chysomelidae*) in the region of Vicosa, Minas Gerais, Brasil. *Revista Biol. Trop. 50*, 117–120.

Silvie, P., Dedryver, C.A., Tanguy, S., **1990**. Experimental application of *Erynia neoaphidis* (Zygomycetes Entomophthorales) mycelium on aphid populations of lettuces in a commercial glasshouse study of the inoculum spread by isoenzyme profiles. *Entomophaga 35*, 375–384.

Six, D., Mullens, B., **1996**. Seasonal prevalence of *Entomophthora muscae* and introduction of *Entomophthora schizophorae* (Zygomycotina: Entomophthorales) in *Musca domestica* (Diptera: Muscidae) populations in California dairies. *Biol. Cont. 6*, 315–323.

Smith, S.M., Moore, D., Karanja, L.W., Chandi, E.A., **1999**. Formulation of vegetable fat pellets with pheromone and *Beauveria bassiana* to control the larger grain borer, *Prostephanus truncatus* (Horn). *Pest. Sci. 55*, 711–718.

Smits, J.E., Johnson, D.L., Lomer, C.J., **1999**. Pathological and physiological response of ring-necked pheasant chicks following dietary exposure to the fungus *Metarhizium flavoviride*, a biocontrol agent for locusts in Africa. *J. Wildlife Dis. 35*, 194–203.

Soper, R.S., Delyzer, A.J., Smith, L.F.R., **1976**. The genus *Massospora* entomopathogenic for cicadas. Part 2. Biology of *Massospora levispora* and its host *Okanagana rimosa* with notes on *Massospora cicadina* on the periodical cicadas. *Ann. Entomol. Soc. Am. 69*, 89–95.

Sopp, P.I., Gillespie, A.T., Palmer, A., **1989**. Application of *Verticillium lecanii* for the control of *Aphis gossypii* by a low-volume electrostatic rotary atomiser and a high-volume hydraulic sprayer. *Entomophaga 34*, 417–428.

St. Leger, R.J., **1993**. Biology and mechanisms of insect-cuticle invasion by deuteromycete fungal pathogens. In: Beckage, N.C., Thomson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects Vol. 2. Pathogens. Academic Press, New York, pp. 211–229.

St. Leger, R.J., **2001**. Notification of intent to release a transgenic strain of *Metarhizium anisopliae* (document submitted by the University of Maryland as required by FIFRA) www.epa.gov/pesticides/biopesticides/other-docs/release_notification.htm (16/08/01).

St. Leger, R., Screen, S., **2001**. Prospects for strain improvement of fungal pathogens of insects and weeds.

In: Butt, T.M., Jackson, C.W., Magan, N. (Eds.), Fungi as Biocontrol Agents. CABI Publishing, Wallingford, pp. 219–237.

St. Leger, R.J., Staples, R.C., Roberts, D.W., **1991**. Changes in translatable mRNA species associated with nutrient deprivation and protease synthesis in *Metarhizium anisopliae*. *J. Gen. Microbiol. 137*, 807–815.

St. Leger, R.J., Charnley, A.K., Cooper, R.M., Slots, J., **1986**. Enzymatic characterization of entomopathogens with the API ZYM system. *J. Invertebr. Pathol. 48*, 375–376.

St. Leger, R.J., Frank, D.C., Roberts, D.W., Staples, R.C., **1992a**. Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *Eur. J. Biochem. 204*, 991–1001.

St. Leger, R.J., Joshi, L., Bidochka, M.J., Roberts, D.W., **1996**. Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc. Natl Acad. Sci. USA 93*, 6349–6354.

St. Leger, R.J., Allee, L.L., May, R., Staples, R.C., Roberts, D.W., **1992b**. World-wide distribution of genetic variation among isolates of *Beauveria* spp. *Mycol. Res. 96*, 1007–1015.

St. Leger, R.J., Shimizu, S., Joshi, L., Bidochka, M.J., Roberts, D.W., **1995**. Co-transformation of *Metarhizium anisopliae* by electroporation or using the gene gun to produce stable GUS transformants. *FEMS Microbiol. Lett. 131*, 289–294.

Steenberg, T., Langer, V., Esbjerg, P., **1995**. Entomopathogenic fungi in predatory beetles (Col.: Carabidae and Staphylinidae) from agricultural fields. *Entomophaga 40*, 77–85.

Steinhaus, E.A., **1975**. Disease in a Minor Chord. Ohio State University Press, Columbus.

Steinkraus, D.C., Slaymaker, P.H., **1994**. Effect of temperature and humidity on formation, germination and infectivity of *Neozygites fresenii* (Zygomycetes: Neozygitaceae) from *Aphis gossypii* (Homoptera: Aphididae). *J. Invertebr. Pathol. 64*, 130–137.

Steinkraus, D.C., Geden, C.J., Rutz, D.A., **1993**. Prevalence of *Entomophthora muscae* (Cohn) Fresenius (Zygomycetes: Entomophthoraceae) in house flies (Diptera: Muscidae) on dairy farms in New York and induction of epizootics. *Biol. Contr. 3*, 93–100.

Steinkraus, D.C., Hollingworth, R.G., Boys, G.O., **1996**. Aerial spores of *Neozygites fresenii* (Entomophthorales: Neozygitaceae): density, periodicity, and potential role in cotton aphid (Homoptera: Aphididae) epizootics. *Environ. Entomol. 25*, 48–57.

Steinkraus, D.C., Howard, M.N., Hollingsworth, R.G., Boys, G.O., **1999**. Infection of sentinel cotton aphids (Homoptera: Aphididae) by aerial conidia of *Neozygites fresenii* (Entomophthorales: Neozygitaceae). *Biol. Cont. 14*, 181–185.

Strasser, H., Vey, A., Butt, T., **2000**. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? *Biocont. Sci. Technol. 10*, 717–735.

Suzuki, A., Kanaoka, M., Isogai, A., Murakoshi, S., Ichinoe, M., *et al.*, **1977**. Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. *Tetrahedron Lett. 25*, 2167–2170.

Swanson, D., **1997**. Economic feasibility of two techniques for production of a mycopesticide in Madagascar. *Mem. Entomol. Soc. Canad. 171*, 101–113.

Sweeney, A.W., **1981**. Preliminary field tests of the fungus *Culicinomyces* against mosquito larvae in Australia. *Mosq. News 43*, 290–297.

Sweeney, A.W., **1985**. The potential of the fungus *Culicinomyces clavisporus* as a biocontrol agent for medically important Diptera. In: Laird, M., Miles, J.W. (Eds.), Integrated Mosquito Control Methodologies. Academic Press, New York, pp. 269–284.

Tabashnik, B.E., **1994**. Evolution of resistance to *Bacillus thuringensis*. *Annu. Rev. Entomol. 39*, 47–79.

Tanada, Y., Kaya, H.K., **1993**. Insect Pathology. Academic Press, San Diego.

Terry, B.J., Liu, W.C., Cianci, C.W., Proszynski, E., Fernandes, P., *et al.*, **1992**. Inhibition of herpes simplex virus type 1 DNA polymerase by the natural product oosporein. *J. Antibiotics 45*, 286–288.

Thomas, M.B., Blanford, S., Lomer, C.J., **1997**. Reduction of feeding by the variegated grasshopper, *Zonocerus variegatus*, following infection by the fungal pathogen, *Metarhizium flavoviride*. *Biocontr. Sci. Technol. 7*, 327–334.

Thomas, M.B., Watson, E.L., Valverde-Garcia, P., **2003**. Mixed infections and insect-pathogen interactions. *Ecol. Lett. 6*, 183–188.

Thomsen, L., Eilenberg, J., **2000**. Time-concentration mortality of *Pieris brassicae* (Lepidoptera: Pieridae) and *Agrotis segetum* (Lepidoptera: Noctuidae) larvae from different destruxins. *Environ. Entomol. 29*, 1041–1047.

Thorvilson, H.G., Pedigo, L.P., Lewis, L.C., **1985**. Soybean leaf consumption by *Nomuraea rileyi* (Fungi: Deuteromycotina)-infected *Plathypena scabra* (Lepidoptera: Noctuidae) larvae. *J. Invertebr. Pathol. 46*, 265–271.

Todorova, S.I., Cote, J.C., Martel, P., Coderre, D., **1994**. Heterogeneity of two *Beauveria bassiana* strains revealed by biochemical tests, protein profiles and bioassays on *Leptinotarsa decemlineata* (Col.: Chrysomelidae) and *Coleomegilla maculata lengi* (Col.: Coccinellidae) larvae. *Entomophaga 39*, 159–169.

Toscano, N.C., Reeves, E.L., **1973**. Effect of *Aspergillus flavus* mycotoxin on *Culex* mosquito larvae. *J. Invertebr. Pathol. 22*, 55–59.

Trzebitzky, C., Lochelt, S., **1994**. Wiedererkennung rückisolierter Stämme von *Beauveria brongniartii* (Sacc.) Petch aus freilandversuchen durch isoenzymanalyse. *Z. Pflanzenkr. Pflanzensch. 101*, 519–526.

Typas, M.A., Mavridou, A., Kouvelis, V.N., **1998**. Mitochondrial DNA differences provide maximum intraspecific polymorphism in the entomopathogenic fungi

Verticillium lecanii and *Metarhizium anisopliae*, and allow isolate detection/identification. In: Bridge, P., Couteaudier, Y., Clarkson, J. (Eds.), Molecular Variability of Fungal Pathogens. CAB International Press, Wallingford, pp. 227–237.

Tyrrell, D., **1987**. Induction of periodic mortality in insect larvae infected with *Entomophaga aulicae*. In: Ann. Meeting Soc. Invertebr. Pathol. Gainsville, Fla., p. 53.

Tyrrell, D., **1988**. Survival of *Entomophaga aulicae* in dried insect larvae. *J. Invertebr. Pathol. 52*, 187–188.

Upadhyay, R.K. (Ed.), **2003**. Advances in Microbial Control of Insect Pests. Kluwer Academic/Plenum, New York.

Uziel, A., Kenneth, R.G., **1991**. Survivial of primary conidia and capilliconidia at different humidities in *Erynia* (subgen. *Zoophthora*) spp. and *Neozygites fresenii* (Zgyomycotina: Entomophthorales), with special emphasis on *Erynia radicans*. *J. Invertebr. Pathol. 58*, 118–126.

Veen, K.H., **1968**. Reserches sur la maladie, due a *Metarrhizium anisopliae* chez le criquet perlerin. *Med. Landbouwbogeschool Wageningen 68*, 1–77.

Vega, F.E., Dowd, P.F., Lacey, L.A., Pell, J.K., Jackson, D.M., *et al*., **2000**. Dissemination of beneficial microbial agents by insects. In: Lacey, L.A., Kaya, H.K. (Eds.), Field Manual of Techniques in Invertebrate Pathology. Kluwer, Dordrecht, pp. 153–177.

Vestergaard, S., Cherry, A., Keller, S., Goettel, M., **2003**. Hyphomycete fungi as microbial control agents. In: Hokkanen, H.M.T., Hajek, A.E., (Eds.), Environmental Impacts of Microbial Insecticides. Kluwer, Dordrecht, pp. 35–62.

Vey, A., Hoagland, R.E., Butt, T.M., **2001**. Toxic metabolites of fungal biocontrol agents. In: Butt, T., Jackson, C., Magan, N. (Eds.), Fungal Biocontrol Agents. CABI Publishing, Wallingford, pp. 311–346.

Viaud, M., Couteaudier, Y., Levis, C., Riba, G., **1996**. Genome organization in *Beauveria bassiana*: electrophoretic karyotype, gene mapping, and telomeric fingerprint. *Fungal Genet. Biol. 20*, 175–183.

Viaud, M., Couteaudier, Y., Riba, G., **1998**. Molecular analysis of hypervirulent somatic hybrids of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria sulfurescens*. *Appl. Environ. Microbiol. 64*, 88–93.

Vicentini, S., Faria, M., de Oliveira, M.R.V., **2001**. Screening of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) isolates against nymphs of *Bemisia tabaci* (Genn.) biotype B (Hemiptera: Aleyrodidae) with description of a new bioassay method. *Neotrop. Entomol. 30*, 97–103.

Vidal, C., Fargues, J., Rougier, M., Smits, N., **2003**. Effect of air humidity on the infection potential of hyphomycetous fungi as mycoinsecticides for *Trialeurodes vaporariorum*. *Biocontr. Sci. Technol. 13*, 183–198.

Villacarlos, L.T., Meija, B.S., Keller, S., **2003**. *Entomophthora leyteensis* Villacarlos & Keller sp.nov. (Entomophthorales: Zygomycetes) infecting *Tetraleurodes acaciae* (Quaintance) (Insecta, Hemiptera: Aleyrodidae) a recently introduced whitefly on *Gliricidia sepium* (Jaq.) Walp. (Fabaceae) in the Philippines. *J. Invertebr. Pathol. 83*, 16–22.

Villani, M.G., Allee, L.L., Diaz, A., Robbins, P.S., **1999**. Adaptive strategies of edaphic arthropods. *Annu. Rev. Entomol. 44*, 233–256.

Ward, M.D., Sailstad, D.M., Selgrade, M.K., **1998**. Allergic responses to the biopesticide *Metarhizium anisopliae* in Balb/c mice. *Toxicol. Sci. 45*, 195–203.

Washino, R.K., **1981**. Biocontrol of mosquitoes associated with California rice fields with special reference to the recycling of *Lagenidium giganteum* Couch and other microbial agents. In: Laird, M. (Ed.), Biocontrol of Medical and Veterinary Pests. Praeger, New York, pp. 122–139.

Watson, D.W., Mullens, B.A., Petersen, J.J., **1993**. Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales). *J. Invertebr. Pathol. 61*, 10–16.

Weseloh, R.M., Andreadis, T.G., **1997**. Persistence of resting spores of *Entomophaga maimaiga*, a fungal pathogen of the gypsy moth, *Lymantria dispar*. *J. Invertebr. Pathol. 69*, 195–196.

Wraight, S.P., Carruthers, R.I., **1999**. Production, delivery and use of mycoinsecticides for control of insect pests of field crops. In: Hall, F.R., Menn, J.J. (Eds.), Methods in Biotechnology, Vol. 5: Biopesticides: Use and Delivery. Humana Press, Totowa, pp. 233–269.

Wraight, S.P., Jackson, M.A., de Lock, S.L., **2001**. Production, stabilization and formulation of fungal biocontrol agents. In: Butt, T.M., Jackson, C.W., Magan, N. (Eds.), Fungi as Biocontrol Agents. CABI Publishing, Wallingford, pp. 253–287.

Yamamoto, M., Aoki, J., **1983**. Periodicity of conidial discharge of *Erynia radicans*. *Trans. Mycol. Soc. Japan 24*, 487–496.

Zabra, A., Piatkowski, J., Greb-Markiewicz, B., Bujak, J., **1996**. Secondary metabolites produced by entomopathogenic fungi of the genera *Zoophthora* and *Paecilomyces*. *Bull-OILB-SROP 19*, 196–199.

Zare, R., Gams, W., **2001**. A revision of *Verticillium* section *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium*. *Nova Hedwigia 73*, 1–50.

Zare, R., Gams, W., Culham, A., **2000**. A revision of *Verticillium* sect. *Prostrata*. I. Phylogenetic studies using ITS sequences. *Nova Hedwigia 71*, 465–480.

Zattau, W. C., McInnis, T., Jr., **1987**. Life cycle and mode of infection of *Leptolegnia chapmanii* (Oomycetes) parasitizing *Aedes aegypti*. *J. Invertebr. Pathol. 50*, 134–145.

Zimmermann, G., **1986**. The "*Galleria* bait method" for detection of entomopathogenic fungi in soil. *Z. Ang. Entomol. 102*, 213–215.

Zimmermann, G., Papierok, B., Glare, T., **1995**. Elias Metschnikoff, Elie Metchnikoff or Ilya Ilich Mechnikov (1845–1916): a pioneer in insect pathology, the first describer of the entomopathogenic fungus *Metarhizium anisopliae* and how to translate a Russian name. *Biocont. Sci. Technol. 5*, 527–530.

6.12 Pheromones: Function and Use in Insect Control

T C Baker and J J Heath, Pennsylvania State University, University Park, PA, USA

6.12.1. Introduction

6.12.1.1. Basic and Applied Research on Pheromones

During the 45 years since the identification of the first pheromone by Butenandt *et al.* (1959), insect pheromones have achieved significant stature in agricultural and urban pest management systems worldwide. If a pest species is known to use a sex pheromone to communicate, one of the most important initial efforts in trying to establish a workable pest management strategy is to explore that pheromone system to try to develop a useful synthetic pheromone attractant.

Since the mid-1960s, basic research has advanced our understanding of the biochemical, neurophysiological, and behavioral underpinnings of insect pheromone communication systems to a degree that none of us in the field could have years ago imagined. However, these advances have to this point not played a significant role in the development of useful and commercially successful applied pheromone tools for pest management. Basic pheromone research has provided valuable after-the-fact insight into why certain pest management applications of pheromones may be working better than others. This insight is now ready to be transformed into predictive knowledge that can, and should, be used to improve existing applied pheromone technologies and to create new ones.

The applied pheromone technologies and products currently in use were developed by applied entomologists using repeated trial-and-error field trials that have shown what works and what does not work. Improved pheromone dispenser technologies resulted from engineering and manufacturing advances that were made mainly by research and development teams at commercial companies. Chemical ecology techniques, which are at the heart of any initial investigations aimed at isolating

and identifying pheromones, have advanced in sophistication and sensitivity over the years, and have played an essential role in identifying several thousand pheromone components. Most pheromone identifications, however, have been initiated by a need for pest management tools and not for basic exploration of communication systems.

Basic research on insect pheromone olfaction has provided knowledge about animal olfaction that arguably has been as valuable as similar lines of research performed on vertebrates (Hildebrand and Sheppard, 1997). This research has been illuminating and exhilarating; now, many of the new insights that have emerged need to be reconnected with efforts toward application.

There are many reasons why insects have received such intense focus for research on pheromones. Certainly a major reason, apart from the simplicity and accessibility of their behavioral, olfactory, and pheromone production systems, is that so many insect species are pests. Also, society's awareness of the overuse of insecticides and the environmental damage they were imparting accelerated during the mid-1960s, and along with it emerged a hope that pheromones might be a safe and effective alternative to insecticides. It is important in this chapter to bring awareness to readers of the successful commercial use and grower acceptance of pheromone-based products. Many who have had the privilege to perform basic pheromone research realize that to a large degree we have been able to do so because of the exciting promise that pheromones presented to society early on that they might replace existing insecticides.

The purpose of this chapter is, however, not to merely summarize the many commercial successes that pheromones have had or to note the inroads they have made into being accepted as reliable tools for population suppression of key pests. Rather, it is to bring together some of the basic knowledge about insect behavior and olfaction from the past 30-plus years of knowledge the authors have gained from the successful field uses of pheromones over the same period. A few new ideas are also suggested for increasing the efficacy and economic competitiveness of pheromones in some pest management arenas.

Successful use of pheromones in insect control depends upon successfully controlling insect behavior through the emission of synthetic pheromone blends. The behaviors that are most amenable to manipulation are those that involve long-distance attraction. There is more power over the target insects with long-range attraction because human control over the behavior reaches far out into the field from every pheromone emission source. Thus, our focus is mainly on sex pheromones in this chapter. It is evident that basic and applied research on sex pheromones historically has involved mainly two groups, the Lepidoptera and Coleoptera, and therefore dwelt mostly on these two taxa. Although this emphasis is necessary, many of the principles developed from research on these two insect orders should be transferable to the pheromone systems of other groups.

6.12.2. Brief Overview of Insect Sex Pheromone Communication Systems

Sex pheromones in insects include a variety of molecules from large, but volatile, aliphatic molecules to small cyclic monoterpenoids (**Figure 1**). Although the vapor pressures of many of these compounds are very low (**Table 1**), many behavioral and electrophysiological experiments over the years have shown that insects do indeed smell these compounds even at extremely low concentrations (Kaissling, 1974, 1987, 1990; Priesner *et al*., 1986; Szöcs *et al*., 1989; Cowles *et al*., 1996; Minor and Kaissling, 2003). Much of the information to follow has come more or less directly from an excellent recent book edited by Hardie and Minks (1999) that reviews nonlepidopteran pheromones.

6.12.2.1. Lepidoptera

Lepidopteran pheromones were the first to be widely studied and include a huge collection of mostly female-based pheromones. Female moths typically produce long-range, fatty acid-derived molecules (e.g., (**11**) in **Figure 1**) that function over long distances, whereas male moths tend to produce close-range courtship compounds that are often very similar in structure to plant secondary metabolites (e.g., (**3**) in **Figure 1**) (Baker, 1989a; Birch *et al*., 1990). In contrast to moths, butterflies tend to use color and motion cues more than pheromones to find mates. Butterfly pheromones are generally restricted to close-range or contact pheromones (Baker, 1989a). Moth pheromones have been cataloged in The Pherolist, a free database.

The majority of moth pheromones identified thus far are blends of 10- to 18-carbon-long straight-chain primary alcohols, acetates, or aldehydes. However, one interesting second major class of moth pheromone components is found in noctuids, geometrids, arctiids, and lymantriids comprises polyene hydrocarbons and epoxides (e.g., (**10**) and (**13**) in **Figure 1**) (review: Millar, 2000). For the majority of moth species, after synthesis of the fatty acyl chain to either 16 or 18 carbons in length, these pheromone

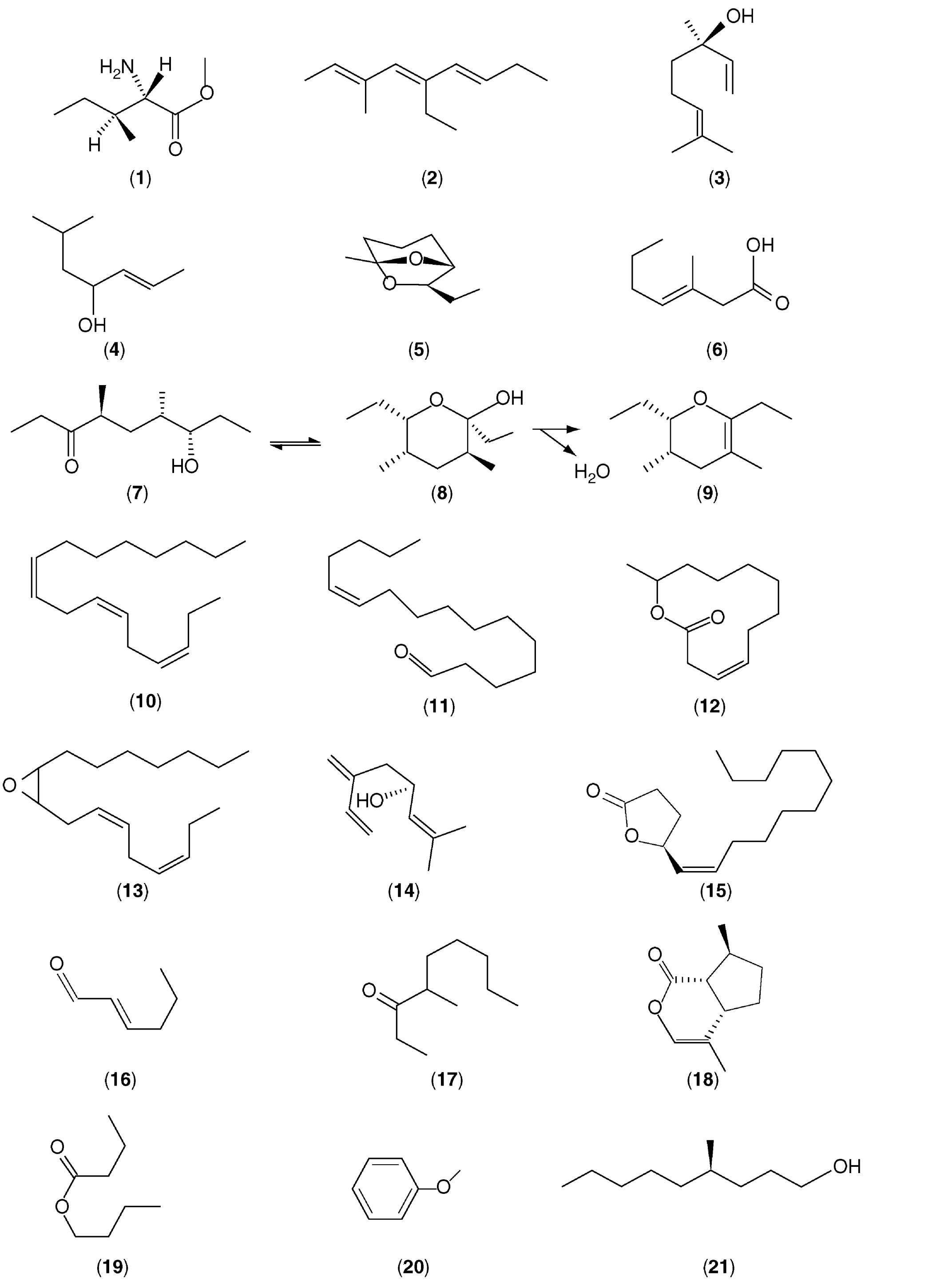


Figure 1 Pheromone component structures for various insects of the orders Lepidoptera, Trichoptera, Coleoptera, and Hemiptera. (**1**), L-isoleucine methyl ester; (**2**), (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene; (**3**), *R*-linalool; (**4**), (2*E*,4*S*)-6-methyl-4-heptenol; (**5**), (1*R*,5*S*,7*R*)-*exo*-brevicomin; (**6**), (*E*)-3-methyl-3-heptenoic acid; (**7**), (4*S*,6*S*,7*S*)-serricornin; (**8**), serricornin (cyclic); (**9**), anhydroserricornin; (**10**), (*Z*,*Z*,*Z*)-3,6,9-heptadecatriene; (**11**), (*Z*)-11-hexadecenal; (**12**), (*Z*)-3-dodecen-11-olide; (**13**), (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheptadecadiene; (**14**), (*S*)-(+)-ipsdienol; (**15**), (*S*,*Z*)-5-(+)-(1-decenyl)oxacyclopentan-2-one; (**16**), (*E*)-2-hexenal; (**17**), 4-methylnonan-3-one; (**18**), (+)-(4a*S*,7*S*,7a*R*)-nepetalactone; (**19**), butyl butyrate; (**20**), anisole; (**21**), (*R*)-4-methyl-1-nonanol.

Table 1 Vapor pressures of some insect pheromone components; vapor pressures of some plant-related volatiles are also listed

Name[a]	*Vapor pressure at 25°C*[b]	*Registry number*[c]	*Insect order*
(*E*,*Z*)-3,13-18:OH	0.000000557	66410-28-4	Lepidoptera
(*Z*)-11-16:COOH	0.00000187	2271-34-3	Lepidoptera, precursor
(*Z*)-9-16:COOH	0.00000282	373-49-9	Lepidoptera, precursor
Linoleic acid	0.00000354	60-33-3	
(*Z*)-11-16:Ac	0.0000493	34010-21-4	Lepidoptera
(*Z*)-11-16:OH	0.0000597	56683-54-6	Lepidoptera
(*Z*)-9-14:Ac	0.000136	16725-53-4	Lepidoptera
(*Z*)-11-16:Ald	0.000253	53939-28-9	Lepidoptera
(*Z*)-11-14:OH	0.000445	34010-15-6	Lepidoptera
(*Z*)-9-14:Ald	0.00135	53939-27-8	Lepidoptera
(*Z*)-11-14:Ald	0.00213	35237-64-0	Lepidoptera
Linalool	0.0905	78-70-6	Coleoptera, Heteroptera
Hexanoic acid	0.158	142-62-1	Coleoptera
Pentanoic acid	0.452	109-52-4	Coleoptera
(*Z*)-3-hexenol	1.04	928-96-1	Lepidoptera
Limonene	1.54	138-86-3	
β-Ocimene	1.56	13877-91-3	

[a]Abbreviated chemical names, e.g., ''(*E*,*Z*)-3,13, 18:OH'' is shortened form of (*E*,*Z*)-3,13-octadecadien-1-ol. Ac, acetate; OH, alcohol; Ald, aldehyde.
[b]Vapor pressures were taken from Scifinder Scholar.
[c]Registry numbers are given to allow searching for additional properties in chemical abstract services.

component precursors begin to achieve some species–specific structural differences that are imparted by one or more desaturases that place double bonds at specific locations in the fatty acyl chain (Tillman *et al*., 1999; Roelofs and Rooney, 2003). The desaturation step is, depending on the species, either preceded or followed by β-oxidation to reduce the chain length of the molecule. Merely reversing the two-step sequence in which these two enzyme systems work creates most of the major differences in pheromone component structures in moths (Roelofs and Rooney, 2003). Final species–specific structural differences result from the final biosynthetic step, conversion of the fatty acyl molecule to its active functional group by reductases and oxidases.

A key neuropeptide named pheromone biosynthesis activating neuropeptide (PBAN) has been found in females of all pheromone-producing moth species thus far. PBAN stimulates both the behavioral release of pheromone (i.e., calling behavior) and the biosynthesis of the pheromone components (Nation, 2002). A receptor for PBAN has recently been characterized in *Helicoverpa zea* by Choi *et al*. (2003) and is a membrane-bound heptahelical G protein-coupled receptor (i.e., a seven-transmembrane GPCR). The PBANs of most species are extremely similar in structure, and injection of one species' PBAN into the female of a second species often causes enhanced pheromone production in the injected female, even across families (Raina and Menn, 1987).

6.12.2.2. Trichoptera

Trichopterans are closely related to Lepidoptera. In fact, on a family level phylogeny they form a monophyletic group with the lepidopterans; that is, they are the sister group to Lepidoptera (Ross, 1964). Trichopteran pheromones, like their lepidopteran counterparts, are produced by females and attract males from a distance. However, the limited number of trichopteran sex pheromone components that have been identified do not share the most common features of their lepidopteran counterparts. Trichopteran pheromone components are, however, similar in structure to those of some primitive lepidopterans of the family Eriocraniidae (Zhu *et al*., 1995; Kozlov *et al*., 1996). In only three species have putative pheromones been shown to have behavioral activity in the trichopterans (Löfstedt *et al*., 1994; Bjostad *et al*., 1996) and all three are produced by the paired set of sternal glands that secrete their products onto the fifth abdominal sclerite through a dorsolateral opening on each side of the sclerite (Ivanov and Melnitsky, 1999). Bjostad *et al*. (1996) identified 6-methylnonan-3-one (similar to (**17**) in **Figure 1**) as the major component of the sex pheromone of *Hesperophylax occidentalis*, and Löfstedt *et al*. (1994) found nonan-2-one and heptan-2-one to be the major components of the sex pheromones of *Hydropsyche angustipennis* and *Rhyacophila fasciata*, respectively.

Although nothing is known yet about the behavioral impact of the differences in pheromone

chirality that these trichopteran pheromone components have, chirality has been shown to be important to the activity of receptor neurons in individual olfactory sensilla (Larsson and Hansson, 1998). In some species these compounds had been implicated as being defensive secretions (see references in Ivanov and Melnitsky, 1999), but the first behavioral studies definitively demonstrated their roles as sex pheromones (Wood and Resh, 1984; Resh and Wood, 1985). No information is available regarding biosynthesis of these pheromones or endocrine regulation. Work on this group may not proceed as quickly as it has in others because trichopterans are not agricultural pests. However, they are important indicators of environmental quality (Brigham and Sadorf, 2001) and monitoring their presence with pheromone traps may be more convenient and efficient than stream sampling.

6.12.2.3. Coleoptera

Coleopteran pheromones have mainly been identified for scarabs (Scarabaeidae) (Leal, 1999), sap beetles (Nitidulidae) (Bartelt, 1999a), scolytids (Scolytidae) (Schlyter and Birgersson, 1999), weevils (Curculionidae) (Bartelt, 1999b), and stored-product beetles (Anobiidae, Dermestidae, Bruchidae, Tenebrionidae, Bostrichidae, Cucujidae, and Silvanidae) (Plarre and Vanderwel, 1999). Recently, much of the literature regarding beetle pheromones and all other insect pheromones has been compiled into one site (Pherobase). This site is likely the most extensive database of pheromones of insects currently freely available.

6.12.2.3.1. Scarabaeidae Scarab pheromone components are generally of four types: fatty acid-derived lactones ((**15**), **Figure 1**); straight-chain unsaturated aldehydes, alcohols, ketones, and esters (similar to 16, but with nine carbon atoms; **Figure 1**); terpenoids ((**3**), **Figure 1**); amino acid derivatives ((**1**), **Figure 1**); or phenolics ((**20**), **Figure 1**). They are produced by either unicellular epidermal glands on the posterior ventral sclerites or by an everted soft tissue sac on the last few segments of the abdomen, similar to the glands of higher lepidopterans. There seems to be an interesting segregation of chemistry among these two gland types that is also manifested at the subfamily level. The everted soft tissue sacs produce mainly terpenoids or amino acid-derived compounds and are associated with the Melolonthinae, whereas the unicellular glands produce the fatty acid derived compounds and are mainly associated with the Rutelinae (Leal, 1999). Not all the Melolonthinae use eversible glands; females in the genus *Macrodactylus* have never been observed everting a gland from the tip of their abdomens (Heath *et al.*, 2002). The lack of obvious calling behavior as well as the lack of cuticular pores in this genus may present a unique opportunity to study the evolution of sex pheromones in melolonthine beetles. In some scarabs the pheromone seems to be synergized by the presence of plant volatiles. For example, the Japanese beetle pheromone (Tumlinson *et al.*, 1977) ((**15**), **Figure 1**) captures twice as many beetles when selected plant compounds are blended and coemitted with it (Klein *et al.*, 1981).

6.12.2.3.2. Nitidulidae Sap beetle (Nitidulidae) pheromones are mainly known as a result of a series of studies conducted by Bartelt and colleagues (Bartelt, 1999a) on species in the genus *Carpophilus*. The pheromones, which attract both sexes, are produced by male glandular cells in the posterior of the abdomen that secrete their contents into tracheal tubes. The chemical structures of the pheromone molecules are of polyunsaturated, multibranched hydrocarbons ((**2**), **Figure 1**). Seventy-five times more *Carpophilus hemipterus* beetles are attracted to the blend of pheromone plus food odor than to either pheromone or food odor alone (Bartelt *et al.*, 1994). In fact, a high level of attraction synergism between food and pheromone odors exists throughout the genus *Carpophilus* (Bartelt, 1999a). These pheromone components seem to be biosynthesized through the use of acetate, propionate, and butyrate acyl units (Petroski *et al.*, 1994; Bartelt and Weisleder, 1996). The initial condensation reaction seems to differ significantly from the typical fatty acid synthesis with malonyl CoA (Petroski *et al.*, 1994). In some methyl-branched pheromones, the branched units are derived ultimately from amino acids (Tillman *et al.*, 1999). Possible hormonal and neurohormonal mechanisms regulating initiation and termination of biosynthesis remain unexplored at present.

6.12.2.3.3. Curculionidae A significant number of weevil (Curculionidae) pheromones have been identified (review: Bartelt, 1999b), and many of these have contributed to highly successful integrated pest management (IPM) of weevil pest species through their effectiveness in mass trapping (see Section 6.12.5.3.1). The majority of the known systems involved are male-emitted and the pheromones are often strong enough to attract both sexes from tens of meters away (Bartelt, 1999b and references therein). The structures of the pheromone molecules seem very diverse. However, they share a common mode of action in that, as in the *Carpophilus* sap beetles, the attraction of weevils by the pheromone is synergized by coemission of plant compounds. For example, all six palm weevil pheromones of

Rhynchophorus spp., (e.g., (**4**), **Figure 1**), are synergized by the presence of plant material such as sugarcane stalks (Oehlschlager *et al.*, 1993). In the Andean potato weevil, *Premnotrypes suturicallus*, a severe pest in the Peruvian Andes, field-collected *P. suturicallus* females must be actively feeding on a host plant to attract males (J.J. Heath *et al.*, unpublished data). It is not well known how or where weevil pheromones are produced, but there is evidence from *R. palmarum* males that they are synthesized in glands in the prothorax and exuded from the mouth (Sánchez *et al.*, 1996). Studies on the mechanisms of biosynthesis and regulation are also lacking.

6.12.2.3.4. Scolytidae Bark beetles (Scolytidae) pheromones were among the earliest studied and identified in insects (Silverstein *et al.*, 1966, 1968; Kinzer *et al.*, 1969; Bedard *et al.*, 1970). They were also, along with lepidopterous pheromones, the group of pheromones that showed the most promise in large-scale field tests to be effective in suppressing or manipulating populations of the devastating pests present in this family (Bedard and Wood, 1981; Lanier, 1981).

Recent work on this group (review: Schlyter and Birgersson, 1999) has not completely resolved a long-standing discussion of whether bark beetle pheromones are biosynthesized *de novo* or are just slightly modified (detoxified) host compounds. In at least two genera, *Tomicus* and *Dendroctonus*, it is the females that locate a host tree, begin boring galleries for oviposition, and in the process produce a long-distance pheromone that results from detoxified host compounds and host resin constituents. *Dendroctonus ponderosae* females release (−)-trans-verbenol, which attracts males. Once males arrive they produce (+)-*exo*-brevicomin ((**5**), **Figure 1**), which acts as an aggregation pheromone (i.e., both sexes are attracted). *Tomicus minor* females use a similar system.

Females of *Dendroctonus brevicomis*, alternatively, start producing (+)-*exo*-brevicomin as the first-arriving beetles burrow into the host tree to initiate galleries. Once they have reached the phloem tissue a resin containing myrcene, a hydrocarbon similar in structure to linalool ((**3**), **Figure 1**), begins to flow. The blend of these two compounds is attractive to males. Once males arrive they begin producing (−)-frontalin. This three-component blend is now attractive to both males and females. It has also been shown that myrcene is converted to (+)-ipsdienol ((**14**), **Figure 1**) simply by passing myrcene-laden air over *Ips paraconfusus* (Byers, 1995 and references therein). Male *D. brevicomis* and many other *Ips* spp. also produce (+)-ipsdienol, which has an antagonistic effect in field studies with *D. brevicomis* (Bertram and Paine, 1994).

Regardless of whether bark beetle pheromones are synthesized entirely *de novo* or their the mode of production involves the beetle making relatively small modifications of existing plant-derived compounds, one feature remains common throughout the bark beetles: pheromone-based attraction is synergized by coemission of plant volatiles. This is true for species in the well-studied genera *Dendroctonus*, *Ips*, and *Scolytus* as well as for species in other genera such as *Conophthorus coniperda* in which pioneering females produce 2*R*,5*S*-pityol while burrowing into the tree. Attraction is synergized by α-pinene and other monoterpenes (Schlyter and Birgersson, 1999).

6.12.2.3.5. Stored-product beetle pests Stored-product pests, including the families Anobiidae, Dermestidae, Bruchidae, Tenebrionidae, Bostrichidae, Cucujidae, and Silvanidae, have an interesting array of pheromones aptly reviewed by Plarre and Vanderwel (1999). These include both female-produced pheromones that only attract males, and also male-produced aggregation pheromones. Anobiid females produce cyclic compounds that can rapidly interconvert between different forms. For example, the acyclic form of serricornin ((**7**), **Figure 1**) is in equilibrium with the cyclic form ((**8**), **Figure 1**), which can then lose water to form anhydroserricornin ((**9**), **Figure 1**) (Mori *et al.*, 1984). Dermestid females produce unsaturated fatty acids and derivatives thereof that serve as pheromone components in this group (Silverstein *et al.*, 1967). The dermestid pheromone components are similar to those of moths ((**11**), **Figure 1**). Bruchid females emit short-chain, branched, unsaturated acids as their pheromone components that attract males ((**6**), **Figure 1**).

In most of the species of stored-product beetle pests, pheromones are produced by glands on or between abdominal segments. The genus *Tribolium* may be an exception. The male-produced aggregation pheromone, 4,8-dimethyldecanal (Plarre and Vanderwel, 1999 and references therein), in *T. castaneum* is apparently produced in glands located on the ventral side of the prothoracic femurs (Faustini *et al.*, 1981). The production of the *Tenebrio molitor* female-produced pheromone, (*R*)-4-methyl-1-nonanol ((**21**), **Figure 1**), is regulated by juvenile hormone III (Menon, 1976). The bostrychids use branched esters very similar in structure to the pheromones of some heteroptera (e.g., (**19**) in **Figure 1**). Cucujidae and Silvanidae use macrocyclic lactones likely produced by fatty acid biosynthesis ((**12**), **Figure 1**).

6.12.2.4. Homoptera

Knowledge of homopteran sex attractant pheromones comes from two agriculturally important groups, the scale insects and the aphids. Most of the species of aphids studied thus far use a blend of nepetalactone and nepetalactol compounds as their sex pheromone (Pickett *et al*., 1992; Hardie *et al*., 1994). For instance, the sex pheromone of the aphid *Aphis fabae* is a 29 : 1 blend of (+)-(4a*S*,7*S*,7a*R*)-nepetalactone and (−)-(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Pickett *et al*., 1992). These are monoterpenoids in the cyclopentanoid series ((**18**), **Figure 1**) and are produced by females in glands on the tibiae of the hind legs (Pettersson, 1971; Hardie *et al*., 1999). Although the nepetalactones and related compounds are found in plants of the genus *Nepeta*, biosynthesis and regulation are not well understood in either kingdom (Hardie *et al*., 1999). There is considerable research effort at present being made to explore the pheromone systems of an increasing number of aphid species for use in pest management. There is usually only one sexually communicating generation, and so this generation represents a vulnerable target for aphid control via pheromones. If mating and hence egg-laying by the sexually reproducing females could be disrupted with pheromones, there could possibly be significant population suppression effects.

One other aspect of aphid pheromone communication is their use of alarm pheromones. Interestingly, the main component of most aphid alarm pheromones is the branched chain sesquiterpene (*E*)-β-farnesene, whereas the alarm pheromone for the spotted alfalfa aphid is the cyclic sesquiterpene (−)-germacrene-A (Hardie *et al*., 1999). Dawson *et al*. (1987) found that (*E*)-β-farnesene, the alarm pheromone of the turnip aphid, *Lipaphis erysimi*, was not effective unless isothiocyanates were added to the blend, suggesting a host-plant volatile, context-dependent effect of (*E*)-β-farnesene.

6.12.2.5. Heteroptera

Heteropterans produce a wide array of compounds, often in unusually large quantities compared with pheromones of other groups of insects (reviews: Aldrich, 1995; McBrien and Millar, 1999). These compounds are produced by a number of different glands that can be found in at least six different parts of the body, including metathoracic glands, dorsal abdominal glands, sternal glands, unicellular glands, subrectal glands, and ventral abdominal glands (Aldrich, 1995). Metathoracic glands are mostly noted for their role in production of defensive secretions in adult bugs, but these glands are known to be sexually dimorphic and do produce pheromones in some species. The secretions from this paired set of glands most often contaminate pheromone extractions. Dorsal abdominal glands most often produce the defensive secretions of nymphs and then they disappear by adulthood. However, these have sometimes been shown to remain functional in adults where they then are involved in pheromone production. The sex pheromone systems of heteropterans are generally male-emitted, but there are a few examples of female-emitted systems. Aldrich (1995), in his comprehensive review of the diversity of heteropteran pheromones, has highlighted the need for focused research in this group that includes many important pest species as well as beneficial predatory species that are useful biological control agents.

Heteropteran pheromones tend to be either a blend of esters (e.g., butyl butyrate, (**19**), **Figure 1**) or an ester and a short-chain aldehyde or alcohol (Smith *et al*., 1991; Leal *et al*., 1996). These esters, aldehydes, and alcohols (e.g., (**16**) and (**19**) in **Figure 1**) are also often combined with a terpenoid ((**3**), **Figure 1**) to form a pheromone. For example, McBrien *et al*. (2002) showed that a conjugated triunsaturated methyl ester, when combined with one or more of three sesquiterpenes, was attractive to the stink bug *Thyanta pallidovirens*. There is little known about pheromone biosynthesis in this group, or of the hormones involved in the regulation of production in the Heteroptera.

6.12.3. Pheromone-Mediated Behavior

6.12.3.1. Research on Olfaction and Orientation

Two major basic lines of investigation have been taken to understand how pheromones cause insects to be “attracted” and arrive at the source. One has focused on orientation movements that are evoked by exposure to the correct pheromone blend for a particular species. By necessity, studies of insect pheromone-mediated orientation have been concerned with flight control, which ultimately needs to be understood with regard to how a flying insect stabilizes its flight in wind in all three possible planes of movement in space (David, 1982, 1986). The second major line has focused on studies of insect olfaction. It is the exposure to the correct blend of pheromone components, and not partial blends or suboptimal blend ratios, that most intensely turns on programs that change an insect’s movements and maximize attraction toward the source. Because the reception and integration of the pheromone blend’s quality modulates these programs, insect pheromone

olfaction has been an equally important and rewarding line of inquiry, and one that has received much funding support from federal funding agencies.

6.12.3.2. Understanding Pheromone-Mediated Orientation

6.12.3.2.1. Importance of the fine-scale structure of pheromone plumes to orientation behavior Wright (1958) recognized, in contrast to Bossert and Wilson's time-averaged "active space" depiction and modeling (Bossert and Wilson, 1963), that an odor plume is composed of strands of odor that issue from the surface of the odor source. The strands are sheared from the release surface and drift downwind where they become stretched, twisted, and more tortuous as they are ripped apart into substrands during their journey farther downwind (Murlis, 1986, 1997). These disjointed substrands interspersed with pockets of clean air comprise what we call the odor plume. However, it was not until intermittency of stimulation from the plume was shown to promote sustained upwind flight (Kennedy *et al*., 1980, 1981; Baker *et al*., 1985) and subsequently when moths were shown to have the ability to react fast enough during upwind flight to the subsecond changes in pheromone concentration (Baker and Haynes, 1987) that one began to understand that these individual pheromone strands and pockets of clean air are what are producing the sustained upwind flight behavior that is called attraction (Mafra-Neto and Cardé, 1994; Vickers and Baker, 1994). Thus, the major advance in basic understanding of insect pheromone-mediated behavior over the past 20 years is that with but one exception (Justus and Cardé, 2002), rapid increases in flux from individual plume strands and decreases from the clean air pockets between strands are essential to producing reiterative upwind surges that result in the attraction of male moths to pheromone sources.

Concerted attempts to apply this new knowledge have not yet been made, but this is an area of research that may offer the best chance for improving the efficacy and economic competitiveness of pheromones in pest control. Wyatt (1997) reviewed the advances in basic and applied pheromone research that had been made through the mid-1990s and provided a series of suggestions as to the types of research and extension efforts that should be undertaken to facilitate the more widespread commercial use of pheromones for direct control of insect pests. One objective of this chapter is to provide an updated snapshot of what has been learned from the continued successful commercial use of pheromones in recent years and also to review some recent advances in basic knowledge about the behavior and neurophysiology of insect olfaction to suggest new ways to improve and optimize the field use of insect pheromones for pest control. Biochemical and molecular aspects of insect olfaction are reviewed in depth by Vogt (see **Chapter 3.15**), and so attempt is not made to cover the many advances made in this important area.

The focus is mainly on sex pheromones because these have achieved the widest commercial utility. The treatment of the basic neuroethological mechanisms involved in olfaction and attraction is restricted mainly to the Lepidoptera, because research on this group has provided the most comprehensive and probably the most useful model by which to understand odor-mediated attraction of all insects. Based on current knowledge about olfactory pathways and flight responses of insects in other orders, there is no reason to suspect that the mechanisms of olfaction or in-flight orientation generally used by insects will be significantly different from those used by Lepidoptera.

6.12.3.2.2. Optomotor anemotaxis and self-steered counterturning: two indirect reactions to pheromone The insight and experience of John S. Kennedy began to free pheromone researchers of their preconceptions about attraction being a chemo-orientation process involving steering with regard to odor gradients (Kennedy and Marsh, 1974; Kennedy, 1977, 1983, 1986; Marsh *et al*., 1978; Kennedy *et al*., 1980, 1981). This allowed a better understanding of insects' responses to pheromones and other odors as being an *indirect* response to odor exposure that turns on steering programs with respect to *wind*, and *not* to odor. It was also discovered that a second indirect response is also turned on in moths by pheromone, an internally generated program of side-to-side oscillations that is switched on and takes place even in the absence of wind, persisting for many seconds after pheromone is removed (Baker and Kuenen, 1982; Kuenen and Baker, 1983).

Since those initial landmark studies by Kennedy's group it has been possible to learn more precisely how, when a flying insect encounters a series of odor strands of the right quality, these two programs are performed. In the wind-steering program, a visually mediated response called optomotor anemotaxis, the moth compensates for any off-line displacement due to wind direction, or for any changes in progress over the ground that arise due to changes in wind velocity. During pheromone stimulation, the optomotor anemotactic response keeps the insect heading and progressing more or less directly upwind. The other, nonanemotactic program (Baker and Kuenen,

1982; Kuenen and Baker, 1983) is a "counterturning" pattern of rapid left–right reversals of direction (Kennedy, 1983, 1986) which is performed concurrently with anemotaxis. Counterturning frequency increases upon contact with pheromone and coincides with more upwind-oriented optomotor anemotactic steering (Baker and Haynes, 1987). Counterturning frequency slows down upon loss of pheromone at the same time that the clean air causes the anemotactic program to allow more crosswind, transverse optical image flow, to result in slower-reversing, greater amplitude crosswind casting flight (Kennedy, 1983, 1986; Baker, 1985, 1989b, 1989c).

6.12.3.2.3. Altitude control Wind is the horizontal motion component of a moving air mass, and optomotor anemotaxis only explained steering with respect to wind to result in attraction (upwind displacement). A flying insect traversing pheromone plume strands also needs to navigate in a third dimension, i.e., with regard to its altitude. David (1982, 1986) provided experimental evidence that altitude control is accomplished via optomotor compensation for the apparent up or down movement of objects. He showed that these responses are performed by *Drosophila hydei* by changing their wing beat frequency to regulate the total flight forces that the wings generate when moving visual cues give them the sensation that they are rising or sinking. This vertical flight-force response system is intimately coupled with changes in body pitch angle that the insect performs in response to changes in the horizontal component of optomotor feedback that arise from changes in total flight force (e.g., wing beat frequency) (David, 1982, 1986). Studies of flight tracks of several moth species, which represent the total pheromone-mediated flight system involving both counterturning and anemotaxis, have shown that when a moth is casting widely following loss of pheromone, its vertical (up-and-down) excursions are correspondingly greater (VonKeyserlingk, 1984; Baker, 1989c). When the insect is surging upwind following repeated contact with pheromone strands, its vertical motion is much reduced as is its side-to-side movement (VonKeyserlingk, 1984; Baker, 1989c) due to high-frequency counterturning, with more thrust being generated to carry the insect upwind at a faster rate (David, 1986).

6.12.3.2.4. Subsecond reaction times of the two programmed responses to pheromone Intermittency of stimulation from the strands of pheromone and pockets of clean air that comprise a pheromone plume was shown to be required for at least two species of moths to promote sustained upwind flight of males (Kennedy *et al.*, 1980, 1981; Baker *et al.*, 1985). Soon after, it was found that male oriental fruit moths were able to react within 1/6 of a second to changes in pheromone concentration (Baker and Haynes, 1987). It was now possible to hypothesize that each encounter with an individual pheromone strand or pocket of clean air produces subsecond reactions in male moth behavior that, when reiterated during multiple encounters with strands and pockets of clean air in a plume, promote successful sustained upwind flight to the pheromone source (Baker and Haynes, 1987; Baker, 1990; Kaissling and Kramer, 1990).

The research progress resulting from many experiments aimed at understanding behavioral responses of moths to pheromone (reviews: Kennedy, 1983, 1986; Baker, 1985, 1986, 1989b; Willis and Arbas, 1991, 1997; Arbas, 1997; Baker and Vickers, 1997; Cardé and Mafra-Neto, 1997) brought the field to the point of being able to embrace a comprehensive understanding of how male moths locate sources of sex pheromone. A series of experiments using two different lepidopteran species from different families (Mafra-Neto and Cardé, 1994; Vickers and Baker, 1994) confirmed that the split-second reactions to individual odor strands are, in fact, the hypothesized upwind surges (Baker, 1990; Kaissling and Kramer, 1990) that are strung together during repeated contacts with strands in a natural plume to produce sustained upwind flight in response to pheromone. The intermittency provided by pheromone strands has been shown, in three of the four moth species that have been experimentally examined for this, to be essential to producing reiterative upwind surges that result in attraction of moths (Kennedy *et al.*, 1980, 1981; Baker *et al.*, 1985; Justus and Cardé, 2002).

The timescale over which the moths' reactions to pheromone strands and clean air occur is remarkably small. The behavioral responses to both the onset and loss of filaments can be as fast as 0.15 s (*Grapholita molesta*: Baker and Haynes, 1987), but usually are between 0.3 and 0.6 s (*Heliothis virescens*: Vickers and Baker, 1996, 1997; *Helicoverpa zea*: Quero *et al.*, 2001; and *Antheraea polyphemus*: Baker and Vogt, 1988). In studies of host-odor responses by flying female moths, only the latency of the casting flight response to loss of the odor has been measured, and its time course is similar to that of the latency for pheromone loss, 0.7 s (Haynes and Baker, 1989).

The speed of the reaction of moths to encounters with a pheromone strand or clean air pocket is not limited apparently by the speed of olfactory pathway processing; the same latency to a change in

movement occurs when visual cues related to wind-shift are experimentally generated instead of odor-loss cues (Baker and Vickers, 1994). The current evidence thus indicates that for responses to pheromone or host odor, the shape of the insect's flight track is determined by the frequency of encounters with filaments relative to encounters with pockets of clean air, and these encounters determine the relative proportions of upwind surging and casting, respectively (Baker, 1990; Kaissling and Kramer, 1990; Mafra-Neto and Cardé, 1994; Vickers and Baker, 1994; Kaissling 1997).

There is no evidence from other insect species in other orders that their in-flight response mechanisms to pheromone or other odors is different from that of moths. But further research examining the behavioral responses of other groups will be enlightening and essential to testing just how universally applicable these mechanisms are to insect odor-mediated flight.

6.12.3.2.5. Relevance of fast pheromone-strand reaction times to field attraction The rapid response to an odor filament is important because the strand has been shed from the odor source and, in higher velocity airflow, continues to travel in a more or less straight line away from the source (David *et al*., 1982; Elkinton and Cardé, 1987). This trajectory allows the responding insect to move in a straight line toward the source if it flies upwind when it smells the odor: the insect can locate the odor source by steering into and progressing upwind when it smells the correct odor. However, it is just as important for the insect to respond quickly to clean air as it is to an odor filament, because large-scale turbulence produces large swings in the wind direction that dictates along which lines the strands are flying away from the source (David *et al*., 1982). If the insect continues to move upwind into these wind-swing-produced large pockets of clean air for any length of time, it will more rapidly distance itself from the odor plume it has just lost contact with, as well as steer more off-line relative to the odor source (David *et al*., 1982; David and Kennedy, 1987; David and Birch, 1989; Baker, 1990).

A rapid cessation of upwind progress in response to odor loss, coupled with rapid and increasingly lengthy side-to-side oscillations in response to odor loss, allows the insect to increase its ability to regain contact quickly with the odor strands that have swung to one side or the other of its body. Because the insect has no way of telling to which side the odor has gone, this casting flight provides an unbiased response to one side of the wind line or the other in clean air, either of which could be the wrong side and result in failure to relocate the plume. The cessation of upwind progress during casting also prevents the insect from plunging too deeply and off-line from the source into a large pocket of clean air that is now moving off-line from the insect's previous toward-source line.

There is experimental evidence in which odor exposure to the insect or ground pattern movement is manipulated that odor-mediated optomotor anemotaxis is used by a variety of species besides Lepidoptera during odor-mediated upwind flight, including flies (*Drosophila*: David, 1982; *Tsetse*: Gibson and Brady, 1988, 1991). Similarities between male moths' reactions to gain and loss of sex pheromone odor and female moths' reactions to gain and loss of host volatile odor are striking (Haynes and Baker, 1989). Thus, knowledge about pheromone-mediated orientation mechanisms should help us better understand how to manipulate not only pheromone-mediated behavior of moths, but also odor-mediated behavior of male and female insects of all types, for better insect control.

6.12.3.3. Pheromone Blend Quality and Upwind Flight Behavior

The blend ratio of pheromone components for evoking optimal attraction and source contact by male insects is the one that most closely approximates the natural ratio emitted by females. Linn *et al*. (1986a) convincingly demonstrated for several species that males' behavioral thresholds for the optimal blend are lower than they are for partial blend compositions or suboptimal ratios. They also showed in the field that the optimal blend causes the initiation of upwind flight in more males from greater distances from the source than do blends comprised of suboptimal ratios or that from which some components are missing (Linn *et al*., 1986b), indicating that the threshold for upwind flight attraction to the optimal blend is lower than that for suboptimal or partial blends. As pointed out by Linn *et al*. (1986a, 1986b) and reiterated by Baker (1989b), these results showed that we must discard a previous view of how blends are used by males. The previous model proposed that there was a stepwise use by males during upwind flight of first one component in a female's blend when they are far downwind, and then two, three, and successively more components as they get closer to the source (Cardé *et al*., 1975; Nakamura, 1981; Bradshaw *et al*., 1983).

Recent studies have demonstrated that blend-quality reactions take place on a strand-by-strand basis, within fractions of a second. Vickers and Baker (1997) showed for *H. virescens* and Quero *et al*. (2001) using *H. zea*, that the duration and

length of males' upwind surging reactions to single strands of pheromone depend on their quality. Strands of the correct pheromone blend tainted with small amounts of heterospecific antagonist are poorer in intensity than are the surges to strands of the pure pheromone blend alone.

The blend of pheromone molecules must arrive simultaneously on the male's antenna, i.e., they must be perfectly blended in each strand, to have their optimal effect. This was demonstrated when male *H. virescens* were shown to be able to discriminate experimentally generated incompletely mixed strands of their major and minor pheromone components from strands that were completely mixed (Vickers and Baker, 1992). They can also discriminate experimentally generated strands of pheromone that are incompletely mixed with heterospecific antagonist from those in which each and every strand contains both antagonist and pheromone (Fadamiro and Baker, 1997; Baker *et al*., 1998a; Fadamiro *et al*., 1999a). This level of plume-strand quality temporal resolution was first suspected in other species using two natural and confluent plumes of pheromone and antagonist placed only tens of centimeters apart (Witzgall and Preisner, 1991; Liu and Haynes, 1992; Rumbo *et al*., 1993).

The propensity of insects to be attracted optimally to pheromone blends most closely approximating those emitted by the opposite sex is therefore attributable to their repeated strong surging reactions to individual strands. Thus, the higher quality surging that occurs in response to each strand of pheromone that is of optimal blend quality will, during repeated contact with natural strands in a pheromone plume, promote more sustained and intense upwind flight and displace a male farther upwind at a faster rate from greater distances downwind than will the surges in response to suboptimal blend strands. Presumably, the long-lasting casting response in a large pocket of clean air will also be of longer duration after contact with optimal blend-strands rather than suboptimal ones.

6.12.4. Pheromone Olfaction

6.12.4.1. Optimizing Flux Detection

It has become clear over the past 20 years that, as emphasized by Kaissling (1998), the insect olfactory system is designed to optimize flux detection and not to measure concentration. New understandings of the biochemical, neurophysiological, and molecular processes that contribute to the propagation of behavior after contact with airborne pheromone plumes show that the emphasis is on processing speed related to temporal resolution of the strand encounters. All the steps in the olfaction process work rapidly in succession to report pheromone strand encounters to higher centers (Vickers *et al*., 2001), from the biochemistry of transduction (see **Chapter 3.15**) to action potential propagation, to integration of the signal at the antennal lobe level, and finally to the output patterns sent to higher central nervous system (CNS) levels. Along at least some specialized pathways in this succession of synapses, the reporting of changes in flux is preserved and seems necessary to promote repeated neuronal excitation and quickly modulate behavior.

6.12.4.2. Elements of Pheromone Olfactory Pathways in Insects

The pheromone olfactory systems of insects are exemplified by that which exists in the Lepidoptera. Decades of research on moths has allowed knowledge about animal olfaction in general to be developed that rivals what is known in the vertebrates (Hildebrand and Shepard, 1997). Although the receptor organs used by different orders of insects to begin transducing pheromone molecules into action potentials vary in type, shape, and size (Kaissling, 1974, 1987; Steinbrecht, 1987, 1999), the integrative pheromone olfactory pathways after transduction that lead to motor output are strikingly similar to those in the Lepidoptera in the most well-studied groups such as cockroaches (e.g., Boeckh and Ernst, 1983; Boeckh *et al*., 1984). Thus, pheromone olfaction in the Lepidoptera at length to provide an overview of a typical insect pheromone olfaction system is discussed. A thorough review of all aspects of the pathways involved in insect olfaction is provided by many authors in Hansson (1999).

6.12.4.2.1. Olfactory receptor neurons and their perireceptor environments In the Lepidoptera, pheromone molecules in a plume-strand contact the thousands of extra-long receptor hairs (trichoid sensilla) on the antennae of male moths that are the primary pheromone receptor organs (Kaissling, 1974, 1987; Steinbrecht, 1987, 1999). In some families in the Coleoptera such as the Scarabaeidae, the pheromone-component-sensitive receptor neurons apparently reside in other types of olfactory receptor organs. The word "apparently" must be used here because it is virtually impossible to identify from which type of sensillum one is recording when using the tungsten-type electrodes that are requisite for recording from these tiny and often densely packed sensilla. For scolytid bark beetles, short trichoid or basiconic sensilla (short stubby hairs) have been implicated in sex pheromone reception (Dickens and Payne, 1978; Dickens, 1979). In

scarab beetles, coeloconic (pit-peg) and placoid (flat, plate-like) sensilla that are arrayed in various patterns on the lamellar hair surfaces are suspected to house the pheromone-sensitive receptor neurons (Mustaparta, 1984; Leal and Mchizuki, 1993; Kim and Leal, 2000; Ochieng *et al.*, 2002).

After landing on a hair's surface, the pheromone molecules enter the interior of the hair (or other types of sensilla in other groups) through pores and associated tubules and traverse a perireceptor gel comprised mainly of pheromone binding protein until they arrive at the membrane-bound pheromone receptor on the dendrite of an olfactory receptor neuron (ORN). Usually there are two, but sometimes three, pheromone-component-sensitive ORNs that are cocompartmentalized within one sensillum. For a thorough review of the biochemical events involving binding proteins, pheromone degrading enzymes, and the receptors themselves (see **Chapter 3.15**). A depolarization of the dendritic membrane of the ORN results if the pheromone component molecule has the correct geometry and electron distribution due to various double bonds and functional groups (e.g., acetate, alcohol, aldehyde, or ketone) to allow it to fit optimally into the protein pocket that is the receptor.

It is a point of debate as to whether the binding-protein-plus-pheromone-component molecule complex or the pheromone-component molecule itself binds to the "pheromone receptor" (see **Chapter 3.15**). A G protein coupled system cascade results in ion channel changes that result in dendritic depolarization and action potential generation down the axon to the brain.

This process is occurring simultaneously in the receptor hairs that contain ORNs that are specifically tuned to the other components of the pheromone blend. Often, as in the Tortricidae, the ORNs tuned to the minor pheromone components reside in the same hairs as those tuned to the major component (Akers and O'Connell, 1988). The minor components arrive on the antenna in the same plume-strands as the major component, and the pheromone components blended in each strand at particular ratios are immediately transduced to action potentials by their respective, component specifically tuned ORNs. The blend composition in each strand is therefore broken down into its component parts and the composition reported to neurons farther down the line by the differential activities of the different types of ORNs responding selectively to their own optimal ligands (Todd and Baker, 1999). The process is remarkable in that the across-antennal response of differentially tuned ORNs to each odor strand is basically a split-second gas chromatographic readout of the odor strand composition that is repeated two or three or more times a second as long as the insect remains in contact with the plume (Vickers *et al.*, 2001).

The differential responsiveness of the ORNs to compounds is imparted by the peculiar receptor site proteins on the dendrites and perhaps even by the selectivities of binding proteins that bathe the ORNs as a gel (Stengl *et al.*, 1999) (see **Chapter 3.15**). The result is that the ORNs respond optimally only to the molecules in the blend to which they are differentially tuned (and also to which the binding protein binds and delivers to the ORN's dendrite), even though the complete blend of compounds has adhered to and possibly entered the hair lumen with its own binding protein milieu. The abundance of each pheromone component in each strand is thus first represented in the relative activities of the neurons specifically tuned to them. The action-potential activities of these thousands of neurons responding to their specific portion of the two- or three-component blend then stream down in separate lines, apparently without cross-communication with other axons in the antennal nerve, to finally arrive at the macroglomerular complex (MGC) in the antennal lobe located at the base of the antenna (Matsumoto and Hildebrand, 1981; Christensen and Hildebrand, 1988; Christensen, 1989; Hildebrand and Shepard, 1997; Hansson and Christensen, 1999).

6.12.4.2.2. Glomeruli in the MGC The MGC comprises varying numbers of glomeruli, which are knots of neuropil made up primarily of axonal branches from antennal sensory neurons but also the fibers and arborizations of interneurons that synapse with them and with each other. The MGC is found only in males and receives information exclusively from sex-pheromone-sensitive antennal neurons (Matsumoto and Hildebrand, 1981; Boeckh and Ernst, 1983; Boeckh *et al.*, 1984; Hildebrand and Shepard, 1997). Antennal neurons that respond to plant- and flower-derived compounds terminate in what are termed ordinary glomeruli located more ventrally in the antennal lobes of both males and females (Hansson, 1995; Hildebrand and Shepard, 1997; Anton and Homberg, 1999; Hansson and Christensen, 1999).

Studies by Hansson *et al.* (1992) using activity-dependent uptake of cobalt to stain ORNs and following them to the MGC revealed that each MGC subcompartment in the antennal lobe of the turnip moth, *Agrotis segetum*, exclusively handles incoming information from the antenna about only one pheromone component or a known behavioral antagonist. Earlier, proof that there is an orderly and

reliable position of ordinary glomeruli in the insect brain from individual to individual was known from anatomical work on a moth and butterfly species (Rospars, 1983). However, it was the discovery that there are functionally, as well as spatially, distinct MGC subcompartments that receive pheromone-component-specific information from the antennal ORNs that was a breakthrough for understanding animal olfactory processing (Hansson *et al.*, 1991, 1992). Results using cobalt staining of ORNs on other moth species mirrored those of Hansson *et al.* (1992), Berg *et al.* (1995), Christensen *et al.* (1995a), Ochieng *et al.* (1995), Todd *et al.* (1995); review: Hansson *et al.* (1995).

Using a new technique involving calcium-sensitive dye developed by Galizia and colleagues for the honeybee, *Apis mellifera* (Galizia *et al.*, 1997, 1998, 1999; Sachse *et al.*, 1999) allowed for the dramatic visualization of the activities of populations of whole populations of lepidopteran ORNs projecting their pheromone-component-specific excitations to their target glomeruli (Galizia *et al.*, 2000; Carlsson *et al.*, 2002; Carlsson and Hansson, 2003). These studies confirmed, on an ORN population-wide stimulation basis, the results of cobalt staining of individual ORNs that showed projections of physiologically identified ORNs tuned to one pheromone component or antagonist projecting to a single glomerulus in the MGC. The interpretation of the calcium dye excitation as being due mostly to the predominance of signal being provided by calcium released in the glomeruli from the cholinergic ORN terminals was at first a matter of debate. But the correspondence of target glomeruli from calcium dye visualization (Galizia *et al.*, 2000; Carlsson and Hansson, 2003) with the target glomeruli to which individual cobalt-stained ORNs had been shown to project to (Ochieng *et al.*, 1995; Berg *et al.*, 1998) was confirmed.

The complete blend of odor in each pheromone plume-strand is represented by a particular balance of neuronal activity across the glomerular subcompartments of the MGC. Early researchers in olfaction and taste studies realized that odor quality discrimination must result from the ratios of activity being weighed across the many different types of receptor neurons (Boeckh, 1967), a phenomenon called "across-fiber patterning" (Dethier, 1972). With recent advances, we can, however, now understand that across-fiber patterning can be considered to be an across-glomerular pattern (Galizia *et al.*, 1998), as anticipated by Rospars (1983).

6.12.4.2.3. Across-glomerular pattern enhancement by local interneurons in the MGC The across-glomerular pattern of relative intensities is initially enhanced by the activities of the first level of interneurons that reach into these glomerular pools of pure, component-specific ORN activity. These first interneurons are called "local" interneurons (Matsumoto and Hildebrand, 1981; Boeckh and Ernst, 1983; Anton and Hansson, 1999; Anton and Homberg, 1999; Hansson and Christensen, 1999) because they spread locally across the antennal lobe like a net, sending no axons out of the antennal lobe. They interconnect the glomerular subcompartments within the MGC and also to ordinary glomeruli receiving input from ORNs about nonpheromone-related volatiles (Matsumoto and Hildebrand, 1981; Anton and Homberg, 1999).

The integrative, discrimination-enhancing ability of local interneurons is indicated by the fact that for most of them, their neurotransmitter is γ-aminobutyric acid (GABA), which imparts inhibition on neurons upon which they synapse (Anton and Homberg, 1999). A greater acetylcholinergic excitation of a local interneuron by antennal ORN synapses in one component-specific glomerulus will therefore impart a greater amount of GABA-ergic inhibition that spreads to the other, neighboring subcompartments (Anton and Homberg, 1999).

Research on the American cockroach (*Periplaneta americana*) MGC synaptic interconnections clearly indicates that inhibition also apparently can be imposed by local interneuron fibers onto the incoming signals from ORN axons, as well as onto projection interneurons and to other local interneurons within one glomerulus (Distler and Boeckh, 1996, 1997a, 1997b; Anton and Homberg, 1999). Whatever pattern across glomeruli that would have occurred due to the inputs to the antennal lobe glomeruli from the ORNs responding to the blend components might now be enhanced by lateral inhibition imparted by these local interneurons (Christensen *et al.*, 1993). Interestingly, the various local interneuron synapses occurring within and between glomeruli might result in some excitation exiting the antennal lobe via projection interneurons due to local interneuron-imposed disinhibition of previously inhibited interneurons (Christensen *et al.*, 1993, 1998a, 1998b; Anton and Homberg, 1999). The glomeruli from which excitatory projection interneuron output emerges can also apparently be shifted by the inhibitory–disinhibitory activities of local interneurons so that the output locations do not necessarily correspond to the input locations of ORNs (Anton and Hansson, 1994, 1999).

6.12.4.2.4. MGC projection interneurons In the male moth (and other insects), pheromone odor

blend information emerges from the MGC and travels downstream to locations deeper in the brain through a very small number of interneurons (Boeckh *et al.*, 1984). These are called projection interneurons, because they project out of the MGC and arborize with interneurons in higher brain centers. Unlike local interneurons, projection interneurons usually arborize only in one or a limited number of subcompartments in the MGC and generally travel to the far back of the brain where they synapse with neurons in the mushroom body (Christensen, 1989; Hildebrand and Shepard, 1997; Hansson and Christensen, 1999) before continuing on to synapse with other neurons in the extreme lateral portion of the protocerebrum. Protocerebral interneurons themselves can respond in enhanced manner to optimal blends (Kanzaki *et al.*, 1991; Kanzaki and Shibuya, 1992).

The interneuronal level in the MGC at which blends have been shown most frequently to be uniquely responded to is at the projection interneuron level (but see below how mixture interactions of ORNs might confound these interpretations). However, there are many projection interneurons that respond only when a particular individual component is present, regardless of whether it is part of a blend (Christensen, 1989; Christensen *et al.*, 1991, 1995b, 1996; Hansson *et al.*, 1991; Hildebrand and Shepard, 1997; Hansson and Christensen, 1999; Vickers and Christensen, 2003). But then again there are even slight differences in pheromone blend ratio, not just composition, that are responded to selectively by blend-sensitive interneurons within the antennal lobe (Wu *et al.*, 1996).

Blend-integrating responses carried by projection interneurons are known to be of at least two different types. One type involves an increased initial spike frequency plus a long-lasting excitation that occurs in response only to the presence of a blend of two components on the antenna and not to each component presented individually. Such blend-dependent excitation responses have now been discovered in antennal lobe interneurons of several species of moths, including *Manduca sexta*, *H. virescens*, and *H. zea* (Christensen, 1989; Christensen *et al.*, 1991; Hansson *et al.*, 1991; Berg *et al.*, 1998; Hansson and Christensen, 1999).

However, another very important type of blend-integrating response also is known from *M. sexta* and other moths, in which the presence of the blend on the antenna sharpens the onset and offset of the train of spikes emerging from these interneurons, resulting in a more highly phasic burst than would otherwise occur in response to either component alone. The temporal sharpening occurs due to a combination of inhibition from neuronal elements responding to one of the two pheromone components and excitation from those responding to the other (Christensen and Hildebrand, 1988; Christensen, 1989; Christensen *et al.*, 1991, 1998a, 1998b). This suggests that the blend uniquely sharpens the time courses of the plume-strand related successive trains of action potentials streaming toward the mushroom body and lateral protocerebrum. Thus two different types of blend-integrating information, based on the temporal aspects of the response – either highly phasic-sharpened, or more long-lasting and tonic – travel out through the projection interneurons to the brain.

As discussed above, it is just as behaviorally important for males to respond to the pockets of clean air between pheromone filaments as to the pheromone filaments themselves because clean wind no longer points toward the source, and the moth continuing upwind for too long upon flying into clean wind can find itself flying off-line from the source. The interneuronal pathways in the insect pheromone olfaction system do seem to provide ample ways to integrate and send highly phasic information related to pheromone filament contact all the way through to higher centers (Christensen and Hildebrand, 1988; Anton and Homberg, 1999; Vickers *et al.*, 2001; Vickers and Christensen, 2003). Therefore, the need to quickly turn off the excitation caused by a plume-strand to allow a male to change its behavior at the first hint of clean air is reflected in the highly irregular, plume-filament correlated responses of interneurons in the MGC of the antennal lobe when placed in natural pheromone plumes (Vickers *et al.*, 2001).

6.12.4.2.5. Mushroom body–antennal lobe-derived oscillations There is also evidence that inhibitory feedback loops between the mushroom body and the antennal lobe can cause oscillations to occur that may impose repetitive and very short time-windows within which the ratios of activities within glomeruli can be most accurately reported by the integrative circuits out of which projection interneuron excitation occurs (Laurent, 1996; Laurent *et al.*, 1996; Stopfer *et al.*, 1997, 1999). These oscillations have been demonstrated to facilitate discrimination of odor blend quality in the honeybee (Stopfer *et al.*, 1997).

6.12.4.2.6. Protocerebral interneuron activity The blend integration already occurring in the MGC does not mean that the sensation of blend cannot also be promoted by integration farther downstream by protocerebral interneurons (Kanzaki *et al.*, 1991; Kanzaki and Shibuya, 1992), or even by descending interneurons, which could weigh the

ratios of activity of component-specific projection interneurons and also integrate them with other inputs such as visual flow-field information (Olberg and Willis, 1990). Because ORNs are now known to be capable of responding to blends either through mixture suppression (Nikonov and Leal, 2002) (see Section 6.12.4.4) or mixture enhancement (O'Connell *et al*., 1986; Ochieng *et al*., 2002), the possibility cannot be ruled out that at least a portion of what seem to be blend-sensitive projection interneurons may merely be those same interneurons relaying an ORN-derived mixture response that originated farther upstream at the antennal level.

6.12.4.3. Pheromone-Sensitive ORNs as High-Fidelity Reporters of Plume-Strand Encounters

Based on the need for fast behavioral reaction times to individual strands of pheromone and pockets of clean air between the strands, the peripheral olfactory receptor neurons of flying insects should be built to report information to the brain about the odor blend such that it can rapidly discriminate odor quality on a strand-by-strand basis. At least some populations of ORNs, therefore, should be built to very rapidly detect and respond to the onset of each odor strand, and interneurons in the antennal lobe farther downstream should preserve and even enhance the reporting of the temporal aspects of the signal so as to be able to report this information to higher centers and then to motor neurons. But there also should be some kind of long-lasting response in the olfactory pathways that persists for many seconds after an encounter with a pheromone strand to promote sustained casting flight in clean air that will optimize an insect's chances of bridging the long clean-air gaps between odor strands that occur during large, sudden swings in wind direction. Another function of the fast response time of the ORNs would be to report sufficiently fine-grained information that the insect can discriminate a plume in which every filament contains a complete blend of odor from one in which the blend is partitioned into separate filaments (Baker *et al*., 1998a; Todd and Baker, 1999).

6.12.4.3.1. Importance of fast-phasic response for reporting changes in flux Insect sensory physiologists early on noted the phasic–tonic nature of the time course of typical ORN-generated action potential spike trains after stimulation with pheromone components (Kaissling, 1974, 1986, 1987). The phasic–tonic profile is characterized by the first portion of action potential output being a sharp burst that is shorter than, and does not correlate with, the longer-lasting stimulus (Kaissling, 1974, 1986, 1987). The remainder of the response is a tonic, residual low level of above-background firing that may not mirror the stimulus, especially at the highest dosages. The lack of correspondence of both the phasic and tonic portions of the receptor response have been shown not to be artifacts resulting from the techniques used to deliver the odorants (Kaissling, 1987).

Pheromone-sensitive ORNs do, however, display a wide variation in the phasic or tonic elements of their responses to pheromone components (Kaissling, 1974, 1986, 1987; Almaas *et al*., 1991; Berg *et al*., 1995; Berg and Mustaparta, 1995). There are examples of both highly phasic and highly tonic ORNs in the same species. For example, Berg *et al*. (1995) found that the receptors on male *H. virescens* antennae that were sensitive to (*Z*)-9-tetradecenal (*Z*9-14:Ald) responded in a more phasic manner than those sensitive to (*Z*)-11-tetradecenal (*Z*11-14:Ald). Rumbo (1983) and Rumbo and Kaissling (1989) found differences in the durations of response of antennal neurons tuned to different components of the sex pheromones of the light brown apple moth, *Epiphyas postvittana*, and *A. polyphemus*, respectively. The differences were usually related to the type of ORN, with the ORN tuned to the major component being the more phasic, and the ORN tuned to one of the minor components firing more tonically.

The propensity of insect olfactory receptor neurons to fire in a phasic versus a tonic pattern is related to their relative rates of adaptation and disadaptation (Kaissling, 1987). In general, phasic neurons are fast to disadapt and are able to track (respond to) the presence of pheromone that is repeatedly and rapidly pulsed at 10 Hz (Almaas *et al*., 1991) or even more than twice that (Bau *et al*., 2002). More tonically firing neurons are slower to disadapt, sometimes taking minutes or tens of minutes to do so, and cannot respond to a new pulse of pheromone during this time (Rumbo, 1983; Rumbo and Kaissling, 1989). Even for fast adapting–disadapting receptor neurons, disadaptation is not necessarily absolute, and the tracking response to rapidly repeated stimuli does not report accurate information about the concentration. The number of action potentials in response to each pulse, reflecting the concentration of odorant in each pulse, is significantly reduced under rapid stimulation, but the fidelity of response with regard to the temporal presence or absence of the pheromone is accurate. Thus, the sensitivity of a receptor unit in response to an odorant is not absolute and is subject to adaptation–disadaptation mechanics (Kaissling, 1986,

1987; Kaissling and Kramer, 1990). As emphasized by Kaissling (1998), it is the change in flux, which has a temporal element, and not concentration, which does not, that ORNs are specialized to report.

Contact with the strands of odorant within a natural plume is on a highly irregular basis in time, as shown both from the work of Murlis (1986, 1997) by using an idealized receptor (ion detector) and odor mimic (ionized air) as well as from the recordings of real odor plumes in the field and laboratory by using insect antennae or their ORNs as detectors (Baker *et al*., 1988; Haynes and Baker, 1989; Hansson and Baker, 1991; Vickers and Baker, 1994; Willis *et al*., 1994; Vickers *et al*., 2001; Bau *et al*., 2002). The adaptation–disadaption characteristics of receptors are concentration- and time-dependent (Kaissling, 1986, 1987; Almaas *et al*., 1991; Hansson and Baker, 1991; Berg *et al*., 1995), and these two aspects affect receptors' abilities to report to the CNS the presence of all of the strands of odorant that are contacted in a natural plume.

Murlis (1986, 1997) emphasized that a form of temporal averaging of the fine structure of an odor plume would occur when two equally concentrated strands contact the receptor too rapidly, resulting in the second strand evoking no action potentials from the already adapted ORN that had not recovered from responding to the first strand. Alternately, contact with a low-concentration strand could follow a high-concentration strand after an appropriately long time interval, but due to adaptation from the highly concentrated first strand, again evoke no action potentials. The adaptation–disadaptation related propensity of the ORN to adjust to both concentration and frequency thus ensures that the CNS receives high-fidelity inputs concerning plume-strand arrival over a wide dynamic range in turbulence-induced plume structures and reports them to higher centers (Vickers *et al*., 2001). The integrative mechanisms in the CNS (e.g., for odor-quality discrimination and enhancement of phasic inputs) (Christensen and Hildebrand, 1988; Christensen, 1989) thus is buffered from outside variance in the plume, and behavioral response likewise attains a higher fidelity over such wide-ranging differences in plume-strand flux.

Limits in the dynamic range of one type of pheromone-component-specific ORN to report the arrival of strands containing the component to which it is tuned compared with the capabilities of others tuned to other components has been shown to be important in natural pheromone plumes and has been correlated with changes in behavior (Baker *et al*., 1988; Hansson and Baker, 1991). Excessive concentrations of the three-component pheromone blend of *A. segetum* were shown to differentially impair the ability of only the (*Z*)-5-dodecenyl acetate (*Z*5-10:Ac) receptor neurons to disadapt and fire in response to repeated contact with filaments in a natural plume (Baker *et al*., 1988; Hansson and Baker, 1991). The competencies of neurons tuned to both *Z*7-12:Ac and *Z*9-14:Ac to fire in this very same plume were completely unaffected. Thus, the excessive plume concentration caused a distorted ratio of firing from the three receptor types, with now only two types firing when there should have been three. The responses of all three neuronal types to either a 10- or 100-fold lower pheromone-source loading were correlated with high levels of completed upwind flights to the source. Both *Z*7-12:Ac and *Z*9-14:Ac would be emitted at lower concentrations than *Z*5-10:Ac due to their lower volatility, and this would explain in part the ability of the receptors tuned to these two components to avoid becoming adapted to the very same frequency of filament contacts that produced adaptation of the *Z*5-10:Ac-tuned neuron (Hansson and Baker, 1991).

The importance of a phasic reporting of stimulus arrival by ORNs to the promotion of sustained upwind movement was further emphasized in experiments that directly manipulated the outputs of ORNs (Kaissling *et al*., 1989; Kramer, 1992). Kaissling *et al*. (1989) found that a hydrocarbon mimic of bombykol, the pheromone of the silkworm, *Bombyx mori*, caused highly tonic firing of the antennal receptor neurons tuned to bombykol that lasted several minutes, and that this tonic response was correlated with no upwind walking toward the source. Kaissling *et al*. (1989) then found that the plant-related compound linalool effectively hyperpolarized this neuron and prevented action potentials from occurring whenever it was puffed over the sensilla. When Kaissling *et al*. (1989) first stimulated the receptor neurons with the pheromone analog to induce high levels of tonic firing, and then interrupted the firing with intermittent puffs of linalool, they could create repeated, regular bursts of firing from the bombykol neurons that was indistinguishable from repeated puffs of bombykol itself. They reasoned that this highly artificial stimulus that does not contain the pheromone but mimics a reiterative pattern of phasic reporting of pheromone stimulation from the pheromone-sensitive neuron should be a behaviorally effective stimulus that should result in upwind walking by intact male moths. Kramer (1992) found that was indeed the case. High levels of upwind walking to the source occurred in response to this

unnatural stimulus and not to the uninterrupted tonic stimulus.

6.12.4.4. Mixture Interactions between ORNs Cocompartmentalized within Sensilla

There has been emerging evidence in recent years that insects can discriminate between two overlapping odor plumes due to the incomplete mixing of their intertwined strands (Witzgall and Preisner, 1991; Liu and Haynes, 1992; Vickers and Baker, 1992; Rumbo *et al.*, 1993; Baker *et al.*, 1998a). Wind-tunnel bioassays using experimentally generated, pulsed plumes have demonstrated that two pheromone-related odorants are not as effective when they are presented in staggered manner (in every other pulse) compared with when they are coincident in every pulse (Vickers and Baker, 1992; Fadamiro and Baker, 1997). *Helicoverpa zea* males demonstrate a startling ability to distinguish completely coincident strands of pheromone and heterospecific antagonist from those whose strands are separated by only 1 mm (Baker *et al.*, 1998a; Fadamiro *et al.*, 1999a). Baker *et al.* (1998a) and Todd and Baker (1999) pointed out that cocompartmentalization of the ORNs that are differentially tuned to these odorants (Cossé *et al.*, 1998) should optimize both the spatial and the temporal odor quality resolution ability of the animal and that on-site processing of mixtures within each sensillum would be the most likely mechanism to explain this high resolution of odor strand arrival.

Neurophysiological support for this hypothesis comes from a recent report by Nikonov and Leal (2002) in which the firing of pheromone-component-sensitive ORNs on the antennae of the Japanese beetle, *Popillia japonica*, was shown to be suppressed when a known behavioral antagonist was puffed in a perfect mixture simultaneously from a single odor cartridge across the antenna. They recorded from sensilla that always house two cocompartmentalized ORNs, one being tuned to the pheromone component and the other tuned to the antagonist (the opposite enantiomer of the pheromone component). Mixture suppression of firing by the pheromone component sensitive ORN was not observed, however, when the "mixture" of the two enantiomers consisted of simultaneous puffs from two different pipettes separated by only 1–2 mm, creating a time lag of only 1.5–3 ms (Nikonov and Leal, 2002). On-site processing of the mixture was thus found to occur in the Japanese beetle sex pheromone olfaction system and would result in reduced reporting of strands of odor containing both pheromone and antagonist to the antennal lobe.

Pheromone component sensitive ORNs on *H. zea* antennae exhibit distinct and significant responses to blends of the pheromone component mixed with certain plant volatiles (Ochieng and Baker, 2002). These plant volatiles mixed with the major *H. zea* pheromone component, (*Z*)-11-hexadecenal, enhance firing frequency during the phasic as well as tonic portions of the response of ORNs tuned to this component (Ochieng and Baker, 2002). The simultaneous arrival of the two odorants is necessary for the mixture-enhanced firing to occur (Ochieng and Baker, unpublished data). These results suggested the possibility that males' behavioral sensitivity and upwind flight response to pheromone might be elevated by the coemission of certain plant volatiles with the pheromone (Ochieng and Baker, 2002). However, a wide range of plant-related compounds as well as pheromone-related compounds mixed with (*Z*)-11-hexadenal are able to enhance firing of this ORN (Ochieng and Baker, unpublished data), and so it is unclear what role such an unspecific mixture response could play in the real world.

Until recently, little attention has been paid to the possibility of mixture interactions occurring at the ORN level, even with regard to their responses to combinations of sex pheromone components that are known to be behaviorally agonistic (O'Connell *et al.*, 1986; Akers and O'Connell, 1988). Likewise, a few sensory physiological studies have involved pheromone sensitive ORN responses to mixtures of pheromones and plant volatiles (Van der Pers and Den Otter, 1978; Van der Pers *et al.*, 1980). The knowledge that enhanced firing can occur on-site by primary olfactory receptor neurons in response to mixtures should provide a note of caution to researchers working at the antennal lobe level. It can no longer automatically be concluded that mixture interactions recorded from projection neurons are due entirely to blend integration occurring within the antennal lobe. Many of these "blend sensititive" interneurons may be merely relaying to higher centers the unprocessed, blend-enhanced activity that has already taken place far upstream, within the sensilla on the antennae (Todd and Baker, 1999).

6.12.4.5. Background Noise and Resolution of Odor Strands in Overlapping Plumes

Under natural field conditions, strands of pheromone are transported in air masses that are comprised also of complex mixtures of other volatile chemicals, including those originating from surrounding vegetation. Plant-related volatiles are an integral part of the pheromone systems of the majority of

beetle species that have been investigated thus far (Byers, 1995; Bartelt, 1999a, 1999b; Leal, 1999) (see Section 6.12.2.3). Also, plant-derived volatiles are used in the courtship pheromones of moths (Birch *et al.*, 1990; Landolt and Philips, 1997). The question of how mixtures of pheromone components or plant volatiles are reported by ORNs to higher centers is of increasing importance to understanding how insects' olfactory systems can discriminate pheromone plus plant odor plume strands and respond behaviorally to natural pheromone blend signals.

In moths, a few field-trapping studies have demonstrated that certain groups of plant volatiles added to sex pheromone can increase the capture of males, including *H. zea*, in sticky traps (Dickens *et al.*, 1990; Light *et al.*, 1993). However, it is not clear whether the enhancement of male attraction to the traps was due to separate enhanced responses of male moths to either the sex pheromone or the plant volatile portion, or to a response to the total blend that may in its entirety represent a unique odor quality.

The behavioral and evolutionary implications of fine-grained spatiotemporal resolution of odor strands first of all with respect to pheromone-related compounds such as pheromone components emitted by nonconspecific females that antagonize upwind flight have been considered (Baker *et al.*, 1998a; Fadamiro *et al.*, 1999a; Todd and Baker, 1999). Strands of heterospecific antagonistic pheromone components should antagonize upwind flight only if they occur as a complete mixture with conspecific pheromone in each and every strand, which would indicate that the strands originated from a single point source, i.e., a nonconspecific female. They should not be as effective in antagonizing upwind flight if they are not perfectly admixed in every filament, with some filaments containing only pure pheromone, others containing antagonist as well. But what about strands of plant volatiles, or more uniform clouds of plant volatile background odor?

If certain plant volatiles do play a role in modulating responses of males to female-emitted pheromone, it is not so clear what the olfactory system would be doing to optimize these responses. Plant odors in the field would seem to be ubiquitous and serve as a rather tonic fog of average chemical "noise" in which pheromone strands in a plume would travel and be detected by the male ORNs. However, there are many ways in which stronger point sources of plant volatiles can occur, the most obvious being flowers, fruits, and seeds (also corn silk) as strong point source plume generators. Other strong point sources can be generated by direct larval insect feeding damage to leaves (Turlings *et al.*, 1995; Alborn *et al.*, 1997; De Moraes *et al.*, 1998), and still other strong sources can be entire plants that have been induced to produce bursts of volatiles over the their entire surface by the systemic effect of insect saliva "elicitors" on the plants' biochemical machinery (Turlings *et al.*, 1995; Alborn *et al.*, 1997; De Moraes *et al.*, 1998).

There are many ways that any of these point source plumes of plant volatiles could be mixed with strands of pheromone from an adult insect. Some of these situations would see pheromone-plus-plant volatiles issuing as fully mixed strands from relatively strong point sources such as from bark beetle gallery holes, or from weevil feeding-wounding (J.J. Heath *et al.*, unpublished data). It would seem that the simultaneous emission of plant volatiles and pheromone from small, adult-generated point sources corresponds with the enhanced pheromone-plant-volatile attraction seen in the majority of studies of species of Scolytidae and Curculionidae. With moths, it is not as clear that there can be many situations where females can generate fully mixed strands of pheromone and plant volatiles from a small point source. One major difficulty here is that for most species in nature we do not know where females go to call. We do know that they will call from almost any surface, natural or unnatural, and in the presence of plant odors as well as in their absence. With no strong requirement having been observed for female moth calling in the presence of plant volatiles, it makes sense that only a very few studies have shown a significant enhancement of male attraction to pheromones by adding plant volatiles.

In natural situations, regardless of whether the calling female is sitting directly on a flower, fruit, or damaged leaf, it seems unlikely that perfectly mixed strands of pheromone plus plant volatiles having the same temporal flux dynamics will arrive simultaneously on males' antennae. Thus, it is questionable as to whether there is the same need in moths for fine-grained resolution of plant odor and pheromone odor strands as there might be in the Coleoptera, where plant-odor–pheromone mixture enhanced attraction is prevalent and strong point sources emitting plant volatiles plus pheromone are prevalent. But the tolerances of olfactory systems and behavioral responses for synchronous versus asynchronous plant-odor-plus-pheromone plume-strand arrival in different species' communication systems have not been explored. In future neuroethological studies of insect pheromone–plant-volatile mixture interactions, attention needs to be paid to the fine-grained resolution of odor strands and the natural plant-plus-pheromone odor

plume generation occurring for each species to understand how these communication systems may have been evolutionarily shaped.

6.12.4.6. Malleability of Receptor Tuning in Evolution of Sex Pheromones

That the relative abundance of differentially tuned ORNs on insect antennae can change over time is graphically illustrated by the findings of George and Nagy (1984), who found that after 20 years of rearing the same culture of oriental fruit moth, the number of sensilla trichodea housing pheromone ORNs on male antennae decreased to one-half of the original, wild-type number. In the noctuid moth species *A. segetum*, over a wide geographic range encompassing Europe and Africa, variation in the ratios of pheromone components emitted by females from different regions is mirrored by the ORN system of males (Löfstedt *et al.*, 1986; Hansson *et al.*, 1990). In *A. segetum*, the proportional abundance of ORNs tuned to *Z*5-10:Ac, *Z*7-12:Ac, and *Z*9-14:Ac is at least coarsely directly proportional to the proportions of these compounds found in gland extracts of females (Löfstedt *et al.*, 1986; Hansson *et al.*, 1990; Löfstedt, 1990). If the vapor pressures of the components were to be considered as well, the degree of correspondence between emitted proportions of components and the proportion of ORNs tuned to these components would certainly be even more highly correlated. Thus, there seems to be a good degree of ability of the ORNs of moths that are tuned to different components to change their abundance on insect antennae fairly quickly in ecological time. There is also evidence that ORN response profiles can be modified by factors from the brain, as suggested by the cross-species antennal transplant studies of Ochieng *et al.* (2003).

How this malleability is accomplished across a population of differentially tuned ORNs on antennae is unclear, but mutations in some insect olfactory communication systems have provided some insight. Mutations in the ORNs of *Drosophila* have been correlated with changes in olfactory mediated behavior (Carlson, 1996; deBryune *et al.*, 1997). These results and others in the area of the neurogenetics of olfaction are discussed thoroughly in the recent review chapter by Stocker and Rodriguez (1999). The *Drosophila* findings show that mutations affecting an ORN can involve the silencing of the ORN, in addition to the altering of the response spectra of other neurons (deBryune *et al.*, 1997). The changes in ORN response profiles were discovered after behavioral evidence in the mutants indicated that significant olfactory changes must have occurred.

Recently, it has been hypothesized that major shifts in moth pheromone blends might occur when previously unexpressed genes controlling pheromone component double bond position become expressed in some females in a population (Roelofs *et al.*, 2002). The new, altered blend might be attractive to a very few males in the population that have the capability of responding to both this new blend and the old blend, and through a process of asymmetric tracking over generations by males (Phelan, 1997), a new population of males and females communicating with this new blend would be formed (Baker, 2002; Roelofs *et al.*, 2002; Roelofs and Rooney, 2003). The degree to which pheromone-sensitive ORNs would be able to shift their tuning properties to correspond to and track, over generations, the new pheromone components is - important for such a shift in pheromone blend communication to occur.

It does seem that in some moth species that, compared with the rest of the species in their group, use unusual pheromone components (Löfstedt *et al.*, 1991; Löfstedt, 1993), the male pheromone-sensitive ORNs display correspondingly unusual response spectra (Löfstedt *et al.*, 1990). The examination of pheromone-sensitive ORN tuning properties within species as it relates to the ability to potentially accept odd pheromone molecules should be informative in future studies after the exciting findings of Roelofs *et al.* (2002). The degree to which pheromone blends can suddenly shift via expression of pseudogenes (Roelofs *et al.*, 2002) followed by asymmetric tracking by responding males (Phelan, 1997) can be first ascertained by studying the variation in pheromone-sensitive ORN tuning curves within, and then between, two or more targeted species within a group (Löfstedt *et al.*, 1990). Attention also needs to be paid to ORNs that are responsive to heterospecific antagonists, because these are the ones that may be involved in completing the asymmetric tracking process by preventing the continued attraction of broadly tuned males to females of the parent population over generations (Baker, 2002).

6.12.5. Use of Pheromones in Insect Control

6.12.5.1. Monitoring Established Populations

The most widespread and nearly ubiquitous use of pheromones has been for the monitoring of pest species' adult populations. Comprehensive IPM programs that were initiated in New York State and Michigan during the early 1970s were based

on good, species-specific monitoring traps for the complex of tortricid moth pests that impose direct and indirect damage to fruit. Tree fruit IPM was impacted positively, resulting in elimination of prophylactic calendar-date spray schedules and the introduction of timed applications of short-residual insecticides. During the mid-1970s to late 1980s, insecticide applications were reduced by more than 50% in New York State, Michigan, and the Pacific Northwest due to monitoring programs that improved decision-making about the need to spray insecticides (Madsen, 1981) as well as their timing. Such programs have become even more refined over the years, with concomitant further reductions in insecticide applications being documented (Agnello *et al*., 1994).

Monitoring of leafroller pests coupled with computer assisted degree-day models (Riedl and Croft, 1974; Riedl *et al*., 1976) allowed sprays to be timed for optimum efficacy against eggs and first instars on such key pests as the codling moth, *Cydia pomonella*, and oriental fruit moth. Spray-or-no-spray decisions based on abundance of adults in monitoring trap grids were also made possible by effective, standardized monitoring traps and deployment schemes developed against some pests such as the codling moth (Madsen, 1981). On other crops in the United States as well as around the world, pheromone monitoring traps have asserted themselves into IPM programs as essential elements to the success of these programs. Their use is such an integral and accepted part of IPM that we do not further review their use.

6.12.5.2. Detection and Survey Programs for "Exotic" Pests

Whereas we usually think of exotic pest species as being those originating from overseas, oftentimes the more distinct threat to agricultural crops comes from immigrating populations from one geographic area within a country into a different location. Pheromone traps have played a large role in detecting influxes of adult pest species from one region to another and also even from noncrop areas into crop areas. Survey programs involving grids of pheromone traps are used so routinely to report and track the yearly arrival of migrating adult populations of insects such as the black cutworm, *Agrotis ipsilon*, in the Midwest (Showers *et al*., 1989a, 1989b) or the spread of expanding populations such as the gypsy moth (Elkinton and Cardé, 1981) that it has become an essential part of our arsenal of tools for tracking and ameliorating pest movement related threats. A few examples of how pheromone trapping survey-detection systems have provided essential information that has aided in the suppression of migrating or expanding populations are reviewed.

6.12.5.2.1. Pink bollworm The pink bollworm, *Pectinophora gossypiella*, is a key worldwide pest of cotton. In the southern California desert valleys and in Mexico, its presence has altered IPM schemes since the 1960s, necessitating the application of 10–15 extra insecticide sprays per hectare per season. In the San Joaquin (Central) Valley in California, growers do not have to apply insecticides against this pest because it is not endemic, and therefore they enjoy much greater profit than their southern California counterparts. Indeed, many have attributed the near complete demise of cotton agriculture in southern California over the past two decades to the presence of the pink bollworm. The pink bollworm has not yet become established in the San Joaquin Valley, in large part due to an intensive grid of detection-survey traps that is deployed every year in a program run by the California Department of Food and Agriculture (CDFA) and supported by bale-tax "check-off" receipts from the grower group, the Cotton Pest Control Board.

Every summer, adult pink bollworms are blown into the Central Valley from Mexico and the southern California desert regions on tropical storm systems. The threat of these "blow-ins" mating and depositing eggs is huge; that they could survive and flourish to become established in the 284 000 ha of cotton grown in the valley is not disputed. The CDFA monitors an extensive grid of pheromone traps averaging approximately one trap per square mile, but extending at various densities throughout the Central Valley (Baker *et al*., 1990). When a male moth is discovered in a trap, an airdrop of sterile moths is implemented, with the plane being guided to the affected set of cotton fields, and the jettisoning of sterile moths from the plane all precisely recorded and verified by global positioning system computer-monitored tracking systems back at a home base. Sterile males are reared at the USDA–APHIS sterile pink bollworm moth-rearing facility in Phoenix. The sterile moths are dyed pink by the diet they ingest at the rearing facility, and so the many sterile males captured in the pheromone traps are easily distinguished from nondyed blow-ins. Quality control is also implemented by project leaders to confirm the vigilance of the many scouts who are employed each summer to check the traps. At predetermined and random times a nondyed male is placed in a trap and the discovery of this planted blow-in by the scout is awaited.

It is assumed that the presence of a nondyed male moth in a trap is indicative of females in the same area, and that the threat of an increasing endemic population of pink bollworms becoming established is real. The airdrops of sterile moths are aimed at creating a ratio of at least 60 : 1 pink:normal-colored (sterile : wild) male captures in the pheromone traps. This has proven to be an effective ratio for the sterile insect technique to eventually eliminate the nascent endemic population in location after location, year after year. When the 60 : 1 ratio cannot be attained, either by too many wild males present or too few sterile individuals being produced at a particular time by the sterile moth rearing facility, the CDFA then applies pheromone mating disruption. In the late 1980s, up to 2025 ha of cotton each year in the Central Valley received mating disruptant formulation applications in this context (Baker *et al.*, 1990). Monitoring and sanitation of this exotic pest under this program has provided a significant savings to growers and to the environment, allowing cotton to continue to be grown in central California with minimal insecticide load and maximum profits. The program is still operating successfully.

6.12.5.2.2. Boll weevil The boll weevil, *Anthonomus grandis*, has historically been one of the most economically damaging pests of agriculture in the United States. Its invasion into all of the southeastern states by 1917 from Mexico through Texas (Ridgway *et al.*, 1990) is widely considered to have caused the severe decline that occurred in cotton production in this region through the 1960s. Even now, it continues to be considered one of the major agricultural insect pests in the United States.

The identification of the male-emitted sex pheromone of the boll weevil by J.H. Tumlinson and colleagues in the late 1960s (Hardee *et al.*, 1967a, 1967b; Tumlinson *et al.*, 1968, 1969, 1970, 1971) resulted in the isolation and identification of a four-component pheromone (Tumlinson *et al.*, 1969, 1970, 1971). The components were identified as: (1*R*-*cis*)-1-methyl-2-(1-methylethenyl)cyclobutaneethanol; (*Z*)-2-(3,3-dimethylcyclohexylidene)ethanol; (*Z*)-(3,3-dimethylcyclohexylidene)acetaldehyde; and (*E*)-(3,3-dimethylcyclohexylidene)acetaldehyde. This blend was named (thankfully!) "grandlure." Numerous field trapping tests determined the requirement that all four components need to be present for optimal activity. The range of ratios that could be tolerated in achieving optimum trap capture of weevils was also determined (Coppedge *et al.*, 1973; Hardee *et al.*, 1974). An effective trap design was developed (Mitchell and Hardee, 1974) that allowed survey-detection and mass trapping studies to be initiated.

A program was subsequently initiated in Texas to begin large-scale efforts at suppressing boll weevil populations with stalk destruction, whose efficacy at reducing populations is monitored and confirmed by widespread deployment of pheromone traps (Ridgway *et al.*, 1990). However, the most widespread and effective use of the boll weevil pheromone traps has been in the highly successful population suppression program in the southeastern states. Initial trials were begun in the late 1970s in Virginia and northern North Carolina, consisting of surveillance and suppression through widespread pheromone trapping. Release of sterile boll weevils and also diapause control procedures (insecticide applications to kill adult weevils before cotton begins fruiting) were triggered if pheromone trap captures exceeded two to five weevils per trap (Ridgway *et al.*, 1990). Over the years of this program in this initial region, boll weevil capture levels declined to near-zero.

The success of the program in Virginia and northern North Carolina allowed the "boll weevil eradication" program to be expanded to the remainder of North Carolina and all of South Carolina in 1983. In 1987, the program was expanded further to include Georgia and parts of Alabama and Florida. The intensive dependence on pheromone traps as the key tool used in this program is indicated by the numbers of traps and lures used. In 1988 alone, approximately 590 000 traps were deployed and more than 8.25 million pheromone dispensers were used in these southeastern states (Ridgway *et al.*, 1990).

The pheromone traps served as a guide for application of insecticides, and this use of traps predominated during the initial years of the program when weevil densities and trap captures were high (**Table 2**). Beginning in year 2, if trap captures exceeded an average of 0.1 weevil per trap, insecticides were applied. However, if capture levels were lower than this, mass-trapping alone was considered adequate for population suppression (Ridgway, 1990). During the last 2 years of the second phase of the project, mass trapping was nearly exclusively used, due to the capture of so few weevils in most of the hectarage (**Table 2**). The effect of the program on weevil population densities over the first 4 years of the program was dramatic (**Table 2**). Whereas in the first year virtually no fields out of the 2800 fields sampled captured zero weevils, by year 4 more than 99.9% of the fields had zero captures. During the first year more than 95% of the fields had pheromone trap captures exceeding five weevils per trap, but by year 4 only 0.6% of the fields had trap captures exceeding this level (**Table 2**).

Table 2 Boll weevil captures in successive years of the expanded boll weevil eradication zones in North Carolina and South Carolina

Year	*Area (ha)*	*Number of fields*	*Percentage of fields capturing indicated numbers of weevils*		
			0	*1–5*	*>5*
1983					
North Carolina	6 600	800	0	1	99
South Carolina	21 240	2000	2	5	93
1984					
North Carolina	9 200	1000	21	35	44
South Carolina	32 000	2300	22	25	53
1985					
North Carolina	9 400	1000	90	7	3
South Carolina	37 000	4300	81	12	7
1986					
North Carolina	8 600	1000	>99.9	0	0
South Carolina	34 400	4200	97.3	2.3	0.4

The impact of the program was significant, in terms of allowing cotton production to once again expand and flourish, in terms of the reduction in insecticides applied per hectare (savings of \$69–74 ha^{-1} in Virginia, North Carolina, and South Carolina), and in terms of the increased yield value of the harvested cotton (increase of \$85 ha^{-1}) (Ridgway *et al*. 1990).

6.12.5.3. Mass Elimination of Insects from the Population by Using Pheromones

Early research in the applied uses of pheromones experimented with mass trapping (Roelofs *et al*., 1970). When experiments, mostly with adult moths, resulted in little or no suppression of populations, explanations as to the lack of effect focused on the fact that only males were being trapped, and various mathematical models were offered that showed the potential futility in using mass trapping with female-produced pheromones that attract and trap less than 95% of the males (Roelofs *et al*., 1970). It was, however, recognized that mass-trapping could be an effective method if good attractants for females could be identified (Lanier, 1990). Such attractants were already known for bark beetle species of the genus *Ips*, in which it is the males that locate the host trees, build galleries, and then emit pheromone to attract females. This male-produced sex pheromone also coincidentally attracts other (opportunistic) males, who fly to the trees on which any newly arriving females might be able to be mated with before they locate the male producing the pheromone (Borden, 1985).

Although this approach to using pheromones for direct control was largely ignored by many groups of pheromone researchers as a tenable population suppression method, Borden (1994) stated, "I contend that mass trapping has acquired an unjustifiably bad reputation." Borden, along with several other groups of bark beetle pheromone researchers were instrumental in exploring various forms of mass trapping to come up with successful demonstration plot programs for a variety of bark beetle pest species (Bedard and Wood, 1981; Borden and McLean, 1981; Lanier, 1981). Commercially successful programs then evolved from some of these programs.

Only a few moth sex pheromone researchers continued to explore another form of mass trapping (attract-and-kill) involving attracting only males to synthetic female sex pheromone sources laced with small amounts of insecticide (Charmillot *et al*., 1996; Charmillot and Hofer, 1997). This technique has begun to enjoy entry into the marketplace in recent years.

Finally, research and development of mass trapping systems for a variety of highly damaging tropical weevil species by using male-produced sex pheromones has resulted in highly successful commercial mass-trapping systems for suppressing populations of these species (Oehlschlager *et al*., 1992a, 1993, 1995, 2002). A few examples of the development of research successes into successful commercial products or governmental programs that have utilized various forms of mass trapping, including attract-and-kill will be discussed.

6.12.5.3.1. Mass trapping In the late 1970s populations of the spruce bark beetle, *Ips typographus*, were increasing yearly and threatening the very existence of the paper and lumber industries of Sweden and Norway. Because of the existence of a highly effective synthetic pheromone blend and the desperation of the industry, the governments of these two countries agreed to subsidize a mass-trapping program that would include virtually all the hectares of forest land affected or that could be affected by this pest. In 1979, more than 600 000 traps baited with the male-produced sex pheromone ("aggregation pheromone") were deployed in Norway, and more than 300 000 traps were deployed in Sweden, often with the help of local citizens who agreed to place them in the forests near their summer cottages in the northern areas of the countries. More than 4.5 billion beetles were trapped during the summer of 1979. The following year, the number of trees killed by *I. typographus* in Norway remained the same as during 1979 or was

slightly reduced, and the program was deemed highly successful because a large increase had been expected. In Sweden, there was a great reduction in the number of trees killed (Lie and Bakke, 1981). In both countries, this was the first year in many that tree mortality had not continued to increase. The forest industry was saved, and populations declined in the succeeding years. No replication or check plots could be used to assess this program due to the areawide (country-wide) deployment of the traps; however, this was not a scientific experiment, it was a desperate measure whose implementation correlated with the first nonincrease in the population in years.

The American palm weevil, *Rhynchophorus palmarum*, is a highly damaging pest of oil and coconut palms in the American tropics, and in Central and South America. A related species, *R. ferrugineus* is a major pest of oil and other palms in the Middle East. Larvae of *R. palmarum* cause direct damage when they bore into the trunks of the trees, but they also are a vector of red ring disease, which is caused by a nematode, *Rhadinaphelenchus cocophilus*. In the early 1990s, losses of trees either due to the weevil itself or to red ring disease (which necessitates removal of the infected trees) often routinely reached 15%. Due to the threat to the oil palm industry posed by this weevil in Costa Rica, experiments aimed at mass trapping the adult weevils were undertaken. An effective male-emitted sex pheromone (Rochat *et al*., 1991; Oehlschlager *et al*., 1992b) plus food attractant bait had been developed (Chinchilla and Oehlschlager, 1992; Oehlschlager *et al*., 1993) which attracts primarily females but also males, that are trapped in inexpensive bucket-type traps. The traps are baited with the pheromone, 2-methylhept-5-en-4-ol (Oehlschlager *et al*., 1992b) plus a stalk of sugarcane laced with carbofuran. Previous experiments had shown that neither sugarcane alone nor pheromone alone attracted significant numbers of weevils, whereas the combination attracted and trapped large numbers (Oehlschlager *et al*., 1992a, 1993).

In 1991 in a 30 ha stand of oil palms in a highly infested plantation in Costa Rica, 219 bucket traps baited with pheromone plus insecticide-laced sugarcane were placed at eye level on the trunks of oil palm trees in a 30 ha plot within the 1256 ha plantation. Over the course of the year, the number of weevils captured per trap per week declined from an initial 15 per trap to fewer than two per trap (Oehlschlager *et al*., 1995, 2002). In contrast, in the surrounding survey areas of the plantation, the number of weevils per trap per week increased from approximately 15 to an average of about 60 for the subsequent months, and ending at more than 20 per trap per week at sampling termination. Incidence of red ring disease in the mass trapping plot decreased in the year after initiation of the mass trapping, to level approximately only 20% of the previous levels and one-half that in the surrounding untreated stands (Oehlschlager *et al*., 1995, 2002).

Plantation-wide mass trapping was undertaken at the same time on two major oil palm plantations in Costa Rica, one comprising 6514 ha and the other comprising 8719 ha (Oehlschlager *et al*., 2002). Four-liter bucket traps baited as described above with pheromone plus insecticide-laced sugarcane stalks were deployed at chest level at a density of only one trap per 6.6 ha. The results in the two plantations were virtually identical (**Figure 2**). Before the mass trapping program was implemented, normal sanitation procedures involving removal of red ring disease-infected palms had failed to reduce the incidence of this disease. Once mass trapping was added to the system, after 1 year the incidence of diseased trees needing to be removed dropped by nearly 80%, from about 10 000 diseased trees removed to 2000 (Oehlschlager *et al*., 2002) (**Figure 2**). During that first year (1992–93) more than 200 000 weevils were trapped. In each successive year, the incidence of diseased trees declined until in 2001 only 50 diseased trees needed to be removed through sanitation (Oehlschlager *et al*., 2002) (**Figure 2**).

Subsequent commercial development of the *R. palmarum* mass trapping system has seen it be transformed into one of the most important pest management tools for weevil pests worldwide. Approximately 25 000 ha of palm plantings is estimated to be under *R. palmarum* mass trapping control yearly in Central and South America (A.C. Oehlschlager, personal communication). The density of traps now used is now only one trap per 7 ha. In addition to the ability of this male-based pheromone to attract females, and males, the reasons given for the success of mass trapping against *R. palmarum* (Oehlschlager *et al*., 2002) are first, that although the weevils cause great direct and indirect damage and induce tree mortality, they are present in relatively small numbers. Second, they have a long adult life, and so the capture of even low numbers steadily throughout the year can remove a large proportion of a generation and have a significant impact on population growth. Third, they are strong flyers, which allows the pheromone traps to be widely spaced (Oehlschlager *et al*., 2002).

These characteristics favoring success of mass trapping also seem to pertain to the other tropical

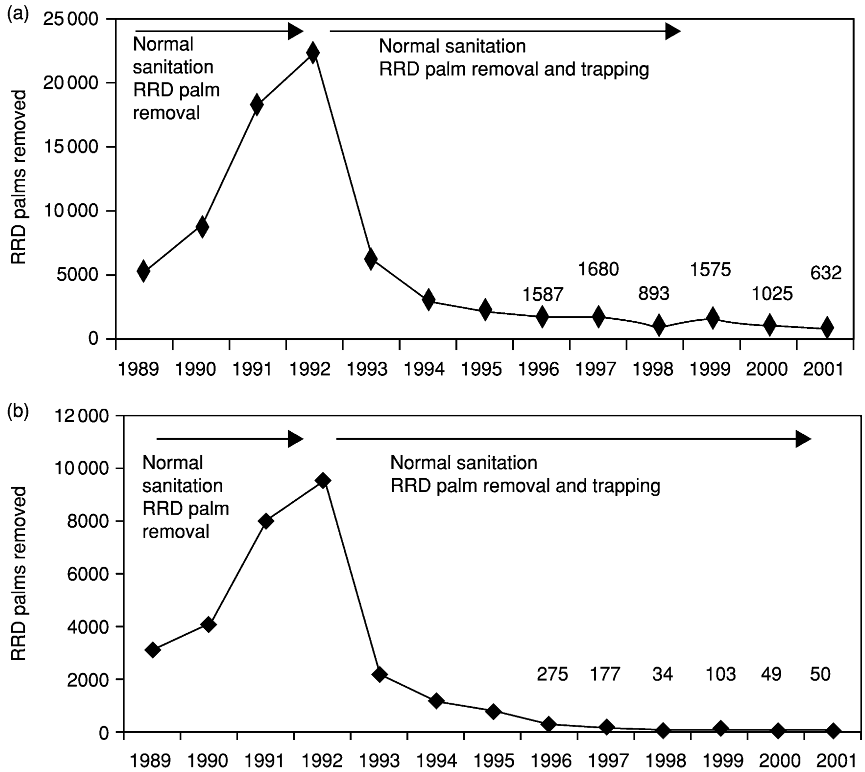


Figure 2 Incidence of red ring disease (RRD) vectored by the *Rynchophorus palmarum* weevil in oil palms in Costa Rica, before and after mass trapping of the weevils, as measured by the number of diseased trees that had to be removed each year from two plantations, (a) and (b), from 1989 to 2001. Plantation (a) comprised 6514 ha and plantation (b) totaled 8719 ha. From 1989 to 1992, before the implementation of mass trapping of the weevils, disease incidence steadily rose. During this period only normal sanitation and diseased palm tree removal were used to try to control the weevils. After 1992, when pheromone mass trapping was added to the program, the incidence of red ring disease declined dramatically. (Adapted from Oehlschlager, A.C., Chinchilla, C., Castillo, G., Gonzalez, L., **2002**. Control of red ring disease by mass trapping of *Rhynchophorus palmarum* (Coleoptera: Curculionidae). *Fla. Entomol. 85*, 507–513.)

pest weevil species. Other highly successful and effective commercial mass trapping systems using male-emitted sex pheromones are in place for these other species. For *R. ferrugineus*, more than 35 000 ha of palm plantings are treated with mass trapping every year in the Middle East. For another pest of palm, *Oryctes rhinoceros*, more than 50 000 ha yearly are under a mass trapping program in the Middle East (A.C. Oehlschlager, personal communication).

An estimated 10 000 ha of commercially grown bananas in the American tropics is under mass trapping programs every year against the banana weevil, *Cosmopolites sordidus* (A.C. Oehlschlager, personal communication). A simple and inexpensive pheromone trap plus this species' pheromone is used in only about four traps per hectare, but here the traps are placed on the ground because the weevils must walk into them to be captured in pit-fall manner. The traps are moved in a line week by week through the banana plantations to trap-out the weevils, and so the actual density of traps per hectare throughout the entire plantation that accomplishes weevil trap-out is extremely small.

Mass trapping also is being used extensively in palmito palm, which produces a delicacy, palm hearts. In palmito palm plantations, a combination lure for *R. palmarum* and the West Indian sugarcane weevil, *Metamasius hemipterus*, is used; the pheromones of the two species, 2-methylhept-5-en-4-ol and 2-methyl-4-heptanol (Perez *et al.*, 1997), do not interfere significantly with attraction and significantly lower the incidence of damage when placed in the same bucket traps at a density of four traps per hectare in this crop (Alpizar *et al.*, 2002). Hence, this "combination trap" kind of mass trapping program aimed at two species at once is now showing success at trapping and removing two damaging species of weevils from the population (Bulgarelli, 2002).

The ambrosia beetle, *Trypodendron lineatum*, is a worldwide pest in industrial timber processing areas, which, along with two other such beetles, *Gnathotrichus sulcatus* and *G. retusus*, causes degradation in lumber value that in the early 1990s was

estimated in to be more than $100 million per year in British Columbia (Lindgren and Fraser, 1994). This estimate ignores other losses, including export restrictions and modified harvesting and processing needed for the damaged timber. A major pheromone component of *T. lineatum*, 3,3,7-trimethyl-2, 9-dioxatricyclo [3.3.1.0^{4,7}] nonane ("lineatin"), had been isolated and identified from female frass in 1977 (MacConnell *et al*., 1977) and its field activity confirmed (Borden *et al*., 1979). The pheromone of *G. sulcatus* was identified from the volatiles of boring males as a 65 : 35 ratio of (*S*)-(+)- and (*R*)-(−)-6-methyl-5-hepten-2-ol (sulcatol) (Byrne *et al*., 1974), and the blend of both enantiomers was requisite to attraction (Borden *et al*., 1976). *Gnathotrichus retusus*, in contrast, was found to use only the (*S*)-(+)-enantiomer, which was identified from the dust of boring males (Borden *et al*., 1980a). Small amounts of the *R*-(−)-enantiomer in the blend were found to reduce attraction to the (*S*)-(+)-enantiomer (Borden *et al*., 1980a).

With the addition of host volatiles, synthetic versions of this female-emitted pheromone were highly effective at attracting males and females as well (Borden *et al*., 1980b). Research efforts towards developing a mass trapping program for keeping lumber yard operations sanitized from attack by all three ambrosia beetle species demonstrated the efficacy of this approach (Borden and McLean, 1981). Before the pheromone-based mass trapping program was available, many timber processing facility managers had used a trap-crop approach in protecting their yards, placing log piles in strategic locations around the yards and then collecting and destroying them (and the beetles) in pulp-chippers (Borden, 1990). The pheromone-based mass trapping IPM strategy evolved first from baiting log piles with pheromones, and then to enhancing beetle attraction to the piles, and finally to a pheromone-trap-only system that was made possible by the development of the easily used and highly effective multiple-funnel trap (Lindgren, 1983; Lindgren and Borden, 1983; Lindgren *et al*., 1983; Borden, 1990).

Several companies successfully marketed this ambrosia beetle mass trapping system, which is still in use commercially. One evaluation of the history of its use in one timber-sorting area on Vancouver Island showed that in more than 12 years, at least 16.5 million beetles had been captured, while keeping infestations to a minimum at an acceptable cost and economic benefit (Lindgren and Fraser, 1994). A key element to the companies' success, and the efficacy of the mass trapping programs, was that clients were required to purchase a service contract to accompany the sales of the traps and lures for the three species. This ensured that the appropriate vigilance and consistency in trap efficacy were maintained (Borden *et al*., 2001).

It is clear that the once-discounted technique of mass trapping using either male-produced or female-produce pheromones has become a newly appreciated, highly effective, environmentally friendly, and relatively inexpensive means of suppressing populations of certain pest species whose pheromone communication systems and bionomical characteristics make them susceptible to this approach. The next section dealing with attract-and-kill is yet another example of possible ways to gain control over pest populations with sex-attractant pheromones.

6.12.5.3.2. Attract-and-kill In the early 1980s many cotton growers in the southern California desert valleys who had been successfully using the hollow-fiber sex pheromone mating disruption system against the pink bollworm started experimenting with reducing the amount of active ingredient (a.i.) per acre, often by one-half or more, and adding small amounts of pyrethroid to the sticky glue in the tank mix that adheres the fibers to the cotton leaves when flung from the specialized aerial application mechanism (Baker *et al*., 1990). The apparent success of this technique in keeping damage low with reduced amounts of a.i. signaled the beginning of the concept of "attract-and-kill." Observations showed that males indeed were attracted all the way to the pyrethroid-impregnated stickum on the fibers (Haynes *et al*., 1986; Baker *et al*., 1990; Miller *et al*., 1990) and that they were killed or else rendered incapable of flying upwind to subsequent sources in the following days due to sublethal toxic effects (Haynes *et al*., 1986).

Pierre Charmillot and colleagues, after years of meticulous experiments in codling moth mating disruption with small apple orchards with irregular borders and high population hot spots (Charmillot, 1990), began experimenting with attract-and-kill in isolated apple orchard blocks (Charmillot *et al*., 1996; Charmillot and Hofer, 1997). In their studies, they used a formulation called Sirene CM (Ciba Corporation) that consisted of very small viscous gel droplets (0.05 ml) containing small amounts of permethrin (3 mg) and 0.08 mg of codling moth pheromone. Droplets were applied at between 1600 and 5000 drops ha^{-1} and after either one of the treatments, damage at harvest was 0.24%, 0.32%, 1.1%, and 1.14% in four orchards.

Two orchards that received a second year of attract-and-kill had negligible larval attack and very

low hibernating larval levels on the trunk (less than one larva per tree). These experiments showed that this technique is effective in keeping damage from this pest at acceptable levels using attract-and-kill alone. The technique works especially well in small (ca. 1 ha) blocks of apple that are not amenable to treatment with standard mating disruption formulations due to their large border-length-to-area characteristics. Proof that the attract-and-kill mechanism and not just mating disruption was the cause of the population suppression came from a second set of experiments in which the pheromone-impregnated droplets either did or did not contain small amounts of insecticide. The plots to which the insecticide-containing droplets were applied showed significantly reduced trap capture and tethered female mating reduction, whereas plots that received droplets containing pheromone without insecticide exhibited virtually no monitoring trap capture reduction and no reduction in tethered female mating (Charmillot *et al.*, 1996; Charmillot and Hofer, 1997).

One major conclusion that can be drawn from these successful studies using the codling moth is that it is possible to use mass trapping (attract-and-kill) against only males and achieve successful population suppression. The innovative bent of certain cotton growers and at least one determined codling moth researcher showed that the initial negative conclusions of early pheromone researchers concerning mass trapping of male moths were premature, or at least based on incompletely understood assumptions. At present, a commercially successful attract-and-kill product against the codling moth based on Charmillot's work and through substantial development efforts by CIBA Corporation is being marketed as "Last Call" in North America by IPM Technologies, Inc. Other attract-and-kill Sirene products using the pheromones of other species are being developed against other pests.

For attract-and-kill to work, obviously an optimally attractive pheromone blend formulation is required that will attract the males of the target species all the way to the source so that they contact the insecticide-laced matrix. This, and the need for so many droplets to be applied and retain their contact toxicity for a long period, are some of the exacting requirements necessary to be met for attract-and-kill to successfully take its place as a useful and successful pheromone-mediated population suppression tool.

6.12.5.4. Mating Disruption

6.12.5.4.1. Evolution of mating disruption technique Mating disruption is the technique by which pheromone is dispensed into a pest habitat in sufficient amounts to reduce the ability of males to find females, or vice versa. In the earliest days of mating disruption trials, it was thought that it was essential to dispense the pheromone such that a ubiquitous cloud of pheromone pervaded every cubic meter of habitat to disrupt mate-finding communication. Most trials concentrated on the use of microcapsules sprayed through conventional pesticide sprayers to achieve uniform coverage. Then, the first mating disruption formulation to be registered by the Environmental Protection Agency (EPA), the Conrel hollow fibers, was developed in 1978. It used hollow microfibers ca. 2 cm in length. These fibers were distributed over cotton fields by using airplanes equipped with specialized applicator technology. These dispensers, once they landed on and stuck to the cotton leaves, emitted volatilized pheromone by means of an open capillary tube technique and were effective when applied at an estimated density of one fiber m^{-2}. The evolution of dispensers into a yet stronger point source emission mode and further away from the original uniform fog mode occurred in the mid-1980s with the commercial appearance of the Shin-Etsu "ropes." These were made up of the pheromone of the target species residing in sealed polyethyelene tubes that were hand-applied in a twist-tie manner around plant stems. The reduction in the number of point sources per hectare from tens of thousands (fibers) to now only 1000 ha^{-1} was achieved with fewer but higher strength point sources with no loss of efficacy.

Still, researchers had not paid attention to the results of an important set of experiments conducted by Farkas *et al.* (1974), who followed up on initial intriguing results from Shorey *et al.* (1972) which showed that the number of point sources could be reduced to only a few per hectare if they were made to emit at very high rates so that the total amount of pheromone emitted per hectare remained at as high a rate as when many more, but weakly emitting, point sources were deployed. Some researchers started experimenting with this concept in the 1990s, and formulations using only 10–15 dispensers per hectare are now available commercially. The initial research on these involved the use of "active" emitters, which were machines that could be programmed to project aerosolized pheromone solution into the air and onto absorbent pads, which would then emit high amounts of pure pheromone of the target insect (Mafra-Neto and Baker, 1996a; Shorey and Gerber, 1996a, 1996b; Shorey *et al.*, 1996; Baker *et al.*, 1997; Fadamiro *et al.*, 1999b).

The initial concept was that the machines could be programmed to emit pheromone only during the

time of activity of the target (moth) insect pest's sexual activity. Efficacy in disrupting sex pheromone communication of a number of different species was clearly demonstrated (Mafra-Neto and Baker, 1996a; Shorey *et al.*, 1996; Shorey and Gerber, 1996a, 1996b; Baker *et al.*, 1997a, 1997b; Fadamiro *et al.*, 1998, 1999b). Then, it became evident that the emission of pheromone, whether from the pads or from surrounding foliage onto which the pheromone solution absorbed, was a constant, passive emission phenomenon. The MSTRS Technologies, Inc., dispensers that had previously been of the active-spray type using aerosolized pheromone evolved into high-release-rate passive-emitting dispensers. The MSTRS system is currently commercially available for use against several pest species, and dispensers are deployed at densities ranging from 10 to 20 ha^{-1}. One original machine-driven aerosolized dispenser system Suterra LLC's is also commercially available and in use against the coding moth.

6.12.5.4.2. Behavioral mechanisms involved in successful mating disruption A very few important studies have provided behavioral observations that have helped understand how moths' abilities to locate sources of female sex pheromone are diminished by the application of mating disruption dispensers. At least four mechanisms have been implicated when the disruptant used is an effective synthetic blend mimicking the natural blend (Bartell, 1982; Cardé, 1990; Cardé and Minks, 1995):

1. Attraction-competition, in which males are attracted some distance toward the disruptant dispensers and waste time flying around in response to these plumes.
2. Sensory adaptation or habituation, in which exposure to the synthetic disruption blend raises the threshold of response (lowers sensitivity) to subsequent plumes.
3. Camouflage of natural female pheromone plumes by the strong plume-strands emanating from the synthetic dispensers.
4. Advancement of the periodicity of activity of males such that they begin responding to the synthetic disruptant dispensers before females begin to call.

These mechanisms are not thought of as operating independently of one another, but rather most of the time they act in concert, depending on the dispenser type that is used and the environmental conditions (Cardé, 1990; Cardé and Minks, 1995).

There is evidence from many studies that what has now been more aptly termed "competition" (Cardé and Minks, 1995) but had previously been labeled "confusion" or "false trail-following," does in fact occur in mating disruption-treated fields, especially those receiving discrete point sources of dispensers such as hollow fibers or ropes. It is unknown, but it is regarded as unlikely, that male moths in fields treated with sprayable microcapsules, creating a nearly uniform fog of pheromone from the closely spaced, weak point source emitters, would be subject to the competition mechanism. The majority of species that have been tested for their responses to uniform clouds of pheromone quickly habituate when released in the pheromone fog, and cease upwind flight after only 1 or 2 s, reverting to cross-wind casting within the cloud (Kennedy *et al.*, 1980, 1981; Willis and Baker, 1984; Baker *et al.*, 1985; Justus and Cardé, 2002).

However, for disruptant formulations that use slightly more widely spaced and stronger point sources, attraction competition imposed by these sources on males should be more likely to come into play, and has been observed in the field (Miller *et al.*, 1990; Cardé *et al.*, 1993). The flight tracks of pink bollworm males have been recorded (Cardé *et al.*, 1997) in large wind tunnels placed over disruptant-treated cotton fields, as they flew upwind in response to the pheromone emitted by Shin-Etsu ropes that had been in the field for 7 days (as well as to those that had been in the field for only 24 h). Males that had been preexposed to the ropes within disruptant-treated fields for 24 h, as well as those males having had no preexposure, flew upwind to the rope dispensers, with the only difference being that the percentage of the 24 h preexposed males doing so was significantly reduced (Cardé *et al.*, 1997). The flight tracks of males flying upwind in response to the ropes appeared similar to the tracks of males flying upwind to much lower strength rubber septum monitoring trap lures, with the tracks of males flying to the ropes exhibiting a slower ground speed and more tortuous paths. The males were observed to land on or near (within 50 cm of) the rope dispensers and then become quiescent for long periods, implicating a second mechanism, habituation of the olfactory pathways, as having occurred after attraction competition.

Habituation is not an all-or-nothing phenomenon. The degree to which the olfactory pathways are habituated depends on the dose of pheromone applied during preexposure. Mafra-Neto and Baker (1996b) demonstrated, using a graded series of preexposure dosages of the pheromone of the almond moth, *Cadra cautella*, that lower habituating do-

sages reduced males' ability to respond subsequently to normal-strength pheromone plumes but that the usual percentage of these males could fly upwind and contact the source if stronger loadings of pheromone were used on the test lure. Thus, the degree of habituation depends on the concentration of the preexposure regime, as well as on the subsequent attractant lure, demonstrating that habituation behaves in a graded manner. The habituating concentration elevates the subsequent response threshold, and the highest habituating concentrations elevated the threshold of tested males to the greatest degree, requiring extremely strong attractant lure dosages to achieve any subsequent attraction whatsoever (Mafra-Neto and Baker, 1996b).

Habituation can occur without attraction-competition being evoked at all. Female pink bollworm produce and emit approximately a 50 : 50 ratio of (*Z,E*)-7,11-hexadecadienyl acetate and (*Z,Z*)-7,11-hexadecadienyl acetate (Hummel *et al*., 1973). The 1 : 1 ratio of the two isomers captures the most males in field trapping experiments, and neither the (*Z,Z*) nor the (*Z,E*) isomer alone traps significant numbers of males (Flint *et al*., 1979). The pure (*Z,Z*) isomer does not even elicit significant levels of upwind flight in laboratory wind tunnels (Linn and Roelofs, 1985). In a series of experiments, Flint and colleagues experimented with applying disruptant formulations, either Hercon laminated flakes or Shin-Etsu rope dispensers emitting only the pure (*Z,Z*) isomer (Flint and Merkle, 1983, 1984; Flint *et al*., 1988). Monitoring traps containing lures emitting a series of blend ratios from 0 : 100 to 100 : 0 of the two isomers were deployed throughout the disruptant-treated fields. Trap catch was totally shut down in the fields treated with the 50 : 50 ratio-emitting hollow fibers. Likewise, trap catch was also nearly totally suppressed by the pure (*Z,Z*) isomer-emitting dispensers. However, in these (*Z,Z*) isomer-treated fields there was a significant capture of males, albeit small, but the captures were significantly shifted to the monitoring traps emitting a 90 : 10 ratio of (*Z,Z*) to (*Z,E*). Males apparently were becoming habituated to the pure (*Z,Z*) isomer alone, and the monitoring lures emitting the highly enriched (*Z,Z*) to (*Z,E*) blend were now able to attract pink bollworm males as if the blend now was perceived as being more similar to 50 : 50 in these disrupant-treated fields.

Because the dispensers emitting the pure (*Z,Z*) isomer will not have been able to cause any attraction (and therefore no competition), the conclusion must be that sensory adaptation or habituation of the sensory pathways alone must be the mechanism acting to cause the near-elimination of trap capture in the (*Z,Z*) isomer treated disruption plots. Importantly, even though strong point source dispensers were used (especially the Shin-Etsu ropes), enough pheromone wafted through the fields from such sources that habituation alone without attraction significantly affected male behavior. Linn and Roelofs (1981) had demonstrated, using the oriental fruit moth, the ability of single components to exert a component-specific habituation on a particular sensory pathway specific for that component. The result was, as in the pink bollworm mating disruption experiments, that males that had been preexposed and habituated to only one (nonattractive) component were subsequently optimally attracted to multicomponent blends that needed to be enriched with excessive amounts of that component. The artificially enriched blend would now be perceived as the normal unenriched blend to which nonhabituated males are optimally attracted.

A third mechanism, "plume camouflage," has also been implicated as a mechanism involved in mating disruption (Cardé *et al*., 1993; Cardé and Minks, 1995). The amount of pheromone in the plumes from the disruption dispensers is thought either to overwhelm the strength of the signal from the female-equivalent source or possibly to dampen the amplitude of ups-and-downs in plume-strand flux over the antenna; the plume's fine-scale structure has been shown to be essential in eliciting reiterative upwind surges from strand to strand that promote sustained upwind flight (Mafra-Neto and Cardé, 1994; Vickers *et al*., 1994). Using their large field wind tunnels situated in cotton fields, Cardé *et al*. (1993) concluded that the reduced ability of male pink bollworms to fly upwind to and be trapped in monitoring traps in some of their experimental regimes in the tunnels was due to the ambient emitted amounts of pheromone from pheromone dispensers present in the fields that camouflaged the weaker plumes from the monitoring traps. As they pointed out (Cardé *et al*., 1997), it is difficult to prove that such a mechanism is working independently of habituation and also competition, but the measurements of pheromone concentration by means of field electroantennograms seemed to support their conclusions that camouflage was indicated as the most likely dominant mechanism in some special experimental regimes (Cardé *et al*., 1993). Camouflage using uniform clouds of pheromone does not seem sufficient for preventing upwind flight of moths in response to the intermittent flux provided by point source plumes (Kennedy *et al*., 1980, 1981; Willis and Baker, 1984; Schofield *et al*., 2003).

Finally, it has been well known since the early days of pheromone research that in a large number of moth species, the diel periodicity of male responsiveness to pheromone is broader than the female calling period (Cardé and Baker, 1984). For pink bollworm, this includes an advanced responsiveness of males to synthetic pheromone lures that precedes the normal time of sexual activity to females in the field (Breasley and Adams, 1994; Schouest and Miller, 1994). The observations of Cardé *et al.* (1997) in their large field wind tunnels also seemed to indicate that not only were freshly released males able to respond in a precocious burst of activity just after sunset but also males that had been exposed to field levels of mating disruptant for 24 h exhibited an advanced period of responsiveness compared with native males. Such behavior would work to the advantage of mating disruptant formulations, causing males to be exposed or to expose themselves through attraction competition with habituating amounts of pheromone before females would even begin calling.

Cardé and Minks (1995) and Cardé *et al.* (1997) suggested that combinations of mechanisms will likely be operating in concert to various degrees, depending on the dispersion of pheromone by a particular formulation, to reduce males' abilities to respond to pheromone plumes from their females. For example, when males are being "confused" and flying upwind in the plumes of synthetic pheromone emitted by hollow fibers (Haynes *et al.*, 1986; Miller *et al.*, 1990) or Shin-Etsu ropes (Cardé *et al.*, 1997), they are receiving high-concentration contacts with the strands of pheromone in those plumes, and habituation of the sensory pathways is occurring as a result of the male remaining in upwind flight and continuing to maintain contact with those strong pheromone strands.

This combination of attraction and habituation has been invoked as the mechanisms explaining why the very high concentration, low point source density dispensers such as the "puffers" and MSTRS dispensers work despite their very wide spacing (Baker *et al.*, 1998b). The very highly concentrated pheromone strands in a plume from a single such dispenser can be effective at great distances downwind, causing males that contact them to begin flying upwind while maintaining contact with the plume. As the males proceed upwind, they continue to "dose" themselves with highly concentrated pheromone strands, and at some point after a prolonged upwind flight to the source or near the source, the males become habituated to the pheromone and cease upwind flight, as has been observed for males flying upwind to the lower emission rate, less widely spaced Shin-Etsu ropes (Cardé *et al.*, 1997).

As formulations use increasingly widely spaced, high emission rate means of emitting pheromones, optimal attraction using the blends most closely approximating the natural female blend will become necessary to prolong the time a male spends locked onto the plume, dosing himself with high amounts of pheromone to become habituated. At the other extreme, sprayable microcapsule formulations will likely depend very little, if at all, on the attraction mechanism, relying almost exclusively on habituation or plume camouflage. Regardless of the type of formulation, it is expected that all should be able to take advantage of the advanced period of sexual responsiveness that males exhibit before females begin to emit pheromone.

6.12.5.4.3. Mechanisms by which mating disruption suppresses population growth It used to be assumed, and is still assumed by many people working in chemical communication, that successful mating disruption can only occur if the majority of females in a population are prevented from mating after the application of a mating disruption formulation. In reality, in all but a handful of the huge number of mating disruption field trials that have been conducted over the years, mating disruption itself has never been directly assessed. That is, the mating success of feral, freely flying females has rarely been evaluated. The use of tethered females or clipped-wing females placed on "mating tables" or at stations deployed throughout the disruption plot and then dissected for the presence (mated) or absence (unmated) of spermatophores does not assess what is really happening to feral females that have the ability to fly freely throughout the area. The tethered female technique gives the illusion of being a robust, real-world assessment of mating disruption efficacy, and although it is one of many good indicators, it is especially deficient when the insects distribute themselves unevenly within the habitat due to environmental factors such as heat, humidity, and wind. Unless the researcher knows ahead of time the locations where adults typically are most densely clumped and can tether the females there, the ability of the disruptant formulation to keep males from finding females will be overestimated.

The results of the few studies that have assessed the mating success of freely flying feral females are illuminating. For the highly successful and grower-accepted Shin-Etsu rope formulation used against the oriental fruit moth, Rice and Kirsch (1990) found that in plot after plot treated with mating

disruptant, females' abilities to mate at least once in disruption-treated plots was only suppressed at most 50% relative to check plots during a flight. Just as commonly, this ability was as little as 15–20% suppressed, despite the reduction of fruit damage in these plots to acceptable levels comparable with those when the standard insecticide regime was used (Rice and Kirsch, 1990). Thousands of females were captured in terpinyl acetate bait pails and analyzed for the presence or absence of spermatophores in their bursae copulatrices in both the disruptant treated plots and the check plots. This study was the first indication that for successful population suppression and damage reduction, it is not necessary to keep females unmated throughout their adult lives. Vickers *et al*. (1985) reported similar results for oriental fruit moth feral female mating success in Australian mating disruptant treated plots (23% mated females in mating disruption plots versus 90% in check plots).

Knight (1997) released six batches of 6000 sterilized codling moth females into disruption treated and check orchards and recaptured them in light traps or passive interception traps to dissect them and to look for the presence of male spermatophores to assess females' success at mating. The disruption formulation used was Isomate-C+, a Shin-Etsu rope-type dispenser system that had already been showing success at suppressing populations of codling moths in the Pacific Northwest. Again, as for the oriental fruit moth, after several days, more than 40% of codling moth females from the disruptant treated plots contained spermatophores, showing that they had mated, despite the application of this highly successful formulation. However, Knight (1997) noticed that females took longer to reach this level of mating success (4 days) in the disruption plots than they did in the check plots (1 day), and introduced the concept that perhaps successful population suppression through the application of mating disruptants requires only that the formulation delay mating, not suppress it entirely. Laboratory studies that prevented codling moth females from gaining their first mating by from 1 to 10 days showed that females whose first mating was delayed by as little as 3 days had reduced fertility and fecundity compared with those who had gained their first mating by day 1 or 2. When female first mating was delayed by 8 to 10 days, the number of fertile eggs laid was only 20% of that of females that had mated on day 1 or 2 (Knight, 1997).

The delayed mating concept was again implicated in studies on European corn borer *Ostrinia nubilalis* mating disruption (Fadamiro *et al*., 1999b). In these studies, analyses were made of the bursae copulatrices of more than 2400 feral females that were captured by hand-netting during the daylight hours as they were flushed from their grassy aggregation areas. The data showed that during each of the two summer flights, 100% of the females eventually became mated despite the application of high release rate, low point source density dispensers (Fadamiro *et al*., 1999b). What was interesting was that at the beginning of the first flight ca. 50% of the females remained virgin in the mating disruption plots, but the mating success of females in these plots eventually reached 100% during the ensuing weeks as the flight proceeded (**Figure 3**). They attained this 100% mated status more slowly than did females in the check plots, which were 100% mated beginning at day 1 (**Figure 3**).

Analysis of the frequency of mating by the European corn borer females captured in the disruptant treated plots versus those from the check plots showed that throughout the entire flight females from the disruption plots were attaining matings at a significantly lower rate than those from the check plots (Fadamiro *et al*., 1999b) (**Figure 4**). The mating disruptant was impairing the ability of females to attract and mate with males on a constant, daily basis but it did not completely eliminate mating. The application of this MSTRS formulation has subsequently been shown to reduce damage to corn by an average of 50–70% in various trials (Baker, unpublished data). Thus, as demonstrated in studies on the oriental fruit moth (Rice and Kirsch, 1990) and codling moth (Knight, 1997), mating disruption success does not require keeping the population of females virgin, but rather just needs to impede females' ability to attract males and attain their first or even subsequent matings. Thus, we must now expect to only retard, not eliminate, mating by females as a benchmark for successful population suppression through the application of mating disruption formulations.

How would delayed mating produce such significant population reductions? A follow-up study by Fadamiro and Baker (1999) investigated the effects of several manipulations on the timing and frequency of European corn borer females' matings during their lifetimes. With regard to delaying the first mating of these females, a delay of first mating to day 4 compared with day 1 produced a greater than 30% overall reduction in the number of fertile eggs laid during their 16-day lifetimes. Further delaying first mating until day 7 resulted in no fertile eggs laid at all, even though these females lived for 16 days total (Fadamiro and Baker, 1999). These results mirrored those of Knight (1997) that experimentally

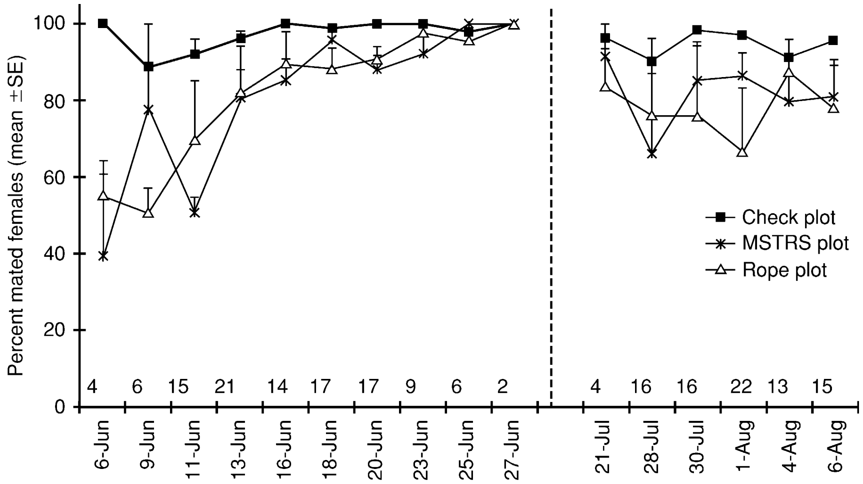


Figure 3 Delayed mating imposed by mating disruption of the European corn borer (*Ostrinia nubilalis*) in Iowa as measured by the percentage of feral, free-flying European corn borer females that were found to have mated at least once on the indicated dates in mating disruptant treated plots or in untreated check plots. Females were captured by hand-netting on the dates shown in grassy areas within and between corn fields and examined for the presence of male spermatophores. During the first of the two flight periods (left panel) 50% of the first females to emerge were kept unmated by the mating disruption treatments, either MSTRS aerosol-based spray machines (MSTRS plot), or by Shin-Etsu polyethylene tubes (rope plots). Over the course of the flight, the percentage of mated females increased to finally reach 100% by the end of the flight period. In check plots the proportion of mated females was nearly 100% from the first days of the flight and continued at that level throughout. During the second flight (right panel), hand-netting did not begin early enough to measure mating success at the beginning of the flight and ended before the flight period ceased. (Reproduced from Fadamiro, H.Y., Cossé, A.A., Baker, T.C., **1999b**. Mating disruption of European corn borer, *Ostrinia nubilalis* by using two types of sex pheromone dispensers deployed in grassy aggregation sites in Iowa cornfields. *J. Asia-Pacific Entomol. 2*, 121–132.)

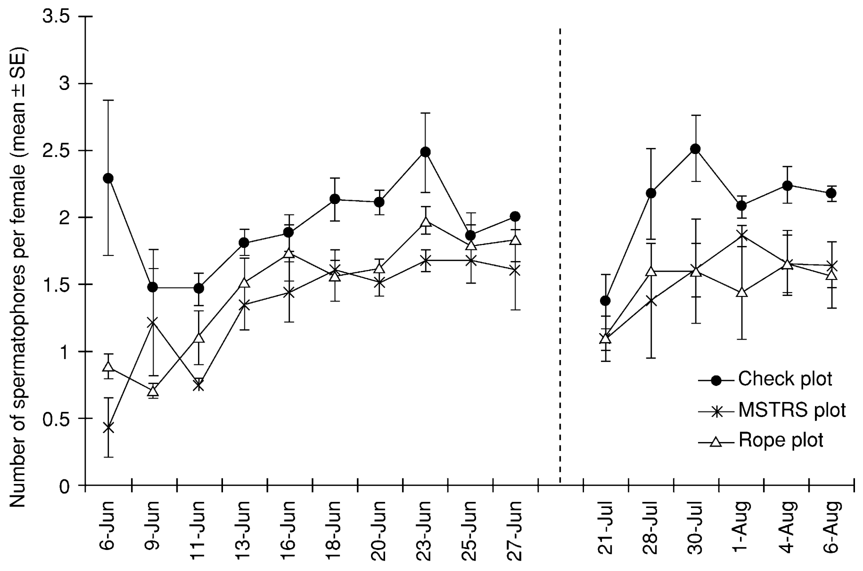


Figure 4 The frequency of mating by European corn borer females in disruptant-treated fields (''MSTRS plot'' or ''rope plot'') is lower than in untreated check plots, as measured by the mean number of male spermatophores found in females collected by hand-netting on the indicated dates. Females' ability to attain matings on any date during the first (left panel) or second flights (right panel) was impaired continuously throughout the flights by the presence of mating disruption dispensers. Thus not only is a female's ability to mate for the first time delayed by mating disruptant dispensers, but so is the second mating. The propensity of females in check plots to mate more than once also suggests that one mating is not sufficient to lay a full complement of fertile eggs. (Reproduced from Fadamiro, H.Y., Cossé, A.A., Baker, T.C., **1999b**. Mating disruption of European corn borer, *Ostrinia nubilalis* by using two types of sex pheromone dispensers deployed in grassy aggregation sites in Iowa cornfields. *J. Asia-Pacific Entomol. 2*, 121–132.)

delayed the first matings of codling moth females (see above).

Fadamiro and Baker (1999) also investigated the effects of reducing multiple mating, because the majority of females from the check plots in the field experiments had mated two or more times (two or more spermatophores), implying that there is an advantage for feral females to obtain a second mating. Therefore, another set of experiments was performed to examine the effects of allowing females to mate more than once (Fadamiro and Baker, 1999). Females allowed to mate only once at day 2 laid only 50% as many fertile eggs during their lifetimes as those that had been allowed to mate twice. Thus, another mechanism by which mating disruption of this species can help suppress populations is by significantly reducing fertile egg production by impeding females' ability to achieve a second mating. When one considers other factors such as mortality of females over time due to predation and other natural causes, these ancillary factors could conceivably augment the fertility effects produced by delayed mating and result in even more profound levels of population reduction.

6.12.5.4.4. Assessing the efficacy of mating disruption

6.12.5.4.4.1. Damage reduction Of primary importance to growers and to companies marketing mating disruption products is the ability of a formulation to reduce crop damage to acceptable levels. In this context, "successful" mating disruption means assessing crop damage in pheromone treated plots versus check plots and finding that damage in mating disruption plots is lower. This process is problematic, but considered by many to be a major outcome. The assessment is relatively straightforward, but it is essential that plots be large enough to reduce the probability that significant numbers of gravid females can fly in from nearby untreated plots and confound the damage data in the pheromone treated plots.

The choice of an appropriately large size for the plots depends on the propensity of females of a particular species to move long distances . It also depends on the damage acceptance levels for a particular pest, with direct-damage pests such as codling moth having lower damage tolerances than indirect-feeding pest species that may have higher damage acceptance levels. As a starting point for all species, 2 ha plots could be considered to be a minimum size for assessing damage reduction. But for direct-feeding pests and perhaps also a greater tendency of females to fly far, 10 ha would be a safer plot size for such testing. True replication, with check plots having to be many hundreds of meters away from the treated plots to reduce the possibility of drift effects, is difficult to attain, if not questionable with regard to ideal statistical design. However, these are the constraints under which such experiments need to be designed.

For tree fruit crops such replication is achievable and less scientifically debatable, but for field crops such as corn, it is virtually impossible to meet all the objections that arise when trying to compare damage and crop yield. This is because, for instance for corn, finding plots planted to identical cultivars is not the entire problem; differences in planting date and crop phenology can confound comparisons among identical cultivars. In corn, oviposition by European corn borer females is notoriously affected by the height of the corn, which in turn is affected by planting date or temperature differences between fields located even a few miles apart. A difference of only 2 or 3 days in planting date can produce 0.3 or 0.6 m differences in plant height, and so even if two fields are chosen for an experiment that have been planted to the same cultivar, damage may differ significantly between the two fields merely because the taller plants in the earlier planted field are targeted preferentially by ovipositing females.

Being an indirect measure, damage assessment evaluates the end result of many processes that are of interest to those operating in commercial integrated pest management and agronomic arenas. Conclusions that mating disruption was "successful" in the context of suppressing damage and being cost-effective can be arrived at without knowing exactly to what degree the formulation affected the behavior of males to reduce mating by freely flying feral females.

6.12.5.4.4.2. Assessing reduction of mating by use of tethered females Considered over many years to be the ultimate test of mating disruption efficacy, tethering females either on a thread or by clipping their wings and placing them on open arenas so that they cannot move from the location at which they are placed has been a good tool in assessing mating disruption efficacy (Evendon *et al.*, 1999a, 1999b). However, although it looks as if it is a direct and appropriate assessment tool, it is actually only one of several other indirect measures, and it can be argued that these other measures may be even better indicators than are tethered females as to what is happening to the actual female population in the field.

Tethered females are placed on stations in the field and allowed to remain overnight to determine whether they are able to attract and mate with males either in disruptant treated fields or in check fields.

They are retrieved and dissected in the laboratory to look for the presence of spermatophores, which, if present, would mean that one or more males had been able to detect, locate, and mate with the female by means of her pheromone. One limitation to this technique is that tethering or otherwise restraining females limits their ability to find the best location in the foliage to try to attract males. It is true that a relative difference in mating by tethered females in check plots compared with disruptant treated plots can be valuable, but translating this directly into conclusions about the absolute ability of a disruption formulation to similarly reduce the matings of feral females is inadvisable.

For example, if the adult moths in the natural population reside in a more clumped distribution in some preferred substructure of the vegetative habitat, placing females on artificial stations outside of each of these clumps will overestimate the efficacy of the disruption formulation with regard to preventing mating of feral females. The formulation in effect will only be being assessed for its ability to prevent the tethered females from attracting males out of their aggregation sites, and this long distance attraction will be easier to disrupt than will be the disruption of males from locating females that are within the same clumps as the males.

Another limitation to this technique is that researchers only get an all-or-nothing, yes-or-no indication from each female as to how well the disruptant is working in the field. Once a female mates with the first male arriving at her station, she emits no more pheromone and the ability to assess disruption of communication using that female ends. The data are binomial, with no opportunity for a graded assessment from individual females as to how many males they could have attracted had they been calling for the entire activity period that night and not mated.

6.12.5.4.4.3. Trap capture of males by using caged, calling females In this technique, a few virgin females are placed in small screen cages within which is a sugar water source to keep the females alive and hydrated (Baker *et al*., 1997b). The cage is situated within a sticky trap, and so males that are attracted to the calling virgin females become ensnared before reaching the females. Males that do manage to land on the cage containing the females without getting trapped will still not be able to mate with any female in the cage. This technique has many advantages over other indirect measurement techniques such as the use of tethered females.

First, as with tethered females, the caged females are emitting their natural blend at its natural emission rate. However, unlike tethered females, the caged females in traps provide a graded assessment of their ability to attract males. If males are able to get close enough to the calling females that they can be trapped, they certainly would have mated if given that opportunity at such close range. The final step, mating, is an unnecessary one to examine because if the disruptant was not able to prevent a male's long distance orientation to the female's plume, certainly it will not be sufficient to stop the male's orientation to the female over the last 10–20 cm or so. Thus, this technique allows a more robust assessment of females' ability to attract males in disruption treated plots than is available from seeing whether tethered females mate.

6.12.5.4.4.4. Trap capture of males by using lures emitting synthetic pheromone blends The most widely used technique for assessing the disruption of mate-finding communication by disruptant formulations is the use of standard pheromone monitoring traps in which are placed synthetic pheromone lures (Rice and Kirsch, 1990; Knight, 1997; Staten *et al*., 1997; Baker *et al*., 1998b; Fadamiro *et al*., 1998, 1999b). This technique can be as informative as the use of caged calling females if the lure that is used has been shown previously in untreated check plots to be able to attract equivalent numbers of males as do caged calling females. Using synthetic lures that are either too strong or too weak (or emit a suboptimal blend composition) will provide data that are not relevant to what is happening with calling females' ability to attract males. However, if a synthetic blend composition emitted at levels that approximate the attraction ability of caged females is used, then conclusions about the ability of a formulation to disrupt attraction to calling females can be valid.

Again, the advantage of assessing trap capture reduction is that it provides a robust, graded data set from these continuously emitting sources. The disruption formulation is challenged throughout the attraction period each night or evening for its ability to continuously suppress the ability of males to locate "females." Using monitoring traps baited with a good synthetic lure is also significantly easier and less problematic than is the use of live, calling females.

Although assessing mating disruption efficacy by means of synthetic pheromone baited traps is an optimal technique when the appropriate synthetic monitoring lures have been developed, it does not mean that there will always be concomitant demonstration of damage reduction when successful trap shutdown occurs. There are many other factors that play into the latter, and there are several species

of moths, including the obliquebanded leafroller, *Choristoneura rosaceana*, and the peach twig borer, *Anarsia lineatella*, where trap capture is routinely reduced by 95% or more, and little to no effect is seen on the damaging population. Perhaps this has something to do with some of these species having aggregation or mating site habits that have been overlooked and the monitoring traps only measure the ability of the disruptant to prevent males from flying out of their aggregated area out to the monitoring traps. Alternatively, perhaps the disruption plots have not been large enough to prevent immigration of gravid females, whose propensity to move great distances perhaps may have been underestimated in these species.

Regardless, trap capture data have proven to be useful indicators in the early tests performed in the development of disruption formulations; they measure the potential of a formulation to be effective and provide graded data sets that are more informative and easier to obtain. In addition, such tests are useful in determining the longevity of a formulation with regard to its effect on male behavior under natural temperature and wind conditions, which is more relevant to measuring the longevity of a formulation than are emission rate data.

6.12.5.4.4.5. Wind tunnel and other laboratory tests Although there have been many attempts to assess the efficacy of a potential mating disruption blend or formulation in the wind tunnel over the years, the major contribution of such studies has been to elucidate, after the fact, possible behavioral mechanisms that are at work in producing successful disruption. The wind tunnel enables researchers to observe and record individual male behavior in response to disruptant dispensers, or the dispensers' ability to interfere with responses to normal strength pheromone plumes. A key example is the work of Cardé *et al*. (1993, 1997) in investigating the behavior of pink bollworm males when exposed to hollow fibers or Shin-Etsu ropes (see Section 6.12.5.4.2). Evendon *et al*. (2000) were able to deduce from a series of laboratory wind tunnel experiments the possible mechanisms that could be at work in disrupting *C. rosaceana* sex pheromone communication in the field.

As useful and informative as these experiments can be, however, it is impossible in a laboratory wind tunnel to create a spatial array of dispensers that mimics the plume dynamics and plume flux exposures that a male would encounter farther downwind under shifting wind conditions in the field. Disruption dispenser arrays that in the field need to be spaced even as moderately widely as the ropes (and especially with yet more widely spaced, extremely high emission rate dispensers such as MSTRS and "puffers") cannot be scaled down to laboratory wind tunnel size. Wind tunnel tests have been useful to explore some of the physiological and behavioral limits that certain kinds of close-range exposure to plumes of various strengths and structures impose on males' olfactory pathways.

6.12.5.4.5. Pheromone component blend composition in the disruptant formulation In considering the cost of formulating and registering pheromone blends for commercial use, it is crucial to balance efficacy of the registered blend with the cost of the product. If an effective product is too expensive and is not able to be competitive with other available products, it will not be purchased and will "fail," despite its good biological activity and despite all the applied research that has been performed. In general, the fewer components that need to be formulated and still retain good efficacy, the less expensive and more competitive the final product will be. However, the cost of the product is also dependent on the amount of a.i. that needs to be applied per hectare, and so a blend formulation that is more effective at lower amounts of a.i. for a longer period under field conditions may prove to be more economically competitive, despite the inclusion of more pheromone components. For a multicomponent pheromone, how many of the minor components play a significant role in effective disruption and how many can therefore be left out of a final formulated product must be decided.

Minks and Cardé (1988) and Cardé and Minks (1995) reviewed results from many key sex pheromone communication disruption experiments and concluded that for a given species, the synthetic blend compositions and ratios most closely mimicking the natural blend for that species should be the most effective mating disruptants at a given dose per hectare because they will be able to make use of more of the mechanisms (see Cardé and Minks, 1995) that result in disruption than will suboptimal, partial blends or off-ratios. Although the majority of field experimental evidence supports this conclusion, this does not mean that one or more of the more minor components, due to their more subtle effects, might be able to be eliminated from the final formulated product and still retain sufficient efficacy.

Also, there are a few apparent exceptions to Cardé and Minks' general rule. Evendon *et al*. (1999a, 1999b, 1999c, 2000) have thoroughly explored sex

pheromone communication in the western population of the obliquebanded leafroller. They performed experiments exploring the underlying mechanisms likely involved in disruption and also conducted field tests in small plots by using different blend compositions that affect both *C. rosaceana* and a sympatric leafroller pest, the threelined leafroller, *Pandemis limitata*. Their results and insightful discussions have been in instructive in helping to conceptually and experimentally guide researchers as to how to pick apart the many mechanisms that may, depending on the species, play larger or smaller roles in successful sex pheromone communication disruption.

Evendon *et al.* (1999a) found in small plot (0.1 ha) field experiments that the four-component sex pheromone blend of *C. rosaceana* emitted from Conrel hollow fiber tapes deployed at a density of 500 dispensers per ha was effective in reducing successful mating of tethered females to a level of only 5% mating, but it was no more successful than a partial three-component or even a two-component blend containing the major component, (*Z*)-11-tetradecenyl acetate plus (*E*)-11-tetradecenyl acetate emitted at the same rate per hectare. Even at emission rates that they determined were at threshold levels for suppressing mating with tethered females or by deploying disruptant dispensers at reduced densities, the two-component blend was as effective as the four-component blend in suppressing mating. Tests of the competition attractiveness of the disruptant dispensers themselves via tests of attraction of males into traps showed that the two- and three-component hollow fiber tapes were ineffective in attracting males to traps, whereas the four-component fiber tapes resulted in high male trap captures.

Other studies (Evendon *et al.*, 1999b, 1999c, 2000) had demonstrated, in detailed wind tunnel and field experiments, that the attraction competition mechanism as well as long-term habituation were likely not very significant contributors to communication disruption in this species. Rather, sensory adaptation plus plume camouflage seemed to be implicated as playing more dominant roles in thwarting attraction to females. Importantly, even disruptant blends that contained the large amount of the heterospecific antagonistic compound, (*Z*)-9-tetradecenyl acetate, that is emitted by *P. limitata* females were as effective as conspecific blends in disrupting attraction to *C. rosaceana* females. Even these, too, were unattractive to *C. rosaceana* males and would therefore not be able to work via the competition mechanism (Evendon *et al.*, 1999b, 1999c). The ability of *P. limitata* males to locate and mate with tethered conspecific females is likewise disrupted as effectively by *C. rosaceana*-related blends emitting no (*Z*)-9-tetradecenyl acetate, and these are not effective for attracting *P. limitata* males.

The results emphasize again that even with moderately strongly emitting and moderately widely spaced dispensers (Evendon *et al.*, 1999b, 1999c), mechanisms other than attraction competition seem to have been dominating in these studies to result in disruption of sex pheromone communication in these two species. The inclusion of heterospecific antagonists in disruption dispensers was similarly found to be as effective as the conspecific blend in mating disruption studies of the pea moth, *Cydia nigricana* (Bengtsson *et al.*, 1994; Witzgall *et al.*, 1997). Because in Evendon *et al.*'s studies (Evendon *et al.*, 1999a, 1999b, 1999c, 2000), sensory adaptation and also perhaps plume camouflage were shown to be so strongly implicated as the dominant disruption mechanisms, the vulnerability of *C. rosaceana* males to sensory adaptation alone (Evendon *et al.*, 2000) may explain why nonoptimal, nonattractive blends can be so effective at sex pheromone communication disruption. Indeed, other studies using partial and completely unattractive blends dispensed in moderately strong and widely spaced dispensers have shown that effective disruption can be attained (Flint and Merkle, 1983, 1984); presumably, this comes from mostly sensory adaptation or habituation to the ambient pheromone that the unattracted males are exposed to.

Previous studies on a different, eastern population of *C. rosaceana* (Roelofs and Novak, 1981) contrast sharply with results from the western population (Evendon *et al.*, 1999a). Roelofs and Novak (1981) found that the optimal, natural female-emitted three-component blend for eastern *C. rosaceana* imposed better disruption than did partial blends. Evendon *et al.* (1999a) used tethered females for their assessment of disruption and as the authors themselves pointed out, Roelofs and Novak used the "more robust" (Evendon *et al.*, 1999a) assessment of measuring the numbers of males captured. As discussed above, tethered females provide a binomial, mated–unmated measure that does not allow for a graded assessment of how many more males would have been attracted to the females had they not mated with the first one arriving.

The mechanisms affecting mating disruption will operate to different degrees in different species under different environmental conditions and pheromone disruptant deployment schemes (Bartell, 1982; Minks and Cardé, 1988; Cardé, 1990; Cardé and Minks, 1995). There do seem to be differences in the behavior and neurophysiology of the various species that have been targeted and these may allow us to use surprisingly unattractive blends

for effective mating disruption. Whether this is always related to each species' vulnerability to sensory adaptation and plume camouflage as opposed to other mechanisms, the studies discussed above suggest that this is one possible conclusion to be made. The only way to optimize a disruptant formulation with regard to the blend used as the a.i., the dispenser loading and longevity, dispenser deployment density, and the overall cost and efficacy, is with extensive trial-and-error field testing.

6.12.5.4.6. Successful mating disruption in population control by using commercial dispensers

6.12.5.4.6.1. Codling moth In the final two decades of the 1900s, apple and pear growers in California and the Pacific Northwest were facing a variety of factors making untenable the continued use of conventional broad spectrum insecticides. Among these factors were the increasing development of resistance of the codling moth to organophosphate insecticides, and also new federal legislation, the Food Quality Protection Act (FQPA), that would require review and reregistration of traditional broad spectrum insecticides such as the organophosphates (Brunner *et al*., 2001). During this same period, beginning with initial small plot studies (Cardé *et al*., 1977; Charmillot, 1990) using hollow fiber and other dispensers, research on mating disruption of the codling moth had proceeded to the point where effective population suppression using improved, long-lasting mating disruption formulations such as Shin-Etsu ropes seemed feasible (Knight, 1996; Gut and Brunner, 1998).

Due to the reduced efficacy of traditional insecticide applications, the need for more insecticide applications to gain effective control, and the looming specter of FQPA legislation, growers in the early 1990s began using commercially available mating disruption formulations. The number of hectares that was treated with mating disruptants against the codling moth grew from a few hundred in 1990 to approximately 5000 in 1994. Due in part to the increasing grower acceptance of this population management tool, a Codling Moth Areawide Management Program (CAMP) was proposed and then approved. Five sites in the Pacific Northwest were identified, with three of them in Washington State and one each in California and Oregon. A total of approximately 1240 ha was included for codling moth mating disruption application over all five sites.

One overall goal of this study was to achieve an 80% or greater reduction of the use of broad spectrum conventional insecticides by the end of the 5-year program. A subsidy was provided to all participating growers of \$125 ha^{-1} for the first 3 years of the project to help defray the cost (\$275 ha^{-1}) of the mating disruption treatments (Brunner *et al*., 2001). For the final 2 years, growers had to pay the full cost of the treatments themselves. Standard high-dose codling moth monitoring traps were used to assess trap capture reductions, and damage assessments were made for all blocks within the CAMP project and compared with fruit from non-CAMP participating grower blocks.

The largest site in the project was at Howard Flat, Washington, more than 440 ha. Data from this site are representative of the trends in the other four CAMP sites (Brunner *et al*., 2001), although their differences in weather, topography, and horticultural practices were many, including the fact that the California and Oregon sites were planted mainly to pears. In 1995, the first year of the CAMP program, codling moth mating disruption formulations (mostly the Shin-Etsu ropes, IsoMate CM) were applied to the CAMP hectarage, and at the end of the year in Howard Flat, trap capture of males had been reduced by 80% and fruit damage at harvest averaged 0.55% for the entire hectarage of the project. The previous year's at-harvest damage had averaged 0.8%. Moreover, the number of conventional insecticide sprays targeting the codling moth averaged only 1.2 ha^{-1}, whereas during the previous, non-CAMP year it had averaged 2.9 ha^{-1} (Brunner *et al*., 2001).

During 1996, the average trap capture of males was reduced further from the levels in 1995, with only 1.5 moths captured per trap compared with 8.8 per trap in 1995. Damage at harvest was reduced to only 0.2% and most of the damage (sometimes reaching 25%) occurred in the few blocks of trees at Howard Flat in which one or two growers had refused to participate in the CAMP program. Higher damage also occurred in mating disruption treated blocks of trees of CAMP participants that bordered these untreated, high-infestation sources. In subsequent years these blocks of trees were leased by neighboring growers who wanted to eliminate these last remaining codling moth infestation foci, and these hot spot infestations were subsequently fully reduced. During 1996, the number of insecticide applications against codling moth fell further to only 1.1 application per hectare (Brunner *et al*., 2001).

During the next 2 years, trap capture fell further to exceptionally low levels, with 90% of the traps capturing zero males during the entire 2 years. During 1998, the average number of insecticide applications fell to only 0.5 application per ha and

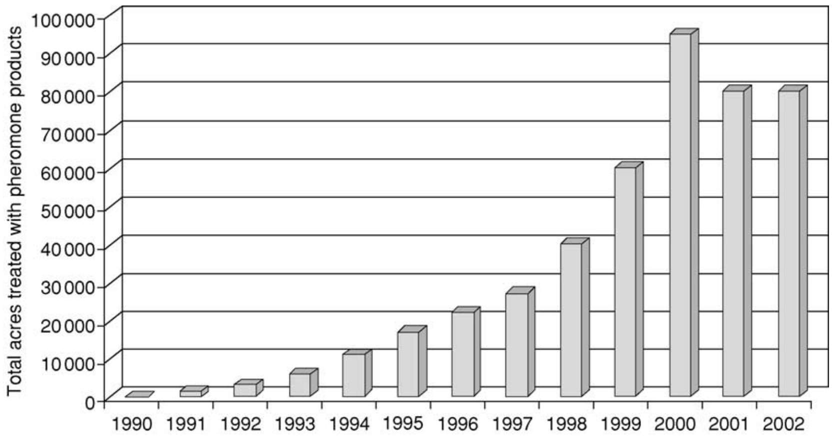


Figure 5 The number of acres treated each year with codling moth (*Cydia pomonella*) commercial mating disruption products in Washington State, USA from 1990 to 2002 (Brunner *et al*., 2001; Years 2001 and 2002 provided by Brunner, personal communication). In 1995 the Codling Moth Areawide Management Project (CAMP) began, with subsidized mating disruption applications provided to growers. The three CAMP sites in Washington State comprised a constant 760 ha during the 5 years of the program (1995–99).

crop damage at harvest reached an all-time low of between 0.01% and 0.03% damaged fruit. These levels were accomplished in 1998 with one-half the density of dispensers per hectare, because this was the first year without a cost subsidy from the program. Also, mating disruption had been so effective in reducing codling moth populations the first 3 years that there was little perceived threat of incurring increased damage by reducing dispenser density. By doing so, growers kept their expenses the same as during the previous 3 years.

It is clear from the continued increase in acreage using commercial codling moth mating disruption formulations in Washington State (**Figure 5**), that growers statewide were satisfied with the population suppression and their economic balance sheets involved in using codling moth mating disruption. The CAMP program served as a focal point for providing mating disruption efficacy data that guided growers statewide as to what was happening on their own mating disruption treated acreage. Not only did the growers in the CAMP program achieve a 75% reduction in insecticide applications while reducing damage to unprecedented levels, but also secondary pests did not arise with the reduced insecticide pressure, as had initially been feared would occur. On the contrary, comparison plots under conventional practice experienced higher levels of secondary pests and often lower levels of beneficial insects and mites than did the CAMP program plots (Brunner *et al*., 2001).

6.12.5.4.6.2. Pink bollworm A similar history of successful area wide use of sex pheromones for mating disruption exists for another key agricultural pest, the pink bollworm (Staten *et al*., 1997). The Parker Valley of Western Arizona, planted to more than 10 000 ha of cotton, was overrun with pink bollworms that had developed resistance to every insecticide on the market. The grower groups in the valley mandated an areawide program of using mating disruption against this pest, and the entire valley had various commercial mating disruption formulations applied from first moth emergence through to August. The formulations included the Shin-Etsu ropes and (the formerly Conrel, now Scentry) hollow fibers. The fibers had been commercially available since 1978, and growers were already knowledgeable about them and about the ropes as well. However, before the start of the program in 1989, virtually no hectarage in Parker Valley had had mating disruptants applied, even though their use had been prevalent in neighboring southern California desert valley cotton hectarage (Staten *et al*., 1997).

As in the codling moth CAMP program, damage diminished year by year in the Parker Valley with continued area wide application of pheromone mating disruption formulations. By 1993, the average season-long capture of males in monitoring traps in Parker Valley was reduced to only 0.5 moth per trap, whereas the statewide average in conventional practice farms grew to greater than 30 per trap (averaging 23 moths per trap for all 3 years). Most importantly, whereas the percentage of infested bolls out of the tens of thousands that were sampled each year was more than 25% during mid-August of year 1, during the same August period in year 2 (1990) damage was only 5.9% (Staten *et al*., 1997) (**Table 3**). At the mid-August point of year 3 damage was only 0.03% and by 1993, out of more than 22 000 bolls sampled season-long, not a single

Table 3 Pink bollworm damage to cotton bolls in Parker Valley mating disruption project, Arizona, USA

	Larvae per 100 bolls				
Week of	*1989*	*1990*	*1991*	*1992*	*1993*
9 July		0.3		0.19	0
16 July		0.6	0.6	0.09	
23 July	3.6	1.4	0.03	0.95	
30 July	7.7	2.7	0.09	0.61	0
6 Aug.	17.9	1.5	0.03	0	0
13 Aug.	25.9	5.9	0.03	2.45	0
27 Aug.	36.4	20.6	1.6	1.19	0
3 Sept.	34.5	10.9	1.9	1.6	0
10 Sept.	21.6	10.4	3.7	1.78	0
17 Sept.	28.4	33.3	6.6		0
Total bolls sampled per year	23 847	31 630	21 675	25 603	22 852

infested boll was found (0% damage) (Table 3). In contrast, the central Arizona average infestation rate for conventionally insecticide treated hectarage in 1993 was 9–10% by mid-August (Staten *et al.*, 1997).

6.12.5.4.6.3. Other successes and some limitations Growers of peaches have had mating disruption successes similar to the above, by deploying Shin-Etsu polyethylene tubes over a wide area against the oriental fruit moth in Australia (Vickers, 1990), in California (Rice and Kirsch, 1990), and in South Africa (Barnes and Blomefield, 1997). The above results from areawide mating disruption against three different species and cropping systems indicate that efficacious mating disruption formulations applied on large areas over a 3-year period can result in the near elimination of pest population problems. During year 1 of a program, high pest populations may not appear to be well controlled, but during year 2, the mating disruption effects from year 1 become evident. Lower populations are present at the beginning of the season, and mating disruption during year 2 is better able to exert its full effect. By years 3 and 4, populations have often been reduced to such low levels that growers decide that they can apply reduced rates of the disruption formulations. This has its dangers in that it can result in less effective mating disruption, allowing populations to build back up again.

Charmillot (1990) summed up lessons he and his coworkers learned concerning when it is that mating disruption becomes less effective for codling moth population suppression. His lessons regarding codling moth are applicable to mating disruption efforts on other pests as well. First, mating disruption works best when applied on an area wide basis and is not advisable on very small hectarage (less than 1 ha). The crop borders represent vulnerable edges for immigration of mated females from adjoining untreated crop areas, and they also serve as a zone that concentrates females along the borders when there is no nearby hectarage of the same crop. Due to geometry, small hectarage accentuates the problem because the edge-to-area ratio becomes greater. Also, the use of more widely spaced dispensers requires that borders be given special attention to reduce the presence of pheromone-free clean air "holes" along the borders; this can be accomplished either by decreasing the spacing between dispensers, or else through the use of different dispenser technology along borders, such as sprayable microcapsules. Mating disruption will be more problematic when crop borders are especially irregular or when the fields are too elongated; in these cases again the crop's edge-to-area ratio becomes too high. This important effect of field geometry is especially exaggerated for very widely spaced high-release-rate disruption formulations.

6.12.5.4.7. Constraints to successful adoption of mating disruption

6.12.5.4.7.1. Upfront cost A primary obstacle to the use of pheromone mating disruption on any crop is that it must be applied no later than the start of the first adult flight period, that is, before a grower knows for sure that there is even going to be a pest problem that season. The requirement of this upfront investment in pheromone puts mating disruption at a disadvantage compared with curative pest management tools that provide a wait-and-see option. Insecticide applications can be made after a preliminary assessment of the population density during the first flight of adults or even after oviposi-

tion when larval damage can be assessed. Pheromone mating disruption cannot be used curatively that way.

In the areawide mating disruption successes discussed above, growers had no other recourse other than mating disruption because their usual curative insecticide tools had been rendered ineffective due to pest resistance. Here, mating disruption no longer represented an upfront cost, but rather became a viable option that growers readily embraced and accepted.

6.12.5.4.7.2. Appearance of secondary pests Except for a few small groups or pairs of species, pheromones are extremely species-specific. The replacement of broad spectrum insecticides in some IPM systems with mating disruption that targets only one species in a pest complex can lead to increases in the populations of species that had been only secondary pests before mating disruption was used (Rice and Kirsch, 1990). No pheromone disruption formulation has been created thus far that functions effectively as a "broad-spectrum" formulation. Evendon *et al*. (1999b, 1999c) experimented with single blend formulations targeting *C. rosaceana* and *P. limita*, with some success. Perhaps there are other situations where effective multispecies blend formulations can be developed, although there are still none that have attained commercial success.

Choristoneura rosaceana disruption seems to depend less on attraction competition and habituation, and more on sensory adaptation (Evendon *et al*., 2000). It remains to be seen whether development of multispecies disruptant formulations can be successful on more sets of species. Certainly when widely spaced, high-emission-rate dispenser systems are used there should be less of a chance for this to work, because attraction competition that facilitates habituation seems to be more essential for success with these formulations (Baker *et al*., 1998b).

6.12.5.4.7.3. Cost of mating disruption formulations Pheromone mating disruption formulations have to this point been expensive compared with curative applications of insecticides. Although growers of many crops are now well aware that mating disruption "works" as a pest management tool, pheromones' expense plus the upfront nature of the cost has put them at a disadvantage relative to curative pest management tools. The a.i., the pheromone itself, is the most expensive part of a formulation. Costs per gram of even the least expensive pheromone components are approximately \$1.00, and many other more expensive major pheromone components cost \$3–20 g^{-1}. Formulating the a.i. into specialized dispensers adds to the cost, but nevertheless, if cheaper organic synthetic routes to the a.i.s can be developed, costs of the final formulated products could be reduced substantially.

Unfortunately the best and cheapest routes for large scale syntheses have apparently been discovered and are already the ones in use by pheromone manufacturers. Therefore, finding other ways to emit behaviorally effective amounts of the active ingredients more judiciously while maintaining efficacy and extending the longevity of dispensers under field conditions is a much-needed area of research. Below, we present some ideas for how to do this as we try to bring together some of the advances in recent basic knowledge about male moth upwind flight behavior with applied aspects of pheromone use in the field.

6.12.6. New Frontiers

6.12.6.1. New Ideas for Reducing the Cost of Pheromone Mating Disruption

6.12.6.1.1. Widely spaced, high emission rate dispensers Widely spaced, high emission rate dispensers offer an advantage for future cost-saving measures to make mating disruption more economically competitive with other tools such as insecticides. Because these dispensers can be deployed at very low densities, from one to 15 dispensers per ha (Shorey and Gerber, 1996a, 1996b; Shorey *et al*., 1996; Baker *et al*., 1997b, 1998b; Fadamiro *et al*., 1998, 1999b), the labor cost for their deployment is lower than for other hand-applied dispenser types. Importantly, widely spaced dispensers are the only type whose density per hectare goes down as the area of crop they are applied to goes up, regardless of starting grid-spacing distance. This is at first not easy to comprehend because it is counterintuitive.

In the example shown in **Figure 6**, high emission rate dispensers are hypothetically deployed on a 1 ha orchard, using a 25 m spacing between dispensers (center square, large dots). The density needed to treat the 1 ha orchard calculates out to be 25 dispensers per ha. However, if a 9 ha orchard needed to be treated using this same 25 × 25 m grid pattern, the cost per hectare is reduced, because over the 9 ha plot the density has been reduced to 18.8 dispensers per ha (**Figure 6**, second square, small dots). For a 25 ha orchard, the cost per hectare is reduced further as the density falls to 17.6 dispensers per ha, still keeping this same grid spacing (**Figure 6**, outermost square, no dots); and to treat 100 ha the dispenser density goes down to 16.8 dispensers per ha.

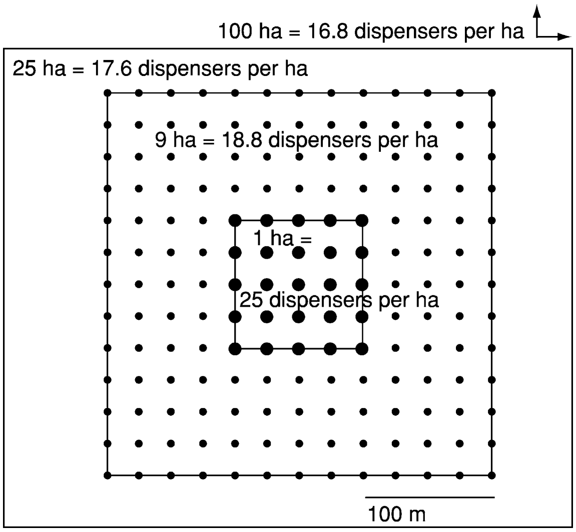


Figure 6 Widely spaced, high emission rate dispensers are the only type of dispenser whose density per hectare diminishes significantly as the area of crop they are applied to increases. In this example, such dispensers are deployed with a 25 m spacing on 1 ha (interiormost square, large dots); here dispenser density calculates to 25 dispensers per ha. Treating 9 ha (intermediate square, smaller dots) while keeping the same 25 m dispenser spacing results in a reduced density of 18.8 dispensers per ha. Enlarging the treated area still further to 25 ha (outer square) and 100 ha (beyond outer square) while keeping the same grid spacing of dispensers results in even lower dispenser densities of 17.6 ha^{-1} and 16.8 ha^{-1}, respectively.

If the starting dispenser grid pattern is less dense than this, the reduction to low densities upon scaling up to very large hectarage is even more striking. Starting with a dispenser spacing twice the above (50×50 m), treating the 1 ha orchard with a grid pattern requires a density of 9 dispensers per ha. Treating a 9 ha orchard reduces the density to 5.4 per ha, and treating 25 ha requires only 4.8 dispensers per ha. The 100 ha orchard needs only 4.4 dispensers per ha.

In the Farkas *et al.* (1974) initial experiments demonstrating that widely spaced, high dispenser loadings work as well as closely spaced, low loadings, they emphasized that the amount of active ingredient per hectare should be maintained. This aspect needs to be explored more with many more trials, because if this turned out to be a strict requirement, then no cost savings from reduced dispenser number per hectare would be gained from this surprising phenomenon of geometry that provides a numerical advantage to wide spacing. But as emphasized in the discussions above, it is not the overall concentration of pheromone that moths respond to, it is the changes in flux provided by the pheromone plume-strands. Therefore there may be previously unrealized opportunities to further reduce the cost of pheromone per hectare by designing dispensers that optimize, with regard to plume-strand strength, what is emitted and not what is applied.

6.12.6.1.2. Strong plume strand emitting dispensers The existing dispensers that have been used for insect mating disruption have emphasized emission aspects related to field longevity that are calculated on the total amount of pheromone emitted per hectare per unit time. Work on improving these previous devices has largely ignored recent findings that have shown that males fly upwind in response to individual strands of pheromone comprising the fine structure of the plume.

An insect's antennae can only sample a small volume of an odor plume, and the strands and pockets of clean air give the olfactory neurons on the antenna rapid fluctuations in stimulation (2–10 Hz). Mating disruption dispensers and deployment spacing schemes that can maximize changes in flux will be able to elicit attraction competition from farther downwind than will less well-designed types. Olfactory pathway habituation, which accompanies attraction competition (Cardé *et al.*, 1997), is known to be optimally imparted by strong pulses of pheromone and not by continuous stimulation, which only will produce sensory adaptation (Bartell and Lawrence, 1977a, 1977b; Bartell, 1982).

It became apparent while working on optimizing the emission rate from MSTRS high release rate pads that, within a few days, the pads became loaded with an amount of pheromone beyond which adding more pheromone did not further increase the emission rate. If the size of the pad were to be increased, the pad's total emission rate would be raised proportionately. However, we also realized that because the insects' antennae are so small, any increase in the diameter of the pad will not increase the strength of the individual pheromone strands that contact the antennae. The amount emitted per dispenser, or per hectare per unit time, will seem to researchers to create greater and more powerful amounts of pheromone to affect behavior, but in fact it will not at all increase the amount that strikes an insect's antenna.

It became clear during our studies that smaller point sources will be better at generating large plume-strand flux, but only if the emission rate of the small point source can be kept the same as the rate that large surface area dispensers emit across their total area. The effect will be to pack more molecules into a smaller space, thereby increasing pheromone strand flux. One way to accomplish this

would be to add energy to the small point source that would generate heat and increase the evaporation rate. Energy could also be used to generate ultrasonic vibration of the point source and increase evaporation rate by increasing air movement over the source. However, both such types of dispenser might be unnecessarily expensive and subject to technical malfunction.

The authors propose that pheromone flux of individual strands can be increased very simply by using an entirely passive technique that allows strands to be enriched before they are sheared from the downwind end of the dispenser surface. The dispenser needs to be asymmetrically shaped and either of a planar or cylindrical design. It then needs to be suspended so that it is allowed to align itself parallel to (along) the wind line. Each parcel of air that passes over the dispenser–surface air boundary will progressively accumulate more molecules as it traverses along more of the dispenser surface, enriching the parcel with increasingly higher concentrations of pheromone before the resulting odor strand is shed from the dispenser's downwind end.

Measurements of plume-strand flux downwind of a rectangular planar dispenser taken in our laboratory using a solid phase microextraction (SPME) fiber approximately the size of a moth antenna have confirmed the large strand enrichment effect of orienting the plane along the wind line (parallel to the wind) (Baker and Park, unpublished data). A greater than 40-fold increase in flux across the SPME fiber (across a moth antenna) resulted, compared with a cross-wind orientation (perpendicular to the wind line). As expected, there were no apparent differences in emission rate from the dispenser when the overall release rates of the planar dispensers were measured using the older method of drawing air over the dispensers housed in a wide tube and collecting the entire volume of the tube onto a cartridge filled with Tenax adsorbent beads (Baker and Park, unpublished data).

From this concept, it follows that if such a planar dispenser (or a cylindrical one) is made much longer than it is wide and its long axis is then aligned along the wind line, pheromone strand concentration and plume flux will be proportionately increased according to dispenser length. Therefore, this method for passively creating the strongest possible odor strands anticipates that odor strand flux can be maximized by using a more linear shaped dispenser, and then keeping the longest axis of its release surface (planar, cylindrical) aligned parallel to the wind. Under variable wind directions, as occur in the field, this wind alignment can be done simply by the use of a wind vane that rotates the emission

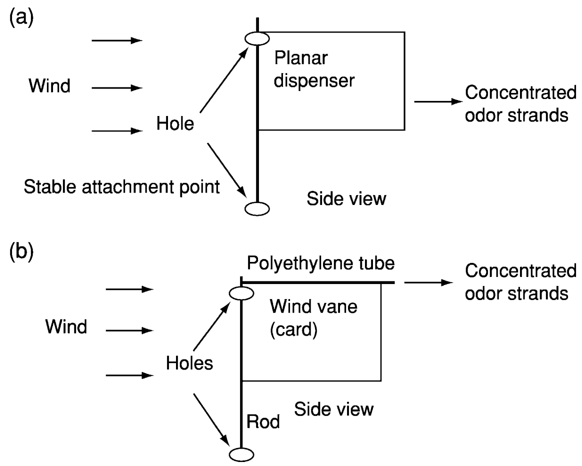


Figure 7 An illustration of how to easily and passively (without the addition of heat or other energy) enrich the strength of the pheromone signal (odor strand) that emanates from a dispenser. The dispenser must be planar-shaped (a) or cylindrical–tubular-shaped (b). The dispenser should be allowed to rotate freely so that the dispenser's long axis will align along the wind line. This can be accomplished by attaching the tubular dispenser to a wind vane (b), or else by allowing the planar dispenser to function as a wind vane (a). It follows from this priniciple that the amount of enrichment for a given evaporative surface area will be further optimized by making the dispenser much long than it is wide, with the increase in strand concentration being proportional to the increase in dispenser length that is aligned along the wind line.

source to follow all the changes in wind flow direction to keep the surface perfectly aligned to allow odor concentration to occur along the long axis (Figure 7b). Alternatively, the long axis of a planar dispenser itself can be allowed to swing in the wind to act as a wind vane, by using single attachment points to a stable surface and either wire, string, or swivel-type rotating fasteners to allow the dispenser to swivel freely in the wind (Figure 7a).

The authors emphasize that this concept for increasing plume flux by increasing odor strand concentration differs from the multitude of previous studies over the years that have used planar filter paper dispensers aligned cross-wind versus parallel to the wind to widen or narrow the plume (Mafra-Neto and Cardé, 1994), or else have used obstacles upwind of the source (Willis *et al.*, 1994) to create different plume structures or plume concentrations. In such previous studies, the effort was only to preserve the plume-strand structure and flux *after* the strands had already been shed from the dispenser. Previous work on optimizing plume shape in the field downwind of pheromone traps likewise focused on minimizing turbulence and preserving plume structure (Lewis and Macaulay, 1976), and not on enriching pheromone strand concentration before pheromone leaves the dispenser surface. This

present method suggests a novel way to enrich odor strands *before* they are shed from the dispenser, thereby maximizing the flux of pheromone in individual odor strands and hence maximizing the resulting changes in flux across the antenna stationed within a plume.

It is realized that the highly concentrated plume-strands from such wind-aligned dispensers are vulnerable to break-up by the turbulent mixing imparted as they travel through surrounding foliage, and their ability to affect male behavior far downwind might be compromised. Nevertheless, there may be ways to take advantage of this system, especially in the deployment of mating disruption dispensers above canopy level against pests of field crops. Over short grass, the ability of such small point source generated strands to stay highly concentrated as they travel downwind has been demonstrated; very strong strands of ionized air do occur at great distances from the source, but not as frequently as they do close to the source (Murlis, 1986, 1997). It is possible that extremely long length, strong plume-strand emitting dispensers can be deployed on wind vanes from very few locations outside very large fields to protect the interiors.

The high emission rate, small point source concept also is instructive with regard to the evolution of lepidopterous female pheromone glands. Female moths likely have, as hypothesized by Greenfield and Karandinos (1979), been selected to emit very little pheromone per unit time because judicious emission of small amounts of pheromone is a form of sexual selection that allows females to discriminate for only the fittest (most sensitive) males that are able to locate females from far downwind.

We would propose in addition that females may be able to economize and use such low amounts of pheromone because they emit it from an extremely small piece of tissue situated on the tip of their abdomens. Although the amount of time-averaged pheromone emitted seems to be very small, the plume-strands that are shed are perhaps optimized with regard to the flux over the male's antenna that they produce. Studies aimed at measuring plume-strand flux from calling female moths on various plant species and foliage substrates may be very informative in investigating this idea further.

The confluence of applied and basic pheromone research and the synergy of ideas is apparent from new lines of inquiry such as these. We must continue to create new blends in this manner.

References

Agnello, A.M., Kovach, J., Nyrop, J.P., Reissig, W.H., Breth, D.I., *et al.*, **1994**. Extension and evaluation of a simplified monitoring program in New York apples. *Am. Entomol. 40*, 37–49.

Akers, R.P., O'Connell, R.J., **1988**. The contribution of olfactory receptor neurons to the perception of pheromone component ratios in male redbanded leafroller moths. *J. Comp. Physiol. A 163*, 641–650.

Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H., *et al.*, **1997**. An elicitor of plant volatiles from beet armyworm oral secretion. *Science 276*, 945–949.

Aldrich, J.R., **1995**. Chemical communication in the true bugs and parasitoid exploitation. In: Cardé, R.T., Bell, J.W. (Eds.), Chemical Ecology of Insects, vol. 2. Chapman and Hall, London, pp. 318–363.

Almaas, T.J., Christensen, T.A., Mustaparta, H., **1991**. Chemical communication in heliothine moths. 1. Antennal receptor neurons encode several features of intra- and interspecific odorants in the male corn earworm moth *Helicoverpa zea. J. Comp. Physiol. A 169*, 249–258.

Alpizar, D., Fallas, M., Oehlschlager, A.C., Gonzalez, L.M., Chinchilla, C.M., *et al.*, **2002**. Pheromone mass trapping of the West Indian sugarcane weevil and the American palm weevil (Coleoptera: Curculionidae) in palmito palm. *Fla. Entomol. 85*, 426–430.

Anton, S., Hansson, B.S., **1994**. Central processing of sex pheromone, host odour, and oviposition deterrent information by interneurons in the antennal lobe of female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Comp. Neurol. 350*, 199–214.

Anton, S., Hansson, B.S., **1999**. Physiological mismatching between neurons innervating olfactory glomeruli in a moth. *Proc. Roy. Soc. B 266*, 1813–1820.

Anton, S.A., Homberg, U., **1999**. Antennal lobe structure. In: Hansson, B. (Ed.), Insect Olfaction. Springer, Berlin, pp. 97–124.

Arbas, M.E., **1997**. Neuroethological study of pheromone-modulated responses. In: Carde, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 320–329.

Baker, T.C., **1985**. Chemical control of behaviour. In: Kerkut, G.A., Gilbert, L.S. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Pergamon, Oxford, pp. 621–672.

Baker, T.C., **1986**. Pheromone-modulated movements of flying moths. In: Payne, T.L., Kennedy, C., Birch, M.C. (Eds.), Mechanisms in Insect Olfaction. Clarendon Press, Oxford, pp. 39–48.

Baker, T.C., **1989a**. Origin of courtship and sex pheromones of the oriental fruit moth and a discussion of the role of phytochemicals in the evolution of lepidopteran male scents. In: Chou, C.H., Waller, G.R. (Eds.), Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones. Academia Sinica, Taipei, pp. 401–418.

Baker, T.C., **1989b**. Sex pheromone communication in the Lepidoptera: new research progress. *Experientia 45*, 248–262.

Baker, T.C., **1989c**. Pheromones and flight behavior. In: Goldsworthy, G.J., Wheeler, C.H. (Eds.), Insect Flight. CRC Press, Boca Raton, FL, pp. 231–255.

Baker, T.C., **1990**. Upwind flight and casting flight: complementary phasic and tonic systems used for location of sex pheromone sources by male moths. In: Proc. 10th Intl. Symp. Olfac. Taste, pp. 18–25.

Baker, T.C., **2002**. Mechanism for saltational shifts in pheromone communication systems. *Proc. Natl Acad. Sci. USA 99*, 13368–13370.

Baker, T.C., Dittl, T., Mafra-Neto, A., **1997b**. Disruption of sex pheromone communication in the blackheaded fireworm in Wisconsin cranberry marshes by using MSTRS™ devices. *J. Agric. Entomol. 14*, 449–457.

Baker, T.C., Fadamiro, H.Y., Cossé, A.A., **1998a**. Moth uses fine tuning for odor resolution. *Nature 393*, 530.

Baker, T.C., Fadamiro, H.Y., Cossé, A.A., **1998b**. Widely-spaced, high-emission-rate pheromone sources suppress mating of European corn borer females. In: Zalucki, M.P., Drew, R.A.I., White, G.G. (Eds.), Proc. 6th Australasian Applied Entomological Research Conference, pp. 279–288.

Baker, T.C., Hansson, B.S., Löfstedt, C., Löfqvist, J., **1988**. Adaptation of antennal neurons in moths is associated with cessation of pheromone-mediated upwind flight. *Proc. Natl Acad. Sci. USA 85*, 9826–9830.

Baker, T.C., Haynes, K.F., **1987**. Manoeuvres used by flying male oriental fruit moths to relocate a sex pheromone plume in an experimentally shifted wind-field. *Physiol. Entomol. 12*, 263–279.

Baker, T.C., Kuenen, L.P.S., **1982**. Pheromone source location by flying moths: a supplementary non-anemotactic mechanism. *Science 16*, 424–427.

Baker, T.C., Mafra-Neto, A., Dittl, T., Rice, M.A., **1997a**. Novel controlled release device for disrupting sex pheromone communication in moths. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 20*, 141–149.

Baker, T.C., Staten, R.T., Flint, H.M., **1990**. Use of pink bollworm pheromone in the southwestern United States. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 417–436.

Baker, T.C., Vickers, N.J., **1994**. Behavioral reaction times of male moths to pheromone filaments and visual stimuli: determinants of flight track shape and direction. In: Kurihara, K., Suzuki, N., Ogawa, H. (Eds.), Olfaction and Taste, vol. 11. Springer, Tokyo, pp. 838–841.

Baker, T.C., Vickers, N.J., **1997**. Pheromone-mediated flight in moths. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 248–264.

Baker, T.C., Vogt, R.G., **1988**. Measured behavioral latency in response to sex-pheromone loss in the large silk moth *Antheraea polyphemus*. *J. Exp. Biol. 137*, 29–38.

Baker, T.C., Willis, M.A., Haynes, K.F., Phelan, P.L., **1985**. A pulsed cloud of sex pheromone elicits upwind flight in male moths. *Physiol. Entomol. 10*, 257–265.

Barnes, B.N., Blomefield, T.L., **1997**. Goading growers towards mating disruption: the South African experience with *Grapholita molesta* and *Cydia pomonella* (Lepidoptera, Tortricidae). *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 20*, 45–56.

Bartell, R.J., **1982**. Mechanisms of communication disruption by pheromone in the control of Lepidoptera: a review. *Physiol. Entomol. 7*, 353–364.

Bartell, R.J., Lawrence, L.A., **1977a**. Reduction in responsiveness of male apple moths, *Epiphyas postvittana* to sex pheromone following pulsed pheromonal exposure. *Physiol. Entomol. 2*, 1–6.

Bartell, R.J., Lawrence, L.A., **1977b**. Reduction in responsiveness of male light-brown apple moths, *Epiphyas postvittana*, to sex pheromone following pulsed pre-exposure to pheromone components. *Physiol. Entomol. 2*, 89–95.

Bartelt, R.J., **1999a**. Sap beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 69–89.

Bartelt, R.J., **1999b**. Weevils. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 91–112.

Bartelt, R.J., Velter, R.S., Carlson, D.G., Baker, T.C., **1994**. Responses to aggregation pheromones for five *Carpophilus* species (Coleoptera: Nitidulidae) in a California date garden. *Environ. Entomol. 23*, 1534–1543.

Bartelt, R.J., Weisleder, D., **1996**. Polyketide origin of pheromones of *Carpophilus davidsoni* and *C. mutilatus* (Coleoptera: Nitidulidae). *Bioorgan. Medic. Chem. 4*, 429–438.

Bau, J., Justus, K.A., Cardé, R.T., **2002**. Antennal resolution of pheromone plumes in three moth species. *J. Insect Physiol. 48*, 433–442.

Bedard, W.D., Wood, D.L., **1981**. Suppression of *Dendroctonus brevicornis* by using a mass-trapping tactic. In: Mitchell, E.R. (Ed.), Management of Insect Pests with Semiochemicals. Plenum, New York, pp. 103–114.

Bedard, W.D., Silverstein, R.M., Wood, D.L., **1970**. Bark beetle pheromones. *Science 167*, 1638–1639.

Bengtsson, M., Karg, G., Kirsch, P.A., Löfqvist, J., Sauer, A., *et al.*, **1994**. Mating disruption of pea moth *Cydia nigricana* F. (Lepidoptera: Tortricidae) by a repellent blend of sex pheromone and attraction inhibitors. *J. Chem. Ecol. 20*, 871–887.

Berg, B.G., Almaas, T.J., Bjaalie, J.G., Mustaparta, H., **1998**. The macroglomerular complex of the antennal lobe in the tobacco budworm moth *Heliothis virescens*: specified subdivision in four compartments according to information about biologically significant compounds. *J. Comp. Physiol. A 183*, 669–682.

Berg, B.G., Mustaparta, M., **1995**. The significance of major pheromone components and interspecific signals as expressed by receptor neurons in the oriental tobacco

budworm moth, *Helicoverpa assulta*. *J. Comp. Physiol. A 177*, 683–694.

Berg, B., Tumlinson, J.H., Mustaparta, H., **1995**. Chemical communication in heliothine moths. 4. Receptor neuron responses to pheromone compounds and formate analogues in the tobacco budworm moth *Heliothis virescens*. *J. Comp. Physiol. A 177*, 527–534.

Bertram, S.L., Paine, T.D., **1994**. Response of *Dendroctonus brevicornis* LeConte (Coleoptera: Scolytidae) to different release rates and ratios of aggregation semiochemicals and the inhibitors verbenone and ipsdienol. *J. Chem. Ecol. 20*, 1617–1629.

Birch, M.C., Poppy, G.M., Baker, T.C., **1990**. Scents and eversible scent structures of male moths. *Annu. Rev. Entomol. 35*, 25–58.

Bjostad, L.B., Jewett, D.K., Brigham, D.L., **1996**. Sex pheromone of caddisfly *Hesperophylax occidentalis* (Banks) (Trichoptera: Limnephilidae). *J. Chem. Ecol. 22*, 103–121.

Boeckh, J., **1967**. Inhibition and excitation of single insect olfactory receptors, and their role as a primary sensory code. In: Hayashi, T. (Ed.), Olfaction and Taste, vol. 2, pp. 721–735.

Boeckh, J., Ernst, K.D., **1983**. Olfactory food and mate recognition. In: Huber, F., Markl, H. (Eds.), Neuroethology and Behavioral Physiology. Springer, Berlin, pp. 78–94.

Boeckh, J., Ernst, K.-D., Sass, H., Waldow, U., **1984**. Anatomical and physiological characteristics of individual neurons in the central antennal pathway of insects. *J. Insect Physiol. 30*, 15–26.

Borden, J.H., **1994**. Future of semiochemicals for the management of bark beetle populations. In: US Forest Service Gen. Tech. Rep. PSW-150.

Borden, J.H., **1990**. Use of semiochemicals to manage coniferous tee pests in western Canada. In: Ridgway, R., Silverstein, R.W., Inscoe, M. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 281–314.

Borden, J.H., **1985**. Aggregation pheromones. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 9. Pergamon, Oxford, pp. 257–285.

Borden, J.H., Chong, L., Gries, R., Pierce, H.D., Jr., **2001**. Potential for nonhost volatiles as repellents in integrated pest management of ambrosia beetles. *Int. Pest Mgt Rev. 6*, 221–236.

Borden, J.H., Chong, L., McLean, J.A., Slessor, K.N., Mori, K., **1976**. *Gnathotrichus sulcatus*: synergistic response to enantiomers of the aggregation pheromone sulcatol. *Science 192*, 894–896.

Borden, J.H., Handley, J.R., Johnston, B.D, MacConnell, J.G., Silverstein, R.M., *et al.*, **1979**. Synthesis and filed testing of 4,6,6-lineatin, the aggregation pheromone of *Trypodendron lineatum* (Coleoptera: Scolytidae). *J. Chem. Ecol. 5*, 681–689.

Borden, J.H., Handley, J.R., McLean, J.A., Silverstein, R.M., Chong, L., *et al.*, **1980a**. Enantiomer-based specificity in pheromone communication by two sympatric *Gnathotrichus* species (Coleoptera: Scolytidae). *J. Chem. Ecol. 6*, 445–456.

Borden, J.H., Lindgren, B.S., Chong, L., **1980b**. Ethanol and α-pinene as synergists for the aggregation pheromones of the two *Gnathotrichus* species. *Can. J. For. Res. 10*, 290–292.

Borden, J.H., McLean, J.A., **1981**. Pheromone-based suppression of ambrosia beetles in industrial timber processing areas. In: Mitchell, E.R. (Ed.), Management of Insect Pests with Semiochemicals. Plenum, New York, pp. 133–154.

Bossert, W.H., Wilson, E.O., **1963**. The analysis of olfactory communication among animals. *J. Theoret. Biol. 5*, 443–469.

Bradshaw, J.W.S., Baker, R., Lisk, J.C., **1983**. Separate orientation and releaser components in a sex pheromone. *Nature 304*, 265–267.

Breasley, C.A., Adams, C.J., **1994**. Relationships between environmental factors and capture times of male pink bollworm (Lepidoptera:Gelechiidae) moths in traps baited with sex pheromone. *J. Econ. Entomol. 87*, 986–992.

Brigham, A.R., Sadorf, E.M., **2001**. Benthic invertebrate assemblages and their relation to physical and chemical characteristics of streams in the eastern Iowa basins, 1996–98. In: US Geological Survey, Water-Resources Investigations Report 00–4256, Iowa City, IA.

Brunner, J., Welter, S., Calkins, C., Hilton, R., Beers, E., *et al.*, **2001**. Mating disruption of codling moth: a perspective from the Western United States. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 25*, 207–215.

Bulgarelli, J., **2002**. Pheromone mass trapping of the West Indian sugarcane weevil and the American palm weevil (Coleoptera:Curculionidae) in palmito palm. *Fla. Entomol. 85*, 426–430.

Butenandt, A., Beckermann, R., Stam, D., Hecker, E., **1959**. Über den sexuallockstoff des seidenspineers *Bombyx mori*: Reindarstellung und konstitution. *Z. Naturforsch. 14*, 283–284.

Byers, J.A., **1995**. Host-tree chemistry affecting colonization in bark beetles. In: Cardé, R.T., Bell, W.J. (Eds.), Chemical Ecology of Insects, vol. 2. Chapman and Hall, New York, pp. 154–213.

Byrne, K.J., Swigar, A.A., Silverstein, R.M., Borden, J.H., Stokkink, E., **1974**. Sulcatol: population aggregation pheromone in the scolytid beetle, *Gnathotrichus sulcatus*. *J. Insect Physiol. 20*, 1895–1900.

Cardé, R.T., **1990**. Principles of mating disruption. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 47–71.

Cardé, R.T., Baker, T.C., **1984**. Sexual communication with pheromones. In: Bell, W.J., Cardé, R.T. (Eds.), Chemical Ecology of Insects. Chapman and Hall, London, pp. 355–383.

Cardé, R.T., Baker, T.C., Castrovillo, P.J., **1977**. Disruption of sexual communication in *Laspeyresia pomo-*

nella (codling moth), *Grapholitha molesta* (oriental fruit moth) and *G. prunivora* (lesser appleworm) with hollow fiber attractant sources. *Entomol. Exp. Applic. 22*, 280–288.

Cardé, R.T., Baker, T.C., Roelofs, W.L., **1975**. Behavioral role of individual components of a multichemical attractant system in the oriental fruit moth. *Nature 253*, 348–349.

Cardé, R.T., Mafra-Neto, A., **1997**. Mechanisms of flight of male moths to pheromone. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 275–290.

Cardé, R.T., Mafra-Neto, A., Staten, R.T., Koch, U., Färbert, P., **1993**. Evaluation of communication disruption in the pink bollworm in field wind tunnels. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 16*, 23–28.

Cardé, R.T., Mafra-Neto, A., Staten, R.T., Kuenen, L.P.S., **1997**. Understanding mating disruption in the pink bollworm moth. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 20*, 191–201.

Cardé, R.T., Minks, A.K., **1995**. Control of moth pests by mating disruption: successes and constraints. *Annu. Rev. Entomol. 40*, *559–585*.

Carlson, J., **1996**. Olfaction in *Drosophila*: from odor to behavior. *Trends Genet. 12*, 175–180.

Carlsson, M.A., Galizia, C.G., Hansson, B.S., **2002**. Spatial representation of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Chem. Senses 27*, 231–244.

Carlsson, M.A., Hansson, B.S., **2003**. Dose-response characteristics of glomerular activity in the moth antennal lobe. *Chem. Senses 28*, 269–278.

Charmillot, P.J., **1990**. Mating disruption technique to control codling moth in western Switzerland. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 165–182.

Charmillot, P.J., Hofer, D., **1997**. Control of codling moth, *Cydia pomonella* L., by an attract and kill formulation. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 20*, 139–140.

Charmillot, P.J., Pasquier, D., Scalco, A., Hofer, D., **1996**. Essais de lutte contre le carpocapse *Cydia pomonella* L. par un procédé attracticide. *Mitt. Schweiz. Entomol. Ges. 69*, 431–439.

Chinchilla, C.M., Oehlschlager, A.C., **1992**. Capture of *Rhynchoporus palmarum* in traps baited with the male-produced aggregation pheromone. *Agric. Serv. Devel. Oil Palm Papers 5*, 1–8.

Choi, M.Y., Fuerst, E.J., Rafaeli, A., Jurenka, R., **2003**. Identification of a G protein-coupled receptor for pheromone biosynthesis activating neuropeptide from pheromone glands of the moth *Helicoverpa zea*. *Proc. Natl Acad. Sci. USA 100*, 9721–9726.

Christensen, T.A., **1989**. The coding of pheromones and pheromone blends in the antennal lobes of moths. In: Erber, J., Menzel, R., Pfluger, H.J., Todt, D. (Eds.), Neural mechanisms of Behavior. Georg Thieme Verlag, Stuttgart, pp. 249–250.

Christensen, T.A., Harrow, I.D., Cuzzocrea, C., Randolph, P.W., Hildebrand, J.G., **1995a**. Distinct projections of two populations of olfactory receptor axons in the antennal lobe of the sphinx moth *Manduca sexta*. *Chem. Senses 20*, 313–323.

Christensen, T.A., Heinbockel, T., Hildebrand, J.G., **1996**. Olfactory information processing in the brain: encoding chemical and temporal features of odors. *J. Neurobiol. 30*, 82–91.

Christensen, T.A., Hildebrand, J.G., **1988**. Frequency coding by central olfactory neurons in the sphinx moth *Manduca sexta*. *Chem. Senses 13*, 123–130.

Christensen, T.A., Mustaparta, H., Hildebrand, J.G., **1991**. Chemical communication in heliothine moths. 2. Central processing of intra- and interspecific olfactory messages in the male corn earworm moth *Helicoverpa zea*. *J. Comp. Physiol. A 169*, 259–274.

Christensen, T.A., Mustaparta, H., Hildebrand, J.G., **1995b**. Chemical communication in heliothine moths. 6. Parallel pathways for information processing in the macroglomerular complex of the male tobacco budworm moth *Heliothis virescens*. *J. Comp. Physiol. A 177*, *545–577*.

Christensen, T.A., Waldrop, B.R., Harrow, J.D., Hildebrand, J.G., **1993**. Local interneurons and information processing in the olfactory glomeruli of the moth *Manduca sexta*. *J. Comp. Physiol. A 173*, 385–399.

Christensen, T.A., Waldrop, B., Hildebrand, J.G., **1998a**. GABAergic mechanisms that shape the temporal response to odors in moth olfactory projection neurons. *Ann. N. Y. Acad. Sci. 855*, 475–481.

Christensen, T.A., Waldrop, B., Hildebrand, J.G., **1998b**. Multitasking in the olfactory system: context-dependent response to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *J. Neurosci. 18*, 5999–6008.

Coppedge, J.R., Bull, D.L., House, V.S., Ridgway, R.L., Bottrell, D.G., *et al.*, **1973**. Formulations for controlling the release of synthetic pheromone (grandlure) of the boll weevil. 2. Biological studies. *Environ. Entomol. 2*, 837–843.

Cossé, A.A., Todd, J.L., Baker, T.C., **1998**. Neurons discovered in male *Helicoverpa zea* antennae that correlate with pheromone-mediated attraction and interspecific antagonism. *J. Comp. Physiol. A 182*, *585–594*.

Cowles, R.S., Millar, J.G., Perry, E.J., McElfresh, J.S., Garrison, N., **1996**. Identification of the sex pheromone of the western poplar clearwing moth (Lepidoptera: Sesiidae). *Environ. Entomol. 25*, 109–113.

David, C.T., **1982**. Compensation for height in the control of groundspeed by *Drosophila* in a new, "barber's pole" wind tunnel. *J. Comp. Physiol. A 147*, 485–493.

David, C.T., **1986**. Mechanisms of directional flight in wind. In: Payne, T.L., Birch, M.C., Kennedy, C. (Eds.), Mechanisms in Insect Olfaction. Oxford University Press, Oxford, pp. 49–52.

David, C.T., Birch, M.C., **1989**. Pheromones and insect behaviour. In: Jutsum, A.R., Gordon, R.F.S. (Eds.), Insect Pheromones in plant Protection. Wiley, New York, pp. 17–35.

David, C.T., Kennedy, J.S., **1987**. The steering of zigzagging flight by male gypsy moths. *Naturwissenschaften 74*, 194–195.

David, C.T., Kennedy, J.S., Ludlow, A.R., Perry, J.N., Wall, C., **1982**. A re-appraisal of insect flight towards a point source of wind-borne odor. *J. Chem. Ecol. 8*, 1207–1215.

Dawson, G.W., Griffiths, D.C., Pickett, J.A., Wadhams, L.J., Woodcock, C.M., **1987**. Plant-derived synergists of alarm pheromone from turnip aphid, *Lipaphis (Hyadaphis) erysimi* (Homoptera, Aphidae). *J. Chem. Ecol. 13*, 1663–1671.

de Bruyne, M., Clyne, P., Carlson, J.R., **1997**. Olfactory receptor cells on the maxillary palps of *Drosophila*: a role for a POU-domain neuronal transcription factor in odor coding. In: Proc. 5th Eur. Symp. Taste Olfaction, Villasimius, Italy, pp. 15–16.

De Moraes, C.M., Lewis, W.J., Paré, P.W., Tumlinson, J.H., **1998**. Herbivore-infected plants selectively attract parasitoids. *Nature 393*, 570–573.

Dethier, V.G., **1972**. A surfeit of stimuli: a paucity of receptors. *Sci. Am. 59*, 706–715.

Dickens, J.C., **1979**. Electrophysiological investigations of olfaction in bark beetles. *Bull. Soc. Entomol. Suisse 52*, 203–216.

Dickens, J.C., Jang, E.B., Light, D.M., Alford, A.R., **1990**. Enhancement of insect pheromone responses by green leaf volatiles. *Naturwissenschaften 77*, 29–31.

Dickens, J.C., Payne, T.L., **1978**. Structure and function of the sensilla on the antennal club of the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Coleoptera: Scolytidae). *Int. J. Insect Morphol. Embryol. 17*, 251–265.

Distler, P.G., Boeckh, J., **1996**. Synaptic connection between olfactory receptor cells and uniglomerular projection neurons in the antennal lobe of the American cockroach, *Periplaneta americana*. *J. Comp. Neurol. 370*, 35–46.

Distler, P.G., Boeckh, J., **1997a**. Synaptic connections between identified neuron types in the antennal lobe glomeruli of the cockroach, *Periplaneta americana*. 1. Uniglomerular projection neurons. *J. Comp. Neurol. 378*, 307–319.

Distler, P.G., Boeckh, J., **1997b**. Synaptic connections between identified neuron types in the antennal lobe glomeruli of the cockroach, *Periplaneta americana*. 2. Local multiglomerular interneurons. *J. Comp. Neurol. 383*, 529–540.

Elkinton, J.S., Cardé, R.T., **1981**. The use of pheromone traps to monitor distribution and population trends of the gypsy moth. In: Mitchell, E.R. (Ed.), Management of Insect Pests with Semiochemicals. Plenum, New York, pp. 41–55.

Elkinton, J.S., Cardé, R.T., **1987**. Pheromone puff trajectory and upwind flight of the male gypsy moth in a forest. *Physiol. Entomol. 12*, 399–406.

Evendon, M.L., Judd, G.J.R., Borden, J.H., **1999a**. Pheromone-mediated mating disruption of *Choristoneura rosaceana*: is the most attractive blend really the most effective? *Entomol. Exp. Applic. 90*, 37–47.

Evendon, M.L., Judd, G.J.R., Borden, J.H., **1999b**. Mating disruption of two sympatric, orchard-inhabiting tortricids, *Choristoneura rosaceana* and *Pandemis limitata* (Lepidoptera: Tortricidae), with pheromone components of both species' blends. *J. Econ. Entomol. 92*, 380–390.

Evendon, M.L., Judd, G.J.R., Borden, J.H., **1999c**. Simultaneous disruption of pheromone communication in *Choristoneura rosaceana* and *Pandemis limitata* with pheromone and antagonist blends. *J. Chem. Ecol. 25*, 501–517.

Evendon, M.L., Judd, G.J.R., Borden, J.H., **2000**. Investigations of mechanisms of pheromone communication disruption of *Choristoneura rosaceana* (Harris) in a wind tunnel. *J. Insect Behav. 13*, 499–510.

Fadamiro, H.Y., Baker, T.C., **1997**. *Helicoverpa zea* males (Lepidoptera: Noctuidae) respond to the intermittent fine structure of their sex pheromone plume and an antagonist in a flight tunnel. *Physiol. Entomol. 22*, 316–324.

Fadamiro, H.Y., Baker, T.C., **1999**. Reproductive performance and longevity of female European corn borer, *Ostrinia nubilalis*: effects of multiple mating, delay in mating, and adult feeding. *J. Insect Physiol. 45*, 385–392.

Fadamiro, H.Y., Cossé, A.A., Baker, T.C., **1999a**. Fine-scale resolution of closely spaced pheromone and antagonist filaments by flying male *Helicoverpa zea*. *J. Comp. Physiol. A 185*, 131–141.

Fadamiro, H.Y., Cossé, A.A., Baker, T.C., **1999b**. Mating disruption of European corn borer, *Ostrinia nubilalis* by using two types of sex pheromone dispensers deployed in grassy aggregation sites in Iowa cornfields. *J. Asia-Pacific Entomol. 2*, 121–132.

Fadamiro, H.Y., Cossé, A.A., Dittl, T., Baker, T.C., **1998**. Suppression of mating by blackheaded fireworm (Lepidoptera: Tortricidae) in Wisconsin cranberry marshes by using MSTRS^{TM} devices. *J. Agric. Entomol. 15*, 377–386.

Farkas, S.R., Shorey, H.H., Gaston, L.K., **1974**. Sex pheromones of Lepidoptera: the use of widely separated evaporators of looplure for the disruption of pheromone communication in *Trichoplusia ni*. *Environ. Entomol. 3*, 876–887.

Faustini, D.L., Burkholder, W.E., Laub, R.J., **1981**. Sexually dimorphic setiferous sex patch in the male red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae): site of aggregation pheromone production. *J. Chem. Ecol. 7*, 465–480.

Flint, H.M., Balasubramanian, M., Campero, J., Strickland, G.R., Ahmad, Z., *et al.*, **1979**. Pink bollworm responses of native males to ratios of *Z*,*Z* and *Z*,*E*-isomers of gossyplure in several cotton growing areas of the world. *J. Econ. Entomol. 72*, 758–762.

Flint, H.M., Curtice, H.J., Yamamoto, A., **1988**. Pink bollworm (Lepidoptera: Gelechiidae): further tests with (*Z*,*Z*)-isomer of gossyplure. *J. Econ. Entomol. 81*, 679–683.

Flint, H.M., Merkle, J.R., **1983**. Pink bollworm (Lepidoptera: Gelechiidae): communication disruption by pheromone composition imbalance. *J. Econ. Entomol. 76*, 40–46.

Flint, H.M., Merkle, J.R., **1984**. The pink bollworm (Lepidoptera: Gelechiidae): alteration of male response to gossyplure by release of its component *Z*,*Z*-isomer. *J. Econ. Entomol. 77*, 1099–1104.

Galizia, G.G., Joerges, J., Kuttner, A., Faber, T., Menzel, R., **1997**. A semi-*in-vivo* preparation for optical recording of the insect brain. *J. Neurosci. Method. 76*, 61–69.

Galizia, G.G., Nagler, K., Holldobler, B., Menzel, R., **1998**. Odour coding is bilaterally symmetrical in the antennal lobes of honeybees (*Apis mellifera*). *Eur. J. Neurosci. 10*, 2964–2974.

Galizia, G.G., Sachse, S., Mustaparta, H., **2000**. Calcium responses to pheromones and plant odours in the antennal lobe of the male and female moth *Heliothis virescens*. *J. Comp. Physiol. A 31*, 1049–1063.

Galizia, G.G., Sachse, S., Rappert, A., Menzel, R., **1999**. The glomerular code for odor representation is species specific in the honeybee, *Apis mellifera*. *Nat. Neurosci. 2*, 473–478.

George, J.A., Nagy, B.A.L., **1984**. Morphology, distribution and ultrastructural differences of sensilla trichodea and basiconica on the antennae of the oriental fruit moth, *Grapholitha molesta* (Busck) (Lepidoptera: Tortricidae). *Int. J. Insect. Morphol. Embryol. 13*, 157–170.

Gibson, T. Brady J., **1988**. Flight behaviour of tsetse flies in host odour plumes: the initial response to leaving or entering odour. *Physiol. Entomol. 13*, 19–42.

Gibson, G., Packer, M.J., Steullet, P., Brady, J., **1991**. Orientation of tsetse flies to wind, within and outside host odor plumes in the field. *Physiol. Entomol. 16*, 47–56.

Greenfield, M.D., Karandinos, M.G., **1979**. Resource partitioning of the sex communication channel in clearwing moths (Lepidoptera: Sesiidae) of Wisconsin. *Ecol. Monogr. 49*, 403–426.

Gut, L.J., Brunner, J.F., **1998**. Pheromone-based management of codling moth (Lepidoptera: Tortricidae) in Washington apple orchards. *J. Agric. Entomol. 15*, 387–406.

Hansson, B.S., **1995**. Olfaction in Lepidoptera. *Experientia 51*, 1003–1020.

Hansson, B.S., **1999**. Insect Olfaction. Springer, Berlin.

Hansson, B.S., Almaas, T.J., Anton, S., **1995**. Chemical communication in heliothine moths. 5. Antennal lobe projection patterns of pheromone-detecting olfactory receptor neurons in the male *Heliothis virescens* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A 177*, 535–543.

Hansson, B.S., Baker, T.C., **1991**. Differential adaptation rates in a male moth's sex pheromone receptor neurons. *Naturwissenschaften 78*, 517–520.

Hansson, B.S., Christensen, T.A., **1999**. Functional characteristics of the antennal lobe. In: Hansson, B. (Ed.), Insect Olfaction. Springer, Berlin, pp. 125–161.

Hansson, B.S., Christensen, T.A., Hildebrand, J.G., **1991**. Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta*. *J. Comp. Neurol. 312*, 264–278.

Hansson, B.S., Lungberg, H., Hallberg, E., Löfstedt, C., **1992**. Functional specialization of olfactory glomeruli in a moth. *Science 256*, 1313–1315.

Hansson, B.S., Toth, M., Löfstedt, C., Szöcs, G., Subchev, M., *et al.*, **1990**. Pheromone variation among eastern European and a western Asian population of the turnip moth *Agrotis segetum*. *J. Chem. Ecol. 16*, 1611–1622.

Hardee, D.D., McKibben, G.H., Rummel, D.R., Huddleston, P.M., Coppedge, J.R., **1974**. Response of boll weevils to component ratios and doses of the pheromone, grandlure. *Environ. Entomol. 3*, 135–138.

Hardee, D.D., Mitchell, E.B., Huddleston, P.M., **1967a**. Field studies of sex attraction in the boll weevil. *J. Econ. Entomol. 60*, 1221–1224.

Hardee, D.D., Mitchell, E.B., Huddleston, P.M., **1967b**. Procedure for bioassaying the sex attractant of the boll weevil. *J. Econ. Entomol. 60*, 169–171.

Hardie, J., Minks, A.K. (Eds.) **1999**. Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford.

Hardie, J., Pickett, J.A., Pow, E.M., Smiley, D.W.M., **1999**. Aphids. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects associated with Agricultural Plants. CAB International, Wallingford, pp. 227–250.

Hardie, J., Visser, J.H., Piron, P.G.M., **1994**. Perception of volatiles associated with sex and food by different adult forms of the black bean aphid, *Aphis fabae*. *Physiol. Entomol. 19*, 278–284.

Haynes, K.F., Baker, T.C., **1989**. An analysis of anemotactic flight in female moths stimulated by host odour and comparison with the males' response to sex pheromone. *Physiol. Entomol. 14*, 279–289.

Haynes, K.F., Li, W.G., Baker, T.C., **1986**. Control of pink bollworm moth (Lepidoptera: Gelechiidae) with insecticides and pheromones (attracticide): lethal and sublethal effects. *J. Econ. Entomol. 79*, 1466–1471.

Heath, J.J., Williams, R.N., Phelan, P.L., **2002**. Aggregation and male attraction to feeding virgin females in *Macrodactylus subspinosus* (F.) (Coleoptera: Scarabaeidae: Melolonthinae). *Environ. Entomol. 31*, 934–940.

Hildebrand, J.G., Shepard, G.M., **1997**. Mechanisms of olfactory discrimination: converging evidence for

common principles across phyla. *Annu. Rev. Neurosci. 20*, 595–631.

Hummel, H.E., Gaston, L.K., Shorey, H.H., Kaae, R.S., Byrne, K.J., *et al.*, **1973**. Clarification of the chemical structure of the pink bollworm sex pheromone. *Science 181*, 873.

Ivanov, V.D., Melnitsky, S.I., **1999**. Structure of sternal pheromone glands in caddisflies (Trichoptera). *Entomol. Rev. 79*, 926–942.

Justus, K.A., Cardé, R.T., **2002**. Flight behaviour of two moths, *Cadra cautella* and *Pectinophora gossypiella*, in homogeneous clouds of pheromone. *Physiol. Entomol. 27*, 67–75.

Kaissling, K.E., **1974**. Sensory transduction in insect olfactory receptors. In: Aenicke, L. (Ed.), Biochemistry of Sensory Functions. Springer, Berlin, pp. 243–273.

Kaissling, K.E., **1986**. Temporal characteristics of pheromone receptor cell responses in relation to orientation behaviour of moths. In: Payne, T.L., Birch, M.C., Kennedy, C.E.J. (Eds.), Mechanisms in Insect Olfaction. Clarendon Press, Oxford, pp. 193–199.

Kaissling, K.E., **1987**. R.H. Wright lectures on insect olfaction. In: Colbow, K. (Ed.), Insect Olfaction. Simon Fraser University Press, Burnaby, BC, Canada, pp. 28–32.

Kaissling, K.E., **1990**. Antennae and noses: their sensitivities as molecule detectors. In: Torre, V., Cervetto, L., Borsellino, A. (Eds.), Sensory Transduction. Plenum, New York, pp. 81–97.

Kaissling, K.E., **1997**. Pheromone-controlled anemotaxis in moths. In: Lehrer, M. (Ed.), Orientation and Communication in Arthropods. Birkhauser-Verlag, Basel, pp. 343–374.

Kaissling, K.E., **1998**. Flux detectors versus concentration detectors: two types of chemoreceptors. *Chem. Senses 23*, 99–111.

Kaissling, K.E., Kramer, E., **1990**. Sensory basis of pheromone-mediated orientation in moths. *Verhandl. Deutsch. Zool. Gesell. 83*, 109–131.

Kaissling, K.E., Meng, L.Z., Bestmann, H.J., **1989**. Responses of bombykol receptor cells to (*Z,E*)-4,6-hexadecadiene and linalool. *J. Comp. Physiol. A 165*, 147–154.

Kanzaki, R., Arbas, E.A., Hildebrand, J.G., **1991**. Phsyiology and morphology of protocerebral olfactory neurons in the male moth *Manduca sexta*. *J. Comp. Physiol. A 168*, 281–298.

Kanzaki, R., Shibuya, T., **1992**. Long-lasting excitation of protocerebral bilateral neurons in the pheromone-processing pathways of the male moth *Bombyx mori*. *Brain Res. 587*, 211–215.

Kennedy, J.S., **1977**. Olfactory responses to distant plants and other odor sources. In: Shorey, H.H., McKelvey, J.J., Jr. (Eds.), Chemical Control of Insect Behavior. Wiley, New York, pp. 67–91.

Kennedy, J.S., **1983**. Zigzagging and casting as a programmed response to wind-borne odour: a review. *Physiol. Entomol. 8*, 109–120.

Kennedy, J.S., **1986**. Some current issues in orientation to odour sources. In: Payne, T.L., Kennedy, C.E.J., Birch, M.C. (Eds.), Mechanisms in Insect Olfaction. Clarendon Press, Oxford, pp. 11–25.

Kennedy, J.S., Ludlow, A.R., Sanders, C.J., **1980**. Guidance system used in moth sex attraction. *Nature 295*, 475–477.

Kennedy, J.S., Ludlow, A.R., Sanders, C.J., **1981**. Guidance of flying male moths by wind-borne sex pheromone. *Physiol. Entomol. 6*, 395–412.

Kennedy, J.S., Marsh, D., **1974**. Pheromone-regulated anemotaxis in flying moths. *Science 184*, 999–1001.

Kim, J.-Y., Leal, W.S., **2000**. Ultrastructure of pheromone-detectin sensillum placodeum of the Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae). *Arthropod Struct. Devel. 29*, 121–128.

Kinzer, G.W., Fentiman, A.F., Page, T.F., Foltz, R.L., Vité, J.P., *et al.*, **1969**. Bark beetle attractants: identification, synthesis, and field bioassay of a new compound isolated from *Dendroctonus*. *Nature 221*, 477–478.

Klein, M.G., Tumlinson, J.H., Ladd, T.L., Jr., Doolittle, R.E., **1981**. Japanese beetle (Coleoptera: Scarabaeidae): response to synthetic sex attractant plus phenethyl propionate: eugenol. *J. Chem. Ecol. 7*, 1–7.

Knight, A.L., **1996**. The impact of codling moth (Lepidoptera: Tortricidae) mating disruption on apple pest management in Yakima Valley, Washington. *J. Entomol. Soc. Br. Columbia 92*, 29–38.

Knight, A.L., **1997**. Delay of mating of codling moth in pheromone disrupted orchards. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 20*, 203–206.

Kozlov, M.V., Zhu, J., Philipp, P., Francke, W., Zvereva, E.L., *et al.*, **1996**. Pheromone specificity in *Eriocrania semipurpurella* (Stephens) and *E. sangii* (Wood) (Lepidoptera: Eriocraniidae) based on chirality of semiochemicals. *J. Chem. Ecol. 22*, 431–454.

Kramer, E., **1992**. Attractivity of pheromone surpassed by time-patterned application of two nonpheromone compounds. *J. Insect Behav. 5*, 83–97.

Kuenen, L.P.S., Baker, T.C., **1983**. A non-anemotactic mechanism used in pheromone source location by flying moths. *Physiol. Entomol. 8*, 277–289.

Landolt, P.J., Phillips, T.W., **1997**. Host plant influences on sex pheromone behavior of phytophagous insects. *Annu. Rev. Entomol. 42*, 371–391.

Lanier, G.N., **1981**. Pheromone-baited traps and trap trees in the integrated management of bark beetles in urban areas. In: Mitchell, E.R. (Ed.), Management of Insect Pests With Semiochemicals. Plenum, New York, pp. 115–131.

Lanier, G.N., **1990**. Principles of attraction-annihilation. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 25–49.

Larsson, M.C., Hansson, B.S., **1998**. Receptor neuron responses to potential sex pheromone components in the caddisfly *Rhyacophila nubila* (Trichoptera: Rhyacophilidae). *J. Insect Physiol. 44*, 189–196.

Laurent, G., **1996**. Dynamical representation of odours by oscillating and evolving neural assemblies. *Trends Neurosci. 19*, 489–496.

Laurent, G., Wehr, M., Davidowitz, H., **1996**. Temporal representations of odors in an olfactory network. *J. Neurosci. 16*, 3837–3847.

Leal, W.S., **1999**. Scarab beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 51–68.

Leal, W.S., Mochizuki, F., **1993**. Sex pheromone reception in the scarab beetle Anomala cuprea. *Naturwissensch. 80*, 278–281.

Leal, W.S., Ueda, Y., Ono, M., **1996**. Attractant pheromone for male rice bug, *Leptocorisa chinensis*: semiochemicals produced by both male and female. *J. Chem. Ecol. 22*, 1429–1437.

Lewis, T., Macaulay, E.D.M., **1976**. Design and elevation of sex-attractant traps for the pea moth, *Cydia nigricana* (Steph.), and the effect of plume shape on catches. *Ecol. Entomol. 1*, 175–181.

Lie, R., Bakke, A., **1981**. Practical results from the mass trapping of *Ips typographus* in Scandanavia. In: Mitchell, E.R. (Ed.), Management of Insect Pests with Semiochemicals. Plenum, New York, pp. 175–181.

Light, D.M., Flath, R.A., Buttery, R.G., Zalom, F.G., Rice, R.E., *et al.*, **1993**. Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera). *Chemoecology 4*, 145–152.

Lindgren, B.S., **1983**. A multiple funnel trap for scolytid beetles. *Can. Entomol. 115*, 299–302.

Lindgren, B.S., Borden, J.H., **1983**. Survey and mass trapping of ambrosia beetles (Coleoptera: Scolytidae) in timber processing areas on Vancouver Island. *Can. J. For. Res. 13*, 481–493.

Lindgren, B.S., Borden, J.H., Chong, L., Friskie, L.M., Orr, D.B., **1983**. Factors influencing the efficiency of pheromone-baited traps for three species of ambrosia beetles. *Can. Entomol. 115*, 303–313.

Lindgren, B.S., Fraser, R.G., **1994**. Control of ambrosia beetle damage by mass trapping at a dryland log sorting area in British Columbia. *For. Chron. 70*, 159–163.

Linn, C.E., Jr., Campbell, M.G., Roelofs, W.L., **1986a**. Male moth sensitivity to multicomponent pheromones: critical role of female-released blend in determining the functional role of components and active space of the pheromone. *J. Chem. Ecol. 12*, 659–668.

Linn, C.E., Jr., Campbell, M.G., Roelofs, W.L., **1986b**. Pheromone components and active spaces: what do moths smell and where do they smell it? *Science 237*, 650–652.

Linn, C.E., Jr., Roelofs, W.L., **1981**. Modification of sex pheromone blend discrimination in male oriental fruit moths by pre-exposure to (*E*)-8-dodecenyl acetate. *Physiol. Entomol. 6*, 421–429.

Linn, C.E., Jr., Roelofs, W.L., **1985**. Response specificity of male pink bollworm moths to different blends and dosages of sex pheromone. *J. Chem. Ecol. 11*, 1583–1590.

Liu, Y.B., Haynes, K.F., **1992**. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol. 18*, 299–307.

Löfstedt, C., **1990**. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl. 54*, 199–218.

Löfstedt, C., **1993**. Moth pheromone genetics and evolution. *Phil. Trans. Roy. Soc. B. 240*, 167–177.

Löfstedt, C., Hansson, B.S., Dijkerman, H.J., Herrebout, W.M., **1990**. Behavioral and electrophysiological activity of unsaturated analogues of the pheromone tetradecyl acetate in the small ermine moth *Yponomeuta rorellus*. *Physiol. Entomol. 15*, 47–54.

Löfstedt, C., Hansson, B.S., Petersson, E., Valeur, P., Richards, A., **1994**. Pheromonal secretions from glands on the 5th abdominal sternite of hydropsychid and rhyacophilid caddisflies (Trichoptera). *J. Chem. Ecol. 20*, 153–171.

Löfstedt, C., Herrebout, W.M., Menken, S.B.J., **1991**. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology 2*, 20–28.

Löfstedt, C., Löfqvist, J., Lanne, B.S., van der Pers, J.N.C., Hansson, B.S., **1986**. Pheromone dialects in European turnip moths *Agrotis segetum*. *Oikos 46*, 250–257.

MacConnell, J.G., Borden, J.H., Silverstein, R.M., Sokkink, E., **1977**. Isolation and tentative identification of lineatin, a pheromone from the frass of *Trypodendron lineatum* (Coleoptera: Scolytidae). *J. Chem. Ecol. 3*, 549–561.

Madsen, H.F., **1981**. Monitoring codling moth populations in British Columbia apple orchards. In: Mitchell, E.R. (Ed.), Management of Insect Pests with Semiochemicals. Plenum, New York, pp. 57–62.

Mafra-Neto, A., Baker, T.C., **1996a**. Timed, metered sprays of pheromone disrupt mating of *Cadra cautella* (Lepidoptera: Pyralidae). *J. Agric. Entomol. 13*, 149–168.

Mafra-Neto, A., Baker, T.C., **1996b**. Elevation of pheromone response threshold in almond moth males pre-exposed to pheromone spray. *Physiol. Entomol. 21*, 217–222.

Mafra-Neto, A., Cardé, R.T., **1994**. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature 369*, 142–144.

Marsh, D., Kennedy, J.S., Ludlow, A.R., **1978**. An analysis of anemotactic zigzagging flight in male moths stimulated by pheromone. *Physiol. Entomol. 3*, 221–240.

Matsumoto, S.G., Hildebrand, J.G., **1981**. Olfactory mechanisms in the moth *Manduca sexta*: response characteristics and morphology of central neurons in the antennal lobes. *Proc. Roy. Soc. B 213*, 249–277.

McBrien, H.L., Millar, J.G., **1999**. Phytophagous bugs. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 277–304.

McBrien, H.L., Millar, J.G., Rice, R.E., McElfresh, J.S., Cullen, E., *et al.*, **2002**. Sex attractant pheromone of the red-shouldered stink bug *Thyanta pallidovirens*: a pheromone blend with multiple redundant components. *J. Chem. Ecol. 28*, 1797–1818.

Menon, M., **1976**. Hormone–pheromone relationships in the beetle, *Tenebrio molitor. J. Insect Physiol. 22*, 1021–1023.

Millar, J.G., **2000**. Polyene hydrocarbons and epoxides: a second major class of lepidopteran sex attractant pheromones. *Annu. Rev. Entomol. 45*, 575–604.

Miller, E., Staten, R.T., Nowell, C., Gourd, J., **1990**. Pink bollworm (Lepidoptera: Gelechiidae): point source density and its relationship to efficacy in attracticide formulations of gossyplure. *J. Econ. Entomol. 83*, 1321–1325.

Minks, A.K., Cardé, R.T., **1988**. Disruption of pheromone communication in moths: is the natural blend really most efficacious? *Entomol. Exp. Appl. 49*, 25–36.

Minor, A.V., Kaissling, K.E., **2003**. Cell responses to single pheromone molecules may reflect the activation kinetics of olfactory receptor molecules. *J. Comp. Physiol. A 189*, 221–230.

Mitchell, E.B., Hardee, D.D., **1974**. In-field traps: a new concept in survey and suppression of low populations of boll weevils. *J. Econ. Entomol. 67*, 506–508.

Mori, M., Chuman, T., Kato, K., **1984**. Cyclic hemiacetal and acyclic chain: the two forms of serricornin. *Tetrahedron Lett. 25*, 2553–2556.

Murlis, J., **1986**. The structure of odor plumes. In: Payne, T.L., Kennedy, C.E.J., Birch, M.C. (Eds.), Mechanisms in Insect Olfaction. Clarendon Press, Oxford, pp. 27–38.

Murlis, J., **1997**. Odor plumes and the signal they provide. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 221–231.

Mustaparta, H., **1984**. Olfaction. In: Bell, W.J., Cardé, R.T. (Eds.), Chemical Ecology of Insects. Chapman and Hall, London, pp. 37–70.

Nakamura, K., **1981**. The mate searching behavior of *Spodoptera litura* and components of its sex pheromone. In: Kioza, M. (Ed.), Regulation of Insect Development and Behaviour. Wrocław Technical University Press, Wrocław, Poland.

Nation, J.L., **2002**. Insect Physiology and Biochemistry. CRC Press, Boca Raton, FL.

Nikonov, A., Leal, W.S., **2002**. Peripheral coding of sex pheromone and a behavioral antagonist in the Japanese beetle, *Popillia japonica. J. Chem. Ecol. 28*, 1075–1089.

Ochieng, S.A., Anderson, P., Hansson, B.S., **1995**. Antennal lobe projection patterns of olfactory receptor neurons involved in sex pheromone detection in *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Tissue Cell 27*, 221–232.

Ochieng, S.A., Baker, T.C., **2002**. Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea. J. Comp. Physiol. A 188*, 325–333.

Ochieng, S.A., Poole, K., Roelofs, W.L., Vickers, N.J., Linn, C.E., Jr., *et al.*, **2003**. Unusual pheromone receptor neuron responses in heliothine moth antennae derived from inter-species imaginal disc transplantation. *J. Comp. Physiol. A 189*, 19–28.

Ochieng, S.A., Robbins, P.S., Roelofs, W.L., Baker, T.C., **2002**. Sex pheromone reception in the scarab beetle *Phyllophaga anxia* (Coleoptera: Scarabaeidae). *Ann. Entomol. Soc. America. 95*, 97–102.

O'Connell, R.J., Beauchamp, J.T., Grant, A.J., **1986**. Insect olfactory receptor responses to components of pheromone blends. *J. Chem. Ecol. 12*, 451–467.

Oehlschlager, A.C., Chinchilla, C., Castillo, G., Gonzalez, L., **2002**. Control of red ring disease by mass trapping of *Rhynchophorus palmarum* (Coleoptera: Curculionidae). *Fla. Entomol. 85*, 507–513.

Oehlschlager, A.C., Chinchilla, C.M., Gonzalez, M., **1992a**. Management of the American palm weevil (*Rhynchophorus palmarum*) and the red ring disease in oil palm by pheromone-based trapping. *ASD Oil Palm Papers 5*, 15–23.

Oehlschlager, A.C., Chinchilla, C.M., Gonzales, L.M., Jiron, L.F., Mexzon, R., *et al.*, **1993**. Development of a pheromone-based trapping system for *Rhynchophorus palmarum* (Coleoptera: Curculionidae). *J. Econ. Entomol. 86*, 1381–1392.

Oehlschlager, A.C., McDonald, R.S., Chinchilla, C.M., Patschke, S.N., **1995**. Influence of a pheromone-based mass-trapping system on the distribution of *Rhynchophorus palmarum* (Coleoptera: Curculionidae) in oil palm. *Environ. Entomol. 24*, 1005–1012.

Oehlschlager, A.C., Pierce, H.D., Morgan, B., Wimalaratne, K., Slessor, N., *et al.*, **1992b**. Chirality and field testing of Rhynchophorol, the aggregation pheromone of the American palm weevil. *Naturwissenschaften 79*, 134–135.

Olberg, R.M., Willis, M.A., **1990**. Pheromone modulated optomotor response in male gypsy moth, *Lymantria dispar* L.: directionally selective visual interneurons in the ventral nerve cord. *J. Comp. Physiol. A 167*, 707–714.

Park, K.C., Baker, T.C., **2002**. Improvement of signal-to-noise ratio in electroantennogram responses using multiple insect antennae. *J. Insect Physiol. 48*, 1139–1145.

Park, K.-C., Ochieng, S.A., Zhu, J.W., Baker, T.C., **2002**. Odor discrimination using insect electroantennogram responses from an insect antennal array. *Chem. Sens. 27*, 343–352.

Perez, A.L., Campos, Y., Chinchilla, C.M., Gries, G., Gries, R., *et al.*, **1997**. Aggregation pheromones and host kairomones of the West Indian sugarcane weevil, *Metamasius hemipterus*. *J. Chem. Ecol. 23*, 869–888.

Petroski, R.J., Bartelt, R.J., Weisleder, D., **1994**. Biosynthesis of (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene: the aggregation pheromone of *Carpophilus freemani* (Coleoptera: Nitidulidae). *Insect Biochem. Mol. Biol. 24*, 69–78.

Pettersson, J., **1971**. An aphid sex attractant. *Entomol. Scand. 2*, 81–93.

Phelan, P.L., **1997**. Genetics and phylogenetics in the evolution of sex pheromones. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 563–579.

Pickett, J.A., Wadhams, L.J., Woodcock, C.M., **1992**. The chemical ecology of aphids. *Annu. Rev. Entomol. 37*, 67–90.

Plarre, R., Vanderwel, D.C., **1999**. Stored-product Beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 149–198.

Priesner, E., Witzgall, P., Voerman, S., **1986**. Field attraction response of raspberry clearwing moths, *Pennisetia hylaeiformis* Lasp. (Lepidoptera: Sesiidae), to candidate pheromone chemicals. *J. Appl. Entomol. 102*, 195–210.

Quero, C., Fadamiro, H.Y., Baker, T.C., **2001**. Responses of male *Helicoverpa zea* to single pulses of sex pheromone and behavioural antagonist. *Physiol. Entomol. 26*, 106–115.

Raina, A.K., Menn, J.J., **1987**. Endocrine regulation of pheromone production in Lepidoptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 159–174.

Resh, V.H., Wood, J.R., **1985**. Site of sex pheromone production in three species of Trichoptera. *Aquat. Insects 7*, 65–71.

Rice, R.E., Kirsch, P., **1990**. Mating disruption of oriental fruit moth in the United States. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 193–211.

Ridgway, R.L., Inscoe, M.N., Dickerson, W.A., **1990**. Role of the boll weevil pheromone in pest management. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 437–471.

Riedl, H., Croft, B.A., **1974**. A study of pheromone trap catches in relation to codling moth (Lepidoptera: Olethreutidae) damage. *Can. Entomol. 112*, 655–663.

Riedl, H., Croft, B.A., Howitt, A.G., **1976**. Forecasting codling moth phenology based on pheromone trap catches and physiological-time models. *Can. Entomol. 108*, 449–460.

Rochat, D.C., Lettere, M., Ducrot, P.H., Sagatti, P., Renou, M., *et al.*, **1991**. Male-produced aggregation pheromone of the American palm weevil, *Rhynchophorus palmarum*: collection, identification, electrophysiological activity and laboratory bioassay. *J. Chem. Ecol. 17*, 2127–2124.

Roelofs, W.L., Glass, E.H., Tette, J., Comeau, A., **1970**. Sex pheromone trapping for red-banded leaf roller control: theoretical and actual. *J. Econ. Entomol. 62*, 1161–1167.

Roelofs, W.L., Novak, M.A., **1981**. Small-plot disorientation tests for screening potential mating disruptants. In: Mitchell, E.R. (Ed.), Management of Insect Pests with Semiochemicals. Plenum, New York, pp. 229–242.

Roelofs, W.L., Liu, W., Hao, G., Jiao, H., Rooney, A.P., *et al.*, **2002**. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl Acad. Sci. USA 99*, 13621–13626.

Roelofs, W.L., Rooney, A.P., **2003**. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl Acad. Sci. USA 100*, 9179–9184.

Rospars, J.P., **1983**. Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth, *Mamestra brassicae*, and a butterfly, *Pieris brassicae*. *J. Comp. Neurol. 220*, 80–96.

Ross, H.H., **1964**. Evolution of caddisworm cases and nets. *Am. Zool. 4*, 209–220.

Rumbo, E.R., **1983**. Differences between single cell reponses to different components of the sex pheromone in males of the light-brown apple moth (*Epiphyas postvittana*). *Physiol. Entomol. 8*, 195–201.

Rumbo, E.R., Deacon, S.M., Regan, L.P., **1993**. Spatial discrimination between sources of pheromone and an inhibitor by the light-brown apple moth *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae). *J. Chem. Ecol. 19*, 953–962.

Rumbo, E.R., Kaissling, K-E., **1989**. Temporal resolution of odor pulses by three types of pheromone receptor cells in *Antheraea polyphemus*. *J. Comp. Physiol. A 165*, 281–291.

Sachse, S., Rapper, A., Galizia, G.G., **1999**. The spatial representation of chemical structures in the antennal lobe of honeybees: steps toward the olfactory code. *Eur. J. Neurosci. 11*, 3970–3982.

Sánchez, P., Cerda, H., Cabrera, A., Caetano, F.H., Materán, M., *et al.*, **1996**. Secretory mechanisms for the male produced aggregation pheromone of the palm weevil *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae). *J. Insect Physiol. 42*, 11–12.

Schlyter, F., Birgersson, G.A., **1999**. Forest beetles. In: Hardie, J., Minks, A.K. (Eds.), Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, pp. 113–148.

Schofield, S.W., Justus, K.A., Mafra-Neto, A., Cardé, R.T., **2003**. Flight of male *Cadra cautella* along plumes of air and pheromone superimposed on backgrounds of pheromone. *Entomol. Exp. Appl. 109*, 173–181.

Schouest, L.P., Miller, T.A., **1994**. Automated pheromone traps show male pink bollworm (Lepidoptera: Gelechiidae) mating response is dependent on weather conditions. *J. Econ. Entomol. 87*, 965–974.

Shorey, H.H., Gerber, R.G., **1996a**. Use of puffers for disruption of sex pheromone communication of codling moths (Lepidoptera: Tortricidae) in walnut orchards. *Environ. Entomol. 25*, 1398–1400.

Shorey, H.H., Gerber, R.G., **1996b**. Disruption of pheromone communication through the use of puffers for

control of beet armyworm (Lepidoptera: Noctuidae) in tomatoes. *Environ. Entomol. 25*, 1401–1405.

Shorey, H.H., Kaae, R.S., Gaston, L.K., McLaughlin, J.R., **1972**. Sex pheromones of Lepidoptera. XXX. Disruption of sex pheromone communication in *Trichoplusia ni* as a possible means of mating control. *Environ. Entomol. 1*, 641–645.

Shorey, H.H., Sisk, C.B., Gerber, R.G., **1996**. Widely separated pheromone release sites for disruption of sex pheromone communication in two species of Lepidoptera. *Environ. Entomol. 25*, 446–451.

Showers, W.B., Smelser, R.B., Keaster, A.J., Whitford, F., Robinson, J.F., *et al.*, **1989a**. Recapture of marked black cutworm (Lepidoptera: Noactuidae) males after long-range transport. *Environ. Entomol. 18*, 447–458.

Showers, W.B., Whitford, F., Smelser, R.B., Keaster, A.J., Robinsons, J.F., *et al.*, **1989b**. Direct evidence for meteorologically driven long-range dispersals of an economically important moth. *Ecology 70*, 987–992.

Silverstein, R.M., Brownlee, R.G., Bellas, T.E., Wood, D.L., Browne, L.E., **1968**. Brevicomin: principal sex attractant in the frass of the female western pine beetle. *Science 159*, 889–891.

Silverstein, R.M., Rodin, J.O., Burkholder, W.E., Gorman, J.E., **1967**. Sex attractant of the black carpet beetle. *Science 157*, 85–87.

Silverstein, R.M., Rodin, J.O., Wood, D.L., **1966**. Sex attractants in frass produced by male *Ips confusus* in ponderosa pine. *Science 154*, 509–510.

Smith, R.F., Pierce, H.D., Jr., Borden, J.H., **1991**. Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *J. Chem. Ecol. 17*, 1437–1447.

Staten, R.T., El-Lissy, O., Antilla, L., **1997**. Successful area-wide program to control pink bollworm by mating disruption. In: Cardê, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 383–396.

Steinbrecht, R.A., **1987**. Functional morphology of pheromone-sensitive sensilla. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 353–384.

Steinbrecht, R.A., **1999**. Olfactory receptors. In: Eguchi, E., Tominaga, Y. (Eds.), Atlas of Arthropod Sensory Receptors. Springer, Tokyo, pp. 155–176.

Stengl, M., Zeigelberger, G., Boekhoff, I., Krieger, J., **1999**. Perireceptor events and transduction mechanisms in insect olfaction. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 49–66.

Stocker, R.F., Rodrigues, V., **1999**. Olfactory neurogenetics. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 283–314.

Stopfer, M., Bhagavan, S., Smith, B.H., Laurent, G., **1997**. Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature 390*, 70–74.

Stopfer, M., Wehr, M., MacLeod, K., Laurent, G., **1999**. Neural dynamics, oscillatory synchronization, and odour codes. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 163–180.

Szöcs, G., Tóth, M., Sziráki, G.Y., Schwarz, M., **1989**. 2,13- and 3,13-octadecadienyl compounds composing sex attractants for tineid and sesiid moths (Lepidoptera). *Biochem. System. Ecol. 17*, 417–422.

Tillman, J.A., Seybold, S.J., Jurenka, R.A., Blomquist, G.J., **1999**. Insect pheromones: an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol. 29*, 481–514.

Todd, J.L., Baker, T.C., **1999**. Function of peripheral olfactory organs. In: Hansson, B.S. (Ed.), Insect Olfaction. Springer, Berlin, pp. 67–96.

Todd, J.L., Anton, S., Hansson, B.S., Baker, T.C., **1995**. Functional organization of the macroglomerular complex related to behaviourally expressed olfactory redundancy in male cabbage looper moths. *Physiol. Entomol. 20*, 349–361.

Tumlinson, J.H., Hardee, D.D., Minyard, J.P., Thompson, A.C., Gast, R.T., *et al.*, **1968**. Boll weevil sex attractant: isolation studies. *J. Econ. Entomol. 61*, 470–474.

Tumlinson, J.H., Gueldner, R.C., Hardee, D.D., Thompson, A.C., Hedin, P.A., *et al.*, **1970**. The boll weevil sex attractant. In: Beroza, M. (Ed.), Chemicals Controlling Insect Behavior. Academic Press, New York, pp. 41–59.

Tumlinson, J.H., Gueldner, R.C., Hardee, D.D., Thompson, A.C., Hedin, P.A., *et al.*, **1971**. Identification and synthesis of the four compounds comprising the boll weevil sex attractant. *J. Org. Chem. 36*, 2616–2621.

Tumlinson, J.H., Hardee, D.D., Gueldner, R.C., Thompson, A.C., Hedin, P.A., *et al.*, **1969**. Sex pheromones produced by male boll weevil: isolation, identification, and synthesis. *Science 166*, 1010–1012.

Tumlinson, J.H., Klein, M.G., Doolittle, R.E., Ladd, T.L., Proveaux, A.T., **1977**. Identification of the female Japanese beetle sex pheromone: Inhibition of male response by an enantiomer. *Science 197*, 789–792.

Turlings, T.C.J., Loughrin, J.H., McCall, P.J., Röse, U.S.R., Lewis, W.J., *et al.*, **1995**. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl Acad. Sci. USA 92*, 4169–4174.

Van der Pers, J.N.C., Den Otter, C.J., **1978**. Single cell responses from olfactory receptors of small ermine moths to sex attractants. *J. Insect Physiol. 24*, 337–343.

Van der Pers, J.N.C., Thomas, G., Den Otter, C.J., **1980**. Interactions between odours and pheromone reception in small ermine moths (Lepidoptera: Yponomeutidae). *Chem. Senses 5*, 367–371.

Vickers, N.J., Baker, T.C., **1992**. Male *Heliothis virescens* sustain upwind flight in response to experimentally pulsed filaments of their sex-pheromone. *J. Insect Behav. 5*, 669–687.

Vickers, N.J., Baker, T.C., **1994**. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Natl Acad. Sci. USA 91*, 5756–5760.

Vickers, N.J., Baker, T.C., **1996**. Latencies of behavioral response to interception of filaments of sex pheromone and clean air influence flight track shape in *Heliothis virescens* (F.) males. *J. Comp. Physiol. A. 178*, 831–847.

Vickers, N.J., Baker, T.C., **1997**. Chemical communication in heliothine moths. VII. Correlation between diminished responses to point-source plumes and single filaments similarly tainted with a behavioral antagonist. *J. Comp. Physiol. 180*, 523–536.

Vickers, N.J., Christensen, T.A., **2003**. Functional divergence of spatially conserved olfactory glomeruli in two related moth species. *Chem. Senses 28*, 325–338.

Vickers, N.J., Christensen, T.A., Baker, T.C., Hildebrand, J.G., **2001**. How do odour plume dynamics influence the brain's olfactory code? *Nature 410*, 466–470.

Vickers, R.A., **1990**. Oriental fruit moth in Australia and Canada. In: Ridgway, R.L., Silverstein, R.M., Inscoe, M.N. (Eds.), Behavior-Modifying Chemicals for Insect Management. Dekker, New York, pp. 183–192.

Vickers, R.A., Rothschild, G.H.L., Jones, E.L., **1985**. Control of the oriental fruit moth, *Cydia molesta* (Busck) (Lepidoptera: Tortricidae), at a district level by mating disruption with synthetic female pheromone. *Bull. Entomol. Res. 75*, 625–634.

Von Keyserlingk, H.C., **1984**. Close range orientation of flying Lepidoptera to pheromone sources in a laboratory wind tunnel and the field. *Meded. Fac. Landbouwwet. Rijksuniv. Gent. 49*, 683–687.

Willis, M.A., Arbas, E.A., **1991**. Odor-modulated upwind flight of the sphinx moth, *Manduca sexta* L. *J. Comp. Physiol. 169*, 427–440.

Willis, M.A., Arbas, E.A., **1997**. Active behavior and reflexive responses: another perspective on odor-modulated locomotion. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 304–319.

Willis, M.A., Baker, T.C., **1984**. Effects of intermittent and continuous pheromone stimulation on the flight behaviour of the oriental fruit moth, *Grapholita molesta*. *Physiol. Entomol. 9*, 341–358.

Willis, M.A., Murlis, J., Cardé, R.T., **1994**. Effects of pheromone plume structure and visual stimuli on the pheromone-modulated flight of male gypsy moths (*Lymantria dispar*) (Lepidoptera: Lymantriidae) in a forest. *J. Insect Behav. 7*, 385–409.

Witzgall, P., Priesner, E., **1991**. Wind-tunnel study on attraction inhibitor in male *Coleophora laricella* Hbn. (Lepidoptera: Coleophoridae). *J. Chem. Ecol. 17*, 1355–1362.

Witzgall, P., Unelius, R.C., Rama, F., Chambon, J.-P., Bengtsson, M., **1997**. Mating disruption of pea moth, *Cydia nigricana*, and codling moth, *C. pomonella*, using blends of sex pheromone and attraction antagonists. *Int. Org. Biol. Integr. Control Noxions Anim. Plants West Palearctic Reg. Sect. Bull. 20*, 207–215.

Wood, J.R., Resh, V.H., **1984**. Demonstration of sex pheromones in caddisflies (Trichoptera). *J. Chem. Ecol. 10*, 171–175.

Wright, R.H., **1958**. The olfactory guidance of flying insects. *Can. Entomol. 98*, 81–89.

Wu, W.-Q., Anton, S., Lofstedt, C., Hansson, B.S., **1996**. Discrimination among pheromone component blends by interneurons in male antennal lobes of two populations of the turnip moth, *Agrotis segetum*. *Proc. Natl Acad. Sci. USA 93*, 8022–8027.

Wyatt, T.D., **1997**. Putting pheromones to work: paths forward for direct control. In: Cardé, R.T., Minks, A.K. (Eds.), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 445–495.

Zhu, J., Kozlov, M.V., Philipp, P., Francke, W., Löfstedt, C., **1995**. Identification of a novel moth sex pheromone in *Eriocrania cicatricella* (Zett.) (Lepidoptera: Eriocraniidae) and its phylogenetic implications. *J. Chem. Ecol. 21*, 29–43.

Relevant Websites

http://www.nysaes.cornell.edu – The Pherolist, a data base catalog of moth pheromones.

http://www.pherobase.com – Pherobase, a compilation of data on insect pheromones.

http://www.mstrs.com – MSTRS Technologies, Inc.